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Changes in Lipid Droplet Localization During Embryogenesis of the Silkworm, *Bombyx mori*

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Lipid droplets are considered one of the most important energy sources in lepidopteran eggs during late embryogenesis, but the process of their incorporation into the embryo is as yet unknown. The present study focused on the process of transition of lipid droplets from the extraembryonic yolk to the embryo of the silkworm *Bombyx mori*, using morphological and biochemical approaches. The morphological study revealed that the incorporation of lipid droplets from the extraembryonic yolk into the embryo occurs at three points and in three different ways during the development of the embryo. Some lipid droplets were translocated directly from the extraembryonic yolk to the embryo before the blastokinesis stage. However, the majority of lipid droplets together with the other components of the extraembryonic yolk were incorporated in the embryo via both morphogenetic inclusion during dorsal closure and ingestion of the extraembyonic yolk by the developing caterpillar prior to hatching. Similar results were obtained from the biochemical study. Thus, we propose that there are three steps in the incorporation of lipid droplets from the extraembryonic yolk into the embryo. In addition, morphological and biochemical data concerning the total amount of lipid droplets in the egg suggested that lipid droplets were mainly consumed during late embryogenesis, seeming to synchronize with tracheal development.

Key words: *Bombyx mori*, egg, embryonic development, lipid droplet, triglyceride

INTRODUCTION

In oviparous animals, the nutrients needed for embryogenesis are only supplied from those stored in the egg (Postlethweit and Giorgi, 1985). The three major types of nutrient contained in eggs are proteins, carbohydrates, and lipids. Generally, during embryogenesis proteins are used as sources of amino acids (McGregor and Loughton, 1977), whereas carbohydrates and lipids are used as energy sources (Steele, 1981; Beenakkers et al., 1981).

In insects that have a centrolecithal egg, a large amount of the nutrients are contained in the center of the egg. During oogenesis, these nutritious materials are incorporated into oocytes in the ovarioles: proteins are stored in the yolk granules (Raikhel and Dhadialla, 1992), carbohydrates are stored in glycogen granules (Gutzeit et al., 1994; Yamashita and Hasegawa, 1985), and lipids are stored in lipid droplets in the oocyte cytoplasm (van Antwerpen et al., 2005). Once fertilization has occurred, the zygote nuclei divide several times and form numerous energids. Some energids move toward the egg surface and form the blastoderm, whereas others form the extraembryonic yolk tissues. The extraembryonic yolk consists of the serosa and yolk cells (Miya et al., 1972; Nagy et al., 1994) or vitellophages (Giorgi and Nordin, 1994). The formation of yolk cells during early embryogenesis has been well documented in the silkworm, *Bombyx mori* (Takesue et al., 1976). Generally, yolk cells have many yolk granules, lipid droplets, and glycogen granules (Miya et al., 1972; Fausto et al., 1994). Therefore, yolk cells are considered to play an important role in the storage of nutrients in insect eggs.

From the point of energetic efficiency, lipids have higher energetic values than carbohydrates. It has been calculated that 1 mg of lipid delivers as much energy as 8 mg of stored glycogen; thus lipid is a more desirable material than carbohydrate for weight economy (Beenakkers et al., 1981).

Lipid droplets in yolk cells are assumed to be incorporated into the embryonic tissues during insect embryogenesis (Kawooya et al., 1988), but there are no studies describing when and how lipid droplets from yolk cells are incorporated into the embryo; morphological studies are required to elucidate this process. The fate of lipid droplets in the yolk cells could help in the understanding of lipid metabolism during insect embryogenesis. This study investigates the eggs of the silkworm, *B. mori*, since these have a particularly high lipid content, amounting to about 12% of their wet weight (Gilbert, 1967) and 27.1% of their dry weight (Nakasone, 1979). Here, we describe changes in the localization of lipid droplets between yolk cells and the embryo during embryogenesis by using morphological and biochemical methods.

MATERIALS AND METHODS

Animals

A standard strain (Daizo) of *Bombyx mori* was used. Under normal conditions, moths lay diapause and /or non-diapause eggs. In order to obtain "parental moths," which only laid non-diapause eggs, we first reared parents in three sequential steps. First,

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selected eggs were kept at 15°C under complete darkness during their embryonic period. Second, the larvae were fed on mulberry leaves and kept under a long-day (light 16 h : dark 8 h) light regime at 25°C for about 1 month. Third, pupae were kept under dark conditions at 25°C during the pupal phase over a period of 10 days (Yamashita and Hasegawa, 1985). After these sequential treatments, experimental non-diapause eggs were obtained from these "parental moths." Within 1 hr after oviposition, eggs were collected and kept at 25°C under dark conditions until hatching. Larvae hatched out on day 9 after oviposition under these experimental conditions. The developmental stages of the embryos were defined morphologically by referring to Ohtsuki (1979), Yamashita and Yaginuma (1991), and Singh et al. (2002).

Histochemistry

At least five eggs were collected every 24 h after oviposition, until hatching. In order to facilitate the rapid penetration of the fixative solution the chorion surrounding the egg was removed by using a fine needle (27G×3/4, Termo Co., Japan) in Grace's insect culture medium (DAIGO, Nippon Seiyaku Co., Japan) under a dissection microscope. These dechorionated eggs were fixed with 4% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.4) for 24 h at 5°C, and rinsed three times at 30-min intervals with cacodylate buffer. Paraffin sections were prepared using a standard preparation process, as previously described by Yamahama et al. (2003), and stained with hematoxylin and eosin. Frozen sections were prepared as follow. The fixed eggs were treated following a standard cryoprotectant series; briefly, the eggs were incubated in 10% sucrose in 0.01 M phosphate-buffered saline (PBS, pH 7.4) for 5 h at 5°C, and the concentration of sucrose was gradually increased to 20%. After this treatment the eggs were immersed in an embedding medium (OCT compound, Miles Scientific Co., USA) and were frozen by using dry ice. Sections (6 μm) were made through the median plane of each egg by using a cryostat (OTFAS/D/XY, Bright Instrument Co., UK). The frozen sections were stained with lipid dyes after drying for 30 min at room temperature.

The frozen sections were stained with oil red O (Merck, Germany) and/or with hematoxylin (Merck, Germany). Slides were mounted in an aqueous mounting medium (Geltol, Immunon, USA). The specimens were observed with a light microscope (AHBS3, Olympus Co., Japan), and digital images were obtained using an attached CCD camera (DP-20, Olympus Co., Japan). Sections from three individual eggs on each day were observed, and pictures were taken in order to calculate color differences.

Image data analyses

Digital images from the specimens stained with oil red O were analyzed with Adobe Photoshop (ver. 7.0.1) by using the histogram function to count the number of pixels in an area. The area stained red by oil red O showed a typical pixel value (R, 175 pixel; G, 65 pixel; B, 65 pixel), which was clearly different from the pixel values of the rest of the egg. The ratios of the red area in the extraembryonic yolk to that in the whole egg or in the embryonic area were calculated for each egg. The ratio of the total red area to the area of the whole egg was also obtained. Data were expressed as averages with standard error for 3 eggs. The values were analyzed using Student's *t*-test. P values<0.05 were judged as significant.

Thin-layer chromatography

Thin-layer chromatography was used to examine the triglyceride composition of embryos and extraembryonic yolk, respectively. Twenty eggs were collected every 24 h after oviposition until hatching. Half of these developing eggs were dechorionated in Grace's insect culture medium under a dissection microscope. These dechorionated eggs were put into 200 μl of 25 mM Hepes buffer containing 150 mM NaCl (pH7.0) and were gently dissected into embryo and extraembryonic yolk by using fine needles (27G×3/4, Termo Co., Japan) under the dissection microscope. Ten of each part (embryo or remaining extraembryonic yolk) were collected and were homogenized with 100 μl of buffer using a Teflon pestle on ice. Ten whole, developing eggs, including the chorion, were washed briefly with ethanol and were also homogenized. Lipids contained in all of the the homogenized substances were extracted in chloroform-methanol (2:1, by volume) following the method of Folch et al. (1957). Neutral lipids were separated by thin-layer chromatography (TLC) (HPTLC silicagel 60, Merck Co., Germany) using hexane/diethylether/acetic acid (80:20:1, by volume) as the eluent, and were observed under ultraviolet light (365 nm) by using a transilluminator after spraying the plate with 0.005% primulin (MP Biomedicals Inc., France) in 80% acetone. Each lipid spot was quantitatively analyzed by using Image-J (ver. 1.38, NIH, USA) with tripalmitin (T-5888, Sigma, USA) as the standard, and the amount of TG in 10 eggs was expressed in tripalmitin equivalents. Statistical analysis was performed with Student's *t*-test.

RESULTS

Fig. 1A shows a section through a developing egg in early organogenesis (stage 17); appendage development in the gnathal and thoracic segments has started. The whole egg is enveloped by the serosa; the amnion and the amnion cavity are formed; and there are two different kinds of tissue: embryonic tissue and extraembryonic yolk. The shape and color of cells in the embryonic tissues differ from those of the cells in the extraembyonic yolk. The extraembryonic yolk is filled with many yolk cells. The shape of each yolk cell is round, with a diameter of ca. 40–60 μm. Many of the granules arranged beneath the surface of the yolk cell membrane are yolk granules. Non-stained areas can be observed around the nuclei at the center of the yolk cells.

Using the serial frozen sections of the developing eggs in early organogenesis (stage 17), we compared the color of each cell stained by oil red O (Fig. 1B), by hematoxylin (Fig. 1C), or by oil red O and hematoxylin (Fig. 1D) in order to examine the localization of lipid droplets in the developing egg. Sections single-stained with oil red O exhibited a red color, whilst those single-stained with hematoxylin exhibited a blue-violet color. Sections double-stained with oil red O and hematoxylin exhibited both red and blue-violet coloration. Dark-red spots in an area stained by oil red O (indicated by arrowheads) might be an artifact caused by undissolved fine dye crystals that were not removed by paper filtration. Comparison of these serial sections indicated that the parts stained with oil red O were various in form, round or irregular shapes, large or small, and exhibited a liquid quality. These seemed equivalent to lipid droplets as previously described (Miya et al., 1972). Oil red O did not stain inside the yolk granules. The parts that were blue-violet and stained with hematoxylin were yolk granules, yolk nuclei, and embryonic tissues, and did not overlap with oil red O-stained areas (Fig. 1C). Thus, hematoxylin acted as a counterstain for oil red O. Lipid droplets were mainly localized in the cytoplasm of the extraembryonic yolk, and some of them were localized in embryonic tissues. Lipid droplets in the embryonic tissues were smaller than those in the extraembryonic yolk.

The transition in the localization of lipid droplets in the eggs during embryogenesis is shown in frozen sections (Fig. 1D–H). In the early organogenesis stage (stage 17, Fig. 1D), the extraembryonic yolk occupied a greater area of the egg

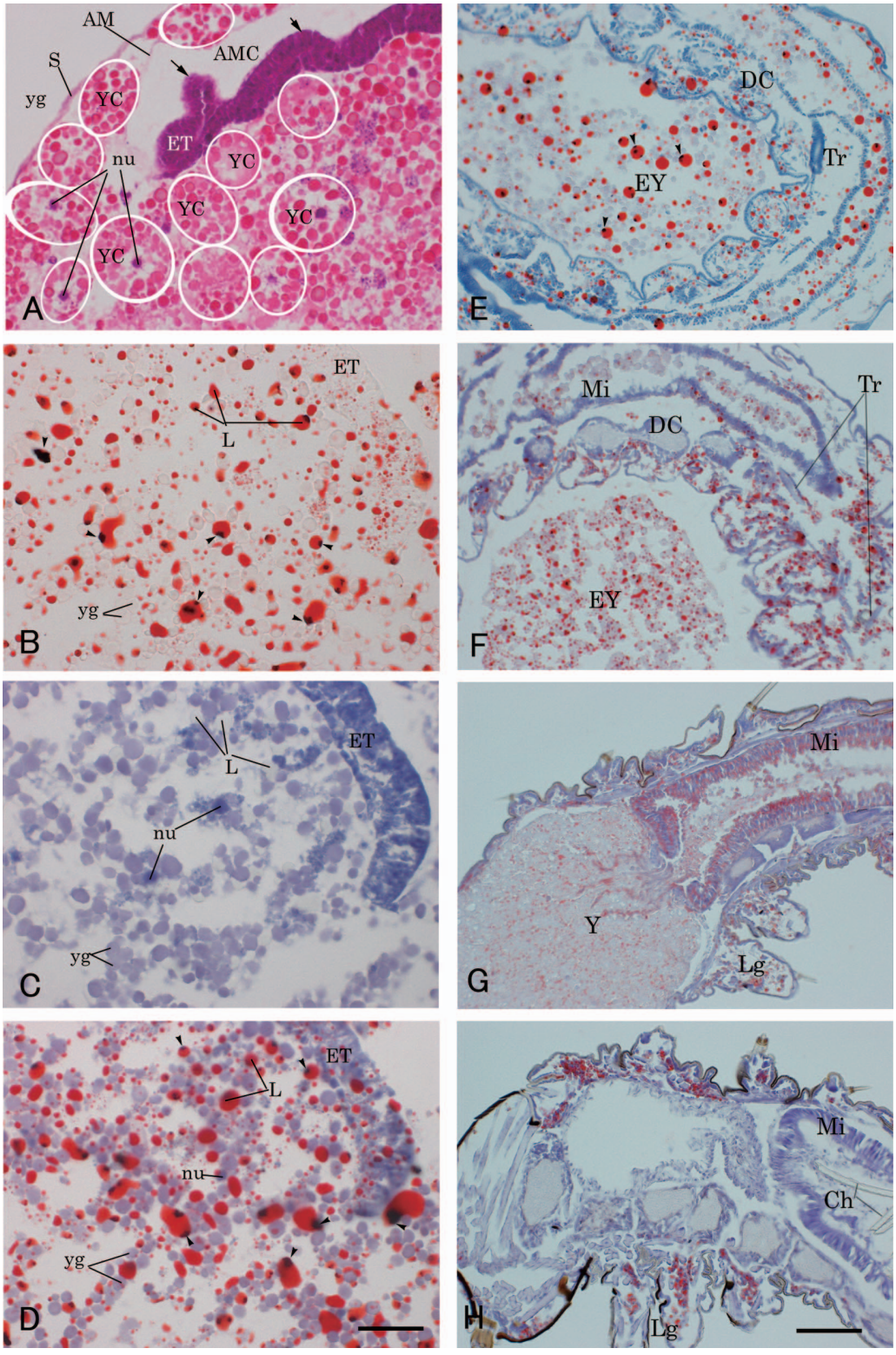

Fig. 1. Morphology of the *Bombyx mori* egg during embryogenesis. **(A)** Paraffin section of a developing egg in the early organogenesis stage (stage 17) stained with hematoxylin and eosin. Yolk-cell areas are circled in white. **(B–D)** Serial frozen sections at stage 17 stained with oil red O and/or hematoxylin. **(B)** Section stained with oil red O. **(C)** Section stained with hematoxylin. **(D)** Section double-stained with oil red O and hematoxylin. **(E–H)** Changes in the localization of lipid droplets in the egg during embryogenesis. **(E)** Embryonic reversal stage (stage 22). **(F)** Head pigmentation I stage (stage 26). **(G)** Body pigmentation I stage (stage 28). **(H)** Hatching larva (stage 30). A–D are at the same magnification, and the scale bar in D indicates 50 μm. E–H are at the same magnification, and the scale bar in H indicates 100 μm. ET, embryonic tissue; EY, extraembryonic yolk; YC, yolk cell; yg, yolk granule; nu, nucleus; L, lipid droplet; S, serosa; AM, amnion; AMC, amnion cavity; arrows, appendages; DC, developing caterpillar; Tr, tracheae; Mi, mid-intestine; Y, yolk; Ch, chorion; Lg, leg; arrowheads, remaining oil red O dye crystals.

than the embryo. Most lipid droplets were localized in the extraembryonic yolk, whereas a small number of lipid droplets were observed in the embryonic tissues. In the early blastokinesis stage (stage 21A), the embryo became invaginated toward the center of the egg, and several lipid droplets were observed in both the embryonic tissues and the extraembryonic yolk (data not shown). At the end of the embryonic reversal stage (stage 22), embryonic dorsal closure and embryonic reversal were completed; half the red dots were observed in the developing embryos, and the other half remained in the extraembryonic yolk (Fig. 1E).

At the head pigmentation I stage (stage 26), the embryos adopted caterpillar-like shapes (the developing caterpillar); the extraembryonic yolk still remained outside the developing caterpillars (Fig. 1F). During this stage, the developing caterpillars were often observed slowly moving their mandibular organs and legs inside the chorion. Ingestion behavior of these developing caterpillars was observed (data not shown). They bit and swallowed the remaining extraembryonic yolk by sticking their head into the yolk, and eventually ingested the serosa covering their body. At the body pigmentation I stage (stage 28), the developing caterpillars showed complete larval shapes and their skins developed the natural black pigmenation; no extraembryonic yolk was found outside the embryo in the egg. The foreguts and midguts of the developing caterpillars were filled with yolk contents, and many red dots were observed in the gut tract and in the gut tissues (Fig. 1G). At the hatching stage (stage 30), the first instar larvae were hatched from the chorion. No yolk or lipid droplets were observed in their gut, whereas some pieces resembling chorion were found in their midguts. A few remaining red dots were observed in the caterpillar leg tissues and the dorsal thorax (Fig. 1H).

Fig. 2 shows the changes in the localization of lipid droplets between the embryo and the extraembyonic yolk during the whole process of embryogenesis. The percentages of the characteristic red area in the embryo and in the extraembryonic yolk against the whole red area were calculated from the morphological observations. At the start of development, the percentage of total lipid area was about 15%. The percentage of lipid area in the embryos gradually increased and the extraembryonic lipid area decreased through the progress of development, while the total percentage of lipid area did not change significantly. At the completion of the embryonic reversal stage (stage 22), the percentage of lipid droplet area in the embryonic tissues was almost the same as in the extraembryonic yolk. From stage 22 to the head pigmentation I stage (stage 26), the percentage of lipid droplets in the embryo was higher than that in the extraembryonic yolk, and the amount of lipid as a percentage of total area started to decrease linearly from stage 22. During this period, the formation of the trachea was observed (Fig. 1F). At the head pigmentation II stage (stage 27), all of the extraembryonic yolk was stuffed into the midgut, and no lipid droplets exterior to the developing caterpillar were observed. At the hatching stage (stage 30), the percentage of lipid droplets was dramatically decreased.

TLC analyses were performed to examine changes in the amount of triglyceride (TG) during embryogenesis. Lipid extracted showed three major bands that had similar Rf values to tripalmitin (Fig. 3). The amount of each of the three major bands decreased similarly during embryogenesis. The total amount of TG in the whole egg decreased gradually from stage 21A to 26, dropped sharply at stage 27, and had

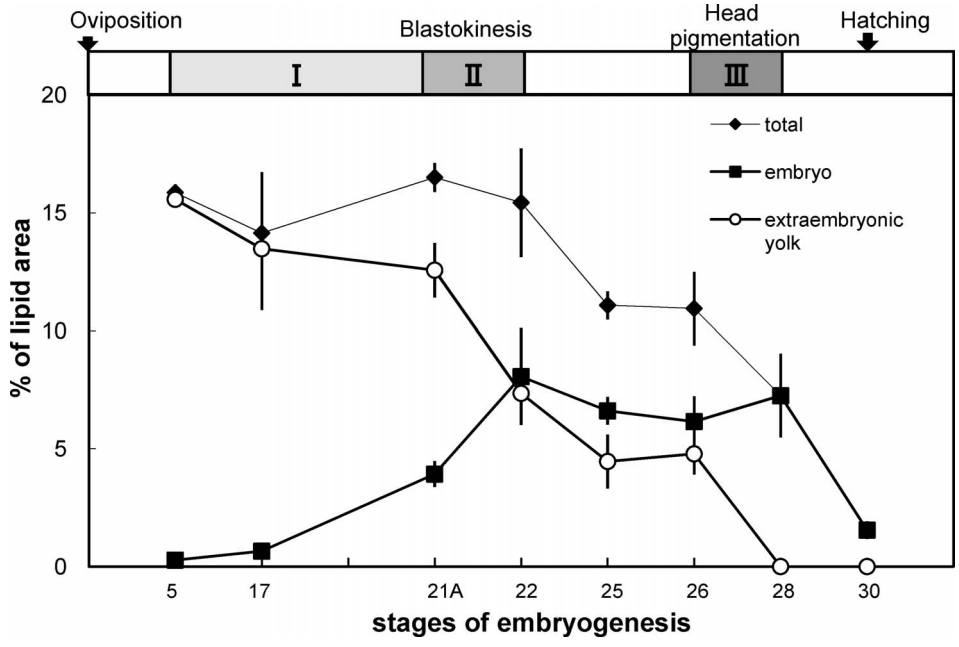

Fig. 2. Changes in the lipid area stained with oil red O between the embryo and extraembryonic yolk during embryogenesis. Black squares indicate the percentage of lipid area in the embryonic tissues. Open circles indicate the percentage of lipid area in the extraembryonic tissues. Black diamonds indicate the total percentages of lipid, by area, in the embryo and extraembryonic yolk. Sampling was performed every 24 h and is shown for each developmental stage. Roman numerals in the top row indicate the three phases of lipid incorporation into the embryo. Each datum point with error bar represents a mean±standard error (n=3).

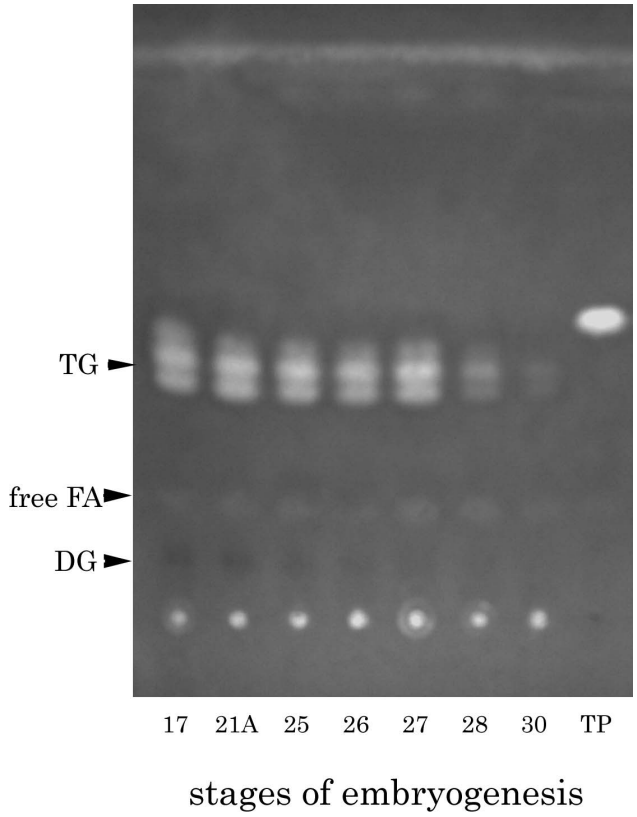

Fig. 3. Results of TLC to detect triglyceride in the whole egg during embryogengesis. Lane numbers at the bottom indicate stages of embryogenesis. TP, tripalmitin; TG, triglyceride; free FA, free fatty acid; DG, diglyceride.

almost disappeared by stage 30 (Figs. 3, 4). Amounts of TG in the embryo and in the extraembryonic yolk were compared (Fig. 4). The amounts of TG in the extraembryonic yolk were larger than that in the embryo during early embryogenesis; they dropped sharply after stage 22, and disappeared entirely during stages 26–27. In contrast, the amounts of TG in the embryo were small from early embryogenesis to blastokinesis and reached a maximum at stage 27. Most TG in the embryo had finally disappeared by stage 30.

DISCUSSION

Lipid is one of the most important energy sources for insect embryogenesis. Neutral lipids, such as TG, have higher energetic values than carbohydrate; thus, lipid is a more desirable material than carbohydrate and is mainly used during late embryogesis (Beenakkers et al., 1981). TGs are stored as lipid droplets in the oocyte cytoplasm during oogenesis (van Antwerpen et al., 2005). Many lipid droplets localize in the yolk cells early in egg development in *Bombyx mori* (Miya et al., 1972). Therefore, it is highly probable that lipid droplets in the yolk cells are incorporated into the embryo and are consumed late in embryogenesis. To investigate the mechanism of energy supply during insect embryogenesis, it is important to understand changes of the localization of lipid droplets in the egg during the whole process of embryogenesis.

Since lipid droplets are quite soluble in organic solvents such as alcohol or xylene, paraffin sections are not suitable for the observation of lipid localization. In order to observe localization of lipid droplets in *Bombyx* eggs during embryogenesis, we used frozen sections. The process of preparing frozen sections does not require dehydration with organic solvents. Serial frozen sections were stained with oil red O,

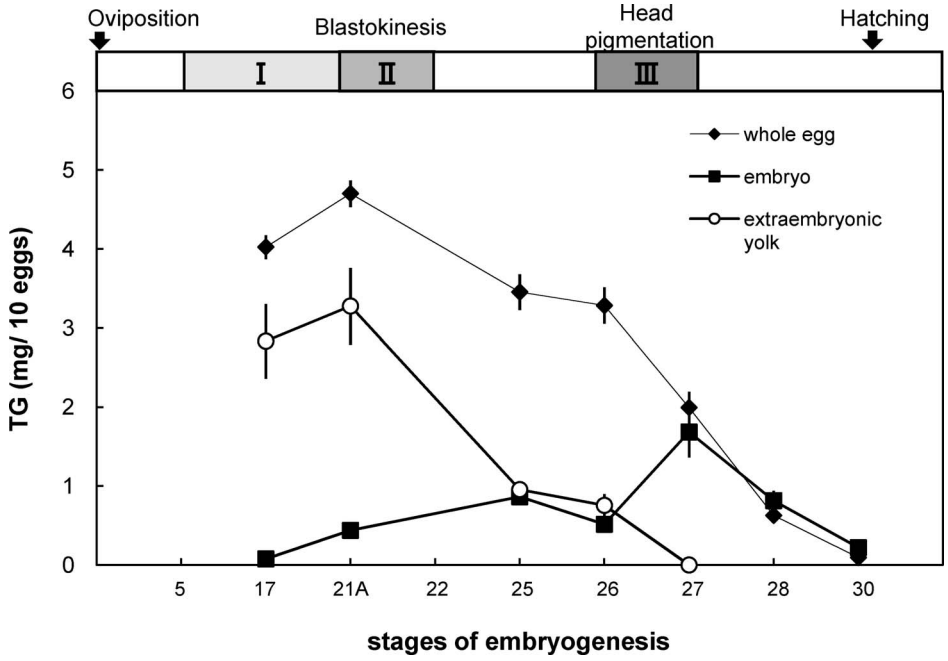

Fig. 4. Changes in the amount of triglyceride (TG) in the whole egg, in the embryo, and in the extraembryonic yolk during embryogenesis. The amount of each sample was quantified by the amount of tripalmitin. Black squares indicate the amount of TG in the embryonic tissues. Open circles indicate the amount of TG in the extraembryonic tissues. Black diamonds indicate the amount of TG in the whole egg. Sampling was performed every 24 h and is shown for each developmental stage. Roman numerals in the top row indicate the three phases of lipid incorporation into the embryo. Each datum point with error bar represents a mean±standard error (n=3).

hematoxylin, or both oil red O and hematoxylin. Oil red O specifically stains regions with fatty acids and neutral lipids with a red color (Lillie and Ashburn, 1943). Hematoxylin stains proteins and nucleic acids (Puchtler et al., 1986, Bettinger and Zimmermann, 1991), but does not stain lipid droplets (Fig. 1C). Using this staining method, it was demonstrated that there were a lot of lipid droplets around the yolk granules in yolk cells. These results correspond well with a previous electron-microscopic report that there are many lipid droplets and yolk granules in the yolk-cell cytoplasm in *B. mori* (Miya et al., 1972). Moreover, frozen sections stained with oil red O allowed observation of the overall distribution of lipid droplets in the whole egg during the embryogenesis, which has scarcely been observed by TEM.

The manner in which lipid from the extraembryonic yolk is incorporated into the embryo differs slightly among species. In Diptera, all of the extraembryonic yolk is enclosed by the amnioserosa and is then finally incorporated into the midgut during dorsal closure (Hartenstein, 1993). On the other hand, in Lepidoptera, initial incorporation of the extraembryonic yolk into the embryo takes place during dorsal closure, but some extraembryonic yolk remains (Truman and Riddiford, 1999; Lamer and Dorn, 2001). A second incorporation occurs through ingestion of the extraembryonic yolk by the developing caterpillar itself before larval hatching (Yamashita and Yaginuma, 1991; Dorn et al., 1987; Truman and Riddiford, 1999). Our morphological data suggest that the transition of lipid droplets from the extraembryonic yolk to the embryo during embryogenesis reflects the incorporation of extraembryonic yolk into the embryo. Moreover, we observed that some lipid droplets were localized in the embryonic tissues before blastokinesis. We can divide the process of incorporation of these lipid droplets into three steps (Fig. 2). The first step (I) occurred during early embryogenesis, before blastokinesis; the total lipid content in the egg was almost constant, whereas the quantity of lipid droplets in the embryos gradually increased, and that in the extraembryonic area decreased. The second step (II) occurred during blastokinesis (stages 21A–C); the lipid droplets associated with the extraembryonic yolk were incorporated into the midgut by the dorsal closure. The third step (III) was observed in developing caterpillars during head pigmentation stages 26–27; the lipid droplets associated with the extraembryonic yolk were incorporated by developing caterpillars through ingestion of the extraembryonic yolk.

It has been shown that the lipids stored in an insect's eggs consists mostly of TG (Beenakkers et al., 1981; van Antwerpen et al., 2005), and in accordance the majority of the lipid in the eggs of *B. mori* is also TG, as shown by biochemical analyses of lipid components (Nakasone, 1979; Miura and Shimizu, 1987). We extracted lipid components from *B. mori* eggs in the same manner as Nakasone (1979). The extracted lipids were separated by TLC by using eluents that can separate neutral lipid components into TG, free fatty acid, and diglyceride. The major components migrated faster than free fatty acid or diglyceride. The Rf values of the major components were similar to that of tripalmitin, a kind of TG. In reviewing our data and previous reports, it is obvious that the major bands were TG. The TG bands seem to contain at least three different kinds of TGs. Nakasone (1979) reported several varieties of TGs in the *B. mori* egg.

When the amount of TG in the egg was examined during embryogenesis by means of TLC analyses, TG was found to be almost constant prior to blastokinesis. The amount gradually decreased during blastokinesis, reaching half the initial volume during the head pigmentation stage, and was finally almost all gone within the hatching larva. From the morphological study, the timing of TG reduction seems to be synchronized with the timing of reduction of the percentage lipid area. Whilst the transitional pattern of the amount of TG distributed between the embryo and extraembryonic yolk, extracted by dissection, was also similar to that found in the morphological study, the sum of TG in the embryo and extraembryonic yolk was not equal to the amount of TG extracted from whole eggs. This discrepancy in TG content between these separate samples may have been due to some technical aspect of the egg dissection process. Nonetheless, the TLC data convincingly support the results of the morphological analysis with respect to the localization of lipid droplets in the egg during embryogenesis.

Previous studies suggest that lipids are mainly used as energy sources for late insect embryogenesis (Beenakkers et al., 1981). Nakasone (1979) demonstrated that the decrease in the total amount of lipid contents during embryogenesis in *B. mori* was mainly due to a decrease in the amount of TG. According to Miura and Shimizu (1987), TG content gradually decreased from day 5 after oviposition, and the respiratory quotient values were close to 1.0 from the early to middle stages of embryogenesis (until day 4 after oviposition); from that point on, the values decreased to about 0.8, suggesting that carbohydrate was used as a source of energy throughout development in *B. mori*, but that lipid was used during late embryogenesis. Our results correspond to these studies, since most TG in the developing egg was consumed by late embryogenesis. In this phase, the developing caterpillar began to move inside the chorion. Our results indicate that lipid consumption seems to be synchronized with tracheal development in the developing caterpillars. To obtain energy as ATP from lipid, β-oxidation must occur following aerobic metabolism in the mitochondrial crista (Beenakkers et al., 1981). From stages 24 to 25, the trachea becomes well developed (Ohtsuki, 1979; Yamashita and Yaginuma, 1991; Singh et al., 2002); thus the developing caterpillars could obtain sufficient oxygen for their mitochondria. During late embryogenesis, lipid may be consumed using oxygen obtained through the newly developed trachea.

During the third step (stages 29–30), larvae must need a greater energy supply until they start to feed on mulberry leaves after hatching. Energy supplied by lipid consumption probably supports active larval movements. Accordingly, TG consumption reaches a maximum during this period.

As far as we know, this is the first observation of lipid-droplet intake separated into three steps. In particular, the first step, the translocation of some lipid droplets to embryonic tissues prior to blastokinesis, is remarkable. Whilst the incorporation of the majority of lipid droplets into the embryo is synchronized with the incorporation of extraembryonic yolk during the second and third steps, in the first step lipid droplets seem to be absorbed directly into the embryonic tissues. During the second and third steps, lipid droplets are probably absorbed via the midgut epithelia after the incorporation of the extraembryonic yolk into the midgut. Thus, the

manner of incorporation in the first step is quite different from that in the other steps.

Many studies have investigated the changes in lipid components during insect embryogenesis (Benakkers et al., 1981; Nakasone, 1979; Miura and Shimizu, 1987); however, there has been little attention to how and when lipid droplets are incorporated into the embryo. The present study demonstrates, both morphologically and biochemically, changes in the localization of lipid droplets during embryogenesis. It is notable that incorporation of lipid droplets starts during early embyogenesis. It is possible that lipid droplets incorporated in the first step are stored and used for subsequent development. The precise mechanism for the uptake of lipids from the droplets by embryonic tissues, either from the surrounding yolk cells during the first step, or from the incorporated extraembryonic yolk inside the midgut during the second and third steps, is still unknown. It is possible that the transport of TG from the lipid droplets to the embryonic tissues involves lipid carrier proteins, such as lipophorin (Chino and Downer, 1982; Kawooya et al., 1988). Further morphological studies by TEM are required to investigate these mechanisms of lipid transport.

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