Chapitre II Caractérisation de la phéromone des butineuses, l'Oléate d'Ethyle transmission, dynamique et impact du stress sur sa production.

Avant propos :

La reine, le couvain et les butineuses interviennent dans la maturation comportementale des ouvrières en produisant respectivement la QMP, la BEP, et l'EO. L'EO participe à l'autorégulation de la division du travail par les butineuses pour maintenir un juste ratio nourrices/butineuses dans la colonie.

La régulation des ouvrières par cette phéromone est majeure pour la colonie. Nous avons étudié certains traits de son histoire de vie dans la colonie.
Nous avons tenté de valider l'hypothèse de la transmission de l'EO des butineuses aux nourrices par l'intermédiaire du nectar, du pollen et de la cuticule des butineuses. La quantité d'EO contenue dans ces différents compartiments a été analysée. Puis nous nous sommes intéressés à la dynamique de l'EO dans la colonie en analysant sa production chez les ouvrières à travers la saison de floraison (Mai à septembre) (influence de l'environnement) et chez deux races d'abeilles *Apis mellifera mellifera* et *ligustica* (influence de la race).

Ensuite, nous avons analysé l'effet de stress externes à la colonie sur la production de cette phéromone. Une modification de la production de cette phéromone (augmentation ou diminution) par un stress externe, peut perturber l'équilibre nourrices/butineuses. Le CCD (syndrome d'effondrement des colonies = colony collapse disorder) présente des symptômes bien décrits : un dépeuplement massif des abeilles, présence de couvain et de réserves conséquents, et une reine entourée d'une poignée de jeunes abeilles. Ce dépeuplement massif pourrait être dû à une modification des mécanismes phéromonaux de la colonie d'abeilles. Nous avons travaillé sur l'impact de l'imidaclopride (un pesticide utilisé actuellement en agriculture) et de *Nosema* spp. (un champignon parasite de l'abeille) sur la production de l'EO par les abeilles.

Distribution of the Forager Pheromone (Ethyl Oleate) in the Honey Bee Colony

Résumé :

Les butineuses émettent une phéromone, l'EO, qui permet l'autorégulation du développement comportemental des ouvrières (nourrices) de la colonie par contact. Un nombre important de butineuses inhibe le développement comportemental des nourrices et inversement, un faible nombre de butineuses accélère la maturation des nourrices.
Des facteurs internes et externes au nid peuvent moduler les besoins en butineuses, et donc le ratio nourrices/butineuses. Nous avons voulu savoir si une possible fluctuation de l'EO pourrait être une réponse à des contraintes environnementales et des modifications du nid pour ajuster le ratio nourrices/butineuses. Nous avons également clarifié le mode de transfert de cette phéromone entre butineuses et nourrices.

Une étude de la quantité d'EO de différentes parties des butineuses, la tête, le thorax, l'abdomen, la cuticule, les pelotes de pollen, le nectar et le jabot a montré que le pollen des butineuses est un vecteur de l'EO ainsi que la cuticule des butineuses.
Parallèlement, pour étudier la dynamique de l'EO, des butineuses de pollen et de nectar ainsi que des nourrices ont été prélevées chaque mois durant deux années à chaque période de floraison (Mai à Septembre) dans trois ruches génétiquement différentes. Une augmentation de production du taux d'EO a été observée durant la période estivale. L'origine génétique des abeilles ne semble pas impliquée, alors que les conditions environnementales semblent jouer un rôle majeur.

Les contacts cuticulaires ou cuticule-antenne entre butineuses et nourrices sont nécessaires à la transmission de la phéromone inhibitrice et le pollen peut servir de stockage de la molécule, mais être également un vecteur lors de son ingestion et de son contact avec les abeilles nourrices.
La dynamique de production de l'EO montre une fluctuation en réponse aux contraintes environnementales. Il est nécessaire de savoir si les nourrices adaptent leur sensibilité à la phéromone et ajustent leurs développements comportementaux en fonction des fluctuations d'EO.

Distribution of the Forager Pheromone (Ethyl Oleate) in the Honey Bee Colony

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Key words: *Apis mellifera*, pheromone, nurse, forager, social regulation

En preparation.

Abstract:

Ethyl Oleate (EO) is a major primer pheromone of the honey bees produced by the foragers to delay the onset of foraging in younger individuals (hive bees) via a mechanism that requires physical contact. This signal is important in regulating the best ratio of foragers / hive bees to optimize colony development. But the pace of bee maturation depends on both internal (colony) and external (environment) factors. We asked whether colony or environmental factors could change the EO production, and clarified the mode of transmission of EO between foragers and hive bees.

Variation in EO production occurs in the nest during the active season. Genetics origin of bees does not seem to be involved, whereas environmental conditions appear to play a major role.
Foragers have 26% of the total forger EO amount on their cuticle and the pellets of pollen also contain EO. Thus hive bees could perceive EO by cuticle or antennae contact with forgers and by assimilating or manipulating the pollen in the hive.

The next challenge is to understand if workers adapt their behavioural maturation trough differential sensitivity to EO variation in the colony. Chemical communication in the honey bee reveals an increasingly complex language with regards to the variation in signal production and nest regulation.

Introduction:

Animals communicate between each other using visual, acoustic, tactile and chemical signals (Wilson, Bossert, 1963). Among the different means, chemical communication between individuals is widespread among insects, and highly developed among social insects.
In the honey bee, a eusocial insect, communication by pheromones is used by the individual to indicate its needs but also to maintain the homeostasis of the colony (Le Conte, Hefetz, 2008; Slessor *et al.*, 2005a). Progress has been made toward decoding the honey bee chemical language. It is now known that pheromones in honey bees modulate individual interaction through their action on behavioral genes and individual physiology (Alaux *et al.*, 2009; Grozinger, Robinson, 2007). Recent studies have presented mechanisms of pheromone

integration and processing in the bee brain, and new descriptions of pheromone receptors (Wanner *et al.*, 2007) and biosynthesis (Malka *et al.*, 2009a) have been made. However, most of our knowledge about social insect pheromones involves the effect of releaser and primer compounds on workers but little is known about pheromone dynamics and translocation in the colony. In order to respond to variations inside and outside of the nest, workers change and coordinate their activities in relation to colony needs using a wide variety of information sources including pheromone variation (Robinson, 1992). The study of dynamic and translocation of pheromones becomes essential to improve the knowledge of bee communication and colony adaptation. When looking at the importance of synergy, dose and context, pheromone communication in honey bees appears to be remarkably complex (Slessor *et al.*, 2005b). The analysis of each pheromone is then necessary to understand this specific language and its role in bee social regulation.

In the colony, the queen mandibular pheromone (QMP) and the 9-ODA (9-oxoxdec-2-enoic acid, one compound of the QMP) are involved in regulating many social functions and continue to be highly studied (Barbier, Lederer, 1960; Butler *et al.*, 1962; Peters *et al.*, 2010; Slessor *et al.*, 1988; Slessor *et al.*, 2005a). But now, there are other important mechanisms (or social functions) known in the honey bee colony in which pheromones are involved.

Age-related division of labor among workers plays a major role in the organization of many insect societies (Robinson 1992), where division of labor is used to maximize colony development rate and reserve accumulation. In honey bees, to optimize colony development, an effective ratio of foragers (old bees) to hive bees (young bees) is required. The colony has a feedback mechanism between nurses and foragers, particularly adapted to regulate the size of the colony. One key of this self-regulating workforce is a social pheromone: Ethyl Oleate (EO) which is produced by the foragers (Leoncini *et al.*, 2004b). The EO signal slows down the natural progression of workers from hive bees to foragers (Leoncini *et al.*, 2004b). The queen (Keeling, Slessor, 2005) and the brood (Le Conte *et al.*, 1990) also produce EO in the colony but the main social regulation of the workforce appears to come from foragers (Huang, Robinson, 1996). When foragers are lost, hive bees are less inhibited in their maturation and they forage precociously. If a colony has too many foragers, the age progression of young bees slows down more than usual, until the correct proportion of foragers to in-hive sectors is reached.

EO is an important pheromone inside the colony to regulate bee behavioural development. But the pace of bee maturation depends on colony conditions, and the environmental, physiological and genetic parameters involved in this complex mechanism (Robinson, 1992). In addition, honey bees are able to respond to changes in colony needs by altering their typical patterns of age polyethism. This flexible system of division of labor is very important to colony fitness because the development of a bee colony continues despite constant changes in environmental (external and colony) conditions (Robinson, 1992). Also, genotypic differences in age-related division of labor in colonies occur in different honey bee subspecies. Bees of various genotypes have different development strategies: subspecies that invest quickly in foraging and subspecies that invest slowly in foraging (Giray, Robinson, 1994; Winston, Katz, 1982). This flexibility of colony structure (ratio foragers / hive bees) suggests a possible variation of EO rate in the colony.

We studied, during 5 months of the beekeeping season, two subspecies of honeybee *Apis mellifera mellifera* and *Apis mellifera ligustica* that differ in their development strategies (Brillet *et al.*, 2002) and genetic patterns (Whitfield *et al.*, 2006a) to investigate the regulatory network that controls colony responses that set the right ratio foragers / hive bees. This study investigated whether there is variation in EO production between the different lineages in response to their different strategies of development or to the environmental conditions.

Social regulation of bee behavioural maturation requires physical contact among bees. Older bees separated from younger bees via a screen that permits some forms of physical contact (food transfer, antennal contact, and licking) are able to inhibit behavioural maturation, but not when they are separated via double screen that prevents these interactions (Huang *et al.*, 1998; Leoncini *et al.*, 2004a). The transmission of EO is not well understood yet according to previous results, trophallaxis between nurse and forager, a form of food exchange but also thought to be a prominent means of communication in insect societies (Korst, Velthuis, 1982), seems to be the way to delay nurse maturation in the colony. But, as these results are not clear, we investigated how foragers could transfer EO to nurse bees in the colony. We analysed the EO level within food transferred between foragers directly to nurses; the nectar and indirectly; the pollen. Also because contacts (cuticle-cuticle or antennae) between nurses and foragers are frequent, we analysed the EO level on the forager cuticle, to see if EO could be exchanged by passive contact (friction) between the thousand of individuals in the colony.

The characterisation of this pheromone could improve our comprehension of bee social regulation and the importance of the proper ratio between foragers and hive bees.

Materials and Methods

EO chemical analysis

All samples were prepared in a solution A of 1.9 ml of iso-hexane with the addition of 100 µl of two internal standard solutions at 10 ng/µl (arachidic acid methyl ester and methyl heptadecanoate, Sigma-Aldrich, France).

Total EO amount was analyzed on different samples (pool of 5 bees, load of pollen…). Each sample was crushed with a glass rod during 2 min at 4°C in the solution A and centrifuged for 20 min at 4°C (2,500 g). The cuticular extracts were prepared by rinsing 5 bees in the solution A for 1 min at 4°C.

Then the supernatant was collected and applied to a silica column (silica gel 60, particle size 40–63 µm, 230–400 mesh). The first fraction was eluted in 3 ml of a solvent mixture (98.5% iso-hexane, 1.5% diethyl ether). Then, the second fraction containing the EO was eluted in 3 ml of a second solvent mixture (94% iso-hexane, 6% diethyl ether). 1 ml of this fraction was concentrated to 10 µl under nitrogen stream and 1 µl was injected into a gas chromatograph (2014, Shimadzu, Japan) equipped with a split-splitless inlet, a flame ionization detector and a capillary column Omegawax 100 (10 m x 0.10 mm, 0.10 µm film thickness). The samples were injected in split mode. Hydrogen was used as carrier gas with a column flow of 0.52 ml/min. Oven temperature was set at 90°C for 1 min, raised to 195°C at 40°C min^{-1}, stabilized for 3 min, then augmented to 210°C at 1°C min^{-1}, stabilized again for 2 min then increased to 270°C at 40°C min^{-1} and held at 270°C for 3 min. Identification and quantification of EO was based on retention times of EO synthetic compound (Sigma-Aldrich, France) and by comparison of internal standard area respectively using gas chromatography solution program (Shimadzu, Japan). The EO confirmation was done by a mass spectrometer (CP2010, Shimadzu, Japan) operated in the electron impact mode at 70 eV with continuous scans (every 0.2 sec) from a mass to charge ratio (m/z) of 70 to 400.

EO transmission (forgers to hive bees)

To gain insight into the mode of transfer of EO among bees in a colony, we measured EO levels in different honey bee parts: head, thorax, abdomen, cuticle, but also in the food transfer to nurse by the forager: the nectar and the pollen. Foragers used in the different experiments came from a typical field colony headed by a naturally mated queen. Foragers were caught by closing the hive entrance and immediately frozen at -20°C; before EO analysis. Each sample was analyzed for total EO levels with the methods described above.

First, to know the distribution of EO in foragers, we analysed EO levels in the 3 parts of foragers: head, thorax and abdomen. As a control we also measured the EO level of intact foragers. Ten groups of 5 bees were dissected for three lots of 10 samples of each honey bee sections (body parts) and 10 groups of 5 bees were kept unchanged (control). Before analyzing the EO the different samples were weighed in order to have the mean amount of EO per sections and also per mg of each section.

Then we studied EO on the cuticle of honey bee. Ten groups of 5 foragers were analysed for total EO amount as a control and cuticular extracts (rinse the cuticle) were made on 10 groups of 5 foragers to know the percentage of EO found on the cuticle.

Also the quantity of EO in nectar of foragers was examined. The EO level in the nectar sampled directly from the forager crop and the nectar regurgitated by the forager were quantified on two different groups because foragers could add secretions to the nectar from the hypopharyngeal gland during trophallaxis (Simpson, 1960; Simpson *et al.*, 1968). The abdomens of 6 groups of 5 foragers were dissected, an incision was made in order to clear the crop, and then with a syringe the crop content was sampled. The crop envelope was analyzed separately. In a second group of bees (6x5 foragers), nectar regurgitation was recovered by applying pressure on the abdomen with a forceps and the regurgitated nectar was collected with a microcapillary pipette and put in a 2mL vial. A third group of foragers (6x5) was analysed for the total amount of EO as a control.

In a final step the pollen load of foragers was considered as a potential transfer of EO between nurse and forager. Pollen is in contact with the forager cuticle before the formation of a pollen load and foragers add oral fluids during pollen handling (Winston, 1987). The pollen could be a way to distribute EO in the colony. We tested this hypothesis by an EO analysis of pollen

load, bee bread (stock of pollen inside the hive) and pollen-carrying forager with pollen load removed (control). In two hives, one day before the experiment at the end of the day an empty frame inside the hive was added. The following day in the afternoon a wire screen was placed in front of the hive entrance, 50 pollen foragers blocked from entering were caught and separated from their pollen load, all the samples were immediately frozen. Then the frame from the previous day was removed and the fresh bee bread collected from comb cells. We analysed EO on 10 groups of 5 foragers without their pollen load, 14 samples of 10 pollen loads, and 10 samples of 10 bee breads. Each sample of beebread and pollen was weighed before analysis.

In addition, as a control for the pollen experiment, we measured EO rates in the pollen of different flowers to be certain that EO came from the foragers. We picked anthers of the five important flowers in blossom (*Onobrychis viciifolia*, *Pyracantha coccinea*, *Cucumus melo*, *Phacelia tanacetifolia*, *Carduus pycnocephalus*). For each type of flower, five samples were made with 30 to 100 mg of flower anther and analysed for EO trace.

Differences in EO levels on the different parts of workers were analzyed by a Kruskal-Wallis ANOVA test followed by Mann–Whitney U post-hoc tests. The differences in the EO level inside the bee bread and the pollen load were analysed by a Mann–Whitney U tests.

EO variation on beekeeping season and on honey bee genetic sources

We followed the EO levels in three hives every month from May until September on three groups of bees: pollen foragers, nectar foragers and nurses. The hives were placed in the same apiary (same environment), and were distant in genetic profile (*A. m. mellifera*, *A. m. ligustica* and one hybrid hive headed with a natural mated queen).

To obtain nurses of 10 days in age, we used one day old bees. Eleven days before the bee sample, one frame containing last nymphal stage was removed on each hive, and placed separately in an incubator (33°C, 60%RH). The day after one-day-old bees appeared on the comb. These one-day-old bees were marked with an appropriate paint and reintroduced into their initial hives.

To sample the bees, the nurses (painted bees of 10 days old) were caught inside the hive on an open brood frame. After, the hive entrances were closed, foragers that returned after their foraging trip accumulated on the screen. The pollen foragers (bees with pollen loads) and nectar forgers (bees with distended abdomen) were captured with special forceps and placed separately in two boxes. All samples were immediately frozen at -20°C.

Four groups of 5 bees of each modality (pollen foragers, nectar foragers and nurses) were analysed for EO level every month on the three hives. We performed the experiments two consecutive years on three different hives for the two races and on the same hybrid hive. The temperatures were followed from May until September.

The results of the two-year experiment were first analysed separately by a two-way ANOVA (hives and bee groups) followed by a Fisher post-hoc test. The level of EO was transformed: y'=ln(y+1) to attain variance homogeneity. Then the difference in EO levels between the first and the second years was analysed by a Mann–Whitney U tests, and the differences in the EO level by month by a Kruskal-Wallis ANOVA test.

Results

EO distribution in forager

Forager analyses:

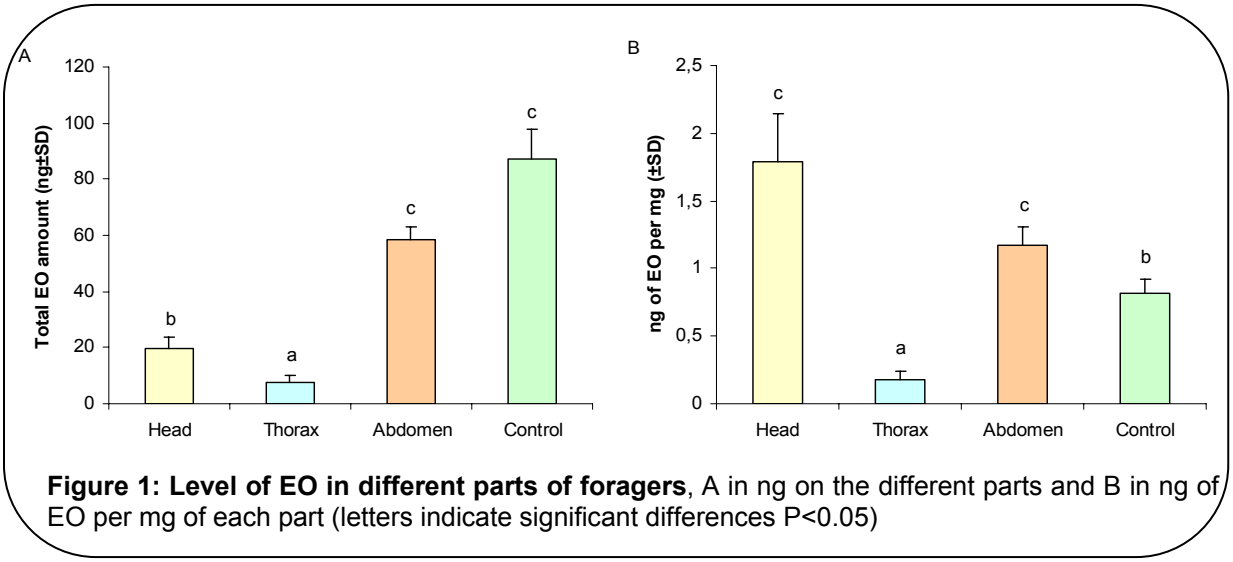

Figure 1: Level of EO in different parts of foragers, A in ng on the different parts and B in ng of EO per mg of each part (letters indicate significant differences P<0.05)

Analysis of the different forager parts showed a significant difference in EO distribution in the three sections (Fig.1): EO amount per section (N =30 H =18.1, df =3, P <0.001) and EO amount per mg of each section (N =30 H =16.31, df =3, P <0.001). Considering the total EO amount per section, the majority of EO was found in the abdomen (69%) (58.34±4.91 ng of EO). This quantity is significantly higher in comparison to the two other parts: the head 23% (19.75± 3.74 ng of EO) (Z = −0.302, P <0.01) and the thorax 9% (7.52± 2.63 ng of EO) (Z =

−3.780, P <0.001). At the EO concentration per mg of each sections, EO was equally concentrated in the head (1.74±0.36 ng of EO/mg) and in the abdomen (1.18±0.13 ng of EO/mg) (Z = −1.436, P =0.15) and significantly less abundant in the thorax (0.18± 0.06 ng of EO/mg) (head vs thorax Z = −0.317, P <0.01 and abdomen vs thorax Z = −0.363, P <0.01).
The EO amount on the cuticle (16.75± 3.74 ng of EO) was 26% percent of the total EO amount found in a forager (64.99± 9.04 ng of EO) (Fig.2).

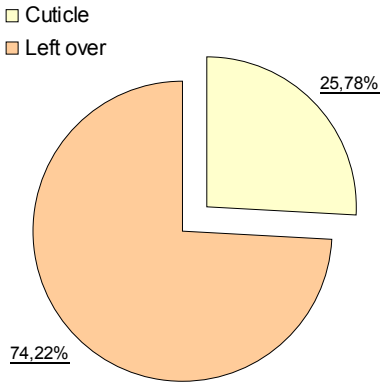

Figure 2: Percentage of EO on the forager cuticle in comparison to the total amount of EO in one forager.

Pollen and nectar analyses:

The nectar did not contain any trace of EO in our experiments; no trace of EO was found in the nectar inside the crop nor in the nectar regurgitated (Table 1). EO was only found in the crop's envelope which represented 12% of the total amount of EO in a forager.

Table 1: EO level in nectar forager and in the different parts of nectar forager.

	OE rates (ng)	SD
Crop envelope	8,89	4,03
Nectar inside crop	0,00	0,00
Regurgitate nectar	0,00	0,00
Nectar forager	72,84	26,28

Unlike the nectar, the pollen contained EO. The EO amounts per 100 mg of pollen load or bee bread were the same (hive 1: Z = −1.757, P =0.07 and hive 2: Z = −1.197, P =0.23).Two pollen loads (4.5±1.2 ng of EO) represented 17% of the total amount in the pollen foragers (21.9±3.8 ng of EO). We did not find EO in any of the pollen extracted from the appropriate flowers.

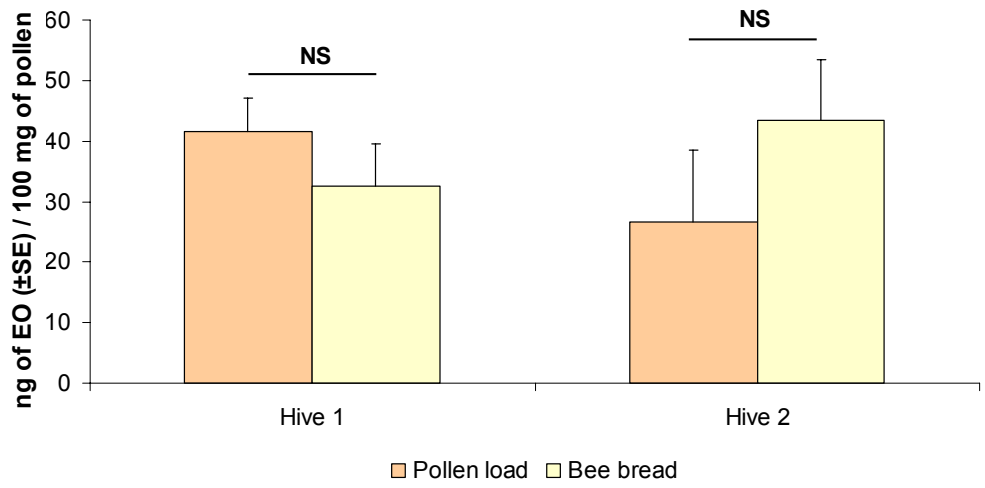

Figure 3: Total EO amount in 100mg of pollen pellets and bee bread from two hives.

EO variation during the beekeeping season

We did not find any significant hive effects on EO titers per bee, but we found differences according to bee groups (nectar foragers, pollen foragers and nurses) in 2008 (Hives: $F_{2,179}$=0.95, P=0.39, Bee groups : $F_{2,179}$=39.58, P<0.001, Interaction : $F_{4,359}$= 1.19, P=0.32) and 2009 (Hives: $F_{2,179}$=2.70, P=0.07, Bee groups : $F_{2,179}$=48.01, P<0.001, Interaction : $F_{4,359}$= 1.40, P=0.23). Because no differences were found between the EO rates on the three hives, the results were pooled (Fig 4). In 2008, nectar foragers had a significant higher level of EO in comparison to pollen foragers and nurses (Nectar vs Pollen: P<0.001, Nectar vs Nurse: P<0.001), and pollen foragers had higher levels of EO than nurses (Pollen vs Nurse: P<0.001). In 2009 the EO level of pollen and nectar foragers were the same (Nectar vs Pollen: P=0.21) but were significantly higher than in nurses (Nectar vs Nurse and Pollen vs Nurse: P<0.001).

The EO honey bee titers change with years (Z=-4.68, P< 0.001) and with months in 2008 (N = 360, H = 43.32, df = 4, P < 0.001) and in 2009 (N = 360, H = 23.8, df = 4, P < 0.001).

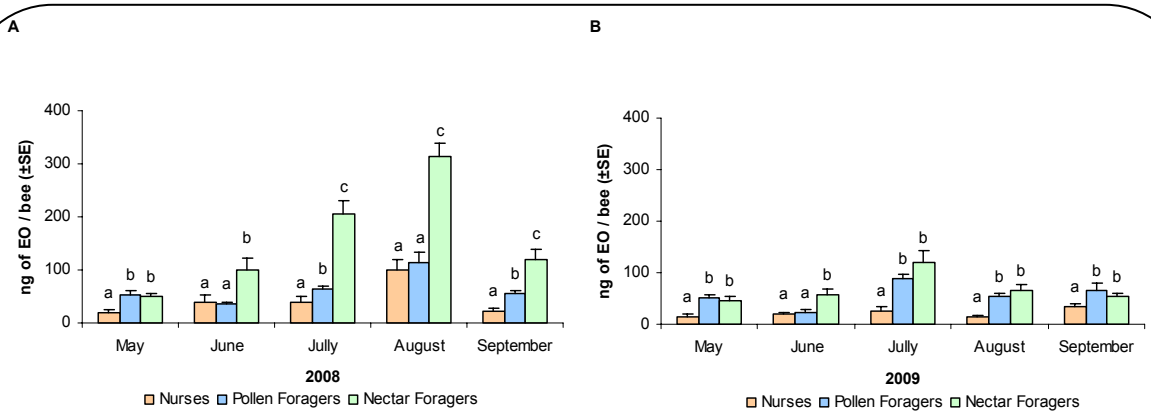

Figure 4: EO amount during seasons 2008 (A) and 2009 (B) in nurses, pollen and nectar foragers. a, b, c denote significant differences P<0.05 between the three group of bee each month.

Discussion

EO is a major pheromone in social regulation of honey bee, being a part of the signal expressed by forager to slow down the maturation of nurses (Leoncini *et al.*, 2004b). The two modes of EO transmission by foragers to nurses seem to be by cuticle contact (antennae and friction) and through the pollen. Variation in EO production occurs in the colony during the beekeeping season. In our experimental conditions the colony genetic origin (race) did not matter, whereas environmental conditions played a major role in this pheromonal variation. Specifically, the season and the foraging specialization of the bees had an effect on EO titers.

Physical contact is required for older bees to inhibit endocrine and behavioral development in younger bees (Huang *et al.*, 1998). EO, as a contact pheromone due to its weak volatility, must be transmitted during food transfer, antennal contact, or licking. Workers seem to perceive colony information via stimuli emanating from the nest during "patrolling" behaviour (Lindauer, 1952). It seems to be also conveyed during trophallaxis (Free, 1965). After measuring its rates in the different parts of foragers, EO appears to be equally distributed in the head and the abdomen and significantly less abundant in the thorax. EO on the cuticle is about 26 % of the total amount of EO in foragers. Thus the EO transfer can be done by cuticle-cuticle or antennal-cuticle contact.
In contrast, the nectar, inside the crop or regurgitated, has no trace of EO, whereas the crop envelope alone contains 12% of the total amount of EO in foragers. The trophallaxis of nectar doesn't seem to be the mode of EO transfer as suggested by Leoncini *et al.* (2004b). During trophallaxis both workers touch each other frequently by antennal contact (Korst, Velthuis, 1982), which supports, because EO is on the cuticle, a possible transfer of EO during trophallaxis through this antennal contact. In comparison, 9ODA is found at 0.67% on the queen cuticle, which is approximately equal to 1000ng of 9ODA, and workers surrounding the queen after 30s have 30 ng of 9ODA (Naumann *et al.*, 1991). Foragers have approximately a total EO level of 100 to 150 ng, with 25 to 37.5 ng on the cuticle, which would allow, in a single contact of 30 sec, nurses to remove 1ng of EO from the foragers.

EO is found on pollen load (17% of the total forager EO amount), in the same proportion as in the bee bread and not found in the pollen of flowers (anthers). So we suggest that a part of EO is provided by pollen foragers in the pollen pellets and the bee bread. Pollen is gathered from

floral anthers by active movements of the legs and the proboscis scraping the anthers, as well as by pollen which drops passively onto the body hairs of workers (Winston, 1987). Subsequently, as the pollen foragers brush their proboscis, clean pollen from their head and their thorax with their forelegs, EO can be added passively by the transfer of EO found on the cuticle. EO can also be directly added in the regurgitation of honey and other substances for the formation of the pollen pellets by the foragers.

When the pollen foragers finish their pollen collecting and return to the hive, they remove their pellets of pollen from their basket directly into pollen cells. Then others workers (hive workers considered as nurses) pack the pellets for storage using their mandibles and forelegs to press the pollen (Winston, 1987). Pollen nourishment during the first 8-10 days of workers life is essential for proper post-emergence glandular development and growth of internal structures (Haydak, 1970). Therefore, nurses eat and are in contact with pollen, and presumably assimilating EO. The pollen is a mode of EO transmission inside the colony, but also a possible way to store EO. In the colony, when pollen foraging amplifies, the amount of beebread increases which also increases the quantity of EO inside the colony. As the function of EO is to delay the age at onset of foraging, the accumulation of EO inside the beebread could be a signal to decrease the foraging activity, by delaying the maturation of nurses.

The augmentation of the amount of stored pollen in the colony concurrently increases brood rearing and decreases pollen foraging activity to a homeostatic set point (Fewell, Winston, 1992). In addition, pollen foraging decreases only when foragers have direct access to stored pollen (Dreller *et al.*, 1999). Therefore, EO could be the inhibitory signal of foraging, first by decreasing the behavioural maturation of nurses to reduce foraging activities, and second by having a direct action on pollen foraging. This second hypothesis needs to be tested.

The modification of colony age structure is one aspect of the environment that changes throughout the year owing to changes in worker age at first foraging. Different factors can alter the division of labour of a colony including internal and external hive factors (Robinson, 1992). Here we demonstrated that the EO level varies in the colony between months of the beekeeping season. That would suggest a difference in the control of nurse behavioural development by foragers over the months. The EO level is weak at the beginning of the season when the demand in foraging is high due to an abundance of flowers in the area. Then EO level increases in July and August when fewer flowers are found in the area, thus the demand for a forager force is less. When in September there is a renewal of flowers blooming in the area, the forager workforce increases and the EO level decreases. Environmental

conditions seem to play a major role in the workers' behavioural maturation, and correspondingly, the ovary, vitellogenin titre, and JH level of worker bees respond to changing environmental conditions (Huang & Robinson 1995; Amdam et al. 2004a; Linksvayer et al. 2009) and then to worker' pheromonal levels.

Racial differences are important components of the division of labour among worker honey bees. *A. m. ligustica* bees have faster rates of behavioural development than *mellifera* (Brillet *et al.*, 2002), with a demonstrated genetic mechanism conferring these differences (Whitfield *et al.*, 2006a), yet their production of EO is the same. Thus it seems that racial differences, between *ligustica* and *mellifera* nurses, in the sensitivity to the social inhibitor could be responsible for differences in rates of behavioural development. Racial differences in sensitivity to social factors have been previously observed as genotypic differences in response thresholds to task-related stimuli (Beshers et al. 1999).

Recent studies have demonstrated that *Nosema* spp. (honey bee parasite) significantly alter EO production in the honey bee colony (Dussaubat *et al.*, 2010). Therefore it is now important to determine if bees are sensitive to this variation in EO level in the colony and adjust their maturation to a lower ratio of foragers to hive bees in response to the disease (which causes an increase of EO titers) or in response to a loss in foragers (which causes a decrease of EO titers). Studies on QMP show that workers discriminate between different queen's pheromone extracts (Kocher *et al.*, 2010; Kocher *et al.*, 2009). Workers are more attracted by pheromone extracts of queens with higher levels of ovary activation (Kocher *et al.*, 2009). In addition there are strong genetic and seasonal components to QMP response by worker honey bees (Pankiw *et al.*, 1994; Pankiw *et al.*, 1995). Thus we predict that workers are also sensitive to EO fluctuation but new studies are needed to understand if bees respond to EO differentially during the season and to different levels of EO.

Honey bee colonies seem to adjust their EO production to environmental changes. In the honey bee colony, production of a signal is modulated and responders are able to perceive this variation and adjust their responses (Hoover *et al.*, 2005b; Kocher *et al.*, 2009; Pankiw *et al.*, 1994). The complexity of the chemical communication in honey bee reveals a more complex language between emitters and receivers than previously thought, regarding the variation in signal production and responses by workers in the colony.

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Transition :

La découverte d'une production dynamique de la phéromone EO en fonction de la saison renseigne le fait que les abeilles puissent s'adapter aux conditions externes de la ruche. La transmission de cette molécule par contact indique que tous les individus actifs ou inactifs recoivent l'information par simple contact dans la colonie. Ainsi, il est possible que lorsqu'un certain seuil de présence de cette phéromone est dépassé (après un certain nombre de contacts, accumulation d'EO sur la cuticule), l'abeille répond à ce stimulus.
La transmission par le pollen permet un stockage de la phéromone dans la colonie et constitue un moyen d'indiquer les besoins en butineuses : une réserve accrue de pollen dans la ruche revient à un fort taux d'EO dans la colonie, ce qui induirait un ralentissement de la maturation des nourrices.

L'EO est une phéromone majeure dans la régulation du nombre de butineuses et de nourrices pour le développement de la colonie, une modification de son taux « normal » peut déstabiliser les mécanismes de la communication dans la colonie. Après avoir analysé la dynamique et la transmission de l'EO dans la colonie, les résultats de l'article suivant présentent l'impact de stress externes, un pesticide et une maladie de l'abeille, sur la production de l'EO. Aujourd'hui, les causes importantes de mortalité d'abeilles de par le monde semblent être d'origine multifactorielle. Nous avons voulu savoir si des facteurs de stress peuvent désorganiser la communication chimique et expliquer en partie les syndromes d'affaiblissement des colonies.

Nosema spp. Infection Alters Pheromone Production in Honey Bees (*Apis mellifera*)

Résumé :

Chez les insectes sociaux, et notamment chez l'abeille domestique, les phéromones participent activement à l'homéostasie du groupe. Les parasites ou les maladies peuvent modifier les taux hormonaux de leur hôte. Dans la littérature, la colonie peut être comparée à un superorganisme, les abeilles sont vues comme les cellules et les phéromones comme des hormones. Dans un organisme, un stress peut entrainer un bouleversement hormonal induisant de grandes complications. Nous avons donc voulu savoir si, comme pour les hormones, des stress pouvaient modifier les taux de phéromones dans la colonie d'abeilles et perturber son fonctionnement.
Des facteurs de stress environnementaux, tels que les pesticides ou des maladies, peuvent affaiblir les colonies d'abeilles. Nous avons alors analysé l'effet de l'imidaclopride, un pesticides de la famille des néonicotinoïdes largement utilisés en agriculture, et l'effet *Nosema* spp., un parasite généraliste de l'abeille, sur la production d'EO par les ouvrières.

Des abeilles ont été exposées en cagette au pesticide et au pathogène. Après 10 jours, les taux d'EO des abeilles traitées et des contrôles ont été analysés. Contrairement à l'imidaclopride, *Nosema* spp. modifie la production de l'EO. Le niveau d'infection des abeilles par *Nosema* spp. est positivement corrélé avec leur niveau de production d'EO.

En conséquence, comme l'EO est impliqué dans la régulation de la division du travail des ouvrières, nos résultats suggèrent que des augmentations de production d'EO par des abeilles infestées par le parasite peuvent perturber la communication chimique de la colonie et donc son homéostasie.

Nosema spp. Infection Alters Pheromone Production in Honey Bees (*Apis mellifera*)

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Key words: Primer pheromone, honey bee, ethyl oleate, *Nosema* spp., imidacloprid.

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Abstract:

Pheromones in social insects play a key role in the regulation of group homeostasis. It is well-established that parasites can modify hormone signaling of their host, but less is known about the effect of parasites on pheromone signaling in insect societies. We thus tested in honey bees (*Apis mellifera*) the effect of the widespread parasite *Nosema* spp. on the production of ethyl oleate (EO), the only identified primer pheromone in honey bee workers. Since environmental stressors like pesticides can also weaken honey bees, we also analyzed the effect of imidacloprid, a neonicotinoid widely used in agriculture, on EO production. We show that, contrary to imidacloprid, *Nosema* spp. significantly altered EO production. In addition, the level of *Nosema* infection was positively correlated with the level of EO production. Since EO is involved in the regulation of division of labor between workers, our result suggests that the changes in EO signaling induced by parasitism have the potential to disturb the colony homeostasis.

Introduction

Analogous to the hormones that control the organism homeostasis, pheromones in social insects play a key role in the regulation of group homeostasis. However, the homeostasis of both organisms and insect societies can be threatened by parasite infection. For example, in mammals there is clear evidence that parasites can modify the endocrine system of the host to favor their development and reproduction (Escobedo *et al.*, 2005). In honey bees, the cuticular hydrocarbon profile involved in social recognition can be altered by an activation of the immune system (Richard et al. 2008) or parasitization by the mite *Varroa destructor* (Salvy *et al.*, 2001). However, it is not known whether, analogous to the modification of hormone signaling in the organism, parasites can affect pheromone signaling in insect societies.

To answer this question, we asked whether the microsporidia *Nosema* spp., potentially involved in the worldwide honey bee losses (Higes *et al.*, 2008), could affect the production of pheromone in workers. We analyzed the production of the only indentified primer pheromone in workers: ethyl oleate (EO), which regulates worker behavioral maturation (i.e. inhibits the transition from inside-nest tasks performed by young bees (nurse) to foraging tasks performed by old bees (forager) (Leoncini *et al.*, 2004b). The focus was done on primer pheromone because they are essential to the regulation of social behaviours and colony

homeostasis. Therefore, a modification in their production could affect the whole colony organization and endanger its survival. Since the survival of honey bees can be threatened by other stressors, like pesticides, we also tested the effects of a neonicotinoid (imidacloprid) widely used in agriculture on EO production. Pesticides are known to disrupt pheromone perception but they can also affect their production (Desneux *et al.*, 2007).

Methods and Materials

Nosema infection and imidacloprid exposure. This experiment was part of a larger study described in details by Alaux et al. (2010a). Briefly, in order to test the effect of *Nosema* infection and/or imidacloprid exposure, one-day old bees were reared in cages and split into four experimental groups: control groups, groups infected with *Nosema*, groups exposed to imidacloprid and groups both infected with *Nosema* and exposed to imidacloprid. For each experimental group, 3 colonies were used, with 2 cage replicates for each colony (N=120 bees per cage). For the *Nosema* infection, bees were individually fed at the beginning of the experiment, with a sugar solution containing 200,000 spores (Alaux *et al.*, 2010a). Spores were previously isolated from infected colonies as in Higes et al. (2007) and genetic analysis showed that our bees were infected with both *N. apis* and *N. ceranae* (see Alaux *et al.*, 2010a). For the pesticide exposure, caged bees were chronically exposed 10 hr per day to imidacloprid by ingesting a sugar solution containing 7 µg/kg of imidacloprid (concentration encountered in the environment) (see Alaux *et al.*, 2010a). The solution was replaced each day. After 10 days, bees were collected and stored at -20°C in order to measure the level of EO and *Nosema* infection.

EO quantification. Pools of 5 bees were analyzed. Whole-body extracts were prepared in 1.9 ml of iso-hexane with the addition of 100 µl of two internal standard solutions at 10 ng/µl (arachidic acid methyl ester and methyl heptadecanoate, Sigma-Aldrich, France). Samples were crushed with a glass rod during 2 min at 0°C and centrifuged for 20 min at 4°C (2,500 g). The supernatant was collected and applied to a silica column (silica gel 60, particle size 40–63 m, 230–400 mesh). The first fraction was eluted in 3 ml of a solvent mixture (98.5% iso-hexane, 1.5% diethyl ether). Then, the second fraction containing the EO was eluted in 3 ml of a second solvent mixture (94% iso-hexane, 6% diethyl ether). 1 ml of this fraction was concentrated to 10 µl under a nitrogen stream and 1 µl was injected into a fast gas

chromatograph (2014, Shimadzu, Japan) equipped with a split-splitless inlet, a flame ionization detector, and a capillary column Omegawax 100 (10 m x 0.10 mm, 0.10 µm film thickness). The samples were injected in split mode. Hydrogen was used as carrier gas with a column flow of 0.52 ml/min. Oven temperature was set at 90°C for 1 min, raised to 195°C at 40°C min^{-1}, stabilized for 3 min then augmented to 210°C at 1°C min^{-1}, stabilized again for 2 min then increased to 270°C at 40°C min^{-1} and held at 270°C for 3 min. Identification and quantification of EO was based on retention times of EO synthetic compound (Sigma-Aldrich, France) and by comparison of internal standard area respectively using gas chromatography solution program (Shimadzu, Japan). The EO confirmation was done by a mass spectrometer (CP2010, Shimadzu, Japan) operated in the electron impact mode at 70 eV with continuous scans (every 0.2 sec) from a mass to charge ratio (m/z) of 70 to 400.

Nosema spore counting. Since *Nosema* is an intestinal parasite, the honey bee intestinal tract was dissected and macerated in distilled water as in Higes et al. (2007). Then, the spore concentration from the suspension was determined using a haemocytometer.

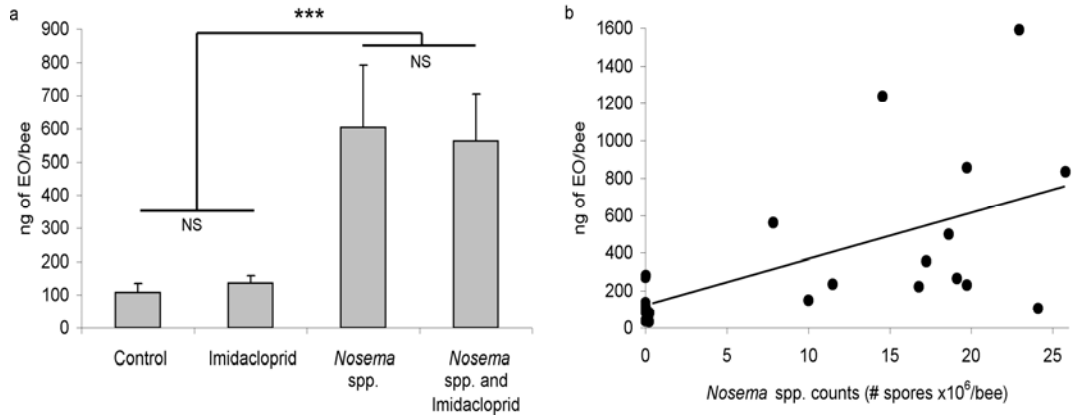

Figure 1: Effect of *Nosema* infection and/or imidacloprid exposure on EO production in honey bee workers. a EO level for each experimental group. Two pools of 5 bees per cage were analyzed, with 2 cages per treatment. The experiment was replicated on 3 colonies giving a total of N=60 bees per treatment. Treatment and colony effects were determined using two-way ANOVA on log-transformed values followed by Fisher post-hoc tests. There was a significant treatment effect on EO production ($F_{3,47}=17.35$, $P<0.001$). Bees infected with *Nosema*, with or without an exposure to imidacloprid, had a higher level of EO than control and imidacloprid-exposed bees ($P<0.001$ for each comparison). However, imidacloprid did not affect EO production (control vs. imidacloprid-exposed bees: $P=0.81$; *Nosema*-infected bees vs. *Nosema*-infected and imidacloprid-exposed bees: $P=0.14$). There was also a significant effect of colony origin ($F_{2,47}=4.59$, $P=0.017$) but no significant interaction with the treatments was found ($F_{6,95}=0.88$, $P=0.52$) demonstrating a consistent effect of the treatments. Data show mean±SE. *** and NS denote significant ($P<0.001$) and non-significant differences between treatments, respectively. b Relation between EO production and the level of *Nosema* infection. There was a significant positive correlation between the quantity of EO produced and the number of *Nosema* spores infecting bees

Results and Discussion

Nosema infection caused a significant increase in EO production compared to non-infected groups (Fig. 1a), demonstrating that pheromone production can be modified by environmental stressors. However, EO production in imidacloprid-exposed bees did not differ significantly from non-exposed bees (Fig. 1a). Neonicotinoids target the nicotinic acetylcholine receptors and so can affect neural function (Decourtye *et al.*, 2004) but here no effect was found on pheromone production. Studies with different pesticides are needed to determine whether this absence of modification we observed is a general phenomenon.

One would expect that *Nosema* infection induces a cost to pheromone production. Contrary to this expectation, parasitized bees produced more EO than healthy bees. Since, EO is present at higher levels in foragers compared to nurses (Leoncini *et al.*, 2004b) and *Nosema* causes a precocious onset of foraging (Wang, Mofller, 1970), the EO increase might reflect a forager profile of infected bees compared to control bees. However, further investigations tend to show that the EO increase is not just a consequence of a forager profile. First, the level of EO in parasitized bees was 6 times higher than healthy bees, which is greater than the difference naturally found between nurses (young bees) and foragers (old bees) (100±19 ng EO/nurse, n=60 and 213±25 ng EO/forager, n=120, unpublished data from n=3 colonies, A. Maisonnasse). Second, there was a positive and significant relationship between EO level and the number of *Nosema* spores per bee (Fig. 1b), showing that the EO increase is not an all-or-nothing response but is linked to the level of *Nosema* infection.

Even if the earlier onset of foraging could be a bee response to decrease the *Nosema* load within the hive, the higher EO level in infected bees has the potential to disturb the colony organization. The abnormally high level of EO could mislead the colony on the actual number of foragers and delay the onset of foraging in non-infected nurses, but it is not know how infected bees, who accelerate they behavioral maturation, would react to the high inhibitory effects of EO. On the other hand, since *Nosema* infection decreases worker lifespan (Higes *et al.*, 2007), a loss of EO in the colony can also be expected and accelerate nurse maturation. Field studies are needed to determine the actual response of the colony and whether a failure in pheromone communication induced by parasitism or a disease can lead to the colony collapse.

To our knowledge, this is the first demonstration that parasites can modify pheromone production in insect societies. Therefore, our finding indicates that pathogens, besides their effect at the individual level, can also cause damage at the social level.

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Transition :

La production de l'EO varie en fonction de l'environnement de la ruche et le parasite *Nosema* spp. augmente anormalement sa production (Fig. 12). La prochaine étape de cette étude consiste à comprendre si les variations de production de l'EO engendrent des variations de réponse des ouvrières et un changement dans leur développement comportemental.

L'étude complète d'une phéromone est difficile en raison de la complexité de la société d'abeilles (contexte, synergie…). La phéromone la plus étudiée chez l'abeille est la QMP et plus particulièrement le 9-ODA. Malgré les connaissances des effets pleiotropiques de cette phéromone, il semblerait que cette phéromone ne soit pas la seule utilisée par la reine pour contrôler la colonie. Dans le chapitre suivant nous avons étudié la possibilité d'un signal redondant chez la reine qui lui permettrait d'appuyer son rôle d'individu central de la colonie.

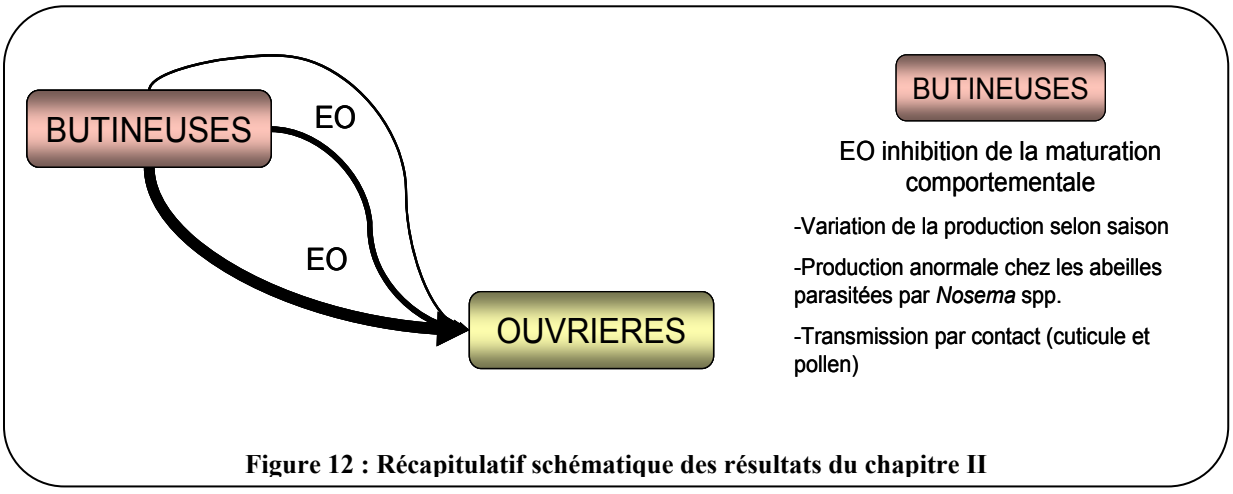

Figure 12 : Récapitulatif schématique des résultats du chapitre II