Chromatin (1992) 1:185-194 Author manuscript

Effects of azadirachtin on Balbiani ring gene activities and development of *Acricotopus lucidus* (Diptera, Chironomidae)

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Abstract

The treatment of larvae of the chironomid *Acricotopus lucidus* with azadirachtin effects the regression of cell-specific Balbiani rings on the polytene chromosomes of the salivary glands. As a result the larvae are no longer able to secrete adherent proteins and to build new tubes. The dose and time dependent effect of azadirachtin on the Balbiani rings BR1 and BR2, and on the development of *A. lucidus* was investigated. At concentrations from 5 to 10 µg/ml, azadirachtin showed strong moult-disturbing properties. Treated animals were not able to pass through larval or larval-pupal moults.

Introduction

Azadirachtin, a tetranortriterpenoid from the neem tree, *Azadirachta indica* (Meliaceae), possesses growth and development inhibiting properties in insects due to the disturbance of their endocrine system (Sieber and Rembold, 1983; Garcia et al., 1984; Dorn et al., 1986). The co-ordinated action of hormones is essential for normal moulting and metamorphosis. The target sites of azadirachtin are most probably located in the central nervous system (Rembold et al., 1989; Subrahmanyam and Rembold, 1989). Due to this potential to influence the hormonal system of insects, Dorn et al., (1986) regarded azadirachtin ‘as a tool in the study of hormonally regulated processes‘.

The regulation of gene activities in insects by ecdysteroids was impressively demonstrated on changes of puffing patterns of the polytene salivary gland chromosomes of dipteran larvae (Clever and Karison, 1960; Becker, 1962; Ashburner et al., 1974; for review, Lezzi and Richards, 1989).

In the chironomid *Acricotopus lucidus* the effect of ecdysone on gene activity is clearly visible in the regression of huge cell-specific puffs, the Balbiani rings (BRs), in the anterior lobe (AL) of the larval salivary gland (Panitz, 1964; Panitz ei al., 1972). The specific BRs in the main

and the side lobe (ML, SL) of the gland remain unaffected by ecdysone. These BRs are always expanded during larval development (Mechelke, 1953; Panitz, 1972; Staiber and Behnke, 1985.

In the present study the effect of azadirachtin on the gene activities of the larval salivary gland chromosomes and on the development of *A. lucidus* was investigated.

Materials and methods

A laboratory stock of *Acricotopus lucidus* (Diptera, Chironomidae) was used in the present study. The cells of the main lobe (ML) and the side lobe (SL) of the larval salivary gland have identical puffing- and BR-patterns in this stock. Rearing conditions and development of *A. lucidus* were described earlier (Panitz, 1964; Staiber and Behnke, 1985). Azadirachtin (purchased from Roth, Karlsruhe, Germany) was dissolved in absolute ethanol (1 mg/200 µl; portions of 40 µl were stored in a freezer at -20°C), followed by further dilutions with tap water to different concentrations ranging from 0.001 to 10 µg/ml.

The larvae were kept at 10-12°C in daylight in glass vessels containing 10 ml or 20 ml azadirachtin solution and with some *Spirogyra* algae as food and material to build tubes. In the experiments to test the developmental response of *A. lucidus* to azadirachtin, the test-solution was exchanged after 1 week with tap water. Salivary glands were prepared in Firling medium as described by Firling and Kobilka (1979), fixed in ethanol-acetic acid (3:1), stained in carmine-acetic acid for about 12-16 h and in orcein acetic-lactic acid for about 1.0-1.5 h, and were then squashed in a small drop of the latter stain.

The experiments for testing the influence of azadirachtin on BR-gene activity were carried out blind, i.e. the salivary gland squash preparations were encoded, and the examining and judging person did not know whether a preparation came from a larva treated with azadirachtin or from a control larva.

Four BR-size ciasses, indicating the BR-activity, were distinguished: class 3, BR maximal expanded (Figure 2); class 2, BR slightly regressed (Figure 3); class 1, BR strongly regressed (Figure 4); and class 0, BR completely regressed (Figure 5). Both glands of a larva were squashed together on a slide. Twenty ML and SL nuclei of each squash preparation were chosen and the BR-size classes of BRl and BR2 were determined. The BR-size classes of those larvae treated with the same concentration or for a definite time were listed and the percentage of BR1 and BR2 exhibiting class 2 and 3 were calculated.

Results

After treatment with *5* and 10 µg/ml azadirachtin for 48-72 h larvae of *A. lucidus* were no longer able to build new tubes. The anterior lobes (ALs) of the salivary glands of such larvae were normally filled and sized, whereas the MLs and the SLs were empty of secretion and appear shrivelled (Figure 1). In squash preparations of these glands the ML/SL-specific BRs 1 and 2

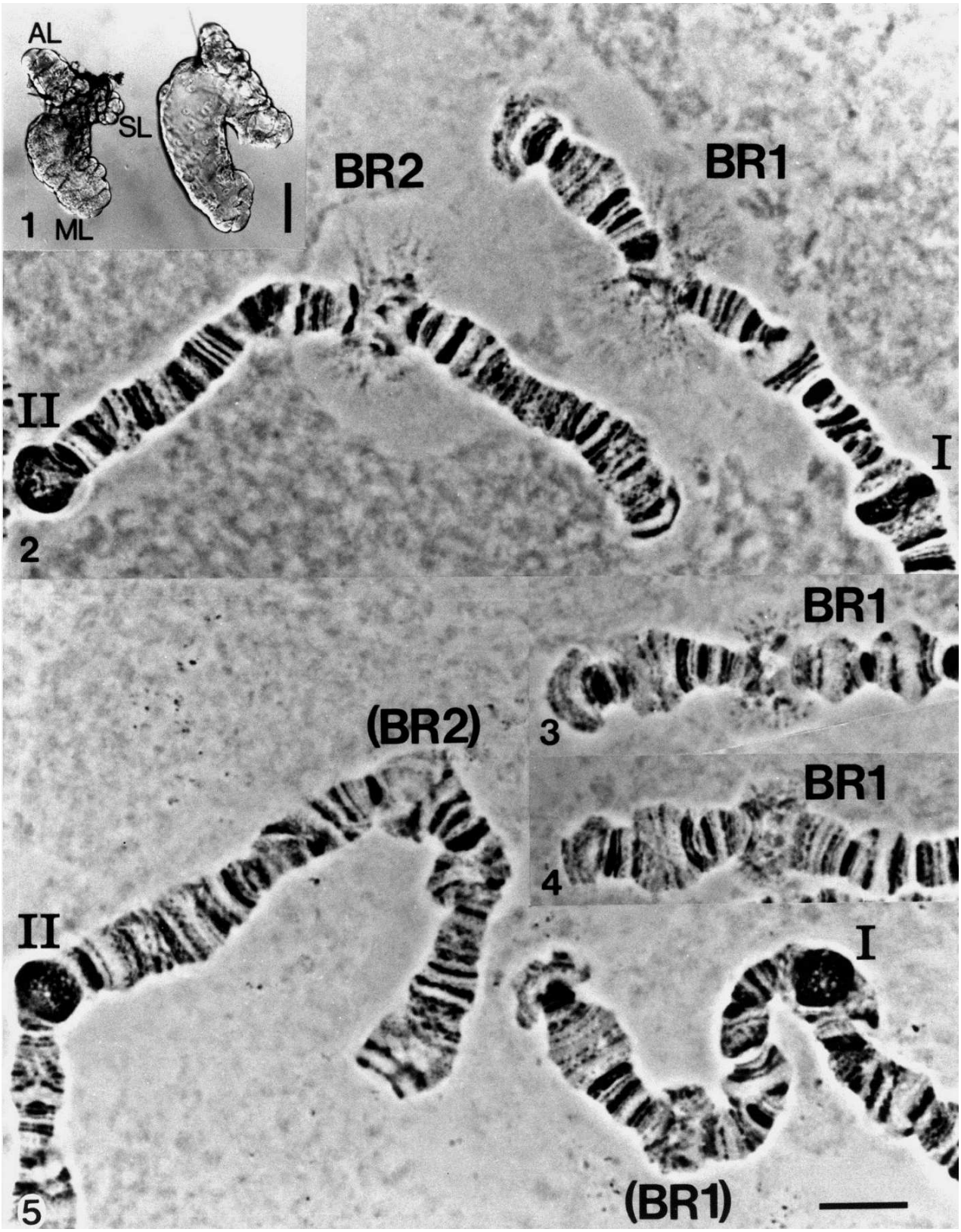

Figure 1 Salivary glands from a 4th instar larva of *A. lucidus* treated with 10 µg/mIlazadirachtin for 72 h (left), and from an untreated control larva (right). The anterior lobe (AL) of the treated larva is filled with a secretion, while the main and the side lobes (ML and SL) are empty. Unfixed glands. Differential interference contrast microscopy. Bar scale represents 200 µm.
Figure 2 Maximal expanded ML/SL-specific Balbiani rings BR1 and BR2 (activity dass 3) from an untreated control 4th instar larva.
Figure 3 Slightly regressed BR1 (activity dass 2).

Figure 4 Strongly regressed BR1 (activity dass 1).
Figure 5 Completely regressed BR1 and BR2 (activity dass 0) from a 4th instar larva treated with 5 µg/ml azadirachtin for 120 h. Bar scale represents 10 µm. Figures 2 to 5 have the same magnification.

were regressed or their size was strongly reduced, while the AL-specific BRs were expanded. BR1 and BR2 represent cell-specific active gene loci, in which mRNAs for the secretory proteins of ML and SL are synthesized. The proteins are necessary to glue algae into protective tubes.

Generally, the size of a definite puff or BR is correlated with its transcriptional activity (Pelling, 1964), i.e. a larger size indicates a higher rate of mRNA-synthesis and a smaller size a lower rate. To quantify the effect of azadirachtin-treatment on the BRs of *A. lucidus,* four BR-size classes were determined, ranging from dass 3, maximal expanded BR, to dass 0, completely regressed BR (Figures 2-5).

The time-dependent correlation of BR-regression of larvae treated with 10 µg/ml azadirachtin is shown in Figure 6.

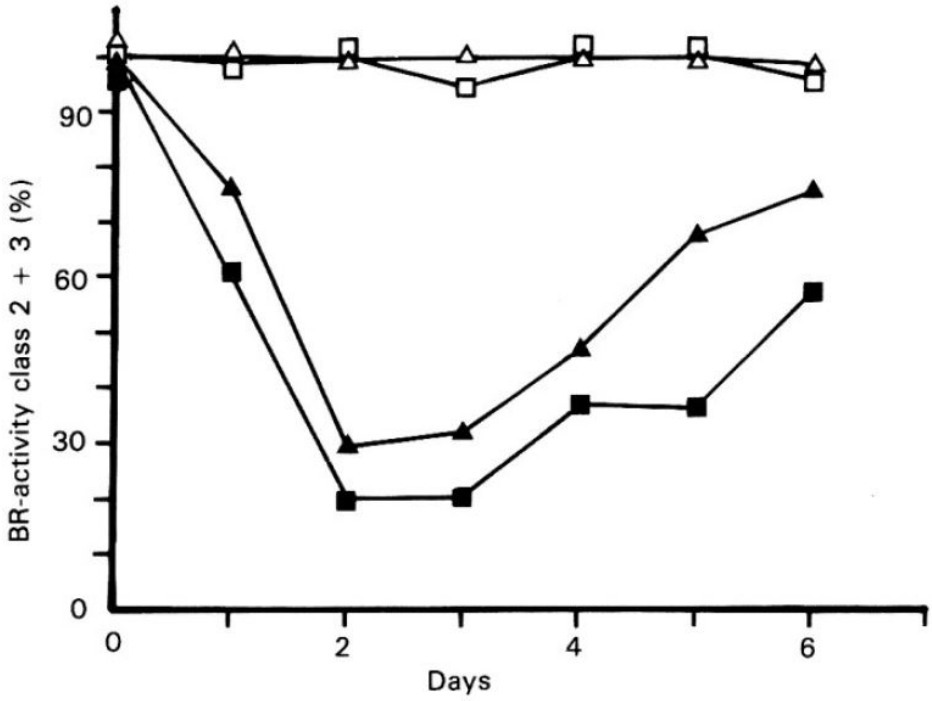

Figure 6 Changes of BR1 and BR2 activity ciasses after treatment of 4th instar larvae of *A. lucidus* with 10 µg/ml azadirachtin. Groups of fifteen treated larvae and of ten control larvae per time. Twenty salivary gland cells per larva were examined. Control larvae: Δ , BR1; □ , BR2. Azadirachtjn-treated larvae: ▲ , BR1; ■ , BR2

A strong reduction of BR1/BR2-activities was observed 1 day after treatment commenced. After 2 days 29.3% of the BR1s and 20% of the BR2s have a BR-size dass 2 or 3. In other words, 70% of the BR1 and 80% of the BR2 were completely regressed (class 0) or were still weakly active (class 1). Nearly the same situation was found after 3 days. After 4 days the percentages of BR1 and BR2 showing size class 2 or 3 rose again and reached 75.6% (BR1) and 57.2% (BR2) after 6 days. Nevertheless, the larvae were unable to form new tubes. After 18 days in a sample of ten

4th instar larvae and prepupae treated with 10 g/ml azadirachtin for 1 week, the percentage of BR-class 2 and 3 was found to be only 6.5% for BR1 and only 5.5% for BR2.

In cells in which BR1 and BR2 were completely regressed, other ML/SLspecific puffs, e.g. 2(B 73) and 6(G 53) (Staiber and Behnke, 1985), were also regressed. lt seems that all ML/SL-specific gene activities were switched off by the azadirachtin-treatment. After 4 days‘ treatment, in some ML/SL nuclei the size of the nucleolus was slightly reduced, but large amounts of nucleolar material were still present. In the AL the specific BR4 was not affected by azadirachtin (10 µg/ml). In all 4th instar larvae examined, the BR4 was maximally expanded (class 3), while in a few larvae the AL-specific BR3 showed size class 2, in rare cases even class 1.

To investigate the effect of different concentrations of azadirachtin on the BR1/BR2-activity and on the development of *A. lucidus,* 4th instar larvae were treated for 96 h with different concentrations ranging from 0.01 to 10 µ*g*/ml.

An effect of azadirachtin on BR1/BR2-activity was clearly visible at 1 µg/ml (Figure 7). At this concentration 63.5% of the BR1s and 55% of the BR2s showed BR-size dass 2 or 3. The lowest percentages of classes size 2 and 3 were found at 2.5 μg/ml (BR1, 31%; BR2, 22.5%) and at 5 µg/ml (BR1, 26%; BR2, 29%; percentage of classes size 2 and 3). At the 10 µg/m1 azadirachtin-concentration somewhat higher BR-activities were determined (BR1, 46%; BR2, 35%).

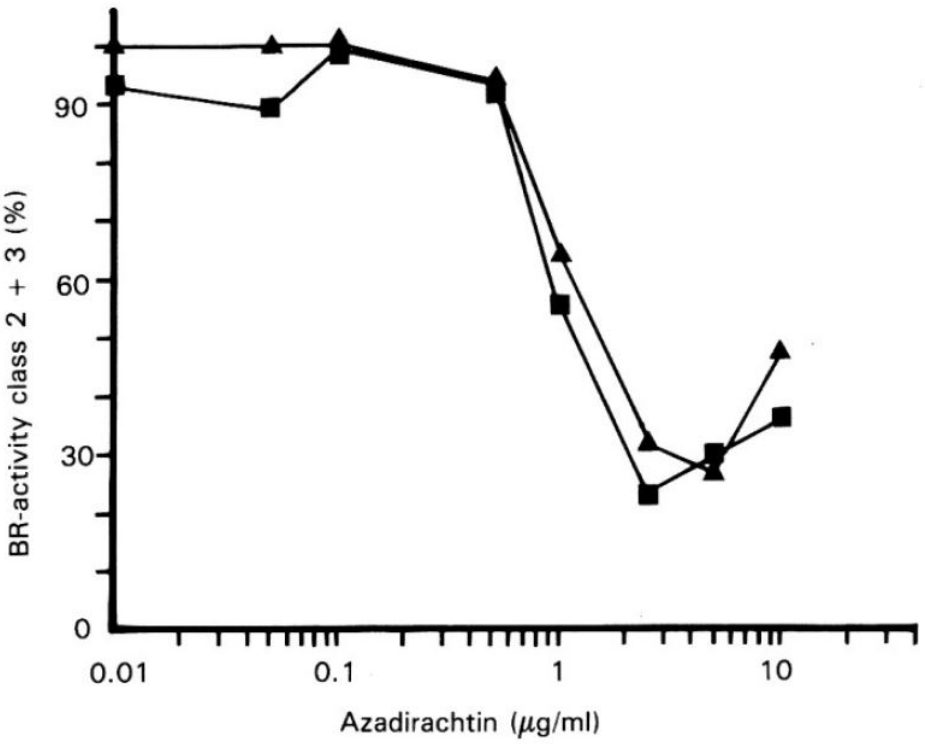

Figure 7 Effect of different azadirachtin concentrations on BR1 and BR2 activity ciasses of *A. lucidus* larvae after 4 days of treatment. Groups of ten larvae per concentration. Twenty salivary gland ML/SL-nuclei per larva were examined. ▲, BR1; ■, BR2.

The animals began to respond with developmental disturbances at a concentration level of 1 µg/ml (Table 1). At this concentration most of the larvae still pupated, but only about half of the animals developed to imagines, and so passed through the pupal-imaginal moult. At 5 µg/m1 about 75% of the animals died before pupation and none developed to an imago. At the 10 µg/ml azadirachtin concentration no animal was able to pass through the larval pupal moult. Most died as larval-pupal-intermediates. The animals were not able to leave the 4th larval-stage exuvia.

Table 1 Developmental response of *A. lucidus* 4th instar larvae to different concentrations of azadirachtin

Azadirachtin concn (µg/ml)	Number of insects developing to: Larval instar (4th)	Prepupa	Pupa	Imago
0 (control)	—	—	1	19
0.001	—	—	2	18
0.005	—	—	—	20
0.01	—	—	3	17
0.05	—	—	2	18
0.1	—	1	1	18
0.5	—	1	2	17
1.0	—	4	5	11
5.0	—	16	4	—
10.0	—	20	—	—

Twenty insects for each azadirachtin concentration. Between the 4th larval instar and prepupa no moulting occurs. The time 1 rom start of test to pupal-imaginal moult or to death of the last animal was 40 day

During experiments the 4th instar larvae were kept in the azadirachtin solutions for 1 week. Then they were raised in normal tap water. Nevertheless, the effect of azadirachtin was manifest about 3-4 weeks later in disturbing the larval-pupal moult. In the development of the imaginal discs of wings and legs no cessation or change was observed.

The results presented in Table 1 demonstrate that azadirachtin inhibited at 5-10 µg/ml the larval-pupal moult or the pupal-imaginal moult. To test whether azadirachtin also affects larval moults, 3rd instar larvae were selected and were treated with 1, 5 and 10 µg/ml azadirachtin (Table 2). At 1 µg/ml all larvae still passed through the moult from 3rd to 4th larval instar, but two thirds of the animals died then as larval-pupal intermediates and one third as pupae. Not one of them developed to an imago. At 5 µg/ml two larvae and at 10 µg/ml only one larva could carry out the moult to the 4th larval instar. The other larvae died during the 3rd larval instar. Possibly the former larvae had already initiated the moulting process when they were transferred into the azadirachtin solution and could still leave their old cuticle, but none of these animals pupated.

Table 2 Effect of azadirachtin applied to 3rd instar larvae on development of *A. lucidus*

Azadirachtin concn (µg/ml)	Number of insects developing to: Larval instar (3rd)	Larval instar (4th) + prepupa	Pupa	Imago
0 (control)	—	1	1	13
1	—	10	5	—
5	13	2	—	—
10	14	1	—	—

Fifteen insects for each azadirachtin concentration. The time from start of test to pupal-imaginal moult or to death of the last animal was 51 day.

In preliminary experiments on *Chironomus thummi* no larva treated with 4 µg/ml azadirachtin developed to an imago. Whether there is also an effect on gene activities of the polytene salivary gland chromosomes of *C. thummi* was not investigated.

When *A. lucidus* 4th instar larvae, which were treated with 0.01-10 µg/ml azadirachtin, developed to prepupae, the normal regression of the ecdysone-sensitive AL-specific BRs 3 and 4, as well as the regular sequential activation of new AL-specific puffs, was observed (Mechelke, 1953; Panitz, 1964; Panitz et al., 1972; Staiber and Behnke, 1985). Azadirachtin had no anti-feed effect in *A. lucidus,* which is in contrast to such an effect in *Locusta* and *Rhodnius* (Garcia *et al.,* 1984; Sieber and Rembold, 1983). In the range from 0.01 to 10 µg/m1 azadirachtin for all larvae which were dissected for salivary gland preparation, freshly consumed *Spirogyra* algae were found in the oesophagus and/or in the midgut. No starvation of the larvae was observed.

Discussion

During normal development of *A. lucidus* larvae, the ML/SL-specific BRs 1 and 2 are fully expanded and regress first just before pupation and the histolysis of the salivary glands (see Figure 4 in Staiber and Behnke, 1985). The treatment of larvae with 1-10 µg/ml azadirachtin induced the regression of BR1 and BR2. The larvae lost the ability to build new tubes because they were no longer able to synthesize adhesive proteins. After 48 h of azadirachtin incubation, larvae were found in which in all the ML/SL-cells of a gland the BRs were completely regressed, while in the AL-cells the specific BRs 3 and 4 were fully expanded.

These observations demonstrate that azadirachtin can effect changes of gene activities, in this case the selective inactivation of cell-type specific gene loci. The regression of BR1 and BR2 may be induced by a direct action of azadirachtin on the ML/SL-cells or by interference with hormonal mechanisms which then affect ML/SL-gene activity. Further investigations are

necessary to understand the exact mode of interaction between azadirachtin and the BR1/BR2-inactivation.

Azadirachtin-treatment of 3rd and 4th instar larvae caused the death of the animals in the larval or larval-pupal moults. Azadirachtin most probably affected the co-ordinating action of the hormones involved in the moulting processes (Garcia *et al.,* 1990; Subrahmanyam and Rembold, 1989). Moreover, Rembold et al. (1989) demonstrated that dihydroazadirachtin A is concentrated more in or on the corpus cardiacum than in the brain, so that the former is probably a target of azadirachtin. The corpus cardiacum is a neurohaemal organ in which trophic hormones originating from neurosecretory cells in the brain are stored and released, and it therefore plays an important role in the regulation of developmental hormones. Regular regression of the ecdysone-sensitive BRs 3 and 4 was also found in azadirachtin-treated animals, when developing from 4th larval instars to prepupae, indicating a regular increase of the ecdysone titre in the haemolymph.

Another way to influence BR-activities of ML and SL was found by Panitz (1967). The incubation of *A. lucidus* larvae in a solution of 1,500 µg/m1 gibberellin A3 effects the regression, especially of the BR2 (Baudisch and Panitz, 1968). After 48 h about 30-40% of the BR2s and also about 10% of the BR1s were regressed or in regression. In the other still expanded BR2s no ^{3}H-uridine incorporation, i.e. no mRNA-synthesis, was found. The expanded BR1s showed a somewhat reduced ^{3}H-uridine incorporation. The AL-specific BRs 3 and 4 were not affected by the gibberellin-treatment. The larvae were no longer able to build tubes. lt was also demonstrated that the BR2 codes for a secretory protein contained hydroxyprolin, and that the hydroxyprolin-synthesis was already inhibited after 30 min gibberellin-incubation. The inhibition of BR2-mRNA-synthesis by gibberellin was reversible (Baudisch, 1977).

Panitz (1967) supposed that gibberellin does not influence the BR2 in a direct way. Whether there exists a relation between the mechanism of BR2- regression induced by gibberellin and the mechanism of BRl/BR2-regression induced by azadirachtin is unknown. Gibberellin-treatment mainly causes the regression of the BR2. The effect on BR1 is much smaller. Such is not the case in the azadirachtin-treatment. Thereby both BRs are affected. Panitz (1967) also found no visible effect of gibberellin on the other puffs beside the BRs, while azadirachtin affects the regression of other ML-specific puffs. A 150- to 600-fold concentration of gibberellin A3 compared with azadirachtin (1,500 µg/ml to 2.5-10 µg/ml) was necessary to obtain the same effect. Baudisch and Panitz did not investigate whether gibberellin A3 also influences the moults or the development of *A. lucidus.*

lt is important to recognise that in the natural environment it would be hazardous for an *A. lucidus* population if the larvae were unable to build protective tubes after azadirachtin treatment. The larvae would then become victims of their natural enemies.

Both effects, the BR1/BR2-regression and disturbance of the moulting processes, were evident following treatment with 1 µg azadirachtin per ml water. The effect on the BRs was visible 1 day after the onset of treatment, whereas the other effect was first visible at the time of the next moult. lt may be that the *A. lucidus* BR-gene activity system can be used to test substances in respect of their ability to influence the hormonal balance in insects.

Acknowledgements

The author gratefully acknowledges the support of Professor F. Mechelke and Professor C. U. Hesemann, and the help of Dr Irmgard Wech.

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