



Effect of different levels of Iron on the development and structure of hypopharyngeal glands of honeybee workers

Dalal M Aljedani

Department of Biology, College of Science, University of Jeddah, Jeddah, Saudi Arabia

Abstract

The hypopharyngeal glands (HG), the most important organ in Hymenoptera, are crucial for insect physiology. The royal jelly that is fed to queens and larvae is made possible by the (HG) in honeybee worker bees. This study examined the effects of inorganic iron (Fe) addition on the development and structure of the (HG). We tested the effects of high iron supplementation on *Apis mellifera jemenatica* hypopharyngeal gland growth during the off-season using various concentrations of inorganic iron. With three hives in each group, we randomly divided 12 beehives into the following four treatment groups: Fe0 (without any additional Fe), Fe25, Fe50, and Fe100 (each with an additional 25, 50, and 100 mg L⁻¹ Fe). The treatment lasted three months. Each group's six-day-old nursing bees were sectioned, and the sections were then viewed under a microscope to study the morphology of the glands. A reliable measure of whether Fe supplementation levels surpassed the dietary requirements for iron that might alter HPG development supplied to nursing bees is the mass and size of the HGs. The formation of HPG may be impacted by iron, according to ANOVA results. (*Apis mellifera* L.) nutritional requirements were exceeded by Fe supplementation levels, which had a significant impact on HG mass and acini size. In the current investigation, we discovered the impact of various Fe supplementation levels on nursing honeybee workers' HGs development and acini size.

Keywords: beekeeping, exocrine gland, hypopharyngeal gland, iron, mineral supplementation, morphology, nutrition

Introduction

Like all living things, honeybees (*Apis mellifera* L.) need nutrition in amounts and types sufficient for their everyday activity and developmental needs. Their dietary requirements are entirely met by floral resources, which contain amino acids and phytochemicals. Since pollen is the primary supply of proteins, amino acids, minerals, and lipids in honeybee colonies, its composition directly affects their nutritional status. These pollen-derived nutrients may have an impact on how the hypopharyngeal glands grow (HPGs) [1], situated on the worker nurse bees' heads and in charge of producing royal jelly (RJ) [2]. The HPGs are in charge of generating a secretion that is high in protein [3].

The paired hypopharyngeal glands, which are found in the heads of both worker and queen honeybees but are only fully formed in workers, are feeding glands. These acinous, age-dependent structures are dispersed in several loops directly behind the compound eyes on the front of the skull. The biggest gland in the worker bee's head cavity, these glands have 1000 or more pear-shaped lobules linked to a lengthy duct [4]. The components of royal jelly, which are supplied to larvae and the queen, are produced and released by the hypopharyngeal gland. When bees first emerge, these glands are underdeveloped. By the time bees are fed (6–12 days later), these glands are fully formed with huge active secreting acini. By the time bees begin foraging, these glands have degenerated [5].

The anatomical changes in the hypopharyngeal gland that take place as worker bees mature and/or take on new activities have been the subject of several investigations [6]. Since they are crucial for creating royal jelly, they are studied. Few research, however, has examined how the nutrition provided to honeybees affects how their glands

grow [7]. The nutritional state of the bees, seasonal circumstances, geographic origin, and pesticide exposure all have an impact on HPG formation [8].

Nutrition is recognized to have a direct impact on an animal's survival, health, and ability to resist disease [9]. Due to their crucial functions as structural components and as enzyme cofactors, minerals are among the nutrients required to sustain life [10]. Iron is the most crucial mineral for the growth of insects among the necessary minerals. Along with phenylalanine and tyrosine, several iron-containing enzymes play crucial roles in DNA and RNA production as well as melanization, which is the process of developing the immune system. Examples of these enzymes are ribonucleotide reductase and RNA polymerase I [11]. Additionally, significant iron-rich granules are found in the abdomen to aid bees in orienting themselves about the earth's magnetic axis, which increases foraging productivity and increases swarm survival [12]. Therefore, dietary deficits may affect the quality of royal jelly, the health of the queen, and the emergence of the swarm [13].

Understanding the function of proteins and metal intrinsic identification, targets of the proteome, and metalloproteomic studies can help with regulating the diet of honeybees [14]. Ionic iron has two qualities that insects must balance: it is both a vital nutrient and a deadly poison. Iron is required for oxidative metabolism's catalysis, but it also must be kept under control to prevent harmful oxidative reactions. The storage protein ferritin and the serum iron transport protein transferrin have evolved into different forms in insects. These proteins may perform distinct tasks in insects than they do in other types of life. In insects, iron regulates protein synthesis like vertebrates' in terms of translational regulation [11]. The present study has aimed to see the effect

of various levels of Iron-induced on the development and histology of the hypopharyngeal gland in Honeybee colonies *Apis mellifera jemenatica* in Saudi Arabia conditions.

Materials and Methods

Study Area

At the Beekeeping Production Area of the Faculty of Environmental Sciences research station at KAU of Saudi Arabia, the experiment was conducted in the apiary in June, July, and August of 2022. on honeybee workers (*Apis mellifera jemenatica*) (Hymenoptera: Apidae). After then, it was transferred to the Entomology Laboratory at the University of Jeddah.

Experimental Groups

Twelve *Apis mellifera jemenatica* beehives were divided into four treatments, each with three hives, and their breeding and feeding frame counts were standardized.

Fe0: control strategy, no iron supplemental therapy

Fe25: 25 mg L⁻¹ of supplementing with iron

Fe50: 50 mg L⁻¹ of supplementing with iron

Fe100: 100 mg L⁻¹ of supplementing with iron

As a food supplement, sugar syrup was used in all regimens. The other treatments got varied levels of the necessary amounts of inorganic iron, whereas the Fe0 treatment received no extra inorganic iron. The recommendations of [15], who suggested supplementing with 50 mg L⁻¹ of iron, served as the foundation for these criteria. After the trial period, the bees were collected. The iron source utilized for this experiment was ferrous sulfate heptahydrate (FeSO₄.7H₂O, 20% iron), which was provided via a Boardman feeder and diluted in sugar syrup at a 1:1 (m/v) ratio using commercial crystal sugar (500 mL per week) [16].

Development of hypopharyngeal glands

Bee sampling was performed based on the methodology described by [17]. Then 80 worker bees at the age of 6 days were taken from a random hive frame in June, July, and August for each treatment. From each treatment, a frame containing pupae was taken out and each one was wrapped in a tissue. The frames were kept in an incubator until the individuals emerged at a temperature of around 30 °C and relative humidity of about 60%. The newly formed worker bees were released into their mother colonies after being tagged in the thorax with a non-toxic pen. Nursing bees were removed at 6 days old using entomological forceps and immediately placed in a plastic tube with openings to allow air to enter. They were then put to death by being cut off with a knife after being put to sleep with CO₂ before having their heads placed in 4% formaldehyde for 24 hours to improve exoskeleton permeability. The bees' heads were then cleaned under running water for 24 hours before being immersed in 70% ethyl alcohol until they were processed for histological examination [18].

Histological analysis

Histological examinations were conducted using a modification of the approach outlined by [19]. Cuts were performed on the front and posterior sections of the heads

before the samples were treated with ethanol in a progressive series at 70%, 80%, 90%, and 95 percent (removing a layer of chitin that was about 1 mm thick). The heads were then embedded in methacrylate resin (HistoResin®, Leica, Heidelberg, Germany), and added to plastic molds with the assembly resin, forming blocks to produce slides. Wooden blocks were bonded together. Using an automated rotary microtome, the blocks were divided into 3- μ m parts (Leica RM2155, Germany). From each block, serial slices were cut, which were then immediately placed on glass slides. Each block produced an average of 13 slides, with each blade producing 12 slices. Hematoxylin-eosin was used to stain the slides containing the sections, which were then oven dried before being covered with coverslips and mounted with Entellan®. The sections were photographed under the microscope using a digital camera, and the Leica Q-win Plus® image analysis program was used to examine the results.

Measurement of the development of hypopharyngeal glands

Using a digital camera (Leica DC300FX) connected to a microscope (Leica 2 D. C. B. de Barros *et al.* DMLB80), the slices were photographed, and the photos were examined using image analysis software (Leica Q-win version 3 for Windows). For bees in each of the experimental groups, the average size of the reservation and the height of the secretory cells in the hypopharyngeal glands were measured.

Statistical Analysis

ANOVA was used to compare the data of hypopharyngeal glands, and Tukey's test was used to confirm that the means differed when $p < 0.05$ [20].

Results

The most significant organ in Hymenoptera, are hypopharyngeal glands (HGs), which are essential to insect physiology, in honeybee (*Apis mellifera* L.) generates royal jelly (RJ) in its hypopharyngeal gland (HG), which is necessary for feeding and caring for queens and broods, this study was conducted to test the addition of different levels of iron and its effect on the hypopharyngeal gland of honeybee individual workers.

Development of hypopharyngeal glands

In honeybees, HGs contain bilaterally paired structures in the head and between compound eyes in front of the brain. Thousands of secretory units connected to the worker bees' secretory duct make up each gland. Additionally, morphological, and physiological changes occur in the bees' HG. In a worker bee, the HG acini are morphologically fully formed after around 6 days Fig.(1). The results showed that the hypopharyngeal gland area in honeybee colonies that feed on different levels of iron (Fe25, Fe50, and Fe100) decreased significantly compared with the control that doesn't add iron in feeding (Fe0), that were hypopharyngeal glands and arrangement of acini (a), around a collecting duct (cd), a connection of acinus to the collecting duct via a cable-like cluster of canal cells (cc), Fig.(2).

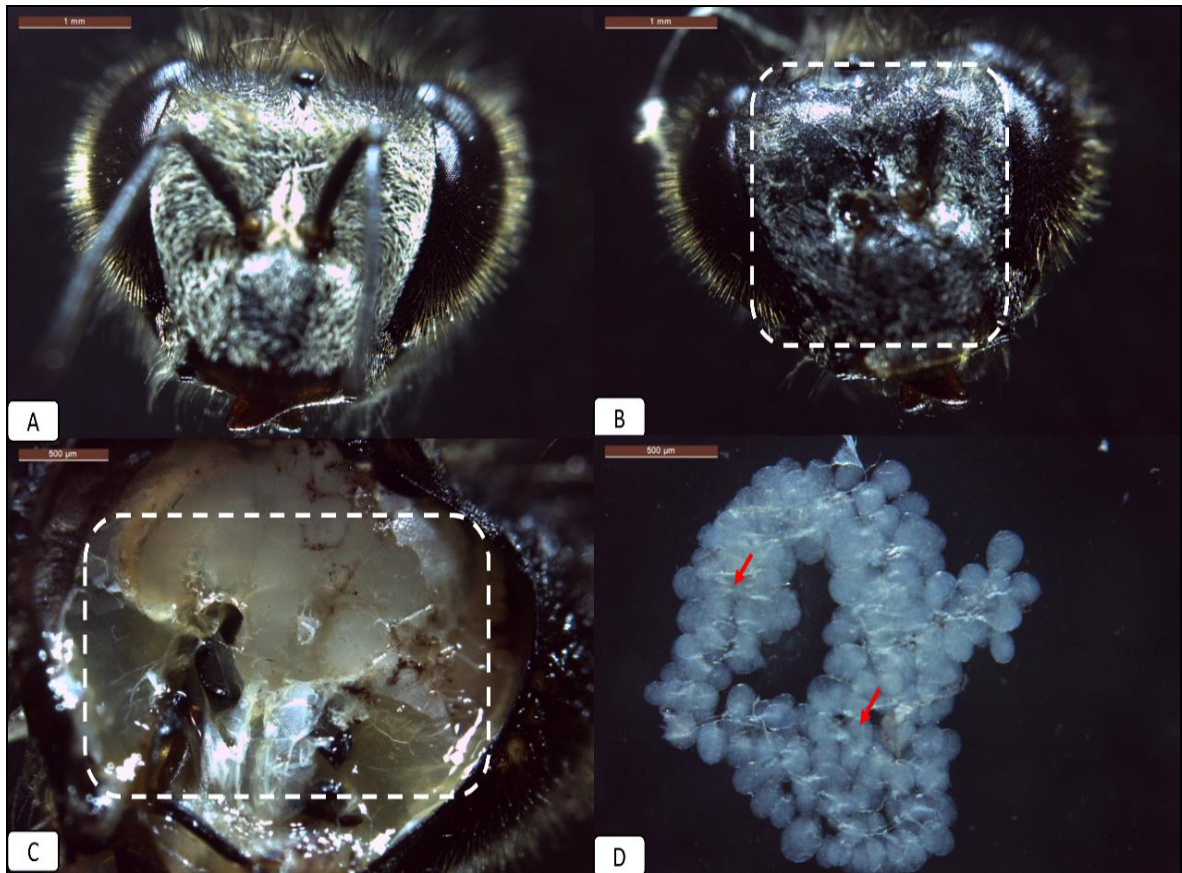


Fig 1: A nursing honeybee's hypopharyngeal glands are being dissected. Hypopharyngeal glands are placed on the head's lateral sides (arrows indicate acini), and the head is fixed on a paraffin dissecting plate in the images above (A, B, and C) (D). Scale bars: 1 mm (A, B), 500 m (C, D).

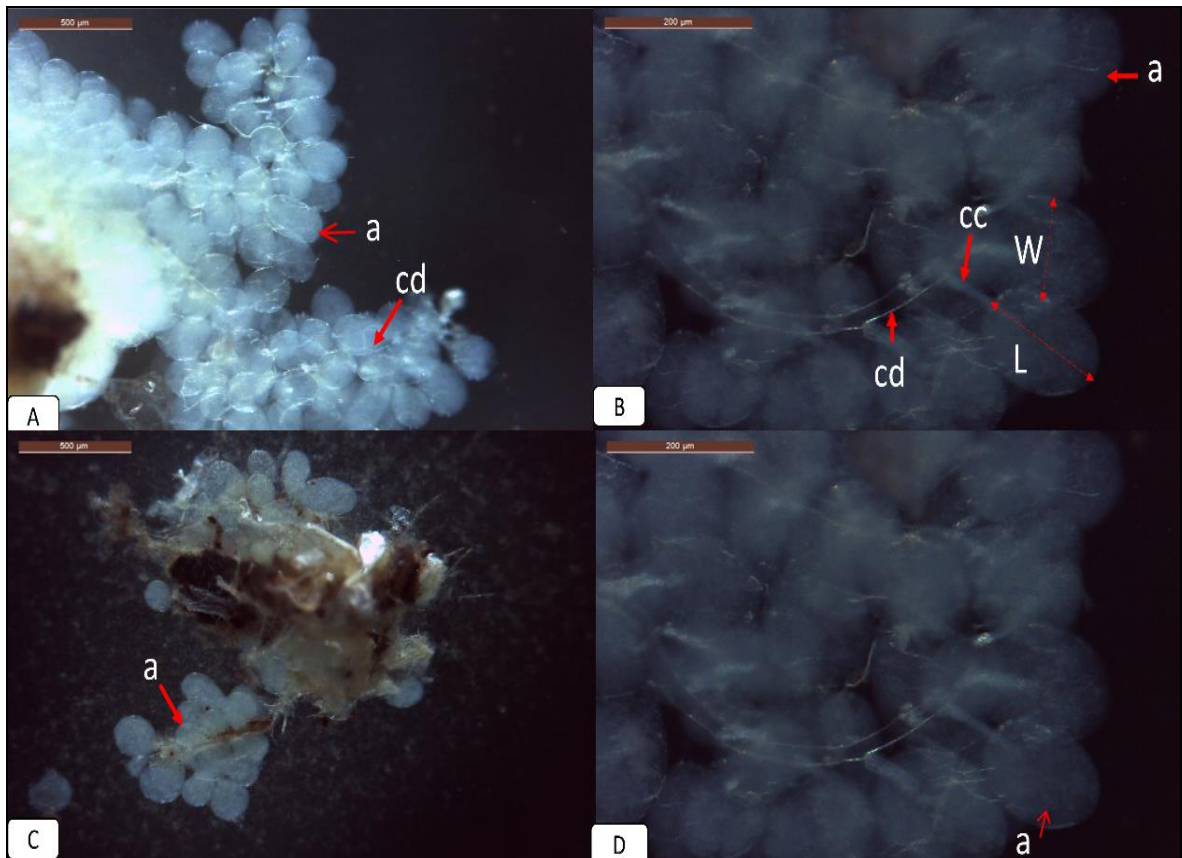


Fig 2: Honeybee worker hypopharyngeal glands photographed under a light microscope. Colonies that feed without Fe addition) (A&B), colonies that feed on various iron levels (C, D). Used symbols: hypopharyngeal glands and arrangement of acini (a), around a collecting duct (cd), a connection of acinus to the collecting duct via a cable-like cluster of canal cells (cc), The length (L) and width (W) of an acinus. Scale bars = (A, C)500 μm, (B, D)200 μm.

Histological analysis

Under a light microscope, the hypopharyngeal gland acini are seen in Figure 3. Each image shows a different worker bee condition, including Fe0 (A), Fe25 (B), Fe50 (C), and Fe100 (D). Histology of the secretory units indicated the fully grown gland and the thin extracellular gap (ES) between adjacent acinus (AC) cells visible separating them from one another. In the control, hypopharyngeal gland acini frequently have a spherical morphology and keep it throughout their development (Fig. 3-A). The composition of the hypopharyngeal gland acini of honeybees is affected by different concentrations of iron; when exposed to Fe25, it shrinks in size while maintaining its shape and a clear nucleus is found (N); and when examined under a microscope, it can be seen that the secretory cell's cytoplasm

contains different numbers of secretory vesicles (VS) and narrow extracellular space (ES) that are almost unaffected by iron (Fig. 3-B). In bees treated with Fe50, the secretory units of the hypopharyngeal gland had fewer secretory cells and a large extracellular gap (ES) between adjacent acinar cells, both of which indicated that the secretory duct (SD) had been affected (Fig. 3-C). While in (Fig. 3-D) iron-treated honeybees Fe100 display a more radical shift by the damaged nucleus (N) and the shrinking plasma membrane, the alteration in these acini (AC) gland bees is far more severe than any other condition. There are almost no vesicles (VS) left, secretion completely stops, and iron-treated bees Fe100 display a significantly reduced plasma membrane thickness.

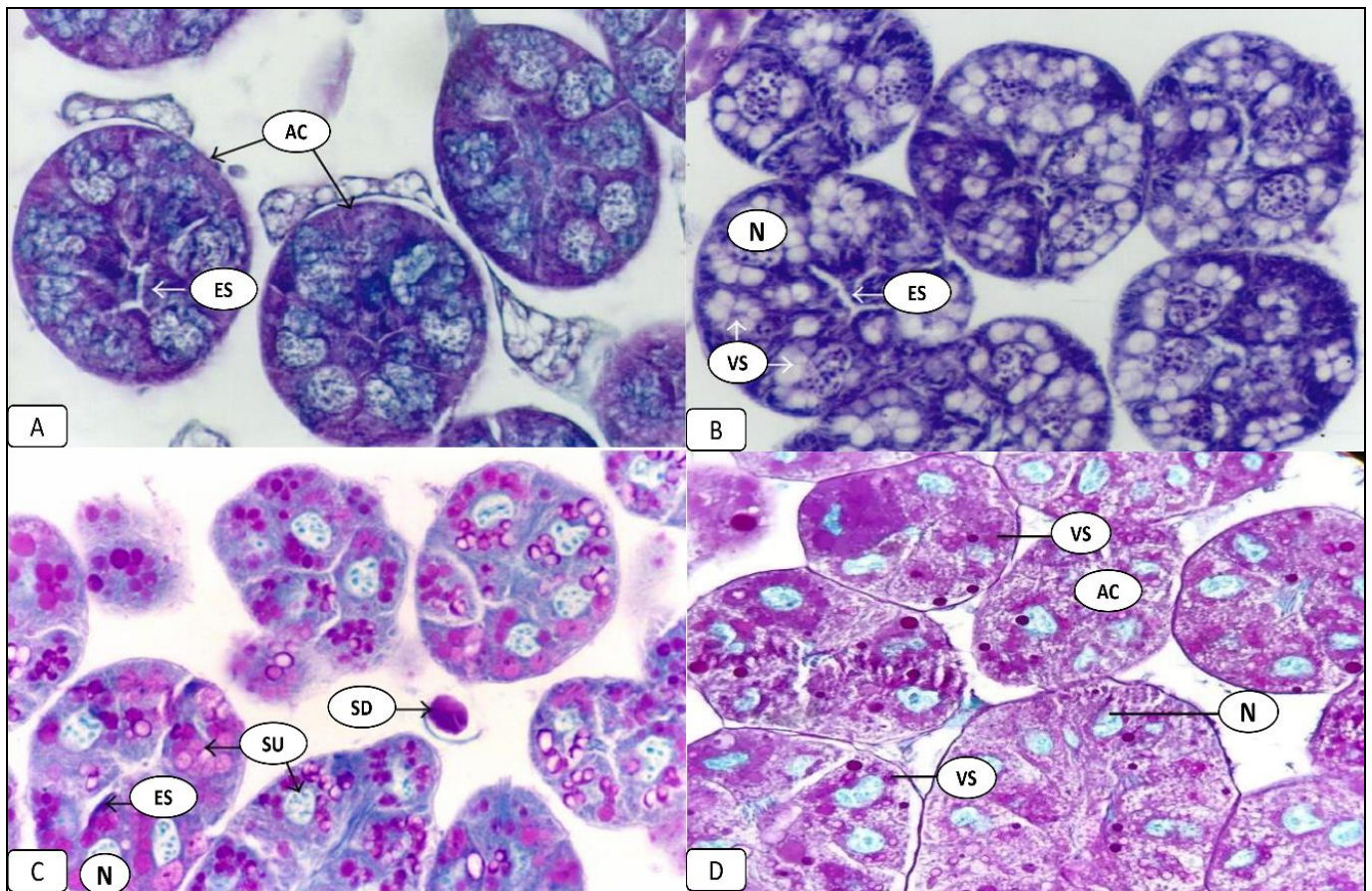


Fig 3: Histological structure of hypopharyngeal gland acini of honeybee workers. Fe0 (without Fe addition) (A), treatment Fe25 (25 mg L⁻¹ Fe) (B), treatment Fe50 (50 mg L⁻¹ Fe) (C), and treatment Fe100 (100 mg L⁻¹ Fe) (D). Used symbols: Acinus (AC); Extracellular space (ES); Nucleus (N); Secretory unit (SU); Secretory duct (SD); Vesicle (VS). (Scale bar 100x).

Measurement of the development of hypopharyngeal glands

In the colonies that got either an iron-free diet, the control group had a medium acini region that was generally typical (Length (L), Width (W)) (μm) ($L=154.04\pm 2.11^a$, $W=94.85\pm 2.06^a$) compared to treatments that involved taking an iron supplement in the off-season. The size of the hypopharyngeal gland (mean \pm S.E.) of honeybee workers (*Apis mellifera* L.) was affected after exposing it to different iron concentrations, Fe25, Fe50, and Fe100 treatments significantly. The results of the ANOVA analysis of the

entire set of data by Tukey's test, $P < 0.05$, indicate that means followed by distinct letters are statistically different. So, this revealed that the HPG acini demonstrated reduced size, with an iron concentration (Fe 25) on day 6 significantly influenced by the level of iron ratio ($L=143.61\pm 1.65^b$, $W=87.90\pm 2.19^a$). But with increasing iron concentrations, the impact of these factors interactions in Fe50 ($L=121.30\pm 2.02^c$, $W=68.03\pm 0.79^b$). treatments influenced significantly, On the other hand, the acini in honeybees fed with a high-concentration (Fe100)

($L=90.61\pm 1.82^d$, $W=64.72\pm 2.05^b$). diet with iron than in those fed with a low-concentration diet, (Fig 4).

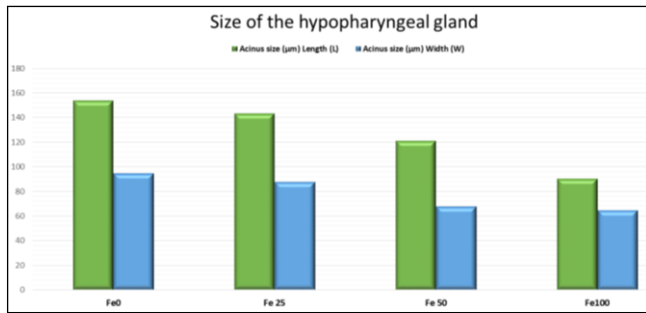


Fig 4: Size of the hypopharyngeal gland of honeybee workers (*Apis mellifera* L.) after exposing it to different iron concentrations.

Discussions

Numerous biotic and abiotic stress factors might harm how HGs grow. Honey bee HGs are age-dependent structures with variable acini sizes that are linked to various social behaviors⁸. Many studies have examined the impact of nutrition on lone honey bees kept in cages but not on entire colonies living in the wild^[21], which may have different outcomes. Therefore, to simulate the natural reality of beekeeping, iron supplementation was implemented in the beehives assigned to the field in this study. Because there is a natural decrease in food supplies in the environment and it is advised that beekeepers provide artificial food to the colonies, the field experiments were carried out off-season.

Both among the same honeybee workers and in other honeybee races, the HGs are investigated. Workers' honeybees have well-developed secretory cells, but pupal secretory cells are asymmetrical in structure and contain a low quantity of protein and carbs. As the glands gradually get smaller in the forager stage, the number of secretory vesicles increases^[22]. To improve the production of royal jelly (RJ) by HGs and to understand its chemical makeup^[23]. Moreover, Pollen is the primary source of inorganic elements for honey bees, and according to^[24] when compared to bees raised under good foraging conditions, bees raised amid pollen shortages possess comparable amounts of the majority of nutrients. For future research into the mineral requirements of honey bees, the authors suggested a diet of 1000 ppm of potassium, 500 ppm of calcium, 300 ppm of magnesium, and 50 ppm each of sodium, zinc, manganese, iron, and copper^[25]. This is consistent with our investigation into hypopharyngeal gland acini, which revealed that they grow spherical and maintain a constant shape throughout Control despite shrinking in size when bees are subjected to various doses of iron. This is evident in Fe25-treated bees, who have smaller acini and acini that are malformed in size and shape. In control bees, the secretory cycle often alters with age and shifts to smaller, more dense vesicles. But at Fe50, iron-treated bees exhibit a more dramatic change by secreting a lot of granular vesicles. These granular honeybees have previously been treated with Fe100. Compared to any other condition, the alteration in these gland bees is far severe. When there are almost no vesicles remaining, secretion stops.

The individual survival and population dispersion of insects in the natural world are significantly impacted by food and water limitations. Nearly all cellular physiological and metabolic activities depend on iron. The honeybee (*Apis*

mellifera L.) is used in the study by^[26] as a model to examine the effects of famine without food and water on iron metabolism in insects, according to those results, bees had a significant iron overload, unbalanced iron homeostasis, and a high death rate. Particularly when malnutrition led to iron excess in the thorax and fat body. However, due to the lack of floral resources and nutritional inadequacies of the few available resources, these food needs are typically not satisfied during the off-season, which harms the swarm's growth in terms of food quality and quantity. Bees forage for food, and the effectiveness of foraging and the caliber of the food produced are both related to how well-fed each bee is^[27].

In nature, bees consume floral resources like nectar and pollen, with concentrations that may vary depending on the geography and plants present in the area around the apiary. Concentrations of Fe in the honey range from 0.08 to 233.0 mg kg⁻¹ to 11.1 to 520.0 mg kg⁻¹ in bee pollen^[28]. Several superparamagnetic (SPM) and/or single domain (SD) magnetite particles have been described using direct imaging and indirect magnetometry techniques, and studies have provided behavioral and physical evidence implicating the honeybee abdomen as the location of an iron-based magnetoreceptor system (reviewed by^[29]). In study by^[30], They established that the majority of the iron is concentrated in the abdomen and accumulates quickly after emerging from the comb by conducting a thorough examination of iron buildup in honeybees raised in natural hive circumstances. It is strange that only five days after eclosion, when activities outside the hive would need a magnetic sense, the abdominal total iron complement reaches and sustains such high levels. The waggle dance is used to communicate directions for feeding sites to other hive members^[31], a phenomenon previously linked to magnetic cues^[32].

Early magnetic sense development in young workers may allow them to decipher these cues from their hive members. The quick iron buildup in the abdomen seen in the study by^[30], is in agreement with findings made utilizing proton-induced X-ray emission (PIXE) to measure the amount of iron in the fat body of honeybees raised in lab^[33]. Iron levels in the fat body of bees fed both iron-rich and iron-deficient diets matched those of wild foraging bees well before foraging behaviors began, according to the PIXE data. In addition, only 9 days after eclosion, the number of iron granules per trophocyte cell reached a plateau. While it is commonly believed that eating a diet high in iron causes the body's iron to accumulate,^[34] Some have hypothesized that iron buildup in this tissue is a "directed activity" for a purpose different than simply storing extra iron from dietary sources due to the quick and early saturation of iron inside the fat body³³.

Conclusions

The hypopharyngeal glands in honeybees that feed on different levels of iron were found to have much less surface area and height, this study concludes that adding inorganic iron (Fe) to the bee diet in the amounts utilized in this investigation negatively affects the growth of the bees. Therefore, our research is crucial for future investigations into the impact of the environment and nutrition on the shape and development of the hypopharyngeal gland.

Author Agreement

There are no competing interests listed by the author.

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