

The basis for colorless hemolymph and cocoons in the *Y*-gene recessive *Bombyx mori* mutants: a defect in the cellular uptake of carotenoids

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Abstract

Bombyx mori is an excellent model for the study of carotenoid-binding proteins (CBP). In previous papers, we identified and molecularly characterized a CBP from the *Y*-gene dominant mutants. In the present study, we attempted to correlate and establish lipid metabolism and distribution in these mutants. When [³H]-triolein was fed to the mutants, typical patterns of uptake of labeled fatty acids from midgut to hemolymph and subsequent delivery to fat body and silk glands were obtained in all mutants. Further analysis of lipid and carotenoid profiles revealed that the yellow coloration in the hemolymph associated with lipophorin is not attributed to a difference in lipophorin concentrations among the mutants, nor to its lipid composition, but rather to its carotenoid content. Lipophorin of the *Y*⁺*I* mutant exhibited the highest concentration of total carotenoids of 55.8 μg/mg lipophorin compared to 3.1 μg/mg in the +*Y*⁺*I* mutant, 1.2 μg/mg in the *YI* mutant and 0.5 μg/mg in the +*YI* mutant. Characteristic retention time in HPLC of the different classes of carotenoids of lipophorin identified the presence of lutein as the major chromophore (62–77%), followed by β-carotenes (22–38%). Although lutein and β-carotene content of mutants' lipophorin differed significantly, the ratio of lutein to β-carotene of 3:1 was not different among mutants. Similarly, lipid compositions of mutant silk glands were not significantly different, but carotenoid contents were. The significantly high concentration of lutein in the *Y*⁺*I* mutant silk gland represented more than 160-fold increase compared to +*Y*⁺*I* mutant (*p* < 0.001). In this report, we conclude that lipid metabolism in the mutants is not defected and that the molecular basis for colorless hemolymph and cocoons is a defect in the cellular uptake of lutein associated with the *Y*-gene recessive mutants.

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1. Introduction

The mechanism by which dietary triacylglycerols (TAG) are absorbed and transported to the fat body has been well characterized in the tobacco hornworm, *Manduca sexta* (Canavoso and Wells, 2000; Ryan and

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Horst, 2000; Tsuchida and Wells, 1988). In *M. sexta* larvae, dietary TAG are completely hydrolyzed to free fatty acids (FA) in the midgut prior to absorption by the lumen epithelial cells (Canavoso and Wells, 2000; Tsuchida and Wells, 1988); subsequently, absorbed FA are incorporated into phospholipid, diacylglycerols (DAG) and TAG (Canavoso et al., 2004). Lipolysis of triolein is supported by the identification of a *M. sexta* midgut lipase that hydrolyzes TAG into monoacylglycerol (MAG) and FA (Rubiolo and Wells, unpublished work). In addition, the high pH in the midgut lumen of *M. sexta*, as is the case in other Lepidoptera, supports acyl migration and subsequent hydrolysis, which leads to free glycerol and FA. This mechanism assures maximal absorption of FA from the midgut lumen, while maintaining a low intracellular concentration of both FA and DAG, both of which are toxic at high concentrations. By the action of a lipophorin receptor and lipid transfer particle (LTP), dietary lipids, in the form of DAG, are transferred to circulating lipophorin that delivers its lipid cargo to target tissues. Mobilization of dietary lipid to hemolymph does not require the synthesis of new lipophorin but, rather, circulating lipophorin is able to load its DAG cargo from the midgut cells. Insect lipophorin transports a wide variety of other lipids, including cholesterol (Jouni et al., 2003; Jouni et al., 2002a, b;), phospholipid (Atella et al., 1995) and carotenoids (Jouni and Wells, 1996; Tsuchida et al., 2004) from sites of absorption in the midgut, to sites of storage and utilization in the fat body.

Carotenoids can be classified into two groups: non-polar hydrocarbons called carotenes, such as β -carotene and lycopene; and polar, oxygen-containing hydrocarbons called xanthophylls (Kiefer et al., 2002). Xanthophylls include zeaxanthin, lutein, cryptoxanthin and canthaxanthin. Carotenoids presence in human tissues (Granado et al., 2003) and in Lepidoptera (Jouni and Wells, 1996) is entirely of dietary origin. However, the eye of *Periplaneta americana* synthesizes carotenoids de novo when its food is deficient in these compounds (Shukolyukov and Saakov, 2001). Lutein has been shown to display biological activities in disease prevention of common chronic human diseases such as cancer, age-related macular degeneration and cardiovascular disease (Mayne, 1996). In *Drosophila*, lutein has a provitamin A activity (Kiefer et al., 2002), a role that is not present in humans (Granado et al., 2003), and has not been established in other insects. As carotenoid absorption, metabolism and utilization have not been well studied, we have been using the silkworm, *Bombyx mori* (*B. mori*), as a model system to characterize these mechanisms.

Three genes whose products control the uptake and transport of carotenoids have been identified by genetic linkage mapping. The first gene identified is the *Y*-gene (yellow hemolymph), which controls uptake of carotenoids from the midgut epithelium; thus, larvae of

mutants with the $+^Y$ phenotype cannot absorb dietary carotenoids. The second gene is the *I*-gene (yellow inhibitor, suppresses *Y*-gene products). This controls transfer of carotenoids from midgut epithelium to the hemolymph and is associated with lipophorin; thus, larvae of mutants with *I*-gene have colorless hemolymph. The third gene is the *C*-gene (golden yellow cocoon); it controls the uptake of carotenoids from hemolymph to the middle parts of the silk gland. Only larvae with the phenotype $Y+^I C$ make yellow cocoons. All other gene combinations make white cocoons (Tazima, 1978). Recently, we identified two carotenoid-binding proteins (CBPs) from the larval wild type: a lutein-binding protein (LBP) was purified from the midgut (Jouni and Wells, 1996), and a CBP was purified from the silk gland (Tabunoki et al., 2002). Deduced amino acid sequence revealed that CBP is a novel member of the steroidogenic acute regulatory (StAR) protein family with the unique structural feature of a StAR-related lipid transfer domain known to aid in lipid transfer and recognition. Immunocytochemistry revealed that CBP expression is restricted to the *Y*-gene dominant mutants. This 33 kDa protein is uniformly expressed along the brush border of the epithelial cells of the midgut and along the distal membrane of the middle silk gland, typifying its function in aiding absorption and redistribution of dietary carotenoids (Tsuchida et al., 2004). *B. mori* mutants are attractive candidates for investigating the molecular bases necessary for carotenoid metabolism; however, it is important to verify whether $+^Y$ mutants, recessive of *Y* gene, are able to absorb other lipids besides carotenoids normally and transfer them to lipophorin in the hemolymph.

In this report, we are presenting a first attempt to correlate and establish lipid metabolism and distribution in *B. mori* of the *YI* mutants. We show that lipid metabolism in the mutants is normal and that the molecular basis for colorless hemolymph and cocoons is a defect in the cellular uptake of lutein.

2. Materials and methods

2.1. Materials

[^3H]-triolein was purchased from NEN (Boston, MA). Standards for α -carotene, β -carotene, lutein, DAG, TAG, hydrocarbons, cholesterol and phospholipids were obtained from Sigma. Benzamidine, PMSF, protease inhibitor mixture III and protease inhibitor mixture complete were purchased from Calbiochem (La Jolla, CA). Horseradish peroxidase- and alkaline phosphatase-conjugated anti-rabbit IgG goat serum were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). All other chemicals were of analytical grade.

2.2. Insects

All *B. mori* mutants were reared on fresh mulberry leaves at 25 °C on a 12 h light/12 h dark photoperiod. The four mutants of *B. mori* used have the following genotypes: YI , $Y+^I$, $+^Y I$ and $+^Y +^I$. The Y strains can transfer carotenoids from midgut lumen to midgut epithelium, while the $+^Y$ strains cannot. The I strains cannot transfer carotenoids from the midgut epithelium to lipophorin, while the $+^I$ strains can.

2.3. Isolation of lipophorin

Hemolymph of *B. mori* mutants from 2–3-day-old fifth-instar larvae was collected in ice-cold bleeding solution (PBS:10 mM phosphate buffer, pH 6.5, containing 150 mM NaCl, 2 mM Na₂EDTA, 10 mM glutathione and 3 mM NaN₃) by puncturing a proleg and gently pressing the abdomen. Hemolymph was centrifuged for 5 min at 800g (4 °C) to remove hemocytes and then subjected to two steps of KBr gradient ultracentrifugation as described previously (Prasad et al., 1986; Shapiro et al., 1984). Twenty milliliters of hemolymph was adjusted to a density of 1.31 g/ml with KBr; then 20 ml of this solution was overlaid with 20 ml of 0.9% NaCl, followed by ultracentrifugation at 50,000 rpm for 16 h (Shapiro et al., 1984). Densities were determined along the gradient. Fractions containing lipophorin were combined and dialyzed against PBS buffer. The purity of the lipophorin preparations was confirmed by SDS–PAGE analysis (Laemmli, 1970). Under these conditions >95% of the label was recovered in the DAG–lipophorin moiety.

2.4. Time course studies

B. mori mutants of day 2–3 fifth-instar larvae were fed [³H]-triolein (2 μCi/ animal) on a small piece of mulberry leaf. At different time intervals (10, 30, 60 and 120 min), hemolymph was collected and lipophorin was isolated as described above. Midgut contents were emptied, and midgut tissues were washed three times with ice-cold PBS buffer containing 1 mM benzamidine. Similarly, the fat body and silk glands were carefully dissected and washed extensively with PBS buffer. All tissues were dried on a piece of Kimwipes and weighed. Individual tissues were homogenized in five volumes of PBS containing protease inhibitor cocktail III in a polytron homogenizer for 30 s and used for lipid extraction.

2.5. Quantification of lipophorin by radial immunodiffusion

Antibody of lipophorin was obtained from Japanese White rabbits injected with fifth-instar lipophorin and adjuvant (MPL + TDM + CWS emulsion, RIBI) mix-

ture. For radial immunodiffusion assay, different concentrations of lipophorin (1–80 μg) were loaded on 1% agarose gel containing 2% lipophorin antibody. The components of the gel were allowed to diffuse for 2 days at 4 °C; then the diameters of the precipitated curves were measured and utilized to generate a lipophorin standard curve (Tsuchida et al., 1987). To measure the concentration of lipophorin in the different mutants, 15 μl hemolymph was used in the radial immunodiffusion assay. The diameters of the precipitated curves were used to obtain the concentration of lipophorin utilizing the lipophorin standard curve.

2.6. Hemolymph and tissue lipid analyses

KBr purified mutant lipophorins (5 mg) and individually homogenized tissues were used for lipid extraction following the method of Bligh and Dyer (1959). Extracted lipids were separated by thin-layer chromatography (TLC) on silica gel using hexane–ethyl ether–formic acid 70:30:3 (v/v/v) as a solvent system (Henderson and Tocher, 1992). The MAG, DAG, FA, TAG and PL fractions were quantified by flame ionization detector (FID) (Iatroscan) via comparison to a known amount of an internal standard (cholesteryl acetate) (Hiraoka and Katagiri, 1992).

2.7. Analysis of carotenoid composition

All solvents were flushed with nitrogen gas. Samples of lipophorin or silk gland (0.5 ml) were added to 1 ml ethanol and 0.5 ml distilled water. Then 6 ml of the *n*-hexane was added to the mixture followed by vortexing for 5 min. The organic phase was removed and purged with nitrogen gas, and anhydrous sodium sulfate was added to remove water. Five milliliters of the organic phase was dried in a rotary evaporator, resolubilized in 0.2 ml of ethanol, and used for carotenoid analyses and quantification using HPLC. Extracted carotenoids were injected onto an Intersil ODS 3.5 μm column (4.6 × 150 mm²) equilibrated with acetonitrile: methanol:tetrahydrofuran (58:38:7, v/v/v). Eluted components were detected by a Shimadzu SPD-10AV detector and compared with a mixture of carotenoid standards of α -carotene, β -carotene and lutein.

2.8. Determination of protein concentration

Protein concentration was determined by either the bicinchoninic acid assay (BCA) or Bradford (Bradford, 1976) with bovine serum albumin (BSA) as a standard.

Statistical tests were performed using GraphPad Prism version 3.00 for Windows (GraphPad Software, San Diego, CA). The results are expressed as percentages ± SEM. $p < 0.05$ was considered as a significant difference between means.

3. Results

3.1. Mobilization and storage of dietary fatty acids in *B. mori* mutants

To determine whether significant differences in dietary lipid metabolism exist in *B. mori* mutants, the insects were fed a single dose of [³H]-triolein (2 μCi/ animal) at different time intervals (10, 30, 60 and 120 min), and the hemolymph, midgut, fat body and silk glands were analyzed for the distribution of labeled fatty acids (short-term study). In all mutants, the amount of radioactivity in the midgut increased immediately after feeding and reached a maximum at 30 min, and then decreased sharply thereafter (Fig. 1A). Although significantly more radioactivity was detected in the *Y+I* mutant, the same pattern of triolein uptake was obtained in all other mutants. At the end of the 2 h feeding period, the amount of label in the midgut greatly reduced, suggesting there is little storage of the lipid in

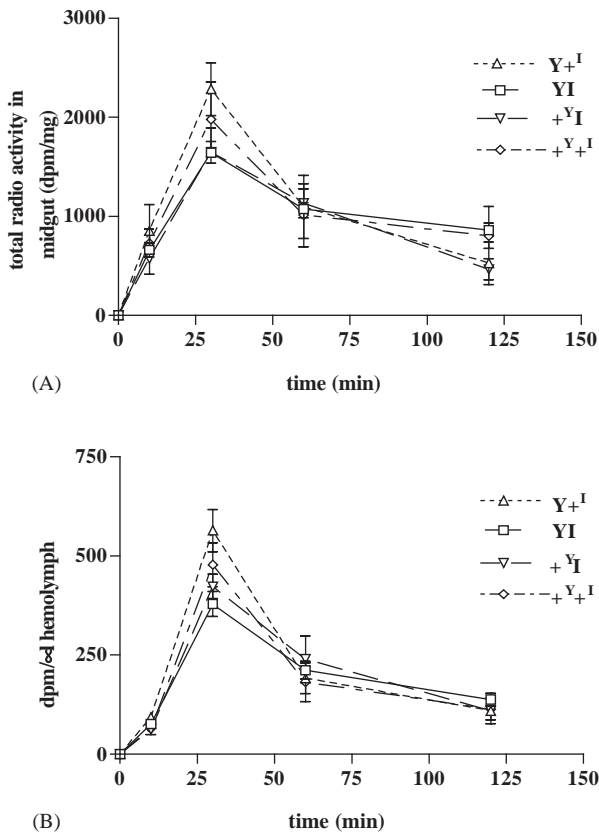


Fig. 1. Time course of the distribution of labeled total lipids in midgut and hemolymph. *B. mori* mutants of 2–3-day-old fifth-instar larvae were fed [³H]-triolein (2 μCi/ animal) on a small piece of mulberry leaf. At different time intervals (10, 30, 60 and 120 min), midgut (A) and hemolymph (B) were collected and the radioactivities were measured using a liquid scintillation counter. Data is presented as average of radioactivity dpm/mg of midgut protein or dpm/μl of hemolymph ± SEM (n = 3–4).

this tissue. Similarly, the amount of radioactivity in the hemolymph increased sharply after feeding, and reached a maximum within 30 min in all mutants (Fig. 1B). At the end of the 2 h feeding period, the majority of the label in the hemolymph decreased to very low levels, suggesting the uptake of the label by other tissues.

Fig. 2 shows the uptake of radioactive lipids in fat body and silk gland of mutants after feeding 2 μCi of triolein. Incorporation of labeled fatty acids in the fat body increased linearly with increasing time. At peak radioactivity in the midgut (30 min), the amount of incorporated radioactivity in the fat body was 7–10-fold less than that of the midgut. However, at the end of the short-term feeding study, the amount of radioactive lipid in the fat body was 100–200-fold higher than the midgut, suggesting the storage of lipids in this organ. Similarly, the uptake of radioactive fatty acids increased linearly with time in the silk glands in all mutants, demonstrating again the accumulation of lipids in the organ, although to a lesser extent than in the fat body. We conclude from these experiments that the short-term mobilization of dietary lipids is the same in all mutants.

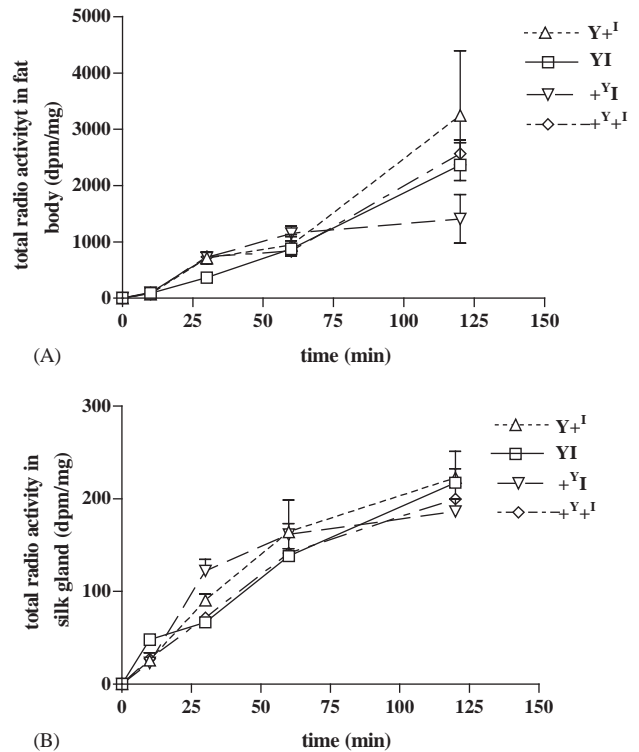


Fig. 2. Time course of the distribution of labeled total lipids in fat body and silk glands. *B. mori* mutants of 2–3-day-old fifth-instar larvae were fed [³H]-triolein (2 μCi/ animal) on a small piece of mulberry leaf. At different time intervals (10, 30, 60 and 120 min), fat body (A) and silk gland (B) were collected, homogenized and the radioactivities were measured using a liquid scintillation counter. Data are presented as average of radioactivity dpm/mg protein of fat body or silk gland ± SEM (n = 3–4).

3.2. Lipid and carotenoid composition of lipophorin

When wild-type larval *B. mori* hemolymph was subjected, for 4 h, to density gradient ultracentrifugation, two yellow peaks corresponding to LTP and lipophorin were observed (Tsuchida et al., 1998). In contrast, when mutant hemolymph is subjected to 4 h ultracentrifugation, only one yellow band corresponding to lipophorin is observed in all mutants. The density region corresponding to LTP was colorless in all mutants except in the $Y+^I$ mutant (data not shown). Further purification of lipophorin, using overnight ultracentrifugation, demonstrated the presence of a yellow band with different intensities among mutants (Fig. 3). The color intensity of this band exhibited the following pattern: $Y+^I > > +^Y+^I > YI > +^YI$. It is conceivable that either the concentration of lipophorin differs significantly among mutants where coloration is faint or, alternatively, the same amount of lipophorin is present in the mutants, but lipophorin does not carry a sufficient amount of carotenoids to give the yellow coloration.

To distinguish between these two possibilities, lipophorin composition and concentration of carotenoids were determined (Tables 1–3). Lipophorin concentrations were not significantly different among mutant hemolymphs ($p > 0.05$, Table 1). Similarly, the optical density of lipophorin (1.144–1.145 g/ml) was the same for all mutants. This infers that the total protein to lipid ratio of 2.6–2.8 was not significantly different ($p > 0.05$). However, the ratio of absorbance of purified lipophorin at 450 nm (carotenoids) to 280 nm (protein) was significantly higher in the $Y+^I$ mutant compared to other mutants ($p < 0.001$). In the $Y+^I$ mutant, this ratio was 3.9, and represents a 27-, 48- and 13-fold higher ratio than in the YI , $+^YI$, and $+^Y+^I$ mutants, respectively.

Analyses of protein and lipid composition of purified lipophorins obtained from third-day fifth-instar larvae,

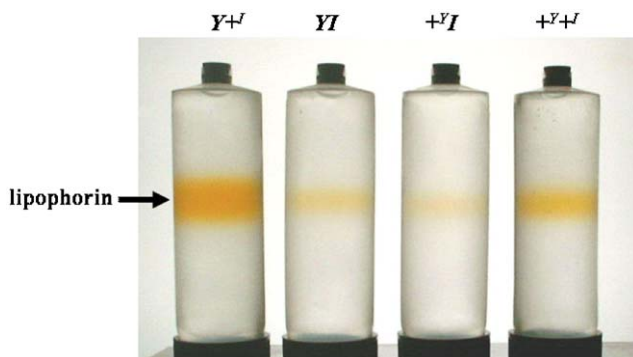


Fig. 3. KBr density gradient ultracentrifugation of mutants' lipophorin. Hemolymphs (20 ml) were collected from 2–3-day-old fifth stadium larvae, centrifuged for 5 min at 1000g (4 °C) to remove hemocytes and then subjected to two steps of KBr gradient ultracentrifugation. The yellow bands correspond to lipophorin.

Table 1
Lipophorin characterization of *B. mori* mutants

	$Y+^I$	YI	$+^Y+^I$	$+^Y+^I$
Concentration (µg/ml)	3.10 ± 0.62	3.23 ± 0.39	3.00 ± 0.45	2.96 ± 0.34
Density (g/ml)	1.144	1.144	1.145	1.145
$A_{450/280}$	3.9767	0.146	0.0817	0.3105

Larval lipophorins were isolated from mutants of third-day fifth-instar larvae by KBr gradient ultracentrifugation as described in Section 2. Lipophorin concentrations were determined by immunodiffusion. $A_{450/280}$ represents the absorbance at 450 nm (carotenoids) versus 280 nm (protein).

Table 2
Percentage of protein and lipid composition of mutant lipophorin

	$Y+^I$	YI	$+^YI$	$+^Y+^I$
Protein (%)	59.7 ± 0.1	60.6 ± 1.5	65.7 ± 2.0	64.4 ± 2.3
Lipid (%)	40.3 ± 0.1	39.4 ± 1.5	34.3 ± 2.0	35.6 ± 2.3
Hydrocarbon	11 ± 1.0	8.1 ± 0.1	9.1 ± 0.6	6.5 ± 0.4
Triacylglycerol	2.8 ± 0.5	2.1 ± 0.1	2.1 ± 0.7	2.7 ± 0.4
Diacylglycerol	37.9 ± 1.6	43.5 ± 1.8	35.3 ± 1.1	33.4 ± 1.6
Cholesterol	13.6 ± 1.5	10.6 ± 0.2	16.5 ± 2.3	18.3 ± 0.4
Phospholipid	34.8 ± 5.0	35.6 ± 2.0	37 ± 1.1	29.1 ± 0.6

Mutants' lipophorins were isolated from third-day fifth-instar larvae by KBr gradient ultracentrifugation. Different classes of lipids were separated on thin-layer chromatography and quantified by Iatrosan; refer Section 2.6 for details. Data is presented as percentage \pm SEM of 3–4 determinations.

Table 3
Carotenoid content of lipophorin of *B. mori* mutants

	$Y+^I$	YI	$+^YI$	$+^Y+^I$
Total carotenoids (µg/mg)	55.8 ± 4.7^1	1.2 ± 0.7^a	0.5 ± 0.1^a	3.1 ± 0.7^a
Weight (%)				
Lutein	77.1 ± 8.0	62.2 ± 11.6	68.1 ± 2.4	68.9 ± 2.1
α -Carotene	1.1 ± 2.0	0	0	1.1 ± 0.1
β -Carotene	21.8 ± 8.8	37.8 ± 11.6	31.9 ± 2.4	30.8 ± 2.6

Carotenoid profile of mutants' lipophorins obtained from third-day fifth-instar larvae were analyzed and quantified using HPLC as described in Section 2. Values represent average \pm SEM of 3–4 determinations.

^aSignificantly different from $Y+^I$, $p < 0.001$.

reared on fresh mulberry leaves (long-term feeding), demonstrated that mutant lipophorins were composed of 60–65% protein and 35–40% lipid (Table 2). Further analyses of the lipid profile revealed that phospholipids and DAG accounted for the majority of the lipid in lipophorin, whereas TAG were the minor component. Taken together, we conclude that the faint coloration of lipophorin is not due to differences in the amount of

protein or lipid composition, but rather to low chromophore (carotenoids) concentration.

To investigate the nature and abundance of the lipophorin-associated yellow chromophore, isolated lipophorin was extracted with ethanol and hexane and analyzed by HPLC (Table 3). Lipophorin of the $Y+^I$ mutant exhibited the highest concentration of total carotenoids with 55.8 $\mu\text{g}/\text{mg}$ lipophorin compared to 1.2 $\mu\text{g}/\text{mg}$ in the YI mutant, 0.5 $\mu\text{g}/\text{mg}$ in the $+^YI$ mutant and 3.1 $\mu\text{g}/\text{mg}$ in the $+^Y+^I$ mutant. The amount of carotenoid confirms the data obtained in Fig. 3, where the color intensity of lipophorin was highest in the $Y+^I$ mutant and lowest in the $+^YI$ mutant. This further confirms the efficiency of carotenoid absorption from the midgut and its transport to hemolymph in the $Y+^I$ mutant that expresses CBP. Characteristic retention times in HPLC of the different classes of carotenoids of lipophorin identified the presence of lutein as the major chromophore (62–77%), followed by β -carotene (22–38%). Although lutein and β -carotene contents of mutant lipophorin differed significantly, the 3:1 ratio of lutein to β -carotene was not different among mutants.

3.3. Lipid and carotenoid composition of silk glands

Unlike the composition of lipophorin carotenoids, mutant silk glands contain exclusively lutein (Table 5). The concentration of lutein is significantly higher in $Y+^I > > > YI > +^YI > +^Y+^I$, a pattern that reflects the yellow color intensity of the mutant silk glands and not its concentration in the hemolymph. The significantly higher concentration of lutein in the $Y+^I$ mutant represents more than 160-fold increase compared to the $+^Y+^I$ mutant ($p < 0.001$). The silk gland of the YI mutant contains 8- and 26-fold higher lutein than the $+^YI$ and $+^Y+^I$ mutants, respectively ($p < 0.05$).

To investigate whether other classes of lipids are different in the silk gland of the mutants, the tissues obtained from third-day fifth-instar larvae, reared on fresh mulberry leaves, were extracted and lipids were separated by TLC (Table 4). In all mutants, phospholipids were the most abundant component. However, in the $Y+^I$ mutant, phospholipid (74.5+3.2%) was significantly lower compared to other mutants ($p < 0.05$) and this decrease was at the expense of a significant increase in its TAG content ($p < 0.05$). We conclude that lutein represents the major difference seen in the long-term storage of lipids in mutant silk glands (Table 5).

4. Discussion

B. mori is an excellent model for the study of CBPs. Three genes (*Y*, *I* and *C*) whose products control the

uptake and transport of carotenoids have been identified by genetic linkage mapping. The *Y*-gene controls the uptake of carotenoids from the midgut epithelium; thus, larvae of mutants with the $+^Y$ phenotype cannot absorb dietary carotenoids. The *I*-gene (yellow inhibitor) controls the transfer of carotenoids from midgut epithelium to the hemolymph associated with lipophorin. The *C*-gene controls the uptake of carotenoids from hemolymph to the middle parts of the silk gland. Only larvae with the phenotype $Y+^IC$ make yellow cocoons. All other gene combinations make white cocoons. Analyses of these mutants offer great promise for analyzing the pathways of carotenoid transport, mobilization and storage.

Prior to undertaking the task of fully characterizing carotenoid metabolism in *B. mori*, we need to (a) determine whether a single ingested dose of [^3H]-labeled TAG for 2 h (short-time study) is metabolized differently in the four mutants, and (b) compare carotenoids and lipid profiles of the last fifth-instar mutant larvae reared on fresh mulberry leaves (long-term study).

4.1. Lipid metabolism in *B. mori* mutants

B. mori, like other Lepidoptera, are continuous feeders; they accumulate their energy stores during the larval instars (active feeding period). The largest consumption of mulberry leaves and growth occur at the fifth instar, which lasts for 5 days. It is generally agreed that a major fate of dietary lipid is storage in the fat body and the subsequent utilization as fuel to sustain life, flight and reproduction (Canavoso et al., 2004; Nakasone and Itoh, 1967), but this process has not been studied in *B. mori*.

When mutants were fed a single dose of triolein, radioactivity in the hemolymph appeared within minutes, inferring that mobilization of absorbed lipids from midgut cells to lipophorin is a very fast process. MAG contributed very little to the radioactivity in the midgut lumen, whereas TAG and phospholipids were the major contributors (data not shown). It is possible that dietary triolein was hydrolyzed to FA and glycerol in the lumen prior to its absorption, a mechanism that is well established in *M. sexta*. Absorbed FA are stored in the midgut cells in a metabolically active TAG pool, which provides lipophorin with its DAG. In support of this mechanism is the progressive and significant increase of DAG in the hemolymph immediately after feeding and in the accumulation of radioactivity in the fat body, which accounted for 65% of the fed triolein.

Since the short-term uptake of dietary lipids did not show any differences in lipid metabolism among the mutants, it was of interest to measure and compare lipid content of the last larval instar, reflecting the long-term processing of lipids.

Table 4
Lipid composition of mutant silk glands

	$Y+I$	YI	$+YI$	$+Y+I$
Total lipids ($\mu\text{g/g}$)	2730.1 \pm 251.8	1963.9 \pm 353.8	2061.3 \pm 614.4	2152.6 \pm 191.7
Weight (%)				
Hydrocarbon	2.5 \pm 1.0 ^a	0.3 \pm 0.2 ^a	0.4 \pm 0.2 ^a	0.2 \pm 0.2 ^a
Triacylglycerol	14.3 \pm 3.1 ^b	6.7 \pm 1.6 ^b	4.5 \pm 1.2 ^b	3.9 \pm 2.3 ^b
Diacylglycerol	4.9 \pm 1.7	5.4 \pm 1.7	4.8 \pm 1.2	3.3 \pm 1.3
Cholesterol	3.7 \pm 1.2	4.4 \pm 0.8	4.7 \pm 0.6	4.5 \pm 1.4
Phospholipid	74.5 \pm 3.2 ^{a,c}	83.2 \pm 2.1 ^c	85.4 \pm 0.6 ^c	88.1 \pm 1.9 ^a

Silk glands of the YI mutants were isolated from third-day fifth-instar larvae, homogenized in PBS and a 0.5 ml sample was used for lipid extraction following the method of Bligh and Dyer (1959). Different lipid components were separated by TLC and quantified by Iatroscan. Data are presented as percentage \pm SEM of 3–4 determinations.

^{a,b}Significantly different from $Y+I$, $p < 0.01$ and $p < 0.05$, respectively.

^cSignificantly different from YI , $p < 0.05$.

Table 5
Carotenoid content of silk glands of *B. mori* mutants

	$Y+I$	YI	$+YI$	$+Y+I$
Total Carotenoids ($\mu\text{g/g}$)	65.0 \pm 7.8 ^a	10.5 \pm 2.6 ^{a,b}	1.3 \pm 0.2 ^{a,b}	0.4 \pm 0.2 ^{a,b}
Weight (%)				
Lutein	98.4 \pm 0.5	98.7 \pm 0.6	99.8 \pm 0.3	100 \pm 0.1
β -Carotene	1.6 \pm 0.5	1.3 \pm 0.6	0.2 \pm 0.3	0

Carotenoid profile of mutants' silk glands obtained from third-day fifth-instar larvae were analyzed and quantified using HPLC as described in Section 2. Values represent average \pm SEM of 3–4 determinations.

^aSignificantly different from $Y+I$, $p < 0.001$.

^bSignificantly different from YI , $p < 0.05$.

4.2. Protein and lipid characterization of mutant hemolymph

Mutant lipophorins are composed of 60–65% protein and 35–40% lipid, of which DAG (33–43%) and phospholipids (29–37%) constitute the predominant lipids. These results are similar to results obtained in other Lepidoptera, including *M. sexta* and the wild type of *B. mori*. At this time, we do not know why mutant lipophorins contain more cholesterol (11–18% of total lipid) compared to *M. sexta* (~3% of total lipid).

The most notable difference among mutant lipophorins is the carotenoid content, giving the hemolymph its yellow coloration. One would expect the hemolymph of the Y -dominant gene containing mutants to have a bright yellow color compared to the Y -recessive gene containing mutants, as CBP would aid in the absorption of more lutein. However, the intensity and the concentration of lipophorin carotenoids exhibited the following pattern: $Y+I > > > +Y+I > > YI > +YI$. One possible explanation could be due to the presence of a different CBP, rather than Y -gene product, which facilitates

carotenoids absorption. Another explanation is that in the $+Y+I$ mutant, as in the other mutants, lutein leaks out or is taken up from the lumen into epithelial cells of the midgut by simple diffusion and consequently into the hemolymph associated with lipophorin. However, in the $+I$ gene mutants the inhibitory protein that prevents the transfer of lutein from the midgut epithelial into hemolymph is absent, and thus the leakage or the passive diffusion of lutein is not restricted, as is the case in the I -dominant genes of the YI and $+YI$ mutants.

Of particular note is the elevated levels of lipophorin carotenoids (55 $\mu\text{g/mg}$) in the $Y+I$ gene containing mutant compared to the wild type (33 $\mu\text{g/mg}$) (Tsuchida et al., 1998). Not only do the $Y+I$ mutants possess more carotenoids, but the composition of the carotenoids also differed significantly between the two strains. This could be due to the high carotenoid content of fresh mulberry leaves fed to the mutants, compared to the artificial diet fed to the wild-type animals. Qualitative and quantitative distribution of carotenoids in insects depends on the host plant, pH of the midgut, and the presence of other carotenoids in the diet. β -Carotenes, in both the *Helicoverpa zea* larvae (Eichenseer et al., 2002) and *Periplaneta americana* (Shukolyukov and Saakov, 2001), have been shown to depend on the host plant.

Further analyses of hemolymph proteins indicate that all mutants possess the same amount of LTP and lipophorin, illustrating the absence of a correlation between the yellow color attributed to LTP and lipophorin and their hemolymph concentrations. As in the wild type (Tsuchida et al., 1998), LTP of the $Y+I$ larvae exhibited a visible absorbance spectrum, with typical carotenoid features, reflecting the high concentration of hemolymph carotenoids in this mutant. Carotenoid-associated LTP is attributed to LTP function in carotenoid transfer between lipophorins (Tsuchida et al., 1998). Carotenoid-associated LTP is not unique to *B. mori*; locust LTP also contains significant amounts of hydrocarbons (Hirayama and Chino, 1990).

4.3. Lipid characterization of *B. mori* mutants

The high expression of CBP in the midgut (972 ± 250 ng/mg protein) and silk glands (9939 ± 2056 ng/mg protein), occurring during the highest consumption of food and carotenoids, may indicate that CBP plays an important role in absorption and redistribution of lutein (Tsuchida et al., 2004). *B. mori* mutants develop normally and have no significant variations in their lipid metabolism, except for reduced carotenoids, supporting the notion that absence of CBP in the *Y*-gene recessive containing mutants appears not to interfere with lipid metabolism in general. In *Drosophila*, a class B scavenger receptor has been shown to play an important role in the redistribution and delivery of carotenoids from adipose tissues to the developing eyes for its subsequent usage in the synthesis of the visual chromophore (Kiefer et al., 2002). A non-lethal mutant of this receptor, *ninaD*, has been identified in *Drosophila*. Flies with the homozygous *ninaD* mutant are blind, a result of vitamin A deficiency. Similar to the *B. mori* mutants, the *ninaD* mutant flies develop normally and possess no significant alterations in lipid contents other than reduced carotenoids (Kiefer et al., 2002). The same authors suggest that the lack of alterations in lipid profiling may be explained by the specificity of the *ninaD* function for carotenoids, which differentially affects the utilization of β -carotene and zeaxanthin.

Of particular interest is the exclusive presence of lutein in the silk gland compared to lutein and β -carotene (3:1 ratio) in lipophorin. In Lepidoptera, dietary carotenoids are absorbed and stored with little modification compared to other insect orders (Feltwell, 1978; Kayser, 1985). However, a bias to sequester oxygenated xanthophylls, particularly lutein, the most abundant carotenoid in most flowering plants, has been documented in Lepidoptera (Carroll et al., 1997; Czeczuga, 1986; Feltwell and Rothschild, 1974; Feltwell and Valadon, 1972; Valadon and Mummery, 1978). The lutein of lipophorin could be more available for transport than β -carotene. Localization of carotenoids in lipophorin has not been investigated, but it is reasonable to assume that significant amounts of lutein, an oxygenated carotenoid, could be located in the phospholipid surface of lipophorin. This assumption is based on the report that the more polar lutein is preferentially located in the phospholipid surface, whereas the less polar β -carotene is present in TAG core of lipid droplets (Tyssandier et al., 2001). In addition, a significant amount of DAG, less polar than lutein, has been found on the lipophorin surface of *M. sexta* (Soulages et al., 1994). Another possibility for the exclusive presence of lutein in silk glands is the well-marked inverse relationship between carotenoid hydrophobicity and transfer efficiency (Tyssandier et al.,

2001). Or perhaps, CBP has an inherent molecular specificity for lutein. Genetic linkage mappings have identified other CBPs that specifically absorb β -carotenes and produce pink-colored cocoons in *B. mori* mutants.

Invertebrate models in which carotenoid deficiency is not lethal allow the isolation of viable mutants with affected carotenoid absorption and transport pathways. Using the wild type, we identified and molecularly and immunocytochemically characterized CBP as the *Y*-gene in *B. mori* (Tabunoki et al., 2002; Tsuchida et al., 2004). We now present the first attempt to correlate and establish lipid metabolism and distribution in *B. mori* using the *YI* mutants. Lipid analyses provide genetic and functional evidence that the *Y*-gene product is essential for the cellular uptake of dietary lutein into cells of target tissues. We also show that the molecular basis for colorless hemolymph and cocoons is a defect in the cellular uptake of carotenoids, and that the loss of CBP in the $+^Y$ gene containing mutants seems not to interfere with the mutants' lipid metabolism in general.

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