

Haemocytes profile of Indian Honey bee *Apisindica* of District Amethi, Uttar Pradesh, India

¹Saleem Ahamad Khan, ²Rajneesh Tripathi

Department of Zoology

Shri Jagdeesh Prasad Jhabarmal Tiberawala University, Jhunjhunu, Rajasthan, India

Abstract

The honey bee, *Apisindica* is a delicate and sensitive Hymenoptera insect, which has been domesticated for honey production and bee wax. Due to continuous domestication, this Indian 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 different types of haemocytes. Haemocytes are found circulating freely in the haemolymph or adhering to internal 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 levels have sex and caste-specific characteristics. PO activity reached a plateau within the first week of adult life, and in queen 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 hemocyte in 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 females whereas the males of this species had only PRs and PLs. Haemocyte numbers in the hemolymph 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, *Apisindica*, Amethi, Uttar Pradesh, India.

1. Introduction

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 favourable ecological conditions and habitat for various economically important insect species and also supports a good number of species diversity [1]. In [2] SaleemAhmad, Rajneesh Tripathi presented the important information on the morphological variation in various forms of silkmothBombyxmori in different seasons from the Amethi regions of Uttar Pradesh, India, which is an important parameter in racial investigation of this species of silkmoth. 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% [2], with increasing concern regarding bee health also Amethi and other locations [3], [4]. This situation threatens the global food supply for an expanding human population. The cause for this loss appears to be multifactorial [5] 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 [6], 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 be evaluated and understood. Previous studies in honeybees demonstrated that foragers undergo a dramatic reduction in circulating hemocytenubers, a reduction in hemolymph volume, and analmost total loss of the capability to form nodules inresponse to bacterial challenge. Froman evolutionary perspective, hemocyte reduction in honeybeeforagers 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 ofhemocyte 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 reversepattern of vitellogenin and juvenile hormone compared toother works [4].

1.1 Honey bee immunity

The immune systems of insects have some similarities with innate defense strategies in mammals [4], which can be broadly separated 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 immunologic efficiency, or vulnerability to infection. Insect hemocytes are a central component of their cellular host defense, wherein mechanisms of phagocytosis, nodulation, encapsulation, and melanization have been described. Despite the importance of honey bee hemocytes in resisting disease and several fruitful studies involving this topic, a number of details about cell types, 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 [3]. When compared with solitary insects, they may utilize additional strategies 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 some aspects of immune defense in their later adult life, in exchange for other capabilities. Even as many of these details continue to emerge, it is now evident that bee colonies that succumb to infectious agents herald mechanisms of disease that breach natural immune surveillance and control.

1.2 Hemocyte subsets

Examination of insect hemocyte subsets has been carried out in several species, including the fruit fly (*Drosophila melanogaster*, mosquito, and silkworm, as well as in a variety of other invertebrates, including shellfish, arachnids, crustaceans, and ascidian tunicates. Fundamental differences in hemolytic types, combined with a paucity of probes for specific cell types, has hindered development of a classification scheme that can be broadly applied across insect orders [2]. We hypothesize that in honey bees, stimuli from infectious agents, xenobiotics, nutritional changes, migratory beekeeping practices, seasonal variation, age, and social caste may be reflected in the abundance and types of their hemocytes. A refined understanding of cellular immunity in honey bees could provide new views on their metabolic responses to a spectrum of challenges; some of which threaten their survival.

2. Materials and Methods

2.1 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 a private apiary in Amethi.

2.2 Bees

Honeybee workers, queens, and drones originated from Apismellifera Amethi region. For the subsequent two age series, 1-day-old bees from different source colonies were paint marked and introduced into the same healthy foster colony. Samples for the two worker 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 individuals were examined. For the analysis of queens, we collected 6-month (hemocytes (H): $n = \frac{1}{4} \times 3$, PO: $n = \frac{1}{4} \times 4$), 1-year (H: $n = \frac{1}{4} \times 4$, PO: $n = \frac{1}{4} \times 4$), and 2-year-old (H: $n = \frac{1}{4} \times 1$, PO: $n = \frac{1}{4} \times 2$) queens from colonies that were scheduled for artificial queen exchange. We artificially reared 1-day-old queens (H: $n = \frac{1}{4} \times 8$, PO: $n = \frac{1}{4} \times 7$).

2.3 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 of individual bees were homogenized in liquid nitrogen and then incubated in 1 ml of ice-cold bee saline for 1 hour at room temperature and an additional 24 hour at 4 degree Celsius. After centrifugation, 20 ml of the supernatant were mixed with 140 ml of distilled water, 20 ml of sodium phosphate buffer, and 20 ml L-Dopa (Sigma D-9626, 4 mg/ml deionized water) as a substrate. PO activity at 30 °C was measured at 490 nm for 20 min in 1-min intervals (MRX Microplate Absorbance Reader, Dynex Technologies). For each bee, we performed two independent measurements and determined an average O.D. (V_{max}) for the two reactions.

2.4 Reagents

Unless indicated otherwise, all reagents and chemicals were obtained 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, 25 Russian 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.

3. Results

3.1. 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).

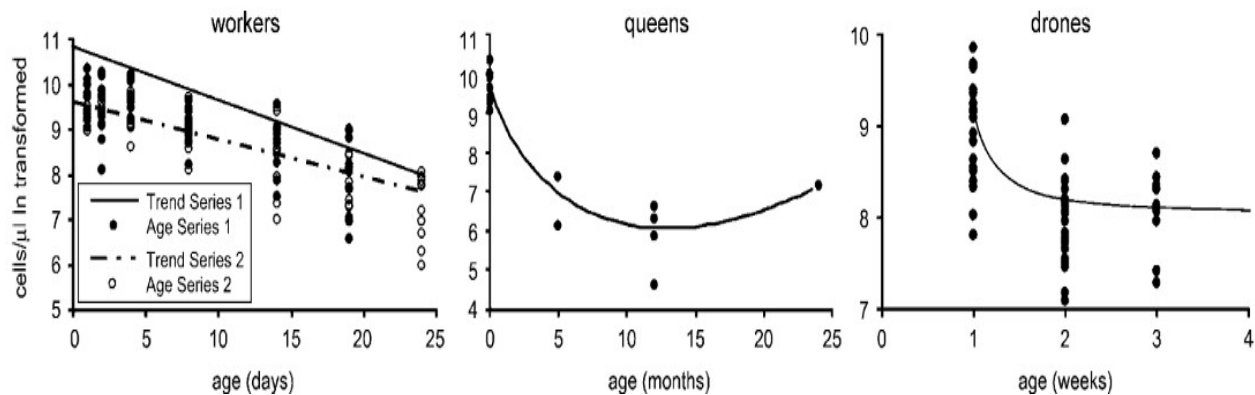


Figure-1: Change of hemocyte number in worker, queen and drone honeybees in relation to age

In the SCC experiment, hemocyte numbers in old nurses and foragers were much smaller compared to their younger counterparts ($P \leq 0.05$), 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).

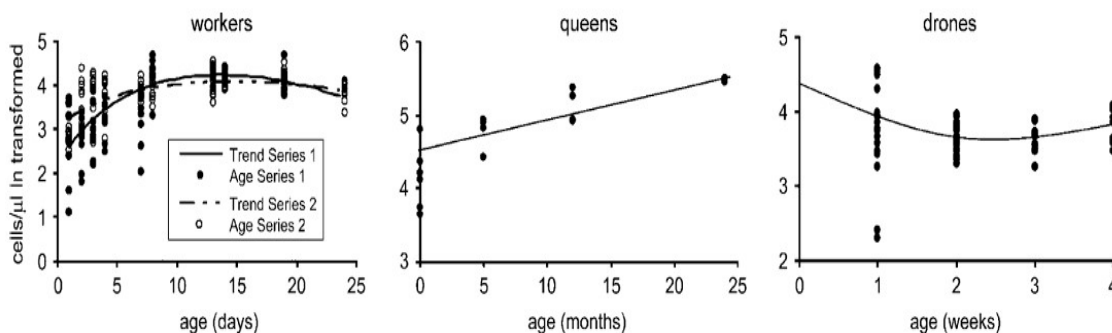


Figure-2: Change of phenoloxidase activity in worker, queen and drone honeybees in relation to age

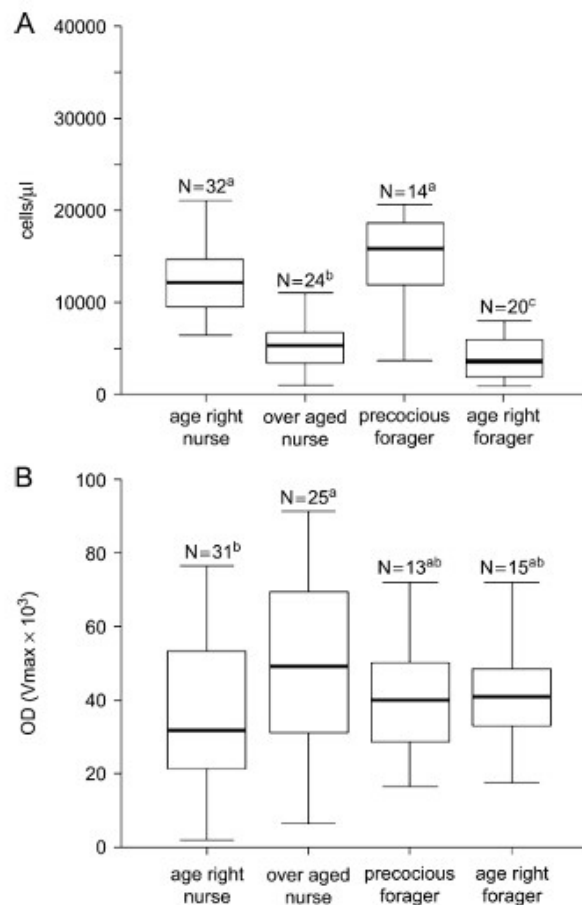


Figure-3: Hemocyte number and phenoloxidase activity in workers of a single cohort colony: age-right nurses (5–11 days), over-aged nurses (23–28 days), precocious foragers (5–11 days), and age-right foragers (23–28 days). Values with the same superscript letter are not significantly different.

4. Discussion

The outcomes of our experiments support our hypothesis that abandoning hemocytic immunity is not restricted to worker honeybees but that all adults undergo hemocyteloss but do not lose 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-day experiments 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 Varroa mites 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.

5. 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 dragonflies, *Lestes viridis*, 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 honey bees, 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.

References:

- [1] Saleem Ahamad Khan, Rajneesh Tripathi, Indu Singh, B K Gupta, "A study on biodiversity of insects, species richness, lavishness and distribution arrangement in the particular region of District Amethi, Uttar Pradesh, India", *IJATTMAS*, 2016, Vol. 2 (1), pp. 61-70
- [2] Saleem Ahamad, Rajneesh Tripathi, "Identification and morphological characterisation of Silkworm *Bombyx mori* from the region of Amethi, Uttar Pradesh, India", *International Journal of Zoology Studies*, Volume 1; Issue 4; May 2016; Page No. 18-21
- [3] Amdam, G.V., Aase, A.L., Seehuus, S.C., Fondrk, K.M., Norberg, K., Hartfelder, K., 2005. Social reversal of immunosenescence in honey bee workers. *Experimental Gerontology* 40, 939–947.
- [4] Calderone NW (2012) Insect pollinated crops, insect pollinators and US agriculture: trend analysis of aggregate data for the period 1992–2009. *PLoS One* 7: e37235.
- [5] Steinhauer NA, Rennich K, Wilson ME, Caron DM, Lengerich EJ, et al. (2014) A national survey of managed honey bee 2012–2013 annual colony losses in the USA: results from the Bee Informed Partnership. *J Apicul Res* 53: 1–18.
- [6] Dainat B, Vanengelsdorp D, Neumann P (2012) Colony collapse disorder in Europe. *Environ Microbiol Rep* 4: 123–125.

- [7]Pirk CWW, Human H, Crewe RM, Vanengelsdorp D (2014) A survey of managed honey bee colony losses in the Republic of South Africa-2009 to 2011. *J Apicul Res* 53: 35–42.
- [8]Vanengelsdorp D, Speybroeck N, Evans JD, Nguyen BK, Mullin C, et al. (2010) Weighing risk factors associated with bee colony collapse disorder by classification and regression tree analysis. *J Econ Entomol* 103: 1517–1523.
- [9]Rosenkranz P, Aumeier P, Ziegelmann B (2010) Biology and control of Varroa destructor. *J Invertebr Pathol* 103 Suppl 1: S96–119.