

## OSMOREGULATION IN *DROSOPHILA MELANOGASTER* SELECTED FOR UREA TOLERANCE

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### Summary

Animals may adapt to hyperosmolar environments by either osmoregulating or osmoconforming. Osmoconforming animals generally accumulate organic osmolytes including sugars, amino acids or, in a few cases, urea. In the latter case, they also accumulate 'urea-counteracting' solutes to mitigate the toxic effects of urea. We examined the osmoregulatory adaptation of *Drosophila melanogaster* larvae selected to live in 300 mmol l<sup>-1</sup> urea. Larvae are strong osmoregulators in environments with high NaCl or sucrose levels, but have increased hemolymph osmolarity on urea food. The increase in osmolarity on urea food is smaller in the selected larvae relative to unselected control larvae, and their respective hemolymph urea concentrations can account for the observed increases

in total osmolarity. No other hemolymph components appear to act as urea-counteractants. Urea is calculated to be in equilibrium across body compartments in both selected and control larvae, indicating that the selected larvae are not sequestering it to lower their hemolymph osmolarity. The major physiological adaptation to urea does not appear to involve increased tolerance or improved osmoregulation *per se*, but rather mechanisms (e.g. metabolism, decreased uptake or increased excretion) that reduce overall urea levels and the consequent toxicity.

Key words: *Drosophila melanogaster*, urea, osmolarity, osmoregulation, hyperosmotic stress, haemolymph.

### Introduction

Hyperosmotic environments create osmotic pressure, favoring the movement of water out of the animal. This, in turn, can cause cell shrinkage and disturb many essential cellular processes. Sea water is the best known hyperosmotic environment, containing high levels of sodium chloride, but other environments can also be hyperosmotic. Some lakes and ponds contain high levels of other inorganic salts. Microbes may encounter high osmolarities in the tissues of their host, and plant nectaries can have significantly higher osmolarities than the hemolymph of the insects that live in them (Nicolson, 1994, 1998).

Studies of animals in saline environments have produced two models of how animals meet the osmoregulatory challenge of a hyperosmotic environment. Some animals osmoregulate, maintaining relatively constant cellular and blood (or hemolymph) concentrations, even against large osmotic gradients. Hyporegulating animals, including marine teleosts, brine shrimp *Artemia salina* and saline-water mosquitoes, compensate for the osmotic loss of water by drinking the external medium. Excess salts are then excreted through the gills in marine organisms or secreted into the urine in the insect rectum (Bradley, 1987; Holliday et al., 1990; Kirschner, 1993).

An alternative strategy is to osmoconform to the external environment (Kinne, 1993; Somero and Yancey, 1997).

Organisms that use this strategy usually accumulate one or more small organic osmolytes, such as zwitterionic amino acids or polyhydric alcohols (Burton and Feldman, 1982; Yancey et al., 1982; Kinne, 1993). These organic osmolytes are used because they are relatively non-perturbing to cellular processes, even at high concentrations (Yancey et al., 1982; Somero and Yancey, 1997). Sharks and other cartilaginous fishes accumulate a perturbing osmolyte, urea, in order to osmoconform to sea water (Smith, 1936; Yancey et al., 1982). Urea also accumulates in tissues of other animals, including mammals and frogs, and consequently in the microorganisms in these animals (Chambers and Kunin, 1985; Somero and Yancey, 1997). These organisms also possess 'urea-counteracting solutes' [including trimethyl amine oxide (TMAO), glycerophosphoryl choline (GPC), glycine betaine and some polyol sugars] that contribute to osmolarity and may mitigate the toxic effects of urea (Somero and Yancey, 1982; Lin and Timasheff, 1994).

Organic osmolytes are found intracellularly in most animals, while the extracellular fluid may be high in salts or, in the case of many marine invertebrates, reflect the composition of the environment (Gilles, 1979; Bagnasco et al., 1986; Yancey, 1988; Lien et al., 1993; Wright and Purcell, 1997). In contrast, elasmobranchs and brackish-water

mosquitoes also accumulate organic osmolytes in their blood and hemolymph (Yancey et al., 1982; Garrett and Bradley, 1987; Kirschner, 1993). It has been proposed that this allows control of the chemical composition of the extracellular fluid in nonmarine environments where ion composition and ratios may differ from those of sea water (Garrett and Bradley, 1987).

We are studying the physiological basis of adaptation to hyperosmotic stress in laboratory populations of the fruit fly *Drosophila melanogaster* selected for tolerance of 300 mmol l<sup>-1</sup> urea in their larval food (Joshi et al., 1996). This experimental system mimics natural aqueous environments because *D. melanogaster* larvae live in a semi-aqueous closed environment (individual rotting fruit in the wild and food-containing vials in the laboratory). They cannot use behavioral avoidance to escape the effects of a new compound, including any resulting osmotic stress, much as microorganisms must contend with their environment in a host and marine organisms cannot avoid the ocean's osmolarity.

For the laboratory selection approach to be successful, the stressor used should be novel or extreme (fatal to a fraction of the population) so that adaptations evolve during the course of selection. Urea is almost certainly a novel chemical for *D. melanogaster* larvae since they neither produce it as nitrogenous waste nor are likely to encounter it in fruit (Borash et al., 1998). Therefore, normal *D. melanogaster* are most likely to lack any adaptations to cope with urea, and selection for urea tolerance should produce detectable changes in the selected populations.

Using a laboratory selection approach allows us to avoid any ambiguities involved in assigning adaptive values to differences in interspecific comparisons or to responses to acutely altered environments (Garland and Adolph, 1994; Garland and Carter, 1994). For example, changes in organic osmolyte levels in the rat brain during hypernatremia parallel those seen in the kidney, but the changes are much smaller, of the order of a few millimolar. While some have proposed that this is adaptive, others have argued that such small changes would not be important (Lien et al., 1990; Somero and Yancey, 1997). In the laboratory selection system, we have control populations of *D. melanogaster* originating from the same ancestral stock that have not been exposed to urea. By comparing the responses of the control and selected populations to acute urea exposure, physiological responses that have evolved due to urea selection can be distinguished from those responses that existed *a priori* and may not be specifically adapted for urea tolerance. Thus, we define an adaptation to urea as a response that occurs in all the selected populations but is absent from their control populations.

This paper focuses on the osmotic aspects of urea exposure, and work currently in progress is examining adaptations to the toxic effects of urea such as denaturation and damage to proteins (Somero and Yancey, 1997). Our experiments were designed to examine the osmotic consequences of an osmoregulatory adaptation to hyperosmotic stress caused by urea exposure. We measured the hemolymph osmolarity of

larvae reared in different hyperosmotic environments to investigate whether the nature of the external osmolyte influenced their osmoregulatory response, and asked whether adaptation to urea granted enhanced tolerance to other hyperosmotic media. We analyzed the hemolymph and total body levels of urea to determine whether it was present in the larvae. We examined whether any of the normal components of *D. melanogaster* hemolymph (amino acids, inorganic ions and trehalose) demonstrated urea-counteraction by assessing whether their concentrations changed in the presence of urea and whether the magnitude of the change differed between control and selected populations. We analyzed the osmolarity and composition data for changes in osmolarity in the selected populations that were not accounted for by these normal components and would be suggestive of a novel osmolyte being accumulated.

## Materials and methods

### *Experimental populations and selection protocol*

In 1992, five outbred baseline (B) populations of *Drosophila melanogaster*, derived from an ancestral population (Ives), were each split into two populations, one of which was exposed to urea during the larval period ('selected', MX), while the other was fed standard culture food ('control', UU; Joshi et al., 1996). Thus, each experimental population is more closely related to a control population founded from the same base population than to the other experimental populations (Fig. 1A). This design results in fivefold replication in the comparison between selected and control populations. The two major forces that may lead to differentiation of populations are random drift, including founder effects, and natural selection. Since drift is a random force, it is unlikely to produce the same type of genetic differentiation multiple times. Thus, strong but inconsistent differences among our replicates would suggest drift as the driving force. Natural selection in large populations is deterministic and thus should produce consistent genetic differentiation among replicate populations (Rose, 1984; Rose et al., 1996).

All populations were maintained at 25 °C on a 24 h light regime, with generation times of approximately 2 weeks. The selected populations were reared at low density on banana-molasses medium with urea added. Adults were maintained in cages with normal banana-molasses food and were not exposed to urea. Control flies were raised under an identical regime, except that larvae were fed normal food (Fig. 1B). Initially, larvae from the selected populations were reared in 200 mmol l<sup>-1</sup> urea food. At generation 5, the concentration was increased to 233 mmol l<sup>-1</sup>, then to 283 mmol l<sup>-1</sup> at generation 15 and to 300 mmol l<sup>-1</sup> at generation 25. 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 in which they are placed (Rose et al., 1996).

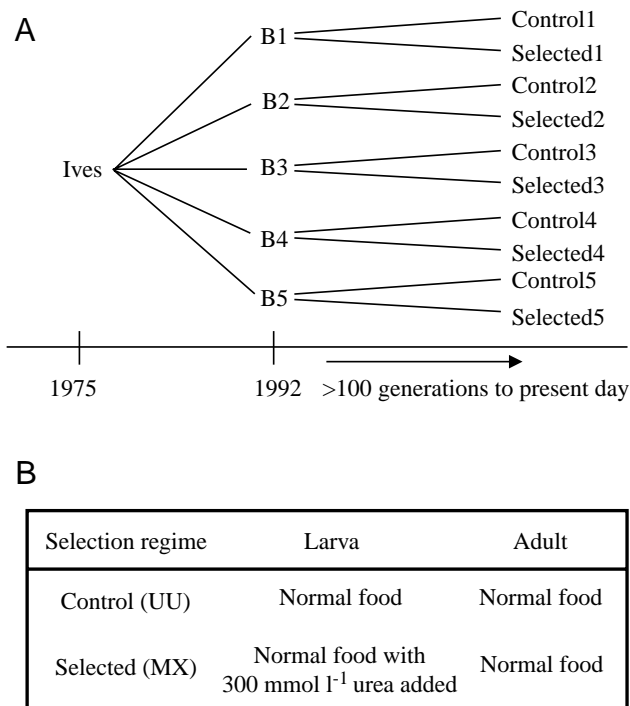


Fig. 1. Relationships of study populations and selection protocol. (A) Genetic relationships of selected (MX) and control (UU) populations. Each pair of populations sharing the same number is descended from the same B population of 1992. Thus, the five selected populations have evolved independently. (B) Selection protocol for urea resistance. Selected populations were fed 200 mmol l<sup>-1</sup> urea initially; this amount was increased to 300 mmol l<sup>-1</sup> over 20 generations. They have been maintained on 300 mmol l<sup>-1</sup> urea for over 100 generations.

The selected flies have been reared on urea food for over 100 generations. Larvae in the urea-adapted populations can develop into adults on food containing 300 mmol l<sup>-1</sup> urea while normal larvae grow and pupate on this food but fail to eclose into adults (Shiotsugu et al., 1997). The egg-to-adult viability on 300 mmol l<sup>-1</sup> urea food is nearly five times higher in the selected populations than in their controls. 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.

#### Experimental design and statistical analysis

Prior to experimentation, the selected and control populations were raised under identical, non-selective conditions to remove parental and grandparental effects. The experimental generation consisted of four groups, each replicated fivefold: control populations reared on normal and 300 mmol l<sup>-1</sup> urea food, and urea-selected populations reared on normal and 300 mmol l<sup>-1</sup> urea food. All measurements were performed on third-instar wandering larvae, the stage just prior to pupation. Two-way analyses of variance (ANOVAs) on population means were used to examine the effects of selection treatment and food type 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  $\pm$  s.d. of ( $N=5$ ) populations. All analyses were performed using Minitab v10 or SYSTAT for Windows.

#### Urea content assays

Four groups of ten larvae were pooled from each experimental group for measurement of whole-body urea levels. Larvae were homogenized in 500  $\mu$ l of 160 mmol l<sup>-1</sup> Tris-Cl, pH 7.6, in a microcentrifuge tube using a glass pestle. Homogenates were centrifuged, and the supernatant removed for analysis. Urea and ammonia contents were determined using an enzyme-based endpoint assay that measures the oxidation of NADH at 340 nm (Mondzac et al., 1965). Ammonia and urea assays on the samples were performed separately, and ammonia values were subtracted from the urea assays to determine urea content. The reaction mixture for the urea assay consisted of 100 mmol l<sup>-1</sup> K<sub>2</sub>HPO<sub>4</sub> buffer, pH 8.0, 2 mmol l<sup>-1</sup> EDTA, 30 mmol l<sup>-1</sup>  $\alpha$ -ketoglutarate, 0.15 mmol l<sup>-1</sup> NADH, 6.8 units of urease and supernatant. The ammonia assay reaction mixture was identical, except that urease was omitted. Blanks consisted of the reaction mixtures with buffer added. Control solutions of 294  $\mu$ mol l<sup>-1</sup> ammonia and 2 mmol l<sup>-1</sup> urea were assayed along with samples. Initial absorbances of samples were measured at 340 nm on a Perkin-Elmer spectrophotometer. The reaction was initiated by the addition of 6 units 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 levels. Values are expressed as nmol mg<sup>-1</sup> larval wet mass. 1 unit converts 1  $\mu$ mole of substrate to product per minute at 25 °C at pH 7.3.

For measurements of hemolymph urea and ammonia levels, hemolymph samples from ten larvae were collected and pooled, diluted 50-fold in 160 mmol l<sup>-1</sup> Tris-Cl, pH 7.6, and assayed using the same procedure as for homogenates. All reagents were purchased from Sigma Chemical Co. and Boehringer-Mannheim.

#### Water content measurements

Four replicates of ten third-instar wandering larvae were collected for each experimental group. Larvae were briefly rinsed and blotted dry. They were placed in pre-weighed microcentrifuge tubes, immediately weighed and placed in a drying oven for several days to 1 week. Larvae were then weighed again and the dry mass subtracted from the wet mass to determine the amount of water in the samples. Water content is expressed as the percentage of wet mass consisting of water.

#### Hemolymph and food osmolarity measurements

Hemolymph was collected from individual third-instar wandering larvae under a dissecting microscope. A larva was placed on Parafilm and the cuticle was torn with fine forceps. As hemolymph leaked out, it was immediately collected using a pulled microcapillary tube containing oil. Under these

conditions no clotting of the hemolymph was observed. Approximately 5 nl of hemolymph were suspended in oil on a temperature-controlled sample plate under a microscope. The osmolarity of the samples was determined by measuring the melting point depression using a nanoliter osmometer (Clifton, Rochester, NY, USA; Garrett and Bradley, 1987). Eight individuals were assayed from each population on both normal and urea food (four experimental groups; total 160 larvae). Each sample was measured in triplicate, along with standards.

To test the effect of rearing on foods containing other osmolytes, one control and one selected population were reared on normal food and food containing 300 mmol l<sup>-1</sup> sucrose or 300 mmol l<sup>-1</sup> NaCl. The hemolymph osmolarity of seven larvae from each population on each food type were assayed as described above (total 42 larvae).

Osmolarity values of normal food and food containing 300 mmol l<sup>-1</sup> urea, 300 mmol l<sup>-1</sup> sucrose or 300 mmol l<sup>-1</sup> NaCl were determined using vapor pressure osmometry. Food samples were collected from vials using a spatula, smeared onto filter paper discs and their osmolarity quickly measured using a Wescor 5100C vapor pressure osmometer (Garrett and Bradley, 1987).

#### Ion measurements

Hemolymph (1 µl) was collected from pooled hemolymph of 6–8 larvae and immediately diluted with 1 ml of 3.97 g l<sup>-1</sup> KCl for Na<sup>+</sup> measurement, 4 ml of 1.27 g l<sup>-1</sup> CsCl for K<sup>+</sup> measurement or 1 ml water for Cl<sup>-</sup> measurement. Samples were stored in sealed tubes at 4 °C until assayed, either on the same day as collection or the following day. Na<sup>+</sup> and K<sup>+</sup> concentrations were determined by measuring absorbance with a Varian AA-275 atomic absorption spectrophotometer (Garrett and Bradley, 1987). While flame photometric measurements include any K<sup>+</sup> sequestered in hemocytes, Stewart et al. (1994) reported similar values using an ion-selective electrode technique, suggesting that the contribution of hemocyte K<sup>+</sup> to total K<sup>+</sup> levels is small in *D. melanogaster*. Cl<sup>-</sup> concentrations were determined by mixing hemolymph samples with a ferric nitrate/mercuric isothiocyanate solution and measuring absorbance at 560 nm on a Perkin-Elmer spectrophotometer (Gonzalez et al., 1998). Three samples from each population in each experimental group were assayed for Na<sup>+</sup> and K<sup>+</sup> concentration and four were assayed for Cl<sup>-</sup> concentration.

#### Amino acid determinations

Hemolymph (1 µl) was collected from 6–8 pooled larvae as above. Samples were diluted with 250 µl of 80% ethanol, centrifuged and 100 µl of supernatant was shipped to Dr Audree Fowler at the UCLA School of Medicine for analysis. The samples were lyophilized, derivatized with phenylisothiocyanate and analyzed on a reverse-phase column (Novapak) using a sodium acetate/acetonitrile gradient (Cohen and Strydom, 1988). Sample peaks were compared with peaks of known amino acid standards for identification and determination of concentration. One sample was collected from each population in each experimental group.

#### Trehalose measurements

Hemolymph (2 µl) was collected from 8–10 pooled larvae as above. Samples were suspended in mineral oil and stored at -70 °C until assayed. Samples were thawed on ice, 1 µl was withdrawn and diluted with 3 µl of distilled water. A sample of the dilution (1 µl) was incubated overnight at room temperature (25 °C) with 100 µl of amyloglucosidase (0.8 mg ml<sup>-1</sup>) in distilled water to hydrolyze the trehalose to glucose (Parrou and Francois, 1997). Glucose concentration was measured using glucose assay kit 510A from Sigma Chemical Co, St Louis, MO, USA. Trehalose and glucose standards were used to quantify trehalose concentrations in the hemolymph samples. One sample was collected from each population in each experimental group.

## Results

### Effect of food type and selection treatment on larval urea content

The type of food the larvae were reared on and the selection history of the population affected larval urea content (two-way ANOVA,  $P < 0.001$  for food type, selection treatment and food type  $\times$  selection treatment). On normal food, control and selected populations contained equivalent amounts of urea ( $6.0 \pm 0.3$  nmol urea mg<sup>-1</sup> larva and  $5.6 \pm 0.3$  nmol urea mg<sup>-1</sup> larva, respectively; Tukey's HSD test,  $P = 0.459$ ; Fig. 2). When reared on 300 mmol l<sup>-1</sup> urea food, control larvae contained  $125.6 \pm 6.5$  nmol urea mg<sup>-1</sup> larva, while selected larvae contained significantly less urea,  $79.1 \pm 7.3$  nmol mg<sup>-1</sup> larva (Tukey's HSD test;  $P < 0.001$ ; Fig. 2).

No urea was detectable in the hemolymph of either control or selected larvae reared on normal food. When reared

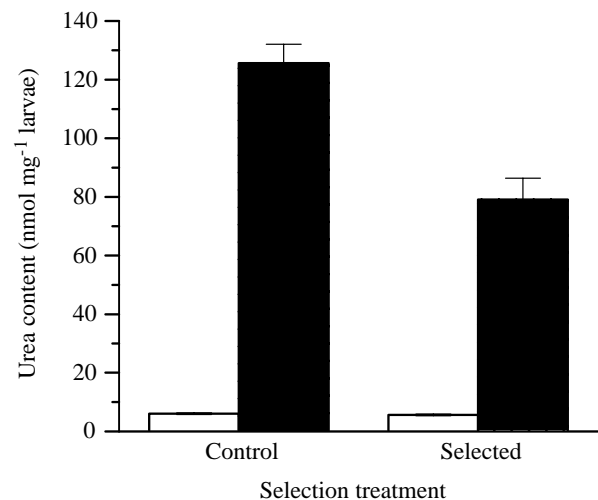


Fig. 2. Effect of food type and selection regime on larval urea content. Larvae reared on normal food do not contain significant levels of urea (open columns). When *D. melanogaster* larvae are reared on food containing 300 mmol l<sup>-1</sup> urea, they do not exclude urea from their bodies (filled columns). However, urea-resistant larvae have lower levels of urea than the controls. Values are means  $\pm$  s.d. ( $N = 5$  populations).

Table 1. *Effect of selection and food type on hemolymph composition*

Food type		Control larvae		Selected larvae	
		Normal food	Urea food	Normal food	Urea food
Ions (mmol <sup>-1</sup> )	Na <sup>+</sup>	51.1±5.7	53.2±6.8	53.8±7.4	52.4±8.8
	K <sup>+</sup>	51.8±9.4	52.8±11.8	48.5±7.4	48.8±4.1
	Cl <sup>-*</sup>	56.1±10.5	49.3±6.6	56.1±8.2	51.1±7.9
Organic osmolytes (mmol <sup>-1</sup> )	Amino acids	57.1±6.1	69.1±6.2	60.0±10.3	64.3±12.2
	Trehalose*	75.3±3.8	88.7±12.5	86.9±7.5	93.6±10.8
	Urea*	–	180.8±9.2	–	102.6±15.9
Sum		291.4	493.9	305.3	413.8

Larvae were reared on normal food or food + 300 mmol<sup>-1</sup> urea.

Values are means ± s.d. *N*=5 populations.

\*Significant effect of food type on concentration of osmolyte (ANOVA; *P*<0.001 for urea, *P*<0.003 for Cl<sup>-</sup>, *P*=0.028 for trehalose).

on 300 mmol<sup>-1</sup> urea food, selected larvae contained 102.6±15.9 mmol<sup>-1</sup> urea in their hemolymph, while control larvae had significantly more urea, 180.8±9.2 mmol<sup>-1</sup> (ANOVA; *P*<0.001; Table 1).

#### *Effect of selection and food type on water content*

Selection history and food type significantly affected larval water content (ANOVA; selection history, *P*=0.011; food type, *P*=0.002; interaction, *P*=0.001), although the magnitude of the changes was small. Control larvae contained 78.4% water when reared on normal food but 76.2% water when reared on urea food. Selected larvae had 78.1% water content, regardless of food type (data not shown).

#### *Effect of food type and selection treatment on food and hemolymph osmolarity*

Addition of 300 mmol<sup>-1</sup> urea, sucrose or NaCl significantly increased food osmolarity (Fig. 3; ANOVA, *P*<0.001). Addition of sucrose or NaCl to food did not affect hemolymph osmolarity (Fig. 3; *P*>0.16 for the effects of all factors). Hemolymph osmolarity was significantly affected by selection treatment and the presence or absence of 300 mmol<sup>-1</sup> urea in the food (two-way ANOVA; *P*<0.001 for all terms). On normal food, the osmolarities of the control and selected flies were 390±5.5 mosmol<sup>-1</sup> and 396±15.9 mosmol<sup>-1</sup>, respectively (Fig. 3). Both populations had significantly higher hemolymph osmolarity when reared on urea food (Tukey's HSD test; *P*<0.001). The control population on urea food had a higher osmolarity (612±28.5 mosmol<sup>-1</sup>) than the selected larvae (513±35.2 mosmol<sup>-1</sup>) under the same conditions (Tukey's HSD test; *P*<0.001; Fig. 3).

#### *Effect of food type and selection treatment on hemolymph ion concentrations*

Hemolymph Na<sup>+</sup> and K<sup>+</sup> concentrations were unaffected by either selection treatment or food type (Table 1; two-way ANOVAs; *P*>0.07 for all factors for both cations). Hemolymph Cl<sup>-</sup> concentrations were significantly lower on urea food in both selection treatments (Table 1; two-way ANOVA; *P*<0.003).

#### *Effect of food type and selection treatment on hemolymph amino acid composition*

There was no significant effect of food type on the total free amino acid (FAA) pool in the hemolymph, although both control and selected larvae tended to have a higher total amino acid concentration on urea food compared to normal food (*P*=0.064; Table 1). Thirteen amino acids were detected in the hemolymph (in order of most to least abundant: glycine, proline, histidine, alanine, threonine, serine, arginine,

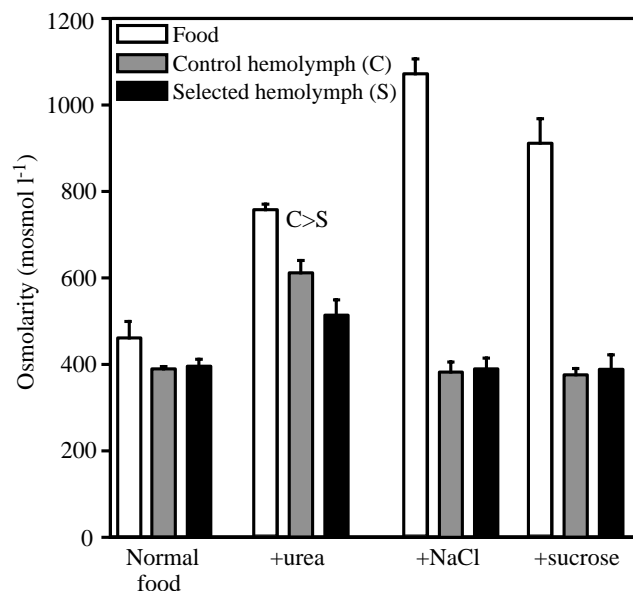


Fig. 3. Hemolymph osmolarity depends on food composition and selection treatment. Control and selected larvae were reared on normal food or on food containing 300 mmol<sup>-1</sup> urea, NaCl or sucrose. Osmolarity values of food and hemolymph samples were determined as described in Materials and methods. Larvae can osmoregulate against large osmotic gradients when the osmolyte is sugar or salt, but not when it is urea. Urea-resistant larvae are less affected by rearing on urea food. Values are means ± s.d. *N*=5 populations (for normal and urea food); *N*=7 individuals of control population 4 and selected population 4 (for NaCl and sucrose foods). C>S, significant difference (*P*>0.001; Tukey's HSD test)

methionine, tyrosine, valine, glutamate, isoleucine and aspartate). The first four amino acids accounted for approximately 76% of the total FAA pool in all experimental groups. The last three accounted for approximately 4% of the total FAA pool.

Levels of arginine, alanine, proline, methionine and isoleucine were all significantly affected only by food type (ANOVA;  $P < 0.005$  for all), although the precise response varied among amino acids (Fig. 4). Arginine and methionine concentrations were lower on urea food than on normal food in both control and selected larvae. Alanine, proline and isoleucine concentrations were all higher on urea food.

Valine concentrations were significantly higher in the selected larvae than in the control larvae, regardless of food type (ANOVA;  $P = 0.018$ ). Glutamate concentration was significantly lower in selected larvae ( $P = 0.013$ ), but increased similarly in both control and selected larvae reared on urea food (food type,  $P = 0.001$ ; interaction term,  $P = 0.369$ ).

Two amino acids, serine and threonine, had significant selection treatment  $\times$  food type interaction terms ( $P < 0.001$  and  $P = 0.015$ , respectively). Serine concentrations were similar in selected larvae on both food types. However, serine levels were higher in control larvae reared on urea food than in those reared

on normal food. Threonine levels in control larvae were lower on normal food than on urea food, while threonine concentrations in selected larvae were higher on normal food than on urea food.

There also were two unidentified peaks in the HPLC analysis, one that eluted slightly earlier than serine and one that had a retention time between that of serine and glycine. These peaks may be hexoseamines, which elute near serine under the conditions used (Cohen and Strydom, 1988). While their exact concentrations cannot be calculated without knowing their identities, we estimated their concentrations by assuming that they contained only one amine group and compared their peak areas with those of the adjacent amino acid peaks (serine and glycine). This assumption maximizes the concentration and osmotic effect of the peaks. The amount of the first peak was unaffected by food type, but was slightly lower in the selected larvae than in the controls ( $2.3 \text{ mmol l}^{-1}$  and  $2.0 \text{ mmol l}^{-1}$ , respectively; ANOVA,  $P = 0.013$ ). The concentration of the second peak between serine and glycine did not differ between the control and selected larvae but decreased in both groups from approximately  $14 \text{ mmol l}^{-1}$  on normal food to  $9 \text{ mmol l}^{-1}$  on urea food (ANOVA,  $P < 0.001$ ).

#### Effect of food type and selection treatment on hemolymph trehalose concentration

Hemolymph trehalose concentration was significantly increased on urea food *versus* normal food (Table 1; ANOVA,  $P = 0.028$ ), and tended to be higher in the selected larvae, but not significantly so (ANOVA,  $P = 0.068$ ).

## Discussion

### Response to hyperosmolar environments

Our findings are consistent with previous work on *D. melanogaster* hemolymph ion composition and osmotic regulation. Croghan and Lockwood (1960) found that *D. melanogaster* larvae were able to maintain similar hemolymph osmolarities on food containing 7% NaCl or KCl. The results of our experiments with different foods support these previous findings. Our standard laboratory food is iso-osmotic to slightly hyperosmotic with respect to the larval hemolymph, and additional NaCl or sucrose raised its osmolarity significantly. Despite the high environmental osmolarity of these supplemented foods, both control and selected larvae had almost normal hemolymph osmolarity values when reared on these foods. Viability for both selection treatments on sucrose- or NaCl-supplemented food was normal or near normal, although development time was slightly slower than normal in both selection treatments (V. A. Pierce, L. D. Mueller and A. G. Gibbs, unpublished observation). Thus, *D. melanogaster* larvae possessed the ability to regulate hemolymph osmolarity before laboratory selection was applied to the populations.

Hemolymph osmolarity increased significantly in both control and selected larvae in the presence of the novel compound, urea. Selection for more than 100 generations has reduced the hemolymph osmolarity of the selected larvae on

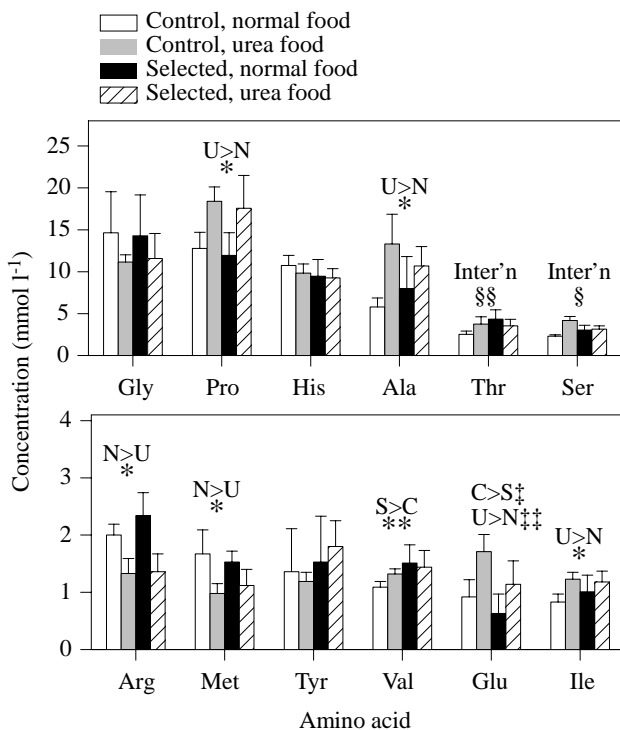


Fig. 4. Effect of food type and selection regime on hemolymph amino acid composition. Concentrations of the 12 most abundant amino acids are shown. Values ranged from  $0.63 \text{ mmol l}^{-1}$  to  $18.4 \text{ mmol l}^{-1}$ . Nine amino acids were significantly affected by selection regime ( $C > S$ ), food type ( $U > N$ ) or an interaction of the two factors (Inter'n). C, control; S, selected; N, normal food; U, urea food (ANOVA;  $*P < 0.005$ ;  $**P = 0.018$ ;  $†P = 0.013$ ;  $‡P = 0.001$ ;  $§P < 0.001$ ;  $§§P = 0.015$ ). Values are means  $\pm 1$  S.D. ( $N = 5$  populations).

urea food, but their osmolarity values have still not returned to normal. Despite their ability to osmoregulate in the presence of other osmolytes, urea-selected larvae do not down-regulate other components sufficiently to achieve normal osmolarity on urea food.

#### *Effect of selection on hemolymph composition*

Hemolymph ion composition on normal food was similar to previous reports (Stewart et al., 1994) in both selected and control populations. *D. melanogaster* larvae generally have a high  $[K^+]:[Na^+]$  ratio, and our use of banana-based food may contribute to  $K^+$  concentrations at the upper range of reported values (Croghan and Lockwood, 1960). Total amino acid concentrations were lower in our larvae than previously reported, but this may reflect the protein content of different foods or differences in measurement methods (Larrivee, 1979; cited by Van der Meer and Jaffe, 1983). Nervous function is probably protected from the effects of the relatively high  $K^+$  and glutamate concentrations by a neuroepithelium (Evans and Crossley, 1974; Gillot, 1980; Irving et al., 1976).

The change in hemolymph osmolarity on urea food raises the question of how hemolymph composition changes in response to urea exposure. There are three possible models. Under an osmoconforming model, one or more organic osmolytes may be increased to maintain osmotic balance with the medium. Levels of amino acids and trehalose, both of which are present in *D. melanogaster* hemolymph, have been shown to increase in response to hyperosmotic stress (Burton and Feldman, 1982; Garrett and Bradley, 1987). A second model is that increased osmolarity is due simply to the presence of urea in the hemolymph. A third, urea-counteractant, model assumes that urea is present in the hemolymph and predicts that concentrations of urea-counteracting solutes will be up-regulated in response to urea. Urea-counteracting solutes stabilize proteins and thus compensate for the denaturing effects of urea. They should be most effective at particular ratios where their stabilizing effects just balance the denaturing effects of urea, such as 1:2 for [TMAO]:[urea] (Chamberlin and Strange, 1989; Lin and Timasheff, 1994). This counteractant may be a normal component of the hemolymph or, in the selected larvae, a novel chemical not normally found in the hemolymph. Other components may be down-regulated to reduce the effect on total osmolarity.

Our analyses do not support the osmoconforming model. Larvae do not osmoconform on NaCl or sucrose food. On urea food, changes in all normal components were small, with no increase greater than  $13 \text{ mmol l}^{-1}$  (Table 1). In addition, the changes are similar in both control and selected larvae and thus cannot account for the hemolymph osmolarity difference between them that is observed on urea food. Thus, these components alone cannot explain the changes in hemolymph osmolarity and an osmoconforming model, as described for brackish-water mosquitoes (Garrett and Bradley, 1987), does not seem to apply to these larvae.

The data on urea concentrations in the hemolymph support either the second model of passive osmolarity increase due to

urea or the third, urea-counteractant, model. Larvae from both selection treatments have significant amounts of urea in their hemolymph when reared on urea food, but the selected larvae contain approximately 60 % of the levels of the control larvae. Thus, osmolarity on urea food should increase less in the selected larvae, which we observe.

The second model assumes that urea has no effect on the composition of the rest of the hemolymph (the contribution of the other components to osmolarity is the same) and that the osmolarity effect of urea is equal to its concentration. Thus, the sum of hemolymph osmolarity on normal food and the urea concentration in the hemolymph should equal the hemolymph osmolarity on urea food. This predicted osmolarity is very similar to the actual osmolarity on urea food (Table 2). The difference between the predicted and observed osmolarity is  $41 \text{ mosmol l}^{-1}$  in the control larvae and  $14 \text{ mosmol l}^{-1}$  in the selected larvae, which is within the standard deviation of the values used in the calculation. The agreement between the predicted and measured values tends to support the second model rather than the third model, but decreased concentrations of normal components could permit increases in urea-counteractants without changing the total osmolarity of the non-urea portion.

The urea-counteractant model requires increases in levels of urea-counteracting solutes and decreases in levels of other hemolymph components to maintain the pattern of osmolarity observed. Trehalose and certain amino acid concentrations increase significantly, as expected for urea counteractants. However, the magnitudes of the changes are small and unlikely to be physiologically important. Trehalose concentration only increased by  $13 \text{ mmol l}^{-1}$  in control larvae and by  $7 \text{ mmol l}^{-1}$  in selected larvae. Under the model of urea counteraction, the effects of urea and its counteractants should sum algebraically, and such small percentage changes (17 % and 8 %, in control and selected larvae respectively) in concentration should not dramatically alter the effectiveness of the counteractant (Lin and Timasheff, 1994). The control larvae, which have 93 % as much trehalose as the selected larvae, suffer much higher mortality on urea food. Furthermore, although trehalose has not been tested directly, no polyol sugars examined have demonstrated any substantial urea-counteracting properties (Somero and Yancey, 1997).

Amino acids are a major component of hemolymph, but no

Table 2. Predicted versus observed hemolymph osmolarity of larvae grown on urea food

Hemolymph osmolarity ( $\text{mosmol l}^{-1}$ )	Control larvae	Selected larvae
Normal food	$390 \pm 5.5$	$396 \pm 15.9$
Urea food:		
Predicted	571	499
Observed	$612 \pm 28.5$	$513 \pm 35.2$
Unaccounted difference	41	14

Values are means  $\pm$  s.d. ( $N=5$  populations).

one amino acid is present at concentrations higher than  $17\text{ mmol l}^{-1}$ . Those that increase on urea food still have concentrations below  $5\text{ mmol l}^{-1}$ . Their ratios with urea are so low that they are probably ineffective at counteracting urea, even if they were capable of doing so chemically. Thus, none of the hemolymph amino acids (or the two unidentified peaks) appears to be used by *D. melanogaster* larvae for urea counteraction. The changes in amino acid concentrations that do occur may be the consequence of impaired homeostatic mechanisms rather than adaptive responses.

If the selected larvae have evolved a novel urea-counteracting solute, then they must have down-regulated normal hemolymph components. The only measured component that decreased significantly was  $\text{Cl}^-$  concentration. The decrease in  $\text{Cl}^-$  concentration was  $6\text{ mmol l}^{-1}$ , which would offset only a very small amount of counteractant. The remaining osmolarity that is unaccounted for is approximately  $90\text{ mosmol l}^{-1}$  in all experimental groups (range:  $74\text{--}108\text{ mosmol l}^{-1}$ ). The counteracting solute would thus have to replace a substantial proportion of the normal components that contribute to this unidentified portion (magnesium, phosphate, bicarbonate, proteins, peptides, etc.), which seems unlikely. This indirectly suggests that the selected larvae have not evolved accumulation of some other unidentified osmolyte, such as GPC or TMAO, to act as a urea-counteractant in their hemolymph.

Analysis of hemolymph composition suggests that the second model, that of simple urea accumulation, best explains the observed hemolymph osmolarity data. The increased osmolarity on urea food is due to the presence of urea in the hemolymph, with little alteration in concentrations of other components. The selected larvae have lowered their hemolymph osmolarity by evolving mechanisms that reduce the amount of urea in their hemolymph.

#### *Presence of urea in the larvae*

The reduction in hemolymph urea concentration in selected larvae could be achieved by overall reductions in urea levels in the body or by sequestering of the urea in a specific compartment of the body, which is the way that some metal ions are stored. Whole-body measurements of urea show a pattern similar to that of the hemolymph; urea levels in the selected larvae were approximately 60% of the levels observed in the control flies. We calculated 'whole-body' urea concentrations for both control and selected larvae using our measurements of larval urea amount, wet mass and percentage water content. Whole-body urea concentrations were calculated as: (amount of urea  $\times$  larval wet mass)/(wet mass  $\times$  fractional water content), assuming that  $1\text{ }\mu\text{l}$  of water weighs  $1\text{ mg}$ . The control larvae had an overall calculated urea concentration of  $165.5\text{ mmol l}^{-1}$  body water, compared with the measured hemolymph concentration of  $180.8\text{ mmol l}^{-1}$  (Table 1). The calculated urea concentration of the selected larvae is  $101.5\text{ mmol l}^{-1}$  body water, compared with a measured hemolymph concentration of  $102.6\text{ mmol l}^{-1}$  (Table 1). The similarity between measured hemolymph concentrations and

calculated 'whole-body' urea concentrations suggest that urea levels have been reduced throughout the tissues of the selected larvae, rather than distributed differently among body compartments. Thus, the primary physiological adaptation of the selected larvae involves a mechanism that reduces their steady-state levels of urea.

#### *Regulation of osmotic responses*

Osmoregulatory responses may be triggered by total osmolarity, by changes in cell volume or be solute-specific. Okazaki et al. (1997) outlined a model of hyperosmolar response in which the response is triggered by cell shrinkage rather than by hyperosmolarity directly. High external concentrations of non-permeant osmolytes, such as  $\text{Na}^+$ , would cause cell shrinkage and trigger osmoregulatory responses, while high concentrations of cell-permeable osmolytes, such as urea or glycerol, would not. This model would predict that high external concentrations of  $\text{NaCl}$  would affect cell volume and thus trigger osmotic responses that would maintain normal osmolarity. In contrast, these responses would not be triggered by urea or sucrose food, and hemolymph osmolarity would rise.

Our results agree with the predictions for  $\text{NaCl}$  and urea food, but not sucrose food. Hemolymph osmolarity was normal on  $\text{NaCl}$  and increased on urea food. However, hemolymph osmolarity was normal on sucrose food, suggesting either that sucrose, or its component sugars, should not be considered permeant as they usually are (sugars cross membranes *via* protein carriers, not through the lipid bilayer as urea or glycerol are thought to do), or that this model inadequately explains the control of osmoregulatory responses in *D. melanogaster*.

Osmoregulatory models may have to consider the specific nature of the solute. It is possible that, without the ability to control the movement of a novel permeant compound, larvae may allow osmolarity to rise rather than decrease the concentrations of normal hemolymph components. Alternatively, larvae may regulate the concentration of normal components individually instead of total osmolarity and thus 'ignore' novel solutes.

#### *Alternative strategies for coping with urea exposure*

Our data suggest that the hyperosmolar environment itself is not a problem for the larvae, but that mortality may be due to accumulation of urea in their bodies, particularly intracellularly. Urea is known to perturb many cellular processes, including enzyme catalysis, translation and transcription (Somero and Yancey, 1997). Despite this, there is no evidence that the larvae have evolved urea-counteracting solutes. Trehalose and amino acids may not possess sufficient urea-counteracting properties for selection to favor altering their regulation. These flies may lack the genes that would allow them to synthesize novel solutes with urea-counteracting properties.

Instead, the data indicate that the major evolutionary response observed in the selected populations has been a



decreased steady-state level of urea. This would reduce the level of intracellular urea and thus the amount of damage it would cause. There are three possible ways this decrease may be achieved. The first is to decrease urea uptake from the environment. The second possibility is that the selected larvae have increased their rate of excretion of urea. The third possibility is for the larvae to metabolize the urea that enters to something less harmful or that can be excreted by existing transport systems. 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 under the same conditions as the control larvae. Current work is focused on identifying which of these mechanisms has evolved.

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