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Pigmentation Restored in Mutant Laboratory Strain of the Lady Beetle *Coleomegilla maculata* through Dietary Supplementation

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Abstract

A laboratory colony of *Coleomegilla maculata* (DeGeer), *ye*, selected for a pigmentation deficiency, was restored to near wild type cuticle coloration by adding crushed heads and wings of the red colored parental strain to the diet. While the wings and other colored portions of the cuticle regained the red color, the eyes of the pigmentation deficient insects were not changed from the pale mutant form. Plant derived carotenes lycopene and beta-carotene did not restore the mutant beetles to a visibly distinguishable red color. An additional pigmentation deficient mutant strain, *gold*, partially recovered red cuticle color when provided with diet containing pigmented insect particles. This work represents the first rescue of a color phenotype in a lady beetle.

Keywords

1. Introduction

Many species of lady beetles (Coleoptera: Coccinellidae) are characterized by bright red or yellow coloration, often with dark patterning. The red colors of lady beetles are generally accepted to be carotenoid pigments. The species *Coleomegilla maculata* (DeGeer) (Coleoptera: Coccinellidae) is commonly called the pink lady beetle or the twelve-spotted lady beetle. *C. maculata* is a beneficial omnivorous beetle ubiquitous in North American

agroecosystems. The name "pink lady beetle" describes the typical color hues attributed to *C. maculata*: dark spots on a lighter background that is pink to red or orange.

A stable homozygous strain of beetles with pale yellow coloration in the cuticle, *ye*, was selected from specimens collected in Mississippi [1] and established as a robust continuous laboratory colony. The *ye* strain also had no visibly apparent pigments in the eyes. The mutation resulting in the phenotype was inherited as a single autosomal allele, and other than the distinctive coloration the mutant insect colony was apparently healthy [1]. An additional phenotype appearing nearly as pale in the colored portion of the cuticle, but maintaining dark eye coloration similar to the wild type, was observed and selected for over 30 generations. The second selected stable homozygous strain was named *gold* [2]. This strain was based on inheritance of two alleles that were both autosomal, and apparently independent of the *ye* allele. The *gold* insects were less fit as indicated by smaller size/mass and lower egg production compared to wild type and *ye* beetles [2]. Establishment of the two distinct genetic strains apparently lacking red pigments as heritable characters suggested that the red color was enzymatically controlled, and that critical steps in the production, packaging, or transport of the red pigment were disrupted by genetic malfunction in the naturally occurring mutant alleles present in the *ye* and *gold* strains.

To begin the process of clarifying the nature of the *ye* and *gold* mutant strains, I designed experiments incorporating candidate compounds into insect diet. Digital images of the adult mutant insects fed these diets were collected, and digital color signatures of the resulting pigmentation characteristics were analyzed. These rescue experiments are the first efforts of their kind performed using a beetle from the family Coccinellidae.

2. Methods

Insects were reared as described previously [3], and maintained in growth chambers set for 14 h/10 h photophase/scotophase lighting. Temperatures were 24°C during photophase and 21°C during scotophase. Individuals used for experiments were kept isolated in small dishes (100 mm D × 26 mm H) with fine mesh glued into the lid to permit ventilation. Live pupae were weighed individually using a Sartorius CP2P-F analytical balance, and those with a mass between 15 - 20 mg were used for adult experiments.

Digital images were collected using a Nikon digital camera, DMX 1200, with factory supplied ACT-1 software. The camera was mounted on a Nikon Stereomicroscope SMZ1500 (Nikon Corporation, Tokyo, Japan) with aperture fully closed to provide maximum depth of field. Shutter speeds varied from 1/20 to 1/100 sec depending on magnification. Subjects were illuminated by a high intensity fiber optic illuminator, Nikon model NI-150 with dual gooseneck light guides from two opposing sides to provide consistent color readings. Specimens were rotated to collect images that could be sampled for color analysis. Sections of cuticle that were neither in shadow nor reflecting from the light source were selected for color analysis. Images used to analyze cuticle color were cropped to include only the pink or yellow sections of the elytra (forewings) and converted to .jpg files then analyzed using RGB software [4]. Four sections of each sampled individual whole insect were used for color analysis, a single insect was considered a replicate for statistical analysis. Color samples were cropped from colored areas between the melanized spots on the elytra.

Experimental diets were prepared from ground bee pollen, Brewers yeast, *Ephestia* eggs or *Lygus* eggs, and supplemented with 10% w/v one of the following: lycopene, beta-carotene, riboflavin (vitamin B2), folic acid, or pigmented particles of wild type *C. maculata* mature adults. The concentrations of the dietary supplements were as formulated for human consumption; the lycopene was collected from 30mg gel caps, the beta-carotene was collected from 15mg gel caps, the riboflavin was crushed 100mg tablets, and the folic acid was crushed 400ug tablets (General Nutrition Center, Pittsburg, PA, USA). Diet without supplementation (standard diet) was used as negative control. In a separate experiment, l-kynurenine (MP Biomedicals, LLC, Solon, OH USA) was incorporated into diet at a concentration of 100 µg/ml. Colored cuticle particles were prepared by removing wings and heads from fresh red (wild type) beetles and grinding them with a mortar and pestle. A preliminary experiment using 10% w/v crushed head and wings in the diet restored a visually distinct pink color to *ye* insects when fed to either larvae or newly eclosed adults. Because this concentration was effective in rescuing the color phenotype, and the preliminary test subjects survived the diet treatment, other diet treatments were supplemented at 10% w/v.

Data were analyzed by analysis of variance (ANOVA) using SigmaPlot, version 12 software (SSI, San Jose, CA USA). Without transformation data passed normality testing using Shapiro-Wilk or Kolmogorov-Smirnov methods. The Holm-Sidak method using multiple comparison versus control was used for six diet studies; the

standard diet without any supplementation fed to *ye* strain insects was used as the control group. For the experiment testing rescue of the gold strain, the Holm-Sidak multiple comparison was implemented without specifying a control group (all pairwise).

3. Results

Based on the hypothesis that the *ye* phenotype was derived from lack of a specific pink pigment, a diet including the pigment should result in restoration of the pink (wild type) color. If, on the other hand, the phenotype was due to lack of a pigment precursor in the laboratory diet, providing sufficient precursor in the diet should restore the color. In a preliminary pilot experiment, particles of wild type (wt) insect wings and heads, the red colored parts of the adult insects, were incorporated into insect diet, and fed to *ye* larvae. When the larvae metamorphosed into adults, the colored portions of the cuticle were visibly pink. In a second pilot experiment, kynurenine was incorporated into diet fed to *ye* larvae, resulting in no visually apparent change in phenotype (data not shown). Controlled experiments were performed incorporating readily available dietary supplements, including carotenoid compounds, into insect diet, and providing that diet to insects either after adult eclosion or throughout the juvenile stages. Negative control was a standard diet fed to *ye* insects, and positive control was the diet incorporating wt red insect particles (rescue diet) fed to *ye* insects. Visual examination confirmed that a pink color was recovered from the rescue diet in both feeding experiments. The empirically visible characteristics were supported by color analysis. Digital images of representative specimens of the experimental insects, *ye* strain provided with the six diets after adult eclosion, are shown in **Figure 1**. The adults are aged 25 days post imaginal ecdysis. The experimental pigmentation, ranging from pale to dark yellow or pink in the case of the adults fed the rescue diet is shown diagrammatically, with a diagrammatic representation of the wild type red color included for reference. As shown in **Table 1**, digital color analyses further clarified that red (t = 6.276, *p* < 0.001) and green (t = 7.163, *p* < 0.001) color components in the group fed rescue diet were significantly different from control in the experiment using adults (Holm-Sidak method using multiple comparison versus control, df = 5; standard diet without any supplementation was used as control group). In the experiment using juveniles, all color components in the group fed rescue diet were significantly different from the *ye* control specimens fed a standard diet: red (t = 10.797, *p* < 0.001), green (t = 8.778, *p* < 0.001), and blue (t = 5.418, *p* < 0.001). Specimens fed diet supplemented with plant derived carotenes, lycopene and *β*-carotene, did not recover a pink color

Diet supplements:
1-Lycopene
2-Negative control
3-Folic Acid
4-Vitamin B2 (Riboflavin)
5-wild type (wt) Insect Parts
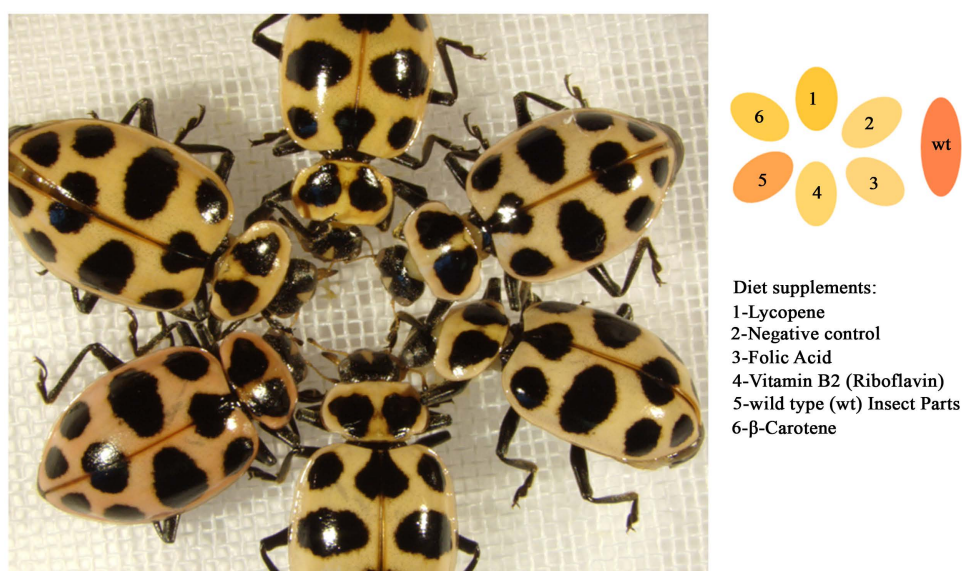6-β-Carotene

Figure 1. Representative specimens of the yellow (*ye*) strain of *Coleomegilla maculata* fed diet supplemented with five materials, an unsupplemented diet (negative control). Specimens were fed experimental diets immediately and continuously after adult eclosion (aae); image was collected 25 days (aae). Legend to the right shows the numbering of the specimens arranges as in the image, with color backgrounds matching the color percentages of the samples as measured, and adjusted to color saturation. The ellipse to the right labeled wt represents the average saturated color of the wild type (wt) strain.

Table 1. Color components (red, green, blue) of colored samples of elytra from specimens of *Coleomegilla maculata*, strain *ye*, provided with supplemented diets. An asterisk (*) indicates significantly different from control. Values are percentages plus or minus standard error.

	Fed rescue diet after adult emergence				Fed rescue diet after egg hatch			
Supplement	Percent red	Percent green	Percent blue	Sample size	Percent red	Percent green	Percent blue	Sample size
Control	43.114 ± 0.491	35.657 ± 0.109	21.243 ± 0.483	7	43.350 ± 0.406	36.300 ± 0.087	20.333 ± 0.485	12
Folic Acid	42.757 ± 0.285	35.957 ± 0.102	21.286 ± 0.360	7	43.167 ± 0.426	36.044 ± 0.128	20.789 ± 0.519	9
Riboflavin	43.400 ± 0.586	36.300 ± 0.156	20.314 ± 0.707	7	44.171 ± 0.814	37.114 ± 0.243*	18.700 ± 1.056	7
Lycopene	46.586 ± 0.280*	36.671 ± 0.278*	16.771 ± 0.403*	7	50.063 ± 0.544*	38.9975 ± 0.208*	10.925 ± 0.692*	8
β-Carotene	45.300 ± 0.575*	36.571 ± 0.274*	18.143 ± 0.792*	7	47.555 ± 0.589*	38.391 ± 0.277*	14.055 ± 0.715*	11
WT elytra	46.543 ± 0.690*	33.714 ± 0.191*	19.757 ± 0.528	7	51.245 ± 0.619*	33.000 ± 0.197*	15.736 ± 0.561*	11

by visual inspection, but did appear deeper yellow (**Figure 1**). Color components were significantly different (**Table 1**). Adult insects fed diet supplemented with *β*-carotene had significantly higher red percentage (t = 3.741, p = 0.003), significantly higher green percentage (t = 3.265, p = 0.010), and significantly lower blue percentage (t = 3.894, p = 0.003), while insects fed the *β*-carotene diet as juveniles had significantly different color values in all components: red (t = 5.750, p < 0.001), green (t = 13.645, p < 0.001), and blue (t = 7.401, p < 0.001). Adult insects fed diet supplemented with lycopene had significantly different color values in all components: red (t = 6.024, p < 0.001), green (t = 3.344, p = 0.011), and blue (t = 5.545, p < 0.001). Insects fed the lycopene diet as juveniles also had significantly different color values in all components: red (t = 8.395, p < 0.001), green (t = 9.887, p < 0.001), and blue (t = 10.142, p < 0.001). Mutant *ye* insects fed diet supplemented with riboflavin as juveniles differed significantly in the green color component (t = 2.867, p = 0.019), but other color components were not significantly different from the *ye* control; *ye* insects fed diet supplemented with riboflavin as adults and those fed diet supplemented with folic acid as both adults and juveniles did not have differences in color (t < 1.00, p > 0.05). A diagrammatic representation of the colors exhibited by the *ye* insects fed supplemented diet throughout their juvenile stages is presented as **Figure 2**.

An additional experiment comparing rescue using both pigmentation defective strains, *ye* and *gold*, is illustrated in **Figure 3**, and color analysis results are shown in **Table 2**. The two mutant strain colors did not differ in any of the three color components when fed standard diet, but the colors of the specimens fed the rescue diet differed significantly in all components with the exception of the blue component for the rescued *gold* strain. All color components, red (F = 21.101, df = 3, p < 0.001), green (F = 97.184, df = 3, p < 0.001), and blue (F = 8.305, df = 3, p = 0.003) varied significantly among the treatment groups.

No restoration of color in the eyes of *ye* adults was ever visually apparent, and color analyses were not performed. An expanded view of the heads, with eyes indicated by arrows, is provided in **Figure 3(b)** (*ye* strain) and **Figure 3(c)** (*gold* strain).

4. Discussion

Research focusing on insect pigments has provided insight into basic genetics, cell biology, and biochemistry. While most foundational research has utilized the model insect *Drosophila melanogaster* [5], research on insect carotenoid metabolism has been performed in the silk moth *Bombyx mori* [6]. While many lady beetles are notable for brilliant red pigmentation, the biochemistry and molecular genetics of these pigments are largely unknown. The red colors of the elytra of the lady beetle *Coccinella septempunctata* are attributed to numerous forms of carotenoids including lycopene and *β*-carotene [7]. The inheritance, biochemistry, and molecular genetics of insect pigmentation have been an active and vital area of study for over a century. Basic genetic inheritance and sex linkage were first demonstrated in *D. melanogaster* based on eye colors. Since then hundreds of color mutations have been studied in many organisms including humans. By crossing selected mutant strains of *D. melanogaster*, the functions of specific pigments in the fly photoreceptor system were deduced [8]. The specific steps of the enzymatic pathways resulting in white, red, orange, and brown eye color mutant phenotypes in
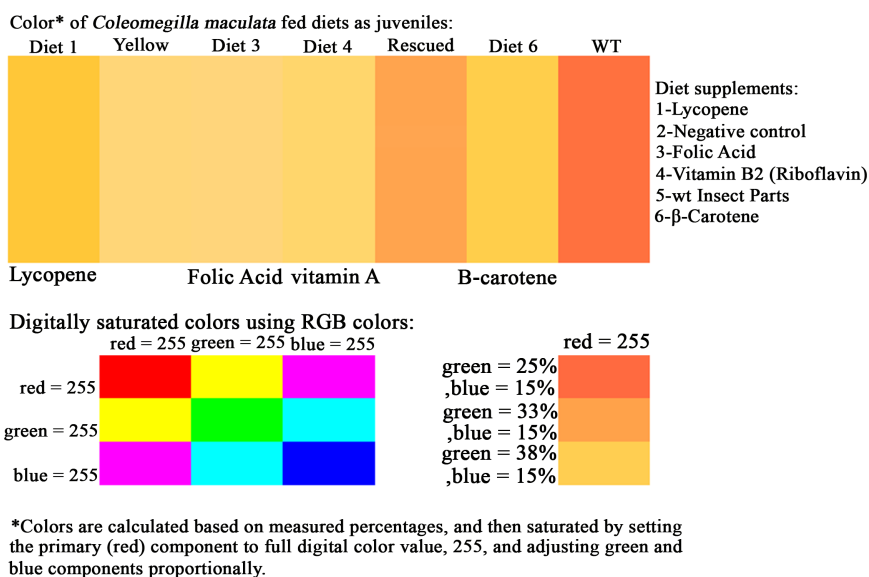
Color* of *Coleomegilla maculata* fed diets as juveniles:

Diet 1   Yellow   Diet 3   Diet 4   Rescued   Diet 6   WT

Diet supplements:
1-Lycopene
2-Negative control
3-Folic Acid
4-Vitamin B2 (Riboflavin)
5-wt Insect Parts
6-β-Carotene

Lycopene   Folic Acid   vitamin A   B-carotene

Digitally saturated colors using RGB colors:
red = 255 green = 255 blue = 255

red = 255

red = 255

green = 255

blue = 255

red = 255
green = 25%
,blue = 15%
green = 33%
,blue = 15%
green = 38%
,blue = 15%

*Colors are calculated based on measured percentages, and then saturated by setting the primary (red) component to full digital color value, 255, and adjusting green and blue components proportionally.

Figure 2. Measured mean colors of ye strain *C. maculata* fed experimental diets throughout juvenile development, and continued as adults. Color bars are digitally adjusted to saturated color as described in text*. Saturated color bars are provided for comparison below and to the left; samples of colors incorporating scaled green components for comparison are shown below and to the right.
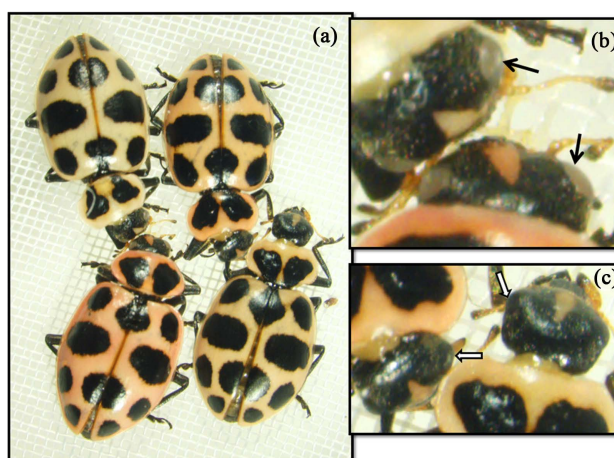

Figure 3. Representative specimens of *ye* and *gold* strain *C. maculata* fed diet supplemented with or without rescue diet that included crushed particles of wild type red pigmented cuticle, primarily elytra and heads. (a) Clockwise from upper left: *ye* control, *gold* rescue, *gold* control, *ye* rescue; (b) Heads of *ye* insects, control above, rescue below. Arrows indicate compound eyes lacking pigment; (c) Heads of *gold* insects, control right, rescue left. Arrows indicate pigmented compound eyes.

Table 2. Color components (red, green, blue) of colored image samples of elytra from specimens of *ye* and *gold* strains *Coleomegilla maculata* provided with standard diet or diet supplemented with particles of wild type red pigmented cuticle (rescue diet). Identical letters indicate values not significantly different; alphabetical order of letters indicates difference from *ye* standard diet value. Values are percentages plus or minus standard error.

Treatment (strain diet)	Percent red	Percent green	Percent blue	Sample size
ye standard	36.475 ± 0.525 A	35.100 ± 0.041 a	28.450 ± 0.548 a	4
gold standard	38.100 ± 0.196 AB	34.750 ± 0.065 a	27.125 ± 0.266 a	4
ye rescue	42.325 ± 0.461 C	32.575 ± 0.131 c	25.100 ± 0.739 b	4
gold rescue	39.700 ± 0.807 B	33.875 ± 0.170 b	26.400 ± 0.660 ab	4

D. melanogaster have been characterized biochemically [9] and through molecular genetic methods [10]. Many steps of the biochemical synthesis pathways for pigments in many other insects have been identified, and more are being found and characterized regularly. For example, a mutant strain of the silk moth *Bombyx mori* characterized by abnormal pigmentation in the eyes and eggs was used to identify a novel function of a major facilitator transporter gene thought to catalyze a terminal step in pigment biosynthesis [11]. A robust transcriptome analysis of two colorful spiders provided a fundamental set of pigment related genes in non-insect arthropods [12].

Insect pigments may be categorized chemically as ommochromes or pteridines, which are usually associated with eye color; melanins which are associated with dark or black cuticle; and carotenes in some lady beetle wings [7]. In the beetle genetic model organism *Tribolium castaneum*, several pigment genes that are orthologs to characterized *D. melanogaster* genes have been identified, including eye color pigments that result in red or loss of red in the mutant fly phenotype [13]. Interestingly, loss of function experiments in beetles using genes encoding tryptophan-to-ommochrome pathway enzymes resulted in white eyed beetles [14]. Further studies using ATP-binding cassette (ABC) transmembrane transporter genes in *T. castaneum* also resulted in loss of eye pigments rather than red or pink eyes, supporting the conclusion that eye pigments in this beetle were composed only of ommochromes [13]. Enzymes resulting in cuticle darkening and hardening have been characterized in *T. castaneum*, and a black beetle phenotype similar to the spontaneous mutant strain *ebony* was produced by gene suppression [15].

Phenotype rescue experiments are crucial tools in genetic discovery. In genetic model organisms, rescue is most often performed by providing a protein coding construct that restores a genetic deficiency. For example, the *D. melanogaster* gene encoding the enzyme kunurenine hydroxylase, *cinnabar* (*cn*) was injected as a plasmid into a white eyed mutant strain of the mosquito *Aedes aegypti*, and resulted in partial restoration of eye color. The mosquito eye color was also restored by providing 3-hydroxykynurnine, an ommochrome eye color precursor, to juveniles [16]. A concentration of 100µg/ml kynurenine preparations was sufficient to rescue eye color in *D. melanogaster* with null mutations in genes of the ommochrome biosynthetic pathway [16], but this concentration of l-kynurenine did not affect *ye* mutants when provided in the diet. This suggests that the tryptophan oxygenase gene is probably not responsible for the *ye* phenotype, and that eye and wing colors in lady beetles may not be ommochrome pigments. Prior to gene sequencing, transplantation of larval eye discs into *D. melanogaster* eye color mutant strains proved the gene to enzyme correlation [17].

The experiments presented here show that the colors diffused throughout the colored portion of the *C. maculata* cuticle can be influenced by diet. The compounds selected as dietary supplements were two plant derived carotenoids, lycopene and *β*-carotene, and two B vitamins, riboflavin and folic acid. Lycopene is a red pigment responsible for red colors of fruits and vegetables, and *β*-carotene is an orange pigment. Both riboflavin and folic acid are yellow compounds. Carotenoids provided in the form of lycopene and *β*-carotene changed the character of the elytra color; the increases in the red and green components of the red/green/blue (rgb) spectrum resulted in a color shift toward more vibrant yellow (**Figure 1** and **Figure 2**). However, only the specific compound provided by particles of red pigment from the wt adult lady beetles restored red to the elytra, characterized by a decreased green component of the rgb spectrum (**Table 1** and **Table 2**, **Figures 1-3**). Thus the red pigment in the cuticle of *C. maculata* is a compound other than lycopene or *β*-carotene. The nature and mechanisms for the synthesis and deposition of the lady beetle pigment are still unknown, but the pigment is clearly able to diffuse to the cuticle but not the eyes.

There is not yet a genetic model species from the family Coccinellidae. Selected laboratory strains with visible phenotypic markers are excellent tools for gene discovery through forward genetics. The *ye* strain of *C. maculata*, a distinctly visible phenotype, is well suited for research on novel genes involved in carotenoid biosynthesis pathways. While the *gold* strain confirmed the rescue results tested in the *ye* strain, the delicate nature of the gold insects prevented full replication of the diet supplementation studies performed in the *ye* strain. Sixteen carotenoid compounds have been specifically described from one species of lady beetle, *Coccinella septempunctata* (http://carotenoiddb.jp/ORGANISMS/Coccinella_septempunctata.html, accessed 25 February 2016) [18], but the identity of many forms remains unknown [12]. Recently, a gene encoding carotene synthesis was identified in the pea aphid [19], and using sequence comparison to that gene a similar gene was discovered in two-spotted spider mite, *Tetranychus urticae* [20]. The genes encoding the biosynthetic or transport pathway or pathways for coccinellid pigments are completely unknown.

Clearly there is a genetic mechanism in *C. maculata* that converts a precursor to a unique red pigment that is

then distributed to the forewings, or elytra, of the adult insect. The form and function of this pigment in the eye remains a compelling unanswered question.

Acknowledgements

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