1998

The Role of the Comstock-Kellogg Glands in Egg Tanning in Romalea guttata

Elizabeth A. Elasser '98
Illinois Wesleyan University

The Role of the Comstock-Kellogg Glands in Egg Tanning in *Romalea guttata*

Elizabeth A. Elsasser

Senior Honors Research Project

Illinois Wesleyan University

Abstract

Freshly laid eggs of the Eastern Lubber grasshopper, *Romaleaguttata*, are yellow and soft-shelled, but within several hours the eggs become hardened and dark brown. In a similar process, calyx and oviduct secretions, which form the egg pod, become frothy, darkened, hardened, and water insoluble during oviposition. It has been suggested that secretions from the paired Comstock-Kellogg glands accelerate tanning of both the eggs and egg pod foam. I investigated the effects of Comstock-Kellogg gland secretions on the rate of egg and egg pod tanning during egg pod production in the female Eastern Lubber grasshopper, *R. guttata*. Eggs streaked with Comstock-Kellogg gland secretions, macerated CK gland, macerated intersegmental membrane, or hemolymph took significantly less time to tan than non-streaked eggs or eggs streaked with deionized water. Females lacking CK glands laid eggs that took significantly longer to tan than eggs from sham-operated or unoperated females. These results suggest that the Comstock-Kellogg glands do serve to accelerate tanning, but the chemical composition of the CK secretions may be common to other grasshopper tissues.

Introduction

Insects are one of the most successful group of animals to inhabit the earth, living in almost every habitat. Their success is due, in part, to their ability to produce many offspring in a short amount of time. This rapid reproductive cycle has been utilized by geneticists studying gene inheritance. Grasshoppers and locusts (Order Orthoptera), have been widely studied because of their economic importance. Because these organisms are typically considered to be crop pests, their reproductive and life history patterns are of great importance when designing pest control measures.

Different species of grasshoppers exhibit various ovipositional characteristics, yet some similarities do exist. Many grasshoppers deposit their eggs underground in a packet called an egg pod composed of eggs embedded in foamy material derived from the calyx (i.e., the common chamber at the base of the ovarioles) and oviducts (Fig. 1) (Stauffer and Whitman, 1997). The primary oocytes develop and mature in synchrony and are laid in unison (Stauffer and Whitman, 1997). Oviposition in the Eastern Lubber grasshopper, *Romalea guttata* involves the same sequence of events as in other grasshoppers. The female digs with her four ovipositor valves into the soil or sand, extending her abdomen as she progresses, and scrapes out a chamber at the bottom. A viscous liquid passes out through the gonopore between the set of four valves. The female's abdomen contracts in a rhythmic pattern followed by the quick opening of the valves. The ventral valves slide through the dorsal valves while the egg guide and genital plate simultaneously slide through the ventral valves. The abdomen then expands and the process repeats (Elsasser, personal observation). These actions result in the production of a milky white froth that is released prior to the release of each egg. The eggs are released individually until about 50 eggs have been laid. The female then caps the batch of eggs with additional froth and removes herself from the hole.

Freshly laid eggs of *R. guttata* are yellow and soft-shelled, but within several hours, the eggs become hardened and dark brown (i.e., tanning) (Fig. 2). Calyx and

oviduct secretions that form the egg pod undergo a similar process. The secretions are yellow, thick liquids while in the body of the female, but they become frothy during oviposition. The secretions subsequently become darkened, hardened, and water insoluble (Fig. 3). After hardening, the foam maintains the orientation of the embryos by binding the eggs together so the heads of the embryos point upward (Stauffer and Whitman, 1997). This orientation allows the newly hatched instars to climb toward the opening of the hole housing the egg pod. In addition, the foam binds the eggs together, prevents collapse of the hole, facilitates gas exchange and reduces water loss (Ewer, 1977; Schmidt, 1988). Accelerated tanning may increase embryo survivorship in the rapidly changing environmental conditions of the Southeastern and South Central United States. The egg pod may be occasionally subjected to periods of submersion, or conversely, extremely dry conditions. A hardened egg pod would be resistant to both flooding and desiccation, and therefore be of great advantage to the animals.

Although little is known about egg tanning in grasshoppers, initial work by Eisner (1966) suggested that an exocrine secretion is added to the calyx and oviduct secretions and serves to accelerate the tanning process of both the eggs and egg pods of *R. guttata*. These secretions are supplied by a pair of glands, the Comstock-Kellogg glands, located adjacent to the gonopore (Fig. 4). Oviposition results in the total depletion of the stored Comstock-Kellogg (CK) secretions, while other activities including mating and defecation produce no visible loss of the secretions (Eisner, 1966). This was the first indication that this pair of glands might be involved in the process of oviposition. Crystals of methylene blue dye added to the CK secretions resulted in subsequent egg pods that were distinctly blue, suggesting that the CK secretions are incorporated into the secretions that form the egg pod (Eisner, 1966). Streaking of eggs with CK secretions reduced the tanning time of the eggs from several hours down to less than one hour. The CK secretions were found to not only accelerate the tanning of eggs of *R. guttata*, but were also shown to hasten the darkening of the egg pod foam (Eisner, 1966).

Despite the apparent acceleration of tanning that occurs when CK secretions are added to the eggs, egg pods laid by females from which the CK glands had been removed tanned at a normal rate (Eisner, 1966). Eisner (1966) also found that blood from the partially healed wounds accelerated tanning as effectively as the CK secretions when applied to eggs. Insect blood, upon exposure to air or foreign substances, will undergo an enzymatic melanization process in which tyrosine-derived quinones (Evans, 1965) and perhaps some proteins polymerize to form darkened clots (Cottrell, 1964). In light of this, it is likely that the active factor in the CK secretion is largely composed of protein (Eisner, 1966). A protein component was found in the CK secretions that could be inactivated by heat treatment at 100°C for 20 min. (Eisner, 1966).

The female Eastern Lubber grasshopper, *R. guttata*, was examined to gain knowledge of the poorly understood process of egg tanning. Although Eisner's work suggested that CK gland secretions accelerate egg and egg pod tanning, the data were unreported in his work. The purpose of this study was to determine if the Comstock-Kellogg glands produce a compound capable of accelerating egg tanning and therefore facilitating increased embryo survivorship.

Methods

Study organism

Female *Romaleaguttata*, were used during the duration of the study. In nature, these animals are found in the Southeast and South Central United States in swamp and marsh areas such as the Everglades (Rehn & Grant, 1959) (Fig. 5). The animals are large (up to 7 cm and 9 g), flightless and typically move less than 100 yards/yr. (Whitman, 1988). Instead of a flight response to danger, the grasshoppers release noxious chemicals, including phenols and quinones, from glands posterior to the pronotum (Jones, *et al.*, 1986). In addition to the metathoracic gland secretions, these animals contain internal toxins capable of inducing vomiting in predators including blue jays (*Cyanocitta cristata*), mocking birds (*Mimus polyglottos*), robins (*Turdus migratorius*), brown thrashers (*Toxostoma rufum*) (Whitman, 1988), and some lizards such as anoles (*Anolis carolinensis*) (Whitman & Yosef, 1992). In addition, *R. guttata* exhibit aposematic coloration (red and black wings, yellow and black exoskeleton) to ward off predators (Whitman, 1990; Whitman & Yosef, 1992).

The typical life cycle includes five instar (larval) stages (Rehn & Grant, 1959). The time from fifth instar to adult is approximately 12 days and the females may begin laying eggs approximately 30 days after eclosion to the adult (Whitman, pers. comm.). Once males have mated with females, they will typically guard the female (i.e., remain on her back) for up to five days to prevent other males from copulating with the female (Elsasser, pers. observ.).

All grasshoppers were obtained from third generation laboratory stock. The original lab animals were obtained from Copeland, FL in the summer of 1996.

Rearing

The study was conducted from May 1997 to April 1998. Female grasshoppers, ranging in age from newly eclosed to approximately two months after eclosion, were fed *ad*

libitum on Romaine lettuce and oatmeal twice daily. The diet was supplemented with green onions, oranges, green beans, and apples when available. Uneaten food was removed at the beginning of each day. The hoppers were kept at ambient room temperature, ranging from 28-30°C (light) and 23-24°C (dark), under a photoperiod of 14:10 (light:dark). All animals were kept in wire cages measuring 45 cm. x 45 cm. x 30 cm., or 42 cm. x 42 cm. x 45 cm.

Experiment 1: Effects of CK gland removal

Thirty females taken from the newly eclosed stock population were randomly assigned to either undergo surgery to remove the CK glands, to undergo sham surgery, or to remain unoperated. All operations were performed 5-10 days after eclosion and all females were anesthetized with CO_2 immediately prior to surgery. The Comstock-Kellogg glands were easily exposed by extending the abdomen and pulling the genital plate anteriorly. The glands were removed with iridectomy scissors and a drop (approx. 20μl) of 1 mg/1.0 ml ampicillin sodium salt was applied to reduce the risk of infection. In order to control for surgical effects, sham surgeries were performed in which two small incisions were made in the tissue surrounding the spermathecal opening. A drop of ampicillin was applied as described above.

Females exhibiting pre-oviposition behavior as described by Stauffer and Whitman (1997), were removed from the stock cage and placed in plastic containers (10 cm. x 7 cm.) with a narrow opening 1.2 cm. wide. The plastic container with the female was then placed directly over the opening between two glass plates, 12.5 cm. x 15 cm., separated by wood. The space between the glass plates had been filled with fine, white sand moistened with deionized water to approximately 15% of saturation prior to the addition of the female. This apparatus allowed for easy visualization of the laying process (Fig. 6). The females oviposited in an area maintained at 29°C with constant light. Two eggs chosen randomly were observed during oviposition. The time for each egg to reach a dark brown color

standard was recorded, and the two times were averaged for data analysis. The standard was chosen as the best match for the color of approximately 95% tanned eggs. The color changes were determined by direct comparison of the color card to the eggs at five minute intervals. Data were analyzed using a one-way ANOVA followed by a Bonferroni's post-hoc test (Norman and Streiner, 1994).

Experiment 2--Egg streaking experiment

In order to determine if CK secretions alone affected egg tanning, eggs surgically removed from gravid female *R. guttata* were streaked with Comstock-Kellogg secretions, ground Comstock-Kellogg gland, macerated intersegmental membrane, hemolymph, or deionized water. The waxy CK secretions were scraped from the surface of the CK glands and gently applied to the surface of the eggs. Once the eggs were streaked, they were placed in tissue culture dishes to maintain 100% humidity and were placed in a water bath at 28°C. The time necessary for darkened brown patches to appear was recorded. Data were analyzed using a one-way ANOVA followed by a Bonferroni's post-hoc test (Norman and Streiner, 1994).

Results

Experiment 1: Effect of CK gland removal on egg tanning time

There was a significant effect of surgical treatment on egg tanning time (ANOVA; $F_{2,22}$=16.45, p<0.00001). Eggs from females lacking CK glands took significantly longer to tan than eggs from sham-operated females or unoperated females. Eggs from unoperated females took significantly longer to tan than eggs from sham operated females (Fig. 7).

Experiment 2: Egg streaking experiment

There was a significant effect of streaking treatment on spotting time of eggs (ANOVA; $F_{5,118}$=281.92, p<0.00001). Eggs streaked with hemolymph, CK secretions, macerated CK gland, or macerated intersegmental membrane took significantly less time to tan than eggs streaked with nothing or deionized water (Fig. 8).

In a preliminary experiment, it was found that if the eggs were initially rinsed in a Ringer's solution prior to streaking with the various compounds, the eggs failed to tan. This indicates that there is some component of the calyx and oviduct secretions involved in the tanning reaction.

Discussion

The fact that eggs from females lacking CK glands took significantly longer to tan than eggs from sham-operated or unoperated females supports the work of Eisner (1966) that suggested the CK glands are involved in egg and egg pod tanning. CK glands likely produce a substance that has enzymatic activity increasing the rate of the melanization reaction. Accelerated tanning may be critical to embryo survivorship in the rapidly changing environment of Southeastern and South Central United States. The eggs and egg pod may be occasionally subjected to periods of extremely dry conditions, or conversely,

periods of submersion. A hardened and melanized egg pod would be resistant to both flooding and desiccation, and therefore be a great advantage to eggs from these animals.

Because the eggs from the sham-operated animals took significantly less time to tan, it is obvious that there was a surgical effect. It is possible that this result is related to the scar tissue produced by surgery. These animals were inspected after laying and had small amounts of scar tissue at the sites of incision. As was shown by the streaking experiment (see discussion below), hemolymph can accelerate egg tanning. It is likely that the eggs or calyx secretions produced by the sham-operated females came in contact with the scar tissue and this resulted in the decreased tanning time. However, females that had the CK glands surgically removed also had scar tissue. By the reasoning above, this would decrease the tanning time, when in fact, the eggs produced by these females took significantly more time to tan. Apparently, the complete lack of the CK glands overwhelmed the rate enhancing effect of hemolymph.

The results of the egg streaking experiment suggest that the chemical composition of the CK gland secretions may be similar to that of other grasshopper tissues. It is possible that the melanization process may be the result of a polymerization reaction of tyrosine-derived quinones and some proteins similar to the formation of darkened clots that result from the exposure of hemolymph to air or other foreign substances (Evans, 1965). It is also possible that the secretion is the enzyme dopa decarboxylase, or a closely related compound. Dopa decarboxylase is the enzyme utilized in the conversion of dopa to dopamine in the pathway of formation of sclerotins and melanins (Fig. 9). Various derivatives of dopamine are used in crosslinking reactions of the insect cuticle which leads to scleritization (Brunet, 1980). In the tobacco hornworm, *Manduca sexta*, dopa decarboxylase is involved not only in cuticular scleritization (Hopkins *et al.*, 1984) but also in larval cuticular melanization (Hiruma & Riddiford, 1985; Hori *et al.*, 1984; Hiruma *et al.*, 1985). Diphenoloxidase is the enzyme responsible for the conversion of dopa to

dopaquinone. Dopaquinone can be converted melanin, therefore, the CK glands may alternatively produce diphenoloxidase or a related compound (Hori, *et al.*, 1984).

Because of the surgical effect shown in this paper, it is uncertain if the CK glands are solely responsible for the increased rate of egg and egg pod tanning. In future experiments, it may be beneficial to cauterize the glands and perform sham surgeries with a cauterizing device. It is possible that this method may reduce the amount of scar tissue and remove the surgical effect produced in this study. In addition, work needs to be done to determine the exact composition of the CK secretions and the mechanism by which the secretions interact with the eggs to bring about tanning.

References

Brunet, P.C.J. (1980). The metabolism of the aromatic amino acids concerned in the cross-linking of insect cuticle. *Insect Biochem.* **10**, 467-500.

Cottrell, C. B. (1964). Advan. Insect Physiol. Vol. 2. Academic Press, NY.

Eisner, T. (1966). Tanning of grasshopper eggs by an exocrine secretion. *Science.* **152**(3718), 95-97.

Evans, J. J. T. (1965). Thesis, Harvard University.

Ewer, D. W. (1977). Two functions of the foam plug of acridid egg pods. *Acrida.* **6**, 1-17.

Hiruma, K., Riddiford, L. M., Hopkins, T. L., and Morgan, T. D. (1985). Roles of dopa decarboxylase and phenoloxidase in the melanization of the tobacco hornworm and their control by 20-hydroxyecdysone. *J. Comp. Physiol. B.*

Hiruma, K., and Riddiford, L. (1985). Hormonal regulation of dopa decarboxylase during a larval molt. *Developmental Biology.* **110**, 509-513.

Hori, M., Hiruma, K., and Riddiford, L. M. (1984). Cuticular melanization in the tobacco hornworm larva. *Insect Biochem.* **14**, 267-274.

Jones, C. G., T. A. Hess, D. W. Whitman, P. J. Silk, and M. S. Blum (1986). Idiosyncratic variation in the chemical defenses among individual generalist grasshoppers. *J. Chem. Ecol.* **12**, 749-761.

Rehn, J. A. G. and Grant, H. J. (1959) A review of the Ramaleinae (Orthoptera: Acrididae) found in America north of Mexico. *Proc. Acad. Nat. Sci. Phil.* **111**, 109-271.

Schmidt, G. H. (1988). Behavioral peculiarities in *Acrotylus patruelis*. *Annals of Entomology*, **6** (2), 35-47.

Stauffer, T. W. and Whitman, D. W. 1997. Grasshopper oviposition. In *Economics of Grasshoppers, Katydids, and Their Kin.* (S. Gangwere & M. Muralirangan eds.). CAB International, Wallingford, UK 550. pp. 231-280.

Whitman, D. W. (1988). Allochemical interactions among plants, herbivores, nad their predators. In Novel Aspects of Insect-Plant Interactions. (P. Barbosa & D. Letoumeau, eds) pp. 11-64. John Wiley, NY.

Whitman, D. W. (1990) Grasshopper Chemical Communication. In *Biology of Grasshoppers*. (R. F. Chapman & A Joern, eds) pp. 357-391. John Wiley, NY.

Whitman, D. W., and R. Yosef (1992). Predator exaptations and defensive adaptations in evolutionary balance: no defense is perfect. *Evolutionary Ecology*. **6**, 527-536.

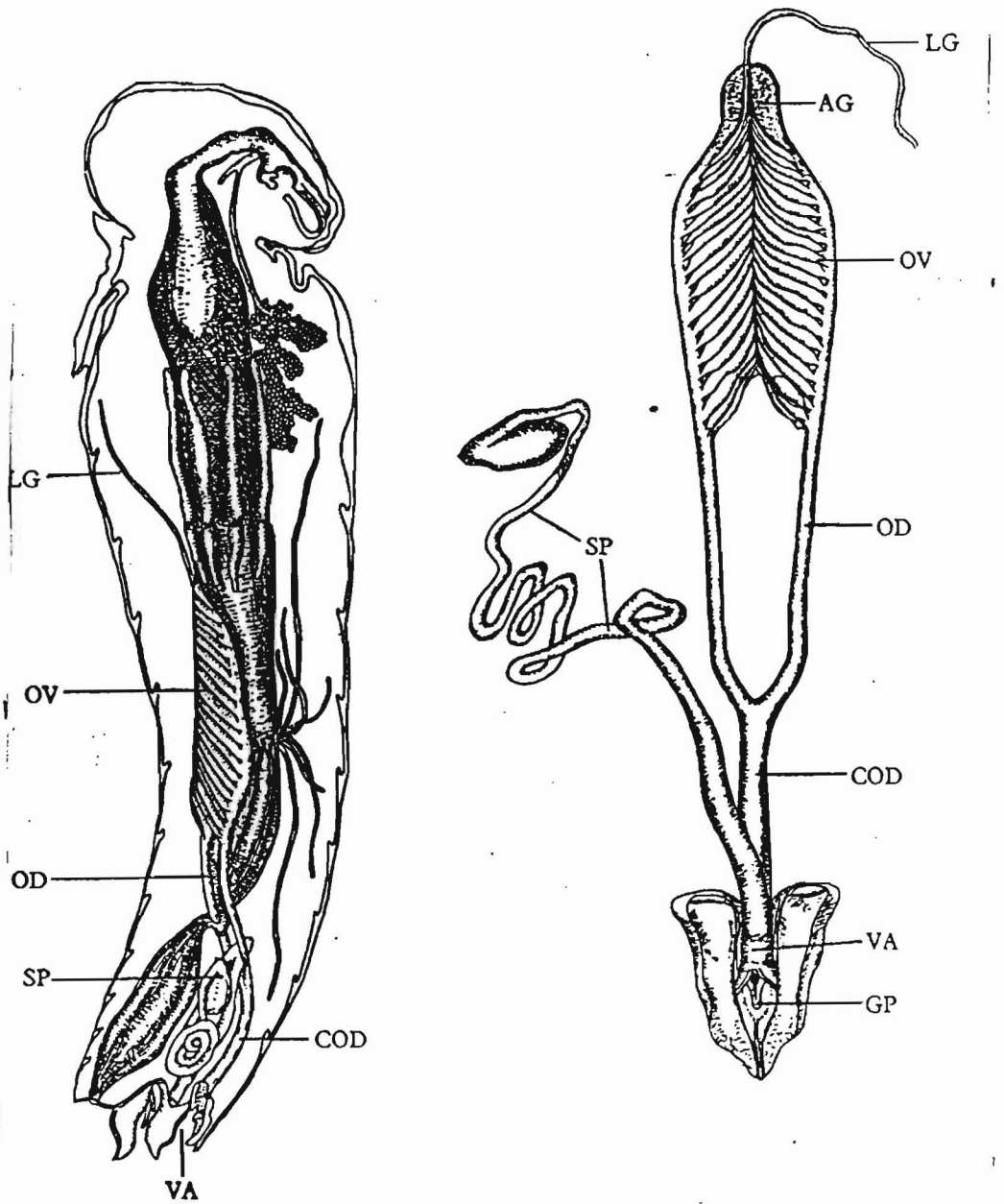


Fig. 1. Locusta, female reproductive organs (Stauffer & Whitman, 1997). LG, ligament; AG, accessory gland; OV, ovary; OD, oviduct; COD, commonoviduct; SP, spermatheca; VA, vagina; GP, gonopore

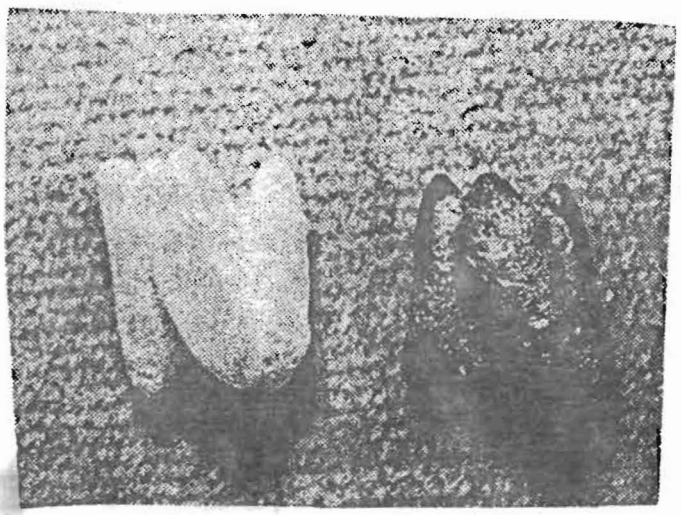


Fig. 2. Eggs from a gravid female. Eggs on the right were stirred in oviductal fluid containing CK gland secretions. Eggs on the lefte were stirred in the same oviductal fluid without the CK secretions (Eisner, 1966).

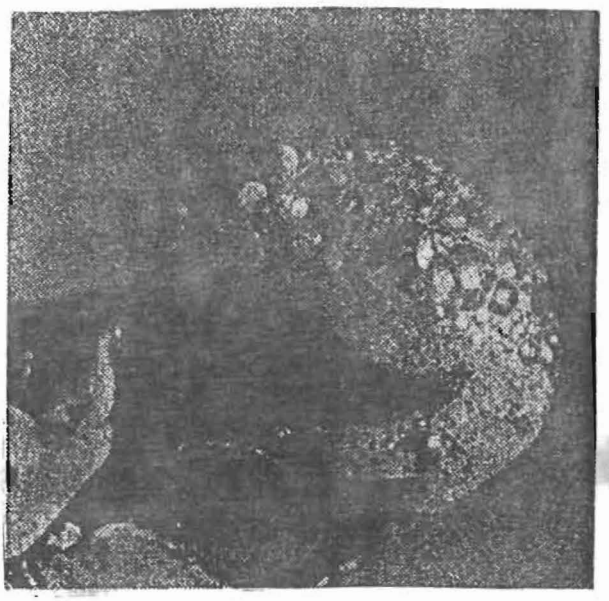


Fig. 3. Tip of the abdomen of a female grasshopper showing the frothy oviductal secretions produced by the movements of the ovipositor valves (Eisner, 1966).

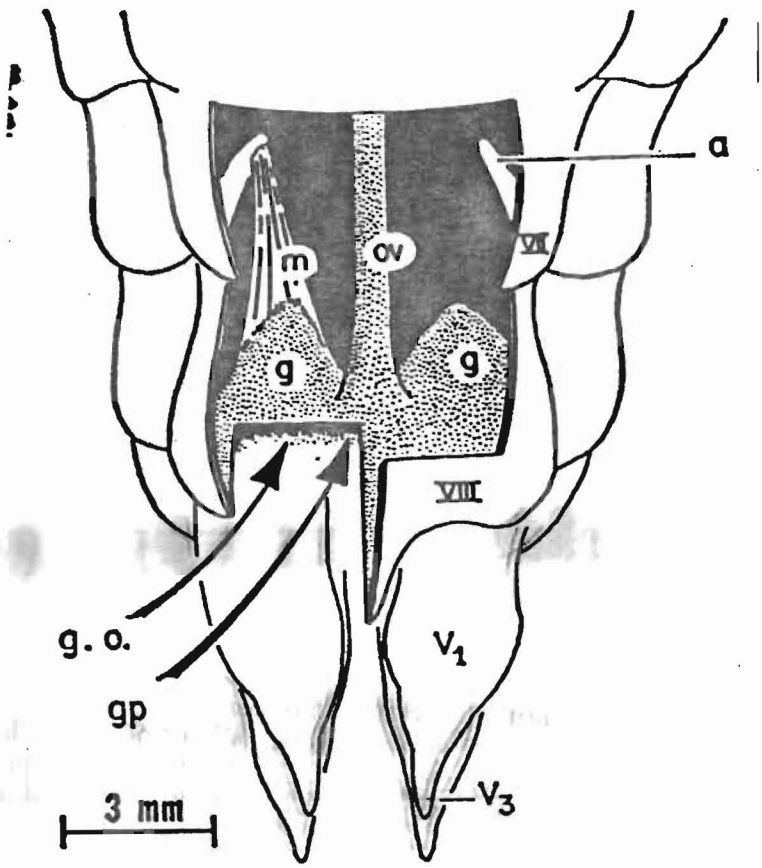
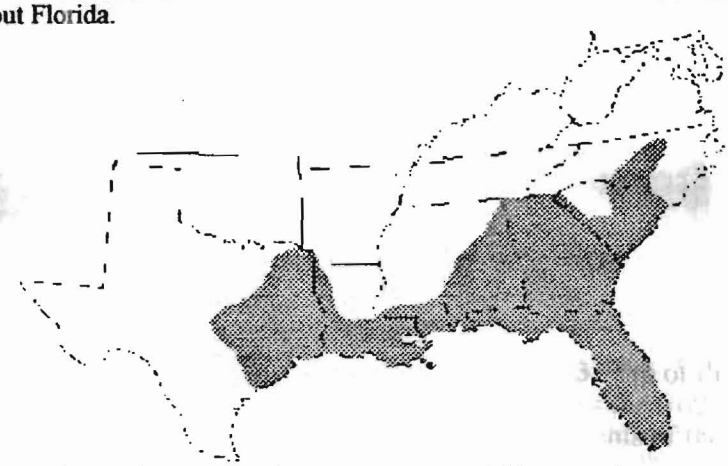


Fig. 4. Ventral view of tip of abdomen of female Romalea (Eisner, 1966). g, CK glands; g.o. CK gland opening; ov, median oviduct; m, muscle connecting the CK gland to a skeletal process (a); gp, opening to the gonopore through which the eggs emerge; roman numeral designate the ventral exoskeletal plates; V_1, first valves of the ovipositor; V_3, third valves of the ovipositor.

Fig. 5.

Distribution

The eastern lubber grasshopper is limited to the southeastern and south central portion of the United States. The northern boundary is central North Carolina west through southern Tennessee, Georgia, Alabama, Mississippi, Louisiana, Arkansas, to Texas. It occurs throughout Florida.



Distribution of *Romalea guttata*, eastern lubber grasshopper

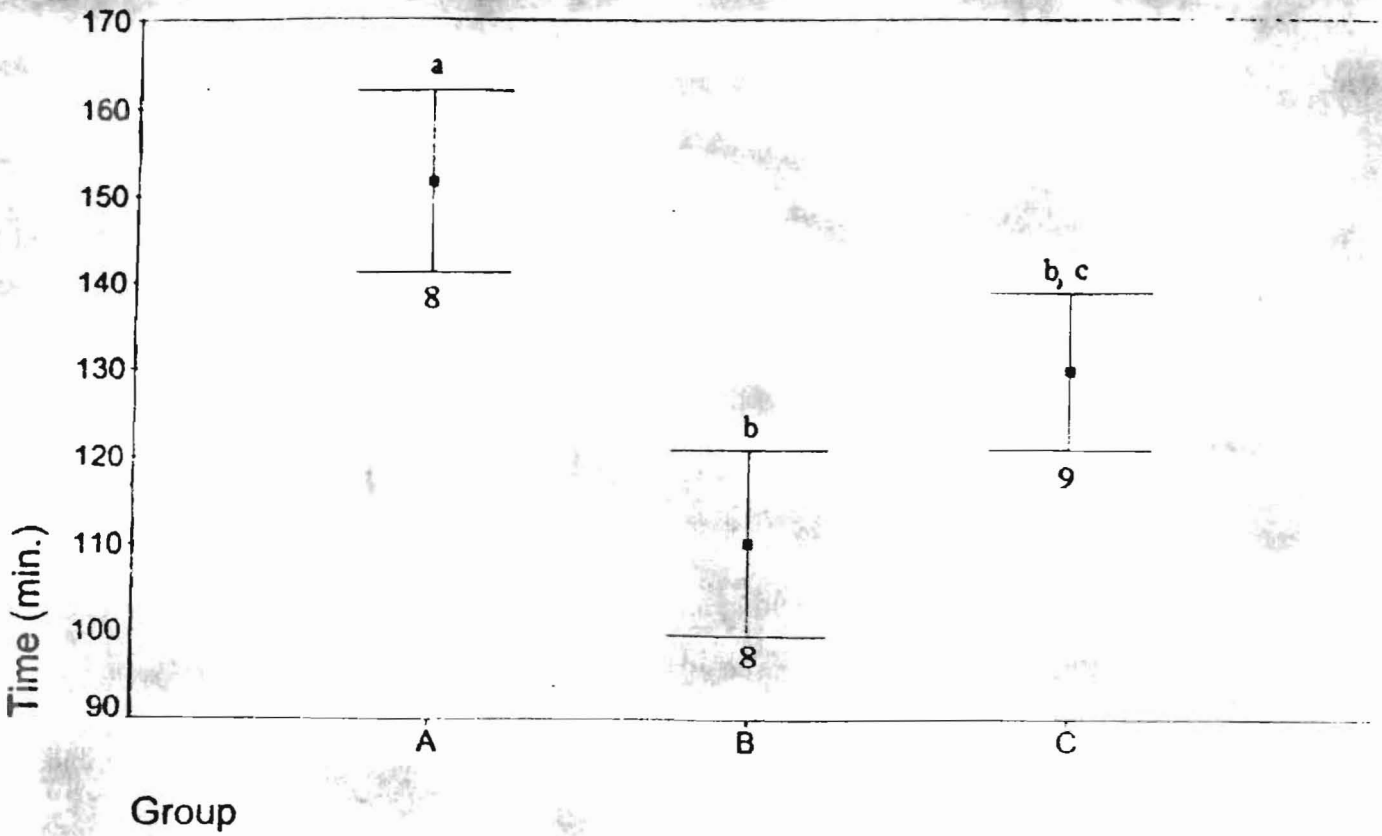


Fig. 7. Effect of surgery on egg tanning time. Bars represent x±2SE. A=removal of CK glands; B=sham surgery; C=unoperated. Means with different letters are significantly different at $p<0.05$. Numbers above bars represent sample size.

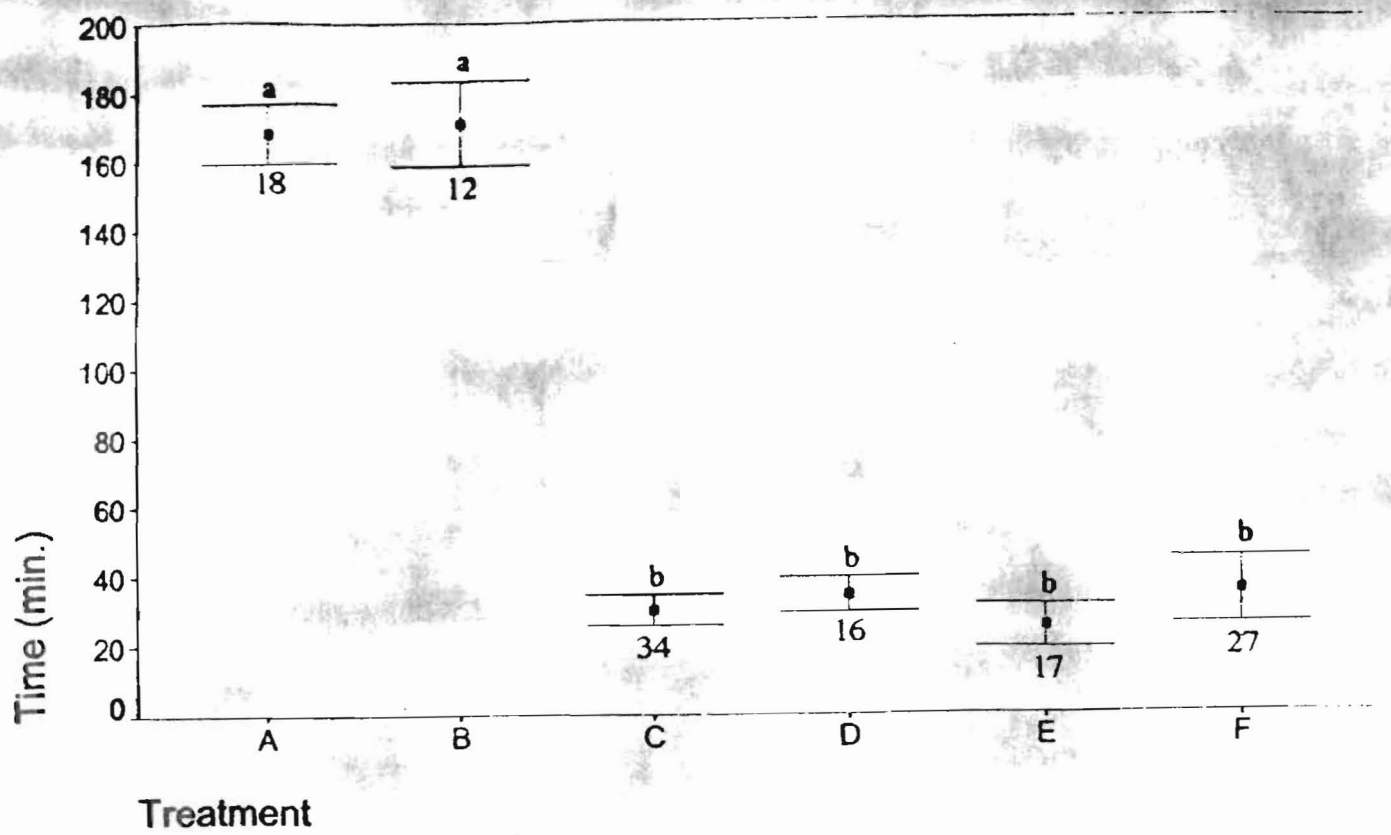


Fig. 8. Spotting times for eggs treated with different compounds. Bars represent $\bar{x}$±2SE. A=nothing added; B= deionized water; C=hemolymph; D=macerated intersegmental membrane; E=macerated CK gland; F=CK secretions. Means with different letters are significantly different at $p<0.05$. Numbers below bars represent sample size.

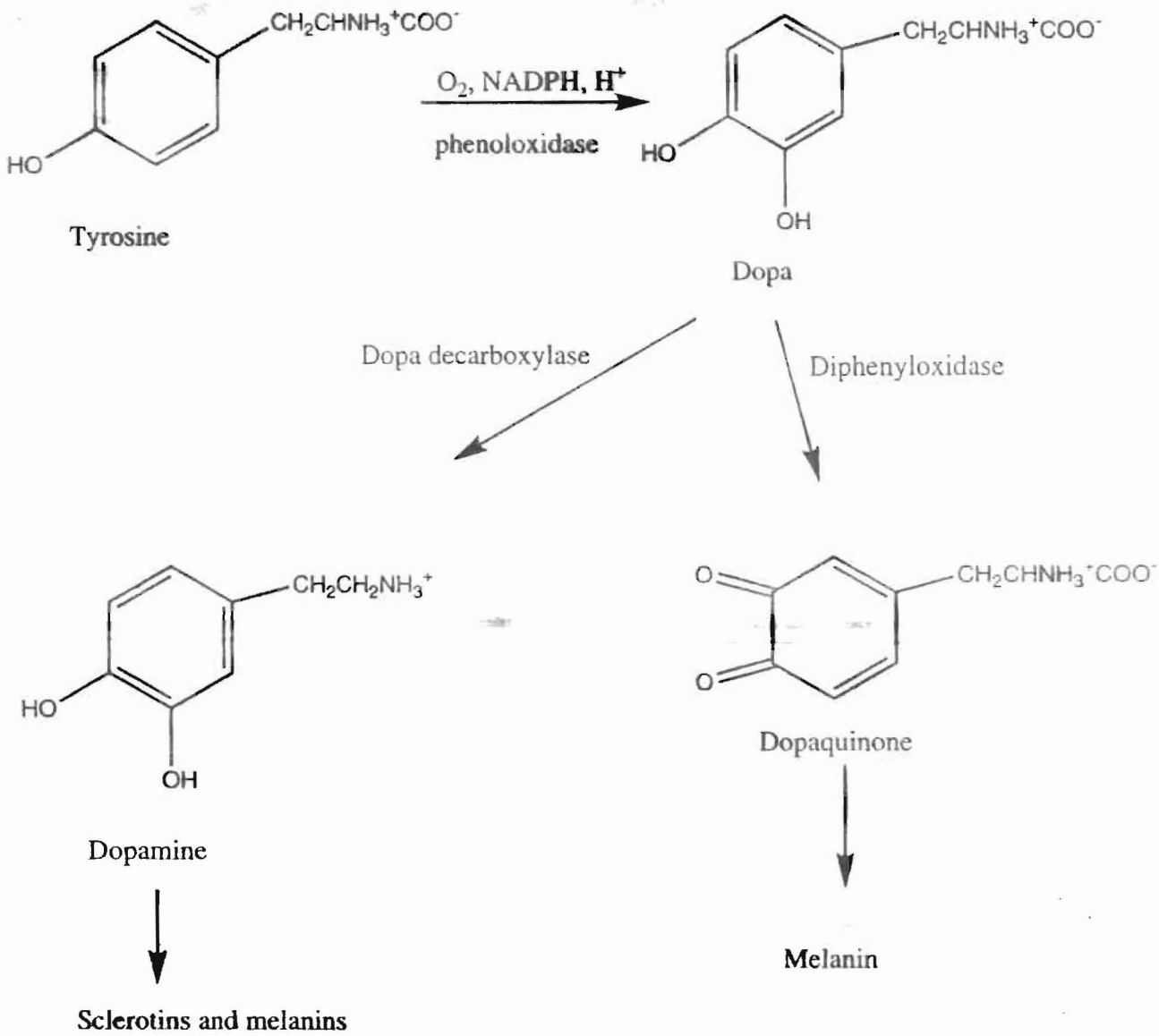


Fig. 9. Biochemical pathway for the production of melanins.