Occurrence of weight gain reduction and inhibition of metamorphosis and storage protein formation in last larval instars of the Mexican bean beetle, *Epilachna varivestis*, after injection of azadirachtin

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Abstract

Injection of the insect growth regulator azadirachtin into 40-h-old last larval instars of *Epilachna varivestis* (Mulsant) inhibited metamorphosis at doses lower than 0.3 μg. Various higher doses (0.3–1.0 μg) caused in addition weight gain reduction, whereas weight gain was almost entirely suppressed after injection of 2.5 μg. Histological and electrophoretical analyses showed that storage protein formation in the fat body, necessary for pupation, did not occur in higher dose treated (1.0 μg) individuals, but it was not affected by low dose (0.1 μg) treatment. It is assumed that azadirachtin causes metabolic defects at higher concentrations.

Introduction

Azadirachtin – a compound from the neem tree *Azadirachta indica* (A. Juss) – is one of the most potent insect growth regulators (IGR) known so far (for surveys see Schmutterer *et al.*, 1981; Schmutterer & Ascher, 1984). Although some insects may be deterred from feeding azadirachtin, its primary action is thought to be on the hormonal system (Sieber & Rembold, 1983; Käuser & Koolman, 1984), leading to inhibition of larval moulting, metamorphosis, or other hormone dependent processes.

After feeding larvae and adults of the Mexican bean beetle, *Epilachna varivestis* with azadirachtin, Steets (1975) observed numerous effects that were partially analyzed by histology and electronmicroscopy (Schlüter & Schulz, 1984; Schulz & Schlüter, 1984). However, feeding did not allow an exact separation of single events from the broad spectrum of phenomena, since the feeding rate and therefore the uptake of azadirachtin were very different. Thus injection of azadirachtin was found more suitable. The objectives of the present study were to separate moulting inhibitory effects from those of weight gain reduction. Furthermore, indications for an interference of azadirachtin with metabolic processes should be found.

Materials and methods

The experiments were carried out on last larval instars (L_4) of *Epilachna varivestis* under constant conditions (65% R.H., 24°C, and LD 16:8 photoperiod). The larvae were fed on bean leaves that were daily replaced with fresh ones. Azadirachtin solutions were prepared at corresponding concentrations with 90% ethanol and each larva was injected 0.5 μl into the left body half, 40 h after moulting to the last instar. Controls were injected with solvent only. For separation of polypeptides by SDS-polyacrylgelelectrophoresis (SDS-PAGE) small pieces of the abdominal fat body were removed from treated and untreated individuals and washed twice in ringer solution. They were homogenized in 50 μl Tris-HCl buffer (pH 6.8, containing SDS, bromphenolblue, and mercaptoethanol) in Eppendorf

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vials with a teflon pestle and by subsequent sonication. The samples were heated at 100°C for 2 min. and centrifuged. The supernatant was either directly used for electrophoresis or stored in a refrigerator at -18°C. Slab gels (12%) were prepared (Laemmli, 1970) and stained with Coomassie brilliant blue in methanol/acetic acid.

Microscopic techniques were described previously (Schlüter, 1984).

Results and discussion

Weight gain reduction. Fig. 1 shows the weight gain after injection of different doses of azadirachtin. The controls reach nearly 50 mg. When entering the prepupal stage (day 5–6) growth stops as larvae stop feeding. Pupation takes place 1.5–2 days later.

Single doses of 0.3, 0.5, 0.65 and 1.0 μg revealed no different effects on weight gain and behaviour. This is especially expressed by the low standard deviation of the means if all values of the four groups were combined (Fig. 1). There was a moderate increase in weight beyond the third day, followed by an equally moderate decline. A more drastic effect was detectable in those individuals that received 2.5 μg azadirachtin; after a very low increase the weight dropped below the starting weight.

There was a significant difference between these larvae and those injected with 0.1 and 0.2 μg only. If the weight values of these individuals were pooled also, no significant difference in weight to the controls were shown (Fig. 1). None of the treated larvae in any of these groups reached the prepupal stage or even pupated.

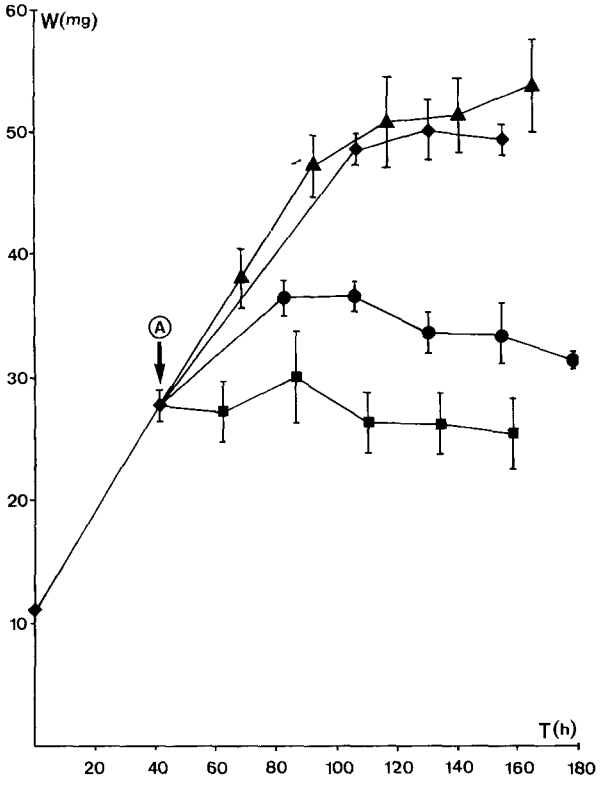

Fig. 1. Weight development after injection of different doses of azadirachtin (A) in 40 h 4th instar larvae (L_4 of *E. varivestis*. ♦ control (solvent only); ▲ 0.1 and 0.2 μg; ● 0.3, 0.5, 0.65, 1.0 μg; ■ 2.5 μg.

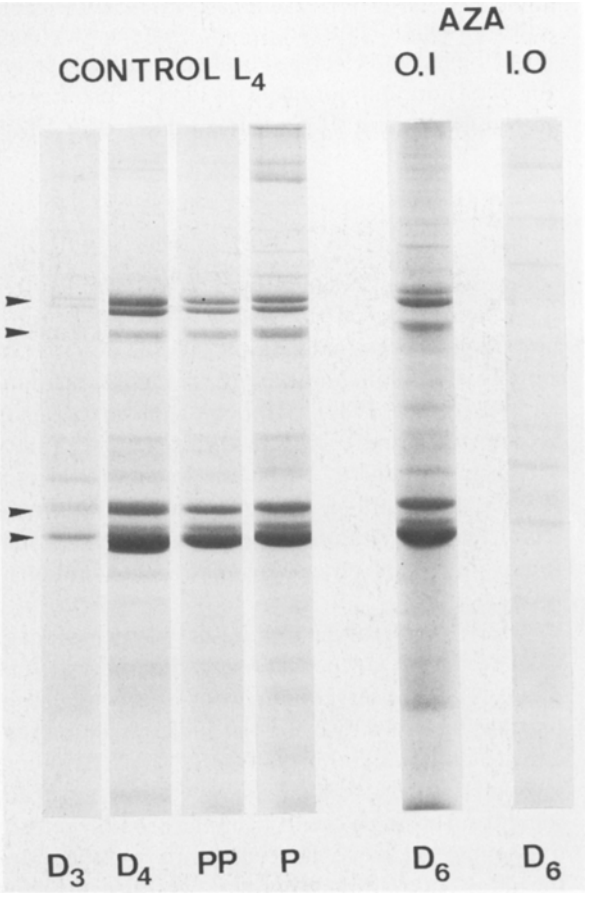

Fig. 2. SDS-PAGE of fat body lobes. Controls of day 3 and 4 (D_3, D_4), of a prepupa (PP) and a pupa (P) on the left. On the right fat body samples of 0.1 and 1.0 μg azadirachtin treated individuals removed on day 6. Bands indicated by arrowheads represent subunits of storage proteins.

There is no detectable difference in feeding behaviour during the first days after treatment. Later on, especially the individuals treated with higher doses showed mobility defects and deficiencies in coordination of the legs, so that they dropped from the leaves.

Weight gain reduction effected by azadirachtin was also found in other insect species (Warthen & Uebel, 1981; Rembold *et al.*, 1982; Gaaboub & Hayes, 1984). However, in contrast to the present case, this is strongly dose-dependent. Moreover, individuals showing reduced weight gain often were still able to moult, as is the case in *Acheta domestica* (Warthen & Uebel, 1981). The present study suggests that azadirachtin action may be twofold: moulting is blocked at low concentrations, whereas higher concentrations cause additional phenomena such as weight gain reduction.

Storage protein formation. The question of reduced weight gain led me to study the conditions of the fat body which is the central organ of intermediary metabolism in insects. Especially in the last larval stage, the fat body plays an important role in metamorphosis preparation: the fat cells accumulate vast amounts of special proteins (storage proteins) that are sequestered from the haemolymph (Locke, 1984). These are needed by the pupa for building up imaginal organs. Late L_4 of *E. varivestis* exhibit fat cells with densely packed protein storage vacuoles (Schlüter, 1984).

Normally, the quantity of fat body storage protein increases drastically after the third day (Fig. 2). Injection of 0.1 μg azadirachtin causes no essential structural alterations in the fat cells for at least the first 8 to 10 days (Fig. 3a). Thereafter increased autophagy and cell death may be observed. Additionally, electrophoresis of the fat body proteins on day 6 shows an almost normal pattern, comparable with that of the prepupa. After injection of 1.0 μg azadirachtin this protein accumulation is suppressed for the most part. Some of the cells degen-

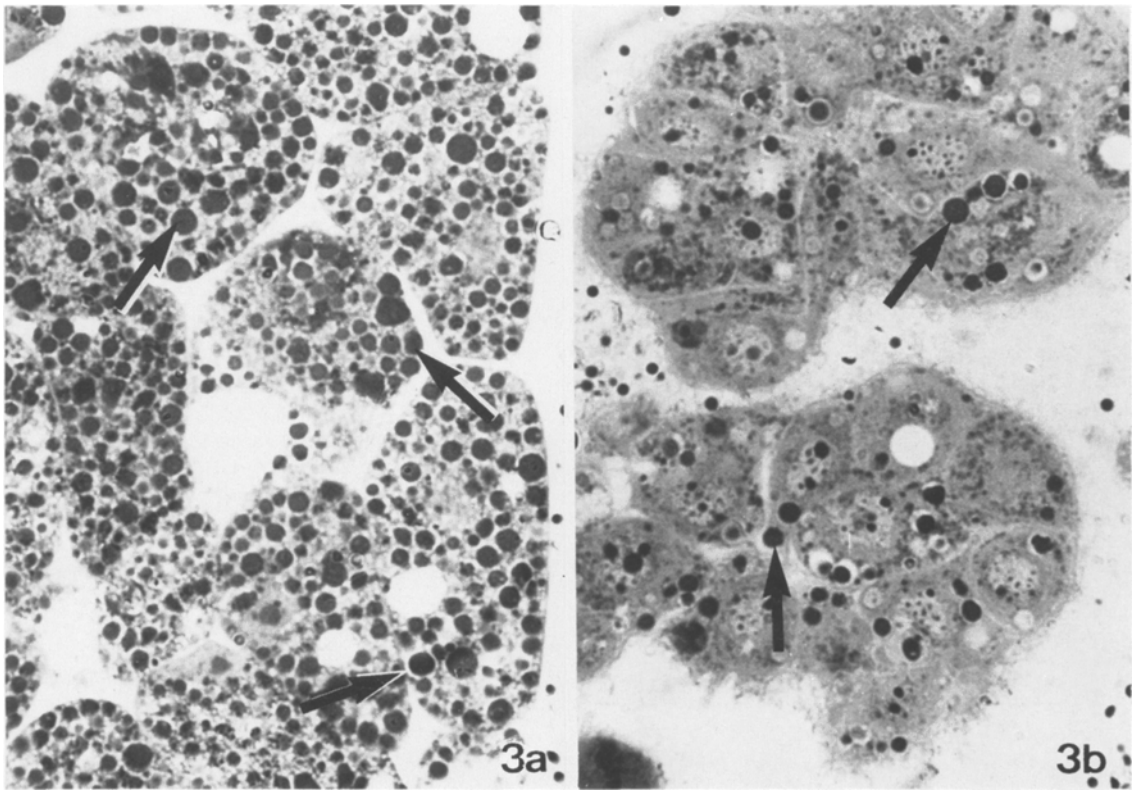

Fig. 3a and 3b. Light microscopic section through 6-day-old L_4 fat body lobes after treatment with 0.1 μg (3a) and 0.5 μg (3b) azadirachtin. Note presence of numerous protein granules in fat cells of 0.1 μg-individuals. Some granules marked by arrows. × 900.

erate as, for example, was observed after feeding azadirachtin (Schlüter & Schulz, 1984). However, the intact fat cells did not show notable protein granule accumulations (Fig. 3b). The lack of storage protein is also supported by electrophoresis (Fig. 2): the pattern is similar to that of normal 3-day-old individuals before protein storage takes place. On no account inhibition of storage protein formation could be effected by reduced feeding activity.

As suggested previously (Schlüter & Schulz, 1984) weight gain reduction may be the consequence of a metabolic defect rather than effected by the antifeedant properties of azadirachtin at higher doses. This was concluded among others from destruction of parts of the fat body. Growth regulating activity that is independent from feeding inhibition was also demonstrated in non-feeding spinning stages of *Ephestia kuehniella* (Rembold *et al.*, 1981). Fagoonee (1984) presumed from his studies on the cabbage webworm, *Crocidolomia binotalis*, that poor weight gain may be referred to energy wasting detoxication processes if azadirachtin is administered at higher concentrations. This may be accepted herein also, without prejudice to metabolic defects.

However, it is not yet clear, whether inhibition of fat body protein accumulation is effected by deficient uptake by the gut epithelium, or by general failure of utilization of food, or by blockage of specific pathways of storage protein synthesis.

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Zusammenfassung

Verminderung der Gewichtszunahme und Hemmung der Metamorphose und der Speicherproteinsynthese beim letzten Larvenstadium des Mexikanischen Bohnenkäfers, Epilachna varivestis, *nach Injektion von Azadirachtin*

Injektion verschiedener Dosen von Azadirachtin in Larven des letzten Stadiums von *Epilachna varivestis* führt zu folgenden Ergebnissen: Niedrige Dosen (0.1–0.2 µg) erzeugen keine Verminderung des Gewichtszuwachses, verhindern aber vollständig die Verpuppung. Bei etwas höheren Dosen (0.3–1.0 µg) tritt zusätzlich eine einheitliche Verminderung der Gewichtszunahme auf, die in diesem Bereich nicht dosisabhängig ist. Es wird vermutet, daß Azadirachtin in höheren Dosen bestimmte metabolische Prozesse in einer bisher unbekannten Weise beeinflußt. Es wird weiterhin gezeigt, daß nach Injektion höherer Dosen die für die Metamorphose wichtigen Speicherproteine im Fettkörper nicht oder nur unzureichend gebildet werden.

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