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Effect of two insect growth regulators on the ecdysteroid contents in eggs of the mealworm

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ABSTRACT. RH-0345 (halofenozide), a bisacylhydrazine derivative, is a nonsteroidal ecdysteroid agonist that mimics the action of the moulting hormones, while KK-42, an imidazole compound, is a potent inhibitor of ecdysteroid biosynthesis. Previous results suggested that the observed reduction of ecdysteroid titre, leading to a reduction of reproductive capacity in the mealworm *Tenebrio molitor*, is due to the direct and rapid action of KK-42 on ecdysteroid biosynthesis. Moreover, RH-0345 was found to increase ecdysteroid production and partially reverse the effects on reproductive events induced by KK-42. Therefore, the present study evaluates the effects of these two insect growth regulators (IGRs) on egg ecdysteroids in mealworms. The IGRs were applied topically (10µg/insect) on newly-emerged adult females. A qualitative and quantitative analysis of ecdysteroids from pooled freshly laid eggs was made by an enzyme-immunoassay (EIA) using two specific antibodies, the rat monoclonal EC 19 antibody to measure for 20-hydroxyecdysone (20E) and the rabbit polyclonal B antibody for ecdysteroids. EIA measurements confirmed the presence of free ecdysteroid and 20E in control and treated series. In addition, the conjugated ecdysteroids were predominant in eggs of untreated mealworms. RH-0345 increased the amounts of 20E relative to the free ecdysteroids. In contrast, KK-42 reduced the amounts of total ecdysteroids, but had no significant effect on the relative hormonal composition of free and conjugated ecdysteroids.

KEY WORDS : Insects, Eggs, Ecdysteroids, Insect growth regulators, RH-0345, Halofenozide, KK-42.

INTRODUCTION

Agrochemical research has resulted in the discovery of novel insecticides that act on selective biochemical sites present in specific insect groups. Good examples are the insect growth regulators (IGRs) such as benzoylphenylurea, which inhibit chitin formation in insects, or other chemicals such as juvenile hormone analogues (JHA) and ecdysteroid agonists, which affect the hormonal regulation of moulting and development processes (ISHAAYA 1990; DHADIALLA et al., 1998; 2005; PALLI & RETNAKARAN 2001). The ecdysteroid agonist RH-0345 (halofenozide) represents a new class of IGRs, which has been developed to provide safe insecticides with high activity in Coleoptera (DHADIALLA et al., 1998; 2005; NAKAGAWA et al., 2001). Similar to the prototype RH-5849 and other ecdysteroid agonists like RH-5992 (tebufenozide) and RH-2485 (methoxyfenozide), this molecule caused in insects a premature and incomplete lethal moult (WING, 1988; WING et al., 1988; SMAGGHE & DEGHEELE, 1994a,b; CARLSON et al., 2001). Various studies revealed that the prototype ecdysteroid agonist RH-5849 presented high activity in species of Lepidoptera and Coleoptera, but later became superseded by RH-5992, RH-2485 and RH-0345, which have enhanced activity against lepidopteran and coleopteran pest insects. The ecdysteroid agonist compounds exert their toxicity by binding to the nuclear ecdysteroid receptor EcR as does the natural insect moulting hormone, 20-hydroxyecdysone (20E) (DHADIALLA et al., 1998; 2005; RIDDIFORD et al., 2000). KK-42 is an imidazole compound considered as an inhibitor of ecdysteroid biosynthesis. KK-42 reduces the ecdysteroid production by ovaries and interferes with reproductive processes in adult females of the mealworm *Tenebrio molitor* (KUWANO et al., 1983; 1985; AMRANI et al., 2004). However, the mode of action of these compounds on reproductive events in insects is not so well understood so far.

Data accumulated on the normal development of ovaries and their regulation provide an experimental basis for investigating IGRs that interfere in the insect's endocrine system, especially for ecdysteroids (SOLTANI-MAZOUNI et al., 1999). As reviewed by HAGEDORN (1985) and LAFONT et al. (2005), in the female adult ecdysteroids are synthesized by the follicle cells in the ovaries and play a major role in ovarian development, vitellogenesis and oocyte/egg maturation. Then the ovarian ecdysteroids, in both free and conjugated forms, are almost entirely taken up by and stored/concentrated in the maturing eggs and may serve as hormonal substrate for embryonic moults during embryogenesis. Many experiments confirm the presence of the free and conjugated forms of ecdysteroids in ovaries of *Schistocera gregaria* (REES & ISAAC, 1984), *Locusta migratoria* (LAGUEUX et al., 1984) and *Manduca sexta* (THOMPSON et al., 1987). It is here an attractive hypothesis that the maternal ecdysteroids are the source of hormone for the embryo during embryogenesis.

To date, few authors have examined the physiological reasons for sublethal effects of IGRs on suppression of the next generation. KOSTYUKOVSKY et al. (2000)

observed that RH-5849 could achieve almost complete control of F_1 adults of *Tribolium castaneum* and *Rhyzopertha dominica* when given to the F_0 generation at 10 ppm. MEDINA et al. (2002) reported that females of *Chrysoperla carnea* lacewings treated with three representative IGRs (the chitin synthesis inhibitor diflubenzuron, the JHA pyriproxyfen and the ecdysteroid agonist RH-5992), accumulated these compounds into the ovaries and eggs, but to a different extent, and although the amount of diflubenzuron was low (74-197pg/egg), it was sufficient to cause egg mortality. Also after treatment with RH-5992 in eggs there existed a reduction of viability (MEDINA et al., 2001). Recently it has been reported that RH-0345 interferes with the reproduction events of *T. molitor* (TAÏBI et al., 2003), and that ovarian follicle cells are sites of biosynthesis of ecdysteroids and protein for egg shell formation (SOLTANI-MAZOUNI & SOLTANI, 1995; SOLTANI-MAZOUNI et al., 1999). In another assay, it was shown that RH-5992 and RH-2485 were able to decrease the mean fecundity and/or fertility of important pest species from several different insect orders (SMAGGHE & DEGHEELE, 1994a,b; SUN & BARRETT, 1999). RH-0345 was the most potent stimulator of the release of hormone into the culture medium by pupal integument explants and by ovaries of *T. molitor* (SOLTANI et al., 1998; 2002). In addition, RH-0345 was able to modify the composition of ecdysteroid amounts in mealworms (TAÏBI et al., 2003). Recently, we reported that RH-0345 is able to partly reverse the depressive effects on reproductive events induced by KK-42 in *T. molitor* (AMRANI et al., 2004).

In this research we used the mealworm *T. molitor*, which is of worldwide importance in stored food products, as a model target beetle. Experiments were undertaken to evaluate the activity of RH-0345 and KK-42 on reproductive events. The compounds were applied topically to adults of *T. molitor* and their effects on ecdysteroid amounts in eggs/embryos were examined.

MATERIALS AND METHODS

Experimental animals

T. molitor pupae from a stock colony were sexed and kept separately until emergence. Adults were collected 0-4h following emergence and reared on wheat flour at 27°C and 80% relative humidity in almost continuous darkness (SOLTANI-MAZOUNI et al., 1999).

Chemicals and treatments

RH-0345 was kindly supplied by Rohm and Haas Research Laboratories (Spring House, PA, USA). KK-42 was kindly provided by Dr. E. Kuwano (Kyushu University, Japan). The compounds were dissolved in acetone (3µl/insect) and administrated topically at 10µg/female on the ventral side of abdomen of newly emerged adult females.

Extraction and enzyme immunoassay of ecdysteroids

EIA measurements have previously been described by TAÏBI et al. (2003). In brief, freshly-laid eggs were collected in control and treated series. Pooled samples, containing the first 30 freshly laid eggs, were submitted to extraction of free ecdysteroids on the one hand and total ecdysteroid on the other with methanol (100%) by sonication and centrifugation (5,000g, 10min). The supernatants containing free and total ecdysteroids were taken and evaporated separately. For determination of the total ecdysteroid amounts (free+conjugated), the samples were further submitted to an enzymatic hydrolysis with porcine liver esterase (EC3.1.1.1., 100,000 units, Sigma Co., St. Louis, IL, USA) (DE REGGI et al., 1992; TAÏBI et al., 2003). Subsequently the extracts were re-extracted using methanolic precipitation of enzymes. Then each individual sample of free and total ecdysteroids was analyzed in duplicate by an EIA using a conjugate of 20E coupled to peroxidase as enzymatic tracer and tetramethyl benzidine as a colour reagent. Quantitative and qualitative analysis of egg ecdysteroids was made by two specific antibodies kindly supplied by Dr. J.-P. Delbecque (University of Bordeaux, France): a rat monoclonal EC 19 antibody to measure 20E amounts, and a rabbit polyclonal B antibody to determine ecdysteroid amounts (DE REGGI et al., 1992). The conjugated amounts of ecdysteroids were deduced by subtracting the amounts of free ecdysteroids from that of total ecdysteroids, both determined with the polyclonal antibody. Data are expressed as pg ecdysteroid equivalents per egg in freshly laid eggs of *T. molitor* females.

Statistical analysis

Results are presented as means ± sd based on four replicates of 30 eggs each. Data were subjected to ANOVA and a *post hoc* LSD test was used to separate means. Statistical analyses were performed using MINITAB 12.21 software (PA Stat College, USA). The significance level was * $p<0.05$, ** $p<0.01$.

RESULTS

The quantitative and qualitative analysis of ecdysteroids in freshly laid eggs, before and after hydrolysis, has been tested by an EIA. The results confirmed the presence of free ecdysteroids with the polyclonal antibody and also of 20E with the monoclonal antibody in all extracts from control and treated series (Fig. 1). In addition, in eggs of untreated mealworms the presence of conjugated ecdysteroids was predominant (Fig. 2). After topical treatment with RH-0345 and KK-42 respectively (10µg/insect), ecdysteroid measurement on extracts of freshly laid eggs showed that RH-0345 increased significantly ($p<0.03$) the amounts of 20E as compared to control series. In contrast, KK-42 caused a significant reduction in ecdysteroid amounts in eggs ($p<0.01$) (Fig. 1).

It can be noted that RH-0345 increased significantly ($p=0.005$) the free ecdysteroids and decreased significantly ($p=0.023$) the conjugated ecdysteroids. However, this ecdysteroid agonist had no significant effect on total ecdysteroids in eggs. KK-42 reduced significantly

($p<0.01$) both total and conjugated ecdysteroids but not the free ecdysteroid form (Table 1). RH-0345 increased significantly the relative importance of free ecdysteroids and decreased significantly the relative importance of conjugated ecdysteroids ($p<0.05$). In contrast, KK-42 had no effect on free ecdysteroid proportions in relation to conjugated ecdysteroids (Fig. 2).

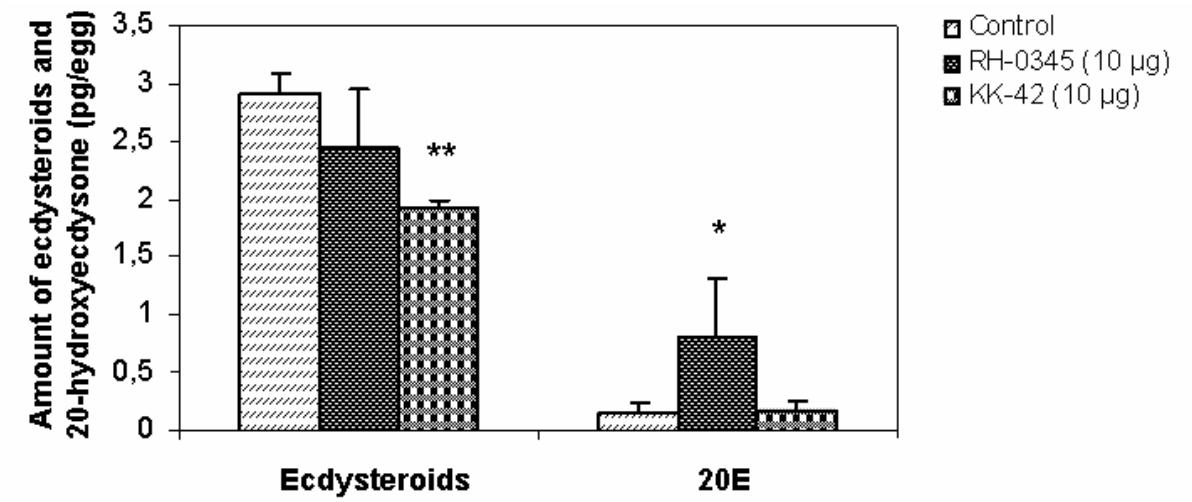

Fig. 1. – Amounts of ecdysteroids detected by polyclonal antibody, and 20-hydroxyecdysone (20E) detected by monoclonal antibody (both in pg/egg), in eggs freshly deposited by females of *Tenebrio molitor* after topical treatment with RH-0345 and KK-42 (10µg/insect). Data are expressed as means ± sd based on four replicates of 30 eggs each. Within the same group, significant differences are indicated by asterisks, * $p<0.05$, ** $p<0.01$.

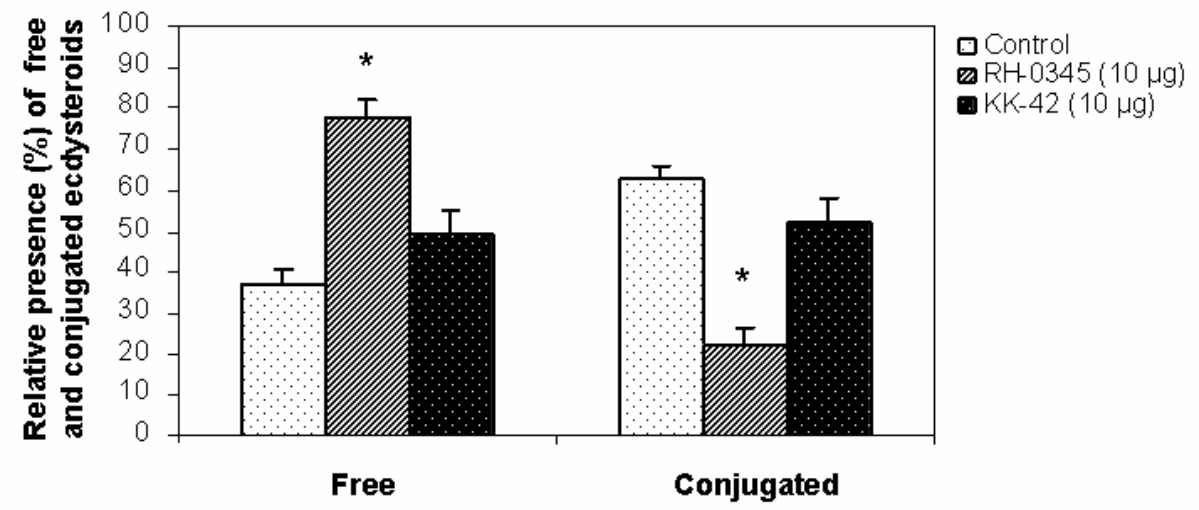

Fig. 2. – The relative presence (%) of free and conjugated ecdysteroids (pg/egg) in eggs freshly deposited by females of *Tenebrio molitor* after topical treatment with RH-0345 and KK-42 (10µg/insect). Data are expressed as means ± sd based on four replicates of 30 eggs each. Within the same group, significant differences are indicated by asterisks, * $p<0.05$.

TABLE 1

In vivo effect of RH-0345 and KK-42, when topically applied at 10µg per female adult, on the amounts of free, conjugated and total ecdysteroids (pg/egg) in eggs freshly laid by *T. molitor* females. Data are expressed as means ± sd based on four replicates of 30 eggs each. Within the same column, values followed by the same letter are not significantly different at the significance level of $p<0.05$

Treatments	Free	Conjugated	Total
Control	1.15±0.13 a	1.93±0.05 c	3.08±0.10 b
RH-0345	2.53±0.84 b	0.72±0.20 a	3.25±0.44 b
KK-42	1.01±0.14 a	1.09±0.13 b	2.10±0.08 a

DISCUSSION

In continuation of our research, the experiments reported here studied in more detail the ovicidal effects of two novel IGRs, the ecdysteroid agonist RH-0345 with a dibenzoylhydrazine structure and the anti-ecdysteroid KK-42 with an imidazole structure. In previous assays RH-0345 and KK-42 were applied at 10µg on female adults; these small amounts did not kill the adults but did cause a reduction in egg viability of about 15% (TAÏBI et al., 2003). We focus here on the significance that the two IGRs may interfere in the ecdysteroid amounts in eggs causing egg mortality through the female adult. In addition, we distinguished between free and conjugated ecdysteroid amounts. From the EIA measurements, we can demonstrate here for the first time that RH-0345 increased the amounts of free ecdysteroids in eggs and decreased that of the conjugated ecdysteroids. But on total ecdysteroid synthesis and accumulation in eggs it had no effect. In contrast, the imidazole compound KK-42 reduced the ecdysteroid amounts in the eggs of *T. molitor*. As reviewed in HAGEDORN (1985) and LAFONT et al. (2005), it is a well-known phenomenon that increases in free ecdysteroids in the eggs result from the release of free hormones from stored maternal ecdysteroids. In adults, free 20E, together with JH, plays an important role in oocyte development, maturation, vitellogenesis and accessory gland development. The concentration of free insect hormones can be modified through interference with the hormone biosynthesis, processing and degradation. As reviewed by LAFONT et al. (2005), free and conjugated forms of ecdysteroids are confirmed in the ovaries of many insects, and it is likely that these maternal ecdysteroids are the source of hormone for the embryo during embryogenesis. Indeed the embryonic ecdysial gland seems not to be necessary for the increase in free ecdysteroid titre or for the accompanying cycles of cuticulogenesis. So any interference in the ecdysteroid hormone response using an ecdysteroid agonist or antagonist would result in abnormal oocyte growth, egg formation and embryogenesis leading to loss of the progeny (SOLTANI et al., 1998; DHADIALLA et al., 1998; 2005). Indeed in previous studies ecdysteroid agonists caused a decline in fecundity and fertility in different insect orders such as Lepidoptera, Diptera, Coleoptera and Orthoptera (WING & RAMSAY, 1989; SMAGGHE & DEGHEELE, 1992; 1994a,b; DHADIALLA et al., 1998; 2005; SWEVERS et al., 1999; SUN et al., 2003; TAÏBI et al., 2003). Also LAWRENCE (1992) found a reduction of protein synthesis and/or incorporation into eggs due to RH-5849 application on *Anastrepha suspense* adults. RH-5992 also caused mortality and the treated females of *Plodia interpunctella* showed smaller ovaries with fewer eggs (SALEM et al., 1997). FARINOS et al. (1999) confirmed the negative effects of RH-0345 on yolk protein accumulation and egg formation in beetle adults (*Leptinotarsa decemlineata*). The latter study also revealed the presence of RH-0345 in the eggs and a reduction in fecundity and/or decrease in progeny survival.

On the mechanisms that may underlie ecdysteroidogenesis and the changes in ecdysteroid amounts, existing data indicate that different factors, hormones and peptides, may influence ecdysteroid biosynthesis (SMAGGHE et al., 1995; GÄDE et al., 1997; GÄDE & HOFFMANN, 2005; LAFONT et al., 2005). Also ecdysteroids themselves play a role in their own biosynthesis via feedback regulation of the PTTH axis in the insect brain, and also via direct action on the ecdysteroid-producing prothoracic glands in the larval stages or the endocrine organs (ovaries, testis) in adults. E and 20E have been reported to stimulate PTTH synthesis and secretion. So for an ecdysteroid agonist compound like RH-0345 we may expect stimulation of free ecdysteroid amounts after treatment. Indeed, SOLTANI-MAZOUNI et al. (2004) reported that the ecdysteroid agonists RH-0345, RH-5849 and RH-5992 increased the ovarian ecdysteroid amounts in adult females after *in vivo* treatment. Also in pupae of *T. molitor*, it was evident that RH-0345 could increase the ecdysteroid titre under *in vivo* conditions and with the use of integument *in vitro* cultures (SOLTANI et al., 2002; TAÏBI et al., 2003). However, as the positive feedback of ecdysteroids happens through an as yet unknown pathway, it also remains a mystery how exactly an ecdysteroid agonist like RH-0345 may have provoked the increase in 20E and free ecdysteroid amounts.

A crucial question that also arises from these studies is why and how the profiles of ecdysteroid titres, free and conjugated, are punctuated with high peaks and deep troughs at specific times during development. Many experiments confirm the presence of the free and conjugated forms of ecdysteroids in ovaries, and it is proposed that these maternal polar conjugated compounds represent storage forms of the hormone, and hydrolysis of these in the developing egg would result in the embryo being exposed to free active hormone (HAGEDORN, 1985; LAFONT et al., 2005). However the mechanisms behind these hormone dynamics are not yet clear, and so the relative reduction of conjugated ecdysteroids due to treatment RH-0345 also cannot yet be explained.

The imidazole compound KK-42 has also been found to reduce the hormonal production in hormone biosynthesis sources from female adult crickets (LORENZ et al., 1995). Interestingly, SOLTANI et al. (1997) also reported a few years later that KK-42 disturbed the growth and the development of oocytes, and in parallel this IGR was found to reduce the amounts of ecdysteroids released into the culture medium by ovaries. The inhibitory action of KK-42 on ecdysteroid production was also observed in prothoracic glands and ovaries under *in vivo* and *in vitro* conditions with the silkmoth *Bombyx mori* (KUWANO et al., 1985). To date several authors have proposed some hypotheses on the mode of action of KK-42. It has been reported to act as an anti- JH, in lowering ecdysteroid levels and/or to induce precocious metamorphosis or diapause termination. The anti-JH activity is more likely interference with the JH synthesis rather than destruction of corpora allata tissue (KUWANO et al., 1983). Herewith, HIRAI et al. (2002) recently reported that the precocious metamorphosis induction by KK-42 correlated with an enhanced expression of BmJH esterase in the fat body, suggesting that KK-42 enhances BmJHE gene expression in the fat body inducing hemolymph JH esterase activity. In addition, to explain the mode of action of KK-42 in ecdysteroidogenesis, SHIOTSUKI & KUWANO (2004) designed an affinity chromatography column system to

detect proteins with high affinity from insect tissues. Although they detected a single receptor protein in the prothoracic glands, the exact underlying mechanism remains unknown. Here, we believe that further research with the availability of the recently characterized and identified Halloween genes, encoding the ecdysteroid-producing cytochrome P450 enzymes, and microarray technologies (WILLIAMS et al., 2002; LAFONT et al., 2005) will provide opportunities to unravel the mechanisms of imidazoles in the hierarchy of ecdysteroid biosynthesis.

In conclusion, the results obtained in this study are the first demonstrating that RH-0345 increased the amounts of both free ecdysteroids and 20E in eggs. In contrast KK-42 reduced the amounts of total and conjugated ecdysteroids. These reported hormonal disturbances in eggs of mealworms help to explain the reduction in egg viability that we scored in previous experiments using RH-0345 and KK-42 on female adults (TAÏBI et al., 2003). However, further research, for instance on the function and dynamics of free and conjugated ecdysteroids in the embryo, is required to better understand the mechanism underlying the ovicidal toxicity of such novel IGRs.

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