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Social reversal of immunosenescence in honey bee workers

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Abstract

A striking example of immunosenescence is seen in the honey bee (*Apis mellifera*) worker caste. The bees' age-associated transition from hive duties to more risky foraging activities is linked to a dramatic decline in immunity. Explicitly, it has been shown that an increase in the juvenile hormone (JH) level, which accompanies onset of foraging behavior, induces extensive hemocyte death through nuclear pycnosis. Here, we demonstrate that foragers that are forced to revert to hive-tasks show reversal of immunosenescence, i.e. a recovery of immunity with age. This recovery, which is triggered by a social manipulation, is accompanied by a drop in the endogenous JH titer and an increase in the hemolymph vitellogenin level. Vitellogenin is a zinc binding glycolipoprotein that has been implicated in the regulation of honey bee immune integrity. We also establish that worker immunosenescence is mediated by apoptosis, corroborating that reversal of immunosenescence emerges through proliferation of new cells. The results presented here, consequently, reveal a unique flexibility in honey bee immunity—a regulatory plasticity that may be of general biological interest.

Keywords

Immunosenescence; Behavioral reversion; Juvenile hormone; Vitellogenin; Apoptosis; Honey bee worker

1. Introduction

Immunosenescence is a systemic reduction of immune efficiency as an organism ages. The adaptive immune response of vertebrates deteriorates, and the immune system of invertebrates shows a comparable decline in function (reviewed by Franceschi et al., 2000; Malaguarnera et al., 2001; Aspinall et al., 2003). Invertebrate immunity is exclusively innate and has been of limited interest to comparative research on immunosenescence. Yet, the finding that innate immune factors play fundamental roles in adaptive immunity has motivated molecular studies in the nematode *Caenorhabditis elegans* and the fruit fly *Drosophila melanogaster* (reviewed by Hoffmann, 2003; Kurz and Ewbank, 2003; Nicholas and Hodgkin, 2004; Pasare and Medzhitov, 2004). The insight that life-history theory has implications for our evolutionary understanding of patterns of immunity in social species (Helle et al., 2004) has further been addressed in the bumble bee *Bombus terrestris* (Doums et al., 2002) and the honey bee *Apis*

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mellifera (Wille and Rutz, 1975; Amdam et al., 2004b). The honey bee, in particular, reveals features that may be of interest to comparative research on aging and immunity.

Worker honey bees are functionally sterile females. They show a distinct pattern of age-related division of labor, in which young workers perform nurse tasks inside the colony, and relatively older workers engage in more risky exterior hive activities such as guarding and foraging. The transition from nurse tasks to foraging activity normally occurs after 18–28 days of adult life (Winston, 1987), and is associated with a marked decline in immunity: an increase in the systemic juvenile hormone (JH) titer induces hemocyte death through nuclear pyknosis (Rutz et al., 1974; Wille and Rutz, 1975; Amdam et al., 2004b). This action cascade results in a dramatic loss of hemocytes, which have several important immunological functions in phagocytosis, encapsulation, nodulation, and wound repair (reviewed by Millar and Ratcliffe, 1994). The immune cells also produce antibacterial peptides, and contain most of the recruitable prophenoloxidase enzyme that catalyses crucial steps in the melanization immune response (Soderhall and Cerenius, 1998). The number of functional circulating hemocytes at any given time, therefore, reflects the organism's capacity to cope with immunogenic challenges (Kraaijeveld et al., 2001; Doums et al., 2002). Consequently, the loss of immune cells in foragers constitutes a dramatic decline in immune efficiency that includes a complete deterioration of the nodulation response (Bedick et al., 2001)—a predominant defense reaction to infection in insects (Franssens et al., 2005).

Honey bee immunosenescence, however, is not a simple function of chronological age because immunity is linked to the workers' behavioral role, which in turn can be affected by the social environment (Miojevic, 1940; Huang and Robinson, 1992, 1996; Pankiw et al., 1998a,b). For example, when ambient conditions become unfavorable, brood rearing ends and recruitment to the foraging population ceases (Huang and Robinson, 1995, 1996). Instead of becoming foragers, young workers enter the '*diutinus*' stage (formerly referred to as the winter bee phenotype, e.g. Maurizio, 1950; Mattila et al., 2001; Omholt and Amdam, 2004). *Diutinus* workers are tolerant to oxidative stress and are long-lived (reviewed by Amdam and Page, 2005). They are characterized by high counts of functional hemocytes (Fluri et al., 1977) and extensive pyknosis is only detected in the subsequent favorable season (Wille and Rutz, 1975), as the bees initiate foraging at an age that may exceed 280 days (Sekiguchi and Sakagami, 1966).

The above findings suggest that immune efficiency is under tight regulatory control in the honey bee due to the systemic effect of JH on immunity. Explicit modulation of individual immunity, however, may show considerable flexibility relative to chronological age because the physiological state of the bee, including its JH titer, appears to be largely controlled by a dynamic intra-colonial milieu driven by cyclic ambient conditions (reviewed by Amdam et al., 2004b). These propositions give rise to a prediction derived from the remarkable plasticity of honey bee workers. Workers can revert from foraging to nurse tasks (Robinson et al., 1992; Huang and Robinson, 1996)—a behavioral shift accompanied by a physiological reversion, which is characterized by a drop in the systemic JH titer and functional redevelopment of the brood food producing hypopharyngeal glands that normally atrophy in foragers (Miojevic, 1940). The dynamic properties of this change could imply that the loss of immunity triggered by JH at onset of foraging is reversible as well.

Here, we establish that reverted foragers show reversal of immunosenescence. The condition is not only accompanied by the known drop in JH titer (Robinson et al., 1992; Huang and Robinson, 1996), but is also associated with an increase in the hemolymph level of vitellogenin—a zinc binding glycolipoprotein previously implicated in the social modulation of honey bee immunity (Amdam et al., 2004b). Our data reveal that the dramatic loss of hemocytes at

transition to foraging is mediated through programmed cell-death, and disclose, for the first time, the full regulatory plasticity of honey bee immunosenescence.

2. Methods

2.1. Bees

The experiment was performed at the University of California, Davis, in June and July 2004. Adult bees emerging from brood cells were obtained as a mix from three wild type colonies of commercial origin, and marked on the thorax with a spot of paint (Testors™ Enamel). One thousand marked bees were introduced into three separate 'host' colonies, which all had a queen and a population of approximately 6000 unmarked workers of diverse ages. Each host colony occupied a hive box that had an additional empty hive unit on top (Fig. 1(A)). Entrance counts of marked bees returning from foraging trips (recorded throughout two 10 min intervals during the peak foraging period: 10.00 am–12.30 pm) were logged until day 25, when the counts leveled off or started to decline for all host colonies.

2.2. Reversion manipulation

A hive manipulation was made to each of the three host colonies to separate marked bees that were foraging from marked bees that still were engaging in hive activities such as nursing. Several meters from the original hive site, the worker bees and the wax combs (frames) of the host colony were transferred into a new hive box (Hive 1) with 3 mm wire mesh screen attached to the bottom (Fig. 1(B)). The host queen was caught and placed into a different hive box (Hive 2) that was carried back to the original hive site and mounted with its entrance pointing in the same direction as the former entryway of the host colony (Fig. 1). No workers, but two frames with larvae and two frames with food (honey and pollen) were placed into Hive 2 with the queen. Hive 1 with the attached screen then was returned to the hive site, positioned on top of Hive 2, and provided with a new entrance 180° from the direction of Hive 2's entryway (Fig. 1(B)). The wire mesh screen enabled continuing contact between the lower box with the queen and the upper unit with workers. Because Hive 1 replaced the empty hive box on top of the host colony, which had been replaced by Hive 2, the visual appearance of the hive site also remained unchanged. The manipulation was made day 26, at 10.00 am, a time of active foraging.

The resulting composite hive (Fig. 1(B)) remained on site throughout the peak foraging hours. During this time, experienced foragers that exited Hive 1 to collect food used their pre-acquired memory of the hive site (reviewed by Hammer and Menzel, 1995) upon returning from the field. Thus, they approached the location via flight-paths that originally lead to the entrance of the host colony. This behavior directed them into Hive 2. Inexperienced bees (i.e. without a visual map of the hive site) that exited Hive 1 to engage in short defecation- or orientation flights, however, primarily returned to Hive 1. At 1.30 pm, the entrance of Hive 1 was closed, and the unit was taken to a location several kilometers away. The remaining Hive 2, consequently, had a queen, larvae, food, and a worker population that, with the possible exception of a small number of randomly drifting bees, consisted of experienced foragers. In this social context, foragers will revert to nursing activities (Robinson et al., 1992; Huang and Robinson, 1996).

The three replicates of the Hive 2 configuration stayed at the original hive site for the remaining experimental period.

2.3. Sample collection

Marked bees were sampled from the Hive 1 and 2 configurations immediately after the reversion manipulations, and subsequently after 4 and 8 days from the Hive 2 configurations.

Foragers were collected when returning from flights during the peak foraging hours, and reverted foragers presumably engaging in nurse tasks were obtained by collecting bees with their heads and thoraxes in cells that contained larvae. This behavior is indicative of nursing activity (Huang and Robinson, 1996). To exclude the possibility that data from the Hive 2 configurations were confounded by any minor presence of randomly drifting bees (see Section 2.2), workers were examined after collection. Only individuals with worn wings (a specific marker for considerable flight activity in studies of bees, e.g. Huang and Robinson, 1996) were included in the study.

Workers were anaesthetized on ice before hemolymph was extracted with Drummond micropipettes ($1 \pm 0.001 \mu\text{l}$). Care was taken to avoid contaminating the samples with tissue fragments and foregut content. Hemolymph ($0.5\text{--}2 \mu\text{l}$) was drawn within 1 h after the bees had been collected (Lin et al., 2004) and stored in $500 \mu\text{l}$ ice cold acetonitrile for JH titer determination, and $1 \mu\text{l}$ hemolymph was dissolved in $50 \mu\text{l}$ TRIS-buffer (20 mM TRIS-HCL, 150 mM NaCl, 5 mM EDTA, pH 7.5, 1 mM phenylmethylsulfonyl fluoride, 5 mM benzamidin, $0.7 \mu\text{M}$ pepstatin, $8 \mu\text{M}$ chymostatin, $10 \mu\text{M}$ leupeptin and $0.8 \mu\text{M}$ aprotinin) for quantification of vitellogenin (Amdam et al., 2004b). Further, $1 \mu\text{l}$ hemolymph was dissolved in $3 \mu\text{l}$ staining solution (16.2 g NaH_2PO_4 , 4.2 g NaOH, 1 g methylene blue in 0.1 l formalin (40%) and 0.9 l $\text{d-H}_2\text{O}$), or processed by the DeadEnd™ Fluorometric TUNEL System (Promega), for classification and count of hemocytes. Finally, the hypopharyngeal glands (paired acinous head glands) were dissected out and transferred to a microscope slide in $2 \mu\text{l}$ Grace insect medium.

2.4. Quantification of physiological characteristics

The size of the hypopharyngeal gland acini (saclike dilations composing the compound hypopharyngeal gland) was determined as the mean diameter in μm of 10 acini under a stereomicroscope with an ocular scale (Crailsheim and Stolberg, 1989).

The JH titer was quantified by a specific radioimmunoassay. Briefly, JH was extracted from hemolymph following the protocol established for honey bee hemolymph (Huang et al., 1994) and toluene was evaporated by vacuum centrifugation before adding the RIA components. The antiserum used in this study was diluted to 1:1250 in phosphate buffer (0.1 M, pH 7.4) supplemented with 0.1% bovine serum albumin fraction V (Sigma-Aldrich), 0.1% rabbit immunoglobulin G (Sigma-Aldrich) and 0.02% sodium azide (Goodman et al., 1995). The assays were performed with tritiated JH, [$10\text{-}^3\text{H(N)}$]-juvenile hormone III (specific activity 19.4 Ci/mmol, NEN Life Science Products), diluted in the phosphate buffer to 6000–6500 cpm per $50 \mu\text{l}$. Juvenile hormone III (Fluka) was used as non-radioactive ligand at a standard solution of $100 \text{ pg}/\mu\text{l}$ in methanol. Standard curves were set up to cover a 50 pg – 10 ng range. JH titers of unknown samples were calculated by log linear regression analysis of standard curve doses (log pg JH-III) on logit binding values.

The vitellogenin titer was determined by vertical SDS-PAGE as described by Lin et al. (1999) using a β -galactosidase (Sigma-Aldrich) standard (Amdam et al., 2004a).

Samples of stained hemocytes were loaded in a cell counter (Bürker, 0.05 mm), and the number of hemocytes in $1 \mu\text{l}$ hemolymph was determined at $400\times$ by light microscopy. Melanized cell aggregates (Bedick et al., 2001) indicative of nodulation (i.e. formation of structures with a central core of entrapped particles, distinct from encapsulation by the absence of zones of flattened and degenerating cells, Millar and Ratcliffe, 1994)—and the presence of putative pathogens (fungi and bacteria) at high concentrations (more than 1000 microbes per μl hemolymph) was recorded. Non-aggregating hemocytes were classified as normal or with pycnotic cell nucleus as described by Rutz et al. (1974), Wille and Rutz (1975), and Price and Ratcliffe (1974).

The hemolymph samples processed by the DeadEnd™ Fluorometric TUNEL System (Promega) were tested for presence/absence of apoptotic hemocytes through the TUNEL staining procedure as recommended by the manufacturer. Positive and negative controls were established and screened as described in the DeadEnd™ Fluorometric TUNEL System protocol (Promega).

2.5. Data analysis

The effects of behavioral phenotype (nursing activity vs. foraging), age (or time after reversion manipulation), and host colony on hypopharyngeal gland diameter, the number of functional hemocytes, and the proportion of nonfunctional immune cells in workers, were determined by MANOVA due to large sample sizes and data that approached normal distributions. Fisher Post-hoc tests were used to establish between-level effects. The data on JH titers and vitellogenin levels did not meet the normality assumption, and were analyzed by Kruskal–Wallis' test. Here, Mann–Whitney *U*-tests were used to identify Post-hoc effects. Host colony was not included as categorical factor for the analyses of the JH- and vitellogenin level. Rather, due to the laborious nature of the titer quantifications, a smaller number of samples were quantified from each colony and the statistical analysis performed on the joint dataset. The effects of behavioral phenotype and age on the occurrence of putative microbial infection and hemocyte nodulation were analyzed by Chi-square tests. All analyses were performed with Statistica 6.0.

3. Results

3.1. Hypopharyngeal gland size

We found a significant effect of behavioral phenotype (MANOVA: $F_{2,344}=360.69$, $P<0.00001$) and age (MANOVA: $F_{2,344}=6.91$, $P<0.005$) on hypopharyngeal gland size as previously established (Huang and Robinson, 1996). Post-hoc analyses revealed that the glands of reverted foragers, independent of age, were of the same size as the glands of the nurse bees that had been removed from the colonies ($P=0.57$, see Fig. 2(A) for information on *n*). The hypopharyngeal glands of the foragers were significantly smaller ($P<0.00001$), but showed a temporal increase in size 4 days after the reversion manipulation ($P<0.00001$). We found no effect of host colony on gland size (MANOVA: $F_{2,344}=2.00$, $P=0.14$), and the data from the three replicates of the setup are thus shown collectively in Fig. 2(A). The presence of functional reverted bees that had engaged in nurse activities during the experimental period was also corroborated by a high number of viable young larvae and pupae in the colonies 8 days after the manipulation (A.L.T.O. Aase, personal observation).

3.2. JH and vitellogenin dynamics

Next, we examined effects on the endogenous JH level and the intrinsic vitellogenin titer. We found a significant effect of behavioral phenotype (Kruskal–Wallis: $H_{2,62}=25.88$, $P<0.00001$) and age (Kruskal–Wallis: $H_{2,62}=10.14$, $P<0.04$) on the JH titer (Fig. 2(B)). Specifically, the JH level of reverted bees was equal to that of nurse bees independent of age (Mann–Whitney *U*-test: $P=0.09$, $df=31$, see Fig. 2(B) for details on *n*), and significantly lower than the JH titer of foragers (Mann–Whitney *U*-test: $P<0.00001$, $df=52$). Four days after the reversion manipulation, moreover, we detected a significant reduction of the JH titer in bees that remained foragers (Mann–Whitney *U*-test: $P<0.02$, $df=31$). This reduction is in accordance with a previous finding (Huang and Robinson, 1996). In addition, however, our data demonstrated that the drop in workers that continue to forage is transient, increasing to the previous, and typical forager level 8 days after the reversion manipulation (Fig. 2(B)).

For the vitellogenin titer, we found a significant effect of behavioral phenotype (Kruskal–Wallis: $H_{2,53}=10.58$, $P<0.005$) but no effect of age (Kruskal–Wallis: $H_{2,53}=5.51$, $P=0.06$). The

hemolymph vitellogenin level increased significantly in reverted bees ($n=9$ and $n=10$, at 4 and 8 days after the hive manipulation, respectively) relative to the workers that continued to forage (Mann–Whitney U -test: $P<0.01$, $df=34$). The vitellogenin concentration of the reverted bees (mean $\pm$ standard error: $6.04 \pm 1.52 \mu\text{g}/\mu\text{l}$), furthermore, was not statistically different from that of the nurse bees ($n=9$) that represent a base line sample (i.e. $8.24 \pm 2.76 \mu\text{g}/\mu\text{l}$, Mann–Whitney U -test: $P=0.28$, $df=28$). Compared to the titer of the foragers ($n=9$) collected at the day of the reversion manipulation (3.66 ± 0.92), the vitellogenin level of the bees that continued as foragers ($n=9$ and $n=6$, after 4 and 8 days, respectively) remained unchanged ($1.36 \pm 0.09 \mu\text{g}/\mu\text{l}$, Mann–Whitney U -test: $P=0.26$, $df=24$).

3.3. Immunity

We established that behavioral phenotype (MANOVA: $F_{2,330}=59.12$, $P<0.00001$) and age (MANOVA: $F_{2,330}=11.67$, $P<0.00001$) significantly affected the number of functional hemocytes in the honey bees (see Fig. 3(A) for information on n). We found that both reverted bees and workers that remained foragers experienced an increase in the number of functional hemocytes 4 days after the reversion manipulation ($P<0.00001$). However, in the bees that remained foragers the positive effect was transient, and after 8 days the number of functional hemocytes showed a significant decline ($P<0.005$), thus representing a mirror image of the JH titer profile. In the reverted bees, on the other hand, the elevated level remained unchanged with age ($P=0.17$). The reverted bees, accordingly, had significantly higher levels of functional hemocytes 8 days after the reversion manipulation ($P<0.01$). The effect of host colony was not significant ($P=0.58$, data shown collectively in Fig. 3(A)).

The proportion of non-functional immune cells (cells with pycnotic nuclei) is another established indicator of honey bee immune integrity (Fluri et al., 1977). Again, we found significant effects of behavioral phenotype and age (MANOVA: $F_{2,329}=109.33$, $P<0.00001$, and $F_{2,329}=92.50$, $P<0.00001$, respectively. See Fig. 3(B) for details on n). Nurse bees and reverted foragers had equal proportions of non-functional cells independent of age ($P=0.67$), and this level was significantly lower than that of foragers ($P<0.00001$) (Fig. 3(B)). Immunohistochemical TUNEL staining further demonstrated that the cells with pycnotic nuclei were apoptotic (Fig. 4), confirming that the dynamics of honey bee immunity is part of a tightly controlled regulatory program—as hypothesized by Amdam et al. (2004b).

Data on the presence of microbes, and nodules consisting of melanized hemocytes (Table 1), suggested that infections occur more frequently in foragers than in nurse bees (Chi-square test: $P<0.00001$). Note, though, that inference from microbial counts is limited—as it is unclear which microbes are legitimate infectious targets of the immune defence (pathogens) and which are not (symbionts, commensals). Nodulation was significantly more frequent in foragers 4 days after the reversion manipulation (Chi-square test: $P<0.005$). This finding indicates that the nodulation immune response, which deteriorates in foraging bees (Bedick et al., 2001), shows transient improvement in association with the corresponding increase in circulated hemocytes that we detected in bees that remained foragers under the reversion regimen (Fig. 3).

4. Discussion

Enlarged hypopharyngeal glands typically synthesize and store brood food (Crailsheim and Stolberg, 1989). Our initial analysis of gland diameter, therefore, verified that our experimental procedure resulted in a differentiation of experienced marked foragers into a population of reverted workers that nursed the brood, and a population of foragers that continued to collect pollen and nectar after the reversion manipulation. This result corresponds to a finding by Huang and Robinson (1996), which suggested that hypopharyngeal glands hypertrophy to nurse bee size after a reversion, while remaining unchanged in bees that continued to forage.

The fact that we, in addition to this result, detected a temporal change in forager gland diameter, may be due to our larger sample size, sampling procedure (we used the mean size of 10 acini, whereas Huang and Robinson obtained data from only one acinus per bee), or the presence of brood in our experimental hives (Huang and Robinson uncoupled the effect of brood per se, as brood pheromones affect worker behaviors, e.g. Pankiw et al., 1998b).

Consequently, we have demonstrated that honey bee workers that are forced to revert from foraging to nurse tasks show reversal of immunosenescence, i.e. a recovery of immunity with age. We also found a transient improvement of immune efficiency in bees that remain foragers under the reversion regimen, as confirmed by a temporary increase in the number of circulating hemocytes (Fig. 3(A)), and an apparent but transient restoration of the nodulation immune response (Table 1). Because apoptosis, as detected by us (Fig. 4), is irreversible (reviewed by Kroemer et al., 1995), the restoration of immunity in reverted bees must be attributable to a rapid recruitment of new hemocytes. Reversal of immunosenescence in honey bee workers, therefore, is likely to involve proliferation in the hematopoietic tissues of the bees, possibly accompanied by cell divisions in the circulating hemocyte population (Millar and Ratcliffe, 1994) or mobilization of cells from storage tissue such as the fat body (Luckhart et al., 1992).

The transient improvement of immunity in foragers, as well as the robust reversal of immunosenescence in the reverted bees, was associated with a drop in the endogenous JH level (Fig. 2(B) vs. Fig. 3(A)). This relationship is in agreement with the previous finding that experimental JH application induces hemocyte pycnosis in the honey bee (Rutz et al., 1974; Wille and Rutz, 1975), and the hypothesized negative association between JH and systemic immunity in insects (Rolff and Siva-Jothy, 2002). Our data also indicate that a robust reversal of immunosenescence is associated with an increase in the hemolymph vitellogenin titer. Vitellogenin is the major circulating zinc binding protein of adult bees, and zinc has a positive effect on worker hemocyte viability in vitro (Amdam et al., 2004b). A rise in the vitellogenin level from the low titers normally encountered in foragers (Engels and Fahrenhorst, 1974), therefore, may imply that the hemolymph zinc concentration increases to a level that has a positive effect on hemocyte number (Amdam et al., 2004b). This action cascade is interlinked with the dynamics of the systemic JH titer, because the high JH level of foragers suppresses vitellogenin synthesis (Pinto et al., 2000) and, thereby, the hemolymph zinc concentration (Amdam et al., 2004b). Our finding that the JH- and vitellogenin levels of reverted workers are equal to those of younger nurse bees is, therefore, in accordance with a model where the synthesis of vitellogenin, and consequently immunity (Amdam et al., 2004b), can be restored by suppression of the intrinsic JH level (as hypothesized by Omholt and Amdam, 2004).

Yet, the transient improvement of immunity in foragers 4 days after the reversion manipulation was not associated with an increase in the hemolymph vitellogenin level. This implies that the hypothesized causal chain JH→vitellogenin→zinc→immune cell integrity, as proposed by Amdam et al. (2004b), is not absolute, and that JH or other factors may affect hemocyte proliferation and death independent of the hemolymph zinc concentration. Another important finding is that the gland size (Fig. 2(A)), the JH titer (Fig. 2(B)) and the proportion of pycnotic cells (Fig. 3(B)) in the reverted bees were indistinguishable from that of nurse bees after as little as 4 days. The number of functional hemocytes in reverted workers, however, had not yet recovered at 8 days after the reversion manipulation (Fig. 3(A)). The result may point to possible constraints on proliferation in the bees' hematopoietic tissues and the rate of division of circulating cells, but can also imply that the number of hemocytes to be mobilized from storage tissues (Luckhart et al., 1992) after the reversion from foraging is limited. The data presented here cannot identify which of these mechanisms are operational. Further studies, therefore, are needed to fully determine how diverse physiological factors contribute to the observed pattern of reversal of immunosenescence in the bee.

Our finding that the dramatic loss of immunity through hemocyte pycnosis is mediated through programmed cell-death provides, for the first time, conclusive evidence of the cellular mechanisms that underlie the loss of immune cells in foraging honey bees (first explored by Wille and Rutz, 1975). The fact that foraging behavior is associated with immunosenescence through hemocyte apoptosis, whereas reversion from foraging to nurse tasks is interlinked with counteracting mechanisms, suggests that the bees embody an explicit potential for discoveries in the interface between immunosenescence and the complex roles of programmed cell-death. Explicitly, the transition from nurse tasks to foraging and the reciprocal reversion to nurse tasks are controlled by the social milieu of the hive (Huang and Robinson, 1996). In essence, this implies that the pathway that triggers hemocyte apoptosis in the honey bee is influenced by social signals. To understand, in full, the machinery that allows the bees' social communication (e.g. via pheromones, Leoncini et al., 2004; Pankiw, 2004) to translate into an apoptotic response may thus be of general biological interest.

An important question in the study of programmed cell-death, furthermore, is why cells die at all in some situations (Golstein et al., 2003). In this connection, it is reasonable to ask why foragers that encounter numerous pathogens on their foraging trips experience a substantial loss of immunity through apoptosis. This question was partially addressed by Amdam et al. (2004b), who interpreted the unique trajectory of immunity in worker bees to reflect a colony-level selection pressure for optimal resource allocation. Insect immunity is costly (Moret and Schmid-Hempel, 2000; Rolff and Siva-Jothy, 2002), and because most bees perish shortly after their transition to the risky foraging stage, selection would not favor investments in forager immune efficiency. Nurse bees, on the other hand, experience low extrinsic mortality risk independent of age, and constitute a key colony-level resource as caregivers for the young brood and the queen (reviewed by Amdam and Page, 2005). It has been suggested that apoptosis has been selected for, in part, because of its superior economic efficiency in situations where the purpose of cell-death is to re-allocate nutrients and energy from less essential cells to tissues with high metabolic demands (Golstein et al., 2003). Relative to nurse bees, foragers have high energetic requirements (Neukirch, 1982; Suarez et al., 1996), and the energy balance of apoptosis in the honey bee may, therefore, provide a new model for dissecting the relationship between programmed cell-death and nutrient re-allocation.

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Fig. 1. Outline of the reversion manipulation. (A) Each original host colony, with a composite population of nurse bees and foragers including our focal 26 day-old marked workers, was split: (B) into a unit predominantly consisting of nurse bees, and a unit predominantly consisting of experienced foragers. Honey bee foragers go through a learning process to locate the hive entrance by visual cues and a sun compass (reviewed by Hammer and Menzel, 1995). Our hive manipulation, therefore, exploited the experienced foragers' pre-acquired memory of the hive site, so that they were directed towards the front entrance of Hive 2 after leaving the rear entryway of Hive 1 in search of nectar and pollen.

Fig. 2. Means and standard deviations for (A) the hypopharyngeal gland diameter (μm) of worker bees obtained from three replicate setups (per replicate, $n=18-20$ for each experimental group), and (B) the juvenile hormone titer ($\text{pg}/\mu\text{l}$) of workers from the same setups (per replicate, $n=3-5$ for each experimental group). Data were collected from 26 day-old nurse bees (from the Hive 1 configurations, see Fig. 1) and 26 day-old foragers (from the Hive 2 configurations) at the day of the reversion procedure (base line). Four and 8 days later, data were collected from the Hive 2 configurations when the workers were 30 and 34 days old, respectively. Note that the bees denoted as nurse bees were removed by the reversion manipulation at base line. Thus, the workers designated as reverted bees at day 4 and 8 were experienced foragers that

reverted to nurse activities. Small letters refer to groups that were significantly different as determined by Fisher Post-hoc test (upper panel) or the Mann–Whitney *U*-test (lower panel), ($P < 0.05$). Numbers inside the bars refer to the total sample size of each experimental group.

Fig. 3.

Means and standard deviations for (A) the number of functional immune cells of worker bees obtained from three replicate setups (per replicate, $n=15-20$ for each experimental group), and (B) the proportion of non-functional, pycnotic hemocytes in the same samples. Data were collected from 26 day-old nurse bees and foragers at base line, and subsequently from reverted bees and continuing foragers after 4 and 8 days. Small letters refer to groups that were significantly different as determined by Fisher Post hoc test ($P<0.05$). Numbers inside the bars refer to the total sample size of each experimental group.

Fig. 4. Slide-mounted forager hemocytes at 40 $\times$ magnification in (A) dark field, and (B) epifluorescence after TUNEL immunohistochemical staining. The lower panels show enlargements of a cluster of hemocytes with pycnotic nuclei as described for the honey bee by Rutz et al. (1974), and Wille and Rutz (1975) in (C) dark field, and (D) epifluorescence, demonstrating that honey bee hemocyte pycnosis corresponds to apoptotic programmed cell-death. Non-apoptotic cells (see methods) did not emit green fluorescence.

Table 1
Number of bees with immune cell nodulations (*Nod*) and microbial infection (*Inf*). Data are shown separately for nurse bees and foragers at the time of the reversion manipulation (base line), and subsequently after 4 and 8 days for bees that reverted to nurse tasks and workers that continued as foragers

Behavioral phenotype	Base line		After 4 days		After 8 days	
	<i>Nod.</i>	<i>Inf.</i>	<i>n</i>	<i>Nod.</i>	<i>Inf.</i>	<i>n</i>
Nurse bees	1	1	53	—	—	—
Reverted workers	—	—	—	0	0	55
Foragers	0	8	52	9	11	59