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# Honeybee queen tergal gland secretion affects ovarian development in caged workers

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**Abstract** – The inhibitory effects of honeybee queen tergal gland secretion on worker ovarian development was studied using a laboratory bioassay with the honeybee races *Apis mellifera capensis* and *A. m. scutellata*. Glass pseudoqueens were treated with daily doses of tergal gland extracts from virgin queens and exposed to queenless experimental groups of caged workers. The control groups of queenless caged workers were exposed to solvent controls. Analysis using loglinear models showed that there were no interactions between treatment, race and cage, with respect to the frequency of developing ovaries. The response was homogeneous among cages and among the two races. The virgin queen tergal gland extracts of both *A. m. capensis* and *A. m. scutellata* inhibited ovarian development in their own workers ($\chi^2 = 8.28$; df = 1; $P = 0.004$). These results indicate that the secretion from the tergal glands can operate as a primer pheromone. © Inra/DIB/AGIB/Elsevier, Paris

***Apis mellifera* / worker reproduction / ovarian development / queen pheromone**

## 1. INTRODUCTION

Honeybees, *Apis mellifera* L., have two female castes: queens which form the reproductive caste; and workers with reduced reproductive capacity as a consequence of caste differentiation. Even though the workers have limited reproductive capacity, there is still competition between the castes over reproduction [33, 45, 46]. In the more primitive social insects, such as the *Polistes* wasps, suppression of worker ovarian development involves overt aggressive dominance interactions [19]. In the more advanced eusocial insects, worker ovarian development is partially inhibited via primer pheromones produced by the queen [59, 65, 66]. Primer pheromones are signals that have

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distinct effects on the physiological state of the target individual [3]. The mandibular gland secretion of *Apis mellifera* has been said to function as an inhibitory pheromone, suppressing the development of ovaries in worker honeybees [66]. However, the efficacy of the mandibular components in producing worker ovarian suppression has not been unequivocally demonstrated [8, 57, 65].

Butler [6] found that extracts from heads of queens almost completely inhibited ovarian development in queenless workers and later showed that 9-ODA in particular inhibited ovarian development of queenless workers [9]. Pain [42], however, demonstrated that ovarian suppression in caged workers was produced by whole queen extracts and not with queen substance (9-ODA) alone. She also showed that inhibition of worker ovary development is dependent on both ingestion of the queen pheromone and antennal contact with the pheromone [42]. Subsequently, Butler and Fairey [8] and Velthuis [57] could only show partial inhibition of worker ovarian development with queen head extracts. In total contrast to the above, Willis et al. [65] demonstrated that the 5-component synthetic mandibular gland secretion does not inhibit ovarian development in workers at any dose. From this, it became clear that additional inhibitory pheromones must be responsible for suppressing ovarian development of workers. To test the probable source of queen inhibitory pheromone, workers have been exposed to various queen body parts and/or to queens whose mandibular glands had been extirpated [56, 57, 59, 62]. Queens of all honeybee races, as well as *A. m. capensis* pseudoqueens, successfully maintained their dominant position in the colonies without their mandibular glands and almost completely inhibited the ovaries of the resident workers [49, 56, 57, 62]. In addition, workers exposed to the tergites of queens, exhibited considerable ovarian suppression [16, 27, 47, 56, 57, 59].

Not only is ovarian development of workers influenced by queen primer pheromones but brood pheromones also regulate this process [2, 36]. Queenless workers exposed to brood, particularly uncapped brood, show very little ovarian development [25, 26, 29, 30, 35]. Besides queen and brood pheromones, laying workers and pseudoqueens also partially inhibit ovarian development in their nestmates [14, 31, 49, 58]. At one level, there is pheromonal suppression, with dominant individuals secreting a pheromonal bouquet similar to that of a queen [14, 49]. At another level, dominant individuals show trophallactic advantage, with the subordinate individuals losing protein which is essential for oogenesis [34]. For the adult worker, the balance between protein availability and the colony's demands will determine the amount of protein necessary for oogenesis [28, 29, 30, 34, 53, 60, 64].

It is clear that numerous factors play a role in preventing ovarian development of the workers in a honeybee colony. Suppression occurs at several levels: pheromonal, environmental and at the level of the individual. The latter includes the following factors. i) The age of the worker, with younger workers having an advantage [27, 37, 54]. However, Delaplane and Harbo [18] have a conflicting opinion and contend that age does not seem to affect the probability of an individual becoming an egg layer, since they found workers of 54-days-old producing as many drones as 15-day-old workers. ii) The number of ovarioles per ovary in each individual may influence the potential ovarian development. The evidence for this relationship is contentious and therefore ovariole number appears to be a poor indicator of potential ovarian development [1, 24, 55, 58]. iii) Genetic variance among subfamilies results in certain individuals being genetically predisposed to develop their ovaries [38, 40, 41, 48].

The research reported here is concerned with the pheromonal effects of the queen, specifically the tergal gland secretions, on

the suppression of ovarian development in worker honeybees. The function of queen tergal gland secretions as a primer pheromone has not been conclusively established [66]. The objective of this study is therefore to examine the inhibitory effects of *A. m. capensis* and *A. m. scutellata* virgin queen tergal gland secretions on *A. m. capensis* and *A. m. scutellata* worker ovarian development, respectively.

## 2. MATERIALS AND METHODS

Tergal glands were dissected out of 7-day-old virgin *A. m. scutellata* ($n$ = 15) and *A. m. capensis* ($n$ = 15) queens and stored in 150 µL of dichloromethane (DCM) at –20 °C (unpublished data). The reason for choosing 7-day-old virgin queens (mating age) was because it has been reported that tergal gland activity reaches a peak at mating age [17, 51]. The tergal gland extracts for all the queens were combined in each trial to overcome individual variability between queens with respect to pheromone production [11].

Thirty 1-day-old *A. m. scutellata* or *A. m. capensis* workers were housed in Liebefeld cages with the pollen-rich food necessary for oogenesis provided ad libitum [34, 58]. Worker bees were obtained from the same colony in order to avoid bias from workers having different rates of ovarian development [24].The cages were accommodated in two separate temperature-controlled rooms (27 °C), one housing 15 control cages and the other with 15 experimental cages. Since exposure to normal light is reported to affect ovarian development, the rooms were kept in darkness and only red light used when necessary [58].

Small glass 'spoons' acting as the pseudoqueen lures were produced from glass Pasteur pipettes with an indentation suitable for a 10-µL aliquot treatment made at one end. The tergal gland extracts were presented at 0.05 queen equivalent (Qeq), where one Qeq represents the average concentration of the tergal gland extract of one virgin queen. This concentration was chosen based on prior results obtained for worker retinue behaviour elicited by queen tergal gland secretion [32]. Young *A. m. scutellata* workers, aged from 1 to 3 days old, were individually selected from the experimental colonies. Fifteen workers were placed in plastic Petri dishes which served as retinue bioassay arenas and exposed to glass pseudoqueen lures that had been treated with varying concentrations of queen tergal gland extracts (0.01–0.1 Qeq). The retinue responses of the workers were quantified and found not to vary significantly among the concentrations and we therefore chose a practical dose of 0.05 Qeq for the bioassays. Workers, in the control cages, were exposed to 10 µL of DCM.

Workers were exposed to daily treatments of queen tergal gland secretions and solvent controls. The treatments were applied to the lure with a microsyringe and the solvent allowed to evaporate before introducing the lure into the cage.

Workers dying within the first 2 days of the experiment were replaced with 1-day-old workers. Workers that died before 8 days were discarded. Therefore, not all the 30 initial workers were always included in the final analyses. However, workers that died from the 8th day onwards were removed and frozen for dissection and ovarian inspection and included in the final analyses. The experiment was terminated at 21 days with all remaining workers frozen for ovarian examination. 'Blind' scoring of ovarian development was performed to ensure impartiality in assessing the status of ovarian development. The stage of development of the ovaries was scored on a scale of I–IV as follows. Stage I ovaries are slender and non-differentiated, referred to as undeveloped ovaries. Stage II development shows the beginnings of differentiation with granular ovarioles. Stage III ovarioles are very granular, have constrictions with developing oocytes, while stage IV ovaries are characterized by having mature oocytes [58]. However, to analyse the results, ovarian development was assigned to two classes: i) undeveloped and ii) developed [because workers only partially developed their ovaries in the cages (stage II to early stage III)].

### 2.1. Statistical analyses

The categorical data were analysed using a three-way loglinear model with ovarian development (two levels: developed and undeveloped), race (two levels: *A. m. capensis* workers and *A. m. scutellata* workers) and cage (41 cages) as the variables [10, 21, 22]. We used the CATMOD procedure on SAS version 6.12 (SAS Institute, Cary, NC).

After running the model, non-significant interaction terms were removed singly, starting from the least significant interaction term, and the

**Table I.** Age of workers in control (solvent) and treatment (tergal gland secretions) groups for the two races.

| | *n* | Median | Range | Median test statistic, $\chi^2$ | *p* |
|---|---|---|---|---|---|
| *A. m. capensis* | | | | 2.472 | 0.1159 |
| control | 133 | 16 | 11–22 | | |
| treatment | 70 | 16 | 8–18 | | |
| *A. m. scutellata* | | | | 2.133 | 0.1442 |
| control | 222 | 11 | 8–22 | | |
| treatment | 178 | 9 | 8–16 | | |

model rerun removing each non-significant term in turn until the best fit model was achieved [21, 23]. The parameter estimates of the model which gave the best fit were then used to calculate indices as the geometric mean of the predicted cell frequencies in the loglinear model multiplied by $e^{\lambda}$ [21, 22]. These indices are interpreted as the percentage to which each level of the variable is either higher or lower than the geometric mean frequency of all the cells in the table.

## 3. RESULTS

The age distributions between the control and treatment groups were highly asymmetrical and so a median test was used to deal with this skewed categorical data [52, 67]. The age of the workers in the control and treatment groups (*table I*) was not significantly different for *A. m. capensis* workers ($\chi^2 = 2.472$, df = 1, $P = 0.116$), nor for the *A. m. scutellata* workers ($\chi^2 = 2.133$, df = 1, $P = 0.442$).

None of the interaction terms in the loglinear model (race × treatment × cage; race × treatment; race × cage; treatment × cage) were significant and each interaction term was removed singly (starting from the least significant interaction term) and the model rerun, removing each non-significant term in turn, until only the main effects remained. The main effects 'race' ($P = 0.665$) and 'cage' ($P = 0.225$) did not contribute significantly to the model. This indicates that the response to the treatment was homogeneous among the cages and among the two geographical races. The final best fit model was thus an independence model with a single significant effect, the treatment ($\chi^2 = 8.28$; df = 1; $P = 0.004$). This demonstrates that tergal gland extracts from virgin *A. m. capensis* queens significantly inhibited ovarian development of caged *A. m. capensis* workers compared with the controls. Similarly, *A. m. scutellata* virgin queen tergal gland extracts significantly inhibited *A. m. scutellata* worker ovarian development (*figure 1*). Therefore, suppression of ovarian development in workers was achieved by tergal gland secretions from their respective queens.

The indices estimated from the model show that the chance of a worker's ovaries developing when exposed to queen tergal gland secretion was approximately 30 % lower than the geometric mean frequency (0.693, *table II*). On the other hand, workers not exposed to queen tergal gland secretion had a 44 % greater chance of developing ovaries than the geometric mean frequency (*table II*).

A percentage of the workers exposed to tergal gland secretion were still able to initiate ovarian development in both races, but they showed significantly reduced development compared to those workers exposed to the solvent control (*figure 1*).

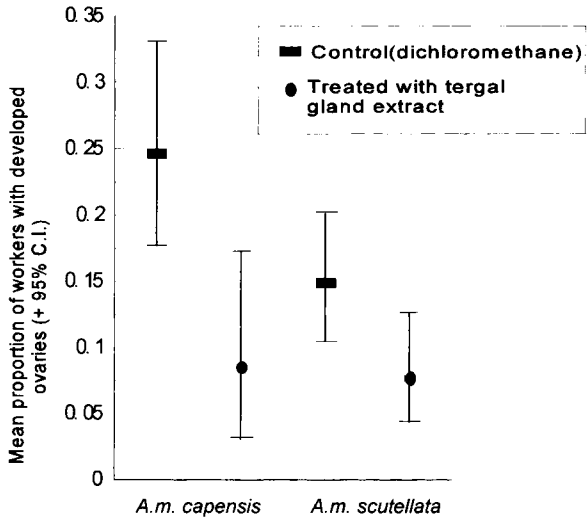


**Figure 1.** The effect of queen tergal gland secretion on ovary development in caged queenless workers of two honeybee races, *A. m. capensis* and *A. m. scutellata.*

## 4. DISCUSSION

Ovarian development in worker honeybees is not entirely controlled by mandibular gland secretions [8, 20, 57, 65]. Likewise, secretions from the tergal glands are not totally effective in suppressing worker ovarian development. However, queen tergal gland secretions were able to significantly inhibit ovarian development in workers of their own race indicating that these secretions behave as a primer pheromone. The less volatile long-chained tergal gland hydrocarbons [51] may not be ideal for long distance communication [62], but they are readily perceived within the hive and therefore could easily act as a persistent signal, responsible for the ovarian inhibition of workers observed in this bioassay [4, 5].

From our results, it is clear that a percentage of the workers exposed to tergal gland secretion were still capable of initiating ovarian development. There are several possible explanations for the tergal gland secretions not suppressing ovarian development completely. First, since live queens are more effective than either mandibular gland pheromone or tergal gland pheromone in inhibiting worker ovarian development, it supports the suggestion that queen control depends on i) secretions from more than one source, and ii) the synergistic functioning of these secretions [5, 7, 57, 60]. Therefore, mandibular gland secretions together with

**Table II.** Effect of tergal gland secretions on worker ovarian development and estimated indices from the loglinear independence model [22].

| Treatment | Number of individuals scored | Frequency of workers with developed ovaries | Estimated index |
|---|---|---|---|
| Control (solvent) | 355 | 66 | 1.444 |
| Tergal gland secretions | 248 | 20 | 0.693 |
| All cells | | | 1.260 |

tergal gland secretions may enhance the inhibitory effect of the queen. Besides mandibular and tergal gland secretions, alternative inhibitory pheromone sources, may act in conjunction with other inhibitory queen pheromones, influencing worker ovarian inhibition.

Second, there is no quantitative data indicating how much tergal gland secretion is produced per queen per day. We know that the secretion is continually produced and released onto the exterior surface of the tergum rather than being stored in a reservoir [47, 57, 61], but the amount of secretion present at any given moment in time is unknown. Therefore, the lack of complete ovarian development in the caged workers may be as a result of being exposed to too low a tergal gland secretion concentration than would typically be necessary for complete ovarian inhibition.

Third, there are conflicting opinions concerning the dominance status of virgin queens. Jay [28] reported that virgin queens had little, if any, inhibitory effect on worker ovarian development, but more recently, DeGrand-Hoffman and Martin [15] demonstrated that virgin queens do partially suppress worker ovarian development. This incomplete dominance by virgin queens is related to the signal they produce, with virgin queens secreting reduced quantities of mandibular gland secretion as well as different proportions of the components compared to mated queens [13, 43, 50]. This is not true for *A. m. capensis* queens which show little change in their mandibular gland secretions with age [12, 13]. Interestingly, however, *A. m. capensis* virgin queen tergal secretion profiles do differ significantly from the profiles of mated queens (unpublished data). It is possible that mated queens may have a stronger inhibitory effect on worker ovarian development, but this has not been unequivocally demonstrated.

Fourth, it is important to be aware of the role played by the glass lures used to introduce the pheromone. In this regard, two issues may be of importance: i) the release of semiochemicals is not only dependent on volatility but texture and chemistry of the body surface play a crucial role in the release of pheromones [39]; ii) movement by the queen may be essential in pheromone distribution [60, 61] and important in signalling her presence to the workers. In these two respects, the use of the glass lure is only an imperfect substitute for the queen effect since queens inhibit worker ovary development both through pheromonal and behavioural dominance [44].

Last, the worker bees were not exposed to brood pheromones and there is strong evidence that the presence of brood is necessary to completely inhibit ovarian development in worker honeybees [2, 26, 29, 30]. However, in this experiment, queen pheromonal effects were being tested exclusively and therefore the absence of brood pheromones was an essential feature of the experimental design.

In queenright colonies, queen and brood pheromones are not solely responsible for preserving the sterility of the workers in the colony. The hypothesis that worker production is being regulated by other workers via worker policing, places the worker caste in an important position to maintain the reproductive hierarchy in colonies [33, 45, 46, 63]. The data presented indicate that queen tergal gland secretions have an effect on worker ovarian development. The degree to which reproductive regulation in honeybees is governed by worker–worker interactions and queen–worker interactions remains unresolved.

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**Résumé – La sécrétion des glandes tergales de la reine d'abeille influe sur le développement ovarien des ouvrières encagées.** Les ovaires des ouvrières d'abeille (*Apis mellifera* L.) sont partiellement inhibés par les sécrétions de la glande mandibulaire de la reine. On a étudié au laboratoire, par un test biologique, les effets inhibiteurs de la sécrétion des glandes tergales de la reine sur le développement ovarien des ouvrières chez les races *capensis* et *scutellata*. Des leurres de reine en verre, réalisés à partir de pipettes Pasteur, ont été traités quotidiennement et présentés à des groupes d'ouvrières orphelines encagées. Les cagettes ont été placées à l'obscurité dans des pièces à température contrôlée (27 °C). Les groupes expérimentaux ($n$ = 15) ont été exposés à des extraits de glandes tergales de reines vierges correspondant à 0,05 équivalent reine. L'extrait a été déposé sur le leurre à l'aide d'une microseringue et on a laissé le solvant, du dichlorométhane, s'évaporer avant d'introduire le leurre dans les cagettes. Les groupes témoins ($n$ = 15) n'ont été exposés qu'au solvant. On a finalement tué par le froid toutes les ouvrières pour examiner leurs ovaires. Le degré de développement ovarien a été évalué et classé de 1 à 4. L'âge des ouvrières des groupes témoins et des groupes traités n'était pas significativement différent (*tableau I*), que ce soit chez *A. m. capensis* ou *A. m. scutellata* (test de la médiane : $p \geq 0{,}116$). L'analyse par des modèles logarithmiques linéaires (*tableau II*) n'a pas montré d'interactions significatives (race × traitement × cage ; race × traitement ; race × cage ; traitement × cage). Les principaux effets cage ($p$ = 0,225) et race ($p$ = 0,665) n'étaient pas statistiquement significatifs et le modèle final qui convient le mieux comprenait l'effet traitement seul qui, lui, était hautement significatif ($\chi^2$ = 8,28 ; df = 1 ; $p$ = 0,004 ; *tableau II, figure 1*). Ces résultats montrent que la sécrétion des glandes tergales peut agir comme phéromone modificatrice mais qu'elle fonctionne vraisemblablement en synergie avec les sécrétions des autres glandes. Le maintien de la stérilité des ouvrières est régulé par de multiples facteurs et la reproduction est finalement régie par des facteurs physiologiques propres à l'ouvrières considérée individuellement. Ce travail a montré que la sécrétion des glandes tergales de la reine peut influencer le développement ovarien des ouvrières qui y sont exposées. © Inra/DIB/AGIB/Elsevier, Paris

***Apis mellifera capensis* / *Apis mellifera scutellata* / ouvrière / développement ovarien / phéromone / glande tergale**

**Zusammenfassung – Das Sekret der Tergaldrüsen der Königin der Honigbienen beeinflußt die Ovarentwicklung bei gekäfigten Arbeiterinnen.** Die Entwicklung der Ovarien der Arbeiterinnen werden durch das Mandibeldrüsensekret der Königin nicht vollständig gehemmt. Der Hemmeffekt der Tergaldrüsen der Königin auf die Ovarentwicklung wurde in einem Laborversuch getestet. Pseudokönigginnen aus Glas, hergestellt aus Pasteurpipetten, wurden täglich beduftet und den gekäfigten, weisellosen Bienengruppen geboten. Die Käfige wurden in dunklen, temperaturgeregelten Räumen (27 °C) gehalten. Die Versuchsgruppen ($n$ = 15) wurden Extrakten aus den Tergaldrüsen von unbegatteten Königinnen ausgesetzt, die einem Aquivalent von 0,05 Königinnen entsprachen. Der Extrakt wurde mit einer Mikropipette auf die Attrappe (Pseudokönigginnen) aufgetragen. Das Lösungsmittel Dichlormethan wurde verdunstet bevor die Attrappe in die Käfige gegeben wurde. Die Kontrollgruppen ($n$ = 15) wurden dem reinen Lösungsmittel ausgesetzt. Der Versuch wurde mit dem Einfrieren aller Arbeiterinnen für die Kontrolle der Ovarien beendet. Der Grad der Ovarentwicklung wurde bewertet und Stadien in den Klassen I–IV festgelegt. Der Hemmeffekt von unbegatteten Königinnen von *A. m. capensis* und *A. m. scutellata* wurde an Arbeiterinnen der entsprechenden Rasse untersucht. Das Alter der Kontrollbienen

(*Tabelle I*) war nicht signifikant unterschiedlich, weder für *A. m. capensis* noch für *A. m. scutellata* (Mediantests: $p^3 = 0.116$). Analysen mit loglinearen Modellen zeigten keine signifikanten Interaktionen (Rasse × Behandlung × Käfig; Rasse × Behandlung; Rasse × Käfig; Behandlung × Käfig. Die Haupteffekte Käfig ($p = 0.225$) und Rasse ($p = 0.665$) waren auch nicht signifikant. Das endgültige am besten passende Modell umfaßt den 'einmal Behandlungseffekt', der hoch signifikant ist ($\chi^2 = 8.28$; df = 1; $p = 0.004$; *Tabelle II; Abbildung 1*). Diese Ergebnisse zeigen, daß das Sekret der Tergaldrüsen als Primärpheromone wirken kann, aber wahrscheinlich synergistisch mit Sekreten von anderen Drüsen wirkt. Der Erhalt der Sterilität der Arbeiterinnen unterliegt einer multifaktorellen Regulation und die Reproduktion ist ultimativ von physiologischen Reaktoren in den individuellen Arbeiterinnen gesteuert. Diese Untersuchung, die die Pheromoneinflüsse der Königinnen (speziell der Sekrete der Tergaldrüsen) auf die Unterdrückung der Ovarentwicklung der Arbeiterinnen betrifft, zeigt daß die Sekrete der Tergaldrüsen der Königinnen die Ovarentwicklung bei den Arbeiterinnen beeinflußt, die diesem Pheromon ausgesetzt waren. 

***Apis mellifera* / Reproduktion von Arbeiterinnen / Ovarentwicklung / Königinnenpheromone**

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