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Pristionchus pacificus, a nematode with only three juvenile stages, displays major heterochronic changes relative to *Caenorhabditis elegans*

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The nematode *Pristionchus pacificus* (Diplogastridae) has been described as a satellite organism for a functional comparative approach to the model organism *Caenorhabditis elegans* because genetic, molecular, and cell-biological tools can be used in a similar way in both species. Here we show that *P. pacificus* has three juvenile stages, instead of the usual four found in other nematodes. Embryogenesis is lengthened and many developmental events that take place during the first juvenile stage in *C. elegans* occur during late embryogenesis in *P. pacificus*. Video imaging and transmission electron microscopy revealed no embryonic moult. The timing of later developmental events relative to the moults differs between *P. pacificus* and *C. elegans*. In addition, the post-embryonic blast-cell divisions display a specific change in timing between the two species, resulting in heterochrony between different cell lineages, such as vulval and gonadal lineages. Developmental events appear to come into register during the last larval stage. Thus, differences in developmental timing between *P. pacificus* and *C. elegans* represent a deep heterochronic change. We designate the three juvenile stages of *P. pacificus* as J1 to J3. Comparison with other species of the family Diplogastridae indicates that this pattern represents an apomorphic character for the monophylum Diplogastridae.

Keywords: *Pristionchus*; *Caenorhabditis*; nematodes; juvenile stages; heterochrony

1. INTRODUCTION

The post-embryonic development of nematodes is accompanied by periodic moulting. The number of four moults is thought to be constant within the entire monophylum (see Malakhov (1994) for a review). In a majority of species, the first-stage juvenile hatches from the ovum and all four larval moults occur in an external environment (outside the egg shell). This type of external moulting is thought to be ancestral in nematodes (Malakhov 1994). However, deviations from this pattern are seen in many different groups of Secernentea as well as in the Mermithidae and Tetradonematidae (for reviews, see Chitwood & Chitwood 1950; Nickle 1984; Anderson 1992). In such cases, embryonization of moulting occurs: among tylenchid phytohelminths, the first moult takes place in the egg and a second-stage juvenile hatches from the ovum later in development (Nickle 1984). In some animal parasites of the Strongylida and Ascaridida, and all Oxyurida, even two larval moults take place in the ovum and a third-stage juvenile hatches (Anderson 1992). Nonetheless, embryonization also occurs in some free-living nematodes, like the diplogastrid *Butlerius degrissei* (Grootaert & Jaques 1979).

Pristionchus pacificus of the Diplogastridae has been described as a satellite organism for functional comparison with the model *Caenorhabditis elegans*. Functional comparative approaches are possible in this species, using genetic and molecular tools. Most of the current work in *P. pacificus* focuses on the genetic and molecular analysis of vulva development and its evolutionary comparison with *C. elegans* (Sommer & Sternberg 1996; Eizinger & Sommer 1997; Jungblut & Sommer 1998; Sommer *et al.* 1998). Here we describe the observation that *P. pacificus* has only three juvenile stages. No embryonic moult has

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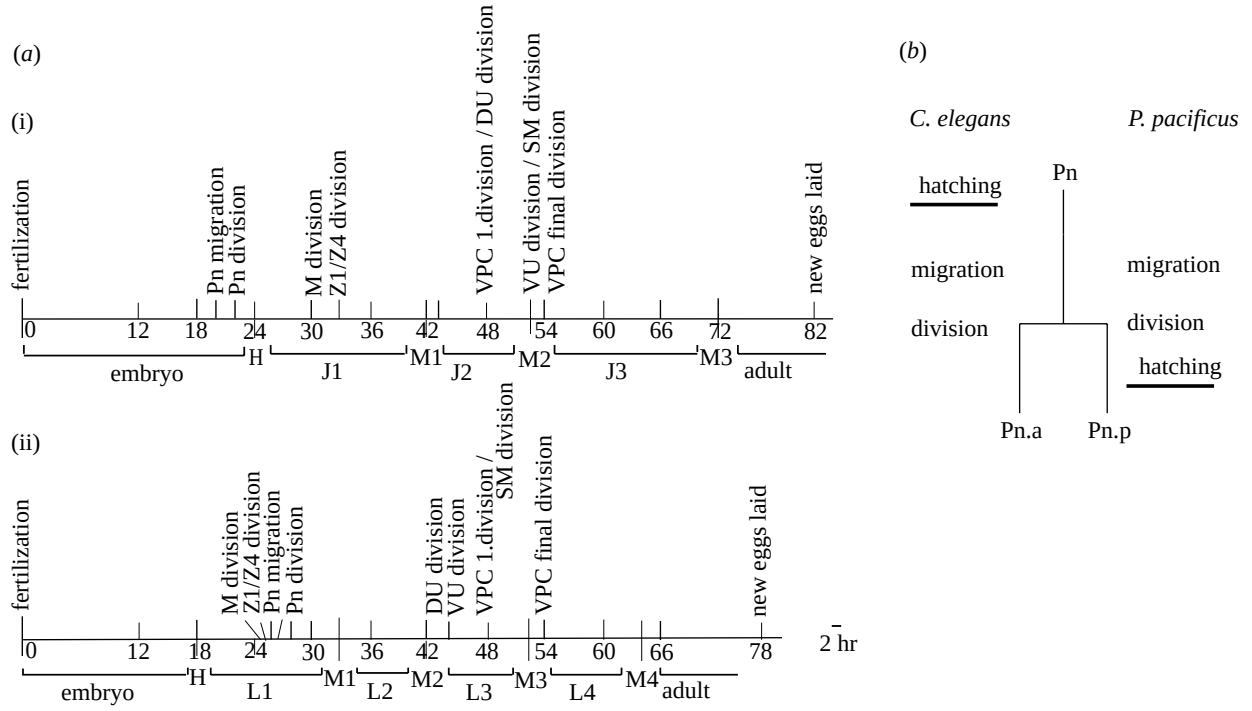

Figure 1. Timetable of developmental events in *C. elegans* and *P. pacificus* (20 °C). (*a*) All aspects of development discussed in the text are summarized in this figure. *C. elegans* data are from Sulston & Horvitz (1977) and Lewis & Fleming (1995). Numbers indicate the number of hours since fertilization. H, hatching; M1, M2, M3 and M4 indicate the moults from one juvenile stage to the next and finally to adulthood; J1–J3 and L1–L4 describe the juvenile stages in (i) *P. pacificus* and (ii) *C. elegans*, respectively. (*b*) Pn cell lineage and timing of events in *C. elegans* and *P. pacificus*. See text for details.

been observed. However, embryogenesis is lengthened, and many developmental events that take place during the first larval stage in *C. elegans* and other Rhabditidae occur during late embryogenesis in *P. pacificus*. We designate the three juvenile stages of *P. pacificus* as J1 to J3. In *C. elegans*, the four larval stages are designated L1 to L4, a terminology that is also used in this study.

2. MATERIAL AND METHODS

(a) *Strains and cultures*

P. pacificus var. *California* was the wild-type strain used in this work. Worms were handled and maintained as described by Sommer *et al*. (1996) and were usually grown at 20 °C. Nomarski observations were made according to standard procedures (Sommer & Sternberg 1996). For details on general worm culture, see also Epstein & Shakes (1995).

(b) *Transmission electron microscopy*

Nematodes were cryoimmobilized by high-pressure freezing according to a protocol of Hohenberg *et al*. (1994). In short, living specimens were sucked into cellulose microcapillaries (200 µm in diameter), and 2 mm-long capillary-tube segments were transferred to aluminium platelets (200 µm in depth) containing 1-hexadecene. The platelets were sandwiched together with platelets without any cavity and then frozen with a high-pressure freezer (Bal-Tec HPM 010, Balzers, Liechtenstein). The frozen capillary tubes were freed from extraneous hexadecene and transferred to 2 ml microtubes with screw caps (Sarstedt no. 72.694) containing the substitution medium precooled to −90 °C. Samples were kept in 1% osmium tetroxide in anhydrous acetone at −90 °C for 16 h, and at −60 °C and −30 °C for 6 h at each step, in a freeze-substitution unit (Balzers FSU 010, Bal-Tec, Balzers, Liechtenstein). After washing with acetone, the samples were infiltrated with Epon and polymerized at 60 °C for 48 h. Ultra-thin sections stained with uranyl acetate and lead citrate were viewed in a Philips CM10 electron microscope at 60 kV.

3. RESULTS

Observation of post-embryonic development in *P. pacificus* revealed that there are only three juvenile stages in this species. In the following sections, we describe characteristic aspects of the vulval and gonadal cell lineages in *P. pacificus* with respect to the timing of moulting and compare the observed pattern with the one known from *C. elegans*. All of the developmental aspects discussed are summarized in figure 1.

(a) *Late embryogenesis in* Pristionchus pacificus

The generation times of *C. elegans* (78 h, 20 °C) and *P. pacificus* (82 h, 20 °C) are very similar. Nonetheless, embryogenesis takes 18 h in *C. elegans* but 24 h in *P. pacificus*. During the last few hours of *P. pacificus* embryogenesis, the P-ectoblasts undertake aspects of their development that are characteristic of the first-stage juvenile (L1) of *C. elegans* (figure 1).

In *C. elegans* and *P. pacificus*, as well as all other nematodes studied so far, there are 12 Pn ectoblast cells and they are named according to their antero-posterior position (P1–P12) (Sulston & Horvitz 1977). These cells are born during embryogenesis in the lateral region of the worm. In *C. elegans*, *ca.* 5 h after hatching, the Pn cells

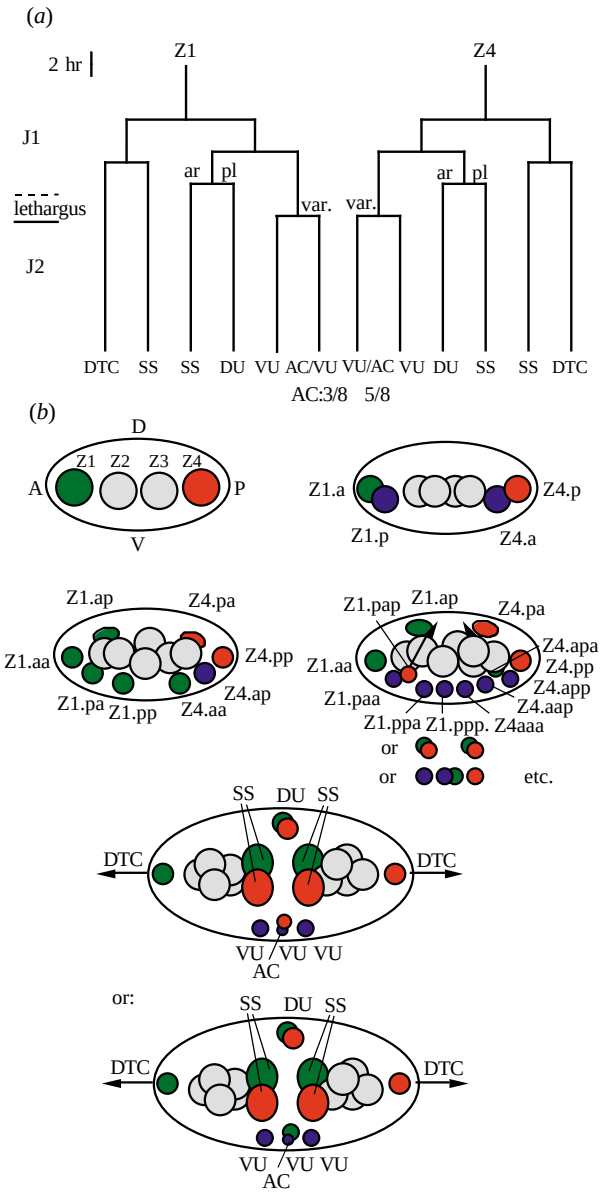

Figure 2. Cell lineage and schematic organization of the gonad in *P. pacificus*. (*a*) The cell lineage is identical between *P. pacificus* and *C. elegans*. DTC, distal tip cell; SS, sheath and spermatheca; DU, dorsal uterus; VU, ventral uterus; AC, anchor cell. The orientation of division is antero-posterior, unless otherwise indicated. Anterior is to the left. Ar/pl, anterior-right to posterior-left division; var., variable orientation. Either Z1.ppp or Z4.aaa can become the anchor cell. (*b*) Organization of the gonad primordium at different developmental stages. Z2 and Z3 are germ line precursors (grey). In comparison with *C. elegans*, a slight difference is found in the configuration of the ventral uterine precursors. In *C. elegans*, if Z1.ppp becomes the AC, Z4.aaa migrates anteriorly on its right side to adopt a position right of Z1.ppa, and Z4.aap remains alone in a posterior position; if Z4.aaa becomes the AC, Z1.ppp migrates posteriorly on its left side to adopt a position left of Z4.aap, and Z1.ppa remains alone in an anterior position (see fig. 6 in Kimble & Hirsh 1979). In *P. pacificus*, irrespective of which of the two becomes the AC, the other one migrates either to the left or right of the AC and does not move beyond it, so that in the final configuration, Z1.ppa is anterior, Z4.aap posterior, and the two other cells left and right of each other in the centre. This might influence further uterine lineages (Newman *et al*. 1995). The two possible configurations indicated (AC on the right or on the left of the central VU) can occur irrespective of whether Z1.ppp or Z4.aaa is the AC. Anterior (A) is to the left, dorsal (D) to the top. Green, right side; red, left side; dark blue, centre. Other abbreviations are as in (*a*).

migrate into their final position in the ventral cord (figure 1*b*). Shortly afterwards, the Pn cells divide asymmetrically, generating 12 neuroblasts, called P(1–12).a, and 12 ectoblasts, called P(1–12).p (Sulston & Horvitz 1977) (figure 1). Pn migration and division are prominent features of the development of the L1 stage in *C. elegans*.

In *P. pacificus*, the 12 Pn cells migrate into the ventral cord after *ca*. 20 h of embryonic development (figure 1*b*). Soon afterwards, the 12 Pn cells divide asymmetrically to generate P(1–12).a and P(1–12).p, still during embryogenesis. Seven out of the 12 Pn.p cells die of programmed cell death, an aspect of *P. pacificus* development that represents an important difference relative to *C. elegans* (Sommer & Sternberg 1996; Eizinger & Sommer 1997). The programmed cell death of P(1–4,9–11).p in *P. pacificus* occurs after *ca*. 22 h and is soon followed by hatching. Taken together, the migration and the asymmetric division of the Pn cells, and the programmed cell death of P(1–4,9–11).p of *P. pacificus*, occur during the last few hours of embryogenesis. The homologous developmental processes in *C. elegans*, Pn migration and division, occur in the first 10 h of the development of the first-stage juvenile (figure 1*b*). Thus, although the overall generation time of *P. pacificus* is somewhat slower than that of *C. elegans*, Pn migration and division occur earlier in *P. pacificus*.

Another developmental event that occurs in the L1 stage in *C. elegans*, but which has occurred at hatching in *P. pacificus*, is the division of the B blast cell, the precursor of the male spicules in the male tail (data not shown).

(b) *First-stage juvenile (J1)*

After hatching, J1 juveniles of *P. pacificus* have 12 neuroblasts, P(1–12).a, and the five surviving ectoblasts, P(5–8,12).p, in the ventral cord region. Soon after hatching, P(1–12).a start to divide, with cell lineages very similar to the corresponding cells in the L1 stage in *C. elegans* (data not shown). As in *C. elegans*, the four central Pn.p cells, P(5–8).p, do not divide further in the J1 stage.

At hatching, the gonad of *P. pacificus* consists of four cells, called Z1–Z4, as in *C. elegans*. Z2 and Z3 give rise to the germ line, whereas Z1 and Z4 form the somatic gonad. In *C. elegans*, Z1 and Z4 divide in the L1 stage and give rise to six cells each (Kimble & Hirsh 1979). These 12 cells rearrange during the L2 stage; cell interactions take place at this time, for instance, those specifying which of Z1.ppp and Z4.aaa becomes the anchor cell (Kimble 1981). In *C. elegans*, the anchor cell is a specialized cell of the somatic gonad that coordinates morphogenesis of epithelia during the development of the uterine–vulval connection (Newman & Sternberg 1996).

The early cell lineage of the somatic gonad in *P. pacificus* is shown in figure 2. Formation of the founder cells of the somatic gonad is very similar between *C. elegans* and *P. pacificus*. As in *C. elegans*, divisions occur in the J1 stage. Cell rearrangements require less time and the final configuration is attained during the first hours of the J2 stage (figure 2). In summary, formation of the somatic

gonad primordium, which happens during the L1 and L2 stages in *C. elegans* (Kimble & Hirsh 1979), occurs mostly during the J1 stage in *P. pacificus* (figures 1 and 2). The larval mesoderm precursor cell M divides shortly before Z1 and Z4 in both species (figure 1*a*).

(c) *Second-stage juvenile (J2)*

Many events occurring during the third larval stage of *C. elegans* happen during the second juvenile stage of *P. pacificus*. (i) The vulva precursor cells (VPCs), P(5–7).p, divide towards the middle of this stage (figure 1*a*). (ii) In the gonad primordium, sheath and spermatheca (SS) and dorsal uterus (DU) precursor cells divide. Their division occurs later relative to VPC division and the moult in *P. pacificus*. (iii) When dauer formation occurs in *P. pacificus*, it is as an alternative developmental pathway to the J2 stage, in contrast to the L3 stage in *C. elegans*. However, dauer formation is similar with respect to the total time of development and VPC divisions in the two species.

However, some developmental events that occur in the J2 stage in *P. pacificus* occur in the L2 stage of *C. elegans*. For instance, the sex myoblast precursors (SM, descendants of the M cell) migrate from their posterior position at birth to the central body region where the vulva will form.

(d) *Third-stage juvenile (J3)*

The third-stage juvenile is the last stage before adulthood in *P. pacificus*. After vulval cell lineages have been completed, the final morphological structure of the vulva develops in this last juvenile stage. At the same time, the uterus has formed as part of the somatic gonad and the connection between the uterus and the outside environment is established through the vulva. In *C. elegans*, all corresponding developmental events occur in the L4 stage.

Again, some events that happen during the L3 stage of *C. elegans* occur at the J3 stage of *P. pacificus*. They are delayed relative to vulval divisions. For instance, the ventral uterus (VU) precursors divide before the VPCs in the middle of the L3 stage in *C. elegans*, but at the time of the final vulval division at the beginning of the J3 stage in *P. pacificus*. The division of the sex myoblasts is delayed similarly (figure 1*a*).

(e) *Transmission electron microscopy and video imaging of* Pristionchus pacificus *eggs*

The analysis of post-embryonic development in *P. pacificus* revealed the existence of only three juvenile stages. One explanation for this phenomenon would be embryonization of moulting. To determine if moulting occurs during embryogenesis, we carried out careful video imaging of embryonic development. With this technology, we were unable to detect any sign of moulting (data not shown). As a thin cuticle might be difficult to detect by light microscopy, we analysed transmission electron microscope sections of late-stage embryos to see if additional thin cuticular structures exist. Figure 3 shows such a late-stage animal after sectioning and transmission electron microscopy (TEM). No sign of an additional cuticle is present. Thus, embryonization of moulting is ruled out by TEM.

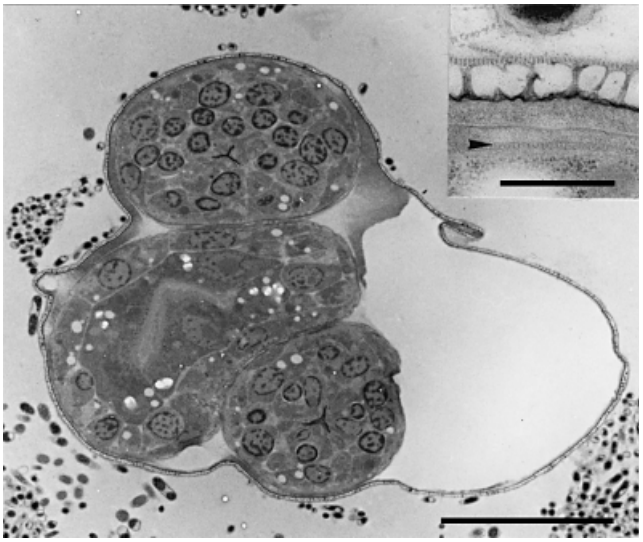

Figure 3. Transmission electron microscope image of a *P. pacificus* egg during late embryogenesis. The section is through a very late *P. pacificus* egg, and it cuts the young larva three times. The inset shows a higher magnification of the egg shell. The arrow points to the cuticle of the J1 juvenile forming in the late egg. No second cuticle is visible as should be in case of internal moulting. Scale bar, 10 μm (0.5 μm in the inset).

4. DISCUSSION

The results described here indicate that *P. pacificus* is a nematode with only three juvenile stages. Four juvenile stages were previously thought to be constant within the entire class, with the only deviation being cases of embryonization of some parasitic nematodes (Chitwood & Chitwood 1950; Nickle 1984; Anderson 1992). In *P. pacificus*, hatching is delayed and developmental events of the L1 stage in *C. elegans* occur in *P. pacificus* partly before hatching and partly during the first juvenile stage. Later on, the timing of developmental events relative to the moults and relative to one another differs between *P. pacificus* and *C. elegans*.

(a) *Evolutionary considerations in the Diplogastridae*

The ventral epidermal cell lineages were analysed in several members of the Diplogasteridae, including species of the genera *Goodeyus, Pseudodiplogasteroides, Diplogasteroides, Diplogaster, Aduncospiculum* and *Koerneria* (Sommer 1997). In all species, Pn migration and division, as well as the Pn.p programmed cell death, occurred during embryogenesis, as in *P. pacificus*. In newly hatched juveniles of all of the species analysed, the five Pn.p cells P(5–8,12).p were in the ventral cord region, indicating that the developmental timing observed in *P. pacificus* represents a common characteristic of the Diplogasteridae. Furthermore, only three juvenile stages were observed in all of the species analysed. These results suggest that the three juvenile stages J1 to J3 represent an apomorphic character for the monophylum Diplogastridae. Such a pattern has not, to our knowledge, been observed previously in any species of the Rhabditidae, Cephalobidae or Panagrolaimidae (Sommer & Sternberg 1995; M.-A. Félix, R. J. Sommer and P. W. Sternberg, unpublished observations).

(b) *Evolutionary changes in the developmental timing and number of moults*

The evolutionary changes described here concern (i) the timing of hatching; (ii) the number of larval stages; and (iii) the timing of developmental events relative to others, hatching and the moults. In *C. elegans*, very little is known about the genetic control of the timing of hatching. Identity of larval stages (i.e. developmental events) and number of moults are both controlled by the so-called 'heterochronic' genes. For instance, in *lin-14* loss-of-function mutants, the Ll-specific lineages are suppressed, and only three moults occur (Ambros & Horvitz 1984). In contrast, the evolutionary change in number of moults in *P. pacificus* is not accompanied by a suppression of developmental events, but by a change in their timing relative to hatching and moults. The nature of the link between developmental events and moults is not known in *C. elegans*.

A salient feature of this comparison between *P. pacificus* and *C. elegans* is the dissociation of timing between different lineages. For instance, Pn cell and B cell development occur much earlier compared with gonad and M development in *P. pacificus* than in *C. elegans* (figure 1). Thus, whereas with respect to vulva development, the *P. pacificus* J2 and J3 stages resemble the *C. elegans* L3 and L4 stages, mesodermal development is shifted relative to these later moults. The timing of gonadal (not M) development seems at least partially independent from other developmental events in *C. elegans*, since distinct mutants affect the timing either of gonadal or of non-gonadal development (Sun & Lambie 1997). Timing is also variable in evolution relative to other developmental events: for instance, early gonadal divisions occur in the Ll stage in *C. elegans*, in-between the Ll and L2 stages in *Panagrellus redivivus*, and as late as the end of the L2 stage in *Cephalobus* or *Panagrolaimus* spp. (Kimble & Hirsh 1979; Sternberg & Horvitz 1981; Félix & Sternberg 1996). The M lineage is not delayed in these species relative to moults and Pn cell development. The later rounds of division then always occur at the end of the L3 stage, in parallel to the vulva and sex muscle lineages.

Taken together, the differences in developmental timing described here for *P. pacificus* and *C. elegans* represent a deep heterochronic change. Heterochrony is a well-established paradigm in evolutionary biology and has been shown to play a major role in developmental evolution (Gould 1977; McKinney 1988). We speculate that the heterochronic changes have been an important evolutionary prerequisite for the alteration of the number of larval stages seen in *P. pacificus*.

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