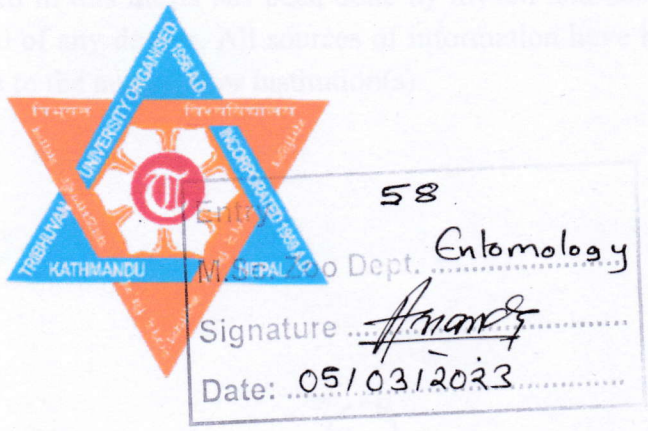
EVALUATION OF HYGIENIC BEHAVIOR AND HEAVY METALS IN ASIAN HONEYBEE *APIS CERANA* FABRICIUS, 1793 IN KASKI, NEPAL



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A thesis submitted
In partial fulfillment of the requirements for the award of the degree of Master of Science in Zoology with special paper entomology

Submitted to
Central Department of Zoology
Institute of Science and Technology
Tribhuvan University
Kirtipur, Kathmandu
Nepal

April, 2023

DECLARATION

I hereby declare that the work presented in this thesis has been done by myself and has not been submitted elsewhere for the award of any degree. All sources of information have been specifically acknowledged by reference to the author(s) or institution(s).

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RECOMMENDATION

This is to recommend that the thesis entitled **"Evaluation of hygienic behavior and heavy metals in Asian honeybee *Apis cerana* Fabricius, 1793 in Kaski, Nepal"** has been carried out by Ravi Timsina for the partial fulfillment of master's degree of Science in Zoology with special paper entomology. This is his/her original work and has been carried out under my supervision. To the best of my knowledge, this thesis work has not been submitted for any other degree in any institutions.

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LETTER OF APPROVAL

On the recommendation of supervisor Prof. Dr. Prem Bahadur Budha this thesis submitted by Ravi Timsina entitled **"Evaluation of hygienic behavior and heavy metals in Asian honeybee *Apis cerana* Fabricius, 1793 in Kaski, Nepal"** is approved for the examination and submitted to the Tribhuvan University in partial fulfilment of the requirements for master's degree of Science in Zoology with special paper entomology.

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CERTIFICATE OF ACCEPTANCE

This thesis work submitted by Ravi Timsina entitled **"Evaluation of hygienic behavior and heavy metals in Asian honeybee *Apis cerana* Fabricius, 1793 in Kaski, Nepal"** has been accepted as a partial fulfillment for the requirements of master's degree of Science in Zoology with special paper entomology.

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Ravi Timsina

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LIST OF ABBREVIATIONS

Abbreviated form	Details of abbreviations
AAS	Atomic Absorption Spectroscopy
CDZ	Central Department of Zoology
DHM	Department of Hydrology and Meteorology
FKB	Freeze-killed brood
HB	Hygienic behavior
NAST	Nepal Academy of Science and Technology
PKB	Pin-killed brood
TU	Tribhuvan University

ABSTRACT

Honeybees have different defensive mechanisms to mitigate impact of pathogens and other stressors. One among such is hygienic behavior expressed by bees, at which upon detection of dead and infected brood, the worker bee removes it from outside the hive. This study concentrates on two major research questions: the hygienic behavior of native honeybees Apis cerana and the prevalence of heavy metals in colony products. The hygienic behavior was studied between two seasons, spring, and autumn of 2022. The effect of environmental factors such as temperature, rainfall, and relative humidity on the manifestation of hygienic behavior was studied. The second study question centers on detecting heavy metals in honey samples from experimentally treated colonies and the relationship between heavy metal concentration and hygienic behavior score. The methods used in this research included a standard freeze-killed brood assay procedure for data gathering and an atomic absorption spectroscopy technique for heavy metal detection in honey samples. The findings indicate that hygienic behavior is an uncommon characteristic in nature and is rarely found in non-selected communities. However, the hygienic behavior score rose at 48-hour testing in both seasons, with one colony having a hygienic behavior of 11% among examined colonies. Likewise, most of the cleaning was done within the first 24-hour period. The chi-squared test showed a significant association between time intervals and empty cell counts in the hive trails ($\chi 2 = 12.25$, df = 1, p = 0.0004653), with more empty cells being created in the first 24 hours than the second 24 hours. None of the environmental variables influenced the hygienic behavior of honey bees. However, survival analysis revealed both uncapping and brood removal rate was higher in autumn tested colonies than spring tested colonies. The research also found heavy metals, especially Lead in honey samples examined. However, no major link was discovered between heavy metals and sanitary behavior. The thesis states that more large-scale research is required to better understand the possible effect of heavy metals on honey bee physiological performance in regions near anthropogenic emissions.

1. INTRODUCTION

1.1 Background of the study

Pollination transfers genetic information between plants, achieved by various means, such as air and living organisms (Hein 2009). Among the different modes of pollination, insect pollination is of utmost importance. Fruits, vegetables, and stimulants crops depend on insects for pollination (Gallai et al. 2009). Furthermore, of these insects involved, Apoidea has the most significant direct impact on pollination (66% of 128 crops)(Lorenzo-Felipe et al., 2020). Honeybees' significance in terms of monetary value is enormous; the value of Apis pollination to directly dependent crops was 11.68 billion dollars in 2009, and 1.15 billion dollars in indirectly dependent crops in the U.S. agriculture system only (Calderone 2012). Apart from pollination services, recent studies have suggested that bees contribute to the fulfillment of 15 out of 17 sustainable development goals (Patel et al. 2021). Even though bees are a critical component of a complex ecosystem and aid directly in acts such as pollination, their population faces a series of stressors that drive their decline. In the honey bee colonies, pests, pesticides, diseases, Varroa mites, Nosema parasites, and beekeeping practices have contributed to the honeybee population's decline (Hristov et al. 2020).

Honey bees are eusocial insects in which over two or more generations are cohabiting, with a collaborative effort to raise young, and there is member division into reproductive and non-reproductive castes (Wilson and Hölldobler 2005). This eusocial living provides tremendous benefits; social living insects dominate the terrestrial earth system (Kocher and Paxton 2014). However, there are expenses of living in a group. Honey bees living in a colony share close genetic relatedness and are involved in extensive interaction with each other (Naug and Camazine 2002). Hence, it is easier for pathogens to attack and transmit diseases in these colony-living organisms. Honeybees have developed a broad set of defense mechanisms to counter these problems. Individual defense (physiological and immunological), pairwise defense, colony defenses, minimizing entry of pathogens, and use of plant resins for colony shielding are some of the established defense mechanisms in honey bees (Evans and Spivak 2010). Hygienic behavior (HB) is the form of colony defense that honey bees adapt to mount a collective defense against parasites and pests; grooming and undertaking are other well-established behavior in honey bees (Evans and Spivak 2010). Honey bees perform HB to counteract brood diseases (Oxley et al. 2010), and this behavior is the same regardless of whether the brood is dead or diseased.

Asian honey bee *Apis cerana* Fabricius, 1793 is a native honey bee species found throughout Nepal and is reared for commercial beekeeping (Aryal et al. 2015). This honey bee is also reared by non-commercial farmers of rural areas. In addition to pollination (Koetz 2013) and other ecological services, these species account for 36% of honey production in the Nepalese market (Thapa et al. 2018). It is only logical to promote the survival of this species. In this time of great insect decline, the population of *A. cerana* is deteriorating (Theisen-Jones and

Bielefeld 2016). Several reasons behind the declining trend include pesticide application to forest conversion. Sac brood disease and Varroa destructor (Anderson and Trueman 2000) hamper *A. cerana* brood (Thapa et al. 2018). Honey bees showcasing superior HB can counteract the detrimental effect of brood diseases. HB in *Apis mellifera* Linnaeus, 1758, is widely reported and integrated into the *A. mellifera* queen breeding program. HB testing ensures that the commercial colonies are resilient to brood pathogens. However, research on the HB behavior of bees and subsequent queen breeding is lacking in Nepal (Thapa et al. 2018). Thus, this research involved the evaluation of HB in *A. cerana* from a commercial apiary in Kaski, Nepal.

Honeybees experience a wide range of stressors that have a detrimental impact on their health. Both biotic(pathogens, predators, pests) and abiotic (pesticides, nutrition) stressors can harm the bee population (Havard et al. 2019). Of late, heavy metal's impact on bees has emerged as another abiotic stressors, but their study is limited (Havard et al. 2019). The findings, however, point out a grim picture regarding the effect of heavy metals on honey bee health. Heavy metals are trace elements found in the environment but are toxic to living beings (Tchounwou et al. 2012). These heavy metal stressors adversely affect social insects like honey bees (Feldhaar and Otti 2020). In honey bees, the heavy metals have an impact on bee cognition (Monchanin et al. 2020), foraging ability (Burden et al. 2019), honey bee health (Feldhaar and Otti 2020) as well as the disturbance in the micro-biome of the honey bees (Rothman et al. 2019). Honey bees can be exposed to these heavy metals via nectar, pollen, dust, and human-induced activities (Johnson 2015). The developing larvae need pollen for essential proteins, vitamins, and lipids during development (Scofield and Mattila 2015). These metals subsequently make their way into the honey bee body, exert their effect on the organism (Goretti et al. 2020), and alter the normal functioning of the bee.

1.2 Objectives

General objective

- Evaluation of hygienic behavior of *Apis cerana* and association of heavy metals with the hygienic behavior.

Specific objectives:

- Evaluation of the hygienic behavior of *A. cerana* in the commercial apiary of Kaski, Nepal and the comparison of hygienic behavior score in between two seasons.
- Impact of environmental variables (temperature, rainfall, relative humidity) on the expression of hygienic behavior of *A. cerana* .
- Evaluation of heavy metals in the honey sample and the association between heavy metal content and hygienic behavior score of the colony.

1.3 Significance of the study

Hygienic behavior is a behavioral response of honey bees that helps them remove pathogens and parasites from the nest (Spivak and Gilliam 1993). Hygienic behavior in honeybees refers to their ability to detect and remove diseased or dead broods from the colony. This behavior is essential for the colony's overall health and can help prevent the spread of disease. While hygienic behavior is extensively studied in the western honeybee, *A. mellifera*, research on hygienic behavior in *A. cerana* is limited. The honeybee species *A. cerana* is native to Asia and is vital for pollination and honey production in the region. Understanding hygienic behavior in *A. cerana* is central to improving the health and productivity of these bees.

Hygienic bees have several advantages over non-hygienic colonies. Studies that accessed the honey production capabilities of hygienic and commercial bees in the apiary and found hygienic bees significantly produced greater honey yield **(**Spivak and Reuter 1998). Hygienic lines of bees are better against American foulbrood (caused by bacteria) and chalkbrood (fungal disease), two brood diseases in the bee colony (Leclercq et al. 2017)**.** Likewise **(**Vung et al. 2020) showed that hygienic bees performed better against the Sacbrood virus when compared to others.

Hygienic behavior and heavy metal exposure are both critical areas of research in the field of environmental health. On the other hand, heavy metals as a potential stressor for honeybees and insects, in general, are recognized, but the research in this area is still in its early stages. Heavy metals are a type of environmental stressor that can harm honeybee health. Heavy metals can accumulate in the environment due to industrial processes and pollution and can be ingested by bees through contaminated food sources. While some studies have examined the effects of heavy metal exposure on honeybee health, this area of research is still relatively new. Understanding the impact of heavy metals on honeybee health is vital for protecting

these critical pollinators and preserving ecosystem health. In conclusion, research on hygienic behavior in *A. cerana* and the impact of heavy metals on honeybee health is necessary. By improving our understanding of these topics, we can take steps to protect honeybees and the ecosystems they support. Hence, this study will help establish the scenario of hygienic behavior in honey bees *A. cerana* in Nepal. This study will also examine environmental variables' effect on the HB score.

1.4 Limitations of the study:

The study was conducted only in a single apiary; time, logistic hurdles, and budget limited this study to a single apiary. Also, the study was undertaken during two seasons (spring and autumn) in 2022. The heavy metal evaluation in the honey sample of tested colonies was limited to Lead (Pb), Cadmium (Cd), Chromium (Cr), and Zinc (Zn). These metals were chosen due to their widespread reporting on hive products and honey bees in the literature review.

2. LITERATURE REVIEW

2.1 Colony organization in honey bees

Due to the complexities in the colony, honey bees are termed "super-organisms," which liken their living to multicellular organisms. Different castes perform distinct functions like specialized cells of multicellular organisms (Moritz and Fuchs 1998). On top of that, honey bees are a cohesive unit that acts together to replicate their genes; colony members possess morphological, physiological, and behavioral distinctiveness (Seeley 1997).

A honey bee colony consists of the reproductive queen and non-reproductive worker caste, and there is further division among workers (Calderone and Page 1988). Workers of honey bees change their duties in a colony as they age (age polytheism); the colony dynamics can influence the assignment, but in general, younger bees perform tasks within the hive while the old are assigned roles outside the colony (Robinson 1992). A worker honey bee performs many errands, including building comb, nursing young ones, cleaning the hive, and foraging for resources (Rothenbuhler 1964). In addition, there are distinct hygienic bees whose task is to perform cleaning behavior to check disease transmission in the colony; these bees detect and remove sick broods (Spivak et al. 2003).

Pathogens and parasites find it ideal for exerting their dominance in colonies of social insects; the high density and genetically similar profile make the social insects vulnerable to the disease-causing organism (Schmid-Hempel 1995). Honeybees have evolved different antics to limit the pathogen's growth and spread and to counter the threat of these pathogens. Eusocial insects like honey bees adopt various defense strategies to counter the infection; allogrooming behavior, prophylactic measures, and other collective efforts to ward off pathogens are typical in all animals living in groups (Cremer et al. 2007). Furthermore, for insects such as honeybees, joint action against the threats helps promote colony health (Simone-Finstrom 2017).

Genes and their protein products help bees protect themselves against diseases (Wilson-Rich et al. 2009). In addition to mechanical, physiological, and immune defenses, honeybees also mount collective protection against parasites and pests; grooming, hygienic behavior, and undertaking are well-established behaviors in honeybees (Evans and Spivak 2010).

Worker bees are responsible for performing hygienic behavior in the colony. Hygienic behavior is a part of the social immune system of honey bees, based on the collective action of worker honey bees (Cremer et al. 2007) against pathogens.

2.2 Hygienic behavior as a part of social immunity in honey bees

Bees behave hygienically to counteract brood disease (Oxley et al. 2010). Such behavior is the same regardless of whether the brood is diseased or dead. However, the behavior gets triggered when hygienic worker bees detect odors associated with different conditions (Spivak and Danka 2021). Honeybees deploy social immunity instead of innate immunity to counter threats (Boutin et al. 2015).

Two distinct processes are associated with bees performing hygienic behavior; First, there is uncapping of infested broods, followed by removing the brood (Lapidge et al. 2002). Social immunity, like hygienic behavior, is influenced by genetics; it has a heritability of 0.65 (Oxley et al. 2010). Rothenburger demonstrated the two-locus basis of genetic behavior in bees performing hygienic behavior in 1964. Two homozygous recessive genes (for uncapping and removal) were asserted as a condition for bees to showcase hygiene (Wilson-Rich et al. 2009). Aside from Rothenburger's view of two major quantitative traits that control hygienic behavior, Spivak and Reuter proposed the existence of a multi locus quantitative mode of inheritance (Oxley et al. 2010). Subsequent research on the genetic basis of hygienic behavior has suggested that multiple quantitative trait loci have a role in this behavior (Boutin et al. 2015). The number of genes involved in hygienic behavior is limited; compared to non-hygienic strains, only 28 genes are over expressed in hygienic strain bees (Boutin et al. 2015).

The role of antennae in odor detection plays a critical role in bees performing hygienic behavior; even though the causes of infestation vary, the bees do not discriminate in their hygienic behavior (Spivak and Danka 2021). Hygienic bees 15-20 days old showed the most significant amount of cleaning behavior, and the antennae of these bees responded to volatile compounds released from the diseased larvae (Arathi et al. 2000). Odorant molecules like β-ocimene and oleic acid (necromone of arthropod taxa) trigger hygienic behavior in honey bees; these two molecules elicit a more significant response in the colony (McAfee et al. 2018)**.** Guarna et al. (2015) found seven high amounts of protein in bees, which showed hygienic behavior. Of these seven, two were odorant-binding proteins (OBP16, OBP18) that point to antennae's role in dead brood detection.

2.3 Evaluation of hygienic behavior in honey bees

There are various bioassays used for quantifying the hygienic behavior of honey bees. The mechanism involved in performing the hygienic behavior of bees involves sacrificing a defined portion of the brood and then recording the cleaned portion of that area by worker bees in a fixed time (Facchini et al. 2019). Leclercq et al. (2018) outlined five main methods to evaluate the hygienic behavior of a colony; these methods involve challenging a colony with pathogens (*Paenibacillus larvae*, *Ascophaera apis*), mechanical damage via pin-killing the brood, freeze-killed brood assay, and Varroa destructor infested brood removal assay.

Pin-killed brood (PKB) removal assay and freeze-killed brood removal assay are the two most common methods used to quantify the hygienic behavior of bees due to their simplicity and ease of application. PKB is a simple yet effective solution for beekeepers to access the hygienic strength of the colony quickly; an entomological pin kills the pupae, and the removal of the dead brood is then observed (Büchler et al. 2013)**.** The pin-killed assay, however, can elicit a response from the colony via the leaked body fluid of pupae (Gramacho et al. 1999). Pin diameter and study time influence the results and make them unreliable (Leclercq et al. 2018).

Freeze-killed brood(FKB) removal assay is a standard method to test the hygienic behavior of the colony; the testing can be via either cutting brood out of the comb and freezing or freezing brood within the comb using liquid ammonia (Büchler et al. 2013)**.** Unlike PKB assay, FKB assay lacks inherent bias (hemolymph leakage does not happen), is convenient to perform, and has no risk of pathogen spread during experimentation (Leclercq et al. 2018)**.**

2.4 Comparison of hygienic behavior in Apis mellifera and Apis cerana

Hygienic bees' ability to check the wide range of brood pathogens makes them economically advantageous to beekeepers (Spivak et al. 2003). The hygienic behavior in *A. mellifera* was initially selected for its ability to resist American foulbrood and chalkbrood diseases: later, the same line of bees was used to see resistance against the parasitic mite Varroa (Spivak and Danka 2021). The behavior of the European honey bee that performs hygienic behavior is well known. Two distinct behaviors occur during the hygienic behavior: uncapping the cells and removing the brood. Middle-aged bees (15–17 days) performed hygienic behavior in the colony (Arathi et al. 2000). In the presence of dead broods, other activities of the bees get reduced (Arathi et al. 2000). Bees that express hygienic behavior also showcased lower Varroa mite loads, but the link between general hygienic behavior and Varroa-sensitive – hygiene has not yet been established (Spivak and Danka 2021).

The diversity of honey bees in Asia is remarkable; it harbors eight native honey bee species, among which *A. cerana* is a middle-sized bee with a multi-comb structure in its nest (Chantawannakul et al. 2016). Compared with the European honey bee (*Apis mellifera*), the Asian honey bee (*A. cerana*) is less affected by pests and pathogens. The social immunity of Asian bees is more robust than European honey bees (Lin et al. 2016). The olfactory detection capabilities of *A. cerana* are superior, and it helps the bee detect odorant molecules and perform better against Varroa mites and chalkbrood disease (Zhao et al. 2015).

2.5 Factors affecting the hygienic behavior of honey bees

Hygienic behavior differs within species and has high narrow-sense heritability (Harpur et al. 2019)**.** Nevertheless, apart from genetics, other environmental factors also influence the hygienic behavior of a bee (Bigio et al. 2013)**.** An inverse relationship between colonies expressing hygienic behavior and the altitude was observed; with an increase in altitude, the hygienic behavior of bees decreased (Masaquiza et al. 2021)**.** The nectar availability and brood amount did not alter the hygienic behavior of the honey bees; however, the interaction of brood, food, and season significantly affected honey bees' hygienic performance (Bigio et al. 2013). Whether new or old, the comb type did not significantly affect the hygienic behavior of bees; after an initial 24 hours, both comb-type yielded similar hygienic results (Pereira et al. 2013)**.** Recently, **(**Wagoner et al. 2019) demonstrated that the brood signal via their cuticular hydrocarbons could trigger hygienic behavior in honey bees.

2.6 Stressors of social insects

Parasitic and infectious agents and predators are the biotic stressors of honey bee health, while pesticides, herbicides, and beekeeping practices are some of the abiotic stressors of honey bees (Havard et al. 2019). Heavy metals are another suit of abiotic stressors impacting the health of honey bees; these metals are harmful due to their high toxicity (El-Seedi et al. 2022). Heavy metals act as stressors affecting brain functions, cognition, and behavior; For instance, selenium affects olfactory learning (Klein et al. 2017). In a study, (Li et al. 2022) reported chronic cadmium exposure led to impaired olfactory learning performance in A. mellifera. Thus, heavy metals are emerging as a new threat to the vulnerable honey bee population.

2.7 Heavy metals and social insects' health

Heavy metals are generally defined as those naturally occurring elements with a high atomic weight and a relatively high density compared to water (Tchounwou et al. 2012). Plants can then take these heavy metals and get into the food sources like pollen and nectar; they may also be airborne and ultimately reach the insects (Feldhaar and Otti 2020). Social insects like honey bees are central foragers where a group of workers bees (foragers) forage nectar, pollen, and other essential component and bring them back to the hive. Gutiérrez et al. (2015) revealed that heavy metals abundant in the environment had bio-accumulated in honey bee bodies. Colony members share these foraged resources for various functions including larval development, adult food, and hive products. The effect of heavy metals on social insects is well documented.

Furthermore, (Sivakoff et al. 2020) examined the effects of urban heavy metal pollution on bumblebee colonies; colonies exposed to greater concentrations of heavy metals, such as lead and zinc, grew more slowly than colonies exposed to lower concentrations. The slow growth is due to the toxic effects of heavy metals on bumblebees, which might adversely influence their ability to forage and reproduce. Likewise, (Rothman et al. 2020) found that exposure to heavy metals and other environmental toxins negatively impacted the health and microbiomes of bumblebees; the study reported the disruption of the bumblebee micro-biome. Likewise, exposure to aluminum and nickel in nectar negatively impacted bumble bee foraging behavior (Meindl and Ashman 2013).

Much of the study on the impact of heavy metals is on bees, especially honey bees and bumblebees. Many studies have shown that exposure to heavy metals and other contaminants can negatively impact honey bee survival, feeding behavior, memory recall, and the functioning of detoxification-related enzymes.

For instance, studies have demonstrated that selenium exposure can impair long-term memory recall and associative conditioning in honey bees (Burden et al. 2016)**,** whereas acute exposure to hazardous heavy metals can change honey bee eating behavior (Burden et al. 2019). Additionally, studies have shown that heavy metals may build up in the hive,

impacting the entire colony's health and brood production (Hladun et al. 2016). Additionally, research has investigated the impact of heavy metals on certain detoxifying enzymes in honey bees, such as catalase and superoxide dismutase. Study by (Gizaw et al. 2020) revealed that exposure to environmental heavy metals can change the expression of genes involved in detoxification in honey bees.

Zinc supplementation can change the transcriptome of honeybee brain tissue, affecting the gene expression of the insects' genes and perhaps affecting their behavior and physiology (Camilli et al. 2022). The microbiome and metabolome of honeybees were studied by (Rothman et al. 2019) to determine how exposure to cadmium and selenate affected them. The study showed that exposure to these heavy metals could disturb the bees' microbiota and metabolome, which has a detrimental impact on their health.

In *A. cerana*, (Wang et al. 2014) found the AccGS gene in response to several abiotic stressors, including heavy metal exposure. The AccGS gene increased in response to these stressors.

3. MATERIALS AND METHODS

The methods employed for data collection were a standard freeze-killed brood assay technique, and the heavy metals in the honey sample were analyzed via atomic absorption spectroscopy technique.

3.1 Research design

3.1.1 Study area

Begnas Beehive Industries Private Limited- apiary of Pokhara metropolitan, Gandaki Province- was chosen for the study. The apiary is situated in the urban area of the Pokhara metropolitan region, and it was ideal to evaluate the impact of heavy metals due to urbanization on the region's bees.

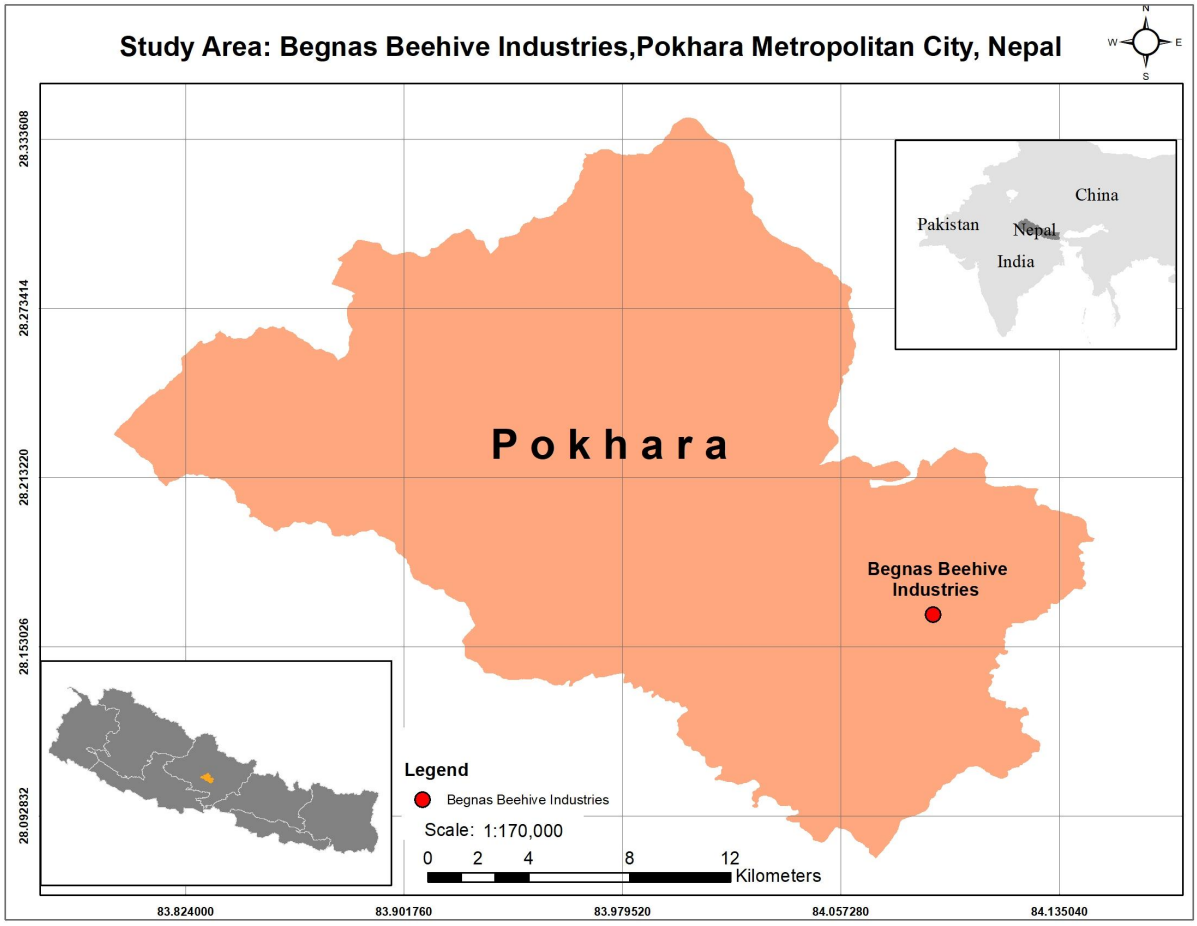


Figure 1. The study site- Begnas Beehive Industries, Pokhara

3.1.2 Study population

A total of nine colonies (five in spring, four in autumn) of Apis cerana were tested. All the tested colonies were free of pests, diseases, and nutritional deficiencies at the time of testing and had six-eight frames of combs in the brood chamber of a commercial hive . A cross-sectional study was carried out in May 2022 and October-November 2022. The hygienic

behavior was evaluated on the sealed brood (specifically, the test is performed on developing purple-eyed pupae); this protocol was followed when evaluating bee hygienic behavior.

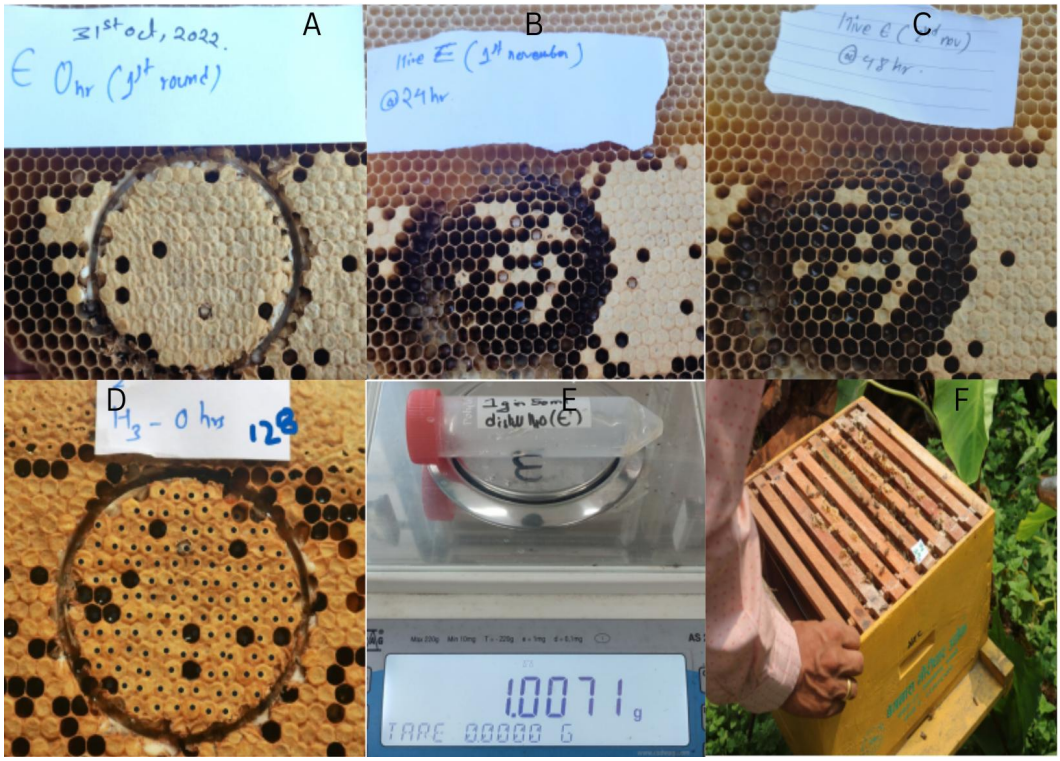
3.2. Method to evaluate hygienic behavior

The hygienic behavior test (a standard Freeze-killed brood assay test) was performed twice on each colony in both seasons (spring and autumn); The procedure for performing the tests were devised in the 1990s by Spivak and Reuter (Leclercq et al. 2018).The procedure consists of the following:

- A thin metal cylinder of 60 mm diameter and 110 mm diameter height was twisted into a comb containing a sealed brood until the midrib.
- Then about 50–60 ml of liquid nitrogen was poured inside the cylinder.
- The remaining (150~200ml) nitrogen was poured (measured in a measuring cylinder).
- The cylinder was removed after a few minutes only after it defrosted (approximately 10–20 min).
- The necessary marking was done to the treated frame. The photograph of the freeze-killed section was taken, and then it was replaced in the colony.
- The treated section was evaluated after 24 and 48 hours: hygienic behavior was calculated.
- The hygienic behavior was calculated as

Hygienic percentage = (no. of the cleaned cap in each time / total number under consideration).

The photographs at zero hours, 24 hours, and 48 hours were taken via a canon eos750D digital single-lens reflection camera. The photos were then imported into the computer and then analyzed. The empty brood cells, uncapped brood cells, and partially removed brood cells were all carefully analyzed.



Picture A-F. An overview of hygienic behavior test in ***Apis cerana*** colony.

3.3 Heavy metal analysis

The honey sample from tested hives was collected in falcon tubes during the same testing period. They were then sealed in a container and kept at normal room temperature until analysis. One gram of honey was dissolved in 50 ml of distilled water for the analysis. It was then analyzed for the presence of heavy metals via Atomic Absorption Spectroscopy (AAS). The analysis was performed in the analytical section of the Nepal Academy of Science and Technology (NAST).

3.4 Climatic variables data collection

The climatic data of both seasons (temperature, rainfall, relative humidity) of the nearest station within 200 m radius of the experimental sites Begnas station (index no 851) were taken from the department of hydrology and meteorology (DHM). The environmental variables data from the freeze-killed treated days were analyzed with the HB score of treated colonies.

3.5 Data Analysis

The hygienic behavior scores were first tabulated in excel. The same was done for the environmental variables. Both data sets were imported into the R programming language and analyzed in the R-studio version 2022.12.0 Build ("Elsbeth Geranium"). Each sealed brood

in the FKB tests were considered individual for survival analysis. The plots were also generated in R–studio.

3.6 Ethical, legal, and social implications

All relevant consent was taken before conducting the research. The procedure was explained to the apiarist before starting the HB testing in the colony. Only after the consent, the test was performed in the colony. Maximum effort was undertaken to harm as little bees as possible.

4. RESULTS

4.1 Freeze-killed brood section in between seasons

The hygienic behavior testing in the colonies was done in two seasons (spring and autumn, 2022). To compare the hygienic behavior among the two seasons, it was crucial that they both had to perform the same amount of cleaning .

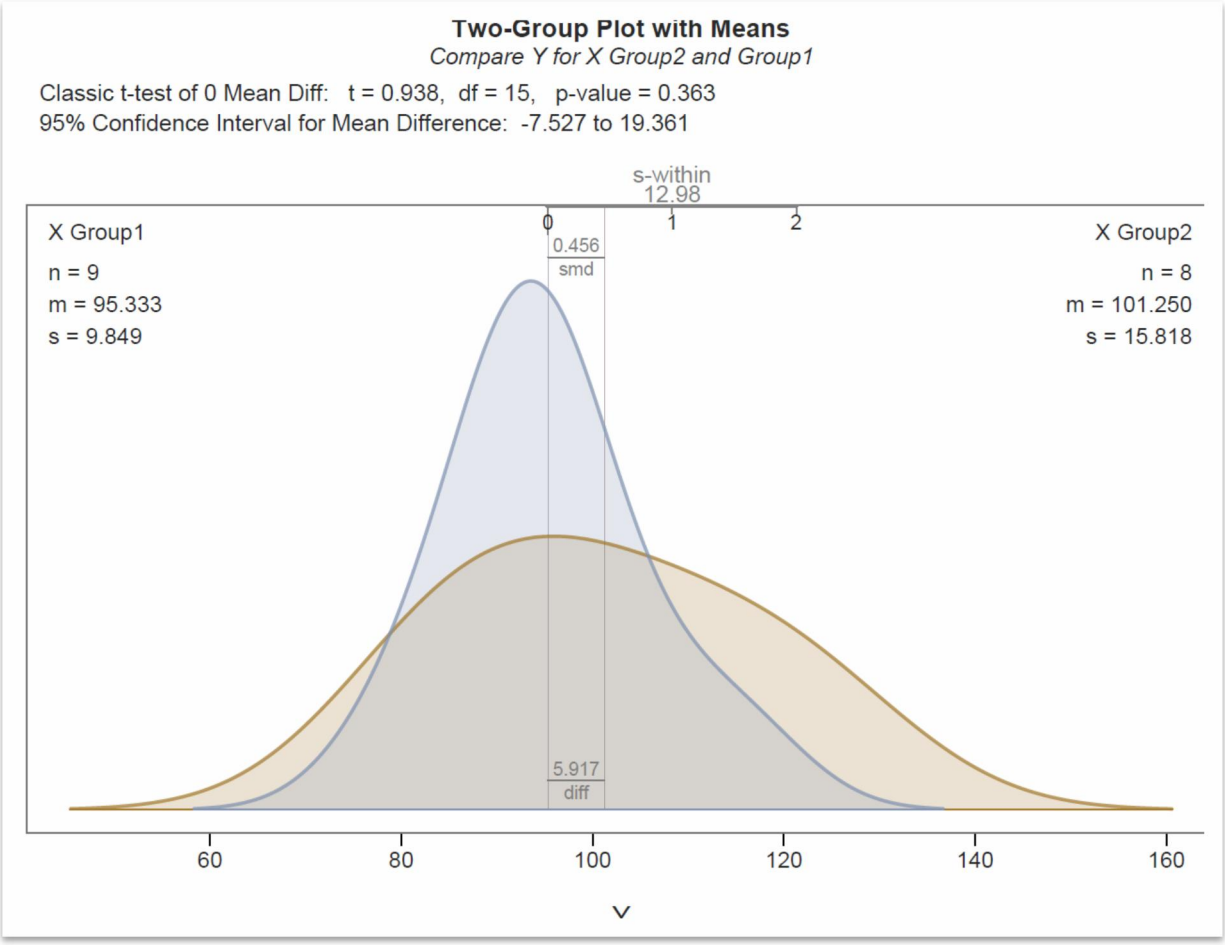


Figure 2. The number of freeze-killed broods in two seasons (X Group 1= spring, X Group 2= autumn).

In the tested *A. cerana* colonies, frozen comb sections during the spring and autumn were **95 ±10.0** and **101 ±16** cells, respectively. The difference in the number of freeze-killed cells that needed to be cleaned by the honey bees was not statistically significant **(**p value= 0.363). Hence, the honey bees had to perform an equal task of cleaning the treated cells in both seasons. Thus, comparison among the score of two seasons could be made.

4.2 Hygienic behavior of *Apis cerana* across seasons

The first objective of this study was to evaluate the hygienic behavior of *A.cerana* across two seasons (spring, and autumn), and to compare the HB score across two seasons. The evaluation was done post 24 hours as well as post 48 hours. The colonies which can remove more than 95% of FKB within 24 hours are considered hygienic colonies. This indicates that colonies can halt the transmission of disease through dead brood and protect the colonies from pathogens.

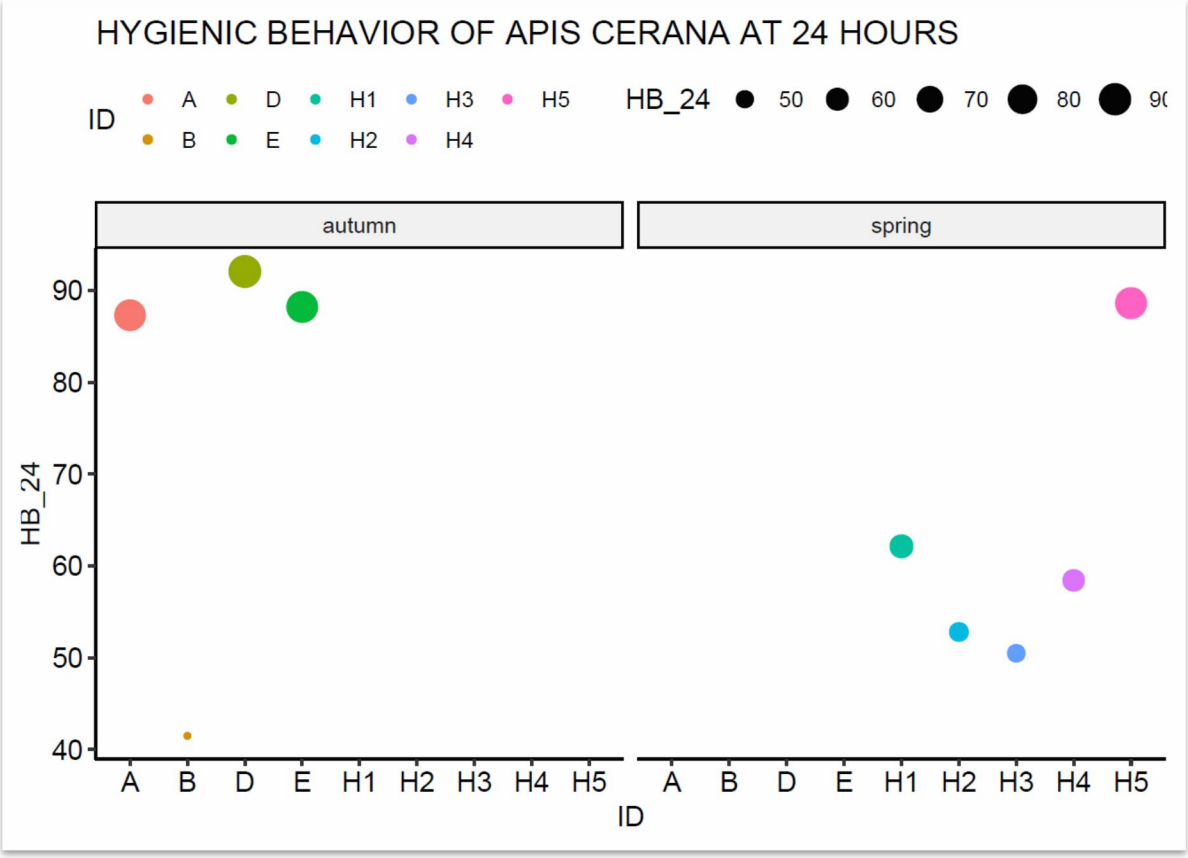


Figure 3. The hygienic behavior score of tested colonies at the 24-hour mark.

None of the tested colonies across both seasons had a hygienic score of over 95%. The lowest score reported was 41.5% (colony B, autumn), while the highest was 92.06 (colony D, autumn).

The evaluation was also carried out post -48 hours. 48 hours is a conventional cut off time to signify if the colonies were hygienic or not. More than 95% of the cleaning of FKB would account to a hygienic colony.

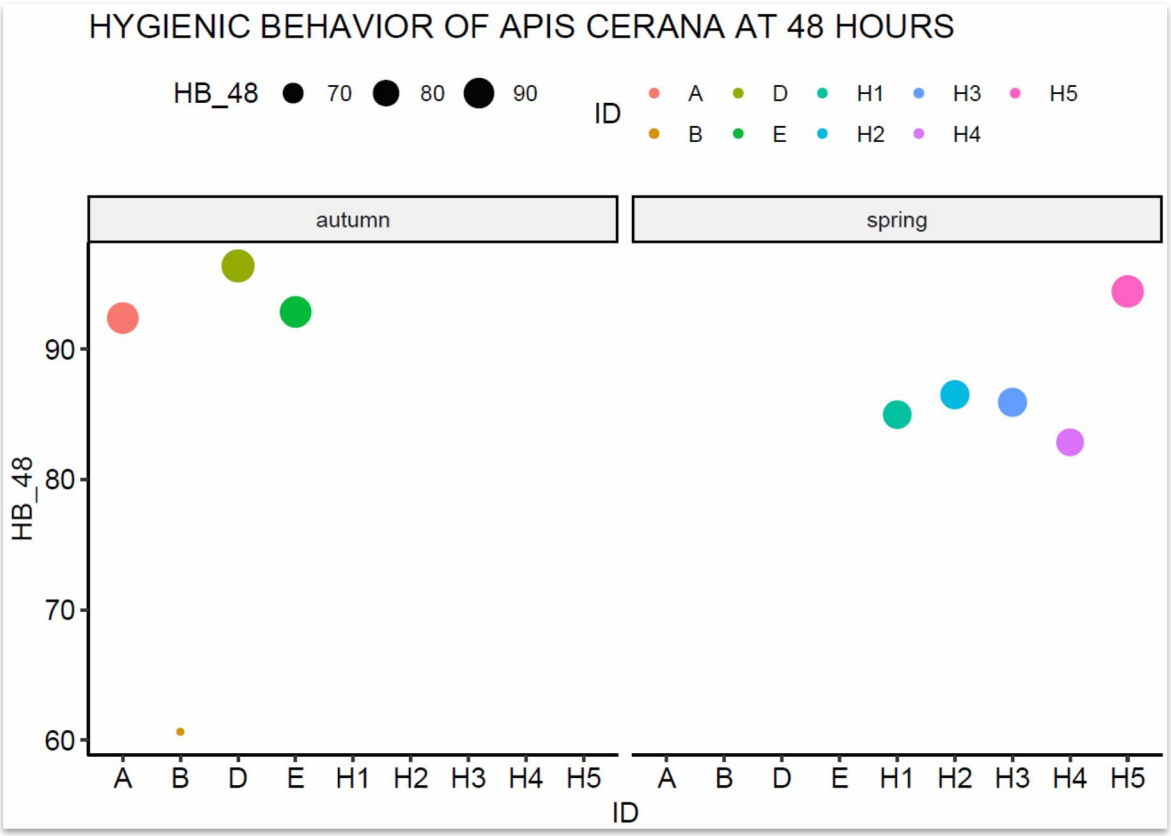


Figure 4. The hygienic behavior score of tested colonies at the 48-hour mark.

One among the nine colonies (Colony D, autumn) had a hygienic score of over 95% at the 48-hour mark. All the tested colonies had cleaned a better amount of freeze-killed brood at the 48-hour mark. On average, a 17.28% increase in hygienic behavior was observed in that time frame.

Honey bees remove the dead larvae after inspection. The trend of brood removal was analyzed by segregating the time under consideration into two distinct phases. These were day one and day two.

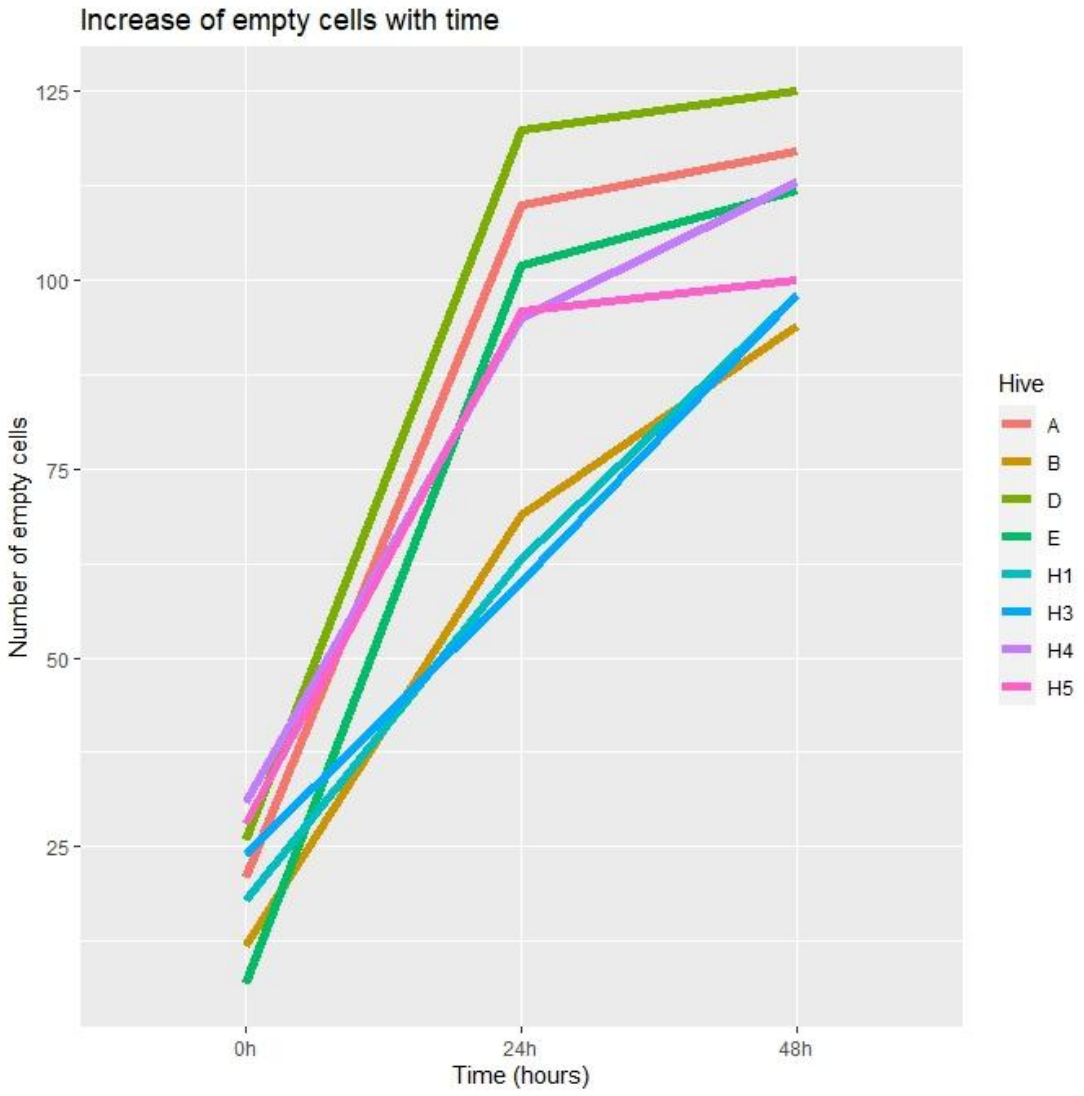


Figure 5. Increase in empty cells of treated section with time.

The figure shows a sharp increase in empty cells within 24 hours in the colonies indicating majority of cleaning is done within the first 24-hour period and that it is vital for limiting the spread of pathogens within the colonies. The chi-squared test showed a significant association between time intervals and empty cell counts in the hive trails ($\chi 2 = 12.25$, df = 1, p = 0.0004653), with more empty cells being created in the first 24 hours than the second 24 hours. The null hypothesis was rejected, and it can be concluded that the time interval has a significant effect on the number of empty cells in the hive trails.

4.3 Hygienic behavior scores across the seasons: do they vary?

As per the objectives, tests were carried out to see if there is difference among HB scores across seasons. The normality of data was not met in autumn season, hence non-parametric test (Mann-Whitney) was used to compare the scores across seasons post 24 as well as 48 hours.

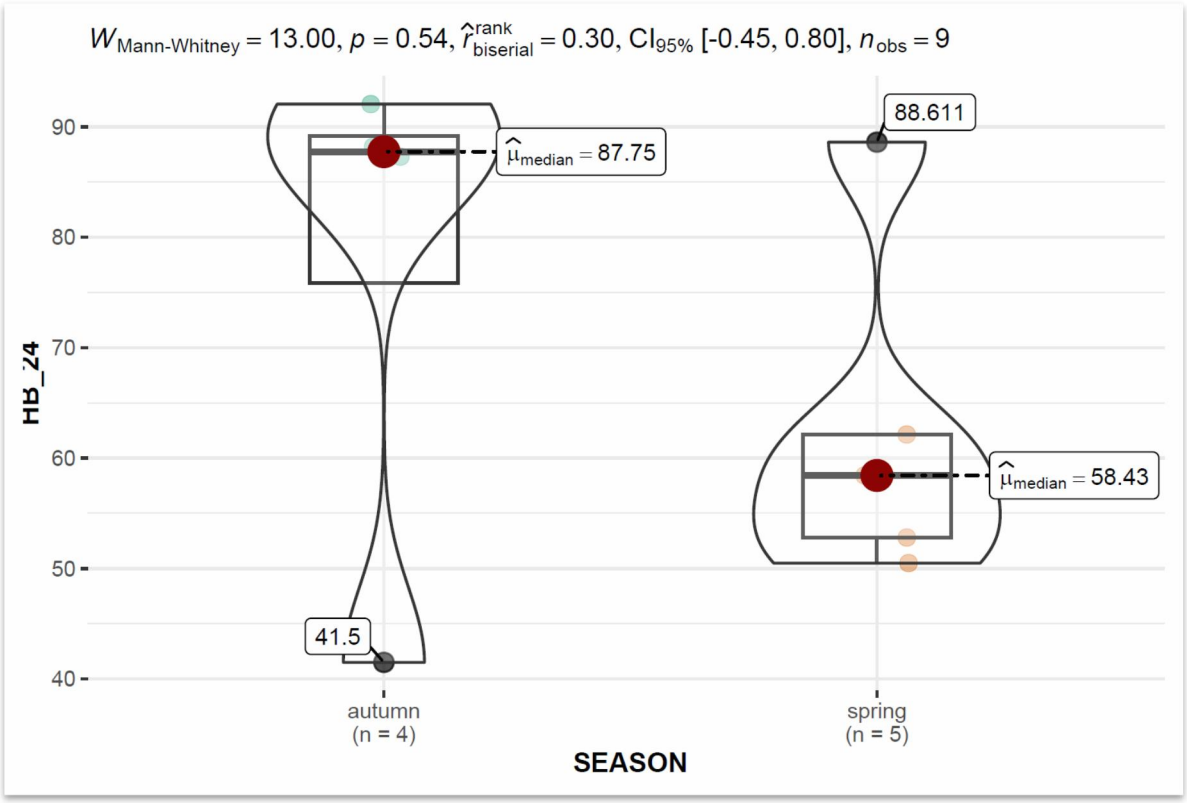


Figure 6. Mann-Whitney test of 24-hour hygienic behavior score.

The statistical analysis results indicate no significant difference between the two groups, as determined by the Wilcoxon-Mann-Whitney U test (Mann-Whitney = 13.00, p = 0.54). The effect size (rbiserial rank) was small (0.30), suggesting only a slight difference between the groups. The 95% confidence interval (CI95%) for the effect size ranged from -0.45 to 0.80, which includes zero, indicating a possibility of no true difference between the groups. Overall, these findings suggest no evidence of a significant difference between the two groups.

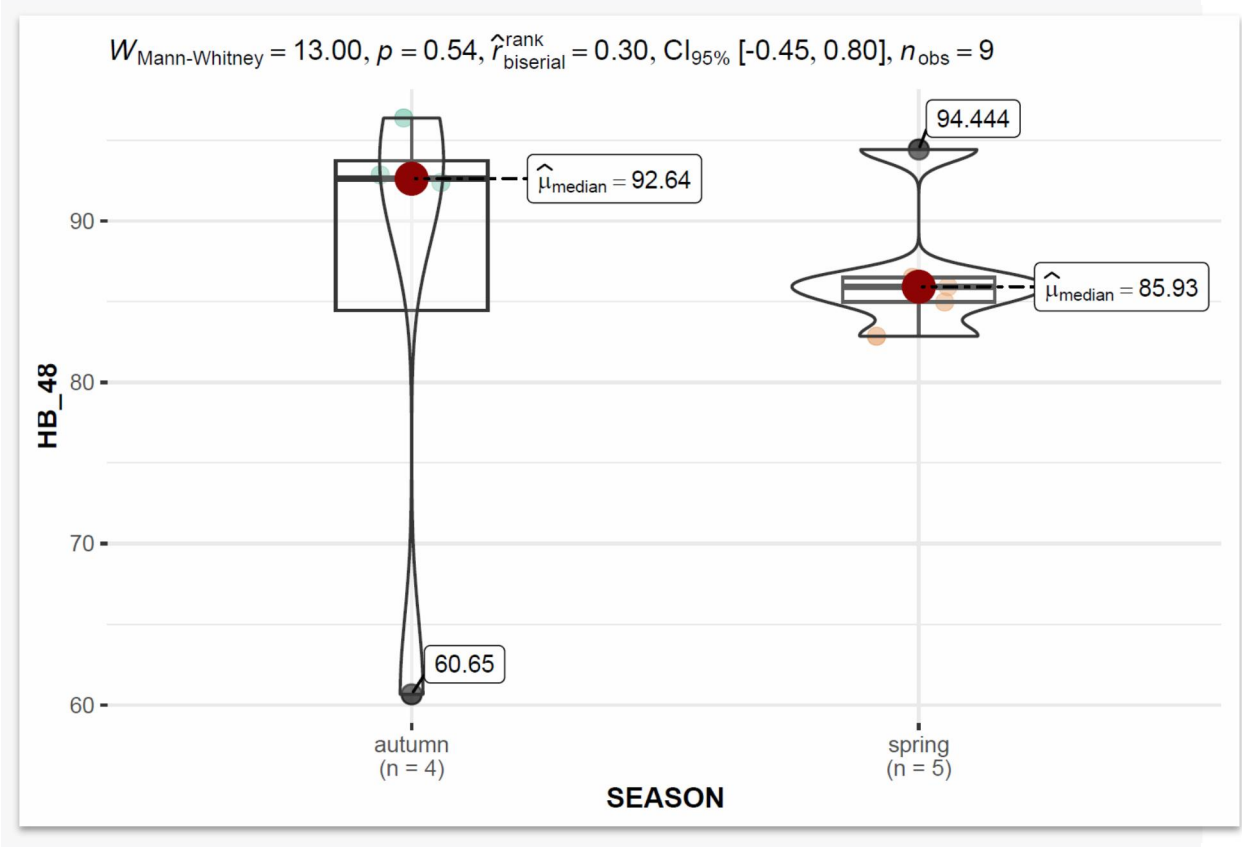


Figure 7. Mann-Whitney test of 48-hour hygienic behavior score.

The statistical analysis results indicate no significant difference between the two groups, as determined by the Wilcoxon-Mann-Whitney U test (Mann-Whitney = 13.00, p = 0.54). The effect size (rbiserial rank) was small (0.30), suggesting only a slight difference between the groups. The 95% confidence interval (CI95%) for the effect size ranged from -0.45 to 0.80, which includes zero, indicating a possibility of no true difference between the groups. Overall, these findings suggest no evidence of a significant difference between the two groups.

In both instances, the analysis revealed that there was no significant difference between the HB score (post 24, and post 48 hour).

4.4 Uncapping and brood removal rate across the seasons:

Survival refers to the ability of honey bees to identify and remove diseased or dead broods from the hive to maintain the colony's health. This process involves identifying a brood infected with a disease or otherwise unhealthy and removing that brood from the hive before the infection can spread. On the other hand, uncapping refers to the physical act of removing the wax cap that covers the brood cell. Worker bees typically do this to feed the developing brood or inspect it for signs of disease.

4.4.1 Brood removal rate among seasons:

Figure 8. Kaplan-Meier plot showing survival probability of brood.

For the autumn season, the survival probability at 24 hours was 0.396, meaning that approximately 40% of honey bee cells survived beyond 24 hours. For the spring season, the survival probability at 24 hours was 0.525, meaning that approximately 52% of honey bee cells survived beyond 24 hours. At 48 hours, the estimated survival probabilities were lower for both seasons, with approximately 21% and 20% of honey bee cells surviving in the autumn and spring seasons, respectively.

In the context of honey bee hygiene, a lower survival probability means that the worker bees remove more cells containing dead or diseased broods, indicating better hygiene in the colony. Therefore, the lower survival probability of cells in the autumn season, compared to the spring season, indicates that the hygienic behavior of honey bees in autumn is more effective at removing dead or diseased broods; This is a positive outcome for the overall health and productivity of the colony.

Mantel- cox test (log-rank test) revealed brood removal experience differs significantly between the "spring" and "autumn" seasons (χ^2= 14.2, df=1, p < 0.001), with the "spring" season associated with a higher survival probability of the dead brood. Thus, there is a greater probability of brood removal in the autumn season.

4.4.2 Uncapping rate among seasons:

Figure 9. Kaplan-Meier plot showing the uncapping rate of brood during seasons.

For the autumn season, approximately 30% of the sealed bees remained capped after 24 hours, and this proportion decreased to 20% after 48 hours. For the spring season,

approximately 40% of the sealed bees remained capped after 24 hours, and this proportion decreased to 16% after 48 hours.

Mantel- cox test (log-rank test) revealed brood uncapping experience differs significantly between the "spring" and "autumn" seasons (χ^2= 4.2, df=1, p=0.04), with the "spring" season associated with a higher capped cell. Thus, there is a greater probability of brood removal in the autumn season.

Therefore, the lower uncapped cells of the treated section in the autumn season compared to the spring season indicates that the hygienic behavior of honey bees in autumn is more effective at removing dead or diseased broods, which is a positive outcome for the overall health and productivity of the colony.

4.5 Effect of environmental variables on the hygienic behavior of *Apis cerana*:

For the fulfillment of the second objective, climatic variables during the time of test were collected from DHM. These variables were

The effect of environmental variables (rainfall, temp, relative humidity) on hygienic behavior and the association, if any, between these variables and hygienic behavior was analyzed.

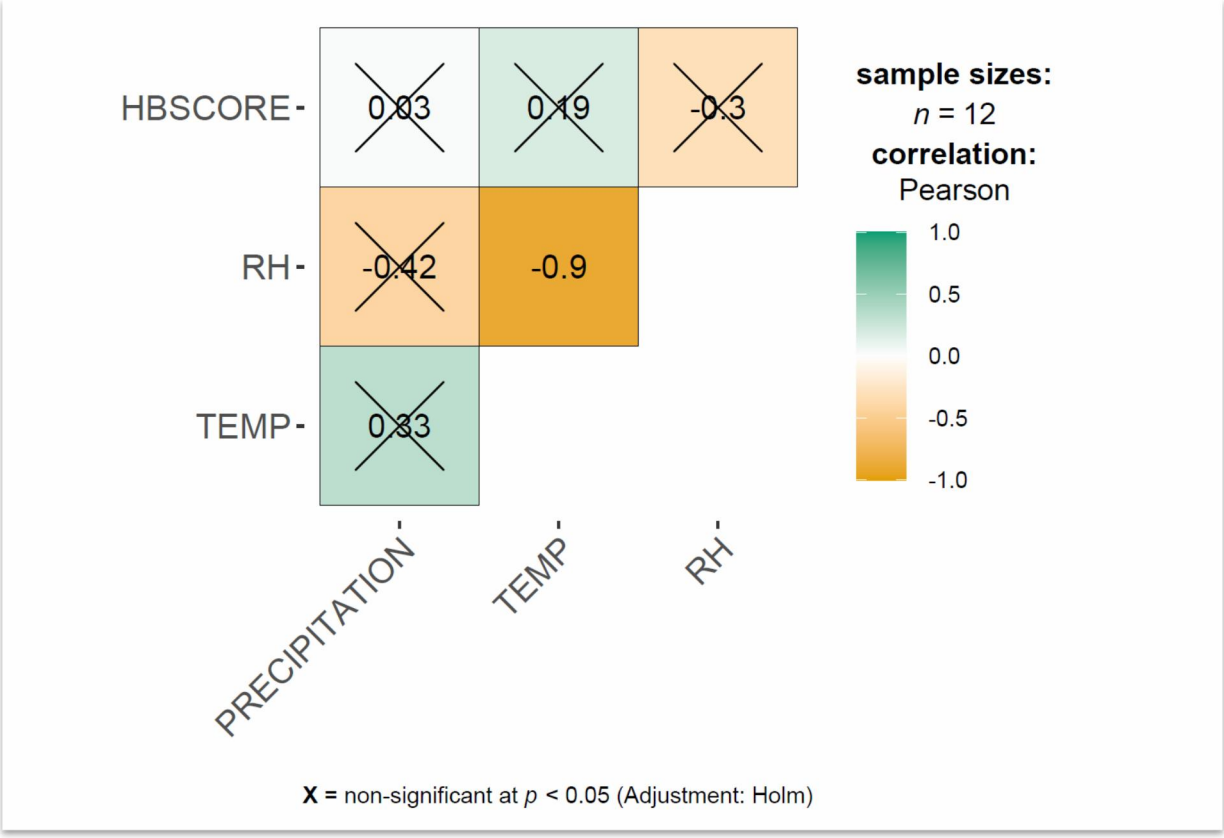


Figure 10. Relationship among environmental variables and hygienic behavior score.

None of the environmental variables tested for the association with hygienic behavior was statistically significant. Pearson's product-moment correlation analysis revealed that there was a weak positive correlation (r = 0.19) between HBSCORE and TEMP, but this correlation was not statistically significant (p > 0.05). Additionally, there was a weak negative correlation (r = -0.3) between HBSCORE and RH, but again, this correlation was not statistically significant (p > 0.05). Finally, there was a moderate positive correlation (r = 0.33) between HBSCORE and precipitation, but this correlation was also not statistically significant (p > 0.05).

4.6 Presence of heavy metals in the honey samples of the tested hive:

For objective three, the heavy metals were evaluated from the collected honey samples of the treated colonies. Sealed honey was collected from the hives when HB test was done to ensure that the honey was from the treated time frame.

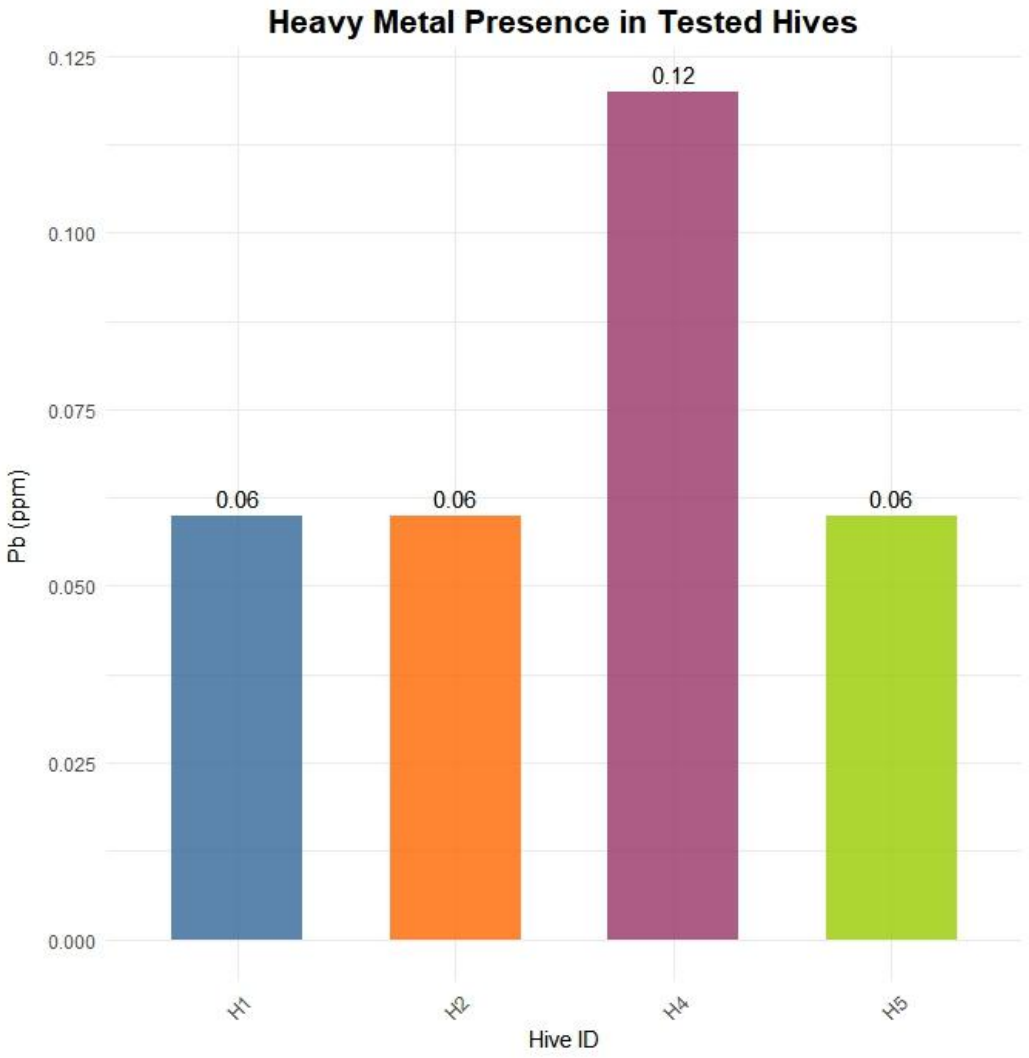


Figure 11. Lead (Pb) Prevalence in the tested honey samples.

All the tested honey samples from the respective hives had a lead (Pb) prevalence. One of the four samples had a cadmium (Cd) presence. The tested samples did not detect other heavy metals (Zinc and chromium). The presence of lead in the honey sample shows that heavy metals make their way into the bee hive.

4.7: Association between heavy metals and hygienic behavior scores:

From the tested colonies, association among the HB score and Lead level if any were evaluated.

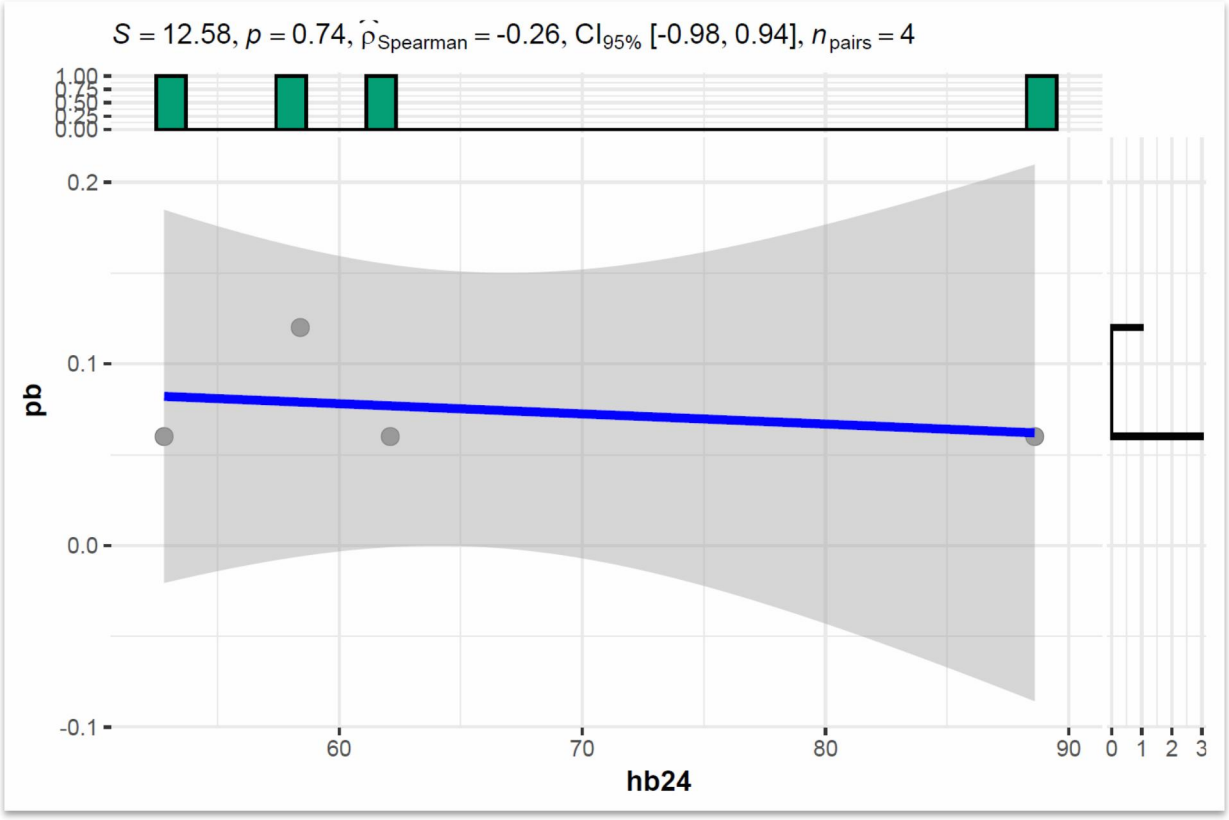


Figure 12. Association between Pb levels and Hygienic behavior scores of tested colonies.

The analysis found a weak negative monotonic relationship (Spearman's rank correlation coefficient = -0.26) between the two variables, suggesting that as one variable increased, the other tended to decrease. However, the p-value of 0.74 indicated that this negative association was not statistically significant. The 95% confidence interval for the true population correlation coefficient was wide [-0.98, 0.94], suggesting high uncertainty about the relationship. Further research with a larger sample size is needed to confirm these findings.

5. DISCUSSION

The objectives of this study were to find out the hygienic behavior of *Apis cerana* and to observe if seasonal differences occur in the behavior. Along with that, the study also addressed the presence/absence of heavy metals within the hive. The literature focusing on hygienic behavior of *A. cerana* is limited; wherever possible the comparison is made with *A. cerana* but in other instances the comparison is done with the European counterpart *Apis mellifera.*

None of the treated colonies at 24-hour mark had a hygienic behavior score of >95% (50.493–88.611 in spring, 41.5–92.06 in autumn). HB is a rare trait in nature, and thus it is scarcely present in non-selected colonies (Bigio et al. 2013). Likewise, the HB score increased at 48-hour interval in both season with one colony having >95% dead brood removal (equates to a HB of 11% among tested colonies). The score of tested colonies themselves did not differ between seasons suggesting seasons do not have prominent roles in determining the HB, and that the test could be performed at any time regardless of the temporal effect. This finding is concurrent with findings from (Bigio et al. 2013) who demonstrated that seasons did not influence the hygienic behavior of *A. mellifera* . However, the study by (Uzunov et al. 2014) revealed that seasons had an influence on this behavior of honeybees (*A. mellifera*).The difference could be due to large scale studied as well as pan-geographical study sites selected for the study whereas this study was confined to a single region. In addition, the species tested in the study was *A. mellifera*, so further large-scale study with *A. cerana* will be required to test if seasons have a say in the hygienic behavior.

The study showed a sharp increase in empty cells within 24 hours in the colonies indicating majority of cleaning is done within the first 24 hour period and that it is vital for limiting the spread of pathogens within the colonies .The chi-squared test showed a significant association between time intervals and empty cell counts in the hive trails ($\chi 2 = 12.25$, df = 1, p = 0.0004653), with more empty cells being created in the first 24 hours than the second 24 hours. This is again in line with the research from (Lin et al. 2016) whose findings suggest that earlier period for dead brood removal remains vital for limiting the pathogens spread within the colony.

Various studies reveal that *A. cerana* is faster than *A. mellifera* in performing hygienic behavior (Lin et al. 2016; Shakeel et al. 2020).The underlying mechanisms to perform hygienic behavior depends on odorant genes and their protein products which acts to trigger the hygienic behavior in worker honey bees. It has been observed that *A. cerana* removes mite infested brood (*Tropilaelaps mercedesae* Anderson and Morgan) faster than *A.mellifera* (Shrestha et al., 2020). This in turn can explain why the native bee is resistant to mites' infection. Although the honey production of *A. cerana* is less compared to *A.mellifera*, its strong resistance against various pathogens should work in its favor for beekeepers to rear this bee in the apiary.

Likewise, the study revealed the possibility of having a hygienic colony of *A. cerana* in commercial colony is rare. So, before breeding for the new queens, a simple test like this can be performed to select the hygienic bees' colonies, and subsequent queen production can be done from the colonies showcasing high hygienic behavior. These colonies can further be pathogenetically challenged to determine their resistance to brood diseases (Spivak and Danka 2021).

The survival analysis of uncapping as well as brood removal rate showed that both processes were faster in autumn tested colonies than spring tested colonies. This result is contemporaneous with study from (Lin et al. 2016). The reason for increased uncapping as well as brood removal in autumn season when compared to spring could be due to the widespread prevalence of Sacbrood virus in autumn. So, the honey bees *A. cerana* may respond strongly to dead and deceased brood during this season. The same phenomenon was reported in the European honey bee which also has increased pathogenic pressure in autumn and winter season.

In this study, environmental variables (precipitation, relative humidity, temperature) did not influence the hygienic behavior of the honey bees. However, study on *A. mellifera* revealed rainfall and altitude significantly impacted the hygienic behavior (Sousa et al. 2016). Comparable work on evaluation of hygienic behavior of honey bees *(A. mellifera)* showed that altitude influenced the hygienic behavior (Masaquiza et al. 2021). While other studies on *A. mellifera* did not find the association between elevation and hygienic behavior (Muli et al. 2014). This study was limited to single sites and hence we could not confer whether the same is true in the case of *A. cerana*. Further study is needed to evaluate if altitude influence the hygienic behavior of Asian honey bees.

This study found the presence of heavy metals in the tested colonies. Lead was found in all the tested samples. This proves that heavy metals are incorporated in the pollinator's ecology. The presence of heavy metals in honeybee hives have been proved in numerous studies (Bortolotti 2003; Celli and Maccagnani 2003; Di Fiore et al. 2022; Ruschioni et al. 2013; Salkova and Panayotova-Pencheva 2014; Skorbiłowicz et al. 2018). This study further proves the notion that environmental contaminants such as heavy metals do make their way into the honeybee's colonies. This study also proves that heavy metals contamination is prevalent in *A. cerana*, the native honey bee of Nepal.

Honey bees including *A. cerana* are central foragers who relies on pollen and nectar for the proper functioning of the colony, during which honeybees may come in contact with heavy metals (Johnson 2015) . In addition, these honeybees needs to overcome major cognitive challenges (acute visual as well as olfactory processing) to gather resources; Stressors like heavy metals can act upon the intricate neuronal dynamics and hamper the foraging ecology of these organism (Klein et al. 2017).

The presence of lead in the honey possesses significant disadvantages to honey bees. Lead are heavy metals whose impacts on pollinators have been documented widely. Numerous physiological disruptions by heavy metals on honey bee health have been reported. A study carried out by (Bromenshenk et al. 1991) discovered that lead presence in the honey bee hive were correlated with deteriorating of hive health. Likewise, (Gizaw et al. 2020) found lead as well as cadmium exposure leads to decrease in foraging activity. The presence of lead in the tested samples in this study point out to the fact that, heavy metals as stressors must be accessed to get a comprehensive picture of factors which are affecting the population of native honey bee of Nepal. Along with existing threats which have wreak havoc in the population of *A. cerana* (pathogens, beekeeping practice, nutritional stress), heavy metals threats need to be considered as a new potential threat to these pollinators' biology.

Even though heavy metals were reported in the tested samples, a significant association between heavy metals and hygienic behavior of tested colonies were not reported. Large scale study across the nation where honeybees are reared near anthropogenic emissions of heavy metals (preferably large metropolitan) could be carried out in near future to better access if heavy metals disturb one or more physiological functioning of honey bees. As hygienic behavior is a part of social immunity, the factors which could potentially disturb the underpinning of this behavior will have a significant negative impact on hive health.

Apis cerana faces series of challenges for their survival; the genetic stock of *A. cerana* is dwindling in Nepal (Aryal et al. 2015). The hygienic behavior of honeybees is also under genetic control; This study showed that a colony with superior hygienic behavior is lacking in commercial colonies. This may be since there is relatively little gene pool available to continue the generation. In the future, genetic improvement of *A. cerana* should be done to ensure the survival of species. This view is concurrent with (Thapa et al. 2018b) who suggested improvement of genetic stocks for better health of the species.

The two most reared honey bee species in Nepal *A. cerana* and *A. mellifera*. Among them *Apis mellifera*, an introduced honey bee species , was established in Nepal in the 1990s (Bhatta, 2018).The impact of *A. mellifera* on *A. cerana* can occur in various instances (shift in apicultural species, competition for resources, exchange of pathogens and pests in species, reproductive competitions, as well as an impact on ecological systems) (Bhatta 2018). Nepal has an estimated 250,000 honeybee hives with a total honey production of about 4000 metric tons of honey (Government of Nepal 2022). Of these, both *A. mellifera* and *A. cerana* contribute to a huge share of honey production. However, the increase in popularity of *A. mellifera* over *A. cerana* for honey production has put extra pressure on the already dwindling native bee population. *Apis mellifera* can yield 20–100 kg honey per hive, which is far greater than 8–15 kg yield from *A. cerana* hive (Devkota 2020); hence commercial bee farming is shifting towards the rearing of *A. mellifera.*

Such scenario points out the grim picture of *A. cerana* in Nepal. Effective intervention to prevent the decline of this species is the need of the hour. This may come in various form; improving the genetic stock of bees, highlighting the importance of this species on pollination of native flora, encouraging bee keepers to adopt and rear the native honey bee species are some of the tasks which could be done to stop the decline of *A. cerana* population in Nepal, and preserve this species.

6. CONCLUSION

The study conducted on *Apis cerana*, the native honey bee of Nepal, focused on understanding their hygienic behavior and the presence/absence of heavy metals within their hives. The study found that hygienic behavior is a rare trait in nature, and thus it is scarcely present in non-selected colonies. The study showed a sharp increase in empty cells within 24 hours in the colonies indicating majority of cleaning was done within the first 24-hour period. Also, there were no significant differences between the two groups in HB score in both 24 hour and 48-hour mark.

Both uncapping and brood removal rates were faster in autumn when compared to spring. This may be since pathogen prevalence in autumn and subsequent winter season is high, and that honey bees have historically evolved to better inspect the colonies and eliminate potential threat as soon as possible.

None of the environmental variables tested for the association with hygienic behavior was statistically significant. The environmental variables such as precipitation, relative humidity, and temperature did not influence the hygienic behavior of *A. cerana*, indicating that their behavior is more consistent and stable.

The study also found the presence of heavy metals in the tested colonies, with lead being present in all the samples. This suggests that heavy metals are incorporated into the pollinator's ecology, and their contamination is a serious issue in A. cerana. The presence of heavy metals in honeybee hives has been proved in numerous studies, indicating the need for further monitoring and mitigation measures to protect their health and well-being.

To conclude, the study on hygienic behavior and heavy metal contamination in *A. cerana* hives found that the trait is rare in nature. Brood removal and uncapping rates were found to differ significantly between spring and autumn seasons. Environmental variables tested did not show a significant association with hygienic behavior. The presence of heavy metals in the hives indicates an issue with their contamination, highlighting the need for further monitoring and mitigation measures to protect the pollinators' health and well-being.

7. RECOMMENDATIONS

The following recommendation could be implemented for betterment of *Apis cerana* populations in Nepal.

- The study recommends performing a simple test to select the hygienic bees' colonies before breeding for new queens to produce colonies with high hygienic behavior.
- Although the honey production of *A. cerana* is less compared to *A. mellifera*, its strong resistance against various pathogens should work in its favor for beekeepers to rear this bee in the apiary.
- The study sheds light on the hygienic behavior and heavy metal contamination in *A. cerana*, indicating the need for further large-scale studies to understand their behavior and protect their health and well-being.
- Effective intervention to prevent the decline of this species is the need of the hour. This may come in various forms, improving the genetic stock of bees, and highlighting the importance of this species on pollination of native flora.

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