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Results of treatments with natural insecticidal substances on the development and physiological state of insects

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Abstract. Reviewed are the results of treatments carried out in the Institute of Agricultural and Environmental Sciences of treatments with natural insecticidal substances on the development and physiological state of insects, using neurotoxical plant extracts (pyrethrins), plant extracts possessing several modes of action (neem) and an Insect Growth Regulator (extract of *Ledum palustre* possessing properties of juvenile hormone). Physiological changes evoked with treatments were monitored by means of complex methods including a constant volume respirometer, infrared actograph, infrared gas analyzer (IRGA),and a microcalorimeter.

Diapausing lepidopterous pupae (*Pieris brassicae, Mamestra brassicae*) exhibited discontinuous gas exchange cycles (DGCs). After treatments of the pupae of *P. brassicae* with neem, the DGCs were abolished, suggesting direct action on the neuromuscular system. The disappearance of DGCs was accompanied with lethal desiccation, supporting the hypothesis that DGC is an adaptation for restricting the respiratory water losses. Cyclic gas exchange was also lost after treatments with neem in adults of *Leptinotarsa decemlineata*. The clear gas exchange cycles in the pupae of *Galleria mellonella, Tenebrio molitor* and *P. brassicae* were also abolished after treatments with extracts of *Artemisia vulgaris*, *Tanacetum vulgare*, *T. roseum* and *L. palustre.* The timing of the normal and failed ecdysis (after treatment with *L. palustre*) as well as length of intercdysial periods in *T. molitor* pupae was measured exactly from calorimetric recordings. The treated pharate pupae transformed into extra-pupal instars, which is a symptom of juvenilizing effect.

Key words: *Tenebrio molitor*, *Galleria mellonella*, *Pieris brassicae, Leptinotarsa decemlineata,* bioinsecticide, discontinuous gas exchange cycles, water loss

INTRODUCTION

The physiological state of insects is usually estimated by standard parameters revealed in respiration, cuticular and respiratory transpiration (water loss rates) and hemolymph circulation (heartbeat patterns). The studies on the physiological state of insects present not only theoretical but also substantial practical interest and value. Chemical stress factors also affect the physiology of insects. The dose-mortality relations of insecticides have been well studied, but the first steps of the poisoning process in a living organism have been poorly investigated. The respiration and transpiration systems are the most vulnerable targets of most of the contemporary insecticides, including the bio-insecticides and plant extracts.

Many plants contain chemicals that are toxic to insects. Throughout history, plant products have been successfully exploited as insecticides, insect repellents, and insect antifeedants (Dethier et al., 1960; Dethier, 1970; Schoonhoven, 1982; Addor, 1995; Mordue (Luntz), 1998). Extracts from the neem tree (*Azadirachta indica*) have emerged as an excellent alternative to synthetic insecticides for plant protection practices. Azadirachtin (referred to below as neem), a steroid-like tetranortriterpenoid derived from neem trees, is a strong antifeedant, repellent and growth regulator for a wide variety of phytophagous insects. Azadirachtin has also shown direct detrimental and histopatological effects on most insect tissues, e.g. muscles, body fat, and gut epithelial cells (Mordue (Luntz) and Blackwell, 1993; Mordue (Luntz), 2004). The direct effect of neem on the muscular system has been studied less.

The main advantages of using a bioinsecticide like neem are reduced toxicity to humans, fast and complete degradation in the environment, low risk for resistance and selective properties for non-target organisms (Schmutterer, 1995).

There is an enormous amount of literature about the influence of the chemical substances on the breathing of insects in their larval and adult stages (see Keister and Buck, 1964; Luik & Viidalepp, 2001). However, the physiological mechanisms on how sub-lethal doses of insecticides affect the breaching in the pupal stage, when no digestion and excretion occur, are almost unknown.

Many insects in adult and pupal stages are characterized by discontinuous gas exchange cycles (DGC), including the pupae and adult of *Leptinotarsa decemlineata* (Tartes et al., 2000; Vanatoa et al., 2006), the pupae of *Pieris brassicae* (Tartes et al., 1999a, 2002) and *Galleria mellonella* (Tartes et al., 1999b).

It is supposed that disturbances in cyclic gas exchange can raise the transpiratory water loss in insects, causing lethal desiccation (see Edney, 1957; Kestler, 1991). In our studies the respiratory and musculatory responses in pupae of *Tenebrio molitor*, *G. mellonella, P. brassicae* and in adult *L. decemlineata* evoked by treatments with bio-insecticides were observed.

MATERIALS AND METHODS

Insects. Eggs of *P. brassicae* (second generation) were collected in the cabbage fields near Tartu (Estonia). The insects were reared at a laboratory in short-day conditions on cabbage leaves (12L: 12D, at 20–22°C). After pupation, the pupae of *P. brassicae* were kept in the state of “permanent” diapause during the winter months at room temperature (18–23ºC) and at ambient air humidity (55–65% RH) in Petri dishes (Harak et al., 1999). *T. molitor* was reared in Petri dishes at 30°C and ca 70%RH in constant darkness. Flour, with the addition of 10% yeast or wheat, was used as the diet. (Kuusik et al., 1993; Kuusik et al., 1995). The pupae of *G. mellonella* were taken from stock cultures, bred on a modified semi-artificial diet at 30°C and in constant darkness (Kuusik et al., 1993; Kuusik et al., 1999). The adults of *L. decemlineata* were collected from the potato fields near Tartu,and were fed in a laboratory until they entered diapause. The diapausing beetles were held at 5…6°C in a refrigerator. Before measuring their respiration and muscular activity, they were adapted to room temperatures (18…22°C) in one day (Kuusik et al., 2001a; Kuusik et al., 2001b).

Bio-insecticides. Extracts of *Heracleum sosnowskyi*, *Nicotiana rusticum*, *Artemisia absinthum, A. vulgaris, Tanacetum vulgare*, *T. roseum*, *Ledum palustre*, Neem Azal T/S (1% Azadirachtin A) from Trifolio-M GmbH, Germany) (Kuusik et al., 1993; Kuusik et al., 1995; Harak et al., 1999; Kuusik et al., 1999; Kuusik et al., 2001a; Kuusik et al., 2001b) and Neem EC (1% Azadirachtin A) from the Indian Neem Tree Company were used. The insects were treated by submersion in plant extracts (dipping method).

Measurements. We recorded the gas exchange cycles and muscular ventilation by means of an electrolytic respirometer-actograph (Kuusik et al., 1993, 1996, 2001b; Tartes et al., 1999, 2000) and by an infra-red gas analyzer (Jõgar et al., 2005). The electrolytic respirometer was combined with either a flow-through respirometer (infra-red gas analyzer) (Fig. 1) or with the microcalorimeter ((Jõgar et al., 2005). For simultaneous recording of tracheal ventilating, gas exchange rhythms, muscular ventilation and heartbeats the respirometer-actograph was also combined with the infrared probe actograph (Kuusik et al., 2001a; Metspalu et al., 2002; Jõgar et al., 2004).

Heat flow rates were registered simultaneously with the respirometric measurements by the twin set-up calorimeter (Harak et al., 1999). The cyclic releases of CO_2 and WLR were simultaneously recorded by means of flow-through respirometry (IRGA) combined with a hygrometer (HygroPalm, Rotronic Company) inserted into the insect chamber. Pupal body masses were determined to 0.1 mg with micro-analytical balances.

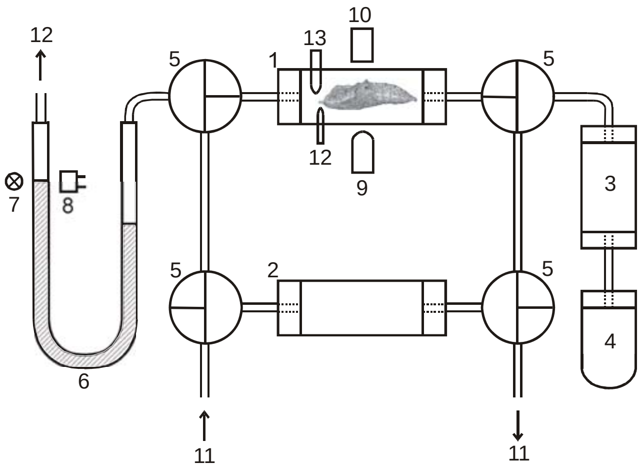

Fig. 1. Schematic diagram of an experimental set-up for respirometry. 1 – An insect chamber that may be switched from still-air electrolytic respirometry to the flow-through respirometry system of the infrared gas analyser (IRGA); 2 – reference chamber of IRGA; 3 – vessel for dry potassium hydroxide; 4 – electrolysis unit; 5 – three-way taps; 6 – glass capillary with ethanol; 7 – light source; 8 – photodiode; 9 – infrared emitter diode; 10 – infrared sensor diode; 11 – connections to the flow-through system; the double lines denote the polyethylene tubings; 12 – fibre-optic oxygen sensor; 13 – hygrometer.

RESULTS

A typical toxic effect caused by various neurotoxic substances (e.g. dipping in *N. rusticum*, *A. vulgaris*, *A. absinthum* or *H. sosnowskyi*) is the abolishing of normal discontinuous gas exchange cycles (DGCs) in pupae of *T. molitor*, *G. mellonella* (Kuusik et al., 1993) and *P. brassicae (*with *T. roseum*) (Harak et al., 1999). The post treatment peaks were similar to the chaotic flutter of relatively high amplitude and frequency (Fig 2.) (Harak et al., 1999). Similar results were recorded in adult *L. decemlineata* after treatment with Neem Azal T/S (neem); DGCs were lost and the beetles showed tidal muscular ventilation resulting in expansive water losses through the spiracles (Kuusik, et al., 2001a). Thus the neem application affected only the regular and larger cyclic emission of carbon dioxide, while the microbursts of this gas were preserved at low standard metabolic rates. Normally the DGCs are synchronized with the opening-closing rhythms of the subelytral cavity. In the beetles treated with neem the subelytral cavity was continuously left semi-open while no regular closing rhythm was evident . (Kuusik et al., 2001b).

After treatment with *T. roseum* in pupae of *P. brassicae* active ventilation was also lost and no bouts of abdominal rhythmic movements were recorded (Harak et al., 1999). The main feature of weak toxication was the lengthening of extracardiac haemocoelic pulsations (EHP) in pupae of *T. molitor* and *G. mellonella*, but in some cases chemical excitation resulted in permanent EHP, which lasted for several hours.

A typical symptom of weak or moderate toxicoses (dipping in *H. sosnowskyi*, *T. vulgare*, *A. absinthum* or *A. vulgaris*) was the strengthening of muscular contractions during abdominal shaking movements and the lengthening of these periods. (Kuusik et al., 1993). Similar results were recorded in pupae of *T. molitor* after treatments with *L. palustre* extract (Kuusik et al., 1995). The Colorado potato beetles responded to the treatments with neem immediately, showing spontaneous contractions of somatic muscles due to the hyperactivity of the neuromuscular system. Due to the muscular activity the metabolic rate increased about two times as compared with the SMR of control individuals (Kuusik et al., 2001a). In the neem treated beetles the influence of heartbeat rates and the regularity of heart pulses were not recorded even in the case of the longest exposition time lasting 30 seconds (Fig. 3) (Kuusik et al., 2001a). After some hours the active ventilating movements ended and now the fully restrained beetles showed continuous gas exchange through the continuously opened spiracles.

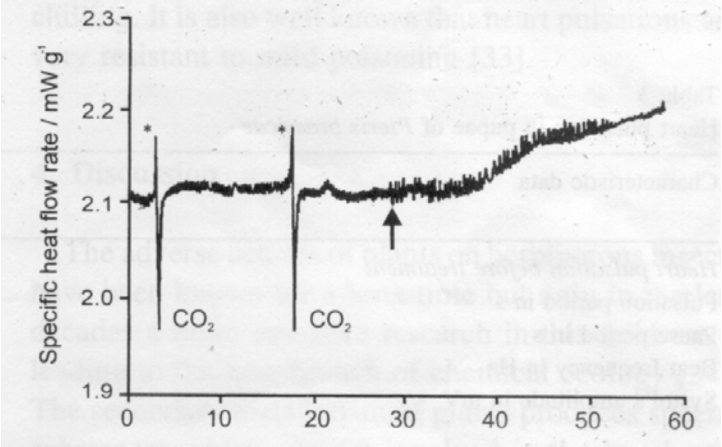

Fig. 2. The calorimetric registration of the abolishing of large CO_2 cycles in a diapausing pupa of *P. brassicae* after treatment (arrow) with the extract of *T, roseum*.

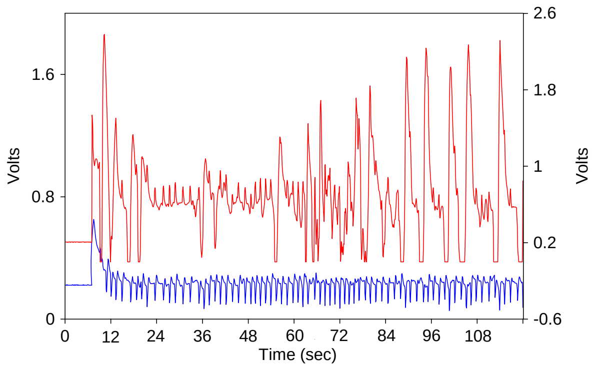

Fig. 3. A typical pattern of muscular hyperactivity (upper trace) caused by treatments with neem preparation in an adult Colorado potato beetle. Note that heart pulsations are not affected by neem (lower trace). An infrared actograph recording from abdominal 3rd segment (upper) and metathoracic segment (lower) simultaneously (Kuusik et al., 2001a).

The right part of the record is typical of nondiapausing pupae. Asterisks indicate the exothermic signal at the abrupt air intake suction into tracheae (Harak et al., 1999).

The normal DGCs were entirely lost and did not resume at all. (Kuusik et al., 2001b). Treatment with Neem EC (neem) showed a clear delayed effect on the skeletal muscles of *P. brassicae* pupae (our unpublished data). After treatment with the *T. roseum* extract the body mass loss of *P. brassicae* and *G. mellonella* pupae increased significantly. All the diapausing pupae who survived the treatment developed into pharate stages, but adult emergence failed (Harak et al., 1999; Kuusik et al., 1999). Before treatments with neem, the daily water loss in *P. brassicae* pupae was 0.6 – 0.9 mg g^{-1}. All *P. brassicae* pupae exposed to the use of the neem preparation died due to desiccation but the time from treatment to death varied individually from 5 to 21 days (our unpublished data).

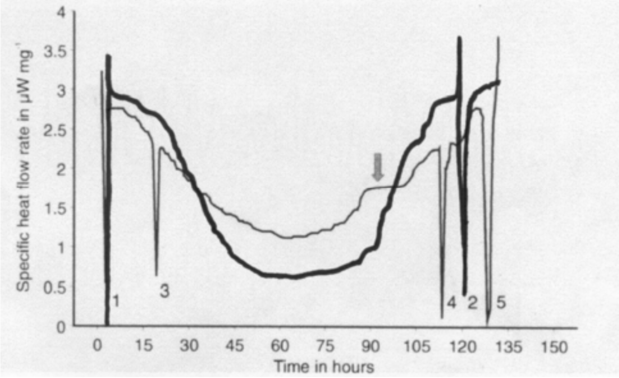

Fig. 4. Metabolic curves of normal pupal development of *T. molitor* (thick line) and inhibited metamorphosis (thin line) due to treatments with *L. palustre* extract. Downward peaks result from liberated exuvial fluid:1, perfect larval-pupal ecdysis (E); 2, pupal-adult ecdysis (E); 3, leaking of exuvial fluid; 4 and 5, partial ecdysis into extra-pupal instars. Upward peaks denote muscular hyperactivity prior to ecdysis. (Kuusik et al., 1995).

The extract of *L. palustre* evoked various morphogenetic effects on pupae of *T. molitor* depending on the time of treatment (Fig. 4). The treated pharate pupae transformed into extra-pupal instars, which is a symptom of juvenilizing effect.

DISCUSSION

The discontinuous gas exchange cycles in insects are thought to be a water-conserving mechanism (Kestler, 1980, 1985). This was the most probable reason why lethal desiccation occurred in the neem-treated *L. decemlineata* beetles and *P. brassicae* pupae. Thus it was evident that one of the immediate and externally invisible side effects of the neem was the failure in the gas exchange resulting in increased transpiration. According to our data in pupae of *P. brassicae* the water was lost mainly during a burst of carbon dioxide, and only a negligible fraction of water was released during the interburst period (Jõgar et al., 2005). In this way it was experimentally demonstrated that cyclic gas exchange restricts the water losses in pupae, and thus the hypothesis that DGC serve as water conserving mechanisms was supported. These results suggested a direct effect of neem on the neuromuscular system. Azadirachtin has also shown direct detrimental and histopatological effects on most insect tissues, e.g. muscles, body fat, and gut epithelial cells (Mordue (Luntz) and Blackwell, 1993; Mordue (Luntz), 2004). The periodically occurring obligatory abdominal pulsations or extracardiac haemocoelic pulsations, recorded in all untreated *P. brassicae* pupae, were lost, together with loss of other active body movements. The spiracles are normally open for the shortest time necessary for efficient respiration, presumably to keep water loss from the tracheal system to a minimum. Spiracle closure results from the sustained contraction of the closer muscle, while opening commonly results from the elasticity of the surrounding tissue when the closer muscle is relaxed. The muscle is controlled by the central nervous system, but may also respond to local chemical stimuli (reviewed in Nation, 2002; Klowden, 2002).

The prolonged periods of vigorous rotation in *G. mellonella* and *T. molitor* pupae were evoked only by highly neurotoxic substances in large doses (*H. sosnowskyi* and *N. rusticum*), and mortality of the treated pupae was supposedly caused by increased transpiratory water losses (Kuusik et al., 1993).

The cessation of the cyclic gas exchange may be regarded as the earliest symptom of poisoning by toxicant in insects (see Kestler, 1991). It can be concluded from the results of this work that various toxic substances in plant extracts can abolish normal DGCs in pupae of all butterfly species studied. According to our preliminary studies sublethal doses of extracts of *T. roseum* stimulate adult development in some lepidopterous pupae associated with toxic influences.

The abolishing of the cyclic gas exchange and lethal desiccation after treatments with neem was also described in adult pine weevil, *Hylobius abietis* (Sibul et al., 2004).

CONCLUSIONS

The respiration and transpiration systems are the vulnerable targets of the used bio-insecticides used. The typical DGCs were also lost in insect species treated with extracts from certain plants. After the loss of DGCs an enhanced water loss occurred due to respiratory transpiration. This result supports the hypothesis that DGC serves as a water conserving mechanism. From the results it may also be concluded that a precondition for effective use of novel methods against a pest insect is extensive knowledge about the physiology of that insect pest.

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