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1/ A technical semi-field methodology to measure the effect of nutrition on honeybee brood rearing

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ABSTRACT

A honeybee colony's well-being is its ability to nurture larvae into healthy adults. Understanding how nutrition supports brood rearing is crucial for developing diets that could aid against environmental threats. Nutritional research on whole colony brood development has been historically challenging due to difficulties documenting the diet's impact on brood production over time. We describe a novel semi-field method to study the influence of nutrition on brood rearing using standardised small colonies formed *de novo* (*ca.* 1500 nurse-age bees and a queen) housed in adapted mating-nucs, placed inside an enclosure and limited to feeding on chemically defined diets. Complete assessments were conducted every fifteen days, assisted by a bespoke device to photograph every frame to measure cell contents. A novel metric describes the number of bees generated per gram of diet consumed, measuring the impact of nutrition on brood rearing and overall colony size.

INTRODUCTION

The honeybee colony's capacity to produce brood and generate healthy young workers is a hallmark of robust colony health [1–3]. The reproduction of honeybees exhibits a haplodiploid eusocial structure where adult individuals (male, reproductive female and unreproductive female) play distinctive roles in colony survival [4,5]. Beyond the queen's polyandrous mating, the population's survival relies on the worker population's ability to maintain larvae, collect food, thermoregulate, and fight off pathogens and pests through their specific division of labour [6,7]. Foragers collect pollen and nectar from flowering plants, which are then processed by nest bees and stored as bee bread or honey, respectively [8–10]. Nurse bees with engorged hypopharyngeal glands, between 3-12 days old, metabolise food to feed jelly to larvae and the queen [9,11–13]. And colony workers regulate the colony's temperature around 35°C for proper brood development [14,15]. These tasks require adequate nutrition and precise coordination between nestmates [16]. However, anthropogenic drivers have changed the diversity and abundance of flowering plants in agricultural and natural landscapes, affecting the honeybee colonies health [17–20]. Starvation conditions are becoming increasingly common, motivating beekeepers and scientists to develop feeds that mimic the composition of floral pollen to feed bees [21–23]. Providing sucrose syrup in the absence of nectar flow, along with protein patties to promote brood rearing [21,24], has raised beekeepers' operational costs related to feeds from 1% in 1976 to 20% in 2018 [25].

Research on whole colony nutrition has been ongoing since the 1930s [26], with literature covering a variety of methods in settings such as open fields [22,27–34], semi-fields [8,23,26,35–40], labs [41–43], and complex indoor bee-flight rooms [44–46]. Among the three primary macronutrients (carbohydrates, fats, and proteins), proteins play a crucial role in promoting brood-rearing [21], and pollen, the most studied protein source for honeybee colonies, has a substantial positive impact [47]. However, certain pollen types, such as dandelion and maise [35,40], are insufficient to support colony growth, and a diverse pollen source is widely regarded as essential for maintaining healthy bee populations [48,49]. When protein sources like pollen are restricted, brood-rearing ceases[50], and if carbohydrate stores, like honey, are absent, the colony enters a period of starvation, putting its survival at stake[19,21,51–53]. Without pollen, colonies cannot raise brood.

The unreliable characteristics of pollen accessibility have led to the development of honey bee diets with protein and many other nutrients but absent from natural pollen – commonly referred to as pollen substitutes. However, these still do not appear to assert a clear benefit in colony health [33]. Pollen substitute diets are less effective than those containing at least 10% natural

pollen in sustaining colony populations over the long term [22,33,54,55]. Furthermore, when feeding these diets under conditions of environmental pollen scarcity, honey bees deplete their internal reserves to support larval development, as these diets often lack essential nutrients such as sterols [21,24,52,56]. It still remains unclear how long a pollen-free diet can support healthy colony populations throughout their seasonal cycle.

A review of 22 independent field studies on pollen substitutes revealed that only five of them confirmed increased brood production[57]. While valuable for understanding real-world conditions, field studies come with significant challenges that can impact the reliability of the research. They are prone to uncontrollable environmental, technical, or genetic variables that may affect brood-rearing more than feeding protein diets [32,58]. For example, access to flowering plants and their pollen, even in small quantities, creates a positive confounding factor in brood rearing, which can mask the actual nutritional effects of the tested pollen substitutes [54]. Methods to restrict access to pollen, such as using pollen traps [33] can harm colony health by damaging workers [59]. Their location may also expose them to plant protection chemicals influencing colony development [60,61]. Likewise, using large colonies in such experiments can lead to the accumulation of other toxins [62] used to control varroa mites, affecting capping rates, larval survival, and adult bee mortality [63,64]. Large colonies are also more challenging in controlling for comb age, which influences the brood rate; new combs have larger brood areas and heavier worker bees than older ones[65], and even the spatial distribution of bee bread within the comb impacts brood-rearing success[66].

In semi-field studies, we can more effectively control pollen access, manage smaller colonies, and precisely measure population size. However, most of these studies use three or fewer colony replicates and only a few tracked brood rearing over time. Typically, colonies are confined in small outdoor cages (< 70 m^3) [67,68], which can lead to higher mortality and lower brood rates [36], with colony size varying from 10,000 bees [68] to 2,000 [40]. Also, the variety of methods of brood counting, such as visual area estimation [69], acetate sheets over a sample area [70], or image analysis tools [71], offer different levels of precision in estimating the diet's impact on brood rearing.

Most importantly, comparing the nutritional effects on brood rearing between whole colony studies is difficult without standardising methods and a standard measurable metric. In contrast, in the field of honey bee toxicology, standard guidelines and metrics are employed to evaluate brood rearing. Such as the Oomen bee brood feeding test [29] and the OECD75 standard guideline [72] are widely used by industry and academic working groups that usually test diets

laced with toxicants, assessing the impact of pesticides on bee brood. These use a key standard endpoint, the brood-termination rate (BTR), which measures the failure of eggs to transition into adult workers [72], thereby evaluating the lethality of the toxicant. Another outcome was the recommendation to use large polytunnels (>100 m^3) to mitigate the identifiable harmful 'caging effect' on bee colonies [66,73–75]. While BTR focuses on the adverse effects of toxicity, comparable standards are needed, in this case, to measure the diet's positive impact in whole-colony nutritional studies.

Therefore, we developed a specific semi-field method to address the need for method standardisation in diet brood-rearing studies and introduced a key standard metric, 'bees per gram': which measures the number of bees produced per gram of diet consumed by the colony. This metric allows comparing the brood-rearing potential of different diets across studies, locations and time. Our approach assesses the effect of diets on brood rearing in free-flying colonies under completely pollen-restricted conditions. Our experimental set-up involved mini-colonies consisting of 1,500 mixed nurse bees, five mini-frames with combs built the same year (without bee bread), and a same-year mated queen. Each mini-colony was housed in repurposed polystyrene, with 0.002 m^3 of nest volume, a dedicated feeding chamber, and enclosed within a large flight arena – a polytunnel with 628 m^3 of flight space or within a 125 m^3 glasshouse. Sucrose solution feeders were dispersed throughout the enclosure to simulate natural nectar foraging behaviour. Experimental brood assessment cycles lasted fifteen days each, where a bespoke device photographed every frame to analyse comb cell contents—empty, honey, or brood. Our approach reduces confounding variables such as environmental pollen access, colony population size, queen age, comb age, foraging behaviour, and agrochemical exposure, enabling the precise determination of the influence of diet on brood-rearing.

In the present study, we developed a minimal colony system through systematic optimisation and showcased key techniques that marginally disturb the colony while providing precise data on brood development. Our practical and reproducible system streamlines the transition from laboratory nutritional studies to practical applications that can directly benefit beekeepers. We hope this method establishes a foundation for standardising future nutrition studies in whole colony populations.

MATERIALS AND METHODS

Nutritional bioassay

Our nutritional bioassay is based on queen-right honey bee colonies exclusively consuming artificial diets, which are then metabolised by nurse bees to feed their worker larvae. The developmental cycle of workers, from egg until emergence, usually takes about 21 days. Our assessments track this cycle almost to its end, from egg until capped pupae - three days before emergence (Fig. 1A).

Our method comprises three parts: acclimation, feeding and brood assessment. An initial set-up phase, not represented in Fig. 1A, is required to first establish the mini-colonies inside the controlled flight enclosures. This initial set-up takes 5 days, where day 0 is the beginning of feeding. The feeding cycles repeat from days 0 to 15. Consumption and the bee population are monitored during the 15-day cycle, and a full brood assessment is conducted at the end. If, in between the 15-day feeding cycles, the mini-colony consumes the diet and has more than two frames of bees, the system progresses through the feeding cycle uninterrupted. However, if the bee population criterion is unmet, it triggers a nurse addition stage and colony acclimation. This is an additional 2-day process that involves adding nurse bees to low-populated mini-colonies, and the system resumes the standard 15-day cycle. This dynamic ensures that the system remains adaptive and maintains a colony baseline throughout its cycle. The method operates in a continuous loop with a repeating 15-day feeding cycle; however, we conducted only one cycle in this study.

PART I - Laboratory diet design

Our chemically defined diets consisted of protein, carbohydrates, fats, sterols, micronutrients, and a maltodextrin filler (with no nutritional value for the bee). We used as a protein source a plant-based - Soy Protein Isolate (Myprotein, UK; 90% purity) product as described in Sereia et al. 2013[76] . Here, we selected six diet treatments varying only in protein content (P): 6% (P6), 12% (P12), 15% (P15), 18% (P18), 25% (P25) and 30% w/w (P30). The percentage (w/w) of the diet total carbohydrate mixture ranged from 38% on P30 to 68% on P6, depending on the diet treatment. The carbohydrates consisted of about two-thirds of a mixture of 80% w/w sugar solution (in an equal mix of glucose and fructose) and one-third sucrose powder (Kent Foods, UK). As the fat source, we used soy lecithin (Optima Lecithin Health & Nutrition, UK) at 6% w/w of the total diet on all treatments as in *Stabler et al, 2021*[77]. We used Cholesterol (Sigma-Aldrich, ≥99%) at 0.1% w/w of the total diet as the sterol source, adapted from Herbert and Svoboda's 1980 diets[78,79]. We then mixed all ingredients into a dough and kneaded them into 50-60 g (5 mm thick) patties. To test the pliability and texture of the dough formulations (structure tests), we further tested them by placing samples on top of a plastic mesh (⌀ 1 cm)

inside an incubator at 34°C and 60% humidity (simulating in-hive conditions). After 24h, we discarded the diet formulations that absorbed excessive moisture and turned into sludge. We finally selected diets that successfully passed the structure test and that had been previously tested in our lab for palatability, consumption, and bee survival (> over 90%) in ventilated acrylic boxes (unpublished data), monitored over a week as in de Sousa et al. (2022)[80].

PART II - Mini-colony preparation and set-up

We assembled 36 mini-nucleus styrofoam boxes with dimensions 24 x 15 x 16.5 cm (Apidea Vertriebs AG, Switzerland), fitted with three plastic mini-frames with an 11 x 11 cm new wax foundation and one internal liquid feeder with clay pebbles inside to prevent bees from drowning. Each mini-nuc (mini-nucleus box) was number tagged and painted in separate colours to facilitate homing behaviour [81]. To measure enclosed temperature fluctuations, we added a temperature sensor data logger (Omega, OM-EL-USB-1) to the food chamber of a small subset of mini-colonies.

We began on day -5 by collecting a selection of mixed nurse bees from our apiary by gently shaking open-brood frames covered with nurse bees into our custom-made "wooden nurse collection box" (37.4 x 42.2 x 31.4 height cm) (Fig.1C). This box covered by insect metal mesh, had three internal frames, spaced at 6 cm one from the other, allowing bees to suspend in cluster formation to dissipate heat in the field. A detachable acrylic funnel sits on top, facilitating the brushing of nurse bees into it. Nurse bees were brushed while lightly sprinkling with a 33% sugar solution laced with a couple of drops of lemongrass oil (Neal's Yard, London) to prevent them from flying and to encourage nestmate formation [82]. To populate each mini-nuc, nurse bees must be brushed from 1.5 frames of open brood. Once complete, the box rests for one to three hours inside a dark room at 10 °C to promote cluster formation.

Next, we turned each mini-nuc bottom up, sliding open the floor panel and exposing the three plastic mini-frames with the foundation. Lining all Apideas in a row, we sequentially placed on each a caged mated queen with candy (Candito per Api, PIDA, Italy) between the 2nd and 3rd frames. We used a 500 mL glass beaker to shave the bees off the collection box frames, filled each mini-nuc with 450 mL of mixed nurse bees (approx. 1,018) inside, and then slid closed the floor panel. Once all mini-colonies were formed *de novo* with nurse bees and a mated queen, we fed 350 mL of 1:1 (water to sugar) syrup into the internal feeder. We placed all mini-colonies housed in Apideas inside a ventilated dark room at 10°C for 48h for further nestmate and colony formation.

PART III - Mini-colony acclimatisation to semi-field enclosures

We conducted two experiments, the first in a glasshouse with 30 colonies and the second in a polytunnel with 6 colonies. After the 48 hours of nestmate formation (day -3), at sunset, we released 30 mini-colonies inside the two-room glasshouse (l: 10 m x w: 5 m x h: 2.5 m) with 125 m^3 of total flight volume to acclimate to the semi-field setting and to test our five increasing stepwise protein diets (P6, P12, P18, P25, P30). The repurposed glasshouse consisted of glass panels with a galvanised steel frame structure. Each of the two rooms of the glasshouse had a long central table where we placed 15 mini-colonies in groups of three (triangle shape) with entrances pointing in different directions. Due to the inherent heating capacity of the glasshouse infrastructure and to promote air circulation, we installed a mesh shade covering the whole area of the glass ceiling, two commercial extraction fans on both ends of the glasshouse, three portable aircon units, and three oscillating pedestal fans.

The second experiment was conducted in a purpose-built polytunnel (NP Structures, UK) measuring 18.4 m x 8.23 m x 4.15 m, providing six times more flight volume (628.4 m^3) than the existing glasshouse. The polytunnel was covered in white insect mesh, and we repeated the same method with the (P15) diet using 6 new mini-colonies, which were placed in different directions in two groups of three, each on two tables, as described above.

After 24h of releasing the mini-colonies inside the semi-field enclosures (day -2, see Fig 1.A), we expanded their nest volume by removing the internal liquid feeder, adding two frames with wax foundation, and a top solid food chamber. We then top-up with an extra 250 mL of mixed nurse bees (approximately 566 bees) from our "nurse collection box" (Fig.1C). The solid food chamber, used for top-up nurse bees and placing our protein diets, is made from an APIDEA super feeder (700 mL), into which we cut a central hole (10 x 8 cm) and covered it with a green plastic mesh (12.5 x 9.5 cm, ⌀1 cm) (Fig.1B). Diet patties were placed over the mesh, 1 cm above the brood frames. Bees could access the food from below and move within the food chamber (Fig.1B) to consume the diet.

Next, we suspended two artificial flowers per mini-colony throughout the enclosures, made from adapted 250 mL infant feeding bottles by piercing five ⌀0.5 mm holes in its nipples and adding colourful velvet pipe cleaners for bee identification and grip (Fig.1 D). Each bottle was filled with 250 mL of 33% sucrose solution and hung upside down, functioning as a gravity feeder to promote nectar foraging. Our artificial flowers restricted bees' drinking access to only five at a time, preventing hoarding behaviour. The sucrose concentration in our feeders is in the mid-range of nectar of flowers typically visited by honey bees [83], and extends foraging activity

within the enclosures relative to using higher sucrose concentrations. Additionally, such syrup concentration maintains high internal humidity favourable to brood development in summer and engages nest bees in activities of nectar-to-honey conversion. Drawbacks of low syrup concentration were noted during periods of continuous rain and cold within the polytunnel, leading to an outbreak of chalkbrood, consistent with findings by Flores et al [84]. We washed and replenished feeding bottles per week and as needed. We also placed four tubs with fresh water and support rocks in each corner of the enclosures for water supply.

Our mini-colonies were considered ready when they consisted of a single 5-frame brood chamber with the additional top food chamber (Fig. 1B). Internally, colonies contained five frames populated with bees (approximately 1,500 individuals), sugar syrup reserves, and brood exclusively at the egg stage produced by a mated queen.

PART IV - Mini-colony nutritional bioassay for brood assessment, colony weight and population size estimation

We assigned *n*=6 mini-colonies to one of each of five diet treatments (P6, P12, P18, P25, and P30) to the glasshouse enclosure and (P15) to the polytunnel enclosure. The assignment criteria of diet to mini-colony aimed to maintain a balanced population size distribution and entrance direction among treatments and reduce bias on sun exposure, orientation, and colony population. Before feeding the mini-colonies with the protein diets, we recorded the initial weight of each mini-colony with a field scale (Ohaus Scout Pro Portable). We also recorded the number of bee seams (0 to 6 bee seams - clusters of bees occupying the spaces between two combs, or comb and hive wall) as per *Chabert et al, 2021* [85] by photographing the colony from above to estimate the population size of each mini-colony. Each bee seam was estimated to represent approximately 300 bees to calculate the colony size.

For all treatments, we weighed each diet to 50-60 grams of dough, rolled them into 5 mm thick patties, and placed them inside the food chamber over the green mesh. This marks the start of our nutritional bioassay experiment (day 0). A week later (day 7), with minimal disturbance to the colony, we removed the patty and registered population size and diet consumption by tracking changes in the patty's weight for all mini-colonies and diet treatments (Fig.1A), taking an observation note if bees were eating the patty. The treatments are discontinued if nurse-aged bees avoid the diet altogether, indicated by marginal changes in diet weight or 'negative consumption' – i.e. when patties are not eaten and instead gain weight due to moisture absorption. In both experiments, the feeding cycle proceeded since consumption was observed in all treatments. Therefore, in our study, a fresh patty was provided, and its weight was

recorded before adding the new patty. The mini-colonies were weighed to estimate changes in sugar stores.

On day 15 of our bioassay experiment (one brood cycle), we performed a complete mini-colony assessment on all treatments. We assessed colony weight, population size, honey stores, diet consumption and brood stages on all frames (Fig. 1 A). We measured the brood stages by photographing both sides of every frame using a bespoke photographic-acrylic device (Fig. S1 - supplementary). The device consisted of a custom-made acrylic box (internal dimensions: 22.3 x 14.5 x 13.2 cm, ⌀ 4 mm of thickness) that could be mounted on top of an open mini-colony rim, with a one-frame stand at its centre. On each end, it displayed two RPi cameras (64MP Autofocus Camera, Arducam) focused on the frame's centre, with both cameras connected to a Raspberry Pi 4 (designated as the imager). Another Raspberry Pi 3 (named "controller") was connected to a touchscreen display (Official Raspberry Pi 7" Touchscreen Display) and to the "imager" via an Ethernet cable (Fig. 1E). The "controller" controlled the "imager" remotely via SSH using a shell script software "image-experiment.sh". Detailed set-up instructions and the code used are available at github.com/carandraug/beehive-imaging under the GNU All-Permissive License. The device recorded the time taken for the complete assessment (five frames, 10 pictures per mini-colony serially numbered); an experienced researcher spent, on average, 5 minutes and 41 seconds per mini-colony from setting the device to removing it. We then visually counted the number of capped brood cells in each image using available software (Adobe Creative Cloud 2019-2024, version 6.4.0.361, PS -Count Tool). At this stage of our experiment, we measured the influence of nutrition during the "one-capped bioassay cycle" (Fig.1A). We defined each bioassay cycle as the period when all capped brood was originated from eggs that were laid under the experimental procedure. At the start of the feeding period, all treatments started at the same brood stage (eggs) at day 0, with no capped cells present.

Not all of the eggs that the queen lays will develop into adults. Nutritional stress may lead to oophagy, the consumption of eggs by nurse bees, and up to cannibalistic consumption of the young larvae [21]. Developing brood that is sick may be identified by nurse bees, which will open capped cells and remove the sick bee [86]. However, a healthy brood that reaches the capped stage will likely emerge as an adult. In the subsequent 15-day bioassay cycles, the previously capped brood will emerge, the newly laid eggs will turn into pupae, and a new cycle begins, guaranteeing that we are not double counting the same capped cells or missing capped cycles. Hence, we can continue the experiment for additional bioassay cycles by sequentially repeating the procedures from Day 7 and Day 15, measuring each newly capped cell that is in the stage

between the pre-pupae and the point before the pupae emerge. In this study, we terminated the experiment after the first bioassay cycle.

Data, Statistics and Power Analysis

Statistical analyses of diet consumption, capped-brood-cell counts, protein consumption, and "bees per gram" were conducted in Python 3.12.4 (www.python.org) within VS Code, with assistance from GitHub Copilot. Models and related functions from Statsmodels 0.14.1 (www.statsmodels.org) with numpy 2.2.3 and pandas 2.2.3 were used for all tests. We removed three mini-colony replicates from the analyses that lost their queen and had zero capped brood at the end of the 15-day bioassay.

To analyse diet consumption during the nutritional bioassay, we measured the total diet consumed over the 15-day period. Protein ingestion was estimated by multiplying each diet's protein percentage by its total consumption. We then calculated our metric, 'bees per gram,' by dividing the number of capped brood cells by the total diet consumed. This metric represents the number of bees reared per gram of diet that is consumed, based on the assumption that each capped cell will develop into a viable adult bee. A Pearson correlation matrix was used to assess relationships between the independent variable (diet type) and dependent variables, including capped brood, total diet consumption, protein ingestion, population size, and 'bees per gram.'

Our main objective was to evaluate the effects of nutrition, specifically protein content, on brood development. We explored the relationship between protein percentage as a continuous variable with our response variables—consumption, capped brood cells, and 'bees per gram' by using the Ordinary Least Squares (OLS) regression model from the Statsmodels 0.14.1 package. We estimated the intercept of the regression line to make it more accurate for our biological data. Key outputs include: the coefficient (coef), which indicates the strength and direction of the protein influence; the p-value and the R^2 value, which quantifies how much of the variation in the response is explained by protein. We also used the OLS to perform a power analysis to estimate: i) the probability of accurately detecting the protein effect on the brood - power (1 - β) and ii) considering that in future studies, we require an 80% degree of confidence – what is the sample size needed. We used Cohen's f formula$\sqrt{(r^2}/(1-r^2))$ to calculate the effect size and set the alpha level at 0.05.

The relationship between bee population size and capped brood was analysed by averaging population size between the three time-points assessed during the feeding period: day 0, day 7

and day 15. Population trends were analysed using Graphpad prism 10.4.1. To analyse population trends throughout the days and population differences between diets, we employed its mixed-effects model with Restricted Maximum Likelihood (REML) estimation at a significance level of 0.05, assuming that the variance between treatments was equal. Specifically, the mixed-effects model evaluated population differences between diet treatments inside the glasshouse (P6, P12, P18, P25, P30) on each assessment day and assessed population changes over time for each diet. This analysis covered the feeding period (days 0, 7, and 15) and the acclimation period (days -2 and -1). We then used Tukey's post-hoc multiple comparison test to determine population differences between days and treatments. Since population size in mini-colonies can be a confounding factor for rearing capped brood, and since we observed absconding events among mini-colonies inside the glasshouse, we set criteria to identify potential outliers. Specifically, if a mini-colony showed a significant difference in population size in our mixed-effects model between Day 0 and Day 15, and its capped brood measurement had a z-score exceeding the factor of 2, meaning a data point beyond two standard deviations from the mean, we have considered that measurement an outlier. The z-score (z) for each data point (x) was calculated using the formula: $z = (x - \mu)/\sigma$. Where (μ) is the mean of the data and (σ) is the standard deviation. Those outliers were not accounted for in statistics but are represented in figures.

To analyse the relationship between the two enclosures, polytunnel versus glasshouse, we selected a subset of our glasshouse data, the P18 diet, to compare it to the diet that was tested inside the polytunnel, the P15 diet. Here, we used a simple t-test to compare these two groups' means and establish if the difference between them is statistically significant. In this case, we used Graphpad prism 10.4.1 t-test model.

RESULTS AND DISCUSSION

Protein-rich diets increase both diet consumption and protein intake.

The mini-colony's access to pollen was entirely restricted and absent from all comb stores, with protein provided solely through the experimental diets delivered to the mini-nucs throughout the 15-day bioassay cycle. We performed an Ordinary Least Squares (OLS) model to evaluate the influence of protein percentage on diet consumption (Fig. 2A). The OLS showed that protein-rich diets positively affected diet consumption ($\beta = 1.33$, $p < 0.005$), indicating that for each protein percentage increase in our diets, our mini-colonies will eat 1.3 grams more. The model explained 35% of the variance in diet consumption ($R^2 = 0.35$, $F(1,25) = 13.6$, $p = 0.001$.) Additionally, the Pearson correlation demonstrated a positive correlation between protein-rich

360 diets and diet consumption (*r(25)* = 0.59, *p<0.005*). Although protein strongly influences diet
361 consumption, it doesn't necessarily mean colonies will neglect diets that are entirely absent of
362 protein. For example, other dietary components, such as carbohydrates, can also significantly
363 influence food collection and consumption in honey bee colonies[87–89] .

364 Since diet consumption and protein intake are inherently linked in our experiment, mini-
365 colonies that consumed more of the protein-rich diets also ingested more protein (Fig. S1).
366 Here, the OLS model showed a strong fit, explaining 80% of the variance in protein intake (R^2 =
367 0.80, F(1,25) = 104.2, p < 0.001). The regression coefficient indicated that for every gram of diet
368 consumed, colonies ingested approximately 0.35 g of protein (β = 0.35, p < 0.001). A Pearson
369 correlation further confirmed a strong positive association between diet consumption and
370 protein intake (r(25) = 0.90, p < 0.005) (Fig. S1). These results indicate that mini-colonies
371 consumed more and ingested significantly more protein when offered protein-rich diets (Figs.
372 2A, S1), suggesting that dietary protein may have both phagostimulatory and metabolic effects
373 that enhance consumption.

374 **Protein-rich diets strongly influence the number of capped brood cells and produce**
375 **significantly more bees per each gram of diet consumed.**

376 Protein plays a critical role in the ability of a honey bee colony to produce healthy young workers
377 as brood rearing ceases without an adequate protein source. One of our primary objectives was
378 to assess how dietary protein content influences brood production and whether a stepwise
379 increase in protein percentage drives greater protein intake and brood rearing. As shown in
380 Figure 2B, we observed a clear positive relationship between dietary protein percentage and the
381 number of capped brood cells. The OLS demonstrated a strong fit, explaining 76% of the
382 variance in capped brood production (R^2 = 0.76, F(1,25) = 78.61, p < 0.001). For every 1%
383 increase in dietary protein, colonies produced an average of 11 additional capped cells (β =
384 10.9, p < 0.001). A power analysis based on our sample size of 27 mini-colonies indicated a 99%
385 probability (power = 0.99) of detecting a protein effect within the tested range. If in future
386 studies a power of 80% were acceptable, only five colonies (N = 5), one per diet level, would be
387 needed to detect a similar effect.

388 A consistent trend was observed for the relationship between protein intake and brood
389 production (Fig. 2C). The OLS model showed a strong fit, explaining 74% of the variance in
390 capped brood (R^2 = 0.74, F(1,25) = 69.56, p < 0.001). For every gram of protein consumed, mini-
391 colonies produced an average of 12 additional capped brood cells (β = 12, p < 0.001). Protein-
392 rich diets had a marked impact on brood production. Colonies fed the highest-protein diet (P30)

393 produced more than twice as many capped brood cells as those on P18, and nearly ten times
394 more than colonies on the lowest-protein diet (P6). These findings support our hypothesis that
395 total protein intake—rather than overall diet consumption—is the primary driver of brood
396 development. Diets with higher protein content significantly enhanced brood production,
397 suggesting that increased protein availability improves colony productivity by increasing worker
398 numbers, which may in turn benefit overall colony health.

399 The ability to precisely quantify both diet consumption and the number of capped brood cells
400 enabled us to estimate diet efficiency, defined by our key metric: bees per gram—the number of
401 bees produced per gram of food consumed (Fig. 2D). The mini-colonies fed diets with low
402 protein content like (P6) and (P12) generated less than one bee per gram, whereas protein-rich
403 diets like (P25) and (P30) reared four times more bees per gram of diet, 4.39±2.78 and 4.43±1.21
404 bees/g, respectively. This relationship between protein and brood is consistent with previous
405 studies, such as Ricigliano et al. [22] and others [30,34,90–94], demonstrating a positive relationship
406 between protein supplementation and brood development. Similarly, Li et al. (2012) [95] identified
407 pollen substitutes containing 30-35% protein as the most influential factor for promoting bee
408 development.

409 To our knowledge, this brood bioassay method is the first to estimate the number of bees a
410 protein diet can produce and evaluate the influence of protein on brood development in a
411 stepwise approach. The novelty lies in demonstrating that a stepwise increase in dietary protein
412 content leads to increased diet consumption (Fig. 2A), and that greater consumption of protein-
413 rich diets results in a compounded intake of total protein (Fig. S1). Furthermore, consumption of
414 protein-rich diets was associated with a higher number of capped brood cells produced (Figures
415 2B and 2C), ultimately improving brood production efficiency, as evidenced by a greater number
416 of bees produced per each gram of diet consumed (Fig. 2D). Future studies should incorporate
417 a positive control using pollen-based diets and a negative control consisting solely of
418 carbohydrates.

419 **Supplementation of nurse bees and population dynamics in mini-colonies: the population**
420 **was affected by supplementation but not by diet treatments.**

421 Mini-colony populations were formed by mixing unrelated nurse bees into a single group,
422 orphaning them by keeping them queenless for three hours and spraying them with sugar syrup
423 laced with an artificial odour, such as lemongrass [96]. This method proved helpful in forming *de*
424 *novo* colonies of specific sizes. All mini-colonies were established by measuring 700 millilitres
425 of mixed-aged bees (approximately 200 grams); we estimated that there are approximately 787

426 bees per 100 grams and 226 bees per 100 millilitres. This estimation was based on calculating
427 the average number of bees per 100 grams of weight and per 100 millilitres of volume from 28
428 research papers, including our lab measurements. At the start, we estimated a population of
429 about 1,580 bees per mini-colony - an average of 5.2 bee seams, where each seam represents
430 approximately 300 bees.

431 Since the release into the enclosure on day -5, we measured population sizes during the
432 acclimation period on days -2, -1, and 0. We standardised colony populations prior to the
433 feeding period, where colonies with fewer than 1,500 total bees were supplemented with
434 orphan nurse bees until each colony contained 1,400–1,500 nurse bees (Fig. 3A). On day -2, at
435 least 24 colonies required substantial supplementation with a mean initial population size of
436 1,050 ± 146 bees (ranging from P6 to P25 treatments). One day after supplementation (day -1),
437 the population of all colonies increased to an average of 1,530 ± 202 bees. However, a slight
438 reduction in population was observed across all mini-colonies between day -1 and day 0, likely
439 due to bee disorientation and subsequent bee losses within the enclosure. Despite this
440 reduction, supplementation successfully increased the population size across all treatments,
441 with a mean of 1,422 ± 195 bees recorded on day 0, closely approaching the target. Statistical
442 analysis demonstrates significant differences in population sizes between days -2 and 0 (Fig.
443 3A; mixed-effects model, three-time points; $P < 0.0001$). This supplementation technique
444 effectively increased the number of nurse bees in underpopulated mini-colonies and
445 standardised colony populations before the onset of the feeding period on day 0. Previously, we
446 attempted to avoid this supplementation step by stocking all mini-colonies from the onset (day
447 -5) with more than 1,500 bees by adding extra bees (>1500) and starting with five frames in each
448 mini-nuc instead of three. However, due to ventilation constraints in the Apideas and the need
449 to use a large top feeder rather than a smaller internal feeder, the bees had great difficulty both
450 ventilating the nuc and consuming syrup during the crucial 48-hour cold and dark
451 nestmate/colony formation period. This variation resulted in high colony mortality; hence, we
452 opted for the intermediate step of adding bees on day -2 according to our methods.

453 During the bioassay feeding period, between day 0 and day 15, we did not anticipate significant
454 differences in population size over such a short time frame unless variations in adult mortality
455 were present. No statistical differences were found when comparing population sizes between
456 diets (Fig. 3B; mixed-effects model, *ns* Diets $P > 0.05$). Additionally, no strong correlations were
457 found between population size and any of our key metrics: protein content (Pearson's $r(4) =$
458 0.08, $P > 0.05$), consumption (Pearson's $r(4) = 0.18$, $P > 0.05$), and capped brood (Pearson's $r(4)$
459 $= 0.48$, $P > 0.05$). Additionally, when comparing population trends during the 15 days within

each diet, most treatment diets (P6, P12, P25, P30) showed no significant population changes (Fig. 3B; Tukey's HSD, Days 0, 7 and 15, $P > 0.05$), as expected from the experiment's short duration. However, mini-colonies in the P18 did increase their population from day 0 to day 15 (Fig. 3B; mixed-effects model, $P < 0.05$, Tukey's HSD, Days 0,7 and 15 $P < 0.05$), most likely due to queenless bee colonies absconding randomly to these colonies rather than brood emergence.

Regarding behavioural observations, orphaned nurse bees exhibited behaviours similar to natural swarms, forming cohesive clusters with minimal flight activity and showing high plasticity in nest-mate affiliation. The use of the 'nurse collection box' proved an effective tool for forming new colonies and replenishing mini-colony populations. Aggression toward non-nestmate nurses at hive entrances was observed in a handful of mini-colonies but subsided within two hours. Supplementing orphaned nurse bees and bee population variations due to absconding can influence colony nutritional physiology; therefore, it is important to time the supplementation across treatments and register any absconding events.

Enclosure systems strongly influence ambient temperature and significantly influence brood health.

The method described was developed over five years, with initial trials of varying mating-nuc type, enclosures, colony size, and feeder configurations until it reached the refined stage presented here. Maintaining the daily average ambient temperature inside the enclosures below 37.5°C is crucial, as higher temperatures affect brood survival [15]. For example, Xu *et al., 2023* [97] have sown that pre-pupae in the developmental stages (days 6 to 9) and pupae exhibit high susceptibility to hive temperatures of 40°C, with increased mortality rates and bees emerging with shortened proboscises and forewings.

Trials tested inside the glasshouse proved significantly challenging in hampering down environmental temperatures. Throughout the optimisation of our methods, we have employed shading nets, misting systems, air conditioning units, pedestal fans, and two horizontal industrial extraction fans; however, these measures were insufficient to prevent some queen losses and absconding during heatwaves *(n=3)*. Additionally, the ground floor cover type can impact the temperature and air sanitation within the enclosed space. Ground covers made of concrete slabs have high thermal mass, which adds a challenge to cooling the environment. Other drawbacks are the accumulation of bee carcasses, which increased the researcher's susceptibility to heat stress and bee dust-related hay fever, necessitating regular cleaning.

492 In July 2019, the glasshouse ambient readings from the supporting tables (n=2) and in-hive
493 temperatures (readings from the food chamber, n=2) were measured every 5 min during the
494 hottest days with USB temperature data loggers (Omega Engineering) (Fig.4). Peak ambient
495 temperatures reached 45°C inside (Fig. 4A). Adult workers demonstrated greater heat stress
496 resilience than brood and queens. Three queens died during the July heat wave that coincided
497 with our bioassay cycle. Despite the losses, the remaining mini-colonies were capable of
498 regulating their internal temperature, maintaining a more stable and less variable environment,
499 as demonstrated by the lower standard deviation (SD) between the inside (6.31°C) and the
500 outside (10.32°C) temperature, as well as the smaller interquartile range (IQR); inside (11.5°C)
501 compared to the outside (20.1°C). The misting system effectively mitigated some heat stress,
502 activated from 12h30 until 16h00 in 30-minute bursts, reducing the ambient temperature from
503 43°C to 30°C (Fig.4A). Once it stopped, the temperature rose to 40°C.

504 The type of enclosure inherently creates different environmental conditions, significantly
505 influencing the mini colonies' capacity to rear their brood (Fig. 5A). In our analysis, we excluded
506 one replicate from the (P18) diet group because its capped brood had a z-score of 2.213,
507 exceeding two standard deviations from the mean, and the mini-colony showed a significant
508 population increase from Day 0 to Day 15. This met both of our criteria for identifying outliers.
509 Still, the mini-colonies inside the polytunnel were able to rear twice as many capped brood
510 cells compared to those inside the glasshouse (Fig. 5A; unpaired t-test, $P<0.0001$), despite
511 similar consumption and population sizes (Fig. 5B and 5C; unpaired t-test, ns, $P>0.05$). The
512 main methodological differences between the two locations were the type of enclosure used to
513 house the mini-colonies during the bioassay and the diet protein percentage. As previously
514 discussed, protein contents influenced brood-rearing capacity (Fig. 2B and 2D). However, we
515 tested a lower protein diet inside the polytunnel (P15) that, nonetheless, had greater brood
516 rearing with a 59% increase in capped cells when compared to the glasshouse (Fig. 5A). This is
517 most likely due to the consistently higher temperatures and poorer ventilation inside the
518 glasshouse, which reached 40°C during the bioassay period. O'Connell *et al.* (2024) [98] and
519 others [99] also reported the detrimental effects of high temperatures on colony physiology and
520 brood care.

521 **Considerations on temperature control, enclosure design and optimisation.**

522 In the purpose-built polytunnel (628 m^3), the ground was intentionally covered with perennial
523 ryegrass, improving heat absorption and ground sanitation – bee carcasses naturally and
524 quickly decomposed. Flowering was prevented by regular mechanical mowing any appearance

525 of flower buds. Furthermore, other differences between the two enclosures might have
526 additional impacts on the well-being of the mini-colonies. For example, flight volume was higher
527 inside the polytunnel, which also had better ventilation due to its netted enclosure. The
528 polytunnel ground had a natural composition of ryegrass, in contrast to the concrete slabs
529 inside the glasshouse. Therefore, consideration should be given to the type of enclosure and
530 materials used for these experiments, and we suggest, when possible, using an enclosure that
531 can closely resemble natural environments, such as our large polytunnels instead of
532 glasshouses.

533 Other observations made inside the enclosures were foragers' ability to collect sugar syrup from
534 various feeder types and deliver it to their colony. Feeders providing ample drinking space were
535 emptied within hours, causing some mini-colonies to fill rapidly. When we supplied high-
536 concentration syrup (>50% carbohydrates), we noticed a reduction in the area available for
537 queens to lay eggs – in these mini-colonies, sugar storage can rapidly occur in the middle
538 frames limiting the number of empty cells available to egg laying and potential brood rearing. A
539 syrup concentration of 33% carbohydrates, similar to the nectar found in wildflowers[100], was
540 preferred. The current artificial flowers function as gravity feeders. Developing a mechanism for
541 timed nectar release is expected to prolong foraging activity and potentially reduce the
542 incidence of foragers becoming trapped against the enclosure walls and in "light traps" by
543 keeping them engaged in the artificial flowers. Future studies should explore methods to
544 mitigate these traps through improved light dispersion shading techniques, including mitigating
545 heat shocks during the summer by applying better cooling systems.

546 **Development and optimisation of a minimally disruptive image-capturing system for**
547 **standardising honey bee nutritional studies**

548 Image analysis is a powerful tool for evaluating colony development, and several machine
549 learning models have been developed to measure comb cell contents accurately[101], such as
550 DeepBee[71] or adapted ImageJ models[102]. However, as crucial as developing these models is, an
551 optimised image-capturing mechanism is essential. The need to shake colony frames to release
552 adult bees for image capture, combined with sudden comb temperature fluctuations, can result
553 in high stress levels for both adults and brood, negatively impacting colony health. The novel
554 device explicitly constructed for this bioassay method sits on top of the mini-colony, allowing us
555 to optimise image timing and minimise disruptions to our mini-colonies during the 15-day
556 complete assessment cycle. Frames with adult bees are removed and shaken sequentially to
557 the inside of the acrylic box, guaranteeing that all nurse bees are placed back in their hive with

frames to support them. The acrylic used to construct the box was off-the-shelf anti-glare Perspex acrylic with UV protection, similar to what museums use to protect picture frames. This provides some UV protection to honey bee eggs and larvae, sensitive to UV irradiation[103].

The image capture speed on both sides of the frame is approximately ≈1.19 min per frame. A single experienced researcher can complete a full assessment of each mini-colony in (≈ 6.35 min), less than 10 min per mini-colony. In contrast, previously, two were needed to speed up the process and reduce colony disruption. To the best of our knowledge, this is the quickest and least disruptive method to capture images across all honey bee colony frames. The image's size is ≈ 2.2Mb, and its quality is assessed by visually confirming it at the touchscreen display. We repeated image capture if images turned out of focus, stray bees covered the combs or environmental light impeded a clear image (Fig. S2.). We captured 800 frame images in one trial, and 39 were out of focus or poor quality. Counting cells with eggs and larvae remains challenging, as the device only captures the full depth of the comb located at the centre of the mini-colony frame, about 100 cells per frame side. This is due to the natural individual cell shape that has an hexagonal structure tilted upwards at 9-14° and with depth of aprox. ≈12mm. In contrast, capped brood and honey cells are easily quantifiable by visually counting each comb cell surface.

The double Raspberry Pi set-up was required because the Raspberry Pi 4 cannot reliably and simultaneously control the Arducam 64MP Hawkeye cameras and its touchscreen display. Therefore, the two cameras were connected to one RPi, the touchscreen display was connected to the second RPi, and an Ethernet cable connected both RPi together. The RPi with both cameras was designed as the "imager", and the RPi with the touchscreen was designed as the "controller". The controller Pi controls the imager Pi remotely and displays the acquired images for review. The capture speed and the acrylic box protect the combs from environmental temperature and UV exposure, which is especially relevant in frames containing brood. However, improvements are still possible in further iterations to increase capture speed and image quality. For example, installing additional cameras would allow us to capture comb edges more effectively, or a scanning method (with a single camera) would enable us to examine each frame from top to bottom. Also, making the system completely wireless for higher flexibility and manoeuvring in field-type of settings. It would also be possible to optimise the software code to enable the device to capture images on both frame sides, optimising speed simultaneously. Future enhancements could also incorporate artificial lighting on each side to better capture honey bee larvae and include small temperature sensors in the acrylic box to warn the

researcher if local micro-temperatures that harm the brood are extremely low or high. We hope this method establishes a foundation for standardising future nutritional studies as a whole.

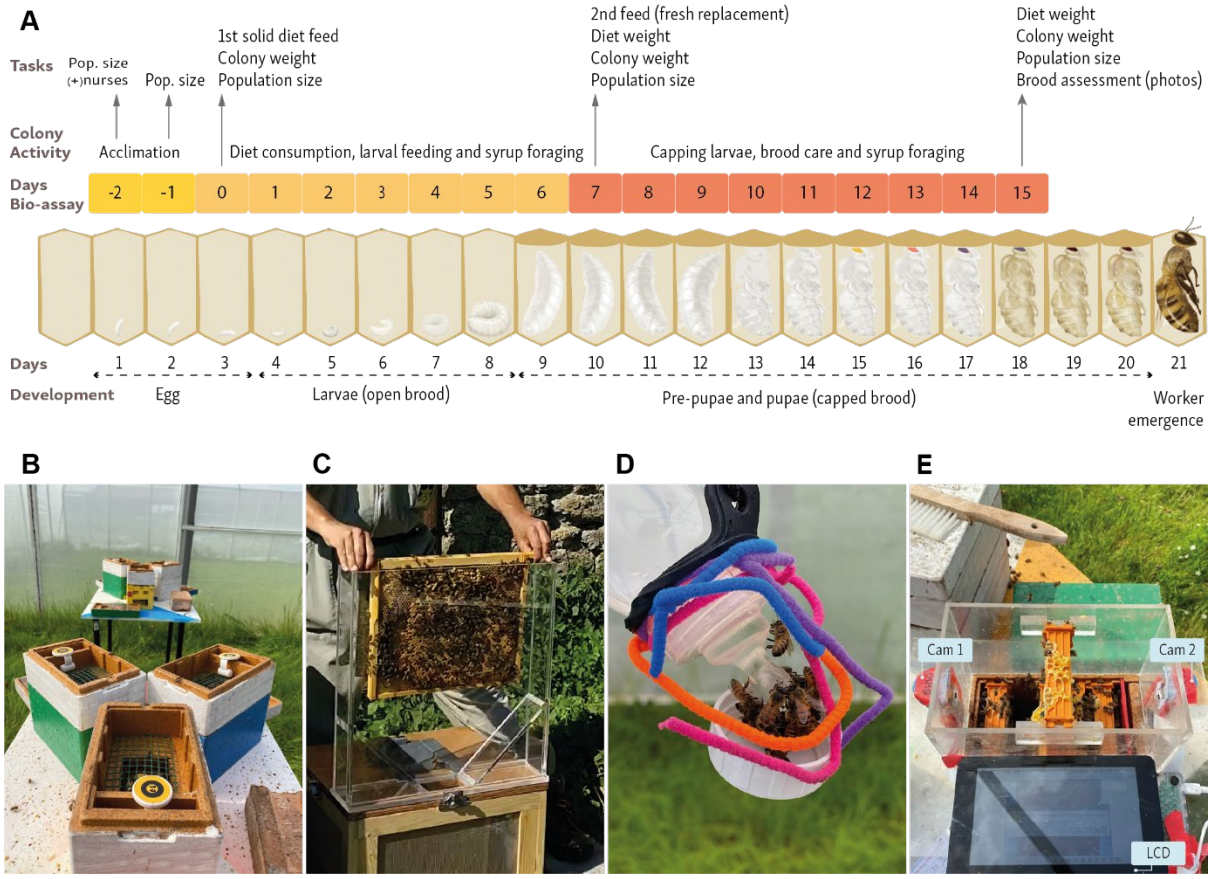

Fig. 1. The brood assessment bioassay protocol. (A) The chart matrix displays the honey bee development stage each day, stacked with bioassay protocol days; negative days represent the acclimation period, and days 0-15 represent the feeding period with a 15-day cycle and a final brood assessment. From top to bottom, the matrix summarises the main tasks to be performed at each time point. At day 0, the main tasks include assessing colony weight, population size and feeding each with a solid diet (patty). On day 7, we recorded the colony weights, population size and the weight change in the patty to estimate diet consumption. Finally, on day 15 (one brood cycle), we assessed the same metrics plus a full brood observation by photographing every frame across all treatments for capped brood count. It also describes the mini-colony's main activity: after acclimation inside the semi-field enclosure, it progressively consumes the diet to feed the larva, forages for nectar syrup, and ultimately takes care of its brood. All treatments started at the same brood stage – egg. **(B)** We distributed the mini-colonies in groups of three nucs in a triangle-shaped configuration; the picture displays the solid food chamber with the mesh supporting diet and a temperature sensor. **(C)** Nurse bees are collected and placed into the "nurse collection box" to top up each mini-colony before the experiment starts. **(D)** Honey bees forage on artificial flowers with attached coloured velvet wires and bottle caps for bee support and grip. **(E)** A novel custom-made device built from acrylic sheets made into a box stand supports a Raspberry Pi LCD and two cameras and displays a bee frame ready to be photographed.

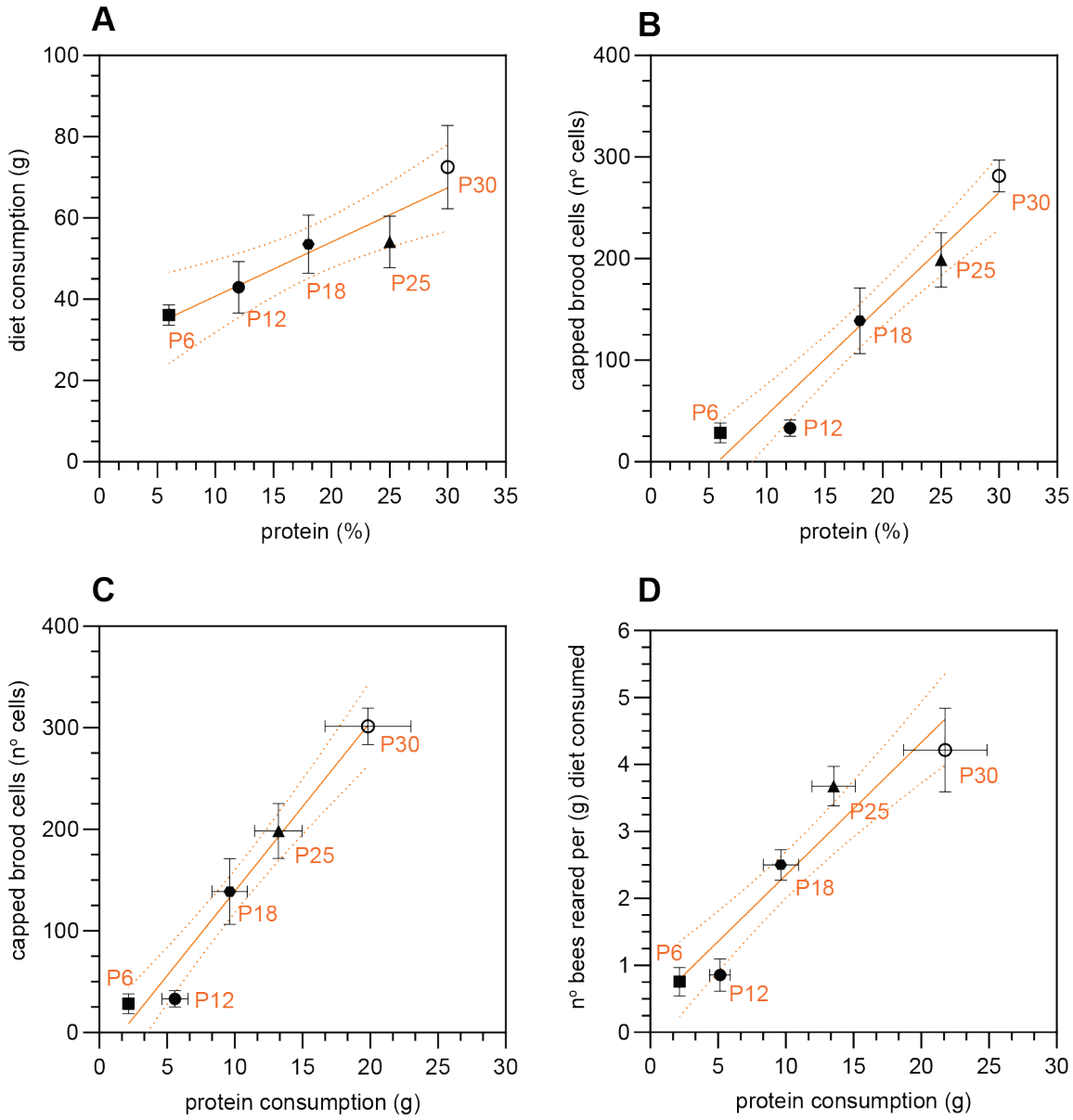

Fig. 2. Protein content proportionally influences diet consumption, capped-brood rates and the number of bees reared in mini-colonies. Data points in all figures show the average results for each of the five diets varying in protein content (% w/w) inside the glasshouse: P6 (6%), P12 (12%), P18 (18%), P25 (25%), and P30 (30%) - over the 15-day period (one bioassay cycle). Orange lines depict the regression lines with 95% degree confidence between different diet types. Error bars in SEM, *n=5* mini-colonies per (P6), (P12), (P30) diet; and n=*6* mini-colonies (P18), (P25) diet. **(A)** The relationship between dietary protein percentage and total diet consumption over the 15-day period. A positive linear association was found, with the OLS model showing that higher protein content is associated with greater consumption. **(B)** Relationship between dietary protein percentage and the total number of capped brood cells counted at the end of the 15-day period. A positive association was found, showing that higher protein content significantly predicted more capped cells. **(C)** The relationship between protein intake (grams) and the number of capped-brood cells (count) shows a positive association with protein-rich diets, leading to higher protein intake and a higher number of capped cells. Our linear regression shows that for every gram of protein ingested, mini-colonies can generate 12 more bees **(D)** The relationship between protein intake and our metric "bees per gram" (number of bees reared per gram of diet consumed). Mini-colonies on protein-rich diets like P30 were, on average, able to rear 4 bees per gram of diet consumed, while lower protein-rich diets (P6 and P12) produced fewer than one bee per gram of food consumed.

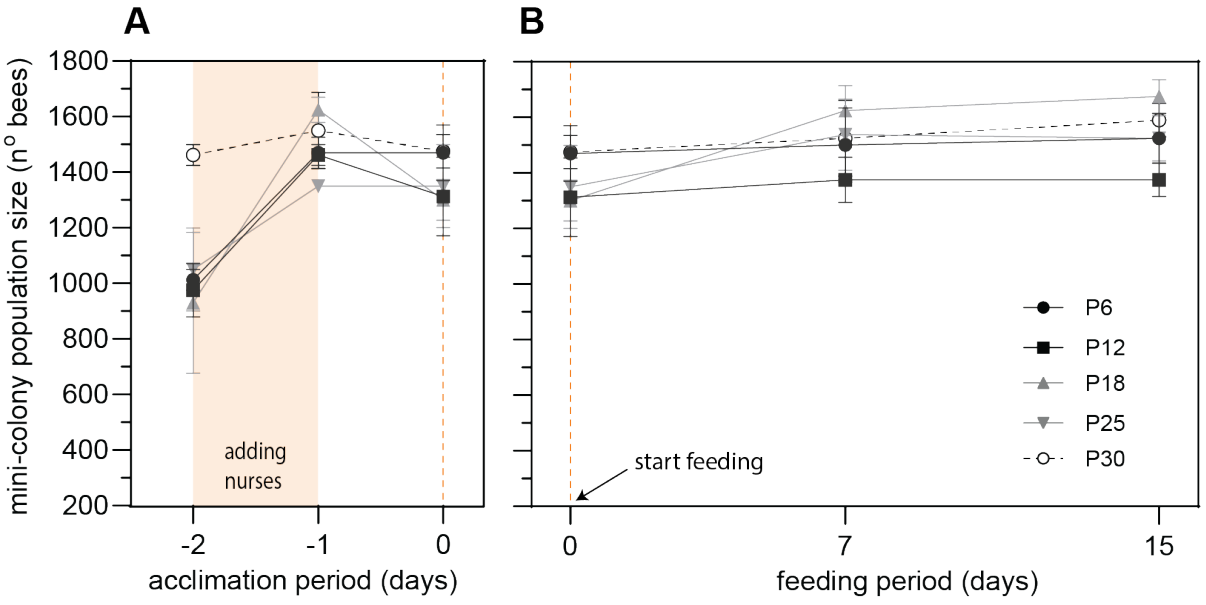

Fig. 3. Population size remains stable during the feeding period. (A) Population dynamics inside the enclosure (glasshouse) during the acclimation period highlighting the addition of extra orphan nurse bees between day -2 and day -1 (shaded area) (~500 bees) and start feeding day 0 (dashed orange line). Nurse addition increased the low populated colonies (Mixed-effects model (REML), Days $P<0.0001$), standardising colony populations between mini-colonies and diet treatments at the start of the protocol (day 0) with similar population sizes 1,400-1,500 bees (*ns*, Mixed-effects model (REML), Day 0 P>0.005). **(B)** Population size inside the glasshouse over the 15-day feeding cycle remained stable on most protein diets except on P18. There were statistical differences in population trends from Day 0 to Day 15, on the P18 diet (Mixed-effects model (REML), Days $P<0.05$, Tukey HSD; $P<0.005$) due to absconding bees randomly entering these colonies. There were no statistical differences between bee populations based on the diet type (*ns*, Mixed-effects model (REML), DIET $P>0.05$).

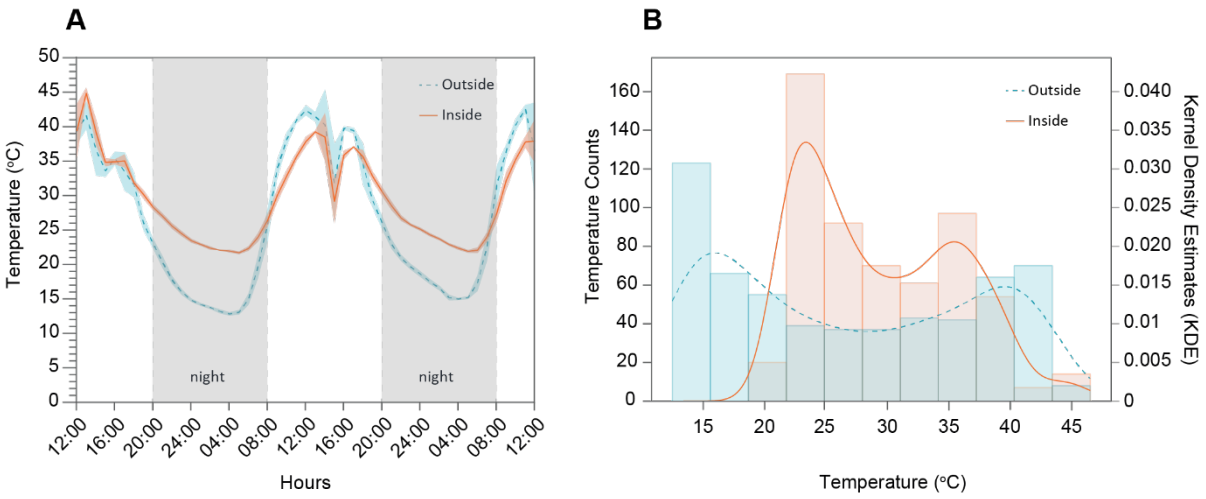

Fig. 4. The mini-colony population was able to buffer environmental temperature fluctuations. In both figures, we show temperature fluctuations inside the mini-colony (orange) (n=2) and outside (blue) (n=2) over a 60-hour (2.5 days) period during a July heatwave inside the glasshouse. In orange, temperature readings were recorded from inside the food chamber; in blue, temperature readings were recorded from supporting tables **(A)** Maximum environmental temperatures reached 45°C during daytime, and the minimum temperature was 14°C during night time (grey shaded area). The temperature drop at 16:00 resulted from the misting system's action starting at 11:00 until 20:00, in 30-minute bursts, to cool the environment. Mini-colonies are better equipped to buffer against lower environmental temperatures than higher temperatures. **(B)** Histogram temperature variation between the inside (orange) and outside (blue), the columns represent the temperature histogram count chart (bin width =3), and the lines represent kernel density estimates (KDE), which are probabilities based on the histogram data. The hive's internal temperature was more stable than the outside temperature, with a shorter range and less variability. The

outside temperature top percentile ranged between 36°C and 42°C. Inside the colony, the temperature mean was 29.5°C, providing an optimal range for brood development.

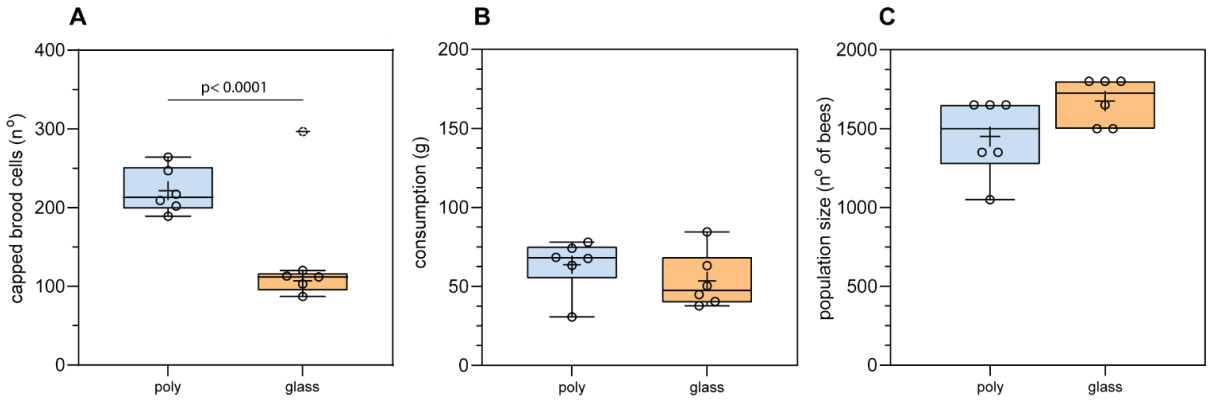

Fig.5. Semi-field conditions can influence capped brood production in mini-colonies. Comparison of mini-colony development inside the polytunnel (blue) and glasshouse (orange) during the first 15 days – one bioassay cycle. Colonies inside the polytunnel (blue) (n=6) were fed a diet with 15% protein (P15) (n=6), and inside the glasshouse (orange) were fed a diet with 18% protein (P18). **(A)** Though mini-colonies in the polytunnel consumed diets lower in protein content compared to those P18 in the glasshouse, the number of capped brood cells inside the polytunnel was two times higher than P18 mini-colonies *(unpaired t-test p<0.0001)*. The dashed circle represents our flagged outlier, which was not considered in our statistical analysis. **(B)** Mini-colonies inside the polytunnel and the glasshouse showed similar diet consumption (ns), ~60g during the first 15 days. **(C)** Mini-colony population size (ns) was also similar between both groups, with a mean difference of 225 bees.

SUPPLEMENTARY:

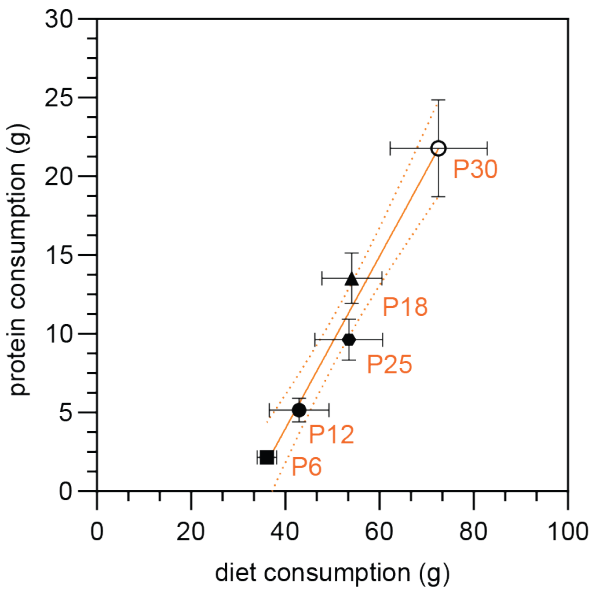

Fig. S1. Protein-rich diets lead to greater diet consumption and protein intake. Data points show the average results for each of the five diets varying in protein content (% w/w) inside the glasshouse: P6 (6%), P12 (12%), P18 (18%), P25 (25%), and P30 (30%) - over the 15-day period (one bioassay cycle). Orange lines depict the regression lines with 95% degree confidence between different diet types. Error bars in SEM, *n=5* mini-colonies per (P6), (P12), (P30) diet; and n=6 mini-colonies (P18), (P25) diet. The relationship between total diet consumption (x-axis) and protein intake (y-axis), where a positive association was observed - mini-colonies consumed more food and ingested significantly more protein when offered protein-rich diets.

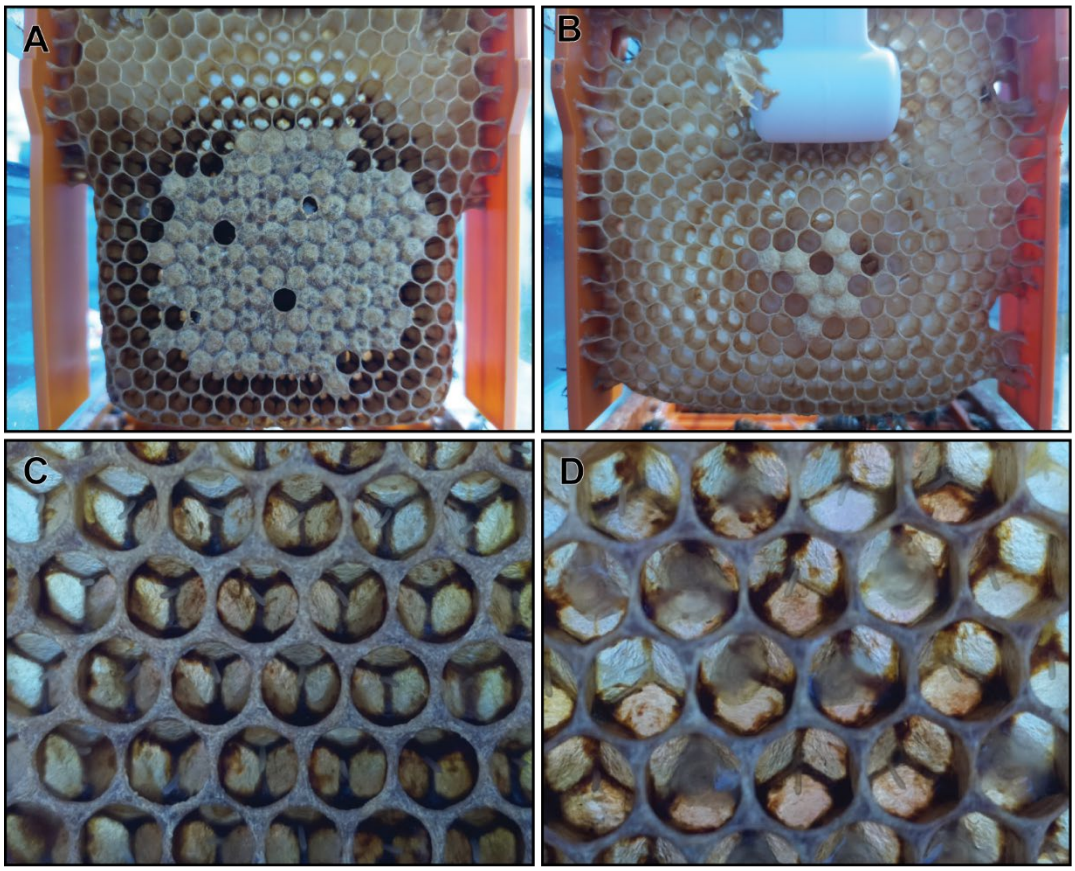

Fig. S2. Sample pictures taken from the photographic-acrylic box device for imaging mini-colony frames. (A) Original size picture showing a mini-colony frame with mostly capped brood and one uncapped, white-eyed pupae. (B) Original size picture showing a mini-colony frame with a white bee sensor on top, nine capped cells and several uncapped larvae. (C) Due to the concave shape of single comb cells, the zoomed-in central section picture on the mini-colony frame can display eggs, in ≈100 cells, to distinguish between empty and egg cell load . (D) zoomed-in central section picture on mini-colony frame demonstrating eggs and 1-2-day-old larvae, facility to distinguish cell load brood development in ≈100 cells.

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Competing interests: The authors, Rui FS Gonçalves, Raquel T. de Sousa, Daniel Stabler, Geraldine A. Wright, and Sharoni Shafir, are shareholders in Apix Biosciences, a bee-nutrition university spin-off registered in Belgium. The authors declare that they have no other competing interests. David MS Pinto has no competing interests to declare. All authors have read and approved the manuscript for submission.

Author contributions: RFSG (Rui Gonçalves), **RTDS** (Raquel T. de Sousa), **DS** (Daniel Stabler), **DMSP** (David M.S. Pinto), **GAW** (Geraldine A. Wright), and **SS** (Sharoni Shafir) contributed as follows: Conceptualization—RFSG, RTDS, GAW, SS; Methodology—RFSG, RTDS,

DS, DMSP, GAW, SS; Software—DMSP; Validation—RFSG, RTDS, GAW, SS; Formal analysis—RFSG, SS; Investigation—RFSG, RTDS, DS; Resources—GAW, SS; Data curation—RFSG, RTDS; Writing (original draft)—RFSG, SS; Writing (review & editing)—RFSG, RTDS, DS, DMSP, GAW, SS; Visualization—RFSG; Supervision—GAW, SS; Project administration—GAW, SS; Funding acquisition—GAW, SS.

References:

1. vanEngelsdorp, D., Evans, J.D., Saegerman, C., Mullin, C., Haubruge, E., Nguyen, B.K., Frazier, M., Frazier, J., Cox-Foster, D., Chen, Y., et al. (2009). Colony Collapse Disorder: A Descriptive Study. PLoS ONE *4*, e6481. https://doi.org/10.1371/journal.pone.0006481.

2. Straub, L., Williams, G.R., Pettis, J., Fries, I., and Neumann, P. (2015). Superorganism resilience: eusociality and susceptibility of ecosystem service providing insects to stressors. Curr. Opin. Insect Sci. *12*, 109–112. https://doi.org/10.1016/j.cois.2015.10.010.

3. Mattila, H.R., and Seeley, T.D. (2007). Genetic Diversity in Honey Bee Colonies Enhances Productivity and Fitness. Science *317*, 362–364. https://doi.org/10.1126/science.1143046.

4. Kennedy, P., and Radford, A.N. (2020). Sibling quality and the haplodiploidy hypothesis. Biology Lett. *16*, 20190764. https://doi.org/10.1098/rsbl.2019.0764.

5. Joshi, C.H., and Wiens, J.J. (2023). Does haplodiploidy help drive the evolution of insect eusociality? Frontiers Ecol. Evol. *11*, 1118748. https://doi.org/10.3389/fevo.2023.1118748.

6. Wild, B., Dormagen, D.M., Zachariae, A., Smith, M.L., Traynor, K.S., Brockmann, D., Couzin, I.D., and Landgraf, T. (2021). Social networks predict the life and death of honey bees. Nat. Commun. *12*, 1110. https://doi.org/10.1038/s41467-021-21212-5.

7. Page, R.E., and Robinson, G.E. (1991). The Genetics of Division of Labour in Honey Bee Colonies. Adv. Insect Physiol. *23*, 117–169. https://doi.org/10.1016/s0065-2806(08)60093-4.

8. DeGrandi-Hoffman, G., Corby-Harris, V., Carroll, M., Toth, A.L., Gage, S., deJong, E.W., Graham, H., Chambers, M., Meador, C., and Obernesser, B. (2021). The Importance of Time and Place: Nutrient Composition and Utilization of Seasonal Pollens by European Honey Bees (Apis mellifera L.). Insects *12*, 235. https://doi.org/10.3390/insects12030235.

9. Johnson, B.R. (2003). Organization of work in the honeybee: a compromise between division of labour and behavioural flexibility. Proc. R. Soc. Lond. Ser. B: Biol. Sci. *270*, 147–152. https://doi.org/10.1098/rspb.2002.2207.

10. Gordon, D.M. (1996). The organization of work in social insect colonies. Nature *380*, 121–124. https://doi.org/10.1038/380121a0.

11. Crailsheim, K. (1992). The flow of jelly within a honeybee colony. J. Comp. Physiol. B *162*, 681–689. https://doi.org/10.1007/bf00301617.

731 12. Wang, Y., Ma, L., Zhang, W., Cui, X., Wang, H., and Xu, B. (2016). Comparison of the
732 nutrient composition of royal jelly and worker jelly of honey bees (Apis mellifera).
733 Apidologie *47*, 48–56. https://doi.org/10.1007/s13592-015-0374-x.

734 13. Johnson, B.R., and Frost, E. (2012). Individual-level patterns of division of labor in
735 honeybees highlight flexibility in colony-level developmental mechanisms. Behav. Ecol.
736 Sociobiol. *66*, 923–930. https://doi.org/10.1007/s00265-012-1341-4.

737 14. Jones, J.C., Myerscough, M.R., Graham, S., and Oldroyd, B.P. (2004). Honey Bee Nest
738 Thermoregulation: Diversity Promotes Stability. Science *305*, 402–404.
739 https://doi.org/10.1126/science.1096340.

740 15. Stabentheiner, A., Kovac, H., and Brodschneider, R. (2010). Honeybee Colony
741 Thermoregulation – Regulatory Mechanisms and Contribution of Individuals in Dependence
742 on Age, Location and Thermal Stress. PLoS ONE *5*, e8967.
743 https://doi.org/10.1371/journal.pone.0008967.

744 16. Johnson, B.R. (2010). Task partitioning in honey bees: the roles of signals and cues in
745 group-level coordination of action. Behav. Ecol. *21*, 1373–1379.
746 https://doi.org/10.1093/beheco/arq138.

747 17. Steinhauer, N., Kulhanek, K., Antúnez, K., Human, H., Chantawannakul, P., Chauzat,
748 M.-P., and vanEngelsdorp, D. (2018). Drivers of colony losses. Curr. Opin. Insect Sci. *26*,
749 142–148. https://doi.org/10.1016/j.cois.2018.02.004.

750 18. Potts, S.G., Biesmeijer, J.C., Kremen, C., Neumann, P., Schweiger, O., and Kunin, W.E.
751 (2010). Global pollinator declines: trends, impacts and drivers. Trends Ecol. Evol. *25*, 345–
752 353. https://doi.org/10.1016/j.tree.2010.01.007.

753 19. Dolezal, A.G., and Toth, A.L. (2018). Feedbacks between nutrition and disease in honey
754 bee health. Curr. Opin. Insect Sci. *26*, 114–119. https://doi.org/10.1016/j.cois.2018.02.006.

755 20. Klein, S., Cabirol, A., Devaud, J.-M., Barron, A.B., and Lihoreau, M. (2017). Why Bees
756 Are So Vulnerable to Environmental Stressors. Trends Ecol. Evol. *32*, 268–278.
757 https://doi.org/10.1016/j.tree.2016.12.009.

758 21. Brodschneider, R., and Crailsheim, K. (2010). Nutrition and health in honey bees.
759 Apidologie *41*, 278–294. https://doi.org/10.1051/apido/2010012.

760 22. Ricigliano, V.A., Williams, S.T., and Oliver, R. (2022). Effects of different artificial diets
761 on commercial honey bee colony performance, health biomarkers, and gut microbiota. BMC
762 Vet. Res. *18*, 52. https://doi.org/10.1186/s12917-022-03151-5.

763 23. Robinson, F.A., and Nation, J.L. (1966). Artificial Diets for Honey Bees, Apis mellifera.
764 The Florida Entomologist *49*, 175–184. https://doi.org/10.2307/3493440.

765 24. Noordyke, E.R., Santen, E. van, and Ellis, J.D. (2021). Tracing the Fate of Pollen
766 Substitute Patties in Western Honey Bee (Hymenoptera: Apidae) Colonies. J. Econ. Èntomol.
767 *114*, 1421–1430. https://doi.org/10.1093/jee/toab083.

25. Sumner, D., and Champetier, A. (2018). Economics of the supply functions for pollination and honey: Marginal costs and supply elasticity. In Invited paper presented at the 2019 Annual Meeting of the Allied Social Sciences Association (ASSA), January 4-6, 2019 in Atlanta, GA https://doi.org/10.22004/ag.econ.281164.

26. Haydak, M.H., and Tanquary, M.C. (1943). Pollen and Pollen Substitutes in the Nutrition of the Honeybee. University of Minnesota Agricultural Experiment Station, Technical Bulletin 160.

27. DeGrandi-Hoffman, G., Chen, Y., Rivera, R., Carroll, M., Chambers, M., Hidalgo, G., and Jong, E.W. de (2016). Honey bee colonies provided with natural forage have lower pathogen loads and higher overwinter survival than those fed protein supplements. Apidologie *47*, 186–196. https://doi.org/10.1007/s13592-015-0386-6.

28. Harbo, J.R. (1986). Effect of Population Size on Brood Production, Worker Survival and Honey Gain in Colonies of Honeybees. J. Apic. Res. *25*, 22–29. https://doi.org/10.1080/00218839.1986.11100687.

29. OOMEN, P.A., RUIJTER, A.D., and STEEN, J.V.D. (1992). Method for honeybee brood feeding tests with insect growth-regulating insecticides. EPPO Bull. *22*, 613–616. https://doi.org/10.1111/j.1365-2338.1992.tb00546.x.

30. Mattila, H.R., and Otis, G.W. (2006). Influence of Pollen Diet in Spring on Development of Honey Bee (Hymenoptera: Apidae) Colonies. J. Econ. Èntomol. *99*, 604–613. https://doi.org/10.1603/0022-0493-99.3.604.

31. Sagili, R.R., and Breece, C.R. (2012). Effects of Brood Pheromone (SuperBoost) on Consumption of Protein Supplement and Growth of Honey Bee (Hymenoptera: Apidae) Colonies During Fall in a Northern Temperate Climate. J. Econ. Èntomol. *105*, 1134–1138. https://doi.org/10.1603/ec11437.

32. Ricigliano, V.A., Mott, B.M., Floyd, A.S., Copeland, D.C., Carroll, M.J., and Anderson, K.E. (2018). Honey bees overwintering in a southern climate: longitudinal effects of nutrition and queen age on colony-level molecular physiology and performance. Sci. Rep. *8*, 10475. https://doi.org/10.1038/s41598-018-28732-z.

33. Mortensen, A.N., Jack, C.J., Bustamante, T.A., Schmehl, D.R., and Ellis, J.D. (2018). Effects of Supplemental Pollen Feeding on Honey Bee (Hymenoptera: Apidae) Colony Strength and Nosema spp. Infection. J. Econ. Èntomol. *112*, 60–66. https://doi.org/10.1093/jee/toy341.

34. Noordyke, E.R., Santen, E. van, and Ellis, J.D. (2022). Evaluating the strength of western honey bee (Apis mellifera L.) colonies fed pollen substitutes over winter. J. Appl. Èntomol. *146*, 291–300. https://doi.org/10.1111/jen.12957.

35. Herbert, E.W., Bickley, W.E., and Shimanuki, H. (1970). The Brood-Rearing Capability of Caged Honey Bees1 Fed Dandelion and Mixed Pollen Diets2. J. Econ. Èntomol. *63*, 215–218. https://doi.org/10.1093/jee/63.1.215.

36. Herbert, E.W., and Shimanuki, H. (1978). Effect of the Size of Outdoor Flight Cages on Brood Rearing and Food Consumption by Honeybees. J. Apic. Res. *17*, 114–117. https://doi.org/10.1080/00218839.1978.11099915.

37. Standifer, L.N., Haydak, M.H., Mills, J.P., and Levin, M.D. (1973). Value of three Protein Rations in Maintaining Honeybee Colonies in Outdoor Flight Cages. J. Apic. Res. *12*, 137–143. https://doi.org/10.1080/00218839.1973.11099741.

38. Campana, B.J., and Moeller, F.E. (1977). Honey Bees: Preference for and Nutritive Value of Pollen from Five Plant Sources123. J. Econ. Èntomol. *70*, 39–41. https://doi.org/10.1093/jee/70.1.39.

39. Bakker, F., and Calis, J. (2014). A semi-field approach to testing effects of fresh or aged pesticide residues on bees in multiple-rate test designs. Bulletin of Insectology.

40. Höcherl, N., Siede, R., Illies, I., Gätschenberger, H., and Tautz, J. (2012). Evaluation of the nutritive value of maize for honey bees. J. Insect Physiol. *58*, 278–285. https://doi.org/10.1016/j.jinsphys.2011.12.001.

41. Arien, Y., Dag, A., Yona, S., Tietel, Z., Cohen, T.L., and Shafir, S. (2020). Effect of diet lipids and omega-6:3 ratio on honey bee brood development, adult survival and body composition. J. Insect Physiol. *124*, 104074. https://doi.org/10.1016/j.jinsphys.2020.104074.

42. Fine, J.D., Shpigler, H.Y., Ray, A.M., Beach, N.J., Sankey, A.L., Cash-Ahmed, A., Huang, Z.Y., Astrauskaite, I., Chao, R., Zhao, H., et al. (2018). Quantifying the effects of pollen nutrition on honey bee queen egg laying with a new laboratory system. PLoS ONE *13*, e0203444. https://doi.org/10.1371/journal.pone.0203444.

43. Mortensen, A.N., and Ellis, J.D. (2018). The effects of artificial rearing environment on the behavior of adult honey bees, Apis mellifera L. Behav. Ecol. Sociobiol. *72*, 92. https://doi.org/10.1007/s00265-018-2507-5.

44. Czoppelt, C., Sharma, G.K., and Rembold, H. (1980). Behaviour of Honeybee Colonies Under Controlled Environmental Conditions in a Flight Room. J. Apic. Res. *19*, 232–241. https://doi.org/10.1080/00218839.1980.11100030.

45. Pernal, S.F., and Currie, R.W. (2001). Improved Flight and Rearing Room Design for Honey Bees (Hymenoptera: Apidae). J. Econ. Èntomol. *94*, 793–805. https://doi.org/10.1603/0022-0493-94.4.793.

46. LOPER, G.M., and BERDEL, R.L. (1980). A nutritional bioassay of honeybee brood-rearing potential. Apidologie, 181–189. https://doi.org/10.1051/apido:19800208.

47. Keller, I., Fluri, P., and Imdorf, A. (2005). Pollen nutrition and colony development in honey bees: part 1. Bee World *86*, 3–10. https://doi.org/10.1080/0005772x.2005.11099641.

48. Frias, B.E.D., Barbosa, C.D., and Lourenço, A.P. (2016). Pollen nutrition in honey bees (Apis mellifera): impact on adult health. Apidologie *47*, 15–25. https://doi.org/10.1007/s13592-015-0373-y.

49. Pasquale, G.D., Alaux, C., Conte, Y.L., Odoux, J.-F., Pioz, M., Vaissière, B.E., Belzunces, L.P., and Decourtye, A. (2016). Variations in the Availability of Pollen Resources Affect Honey Bee Health. PLoS ONE *11*, e0162818. https://doi.org/10.1371/journal.pone.0162818.

50. Mattila, H.R., and Otis, G.W. (2007). Dwindling pollen resources trigger the transition to broodless populations of long-lived honeybees each autumn. Ecol. Èntomol. *32*, 496–505. https://doi.org/10.1111/j.1365-2311.2007.00904.x.

51. Schulz, D.J., Vermiglio, M.J., Huang, Z.Y., and Robinson, G.E. (2002). Effects of colony food shortage on social interactions in honey bee colonies. Insectes Sociaux *49*, 50–55. https://doi.org/10.1007/s00040-002-8279-x.

52. Scofield, H.N., and Mattila, H.R. (2015). Honey Bee Workers That Are Pollen Stressed as Larvae Become Poor Foragers and Waggle Dancers as Adults. PLoS ONE *10*, e0121731. https://doi.org/10.1371/journal.pone.0121731.

53. Branchiccela, B., Castelli, L., Corona, M., Díaz-Cetti, S., Invernizzi, C., Escalera, G.M. de la, Mendoza, Y., Santos, E., Silva, C., Zunino, P., et al. (2019). Impact of nutritional stress on the honeybee colony health. Sci. Rep. *9*, 10156. https://doi.org/10.1038/s41598-019-46453-9.

54. Lamontagne-Drolet, M., Samson-Robert, O., Giovenazzo, P., and Fournier, V. (2019). The Impacts of Two Protein Supplements on Commercial Honey Bee (Apis mellifera L.) Colonies. J. Apic. Res. *58*, 800–813. https://doi.org/10.1080/00218839.2019.1644938.

55. Wright, G.A., Nicolson, S.W., and Shafir, S. (2018). Nutritional Physiology and Ecology of Honey Bees. Annu. Rev. Èntomol. *63*, 1–18. https://doi.org/10.1146/annurev-ento-020117-043423.

56. Powell, J.E., Lau, P., Rangel, J., Arnott, R., Jong, T.D., and Moran, N.A. (2023). The microbiome and gene expression of honey bee workers are affected by a diet containing pollen substitutes. PLOS ONE *18*, e0286070. https://doi.org/10.1371/journal.pone.0286070.

57. Noordyke, E.R., and Ellis, J.D. (2021). Reviewing the Efficacy of Pollen Substitutes as a Management Tool for Improving the Health and Productivity of Western Honey Bee (Apis mellifera) Colonies. Front. Sustain. Food Syst. *5*, 772897. https://doi.org/10.3389/fsufs.2021.772897.

58. Peirson, M., Ibrahim, A., Ovinge, L.P., Hoover, S.E., Guarna, M.M., Melathopoulos, A., and Pernal, S.F. (2023). The effects of protein supplementation, fumagillin treatment, and colony management on the productivity and long-term survival of honey bee (Apis mellifera) colonies in Canada. https://doi.org/10.1101/2023.07.10.548417.

59. Dubois, E., Reis, C., Schurr, F., Cougoule, N., and Ribière-Chabert, M. (2018). Effect of pollen traps on the relapse of chronic bee paralysis virus in honeybee (Apis mellifera) colonies. Apidologie *49*, 235–242. https://doi.org/10.1007/s13592-017-0547-x.

60. Fisher, A., DeGrandi-Hoffman, G., Smith, B.H., Ozturk, C., Kaftanoglu, O., Fewell, J.H., and Harrison, J.F. (2021). Field cross-fostering and in vitro rearing demonstrate negative

effects of both larval and adult exposure to a widely used fungicide in honey bees (Apis mellifera). Ecotoxicol. Environ. Saf. *217*, 112251. https://doi.org/10.1016/j.ecoenv.2021.112251.

61. Souza, D.D., Urbanowicz, C., Ng, W.H., Baert, N., Fersch, A.A., Smith, M.L., and McArt, S.H. (2024). Acute toxicity of the fungicide captan to honey bees and mixed evidence for synergism with the insecticide thiamethoxam. Sci. Rep. *14*, 15709. https://doi.org/10.1038/s41598-024-66248-x.

62. Morales, M.M., Ramos, M.J.G., Vázquez, P.P., Galiano, F.J.D., Valverde, M.G., López, V.G., Flores, J.M., and Fernández-Alba, A.R. (2020). Distribution of chemical residues in the beehive compartments and their transfer to the honeybee brood. Sci. Total Environ. *710*, 136288. https://doi.org/10.1016/j.scitotenv.2019.136288.

63. Castle, D., Alkassab, A.T., Bischoff, G., Steffan-Dewenter, I., and Pistorius, J. (2022). High nutritional status promotes vitality of honey bees and mitigates negative effects of pesticides. Sci. Total Environ. *806*, 151280. https://doi.org/10.1016/j.scitotenv.2021.151280.

64. Barascou, L., Sene, D., Barraud, A., Michez, D., Lefebvre, V., Medrzycki, P., Prisco, G.D., Strobl, V., Yañez, O., Neumann, P., et al. (2021). Pollen nutrition fosters honeybee tolerance to pesticides. R. Soc. Open Sci. *8*, 210818. https://doi.org/10.1098/rsos.210818.

65. Berry, J.A., and Delaplane, K.S. (2001). Effects of comb age on honey bee colony growth and brood survivorship. J. Apic. Res. *40*, 3–8. https://doi.org/10.1080/00218839.2001.11101042.

66. Wang, M., Braasch, T., and Dietrich, C. (2020). Reduction of variability for the assessment of side effects of toxicants on honeybees and understanding drivers for colony development. PLoS ONE *15*, e0229295. https://doi.org/10.1371/journal.pone.0229295.

67. Sonter, C.A., Rader, R., and Wilson, S.C. (2019). A new, practicable and economical cage design for experimental studies on small honey bee colonies. J. Exp. Biology *222*, jeb200998. https://doi.org/10.1242/jeb.200998.

68. Corby-Harris, V., Bennett, M.M., Deeter, M.E., Snyder, L., Meador, C., Welchert, A.C., Hoffman, A., Obernesser, B.T., and Carroll, M.J. (2021). Fatty acid homeostasis in honey bees (Apis mellifera) fed commercial diet supplements. Apidologie *52*, 1195–1209. https://doi.org/10.1007/s13592-021-00896-0.

69. Delaplane, K.S., Steen, J. van der, and Guzman-Novoa, E. (2013). Standard methods for estimating strength parameters of Apis mellifera colonies. J. Apic. Res. *52*, 1–12. https://doi.org/10.3896/ibra.1.52.1.03.

70. Ko, C.-Y., Chen, Y.-W., and Nai, Y.-S. (2017). Evaluating the Effect of Environmental Chemicals on Honey Bee Development from the Individual to Colony Level. J. Vis. Exp. https://doi.org/10.3791/55296.

71. Alves, T.S., Pinto, M.A., Ventura, P., Neves, C.J., Biron, D.G., Junior, A.C., Filho, P.L.D.P., and Rodrigues, P.J. (2020). Automatic detection and classification of honey bee

comb cells using deep learning. Comput. Electron. Agric. *170*, 105244. https://doi.org/10.1016/j.compag.2020.105244.

72. OECD (2014). Guidance Document on the Honey Bee (Apis Mellifera L.) Brood test Under Semi-field Conditions. OECD Ser. Test. Assess. https://doi.org/10.1787/9789264085510-en.

73. Szczesniak, B., Pilling, E., Bocksch, S., Becker, R., and Lückmann, J. (2012). ICP-PR Bee Brood Working Group – Variability of brood termination rates in reference to validity criteria and limited effectiveness of method improvement in honeybee semi-field studies (OECD GD 75). Hazards Pestic. bees : 13th Int. Symp. ICP-PR Bee Prot. Group 18 - 20 Oct. 2017, València (Spain) - Proc. -, 111–115. https://doi.org/10.5073/jka.2018.462.030.

74. Lückmann, J., and Schmitzer, S. (2019). The Oomen bee brood feeding test – revision of the method to current needs and developments. EPPO Bull. *49*, 137–146. https://doi.org/10.1111/epp.12553.

75. Becker, R., and Lückmann, J. (2017). Detailed brood evaluation under field conditions: advantages and disadvantages. Hazards Pestic. bees : 13th Int. Symp. ICP-PR Bee Prot. Group 18 - 20 Oct. 2017, València (Spain) - Proc. -, 95–98. https://doi.org/10.5073/jka.2018.462.023.

76. Sereia, M.J., Toledo, V. de A.A. de, Furlan, A.C., Faquinello, P., Costa-Maia, F.M., and Wielewski, P. (2013). Alternative sources of supplements in Africanized honeybees submitted to royal jelly production. Acta Sci. Anim. Sci. *35*, 165–171. https://doi.org/10.4025/actascianimsci.v35i2.16976.

77. Stabler, D., Al-Esawy, M., Chennells, J.A., Perri, G., Robinson, A., and Wright, G.A. (2021). Regulation of dietary intake of protein and lipid by nurse-age adult worker honeybees. J. Exp. Biol. *224*, jeb230615. https://doi.org/10.1242/jeb.230615.

78. Svoboda, J.A., Thompson, M.J., Herbert, E.W., and Shimanuki, H. (1980). Sterol utilization in honey bees fed a synthetic diet: Analysis of prepupal sterols. J. Insect Physiol. *26*, 291–294. https://doi.org/10.1016/0022-1910(80)90136-5.

79. Herbert, E.W., Svoboda, J.A., Thompson, M.J., and Shimanuki, H. (1980). Sterol utilization in honey bees fed a synthetic diet: Effects on brood rearing. J. Insect Physiol. *26*, 287–289. https://doi.org/10.1016/0022-1910(80)90135-3.

80. Sousa, R.T. de, Darnell, R., and Wright, G.A. (2022). Behavioural regulation of mineral salt intake in honeybees: a self-selection approach. Philos. Trans. R. Soc. B *377*, 20210169. https://doi.org/10.1098/rstb.2021.0169.

81. Capaldi, E.A., and Dyer, F.C. (1999). The role of orientation flights on homing performance in honeybees. J. Exp. Biol. *202*, 1655–1666. https://doi.org/10.1242/jeb.202.12.1655.

82. Slessor, K.N., Winston, M.L., and Conte, Y.L. (2005). Pheromone Communication in the Honeybee (Apis mellifera L.). J. Chem. Ecol. *31*, 2731–2745. https://doi.org/10.1007/s10886-005-7623-9.

83. Pamminger, T., Becker, R., Himmelreich, S., Schneider, C.W., and Bergtold, M. (2019). The nectar report: quantitative review of nectar sugar concentrations offered by bee visited flowers in agricultural and non-agricultural landscapes. PeerJ *7*, e6329. https://doi.org/10.7717/peerj.6329.

84. Flores, J.M., Ruiz, J.A., Ruz, J.M., Puerta, F., Bustos, M., Padilla, F., and Campano, F. (1996). Effect of temperature and humidity of sealed brood on chalkbrood development under controlled conditions. Apidologie *27*, 185–192. https://doi.org/10.1051/apido:19960401.

85. Chabert, S., Requier, F., Chadoeuf, J., Guilbaud, L., Morison, N., and Vaissière, B.E. (2021). Rapid measurement of the adult worker population size in honey bees. Ecol. Indic. *122*, 107313. https://doi.org/10.1016/j.ecolind.2020.107313.

86. Leclercq, G., Pannebakker, B., Gengler, N., Nguyen, B.K., and Francis, F. (2017). Drawbacks and benefits of hygienic behavior in honey bees (Apis mellifera L.): a review. J. Apic. Res. *56*, 366–375. https://doi.org/10.1080/00218839.2017.1327938.

87. Paoli, P.P., Donley, D., Stabler, D., Saseendranath, A., Nicolson, S.W., Simpson, S.J., and Wright, G.A. (2014). Nutritional balance of essential amino acids and carbohydrates of the adult worker honeybee depends on age. Amino Acids *46*, 1449–1458. https://doi.org/10.1007/s00726-014-1706-2.

88. Hendriksma, H.P., Toth, A.L., and Shafir, S. (2019). Individual and Colony Level Foraging Decisions of Bumble Bees and Honey Bees in Relation to Balancing of Nutrient Needs. Front. Ecol. Evol. *7*, 177. https://doi.org/10.3389/fevo.2019.00177.

89. Bouchebti, S., Wright, G.A., and Shafir, S. (2022). Macronutrient balance has opposing effects on cognition and survival in honey bees. Funct. Ecol. *36*, 2558–2568. https://doi.org/10.1111/1365-2435.14143.

90. Jr., E.W.H., Shimanuki, H., and Caron, D. (1977). Caged honey bees (Hymenoptera, Apidae): comparative value of some proteins for initiating and maintaining brood rearing. Apidologie *8*, 229–235. https://doi.org/10.1051/apido:19770302.

91. Herbert, E.W., and Shimanuki, H. (1978). Consumption and Brood Rearing by Caged Honeybees Fed Pollen Substitutes Fortified with Various Sugars. J. Apic. Res. *17*, 27–31. https://doi.org/10.1080/00218839.1978.11099895.

92. Herbert, E.W., and Shimanuki, H. (1982). Effect of population density and available diet on the rate of brood rearing by honey bees offered a pollen substitute. Apidologie *13*, 21–28. https://doi.org/10.1051/apido:19820103.

93. Jr, E.W.H., and Shimanuki, H. (1983). Effect of mid-season change in diet on diet consumption and brood rearing by caged Honey Bees. Apidologie *14*, 119–125. https://doi.org/10.1051/apido:19830206.

94. Steen, J. van der (2007). Effect of a home-made pollen substitute on honey bee colony development. J. Apic. Res. *46*, 114–119. https://doi.org/10.1080/00218839.2007.11101377.

95. Li, C., Xu, B., Wang, Y., Feng, Q., and Yang, W. (2012). Effects of dietary crude protein levels on development, antioxidant status, and total midgut protease activity of honey bee (Apis mellifera ligustica). Apidologie *43*, 576–586. https://doi.org/10.1007/s13592-012-0126-0.

96. Downs, S.G., Ratnieks, F.L.W., Jefferies, S.L., and Rigby, H.E. (2000). The role of floral oils in the nestmate recognition system of honey bees (Apis mellifera L.). Apidologie *31*, 357–365. https://doi.org/10.1051/apido:2000127.

97. Xu, X., Du, X., Zhou, S., Zhou, B., Lai, K., Wang, Q., Li, H., Zhu, C., Xu, H., Zhang, X., et al. (2023). Impacts of high temperature during early capped brood on pupal development and the size of appendages in adult workers Apis cerana. Front. Ecol. Evol. *11*, 1144216. https://doi.org/10.3389/fevo.2023.1144216.

98. O'Connell, D.P., Baker, B.M., Atauri, D., and Jones, J.C. (2024). Increasing temperature and time in glasshouses increases honey bee activity and affects internal brood conditions. J. Insect Physiol. *155*, 104635. https://doi.org/10.1016/j.jinsphys.2024.104635.

99. Morimoto, T., Kojima, Y., Toki, T., Komeda, Y., Yoshiyama, M., Kimura, K., Nirasawa, K., and Kadowaki, T. (2011). The habitat disruption induces immune-suppression and oxidative stress in honey bees. Ecol. Evol. *1*, 201–217. https://doi.org/10.1002/ece3.21.

100. Kim, W., Gilet, T., and Bush, J.W.M. (2011). Optimal concentrations in nectar feeding. Proc. Natl. Acad. Sci. *108*, 16618–16621. https://doi.org/10.1073/pnas.1108642108.

101. Rathore, N., and Agrawal, D. (2024). Automated precision beekeeping for accessing bee brood development and behaviour using deep CNN. Bull. Èntomol. Res. *114*, 77–87. https://doi.org/10.1017/s0007485323000639.

102. Jeker, L., Schmid, L., Meschberger, T., Candolfi, M., Pudenz, S., and Magyar, J.P. (2012). Computer-assisted digital image analysis and evaluation of brood development in honey bee combs. J. Apic. Res. *51*, 63–73. https://doi.org/10.3896/ibra.1.51.1.08.

103. Beard, R.L. (1972). Lethal Action of UV Irradiation on Insects. J. Econ. Èntomol. *65*, 650–654. https://doi.org/10.1093/jee/65.3.650.