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Differentiation and Behaviour of Primordial Germ Cells during the Early Embryonic Development of *Parnassius glacialis Butler*, *Luehdorfia japonica* Leech and *Byasa (Atrophaneura) alcinous alcinous* Klug (Lepidoptera: Papilionidae)

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Synopsis

Differentiation and behaviour of the primordial germ cells of *Parnassius glacialis*, *Luehdorfia japonica* and *Byasa (Atrophaneura) alcinous alcinous* are described. The primordial germ cells of *P. glacialis* are formed through mitotic divisions and ivagination of the cells at mid-line of the region about one-third of ventral plate, before completion of formation of serosa. Those of *L. japonica* and *B. alcinous alcinous* appear in a group at the regions about one-third of the germ band before the inner layer formation. When the inner layer has formed, the primordial germ cells are surrounded by the mesodermal cells and come to be undistinguishable from surrounding cells. After the segmentation of ectodermal part of embryo and the metamerization of inner layer, the primordial germ cells divide into two lateral groups in the fourth to sixth abdominal segments, and then they are carried to the lateral-inner sides of the ectodermal parts with the mesodermal cells. During this process, the primordial germ cells grow and assume their characteristic appearance. Their number on each side in the fourth to the sixth abdominal segment varies more or less among the species and individuals. One to four primordial germ cells are counted on one side of each segment in *P. glacialis*, one to three in *L. japonica* and two to five in *B. alcinous alcinous*.

Introduction

Differentiation and behaviour of the primordial germ cells are the subject of study attracting interest of insect embryologists. A considerable number of researches on the germ cells of the lepidopteran insects have been done. The time and the manner of primordial germ cell formation, however, differ remarkably among the species or the author's opinion, and there remain some uncertain problems concerning the differentiation and behaviour of the primordial germ cells in the Lepidoptera. Different opinions and uncertain problems are probably caused by the difficulty to distinguish the primordial germ cells from the somatic cells in the early stage of embryonic development.

In the present paper, the author describes the formation and the behaviour of primordial germ cells in *Parnassius glacialis*, *Luehdorfia japonica* and *Byasa (Atrophaneura) alcinous alcinous*.

Materials and Methods

To obtain eggs for the present study, pregnant females of *Parnassius glacialis* Butler, *Luehdorfia japonica* Leech and *Byasa (Atrophaneura) alcinous alcinous* Klug were captured in Gifu Prefecture in 1980 to 1984. Eggs of *P. glacialis* were laid in May, those of *L. japonica* in April and those of *B. alcinous alcinous* in June, and kept at room temperature in the laboratory.

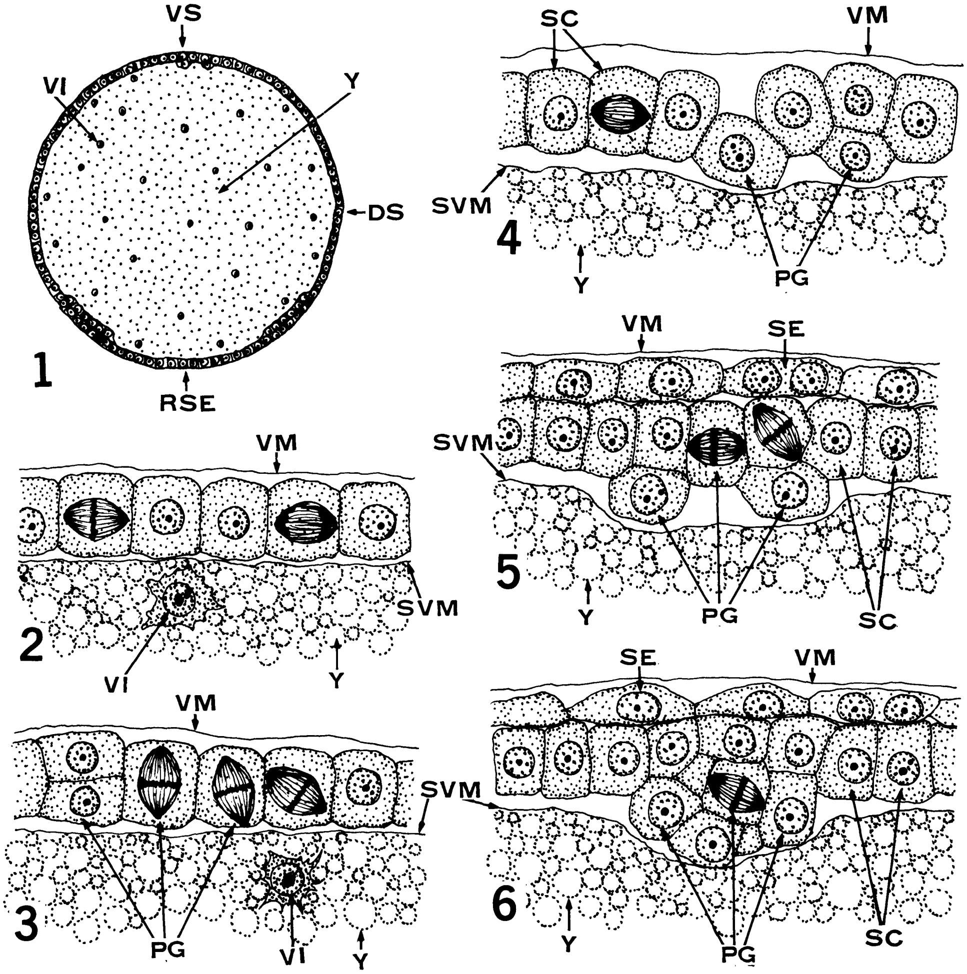
The eggs at various ages were fixed in Carnoy's fluid for 30—40 min or alcoholic Bouin's fluid for 60—90 min. After fixation, the eggs removed of their chorion were dehydrated, embedded in paraffin, sectioned in 8 μ m, and stained with Delafield's haematoxylin and eosin.

Observations

For the convenience of description, the duration of the developmental period, from oviposition to formation of rudiments of appendages, is divided into seven stages as shown in Table 1.

Table 1. Time table of the early embryonic development of *Parnassius glacialis*, *Luehdorfia japonica* and *Byasa (Atrophaneura) alcinous alcinous* at room temperature.

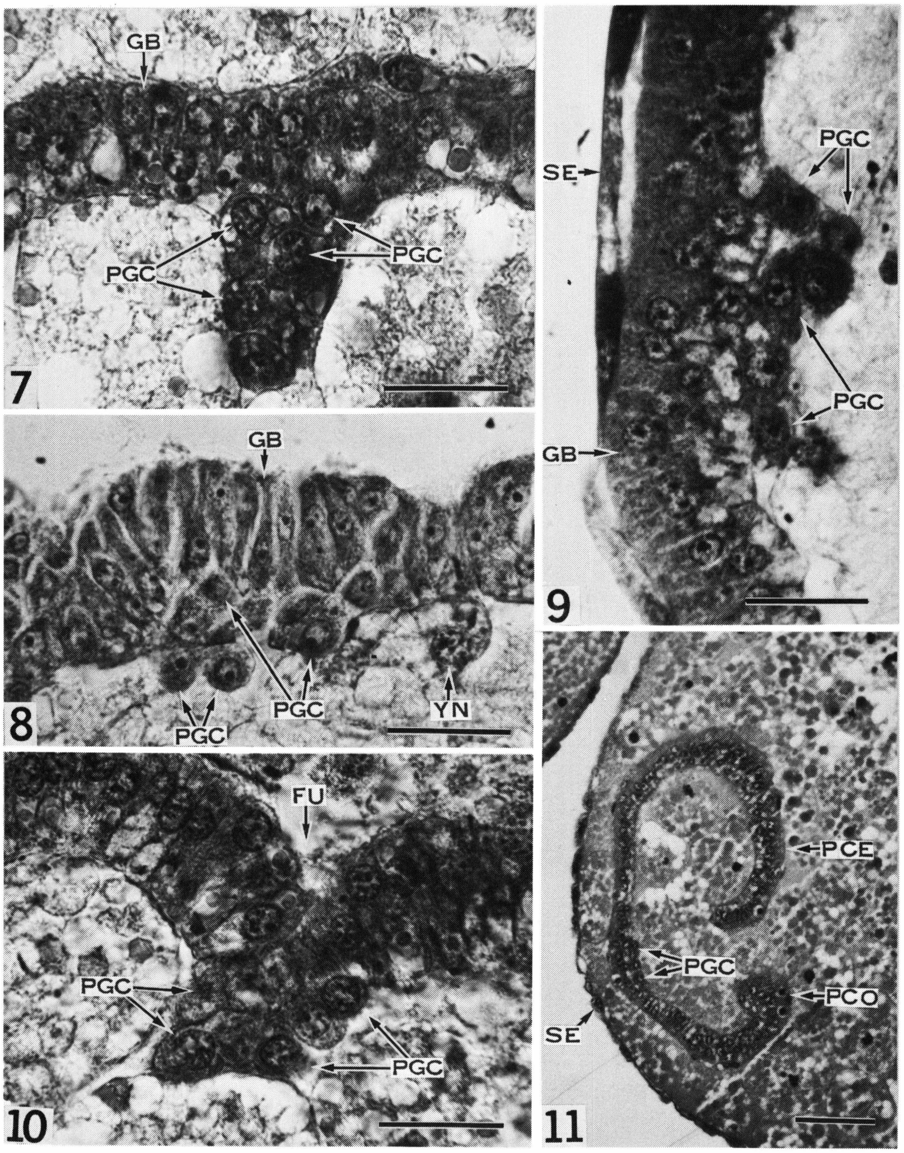
Stage	State of Development	Approximate Age of Eggs		
		P. glacialis (20-23°C)	*L. japonica* (12-15°C)	*B. alcinous* (25-30°C)
1	*Maturation and Fertilization*	0- 1 *hr*	0- 1 *hr*	0- 1 *hr*
2	*Cleavage*	2-16 *hr*	2-14 *hr*	2- 8 *hr*
3	*Formation of Blastoderm and Appearance of Primary Dorsal Organ*	17-20 *hr*	15-18 *hr*	9-12 *hr*
4	*Formation of Ventral Plate and Serosa*	21-24 *hr*	19-23 *hr*	13-15 *hr*
5	*Formation of Germ Band and Anatrepsis Formation of Protocephalon and Protocorm*	1- 1.5 *days*	1 *day*	16-20 *hr*
6	*Formation of Inner Layer and Segmentation of Ectoderm and Mesoderm*	2- 3.5 *days*	1.5- 2 *days*	1 *day*
7	*Embryo Longest in Pre-revolution Stage Formation of Rudiments of Appendages and of Neural Groove*	4- 6 *days*	3- 5 *days*	1.5-2 *days*



Figs. 1—6. Formation of primordial germ cells in *Parnassius glacialis*.

1. Cross section of about 23 hr-old egg through a region about one-third of the egg length from its posterior end. 2. Cross section through the lateral side of ventral plate at about 23 hr after oviposition, showing the mitotic figures of the somatic cells. 3, 4. Cross sections through the ventral side of ventral plate at the same stage of Fig. 2, showing the mitotic figures (Fig. 3) and the invagination (Fig. 4) of primordial germ cells. 5, 6. Cross sections through the ventral side of ventral plate at about 27 hr after oviposition (Fig. 5) and at the slightly later stage than that shown in Fig. 5 (Fig. 6).

DS, dorsal side of ventral plate; PG, primordial germ cell; RSE, rudimentary serosa; SC, somatic cell; SE, serosa; SVM, secondary vitelline membrane; VI, vitellophagus; VM, vitelline membrane; VS, ventral side of ventral plate; Y, yolk.



Figs. 7—11. Primordial germ cells of embryo of *Parnassius glacialis*, *Luehdorfia japonica* and *Byasa alcinous alcinous*.
7. Sagittal section of 45 hr-old embryo of *P. glacialis*, showing a cluster of primordial germ cells. 8. Cross section of 12 hr-old embryo of *B. alcinous alcinous*, showing primordial germ cells. 9. Sagittal section of 24 hr-old embryo of *L. japonica*, showing primordial germ cells. 10. Sagittal section of 2. 5 day-old embryo of *P. glacialis*, showing a furrow at the region where the cluster exists. 11. Sagittal section of 2. 5 day-old embryo of *P. glacialis*, showing the region in which primordial germ cells are located.
GB, germ band; FU, furrow on the ventral surface of germ band; PCE, protocephalon; PCO, protocorm; PGC, primordial germ cell; SE, serosa; YN, yolk nucleus. Scales: 30 μm in Figs. 7—10, 100 μm in Fig. 11.

1. *Parnassius glacialis*

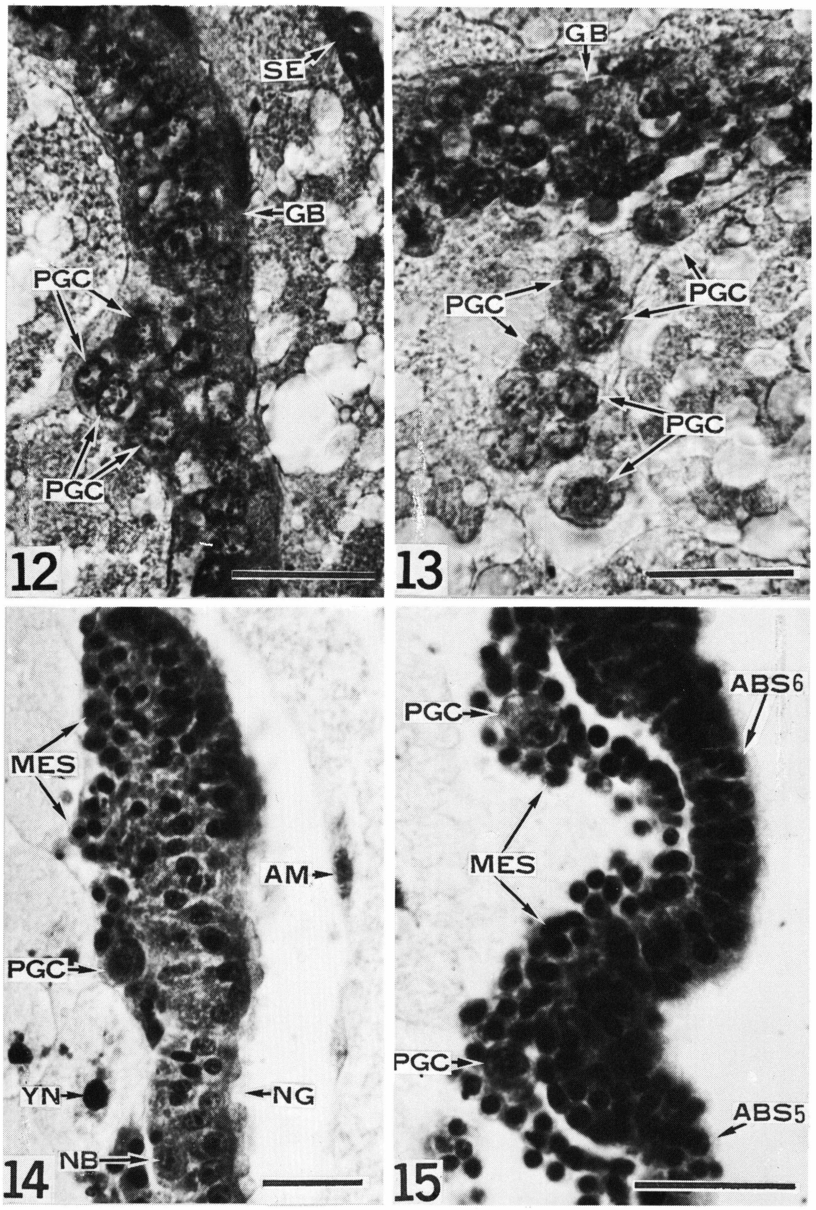
Like in other lepidopteran eggs, there are neither polar granules nor pole cells in the egg of this species.

During stage 4, the equatorial region of the blastoderm becomes thickened to form a ventral plate, and then the ventral plate is covered with the extra-embryonic area, the developing serosa (Fig. 1). At this time, the cells of ventral plate are cuboidal in shape and continue to increase in number by cell proliferation. Several mitotic figures are found at the mid-line of the region at about one-third of the ventral plate. These mitotic figures are of primordial germ cells and are clearly distinguishable from those of the somatic cells by the location and the direction of spindles. The mitotic spindles of primordial germ cells do not show any definite directions (Fig. 3), while those of ~~the~~ somatic cells are always parallel to the surface of the ventral plate (Fig. 2). This is the first appearance and multiplication of the primordial germ cells. A few cells, invaginating from the ventral plate into the yolk, are found in same material. They are also the primordial germ cells. At the region where the primordial germ cells invaginate into the yolk, a furrow is formed between the cells (Fig. 4). In other cases, two-cell layer produced by the divisions and one or more primordial germ cells lying in close contact with the ventral plate (Figs. 3—5) were observed. The feature of primordial germ cells is quite the same as that of invaginating cells, namely those cells are polygonal and have a distinct nucleus with one or two nucleoli and many tiny chromatin granules. The morphological difference between primordial germ cells and somatic cells is not yet remarkable except the shape. The nuclei of the former, however, are slightly larger than those of the latter and are almost the same as those of the yolk cells. Thus the primordial germ cells are clearly distinguishable from the somatic cells before the completion of serosa.

At the next step, the furrows resulted by invagination of the primordial germ cells are completely closed, but yet the arrangement of somatic cells at this position is irregular.

By the middle stage 5, in the cup-like embryo sunk into the yolk, multiplied primordial germ cells aggregate and make a group or cell-mass at the region where they differentiated originally. At this time the mitotic figures of primordial germ cells are still seen (Fig. 6). In a sagittal section, in the latter half of stage 5, the embryo shows C-shape and has a remarkable cluster of primordial germ cells, protruding into the yolk (Fig. 9). The proliferation of primordial germ cells is lost at this stage. The number of primordial germ cells in a cluster is 25 — 35, shows a remarkable variation among the individuals. Each nucleus of primordial germ cells is about 9 — 10 μm in diameter, is slightly larger than that of somatic cells and has one or two distinct nucleoli stained with haematoxylin. Difference of affinity for dyes is not yet remarkable between the primordial germ cells and the somatic cells. Position and structure of the cluster remain for a while.

At the beginning of stage 6, the pot-like embryo is deeply embedded by the yolk in its ventral side. In cross and sagittal sections the embryo is C-shaped. The primordial germ cells are located at the middle portion of protocorm of the embryo (Fig. 11). At this stage a shallow furrow is formed temporally, at the region where the cluster exists (Fig. 10). In advance of development, the embryo turns sideways in the posterior half



Figs. 12—15. Primordial germ cells of *Parnassius glacialis* and *Luehdorfia japonica*.
12, 13. Sagittal sections of the protocorm of embryo of *P. glacialis*, showing a group of primordial germ cells at the same stage in Fig. 11. (12) and at the slightly later stage than that in Fig. 12 (13). 14. Cross section of the fifth abdominal segment of *L. japonica* at about five days after oviposition, showing the migrating primordial germ cell. 15. Longitudinal section of the fifth and the sixth abdominal segments of *L. japonica* at the same stage in Fig. 14, showing primordial germ cells surrounded by mesodermal cells.
ABS5, 6, fifth and sixth abdominal segments; AM, amnion; ECT, ectoderm; GB, germ band; MES, mesoderm; NB, neuroblast; NG, neural groove; PGC, primordial germ cell; SE, serosa; YN, yolk nucleus. Scales: 30 μm.

of the egg. During this process the connection among the primordial germ cells is gradually lost and rearrangement of the cells occurs to form a cell-mass in close contact with the inside of the embryo (Fig. 12), and in some cases the germ cells lie apart from the wall of the embryo in a space between the spherical yolk-masses (Fig. 13).

Early in stage 6, just before the beginning of the inner layer formation, a shallow groove, *i. e.* primitive groove, makes its appearance along the mid-line of the embryo. The primordial germ cells stay as a single cell-mass at their original position, but in rare cases they are divided into two lateral groups by the groove. As the middle part, *i. e.* middle plate sinks inside and forms the inner layer, the primordial germ cells become scattered in the limited area and are surrounded by the mesodermal cells. It becomes difficult to discriminate the former from the latter at this stage, because the structural differences between them are not yet distinct, except the size of nuclei and the amount of cytoplasm.

Up to the middle of stage 6, the ectodermal parts of embryo undergo segmentation and the mesodermal cells extend gradually to the lateral side along the inner surface of the ectoderm. The author failed to trace the behaviour of primordial germ cells during the latter half of stage 6.

In stage 7, as the formation of neural groove progresses, the mesoderm is divided into lateral groups, and primordial germ cells are also divided into two symmetrical groups by this groove. Then the germ cells are carried to the lateral sides of the embryo together with the mesodermal cells. In this stage the primordial germ cells, localized in the fourth to the sixth abdominal segment, become to be characterized, that is, they are of light appearance, much larger in size than those of mesodermal cells because of rich cytoplasm and a large nucleus with one or two dark-stained nucleoli and many tiny chromatin granules. The number of primordial germ cells on each side and in each segment varies more or less among the individuals. For example, in one embryo, two, three and two cells were counted on one side of the fourth to the sixth abdominal segment respectively, and one, two and two on the other side. In other embryo, their number was three, three and two on one side, and none, four and two on the other.

The primordial germ cells do not increase in number since they were differentiated.

2. *Luehdorfia japonica*

Neither the polar granules nor the pole cells are found in the early stage of development. The first differentiation of the primordial germ cells could not be observed.

Late in stage 4, the broad equatorial zone, *i. e.* ventral plate, comes to be distinguished and the cells of this region divide actively to form the columnar cells during the formation of the serosa. The germ band increases in length, diminishing its width, and becomes L-shaped in a sagittal section. It encircles three-fouths of the equator of the egg. At this stage several cells, between the germ band and the yolk, are observed at the region about one-third of the germ band from its posterior end (Fig. 9). These are the primordial germ cells.

At the end of stage 5, the germ band differentiates to the protocephalon and the protocorm, and their ends curve into the yolk. The yolk undergoes cleavage to form large spherical masses. At this time 20－30 primordial germ cells between the embryo and the yolk spherical masses are observed. These cells do not distribute separately, but assemble as a group or cell-mass. Each cell is almost spherical in shape, about 16 μm in diameter and has a distinct nucleus with one or two nucleoli deeply stained.

During stage 6, the embryo assumes the coiled form, directs to the anterior pole of the egg, and lies wrapped around the yolk. The primordial germ cells lie at the region one-fifth of the embryo from its posterior end, along the mid-line. They are distributed somewhat separately, closely contacting with the inside of embryo. As the formation of inner layer progresses, the primordial germ cells come to be embedded in the mass of inner layer and to be undistinguishable from neighbour cells.

At the middle of stage 6, the embryo sinks deeply into the yolk. The segmentation of ectoderm and inner layer take place. At the next step the mesodermal cells spread laterally and divide into two lateral groups. The primordial germ cells with large nuclei, about 8 μm in diameter, make their appearance on the both sides in the fourth to the sixth or the fifth to the sixth abdominal segment (Fig. 14). In the longitudinal sections they lie at the anterior half of each segment (Fig. 15). The number of primordial germ cells is five or six on one side of the body, namely one to three on one side of each segment. The divisions of primordial germ cells are not found until the stage of gonad formation.

3. *Byasa alcinous alcinous*

In a newly laid egg and the early stage of development, *i. e.* from cleavage to the differentiation of embryonic and extra-embryonic areas, the primordial germ cells are not found.

At the end of stage 4, the germ band lying on the ventral side of the egg, is C-shaped in sagittal section and encircles two-thirds of the egg periphery in cross section through the equator of the egg. Twenty-five to 35 primordial germ cells are observed at the region about one-third of the germ band from its posterior end, along the mid-line. They lie in close contact with irregularly arranged, columnar cells of the germ band, making a cell-mass loose assembled as observed in *L. japonica* (Fig. 8).

When the differentiation of protocephalon and protocorm takes place in the middle of stage 5, the mass of primordial germ cells is located between the embryo and the spherical yolk masses at the region about one-forth of embryo from its posterior end. Each primordial germ cell has a distinct nucleus which is as large as that of somatic cells, and it does not represent remarkable characteristic as seen in the later stage of development.

By the end of stage 5, the elongated embryo, directing its cephalic part to the anterior pole of the egg, occupies the periphery from the lateral side to the posterior pole of the egg. The primordial germ cells are in close contact with the inner surface of embryo as a single cell-mass, and some of them lie among the somatic cells at the posterior region of the embryo. This state is similar to that of *L. japonica*.

In the early part of stage 6, the middle part of the embryo sinks into the yolk, and finally the inner layer is formed. Through this process the primordial germ cells are embedded in the mass of inner layer and come to be undistinguishable from the other cells.

By the end of stage 6, the segmentation of ectodermal part and of mesoderm of the embryo takes place. At this time the primordial germ cells begin to spread laterally with the mesodermal cells, and then they are completely divided into two symmetrical groups by a neural groove. In stage 7, the typical primordial germ cells become clear observable on both sides of the fourth to the sixth abdominal segment. The number of them varies among the individuals as that of other species. Table 2 shows the distribution and the number of primordial germ cells on each side and segments at the stage of gonad formation.

Table 2. Number and distribution of primordial germ cells in the fourth to the sixth abdominal segment of *Byasa alcinous alcinous*.

Abdominal Segment / Sample Number	Number and Distribution of Primordial Germ Cells			Total Number of Primordial Germ Cells
	4th	5th	6th	
1	3	4	5	12
	5	4	4	13
2	5	5	4	14
	4	5	4	13
3	3	4	2	9
	2	5	4	11
4	4	5	5	14
	4	5	4	13
5	3	4	4	11
	3	5	4	12

Discussion

Many investigations concerning the period and the process of germ cell formation in insects have been done. Results obtained, however, differ remarkably according to the species, families and orders of insects. Though considerable number of works on these subjects have been also reported in the Lepidoptera (Toyama, 1902, in *Bombyx*; Schwangart, 1905, in *Endromis*; Yamauchi, 1917, in *Antheraea*; Johannsen, 1929, in *Diacrisia*; Eastham, 1930, in *Pieris*; Sehl, 1931, *Ephestia*; Lautenschlager, 1932, in *Solenobia*; Saito, 1937, in *Antheraea*; Kawaguch and Miya, 1943, in *Bombyx*; Miya, 1950, 1951, 1952, 1953, 1957, 1958, 1959, 1975, in *Bombyx*; Presser and Rutschky, 1957, in *Heliothis*; Okada, 1960, in *Chilo*; Bassand, 1965, in *Zeiraphera*; Anderson and Wood, 1968, in *Epiphyas*; Tanaka, 1970, in *Ancylolomia*; Ando and Tanaka, 1980, in *Endoclita*; Kobayashi, 1983, in *Neomicropteryx*; Kobayashi and Ando, 1984, in *Neomicropteryx*; Tanaka, 1985, in *Amata*), the author's opinions are not always the same among different species or even in the same species.

Nelsen (1934) and Johannsen and Butt (1941) classified the insects into six and five groups respectively, considering the time of primordial germ cell appearance. Furthermore, Miya (1958) divided insects into several groups according to the process and the position of germ cell differentiation. In his classification the lepidopterous insects belong to three groups. Namely first, primordial germ cells differentiate at or near the posterior pole of egg, but polar granules are obscure. Next, primordial germ cells differentiate, or probably differentiate, at the definite region of ventral side of egg, and they appear in a group and scatter in several segments later. Last, it is the same as the second type, but primordial germ cells are already scattered in several segments at the time of appearance. Almost lepidopterous insects, including *Parnassius*, *Luehdorfia* and *Byasa* belong to the second type.

The last type is known in *Antheraea* (Yamauchi, 1917; Saito, 1937), *Diacrisia* (Johannsen, 1929), *Pieris* (Eastham, 1930) and *Neomicropteryx* (Kobayashi, 1983; Kobayashi and Ando, 1984). Generally, newly differentiated primordial germ cells have not yet striking feature, and the typical features of them make during the proceeding of development. For instance, in *P. glacialis*, *L. japonica* and *B. alcinous alcinous* the primordial germ cells become remarkable at the time when the mesodermal cells spread laterally, along the inner surface of ectoderm, after the segmentation of ectodermal part of embryo and of inner layer. Thus there is a time lag between the first appearance and the emergence of striking feature of primordial germ cells.

In *Bombyx mori*, Kawaguchi and Miya (1943) described that primordial germ cells originated as a group of cells from the primitive ectoderm before the mesoderm formation, and Miya (1950, 1951, 1952, 1953, 1958, 1959) made it clear that they were formed through the process of invagination at the ventral side of germ band when the blastoderm differentiated to the germ band and extra-embryonic region. Moreover, he (1950, 1957, 1958) presumed, by the results of cauterization experiments, that the cells destined to be differentiated to the primordial germ cells were predetermined in the blastoderm stage. In *Heliothis zea*, Presser and Rutschky (1957) and in *Epiphyas postvittana*, Anderson and Wood (1968) also observed the primordial germ cell differentiation through the invagination process. Therefore, it seems to be the dominant opinion that the first multiplication of primordial germ cells is finished during the blastoderm stage, and then they are distinguished from the somatic cells through the invagination process shortly after germ band formation. Recently Tanaka (1985), in *Amata fortunei*, reported that several mitotic figures distinguished from those of the somatic cells existed at the mid-line near the center of newly formed germ band, and they show the first appearance of the primordial germ cells.

In *P. glacialis*, the morphological differences between the primordial germ cells and the somatic cells were not yet recognized at the stage when the blastoderm began to differentiate to the ventral plate and the rudimentary serosa. In the next stage, however, the multiplication and the invagination of primordial germ cells underwent in the region about one-third from the posterior end of ventral plate, and then they aggregated to form a single cell-mass consisting of 25 – 35 cells. These facts, as observed in *A. fortunei* and in *P. glacialis*, suggest that several cells localized at the definite region of the blastoderm are destined to differentiate to the primordial germ cells, that is, the primordial germ cells are predetermined at the blastoderm stage as pointed out by Miya in *Bombyx mori*.

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