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A comparison of 'Empire' apple fruit size and anatomy in unthinned and hand-thinned trees

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SUMMARY

A stereological method was developed to analyze the anatomical features of fresh 'Empire' apple fruit sectors cut in the transverse equatorial plane. Fruits were from unthinned trees or trees hand-thinned to one fruit per cluster at −7, 0, 10, 20 or 40 d after full bloom. At final harvest (140 DAFB), fruits representing the size range within each treatment were analyzed for the effects of thinning on fruit size, weight and cortex anatomy, namely, parenchyma cell size, cell number and the proportion of cortex volume occupied by intercellular space (IS). A dissecting stereobinocular microscope fitted with a ten-by-ten reticule was used to count cells and proportion of IS in three fields in each of two cortex sectors per fruit. Cell volume in each field was derived by knowing only the grid area, a point-count for proportion of IS and a count of cell numbers within the grid. Fruit size and weight decreased as thinning was prolonged and unthinned trees had the smallest fruit. Within a thinning treatment, fruit size was positively correlated with cortex cell number, not with cell size or proportion of IS. This also held between treatments: unthinned trees had smaller fruit with fewer cells than did larger fruit from thinned trees, and fruit of trees thinned near bloom were larger with more cells than those of trees thinned later. For 'Empire', fruit thinning appeared to increase fruit size by allowing remaining fruits to continue cell division under less competition during the first weeks after bloom, and not by extending the cell division period, increasing cell size or increasing proportion of IS.

THE 'Empire' apple is a high quality, dessert type fruit which accounts for an increasing share of the Northeastern USA apple crop each year. In some years, 'Empire' fruit size is below market requirements (i.e. fruit <70 mm diameter). The cash value of any fresh-market apple cultivar is primarily determined by size more than any other fruit character except colour (Bergh, 1985; Heinicke, 1985; Schotzko, 1985). Fruit size can be optimized by appropriate nutrition, pruning and fruit thinning, although yields are reduced by thinning.

Thinning apples to increase fruit size has become a common practice based on many studies (Denne, 1960; Martin and Lewis, 1952; Quinlan and Preston, 1968; Westwood, 1978; Westwood, Batjer, and Billingsley, 1967; Williams and Edgerton, 1981; Williams, 1985). Thinning may stimulate fruit growth by influencing cell division (rate or duration), enhancing cell enlargement, producing more or proportionately more intercellular space (IS), or some combination of these processes (Bergh, 1985, 1990; Denne, 1960; Martin and Lewis, 1952; Martin, Lewis and Cerny, 1964; Quinlan and Preston, 1968; Sharples, 1968; Westwood, Batjer, and Billingsley, 1967). The contribution of IS to increase in apple fruit volume is not as well understood as that for cell size or cell number. Previous efforts to measure IS as a major contributor to apple fruit size are indirect (Calbo and Sommer, 1987; Reeve, 1953). More direct methods are being developed, for example, digital imaging of sectioned material (Ruess and Stösser, 1993). IS may contribute to size increase in apple (Skene, 1966), and IS is implicated as a major contributor to much of the late-season

expansion of apple fruit (MacArthur and Wetmore, 1941). There is a need to examine cell volume, cell size and proportion of IS simultaneously in flesh samples of apple.

This investigation considers the effect of time of hand-thinning of fruit on the histological development of the 'Empire' apple. Each thinning treatment provided a range of fruit sizes induced by the time of thinning and the cropload which can be used to analyze the anatomical aspects of size development. Specific objectives were to resolve the relationship between time of hand thinning and histology of mature fruit, namely, cortex cell size, cell numbers and proportion of IS. Our hypothesis was that increased size results primarily from the proliferation of cells stimulated by thinning, and that the earlier the thinning the greater the effect on stimulating cell division and total cell number. The study was undertaken to understand better the contribution of orchard management and fruit cellular adjustments to greater fruit size in 'Empire' apple, as well as to examine methods by which anatomical features could be assessed accurately and efficiently.

MATERIALS AND METHODS

A thinning experiment was established in 1987 at Geneva, NY, in a ten year old commercial orchard of 'Empire'/M.9 trees trained as slender spindles. At each of five times relative to full bloom, trees were hand-thinned to single-fruited clusters: at –7 days ("pink cluster"), at full bloom, or at 10, 20, or 40 d after full bloom (DAFB). Nineteen additional trees served as unthinned controls. A randomized complete block design included 19 replications with single trees serving as experimental units. Fruit from all trees were harvested on September 18 (140 DAFB). To determine the effect of thinning date on fruit histology, 10 of the 19 trees per treatment were selected randomly, and five flower or fruit clusters were tagged on each tree when thinning occurred. Each tagged cluster was hand-thinned to its single king (central) fruit to minimize within-cluster position effects on size. Tagged clusters were on well exposed branches on the southern half of each tree. Although we started the investigation with 50 tagged fruit, not all remained on the trees at final harvest. At 35 DAFB, 15 of the tagged fruit were harvested randomly in each treatment for another study, while additional fruit fell as the season progressed.

At September harvest, the treatment mean fruit weights for the orchard pack-out were less than the treatment mean fruit weights for the histological samples but the relative differences were similar between treatments (Tables I and II). The same situation was found for mean fruit diameters. The histological samples for each treatment provided the variation in fruit sizes needed in this study to examine the interrelationships of fruit size and anatomical characters.

At final harvest, the diameter and weight of

TABLE I

Effect of hand-thinning to single-fruited clusters and no fruit thinning on fruit set, cropload and final fruit size of 'Empire'/M.9 apple trees in 1987

Thinning date (DAFB)	Fruit set (No. fruit per flower cluster)	Crop load (No. fruit per cm^2 TCA	Fruit size (g)	Fruit size adjusted for crop load (g)
–7	0.63	2.7	149	144
0	0.66	2.1	155	146
10	0.89	4.0	137	139
20	0.94	4.8	133	138
40	0.91	3.7	133	133
Unthinned	1.38	4.7	117	124
$LSD_{0.05}$ for Unthinned vs. Thinned	0.12	1.2	6	6
Regressions with thinning date (excluding unthinned treatment):				
Linear	$r^2 = 0.34^{**}$	$r^2 = 0.24^{**}$	$r^2 = 0.54^{**}$	$r^2 = 0.16^{**}$
Quadratic	$r^2 = 0.44^{**}$	$r^2 = 0.31^{**}$	$r^2 = 0.57^{**}$	n.s.
Regressions with thinning date (including unthinned treatment):				
Linear	$r^2 = 0.66^{**}$	$r^2 = 0.21^{**}$	$r^2 = 0.62^{**}$	$r^2 = 0.36^{**}$
Quadratic	n.s.	$r^2 = 0.26^{*}$	$r^2 = 0.67^{**}$	$r^2 = 0.38^{**}$

n.s. *, **Not significant or significant at $P = 0.05$ or 0.01, respectively.

TABLE II

Effect of hand-thinning to single-fruited clusters and no fruit thinning on fruit weight and size and on cortex anatomical characters of 'Empire' apples sampled at final harvest 18 September 1987. N = 10 fruit per treatment sampled across range of fruit size

Thinning date (DAFB)	Fruit weight (g)	Fruit diameter (mm)	Fruit volume (cm^3)	Cortex volume (cm^3)	Fruit proportion cortex (%)	Cell volume ($\mu m^3 \times 10^6$)	Proportion IS (%)	Cell number ($\times 10^6$)	Cells per mm^3
−7	169	74.8	223	182	82.0	2.520	33.5	47.86	272
0	187	78.3	253	204	81.0	2.485	34.9	54.12	271
10	162	74.1	214	175	81.7	2.397	34.4	48.32	282
20	149	71.5	192	160	83.2	2.658	32.1	41.02	263
40	158	72.8	204	166	81.6	2.515	33.1	43.80	268
Unthinned	112	64.9	147	114	78.3	2.320	33.4	32.36	297
Unthinned vs Thinned									
$P>F$	0.000	0.000	0.000	0.000	0.006	0.025	0.597	0.000	0.013
Regressions with thinning date (excluding unthinned treatment):									
Linear	$r^2 = 0.08$*	$r^2 = 0.11$*	$r^2 = 0.11$*	$r^2 = 0.11$*	$r^2 = 0.00$ n.s.	$r^2 = 0.01$ n.s.	$r^2 = 0.01$ n.s.	$r^2 = 0.11$ n.s.	$r^2 = 0.01$ n.s.
Quadratic	$r^2 = 0.10$ n.s.	$r^2 = 0.12$ n.s.	$r^2 = 0.13$ n.s.	$r^2 = 0.12$ n.s.	$r^2 = 0.01$ n.s.	$r^2 = 0.01$ n.s.	$r^2 = 0.03$ n.s.	$r^2 = 0.11$*	$r^2 = 0.01$ n.s.
Regressions with thinning date (including unthinned treatment):									
Linear	$r^2 = 0.38$***	$r^2 = 0.40$***	$r^2 = 0.37$***	$r^2 = 0.42$***	$r^2 = 0.11$**	$r^2 = 0.06$ n.s.	$r^2 = 0.01$ n.s.	$r^2 = 0.35$***	$r^2 = 0.08$*
Quadratic	$r^2 = 0.38$ n.s.	$r^2 = 0.40$ n.s.	$r^2 = 0.38$ n.s.	$r^2 = 0.43$ n.s.	$r^2 = 0.13$ n.s.	$r^2 = 0.09$ n.s.	$r^2 = 0.00$ n.s.	$r^2 = 0.36$ n.s.	$r^2 = 0.11$ n.s.

n.s. *, **, ***Not significant or significant at $P = 0.05$, 0.01, or 0.001, respectively.

all remaining tagged fruit were measured. Numbers available were 26, 23, 28, 23, 41 and 22 fruit for −7, 0, 10, 20, 40 DAFB and controls, respectively. For the histological analysis, ten were selected to represent the range in size for each treatment. After measuring fruit diameter with a band loop caliper, each selected fruit was sectioned equatorially, approximately at the level where seed cavities were largest (Figure 1A). The radial increment of seed cavity, pith and cortex tissues in the thickest and thinnest of the five ovarian sectors was measured to reconstruct their respective volumes as concentric spheres. Further histological analysis was confined to the cortex tissue in each treatment because samples revealed that approximately 80% of mature 'Empire' fruit volume is cortex, the remainder being pith and seed cavity (Table II). This percentage was also stated as a fruit character for several varieties by Ruess and Stösser (1993). The thickest and thinnest cortex sectors of each fruit were then removed (Figure 1A) and carefully recut in the transverse plane with a single sweeping stroke of a double-edged razor, producing a surface having very cleanly cut cells with minimal distortion (Figure 1B). The cell walls at the surface of each slice were stained by immersing sectors for three minutes in 5% aqueous tannic acid, rinsing briefly in tap-water, and immersing in 1% ferric ammonium sulfate (iron alum) for one minute. After a second water rinse the sectors were pinned to the bottom of water-filled dishes and examined at a magnification of ×70, using a Wild M5A dissecting microscope fitted with a 10 × 10 reticule (grid) placed within an ocular (Figure 1C). Three non-overlapping populations of cells were sampled along the radius of each cortex sector, at 1/4, 1/2 and 3/4 the cortex thickness (Figure 1B). Cortex thickness was taken as the length measured between epidermis and the main vascular bundle serving a sepal (Figure 1A). The ten major vascular bundles of the flesh mark the cortex-pith boundary.

In this study and in preliminary studies of apple fruit histology (Goffinet and Maloney, 1987) we used stereological (morphometric) methods for determining the composition of sectioned tissues. *Stereology* is herein defined as the determination of the three-dimensional composition of a tissue by a statistically based sampling of chosen components at the cut surface of slices of the tissue. The basis of the method is explained by Aherne and Dunnill (1982), Weibel (1973, 1979), and Toth (1982). Specifically, in our study cell volume, cell number and proportion of IS were determined for 'Empire' fruit tissues overlain by the 10 × 10 ocular grid as follows. For our purpose a cube of apple cortex tissue of known size (1.242 mm^3) consists of IS and a popula-

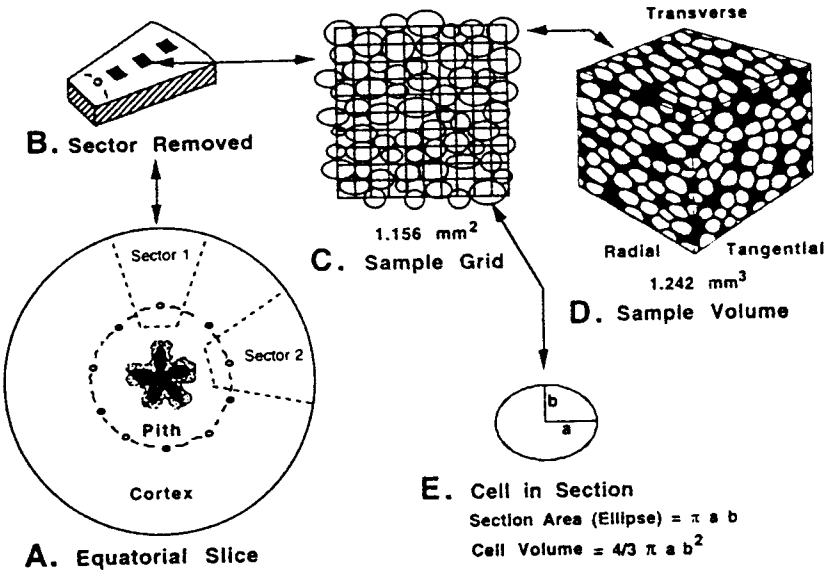

FIG. 1
Sampling technique for derivation of 'Empire' apple cell number, cell volume and proportion of intercellular space (IS). 1A) Equatorial transverse section showing delimitation of seed cavity, surrounding pith, and cortex separated from pith by ten vascular bundles. 1B) Cortex sector, re-cut transversely by razor blade, providing three sample areas (black squares) for analysis at 1/4, 1/2 and 3/4 the radial thickness of the cortex. 1C) Sampled area, overlain with a 10×10 ocular grid (viewed area = 1.156 mm^2) fitted to a dissecting microscope. Using only the upper focal plane of the microscope, cells within the grid were counted and 100 line intersections were used to estimate directly the proportion of IS. 1D) Extrapolation of the grid area to three dimensions, giving 1.242 mm^3, showing IS (black) and radially elongate shape of parenchyma cells in radial and transverse planes. 1E) Cell shape, a prolate spheroid whose length lies in the fruit radial plane and whose length is 1.28 times cell width or height. Cortex cells in a transverse fruit section thus appear in each grid as ellipses whose average volume can be determined from counts of cell number and proportion of IS within the grid.

tion of cells (predominantly large vacuolate parenchyma) of some average size, shape and number (Figure 1C). If cell size is estimated from section analysis, an accurate estimate of cell shape is necessary, although difficult (Weibel, 1973). We estimated cortex parenchyma cell shape in 'Empire' to be a prolate spheroid after preliminary tissue sampling in the three planes during 1986 (Figure 1C–1E). The long dimension of these cells lay in the radial plane, and the ratio of long to short cell axes averaged 1.278. In the present study the equatorial transections taken for cortex histology were of advantage because they allowed us to average data taken from thickest and thinnest cortex sectors, and allowed us to see the proportionality of the radially elongate cells in the section plane (Figure 1C). This is critical to the stereological method, as cortex cell volume was calculated from the elliptical areas of these cells as seen in the transections (Figure 1C, 1E).

Proportion of IS

The 10×10 ocular grid of the stereomicroscope was superimposed on the uppermost cut surface of the cortex sectors (Figure 1C). No further focusing was made during analysis of the tissue overlain by the grid; in fact, the stereological method depends on a minimal section thickness used (Aherne and Dunnill, 1982; Weibel, 1973; Toth, 1982). The locations of 100 of the grid's line intersections were used to determine the proportion of IS for the absolute area covered by the grid, with a grid area of 1,155,600 μm^2 at $\times 70$ magnification. This proportion was extrapolated to a cube of tissue having the grid's dimensions, or 1.242 mm^3 (Figure 1D). This was appropriate because preliminary samples of mature 'Empire' cortex tissues showed their tangential and transverse sections had almost identical proportions of IS. The proportions of IS of the six grid areas (three each for thick and thin cortex sectors) were combined and a

mean volume percentage IS was determined for each fruit. The means taken from the ten sampled fruit in each treatment were in turn averaged to give an overall determination of mean volume percentage of IS in the cortex.

Cell volume

Cell volume in μm^3 was derived in the following way for each tissue region overlain by the ocular grid. The section area of a prolate spheroid cut through its long axis is πab and the volume of the spheroid is $4\pi ab^2 \div 3$, where a and b are respectively the long and short radii of the spheroid (Figure 1E). By knowing the absolute area of the grid and the estimate of that proportion occupied by IS, we estimated the grid area occupied by sectioned cells as: Grid Area × (1.0 − Proportion IS). The mean elliptical area occupied by each cell in a grid is the above product divided by the number of cells counted in the grid, found by counting all cells with 50% or more of their section area within the grid border.

Cell radii a and b were needed to determine cell volume. However, in our method one need not know each cell's absolute dimensions, but only the ratio of long and short dimensions. As mentioned, a prolate spheroid's area in its median-longitudinal plane is: $A = \pi ab$, where $\alpha = 1.278b$. Thus, mean elliptical cell area, A in μm^2, is:

$A = \pi ab = [(1{,}155{,}600\ \mu m^2)(1.0 - \text{Proportion IS}) \div \text{Grid Cell No.}]$

Substituting $1.278b$ for a, and solving for b in μm, this equation becomes:

$b = \{[1{,}155{,}600\ \mu m^2(1.0 - \text{Proportion IS})] \div [1.278\pi\ (\text{Grid Cell No.})]\}^{-2}$

Since *Cell Volume* $= 4\pi(1.278b)b^2 \div 3 = 5.3533\ b^3$, then:

Cell Volume in $\mu m^3 = 5.3533\ [(A \div 1.278\pi)^{-2}]^3 = 0.6654\ [A^{-2}]^3$

By further substitution:

Cell Volume $= 0.6654\ \{[1{,}155{,}600\ \mu m^2(1.0 - \text{Proportion IS}) \div \text{Grid Cell No.}]^{-2}\}^3$

Although cumbersome in derivation, the result is that the cell volume in μm^3 can be derived by knowing only the grid area, a point-count for proportion of IS and a count of cell numbers within the grid. The cell volumes found for the six grid areas per fruit were combined and a mean value determined for the cortex of each fruit. The ten means taken from the ten sampled fruit in each treatment were averaged to provide an overall determination of mean cortex cell volume for the treatment.

Cortex volume

Cortex volume was taken as the difference between a spherical fruit volume based on fruit diameter and the spherical volume of the seed cavity plus pith region based on their combined radial measurements. The basal and apical depressions of the fruit were disregarded: 'Empire' fruit shape is generally spherical. The derived cortex volumes for the ten sampled fruit of each treatment were averaged to provide an overall determination of mean cortex volume.

Total cortex cell number

The number of cells in the entire cortex of a given fruit was derived from the fruit's mean values for cortex cell volume and proportion of IS, and the calculated cortex volume. Thus:

Total Cortex cells = (Cortex Vol.)(1.0 − Proportion IS) ÷ Cell volume

Resulting values for the ten fruit within a treatment were averaged to represent the treatment mean.

Cortex Cell Packing

Cells per mm^3 was calculated for each fruit by dividing one cubic mm ($1.0 \times 10^9\ \mu m^3$) by the fruit's mean cortex cell volume. Intercellular space volume was first excluded to adjust the projected volume to that actually occupied by cells. Thus:

Cells per $mm^3 = (1.0 - \text{Proportion IS})(1.0 \times 10^9\ \mu m^3) \div$ Cell Vol. in μm^3

A grand mean was also calculated for the ten fruit per treatment.

Statistical analysis of the gross and anatomical characters determined for the fruit of each treatment was performed with the SAS statistical program (SAS Institute Inc., Cary, North Carolina, USA). The intent of the histological investigation was to determine, first, if thinning affected fruit size and anatomy and, secondly, if differences in fruit anatomy could be affected by time of thinning.

RESULTS

The field data show that the thinning treatments reduced fruit set from a high of 1.38 fruits per cluster for the unthinned treatment to 0.63 fruits per cluster for the −7 DAFB treatment (Table I). Regression analysis showed a significant linear increase in set as thinning date was delayed. Final cropload values were reduced for the early thinning dates but were not different from the unthinned control for the later three timings (Table I). Thus, although the thinning treatments reduced fruit set, the later thinning treatments had a higher initial bloom resulting in similar croploads as the control. The higher initial bloom was the result of the carry-over effect of the same treatments applied to the trees the previous two years (unpublished data). Fruit from trees thinned at bloom were the largest among the treatments. As thinning was delayed after bloom, there was a linear decrease in size (Table I) [Fruit size (g) = 158 − 0.21 DAFB].

Given the known effect of cropload on fruit size, the simple regression of fruit size and date of thinning is misleading because of differences in cropload related to time of thinning. Using covariate analysis we adjusted fruit size of each of the treatments to remove the effect of cropload. Adjusted fruit size showed the largest fruits were obtained when thinning was done at either −7 DAFB or full bloom (Table I). Regression analysis again showed a significant linear decrease in fruit size associated with later thinning [Fruit Size (g) = 142 − 0.14 DAFB].

When the unthinned treatment was excluded from the regression analyses, similar results were obtained except that in all cases the slope was steeper.

The anatomical data showed that mature 'Empire' fruit size, fruit weight, cortex volume and cell number generally decreased as hand-thinning was prolonged after bloom (Table II). Among thinned trees, bloom-thinned trees had fruit with more cells than did fruit of other treatments, but fruit of bloom-thinned trees could not be distinguished from fruit of other treatments on cell size or proportion of IS in cortex tissue. Fruit of unthinned trees differed significantly from those of all thinning treatments by being smaller and lighter, having less flesh volume, especially cortex, and having fewer cortex cells. Cortex cell packing (cells per cubic mm) was also greater in unthinned trees. Cortex cell volume was similar for unthinned and thinned treatments, while proportion of cortex IS was identical for all treatments.

Within thinning treatments, linear regressions of fruit weight, fruit diameter, fruit volume and cortex volume against thinning date were significant, but quadratic regressions were not significant (Table II). No linear nor quadratic regressions of fruit proportion cortex, cortex cell volume, proportion of IS, cell number and cell packing against thinning date were significant (Table II).

If we included the unthinned trees as part of the data set to determine to what extent a treatment thinned at 140 DAFB would contribute to the features we analyzed, regressions of gross or anatomical measurements against thinning date fit linear models (Table II). Exceptions were cell volume and proportion of IS, whose linear regressions remained non-significant.

In considering the entire range of fruit weight and size across the ten fruit and six treatments providing the histological data, there was a positive and significant linear relationship between fruit weight and diameter (Figure 2). A positive and significant linear relationship was also found when apple fruit weight was regressed against fruit diameter within each treatment (r^2 = 0.89 to 0.99). Because of this association, either fruit weight or size could be used to assess anatomical characters relating to fruit size within the range observed.

The cortex volume of 'Empire' fruit contributes approximately 80% of total fruit volume (Table II). Across the fruit of all treatments there was a positive and significant linear relationship between fruit weight and cortex volume calculated as a spherical shell (Figure 3). There was also a positive and significant linear relationship within each treatment between fruit weight and cortex volume (r^2 = 0.79 to 0.97). Thus, we limited further analysis of anatomical contributions to fruit size to cortex characteristics.

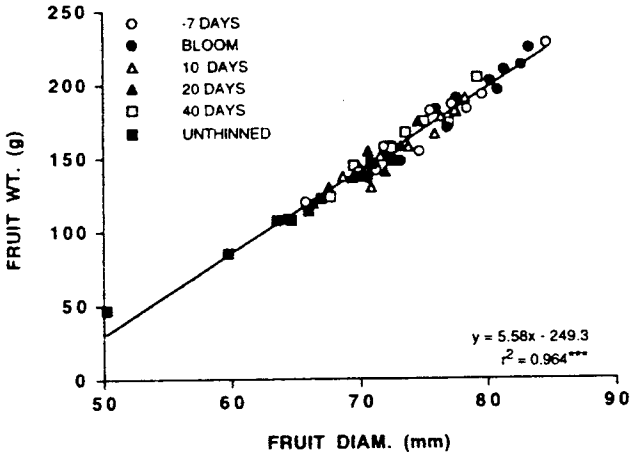

FIG. 2
Least-squares linear regression of fruit weight plotted against fruit diameter for 60 'Empire' fruit at final harvest. Ten fruit per treatment were taken across the range in fruit size resulting from trees hand-thinned at various dates relative to DAFB or from unthinned trees.

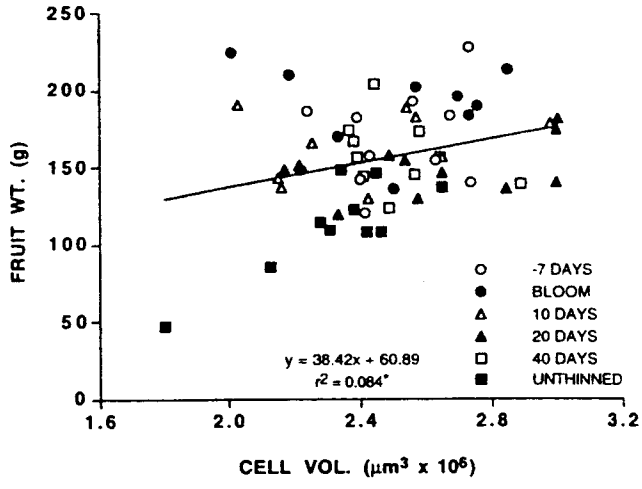

FIG. 4
Least-squares linear regression of fruit weight plotted against cell size within the cortex for 60 'Empire' fruit at final harvest. Ten fruit per treatment were taken across the range in fruit size resulting from trees hand-thinned at various dates relative to DAFB or from unthinned trees.

The relationship between final fruit weight and cortex cell size, proportion of IS, and cell number were each plotted for the 10 fruit sampled in each treatment (Figures 4, 5, 6, respectively). In each figure a single least-squares regression line was fitted through all 60 data points to show the major trends in the data.

There was almost no effect of cell size within cortex tissue on fruit weight (Figure 4). However, in the case of unthinned trees, fruit weight was significantly and positively affected by cell size ($r^2 = 0.71$). The proportion of IS in cortex tissue also had little affect on fruit weight (Figure 5).

Cortex cell number significantly affected fruit weight (Figure 6). Even though a least-squares linear regression closely fits the 60-fruit sample, an even better fit was shown by a second-order regression within the range of the data (Figure 6). The second-order fit also disclosed that as cell numbers increased there was not a similar increase in fruit weight, and that an asymptote of 220 g to 230 g could be extrapolated as an approximate maximum

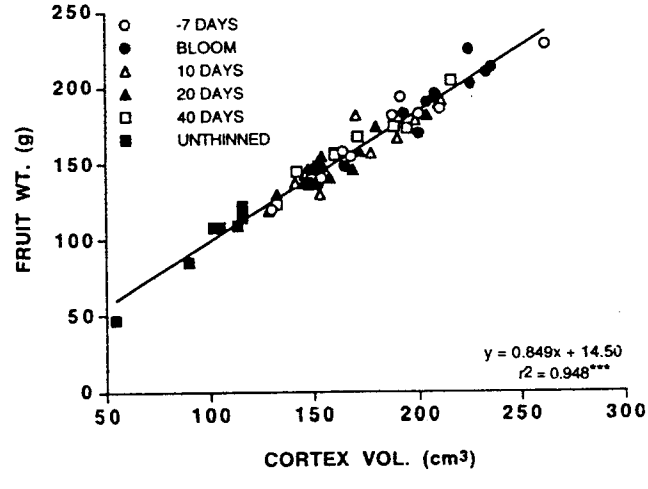

FIG. 3
Least-squares linear regression of fruit weight plotted against cortex volume for 60 'Empire' fruit at final harvest. Ten fruit per treatment were taken across the range in fruit size resulting from trees hand-thinned at various dates relative to DAFB or from unthinned trees.

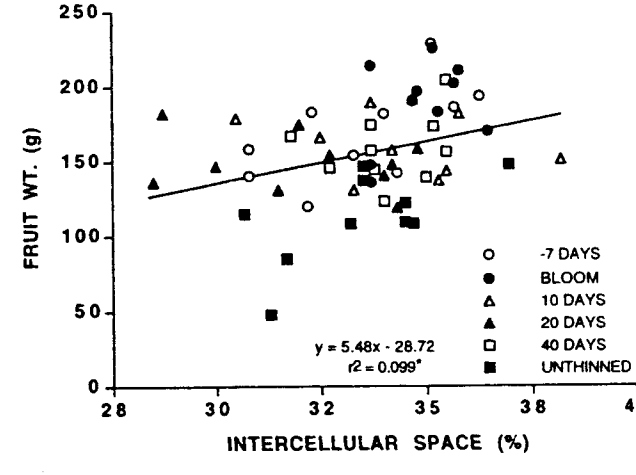

FIG. 5
Least-squares linear regression of fruit weight plotted against proportion of intercellular space within the cortex for 60 'Empire' fruit at final harvest. Ten fruit per treatment were taken across the range in fruit size resulting from trees hand-thinned at various dates relative to DAFB or from unthinned trees.

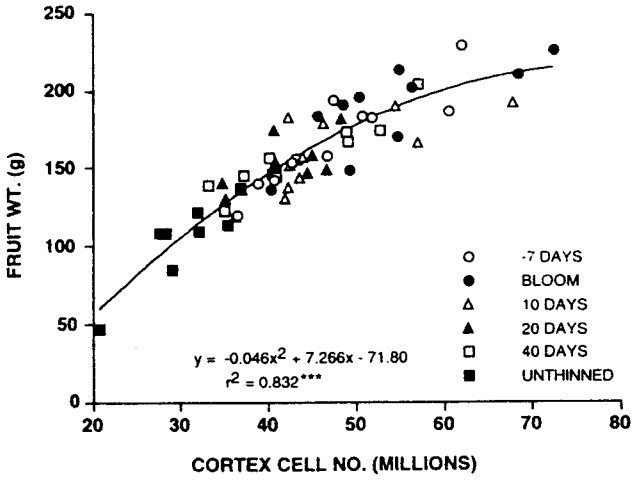

FIG. 6
Least-squares quadratic regression of fruit weight plotted against cell number within the cortex for 60 'Empire' fruit at final harvest. Ten fruit per treatment were taken across the range in fruit size resulting from trees hand-thinned at various dates relative to DAFB or from unthinned trees.

weight. Nevertheless, the largest fruit consistently had greatest cell numbers, whereas the same could not be said about the relationship between fruit weight and cortex cell volume or proportion of IS. The largest fruit resulting from bloom-thinning contained 50 million more cortex cells than did fruit of unthinned trees. No fruit examined histologically contained more than 50 million cortex cells if from trees thinned later than 10 DAFB. No fruit from unthinned trees had more than 42 million cortex cells.

DISCUSSION

For a given treatment the mean fruit size and weight were considerably larger and heavier in the samples taken for histology than in the fruit taken from the field as the entire orchard pack-out (cf. Tables I and II). This was expected because only the most competitive king fruit were left on well-exposed spurs yielding the histological samples. The sample from the whole orchard presumably contained many spurs thinned to a single lateral fruit and many fruit were in shaded areas on the trees. Nevertheless, the orchard packout and the fruit taken for histology had similar relative rankings for mean fruit size and weight between treatments. We have noticed in other years that thinning at bloom significantly increased fruit size compared with thinning just one week earlier or later (unpublished data).

The regressions of fruit size and date of thinning done with the histology samples showed a similar negative relationship as did the field samples. It was not possible to use cropload directly as a covariate with the histology samples since they were selected to represent the range of fruit sizes within each treatment and were not connected to the field replications. However, adjusting the sample means of fruits used for histology by the same percentage change as the field sample means, and then regressing the adjusted means with thinning date, resulted in a high linear correlation with a negative slope of 0.35 g per day after full bloom.

'Empire' apple fruit weight and size were positively and linearly correlated. Also, because fruit weight and size were each positively and linearly correlated with cortex volume, we expected the anatomical characters of the cortex to provide adequate information on the effects of hand-thinning on anatomical determinants of fruit size. Indeed, about 80% of 'Empire' fruit volume is cortex tissue. Thus, in making anatomical comparisons of thinning effects, we concentrated on the size, number and packing of the large vacuolate parenchyma cells of the cortex, and vascular tissue was excluded from the analysis.

Preliminary sampling of the three section planes of mature 'Empire' fruit indicated the parenchyma cells in 'Empire' apple cortex were prolate spheroids whose long axes lay in the radial direction, and the proportion of IS appeared similar in all planes. We could have used either transverse or longitudinal sections to display cortex cells in proper proportion for our microscopic analysis; however, we chose the transverse plane because samples of cell populations could be taken easily in any, or all, of the five ovarian sectors comprising the fruit.

Many studies demonstrate that apple fruit thinning promotes greater size by promoting cell division (or prolonging it) and/or cell enlargement. There is some evidence that large-celled apple fruit may be more subject to storage disorders (Martin *et al.*, 1965). This has not been substantiated by other studies on

storage breakdown (Sharples, 1968) or on tissue separation during saucing (Reeve, 1953). Certainly cell division is important, as it sets up the potentially large differences between fruit sizes under various thinning practices, if no differences in cell size occurs between treatments. Westwood (1978) stated that early thinning would tend to increase cell number but decrease cell size; while heavy thinning may increase cell size. During fruit histogenesis, cell expansion, intercellular space formation and cell division work in consort on fruit tissues. Thus, as one growth variate undergoes flux, there may be compensation by changes in others, such that not only fruit size is worked upon, but also fruit shape.

Martin and Lewis (1952) found that as crop load decreased in five apple cultivars, fruit weight increased along with average cell size and number. Bergh (1990) found as crop load increased, cell number and fruit size decreased. Denne (1960) noted in 'Cox's Orange Pippin' and 'Miller's Seedling' that early pre-bloom thinning allowed apples to be larger, with larger and more cells, than did no thinning. It is interesting to note that Martin *et al.* (1964) found thinning 'Jonathan' apples with NAA did not increase cell number, although cell number was increased after hand-thinning or by use of 3% sodium dinitro-o-cresylate. Kinetin sprays also did not promote cell division. They also obtained greatest cell numbers by thinning at blossom time. They suggested the major factor controlling cell number is the amount of reserves carried over from the previous season. Thinning sprays during the season do not affect the seasonal carry over and thus could not be expected to increase fruit cell numbers. Along these lines, Bergh (1985) found for 'Starking Delicious' that a heavy crop the previous season would reduce fruit size and act to reduce cortex cell number, and the potential reduction was already evident in differentiating flowers the previous fall.

Quinlan and Preston (1968) showed in 'Sunset' apple that severe hand thinning (leaving only 5% of all blossoms) at pink, 1, 2, or 3 weeks AFB increased fruit size because of increased cell numbers alone, rather than by the additional influence of cell enlargement. Also, the earlier they thinned, the greater the growth curve for fruit diameter over the season. Westwood, Batjer, and Billingsley (1967) presented evidence that smaller 'Delicious' fruits had fewer and smaller cells than did larger fruits. Centre-bloom fruits were also larger than side-bloom fruits of a given weight and had larger cells and a lower specific gravity than did side-bloom fruits. However, such centre-bloom fruits had no more cells than did side-bloom fruits. Nevertheless, early (one week AFB) heavy hand-thinning resulted in slightly more cells in large-sized fruit on thinned vs. unthinned trees. Smaller fruit did not show the same response. Marguery and Sangwan (1993) also discovered that cell numbers of central and side fruit generally were similar within one year old spurs, but fruit on two year old spurs were larger and had more cells than did fruit on one year old spurs.

Our investigation clearly shows that within a thinning treatment, increased 'Empire' fruit size is due primarily to increased cell numbers and not to greater cell size or to greater proportion of IS. This is also true between treatments: unthinned trees have smaller fruit which have fewer cells than do larger fruit from thinned trees; and fruit of trees thinned near bloom are larger and have more cells than those of trees thinned later. It appears that, no matter what the hand-thinning date, any treatment will display a fruit size-cell number relationship, while this cannot be said of cell volume or proportion of IS. Bain and Robertson (1951) found a strong linear relationship between fruit weight and fruit cell number. We also found this to be the general case for fruit within a thinning treatment; however, we found a curvilinear, almost asymptotic increase in fruit weight when regressed against cell numbers when using all data from all 60 fruit. Within the range of the data set we fit a second-order equation. It is clear, nonetheless, that there is a limit to the contribution of cell division to increase in fruit growth, because mature fruit size and weight tend toward an upper asymptote even as cell numbers may continue to increase in the very largest fruit.

Although results from any treatment show an increase in fruit weight with increase in cell

number, fruit from trees thinned beyond 10 DAFB and fruit from unthinned trees did not develop beyond a weight of 190 g or 50 million cortex cells. Fruit thinned earlier were able to increase in weight and cell number, presumably because early thinning allows fruit to continue cell division later in fruit development. However, in fruit from trees thinned early, the increase in fruit size appeared to diminish even as cortex cell numbers continued to increase (Figure 6). It appears that for fruit size or weight to continue to increase, factors other than cell number alone would have to contribute to the biological (and field management) process of size control.

Cell division slows markedly after anthesis. Bergh (1990) found the greatest rate of cell division in the apple cortex occurred about two weeks AFB. Coombe (1976) suggests that cells of the incipient apple flesh must double 21 times before anthesis, but only four to five times thereafter. In counting cells radially across the fruit flesh, we have also found the mature thickness of the flesh of 'Empire' results from only three to four cell generations from bloom date, and by three to four weeks AFB cell enlargement predominates (unpublished data). Coombe (1976) calculated that mature apple fruits had about 40 million cells, while Bergh (1990) calculated cortex cell numbers of 37–59 million, depending on cultivar and date of thinning. Our method appears to derive similar cell numbers in that we estimate 32–54 million cortex cells for mature 'Empire' fruit. Our method is advantageous because all data can be collected from a single fresh fruit in less than an hour, using unsophisticated equipment available in most histology laboratories anywhere in the world.

In related developmental studies of apple, the correlation between final 'Empire' fruit size and earlier fruit size became significant only after four to five weeks AFB; however, the best correlation between the relative growth *rate* of young fruit and final fruit size occurred in the period three to four weeks AFB, after which it diminished (Lakso and Goffinet, 1987). These early influences presumably result while the "cell division phase" is still in progress and while cells are small.

Knowledge of the relationship between IS and apple fruit volume remains rudimentary. In developmental studies of apple fruit, IS has usually been calculated by specific gravity determinations and with fruits weighed while immersed in water, that is indirectly, and not from microscopic examination (See references in Blanpied and Wilde, 1968). Calbo and Sommer (1987) estimated intercellular volume to be approximately 17% of total tissue volume in 'Gravenstein' apple, pear and other fruits, as well as in potato. Their method related the resistance of the tissue to evacuation of its contained air to the proportion of intercellular volume. Calculations were based on the volume of water displaced as a vacuum was applied above submerged pieces of tissue. An important admission by Calbo and Sommer was that removal of all the air is very difficult and even small gas leaks in the apparatus could cause some large errors. In somewhat similar fashion Reeve (1953) estimated apple IS volume by weighing submerged fruit in water or various concentrations of sucrose, vacuum infiltrating the tissues, then reweighing. Typical values for proportion of IS lay in the range of 20% to 25% for the middle flesh region of several varieties. However, Reeve did not include the smallest IS in his estimates.

Ruess and Stösser (1993) discussed a quantitative analysis of the IS system of apple fruits based on stereological analysis of plastic-embedded sections. Such direct anatomical methods are an important step in visualizing the three-dimensional structure of the IS system in apple fruit and in discerning differences imposed by crop management, such as fruit thinning. Their estimates of IS volume within the central cortex of mature fruits ranged from 17% to 27% in five varieties. Our stereological method estimates that the proportion of mature fruit cortex occupied by IS ranges from 32–35% in 'Empire'. This is substantially greater than the proportion found by the above authors; however, none has worked on 'Empire', so the cause of this difference cannot be explained fully.

Although Reeve (1953) and MacArthur and Wetmore (1941) discussed the possibility that the late-season increase in apple fruit size results from increased proportion of IS, they

provided no histological evidence that IS actually increased late in the season. Skene (1966) measured proportion of IS during development in apple and found no evidence for the above views; however, Skene's "volume of air-spaces" included the seed cavities. Skene (1941) had previously argued that air spaces grow in constant proportion to the fruit as a whole. Ruess and Stösser (1993), in contrast, give compelling anatomical evidence that the IS volume of apple fruit continues to increase well into postharvest storage.

We found no evidence in our study to support the view of Ruess and Stösser (1993) that, within a variety, there is a positive correlation between fruit size and intercellular space volume. They described results from evaluating nine varieties, none being 'Empire' or a parent of 'Empire'. We found little increase in proportion of IS with increase in 'Empire' apple fruit size or weight, and the proportion of IS explained very little of the variance in size or weight. Nor did our findings agree with those of Bain and Robertson (1951), that there is a close curvilinear relationship between fruit size and proportion of IS and that larger fruit have larger, more loosely packed cells.

Because our method attempts to use the stereological approach on rather thick transverse slices of tissue viewed with relatively low-magnification, optical focusing on the uppermost surface of the slice is critical. The focus must not be changed once cell counting and IS counting have begun for a given tissue region. Because of the great depth of field in this system, great care must also be made to mentally defocus any cells or IS seen below the cut surface. This is the greatest challenge to our method because, without careful cross checking, two observers may not resolve the same cell number or proportion of IS in a given sample. Careful training of operators, with consistent cross checking, decreases this discrepancy. The fruit preparations in our study were observed by only one trained observer. We have recently repeated our method using 'Empire' fruit harvested in 1993, in which two operators calculated statistically similar results for cell size, number and proportion of IS. No method of estimating fruit cell size and number will be error free. In the case of treatments affecting fruit size, it is the relative difference in fruit anatomy between treatments that is of critical importance to a study. Our method does have the advantage in that estimates can be derived very quickly for mature fresh fruit, without the need for chemical fixation, embedding in plastic, thin sectioning, and digital scanning.

In summary, the greatest increase in 'Empire' fruit size occurs in hand-thinned trees with bloom or near-bloom thinning. Fruit size and weight at harvest decrease as the post-bloom thinning date is prolonged. Decrease in size has an anatomical basis, whether within a range of fruit size resulting from a given thinning treatment or across all thinning treatments combined. That is, smaller fruit have fewer cells, while cell size and proportion of IS are each relatively unimportant in fruit size variation. Prolonging thinning reduces fruit size primarily because trees are thinned after the major cell division period. Fruits thinned before the end of this period can have the advantage of continuing cell division in the absence of nearby competing fruit.

We are now able to determine more precisely how adjustments in the time and severity of thinning affect 'Empire' fruit size, and how fruit size relates to changes in cell size, cell number and IS at harvest. Thinning date has a great influence on size of fruit and cell number is the primary anatomical character correlating with increase in fruit size in this variety. Thinning at or near bloom produces the largest fruits with the most cells. Current and future studies will test the effects of hand thinning and chemical thinning with NAA and BA on fruit size and anatomy. These studies will incorporate refinements in the stereological method, especially in the modelling of cortex shape, cell shape and intercellular space configurations in the three dimensions.

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