Evolution of ammonia and urea tolerance in *Drosophila melanogaster*: resistance and cross-tolerance

Daniel J. Borash [a,d], Valerie A. Pierce [b,d], Allen G. Gibbs [c,d], Laurence D. Mueller [d,*]

[a] *Department of Systems Science–Biology, University of Tokyo, Komaba, Meguro-ku 153-8902, Japan*
[b] *Department of Biology, 6S-143, College of Staten Island, 2800 Victory Blvd, Staten Island, NY 10314, USA*
[c] *Department of Ecology and Evolutionary Biology, University of Arizona, Tuscon, AZ 85721, USA*
[d] *Department of Ecology and Evolutionary Biology, University of California, Irvine, Irvine, CA 92697-2525, USA*

Abstract

We examined whether populations of *Drosophila melanogaster* could evolve a genetically based tolerance to high levels of toxic compounds (urea or ammonia) added to their larval food medium. We also examined whether tolerance to one compound may impart cross-tolerance to other compounds. Five populations selected for ammonia tolerance (AX), five populations selected for urea tolerance (UX), and five unselected controls (AUC) were assayed for developmental time, viability, and female fertility. These characteristics were measured on each of the 15 populations reared on one of three larval food conditions (plain banana-molasses, 0.35 M NH_4Cl, or 0.266 M urea). On urea-supplemented media, the urea-selected populations developed fastest and expressed the highest viability; the ammonia-selected populations developed significantly faster and had a higher viability than the controls. Similarly, on ammonia-supplemented media, the ammonia-selected populations developed fastest and expressed the highest viability; the urea-selected populations developed significantly faster and had a higher viability than the controls. This suggests that a cross-tolerance exists for resisting different toxic compounds. Urea-selected females reared on urea-containing food media displayed superior fecundity, without any observable cross-tolerance effect. When all populations were reared on food containing 0.266 M urea, the urea-selected populations had the lowest levels of urea in their tissues. All populations reared on food containing 0.37 M ammonia or 0.266 M urea, contained more ammonia in their tissues than did populations reared on plain food. © 2000 Elsevier Science Ltd. All rights reserved.

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In nature, insects may be exposed to a wide variety of toxic substances. These may include feeding deterrents synthesized by plants, heavy metals, insecticides, etc. The evolution of resistance to toxins has been studied extensively. In the case of compounds which affect one or a few specific enzymes, selection will favor those individuals with resistant isoforms. Thus, only one or a few genes may be under strong selection, which will differ depending on the toxin, and selection for resistance to one compound is not generally expected to confer resistance to others.

Many substances, such as heavy metals, may have wide-ranging effects on many biochemical and physiological processes. Mechanisms of resistance are likely to be polygenic, and may confer resistance to other toxins as well. For example, selection for decreased permeability of the cuticle may prevent the entry of a wide range of substances, in addition to the compound used as a selective agent. In this case, we may predict that insects will evolve cross-tolerance to multiple toxins. Hoffmann and Parsons (1991) documented several cases in which cross-tolerance to different stresses occurs. Many of these stresses trigger the stimulation of heat-shock proteins (Feder, 1998), or stress metabolites in the case of plants.

As part of our research program in density-dependent selection, we have examined how populations of *Drosophila melanogaster* adapt to high levels of environmental urea and ammonia. Both of these nitrogenous com-

* Corresponding author. Tel./fax: +1-949-824-4744.

E-mail addresses: pierce@postbox.csi.cuny.edu (V.A. Pierce), agibbs@mail.ar1.arizona.edu (A.G. Gibbs), ldmuelle@uci.edu (L.D. Mueller).

pounds have been reported to accumulate in the food of crowded *Drosophila* cultures (Botella et al., 1985; Borash et al., 1998). They do not appear to target any specific biochemical processes, but instead have general cytotoxic effects. Urea is a protein denaturant (Somero and Yancey, 1997), and larvae reared on urea-containing media have increased levels of proteins containing isoaspartyl residues, a form of protein damage (David et al., 1999). Ammonia's effects are less well understood. It appears to be neurotoxic in vertebrates (Cooper and Plum, 1987) and may affect pH regulation.

Previous work has demonstrated that populations reared under crowded larval conditions evolve increased resistance to both urea (Joshi et al., 1996) and ammonia (Borash, unpublished data), although only ammonia accumulates to significant levels in larval cultures (Borash et al., 1998). This suggests that evolved resistance to ammonia and urea may involve some of the same physiological mechanisms. To investigate this possibility, we subjected populations to selection for resistance to these compounds. We report here the effects of urea and ammonia on life history characters (survival, development time, and fecundity) in urea-selected, ammonia-selected, and control populations of *D. melanogaster*. Selected populations exhibited the greatest fitness in their respective treatment conditions, but also showed increased tolerance to the other compound, relative to unselected controls.

1. Methods and materials

1.1. The populations

Fifteen populations were derived (Fig. 1) from the five UU populations (Joshi and Mueller, 1996). The UU

Derivation Of Selected and Control Populations

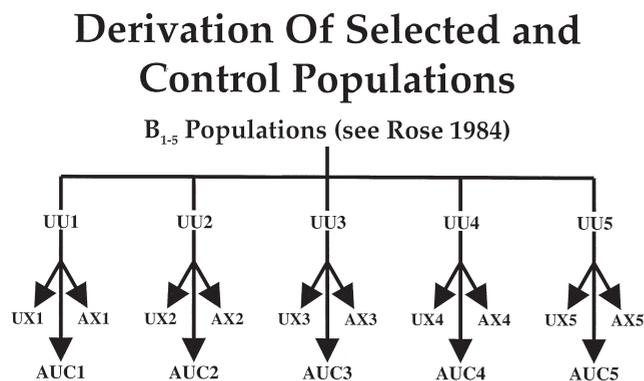### B$_{1-5}$ Populations (see Rose 1984)

Fig. 1. Derivation of the ammonia-tolerant (AX), urea-tolerant (UX), and unselected controls (AUC) selection regimes. All selection regimes are five-fold replicated, maintained at large population sizes (>750), with discrete generations. These populations were created in the Fall of 1996. Both selection regimes and the control populations were derived from a five-fold replicated set of populations called UU, which have a 3-week generation time, and are reared at low larval and adult densities. The UU populations were derived in 1990, from the Rose B populations (Rose, 1984; Chippindale et al., 1994, 1996).

populations (UU1 ... UU5), derived from established Rose B populations (Rose, 1984), were initiated in September 1991, with all UU populations maintained on banana-molasses food (Rose, 1984) at 25°C (24 h light) and uncontrolled humidity, and having a generation time of approximately 3 weeks. The UU populations were uncrowded as larva (~60–80 eggs per 8-dram vial), with emergent adults kept at a low density of approximately 50–60 flies per 8-dram vial, and transferred to fresh food every other day for approximately 1 week. N_e for each UU line was >1000 every generation.

In Fall 1996, each of the five ammonia-selected (AX) populations were initiated with 60 vials containing 60–80 eggs each. Eggs were collected on small pieces of non-nutritive agar, which would not affect the food level or ammonia concentration of the individual vial. Plastic sleeves were inserted in the vials, so pupae could be removed before eclosion. After >90% of the visible larvae had pupated, the sleeves were removed and placed into plexi-glass cages, with a standard banana-molasses food plate. After the majority of adults had eclosed, yeasted food plates were inserted into the cages, to stimulate female oviposition. Thus, only the larvae were exposed to the ammonia food.

Ammonium chloride (pH ~5.5, equivalent to the standard pH of the banana-molasses food pH) was added to standard banana-molasses food medium, after it had cooled to 48°C, and an anti-fungal agent was added to the food. In order to homogenize the mixture as thoroughly as possible before dispensing it into vials, the supplemented media was placed upon a magnetic stirring apparatus, which rapidly stirred the banana-molasses–ammonia mixture as it was dispensed into vials. The levels of ammonium chloride were increased every few generations (Fig. 2), when it was observed that a great proportion of larvae were surviving to adulthood. The UX selection regime was maintained in a similar fashion, with the exception that urea (pH ~5.5, equivalent to the standard banana-molasses food pH) was added to the banana-molasses food, instead of NH_4Cl. The AUC (Ammonia–Urea Control) regime served as a control — the larvae not being exposed to urea or ammonia.

During the larval feeding phase, all populations were maintained in incubators under constant conditions (25°C, 24 h light, and ambient humidity). Before any experiments were performed, all populations were removed from selection for two generations and reared under identical larval and adult conditions to remove any environmental or maternal effects, which may confound the observance of genetic differences between any of the populations. Assays were performed after ten and 21 generations of selection.

1.2. Urea and ammonia content assays

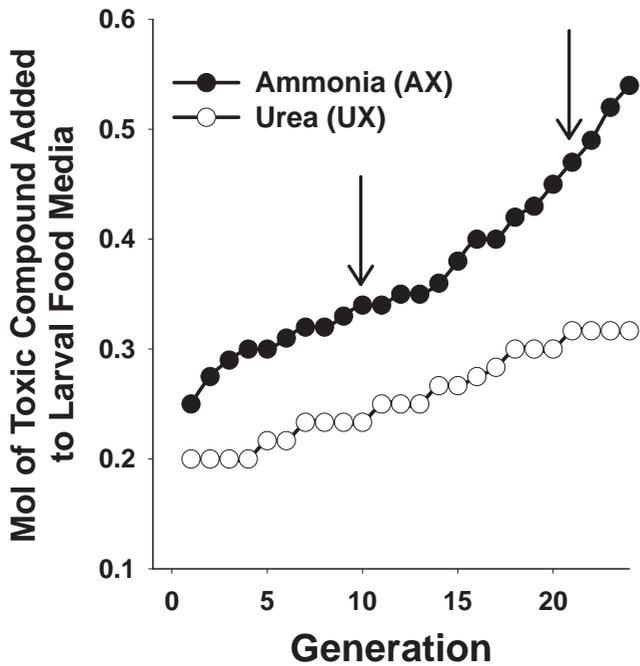Three groups of ten third-instar larvae were pooled from each experimental group in order to measure

Fig. 2.   Increase in environmental levels of ammonia and urea during selection. The levels of supplemental nitrogenous material in the larval culture were increased whenever it appeared that a large majority of adults were surviving the exposure to either ammonia or urea during the larval phase. Arrows indicate generations at which experiments were performed.

whole-body urea levels. Larvae were weighed and homogenized in 500 μl of 0.16 M TrisCl (pH 7.6) in a microcentrifuge tube using a glass pestle. Homogenates were centrifuged and the supernatant removed for analysis. The urea and ammonia contents were determined using enzyme-based endpoint assays that measured the oxidation of NADH at 340 nm (Mondzac et al., 1965). The urea assay also detects ammonia, so ammonia values were subtracted from the urea assays to calculate urea content. The reaction mixture for the urea assay consisted of 0.1 M phosphate buffer, pH 8.0, 2 mM EDTA, 37 mM α-ketoglutarate, 0.32 mM NADH, 5.4 U urease and supernatant. The ammonia assay reaction mixture was identical, except that urease was omitted. Blanks consisted of the reaction mixtures without any added samples. Control solutions of 294 μM ammonia and 2 mM urea were assayed along with samples. Absorbance of samples was measured at 340 nm on a Molecular Devices microplate reader. The reaction was initiated by the addition of 6 U GLDH and allowed to proceed for 1 h at room temperature. After 1 h, absorbance at 340 nm was measured again, and the difference, after subtraction of blank values, was used to calculate ammonia or urea content. All values are expressed per mg larval wet weight. All reagents were purchased from Sigma Chemical Co. or Boehringer-Mannheim.

1.3. Developmental time and viability

From each replicate of each selection regime, exactly 60 eggs, which were laid over a 5-h period, were collected on a piece of non-nutritive agar, and placed into an 8-dram vial with 5 ml banana-molasses food supplemented with one of the following treatments: 0.35 M NH_4Cl, 0.266 M urea, or no supplements added to the food. Each condition was replicated eight times for each of the 15 populations.

Every 8 h from the start of adult emergence, flies were removed from the vials, using CO_2 anesthesia, and the time and gender of each fly were recorded. Checks continued every 8 h until >90% of the adults reared as larvae on plain food had emerged. As development was significantly slower and the number of surviving adults was considerably less on both ammonia and urea supplemented foods, checks were performed every 12 h for approximately the next 10 days. After a period of 48 h, in which no flies had emerged from a given population treatment, checks ceased for that population treatment.

1.4. Fecundity

Several groups of 60–80 adults, culled from the peak period of eclosion during the developmental time assay, were placed into fresh food vials with a liberal amount of live yeast paste smeared on the side of the vial. The adults were transferred after 2 days to a new vial with fresh yeast paste. After 4 days of conditioning on food supplemented with live yeast, a single male and female were placed into a vial containing charcoal food for egg laying. Each treatment was replicated 20 times for each of the 15 populations. After 24 h, the adults were discarded and the eggs in each vial were counted.

1.5. Statistics

1.5.1. Ammonia and urea contents
Variables were log-transformed as necessary to meet the assumptions of normality and homoscedasticity of analysis of variance (ANOVA). Three-way ANOVA on population means were used to examine the effects of selection treatment, food type, population and the interaction of these terms on the traits measured. Tukey's HSD tests were performed to make post hoc comparisons among groups. All data are presented as means of $n=5$ populations ± 1 standard deviation. All analyses were performed using Minitab v10 or SYSTAT for Windows.

1.5.2. Fitness-related traits
SAS for Windows, version 6.0.8 was used to perform ANOVA to determine the significant effects. The selection regime (AUC, AX, and UX) and larval food condition (plain, 0.35 M NH_4Cl, and 0.266 M urea) were

treated as fixed effects. The selection regime replicate was treated as a random effect, because of the common origin of the selection regimes from the UU populations. Viability data were arc-sin transformed prior to analysis. Multiple comparisons were performed using the Tukey–Kramer and/or Scheffe methods.

2. Results

2.1. Effect of food type and selection treatment on larval ammonia and urea content

The type of food fed to the larvae and the population of origin affected larval ammonia content (three-way ANOVA, $P<0.001$ and $P=0.001$, respectively). There was no effect of selection treatment on ammonia content (Fig. 3). The larvae fed on 0.37 M ammonia food had about three times more ammonia than those fed on normal food. When fed on 250 mM urea food, all populations had ammonia levels similar to those of the ammonia food.

The type of food the larvae were reared on and the selection history of the population affected larval urea content (three-way ANOVA, $P=0.002$ for selection treatment, $P<0.001$ for food type and food type × selection

treatment interaction). On normal food and ammonia food, all populations contained less than 1 nmol/mg of urea. All populations had significant amounts of urea in their bodies when reared on urea food. However, the urea-selected populations had less urea than the control and ammonia-selected populations (82.54 ± 20.6 nmol/mg in the urea-selected larvae versus 104 ± 20.7 nmol/mg and 106 ± 17.3 nmol/mg in the control and ammonia-selected larvae, respectively). Post hoc tests indicated that control and ammonia-selected larvae had significantly more urea in their bodies than urea-selected larvae when they were reared on 250 mM urea food ($P<0.001$).

2.2. Developmental time

After ten generations of selection, AUC, AX, and UX lines developed from egg to adult at the same rate when reared on plain food. When reared as larvae on urea-supplemented media, females (Fig. 4A) and males (Fig. 4B) from the UX lines eclosed significantly faster than the AX lines, which were significantly faster than the AUC lines. When reared as larvae on ammonia-supplemented media, females (Fig. 4A) and males (Fig. 4B) from the AX lines eclosed significantly faster than the UX lines, which were significantly faster than the AUC lines. Similar results were obtained after 21 generations of selection. Each selection regime developed fastest when reared as larvae on the food medium to which they had been selected (e.g. UX on urea-supplemented food), while expressing incomplete cross-tolerance when reared on the other supplemented media.

2.3. Viability

After ten generations of selection, both selection regimes showed no egg-to-adult viability differences compared to the unselected controls when assayed on plain food (Fig. 5). As expected, the UX lines showed a much higher viability than either the AX or AUC lines when reared on urea food. In addition, the AX populations were superior to the AUC populations when reared on urea food. On ammonia food, the AX lines showed greater viability than either the UX or AUC lines when reared on ammonia food, with the UX populations possessing a superior egg-to-adult survivorship than the AUC populations. Similar trends were also seen in the generation 21 assay.

2.4. Fecundity

At generation ten, no differences were seen in female fecundities between either of the selection regimes and the control populations when larva had been reared on either plain food or ammonia-supplemented food. The UX populations had a superior fecundity when reared on urea food compared to either the AX or AUC popu-

Larva reared on:
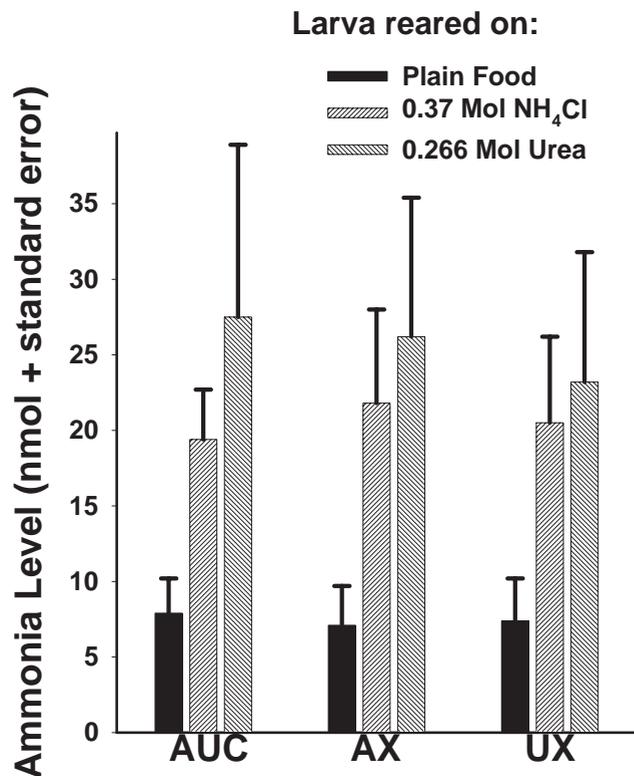
■ Plain Food
▨ 0.37 Mol NH₄Cl
▧ 0.266 Mol Urea

Fig. 3. Ammonia levels of larvae reared on different food types. Third-instar larvae reared on ammonia- or urea-supplemented food had higher ammonia concentrations than larvae reared on normal food. Data are means of $n=5$ populations (+SE).
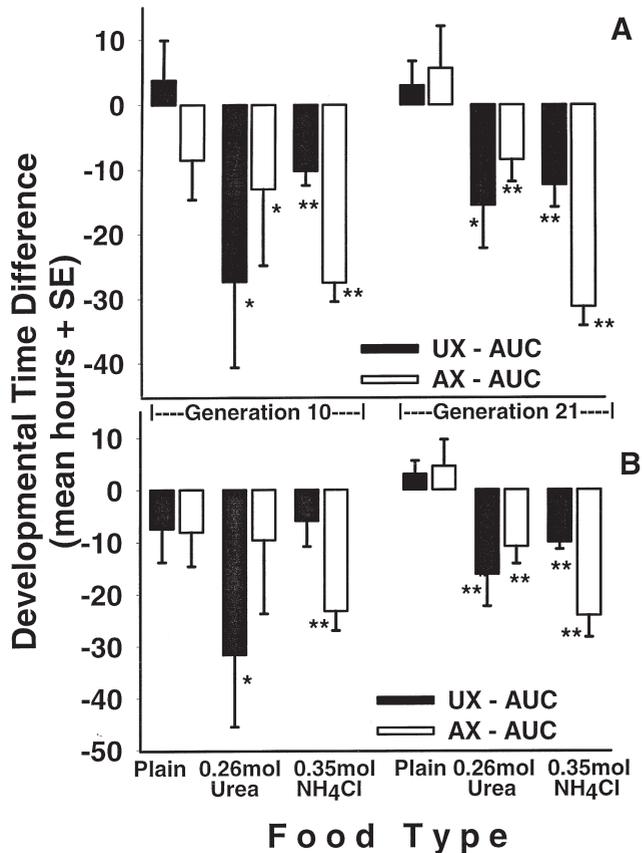
Fig. 4.   Effects of larval exposure to ammonia and urea on developmental time. (A) Female developmental time. (B) Male developmental time. All populations were measured on one of three food types: (1) plain banana food, (2) banana food supplemented with 0.266 mM urea, or (3) banana food supplemented with 0.350 mM ammonium chloride. The negative time units on the y-axis denote the mean difference in developmental time between five pairs of selected populations (either AX or UX) and their respective AUC control populations. A greater negative value signifies that selected flies develop faster on the particular food type assayed. The error bars around the mean are standard errors of the five replicate populations comprising each selection regime. *$P<0.05$, **$P<0.01$.
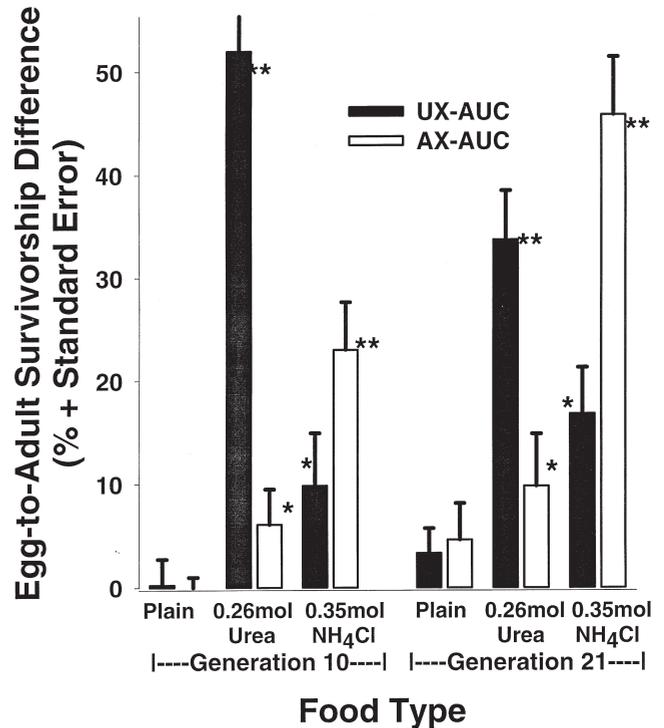

Fig. 5.   The impact of larval exposure to ammonia and urea on egg-to-adult survivorship. Survivorship was measured on one of three food types: (1) plain banana food, (2) banana food supplemented with 0.266 mM urea, or (3) banana food supplemented with 0.350 mM ammonium chloride. The positive percentage units on the y-axis denote the mean difference in viability between five pairs of selected populations (either AX or UX) and their respective AUC control populations. A greater positive value signifies a superior viability of the nitrogenous compound tolerant population, relative to that of the AUC controls, on that particular food type assayed. The error bars around the mean are standard errors of the five replicate populations comprising each selection regime. *$P<0.05$, **$P<0.01$.

lations. The AX populations did not display a significant cross-tolerance for female fecundity when reared as larvae on either ammonia- or urea-supplemented food media. Similar results were observed in the generation 21 assay (Fig. 6).

3. Discussion

Studies of resistance to toxic chemicals, such as pesticides, have usually considered compounds which target a specific enzyme or biological process. Urea and ammonia differ from these in their wide-ranging effects on organismal and cellular physiology (Somero and Yancey, 1997). Previous work has shown that *Drosophila* populations can evolve resistance to high levels of environmental urea (Shiotsugu et al., 1997). Our goal in

this study was to determine whether *D. melanogaster* could also adapt to high levels of ammonia, and whether selection for resistance to one compound could result in resistance to the other. This would suggest that some mechanisms of chemical resistance may confer broad tolerance to many toxins.

3.1. Ammonia and urea contents

Larval ammonia content shows different patterns among the food types and probably reflects differences in nitrogen excretion. *Drosophilia* larvae excrete ammonia and thus should have physiological mechanisms in place to excrete excess ammonia. Even on 0.37 M ammonia chloride food, internal concentrations of ammonia are less than one-tenth of those of the external medium (Fig. 3). In addition, comparably high ammonia levels occur when the larvae are reared on urea food relative to the levels of ammonia detected when larvae are reared on plain food. These results, plus the lack of differentiation between the AX and other populations,
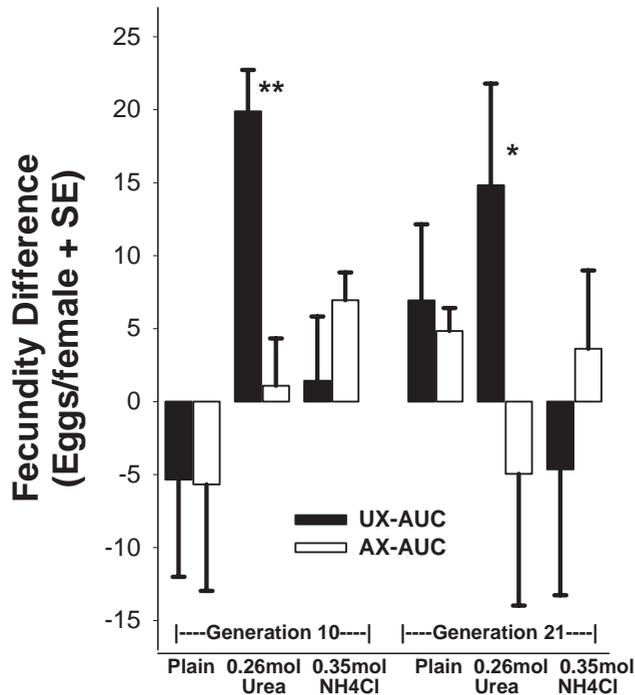
Fig. 6. The impact of larval exposure to ammonia and urea on female fecundity. Fecundity was measured on one of three food types: (1) plain banana food, (2) banana food supplemented with 0.266 mM urea, or (3) banana food supplemented with 0.350 mM ammonium chloride. The units on the *y*-axis denote the mean difference between five pairs of selected populations (either AX or UX) and their respective AUC control populations. A greater positive value signifies a superior fecundity of the nitrogenous compound tolerant population, relative to that of the AUC controls, on that particular food type assayed. The error bars around the mean are standard errors of the five replicate populations comprising each selection regime. *$P<0.05$.

suggest that the elevated ammonia levels of larvae fed on ammonia food reflect general disruptions of homeostasis, rather than an accumulation of ammonia because of a strong gradient.

In contrast to ammonia, urea is probably a novel compound for the larvae. They do not produce it, nor are they likely to encounter it in their environment. Thus, the larvae may lack physiological mechanisms to specifically handle urea. While urea is nearly indetectable in the larvae fed on normal or ammonia food, urea accumulates to high levels when the larvae are reared on 250 mM urea food. The UX larvae have significantly less urea than the other populations, indicating that adaptation to the urea food has involved physiological changes that result in lower steady-state urea levels. Pierce et al. (1999) found that even greater differences in ammonia and urea content occurred in populations which had been selected for extreme levels of urea for >100 generations (see Shiotsugu et al., 1997, for a description of these populations). Thus, at least some of the mechanistic bases of urea and ammonia adaptation differ. Whether the cross-tolerance observed in the differently-selected populations is because of genetic corre-

lations between these different stress traits, or because these different mechanisms are able to serve as general stress tolerance mechanisms, remains to be determined.

3.2. Analysis of fitness-related traits

All populations were removed from selection for two generations prior to the experiments, thereby eliminating non-genetic artifacts, such as maternal and environmental effects, from confounding the results. Thus, phenotypic differences between populations reflect genetic differences that have arisen due to the selection regimes. The UX populations displayed higher viability, faster developmental time, and greater adult female fecundity when reared on food supplemented with urea, compared to the other populations reared, as larvae, on urea. The AX populations also displayed higher viability and faster developmental time when reared, as larvae, on food supplemented with ammonium chloride, compared to the other populations. However, the AX populations were equal to the UX populations and the AUC unselected controls, in terms of female fecundity, when they were reared on food supplemented with ammonium chloride.

A potential factor in our experiments was variation in larval crowding caused by greater mortality of larvae reared in the presence of ammonia and urea. In populations which typically experience a crowded larval culture, those adults which emerge later from culture experience higher levels of ammonia as larvae (Borash et al., 1998). This later-emerging subpopulation also shows correspondingly greater viability when reared on food supplemented with either ammonia or urea. We do not feel that larval density effects were a factor in our experiments for two reasons: (1) urea typically causes mortality during the pupal stage and would not affect larval densities; (2) larval densities were relatively low under all conditions (no more than 60–80 per vial in control treatments). This is an order of magnitude lower than the densities required to produce detectable life-history evolution, so that all treatment groups would have been uncrowded (Mueller et al., 1993).

While larval traits (developmental time and viability) seemed heavily differentiated by the selection regime, the adult characteristic examined (fecundity) was not differentiated in AX populations reared on any of the three food types. As fecundity is generally a character under strong selection, it is possible that only the UX adults recover from their larval exposure to urea.

3.3. Cross-tolerance to toxic substances

The AX populations displayed partial cross-tolerance in terms of egg to adult survivorship and developmental time, when they were reared on urea-supplemented food. Additionally, the UX lines show cross-tolerance relative to the controls in terms of larva to adult survivorship,

developmental time, and female fecundity, when reared on ammonia-supplemented food. This result suggests that the genes controlling the ability to tolerate one compound also exert some pleiotropic control over the tolerance to other toxic compounds. Traits such as egg-to-adult survivorship, developmental time, and fecundity, like many life history characteristics, are quantitative traits that are genetically correlated (Chippindale et al. 1994, 1996. The response to selection for increased toxic compound tolerance may be restrained due to the genetic correlations between characters. While most chemicals that are toxic (i.e. pesticides) to an organism tend to affect one or a few targets (Russell et al., 1990; Morton, 1993), urea and ammonia are globally detrimental to the organism. Thus, populations must evolve to withstand the many damaging effects of these compounds. Tolerance to toxic compounds is governed by a suite of genes conveying tolerance to toxic substances rather than the particular compound the organism was originally under selection to tolerate. Furthermore, tolerance may involve a process of a shared physiological mechanism responding to selection (e.g. decreases in cuticular or membrane permeability). We have already initiated a more detailed examination of the physiological basis of tolerance in these populations (Pierce et al., 1999; David et al., 1999). These populations may also serve as a model system to study the mechanisms of adaptation to pollution and toxic compounds.

Acknowledgements

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