カイコ発生卵における脂肪滴含有状態の超微形態学的検討

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はしがき

動物の胚発生において、胚子の成長には栄養成分はもちろん、エネルギーの適切な供給が必要不可欠である。胎盤を介して母体から絶えず供給を受ける哺乳類（胎生生物）とは異なり、昆虫類をはじめとする地球上に生息する大部分の動物種（卵生生物）は、発生開始から採餌を開始するまでの間、胚発生に必要な全てのエネルギーを脂肪やグリコーゲンなどの卵に予め貯蔵された物質に依存している。従って、卵生生物の胚発生過程における胚子へのエネルギー供給は、卵黄タンパク質の分解機構と同様に厳密な発生プログラムの調節下におかれている。

昆虫類の卵は、栄養成分に富み、グリコーゲン顆粒や脂肪滴が未受精卵の段階から卵内に含まれている。胚発生の開始直後から、胚子は、グリコーゲン顆粒や脂肪滴を細胞内に取り込み分解し、エネルギーとして利用している。グリコーゲンは主に胚発生前半に、中性脂肪は胚発生後半に消費される事が報告されている。円滑な胚発生遂行のためには、この限られた資源を効率良く組織内部に取り込んで維持し、適切なタイミングと場所（組織）で利用することが重要であるが、その詳細については殆ど解明されていない。その仕組みを明らかにする事は、昆虫類をはじめとする全ての卵生生物の胚発生現象の解明において非常に重要である。

本研究課題の目的は、卵生生物の栄養分・エネルギー源供給機構の解明の一環であり、脂肪滴を多く含むカイコ卵を用い、カイコ発生卵に含まれる脂肪滴の分布を正しく把握する事にある。昆虫の胚発生過程における脂肪滴の取り込みと消費機構を明らかにするとともに、本研究遂行のために必要な電子顕微鏡試料作製方法の確立を目指した。

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<u>山濱由美</u>、村中祥悟、熊切葉子、保智己、針山孝彦
カイコ卵の初期発生段階における脂肪滴動態の形態学的解析と超微細構造観察法の検討
日本動物学会第 78 回大会（弘前）平成 19 年 9 月

研 究 成 果

はじめに

卵生生物の胚発生過程において、卵に蓄積されているエネルギー源であるグリコーゲン顆粒や脂肪滴の胚体内への取り込みは、その後の胚の生存に関わる重要な現象である。特に、脂肪滴はエネルギー効率も高く、胚発生後半の主要なエネルギーとして利用されるが、胚発生過程における胚体内への脂肪滴の取り込みについてはあまり研究が行われていない。

本研究課題では、カイコ胚発生過程において、胚体外卵黄に含まれる脂肪滴が、いつ、どのように胚組織内へと取り込まれるかを解明することを目的として行った。その成果を(1)「Changes in lipid droplet localization during the embryogenesis of the silkworm, *Bombyx mori*」として以下に報告する。

カイコ卵は、硬い卵殻に包まれ、大量の栄養物質を含むがゆえに、電子顕微鏡試料作製が非常に困難な材料である。本研究課題では、流動性のある脂肪滴を超微形態学的に検討するためには、急速凍結法により卵細胞内部の脂肪滴を瞬間的に凍結固定することが有効な方法であると考え、液体窒素への投入や液化プロパンなどへの投入等、様々な急速凍結法による試料作製を試みてきた。しかし、細胞内に氷晶が形成されることによる組織破壊が起こるために、充分な電子顕微鏡観察像が得られなかった。そこで本研究課題では、無氷晶凍結が可能な高圧凍結・凍結置換法をカイコ卵の電子顕微鏡試料作製に適用することにより、脂肪滴などの成分流失を極力抑え、容易で、かつ安定した試料作製方法を確立した。さらにこの方法を用い、カイコ初期発生過程での脂肪滴の局在について検討を行ったので、(2)「高圧凍結・凍結置換法を用いたカイコ発生卵の電子顕微鏡試料作製法と、カイコ初期発生過程における脂肪滴含有状態の電子顕微鏡像」として以下に報告する。

(1) Changes in lipid droplet localization during the embryogenesis of the silkworm, *Bombyx mori*

Abstract

Lipid droplets in Lepidopteran eggs are considered one of the most important energy sources 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 the tracheal development.

Keywords: *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, proteins are used as sources of amino acids (McGregor and Loughton, 1977), whereas, carbohydrates and lipids are used as energy sources during embryogenesis (Steele, 1981, Beenakkers *et al.*, 1981).

In insects which 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 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 investigate the manner in which lipid droplets are incorporated into the embryo during insect embryogenesis. 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 they have a particularly high lipid content, which amounts to about 12% of their wet weight (Gilbert, 1967) or 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 using morphological and biochemical methods.

MATERIALS AND METHODS

Animals

A standard strain (Daizo) of the silkworm *Bombyx mori* was used. Under normal conditions, moths lay diapause and /or non-diapause eggs. In order to obtain "parental moths", that only layed non-diapause eggs we first reared parents using three sequential steps. First, selected eggs were kept at 15℃ under complete darkness during their

embryonic period. Second, the larvae were fed on mulberry leaves and kept under a long-day (Light 16 : Dark 8) light regime at 25℃ for about 1 month. Third, pupae were kept under dark conditions at 25℃ 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℃ under dark conditions until hatching. Larvae hatched out on the 9th day 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 5 eggs were collected every 24 h after oviposition, until hatching. In order to facilitate the rapid penetration of the fixative solution the chorion, which surrounds the egg, was removed using a fine needle (27×3/4G, Termo Co. Ltd., Japan) in Grace's insect culture medium (DAIGO, Nippon Seiyaku Co.Ltd., 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℃ in the refrigerator, 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℃, and the concentration of sucrose was gradually increased to 20%. After this

treatment eggs were immersed in the embedding medium (OCT compound, Miles Scientific Co.Ltd., USA), and were frozen using dry ice. Sections (6 μm) through the median plane of each egg were made 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. Ltd., Japan), and digital images were obtained using an attached CCD camera (DP-20, Olympus Co.Ltd., 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), using the histogram function, by counting 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. Ratios of the total red area to the area of the whole egg were also obtained. Data were expressed as averages with standard error for 3 eggs. The values were analyzed using the 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 in 200 μl of 25 mM Hepes buffer containing 150 mM NaCl (pH7.0), and were gently dissected into embryo and extraembryonic yolk using fine needles (27×3/4G, Termo Co. Ltd., 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.Ltd, Germany) using hexane/diethylether/acetic acid (80:20:1, by volume) as an eluent, and were observed under ultraviolet light (365 nm) using a transilluminator after spraying the plate with 0.005% primulin (MP Biomedicals Inc., France) in 80% acetone. Each lipid spot was quantitatively analyzed using Image-J (ver.1.38, NIH, USA) with tripalmitin (T-5888, Sigma, USA) as a standard. Therefore the amount of TG in ten eggs was expressed in tripalmitin equivalent. Statistical analysis was performed using Student's *t*-test.

RESULTS

Figure 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 and the diameter is 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 (Figure 1B), by hematoxylin (Figure 1C), or by oil red O and hematoxylin (Figure 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 by the unsolved fine dye crystals which could not remove 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. Those parts which 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 (Figure 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 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 using frozen sections (Figure 1D-H). At the early organogenesis stage (stage 17, Figure 1D), the extraembryonic yolk occupied a greater area of the egg than the embryo. Most lipid droplets were localized in the extraembryonic yolk area, whereas a small number of lipid droplets were observed in the embryonic tissues. At the early blastokinesis stage (stage 21A), the embryo became invaginated toward the center of the egg and several lipid droplets were observed both in the embryonic tissues and the extraembryonic yolk area (data not shown). At the end of the embryonic reversal stage (stage 22), embryonic dorsal closure and embryonic reversal were completed; half of the red dots were observed in the developing embryos, and the other half remained in the extraembryonic yolk (Figure 1E).

At the head pigmentation I stage (stage 26), the embryos adopted caterpillar-like shapes (the developing caterpillar); the extraembryonic yolk still remained outside of the developing caterpillars (Figure 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 became black naturally; 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 (Figure 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 (Figure 1H).

Figure 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 trachea was observed (Figure 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 the changes in the amount of the triglyceride (TG) during embryogenesis. Lipid extracted showed three major bands which had similar Rf value to tripalmitin (Figure 3). The amount of each 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 almost disappeared by stage 30 (Figure 3, 4). Amounts of TG in the embryo and in the extraembryonic yolk were compared (Figure 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 stage 26 - 27. In contrast, the amounts of TG in the embryo were small from early embryogenesis to blastokinesis, and reached a maximum amount of TG at stage 27. Finally most of TG in the embryo had 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 in the early 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 during late 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 easily 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 using organic solvents. Serial frozen sections were stained by oil red O, by hematoxylin, and both oil red O and hematoxylin, respectively. 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 it does not stain lipid droplets (Figure 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 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 the 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 suggests 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, it was 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 (Figure2). The first step (I) is 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. During blastokinesis (stage 21A-C), the second step (II) occurred: the lipid droplets associated with the extraembryonic yolk were incorporated into the midgut by means of their dorsal closure. The third step (III) was observed in developing caterpillars during head pigmentation stages (stages 26 to 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 a biochemical analysis of lipid components (Nakasone, 1979, Miura and Shimizu, 1987). We extracted lipid components from *B .mori* eggs by the same manner of Nakasone (1979). The extracted lipids were separated by TLC using the eluents which can separate the neutral lipid components into TG, free fatty acid, and diglyceride. The major components migrated faster than free fatty acid and diglyceride. The Rf value of 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 composed of TG. The TG bands seem to contain at least three different kinds of TGs. Varieties of TGs in *B. mori* egg has been reported by Nakasone (1979).

When the amounts of TGs in the egg were examined during the embryogenesis by means of TLC analyses, TG was almost constant prior to blastokinesis. The amount gradually reduced during blastokinesis, reaching half volume during the head pigmentation stage, and was finally almost all gone within hatching larva. From the morphological study the timings of TG reductions seem to be synchronized with the timing of reduction of percentage lipid area. Whilst the transition pattern of the amount of TG between the embryo and extraembryonic yolk, extracted using dissection, were also similar to those found in the morphological study, the sum of TGs in the embryo and extraembryonic yolk was not equal to the amount of TGs extracted from whole eggs. The loss of TG in these separate samples may perhaps be accounted for by some technical aspect of the egg dissection process. Nonetheless, it is convincing that the TLC data supports the results of 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 was mainly due to a decrease in the amount of TG in *B. mori*. According to Miura and Shimizu (1987), TG content gradually decreased from the 5th day after oviposition, and the respiratory quotient values were close to 1.0 from the early to middle stage of embryogenesis (until the 4th day 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,

but that lipid was used during late embryogenesis in *B. mori*. Our results correspond to these studies, since most of 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 the stages 24 to 25, the trachea become 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 (stage 29-30), larva must need a greater energy supply until they start to feed on mulberry leaves by themselves after hatching. Energy supplied by lipid consumption probably supports the 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 for the first step is quite different from the other steps.

Many studies have been performed to investigate 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 the changes of the localization of lipid droplets during embryogenesis both morphologically and biochemically. 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 droplet to the embryonic tissues involves lipid carrier proteins, such as lipophorin (Chino and Downer, 1982, Kawooya *et al.*, 1988). Further morphological – TEM studies are required to investigate these mechanisms of lipid transport.

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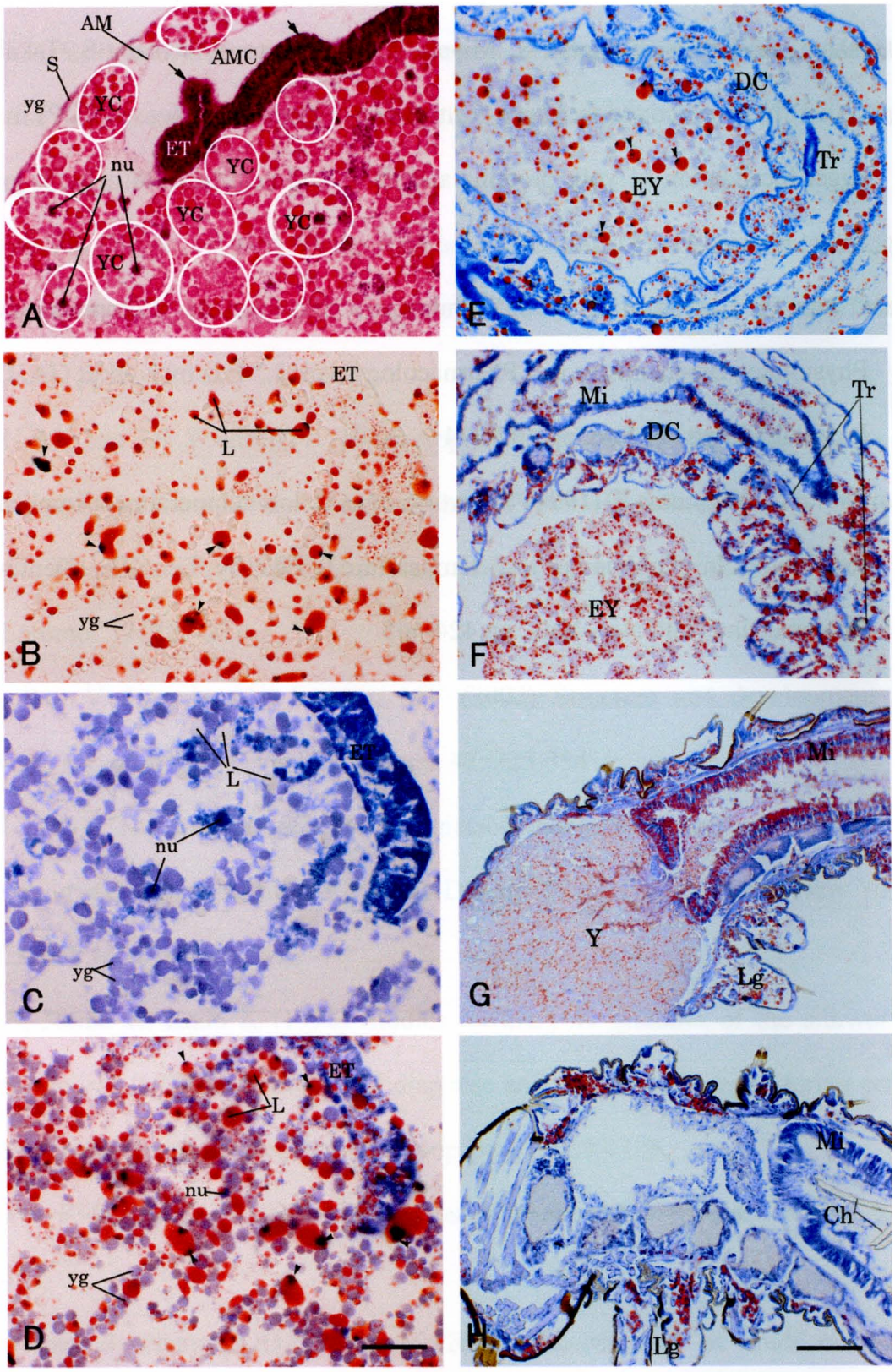

Figure 1: Morphological studies of the *Bombyx* egg during the embryogenesis. A: Paraffin section of a developing egg in early organogenesis stage (stage 17) stained with hematoxylin and eosin. White line circles indicate the yolk cell area. B-D: serial frozen sections at stage 17 stained with oil red O and/or hematoxylin. B was stained with oil red O. C was stained with hematoxylin. D was 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 indicates 50 μm in D. E-H are at the same magnification and the scale bar indicates 100 μm in H. ET: embryonic tissue, EY: extraembryonic yolk, YC: yolk cell, yg: yolk granule, nu: nucleus, L: lipid droplet, S: serosa, AM: amnion, AMC: amnion cavity, arrow: appendage, DC: developing caterpillar, Tr: tracheae, Mi: mid-intestine, Y: yolk, Ch: chorion, Lg: leg, arrowhead: remained oil red O dye crystal.

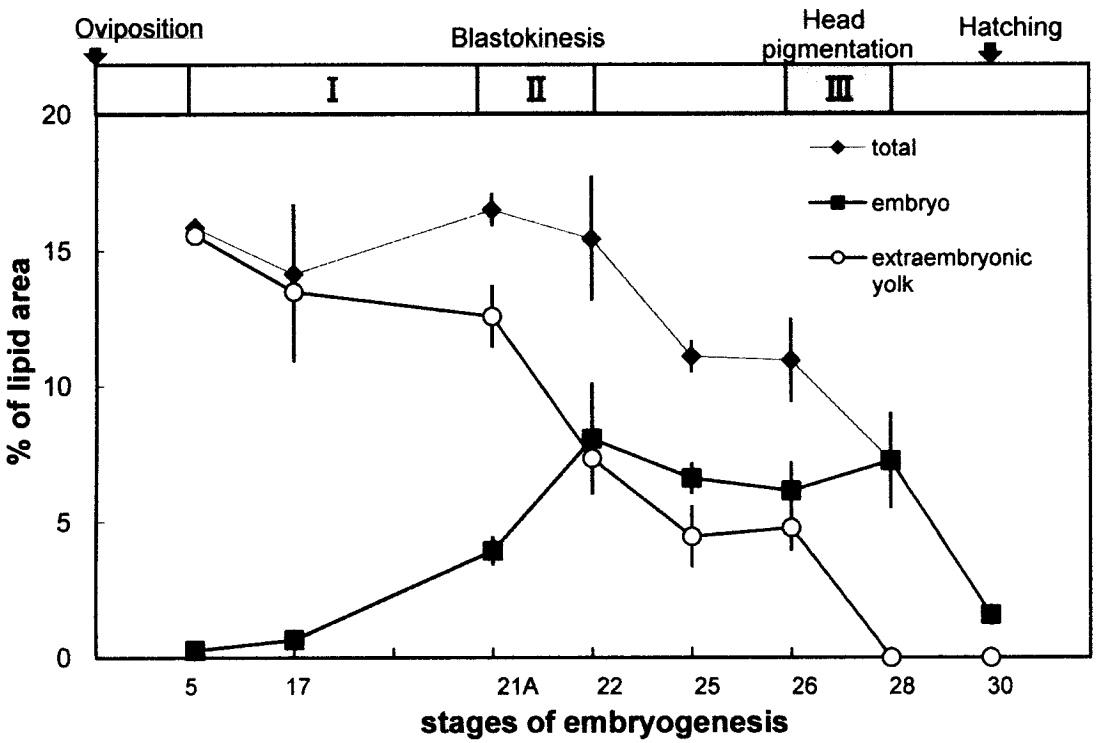

Figure 2: Changes in the lipid area stained with oil red O between the embryo and extraembryonic yolk during embryogenesis. Black squares indicate the percentage of lipid area in the embryonic tissues. Open circles indicate the percentage of lipid area in the extraembryonic tissues. Black diamonds indicate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

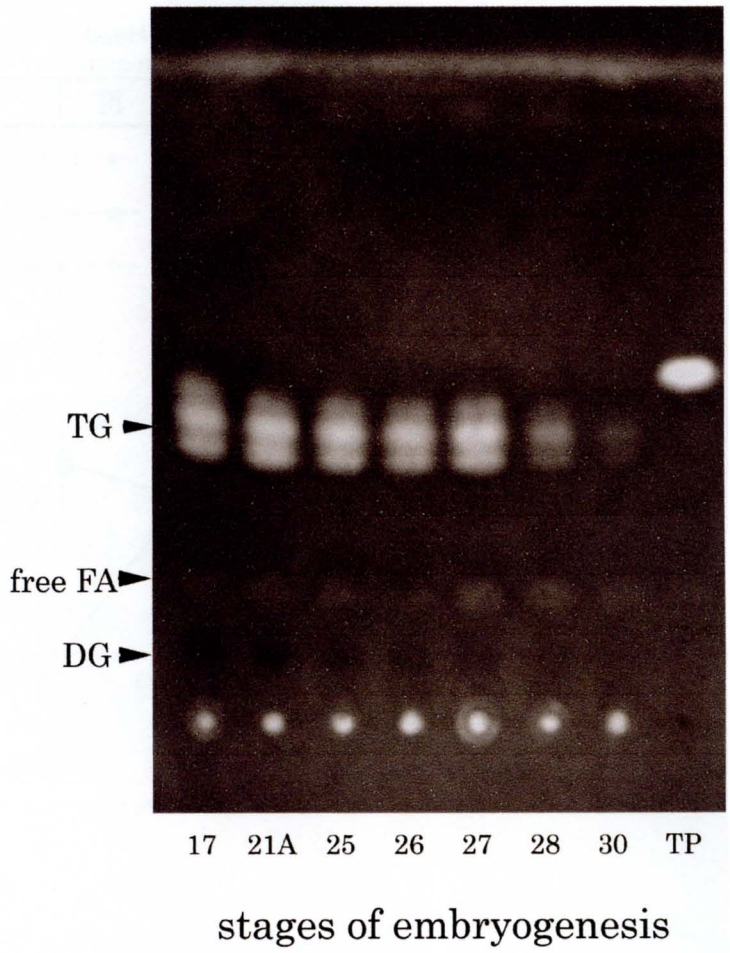

Figure 3: TLC study for triglyceride in the whole egg during embryogengesis. Each lane number shows the stages of embryogenesis and TP shows tripalmitin. TG: triglyceride, free FA: free fatty acid, DG: diglyceride.

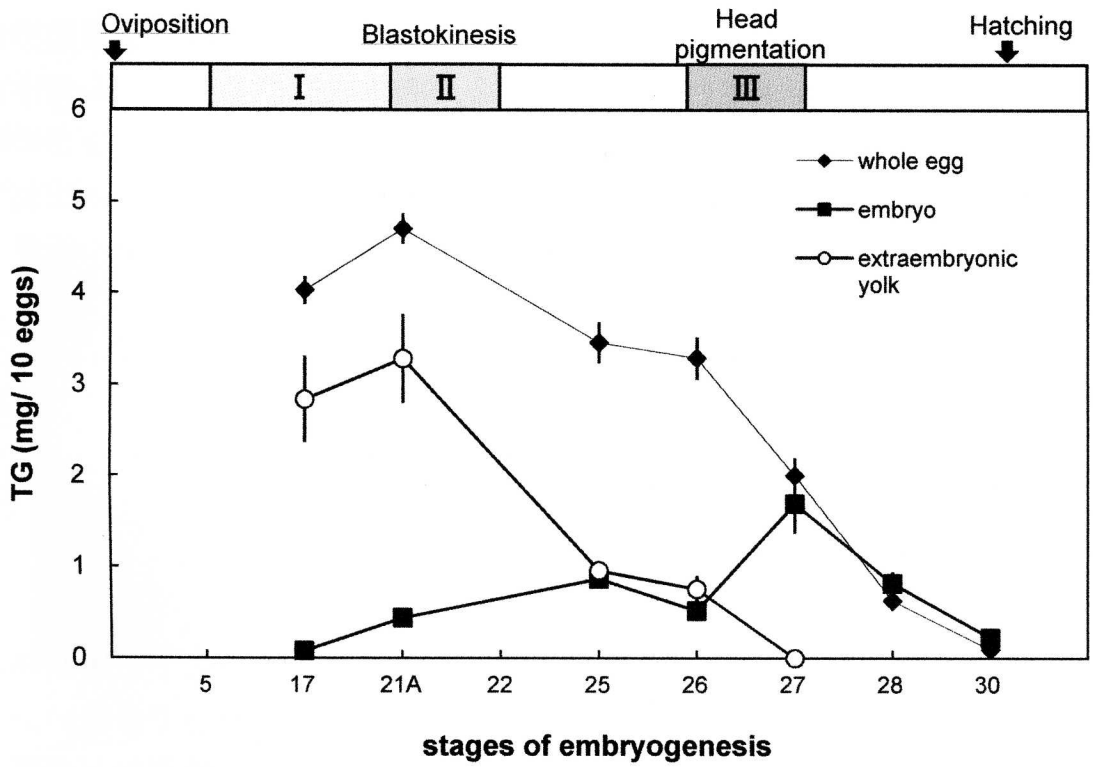

Figure 4: Changes in the amount of triglyceride (TG) in the whole egg, in the embryo, or in the extraembryonic yolk during embryogenesis. The amount of each sample was quantified by the amount of tripalmitin. Black squares indicate the amount of TG in the embryonic tissues. Open circles indicate the amount of TG in the extraembryonic tissues. Black diamonds indicate the amount of TG in the whole egg. Sampling was performed every 24h and is shown for each developmental stage. Numbers in the top column shows three phase of lipid incorporation into the embryo. Data shows mean ± standard error (n=3).

(2) 高圧凍結・凍結置換法を用いたカイコ発生卵の電子顕微鏡試料作製法と、
カイコ初期発生過程における脂肪滴含有状態の電子顕微鏡像」

カイコ卵は、昆虫卵の中でも特に試料作製が困難な材料である。その理由として、①硬い卵殻をもつ、②卵細胞は非常に軟らかく壊れやすい、③液体成分（水溶性・脂溶性）を多量に含む、ことなどがあげられる。カイコ卵の電子顕微鏡（以下、電顕）観察は、1960～70 年代に宮 慶一郎博士（岩手大学農学部）らの研究グループによる優れた研究がなされていたが、カイコ卵の電顕観察には高度に熟練した技術が要求されるだけでなく、液体成分があったであろう部分の構造が充分に維持されず、得られた観察像にばらつきが生じる事などの理由から、現在では殆ど行われていない。一方で近年、カイコ卵を用いた生化学的・分子生物学的解析の進展に伴い、形態学的な解析の重要性が高まってきている。私たちは、試料作製上のいくつかの技術的な問題を解決し、より簡単で再現性の高いカイコ卵の電顕試料作製方法を確立することにより、カイコ発生卵に含まれる脂肪滴の局在を検討した。発生過程での脂肪滴の局在性の変化については現在解析中であるが、本研究で確立したカイコ卵の試料作製方法についての成果を以下に記述する。

卵殻の除去

カイコ卵を電顕で観察するためには、まず卵殻を固定前に除去する事が必要である。カイコの卵殻は非常に硬く、殻の外側から固定液は浸透できない。また、強酸、強アルカリ、次亜塩素酸ナトリウム、タンパク分解酵素などの薬品処理を行っても溶解しない。従って、電解研磨したタングステン針などを用いて物理的に除去する方法が長年用いられてきたが、卵殻内部の卵細胞は非常に軟らかく壊れやすいため、物理的な卵殻除去には熟練を要する。本研究では、カイコ卵を虫ピンの頭に瞬間接着剤で固定し、実体顕微鏡下でグレース昆虫培養液中に移し、医療用のディスポーザブル注射針(27G×3/4, テルモ)を用いて卵殻のみを切り裂くようにして除去した(図 1 参照)。この方法は比較的容易に卵殻を除去することができ、卵殻を除去された卵は、培養液中で気管形成期前後まで成長した。

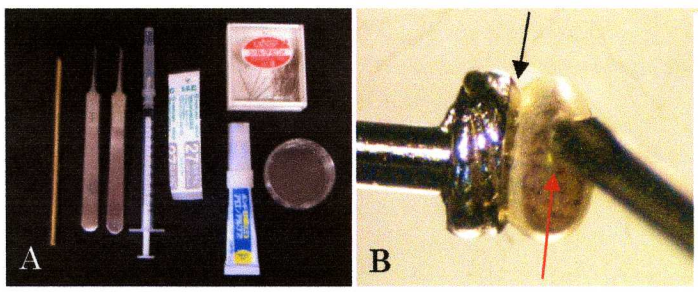

図 1　カイコ卵の卵殻除去
A; 卵殻除去に必要な器具
(左側より、ヘアループ，ピンセット 2 本，ディスポーザブル注射針(1ml 用)，注射針(27G×3/4)，虫ピン，瞬間接着剤，プラスティックシャーレ)、この他に必要な物品；実体顕微鏡，パスツールピペット
B; 卵殻除去の方法（実体顕微鏡下での作業）
虫ピンの頭部に卵を接着し（黒矢印）、実体顕微鏡下でグレース昆虫培養液満たしたシャーレ内で卵殻を注射針で縦方向（赤矢印）に切り裂くようにして卵殻を除去する。

高圧凍結・凍結置換

高圧凍結は、奈良女子大学理学部に設置されている高圧凍結装置（EM-PACT, Leica, Germany）を使用した。高圧凍結用の試料台は銅製の円盤で、中央の溝（直径 1.2mm, 深さ 200μm）に試料を置き、試料台の上下から挟み込むようにして試料ホルダーに装着する構造である。従って、この試料台の溝より大きい試料はこの装置で凍結させる事ができない。カイコ卵はこの試料台の深さより若干大きいため、フライス盤を用いて市販の試料台の溝をさらに 175 μm 掘り下げたものを準備した(図 2 参照)。この加工試料台（直径 1.2 mm, 深さ 375μm）を用いて高圧凍結を行ったところ、未加工の試料台と同様の 1990~2030 気圧の高圧状態を示し、加工試料台での高圧凍結が可能であることが示されたので、以降の試料作製には全て加工試料台を用いた。

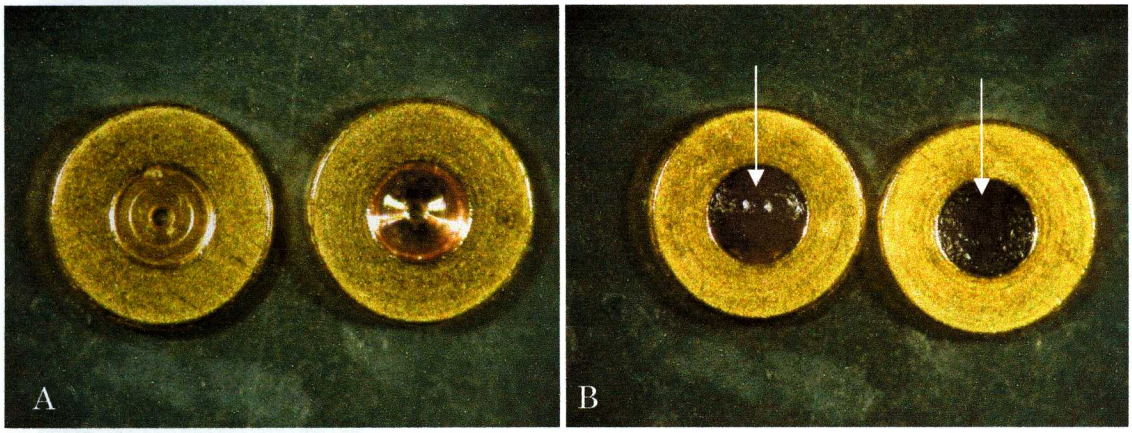

図 2　高圧凍結用試料台の金属加工
A; 未加工の試料台（左）と加工した試料台（右）
B; 卵殻除去卵を試料台に載せた状態
未加工の試料台（左）、加工した試料台（右）、卵殻除去卵(矢印)
未加工の試料台では卵は溝から盛り上がっているが、
加工した試料台では卵は溝に納まっている。

カイコ卵は、凍結直前に卵殻を除去し高圧凍結を行い（**未固定卵**）、液体窒素に入れた状態で浜松医大まで運搬し、本研究費補助金で導入した凍結置換装置(EM-AFS, Leica, Germany)を用い、オスミウム・アセトンによる凍結置換作業を行った。その後、Quetol 812 樹脂に包埋し、ウルトラミクロトーム(UCT, Leica, Germany)を用いて超薄切片を作製し、透過型電子顕微鏡(JEM-1220, JEOL, Japan)で観察を行った。比較として、通常の試料作製方法で作製された試料の観察像も示した（図 3 参照)。各試料作製方法による休眠卵の卵黄細胞の電顕像を比較すると、通常法で作製された試料では、卵黄細胞の内部には卵黄顆粒と核、ミトコンドリアなどが観察されたが、細胞内に無構造の部分が多く見られた。一方、高圧凍結・凍結置換法で作製された未固定卵の試料では、卵黄顆粒膜の一部破損が見られたが、通常法の試料で卵黄細胞内部の成分が消失していたと思われる部分に、多数の脂肪滴や細胞質基質が観察された。

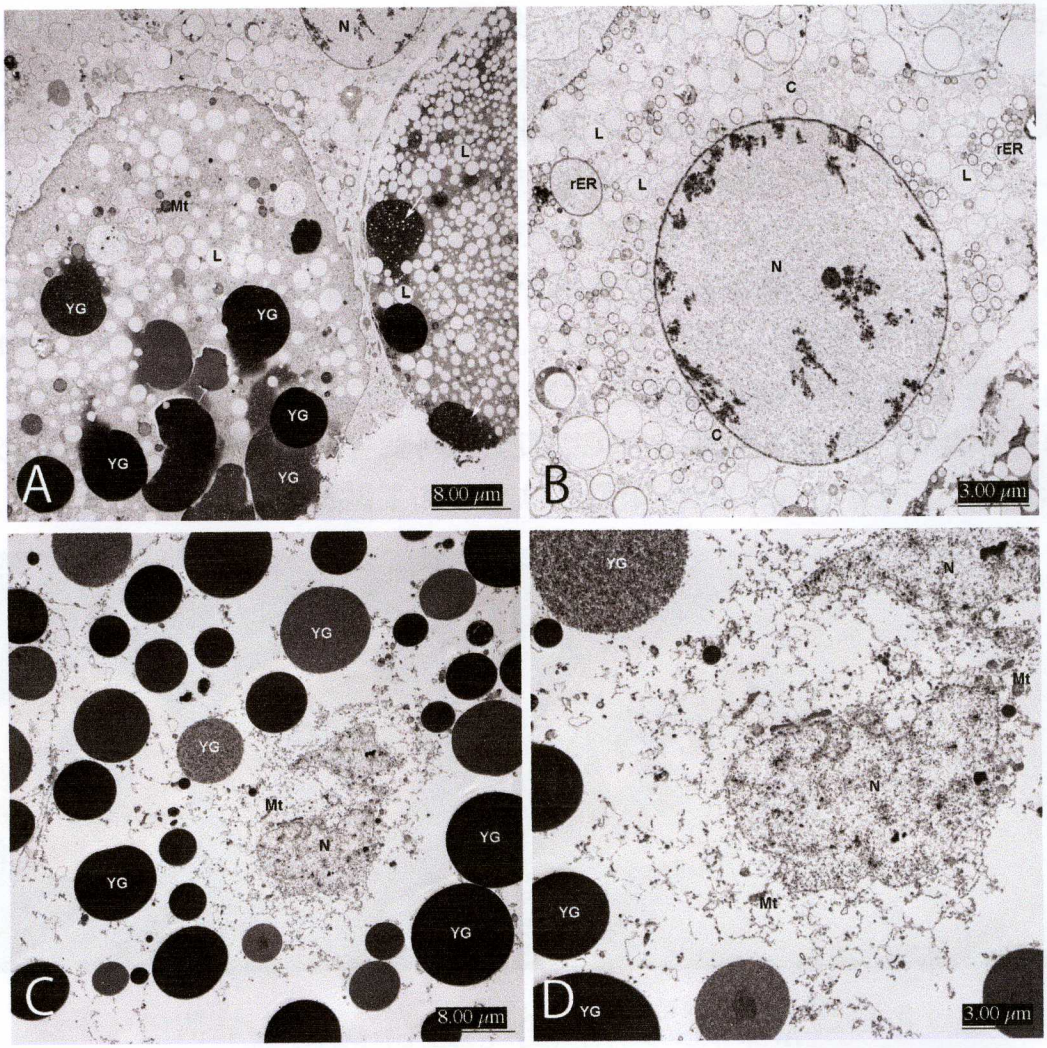

図 3　試料作製方法の違いによるカイコ休眠卵の電子顕微鏡観察像の差異
A, B; 高圧凍結・凍結置換法による（未固定卵）, C, D; 通常の試料作製方法,
B, D はそれぞれ A, C の拡大像
YG; 卵黄顆粒，N; 核，Mt; ミトコンドリア，rER; 粗面小胞体，L; 脂肪滴

また、高圧凍結前に 2%グルタールアルデヒド固定液により前固定を行った場合（固定卵）では、未固定卵の像と比較して、細胞質基質構造が壊れる傾向が見られた。特に、グリコーゲン顆粒などが消失する傾向があり、凍結前の固定作業中に流失したものと考えられる。従って、カイコ卵は、固定などの試料作製中に流出する成分が多量に含まれていることが示唆されることから、高圧凍結直前に卵殻を除去し、未固定のままで高圧凍結した試料が、最も生体状態を反映した電顕観察像である事が示された（図 4）。

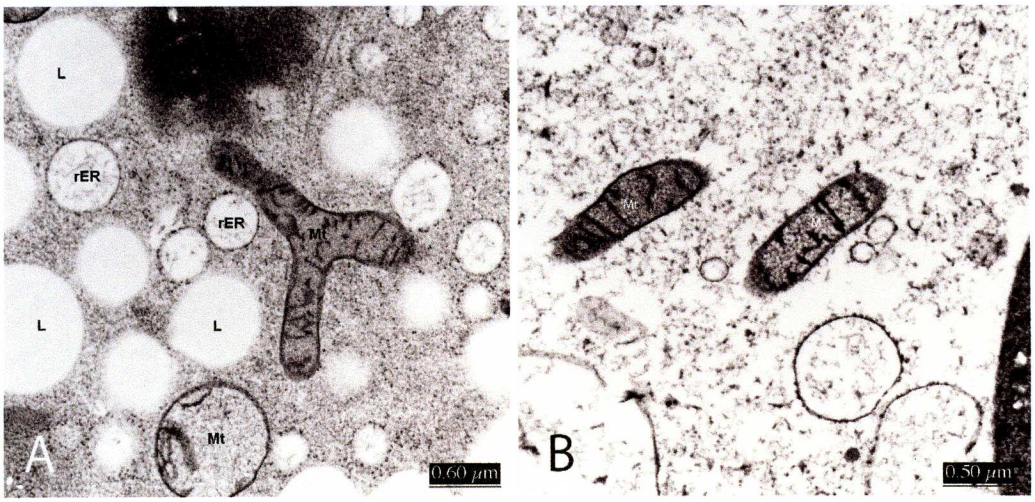

図 4　高圧凍結・凍結置換法で作製されたカイコ休眠卵卵黄細胞の超微細構造
A: 未固定卵, B: 固定卵 (2%グルタールアルデヒド前固定)
Mt; ミトコンドリア, L; 脂肪滴, rER; 粗面小胞体

カイコ初期発生過程における脂肪滴含有状態の電子顕微鏡像

研究（1）で示したように、発生過程において卵内に蓄積されていた脂肪滴は、胚体外卵黄から胚組織へと取り込まれ、孵化直前の発生後期に消費されている。胚体外卵黄に含まれる脂肪滴は、胚運動期以前(第一段階)、胚反転完了期(第二段階)、点青期(第三段階)の三段階で胚体内へと取り込まれるのである。ここでは、高圧凍結・凍結置換法を用いた試料作製方法により、初期発生過程におけるカイコ卵の脂肪滴含有状態を観察した。その成果については現在解析中であるが、その観察の一端として、第一段階の始めである胚盤葉期(stage 3)の胚組織と第二段階の胚反転完了期(stage 22)の幼虫体内の電子顕微鏡観察像を示す（図 5)。第一段階の胚盤葉細胞では、細胞内に脂肪滴がグリコーゲン顆粒と共に含まれていることが観察された。また、第二段階の胚反転完了期では、dorsal closure によって中腸内部に取り込まれた卵黄細胞と、発達中の中腸細胞、体腔内の脂肪体、それぞれの組織内部に脂肪滴が観察された。このことから、第二段階の発達中の幼虫体内において、中腸内の卵黄細胞の脂肪滴は、中腸内で分解・吸収され、体腔の脂肪体へと輸送されていることが示唆された。同時に、中腸細胞は形態学的に未分化な状態でも胚発生段階の早い段階から脂肪滴の輸送に関して機能的に働いていることが示唆された。

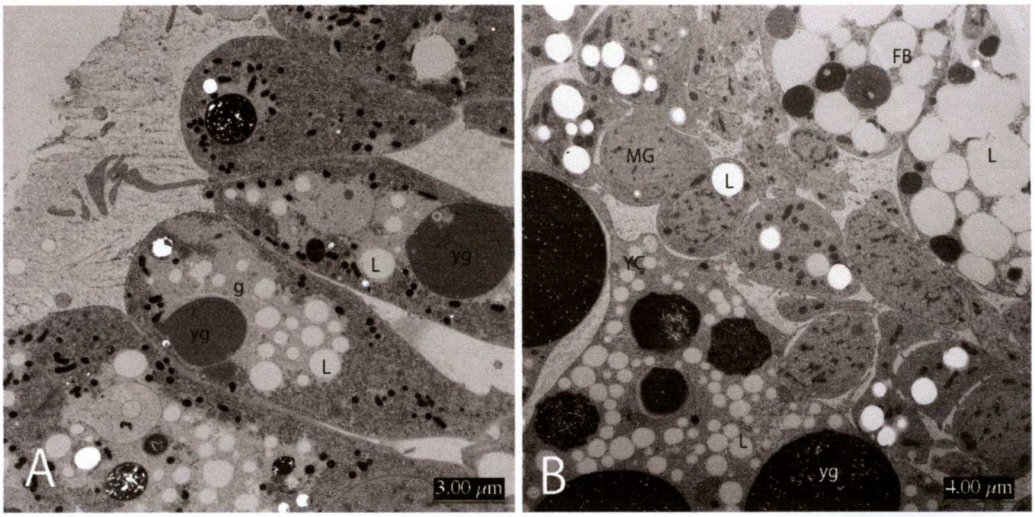

図 5　高圧凍結・凍結置換法を用いたカイコ発生卵の電子顕微鏡観察像
A; 胚盤葉形成期の胚組織,　B; dorsal closure 完了期の幼虫体内
MG; 中腸, YC; 卵黄細胞, FB; 脂肪体,
L; 脂肪滴, yg; 卵黄顆粒, g; グリコーゲン顆粒

本研究課題成果のもたらした今後の電子顕微鏡観察への展望と期待

本研究課題で得られた成果は、難しかったカイコ卵の試料作製をより確実で簡単なものとした事のみにとどまらない。第一に、通常法による電顕観察では捉えにくかった脂肪滴や細胞質基質の観察を容易なものとし、化学固定等によるアーティファクトの少ない、生体内の状態をより反映した超微細構造の観察が可能になった事が挙げられる。さらに、高圧凍結法は細胞・組織を無氷晶状態で凍結させる事ができるため、生きた状態の細胞・組織を瞬間的に凍結固定し、生体内での反応を超微形態学的に捉えることが可能である。また、試料台の制約により凍結可能な試料の大きさが限られていたため、これまではあまり多くの組織等の電顕観察に用いられることはなかったが、今回の検討で試料台を加工することにより、わずかでも凍結可能な試料サイズが大きくなった事は、非常に意義深い。たとえわずかな大きさの拡張であっても、高圧凍結が適用できる標本が大きくなったことにより、カイコ卵はもちろんの事、高圧凍結が可能になった標本が増えたからである。

本研究課題で得られた成果をもとに、今後、高圧凍結・凍結置換法を用いた電顕試料作製は、電子顕微鏡観察の主流となることが予想される。