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Autor: Rutz, Walter / Gerig, Luzio / Wille, Hans

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A bioassay for juvenile hormone (JH) effects of insect growth regulators (IGR) on adult worker honeybees

PRELIMINARY REPORT

WALTER RUTZ, LUZIO GERIG, HANS WILLE and MARTIN LÜSCHER
Federal Dairy Research Station, Apicultural Section, CH-3097 Liebefeld-Bern, Switzerland
Division of Animal Physiology, Zoological Institute of the University of Bern, CH-3012 Bern, Switzerland

A relatively simple bioassay for JH-action on adult honeybees is described, using characteristic effects which all seem to indicate that JH shifts the physiology of the hive bee to that of the field bee.

Prüfung von Insekten-Wachstums-Regulatoren (IWR) auf Juvenilhormon (JH) – Wirkung bei der adulten Bienenarbeiterin

Es wird ein relativ einfaches Prüfverfahren beschrieben, bei dem charakteristische JH-Effekte benützt werden, die alle darauf hinzuweisen scheinen, dass JH für eine Änderung des physiologischen Zustands von demjenigen der Stockbiene zu demjenigen der Flugbiene verantwortlich ist.

INTRODUCTION

The increasing interest in insect growth regulators (IGR) as insecticides and in their possible use in agriculture leads to the necessity of simple routine laboratory methods which allow to test the juvenile hormone (JH) effects in adult worker honeybees (for the effects on brood-rearing by spraying IGRs see BEETSMA, 1974). The methods described in this paper are based on our findings that JH-III-application is followed by pycnosis in a high proportion of the leucocytes, by a decrease of the haemolymph protein titre (RUTZ, GERIG, WILLE and LÜSCHER, in preparation), by the degeneration of the hypopharyngeal glands (GAST, 1967) and by a reduction of the life-span.

MATERIAL AND METHODS

Freshly emerged 12–24 h old workers are kept at 30° C with and without queens in modified Liebefeld cages in groups of at least 50. The IGR can be fed or injected. As food the IGR is mixed with sunflower margarine and given to a pollen paste (0,1% margarine with IGR, 66% dried pollen, 33,9% sugar syrup-50%). Approximately 10% ether is added and after thorough stirring evaporated. A fresh paste is made every day. The daily uptake of IGR is determined by weighing the pollen consumed. In addition each group is fed 30% sugar syrup. For injection the IGR is applied in a mixture of sterile, neutralized olive oil/mineral oil 1:1. Per bee 0,18 µl are injected into the abdomen on the day of emergence. On day 8 haemolymph is collected with a thin glass capillary by puncturing the abdomen dorsally between tergites 3 and 4. The vial for

collecting the pooled haemolymph of at least 25 bees is kept in icewater to prevent melanization.

For *haemocyte counting* haemolymph of queenright or queenless workers can be used. With a precision constriction pipet 1 µl of haemolymph of a pooled sample of at least 25 workers is put on a glass slide and a round smear of about $^1/_2$ cm^2 is made. After drying we fix for 5 min. in absolute methanol and stain for 30 min. in 2% Giemsa stain in unbuffered distilled water. The leucocytes and pycnotic leucocytes are counted diametrically through the whole smear at a magnification of 600×. The ratio leucocytes/pycnotic leucocytes is needed. Normal and pycnotic leucocytes are defined as follows:

Normal leucocytes are haemocytes of oval or elliptic form. Their length of 8–14 µm increases with age, the width is 4–8 µm. The cytoplasm stains weakly with Giemsa for the first 17 days. The fine cytoplasmatic membrane is clearly visible. The ovoid nucleus lies in the centre and reveals no membrane under the light microscope. The length of the nucleus is 4–8 µm, its width 3–4 µm. The chromatine forms small stretched well distinguishable blocks, which show strong light refraction in phase contrast microscopy (WILLE and VECCHI, 1966).

The *pycnotic leucocytes* consist almost entirely of a spherical or ovoid nucleus without visible membrane with a diameter of 3,4–4,3 µm. In the phase contrast microscope the pycnotic leucocytes show a central bright zone in their early stage. Later the whole nucleus shows an uniformly strong light refraction. The cell membrane is broken and the cytoplasm is usually lacking or present in traces only which stick more or less to the nucleus. Sometimes remains of the cell membrane are seen sticking to plasmatic fragments. WILLE and VECCHI (1974). Figure 1 shows 3 pycnotic, one intermediary and 4 normal leucocytes.

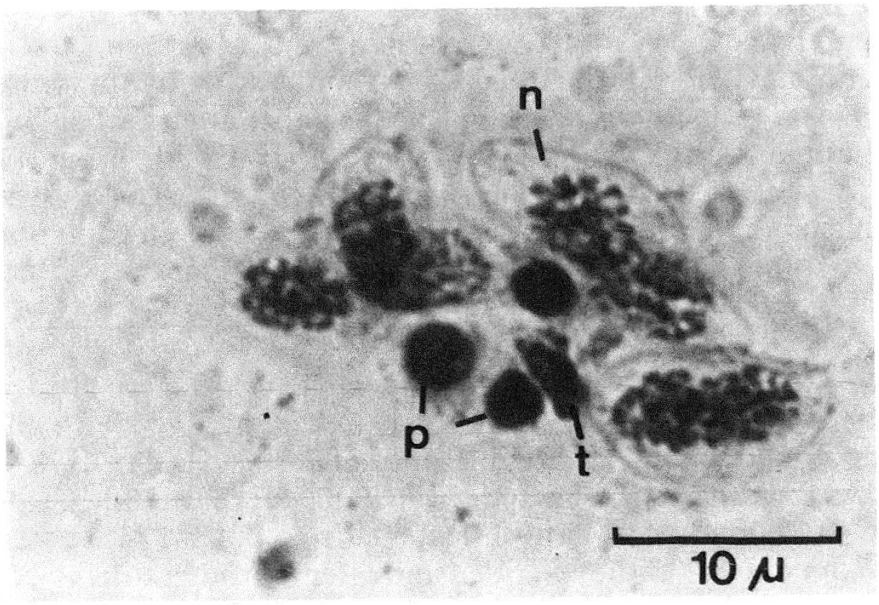

Fig. 1 Normal leucocytes and pycnotic leucocytes of 8-day-old queenright workers.
n normal leucocytes
p pycnotic leucocytes
t transition form of leucocyte.

For the determination of the *haemolymph protein titre* haemolymph of queenright or queenless workers may be used (RUTZ and LÜSCHER, 1973). 5 µl

of a pooled sample of centrifuged haemolymph are given to 1 ml of 1,7% NaCl solution. Measuring the absorbance directly at 280 nm UV proved to be a practicable and fast method for screening purposes. We also used the method of LOWRY et al. (1951). In both cases a standard curve with bovine serum albumin is established.

For the evaluation of the *state of the hypopharyngeal glands* the front part of the head capsule is cut away with a razor blade and the glands are taken out with fine forceps. The size of the acini is estimated and the glands are photographed.

Longevity tests are run until all bees have died. (For methods see WILLE, 1973).

RESULTS

As an example of the test we shall now present the results obtained in laboratory experiments with the IGR Triprene (ethyl-11-methoxy-3,7,11-trimethyldodeca-2,4-dienethiolate) of Zoecon Corporation[1]. These results will be compared with those obtained after injection of racemic C_{16}-JH (JH-III) into workers kept in large queenright colonies. JH-III was used because this seems to be the active hormone in the honeybee (TRAUTMANN et al., 1974).

Leucocyte counts: In Table 1 the total number of leucocytes per μl haemolymph are listed for worker bees after injection of JH-III. The difference between the ratios in experimentals and controls is highly significant with $p < 0{,}0005$ (χ^2-test). 1 μg of JH-III injected on day 0 and day 4 also caused a statistically significant decrease of normal leucocytes and an increase in pycnotic leucocytes. Even a single dose of 1 μg JH-III injected on day 0 produced statistically significant results when examined on day 8 with $p < 0{,}01$ (publication in preparation).

Table 1
Number of leucocytes found in 1 μl of pooled samples of haemolymph of 8-day-old workers which were injected with JH-III at the rate of 1 μg on days 0, 2, 4 and 6. The workers were kept queenright in large colonies.

JH-III-injected			Controls		
Normal leucocytes	Pycnotic leucocytes	Ratio	Normal leucocytes	Pycnotic leucocytes	Ratio
2558	4932	1.93	7021	640	0.10
1307	2895	2.22	5603	364	0.07
1385	2770	2.00	5120	410	0.08
1315	4372	3.33	8283	1445	0.17
1492	4675	3.13	5754	518	0.09
2507	3356	1.34	4821	760	0.16
			7144	1514	0.21
	mean	2.32 ± 0.31		mean	0.13 ± 0.02

Injections with Triprene leads to the same effects (Table 2). The χ^2-test reveals highly significant differences between experimentals and controls.

[1] We are thankful to Zoecon Corp., Palo Alto, Calif. for supplying various IGRs and JH-III.

Table 2
Number of leucocytes found in 1 µl of pooled samples of haemolymph of 8-day-old queenright workers injected on day 0.
Workers kept in modified Liebefeld cages.

1 µg of Triprene injected per worker			Controls		
Normal leucocytes	Pycnotic leucocytes	Ratio	Normal leucocytes	Pycnotic leucocytes	Ratio
159	4200	26.42	1393	2786	2.00
492	7763	15.78	524	2564	4.89
479	6793	14.18	295	1566	5.31
405	8661	21.38	269	3324	12.36
123	6984	56.78	1647	1033	0.62
	mean	26.91 ± 7.77		mean	5.04 ± 2.03

The differences in the total number of haemocytes and in the ratios in Tables 1 and 2 are due to the differences in keeping the workers and to seasonal changes (WILLE, 1973).

Haemolymph protein titre: Table 3 shows that JH-III decreases the haemolymph proteins. The difference between the oil treated controls and the JH-III injected workers is highly significant with $p < 0{,}0005$ (t-test).

Table 3
Protein titre in haemolymph of 8-day-old workers after injection with JH-III at the rate of 1 µg on day 0 and 4.
The workers were kept queenright in large colonies.

JH-III-injected		Oil-injected	
	47.1 µg BSA		81.1 µg BSA
	43.4		63.2
	47.1		65.0
	43.5		81.2
	48.1		72.6
	48.2		64.1
mean	46.23 ± 0.90	mean	71.20 ± 3.43

The haemolymph protein titres are indicated in equivalents of bovine serum albumin (BSA) per µl haemolymph (UV-method).

Figure 2 shows the effect of Triprene on the titre of the serum proteins. There is a negative logarithmic correlation between the protein titre and the IGR dose.

Morphology of the hypopharyngeal glands: In Fig. 3 the glands of the workers treated with JH-III are compared with the controls. Under the influence of the hormone the glands degenerate and show pyriform acini. Granulalike structures appear within the acini and the duct becomes clearly visible. All IGRs we have examined until now have affected the morphology of the hypopharyngeal glands, however the dosage needed for the same response varied considerably. Fig. 4 shows the effects of Triprene.

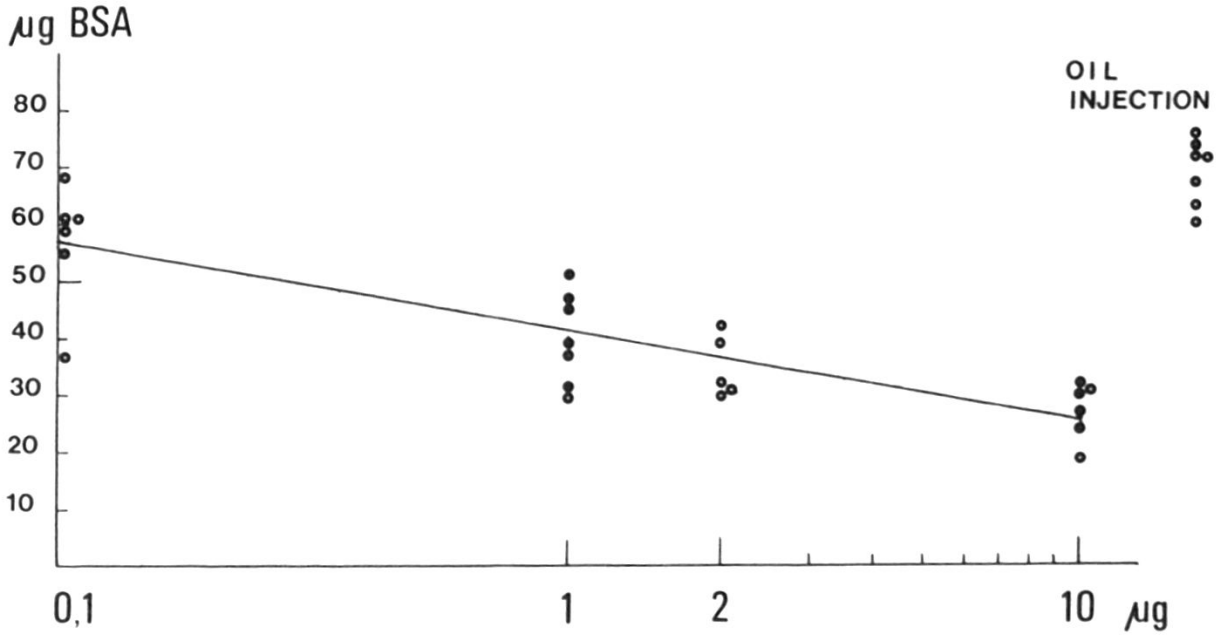

Fig. 2 Protein titre per µl haemolymph in 8-day-old queenright workers after injection with Triprene.
The straight line represents the calculated regression.
Abscissa: injected dose.
Ordinate: haemolymph proteins in equivalents of µg of bovine serum albumin (BSA) per µl haemolymph (UV-method).

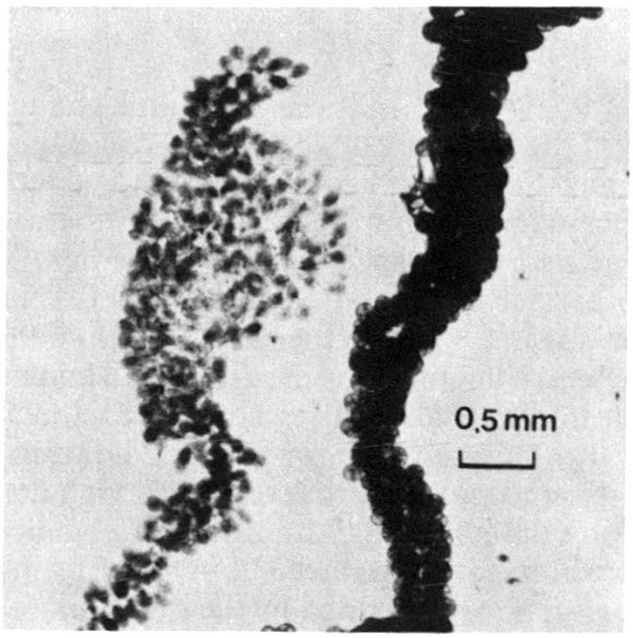

Fig. 3
Right: 8-day-old hypertrophied hypopharyngeal gland of a queenright worker injected with oil on days 0 and 4.
Left: 8-day-old degenerated hypopharyngeal gland of a queenright worker injected with 1 µg JH-III on days 0 and 4.

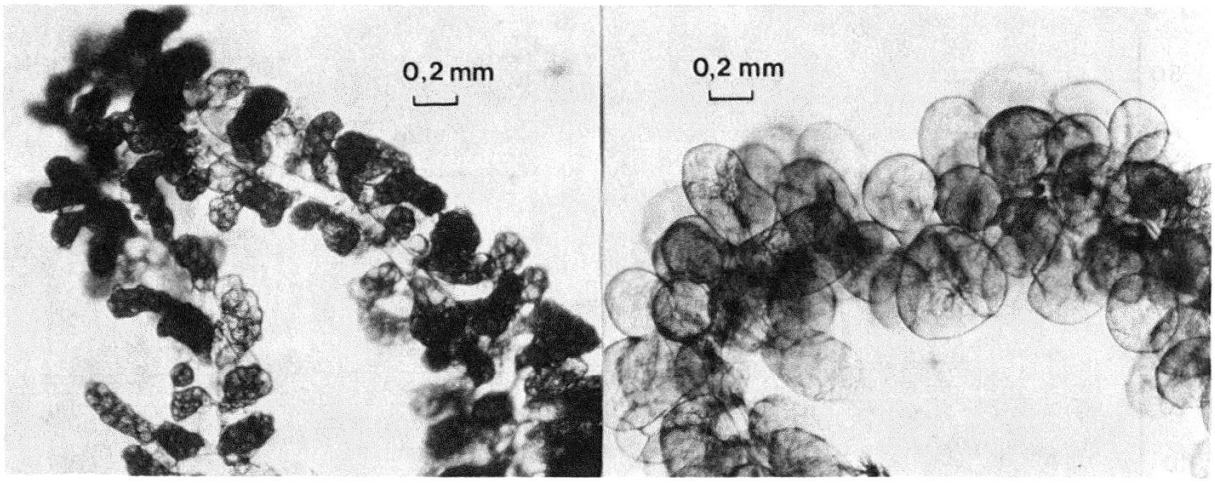

Fig. 4
Right: 4-day-old hypertrophied hypopharyngeal gland of a queenright worker injected with oil on day 0.
Left: 4-day-old degenerated hypopharyngeal gland of a queenright worker injected with 1 μg Triprene on day 0.

Longevity: Most IGRs shorten the longevity significantly. 1 μg of Triprene injected per queenless worker shortened the longevity by 17.9 ± 7.3%. (For longevity tests see JAYCOX, SKOWRONEK and GUYNN, 1974).

DISCUSSION

JH-III injected into workers induces profound changes in physiology. Relatively high doses of the hormone (1 μg per bee) cause the regression of the food glands which means that the worker loses its ability to feed the larvae and is forced to assume other duties within the colony. Furthermore under the influence of the hormone many leucocytes change into pycnotic forms which are inactive in r-RNA synthesis (RUTZ, GERIG, WILLE and LÜSCHER, in preparation) much earlier than they normally are. WILLE and VECCHI (1966) considered the normal leucocyte as a type of haemocyte out of which all known forms of haemocytes differentiate. The hormone also causes a decrease of the total protein in the haemolymph. A low titre of haemolymph proteins however is normal in old field-bees. Therefore it seems probable that relatively high doses of JH initiate a process of aging, shifting the physiology from that of hive bees to that of field-bees. This probably accounts for the reduced life-span. Further evidence for this interpretation of the JH-action is provided by the detection of an increasing JH-titre with the Galleria test during the life of the normal adult worker bee.

In conclusion a combined laboratory examination of the haemolymph proteins, of the morphology of the leucocytes, of the morphology of the hypopharyngeal glands and of the longevity proved to be a simple and reliable method for characterizing the effects of IGRs of hormonal nature. A combined method is particularly useful because some IGRs have characteristic differential effects upon the various criteria.

However, it should be emphasized that our laboratory results with injections of relatively high doses can not be interpreted as having any direct bearing on possible effects of sprayed hormonal IGRs in the field.

Diskussion

Injektionen von JH-III rufen bei der adulten Bienenarbeiterin tiefgreifende physiologische Veränderungen hervor. Der Blutproteinspiegel sinkt, die normalen Leukozyten werden zu pyknotischen Leukozyten, die Futtersaftdrüsen degenerieren, und die Lebensdauer wird herabgesetzt. Eine kombinierte Prüfungsmethode erweist sich als vorteilhaft, da verschiedene IWR nicht alle charakteristischen JH-Wirkungen in gleichem Masse ausüben. Die beobachteten Effekte scheinen dafür zu sprechen, dass JH für eine Umstimmung der Physiologie der Stockbiene zu derjenigen der Flugbiene verantwortlich ist.

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