Embryonic development and organogenesis of Chinese giant salamander, *Andrias davidianus* *

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Abstract The morphology and organogenesis of Chinese giant salamander, *Andrias davidianus*, in its different developmental periods and stages are described in detail, which provides an intact criterion for distinguishing different stages of its developmental process. Based on the external morphological and internal histological features, six periods including 20 stages of organogenesis of Chinese giant salamander are established, which are cleavage period, blastula period, gastrula period, neurula period, organogenesis stage and hatching stage. Generally, the embryonic development of Chinese giant salamander is consistent with those of Eastern newt, *Cynops orientalis*, and Black spots frog, *R. nigromaculata*. However, they have some differences in the early cleavage process and the development of digestive system. The cleavage of Chinese giant salamander, *A. davidianus* is not a discoidal division type, which is different from other species reported. And the first three cleavages being meridional and a retardant development of its digestive system without halter and sucker existing are the evident features of the embryonic development of Chinese giant salamander.

Keywords: Chinese giant salamander, *Andrias davidianus*, embryogenesis, cleavage, organogenesis.

Amphibian is a transitional animal class for the vertebrates from aquatic to terraneous, the research of whose embryonic development is significant for exploring vertebrates' evolution and studying the comparative embryology. At the present time, the major research of amphibian embryology focuses on *Anuran* and *Salamandra*, and the study of Eastern newt, *Cynops orientalis*, is a notable one. Cai[1] has established a system to distinguish different stages of the embryonic development of Eastern newt, and illustrated a series of detailed pictures of its morphology and organogenesis. And *Cryptobranchidae* is a group of archaic vertebrates on the edge of depopulation[2], among which are North American hellbender (*Cryptobranchus*), Japanese giant salamander (*Andrias japonicus*), and Chinese giant salamander (*Andrias davidianus*). There are no publications about the embryonic development of North American hellbender and Japanese giant salamander, but some embryonic studies have been carried out on Chinese giant salamander, although the study is limited and restricted to primary observation of embryonic appearance, and the stage distinguishing system is obscure with a lacking of organogenesis study[3—5]. Recently, our lab has made some research progress in the artificial reproduction of Chinese giant salamander, with which a continual and systematic study on its morphological and histological features has been carried out. It will help us understand its early cleavage and organogenesis process, and finally reveal the evolution of *Cryptobranchidae* and *Amphibian*.

1 Materials and methods

1.1 Materials

The artificial reproduction and incubation of Chinese giant salamander were carried out at Base of Artificial Reproduction of Chinese Giant Salamander, an affiliated agency to our lab. At first, the sexually matured parents were treated with artificial reproduction to get the matured eggs and sperms, then the fertilized eggs were obtained by artificial insemination. Then the fertilized eggs were hatched by incubating in flowing water in the incubation pools (temperature 20 ± 1 ℃, concentration of dissolved oxygen 5 ± 1 mg/L, pH 7.4—7.6). The illumination time was about one hour per day (exposure rate, 4 lx).

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1.2 Methods

The methods for observing and recording morphological features are as follows. For the embryos between fertilization and multi-cell stage, the observation and report were made every 2 hours. For the embryos between blastula stage and tail bud stage, the observation and report were made every 8 hours. For the embryos between branchia plate stage and hatching stage, the observation and report were made every 12 hours. Each embryonic developmental stage was decided by the time when more than a half of the embryos were in the same stage. Totally about 1100 embryos were morphologically studied from year 2003 to year 2005. The histological studies investigated 56 embryos from 1100 during the whole embryonic developmental process. The early embryos were fixed in Smith's fluid and dehydrated by ethanol and tertiary alcohol. The embryos after the forelimb bud stage were fixed in Bouin's solution and dehydrated by ethanol. All the embryonic samples were embedded in paraffin, sectioned (6—10 μm), and stained with hematoxylin-eosin. Nikon SMZ800 and Olympus XTL-Ⅱ stereoscope systems were used to investigate the exterior appearance of the early embryos, and a Sony R1 digital camera was used to take pictures. For tissue sections, micrographs were taken by a Nikon E600 micro-photographic shoot system.

The distinguishing of the embryonic developmental stages of Chinese giant salamander was based on the morphological and histological characters referring to the standard for *Cynops orientalis*[Ⅱ] and *Rana nigromaculata*[6].

2 Results

The whole process of the embryonic development of Chinese giant salamander could be divided into 6 periods, which could be further divided into 20 stages (Table 1).

Table 1. Different periods of the embryonic development of Chinese giant salamander

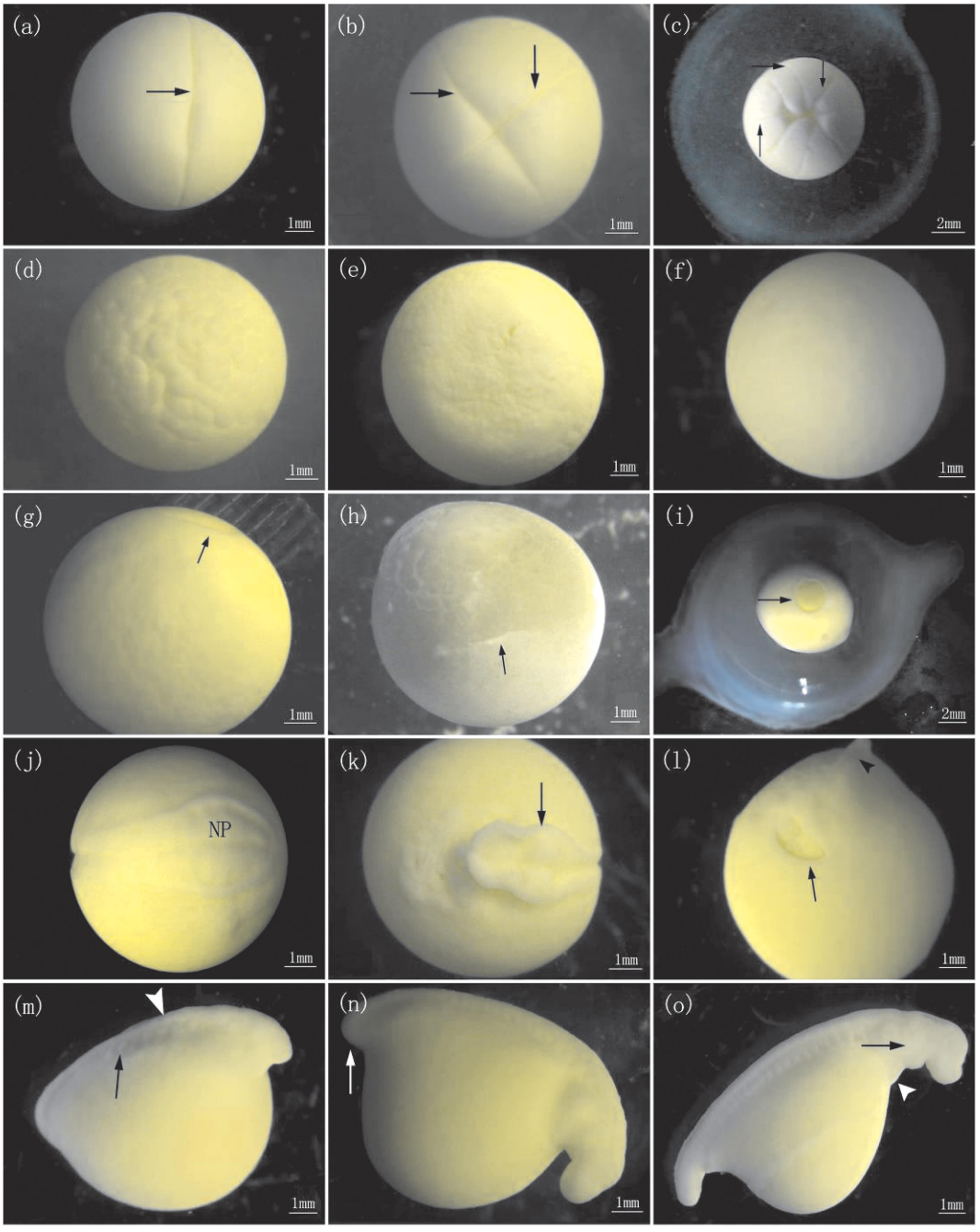
Periods	Stages	Time after fertilization (h)	Characteristics
Cleavage	2-cell	18—22	Cleavage of fertilized egg
	4-cell	22—26	2 cleavage grooves
	8-cell	26—30	4 cleavage grooves
	Multi-cell	30—48	Cleavage cavity
Blastula	Early blastula	48—90	Blastocoel
	Late blastula	90—128	Expanded blastocoel with 2—3 layers of cells on the top
Gastrula	Early gastrula	128—172	Dorsal lip
	Middle gastrula	172—216	Expanded archenteric cavity and lateral lip
	Late gastrula	216—240	Disappearance of blastocoel and yolk plug formation
Neurula	Neural plate	240—256	Neural plate and primary body cavity
	Neural folds	256—280	Neural fold and somites
	Neural tube	280—300	Forebrain and hindbrain differentiation, optic vehicle/pharynx/nephridium/intestine
Organogenesis	Tail bud	300—356	Heart anlage and tail bud
	Branchial plate	356—412	Branchial plate and pigment in body, atrium and ventricle differentiation
	Early forelimb bud	412—468	Auditory vesicle/diencephalons/rhomb encephalon/branchial branches/liver/forelimb, beating heart, dorsal chorda vacuolization
	Late forelimb bud	468—516	Lens/olfactory pit/thyroid gland
	Early branchia-circulation	516—540	Eye pigment/branchia-circulation, transparent ventral vessel networks
	Late branchia-circulation	540—596	Increased branchial branches, mandible/stomach, abundant body pigment
	Tail circulation	596—676	Vitreous lens, feather-like branchia, tail circulation, abundant pigment in the ventral
Hatching	Posterior limb bud	676—768	Posterior limb bud/anus, dorsal skin glands

2.1 Cleavage period

The eggs of Chinese giant salamander were about 5 to 8 mm diametrically, with no pigment existing. And the abundance of yolk made them yellowish, although in the nearby of the animal pole was an undertone. The egg was wrapped by a gel envelope, which would expand after hydration and made the perivitelline space enlarge.

About 20 hours after fertilization, a cleavage groove appeared in the animal pole, indicating the first cleavage. The first cleavage was a meridional one, and the cleavage groove deepened in the animal

pole, which extended to the plant hemisphere gradually and disappeared there (Fig. 1(a)). It took about 4 to 8 hours for the fertilized egg to finish the first cleavage. The second cleavage appeared two hours after the appearance of the first before it finished. And the second cleavage was also meridional, vertical to the first one (Fig. 1(b)). The third cleavage was meridional too, and the two cleavage grooves were mutually vertical, making the eight cells in the early embryo look like a sign of ✳ (Fig. 1(c)). The fourth cleavage was latitudinal and close to the animal pole. The fourth cleavage in the eight cells was non-synchronous and irregular with different dividing speed for each cell. The cleavages happened every 2 to 4 hours in the early developmental time, whose speeds were quickened gradually in the following development. Subsequently, a thickly-woven groove of cleavages appeared in the animal hemisphere, with only several being distinguished in the plant hemisphere.



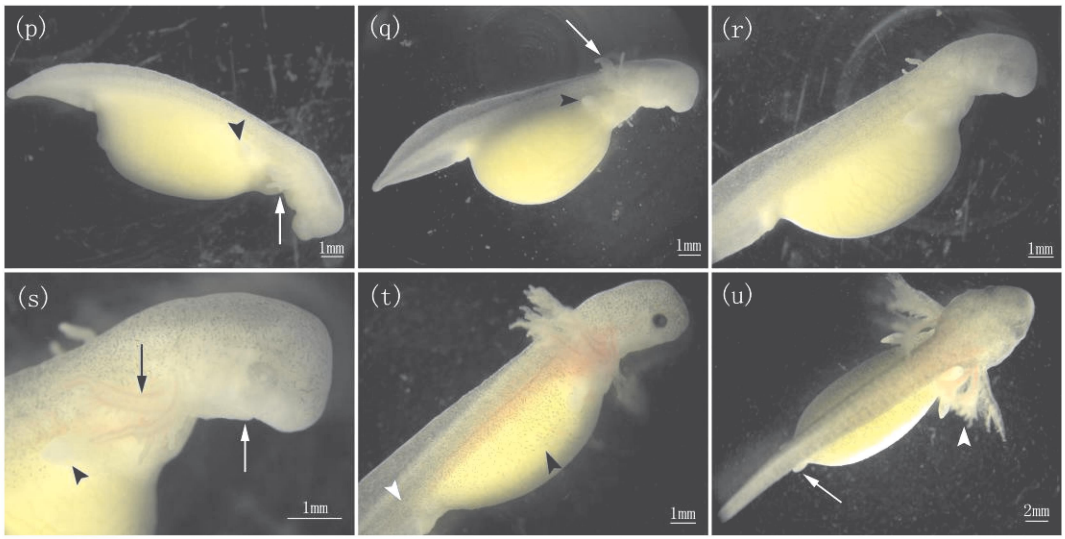


Fig. 1. Morphology of Chinese giant salamander. (a) 2-cell stage, arrowhead shows cleavage canal; (b) 4-cell stage; (c) 8-cell stage; (d) multi-cell stage; (e) early blastula stage; (f) late blastula stage; (g) top of early gastrula stage; (h) top of early gastrula stage (after fixed); (i) ventral of late gastrula stage; (j) neural plate stage, NP shows neural plate; (k) neural folds stage, arrowhead shows the neural folds; (l) bottom of merging neurula, long arrowhead shows blastopore, short arrowhead shows neural tube; (m) neural tube stage, arrowhead shows segment, short-arrowhead shows neural tube; (n) tail bud stage, arrowhead shows tail bud; (o) branchial plate stage, arrowhead shows branchial anlage, short-arrowhead shows heart; (p) early forelimb bud stage, arrowhead shows branchial bud and forelimb bud; (q) late forelimb bud stage; (r), (s) branchia circulation stage, black arrowhead shows branchias, white arrowhead shows mandible; (t) tail circulation stage, white arrowhead shows tail blood vessel, black arrowhead shows ventral pigment; (u) posterior limb bud stage, arrowhead shows posterior limb bud, short-arrowhead shows feather-like branchias.

2.2 Blastula period

The embryonic blastula period began on the third day after fertilization (Fig. 1(e), (f)), specified in the appearance of a big cavity near the topmost animal pole, which was then nominated as blastocoel (Fig. 2(a)). And in the plant hemisphere, a lot of rimous grooves appeared. The cells located above the blastocoel had obscure boundaries, with evident nuclei while small and little yolk particles existing in the cells. However, the cells under the blastocoel had a bigger size, and their nuclei were embedded in abundant yolk (Fig. 2(b)).

2.3 Gastrula period

On the sixth day after fertilization, a shallow horizontal groove appeared slightly below the equatorial surface of the embryo of Chinese giant salamander, the location above which was nominated as dorsal lip, indicating the beginning of gastrula period (Fig. 1(g), (h); Fig. 2(c)). In the following days, the short dorsal lip was prolonged to an arc, and at the same time, the gastrula cavity expanded while the blastocoel disappeared gradually (Fig. 2(e)). Then the embryonic axis rotated gradually, and the arc merged into a circular ring with the big yolk plug blocked midst (Fig. 1(i)). The yolk plug was then reduced to the tail end, which would finally disappear completely at the time of neural tube stage.

2.4 Neurula period

Neural plate stage: a scoop-like dorsal neural plate with a wide end in the front and a narrow end in the rear appeared (Fig. 1(j); Fig. 2(f)), while abundant yolk still existed in the neural plate and in the tissues around it (Fig. 2(g)).

Neural folds stage: the neural folds appeared when the edges of both sides of neural plates rose (Fig. 1(k); Fig. 2(h)), with abundant yolk stuffed inside the folds (Fig. 2(i)). And about 6 to 8 pairs of somites formed in the late phase of neural folds stage. With the embryo developing, both sides of the neural folds would merge up, adhere and fuse at the dorsal central line, while the cells in the center would sink to form a hollow tube.

Neural tube stage: at this period the neural tube was closed completely (Fig. 1(m)), and the embryo in the gel envelop changed the direction to side-lie. The front neural tube expanded and projected outward from the circular embryo, and some hunches appeared there. About 10 to 12 pairs of somites had formed in this period, with the appearance of kidney anlage under the somites and optic vehicles, forebrain

and hind-brain appearing in the front part of neural tube (Fig. 2(j)). And pharynx, dorsal chorda and nephridium could be observed, with a thin and long intestine located at the rear of the embryo (Fig. 2(k), (l)).

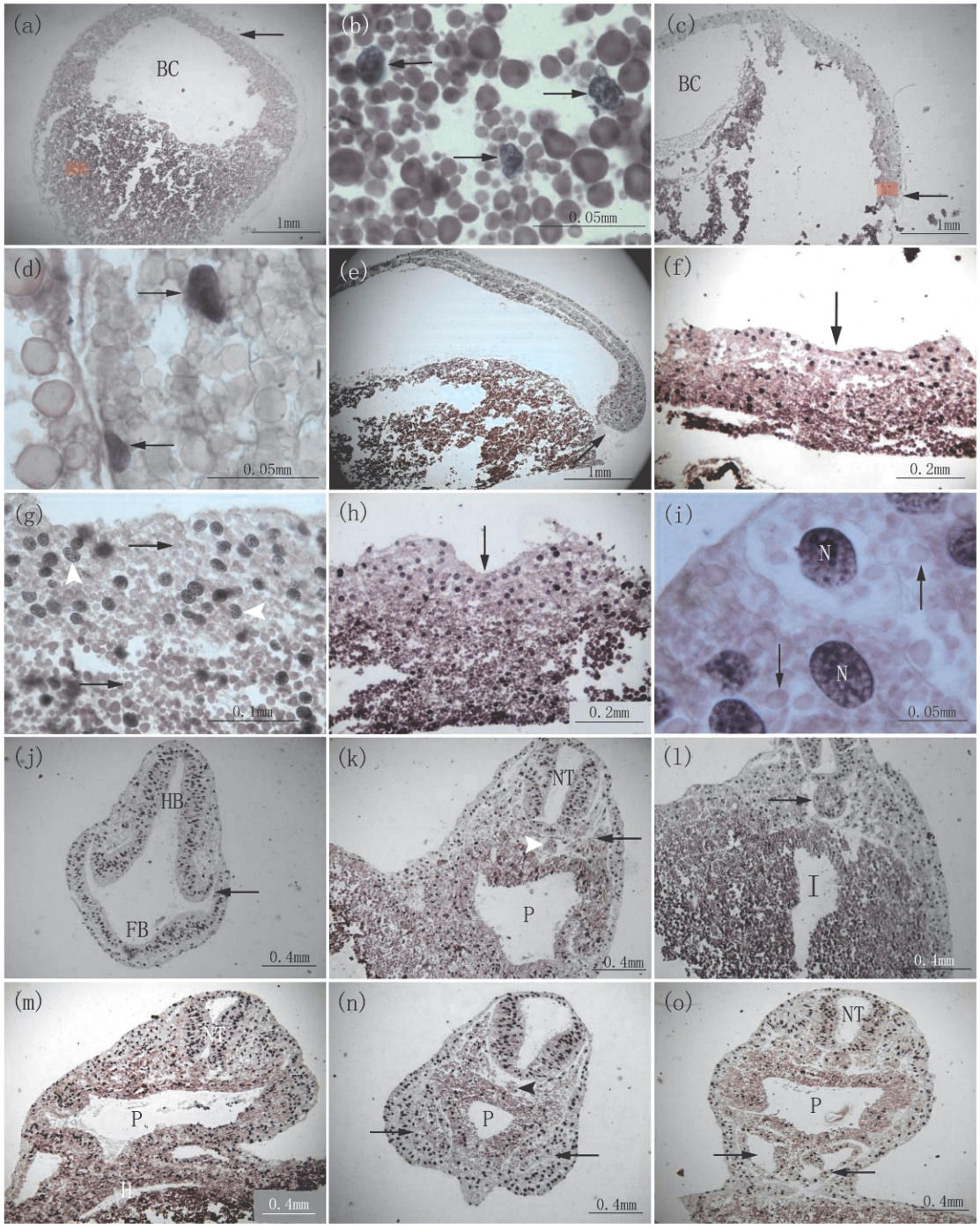


Fig. 2. Histological features of Chinese giant salamander. (a) Blastula stage, BC shows blastocoel, arrowhead cells on the top, red region is an amplification of (b); (b) amplification of blastula plant hemisphere, arrowheads show nuclei; (c) early gastrula stage, BC shows blastocoel, arrowhead shows dorsal lip, left cavity is a feint for yolk falling off, and not gastrula cavity, red region is an amplification of (d); (d) dorsal lip in gastrula, arrowheads show nuclei; (e) late gastrula stage, arrowhead shows dorsal lip; (f) neural plate stage, arrowhead shows the neural plate; (g) amplification of neural plate, short arrowhead shows nucleus and arrowhead shows yolk; (h) neural folds stage; (i) amplification of the neural folds, N shows nucleus, arrowhead shows yolk; (j) neural tube stage, arrowhead shows optic vesicle, FB shows forebrain, HB shows hindbrain; (k) neural tube stage, short-arrowhead shows solid dorsal chorda, NT shows neural tube, P shows Pharynx; (l) neural tube stage, arrowhead shows solid dorsal chorda and I shows intestine; (m) tail bud stage, H shows heart anlage; (n) branchial plate stage, arrowhead shows branchia plate, short arrowhead shows dorsal chorda; (o) branchial plate stage, arrowhead show atrium and ventricle.

2.5 Organogenesis period

Tail bud stage: the embryonic head expanded evidently, with some distinguishable optic vehicles being observed by naked eyes. Along with the development of the brain, the whole neural tube extended and bended rapidly, forming an almost orthogonal curve, which was called brain curve (Fig. 1(n)). At the same time, the rear end of the embryo turned into a tail bud, projecting outward from the circular surface of the embryo, and heart anlage also appeared at this time (Fig. 2(m)).

Branchia plate stage: the optic vehicles projected outward while its surrounding region sank inward. The branchia anlages upheaved and gradually differentiated into branchial arch (Fig. 1(o); Fig. 2(n)). At this stage, the thorax expanded further and the heart differentiated into atrium and ventricle (Fig. 2(o)). And the number of somites was 24 pairs for this stage. Some pigment dots appeared in the dorsal skins and the thin tail bud ended roundly.

Forelimb bud stage: the sign of this stage was the appearance of forelimb bud. The branchial arch differentiated into three smallish branchial buds (Fig. 1(p), (q); Fig. 3(c), (d), (f)). And the thorax became transparent, while the heart began to beat (about 25 times per minute in early stage). At this stage, the embryo could twist slowly. Some embryonic parts appeared, such as optic cup, lens, olfactory pit (Fig. 3(a)), auditory vesicle, midbrain, rhombencephalon, brain ganglion, thyroid gland and liver (Fig. 3(b), (c), (e)). Vacuolization of the dorsal chorda began and the three pairs of the branchial buds extended and ramified. The pigment increased in the embryo, while the ventral cells with abundant yolk divided rapidly, which made the multiple nuclei distinguishable. And in the late time of this stage, the beating rhythm of the heart was 30 to 40.

Branchia-circulation stage: by a stereoscope, the heart-branchia circulation could be observed, and a beating rhythm between 40 and 50 was estimated. The head and branchia developed well (Fig. 1(s); Fig. 3(h), (i)), compared with those in the forelimb bud stage, with three to five pairs of branches appearing on each branchia anlage and some erythrocytes flowing in the transparent capillary vessels. A network of blood vessels could be observed in the ventral, and a slight pigment was present in the eyes, while the pigments in the head and dorsal spread in a larger region (Fig. 1(r), (s)). In the late developmental phase of this stage, the beating rhythm of the heart increased to 50—55, and some other features could be detected, including five to eight pairs of the branches in each branchial bud, the slightness of the pigment in the eyes, the wiry appearance of the forelimb bud, and the appearance of mandible and stomach (Fig. 3(j)).

Tail circulation stage: tail circulation and mandible movement could be observed at this stage. The wall of brain became thicker and lens transparent, while the pigment spread widely in the ventral (Fig. 1(t); Fig. 3(k), (l)). The larva at this stage was specified as strong muscle, well-developed skeleton and frequent movement. And another feature of this stage was that, some embryos might get out of envelops in advance, and the newborn tadpoles could survive more likely than the larvae before this stage.

2.6 Hatching period (Posterior limb bud stage)

About 31 days after fertilization, the beating rhythm of the embryonic heart turned to 55, and six to eight pairs of branches existed in each branchia with an appearance of feather. The head and dorsal part of the embryo appeared a wine color with thick pigments and blood vessels in it. About a half of the ventral region had some pigments, with abundant yolk. Some features for this period included the appearance and extension of the posterior limb buds, the appearance of skin gland, the appearance of anus (Fig. 1(u); Fig. 3(m), (n), (o)), the extension and thickening of the forelimb buds with some pigments. In this period, the forelimbs had differentiated into two toes. The tadpoles had a wide dorsal fin and a narrow tail fin, and also two black eyes. The tadpole could twist constantly to get out of the gel envelop completely, when the outer gel expanded and loosened. The newborn tadpole was about 3.5 cm long, with abundant yolk in the ventral cells and the dorsal appearing brown and black. The branchias were well developed and about 1 cm long on each side without halter and sucker at this stage. The newborn tadpole could level-lie or side-lie steadily at the bottom of the incubator, and swam directly by swaying its tail fin.

About one month later, the newborn Chinese giant salamander finished the extension of forelimbs and posterior limbs, and the differentiation of its toes finished gradually, while the ventral yolk was exhaust-

ed. With the developing process going, the lung could develop gradually, while the branchias degenerated about eight months later, and it took about one to two years for the degeneration of the branchia to complete and then lung was the only respiration organ.

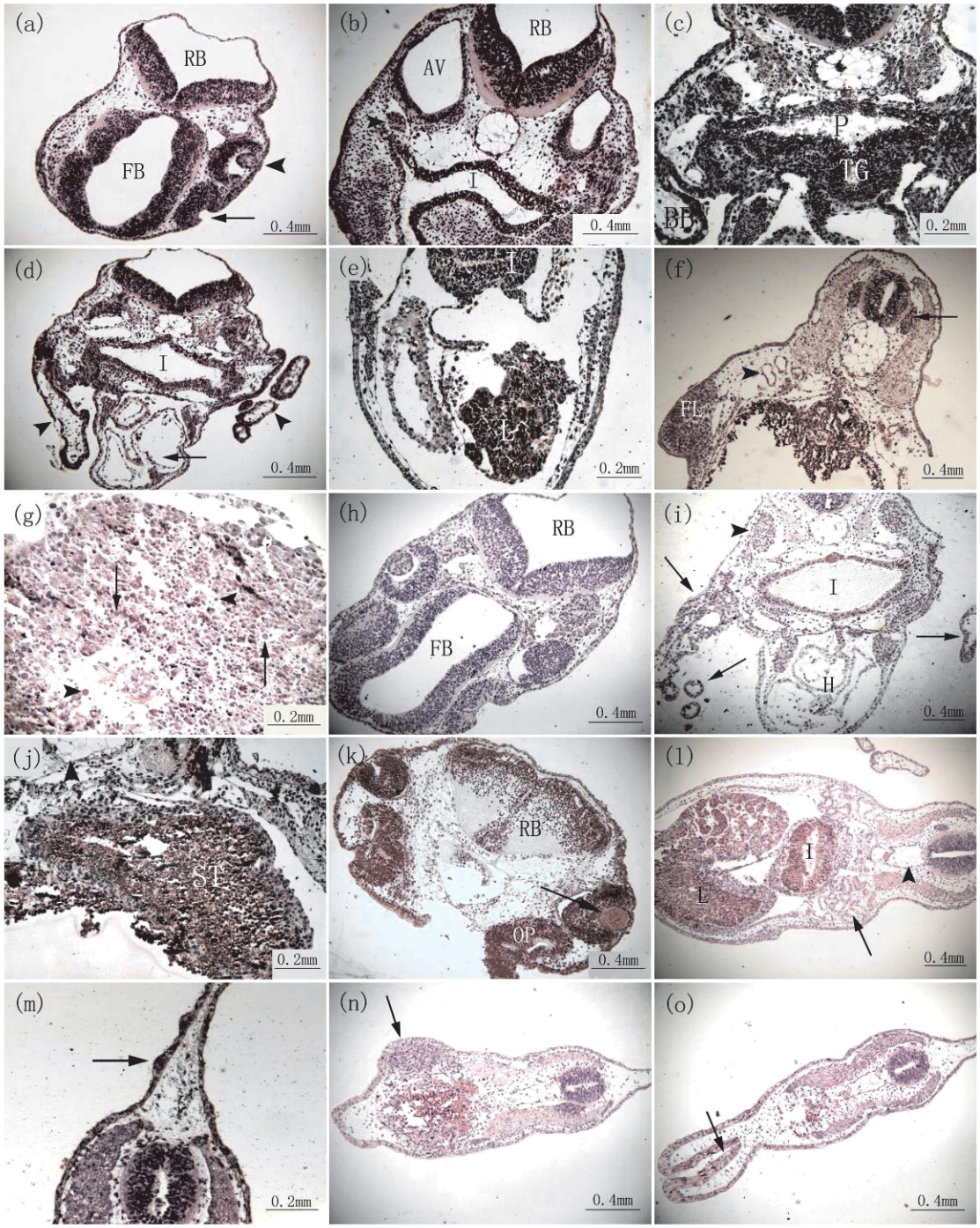


Fig. 3. Histological features of Chinese giant salamander (continued). (a) Forelimb bud stage, RB shows rhomb encephalon, arrowhead shows olfactory pit, short-arrowhead shows optic cup and lens; (b) forelimb bud stage, AV shows auditory vesicle, short-arrowhead show cranial nerve; (c) forelimb bud stage, TG shows thyroid gland, P shows Pharynx, BB shows branchial bud; (d) forelimb bud stage, arrowhead shows heart, short-arrowheads show branchial buds, I shows intestine; (e) forelimb bud stage, L shows liver; (f) forelimb bud stage, arrowhead show neural crest, short-arrow shows nephridium, FL shows forelimb bud; (g) ventral section of forelimb bud stage, arrowhead shows nucleus, short-arrowhead shows yolk; (h) head of branchia circulation stage; (i) branchia circulation stage, arrowhead show branchial buds and branches, short arrowhead shows cranial nerve; (j) branchia circulation stage, ST shows stomach; (k) tail circulation stage, arrowhead shows the vitreous lens, OP shows olfactory pit, RB shows well-developed rhomb encephalon; (l) tail circulation stage, L shows liver; (m) posterior limb bud stage, arrowhead shows dorsal skin gland; (n) and (o) posterior limb bud stage, they show posterior limb bud and anus.

3 Discussion

3.1 The cleavage mode of Chinese giant salamander

Some publications have already reported that after fertilization, the egg cleavage of Chinese giant salamander is a classic discoidal division type, and the division is only restricted to the area of blastoderm, whose appearance is white and cap-shaped[3—5]. Our lab has done a series of observations in the past three years to study the early development of Chinese giant salamander, and we have found that after fertilization, the eggs of Chinese giant salamander do not upheave a blastoderm. The cleavage grooves can be observed from the animal pole to the plant hemisphere in the cleaving eggs. Some shallow and chap-like cleavage grooves can be detected in the plant hemisphere in the blastula embryo, which is a confirmation of the data reported by other scientists[4,5]. And no yolk sac can be observed in the embryo of Chinese giant salamander, although abundant yolk exists in the cells on the top wall of the blastocoel and a lot of nuclei can be detected in the cells in the plant hemisphere below the blastocoel. At the same time, all the cells originating from ectoderm are rich in yolk, despite of their different developmental periods and stages, such as neurula period, tail bud stage, branchia plate stage and forelimb bud stage. And it is well known that as a discoidal division type, the cleavage grooves should be restricted only to the area of blastoderm, and the yolk is not involved in the division[7]. However, our data points to a conclusion that the cleavage of Chinese giant salamander, *A. davidianus* is not a discoidal division type, but to some extent, more similar to the holoblastic division type of Black dots frog, *Rana nigromaculata* and Eastern newt, *Cynops orientalis*, although in the early embryos of Chinese giant salamander, the cell boundaries can not be distinguished, especially in the tissues wealthy of yolk, which only shows a lot of nuclei. It is very interesting that for the ectoderm tissues with a very small amount of yolk, the cell boundaries can be easily detected, while for the endoderm-originated tissues, the obscure cell boundaries can be observed more and more easily with the consumption of yolk going. To answer the question that whether the existence of yolk in the early embryo interferes with our observation of the cell boundaries or the abundant yolk directly counteracts the divisions, we need further and more investigations.

All the first three divisions of the early embryo of Chinese giant salamander are meridional cleavage and the forth division is an irregular latitudinal one, which is different from Black dots frog and Eastern newt, for the third and forth divisions of the latter two are respectively latitudinal and meridional[1,6]. It is reported that for the teleostean, *Acipenser schrenckii*[8], whose early cleavages are unequal divisions, its first three cleavages are all meridional while the forth is latitudinal. And Liu[8] thought that this specific cleavage mode is a transitional one for the vertebrates from teleostean to amphibian. And the cleavage process of Chinese giant salamander is similar to that of *Acipenser schrenckii*, indicating that evolutionally perhaps Chinese giant salamander is closely related with teleostean.

Usually, under the temperature of 20 ℃, it respectively takes 3, 6 and 24 hours for most teleosteans[9], Black dots frog[10] and Eastern newts[11] to develop from fertilization to early blastula period. But in the case of Chinese giant salamander, it is 70 hours. And the slow division speed of the early embryonic cells is the direct reason for the long developmental time for the embryo of Chinese giant salamander (about 700 hours). And remarkably, to increase the embryonic livability of Chinese giant salamander in the artificial developing process, finding an optimum water temperature and egg density, increasing the concentration of soluble oxygen, and keeping the water clean and apart from microorganism infection are very important.

3.2 The features of organogenesis of Chinese giant salamander

The development of Chinese giant salamander and Eastern newts has some common features, such as the differentiation of brain, the organogenesis of eyes, olfactory pit, auditory vesicle, nephridium and genital crest (Fig. 4). And at the branchia plate stage, both atrium and ventricle differentiate, and heart beating and blood circulation appear at the same early forelimb bud stage, although the heart anlage of Chinese giant salamander appears a little bit later than Eastern newt. However, the organogenesis of digestive system differs in these two amphibians. The embryo of Eastern newt has already developed pharynx, liver, thyroid gland and anus at neural tube stage, tail bud stage and gill plate stage. While in the case of Chinese giant salamander, the situation is different.

Although the pharynx and intestine had formed at the stage of neural tube, the development of the digestive system was not continued until liver and thyroid gland developed ped at the forelimb bud stage. And anus appeared at hatching stage in the embryonic development of Chinese giant salamander. The slow development of the digestive system of Chinese giant salamander may closely relate with the existence of rich yolk, for the abundant yolk in different tissue cells can provide enough energy and nutrition for the embryonic development after the tadpole has come out of the gel envelope. The yolk can not be exhausted until about 30 days after the tadpole gets out of the envelope, when the digestive system has been well developed, which is also a perfect time for culturing. An immediate culturing right after the hatching period may result in death of the larvae, although the tadpoles can open their mouths and have the ability to swallow after the hatching period.

Most amphibians develop a kind of temporary organs in the head in the hatching period, including halter for Eastern newt[11], and sucker for anurans. The halter is a stick-shaped projecting organ, whose function is suggested as helping tadpoles maintain balance, while the sucker can excrete mucus of glycoprotein and help tadpoles adhere to objects in water[12]. It is very interesting that neither halter nor sucker can be observed in the tadpoles of Chinese giant salamander. And it is suggested that, since the tadpoles of Eastern newt are about 1.2 cm[11] long and the tadpoles of Black dots frogs 1.1 cm long[6], either halter or sucker is necessary for them to maintain balance in water. While in the case of Chinese giant salamander, its tadpole is 3.5 cm long and having a spoon-shaped abdomen and a big size body, so a well developed branchia (1 cm) can effectively help it maintain balance. And in the end, we suggest that the lacking of temporary halter and sucker in tadpoles of Chinese giant salamander is a feature of its own structural adaptation.

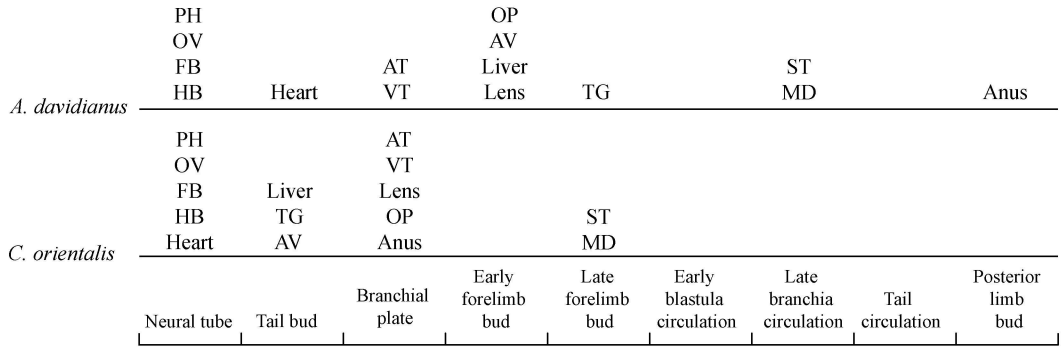


Fig. 4. Comparison of organogenesis between *A. davidianus* and *C. orientalis*. Abbreviations: PH, pharynx; OV, optic vesicle; FB, forebrain; HB, hindbrain; AT, atrium; VT, ventricle; OP, olfactory pit; AV, auditory vesicle; TG, thyroid gland; ST, stomach; MD, mandible.

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