Short- and long-term effects of environmental urea on fecundity in *Drosophila melanogaster*

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Previous studies have shown that exposure to urea-supplemented food inhibited fecundity in *Drosophila* females, and that this inhibition was not expressed when females were given a choice between regular and urea-supplemented food as an oviposition substrate. We assayed fecundity, on both regular food and urea-supplemented food, at 5, 15 and 25 days post eclosion on females from ten laboratory populations of *Drosophila melanogaster*. The females assayed came from one of two treatments; they were maintained as adults on either regular or urea-supplemented food. We found that exposure to urea-supplemented food inhibited fecundity, relative to the levels exhibited on regular food, regardless of whether the urea was present in the assay medium, or in the medium on which the flies were maintained over the course of the experiment, thereby suggesting that urea has both a long-term (possibly physiological) as well as a short-term (possibly behavioural) inhibitory effect on fecundity of *Drosophila* females. We also tested and ruled out the hypothesis that prior yeasting could ameliorate the inhibitory effect of urea in the assay medium on fecundity, as this was a possible explanation of why flies given a choice between regular and urea-supplemented food did not exhibit a preference for regular food in a previous study.

Introduction

Laboratory populations of *Drosophila* spp. are commonly used for ecological and evolutionary studies (recent reviews by Mueller 1995; Rose *et al* 1996; Joshi 1997), thereby rendering their laboratory ecology an issue of fairly wide interest. One of the important aspects of the ecology of a crowded *Drosophila* culture is the accumulation of metabolic wastes such as urea, uric acid and ammonia in the medium (Budnik and Brncic 1976; Botella *et al* 1985; Mueller 1995; Joshi *et al* 1996a; Borash *et al* 1998). Egg laying behaviour of *Drosophila* females has been known to be affected by the presence and density of larvae in the culture for quite a long time. At low to moderate larval density, females prefer to lay eggs on food already colonized by larvae (Del Solar and Palomino 1966), whereas in crowded cultures, female fecundity declines as the age and density of larvae in the medium increases (Chiang and Hodson 1950). Moreover, the presence of larval metabolic wastes in the medium has also been shown to inhibit egg-laying by *Drosophila* females, even in the absence of larvae (Aiken and Gibo 1979). More recently, an increasing degree of inhibition of fecundity over the course of 30 days of adult life was reported in flies maintained as adults on food supplemented with urea (Joshi *et al* 1996b).

Unfortunately, in the study by Joshi *et al* (1996b) the effects of urea-supplemented food as a treatment and as the assay medium were confounded; flies maintained as adults on regular food were assayed for fecundity on regular food, while those maintained on urea-supplemented food were assayed on urea-supplemented food. Consequently, it was not clear whether the reduced

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fecundity of the flies maintained on urea-supplemented food was due to (i) a long-term, possibly physiological, effect of urea, or (ii) a short-term (i.e., within the 24 h duration of the assay), possibly behavioural, response to the presence of urea in the assay food medium, or (iii) a combination of both (Joshi *et al* 1996b). There was, however, some indication that a long-term effect of being maintained on urea-supplemented food was at least partly responsible for the observed reduction in fecundity (Joshi *et al* 1996b).

In a further study, it was seen that female flies given a choice of regular and urea-supplemented food for egg laying did not exhibit any significant preference for regular food (Joshi *et al* 1997). This could suggest that urea does not induce a short-term reduction of fecundity in *Drosophila* females, indicating that the observation of Joshi *et al* (1996b) could be entirely due to a long-term effect of exposure to urea. On the other hand, the treatment of flies prior to assaying fecundity differed between the two studies. In the study on fecundity, flies were not given any live yeast during the course of the experiment (Joshi *et al* 1996b). In the oviposition preference study, however, flies were maintained on non-nutritive agar medium supplemented with live yeast prior to the assay, in order to ensure that the flies were not given prior exposure to the regular food medium which was to be one of the choices in the assay (Joshi *et al* 1997). Thus, it is possible, at least in principle, that the lack of discrimination between regular and urea-supplemented food seen in the oviposition preference study (Joshi *et al* 1997) was a consequence of the flies carrying a heavy egg-load because they were given live yeast, which is known to stimulate fecundity in *Drosophila* (Mueller 1988; Mueller *et al* 1991).

In the present study, we used the same populations of *Drosophila melanogaster* used by Joshi *et al* (1996b; 1997) in an attempt to assess whether urea has a short-term (possibly behavioural) effect on fecundity, and whether the short- and long-term effects of urea on fecundity interact in any way. We also tested the hypothesis that prior yeasting could ameliorate the inhibitory effect of urea in the assay medium on fecundity.

2. Materials and methods

2. *Experimental populations*

Five populations of *D. melanogaster* selected for increased larval tolerance to urea for ~90 generations (MX populations) and five matched controls (MC populations) were used in this study. The derivation and maintenance of these populations are described in detail elsewhere (Joshi *et al* 1996a, b; Shiotsugu *et al* 1997), so we restrict ourselves here to details pertinent to the present study. The experimental populations were raised as larvae at low densities (50–100 eggs per 8 dram vial) on banana-molasses food (MC populations) or banana-molasses food supplemented with 18 g/l urea (MX populations). Plastic sleeves were inserted into these vials onto which larvae would crawl up to pupate. The sleeves with pupae on them would then be removed from the larval vials, cleansed of any food sticking to them, and placed into plexiglass cages ($25{\cdot}5 \times 20 \times 14{\cdot}4$ cm^3) containing yeasted banana-molasses food. Thus, only larvae and not adults in the MX populations were exposed to urea. All populations were maintained under constant light at 25°C, and had a generation time of 3 weeks.

2.2 *First fecundity assay*

This assay was done in order to distinguish between the long-term effects of exposure to urea in holding vials and short-term (behavioural) effects that urea present in the assay medium may have on egg-laying behaviour. Prior to initiating the assay, all ten MX and MC populations were passed through two generations of identical rearing (under MC conditions) to avoid any differences between selection regimes stemming from selection-regime specific environmental or maternal effects. For assaying fecundity, freshly eclosed virgin flies from all test populations were collected and placed into 8 dram vials with about 3 ml of food medium, at a density of four males and four females per vial. From each population, seven such vials each were set up with banana-molasses food (henceforth, regular food) and regular food supplemented with 18 g/l urea (henceforth, urea-food), respectively. Flies were transferred to fresh vials every third day, and fecundity was assayed three times, at 5, 15 and 25 days post-eclosion, respectively. From each population × food-type (regular versus urea food) combination, at each assay time, eight females were assayed for fecundity on regular food, and eight on urea food. To assay fecundity, one male and one female were placed in a vial containing ~3 ml of the appropriate food medium and allowed to lay eggs for 24 h. After 24 h, the flies were placed back into the vials in which they were being maintained at a density of four females and four males. As far as possible, we tried to assay the same 16 females from each population × food-type combination at the three assay times; any deaths among these flies were compensated for by using back-up flies from the other holding vials being maintained for that population × food-type combination.

2.3 *Second fecundity assay*

This assay was done in order to see whether prior exposure to yeast altered the subsequent effect of urea in the assay medium on egg-laying. Since the first assay revealed no difference between the two selection regimes

(see § 3), the second assay was conducted on only the five MC populations. For each population, eclosing flies from vials that had been set up with 60–80 eggs per vial were collected into cages and subjected to two treatments for 4 days. In the yeasted treatment, the flies were given a Petri dish of regular food supplemented with a generous dab of live yeast paste, whereas in the unyeasted treatment, just a Petri dish of regular food was supplied. The food in the cages was changed on the second day. After 4 days post-eclosion, fecundity was assayed on regular food and on urea food, using the same protocol as in the first assay. Fecundity on each of the two food types was assayed on 20 females from each population × treatment combination.

2.4 *Statistical analyses*

Untransformed fecundity data from the first assay were subjected to a mixed-model analysis of variance (ANOVA). Due to the pattern of relatedness among the MX and MC populations (Shiotsugu *et al* 1997), pairs of MX and MC populations, matched by subscripted indices, were treated as random blocks in the ANOVA. Selection (MX versus MC), treatment (whether the flies were maintained on regular food or urea food in the holding vials over the course of the experiment), environment (whether fecundity was assayed on regular food or urea food) and time (day 5, 15, or 25 post eclosion) were treated as fixed factors, crossed with blocks. Data from the second assay were ln-transformed to equalize cell variances (fecundities of 0 were taken as being 1) and then subjected to a mixed model ANOVA in which treatment (yeasted versus unyeasted) and environment (assay medium: regular versus urea food) were treated as fixed factors crossed with blocks. All ANOVAs were implemented using the GLM procedure of SAS (version 6·08) for Windows.

3. Results

3. *First fecundity assay*

The presence of urea in the food medium, whether in the holding vials or in the assay vials, had an inhibitory effect on fecundity (figure 1). In general, the fecundity of flies maintained on regular food was higher than that of flies maintained on urea food (figure 1, table 1: significant effect of treatment). Similarly, flies assayed on urea food tended to have reduced fecundity as compared to those assayed on regular food (figure 1, table 1: significant effect of environment). A significant effect of time was also seen in the ANOVA (table 1): fecundity at 5 days differed significantly from that at 15 and 25 days post eclosion (pair-wise t-tests, $P < 0{\cdot}005$), whereas fecundities at 15 and 25 days post eclosion were not significantly different (t-test, $P = 0{\cdot}063$). The ANOVA also revealed a significant treatment × time interaction (table 1). Basically, in flies maintained in holding vials

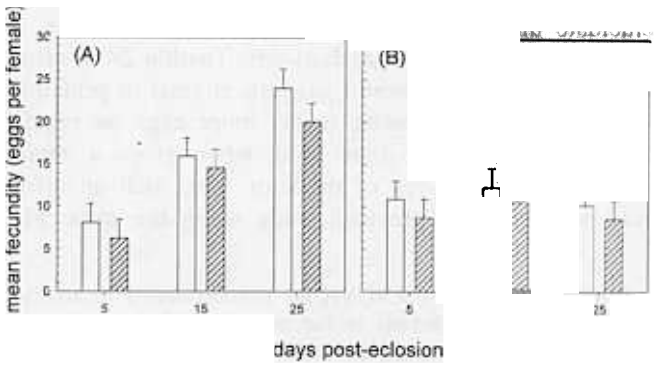

Figure 1. Mean fecundity of MX and MC flies assayed on either regular food (0 g/l urea) or urea food (18 g/l), after being maintained as adults on either (**A**) regular food, or (**B**) urea food. The error bars represent 95% confidence intervals about the mean of the 10 MX and MC populations at each maintenance medium × assay medium × assay time combination, and were calculated using least squares estimates of the standard errors of the appropriate cell means in the randomized block ANOVA. Data for MX and MC populations was pooled as the ANOVA revealed no significant effect of selection regime, or of any interaction of selection regime with any of the other fixed factors. (□), Assayed on 0 g/l urea; (▨), assayed on 18 g/l urea.

Table 1. Results of ANOVA on fecundity in the MX and MC populations in the first fecundity assay.

Source	*df*	MS	*F*	*P*
Selection (Sel)	1	171·71	1·57	0·2787
Treatment (Treat)		4909·64	29·23	0·0057
Environment (Env)	1	1052·54	15·16	0·0176
Time	2	4158·42	23·19	0·0005
Sel × Treat		867·77	2·04	0·2262
Sel × Env	1	219·44	1·03	0·3679
Sel × Time	2	75·67	0·79	0·4880
Treat × Env	1	18·33	0·29	0·6178
Treat × Time	2	4486·86	54·69	0·0001
Env × Time	2	27·89	0·72	0·5146
Sel × Treat × Env		0·96	0·01	0·9254
Sel × Treat × Time	2	14·07	0·28	0·7606
Sel × Env × Time	2	104·34	1·38	0·3056
Treat × Env × Time	2	49·52	1·02	0·4016
Sel × Treat × Env × Time	2	16·21	0·16	0·8572
Error	808	65·59		

Selection refers to MX or MC, treatment to maintenance regime of assay flies as adults (maintained on food with or without urea), and environment refers to assay medium (with or without urea). The effects of block, and several interactions involving block were significant, but random factors, and their interactions have been omitted from the table for brevity because our primary interest was in the fixed factors and their interactions.

on regular food, fecundity increased over time (figure 1A), with fecundities at 5, 15, and 25 days being significantly different from each other (pair-wise *t*-tests, $P < 0.0005$). On the other hand, flies maintained on urea food did not show any significant difference in fecundity at the three assay times (pair-wise *t*-tests, $P > 0.05$) (figure 1B). Neither selection regime, nor any interaction involving selection regime and any of the other fixed factors, had a significant effect on fecundity (table 1).

3.2 *Second fecundity assay*

As expected, fecundity of yeasted flies was significantly higher than that of unyeasted flies, and fecundity assayed on regular food was higher than that on urea food (figure 2, table 2). There was no significant effect of the treatment × environment interaction (table 2), clearly suggesting that yeasting did not interact with the effect of urea in the assay medium. At a qualitative level, it was in the yeasted treatment that a larger difference was seen between fecundity on the two types of assay medium (figure 2).

4. Discussion

Laboratory studies such as these are important for an understanding of the 'laboratory ecology' of *Drosophila*: a matter of some significance given the number of evolutionary studies based on laboratory *Drosophila* populations. The results from the first fecundity assay clearly confirm the observation by Joshi *et al* (1996b) that long-term exposure to urea leads to an inhibition of fecundity, relative to that on normal food, and that this inhibition is accentuated with increasing time of exposure to urea food. The data also support an earlier observation (Joshi *et al* 1996b) that selection for increased larval tolerance to urea does not seem to affect the response of adult components of fitness to urea-supplemented food. Moreover, our results also show that there is a short-term effect of urea on fecundity, manifested as a reduction of fecundity when assayed on urea food, as compared to regular food (table 1, figure 1). The data, of course, do not permit a finer assessment of the causes underlying these short- and long-term effects. It is possible that the observed long-term effects of urea may be due to reduced feeding and hence delayed maturation of eggs, or it may simply be due to retention of otherwise mature eggs. Similarly, the short-term effect of urea could have a behavioural component, but the data do not unequivocally implicate behaviour as being the only causal factor. Indeed, both results might be accounted for by urea having a repellent effect on flies. For example, suppose that flies given urea-supplemented food for oviposition are not attracted to it; they will therefore lay fewer eggs. On the other hand flies kept for extended periods on such food will, if repelled by urea, not feed as much and therefore produce fewer eggs for subsequent oviposition. Further studies on egg loads in the ovaries of flies maintained on urea and normal food may help elucidate the exact causes of these observed effects.

The existence of such a short-term (within 24 h) effect of urea on fecundity would suggest, at least in principle, that females should prefer to lay more eggs on regular food, as compared to urea food, when given a choice between the two types of medium. Yet, such an effect was not seen in a previous study using the same MX

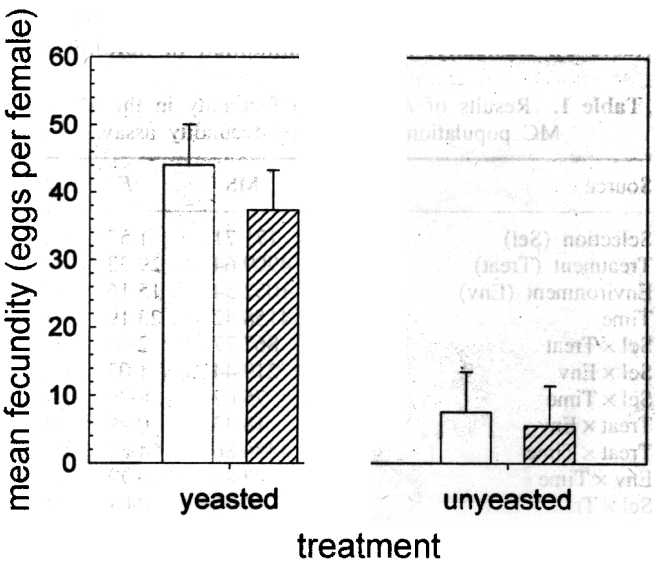

Figure 2. Mean fecundity of MC flies assayed on either regular food (0 g/l urea) or urea food (18 g/l), after being maintained as adults on either regular food, or regular food supplemented with yeast. The error bars represent 95% confidence intervals about the mean of the five MC populations at each treatment × assay medium combination, and were calculated using least squares estimates of the standard errors of the appropriate cell means in a randomized block ANOVA conducted on untransformed data. (□), Assayed on 0 g/l urea; (▨), assayed on 18 g/l urea.

Table 2. Results of ANOVA on ln-transformed fecundity in the MC populations in the second fecundity assay.

Source	*df*	MS	*F*	*P*
Block	4	1·71	3·85	0·0044
Treatment (Treat)		368·00	436·99	0·0001
Environment (Env)	1	7·50	14·13	0·0198
Block × Treat	4	0·84	1·89	0·1107
Block × Env	4	0·53	1·19	0·3126
Treat × Env	1	0·60	0·47	0·5300
Block × Treat × Env	4	1·28	2·87	0·0230
Error	380	0·44		

Treatment refers to maintenance regime of assay flies as adults (yeasted or non-yeasted), and environment refers to assay medium (with or without urea).

and MC populations (Joshi *et al* 1997). Similarly, fecundities of *D. melanogaster* females on three different food media have recently been found to be uncorrelated with oviposition preferences in pair-wise choice trials between the same three media (Sheeba *et al* 1998).

The lack of a significant interaction between treatment and environment in the second fecundity assay (table 2) is interesting. It clearly rules out one simple possible explanation for the lack of discrimination between regular and urea-supplemented food seen in the oviposition preference study of Joshi *et al* (1997). If prior yeasting could ameliorate the inhibitory effect of urea in the assay medium on fecundity, as a consequence of increased egg-load of females given yeast, then one would expect to have seen a greater inhibitory effect of urea on fecundity in unyeasted flies. In fact, the trend seen, although not statistically significant, actually shows exactly the opposite: yeasted flies underwent a greater inhibition of fecundity when assayed on urea food, as compared to unyeasted flies (figure 2). It is, thus, clear that there is more to the difference in egg laying behaviour under choice and no-choice situations than can be explained simply by invoking differences in pre-assay maintenance conditions. These findings suggest that there is a qualitative difference in the manner in which ovipositing *Drosophila* females react to environmental cues under situations where the cues are presented one at a time, versus simultaneously.

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