FORAGING BEHAVIOUR AND PHYSIOLOGY OF BEES: IMPACT OF INSECTICIDES

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LIST OF THE ORIGINAL PUBLICATIONS

The thesis is a summary of the following papers, which are referred to by Roman numerals in the text. The papers are reproduced by kind permission of the following journals or publishers: Society of Chemical Industry (I), European Journal of Entomology (III) and Apidologie (IV).


ABBREVIATION

a.i. active ingredient
CCD colony collapse disorder
DGC discontinuous gas exchange cycles
$\text{FO}_2$ Oxygen flux
FO flutter-open cycle
FV flutter-ventilation cycle
IR infrared
IRA infrared actograph
IRGA infrared gas analyser
ppm parts per million
PSV passive suction ventilation
RH relative humidity
$V_{CO_2}$ rate of CO$_2$ emission
1. INTRODUCTION

Pollination is one activity in the insect-plant relationship which offers advantages to both interactors. For plants, the benefit involves fast transportation of the pollen grains; for insects, pollen and nectar represent food. In the process, the pollen from anthers must be transported to the stigmas of the same or different flowers. Even plants which have flowers capable of self-pollination benefit from cross-pollination, as more pollen grains are transported to the stigmas. The interaction of plant and pollinator ensures abundant, high quality plants and seeds.

For these reasons, the decrease in the numbers of bees in the agricultural landscapes is an alarming tendency (Thomson, 2001, Biesmeijer et al., 2006, Gabriel, Tscharntke, 2007). Agricultural practice including the intensive use of pesticides has been cited as one reason for the decline (Osborne et al., 1999, Miranda et al., 2003). Bumble bees are more vulnerable than honey bees; for a long period in late spring, when only the queens are collecting food, their colonies are smaller, and they do not have the trophallaxis that could diminish the amount of pesticide residues possibly reaching the larval food. Furthermore, their foraging behaviour differs from that of honey bees (Alford, 1975). The restrictions for applying insecticide often take the foraging timetable of honey bees into account (e.g. spraying is allowed early in the morning or late in the evening), but this is exactly the time when bumble bees are most active (Thompson, 2001).

Pesticides have been linked to some new bee diseases, such as Colony Collapse Disorder (CCD) found in the USA. The primary symptom is a sudden disappearance of adult bee populations without any accumulation of dead bees: only the queen and a few young workers remain even though brood, pollen and honey are present, and there is little or no evidence of hive-robbing or other diseases (Barrionuevo, 2007, Bradshaw, 2007, McDonald, 2007). Although there are no scientific studies on the causes of CCD yet, the residual accumulation of pesticides in the hives has been considered as a possibility. The pesticide residue may cause orientation disorders in young bees resulting in a loss in navigational ability: they simply get lost out on the fields and never return to their hives (Engelsdorp et al., 2007).
Even though the effects of pesticides on bees are severe (see for review Thompson, 2001, 2003), big gaps still exist in scientific research on the topic. Most research is restricted to laboratory experiments that may not adequately reflect the situations on fields (Thompson 2001, 2003). Many factors affect the movement and transmission of pesticides in the open: wind, temperature, the combining of different pesticides, the attractiveness of a particular food plant species for pollinators. The toxicity of pesticides on arthropods is mostly studied on the basis of mortality (Thompson, 2003), but pesticide toxicity is a complex question. For example, contact with a pesticide may cause knockdown which might be reversible if death does not occur due to dehydration (Jagers op Akkermans et al., 1999a). Some pesticides (e.g. pyrethroids, azadirachtin) affect the muscles of the insect (Mordue (Luntz), 1993), possibly leading to difficulty in controlling the spiracular openings or an inability to move.

The physiological conditions of insects accurately reflect the effects of chemical compounds. Therefore, to gain a better understanding of complex processes following pesticide poisoning of arthropods, it is necessary to investigate the physiology and toxicokinetics, as well as behaviour, of the animals affected with pesticides.
2. REVIEW OF THE LITERATURE

In the present study I consider the reasons for pollinator decline, with attention to different aspects of the foraging behaviour of bees, focusing on honey bees and bumble bees. I include a brief overview of insect respiration and its reaction to pesticides.

2.1. Pollinator decline in agricultural landscapes

In Europe the agricultural landscapes are over 2000 years old (Groppali, 1993). Many species of wildlife have adapted to these managed landscapes, resulting in anthropogenic species-rich ecosystems (Kleijn et al., 2006). The presence of relatively small semi-natural areas help to maintain the richness of pollinator species in general and increases the abundance of pollinators in surrounding intensively farmed areas (Michener, 2000, Öckinger, Smith, 2007).

The intensification of agriculture has lead to a rapid loss of species-richness in farmlands (Benton et al., 2003). The populations of both wild plants and wild bees have been declining during the last few decades (Robinson, Sutherland, 2002, Goulson, Darvill, 2004, Carvell et al., 2006). Several factors have been suggested as possible contributors to these declines, including competition from the honey bee, changes in climate, and the effects of predators and parasites (Williams, 1986). However, the principal factor is likely to have been the loss and degradation of habitats and critical food resources due to changes in land-use and agricultural practices (Goulson et al., 2005, Williams, 2005).

Common agricultural practice involves intensive use of pesticides, which have both direct and indirect effects on organisms. A direct effect is the toxicity of the compounds to insects; indirectly, the pesticide use can lead to a decline in biodiversity as well as sub-lethal effects on the colonies, on the brood and on the behaviour of bees. The pesticide production process also involves laboratory experiments investigating the toxicity of the compounds on different organism groups, and there are restrictions on pesticide use intended to limit its harmful consequences for both beneficial and non-target organisms. Yet there is evidence that,
in the case of bees, results of laboratory or semi-field tests may not reflect the conditions on open fields (Thompson, 2001, 2003). In order to reverse the damage to biodiversity, farmers are being compensated for their efforts to use more nature-friendly agricultural techniques (Kleijn et al., 2006, Carvell et al., 2007).

2.2. Pollinator foraging behaviour

2.2.1. Pollen and nectar as food resources

In the mutual relationship of pollination, the interactors have an inherent conflict of interests: plants need bees as pollen vectors but bees only use flowers as a food source. Originally plants offered pollen as food; nectar, which has a water component, evolved secondarily, to provide a food resource that is less costly to the plant.

Bees use pollen as the prime protein source for raising larvae. Unlike bumble bees, honey bee larvae do not consume pollen directly throughout their entire development. During their early development, they are fed by glandular secretions of adult workers, who eat pollen both to feed the larvae (Dobson, Peng, 1997, Hrassnigg, Crailsheim, 1998, Babendreier et al., 2004) and to satisfy their own protein needs (Smeets, Duchateau, 2003).

Chemically the pollen consists of proteins, starch, carbohydrates, lipids and pectines (Runge, 1973). Pollen grains consist of three layers: the outer exine (pectines and lipids), the intine (starch and carbohydrates), and the inner protoplasm, which contains mostly proteins (Dobson, 1991). The exine layer greatly resists digestion, but is perforated by pores that lead to the intine (Roulston, Cane, 2000). Storing the pollen helps to ease the digestibility of the pollen grain when it comes into contact with nectar and enzymes (Dobson, Peng, 1997, Roulston, Cane, 2000, Human, Nicolson, 2006).

The nutritional values of pollens vary among plant species and for insect species. Food preference may be based not only on the chemical composition (Dobson, 1988, Kim, Smith, 2000, Roulston, Cane, 2000, Cook et al., 2003, Markowicz Bastos, et al. 2004), protein concentration (Rasheed, Harder, 1997, Roulston et al., 2000, Roulston, Cane, 2000,

Nectar is a liquid substance that serves both as an attraction to the bee and as a reward for the pollination service (Baker, Baker, 1973). Of the several hypotheses about the origin of floral nectar, the most widely held is the “leaky phloem” hypothesis: the phloem is also the sugar-rich solution that provides flowers with nutrients (Barrera, Nobel, 2004). In addition to water and sugars, nectar contains small quantities of amino acids, lipids, antioxidants, minerals and secondary metabolites (Faegri, van der Pijl, 1979, Gardener, Gillman, 2001a, Gardener, Gillman, 2002, Barrera, Nobel, 2004). The presence and amount of trace elements in the nectars may explain why some sugar-rich nectars are not or less attractive to bees (Adler, 2000, Afik et al., 2006). The exact chemical composition of nectar varies among the plant species (Faegri, van der Pijl, 1979, Adler, 2000), flower types and even the soil properties of a particular location (Waser, Mitchell, 1990, Gardener, Gillman, 2001b). For bees, nectar is important as an easily assimilable energy source (Faegri, van der Pijl, 1979) and as the proper medium for supporting the digestion of pollen grains (Roulston, Cane, 2000).

2.2.2. Flower odour as the basis for searching food

Olfactory signals have been thought to be the most important signals for the bees’ recognition of food sources while foraging (Kriston, 1973, Menzel, et al., 1993). However, scientists have argued the prevalence of colour versus scent. The priority of colour over scent has been found in halictid bees (Roy, Raguso, 1997) and in one butterfly species (Ômura, Honda, 2005).

The chemical signals may act directly as long or short distance attractants, or may function as indirect cues: young bees remember the smell of the food they ate inside the hives and search for it during their first foraging trip. Bees are able to differentiate a large number of olfactory signals and learn to predict foods offer rewards and which do not (Menzel et al., 1993, Laska et al., 1999, Gumbert, Kunze, 2001). Since floral odours are blends containing tens to hundreds of components which vary in quality
or quantity over time and in space, the generalization process is fundamental for bees’ survival (Sandoz et al., 2001). From the floral extracts, comprising multiple components, bees restrict the number of scent components they use in their searching (Pham-Delegue et al., 1997, Laloï et al., 2000). It is considered possible that bees may avoid plants treated with pesticides due to the repellent odours of the compounds (Shires et al., 1984).

### 2.2.3. Flower constancy

Of all flower-visiting insects, bees exhibit a high level of flower constancy; in fact, it is their behavioural specialty. They must gather food not only to feed themselves but also their brood.

Foraging bees often show a kind of flower constancy favoring some and bypassing others that might offer rewards (Free, 1970, Gegear, Thomson, 2004). It has been claimed that bees have no innate flower colour preference (Waser, Price, 1983, Gumbert, Kunze, 2001) but develop them over time, testing various flower types until they find one that offers a reward (Pohtio, Teräs, 1995). It is suggested that flower constancy may have evolved to save energy and/or time for the foragers (Free, 1970, Dukas, 1995, Gegear, Thomson, 2004). The level of flower constancy varies among bee species even belonging to the same genera (Free, 1970). Complex flowers require more handling skills than do simple flowers. A bee that is morphologically suited to the flower shape is able to learn the easiest way to obtain a reward (Laverty, 1994, Gegear, Laverty, 1998, White et al., 2001).

Both memory and the learning capacity of insects are usually under-estimated. Studies in honey bees (under both natural and laboratory conditions) demonstrate that learning is fast and comprises various levels of cognitive processing, such as generalization, categorization, concept formation, configuration, and context-dependency (Menzel, Giurfa, 2001, Gegear, Thomson, 2004).

The honey bee’s memory is rich, highly dynamic, and long-lasting (Menzel, 1999). Despite that, it is probably not feasible for them to store the locations of several hundred individual flowers (Goulson, 2000). Although they may restrict the number of flower choices due to their
limited memory patterns (Dukas, 1995, Menzel, 2001) they also create new scents to improve their foraging. When collecting nectar or pollen, they deposit short-lived repellent odours on the flower corolla to ease demands on memory (Cameron, 1981, Free, Williams, 1983, Goulson et al., 2000, Goulson et al., 2001, Stout, Goulson, 2001). These marks can be used intra- or inter-specifically to avoid visiting empty flowers (Goulson et al., 2000, Goulson et al., 2001, Gawleta et al., 2005). However, bees do not rely entirely on those pheromone scent marks. They sometimes encounter situations when it could be profitable to ignore the scent marks and probe the newly visited flowers (Saleh et al., 2006).

2.2.4. The sub-lethal effects of pesticides on the behaviour of bees

The effects of pesticides on non-target organisms have been studied extensively. It is obligatory for chemical companies to provide mortality data for their products for all larger organism groups. But, despite research data indicating the severe mortality rate on insects, less attention has been paid to the sub-lethal effects.

Application of insecticides is often allowed during the flowering period of a given crop. However, even when insecticides are not sprayed on flowers, the residues of the compounds still contaminate nectar and pollen in sub-lethal doses via both active and passive transport (Thompson, 2001). Many insecticides have been described as safe to bees because they do not kill them, although sub-lethal doses may result in a decrease in their foraging and navigational abilities (Gels et al., 2002). Under certain circumstances, the sub-lethal effects may cause more harm than lethal doses since they affect the survival of the brood and colony.

In the colonies of social insects the division of labour plays an important role. Each worker has specific, often age-dependent tasks. Treatment of honey bees with juvenile hormone analogues, (synthetic hormone-like compounds used as insecticides), results in a decreasing ability of young emerging bees to feed larvae, due to the early degeneration of the hypopharyngeal glands and precocious foraging ability (Tasei, 2001). Changes in the division of labour of honey bees - decreased house cleaning abilities, delayed onset and duration of foraging and handling of nectar – have also been recorded (reviewed by Thompson, 2003). Organophosphate insecticides may decrease the longevity of honey bees
(Johansen, Mayer, 1990). Juvenile hormone analogues also affected the overwintering of colonies (Thompson et al., 2005).

Foraging depends on the bee’s ability to discriminate odours, to learn, to communicate, and to orientate to its environment; altering these systems may result in a decrease in foraging. The bees’ orientation and communication ability have been found to be affected by sub-lethal doses of organophosphorus (Schricker, Stephen, 1970), synthetic pyrethroids (Cox, Wilson, 1984, Vandame et al., 1995) and at least one neonicotinoid (Bortolotti et al., 2003). Pyrethroids and neonicotinoids have also been shown to affect both foraging activity (Thompson, 2003) and learning capacities (Decourtye et al., 1999, 2003, Guez et al., 2001, Ramirez-Romero et al., 2005). Pyrethroids may also affect thermoregulation (Jagers op Akkerhuis et al., 1999b, Belzunces et al., 2001); in cooler climates, that can lead to decreased flying ability. The decrease in foraging and in returning foragers reduces the brood production (Thompson, 2003), and weakens a colony’s potential for surviving the winter.

The reduction of the brood may have more damaging consequences for honey bees than simply the moderate loss of foragers (Haynes, 1988). Aside from brood mortality there can be changes in larval development (both prolonged development time and malformations may occur) due to the contamination of the food by pesticides (Tasei, 2001). Some organophosphates have affected the queen’s status or have interfered with a colony’s ability to requeen itself (Stoner et al., 1985, Thompson et al., 2005). In solitary bees, pyrethroids have been found to affect the queen’s fecundity (Tasei et al., 1988). Neonicotinoids (Tasei et al., 2000) and organophosphates (Johansen, Mayer, 1990) have decreased the bumble bees’ brood production.

In addition to ignoring the sub-lethal effects of insecticides, there exists the problem of extrapolating data from honey bees to bumble bees. Pesticide risk assessments for honey bees are based on hazard ratios which rely on application rates and toxicity data that are unlikely to be appropriate for bumble bees. Bumble bees are active at different times and on different crop species and, therefore, are likely to have different exposure profiles. Unlike honey bees, deaths of bumble bees due to pesticides are unlikely to be reported, since the bees are not kept domestically and die in small numbers (Thompson, Hunt, 1999).
2.3. Discontinuous gas exchange in insect physiology

Understanding pesticide effects in the field also requires insight into the effects on different physiological functions, e.g. respiration. In the case of bees it is hard to examine the effects of pesticides on respiration patterns because there is little data on the respiration patterns at all.

Since water is a key element in every living organism, it is probable that most insects have (Klowden, 2002) evolved specific patterns to prevent excessive water loss. Resting insects often exhibit discontinuous gas exchange cycles (DGC), a function of which may be reduction of respiratory water loss (Hadley, 1994) through the large inner surface of the tracheal system.

In the state of discontinuous gas exchange, the spiracles are closed most of the time. At low oxygen rates the spiracular valves flutter, allowing oxygen to enter the tracheal system. As larger amounts of carbon dioxide accumulate in the tracheae, the spiracles open and allow the gas to escape. Thus as compared with continuous respiration, loss of carbon dioxide along with evaporated water occurs only discontinuously during the brief open phases of the spiracles. Water balance is maintained because the amount of metabolic water generated by hydrolysis of stored fats equals the amount of water lost during this discontinuous respiration (see review from Hadley, 1994).

There are different arguments about the origin of DGC, as reviewed by Chown (2002). Lighton (1994) found respiratory water loss to be such a small part of total water loss that it is unlikely to have a negative effect on soft-bodied insects. Kestler (1980, 1982) and Barnhart and McMahon (1987) have stressed the importance of passive suction ventilation (PSV) in reducing water loss in insects. Essentially, these authors stated that over the entire gas exchange cycle, it is CO₂ release that limits the extent to which ventilation and, consequently, water loss can be minimized. There are also hypotheses that DGC serves as an adaptation for coping with hypercapnia and/or hypoxia in soil-living insects (Lighton, 1998, Vogt, Appel, 2000, Lighton et al., 2004).

The existence and the precise pattern of DGC depend on the environment and life stage of the individuals. For example, bumble bee (B. terrestris) queens have been found to exhibit discontinuous ventilation cycles.
at room temperature in different life stages, before and after hibernation (Beekman, Stratum, 1999). The distinct environmental conditions that the queen was exposed to affected the frequency and amount of carbon dioxide emitted. In honey bee workers the temperature also affected the DGC (Lighton, Lovegrove, 1990). Kuusik and co-workers (2002) studied the respiratory patterns of bumble bee (*B. terrestris*) workers at low temperatures when they were restrained and motionless, to rule out their high activity at room temperature.

2.4. Sub-lethal effect of pesticides on DGC

The patterns of DGC have been used for characterizing the physiological state of an insect, while several stress factors including the chemical ones can affect it (Kestler, 1991). Although knowledge about the sub-lethal effects of pesticides is scarce, it is known that treatments of arthropods with pyrethroids cause neurotoxic effects in parts of the nervous system, including the central nervous system and sensory, motor or neurosecretory neurons (Corbett, 1974, Jagers op Akkerhuis *et al.*, 1995). Because the closing and opening of spiracular valves is controlled by the nervous system, the neurotoxic effects may also include interference by DGC. Pyrethroids as well as many other insecticides also induce increased water loss in arthropods (Gerolt, 1976, 1983). Water loss is induced by producing the diuretic hormones after pyrethroid poisoning (Jagers op Akkerhuis *et al.*, 1999a) in insects’ larvae and adults, a process which could be reversible if the insect could replenish its water reserves. Since the pyrethroids often affect motion, as well, causing the knockdown effect, death may come through desiccation (Jagers op Akkerhuis *et al.*, 1995, Jagers op Akkerhuis *et al.*, 1999a, Thompson, 2003). In pupae of cabbage butterfly *Pieris brassicae*, after the treatment with original pyrethrum, the DGCs disappeared and the metamorphosis was disrupted (Harak *et al.*, 1999, Jõgar *et al.*, 2006). Insects exposed to spinosad experience hyper-excitation of the nervous system, followed by inhibition of neural firing (Salgado, 1998).
3. AIMS OF THE STUDY

Maintaining pollinators’ communities in agricultural landscapes enhances high quality food production. As previously discussed, the knowledge of the sub-lethal effects of pesticides on pollinators is scarce, but it is important for understanding the reasons for the global decline of pollinators. The present thesis aims to clarify this gap in our knowledge.

My overall goals were twofold: First, we sought answers to two questions: 1) Do bees avoid pesticides in their food plant choice? (I) and 2) Do sub-lethal doses of pesticides in food affect their foraging behaviour? (II). Secondly, we explained the respiration mechanisms in untreated bumble bees (III, IV) and examined the changes in these patterns after the treatment with a sub-lethal dose of alpha-cypermethrin (V).
4. MATERIAL AND METHODS

4.1. Field experiments: foraging behaviour of bees

4.1.1. Study sites and subjects

The field work was conducted on the experimental fields of the Estonian University of Life Sciences and on the seed production field of Pilsu Farm in Tartu County, Estonia (I) and in Jõgeva Plant Breeding Institute, Jõgeva County, Estonia (II). For testing the effect of alpha-cypermethrin on honey bees, spring oilseed rape *Brassica napus* var *oleifera* fields were used in 2003, 2004 and 2005 (I). In Pilsu six honey bee colonies were brought to the crops. In 2003 and 2005 in Jõgeva, three adjacent leguminous crop fields (hybrid lucerne *Medicago x varia* Mart., white clover *Trifolium repens* L. and red clover *T. pratense* L.) were used for testing the effect of azadirachtin on the foraging of bumble bees (II). Bumble bee hives (NATUPOL) were obtained from Koppert Biological Systems B.V. (the Netherlands).

4.1.2. Impact of insecticides on the foraging of bees

**Alpha-cypermethrin**

The impact of the treatment of food plants with alpha-cypermethrin on honey bee foraging was studied during two experiments. In the first experiment, we studied the number of honey bees foraging on small patches of spring oilseed rape treated with the insecticide once or twice to determine whether honey bees discriminate between differently treated plants. The observation area consisted of a 5 ha field of summer wheat where a regular array of patches (1m x 10m) of spring oilseed rape was sown. The second experiment was carried out on a seed-production crop of spring oilseed rape to test the abundance of honey bees before and after insecticide application. In both experiments, the rape cultivar was ‘Maskot’ and a commercial formulation of alpha-cypermethrin (Fastac, a.i. 50 g/l) was used at a rate of 0.15 l/ha (I).

**Azadirachtin**

In the first experiment in 2003 four pairs of colonies were placed at 0, 400, 800 and 1200 meters from leguminous fields. There were no other
superabundant food resources in the radius of 2500 m from leguminous fields. In the second experiment in 2005 the effect of azadirachtin on bumble bees’ foraging behaviour was tested. The experimental design was the same as in 2003. Prior to taking the colonies onto the field the test colonies (one out of each pair) were fed with a sublethal dose of azadirachtin (0.01 ppm in the food) for a three-week period. In both experiments pollen loads from both hind legs from 30 homing bumble bees per colony were removed during three consecutive days. 200 pollen grains out of each sample were identified by light microscopy (II).

4.2. Laboratory experiments: the physiology of bumble bees

4.2.1. Subjects

The last instar larvae (III) and early-, mid- and late-stage pupae (IV) were tested to determine their respiration patterns. The bumble bee foragers (V) were used for studying the effect of a sublethal dose of alpha-cypermethrin on the respiration cycles.

4.2.2. Laboratory equipment and measurements

Flow-through respirometry
An infrared gas analyser (IRGA, Infralyt-4, VEB, Junkalor, Dessau), adapted for entomological research, was used to record the CO$_2$ signals. The IRGA was calibrated at different flow rates by means of calibration gases (Trägergase, VEB, Junkalor, Dessau), with gas injection. The rate of carbon dioxide release was measured (VCO$_2$ ml h$^{-1}$) (III, IV, V). All measurements were made at 25°C. Approximately 70% or 95% relative humidity (RH) was maintained by means of moistened filter paper strips inside the chamber. The insect chamber could be switched either to the IRGA or to the electrolytic respirometer (III, IV) without disturbing the insect, as seen in Figure 1 (see also Martin et al., 2004).

Opto-cardiography (III, IV)
The IRGA was combined with an infrared (IR) cardiograph for insects, which we refer to as the opto-cardiograph. This recorded not only cardiac (heart) pulses, but also all extracardiac abdominal muscular contractions, including ventilatory contractions. An IR-emitting diode was
placed on one side of the chamber near the ventral side of the abdomen, and an IR sensitive diode was placed on the opposite side of the chamber (see Metspalu et al., 2001, 2002; Kuusik et al., 2002). The light from the IR-diode was modulated by the contractions of the heart and skeletal muscles. The level of output voltage reflected the vigour of the muscular contractions of the insect (see Hetz et al., 1999).

**Constant-volume respirometry (IV)**
A differential electrolytic micro-respirometer-actograph was used for the sensitive recordings of gas exchange cycles and microcycles (Kuusik et al., 1992, Tartes, Kuusik, 1994, Tartes et al., 1999, 2000, 2002). This closed-system and constant volume micro-respirometer allowed simultaneous recording of metabolic rate, discrete CO$_2$ releases (bursts), rapid intakes of air into the tracheae referred to as passive suction ventilation (PSV) in microcycles, and active abdominal movements. The rates of generation of oxygen by electrolysis are indicated on graphs as oxygen flux ‘F0$_2$ (mL O$_2$ h$^{-1}$)’. They represent also the recorded transient mL rate changes of CO$_2$ release or air intake as indicated.

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**Figure 1.** A scheme of the electrolytic respirometer that may be switched to the flow-through system of an infrared gas analyser (IRGA). (1) Glass capillary with ethanol; (2) light source; (3) photodiode; (4) tubing to the compensation vessel (a thermos bottle 0.5 L); (5) an electrolysis unit with Cu and Pt electrodes; (6) air inflow from the IRGA; (7) insect chamber with infrared-emitting diode (A) and infrared-sensor diode (B); (8) air outflow to the IRGA; (9) vessel for potassium hydroxide.
The respirometer ensures continuous replacement of consumed oxygen with electrolytically produced oxygen. The insect itself plays an active role in this self-regulating system. Rapid changes of pressure in the insect chamber, caused by active body movements of the insect, or other rapid events, will lead to corresponding rapid changes in the electrolysis current reflected as spikes on recordings. Carbon dioxide release causes a rise of the liquid meniscus in the left side of the U-shape capillary (Figure 1), thus, the photodiode is screened from the light beam. This event causes a temporary decrease in the electrolysis current and oxygen generation. In this way, CO$_2$ bursts are not measured but only indicated on the respirogram as clear downward peaks lasting several minutes (Figures 2, 3A, IV), and these peaks we refer to as bursts of carbon dioxide. A 15% potassium hydroxide solution was used to absorb the CO$_2$.

**Treatment of foragers with a sublethal dose of alpha-cypermethrin (V)**

In the present experiment a commercial formulation of alpha-cypermethrin (Fastac, a.i. 50 g/l) was used. The preparation was diluted with distilled water to $3.7 \times 10^{-4}$ % of a.i., which is a tenfold lower dose than that recommended for use in fields against pests. The respiration rate and the frequency of bursts of CO$_2$ releases of _B. terrestris_. foragers were measured at 4–5 °C at a humidity of about RH = 2% and RH = 95% for four hours. After the first two hours, the bees were dipped in distilled water or in the alpha-cypermethrin solution for 10 seconds. Following dipping, each bee was dried for 1 minute on filter paper and placed back into the respiration chamber. After the end of the experiments all bees were kept at 4 °C at RH = 50–60% and their behaviour was observed for one week.

**4.2.3. Data acquisition and statistics**

Computerized data acquisition and analysis were performed using DAS 1401 A/D hardware (Keithley, Metrabyte, USA) with a 10 Hz sampling rate. The two bipolar channels allowed the recording of two events simultaneously (III). The mean metabolic rate was automatically calculated by averaging data over a period involving at least 3 periods of activity or at least 12 cycles of gas exchange; i.e., a period lasting at least 1 hour (IV, V).

In the statistical analyses the software package STATSOFT 7.0 was used.
For discovering the correlations between two factors Spearman correlation was used. In comparing the proportions between two groups $\chi^2$-tests were used (II). In comparing the mean values between the groups t-tests (I, IV, V) and ANOVA F-tests were used (II, V). If the experimental design was more complicated, the repeated measures ANOVA (IV) and nested ANOVA (III) were used. In the text the mean ± standard deviation (III, IV) and the mean ± standard error of means (V) are shown. The level of significance for all tests was $P \leq 0.05$. 
5. RESULTS

5.1. The effect of insecticides on bee foraging

**Alpha-cypermethrin**

In each experimental year, there was no significant difference in the number of bees per 1000 flowers between the treatments either during the whole observation period (Figure 2) (2003: $F_{2,57} = 0.3$, $P = 0.8$; 2004: $F_{2,33} = 0.7$, $P = 0.5$; 2005: $F_{2,81} = 0.04$, $P = 0.9$), or on the first observation day, i.e. 24 h after the second spraying (2003: $F_{2,9} = 0.5$, $P = 0.6$; 2004: $F_{2,9} = 1.6$, $P = 0.3$; 2005: $F_{2,9} = 0.2$, $P = 0.8$). Yet there was a significant difference in total number of bees between the years ($F_{2,177} = 3.7$, $P = 0.03$). Flower densities differed significantly between the treatments in all years (2003: $F_{2,57} = 5.2$, $P = 0.008$; 2004: $F_{2,33} = 8.4$, $P = 0.001$; 2005: $F_{2,81} = 8.2$, $P = 0.001$). An interesting trend was found: in the case of lower flower densities, the number of bees did not depend on the number of flowers but statistically significant positive correlations became apparent at a certain level of flower density (Figure 3).

The number of honey bees per 1000 flowers did not differ between alpha-cypermethrin-treated and untreated crops either one week before ($t = 1.7$, $df = 12$, $P = 0.12$) or one week after ($t = 0.2$, $df = 12$, $P = 0.9$) the application of the insecticide (Figure 4). However, 24 h after spraying, the number of honey bees per 1000 flowers for the treated crop was significantly higher than for the untreated crop ($t = 4.4$, $df = 12$, $P = 0.001$). We investigated whether these differences in the abundance of honey bees between the crops are induced by the differences in flower densities. Indeed, in the middle of the flowering period (counted 24 h after spraying) the density of flowers in the treated crop was significantly higher than in the untreated crop ($t = 2.2$, $df = 12$, $P = 0.048$). At the same time, the number of oilseed rape flowers did not differ significantly between the untreated and treated crops at the beginning and at the end of the flowering period (accordingly: $t = 1.5$, $df = 12$, $P = 0.2$; $t = 0.04$, $df = 12$, $P = 0.9$). When comparing the abundance of honey bees on the observation days, the number of bees was significantly lower for both crops 24 h after spraying (untreated: $F_{2,18} = 16.4$, $P = 0.001$; treated: $F_{2,18} = 3.3$, $P = 0.05$) (Figure 4).
Figure 2. The number of honey bees per 1000 flowers on oilseed rape crops treated with alpha-cypermethrin and not treated with alpha-cypermethrin, a) 2003; b) 2004; c) 2005. Means with standard error are given.
Figure 3. The Spearman’s correlations between the number of honey bees and the number of flowers on the experimental plots (10 m²). a) 2003; b) 2004; c) 2005; * – $P < 0.05$; n.s. – not significant.
There were statistically significant differences between the bumble bee hives treated with neem EC and the control hives (Figure 5). When the hives were placed close to the food source, the bumble bees from the neem-treated colony gathered significantly more pollen from the crops ($\chi^2 = 4.6, P = 0.03$). When the colonies were farther away, the treated bees gathered significantly less pollen from these crops (from 400 to 1200 m accordingly: $\chi^2 = 435, P < 0.001; \chi^2 = 1093, P < 0.001; \chi^2 = 477, P < 0.001$).

Figure 4. The number of honey bees per 1000 flower on three observation days on seed production crops adjacent to each other. Means with standard error and standard error *1.96 are given. *** – $P < 0.001$, n.s. – not significant.

Azadirachtin
There were statistically significant differences between the bumble bee hives treated with neem EC and the control hives (Figure 5). When the hives were placed close to the food source, the bumble bees from the neem-treated colony gathered significantly more pollen from the crops ($\chi^2 = 4.6, P = 0.03$). When the colonies were farther away, the treated bees gathered significantly less pollen from these crops (from 400 to 1200 m accordingly: $\chi^2 = 435, P < 0.001; \chi^2 = 1093, P < 0.001; \chi^2 = 477, P < 0.001$).

Figure 5. The proportions of leguminous pollen in the samples (N = 30) of 200 grains in four distances from the main food source.
5.2. Bumble bee physiology

The respiration of bumble bees has been studied insufficiently until now. In the following chapter I describe the normal respiration patterns of untreated healthy bumble bee larvae and pupae.

5.2.1. Respiration patterns of bumble bee larvae

The last instar larvae of *B. terrestris*, 2–3 days before pupal ecdysis, displayed bouts of two types of extracardiac abdominal contractions, here referred to as pulsations and pumping (Figure 6). Bouts of abdominal pulsations were both short and long. In most individuals (N = 7) they were short, lasting 4–6 minutes, and consisting of 25–30 contractions of almost uniform frequency (30–40 strokes/min). However, in some individuals (N = 3) these bouts were longer, lasting 14–20 minutes with 60–80 contractions of decreasing frequency: high (43–53 per min), then (30–40 per min), and lowest, (25–30 per min) (Figure 2; III).

The respiratory responses to the bouts of abdominal pulsations were recorded on the IRGA as a small rise (about 5%) in carbon dioxide release (peak) (Figure 5). The mean rate of CO₂ release was 0.75 ± 0.11 ml g⁻¹ h⁻¹ (N = 10 individuals). The bouts of abdominal pumping were more vig-

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**Figure 6.** The lower trace (Volts) is a typical opto-cardiographic recording of the periodically occurring extracardiac abdominal pulsations (grey bars) and abdominal pumping (asterisks) in a larva (415 mg) of *Bombus terrestris* 2 days before pupal ecdysis. The upper trace is a simultaneous recording of an infrared gas analyser (VCO₂), demonstrating the respiratory responses to the bouts of abdominal pulsations; note that abdominal pumping results in minor spikes on the recording trace.
orous and slower (8–12 strokes/min) than the bouts of pulsations. They
were also less regular, in terms of the amplitude of the contractions and
the intervals between the bouts (Figure 6; III).

The bouts of pulsations and pumping occurred independently of each
other and sometimes overlapped (Figures 3–4; III). The amplitude of
the pumping contractions was 2–8 times larger than that of the pulsa-
tion contractions.

5.2.2. Respiration patterns of bumble bee pupae (IV)

Cyclic release of carbon dioxide and active ventilation
During the first 2–3 hours of recording, the pupae enclosed within a
cocoon displayed activity periods recorded as irregular spikes both on
the respirograms and on the recordings of an infrared actograph (Figure
2A; IV). After 3–4 hours of recording, the irregular bouts of vigorous
abdominal contractions ceased and cleared, and regular bursts of CO₂
appeared with the frequency of 4–10 (mean 7.3 ± 1.9) per h (Figure 2B;
IV). In addition, the cyclic gas exchange was not interrupted by irregular
bouts of vigorous abdominal contractions.

In mid-stage pupae the abdominal contractions during the ventilation
periods tended to coincide with the bursts of carbon dioxide, with many
of the contractions occurring in groups (bouts) at the same time as the
bursts of carbon dioxide (Figure 3A, B; IV). However, during some inter-
brust periods, single movements occurred. About 20% of the mid-stage
pupae displayed clear DGC, where the bursts of CO₂ were accompanied
by abdominal ventilating movements. In late stage pupae, all abdominal
contractions, interpreted as active ventilation, were strongly associated
with the bursts of carbon dioxide (Figure 7).

Metabolic rate and water loss
Late stage pupae enclosed in cocoons exhibited significant differences
in metabolic rate between active and resting periods (1.29 ± 0.27 mL
O₂ g⁻¹ h⁻¹ and 1.07 ± 0.28 mL O₂ g⁻¹ h⁻¹, respectively; Student’s t-test,
t = 2.54, df = 38, P < 0.05; N = 4 pupae each with 5 activity and 5 resting
periods). Thus the metabolic rate during intermittent activity was about
20% higher than during resting. After 4–5 hours of recording when no
active periods occurred, the standard metabolic rate for enclosed mid-
stage pupae and late stage pupae was determined to be 0.35 ± 0.08 mL O$_2$ g$^{-1}$ h$^{-1}$ and 0.82 ± 0.12 mL O$_2$ g$^{-1}$ h$^{-1}$, respectively.

The metabolic rate during continuous activity of pupae removed from cocoons did not differ from that of enclosed pupae during their active periods (1.52 ± 0.46 mL O$_2$ g$^{-1}$ h$^{-1}$ and 1.29 ± 0.27 mL O$_2$ g$^{-1}$ h$^{-1}$, respectively, N = 20 pupae of both group; Student’s t-test, $t = 1.8$, df = 38, $P = 0.07$). Water loss in late stage pupae within cocoons differed significantly from that of late stage pupae removed from their cocoons (0.21 ± 0.04 mg g$^{-1}$ day$^{-1}$ and 0.34 ± 0.05 mg g$^{-1}$ day$^{-1}$, respectively, N = 10 pupae; repeated measures ANOVA, $F_{1,9} = 91.0$, $P < 0.01$).

5.3. The effect of low sub-lethal dose of alpha-cypermethrin on the DGC of bumble bee foragers

Respiration rate
The respiration rate of the bumble bees was affected by the humidity of the environment (ANOVA: $F_{1,16} = 13.2$, $P = 0.002$). In dry conditions (RH = 2%) the mean rate of CO$_2$ emitted by the bumble bee foragers was significantly lower ($0.0738 ± 0.014$ ml h$^{-1}$) than in moist conditions (RH = 95%) ($0.1295 ± 0.011$ ml h$^{-1}$). Treatment itself did not affect
the respiration rate (ANOVA: $F_{1,16} = 0.11, P = 0.7$) of the bees, nor did the respiration rate change considerably after dipping the bees into distilled water or the alpha-cypermethrin solution (ANOVA: $F_{1,16} = 0.9, P = 0.3$).

**Frequency of DGC**
The bumble bee workers exhibited clear cycles of discontinuous gas exchange when resting in low temperature conditions (Figure 1; V). In dry air, the frequency of bursts of CO$_2$ releases was the highest ($4.9 \pm 1.01$ bursts h$^{-1}$) in the untreated bumble bees and the lowest in the alpha-cypermethrin treated bees ($0.36 \pm 0.17$ bursts h$^{-1}$). A significant difference in the frequency of the bursts of CO$_2$ releases before and after treatment became apparent only in the case of alpha-cypermethrin treatment in dry conditions (RH = 2%) ($t = 4.25, df = 4, P = 0.01$) (Figure 8). At high humidity (RH = 95%) alpha-cypermethrin treatment did not affect the frequency of bursts of CO$_2$ releases ($t = 2.27, df = 4, P = 0.09$), nor was it affected by treatment with distilled water in either condition (RH = 2%: $t = 2.48, df = 4, P = 0.07$; RH = 95%: $t = 0.53, df = 4, P = 0.6$).

**After-effect of the treatment on bumble bees**
The bumble bees treated with alpha-cypermethrin maintained the normal resting position, standing upright only when they were exposed to high humidity, RH = 95%. However, at low air humidity, RH = 2%, the treated bees were lying on their backs and appeared to be dead. After returning them to humid conditions, the bees recovered within a couple of hours but survived at low temperatures only 2–4 days after the experiment. The bees treated with distilled water did not lie down and survived in humid conditions and at low temperature for at least one week.
Figure 8. The effect of dipping the bumble bee foragers (N = 5 for each treatment) into distilled water or into the alpha-cypermethrin solution (3.7 x 10^{-4} \%) on the frequency of bursts of CO_{2} releases in RH = 2\% (A) and RH = 95\% (B). The means, SE of the means and SE * 1.96 of the means are presented. * \( P < 0.05 \); n.s. = not significant (paired t-test).
6. DISCUSSION

6.1. The effect of insecticides on the foraging of bees

Our results tend to confirm that alpha-cypermethrin does not repel honey bees in field conditions. If any repellency does occur with respect to this insecticide, the attractiveness of the flower resource is likely to override it. Pyrethroids are known as the insecticides most repellent to bees (Thompson, 2003). Pyrethroid repellency can also reduce the foraging activity of bees (Mueller-Beilschmidt, 1990). Alpha-cypermethrin has been reported to maintain repellency to bees for 48 h after treatment (Thompson, 2001). However, most studies on repellency have been performed in laboratory or semi-field conditions. In field conditions, the repellency of pyrethroids may be lower than suggested by semi-field experiments (Thompson, 2003). In field studies by Mayer and Lunden (1999), bees were not repelled by alpha-cypermethrin applied at the field rate to flowering oilseed rape. Shires et al. (1984) found that, when sprayed on oilseed rape during periods of peak honey bee foraging activity, cypermethrin caused a slight decline in the level of foraging and in the levels of collected pollen (Thompson, 2003). The detection of relatively high residues of cypermethrin in honey and wax (Thompson, 2003) also raised questions about its repellent qualities.

We could not find any difference in the numbers of foraging honey bees between the patches treated with alpha-cypermethrin and those not treated with the insecticide (I). The result persisted through three observation years regardless of varying flower and honey bee densities. No repellent effect of the insecticide on honey bees was found even 24 h after spraying. Instead, the density of oilseed rape flowers most likely played a major role in choosing the foraging area (I). The relative number of honey bees was connected with floral density: on dense observation plots, the numbers of bees and flowers were positively correlated, whereas on sparse patches no such correlation was found (I). According to the theory of optimal foraging, animals distribute themselves among differently rewarding food resources so that the average amount of food per specimen remains equal (Alonso et al., 1995). Despite the theory, the attractiveness of dense patches was higher than that of sparse foraging patches.
The alpha-cypermethrin formulation Fastac is commonly used to control pollen beetles in oilseed rape. Controlling this pest contributes to higher flower densities as the damage caused by the larvae to the flowering structures is prevented. Therefore, treated crops may often have high flower densities and, therefore, are more attractive to bees than crop areas damaged by the beetle. In field conditions, honey bees can become contaminated with the residues of alpha-cypermethrin even if the hives have been kept closed for some time after spraying. The foraging ability of honey bees depends on its physiological state. Therefore, it is evident that reliable data are needed with respect to the effects of sublethal doses of the insecticide on the transpiration and respiration of the bees.

Neem preparations are said to be safe for honey bees (National Research Council, 1992), but the effect on bumble bees has been studied less. In our field experiment (II), using ten times lower than the sublethal dose (0.01 ppm), there was an effect on the foraging behaviour of bumble bees. In the experiments of Melathopoulos and co-workers (2000) honey bee adults were not affected by different dosages of the neem preparations, but the honey bee larvae were susceptible to the compounds: after using high doses, different malformations occurred when young bees emerged from the cocoons. Naumann and co-workers (1994) found the detrimental effect of neem preparation (0.1 ppm) for honey bees in a laboratory experiment. The species earth bumble bee *B. terrestris* has longer mean foraging distances than many other bumble bee species and has been considered to be a spatial generalist (Walther-Hellwig, Frankl, 2000). The treated bumble bees gathered more pollen from the close crops than the untreated control bees. In the case of longer distances, the treated bees foraged less on the superabundant food source and visited more wild plants near their nests.

Compared to synthetic pesticides, the botanical pesticides should be safer for pollinators and using them could retard the vanishing of natural pollinator species. Still, the bees can carry the contaminants sprayed on the flowering crops into the nests and feed them to the larvae. Even very small changes in the behaviour may affect the survival rate of colonies, especially in the intensely managed agricultural areas, where the distances between food sources are longer.
6.2. Bumble bee physiology

6.2.1. Respiration patterns of bumble bee larvae

According to Slama (1984, 1988, 1999), abdominal pulsations occur in all postembryonic developmental stages of insects, including the larval stage. The respiratory pattern found in the larvae of *B. terrestris* in this study supports this view.

This study clearly demonstrated that the last instar larvae of the bumble bee, *B. terrestris*, display regular periods of extracardiac abdominal contractions (III). Two types of contractions were found: 1) extracardiac pulsations, the weak movements of the abdominal segments not discernible to the naked eye, and 2) visually observable vigorous abdominal pumping. Sometimes the abdominal pumping and extracardiac pulsations occurred at the same time, indicating that the difference between the two types of contractions is not purely quantitative.

Respiration by the larvae was continuous and the small peaks on the IRGA recording trace were not discontinuous gas exchange cycles (DGCs). In actual DGC, the CO$_2$ level falls to zero or close to zero between successive bursts of CO$_2$ i.e. during the interburst period. In the current study, only about 5% more CO$_2$ was released during the bouts of abdominal pulsations than between the bouts.

Extracardiac pulsations are responsible for tracheal ventilation, being directly associated with mechanical emission of gas from certain spiracles (Slama, 1999, Slama, Neven, 2001). In the current study the spiracular movements were not studied and the exact role of the periodically occurring abdominal contractions in gas exchange remained unclear. The respiratory responses to abdominal pumping were essentially weaker than those of the extracardiac pulsations, which occurred with much higher frequency than pumping. Obviously, the spikes on the IRGA recording were at least partly due to the metabolic cost of the muscular activity.

6.2.2. Respiration patterns of bumble bee pupae

Clear gas exchange cycles were recorded both in mid-stage and in late-stage bumble bee pupae (IV). Enclosed in the cocoon, the activity peri-
ods gradually shortened, alternating with inactivity when short relatively frequent gas exchange cycles were displayed (9–14 per h) in mid-pupae and late pupae. Furthermore, the activity periods were lost and regular larger bursts of CO$_2$ appeared (4–10 per h).

Previous studies have shown that adult foragers of $B. terrestris$ exhibit DGC where all bursts of CO$_2$ are accompanied by active ventilating movements with a frequency of about two cycles per hour, when measured at 5 °C (Kuusik et al., 2002), and that pre-diapause queens of $B. terrestris$ exhibit two large DGC per hour at 18 °C (Beekman, Stratum, 1999).

In mid-stage pupae, weak abdominal contractions tended to coincide with the bursts; i.e., FV cycles were observed, however there occurred also FO cycles, where no ventilating movements were recorded. In late stage pupae, only FV cycles were recorded, where all bursts of CO$_2$ were accompanied by movements of active ventilation with the frequency of 50–60 movements per hour.

Insect pupae exhibit great diversity in their patterns of obligatory abdominal movements and in the coordination of these movements with gas exchange cycles (Tartes et al., 2002). Regular bouts of weak abdominal movements have been described in pupae of the great wax moth $Galleria mellonella$ (Kuusik et al., 1996, Tartes et al., 1999) but in this species bouts occurred independently of the short gas exchange cycles. Pupae of the Colorado potato beetle $Leptinotarsa decemlineata$ exhibited large, small and microbursts of CO$_2$ but only the large bursts were associated with active ventilation bouts (Tartes et al., 2000, Kuusik et al., 2001).

In the mid-stage pupae of $B. terrestris$, we observed that not only CO$_2$ release but also O$_2$ uptake was intermittent. Each small burst in mid-stage pupa began with an inflow of O$_2$ into the tracheae. Similar patterns of intermittent O$_2$ uptake have also been recorded from the pupae of $G. mellonella$ (Kuusik et al., 1996) and of $Pieris brassicae$ (Harak et al., 1999) using the same type of electrolytic respirometers. Intermittent O$_2$ uptake has also been reported from adults of the ant $Formica polyctena$ (Martin et al., 2004) and several other insect species, mostly Coleoptera (Punt et al., 1957, Lighton, 1988, Bartholomew et al., 1985).
It is well known that several stress factors may abolish the normal gas exchange cycles or cause their irregularity (Kestler, 1991, Möbius et al., 1996). Most of the *B. terrestris* pupae removed from their cocoons displayed an irregular pattern of gas exchange and body movements due to the abnormal environment, handling and apparatus stress. These activity periods sometimes alternated with resting periods, which were too short to observe normal patterns of gas exchange and obligatory body movements.

Water loss of pupae after their removal from the cocoons rose noticeably due to the almost continuous activity. However, the cocoon itself could retard the transpiratory water loss from the pupa suggesting a vapour gradient between the saturated stagnant layer around the pupa and the ambient air which runs through the cocoon.

In our study, the simultaneous IRA recordings and respirograms allowed easy discrimination of the periods of activity and inactivity. From our results we also concluded that the respiration pattern and the obligatory abdominal movements in the pupal stage of bumblebees should be studied without removing the pupa from the cocoon to avoid the stress induced by handling, apparatus and environment.

### 6.3. Respiratory response of bumble bee foragers to the sub-lethal dose of alpha-cypermethrin

Discontinuous ventilation has often been cited as being an adaptive mechanism for minimizing respiratory water loss especially for pupae (Hadley, 1994, Klowden, 2002). Along with adults of many insect species (Klowden, 2002), cyclic respiration has also been found in bumble bee queens (Silvola, 1984, Beekman, Stratum, 1999) and foragers (Kuusik et al., 2002). The above studies proved that in rest conditions bumble bees change from continuous to discontinuous ventilation; the latter is thought to prevent excessive water loss through the spiracles (Kestler, 1984, Hadley, 1994, Lighton, 1994, 1996).

Our experiments (V) showed that the CO₂ emitting rate of the *B. terrestris* foragers was higher in humid conditions compared with dry conditions. In addition, the frequency of bursts of CO₂ releases was lower not only after treatment of the bumble bees with the weak alpha-cypermeth-
rin solution but also after treatment with distilled water. This supports the theory that the DGC can be used as a water saving mechanism.

The rate of CO$_2$ emission in the bumble bee foragers kept in dry air was low (mean 0.074 ± 0.01) in comparison with corresponding rate for those kept in moist air (mean 0.130 ± 0.01). Alpha-cypermethrin treatment after 2 hours of the experiment reduced the frequency of bursts of CO$_2$ releases almost to zero. The reason for the significant decrease in DGC after treatment with the pyrethroid insecticide at RH = 2%, but not at RH = 95%, remains unclear. It does prove, however, that the effect of the chemical is definitely environmental-specific and shows that laboratory data obtained by defined conditions must be considered critically. Yet the effect of the pesticide on the survival of the foragers was evident, as all of them died earlier than the ones treated with distilled water. The synthetic pyrethroids act on the insects’ nervous systems and cause disorders in neuronal functions (Corbett, 1974). Deltamethrin has been found to cause concentration-dependent hyper-excitation of the respiratory motoneurons (Zafeiridou, Theophilidis, 2006) and the treatment of $P.$ brassicae pupae with original pyrethrum caused the disappearance of DGC cycles (Harak et al., 1999). If the disappearance of DGC was the cause of the earlier death of bumble bees in our experiment, it indicates the importance of DGC in the water-saving mechanism. Since the effect (toxic?) level of many insecticides is dependent upon environmental factors (Jagers op Akkerhuis et al., 1999b), we deduce that contact with sublethal doses of alpha-cypermethrin may cause the lethal after-effect under certain environmental conditions. The co-effects of climate conditions and toxins could lead to dehydration if the bee were not able to reach its nest.

During their life cycle, bees encounter widely varying humidity conditions; the variations strongly affect their physiology. Climate conditions in the nest of bumble bees are stable and provide optimum temperature and relative humidity levels for development of the immature offspring. In many eusocial species the brood is highly sensitive to temperature extremes and perishes soon if the nest’s humidity is not regulated (Moritz, Crewe, 1988). Therefore approximately 40% relative humidity is maintained in the brood chamber in honey bee hives (Human et al., 2006). In bumble bee nests, the relative humidity is 50-60% (Heemert et al., 1990), which is much higher than the humidity level that they encounter when foraging on hot, sunny midsummer days. In regions with
temperate climate, the relative humidity outside the hives may vary on a large scale, from 70–80% in early morning to 12–20% during the afternoon on particularly warm and dry days. Temperature also varies widely from day to night: in early summer, daily maximum temperatures may reach approximately 25°C, while by night, temperatures may still remain quite low, around 10°C. These fluctuations increase the bees’ sensitivity to pesticides.

There is evidence that synthetic pyrethroids may cause orientation problems for bees so that they may never return to the hive (Cox, Wilson, 1984). This has been suggested as one of the reasons which caused Colony Collapse Disorder (CCD), a newly discovered disorder which was found in the southern states of the USA in 2006 (Engelsdorp et al, 2007). In CCD, a great number of colonies with the queen, the brood and food supplies simply run out of workers. It is not known whether similar symptoms have been found in wild bees, but our experiment showed that sub-lethal doses of pesticides may affect bees on the physiological level. The effects of the insecticide may not be observable in a short time frame but subsequently do affect the survival of both the individual and the colony. More detailed research is needed about the short- and long-term effects of sub-lethal doses of pesticides on the physiology of insects.
1. This study showed that there was no difference in the number of foraging honey bees between the patches treated with alpha-cypermethrin and those not treated with the insecticide (I). No repellent effect of the insecticide on honey bees was found even 24 h after spraying. It seems that bees are not able to recognize pesticides as warnings of hazards. The density of oilseed rape flowers most likely played a major role in choosing the foraging area.

2. The results of my work showed that exploitation of the foraging area was affected by sub-lethal doses of azadirachtin fed with larval food to bumble bees (II). Even very small changes in the behaviour may affect the survival rate of colonies, especially in the intensely managed agricultural areas, where the distances between food sources are longer.

3. In the larvae of *B. terrestris*, the periodically occurring abdominal contractions play an essential role in respiration and/or haemolymph circulation. The larvae did not show discontinuous gas exchange cycles (III), nor did the early stage pupae (IV). The regular DGC appeared in the mid-stage pupae. In late stage pupae the bursts of CO$_2$ are associated with active ventilation (IV).

4. The sub-lethal dose of alpha-cypermethrin decreased the number of regular bursts DGC almost to zero (V). The effects of the insecticide may not be observable in a shorter perspective but subsequently threaten the survival of both the individual and the colony.

5. Pollinators have evolved to recognize different signals and react respectively. The presence of pesticides on the fields is a very recent phenomenon from the evolutionary perspective; therefore no co-evolving has occurred and the pollinators are not always able to recognize the hazards. The sub-lethal doses of pesticides that bees encounter affect the foraging behaviour and physiological states of the insects, therefore is one of the reasons for the decline in global pollination.
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Käesoleva doktoritöö eesmärgiks oli vähemalt osaliselt täita seda tühikut meie teadmistes. Esmalt uurisin ma kas mesilased väldivad pestitsiididega töödeldud taimi (I) ja kas subletaalsete pestitsiididoodide esinemine mesilasevastsete toidus mõjutab hiljem nende korjekäitumist (II). Teiseks selgitasin ma välja, missuguseid hingamisrütme tervetel, pestitsiidega mitte kokku puutunud kimalastel esineb (III, IV) ja kas peale subletaalse doosiga kokku puutumist toimub rütmides muutusi (V).

Minu uurimistöö tulemused näitavad, et mesilased ei erista insektitsiitidiga töödeldud taimi töötlemata taimedest (I). Alfa-tsüpermetriini oletatatav repellents efekti ei täheldatud isegi kõigist 24 tunni möödumisel pritsimesest. Pigem mõjutas mesilaste toidutaime valikut õite tiheus. Selgus...
ka, et vastsena asadirahtiini subletaalse doosiga kokku puutunud kimalaste korjekäitumine oli erinev vürreldes mitte kokku puutunud kimalastega (II). Isegi väga väikesed käitumuslikud muutused võivad mõjutada perede püsimajäämist, seda aga eriti intensiivse põllumajanduspraktika aladel, kus vahemaa erinevate toidutaimede vahel on vahel.

Meie uurimised näitasid, et kimalast vastsetel ja varases nuku staadiumis esines pidev hingamine (III, IV), mida toetas perioodiline aktiivne ventileerimine lihastöö abil. Regulaarne katkendlik hingamine esines keskmise ja vanema staadiumi nukkudel. Hilises nukustaadiumis olid süsihappegaasi väljalasked seotud aktiivse ventileerimisega (IV).

Subletaalse alfa-tsüpermetriini doosiga töötlemise tulemusel kadusid kui- vas õhus korjekimalastel regulaarsed süsihappegaasi väljalasked peaaegu täiesti. Niiskes õhus sellist erinevust ei ilmnenud (V). Pestitsiidide mõjud ei pruugi alati katses tähendatavat olla, kuid pikaajalises perspektiivis mõjutavad putukate ellujäämist nii individuaalsel kui pere tasandil.

Tolmeldajate reageeringud erinevate toitumiskäitumist mõjutavatele signaalidele on evolutsioonis kinnistunud. Pestitsiididest tulenev oht on ajalises möttes väga uus ning tolmeldajad ei ole veel kohastunud neid ära tundma ja vältima. Seega võib pestitsiidide subletaalsete kogustega kokku puutumine mõjutada nii putukate käitumist kui füsioloogiat, olles seega üheks pöhjuseks globaalsele tolmeldajate kriisile.
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PUBLICATIONS
Impact of alpha-cypermethrin on honey bees foraging on spring oilseed rape *Brassica napus* flowers in field conditions

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Abstract: Cruciferous oil-bearing crops have gained in importance worldwide. The expansion of the growing area of these crops has caused a proliferation of pests. Exposure to organophosphate, carbamate, and pyrethroid insecticides has been associated with bee poisonings in food crops. This study examines the repellent effect of alpha-cypermethrin on the number of foraging honey bees *Apis mellifera* L. on spring oilseed rape *Brassica napus* L. var. *oleifera* fields. The first experiment was conducted on differently sprayed 10m² experimental plots, where alpha-cypermethrin was applied at different times. Another experiment was conducted on a 4ha seed production field divided into two parts: one part was treated with alpha-cypermethrin and the other was not treated with this insecticide. The results show that there was no difference in the number of honey bees between alpha-cypermethrin-treated and untreated patches. The result persisted through three observation years regardless of varying flower and honey bee densities. No repellent effect of the insecticide on honey bees was found even 24 h after spraying. The density of oilseed rape flowers most likely played a major role in choosing the foraging area.

Key words: alpha-cypermethrin, *Brassica napus* L. var. *oleifera*, *Apis mellifera* L., foraging
1 Introduction

The continuous growth of the human population has increased the need for agricultural products. During the last fifty years the growing area of cruciferous oil-bearing crops has greatly increased. Vegetable oils are needed not only in food production but also as a raw material for fuel. Northern agricultural areas are unsuitable for the effective cultivation of most oil crops but oilseed rape, *Brassica napus* L. var. *oleifera*, is easy to establish and grow in northern temperate climates.

A major problem with cultivating spring oilseed rape in northern Europe is that damage caused by the key pest, the pollen beetle *Meligethes aeneus* F., is increasing. Hokkanen\(^2\) has explained the increase in the number of pollen beetle with ecological changes: initially, when the host plants were sparse, the high reproductive rate of the insect was of no benefit for it; when the number of host plants became unlimiting, however, their high fecundity became advantageous. Due to the increased occurrence of pests in oilseed rape, the use of pesticides has become an almost inevitable part of cultivating these crops.

Oilseed rape plants are very attractive to pollinating insects.\(^3,4\) In the case of conventional farming where pesticides are widely used, the high attractiveness of a plant species may enhance the hazards of pesticide poisoning to bees. Bee poisoning incidents have been frequently associated with exposure to pesticides.\(^5,6\) Bees may come into contact with poisoning compounds through contaminated flower resources, direct contact with poison or exposure to residues.\(^6\)

Application of insecticides is often not permitted during the flowering period of a given crop. Even when insecticides are not sprayed on flowers but on flower buds, the residues of the compounds still contaminate nectar and pollen in sublethal doses via both active and passive transport.\(^5\) Many insecticides have been described as safe to bees because they do not kill them, although sublethal doses may affect pollinators by decreasing their foraging and navigation abilities.\(^6\) Some pesticides do not affect adult bees, but affect brood so that young adults emerging from cocoons may have malformed wings or other deformations.\(^7\) However, some insecticides may be regarded as safe because they repel bees, although in some instances, such as in the case of oilseed rape, the attractiveness of a food resource may override the repellent effect.\(^8\) The effect of cultivation
methods on the abundance of bees has been studied at landscape scale. Morandin and Winston\textsuperscript{9} have shown that the abundance of bees within organic crops is higher than in conventional and genetically-modified crops. One of the explanations they have offered for this observation is that organic crops are smaller in area and therefore their environment could be more suitable for natural bee populations. These results concur with the studies by Mänd \textit{et al.}\textsuperscript{10} and Sepp \textit{et al.}\textsuperscript{11} However, there is a lack of data concerning bee abundance on insecticide-treated and untreated crops when the crops are situated next to each other. Alpha-cypermethrin is a non-systemic insecticide with contact and stomach action that may reduce bees’ foraging ability\textsuperscript{12} and is repellent to them for 48 hours.\textsuperscript{5} Hence it can be assumed that, due to repellency, the number of honey bees should be lower on insecticide-treated food resource patches for at least 24 h after treatment. This study examines the repellent effect of alpha-cypermethrin on the number of foraging honey bees on spring oilseed rape fields.

2 Materials and methods

Two field experiments were carried out on spring oilseed rape crops to study the repellent effect of the insecticide alpha-cypermethrin on the density of honey bees. The experiments were conducted near Tartu, Estonia, in 2003–2005. In both experiments, the rape cultivar was ‘Maskot’ and a commercial formulation of alpha-cypermethrin (Fastac, a.i. 50 g/l) was used at a rate of 0.15 l/ha.

2.1 Experiment 1: effect of alphacypermethrin treatment intensity on the number of honey bees

This experiment was performed to evaluate the impact of alpha-cypermethrin on the number of foraging honey bees on small patches of spring oilseed rape treated once or twice (at different times) with the insecticide. The observation area consisted of a 5 ha field of summer wheat where a regular array of patches of spring oilseed rape was sown. The design of the experiment was a randomized block with twelve 1 x 10 m\textsuperscript{2} plots with a distance of 1 m between each. Three treatments were used: unsprayed, once sprayed and sprayed twice, each replicated four times. In the sprayed once treatment the insecticide was applied when rape plants were in the growth stage of 2-4 true leaves (GS 10\textsuperscript{13}). On the twice-sprayed treatment, the first spray was applied at the same
time as the once-sprayed plots with an additional application at the stage of first flowers (GS 61-62). The insecticide was applied using a manually operated sprayer and during spraying plastic screens prevented the contamination of neighbouring plots. The insecticide treatments were conducted only on days when wind speed did not exceed 1-2 m s\(^{-1}\). The cultivation methods between the treatments were identical.

In all years, the observation period lasted throughout July, i.e. the flowering period of oilseed rape. The lengths of flowering periods differed according to weather conditions and lasted from two weeks (2004) to three and a half weeks (2005). During bee counts, the observer walked slowly along the plot and recorded all honey bees foraging on the oilseed rape. The number of open flowers was determined on 1 m\(^2\) quadrats within each plot. Counts were made twice weekly during the flowering period starting at 24 h after the last spray application. All bee counts were made on days when there was no rain, fog or strong wind and air temperature was over 16\(^{\circ}\) C at around midday (11.00–16.00).

2.2 Experiment 2: honey bee abundance before and after alpha-cypermethrin treatment

The second experiment was carried out on a seed-production crop of spring oilseed rape to test the abundance of honey bees before and after insecticide application. The experiment was conducted in July 2003. A spring oilseed rape field (4 ha) was divided into two parts (approx. 2 ha): one part was treated with alpha-cypermethrin and the other was left untreated. Within both fields, seven 1 x 10 m\(^2\) observation plots were marked. Six honey bee colonies were brought close to the crops (200 m away) two days before flowering started (late bud stage, GS 60). The insecticide was applied using a motorized field sprayer when the plants were at the mid-flowering stage (GS 65-66). During spraying, wind speed did not exceed 1-2 m s\(^{-1}\). To prevent direct poisoning of honey bees, the hives were closed before the insecticide application and kept closed for 24 hours. No visible mortality was detected in close proximity to the hives during the experiment. The counting of flowers (on 1 m\(^2\) per plot) and bees (on the whole plot, 10 m\(^2\)) was made 8 days before, one day after and 8 days after the insecticide treatment using the methods described above.
2.3 Data analysis

To test for the effects of the treatments and years on the number of flowers and the number of bees, one-way and two-way analysis of variance (ANOVA) was used. The number of flowers on different observation plots varied both from day to day and throughout the flowering period. Therefore, when estimating the mean density of honey bees, their number was not taken per unit area but per 1000 flowers. Because the data of the first experiment were not distributed normally, Spearman’s correlation was used to test for correlation between number of flowers and number of bees. To compare the abundance of bees and flowers on seed production crops, the t-test was used. The accepted level of significance was 5% in all cases.

3 Results

3.1 Experiment 1: effect of alphacypermethrin treatment intensity on the number of honey bees

In all years, there was no significant difference in the number of bees per 1000 flowers between the treatments either during the whole observation period (Fig. 1) (2003: $F_{2,57} = 0.3$, $P = 0.8$; 2004: $F_{2,33} = 0.7$, $P = 0.5$; 2005: $F_{2,81} = 0.04$, $P = 0.9$), or on the first observation day, i.e. 24 h after the second spraying (2003: $F_{2,9} = 0.5$, $P = 0.6$; 2004: $F_{2,9} = 1.6$, $P = 0.3$; 2005: $F_{2,9} = 0.2$, $P = 0.8$). Yet there was a significant difference in total number of bees between the years ($F_{2,177} = 3.7$, $P = 0.03$). Flower densities differed significantly between the treatments in all years (2003: $F_{2,57} = 5.2$, $P = 0.008$; 2004: $F_{2,33} = 8.4$, $P = 0.001$; 2005: $F_{2,81} = 8.2$, $P = 0.001$). An interesting trend was found: in the case of lower flower densities, the number of bees did not depend on the number of flowers but statistically significant positive correlations became apparent at a certain level of flower density (Fig. 2).

3.2 Experiment 2: honey bee abundance before and after alpha-cypermethrin treatment

The number of bees per 1000 flowers did not differ between the untreated and treated crops either one week before ($t = 1.7$, $df = 12$, $P = 0.12$) or one week after ($t = 0.2$, $df = 12$, $P = 0.9$) the application of the insecticide (Fig. 3). However, 24 h after spraying the number of honey bees per 1000 flower for the treated crop was significantly higher than for the
untreated crop ($t = 4.4, df = 12, P = 0.001$). We investigated whether these differences in the abundance of honey bees between the crops are induced by the differences in flower densities. Indeed, in the middle of the flowering period (counted 24 h after spraying) the density of flowers in the treated crop was significantly higher than in the untreated crop ($t = 2.2, df = 12, P = 0.048$). At the same time, the number of oilseed rape flowers did not differ significantly between the untreated and treated crops at the beginning and at the end of the flowering period (accordingly: $t = 1.5, df = 12, P = 0.2$; $t = 0.04, df = 12, P = 0.9$). When comparing the abundance of honey bees for the observation days, the number of bees was significantly lower for both crops 24 h after spraying (untreated: $F_{2,18} = 16.4, P = 0.001$; treated: $F_{2,18} = 3.3, P = 0.05$) (see Fig. 3).

4 Discussion

This study showed that there was no difference in the number of foraging honey bees between the patches treated with alpha-cypermethrin and those not treated with the insecticide. The result persisted through three observation years regardless of varying flower and honey bee densities. No repellent effect of the insecticide on honey bees was found even 24 h after spraying. The density of oilseed rape flowers most likely played a major role in choosing the foraging area.

Pyrethroids are known as the insecticides most repellent to bees. Pyrethroid repellency can also reduce the foraging activity of bees. Alpha-cypermethrin has been reported to maintain repellency to bees for 48 h after treatment. However, most studies on repellency have been performed in laboratory or semi-field conditions. In field conditions, the repellency of pyrethroids may be lower than suggested by semi-field experiments. In field studies, Mayer and Lunden did not find any repellency to bees for alpha-cypermethrin applied at the field rate to flowering oilseed rape. Shires et al. found that, when sprayed on oilseed rape during periods of peak honey bee foraging activity, cypermethrin caused a slight decline in the level of foraging and in the levels of collected pollen. Evidence for repellency may also be questioned by the detection of relatively high residues of cypermethrin in honey and wax. Our results tend to confirm that alpha-cypermethrin does not show repellency for honey bees in field conditions. If any repellency does occur with respect to this insecticide, the attractiveness of the flower resource may override it.
The results of the first experiment showed that application of the insecticide at the beginning of flowering had no effect on the number of foraging honey bees per unit of flowers. Irrespective of the variable number of bees available in different years, the trends remained the same. The relative number of honey bees was connected with floral density: on dense observation plots, the numbers of bees and flowers were positively correlated, whereas on sparse patches no such correlation was found. According to the theory of optimal foraging, animals distribute among differently rewarding food resources so that the average amount of food per specimen remains equal.\textsuperscript{17} Despite the theory, in the first experiment, dense patches of oilseed rape were even more attractive for the bees. The data of the second experiment also uphold the result that the bees visited rich food patches more often than expected on the basis of flower resources.

Rape plants are known to be a favoured food source for bees due to their high nectar production rate\textsuperscript{3} and valuable pollen amino acid content.\textsuperscript{4} It is also known that honey bees recruit nestmates to profitable foraging sites. Newly recruited bees fly directly from the hive to the vicinity of a food source, and then proceed to search for its exact location using odour and other cues.\textsuperscript{18} The patches with higher flower densities may trigger more recruitments of nestmates on fields as it might have occurred in our second experiment. However, in the first experiment this could hardly affect the results because the area itself, and the experimental patches, were too small and situated between each other to permit exact identification of profitable small patches through waggle dance.

In the second experiment, 24 h after spraying there was a decline in the number of foragers not only on the treated- but also on the untreated crop when compared to the rest of the observation days. As the abundance of honey bees decreased on both fields, it can be assumed that this was not related to treatment but more likely to climatic conditions and/or the start of flowering of some other attractive food plant species (e.g. leguminous) nearby. The end of July is the period when the aftermath of clover starts flowering on pastures or meadows and may attract bees away from rape crops.

Coming into direct contact with alpha-cypermethrin, or its residues, may cause death or sub-lethal effects in bees. The contact may be either direct (residues on leaf surfaces) or indirect (spray contamination of the
nectar or pollen). It has been shown that the residues on leaf surfaces are toxic for more than 3 days following insecticide application and may kill up to 25% of bees that come into contact with them. There is at least one study that shows the presence of the residues of alpha-cypermethrin in small quantities (0.01 mg/kg) in the pollen of oilseed rape after insecticide application (10 g ai/ha). The compound has an LD$_{50}$ of 0.319 µg ai/bee. There is also evidence for the existence of alpha-cypermethrin residues in dead honey bees. Our experiments indicate that honey bee food crop preference does not depend on the presence of insecticide residues on flowers but rather on the flower abundance of the crop plant. The alpha cypermethrin formulation Fastac is commonly used to control pollen beetles in oilseed rape. Controlling this pest contributes to higher flower densities as the damage caused by the larvae to the flowering structures is prevented. Therefore, treated crops may often have high flower densities and, therefore, are more attractive to bees than crop areas damaged by the beetle. In field conditions, honey bees can become contaminated with the residues of alpha-cypermethrin even if the hives have been kept closed for some time after spraying. The foraging ability of honey bees depends on its physiological state. Therefore, it is evident that reliable data are needed with respect to the effects of sublethal doses of the insecticide on the transpiration and respiration of the bees.

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6 References


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Figure 1.

Untreated | Once-treated | Twice-treated
---|---|---
0 | 10 | 20
10 | 20 | 30
20 | 30 | 40
30 | 40 | 50

F(2;57) = 0.3; p = 0.8

a) Untreated | Once-treated | Twice-treated
---|---|---
0 | 10 | 20
10 | 20 | 30
20 | 30 | 40
30 | 40 | 50

F(2;33) = 0.7; p = 0.5

b) Untreated | Once-treated | Twice-treated
---|---|---
0 | 10 | 20
10 | 20 | 30
20 | 30 | 40
30 | 40 | 50

F(2;81) = 0.04; p = 0.9

c) Untreated | Once-treated | Twice-treated
---|---|---
0 | 10 | 20
10 | 20 | 30
20 | 30 | 40
30 | 40 | 50

F(2;81) = 0.04; p = 0.9
Figure 2.
Figure 1. The number of honey bees per 1000 flowers on oilseed rape crops treated with alpha-cypermethrin and not treated with alpha-cypermethrin, a) 2003; b) 2004; c) 2005. Means with standard error are given.

Figure 2. The Spearman’s correlations between the number of honey bees and the number of flowers on the experimental plots (10 m²). a) 2003; b) 2004; c) 2005; * – $P < 0.05$; n.s. – not significant.

Figure 3. The number of honey bees per 1000 flower on three observation days on seed production crops adjacent to each other. Means with standard error and standard error $*1.96$ are given. *** – $P < 0.001$, n.s. – not significant.
The effect of Neem EC on pollen forage of the bumble bee Bombus terrestris L.

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Abstract

The decrease in the number of natural pollinators impels scientists to work out more environmentally friendly pest control methods. The aim of this study was to investigate the effect of a sublethal dose of a new biological insecticide Neem EC (1% azadirachtin) on the bumble bee Bombus terrestris L. pollen forage. The results of the study show that sublethal doses of azadirachtin affect the foraging distances of bumble bees. Although neem preparations are said to be safe for bees, they do affect the foraging behaviour and flight distances of bumble bees.

Key words: bumble bees, neem, pollen forage, legumes

Introduction

The seed yield of leguminous crops depends largely on the pollination. Flower morphology of many leguminous plant species causes the incapability for self-pollination or wind pollination (Free, 1993). Any kinds of bees, including bumble bees, belong to the important pollinators of these crops in northern hemisphere (Corbet et al., 1991).

In last decades a decrease in the number of bumble bees and other wild bees has been discovered in the agricultural landscapes of many European countries (Goulson, 2005). The intensive use of pesticides has been held partly responsible for this (Osborne, et al., 1999; Miranda et al., 2003). Once contaminated by a certain insecticide, the bee may be killed by a lethal dose, or carry the insecticide to the nests through contaminated nectar or pollen (Miranda, et al., 2003). Inside the nests the contaminated food will be fed to the larvae, which could cause various malformations in pupae and new adults (Thompson, 2003). Bumble bees are often more vulnerable than honey bees because for a long period in late spring, when only the queens are collecting food, their colonies are smaller, they don’t have the trofallaxis and their foraging behaviour differs from that of honey bees (Alford, 1975). The restrictions of how to apply the insecticide often regard the foraging time of honey bees (e.g. spraying is allowed early in the morning or late in the evening), but this is exactly the time when bumble bees are most active (Thompson, 2001).

The need for more environmentally friendly pest management is growing continuously. There are several new pesticides e.g. neem preparations, Quassia amara L. extracts, spinosad etc., that have been introduced for commercial use in recent times. Many of these are recommended to use also in the ecological farms. Neem preparations (active ingredient: azadirachtin) are said to be safe for bees due to fast degradation time and low concentration they can get into contact with.
tests have shown that only extremely high doses can affect honey bees through the feeding of contaminated nectar to the larvae (Natural Research Council, 1992, Naumann, Isman, 1996). In this particular experiment only small beehives showed insect growth-regulating effects, but medium-sized and large bee populations were unaffected. Honey bee larvae are less susceptible to the azadirachtin-enriched neem pesticides than other insect species (Melathopoulos et al., 2000).

The studies about the effect of neem preparations on bees as non-target species have been mostly directed on honey bees. Neem preparations are safe for adult honey bees at the dosages likely to be encountered in field applications and the bees are not repelled by the neem application (Naumann et al., 1994). Thus they can bring the contaminated nectar and pollen into hives and feed it to the larvae in the case of the application on the flowering crop. There is not much research made on wild bees, although these constitute an important pollinator group. The aim of the study was to test the effect of neem preparation on the foraging behaviour of bumble bee *Bombus terrestris* L. workers that had previously been fed with the sublethal dose of the insecticide.

**Material and methods**

The study was conducted during the flowering period of leguminous crops in 2003 and 2005 in Jõgeva Plant Breeding Institute, Jõgeva County, Estonia. The experimental area was situated in intensely cultivated arable land. In both years three superabundant flower resources were available: hybrid lucerne *Medicago x varia* Mart., red clover *Trifolium pratense* L. and white clover *Trifolium repens* L. The fields were situated next to each other in the area of 1.5 ha.

The bumble bee hives were obtained from Koppert Biological Systems (Koppert B.V., Postbus 155, 2650 AD Berkel en Rodenrijs, Netherlands). The bumble bee hives NATUPOLE contain colonies with the queen, workers, brood and larvae of earth bumble bee *B. terrestris*. In 2003 four pairs of colonies were placed at 0, 400, 800 and 1200 meters from leguminous fields to establish the foraging behaviour of bumble bees. The area was surrounded by cereal crops. In 2005 the design of the field experiment was the same. This year prior to taking the colonies onto the field the test colonies were fed with a sublethal dose of azadirachtin for a three-week period. The original food source of the commercially produced colonies was substituted separately with water and the mixture of fresh pollen and sugar solution (30%). In case of the test colonies (one of each pair) a sublethal dose (0.01 ppm in the food) of Neem EC was added to the food. For the experiment we used Neem EC obtained from India (M/S RYM Exports – The Indian Neem Tree Company). The preparation of Neem EC (1% azadirachtin) was diluted with distilled water.

The pollen loads from both hind legs were removed from 30 homing bumble bees per colony during three consecutive days. The pollen loads were dried and later analyzed in the laboratory. Acetolysis (Kearns, Inouye, 1993) was used to separate the pollen grains for identification and counting. Acetolysis removes the protoplasm and other organic debris leaving the exine very clean, which makes the pollen grains well suited for studies of sculpturing by light microscopy. 200 pollen grains out of each sample were identified by light microscopy. The Chi-square test
(STATISTICA 7.0) was used to find out the differences in the proportions of leguminous pollen occurred in the samples.

**Results**

In 2003 the bumble bees from nests in the same places collected similar proportions of pollen from three food sources (hybrid lucerne, red clover and white clover) (Figure 1). There were no statistically significant differences between the hives in each pair (from 0 to 1200 m: \( \chi^2=3.5 \ p=0.1; \ \chi^2=0.3 \ p=0.6; \ \chi^2=3.4 \ p=0.1; \ \chi^2=1.9 \ p=0.2 \)). The amount of legume pollen decreased constantly as the distance from the crops increased.

In 2005 there were statistically significant differences between the treated and control hives (Figure 2). When the hives were placed close to the food source, the bumble bees from neem treated colony gathered significantly more pollen from the crops (\( \chi^2=4.6 \ p=0.03 \)). When the colonies were further, the treated bees gathered significantly less pollen from these crops (from 400 to 1200 m accordingly: \( \chi^2=435 \ p<0.001; \ \chi^2=1093 \ p<0.001; \ \chi^2=477 \ p<0.001 \)).

![Figure 1](image1.png)

Figure 1. In 2003 the proportions of leguminous pollen in the samples (N=60) of 200 grains in four distances from the main food source

![Figure 2](image2.png)

Figure 2. In 2005 the proportions of leguminous pollen in the samples (N=30) of 200 grains in four distances from the main food source
Discussion

Pollen of entomophilous plants has higher caloric values than pollen of wind pollinated plants (Petanidou, Vokou, 1990). Leguminous plants have relatively high content of amino acids in the pollen (Roulston et al., 2000) and it is useful for bees to fly longer distances for foraging it. Hybrid lucerne and red clover are commonly used as food plants by bumble bees, white clover has less reward in the flowers, but in case of high competition for food resources it is visited often (Free, 1993). The number of flower visitors also depends largely on the adjoining cultures or wild plants. Large areas with massively flowering plants attract bees more than smaller areas.

The insect flight is one of the most expensive activities performed in the animal kingdom (Wolf et al., 1999). The task of the foraging bee is to collect as much of nectar and pollen as possible with the shortest time and energy costs. Therefore the bees use memory very much: they remember the colour (White et al., 2001) and smell of the profitable flowers (Dobson, 1987) and learn how to handle these in the most efficient way (Møller, Eriksson, 1995, White et al., 2001). On the basis of the knowledge they develop flower constancy to save energy (Roulston et al., 2000).

The results of the experiment in 2003 showed that bumble bees from nests in the same places collected similar proportions of pollen from legume crops. Bumble bees have large individual differences in the foraging behaviour (Chittka, Thomson, 1997). They do not have the same recruitment methods as honey bees, but have indirect ways of pronouncing about profitable food sources. They remember the pollen and nectar odors and colors that they were fed with as larvae or indoor workers (Dobson, 1987). They leave scent-marks on flowers to recognize them as profitable or unprofitable (Stout, Goulson, 2001). On average they use similar food resources also because they have relatively restricted flying distances compared with honey bees (Walther-Hellwig, Frankl, 2000). Still all of them have the same task: to find the optimal foraging strategy and this makes them behave similarly.

Neem preparations are said to be safe for honey bees (National Research Council, 1992), but the effect on bumble bees has been studied less. In the experiments of Melathopoulos and coworkers (2000) honey bee adults were not affected by different dosages of the neem preparations. The honey bee larvae were susceptible to the compounds and in case of high level doses different malformations occurred when the young bees emerged from the cocoons. The latter appeared only in the case of high dosages. Naumann and coworkers (1994) have found the deterring effect of neem preparation (0.1 ppm) for honey bees. In our experiment in 2005 ten times lower sublethal dose (0.01 ppm) was used, but still the effect on the foraging behaviour of bumble bees was found. The species earth bumble bee *B. terrestris* has longer mean foraging distances than many other bumble bee species and has been considered as a spatial generalist (Walther-Hellwig, Frankl, 2000). Our results showed that the flight distances must have shortened due to the toxic compounds in the larval food. The treated bumble bees gathered more pollen from the close crops than the untreated control bees. In case of longer distances, the treated bees foraged
less on the superabundant food source and visited more wild plants in the neighborhood of the nests.

The biopesticides are safer for pollinators and using them could retard the vanishing of natural pollinator species. Still when spraying on the flowering crops the bees can carry the contaminants into the nests and feed them to the larvae. Even very small changes in the behaviour may affect the survival rate of colonies, especially in the intensely managed agricultural areas, where the distances between food sources are longer.

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References

Regular periods of abdominal contractions recorded from larvae of the bumblebee, *Bombus terrestris* (Hymenoptera: Apidae)

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Abstract. Using an opto-cardiograph combined with an infrared gas analyzer regular bouts of abdominal contractions were recorded from last instar larva of *Bombus terrestris*. The rate of CO<sub>2</sub> release was about 0.7 ml g<sup>-1</sup> h<sup>-1</sup>. The bouts of contractions were of two types: weak extracardiac pulsations and vigorous pumping. The frequencies of pulsations and pumping were 25-35 per min and 8-12 per min, respectively. Bouts of extracardiac pulsations and abdominal pumping were independent of each other and sometimes overlapped. Cardiac contractions (heartbeats) were continuous (57-63 pulses/min). This study shows that the periodically occurring abdominal contractions play an essential role in respiration and/or in haemolymph circulation in larvae of *B. terrestris*.

INTRODUCTION

In adult and pupal stage of many insects, visible abdominal contractions are known to actively ventilate the tracheal system (Buck, 1962; Miller, 1974, 1981; Mill, 1985, for reviews). These vigorous abdominal contractions are known as abdominal pumping.

Sláma (1976) described a repeated series of pulsations regulated by the autonomic nervous system (coelopulse), resulting in changes in hydrostatic pressure within the insect body, which he termed extracardiac haemocoelic pulsations. The pulsations are often microscopic invisible movements of some elastic body segments. According to Sláma (2000), “the pulsations are produced by coordinated rhythmic contractions of intersegmental (dorsocentral in certain species) muscles”. These extracardiac pulsations have been described in various developmental stages in a number of insect species. These pulsations are controlled by the autonomic nervous system (coelopulse), which regulates important physiological functions including gaseous exchange through the spiracles and haemolymph circulation (Sláma 1999, for reviews).

In many insects, discontinuous gas exchange cycles or DGCs are associated with active ventilatory movements of the abdomen, during which emission of carbon dioxide is discontinuous (Kestler, 1985; Sláma, 1988; Lighthoon, 1994, 1996, for reviews).

Although active ventilation has been extensively studied in the pupal and adult stages of insects, little is known about it in the larval stages of terrestrial holometabolous insects. Gas exchange in soft-bodied larvae was thought to be by diffusion alone and not by active ventilation (Krogh, 1920, Wasserthal, 1996). However, ventilatory movements can be very weak and therefore may have been overlooked. Special techniques are now available to study such weak movements. Extracardiac pulsations have been reported from the tip of the abdomen using an electronic transducer (Sláma, 1984), or by means of an infrared cardiograph referred to as an opto-cardiograph (Sláma, 2003; Kuusik et al., 2002). Extracardiac pulsations have been recorded also using an electrolytic respiriometer-actograph (Kuusik et al., 1994, 1996).

Abdominal contractions have been recorded in the late-spinning larvae of *Galleria mellonella* (Sláma, 1984). The last instar larvae of *G. mellonella* in the wandering stage showed almost continuous rhythmic movements with short pauses, when immobile (Kuusik et al., 1991). Sláma (1999) recorded in prepupae of *Cossus cossus* continuous radial contractions of the larval somatic muscular sheath with a periodicity of 13–15 contractions per minute. He demonstrated that simple passive diffusion alone could not meet the respiratory requirements of the larva; active ventilation is also needed. In *Drosophila melanogaster*, the extracardiac contractions produced by larval somatic muscles or oral armature are very strong (Sláma & Farkas, 2005).

The aim of the present study was to investigate the rhythmic abdominal movements and their respiratory responses in mature larvae of *Bombus terrestris*, using non-invasive methods that allowed recording of abdominal movements and respiration simultaneously without the removal of the larva from its cocoon.

MATERIAL AND METHODS

Insects

A commercial colony (Natopol hives) of the bumblebee *B. terrestris* was purchased from Koppert Biological Systems B.V. (the Netherlands). The colony was kept in a nest box in the laboratory at a temperature of 22–25°C. The temperature in the nest box around the brood was 28–30°C and the air humidity was 70–80% RH. All manipulations of the colony were carried out under red light. The colony was supplied with unlimited amounts of water, sugar or honey solution and fresh pollen col-
Fig. 1. The lower trace (Volts) is a typical opto-cardiographic recording of the periodically occurring extracardiac abdominal pulsations (grey bars) and abdominal pumping (asterisks) in a larva (415 mg) of Bombus terrestris 2 days before pupal ecysis. The upper trace is a simultaneous recording of an infrared gas analyser (VCO₂), demonstrating the respiratory responses to the bouts of abdominal pulsations; note that abdominal pumping results in minor spikes on the recording trace.

lected from honeybee colonies. The larvae were tested soon (4-5 h) after the brood cells were covered. A total of ten mature larvae (390–460 mg) were tested. Neither the colony, nor its larvae were parasitized or infected by disease.

**Flow-through respirometry**

An infrared gas analyser (IRGA, InfraLyt-4, VEB, Junkalor, Dessau), adapted for entomological research, was used to record the bouts of abdominal contractions via the CO₂ signals. IRGA was calibrated at different flow rates by means of calibration gases (Trägergase, VEB, Junkalor, Dessau), with gas injection. Air flow rate was 3.6 l per h. The rate of carbon dioxide release was measured (VCO₂ ml h⁻¹). All measurements were made at 25°C. The humidity (% RH) and temperature inside the insect chamber were recorded on the PC monitor using the Humidity and Temperature Display Instrument for digital HygroClip probes (HygroPalm, Rotronic Company) referred to as a hygrometer. A humidity of about 70% RH was maintained by means of moistened filter paper strips inside the chamber.

**Opto-cardiography**

The IRGA was combined with an infrared (IR) cardiograph for insects, which we refer to as the opto-cardiograph. This recorded not only cardiac (heart) pulses, but also all extracardiac abdominal muscular contractions, including ventilatory contractions. An IR-emitting diode was placed on one side of the chamber near the ventral side of the abdomen, while an IR-sensitive diode was placed on the opposite side of the chamber (see Metsalu et al., 2001, 2002; Kuusik et al., 2002). The light from the IR-diode was modulated by the contractions of the heart and skeletal muscles. The level of output voltage reflected the vigour of the muscular contractions of the insect (see Hetz et al., 1999). Contractions of the abdomen were represented by downward spikes on the recordings, while muscular relaxations were directed upward. To avoid confusion between extracardiac pulsations and heartbeats, the IR sensor was placed on the larval body side of the respiratory chamber. When the IR sensor was placed upon the dorsal side of the larva then heartbeats and other body movements were recorded in parallel.

Fig. 2. Detail of the lower trace in Fig. 1 showing the different frequencies and amplitudes of the contractions during a bout of extracardiac abdominal pulsations and abdominal pumping in a larva of Bombus terrestris.

**Data acquisition and statistics**

Computerized data acquisition and analysis were performed using DAS 1401 A/D hardware (Keithley, Metabyte, USA) with a 10 Hz sampling rate. The two bipolar channels allowed the recording of two events simultaneously.

Tests were performed using the statistics package StatSoft ver. 7, Inc./USA. Values are shown as means ± standard deviations. Statistical comparisons were performed with nested ANOVA followed by multiple comparison procedures (Tukey test). Probabilities of P < 0.05 were considered significant.

**RESULTS**

The last instar larvae of B. terrestris, 2–3 days before pupal ecysis, displayed bouts of two types of extracardiac abdominal contractions, here referred to as pulsations and pumping (Fig. 1). Bouts of abdominal pulsations were either short or long. In most individuals (n = 7) they were short, lasting 4–6 min, and consisting of 25–30 contractions of almost uniform frequency (30–40 strokes/min). However, in some individuals (n = 3) these bouts were longer, lasting 14–20 min with 60–80 contractions. These longer bouts started with contractions of high frequency (43–53 per min), followed by contractions of a lesser frequency (30–40 per min) and ended with contractions of a lower frequency (25–30 per min) (Fig. 2).

The respiratory responses to the bouts of abdominal pulsations were recorded on the IRGA as a small rise (about 5%) in carbon dioxide release (peak) (Fig. 1). The mean rate of CO₂ release was 0.75 ± 0.11 ml g⁻¹ h⁻¹ (n = 10 individuals).

The bouts of abdominal pumping were more vigorous and slower (8–12 strokes/min) than the bouts of pulsations. They were also less regular, in terms of the amplitude of the contractions and the intervals between the bouts (Fig. 1).

The bouts of pulsations and pumping occurred independently of each other and sometimes overlapped (Figs 3–4). The amplitude of the pumping contractions was 2–8 times larger than that of the pulsation contractions.

The bouts of abdominal pumping produced far smaller peaks on the recording trace than those caused by the
Fig. 3. An opto-cardiographic recording of a larva of *Bombus terrestris* showing three bouts of extracardiac pulsations. The first two bouts of pulsations (smaller spikes) are overlapped by a bout of abdominal pumping (larger spikes).

bouts of extracardiac pulsations despite the larger amplitude of their strokes (Figs 1–2). Between the peaks caused by the bouts of pulsations, peaks due to decreasing CO₂ emission were recorded (Fig. 1); however, the origin of these downward peaks remains unknown.

The heartbeats of the larvae were continuous, with a frequency of about 1 Hz (58–62 pulses per min) at 25°C. On the opto-cardiographic recording trace, the amplitude of heartbeats was at least 4 times shorter than that of the abdominal pulsations (Fig. 4).

Heartbeats, extracardiac pulsations and abdominal pumping were also clearly distinguishable due to their different frequencies: 60.2 ± 1.6, 26.9 ± 4.1 and 9.8 ± 1.7 strokes per min, respectively (nested ANOVA, \( F_{2,81} = 726.1; P < 0.001 \); Tukey test, \( P < 0.05 \)).

**DISCUSSION**

This study clearly demonstrated that last instar larvae of the bumblebee, *B. terrestris*, display regular periods of extracardiac abdominal contractions. Two types of contractions were found: weak movements of the abdominal segments not discernible to the naked eye, the extracardiac pulsations, and visually observable vigorous abdominal pumping. The bouts of abdominal pumping were shorter than those of the pulsations and the frequency of pumping contractions was at least five times slower than that of the pulsations. Sometimes the abdominal pumping and extracardiac pulsations occurred at the same time, indicating that the difference between the two types of contractions is not purely quantitative.

Respiration by the larva was continuous and the small peaks on the IRGA recording trace were not discontinuous gas exchange cycles (DGCs). In real DGC, the CO₂ level falls to zero or close to zero between successive bursts of CO₂ i.e. during the interburst period. In the current study, only about 5% more CO₂ was released during the bouts of abdominal pulsations than between the bouts.

Previous studies have shown that extracardiac pulsations are responsible for tracheal ventilation, being directly associated with mechanical emission of gas from certain spiracles (Šlámě, 1999; Šlámě & Neven, 2001). In the current study the spiracular movements were not studied and the exact role of the periodically occurring abdominal contractions in gas exchange remained unclear. The respiratory responses to abdominal pumping were essentially weaker than those to the extracardiac pulsations, which were made with much higher frequency than pumping. Obviously, the spikes on the IRGA recording were at least partly due to the metabolic cost of the muscular activity.

Periodic active ventilation was previously recorded in early pupae of *B. terrestris*, while in old pupae and adults the emission of CO₂ is discontinuous (DGC) and always accompanied by active ventilation (Kuusik et al., 2002; Mänd et al., 2005). By comparison, the abdominal contractions in the pupal stage of *B. terrestris* are not as uniform and regular as those in the larval stage. Thus, in *B. terrestris* the patterns of active ventilation in the larvae are similar to those found in the pupae. By contrast, continuous extracardiac pulsations occur in the larvae of *G. mellonella* and *C. cossus* (Šlámě, 1984, 1999). The patterns of active ventilation in larvae of *B. terrestris* are similar to the periodically occurring ventilation movements in pupae.

In the larvae of *B. terrestris* abdominal pulsations, pumping and heartbeats are easily distinguishable by their different frequencies and relative amplitudes. In addition, ventilatory movements occur in bouts and the heartbeats are continuous in the larvae of other holometabolous insects (Wasserthal, 1996).

According to Šlámě (1984, 1988, 1999), abdominal pulsations occur in all postembryonic developmental stages of insects, including the larval stage. The respiratory pattern found in the larvae of *B. terrestris* in this study supports this view.
CONCLUSION

Active body movements were recorded in the last instar larvae of *B. terrestris*, 2–3 days before pupal ec dysis. These consisted of two different forms of extracardiac abdominal movement: regular and uniform abdominal pulsations and less regular abdominal pumping. These movements were not continuous but occurred in bouts. The two forms of abdominal contractions appeared to be fully independent of each other and thus regulated independently by nerve centres. The respiratory responses of abdominal pulsations were represented as discrete peaks on the IRGA recording, whereas the periods of pumping were scarcely noticeable on the same recording.

These results suggest that obligatory bouts of abdominal contractions, pulsations and pumping, are involved in respiration and/or circulation in the larvae of *B. terrestris*. There is need of further investigations to elucidate the function of abdominal pulsations and pumping in the larvae of this species.

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Discontinuous gas exchange cycles and active ventilation in pupae of the bumblebee Bombus terrestris

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Abstract – Discontinuous release of CO₂ (bursts) or discontinuous gas exchange cycles (DGC), metabolic rate (MR) and ventilation movements were simultaneously recorded from the pupae of the bumblebee Bombus terrestris by means of an electrolytic respirometer and an infrared gas analyser (IRGA) combined with an infrared actograph (IRA). After recovering from stress, the early stage pupae showed irregular continuous respiration, mid-stage pupae displayed regular DGC. The bursts of CO₂ release tended to coincide with abdominal contractions. In late stage pupae all bursts of CO₂ were associated with active ventilation. During interburst periods, spikes appeared on the respiromgrams interpreted as micro-cycles of passive suction ventilation (PSV). After removal from their cocoons, the pupae exhibited frequent periods of muscular activity due to stress. Water loss of pupae inside cocoons was significantly less than that from pupae without cocoons.

Bombus terrestris L. / passive suction ventilation / standard metabolic rate / respirometry

1. INTRODUCTION

Bumblebees, such as Bombus terrestris L. are known to be useful pollinators. The pollinating role of bees is vital to both agricultural and natural ecosystems, and in most temperate regions bumblebees are an important and sometimes indispensable component of the pollinator complex (Osborne and Williams, 1996). Their activity is dependent on their physiological state and health and it is therefore important to develop appropriate methods for assessing their physiological condition.

The physiological state and health of insects are usually estimated by measuring their standard metabolic rate (SMR). This is defined as a value measured at a particular temperature when an insect is quiet and inactive, is not digesting a meal and is not exposed to any stress (Withers, 1992). The pattern of gas exchange is another essential parameter indicating the physiological resting state of adult insects and pupae (Kestler, 1971). Many insects display a discontinuous gas exchange cycle (DGC), which means that CO₂ is released in bursts and uptake of O₂ often occurs cyclically (see reviews by Miller, 1981; Kestler, 1985; Slama, 1988; Lighton, 1994, 1996; Wasserthal, 1996). From spiracular activity, the gas exchange cycle can be described as having three phases: closed (C), flutter (F) and burst (B). C phase indicates that the spiracles are closed. F phase means that the spiracles open and closed rapidly and O₂ enters the tracheal systems by bulk
flow or by diffusion or a combination of the two. During the B phase a sudden release, or burst, of carbon dioxide occurs. Moreover, cyclic CO$_2$ release is a sensitive indicator of physiological stress in insects (Kestler, 1991).

Some studies have addressed gas exchange in adult bumblebees, mainly in hibernating queens (Silvola, 1984; Beekman and Stratum, 1999), or in workers at low temperatures (Kuusik et al., 2002). Adult bumblebees are almost continuously active and it is therefore difficult to measure their standard metabolic rate or their gas exchange patterns during normal activity at ambient temperature.

To date, information on gas exchange patterns and ventilation movements in bumblebee pupae within their cocoons have not been reported. It can be presumed that, when enclosed within the cocoon, the pupae are only weakly exposed to stress factors. To measure the gas exchange patterns and ventilation movements of pupae within cocoons, devices which allow the recording of gas exchange and abdominal contractions through the cocoon must be used.

This paper reports a study of the metabolic rate (MR), gas exchange patterns and ventilation movements in _B. terrestris_ pupae inside their cocoons using combined non-invasive methods which allow simultaneous recording of these different activities at temperatures prevailing in the brood of bumblebee colonies.

### 2. MATERIALS AND METHODS

#### 2.1. Insects and weighing

The colonies (Natupol hives) of the bumblebee _B. terrestris_ were purchased from Koppert Biological Systems B.V. (the Netherlands). The colonies were kept in nest boxes in the laboratory at the temperature of 22–25 °C. All manipulations were carried out under red light. The temperature in the brood chambers was 28–30 °C. All colonies were supplied with unlimited amounts of sugar or honey solution and fresh pollen collected from honey bee colonies. Neither colonies nor pupae used in this study were parasitised or infected by diseases.

For the experiments, pupae weighing at least 120 mg were selected. The pupae used were of three age groups: (1) 25 early stage pupae with non-pigmented eyes and white body, (2) 35 mid-stage pupae with pigmented eyes and non-pigmented (white) body and (3) 30 late stage pupae with pigmented bodies.

After respirometry pupae with the cocoons removed were weighed to 0.1 g on an analytic balance. Body mass loss was measured gravimetrically, but then the pupae were weighed to 0.01 mg using a microanalytic balance. During the measurements of mass loss, the pupae were kept at 22.0 ± 0.5 °C in ambient room humidity (55–65% RH). The gravimetric method assumes that mass loss and water loss are equivalent in the pupal stage when there is no intake of food or water (see Hadley, 1994). Hence, assuming fat metabolism, pupal mass loss will be referred to below as water loss. All recordings were made at 28 °C, which is the normal temperature for bumblebee brood (Heinrich, 1974).

#### 2.2. Constant-volume respirometry

A differential electrolytic microrespirometer-actograph was used for the sensitive recordings of gas exchange cycles and microcycles (Kuusik et al., 1992; Tartes and Kuusik, 1994; Tartes et al., 1999, 2000, 2002). This closed-system and constant volume micro-respirometer allowed simultaneous recording of metabolic rate, discrete CO$_2$ releases (bursts), rapid intakes of air into the tracheae referred to as passive suction ventilation (PSV) in microcycles, and active abdominal movements. The rates of generation of oxygen by electrolysis are indicated on graphs as oxygen flux $F_{O_2}$ (mL O$_2\cdot$h$^{-1}$). They represent also the recorded transient mL rate changes of CO$_2$ release or air intake as indicated.

The respirometer ensures continuous replacement of consumed oxygen with electrolytically produced oxygen. The insect itself plays an active role in this self-regulating system. Rapid changes of pressure in the insect chamber, caused by active body movements of the insect, or other rapid events, will lead to corresponding rapid changes in the electrolysis current reflected as spikes on recordings. Carbon dioxide release causes a rise of the liquid meniscus in the left side of the U-shape capillary (Fig. 1), thus, the photodiode is screened from the light beam. This event causes a temporary decrease in the electrolysis current and oxygen generation. In this way, CO$_2$ bursts are not measured but only indicated on the respirogram as clear downward peaks lasting several minutes (Figs. 2, 3A), and these peaks we refer to as bursts of carbon dioxide. A 15% potassium hydroxide solution was used to absorb the CO$_2$.

Most measurements were made using pupae enclosed within their cocoons, although some patterns were compared with those of enclosed pupae after their removal from cocoons (N = 12). When the smallest volume changes in the insect chamber due to passive suction ventilation (PSV) were to be recorded using high resolution, then a small aperture...
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(about 1 mm$^2$) was made in the cocoon near the head. The pupae without cocoons in the transparent insect chamber were sometimes visually observed for body movements using up to 40× magnification of a stereomicroscope (Olympus SZ 40).

2.3. Flow-through respirometry

The infrared gas analyser, or IRGA (Infralyt-4, VEB, Junkalor, Dessau), was used to prove that the presumed CO$_2$ signals; i.e., the downward peaks on the recording of the electrolytic respirometer, were actually due to CO$_2$ bursts and to measure them quantitatively (Fig. 3B). IRGA was calibrated at the different flow rates by means of calibration gases (Trägergase, VEB, Junkalor, Dessau) and with gas injection. Air flow rates from 3.6 to 10.8 L per h were used; the lower air flow rates gave higher sensitivity. The insect chamber could be switched either to the IRGA or to the electrolytic respirometer without disturbing the insect as seen in Figure 1 (see also Martin et al., 2004).

2.4. Infrared actography

The electrolytic respirometer and IRGA both were combined with an infrared (IR) cardiograph, which we refer to as the IR actograph (IRA), because it records not only heart pulses but also all other abdominal contractions. An IR-emitting diode was placed on one side of the chamber near the ventral side of the abdomen, while an IR-sensitive diode was placed on the opposite side of the chamber. The light from the IR-diode was modulated by the contractions of the heart and skeletal muscles. The level of output voltage reflected the vigour of the muscular contractions of the insect (see Hetz, 1994; Hetz et al., 1999; Metspalu et al., 2001, 2002; Kuusik et al., 2002). Abdominal contractions resulted in downward spikes, muscular relaxations were directed upward.

2.5. Data acquisition and statistics

Computerized data acquisition and analysis were performed using DAS 1401 A/D hardware (Keithley, Metrabyte, USA) with a 10 Hz sampling rate. The mean metabolic rate was automatically calculated by averaging data over a period involving at least 3 periods of activity or at least 12 cycles of gas exchange; i.e., a period lasting at least 1 hour.

Means, standard deviations and the number of observations (N) are reported. Tests were performed using a statistic package StatSoft ver. 6, Inc./USA. Means were compared using Student’s t-test or by repeated measures ANOVA, after testing for homogeneity of variances. The level of significance for all tests was $P \leq 0.05$.

3. RESULTS

3.1. Cyclic release of carbon dioxide and active ventilation

During the first 2–3 hours of recording, the pupae enclosed within a cocoon, displayed activity periods recorded as irregular spikes on both on the respirograms and on the recordings of infrared actograph (Fig. 2A). The irregular pattern comprised several events: abdominal contractions, heartbeats and small bursts of CO$_2$. These were quite similar to those previously recorded from pupae after removal from their cocoons. However, the resting periods of pupae within cocoons became gradually longer lasting 20–50 min alternating with activity (Fig. 2A). During the resting period, clear small fluttering bursts of CO$_2$ release in microcycles were recorded from mid-pupae with a frequency of 9–14 bursts per hour (mean 12.6 ± 2.5 bursts h$^{-1}$), with each burst lasting 1–2 minutes (Fig. 2A). In young pupae we did not detect the clear cycles of gas exchange.
Figure 2. (A) A typical pattern of muscular activity (solid line) alternating with the resting periods (dotted line) in a mid-stage pupa of *Bombus terrestris* (525 mg) enclosed within a cocoon. The lower trace (left axis) is a recording of the electrolytic respirometer; the upper trace (right axis) is a simultaneous recording of infrared actograph. (B) A detail of the right part of the upper figure: a resting period with bursts of CO$_2$; note that most of the bursts begin with an upward spike (indicated by asterisks) due to an inspiration stroke (lower trace, a recording of electrolytic respirometer). The upper trace is the simultaneous recording of infrared actograph; note that each inspiration stroke is associated with an abdominal movement. A short ventilation bout is denoted by arrow. The horizontal line is a period of heartbeat. (C) A detail of the upper figure made with higher time resolution.
After 3–4 hours of recording, the irregular bouts of vigorous abdominal contractions ceased and clear and regular bursts of CO$_2$ appeared with the frequency of 4–10 (mean $7.3 \pm 1.9$) per h (Fig. 2B). In addition, the cyclic gas exchange was not interrupted by irregular bouts of vigorous abdominal contractions.

After removal from the cocoon, the pupae showed long periods of abdominal contractions or activity, alternating with short resting periods. The latter usually lasted less than 5 minutes, and were thus too short to monitor gas exchange rhythms and other events. The patterns of activity were quite similar to those recorded in pupae inside cocoons (Fig. 2A). However, there occurred also mid-stage pupae and late stage pupae displaying clear cyclic gas exchange soon after removal from their cocoons.

Many (about 60%) of the small bursts began with an inspiration movement of the abdomen and oxygen uptake. This was reflected on the recording trace as an upward peak and simultaneously on the recording of infrared actograph as a spike due to a muscular contraction-relaxation or abdominal compression-decompression. Sometimes a short bout of 2–4 strokes of ventilation movements occurred during the final part of the burst of carbon dioxide (Fig. 2C).

In mid-stage pupae the abdominal contractions during the ventilation periods tended to coincide with the bursts of carbon dioxide, with many of the contractions occurring in groups (bouts) at the same time as the bursts of carbon dioxide (Fig. 3A, B). However, during some interburst periods, single movements occurred. About 20% of the mid-stage pupae displayed clear DGC, where the bursts of CO$_2$ were accompanied by abdominal ventilating movements. In late stage pupae, all abdominal contractions, interpreted as active ventilation, were strongly associated with the bursts of carbon dioxide (Fig. 4).

### 3.2. Passive suction ventilation (PSV)

PSV occurred between the CO$_2$ bursts in flutter periods when air was sucked periodically into the tracheae when the spiracles were slightly opened for a fraction of a second (0.2–0.3 s) and then when they closed, after which a slight negative intra-tracheal pressure developed. This type of gas exchange formed a typical pattern on the respirogram: an almost vertical upward spike due to the microopening of the spiracles, followed by a descending trace after the closure of the spiracles (Fig. 5) during micro-constrictions where even the minute flutters (nano-cycles) could be resolved. Visual observations through the microscope during recording showed telescoping movements of the abdominal segments: rapid retraction followed by slow passive contraction due to internal O$_2$ consumption and tracheal and abdominal collapse.

This typical pattern of PSV was most clearly recorded in mid-stage pupae when the interburst periods lasted 5–8 minutes. The PSV was not detected in the early stage pupae, probably because their soft integument does not allow development of negative intratracheal pressure. The frequency of PSV ranged from 80 to 115 (mean 96 ± 12) per hour. The corresponding CO$_2$ release is shown in Figures 3 and 4 as the typical flutter period pattern.

### 3.3. Metabolic rate and water loss

Late stage pupae enclosed in cocoons exhibited significant differences in metabolic rate between activity and resting periods ($1.29 \pm 0.27$ mL O$_2$ g$^{-1}$ h$^{-1}$ and $1.07 \pm 0.28$ mL O$_2$ g$^{-1}$ h$^{-1}$, respectively; Student’s t-test, $t = 2.54$, df = 38, $P < 0.05$; N = 4 pupae each with 5 activity and 5 resting periods). Thus metabolic rate during intermittent activity was about 20% higher than during resting. After 4–5 hours of recording when no activity periods occurred, the standard metabolic rate for enclosed mid-stage pupae and late stage pupae was determined to be $0.35 \pm 0.08$ mL O$_2$ g$^{-1}$ h$^{-1}$ and $0.82 \pm 0.12$ mL O$_2$ g$^{-1}$ h$^{-1}$, respectively.

The metabolic rate during continuous activity of pupae removed from cocoons did not differ from that of enclosed pupae during their activity periods ($1.52 \pm 0.46$ mL O$_2$ g$^{-1}$ h$^{-1}$ and $1.29 \pm 0.27$ mL O$_2$ g$^{-1}$ h$^{-1}$, respectively, N = 20 pupae of both group; Student’s t-test, $t = 1.8$, df = 38, $P = 0.07$).

Water loss in late stage pupae within cocoons differed significantly from that of late stage pupae removed from their cocoons ($0.21 \pm 0.04$ mg g$^{-1}$ day$^{-1}$ and $0.34 \pm 0.05$ mg g$^{-1}$ day$^{-1}$, respectively, N = 10 pupae; repeated measures
Figure 3. (A) A simultaneous recording of infrared actograph (upper trace, Volts) and electrolytic respirometer (lower trace) showing a coinciding trend of the abdominal contractions with the bursts of CO₂ release in mid-stage pupa of *B. terrestris* (181 mg). Between 18–20 min a burst of carbon dioxide occurs, which is not associated with active ventilation, the other bursts are accompanied with abdominal ventilating movements. (B) The bursts of CO₂ release recorded in the same pupae after switching the electrolytic respirometer to the infrared gas analyser. An activity period is seen between 30 and 40 min of the recording.

Figure 4. A simultaneous recording of infrared actograph (upper trace, Volts) and infrared gas analyser (lower trace) representing the regular bursts of carbon dioxide in a late stage pupa of *B. terrestris* (280 mg). Note that the clear pattern of active ventilation (upper trace) coincided with bursts of CO₂ (lower trace).
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ANOVA, $F_{1,9} = 91.0, P < 0.01$). Water loss of pupae after their removal from the cocoons rose noticeably due to the almost continuous activity. However, the cocoon itself could retard the transpiratory water loss from the pupa suggesting a vapour gradient between the saturated stagnant layer around the pupa and the ambient air which runs through the cocoon.

4. DISCUSSION

In the present study, we used methods, which allowed simultaneous recording of metabolic rate and the normal patterns of the gas exchange and ventilatory movements of pupae of the bumblebee *B. terrestris* that had not been removed from their cocoons, which minimized stress to the insect.

It is well known that several stress factors may abolish the normal gas exchange cycles or cause their irregularity (Kestler, 1991; Möbius et al., 1996). Most of the *B. terrestris* pupae removed from their cocoons displayed an irregular pattern of gas exchange and body movements due to the abnormal environment, handling and apparatus stress. These activity periods sometimes alternated with resting periods, which were too short to observe normal patterns of gas exchange and body obligatory movements.

In this study, clear gas exchange cycles were recorded both in mid-stage and in late stage bumble bee pupae. Enclosed in the cocoon, the activity periods gradually shortened alternating with inactivity when short relatively frequent gas exchange cycles were displayed (9–14 per h) in mid-pupae and late pupae. Further the activity periods were lost and regular larger bursts of CO$_2$ appeared (4–10 per h).

Previous studies have shown that adult foragers of *B. terrestris* exhibit DGC where all bursts of CO$_2$ are accompanied by active ventilating movements with a frequency of about two cycles per hour, when measured at 5 °C (Kuusik et al., 2002), and that pre-diapause queens of *B. terrestris* exhibit two large DGC per hour at 18 °C (Beekman and Stratum, 1999).

In mid-stage pupae, weak abdominal contractions tended to coincide with the bursts; i.e., FV cycles were observed (Fig. 3A), however there occurred also FO cycles, where no ventilating movements were recorded. In late stage pupae (Fig. 4), only FV cycles were recorded, where all bursts of CO$_2$ were accompanied by movements of active ventilation with the frequency of 50–60 movements per hour.

Insect pupae exhibit a great diversity in their patterns of obligatory abdominal movements and in the coordination of these movements with gas exchange cycles (Tartes et al., 2002). Regular bouts of weak abdominal movements...
have been described in pupae of the great wax moth *Galleria mellonella* (Kuusik et al., 1996; Tartes et al., 1999) but in this species bouts occurred independently of the short gas exchange cycles. Pupae of the Colorado potato beetle *Leptinotarsa decemlineata* exhibited large, small and microbursts of CO$_2$ but only the large bursts were associated with active ventilation bouts (Tartes et al., 2000; Kuusik et al., 2001).

In the mid-stage pupae of *B. terrestris*, we observed that not only CO$_2$ release but also O$_2$ uptake was intermittent. Each small burst in mid-stage pupa began with an inflow of O$_2$ into the tracheae. Similar patterns of intermittent O$_2$ uptake have also been recorded from the pupae of *G. mellonella* (Kuusik et al., 1996) and of *Pieris brassicae* (Harak et al., 1999) using the same type of electrolytic respirometers. Intermittent O$_2$ uptake has also been reported from adults of the ant *Formica polyctena* (Martin et al., 2004) and several other insect species, mostly Coleoptera (Punt et al., 1957; Lighton, 1988; Bartholomew et al., 1985).

We recorded clear and real cycles of gas exchange in late stage pupae and even in pupae during mid-stage pupal development. We observed a typical pattern of PSV in microcycles of micro-constrictions, and micro-openings during the interburst periods of mid-stage pupae in *B. terrestris* are also probably typical. A similar pattern of PSV has been recorded from adult ants (foragers) of the species *F. polyctena* (Kuusik et al., 2004). The PSV acts as a water conserving mechanism in many insects (see Kestler, 1978, 1980, 1982, 1985). In contrast to flow-through respirometry we can measure for the first time the combined O$_2$ uptake by convection and by simultaneous diffusion as described in the theory of diffusive convective gas exchange by Kestler (1985). Figure 3 shows that not more than 21% of the volume of tracheal collapse in the micro-constriction period can be replaced by convection as the inflowing air contains only 21% oxygen. The rest or even more must be diffusion, which is modified by convection.

Herford (1938) observed PSV as the pulsations of tracheal collapse first in the soft skinned transparent flea. The tracheal collapse is caused by the faster decrease of the tracheal oxygen pressure (P$_{O_2}$) than the rise in the pressure of carbon dioxide (P$_{CO_2}$), which causes a pressure fall. It leads to an abdominal collapse as the compliant system shows higher volume than pressure change also observed in hard skinned *Hyalophora cecropia* and *Attacus atlantis* pupae. The lack of micro-cycles and PSV at higher SMR in discontinuous gas exchange cycle of early and late stage pupae is probably due to the fact that the spiracles start the micro-opening from a minimal basal opening width, which means that rapid air suction inflow into the tracheae can not occur (Kestler, 1985).

In our study, the simultaneous IRA recordings and respiromgrams allowed easy discrimination of the periods of activity and inactivity. From our results we also concluded that the respiration pattern and the obligatory abdominal movements in the pupal stage of bumblebees should be studied without removing the pupa from the cocoon to avoid the stress induced by handling, apparatus and environment.

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Résumé – Cycles d’échanges gazeux et ventilation active chez les nymphes du bourdon *Bombus terrestris*. Les bourdons sont connus pour être d’utiles pollinisateurs. Leur activité dépend de leur état physiologique et de leur santé et il est donc important de mettre au point des méthodes appropriées pour évaluer leurs conditions physiologiques. On ne dispose pas à l’heure actuelle de données sur les schémas d’échanges gazeux, ni sur les mouvements de ventilation chez les nymphes de bourdons à l’intérieur de leur cocon. Cet article rend compte d’une étude portant sur le taux métabolique (MR), les schémas d’échanges gazeux et les mouvements de ventilation chez les nymphes de *Bombus terrestris* L. à l’intérieur de leur cocon. Nous avons enregistré simultanément le taux métabolique basal (SMR), l’émission discontinue de bouffées et de micro-bouffées de CO$_2$ et la consommation cyclique d’oxygène, i.e. les cycles discontinus d’échanges gazeux (DGC), ainsi que les mouvements de ventilation sur des nymphes d’âge moyen et des nymphes âgées de *B. terrestris*, sans les extraire de leur cocon. Un actographe-microrespiromètre électrolytique a été utilisé pour enregistrer
After being extracted from their cocoons, the nymphs presented a typical profile on the respirogram (Fig. 5). The mode of gas exchange formed the typical pattern during the diurnal cycles (DGC) as well as the active ventilation (Fig. 4). The nymphs were younger, with a more continuous gas exchange. The standard metabolic rate (SMR) was observed in all CO₂ emissions in the respirometry (IRGA), so the respirometer electrolytically was not interrupted for the insect (Fig. 1).

This study allowed registration of the first respiratory cycles and active ventilation in bumblebee pupae. The CO₂ emission was always within the cocoons for the insects without disturbing them (Fig. 1). In the late pupal stages, the IRGA and the electrolytically coupled actograph (IRA). The respirometer was set up inside the insect chambers between the nymphs of different ages. In the middle and late pupal stages, a periodic cycle of gas exchange was observed in the respirogrammes (Fig. 5). After being extricated from their cocon, the nymphs lost water significantly more than the nymphs did not extract the cocoon, thus avoid the stress induced by the manipulation, the apparatus, and the environment.

**Bombus terrestris** / *échanges gazeux discontinus / ventilation passive par succion / taux métabolique de base / respirométrie*


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The effect of a sublethal dose of alpha-cypermethrin on the cyclic gas exchange of bumble bee *Bombus terrestris* foragers

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Abstract

Using a system of flow-through CO₂ respirometry, the effect of sublethal doses of alpha-cypermethrin on bumble bee *Bombus terrestris* foragers was studied. We found an increase in CO₂ emission rate at high relative humidity (RH=95%) (0.130±0.01 ml h⁻¹), compared to low relative humidity (RH=2%) (0.074±0.01 ml h⁻¹). Treatment with alpha-cypermethrin decreased significantly the frequency of bursts of CO₂ releases during discontinuous gas exchange (DGC) in dry conditions. In humid conditions no significant differences were found.

**Key words:** gas exchange, bumble bee, alpha-cypermethrin

1. Introduction

In agricultural landscapes the abundance of native pollinators has been decreasing rapidly during recent decades (Benton et al., 2003, Carvell et al., 2006). As one of the probable reasons, intensive use of pesticides has been argued (Wickramasinghe et al., 2004). As in commercial pesticide production data are needed about the mortality of non-target organisms, lethal doses have been established and consequent restrictions on the application of pesticides have been set. Yet sublethal, imperceptible doses affect non-target organisms in several ways (see for review Thompson 2001, 2003) and further research should also focus on the effects of sublethal doses on the physiological states of insects.

In resting insects often exhibit discontinuous gas exchange cycles (DGC) (Hadley, 1994). In this state the spiracles are closed most of the time. At low oxygen rate the spiracular valves flutter, allowing oxygen to enter the tracheal system. As larger amounts of carbon dioxide accumulate in the tracheae, the spiracles open and allow the gas to escape. Thus in comparison with continuous respiration, loss of carbon dio-
ide along with evaporated water occurs only discontinuously during the brief open phases of the spiracles. Water balance is maintained because the amount of metabolic water generated by hydrolysis of stored fats equals the amount of water lost during this discontinuous respiration (see reviews: Hadley, 1994, Klowden, 2002).

There are different arguments about the origin of DGC, as reviewed by Chown (2002). Lighton (1994) found respiratory water loss to be such a small part of total water loss that it is unlikely to have a negative effect on soft-bodied insects. Kestler (1980, 1982) and Barnhart and McMahon (1987) have stressed the importance of passive suction ventilation (PSV) in reducing water loss in insects. Essentially, these authors stated that over the entire gas exchange cycle, it is CO₂ release that limits the extent to which ventilation and, consequently, water loss can be minimized. There are also hypotheses that DGC serves as an adaptation for coping with hypercapnia and/or hypoxia in soil-living insects (Lighton, 1998, Vogt, Appel, 2000, Lighton et al., 2004).

The existence and the precise pattern of DGC depend on the environment and life stage of the individuals. For example, bumble bee (B. terrestris) queens have been found to exhibit discontinuous ventilation cycles at room temperature in different life stages, before and after hibernation (Beekman, Stratum, 1999). The distinct environmental conditions that the queen was exposed to affected the frequency and amount of carbon dioxide emitted. In honey bee workers the temperature also affected the DGC (Lighton, Lovegrove, 1990). Kuusik and co-workers (2002) studied the respiratory patterns of bumble bee (B. terrestris) workers at low temperatures when they were restrained and motionless, to rule out their high activity at room temperature.

The patterns of DGC have been used for characterizing the physiological state of an insect, while several stress factors including the chemical ones can affect it (Kestler, 1991). Although knowledge about the sub-lethal effects of pesticides is scarce, it is known that treatments of arthropods with pyrethroids cause neurotoxic effects in parts of the nervous system, including the central nervous system and sensory, motor or neurosecretory neurons (Corbett, 1974, Jagers op Akkerhuis et al., 1995). Because the closing and opening of spiracular valves is controlled by the nervous system, the neurotoxic effects may also include interference by DGC. Pyrethroids as well as many other insecticides also induce increased water loss in arthropods (Gerolt, 1976, 1983). Water loss is induced by producing the diuretic hormones after pyrethroid poisoning (Jagers op Akkerhuis et al., 1999a) in insects’ larvae and adults, a
process which could be reversible if the insect could replenish its wa-
ter reserves. Since the pyrethroids often affect motion, as well, causing
the knockdown effect, death may come through desiccation (Jagers op
Akkerhuis et al., 1995, Jagers op Akkerhuis et al., 1999a, Thompson,
2003). In pupae of cabbage butterfly *Pieris brassicae*, after the treatment
with original pyrethrum, the DGCs disappeared and the metamorphosis
was disrupted (Harak, et al., 1999, Jõgar et al., 2006). Insects exposed to
spinosad experience hyper-excitation of the nervous system, followed by
inhibition of neural firing (Salgado, 1998).

Bumble bees are valuable pollinators that are endangered by vari-
ous human activities. The sublethal effects of pesticides on adult foragers
may not be observable without special methods used in experiments of
insect physiology. Therefore the aim of the present research was to study
the effect of a sublethal dose of alpha-cypermethrin on discontinuous gas
exchange cycles of bumble bee *B. terrestris* foragers.

2. Material and Methods

2.1. Bumble bees

The colonies (Natupol hives) with healthy and non-infected bumble
bees *B. terrestris* were purchased from the Koppert Biological Systems
B.V. (the Netherlands). The hives were kept at room temperature. The
bumble bees were fed with dried pollen and a sugar solution (30%).
Foragers were caught from the hives directly before using them in the
experiment, so they had no handling. To ensure that the individuals were
indeed foragers, we let them leave the nest through the tunnel in a way
common for *B. terrestris* in the nature.

2.2. Respirometry

An infrared gas analyser (IRGA, Infralyt-4, VEB, Junkalor, Dessau),
adapted for entomological research, was used to record the CO$_2$ signals.
IRGA was calibrated at different flow rates by means of calibration gases
(Trägergase, VEB, Junkalor, Dessau), with gas injection (look also Kuu-
of carbon dioxide release was measured ($V_{CO_2}$ ml h$^{-1}$) at an air flow rate
of 3.6 l h$^{-1}$. The CO$_2$ and H$_2$O were eliminated from the air, used in the
flow-through system, by drierit and a molecular sieve. High humidity
level was achieved secondarily by using wet filter paper inside the respira-
tion chamber. CaCl$_2$ was used to remove H$_2$O vapour from the system before the air entered the infrared gas analyser.

2.3. Treatments

In the present experiment a commercial formulation of alpha-cypermethrin (Fastac, a.i. 50 g/l) was used. The preparation was diluted with distilled water to $3.7 \times 10^{-4}$ % of a.i., which is a tenfold lower dose than that recommended for use in fields against pests. The respiration rate and the frequency of bursts of CO$_2$ releases of _B. terrestris_ foragers were measured at 4-5 °C at a humidity of about RH = 2% and RH = 95% for four hours. After the first two hours, the bees were dipped in distilled water or in the alpha-cypermethrin solution for 10 seconds. Following dipping, each bee was dried for 1 minute on filter paper and placed back into the respiration chamber. After the end of the experiments all bees were kept at 4 °C at RH = 50–60% and their behaviour was observed for one week. In the statistical analysis of the data the paired t-test and factorial ANOVA (STATISTICA 7.0) were used. All mean values are presented with ± Standard error.

3. Results

3.1. Respiration rate

The respiration rate of the bumble bees was affected by the humidity of the environment (ANOVA: $F_{1,16} = 13.2$, $P = 0.002$). In dry conditions (RH=2%) the mean rate of CO$_2$ emitted by the bumble bee foragers was significantly lower ($0.0738 \pm 0.014$ ml h$^{-1}$) than in moist conditions (RH=95%) ($0.1295 \pm 0.011$ ml h$^{-1}$). Treatment itself did not affect the respiration rate (ANOVA: $F_{1,16} = 0.11$, $P = 0.7$) of the bees, nor did the respiration rate change considerably after dipping the bees into distilled water or the alpha-cypermethrin solution (ANOVA: $F_{1,16} = 0.9$, $P = 0.3$).

3.2. Frequency of DGC

The bumble bee workers exhibited clear cycles of discontinuous gas exchange when resting in low temperature conditions (Figure 1). In dry air, the frequency of bursts of CO$_2$ releases was the highest ($4.9 \pm 1.01$ bursts h$^{-1}$) in the untreated bumble bees and the lowest in the alpha-cypermethrin treated bees ($0.36 \pm 0.17$ bursts h$^{-1}$). A significant difference
in the frequency of the bursts of CO$_2$ releases before and after treatment became apparent only in the case of alpha-cypermethrin treatment in dry conditions (RH=2%) (t = 4.25, df = 4, P = 0.01) (Figure 2). At high humidity (RH=95%) alpha-cypermethrin treatment did not affect the frequency of bursts of CO$_2$ releases (t = 2.27, df = 4, P = 0.09), nor was it affected by treatment with distilled water in both conditions (RH=2%: t = 2.48, df = 4, P = 0.07; RH=95%: t = 0.53, df = 4, P = 0.6).

3.3. After-effect of the treatment on bumble bees

The bumble bees treated with alpha-cypermethrin maintained the normal resting position, standing upright only when they were exposed to high humidity, RH = 95%. However, at low air humidity, RH = 2%, the treated bees were lying on their backs and appeared to be dead. After returning them to humid conditions, the bees recovered within a couple of hours but survived at low temperatures only 2–4 days after the experiment. The bees treated with distilled water did not lie down and survived in humid conditions and at low temperature for at least one week.

4. Discussion

Discontinuous ventilation has often been cited as being an adaptive mechanism for minimizing respiratory water loss especially for pupae (Hadley, 1994, Klowden, 2002). Along with adults of many insect species (Klowden, 2002), cyclic respiration has also been found in bumble bee queens (Silvola, 1984, Beekman, Stratum, 1999) and foragers (Kuusik, et al., 2002). The above studies proved that in rest conditions bumble bees change from continuous to discontinuous ventilation; the latter is thought to prevent excessive water loss through the spiracles (Kestler, 1984, Hadley, 1994, Lighton, 1994, 1996).

Our experiments (V) showed that the CO$_2$ emitting rate of the $B.$ terrestris foragers was higher in humid conditions compared with dry conditions. In addition, the frequency of bursts of CO$_2$ releases was lower not only after treatment of the bumble bees with the weak alpha-cypermethrin solution but also after treatment with distilled water. This supports the theory that the DGC can be used as a water saving mechanism.

The rate of CO$_2$ emission in the bumble bee foragers kept in dry air was low (mean 0.074 ± 0.01) in comparison with corresponding rate for those kept in moist air (mean 0.130 ± 0.01). Alpha-cypermeth-
rin treatment after 2 hours of the experiment reduced the frequency of bursts of CO$_2$ releases almost to zero. The reason for the significant decrease in DGC after treatment with the pyrethroid insecticide at RH = 2%, but not at RH = 95%, remains unclear. It does prove, however, that the effect of the chemical is definitely environmental-specific and shows that laboratory data obtained by defined conditions must be considered critically. Yet the effect of the pesticide on the survival of the foragers was evident, as all of them died earlier than the ones treated with distilled water. The synthetic pyrethroids act on the insects’ nervous systems and cause disorders in neuronal functions (Corbett, 1974). Deltamethrin has been found to cause concentration-dependent hyper-excitation of the respiratory motoneurons (Zafeiridou, Theophilidis, 2006) and the treatment of *P. brassicae* pupae with original pyrethrum caused the disappearance of DGC cycles (Harak, *et al*., 1999). If the disappearance of DGC was the cause of the earlier death of bumble bees in our experiment, it indicates the importance of DGC in the water-saving mechanism. Since the effect (toxic?) level of many insecticides is dependent upon environmental factors (Jagers op Akkerhuis *et al*., 1999b), we deduce that contact with sublethal doses of alpha-cypermethrin may cause the lethal after-effect under certain environmental conditions. The co-effects of climate conditions and toxins could lead to dehydration if the bee were not able to reach its nest.

During their life cycle, bees encounter widely varying humidity conditions; the variations strongly affect their physiology. Climate conditions in the nest of bumble bees are stable and provide optimum temperature and relative humidity levels for development of the immature offspring. In many eusocial species the brood is highly sensitive to temperature extremes and perishes soon if the nest’s humidity is not regulated (Moritz, Crewe, 1988). Therefore approximately 40% relative humidity is maintained in the brood chamber in honey bee hives (Humar, *et al*., 2006). In bumble bee nests, the relative humidity is 50-60% (Heemert *et al*., 1990), which is much higher than the humidity level that they encounter when foraging on hot, sunny midsummer days. In regions with temperate climate, the relative humidity outside the hives may vary on a large scale, from 70-80% in early morning to 12-20% during the afternoon on particularly warm and dry days. Temperature also varies widely from day to night: in early summer, daily maximum temperatures may reach approximately 25°C, while by night, temperatures may still remain quite low, around 10°C. These fluctuations increase the bees’ sensitivity to pesticides.
There is evidence that synthetic pyrethroids may cause orientation problems for bees so that they may never return to the hive (Cox, Wilson, 1984). This has been suggested as one of the reasons which caused Colony Collapse Disorder (CCD), a newly discovered disorder which was found in the southern states of the USA in 2006 (Engelsdorp et al, 2007). In CCD, a great number of colonies with the queen, the brood and food supplies simply run out of workers. It is not known whether similar symptoms have been found in wild bees, but our experiment showed that sub-lethal doses of pesticides may affect bees on the physiological level. The effects of the insecticide may not be observable in a short time frame but subsequently do affect the survival of both the individual and the colony. More detailed research is needed about the short- and long-term effects of sub-lethal doses of pesticides on the physiology of insects.

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Figure 1.
Figure 2.
Figure 1. Recording of the infrared gas analyser ($V_{CO_2}$), demonstrating the release of $CO_2$ by a bumble bee $B. terrestris$ forager at low temperature in dry conditions (RH=2%) before (A) and after (B) alpha-cypermethrin treatment.

Figure 2. The effect of dipping the bumble bee foragers (N=5 for each treatment) into distilled water or into the alpha-cypermethrin solution (3.7 x 10^{-4} %) on the frequency of bursts of $CO_2$ releases in RH=2% (A) and RH=95% (B). The means, SE of the means and SE*1.96 of the means are presented. * - p<0.05; n.s. – not significant (paired t-test).

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1.3. Papers in Estonian and in other peer-reviewed research journals with a local editorial board:


3.2. Papers published in books by Estonian or foreign publishers not listed in the ISI Web of Proceedings:

Karise, R. 2005 Ausus ja valetamine taim-putuka suhetes. TÜ Schola Biotheoretica XXXI, 115–121.

3.4. Papers published in the proceedings of international conferences:

Sustainable Agriculture, Agrienvironment and Food Technology. Volos, 367–372