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HOW DOES REGIONAL EPISODES OF EXTREME WEATHER CHANGE, INFLUENCE THE HAEMOCYTES PROFILE OF INDIAN HONEY BEE, *Apis indica* OF DISTRICT AMETHI, UTTAR PRADESH, INDIA

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ABSTRACT

The honey bee, *Apis indica* is a delicate and sensitive Hymenoptera insect, which hasbeen domesticated for honey production and bee wax. Due to continuous domestication, thisIndian bee becomes susceptible to various diseases. The insect immune response consists of two tightly interconnected components, the cellular and the humoral responses. The cellular response is mediated by haemocytes and involves responses such as phagocytosis, encapsulation, and clotting. During the course of infection the cellular defense mechanism is mediated by differenttypes of haemocytes. Haemocytes are found circulating freely in the haemolymph or adhering tointernal organs such as the fat body or the digestive tract of the insects. Different types of haemocytes were found in the haemolymph of insects as leucocytes and pycnonucleocytes. We found that in all three adult phenotypes hemocyte number is dramatically reduced in early adult life. In contrast, we found that the dynamics of PO-activity level shave sex and caste-specific characteristics. PO activity reached a plateau within the first week of adult life, and in queens enzyme levels continuously increased with age and reached levels twice as high as those found in workers. PO-activity levels slightly declined with age in drones. These data support our hypothesis, from which we infer that the previously reported reduction of hemocytein foragers is not worker specific but represents a general phenomenon occurring in all honeybee adult phenotypes. The total haemocyte counts and the differential haemocyte counts vary in the different life stages. In the haemolymph of Apisindica free haemocytes contained PRs, PLs, GRs and a special type of cell in the femaleswhereas the males of this species had only PRs and PLs. Haemocyte numbers in the haemolymph of any particular insect may vary depending on various factors, such as disease and meteorological factors, including altitude. The results show significant variations of different haemocyte in the various life forms of Apisindica. These investigations may be very useful in planning rearing strategies for commercial species of Honey bee.

KEYWORDS: Haemocytes, Indian Honey Bee, *Apis indica*

The western honey bee (*Apis mellifera*) contributes to about one third of the food supply for humans. Commercial-scale production of almonds, certain fruits (apple, apricot, peach, and cherry) and some vegetables (cucumbers and melons), would not be possible without their role in pollination. The study area provides favorable ecological conditions and habitat for various economically important insect species and also supports a good number of species diversity (Khan *et al.,* 2016). In (Khan and Tripathi, 2016) presented the important information on the morphological variation in various forms of silk mothBombyx mori in different seasons from the Amethi regions of Uttar Pradesh, India, which is an important parameter in racial investigation of this species of silk moth. In recent decades, honey bee colonies have declined in most agricultural areas worldwide. During the 2014–2015 season, colony loss for the average India beekeeper was 47.2% (Khan and Tripathi, 2016), with increasing concern regarding bee health also Amethi and other locations (Amdam *et al.,* 2005) (Calderone, 2012). This situation threatens the global food supply for an expanding human population. The cause for this loss appears to be multifactorial (Steinhauer *et al.,* 2014) and has defied clear definition.

Considerable effort is being devoted to understanding threats that impact normal function of honey bee colonies. Pathogens such as Varroa destructor mites (Dainat *et al.,* 2012) tracheal mites, two species of Nosema intestinal parasites bacteria, fungi, and viruses are now recognized to infect honey bees and threaten their survival. Application of xenobiotics (pesticides, herbicides, and fungicides) has also been implicated in the decline of honey bee colonies. In addition, climate change, variability in nutritional sources for bees, and trends toward migratory beekeeping create additional stress on managed hives. Our ability to mitigate stress factors of honey bees will require a better understanding of their defense and response mechanisms. At this time, however, few metrics are available upon which changes in honey bee metabolism can

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be evaluated and understood. Previous studies in honeybees demonstrated that for-agers undergo a dramatic reduction in circulating hemocyte numbers, a reduction in hemolymph volume, and analmost total loss of the capability to form nodules in response to bacterial challenge. From an evolutionary perspective, hemocyte reduction in honey bee for agers may represent a colony-level strategy oftrading off costs of immunity against energy reserves of thewhole colony. As foragers already face a high mortality rate, e.g. due to predation, reduction ofimmune competence might not substantially increasemortality risk for the individual but may be beneficial tothe colony, as it saves energy costs needed to maintain cellular immunity. Correspondingly, loss of hemocytes may be an integral part ofivision of labor regulated by vitellogenin and juvenile hormone. Both these hypothesesimply two important predictions about dynamics of hemoctye numbers in honeybee female castes: in workers, hemocyte number should be task- and not age-dependent, and in queens, which are long-lived and show a reverse pattern of vitellogenin and juvenile hormone compared toother works (Calderone, 2012).

Honey Bee Immunity

The immune systems of insects have some similarities withinnate defense strategies in mammals (Calderone, 2012), which can be broadlyseparated, into cellular and humoral (soluble) components. When compared with other insects such as Drosophila and Anopheles, honey bees have only about one third the number of genes devoted to immunity, suggesting either their immunologicefficiency, or vulnerability to infection. Insect hemocytes area central component of their cellular host defense, where in mechanisms of phagocytosis, nodulation, encapsulation, and melanization have been described. Despite the importance of honey bee hemocytes in resisting disease and several fruitfulstudies involving this topic, a number of details about celltypes, numbers, and response to challenge are lacking. Therefore, one goal of our study was to extend the work of others who have shown differences between hemocyte types in honey bees. Honey bees require complex immune defense mechanisms (Amdam *et al.,* 2005). When compared with solitary insects, they may utilize additionalstrategies that limit spread of infection through close contact in society members. Also, considerable interest has focused on the possibility that honey bees sacrifice or suppress someaspects of immune defense in their later adult life, in exchange forother capabilities. Even as many of these detailscontinue to emerge, it is now evident that bee colonies thatsuccumb to infectious agents herald mechanisms of disease thatbreach natural immune surveillance and control.

Hemocyte Subsets

Examination of insect hemocyte subsets has been carried out inseveral species, including the fruit fly (Drosophila melanogaster, mosquito, and silkworm, as well as in a variety of other invertebrates, including shellfish, arachnids, crustaceans, and ascidiantunicates. Fundamental differences in hemolytic types, combined with a paucity of probes for specific cell types, has hindered development of a classification scheme that can bebroadly applied across insect orders (Khan and Tripathi, 2016). We hypothesize thatin honey bees, stimuli from infectious agents, xenobiotics, nutritional changes, migratory beekeeping practices, seasonalvariation, age, and social caste may be reflected in the abundance and types of their hemocytes. A refined understanding of cellularimmunity in honey bees could provide new views on theirmetabolic responses to a spectrum of challenges; some of whichthreaten their survival.

MATERIALS AND METHODS

Ethics Statement

Studies did not involve endangered or protected species. Following obtaining permission from the owners of the private apiaries, approximately 120 honey total honey bees were collected from a private apiary in Amethi and approximately 1100 total were collected from aprivate apiary in Amethi.

Bees

Honeybee workers, queens, and drones originated from Apismellifera Amethi region. For the subsequent two age series, 1-day-old bees fromdifferent source colonies were paint marked and introducedinto the same healthy foster colony. Samples for the twoworker age series were collected over 3 weeks. For hemocyte number, we obtained measurements for 1–4, 7,8, 12, 13, 18, and 23 days of age, and for PO-activity determinations, we obtained measurements for 1–17, 19,22, and 24 days of age. For each day of sampling, we determined hemocyte number and PO activity for 10 individuals and at any time point new

individual's were examined. For the analysis of queens, we collected 6-month (hemocytes (H): n ¼ 3, PO: n ¼ 4), 1-year (H: n ¼ 4, PO:n ¼ 4), and 2-year-old (H: n ¼ 1, PO: n ¼ 2) queens fromcolonies that were scheduled for artificial queen exchange. We artificially reared 1-day-old queens (H: n ¼ 8, PO:n ¼ 7).

Hemocyte Number and Phenoloxidase (PO) Activity

Hemocyte number was estimated using a hemocytometer and phase-contrast microscopy. Cell counts were done 5–10 min after filling the hemocytometer. For the measurement of PO-activity levels, thoraces ofindividual bees were homogenized in liquid nitrogen andthen incubated in 1 ml of ice-cold bee saline for 1 hour at roomtemperature and an additional 24 hour at 4 degree Celsius. After centrifugation, 20 ml of the supernatant were mixed with140 ml of distilled water, 20 ml of sodium phosphate buffer, and 20 mlL-Dopa (Sigma D-9626, 4 mg/ml deionized water) as a substrate. PO activity at 30 1C was measured at 490 nm for 20 min in 1-min intervals (MRX Microplate Absorbance Reader, Dynex Technologies). For each bee, wereformed two independent measurements and determined an average O.D. (Vmax) for the two reactions.

Reagents

Unless indicated otherwise, all reagents and chemicals wereobtained from either Fisher Scientific or Sigma Aldrich. Honey bees: Colonies of honey bees were housed in standard 10-frame, deep-body Langstroth hives. Bees were collected from the top edge of honeycombs, above and away from the brood-rearing area. This location was chosen for consistency, and to reduce the chance of collecting hatchlings and nurse bees which may represent different phenotypes of hemocyte subsets. We consulted a reference for standard methods in honey bee research for guidance on hive and bee sampling and for anesthesia of bees by chilling. Our studies included flow cytometry and Wright-stained slides for microscopy involving 25 Carnolian bees from one hive, 25Russian bees from another hive, 416 Buckfast-Italian cross bees from three separate hives, and 168 Italian bees from two additional hives, providing a total of 634 individual bees. Our analyses included different bee strains to represent some of the more common managed genetic lines. Data produced by these experiments did not identify strain-specific differences in hemocytes. A greater number of hives might be needed to investigate that possibility in detail. Examination of hemocyte subsets in bees with respect to numbers of Varroa mites was restricted to three colonies of Buckfast-Italian bees housed in modern equipment purchased from Mann Lake Bee Company in Hackensack, MN.

RESULTS

Changes in Hemocyte Number in Workers, Queens and Drones

In both worker age series, hemocyte number continuously decreased with age (Figure-1; linear regression analysis: age series 1 adjusted R^2 = 0.89, P<0.002; age series 2 adjusted R^2= 0.94, P = 0.0001). Similar to workers, hemocyte number in queens and drones also declined sharply, and non-linearly, with age, (queens: R^2= 0.9997, P<0.0001; drones: R^2=0.89, P<0.025; Figure-1).

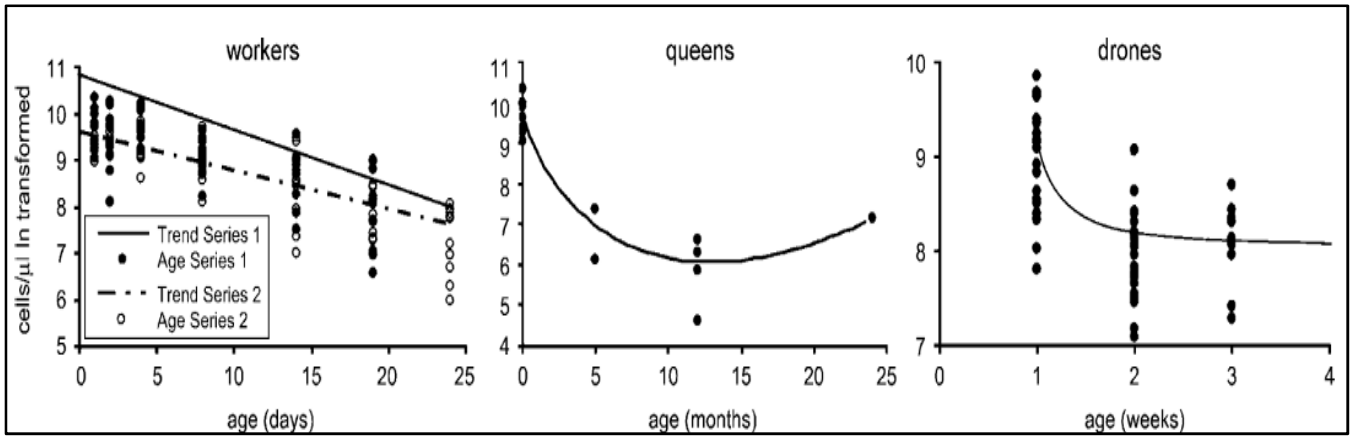

Figure 1: Change of Hemocyte Number in Worker, Queen and Drone Honeybees in Relation to Age

In the SCC experiment, hemocyte numbers in oldernurses and foragers were much smaller compared to their younger counterparts (P<=0.05), indicating that the overall hemocyte decline is age- but not task-dependent. Although numbers of hemocytes were already small in over-aged nurses and age-right foragers, over-aged nurses have a significantly higher number of hemocytes compared to foragers of similar age (Figure-3a).

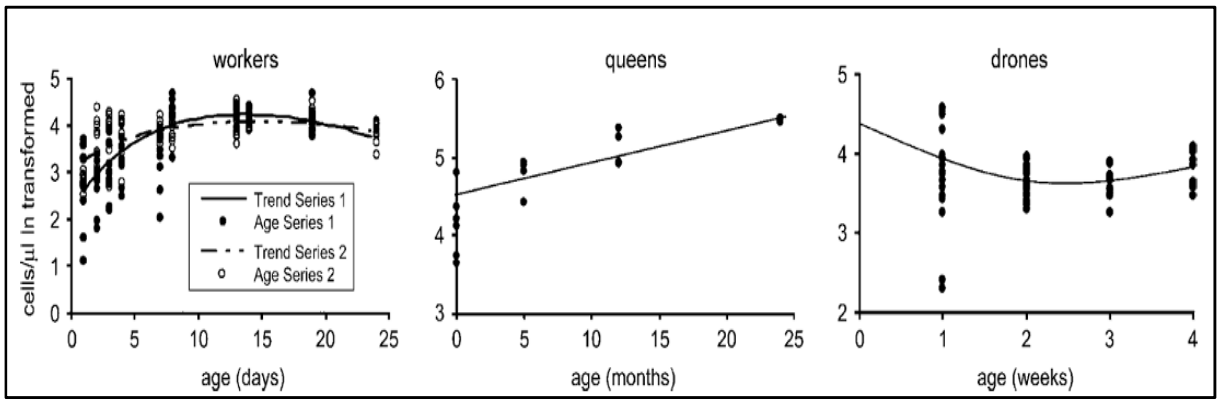

Figure 2: Change of Phenoloxidase Activity in Worker, Queen and Drone Honeybees in Relation to Age

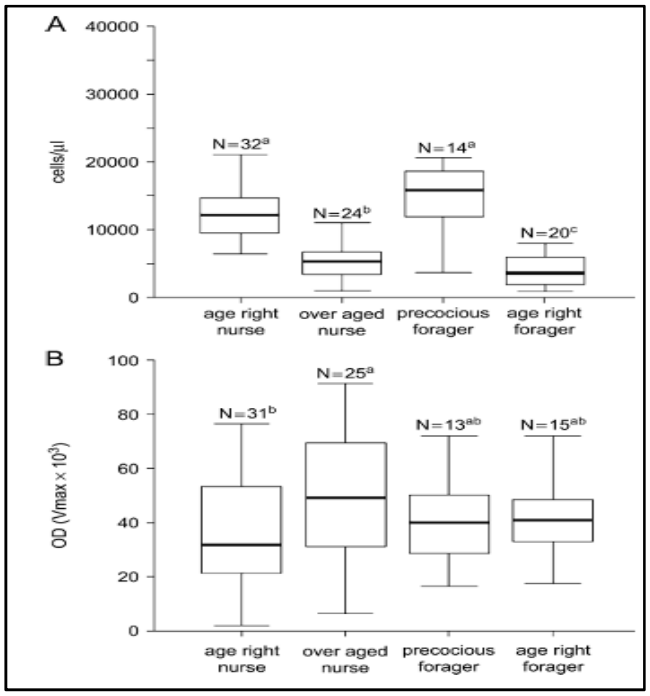

Figure 3: Hemocyte Number and Phenoloxidase Activity in Workers of A Single Cohort Colony: Age-Right Nurses (5–11 days), Over-Aged Nurses (23–28 days), Precocious Foragers (5–11 days), and Age-Right Foragers (23–28 days). Values with the Same Superscript Letter are Not Significantly Different.

DISCUSSION

The outcomes of our experiments support our hypotheses that abandoning hemocytic immunity is not restricted to worker honeybees but that all adults undergo hemocyte loss but do not loose PO activity. Several points are germane. First, hemocyte populations declined with increasing age in workers, queens, and drones. Second, constitutive PO activity increased with age in workers and queens and declined slightly throughout the 30-dayexperiments in drones. Third, the SCC experiment

revealed that the changes in hemocyte populations were related to age and not to task. We infer our data are consistent with the idea that adult honeybees of all castes abandon hemocytic immunity while retaining constitutive PO-based immunity. A worldwide effort is underway to mitigate global losses of honey bees, though a comprehensive understanding of this problem has proven elusive. Current evidence indicates Varroamites contribute to colony loss, as mites may induce immune suppression in bees, as well as transmit viral pathogens. Bacterial infection may further predispose immune deficiency in honey bees. In spite of this situation, relatively few studies are focused on cellular defense mechanisms in honey bees. These concerns suggest a need for better strategies to monitor hemocyte subsets in honey bees and to better define the contribution of pathogens in colony failure.

CONCLUSION

In summary, we report a rapid method of examining honey bee hemocyte profiles that may be sensitive to conditions that impact their health and social structure. Expansion of this approach to connect indices of honey bee hemolymph with stress factors will provide a better understanding of their susceptibility to challenge, disease, and hive failure. Further studies are needed to unravel these complex relationships. Found that sexually mature male and female dragon flies, Lestesviridis, captured in the wild, had higher hemocyte counts and lower PO activity than their newly emerged counterparts, demonstrating for the first time that these two immune measures do not necessarily correlate with each other and might even be regulated according to specific conditions. In the case of honeybees, our study suggests that the marked drop in hemocytes is coupled with increased PO activity, indicating a programmed change in immune functions, from cellular-based to PO-based immunity.

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