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ORIGINAL RESEARCH ARTICLE

Physiological susceptibility and hygienic behaviour affect chalkbrood disease incidence in worker and drone larvae in honey bees (*Apis mellifera* L.)

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Summary

Chalkbrood disease is a mycosis of honey bee (*Apis mellifera*) larvae caused by the heterothallic fungus *Ascophaera apis*. In infected colonies it is often found that drone larvae are more affected than worker larvae and this difference is attributed to the higher probability of chilling of drone brood. Given the great differences found between worker and drone larvae, however, it would be expected to find differential physiological resistance to chalkbrood disease. Additionally, if hygienic behaviour of adult bees (uncapping of cells containing dead brood and its subsequent removal) is expressed differently in worker and drone cells, it may differentially affect the proportion of mummified larvae of each sex. The goal of this research was to determine how physiological resistance and hygienic behaviour affect chalkbrood incidence in worker and drone larvae. Firstly, the proportion of worker and drone larvae mummified after receiving food containing *A. apis* spores was determined. Then hygienic behaviour in relation to dead brood of both sexes was evaluated. It was found that drone larvae mummify and have fruiting bodies in a greater proportion than worker larvae and that hygienic behaviour is more efficient in relation to worker rather than drone cells. These results indicate that physiological susceptibility and hygienic behaviour are two factors that could explain satisfactorily the greater proportion of mummified drones found in diseased colonies.

La susceptibilidad fisiológica y el comportamiento higiénico afectan la incidencia de la cría yesificada en larvas de obreras y zánganos en las abejas melíferas (*Apis mellifera* L.)

Resumen

La cría yesificada es una micosis de las larvas de las abejas melíferas (*Apis mellifera*) causada por el hongo *Ascosphaera apis*. En colonias enfermas se suele encontrar que las larvas de zánganos son más afectadas que las de obreras atribuyéndose esta diferencia a la mayor probabilidad de enfriamiento de la cría de zánganos. Sin embargo, ante las marcadas diferencias entre las larvas de obreras y zánganos, cabría esperar encontrar resistencia fisiológica diferencial a la cría yesificada. Por otro lado, si el comportamiento higiénico de las abejas adultas (desoperculado de las celdas que contienen crías muertas y su posterior remoción) se expresa diferente frente a la cría de obreras y zánganos, éste podría afectar diferencialmente la proporción de larvas momificadas de cada sexo. El objetivo de esta investigación fue determinar cómo la resistencia fisiológica y el comportamiento higiénico afectan la incidencia de la cría yesificada en las larvas de obreras y zánganos. Para ello, primeramente se determinó la proporción de larvas de obreras y zánganos momificadas luego de recibir alimento conteniendo esporas de *A. apis*. Posteriormente se evaluó el comportamiento higiénico de las abejas frente a cría muerta de ambos sexos. Se encontró que las larvas de zánganos momifican y presentan cuerpos fructíferos en mayor proporción que las larvas de obreras y que el comportamiento higiénico de las abejas es más eficiente frente a la cría muerta de obreras que de zánganos. Estos resultados indican que la

susceptibilidad fisiológica y el comportamiento higiénico son dos factores que podrían explicar satisfactoriamente la mayor proporción de zánganos momificados encontrados en colonias enfermas.

Keywords: *Apis mellifera*, chalkbrood, *Ascosphaera apis*, resistance, hygienic behaviour, haploid susceptibility

Introduction

Ascosphaeriosis or chalkbrood disease is an infection of honey bee (*Apis mellifera* L.) larvae caused by the heterothallic fungus *Ascosphaera apis* (Olive and Spiltoir). The fungal spores enter the larvae through food and germinate at the distal end of the gut when cells are capped (8-9 days old). The mycelia expand fast, break through the peritrophic membrane, and three days later reach the larval surface continuing their aerial growth. Affected larvae become a dry and hard structure (mummy) keeping the white colour of mycelium or acquiring a grey-white colour if fruiting bodies are formed (Bailey and Ball, 1991; Gilliam and Vandenberg, 1997; Aronstein and Murray, 2010).

According to Bailey and Ball (1991), worker larvae are more susceptible to chalkbrood disease when they take in food with *A. apis* spores between the third and fourth day of age and suffer chilling two days later, immediately after their cells are capped. Puerta *et al*. (1994) and Flores *et al*. (1996) confirm that brood chilling, at least for a few hours, is essential for larvae to mummify, although it may occur 24 hours before or after cells are capped. Chalkbrood disease occurs mainly in spring when, due to increased egg laying of the queen, the relationship between adult bees and brood decreases, increasing the probability that larva become chilled (Bailey and Ball, 1991). Given this, Bailey and Ball (1991) suggest that most affected brood is in the periphery of the breeding area, especially drone brood which is usually located in that area.

Worker and drone larvae differ besides their location within the nest, in size, in anatomical and physiological characteristics, in nutritional requirements, in rate of growth, in cycle duration, in cell size and in ploidy (Snodgrass, 1956; Winston, 1987; Hrassnigg and Crailsheim, 2005). Based on all these aspects it would be expected that worker and drone larvae exhibit differential resistance to *A. apis* regardless of the likelihood of chilling. This kind of difference between castes has been clearly confirmed in honey bees for at least two well known parasites. For example, drone larvae are more resistant than worker or queen larvae to *Paenibacillus larvae* spores, the bacterium that causes American foulbrood, possibly due to different diets between the castes (Rinderer and Rothenbhuler, 1969). In contrast, drone brood is parasitized by the ectoparasitic mite *Varroa destructor* much more than worker brood for reasons that are not yet determined (Trouiller *et al*., 1992; Boot *et al*., 1995; Calderone and Keunen, 2001, 2003).

Honey bee hygienic behaviour (uncapping of cells that contain dead brood and its subsequent removal) constitutes a good mechanism of resistance to brood diseases, including chalkbrood disease (Gilliam *et al*., 1988; Spivak and Gilliam, 1993; Spivak and Reuter, 1998; Palacio *et al*., 2010; Invernizzi *et al*., 2011). Thus, this behaviour could also be affecting the differential manifestation of chalkbrood disease symptoms. If workers express hygienic behaviour differentially in relation to worker and drone cells, the proportions of visible mummies of both sexes will be affected. The fact that drone brood is larger and develops in larger marginal cells with thicker opercula than workers, suggest that worker hygienic behaviour will be less efficient on drone cells than on worker cells. The aim of this study was therefore to analyse worker and drone larval susceptibility to chalkbrood disease and to evaluate worker hygienic behaviour on cells of both castes.

Materials and methods

Trials were carried out from December to February (Austral Summer) using colonies of hybrid bees in Langstroth hives originated from crossings between *A. m. mellifera* with *A. m. scutellata* (Diniz *et al*., 2003; Collet *et al*., 2006). None of the colonies used presented chalkbrood disease symptoms. To obtain a large number of drone larvae, a small frame (12 cm deep) was put in the brood chamber of each colony to make the bees construct drone comb. This technique is frequently used to attract and then eliminate the parasitic mite *V. destructor*, using mites' preference to parasite drone brood (Charriére *et al*., 2003).

Susceptibility of worker and drone larvae to *A. apis*

To test the susceptibility of worker and drone larvae to *A. apis*, two trials were done varying the way in which larva were supplied with *A. apis.*

Massive contamination of colonies with *A. apis*

In these massive contamination trials, five colonies of *A. mellifera* were used. From each colony, a comb with worker brood and a comb with drone brood were removed. An area of cells containing larvae younger than two days was defined on each comb, taking care that both castes were of the same age. These combs were placed together at the centre of the brood chamber. When brood was between the third and fourth day of age, colonies were fed with syrup containing *A. apis* using two external feeders of 0.5 l containing 15 white and 15 black mummies blended in a 1:1 (weigth:volume) sugar and water syrup. Within the next 24 hours after cell capping (5-6-day-old larvae)

the combs with worker and drone brood were removed from the hive and put into an incubator at 30ºC for three to four days. Cells were then manually uncapped recording the number of healthy and mummified larvae. The presence and absence of fruiting bodies in mummified larvae was also recorded.

Individual feeding of larvae with *A. apis*

The individual feeding trials were done using seven colonies. Combs containing worker and drone larvae of the same age were placed at the centre of the brood chamber. When larvae were approximately 5 days old and their cells were close to being capped, they were individually fed with 10 µl of an *A. apis* spore suspension (water and honey in a 3:2 volume proportions). Larvae from colonies No 1, 2 and 3 received 3.76 x 10^5 spores, while larvae from colonies No 4, 5, 6 and 7 received 5.56 x 10^5 spores. Combs were returned to the colonies to be withdrawn the next day, with experimental cells already capped, and put into an incubator at 30ºC for three to four days. Healthy larvae and mummies were recorded as before.

Hygienic behaviour in worker and drone cells

To assess bee hygienic behaviour in worker and drone cells simultaneously, prepupae (younger than 13 days) of both types of brood were killed by stabbing an entomological pin through the operculum (Newton and Ostasiewski, 1986). Combs containing the experimental brood (normally two) were then placed in the centre of the brood chamber. Twenty four hours later capped cells, clean cells and cells with brood remains (without taking into account the size of these remains) were counted. From these records the Uncapping Rate (UR) and the Cleaning Rate (CR) were defined as follows:

$$UR(\%) = \frac{\text{(Initial No. of capped cells - Final No. of capped cells)}}{\text{Initial No. of capped cells}} \times 100$$

$$CR(\%) = \frac{\text{Final No. of clean cells}}{\text{Initial No. of capped cells}} \times 100$$

Two groups of colonies were used, differentiated by the location of drone cells employed in the hygienic behaviour tests. In the first group of 12 colonies, one test per colony was conducted using drone cells built naturally in the margins of the combs. The number of worker experimental cells ranged between 79 and 122 (104.4 ± s.e. 13.0) and that of drones between 28 and 120 (70.0 ± s.e. 31.1). In almost all hygienic behaviour tests, the number of drone cells used was lower than that of workers. This was due to the low presence of drone cells clustered on the comb margins.

In the second group of seven colonies, 19 hygienic behaviour tests were done (1-5 tests per colony) choosing drone cells located centrally (again using a comb with drone cells built under the bar of a small frame). The number of worker experimental cells ranged between 77 and 188 (114.3 ± s.e. 27.5) and that of drones between 33 and 139 (86.4 ± s.e. 27.9).

Statistical analysis

Susceptibility of worker and drone larvae to *A. apis*, and hygienic behaviour in worker and drone cells, were both analysed in each colony using the G test with the Yates correction for continuity in a 2 x 2 contingency table (Zar, 1997).

Results

Susceptibility of worker and drone larvae to *A. apis*

Massive contamination of colonies with *A. apis*

In response to the feeding of colonies with syrup containing *A. apis,* drone larvae mummified in a higher proportion than those of workers in all colonies tested (Fig. 1a). There were also differences between sexes in the proportion of mummies that had fruiting bodies, being higher in drone brood. Only mummies of colony No. 1 did not show this trend (Fig. 1b).

Individual feeding of larvae with *A. apis*

After supplying drone and worker larvae with the same amount of *A. apis* spores, the proportion of total mummification was lower than in the previous experiment (21.5 and 4.8% compared to 61.1 and 24.7% for drones and workers, respectively). Nevertheless, four of the seven colonies showed the trend of a higher mummification in drone larvae; in the other colonies there was no difference (Fig. 2). The number of drone and worker mummies that presented fruiting bodies was very low in each colony (1-12 drone mummies, 0-3 worker mummies), so it was not possible to analyse the data statistically (not shown).

Hygienic behaviour in worker and drone cells

In most colonies, the trend was to preferentially uncap and clean cells containing dead workers rather than dead drones, regardless the location of drone cells in the breeding nest (Fig. 3a, b; Fig. 4a, b). In tests in which drone brood was located in comb margins, six of the 12 colonies showed a higher UR in worker cells than in drone cells; in the remaining colonies there were no differences (Fig. 3a). Eight colonies showed a higher CR in worker cells than in drone cells, in three colonies there was no difference, and in just one, bees preferably cleaned drone cells (Fig. 3b).

In the seven colonies with drone cells located centrally in the brood nest (19 tests), UR was higher in worker cells in 11 tests, in five tests there was no difference and in three tests workers uncapped more drone cells than worker cells (Fig. 4a). Regarding the CR, in 13 tests bees cleaned more worker cells than drone cells, in three tests there was no difference, and in four tests drone cells were preferably cleaned (Fig. 4b).

Fig. 1. a. Proportion of drone and worker larvae that mummified after colonies were massively supplied with food contaminated with *A. apis.* Asterisks indicate significant differences for G test. **: P < 0.01; ***: P < 0.001.

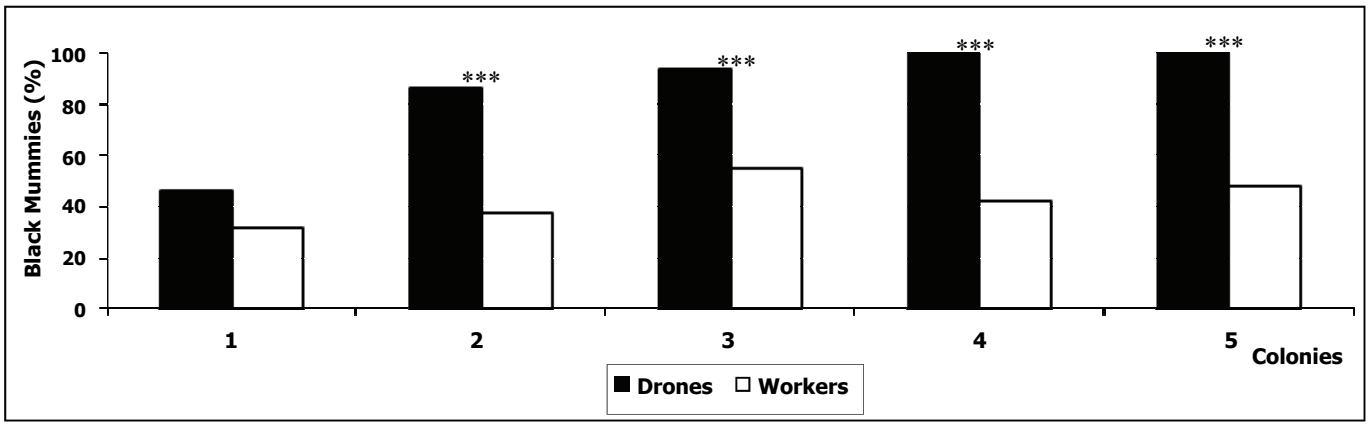

Fig. 1. b. Proportion of drone and worker mummies that presented fruiting bodies after colonies were massively supplied with food contaminated with *A. apis*. Asterisks indicate significant differences for G test. **: P < 0.01; ***: P < 0.001.

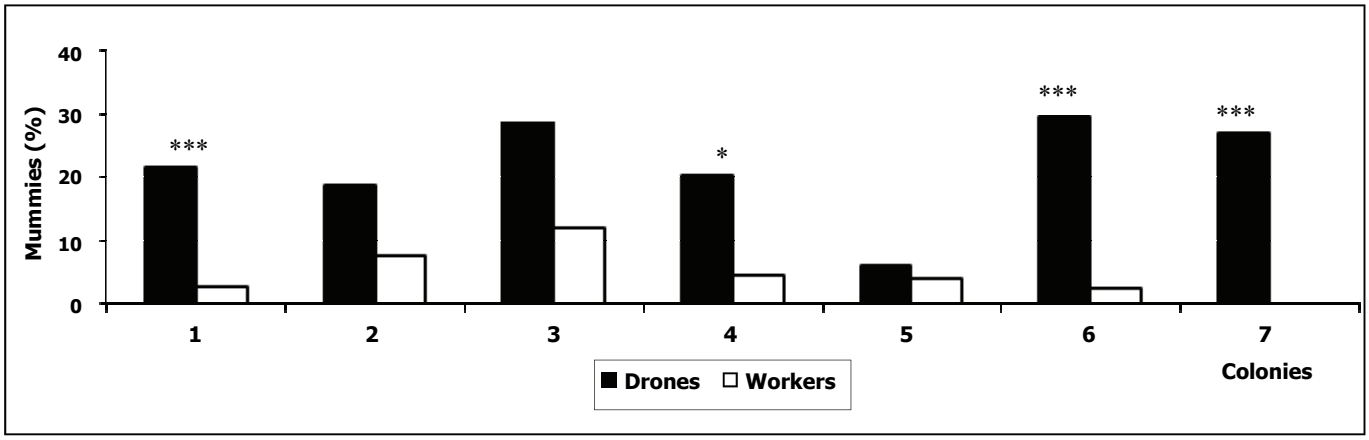

Fig. 2. Proportion of drone and worker larvae that mummified after supplying larvae individually with food contaminated with *A. apis.* Asterisks indicate significant differences for G test. *: P<0.05; **: P < 0.01; ***: P < 0.001.

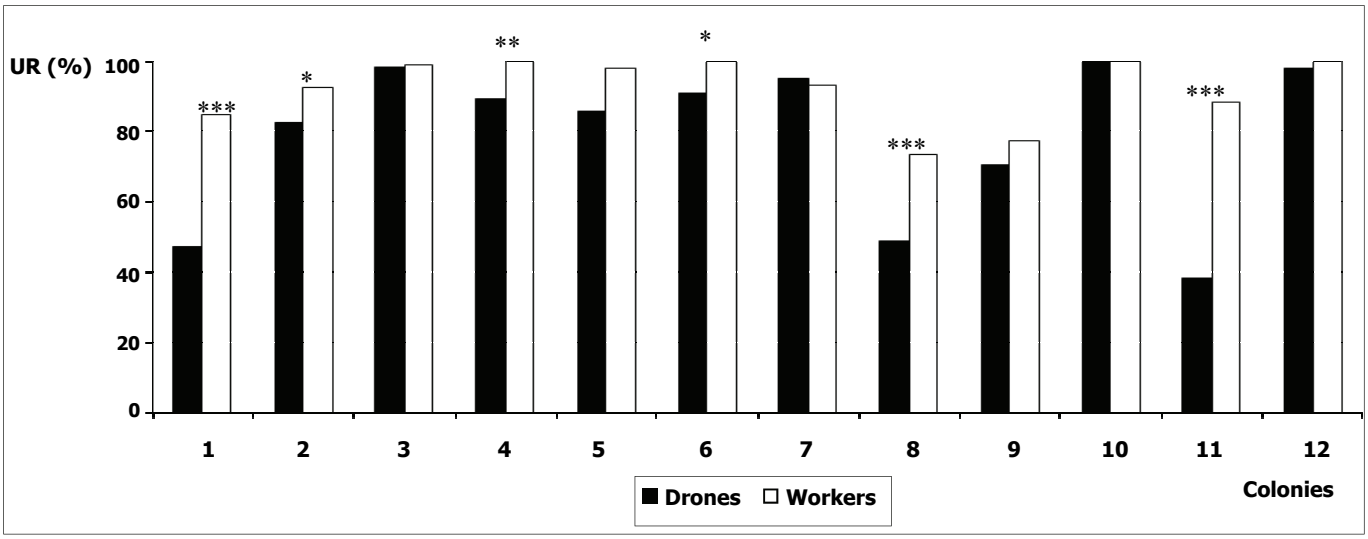

Fig. 3. a. Uncapping Rate (UR) values in drone and worker cells with drone cells located in the margins of the brood area. Asterisks indicate significant differences for G test. *: P < 0.05; **: P < 0.01; ***: P < 0.001.

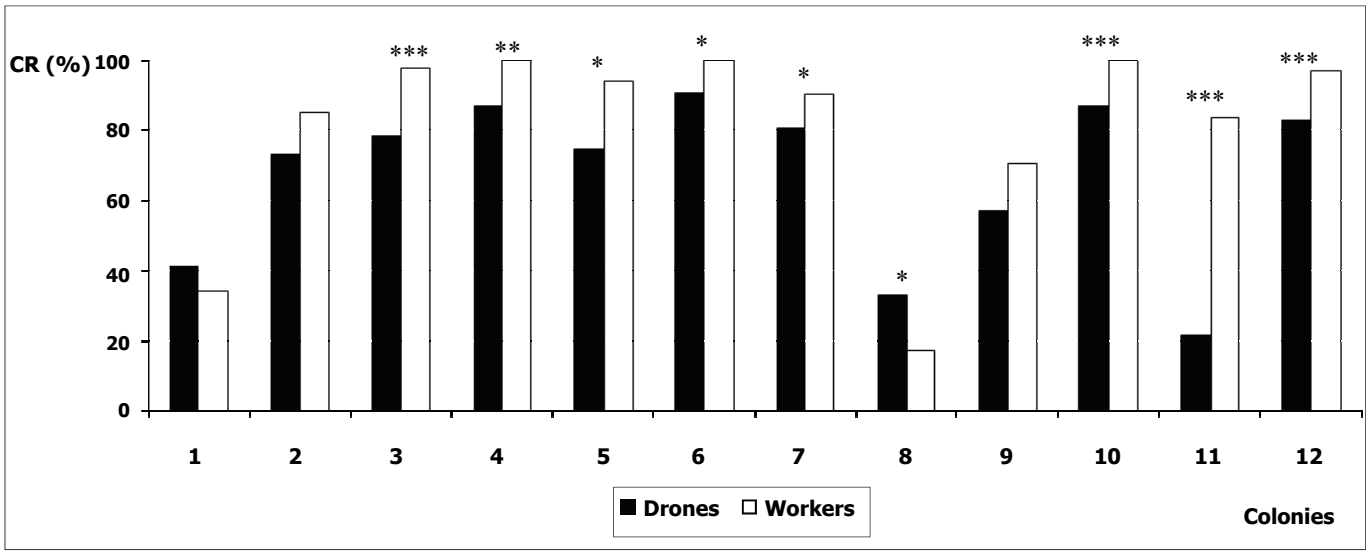

Fig. 3. b. Cleaning Rate (CR) values in drone and worker cells with drone cells located in the margins of the brood area. Asterisks indicate significant differences for G test. *: P < 0.05; **: P < 0.01; ***: P < 0.001.

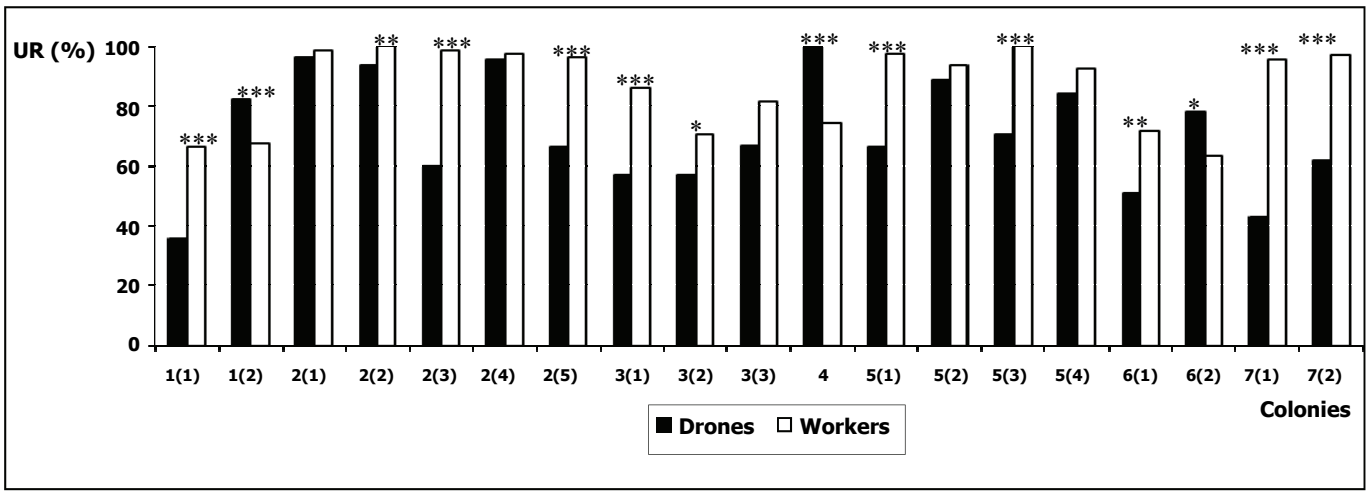

Fig. 4. a. Uncapping Rate (UR) values in drone and worker cells with drone cells located in the centre of the brood area. Asterisks indicate significant differences for G test. *: P < 0.05; **: P < 0.01; ***: P < 0.001.

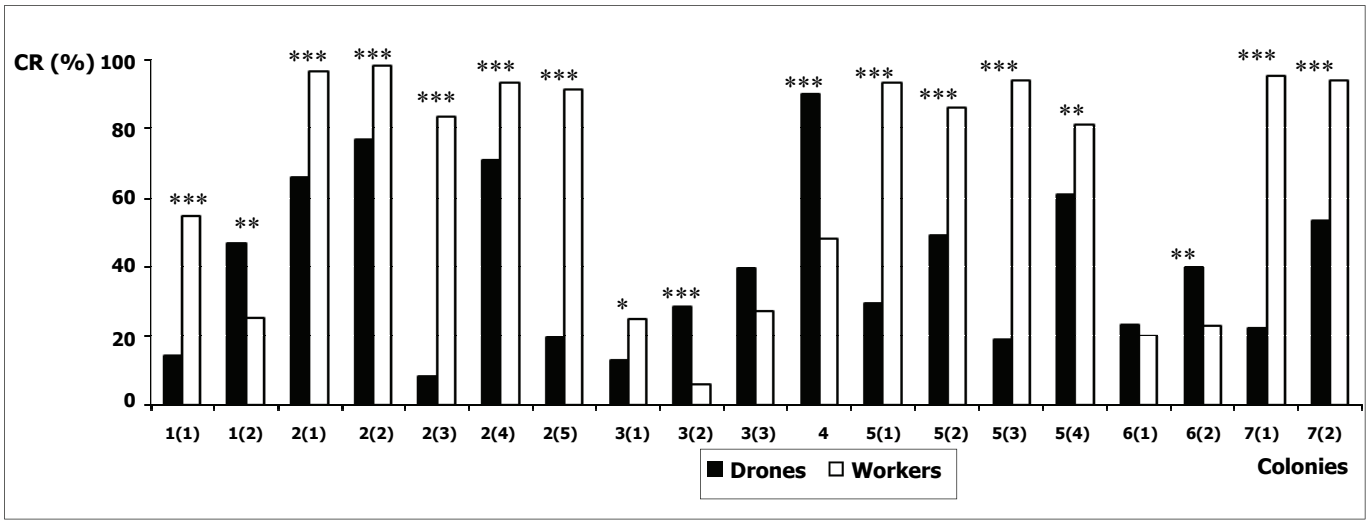

Fig. 4. b. Cleaning Rate (CR) values in drone and worker cells with drone cells located in the centre of the brood area. The numbers in brackets indicate the number of hygienic behaviour tests done. Asterisks indicate significant differences for G test. *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$.

Discussion

Etiological descriptions of chalkbrood disease note that drone larvae are more prone to infection than worker larvae because they are more likely to get chilled due to the marginal location that they usually have (Bailey and Ball, 1991; Gilliam and Vandenberg, 1997). In light of our results and considering the methodologies used, this argument is insufficient to explain the higher mummification of drone larvae. Firstly, drone larvae located along with those of workers in the centre of the brood chamber were used. In addition, experiments were performed with high ambient temperature and using highly populated colonies. Thus, bees must have kept the same temperature in drone and worker experimental brood until they were removed from the hive and taken to the incubator. Differential mummification of drone and worker larvae must therefore be due to other reasons than chilling.

Feeding is one of the factors that *a priori* could explain the higher susceptibility of drones to chalkbrood disease. Adult bees provide more food to drone larvae than to worker larvae due to their larger size, which requires a greater number of feeding events (Calderone and Keunen, 2001; Hrassnigg and Crailsheim, 2005). Additionally, the larval stage lasts a little longer in drones than in workers (6.3 and 5.5 days on average, respectively) so drone larvae receive food for a slightly longer period (Winston, 1987). The greater amount of food supplied to drones by dozens of nurse bees increases the likelihood of ingesting food contaminated with spores of *A. apis*, especially when in the last two days prior to the capping of cells larvae receive increasing amounts of pollen (Hrassnigg and Crailsheim, 2005). Pollen reserves are an important reservoir of fungal spores (Gilliam *et al.*, 1983, 1988; Flores *et al.*, 2005). This explanation would be consistent with results obtained in the first experiment, when larvae were fed in an indirect and massive way. Results were, however, similar in four of the seven colonies in which both types of larvae received the same amount of spores. The other three colonies, mainly No. 2 and 3, maintained the trend, but the low number of mummies did not allow statistical analysis of the result. The higher mummification of drone brood could therefore not lie only in the greater amount of contaminated food that drones received.

Drone and worker larvae differ in two aspects that can affect the development of *A. apis*. Firstly, as already mentioned, the drone larval stage is slightly longer than that of workers. As a consequence, the susceptible period (between the third and fourth day of age in worker larvae (Bailey and Ball, 1991) that favours spore germination and fungal growth, might be extended in drone brood increasing the likelihood of infection.

Another aspect that may play an important role in brood mummification is the ability of larval food to affect germination and / or growth of *A. apis* mycelia. Rinderer and Rothenbuhler (1969), studying the resistance to *P. larvae* in different castes, found that drone larvae are the most resistant, followed by workers and finally queens. These researchers associate this order of resistance to decreasing pollen consumption by the larvae of the three castes. While feeding of worker larvae have been very much studied, there is little information about drone feeding. It is known that brood food supplied to drones contains greater diversity of proteins than that given to workers, and that nectar and pollen input is higher in drones (Winston, 1987; Hrassnigg and Crailsheim, 2005). Differences in diet composition could be associated to the development of *A. apis*, in this case with opposite results to those obtained by Rinderer and Rothenbuhler (1969) with *P. larvae*.

A genetic factor that may contribute to differential resistance to chalkbrood disease is the fact that drones are haploid and the females diploid. Regarding the incidence of ploidy on susceptibility to diseases,

O'Donnell and Beshers (2004) proposed the hypothesis that males of species with haplo-diploid systems of sex determination, like Hymenoptera, should have lower resistance to diseases (haploid susceptibility) compared with diploid females. Their rationale was that heterozygosity at loci that determine disease resistance benefits host fitness, especially when alleles are codominant. Haploid males lack individual genetic variability. When heterozygosity affects characteristics associated with resistance, such as behavioural or immune responses, haploid males are at disadvantage compared with diploid workers. According to the authors of this hypothesis, the greater male susceptibility to disease is a factor that shapes social behaviour in hymenopteran colonies in order to prevent the spread of pathogens and parasites. In this sense, our results seem to support the haploid susceptibility hypothesis. As described above, however, other factors may be influencing the higher susceptibility of drones to chalkbrood disease.

Another important result obtained in the first experiment is that the ratio between mummies with fruiting bodies and those without is usually much higher in drones than in workers. *A. apis* is an heterothallic fungus that only sporulates when mycelia of opposite sex come together. Increased presence of fruiting bodies in drone larvae must respond to a higher probability of germination and development of mycelia of different sign due to the greater amount of food drones receive. The likelihood of consuming spores of fungus of a different sign could be higher with massive contamination than with individual feeding. Coincidentally, the greater amount of drone larvae with fruiting bodies was found in the first experiment, possibly due to the increased availability of contaminated food.

Worker bees have the same type of behavioural response when faced with worker and drone cells containing dead brood, but this response is clearly faster in the first situation. Thus, considering all evaluations (n = 31), UR was higher in worker cells than in drone cells in 17 evaluations and just in three of them UR was higher in drones cells. Besides, CR was higher in worker cells than in drone cells in 21 evaluations and in five it was higher in drones cells. Nevertheless, it should be borne in mind that adult bees can remove dead larvae only after cells have been uncapped, so that CR is largely subjected to UR. This may explain the results found in colonies No. 8 (Fig. 3) and No. 3 (2) (Fig. 4), where UR and CR did not follow the same trend. In these cases perhaps bees were more prone to cannibalize the exposed dead drone brood than to continue uncapping the remaining cells.

Gramacho (1999) found that both Africanized bees (*A. m. scutellata*) and European bees (*A. m. carnica*) had more efficient hygienic behaviour in worker cells than in drone cells after killing the brood with an entomological pin. Bees cleaned most worker cells in just 24 hours while cleaning drones cells needed more than 48 hours. The amount of larval remains found after 24 hours was four to five times higher in drone cells than in worker cells.

The tendency of bees to clean worker cells faster than drone cells can be explained, as Gramacho (1999) did, by invoking proximal factors such as size of brood, cell dimensions and capping thickness that is higher in drones than in workers. When cells are capped, the weight of worker and drone larvae is 144-162 mg and 262-419 mg respectively, and cell size in European races is 5.2-5.4 mm and 6.2-6.4 mm respectively (Winston, 1987; Hrassnigg and Crailsheim, 2005).

Results may also be explained invoking evolutionary factors, however. As the uncapping of cells takes place once bees have noticed the death of the brood, the differences in the UR would indicate that worker death is detected faster than drone death. This could happen if adult bees examined worker cells more or better than drone cells increasing the probability of detecting the death of worker brood. Another explanation could be that bees facing the death of both kinds of brood, choose to remove worker larvae. In any case, differencial response would indicate that adult workers would give more importance to the care of worker brood than to drone brood. Maintenance and growth of a colony of honey bees (as in other social Hymenoptera) depends almost exclusively on the activities of the workers without any substantial contribution of males that are of seasonal occurrence (Michener, 1974). If therefore, hygienic behaviour helps in the survival of worker brood preventing the spread of a pathogen that preferentially attacks this caste, for example *P. larvae*, the greater care of worker brood would have a clear adaptive significance.

Finally, our results can be interpreted in the context of the previously mentioned haploid susceptibility hypothesis (O'Donnell and Beshers, 2004). According to the authors of this hypothesis, in social species colony members should have behaviours aimed at maintaining colony health by preventing males from contracting and spreading diseases. If the bees' strategy to prevent the increase of disease or parasites would be leaving the affected brood to die in the cells without removing the operculum, the findings would be consistent with the haploid susceptibility hypothesis. In this sense, Boecking (1999) found in *A. cerana*, the species more closely related to *A. mellifera*, that bees eventually cover with wax the pore that usually occurs in the opercullum of drone cells. Inspection of the contents of these cells showed that all immature drones were dead having symptoms of bacterial or viral diseases. Dead drones parasitized with the mite *V. destructor* were also found in one of the populations studied. A test of hygienic behaviour was performed in one of the colonies by killing worker and drone brood with an entomological pin. Results showed that after 24 hours bees had cleaned 100% of workers cells but only 51.9% drone cells. To this author, the reason why *A. cerana* does not remove diseased or parasitized drone pupae is the density and hardness of the opercullum of drone cells which prevents bees from detecting the brood condition. The author,

however, does not exclude the possibility that these bees actively avoid the removal of affected drone brood. Perhaps for this reason, in *A. cerana*, the original host of *V. destructor*, the mite reproduces exclusively in drone cells (few and of seasonal occurrence) so that the population remains at low levels without compromising colony viability. If mites enter a worker cell to reproduce, worker bees quickly eliminate it by removing the larva from the cell (Rath and Drescher, 1990; Rosenkranz and Tewarson, 1992). It is possible that results found in the present study reflect no more than the expression in *A. mellifera* of an ancestral character that it shares with *A. cerana*.

In conclusion, this study has found that differences in susceptibility between drone and worker larvae to *A. apis* and differences in the hygienic behaviour of adult bees in relation to brood of both sexes can satisfactorily explain the higher proportion of drone mummies found in colonies with chalkbrood disease. From the standpoint of colony health, and agreeing with the haploid susceptibility hypothesis (O'Donnell and Beshers, 2004), drone brood seems to be a high risk population inside the colony where *A. apis* has a high probability of developing and forming spreading spores.

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