

Cellular Consequences in the Brain and Liver of Age-Specific Selection for Rate of Development in Mice

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ABSTRACT

Changes in cell number (hyperplasia) and cell size (hypertrophy) in the brain and liver are described for mice subjected to 24 generations of age-specific restricted index selection for rate of development in body weight. One selection treatment (*E*) altered rate of development between birth and 10 days of age, another treatment (*L*) involved changes in rate of development between 28 and 56 days of age, while a third control treatment (*C*) involved random selection. Each selection treatment was replicated three times. These age-specific selection treatments focused on intervals during ontogeny when different developmental processes (hypertrophy or hyperplasia) were more predominant in the control of growth. Significant changes in brain and liver weight occurred at both 28 and 70 days of age. Early selection (*E*) generated significant changes in the number of cells in the brain while later selection (*L*) had no effect since the brain had stopped growth before selection was initiated. For the liver, early and late selection produced significant effects on both cell number and cell size. These results describe the dynamic and multidimensional aspects of selection in terms of its ability to alter different cellular and developmental components of complex morphological traits.

MAMMALIAN growth is a dynamic, multidimensional process involving genetic and developmental interactions at many levels of organization. Studies on the genetic architecture of growth in rodents suggest complex patterns of age- and tissue-specific gene expression where certain loci are active at one phase of ontogeny and not during other phases. The evolutionary ramifications of such a mosaic of temporal and spatial gene expression patterns on direct and correlated response to selection in complex, polygenically controlled traits could be considerable.

The underlying control of mammalian growth involves many different processes and mechanisms (Figure 1). Distinct sets of genes regulate early and late growth in mice. Cheverud *et al.* (1996) observed 5 chromosomally distinct quantitative trait loci (QTL) with effects restricted to early growth while ~12 QTL influenced later growth. The effect of loci affecting early growth decreases by ~6 wk of age while those influencing later growth first appear between 3 and 6 wk of age. Indeed, these authors found 7 QTL affected body weight at 7 days of age while 17 were involved at 70 days of age. Cheverud *et al.*'s findings corroborate a number of experimental analyses showing ontogenetic changes in additive and nonadditive genetic variances and co-

variances for a number of traits in rodents. For example, Atchley and Zhu (1997) found that new additive genetic variance for body weight arises during ontogeny in mice corresponding to temporal patterns of gene expression as described by Cheverud *et al.* (1996).

Such differential gene expression patterns parallel the actions of hyperplasia, hypertrophy, and apoptosis, which are cellular processes that operate during different time periods in ontogeny. In many organs, early growth is characterized by cell proliferation (hyperplasia) with little cell enlargement, while late growth is determined by cell enlargement (hypertrophy; Enesco and Leblond 1962; Winick and Noble 1965; Goss 1966; Falconer *et al.* 1978). Hyperplasia and hypertrophy are connected by a transitional phase during which time both processes occur. Acting in concert is apoptosis or programmed cell death, a genetically regulated, signal transduction-dependent process that provides a precise regulation of cell numbers during growth and development. Apoptosis has been conserved over a wide range of phyla.

Indeed, these various cellular processes can exhibit age-specific episodes of positive and negative interaction and are probably involved with compensatory growth patterns (*e.g.*, Riska *et al.* 1984). Hyperplasia, hypertrophy, and apoptosis are under separate genetic control, have different underlying causal components of variability, and therefore each should respond to selection, either separately or in concert (Baserga 1981; Corradino 1982; Veneziale 1985; Vaux 1993; Cheverud *et al.* 1996; Yuan 1996; King and Cidlowski 1998).

This article is dedicated to our friend and colleague Bruce Riska whose early medical retirement deprived us of a creative and imaginative colleague.

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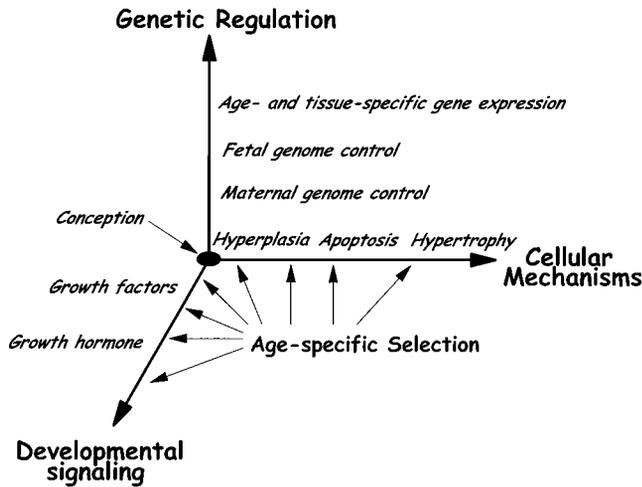


Figure 1.—A schematic summary of the multidimensional nature of the control of mammalian growth and age-specific selection. Each axis summarizes ontogenetic changes in one aspect of growth. Genetic regulation shows that initially fetal development is under control of the maternal genome but the fetal genome itself assumes control followed by age- and tissue-specific patterns of gene expression. Selection may act at several points during ontogeny and on various developmental components.

Paralleling ontogenetic differences in gene expression and cellular architecture are differences in “developmental signaling.” Prenatal and early postnatal growth in mammals occurs in an environment dominated by various cellular growth factors and in the absence of growth hormone. Insulin-like growth factors, such as IGF-2, exist in high concentrations and appear to dominate the regulation of various organs in rodents until ~18 days of age when growth hormone assumes a predominant role (Atchley *et al.* 1984; Cheverud *et al.* 1996).

Superimposed upon these genetic, cellular, and developmental effects are the epigenetic consequences of maternal effects (*e.g.*, Falconer 1965; Willham 1972; McLaren 1981; Atchley *et al.* 1991, 1994; Falconer and Mackay 1996). Early in ontogeny there is a transition from maternal control of fetal development to control by fetal genes. However, a significant uterine and postnatal maternal influence that is separate from the direct transmission of maternal nuclear genes may still persist that significantly influences development (Falconer 1965; Willham 1972; McLaren 1981; Atchley *et al.* 1991). Indeed, uterine and postnatal maternal effects may condition the expression of fetal genes during development (Atchley and Hall 1991; Atchley *et al.* 1994). Uterine maternal effects include heritable and nonheritable maternal attributes and are usually most pronounced early in postnatal ontogeny and decrease in importance as the organisms age. However, some maternal effects can be long-lived (*e.g.*, Atchley *et al.* 1991).

A series of developmental quantitative genetic models

have been proposed by our research group to better understand the evolution of complex morphological structures (Atchley 1984a,b, 1987, 1990, 1998; Atchley *et al.* 1984, 1991, 1994; Riska and Atchley 1985; Atchley and Hall 1991). These models stress the dynamics during ontogeny of the cellular components of growth and morphogenesis. Indeed, many authors have stressed the importance of evolutionary changes in hyperplasia, hypertrophy, and apoptosis in understanding the origin and evolution of complex morphologies (*e.g.*, Enesco and Leblond 1962; Winick and Noble 1965; Eisen *et al.* 1978; Falconer *et al.* 1978; Katz 1980; Del one *et al.* 1987; Hall 1992). What has been lacking is the experimental verification of many critical parts of these models.

A poorly understood aspect of the evolution of complex morphologies is the consequence of age-specific selection applied to a mosaic of developmentally varying structures. Change by selection in the overall phenotype of a complex trait like body weight is easily observed and studied. Less easily observed or understood is which underlying developmental components of a complex trait have been changed as the result of selection. As noted in Figure 1, age-specific selection can affect different component parts in growth and morphogenesis, depending upon when during ontogeny selection occurs. Further, developmental homoplasy may result where selection on different components of development produces phenotypically identical adult organisms (Atchley *et al.* 1997). Finally, age-specific selection can alter the genetic associations among traits depending upon the patterns of gene expression during ontogeny when selection was occurring.

Herein, we examine some cellular aspects of response to selection in a series of mouse lines selected for early and late rates of development in body weight. The *raison d'être* for this long-term selection experiment was to explore the cellular and developmental consequences for age-specific selection, to ascertain whether similar phenotypes could be generated through quite different genetic and developmental mechanisms, and to test evolutionary models about brain-body size evolution.

In the present analyses, we evaluate the overall hypothesis that age-specific selection can differentially alter relevant developmental components that comprise complex traits. Specifically, we evaluate the following hypotheses:

1. Selection early in postnatal ontogeny has a significant impact on cell number rather than cell size. This effect will be particularly pronounced in the brain since it develops early in ontogeny. This is a test of the hyperplasia hypothesis proposed by Riska and Atchley (1985) to explain some of the different patterns of brain-body size evolution previously described by Jerison (1973), Gould (1975), Lande (1979), Shea (1983), and others.

2. Selection on rate of development occurring later in ontogeny will have little impact on traits that undergo growth prior to the onset of selection.
3. Selection occurring later in ontogeny will have more of an impact on cell size than cell number.
4. Traits with different growth patterns will be differentially affected by age-specific selection.

MATERIALS AND METHODS

The present experiments were performed using mice from an ongoing long-term selection experiment. Five selection regimes are involved: E^+L^0 [E^+] was selected to increase early body weight gain (rate of change from birth to 10 days of age) while holding late weight gain (28 to 56 days) constant; E^-L^0 [E^-] was selected to decrease early gain while holding late gain constant; E^0L^+ [L^+] was selected to increase late gain while holding early gain constant; E^0L^- [L^-] was selected to decrease late gain while holding early gain constant; and E^0L^0 [C] was the randomly-selected control line. Each line was replicated three times for a total of 15 genetically independent lines. A more detailed description of the lines and the direct results of selection through generation 15 can be found in Atchley *et al.* (1997).

Herein, we examine cell number and cell size in brains and livers of 28- and 70-day-old animals. For analyses of cellular traits in 70-day-old mice, one male and one female were chosen from each of the 12 litters in each line and replicate (5 lines \times 3 replicates \times 12 litters \times 2 sexes \times 1 individual = 360 individuals) in generation 23. For analyses of cellular traits at 28 days of age, 5 females and 5 males randomly chosen from separate litters within each selection line and replicate were mated (75 pairings) for a total of 223 mice in generation 24. The use of mice from two successive generations was necessary because of the need to maintain the ongoing selection experiment. Because of the types of hypotheses being evaluated, there should be little if any impact from using mice from two successive generations.

Data collection: Mice were assigned unique identification numbers by toe-clipping at 6 days of age. Body weights were recorded at birth, 10, and 28 days of age for both experiments, and at 56 and 70 days of age for the 70-day analyses. Twenty-eight days of age represented the end of the early selection interval. We recorded data on the late-selected mice at 70 days of age simply to achieve data that were compatible with those of our several other experiments on ICR mice (*e.g.*, Atchley *et al.* 1984; Riska and Atchley 1985).

Litters were standardized at birth to eight pups and an even sex ratio. Litters with fewer than eight pups were augmented with excess pups from other litters. Measurements on substitute pups were not included in the analyses.

Cell number and cell size at 28 and 70 days of age were estimated for brain and liver tissue. These organs were chosen because they have quite different times of origin, differentiation, and patterns of growth (Altman and Katz 1962; Kobayashi 1963). The brain is one of the first organs to begin growth and differentiation in mammals, and its size is allometrically related to early body size (Atchley *et al.* 1984; Riska and Atchley 1985). The liver develops later in ontogeny and because of its role in metabolism, the size of the liver is highly correlated with later body size (Eisen 1986).

Estimates of the number and sizes of cells in organs or tissues can be obtained by direct counts of cells or nuclei or by using indirect estimates obtained by measuring the amount of DNA. Many researchers (*e.g.*, Enesco and Leblond 1962; Winick and Noble 1965) found it more efficient to estimate

cell numbers by measuring DNA amount as opposed to directly counting cells or nuclei because this approach could be applied quickly to large samples. It is assumed that the amount of DNA per cell is constant for diploid cells of mammals (Baserga 1995). In the present study, DNA amount in the liver and brain were estimated using the method of Labarca and Paigen (1980), which permits crude tissue homogenates to be used, thereby eliminating a DNA purification step. Presence of RNA does not interfere with this assay, and as little as 10 ng of DNA can be detected quickly and reliably.

For both brain and liver, total DNA per organ was calculated as total DNA_(ng)/organ = (DNA concentration \times volume of liquid measured)/dilution percentage). Total amount of DNA in each mammalian cell is assumed to be constant (6×10^{-6} ng per cell; Baserga 1995) so cell number was calculated as the total DNA_(ng)/ 6×10^{-6} (ng per cell).

From the calculation of total DNA, an estimate of cell size was determined by dividing the total organ weight by the number of cells in that organ. Extracellular components such as connective tissue could affect the estimate of cell size; however, this error would not be expected to differentially affect the groups (Falconer *et al.* 1978). Our measure of cell size, therefore, is the weight of organ associated with each nucleus, expressed in picograms (grams $\times 10^{-12}$).

Mice were weighed and sacrificed at 28 days or 70 days. Entire brains and livers were removed, weighed, and placed into individual tubes containing 10 ml phosphate-saline buffer (0.05 M NaPO₄, 2.0 M NaCl, 2 mM EDTA, pH 7.4). Each organ was homogenized and tubes containing crude cellular homogenates were brought to a volume of 40 ml. Calf thymus DNA (200 ng/ μ l concentration) was the standard DNA sample that was measured along with each group of mouse brain and liver samples. Cell number and size were estimated from measures of total DNA by the Hoechst 33258 assay using a fluorescence spectrophotometer (Brunk *et al.* 1979; Labarca and Paigen 1980). Two independent measurements were taken on each tissue sample to determine the repeatability of the procedure. The measured fluorescence units (FU) were plotted against DNA concentration, and only those points ranging in a straight line were used to calculate the standard slope by regression. Concentrations of DNA were determined for each sample on the basis of the slope of the line generated by the standard curve for that particular group of measurements.

Statistical analyses: At 28 days of age, two individuals of each sex from each litter were examined to estimate the litter effect. Our statistical model was modified appropriately for the 28-day analyses (Model 1),

$$Y_{ijklmn} = \mu + \alpha_i + \beta_{j(i)} + \gamma_k + \delta_l + \chi_{m(ij)} + \varepsilon_{ijklmn}$$

where Y_{ijkln} is the observation for individual n of sex k in replicate j of line i and measured in group l , and $\chi_{m(ij)}$ is the term for litter effect. Interactions among effects were not significant for either 28-day or 70-day analyses and these terms subsequently were dropped from the models.

Data for 70-day body weight, brain weight, liver weight, total DNA, cell number, and cell size for each organ were fit to Model 2,

$$Y_{ijkln} = \mu + \alpha_i + \beta_{j(i)} + \gamma_k + \delta_l + \varepsilon_{ijkln}$$

where all of the variables represent the same effects as in Model 1. Two independent estimates were made of the cell number and cell size traits in order to measure repeatability. The product-moment correlation coefficient for the two measures estimates the extent of repeatability. The correlations between the two measures were statistically highly significant ($P < 0.01$). All subsequent analyses were carried out on the average of these two estimates. Litter effects were not included

TABLE 1

Line means and least significant difference (LSD) tests showing divergence in body weight at 28 and 70 days of age in selected lines of mice for brain and liver size at 28 and 70 days of age

28 days		70 days	
Body weight (g)	LSD	Body weight (g)	LSD
18.105	<i>E⁻</i>	28.161	<i>L⁻</i>
21.002	<i>L⁺</i>	33.677	<i>E⁻</i>
22.071	<i>L⁻</i>	33.897	<i>C</i>
22.764	<i>C</i>	40.535	<i>E⁺</i>
27.081	<i>E⁺</i>	41.094	<i>L⁺</i>
<i>1.759</i>		<i>3.220</i>	

Traits within each age are ranked and those connected by a vertical line are not statistically different at $P < 0.05$ by a LSD test. Pooled standard errors are given in italics for each trait.

in the 70-day model because only one individual of each sex from each litter was examined.

Analysis of variance (ANOVA) was carried out on each trait using the general linear models procedure (SAS Institute 1988). The presence of sexual dimorphism and differences among selection lines was evaluated using least-squares means. Because of the large numbers of comparisons in the correlation analyses, we employed the Bonferroni adjustment procedure, which adjusts the significance levels of statistical tests to more accurate levels of type one error rates. However, this procedure sometimes reduces one's ability to reject a null hypothesis.

To examine how selection influenced the covariance structure in each selection line, correlation coefficients were computed between the two organ weights and between these traits and body weight at various ages. Product-moment correlation coefficients between organ weights and their components were computed with body weights at varying ages. These values were used to investigate the relationship between body and organ growth through ontogeny.

RESULTS

Effect of selection for rate of development in body weight: Line means and ANOVA results comparing all possible pairs of means are shown in Table 1. Both *E* and *L* treatments differed significantly from *C*. For 28-day-old mice, the *E⁺* lines were significantly heavier while the *E⁻* lines were significantly lighter in body weight compared to the controls. *E⁺* and *E⁻* differed by an average of 9 g in 28-day body weight. The late-selected (*L⁺* and *L⁻*) and control lines were intermediate in value between *E⁺* and *E⁻*. *C* and *L⁻* did not differ significantly in 28-day body weight, and *L⁺* weighed significantly less than *L⁻* and *C*.

At 70 days of age early-selected lines differed by 7 g and late-selected lines differed by 13 g. The two up-selected lines (*E⁺* and *L⁺*) had similar 70-day body weights, while *C* was approximately intermediate between *L⁺* and *L⁻* (Table 1).

TABLE 2

Line means and LSD tests for brain and liver size at 28 and 70 days of age

Brain weight (g)				Total brain DNA (mg)			
28 days		70 days		28 days		70 days	
0.420	<i>E⁻</i>	0.458	<i>E⁻</i>	14.569	<i>E⁻</i>	13.875	<i>E⁻</i>
0.456	<i>L⁺</i>	0.482	<i>L⁻</i>	15.292	<i>L⁺</i>	14.363	<i>L⁻</i>
0.460	<i>L⁻</i>	0.496	<i>L⁺</i>	15.806	<i>L⁻</i>	14.500	<i>L⁺</i>
0.462	<i>C</i>	0.498	<i>C</i>	16.189	<i>C</i>	14.959	<i>C</i>
0.511	<i>E⁺</i>	0.533	<i>E⁺</i>	16.575	<i>E⁺</i>	16.128	<i>E⁺</i>
<i>0.019</i>		<i>0.028</i>		<i>1.043</i>		<i>1.200</i>	
Brain cell no. ($\times 10^6$)				Brain cell size (pg)			
28 days		70 days		28 days		70 days	
2428	<i>E⁻</i>	2313	<i>E⁻</i>	0.1745	<i>C</i>	0.1995	<i>E⁺</i>
2549	<i>L⁺</i>	2394	<i>L⁻</i>	0.1759	<i>E⁻</i>	0.2003	<i>E⁻</i>
2634	<i>L⁻</i>	2417	<i>L⁺</i>	0.1772	<i>L⁻</i>	0.2027	<i>L⁻</i>
2698	<i>C</i>	2493	<i>C</i>	0.1814	<i>L⁺</i>	0.2032	<i>C</i>
2762	<i>E⁺</i>	2688	<i>E⁺</i>	0.1893	<i>E⁺</i>	0.2075	<i>L⁺</i>
<i>172</i>		<i>200</i>		<i>0.01</i>		<i>0.02</i>	
Liver weight (g)				Total liver DNA (mg)			
28 days		70 days		28 days		70 days	
1.585	<i>E⁻</i>	2.033	<i>L⁻</i>	74.049	<i>E⁻</i>	87.125	<i>L⁻</i>
1.827	<i>L⁻</i>	2.297	<i>C</i>	83.942	<i>L⁺</i>	98.475	<i>E⁻</i>
1.875	<i>C</i>	2.420	<i>E⁻</i>	88.313	<i>C</i>	104.050	<i>C</i>
1.896	<i>L⁺</i>	2.721	<i>E⁺</i>	88.930	<i>L⁻</i>	111.775	<i>E⁺</i>
2.232	<i>E⁺</i>	2.908	<i>L⁺</i>	102.205	<i>E⁺</i>	116.525	<i>L⁺</i>
<i>0.181</i>		<i>0.313</i>		<i>10.756</i>		<i>15.759</i>	
Liver cell no. ($\times 10^6$)				Liver cell size (pg)			
28 days		70 days		28 days		70 days	
12,304	<i>E⁻</i>	14,521	<i>L⁻</i>	0.1274	<i>L⁻</i>	0.1374	<i>C</i>
13,990	<i>L⁺</i>	16,411	<i>E⁻</i>	0.1318	<i>C</i>	0.1416	<i>L⁻</i>
14,719	<i>C</i>	17,343	<i>C</i>	0.1333	<i>E⁻</i>	0.1482	<i>E⁺</i>
14,822	<i>L⁻</i>	18,629	<i>E⁺</i>	0.1345	<i>E⁺</i>	0.1500	<i>E⁻</i>
17,034	<i>E⁺</i>	19,420	<i>L⁺</i>	0.1392	<i>L⁺</i>	0.1525	<i>L⁺</i>
<i>1,792</i>		<i>2,626</i>		<i>0.01</i>		<i>0.02</i>	

Traits within each age are ranked and those connected by a vertical line are statistically homogeneous by an LSD test. Pooled standard errors are given in italics for each trait.

The *E⁺* and *L⁺* selection treatments both significantly increased body weight compared to the controls. Indeed, there is no significant difference between *E⁺* and *L⁺* lines in body weight and, in some *E⁺* and *L⁺* replicates, 70-day-old body weights are identical to the second decimal place. Thus, seemingly identical complex phenotypes (70-day-old body weight) are produced by two different developmental processes. This phenomenon has been termed "developmental homoplasy" by Atchley *et al.* (1997).

Effect of selection for rate of development on organ weights: Among these five selection treatments, *E⁺* mice had the largest and *E⁻* the smallest 28-day brain weights (Table 2). The difference between these lines was 0.09 g ($P < 0.05$). The late-selected and control lines were

intermediate between the early-selected lines for brain weight. No significant difference in 28-day-old brain weight existed among *C* and *L* lines.

Brain weight at 70 days of age had a similar ranking in that E^+ had the largest brain while E^- had the smallest. *C* and *L* lines were intermediate. However, L^- differed significantly from both L^+ and *C* by 0.01 g ($P < 0.05$). Thus, early selection produced the largest and smallest brains compared to the late selection and control lines at both 28 and 70 days of age.

Line means for liver weight at 28 days were of similar rank as those for body and brain weights at that age, *i.e.*, E^+ was 0.6 g heavier than E^- , with the two late-selected lines and the control intermediate and not significantly different from each other (Table 2). At 70 days of age, all lines differed significantly in liver weight. L^+ had the heaviest liver and the difference between the two late-selected lines is 0.9 g. Means for the early-selected lines were intermediate between the divergent late-selected lines, and livers from E^+ were 0.3 g heavier than those from E^- ($P < 0.05$). Thus, late selection produced the most divergent liver weights with the early selected lines and the controls being intermediate in size.

DNA values, cell number, and cell size: Because total DNA per organ was converted to total cell number using a constant multiplier, rankings of line means and tests of significance for cell number are identical to those for total DNA per organ. E^+ had the most brain cells at 28 days of age and E^- had the fewest. Twenty-three generations of selection generated an average difference of 334×10^6 cells ($P < 0.05$, Table 2). E^+ and *C* did not differ significantly; however, E^- was significantly different from both E^+ and *C*. At 28 days of age, L^- had slightly more (85×10^6) brain cells than L^+ . *C* and L^- did not differ significantly but L^+ differs significantly from both in total number of brain cells.

Similarly, at 70 days of age, E^+ and E^- lines were most divergent in number of brain cells (375×10^6 cells). No significant difference in brain cell number was found between the two late-selected lines. The number of brain cells for the control line was slightly larger than that for the two late-selected lines. The early selection regime generated significant differences in the number of brain cells at both 28 and 70 days of age. The late selection regime did not produce such obvious results.

Line means for total liver cell number at 28 days of age rank similarly to brain cell number at the same age. E^+ had 4730×10^6 more cells than E^- , L^+ had 832×10^6 more cells than L^- , and *C* had a mean intermediate of L^+ and L^- but not significantly different from either. At 70 days of age, the largest difference in liver cell number was between the two late-selected lines (L^+ had 4899×10^6 cells more than L^-). Liver cell number differences between two early-selected lines at 70 days were essentially half those at 28 days, at 2218×10^6 cells. The

control line had a mean intermediate between the “up” (E^+ , L^+) and “down” (E^- , L^-) selection regimes.

With regard to cell size, significant differences were present for brain cells at both 28 and 70 days of age. At 28 days of age, the difference between E^+ and E^- was 0.014 pg, and the difference between L^+ and L^- was 0.004 pg ($P < 0.05$). There were no significant differences among E^- , L^- , and *C*. At 70 days of age, there were no differences between the *E* lines, no differences between the *L* lines, and no difference between any of the selection lines and the control line ($P > 0.05$).

Differences for liver cell size at 28 days of age between L^+ and L^- were significant ($P < 0.05$). There were no significant differences in liver cell size among the early-selected lines and the control line at this age. At 70 days of age, in L^+ and L^- the differences in liver cell size were of the same magnitude as those at 28 days of age; however, unlike the 28-day results, E^+ and E^- had significantly larger liver cells than *C* at 70 days ($P < 0.05$), but two *E* lines were not different from one another (Table 1).

Apoptosis in the brain: The results given in Table 2 show that, depending upon selection line, brain weight increased 4–9% between 28 and 70 days of age, which was accompanied by a 5–16% increase in cell size during the same interval. However, the estimated number of cells decreased by 3–10% during this interval. Several possible explanations for this finding exist. First, the results could be due to variation in laboratory technique since the 70-day-old brains were analyzed before the 28-day-old brains. Consequently, our technique could have become more efficient and accurate by the time the 28-day-old brains were examined. To ascertain if technical variation in DNA measurement was responsible, 20 samples from each age were simultaneously remeasured for DNA content using the same standard DNA curve that was used to calculate total DNA in the samples. When this was done, there was no statistically significant difference between the two ages.

Since apoptosis is recognized as a prominent event during development of the vertebrate nervous system, we assume that the systematic decrease in the number of brain cells seen in these selection lines reflects this general process of programmed cell death that occurs in the brain as organisms age (*e.g.*, Raff *et al.* 1993; Leist and Nicotera 1997; Thomaidou *et al.* 1997; King and Cidlowski 1998).

Sexual dimorphism: Tests for sexual differences for all traits are provided in Table 3. Male mice were significantly heavier than females at both ages ($P < 0.05$). At 28 days of age, males had heavier brains and livers than females ($P < 0.05$), and the number and size of the liver cells were greater in males. However, there was no significant sexual dimorphism in brain cell number or size. At 70 days, males had significantly larger values for all traits except brain size and brain cell number.

Because male mice have significantly greater body

TABLE 3
Trait least-squares means and results from tests for sexual dimorphism

Trait	28 days		70 days	
	ANOVA model	ANCOVA model	ANOVA model	ANCOVA model
Body weight (g)	20.39/24.04**	NA	32.43/38.51**	NA
Brain weight (g)	0.456/0.466**	<i>0.465/0.457*</i>	0.494/0.493	<i>0.500/0.487**</i>
Brain cell no. ($\times 10^6$)	2,619/2,634	<i>2,665/2,586*</i>	<i>2,485/2,436*</i>	<i>2,498/2,423**</i>
Brain cell size (pg)	0.177/0.180	0.177/0.180	0.201/0.204*	0.202/0.203
Liver weight (g)	1.679/2.089**	1.792/1.971**	2.279/2.673**	2.491/2.462
Liver cell no. ($\times 10^6$)	13,549/15,935**	14,419/15,015	16,732/17,802**	<i>17,807/16,730**</i>
Liver cell size (pg)	0.128/0.135**	0.129/0.135*	0.138/0.153**	0.141/0.151**

Presented are results from both analyses of variance and analyses of covariance, where either 28-day or 70-day body weight was used as a covariate. Means after the slash are for males and means before the slash are for females. For each age group and model, pairs of means with asterisks indicate significant sexual dimorphism for that trait. Pairs of means in italics indicate a greater mean for females; otherwise, means for males were greater.

* $P < 0.05$; ** $P < 0.01$. NA, not available.

size and weight, we asked if sexual differences in organ size and its components result from a geometric scaling phenomenon related to larger body size in males. Analysis of covariance using body weight as a covariate was carried out to test whether significant differences exist when the two sexes are at the same body weight (Table 3). At 28 days of age, females had higher values for brain weight and cell number while males still had significantly heavier livers and liver cell size than females. At 70 days of age, females had significantly heavier brains, more brain cells, and more liver cells than males. Males had significantly larger liver cell size. Thus, significant sexual dimorphism exists in organ weights and their components.

Correlation analyses: Product-moment correlation coefficients among brain, liver, and body weights were computed at various ages to explore the impact of selection on rate of development. Tables 4 and 5 show correlations for 28- and 70-day-old mice between brain, liver, and body weight within each selection line as well as pooled over all selection lines.

Brain and liver weight were significantly correlated ($R = 0.57$, $P < 0.05$) for the 28-day-old mice. Correlations within each of the selection lines describe the impact of selection on the intercorrelations of these two traits. The correlations between brain and liver weight were highly significant in all lines except E^+ and L^- . At 70 days of age, the extent of association between brain and liver weight decreased considerably. The pooled R value ($R = 0.27$) was about half that seen for the 28-day mice and within selection lines only L^- and C exhibited a significant correlation between brain and liver weight.

Correlations between 28-day brain and liver weights and their components with body weight at 10 and 28 days of age are shown in Table 4. Twenty-eight-day brain and liver weights were highly correlated with both 10-

and 28-day body weight, although the largest values were with 28-day body weight. Twenty-eight-day brain weight was significantly correlated with 10-day body weight in E^- and L^+ and with 28-day body weight in E^+ , E^- , and L^+ . For brain cell number, there were no significant correlations with 10- or 28-day body weight. At 28 days of age, the E^+ line had the highest correlation between brain cell number and 28-day body weight but this value did not reach the Bonferroni adjusted critical value for significance at $P < 0.05$. There was a significant correlation of brain cell size with 28-day body weight for E^- and L^+ .

Liver weight at 28 days was significantly correlated with 10-day body weight in E^- , L^+ , and C , and all selection lines showed a significant correlation between 28-day liver weight and 28-day body weight. Twenty-eight-day liver cell number was highly correlated with 10-day body weight in all lines except E^+ and with 28-day body weight in all lines but E^- and L^+ .

Table 5 gives the correlations of brain and liver weight as well as organ weights with body weights at 10, 28, 56, and 70 days for the 70-day-old mice. Since the brain stopped growing early in postnatal ontogeny, it was not unexpected that the correlations of 70-day-old brain with body weights were much lower than those for 28-day brain weight. Thus, the pooled correlations between 70-day brain weight and body weight at different ages decreased from 0.65 at 10 days to 0.38 at 70 days. Within selection lines, 70-day brain weight and body weight at various ages generally were not significantly correlated or were decreasing in correlation over time, e.g., E^- . All correlations of 70-day brain weight and 70-day body weight were < 0.4 .

For brain cell number, the pooled correlations also showed a decreasing pattern with age. The only exception was in L^- , where there was an increased correlation between 70-day brain weight and body weight at differ-

TABLE 4
Correlation coefficients for both organ weights and cellular traits with body weight at 28 days

Line	Brain weight		Brain cell no.		Brain cell size		Liver weight		Liver cell no.		Liver cell size	
	wt10	wt28	wt10	wt28	wt10	wt28	wt10	wt28	wt10	wt28	wt10	wt28
E ⁺ (44)	0.279	0.663	0.095	0.408	0.011	-0.203	0.071	0.752	0.162	0.612	-0.122	-0.062
E ⁻ (48)	0.554	0.659	-0.138	-0.153	0.276	0.576	0.594	0.785	0.518	0.237	0.065	0.467
L ⁺ (40)	0.315	0.835	-0.138	-0.153	0.276	0.576	0.645	0.825	0.518	0.237	0.065	0.467
L ⁻ (43)	-0.122	0.211	0.263	0.058	-0.321	0.027	-0.017	0.812	0.375	0.656	-0.459	-0.052
C (48)	-0.016	0.271	0.269	0.115	-0.262	0.012	0.587	0.939	0.665	0.630	-0.253	0.071
Pooled (223)	0.584	0.739	0.325	0.311	0.074	0.186	0.606	0.863	0.606	0.641	-0.153	0.068

Correlations are computed on the basis of models 1 or 2 as described in the text. Numbers in parentheses indicate sample size for each analysis. Significance values for $P < 0.05$ using the Bonferroni adjustment procedure are shown in italics.

ent ages. In L^- , the correlation between 70-day brain and 70-day body weight was 0.8 ($P < 0.01$). The only significant correlation of 70-day brain cell size was with 70-day body weight for line L^- .

The correlation patterns for 70-day liver weight showed an opposite trend to brain weight. Liver weight was less highly correlated with earlier body weights and the correlations of liver weight with body weight *increased* over time. The pooled correlations range from 0.26 with 10-day body weight to 0.86 with 70-day weight. This pattern was seen for each selection line.

DISCUSSION

Debates about the evolution of brain-body size relationships among mammals have occurred for decades (*e.g.*, Jerison 1973; Gould 1975; Lande 1979; Shea 1983; Atchley *et al.* 1984; Riska and Atchley 1985 and references therein). One of the more interesting aspects of these discussions has been to discover the mechanisms that produce the steeper brain-body size allometric slopes seen between distantly compared to closely related taxa (Atchley *et al.* 1984; Riska and Atchley 1985). Based upon extensive quantitative genetic data, Atchley *et al.* (1984) and Riska and Atchley (1985) suggested that the steeper allometric slope of brain-body size might be the result of selection operating early in ontogeny when growth was occurring primarily by increases in cell number rather than later when selection favors changes in cell size. Selection early in ontogeny might occur associated with large changes in body size. These authors provided extensive data from genetic correlations between brain size and early and late body weight gain to substantiate their hypothesis. However, this hypothesis has never been experimentally validated.

Quantitative genetic parameter estimates: Response to selection should be predictable from estimates of relevant quantitative genetic parameters, *e.g.*, narrow-sense heritability and additive genetic correlations, for the traits in question. Heritability estimates for body weight in these selected lines of mice are reported in Atchley *et al.* (1997) and these estimates are comparable to other studies on ICR mice (*e.g.*, Atchley *et al.* 1984).

Using large sample estimates of relevant genetic parameters of traits in random-bred ICR mice, we have previously shown that brain size has narrow-sense heritabilities of 0.64 (± 0.19) and 0.61 (± 0.12) at 38 and 70 days of age, respectively (Atchley *et al.* 1984). The additive genetic correlation between brain size at 38 days of age and body weight gain up to 14 days of age is 0.73 (± 0.13). The correlation was -0.74 (± 0.14) for 38-day brain size and weight gain between 21 and 38 days of age. For 70-day-old brain size, the genetic correlation with body weight gain up to 14 days of age is 0.59 (± 0.12) but -0.56 for weight gain between 21 and 70

TABLE 5
Correlation coefficients for both organ weights and cellular traits with body weight at 70 days

Line	Brain weight				Brain cell no.				Brain cell size			
	wt10	wt28	wt56	wt70	wt10	wt28	wt56	wt70	wt10	wt28	wt56	wt70
<i>E</i> ⁺ (72)	0.184	0.223	0.184	0.154	0.126	-0.008	-0.064	-0.038	0.011	0.155	0.188	0.149
<i>E</i> ⁻ (72)	<i>0.737</i>	<i>0.684</i>	<i>0.425</i>	0.379	<i>0.498</i>	<i>0.414</i>	0.264	0.238	0.014	0.055	0.023	0.029
<i>L</i> ⁺ (72)	<i>0.433</i>	0.362	0.080	0.062	0.105	0.157	-0.024	-0.043	0.109	0.022	0.058	0.076
<i>L</i> ⁻ (72)	0.132	0.242	0.367	<i>0.396</i>	0.017	<i>0.609</i>	<i>0.657</i>	<i>0.803</i>	0.118	0.225	0.214	0.292
<i>C</i> (72)	<i>0.412</i>	0.195	0.185	0.159	0.162	-0.015	0.014	0.067	0.056	0.139	0.091	0.014
Pooled (360)	<i>0.647</i>	<i>0.579</i>	<i>0.429</i>	<i>0.383</i>	<i>0.425</i>	<i>0.325</i>	0.225	0.191	0.027	0.081	0.079	0.085

Line	Liver weight				Liver cell no.				Liver cell size			
	wt10	wt28	wt56	wt70	wt10	wt28	wt56	wt70	wt10	wt28	wt56	wt70
<i>E</i> ⁺ (72)	0.161	<i>0.612</i>	<i>0.809</i>	<i>0.801</i>	0.238	0.310	<i>0.434</i>	<i>0.411</i>	-0.078	0.271	0.335	0.356
<i>E</i> ⁻ (72)	0.165	0.278	<i>0.519</i>	<i>0.560</i>	<i>0.383</i>	0.287	0.375	<i>0.401</i>	-0.269	-0.075	0.039	0.086
<i>L</i> ⁺ (72)	<i>0.443</i>	<i>0.635</i>	<i>0.772</i>	<i>0.798</i>	<i>0.416</i>	<i>0.399</i>	0.367	<i>0.410</i>	0.010	0.159	0.331	0.323
<i>L</i> ⁻ (72)	0.284	<i>0.609</i>	<i>0.657</i>	<i>0.803</i>	0.207	<i>0.472</i>	<i>0.539</i>	<i>0.677</i>	0.078	0.129	0.106	0.120
<i>C</i> (72)	0.393	<i>0.631</i>	<i>0.829</i>	<i>0.878</i>	0.255	0.377	<i>0.398</i>	<i>0.387</i>	-0.059	0.039	0.124	0.162
Pooled (360)	<i>0.258</i>	<i>0.495</i>	<i>0.819</i>	<i>0.862</i>	<i>0.313</i>	<i>0.396</i>	<i>0.573</i>	<i>0.597</i>	-0.088	0.079	0.231	0.254

Correlations are computed on the basis of models 1 or 2 as described in the text. Numbers in parentheses indicate sample size for each analysis. Significance values for $P < 0.05$ using the Bonferroni adjustment procedure are shown in italics.

days of age (Atchley *et al.* 1984; Riska and Atchley 1985). There is a change in sign in the genetic correlation between brain size and early *vs.* later rates of development. The additive genetic correlation between adult brain size and age-specific body weight is high initially (0.60, ± 0.12) between 70-day brain weight and 14-day body weight. This value decreases to 0.38 (± 0.16) between 70-day brain and body weight. The dynamics of these genetic correlations strongly suggest that the underlying genetic associations between brain and body size are changing significantly during postnatal ontogeny. Similar results are available for data from rats, *e.g.*, Atchley (1984a,b) and Riska and Atchley (1985).

Other authors have shown that the developmental components of the brain also exhibit high heritabilities. Williams *et al.* (1996), for example, have shown that broad-sense heritability of retinal ganglion cell number is ~ 0.8 .

Divergence in brain and liver size: This experiment describes the cellular consequences of age-specific selection in two different organs, *i.e.*, the brain and liver, that grow under quite different controls. Nervous system growth begins very early in ontogeny and the developing brain actually provides the mold for the final shape of the bones of the skull and craniofacial region. Growth factors, such as insulin-like growth factors, exercise a major control on brain growth because growth hormone in rodents does not become involved in growth regulation until ~ 18 days postnatal age. In the mouse, the brain reaches its mature size at ~ 16 days postnatal development (Kobayashi 1963). Thus, the brain has completed the majority of its growth before growth hormone becomes involved in growth regulation. Liver

growth and differentiation, on the other hand, begin later in ontogeny and continue in mice until ~ 50 days of age, which is well past when the brain has ceased to grow. Much of liver growth occurs in the presence of growth hormone. Further, liver growth is accompanied by changes in ploidy levels, which are related to age and weight (Epstein 1966).

The results described here clearly show that significant divergence has occurred in brain size between *E*⁺ and *E*⁻ lines at both 28 and 70 days of age. Further, *E*⁺ has significantly more brain DNA and has more brain cells than line *E*⁻ at both 28 and 70 days of age. The *E* lines were selected for growth rate between birth and 10 days of age, when growth is hypothesized to proceed mainly through cell proliferation. *E*⁺ brains also have larger cells than *E*⁻ at 28 days. While significant differences are found in both cell number and cell size for the 28-day-old mice, 99% of the changes in brain size were the result of changes in cell number.

For late-selected mice (*L*), selection occurred after all major growth activity in the mouse brain had ceased. No significant divergence in brain weight between the *L* lines or between the *L* and *C* lines was found at 28 days. Thus, selection for increased or decreased rate of development in body weight between 28 and 56 days of age had no significant correlated effect on brain weight at 28 days of age or beyond. These results provide important documentation about the relative efficacy of selection on complex traits whose various developmental components start and stop growth at different times.

In spite of only a modest amount of divergence in brain weight (3%) at 70 days of age, significant differences were noted in brain cell number and size (*L*⁻ >

L^+ for cell number while $L^+ > L^-$ for cell size). At 70 days of age, L^+ brains were significantly larger than L^- brains. However, there is no difference between the L lines for either brain cell number or cell size at 70 days. The difference in brain weight between L^+ and L^- is not large (0.014 g) relative to the differences between E^+ and E^- . Consequently, the lack of differences between L^+ and L^- mice in cell number or size is probably a function of the small magnitude of the difference relative to the variance of the measurements. For this reason, we do not ascribe real biological meaning to the significant but small difference in brain weight between the L^+ and L^- lines.

These results on the components of brain size validate the hypothesis proposed by Atchley *et al.* (1984) and Riska and Atchley (1985) that selection for rate of development in body weight during early postnatal ontogeny in rodents significantly affects cell number in the brain. This is a correlated response in brain size to changes in body size during this interval and these changes in brain size are primarily a function of changes in brain cell number. Selection occurring after the brain has ceased the major part of its growth will have little effect on changes in brain size. As will be documented elsewhere (W. R. Atchley, unpublished results), selection early in ontogeny affecting cell number in the brain provides a steeper allometric slope for brain-body size.

Growth in the liver is quite different from that of the brain and, as a result, one might expect that selection will have quite different effects on the liver compared to the brain. At 28 days, significant divergence between lines for liver weight was present only between the E lines and all of the divergence between E^+ and E^- was in the number of liver cells. No significant difference in liver cell size was seen at 28 days between E^+ and E^- . At 28 days of age, there were no significant differences in liver weight between L lines, or between L and C . Interestingly, in spite of the lack of significant difference in liver weight, L^- had significantly more cells than L^+ at this age but L^+ had significantly larger cells. Apparently, these two traits were effectively canceling out each other with regard to their contribution to divergence in liver weight.

For 70-day liver weight, the two L lines were quite different from each other and were more divergent than the two E lines. L^+ livers weighed almost 1 g more than L^- livers. The L^+ line had significantly greater liver cell number and liver cell size compared to L^- .

These results demonstrate that response to selection for such cell traits is a complicated process involving the time during ontogeny at which selection occurs and the patterns of growth of the organs. The brain starts and stops growth early in ontogeny. Thus, only selection early in ontogeny appears to have significantly affected brain size. On the other hand, the liver begins growth later and continues much longer in ontogeny. Conse-

quently, the liver responded well to both regimes of selection.

Explanations of how selection has affected the cellular components of liver growth are complicated by the presence of endopolyploidy in the liver (Epstein 1966). With endopolyploidy, the amount of DNA in the cell increases in multiples of haploid DNA amounts. However, polyploidy may not bias the conclusions because we are contrasting the weight of the liver and its cellular components *between selection lines but at the same age*. Polyploidy becomes more of an issue if comparisons are made between different organs with different polyploidy levels or between organs at disparate ages. Our assumption would be suspect if selection for growth rate affected the time of onset and the rate of polyploidization in different selection lines. The liver is a highly metabolic organ involved with the synthesis of many gene products. It is feasible that selecting for increased rate or development or larger body size might influence the time at which polyploidization is initiated and its rate. Answering this question is beyond the scope and methodology of this article; however, it is certainly of interest in future analyses on these and other similar selection lines.

Overall conclusions: These results clarify several aspects of the response to selection for developmental traits: First, each complex structure like the brain has a particular overall growth trajectory during ontogeny and the shape of this trajectory is a function of the dynamics of its component parts (Atchley and Hall 1991). The shape of the growth trajectory of each organ reflects when the various component parts initiate and terminate growth, the rates and pattern of changes of these various components, and the interactions of these features (Atchley 1987; Atchley and Hall 1991). Under such a scenario, altering the time during ontogeny in which selection operates should result in different component parts being altered, which, in turn, will have an impact on the shape of the growth trajectory of the organ. Clearly, these results show that age-specific selection does change different developmental components of complex traits, which in turn have a significant impact on the shape of the growth trajectory of the entire complex structure. The impact of age-specific selection on growth trajectories of body weight for these selection lines has been examined in considerable detail (Rhees and Atchley 2000).

Second, for both brain and liver, the early growth phase (rapid growth) was due primarily to the multiplicative aspects of cell proliferation, and the decreased rate of growth later in ontogeny was due to an increase in cell size as well as a decrease and eventual cessation of cell proliferation.

Besides the early- and late-selected lines, we also examined growth traits in the randomly-selected control line (C). Expected means of all traits in this line should be intermediate between the up and down selection

regimes, but observations in some traits were not as expected. There are several possible explanations for this. First, random drift was highly significant for all traits, and it is possible that line *C* was more affected by drift than were the selection lines, since the controls were not under selection pressure. Another possible explanation is inbreeding depression, and this explanation is particularly relevant since most of the trait means for *C*, which were not intermediate between the up- and down-selected lines, were lower than expected (*i.e.*, 70-day liver weight). One final possibility is that compensatory growth has occurred in the down-selected lines, such that they "bounced back" after selection pressure was relaxed. In other words, *E*⁻ lines after 10 days or *L*⁻ lines after 56 days might have increased their growth rates so that their body weights were actually greater than those for *C*.

Several interesting and somewhat unexpected results were obtained from the study. For example, at 70 days of age significant sexual dimorphism occurs in the number of brain cells with female mice having more brain cells than males. After adjustments were made for differences in body weight by analysis of covariance, females in this experiment at 70 days of age were found to have significantly larger brains. Such sexually dimorphic results have not been previously reported, possibly because many of these experiments used much smaller sample sizes in their statistical analyses.

Another significant finding was that in spite of the brain being larger at 70 days of age, total brain DNA and the number of cells were higher at 28 compared to 70 days of age. We believe that this variation in DNA amount and cell number arises from differential rates of cell death in these selection lines. That is, cell death is proportional to organ size or growth rate. The number of cells in nervous tissue is produced in excess early in ontogeny and then decreases through programmed cell death as the animal ages (Raff *et al.* 1993). In chicks, for example, the number of brain cells declines as the result of patterns of cell death after ~20 days of age (Zamenhof and Marthens 1979). Our finding of fewer cells at 70 compared to 28 days of age might not be surprising with age-related cell death in mouse brains. Indeed, an important hypothesis that should be considered in future work is whether selection for changes in growth rate early and late in ontogeny also changes the rates of programmed cell death in addition to changing rates of cell multiplication and enlargement. Indeed, evolutionary models of cellular change may include changes in rates of programmed cell death (Atchley 1987, 1990).

In addition to simply changing trait means, different regimes of age-specific selection may also alter the genetic associations among traits. Thus, we performed correlation analyses for traits with body weight at 10, 28, and 56 days of age. Brain weight was significantly correlated with body weight at 10 and 28 days of age,

while liver weight was significantly correlated with body weight at 56 days of age. There were significant correlations between brain cell number and 10-day body weight, brain cell size and 28-day body weight, and liver cell number and cell size with 56-day body weight. Examining correlations among traits for the various selection lines, it is apparent that selection has had a differential effect on trait associations. These analyses indicate that selection applied at different ages and in different directions not only differentially alters organ cellular components but also changes the genetic associations among the various component traits.

Two other studies are relevant to our work. Falconer *et al.* (1978) tested the hypothesis that selection for body weight would change cell number as well as cell size (mass). The experiment compared mice selected for divergent 6-wk body weight with a control line. For 42-day-old mice, they found for lung and spleen that 70% of the size difference between selected and control lines was due to cell number differences and 30% was due to cell mass, but for liver and kidney, size difference was due equally to cell number and cell mass. Our results reported here demonstrate that cell number and cell size contribute differentially to body or organ size at different time periods during ontogeny. Therefore, selection for growth rates at different ages may act on different genes and elicit different response to selection at the cellular level.

Cheverud *et al.* (1996) mapped QTL and estimated their effects on age-specific body weight and weight gain in F₂ progeny of two inbred mice strains using microsatellite DNA markers. They suggested that different genetic and physiological systems operate during early and late growth. Our results are essentially in agreement with these findings. We have shown that age-specific selection occurring at different periods during ontogeny will change distinct cellular components of growth. A QTL mapping study will help define those loci involved in age-specific growth patterns and identify those loci controlling the various cellular aspects of mammalian growth. Such studies will provide a deeper understanding of the genetic and developmental models that best characterize mammalian growth and development.

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