



## Short Communication

## “Entombed Pollen”: A new condition in honey bee colonies associated with increased risk of colony mortality

Dennis vanEngelsdorp<sup>a,\*</sup>, Jay D. Evans<sup>b</sup>, Leo Donovall<sup>a</sup>, Chris Mullin<sup>c</sup>, Maryann Frazier<sup>c</sup>, James Frazier<sup>c</sup>, David R. Tarpy<sup>d,1</sup>, Jerry Hayes Jr.<sup>e</sup>, Jeffery S. Pettis<sup>b</sup>

<sup>a</sup> Pennsylvania Department of Agriculture, Penn State University, 2301 North Cameron Street, Harrisburg, PA 17074, United States

<sup>b</sup> Bee Research Lab, USDA-ARS Bldg. 476, BARC-East Beltsville, MD 20705, United States

<sup>c</sup> Penn State University, 501 ASI Building, University Park, PA 16802, United States

<sup>d</sup> North Carolina State University, Department of Entomology, Campus Box 7613, Raleigh, NC 27695-7613, United States

<sup>e</sup> Florida Department of Agriculture and Consumer Services, 1911 South West 34th Street, P.O. Box 147100, Gainesville, FL 32614-7100, United States

## ARTICLE INFO

## Article history:

Received 9 October 2008

Accepted 30 March 2009

Available online 8 April 2009

## Keywords:

*Apis mellifera*

Colony collapse disorder

Bee bread

Honey bee

Pesticide residues

Physical encapsulation

Entombed pollen

## ABSTRACT

Here we describe a new phenomenon, entombed pollen, which is highly associated with increased colony mortality. Entombed pollen is sunken, capped cells amidst “normal”, uncapped cells of stored pollen, and some of the pollen contained within these cells is brick red in color. There appears to be a lack of microbial agents in the pollen, and larvae and adult bees do not have an increased rate of mortality when they are fed diets supplemented with entombed pollen *in vitro*, suggesting that the pollen itself is not directly responsible for increased colony mortality. However, the increased incidence of entombed pollen in reused wax comb suggests that there is a transmittable factor common to the phenomenon and colony mortality. In addition, there were elevated pesticide levels, notably of the fungicide chlorothalonil, in entombed pollen. Additional studies are needed to determine if there is a causal relationship between entombed pollen, chemical residues, and colony mortality.

© 2009 Published by Elsevier Inc.

Honey bee populations have been declining rapidly over the past 40 years (NRC, 2007). Much of this decline, particularly over the last two decades, can be attributed to known causes such as the parasitic mite *Varroa destructor*. More recently, however, extensive losses of honey bee colonies in the continental United States have been attributed to a poorly understood phenomenon referred to as Colony Collapse Disorder or CCD (vanEngelsdorp et al., 2007, 2008). CCD is defined by a specific set of symptoms, including the rapid loss of the adult population with no dead bees in or in proximity to the hive (Cox-Foster et al., 2007). In an attempt to elucidate the causes of poor colony health in general, and CCD in particular, two longitudinal studies were initiated in the spring of 2007. One of these studies monitored three US migratory beekeeping operations, while the other studied the effects of various comb treatments on 200 colonies established from packaged bees imported from Australia.

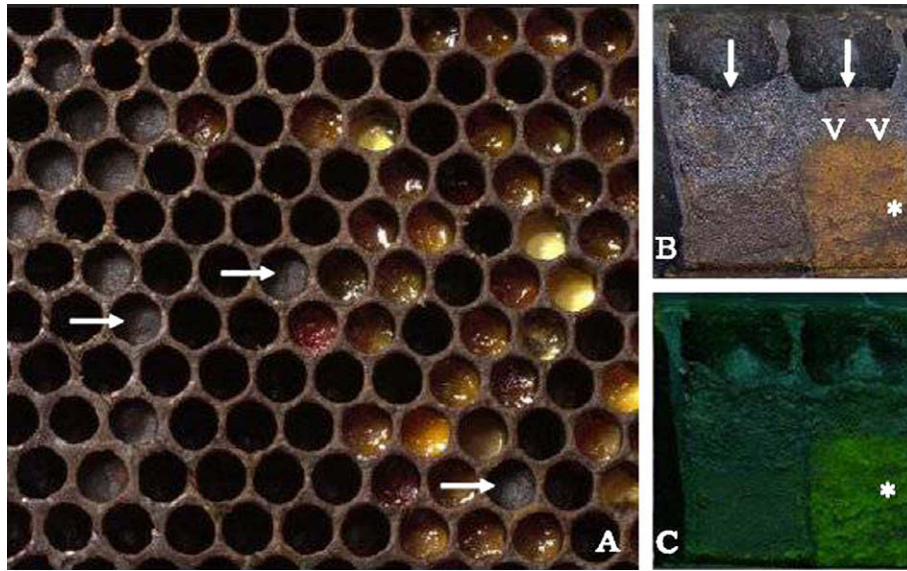
Pollen is a honey bee colony's only source of protein, lipids, vitamins, and minerals. Forager bees collect pollen from flowering plants, pack it on their hind legs, transport it back to the colony, and deposit it in the wax comb near the brood nest. Food-handler bees then add an assortment of enzymes and honey to the stored pollen to help preserve it and make it available for eventual consumption as “bee bread” (Chauvin and Lavie, 1956). Bee-bread provisions are easily identified in the combs as they remain uncapped and are often brightly colored, reflecting the diversity of floral sources visited by pollen-collecting bees.

“Entombed” pollen, a condition described here for the first time, is bee bread covered by a sunken capping (Fig. 1A). At least some of the pollen stored in these cells is brick red in color (Fig. 1B); this brick red pollen does not fluoresce under ultraviolet light (Fig. 1C). In most cases the entire cell content is brick red in color, however in some rare instances the characteristic color appears only on the top section of cells (Fig. 1B and C). In subsequent, unrelated surveys of colonies in Florida and Pennsylvania, similarly capped bee bread was observed, but the cells did not contain pollen that was characteristically brick red in color (hereafter referred to as “capped pollen”). Melting point tests revealed that the cappings on entombed pollen were composed mostly of propolis but did contain beeswax ( $n = 21$ ). In contrast, the cappings on capped

\* Corresponding author.

E-mail addresses: [dennis.vanengelsdorp@gmail.com](mailto:dennis.vanengelsdorp@gmail.com) (D. vanEngelsdorp), [jay.evans@ars.usda.gov](mailto:jay.evans@ars.usda.gov) (J.D. Evans), [ldonovall@state.pa.us](mailto:ldonovall@state.pa.us) (L. Donovall), [CAMullin@psu.edu](mailto:CAMullin@psu.edu) (C. Mullin), [mfrazier@psu.edu](mailto:mfrazier@psu.edu) (M. Frazier), [jfrazier@psu.edu](mailto:jfrazier@psu.edu) (J. Frazier), [david\\_tarpy@ncsu.edu](mailto:david_tarpy@ncsu.edu) (D.R. Tarpy), [hayesg@doacs.state.fl.us](mailto:hayesg@doacs.state.fl.us) (J. Hayes), [jeff.pettis@ars.usda.gov](mailto:jeff.pettis@ars.usda.gov) (J.S. Pettis).

<sup>1</sup> Fax: +1 919 515 7746.



**Fig. 1.** 'Entombed' pollen (→) is readily identified as having sunken, wax-covered cells amidst "normal", uncapped cells of bee bread (A). Unlike capped honey and brood cells, the entombed cells are capped below (↓) the comb surface, appearing to be sunken into the cell (B). At least some of the pollen contained within these cells is brick red in color, and this pollen does not fluoresce under ultraviolet light like most non-red colored pollen (\*, C). In rare cases, cells contained the characteristic red, non-fluorescing pollen on top of otherwise normal-looking, fluorescing pollen (BV). (For interpretation of color in Fig. 1, the reader is referred to the web version of this article.)

pollen were made up of propolis alone ( $n = 18$ ). Further comparative examinations of the contents of cells classified as entombed, capped, and normal revealed that all cells contained pollen grains from a variety of different floral sources. However, normal and capped cells contained full pollen grains, while entombed cells contained only empty pollen grain husks.

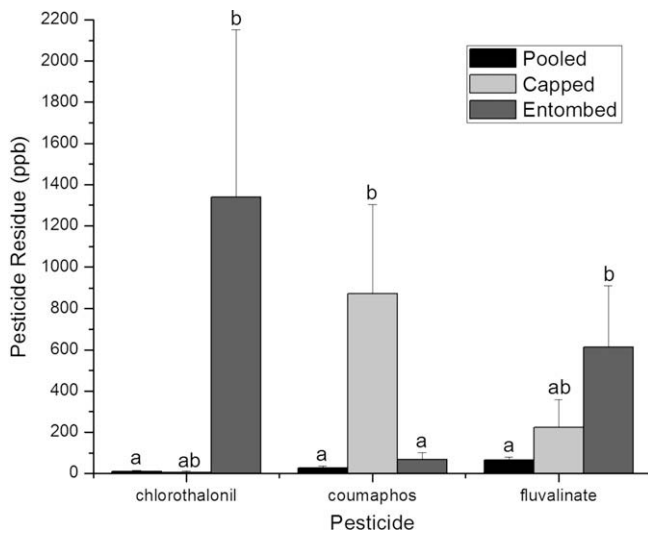
The first study monitored three migratory beekeeping operations that transported honey bees among Florida, Pennsylvania, New Jersey, New York, Massachusetts, and Maine for the purposes of crop pollination, honey production, or both. In all, 60 colonies (~20 from each operation) were monitored and periodically evaluated between March 2007 and January 2008, so that each colony was examined and sampled a total of 7–8 times. Appreciable amounts of entombed pollen were first observed in these colonies in June 2007; 40.5% ( $n = 49$ ) of monitored colonies had the condition with some having more than 100 cells of entombed pollen. By November 2007, colonies with entombed pollen had a higher rate of mortality (43%) than those without entombed pollen (20%; Fisher's exact test,  $P < 0.05$ ). The presence of entombed pollen in colonies in June represented a relative mortality risk of 3.1. Only 1 of 20 colonies with entombed pollen in June died with symptoms indicative of CCD, thus entombed pollen is not likely associated with CCD. Capped pollen was not observed in any of the colonies in this longitudinal study, so future studies that quantify its effect, if any, on colony health are needed.

The second longitudinal study monitored 200 colonies that had been established with packages of bees imported from Australia in March of 2007. The packaged bees were introduced following standard practices into previously used beehives belonging to one of four treatment groups: (1) combs from colonies that had recently died while exhibiting CCD-like symptoms ('non-irradiated'); (2) combs from colonies that had recently died while exhibiting CCD-like symptoms and were subsequently irradiated ('irradiated'); (3) combs from colonies that died while exhibiting CCD-like symptoms and were subsequently fumigated with acetic acid ('acetic acid'); and (4) combs from seemingly healthy colonies that had only previously been used for honey storage ('honey comb'). By August 2007, the incidence of entombed pollen differed significantly among treatment groups ( $F = 6.60$ ,  $df = 3$ ,  $P < 0.001$ ), with

the honey comb control group having fewer colonies with entombed pollen (10.7%,  $n = 28$ ) than all other treatment groups (non-irradiated: 53.4%,  $n = 58$ ; irradiated: 52.9%,  $n = 34$ ; acetic acid: 59.3%,  $n = 27$ ). These observations suggest that the occurrence of entombed pollen is associated with comb type and that comb treatment did not remove any risk factors found in comb from colonies that died from CCD.

Because irradiation had no measurable effect on the incidence of entombed pollen, the underlying cause of the phenomenon does not appear to be pathogenic. This supposition is further supported by attempts to quantify and compare the fungal and bacterial loads in entombed and normal pollen. Generic primers for bacteria (16rRNA) and fungi (ITS; Evans, 2006) were used to screen Chelex-extracted DNA from all pollen sources. Levels of both bacteria and fungi were undetectable in all samples following 35 cycles of PCR. Additional assays were then conducted to test for possible inhibition of PCR by pollen compounds, using standard (bacterial) controls for PCR efficiency. While all pollen extracts inhibited PCR to some extent, those from the entombed pollen samples were inhibitory at a 10-fold lower concentration than were extracts from apparently normal pollen. The agent(s) behind this inhibition was heat stable and water soluble. This factor need not be involved with bee disease but will be a factor in attempts to quantify microbial associates of entombed pollen. The fact that no bacterial or fungal microbes were identified in this survey might also reflect the extraction method: Chelex extractions are most sensitive for vegetative cells and we might have under-reported dormant spores in pollen.

Pesticide levels were determined (Mullin et al., in preparation) for each pollen type: entombed pollen ('entombed',  $n = 6$ ); capped pollen ('capped',  $n = 6$ ); seemingly normal pollen from colonies in which entombed pollen occurred ('normal',  $n = 11$ ); and seemingly normal pollen from colonies lacking entombed pollen ('control',  $n = 3$ ). In total, 30 different pesticides and metabolites were found in the samples. The most commonly occurring pesticides were the miticides coumaphos (detected in 100% of samples) and fluralinate (detected in 96% of samples) and the fungicide chlorothalonil. Chlorothalonil was found in 100% of the samples of entombed pollen, but only in 45.5% of samples of normal pollen, 16.7% of sam-



**Fig. 2.** Comparison of pesticide levels in normal and control (pooled;  $n = 14$ ), capped ( $n = 6$ ) and entombed ( $n = 6$ ) pollen. Differences in the residue levels for a given pesticide (Tukey-Kramer HDS,  $P < 0.05$ ) are indicated by different letters above bars.

ples of capped pollen, and 0% of samples of control pollen (Fisher's exact test,  $P < 0.01$ ). However, amounts of this fungicide were more than 40-times higher in entombed pollen compared to any other type of pollen (Fig. 2). Levels of chlorothalonil, coumaphos, and flufenoxystrobin were either numerically or significantly lower in normal and control pollen (samples were pooled for analysis) when compared to entombed and capped pollen (Fig. 2; Coumaphos:  $F = 6.60$ ,  $df = 23$ ,  $P = 0.005$ ; Chlorothalonil:  $F = 4.71$ ,  $df = 23$ ,  $P = 0.019$ ; Flufenoxystrobin:  $F = 4.55$ ,  $df = 23$ ,  $P = 0.021$ ).

Survivorship studies were performed to determine the potential toxicity of entombed pollen on adult bees. Approximately 30 twenty-hour-old adult workers were introduced into a  $16.5 \times 12.25 \times 13.35$  cm cage and fed 50% sucrose solution and either: (1) seemingly normal pollen collected from a colony lacking entombed pollen ('positive control'); (2) seemingly normal pollen collected from a colony in which entombed pollen occurred ('normal'); (3) entombed pollen ('entombed'), or (4) no pollen ('negative control'). Each treatment was replicated three times using bees from three different colonies. Bees in all groups consumed the provided pollen equally. Bees feeding on pollen, regardless of pollen type, had a higher survivorship rate than those bees feeding on sucrose solution alone ( $\chi^2 = 93.84$ ,  $df = 3$ ,  $P < 0.001$ ), but survivorship did not differ among the pollen-fed treatments.

To test for effects of entombed pollen on larval growth, groups of eight larvae were reared *in vitro* from the first instar to the prepupal stage (8 days) following Kucharski et al. (2008). Supplemental food consisted of 1 ml of 66% royal jelly, 10% fructose, and 10% glucose supplemented with either 50 mg of entombed pollen (three replicates), 50 mg of seemingly normal pollen (six replicates), or no additional pollen (three replicates). Survival of the larvae did not differ significantly among the three diets, although a one-tailed Fisher's exact test between larvae reared on entombed versus normal pollen bordered on significance (35/48 survivors on normal pollen, 13/24 survivors on entombed pollen;  $P = 0.09$ ). Pollen-free aqueous extracts were then prepared

from the same normal ( $n = 6$ ) and entombed ( $n = 3$ ) pollen sources by suspending 0.1 g of pollen in 1.0 ml distilled water for 48 h, pelleting the pollen grains by centrifugation and adding 50  $\mu$ l of the supernatant to 950  $\mu$ l of the larval diet described above. Again, there was a trend toward lower survival for larvae raised on a diet with entombed-pollen extract (15/24 survivors versus 39/48 survivors when fed an extract of normal pollen and 18/24 survivors on a pollen-free diet), but this difference only bordered on significance ( $P = 0.09$ ).

These results provide compelling evidence that entombed pollen indicates exposure to a risk factor that is detrimental to honey bee colony survival. Entombed pollen is not directly responsible for increased mortality, as there is no significant reduction in the longevity of larvae or adult bees fed entombed pollen. Nor is entombed pollen directly associated with CCD, as few of the dead colonies showed symptoms that define this condition. The higher rates of entombed pollen documented in colonies established on old brood comb suggests that one potential factor may be the accumulation of pesticides (Wallner, 1995; Frazier et al., 2008). Of particular note is the fungicide chlorothalonil, which was ubiquitously detected in entombed pollen samples. This fungicide may be responsible for the diagnostic color change observed in entombed pollen, as it is highly reactive and forms metabolites that may lead to colored products (Chaves et al., 2008).

This is the first study to document "capped" and "entombed" pollen. This study did not examine the possible risk associated with the presence of capped pollen, however did document increased mortality associated with the presence of entombed pollen. Chlorothalonil appears to be linked to the entombed pollen, but it cannot fully explain pollen-capping behavior; a majority of cases of capped (but not entombed) pollen did not exhibit detectable levels of chlorothalonil. Considering the increased risk of colony mortality associated with the presence of entombed pollen, continued research should be conducted to elucidate the role and potential threats of this condition.

## References

- Chauvin, R., Lavie, P., 1956. Recherches sur la substance antibiotique du pollen. *Ann. Inst. Pasteur* 90, 523–527.
- Chaves, A., Shea, D., Danehower, D., 2008. Analysis of chlorothalonil and degradation products in soil and water by GC/MS and LC/MS. *Chemosphere* 71, 629–638.
- Cox-Foster, D.L., Conlan, S., Holmes, E.C., Palacios, G., Evans, J.D., et al., 2007. A metagenomic survey of microbes in honey bee colony collapse disorder. *Science (Washington)* 318, 283–286.
- Evans, J.D., 2006. Beepath: an ordered quantitative-PCR array for exploring honey bee immunity and disease. *J. Invertebr. Pathol.* 93, 135–139.
- Frazier, M., Mullin, C., Frazier, J., Ashcraft, S., 2008. What have pesticides got to do with it? *Am. Bee J.* 148, 521–523.
- Kucharski, R., Maleszka, J., Foret, S., Maleszka, R., 2008. Nutritional control of reproductive status in honeybees via DNA methylation. *Science* 319, 1827–1830.
- Mullin, C.A., Frazier, M., Frazier, J.L., Ashcraft, S., Simonds, R., et al., in preparation. Pesticides and honey bee health: high levels of acaricides and crop protection chemicals in US apiaries.
- National Research Council (NRC), Committee on the Status of Pollinators in North America, 2007. Status of Pollinators in North America. The National Academies Press, Washington, DC.
- van Engelsdorp, D., Hayes Jr., J., Underwood, R.M., Pettis, J., 2008. A survey of Honey Bee Colony Losses in the U.S., Fall 2007 to Spring 2008. *PLoS ONE* 3: e4071.
- van Engelsdorp, D., Underwood, R., Caron, D., Hayes Jr., J., 2007. An estimate of managed colony losses in the winter of 2006–2007: a report commissioned by the Apiary Inspectors of America. *Am. Bee J.* 147, 599–603.
- Wallner, K., 1995. Nebeneffekte bei Bekämpfung der Varroamilbe. Die Rückstandssituation in einigen Bienenprodukten. *Bienenwatter* 116, 172–177.