Early-stage honeycomb contains non-uniform cells which are subsequently optimised

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Abstract

The comb of honeybees has long been admired for its even hexagonal layout, but such a regular geometry is possible only if it comprises isodiametric cells arranged in a regular pattern of one surrounded by six others. The support structure of a natural nest will not provide a horizontal smooth surface and so will force irregularities, as will the merging of individual tongues of comb, introduction of accidental errors or the inclusion of larger drone cells. These perturbations force within regular comb the construction of non-equilateral or non-equiangular cells, or those that are non-hexagonal. Using automated image analysis, we extracted cell geometry from naturally built comb and that built on wax stimuli at different stages during comb construction. We show that, when faced with disruption, the bees initially build cells that are uneven and irregular, but that following further construction effort they bees reform those cells. The eventual comb exhibited increased homogeneity of cell area; cells that were more equilateral and equiangular. Overall regularity, or progressive transition of cells from one size or alignment to another arose through adjustments to each cell after the initial steps of comb construction.

Introduction

Within natural honeycomb, the hexagonal face shows 3-way cell intersections with typical 120° wall junctions, where the walls are equally spaced around 360° in a two-dimensional layout. At the interface between cells on either side of the comb, there are four-way cell intersections; one cell from one face centred at the intersection of three cells from the other face. At that four-way junction, the dihedral angles are all 120° where the walls are equally spaced around 360° in a 3-dimensional layout. The bees seem highly adept at building comb where cell walls are evenly distributed, and the experiments described in this chapter were designed to examine the mechanisms whereby they achieved such an outcome.

We hypothesise that a stigmergic reaction to form a convex, spheroid, cell will be triggered by conditions where the current state of a cell's construction would be recognised as an enclosure. Wherever cells are sufficiently close as to be in contact with each other, the construction of each will interfere with the construction of the other(s). Any wall common to two cells will inevitably be subject to the influence being exerted in both cells. Cell walls can have two cells in common, but junctions between walls can arise through contact between multiple cells: 2, 3, 4 or, occasionally, more. If behaviour were to proceed according to this hypothesis, thenwe

predict that for all cells that meet at a single junction, the angles between pairs of walls would equalise.

To test this prediction, pieces of wax were placed in hives and left for the bees to build honeycomb upon them. These samples were inspected periodically, and the resulting cell structures were measured to determine the curvature of cell walls under conditions of isolation compared with those built between two cells. Side walls between cells on a single face of the comb and base walls between cells on opposing sides of the comb were measured and compared during these experiments.

The experiments described here test our prediction by creating atypical conditions. If our prediction is correct then, as a result of equalisation, non-120° inter-wall angles should be formed under conditions where the angular range is other than 360° or the number of intersecting cells is not two. We tested this prediction further by experiments that allow conditions to change over time, with the expectation that the eventual state of equalisation will also change accordingly.

Methods

Hive handling and recording

Hives

Three British Nation hives housing locally reared honeybees, *Apis mellifera* were used in these experiments. The frames carrying the experimental stimuli were positioned at the edge of the brood zone.

Instigation of comb construction

Comb construction was encouraged by *ad libitum* feeding with 1:1 sucrose solution.

Wax

Wax used to form the various stimuli for these experiments had been recovered during previous seasons from hives within the same apiary was formed into sheets of either 0.5 mm-0.6 mm or 1.0 mm-1.2 mm thickness.

Preparation of stimuli

The stimuli used to study comb building behaviour were formed from flat sheets of wax placed vertically within the hive mounted under the top bar of an otherwise empty frame. Experiment specific stimuli were prepared, shaped as required and welded to the vertical tab. The exact designs used for the stimuli are detailed below.

Experiment-specific stimulus methods

The experiments in this chapter were designed to test the prediction that cell geometry will equalise. Briefly, bees should attempt to build a cell shaped as a cylindrical tube, and where this construction influences (and is influenced by) the same attempt being made for an adjacent cell, walls meeting at a junction will be arranged to equally sub-divide the available angular range. These experiments, i to iii, each tested the prediction for optimisation under different conditions. These conditions were as follows:

- i – Linear barrier progression, angular equalisation between two cells and subsequent inclusion of a third cell
- ii – Flat-bottomed cells, single base element between directly opposing cells
- iii – Tongue uniting, alterations to cell structure effected after comb tongues merged.

If the prediction is correct then the distribution of available space, angular or area, will be redistributed as conditions change, for all three conditions.

Experiment i - Linear barrier progression

This experiment was to test the prediction that the internal angle between two walls of a cell will be arranged in such a way that the available angular range would be distributed equally between all the cells that meet at a junction. This experiment examined the construction of two cells against a barrier, with the wall built between these cells meeting with the barrier (Figure 0-1 C). This would give an available range of 180°, which would be shared equally between paired cells. If this prediction is correct, the angle to the barrier for paired cell walls would be statistically equivalent to 90°.

Further construction would form cells to the other side of the seed strip and the strip would be eroded. Thus, the wall intersection would come to involve three cells, and encompass the full 360° or 120° per wall-to-wall junction (Figure 0-1 D-E). If this prediction is correct, the cell corner angles at triple cell junctions will differ from those at paired cells more than would be expected by chance. Furthermore, the angle at triple-cell junctions will be statistically equivalent to 120°.

The test pieces were fabricated using a backplane, approximately 25 mm by 40 mm and 0.5 mm thick, onto the face of which seed strips were added. The seed strips were 2-3 mm in height and 1-1.2 mm in width, and were prepared by cutting strips from a flat sheet of wax formed by dipping a flat mould in molten wax; dipped six times in this case. The seed strips were welded orthogonally onto the wax backplane and small, 4 mm-diameter depressions were pressed into the backplane close to the strips, to further encourage the construction of cells in this area. Presentation of the substrate causes the initial construction of cells to be restricted to two dimensions and the available angular range becomes some portion of 360°. The addition of the barrier strip applies an additional constraint, initially restricting the range to 180°.

The expected first step of construction was that a single cell would be built against the barrier, with the barrier itself forming one of the cell walls. An adjacent wall would then be built branching away from the seed strip, initially without a cell to the other side. Based on my thesis, the isolated wall should form part of a circle that intersects the seed wall. The angle at which the circle arc met the seed strip would therefore be greater than 90°, but no larger than 120°. As construction continued, an adjacent cell would be built, meaning angular equalisation would occur between two cells while the seed strip would constrain the available range to 180°. This constraint should lead to the angle between the seed strip and the wall being 90°. Eventually, construction would form cells to the other side of the seed strip and the strip would be eroded. Thus, the wall intersection would involve 3 cells, and encompass the full 360° range (or 120° per wall-to-wall junction).

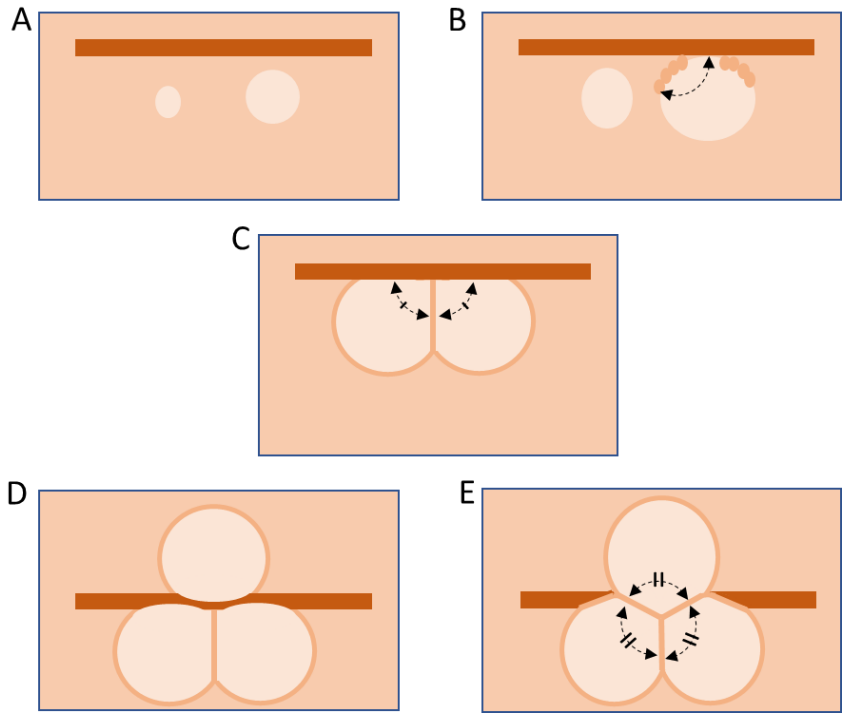

Figure 0-1 Progression of cells built against a barrier. A: initial construction focussed on pits located close to the barrier that will grow until a cell is built, exploiting the barrier as one of its walls. B: the next side wall will branch from the barrier at an angle between 90° and 120° .C: as construction continues, a second cell built adjacent to the first will influence the common wall, dividing the available 180°angle into two internal angles. D: after further construction on the opposite side of the barrier, the barrier will be eroded. E: the 3-way influence will divide the range, a full circle, into three eq internal angles.

Experiment ii - Flat-bottomed cells

Experiment ii was designed to test the prediction that the internal angle between two walls of a cell would be arranged to distribute the available angular range equally between the cells meeting at the junction. In this case, the junction will involve two cells that share a base, with one cell on each face of the comb. In this arrangement, the angle between the side walls of the cells that extend in opposite directions from the backplane is 180°, and the base, which serves as a division between the two cells, would therefore lay at 90° to the side wall. If this prediction is correct, the angle between the cell base and side walls will diverge from 90° by less than expected by chance.

The stimulus used was a flat piece of wax approximately 25mm by 40mm and 1mm thick. Shallow depressions were pressed into each face, arranged as an approximation of the layout and spacing of worker cells. The depressions were positioned so that those on each face were opposite each other to encourage the construction of cells positioned thus.

A thick wax backplane was used here to delay or avoid a dome being raised on one side of the backplane as a consequence of cell construction on the other. Such domes would likely displace cells formed on the, second, face which would reduce the occurrence of cells in direct opposition.

Once prepared, the sample stimuli were placed in a hive for 6-10 hours until cells had been drawn to a depth of greater than 5mm and the cell bases had been thinned.

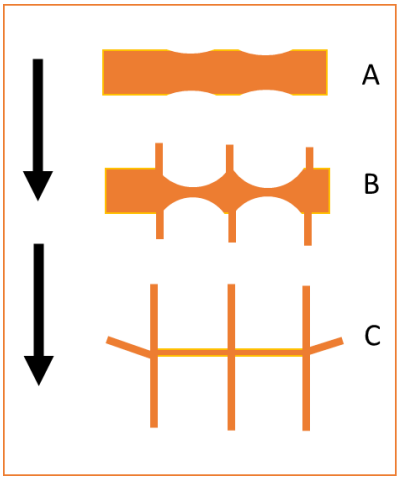

Figure 0-2 Flat-bottomed cell production and selection. A: a diagram showing the initial indentations made in the backplane to guide the alignment of cells. B: the construction was expected to proceed to initial cell construction via excavation of the guide pits and deposition of wax at their edges. C: further cell construction would then thin the substrate and extend the cell walls. Cell location would be guided by the stimuli, ideally leading to cells in opposition. However, inaccuracies in preparation and variations in comb construction would likely result in differing degrees of overlap.

Experiment iii - Tongue uniting

Experiment iii investigated whether the proposed outcome of the prediction that the internal angle between two walls of a cell will be arranged to equally distribute the available angular range between the cells that meet at the junction, would arise from abnormal initial conditions. Discrete tongues of comb, built independently, would likely be separated by a non-integer number of cell widths and would lie at distinct orientations. As two such tongues were extended and so came into contact, cells at the interface would be misaligned. Thus, the interface would comprise cells with a higher-than-average degree of irregularity. If the prediction is correct, then following further construction, the irregularities in cell structure will reduce from their initial levels by more than would be expected by chance.

Construction of comb in several distinct places was stimulated by preparing frames that carried four strips of wax approximately 25mm by 5mm and 0.5mm thick bonded, using molten beeswax, to the top bar of a hive frame.

Experiment-specific measurements

Experiment i - Linear barrier progression

Cell construction against a straight wall stimulus was measured in three situations. The first was where adjacent cells (each of a depth <5 mm) had been built against one side of the stimulus. The second was where walls were found between adjacent cells built against a barrier formed by the

wooden frame. The third situation was where cell construction had progressed sufficiently for a wax barrier to have been eroded and the cells to be built to a depth of several millimetres; a condition that was typically associated with an extended comb covering the test piece.

A cell's side walls were measured from the photographic records taken of each face of the subject comb, using the coordinates of points marked on the photographs. The author's frame comparison tool, FormImageCompare, includes a feature to measure 'bend'. This feature allows the user to mark two points at the ends of two walls, or parts of a barrier, that meet at a cell corner, followed by the wall junction 'centre'. The software also captures user-placed marks indicating two ends of the third element of the junction, the inter-cell wall. The custom software used the locations of the marks to calculate the orientation of each half of the barrier, as well as that of the wall. From these, the angles between each could be calculated – w, the angle between the wall and the barrier element (clockwise from the wall to the first element of the barrier) and 'b' the obtuse angle between two halves of the barrier (Figure 0-3).

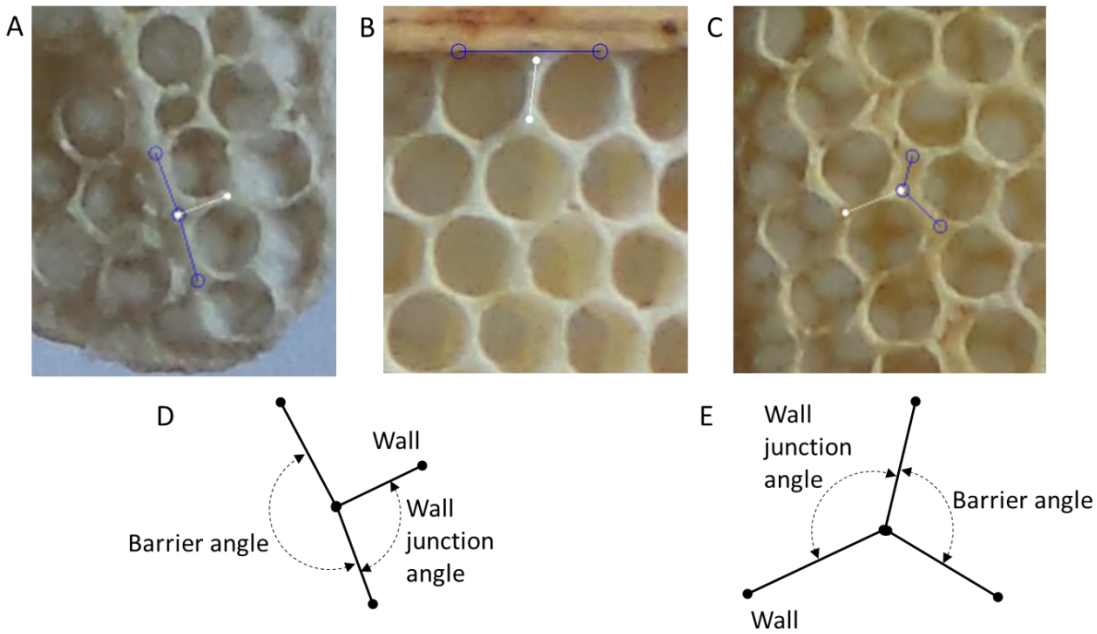

Figure 0-3 Example measurements taken of cells adjacent to linear barriers. A: an image showing a wall between a pair of cells against a wax barrier stimulus. B: the wall between a pair of cells built against a fixed barrier, the wooden frame. C: a later image of the cells against the wax barrier when further construction work has moved the alignment of the cell walls. D: showing the attributes of the feature that were measured, the barrier angle and the wall to barrier angle. E: measurements taken of the situation where the barrier had become eroded, resulting in a more pronounced angle at the barrier.

Experiment ii - Flat-bottomed cells

The cell bases selected for measurement were those within cells that were aligned base-to-base and showed a high degree of overlap, sufficient that

when viewing the cell base via transmitted illumination no gap was perceptible between the side walls and those of the opposing cells (Figure 1-4).

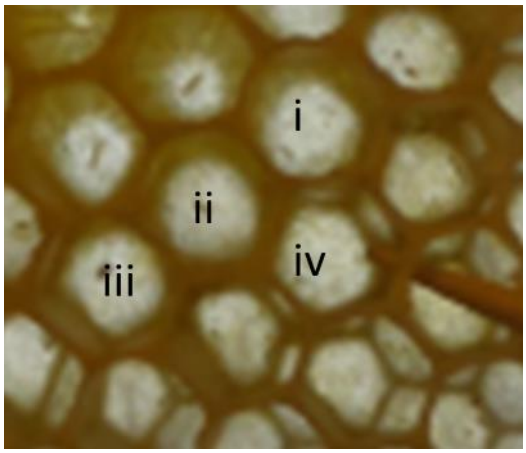

Figure 0-4 Flat-bottomed cell selection. The image shows, in transmitted illumination, an example collection of cells. Of these, the cells marked I, ii and iii were selected for measurements, but iv and all others were rejected as they showed insufficient overlap.

Measurements of the bases were made using a multi-point depth gauge, which was placed into the cell to be measured, withdrawn and then photographed alongside a 10 mm gauge. Measurements obtained from the photograph yielded the depth at seven points (d_0-d_6) across the cell six of which (d_1-d_6) were adjusted to provide heights of the cell base profile relative to pin 0, hence $d_0 = 0$.

The control samples used for comparison were baes of cells within section of natural comb built alongside the experimental sections of comb.

Experiment iii - Tongue uniting

For this experiment, several measurements related to cell geometry were extracted from the photographic records with the assistance of software image analysis.

The photographs used for the initial state were those that recorded comb construction when the individual tongues neared each other (with less than a single cell diameter separation) or where the tongues had made contact but had been separated in a previous photograph recording its state (taken 4 hours prior or less). Second-stage photographs were also required, which recorded the areas of tongue contact at least 6 hours later. This revealed any post-merger condition of the cells.

Each image was initially converted from colour to monochrome, after which it was enhanced by a combination of darkening (subtraction of a fixed value from every pixel) and increasing contrast (multiplication of each pixel value). The third image processing step was to apply a threshold that caused each output pixel to become either white or black, depending on the corresponding pixel brightness relative to a local reference value. The localised reference was computed using a Gaussian filter over an area of 63x63 pixels, which equates to approximate the size of a cell within the image. The final step of automated processing was to compute contours,

i.e., the external boundary of a black zone. Several thousand contours would typically be computed for each image, of which several hundred were selected as being representative of cells based on the area within the contour and their proximity to each other.

Each selected contour was processed to compute an external hull, i.e., a shape comprising only convex external vertices. The convex hull was then processed to find its centroid, which was computed as the first-order moment divided by the zeroth for both x and y coordinates.

Image processing was performed using my custom software to compute the coordinates of each cell centre. With this software, it was possible, in real-time, to control the levels used for brightness, contrast, threshold and contour magnitude while the software displayed the resulting array of computed cell centres overlaid onto the original image (Figure 0-5 C). Therefore, the operator could make any adjustments necessary to yield the most complete cell recognition possible. The interactive nature of this software allowed optimisation of the filter values without resorting to complex and error-prone automation, nor suffering the potential loss of information that might otherwise result from poorly chosen fixed values.

Processing each image yielded coordinates for more than 95% of the cells; a useful reduction of effort compared with manual processing. Nonetheless, some correction of omissions and errors was necessary, requiring an additional software method to be written which allowed the operator to mark erroneous cell centres or connections for removal, and to add missing centres and connections.

Using the coordinates of each cell centre, the software computed further aspects of each cell's geometry using the centres of adjacent cells. Following a line connecting the centre of one cell to each adjacent cell, the software inspected the pixels between the two cell centres and the maximum illumination was interpreted as the edge of the cell. The intersection between the cell connection line and the periphery of the computed contour for that cell provided an additional estimate of the location of the cell edge, with the adopted value being the mean of the two.

The number of adjacent cells was assumed to equal the number of walls for each cell, and each wall was assumed to pass through the cell edge, as computed above. Cell walls were also assumed to be orthogonal to the cell centre-to-centre connection, hence the line of each cell wall could be calculated. In turn, the location of cell corners could be derived as the intersection between two cell walls. The resulting cell corner locations were then used to define the geometry of the cell. From this, it was possible to derive the area of each cell, the length of each of its walls, and the orientation of its walls.

These calculations were performed in terms of image pixels, but a standard scale would be required for the analysis of cell geometry and to compare the values obtained from one image with those of another. An image scale, the conversion factor from pixels to millimetres, was calculated from the locations of two marks, placed by the operator, at the top and bottom corners of the frame sidebar. These two locations had been measured to be 114 mm apart.

This experiment was designed to measure honeycomb cells in the regions around the junction between two tongues of comb, meaning that these

regions needed to be specified. While inspecting an image, typically the earlier of a pair that showed the tongues either in contact or close to it, the operator was able to mark each end of a tongue merger junction. The software used these two locations to define the centre line of a zone of interest and, using these, computed a rectangle measuring 30 mm on either side of the centre line. Cells with centres contained within that rectangle were those sampled.

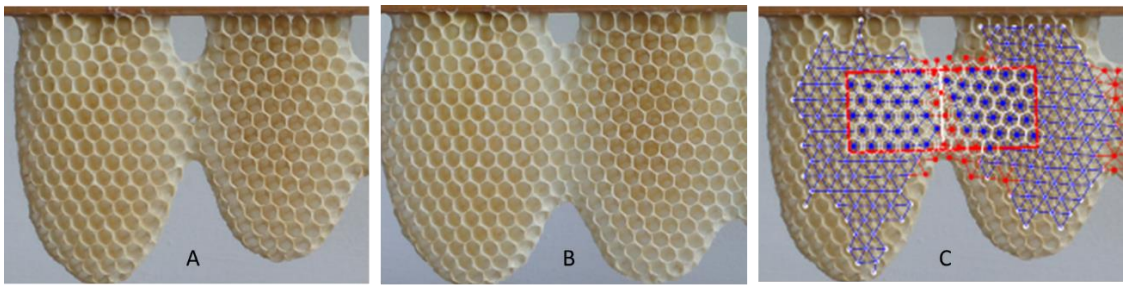

Figure 0-5 Merged tongues of the comb. A: tongues recently merged. B: the same comb with an additional 10 hours of construction. C: the software identified cell structure including the area marked for analysis.

The software provides the ability to reshape one of a pair of images to align the features of both. The same transformation operation can translate coordinates from both images into a common frame of reference. Each cell in one image was therefore compared with the cell locations found in the other, making it possible to pair the cells between both images, and so allowing cell-by-cell comparison of geometry.

The raw data, comprising coordinates in both pixels and millimetres, was exported using an image processing tool and saved as comma-separated values (CSV) in text files comprising one line for each cell in each photograph. The tool also exported the distance from the centre of each cell to the tongue junction line on which the cell selection was based. Cell centre to junction line values were negative for those lying to the left of the line, and positive for cells to the right.

Analysis

Data obtained from the images, using FormImageCompare, was subsequently processed using custom scripts written in R, executed on R runtime version 3.6.3 within RStudio version 1.3.1093.

Experiment i - Linear barrier progression

The ‘bend’ feature of the image measurement software FormImageCompare provided orientations from the two halves of the barrier and the junction wall and the angles between them ‘w’ and ‘b’.

The value of the wall to barrier angle (w) was assessed by comparison with the predicted values of 90° or 120° for 2 or 3-way cell wall junctions, respectively (Figure 0-6). For measurements of a wall against a barrier (either wax or wooden), the wall to barrier angle *w* was compared with a predicted value of 90°. For three-way junctions, all three angles were calculated, using w and the barrier angle (b) and the anti-clockwise wall to

barrier angle (w'). These angles were compared with a predicted value of 120°.

Comparison between the measured angles and the predicted value (either 90° or 120°) used the R function TOSTone() to perform a Two One-Sided Test (TOST) with the Smallest Effect Size of Interest (SESOI) of 0.5 standardised mean difference, Cohen's D.

Experiment ii - Flat-bottomed cells

Measurements taken of the bases using the multi-point depth gauge yielded the depth at seven points (d_0-d_6) across the cell relative to pin 0, hence $d_0 = 0$. Linear regression was used to calculate a straight line to fit the points of the profile. The measurement of flatness was the maximum displacement from the regression line of any probe depth.

The base angle, between the line of the base and the vertical side of the cell, was calculated for the sample set using the gradient of the regression line. The equivalent metric for the control set was calculated by taking the gradient of lines through the two halve of the base profile. The points for these two lines were from d_0 and from d_6 to the deepest location, typically d_3 or d_4.

Comparison between the measured base inclination and the predicted value of 90° used the R function TOSTone() to perform a Two One-Sided Test (TOST) with the Smallest Effect Size of Interest (SESOI) of 0.5 standardised mean difference, Cohen's D.

Experiment iii - Tongue uniting

The data for the cells within the zones of interest was used to calculate the four measurements described below. For each cell, the measurement value was plotted against the distance of that cell from the merger junction, providing a visual indication of any relationship between the regularity of cell geometry and separation from the junction. These graphs were prepared separately at the point of merger, stage 1, and following further construction, stage 2, to indicate any change in the pattern. Whether a change had occurred between the two stages was assessed by sampling those cells within a band of 10 mm to either side of the tongue junction and testing stage 1 samples compared with stage 2 samples. The metrics were not normally distributed and therefore a Wilcoxon test was performed using the R function wilcox.test(). If prediction P5.1 is correct, then following further construction from stage 1 to stage 2, the irregularities in cell structure will have reduced from their initial levels more than expected by chance.

Cell separation. The distance to each neighbouring cell was calculated using the coordinates for each cell. For each cell, the standard deviation of these distances was computed as a measure of regularity (or otherwise) of the cell. The measurements included only inter-cell distance to a cell further from the junction.

Corner angle deviation. Using the computed coordinates for each corner of a cell, the angle at each corner was calculated. The number of sides for the

cell allowed the ideal angle at each corner to be determined, assuming each to be equal for an ideal, regular, cell. The calculated measure of irregularity for each cell was the standard deviation of the difference between the ideal angle separation and that measured for each corner.

Wall length. The coordinates for each corner of a cell were used to calculate the length of the cell's walls. The calculated measure of irregularity for each cell is the standard deviation of its wall lengths. The samples include only those walls for which the centre lay further from the junction than the cell centre.

Cell area. The coordinates for each corner of a cell were used to calculate cell area.

Cell by cell comparison

For those cells that could be identified from both photographs, it was possible to directly compare each irregularity metric at the two stages and obtain the amount of change cell by cell, revealing detailed cell-specific changes from Stage 1 to Stage 2

The image processing described above calculated the coordinates for each cell and its structural elements. Initially, these calculations used pixel coordinates but were subsequently converted to vertical and horizontal offsets in millimetres. Additionally, the coordinates for the stage 2 photographs were adjusted, by matching the four reference locations in each image to align with those of the stage 1 photographs. This alignment allowed the software to match cells from each set: those that appeared in the stage 1 photographs with those from stage 2. Coordinate matching used a tolerance of 2.5 mm (half a cell width.

Results

Experiment i – Available angular range was divided equally between the cells at a junction

Measurements were made for 73 cell walls shared between two adjacent cells and connected to a stimulus barrier (Figure 0-6). The corner angles were measured as 90.9°± 7.0° (means ± standard deviation, throughout). Measurements were also taken for 72 cells from the top row of comb built against the frame (Figure 0-6), with the corner angles measured at 90.0± 5.53. For the 75 cells where the barrier had been eroded (Figure 0-6), the corner angles were measured as 119.4° ± 6.46.

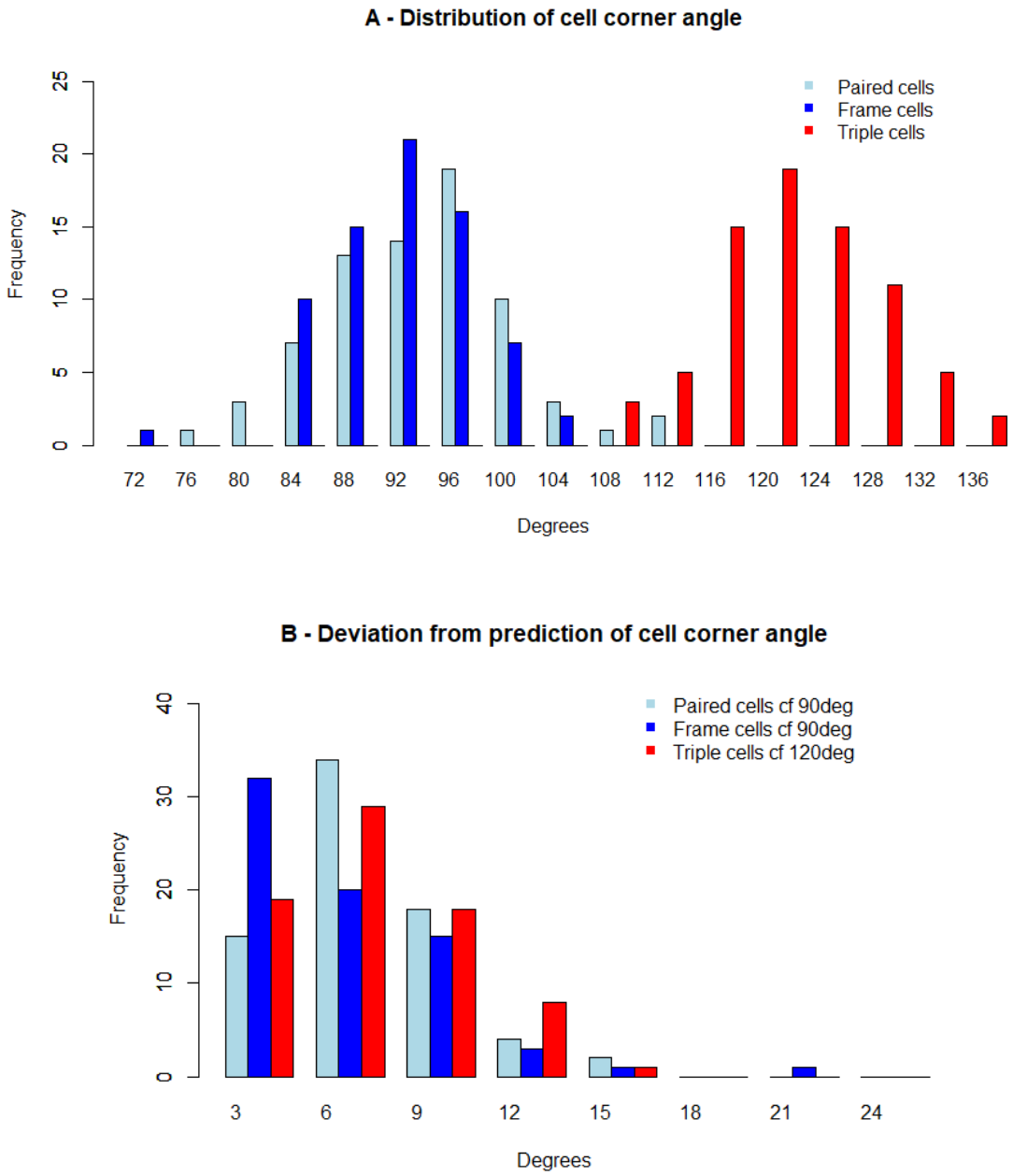

Figure 0-6 Cell corner angle measurements. A: distribution of the angle at cell corners in three configurations. Paired cells are those at a junction with the barrier where the wall was between two cells (N= 73 and mean = 90.95°, predicted to be 90°). Those labelled 'Frame cells' are wall junctions abutting the wooden frame, (N= 72 and mean = 89.96°, predicted to be 90°). "Triple cell" measurements were taken from the 3-way wall junctions where the barrier had been eroded (N= 75 and mean = 119.40°, predicted to be 120°). B: the deviation of those cell corner angles from the predicted angle, for the three conditions, means of 5.24°, 4.21° and 5.29°.

Samples for each junction type, paired, triple and frame, were compared for equivalence to the predicted values, 90°, 120° and 90° respectively, using a Two One-Sided Test (TOST) with Smallest Effect Size of Interest (SESOI) of 0.5 standardized mean difference, Cohen's d (Figure 0-7) revealed that for paired cells the corner angles were equivalent to 90° (P=0.0013/0.249;

probability of different from reference/probability of equivalent to reference), paired cell corners against the frame were equivalent to 90° (P=0.00004/0.955), triple cell corners were equivalent to 120° (P=0.00036/0.42), and paired cells corners were equivalent to frame cell corners (P=0.020/0.346).

These results demonstrate that the angles between cell walls are balanced to equalise the use by each cell of the available angular range, as predicted.

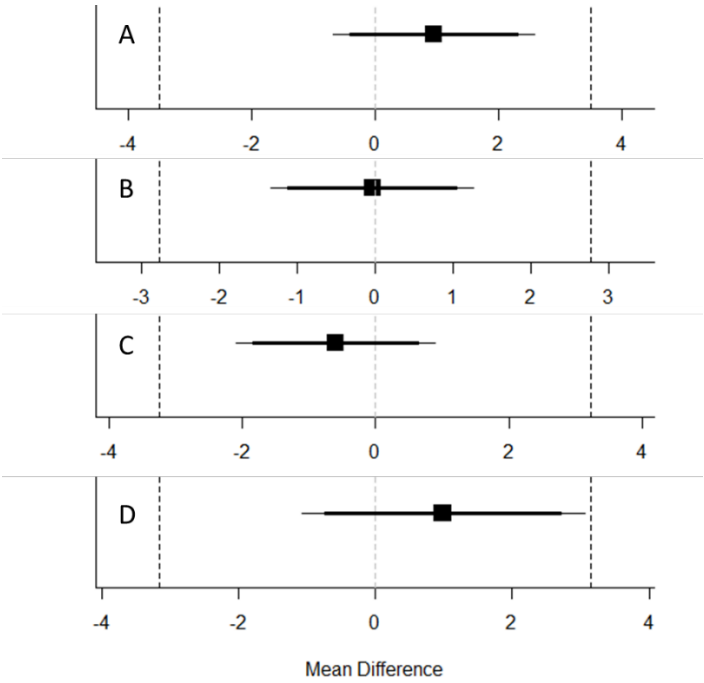

Figure 0-7 Equivalence testing using TOST. each with the SESOI of 0.5 Cohen's D. A: the result of comparing two-cell wall corner angles with 90°. B: two-cell corners adjacent to the frame compared with 90°. C: three-cell corners compared with 120°. D: comparison of two-cell wall corner angles with two-cell corners adjacent to the frame. All four tests show equivalence as the observed effect is statistically not different from zero and statistically equivalent to zero.

Experiment ii – Opposing cells formed a flat orthogonal common base

Measurements of the opposing cells and the samples from natural comb sampled the profile of the base at seven points. The comparison of base shapes within one population compared with the other can be seen in Figure 0-8. These show the relative height for each of the seven probe points where the values were levelled hence the heights at points 0 and 6 are both zero and intermediate points adjusted accordingly. The heights are presented as boxplots showing median, interquartile range and outliers.

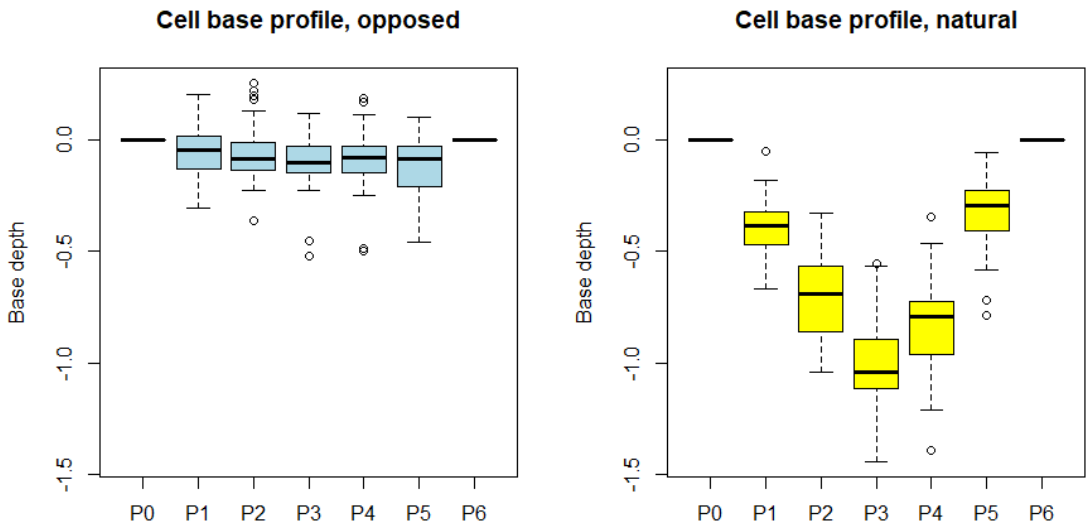

Figure 0-8 Base profiles comparing the bases of opposing cells with those formed naturally. The seven values labelled as probe points (p0-p6) map onto the height of the corresponding element of the probe array.

Measurements were made of the bases of 42 cells, each in opposition with a cell on the other face of the comb. The maximum distance between the base and the line of regression through the 7 points on the profile was 0.13 ± 0.06 mm, which was significantly less than that measured for cells within natural comb (0.58 ± 0.11 mm; $t_{2=65}$ = 22.59, P <0.00001; Figure 0-9).

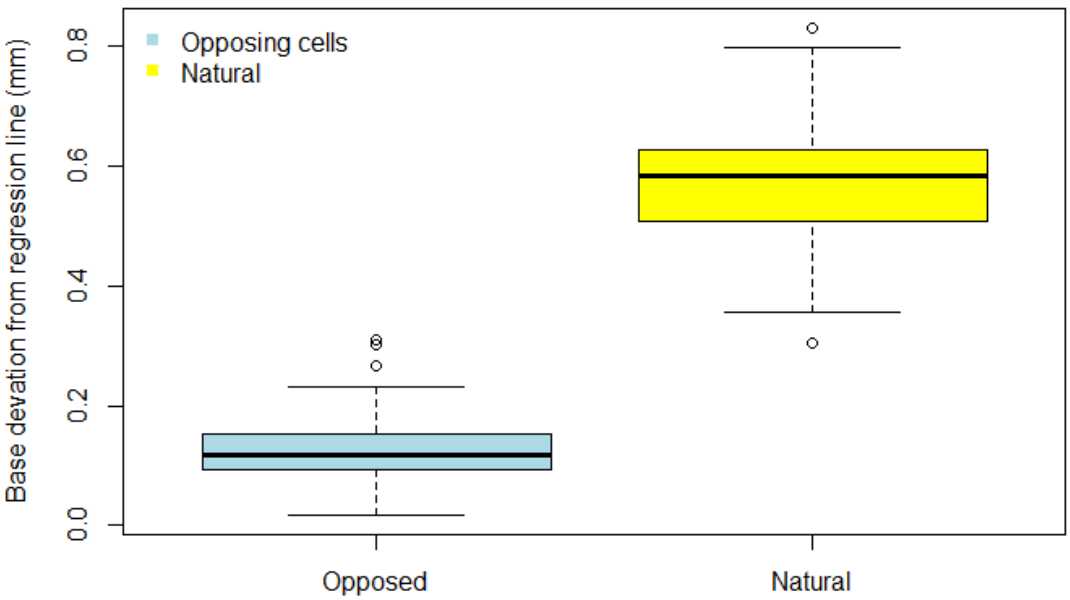

Figure 0-9 Cell base deviation from flat for opposed cells and for cells on natural comb. Flatness as measured by the maximum deviation from a regression line

through the seven depths acquired with the multi-point probe. The results shown are for opposing cell bases (N=42, mean=0.13) and for the bases of natural cells (N=43, mean=0.58).

The slope of the base relative to the side walls of its cell for those in opposition was measured as 90.6°± 5.4°, which was significantly less than that for each side of a base from a natural cell (116.2°± 5.4°; $t_{2=65}$ = 22.59, P <0.00001; Figure 0-10).

Equivalence of the angle of bases to the side wall of cells in opposition compared to the predictions was assessed using a TOST with SOSEI of 0.5 standardised mean difference, Cohen's d. The base angles measured from opposed cells were equivalent to 90° (P=0.0083/0.745; probability of different from reference/probability of equivalent to reference).

These results confirm that the angles between cell walls are balanced to equalise the use by each cell of the available angular range, as predicted.

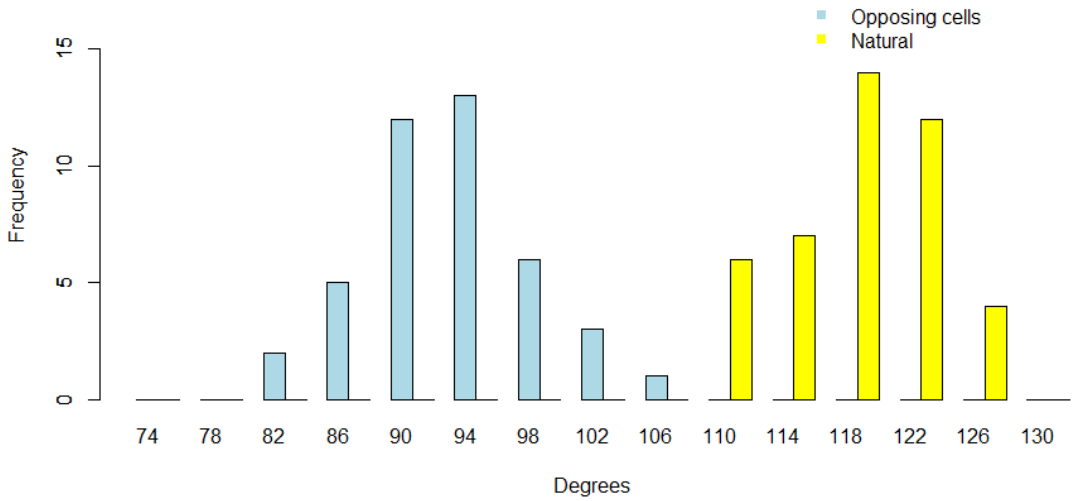

Figure 0-10 The distribution of angles between the base of a cell and its side walls as measured for cells in opposition (n=42, 90.6°± 5.4°) are distributed distinctly when compared with those for natural cells (n=43, 116.2°± 5.4°).

Experiment iii – Irregularities at a comb merger reduced after further construction

The prediction states that irregular cell structure will be modified to smooth irregularities by additional builder activity, which will ultimately lead to increased uniformity. These experiments measured the geometry of cells at and around the line where two tongues came into contact and compared early irregularities with those found later. The geometric characteristics used for these comparisons were separation, corner angle, wall length and cell area.

The measurements gathered for each cell relate to either surrounding cells or cell walls. Such measures are by definition shared with adjacent cells: for example, the value for the length of a wall would be common to the

collection of values for both cells separated by a single wall. To avoid duplication of results, all such values were retained only where the cell aspect in question lay in the direction away from the junction.

Comparison of the centre of each cell with the tongue junction line allowed the software to calculate, for each cell, a distance from the junction. All measurements of irregularity were at a maximum for small distances, i.e., cells close to the junction. It was the cells within this region (up to one cell's width from the junction) where the features of cell geometry were compared.

Each cell measurement was calculated from data recording the cells found at an earlier stage (stage 1) and from a separate image recording at a later stage (stage 2). The sections below show, for each of those aspects of geometry, a chart plotting the property on the Y-axis against the distance of the cell centre from the junction on the X-axis. Two such charts are presented for each measurement: first, the values at stage 1, and then at stage 2. The third chart shows the distribution of the values for the cells that fall within two cells' width of the junction (10 mm) as a pair of boxplots; one for stage 1 and the other for stage 2. In total, measurements were taken from 458 cells at stage 1 and subsequently from 429 cells at stage 2.

Cell separation

The standard deviation of distance from a cell to its neighbours at stage 1 was 0.52 ± 0.26 mm, which was significantly greater than that at stage 2 (0.37 ± 0.21 mm; P <0.00001; Figure 0-11). This demonstrated that cell wall position altered over time to balance the space allocation between adjacent cells, as predicted.

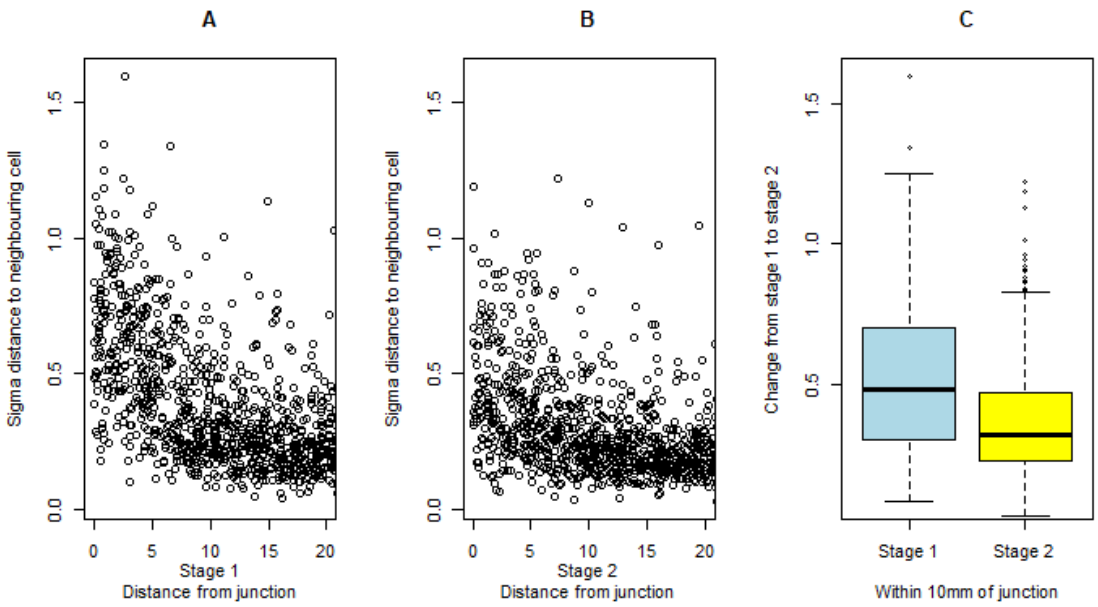

Figure 0-11 Distribution of inter-cell distance relative to the distance from the tongue junction. A: standard deviation of the distance between a cell and its neighbours at stage 1, for each cell, plotted against the distance between the cell and the junction. B: the same metrics but measured at stage 2. C: sampling only those cells within 10mm of the junction, a boxplot of measurements taken at stage 1 and those taken at stage 2 showing the comb had become significantly less irregular.

Corner angle deviation

The standard deviation of corner angles around a cell at stage 1 was 0.15 ± 0.09 rad, which was significantly greater than that at stage 2 (0.10 ± 0.06 rad; P <0.00001; Figure 0-12). This suggested that cell walls were repositioned to even the perimeter, as predicted.

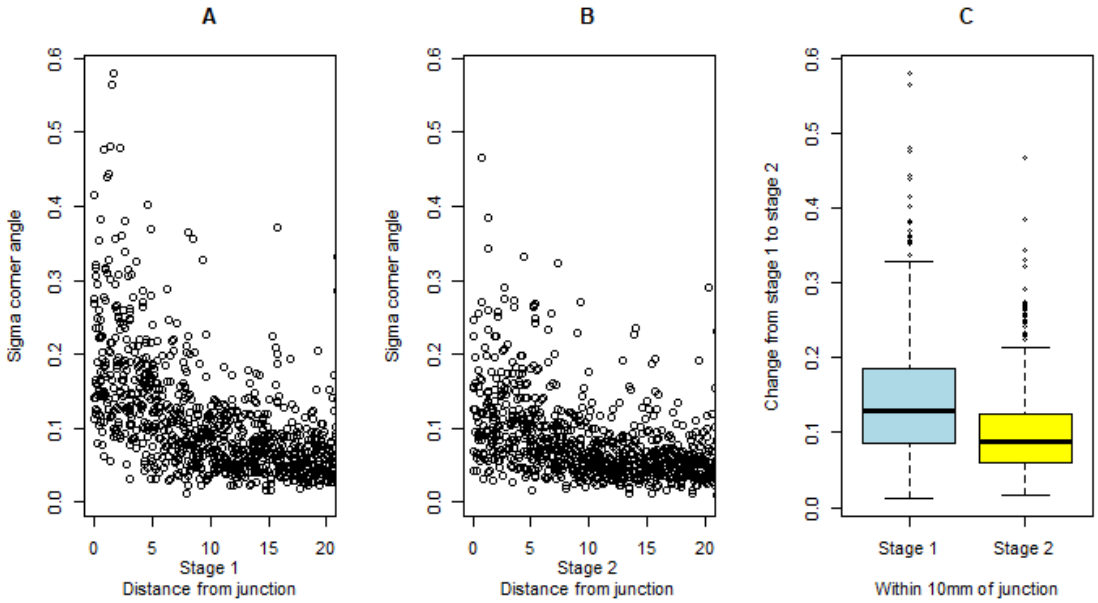

Figure 0-12 Distribution of inter-cell angle error relative to the distance from the tongue junction. A: standard deviation of the difference between the angles at the corners of a cell from that expected at the corner of a regular polygon at stage 1, for each cell, plotted against the distance between the cell and the junction. B: the same metrics but measured at stage 2. C: sampling only those cells within 10mm of the junction, a boxplot of measurements taken at stage 1 and those taken at stage 2 showing the comb had become significantly less irregular.

Wall length

The standard deviation of the length of walls around a cell at stage 1 was 0.72 ± 0.35 mm, which was significantly greater than that at stage 2 (0.55 ± 0.26 mm; P <0.00001; Figure 0-13). This demonstrated that cell walls were repositioned to even the perimeter, as predicted.

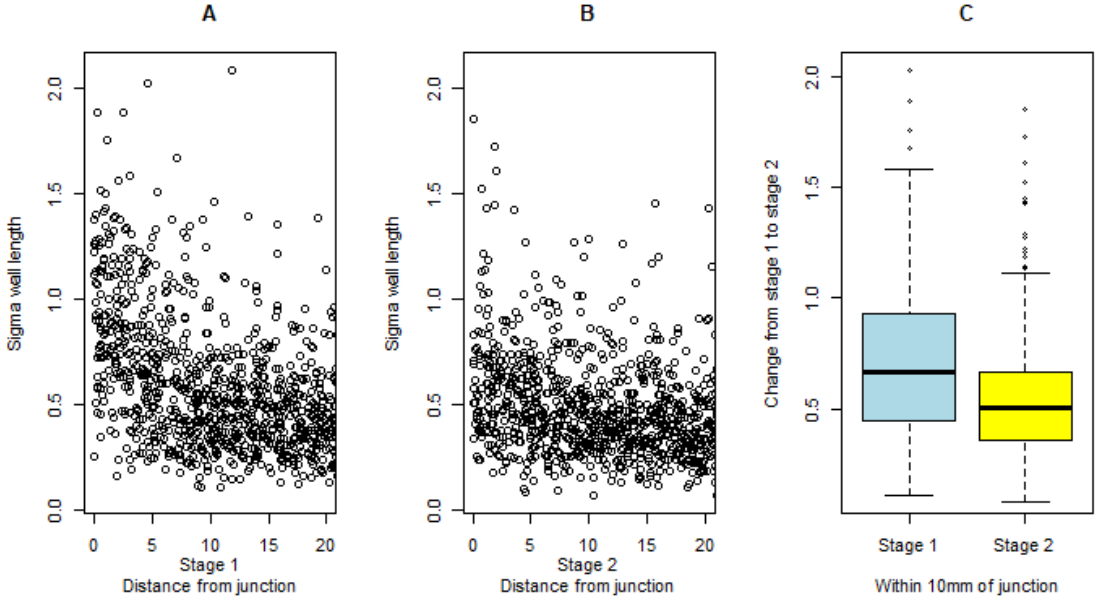

Figure 0-13 Distribution of cell wall lengths related to the distance from the tongue junction. A: standard deviation of the lengths of the walls around a cell at stage 1, for each cell, plotted against the distance between the cell and the junction. B: the same metrics but measured at stage 2. C: sampling only those cells within 10mm of the junction, a boxplot of measurements taken at stage 1 and those taken at stage 2 showing the comb had become significantly less irregular

Cell area

The standard deviation of the length of walls around a cell at stage 1 was 20.8 ± 5.0 mm^2, which was significantly less than that at stage 2 (22.9 ± 4.1mm^2; P <0.00001; Figure 0-13). This demonstrates that cell walls were repositioned to even the perimeter, as predicted.

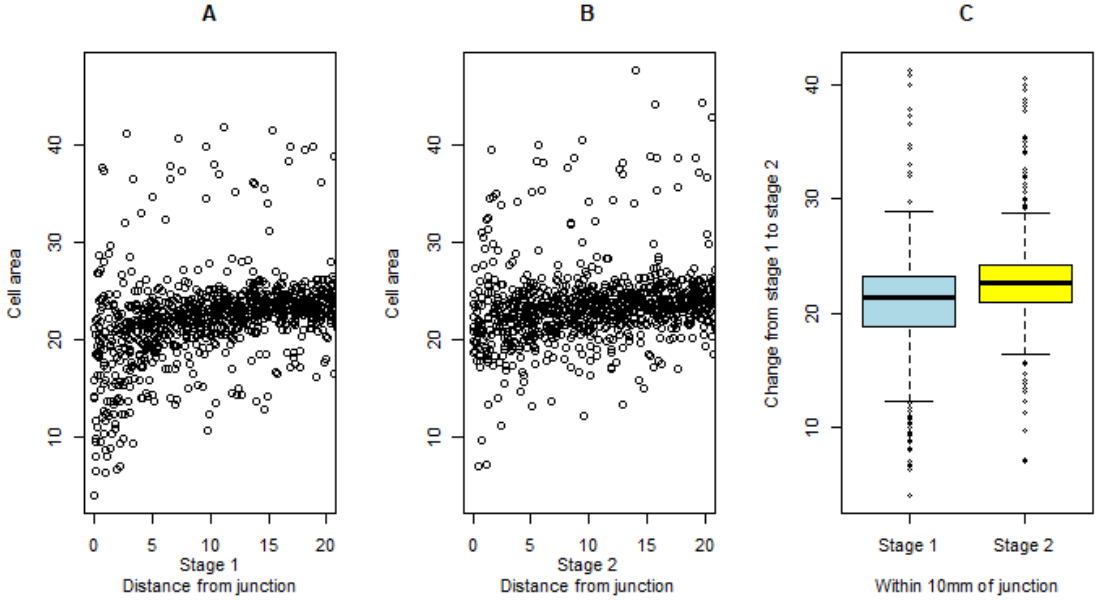

Figure 0-14 Distribution of cell area as a function of distance from the tongue junction. A: the area of a cell at stage 1 plotted against the distance between the cell and the junction. B: the same metrics but measured at stage 2. C: sampling only those cells within 10mm of the junction, a boxplot of measurements taken at stage 1 and those taken at stage 2 showing the cell area had changed significantly and had become more regular.

Cell-by-cell comparison

The results presented in the section above treated the measurement cells at stage 1 and stage 2 as two samples of cells to show how the two samples differed, reflecting any difference in the overall populations. Differences between the sample means could be the result of many small changes or be dominated by a small number of large differences. This section presents how the measurements differed from stage 1 to stage 2 for each cell.

To pair cells at stage 1 with those at stage 2, the coordinates for cells within one set were matched to those on the other. A proximity tolerance of 2.5mm, approximately half a cell diameter, was allowed. This resulted in the pairing of 409 of 419 (97.6%) of the cells from stage 1 with 409 of 426 (96.0%) of those from stage 2.

Corner angle deviation

Measurements were taken of the 409 cells positioned within 10 mm of the tongue junctions that were identifiable at both stage 1 and stage 2. Of those cells, variation in corner angles was reduced between stage 1 and stage 2 in 282 cells (69%). A significant change towards the sample mean at stage 2 was found ($t_{408} = 10.53$, $P < 0.00001$), with 288 cells (70%) found to be closer (Figure 0-15).

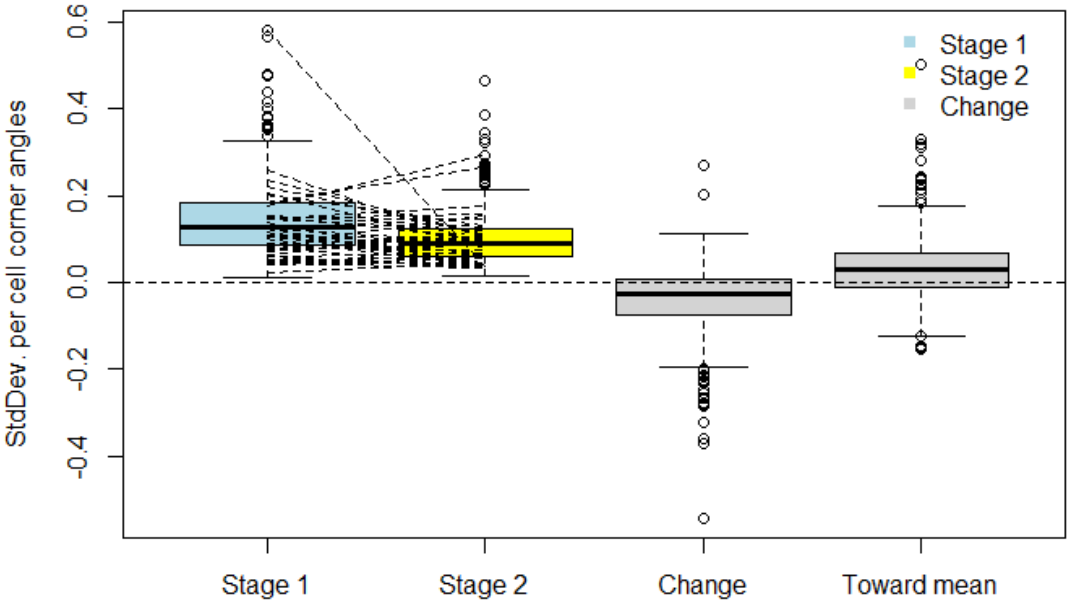

Figure 0-15 Distribution of cell corner angle irregularity at stages 1 and 2. The solid line on each plot shows the median value, while the box shows the inter-quartile range for the samples. Additional lines from stages 1 to 2 show the individual cell changes. For clarity, only 10% of the changes are shown. The grey boxes show the distribution of change for each cell (282 out of 409 showed reduced irregularity) and the distribution of change towards the sample mean (288 out of 409 changed toward the mean).

The change in the irregularity of corner angles between stage 1 and stage 2 can be seen in Figure 0-16. The points are plotted along the X-axis using the difference between the metric value at stage 1 and the mean at stage 2 and along the Y-axis using the change in the metric. Cells further from the eventual mean changed most towards that mean.

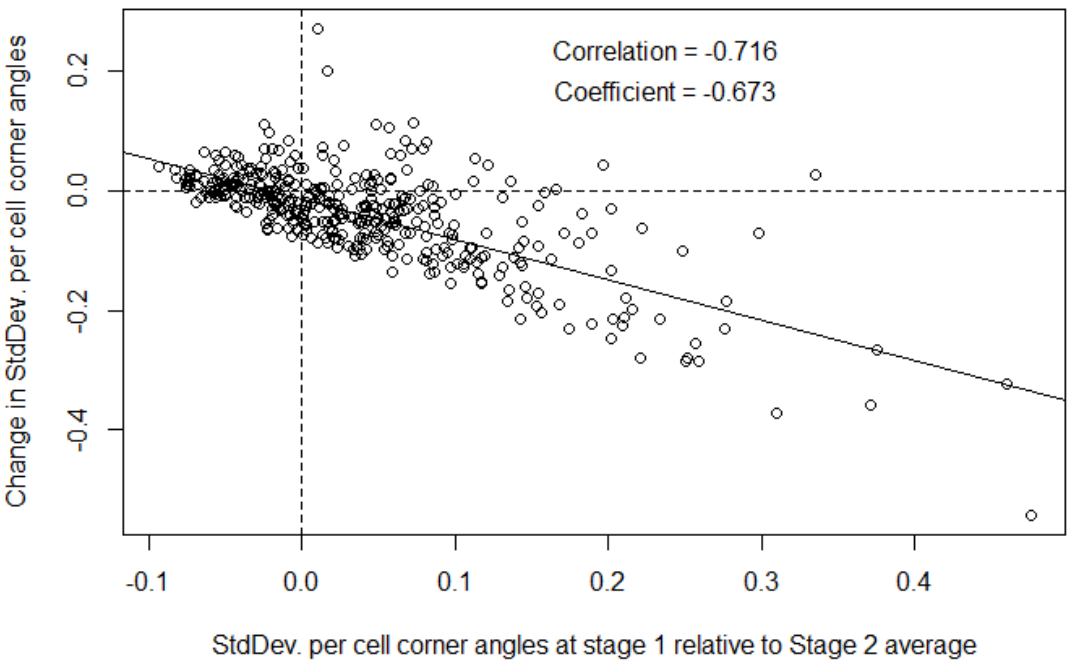

Figure 0-16 At stage 1, for each cell, the cell corner angle irregularity is plotted against the change in that measure between stage 1 and stage 2. The points are overlayed by a regression line relating the values and indicating that those cells with greater irregularity underwent the larger improvement.

Cell area

Measurements were taken of the 409 cells that lay within 10 mm of the tongue junctions that could be identified in both stage 1 and stage 2 images. Of those 409 cells, cell area increased between stage 1 and stage 2 in 275 (67%). A significant change towards the sample mean at stage 2 was identified (t_{408} = 10.53, P < 0.00001), with 289 cells (71%) found to be closer.

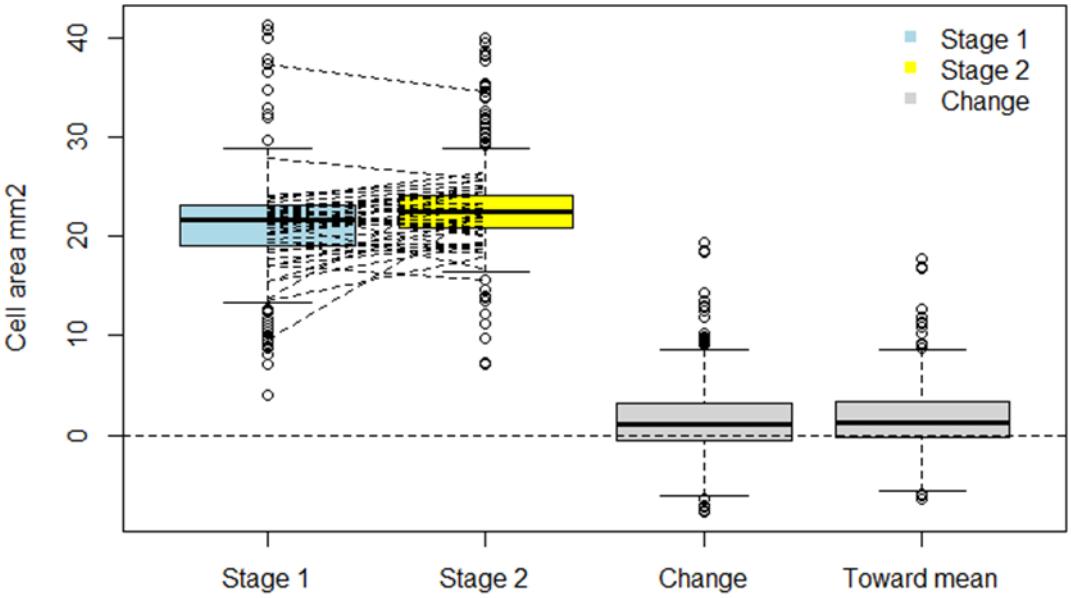

Figure 0-17 Distribution of cell area at stages 1 and 2. The solid line on each plot shows the median value, while the box shows the inter-quartile range for the samples. Additional lines from stages 1 to 2 show the individual cell changes. For clarity, only 10% of the changes are shown. The grey boxes show the distribution of change for each cell (275 out of 409 showed reduced irregularity) and the distribution of change towards the sample mean (289 out of 409 changed toward the mean).

The change in cell area between stage 1 and stage 2 can be seen in Figure 0-18. The points are plotted along the X-axis using the difference between the metric value at stage 1 and the mean at stage 2 and along the Y-axis using the change in the metric. Cells further from the eventual mean changed most towards that mean.

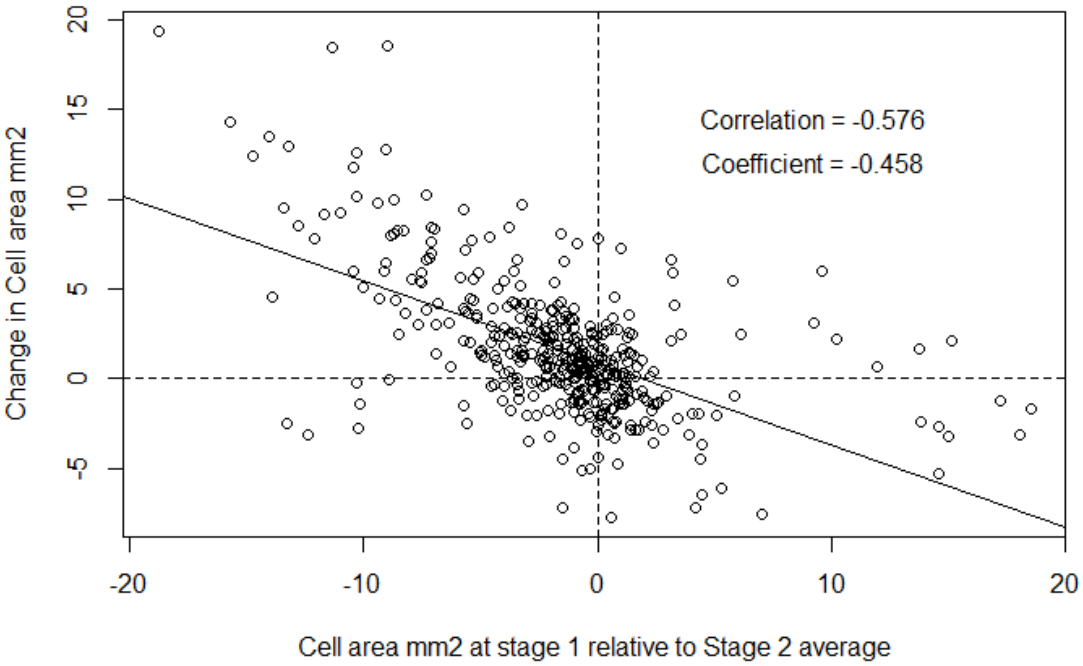

Figure 0-18 Cell area (mm^2) change related to deviation from mean. At stage 1, for each cell, the cell area (compared with the mean) is plotted against the change in cell area between Stage 1 and Stage 2. The points are overlayed by a regression line relating the values and indicating a trend toward the mean where undersized cells became larger and oversized ones were made smaller.

Discussion

Honeycomb has a pleasingly regular form, comprised of almost flat blades formed from nearly regular hexagons. The regular size of these hexagons leads to clear rows of cells that run almost entirely straight, and these features all contribute to an appearance of a strictly defined, intentional architectural structure. This regularity of comb has attracted considerable comment and discussion, with perhaps the earliest analysis of honeycomb being attributed (Heath 1921) to Pappus of Alexandria. In his preface to Book V of 'Mathematical Collections', he observes that honeybees make “honeycombs,(with cells) all equal, similar and contiguous to one another… they have contrived this by virtue of a certain geometrical forethought. … the figures must be such as to be contiguous …Bees, then, know just that the hexagon is greater than the square and the triangle and will hold more honey for the same expenditure of material used in constructing the different figures.” However, on closer inspection, these aspects of comb are only *approximately* regular. Each cell, and indeed each wall of a cell, is formed subject to the builders’ ability to respond to local features, which invariably leads to local inaccuracies. These contribute to the slight variability seen between cells across a comb.

Kepler (1611) also wrote of the bees’ choice of a hexagon to facilitate tessellation of cells across the plane of honeycomb, but then went further and explored the bases of honeycomb cells. His geometric analysis of

these resulted in the first description of a rhombic dodecahedron, a regular solid with internal angles of 120° and therefore one that tessellates in 3 dimensions. He considered how the hexagonal outline, and the pyramidal bases, of cells, may be formed and included examples of naturally occurring semi-rigid objects deformed in a geometrically regular fashion. His first example was of peas in a pod. The pod linearly constrains the peas with the result that they each adopt a flat face that is orthogonal to the line of constraint. This example from Kepler compares well with the outcome of experiment 5ii, where the angular range was restricted to 180° and when distributed between two cells resulted in corner angles of 90°.

The illustration used by Kepler to suggest how a cell base may be formed begins with a closely packed, two-dimensional array of cannon-balls, onto which another ball would be placed. This additional ball would settle between three balls on the lower level where, if it were to be pressed down (and if balls were sufficiently malleable) then the top ball would become pointed at the centre and would form 3 flat faces at the points of contact with three supporting balls. Kepler suggested pomegranate seeds as an example of 3-dimensional compaction that results in a form similar to the triple rhombic form of a cell base. When pomegranate seeds are packed together, one growing against 3 on another layer, the resulting surface comprises 3 rhombi.

The interface comprising three rhombi at the base of cells where two - one from each face - are cojoined, as with these pomegranate seeds, could adopt almost any major angle from slightly less than 120° for nearly flat bases, to approaching zero as the pyramid becomes increasingly pointed. The angle was measured as 109° 28’ by Huber (1814:109). Kepler (1611) suggested that bees use a rhombic dodecahedron on the grounds of economy, stating that “rhomboids are roomier than cubes”, but his analysis went no further. However, Huber (1814:106) investigated the matter of efficiency and cites Réaumur, who consulted the “skilled geometrician Mr Koenig” who calculated that an angle of 109° 16’ would improve the ratio of storage volume to the quantity of wax. More recently, Tóth (1964) demonstrated that it is most efficient for the base to be constructed as a truncated octahedron comprising two rhombi and two hexagons, which would result in a 0.35% saving of wax to form the base at a cost of increased geometric complexity.

Our experiment i for triplets of cells positioned within an angular range of 360° provides an alternative suggestion, demonstrating that bees will build cells with a dihedral angle of 120°. When applied to three cells in a 2-dimensional arrangement, the result is a triple junction within the tessellated hexagons visible on the face of the comb. However, when the corner angles are equalised between four cells in a 3-dimensional arrangement, the results from experiment i suggest that each corner will settle at 120° and, therefore, that the base will adopt the shape of a regular rhombic dodecahedron. In this case, the rhombi angles can be calculated to be $2 * \mathrm{Cos}^{-1}(1/\sqrt{3})$ or 109° 28.27’. This implies that bees do not construct comb in a manner aimed at maximising efficiency, and the more parsimonious explanation is that they simply balance the angular space between the cells.

The discussions above concern the depth of the basal pyramid being either exactly, or close to, a section of a rhombic dodecahedron. However, experiment ii caused cells with flat bases to be constructed. Such cells

differ from those normally found in naturally produced comb and seem less economical by providing considerably less volume for a quantity of wax but were nonetheless predictable. The prediction does not state that every angle within a cell should be 120°; rather, it predicts that the angle will be an integer fraction of the total range. For a range of 180° and where the number of cells is two, then the wall angle would be predicted to be 90° – the outcome of experiment ii. Flat bases were formed where cells lay in direct opposition, while normal rhombic bases were formed where cells overlapped by half their width, with the centre of a cell on one face positioned opposite the triple junction on the other face. The prediction explains how both situations arise, but where the cells are positioned in different arrangements, the same mechanism explains how alternative base structures would be formed. Figure 0-19 shows a range of base forms including pyramidal bases, flat bases and some that approximate the truncated octahedron which Tóth (1964) suggested as an improvement. In all cases, one element of the base, a flat side, would be formed where a cell on one face overlaps one on the opposite face. Therefore, the arrangement of base faces, and thus the geometry of the base, depends on the alignment of cells between the two faces. A similar range of base styles was present when naïve bees built comb without prior experience in doing so (Oelsen and Rademacher 1979). The comb built by these bees lacked rotational alignment between cells on each face, and this resulted in a circular pattern of cell overlap, which was described as 'floral' by the authors.

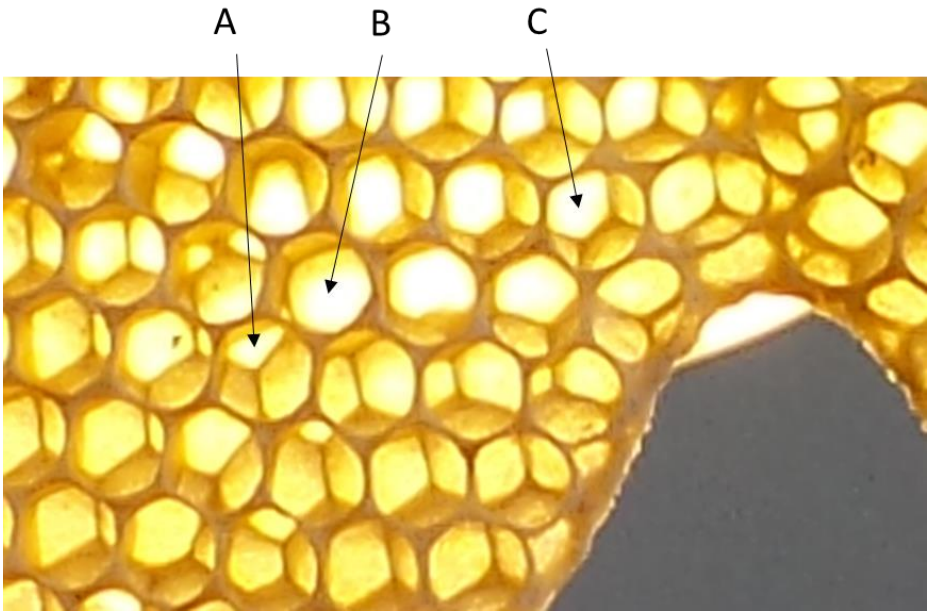

Figure 0-19 Varied base forms resulting from cell alignment. Cell A is aligned with those opposite in a near-conventional fashion so has a rhombic base. The cells (B) are directly opposite each other causing a flat base and the centre of cell C is aligned above the side wall of opposite cells and the resulting base is a section of a truncated octahedron. The pattern of cell overlap around B is similar to that described as 'floral' by Oelsen and Rademacher.

Experiments i, ii and iii show that the geometry of cells is adjusted during construction so that these features such as wall length, angles between walls at junctions and the area of adjacent cells are locally balanced. However, comb also exhibits regularity of structure on a larger scale, for

example, where bees build multiple blades of comb, these are commonly separated by a gap of constant width of approximately 9 mm depending on the morphology of the species of bee (Langstroth 1853). However, the multiple blades began at several locations, each built downwards from an initial wax deposit placed somewhere on the underside of the supporting structure, and this occurred *without* any coordination. Some will therefore have been spaced too widely and some too closely, yet the comb settles to the correct spacing. In a previous study of cell irregularities (Hepburn and Whiffler 1991), the authors describe how additional comb sections will be built within spaces that are large enough, and extensions to tongues will be adjusted where a gap becomes too small. These macroscopic adjustments can be explained, like the equilibrium observed in these experiments, by considering pressure that may be applied to the workers constructing a part of the comb through contact with workers engaged in the construction of adjacent tongues of comb. A tendency by a worker to retreat from direct contact would impact the shape and hence location, of each cell during its construction.

Cell adjustment has been observed where bees were offered wax foundations on which to build comb where the indentations included those of an inappropriate scale but has not been fully explained (Yang et al. 2010). It was found that the larger bees, *A. mellifera*, built cells on foundations with indentations that were spaced too closely (being appropriate for *A. cerana*) but did so by extending the initial guide. The results from experiment 5iii suggest the explanation for this was that cells were progressively adjusted towards the appropriate size. Hence, the cells would be guided initially by the foundation, but the authors observed that walls were subsequently relocated to form cells of the correct size for a worker at the expense of comb scale regularity.

Coercing bees to build comb on inappropriate foundations creates the conditions for comb construction to start irregularly over a wide area. A strip of irregular cells is also created when two tongues of comb merge, although ‘collide’ may be the *mot juste*. Cell level irregularities at such intersections were studied, and metrics including wall length, cell area and orientation were found to change smoothly across the area of four or five cells on either side of a junction (Smith et al. 2021). The authors suggested that the bees could anticipate the narrowing gap and could, by touch, determine the relative alignment on either side and hence build cells of a suitable transitory form. This conjecture appears to be contradicted by the results of experiment iii which showed that, at the time of contact, the cells close to the junction were irregular. After further construction had taken place, experiment iii showed that irregularities were significantly reduced. The suggestion by Smith *et a*l. that the behaviour of the bees was modified by the proximity of one tongue edge to another appears unlikely, hypothesis III does however explain how continued construction leads to equalisation between cells by reducing cell-to-cell disparity, leading to their findings of a gradual change across a tongue junction.

We have examined the response to conditions that occur during the later stages of cell construction. The results show how the interaction between two or more cells in contact with each other equilibrates so that cell walls are of equal length, corner angles settle at 120° and, emergent from these features, cells become hexagonal prisms with rhombic bases. In conclusion, qualitative stigmergy describes how different patterns of behaviour exhibited by an animal, in this case, *A. mellifera*, are triggered as

responses when confronted by distinct conditions. While striving to build cells that conform to an ideal of a cylindrical tube with a rounded base, the constraints imposed by adjacent cells compel a self-organised mutual compromise that results in the available space and angular being shared equally between connected cells.