

GENE EXPRESSION AND ENZYMATIC REGULATION OF HONEY PRODUCTION IN HONEY BEES UNDER THERMAL AND PESTICIDE STRESS: A REVIEW

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Abstract

Honey production in *Apis mellifera* is an actively regulated physiological process centered on the hypopharyngeal and salivary glands, driven by enzymes including invertase and glucose oxidase whose expression is tightly coordinated with worker developmental stage, behavioral caste, and hormonal signaling. This review synthesizes current molecular and enzymatic understanding of honey production, then examines how thermal and pesticide stress disrupt these pathways individually and, where evidence permits, in combination. Thermal stress alters gland protein synthesis and metabolic gene expression, while sublethal pesticide exposure downregulates key honey-production genes while activating detoxification pathways, suggesting a physiological trade-off. Limited but consistent evidence indicates these stressors interact synergistically rather than additively. The review identifies three priority gaps: the near-absence of gland-specific (as opposed to whole-body) combined-stressor studies, the need for CRISPR-based functional validation of candidate genes, and limited comparative data between *Apis mellifera* and *Apis cerana*. Closing these gaps would extend current single-stressor, whole-body findings toward a mechanistic, gland-level understanding directly relevant to colony health and honey yield under increasing environmental pressure.

1. INTRODUCTION

Honey bees (*Apis mellifera*) are one of the most economically and ecologically important insects in the world and are responsible for a significant amount of pollination worldwide, as well as producing honey, which is a product with a great biochemical complexity. The honey bee is one of

the most important insect pollinators of monoculture food crops worldwide, adding over \$15 billion in economic value to agriculture each year in the United States and more than \$200 billion worldwide (Wu et al., 2020).

The amount of honey produced in the world is indicative of the size and location of this industry.

Honey production is the major concern of China as it produces about 24% of the world's honey and exports about 18% of the world's honey (FAO, 2018). In China, honey production is conducted in several provinces, each having its own floral sources, and beekeeping is still playing a role in the rural life and in improving the income of smallholders as well as providing significant contribution to the national and export market. It's a scale of production that not only allows for understanding in terms of basic biology but also directly applicable to a significant national agricultural sector.

Honey production is not just a simple process of storing nectar, but an enzymatic process that is mostly performed in the hypopharyngeal and salivary glands of worker bees. The hypopharyngeal glands produce brood food proteins like royal jelly in young nurse bees and the enzymes responsible for processing the nectar to honey (alpha-glucosidase, glucose oxidase, invertase, amylase and lipase) in older forager bees (Ueno et al., 2015). Two of the hypopharyngeal gland enzymes secreted serve a particular function: Invertase breaks down sucrose, and glucose oxidase protects the young honey from the contamination of microorganisms (Sahin et al., 2020).

Such a change in the function of this gland is well correlated with the change in the worker bee's role in the colony and is well controlled at the molecular and hormonal level. Genes that were found to be more highly expressed in nurse bees than in forager bees were directly linked to the worker caste's functional role, not just to worker age, for instance, *Ambuffy*, *AmMMP1*, *mrjp2*, and *Hbg3* (Ueno et al., 2015). In both ecdysone-regulated and juvenile hormone (JH)-regulated genes, the expression is higher in foragers than that in nurse bees, and experimental manipulation of glands with hormones can switch the gene expression profile between a nurse bee and a forager bee (Ueno et al., 2015).

Microbial pathogens, parasites, viruses, and environmental stressors are disrupting these finely-tuned molecular pathways and honey bee declines are now known to be due to the cumulative impact of multiple interacting stressors. Honey bee colony

losses are known to be caused by multiple stressors (habitat loss, flower shortages, exposure to agrochemicals, parasites and others) often acting synergistically rather than additively, and the exposure of honey bees to pesticides as well as nutritional stress can have a detrimental effect on immune responses and thus increase their susceptibility to other stressors (Goulson et al., 2015). The authors conclude that climate change predictions are likely to exacerbate these stresses on bee health (Goulson et al., 2015) and highlight the need to understand the interaction between thermal stress and the other stressors that are known to affect colony viability.

One of the most significant factors at the physiological level is temperature-stress, which has been demonstrated to cause differential gene expression of protein synthesis and metabolic regulation genes, and has been linked to a decrease in gland function, and directly to the royal jelly production potential (Maigoro et al., 2025). The hypopharyngeal gland activity of foragers under extreme heat stress is lower than that of non-foragers under the same stress, and the difference in hypopharyngeal gland activity is correlated with the difference in protein synthesis rates (Maigoro et al., 2023).

The second major disruptive pressure is through pesticide exposure. While the impacts of pesticides on honey bee mortality in the field have been described as minimal, sublethal exposures can impact honey bee behavior, foraging prowess, learning, individual and colony development (Wu et al., 2020). Cytochrome P450 enzymes, like the neonicotinoid metabolizing enzyme CYP9Q3, have been identified as important detoxification mechanisms that allow bees to metabolize specific insecticides such as thiacloprid (Haas et al., 2022); however, detoxification capacity and the responses of these enzymes are highly variable depending on the class of insecticide and the exposure conditions. Furthermore, synergic effects between pesticides and other stressors such as pathogens and parasites have been discovered as well (Wu et al., 2020; Chmiel et al., 2020), which means that individual-stressor experiments may underestimate the effects of the real world on the gland and honey production.

Although there are a number of individual bodies of literature focusing on either thermal stress or pesticide exposure, and there is a growing ecological literature that shows environmental stressors rarely occur independently (Goulson et al., 2015), comparatively few studies have addressed how temperature and pesticide exposure interact and/or synergise at the molecular level within the glands directly involved

in making honey. This review compiles current information on the molecular and enzymatic basis of honey production in the honey bee and highlights the specific areas of interest regarding the expression of genes in response to thermal stress and pesticide stress that still require research, especially in the context of multi-stress interactions at a gland level.

Table 1. Summary of key studies underpinning this review's discussion of thermal and pesticide stress effects on honey bee gland and honey-production physiology.

Stressor(s)	Model System	Key Finding Relevant to Gland or Honey-Production Function	Study
Baseline hormonal	/ A. mellifera, HG transcriptome	Nurse-to-forager HG gene expression shift is behavior-linked and hormonally (JH/ecdysones) controlled.	Ueno et al., 2015
Baseline developmental	/ A. mellifera, HG proteome	Mapped MRJP and secretory protein expression across HG development; linked acinus diameter to royal jelly yield.	Feng et al., 2009; Li et al., 2010
Thermal	A. mellifera, HG transcriptome	Temperature stress alters protein-synthesis and metabolic gene expression in the HG, impairing gland function.	Maigoro et al., 2023, 2025
Thermal	A. mellifera, whole body / HG	HSP70, HSP90, and GRP78 induced by heat, peaking at 45°C in gland and fat body tissue.	Koo et al., 2015
Thermal (baseline)	A. mellifera, colony	Establishes 33-36°C brood-nest thermoregulatory range that gland activity operates within.	Stabentheiner et al., 2010
Pesticide	A. mellifera, HG / whole body	Larval imidacloprid exposure decreases HG size and downregulates MRJP genes in emerging adults.	Wu et al., 2017 (Insect Biochem. Mol. Biol.)
Pesticide (detox.)	A. mellifera, P450 pathway	CYP9Q3 identified as a conserved detoxification enzyme metabolizing neonicotinoid insecticides.	Haas et al., 2022
Pesticide (co-exposure)	A. mellifera, P450 pathway	Sterol-inhibiting fungicides potentiate insecticide toxicity by blocking the same P450 detox pathway.	Schuhmann et al., 2022
Thermal Pesticide (combined)	x A. mellifera, colony	Combined heat + imidacloprid synergistically alters HSP expression and metabolic transcriptome beyond either stressor alone.	Kim et al., 2022
Thermal Pesticide (combined)	x A. mellifera, gland-relevant genes	Non-optimal temperature and imidacloprid combine to downregulate vitellogenin and mrjp1 beyond single-stressor effects.	Alburaki et al., 2023
Pesticide Pesticide	x A. mellifera, HG	Combined amitraz + dinotefuran directly impairs HG morphology – confirms	Esmaily et al., 2025

(combined)	morphology	feasibility of gland-level combined-stress study design.	
Pesticide (lab vs. field)	A. mellifera, HG / immune genes	Lab and field settings diverge in immune response despite similar HG size reduction – context-dependency caution for future work.	De Smet et al., 2017

2. Physiological Basis of Honey Production

2.1 Hypopharyngeal Gland Anatomy and Development

Hypopharyngeal glands (HGs) are bilaterally located, paired exocrine glands in the head of worker honey bees in front of the brain, between the compound eyes (Ahmad et al., 2021). Thousands of collecting units, each consisting of a secretory cell and a duct cell, make up each gland (Klose et al., 2017). The collecting duct is the smaller of the two ducts and has an inner lining of a cuticular layer, measuring about 60 micrometers in width, with each HG containing about 800 acini each of which contains 6-20 two-cell secretory units (Ahmad et al., 2021). These morphological characteristics (a secretory cell with an intracellular canaliculus that discharges its product into the plasma) make the hypopharyngeal gland a Class III insect dermal gland (Klose et al., 2017).

The secretory cell is joined to the duct cell in each secretory unit by a "tube-joint" at the open end of the canaliculus, which is lined by septate and adherens junctions that separate the canaliculus from the remaining plasma membrane of the duct cell (Klose et al., 2017). The canaliculus is a membrane-bounded blind-ended tubule that coils inside the secretory cell, and is lined on its lumen by a thin fenestrated cuticular lining called the end apparatus through which the secretory products pass before entering the duct cell and subsequently the collecting duct (Klose et al., 2017).

Glands start their development at the pupal stage. The hypopharyngeal gland primordium undergoes cell proliferation on the first day of pupal development and the epithelium reorganizes into three-celled units: a prospective duct cell, an accessory cell and a prospective secretory cell, and clusters of 5-15 three-celled units develop into a single acinus and its associated canal bundle, whereas cells between clusters form the collecting duct (Klose et al., 2017). During the second half of pupal development, the third cell (the accessory cell) undergoes programmed cell death (apoptosis) and the two remaining cells develop into the secretory cell and duct cell of the mature unit (Klose et al., 2017). The gland starts to look similar to the adult at mid-pupae, and the secretory cell then forms the characteristic canaliculus by progressive invagination and extension of the apical cell membrane through targeted exocytosis, followed by reorganisation of the membrane-associated F-actin cytoskeleton (Klose et al., 2017). Once adult, the gland structure and secretory activity also vary significantly with age and functional role, following a nurse-forager gradient in the adults. The development and function of the gland is influenced by several factors, such as the race of the honey bee, nutrition, colony size, season, exposure to pesticides and Varroa infestation (Ahmad et al., 2021), rendering it a physiologically sensitive structure suitable for the study of the effects of environmental stress.

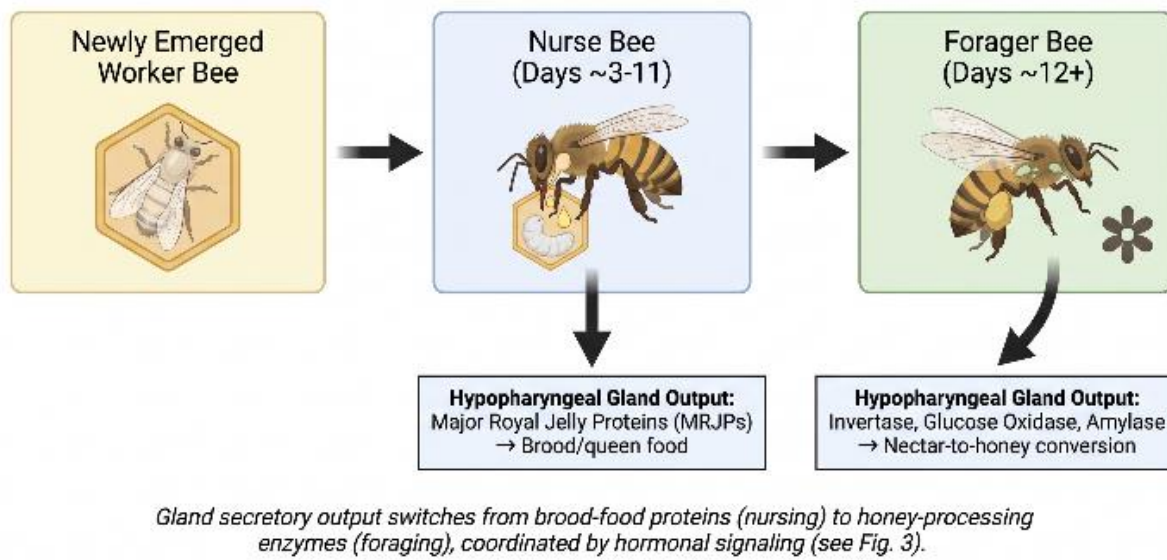


Figure 1. Functional shift of the hypopharyngeal gland across worker developmental stages.

2.2 Role of Salivary and Mandibular Glands

2.2.1 Salivary Glands

Honey bees have two types of salivary glands with different functions: cephalic (post-cerebral) salivary gland and thoracic salivary gland. Thoracic salivary gland secretes an aqueous solution of digestive enzymes (invertase, glucose oxidase, and amylase) that are involved in the digestion of honey and sugar, and in the moistening of pollen and wax, while the cephalic salivary gland secretes an oily secretion that is primarily used for wax manipulation and lubricating mouthparts (Mazeed et al., 2017). Comparative activity assays for all honeybee subspecies (Mazeed et al., 2017) show that the secretion of invertase is always the highest of all three enzymes in both cephalic and thoracic glands from the honeybee, followed by amylase and then glucose oxidase (GOD), which is the enzyme that catalyzes the terminal step in sucrose degradation.

2.2.2 Mandibular Glands

Both the structure and secretory activity of the mandibular glands (MGs) are caste specific. In queens, MGs produce a pheromone called queen mandibular pheromone (QMP) that contains primarily two omega-1-hydroxylated decenoic acids along with aromatic molecules which control

important aspects of colony social organization such as the worker retinue response, drone attraction, and inhibition of worker ovary development (Wu et al., 2017). The omega-hydroxylated decenoic acids, 10-hydroxy-2-decenoic acid (10-HDA) and its precursor 10-hydroxy-decanoic acid (10-HDAA), are produced by MGs, and make up 60-80% of the total fatty acid composition of royal jelly, directly affecting the growth of larvae (Wu et al., 2017).

2.3 Key Enzymes in Nectar-to-Honey Conversion

2.3.1 Invertase

The hypopharyngeal and salivary glands are the main source of invertase, which breaks down sucrose in nectar to glucose and fructose, the two major sugars found in honey (Sahin et al., 2020). Invertase and diastase, among the enzymes extracted from bees responsible for transforming the nectar into honey, are most active in the honey stomach where the complex sugar molecules are first converted to simpler ones that can be used by the bee for energy production (Harithasree & Vijayasree, 2026). Invertase activity decreases slowly during honey storage and heating, and is regarded as a good parameter to show the freshness and quality of honey (Sahin et al., 2020).

2.3.2 Glucose Oxidase

Glucose oxidase then reacts with the glucose generated by invertase activity to convert it to gluconic acid and hydrogen peroxide (Sahin et al., 2020). This reaction is the key to the antimicrobial activity of honey because the hydrogen peroxide produced helps prevent honey from being contaminated by microbes during storage (Sahin et al., 2020). The activity of glucose oxidase shows a high activity in initial stages of honey formation and do not decrease as much as that of invertase with time, and its behaviour has not been known to be a reliable indicator of the quality of the honey as that of invertase (Harithasree & Vijayasree, 2026; Sahin et al., 2020).

2.3.3 Enzymatic Activity as a Quality Standard

In addition to their biological function in processing nectar, enzymes produced by the glands are also directly relevant to the practical world, as recognized quality markers of honeys around the

world. International honey standards (including the Codex Alimentarius) have set a minimum threshold of 8 DN for diastase activity, where 1 DN is the quantity of diastase that will hydrolyse 1 g of starch in 1 hour at a standardised temperature and pH. Lower-enzyme varieties of honey, such as acacia, have a reduced threshold of 3 DN, provided the hydroxymethylfurfural (HMF) content is less than 15 mg/kg (Raweh et al., 2023). Some related compositional parameters (moisture content ($\leq 21\%$), fructose (31-42%), glucose (23-32%) and HMF (≤ 40 mg/kg)) are also specified in the international standards (Raweh et al., 2023). Enzymatic markers like invertase and diastase are also effective indicators of the secretory output of the glands and the quality of the post harvest processing and establish a link between the molecular biology presented in this paper and the commercial standards for honey grading, applicable to apiculture industries like the one in China.

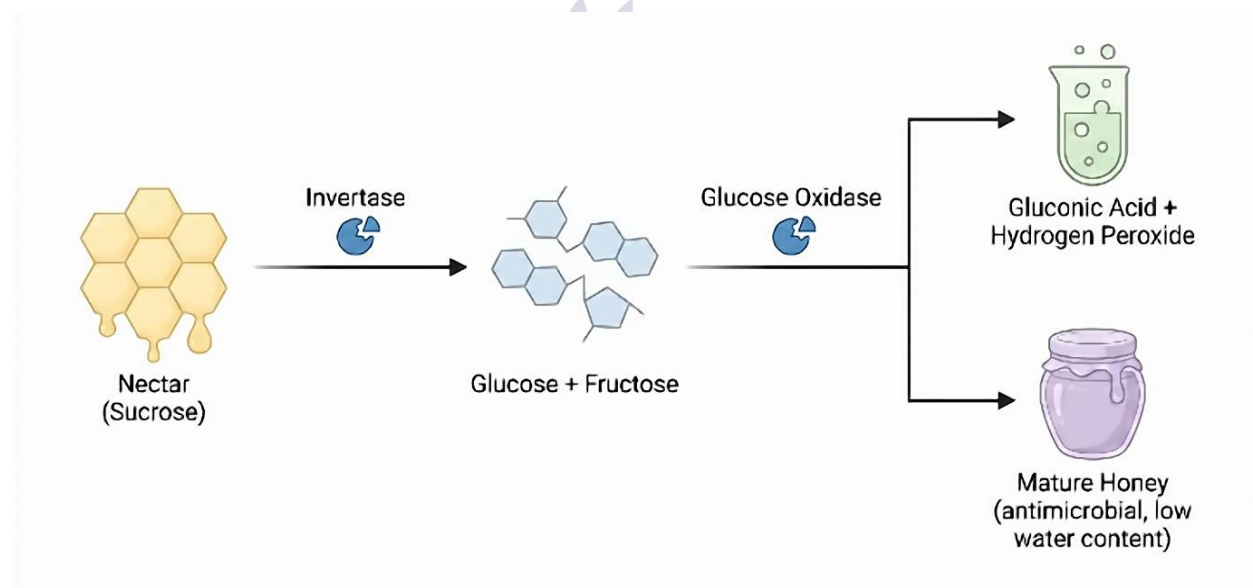


Figure 2. Enzymatic conversion pathway from nectar to honey.

3. Molecular Regulation of Gland Function and Enzyme Expression

3.1 Gene Expression Patterns Across Bee Age and Caste

The expression of hypopharyngeal gland (HG) genes undergoes a significant change during transition to the foraging role. The genes that were

enriched in the nurse bee compared to the forager HGs (Ambuffy, AmMMP1, mrjp2, and Hbg3) were positively correlated with the worker's task at the same age, suggesting that this regulation is linked directly to the tasks rather than to age alone (Ueno et al., 2015). This pattern holds true at a larger scale; comparative transcriptomic analyses

of differential gene expression at three developmental stages (newly emerged worker, nurse and forager) of the HG across three species revealed age/role-dependent gene expression changes were conserved across species (Liu et al., 2014). Perhaps most important is that this behavioral maturation schedule is not set in stone, but is instead known to be accelerated due to colony-level stressors and environmental stressors, in general, so any of the environmental stressors discussed later in this review could in principle affect HG gene expression both directly, and indirectly, by altering the timing of the nurse to forager transition itself.

3.2 Key Regulatory Genes and Pathways

Juvenile hormone (JH) and ecdysone signaling regulates HG physiology with worker behaviour. The forager HGs express higher levels of both ecdysone regulated genes (ecdysone receptor, E74) and JH regulated genes (Methoprene tolerant, Kruppel homolog 1) than the nurse bee HGs, and experimental hormone treatments can alter the gland gene expression profile (Ueno et al., 2015): 20-hydroxyecdysone treatment reduced the expression of both nurse- and forager-selective genes; methoprene (a JH analog) increased forager-selective gene expression and decreased nurse-selective gene expression. This JH-based system is not an independent one, but is downstream from a larger nutrient-sensing system. Insulin/insulin-like peptide signaling (IIS) controls feeding-related behavior and metabolism in honey bees: feeding

or a high fat content increases the levels of insulin-like peptides (ILPs) and decreases adipokinetic hormone (AKH, the bee equivalents of glucagon) production, and itself stimulates the intracellular target of rapamycin (TOR) pathway and the production of JH (Ament et al., 2008). This IIS/TOR/JH axis is known to convert the internal nutritional and metabolic state of each individual nurse into the hormones he or she secretes, which in turn determine the task allocation of his or her colony mates and, through this effect, the expression of the genes of the colony. In honey bees, the yolk precursor protein, vitellogenin (Vg), has been recruited to control honey bee worker division of labor, in addition to its original role in egg production. Vg is very poly-functional in its role in social organization with multiple coordinating effects working through a regulatory feedback loop with JH: high Vg levels are correlated with nursing behavior, whereas low Vg levels and high JH levels dictate a switch to foraging (Nelson et al., 2007). The Vg-JH cycle has now come to be known as a key feedback circuit that connects nutritional state, hormonal signaling and the allocation of tasks in worker bees and which directly influences the hormonal milieu in which the expression of the HG is regulated (Nelson et al., 2007). Notably, Vg and IIS interact antagonistically at the level of regulation; rather, the Vg-JH loop described above and the IIS/TOR nutrient-sensing pathway constitute a single interconnected regulatory network, rather than two systems (Ament et al., 2008).

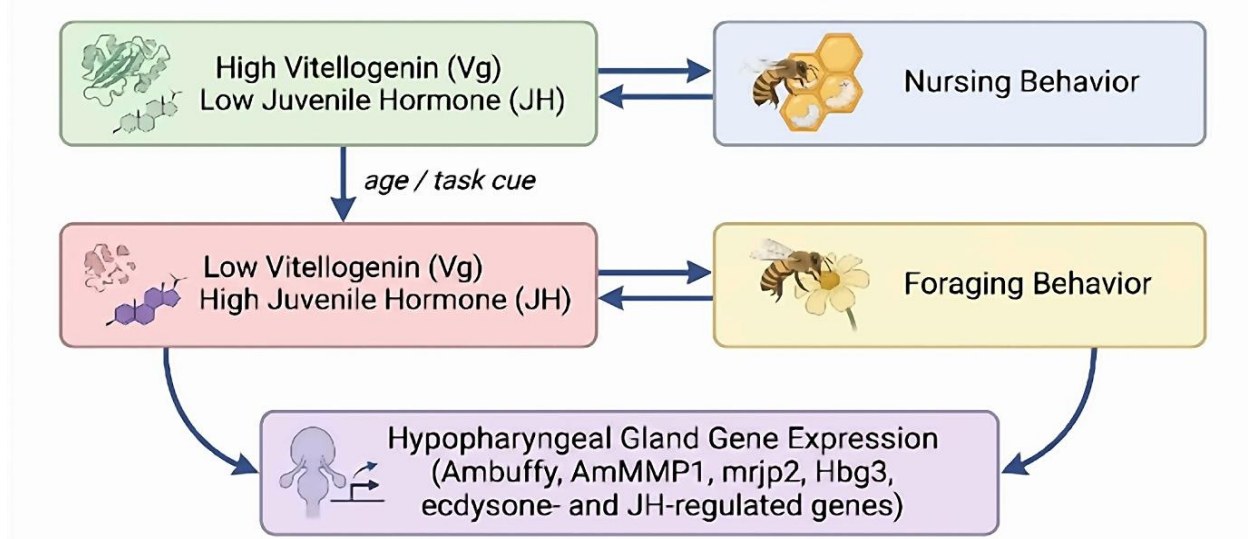


Figure 3. Vitellogenin-Juvenile Hormone feedback loop regulating hypopharyngeal gland gene expression.

3.3 Post-Transcriptional and Epigenetic Regulation

In addition to hormonal control of transcription, the division of labor and the related changes in gene expression is also regulated by post-transcriptional regulators such as microRNAs (miRNAs). Although microRNA knockdown did not directly impact abdominal lipid mass, there were significant downstream changes in gene expression in the brain of adult worker bees that correlated with changes in behavior as the bees matured from nursing to foraging, including altered expression of transcription factors previously linked to the nursing-to-foraging transition (Stuart et al., 2024). This suggests that miRNA-mediated regulation is an extra non-hormonal regulation of the same behavioral maturation process regulated by the JH-Vg-IIS network and can propagate to distant tissues like the brain. Although similar miRNA-level functional studies have not yet been performed specifically in HG tissue, the overall body of evidence suggests that post-transcriptional regulators plays a role in the modulation of HG gene expression in addition to the more well-studied hormonal pathways, and is an under-explored dimension of gland regulation.

3.4 Proteomic and Transcriptomic Findings from Recent Studies

An extensive proteomic and transcriptomic database has been established by a large number of studies directly correlated with HG protein and gene expression changes throughout development at the Institute of Apicultural Research, Chinese Academy of Agricultural Sciences (IAR-CAAS). Proteomic profiling of HG development revealed 87, 76, 85, 74, 71 and 55 proteins at days 1, 3, 6, 12, 15 and 20, which were classified as major royal jelly proteins (MRJPs), carbohydrate and lipid metabolism enzymes, and cytoskeletal proteins, respectively, and with MRJP expression peaking between days 6-12 (Feng et al., 2009). Comparative proteomic analysis between a high producing bee strain and standard Italian bees revealed a positive correlation between the diameter of HG acini and royal jelly production, as the high royal jelly-producing strain produced 5 times more royal jelly than the Italian strain, and had differential protein expression (Li et al., 2010). Since then, this molecular characterization has been expanded to post-translational regulation: phosphoproteomic analysis of HGs from newly emerged bees led to the identification of a protein kinase namely FAM20C, which is likely to play a role in the phosphorylation of royal jelly proteins, with the expression level of its corresponding mRNA and

protein being the highest in the nurse bee HGs (Ahmad et al., 2022). Together, this collection of IAR-CAAS related work shows a transition in the field from cataloguing a particular gene/protein to understanding the mechanisms by which post-translational modifications and strain-specific differences affect the output of these HGs, complementing the hormonal (Section 3.2) and the post-transcriptional (Section 3.3) regulatory steps described above, and combined contributing to an understanding of HG gene expression as a multi-layered system that is susceptible to disruption at several distinct points by environmental stress.

4. Effects of Thermal Stress on Honey Production Pathways

4.1 Baseline Colony Thermoregulation and Gland Vulnerability

Knowing the reason for thermal stress at the gland level depends on knowledge of the narrow temperature range the honey bee colonies keep during natural conditions. Honeybee larvae and pupae are very stenothermic as they need to maintain a brood nest temperature within a narrow range of 33 – 36 °C for optimal development (Khan et al., 2025; . Stabentheiner et al., 2010). This range is maintained by individual and collective responses to cold stress, namely by activation of thoracic flight muscles ("endothermy on demand") and by clustering of young bees to conserve heat; and to heat stress, by water collection and distribution ("evaporative cooling") and by fanning of the wings to increase air circulation. Significant reduction of active thermoregulatory heat production to almost exclusively the two large task divisions in cold stress (Stabentheiner et al., 2010) is a critical point, which led to the definition of age polyethism: young bees (less than two days) do not participate in active thermoregulatory heat production. The number of hypopharyngeal glands active in either brood-food preparation or honey-enzyme production (Section 2.1) is directly associated with the number of non-newly-emerged workers in the colony, which is also directly associated with the colony level thermoregulatory capacity.

4.2 Temperature Effects on Gland Development and Enzyme Activity

Temperature is one of the critical abiotic factors impacting honey bee survival and pollination and plays a key role in colony collapse disorder (CCD) in addition to direct impacts on gland physiology (Maigoro et al., 2025). The analysis of RNA sequencing of HG under low, regular and high ambient temperatures indicated that temperature stress affects the expression of genes associated with protein synthesis and metabolic regulation, which are related to the dysfunction of the gland and the potential for royal jelly production (Ahmad, 2021a; Maigoro et al., 2025). Pathway analysis of the expression changes in the differentially expressed genes (DEGs) highlighted the enrichment of the ECM-receptor interaction and fatty acid metabolism pathways, as well as the downregulation of protein processing in the endoplasmic reticulum, a pathway that is directly related to the production of RJ and gland nutritional output (Maigoro et al., 2025). Heat shock protein (HSP) genes, at the whole organism level, act as molecular indicators of thermal stress. Hsp70, grp78 and hsp90 were shown to be up-regulated with peak expression at 45°C and only hsp90 showed a decreased survival upon exposure to 50°C for 1 hour, whereas hsp40 did not show a significantly different expression pattern (Koo et al., 2015), suggesting that the hypopharyngeal glands and fat bodies are the organs most strongly affected by the changes. This pattern is noteworthy because the normal brood nest temperature is approximately 33-36°C, and there is already measurable induction of HSP in gland tissue for increases of less than 10°C above this normal thermal operating range (Stabentheiner et al., 2010) in advance of any measurable impact on bee survival.

4.3 Gene Expression Changes Under Heat and Cold Stress

When nurse bees are subjected to extreme heat, their HG activity is reduced as compared to nurse bees subjected to no heat stress, and this reduction is correlated with a decrease in protein synthesis rates in the gland (Maigoro et al., 2023). Under temperature-stress conditions, there were

variations of expression of heat-shock protein 90, the marker of the development of the heat-shock response, as well as of gustatory receptor 10 (GR10), which is a marker of the development of the heat-shock response and nutritional performance during nursing, indicating that heat stress has a disruptive effect on the immediate protein-folding defense system and on the nutritional signaling pathways regulating gland development (Maigoro et al., 2023; Maigoro et al., 2025). Cold stress also has a unique, but similar molecular signature, the focus of this study was not on the adult gland tissue, but on developing brood. When *Apis mellifera* brood is raised at suboptimal temperature, their growth rate is reduced and development is arrested, but growth is quickly restored within 24 hours after shifting to the control temperature, through compensatory mechanisms (Ramirez et al., 2021). Rapid alterations in the expression of insulin/insulin-like peptide signalling (IIS) and adipokinetic hormone (AKH) pathway components, and increased levels of the uncoupling protein, MUP2 (Ramirez et al., 2021) are responsible for this recovery. Of particular note is the association of the IIS/AKH pathway in this cold stress recovery pathway, which is the same nutrient-sensing pathway described in Section 3.2 as upstream regulator of JH production and, thus, HG gene expression; this may indicate that the underlying signal architecture of this pathway is shared by both cold stress recovery pathways and hormonal regulation of HG function, although this has not been tested directly in adult HG tissue.

4.4 Colony-Level Consequences

The consequences at the molecular level are measurable, colony consequences. The effects of temperature-stress on gland function are specifically associated to the risk of colony collapse disorder (Maigoro et al., 2025). This brood developmental interruption and death is a direct result of the cold-stress conditions due to colony thermoregulation failure, which is often a downstream effect of a reduction in the adult population and the presence of an increasing young forager caste following higher forager mortality rates (Ramirez et al., 2021). This creates

a feedback loop: molecular effects of environmental thermal stress reduce gland and brood function, weakening the colony thermoregulatory capacity (Section 4.1), and increasing susceptibility to more thermal stress. Of course, the same principle is relevant to the function of the gland in honey bees, namely that its physiology and enzymes are modulated by temperature, but this is the same principle used in earlier studies of the effects of temperature on efficacy of an insecticide against a disease vector; it is the same principle and only the organism (honey bee) and downstream trait (gland function) are different.

5. Effects of Pesticide Exposure on Honey Production Pathways

5.1 Sublethal Pesticide Effects on Gland Gene Expression

Exposure to sublethal levels of pesticides (those that do not kill honey bees outright), has repeatedly been demonstrated to affect honey bee behavior, foraging efficiency, learning, and development of individuals and colonies (Chmiel et al. 2020). More important, the doses employed in most of the laboratory gland studies are not random, but are based on residue measurements made in the field. A four-year field experiment on the monitoring of thiamethoxam residues and its metabolite clothianidin in bee-collected pollen showed that residues, after exposure to treated maize, in bee-collected pollen were generally in the range 1-7 µg/kg, with median values, while residues in hive nectar, honey, and royal jelly were generally at or below 0.5 µg/kg (Pilling et al., 2013; ul Abadin, 2021) – and the gland's own direct secretory product (royal jelly) contained measurable, but low, thiamethoxam residues under real agricultural conditions. Honey bees exposed orally for 10 days exposed to low field-realistic concentrations of neonicotinoids demonstrated measurable physiological disruption at concentrations below those that produce measurable alteration in acetylcholinesterase activity, such as decreased carotenoid and alpha-tocopherol levels and increased lipid peroxidation, suggesting that disruption of the oxidative and antioxidant systems can occur as a sensitive sub-

behavioral effect of neonicotinoid exposure at concentrations below those required to produce classic neurotoxic symptoms (Gauthier et al., 2018). The sublethal concentrations of imidacloprid have shown to have a negative effect on hypopharyngeal gland size in nursing bees, with acinus diameter being a direct measure of the glandular secretory activity (Wu et al., 2017). Likewise, imidacloprid caused a global pattern of changes in gene expression in the heads of newly emerged adult workers when fed during the larval stage, and the expression of a set of major royal jelly proteins (MRJPs) was highly suppressed (Wu et al., 2017). Disruption of the MRJPs is not the only manifestation of gland relevant transcriptomic disruption. Wu et al. (2017) performed RNA-sequencing on sublethal exposure to imidacloprid (0.1, 1, and 10 ppb), which showed that muscle-related genes were significantly down-regulated, while ribosomal protein genes were up-regulated, suggesting that sublethal exposure to neonicotinoids can lead to broad transcriptomic reprogramming of core cellular protein-synthesis machinery and behavioral capacity relevant to secretory tissues, conducted at IAR-CAAS.

5.2 Detoxification Gene Responses (P450s) and Trade-offs with Secretory Function

The enzymatic systems responsible for detoxification of xenobiotics, such as pesticides, in honey bees are mainly cytochrome P450 enzymes. CYP9Q3 has been found to specifically detoxify bees' neonicotinoid insecticide thiacloprid (Haas et al., 2022). This detoxification process is also influenced by gut microbiota composition, with some gut bacterial taxa shown to be able to regulate host P450 gene expression in the digestive tract (Wu et al., 2020). Compared to that of most other insect genomes, *Apis mellifera* has fewer genes in all three categories (Phase I, Phase II and Phase III) of genes involved in the processing of xenobiotics; although this does not imply universal susceptibility to pesticides, it is known to increase the susceptibility of bees to synergistic effects of multiple xenobiotics to which they are exposed at the same time (Berenbaum & Johnson, 2015). Interestingly, up-regulation of

detoxification genes and loss of secretory function seem to be related, and not independent. After larval exposure to imidacloprid, expression of detoxification genes (CYP9Q3) also increased while hypopharyngeal gland size decreased (Wu et al., 2017), indicating a physiological trade-off that resources or cellular capacity used for xenobiotic detoxification is at the expense of gland secretory output.

5.3 Fungicide Co-Exposure and P450 Inhibition: A Complicating Factor

The above mentioned P450 mediated detoxification trade-off depends on the assumption of stress-induced induction and functionality of P450 enzymes. In the field however, exposure is rarely to a single class of pesticide and some fungicides prevent this detoxification process rather than only competing for this capacity. Cycloölation inhibitors (inhibition of sterol biosynthesis pathway) are fungicides with a mode of action that also strongly enhances the toxicity of neonicotinoid and pyrethroid insecticides in bees, since the same class of P450 enzymes are targeted by fungicides in the target fungi and are also responsible for detoxification in bees (Schuhmann et al., 2022). This means that in more realistic field situations where an extended period of time over which neonicotinoids and sterol-biosynthesis-inhibiting fungicides are applied to flowering crops, the same kinds of detoxification enzymes (including CYP9Q3-family P450s) that Section 5.2 shows as compensation for pesticide exposure may also be pharmacologically inhibited, with direct (but currently untested) implications for hypopharyngeal gland function – a scenario that is explored directly in Section 5.2, where the induction of these enzymes is discussed, even when they develop normally.

5.4 Neonicotinoid and Insecticide-Specific Findings

The class of neonicotinoids, and specifically imidacloprid, is the most widely studied class of insecticide for honey bee molecular stress responses. Heat shock protein genes (hsp70, hsp90, grp78) are activated by imidacloprid

exposure in a different manner but in a similar pathway as thermal stress, indicating that both pesticide and thermal stress induce similar cellular stress pathways (Ahmad, 2021b; Koo et al., 2015). Exposure to imidacloprid during the larval stage has also been associated with precocious foraging, as adults exposed to imidacloprid underwent a transcriptomic shift of nurse bees to forager gene expression profiles early in development (Wu et al., 2017). The nurse-to-forager transition period is the time at which HPGs switch from royal-jelly to honey-enzyme secretion, and a premature switch could be a second way that neonicotinoids interfere with honey-production gland development.

6. Interactive and Combined Stress Effects

6.1 Studies Examining Combined Temperature x Pesticide Effects

Although the interaction between temperature and pesticides has not been directly addressed by many studies, a small number have shown consistent results that indicate synergistic rather than additive negative effects on honey bees. This synergistic response is similar to that exhibited by the detoxification biology of the honey bee (see Section 5.2): a weakening of detoxification genes provides a plausible mechanistic explanation for the consistency observed in the honey bee literature that combined stressors exert a more severe effect than single stressors. Kim et al. (2022) conducted a transcriptome analysis of bees exposed to imidacloprid alone, heat stress alone, or both, and imidacloprid upregulates HSP70 and HSP90 genes, which are part of the metabolic pathways, whereas heat stress alone does not disrupt these pathways significantly. This symbiosis applies in particular to genes that are relevant for the glands. In a longitudinal cage study where bees were exposed to three ambient temperatures and three concentrations of imidacloprid, both elevated and reduced bee mortality rates were observed at both non-optimal temperatures, while both vitellogenin (Vg) and major royal jelly protein 1 (mrjp1) were significantly down-regulated at non-optimal temperatures compared to 32°C, which is the optimal temperature for both; most critically,

exposure to imidacloprid alone further reduced the expression of Vg and mrjp1 at 26°C only (Alburaki et al., 2023; Ahmad et al., 2025). A similar study reported that imidacloprid induced changes in brain gene expression were exacerbated only in the cold (Manzi et al., 2020). Interestingly, there are some studies that directly measure the morphology of hypopharyngeal glands for combined-stressor treatments (pesticide x pesticide) but not for temperature x pesticide. A recent study showed that both amitraz and dinotefuran (in isolation and in combination) significantly reduced the expression of the vitellogenin gene and disrupted gene expression related to antioxidant, immune and detoxification processes in bees, and that high concentrations of dinotefuran and dinotefuran together with amitraz directly affected hypopharyngeal gland morphology (Esmaily et al., 2025). This suggests that combined-stressor impact on HG morphology is measurable and additionally can be identified with conventional gland dissection techniques routinely used in the field—there is no methodological hurdle to assessing the effect of temperature x pesticide on the gland specifically, it is a research question. This is further emphasized by the fact that the effects of pesticides on the gland are highly context-dependent, and the need to test the gland outcomes under multiple simultaneous realistic stressors, rather than under a single one in the laboratory. The immune response was significantly different between the laboratory-caged and field-colony bees: although both colony groups had reduced the size of their hypopharyngeal glands and increased the expression of detoxification genes (CYP9Q3, CYT P450), only the bees in the field colonies exhibited an intact immune response with a delayed effect – a trend of decreased brood and colony numbers was observed after the first brood cycle (De Smet et al., 2017). This laboratory-field discrepancy is directly relevant for the interpretation of any future combined temperature x pesticide gland study and is a warning. These findings suggest that there is an urgent need for studies to directly investigate the interactions of thermal and pesticide stress on gene expression of the hypopharyngeal gland and activity of the enzymes

involved in honey production. This is a clearly identified gap: although it has been shown that *Vg* and *mrjp1* get downregulated in response to combined stress at the whole-body level (Alburaki et al., 2023), and the combined action of the pesticides on HG morphology has now been demonstrated (Esmaily et al., 2025), nothing to date has directly measured the combined action of thermal and pesticide stress on the gene expression, enzyme activity and morphology of HG.

6.2 Nutritional Stress as a Compounding Factor

Availability of pollen and pollen quality has long been known to influence stress resilience in honey bees, in conjunction with both pesticides and other stressors, making the nutritional aspect a

third, often ignored axis in which stress may interact. Pollen has been proven to decrease the toxicity of acute and chronic pesticide exposure, with pollen-fed bees exposed to an insecticide having a significantly lower risk of mortality compared to unfed ones, which may be explained in part by the protective effect of vitellogenin against oxidative stress (Barascou et al., 2021). There is no direct literature evidence of a three-way interaction between nutrient deficiency, concurrent thermal stress, and pesticide stress, but nutritional deficiencies may exacerbate the impact of hypo- or hyperthermy on hypopharyngeal gland tissue growth, which, in turn, may affect hypopharyngeal gland function in the honey-production pathway.

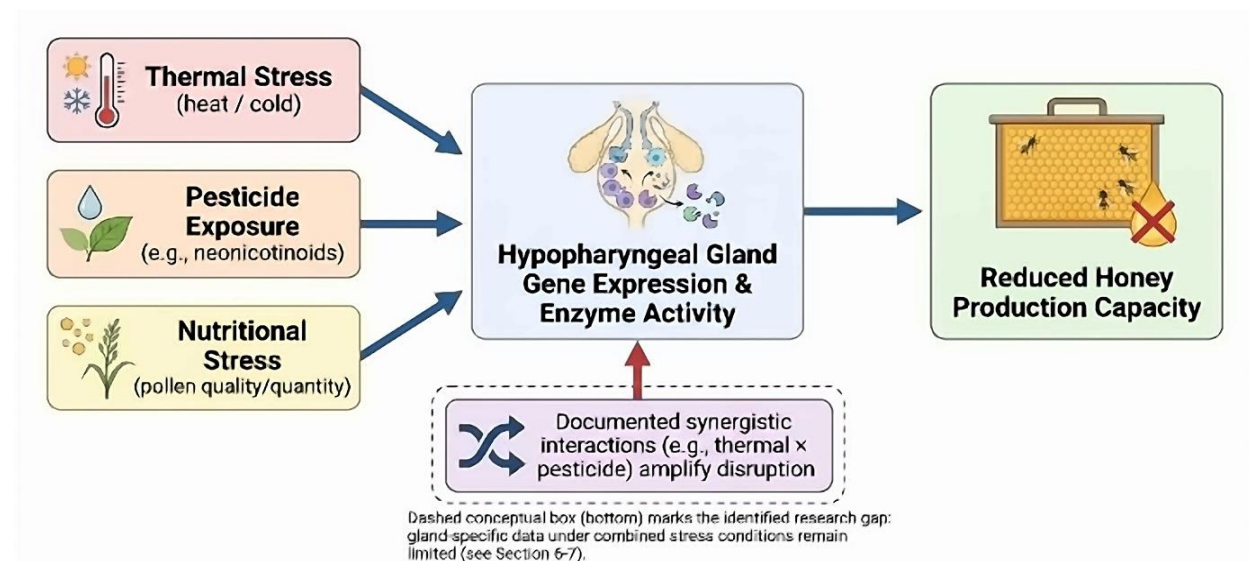


Figure 4. Conceptual model of environmental stressors converging on hypopharyngeal gland function and honey production.

7. Knowledge Gaps and Future Research Directions

7.1 Limited Combined-Stressor (Thermal + Pesticide) Studies

While the direct evidence for temperature-pesticide interaction effects is shown here at the whole-body and colony level (Kim et al., 2022; Alburaki et al., 2023), the pattern of combined exposure already demonstrates that interaction exposure changes the expression of those genes involved in the production of the glands

themselves, such as vitellogenin and *mrjp1*. But, there is no published work that have directly compared the gene expression, enzyme activity or gland morphology in the hypopharyngeals with a combination of thermal and pesticide stress. This is not an isolated exception; even targeted studies to analyze the impacts of multiple stressors have usually been limited to two stressors at a time, and recently, a three-way interaction experiment between malnutrition, agrochemical exposure, and virus infection revealed that the added effect

of stressors was highly dependent on the specific context; under poor nutrition, agrochemical exposure exacerbated the effect of the virus on bee mortality, but under good nutrition, the effect was reversed entirely (Hsieh & Dolezal, 2024). This discovery indicates that a two-factor study using the combination of temperature x pesticide is a necessary first step, but might still be underestimating in vivo sensitivity to the gland when the nutritional status is not utilized as a third variable. However, due to the combinatorial complexity of testing every possible combination of stressors experimentally, there has been a proposal to use mechanistic systems models as an alternative approach to prioritize which stressor combinations might be most biologically critical to test, as opposed to testing combinations essentially at random (Henry et al., 2017). If this "funnel-analysis" logic is applied to gland physiology more specifically, existing single-stressor gland datasets could be used to parameterize predictive models to determine which temperature x pesticide x concentration combinations are most likely to produce detectable gland-level effects, thereby making the eventual complete factorial wet-lab study more efficient to design.

7.2 Need for Functional Validation (e.g., CRISPR-Based Gene Function Studies)

The knowledge of current regulation of genes from glands is largely correlational, meaning that the differences in gene expression have occurred under stress conditions but not necessarily been functionally validated by direct testing of a candidate set of genes. Honeybees are well suited for CRISPR/Cas9 gene editing due to the high efficiency of delivery into the embryo by microinjection of a sgRNA and Cas9 protein and the resulting biallelic knockout of the major royal jelly protein 1 (Mrjp1) gene (Hu et al., 2019). Given that Mrjp1 is directly connected to the secretory activity of the glands there is a clear precedent to expand the approach of knock out of other candidate genes identified in studies of gland stress response, such as detoxification genes (e.g. CYP9Q3) and thermal stress response genes (e.g. hsp90, GR10). The technical scope of these tools has also expanded: The original SpCas9

system edited genomic sites with an NGG PAM sequence, but additional work has since resulted in the development of an integrated multi-nuclease system (incorporating SaCas9 and Cpf1 together with SpCas9) that significantly expanded the number of honeybee gene sequences that could be targeted for editing (Liang et al., 2021). This increased targeting range eliminates one practical drawback that could have prevented the use of one of these candidate gland-stress genes in a functional validation program using CRISPR, and thus provides additional proof of the potential of such a program for the genes identified throughout this review.

7.3 Data Gaps Between *Apis mellifera* and *Apis cerana*

The bulk of the published molecular and proteomic papers cited in this paper have been carried out in the honeybee, *Apis mellifera*. Comparative transcriptomic studies have shown that the expression of the genes significantly differs between *Apis mellifera* and *Apis cerana*, the latter being the Eastern honeybee widely cultivated throughout Asia, with more than 1,300 genes being found to be differentially expressed in gland developmental stages between the two species, especially in *A. cerana* (Liu et al., 2014; Shahid et al., 2025). This difference is not restricted to the gland transcriptome; a direct interspecific comparison revealed that, under extreme high-temperature and humidity conditions, *A. cerana* can withstand higher maximum temperatures than *A. mellifera* before the onset of intolerance, and that the activity of antioxidant and detoxification enzymes both species have increased significantly under combined heat and humidity stress, though the two species diverged in terms of their survival and body-water-loss pattern (Li et al., 2019). However, as far as it is known, there is no literature on environmental stress response of *A. cerana* in relation to honey yield until now, leaving unanswered the question of whether *A. cerana* is more, less, or just as affected as *A. mellifera* at the gland level when exposed to the same combined thermal stress and pesticide conditions described in this review paper.

Research Coverage of Environmental Stressor Effects on Honey Production Pathways

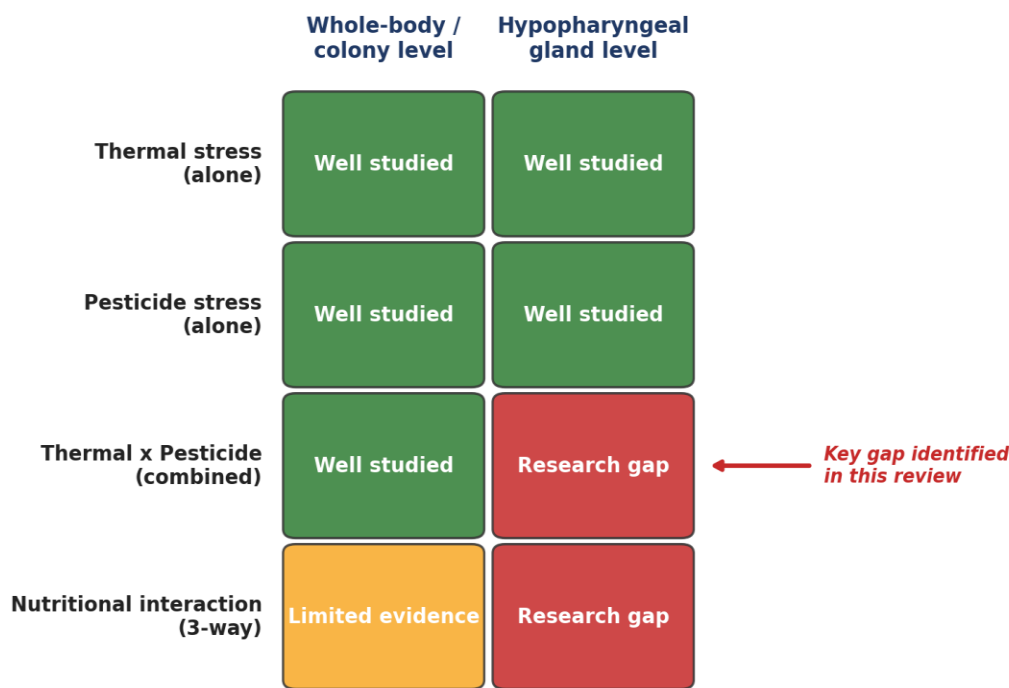


Figure 5. Conceptual synthesis of research coverage across stressor combinations and levels of biological organization, based on the literature reviewed in Sections 4–6. Colour reflects the density of available evidence, not a quantitative meta-analytic result.

8. Conclusion

Honey production in *Apis mellifera* is not a direct result of nectar collection but, rather, it is a regulated physiological process that is controlled by enzymes whose expression is closely correlated with the developmental stage and with the behavioral castes and hormonal environment of the worker bee (Ueno et al., 2015; Sahin et al., 2020). There is a well-defined regulation at the gene and protein level for normal development, which has been significantly contributed by proteomic and transcriptomic studies, especially from IAR-CAAS (Feng et al., 2009; Li et al., 2010; Ahmad et al., 2022). Environmental stressors disrupt gland physiology by different but overlapping molecular mechanisms on this baseline. Thermal stress has been shown to affect the expression of metabolic genes and protein synthesis in the gland itself (Maigoro et al., 2025; Maigoro et al., 2023), and pesticide exposure has

been shown to downregulate genes involved in honey production and upregulate genes involved in detoxification, indicating a physiological trade-off (Wu et al., 2017; Haas et al., 2022). This trade-off is in accord with the evolution of detoxification biology in the honey bee, which has evolved a lower number of xenobiotic-processing genes compared to most insects, making it thus expected that *A. mellifera* would be especially vulnerable towards synergistic, not just additive, effects of multiple stressors acting together (Berenbaum & Johnson, 2015). This explains, at the whole-body level, the synergistic impacts of thermal and pesticide stress on genes that are relevant to glands, as thermal and pesticide stresses individually have various effects on these genes, and at the gland-morphology level, the direct disruption to hypopharyngeal gland structure caused by combined pesticide exposures have already been demonstrated (even if not yet at the whole-body

level) (Kim et al., 2022; Alburaki et al., 2023). One of the greatest gaps in the information from this review is the lack of research specifically targeted to the links between combined thermal and pesticide stress. It is scientifically feasible – the same methods employed in single-stressor studies can be used to address a combined design, as can laboratory versus field comparisons of the effects of single stressors on the gland – and it is growing in importance, as climate change has been identified as a major stressor, and one that is inextricably linked with others, on managed bee colonies around the globe, and there is no overarching methodology that is currently available to support beekeeping to effectively tackle this stressor (Neumann & Straub, 2023). The complementary directions outlined – functional validation using CRISPR-based approaches (Hu et al., 2019), and extending this research to the other species of *Apis*, *Apis cerana* which has already been demonstrated to have diverged from *A. mellifera* on both the gland transcriptome and whole-organism thermal tolerance (Liu et al., 2014; Li et al., 2019) – would further support the mechanistic and applied significance of this research area. The molecular mechanisms underlying honey production are reasonably well known under baseline conditions, but not as well understood under the myriad of environmental stresses bees are increasingly exposed to. This gap can be addressed in a well-defined and consequential direction for future research – one that is directly continuous with and extends this review's synthesis of thermal- and pesticide-stress biology in the honey-production gland, and that involves gland-targeted, multi-stressor, and cross-species research.

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