Invitation to Experimental Evolution: Changes of Morphogenesis of Horseshoe crabs.

伊 藤 富 夫
Tomio ITOW

（平成16年9月29日受理）

Summary

We must experimentally examine the mechanism and process of evolution. The results of experimental embryology often shed light on these problems.

Interspecific grafts of the center cells of horseshoe crab embryos were attempted and were successful. The fact indicated that embryonic inductions are widespread and has common mechanism. It may mean that the Arthropoda are closely related with Vertebrata.

The center cells of horseshoe crab actively divide and form the mesodermal layer which constitute the primordia of body segments. The way that segment number is regulated is an important problem in evolution and embryonic development. Short periods of inhibition of DNA synthesis were found to induce the increase of segment numbers. This indicates the following: Each body segment is formed depend on one or two cell cycles of the center cells. The active sites of genes, which decide characteristics of each segment, shift at S phase of cell cycles.

The experimental separation of mesodermal layer induced the release of crossing of central nervous system and stomodaeum. Therefore attempts to derive Deuterostomia from Protostomia cannot be rejected because of the crossing of the alimentary canal and nervous system.

Introduction

The mechanism of evolution is one of the most exciting problems to be analyzed in biology. When the life cycle of a certain species changes and the species can not reproduce with the origin species, the species rises a degree of the evolution. When we understand the mechanisms of embryonic development, we will know much of value for understanding evolution[1].

For analyzing the mechanism of embryonic development, artificial modifications of the metabolic pathway and the extracellular environment are good methods. The modification of cellular activities such as cell proliferation, differentiation, migration, and detachment are also good methods[2]. Horseshoe crab embryos are useful materials in such experiments, for several

reasons. (1) Many chemical reagents penetrate into the embryo when they dissolve in sea water. (2) As the embryos can be obtained by artificial insemination, we can analyze at one time more than 10,000 embryos at the same developmental stage. (3) When treated at a low temperature (5 to 10 ℃), the embryos stay at the same stage for 1 to 3 months and do not die. The embryos at Stage 7 (the stage of early gastrula) are especially sturdy. (4) The embryos are easily observed, because the eggs are large (1.5 to 4 ㎜) and are vitally stained with neutral red or Nile blue. (5) As the speed of embryonic development is slow, we can treat them with various reagents at specific stages. (6) The body structure is uncomplicated and the process of embryonic development is simple.

The horseshoe crab has been called a living fossil. It is said to be far older than the dinosaurs. After branching off from the trilobite at the beginning of the fossil or Paleozoic era 500-600 million years ago, it reached its present form in the Triassic period of the Mesozoic era 200 million years ago and has changed hardly at all since that time. With the breaking apart and movement of the continents that occurred 200 million years ago, the horseshoe crab was split between the American and Eurasian continents, and it survives today in the east coast of North America (1 species; *Limulus polyphemus*) and in East Asia (3 species; *Carcinoscorpius rotundicauda, Tachypleus gigas* and *T. tridentatus*).

Interspecific Grafts of Center Cells

Surface cleavages occur in horseshoe crab embryos as in other Arthropoda, and the blastopore appears at the early gastrula stage (Stage 7). Surface cells migrate toward the blastopore and enter there. A cell mass is formed beneath the blastopore at the early gastrula stage (Fig. 1).

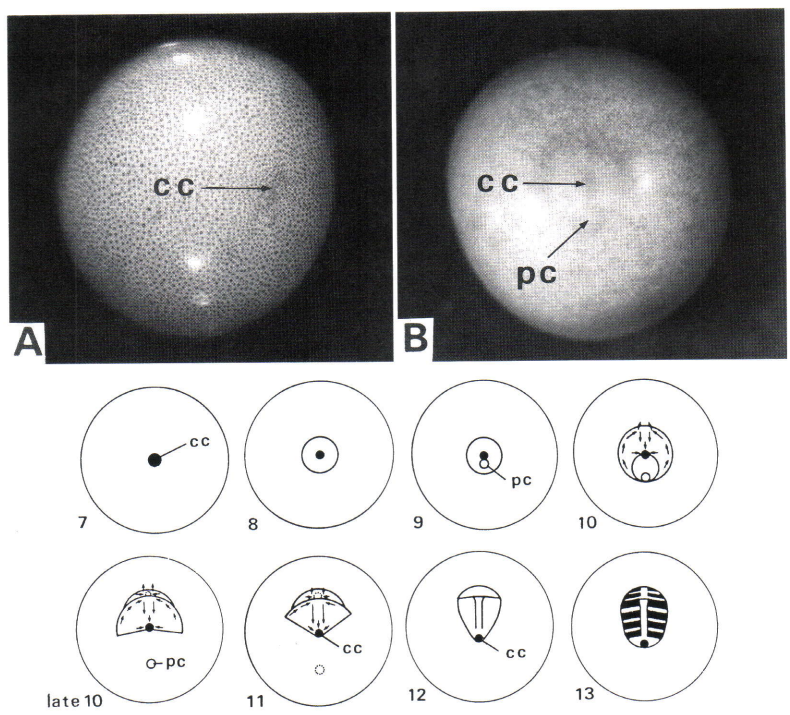

Fig. 1. Development of horseshoe crab embryos. UPPER: *Tachypleus tridentatus* embryo at the early gastrula stage (Stage 7) (A) and at the stage of completion of germ disc (Stage 10) (B). LOWER: Diagramatic representation of embryonic development. Numbers show developmental stages. Arrows indicate the direction of migration of cells. cc, center cells (anterior cumulus); pc, posterior cumulus. (from 4, 11)

The cell mass at the gastrula stage is called the primary cumulus in spider embryos. Morphological observations and previous experimental studies have shown that the germ disc is formed around the cell mass, and that cell mass is later situated at the posterior end of the ventral plate (the embryonic area) and successively forms segment primordia[3-7]. The cell mass at the posterior end of the embryonic area is sometimes called the growth zone.

If all of the cell mass at the early gastrula stage is electrically cauterized, the treated embryo cannot develop further. If the cell mass is divided into two or three pieces by electrocauterization or cell dissociation, the treated embryo develops into a double or triple embryo[8,9]. These experiments suggest that the cell mass plays a central role in embryogenesis, and that it is similar to the mesodermal teloblast of Annelida and Crustacea. Therefore the mass is referred to as the center cells. To find out more about the role of the center cells, we attempted grafting experiments[10-12]. We found that secondary embryos could be induced by intra- and interspecific grafts of center cells, indicating that center cells have the capacities of primary induction. Secondary embryos were formed when center cells from Asian horseshoe crabs *Carcinoscorpius*

rotundicauda were grafted into American horseshoe crabs *Limulus polyphemus* embryos, and when center cells were grafted from *L. polyphemus* into *C. rotundicauda*. In the case of the interspecific grafts, the form and structure of the secondary embryos were the same as those of the host embryos (Fig. 2, Table 1).

Fig. 2. The secondary embryos (upper side). A: An embryo induced after grafting center cells from *Carcinoscorpius rotundicauda* into the same species. B: An embryo induced after grafting center cells from *Limulus polyphemus* into *C. rotundicauda*. The scale bars shows 1 mm. (from 11)

Table 1. Results of grafting in horseshoe crab embryos

DONOR	→	HOST	Total number of operated on embryos	No of embryos developed upto St. 20 (percent of the total)	No of embryos with secondary embryos (percent of the developed)
L. p.	→	*L. p.*	162	83 (51.2)	18 (21.7)
C. r.	→	*L. p.*	211	38 (18.0)	6 (15.8)
C. r.	→	*C. r.*	191	20 (10.5)	4 (20.0)
L. p.	→	*C. r.*	166	22 (13.3)	3 (13.6)
Non-center cells	→	*L. p.*	107	47 (43.9)	0 (0.0)

L. p.: *Limulus polyphemus*, *C. r.*: *Carcinoscorpius rotundicauda*

Injection of homogenized center cells also induced secondary embryos, with the same characteristics as those of secondary embryos induced by intra- and interspecific grafts of whole cells.

When homogenized center cells of the spider *Chiracanthium gratiosum* were injected into the gastrulae of *L. polyphemus*, secondary embryos were induced at 11.4% (Table 2).

Direct transplantation of center cells from *L. polyphemus*, and the microinjection of homogenized center cells into early gastrulae of the frog *Xenopus laevis* induced the formation of secondary embryos in a high proportion of recipients (about 30-40%). The secondary embryos had similar characteristics to those of the host frog embryos. Histological examination showed that the secondary embryos had the structure of eyes, nervous systems and the other tissues (Fig. 3).

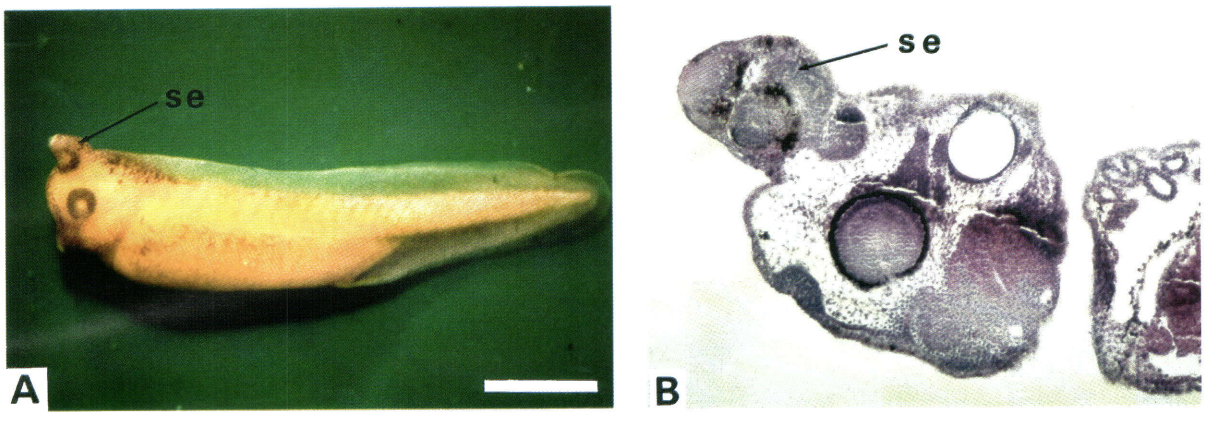

Fig. 3. A: Secondary embryo after grafting of horseshoe crab embryo center cells into a *Xenopus* embryo. B: Histological features of the secondary embryo of " A". The longitudal section of head part is shown. se; secondary embryo. The scale bar shows 1 ㎜.

Our results showed that center cells can induce secondary embryos in animals of a different class.

These facts indicate that horseshoe crab embryos have an induction system similar to the organizer of Amphibia. The horseshoe crab is a primitive arthropod, and its pattern and mechanism of morphogenesis differ from those of more highly evolved Arthropoda such as insects. The embryonic induction system has not been known in insect embryos. The horseshoe crab is thought to possess the primitive arthropodan system of embryonic induction, and the system is related to that of Annelida. Arthropoda are believed to have evolved from Annelida. The induction systems of the primitive Arthropoda and Vertebrata may have originated from a common system. At the very least, we can conclude that the mechanism of embryonic induction is closely similar in both these animal groups.

Table 2. Injection of homogenized center cells and transplantation of crude center cells.

METHODS OF GRAFTINGS	Total number of operated on embryos	No of embryos developed upto St. 20 (percent of the total)	No of embryos with secondary embryos (percent of the developed)
SPIDER EMBRYOS INTO *Limulus* EMBRYOS			
Injection of homogenates	160	70 43.8%	8 11.4%
Transplantation of crude center cells	34	29 85.3%	0 0.0%
HORSESHOE CRAB EMBRYOS INTO *Xenopus* EMBRYOS			
Transplantations of crude center cells	53	39 73.6%	15 38.5%
Injections of homogenates	35	23 65.7%	8 34.8%
Control (injection of non-center cells)	36	20 55.6%	0 0.0%
Control (injection of agar, paraffin & starch)	43	31 74.4%	1 3.1%

The Number of Body Segments — Successive Gene Expression Theory

The number of body segments is a very important characteristic in the classification of animals. We found that inhibitors of DNA synthesis such as hydroxyurea, fluorodeoxyuridine and aphidicolin, affected the differentiation of the segment primordium and increased the number of segments in more than 80% of the horseshoe crab embryos thus treated[13] (Fig. 4).

In normal horseshoe crab embryos, the primordia of abdominal segments are formed from Stage 12 to Stage 19. Each segment primordium of three Asian species is formed for about 2 days (1 day in case of American horseshoe crab, *Limulus polyphemus*). Duration of Stage 12 plus Stage 13 is about 2 days (1 day in case of *L. polyphemus*). Duration of each stage from Stage 14 to Stage 18 is about 2 days (1 day in case of *L. polyphemus*). Duration of Stage 19 is about 6 days (3 days in case of *L. polyphemus*). The 7th segment is the 1st abdominal segment. The 1st abdominal segment primodium is formed at Stage 12 and 13. The 2nd one is formed at Stage 14. The 3rd one is formed at Stage 15. The 4th one, at Stage 16. The 5th one, at Stage 17. The 6th one, at Stage 18. The 7th, 8th and 9th (last) abdominal segment primordia are formed at Stage 19. The one cell cycle is about 1 day.

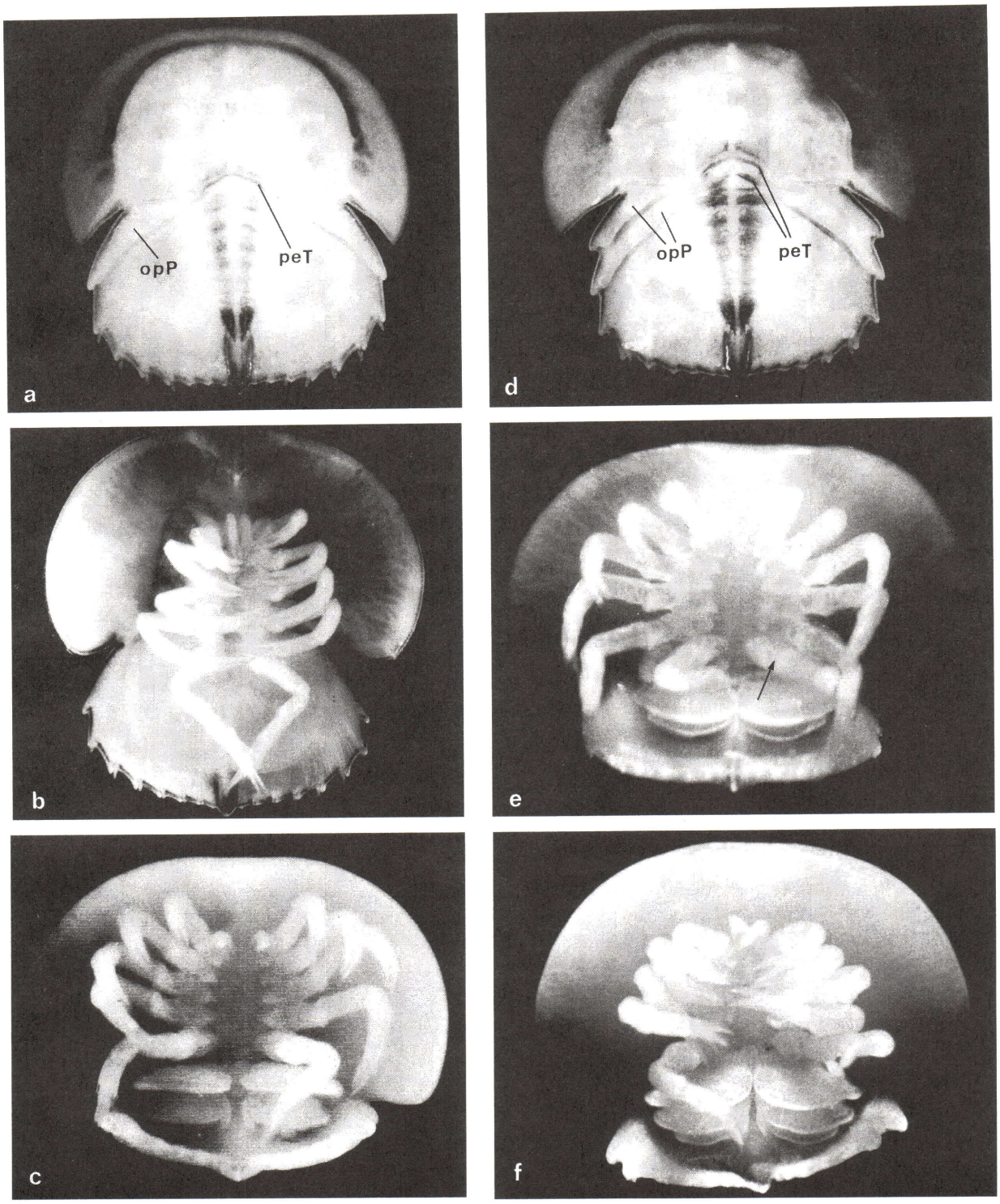

Fig. 4. Examples of normal embryos and those with supernumerary segments. a: Dorsal view of a normal embryo at Stage 21. b: Ventral view of a normal embryo at Stage 21. c: Ventral view of a normal embryo at Stage 20. Six pairs of cephalothoracic appendages and two pairs of large abdominal ones are clearly recognizable. d: Dorsal view of the embryo with supernumerary segments. Note the increase of the posterior end of cephalothorax (PeT) and the opercular pleurite (opP). e: The embryo with supernumerary segments. Note the supernumerary appendages with a structure intermediate between the last cephalothoracic appendage and the first abdominal appendage (arrow). f: The embryo with supernumerary segments. Three pairs of large abdominal appendages can be seen. (from 13)

When embryos at the stage of formation of abdominal segment-primordia (stages 12 to 19) were treated for about a day with inhibitors of DNA synthesis, they developed into embryos with supernumerary segments (Fig. 5). The supernumerary segments have often branchial appendages. Branchial appendages are primitive type of appendages.

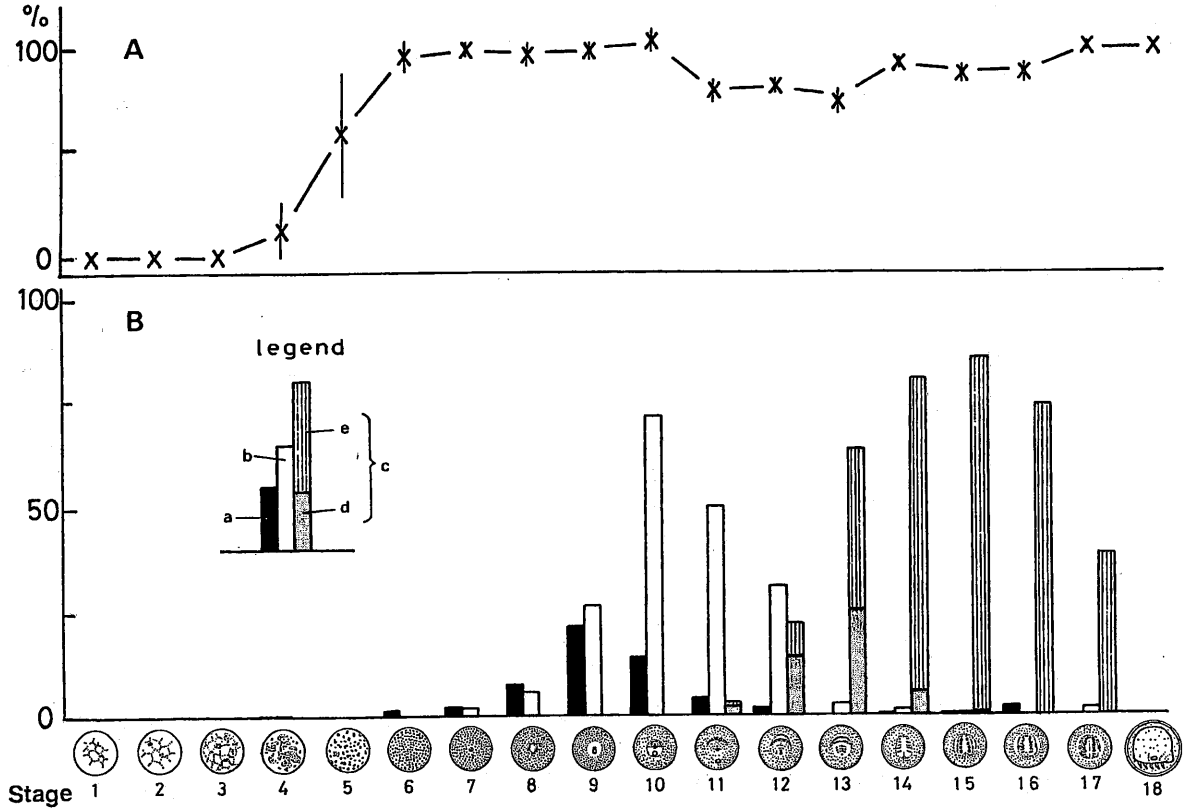

Fig. 5. 24 hr treatment with 10^{-2} M hydroxyurea at different stages. A, The rate of survival embryos; B, the rate of occurrence of different malformations. a, The separate embryos; b, segment-defective embryos; c, embryos with supernumerary appendages; d, embryos with supernumerary " cephalothoracic" appendages; e, embryos with supernumerary " abdominal" appendages. (from 5)

The primordia of segments are formed one by one from the center cells at the posterior end of the embryonic area, and they are determined soon after formation. When DNA synthesis inhibitors are applied, the forming primordium acquires a character intermediate between the anterior segment already determined and the segment next to be determined. The abnormal differentiation is possibly caused by a time lag between DNA synthesis and the rest of metabolism. The specific character of each segment of normal embryos and malformations successively determines that of the next segment. By this route the whole number of segments is established.

Inhibitors stop the cell cycle at the S phase, and block both segmentation and embryonic development, but segmentation recovers in advance of embryonic development. As a result, the transfer of active gene sites becomes incomplete and supernumerary segments are formed.

That is, one or two cells successively divide from the center cells and the cells become the primordium of each segment. The active site of genes of the center cells and the divided cells

succesively transfer at S phase of cell cycle, and the characteristics of each segment is determined according to active gene sites. We called this idea the successive gene expression theory (Fig. 6).

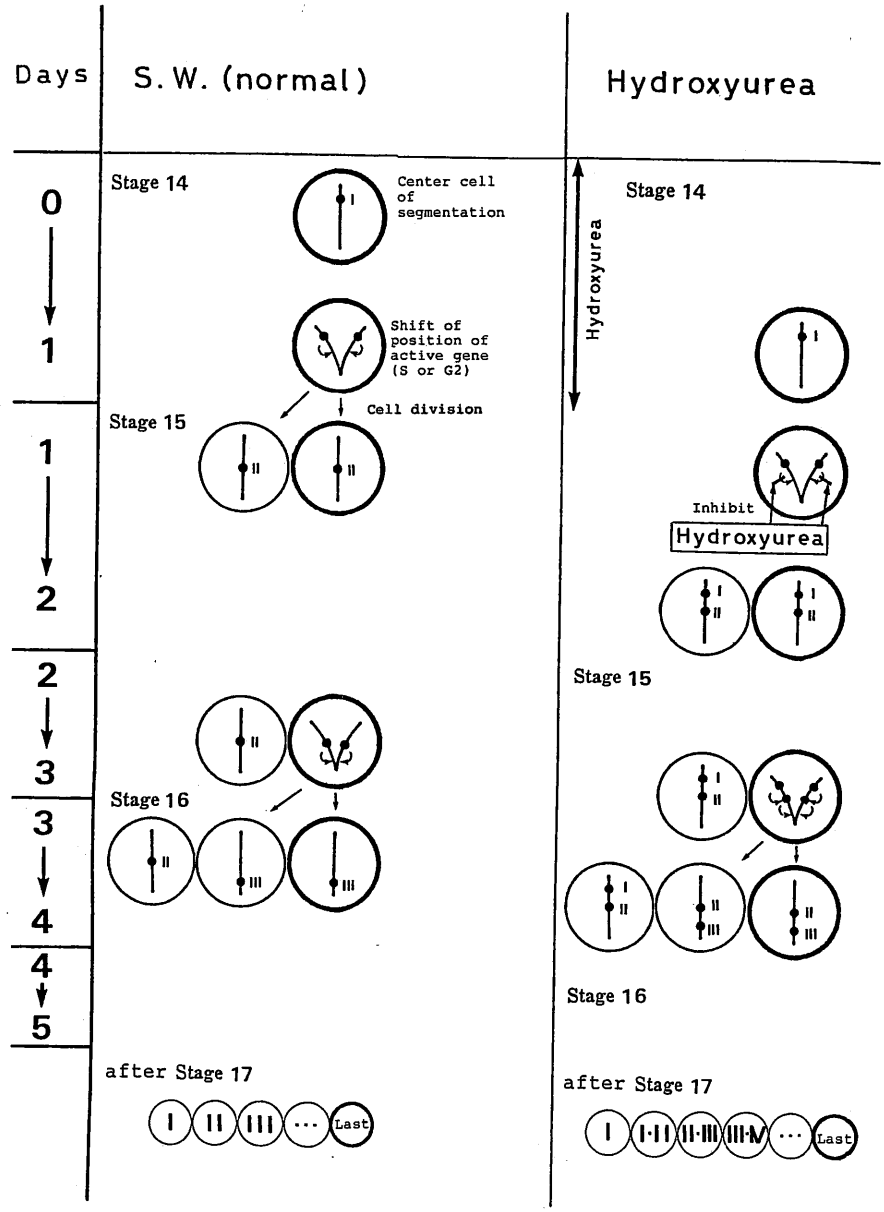

Fig. 6. Hypothetical mechanism of formation of supernumerary segments. This figure, shows different stages of normal embryos and the embryos after the treatment with hydroxyurea. Each diagram shows the character and the active genes in segments and the center cells (growth zone) where segment primordia are formed. A series of genes determine the characters of the segments. These genes appear one by one, according to the formation of segment primordia. The transfer of active genes occurs during the S or G2 period of the cell cycle in the center cells (the circle on the right side of each diagram). When embryos are treated with hydroxyurea, the transfer becomes incomplete. We can think of two explanations: one is that the gene active sites of some cells in the center cells are never transferred, whereas others are normally transferred. The second is that the gene active sites of all cells in the center cells are incompletely transferred. The latter possibility is shown in this graph. (from 5)

Besides, the horseshoe crab egg extract rescues embryos from formation of supernumerary segments after hydroxyurea treatment[14] (Table 3). An extract of horseshoe crab eggs cancels the formation of supernumerary segments induced by hydroxyurea, although the extract does not cancel or slow down the cell cycle after hydroxyurea treatment. The active components of the egg extract are amino acids and related substances, which act antagonistically on hydroxyurea. The supernumerary segments are determined 4 hr immediately after hydroxyurea treatment. This phase corresponds to the S or G_2 phase of cell cycle, and may indicate that the transfer of active gene sites occurs at this time. The egg extract rescues embryos from the abnormal state according to the timing between segmentation and embryonic development.

Table 3. Effects of treatment of embryos with egg extract and amino acids on the supernumerary segment formation. Embryos were treated for 24 hr with 10^{-2} M hydroxyurea at Stage 12-14, and they were simultaneously or later treated for 24 hr with egg extract or amino acids. The mean value of the rate of embryos with supernumerary segments (%) and S.E. are shown. (from 14)

	Embryos with supernumerary segments (A)	Monsters by hydroxyurea as control (B)	A/B x 100	Judgement	Number of treated embryos
Hydroxyurea + Egg extract	18.7 ± 4.6	85.3 ± 2.8	21.9	Rescue	725
Egg extract after Hydroxyurea	28.9 ± 6.1	86.2 ± 3.3	33.5	Rescue	525
Hydroxyurea + Glutamic acid	27.3 ± 27.3	62.3 ± 31.0	43.8	Rescue	50
Glutamic acid after Hydroxuurea	26.2 ± 15.8	77.1 ± 22.9	34.0	Rescue	75
Hydroxyurea + Glycine	11.9 ± 4.8	64.1 ± 24.1	18.6	Rescue	50
Glycine after Hydroxuurea	8.9 ± 3.0	64.1 ± 24.1	13.9	Rescue	50

The genes that determine characteristics of body segments have been found in insects[15,16].

Similar genes have been found in Vertebrata[17,18]. The genes of cephalothoracic segments in Arthropoda are similar to ones specifying neck segments in Vertebrata. The genes of abdominal segments in Arthropoda are similar to ones of the thorax in Vertebrata. Those genes line up on one or a few chromosomes. The relationships of those genes with the genes of horseshoe crabs will be analyzed in future.

Upturned Horseshoe Crabs ("Kabuto" Crab=Helmet Crab) are Armored Fishes ("Kabuto" Fishes) ???

The origin of the Deuterostomia, which includes Vertebrata, is also important problem in the biology of evolution. Some scholars think that the Deuterostomia originated from the Protostomia, which includes Annelida and Arthropoda. The upturned features of Protostomia are similar to Deuterostomia (Fig. 7). The genes of determination of ventral side of Protostomia have made clear to be similar to ones of dorsal side of Deuterostomia[19]. But some other scholars deny this idea because the alimentary canal of Protostomia pass through the middle of the central nervous system. They think that the body plan of Deuterostomia principally differs from that of Protostomia. However, we found the following fact. Attempts to derive Deuterostomia from Protostomia cannot be rejected because of the crossing of the alimentary canal and central nervous system[20].

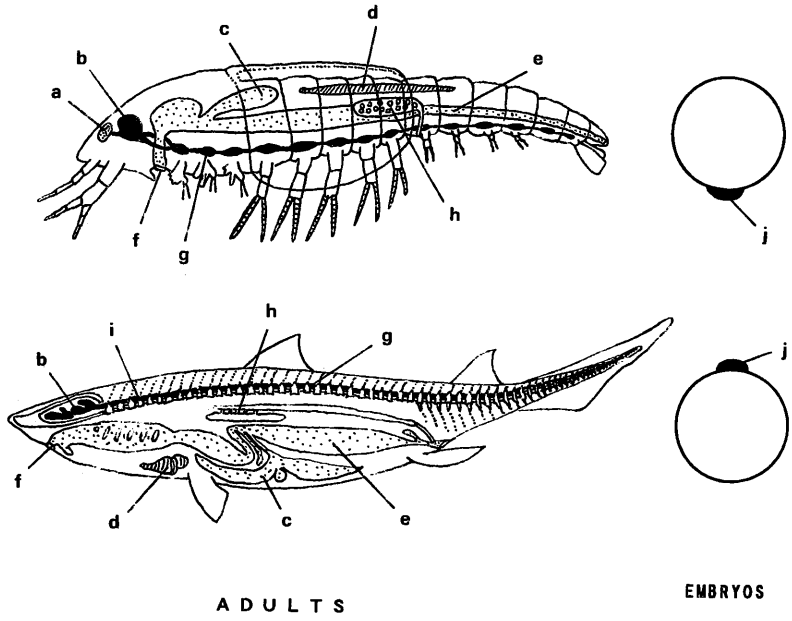

Fig. 7. Adults and embryos of Protostomia (Cruatacea) [Upper figure] and Deuterostomia (Pisces). a, Eye; b, brain; c, liver (mid-gut gland); d, heart; e, alimentary canal; f, mouth; g, central nervous system; h, reproductive gland; i, endoskelton; j, germ disc (blastodisc). Horseshoe crabs have large endoskeleton (cartilage) which are situated at inner side on central nervous system.

Calcium free sea water, $NaHCO_3$ and inhibitors of DNA synthesis blocked the development of mesodermal bands. As the result, the mesodermal bands were separated and the treated embryos developed into the separate embryos. The ventral plate of the separate embryo was divided into anterior region and a posterior region (Fig. 8).

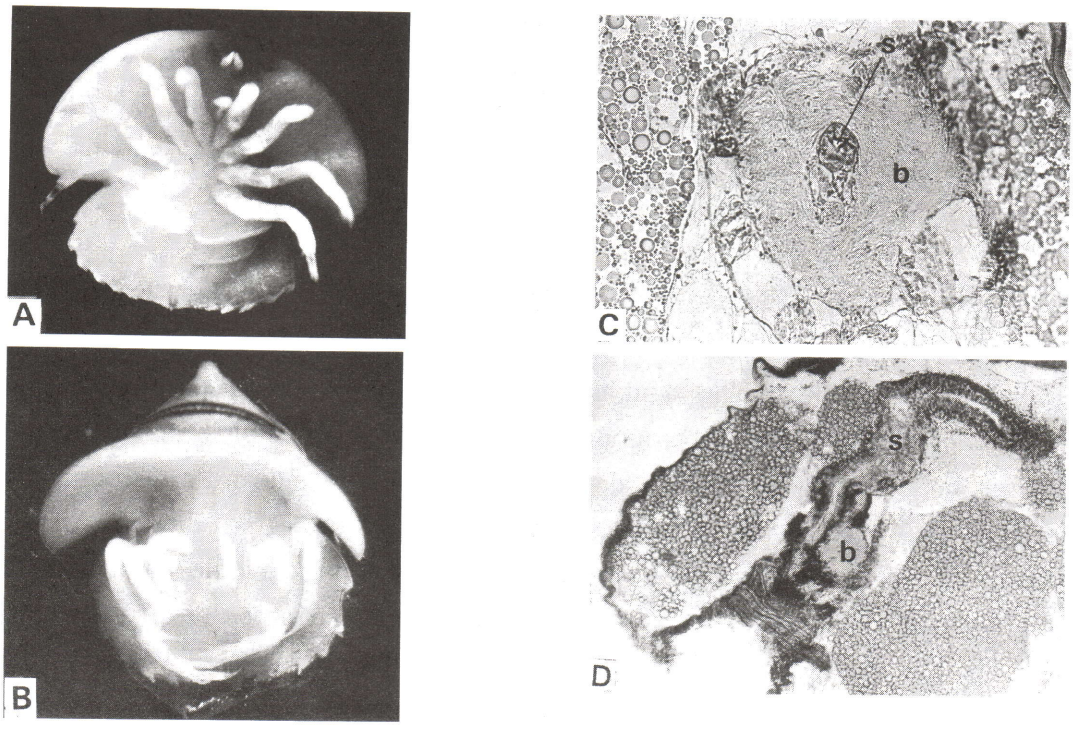

Fig. 8. Examples of separate embryos. A: A separate embryo whose ventral plate is separated at the point between the 1st segment and the 2nd one. B: A separate embryo whose ventral plate is completely separated at the 2nd segment. The 2nd appendages are lost. C: A horizontal section of a separate embryo whose ventral plate is separated at the 2nd segment. The stomodaeum passes through the middle of the brain. D: A longitudinal section of a separate embryo whose ventral plate is separated at the region between the 1st segment and the 2nd one. The crossing of stomodaeum and nervous system is released. b, Brain; s, stomodaeum.

When embryos were treated for 24 hr with calcium free sea water or $NaHCO_3$ at Stages 7, 8 and 9 (stage of enlargement of the germ disc, the gastrula stage), they developed into the separate embryos whose ventral plates were separated mainly at the region between the 3rd and 5th segments. When treated for 24 hr at Stages 10 and 11 (stages of obvious morphogenetic movement), the ventral plates of the treated embryos were separated mainly at the 2nd and 3rd segments. Following treatment for 24 hr with an inhibitor of DNA synthesis at the stage of enlargement of germ disc, the treated embryos developed into separate embryos, whose ventral plates were separated mainly at the 2nd and 3rd segments. The treatment with DNA synthesis inhibitors at the stage of obvious morphogenetic movement did not induce separate embryos (Fig. 5).

When embryos at the stage of enlargement of the germ disc were treated with calcium free sea water or $NaHCO_3$, the connection between cells composing the germ disc was weakened. Their ventral plates were separated mainly at the region between the 3rd and 5th segments of the cephalothorax, which was formed in the process of enlargement of the germ disc. When embryos at the stage of enlargement of the germ disc were treated by inhibitors of DNA synthesis, cell proliferation of the germ disc became incomplete and the cell density of the germ disc decreased in spite of normal spreading of the germ disc. The incomplete germ disc was separated mainly at the 2nd and 3rd segments in the process of obvious morphogenetic movement. During the movement, the embryonic area elongated anteriorly and posteriorly at

the region where the 2nd and 3rd segments had recently been formed (Fig. 1). Calcium free sea water and $NaHCO_3$ directly affected the central region of elongation at the stage of obvious morphogenetic movement. The ventral plates of the treated embryos were separated mainly at the 2nd and 3rd segments.

The form and structure of the separate embryos depended on the position of separation. It was impossible to recognize any difference between embryos induced by different reagents, if the position of separation was the same.

In some of the separate embryos, the stomodaea passed behind the brains. This is the normal position. The stomodaea of some separate embryos passed through the middle of the brains. The crossing of the nervous system and stomodaeum was released in others of the separate embryos.

The release of the crossing was not related directly to the degree of separation, but it was related closely with the position of separation, that is, the extent of the anterior region of the separate ventral plate (Fig. 9). When the anterior region had more than three pairs of appendages, the stomodaeum passed behind the brain and both structures crossed each other. When it was moderate, the stomodaeum passed the middle of the brain. When the anterior ventral plate of the separate embryos had no or only one pair of cephalothoracic appendages, the crossing was released. The size of the posterior region of the separate ventral plate was not related directly to the release of the crossing of the nervous system and stomodaeum.

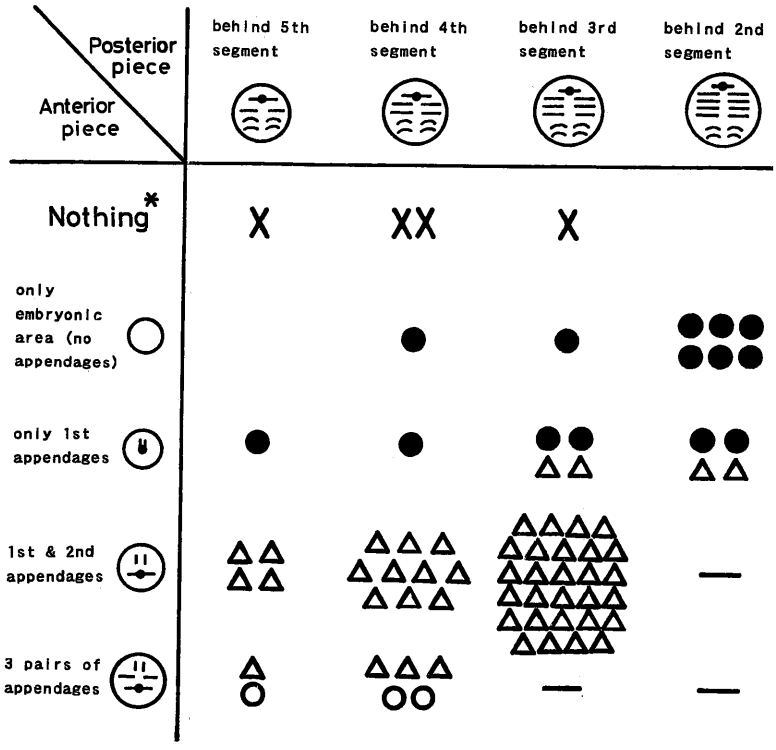

Fig. 9. Release of the crossing of the stomodaeum and nervous system. The schematic diagrams show the features of each piece of the separate ventral plates. Each mark indicates an embryo. × : Embryos having no brains or stomodaea. ● : Nervous systems and stomodaea do not cross each other. △ : Embryos whose stomodaea pass through the middle of the brain. ○ : Embryos whose stomodaea pass behind the brain (normal position). * : Embryos without anterior pieces of separated ventral plates (= no-anterior embryos).

In normal horseshoe crab embryos, the tubular structure of the stomodaeum was recognizable after stage 14 (stage of appearance of appendage primordia). The cell masses of ganglions are observed clearly after Stage 16; the commissures of the ganglions were stained by eosin after Stage 19. The final number of ganglions is settled by Stage 19: This means that the formation of segment primordia is settled by Stage 19.

The stomodaeum passed through the area between the commissure of the first ganglion (1st segment) and that of the second ganglion. The opening of the stomodaeum (mouth) first appeared in front of the 1st segment. The mouth began to migrate posteriorly at stage 18. These results and the fact that the stomodaeum passed through the area between the commissure of the ganglions of the 1st and 2nd segment indicate that the crossing of the nervous system and stomodaeum occurs at stage 18-19.

Prospective cells of the stomodaea and brains were determined before the time of separation of the ventral plates, because regulation did not occur after separation. The prospective cells of the brain were moved by the separation (Fig. 10). When the anterior part of the separate ventral plates was very small, the prospective cells of the brain were situated behind the stomodaeum. As a result the crossing of the nervous system and stomodaeum did not occur.

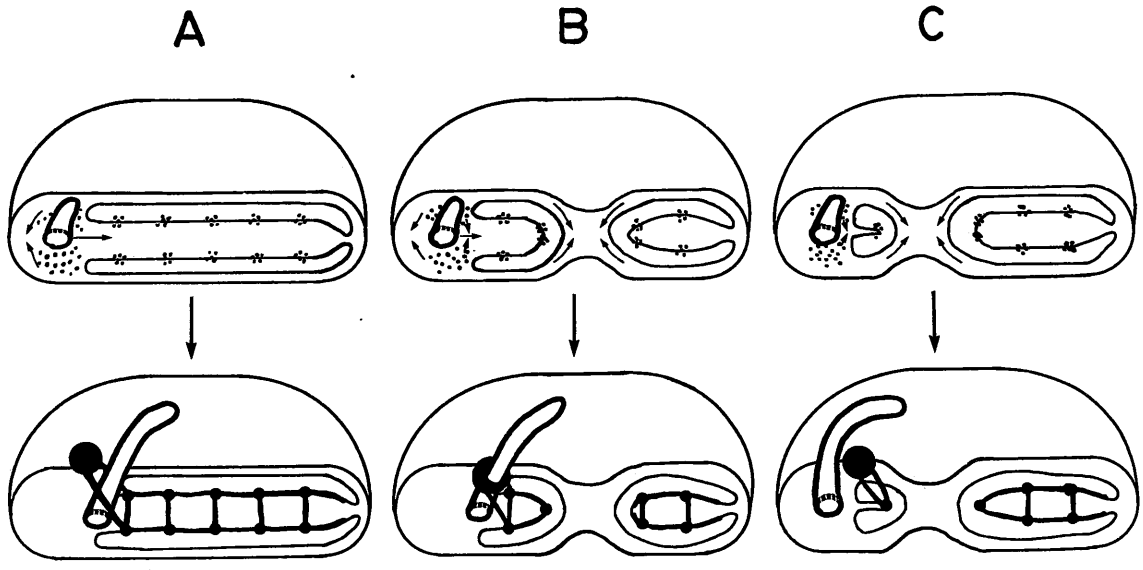

Fig. 10. Diagramatic representations of development of brains, other nervous systems and stomodaea in normal embryos and separate embryos. A: A normal embryo. B: A separate embryo whose ventral plate is separated at the 2nd and 3rd segment. C: A separate embryo whose embryonic area is separated at the 1st segment.

The release of crossing reveals the following four points. (1) The crossing is constructed through the process of clustering of brain cells. (2) It is no determined that the cells of the brain cluster anterior to the stomodaeum; that is, the stomodaeum does not determine the clustering point of the brain. Further, the brain does not determine the pathway of elongation of the stomodaeum. (3) As the position of the mouth is changed easily, it is not appropriate to use it as an indicator of determination of homologous segments among arthropod species[21,22]. (4) The crossing is susceptible to modification in at least one representative Protostomia, the horseshoe

crab. The crossing does not constitute a valid reason for rejecting the idea that Deuterostomia have originated from Protostomia.

Conclusions

We could change the morphogenesis of horseshoe crab embryos. Some of those changes could be related to evolutionary events. Trilobites are thought to be ancestors of horseshoe crabs. They had many segments and branchial appendages. Through the process of evolution, the number of segments decreased and appendages became non branchial types. Our experiments induced the horseshoe crabs to have supernumerary segments. Those segments had branchial appendages. Besides, we could form Deuterostomial type horseshoe crabs. The mechanism and process of evolution must be made clearly by experimental biology of evolution. The great advance of experimental evolution may bring the handling evolution in future. We may be able to see the revival dinosaurs and the revival trilobites[23].

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