and MARSHALL[6]. Test cultures were incubated continuously in medium containing 1 µg/ml α-bungarotoxin, a concentration known to cause irreversible blockade of cholinoreceptors in cultured skeletal muscle[6,7]. Parallel cultures without the toxin were used as controls. Cultures were examined at various stages up to 12 days in vitro. Morphology was observed by phase contrast microscopy; resting membrane potentials and acetylcholine sensitivity were measured by standard electrophysiological techniques; and cholinesterase activity was monitored by a modification of the histological staining method of KOELLE and FRIEDENWALD[8] using acetylthiocholine as substrate.

During the first 2 days development proceeded normally, myoblasts multiplied and fusion began on the second day. At 3 days there was no obvious difference between control and α-bungarotoxin treated cultures (Figure 1, a and b). Fusion of myoblasts and formation of myotubes was unimpaired by the presence of the toxin. Subsequent observation showed that the continued morphological development of muscle fibres in culture was not affected by the toxin.

Resting membrane potential recordings made on myotubes at various stages of development showed the expected rise in mean resting potential in both control and toxin grown cells. There was no significant difference between the pairs of measurements (Table).

The control muscle fibres responded at all stages of development to iontophoretically applied acetylcholine, whereas fibres grown in the presence of α-bungarotoxin were not depolarized by acetylcholine even when the iontophoresis current was increased by a factor of 10 (Figure 2). 5 min exposure to 10^{-3} *M* acetylcholine did not produce any depolarization in a 10-day-culture grown in 1 µg/ml α-bungarotoxin. The absence of response in the α-bungarotoxin treated fibres indicates that the toxin retains its blocking activity throughout the incubation period. It has been shown by others that in cultured muscle receptor synthesis occurs in the presence of α-bungarotoxin and that such newly synthesized receptors are blocked as they occur[9].

The intensity and distribution of the cholinesterase stain in toxin treated fibres was similar to controls at all stages (Figure 3), although at 8 days some of the fibres grown in α-bungarotoxin had more distinctly localized regions of high enzyme activity than did the control fibres. At 10 days localization of enzyme was the same in both control and α-bungarotoxin treated cultures although the intensity of staining in both sets of cultures diminished. Localized areas of cholinesterase activity found in aneural muscle cultures may represent regions which would form motor endplates if innervation were to take place[10].

It appears, therefore, that although the rate of receptor incorporation into cultured skeletal muscle is unaffected by α-bungarotoxin[9], the irreversible blockade of these receptors has little effect on the development of the fibre itself, its resting membrane potential, or its content of cholinesterase.

In contrast to our findings in culture, blockade of cholinoreceptors in developing chick embryos resulted in pronounced dystrophic effects[11-13]. It is possible that in the whole embryo the acetylcholine receptor plays a role in the formation of neuromuscular junctions[10], and that the blockade of the receptor interrupts the functional relationship between nerve and muscle which seems essential for normal development of embryonic muscle in situ. However, in a nerve-free culture system, we have shown that, despite irreversible blockade of acetylcholine receptors, early muscle development continues normally. Our results indicate that myogenesis in culture is not dependent on the presence of functional acetylcholine receptors.

Resting membrane potentials in control and α-bungarotoxin treated cultures

Age of cultures (days)	Resting potentials in control cultures (-mV)	Resting potentials in α-bungarotoxin (-mV)
3	15.3 ± 2.1 (6)	15.1 ± 1.1 (15)[a]
6	28.3 ± 1.3 (19)	30.3 ± 2.1 (14)[a]
10	31.9 ± 1.3 (25)	28.1 ± 0.9 (59)[a]
12	25.7 ± 2.9 (15)	22.2 ± 1.8 (20)[a]

Values are mean ± S.E.M. of the number of fibres shown in parentheses. [a] Difference between control and toxin values not significant at $P > 0.025$, Student's *t*-test.

Résumé. Nous avons étudié le rôle du récepteur cholinergique dans la différentiation du muscle de squelette en culture. En présence continuelle de la α-bungarotoxine, les cellules n'ont pas réagi à l'acétylcholine, mais la myogénèse et l'acétylcholinestérase ont été normales. Nous concluons que la myogénèse en culture ne dépend pas de la présence des récepteurs cholinergiques fonctionnels.

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Inhibition of Metamorphosis by Juvenoids in *Nauphoeta cinerea* (Olivier)

It has been repeatedly proved that juvenoids or juvenile hormone analogues inhibit imaginal differentiation when applied before a critical period in insect metamorphosis. The present article examines the period of sensitivity to juvenoids in the cockroach *Nauphoeta cinerea* (Olivier) (Blattodea, Blaberidae) and compares activities of 18 selected juvenoids on this species.

The stock of *N. cinerea* was maintained at 25 °C and 45–50% r.h. on Larsen's diet and fresh carrots. Groups of 10 last instar nymphs of an age known within 2 days

after the ecdysis were used for the experiments. The juvenoids[1] were administered topically under the wing lobes in 5 µl of acetone. The effects were evaluated after the following ecdysis, which was the imaginal ecdysis in controls.

In the first series of experiments, the groups of 10 nymphs were accommodated in 650 ml jars and kept at 25 °C. The length of the last larval instar varied between 26 and 31 days, the average being 27 days. The nymphs received 50 µg either of ethyl 11-chloro-3,7,11-trimethyl-dodec-2-enoate (compound IV in Table II) or of ethyl 3,7,11-trimethyl-dodeca-2,4-dienoate (compound VII in Table II) at various times after ecdysis. A majority of the treated insects maintained larval characteristics after the imaginal ecdysis. Degree of the juvenilizing effect was classified with a scale ranging from 0 (normal adult) to 5 (perfect superlarva, i.e. a supernumerary larval instar). Degrees 1–4 denoted various intermediate forms between larva and adult (Figure).

Table I demonstrates that administrations of juvenoids on days 1–8 after the last larval ecdysis (exceptionally also on day 12) induced development of superlarvae (effect 5) whereas those performed on days 8–24 after ecdysis caused development of intermediates (effects 1–4). These results indicate that *N. cinerea* responds to juvenoids for about 85% of the length of its last larval instar. A similar conclusion may be drawn for *Blattella germanica* (L.) (Blattellidae) from the data reported by O'FARRELL and STOCK[2]. Since N. *cinerea* and *B. germanica* belong

Table I. Effects produced by 50 µg of ethyl 11-chloro-3,7,11-trimethyl-dodec-2-enoate (IV), 50 µg of ethyl 3,7,11-trimethyl-dodeca-2,4-dienoate (VII), and 5 µl of acetone (used also as a solvent for juvenoids) applied to *N. cinerea* on different days after the last larval ecdysis

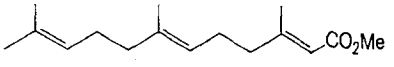
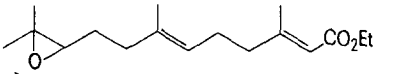
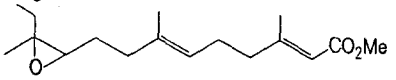
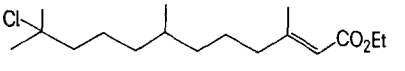
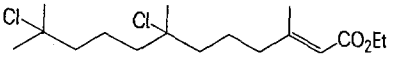
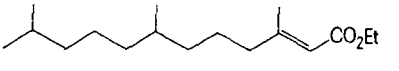
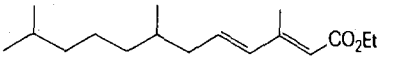
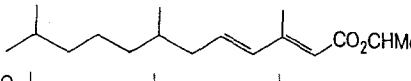
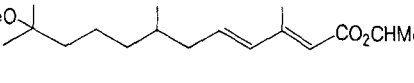
Day of treatment	IV	VII	Acetone
1–4	5	5	5
8	5	4	0
12	4	3	0
16	3	1–2	0
20	1–2	1	0
24	0–1	?	0

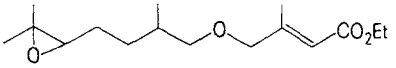
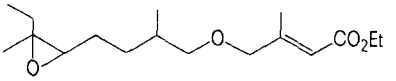
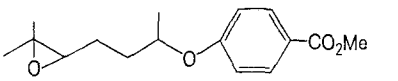
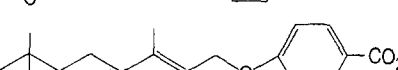
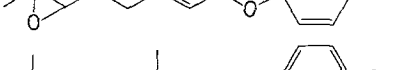
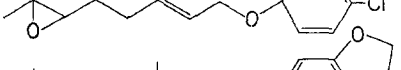
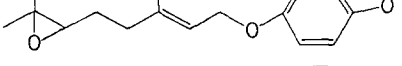
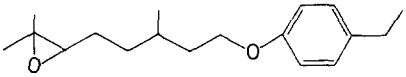
The effects were classified with a scoring system ranging from 0 to 5 degrees (see text and Figure).

[1] The juvenoids used are listed in Tab. II. Compounds I, IV, V, IX–XII, XV and XVIII were kindly provided by Drs. M. ROMAŇUK and V. JAROLÍM of the Institute of Organic Chemistry and Biochemistry, ČSAV, Prague; the remaining substances were obtained by the courtesy of the Zoecon Corporation, Palo Alto, California. All juvenoids were racemic mixtures; the aliphatic compounds contained about $^2/_3$ of the 2-*trans* isomers.

[2] A. F. O'FARRELL and A. STOCK, Life Sci. *3*, 491 (1964).

Table II. Activities of selected juvenoids expressed in doses causing average effects 2.5 (ID_{50}) and 1 (ID_{20})

Compound Number	Strukture	Effective doses (µg) ID_{50}	ID_{20}
I		>500	500
II		>500	200
III		50	1
IV		50	10
V		500	50
VI		50	3
VII		5	0.5
VIII		5	0.3
IX		3	0.05

Compounds Number	Strukture	Effective doses (µg) ID_{50}	ID_{20}
X		>500	100
XI		>500	100
XII		?	50
XIII		40	10
XIV		40	10
XV		>500	100
XVI		500	40
XVII		500	30
XVIII		300	100

to different families of the Blattodea, it seems probable that most cockroaches differ from *Odonata*[3], *Mallophaga*[4], *Orthoptera*, *Heteroptera*, and *Dermaptera*[5] by a very long period of sensitivity to juvenoids.

It is interesting to note that application of pure acetone to *N. cinerea* within 4 days after the last larval ecdysis also induced development of superlarvae. Treatment of older larvae with acetone was ineffective. These results resemble the effect of injury, which also causes development of superlarvae if inflicted at the beginning of the last larval instar[6]. Superlarvae produced both with acetone and juvenoids developed into bigger but otherwise normal adults.

In the second series of experiments, the groups of 10 nymphs were reared in 250 ml beakers and kept at 30 °C and 70% r.h. The nymphs were starved for 3 days after the last ecdysis in order to synchronize their development[7]. Under these conditions, the last larval instear lasted mostly 17–19 days in males and 19–23 days in females (18.7 days when averaged for both sexes).

In an attempt to prolong the larval stage for more than one extra larval instar, we examined the effects of repeated application of juvenoids. To this end, 5 groups of nymphs were treated with 50 µg of ethyl 3,7,11-trimethyldodeca-2,4-dienoate per specimen at the end of the starvation period. All of the 50 treated insects ecdyzed as superlarvae in 12–18 days. 35 of these superlarvae were treated again with the same dose of the juvenoid. Within 16–20 days, 7 of them produced intermediates between larva and adult, and all others ecdyzed as 'second' superlarvae. The 'second' superlarvae were treated with the juvenoid for the 3rd time. Half of them moulted within 20–22 days into giant 'third' superlarvae, the other half ecdyzed as giant adults 2–4 days later.

The 'second' and 'third' superlarvae possessed typical larval features but had large wing buds and some of them showed white spots in the cuticle, which is the typical pigmentation of the adult. The giant adults differed from the normal ones by bigger body size and unusually long wings. Their body weight often surpassed 750 mg as compared to less than 500 mg of the body weight of normal adults. Their wings were always longer than the abdomen while in normal adults the wings reached at the most to the second last abdominal segment.

The activities of different juvenoids were assayed on nymphs 10 days after the last larval ecdysis, i.e. 7 days after the end of the starvation period. The treated insects were affected to different degrees in dependence on the activity of the compound and the dose applied. Exceptionally, the affected insects could not accomplish the moulting process or showed a partial paralysis of their legs. The length of the instar was not related to the degree of elicited effect and ranged from 17 to 19 days, exceptionally from 15 to 23 days (18.3 days an average).

Table II compares doses of different compounds which caused average effects 2.5 and 1 of our scoring system. Since these effects represented a 50% and, respectively, a 20% inhibition of imaginal differentiation, the corresponding doses of juvenoids were called ID_{50} and ID_{20} (ID for inhibition dose). The Table II shows that dienoates VII, VIII, and IX were the most active of all compounds tested. By contrast, the methyl farnesoate (I), which is closely

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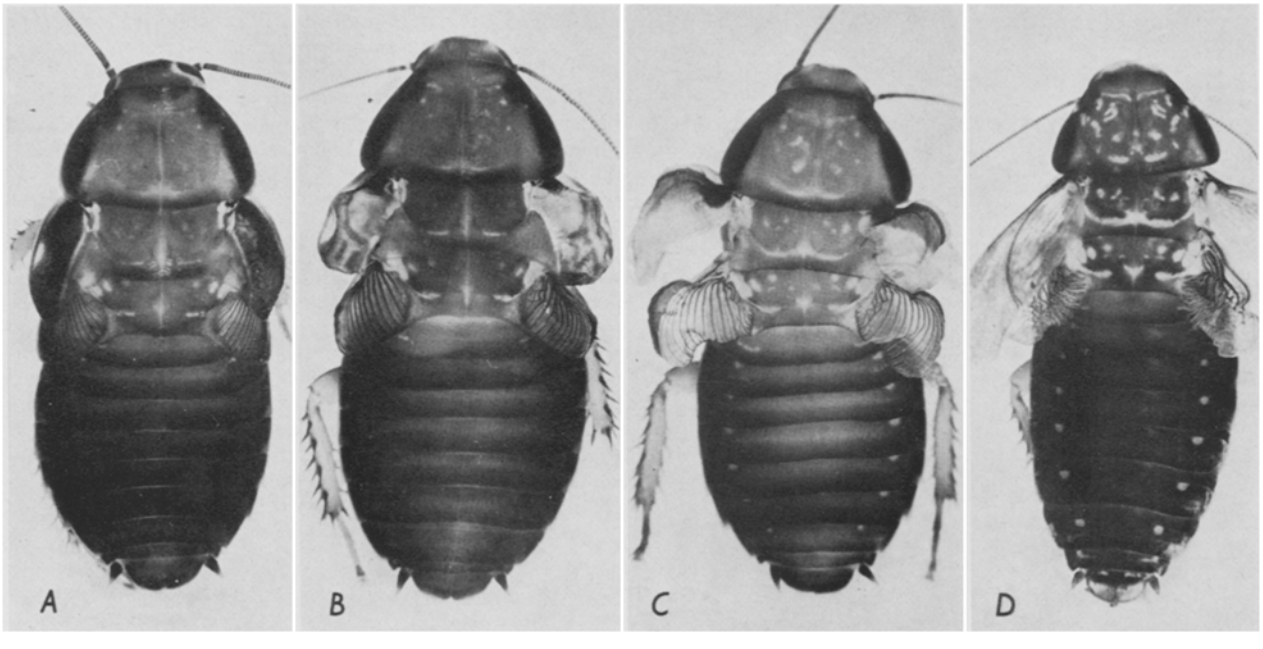

Various effects produced by juvenoids applied to the last instar larvae of *Nauphoeta cinerea:* A) effect 4 (nymphoid showing some differentiation of the hind wings); B) effect 3 (larval-adult intermediate with both pairs of wings partly differentiated but without any well-outlined white spots of adult pigmentation); C) effect 2 (intermediate with incomplete adult pigmentation limited to the dorsal side of the body. The front wings may attain imaginal size); D) effect 1 (adultoid possessing a nearly normal adult pigmentation but with incompletely developed hind wings).

related in structure to the farnesyl methyl ether used in several studies with positive results on cockroaches[2,7,8], showed the lowest activity of all substances examined. The activity of all aromatic compounds tested was also relatively low[9].

In summary, the cockroaches seem to maintain sensitivity to juvenoids for a longer portion of the last larval instar than many other Hemimetabola. The larval development may be prolonged considerably by repeated applications of juvenoids. *N. cinerea* responds to diverse juvenoids but their activities differ several thousand times.

Zusammenfassung. Mit 18 an Larven der Schabe *Nauphoeta cinerea* getesteten JHA wurden Juvenilisationseffekte erzielt. Das letzte Larvenstadium ist während 85% der Stadiendauer sensitiv. Frisch gehäutete Tiere reagieren auch auf das Lösungsmittel (Aceton) allein. Durch wiederholte Applikation von JHA können mindestens 3 zusätzliche Larvenstadien erzeugt werden, die sich zu Imagines mit abnorm langen Flügeln häuten.

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Entomological Institute, Czechoslovak Academy of Sciences, No Folimance 5, CS-12000 Praha 2 (Czechoslovakia), 19 October 1973.

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[10] Permanent address: Department of Entomology, Faculty of Science, Ain-Shams University, Cairo, Egypt.
[11] We acknowledge with many thanks the critical reading of the manuscript by Dr. V. J. A. NOVÁK.

Reduction of Biochemical Polymorphisms in Honeybees (*Apis mellifica*)

For several years electrophoresis has been extensively used for the study of isozyme polymorphisms in many different organisms. A great number of polymorphic loci has been detected in different animals throughout the animal kingdom. The study of biochemical polymorphisms in honeybees (*Apis mellifica*) presented here, was to determine if their genetic system of parthenogenesis has an impact on the percentage of polymorphic loci present in the population. The enzymes malate dehydrogenase (MDH), phosphoglucomutase (PGM) and esterase were tested. They are known to be polymorphic in other species[1-5].

For electrophoresis tissue extracts were made of entire thorax muscles (MDH, PGM) from individual workers and drones. Thorax muscles of workers were homogenized in 0.2 ml of distilled water, of drones in 0.5 ml of distilled water and then centrifuged at 30,000 *g* for 20 min. The clear supernatant material, which had a protein content of 25 mg/ml, was used for electrophoresis on cellulose acetate strips (8 × 8 cm). Haemolymph samples were taken from the dorsal vessel of individual bees and used directly for electrophoretic separation of esterase. Electrophoresis was carried out for all enzymes in a Shannon Universal apparatus at + 5 °C.

Malate dehydrogenase — 5 µl of the tissue extract were applied to the gel-strips and electrophoresis was conducted at 8 mA/240 V for 30 min using a 0.1 *M* veronal-Na buffer (pH 8.4). For enzyme detection, the strips were incubated in the dark at 37 °C in a solution of 0.2 *M* DL-malic acid, 0.001 *M* DPN, 0.000163 *M* phenazine methosulfate, 0.00043 *M* NB-tetrazolium and 0.05 *M tris* (ph 7.0). For the reaction mechanism, see BREWER[6].

Phosphoglucomutase — 5 µl of the tissue extract were applied to the strips and electrophoresis performed at 7 mA/100 V for 80 min using a 0.1 *M tris*-maleic acid buffer (ph 7.6). For staining the strips were incubated at 37 °C in the dark in a solution of 0.0046 *M* glucose-1-phosphate, 0.01 *M* $MgCl_2$, 0.0012 *M* TPN, 0.00033 *M* phenazine methosulphate, 0.00024 *M* MTT-tetrazolium, 0.075 mg G-6-PD/100 ml staining solution and 0.03 *M tris* (ph 8.0).

Esterase — 3 µl of haemolymph were applied to the strips and electrophoresis performed at 7 mA/150 V for 30 min using a 0.3 *M* boric acid buffer (pH 8.0). For enzyme detection the strips were incubated at room temperature in the following staining mixture: 0.006 *M* α-napthyl acetate, 200 mg Blue RR salt/100 ml staining solution and 0.08 *M tris* (pH 7.0)[6].

Bees from 14 different hives were studied, including 4 different races of *Apis mellifica* (see Table). All bees proved to be monomorphic for the 3 enzymes, showing the same banding pattern in workers and drones of all colonies: 1 band of enzyme activity was detected for

Reduction of biochemical polymorphisms in honeybees (*Apis mellifica*)

Colonies		Enzymes					
		PGM		MDH		Esterase	
A. mellifica carnica							
from Frankfurt hives	1	24 W	24 D	12 W	12 D	12 W	12 D
	2	13 W		11 W		16 W	7 D
	3	54 W	52 D	21 W	17 D		
	4	12 W	12 D	12 W	12 D	27 W	36 D
from Munich hives	5	22 W	24 D	22 W	24 D	21 W	9 D
	6	61 W	55 D	12 W	12 D	12 W	12 D
	7			12 W		10 W	
	8	10 W		6 W			
	9			12 W			
	10			12 W			
	11	6 W		5 W			
A. mellifica ligustica	12	12 W		12 W		12 W	
A. mellifica caucasica	13	12 W		12 W		9 W	
A. mellifica fasciata	14	12 W		12 W		10 W	

W, workers; D, drones.

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