An experimental study on the formation of body axes in embryos of the horseshoe crab, *Tachypleus tridentatus*

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ABSTRACT. The dorsal–ventral axis and the anterior–posterior axis of the horseshoe crab are formed independently of the direction of gravity. Several experiments induce the modification of formation of anterior–posterior axis. The results of these experiments show the followings : The anterior–posterior axis is not determined before the appearance of germ disc (early gastrula stage). This character differs from that of insects, and is similar to those of Annelida, Crustacea and Vertebrata. *Cumulus posterior* is not necessary for the formation of the anterior–posterior axis. The axis is determined during the process of spreading of germ disc.

INTRODUCTION

The gravity affects the determination of the dorsal–ventral axis and the anterior–posterior axis (medial body axis) of many animals such as Amphibia (Scultze, 1894; Kirschner et al., 1980; Ubbels et al., 1983; Wakahara, 1986), Aves (Kochav and Eyal–Giladi, 1971) and Pisces (Clavert and Filogamo, 1966). Under the condition of no gravity in the space shuttle, embryos of Aves could not develop. The cause of fault is thought the incomplete formation of body axes. In the embryos of the horseshoe crab (Chelicerata, Arthropoda), the pseudocleavage occurs in the opposite side of direction of gravity (Patten, 1894; Oka, 1943). However, whether the dorsal–ventral axis and the anterior–posterior axis of this animal are determined under the influence of gravity or not have been unknown. In this paper, the influence of the gravity to the differentiation of the body axes, dorsal–ventral and anterior–posterior was examined. In addition, the author tried to modify the anterior–posterior axis by the treatment using chemical reagents and electrocauterization. From these results, the cause of formation of the axis is discussed.

MATERIALS AND METHODS

Males and females of horseshoe crabs *Tachypleus tridentatus* were collected at the beaches of north Kyushu in Japan, and were transferred to Shizuoka University where present studies were carried out.

Influence of gravity: Some unfertilized eggs (spherial, ca.3mm) taken from the living female were put on the slide glass. The eggs easily adhere to the surface of glass by own adhesive substances on the chorion. Such fixed eggs were artificially inseminated and reared in a plastic tray filled the filtered sea water at 28 to 30℃. The sea water was changed everyday (Fig. 1).

Treatments by chemical reagents : (1) The embryos were treated by cycloheximide (the inhibitor of protein synthesis) and actinomycin D (the inhibitor of RNA synthesis) at the stage of surface cleavage (stage 4 and 5).

(2) The cells of embryos at the stage of appearance of germ disc (early gastrula, stage 7) were dissociated by calcium free sea water which was made by adding NaCl instead of $CaCl_2$ in Van't Hoff's artificial sea water. Embryos in periods from stage 8 to stage 10 were also treated by calcium free sea water.

Electrocauterization : The part of medial portion in front of the blastopore was electrically cauterized by the method of Oka (1943) in periods from the stage of spreading of germ disc (stage 9) to the stage of appearance of segment structures (stage 12). By the other experiments, the embryonic area was divided into right half and left half during same stages.

Observation : The normal and treated embryos were vitally stained with neutral red (1/20,000), and the dorsal–ventral axis and the anterior–posterior axis of embryos were ascertained under a stereoscopic microscope. The stages of embryonic development were compared with the normal plate described by Sekiguchi (1973).

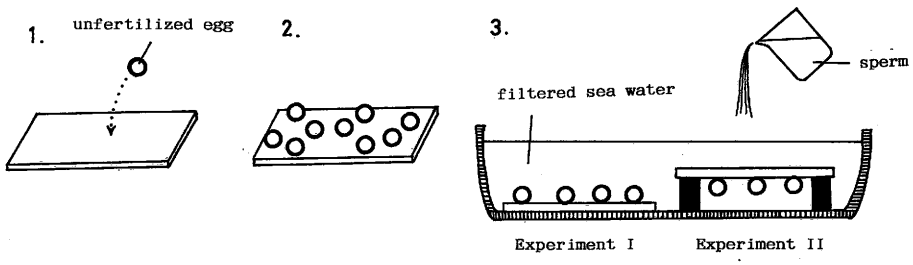

Fig. 1. The adhesion of horseshoe crab eggs on the slide glass (1 and 2), and the insemination of them (3). The eggs were fixed upside and underside of the glass (Experiments I and II).

RESULTS

1. The influence of gravity

The unfertilized eggs were adhered on the slide glass, and they were normally fertilized and developed.

The influence on the determination of the dorsal–ventral axis : The dorsal–ventral axis of the horseshoe crab embryos can be ascertained at stage 7 (early gastrula stage)when the germ disc appears, because the region at which the germ disc appears becomes the future ventral side of the embryo.

When the eggs were partitioned into three equal parts perpendicular to the direction of gravity and the position of a germ disc of each egg was ascertained (Fig.2), germ discus of different eggs appeared in all parts. The results were same in the both case of the eggs adhered on the upper surface of the slide glass(Table 1–Experiment I)and the eggs adhered on the under surface (Table 1 – Experiment Ⅱ).

The influence on the determination of the anterior–posterior axis: The anterior–posterior axis (medial body axis) of the horseshoe crab embryo coincides with the direction of migration of the cell mass composing of *cumulus posterior*(Sekiguchi,

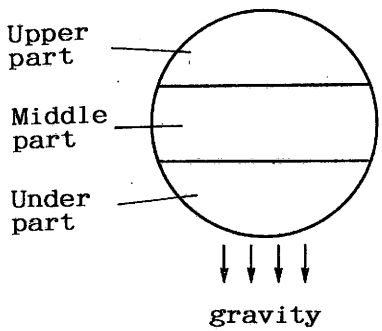

Fig. 2. The classification of the position of germ disc in eggs, for the experiment of Table 1.

Table 1. The position of germ disc in regard to gravity. The position of germ disc is future ventral side, and the dorsal–ventral axis is determined as the result. For the method of experiments, refer to Fig. 1, Fig. 2 and the text.

	Experiment I	Experiment Ⅱ	Total
Upper part	11	12	23
Middle part	9	9	18
Under part	10	8	18

1973; Itow and Sekiguchi, 1980). At stage 9, *cumulus posterior* separates from the central portion of germ disc (*cumulus anterior*)and migrates posteriorly. There is the blastopore at the central portion of germ disc.

When the direction of migration was classified into upward,horizontal and downward direction (Fig. 3), the migration of *cumulus posterior* was observed in all three direction (Table 2-A). When the direction of migration was classified into right, vertical and left direction(Fig. 3),*cumulus posterior* migratedin all three direction (Table 2-B). The above-mentioned results were same in the both cases of the eggs

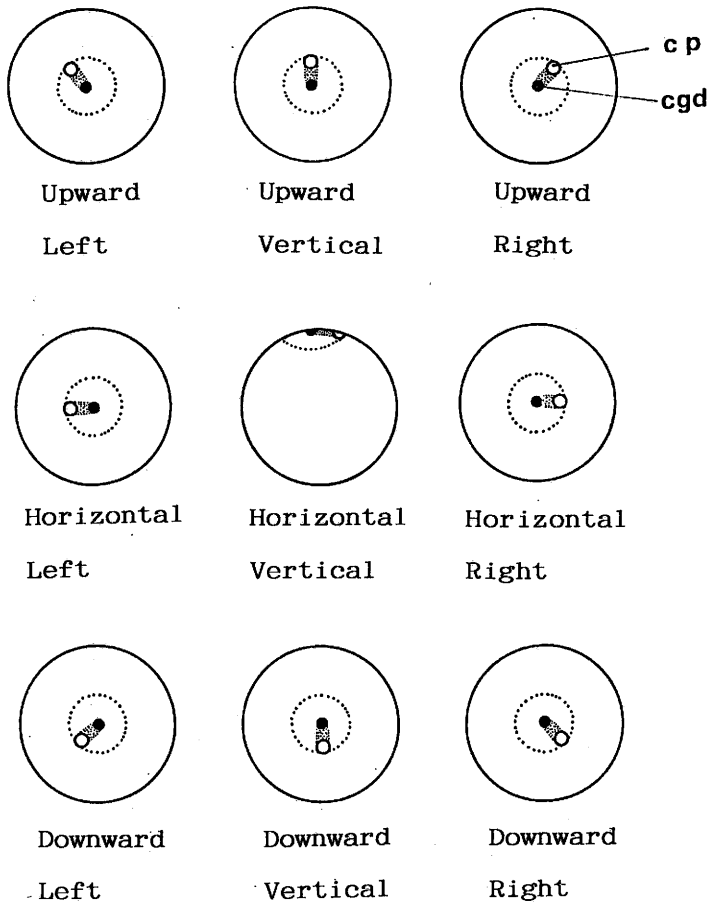

Fig. 3. The classification of the direction of migration of *cumulus posterior*, for the experiment of Table 2. In this figure, the direction of gravity is downward. cgd: the center of germ disc (*cumulus anterior*), that is, the portion of the blastopore. cp: *cumulus posterior*.

adhered on the upper surface of the slide glass (Table 2– Experiment I) and the eggs adhered on the under surface (Table 2 – Experiment II). After all, the direction of migration of *cumulus posterior* can be said to be all way independently of the gravity (Table 2–C).

2. Effects of chemical reagents

(1) When embryos at the stage of surface cleavage (stage 4 and 5) were treated for 24hr by 10 to 100 μg/ml cycloheximide or 25 to 250ng/ml actinomycin D, the germ disc of treated embryos became large and abnormal at stage 7. In about 5% of them, two anterior–posterior axes elongate from the abnormal and large germ disc (Fig. 4).

(2) When embryos are treated by calcium free sea water at stage 7, the cells are dissociated and the multiple monsters are induced (Itow and Sekiguchi, 1979). The process of induction was in detail observed in the present study. The blastoderm layer became torn by the treatment. The tears arose in several undefined regions of the treated embryo and spread in various directions. The germ disc disappeared during the treatment. After returning the treated embryos to normal sea water, the

Table 2. The direction of migration of *cumulus posterior*. The direction of migration is coincided with anterior–posterior axis (medial body axis). For the methods of experiments, refer to Fig. 1, Fig. 3 and the text.

〔A〕

Direction	Experiment I	Experiment II	Total
Upward	11	2	13
Horizontal	5	9	14
Downward	10	8	18

〔B〕

Direction	Experiment I	Experiment II	Total
Left	11	6	17
Vertical	11	6	17
Right	4	7	11

〔C〕

	Left	Vertical	Right	Total
Upward	6	3	4	13
Horizontal	4	7	3	14
Downward	7	7	4	18
Total	17	17	11	45

torn blastoderm recovered. The cells which had composed of the germ disc were reaggregated into some cell-masses. These cell-masses bordered on a common area devoid of cells. Each cell-mass developed into an embryo, and finally multiple monsters were formed(Fig. 5).The position bordering the void space became the posterior end of an embryo and the anterior-posterior axis of each embryo was generally vertical to the tangent of the void space.*Cumulus posterior* was not formed during development of the multiple monsters. The embryos which were treated after stage 9 did not develop into multiple monsters.

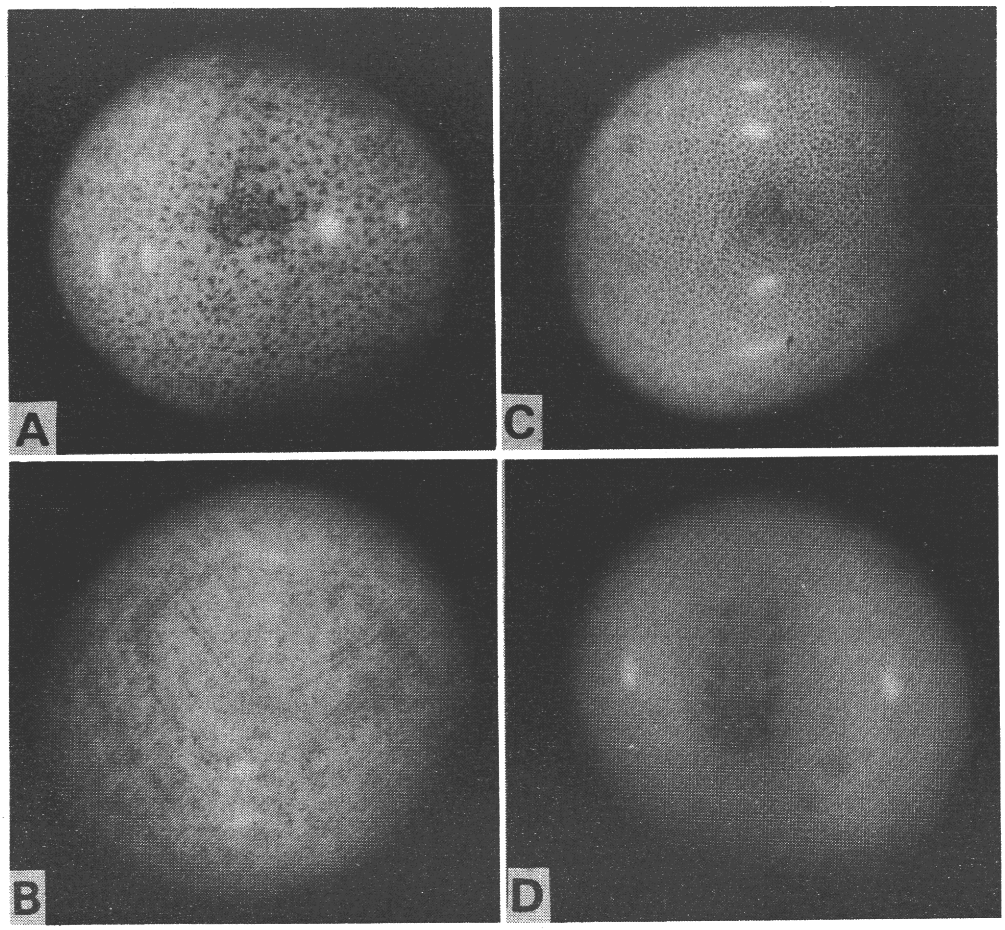

Fig. 4. The treatment by cycloheximide. Embryos were treated for 24 hr by 10-100 μg/ml cycloheximide at stage 4 and 5. A: The embryo at stage 7 after the treatment. The cells composing of germ disc are larger than those of normal embryos, and the size of germ disc is also larger than normal one. B: The embryo at stage 16 after the treatment. Two anterior-posterior axes can be observed. C: A normal embryo for control at stage 7. D: A normal embryo at stage 16.

3. Electrocauterization

When the cells at the medial portion in front of the blastopore at stage 9 were electrically cauterized (Fig. 6), the anterior-posterior axis of treated embryos was often curved at the cauterized portion. The same experiment induced double monsters at the rate of about 10%(Table 3 and Fig.7). The similar experiments at other stages induced defective embryos, but did not induce the curve of axis.

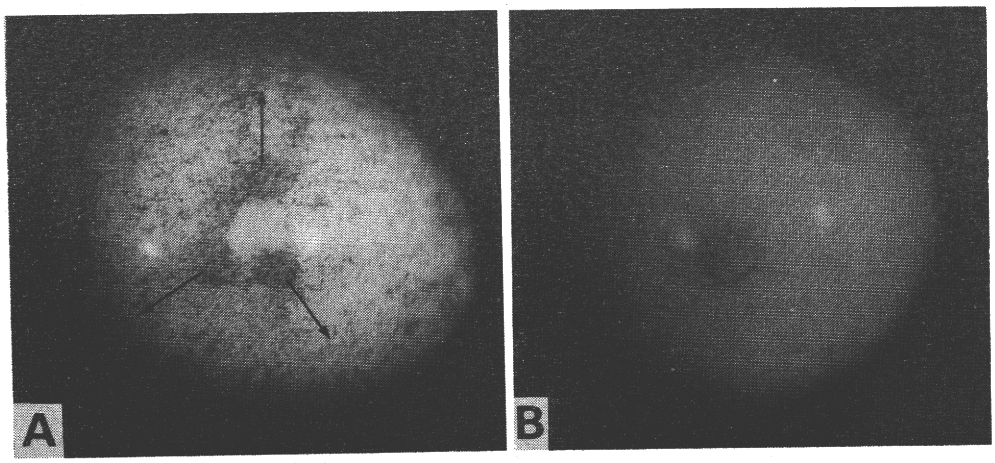

Fig. 5. The appearance of a few cell-masses after the treatment by calcium free sea water. Embryos were treated for two or three days at stage 7. A: The treated embryo with three cell-masses. The embryo developed into a triple monster. Arrows show the direction of anterior-posterior axis of each embryo. In this case, *cumulus posterior* was not observed. B: A normal embryo for control at stage 8.

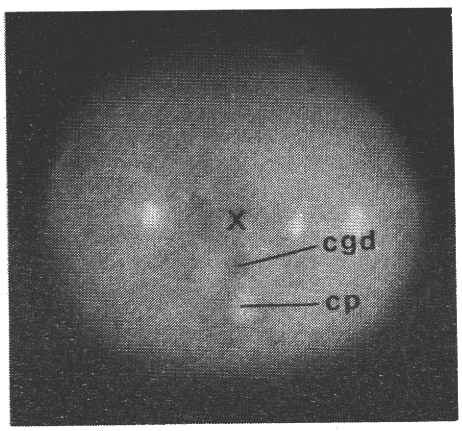

Fig. 6. The method of electocauterization at the medial portion of cells in front of the blastopore. X shows the point of electrocauterization. cgd: the central portion of germ disc (the blastopore). cp: *cumulus posterior*.

Table 3. The result of electrocauterization at the medial portion of cells in front of the blastopore at stage 9. For the methods of experiments, refer to Fig. 6 and the text.

	Number	(%)
Normal embryo	6	(24)
Curve of axis	14	(56)
Duplication and Excess	3	(12)
Partial defects	2	(8)
Not development	15	

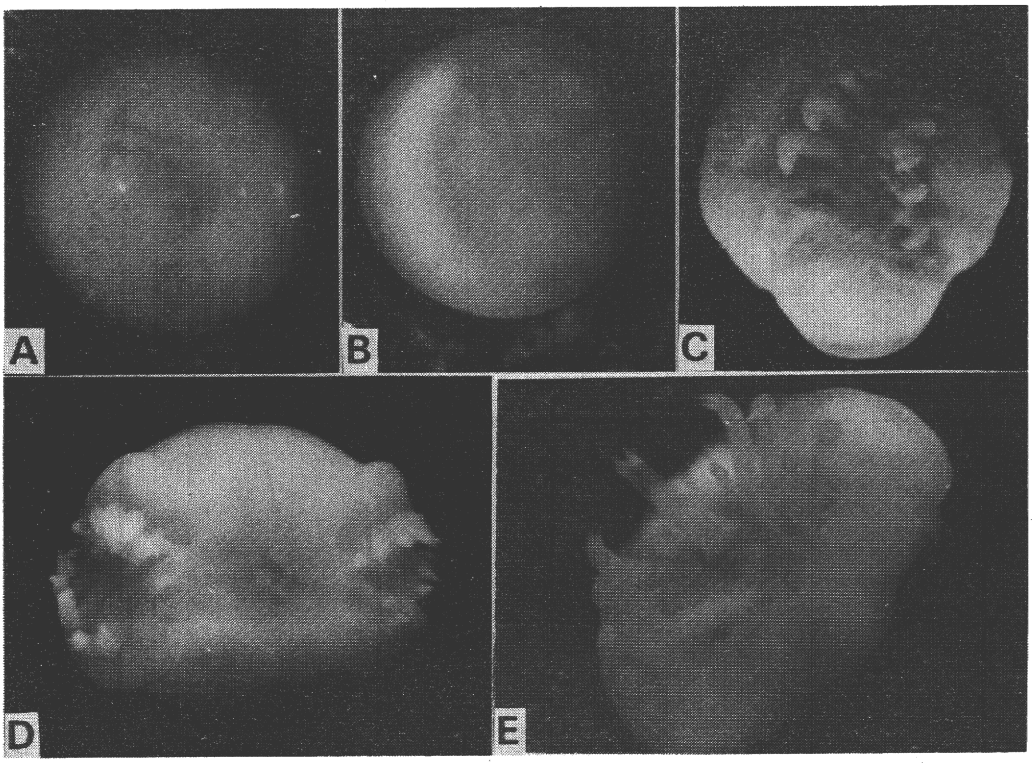

Fig. 7. The malformed embryos induced by the electrocauterization. For the methods, refer to Fig. 6 and the text. A, B and C: The monsters having the curved anterior–posterior axes. D: A double monster. E: The monster showing partial excess. The excess appendage can be observed on the anterior–posterior axis.

When the anterior regions of germ discus of embryos at stage 9 and stage 10 were electrically cauterized to be divided into right and left half, almost of them developed into double monsters which showed anterior duplications. When the same place of the embryos after stage 11 were electrically cauterized, they did not develop into double monsters. The results coincided with the results of Sekiguchi (1966).

DISCUSSION

The position of germ disc becomes ventral side, and the dorsal–ventral axis is determined. The germ disc of the horseshoe crab embryo appeared at unspecified portion in regard to the direction of gravity (Table 1). The phenomenon such as Schultze's double formation, the inverted amphibian eggs develop into double monsters(Schultze, 1894), was not observed in the horseshoe crab embryos. Besides, the rotation of embryonic body in chorion, which is seen in bird embryos and spider ones (Holm, 1952),was not seen in the embryo of horseshoe crab. These facts show that the position of germ disc of the horseshoe crab was not formed under the influence of gravity. The position of germ disc of horseshoe crab embryos was not coupled to the position of the pseudocleavage. The germ disc may appear at the fixed position which was formed before the fertilization.

The direction of the anterior–posterior axis(medial body axis) in the horseshoe crab is also independent of the gravity (Table 2) .

The anterior–posterior axis of the horseshoe crab embryo can be increased in number when the embryo is treated by electrocauterization or chemical reagents at stage 7 (Figs. 4 and 5; Sekiguchi, 1966; Itow and Sekiguchi, 1979). The fact means that the embryos of horseshoe crab have the capacity to form the anterior–posterior axis to all directions after the appearance of germ disc. This character differs from that of long–germ band insects whose anterior–posterior axes are determined before the blastura stage (French, 1988; Sander, 1988). On the other hand, it is similar to those of Annelida (Weisblat et al., 1988), Crustacea (Dohle and Scholtz, 1988) and Vertebrata (Jacobson, 1988).

The formation of anterior–posterior axis is thought to depend on the spreading and aggregation of cells in front of the blastpore at stage 9. Becauce the anterior–posterior axis was often curved when the medial portion in front of the blastopore is electrically cauterized at stage 9(Table 3 and Fig.7).At this stage the mesodermal layer elongates from the blastopore (Itow,1985). The elongation must be curved at the cauterized point. Besides, it is known that two anterior–posterior axes are formed when these cells were experimentally divided into right and left part at stage 9 and stage 10 (Sekiguchi, 1966). These cells migrate and aggregate by the law of minimum free energy and they reach equilibrium, and form the anterior–posterior axis.

The axis may be determined during the process of spreading of germ disc(stage 9

to10), because the axis did not increase in number after the similar electrocauterization after stage 11.

The direction of migration of *cumulus posterior* in normal embryos coincides with the future anterior–posterior axis of embryo, and Holm (1952) said that *cumulus posterior* played the role of primary organizer in the spider embryos. But this study showed the morphogeneses of horseshoe crab embryos proceed normally without the appearance of *cumulus posterior*. The role of *cumulus posterior*, which may be reproductive cells, remains unexplained in this study.

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