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Direct and correlated responses to artificial selection on lipid and glycogen contents in *Drosophila melanogaster*

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Summary

A large outbred population of *Drosophila melanogaster* was subjected to artificial selection on lipid and glycogen storage. In three separate experiments, two replicates underwent sib selection for both increased and decreased storage. In the first study, flies were selected on the basis of total triacylglycerol for ten generations. This experiment resulted in no significant direct response, but there was a significant change in total body weight, underscoring the importance of concern for the allometric relationship between body weight and lipid content. In the second study, selection was performed for 15 generations on the percentage of body composition that was triacylglycerol. A significant direct response was obtained, and the two replicates revealed heritability estimates of 0·40 and 0·43. The third study selected glycogen content for 15 generations, and produced a significant response with heritabilities of 0·25 and 0·31. A series of 12 biochemical and enzyme kinetic traits was examined at five generation intervals in all experiments, and a number of correlated responses were detected. The results are interpreted with respect to the evolutionary constraints on energy storage evolution and the genetic basis of the allometric relationship between body weight and fat content.

1. Introduction

The connection between complex quantitative traits and single gene substitutions is best made when there is some prior understanding of the mechanism for the phenotypic manifestation of the trait. The pursuit of 'candidate genes' in human genetics is an excellent example of this approach, and is perhaps best exemplified by the association between *apoE* genotypes and the risk of coronary heart disease (Davignon *et al.* 1988). Correlated responses at targeted enzymes to selection for a related phenotype have been observed in *Drosophila* eye pigments and enzymes involved in pteridine synthesis (Barthelmess & Robertson, 1970), and selection on fat content in mice has been shown to result in correlated responses in lipogenic enzymes (Asante *et al.* 1989; Hastings & Hill, 1990). We have taken a similar approach in the analysis of lipid and carbohydrate storage in *Drosophila*, with the intention of understanding the evolution of the underlying quantitative genetic basis for these traits. Rather than focusing on a particular candidate gene, a series of related quantitative biochemical traits have been studied. Our approach has been to consider the storage of lipids and glycogen as pools in metabolic pathways, and to quantify the correlations among these storage pools and the activities of enzymes that should affect these pools. Enzyme activities are themselves quantitative traits influenced by many genes, and experiments have shown that the structural locus may account for less than half the variance in activity in a natural population (Laurie-Ahlberg *et al.* 1982; Miyashita & Laurie-Ahlberg, 1984, 1986). While this approach would appear to keep the analysis a step removed from discrete genetics, it gives an advantage in accessing a formal theory that provides some hope in quantifying mechanistic relationships among components of metabolic pathways. Although there are several aspects of the experimental system that depart from the assumptions of this metabolic control theory, the approach has been successful in finding a number of significant correlations between enzyme activities

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and quantities of stored fat and glycogen (Clark & Keith, 1988, 1989; Clark, 1989).

The purpose of the artificial selection experiments reported here is to quantify the realized heritabilities of the storage traits, and to determine the magnitudes of correlated responses of relevant enzyme activities. This provides a means of assessing whether natural selection, acting on storage pool sizes, would result in changes in activities of enzymes in relevant metabolic pathways. The allometric relations among the traits were found to have a significant influence on the outcome of selection.

2. Materials and methods

(i) *Founding population*

In June 1987, 240 gravid female *Drosophila melanogaster* were trapped in the peach orchard of the University of California at Davis Pomology Department near Putah Creek, California, USA. The progeny of the 240 females were pooled and the population was reared in six half-pint bottles in a discrete generation transfer protocol. Naturally occurring lethals and inversions were not scored, and presumably were segregating in the population. Each generation the flies from the six bottles were mixed and distributed into six fresh bottles of medium. The minimum population size during this time was estimated by weight to be 1000. In October 1987, or 10 generations after the flies were first brought into the laboratory, the first artificial selection experiment began. Throughout all experiments, flies were reared in incubators at 25 °C with a 12 h light/12 h dark cycle. The standard medium was Carolina 4-24, which is composed of 86·2% complex carbohydrate, 9·16% protein, 2·40% methylparaben (mould inhibitor) and 0·68% fat.,

(ii) *First lipid selection*

Pairs of flies from the mass population were placed into each of 120 vials and allowed to lay eggs for 4–5 days. On the eleventh day after egg laying began, two groups of 47 healthy vials were chosen from this set, one to found the up (increased lipid) and down (decreased lipid) selected lines of replicate 1, and one to found the two selected lines of replicate 2. When the progeny had emerged and aged 5–6 days, two males from each vial were collected and weighed. Flies were homogenized with a motorized homogenizer using a Kontes pestle and microcentrifuge tubes. Each pair of flies was homogenized in 400 μl of homogenization buffer (0·01 M-KH_2PO_4, 1 mM-EDTA, pH 7·4). The homogenates were spun at 2000 rev/min for 2 min to pellet debris, and the lipid layer was resuspended with the supernatant before being dispensed into microtitre plates. The assay of triacylglycerol content was done with a *V*max kinetic microtitre plate reader (Molecular Devices Corporation), using the Sigma triglyceride test reagent 336-20 as described in Clark & Keith (1989). This procedure hydrolyses triacylglycerols and quantitates the released glycerol enzymically (Bucolo & David, 1973). After adding 200 μl of reagent to each well of the microtitre plates, the sample was incubated at 29 °C for 30 min and the optical density was read immediately at 490 nm with the microtitre plate reader.

A simple computer program indicated the distribution of lipid contents among samples, their ranking, and the selection differential. For the up-selected lines, the adult sibs of the top 10 assayed pairs were taken from these vials and combined in a mass mating bottle and allowed to mate randomly for two days. After that time, single females were distributed into 60 vials, and allowed to lay eggs for 4–5 days to begin the next generation. The down-selected lines were maintained by selecting the lowest 10 vials among the set of 47 assayed each generation. This design allowed both up-selected replicates, a blank (homogenizing buffer only) and a standard (25 μl of 10 mg/dl triglyceride standard) to be tested on one microtitre plate. Similarly, both down-selected replicate populations were tested on a single plate. At generations 0, 5 and 10, the homogenates from each selected population were distributed among a set of 14 replicate microtitre plates for additional assays of glycogen storage and enzyme activities.

(iii) *Second lipid selection (weight-adjusted)*

Preliminary analysis of the first lipid selection experiment indicated a correlated response in body weight, and because of the allometric relation between body weight and lipid content a second lipid selection was begun in July 1988. Flies from the same population were used to found the selections, except that they had now been in culture in the laboratory for 26 generations. Two up-selected and two down-selected replicates were followed, and each generation 47 vials were tested from each replicate. Each homogenate was prepared with 7 adult males (instead of the previous 2 males), and the weights were entered into the computer before the selection of vials for the next generation. The routine then gave the distribution of the percentage of body weight that was lipid, and identified the vials from which the upper (or lower) 10 samples came. Siblings from these vials were pooled in a mass mating bottle for two days, and inseminated females were distributed into vials to found the next generation.

(iv) *Glycogen selection*

Beginning in March 1988, another artificial selection experiment was begun on glycogen content. As before, there were two up-selected replicates and two down-selected replicates, and each replicate was represented

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by 47 vials each generation. From the assays of 10 males from each of the 47 vials, 10 vials were selected for the next generation. The groups of 10 males were homogenized in 1200 μl of homogenizing buffer, and centrifuged at 7000 rev/min for 2 min. The supernatant was dispensed in 25 μl aliquots into microtitre plates. A blank containing only homogenizing buffer was reserved on each plate, as was a standard of 25 μl of 1 mg/ml glycogen dissolved in the homogenizing buffer. The glycogen assay was performed with the kinetic microtitre plate reader by first hydrolysing the glycogen with amyloglucosidase (Boehringer Mannheim 208469 at 93·5 μg/ml) and then quantifying total glucose as described in Clark & Keith (1989). The test reagent contained 0·1 U/ml amyloglucosidase (1,4-α-D-glucan-glucohydrolase E.C. 3.2.1.3), 5 U/ml glucose oxidase, 1 U/ml peroxidase, 0·04 mg/ml *o*-dianisidine dihydrochloride. The reagent was buffered by salts contained in the Sigma preparation of glucose oxidase and peroxidase (PGO enzyme, catalogue no. 510-6). A 200 μl aliquot of reagent was added to the 25 μl samples and incubated at 37 °C for 30 min. Optical density was read at 450 nm, and concentration of glycogen was determined from glycogen standards run with each assay.

The homogenates that had been collected and stored at −70 °C at generations 5, 10 and 15 were tested for a series of enzyme activities including fatty acid synthase (FAS), glucose-6-phosphate dehydrogenase (G6PD, E.C. 1.1.1.49), α-glyceraldehyde phosphate dehydrogenase (GPDH, E.C. 1.1.1.8), glycogen phosphorylase (GP, E.C. 2.4.1.1), glycogen synthase (GS, E.C. 2.4.1.11), hexokinase (HEX, E.C. 2.7.1.1), malic enzyme (ME, E.C. 1.1.1.40), 6-phosphogluconate dehydrogenase (6PGD, E.C. 1.1.1.44), phosphoglucose isomerase (PGI, E.C. 5.3.1.9), phosphoglucomutase (PGM, E.C. 2.7.5.1), and trehalase (TRE, E.C. 3.2.1.28). These assays were also performed on the microtitre plate reader, as described in Clark & Keith (1989).

(v) *Statistical methods*

Estimation of realized heritabilities requires the fitting of data to a model, and the model involves a number of assumptions (Hill, 1971). First, the genetic variances and covariances are assumed to be constant during the course of the selection, and inbreeding is assumed to be negligible. The trait is assumed to be affected by many genes of small effect with no confounding influence of linkage. The environment is assumed to be constant, but small environmental influences are controlled by taking the difference between up- and down-selected populations as the response. The realized heritability is estimated as the regression coefficient of the response against the cumulative selection differential, with both response and selection differential calculated as the difference between the up- and down-selected populations (Hill, 1971).

The significances of correlated responses were assessed by analysis of covariance, fitting the model:

$$Y_{ijk} = \mu + D_i + RD_{ij} + \beta(\bar{w} - w_{ij}) + \epsilon_{ijk},$$

where Y_{ijk} is the measure of the kth sample of the jth replicate of the ith direction. The effects include D_i for the ith direction and RD_{ij} for the jth replicate nested in the ith direction. β is the regression of weight on the response variable, so the contribution of weight is adjusted out of the response. The grand mean is μ and the error term is ϵ_{ijk}.

Allometric relationships between total live weight and lipid or glycogen weights were obtained by fitting the allometric relation $Y = aX^b$, where Y is the lipid weight and X is the total body weight. After logarithmic transformation, this equation becomes log(lipid weight) = β[log(total weight)], and β is the allometric parameter, β was estimated by linear regression of the log-transformed data.

3. Results

The first lipid selection experiment yielded an unexpected result. There was no direct response to lipid storage selection, but the live weight of the down-selected lines decreased significantly (Fig. 1). The lipid content is plotted as a weight-adjusted deviation from the generation mean, and the weight adjusting is accomplished by analysis of covariance. If Fig. 1 were plotted as the raw lipid amounts, there would be a slight increase in the divergence between treatments, but the response would still not appear significant (i.e. no significant regression of raw lipid on cumulative selection differential). If Fig. 1 were plotted as the percentage of the body weight that is lipid (i.e. raw lipid divided by body weight), then the down-selected line, which decreased in body weight, would paradoxically appear to have increased in lipid content. This result underscored the importance of identifying the selected character carefully, and a second lipid selection experiment was designed to correct the problem.

The second lipid selection used lipid contents as a percentage of body weight for the selection criterion, and as Fig. 2 shows, it resulted in a significant direct response, and an absence of a correlated response in body weight. The cumulative selection differential was calculated as the deviation between the up- and down-selected lines of each replicate separately, and adjusted by the intraclass correlation coefficient because this is a sib selection (Hill, 1972). Heritability was calculated as the regression coefficient of cumulative response on the cumulative selection differential (Hill, 1972; Falconer, 1980; Becker, 1984), and was found to be 0·40 and 0·43 for the two replicates.

The glycogen selection experiment was begun before the first lipid selection experiment was completed, so the importance of using weight-adjusted selection criteria was not known. Consequently the glycogen

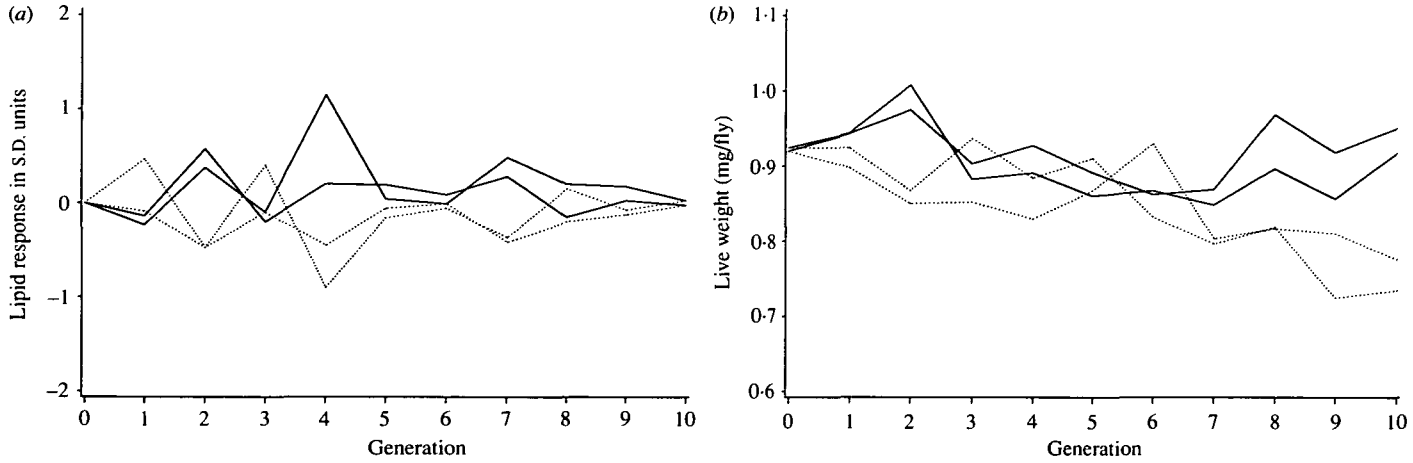

Fig. 1. First triacylglycerol (lipid) selection experiment. (*a*) Mean response of lipid contents, expressed as deviations from the generation mean, in units of standard deviations. (*b*) Mean weights of the two up-selected replicates (——) and the two down-selected replicates (....).

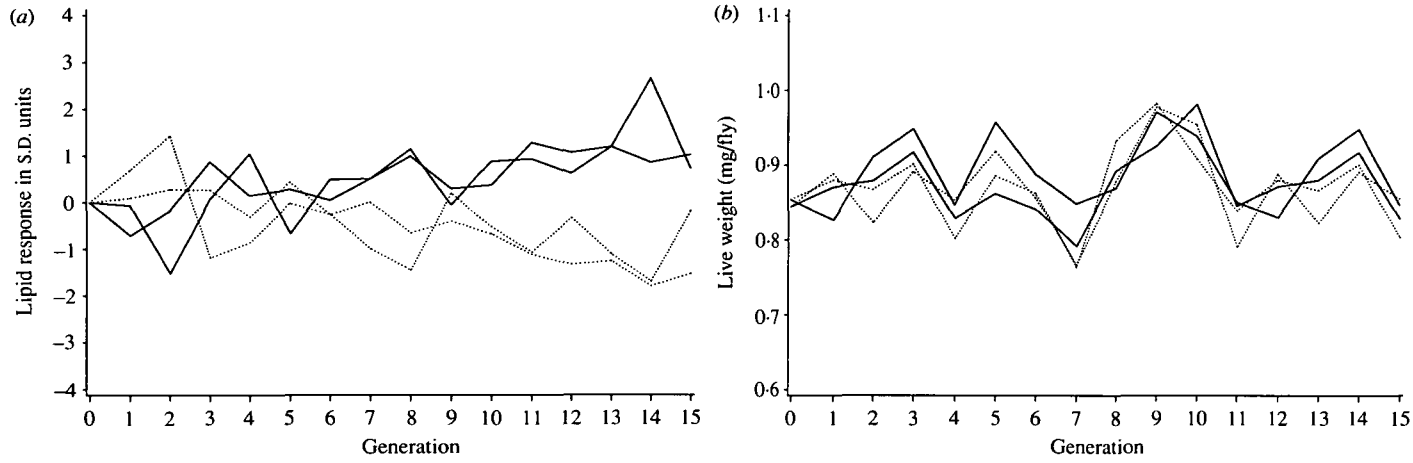

Fig. 2. Second lipid selection experiment. (*a*) Mean response of lipid contents, expressed as deviations from the generation mean, in units of standard deviations. (*b*) Mean weights of the two up-selected replicates (——) and the two down-selected replicates (....).

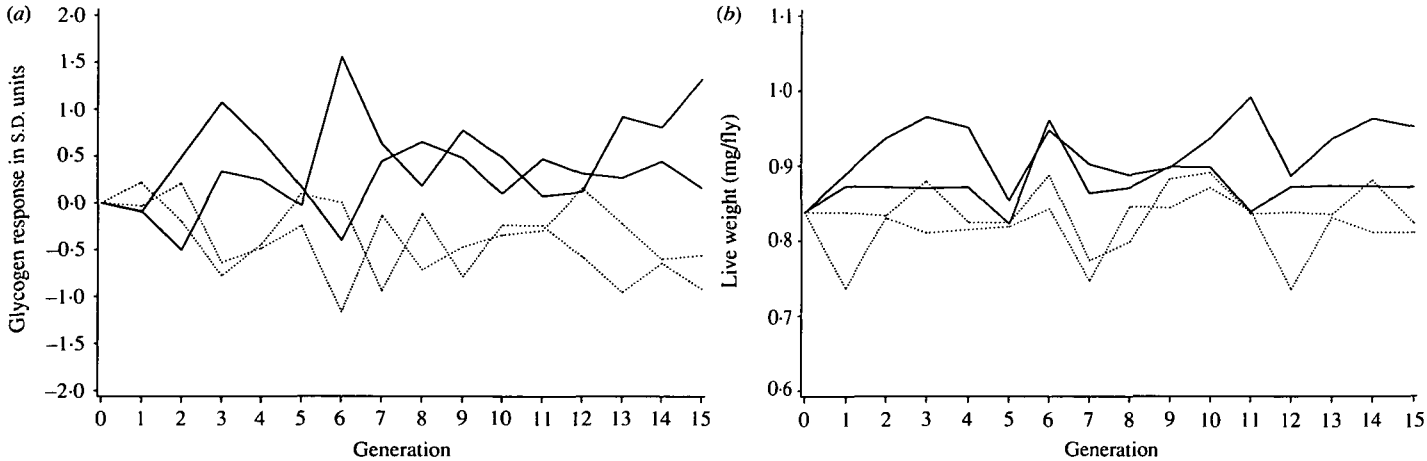

Fig. 3. Glycogen selection experiment. (*a*) Mean response of glycogen contents, expressed as deviations from the generation mean, in units of standard deviations. (*b*) Mean weights of the two up-selected replicates (——) and the two down-selected replicates (....).

selection was based on raw glycogen amounts, rather than weight-adjusted glycogen contents. As Fig. 2 indicates, there was a tendency for the down-selected lines to have a lower body weight, but the difference remained fairly constant throughout the experiment. Despite the body weight difference, there was a significant direct response in glycogen whether calculated as raw glycogen, weight-adjusted glycogen, or percentage of body weight that is glycogen. Fig. 3 displays the response as weight-adjusted deviations

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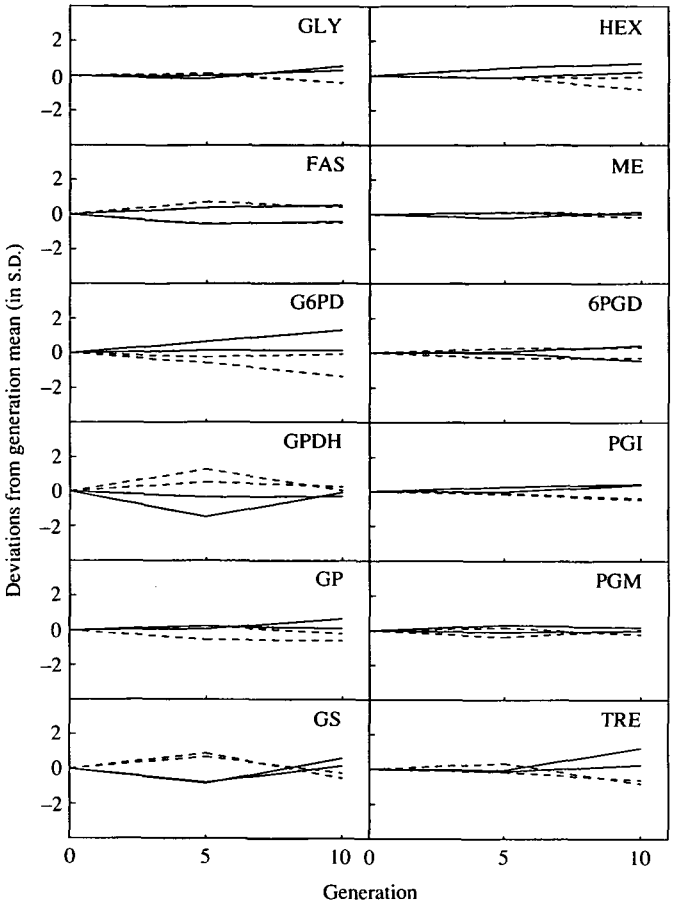

Fig. 4. Correlated responses scored at generations 0, 5 and 10 of the first lipid selection experiment. As in the previous figures, the solid lines are for the up-selected replicates, and the broken lines represent the down-selected replicates.

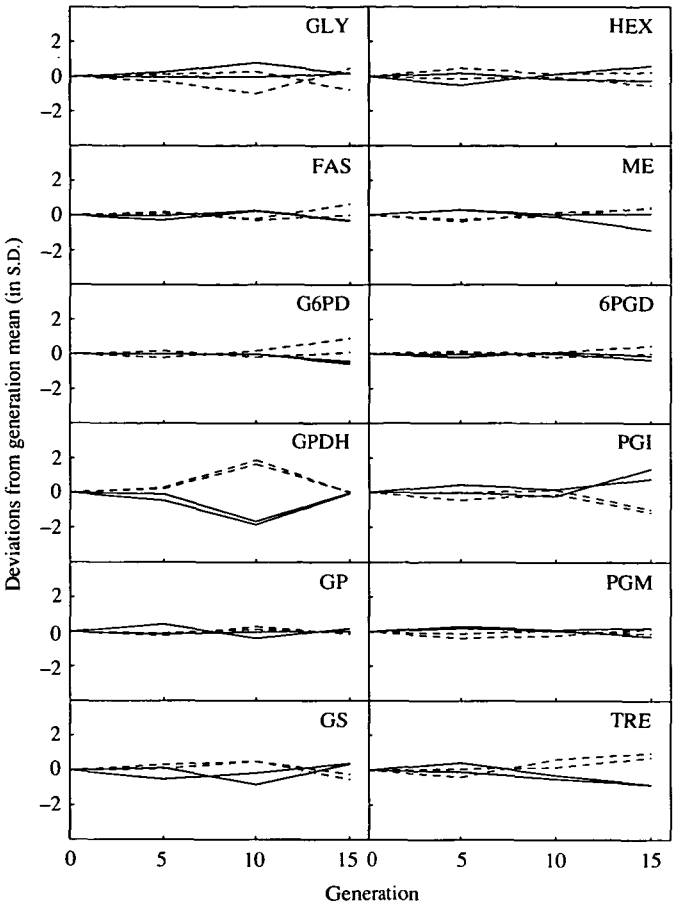

Fig. 5. Correlated responses scored at generations 0, 5, 10 and 15 of the second lipid selection experiment. As in the previous figures, the solid lines are for the up-selected replicates, and the broken lines represent the down-selected replicates.

from the generation mean. When realized heritabilities were calculated as in the second lipid selection, they were found to be 0·25 and 0·31. The observation of lower heritabilities is consistent with the lower total response seen in Fig. 3.

At generations 5, 10 and 15, homogenates had been distributed into sets of microtitre plates for scoring the activities of a number of enzymes. Figs. 4, 5 and 6 show these correlated responses as weight-adjusted deviations from the generation mean. The significance of correlated responses was determined in a conservative fashion by analysis of covariance. The model tests the null hypothesis that there is no difference between the up- and down-selected treatments in the mean weight-adjusted measures, using the among replicate variance error in a nested design (see Methods). The results of these significance tests appear in Table 1. The only correlated response seen in the first lipid selection experiment was an increase in glycogen synthase activity in generation 5 of the down-selected lines. The lack of correlated responses in the first experiment is not surprising considering the lack of a direct response.

The second lipid selection produced a number of significant correlated responses. Fatty acid synthase had a higher activity on the up-selected lines at generation 10, consistent with previous observations that lines of *Drosophila* with high lipid storage often have higher FAS activities. This result is consistent with the position of FAS in lipid metabolism, since higher synthetic rates – all else being equal – would result in increased concentration of a 'downstream' pool. The absence of a difference at generation 15 weakens this conclusion. GPDH and GS showed a similarly strong significant effect at generation 10, which then disappeared by generation 15. These transient responses may be real, and the initial response to lipid selection may result in changes in the metabolic balance which is subsequently restored. PGI and TRE showed significant effects at generation 15, with the up-selected lines having a higher PGI activity and a lower TRE activity.

The glycogen selection experiment also yielded only a few significant correlated responses. PGI had higher activity in the down-selected lines (the opposite of the lipid selection). The only case of a significant reversal in sign was seen in TRE, which began with the up-selected lines having higher activity, but ended with the reverse. While a number of significant genetic correlations were identified by Wilton *et al.* (1982),

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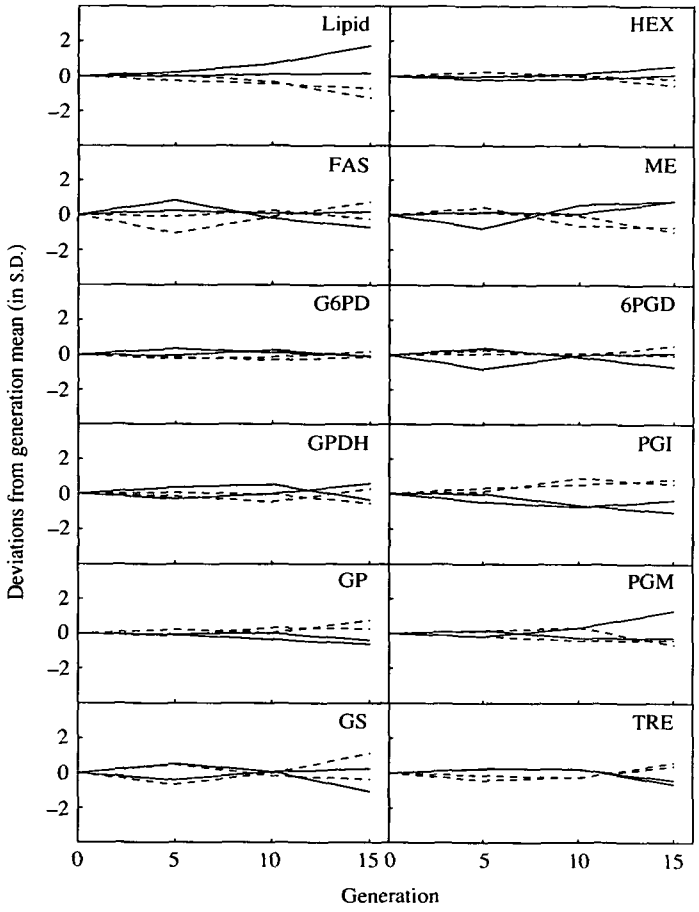

Fig. 6. Correlated responses scored at generations 0, 5, 10 and 15 of the glycogen selection experiment. As in the previous figures, the solid lines are for the up-selected replicates, and the broken lines represent the down-selected replicates.

Table 1. *Results of significance tests for correlated responses. Analysis of covariance was performed at each generation on each character to test whether the direction of selection had a significant effect. The ANCOVA model is described in Methods*

	First lipid selection		Second lipid selection			Glycogen selection		
	5	10	5	10	15	5	10	15
TRI	d	d	d	d	d	—	*	—
GLY	—	—	—	—	—	d	d	d
FAS	—	—	—	**	—	—	—	—
G6PD	—	—	—	—	—	—	—	—
GPDH	—	—	—	**	—	—	—	—
GP	—	—	—	—	—	—	—	—
GS	**	—	—	*	—	—	—	—
HEX	—	—	—	—	—	—	—	—
ME	—	—	*	—	—	—	—	*
6PGD	—	—	*	—	—	—	—	—
PGI	—	—	—	—	*	*	*	*
PGM	—	—	—	—	—	—	—	—
TRE	—	—	—	—	*	—	**	**

* $P < 0{\cdot}05$, ** $P < 0{\cdot}01$, d = direct response.

Clark & Keith (1989) and Clark (1989), the modest direct-selection response (which resulted in part from the relatively weak selection differential imparted by the sib selection), and the conservative statistical test account for the paucity of correlated responses seen here. The consistency of these results of the extracted line studies, the sib analysis of Clark (1990) and of these selection experiments is discussed below.

4. Discussion

Response to artificial selection represents one of the most direct means of demonstrating a genetic basis for a complex character. In the case of individual selection, quantitative genetics theory shows that the response is directly proportional to the heritability. With sib selection, as was practised in this study, the response depends also on the correlation between the tested sibs and those that are actually used to propagate the population. These complications, along with the problems of allometry discussed below, suggest that it would not be unlikely to observe differences between estimates of heritability obtained through parent–offspring regression vs. realized heritability. The estimates of heritabilities of lipid and glycogen content by maximum likelihood, using both parent–offspring and half-sib data, were 0·29 and 0·49 respectively (Clark, 1990), while the realized heritabilities were 0·42 and 0·27. The discrepancy is due in part to the identification of the selection criterion, since the selection on total lipid resulted in no response, while the weight-adjusted selection criterion gave a dramatic response.

The apparent magnitude of genetic correlations was greater in both the extracted chromosome studies and the sib analysis (Clark & Keith, 1988; Clark, 1989). This is not unexpected, for the same reasons that the heritabilities may not be exactly comparable. A consistent correlation that had been observed was between lipid storage and the activity of FAS, and the only significant correlated response observed in this study was a higher FAS activity in up-selected lines at generation 10 of the second lipid-selection experiment. An interesting correlated response occurred in the activity of PGI. Both lipid experiments had an increased activity in the up-selected lines, and the glycogen selection resulted in a decreased activity in the up-selected replicates. The importance of PGI as a glycolytic switchpoint has been emphasized by Watt *et al.* (1983), and warrants further examination as a possibly critical branch point between lipid and carbohydrate storage.

The importance of allometric relations between body weight and body content has been widely appreciated by animal breeders. Fat content is a trait of considerable economic importance to a number of domestic animals, and a number of selection experiments on fat content have been performed on animal models in the laboratory. Mice are representative of

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one aspect of these studies, in that the allometric relation between total body weight and fat content has a slope greater than one (Bailey *et al.* 1988). This means that selecting for increased size, a trait that is often economically desirable, results in a correlated increase in fat content that results in a higher percentage body content that is fat. This correlated response is generally not desirable, and while selection index methods can be devised to weaken the correlation, the fat-deposition characters often have higher heritability than lean body weight characters (Eisen & Prasetyo, 1988). Chickens, turkeys and Japanese quail show a similar allometry of body and fat weights, with heavier birds having a higher percentage of fat (Darden & Marks, 1988; Leenstra, 1988; Leenstra & Pit, 1988). The idea that these allometric relations may themselves be modified genetically is supported by the correlation between fat:body mass ratio and the plasma levels of insulin-like growth factor I (McKnight & Goddard, 1989). The mouse system is especially good for finding biochemical correlates with selection response. Lines of mice selected for high and low fat content (ratio of gonadal fat pad weight to total weight) were found to exhibit significant differences in activities of NADPH-generating enzymes (Asante *et al.* 1989) and enzymes involved in lipogenesis, including fatty acid synthetase (Hastings & Hill, 1990).

The unexpected finding here is that the regression of the log of body weight on the log of lipid yields an allometric parameter (β) of $0{\cdot}55 \pm 0{\cdot}52$ in the first lipid selection and $0{\cdot}43 \pm 0{\cdot}06$ in the second lipid selection. This is quite different from the general pattern for the allometry of body weight and fat content in vertebrate animals. As cited above, most vertebrate animals have an allometric slope that is greater than one, meaning that a doubling in weight results in a more than doubling in fat. The low allometric slope in flies means that as body weight increases, the percentage that is fat decreases. This provides an explanation for the paradoxical response seen in the first lipid selection. As selection for decreased lipid proceeds, body weight decreases. However, flies with lower body weight have a higher weight-percentage of lipid, assuming they fall on the same allometric curve. The most likely reason for this unusual allometry is that heavier flies generally have a higher water content, and the lipid and water contents in *Drosophila* are negatively correlated (Clark & Doane, 1983).

The broad objective of this study is to determine the evolutionary basis for why *Drosophila* store the quantities of lipid and glycogen that they do. Part of the answer will require the determination of whether the differences in storage are related to activities of enzymes in synthetic and degradative metabolic pathways. Experiments have been performed to quantify the variation in the storage traits and possibly relevant enzyme activities, and to determine the magnitude of genetic variation for these traits. The pattern of genetic variances and covariances is affected by evolutionary forces acting on a group of traits, and to some extent it is possible to infer the action of those forces from population samples. When traits are mechanistically related, so that a quantitative model for the interaction of the traits on an ensemble property like fitness can be hypothesized *a priori*, then there is some possibility for model verification. Metabolic control theory provides a framework to allow predictions of properties of metabolic pathways from individual components (Kacser & Burns, 1973, 1979, 1981; Heinrich & Rapoport, 1983), and aspects of the theory may be applicable here. Multivariate selection models allow prediction of response to selection on sets of correlated characters when the genetic variance–covariance matrix is known. We are still a long way from tying all of these approaches together, but the goal of this study was to quantify the genetic relations among the traits, and to begin to determine how the components of a metabolic pathway respond to selection acting on a global property of a specified pathway.

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