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Diet-Induced Nonmelanized Cuticle in Workers of the Imported Fire Ant *Solenopsis invicta* Buren

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Nonmelanized cuticle development was induced in workers of *Solenopsis invicta* by feeding them an insect-free diet. The nonmelanized workers weighed less and had smaller mean headwidths than workers from normal colonies. Although nonmelanized ant colonies appeared to function normally in the laboratory, their attempts at stinging were felt only as "pin pricks." Chemical analysis of venom alkaloids and cuticular hydrocarbons indicated no qualitative differences between nonmelanized and normal workers. Tyrosine, an essential amino acid tanning precursor, was found in adequate quantities in the free amino acid pool of nonmelanized ants. The specific cause of the nonmelanized condition is not known.

Key words: insect diets, worker ants, melanization, venom alkaloids, hydrocarbons

INTRODUCTION

Solenopsis invicta Buren, the red imported fire ant, became a pest ant species in the United States soon after its accidental introduction into Mobile, Alabama in the 1930s. It now infests over 230 million acres in nine southern states and Puerto Rico [1]. *S. invicta* occupies both rural and urban habitats, causing medical problems due to its potent sting and damaging several agricultural crops [2]. The need for applied and basic research on *S. invicta* has demanded techniques to successfully maintain colonies in the laboratory.

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We have reared *S. invicta* for over a decade and presently maintain several hundred colonies [3,4]. These laboratory colonies are used for a multitude of purposes such as studies on behavior, pheromones, nutrition, biocontrol, and pesticide screening. Although many improvements have been made in rearing techniques and diet, we have found that laboratory colonies are not as vigorous as field colonies in terms of worker and colony size and aggressiveness. During our efforts to improve colony vigor, we serendipitously discovered that an insect-free diet caused workers to have nonmelanized cuticle. Subsequently, we conducted the studies reported herein to: (1) verify that the nonmelanized condition could be consistently induced; (2) determine if the condition could be reversed with dietary modifications; and (3) compare the amino acids, cuticular hydrocarbons, and venom components of normal and nonmelanized ants. This paper presents the results of these tests.

MATERIALS AND METHODS

Dietary Studies

Queenright laboratory colonies (10,000 or more workers with queen and brood) reared from newly-mated queens collected in Gainesville, Florida were fed diets composed of hard-boiled chicken eggs, honey:water (1:1), and a third component of either: 1) raw ground beef (n = 5); 2) fried ground beef (n = 5); or 3) cockroaches (n = 3). The biweekly amounts of each dietary component fed to the colonies were eggs (3 g), honey:water (8 ml), and either beef (3 g) or cockroaches (2 g). All colonies were observed weekly for changes in cuticle development. Changes were readily apparent because of the striking differences in coloration of the workers (Fig. 1).

We also conducted tests to determine if the nonmelanized ant colonies would revert to normal colonies by placing 10 of the abnormal colonies on the standard diet with insects. Since the nonmelanized ants appeared smaller, we also randomly selected and evaluated ten of the largest and ten of the smallest workers from normal and nonmelanized ant colonies. The ants were arbitrarily designated as major and minor workers. They were weighed and their headwidth measured under a microscope using a micrometer disc.

A second study was initiated to determine if certain dietary supplements would compensate for the missing ingredients contained in the insects. Thirty mini-colonies consisting of 20 workers and 20 second- and third-instar larvae were formed from colonies on the insect-free diet. The mini-colonies were divided into five equivalent subsets: one subset was fed the nonmelanization diet of fried ground beef (0.5 g), hard-boiled chicken eggs (0.5 g), and honey:water (2 ml), while a second subset received a normal colony diet (with insects). The remaining three subsets were fed the nonmelanization diet with one of the following components added to the honey:water in the proportions indicated: Grace's Insect Tissue Culture Medium (containing 10% fetal bovine serum) (1:4), Yeastolate (1:20), and tyrosine (1:40). All mini-colonies were observed weekly for changes in cuticle development.

Fig. 1. The effects of an insect-free diet on workers of the imported fire ant, *Solenopsis invicta,* as indicated by the three nonmelanized workers (light-tan-colored) as compared to the normal workers (dark-colored).

Biochemical Studies

Free amino acids. Samples of fourth-instar worker larvae from normal *S. invicta* colonies and a sample of fourth-instar larvae from colonies producing only nonmelanized workers were weighed. They were then quick-killed with dry ice and immediately ground and extracted in 10% trichloroacetic acid. The extract was filtered and vacuum evaporated. The oily residue was triturated with hexane or diethyl ether to remove lipids, and the remaining material was dried under a stream of nitrogen. The sample was redissolved in a phosphate or citric acid buffer and the amino acids analyzed with a Beckman 120c Amino Acid Analyzer.

Cuticular hydrocarbons. Workers (100) were collected from each of three normal laboratory colonies and three nonmelanized ant colonies producing only nonmelanized workers. The ants from each colony were weighed and soaked in n-hexane (Baker Resi-Analyzed) for 7 min. The hexane was removed and the ants were quickly rinsed three additional times with hexane. A known amount of internal standard (n-pentacosane, Applied Science Laboratories) was added to the combined hexane washes and the solution evaporated under nitrogen to 100 μl. The concentrate was applied to a silicic acid Pasteur pipet (Biosil 325-mesh) column; the hydrocarbons were eluted

with hexane (7 ml) and the solution concentrated under a stream of nitrogen to about 100 μl. This solution was analyzed by gas chromatography (Varian 3700, flame ionization detector, 1.8 m × 2 mm i.d. glass column packed with 3% OV-17 on 120/140 mesh chromsorb W, Applied Sciences Laboratories, State College, PA isothermal at 220°C). The gas chromatograph peaks were qualitatively identified by direct comparison with standards and quantified using a Varian Vista CDS 401 data processor (Walnut Creek, CA).

Venom alkaloids. Three individual workers from each of three normal laboratory colonies and three nonmelanized ant colonies were weighed. Poison sacs were extirpated from these workers and pooled to provide three replicates of the two worker types. Poison sacs were immediately crushed in vials containing 100 μl of n-hexane (Baker, Resi-Analyzed); docosane (3 μl of a 0.1% hexane solution) was added as an internal standard. This solution was analyzed directly by gas chromatography (1.8 m × 2 mm i.d. glass column packed with Superpak 20M, Analabs, North Haven, CT, oven isothermal at 150°C for 20 min then to 200°C at 10°/min). The piperidine alkaloids were unambiguously identified by their GC properties and comparison with standards. They were quantified with a Varian Vista CDS 401 (Walnut Creek, CA) data processor.

RESULTS

Dietary Studies

Insects were confirmed as the food item that was directly related to the nonmelanized condition, since all colonies on insect-free diets produced transparent workers within 66 days. At the end of the test period (118 days), 99.9% of all workers raised on the insect-free diet were nonmelanized. The few remaining workers were majors and probably were holdovers from the original group of workers, since major workers can live more than 100 days [5]. The nonmelanized worker ants appeared normal except that they were a light tan color (Fig. 1) and, when observed under a microscope, their foregut, crop, and midgut were clearly visible through the cuticle. When touched with a probe, the cuticle was soft and appeared to be unsclerotized. The translucent cuticular condition was even more apparent when the ants were fed sugar solution or vegetable oil containing dyes. The pathway of these substances was easily traced from the mouthparts to the crop or midgut.

When nonmelanized colonies were fed a diet containing insects (cockroaches), all of the newly emerged workers had normal pigmented cuticle after approximately 55 days. The cuticle of nonmelanized workers did not change, indicating the condition was irreversible in adults. These results also have been confirmed in related studies in which we have been able to produce nonmelanized or melanized workers simply by omitting or including insects in the egg-hamburger-honey diet (unpublished data).

Influence of diet on worker size was apparent also in the body weight and headwidth measurements. Normal colony major workers were almost twice as heavy (3.62 ± 0.19 mg vs 1.88 ± 0.09 mg, n = 10; mean ± SE) and their headwidth was greater (1.4 ± 0.02 mm vs 1.0 ± 0.02 mm, n = 10); normal minor workers weighed more (0.68 ± 0.02 mg vs 0.42 ± 0.01 mg, n = 10)

but their headwidth was only slightly greater (0.71 ± 0.02 mg vs 0.62 ± 0.01 mg, n = 10).

The addition of Grace's Tissue Culture Medium, Yeastolate, or tyrosine to the honey-water in the diet at the concentrations used in this test did not prevent the larvae from developing into adults with nonmelanized cuticle. However, because the materials were offered ad libitum in the honey-water, we do not know how much was ingested. The diet with insects resulted in production of normally pigmented workers.

Biochemical Studies

Amino acids. Analysis of the free amino acid pool of nonmelanized versus normal *S. invicta* fourth-instar larvae showed no obvious abnormalities (Table 1). Of particular note was the fact that nonmelanized fourth-instar larvae had adequate amounts of tyrosine, an amino acid required for cuticular melanization.

Cuticular hydrocarbons. A comparison of cuticular hydrocarbons of normal and nonmelanized *S. invicta* workers (Table 2) showed that, qualitatively, nonmelanized workers have the same hydrocarbon pattern as that associated with normal *S. invicta* workers. The variation observed for individual components within a series of different colony replicates or between normal and nonmelanized colonies was typical of that found in previous studies (Vander Meer, unpublished data). Quantitative results indicated that when expressed on a ng/ant basis, nonmelanized ants had almost 2.5 times less cuticular hydrocarbon (121 ± 18.1 ng/ant vs 293 ± 6.2 ng/ant, n = 3; mean ± SE). However, when the weight of the ants is taken into consideration, we found the opposite relationship to be true. Nonmelanized workers had 3.3 times

TABLE 1. A Comparison of the Free Amino Acid Pools of Normal and "Nonmelanized" *S. invicta* Fourth-Instar Larvae

Amino Acid	Normal (mol/g)	Nonmelanized (mol/g)
Lysine	10.53	3.68
Histidine	6.83	3.92
Arginine	3.18	1.76
Aspartic Acid	0.15	6.68
Threonine	—	—
Serine	8.46	9.60
Glutamic Acid	6.02	8.60
Proline	12.75	25.72
Glycine	5.50	8.28
Alanine	8.35	3.72
Half Cysteine	—	—
Valine	4.73	10.00
Methionine	0.97	1.36
Isoleucine	1.73	2.48
Leucine	0.91	6.96
Tyrosine	2.19	6.16
Phenylalanine	4.85	4.52

TABLE 2. Comparison of the Percentages of the Five Major Cuticular Hydrocarbons of "Nonmelanized" and Normal *S. invicta* Workers

Component[a]	Percent component in ant type[b]	
	Normal	Nonmelanized
A	16.43 ± 1.53	14.63 ± 3.95
B	26.01 ± 0.03	28.14 ± 1.76
C	17.34 ± 0.62	21.13 ± 0.41
D	22.03 ± 1.62	15.64 ± 1.22
E	17.91 ± 3.93	17.03 ± 0.59

[a]A, n-heptacosane; B, 13-methylheptacosane; C, 13,15-dimethylheptacosane; D, 3-methylheptacosane; E, 3,9-dimethylheptacosane.
[b]Mean ± standard error (n = 3).

TABLE 3. Percentage of Venom Alkaloid Components From Normal and "Nonmelanized" *S. invicta* workers

Component[a]	Percent component in ant type[b]	
	Normal	Nonmelanized
C11:0	1.0 ± 0.3	1.8 ± 0.7
C13:1	13.6 ± 0.7	13.5 ± 0.9
C13:0	10.6 ± 6.5	21.3 ± 3.4
C15:1	30.6 ± 2.0	35.9 ± 4.7
C15:0	25.7 ± 5.4	26.5 ± 5.4

[a]Alkaloids are 6-methyl-2-alkyl or alkenyl-piperdines. The notation refers to the length and degree of unsaturation of the side chain at the 2-position [19].
[b]Mean ± standard error (n = 3).

more total cuticular hydrocarbon per mg of ant than their normal colony counterpart (698 ± 66.5 ng/mg vs 209 ± 0.88 ng/mg, n = 3; mean ± SE).

Venom alkaloids. Venom alkaloids were qualitatively identical in both normal and nonmelanized *S. invicta* workers (Table 3). There were, however, significant quantitative differences between the two ant types. Nonmelanized workers contained almost twice as much alkaloid per individual as normal workers (16.0 ± 6.3 μg vs 29.4 ± 0.5 μg, n = 3; mean ± SE). The same relationship was found when the total alkaloid was expressed as μg alkaloid per mg of ant (2.4 ± 1.1 μg/mg vs 7.4 ± 0.6 μg/mg, n = 3; mean ± SE).

DISCUSSION

The process of cuticular tanning (melanization), in general, is similar in most insects, including *S. invicta*. Although numerous diphenols have been implicated in the tanning process, N-acetyldopamine quinone is the major tanning agent in insects of all stages, while bursicon, a proteinaceous neurohormone, controls tanning of the cuticle [6–8]. It is apparent from our studies that *S. invicta* diets that lack insects do not provide a necessary component(s) for this normal process of melanization and sclerotization. When meat, eggs,

honey-water, and Grace's Tissue Culture Medium [9], which contained numerous vitamins, amino acids, carbohydrates, and inorganic salts, were provided to the colonies, the result was the same: all workers produced had nonmelanized cuticles. There were other differences besides cuticular tanning observed between those colonies maintained on the standard diet and on the insect-free diet. The transparent workers weighed less and had narrower headwidths, and, although they reacted similarly to normal workers when disturbed, their attempts at stinging were felt only as "pin pricks." Apparently, their stinger was unable to penetrate the skin to inject the venom.

As a first attempt at deciphering the cause of the nonmelanized cuticle phenomenon, we determined the level of the amino acid tyrosine in fourth-instar larvae, since it is a precursor for the formation of melanin [6–8]. The results (Table 1) clearly showed that lack of melanization was not due to low levels of tyrosine in the diet, nor to the insects' inability to absorb tyrosine from their diet.

The species specific hydrocarbons associated with *S. invicta* are ubiquitous to the species and can be found associated with virtually all developmental stages and tissues [10]. They constitute over 70% of the total cuticular lipids of *S. invicta* and have been identified as a series of normal, monomethyl, and dimethyl alkanes [11,12]. They probably play a role in prevention of water loss through the cuticle, in addition to being implicated in species and nestmate recognition [13–14]. The hydrocarbon patterns of four *Solenopsis* species found in the United States are unambiguously different (Vander Meer, unpublished data) and have been useful chemotaxonomic tools [15]. When we compared this class of compounds in normal and nonmelanized *S. invicta* workers, we found that qualitatively the hydrocarbon pattern was the same in both groups (Table 2), and the quantitative variation in peak percentages was not different from variation previously observed (Vander Meer, personal communication). The quantitative results for total hydrocarbon confirm that on a per ant basis, nonmelanized workers have less hydrocarbon than normal workers; however, when the size of the ant is considered, the nonmelanized workers have more hydrocarbon per mg of ant. This is a consequence of increased cuticular surface area for a given weight of ant. Thus, cuticular hydrocarbons apparently are not affected by the lack of cuticle melanization.

It has been demonstrated that the venom alkaloids and the sting apparatus, which is lined externally with cuticle, have a wide variety of functions [16–17]; malfunction of any part of the sting apparatus would compromise the ant's defense, food procurement, and pheromonal communication. Our analysis of the well-characterized venom alkaloids [18] showed no qualitative differences between nonmelanized workers and normal *S. invicta* workers (Table 3). Suprisingly, when the quantity of alkaloid was expressed on a per ant or per weight of ant basis, the nonmelanized *S. invicta* worker had 1.6 to 3 times as much material. So, without question, nonmelanized ants successfully produce and store the toxic piperidine alkaloids. However, the fact that their sting apparatus is not melanized nor hardened makes it highly unlikely that these colonies could survive the competitive rigors of field conditions.

In summary, *S. invicta* colonies fed a diet free of insects developed small, nonmelanized workers that were incapable of stinging. When these colonies were returned to a diet with insects, new melanized workers were observed within 55 days. Chemical analyses of venom alkaloids, cuticular hydrocarbons, and the availability of the amino acid tyrosine, an essential tanning precursor, did not show any abnormalities related to these components. At present, we do not know the specific cause of this phenomenon. Apparently, the ants sequester something from dietary insects that is required for their normal growth and cuticular development. Future studies will address the other areas of the melanization and sclerotization mechanisms in an effort to understand this unique phenomenon and perhaps ultimately use the knowledge in controlling fire ants.

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