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Histomorphological study on embryogenesis of the honeybee *Apis cerana*

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ABSTRACT

As important pollination species, honeybees play substantial impacts on the balance of global ecosystem, including two best-known honeybees *Apis mellifera* and *Apis cerana*. Embryogenesis is a fundamental stage of honeybee development and plays important roles in supporting the whole-life developmental process. However, few studies were reported on honeybee embryonic morphology using egg section, possibly due to the fragility of honeybee eggs and the difficulty of making embryonic sections. In this study, we reported a simply equipped method of frozen sectioning and PI (propidium iodide) staining to show the inner structure and cell distribution of *A. cerena* embryos at the different embryonic developmental stages. We found that the stages of *A. cerena* embryogenesis could also be typically classified into ten developmental stages, which are similar with the sister honeybee species, *A. mellifera*. To be noted, besides the cell distribution in the whole egg, we clearly observed the migration route of embryonic cells during the early embryonic development in *A. cerena*. This study provides a new insight into the whole process of honeybee embryogenesis from the perspective of egg sectioning, a histological basis for genetic manipulation using *A. cerena* eggs, and a reference method for egg sectioning for other insect species.

Introduction

Honeybees are an important group of Hymenoptera insects with complete metamorphosis, which have great economic and ecological values and are closely related to human. The well-known honeybees are *Apis mellifera* and *Apis cerana*. Both honeybees are domesticated and used to produce honeys or other bee products; meanwhile, both honeybees are raised for pollination of crops (Winston, 1991; Aizen and Harder, 2009). The whole development process of honeybees includes four stages: eggs, larvae, pupae and adult. It was widely accepted that honeybee embryogenesis was an important process and of great significance to the late-stage development of honeybees and formation of their social behavior.

The morphological studies of embryonic development of honeybees (*A. mellifera*) began in the late nineteenth and early twentieth century. Optical microscopy was mainly used for morphological observation of honeybee embryos during those days. Through observation under optical microscopy, Nelson (1915) and Schnetter (1934) roughly divided the development of honeybee embryos into eight stages: (1) fertilized eggs, (2) cleavage, (3) blastoderm formation and completion, (4) germ band formation, (5) mesoderm formation, (6) gastrointestinal tract and amniotic membrane formation, (7) basic completion of internal system, and (8) mature embryos. Later, DuPraw (1963) improved the observation method by continuously culturing an *A. mellifera* embryo at a constant temperature of 35.5 °C and observing its complete embryonic development. DuPraw (1967) clearly divided the process of honeybee embryogenesis into ten stages. Each of the stages was further divided into early, middle and late intervals. Overall, 10 developmental stages were defined with a detailed morphology of *A. mellifera* embryos. From then on, many studies on morphology and molecular biology of bee embryos referred to DuPraw's embryonic staging. For examples, Milne et al. (1988) used optical imaging system to take a series of pictures of honeybee embryos and depicted the development of honeybee embryos sorted by DuPraw's embryonic staging times. Dearden et al. (2006, 2014) staged the experimental honeybee embryos according to the scheme of DuPraw. Cridge et al. (2017) also referred to DuPraw's hand-drawing and their staging times to give a dark optical field view of the developing honeybee embryos.

With the development of observation equipment, electron microscopy has been employed to study the morphology of honeybee embryos. Based on the observations in the scanning electron microscope, Fleig and Sander (1985) described the successive changes at the cell surface of fertilized honeybee eggs, the very long process of blastoderm formation and the morphological change and arrangement of peripheral nuclei during this process. They further observed and described the whole process of honeybee embryogenesis based on embryos fixed at

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one hour intervals from oviposition to hatching using the scanning electron microscope (Fleig and Sander, 1986). Fleig and Sander (1988) continued to employ the scanning electron microscope to observe the migration of epithelial cell populations (epithelial expansion or translocation) in the honeybee embryogenesis, which appears to result from various combinations of ameboid movement, cell flattening, cell rearrangement (intercalation) and cell contraction along the free epithelial edge.

Since the late 1980s, many molecular studies of honeybee embryogenesis have been conducted and provided a strong base for understanding honeybee embryonic development (Cridge et al., 2017). And since the 21th century, with the wide use of fluorescence microscopy, many important techniques for visualizing gene expression in honeybee embryos have been developed, such as immunohistochemistry (Dearden, 2006; Dearden et al., 2009) or *in situ* hybridization (Osborne and Dearden, 2005a; Osborne and Dearden, 2005b; Wilson et al., 2014). For example, Dearden used the blue-fluorescent dye 4′,6-diamidino-2-phenylindole (DAPI) to counterstain *in situ* hybridized *empty-spiracles* gene, which revealed the expression of *empty-spiracles* gene at different embryonic stages and also showed the morphologic changing of honeybee embryos (Dearden, 2014). Additionally, the images of honeybee embryos have been usually taken using a stereo microscope with dark-field illumination at different embryonic stages (Cridge et al., 2017).

Most of the above morphological studies on honeybee embryos were focused on *A. mellifera*. There were few studies on the histomorphology of *A. cerana* embryos. It is generally accepted that making sections of honeybee embryos was difficult due to the fragility of honeybee eggs, especially at the early stages. When we looked up and read the relevant literature about honeybee embryogenesis, we seldom saw real images of complete embryonic section, instead hand-drawn images of inner embryonic structure in early literature. Here in this study, we employed a simply equipped method of frozen sectioning and PI (propidium iodide) staining in the section making of *A. cerana* embryos. We successfully made a series of *A. cerana* embryonic sections to show the inner structure and cell distribution of *A. cerena* embryos at ten embryonic developmental stages. These histological developmental atlas of *A. cerana* embryos provides a basic histological basis for the behavioral and neurological studies in the later stages of Asian honeybees.

Materials and methods

Experimental honeybees and ethics statement

The experimental Asian honeybees (*A. cerena*) were reared in the apiary of Honeybee Research Institute, Jiangxi Agricultural University, China (28.46°N, 115.49°E), same as our previous reports (Yan et al., 2016; Liu et al., 2019). All animal procedures were performed in accordance with guidelines developed by the China Council on Animal Care; and protocols were approved by the animal care and use committee of Jiangxi Agricultural University, China.

Collection of honeybee embryos

The *A. cerana* embryos were collected from honeybee colonies in the breeding season of *A. cerana* (the temperature usually ranges from 15 to 30 °C). A 100 μl plastic tip was made as a thin and soft embryo-transferring picker (as shown in Fig. S1). We used the end of the modified picker to softly contact the adhered posterior end of the embryos and gently move the embryos from the bottom of the cell.

To collect age-controlled embryos, mated egg-laying queens were first held in queen cages for 2–3 h, and then placed in the egg- and brood-free areas of a pre-cleaned comb; before the queen was placed, the comb was cleaned overnight by worker bees. During queen ovulation, the queen was confined to the free space of half a comb by a wooden or bamboo fence that only allows worker bees to come in and out but does not allow the queen out. In the process of limiting the queen, the operation of the queen and the colony was as gentle as possible, so as not to frighten the queen and the worker bees. The whole process was carried out in quiet conditions. After four hours, the queen was released from the egg-containing honeycombs. Generally, the queen needs to adapt to the new honeycomb for a period of time (more than 1 h), so the age of honeybee embryos should be about 0–3 h. To gain the appropriate honeybee eggs with specific developmental ages, the cultivation time of embryos in incubator were determined according to this developmental interval. If no embryo was not taken, the queen would be limited for another 4 h until the embryos were collected. Then the honeycomb with embryos was put in a thermostat (35 °C, 85% humidity) and the embryos were cultured to the corresponding age of each developmental stage. Then the embryos were taken for section making.

Observation of fixed embryos

Honeybee embryos were taken carefully and gently without any damage from the honeycomb using a self-made egg-taken picker (Fig. S1), and then fixed in 4% polyformaldehyde solution for 12–24 h. The fixed time varied according to the developmental stages of embryos; usually the shorter developmental time the shorter fixing hour. The fixed embryos at the different developmental stages were transferred to a slide and immersed in the polyformaldehyde solution. Then the immersed embryos were observed and photographed under a microscope.

Frozen section making

The fixed embryos were transferred into 20% sucrose solution for 24–48 h dehydration at 4 °C. The dehydration time varied according to the developmental stages of embryos; usually the shorter developmental time the longer dehydration hour. Due to more protoplasm and more water, the sections of early-stage eggs were much easier to deform and rupture. The dehydrated eggs were moved into a plastic box for embedding. Before egg embedding, the sucrose solution around the eggs was carefully sucked up with filter paper. Then the eggs were filled with optimal cutting temperature (OCT) embedding agent, and no bubbles should be generated. Then the box containing embedded eggs were put into the isopentane pre-frozen on liquid nitrogen surface.

After frozen, the embedded embryos were transferred to slice-making machine (LEICA CM1950) to make frozen sections. The frozen sections with thickness of 5–7 μm were cut out. The sections were placed in a cool place with room temperature below 20 °C for natural drying.

PI staining and microscopy observation

The 50 μg/ml PI staining solution was gently dropped to the dried embryonic section to keep the whole embryo immersed in a thin layer of PI staining solution. When dropping PI staining solution to the section, the action should be gentle. The dropping site was directly above the embryonic section. The dosage of one dropping was within 5 μl just covering the whole embryo and avoiding egg tissue floating. Then, the embryonic section was placed and observed under a fluorescence microscope (Leica, Germany) using green fluorescence as the excitation light. Photographs of honeybee embryonic sections were taken with red fluorescence.

Results

To study the development of *A. cerana* embryos at different stages more clearly, we not only observed the sections of *A. cerana* embryos at each stage, but also collected the pictures of polyformaldehyde-fixed *A. cerana* embryos at each stage under a microscope for comparison with the corresponding sections of *A. cerana* embryos. In the present study,

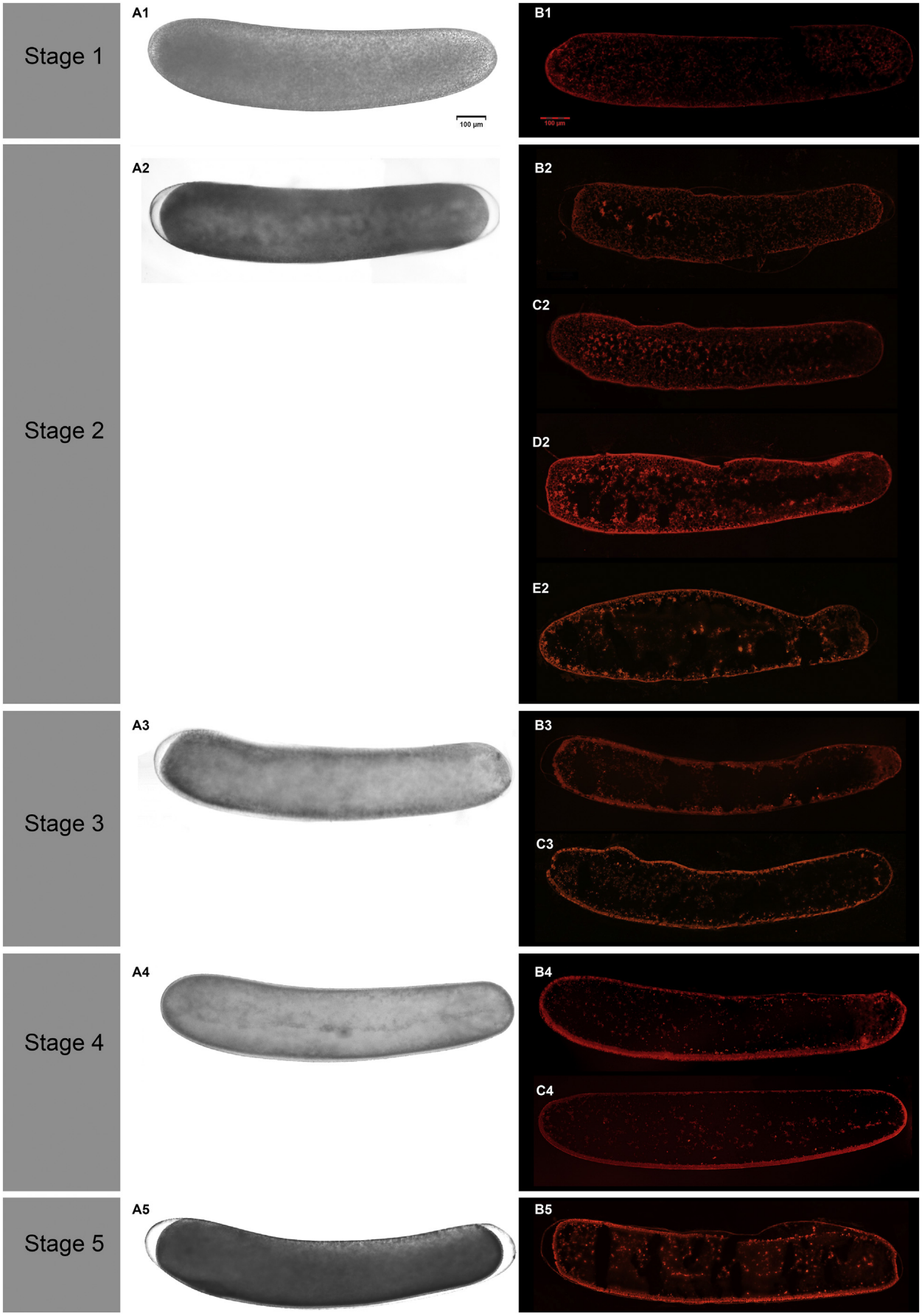

Fig. 1. Morphological observation of *Apis cerana* embryos at stages 1–5 after fixing (A1, A2, A3, A4 and A5) and PI staining (B1, B2, C2, D2, E2, B3, C3, B4, C4 and B5).

the pictures were displayed from a lateral view with the downward abdomen and the upward back.

Stage one

The age of the collected embryos at this stage was 0–4 h. The *A. cerana* embryos looked more like sausages: the interior was homogeneous in texture and there were no gaps at both ends of the eggshell (Fig. 1-A1). From the embryonic section, uniform and light-colored flocculent structures were found (Fig. 1-B1), which were the protoplasm and yolk fluid of the first-stage eggs. At the first stage, the cleavage cells were very few; it was difficult to detect the energids on a section. Because the peak period of cleavage did not reach, the inner nutrients were abundant and the embryonic texture was thick.

Stage two

The age of our collected embryos at the second stage was 5–8 h after oviposition. As shown in Fig. 1-A2, significant protoplast contractions occurred at the cephalic and posterior ends of the embryo, resulting in obvious gaps at both embryonic ends, which was a typical morphological feature of honeybee embryos at the second stage. Meanwhile, a columnar void was observed in the middle axis of the embryo, and the shadows of the gray clumps were seen around the voids, which were possibly the migrating energids. This chart showed that the cleavage cells had completed the migration to the posterior end and were migrating outward along the middle axis of the embryo (Fig. 1-A2). The inner structures of *A. cerana* embryos at the early, middle, late and end intervals of the second stage were shown in Fig. 1-B2, C2, D2 and E2. During the early interval of stage 2, a small number of distinct energids were detected around the region less than one third of the body length near the cephalic side of the embryo (Fig. 1-B2). During the middle interval of stage 2, cells proliferated quickly and the number of energids increased significantly. Most energids were around the middle axis of the embryo; there were more cells in the anterior half of the embryo than in the posterior half (Fig. 1-C2). Several energids have been distributed on the posterior region of the embryo, which indicated that some energids were moving to the posterior end of the embryo. As the developmental time went on, the energids at the later interval of stage 2 (Fig. 1-D2) were closer to the surface of embryo than the middle interval. At the end of stage 2, most energids had migrated into the embryonic periplasm and reached the inner surface of the embryo, only a few of energids remained in the protoplasm (Fig. 1-E2). Taken together, looking at these four images, we could clearly reveal the migration route of energids during the whole developmental time of stage 2.

Stage three

The age of our collected embryos at stage three was about 8–11 h. The typical morphology of honeybee embryo at the early third stage was shown in Fig. 1-A3. Many gemlike protuberances were observed at the cephalic side of the embryo. There were still gaps on both cephalic and posterior ends of the embryo; the gap at the posterior end was smaller than that at the cephalic end; but both gaps were less than those at stage two, which would finally disappear at the end of stage three. The histological sections of *A. cerana* embryos at the early and late intervals of stage three were shown in Fig. 1-B3 and C3, respectively. At the early third stage, most cleavage nuclei were just approaching the embryonic surface, the periplasm around each cleavage nucleus begun to indent. The gemlike protuberances on the embryonic surface were clearly observed at the anterior pole (Fig. 1-B3). While at the late interval of stage three, both gaps at the anterior and posterior ends disappeared, cell boundaries around the cleavage nuclei formed; monolayered embryonic blastoderm cells were visible on the surface of the egg, but there was still no arrangement of monolayer cells at the posterior side of embryo (Fig. 1-C3).

In Fig. 1-B3 and C3, a certain number of cleavage nuclei (about 10%), visible as bright red granules, were found in the yolk fluid of eggs. These cleavage nuclei remained in the interior of the protoplast, did not migrate to the periplasm and would become vitellophages. These nuclei were called as “yolk nuclei” or primary vitellophages. At the later stage of embryonic development, they would play important roles in transformation of the yolk fluid (DuPraw, 1967).

Stage four

At this stage, the age of embryos we collected was 16–22 h. The fixed embryo at this stage resembled the initial morphology at the first stage in appearance.

The liquid-filled gaps at both ends disappeared; the whole surface of the embryo could be seen surrounded by the blastoderm cells; and a dense line or layer was visible between the blastoderm and the yolk mass (Fig. 1-A4). This layer of inner periplasm first appeared in the anterior ventral region of the embryo, then gradually extended to both anterior and posterior ends, and finally surrounded the entire dorsal side.

In Fig. 1-B4, we found that the whole surface of embryo was surrounded by the blastoderm cells. On the ventral side, the structure of interlaced two-layer blastoderm cells was formed; while on the dorsal side, a single layer of blastoderm cells was visible. There was a foggy periplasm surrounding the inner side of the whole blastoderm where the yolk mass intersected; the periplasm on the ventral side was obviously thicker than that on the dorsal side (Fig. 1-B4). The blastoderm cells on the dorsal side do not rearrange and remain single layer structure during the whole stage of embryonic blastoderm; they do not form part of the embryo proper but may contribute to the formation of embryo membrane (amnion-serosa) in the later stage (DuPraw, 1967). Fig. 1-C4 showed a section of *A. cerana* embryo at late stage four. It was visible that the ventral cells of the embryo begun to transit to the regular single cell morphology. The periplasm and cytoplasm were gradually fusing, and the periplasm at this time became thinner than that at the early stage four.

Stage five

The age of the collected embryos at this stage was about 26–29 h. There were obvious fluid-filled spaces in the anterior and posterior ends of the egg again (Fig. 1-A5). The recurrent spaces were first detected at the anterior end and then observed at the posterior end. This embryo was in the late interval of stage five, and the dorsal single-layer cell area was more transparent than the ventral region (Fig. 1-A5). The embryo in Fig. 1-B5 also belonged to the later stage five. The large vacuoles in the blastoderm cells could be clearly visible, which was a typical developmental feature of the blastoderm cells in the later interval of stage five; the whole periplasm of the embryo was relatively uniform (Fig. 1-B5).

Stage six

The age of our collected embryos in this period was about 32–38 h. Fig. 2-A6 showed external morphology of a fixed embryo at the early stage six. There were still fluid-filled spaces at both anterior and posterior ends of the egg. The anterior side of the embryo became a little pointed; and the serosa structure of the single cell layer covering the anterior end was visible, which looks like a transparent cap. A conspicuous, plate-like thickening appeared in the ventroanterior blastoderm, which is the anterior midgut rudiment. The brighter band on the ventral side of the anterior end was the mesoderm which had not been covered by the ectoderm. Another image of fixed embryo in the a-little-later interval of stage six was showed in Fig. 2-B6. It was seen that the cap-like single-cell layer was more obvious and moving down to the ventral region at the anterior end. In the ventral side of the embryo

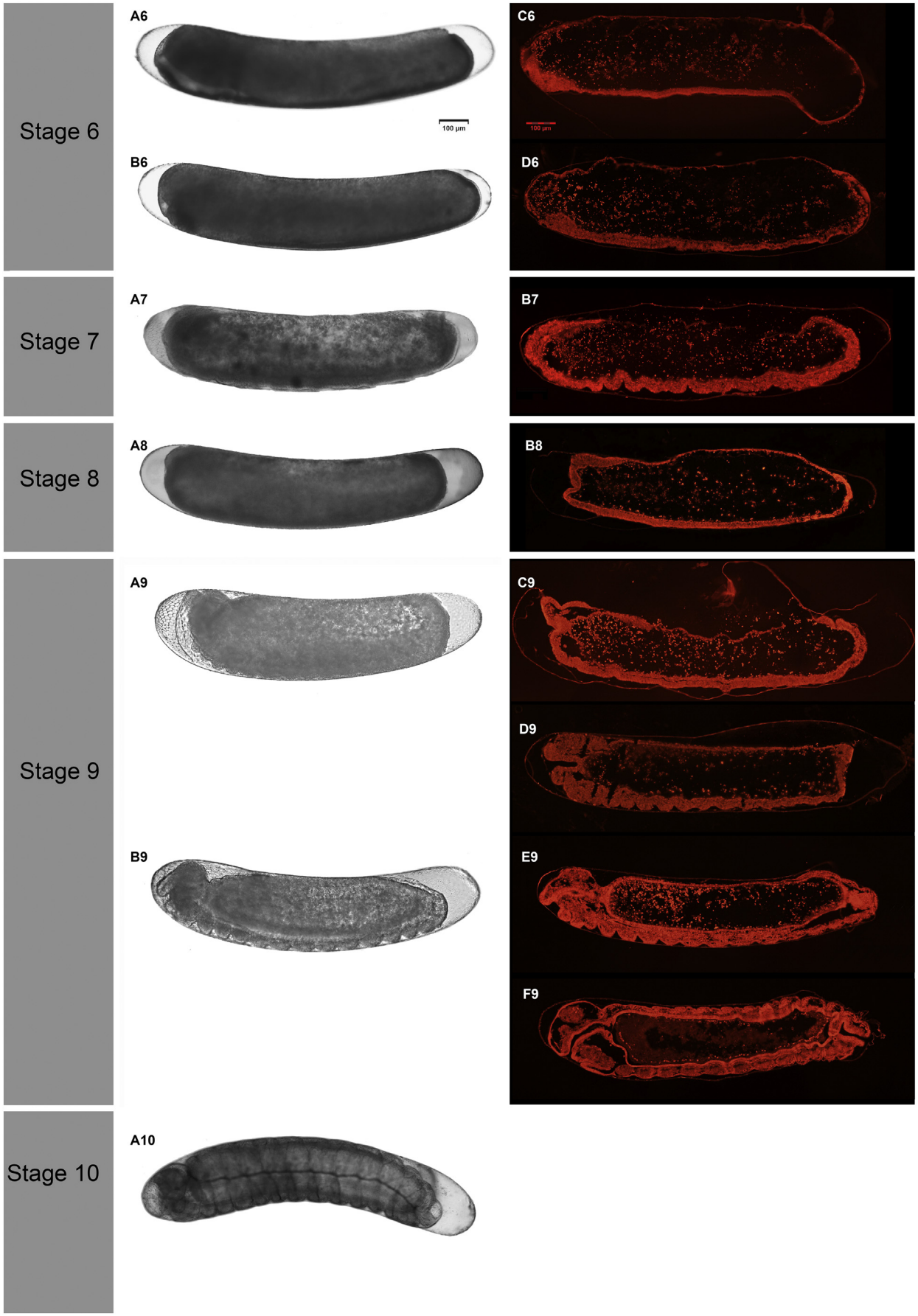

Fig. 2. Morphological observation of *Apis cerana* embryos at stages 6–10 after fixing (A6, B6, A7, A8, A9, B9 and A10) and PI staining (C6, D6, B7, B8, C9, D9, E9 and F9).

head, a large number of ectoderm cells could be seen to form long strips of shadow, and the shadow at the anterior ventral end near to the cap was much thicker.

As shown in the embryonic section of early stage six (Fig. 2-C6), there was a distinctly thicken dish-like structure at the anterior ventral side of the embryo, which was caused by the rapid ventral migration of the blastoderm cells at the anterior end of the protoplast. At the same time, a hood-like single-cell layer was visible between the chorion and the protoplast at the anterior end of the egg, which is the anterior part of the developing embryonic amnion-serosa. In addition, the embryonic amniotic chorion, a single layer of cells similar to that at the anterior end, was forming around the dorsal side of the embryo. By the end of stage 6, the amniotic serosa would surround the entire dorsal side, and the ventral margin of the serosa would coincide with the dorsal margin of the ectoderm. This was the developing embryonic amniotic chorion. Fig. 2-D6 showed the embryonic sections at the later interval of stage six. The ventrolateral ectoderm was gradually covering the abdominal mesoderm from both sides and extending toward the posterior end at the junction of the ventrolateral midline. This extension would terminate after reaching the posterior end and eventually converge with the gradually separated single layer cells (chorion) on the dorsal side. The posterior ends of the embryos in Fig. 2-C6 had some distortion, which was caused by the process of making sections. Its posterior end should be consistent with the posterior ends of the eggs of Fig. 2-A6. It would be blunt and round. The shape of the posterior ends was basically unchanged throughout the sixth stage.

Stage seven

The embryos we collected in stage seven were at the age of 38–44 h. The main developmental events in the seventh stage were the formation of the whole amniotic chorion and its complete separation from the embryo. The whole fixed embryo looked like a canoe, and there were dents in the middle ventral part between the anterior and posterior ends (Fig. 2-A7). A small arc protrusion was seen at the anterior end, which was the shadow of the separated amnion-serosa structure. The embryo of Asian honeybee in Fig. 2-B7 was in the late stage seven. The whole embryo was surrounded by the single cell layer of the amnion-serosa. The ventral part between the anterior and posterior ends had obvious depression. The shape at the anterior end was very similar to that at the posterior end. Based on the embryonic section, the whole embryo was obviously like a canoe. Compared to the sixth stage, the embryonic cells of the progenitor midgut at the anterior and posterior ends proliferated more frequently and the cell layer was obviously thicker.

Stage eight

The age of the collected embryos in this stage was 44–47 h. In the eighth stage, one of the most typical developmental events was that the progenitor of the midgut develops into the midgut. As shown in Fig. 2-A8, the embryonic protoplast contracted sharply during this period, the inner length of the embryo was the shortest in the whole embryonic development stage. The anterior and posterior ends resembled as the water tank, and there were no obvious head and other symmetrical appendages. It was difficult to distinguish the embryonic head and tail, but the space at the anterior end was smaller than that at the posterior end. At the anterior end there was a precursor of the lip formed by the thickening of the ectoderm.

In Fig. 2-B8, a conspicuous protuberance at the anterior end of the embryo seemed as the precursor of the lip. At the dorsal side of the egg, there was no longer a sparse single-celled serous chorionic structure, but a well-arranged single-celled structure. The midgut precursor completed the development of the midgut, and the whole yolk fluid was surrounded by a single layer of cells, forming a complete midgut cavity. And the indentation on the ventral surface from the anterior part to the posterior part disappeared.

Stage nine

The age of our collected embryos at this stage is 47–67 h. The main developmental events in the ninth stage are the formation of body segments and internal tissues. In Fig. 2-A9, a fixed image of Asian honeybee embryo at the early ninth stage was shown. Thirteen body segments and the front lip of the head were clearly visible; while the posterior tail was blunt and round. Fig. 2-B9 showed another fixed embryo at the middle or late ninth stage. It was seen that most of the external morphology of the larvae head was formed, the development of the ventral side was close to that of the larvae, and the tail was changed. The apex begun to form the tail organ, but the dorsal part was not well developed and no obvious thickness of the back was seen. Fig. 2-C9, D9, E9 and F9 showed the early, middle, late and end intervals of stage nine, respectively. The basic structure of the anterior lip just formed was seen in Fig. 2-C9. The body segments were not very clear yet. The dorsal side of the midgut cavity had just formed, so there was only a very thin layer of cells. Fig. 2-D9 showed 13 distinct body segments in the ventral region, which begun to form the embryonic stomatal prototype, the cell layer on the dorsal side of the embryo begun to thicken, the tail begun to change from a circular shape to a slightly protruding shape, and the shape of the tail begun to change. In Fig. 2-E9, we could see clearly the structure of the large-capacity brain, the outline of the mouth was clearer, the rudiments of three pairs of appendages (the mandibles and two maxillae) of the head were basically formed, the tail organs were sharpened, the basic shape had been formed, the back continues to thicken, and the pouch-like midgut cavity was very clear. Fig. 2-F9 showed the complete intracranial structure, clear oral structure and tubular structure of the whole digestive system, the dorsal morphology of the larvae, the bronchial structure from the second to the eleventh ganglia and the clear nerve cord in the abdomen.

Stage ten

The embryos were collected at 67–70 h. The main developmental event in the tenth stage was larvae hatching. A fixed-*in-vivo* view of Asian honeybee embryo at this stage was shown in Fig. 2-A10. Clear blood vessels were visible. The embryo had not yet ruptured the amnion-serosa, but it was ready to hatch. The tissue development of the larvae was basically completed at the end of ninth stage; the hatching processes were mainly carried out in this stage, including muscle activity, head and tail shaking and body rotation. The embryonic section at the tenth stage was same to the one at the end of the ninth stage, so it was not shown.

Discussion

In the morphological studies on honeybee embryos, optical, electron and fluorescence microscopies have been widely used to observe and describe honeybee eggs. It has provided us an abundant morphological data of honeybee embryogenesis and detailed honeybee embryonic stages. However, many of the internal structures of honeybee eggs and the processes of energid migration and blastoderm formation were presented in the form of textual descriptions, photos of the whole eggs and/or hand-drawn drawings. There were few reports on honeybee embryonic sections, especially few *A. cerana* embryonic sections. The reasons might be that the presence of a large number of egg fluids makes it difficult to make sections, especially for early embryos. Recently, we have provided some images of *A. cerana* embryonic sections in the related studies of transcriptome (Hu et al., 2018) and gene editing (Hu et al., 2019), but it was only a part of the whole embryogenesis. In this paper, applying a simple method of making sections, as far as possible to reduce the intermediate steps in the making process, we clearly showed the images of *A. cerana* embryos at ten stages one by one. The whole migration route of energids during early developmental stages of bee eggs were clearly shown through real images of embryonic

sections. Meanwhile, in the whole embryo picture, the formation and migration path of the blastoderm cells into the endoderm, mesoderm and ectoderm, the formation of the intestinal cavity and the formation of the internal organs of the embryo were well reflected. The display of these morphological changes through embryonic sections was not only a supplement but also a good verification to previous morphological studies of honeybee embryos.

Generally, two methods were widely used in making tissue sections in divergent species: paraffin section and frozen section. Previously, we applied a common paraffin sectioning method, an improved paraffin sectioning method of pre-embedding the eggs with swine fat and a frozen sectioning method to make embryonic section and observe their internal structure in *A. mellifera* (Hu et al., 2016). Our results showed that it was very hard to obtain the sections of honeybee eggs directly by the common paraffin sectioning method, mainly because that the process of making paraffin sections contained many immersing and cleaning steps and honeybee embryos were very small and easy to lose. We improved the paraffin sectioning method by pre-encapsulating honeybee eggs with swine fat to avoid egg losing; however, this improved method easily resulted in the whole contraction or collapse of honeybee eggs. We found that this improved method was suitable for local morphological observation of honeybee embryos at the late stages but still difficult to make sections for the early-stage eggs. Instead, the frozen sectioning method would be better to make sections for honeybee embryos, especially for early-stage embryos. It was relatively simple using frozen section method to make honeybee egg sections, which only needs polyformaldehyde fixation and sucrose dehydration processes to prepare honeybee eggs for slicing. The Sucrose dehydration process would also lead to a small contraction of the egg, but it would not change the overall shape and structure. So the completement of the embryonic sections was relatively better compared to the paraffin sections.

In the choice of dyeing method, we did not choose the traditional hematoxylin and eosin (HE) staining method, because this method needs many cleaning steps. Cleaning steps easily lead to the loss of the inner materials (such as yolk mass and vitellophages) in eggs and the incompleteness of embryonic external structure. The PI staining method used in this study almost omitted most of the cleaning processes before microscope observation (Nicoletti et al., 1991). A very small amount of PI staining solution was gently dropped and covered on the embryonic sections and then the sections were observed directly. This treatment avoided huge loss of the inner materials in the eggs and reduced the chance to deform the whole embryonic structure. In our present study, this floating phenomenon of embryonic single layer cells was very hard to avoid. Especially, it was easy to occur in the early *A. cerana* embryo before stage three, the monolayer of blastoderm at the fifth stage and the dorsal amniotic serosa at the seventh stage. Because PI staining was mainly aimed at the specific staining of nuclei, under fluorescence microscope, green fluorescence will stimulate the PI-specific staining of nuclei to emit red fluorescence, but other tissues will also have a relatively light staining, so that the structure of the whole egg could be clearly seen and the overall distribution of embryonic cells with a brighter red fluorescence could be seen. In this study, we gained and showed good section results of *A. cerana* embryos at ten developmental stages. This dyeing method could also be used as a reference in making sections for other similar tissues or insect eggs in the future.

From our fixed pictures and histological pictures, we could see that the key events of embryonic development and the development of *A. cerana* embryo was basically similar to those of *A. mellifera*, and the same treatment of *A. cerana* according to the embryonic development zoning schedule of *A. mellifera* will find that the embryonic development zoning table of *A. mellifera* is basically applicable to the embryonic development zoning of *A. cerana*. In all the morphological and histological sections, it was found that the typical morphological and histological characteristics of each developmental stage of *A. cerana* were similar to those of *A. mellifera*. From our histological study, we could conclude that the time range and histological characteristics of embryonic development of *A. cerana* are basically the same as those of *A. mellifera*. Therefore, the Histomorphology of *A. cerana* embryo in this paper could also be used as a reference for embryonic development section of *A. mellifera*.

Author contributions

Xiao Fen Hu and Zhi Jiang Zeng provided the experimental design and developed the methodology. Xiao Fen Hu and Li Ke completed all the experiments, including sample collection and making embryo slices. Zhi Jiang Zeng provided the financial support for publishing the manuscript. Xiao Fen Hu and Zhi Jiang Zeng wrote the manuscript. All authors read and approved the final manuscript and agreed to be accountable for all aspects of the work.

Declaration of Competing Interest

No conflict of interest exits in the submission of this manuscript.

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The following are the supplementary data related to this article

References

Aizen, M.A., Harder, L.D., 2009. The global stock of domesticated honey bees is growing slower than agricultural demand for pollination. Curr. Biol. 19 (11), 915–918.
Cridge, A.G., Lovegrove, M.R., Skelly, J.G., Taylor, S.E., Petersen, G.E.L., Cameron, R.C., Dearden, P.K., 2017. The honeybee as a model insect for developmental genetics. Genesis 55 (5), e23019.
Dearden, P.K., 2006. Germ cell development in the honeybee (*Apis mellifera*); Vasa and Nanos expression. BMC Dev. Biol. 6 (1), 6.
Dearden, P.K., 2014. Expression pattern of empty-spiracles, a conserved head-patterning gene, in honeybee (*Apis mellifera*) embryos. Gene Expr. Patterns 15 (2), 142–148.
Dearden, P.K., Wilson, M.J., Sablan, L., Osborne, P.W., Havler, M., McNaughton, E., Kimura, K., Milshina, N.V., Hasselmann, M., Gempe, T., Schioett, M., Brown, S.J., Elsik, C.G., Holland, P.W.H., Kadowaki, T., Beye, M., 2006. Patterns of conservation and change in honey bee developmental genes. Genome Res. 16 (11), 1376–1384.
Dearden, P.K., Duncan, E.J., Wilson, M.J., 2009. RNA interference (RNAi) in honeybee (*Apis mellifera*) embryos. Cold Spring Harb Protoc. 6 (6) (prot5228).
DuPraw, E.J., 1963. Techniques for the analysis of cell function and differentiation, using eggs of the honeybee. XVI Int. Congress Zool. 2, 238.
DuPraw, E.J., 1967. The honeybee embryo. In: Wilt, F.H., Wessells, N.K. (Eds.), Methods in Developmental Biology. Thomas Y. Crowell, New York, pp. 183–217.
Fleig, R., Sander, K., 1985. Blastoderm development in honeybee embryogenesis as seen in the scanning electron microscope. Int. J. Insect Morphol. Embryol. 8 (4–5), 279–286.
Fleig, R., Sander, K., 1986. Embryogenesis of the honeybee *Apis mellifera L.* (Hymenoptera: Apidae): an SEM study. Int. J. Insect Morphol. Embryol. 15 (5–6), 449–462.
Fleig, R., Sander, K., 1988. Honeybee morphogenesis: embryonic cell movements that shape the larval body. Development 103 (3), 525–534.
Hu, X., Ke, L., Zeng, Z., 2016. Comparison of three methods for honeybee egg histological section. Acta Agric. Univ. Jiangxiensis 38 (4), 729–733.
Hu, X., Ke, L., Wang, Z., Zeng, Z., 2018. Dynamic transcriptome landscape of Asian domestic honeybee (*Apis cerana*) embryonic development revealed by high-quality RNA sequencing. BMC Dev. Biol. 18 (1), 11.
Hu, X.F., Zhang, B., Liao, C.H., Zeng, Z.J., 2019. High-efficiency CRISPR/Cas9- mediated gene editing in honeybee (*Apis mellifera*) embryos. G3: Genes, Genomes, Genetics g3–400130.
Liu, J.F., Yang, L., Li, M., He, X.J., Wang, Z.L., Zeng, Z.J., 2019. Cloning and expression pattern of odorant receptor 11 in Asian honeybeedrones, *Apis cerana* (Hymenoptera, Apidae). J. Asia Pac. Entom. 22 (1), 110–116.
Milne Jr., C.P., Phillips, J.P., Krell, P.J., 1988. A photomicrographic study of worker honeybee embryogenesis. J. Apicult. Res. 27 (2), 69–83.
Nelson, J.A., 1915. The Embryology of the Honey Bee. Princeton Univ. Press, Princeton, NJ, USA.
Nicoletti, I., Migliorati, G., Pagliacci, M.C., Grignani, F., Riccardi, C., 1991. A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. J. Immun. Methods 139 (2), 271–279.

Osborne, P.W., Dearden, P.K., 2005a. Expression of Pax group III genes in the honeybee (*Apis mellifera*). Dev. Genes Evol. 215 (10), 499–508.
Osborne, P.W., Dearden, P.K., 2005b. Non-radioactive in-situ hybridisation to honeybee embryos and ovaries. Apidologie 36 (1), 113–118.
Schnetter, M., 1934. Morphologische untersuchungen über das differenzierungszen- trum in der embryonal-entwicklung der honigbiene. Z. Morphol. Okol. Tiere 29 (1), 114–195.
Wilson, M.J., Kenny, N.J., Dearden, P.K., 2014. Components of the dorsal-ventral pathway also contribute to anterior-posterior patterning in honeybee embryos (*Apis mellifera*). EvoDevo 5 (1), 11.
Winston, M.L., 1991. The Biology of the Honey Bee. Harvard Univ. Press, Cambridge, MA, USA.
Yan, W.Y., Gan, H.Y., Li, S.Y., Hu, J.H., Wang, Z.L., Wu, X.B., Zeng, Z.J., 2016. Morphology and transcriptome differences between the haploid and diploid drones of *Apis cerana*. J. Asia Pac. Entom. 19 (4), 1167–1173.