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RESEARCH PAPER

A novel and robust method for testing bimodality and characterizing porcine adipocytes of adipose tissue of 5 purebred lines of pig

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ABSTRACT

Adipocyte sizes from adipose tissue of mature animals form a bimodal distribution, thus reporting mean cell size is misleading. The objectives of this study were to develop a robust method for testing bimodality of porcine adipocytes, describe the size distribution with an informative metric, and statistically test hypertrophy and appearance of new small adipocytes, possibly resulting from hyperplasia or lipid filling of previously divided fibroblastic cells. Ninety-three percent of adipose samples measured were bimodal ($P < 0.0001$); therefore, we describe and propose a method of testing hyperplasia or lipid filling of previously divided fibroblastic cells based upon the probability of an adipocyte falling into 2 chosen competing "bins" as adiposity increases. We also conclude that increased adiposity is correlated positively with an adipocyte being found in the minor mode ($r = 0.46$) and correlated negatively with an adipocyte being found in the major mode ($r = -0.22$), providing evidence of either hyperplasia or lipid filling of previously divided fibroblastic cells. We additionally conclude that as adiposity increases, the mode of the major distribution of cells occurs at a larger diameter of adipocyte, indicating hypertrophy.

Introduction

It is well established that populations of adipocytes from adipose tissue of adult animals have a bimodal distribution and are, thus, not normally distributed.[1-9] Even so, to date the standard reported metric of cell size is typically the mean cell size of the distribution. This is despite recommendations by Rogers *et al.*[7] that the normality assumption, at least in Zucker rats, is erroneous. We also believe that it is inappropriate to use a single grand mean cell size as representative of adult adipose cell size distribution specifically because the presence of a secondary mode in the range of measured smaller cells artificially lowers the mean and may be misleading. One would expect an animal with a greater mean adipocyte cell size to have more adiposity, which may not be the case if the cells are bimodally distributed.

Many methods have been proposed to arrive at a simple hypothesis test for bimodality. A discussion of some of them is included in Jackson *et al.*[10] All of those methods discussed rely on the assumption of normality under both the null and alternative hypotheses. As discussed above, however, this condition is not met in our situation. In spite of this, we have the good fortune of data whose empirical distribution is such that a simple and robust hypothesis can be used. This method is described in detail in the Materials and Methods section below. No model distribution is necessary using this method.

The objective of this study was to characterize populations of adipocytes of adipose tissue of 5 different purebred lines of pigs of varying adiposity and to develop a more appropriate statistical method of describing these populations based on the hypothesis that a single grand mean is an inappropriate metric for describing adipocyte populations. We now proceed to the details of this study.

Materials and methods

Animals and diets

All research was approved by the Iowa State University Institutional Animal Care and Use Committee (IACUC). A total of 149 barrows and gilts of 5 different breeds (Berkshire = 40 barrows and 25 gilts; Chester White = 15 barrows and 2 gilts; Duroc = 7 barrows and 4 gilts; Landrace = 6 barrows and 17 gilts; Yorkshire = 17 barrows and 16 gilts) were delivered to the Iowa Swine Testing Station (Ames, IA) and housed in a commercial

slatted finishing barn with 8 pigs randomly assigned per pen. Pigs began the performance test when the pen average pig weight was 31.8 kg. All pigs were fed a 6-phase commercial maize/soy based diet with dried distillers grains with solubles (DDGS) inclusion at 30% of dietary DM. DDGS was removed from the ration for the final 30 d of the finishing period. Pigs were removed from the performance test at a minimal weight of 111.4 kg and transported to Hormel Foods (Austin, MN) for slaughter the following morning.

Adipose tissue cellularity

Following harvest and upon arrival of the split carcasses to the chillbox, adipose tissue samples (about 20 g) were excised from the jowl, the back over the 10th rib, and the midsection of the belly, placed in 0.9% NaCl solution at 37°C, and transported to Iowa State University (Ames, IA) at 37°C for cellularity assays. An approximately 200 mg section of fresh adipose tissue from the back (outer and middle subcutaneous fat), belly, and jowl were fixed in 5 mL of 2% osmium tetroxide in 50 mM collidine hydrochloride buffer at pH 7.6 as described by Hirsch and Gallian[11] approximately 6 hours post slaughter. Connective tissue debris was solubilized with 8 M urea as described by Etherton et al.[12] Adipocytes were freed by washing with a 0.9% NaCl solution containing 0.01% Triton X-100, pH 10, through a 250-μm nylon mesh filter and collected onto a 20-μm nylon mesh filter. Mean and mode adipocyte cell size and cellularity (cells per gram of tissue) were determined using a Coulter Counter (Beckman-Coulter, Brea, CA). The sample diluent was 0.9% NaCl containing 0.01% Triton X-100, pH 10, and the K_d (diameter calibration) of the machine was calibrated to 631.48. Samples were sized and counted by using a 400 μm aperture into 300 size bins ranging from 20 to 240 μm and counted in triplicate. The mean of the 3 measurements was used. The coefficient of variation between replicates was less than 5 percent.

Robust hypothesis testing of adipose bimodality and hyperplasia or lipid filling of previously divided fibroblastic cells

Formal testing of bimodality of each distribution of adipocytes was performed by identifying 2 bins (Fig. 1A), with bin 1 containing the suspected minor mode and bin 2 separating the major and minor modes to be used for comparison. Bin 1 contained measured adipocytes with a diameter between 25.0125 and 40.1052 μm. Bin 2 contained measured adipocytes with a diameter between 50.1565 and 65.3792 μm. For the kth cell, we defined the event $[X_k = 1]$ with probability $p = \Pr[X_k = 1]$ if that cell's diameter falls in bin 1 and the event $[X_k = 0]$ with probability $1 - p = \Pr[X_k = 0]$ if it falls in bin 2. The following natural estimator of (p) was used:

$$\widehat{p} = \overline{X} = \frac{1}{N}\sum_{k=1}^{N} X_k \qquad (1)$$

Let the true mean height of bin 1 be $f_1 = p/b_1$ where b_1 is the width of bin 1. Similarly, the true mean height of bin 2 is $f_2 = (1 - p)/b_2$. We then propose the following simple one-sided hypothesis test: $H_0: f_1 = f_2$ versus $H_1: f_1 > f_2$.

By noting that H_0 is equivalent to $p = b_1/(b_1 + b_2) \triangleq r_b$, this test may be restated as

$$H_0: p = r_b \text{ vs. } H_1: p > r_b$$

In Figure 1A we have $b_1 = b_2$; hence, $r_b = 0.5$. By focusing on only these 2 bins, the size of our data set is reduced to only data included in these bins. Clearly, a key element of this method is in the selection of the bins. In Figure 1A we have the good fortune of selection in a relatively straightforward manner. In general, one would need to acknowledge the tradeoff between using more data associated with larger bins, and having bins so large as to weaken the power of the test. We are undertaking the development of a robust bin width selection metric. However, this development not far enough to bring it to bear on this work.

The test statistic given in (1) is an average of simple 0/1 (i.e. Bernoulli) random variables. In fact, the probability structure of (1) is known exactly. It is a scaled binomial random variable with parameters (N,p). Moreover, for sufficiently large N, (as is the case in this work), it follows from the Central Limit Theorem (CLT) that (1) can be well- approximated by a normal random variable having mean p, and variance $p(1-p)/N$.

Because the sample size, N, associated with the number of cells falling in either of the 2 bins was on average on the order of 6,600 adipocytes counted for a given test, the CLT clearly applies. Hence, the test statistic (1) becomes the standard normal random variable

$$Z = \frac{\widehat{p} - 0.5}{0.5/\sqrt{N}} \qquad (2)$$

There are several tests for multimodality. Two popular tests are the likelihood ratio test[13] and the dip test.[14] The former test requires a specified distribution, while the latter test uses the uniform distribution as the least favorable prior. Both tests are of a general nature, in the sense that there is no a priori knowledge of potential multimodality. In that sense,
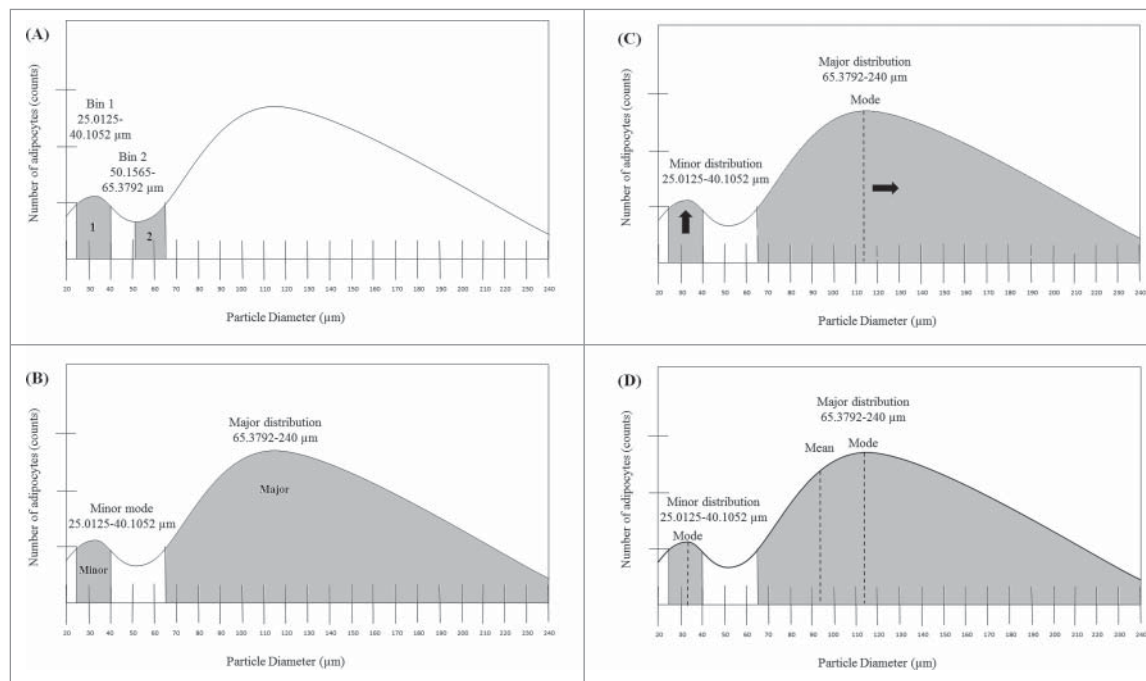
Figure 1. Representation of typical distribution of adipocytes in porcine adipose tissue. (A) Representation of bin 1 and bin 2 (defined in Materials and Methods) used in hypothesis testing for determining unimodality or bimodality of adipocyte distribution. (B) Representation of the "minor" and "major" modes with the "dead zone." (Bin 2 in Fig. 1A; 50.1565–65.3792 μm) (C) Illustration of the behavior of the minor and major modes as an animal increases in adiposity. The minor distribution of cells becomes better defined (i.e., taller), and the mode of the major distribution of cells moves to the right. (D) Illustration of why mean adipocyte size is an inappropriate metric for describing bimodal adipocyte cell distributions. It is clear that the mean is misleading because the presence of the minor mode lowers the value of the mean. The mode of the major distribution and the mode of the minor distribution more accurately represent the most abundant diameter of adipocyte in both distributions.

they are more sophisticated than ours. However, our data strongly supports such a potential. Moreover, unlike the above tests, ours makes no assumptions whatsoever, in regard to cell size distribution. We assume only that it is a continuous distribution, and that the CLT assumption holds.

Other statistical analyses

All statistical analyses other than the test for bimodality were performed by using SAS version 9.3 (SAS Inst. Inc., Cary, NC). Correlations were analyzed by using the CORR procedure of SAS.

Adipocyte cellularity (mean cell size, mode cell size, and adipocytes per gram of adipose tissue) data were analyzed by using the MIXED procedure of SAS with sampling location, breed, sex, sample date (contemporary group), and the interactions of breed × location and sex × location as fixed effects with percentage fat-free lean tissue (%FFL) as a covariate.

All means were separated by using an F-protected least-square means separation and reported as the mean plus or minus the SEM. When significant interaction effects were found, P-values were determined by using

the SLICE command of LSMEANS. Statistical significance was declared at a $P < 0.05$.

Results and discussion

Bimodality of the adipocyte size distribution

For equal representation of lean and fat animals, these data were generated from breeds of growing pigs of varying adiposity as shown in Table 1. Evidence that sample mean adipocyte diameter is an inappropriate metric and that the data are bimodally distributed is shown in Figure 2. Clearly, the most probable cell diameter, which one would expect to be the sample mean in a unimodal distribution, does not occur in the region of the grand mean (∼91 μm averaged across all breeds and locations) but rather in the location of the mode of the major distribution of adipocytes (∼112 μm averaged across all breeds and locations; Fig. 1D). The maximal probability of a particular adipocyte diameter that is located in the minor mode occurs quite near where we would expect to see it based on the minor mode data shown in Table 2 and Figure 1D.

The cells that contribute to the cellularity of the minor mode could be of 3 distinct origins: 1) Nondifferentiated

Table 1. Average daily gain, fat-free lean percentage, and backfat thickness of 5 purebred and one crossbred line of pigs.

Item	Breed[2]						Sex		P-Values		
	Berkshire	Chester White	Crossbred	Duroc	Landrace	Yorkshire	Barrow	Gilt	Breed	Sex	Breed × Sex
ADG, kg	0.80[a] (± 0.01)	0.83[ab] (± 0.02)	0.85[b] (± 0.01)	0.87[b] (± 0.01)	0.79[a] (± 0.01)	0.87[b] (± 0.01)	0.85[a] (± 0.01)	0.82[a] (± 0.01)	<0.001	0.011	0.151
FFL, %[3]	47.94[a] (± 0.41)	50.59[bc] (± 1.15)	53.37[d] (± 0.56)	52.53[cd] (± 0.39)	51.19[b] (± 0.44)	52.39[cd] (± 0.40)	49.33[a] (± 0.24)	53.34[b] (± 0.43)	<0.001	<0.001	0.016
BF, cm[4]	2.346[ab] (± 0.01)	2.366[a] (± 0.04)	2.422[b] (± 0.02)	2.493[c] (± 0.01)	2.467[c] (± 0.01)	2.415[ab] (± 0.01)	2.455[b] (± 0.01)	2.382[a] (± 0.01)	<0.0001	<0.0001	0.0004

Note. [a, b, c, d] Within a row and main effect, means without a common superscript differ (*P* < 0.05).
[1] Standard error in parenthesis below mean value.
[2] *n* = 149 pigs; number of pigs within each breed (number of barrows, number of gilts): Berkshire = 65 (40, 25); Chester White = 17 (15, 2);
Duroc = 11 (7, 4); Landrace = 23 (6, 17); Yorkshire = 33 (17, 16).
[3] FFL = fat-free lean.
[a,b,c,d] Within a row and main effect, means without a common superscript differ (*P* < 0.05).
[4] BF = backfat thickness in cm.

stem cells become differentiated to preadipocytes that begin to accumulate lipid and come to be found in the minor mode, 2) Proliferative pre-adipocytes from embryonic development may begin to add lipid and come to be found in the minor mode (~8 μg to 34 μg of lipid), and 3) Mature cells may proliferate and appear in either the major or minor mode.[15] The cells found in the major mode are of 2 types: 1) Mature adipocytes that have accumulated enough lipid (approximately 144 μg or more of lipid) to be found in the major mode and 2) Mature adipocytes that have proliferated but are still
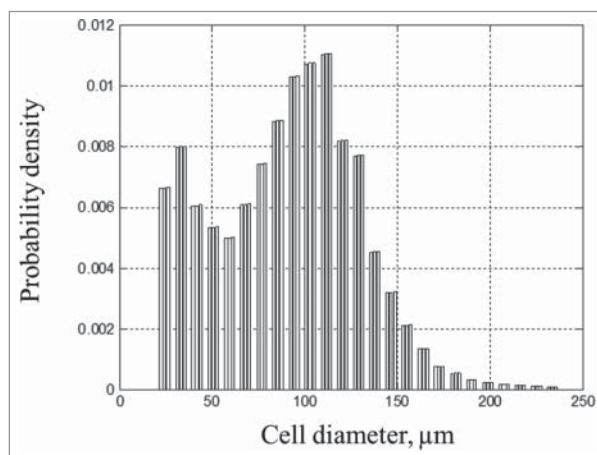
Figure 2. Histogram-based adipocyte diameter probability distribution function. Probability distribution function was generated from 14,703,815 size bins. Probability distribution function of adipocyte diameter is from all breeds, sexes, and anatomic locations of assayed pigs (n = 149 pigs x 3 anatomic locations = 447). In each cluster of bars, the left bar is the lower standard (2-sigma) error, the middle bar is the estimated probability distribution function, and the right bar is the upper standard (2-sigma) error. As in Figure 1C, we would expect that as an animal gains more adiposity the probability of the minor distribution of cells as a whole would increase, and maximum probability of the major distribution of adipocytes would shift to a larger adipocyte diameter. The probability of a chosen bin is the bin width (8.7141 μm) multiplied by the height of that chosen bin.

large enough to be found in the major mode. It is, however, beyond the scope of this methods paper to investigate the origin of the small adipocytes that appear in the minor mode.

Additionally, reporting sample mean cell size is only appropriate if cells are unimodally distributed. In carrying out the hypothesis test for bimodality described in the Materials and Methods section, we observed that, indeed, 93.18% of the 455 measured adipose tissue samples had a bimodal distribution of adipocytes at a *P*-value < 0.05. The additional 6.82% of samples that did not test positive for being bimodal were visually inspected to verify that no secondary mode existed. These samples were, however, not a normal distribution. So, sample mean adipocyte diameter is not the appropriate metric to use for describing distributions of adipocyte diameters. Thus, we propose using adipocyte diameter of the mode of the minor (Table 2) and major (Table 3, Fig. 1D) distributions of cells as a metric for reporting cell distributions for future work. For sake of comparison to traditional metrics of adipocyte size we also have included grand mean adipocyte cell sizes in Table 4.

Adipose tissue cellularity and mean and mode cell size

Cellularity, defined as the number of cells (adipocytes) per gram of adipose tissue, from backfat was significantly and negatively correlated with backfat thickness (*r* = −0.515; Table 5). Adipose tissue cellularity (Table 6) was consistent with, but slightly lower than, cellularity data reported for 114 kg pigs sampled from dorsal subcutaneous neck adipose tissue by Mersmann and Macneil[5] (1.81 × 10^{6} cells × gram[−1]). A possible explanation for our data being slightly lower is that the adipose of the dorsal neck may differ from the adipose of the ventral neck. Significant breed effects in cellularity were found

Table 2. Minor mode adipocyte diameter from 5 breeds of pigs as affected by breed, anatomic location, and sex (μm).[1,2]

	Breed					Sex[3]		P – Value	
Depot	Berkshire	Chester White	Duroc	Landrace	Yorkshire	B	G	Breed × Location	Sex
Back	30.67[X]	31.95[X]	29.12[X]	31.17[X]	30.06[X]	32.85	31.17	0.1960	0.0002
	(± 0.45)	(± 0.84)	(± 1.03)	(± 0.75)	(± 0.64)	(± 0.27)	(± 0.35)		
Belly	33.00[Y]	34.81[Y]	33.67[Y]	34.68[Y]	32.66[Y]			0.1166	
	(± 0.44)	(± 0.92)	(± 1.28)	(± 0.81)	(± 0.64)				
Jowl	32.93[Y]	32.01[Y]	30.70[XY]	30.50[X]	32.29[Y]			0.0900	
	(± 0.45)	(± 0.88)	(± 1.13)	(± 0.83)	(± 0.63)				

Note. [1]n = 149 pigs; number of pigs within each breed (number of barrows, number of gilts): Berkshire = 65 (40, 25); Chester White = 17 (15, 2); Duroc = 11 (7, 4); Landrace = 23 (6, 17); Yorkshire = 33 (17, 16).
[2]Standard error in parenthesis below mean value.
[3]B = Barrow, G = Gilt.
[X, Y, Z] Within a column, means without a common superscript differ ($P < 0.05$).

when comparing back, belly, and jowl adipose depots (P = 0.0004, $P < 0.0001$, and P = 0.0209, respectively) across breeds (Table 6). Differences also were found when comparing anatomic locations within a breed, with the belly always having the numerically greatest cellularity and back always having the numerically least cellularity. It is also worth noting that most of the measured depots had a fairly large standard error of mean (SEM) indicating that there is considerable variability in the cellularity of these measured depots both within and across breeds.

Significant differences for mean adipocyte cell size were only found in belly fat (P = 0.0007) across breeds with Duroc pigs having the smallest mean adipocyte size and Berkshire pigs having the largest. Within Berkshire pigs, the mean cell size of jowl fat adipocytes was significantly smaller in diameter than that in belly and backfat ($P < 0.05$; Table 4).

The adipocyte diameter of the mode of the minor distribution of cells (Table 2; modes described in Fig. 1B) did not vary significantly across breeds but varied significantly within a breed. The general trend of adipocyte diameter of the minor mode was for backfat adipocytes in the minor distribution of cells to be of the smallest diameter and belly fat adipocytes of the minor mode always to be of the largest diameter. It is, however, unclear if the differences in adipocyte diameter of the mode of the minor distribution is of practical significance because the differences were 4 μm or less and because the number of cells that fall into the minor mode is very small in comparison to the cells found in the major mode. In addition, adipocyte diameter of the minor mode is not significantly correlated with adiposity as measured by backfat thickness (data not shown). Therefore, the differences in adipocyte diameter of the minor mode are not likely of any physiologic consequence. Adipocyte diameter of the mode of the minor distribution of cells was also significantly different for barrows

and gilts (approximately 1.6 μm) which, again, is likely not of any physiologic significance.

The adipocyte diameter of the mode of cells found within the major distribution (Table 3) varied significantly within both a breed and anatomic location within sex. As expected, Durocs, one of the leanest breeds of pigs (Table 1), had the smallest adipocyte diameter for the mode of the major distribution of cells (Table 3). Interestingly, Yorkshire and Chester White pigs that were equally as lean as Duroc pigs had an intermediate adipocyte diameter for the mode of the major distribution of cells indicating that adiposity is not the only factor influencing the adipocyte diameter of the mode of the major distribution of adipocytes in Duroc pigs (Table 3). The largest adipocyte diameter of the mode of the primary distribution of cells was found in Berkshire pigs, the breed with greatest adiposity in this experiment. It seems that Duroc pigs may be somewhat of an anomaly as based on this cellularity-related data, likely as a result of genetic differences. When Duroc pigs are ignored, the range between the smallest and largest major mode cell size is only about 5 μm, which might be of little physiologic consequence (Table 3).

Adipose tissue hypertrophy and appearance of small adipocytes in the minor mode

Understanding how adipose tissue development and growth is regulated is of great clinical importance.[16] Adipose tissue growth occurs by hypertrophy, hyperplasia, or both hypertrophy and hyperplasia.[15] Understanding the mechanism of adipose tissue growth is important to basic science and relevant to animal and human health. Adipose growth by hypertrophy is linked with inflammation, fibrosis, and insulin resistance,[17] whereas adipose hyperplasia is linked with increased insulin sensitivity.[17-19] Because the adipose samples we studied were distributed bimodally, we investigated if the nature of this bimodal distribution was evidence of hypertrophy,

Table 3. Major mode adipocyte diameter from 5 breeds of pigs as affected by breed and anatomic location within sex (μm).[1,2]

| | Breed | | | | | Location | | | | | | P – Values | |
| | | | | | | Back | | Belly | | Jowl | | | |
Item	Berkshire	Chester White	Duroc	Landrace	Yorkshire	Barrow	Gilt	Barrow	Gilt	Barrow	Gilt	Breed	Location × Sex
Major Mode[3]	117.18[c] (0.85)	112.15[b] (1.50)	106.14[a] (1.89)	112.80[b] (1.34)	111.01[b] (1.12)	117.03[c] (1.28)	112.03[b] (1.54)	104.43[a] (1.29)	111.83[b] (1.55)	111.91[b] (1.28)	113.91[bc] (1.60)	<0.0001	<0.0001

Note. [1] n = 149 pigs; number of pigs within each breed (number of barrows, number of gilts): Berkshire = 65 (40, 25); Chester White = 17 (15, 2); Duroc = 11 (7, 4); Landrace = 23 (6, 17); Yorkshire = 33 (17, 16).
[2]Standard error in parenthesis below mean value.
[3]Adipocyte diameter of the mode of cells within the major distribution.
[a, b, c]Within a row, means for breeds without a common superscript differ ($P < 0.05$).

Table 4. Mean adipocyte cell size from 5 breeds of pigs (μm).[1,2]

Depot	Breed					P - Value Breed × Location
	Berkshire	Chester White	Duroc	Landrace	Yorkshire	
Back	94.26[Y]	91.05	90.59	93.46	92.46	0.3019
	(±0.88)	(±1.69)	(±2.07)	(±1.45)	(±1.22)	
Belly	95.41[Y,c]	89.02[a,b]	87.42[a]	93.26[b,c]	92.49[b,c]	0.0007
	(±0.88)	(±1.74)	(±2.07)	(±1.48)	(±1.22)	
Jowl	89.15[X]	90.57	92.08	90.73	90.67	0.6873
	(±0.91)	(±1.70)	(±2.07)	(±1.51)	(±1.24)	

Note. [1]n = 149 pigs; number of pigs within each breed (number of barrows, number of gilts): Berkshire = 65 (40, 25); Chester White = 17 (15, 2); Duroc = 11 (7, 4); Landrace = 23 (6, 17); Yorkshire = 33 (17, 16).
[2]Standard error in parenthesis below mean value.
[X, Y, Z]Within a column, means without a common superscript differ ($P < 0.05$).
[a, b, c]Within a row, means without a common superscript differ ($P < 0.05$).

hyperplasia, or both hypertrophy and hyperplasia occurring in pigs of market weight. Because backfat thickness was the indicator of adiposity, it would be inappropriate to correlate attributes of backfat adipose to any depot other than the back (Table 5). As would be expected, mean and mode cell size of the back adipose tissue is correlated negatively to the cellularity of backfat adipose, and adipocyte cell size is positively correlated with backfat thickness indicating that as an animal becomes larger, its adipose tissue is filled with larger adipocytes and fewer adipocytes per gram of tissue (Table 5). When comparing the adipocyte diameter of the minor mode to backfat thickness (Table 5) we found no significant correlation between the 2 measurements indicating that the adipocyte diameter of the minor mode is not dependent on the adiposity of the pig. The ratio of the adipocyte diameter of the minor mode to the adipocyte diameter of the major mode (r_d) was significantly and negatively correlated with backfat thickness (Table 5) and, because the adipocyte diameter of the minor mode is not dependent on the adiposity of the pig, this negative correlation indicates that as the backfat thickness increases the adipocyte

diameter of the major mode becomes larger and the minor distribution of cells becomes more well defined (i.e., more adipocytes represented by the minor mode; Fig. 1C).

Next, we computed an estimate of the ratio of probabilities (r_p), the probability of an adipocyte being in the minor mode divided by probability of an adipocyte being in the major mode. A significant and positive correlation (Table 5) between r_p and backfat thickness indicates that as adiposity increases the probability of an adipocyte being found in the minor mode is greater, which is consistent with adipose hyperplasia or differentiation of pre-adipocytes into nascent adipocytes. The correlation of r_d and r_p to backfat thickness supports the idea that in the finishing phase these pigs were undergoing adipose growth via both hypertrophy and hyperplasia, differentiation of pre-adipocytes to nascent adipocytes, or lipid filling of previously divided fibroblastic cells. The histogram of probabilities associated with adipocyte diameters (Fig. 2) indicates that the maximal adipocyte diameter is limited to approximately 240 μm because the probability of finding an adipocyte of that size or greater drops to

Table 5. Correlations of cellularity and cell size related measures of back adipose tissue.

Items	Data[1]	Data[2]	r	P- Value[3]
Cellularity × 10^6 of backfat to BF[4]	1.09 ± 0.42	2.48 ± 0.79	−0.5157	<0.0001
Cellularity × 10^6 of backfat to mean cell size of backfat (μm)	1.09 ± 0.42	93.21 ± 7.71	−0.6610	<0.0001
Cellularity × 10^6 of backfat to major mode cell size of backfat (μm)	1.09 ± 0.42	117.16 ± 13.75	−0.6491	<0.0001
BF[4] to major mode cell size of backfat (μm)	2.48 ± 0.79	117.16 ± 13.75	0.7818	<0.0001
BF[4] to secondary mode cell size (μm) of backfat	2.48 ± 0.79	30.81 ± 3.10	−0.0681	0.4226
BF[4] to probability ratio of modes of backfat[5]	2.48 ± 0.79	0.19 ± 0.04	0.4266	<0.0001
BF[4] to ratio of mode cell sizes of backfat[6]	2.48 ± 0.79	0.26 ± 0.04	−0.6021	<0.0001
BF[4] to probability of a cell being found in the minor mode of backfat	2.48 ± 0.79	0.15 ± 0.02	0.4601	<0.0001
BF[4] to probability of a cell being found in the in the major mode of backfat	2.48 ± 0.79	0.78 ± 0.03	−0.2228	0.0079

Note. [1]Mean numerical value from first correlate in each line plus/minus standard deviation, n = 143 pigs.
[2]Mean numerical value from second correlate each line plus/minus standard deviation, n = 143 pigs.
[3]P-values for difference from zero.
[4]BF = backfat thickness in centimeters.
[5]Probability of finding an adipocyte in the minor mode divided by the probability of finding an adipocyte in the major mode.
[6]Adipocyte cell diameter of the minor mode divided by the adipocyte cell diameter of the major mode.

Table 6. Adipose tissue cells per gram of tissue of 5 breeds of pig (X 10^6).[1]

| Depot | Breed[2] | | | | | P-Value |
	Berkshire	Chester White	Duroc	Landrace	Yorkshire	Breed × Location
Back	1.01^{a,X}	1.15^{a,X}	1.55^{b,X}	1.07^{a,X}	1.04^{a,X}	0.0004
	(±0.05)	(±0.09)	(±0.11)	(±0.08)	(±0.07)	
Belly	1.35^{a,Y}	1.68^{b,Y}	2.24^{c,Y}	1.63^{b,Z}	1.51^{a,b,Y}	<0.0001
	(±0.05)	(±.10)	(±0.11)	(±0.08)	(±0.07)	
Jowl	1.24^{a,b,Y}	1.45^{c,Y}	1.46^{b,c,X}	1.31^{a,b,c,Y}	1.14^{a,X}	0.0209
	(±0.05)	(±0.09)	(±0.11)	(±0.08)	(±0.07)	

Note. [1]Standard error in parenthesis below mean value.
[2]$n = 149$ pigs; number of pigs within each breed (number of barrows, number of gilts): Berkshire = 65 (40, 25); Chester White = 17 (15, 2); Duroc = 11 (7, 4); Landrace = 23 (6, 17); Yorkshire = 33 (17, 16).
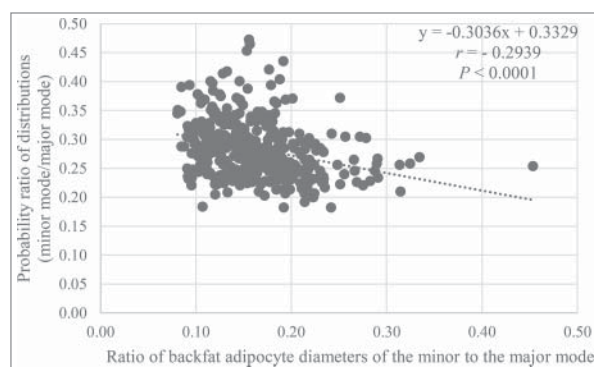X, Y, Z Within a column, means without a common superscript differ ($P < 0.05$).
a, b, c Within a row, means without a common superscript differ ($P < 0.05$).

nearly zero. Thus, the limit of hypertrophy seems to occur when porcine adipocytes reach a diameter of around 240 μm.

More evidence of hyperplasia or differentiation of pre-adipocytes into nascent adipocytes was indicated when comparing r_p to backfat thickness. As backfat thickness increases, r_p increases (Table 5) suggesting that as adiposity increases appearance of small adipocytes in the minor mode increases (i.e., differentiation of preadipocytes to adipocyte, both hyperplasia and differentiation of preadipocytes, or lipid filling of previously divided fibroblastic cells). The notion that these data represent an increase in number of small adipocytes in adipose tissue is supported even further when comparing r_p to r_d (Fig. 3). Figure 3 demonstrates this notion, because these 2 ratios are negatively correlated. As the adipocyte diameter of the major mode becomes larger (or the adipocyte diameter of the

Figure 3. Behavior of the major and minor modes in relation to probability of an adipocyte being found in either the major or minor mode. Relationship (probability ratio of modes) of the area under the curve of the distribution of adipocytes containing minor mode (smaller cell diameter) divided by the area under the curve of the distribution of adipocytes containing the major mode (larger cell diameter) to the ratio of the adipocyte diameter of the mode of the minor adipocyte distribution divided by the adipocyte diameter of the mode of the major adipocyte distribution (ratio of adipocyte diameters of the minor to the major mode).

minor mode becomes smaller), the probability of finding an adipocyte in the distribution of adipocytes containing the minor mode becomes larger (or the probability of finding an adipocyte in the distribution of adipocytes containing the major mode becomes smaller). The results that indicate hyperplasia, differentiation of pre-adipocytes to nascent adipocytes, or lipid filling of previously divided fibroblastic cells is occurring in these pigs at slaughter weight are not consistent with previous research that observed no further hyperplastic growth and only hypertrophic growth was observed in extramuscular fat of Hampshire × Yorkshire and Minnesota 3 × 1 pigs after 83 kg live weight.[20] However, both adipocyte hypertrophy and hyperplasia were observed in both Meishan (~52 kg) and Landrace pigs (~83 kg) at 5 months of age.[20] Traditionally, including the work done by Nakajima and colleagues,[21] researchers have relied on an observed bimodal/biphasic distribution to be evidence of hyperplasia. We have taken that one step further and applied rigorous statistical analyses to describe the bimodal distribution evidence of an increase in the number of small adipocytes gleaned from these distributions by quantitative rather than by qualitative methods. It is possible that in a younger pig you would not see such a prominent minor mode, and the probability of finding an adipocyte in the major mode or minor mode may be reversed. It is worth taking into consideration that these pigs were adolescent in terms of human growth at the time of slaughter and that hyperplasia has been demonstrated in humans to occur through adolescence.[22] So, observing adipose growth by both hypertrophy and an increase in number of small adipocytes at this age of the pig is consistent with data previously reported. Additionally, high fat diets, such as those for the pig, cause hyperplasia in rats.[23] It also has been demonstrated that in mice hypertrophy and hyperplasia occur as a result of fat pad mass increases rather than as a function of age.[6] Finally, variations between individuals occur and that some New Zealand rabbits at one year of age have normally

distributed adipocytes and some have bimodally distributed adipocytes.[24] The authors speculated that the bimodal distribution was a result of recruitment of new small adipocytes. These data reported in our study assume that small cells (located in the minor distribution) are new cells and also rely on only one time point in the life of the animal. However, differentiating between new cells and old small cells is not possible. Future confirmation of in vivo cellular division by BrdU/PCNA[25,26] studies should be done to differentiate between the many ways an adipocyte may be found in the minor mode. These ways include both hyperplasia of adipocytes, differentiation of pre-adipocytes to nascent adipocytes, or lipid filling of previously divided fibroblastic cells and cannot be distinguished by using this indirect method of measuring hyperplasia. Additionally, the osmium tetroxide approach for estimation of cellularity is not without inherent flaws. It is likely, that because the osmium tetroxide approach is specific to lipid-filled adipocytes, it therefore would not identify adipose tissue stem cells that contain no lipid. To identify these stem cells, certain cell-specific markers would need to be applied to characterize the stem cells that contain no lipid; however, that was beyond the scope of this study. Finally, the outer layer of subcutaneous back fat contains a potentially confounding additional depot, which is those adipocytes around the hair follicles that are physiologically distinct from other subcutaneous adipose tissue.[27] Although every effort was made to exclude hair follicles from the adipose tissue used for this study, the possibility remains that some of these physiologically distinct adipocytes also were included in the analysis and therefore the full extent of depot influence must be considered in this type of study. To fully understand how adipose growth progresses, a longitudinal animal study examining regulation and development of adipose tissue over time, and going well into adulthood for the animal, needs to be done to determine if hyperplasia occurs in adulthood.

Summary and conclusions

It was determined that sample mean adipocyte cell size is an inappropriate descriptor of adipocyte cell size because of the bimodal distribution of adipocytes found in most of adipose depots assayed. We recommend that future adipose tissue research include the generalized analysis proposed in this study to quantitatively identify bimodality of adipocyte cell distributions.

Finally, convincing evidence of hypertrophy and increased numbers of small adipocytes (either by hyperplasia or by differentiation of pre-adipocytes into nascent adipocytes) both being a factor in expansion of adipose tissue of pigs at market weight was shown through quantitative methods and should be applied to future adipose tissue growth and development research. Increased numbers of small adipocytes in adipose tissue that is still occurring in pigs of market weight begs the question whether or not hyperplasia of adipose tissue continues on into adult life, particularly in high fat feeding programs. More research will need to be done to determine whether or not hyperplasia of adipose tissue is a factor in adult-life, to identify mechanisms controlling hyperplasia of adipose tissue, and to develop the potential implications to better understand the growth and development of adipose tissue of food-producing animals and of humans.

Abbreviations

BF	Backfat thickness in centimeters
BrdU	Bromodeoxyuridine
DDGS	Dried distillers grains with solubles
DM	Dry matter
FFL	Percentage fat free lean tissue
NaCl	Sodium chloride
PCNA	Proliferating cell nuclear antigen
SEM	Standard error of the mean

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The authors have no potential conflicts of interest to disclose.

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