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A correlative study of experimentally changed first cleavage and Janus development in the trunk of *Platynereis dumerilii* (Annelida, Polychaeta)

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Summary. Among zygotes of *Platynereis dumerilii* treated with cytochalasin B (CCB) prior to first cleavage, a wide variety of developmental effects were observed. One effect is a delay in the first cleavage. Treated embryos may skip the first or even more than one cleavage cycle and become multinucleated. Once these eggs start cleaving their cleavage plane takes the same position as in synchronously fertilized controls. Accordingly, the first cleavage in embryos having skipped the first normal cleavage cycle is meridional and equal, but their second cleavage is equatorial as in the third cleavage in controls. None of the embryos that were observed to skip early cleavages showed normal organogenesis, but developed into vesicle-shaped embryos with little cytological differentiation. Another effect of CCB treatment is altered blastomere size in those embryos which begin cleaving in synchrony with controls. While the majority of treated embryos followed a normal cleavage pattern, i.e. they cleaved at the right time and inequally, some of them cleaved equally or almost equally (adequally). Most of these embryos showed cleavage defects in subsequent cleavage cycles and became abnormal vesicle-shaped embryos. However, some of these embryos cleaving on schedule and equally or adequally developed into juvenile worms showing complete duplication of urites and parapodial rows (0.3% of all treated eggs) and are described as Janus *duplicitates*. This means that the occurrence of *duplicitates* and geometrically altered first cleavage patterns are correlated phenomena. The character and origin of the duplications and the consequences for dorsoventral polarity are discussed.

Key words: Janus *duplicitas* – Unequal cleavage – Cytochalasin B – Dorsoventral polarity – Polychaete

Introduction

In spiralian eggs prior to cleavage only the animal-vegetal axis is determined. Unequal cleavage represents a mechanism by which the ooplasmic constituents are divided in such a way that the second embryonic axis – dorsal/ventral – becomes established and is added to the pre-existing animal/vegetal axis at the two-cell stage. At the four-cell stage the destiny of the four quadrants A, B, C and D is determined (D being the quadrant to develop the mesentoblast).

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Experiments by Penners (1922), Tyler (1930) and Guerrier (1970a, b), in which the first cleavage was equalized mechanically, led to the development of double embryos. This shows that the development of the dorsoventral axis and normal organogenesis both depend on and are fixed by the plane of first cleavage. Together with cell deletion and blastomere isolation experiments (Wilson 1904; Clement 1952; Cather and Verdonk 1979) suppression of division inequality demonstrates the determining character of the unequal cleavage pattern in spiralians.

In normal two-cell stages of *Platynereis*, as well as in *Nereis*, the first cleavage plane runs perpendicular to the dorsoventral axis of the future larva (Wilson 1892; Fischer 1983). The blastomeres AB and CD are extremely different in size. Recent morphometric data show that the AB blastomere contains only 27% of the total ooplasm (Dorresteijn and Fischer, in preparation). In this paper we will present evidence that treatment of the *Platynereis* zygote with cytochalasin B (CCB) (cf. Guerrier et al. 1978) may lead to a more equalized first cleavage, thereby changing the development of the dorsoventral axis, and produces supernumerary structures in the trunk of the worm. The morphology of the young worm is such that safe conclusions on the nature and orientation of supernumerary structures can be drawn. This is of great importance since similar conclusions in earlier experiments were derived from experiments on polychaetes with planktotrophic larvae (*Chaetopterus*, Titlebaum 1928; *Sabellaria*, Novikoff 1940, Render 1983). In these species, malformed embryos stop development as larvae, and are still devoid of distinctive external characters of dorsoventral polarity (mainly parapodia).

Materials and methods

Cultures of *Platynereis dumerilii*, a small nereid of the Mediterranean, were kept in the laboratory under artificial food and light conditions; they can be bred all year round (Hauenschild and Fischer 1969). Under these circumstances the animals grew over a period of several months. Every 2 days mature epitokous animals were collected from the dishes. To avoid bacterial contamination these animals were kept in a solution of 0.130 g streptomycin sulphate and 100,000 IU penicillin pasteurized sea-water for 30 min and subsequently kept in pasteurized sea-water in glass dishes. Before spawning, as the animals swam about, a male and female were put into a separate glass dish with sea-water. Gametes were shed simultaneously and after approximately

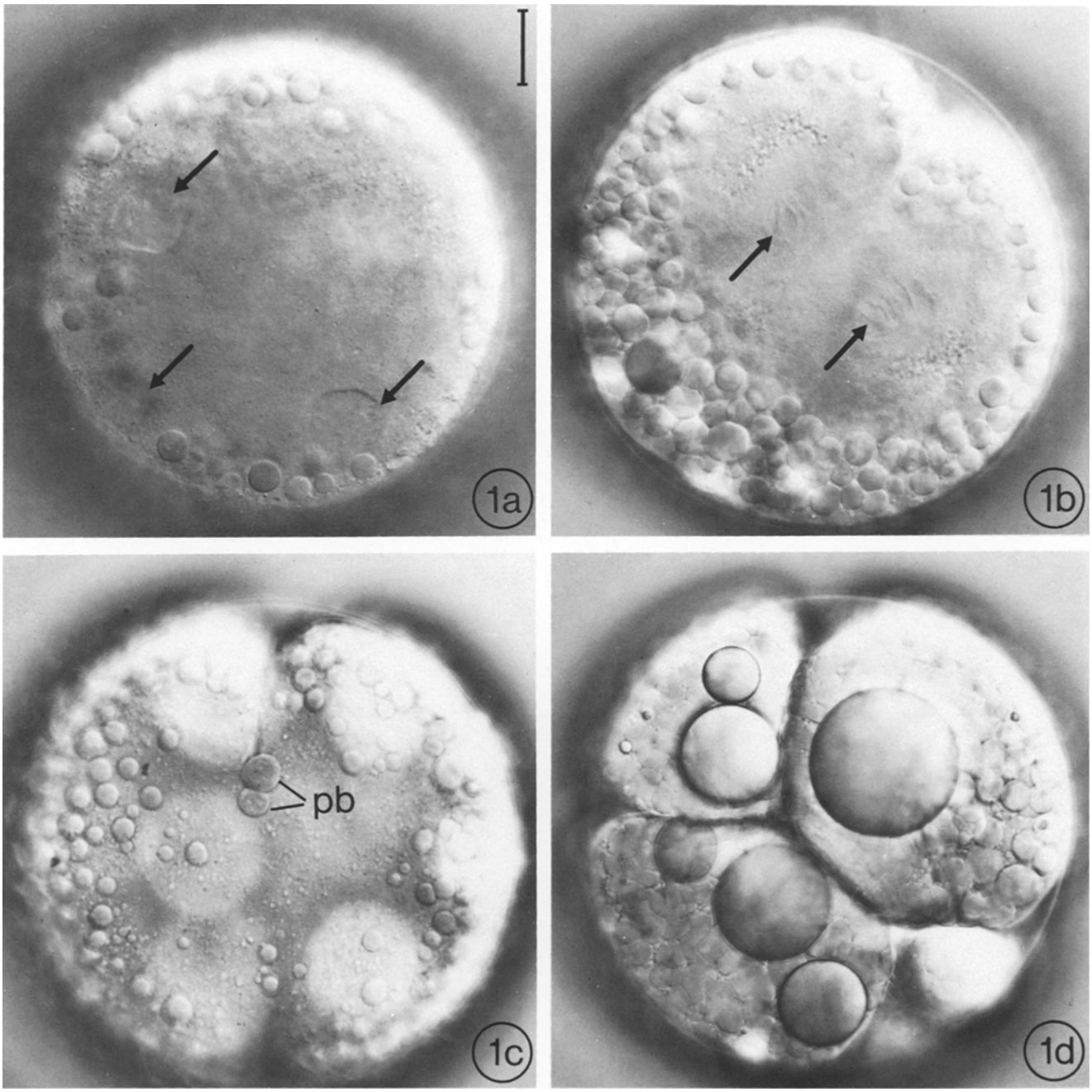


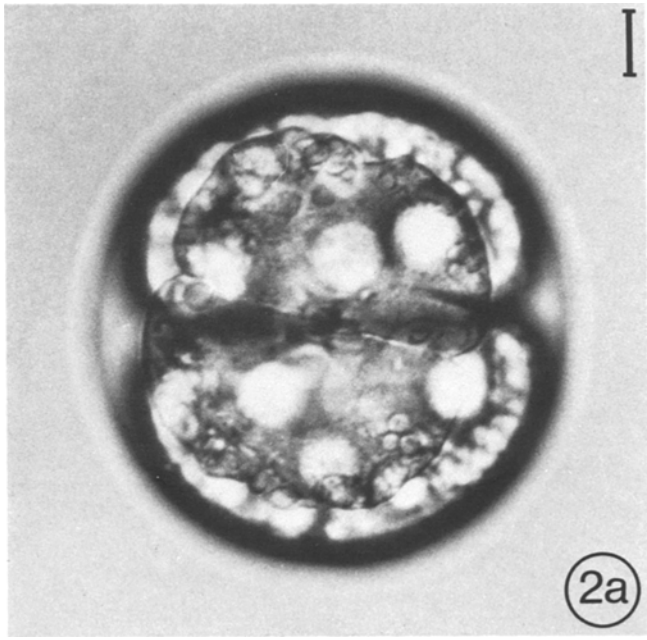
Fig. 1. **a** Uncleaved multinucleated *Platynereis* embryo after cytochalasin B (CCB) treatment, photographed at 142 min after fertilization, where controls had already reached the four-cell stage. *Arrows* indicate the position of three from a total of four nuclei within the clear portion of the ooplasm. **b** Equal first cleavage after previous CCB treatment showing the anaphase with karyomeres (*arrows*) migrating towards the spindle poles (marked by *small vesicles*, presumably of the endoplasmic reticulum). **c** Two-cell stage after CCB-induced equal first cleavage, viewed from the animal pole. Both blastomeres share a clear, yolk-free peripheral cytoplasm close to the animal pole in the centre of the photograph. During undisturbed embryogenesis, this clear cytoplasm would almost exclusively be allocated to the (larger) CD blastomere. (*pb* = polar bodies). **d** Eight-cell stage from a CCB-treated embryo with equalized first cleavage and meridional second cleavage, showing the four macromeres with protein yolk and lipid droplets at the vegetal cross-furrow. The two cross-furrow macromeres are practically of equal size, but significantly larger than the two non-cross-furrow macromeres. *Scale bar* = 20 μm

1 min the surplus of sperm was removed by rinsing the eggs several times. The embryos were kept at 18° C; at this temperature the meiotic divisions were completed 55 and 75 min after fertilization, respectively. In these embryos, first, second and third cleavage took place 100–110, 145–155 and 185–195 min after fertilization, respectively. From a stock of several thousands of eggs a batch (25%) were observed as control embryos. The experiments were performed in two different ways. In the first approach a total of 15,000–20,000 eggs were treated at 75, 80, 85 and 90 min after fertilization. Batches of 100–200 eggs were introduced into solutions of 0.025, 0.05 or 0.1 mg CCB/l seawater, prepared from a stock solution of 1 g/l dimethyl sulfoxide (DMSO) (Cloney 1972). Control experiments us-

ing low concentrations of DMSO have shown that this compound does not influence normal development. After a 5 or 10 min treatment with CCB the eggs were rinsed three times in sterile sea-water. Beginning 100 min after fertilization, samples from the treated and control embryos were taken every 10–15 min and studied under the microscope. The percentages of abnormalities in cleavage geometry and cleavage time were scored. In controls the trochophore larvae hatched from the egg jelly after 1 day of development. Hatching in treated batches appeared less rapid. Part of the trochophores of treated eggs did not hatch until after a 2nd day of development. These larvae were reared for 5–14 days without feeding. The young specimens were anaesthetized in 5% 5,5-diethyl barbituric acid for 15 min and subsequently fixed in 2.5% glutaraldehyde and 0.14 M NaCl in 0.2 M phosphate buffer (pH 7.4) for 0.5–1 h at room temperature. The percentages of abnormalities in these young worms were then compared with the abnormalities during the early cleavage phase.

In another set of experiments 2,716 zygotes of *Platynereis* were treated with either 0.025, 0.05 or 0.1 mg CCB/l for 10 min starting 75 min after fertilization. After rinsing, these eggs were observed until the first cleavage occurred. Unlike controls, in which the first cleavage is synchronous, i.e. all eggs cleave within 2 min, the "first" cleavage in CCB-treated embryos may be extremely delayed. Therefore, all embryos which cleaved within 5–10 min were timed at the moment of their first cleavage and put into separate groups. Those embryos that cleaved adequally or equally during this interval were reared in separate dishes.

All embryos and young worms were studied with an inverted IM 35 microscope using differential interference contrast optics (Zeiss, Oberkochen, FRG). Micrographs were photographed on Copex or Agfapan negative film. Preparation for scanning electron microscopy (SEM) started with the previously mentioned fixation for 1–1.5 h. After rinsing twice for 5 min in 0.14 M NaCl in 0.2 M phosphate buffer (pH 7.4), the specimens were postfixed in a 2% OsO_4 solution containing 1.25% $NaHCO_3$ at the same pH for a period of 2–3 h. After a short rinse in tap water and dehydration in a graded series of ethanol, the specimens were critical-point dried and gold-sputtered. Morphological investigation was performed in an ISI scanning electron microscope (Mountain View, Calif., USA) and photographed on Ilford FP4 film.



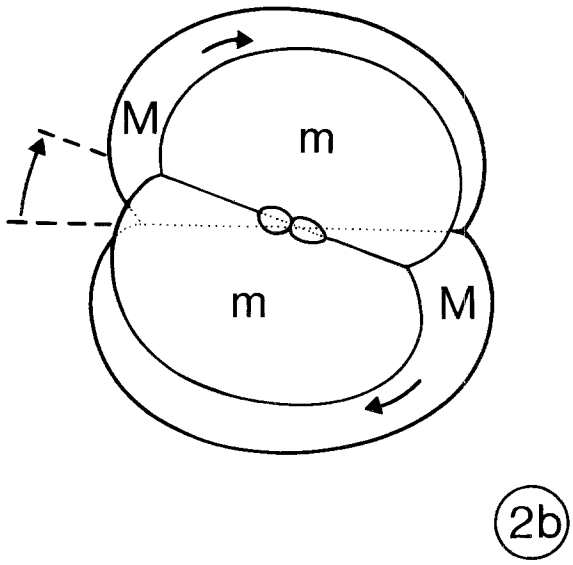
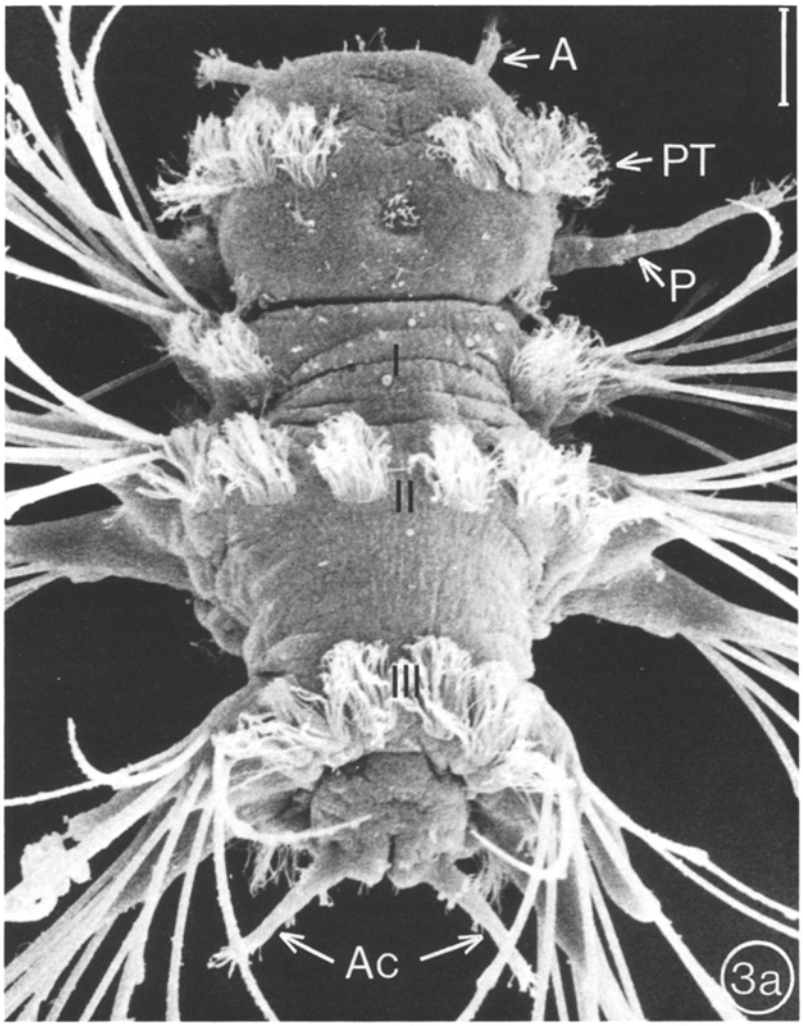


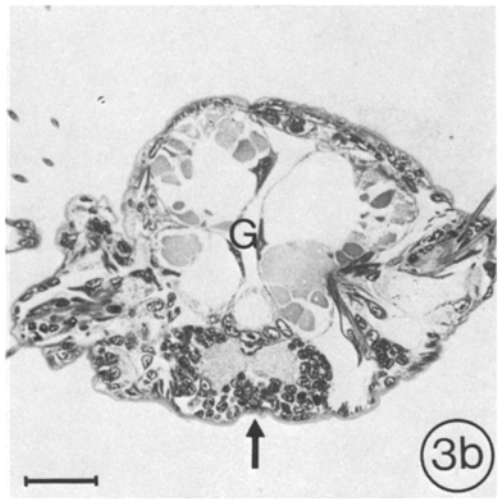
Fig. 2a, b. Cytocholasin B-(CCB-) induced cleavage abnormality of the type usually found in four-cell embryos having skipped the first cleavage cycle. Viewed from the animal pole. First cleavage was meridional and, in this case, equal. Although the normal second cleavage-plante should be meridional as well, in this case the plane of second cleavage is equatorial (in the focal plane). The micromeres (*m*) are formed in a dexiotropic fashion (*arrows*) towards the animal pole. (*M* = macromeres). *Scale bar* = 20 μm

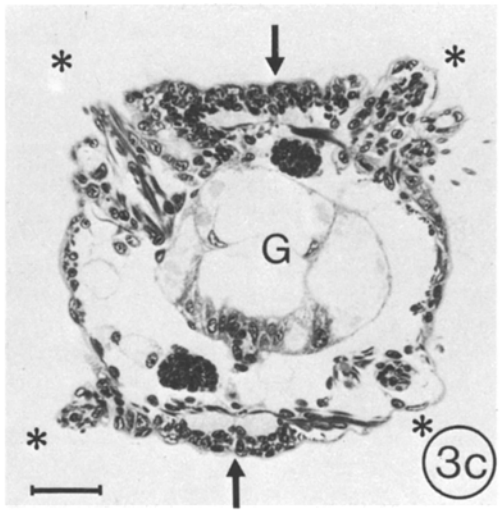
Results

In a first set of experiments, approximately 15,000 eggs from 31 eggs batches, each from a different *Platynereis dumerilii* female, were treated and screened for the various effects of CCB. Some of these treated embryos were screened for abnormal geometry of the first cleavage. In a sample of 2,355 CCB-treated eggs, 766 (32%) showed an equalized first division (Fig. 1b). The highest proportion of equalized two-cell stages was obtained with high concentrations of CCB (0.05 and 0.1 mg/l). No significant difference in the percentage of equally cleaving eggs could be recorded by lengthening the duration of treatment from 5 to 10 min.

Equally dividing CCB-treated embryos usually entered first division with delay, and most equal cleavages were observed around the time of second cleavage of control embryos. Only a few of the equally dividing eggs cleaved simultaneously with or only a little later than unequally cleaving CCB-treated embryos. Characteristically, second cleavage in those embryos that showed their first cleavage at the moment of second cleavage in controls was equatorial, producing two micromeres in the animal direction and two macromeres towards the vegetal pole. The position of the micromeres with respect to the macromeres was such that this cleavage can be considered as a dexiotropic spiral cleavage (Fig. 2a, b). Since the time schedule, orientation


A
PT
P
I
II
III
Ac
3a


G
3b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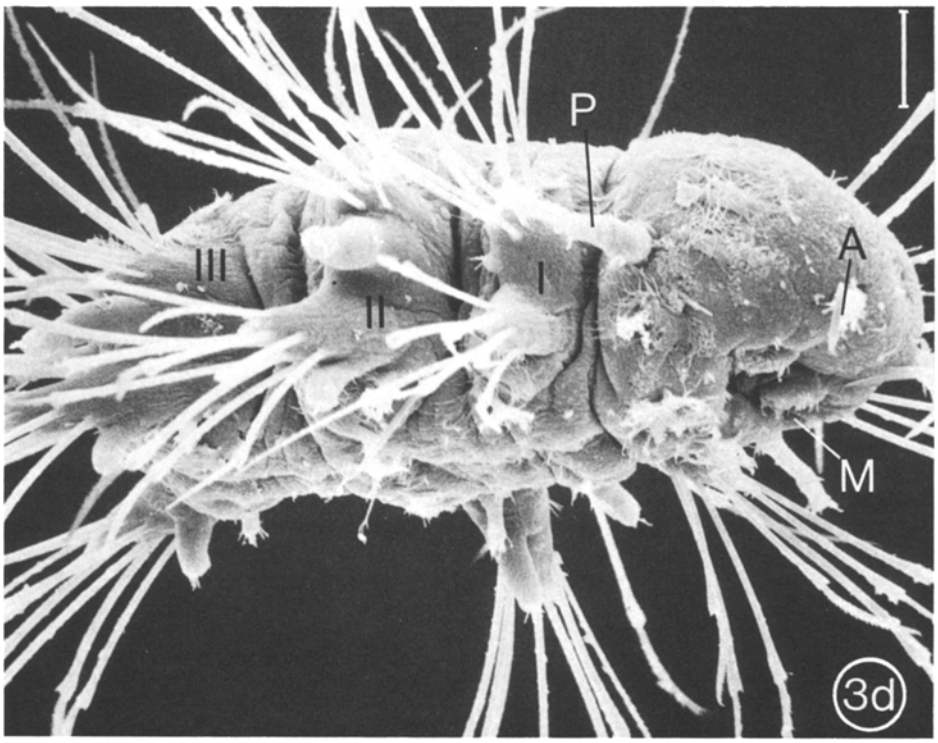
P
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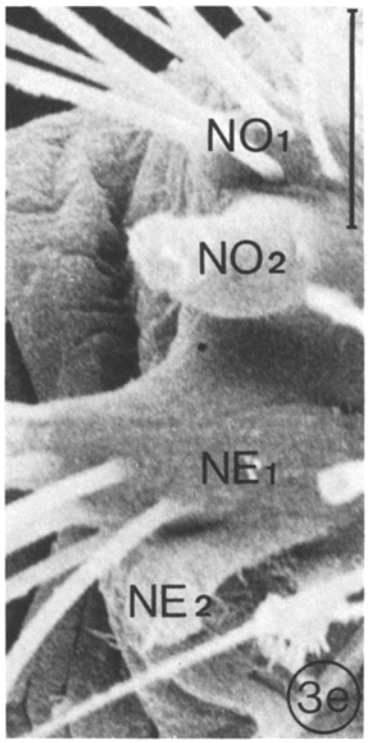

NO1
NO2
NE1
NE2
3e

and spiral properties of this unusual second cleavage were similar to a normal third cleavage, we assume that these embryos had skipped first cleavage. In other eggs both first and second cleavage appeared to be skipped, as their cleavage was equatorial and occurred simultaneously with third cleavage in controls. Embryos that skipped first or both first and second cleavage had multinucleated blastomeres (Fig. 1a) and did not develop a distinct spatial organization. These embryos usually became vesicle-shaped with a few remnants of macromeres inside. Organogenesis was lacking, although light-microscopical sections occasionally showed scattered differentiations as in muscle cells or nerve fibres.

After 4 days of development a control embryo has developed into a young worm (often called "nectochaeta larva") having three trunk segments, each bearing a parapodium on either side (Fischer 1971, 1985). The anal region or pygidium is characterized by two urites (Fig. 3a). The head bears two antennae in front and two peristomial cirri in a lateral position in front of the parapodia of the first segment (Fig. 3a). Apart from the larval eyes in the ventral region of the head two pairs of "adult" eyes can be distinguished in a dorsolateral position. It should be mentioned that a very small percentage of control embryos develop abnormally, e.g. lacking single extremities or bristles of single parapodia; sometimes the head region is slightly reduced. The same percentage of abnormalities of these types was found in the treated groups. These abnormalities will not be discussed.

Out of the approximately 15,000 eggs used in the first experiment, 7,051 young worms did hatch and were screened for malformations. We obtained 44 worms with four rows of parapodia, instead of the normal two, along the longitudinal axis. Of these, 41 also showed duplicated urites. Thus, these larvae show a duplication of trunk structures and are regarded as *duplicitas* specimens. In the second set of experiments our screening methods were more subtle in order to find the group of potential *duplicitas* specimens among the early cleavage stages. We found 7 *duplicitas* specimens among 2,716 treated zygotes. Duplications developed exclusively among those zygotes whose first cleavage was adequal and took place within less than 30 min after first cleavage in controls, i.e. before normal second cleavage. The blastomeres of the two-cell stages after adequal first cleavage differ only slightly in size (although not quantified). Most significantly, both first and second cleavage are meridional. A few of these embryos were observed continuously during several cleavage cycles. Second cleavage was sometimes, but not always, equal in these cases. One embryo, observed until the eight-cell stage, had developed two large quadrants in a diametrical position and two smaller quadrants on either side (Fig. 1d). We are not able to give an exact figure of adequal two-cell stages that produce a four-cell stage at the subsequent cleavage, since we have observed several cases in which the second cleavage-furrow regresses and daughter blastomeres become united again. This phenomenon was even observed to happen during second cleavage in a few treated embryos that had cleaved inequally at the moment of first cleavage. These embryos also develop into abnormal embryos almost completely lacking organogenesis.

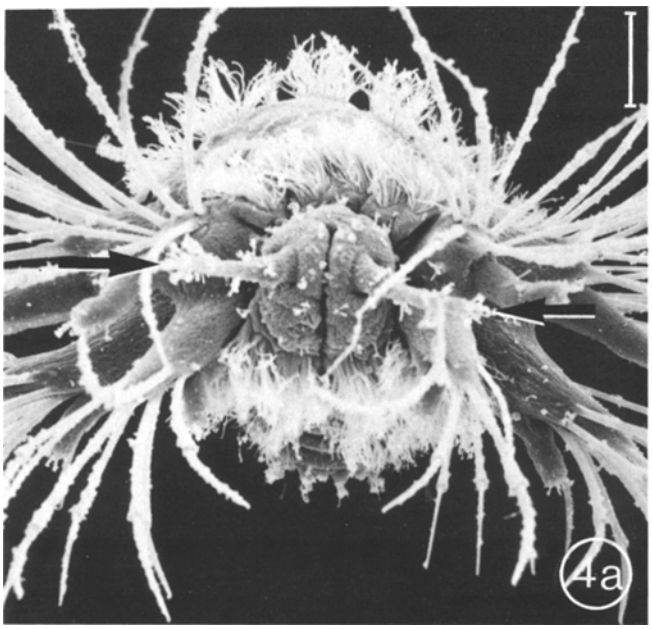
In the following, the regular and specific disturbance of the normal dorsoventral pattern of development in *duplicitas* embryos will be described and discussed in more detail. In *duplicitas* specimens, the trunk is shortened and has a larger diameter than normal. The number of parapodia and setae is doubled. Each normal parapodium in *Platynereis* juveniles has a pronounced dorsoventral polarity (Figs. 3e, 4c, e). The same dorsoventral polarity could be found in each parapodium of *duplicitas* juveniles as well, every single parapodium in each of the four rows disclosing the polarity of the trunk wall like a pointer. Polarity among the parapodia of each single row is always the same, and polarity of one row is mirrored by the neighbouring row, neuropodia thus facing neuropodia and notopodia facing notopodia (Fig. 4b, d, f). Thus, the walls of the four-sided trunk are carrying two dorsal and two ventral sides, each pair of duplications occupying diametrical positions (Fig. 4f). We regard these embryos as *duplicitas* of the *Janus* type.

In order to study the internal organization of the *duplicitates,* serial cross-sections of the trunk were produced and compared with similar sections of control young worms. The position of the nerve cord acts as an inportant marker for the ventral median (Fig. 3b). In cross-sections of the *duplicitas* specimens, malformation always became obvious by the duplication of two distinctive internal tissues, i.e. the ventral nerve cord and the setae sacs. The position of the nerve cord was diametrical (Fig. 3c) and each was flanked by two setae sacs. This shows that external characters in *duplicitas* embryos match the internal organization.

Duplication of trunk appendages in juveniles may also extend into the head region. However, neither the prostomium or the gut as a whole nor the antennae or the palps were ever duplicated. The ventral face of the prostomium, recognizable by the position of the antennae and the mouth, was always on axis with one of the two ventral sides of the duplicated trunk. However, the *peristomial cirri* emerging from the posterior sides of the prostomium were duplicated in 29 out of 41 individuals. This phylogenetically important phenomenon lies outside the scope of this paper and will be published in detail elsewhere.

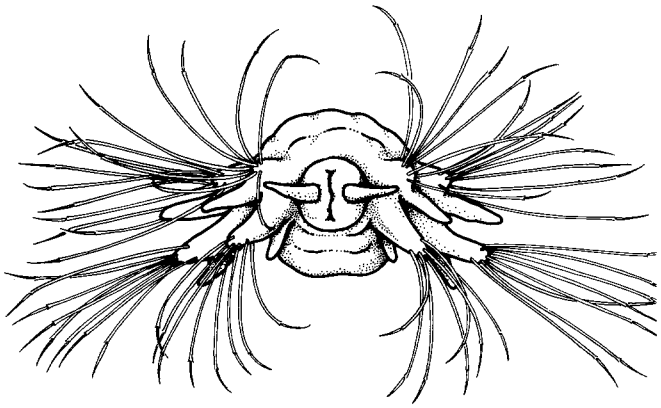
The only other structure of the prostomium found to

Fig. 3. a Dorsal view of a normal three-segmented juvenile. The head region bears two antennae (*A*), two peristomial cirri (*P*) and the prototroch (*PT*). The consecutive segments are marked by *I, II, III.* The anal region (pygidium) is characterized by urites (*Ac*). **b** Light-microscopical cross-section in the second segment of a normal three-segmented young worm. The gut (*G*) is outlined by the remnants of the yolky macromeres and laterally flanked by the setae sacs of the parapodia. The nerve cord is in the midventral position (*arrow*). **c** Cross-section in the second segment of a *duplicitas* young worm. Both above and below the central gut (*G*) a nerve cord (*arrows*) is visible. Each nerve cord is flanked by two parapodia (*asterisks*). **d** Side view of a normal juvenile. The parapodia of the three larval segments are indicated by *I, II, III.* The structure of the second parapodium is magnified in Fig. 3e. (*P* = peristomial cirrus; *A* = antenna; *M* = mouth). **e** This micrograph shows the dorsoventral polarity of the second parapodium. In the dorsal notopodium (*NO1* and *NO2*) there is no dorsal cirrus yet and the most dorsal structure is a pincushion-like protrusion (NO1) bearing setae, followed ventrally by a long lip without bristles (NO2). The ventral neuropodium (*NE1* and *NE2*) is subdivided into a dorsal lip carrying a bundle of setae (NE1) at its tip and a ventral cirrus (NE2). The long dorsal lip and ventral cirrus are lacking in the first pair of juvenile parapodia (see segment I in **d**). *Scale bar* = 20 μm



4a

4b



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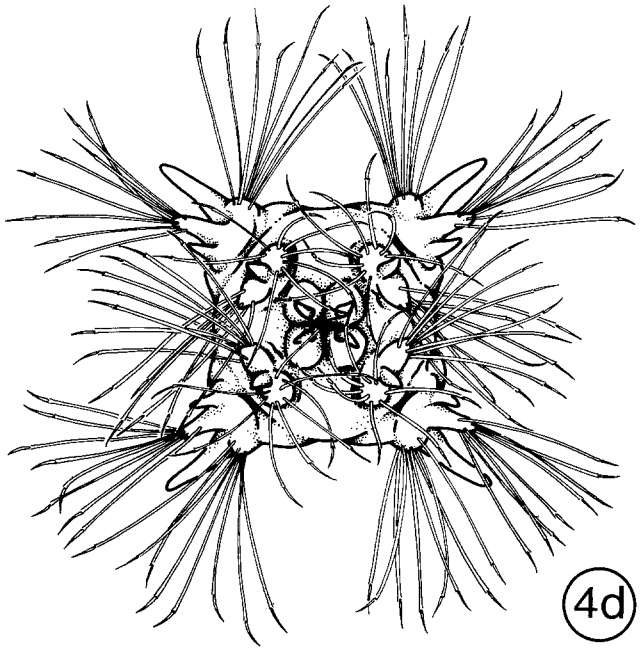

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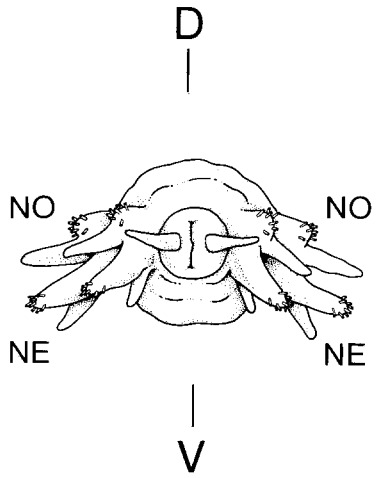

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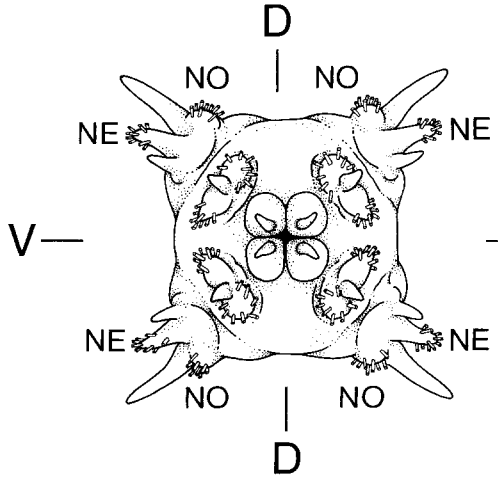

4f

be influenced by the treatment and forthcoming *duplicitas* development were the eyes, with eye numbers ranging from zero to four pairs, including the occurrence of odd numbers.

Discussion

Spiralian embryos whose first cleavage is unequal, either by formation of a polar lobe or by asymmetrical position of the first cleavage spindle, provide very suitable systems for the study of development of dorsoventral polarity. In all these systems a four-cell stage consisting of one larger D blastomere and three smaller blastomeres A, B and C, is formed. These cells develop into the four quadrants of the embryo. The D quadrant contributes to all dorsal and mesodermal structures. In species forming polar lobes the dorsal capacity becomes localized and transmitted by the formation of transient polar lobes at the first and second cleavage (Wilson 1904; Clement 1952; Cather and Verdonk 1974; van Dongen and Geilenkirchen 1975). This localization phenomenon is less obvious in the other type of unequal cleavage. Attempts to equalize first cleavage in such species resulted in the development of so-called double embryos (Tyler 1930; Guerrier 1970b). The final results of these experiments, however, were always derived from the external morphology of early larvae, with only faint indications of dorsoventral polarity at best. *Platynereis* larvae, on the other hand, are lecithotrophic and pass their whole larval life without any food uptake. Thus, even in cases of malformations obstructing food uptake, we could examine the morphogenetic result of CCB treatment in young worms already carrying dorsoventrally polarized body appendages.

In this paper, we provide evidence for the possibility of changing the organization of dorsoventral polarity in *Platynereis* by means of CCB treatment before first cleavage. We observed that CCB influences the time-schedule and geometry of first cleavage. Moreover, treatment at a very early stage caused the development of 51 young worms with a double dorsoventral organization in a total of approximately 18000 treated zygotes. Neither equal cleavage nor double embryos were ever observed in normal egg batches (A. Fischer, unpublished work). Therefore, we stress that equalization of the first cleavage and duplication of dorsoventral polarity are correlated phenomena.

Studies on morphological abnormalities in natural populations of nereids (Boilly et al. 1975) have revealed that 12 adult worms, among several thousands of animals, showed duplication of trunk segments. Polarity and orientation in the duplicated parapodia is fully comparable with that found in our experiments. However, these *duplicitates* in adults must have originated during postlarval development and not during embryogenesis as in our experiments, because *duplicitas* morphology is expressed in the caudal trunk region only. It seems likely that these abnormalities are caused by injuries of the ventral nerve cord (VNC), since duplicated rows of parapodia start in those specimens from bifurcations of the VNC somewhere along the trunk. These results have been confirmed by implanting an additional VNC in *Nereis pelagica* (Wattez-Combaz 1985). Duplication in trunk segments, often leading to double tails, can even be enforced by cutting the VNC in the polychaete *Ophryotrocha puerilis* (Pfannenstiel 1984). These experiments show the role of the VNC in the organization of the trunk. We suggest that the duplication of the VNC in our experiments should be ascribed to a duplication of the precursor cell of the VNC.

The pattern of abnormalities observed in our *Platynereis* juveniles was essentially identical among the 51 young worms, grouped together as *Janus* embryos. Duplications were positioned and orientated harmoniously, i.e. in perfect order with respect to the two (instead of one) planes of mirror symmetry running along the axis of the trunk.

At the moment of first cleavage we were not able to decide which of the treated embryos would become double embryos. Approximately 32% of all treated embryos (including those that did not hatch) were scored as having an equal first cleavage. According to the results of equalized first cleavage in other unequally cleaving spiralians (Tyler 1930; Novikoff 1940; Guerrier 1970a, b; Guerrier et al. 1978) we initially expected to obtain an equivalent percentage of double embryos. However, less than 1% of all treated embryos show the features of double embryos. The apparent discrepancy has been clarified in experiments in which we discovered that embryos with an equalized first cleavage can only develop into double embryos if they start cleavage well before control embryos enter the second cleavage cycle, i.e. on an approximately normal schedule. However, the majority of equal cleavages in treated embryos were observed between 30 and 60 min after first cleavage in controls. This indicates that in most cases the normal first cleavage had been skipped. This supposition is corroborated by light-microscopical observations on the early cell-lineage of equally cleaving embryos. Second cleavage in such embryos is equatorial, spirally dexiotropic and takes place in synchrony with the third cleavage in controls. Such embryos develop into dauerblastula-like embryos with virtually no polarity at all. We have also found embryos that skip two or more cleavages. In embryos in which the first two cleavages had been skipped, the "first" cleavage was always equatorial. This shows that, although the machinery for the cleavage furrow is affected by CCB, the spindle position is preprogrammed for specific moments during development. If cleavages are suppressed, nuclear divisions continue and therefore blastomeres become multinucleated. Apparently, the capacity to regulate the cleavage schedule is lost. Therefore, organogenesis is lacking, a situation that is also observed in polyspermic eggs of *Platynereis*.

Our data indicate that in a small (not quantified) proportion of all treated embryos the geometry of the true first cleavage is altered and size difference between the two daughter blastomeres is reduced or even eliminated. All

Fig. 4. a, b This pair of SEM micrographs shows a normal **a** and a *duplicitas* juvenile **b** viewed from the posterior. Four instead of two urites (*arrowheads*) and four rows of parapodia (*arrowtails*) are seen in the *duplicitas*. Every parapodium has a normal dorsoventral polarity, expressed by the array of notopodial (dorsal) and neuropodial (ventral) structures. **c, d** Drawings of a normal **c** and *duplicitas* juvenile **d**. Same view as in **a, b. e, f** Drawings as in **c** and **d**. The setae have been removed to improve the visual clarification of polarity within the trunk organization. The polarity within each parapodium is indicated by letters, the notopodium (*NO*) being the dorsal portion and the neuropodium (*NE*) being the ventral portion. Therefore, each dorsal side (*D*) is flanked by two notopodia and each ventral (*V*) side by two neuropodia. *Scale bar* = 20 μm

duplicitas embryos developed within this particular group of embryos. As a consequence of altered geometry during first cleavage, different components of the ooplasm are distributed among the two daughter blastomeres in an abnormal fashion. During normal first cleavage of *Nereis* and *Platynereis* embryos, a distinctive clear plasm becomes segregated into the CD blastomere (Wilson 1892; Costello 1945; Dorresteijn and Fischer, in preparation). This cytoplasm is shunted into the future dorsal quadrant and particularly into the first somatoblast 2d. According to Wilson (1892), the 2d blastomere of the *Nereis* embryo will produce the ventral plate, which gives rise to the nerve cord and the parapodial setae sacs. Since Wilson's precise description of organogenesis in *Nereis* shows no detectable differences to our observation on the *Platynereis* system, the blastomere 2d will play the same formative role in *Platynereis*. In fact, nerve cord and parapodial sacs are the most obvious structures that are duplicated within the *duplicitates*. Thus, we assume that the potencies to develop these structures are normally segregated to the CD blastomere after first cleavage. However, if the first cleavage is equalized, the potency is conveyed to both cells of the two-cell stage. Thereby both blastomeres attain the capacity to develop a first somatoblast at fourth cleavage. Figure 1 d illustrates a blastomere configuration of this sort at the eight-cell stage with two large and two small quadrants. This blastomere configuration fits well with the four-cell stage of CDCD embryos of *Dentalium*, after inhibition of polar lobe formation at first cleavage (Guerrier et al. 1978), which also developed into double embryos.

The present results give proof that the first cleavage organizes the dorsoventral polarity, as has been indicated for many other spiralians with unequal cleavage. In *Nereis* as well as in *Platynereis dumerilii* (Wilson 1892; Fischer 1983) the excentrically positioned first cleavage furrow subdivides plasms with differing cytological properties in an unequal manner. We assume that the quantity of certain cytoplasmic components, e.g. the clear cytoplasm in the CD cell, produces differences in morphogenetic information. After experimentally created equal first cleavage, such plasm (Fig. 1 c) and such morphogenetic information is allocated equally instead of asymmetrically to the two daughter blastomeres. The role of specific parts of the ooplasm and the allocation of specific plasms to specific cell lines during the cleavages will be the subject of further studies on the organization of dorsoventral polarity in the *Platynereis* embryo.

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