

ORIGINAL RESEARCH ARTICLE



Computer-assisted digital image analysis and evaluation of brood development in honey bee combs

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Summary

The potential impact of plant protection products on honey bee brood development is of increasing concern. Regulatory authorities therefore now request studies monitoring potential adverse effects on honey bee brood development. Current methods have a number of inherent technical limitations which we have circumvented by computerization of analysis. In this article, we describe the computer-assisted digital image analysis and evaluation method of brood development in honey bee combs. With this tool it is possible to systematically evaluate brood development on the basis of high definition pictures of brood frames taken during semi-field or field scale honey bee trials. The computer-assisted method enables the *post-hoc* analysis of virtually any number of cells, and addresses both the issues of small cell numbers as well as the traceability and verification of the data. The method and software described has been designed and compiled with the intention of providing a tool for the 100% traceable analysis of bee brood studies with gap-free, systematic documentation. This is extremely important when working under Good Laboratory Practice (GLP) conditions. By reducing the out-of-hive time, the method minimizes the impact on the bee brood, and is thus likely to increase the success rate of studies. The availability of digital images allows the *post-hoc* analysis of any number of cells. The automated tracing of the cells under investigation and the automated population of the cell classification data excludes manual data transcription errors and thus significantly improves data reliability.

Análisis de imagen digital asistido por ordenador y evaluación del desarrollo de la cría en los panales de abejas

Resumen

El potencial impacto de los productos fitosanitarios en el desarrollo de la cría de abejas es cada vez más preocupante. Por lo tanto, las autoridades reguladoras solicitan estudios de monitoreo de los posibles efectos adversos sobre el desarrollo de la cría de abejas. Los métodos actuales tienen una serie de limitaciones técnicas inherentes que eludimos gracias a la informatización de los análisis. En este artículo se describe el análisis de imágenes digitales asistido por ordenador y un método de evaluación del desarrollo de la cría de abejas en los panales. Con esta herramienta es posible evaluar sistemáticamente el desarrollo de la cría sobre la base de imágenes de alta definición de cuadros de cría tomadas en ensayos de campo o en semi-campo con las abejas de miel. El método asistido por ordenador permite el análisis post-hoc de prácticamente cualquier número de celdas y permite abordar tanto la cuestión del bajo número de celdas, así como la trazabilidad y la verificación de los datos. El método descrito y el software han sido diseñados y elaborados con la intención de proporcionar una herramienta para un análisis de trazabilidad del 100 % de los estudios de la cría de abejas con documentación sistemática libre de fallos. Esto es extremadamente importante cuando se trabaja en buenas condiciones de laboratorio (BCL). Al reducir el tiempo fuera de la colmena, el método minimiza el impacto en la cría de abejas, y por lo tanto es probable que aumente la tasa de éxito de los estudios. La disponibilidad de imágenes digitales permite el análisis post-hoc de cualquier número de celdas. El sistema automatizado de rastreo de las celdas investigadas y la población automatizado de los datos de clasificación de las celdas no incluye errores de transcripción de datos manual y por lo tanto mejora significativamente la fiabilidad de los datos.

Keywords: *Apis mellifera*, method, honey bee brood, risk assessment, acetate-sheet technique, digital evaluation, image analysis, OECD guidance document 75, OEPP/EPPO guideline No. 170

Introduction

Within the European Union, the potential side effects of plant protection products (PPP) on honey bees (*Apis mellifera* L.) are currently assessed on the basis of the risk assessment guidelines outlined by the European Plant Protection Organisation (EPPO). According to the currently established decision making scheme for the environmental risk assessment of plant protection products (OEPP/EPPO, 2001), a honey bee brood test is required if a product is likely to affect bee brood development. This is usually the case when a product is known to act as an insect growth regulator (IGR) and is applied onto a flowering crop, or if a product is applied onto a flowering crop and the product is known to be transferred into the bee hive in considerable amounts, or if a product is applied as a seed treatment or soil granule and the active ingredient is known to be a systemic compound found in pollen and nectar (OECD, 2007). The effects of PPP can be evaluated under semi-field condition in tunnel studies (OECD, 2007; Schur *et al.*, 2003), under field conditions with the "Oomen" method (Oomen *et al.*, 1992), or a full field trial according to OECD guidance document 75 (OECD, 2007) and OEPP/EPPO guideline No. 170 (OEPP/EPPO, 2001).

The "Oomen" brood test method (Oomen *et al.*, 1992) was designed to test intrinsic larvicidal effects, and bees are exposed to the test compound by feeding the colony with compound treated food, which leads to an unrealistically severe exposure of honey bee colonies. The method is considered as a type of intermediate tier test between the laboratory larval tests (Aupinel *et al.*, 2007 and Huang, 2009) and the semi-field / tunnel-test or the full field trial (OECD Guidance Document 75).

The semi-field brood tunnel test (OECD, 2007; Schur *et al.*, 2003) (Fig. 1) provides a more realistic worst-case exposure than the Oomen method, and bees are exposed to the test compound by being confined to the treated crops using a tunnel. The field test is the highest tier test, where brood effects are tested under realistic exposure conditions.

In all the above mentioned methods, the evaluation of brood development is currently carried out using the "acetate sheet method". In the semi-field brood tunnel test, 100 cells each containing one egg are selected before treatment application. The brood development is monitored 5 times over a 22 days period. Individual cells are selected by marking them on acetate sheets. For cell selection before the application, one brood comb is taken out of each colony to define areas with at least 100 cells containing eggs. The sheet is fixed with needles on the wooden frame and the position on the frame is marked. This procedure allows the accurate positioning of the acetate-sheet in the same position on each of the subsequent observation days. One hundred cells are marked with a permanent marker pen on acetate sheets. Photocopies of the first acetate sheet are used as a positioning device for all subsequent

assessments. On subsequent analysis days, a photocopy of the acetate-sheet is re-positioned on the same comb and the development of each marked cell is assessed by the expert. A colour system is used to label the different stages on the acetate sheets. Finally, the cells' classifications on subsequent evaluation days performed on the different sheets needs to be transcribed manually into spreadsheets for further calculations and evaluations (e.g. brood termination-rate, brood-index and the compensation-index).

According to our measurements, a hive with optimally filled Swiss combs (five frames with inner frame sizes of 346 mm (H) x and 270 mm (w)) can contain up to 37,000 cells, which is in good agreement with previous observations (400 cells / 100 cm²; Imdorf *et al.*, 1987). Under the current guidance document, 100 cells have to be evaluated, corresponding to only 0.27% of such a colony. This low percentage raises concerns regarding the representativeness of a human selected random sample, and the low absolute number (100) of cells causes uncertainties regarding contingency of observations.

For the testing method according to Oomen *et al.* (1992) and for field trials, areas with at least 100 eggs, 100 young larvae and 100 old larvae are selected before treatment similar to the method described above for the semi-field tunnel test. The monitoring and the subsequent assessment of the brood development in the individually marked cells are conducted in the same way as described above for the semi-field tunnel test. Each of the selected larva stages is monitored and assessed until emergence of the honey bees. Due to the different age of the larval stages at the time of selection, the observation period is adapted to the expected developmental time for each larval stage.

The acetate-sheet method has six main limitations: 1. To minimize the impact of the analysis itself on the brood (e.g. UV-rays, temperature and humidity loss of the comb), the number of cells which can be assessed by this technique is limited; 2. The relocation of the cells at the subsequent assessment can be difficult due to comb deformation (wax structure) during the study period; 3. The system does not provide the possibility to re-examine or to verify the classification of cell content; 4. The whole analysis is very time consuming and the manual transcription of the many classification data is a potential source of errors; 5. The age of a honey bee egg can vary between 0 and 3 days, so the actual cell content at the application day for tunnel studies is not clearly defined due to the fact that the marking of the cells containing an egg is done 1 to 2 days before treatment application; 6. Due to the limited sample size (100 comb cells) the statistical power of the data analysis may be poor.

To overcome the above limitations we have developed a computer-assisted digital image analysis and evaluation method for the assessment of brood development in honey bee combs. Preliminary development of this method has been presented (Jeker *et al.*, 2010) but here we describe the method in full for the first time. With this tool it is possible to rapidly evaluate high definition pictures of brood



Fig. 1. Set up of a semi-field brood tunnel test.

frames taken during the trials. The computer-assisted method enables the *post-hoc* analysis of the data in which any number of cells can be evaluated, thus addressing both the issue of the small cell number as well as the traceability and verification of the data. The extent of analysis is therefore only limited by the number of suitable cells and the amount of resources dedicated. The method and software described has been developed and compiled with the intention of providing a tool for a 100% traceable analysis of bee brood studies with gap-free, systematic documentation. This is extremely important when working under Good Laboratory Practice (GLP) conditions.

The method minimizes the impact on bee brood, and increases reliability of the data. The automated transposition of cell coordinates and the automated pooling of the classification data minimizes manual data transcription errors and therefore may significantly improve reliability and the power of statistical analysis.

Materials and methods

Digital pictures of brood

A prerequisite of computer-aided evaluation is the ability to generate high definition pictures of constant quality of the brood at the beginning and during the conduct of the studies.

Before the application of the test substance (brood fixing day 0), one comb with a sufficient number of eggs (or any other developmental stage of interest) is selected. The frame has to be labelled with a pin on each of the long sides and with a tag allowing unequivocal identification. These pins are referenced later as reference points for the transposition of the coordinates in the computer software (see, below and Fig. 2). For photography, the frame is transferred into a custom-built photo box 1.6 x 0.6 x 0.6 m (L x W x H) (Fig. 3) where the comb is fixed in a frame holder (Fig. 4).

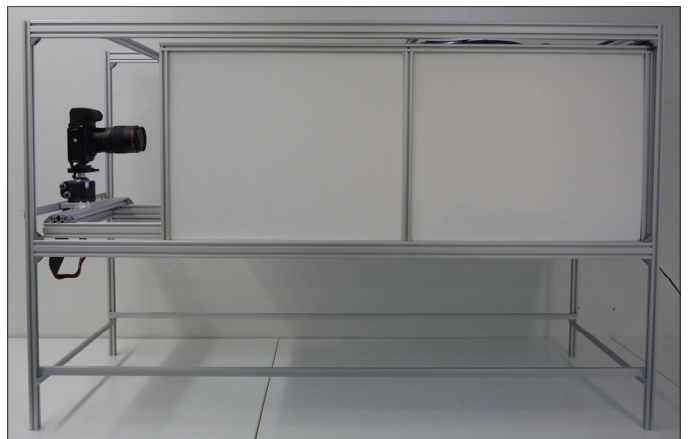


Fig. 2. Photo box (side view).



Fig. 3. Photo box (back view).

The photo box is portable and designed for field use. If necessary, the photo box fits into any estate car, or can be carried by hand close to the honey bee colonies, where a car battery serves as a power supply

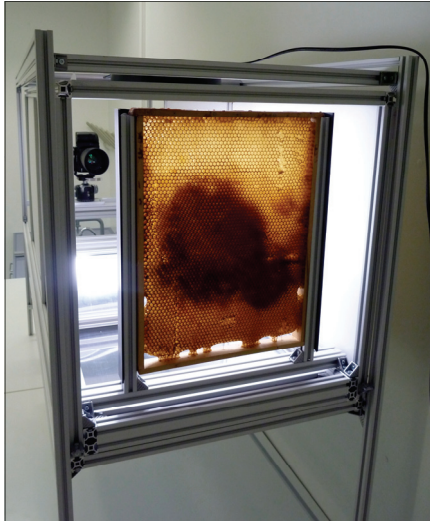


Fig. 4. Frame fixed in the frame holder within the photo box.

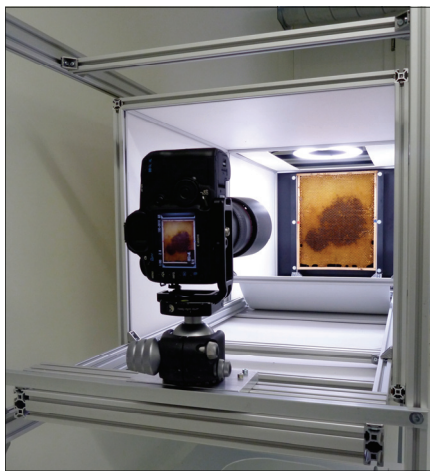


Fig. 5. Inside view of the photo box: Distance between camera lens and comb (fixed in the frame holder 1.2m).

for lighting. The distance between the frame and camera lens should be approximately 1.2 m (Fig. 5). A circular light source with a light temperature of 5500 Kelvin (Fluorescent Studio Light, model: NC – 1327 230 V, 50 Hz, lamp type: NG-954) is used to minimize reflections of the cell contents. For an optimal illumination of the cell contents, a mirror foil is fixed beneath the light source at the bottom of the photo box (Fig. 6). Images are acquired using a full format CMOS digital camera with a macro lens. The camera is connected to and controlled by a computer using Canon EOS utility software. The tool provides a live-view of the camera image on the computer screen and allows the remote adjustment of optimal settings. Once a picture is taken the comb is immediately returned to the original bee colony. The time for taking a picture is approximately one to two minutes so the time that a comb is outside the colony is reduced to a minimum. For the subsequent assessment of other combs and images, the same procedure is used. The selection of the cell area containing brood stages is conducted in the laboratory.

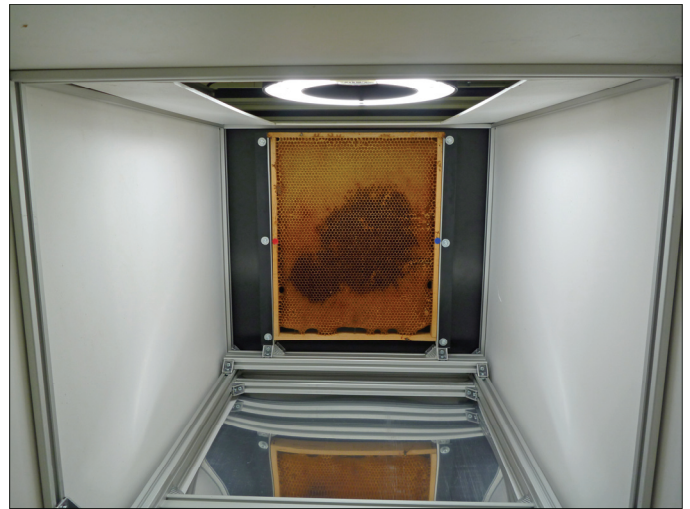


Fig. 6. Inside view of the photo box: circular light source on top and mirror foil, fixed beneath at the bottom of the photo box.

Image acquisition

The quality of image acquisition is decisive for the entire analysis. This is especially true for the image of brood fixing day 0, where the smallest objects (e.g. eggs) have to be identified unequivocally. There are a number of quality and technical requirements, which have to be fulfilled simultaneously in order to obtain images of sufficient quality. These include: sufficient illumination; minimal reflections; objects motionless and in focus; sufficient depth of focus; and adequate resolution.

Frame labelling

To allow for the transposition of the cell-coordinates on images encompassing the entire frame, the frames have to be labelled with a pin on each of the long sides (red and blue dots). These pins are then used as reference points for the transposition of the coordinates in the image analysis software (Fig. 7). In addition, each frame should be labelled with an ID code to enable identification. Both the pins as well as the ID string remain on the frame throughout the study and the frame is photographed in a way allowing the complete view on these items. To complete traceability, the camera is set to include date and time (and GPS signal if applicable) in the image.

Camera requirements

The camera should fulfil the highest technical standards both with regard to electronics as well as optics. We recommend using a professional full size CMOS (Complementary Metal Oxide Semiconductor) camera, e.g. Canon EOS 5D Mark II or an equivalent camera of any other brand. In the past, we have successfully used cameras with less resolution (e.g. Canon EOS 500) but differences are substantial, not only in price. The Canon EOS 5D Mark II with a macro lens Canon EF 100 mm 2.8L Macro IS USM as an example, has a resolution of 5,616 x 3,744 pixels with a length to width ratio of 1.5.

Photographing an entire comb with this camera will deliver images where the bee eggs will have a resolution of about four pixels per egg width.

We also recommend controlling the camera remotely with a computer (e.g. Canon EOS utility software). Having the live view of the image on a computer screen allows for a much more exact adjustment of the optimal parameter. To close the last potential source for image identification errors (relevant for GLP), the camera should be configured to include a date and time string (and any other relevant information if available; e.g. GPS coordinates) in the image. To minimize compression-caused artefacts, yet keeping image sizes reasonable, the images should be saved in a high quality RAW and JPEG format.

Photographic distance

For a reasonable assessment of the cell content, we assumed that at least $\frac{3}{4}$ of the cell floor diameter should be visible. Given the dimensions of a worker bee cell (5.3 mm width x 11 mm depth), the necessary photographic distance fulfilling the above requirement is $11/5.3 \times 0.25/2 = 4.15$ fold the long axis of the image. If using frames with a length-to-width ratio below that of the chip (1.5), the distance can be shortened accordingly. For the Swiss bee frames with a length-to-width ratio of 1.25, this means that the minimal distance should be $4.15/1.5 \times 1.25 = 3.46$ fold the long axis of the frame. Closer photographic distances will work too, but might limit the number of evaluable cells, particularly in the periphery. For these considerations we have ignored the fact that the angle of the cell walls is tilted slightly upwards, and we have assumed that the entire comb is photographed. As much as possible, the photographic distance should be kept constant for a series and we recommend the use of fixed zoom optics (e.g. Canon EF 100 mm 2.8L Macro IS USM),

eliminating possible mistakes which might arise from unintentional variations in zoom settings.

Illumination and elimination of reflection

Illumination intensity inside the light-box should be minimal, yet sufficient to allow the acquisition of pictures within 20 ms. We recommend using the following settings: Aperture 4.0 to 8.0, ISO 200 to 400, exposure bias 15 to 30msec.

Transfer of the images onto the computer and naming of the image files

Once the photos are taken, the images are transferred to the computer using the software provided by the camera manufacturer. If remote control software is used, images might be saved on the computer directly (e.g. no transfer is necessary). In order to enable automated processing, a systematic naming of the image files is mandatory. The generic filename that we use has the pattern "AAAAAA_BB_CCD_EE.jpg", where "AAAAAA" corresponds to the study identifier, "BB" corresponds to two numeric digits identifying the hive (e.g. "05"), "CC" corresponds to two numeric digits identifying the frame within the hive (e.g. "02"), "D" corresponds to the identifier of the frame side (e.g. "a" or "b") and finally, "EE" is the two-digit identifier of the day on which the photography was taken (brood fixing day; e.g. "05"). It is mandatory for each image series that the first image is taken on the brood fixing day 0 (i.e. "EE" = "00") and is named as such. Therefore, the filename of the image of brood fixing day 5 of the "a" side of the frame 3 of the hive 14 of study AB1234 would be "AB1234_14_03a_05.jpg". All image files of a series have to be stored in the same folder. There could be several image series in one folder, although we recommend keeping them in separate folders in order to avoid confusion of the user.

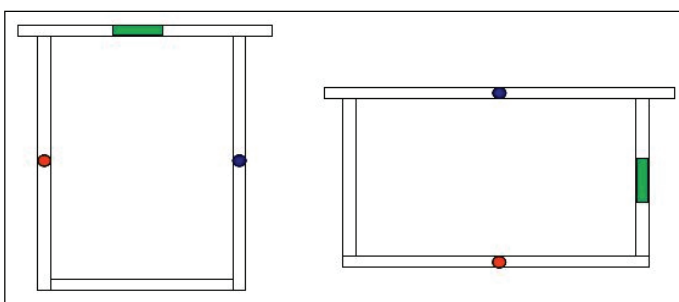


Fig. 7. Labelling of the frames: The frames have to be labelled with two pins (reference points, red and blue dots) and with a unique identifier string (green bar) in order to ensure error-free identification of frame and orientation within the hive. In order to minimize alignment problems arising from optical distortions mainly occurring in the periphery of photographs, the pins should be located on the long side of the frame and image.

Summary of the steps for computer-assisted bee brood analysis

The software is an extension of the JAVA-based freeware image analysis program FIJI, which is a life-science adapted version of ImageJ (Abramoff *et al.*, 2004). Since it is based on JAVA, the program runs platform independently (Linux, Windows, MacOS). To use this program, you have to download and install FIJI (or ImageJ) from the corresponding internet site <http://pacific.mpi-cbg.de/> (or <http://rsbweb.nih.gov/ij/>). For a description of the functions and use of FIJI and/or ImageJ, please refer to the user manual and other useful information available at the same sites.

For the analysis of an image series, three different steps have to be completed: 1. the analysis of the first image; 2. analysis of consecutive images and; 3. finalization and data evaluation of the image analyses.

Analysis of the first image

On the first image (Figs. 10, 11), the user has to select and classify the cells of interest and localize the pins as reference points for the later alignment of consecutive images. The first image is the one taken on the brood fixing day 0. According to the naming convention, the name of this file is "AAAAAA_BB_CCD_00.jpg". Before selecting the very first cell, the user has to delineate the inner border of an example cell and set this as the "standard" diameter of the cell using the menu

Table 1. Colour-coding and number-coding of the cell content.

Brood stage / Stores	Colour	Number
Vacant cell/empty	Brown	0
Egg	Blue	1
Young larva (1 – 3 days old)	Green	2
Old larva (4 – 6 days old)	Red	3
Pupa (capped cell)	Yellow	4
Nectar	Orange	5
Pollen	Deep-pink	6
Dead larva/pupa	Dark-salmon	7
Not characterized (nc)	White	8

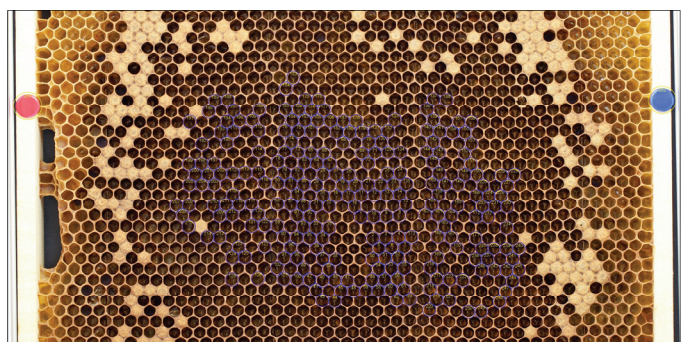
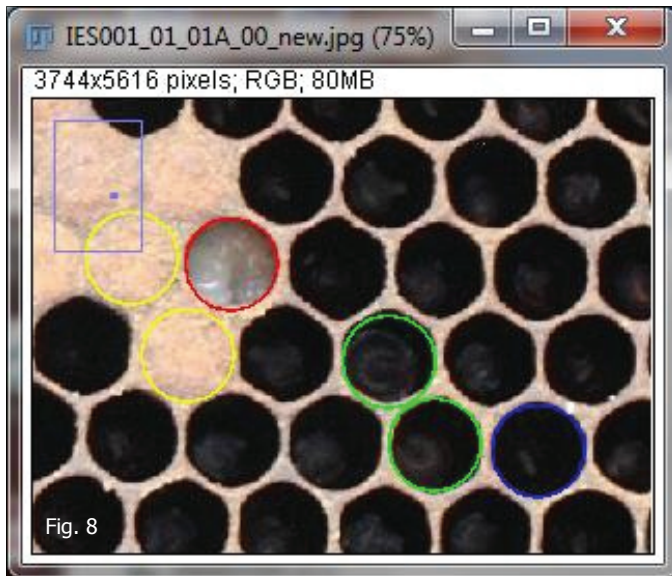


Fig. 10. A section of a brood comb with selected cells (blue circles) each cell contains one egg (BFD 0).

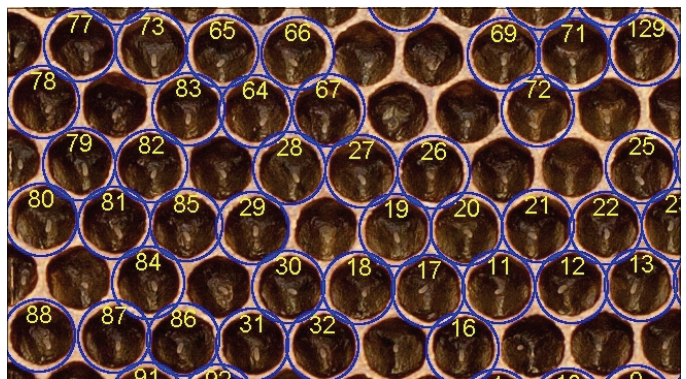
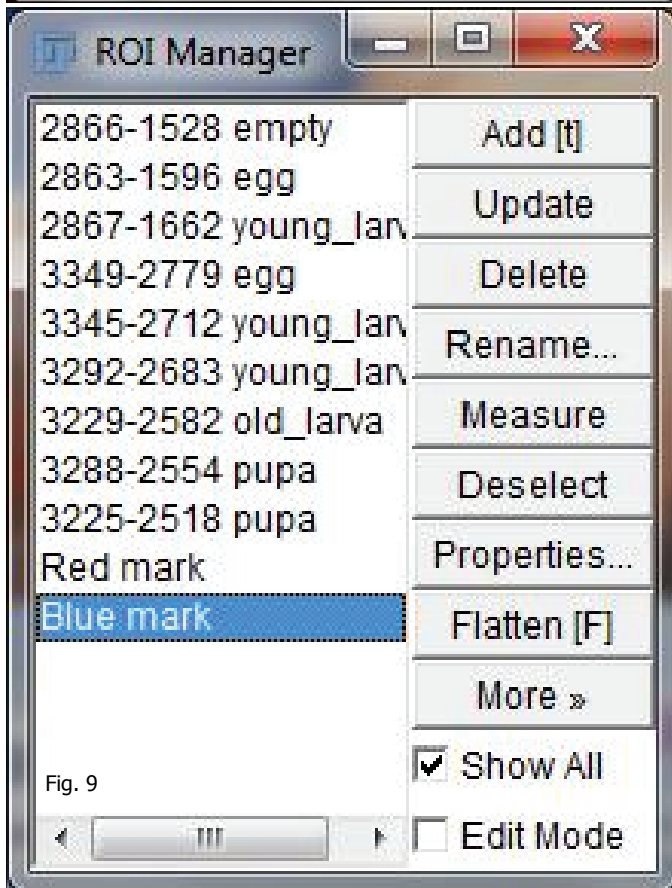


Fig. 11. Close-up section of comb area with selected eggs at BFD 0.

Figs. 8, 9. Selection of the cells and the corresponding entries in the ROI manager. At the end of the cell selection process the orientation hallmarks "Red mark" and "Blue mark" are defined.

command "Set diameter of cell" (F1). This feature can be re-adjusted at any time if needed. To select a cell, the user has to place the cursor on a cell to be selected and press one of the keys on the number pad corresponding to the actual developmental stage. The assignment of the number pad key to the developmental stage is an extended version of the numbering according to the OECD Guidance Document 75 and is listed in Table 1. Upon selection of a cell (e.g. selecting an egg-containing cell by placing the cursor to the cell and pressing "1" on the number keypad), a region of interest (ROI) is generated with a colour-coding according to the recommendation of the OECD Guidance Document 75. The coordinates and the classification information are maintained and displayed within the ROI manager (Figs 8 and 9). If a ROI other than the last one is selected, then pressing a key on the number-pad results in the re-classification

of the selected ROI according the number coding (Table 1). Once the selection of the cells is completed, the orientation reference points have to be indicated by delineating them and invoking the command "Select RED mark" (F2) or "Select BLUE mark" (F3). The analysis of the first image is completed by the command "Save selections" (F4), upon which the computer saves a file with all ROI with the file name "AAAAAA_BB_CCD_00_ROI.zip". In addition, the program creates an image file, where the numbered and colour-coded ROI are "burned" into the image along with other file-, and user-related information. This file is saved as "AAAAAA_BB_CCD_00_selections.jpg" in the working folder.

Analysis of consecutive images

For the analysis of consecutive images (Figs 12 to 16) (i.e. brood fixing day "nn"), only the orientation reference points have to be defined by the user, analogous to that described above. By invoking the command "Transpose cell-coordinates" (F5) the computer transposes the ROIs from the first image (BFD 0) onto the current image. To ensure that the cell content of the transposed ROIs is re-classified, the content label of the transposed ROIs is set to "nc" (i.e. not characterized) during the transposition. The user now

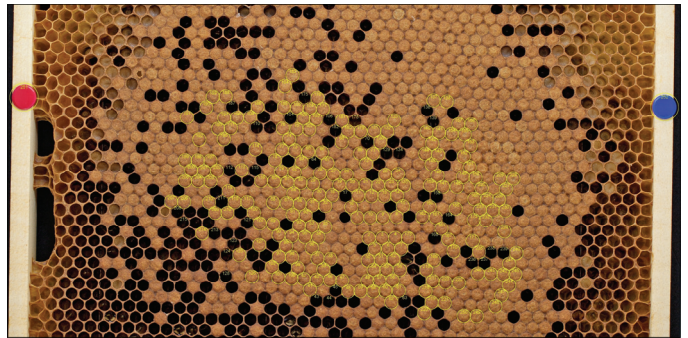


Fig. 14. A section of a brood comb with automatically transposed and assessed cells (coloured circles) containing different brood stages. For a better recognition of the different brood stages on the printouts, the assigned colour-code highlights the cell content (BFD 10).



Fig. 12. A section of a brood comb with automatically transposed and assessed cells (coloured circles) containing different brood stages. For a better recognition of the different brood stages on the printouts, the assigned colour-code highlights the cell content (BFD 6).

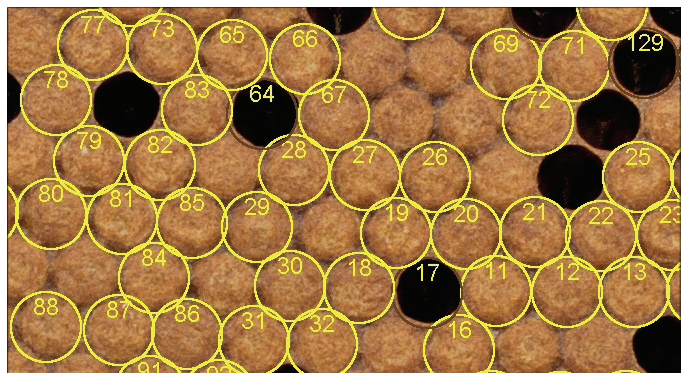


Fig. 15. Close-up section with assessed brood cells BFD 10. Most cells are capped, containing pupae (colour: yellow)

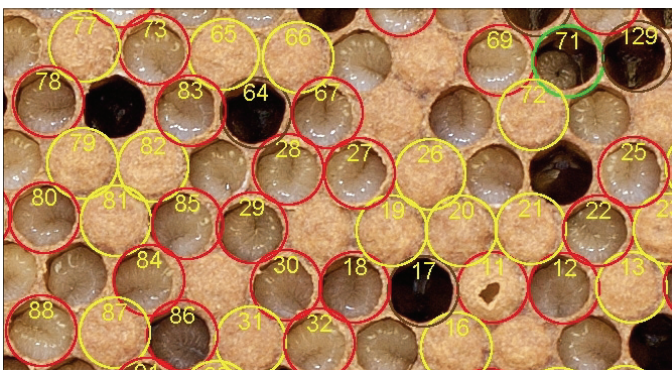


Fig. 13. Close-up section with assessed brood cells BFD 6. Colour: green for young larva, red for old larva, yellow for pupa and brown for empty cells.

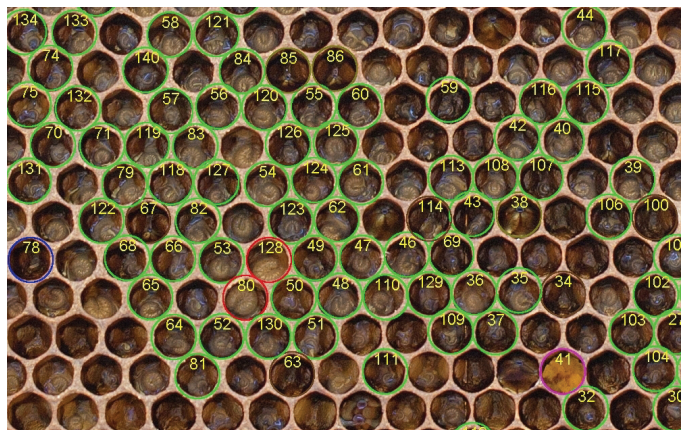


Fig. 16. Close up view of a honey bee comb with numbered and colour-coded assessed cells.

has to classify the cell content, for which the program provides an interface. To re-classify the ROIs, the user has to activate the ROI to start with, either by clicking on it in the ROI manager or by clicking on the ROI boundary displayed on the image. Typically, the re-classification starts with the first ROI. Since now a ROI other-than-the-last-one is selected, the keys of the number-pad behave differently than during cell selection and serve now the re-classification (i.e. pressing the key "2" on the number-pad will re-classify the cell content to "young larva"). To expedite the re-classification process, each time a ROI is re

Bee brood study evaluation

Study: SHOW01 Hive: 05 Frame: 06 Side: A
Bee Brood Analysis, IES LTD, Witterswil, Switzerland.
Ischmid / Thu 18-Aug-2011 09:52:15

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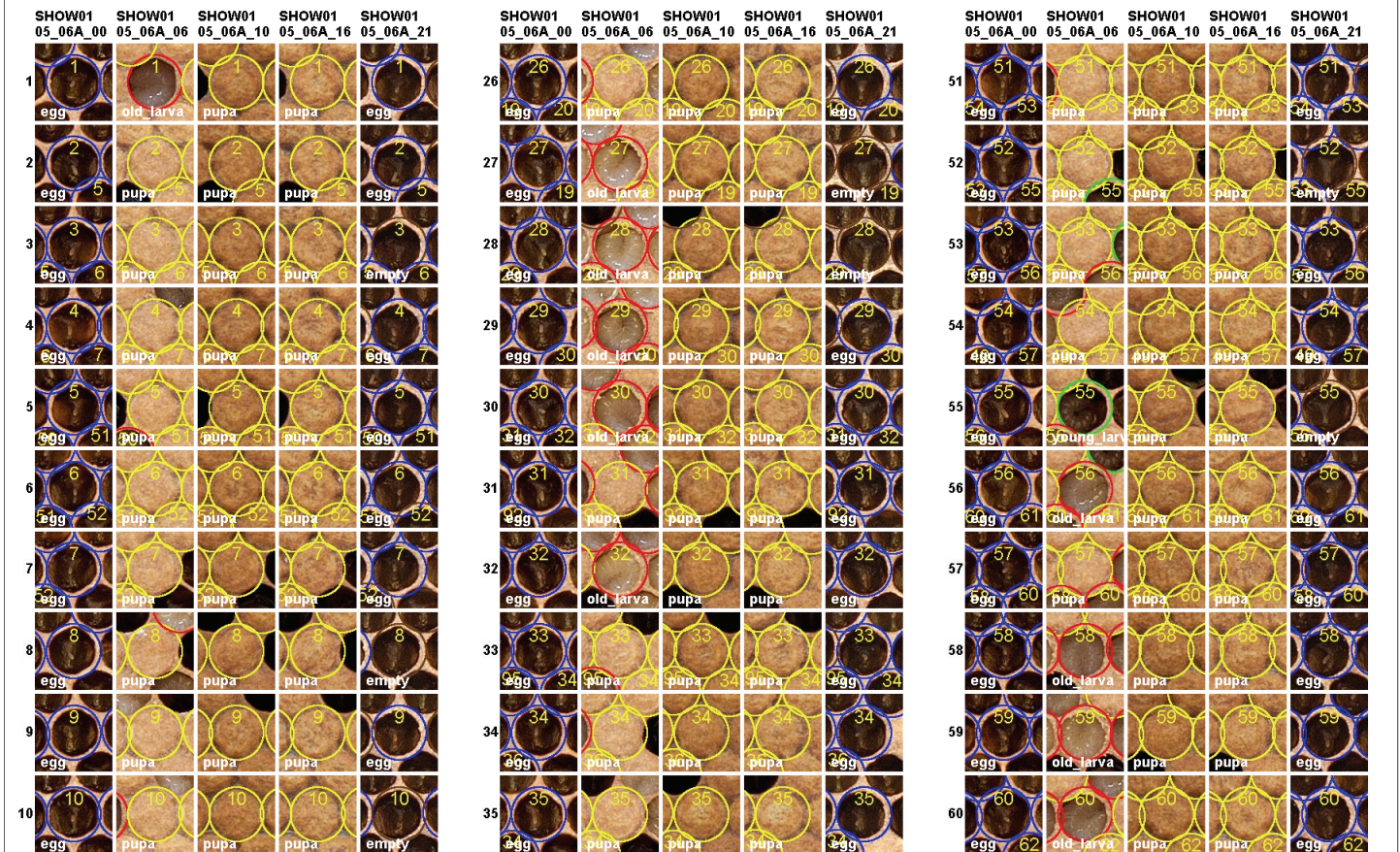


Fig. 17. Example of a generated gallery image. Images of a single cell at each observation day (e.g. BFD 0 / 6 / 10 / 16 and BFD 21) are assembled together side by side.

-classified the program “jumps” to the next ROI and presents it at a zoom factor convenient for visual inspection. The re-classification process is completed and halts if the ROI named “Red mark” is reached. As soon as the re-classification is completed, the process can be finalized by invoking the menu command “Save selections” (F4).

Analogous to that described for the first image, the computer saves a file with all ROI in the working folder and names this file as “AAAAAA_BB_CCD_nn_ROI.zip”. In addition, the program generates an image file with the selections and other file-, and user-related information “burned” into the image and saves this file in the working folder under the name AAAAAA_BB_CCD_nn_selections.jpg.

Finalization and data evaluation

As soon as all images of a series have been processed, the user can initiate the data evaluation by invoking the command “Make gallery” (F6). The program pools the ROI data from the different days into one single ROI raw data file and saves this file as “AAAAAA_BB_CCD_RawData.xls” in the working folder. Next the program populates the classification data of each individual cell from

the different observation days as numerical values according to Table 1 (i.e. “1” for a cell containing an egg). In this table, each cell is represented by a row (e.g. row “AAAAAA_BB_CCD cell 105”) and each observation day is represented by a column (e.g. column “BFD 05” for the brood fixing day 5). Additional columns are generated for the calculation of the brood termination rate (e.g. column “BTR 05”), for the calculation of the brood index (e.g. column “BI 05”) and for the calculation of the compensation index (e.g. column “CI 05”). As a matter of course, as many of each column type are generated as observation days exist. The program evaluates and fills in the corresponding values along the logics described in the OECD guideline. Finally, the total number of cells, the sum of cells with a given content (i.e. “Sum of pupa cells”), the brood termination rate, the brood index and the compensation index are calculated for each of the observation days. The file with the above data is saved as “AAAAAA_BB_CCD_FinalData.xls” in the working folder. At the very end, an image gallery is generated (Fig. 17), where the cut-outs of cells from each observation day are assembled together side by side.

10 min. The time to assess the developmental stages at subsequent assessments is estimated to be 5 min per time point (e.g. BFD 5/10/16/22). The data transfer and evaluation at the computer is automated and takes seconds. Due to the system validation no data transcription and data evaluation check is necessary. The validation of the digital brood assessment method needs to be conducted once and takes about 10 min. The quality assurance check of the assessed images (Figs 12 to 16) is estimated to be 15 min. The digital evaluation of the study-example would therefore take 14 hours. This is approximately half the time compared to the traditional acetate sheet method.

Discussion

Digital evaluation significantly improves the quality of the raw data, accelerates the assessment and allows a full GLP compliant documentation of the data and evaluation. The raw data (images) can be re-evaluated at any time. The current guideline requires the analysis of at least 100 cells. Although this number is clearly too low to reliably detect effects in the range of 10%, the processing time required for the analysis of, say 400 cells by the acetate sheet method would be far too long and would thus have a negative impact on the brood. Therefore up until now, researchers and regulatory authorities have had to accept this trade-off. Reasons for incorporating more cells in the analysis and, therefore, increasing the sample size, are also obvious from the statistical point of view. Current practice of statistical analysis is a two-step procedure, i.e. analysis of variance followed by a multi comparison test (e.g. Tukey, Dunnet). Not infrequently, a multi comparison test may yield ambiguous results and/or fails to detect differences between any pair of means whilst analysis of variance has detected differences. This reflects the fact that the analysis of variance is more powerful than is the multi comparison test (i.e. Type II errors are more likely to occur in multiple comparisons than in performing an analysis of variance). Here, increasing the sample sizes would tend to result in a multi comparison analysis more capable of locating differences among means (Zar, 1999) and, therefore lead to more decisive conclusions as the test would have more power.

Given that standardized conditions are established, photographing of a frame can be done within one to two minutes and the assessment of the images is performed separately. Therefore, the disturbance of the colonies during the study is reduced to a minimum, which improves the quality of the studies. During a *post-hoc* evaluation of cell content, additional parameters could be monitored e.g. food storage, queen fertility (newly laid eggs), morphometric parameters (e.g. larval shape, size) and brood replacement and rearrangement. The raw data of the cells also contains information about the location of the cells within the frame. This information could

enable the analysis of possible cluster effects (e.g. location-, or neighbourhood-dependent effects on larval development).

The weak points of the acetate sheet method (long off-hive times, inability to handle wax deformations, lack of possibility for re-examination and verification, numerous manual data transcription steps, unclear developmental stage at treatment, minimal sample size and low statistical power) are resolved by using the digital brood assessment method.

For tunnel brood studies application of the test compound is usually performed 1 to 2 days after marking of the brood cells containing eggs. Visual age determination of eggs is very difficult and only freshly laid eggs or eggs just before larval hatch can be conclusively recognized as such. The brood stage at the time of application and during the exposure phase is variable. Since only the hatched larvae are fed by the worker bees, a possible exposure of the larvae to contaminated food is not guaranteed. The age specific sensitivity of the brood could also contribute to variable study outcomes. Therefore, a possible effect caused by the pesticide could either be missed or diminished.

Since the image capturing of the brood combs only takes a few minutes, it is possible to acquire the images for the brood fixing day 0 (BFD 0) on the day of the treatment application. This means that the exact proportion of eggs and young larvae at the day of application can be defined. In the current acetate-sheet method, the brood is fixed 1 to 2 days before treatment application (due to the time-consuming brood marking procedure). Therefore, the brood stages within the marked cell area are variable and not defined. This means, using the acetate sheet method, that the mode of action of a product cannot be reasonably determined due to the unknown brood stage at the time of application, whilst it is possible when the brood fixing is conducted at the day of treatment application. The duration of the egg stage during honey bee larval development is four days. Therefore, determination the egg stage once within these four days will provide a four-day-wide window of temporal resolution. This window for the determination of larval development status is clearly too broad, if shifts in brood development of one or two days were to be detected. A duplicate determination of initial brood stage within two days (e.g. once two days before and once on the day of treatment) would allow the definition of two starting populations each with a temporal accuracy of two days (i.e. three-to-four days-old eggs and one-to-two days-old larvae at the time of treatment). This would double the temporal resolution of the study and would increase the interpretability of data.

A fully automated image analysis and cell content recognition is desirable, and indeed the digital image recognition would allow the automated identification of non-capped cells. In such a case, typically 2,500 to 3,500 cells are identified on one comb site of a frame in the Swiss format. Automated cell recognition is, however, only meaningful if automated classification of cell content is reliable. This is

manageable for adult larvae, where the intense white colour makes a distinction possible but is difficult for egg stages, where the minute signals of the egg have to compete with reflection artefacts and have to be detected in a highly variable surrounding (e.g. colour of wax, angle of view, etc.). Our method therefore relies on the user-defined classification of cell content. Applying the manual classification, we found that the manual selection and classification of few hundreds of cells is faster than the classification or deletion of e.g. 3,500 cells resulting from automated cell detection. Therefore the method described is the fastest possible method, allowing the reliable selection and classification of several hundred cells.

Should the classification of all cells of a frame be required in future, the automated cell recognition would provide a reasonable basis for the efficient manual classification of cell contents. We developed this program with the requirement in mind that the classification of the cell content has to be error free. Due to the high variability in background and the frequent occurrence of reflections, the automated detection of egg-containing cells is not 100% reliable. A partial automation of cell content classification (e.g. classification of adult larvae, capped cells and pollen-containing cells) seems, however, to be feasible, leaving the user the task of classifying the remaining ones (cells containing eggs, empty cells, young larvae).

To improve the quality, documentation and data interpretation of honey bee semi-field and field studies with brood evaluation, the digital comb assessment methods should be used for any brood development evaluation. The fully automated alignment and cell content recognition is under development and should be a next step to enhance and improve the quality and significance of honey bee brood studies. The big advantage of the digital evaluation is, however, that increased number of cells can be analysed and that it provides the possibility of complete photographic documentation and re-traceability of any evaluation. All these advantages increase the reliability of the experiment and statistical power of the data evaluation. In view of the qualitative improvement and time saving, we believe that the investment in this technology is absolutely justified.

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