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Research article

Influence of age and caging upon protein metabolism, hypopharyngeal glands and trophallactic behavior in the honey bee (*Apis mellifera* L.)*

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Key words: Apis mellifera, cage condition, hypopharyngeal glands, protein metabolism, food exchange, age.

Summary

Different-aged honey bees were either kept in a cage together with young sisters for eight days or lived in their colony. Following an injection of ^{14}C-phenylalanine (Phe) we measured incorporation of ^{14}C-Phe into head protein and total protein, as well as the size of the hypopharyngeal glands. While confined in a cage for four hours, injected bees (from colony or cage) dispensed the ^{14}C-labelled protein-rich products of their hypopharyngeal glands to recipients. Eight-day-old colony bees had well developed hypopharyngeal glands, whereas at the age of sixteen days the glands had already decreased in size. Young caged bees had smaller hypopharyngeal glands. Colony bees had higher incorporation rates into total protein and head protein than bees living in a cage. Bees of different age classes, irrespective of caging, fed the same number of recipients; but the amount of ^{14}C-labelled protein-rich jelly distributed by caged bees was significantly smaller than that distributed by colony bees. Our results indicate that trophallaxis between young donor workers and newly emerged recipient worker bees is not the key factor for regular development and activity of the hypopharyngeal glands.

Introduction

Social insects with their multiple worker castes are generally admired but occasionally dreaded. The castes perform their activities concurrently and enable colonies to do prodigious amounts of work efficiently (Wilson, 1985).

After honey bees emerge, a worker passes through different stages, defined by special duties within the community (Rösch, 1925, 1930; Lindauer, 1952; Free, 1965; Michener, 1969; Calderone and Page, 1988; Kolmes et al., 1989). The different jobs such as cleaning, producing wax, building combs, rearing brood, or foraging are accompanied by physiological changes in the bees.

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* Dedicated to Achim Lass's daughter Katrin.

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The development of the glands is linked to job performance, for instance depending on whether jelly (= larval food) or wax is synthesized (King, 1933). The hypopharyngeal glands that produce jelly in nurse bees have been investigated thoroughly (Hess, 1942; Maurizio, 1954; Free, 1961; Halberstadt, 1980; Brouwers, 1982; Moritz and Crailsheim, 1987; Takenaka and Kaatz, 1987; Suzuki, 1988; Crailsheim and Stolberg, 1989). The gland's activity is age dependent and closely correlated with worker behavior (reviewed by Wilson, 1971; Michener, 1974).

Estimating the activity of hypopharyngeal glands by an arbitrary classification scale (Maurizio, 1954), by the size of the glands (Hassanein, 1952), or by the total protein content of the glands (Rosça et al., 1972) gives a direct correlation between size and activity. Huang et al. (1989), however, postulated that neither underdeveloped glands nor hypertrophic glands, but rather glands of medium size are more likely to synthesize protein.

In contrast, Hess (1942), Park (1946), Maurizio (1954) and Rosça et al. (1972) propose a correlation between physiology and corresponding behavior depending on age. Usually, the physiological status of body and organs was ignored, and only age was used to estimate the worker bee's duty in the colony.

Suzuki (1988), Crailsheim and Stolberg (1989), Huang (1990) and Kaatz et al. (1990) demonstrated that caging causes a change in the bee's physiology. This approach permits a comparison of colony and caged bee's physiology at the same age.

The experiments described in this paper were intended to investigate the interrelation of trophallactic behavior and protein metabolism. Taking in consideration that the bee's environment can influence glandular activity (i.e., requirement of tending brood or hive mates), we asked whether confinement in a cage might inhibit glandular development and activity, even if just-emerged bees were also present in the cage. In addition, the ability and willingness of bees to share proteinaceous food under caged conditions were tested.

Material and methods

The experiments were performed from July 20th to August 21st, 1992. Bees (*Apis mellifera carnica* POLLM.) were kept queen right in large commercial hives with 20 frames. In all experiments it was necessary to know the exact age of the bees. Therefore bees emerging in an incubator at 34 °C were colour-coded with a pigment dot and returned to their colony.

Polystyrol dishes (Greiner) were used as experimental cages (Fig. 1). They were separated into two unequal areas by a modified piece of a queen excluder. Both fields were provided with two small bowls for honey and water. Only one field was equipped with a small bowl for bee-collected pollen. Bees confined in these containers were reared in the dark at 33 °C and relative humidity of 60 to 70%.

Each experimental cage was provided with one donor bee and eight recipient bees. The caged donor bee was allowed to move around freely and consume water, honey and pollen. All recipient bees were marked with small plastic rods, fixed to their thorax with PATTEX adhesive (Henkel). These allowed them to move within their given field but not to pass the queen excluder. Thus they could consume only

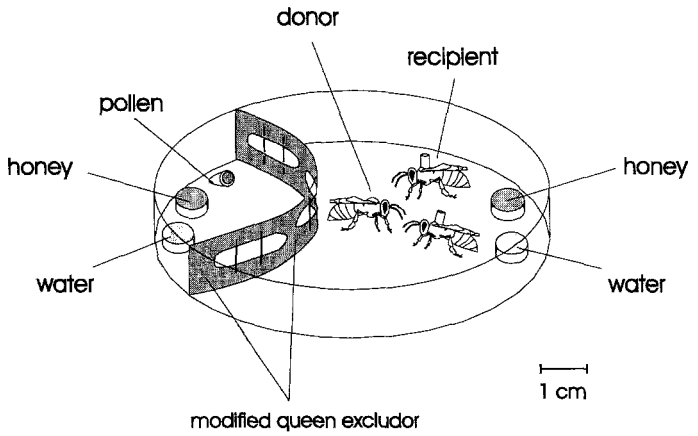

Figure 1. Scheme of cages used

water and honey. The average age of recipient bees was about zero to one day (baby bees). In one series of experiments we also used foragers with a defined status as recipients; these bees were caught at an artificial feeding place.

Bees of different age were caged as donors for eight or six days and their recipients were replaced every second day. One-day-old bees were either caged for eight days or returned to their hive after colour coding. Eight-day-old bees and twenty-day-old bees stayed first in the hive and were then removed and caged for eight and six further days. After the cage period donor bees as well as their hived sisters of the same age were then tested for protein synthesis and trophallactic behavior as described below.

Age classes of caged bees used as donor bees:

- one-day-old bees were caged for eight days, final age *eight* days;
- eight-day-old bees were caged for eight further days, final age *sixteen* days;
- twenty-day-old bees were caged for six further days, final age *twenty six* days;

The bees in the control group were removed from the bee hive around noon at the same time as the bees in the experimental caged group; they were sisters of the caged bees.

Age classes of uncaged bees used as donor bees:

- ***eight, sixteen, twenty*** and ***twenty-six*** days;
  (corresponding to the ages of caged bees before and after their experimental [cage] period [except newly emerged bees]).

All the age classes are given as the minimum age and could be one day more at most.

To determine protein synthesis and trophallactic habit of bees, ^{14}C-phenylalanine was used. Donor bees (uncaged bees and bees after their cage period) were fixed to a piece of wood with two crossed needles. In this position they were injected with 1 µl ^{14}C-phenylalanine (from Amersham, 1.85 MBq/ml, 18.98 GBq/mmol) with a Hamilton microsyringe dorsally between the 5th and 6th abdominal segments. If

haemolymph was squeezed out after the needle was removed, it was blotted gently with a strip of filter paper. This haemolymph was then tested for its ^{14}C content. The result had to be taken into account to calculate the amount of ^{14}C-Phe that was actually injected into the bee and was available for incorporation. Then the wound was sealed with a drop of a warm micture of beeswax and collophonium. Bees stayed in this fixed position for 120 minutes at room conditions.

After injection of ^{14}C-phenylalanine and the 120-minute incorporation period, the donor bee (which either had been caged for the preceding eight days or had just been removed from hive) was caged with eight recipient bees (baby bees or foragers) for four hours. Then the experiment was terminated and the bees were frozen at – 18 °C.

To obtain the hypopharyngeal glands, the caput of the donor bee was separated from the thorax and fixed to a piece of wood with needles. The caput was so cut with scissors that the back half of the caput could be removed. The inside of the head was then moistened with a drop of isotonic (0.5 M) glucose solution. The hypopharyngeal glands were removed with forceps. The acini diameters were measured with a microscope. We chose eight neighbouring acini at one end of the hypopharyneal gland.

To determine the bee's protein synthesis, it was divided into caput, thorax and abdomen. Each part was cut into pieces, placed in 0.3 ml water, homogenized ultrasonifically and mixed with 1.7 ml 96% ethanol. Samples were incubated at 60 °C for 20 min, cooled to 4 °C and centrifuged (2750 g; 5 min). The ^{14}C content was determined in an aliquot of the supernatant. The amount found in those fractions was defined as ^{14}C of free phenylalanine.

The precipitate was washed twice with ethanol, and solubilized in 0.5 ml 2 M NaOH for 1 h at 60 °C. After neutralization with HCl, the sample was mixed with 1.5 ml 96% ethanol and centrifuged (2750 g; 5 min). The amount of ^{14}C found in this fraction was defined as having been incorporated into protein. If those bees were caged with recipients after the injection, the ^{14}C-activity found in the recipients was calculated as protein bound ^{14}C in the head compartment of the donor for the calculation of total bound radioactivity.

To determine proteinaceous jelly transmitted by the donors to recipients ("distributed" jelly), the ^{14}C content of recipients was investigated. Single recipients were frozen with liquid nitrogen and macerated with a glass rod. They were placed in 2 ml 1.5 M NaOH and were incubated at 60 °C for 60 min. After neutralization with HCl, the sample was filled to 3 ml with distilled water. The amount of ^{14}C found in this fraction was defined as "distributed" and considered as protein-bound ^{14}C of the donor bees. This assumption was based on a previous study by Crailsheim (1990), who found that ~75% of distributed ^{14}C found in the recipients was protein bound and that immediately after a trophallactic feeding ^{14}C labelled protein was found in the crop of the recipients.

All ^{14}C-determinations were done with Hydroluma (a liquid scintillation cocktail, from Baker) in a Packard scintillation analyser (1900 CA Tri carb).

From the calculated amount of ^{14}C available for incorporation, the proportional value of protein-bound activity could be defined. In addition, the proportional value of protein-bound activity in the caput could be determined apart from the total amount of protein-bound activity in a donor bee's body.

When ^{14}C could be identified in a recipient bee, this was an indication that the recipient had been fed with food containing ^{14}C-marked proteins by the donor. All measurements of the activity of recipients that resulted in a value more than 50 DPM (decay per minute) were taken into account. As the values measured in a recipient bee must be seen in relation to the total amount of activity in the donor bee, the distributed amount was given as a proportinal value of activity available in the donor bee.

When we calculated the amount of protein-bound activity for a donor bee we added all activity found in her recipients to the fraction of the donor's head or when calculated for the total protein, to the total protein.

Statistical analysis

Means and standard deviation (n = 8–12) are given in the figures. Differences were analysed for statistical significance using nonparametric tests. Mann and Whitney test was used for comparisons between colony bees and caged bees and Wilcoxon test for comparisons within the same group.

Results

Size of the hypopharyngeal glands

The diameter of the acini of the hypopharyngeal glands decreased significantly in colony bees between the age of eight and twenty-six days (Fig. 2). In contrast, acini diameters from caged bees eight days old were smaller than those of caged bees sixteen and twenty-six days old. There were significant differences in the size of the acini when glands of caged bees at their eighth and sixteenth days of life were compared with glands of colony bees. No difference could be found in the diameters of acini in colony bees and in caged bees aged twenty-six days (Fig. 2)

Incorporation of phenylalanine into protein

The incorporation of ^{14}C phenylalanine into the protein of colony bees decreased slightly with increasing age of the bees. Hive bees showed a significantly higher rate of incorporation than caged bees (Fig. 3A). The incorporation rate of caged bees droped increasingly and significantly with age (Fig. 3A).

Two hours after injection of ^{14}C-phenylalanine, about 40% of the radioactivity was found in the protein fraction in the heads of nurses (8 days, living in a colony) (Fig. 3B). It was previously shown in laboratory experiments that ~ 50% of this activity could be found in the hypopharyngeal glands (Crailsheim, 1990).

The ^{14}C in the heads of colony bees showed no statistically significant decrease with advancing age with reference to the total amount of protein-bound activity (Fig. 3B). Nevertheless, the higher activity in the heads of colony bees at eight days in comparison to the activity in the heads of colony bees at the twenty-sixth day of life was significant. (Not illustrated in Fig. 3B.)

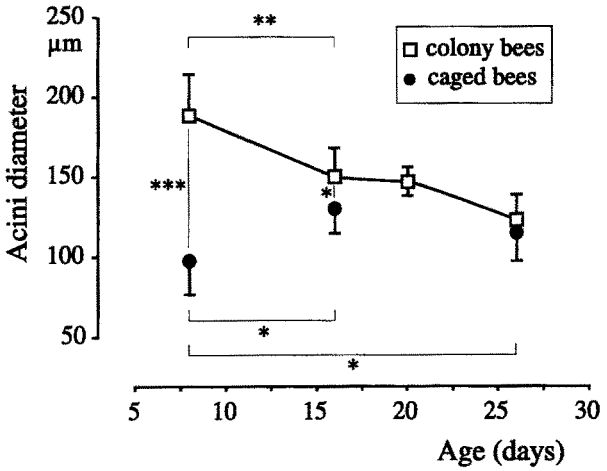

Figure 2. Acini diameter of the hypopharyngeal glands of bees depending on age and keeping conditins. Eight- and 16-days caged bees were caged for 8 days, those 26 days old for 6 days. * indicates significant differences. * $0.01 < p < 0.05$, ** $0.001 < p < 0.01$, *** $p < 0.001$

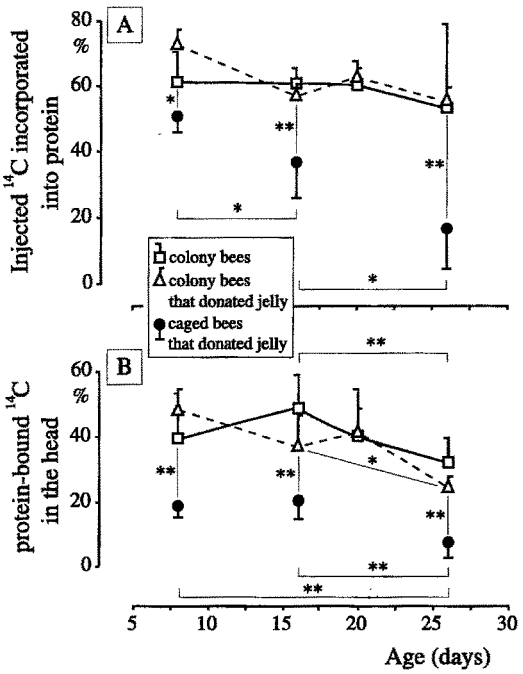

Figure 3. Percentage of ^{14}C incorporated into total protein (A) and percentage of ^{14}C incorporated in the head (B) of donor bees that had lived in the colony or in a cage. All donors were caged for four hours after injection and bees symbolized by triangles and circles were kept with baby bees as recipients. The ^{14}C that was found in the recipients was calculated as part of the protein fraction of the donor's heads. * indicates significant differences. * $0.01 < p < 0.05$, ** $0.001 < p < 0.01$, *** $p < 0.001$

Caged bees of the same age always showed a significantly lower incorporation rate in the head in comparison to the rate for colony bees. Caged bees showed little dependence between incorporation rate into the head and advancing age (Fig. 3B).

A difference between "colony bees" and "jelly-distributing colony bees" was not noticeable, neither regarding the incorportion rate into protein nor regarding the incorporation rate in the head (Fig. 3A, 3B).

Trophallactic parameters

Bees that had already been caged for six or eight days showed a tendency to feed smaller numbers of recipients than colony bees. Eight-day-old caged bees showed a significantly higher feeding rate than older caged bees (Fig. 4).

Bees injected after being taken from the colony always fed significantly (factor of 5) more ^{14}C to their recipients than their same-aged caged sisters. Eight-day-old bees, whether caged or not, did more feeding than older bees; this was significant at least for the oldest group (twenty-six days) (Fig. 5).

Foragers as recipients:

When we caged donors from day eight to sixteen together with foragers instead of baby bees as recipients, we obtained the same results as with the baby-bee recipients (frequency of feeding and amount of transferred ^{14}C; data are not shown).

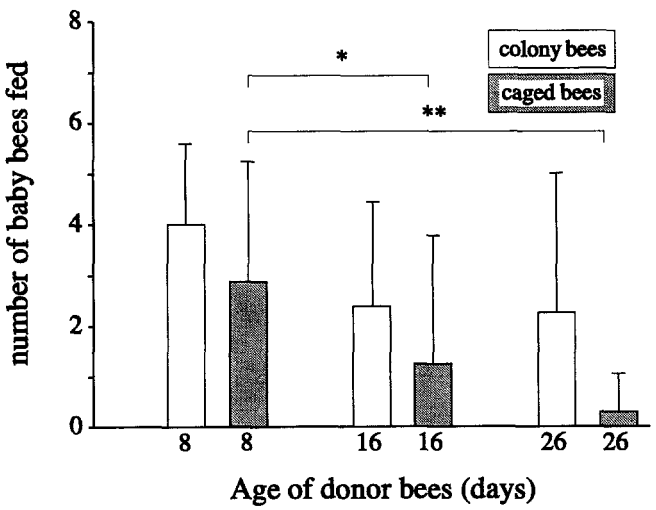

Figure 4. Number of baby bees that received ^{14}C from donors of different ages that had lived in the hive or in a cage. All donors were caged with recipients (eight baby bees) for four hours after injection. * indicates significant differences. * $0.01 < p < 0.05$, ** $0.001 < p < 0.01$, *** $p < 0.001$

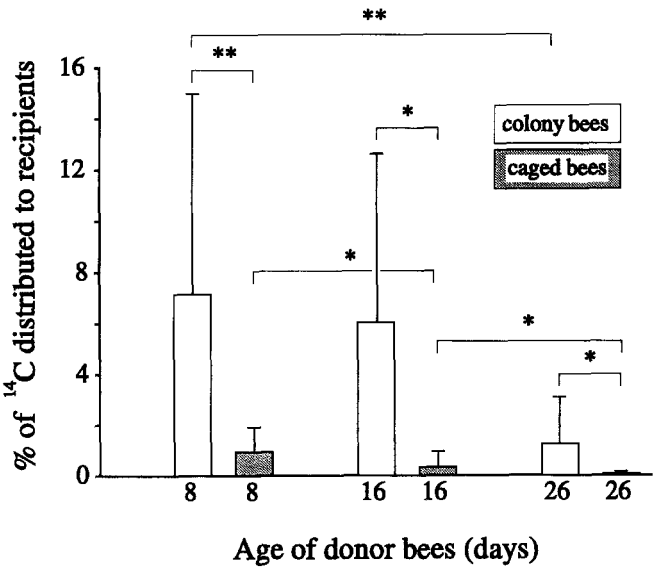

Figure 5. Percentage of ^{14}C "distributed" by the injected donors to baby bees depending on age. Donors had lived in the colony or in a cage with young sisters. All donors were caged with recipients (eight baby bees) for four hours after injection. "Distributed" ^{14}C was related to the amount of ^{14}C-Phe that was injected into donors. * indicates significant differences. * $0.01 < p < 0.05$, ** $0.001 < p < 0.01$, *** $p < 0.001$

Discussion

Hypopharyngeal gland development

It is well established in the literature that young bees that mostly stay in the hive, consume pollen and usually feed larvae, have enlarged hypopharyngeal glands (Hess, 1942; Maurizio, 1954; Moritz and Crailsheim, 1987; Suzuki, 1988). Foragers that leave their hive frequently to collect food have reduced acini diameters (Fluri et al., 1982; Crailsheim and Stolberg, 1989; Suzuki, 1988). According to Moritz and Crailsheim (1987) and Suzuki (1988) these acini diameters are comparable with acinis of just emerged bees.

In this study we also observed the typical reduction of hypopharyngeal glands in colony bees between the ages of eight and twenty-six days. In contrast to them, glands of caged bees of the same age were either underdeveloped (eight days old) or showed more pronounced reduction (age sixteen) (Fig. 2). Similarly under developed glands are described by Crailsheim and Stolberg (1989), who caged fifty just-emerged bees for eight days. In agreement with Figure 2 they also found that those glands were actually smaller than the reduced glands in foragers living in a colony. Interestingly, in our study the presence of recipients, who had no opportunity to consume protein other than that provided by the donors, did not lead to normal development of the donor's glands.

Incorporation of ^{14}C-phenylalanine into protein

The incorportion rate of injected ^{14}C-Phe into protein can be used as an indicator of protein synthesis (Crailsheim, 1990). The injected amount of ^{14}C-phenylalanine in this study was ~ $21.6 \cdot 10^{-2}$ µg which is far less than one percent of the phenylalanine pool in the haemolymph (1.7 µg and 3.7 µg per bee, respectively; Wang and Moeller, 1970; Sinitzki and Lewtschenko, 1971). Nevertheless we have to consider that the phenylalanine pool in the haemolymph of young bees is much higher (double) than in foragers (Sinitzki and Lewtschenko, 1971) while the amount of ^{14}C-phenylalanine injected in our experiments is constant. Because of the relation of nonradioactive phenylalanine in the bee to injected ^{14}C-phenylalanine in the haemolymph, the similar incorporation rate of ^{14}C-phenylalanine in younger and older bees means a higher incorporation of phenylalanine for the younger ones. Thus any decrease of incorporation of ^{14}C with age would be stronger when so calculated.

Actually we found only a very slight decrease in the incorporation of ^{14}C into protein in colony bees with age (Fig. 3A). This can be compared with the results of Crailsheim (1990) using ^{14}C leucine as tracer. In contrast to colony bees, caged bees showed a drastic decrease in incorporation rates of ^{14}C-phenylalanine into the body protein (Fig. 3A), indicating a stronger influence of caging on bees when older. Obviously this did not correlate with the size of the hypopharyngeal gland of caged bees (compare Fig. 3A with Fig. 2).

It is surprising that the underdeveloped glands (Fig. 2) of caged bees eight days old are significantly more active (Fig. 3B) than larger glands in caged foragers (twenty-six days old). This again indicates a greater effect of caging upon older bees, but might also suggest that the acini size of hypopharyngeal glands under non-hive conditions does not necessarily reflect glandular activity. This was also shown for extraordinary hive conditions by Huang (1990) and for winter bees by Brouwers (1982). The latter showed that winter bees' glands remained hypertrophied but with reduced activity. Meanwhile, Huang and Otis (1989) suggested that neither enlarged glands nor reduced glands but rather glands with medium-size acini are most active. In any case, hypopharyngeal glands produce not only jelly but also special proteins like invertase (Maurizio, 1962; Simpson et al., 1968; Halberstadt, 1970) and saccharase. Thus synthesis rates cannot simply be associated with jelly production. Halberstadt (1980) postulated two phases of hypopharyngeal glandular activity: a period of larval food production, and of enzyme production. Kaatz and Takenaka (1987) and Knecht and Kaatz (1990) observed a presumably functional differentiation in the pattern of synthesis: the production of new forager-specific protein begins without a loss of capability to produce larval food proteins.

Glandular activity must vary to suit the bee's requirements (occupational duties) at different ages. The polyethism of bees has been well described by Rösch (1925), Perepelova (1928), Lindauer (1952) and Sakagami (1953). Park (1946) showed that different functions correspond to the bee's physiological age. This physiological age depends on the juvenile hormone titre (see Rutz et al., 1976; Rutz et al., 1977; Robinson, 1985) and this titre is influenced by the queen's pheromone (Kaatz et al., 1990).

With the differences in gland sizes, caging reduces protein synthesis both for total protein and for head protein as well. The presence of a limited number of recipients (i.e. baby bees or foragers) that depended on the donor's jelly did not lead to normal development but protein synthesis nonetheless remained at a higher level in the two younger groups in comparison to that of twenty-six-day-old caged donors, when the smaller volume of the head is considered (5% of body weight but 20% of ^{14}C was incorporated).

Trophallactic behavior

Bees are more likely to transfer food to bees of about the same age (Free, 1957). Moritz and Hallmen (1986) observed that the number of recipients fed decreases with the age of the donor bee. Figure 4 shows the latter effect, which is equally true for colony bees and caged bees (recipients are baby bees). Although the donor bees had different backgrounds (kept in the colony or in a cage) (compare Fig. 2, Fig. 3 A, 3 B) their trophallactic behaviors were similar regarding the number of recipients fed (Fig. 4). Therefore the trophallactic activity of caged bees seems to be less influenced by the caging condition. On the other hand the insignificant finding that all caged age classes fed a lesser number of recipients (Fig. 4) might be caused by the chosen threshold of 50 DPM per recipient as the amount of transferred ^{14}C is much smaller (Fig. 5) in the group of caged donors.

It seems to be contradictory that as compared to caged bees at sixteen days, caged bees eight days old distributed (significantly) more ^{14}C linked to jelly protein (Fig. 5), whereby their acini diameter were (significantly) smaller (Fig. 2), while their glandular synthesis activity was similar (Fig. 3 B). An explanation for this might be the (significantly) higher number of baby bees that were fed by caged bees aged eight days. This more pronounced donating activity might stimulate glandular activity.

Caging with regular food supplementation and the necessity to produce jelly (as demanded by the protein-deprived recipients) caused almost unchanged trophallactic behavior but drastically changed the ability to produce and to transfer jelly. Thus glandular activity is not directly triggered by a limited number of protein demanding recipients and these recipients are very likely not the key element in the regulation of food production. Further experiments at the colony level and cage experiments with more natural components as for instance differently aged recipients or brood will give more differentiated results.

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