ADIPOKINETIC HORMONES IN FIFTH INSTAR *ROMALEA GUTTATA* (ORTHOPTERA: ACRIDIDAE): ACTIVATION OF GLYCOGEN PHOSPHORYLASE DOES NOT PRODUCE HYPERTREHALOSEMIA

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ABSTRACT

Romalea guttata Houttuyn (= *R. microptera* Beavois) is flightless, lethargic, aposematic, and chemically defended. *R. guttata* stores large quantities of two adipokinetic hormone (AKH) family peptides in its corpora cardiaca. In adults, these peptides (Rom-CC-I and Grb-AKH) activate fat body glycogen phosphorylase but are not hypertrehalosemic. Because juvenile *R. guttata* contain sufficient peptide to be bioactive, we sought to determine whether these peptides are hypertrehalosemic, phosphorylase activating, or hyperlipemic in juveniles. Late fifth (= last) instar and adult *R. guttata* activated phosphorylase in response to Rom-CC-I injections. These same individuals showed no hypertrehalosemia in response to Rom-CC-I. We hypothesize that the glycogenolysis pathway is not started by activation of glycogen phosphorylase in response to Rom-CC-I. From fourth instar through third week adult, *R. guttata* showed a slight, statistically insignificant hypolipemia, but clearly no hyperlipemia. *R. guttata* differs from *Locusta migratoria* in that it appears to show neither hypertrehalosemia nor hyperlipemia at any developmental stage.

Key Words: lubber grasshopper; chemical defense; glycogenolysis; adipokinetic hormone

RESUMEN

Romalea guttata Houttuyn (= *R. microptera* Beavois) es una especie que no vuela, es letárgica, es aposemática, y se defiende por medios químicos. *R. guttata* guarda grandes cantidades de dos péptidos de la familia de las hormonas adipokinéticas (AKH) en la glándula corpora cardiaca. En los adultos, estos péptidos (Rom-CC- I and Grb-AKH) activan fosforilasas de glicógeno del cuerpo graso pero no son hipertrehalosémicos. Como los *R. guttata* juveniles contienen suficientes péptidos para que sean bioactivos, hemos tratado de determinar si estos péptidos son hipertrehalosémicos, activadores de fosforilasas, o hiperlipémicos en los juveniles. El quinto (último) instar y el adulto de *R. guttata* activaron la fosforilasa como respuesta a injecciones de Rom-CC-I. Estos mismos individuos no mostraron ninguna hipertrehalosemia como respuesta a Rom-CC-I. Nosotros suponemos que el proceso de glicogenolisis no es iniciado con la activación de la fosforilasa del glicógeno como respuesta a Rom-CC-I. Del cuarto estadío al adulto de tres semanas, los *R. guttata* mostraron una hipolipemia estadísticamente insignificante, pero claramente ninguna hyperlipemia. *R. guttata* difiere de *Locusta migratoria* en que parece no mostrar ni hipertrehalosemia ni hyperlipemia en ninguna fase de su desarrollo.

The Eastern Lubber Grasshopper, *Romalea guttata* Houttuyn (= *R. microptera* Beavois), is seasonally common in the Southeastern US, flightless, lethargic, aposematic, and chemically defended (Whitman et al. 1990). Adult *R. guttata* store large

quantities of two small peptides in their corpus cardiacum (CC; Gäde and Spring 1986). These peptides (Rom-CC-I and Grb-AKH) are members of the adipokinetic / red-pigment concentrating hormone (AKH/RPCH) family by both bioactivity in appropriate test species (Gäde and Spring 1986) and their primary structures (Gäde et al. 1988). Further, the peptides are released under *in vitro* physiological conditions (Spring and Gäde 1991). In adults, the endogenous functions of both Rom-CC-I and Grb-AKH appear to be control of fat body glycogen phosphorylase (GP), converting inactive GP to the active form. In adult *R. guttata*, separate injections of Rom-CC-I and Grb-AKH activated GP in a dose dependent manner (Gäde and Spring 1989). As is true with the well-studied migratory locust (*Locusta migratoria* L.), the activation of GP was not concurrent with hypertrehalosemia (Goldsworthy 1994). Also, the peptides elicited no hyperlipemic response in adult *R. guttata* (Spring and Gäde 1987). In sum, injections of endogenous AKHs into adult *R. guttata* do not appear to affect hemolymph metabolite levels.

Juvenile *R. guttata* contain sufficient Rom-CC-I and Grb-AKH to be biologically active (Spring and Gäde 1991). For example, early fourth instar *R. guttata* contain about 230 pmol Rom-CC-I and about 25 pmol Grb-AKH in their CC (Spring and Gäde 1991). These quantities are much greater than the minimum dosage (≈1 pmol) needed for maximal activation of GP in adults. We hypothesized that because Rom-CC-I and Grb-AKH are not known to play a biologically important role in adult *R. guttata*, they may function to control hemolymph metabolites in juveniles. Alternatively, endogenous AKHs in *R. guttata* may not serve any metabolite control functions that endogenous AKHs serve in other grasshoppers, such as *L. migratoria*.

We asked four primary questions. First, are juvenile *R. guttata* significantly hypertrehalosemic in response to synthetic Rom-CC-I (sRom-CC-I)? Second, do these same grasshoppers activate GP in response to sRom-CC-I? Third, are decapitated *R. guttata* significantly hypertrehalosemic in response to sRom-CC-I? Fourth, are juvenile *R. guttata* hyperlipemic in response to injections of CC homogenates?

Materials And Methods

Experiment 1—Responses of Hemolymph Carbohydrates to sRom-CC-I Injections through Development.

Experimental animals. In April, 1996, we collected first instar *R. guttata* near Lydia, LA, USA. We brought the insects to the laboratory and raised them as described by Whitman (1986). Briefly, we kept *R. guttata* at 30 ± 2°C on a 14L:10D photoperiod. *R. guttata* were fed oatmeal and Purina Cricket Chow® *ad libitum,* Romaine lettuce daily, and green beans, green onions, carrot tops, and apple occasionally.

*Determination of hemolymph carbohydrates***.** We measured changes in hemolymph carbohydrates in response to sRom-CC-I injections at six stages of development: instar 4 day 4 (= L4-d4); L5-d2; L5-d6; L5-d10; adult days 3 and 4 (= Ad-d3); and Ad-d9. To determine the hemolymph carbohydrate concentrations, we collected 2 µl hemolymph samples and measured total carbohydrates as anthrone positive material with trehalose standards (Spik and Montreuil 1964). We then injected each grasshopper with either 5 µl deionized H_2O or 20 pmol sRom-CC-I (Peninsula Laboratories Inc.; San Carlos, CA) in 5 µl deionized H_2O and measured hemolymph carbohydrates again 90 min later.

*Statistics***.** We tested the changes in carbohydrates for statistical differences between treatments with ANOVA and Tukey's post-tests.

Experiment 2—Responses of Active GP to sRom-CC-I Injections through Development**.**

*Experimental animals***.** Following the determination of carbohydrates, we offered each *R. guttata* Romaine lettuce and kept them isolated at 25 ± 2°C overnight until the GP experiments the next day. Half of the grasshoppers injected with deionized H_2O for carbohydrate determination were also injected with deionized H_2O for active GP determination. The remainder of the grasshoppers injected with deionized H_2O for carbohydrate determination were injected with sRom-CC-I for active GP determination. This treatment control influenced our data only once, for L5-d6 grasshoppers. At this developmental stage, insects injected with sRom-CC-I the previous day did not activate GP, but insects injected with deionized H_2O the previous day did activate GP. Because this was the sole influence of our treatment control, we combined the data for clarity of presentation.

Determination of active GP. We assayed active GP by following glycogen breakdown according to the methods of Ziegler et al. (1979) as modified by Gäde and Spring (1989).

*Statistics***.** We tested the changes in percent active GP for statistical differences between treatments by ANOVA with Tukey's post-tests.

Experiment 3—Responses of Hemolymph Carbohydrates to sRom-CC-I Injections in Decapitated Adults**.**

*Experimental animals***.** In August, 1996 we collected adult *R. guttata* from the same collection site used in Experiment 1. These *R. guttata* were kept as described above for three to six days before experimentation.

*Determination of hemolymph carbohydrates***.** The night before an experiment, we decapitated the grasshoppers and sealed the exposed orifices with liquid wax. We measured changes in hemolymph carbohydrates in response to either deionized H_2O injection or sRom-CC-I injection identically to Experiment 1, except that we took hemolymph samples from the coxal membrane.

*Statistics***.** We compared the data using student's t-tests.

Experiment 4—Determination of Hemolymph Lipids in Response to CC Homogenate Injections through Development**.**

Experimental animals. We collected *R. guttata* from April to August, 1994 from the same collection site used in Experiment 1. We fed these grasshoppers lettuce daily and Purina Cricket Chow® *ad libitum*. Grasshoppers were held in the laboratory at least 48 h prior to use. In all other respects, we maintained these grasshoppers identically to the grasshoppers used in Experiment 1.

CC homogenates. We prepared CC homogenates by the method of Gäde and Spring (1989). Our CC homogenates contained both Rom-CC-I and Grb-AKH, the predominant *R. guttata* CC peptides (Spring and Gäde, 1987).

Lipid assays. We measured changes in hemolymph lipids in response to CC preparation injections at six stages of development: instar 3 (= L3); L4; L5; week 1 adults (= Ad-w1); Ad-w2; Ad-w3. We used the method of Spring and Gäde (1987) to determine if *R. guttata*'s competence to CC homogenates with respect to hyperlipemia changes through development. We first collected a hemolymph sample from each grasshopper, and then we injected 5 μl aliquots of test solution (= 0.1 CC-equivalents) intra-abdominally. Second samples were taken 90 min post-injection. We measured total lipids as vanillin-positive material using vegetable oil standards following the method of Zöllner and Kirsch (1962).

Statistics. We tested the changes in lipid concentrations for statistical differences among developmental stages by ANOVA.

RESULTS

Experiment 1—Responses of Hemolymph Carbohydrates to sRom-CC-I Injections through Development.

Changes in hemolymph carbohydrate concentrations after test solution injections varied widely among the six developmental stages examined (Fig. 1). Except for L5-d2 ($P < 0.05$; Tukey's test), injection of sRom-CC-I did not statistically change hemolymph carbohydrate concentrations in comparison to water injection within any developmental stage.

Experiment 2—Responses of Active GP to sRom-CC-I Injections through Development.

Synthetic Rom-CC-I injection activates GP during the developmental period from L4-d4 to Ad-d9; an ANOVA revealed a statistically significant effect of sRom-CC-I injection ($P = 0.009$), but no significant effects for developmental stage ($P = 0.651$) or the interaction of developmental stage and sRom-CC-I injection (0.417; Fig. 2). In general, activation of GP was stronger in the older *R. guttata,* with competence to sRom-CC-I appearing to develop by L5-d10.

Experiment 3—Responses of Hemolymph Carbohydrates to sRom-CC-I Injections in Decapitated Adults

Changes in hemolymph carbohydrate concentrations in decapitated adults that were injected with water ($\bar{x} = 0.0151$ mg/ml; SE = 0.0054; n = 9) did not differ signif-

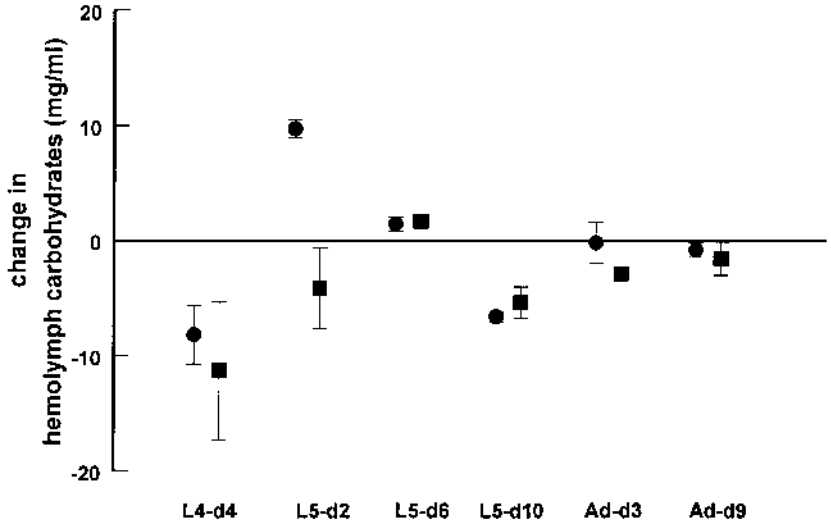

Fig. 1. Changes in hemolymph carbohydrate concentrations in response to injections at six developmental stages of *R. guttata*. Dots represent deionized water injected groups (n = 13-20), and squares represent sRom-CC-I injected groups (n = 7-10). For abbreviations, see text.

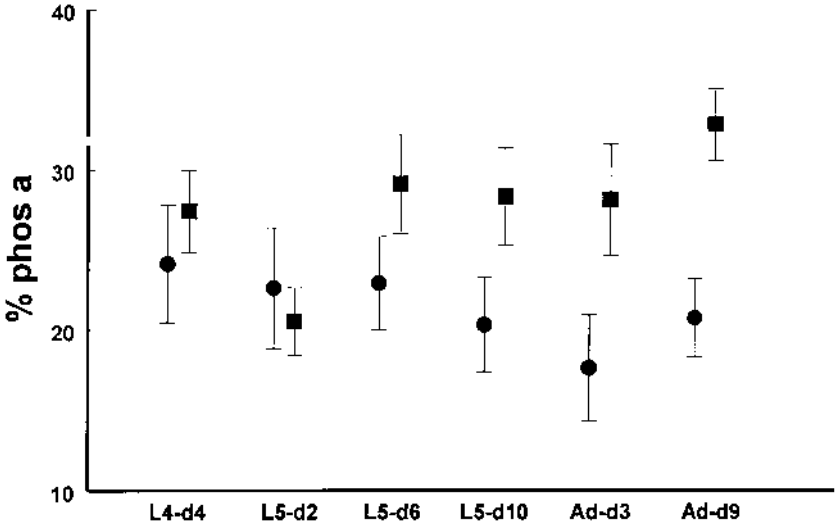

Fig. 2. Percent active fat body glycogen phosphorylase in response to injections in six developmental stages of *R. guttata*. Dots represent deionized water injected groups (n = 7-8), and squares represent sRom-CC-I injected groups (n = 13-17). For abbreviations, see text.

icantly from concentration changes in decapitated adults that were injected with sRom-CC-I ($\bar{x}$ = 0.0091 mg/ml; SE = 0.0038; n = 12).

Experiment 4—Changes in Hemolymph Lipids in Response to CC Homogenate Injections through Development.

Injections of CC extracts produced a hypolipemic affect in all developmental stages from L3 to Ad-w3 (Fig. 3). ANOVA revealed no significant differences in the responses among any of the developmental stages (P = 0.127). In general, adults showed the strongest hypolipemic responses, and larvae showed the weakest hypolipemic responses.

Discussion

Activation of GP but Absence of Hypertrehalosemia

R. guttata significantly activate GP in response to sRom-CC-I injections (Fig. 2). The interaction of developmental stage and sRom-CC-I injection was insignificant; nonetheless, it appears from our data that the competence to AKHs in *R. guttata* develops in the late fifth instar. Regardless of the developmental moment of the onset of competence, it is clear that the older *R. guttata* in our study activated GP in response to sRom-CC-I injections.

R. guttata has no competence to sRom-CC-I with respect to hypertrehalosemia at any developmental stage from L4-d4 through Ad-d9 (Fig. 1). For the well-studied *L. migratoria*, there have been at least two explanations postulated in the literature for this lack of hypertrehalosemia concurrent with activation of GP: 1) not enough glyco-

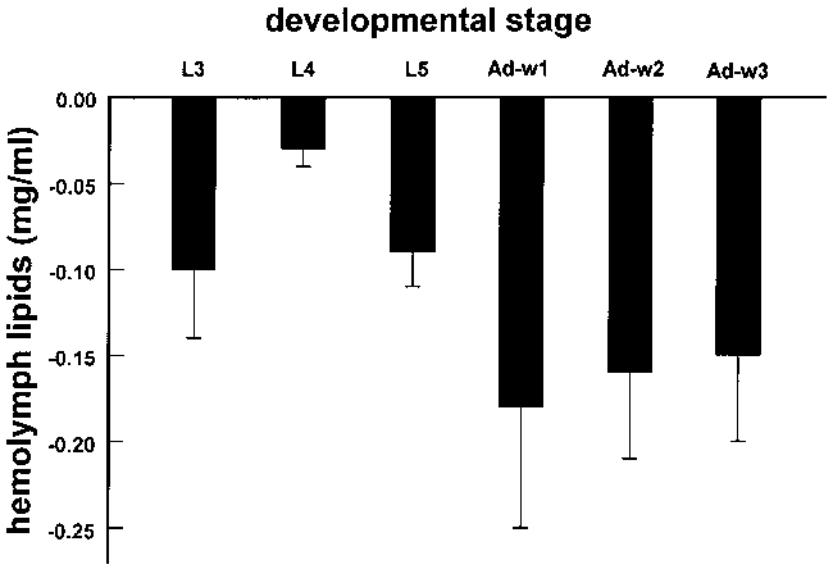

Fig. 3. Changes in hemolymph lipid concentrations in response to injections of corpus cardiaca preparations at six developmental stages (n = 7-10) of *R. guttata*. For abbreviations, see text.

gen in the fat body (Goldsworthy 1994), and 2) inhibition of hyperglycemia by an unspecified "head factor" (Loughton and Orchard 1981). Adult *R. guttata*, especially those fed daily in the laboratory (as ours were), have sufficient glycogen in the fat body to produce a hypertrehalosemic effect (Spring and Gäde 1987). Second, the lack of hypertrehalosemia in decapitated adults (see Experiment 3) suggests that, in *R. guttata*, a head factor is not necessary for the prevention of hypertrehalosemia.

Alternatively, it may be that the additional quantity of GP activated in our experiments (≈ 10%) was not sufficient to induce hypertrehalosemia. We do not believe this to be true for two reasons. First, Gäde and Spring (1989) showed stronger activations of GP (≈ 30%) but still no hypertrehalosemia. Second, the catalytic nature of enzyme function requires only a small change in enzyme activation to produce a large change in metabolite concentrations.

Fifth instar *L. migratoria* are moderately hypertrehalosemic in response to the endogenous Lom-AKH-I (Van Marrewijk et al. 1984), whereas fifth instar *R. guttata* are clearly not hypertrehalosemic in response to sRom-CC-I. In fact, our data suggest that L5-d2 *R. guttata* may be hypotrehalosemic in response to sRom-CC-I. We therefore hypothesize that the glycogenolysis pathway is not started by activation of GP in response to sRom-CC-I in *R. guttata*. *R. guttata* may activate GP for some function other than the mobilization of sugars, but this seems highly unlikely. Barring this explanation, the competence to sRom-CC-I in *R. guttata* appears to be an evolutionary remnant of the development of flight physiology in last instar Acrididae. Importantly, our data suggest that the physiology of *R. guttata*, as well its behavior, is different from other grasshoppers, and that this difference reflects its flightless, lethargic, chemically defended life style.

Hypolipemic Response to CC Homogenates?

R. guttata are slightly hypolipemic in response to CC homogenate injections at developmental stages from L3 through Ad-w3. Rom-CC-I and Grb-AKH are the predominant peptides in *R. guttata* CC homogenates. Hence, at the very least, *R. guttata* are not hyperlipemic in response to injections of endogenous AKHs. In contrast, *L. migratoria* develop competence with respect to hyperlipemia to the synthetic endogenous AKH as L5 (Van Marrewijk et al. 1984). The lack of hyperlipemia in L5 and adult *R. guttata* is further evidence that the physiology of these grasshoppers is different from the physiology of grasshoppers that fly.

Divergent Physiology?

We have shown three ways that *R. guttata* differs physiologically from *L. migratoria*. All three of these differences make sense in light of *R. guttata*'s flightless and lethargic behavior: 1) absence of hypertrehalosemia in late L5 grasshoppers in concert with activation of GP; 2) absence of hypertrehalosemia in decapitated adults of *R. guttata*; 3) absence of hyperlipemia in L5 and adult *R. guttata.* Taken together, these data suggest that *R. guttata* may have diverged physiologically from grasshoppers that can fly, and that *R. guttata's* responses mirror its flightless, lethargic, chemically defended life-style.

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