RESEARCH NOTE

Microenvironmental variation in preassay rearing conditions can lead to anomalies in the measurement of life-history traits

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Introduction

Experiments in ecology and evolution often involve the measurement of traits related to the life history, such as fecundity, stress resistance and duration of various life stages. Since any such trait is expected to exhibit some variance around the mean, a meaningful point estimate can only be derived by taking an average over a large number of replicate measurements. Although all replicates within a treatment should ideally be identical to each other in every respect, it is often impossible to realize this in practice. For example, if one needs to measure the fecundity of a large number of adults, it might not be feasible to procure or generate all of them from a single source or batch such that they share a common environment during preassay rearing. The effects of such preassay variation in macroenvironmental factors on life-history traits have been well studied empirically in laboratory systems such as *Drosophila* (Mueller 1985; Service and Rose 1985; Chippindale *et al*. 1993; Borash and Ho 2001; Prasad *et al*. 2003). However, such variation is not expected to be a severe problem under laboratory conditions, as it is possible to exercise strict control over most known sources of macroenvironmental variation, such as temperature, light and food, across batches. Nevertheless, we still need to address possible effects on assayed traits of differences across batches in microenvironmental factors, which include all those elements that cannot possibly be controlled by an experimenter. For example, in case of a *Drosophila* system in the laboratory, this might include, *inter alia*, density of microflora on the food or minor differences in the space available to the flies; factors that are normally ignored as of trivial import.

In this study, we directly examined the possible effects of microenvironmental variation while generating experimental organisms on the measurement of a life-history trait. We assayed fecundity in the fruit fly *Drosophila melanogaster* by allowing replicate single pairs (one male and one female) to lay eggs for varying lengths of time. We found that the temporal pattern of cumulative fecundity was anomalous when all flies in a particular egg-lay duration treatment were derived from a single vial. We conducted another experiment to show that the anomalous patterns tended to disappear as a consequence of differences due to microenvironmental variation getting averaged out when the flies subjected to a particular egg-lay duration treatment were derived from different vials. We computed an index that reflected this parent-vial-specific effect, and used it to generate predictions about the expected number of eggs laid over time. We then performed a third experiment to independently verify these predictions and found good agreement between the predicted and observed values. These results demonstrate the importance of randomizing across preassay microenvironmental conditions before assaying any life-history-related trait.

Materials and methods

Derivation of the flies

All experiments were conducted on a large outbred population of *D. melanogaster*, the so-called JB_1, that has been maintained in the laboratory on a three-week discrete-generation cycle for more than 200 generations. Details of the maintenance protocol of these flies have been described elsewhere (Sheeba *et al*. 1998) and are not relevant to the present study. Eggs were collected from the JB_1 population by placing a Petri plate containing banana–jaggery medium in the population cage for 24 h. The eggs were then distributed into 16 vials, each containing 70–80 eggs in ~6 ml of medium. The medium in each vial was obtained from a single cooked batch. The adult flies eclosing in these vials were

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transferred to fresh-medium vials on days 12, 14 and 16 after egg collection. All flies that eclosed from a particular vial were collected together and strict one-to-one correspondence was maintained between the egg vials and the adult collection vials. On day 18 after egg collection, the flies were put into vials containing ~6 ml of medium, for three days. Eight of the 16 vials were supplied with excess live yeast paste to boost female fecundity, while the remaining eight vials did not get any nutritional supplement. Thus, all flies in a particular vial (henceforth, parent vial) ultimately came from the same egg vial and presumably experienced similar microenvironmental conditions during their preadult and adult stages, especially during the three-day conditioning period. On day 21 after egg collection, these flies were distributed into fecundity vials for measuring the number of eggs laid over different durations of egg-laying window.

Experiment 1

Twenty fecundity vials, each containing one male and one female fly in ~2 ml of medium, were derived from each of the eight unyeasted parent vials. The flies were then allowed to lay eggs in these vials for durations of 1, 2, 3, 4, 5, 6, 7 or 8 h. All 20 fecundity vials that were set up from a particular parent vial were allotted to the same egg-lay duration treatment. At the end of the assigned time, the adults were discarded and the number of eggs laid in each vial was counted manually under a binocular microscope. A similar protocol was followed for measuring the fecundity of flies from the yeasted parent vials, with the exception that only 10 fecundity vials were set up from each parent vial.

Experiment 2

In this experiment, 16 egg vials, each containing 200–300 eggs were set up, and the flies were handled as explained above (see section Derivation of flies) until day 21 after egg-lay. For both unyeasted and yeasted treatments, seven fecundity vials containing one male and one female each were obtained from each parent vial. Seven different durations of egg-lay window, between 1 and 7 h, were studied in this experiment. Eight fecundity vials, one from each parent vial, were allotted to each egg-lay duration in case of both unyeasted and yeasted treatments. As before, the number of eggs laid in each vial during the egg-lay duration was recorded, after discarding the adults. Thus, experiment 2 differed from experiment 1 in that parent vial was not confounded with egg-lay duration but crossed with it.

Performance index

Using the data from experiment 2, we calculated a statistic that we call the performance index, in the following way:

$$S_i = \frac{1}{T}\left[\sum_{t=1}^{T}\frac{f_{i,t}}{N_t}\right].$$

Here, S_i denotes the performance index of the ith parent vial, T the total number of egg-lay window durations studied (7 in experiment 2), $f_{i,t}$ the number of eggs in the fecundity vial belonging to ith parent vial and tth egg-lay duration window, and N_t the mean number of eggs laid in the tth egg-lay window, averaged across all fecundity vials in that window. This statistic, calculated separately for each parent vial, gives us an estimate of the relative fecundity of the pairs of flies that belonged to a particular parent vial vis-a-vis flies from other parent vials. When a particular value of S_i is multiplied by any N_t, we get a prediction for $E[f_{i,t}]$, the expected number of eggs laid by the flies from the ith parent vial over an egg-lay duration window of t hours. A third experiment was conducted simultaneously to test these predictions arising out of experiment 2.

Experiment 3

The design of this experiment was similar to that of experiment 1 in that all the fecundity vials in a given egg-lay window were derived from a single parent vial. However, there were two major differences: (a) the 16 parent vials used in this experiment were the same ones that were used in experiment 2, and (b) each egg-lay duration treatment consisted of 10 fecundity vials in both the unyeasted and yeasted treatments. The number of egg-lay window durations studied was seven, as in experiment 2.

Results and discussion

Experiment 1

In this experiment, we were measuring the number of eggs laid by single *D. melanogaster* females over an increasing duration of egg-lay. Intuitively, one would expect this number to increase up to a certain point of time and then plateau out. However, under no circumstances would one anticipate a reduction in the cumulative number of eggs laid over successively increasing lengths of time, as seen in this experiment (figure 1). Here we note that there almost seems to be
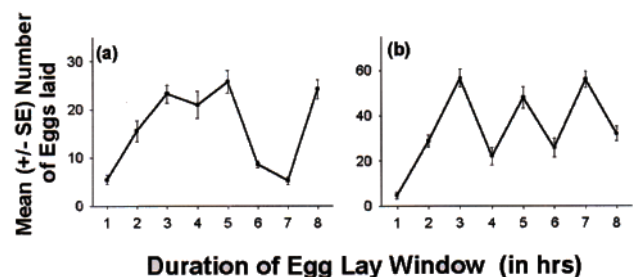

Figure 1. The mean number of eggs laid across successive lengths of time in the two treatments, (a) unyeasted and (b) yeasted, in experiment 1. Since this number is cumulative, the observed trends are unexpected. See text for possible explanations.

a regular oscillation (two-point cycle) in the mean fecundity of the yeasted flies (figure 1b). However, this is most probably a coincidence, as all the means arising out of different egg-lay durations are independent of each other by design in this experiment. Such anomalous results can possibly arise if there is large variation in fecundity among individuals, or alternatively in the presence of some random environmental noise affecting the fecundity vials. These explanations, nevertheless, are unlikely in the present case, as the standard errors across the mean (fecundity) were found to be small (figure 1) and macroenvironmental factors were strictly controlled. The observed pattern of cumulative fecundity (figure 1) could also potentially result from microenvironmental variation leading to a systematic increase or decrease in the fecundity of all pairs of flies that came from a particular parent vial. Experiments 2 and 3 were specifically designed to test this hypothesis.

Experiment 2

In this experiment, each fecundity vial in a particular egg-lay window was derived from a different parent vial. Therefore, in terms of the mean number of eggs laid in a given duration, any major parent-vial-specific variation, if present, is expected to be smoothed by averaging across parent vials within egg-lay window durations. On the other hand, in case there was major among-individual variation in fecundity, one could anticipate some anomalous pattern, as observed in experiment 1. The same argument applies to any random environmental noise affecting the fecundity vials differently, although such an event is unlikely in the controlled laboratory conditions under which the experiments were run.

In experiment 2, the mean number of eggs laid over successively longer durations of time increased initially up to ~4 h and then levelled off (figure 2). This result rules out individual variation or random environmental noise as potential causes of anomaly in experiment 1, but does not directly implicate microenvironmental variation among parent vials for the same. To prove that microenvironmental variation
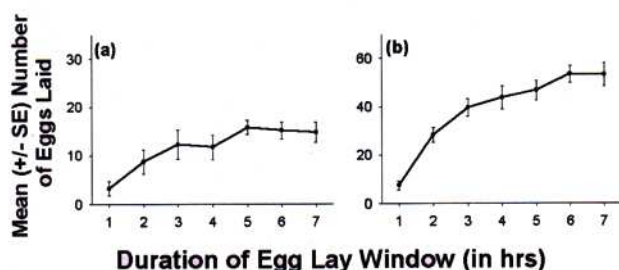
can indeed lead to systematically aberrant cumulative fecundity patterns, we calculated the performance index (S_i) as mentioned above (see section Materials and methods: Performance index). This statistic is an average score for the fecundity of flies that came from the same parent vial, relative to the fecundity of flies from other parent vials. Thus, S_i is expected to reflect the component of variation due to parent-vial-specific differences in microenvironment. Since the same parent vials were used in experiments 2 and 3, we were able to generate independent predictions for the mean number of eggs in a time window in experiment 3. For this, we used the product of S_i and N_t (from experiment 2) for the corresponding egg-lay window of t hours in which the ith parent vial was tested in experiment 3.

Experiment 3

There was considerable agreement between the predicted and the observed values of mean fecundity across different egg-lay window durations (figure 3) and a chi-square test detected no significant difference between the two in either regime (unyeasted, $\chi^2_{(6)} = 3.93$, $P = 0.69$; yeasted, $\chi^2_{(6)} = 4.24$, $P = 0.64$). This ability of S_i to successfully predict the mean fecundity in experiment 3 indicates that microenvironmental variations can systematically affect life-history traits of organisms. It is worth noting that by mimicking the design of experiment 1, we again confront some anomalous patterns in the unyeasted regime (figure 3a). However, no such clear aberrations are observable in the yeasted regime (figure 3b), which most probably happens to be a fortuitous event.

This study demonstrates the artefactual anomalies that can potentially arise due to nonrandom sampling across the microenvironmental conditions over which the experimental organisms have been reared before an assay of life-history related traits. Unfortunately, this aspect is not always taken care of while setting up experiments in ecology or evolution, and most often not reported clearly in the literature. Similar artefactual results might arise while measuring other
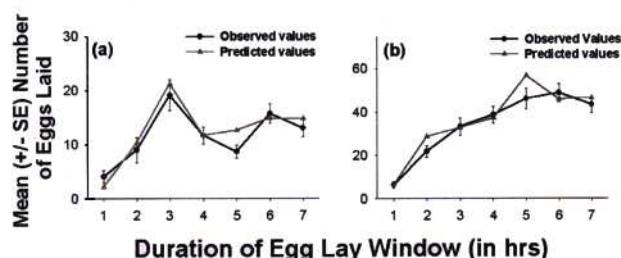

Figure 2. The mean number of eggs (N_t) laid across successive lengths of time in the two treatments, (a) unyeasted and (b) yeasted, in experiment 2. These curves are closer to the intuitive expectations and thus rule out individual variations and random noise as causes of the observed patterns in experiment 1.

Figure 3. The mean number of eggs across successive lengths of time in the two treatments, (a) unyeasted and (b) yeasted, in experiment 3, along with the corresponding predictions from experiment 2. There are no significant differences between the predicted and observed numbers of eggs.

life-history-related traits too, as fecundity is known to be correlated with a host of life-history attributes (Prasad and Joshi 2003). It is noteworthy that this study was conducted in the laboratory under constant temperature, humidity, light, etc. and all flies were treated similarly as far as practicable. It is difficult, if not impossible, to maintain such rigorous standards of control in field or quasinatural studies. Thus, one cannot overemphasize the need for randomization across pre-assay microenvironments before assigning individuals to different experimental treatments for measuring trait values.

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