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MECHANISMS OF UREA TOLERANCE IN UREA-ADAPTED POPULATIONS OF *DROSOPHILA MELANOGASTER*

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Summary

When behavioral avoidance cannot prevent an animal from being exposed to novel environmental toxins, physiological mechanisms must cope with the toxin and its effects. We are investigating the basis of urea tolerance in populations of *Drosophila melanogaster* that have been selected to survive and develop in food containing 300 mmol l^{-1} urea. Previous research has demonstrated that the urea-selected larvae have lower levels of urea in their body than control larvae reared under the same conditions. The current series of experiments focuses on three possible ways of reducing urea levels in the body: urea metabolism, increased urea excretion and decreased urea uptake from the environment. We tested for urea metabolism directly, by assaying for activity of two urea-metabolizing enzymes, and indirectly, by looking for reduced urea content of their medium. To measure urea excretion rates in whole animals, we reared control and urea-selected larvae on urea-containing food (urea food), switched them to normal food and monitored the loss of urea from their hemolymph. We measured urea uptake by rearing control and selected larvae on normal food, switching them to urea food and monitoring the rate of urea appearance in the hemolymph. We found no evidence for urea metabolism by either direct or indirect methods. Control larvae excreted urea at a higher rate than selected, probably because they contained more urea than the selected larvae and thus had a greater gradient for urea loss. The rate of urea uptake in selected larvae was 2 mmol l^{-1} h^{-1} slower than the rate in control larvae, a difference that could account for the measured differences in body urea levels. Thus the selected larvae appear to have adapted to urea exposure primarily by decreasing the ability of urea to enter their body in the first place. The mechanism responsible for this reduction in uptake is uncertain.

Key words: permeability, stress resistance, urea, *Drosophila melanogaster*.

Introduction

Animals must use physiological mechanisms to cope with novel toxins in the environment when behavioral avoidance options are unavailable (such as in aquatic environments) or undesirable (when utilizing a novel food source). There are four possible ways to adapt physiologically to a novel toxin in the environment. The organism can develop tolerance to the toxin either through general stress mechanisms or by developing counteractants to that specific toxin (Duffey, 1980; Yancey et al., 1982; Brattsen and Ahmad, 1986; Somero and Yancey, 1997; Feder, 1999). Alternatively, an animal might evolve the ability to detoxify the chemical into something less harmful or that can be handled by physiological systems already in place (Scott et al., 1998). The organism might also reduce the uptake from the environment or increase its excretion back into the environment (Shah et al., 1983; Pasteels et al., 1986). Animals may also use a combination of these mechanisms (Wen and Scott, 1999).

We are examining which of these possibilities have actually evolved in *Drosophila melanogaster* populations selected for tolerance to 300 mmol l^{-1} urea in their larval food. This system mimics natural circumstances in which toxins are unavoidable, because fruitfly larvae live in patchy semi-aqueous environments (rotting fruit in the wild and food vials in the laboratory) and cannot behaviorally avoid novel toxins. By using replicate laboratory populations deliberately selected to tolerate a novel toxin, we can avoid any ambiguities associated with assigning adaptive values to interspecific differences or to one variable of a complex natural environment (Garland and Adolph, 1994; Garland and Carter, 1994; Rose et al., 1996; Gibbs, 1999). In this system, we define an adaptation as a change that has occurred in all five selected populations but is absent from their sister control populations.

Urea is a general toxin that interferes with fundamental cell processes such as translation, acts as a protein denaturant, and reduces enzyme activity and thermostability (Bowlus and Somero, 1979; Yancey and Somero, 1979; Yancey, 1985; Yancey, 1992; Somero and Yancey, 1997). Urea is almost certainly a novel chemical for fruitflies since they do not produce it and are not likely to encounter it in their food; thus they should not have any pre-existing adaptations to urea.

Despite its toxicity, urea occurs in high levels in a variety of organisms, including sharks, mammals and associated microorganisms (Yancey et al., 1982). These systems where urea occurs naturally offer mechanistic models to test in the urea-adapted fruitflies.

Animals that contain high levels of urea, such as sharks and mammals, produce solutes that appear to counteract the denaturing effects of urea on proteins (Yancey et al., 1982; Lin and Timasheff, 1994; Somero and Yancey, 1997). Previous biochemical and physiological work has demonstrated that the major evolutionary response observed in the urea-selected fly populations has been decreased steady-state levels of urea (Pierce et al., 1999). Tolerance mechanisms would not be expected to reduce the amount of urea present, suggesting that this type of adaptation has not been the major evolutionary response of these populations. Furthermore, analysis of hemolymph properties and composition revealed no evidence for any tolerance mechanisms and the activity of a protein repair enzyme, protein isoaspartyl methyltransferase, showed no evolved change in the selected populations (David et al., 1999; Pierce et al., 1999). Thus there is both direct and indirect evidence to support the idea that tolerance adaptations have not evolved significantly in these populations.

The other three mechanisms, i.e. metabolism, increased excretion and decreased uptake, could all potentially reduce steady-state levels of urea. There are three known urea-metabolizing enzymes: arginase in the ornithine–urea cycle, and allantoicase and urease in the uricolytic pathway. However, neither of these pathways has been rigorously demonstrated to operate in insects (Bursell, 1967; Powles et al., 1972; Sumida et al., 1984). Nutritional requirements of *D. melanogaster* suggest that the urea cycle is incomplete in this species (Bursell, 1967). A gene putatively coding for arginase in *D. melanogaster* has been sequenced but activity has not been demonstrated, and gene knockouts did not have any effect on viability or morphology (Samson, 2000). Only the first enzyme in the uricolytic pathway, urate oxidase, is known to be present *D. melanogaster* (Friedman et al., 1992). The *Drosophila* Genome Project has not identified any genes homologous to allantoicase or urease thus far. Thus these enzymes are probably not normally active in *D. melanogaster* larvae. However, it is possible that the selected larvae have increased the activity of other enzymes, co-opting them to metabolize urea.

The second possibility is that the adapted larvae have decreased the uptake of urea from the environment. Urea may enter the larvae across their body surface or through the gut wall when they ingest the urea food. Larvae possess a chitinous cuticle and foregut that serve as a barrier to many compounds, but it is not known whether it would block urea (Demerec, 1965). The midgut is the site of digestion and absorption of nutrients and thus it must be permeable, at least to some compounds, in order to function (Demerec, 1965). Thus the midgut is the most likely site of entry for urea, but reducing its permeability to urea might impair nutrient uptake. It is interesting to note that the selected larvae develop more slowly on urea food than on normal food and have lower wet mass at pupation (V. A. Pierce, unpublished data). Impaired absorption of nutrients could result in slower growth, but general impairment of metabolic processes by urea could also explain this observation.

The third possibility is that the selected larvae have increased their excretion of urea. In insects, most solutes are thought to pass into the urine *via* protein transporters rather than *via* passive filtration as in vertebrate kidneys. Thus the larvae must possess transporters capable of passing urea in the lumen of their Malpighian tubules and be able to upregulate this activity in order to evolve this mechanism. While urea-specific transporters have been identified in a variety of vertebrates, they would not be expected to occur in *Drosophila* because these insects do not normally encounter urea. *D. melanogaster* excrete their nitrogenous waste as uric acid and ammonia (which is the major product during the larval stage) and must have excretory mechanisms to handle them (Borash et al., 1998). However, urea differs substantially enough in size and chemical properties from uric acid and ammonia, so it is unlikely that those pathways can also be used to excrete urea. Instead, larvae may possess very general transporters to remove foreign compounds, or passive filtration routes may play a greater role than was generally thought under these conditions.

Despite the issues outlined above, conceptually at least one of these three mechanisms, metabolism, decreased uptake or increased excretion, must occur for the selected larvae to have reduced steady-state levels of urea compared to control larvae under the same environmental conditions. The aim of our experiments is to identify which of these mechanisms has evolved.

Materials and methods

Experimental populations and selection protocol

Our study populations consisted of five pairs of selected (MX) and control (UU) populations (Joshi et al., 1996). Each pair was derived from one of five outbred baseline (B) populations in 1992. Thus, each selected population is more closely related to a control population founded from the same base population than to the other selected populations, resulting in fivefold replication of the selection experiment (Fig. 1). All populations are maintained at 25 °C on a 24 h light regime, with generation times of about 2 weeks. The larvae of the selected populations are reared at low density on banana-molasses medium with urea added. Adults are maintained in cages with normal banana-molasses food and are not exposed to urea. Control flies are raised under an identical regime, except that larvae are fed normal food.

These experiments are laboratory natural-selection experiments rather than artificial-selection experiments. That is, the parents that survive and reproduce are not chosen by the experimenter, but rather are those that survive the conditions of the environment they are placed in (Rose et al., 1996). Random drift, including founder effects, and natural selection

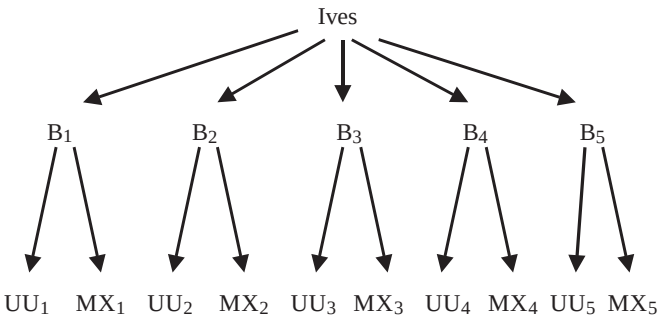

Fig. 1. Genetic relationships of experimental (MX) and control (UU) populations. Each pair of populations sharing the same subscript number is descended from the same stock population. Selected flies have been reared on 300 mmol l^{-1} food during the larval period for over 100 generations.

are the major forces likely to cause differentiation in these populations. Differences produced by random drift would be expected to be inconsistent among treatments while differences due to natural selection should be consistent among selection treatments (Rose, 1984; Rose et al., 1996).

The selected flies have been reared on urea food for over 100 generations. Larvae were initially exposed to 200 mmol l^{-1} urea and the amount was gradually increased to 300 mmol l^{-1} over 35 generations. Larvae in the urea-adapted populations develop into adults on food containing 300 mmol l^{-1} urea, whereas normal larvae grow and pupate but fail to eclose into adults (Shiotsugu et al., 1997). The egg-to-adult viability on 300 mmol l^{-1} urea food is nearly fivefold higher in the selected populations than in their controls.

Prior to experimentation, the selected and control populations were raised under identical, nonselective conditions for two generations to remove parental and grandparental effects (Rose et al., 1996). All measurements were done on third-instar wandering larvae, the stage just prior to pupation. Since even naive larvae survive well until pupation in food with high levels of urea, the use of third-instar wandering larvae for experiments should not be biased by differential mortality of the treatment groups. All data are presented as means ± 1 S.D. of populations (N=5). All statistical analyses were performed using Minitab v. 10 or 12.21.

Urea metabolism assays

Two experimental approaches were used to investigate the possibility that the selected larvae are metabolizing urea. We attempted to assay directly for the activity of two enzymes, urease and arginase, which are known to be involved in urea metabolism in other organisms. Absence of these enzyme activities in our samples could be explained three ways: either these enzymes are not significantly active in *D. melanogaster* and no urea metabolism is occurring, or metabolism by these enzymes is occurring but they require unusual assay conditions to be detected, or urea metabolism may be performed by enzymes from other pathways that are not detected by these assays. To address the latter two possibilities, we also investigated whether the urea content of the culture medium was lower after the larvae had fed on it.

Urease and arginase activities were measured using both kinetic and endpoint assays. For the kinetic assays, ten larvae were homogenized in 45 µl of 0.1 mol l^{-1} Tris-Cl, pH 7.5, 10 mmol l^{-1} KCl, 0.5 mmol l^{-1} EDTA. The reaction mixture for the urease assay consisted of 0.1 mol l^{-1} K_2HPO_4, pH 8.0, 2 mmol l^{-1} EDTA, 1.25 mmol l^{-1} urea, 29 mmol l^{-1} α-ketoglutarate, 0.2 mmol l^{-1} NADH and 0.3 U ml^{-1} L-glutamate dehydrogenase (assay modified from Pierce et al., 1999). The reaction was monitored for 3 min at 340 nm at room temperature on a Perkin–Elmer spectrophotometer. Two negative controls were run: one omitting homogenate and one omitting urea. For the arginase assay, larval homogenate was diluted 1:10 with 0.1 mol l^{-1} Tris-Cl, pH 7.5, 5 mmol l^{-1} $MnCl_2$ and preincubated at 33 °C for 10 min or 30 min. The reaction mixture for the arginase assay consisted of 0.17 mol l^{-1} Tris-Cl pH 8.6, 25 mmol l^{-1} L-arginine, 29 mmol l^{-1} α-ketoglutarate, 0.2 mmol l^{-1} NADH, 2.4 i.u. ml^{-1} urease and 0.3 i.u. ml^{-1} L-glutamate dehydrogenase (Schimke, 1970). Endpoint assays detected the amount of urea consumed by urease or produced by arginase after incubation at 5, 15, 30 or 60 min (Mommsen et al., 1983). After incubation of homogenate or negative controls with the appropriate substrates and cofactors, reactions were stopped by the addition of 25 µl of 60 % perchloroacetic acid. Samples were centrifuged for 5 min and 25 µl was transferred to a tube containing 1 ml of water and 0.5 ml urea reagent (20 % H_2SO_4, 2.8 % H_3PO_4, 0.25 mmol l^{-1} $FeCl_3$, 0.37 mmol l^{-1} thiosemicarbazide, 16.7 mmol l^{-1} diacetylmonoxime). Samples were boiled for 10 min in the dark, allowed to cool and absorbance read at 525 nm on a Perkin–Elmer spectrophotometer. Urea standards of known concentrations were assayed simultaneously. Commercial preparations of arginase and urease (Type III and Type IX) were used as positive controls. All reagents and enzymes were purchased from Sigma Chemical Co.

In the second, non-enzyme-specific approach, we determined whether the urea content was lower in the waste that the larvae excreted than in the food they had ingested. This approach took advantage of the fact that the culture vial is a closed system with respect to urea. The amount of urea present in a culture vial is determined by the urea content of the food poured into the vial initially. If the larvae metabolize urea, then the amount of urea in the vial should decrease over the larval developmental period, as the food becomes mixed with urea-depleted excreta. If the larvae are not metabolizing urea, then the amount of urea in the food should remain constant. By assaying the amount of urea in the food after the larvae have fed on it, we can determine whether or not *Drosophila* larvae metabolize urea and whether the selected larvae do so at higher rate than the controls, regardless of the pathway(s) used.

We assayed food from four vials from each selected population, four vials from each control population, and four vials containing no eggs to control for non-larval urea degradation under our rearing conditions (total number of vials = 44). Each vial contained exactly 2 ml of 300 mmol l^{-1} urea

food, so that treatments started with the same amount of urea. The vials that received eggs contained exactly 40 eggs, resulting in a larval density high enough to ensure that all the food was eaten over the developmental period, without crowding the larvae enough to interfere with their development. After the larvae pupated approximately 6 days later, 0.2 g of food were collected from each vial and assayed for ammonia and urea content using enzyme-based endpoint assays that measure the oxidation of NADH (for details see Pierce et al., 1999). Assays were performed on a Molecular Devices Thermomax microplate reader. Values are expressed as mmol urea g^{-1} food. The effect of treatment on urea content of the food was analyzed using an ANOVA in the GLM procedure in Minitab v. 12.

Urea excretion rate measurements

The urea excretion rate is measured as the rate of urea disappearance from a known volume of hemolymph. To measure the rate of urea loss from control and selected populations, eggs from the experimental generation were placed on 300 mmol l^{-1} urea food and allowed to develop for 5 days. On day 5, while the larvae were feeding as third instars, they were transferred to normal food for 3, 6, or 9 h. Larvae were also transferred to 300 mmol l^{-1} urea food for the same time points to control for the effect of handling. After being allowed to feed for the designated amount of time, ten larvae were collected from the food, rinsed, and 1 µl of hemolymph collected from them as described previously (Pierce et al., 1999). This hemolymph sample was diluted in 99 µl of 160 mmol l^{-1} Tris-Cl, pH 7.5. Three samples of hemolymph were collected from each population at each time point for a total of 60 samples. Hemolymph urea concentration was determined spectrophotometrically on a Molecular Devices 340PC microplate reader as above (Pierce et al., 1999). Larvae that were transferred to urea food for 3, 6 or 9 h (handling controls) were pooled as a zero time point (they spent zero time on normal food) in the data analysis.

The rate of urea loss for each selection treatment is calculated as the slope of the regression of hemolymph urea content on time spent on normal food. To test whether the slope differed between treatments, a modified ANCOVA model was used, in which time on normal food was a covariate and the factors were selection treatment, population (i.e. replicate number) and the interaction of selection and time (Steel and Torrie, 1980). Control and selected populations with the same replicate number were assayed together, thus this factor represents a block effect.

Assuming the loss of urea is passive, then its rate is can be described by the equation:

$$J_{\text{loss}} = P(C_{\text{in}} - C_{\text{out}}), \quad (1)$$

where J_{loss} is the rate of urea loss in mmol l^{-1} h^{-1}, P is a permeability coefficient in h^{-1} that includes all routes of loss in these larvae, C_{in} is the internal urea concentration in mmol l^{-1} at the beginning of the experiment and C_{out} is the external urea concentration in mmol l^{-1}.

Urea uptake measurements

The rate of urea uptake is measured as its rate of appearance in the hemolymph. To measure the rate of urea uptake from control and selected populations, eggs from the experimental generation were placed on standard culture food and allowed to develop for 4 days. On day 4, while the larvae were feeding as third instars, they were transferred to 300 mmol l^{-1} urea food for 3, 6, 9, 12, 15 or 18 h. Larvae were also transferred to normal food for 3, 6 and 9 h to control for the effect of handling. After being allowed to feed for the designated amount of time, ten larvae were collected from the food, rinsed, and 1 µl of hemolymph collected from them as described previously (Pierce et al., 1999). Hemolymph was diluted in 99 µl of 160 mmol l^{-1} Tris-Cl, pH 7.5. Three samples of hemolymph were collected from each population at each time point for a total of 105 samples. Hemolymph urea concentration was determined spectrophotometrically on a Molecular Devices 340PC microplate reader using the method described above (Pierce et al., 1999). Larvae that were transferred to normal food were pooled as a zero time point (they spent zero time on urea food) in the data analysis.

The rate of urea uptake for each selection treatment is calculated as the slope of the regression of hemolymph urea content on time spent on urea food. We tested whether the slope differed between treatments by using a modified ANCOVA model in which time on urea food was a covariate and the factors were selection treatment, population (i.e. replicate number) and the interaction of selection and time. Control and selected populations with the same replicate number were assayed together, thus this factor represents a block effect.

Assuming the uptake of urea is passive, then its rate can be described by the equation:

$$J_{\text{uptake}} = P(C_{\text{out}} - C_{\text{in}}), \quad (2)$$

where J_{uptake} is the rate of urea uptake in mmol l^{-1} h^{-1}, P is a permeability coefficient in h^{-1}, C_{in} is the internal urea concentration in mmol l^{-1} at the beginning of the experiment and C_{out} is the external urea concentration in mmol l^{-1}.

Calculations of mass-specific fluxes and permeability coefficients

Most reported urea fluxes are mass-specific, and permeability coefficient calculations include both mass and the surface area available for diffusion. To make our data more directly comparable with other systems, we assumed that 1 µl of larva weighs 1 mg and substituted this value for volume in our rates of urea loss and urea uptake. Thus we report mass-specific urea fluxes in µmol kg^{-1} h^{-1}.

We assume that the larval gut is the primary site for movement of urea into and out of the body (see Discussion). We calculated total gut surface area for one larva using standard geometrical equations and the following size estimates provided by R. Krebs (personal communication): 0.5 mm, 2 mm and 2 mm for the lengths of the gastric cecae, midgut and hindgut, respectively; and 1 mm, 0.5 mm and

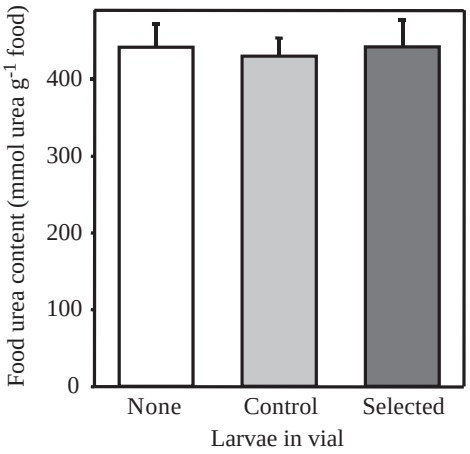

Fig. 2. Effect of larval presence on amount of urea in food. Experimental groups consisted of vials containing either selected larvae, or control larvae or no larvae. 40 larvae were reared in exactly 2 ml of urea food per vial. Food was assayed for urea content after larvae pupated, about 1 week later. Values are means ± 1 S.D., N=5 populations per each experimental group.

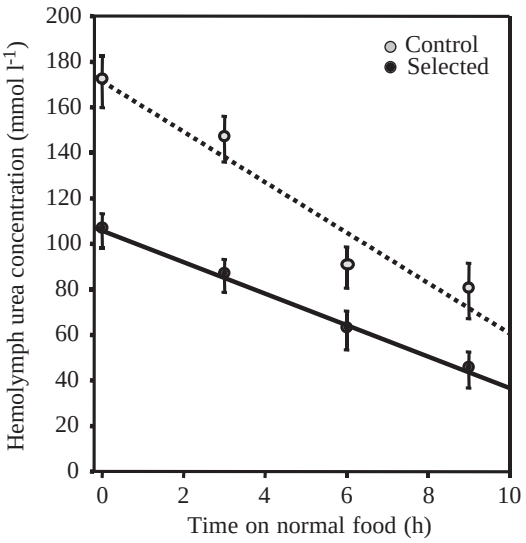

Fig. 3. Effect of selection treatment and time on normal food on urea loss. After rearing on 300 mmol l^{-1} urea food, larvae were switched to normal food or urea food. The zero time point represents larvae switched to urea food as a control for the effect of handling. Hemolymph urea concentrations decrease with increasing time on normal food. The rate of urea loss is significantly greater in the controls than in the selected larvae (10.4 mmol l^{-1} h^{-1} *versus* 7.0 mmol l^{-1} h^{-1}, respectively; values are means ± 1 S.D., N=5, ANCOVA, P<0.001). Note that under a diffusion model, the higher rate of urea loss in the controls can be explained by their higher urea concentration gradient (see text).

0.25 mm for the circumferences of the gastric cecae, midgut and hindgut, respectively. Mass-specific surface area, SA_{ms}, was calculated by dividing by 1.67 mg, the mass of one larva. This calculation ignores any additional and currently unassessed surface area provided by microstructure in the gut.

The mass and surface area-specific urea permeability coefficient, P_s, is calculated as:

$$P_s = R(G \times SA_{ms}), \quad (3)$$

where R is the mass-specific urea flux rate in μmol kg^{-1} h^{-1}, G is the urea concentration gradient in μmol ml^{-1} calculated in the general diffusion equations above, and SA_{ms} is the mass-specific surface area in cm^2 kg^{-1}.

Results

Urea metabolism

Both kinetic and endpoint assays successfully detected activity of our commercially prepared enzymes. However, no urease or arginase activity was detected in our larval samples by either method, regardless of time treatments or variations in the amount of substrate used.

There was no significant effect of treatment on the urea content of the food in the vials after 1 week (P=0.425; Fig. 2). Vials that did not contain larvae had 442.1±30.5 mmol urea g^{-1} food (N=4), vials containing control larvae averaged 430.2±23.8 mmol urea g^{-1} food (N=20) and vials with selected larvae had 442.6±35.5 mmol urea g^{-1} food (N=20).

Rate of urea loss

Hemolymph urea concentrations were significantly affected by selection treatment, population (replicate number), time, and the interaction between time and selection treatment (P<0.001 for all factors; Fig. 3). On urea food, the hemolymph of control larvae contained more urea, 170.5±9.8 mmol l^{-1}, than the hemolymph of selected larvae, which contained 107±9.4 mmol l^{-1}. The rate of urea loss from the hemolymph was significantly higher in the controls than in the selected larvae (10.4 mmol l^{-1} h^{-1} and 7.02 mmol l^{-1} h^{-1}, respectively). The permeability coefficients of the control and selected larvae were similar (0.0622 h^{-1} and 0.0669 h^{-1}, respectively).

Rate of urea uptake

Hemolymph urea concentrations were significantly affected by time on urea food (P<0.001), population (replicate number) (P<0.001) and the interaction between time and selection treatment (P<0.029). Urea levels increased more quickly in the hemolymph of control larvae than selected larvae (8.98 mmol l^{-1} h^{-1} *versus* 7.12 mmol l^{-1} h^{-1}, respectively) and accumulated to higher levels over the time course of the experiment (Fig. 4). The permeability coefficients were 0.0293 h^{-1} for the control larvae and 0.0237 h^{-1} for the selected larvae.

Mass-specific fluxes and permeability coefficents

Mass-specific urea influxes were 7120 μmol kg^{-1} h^{-1} and 8980 μmol kg^{-1} h^{-1} for selected and control larvae, respectively. Mass-specific urea effluxes were 7020 μmol kg^{-1} h^{-1} and 10 400 μmol kg^{-1} h^{-1} for selected and control larvae, respectively. Mass-specific surface area of the gut was

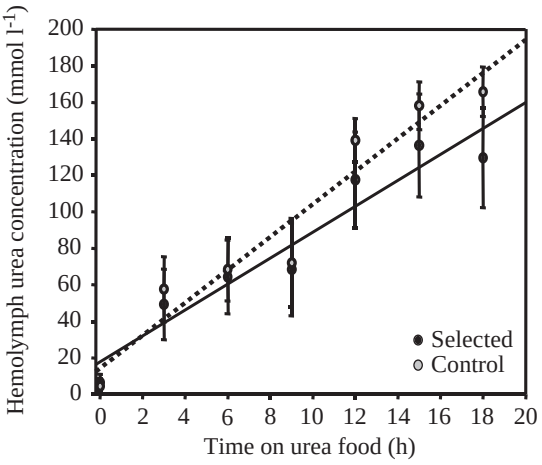

Fig. 4. Effect of selection treatment and time on urea food on urea uptake. After rearing on normal food, larvae were switched to 300 mmol l^{-1} urea food or normal food. The zero time point represents larvae switched to normal food, who thus had no exposure to urea. Hemolymph urea concentrations increase with increasing time on urea food. The rate of urea uptake is significantly greater in the controls than in the selected larvae (8.98 mmol l^{-1} h^{-1} *versus* 7.12 mmol l^{-1} h^{-1}, respectively; values are means ± 1 S.D., N=5, ANCOVA, P<0.029).

calculated to be 119 760 cm^2 kg^{-1}. The concentration gradients listed above were used to calculate the mass- and surface-area-specific permeability coefficients. The permeability coefficient calculated for urea efflux was 0.15×10^{-6} cm s^{-1} in the selected larvae and 0.14×10^{-6} cm s^{-1} in the control larvae. The permeability coefficient for urea influx was 0.055×10^{-6} cm s^{-1} in the selected larvae and 0.069×10^{-6} cm s^{-1} in the selected larvae (Table 1).

Discussion

We investigated three possible mechanisms that could reduce urea accumulation in the bodies of urea-tolerant *D. melanogaster* larvae. Our results indicate that only one of these mechanisms, reduced uptake of urea, appears to be occurring.

Urea metabolism

We found no direct evidence for urea-metabolizing enzyme activity in our larval samples. No urease gene has been identified so far in the *Drosophila* genome and it is generally thought not to be present in animals, so its absence in our studies is not surprising. Furthermore, despite the putative arginase gene identified in *Drosophila*, we could not detect any arginase activity. The *Drosophila* enzyme might have very low activity, might require different assay conditions, or might not function as a urea-cycle enzyme. The *Drosophila* gene is most likely to be the extrahepatic arginase (A-II), which does not usually contribute to the urea cycle, even in ureotelic organisms (Samson, 2000). Thus, even if this enzyme is active, it may not be able to catalyze the reverse reaction and condense urea and ornithine to produce arginine. This is consistent with our previous results showing that larvae reared on urea food had significantly less arginine in their hemolymph than those reared on normal food (Pierce et al., 1999).

If selected larvae were metabolizing urea, then we would have expected to find reduced urea levels in the waste food after 1 week of high-density feeding. Instead, we observed that vials that held selected larvae had the same urea content as vials that contained control larvae and vials that did not contain larvae at all. Thus neither selected nor control larvae appear to be capable of metabolizing urea. Because our measurements are calculated per unit mass of agar-containing food, not per unit volume, we cannot directly compare these data with the starting concentration of 300 mmol l^{-1} urea to determine whether urea degradation occurred over the time course of the experiment. While there may have been microbial degradation of urea under our culture conditions, the urea level does not differ between vials, indicating that any such degradation was the same in all vials, regardless of the presence of larvae. Thus urea metabolism cannot account for the reduced levels of urea observed in the bodies of selected larvae (Pierce et al., 1999).

Table 1. *Urea permeability coefficients for different tissues*

Tissue	Urea coefficient ($\times10^{-6}$ cm^{-1} s^{-1})	Reference
Erythrocytes, human	360	Sha'afi, 1981
Hepatocytes, rat	182	Alpini et al., 1986
Toadfish gill, urea pulse event	7.79	McDonald et al., 2000
Inner medullary collecting duct cells, rat	6.0	Schwartz et al., 1990
Urinary bladder, toad	0.61	Bindslev and Wright, 1976
Gill, toadfish, non-pulse period	0.26	McDonald et al., 2000
Efflux, selected larvae	0.15	Current study
Efflux, control larvae	0.14	Current study
Influx, control larvae	0.069	Current study
Influx, selected larvae	0.055	Current study
Gill, dogfish	0.032	Part et al., 1998

Urea excretion

Urea disappeared from the hemolymph of control larvae at a higher rate than from the hemolymph of selected larvae. This result is the exact opposite of what we would have expected if the selected larvae had evolved an improved ability to excrete urea and thus reduce its accumulation in their tissues. The relatively linear rate of urea loss suggests that it occurs primarily by passive means (Patrick et al., 1997; Wood et al., 1998).

Because the control larvae accumulate urea to higher levels than the selected larvae when reared on urea food, they have a more favorable concentration gradient for diffusion of urea out of the body when they are switched to normal food for these measurements. When the permeability constant is calculated for each selection treatment using the measured concentration gradients and rates of loss, the permeability constants for the

control and selected larvae are very similar; the coefficient for the selected larvae is 7.6 % greater than that for the control larvae. When the permeability of each population is calculated individually to provide estimates of variance, the control and selected larvae permeability coefficients are not significantly different (ANOVA, P=0.49). This suggests that selected larvae have not altered their ability to excrete urea and that the higher rates of loss observed in the control larvae in this experiment are explained by their higher concentration gradients.

It should be noted that under the selective conditions used to create the urea-tolerant flies, the larvae remain on 300 mmol l^{-1} urea food during the entire developmental period. The environmental urea precludes the existence of a favorable outward urea gradient. Under these conditions, changes in the passive loss of urea would not enable the larvae to get rid of more urea and thus would not be selected for.

Urea uptake

Under our experimental conditions, the urea gradient favors its entry from the 300 mmol l^{-1} urea food into the larval body. Urea accumulated at a faster rate in the hemolymph of the control larvae than in the selected larvae. Thus the selected larvae have evolved an adaptation that reduces the rate at which urea enters their body. If all other conditions are equal, reduced rates of entry should result in lower levels of urea in their tissues, which would in turn lower the amount of damage potentially caused by the urea. Eventually, larvae from both selection treatments will reach similar equilibrium concentrations, but over the developmental period of 5–6 days, the rate difference between control and selected larvae could more than account for the observed difference of 58 mmol l^{-1} in third instar larvae.

If urea enters passively, than this difference in uptake rate should be attributable to differences in the permeability of the larvae to urea. The permeability coefficient calculated for the selected larvae is 82 % of the coefficient for the control larvae. When the permeability of each population is calculated individually to provide estimates of variance, the permeability coefficients for control and selected larvae are significantly different (ANOVA, P=0.014). Thus the selected larvae are most likely to have evolved reduced permeability to urea. The possible sites of urea entry can be broadly considered as the gut and the cuticular surface, because the larvae both consume and are immersed in their food. The cuticle and foregut are both lined with chitin and thus may be impermeable, but the midgut must be at least somewhat permeable because it is the site of digestion and nutrient absorption (Demerec, 1965). The hindgut is probably also somewhat permeable if it plays a role in urine modification, as occurs in other insects (Bradley, 1987).

Urea fluxes: comparison with other systems

How do larval urea fluxes compare with fluxes in other organisms? Our larval urea effluxes are similar to the estimated efflux of 10 000 μmol kg^{-1} h^{-1} for teleost gills with a gradient of 350 mmol l^{-1}, but are much higher than the *in vivo* rate of 270 μmol kg^{-1} h^{-1} measured in dogfish with a 350 mmol l^{-1} gradient (Part et al., 1998). However, dogfish retain urea for osmotic balance and their gills are considered to be exceptionally impermeable to urea (Part et al., 1998). Urea efflux in gulf toadfish gills averages 59.9 μmol kg^{-1} h^{-1} with a plasma concentration of only 3 mmol l^{-1} urea (McDonald et al., 2000). Thus our mass-specific urea fluxes are high, but within the same range as animals that are not known to be deliberately retaining urea.

Urea permeability coefficients for the larvae are two- to fivefold higher than the coefficient reported for dogfish gills but lower than coefficients reported for most other cells (Table 1; Part et al., 1998; McDonald et al., 2000). The efflux permeabilities are similar for both selected and control larvae, but the control larvae appear to be more permeable to urea entry than the selected larvae. The discrepancies between the larval influx and efflux permeabilities probably indicate that the surface areas for urea uptake and loss are not identical (e.g. there may be loss of urea but not uptake in the Malpighian tubules), but also may indicate that other factors do not affect the two processes identically.

How is this reduced uptake effected in the urea-resistant larvae? Possible mechanisms would depend on the route of urea entry. Influx of urea through the gut wall may be occurring either transcellularly, or paracellularly, or by some combination of the two routes. If urea is entering paracellularly, then tightening cell junctions in the gut could reduce urea uptake. Transcellular entry probably depends on transporters present in the gut cells; recent work has demonstrated that urea does not easily cross pure lipid bilayers (Walsh, 1997). Many membranes that are permeable to urea contain urea-transporters, including mammalian kidney cells, erythrocytes and toadfish gills (Walsh, 1997). To date, urea transporters have been cloned from mammals, toadfish, sharks and frogs (Walsh et al., 2000, and references therein). While *Drosophila* larvae probably do not have urea transporters, urea may enter through other transporters. Rat and human liver cells appear to export urea through an alternative transporter, aquaporin 9, which has broad specificity for non-charged solutes (Ishibashi et al., 1998; Tsukaguchi et al., 1998). Thus, in our *Drosophila* larvae, urea may enter through transporters normally used for other solutes, and adaptation may involve either downregulating these transporters or altering their activity to reduce their ability to transport urea.

Conclusions

Tolerance responses and metabolism of toxins have received a great deal of attention, especially with regard to the role they play in insecticide resistance (Scott et al., 1998). However, differences in the genetic basis of resistance to natural *versus* man-made toxins have been noted and may reflect differences in physiological mechanisms (Jones, 1998; Wen and Scott, 1999). Resistance to a new insecticide, fipronil, in houseflies is polygenic and appears to be due to both decreased entry of the insecticide and to a second mechanism, either increased

metabolism or increased excretion (Wen and Scott, 1999). We have shown that *Drosophila melanogaster* larvae selected for urea resistance have reduced urea uptake and have less urea in their bodies than control larvae. Resistance in our populations is also polygenic but appears to be limited to a single physiological mechanism, that of decreased entry (Joshi et al., 1996). Our results suggest that the importance of strategies involving reduced entry as a mechanism for toxin resistance may be underestimated.

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References

Alpini, G., Garick, R. A., Jones, M. J. T., Nunes, R. and Tavoloni, N. (1986). Water and nonelectrolyte permeability of isolated rat hepatocytes. *Am. J. Physiol*. **251**, C872–C822.

Bindslev, N. and Wright, E. M. (1976). Effect of temperature on nonelectrolyte permeation across toad urinary bladder. *J. Membr. Biol*. **29**, 265–288.

Borash, D. J., Gibbs, A. G., Joshi, A. and Mueller, L. D. (1998). A genetic polymorphism maintained by natural selection in a temporally varying environment. *Am. Nat.* **151**, 148–156.

Bowlus, R. D. and Somero, G. N. (1979). Solute compatibility with enzyme function and structure: rationales for the selection of osmotic agents and end-products of anaerobic metabolism in marine invertebrates. *J. Exp. Zool*. **208**, 137–152.

Bradley, T. J. (1987). Physiology of osmoregulation in mosquitoes. *Annu. Rev. Ent*. **32**, 439–462.

Brattsen, L. B. and Ahmad, S. (1986). *Molecular Aspects of Insect–Plant Associations.* Plenum Press, New York.

Bursell, E. (1967). The excretion of nitrogen in insects. *Adv. Insect Physiol*. **4**, 33–67.

David, C. L., Pierce, V. A., Aswad, D. W., and Gibbs, A. G. (1999). The effect of urea exposure on isoaspartyl content and PIMT activity in *Drosophila melanogaster*. *Comp. Biochem. Physiol.* **124**B, 423–427.

Demerec, M. (1965). *Biology of* Drosophila, 632pp. New York: Hafner Publishing Co.

Duffey, S. S. (1980). Sequestration of plant natural products by insects. *Annu. Rev. Entomol.* **25,** 447–477.

Feder, M. E. (1999). Organismal, ecological and evolutionary aspects of heat-shock proteins and the stress response: Established conclusions and unresolved issues. *Amer. Zool*. **39**, 857–864.

Friedman, T. B., Burnett, J. B., Lootens, S., Steinman, R. and Wallrath, L. L. (1992). The urate oxidase gene of *Drosophila melanogaster*: Evolutionary changes of sequence and regulation. *J. Mol. Evol*. **34**, 62–77.

Garland, T., Jr and Adolph, S. C. (1994). Why not to do two-species comparisons: limitations on inferring adaptation. *Physiol. Zool*. **67**, 797–828.

Garland, T., Jr and Carter, P. A. (1994). Evolutionary physiology. *Annu. Rev. Physiol*. **56**, 579–621.

Gibbs, A. G. (1999). Laboratory selection for the comparative physiologist. *J. Exp. Biol*. **202**, 2709–2718.

Ishibashi, K., Kuwahara, M., Gu, Y., Tanaka, Y., Marumo, F. and Sasaki, S. (1998). Cloning and functional characterization of a new aquaporin (AQP9) abundantly expressed in the peripheral leukocytes permeable to water and urea but not to glycerol. *Biochem. Biophys. Res. Commun*. **244**, 268–274.

Jones, C. D. (1998). The genetic basis of *Drosophila sechellia*'s resistance to a host plant toxin. *Genetics* **149**, 1899–1908.

Joshi, A., Knight, C. D. and Mueller, L. D. (1996). Genetics of larval urea tolerance in *Drosophila melanogaster*. *Heredity* **77**, 33–39.

Lin, T. Y. and Timasheff, S. N. (1994). Why do some organisms use a urea–methylamine mixture as an osmolyte? Thermodynamic compensation of urea and trimethylamine N-oxide interactions with protein. *Biochemistry* **33**, 12695–12701.

McDonald, M. D., Wood, C. M., Wang, Y. and Walsh, P. J. (2000). Differential branchial and renal handling of urea, acetamide and thiourea in the gulf toadfish *Opsanus beta*: Evidence for two transporters. *J. Exp. Biol*. **203**, 1027–1037.

Mommsen, T. P., Hochachka, P. W. and French, C. J. (1983). Metabolism of arginine, proline, and ornithine in tissues of the squid, *Illex illecebrosus*. *Can. J. Zool*. **61**, 1835–1846.

Part, P., Wright, P. A. and Wood, C. M. (1998). Urea and water permeability in dogfish (*Squalus acanthias*) gills. *Comp. Biochem. Physiol*. **119**A, 117–123.

Pasteels, J. M., Daloze, D. and Rowell-Rahier, M. (1986). Chemical defenses in leaf beetles and their larvae: the ecological, evolutionary and taxonomic significance. *Biochem. Syst. Ecol.* **12,** 395–406.

Patrick, M. L., Part, P., Marshall, W. S. and Wood, C. M. (1997). Characterization of ion and acid–base transport in the fresh water adapted mummichog (*Fundulus heteroclitus). J. Exp. Zool*. **279**, 208–219.

Pierce, V. A., Mueller, L. D., and Gibbs, A. G. (1999). Osmoregulation in *Drosophila melanogaster* selected for urea tolerance. *J. Exp. Biol*. **202**, 2349–2358.

Powles, M. A., Janssens, P. A., and Gilmour, D. (1972). Urea formation in the green vegetable bug, *Nezara viridula*. *J. Insect Physiol*. **18**, 2343–2358.

Rose, M. R. (1984). Laboratory evolution of postponed senescence in *Drosophila melanogaster*. *Evolution* **38**, 1004–1010.

Rose, M. R., Nusbaum, T. J. and Chippindale, A. K. (1996). Laboratory evolution: The experimental wonderland and the Cheshire Cat syndrome. In *Adaptation* (ed. M. R. Rose and G. V. Lauder), pp. 221–242. San Diego: Academic Press.

Samson, M. L. (2000). *Drosophila* arginase is produced from a nonvital gene that contains the *elav* locus within its third intron. *J. Biol. Chem*. **275**, 31107–31114.

Schimke, R. T. (1970). Arginase. In *Methods in Enzymology* (ed. H. Taylor and C. W. Taylor), vol. XVIIA, pp. 313–317. New York: Academic Press.

Schwartz, J. H., Bengele, H. H., McNamara, E. R., and Alexander, E. A. (1990). Vasopressin-enhanced urea transport by rat inner medullary collecting duct cells in culture. *Cell Tissue Kinet.* **23**, 487–493.

Scott, J. G., Liu, N. and Wen, Z. (1998). Insect cytochromes P450: diversity, insecticide resistance and tolerance to plant toxins. *Comp. Biochem. Physiol.* **121**C, 147–155.

Sha'afi, R. I. (1981). Permeability for water and other polar molecules. In *Membrane Transport* (ed. S. L. Bonting and J. J. H. H. M. de Pont), pp. 29–60. Amsterdam: Elsevier/North Holland Biomedical Press.

Shah, P. V., Monroe, R. J. and Guthrie, F. E. (1983). Comparative penetration of insecticides in target and non-target species. *Drug Chem. Toxicol.* **6,** 155–179.

Shiotsugu, J., Leroi, A. M., Yashiro, H., Rose, M. R. and Mueller, L. D. (1997). The symmetry of correlated responses in adaptive evolution: an experimental study using *Drosophila*. *Evolution* **51**, 163–172.

Somero, G. N. and Yancey, P. H. (1997). Osmolytes and cell-volume regulation: physiological and evolutionary principles. In *Handbook of Physiology: Cell Physiology* (ed. J. F. Hoffman and J. D. Jamieson), pp. 441–484. New York: Oxford University Press.

Steel, R. G. D. and Torrie, J. H. (1980). *Principles and Procedures of Statistics: A Biometrical Approach*, 363 pp. New York: McGraw-Hill.

Sumida, M., Haga, K., Tanaka, Y., Shimabukuro, J., Ichida, M. and Matsubara, M. (1984). Developmental changes in urea in the haemolymph (determined by a urease-indophenol method) in hybrid strains of the silkworm, *Bombyx mori*, and the effect of starvation in the fifth instar larvae, fed an artificial diet, on urea level in subsequent development. *Comp. Biochem. Physiol*. **105**A, 563–570.

Tsukaguchi, H., Shayaki, C., Berger, U. V., Mackenzie, B., Devidas, S., Guggino, W. B., van Hoek, A. N. and Hediger, M. A. (1998). Molecular characterization of a broad selectivity neutral solute channel. *J. Biol. Chem*. **273**, 24737–24743.

Walsh, P. J. (1997). Evolution and regulation of urea synthesis and ureotely in (Batrachoidid) fishes. *Annu. Rev. Physiol*. **59**, 299–323.

Walsh, P. J., Heitz, M. J., Campbell, C. E., Cooper, G. J., Medina, M., Wang, Y. S., Goss, G. G., Vincek, V., Wood, C. M. and Smith, C. P. (2000). Molecular characterization of a urea transporter in the gill of the gulf toadfish (*Opsanus beta*). *J. Exp. Biol*. **203**, 2357–2364.

Wen, Z. and Scott, J. G. (1999). Genetic and biochemical mechanisms limiting fipronil toxicity in the LPR strain of housefly, *Musca domestica*. *Pestic. Sci*. **55**, 988–992.

Wood, C. M., Gilmour, K. M. and Part, P. (1998). Passive and active transport properties of a gill model, the cultured branchial epithelium of the freshwater rainbow trout (*Oncorhyncus mykiss*). *Comp. Biochem. Physiol*. **119**A, 87–96.

Yancey, P. H. (1985). Organic osmotic effectors in cartilaginous fishes. In *Transport Processes, Iono- and Osmoregulation* (ed. R. Gilles and M. Gilles-Ballien), pp. 424–436. Berlin: Springer-Verlag.

Yancey, P. H. (1992). Compatible and counteracting aspects of organic osmolytes in mammalian kidney cells *in vivo* and *in vitro*. In *Water and Life: A Comparative Analysis of Water Relationships at the Organismic, Cellular, and Molecular Levels* (ed. G. N. Somero, C. B. Osmond and C. L. Bolis), pp. 19–32. Berlin: Springer-Verlag.

Yancey, P. H., Clark, M. E., Hand, S. C., Bowlus, R. D. and Somero, G. N. (1982). Living with water stress: evolution of osmolyte systems. *Science* **217**, 1214–1222.

Yancey, P. H. and Somero, G. N. (1979). Counteraction of urea destabilization of protein structure by methylamine osmoregulatory compounds of elasmobranch fishes. *Biochem. J.* **182**, 317-323.