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Research article

Long-duration feedings and caste differentiation in *Bombus terrestris* larvae

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Summary. The duration of feedings received by *Bombus terrestris* larvae was studied using video-recordings. In the last days of development all larvae received feedings mainly of long duration. Worker larvae of the third brood received significantly longer feedings than worker larvae reared in the other broods. Throughout the development queen larvae and worker larvae received feedings of similar duration. Male larvae received shorter feedings than both kinds of female larvae. Therefore, the duration of feedings seems to be associated to the sex and stage of development of the larvae.

The causes of the long-duration feedings seem not to be related to the amount of food provided, workers' age and size, to the workers' abdominal contraction or to the amount of pollen in the larval food. Perhaps the feeding duration is caused by the viscosity of the food, which is a consequence of the presence of pollen grains, sugar and glandular material. Although the precise amount of pollen was not measured, the differences in colour showed clearly that the larval food samples contained variable quantities of pollen grains. Some of the samples did not contain any pollen at all.

It is suggested that the duration of feedings may be related (among other factors) to the presence of glandular material (proteins and enzymes) which is added to the larval food. This could be especially important for queen larvae in the last phase of their development. Because they have a long development and are fed with a high frequency they might receive large amounts of these substances. This could help them to grow more efficiently using a relatively smaller amount of pollen than expected.

Key words: Bombus terrestris, bumble bee, larval feeding, caste differentiation.

Introduction

While studying the feeding behaviour of workers and the frequency with which the larvae are fed (Ribeiro and Velthuis, 1997; Ribeiro et al., 1999) we found that the feedings varied greatly in length according to the kind of larva and varied throughout their development. Such variation has also been described for honey bee nurses and in that case it was associated with the composition of the larval food. The long-duration feedings received by worker honey bee larvae after 48 h of development are related to the high amounts of protein and low ratio of glucose/fructose in the worker jelly. Queen larvae, however, receive mainly short feedings during their development and the composition of the royal jelly remains practically constant (Brouwers et al., 1987).

It is admitted that bumble bees (unlike honey bees) do not produce royal jelly to feed the queen larvae and improve their development. One possible reason is that the digestive tracts of bumble bees are unable to deal with as much pollen as honey bees. This could make them unable to produce much protein for the "brood-food" (Bailey, 1954). Another study showed that nurse bees and foragers do not differ in the size of their hypopharyngeal glands or in the amount of pollen they eat, conditions which would be essential for the production of a special food (Free, 1955; Free and Butler, 1959). However, Röseler (1967, 1974) did find differences when examining the hypopharyngeal glands in workers of several ages. Young bees (2–5 days old) had the largest hypopharyngeal glands. But older workers (even older than 30 days) also had large glands when no workers were emerging in the colony anymore. This could explain the results found previously by Free.

The secretion from the bumble bees' hypopharyngeal glands is an acid fluid containing much protein and two enzymes (Palm, 1949). It is possible that this secretion is added to the larval food to help digestion (Free and Butler, 1959;

Röseler, 1974). Proteins secreted by hypopharyngeal glands are probably added to the larval food of stingless bees too, and would be important mainly because of their enzymatic properties (Hartfelder and Engels, 1989; Velthuis and Sommeijer, 1991; Velthuis, 1992; Sommeijer and de Bruijn, 1994).

In *B. terrestris,* queen larvae attain a much larger mass than worker larvae, although they receive a relative small amount of pollen regarding their weight. This suggests they may have an additional source of protein in their diet (Ribeiro, 1994). Indeed, protein (besides that coming from the pollen) was found in the larval food. But because it is present in the food given to all larvae it is not thought to play a major role in caste differentiation (Pereboom and Shivashankar, 1994; Pereboom, 1996).

In this paper the variation in the feedings' duration received by worker, male and queen larvae of *B. terrestris* is reported. In order to know whether the queen and/or the colony conditions could have an influence on the length of feedings the different phases of the colony development were analysed, comparing the feedings received by worker larvae reared in the three broods.

Furthermore, the causes of such variation were investigated. The first is the amount of food: a feeding of long duration could indicate that a large amount of food is being supplied to the larva. The second is the worker age: long duration might be related to the age (and physiology) of the worker, which in its turn could be connected to the composition of the food. Thirdly, the worker size: a small worker (with a small crop capacity) could provide a small amount of food in a short-duration feeding. The length of time may be still associated with the worker crop content: with a totally filled crop the food would be easily expelled. The opposite is expected for an almost empty crop. Then the contents could be released more slowly or with more difficulty. Feedings' duration could also be related to the amount of pollen present in the food. More concentrated food could be more difficult to regurgitate. In honey bees, it was found that the viscosity of the liquid may affect the speed with which the food is transferred (Farina and Núñez, 1991).

Finally the results are discussed as well the possible functions of the long-duration feedings.

Material and methods

B. terrestris colonies were reared under controlled conditions of temperature (28 °C) and relative humidity (60%). (For details of the rearing method see Duchateau, 1985; Duchateau and Velthuis, 1988). Egg cells of first, second and third broods were video-recorded for 4 h a day, during their entire development. Methodological details and the results of the frequencies with which worker, male and queen larvae were fed are described in another paper (Ribeiro et al., 1999). Here the results of one aspect of the behaviour (duration of feedings) of nurse bees is presented as well the investigations concerning its possible causes.

The workers were marked with numbered tags less than 24 h after emergence so that they could be recognised individually and information about their age could be obtained.

Each feeding event was recorded per larva, as was the number of the worker which performed it. The queen may feed young larvae after workers have emerged (Duchateau, 1989; Ribeiro and Velthuis, 1997). Because the main interest was to study the feedings performed by the workers, the feedings performed by the queen were excluded in the analysis of the larvae from the second brood and onwards. Thus, in some cases, in the first days of larval development there were little or no data, what does not necessarily mean the larvae were not fed that day; the feedings could have been performed by the queen. A feeding event can be summarised as follows: the feeder approaches the larva, introduces her mouth parts into the hole in the larval wax envelope, remains motionless for a moment and discharges the food. This food discharge is generally identified by a clear contraction and/or an elevation of the feeder's abdomen (Katayama, 1973, 1975; Pendrel and Plowright, 1981). However, there are feedings that happen with a very slight or almost no contraction of the worker's abdomen. Thus, the entire movement of the abdomen (and not only the actual contraction) was considered as representing the duration of the feeding. The measurements were performed with a chronometer (± 0.01 s). To ensure a high accuracy each feeding was measured at least three times and an average duration was calculated. Sometimes it was not possible to measure the feedings' duration because of the position of the worker (her abdomen was not visible) or because another bee walked over the nurse which was feeding. However, the majority (about 92%) of feedings provided to the larvae were measured.

To find out the reasons for the feedings variable duration two experiments were performed. In the first one, groups of 8–10 recently emerged workers were collected from several colonies, marked with numbered tags and put in observation boxes together with a queen and some larvae. Food (pollen and sugar-water solution at 50%) was provided in abundance. After one to several days the larvae were removed and the experiment started. Other last-instar larvae (many queen larvae and a few worker and male larvae) placed earlier in small honey bee wax cups were weighed (± 0.1 mg) and then introduced into an observation box one at a time. A video-recording was made until the larva was fed by a worker. If the feeding did not occur within 10–15 min another larva was used. After the feeding the wax cup containing the larva was immediately weighed again. The difference in weight indicated the amount of food provided. The worker that fed the larva was collected and preserved in the freezer. The size of the worker was measured by two parameters: her dry weight and the size of her wing's radial cell. The dry weight was obtained after she had been kept for at least 24 hours in an incubator at 100 °C. The length of her wing's radial cell was measured using a stereo-microscope with a graduated ocular. During the analysis of the video tapes, the duration of the feedings was measured. In addition, the intensity of the worker's abdominal contraction was estimated on an arbitrary scale which varied from 1 (small, slight contraction) to 3 (strong, clear contraction).

In a second experiment observation boxes were prepared as before. Last-instar larvae (queens only) were again placed in honey bee wax cups. They were introduced into the box one at a time and a video-recording was made until they were fed. This time the food was collected immediately after it was provided, using labelled disposable capillaries. The amount of pollen in each capillary was not measured directly, but was estimated by evaluating the colour of the larval food. Since all pollen grains provided to the bees were of a similar yellow colour, an arbitrary scale from 0 (no colour, assumed to correspond to the absence of pollen grains) to 3 (dark yellow, believed to contain a large amount of pollen grains) was used. The duration of the feedings and the workers' abdominal contractions were also measured from the tapes.

The statistical analysis was done by a Pearson correlation coefficient, a Kruskal-Wallis test and a Mann-Whitney U-test (Sokal and Rohlf, 1981). All p-values above 0.05 were considered to be not significant.

To compare the feedings' duration throughout the development a Multilevel analysis of variance was used (Goldstein, 1995; computer program by Rasbash and Woodhouse, 1995). This test was chosen because of the different number of dependent observations per individual larva. Due to the large variation found in the feedings' duration per day of development first the best fitting curves per individual were determined and afterwards they were averaged for each larval group. The

resulting models are presented in Figure 2. Because there were not much data in the beginning of the larval development (due to the low number of feedings in this period and/or because many times the queen was the feeder), the comparisons were done from day 9 or 10 of development depending on the group of larvae considered. Initially the worker larvae of the third brood with the worker larvae of the first brood were compared (Fig. 2 a), and then worker larvae of the third brood with worker larvae of the second brood (Fig. 2 b). Then all larvae of the third brood were compared (workers, males and queens; Fig. 2 c). In order to treat all larvae equally (i.e., correcting for the different moments in which each larva has stopped the development) in this analysis the duration of feedings was considered only until the moment the larvae got the maximum feeding frequency (for details see Ribeiro et al., 1999).

Results

The duration of feedings

Initially the workers were compared at different phases of colony development. The variation in the feedings' duration over the total development of worker larvae fed only by the queen and worker larvae fed by workers (in the second and third broods) is shown in Figure 1a. Worker larvae fed by the queen had a lower median (0.64 s) than the worker larvae fed by workers in the second brood (0.67 s) as well as in the third brood (0.70 s). The differences were highly significant ($p = 0.0001$, and $p = 0.0015$ respectively; Mann-Whitney U-test).

Comparing larvae of the third brood, (Fig. 1b) it was found that both types of female larvae had higher medians for the feedings' duration than male larvae (0.70 s for workers, 0.84 s for queens, and 0.62 s for males). The differences were highly significant ($p = 0.0001$, Kruskal-Wallis). Compared to worker larvae and male larvae queen larvae had the highest median ($p = 0.0001$, Mann-Whitney U-test, for both comparisons).

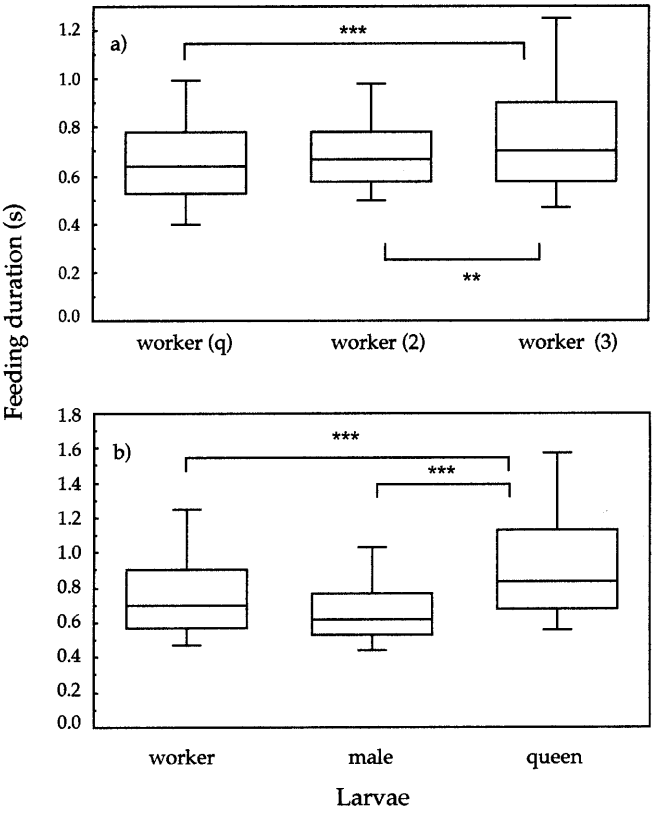

Figure 1. Duration of feedings (s) received by larvae of *Bombus terrestris* over their entire development: a) worker larvae fed only by the queen (N = 327) and by workers in the second (N = 540) and third broods (N = 935); b) worker larvae (N = 935), male larvae (N = 1678) and queen larvae (N = 2103).
Legend: (q): fed only by the queen; (2): second brood; (3): third brood. *** and **: highly significant ($p = 0.0001$ and $p = 0.0015$, respectively; Mann-Whitney U-test). This figure is composed of box-plots which show the percentiles. The three horizontal lines which form each box represent, in the upward direction, 25, 50 and 75% respectively, whereas the end of the lower bar (under the box) represents 5% and the end of the upper bar (above the box) 95%

The duration of feedings throughout the larval development

The curves of the duration of feedings of worker larvae when fed only by the queen (first brood) and of worker larvae when fed only by workers (in the third brood) throughout the development are shown in Figure 2a. Although there is a tendency for the feeding's duration to increase with age in both groups of worker larvae, the increase is much more pronounced for the worker larvae from the third brood. This difference was highly significant ($p < 0.001$, Multilevel analysis).

The same pattern is observed in a comparison of worker larvae of the second and third broods (all larvae fed by workers only; Fig. 2 b). Again a larger increase is observed for worker larvae of the third brood. The difference was significant ($p < 0.01$, Multilevel analysis).

Figure 2 c shows the curves found for the duration of feedings provided for the three types of larva of the third brood. From day 10 the feedings' duration increases more for the female larvae than for the male larvae. Indeed, the difference between worker larvae and male larvae was highly significant ($p < 0.0005$, Multilevel analysis), while the slope of the curves found for worker larvae and queen larvae was similar ($p > 0.05$, Multilevel analysis).

The possible causes of the variation in the duration of feedings

In order to find out in what way the crop content could influence the duration of feedings, sequences of feedings performed by the same worker were observed. Because it was not possible to see the whole colony at the same time, but only a small part covered by the video-camera, only the feedings that took place before the worker disappeared from view were studied. In such cases it was possible to be sure she performed all the feedings with only one crop load. Thus the

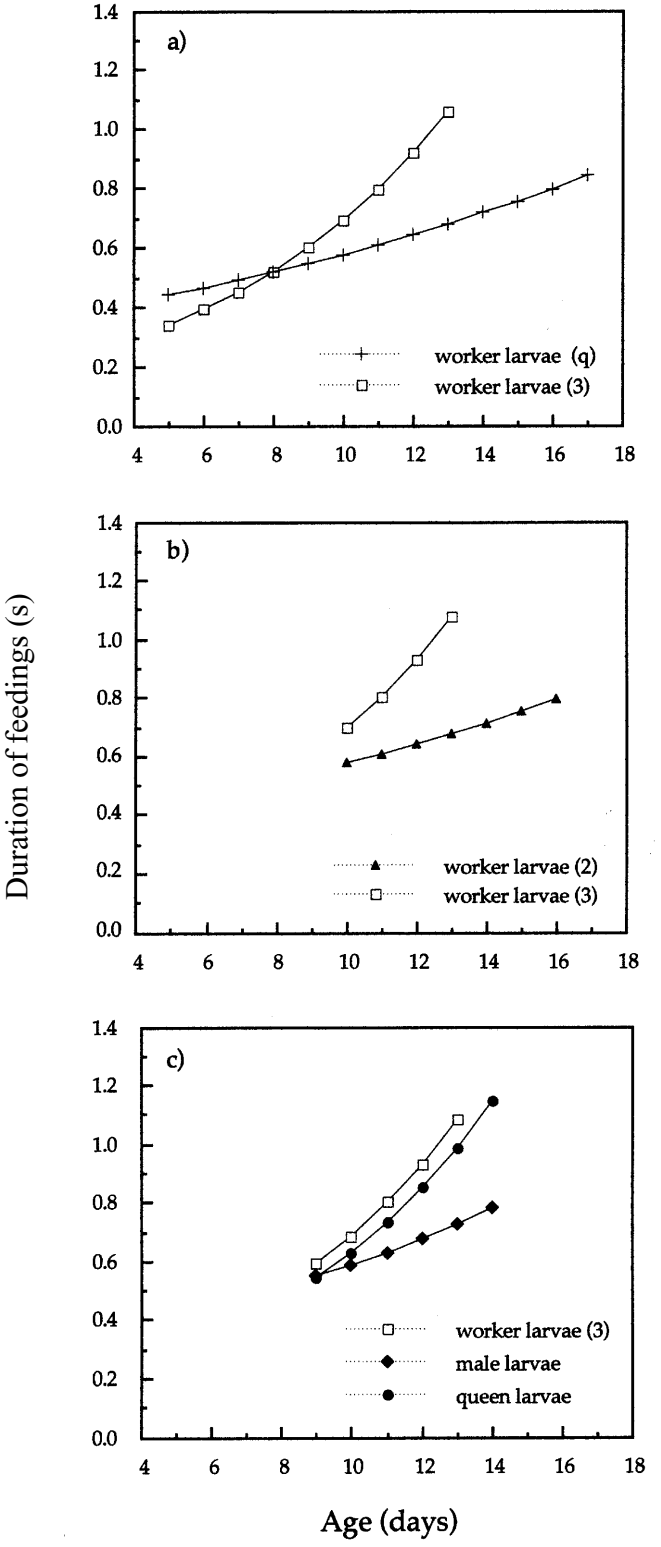

Figure 2. Duration of feedings (s) received by *B. terrestris* larvae during their development: a) worker larvae fed only by the queen (first brood) and worker larvae fed by workers (in the third brood); b) worker larvae fed by workers in the second and third broods; c) worker larvae (third brood), male larvae and queen larvae.
Legend: (q): fed only by the queen; (2), second brood; (3), third brood

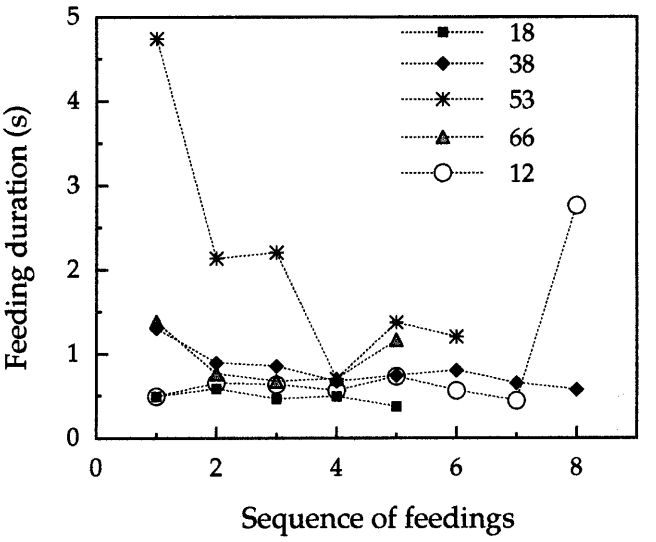

Figure 3. Duration of several feedings (s) performed by *B. terrestris* workers in a sequence, with the same crop load. Each number represents one worker

duration of the first feeding observed was compared with the duration of the second one, and this with the third and so on. As already mentioned, it was expected that the duration of the feedings would increase with the decrease in crop volume. But this was true only in some cases. Sometimes there was a large variation in the length of the feedings. At other times several feedings were performed each with a similar duration. A few examples are given in Figure 3, which relates to workers which fed from 5 to 8 larvae one after the other with the same crop load. It is clear that the feeding pattern of worker 53 was completely opposite to that of worker 12. On the other hand, the duration of feedings performed by some workers (18 and 38) did not change much. Therefore, there seems to be no relation between the duration of feedings and the volume of the crop content.

Further a series of other factors that could influence the duration of feedings was analysed: amount of food provided, age, weight and size (dry weight and wing) of workers (first experiment; N = 85) and presence of pollen (second experiment; N = 69). The results are summarised in Table 1. (Because in some cases there were no data on one or another factor simultaneously, the number of samples in the tables differs from the total mentioned above).

Although the correlation between dry weight and wing's radial cell of the worker is high (in this case, r = 0.823; N = 84), and one factor (wing) would be enough, the dry weight was measured as well. The reason was that other aspects (for example, content of the intestines, ovarian development, etc) could affect the duration of the feedings without modifying the worker's size. However, due to the lower correlations it seems these aspects were not important.

None of the variables analysed showed a high correlation indicating that the duration of feedings is not strongly related to any of these factors.

Table 1. Correlation coefficients between the duration (s) of feedings given to last-instar *B. terrestris* larvae and all other variables measured

Variables compared to feeding duration (s)	Correlation coefficients
Amount of food provided (mg)	0.055 (N = 80)
Worker's age (days)	–0.231 (N = 82)
Worker's dry weight (mg)	–0.035 (N = 82)
Worker's wing radial cell length (mm)	0.010 (N = 82)
Presence of pollen in the larval food	0.102 (N = 57)

N: number of feedings.

Table 2. Correlation coefficients between the intensity of workers' abdominal contractions during the feedings received by last-instar *B. terrestris* larvae and all other factors measured

Variables compared to worker's abdominal contraction	Correlation coefficients
Amount of food provided (mg)	0.178 (N = 80)
Worker's age (days)	–0.167 (N = 81)
Worker's dry weight (mg)	–0.061 (N = 81)
Worker's wing radial cell length (mm)	0.021 (N = 81)
Presence of pollen in the larval food	0.293 (N = 57)

N: number of feedings.

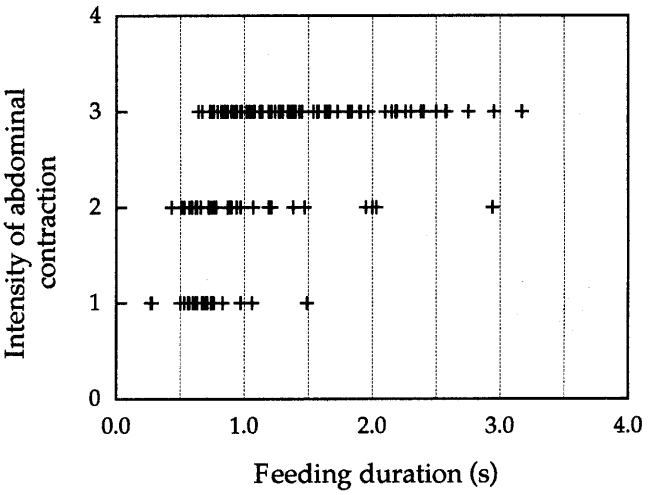

Figure 4. Relation between the duration of feedings (s) and the intensity of the worker's abdominal contraction observed in the feedings (N = 143) provided to the larvae of *B. terrestris*

Table 3. Correlation coefficients between the amount of food provided in the feedings received by last-instar *B. terrestris* larvae and all other factors measured

Variables compared to the amount of food provided	Correlation coefficients
Worker's age (days)	0.155 (N = 81)
Worker's dry weight (mg)	0.105 (N = 81)
Worker's wing radial cell length (mm)	0.178 (N = 81)

N: number of feedings.

The amount of pollen in the larval food samples (N = 64) varied greatly. It was found that 72% of the samples contained pollen grains distributed in the following percentages: 19% (little pollen), 14% (medium amount of pollen) and 39% (much pollen). The remaining samples (28%) were very clear and probably contained no pollen. The difficulty to obtain the food samples with capillaries was variable, probably because of differences in the amounts of food provided and viscosity. In general, food samples which contained much pollen and no pollen at all were the most difficult to collect, indicating that the viscosity of the food was not only caused by the presence of pollen grains.

The worker's abdominal contractions were also tested in relation to the variables mentioned above (Table 2). Once more all the factors had no remarkable correlation with the strength of the worker's abdominal contraction.

Indeed, although feedings of long duration were generally accompanied by a strong abdominal contraction of the worker the same was also observed with short-duration feedings. In addition, long feedings could occur with less pronounced contractions. Figure 4 illustrates the patterns found for the feedings recorded in the two experiments described. To permit better visualisation 3 feedings of extremely long duration were excluded from the figure: 8.19 s; 5.92 s and 6.59 s, with values for the contractions of 3, 3 and 2, respectively.

The amount of food provided also could have some relation to the variables analysed. The correlation coefficients are presented in Table 3. Still no high correlation coefficients were found.

Discussion

Several aspects could be involved in the variable duration of feedings provided to the larvae in the end of colony development (Fig. 2). The first is the environment: with time, the changes in colony conditions (temperature, humidity, worker: larva ratio, food availability, etc.) could affect the

worker's behaviour. However, in the two experiments with small artificially prepared colonies, where the conditions were quite different from the ones of a normally developed colony, feedings of long duration were also observed. This rules out this hypothesis.

The second possibility is that the length of feedings varies as a result of the queen's influence. Röseler (1970, 1975, 1976, 1991) suggested that the queen was able to affect the behaviour (and/or the physiology) of the nurses in such a way that no queen larvae could be reared. One might suppose this inhibitory mechanism could also suppress the long-duration feedings at the beginning of colony development (when the queen is still dominant) and the larvae reared would receive shorter feedings. In the third brood, when the queen decreases her pheromone production (or the pheromone loses its effectiveness), she also could reduce her influence on the workers. They could then start to feed old larvae with feedings of longer duration. Although our results suggest this occurs, only studies of the feedings' duration in old colonies with introduced young queens could test the validity of this idea.

The third hypothesis is that the aspects related to the workers themselves would be the cause of long-duration feedings. But our results showed no high correlation values (Tables 1, 2, 3).

At last, the feedings of long duration may be stimulated by the larvae. Thus, one could expect that queen larvae have received feedings with the longest duration (Fig. 1b) because they take longer to develop than worker and male larvae. But when the duration of feedings throughout the development was analysed it was found that the curves for worker larvae and queen larvae were very close to each other (Fig. 2c). Furthermore, the curve for the feeding duration of the male larvae, which have a longer development than worker larvae, was lower than the one of the worker larvae (Fig. 2c).

Alternatively, feedings' duration could be inversely related to feeding frequency. In this way, queen larvae that are fed with a higher frequency than male and worker larvae (Röseler and Röseler, 1974; Ribeiro et al., 1999) could receive shorter feedings. Our data showed the opposite relationship. Moreover, since amount of food and duration of feedings do not show a high correlation (Table 1), an inverse relationship between number and duration of feedings is not likely to occur.

These facts demonstrate that the duration of feedings the larvae receive is not influenced by their total development time or the frequency with which they are fed.

The duration of feedings showed a clear increase with larval age (Fig. 2). Because this result was found for worker, male and queen larvae (though in different degrees) it seems that the duration of feedings is in general related to the age of all larvae. Several experiments by Le Conte et al. (1994) showed that honey bee workers are able to discriminate between young and old larvae because of their different concentration and composition of the brood pheromone. Although a brood pheromone also occurs in bumble bees (Heinrich, 1974; Gamboa et al., 1987), it is not known whether there is a variation in its composition according to the larval age.

The size of the larvae might also be a cause of the long-duration feedings. However, size alone is certainly not the only factor. If this were the case, male larvae would receive longer feedings than worker larvae because they are larger. Our results showed the opposite pattern.

Our data indicate that nurses treat female and male larvae in different ways. Queen and worker larvae received longer feedings than males (Figs.1b, 2c). Workers may be able to recognise the sex of the larvae because the larvae have sex-specific cuticular hydrocarbons (Greenwood, in Fisher and Pomeroy, 1990). Male larvae also present a different feeding pattern throughout the development; it is possible that their metabolism is different (Ribeiro et al., 1999). The nurses possibly respond to these differences by giving the male larvae shorter feedings.

Therefore, the duration of feedings seems to be associated with the sex and age (stage of larval development and/or size) of the larvae and probably with their different requirements at that moment. Thus, long-duration feedings could be related to food quality. In honey bees long-duration feedings are indeed related to high amounts of protein which are present in the larval food given to worker larvae 48 to 84 h old (Brouwers et al., 1987).

Up till now it has been assumed that the food given to all larvae is of identical composition. The first reason is that workers feed larvae of different sex and caste alternately and successively (Katayama, 1975). Secondly, a previous study showed that in the food provided to worker, male and queen larvae the proportions of protein (from pollen and from another source) and carbohydrates (honey or sugar) were equivalent (Pereboom and Shivashankar, 1994; Pereboom, 1996). However, our results showed that the food composition did vary per feeding. Besides controlling the amount of food per larva, workers are possibly able to adjust its composition, too. In stingless bees, it was also suggested that the concentration of these components is one of the sources of the variation in the larval food (Velthuis and Sommeijer, 1991). Thus, different concentrations of pollen grains, sugar and proteins would result in larval food with variable viscosity, which could affect the length of the feedings and the workers' abdominal contractions.

Another possibility is that the duration of feedings is not related at all to the crop contents, but only to the substances produced by the hypopharyngeal glands. Hypopharyngeal glands do not have a reservoir and therefore, it is assumed that their secretion must be released soon after it is produced (Simpson, 1960). Supposing the discharge of these glands take a long time because it has to pass through long ducts (as suggested for honey bees by Brouwers et al., 1987), this could explain the long duration feedings (and why they were not strongly related to any of the variables measured).

The fact that both worker and queen larvae received longer feedings suggests there is no difference in their food composition. But one must bear in mind that if long feedings do mean a different kind of food, queen larvae continue to

receive this food after worker larvae have pupated (because queens have a longer development time).

It is remarkable that about 28% of the samples did not contain pollen (they had no colour). Many of the feedings therefore probably contained only honey (or sugar-water in this case, and maybe other substances) but no pollen. As a consequence, this could decrease the final amount of pollen received by the larvae to values smaller than expected with a uniform food composition. This also might explain the relatively lower amount of pollen grains ingested by queen larvae in spite of the high frequency with which they are fed (Ribeiro, 1994; Ribeiro et al., 1999).

Furthermore, the enzymes (amylase and invertase) produced by the hypopharyngeal glands could help the larvae to digest carbohydrates and the starch of pollen (Palm, 1949) and consequently enhance their development. This could be specially important for queen larvae because they have a chance of receiving larger amounts of these substances, since they have a longer development and a high feeding frequency. In this way they might be able to use a relatively small amount of pollen and still grow very large (Ribeiro, 1994). Simultaneously, the protein provided in the larval food could promote the final large growth of the queens. Thus queen larvae would be "less dependent" on protein of the pollen to grow. Moreover, because pollen walls are not digested and are accumulated in the closed intestines up to the last larval stage (Velthuis, 1991, 1992) queen larvae would avoid a large accumulation of waste and a consequent restraint of development.

Therefore, contrary to what has been supposed previously, the proteins (and digestive enzymes) produced by hypopharyngeal glands and added to the larval food, might play an important role in caste development and differentiation. They could allow queen larvae to grow more efficiently. This could also represent a considerable economy for the colony. In terms of pollen it could be "less expensive" to rear queens.

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