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Halofenozide affects sexual behaviour, cuticular hydrocarbons and reproduction in the female German cockroach *Blattella germanica* (Dictyoptera, Blattellidae)

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ABSTRACT. Halofenozide, a dibenzoylhydrazine insect growth regulator, was applied topically to female individuals of the most prevalent German cockroach species, *Blattella germanica*, and its effects on sexual behaviour, cuticular hydrocarbons and reproduction were investigated. Dissection of treated females showed clearly reduced numbers of oocytes and volume of basal oocytes. Interestingly, the ecdysteroid amounts were also significantly lower. Characterization of the cuticular hydrocarbons by gas chromatography showed 13 major compounds, including the female contact sex pheromone precursor. It was clear that halofenozide application resulted in significantly lower amounts of all the investigated cuticular components. Finally, behavioural tests revealed that halofenozide treatment of females caused a significant decrease in sexual receptivity of the untreated conspecific males. Thus, exposure to the ecdysteroid agonist can negatively affect the male's response to a calling female, which is linked with and most likely caused by lesser production of female contact sex pheromone and a delay in the up-regulation of ecdysteroid amounts, and in turn this provoked an obvious lower oocyte number and basal oocyte size as measures of reproduction.

KEY WORDS : *Blattella germanica*, Halofenozide, Reproduction, Sexual behaviour, Cuticular hydrocarbons, Ecdysteroids.

INTRODUCTION

In insects, the steroid moulting hormone, 20-hydroxyecdysone (20E), and the sesquiterpenoid, juvenile hormone (JH), play a central role in the regulation of growth, development and reproductive processes, and are considered as potential specific target sites for pest control (DHADIALLA et al., 1998; GÄDE & HOFFMANN, 2005). Due to secondary effects of conventional insecticides on the environment, a class of selective insect growth regulators (IGRs) that mimic or antagonize the action of 20E and JH, has been developed. While success in the discovery of JH mimetics came much earlier, it is only in the last decade that insecticides that act as agonists of 20E, have been discovered (DHADIALLA et al., 2005).

RH-5849 (1,2-dibenzoyl-1-tert-butylhydrazine), tebufenozide (RH-5992) and methoxyfenozide (RH-2485) are the first members of this new class of compounds, namely dibenzoylhydrazines, that induce a precocious and incomplete moulting in several insect orders, especially Lepidoptera (DHADIALLA et al., 1998; 2005). These compounds manifest their biological activity *via* interaction with the ecdysteroid receptor complex in a competitive manner with ecdysteroids and interfere with expression of some genes involved in cuticle synthesis and secretion. Halofenozide (RH-0345) is a novel member of this class of IGRs. Over the last decades, researchers from different laboratories reported the effects of several of these IGRs on the reproductive performance of insects of different orders, especially Lepidoptera and Coleoptera (SMAGGHE & DEGHEELE, 1994; DHADIALLA et al., 1998; HOELSCHER & BARRETT, 2003; TAIBI et al., 2003; AMRANI et al., 2004). Interestingly, negative effects were also reported in females and males of Lepidoptera (SMAGGHE et al., 2004). In addition, recent reports speculated on the behavioural effects induced by ecdysteroid agonists. In this way, HOELSCHER & BARRETT (2003) showed that the male moth's ability to respond to a calling female is negatively affected by treatment with methoxyfenozide, inhibiting the male's locomotory activity, and this, in turn, caused an obvious reduction in reproduction. BARRETT (2008) also demonstrated that exposure of adult moths of the codling moth, *Cydia pomonella*, to methoxyfenozide-treated surfaces resulted in a negative impact on male responsiveness to calling females and synthetic pheromone lures.

The chemistry, biochemistry and behavioural ecology of the sexual communication system of German cockroaches are relatively well understood (GEMENO & SCHAL, 2004). Elaborate mating display is controlled by various volatile and non-volatile (sex) pheromones, but the mechanisms affecting the regulation of these chemicals are still not fully understood due to a lack of sufficient physiological data. SCHAL & SMITH (1990) reviewed the potential role of brain, *corpora allata* and various hormones such as ecdysteroids, in pheromone production in various cockroach species, with most detailed studies in *Blattella germanica* and *Supella longipalpa*. In *B. germanica*, a number of cuticular hydrocarbons serve as biosynthetic precursors to specific contact sex pheromones

that are perceived by antennal sensillae and function as contact primers of sex pheromone triggering behavioural phase transition (BLOMQUIST et al., 2005).

The most prevalent German cockroach *B. germanica* is well known for its high economic and medical importance and its strong resistance to conventional insecticides (SCOTT et al., 1990). Thus new target insecticides are urgently needed to reduce resistance pressure. In this study, adult females of *B. germanica* were tested to evaluate the activity of the ecdysteroid agonist halofenozide on their reproduction. With these experiments we aimed to confirm the ecdysteroid agonistic effects of halofenozide and also to try to understand the action of those molecules on the reproductive process and insect behaviour. This component was firstly tested on the female for its potential activity on ovaries, total ecdysteroid amounts and cuticular hydrocarbon profiles. The observed perturbation of adult sexual behaviour is discussed.

MATERIALS AND METHODS

Insect rearing

Colonies of *B. germanica* were reared in plastic boxes with dog food pellets and water *ad libitum*. They were kept at 27±1°C under a 12h light:12h dark regime and 70% relative humidity (HABES et al., 2006). None of the insects had been in previous contact with any insecticides.

Insecticide and treatment

Halofenozide of technical grade (>95% pure; Rohm and Haas Co., Spring House, PA, USA) was topically applied after dilution in acetone (10µg in 3µl per insect) to newly emerged adult females. Control insects were treated with acetone alone. All the insects were kept under the same conditions as given above. Experiment procedures for each test are outlined below.

Ovarian parameter

Halofenozide was administrated topically to newly emerged females (less than 3 hours after adult emergence). Each treated female was immediately paired with one untreated male in a plastic box (9.5cm x 6.5cm x 2cm) containing food and water. Adult females from control and treated series were sampled at 0, 2, 4 and 6 days during the adult life and their ovaries dissected out. After removal of circumovarian fat body, the numbers of oocytes per pair of ovaries were recorded and the volume of the basal oocyte was determined as described in LAMBREAS et al. (1991). Six to ten replications were done for each series (AMRANI et al., 2004).

Ecdysteroid extraction and quantification

Six days after treatment, ecdysteroids were extracted from individual whole adult female bodies with methanol (2ml) by sonication (2-3min) as previously described (BERGHICHE et al., 2008). Samples were centrifuged at 5,000g for 10min, and the supernatants evaporated. Each extract was resuspended in 500µl of phosphate buffer (0.1M; pH7.4) and analyzed in an enzyme immunoassay (EIA) using a rabbit polyclonal B antibody against 20E coupled to peroxidase as an enzymatic tracer and tetramethyl benzidine as a color reagent. Each experiment was replicated 6 times according to previous publications (AMRANI et al., 2004; ARIBI et al., 2006). Data are expressed in pg 20E equivalents per insect. Antibodies and tracer were kindly supplied by Dr. J.-P. Delbecque (Laboratoire de Neuroendocrinologie, Université de Bordeaux I, France).

Gas chromatography analyses of cuticular hydrocarbons

After 1, 3 and 6 days, insects were frozen for about 5min at -20°C and extracted individually for 5min at room temperature in 1ml of distilled pentane containing 10µg *n*-octadecane (C18) as internal standard (IS). The extracts were stored at -20°C until analysis. Extracts were then concentrated to about 100µl under a nitrogen flow, and aliquots of 1µl analyzed by gas chromatography (GC) using a CP-9000 of Chrompack (Bergen op Zoom, The Netherlands) fitted with a split-splitless injector (30s split, 25ml/min) and a flame-ionisation detector. Injector and detector temperatures were 260°C and 280°C, respectively. The analytical column used was an apolar CP-Sil 5 fused silica capillary column (25m x 0.25mm ID, Chrompack). Helium was used as carrier gas at a velocity of 35cm/s at 120°C. The oven temperature was programmed from 140°C to 280°C at 3°C/min. The signal was recorded and integrated on a computer fitted using Maestro software (Chrompack). Ten repetitions were done for each series. Estimation of the proportion obtained was done with a normalized area quantitative method (EVERAERTS et al., 1997). The quantification (ng per insect) of each component was calculated using the response factor of the internal standard.

Sexual behaviour assay

All the observations were made under a dim red light in plastic boxes (19cm x 13cm x 4cm) during the first hours of the scotophase, which correspond to the maximal period of sexual activity in this species (LIANG & SCHAL, 1993). From the continuous colony, 80 virgin adults (newly ecdysed adults, 40 males and 40 females) were randomly selected and kept separated. In this species, the females are responsible for the male sexual attraction; so, in the treated series, only the females received halofenozide. For the experiments we used insects 6 days into the adult stage when females and males are sexually mature (SCHAL et al., 1997; LIHOREAU et al., 2008). Behaviour observations were made on 20 separate couples. Each insect pair was introduced into an observation box without any anesthesia because CO_2 is known to affect insect behaviour (GUERENSTEIN & HILDEBRAND, 2008; CHAMPION DE CRESPIGNY & WEDELL, 2008). The number of male wing raisings, a characteristic response when a mature virgin female is encountered (LIHOREAU et al., 2008), was determined during a 15min period (KIM et al., 2004; LIHOREAU et al., 2008).

Statistical analysis

Results are presented as the mean ± SEM. Significance of differences between means was estimated by a Stu-

dent's *t*-tests at $p<0.05$. A multivariate analysis (MANOVA) was conducted on relative amounts of the cuticular components in relation to treatment and duration of exposure (KIM et al., 2004). Statistical analyses were performed using MINITAB 12.21 software (Minitab Inc., State College, PA, USA).

RESULTS

Ovarian measurements

As shown in Fig. 1, the number of oocytes per paired ovaries in female controls increased during sexual maturation (0–2 days-old; $p<0.001$), and decreased at days 4 and 6 ($p<0.001$) (beginning of ovulation). Treatment with halofenozide on newly emerged females of *B. germanica* significantly reduced the number of oocytes present at days 2 ($p=0.002$) and 4 ($p=0.016$) compared to controls of the same age. At day 6, the absence of differences between controls and the treated series is probably due to the reduction of oocyte numbers following egg–laying in controls and stable values in the treated series.

Fig. 2 illustrates that the volume of the basal oocyte in controls increased during sexual maturation from 0.0040±0.0003 mm^3 to 0.090±0.004 mm^3, while halofenozide–treated females had significantly smaller volumes at days 2, 4 ($p=0.006$) and 6 ($p<0.001$) (Fig. 2).

Ecdysteroid amounts

During the first 6 days, ecdysteroid amounts in control females reached 7.34±0.57 pg/insect. Halofenozide-treated females (10µg) had about 26% lower ecdysteroid titers (5.46±0.54pg/insect) ($p=0.039$).

Effects on cuticular hydrocarbon profiles

In *B. germanica*, the chemical nature of the cuticular hydrocarbons has previously been described (RIVAULT et al., 2002). In our GC analyses, 25 peaks was detected according to RIVAULT et al. (2002) but, because of low concentrations of some compounds, we chose the 13 major ones that were present at concentrations of >500ng/insect in all the investigated samples (*i.e.*, at 6 day, peak 1: 602±72ng, peak 12: 8334±999ng) (Fig. 3). As described by RIVAULT et al. (2002), the compounds were identified as *n*-heptacosane (peak 1), 9-, 11- and 13-methylheptacosane (peak 2), 5-methylheptacosane (peak 3), 3-methylheptacosane (peak 4), *n*-nonacosane (peak 5), 9-, 11-, 13- and 15-methylnonacosane (peak 6), 7-methylnonacosane (peak 7), 5-methylnonacosane (peak 8), 11,15- and 13,17-dimethylnonacosane (peak 9), 3-methylnonacosane (peak 10), 5,9- and 5,11-dimethylnonacosane (peak 11), 3,7-; 3,9- and 3, 11-dimethylnonacosane (peak 12) and 11-, 13- and 15-methyltriacontane (peak 13). Peak 12 corresponds to the most abundant component of the female contact sex pheromone precursor (CHASE et al., 1992). All the obtained chemical profiles are comparable. Quantitative differences between the control and treated groups at days 1, 3 and 6 were observed (Fig. 4). The concentration of each component did not depend on the length of the treatment: *i.e.*, peak 12: day 1 (1316±175ng), day 3 (1478±286ng), day 6 (1837±300ng). A multivariate analysis revealed that the amounts of all the investigated peaks were significantly lower (MANOVA: $p<0.001$) in the insects treated with halofenozide than in the control insects.

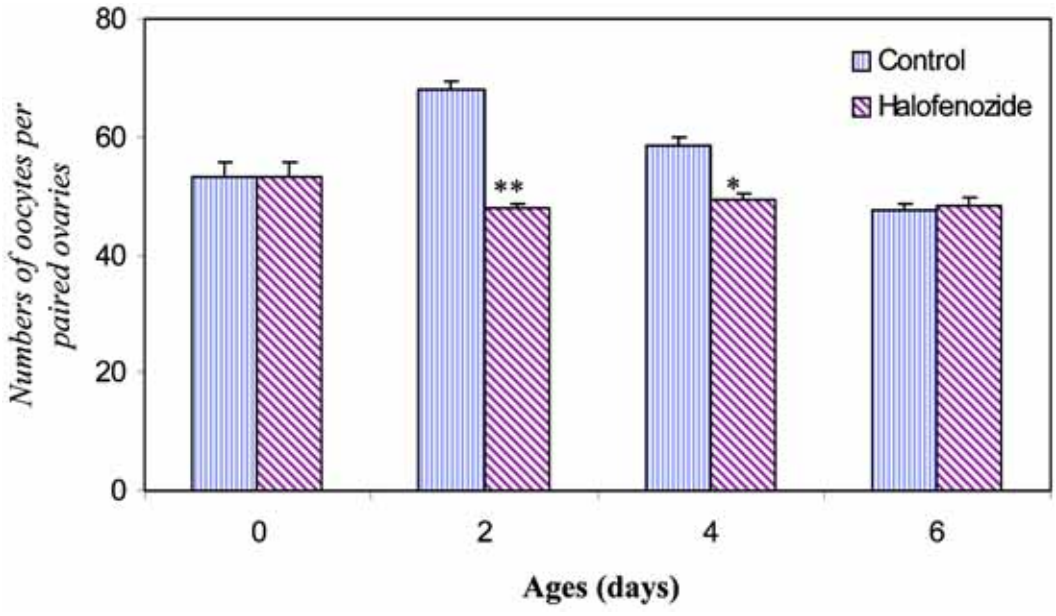

Fig. 1. – Effect of topical application of halofenozide (at day 0) on newly ecdysed female adults of *B. germanica* on the numbers of oocytes per paired ovaries. Value is the mean ± SEM (n=6-10). Asterisks indicate a significant difference between control and treated series of the same age (*: $p<0.05$; **: $p<0.01$; ***: $p<0.001$).

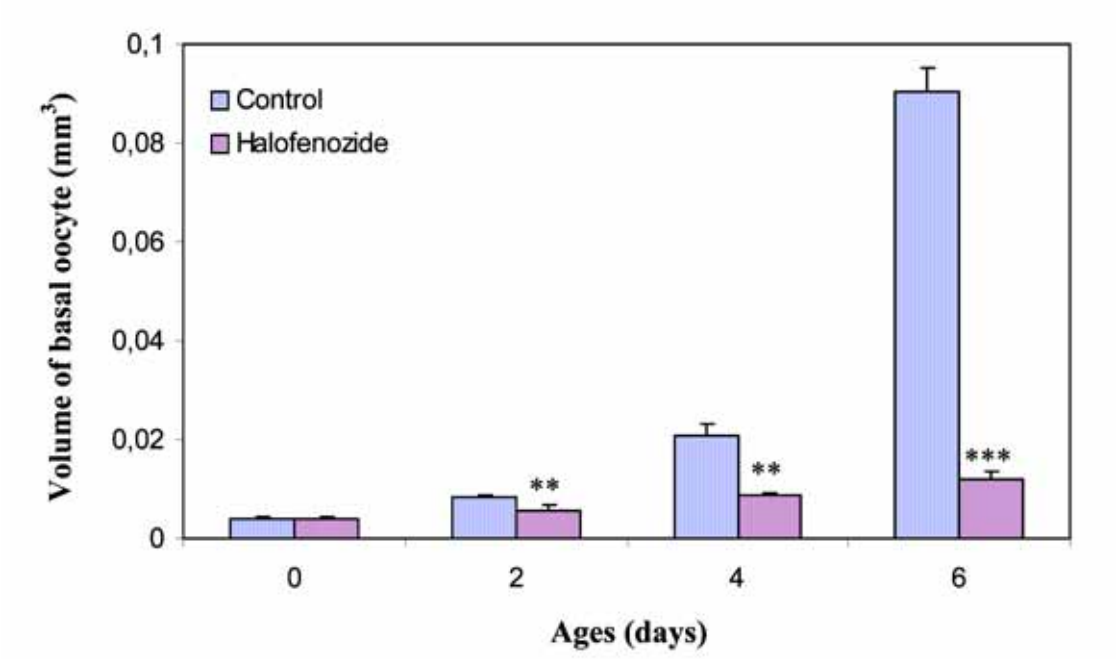

Fig. 2. – Effect of topical application of halofenozide (at day 0) to newly ecdysed female adults of *B. germanica* on the volume of the basal oocyte. Value is the mean ± SEM (n=6-10). Asterisks indicate a significant difference between control and treated series of the same age (*: $p<0.05$; **: $p<0.01$; ***: $p<0.001$).

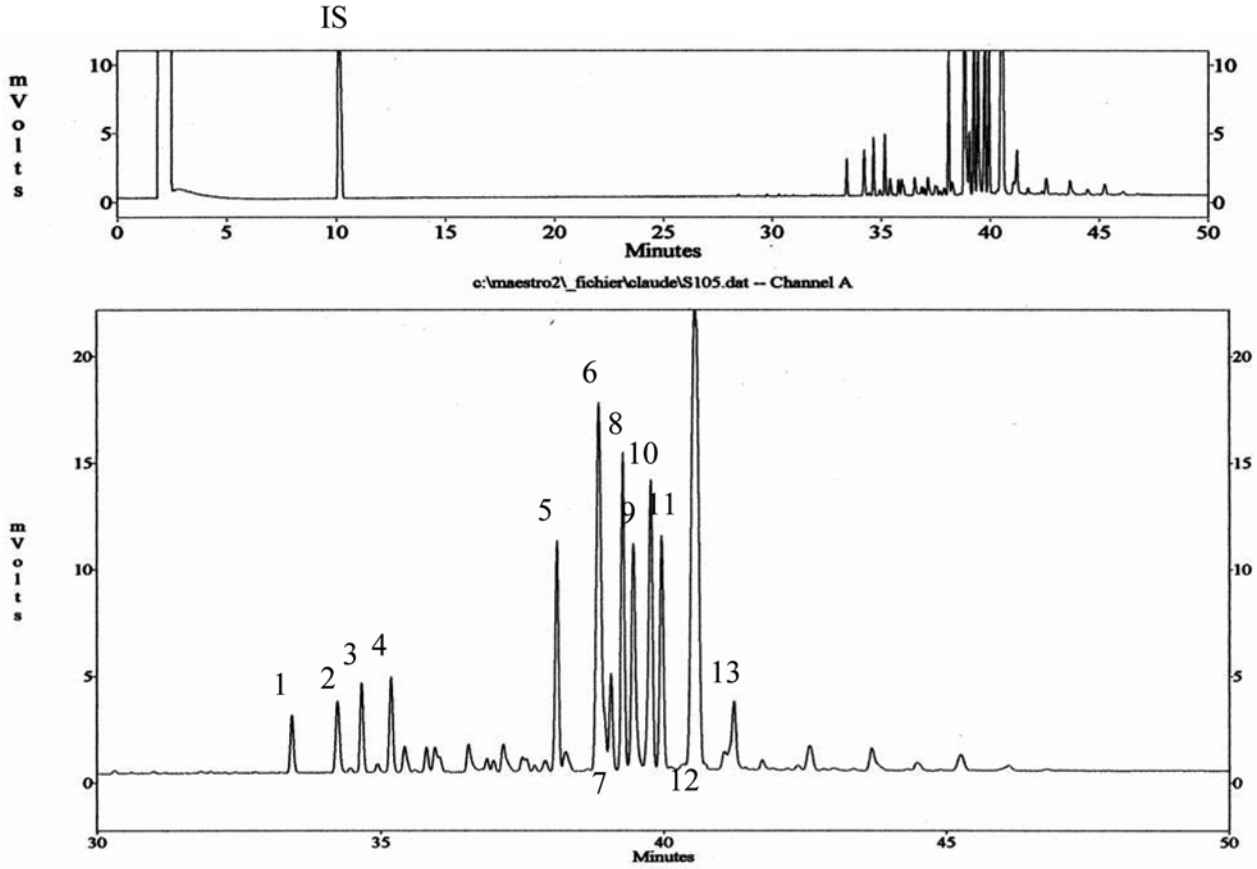

Fig. 3. – Typical gas chromatography profile of an extract of cuticular hydrocarbons of *B. germanica* adults of both sexes. Peaks 1 to 13, see results; IS, internal standard.

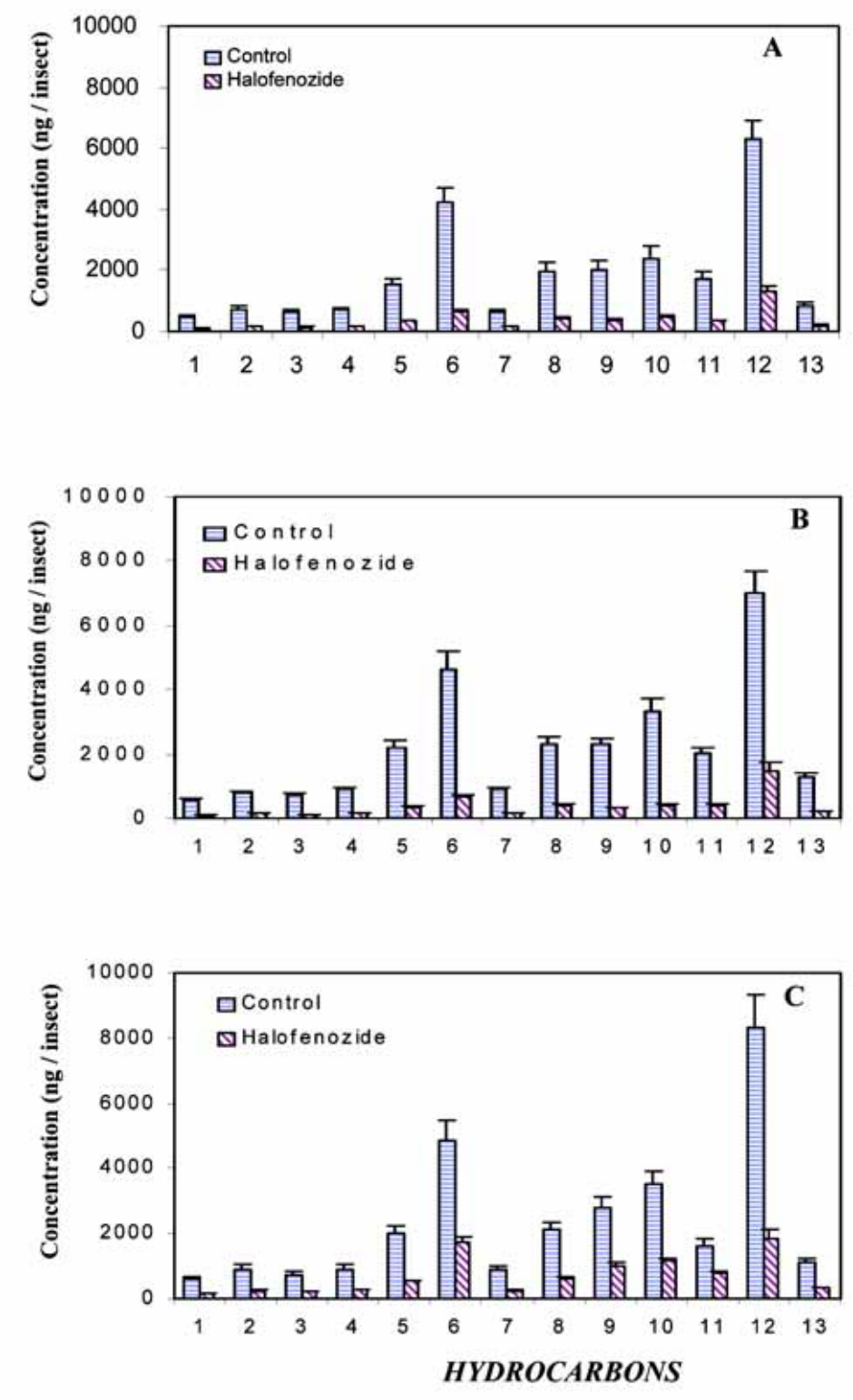

Fig. 4. – Quantification (ng/insect) of the 13 investigated major cuticular hydrocarbons in control and treated females of *B. germanica* of various ages (mean ± SEM, n=10). (A: day 1, B: day 3, C: day 6).

Sexual behaviour

To test the potential "sex-appeal" of a female, we chose to measure the number of male wing-raisings, which are generally correlated with the sexual maturity of the female (LIHOREAU et al., 2008). Wing-raising is a characteristic posture of the male that occurs only after contact with a virgin female. This behaviour does not imply that the female mounts the male to copulate, but it is a good indication of the sexual "excitation" of the male.

We observed (data not shown) that the number of male antennae contacts with a female did not differ significantly between control and treated series. Thus, antennae contact between the sexes is not related to the male wing-raising effect. The 6-day-old males in the controls showed 14.3±1.3 wing-raisings in response to a sexually mature female. It was striking that when halofenozide was applied to the newly emerged females, significantly ($p=0.004$) fewer male wing-raisings (9.1±1.0) were noted.

DISCUSSION

Development and reproduction processes in insects are orchestrated by ecdysteroids, JHs and neuropeptides (GÄDE & HOFFMANN, 2005) and it is clear that any exogenous sources of hormones, synthetic agonists or antagonists that interfere with the homeostasis of the insect hormones can be exploited as novel insecticide targets to disrupt normal development and reproduction of pest insects.

In the present study we proved that treatment of newly ecdysed females of *B. germanica* with the ecdysteroid agonist halofenozide resulted in reduced numbers of oocytes and smaller volume of the basal oocyte during the adult life as compared to controls. It is well known that non-steroidal ecdysteroid agonists cause a decline in reproduction in different insect orders such as a Lepidoptera, Diptera, Coleoptera and Orthoptera (SMAGGHE & DEGHEELE, 1994; DHADIALLA et al., 1998; SOLTANI et al., 1998; SUN et al., 2003; TAIBI et al., 2003; AMRANI et al., 2004). Previous studies using various ecdysteroid agonists have shown that these compounds affect ovarian growth in lepidopteran and coleopteran species (FARINOS et al., 1999; SUN et al., 2003; SMAGGHE et al., 2004). Halofenozide was found to reduce both growth and development of oocytes *in vitro* and the thickness of follicular epithelium *in vivo* in mealworms, suggesting a reduction in the synthetic activity of the follicle by structural alterations and/or biochemical modifications (SOLTANI et al., 1998). In *B. germanica*, as in all cockroaches studied to date, vitellogenesis and cyclic maturation of oocytes depend upon JH III synthesis by the *corpora allata* and a decline in JH synthesis occurs just before ovulation (SCHAL et al., 1997). Consequently, the reduced number and volume of oocytes we observed a few days after halofenozide treatment may be due to interference by this hormone agonist with JH or other neuropeptides that regulate ovulation and reproduction, which can subsequently affect the sex communication system. However, the exact mechanism underlying this negative activity of halofenozide remains unknown so far.

For the prototype compound of halofenozide, RH-5849, it was shown that ecdysteroid titers in treated tobacco hornworm *Manduca sexta* larvae were depressed within 4hr after injection (WING et al., 1988); this implies that ecdysteroids and agonists may exert a negative feedback inhibition on hormone biosynthesis in *Pieris brassicae* (BEYDON & LAFONT, 1983) and *Bombyx mori* (GU et al., 2008). ROMANA et al. (1995) found ecdysteroid titers in the haemolymph and ovary to be relatively low in freshly ecdysed females of *B. germanica*, increasing progressively during vitellogenesis, peaking at choriongenesis, and decreasing after ovulation. Our data from EIA measurements showed clearly that halofenozide caused inhibition of the ecdysteroid titers in 6-day-old adult females of *B. germanica*.

In the current experiments, halofenozide was also found to reduce quantitatively the various major cuticular hydrocarbons of female adults of *B. germanica*. In this species, the cuticular hydrocarbons are synthesized at the level of the oenocytes in the abdominal integument (FAN et al., 2003). In insects, cuticular hydrocarbons are synthesized through elongation of fatty acyl-CoA, fatty acid reduction to aldehydes, and fatty aldehyde conversion to alkanes that contain one carbon less (NELSON & BLOMQUIST, 1995). The aldehyde is decarboxylated to hydrocarbon and carbon dioxide by cytochrome P450 enzyme, which requires NADPH and molecular oxygen (REED et al., 1995). In *B. germanica*, the cuticular hydrocarbons consist of a complex mixture of apolar compounds that contain *n*-alkanes, monomethylalkanes and dimethylalkanes (RIVAULT et al., 2002). In addition, six female sex-specific components were identified (CHASE et al., 1992). The most abundant component of the female contact sex pheromone, 3,11-dimethyl-2-nonacosanone (NISHIDA et al., 1974), is derived through hydroxylation and subsequent oxidation of the abundant cuticular hydrocarbon 3,11-dimethylnonacosane (CHASE et al., 1992). Three components derived from this C29-dimethyl ketone with either a methyl, alcohol, or an aldehyde functionality at the C29 position, are also present. Other components with a C27 skeleton, 3,11-dimethylheptacosanone and its alcohol or aldehyde forms also serve as sex pheromones (JURENKA et al., 1989; MORI, 2008), but those components are less abundant on the female cuticular surface. The behavioural activity of 3,11-dimethylheptacosanone is significantly lower than that of its C29 homologues (ELIYAHU et al., 2004). As shown in our data, the compound represented by peak 12, one of the major precursor of the female sex pheromone components, appeared in higher concentration in 6-day-old treated females. Due to its poor GC response, the high molecular weight contact pheromone, 3,11-dimethyl-2-nonacosanone, which appears in very minute quantities in the female, was not detected in our GC traces. This compound is one of the female components that is responsible for the wing-raising posture of the male. Consequently, the accumulation of peak 12 onto the cuticle of the female may certainly be in close connection with a perturbation of the biosynthesis pathway of the specific female hydrocarbons. Regarding our results, we can hypothesize that the reduced amounts of hydrocarbons observed in halofenozide-treated females might have been caused at different levels of synthesis and at delivery and/or trans-

port of these compounds to the surface of the cuticle. The decrease in cuticular compounds might have a secondary influence on sex recognition in the reproductive process and explain the perturbation noted in the male behaviour.

In cockroaches, pheromone production is coordinated with the gonadotropic cycle and the major gonadotropic hormone (*i.e*., JH) has been recruited to act on several target tissues (BLOMQUIST et al., 2005). Previous studies demonstrated that precocene inhibited pheromone production in this family (CHASE et al., 1992; SCHAL et al., 1994), whereas hydroprene, a JH analogue, increased the female pheromone production (SCHAL, 1988). So, as described in various insect species (SLEDGE et al., 2004; LOMMELEN et al., 2006), we can suggest that the quantitative variation observed in the female *B. germanica* cuticular hydrocarbons after halofenozide treatment could be related to reproduction and/or to other unknown processes; this will be due to the synergistic or inhibitory interactions between 20E, JH and other neurohormones (BELLÈS, 1995). In this way, it has been speculated that 20E appears to regulate fatty acyl-CoA elongase. However, little is known of either the enzymology or the molecular biology of hydrocarbon production (BLOMQUIST et al., 2005). BLOMQUIST et al. (2005) noted that 20E and JH induce and repress the synthesis of specific enzymes at the transcription level of pheromone production. In the female housefly, *Musca domestica,* and possibly in other species of Diptera, it appears that during hydrocarbon sex pheromone biosynthesis, ovarian-produced ecdysteroids regulate the synthesis by affecting the activities of one or more fatty acyl-CoA elongation enzyme(s). In Lepidoptera, sex pheromone biosynthesis is often mediated by the pheromone biosynthesis activating neuropeptide (PBAN) through alteration of enzyme activities at one or more steps prior to, or during fatty acid synthesis or modification of the carbonyl group (TILLMAN et al., 1999). So the decrease of hydrocarbon amounts observed in our experiments after halofenozide treatment could be explained by the inhibition of fatty acyl-CoA elongase.

In the German cockroach, sexually receptive females attract males from a distance with a volatile pheromone, namely blattellaquinone, which is emitted at the level of the pygidium (ABED et al., 1993; NOJIMA et al., 2005). When the male is in contact with a virgin female, he recognizes her by his antennae. The presence of a contact sex pheromone (3,11-dimethyl-2-nonacosanone) on the cuticular surface of the female induces the characteristic male wing-raising behaviour (ELIYAHU et al., 2004). This typical behaviour triggers specialized male tergal glands that induce the female to climb onto the back of the male, and so placing her in a precopulatory position (GEMENO & SCHAL, 2004).

In the present study, we showed that when applied on females, halofenozide disturbed the male wing-raisings. The observed decline of this male posture could result in a lower production or lower perception of the contact female sex pheromone due to the lower amount of cuticular compounds. It is known that females of *B. germanica* can already mate 2 days after emergence, but the maximum copulation was observed at day 13 (ABED et al., 1993). In our experiments, no copulation was noted in control or treated series within the first 6 days and the possibility that halofenozide can interfere at the behavioural level, through control of pheromone production or emission during calling, remains realistic.

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