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Social apoptosis in honey bee superorganisms

Paul Page^{1,*}, Zheguang Lin^{2,*}, Ninat Buawangpong^{3,*}, Huoqing Zheng², Fuliang Hu², Peter Neumann^{3,4,5}, Panuwan Chantawannakul³ & Vincent Dietemann^{1,5}

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Eusocial insect colonies form superorganisms, in which nestmates cooperate and use social immunity to combat parasites. However, social immunity may fail in case of emerging diseases. This is the case for the ectoparasitic mite *Varroa destructor*, which switched hosts from the Eastern honeybee, *Apis cerana*, to the Western honey bee, *Apis mellifera*, and currently is the greatest threat to *A. mellifera* apiculture globally. Here, we show that immature workers of the mite's original host, *A. cerana*, are more susceptible to *V. destructor* infestations than those of its new host, thereby enabling more efficient social immunity and contributing to colony survival. This counterintuitive result shows that susceptible individuals can foster superorganism survival, offering empirical support to theoretical arguments about the adaptive value of worker suicide in social insects. Altruistic suicide of immature bees constitutes a social analogue of apoptosis, as it prevents the spread of infections by sacrificing parts of the whole organism, and unveils a novel form of transgenerational social immunity in honey bees. Taking into account the key role of susceptible immature bees in social immunity will improve breeding efforts to mitigate the unsustainably high colony losses of Western honey bees due to *V. destructor* infestations worldwide.

Honey bee, *Apis* spp., colonies can be regarded as superorganisms, in which cooperating individuals in overlapping generations support functions comparable to those of cells in a multicellular organism¹. Such cooperation can foster the survival of parasite-infested colonies via social immunity^{2,3}, e.g. social analogues of encapsulation⁴ or fever⁵. However, social immunity might be inefficient if parasites shift host species such as in the case of the ectoparasitic mite *Varroa destructor*, which originally infested the Eastern honey bee *A. cerana*, and now infests the Western honey bee, *A. mellifera*, at a global scale⁶. There is general consensus that this ubiquitous mite, together with the viruses it vectors⁶, is the main biotic factor threatening *A. mellifera* colony survival, because infestations usually result in colony death within 2–3 years^{6–9}. This is due to the exponential growth of mite populations sustained by developing worker brood throughout the year and seasonal male brood⁶. In sharp contrast, infested colonies of the original host, *A. cerana*, are surviving. Among several potential resistance traits of *A. cerana*⁶, the apparent absence of mite population growth in association with worker brood is likely to be most significant¹⁰. Other *A. cerana* populations naturally infested with different mite species and haplotypes also appear resistant to the ubiquitous invasive *V. destructor* Korean haplotype infesting sympatric *A. mellifera* in Asia¹¹. Social immunity² is likely to play a major role as resistance mechanism; for instance, adult honey bee workers cooperate to detect and remove worker brood infested by this mite, thereby interrupting its reproduction^{6,12}. The trigger of this hygienic behaviour by workers is based on signals from infested brood¹³ and/or by cues from the parasite¹⁴, on the ability of workers to detect this information as well as on their response thresholds^{11,15,16}. The ability of *A. cerana* workers to perform hygienic behaviour towards *V. destructor*-infested brood is greater than that of the new host, *A. mellifera*¹⁷. However, the mechanisms underlying this major resistance trait of *A. cerana* are unknown. In particular, the crucial role of mite-infested brood in inducing this behaviour has not yet been fully investigated, since all previous studies have been performed in the presence of adult workers, thereby confounding the respective roles of signals/cues, of detection abilities and of response thresholds, which may all contribute to efficient hygienic brood removal.

¹Agroscope, Swiss Bee Research Center, Schwarzenburgstrasse 161, 3003 Bern, Switzerland. ²College of Animal Sciences, Zhejiang University, Yuhangtang Road 866, Hangzhou, China. ³Bee protection laboratory, Department of Biology, Faculty of Science, Chiang Mai University, Chiang Mai, Thailand 50200. ⁴Institute of Bee Health, Vetsuisse Faculty, University of Bern, Bern, Switzerland. ⁵Social Insect Research Group, Department of Zoology and Entomology, University of Pretoria, Private Bag X20, Hatfield, Pretoria 0028, South Africa. ⁶These authors contributed equally to this work. Correspondence and requests for materials should be addressed to H.Z. (email: hqzheng@zju.edu.cn) or P.C. (email: panuwan@gmail.com)

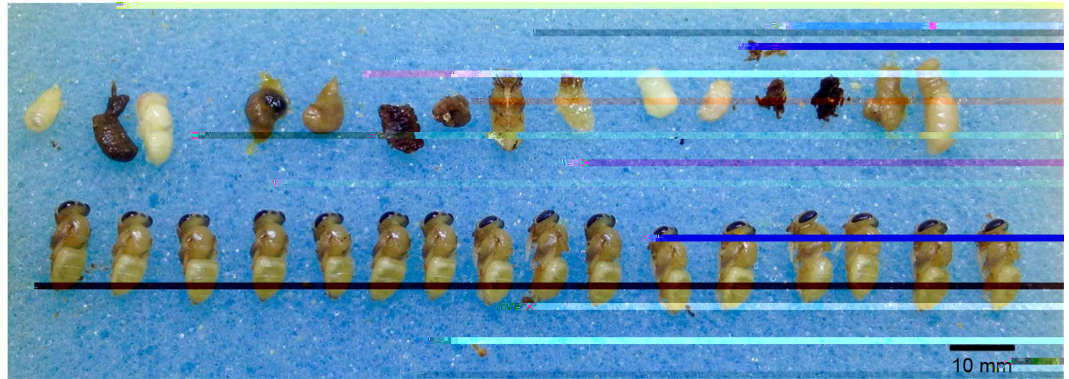


Figure 1. Mite-infested brood (above row) and control brood (bottom row) from an *A. cerana* colony (Phatthalung, Thailand). Individuals were removed from their cells one day before emergence was expected. Most infested individuals stopped development at the larval and prepupal stages and died, Photo by Paul Page.

Here, we took an alternative approach to address the apparent gaps in our understanding of the mechanisms underlying the principal resistance traits of the original host of *V. destructor*, *A. cerana*. The traditional approach to dealing with complexity is to reduce or constrain it. Here, we experimentally reduce the complexity of our system by excluding one level (adult workers), thereby allowing for the first time to distinguish between the effects of brood and adult workers in the hygienic behaviour. By monitoring the development and survival of mite-infested honey bee brood of *A. cerana* and *A. mellifera* colonies in both the absence and presence of adult workers, we separated the respective roles of the developing brood from that of adult nestmates in the resulting hygienic behaviour. We hypothesised that the brood of *A. cerana* is more susceptible compared to *A. mellifera*, thereby providing the basis for a more efficient removal behaviour by adult nestmates. A higher susceptibility of infested or wounded worker brood associated with efficient hygienic behaviour in adult workers is expected to reinforce the social immunity of a honey bee colony infested by brood parasites or pathogens and may contribute to colony survival.

Results and Discussion

We infested 314 worker larvae from 6 colonies in one population of *A. mellifera* and from 4–5 colonies in each of three distant populations of *A. cerana*. For infestations, we used the invasive Korean haplotype of *V. destructor*¹⁸. Wax combs containing infested and non-infested control brood cells were placed in an incubator mimicking hive conditions for optimal brood development¹⁹. The cells were opened one day prior to the expected emergence date of the adult bees and the developmental stages of the individuals were recorded²⁰.

The data shows a striking difference in the effect of parasitism between *A. cerana* and *A. mellifera*. The development of *A. mellifera* worker brood infested by *V. destructor* was similar to the non-infested controls. In contrast, in the three *A. cerana* populations, we found a higher frequency of individuals at early developmental stages in infested worker brood than in control brood (Fig. 1; Supplementary Fig. S1). Overall, the development of infested individuals was significantly delayed in *A. cerana* compared to *A. mellifera* (one-way ANOVA on log-transformed values with a Dunnett's *post hoc* test using *A. mellifera* as control group, $df = 3$, $F = 7.2$, $p = 0.003$, Fig. 2). Whenever we observed larvae or pre-pupae (earliest developmental stages) one day prior to expected emergence, most of them were decomposed and were obviously dead (Fig. 1).

In the presence of workers, and even when assuming an equal ability of both honey bee species to detect signals triggering hygienic behaviour as well as equal response thresholds towards them, this observed difference in brood susceptibility is likely to elicit an earlier and more frequent production of such signals, ultimately leading to more efficient removal behaviour and overall enhanced social immunity. In order to test this hypothesis, we subjected larvae of both species to a benign wounding and either placed them in an incubator to monitor their development or back into their colony of origin. *A. cerana* brood died more quickly and in greater proportions than *A. mellifera* in incubator conditions (Log-rank Mantel test, $df = 1$, $\text{Chi}^2 = 12.8$, $p < 0.001$, Fig. 3a; Supplementary Fig. S2) and in colonies, *A. cerana* workers removed the wounded brood significantly more and faster than *A. mellifera* (Log-rank Mantel test, $df = 1$, $\text{Chi}^2 = 67.8$, $p < 0.001$, Fig. 3b). These results indicate that *A. cerana* brood was affected faster and to a higher degree by wounding than *A. mellifera* and that wounded brood could be detected by workers for a more efficient hygienic removal. The higher proportion of brood removed by the workers in the colonies compared to our estimate of brood death in the incubator (Fig. 3) is likely due to the sensitivity of workers to dysfunctional individuals that could have survived in incubator conditions. Nonetheless, a benign wounding of *A. cerana* larvae appears to be sufficient to yield a reaction analogous to apoptosis in multicellular organisms. Given the use of a sterile pin for the pricking, these results also allow the exclusion of secondary pathogens commonly carried by *V. destructor*⁶ in the mortality observed.

Hence, the significantly higher susceptibility of mite-infested brood of the original host of *V. destructor* leads to more efficient hygienic behaviour, thereby providing a basis for honey bee colony survival to parasitism and constituting an additional resistance trait of the original host of this parasite. Our results provide a most parsimonious explanation for the striking differences in the impact of infestations by the invasive Korean haplotype

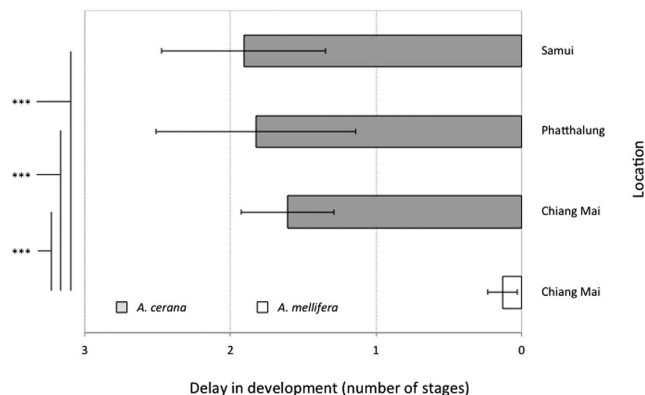


Figure 2. Delay in brood development calculated as the difference in number of developmental stages (x axis) separating infested brood from non-infested (control) brood in three populations of *A. cerana* and one of *A. mellifera* (y axis). The average developmental delays within were compared by using a one-way ANOVA on log-transformed values combined with a Dunnett's *post hoc* test using *A. mellifera* as control group. Values represented are means \pm 1 S.E.M. *** $P < 0.001$.

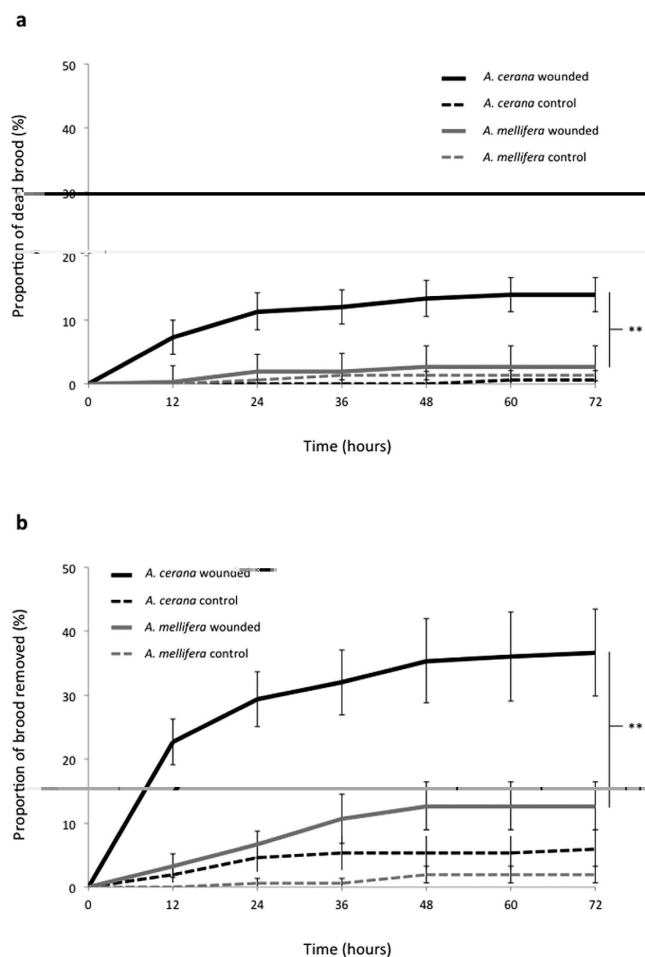


Figure 3. (a) Mortality of wounded brood in *A. cerana* and *A. mellifera* developing in an incubator. The survival status of larvae pricked with a sterile glass-pulled needle and of control larvae was monitored every 12 hours during three days; (b) Removal of wounded brood in *A. cerana* and *A. mellifera* colonies. Larvae pricked with a sterile glass-pulled needle and controls were exposed to workers and their removal via hygienic behaviour was monitored every 12 hours during three days. Brood mortality and removal rates were compared with log-rank Mantel-tests. Values are means \pm 1 S.E.M. ** $P < 0.01$.

155.-9.4(d)25.5(e)71.4(s)075(tr)-8(s0 -0-864(c)473.5(t)7r.5(n)0.8(to 1 W05(8(b)3100)9.15tt-7d9(r5(t9))40/11bT1_0d)2(4inf)0)6151a315nt4_d1f.a

We ranked each individual from 1 (larva) to 6 (mature), depending on its developmental stage at cell opening. Within each replicate, we calculated an average developmental stage for infested and control cells separately and subtracted the former from the latter, in order to obtain a mean developmental delay per colony. All residuals were normally distributed within populations and the variance was homogeneous between populations. We then compared the average developmental delays within each species and each location by using a one-way ANOVA on log-transformed values combined with a Dunnett's *post hoc* test and *A. mellifera* as the control group. Statistical calculations were performed using Systat software (version 13).

Further analyses were performed with generalized linear models (GLMs) applied to the same developmental stage dataset with colony as random factor and using the *lmer* function from the package *lme4*³⁶. Response variables were the frequencies of control and infested individuals at each stage in each colony. They were modelled with Poisson error distributions. Statistical tests were performed in R software (R version 3.2.2)³⁷ and are summarized in the Supplementary Table S1.

Larvae wounding experiments. *Honey bee colonies and brood comb preparations.* We used 5 colonies of *Apis mellifera* and 5 of *Apis cerana* kept in Langstroth hives in an apiary at Zhejiang University, Hangzhou (China) for the larval wounding experiments. On the combs of both species, we identified cells by mapping brood at the L5 stage, as described above. We then replaced the comb into the colony for capping by the workers to occur and identified the capped cells 6 hours after the mapping. We used a fine pulled-glass capillary with a diameter similar to that of the *V. destructor* mite chelicerae ($\varnothing = 50 \mu\text{m}$) to wound the larvae by pricking, simulating the wound induced by *V. destructor* mites feeding on the bee larvae^{38,39}. We inflicted wounding by pressing the tip of the capillary against the cuticle until it gave way. We sterilized the needle with ethanol before each use and replaced it anew for each colony tested. Two experiments were conducted in each species in order to quantify the susceptibility of larvae towards wounding, firstly in the absence of workers and secondly in their presence to compare the removal of wounded brood by workers, i.e. hygienic behaviour.

(1) To measure the susceptibility of wounded larvae, we pricked 30 freshly capped larvae and instead of resealing the wax capping, we removed it and closed the cell with a transparent gelatin cap, allowing for the observation of brood development⁴⁰. As a control, we opened 15 freshly capped cells and sealed them with a gelatin cap without pricking the larvae (controls for the effect of pricking) and we left 15 cells non-manipulated (controls for the effect of gelatin cap on larva survival). We then placed the comb into an incubator at developing brood conditions, i.e. 34.5 °C and 70% RH, and checked the experimental cells every 12 hours during the next three days. At every check, we reported the survival status of the brood in the cells. We considered a larva dead when it turned black and deflated or when it did not stretch in the cell as is typical for the transition to pre-pupal stage. We opened the non-manipulated cells after 72 hours to identify the final state of larvae/pre-pupae.

(2) To measure the hygienic removal of wounded larvae, we opened 30 freshly capped cells and gently pricked the larvae inside of them. As a control, we opened 15 cells without pricking the larvae (positive controls). Wax caps of cells with pricked and non-pricked larvae were then resealed. As a control for the effect of cell opening on removal by workers, we mapped an additional 15 cells that we did not manipulate (negative controls). The comb was then replaced into its original colony and the experimental cells were checked every 12 hours during the next 3 days. At every comb check, we reported the number of mapped brood cells that had been cleaned out by the workers of each species during an interval, i.e. brood removal.

Data and statistical analyses. (1) To assess susceptibility to wounding, we counted the dead and surviving larvae in each species and at each time interval. (2) To compare hygienic removal of wounded brood, we counted the number of wounded brood removed by the workers of each species at each time interval. As the respective mortality and removal rates of the different controls were not significantly different for either experiment (log-rank Mantel test; susceptibility: *A. mellifera*, $df = 1$, $\text{Chi}^2 = 0.34$, $p = 0.56$; *A. cerana*, $df = 1$, $\text{Chi}^2 = 0.0$, $p = 0.99$; hygienic removal: *A. mellifera*, $df = 1$, $\text{Chi}^2 = 2.0$, $p = 0.16$; *A. cerana*, $df = 1$, $\text{Chi}^2 = 1.0$, $p = 0.32$), these data were pooled. The validity of our assays was confirmed by the control mortality being below a 15% threshold⁴¹. We then compared the mortality rates and wounded brood removal rates between bee species by means of log-rank Mantel tests.

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Author Contributions

V.D., P.N., H.Z. and P.P. conceived and designed the study; V.D., H.Z. and P.P. conducted the research with input from P.N., F.H. and P.C.; V.D., P.P., N.B., H.Z. and Z.L. performed the experiments; V.D., P.P. and L.Z. analysed the data; V.D., P.N. and P.P. wrote the manuscript with contributions from all authors.

Additional Information

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