Natural Selection in a Bottle

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ABSTRACT: The study of natural selection in laboratory systems undergoing experimental evolution can provide important insights into the relationship between natural selection and adaptation. We studied selection on the norm of reaction of age at first reproduction in a laboratory population of *Drosophila melanogaster.* This population had been selected on a discrete generation schedule in the laboratory for more than 600 generations. Using genetically marked strains, we studied development time, size, female fecundity, and viability of flies that began development at different times relative to the initiation of each bottle. Only flies that began development within 30 h of the initiation of the bottle were reliably able to eclose before the next transfer. Theory predicts that flies initiating development around this critical time should decrease size at maturity to ensure eclosion by the 14-d deadline, but late flies are not smaller. This result suggests an unknown constraint on response to selection on age at maturity in this population. Ultimately, laboratory systems provide the best opportunity for the study of natural selection, genetic variation, and evolutionary response in the same population.

Keywords: natural selection, laboratory evolution, constraint, life history, development time, age at maturity, norm of reaction.

Our understanding of microevolutionary processes has increasingly come to depend on two complementary sources of information: studies of natural selection in the field and studies of experimental evolution in the laboratory. Field studies have helped to create a new consensus that strong selection is not uncommon (Endler 1986; Kingsolver et al. 2001). Information on selection is being combined with quantitative genetic techniques to make detailed predictions about the course of evolution (Price et al. 1984; Schluter and Smith 1986*a*, 1986*b*; Stratton 1994; Campbell 1996; Dudley 1996*a*, 1996*b*). However, tests of those predictions are difficult and have rarely been carried out.

Experimental studies of adaptation in the laboratory are designed to create strong natural selection whose nature can be inferred (Rose et al. 1996). Populations selected in these ways often show spectacular responses (Bennett et al. 1990; Mueller et al. 1991; Travisano et al. 1995; Rose et al. 1996; Wichman et al. 1999; Sgrò and Partridge 2000). However, these studies have been focused on the responses to selection and not at all on the form of the selection imposed. This approach precludes making or testing quantitative predictions. In addition, the interpretation of the correlated responses and potential trade-offs is very difficult without information on the multivariate selection gradient (Harshman and Hoffmann 2000). Superficially similar experiments often yield contradictory results, leaving open to debate which responses have been directly, if inadvertently, selected for and which are the pleiotropic consequences of responses in the intended targets of selection.

The information provided by field studies of natural selection and laboratory studies of evolution is therefore largely nonoverlapping. Rarely do studies measure both natural selection and evolution, leaving us with rather little information on the relationship between the two. Some have been prepared to assume that this relationship is simple, as exemplified by the use of the breeder's equation of quantitative genetics (Lande 1979; Arnold 1992). However, this assumed simplicity should be distrusted for a number of reasons (Houle 2000; Stern 2000). A few exceptional studies have combined the study of natural selection with observations of evolutionary change (Grant and Grant 1995; Reznick et al. 1996*a*, 1996*b*, 1997; Losos et al. 1997, 2000) with mixed results.

An illustration of the difficulties of relating selection and adaptation is the exceptionally well-studied evolution of guppies (Poeciliopsis) introduced into Trinidadian streams with contrasting predation pressures (Reznick et al. 1996*b*, 1997). The responses to selection are consistent with the authors' a priori expectation of differential age-specific mortality in the two sorts of streams, but direct studies of mortality revealed a pattern of mortality and thus selection very different from that assumed in previous

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work (Reznick et al. 1996*a*). Although this alternative pattern of mortality may yet prove to be a cause of the consistent and rapid responses to selection observed in this system, gaps remain in the interpretation of even this well-studied system.

In this article, we suggest that the study of natural selection in a laboratory setting is the best method of making the link between natural selection and evolution and may thus permit predictive and rigorous study of adaptation. The same control that allows the experimenter to tailor novel selective pressures permits the characterization of those selective pressures with greater accuracy than is generally possible in the field. We characterize some aspects of natural selection in a longtime laboratory population to illustrate the advantages of studying natural selection in the laboratory.

We have made direct measurements of the fitness consequences of variation in the timing of female reproduction in a pair of naturally selected laboratory populations of *Drosophila melanogaster*, derived from a population called IV. The IV population has been maintained in essentially the same environment since the ancestral population was founded from wild North American flies 25 yr and more than 600 fly generations ago. Previous studies have demonstrated adaptation of *Drosophila* to laboratory environments (Frankham and Loebel 1992; Latter and Mulley 1995; Sgrò and Partridge 2000), and we presume that the IV population has also evolved since capture. In this work, however, our purpose is to characterize the current selective environment and the correspondence of life-history traits to their predicted optima, not to infer anything about past responses to selection.

For this population of flies, the most important feature of the laboratory regime is that transfers of adult flies to new medium are made on a schedule that allows the completion of only one generation per transfer. In nature, populations of *D. melanogaster* have overlapping generations. Therefore, part of the adaptive challenge the IV population faces is the change from overlapping to discrete generations. We therefore expect that the timing of metamorphosis is an important target of natural selection. A theoretical framework for predicting the optimal norm of reaction for life-history transitions in such contexts has been well worked out (Rowe and Ludwig 1991; Rowe et al. 1994).

In the IV population, the challenge faced by individuals differs greatly depending on when they start development relative to the age of the culture. An egg that begins development immediately after transfer to new medium has ample time to complete development at a large size. However, an egg laid some days later faces a desperate struggle to complete its development in time for the next transfer. Therefore, the optimal age at maturity must be a function of the time that an egg begins development.

Potential costs and benefits of delaying metamorphosis are clear from previous work on *Drosophila* life history. A major cost of delay is the risk of not eclosing as an adult in the allotted 14-d period. The IV population is crowded, and crowding can cause high mortality at the egg, larval, and pupal stages (Sang 1949*a*, 1949*b*; Chiang and Hodson 1950; Bakker 1961; Kondrashov and Houle 1994). A second likely cost of delay is therefore increased preadult mortality. As the medium is consumed, harmful waste products also build up (Borash et al. 1998), suggesting that growth rate will decline over time. Finally, given that maximal egg production is not reached for a few days after eclosion (Ashburner 1989), females that eclose well before transfer would gain an additional fecundity advantage. This factor could be even more important for males, since matings obtained well before transfer may still benefit them. On the other side of the equation, increasing the larval period increases adult size (Bakker 1969; Nunney 1996), and size is highly correlated with female fecundity (Roff 1981; Zwaan et al. 1995*a*) and male mating success (Partridge et al. 1987; Santos 1996). Late eclosion may also avoid mortality and other costs to the adults before transfer (Joshi et al. 1998*a*, 1998*b*). Finally, females eclosing more than a few days before transfer may lose additional fitness as a result of wastage of eggs.

Our approach to measuring natural selection utilizes two genetically marked populations derived from the original IV population. Marked populations of flies that differ in the starting time of development are followed into adulthood. This method allows us to quantify the importance of most of the hypothesized costs and benefits of altered time of eclosion listed above. Most important, these studies can be performed in the environment in which the IV population has been evolving for more than 600 generations.

Methods

General Experimental Design

The basic design of the experiments is shown in figure 1. In each case, eggs of the genotype of interest, referred to as the "pulse genotype," were introduced into rearing bottles during a brief period of time. Flies of the complementary, or push, genotype were allowed to lay eggs in the same bottles during the rest of the time to provide the background competition that forms an essential part of the selective environment in which the flies evolved. This general approach has been used previously by Sang (1949*b*) in his studies of larval ecology. The introduction of complementary genotypes was accomplished in two dif-

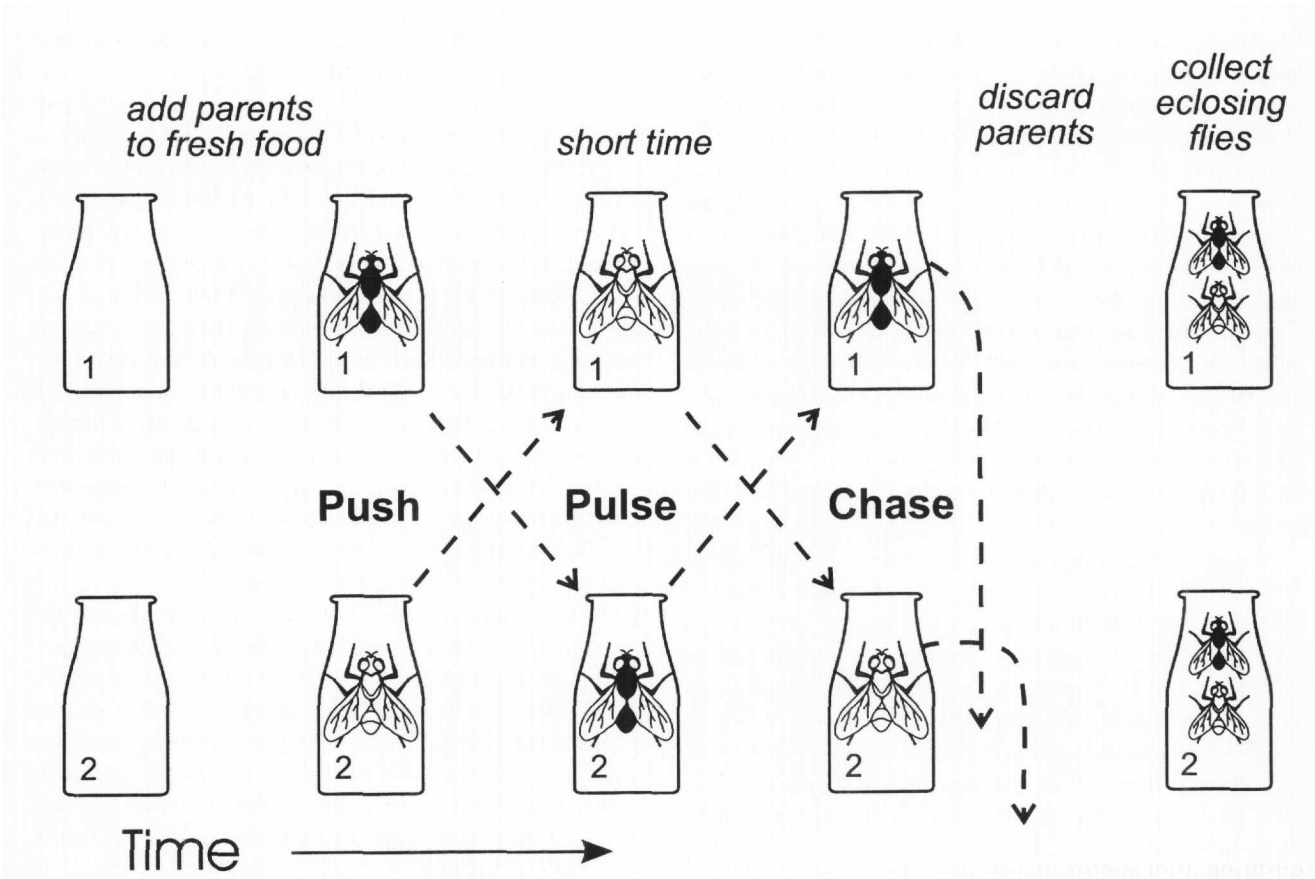

Figure 1: General design of the experiments. Visibly marked, complementary genotypes were established in bottles 1 and 2. Focal offspring of the complementary genotype were then introduced during a short period of time either by transfer of adults (as shown in the figure) or by introduction of eggs. When the adults eclosed, the focal group could be identified and their phenotypes measured.

ferent ways. In our first experiments, we established parallel bottles of each genotype and, at the designated time, reciprocally switched the parental flies into the complementary bottle, as shown in figure 1. We refer to this as the "push-pulse-chase," or PPC, design. In later experiments, we collected batches of eggs from populations of each genotype over a period of 2 h. We then counted out batches of eggs that were placed in each bottle. The push parents were only removed from each bottle during the brief time necessary to place the pulse eggs. These experiments we refer to as the "blob of eggs," or blob experiments. In each experiment, the parents of the push flies were discarded on the sixth day after the bottles were set up so that the offspring could be unambiguously identified and their development time, size, or fecundity characterized. Times are given relative to the introduction of flies into each experimental bottle. Because the genetic marker that differentiates these populations itself has some effects on life history, we expected that natural selection might differ between the two populations.

Stocks and Rearing Conditions

The populations of *Drosophila melanogaster* used in this series of experiments, called IV, consist of descendants of about 200 flies collected by P. T. Ives in Amherst, Massachusetts, in 1975. Since that time, the population has been maintained in 10 half-pint bottles at 25°C on a 14-d transfer schedule. At each transfer, all the adult flies from each bottle are mixed with those of two other bottles in a circular scheme and transferred to fresh medium without anesthesia. In 1976, 50 isofemale lines were derived from this population and checked for inversion polymorphism. Twenty-one lines proved to be homozygous for the standard gene arrangements, and the population was reinitiated by combining these 21 lines (Charlesworth and Charlesworth 1985). The population used in these experiments was maintained in the laboratory of B. Charlesworth until 1992 and in the Houle lab after that time. A spontaneous ebony (*e*) allele was detected in the population in 1992 at a frequency of about 1%. A population

homozygous for *e*, IV*e*, was created by four cycles of mass backcrossing homozygous *e* flies from IV to IV followed by selection for *e* in the F_2 generation. In 1993, each population was split into two replicates. Two replicate wild-type (IV+) and ebony (IV*e*) populations have been maintained under identical conditions since that time.

All stocks have been maintained on a standard brewer's yeast, corn flour, and sucrose medium with propionic acid as a preservative, with the exception that before 1993 cornmeal was used instead of corn flour. Live yeast is never added to the bottles, although yeasts are often present and carried from bottle to bottle by the flies. Because of the generally high density of the IV populations, live yeast rarely becomes noticeable in the cultures. Before 1994, strips of paper towel were added to the bottles, but this practice was discontinued about the time these experiments were started. The populations were on a 20L : 4D photoperiod from 1995 through 1997, although until 1995 they were maintained on a 12L : 12D photoperiod

A few generations before each experiment, samples of flies from the IV*e* and IV+ populations were removed and their offspring reared under uncrowded conditions. One generation before the beginning of each experiment, bottles were initiated with approximately 50 pairs of flies. After about a week, these grandparental flies were discarded. To initiate the experimental bottles, we pooled the offspring from approximately 10 of these and then placed 125 pairs (100 pairs in the blob2 experiment) in a bottle containing fresh medium. Each experiment began with the transfer of flies from the holding bottles into fresh bottles without anesthesia. Flies used to produce the blob eggs were obtained from the same bottles but held at lower density on heavily yeasted food. All experimental flies were reared on the 20L : 4D photoperiod, except in experiment PPC1 where flies were reared in constant light. When necessary, CO_2 anesthesia was used for handling of the flies.

We measured fecundity of groups of female flies by allowing them to lay eggs on unyeasted food for 24 h at 25°C. If the eggs could not all be counted within a few hours, the vials were frozen and the eggs counted later. Deaths and escapes were noted when they occurred, and the corresponding counts were adjusted to a per-female basis.

We report the results of two PPC experiments and three blob experiments. Each experiment differed slightly in its details, as outlined below, so that different aspects of the system could be investigated.

PPC Experiments

PPC1. The reciprocal transfers between pairs of bottles were started at 0, 12, 24, 36, or 60 h after the first flies were placed in each bottle and ended 12 h later. Three bottles of each time-genotype combination were initiated for a total of 30 bottles. Newly emerged adults were collected every 12 h from day 9, when eclosion began, to day 20. Bottles were maintained in constant light so that eclosion times would not be clumped. The sex and genotype of each eclosing fly were recorded. If six or more pulse females eclosed from a bottle on a particular day, up to two vials containing six females were set up for fecundity assays. Six pulse males collected on the same day were also placed in each vial unless not enough emerged, in which case push males eclosing on that day were used. Fecundity vials were transferred until day 26 so that there were at least 7 d of fecundity data for each group. After the fecundity trials, all females and pulse males in each vial were grouped by sex and weighed to the nearest tenth of a milligram. The live weight of pulse males not used in the fecundity vials was also measured at least 2 d after eclosion.

PPC2. Reciprocal transfers were started at 0, 24, 48, and 72 h and ended 12 h later. Two bottles of each genotype-time combination were set up. The emerging flies were collected daily from the onset of eclosion on day 8 until day 20. Fecundity vials containing four pairs of pulse females and pulse and/or push males were set up until day 15 for up to a maximum of four vials for each bottle and collection time. Fecundity vials were transferred every 24 h until day 20. After the fecundity trials, the surviving flies from each vial were weighed by sex. In addition, groups of four pulse females or males collected on days 15–20 were also weighed when they were at least 2 d old.

Blob Experiments

We obtained the pulse flies in these experiments by transferring a known number of eggs into a bottle containing flies of the opposite genotype. Flies used for egg laying were kept at 25°C either in a styrofoam box with petri dishes containing media or in bottles and then transferred to Plexiglas cylinders that fit over a petri dish. After the egg-laying period, pieces of medium containing 100 or 120 eggs were cut from the petri dish and carefully transferred into the surface of the experimental bottles. In the second and third blob experiments, we minimized laying of "held" eggs that initiate development before laying (Milani and Palenzona 1957; King and Sang 1958) by offering the flies fresh unyeasted food 3–4 h before the time when the eggs were needed (the pulse time). These plates were discarded, and a second set of unyeasted dishes was offered to the flies at the appropriate pulse time.

In blob1 and blob2, eggs collected starting at 0, 24, 48, and 72 h were used as pulse flies. Blobs consisted of 120 eggs. Each time-genotype combination was replicated eightfold in blob1 and 10-fold in blob2. Collections of the

offspring were made every 24 h from day 9 to day 23. Emerging flies were sorted and counted, and all of the pulse flies from each collection were weighed in groups. In blob2, the length of one wing of each pulse fly was also measured from a digitized video image.

The purpose of blob3 was to investigate survival trends for eggs laid in the first few hours in a new bottle. Eggs laid from 8 h before to 24 h after the bottle was set up were used as pulse flies. The experiment was set up over 2 d, and bottles with a variety of start times were set up on both days so that day of egg collection and pulse times were not confounded. Overall, 48 bottles were set up in this experiment. Blobs in this experiment consisted of 100 eggs. Adult flies were collected every 24 h from day 9 to day 19.

Results

Numbers of Offspring

Table 1 shows the mean number of push and pulse flies that eclosed in each experiment, genotype, and pulse time treatment. In addition, the columns labeled "Total transfer" show the numbers of flies that eclosed by day 14 from the initiation of the bottles. Analyses of variance of the numbers of push flies eclosing and of the total transfer revealed significant experiment, experiment by genotype, and pulse time nested within experiment effects but no overall effect of genotype (analyses not shown). This pattern suggests that the treatment of flies in each cell had important effects on the number of offspring produced. Factors that could be responsible for these effects include differences in the quality or age of the medium during rearing, during holding, or among batches of experimental bottles or differences in the handling of the flies during the setup of each cell.

The mean number of offspring that eclosed by day 14 is substantially higher than the numbers of push parents in each bottle (250). This was unexpected, since the number 250 was chosen to be near the average number of flies transferred during normal culture of the IV populations (N. Keyghobadi and D. C. Houle, unpublished data). However, the number of transferred offspring was within

Table 1: Means and standard deviations of the number of pulse and push flies eclosed

Experiment and pulse time	N[a]	+			*e*		
		Pulse	Push	Total transfer[b]	Pulse	Push	Total transfer
PPC1:							
6	3	188 ± 57	631 ± 14	554 ± 43	145 ± 56	761 ± 55	657 ± 22
18	3	72 ± 49	719 ± 17	541 ± 67	51 ± 7	790 ± 133	585 ± 48
29	3	135 ± 48	713 ± 199	606 ± 84	101 ± 48	765 ± 133	569 ± 127
42	3	116 ± 17	780 ± 172	589 ± 82	81 ± 62	763 ± 190	591 ± 96
66	3	183 ± 34	844 ± 82	649 ± 78	129 ± 72	774 ± 111	647 ± 93
PPC2:							
6	2	117 ± 40	761 ± 77	620 ± 19	220 ± 36	897 ± 214	641 ± 134
30	2	70 ± 22	794 ± 15	505 ± 26	362 ± 83	636 ± 16	684 ± 45
54	2	112 ± 33	831 ± 4	558 ± 31	199 ± 24	687 ± 49	581 ± 3
78	2	56 ± 1	819 ± 89	502 ± 49	50 ± 9	753 ± 98	523 ± 45
Blob1:							
1	8	54 ± 10	979 ± 98	501 ± 56	8 ± 7	971 ± 116	394 ± 89
21	8	46 ± 4	944 ± 48	690 ± 85	56 ± 9	968 ± 136	557 ± 87
44	8	29 ± 17	889 ± 73	581 ± 52	20 ± 13	918 ± 130	478 ± 72
68	8	14 ± 5	1001 ± 98	621 ± 77	2 ± 3	1016 ± 115	521 ± 70
Blob2:							
1	8	23 ± 14	630 ± 44	444 ± 64	13 ± 12	795 ± 224	616 ± 117
25	8[c]	44 ± 14	935 ± 120	676 ± 136	33 ± 24	857 ± 106	701 ± 63
49	8	15 ± 12	924 ± 112	723 ± 104	.1 ± .3	826 ± 166	721 ± 166
73	8	1 ± 1	851 ± 74	687 ± 104	0 ± 0	935 ± 144	859 ± 153
Blob3:							
1	12	48 ± 11	817 ± 71	581 ± 96	19 ± 6	919 ± 94	668 ± 96
5	6	26 ± 11	760 ± 78	478 ± 118	39 ± 15	965 ± 118	758 ± 102
22	6	40 ± 9	806 ± 70	599 ± 59	47 ± 8	915 ± 45	690 ± 82

Note: Push genotypes: *e* = ebony, + = wild type.

[a] Number of bottles in each pulse time–genotype block.

[b] Total number of push and pulse flies that eclosed by day 14, the normal time of transfer to new bottles.

[c] Only seven bottles with push genotype *e*.

the range of values observed for bottles in the IV populations, which range from approximately 50 to 1,000.

Development Time

Analyses of variance of the development times of pulse flies in each experiment revealed that the sexes never differed in their development times, which is a typical result when flies are reared under competitive conditions (Miller 1964; Zwaan et al. 1991). Therefore, the sexes were pooled for further analyses. Figure 2 shows means for each start time and pulse genotype combination over the five experiments. The pattern of increasing development time as a function of pulse time is consistent among experiments. However, an analysis of the combined data, with experiment and genotype as classification variables and mean start time as a covariate, revealed complex interactions among these variables, suggesting that the slope of the relationship between start time and development time differs among experiments and sometimes among genotypes and that the difference between genotypes also differs among experiments.

A more interpretable summary of these results, based on an analysis of each experiment separately, is shown in table 2. The overall pattern is similar in each experiment, although the details differ. Development time always shows a highly significant increase with pulse time, and usually this relationship was the same for each genotype. This similarity of pattern over experiments, coupled with statistically significant differences among the results of experiments, is characteristic of the results for all traits in this study.

Size

Size was measured in two ways during these experiments. In the two PPC experiments and in blob1, the fresh weights of groups of flies eclosing from each bottle on the same day were recorded. Because female flies typically vary considerably in weight depending on their age and reproductive state, we pay the most attention to the data for male flies. The mean number of flies weighed in each group was 5.9 (SD 1.8) in PPC1, 3.8 (SD 0.6) in PPC2, and 4.4 (SD 4.8) in blob1. The variance in the number weighed was much higher in blob1 because all pulse flies were weighed regardless of the number eclosing on a given day. In blob2, the wing lengths of 700 pulse flies were recorded. For each type of data, we calculated growth rates by dividing the size measure by the development time of the flies.

Analyses of covariance of bottle means for size are shown in tables 3 and 5 and for growth rates in tables 4 and 5. The left side of table 3 shows the ANCOVA tables

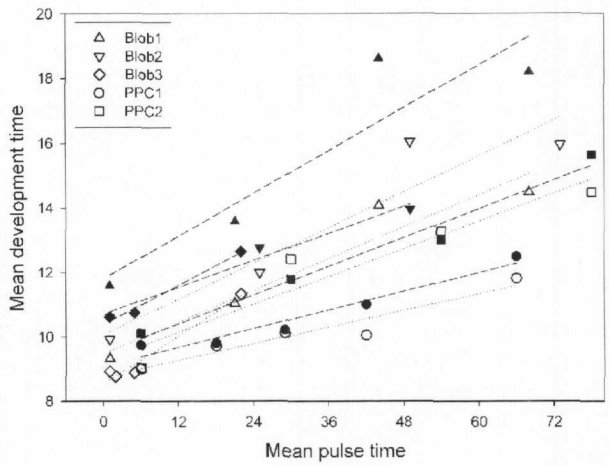

Figure 2: Mean development time for each pulse time, sexes combined. Open symbols denote wild-type pulse flies; solid symbols denote *e* flies. Regressions for wild-type pulses are shown as dotted lines, and those for *e* flies are shown as dashed lines.

for models, including the interaction term between pulse time and genotype, which is sometimes significant; the last two columns show the slope for a model including only genotype and pulse time and not their interaction. Other interaction terms were never significant. In almost all experiments, pulse time and size were significantly negatively related. The exceptions are for female weight in PPC2, where the slope is just slightly <0 (table 3). The highly significant interaction between pulse time and genotype for male weight in PPC2 and blob1 arises because *e* males have a slope near 0, whereas the slopes for + males were significantly negative. For blob2, no significant interactions were found between sex, genotype, and pulse time. The relationship between pulse time and wing length was significantly negative for each genotype. The weight of *e* flies was significantly greater for both sexes in PPC1 and PPC2 when interactions were not included in the models; there were no significant differences in blob1. The wing length of + flies was significantly greater than that of the *e* flies.

The results for growth rate are more straightforward. As shown above, development time increases with pulse time, whereas size decreases. Growth rate combines these two results, and, unsurprisingly, the evidence that growth rate declines with pulse time is very strong in both sexes in every experiment. Male growth rates for weight are shown in figure 3. The differences in growth rate among genotypes follow those for size; *e* had higher growth rate in the PPC experiments, and + had the higher growth rate in the blob experiments.

We also investigated the relationship between size and development time within bottles. To look for overall pat-

Table 2: Analysis of mean development time data

Experiment	Genotype	Pulse time	Genotype × start time	Error	Error df	Parameter estimates ± SE	
						Slope[a]	$(e-+)$[b]
PPC1						.045 ± .004	.62 ± .20
MS	87.4	3,831	2	36.5	26		
P	.13	<.0001	.82				
PPC2						.073 ± .008	.34 ± .41
MS	69.5	6,180.1	25.2	85.2	12		
P	.38	<.0001	.6				
Blob1						.103 ± .007	2.76 ± .28
MS	216.4	6,244.3	357.6	28.1	56		
P	.008	<.0001	0				
Blob2						.101 ± .008	.40 ± .27
MS	30.7	1,253.8	7.6	16.4	40		
P	.18	<.0001	.5				
Blob3						.106 ± .010	1.65 ± .19
MS	661.3	1,536.1	5.2	14.1	44		
P	<.0001	<.0001	.55				

Note: Analysis was carried out in the SAS program GLM, with Type IV sums of squares. Data were weighted by the number of pulse flies eclosing from each bottle. All entries in the ANCOVA, except the error term, have 1 degree of freedom.

[a] Slope of regression of development time on start time.

[b] Mean difference between development time of *e* and + flies.

terns, we calculated the regression slopes within each bottle. No significant effects of sex, experiment, genotype, or pulse time on within-bottle slopes were detected for either weights or lengths. The mean slope in bottle for the three experiments with weight as the measure of size was -0.0225 (± 0.0039, $N = 190$), which is significantly <0 by a *t*-test ($P < .0001$). In blob2, the mean slope for wing length was -0.0102 (± 0.0030, $N = 62$), which is also significantly <0 ($P = .0014$). Thus, within a group of flies that initiated development at roughly the same time, flies that eclosed later were on average smaller than those that eclosed earlier over all experiments.

In summary, flies that began development later were smaller than those that began developing earlier; within a cohort of flies initiating development at the same time, slower-developing flies were smaller than more rapidly developing ones. These results are consistent with many previous experiments with *Drosophila melanogaster* (Sang 1949*a*; Chiang and Hodson 1950; Robertson 1960; Bakker and Nelissen 1963).

Fecundity

The mean fecundity per female in the first 5 d of adult life is shown as a function of mean female weight in figure 4. The residuals from a regression of fecundity on weight will clearly not be normally distributed. Examining the distributions of fecundities within genotypes revealed six potential outliers in PPC1 and three in PPC2, all with above-average fecundity for their weight and genotype. To approximate more closely the conditions in the IV bottles, we did not add live yeast to the medium in these experiments, but microorganisms do sometimes colonize unyeasted vials, which substantially increases fecundity. The presence of outliers with above-average fecundity is consistent with such events. An additional outlier for female weight in PPC2 has very high influence on any regressions. These points are shown with gray fill on figure 4. The presence of these potential outliers was dealt with in two ways. First, a conventional ANCOVA was performed with the potential outliers deleted, as shown in table 6. Second, we fitted a robust linear model to the entire data set using Huber's M-estimator (metric winsorization), with the scale parameter determined by iterated MAD, as implemented in the S-Plus program rlm (Venables and Ripley 1994). In each analysis, we treated pulse time as a factor. The parameter estimates for genotype and weight effects using this model are also shown in table 6. In both the conventional and the robust analysis, *e* flies had significantly higher fecundity than wild-type flies in both experiments, although the robust approach shows a significantly smaller effect than the conventional one. Larger females also had a significant fecundity advantage in both experiments under both analyses. The slopes for the robust analysis are significantly larger, as expected given the elimination of all positive outliers in the conventional analysis. Dropping the outlier for weight in the PPC2 experiment had little effect on the slope in the robust analysis. In both exper-

Table 3: Analyses of mean size data for each bottle

Experiment	Genotype	Pulse time	Genotype × pulse time	Error	Error df	Parameter estimates ± SE	
						Slope[a]	$(e-+)$[b]
Males:							
PPC1						−.0021 ± .0004	.067 ± .016
MS	.3411	2.2255	0	.0645	25		
P	.03	0	.91				
PPC2						−.0006 ± .0003	.103 ± .017
MS	.0759	.3894	.5847	.0661	28		
P	.29	.02	.006				
Blob1						−.0021 ± .0003	−.001 ± .013
MS	.2127	.4735	.2942	.0282	54		
P	.008	0	.002				
Females:							
PPC1						−.0026 ± .0005	.059 ± .021
MS	.2326	1.9357	.0167	.0691	25		
P	.08	0	.63				
PPC2						−.0003 ± .0006	.152 ± .032
MS	.6117	.0987	.5533	.3041	28		
P	.17	.57	.19				
Blob1						−.0025 ± .0008	.004 ± .030
MS	.3043	.6274	.3278	.1925	55		
P	.3	.08	.2				

Note: Analysis was carried out in the SAS program GLM, with Type IV sums of squares. Each observation was weighted by its sample size. All entries in the ANCOVA, except the error term, have 1 degree of freedom.

[a] Slope of regression of weight on pulse time for pooled genotypes.

[b] Mean difference between development time of *e* and + flies, assuming a common slope.

iments, genotypes had homogeneous slopes (analysis not shown). The effect of pulse time was always nonsignificant when female size was included as a covariate in the models.

We also examined how the fecundity of females changes with their adult age in PPC2. Daily fecundity totals differed significantly with age, but only because fecundity on day 1 of adulthood was less than that on other days (data not shown). The relationship of these fecundity schedules to those in the natural bottle environment are unclear, since our measure of fecundity entailed daily transfers to fresh medium, whereas flies in the IV populations are transferred to fresh food only once in their lives.

In summary, fecundity increased with female size, as expected from many previous studies (reviewed in Roff 1981). The differences in start time and development time of individuals did not affect fecundity other than through their effect on size. Under the conditions of our experiments, fecundity is low on the first day of adult life but stable during days 2–7.

Viability

The egg-to-adult viability for each bottle in the three blob experiments is shown in figure 5. The means for each experiment, genotype, and pulse time are connected by lines in the figure. Clearly, experiments and genotype combinations differ in their patterns of viability. Simultaneous analysis of data from all experiments in the S-Plus program glm confirms a highly significant three-way interaction among experiments, genotypes, and pulse times ($\chi^2 = 20.79$, df = 1, $P < .0001$). Similarly, within each experiment, the two-way interactions between genotype and pulse times are all highly significant ($P < .0001$ in each case). Viability at pulse times near 0 is particularly variable, and a great deal of the variation is among experiment–genotype–pulse time combinations. Variance-component analysis of logit transformed data was carried out to compare results for pulse times of 5 h and less with those for pulse times between 21 and 25 h. The variance components for the early pulse times (1.122 among experiment–genotype–pulse time combinations, df = 7; 0.549 within, df = 60) were in each case close to being significantly greater than those for the intermediate pulse time (0.214 among, df = 6; 0.344 within, df = 37) by an *F*-test ($P = .04$ among, $P = .06$ within).

Overall, the data suggest an intermediate optimum pulse time. To test whether the apparent maximum in viability at pulse times near 24 h is real, we fit separate logistic regressions for each experiment-genotype combination for pulse times of 21 h and more and for those ≤25 h. All six

Table 4: Analyses of mean growth data for each bottle

Experiment	Genotype	Pulse time	Genotype × pulse time	Error	Error df	Parameter estimates ± SE: Slope[a]	Parameter estimates ± SE: $(e-+)$[b]
Males:							
PPC1						−.00050 ± .00006	.0030 ± .0026
MS	17	1,289	3	17	25		
P	.33	0	.65				
PPC2						−.00028 ± .00007	.0070 ± .0034
MS	3	598	31	34	28		
P	.79	0	.34				
Blob1						−.00061 ± .00004	−.0130 ± .0017
MS	178	709	15	5	54		
P	0	0	.1				
Females:							
PPC1						−.00064 ± .00008	.0014 ± .0036
MS	11	1,178	8	19	23		
P	.46	0	.52				
PPC2						−.00041 ± .00011	.0106 ± .0057
MS	31	1,357	25	104	28		
P	.59	.001	.63				
Blob1						−.00089 ± .00008	−.0183 ± .0029
MS	261	1,515	5	18	55		
P	0	0	.59				

Note: For details, see table 3. Mean squares have been multiplied by 10^4.

[a] Slope of regression of weight on pulse time for pooled genotypes.

[b] Mean difference between development time of *e* and + flies, assuming a common slope.

regressions for 21 h and greater were highly significant and <0 ($P < .0001$). The regression for the 25-h-and-less data were variable. For the *e* genotype, all three regressions were positive and highly significant (blob1 slope 0.132 ± 0.007; blob2 slope 0.051 ± 0.005; blob3 slope 0.055 ± 0.005). For the + genotype, one slope was significantly negative, one significantly positive, and one not different from 0 (blob1 slope −0.016 ± 0.005; blob2 slope 0.036 ± 0.004; blob3 slope −0.009 ± 0.005). In four of the six experiment-genotype combinations, therefore, there was a significant maximum in viability near 24 h, whereas in one case the maximum viability was clearly at the earliest pulse time in the experiment. An intermediate peak in survivorship was also suggested by the results of Gordon and Sang (1941) and Sang (1949*b*).

Probability of Transfer

In addition to surviving to adulthood, flies in the IV transfer schedule must also eclose in time to be transferred to the next generation of bottles, 14 d after the previous transfer. Figure 6 shows the probability that a fly eclosed within this 14-d window in the three blob experiments. In most cases, flies that started up to 24 h after the bottles are initiated had a very high probability of eclosing within 14 d. By 44 h, however, the probability of eclosion within the time allotted dropped nearly to 0. Substantial numbers of flies completed development in 14 d in only a few bottles. Substantial numbers of points are concealed at 0 for the later pulse times. Again, the probability of transfer is maximal at around 24 h.

We can estimate the probability that a fly eclosed in time for transfer, given that it survived to adulthood in all five experiments, as shown in figure 7. Bottles where no pulse flies eclosed were scored as 0. Again, substantial

Table 5: Analysis of mean wing length and growth rate data for each bottle in blob2

Effect	MS	*P*	Parameter estimates
Wing length:			
Sex	6.0184	0	.186 ± .013
Genotype	.1549	.024	−.032 ± .014
Pulse time	1.1958	0	−.0027 ± .0004
Error	.0291		
Growth rate:			
Sex	.0555	0	.0179 ± .0029
Genotype	.0073	.025	−.0069 ± .0030
Pulse time	.227	0	−.0012 ± .0001
Error	.0014		

Note: All entries in the ANCOVA have 1 degree of freedom, except the error term, which has 80. For additional details, see table 3.

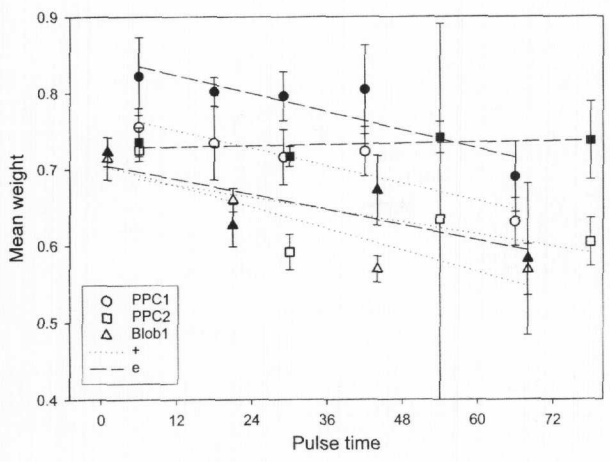

Figure 3: Mean growth rate in weight of adult male flies as a function of pulse time. Symbols as in figure 2.

numbers of points are obscured at 0 for the later pulse times; the same is true at 1.0 for the earlier ones. Most of the points above 0 for the late transfer represent the survival of a very small number of flies, as shown in table 1. Clearly, the probability of transfer dropped precipitously in all experiments. Wild-type flies did slightly better than *e* flies, as expected given their faster development time.

The Body-Size/Growth Rate Norm of Reaction

The abrupt decrease in the probability of eclosion before the transfer of flies to a new bottle between 24 and 48 h (figs. 6, 7) imposes strong selection. Flies seem to respond to their decreasing growth rate as conditions deteriorate by pupating at a smaller size, as demonstrated in "Size." If flies are able to detect cues as to the time of the impending transfer in addition to their own growth rate, cohorts of flies that begin development later should also eclose earlier for a given growth rate. Because growth rate and size are autocorrelated, we tested this expectation using the relationship between development time and body size. Pulse times were split into three groups: the early group, with pulse times of 6 h or less, from which essentially all flies that survive eclose in time for transfer; the middle group, with pulse times between 18 and 30 h, from which most but not all flies eclose in time; and the late group, with pulse times >30 h, from which most flies did not eclose in time for transfer.

A summary of these data for males is presented in figure 8. Too few *e* flies eclosed in the late treatments in blob2 to be useful. Weighted regression lines of size on development time are shown for the early and middle groups, whereas the data for all flies in the late group are shown as triangles. If the expectation that flies should use time cues to alter their size at eclosion is borne out, flies from later pulse times should fall below and to the left of flies from the earlier pulse times in each experiment. Therefore, we expect that the dashed lines, representing the norms of reaction of the middle group, should fall below the solid lines, which represent the norm of reaction of the early group. Although this expectation seems to be borne out in some cases, such as blob2, the overall pattern is not consistent. Similarly, late cohorts should have average residuals that fall below and to the left of the norms of reaction for both the early and middle group. Again, two experiment-genotype combinations seem to show this pattern (PPC1 + and perhaps blob1 *e*), but most definitely do not.

More detailed examination of the results of each ex-

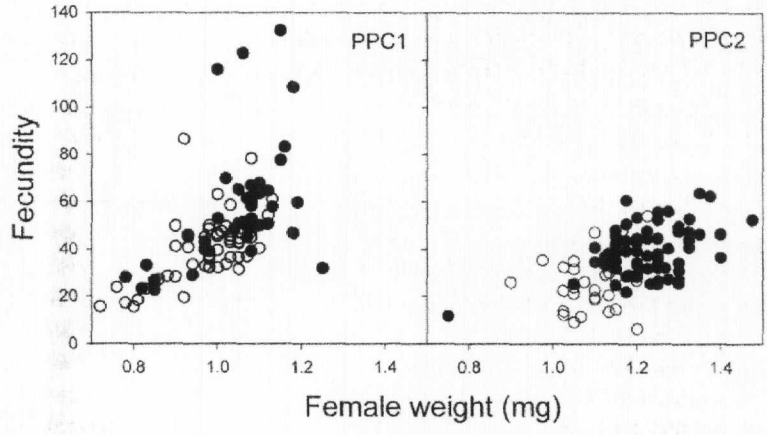

Figure 4: Per-female fecundity as a function of weight. *Left panel*, PPC1. *Right panel*, PPC2. Open symbols denote wild-type pulse flies; closed symbols denote *e* flies.

Table 6: ANCOVA of fecundity data from PPC1 and PPC2

Effect	Conventional ANCOVA				Robust parameters
	df	MS	*P*	Parameters	
PPC1:					
Genotype	1	1,128	0	7.89 ± 2.14	3.97 ± 1.08
Pulse time	4	192	.06		
Weight	1	1,963	<.0001	67.83 ± 13.97	92.22 ± 13.48
Error	85	83			
PPC2:					
Genotype	1	1,476	<.0001	11.46 ± 2.84	3.96 ± 1.5
Pulse time	2	24	.76		
Weight	1	485	.02	31.66 ± 13.66	41.57 ± 14.88
Error	90	90			

periment suggests that the middle-group flies eclosed earlier and smaller, given their growth rate, than those of the early group. For the weights from PPC1, PPC2, and blob1, the average residuals of the middle group from the early regression are negative. Within experiment, sex, and genotype combinations, the residuals of the middle group were significantly less than those of the early group in two experiments, less but not significantly so in six cases, and positive and nonsignificant in the remaining two combinations. For the wing lengths measured in blob2, the overall mean residual was negative, and tests on all four of the sex by genotype combinations showed that the residuals of the middle group were significantly less than those of the early group.

In contrast, the late-group flies consistently eclosed later and larger, given their growth rate, than those of the middle group. For the weights from PPC1, PPC2, and blob1, the average residuals of the late group from the middle group regression are positive. Within experiment, sex, and genotype combinations, the residuals of the late group were significantly greater than those of the middle group in four experiments, greater but not significantly so in two cases, negative and nonsignificant in four combinations, and significantly negative in two combinations. For the wing lengths measured in blob2, the deviations were significantly positive for both sexes in the wild-type flies. Residuals of weights for the late group from the early group regressions were on average positive but less consistently so than those for the middle group. Length residuals of the late group were on average negative and significantly less than those of the early group for wild-type flies in the blob2 experiment.

These data therefore imply that the middle-group flies are altering age at first reproduction relative to that of early-group flies in the direction expected because of natural selection. This result is consistent with these flies' generally high probability of eclosion in time for transfer. In contrast, the late-group flies seem to eclose later and larger than predicted if they are reaching maturity at the optimal time.

Discussion

The purpose of our experiments is twofold. First, we want to estimate the form of natural selection on age at maturity in our laboratory populations of *Drosophila melanogaster*. Our results provide evidence for the quantitative effects of many of the trade-offs involved in determining age at maturity as well as for the variability of selection even in this well-controlled laboratory population. More broadly, we suggest that studies of natural selection in the laboratory are perhaps the most practical way to make testable predictions about the course of evolution.

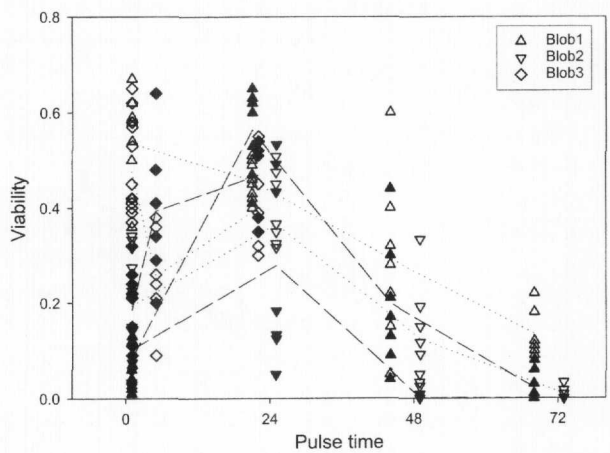

Figure 5: Viability for each bottle as a function of pulse time. Symbols as in figure 2.

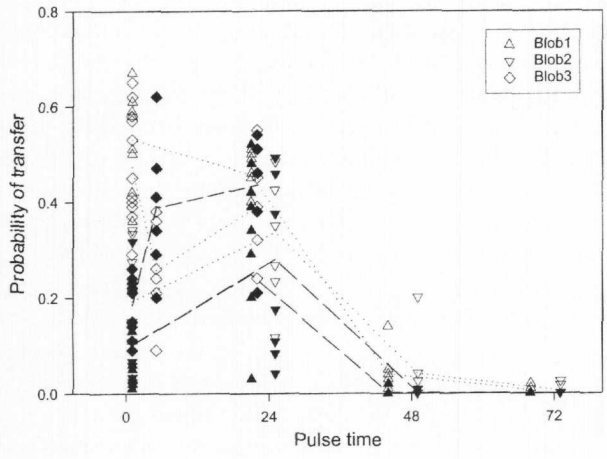

Figure 6: Probability of transfer of pulse flies for each bottle as a function of pulse time. Symbols as in figure 2.

Trade-offs Involved in Determining Age and Size at Maturity

A major factor affecting the fitness consequences of age at maturity in the IV population is the 14-d limit to development imposed by the transfer schedule, which favors rapid development. This selection is exactly analogous to that imposed on a natural population by seasonal environments (Rowe and Ludwig 1991; Newman 1992; Nylin and Gotthard 1998). This constraint is a novel one for *D. melanogaster* populations, although the IV flies had approximately 600 generations to respond to this novel selection pressure before our experiments began. Therefore, one of the most striking results of our experiments is the high proportion of flies that eclose too late to be transferred to the subsequent generation (fig. 7) but at a size well above the minimum size a fly can be. These flies have zero fitness, which obviously would have been greater had they accelerated their development sufficiently. Presumably, there is a limit to how rapidly a fly can develop, yet our data suggest that many of those flies that missed transfer had not approached this limit.

A second potential advantage of accelerated development is the avoidance of preadult mortality. Such mortality reductions can clearly be large because <50% of the embryos eventually reach maturity for all starting times (fig. 5). The pattern of mortality we observed, which was often least at about 24 h and increased rapidly after that time, suggests that the overall mortality rate is the result of complex interactions potentially involving all immature stages. A third potential advantage of early pupation is escape from a deteriorating larval environment that may decrease adult fecundity. Our data suggest that this advantage is unlikely to be important in our population, since the timing of development had no effect on fecundity once the effect of adult size was removed.

On the other side, females derive a clear fecundity benefit from increased size (fig. 4), as expected from many previous experiments (see the first section of this article). We have therefore confirmed that the principal trade-off for females in our populations is between the additional size and fecundity that an individual may gain through further growth and the risk of not being transferred to the next generation's bottles. We also have a quantitative picture of the importance of this trade-off.

Although the costs of eclosing too late are obviously paid by large numbers of flies in our populations, the benefits of longer development are not obvious in our results. Instead of a size benefit to flies that develop longer, our data show that flies that take more time to reach maturity are smaller than more rapid developers, as has been found in many previous studies (Sang 1949*a*; Chiang and Hodson 1950; Robertson 1960; Bakker and Nelissen 1963). For flies that begin development at different times, the negative correlation of size and development time is readily explicable as the result of a decline in the quality of the environment as nutrients are consumed and waste products accumulate. In the case of flies from the same cohort, this negative correlation at first seems counterintuitive. Rowe and Ludwig (1991) and Rowe et al. (1994) have shown that a negative relationship between measures of adult quality or fecundity and age at maturity is expected when individuals face a time constraint on the transition to adulthood. As the time left in which to mature decreases, the best strategy is to increase one's willingness to give up additional growth and the adult fitness it brings in

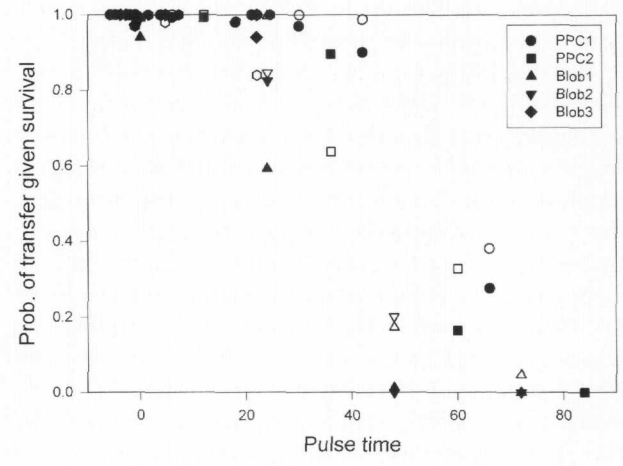

Figure 7: Probability of transfer of pulse flies, given that they survived to adulthood, for each bottle as a function of pulse time. Symbols as in figure 2.

exchange for the opportunity to reproduce. Thus, the negative relationship between development time and size is expected at equilibrium when individual growth rates differ, either because of the quality of the environment (as in the differences between start times) or because of the quality of individuals (as is likely to be involved in the variance in development time within cohorts).

Constraints and the Optimal Life History

Ultimately we wish to make a quantitative prediction of the nature and amount of natural selection acting on these populations after 600 generations in a nearly constant environment. We have a quantitative life-history model (M. Mangel, D. Houle, and L. Rowe, unpublished data) based on these results that will be published elsewhere. This empirically based model allows us to predict selection gradients and ultimately any responses to selection that might continue to occur. Even without these quantitative predictions, however, one aspect of our results is apparently in qualitative disagreement with intuitions based on life-history theory: the increase in development time with starting time shown in figure 2 and the correspondingly large proportion of surviving flies that fail to eclose during the 14-d period allowed, as shown in figure 7.

This observation can be explained in a number of ways. First, the environment that the later flies experience is dictated by the earlier flies. If, as expected, selection favors a more competitive larva early in the culture, this may cause deterioration in the conditions experienced by later larvae. Such a deterioration is expected from the results of Joshi and Mueller (1996) and Santos et al. (1996), which suggest that larvae selected in crowded cultures consumed more resources but used them less efficiently. Selection on flies developing late in the culture cycle is weaker than that on earlier flies because those late flies that do make it into the next generation are smaller and likely to have very low fitness. When individuals are selected in a range of environments, the overall fitness gradient will be heavily weighted toward those environments that give the highest absolute fitness (Donohue et al. 2000). We therefore expect the response of early flies to selection to be faster than that of flies in the later time periods. Thus, the environment late in the culture could be deteriorating at a higher rate than that at which the norm of reaction can respond.

A second explanation for the fatally long development time of many flies is that the flies already develop at some maximal rate or have achieved a minimum size, and further increases in this rate are not possible. Removal of third-instar larvae from food has shown that male flies can eclose at a weight of about 0.3 mg (Sang 1949*a*; Bakker 1959, 1961). In our experiments, the vast majority of the flies eclosing in all cohorts were much heavier than this, arguing against this explanation. However, this minimum size may be misleading, since it does not take into account the impact of size on fitness under competitive conditions. In our populations, the surface of the medium becomes liquefied, and larvae must reach down through this layer while keeping their spiracles above the surface to feed on the more nutritious part of the medium. Thus, later larvae may have to reach a larger and larger minimum size to feed effectively as the liquefied surface of the medium increases in depth. Size might also be constrained by selection on adult males, which we have not studied. For example, males might need to be a minimum size to compete for mates, and therefore males would be more willing than females to pay the costs of additional growth.

A third hypothesis is that there are no cues available to the flies that would indicate the age of the bottle and, therefore, the amount of time remaining until the next transfer. This does not seem likely, since there are a number of predictable changes during the life span of a bottle, such as the buildup of waste products (Borash et al. 1998), changes in texture or nutritional value of the medium (Gordon and Sang 1941), increases in the number and sizes of competitor larvae, and so on. A more plausible version of this hypothesis is that flies are not capable of responding to those cues that are available.

These possible sources of constraint on the optimal norm of reaction suggest additional experiments. For example, the timing of male mating success could have profound effects on male life history, as could adult mortality. One of the principal kinds of environmental variation present in the IV populations is variation in density. Investigation of fitness trade-offs at the low end of the range of densities would indicate whether genotype-density interactions are potentially important and perhaps responsible for the apparent suboptimal state of these populations at high density. The power of the approach we have laid out here is that all of these questions, as well as a host of others, can be addressed using our methods.

Implications for Drosophila *Laboratory Selection Experiments*

The study of natural selection in the laboratory can help to interpret the results of experimental evolution experiments in *D. melanogaster*. For example, Promislow and Tatar (1998) have suggested that much of the response of *Drosophila* populations to selection for delayed senescence (e.g., Luckinbill et al. 1984; Rose 1984; Zwaan et al. 1995*b*; Partridge et al. 1999) may have resulted from the purging of late-acting mutations that have accumulated since the time of domestication. Our results lend credence to this idea because adult flies more than a few days old make no contribution to fitness. The majority of flies eclose <4

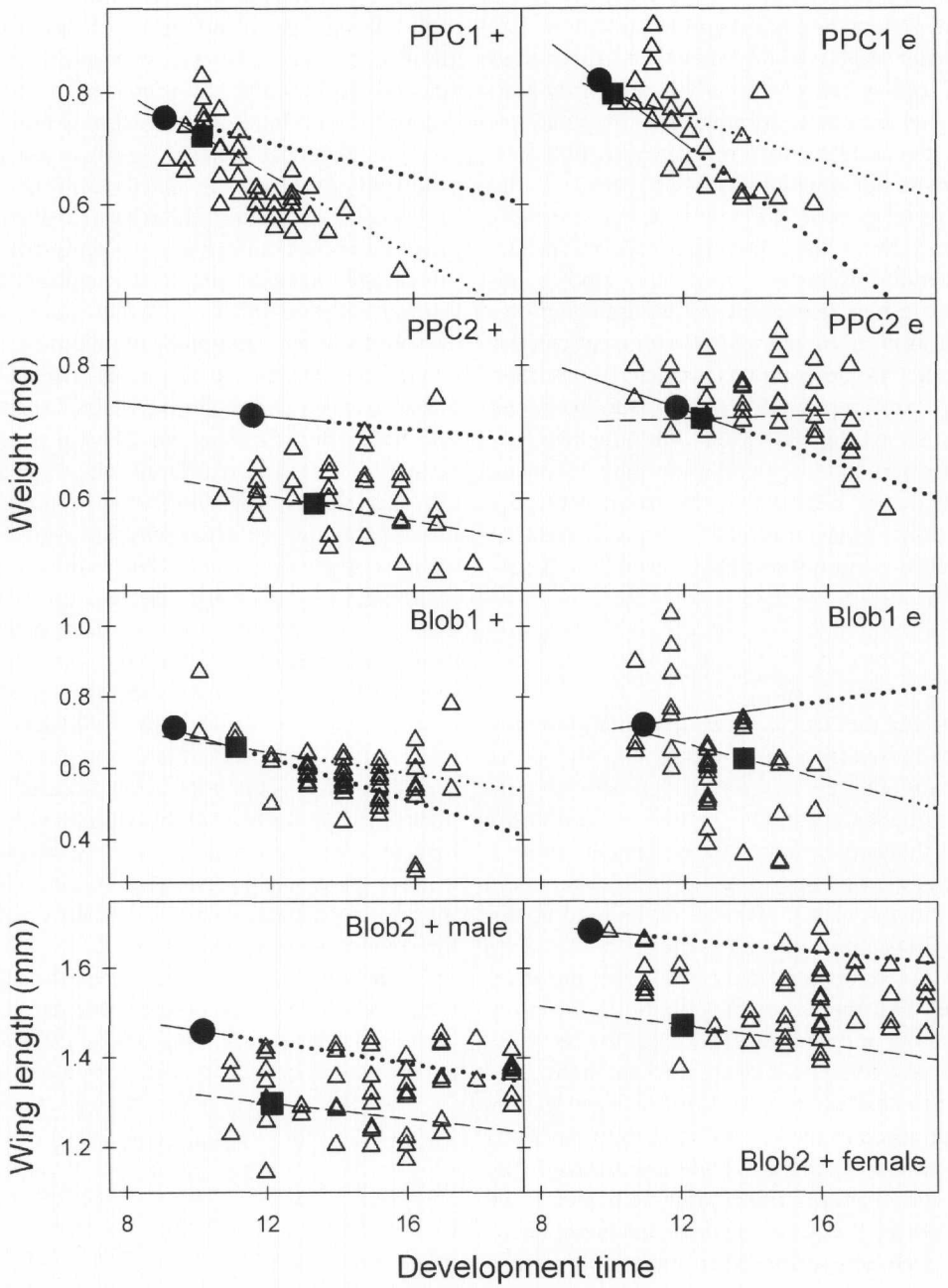

Figure 8: Norm of reaction for body size as a function of development time. In each experiment, the regression of body size on development time for the earliest cohorts of experimental flies (pulse time <6 h) is shown by the solid line. The dashed line shows the regression for flies in the middle cohorts (pulse times between 18 and 30 h). The range of the development times in each of these cohorts is that covered by solid or dashed lines; dotted lines extrapolate these regressions to longer development times. Solid circles show the means for the early cohort; solid squares show that for the middle cohort. Triangles show the individual (or group) estimates of size for flies eclosing from all pulse times later than 30 h.

d before transfer to fresh bottles, and successful reproduction declines to near 0 within 48 h of transfer.

Borash et al. (1998) recently presented evidence for a genetic polymorphism related to culture age in a set of populations maintained at very high densities. The evidence for this polymorphism is from a one-generation selection experiment for long and short development time, replicated over five populations of *D. melanogaster* main-

tained at high density and five at low density. The resulting late-selected lines from the high-density populations had significantly higher larval viability than any of the other lines, while the feeding rate of the early high-density populations was higher than those from the other populations. We think that it is unlikely that an analogous polymorphism is present in our populations. We have shown that variation in realized growth, development, and mortality rates is continuous rather than discrete. Moreover, the rate of decline in conditions varies considerably among cultures and even within a generation so that each genotype is likely to find itself in an array of environments. These are conditions that would promote plasticity rather than polymorphism. Borash et al. (1998) also made the liberal assumption that all mating takes place within the two "environments" of rapid and late developing flies. Modeling of similar cases makes it clear that the parameter space where a stable polymorphism results is markedly reduced under more realistic assumptions about gene flow (Curtsinger et al. 1994; Prout 1999).

Natural Selection in the Laboratory

In the first section of this article, we asserted that the study of natural selection in a laboratory setting may provide us with the opportunity to test the relationship between natural selection and adaptation. Our studies of natural selection support this contention, since our results show a detailed and consistent picture of natural selection on age at maturity in our population, which can be used as the basis for predictions about future changes.

We have encountered two related criticisms of this idea. First, some claim that the nature of selection in the lab is understood a priori and that detailed study of selection itself is unnecessary. We answer with two examples. Although we could obviously predict a priori that selection would favor reproduction early in the 14-d culture period, we were surprised that success would decline from a maximum for eggs laid at 24 h to near 0 only 24 h later. Even robust general predictions can benefit from quantitative refinement. We were also surprised to find evidence that viability was maximized for flies beginning development 24 h after a bottle was initiated. This unexpected result could lead to important insights about evolution in our population if confirmed by further research.

Another argument against the view that natural selection is already well understood in the laboratory points to the many examples where different outcomes result from slight alterations in selective regime, particularly with regard to "indirect" responses to selection (Harshman and Hoffmann 2000). Excellent examples are the studies of the evolution of senescence in *D. melanogaster*. Studies yield inconsistent "indirect" responses to selection for some traits, such as fecundity early in life (e.g., Partridge et al. 1999). In the case of early fecundity, some controversy has resulted, as has a great deal of increasingly careful experimental work aimed at understanding the causes of this inconsistency. One potential cause is unintentional selection; in cases like this, direct study of actual selection gradients of the traits involved would clearly be helpful.

A second criticism of the relevance of studies of natural selection in a laboratory population is that selection in the lab is likely to be so simple that it has little relevance to natural selection in the field. This issue is a serious one and clearly makes laboratory populations poor models for some questions, as are all model systems. The IV population has been insulated from macro- and micropredation and from abiotic stresses for 25 yr, a potential set of selective forces that virtually all natural populations experience. However, selection in the IV population captures essential features of many selection regimes whose effects we do not yet understand. For example, we have clearly demonstrated conflicting selection on aspects of the life history in our population. The effects of such conflicts in evolution are of perennial interest. Similarly, the environment of the IV population captures important sources of environmental variation both within generations (as the environment deteriorates) and among generations (in offspring density). Our population is called on to evolve a norm of reaction for age at maturity very different from that of a wild population, making accessible a host of general questions, such as that of plasticity versus polymorphism and the degree to which phenotypes can be fine-tuned by selection.

We believe that the marriage of the study of natural selection with studies of experimental populations will prove a powerful technique whose applicability will ultimately extend beyond evolution in a bottle.

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