

1 Nutritional aspects of honey bee-collected pollen and constraints on  
2 colony development in the eastern Mediterranean

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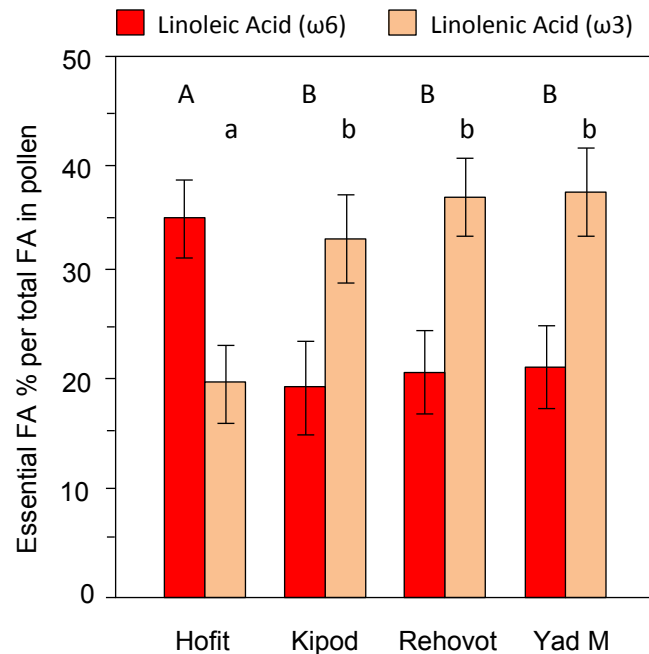
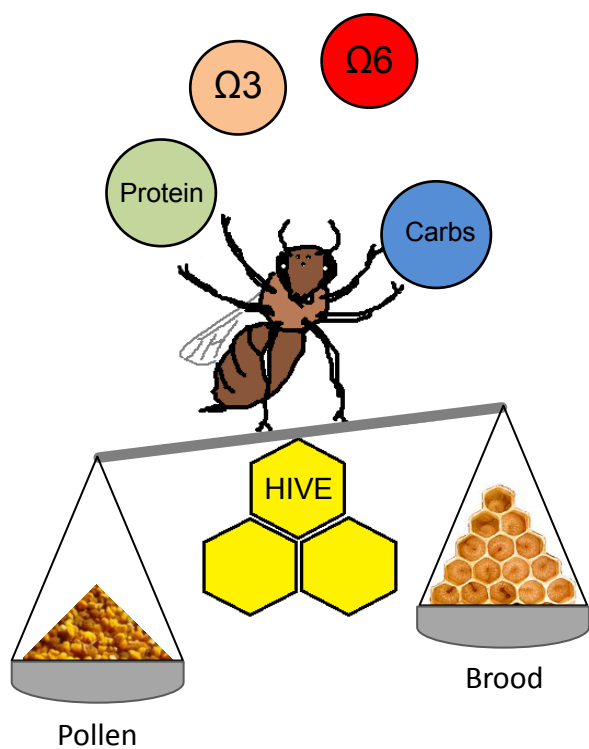
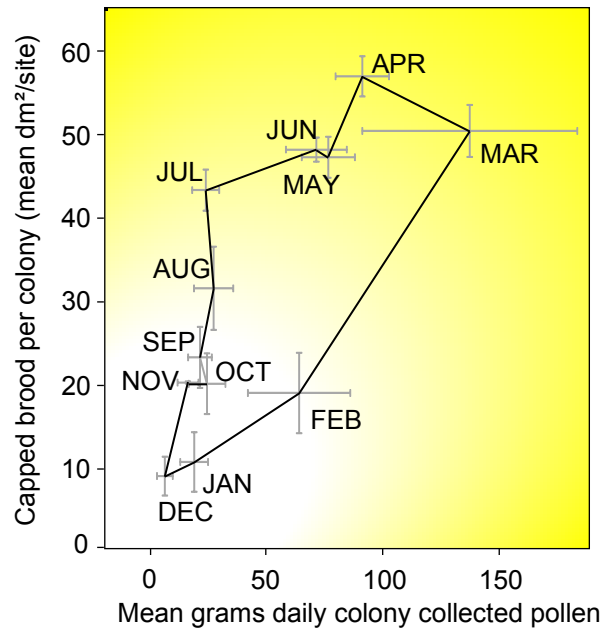
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# \*Graphical Abstract (for review)

## Pollen intake and brood production



- Honey bee colonies in Israel collect a mean 7 kg protein and 0.7 kg fat per year
- At maximal colony development, honey bee queens lay up to 3,300 eggs per day
- Amount and content of collected pollen differ between sites and seasons
- Pollen linoleic acid and protein levels best describe bee production cost
- Colonies seem limited first by climate, then protein and finally specific nutrients

28

29 ABSTRACT

30

31 Pollen is the main protein and lipid source for honey bees (*Apis mellifera*), and nutritionally  
32 impoverished landscapes pose a threat to colony development. To determine colony  
33 nutritional demands, we analyzed a yearly cycle of bee-collected pollen from colonies in  
34 the field and compared it to colony worker production and honey bee body composition, for  
35 the first time in social insects. We monitored monthly bee production in ten colonies at  
36 each of seven sites throughout Israel, and trapped pollen bi-monthly in five additional  
37 colonies at each of four of these sites. Pollen mixtures from each sampling date and site  
38 were analyzed for weight, total protein, total fatty acids (FAs), and FA composition.  
39 Compared to more temperate climates, the eastern Mediterranean allows a relatively high  
40 yearly colony growth of ca. 300,000 to 400,000 bees. Colonies at higher elevation above  
41 sea level showed lower growth rates. Queen egg-laying rate did not seem to limit growth,  
42 as peaks in capped brood areas showed that queens lay a prolific 2,000 eggs a day on  
43 average, with up to 3,300 eggs in individual cases. Pollen uptake varied significantly  
44 among sites and seasons, with an overall annual mean total 16.8 kg per colony, containing  
45 7.14 kg protein and 677 g fat. Overall mean pollen protein content was high (39.8%), and  
46 mean total FA content was 3.8%. Production cost, as expressed by the amount of nutrient  
47 used per bee, was least variable for linoleic acid and protein, suggesting these as the best  
48 descriptive variables for total number of bees produced. Linolenic acid levels in pollen  
49 during the autumn were relatively low, and supplementing colonies with this essential FA  
50 may mitigate potential nutritional deficiency. The essentiality of linoleic and linolenic acids  
51 was consistent with these FAs' tendency to be present at higher levels in collected pollen  
52 than in the expected nutrients in bee bodies, demonstrating a well-developed adjustment  
53 between pollinator nutritional demands and the nutritional value of food offered by  
54 pollinated plants.

55

56 KEYWORDS

57 nutritional homeostasis / pollen trap / omega 3 / omega 6 / food supplement

58

## 59 **1. Introduction**

60

61 Honey bees collect pollen from a wide range of flowering plants, which usually  
62 fulfills their dietary requirements for proteins, lipids, minerals, and vitamins. When pollen is  
63 abundant, a honey bee colony collects between 15 and 55 kg pollen annually (Winston,  
64 1987). Protein levels in the range of 2.5% to 61% have been reported in honey bee-  
65 collected pollen (de Arruda et al., 2013; Odoux et al., 2012; Roulston and Cane, 2000;  
66 Schmidt, 1984; Schmidt and Buchmann, 1999; Yang et al., 2013). Protein is essential for  
67 proper development and function of body tissue, muscles, membranes and glands  
68 (Herbert, 1999). Variability in pollen quantity and quality may pose a challenge for  
69 nutritional homeostasis within the colony. A nutritional deficit may shorten life span, reduce  
70 brood production, inhibit gland development, enhance emergence of diseases and reduce  
71 honey bee weight (Alaux et al., 2010; Schmidt, 1984; Schmidt et al., 1987; Schmidt et al.,  
72 1995).

73 Honey bee foragers collect nectar from plants to produce honey, which is the main  
74 source of carbohydrate nutrition for the colony. Honey is the alimentary resource for adult  
75 honey bees and the developing brood. In addition, the carbohydrates are used as fuel to  
76 thermoregulate the brood nest and as flight fuel for foragers. Carbohydrates are also used  
77 by young honey bees to biosynthesize lipids. For example, honey bees produce wax,  
78 which is the main building material for honeycombs. In Israel, honey bee hives yield 24.3  
79 to 31.3 kg honey annually (Avni et al., 2009). In more temperate regions, honey bee  
80 colonies can collect up to 200 kg honey in a year (Winston, 1987).

81 Pollen is an essential source of lipids for honey bees, used for energy and as a  
82 structural component of cell membranes. Fatty acid (FA) content of pollen ranges between  
83 1% and 20%, depending on the plant species (Brodschneider and Crailsheim, 2010). Most  
84 insects require essential polyunsaturated fatty acids (PUFA) in their diet, and linoleic and  
85 linolenic acids usually satisfy this nutritional need (Canavoso et al., 2001; Cohen, 2004;  
86 Khani et al., 2007; Nurullahoglu et al., 2004; Wang et al., 2006). These FA have multiple  
87 double bonds, starting at the 3<sup>rd</sup> and 6<sup>th</sup> carbon atom from the omega group end, hence  
88 alternatively named omega 3 (18:3 $\omega$ 3 / C18:3 cis-9,12,15 / linolenic acid) and omega 6  
89 (18:2 $\omega$ 6 / C18:2 cis-9,12 / linoleic acid).

90           Pollen is typically rich in water-soluble vitamins, and it provides bees with their  
91 vitamin requirements. In general, as long as pollen is abundant, the honey bee  
92 requirements for these nutrients are satisfied (Brodschneider and Crailsheim, 2010;  
93 Herbert, 1999). Honey bee mineral requirements are the least known of all nutritional  
94 components (Cohen, 2004). Minerals are also obtained by the bees from pollen, which  
95 contains ash at 2.5% to 6.5% of its dry weight. Addition of 1% pollen ash to a synthetic  
96 honey bee diet increased brood rearing significantly, but exceeding the ash content by  
97 more than 2% did not seem to be advantageous (Herbert and Shimanuki, 1978).

98           Nutritional ecology is a rapidly growing field, especially for bee species; a  
99 nutritional deficiency due to anthropogenically altered landscapes could have implications  
100 for the global decline in bee populations. Suboptimal nutrient balance has been argued to  
101 be one of the threats to pollinator populations (Vanbergen et al., 2013). The division of  
102 labor in eusocial insect colonies adds a level of nutritional complexity. In the honey bee  
103 colony, older foragers collect the food, which is consumed by younger workers who then  
104 feed the larvae. Thus, the body composition of emerging adults is indirectly affected by the  
105 diet collected by the colony.

106           In the current study, analyses of pollen content and honey bee body composition  
107 were used to determine honey bee colony nutritional demands. This method is commonly  
108 used by animal nutritionists to determine the animal's nutritional demands and compose a  
109 suitable artificial diet to satisfy them (Kratzer et al., 1994; Leeson and Summers, 2001;  
110 Rock and King, 1967). Here, we applied this method for the first time to a eusocial insect.

111           Long-term studies of honey bee colony pollen collection and nutrition can serve to  
112 elucidate the timing of major colony life-cycle events, such as growth, reproductive  
113 swarming, migration, and senescence. The current objective was to quantify annual  
114 amounts and composition of honey bee-collected pollen (total protein, total FA, and FA  
115 profiles), and compare them to the colony's performance in terms of population growth and  
116 honey bee body composition. Our first hypothesis was that temporal and spatial effects  
117 govern the nutritional contents and amounts of honey bee-collected pollen. Secondly, we  
118 expected honey bee colony growth to be affected by altitude (as a measure of the growth  
119 season), and by pollen quantity and nutritional quality.

120

## 121 **2. Materials and methods**

122

### 123 *2.1. Sites*

124

125 Honey bee colonies (*Apis mellifera ligustica*) were monitored between November  
126 2004 and December 2005 at geographically distinct sites (Fig. 1). These locations had  
127 different habitats and surrounding flora representing the Mediterranean climate (Avni et al.,  
128 2009), which is subtropical with long, hot and dry summers and relatively short, cool, rainy  
129 winters (Köppen climate classification Csa). The sites differed in elevation above sea level  
130 and rainfall (Table 1).

131

### 132 *2.2. Honey bee colonies and assessments*

133

134 Fifteen honey bee colonies were kept at each of the seven sites. At the beginning  
135 of the experiment, test colonies had on average 6.5 populated frames and 25 dm<sup>2</sup> brood  
136 (Supplement 1). Young queens were introduced in the autumn, and all were daughters of  
137 the same mother-queen from the local breeding program of the Ministry of Agriculture  
138 apiary in Tzrifin, Israel. The queens were naturally mated with local drones from Italian  
139 strains. Local beekeeping practice was followed, applying swarm-suppressing measures  
140 such as the use of young queens, the removal of developing queen cells, and the  
141 placement of additional hive boxes on top of the brood frames to increase the amount of  
142 space available for food storage and brood production.

143

144 Every month, we assessed 10 hives per site for comb surface areas of brood and  
145 pollen and honey storage (Supplement 1). The areas were estimated by dividing each side  
146 of each comb into eight squares (10 cm x 10 cm = 1 dm<sup>2</sup>), and counting the number of  
147 squares of each type (Kalev et al., 2002). The growth of each colony over one year was  
148 determined by taking the sum of the daily capped brood amount (see section 2.3),  
149 multiplying it by the number of cells per dm<sup>2</sup> (397 worker cells; based on counting the total  
150 cell number on a comb foundation), and dividing by 12, the number of days that brood  
151 cells are capped (Winston, 1987). From a total 70 colonies ( $n_{\text{observations}} = 802$ ), seven  
152 colonies lost their queen during the year and were excluded from population growth  
analyses. The peak in capped brood area per colony (on any one of the sample days) was

153 used to indicate the queen's egg-laying capacity. For example, a capped brood area of  
154  $60.5 \text{ dm}^2$  contains 24,000 worker cells ( $397 \text{ cells}\cdot\text{dm}^{-2}$ ), thus considering the 12 days  
155 during which the brood is capped, the queen must have ovipositioned an average 2,000  
156 eggs per day in the preceding period.

157 Bottom pollen traps were placed under five hives at each site. As pollen trapping  
158 may influence colony development, the traps were not placed under the hives used for  
159 colony growth monitoring. The traps consisted of a double net with  $0.5 \text{ cm} \times 0.5 \text{ cm}$   
160 openings that allowed the bees to pass through, while trapping the pollen pellets on their  
161 hind legs. Below the nets there was a drawer to collect the fallen pollen pellets. The  
162 percentage of incoming pellets captured by the traps was checked in a separate  
163 experiment: mean ( $\pm \text{SE}$ ) capture rate was  $40.3\% \pm 3.2$ ,  $n = 33$  trials with six colonies  
164 (Avni et al., 2009). The traps were closed for 3 to 5 days every 2 weeks, and the captured  
165 pollen was collected, weighed to the nearest  $0.01 \text{ g}$ , and stored at  $-20 \text{ }^\circ\text{C}$  until analysis.

166 At four of the seven sites (Hofit, Yad Mordechai, Rehovot, Kipod), on each  
167 sampling date, a spoon of trapped pollen from each of the five colonies was pooled into a  
168 single sample for nutritional analysis. Each sample was mixed and crushed mechanically  
169 by mortar and pestle (Pirk et al., 2009), and 2–3 g were used for protein and FA analyses  
170 (see section 2.5).

171 Colonies' daily pollen uptake was calculated as follows: the collected pollen weight  
172 per sample was divided by the number of days of trapping activity (3–5), and divided by  
173 40.3% to correct for the previously reported trap efficiency. Overall, a mean 15.3 samples  
174 were taken per colony, with the pollen traps active for a total of  $52 \text{ days} \pm 2 \text{ SE}$  per colony,  
175 over a total mean period of  $305 \text{ days} \pm 2 \text{ SE}$  ( $n_{\text{colonies}} = 20$ ,  $n_{\text{sites}} = 4$ ).

176

### 177 *2.3. Annual interpolation*

178

179 The sample dates for pollen and brood differed within and between sites. To  
180 enable comparisons over time, and to estimate yearly totals, the data were interpolated  
181 between sampling dates. For each of the test colonies, we fit a kernel smooth curve at  $\alpha$   
182 level of 0.3 for the relationship between amount of capped brood ( $n = 63$  colonies) or  
183 amount of bee-collected pollen ( $n = 20$  colonies) and date, yielding a coefficient of  
184 determination ( $R^2$ ) for each colony. The means of these coefficients were  $R^2_{\text{brood}} = 0.86$   
185 and  $R^2_{\text{pollen}} = 0.72$ , respectively. To cover a full-year cycle, the first brood data points were



186 repeated after 365 days, and the pollen data were set to zero on 1 Dec 2004 and 2005. In  
187 addition, the mean amounts of collected pollen (five colonies per sampling date) were  
188 multiplied by data on sample content (see section 2.5), and thereafter interpolated to gain  
189 estimates on annual colony intake (mean  $R^2 = 0.70$ ).

190

#### 191 *2.4. Honey bee body composition*

192

193 To assess body composition of bees from nutritionally nondeprived colonies, we  
194 kept six colonies in a netted enclosure and fed them pollen patties (50% multifloral pollen  
195 mixed with 50% sugar, w/w) ad libitum. Emerging bees were marked and returned to the  
196 colonies to be collected 8 days later. The body weight ( $n = 212$  bees at 8 days of age),  
197 protein content and FA composition were measured ( $n = 12$ ; 6 colonies x 2 bees) and used  
198 as a reference for expected body composition of field bees (see section 2.5). A worker  
199 honey bee is often considered an adult after emergence from the cell, though during the  
200 first 6 days, substantial growth occurs, expressed as an increase in body weight and a 25-  
201 50% increase of total protein content of (Haydak, 1934). We therefore analyzed the body  
202 composition of 8-day-old adults.

203

#### 204 *2.5. Protein and FA analyses*

205

206 Pollen and honey bee samples were homogenized with water (0.1 g in 1.0 ml  
207 water) in a Mini Bead Beater (Biospec Products, Bartlesville, OK, USA). Samples from the  
208 homogenate (0.1 ml) were diluted 1:5 with water and total protein (mg/g) was detected by  
209 Bio-Rad protein assay (Bradford, 1976), which uses a copper-based reagent to stain  
210 protein. To quantify the proteins, bovine serum albumin was used as a standard.

211 For total FA analysis, 0.1 ml of homogenate (0.1 g in 1 ml water) was subjected to  
212 basic hydrolysis, followed by petrol ether extraction. Nonsaponified matter was removed.  
213 The sample was then transferred to an acidic environment for FA release. A second petrol  
214 ether extraction was conducted, followed by evaporation and methylation with 5% sulfuric  
215 acid in methanol. Then a third petrol ether extraction was conducted for methyl ester  
216 collection. The methyl esters were separated by HP 5890 gas chromatograph with FID. An  
217 internal standard (heptanoic acid, C17:0, Sigma, Israel) was added to each sample to  
218 quantify FA amounts (Sklan and Budowski, 1979). The injector, oven and detector

219 temperatures were 230 °C, and constant 180 °C and 235 °C, respectively. We used a 1/8",  
220 2.5 m stainless-steel packed column filled with GP 10% SP-2330 on 100/120 chromosorb  
221 WAW (Supelco).

222

## 223 2.6. Data analysis

224

225 Values of the monitored colony parameters (honey bee colony growth, pollen  
226 collection, relative levels of pollen constituents) were compared by ANOVA with Tukey's  
227 honestly significant difference test (JMP, Version 10, SAS Institute Inc.). Pollen data were  
228 analyzed on two explanatory variables, season (4 levels) and site (4 levels), by two-way  
229 ANOVA. The main results were considered statistically significant at  $p$ -values below  $\alpha =$   
230 0.05. The pollen fat-content variables (total FA, linolenic and linoleic acids) were not  
231 independent and thus tested at  $\alpha = 0.01$ . The cost of honey bee production was calculated  
232 by dividing the total amount of nutrients taken up by the number of honey bees produced.  
233 We expected nutrients that govern or limit production to show a stable cost per bee; such  
234 nutrients were identified by a low coefficient of variation (CV) for the honey bee production  
235 cost.

236

## 237 3. Results

238

239 The number of new bees produced per colony during the year differed among the  
240 seven sites, with a mean range of ca. 300,000 to 400,000 (Fig. 1;  $F_{6,56} = 10.2$ ,  $p < 0.001$ ), a  
241 minimum of 217,000 and a maximum 520,000 bees per year ( $n = 63$  colonies). Total  
242 honey bee production was lower at higher elevations (Fig. 1;  $R^2 = 0.50$ ,  $F_{1,61} = 42.64$ ,  $p <$   
243  $0.001$ ). The overall egg-laying rate of queens approximated 1,000 eggs per day (on  
244 average 367,000 bees  $\pm$  8,000 SE in a year). The mean of yearly peak for capped brood  
245 area in the 63 colonies was 62.2 dm<sup>2</sup>, corresponding to the queens laying approximately  
246 2,078 eggs per day during peak periods. The two colonies with the highest peaks had  
247 capped broods of 99.5 dm<sup>2</sup> and 89.5 dm<sup>2</sup>, with queens laying 3,300 and 3,000 eggs per  
248 day, respectively.

249 The amount of pollen collected by the honey bees in Israel varied over the seasons  
250 (Table 2). In the absence of rain, pollen collection decreased over the summer, with the  
251 smallest amounts collected in the autumn (Fig. 2). Pollen collection also differed among

252 sites (Table 2), with an annual colony mean of 16.8 kg. The smallest amount of pollen was  
253 collected in Hofit (insignificant by post-hoc test), compared to Yad Mordechai, Rehovot,  
254 and Kipod (Table 2). Similarly significant was the site effect for the yearly amount of  
255 collected pollen ( $F_{3,16} = 3.48$ ,  $p = 0.04$ ), albeit with a post-hoc difference between the  
256 lowest and highest levels (Hofit and Yad Mordechai, respectively, Table 3).

257 In contrast, the content of pollen constituents was stable over the seasons, but  
258 differed significantly among sites (Table 2). In the 58 pollen samples, the protein content  
259 was on average 39.8%, ranging between a minimum 10.6% and maximum 73.0%. The  
260 protein levels differed significantly with a mean 30.4% in Kipod, 40.5% in Yad Mordechai,  
261 40.9% in Rehovot and 45.8% in Hofit (Table 2, Fig. 2, and see also graphical abstract).  
262 Among sites, the lowest annual protein uptake was in Hofit (<6 kg), despite its highest  
263 annual bee production (Table 3).

264 Total FA content in the pollen averaged 3.8% (38.4 mg/g pollen), ranging from a  
265 minimum 2.3% to a maximum 6.6% ( $n = 58$  samples). There was a difference between  
266 sites (Table 2), with means of 3.2%, 3.3% and 3.6% in Hofit, Yad Mordechai and Kipod,  
267 respectively, and a significantly higher content of 5.1% in Rehovot (see also graphical  
268 abstract). On average, a colony collected about ten times more protein than fat per year  
269 (Table 3).

270 Per total amount of FA in the pollen, the three major FAs were palmitic acid (16:0),  
271 linoleic acid (18:2 $\omega$ 6) and linolenic acid (18:3 $\omega$ 3), with mean ( $\pm$  SE) values of 28.3%  $\pm$  6.9,  
272 24.7%  $\pm$  9.7 and 31.6%  $\pm$  10.7, respectively (Fig. 2). Together, these three FAs made up  
273 over 80% of the total FAs. Other FAs detected were: myristic, palmitoleic, stearic, oleic  
274 and arachidonic acids (Table 4). The level of palmitic acid remained relatively stable  
275 throughout the year, whereas linoleic and linolenic acids showed more fluctuations (Fig. 2).  
276 The greater stability of palmitic acid levels (as % in all pollen samples) was expressed in  
277 their lower CV value of 24.4, in comparison to 39.1 and 33.8 for the essential FAs linoleic  
278 and linolenic acid, respectively. The variability in linoleic and linolenic acids was partly due  
279 to a striking difference between Hofit and the other three sites (Fig. 2). In Hofit, linolenic  
280 acid levels were typically low relative to linoleic acid, whereas the opposite pattern was  
281 found at the other sites (Graphic abstract, Table 4).

282 The mean ( $\pm$  SE) honey bee weight, and protein and total FA contents of 12 bees  
283 reared under controlled conditions were 111.4 mg  $\pm$  3.2, 11.1%  $\pm$  0.32 and 1.7%  $\pm$  0.10,  
284 respectively. Fig. 3 shows the total protein and FA contents in the pollen collected by the

285 colonies at each site during the year, and total expected amounts in honey bee bodies  
286 based on the number of bees produced during the year (panes A and B, respectively). At  
287 all sites, the amount of protein in the collected pollen was greater than that in the bees'  
288 bodies. For FAs, however, at some sites the content in the bees was greater than that in  
289 the collected pollen.

290 Table 4 shows the individual FA contents of bee bodies and collected pollen. In  
291 addition, Fig. 4 compares the FA balance between bee bodies and collected pollen. In  
292 general, there was a positive FA budget for palmitic, linoleic and linolenic acids, with more  
293 FA in the collected pollen than in the bee bodies. Hofit was the exception, with negative  
294 budgets for palmitic (nonessential) and linolenic (essential) acids. Other nonessential FAs  
295 were more abundant in the bees than in the pollen that they had collected. From largest to  
296 smallest difference, these included oleic, stearic, palmitoleic, and myristic acids.

297 Totaled over the year, the brood production cost was on average 51.3 mg pollen  
298 per produced honey bee (Table 5). In absolute uptake of pollen contents, the cost showed  
299 the least variation for linoleic acid and protein (lowest CVs; Table 5), and these were  
300 accordingly the best descriptive variables for the total number of workers produced, as  
301 compared to, for example, the gross amount of pollen or linolenic acid (Table 5).

302

#### 303 **4. Discussion**

304

305 Pollen fulfills honey bees' nutritional demands for protein and fat. Whereas the  
306 importance of protein in pollen is well established, that of fat has often been overlooked  
307 (Cohen, 2004). Pollen protein and fat contents differ among plants. Here we measured the  
308 amount and content constituents of honey bee-collected pollens in Israel, and assessed  
309 the nutritional balance between the collected pollen and the number of bees produced in a  
310 colony. Amounts of collected pollen and brood both peaked in spring (see also graphical  
311 abstract). This fits the consensus that colony development and reproduction are mainly  
312 related to the amount of pollen collected, rather than its composition (Avni et al., 2009;  
313 Dimou and Thrasyvoulou, 2007; Schmidt and Buchmann, 1999). Nonetheless, we show in  
314 the present study that data on pollen content can be an excellent predictor of honey bee  
315 production levels, as compared to gross pollen amounts. The smaller CVs for pollen  
316 linoleic acid and protein contents suggested that these parameters best describe the  
317 overall amount of bees produced by colonies on a yearly basis (Table 5).

318

319 *4.1. Total pollen collection and honey bee production*

320

321         There was high seasonal variability in the amount of pollen collected by colonies  
322 (Table 2). Overall, colonies collected an annual average of 16.8 kg pollen. Similarly,  
323 colonies in Europe and North America have been estimated to collect between 13.4 kg  
324 and 55 kg pollen on a yearly basis (Crailsheim et al., 1992; Herbert, 1999; Odoux et al.,  
325 2012; Schmidt and Buchmann, 1999; Winston, 1987). Surprisingly, the average yearly  
326 production of 367,000 bees is notably high; double that of 117,000 to 150,000 worker  
327 brood cells per year (as listed for multiple studies in Table 5 within Crailsheim et al.  
328 (1992)), or 226,596 born worker bees (Page and Metcalf, 1984). A likely driver for the high  
329 annual brood-production level might lie in the subtropical Mediterranean climate in Israel,  
330 which allows for a prolonged brood-rearing season (Avni et al., 2009). High production,  
331 however, may be offset by shorter worker lifespan than in colonies in temperate climates,  
332 where winter (diutinus) workers are less active and live for several months (Amdam and  
333 Page, 2005).

334         At the height of colony growth in our study, the peak brood amounts indicated that  
335 queens laid, on average, 2,078 eggs per day, which is similar to the 1,950 to 2,500 eggs  
336 reported by Harbo (1986) based on brood area. Two colonies peaked with queens having  
337 laid 3,300 and 3,000 eggs per day, respectively, equivalent to 2 eggs per minute.  
338 Beekeepers have reported such observations anecdotally (Wright, 2008), but we believe  
339 that our data provide unprecedented support for the potentially high egg-laying rate of  
340 honey bee queens. This finding suggests that the queen's maximum egg-laying capacity is  
341 not the limiting factor in yearly colony production, but that colony development is limited by  
342 other factors.

343         By dividing the colony's annual pollen uptake by the number of bees produced, we  
344 calculated a pollen cost per produced honey bee (Table 5). The average cost of 51.3 mg  
345 pollen per honey bee produced in Israel reflects high efficiency compared to the previously  
346 reported range of 86 to 188 mg pollen per honey bee (Alfonsus, 1933; Babendreier et al.,  
347 2004; Hrasnigg and Crailsheim, 2005; Rosov, 1944; Wille et al., 1985). This efficiency  
348 may be partly attributed to the pollen content.

349

350 *4.2. Protein content*

351

352           The total amount of pollen collected by colonies in this study was in the low range  
353 of amounts reported for colonies in temperate regions; nevertheless, about twice as many  
354 bees were produced per year. This may be due to the high protein content of the pollen in  
355 Israel (39.8%), almost twice that reported in other studies. To compare with other  
356 Mediterranean countries, Tüylü and Sorkun (2006) reported an average 19.0% protein for  
357 14 bee-collected monofloral pollens from Turkey, and Odoux et al. (2012) report a range of  
358 16 to 29% protein for mixed bee-collected pollens from the south of France. In absolute  
359 protein amounts, Crailsheim et al. (1992) reported a yearly colony uptake of between 2.8  
360 and 3.7 kg of protein per colony, whereas the estimated uptake in the current study was  
361 7.14 kg. These figures illustrate how high protein intake might have enabled production of  
362 double the amount of bees with the same amount of pollen. The relatively high colony  
363 production levels in Israel in relation to the high protein levels could indicate that in more  
364 temperate regions, protein is a limiting nutrient.

365           Whereas pollen protein quantifications may differ according to the analytical  
366 method used (Roulston et al., 2000; Vanderplanck et al., 2014), between March and  
367 September 2005, 11 pollen samples from Amman, Jordan, were collected by Nizar  
368 Haddad's group and analyzed by the same method presented in the current paper (Shafir  
369 et al., 2009): The average protein content was 28.7%, strengthening the finding of  
370 comparably higher pollen protein contents in Israel. Furthermore, since bees may add  
371 variable amounts of nectar to pollen pellets, protein percentage of honey bee-collected  
372 pollen may underestimate the protein percentage in pollen (Roulston et al., 2000). In the  
373 review by Roulston et al. (2000), hand-collected pollen from 62 bee-pollinated plants had  
374 an average 38.1% protein, as analyzed by the Bradford method, similar to our average of  
375 39.8% protein found for 58 honey bee-collected pollen batches.

376           Importantly, the protein analyses in our study were internally consistent, rendering  
377 the comparative analyses, i.e. between seasons and sites, reliable. We found pollen  
378 protein content to be stable over the seasons, despite significant differences among sites  
379 (Table 2). The low pollen protein level in Kipod (see graphical abstract) may have  
380 contributed to the relatively low brood production at that site, in addition to the altitude  
381 effect which significantly reduced colony growth (Fig. 1).

382

383 *4.3. FA content*

384

385           We found a narrow range of 2.3% to 6.6% total FA in bee-collected pollen  
386 mixtures. Similarly, seven mixed bee-collected pollen samples of subtropical origin were  
387 reported to have a narrow range of 4.6% to 6.1% total FA (de Arruda et al., 2013), though  
388 mixed bee pollen of the western Mediterranean was reported to have a much higher and  
389 wider range of 7 to 24% lipids (Odoux et al., 2012).

390           Honey bees collect pollen of mixed sources, from an average six plants at any one  
391 time in Israel (Avni et al., 2009), and an average 11 plants in a subtropical region in Brazil  
392 (Hilgert-Moreira et al., 2014). A study by Schmidt et al. (1987) found that honey bee  
393 survival is optimal when feeding on a blend of five different pollens, compared to  
394 monofloral pollen diets. Hence, balancing by means of a polyfloral diet can be an active  
395 nutritional strategy for honey bees, to mitigate the risk of a particular nutritional source (a  
396 monofloral pollen diet) having a shortfall in essential nutrients. In addition, certain FAs,  
397 such as linoleic, linolenic, myristic and lauric acids, have bactericidal and antifungal  
398 properties that support colony hygiene (Manning, 2001; Manning and Harvey, 2002), and  
399 may therefore be important for disease resistance and colony survival.

400           We used the FA composition of bees fed a rich mixed pollen diet under controlled-  
401 enclosure conditions as a reference to assess honey bee nutritional demands. Although  
402 linolenic acid content in Hofit suggested a deficiency (Fig. 4), this site was not  
403 outperformed by the others in honey bee production (Fig. 1). It is possible that under  
404 deficient conditions, bees are produced that require fewer resources, for example, smaller  
405 bees produced by early capping (Schmickl and Crailsheim, 2001). It is also possible that  
406 microbial interactions, either in the gut or during pollen storage as bee bread, change the  
407 content of the honey bee's food to compensate for the observed potential deficiencies  
408 (Douglas, 2013; Haydak and Vivino, 1950). However, in the absence of actual analyzed  
409 honey bee samples from each site, we have no means of explaining the discrepancy in  
410 linolenic acid levels in Hofit.

411           The essentiality of linoleic and linolenic acids is consistent with the fact that these  
412 FAs were generally (except for linolenic acid in Hofit) present at higher levels in the  
413 collected pollen than in the bees' body mass (Fig. 4). The dominance of palmitic acid, a  
414 substrate for longer FA synthesis, and the two essential linolenic and linoleic acids in bee-  
415 collected pollen, demonstrates a well-developed adjustment between pollinator nutritional  
416 demands and the nutritional value of the food offered by pollinated plants.

417

418 *4.4. Nutritional balancing*

419

420 Some insects can balance their diet to compensate for a lack of essential nutrients  
421 (Dussutour and Simpson, 2009; Mayntz et al., 2005; Raubenheimer and Simpson, 1999;  
422 Simpson et al., 2004). We analyzed the nutritional balance between collected pollen and  
423 produced bees. These data cannot be used to assess whether the bees' body composition  
424 follows the pollens' content profiles, as we did not analyze honey bee samples at all test  
425 sites. In addition, not every potentially lacking nutrient, including essential amino acids,  
426 minerals and vitamins, was monitored in our field study. Nonetheless, the current study  
427 provides insight into the temporal and spatial fluctuations of pollen-derived nutrients for  
428 honey bee colonies.

429 The amount of protein expected in the honey bee's body mass was lower than the  
430 amount in the collected pollen (Fig. 3A). This difference can be explained by the efficiency  
431 of pollen digestion, as a certain extent remains undigested, and thus not all of the protein  
432 in pollen is absorbed by young nurse bees (Crailsheim et al., 1992). The expected total FA  
433 content of the honey bee body mass was sometimes higher than the actual amount of total  
434 FA coming into the colonies via pollen nutrition (Figure 3B). This indicates that bees  
435 biosynthesize certain nonessential FAs, either as nurses while processing the pollen into  
436 worker jelly or during development as larvae, pupae, or young bees (Feldlaufer et al.,  
437 1985; Svoboda et al., 1982).

438 Unlike the storage of honey, which can reach many kilograms, bees have been  
439 reported to store only up to about 1 kg of pollen (Seeley, 1995). The colonies in the current  
440 study stored similar pollen amounts over all sites (Avni et al., 2009), albeit with a seasonal  
441 pattern paralleling the amounts of open and capped brood (Supplement 1). This suggests  
442 that in bees, pollen-intake targets and pollen storage are brood-dependent.

443 The proportion of linolenic acid in bee-collected pollen was generally higher than  
444 that of linoleic acid (Fig. 2). There was a distinct discrepancy in the essential FA content in  
445 Hofit (see graphical abstract). In this context, it is interesting that colonies sometimes  
446 collected relatively high amounts of particular nutrients (Fig. 3), but this was not translated  
447 into the production of more bees. However, high uptake of a specific nutrient may be a  
448 side effect of compensation for deficiency in another nutrient. The limiting nutrient in this  
449 case could be linoleic acid, as the other sites were relatively low in linoleic acid in



450 comparison to Hofit (Fig. 2). Hence, to achieve their target level of linoleic acid, colonies  
451 may have increased pollen uptake. That linoleic acid might be a limiting factor to growth  
452 levels is supported by the production cost per bee, having the lowest CV (Table 5).

453         Knowing which dietary ingredient is in short supply may allow beekeepers to  
454 supplement diets with the lacking nutrient when suboptimal growth conditions are  
455 encountered (e.g., when using honey bee hives for pollination in greenhouses). Food  
456 supplementation is a common practice in animal nutritional sciences (Leeson and  
457 Summers, 2001; Lupatsch et al., 2001), and we believe that it could be adopted more  
458 extensively in apiculture as well. For example, we have shown that linolenic acid levels  
459 can be relatively low in Israel, either spatially, such as in Hofit, or temporally, in autumn  
460 (Table 2). The practice of applying omega 3 supplements to colonies might mitigate the  
461 chances of a potential nutritional deficiency for this essential fatty acid.

462         In summary, in support of our first hypothesis, we found that colony uptake of  
463 pollen can differ both spatially and temporally for pollen quantity and content. We also  
464 found support for altitude, as a measure of the growth season, affecting honey bee colony  
465 growth. The relatively high numbers of bees produced per year in our study in a  
466 subtropical climate compared to those reported in more temperate zones suggest that mild  
467 winter conditions can greatly increase annual honey bee production. This was further  
468 supported in our study, where colonies at higher elevations produced fewer bees than  
469 those at lower elevations. This effect may have important consequences in the face of  
470 global climate changes. We also found that the queens' peak egg-laying rate is about  
471 double that of the mean rate calculated over the full year. This shows that total honey bee  
472 production is limited by additional factors. Our findings do not support the hypothesis of  
473 pollen quantity and nutritional quality affecting honey bee colony growth, at least not on an  
474 annual basis. For example, the site with the highest colony growth had the lowest uptake  
475 of pollen and amounts of protein and FAs (Table 3). However, it seems that relative to  
476 other reports, our colonies generally collected relatively high amounts of total protein, and  
477 were consequently able to produce a relatively large number of bees. When colonies can  
478 forage from relatively diverse landscapes, they may be able to acquire the needed levels  
479 of essential nutrients. The availability of particular nutrients may be low in particular  
480 locations or seasons, but severe nutritional deficiencies are more likely in especially  
481 depleted environments, for example in agricultural monocultures or in greenhouses.

482 Whether bees can then discriminate between pollens based on their nutritional  
483 composition and compensate for such deficiencies deserves further study.

484

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486

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493

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- 645

645

## 646 FIGURE CAPTIONS

647

648 **Fig. 1.** Honey bee colony development was monitored throughout Israel at seven sites,  
649 indicated on the map with closed circles (left pane). Mean ( $\pm$  SE) number of bees born in a  
650 honey bee colony over one year, as based on capped brood cell dynamics in 63 colonies  
651 (right pane). Sites are ordered according to increasing altitude, which correlates negatively  
652 with population growth per colony (linear regression:  $R^2 = 0.50$ ,  $F_{1,61} = 42.64$ ,  $p < 0.001$ ).

653

654 **Fig. 2.** Amount of bee-collected pollen and its relative protein content over time, at four  
655 sites in four seasons (per solstice and equinox, as indicated by the white and gray  
656 backgrounds). In the upper row, left axis shows weight of collected pollen; means with SE  
657 (error bars) are from five colonies; right axis shows protein percentage in pollen samples.  
658 The lower row shows the three most abundant fatty acids, as percentage of the total FA  
659 amount in pollen. Note the reduction in Hofit of both amount of pollen collected over the  
660 year, and contrasting levels of linolenic and linoleic acids, in comparison to the other sites.

661

662 **Fig. 3.** Comparison in four sites in Israel between protein (A) and total fatty acid (FA) (B)  
663 levels in pollen collected by colonies and those calculated to be present in the total  
664 produced bees' body mass. Note that the honey bee protein is accounted for, with more  
665 total protein in pollen than in honey bee bodies, whereas FAs were also derived by means  
666 other than pollen content, for instance by biosynthesis.

667

668 **Fig. 4.** Fatty acid (FA) uptake by colonies over a whole year was compared with the  
669 expected FA composition of all bees produced in a year. The difference between colony  
670 FA intake and output was calculated by subtracting the honey bee body FA contents from  
671 the pollen FA contents. Thus, a positive value indicates that more pollen FA was taken in  
672 than was used for the production of bees. A negative value shows that pollen contained  
673 less of this FA compared to its output in the body mass, indicating its biosynthesis within  
674 the colony. Note that the essential FAs were available in sufficient quantities, with positive  
675 linolenic and linoleic acid values, except in Hofit. The lipid numbers are given in

676 parentheses, indicating the number of carbon atoms and the number of double bonds in  
677 the fatty acid.

678 **Table 1**

679 The experimental sites where honey bee colonies were monitored. Rainfall data were  
680 derived from the nearest weather stations (data.gov.il/ims), with reported total rainfall for  
681 the season 2004–2005.

Site	Hofit	Yad M.	Rehovot	Kabri	Kipod	Kanaf	Fijas
Latitude N	32°23'37"	31°35'25"	31°54'26"	32°01'15"	32°36'18"	32°52'11"	32°41'43"
Longitude E	34°52'30"	34°34'07"	34°48'16"	35°08'50"	35°07'20"	35°41'52"	35°33'05"
Rain (mm)	556	433	466	597	1162	696	902
Elevation (m)	19	45	51	89	237	279	288

682

683 **Table 2**

684 Two-way ANOVA of bee-collected pollen quantity and contents over time and place.  
685 \*Statistically significant considering a threshold at  $\alpha = 0.05$ , or for the interdependent fatty  
686 acid (FA) tests at  $\alpha = 0.01$ . For all significant results in bold, post-hoc results are indicated  
687 (right column). Differences exist between the underlined groupings. The indicated site  
688 effect on collected pollen was not post-hoc significant. Note: Pollen amount is strongly  
689 influenced by season, and pollen contents is typically influenced by site.

Pollen content data	Site effect	Season effect	Post-hoc tests <sup>b</sup>
Pollen collected (g) <sup>a</sup>	$F_{3,51}=3.01, P=0.04^*$	$F_{3,51}=10.6, P<0.001^*$	<u>Sp&gt;Wi&gt;Su,Au</u>
Protein (mg/g pollen) <sup>a</sup>	$F_{3,51}=5.66, P=0.002^*$	$F_{3,51}=0.49, P=0.69$	<u>Ho&gt;Re&gt;Ym&gt;Ki</u>
Total FA (mg/g pollen)	$F_{3,51}=25.4, P<0.001^*$	$F_{3,51}=1.53, P=0.22$	<u>Re&gt;Ki,Ym,Ho</u>
Palmitic acid (% of FA) <sup>a</sup>	$F_{3,51}=4.76, P=0.005^*$	$F_{3,51}=0.14, P=0.94$	<u>Ym&gt;Ki,Ho,Re</u>
Linoleic acid (% of FA) <sup>a</sup>	$F_{3,51}=15.6, P<0.001^*$	$F_{3,51}=2.86, P=0.046$ (ns)	<u>Ho&gt;Ki,Re,Ym</u>
Linolenic acid (% of FA) <sup>a</sup>	$F_{3,51}=19.8, P<0.001^*$	$F_{3,51}=3.83, P=0.015$ (ns)	<u>Ki,Re,Ym&gt;Ho</u>
Linoleic acid (mg/g pollen)	$F_{3,51}=6.60, P<0.001^*$	$F_{3,51}=0.95, P=0.42$	<u>Ho,Re&gt;Ym,Ki</u>
Linolenic acid (mg/g pollen)	$F_{3,51}=41.0, P<0.001^*$	$F_{3,51}=5.89, P=0.002^*$	<u>Re&gt;Ym,Ki&gt;Ho</u> <u>Su,Wi&gt;Sp&gt;Au</u>

690 <sup>a</sup>Effect sizes are illustrated in Fig. 2, and in the graphical abstract. <sup>b</sup>Sp, spring; Wi, winter; Su,  
691 summer; Au, autumn; Ho, Hofit; Re, Rehovot; Ym, Yad Mordechai; Ki, Kipod.



692

693 **Table 3**

694 Estimates per year in mean  $\pm$  SE (no. of colonies in parenthesis), or total estimate per site.  
 695 The overall number of bees produced is given for the seven sites. For four sites, the  
 696 average yearly total pollen amount and the totals for protein and FA contents are listed.  
 697 Pollen content data originate from pooled batches of five sampled colonies per site,  
 698 collected every month. The absolute uptake of each nutrient was interpolated over a  
 699 period of 1 year to assess the total uptake of nutrients (protein and FAs).

Estimate	Overall mean	Hofit	Yad Mordechai	Rehovot	Kipod
Bees * 1,000	367 $\pm$ 8.1 (63)	413 $\pm$ 14 (9)	402 $\pm$ 15 (9)	399 $\pm$ 13 (10)	335 $\pm$ 14 (9)
Pollen (kg)	16.8 $\pm$ 1.2 (20)	11.8 $\pm$ 0.7 (5)	20.9 $\pm$ 2.9 (5)	16.9 $\pm$ 2.2 (5)	17.6 $\pm$ 1.6 (5)
Protein (kg)	7.14	5.88	9.50	7.06	6.13
Total FA (g)	677	358	689	926	735
Palmitic acid (g)	190	104	164	269	225
Linoleic acid (g)	152	129	146	199	135
Linolenic acid (g)	234	74	243	352	244

700

701 **Table 4**

702 Fatty acid (FA) content in worker bee bodies and in pollen samples at four different sites.  
 703 Data are presented as the percentage of total FA content and are reported as means  $\pm$  SE  
 704 (number of samples analyzed is in parentheses). Yearly variation for palmitic, linoleic and  
 705 linolenic acids is illustrated in Fig. 2, and in the graphical abstract. After the trivial names,  
 706 the lipid numbers are given, indicating the number of carbon atoms and the number of  
 707 double bonds in the fatty acid.

Fatty acid		Bees (12)	Hofit (16)	Yad M. (13)	Rehovot (16)	Kipod (13)
Myristic acid	14:0	2.05 $\pm$ 0.11	0.83 $\pm$ 0.11	1.10 $\pm$ 0.11	1.19 $\pm$ 0.14	0.83 $\pm$ 0.11
Palmitic acid	16:0	18.57 $\pm$ 1.11	30.0 $\pm$ 1.79	22.3 $\pm$ 1.96	30.0 $\pm$ 0.58	30.1 $\pm$ 2.05
Palmitoleic acid	16:1	2.36 $\pm$ 0.24	0.07 $\pm$ 0.07	0.36 $\pm$ 0.10	0.42 $\pm$ 0.16	0.27 $\pm$ 0.07
Stearic acid	18:0	12.54 $\pm$ 0.61	3.59 $\pm$ 0.41	5.30 $\pm$ 0.52	2.97 $\pm$ 0.28	4.60 $\pm$ 0.33
Oleic acid	18:1	31.28 $\pm$ 0.90	9.84 $\pm$ 0.89	10.25 $\pm$ 0.82	5.67 $\pm$ 0.49	9.73 $\pm$ 0.58
Linoleic acid	18:2 $\omega$ 6	11.43 $\pm$ 0.72	34.6 $\pm$ 2.26	21.4 $\pm$ 1.73	21.4 $\pm$ 1.93	20.0 $\pm$ 1.90
Linolenic acid	18:3 $\omega$ 3	21.76 $\pm$ 1.06	20.3 $\pm$ 1.73	37.2 $\pm$ 2.00	37.4 $\pm$ 2.39	32.8 $\pm$ 2.22

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Arachidonic acid	20:4	17.04 ± 0.99	0.77 ± 0.16	2.14 ± 0.31	0.98 ± 0.13	1.72 ± 0.22
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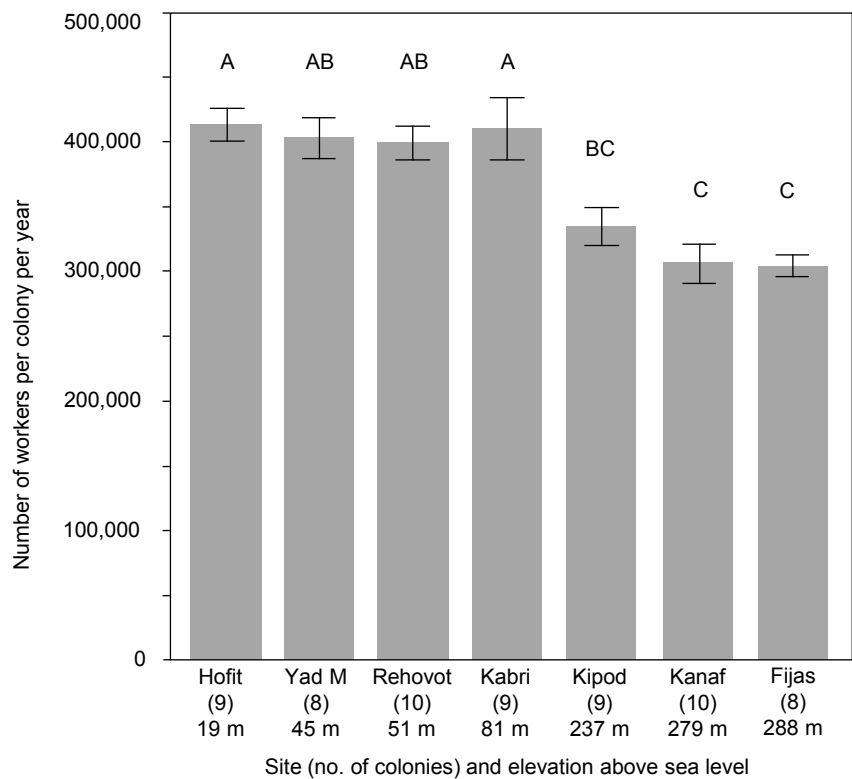
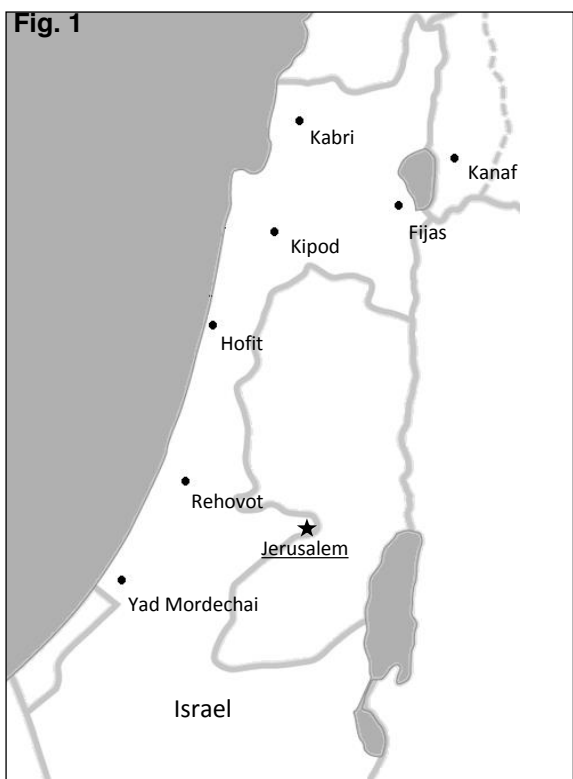
708

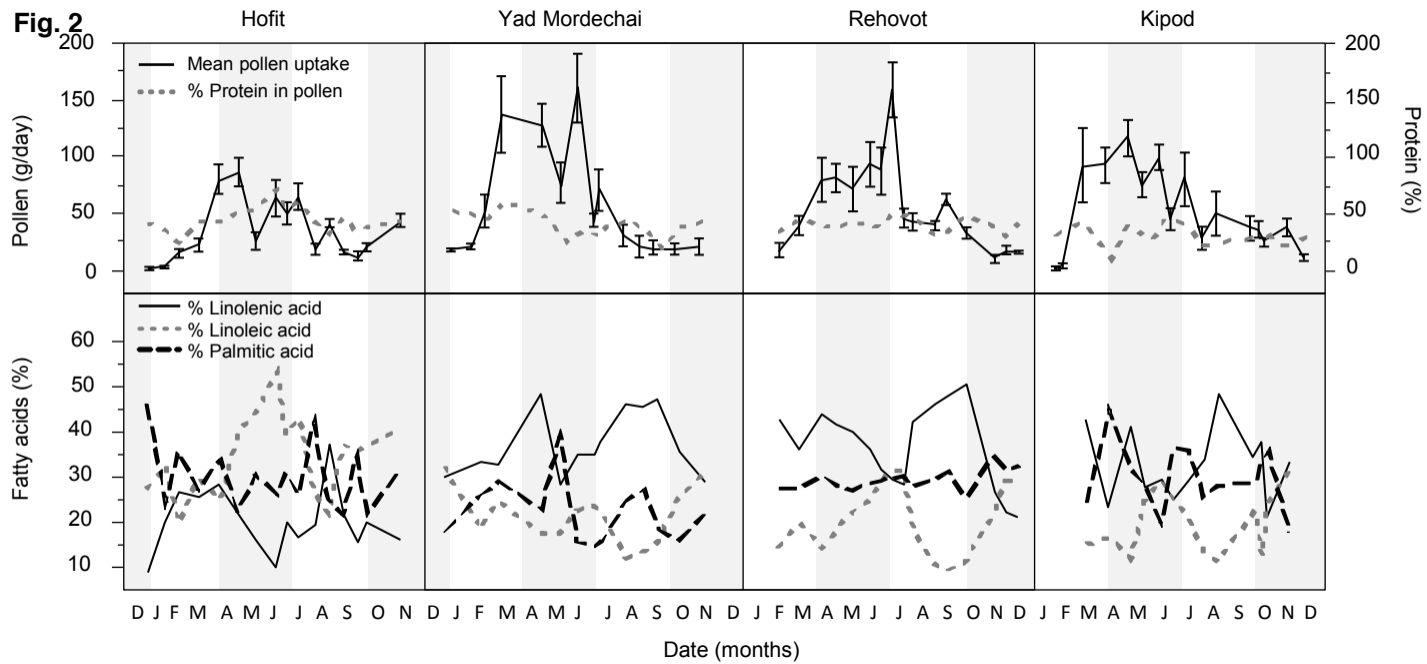
708 **Table 5**

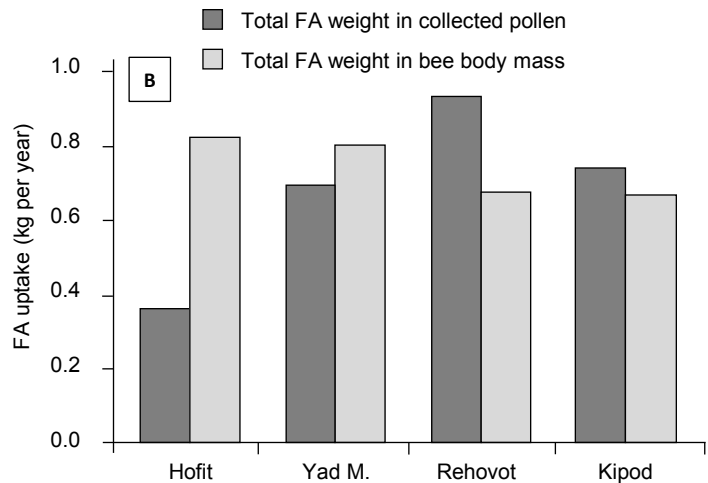
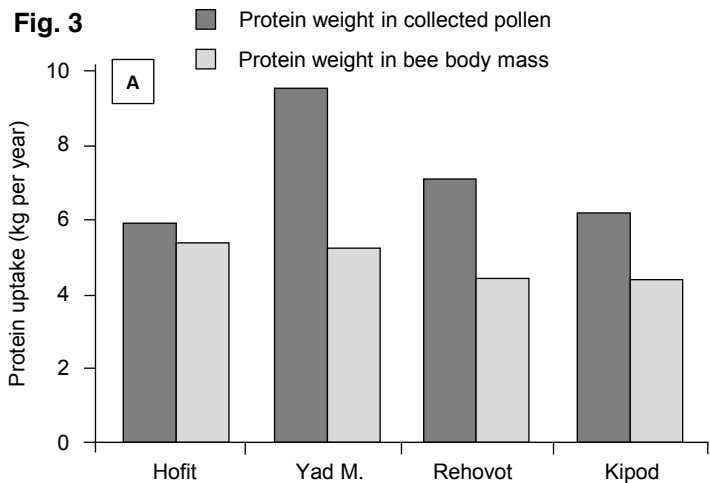
709 Nutritional costs of producing a honey bee at four sites in Israel, and overall mean. The  
710 last column is the coefficient of variation ( $CV = SD/mean$ ), used to compare the costs at  
711 the four sites. The nutrients are listed according to increasing CV value. The nutrient with  
712 the lowest CV may be a limiting factor for growth, as it best describes the production cost  
713 of bees.

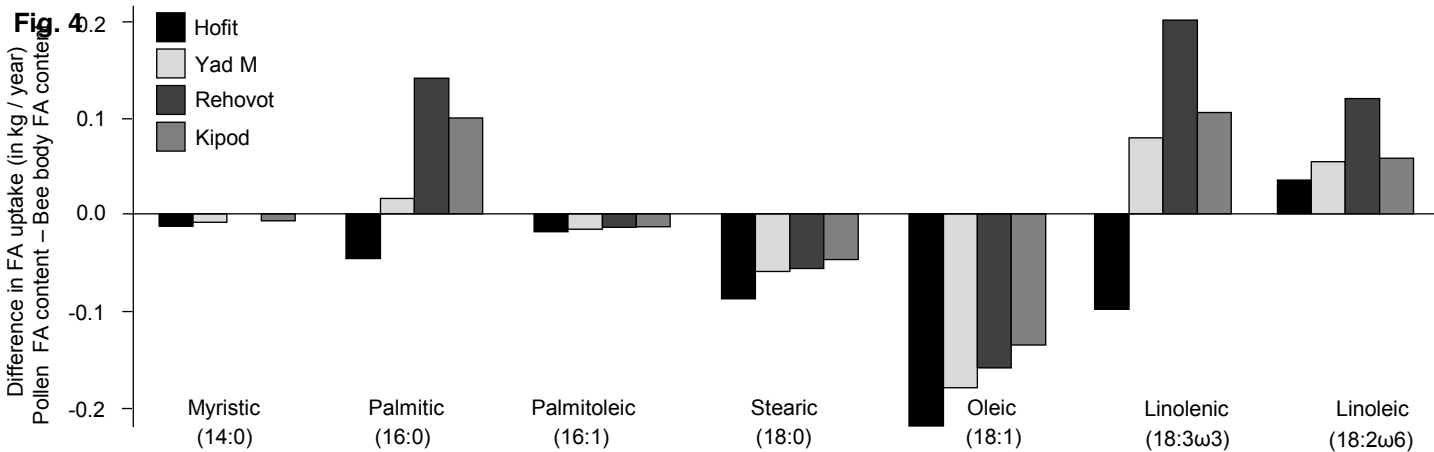
Cost (nutrient per bee)	Overall mean	Hofit	Yad M.	Rehovot	Kipod	CV (%)
mg linoleic acid	0.46	0.37	0.42	0.58	0.47	20.0
mg protein	21.6	16.7	27.6	20.7	21.4	21.0
mg pollen	51.3	33.4	60.8	49.6	61.3	25.5
mg total FA	2.07	1.01	2.00	2.72	2.56	37.1
mg palmitic acid	0.59	0.29	0.48	0.79	0.78	41.5
mg linolenic acid	0.70	0.21	0.71	1.03	0.85	50.4

714



**Fig. 2**

**Fig. 3**

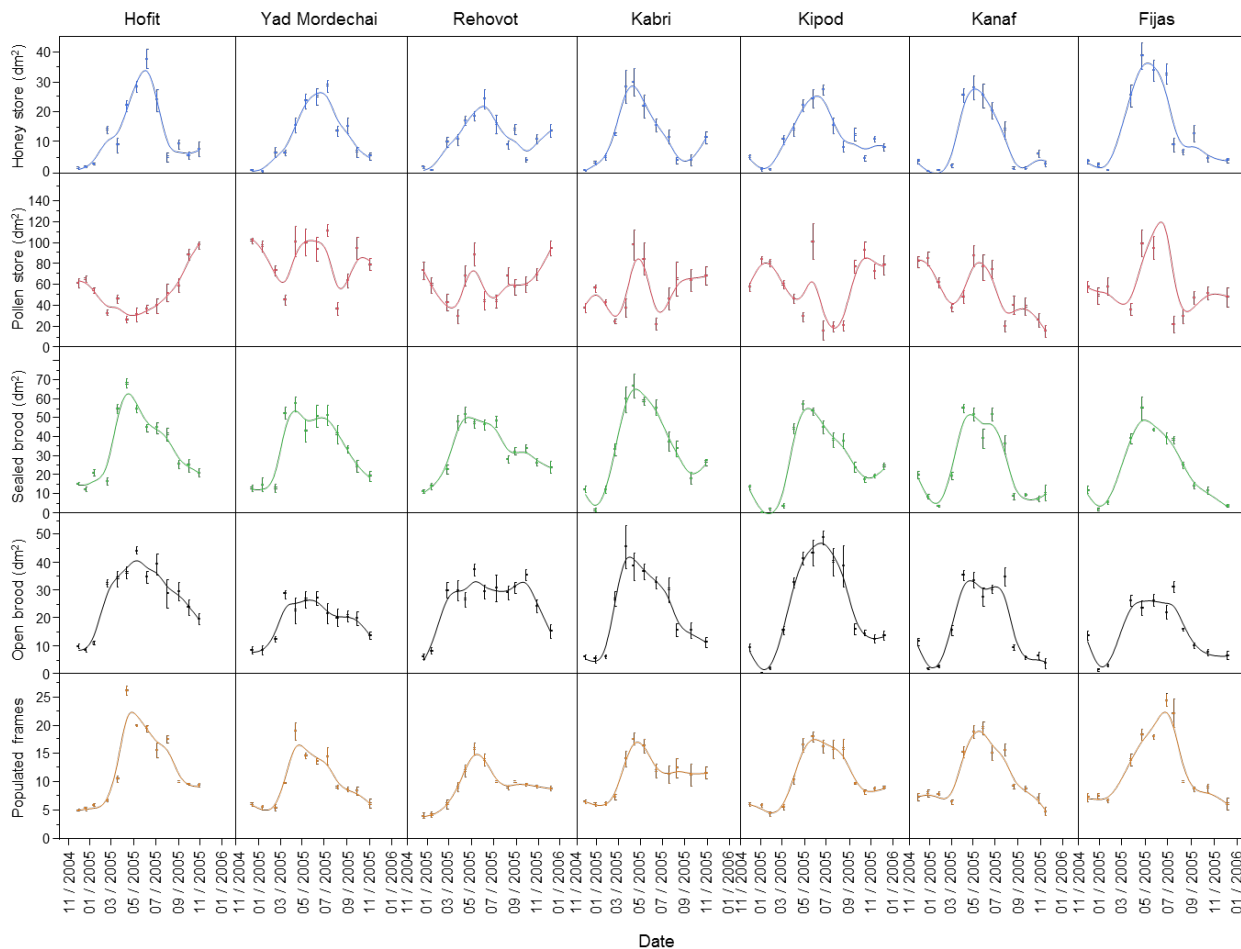


714

715 **Supplement 1**

716

717 **Fig. S1.** Over the course of one year, 802 hive assessments were performed: 70 hives  
 718 over 7 sites with an average of 12 samplings per colony. Below are the average results of  
 719 five variables for seven sites over the year, showing standard errors over the different  
 720 colonies per sampled site and test date. The curves are a LOESS fit. The variables were  
 721 given for total comb side coverage per colony ( $\text{dm}^2$ ): pollen stores (blue), honey stores  
 722 (red), open brood (black) and closed brood (green). The bee population was assessed by  
 724 the number of occupied frames per hive (yellow).



757

**Methodology**

758

Standard procedures were followed, in which one person communicated observations to an  
 759 assistant who recorded the data on a standard worksheet. Every comb side within a colony was  
 760 closely observed. The number of worker cells filled with either stored honey or stored pollen, open  
 761 brood or closed brood was assessed by estimating total comb side coverage to the nearest  $0.5 \text{ dm}^2$   
 762 for each of these variables. For example, when a  $5 \text{ dm}^2$  surface of capped brood was observed,  
 763 though with an estimated 10% empty cells, the total capped brood amount was recorded as  $4.5 \text{ dm}^2$   
 764 coverage for that comb side. Such estimates were performed for every parameter on each comb



765 side. In addition, the total number of frames populated by bees, and whether the colony had a  
766 notable condition such as observable disease symptoms, were noted.