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DIAPAUSE IN THE EARTHWORM, *APORRECTODEA LONGA*: MORPHOLOGICAL
AND QUANTITATIVE X-RAY MICROANALYSIS OF CRYOSECTIONED
CHLORAGOUS TISSUE

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Abstract

The earthworm, *Aporrectodea longa*, which experiences an apparent obligatory physiological resting state ("diapause") in the temperate Summer, was collected monthly from February through to October. Morphological examination of the chloragocytes, and quantitative electron probe X-ray microanalysis of their constituent chloragosome granules prepared by cryoultramicrotomy and air-dried/smearing, were undertaken over this period. Worms entered diapause in May, and emerged from it between August and September. The long-term energy stores in the form of lipid, in addition to the more usual polysaccharide (glycogen) reserves, were accumulated by early diapause; the lipid was gradually consumed during diapause. In addition, the structure and composition of the chloragosomes changed considerably during the annual cycle: there was an accumulation of Ca, P, Zn and S around the period of entry into diapause; Ca, P and Zn were mobilized from the granules as diapause progressed. The adaptive significance of these changes is discussed in the broad context of the different ecophysiological strategies evolved by stenohaline earthworms to resolve the problems posed by dry climatic conditions.

Key words: earthworms, diapause, chloragosomes, morphology, X-ray microanalysis.

Introduction

Earthworms form the dominant component of the animal biomass in many temperate soils, and they participate actively in soil mineralisation and nutrient cycling processes (Edwards and Lofty, 1977; Wallwork, 1983). Earthworms are adapted to a range of different moist terrestrial habitats, but they cannot colonize permanently dry soils because they have evolved from aquatic, stenohaline freshwater ancestors (Sims and Gerard, 1985), and still retain the osmoregulatory features of freshwater animals (Dietz and Alvarado, 1970). Some tropical worms belonging, for example, to the genera *Pheretima* and *Lampito* can survive in relatively dry soils, because they possess specialized enteronephric excretory systems that promote water conservation (Takeuchi, 1980; Wallwork, 1983). Survival during prolonged spells of dry weather in temperate climates can be achieved by combinations of behavioural and physiological adaptations. Two distinct physiological resting states can be distinguished: quiescence and diapause (Olive and Clark, 1978).

Quiescence is a state that all earthworm species can adopt in response to dehydration. It is often characterized by a retreat to deeper soil layers especially in species possessing permanent vertical burrows, the arrest of feeding and reproduction, a regression of primary and secondary sexual structures and, crucially, a progressive loss of tissue water (Olive and Clark, 1978). Diapause, in contrast, is a more discrete physiological state, restricted to certain species, where the individual worms stop feeding, coil into a knotted ball inside a mucus-lined soil "cell", resorb reproductive tissues, undergo caudal regeneration, and despite losing weight do not suffer significant tissue dehydration (Olive and Clark, 1978; Clement and Manavalaramanujam, 1982; Morgan, 1984).

Diapause in earthworms, as in insects (Saunders, 1976), can be either obligatory or facultative. Facultative diapause is triggered by deteriorating environmental conditions, notably a gradual reduction of soil moisture content; but if conditions remain favourable, then activity is uninterrupted. Obligatory diapause is less tied to environmental conditions; worms may spontaneously enter and exit this state,

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presumably according to genetically programmed neuroendocrine rhythms. The distinction between these two forms of diapause is conceptually clear, but experimentally difficult to demonstrate.

The earthworm, *Aporrectodea longa*, enters a definite diapause that lasts for several months in the British summer. Semiquantitative electron probe X-ray microanalysis (EPXMA) of air-dried tissue smears from the chloragogenous tissue of this species, has shown that the multifunctional chloragosome granules undergo major compositional changes before and during diapause (Morgan, 1984). It has been suggested that the temporal pattern of accretion and loss of elements (P, S, Ca, Zn), in these unique storage organelles (Fischer, 1975; Prentø, 1979; Morgan, 1984) during the annual cycle, coincides with the phased nutrient deposition, nutrient consumption and acid-base regulation demands imposed by the protracted resting period.

The objectives of the present study are twofold. First to observe whether, in *A. longa*, the morphology of the chloragocyte cells, and of the intracellular chloragosome granules, change in response to the distinct ecophysiological states that the worm experiences during its entire annual cycle, including diapause. Second, to measure quantitatively by EPXMA the composition of chloragosomes, before and during diapause, to investigate the role of these organelles during diapause.

Materials and Methods

Animals. About 10 individuals of the earthworm, *A. longa*, were collected at monthly intervals, from February to October, by digging and handsorting. The sampling site was a relatively undisturbed garden in Cardiff, South Wales. The February, March, April and October worms were active; July and August (no sample was collected in June) worms were in diapause. About 20 worms were collected in May; approximately half of these were still active, whilst the others had entered the characteristic knotted diapause state. Worms were transferred immediately in their native soil to the nearby laboratory and processed for analysis.

Morphology: electron microscopy. Worms were not "starved" to clear their guts prior to processing. Small pieces of intestine with attached chloragogenous tissue, taken from approximately half-way along the length of the intestine, were fixed in 3% glutaraldehyde in 0.1M phosphate buffer (pH 7.4), post-fixed in 1% Millonig's phosphate-buffered OsO_4 , dehydrated and embedded in Spurr resin. Thin sections 60nm were cut with an LKB Ultratome III and stained with uranyl acetate and lead citrate, and observed and photographed in a JEOL 100S transmission e.m.

Cryomicrotomy. Small pieces of fresh chloragogenous tissue were taken from worms sampled at each time interval, and cryofixed by immersion in a home-made propane plunger (Roos and Morgan, 1990). Specimens were stored under liquid nitrogen until required for sectioning.

Cryosectioning was performed either in a Slee Type TUL Cryostat (chamber temperature,

183K) or in a Reichert Ultracut FC4E Cryoattachment (chamber temperature, 153K). Sections nominally 70nm thickness, were handled and externally freeze-dried as described by Winters and Morgan (1988).

Air-dried smears. These were prepared from 5 "active" worms sampled in April and from 5 "resting" worms collected in May. Smearing was performed on titanium grids as described previously (Morgan, 1984; Winters and Morgan, 1988). Individual granules were analysed.

EPXMA. Analyses were performed in a Philips TEM 300 (80kV; current = 0.2-0.3 nA; livetime = 100s; output count rate 1000 counts per second; specimen tilt angle = 21°) equipped with a Link 30mm² EDS detector and Link 860 Series 2 multichannel analyzer/Quantem FLS (Link Systems Ltd.) analytical system. Quantitation was facilitated with sectioned aminoplastic standards (Morgan and Winters, 1988; Morgan *et al.*, 1989).

Data analysis. Statistical analyses of the data were performed by Epistat, Version 3.0 for the IBM P.C.

Results

Morphology: chloragosome granules

Earlier observations indicated that the chloragosomes of *A. longa* underwent major compositional changes during the annual cycle, and especially in those Summer months during which diapause occurs (Morgan, 1984). Although a direct correlation of compositional and structural changes in these inorganic-rich organelles (Morgan, 1981, 1982, 1984; Morgan and Winters, 1982; Prentø, 1979; Winters and Morgan, 1988) would probably require preparation by a stringent version of a "compromise cryoprotocol", such as freeze-substitution (Roos, 1989; Roos *et al.*, 1990), it was quite apparent that the chloragosome morphology did change seasonally.

In late Winter (February) the granules appeared to be uniformly, and heavily, mineralized; the fenestrated appearance of some granules probably being an artefact of preparation or sectioning (Figure 1). In March (Figure 2) and April (Figure 3) the chloragosomes had irregular profiles, with evidence of the budding or fusion of smaller vesicular bodies, containing a similar matrix, to or from the main body of individual chloragosomes. By the pre-diapause period in May (Figure 4) the chloragosomes were more regularly shaped and possessed a uniform internal appearance, although there was still evidence of vesicular fusion or budding.

Immediately after entry into diapause in May (Figure 5A) the chloragosomes possessed regular ovoid profiles, and most granules contained a core of significantly denser material. By July (Figure 6) and August (Figure 7), approaching the end of diapause, the ovoid shaped chloragosomes had a fairly lightly stippled appearance, and had lost the dense core that they contained during very early diapause.

After emergence from diapause, the chloragosomes of *A. longa* in October (Figure 8) were ovoid in shape, and although they displayed

Diapause in the Earthworm

Table 1. Elemental composition* of the chloragosome granules of *A. longa* determined by EPXMA of freeze-dried cryosections.

Sampling month**	Element (mM kg ⁻¹ dry weight)			
	P	S	Ca	Zn
March (N.D.)	299.6 (20) ± 149.0	162.6 (20) ± 94.8	67.5 (20) ± 36.7	111.6 (20) ± 56.5
May ^T (N.D.)	678.2 (20) ± 168.7	379.9 (20) ± 100.6	94.1 (20) ± 65.7	323.5 (20) ± 86.7
May ^T (I.D.)	919.7 (20) ± 506.9 N.S.	288.4 (20) ± 125.9 P < 0.05	1004.6 (20) ± 553.0 P < 0.001	269.6 (20) ± 156.7 N.S.
July (I.D.)	82.3 (20) ± 66.9	174.1 (20) ± 50.2	86.2 (20) ± 47.7	5.1 (20) ± 10.8
August (I.D.)	458.1 (20) ± 235.2	416.8 (20) ± 200.0	427.0 (20) ± 244.4	46.0 (20) ± 29.2
October (N.D.)	389.8 (20) ± 370.4	64.9 (20) ± 45.2	183.3 (20) ± 214.0	72.3 (20) ± 35.3

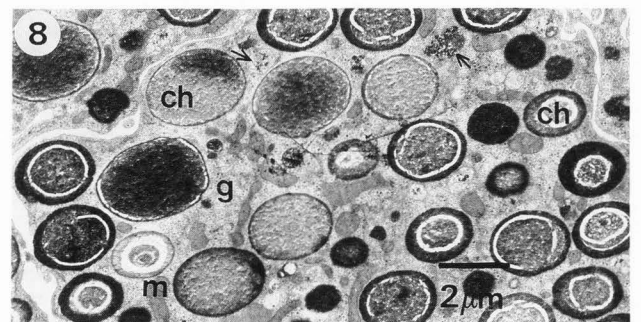
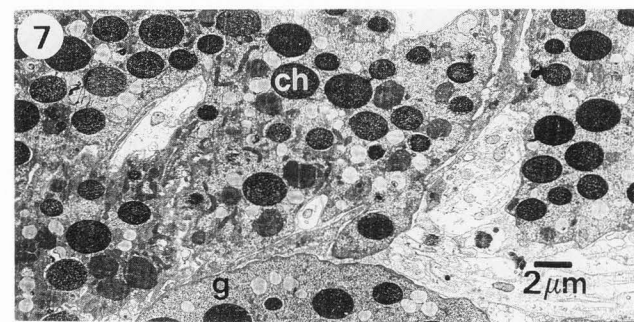
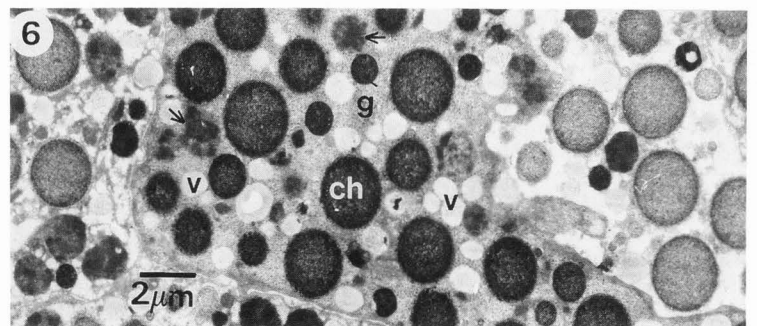
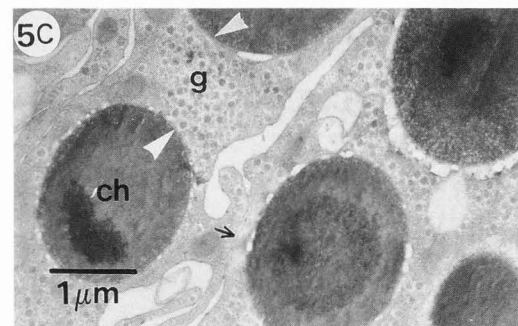
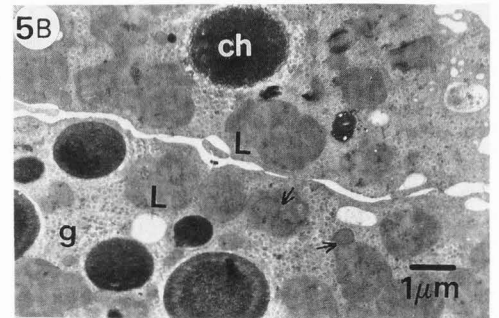
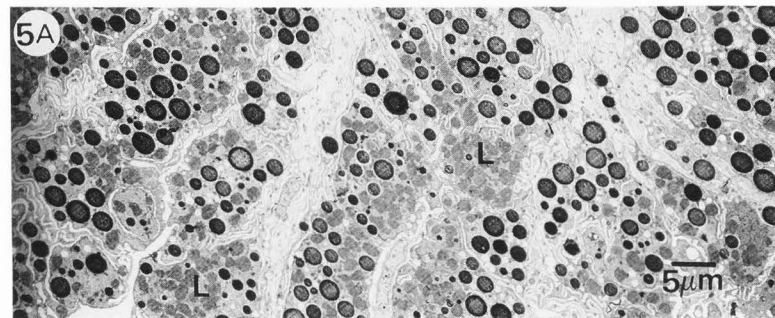
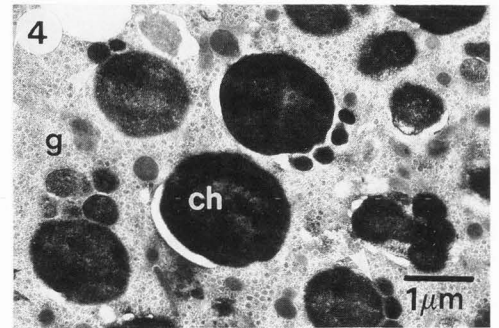
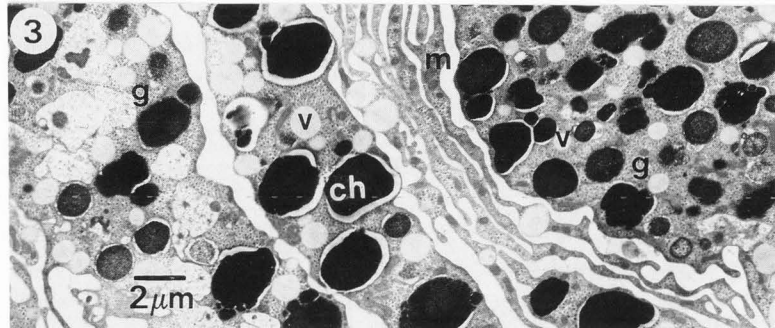
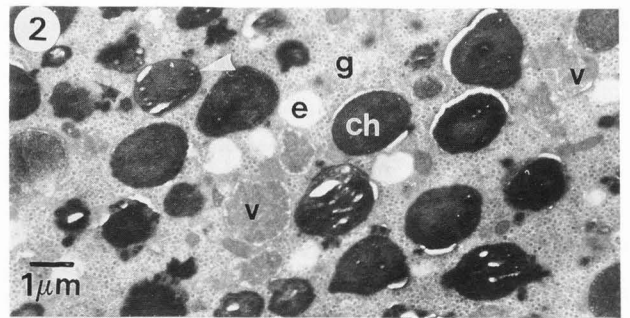
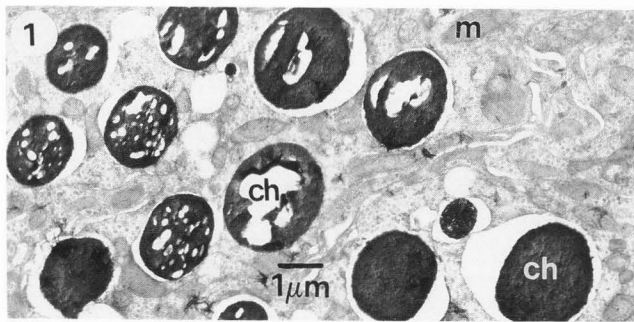
* data expressed as mean ± S.D.; ** April samples were inadvertently lost; N.D. = not in diapause; I.D. = worms in diapause; numbers in parentheses = number of individual granules analyzed.

[^T The N.D. and I.D. samples for the month of May were compared by 2-way Student's t-test; N.S. = non-significant.]

Table 2. Linear regression analysis of the correlations between Ca:P and Zn:S in smeared* and cryosectioned** chloragosome granules from active and resting worms.

		Regression Equation	t	d.f.	P
Ca:P (Cryosections)	ND	y = -2.44 + 1.41x	1.638	18	P=0.11(N.S.)
	ID	y = 89.79 + 0.99x	9.424	18	P < 0.001
Zn:S (Cryosections)	ND	y = 19.56 + 0.77x	4.677	18	P < 0.001
	ID	y = 93.56 + 0.61x	2.358	18	P < 0.05
Ca:P (Smears)	ND	y = 29.00 + 0.24x	7.351	33	P < 0.001
	ID	y = 2.79 + 0.72x	10.084	39	P < 0.001
Zn:S (Smears)	ND	y = 7.03 + 0.47x	11.63	33	P < 0.001
	ID	y = 152.31 + 1.65x	0.299	39	(N.S.)

t - distribution was used to determine if the slope of the regressions differed significantly from zero; * = for smears the active animals (ND = "not diapause") were sampled in April; ** = for cryosections the active animals were sampled in May; for both smears and cryosections the resting animals (ID = "in diapause") were sampled in May; N.S. = non significant.



Figures 1-8: Transmission electron micrographs of resin embedded sections of the chloragogenous tissue from *A. longa* sampled monthly from February to October. **Labelling:** ch, chloragosome granules; g, glycogen rosettes; m, mitochondria.

Figure 1: February sample. Note the heavily mineralized, fenestrated nature, of the chloragosomes.

Figure 2: March sample. Note: the irregular chloragosomes; the membrane limiting the chloragosomes (arrows); glycogen; the irregularly-shaped membrane-limited vacuoles with grainy contents (v); and the pale empty vacuoles (e).

Figure 3: April sample. Many of the chloragosomes are irregularly shaped, the processes of adjacent cells interdigitate, and the empty vacuoles (v) appear to have rows of glycogen particles radiating from them.

Figure 4: May sample - before entry into diapause. Note that the cytoplasm of the chloragocyte is packed with glycogen, and that many of the chloragosomes are surrounded by small dense vesicles which, in places, seem to be fused with the main body of the chloragosome (arrows).

Figure 5: A. May sample - just after entry into diapause. Note that the chloragocytes are packed with irregularly shaped vacuoles (L), which may be lipid-rich. B. Note that the (presumptive) lipid vacuoles (L) have microvesicular contents (arrows). C. The membrane limiting the chloragosome (arrows) and the dense cores of these mineralized organelles are prominent; note also the high density of glycogen and the cell-cell contacts (small arrow).

Figure 6: July sample. The chloragosomes possess homogeneous finely-grained contents. One cell contains some glycogen reserves and several empty vacuoles (v). There is some evidence of the presence of multivesicular vacuoles (arrows), but whether they are the same as the presumptive lipid vacuoles seen earlier in diapause (Figure 5B) is uncertain. Some cells may be disrupted.

Figure 7: August sample - end of diapause. The cells still contain ovoid chloragosomes with grainy contents, glycogen, and many small empty vacuoles. The cell membranes are relatively smooth.

Figure 8: October sample - active worm out of diapause. The chloragosomes possess a concentric structure, and the glycogen content of the cytoplasm is depleted. Note the presence of lysosome-like vesicles with dense, finely granular contents (arrows).

some structural heterogeneity, many possessed a concentric appearance with an outer shell of relatively dense material. These granules were rather reminiscent of those described in the active, non-diapausing earthworm, *Lumbricus terrestris* (Prentø, 1979; Morgan, 1981).

Morphology: chloragocytes

The chloragocytes were rich in glycogen during the pre-diapause (Figures 1-4) and early diapause (Figures 5B and 5C) periods. There was some indication that the amount of glycogen stored in some cells was significantly depleted by late diapause (Figure 6), although the stores in other cells remained relatively high.

In early diapause the cytoplasm of the chloragocytes became packed with irregularly shaped vacuolar structures of unknown aetiology containing relatively electron dense material (Figure 5A). Some of these (presumptive) storage vacuoles were distributed between the chloragosomes, but most were aggregated together in relatively chloragosome-free regions of cytoplasm. At higher magnifications these vacuoles appeared to have microvesicular contents (Figure 5B). By July and August the number of storage vacuoles within the chloragocytes seemed to have diminished, although there were electron-dense multivesicular bodies (Figure 6) present, which may be cytologically identical to the storage vacuoles seen in early diapause.

Elemental composition of chloragosomes: cryosections

EPXMA observations on the composition of cryosectioned chloragosomes (Figure 9) are summarized in Table 1. Four main conclusions emerged from the quantitative data: (i) the Ca and P concentrations peaked after entry into diapause (although the pre- to post-diapause (May) rise in P was not significant) and decreased through diapause; (ii) the Zn concentration peaked around

the time of entry into diapause (May), and decreased through the resting period; (iii) the S concentration peaked just before entry into diapause, but remained fairly high throughout diapause; (iv) the Ca:P molar ratio was low (< 0.5) outside diapause, but approached 1:1 during the resting period.

Positive relationships were found between Ca:P and Zn:S in the chloragosome granules of earthworms sampled immediately before and immediately after entry into diapause; the relationships were statistically significant, except for Ca:P in the pre-diapause period (Table 2).

Two further observations should be made. First, the inter-granule compositional variability was high, indicating that these organelles are a chemically, if not functionally, heterogeneous population. Whether multivariate statistical analysis of a large unbiased sample of chloragosomes (Morgan *et al.*, 1989) would dissect the population into distinct cohorts of granules remains an open question. Second, to describe elemental concentrations peaking "just before" or "just after" entry into diapause is slightly misleading, because the "not in diapause" and "in diapause" samples taken at the same time in May do not necessarily represent physiological continua. It is feasible, for example, that at the point when each worm switched into diapause there was a surge in chloragosomal Ca concentration which persisted into early diapause; if the kinetics of this Ca surge is rapid it could be missed in the pre-diapause samples and recorded as a post-diapause Ca peak.

Elemental composition of chloragosomes: smears

EPXMA observations on the composition of smeared chloragosomes (Figures 10), sampled from "active" April worms and "resting" May worms, are summarized in Table 3. Three conclusions emerged from the data: (i) the chloragosomal Ca

Table 3. Elemental composition* of the chloragosomes from "active" (April sample) and "resting" (May sample) *A. longa* prepared by air-dried/smearing.

	Element (mM kg ⁻¹ dry weight)			
	P	S	Ca	Zn
Not in Diapause (April)	381.5 (35) ± 358.6	160.6 (35) ± 157.5	120.5 (35) ± 109.2	81.9 (35) ± 81.9
Early Diapause (May)	990.6 (41) ± 422.3	447.3 (41) ± 211.7	712.2 (41) ± 355.9	159.6 (41) ± 73.0
	**P < 0.001	P < 0.001	P < 0.001	P < 0.001

* = data expressed as mean ± S.D.; numbers in parentheses = number of individual granules analysed; ** = differences between means tested by 2-tailed Student's t-test.

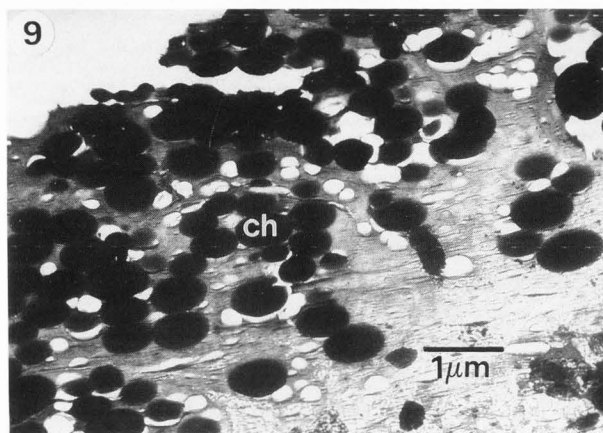
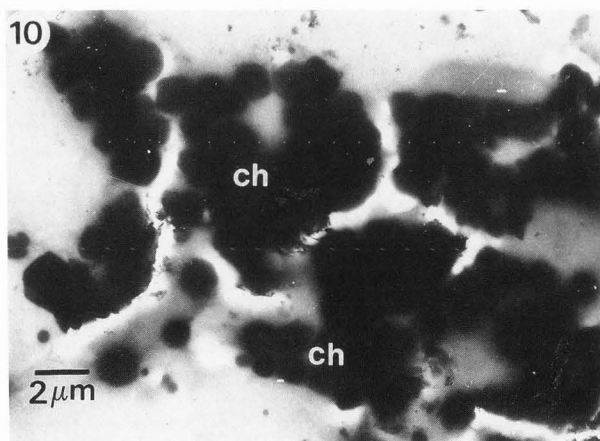


Figure 9: Thin freeze-dried cryosection of the chloragogenous tissue of *A. longa* sampled in March (not in diapause). The ovoid chloragosomes (ch) are readily discernible.

Figure 10: Air-dried smear of fresh chloragogenous tissue from a worm sampled in chloragosomes (ch).



derived from cryosections and smeared chloragosomes, respectively (cf. Tables 1 and 3).

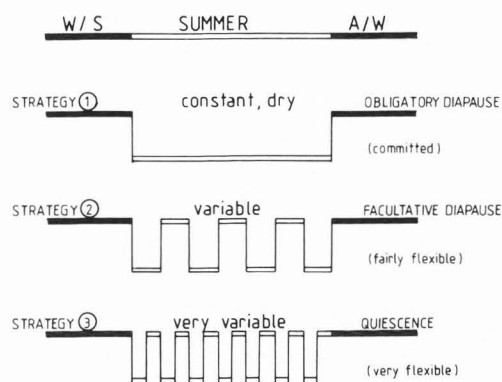
When the data from smears were plotted as frequency histograms (not shown) the change in chloragosomal composition from the active (April) to the early resting (May) phases of the life-cycle was revealed: the April to May shift in the Zn median was from 48 to 154 mM kg⁻¹ dry weight; P, from 202 to 912 mM kg⁻¹ dry weight; Ca, from 88 to 672 mM kg⁻¹ dry weight; S, from 160 to 431 mM kg⁻¹ dry weight.

Discussion

In an earlier paper (Morgan, 1984), involving the semiquantitative EPXMA of smears, it was observed that the composition of the chloragosomes of *A. longa* changed during the life-cycle, and especially in the period immediately before and during Summer diapause. The present quantitative EPXMA analyses substantiated these findings. Furthermore, it was shown that the structure of the chloragocyte cells, including the morphology of their constituent chloragosome granules, also changed considerably with seasonal adaptations.

EPXMA data alone cannot provide direct information about the metabolic or functional significance of the "programmed" elemental changes in the chloragosomes. However, the present morphological and microprobe observations indicated conclusively that the chloragogenous tissue plays important nutrient storage and supply roles during the protracted resting period experienced by *A. longa*. Significantly, Semenova (1967) concluded from histological studies of two related species, *A. rosea* and *A. caliginosa*, that the chloragogenous tissue accumulated high concentrations of glycogen and lipid before

and P concentrations were significantly higher during early diapause; (ii) Zn and S concentrations were also significantly higher in early diapause, compared with the chloragosomes of animals actively feeding approximately one month prior to Summer activity; (iii) there seemed to be no significant difference between the absolute concentration values for Ca, P, Zn and S



SEASONAL ECOPHYSIOLOGICAL ADAPTATIONS

Figure 11: Hypothetical models of the adaptation of different British earthworm species to the problems that may be posed by temperate Summers, when dry soils can limit movement, feeding and reproduction. In Summers that are exceptionally dry for long continuous periods, Strategy 1 (obligatory diapause), as displayed by *A. longa* and characterized by a programmed (?) accretion of nutrient and energy stores, probably provides survival advantages to the individual worm, although reproduction is precluded during diapause. In Summers where the dry period is interrupted periodically by occasional wet spells, Strategy 2 (facultative diapause) may confer an advantage, although the extent of nutrient and energy storage prior to diapause, the rapidity with which these species can resume reproduction after emergence from short-term diapausal rest, is unknown. In exceptionally wet Summers, species displaying Strategy 3 (quiescence) are probably at an advantage, certainly over those species displaying obligatory diapause, because they can exploit the favourable climatic conditions by continuing their feeding and reproductive activities. These various strategies persist in different species, because, for example: (a) the selective advantages frequently change from year to year; and (b) the terrestrial ecosystem is very heterogeneous, offering favourable microhabitats to quiescing species even in extremely dry Summers.

(W/S = Winter/Spring; A/W = Autumn/Winter)

diapause, and that the quantities of these energy reserves decreased as diapause progressed. Glycogen is generally considered to be the main energy-storage molecule in earthworm tissues (Ireland and Richards, 1977). The advantages of supplementing the readily mobilizable glycogen energy reserve with long-term adipose reserves, prior to diapause are self-evident, especially since

the net yield of ATP from the complete oxidation of stearic acid (MW = 284), for example, is 146 units compared with the yield from glucose (MW = 180) of 38 units (Staunton, 1978). The organic matrix of the chloragosome granules contains a mixture of redox pigments, including riboflavin, flavin nucleotides, thiamin, carotenoids and metalloporphyrins (Roots and Johnston, 1966; Fischer, 1975). Since earthworm chloragocytes are not especially mitochondria-rich cells, it is intriguing to speculate about the possible role of the chloragosomes in the catabolism of energy-storage molecules during diapause.

It has been shown (Morgan, 1982; Morgan and Winters, 1982) that the concentrations of Ca and P in the chloragosomes of *A. longa* are significantly lower than in the chloragosomes of *Lumbricus terrestris*, an earthworm that does not enter diapause during the British Summer. It was suggested (Morgan, 1982, 1984) that the lower chloragosomal Ca and P concentrations, coupled with the possession of non-mineralizing calciferous glands by *Aporrectodea* species, resulted in the relatively narrow soil-pH tolerance of this species group. Our present observations do not detract from this hypothesis, but they do indicate that the endogenous pH buffering capacity of the chloragogenous tissue, mediated via stored Ca and P, is elevated by the start of diapause. If, as seems likely, Ca and P are released from the chloragosomes during the progression of diapause, it is pertinent to consider their fate, since the "active" *A. longa* does not possess glands capable of excreting the excess Ca^{2+} (and HCO_3^-) from the body fluids (Morgan, 1982; see also: Kuhle, 1980). Thus, do Ca^{2+} and PO_4^{2-} accumulate in the body fluids of *A. longa* during diapause, or are they eliminated either through the nephridial system or via a diapause-activated calciferous gland?

Caudal regeneration has been shown to be activated during diapause due to the withdrawal of a regeneration-inhibiting hormone with gonadatrophic properties (Olive and Clark, 1978; Clement and Manavalaramanujam, 1982). Could it be that the calciferous glands of *A. longa* are also activated during diapause by release from the inhibitory influences of a neurosecretion? At present there is no answer to the question, although the presence of an anatomically discrete, morphologically complex, but "non-functional" calciferous gland in *A. longa* (Morgan and Chatterjee, unpublished) represents an evolutionary puzzle.

Diapause, in earthworms that display it as a discrete phase in the life-cycle repertoire, is an ecophysiological strategy to overcome the problems imposed by adverse climatic conditions (Figure 11). However, since temperate climates are as a rule very variable, the evolutionary "alternative" strategy of quiescence would appear to be both more flexible and productive. With the trend anticipated in some quarters of long, dry Summers extending northwards in Europe, it will be interesting to see whether diapausing species of earthworms will become increasingly predominant in soils that geochemically favour them, with those species relying on quiescence being confined to microhabitats that are permanently moist.

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Discussion with Reviewers

D. Sigee: The authors state that there seemed to be no difference in chloragosome concentration between cryo and smeared preparations. In view of the high degree of variability, do they feel this conclusion is justified?

G. M. Roomans: As can be judged from the standard deviations, there must be a considerable variation in the elemental composition of granules within a single month. Is this mainly an inter-granule variation or an inter-animal variation?

Authors: Analytical theory indicates that there should be no difference in the measured concentrations in cryosectioned and smeared

preparations of organelles (albeit rather untypical ones!) of the dimensions of earthworm chloragosomes. However, the observed high variability in chloragosome composition is both interesting and troublesome, because it is undoubtedly genuine in that a major proportion of it is due to biological heterogeneity. The analysis of several chloragosomes within a single cell in a cryosection, where the limits of the cell can be accurately delineated, indicates that there is considerable compositional heterogeneity. Morphological studies also indicate that there is considerable structural heterogeneity within a given cell's chloragosome population. These aspects of palpable variability emphasize how little we understand of the formation, fate and function of chloragosomes. However, high variability is troublesome because it presents an analytical challenge akin to the sampling decisions that have to be made in morphometry: how many compartments per cell should be analyzed; how many cells per section; how many sections per specimen; how many tissue specimens per animal; how many animals should be sampled?

D. Sigee: Although the results demonstrate chloragosome changes in relation to diapause, there seems to be no clear evidence that these changes have nutrient implications. What critical evidence is there that chloragogenous tissue is important in nutrient storage and supply?

Authors: The critical evidence that chloragogenous tissue possesses a nutritional function is rather limited and indirect (see papers by: Semenova, 1967; Ireland and Richards, 1977). However, elegant biochemical assays by Prentø (Prentø P, 1987a - Blood sugar, sugar metabolism and related enzymes in the earthworm, *Lumbricus terrestris* L. *Comp. Biochem. Physiol.* 86B: 333-341; Prentø P, 1987b - Distribution of 20 enzymes in the midgut region of the earthworm, *Lumbricus terrestris* L., with particular emphasis on the physiological role of the chloragogenous tissue. *Comp. Biochem. Physiol.* 87B: 135-142) indicate that the tissue is a storage site for glycogen, lipids and phosphate. The tissue is probably also involved with haemoglobin synthesis (Prentø, 1987b), the scavenging of H₂O₂ by cytosolic catalase (Prentø P, 1986 - Cellular and intracellular distribution of catalase and acid phosphatase in the midgut of *Lumbricus terrestris* L.: a cell fractionation study. *Comp. Biochem. Physiol.* 83B: 385-390), and urea biosynthesis during starvation (Prentø P, 1989 - Distribution of arginase and other ornithine cycle enzymes in the gut of the earthworm *Lumbricus terrestris* L., and some physiological and comparative implications. *Comp. Biochem. Physiol.* 93B: 509-515).

R. Wroblewski: Could you correlate your results obtained from the smears and from sections from the same animal? Such values will give a measure of how reliable are the respective methods of preparation and analysis. [An alternative method of analysis of chloragocytes and chloragosomes might be the use of semi-thin and thick freeze-dried cryosections cut in the conventional cryostat. We found it possible by using this type of tissue preparation to identify chloragocytes

and single chloragosomes: Wroblewski R *et al.*, 1979 - Elemental analysis of histochemically defined cells in the earthworm *Lumbricus terrestris*. *Histochemistry* 61: 167-176).

Authors: We have not systematically attempted to determine the correlation that you suggest. The existence of large cell-to-cell, apart from large inter-granule, variations would intrude on the technical purpose of such a study. In essence, it would be difficult to ensure that the analyzed specimens were initially identical even though they were derived from the same animal.

R. Wroblewski: You mentioned that intergranule compositional variability was high and that during May dense cores were visible in the centre of chloragosomes. Could you see the same feature also in the cryosections and possibly analyse these two areas?

Authors: We have not seen dense cores or concentric substructures within any of the cryosectioned granules of *A. longa*, probably because our sections were, on the whole, rather thick. It is also conceivable that some of the substructure seen in fixed, resin sections is an artefact due to the differential extraction of materials. However we have observed dense cores and dense caps in the cryosectioned chloragosomes of the earthworm species, *Dendrodrilus rubidus*, containing Pb accumulated from a contaminated environment; the composition of these features was shown to differ (Winters and Morgan, 1988).

R. Wroblewski: How would your results look if you took into account changes in the hydration state of tissues during ND ("non-diapause") and ID ("In diapause") periods, and presented the data on a wet weight basis?

Authors: This is an intriguing question that cannot be answered at present. We need to know whether the state of hydration of tissues, and of individual cells and their compartments, changes with entry into diapause and with the progress of the resting state. In particular, it would be important to know whether water is preferentially withdrawn from the integumentary epithelial cells, so that the elevated osmotic pressure would tend to reduce losses to the dry surrounding soil.

G. M. Roomans: In mammals, zinc is an important ion in wound repair. Does this element play a similar role in the regeneration of tissue in earthworms?

Authors: Zinc has a well recognised role in the prevention of tissue lesions, and as a stimulant of tissue repair processes, in a number of mammalian systems, although the precise mechanism(s) are rather poorly understood (e.g. Arakawa T *et al.*, 1990 - Effects of zinc L-carnosine on gastric mucosal and cell damage caused by ethanol in rats. Correlation with endogenous prostaglandin E₂. *Digestive Diseases and Sciences* 35: 559-566). Earthworms have a considerable capacity to regenerate lost or damaged segments. They also normally contain high zinc concentrations in their chloragogenous tissue (Morgan, 1982). But it has not been shown that zinc plays a role in earthworm tissue regeneration, although the likelihood that it does so is high.