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Effects of Foodstuffs on Intestinal Length in Larvae of *Rhacophorus arboreus* (Anura: Rhacophoridae)

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ABSTRACT—Correlation between foodstuffs and intestinal length was examined in larvae of *Rhacophorus arboreus* (Anura: Rhacophoridae). The larva, being heterophagous, has a tube-like intestine provided with neither epithelial outfoldings nor villi, and intestinal length is found to be a good morphological index of digestive and absorptive functions of the intestine. The results obtained were summarized as follows: The grown larva fed on boiled spinach had an intestine more than 1.5 times as long as that of a grown larve fed on scrambled eggs. Change of diet from the scrambled eggs to boiled spinach triggered intestinal lengthening in the egg-fed larvae, whose intestine had hardly elongated; and in 4 days after the change of diet, intestinal length became almost equal to that of the larvae fed on boiled spinach from the beginning. The intestinal length of the larvae was proportional to the number of intestinal epithelial cells, irrespective of their diets and developmental ages before the metamorphic climax. Incorporation of [^{3}H]thymidine into DNA of intestinal epithelial cells was the highest in larvae 2 days after the change of diet, but the incorporation was low in larvae whose diet was not changed. These data suggested strongly that many intestinal epithelial cells entered the S-phase of the cell cycle after the change of diet. Labeled mitotic index of intestinal epithelial cells showed a peak at 12 hr after [^{3}H]thymidine administration in the diet-changed larvae. The intestinal lengthening after the change of diet would be brought by approximately 50% of the intestinal epithelial cells having divided once and followed by recovery of their cell size.

INTRODUCTION

It has been often cited in textbooks [1–3] that in general the intestine of plant eaters in longer than that of flesh eaters from teleosts to mammals, although there are many exceptions. In fact, recently Iwata [4] reported that *Carassius auratus grandoculis,* a carnivorous teleost, had the shortest intestine when compared with two herbivorous species, *C. auratus cuvieri* and *Hypophthalmichthys molitrix*. Altig and Kelly [5] compared the length, diameter, and other characteristics of the intestine in anuran larvae among 13 species belonging to 11 genera, and ascertained that the larvae thought to be carnivorous had a shorter and less voluminous intestine than the larvae thought to be herbivorous. Plant food, particularly cellulose, may be difficult to be digested and its products also difficult to be absorbed in vertebrates, so that intestinal lengthening, often accompanied by outfoldings and/or projections of intestinal lining, is believed, at least in part, to represent a functional adaptation to the problems of plant food digestion. However, we have few experimental findings of any correlation between feeding habits and intestinal area capable of absorption in vertebrates, except for experiments with anuran larvae. Oshima's preliminary experiment was cited in Ichikawa's textbook [6] as a personal communication showing that larvae of *Rana nigromaculata* fed on herbivorous diet had a longer intestine than the larvae fed on carnivorous one; and Janes [7] using *R. sylvatica* reported results similar to those of Oshima's experiment. In order to detect whether or not the area of intestinal epithelium actually correlates with the type of foodstuff ingested, a detailed re-examination should be carried out. The present paper deals

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with the results obtained and conclusions drawn from this re-examination.

MATERIALS AND METHODS

Experimental animal

Based on our previous experiences, we used larvae of the Japanese green frog *Rhacophorus arboreus* as an adequate experimental animal because larvae of this frog, having a smoothly lined tube-like intestine, are heterophagous; and the larvae grow not only healthily but in synchronously during the development even under various laboratory conditions. Eggs of the frog are envelopped in a foamy substance and lie scattered in a mass numbering 400–500. Egg-masses hanging on twigs above marshes were collected at Kiyotaki hill near Kyoto in June during its breeding season, and the eggs were made to hatch out in a moist chamber in the laboratory. Under natural field conditions the larvae hatch out in the mass that has become semi-fluid in about one-third of its inner part, gather together in this semi-fluid environment, and then fall into the water by tearing through the lower end of the semi-fluid portion. However, in the laboratory we obtained the hatched larvae by stirring the mass gently in tap water.

We set up every series of experiments with the larvae obtained from one egg-mass that was laid by one female, although 2–3 males were usually involved with the spawing.

Feeding and diets

Feeding of the larvae was started at Iwasawa-Kawasaki stage 36 [8], which begins 4–5 days after hatching when the larvae first exhibit food-searching behavior. The change of diet from the carnivorous to the herbivorous was made at 11–25 days after the initiation of feeding, when the larvae had grown, at room temperature (20–30°C), to stage 38. About 100 young larvae were kept in a plastic aquarium with 4.5 liter of dechlorinated tap water; when they grew up to stage 35–36, the number was reduced to about 50 in order to eliminate the so-called "crowding effect" [9–10]. The tap water of each aquarium was made fresh once a day and the larvae were allowed to feed *ad libitum*.

Frozen boiled spinach bought from a local market was thawed and chopped finely into pieces about 1–2 mm long for the herbivorous diet; and hens' whole eggs were scrambled in a pan without using any cooking oil for the carnivorous diet. Scrambled eggs containing cellulose were prepared by mixing cellulose powder (Type C 300 mesh, Toyo Roshi Co. Ltd., Tokyo) in the eggs before cooking, and cellulose contents were expressed as cellulose powder dry weight in per cent in egg (wet weight).

Morphological measurements, histological examination, and cell counting

Every experimental measurement was provided by 4–10 larvae chosen at random and the data measured were expressed by sample average with its standard deviation. The body length was calipered as the length from snout to vent. After the whole digestive tract was removed from the body cavity onto a piece of moistened filter paper, the tract was uncoiled and laid straight along a ruler by cutting the mesentery. The intestinal length from the esophagus orifice to the posterior end of rectum was measured with the ruler. The intestine of the larva was coiled into a double helix-like structure, which consisted of both descending and ascending coils with the turning point of coils lying midway between esophagus and rectum.

As soon as the measurement of intestinal length was completed, a 10 mm piece of intestine was cut out from 15 to 5 mm anterior to the turning point, which was easily distinguishable, irrespective of larval stage and feeding condition. The piece was fixed in Bouin's fluid modified by Lillie, embedded in paraplast, sectioned at a thickness of 5–8 μm, and finally stained with Ehrlich's hematoxylin and eosin for histological examination.

The remainder of the intestine was used for determination of total number of epithelial cells. The determination procedures were as follows: The intestine was put into a tube with 4 ml of ice-chilled homogenizing solution which contained 10 mM ethylenediamine tetra-acetic acid and 1% citric acid. After a drop of octyl alcohol was added to prevent foaming during homogenization, the intestine was homogenized for 15–30 sec in a

Polytron (Kinematica G.m.b.H., Luzern) equipped with a 10 mm diameter crushing shaft. The homogenate was shaken for 2–3 sec by a thermomixer (Taiyo Mixer OT-1, Taiyo Bussan Co., Tokyo). A 0.5 ml aliquot of the homogenate was mixed with the same volume of staining solution (0.1% crystal violet in 0.1 M citric acid) and shaken well again by the thermomixer, after which a drop of the mixture was mounted on a Thoma's hemocytometer (Erma Optical Works Ltd., Tokyo) for cell counting. The nucleus of the epithelial cell, being the major one in the intestinal cell preparations, was easily distinguished by its elliptic shape from other nuclei, such as the elongated nucleus of smooth muscle cells, small and condensed nuclei of connective tissue cells, and the round flat nucleus of the red blood cells. Counting of epithelial cell number was carried out on 2 aliquots of the homogenate prepared from each of the larvae. The total number of epithelial cells in the whole intestine (A) was estimated by the following formula:

$$A = 4 \times 10^4 \times a \times b \times 1/(b\text{-}10)$$

where a is the average number of epithelial cells contained in 10^{-4} ml and b is the intestinal length expressed in mm. The term $1/(b\text{-}10)$ is included to compensate for the length of intestine (10 mm) removed for histological examination; and the factor 4×10^4, to arrive at the number of epithelial cells in the total volume of homogenate (4 ml).

Tritiated thymidine administration, radioactivity measurement, and autoradiography

In order to elucidate intestinal cell proliferation, we carried out cell kinetics studies using [^{3}H]thymidine as a DNA precursor. After the larvae were anesthetized by immersion in a 0.05% aqueous solution of tricaine (ethyl m-aminobenzoate methanesulfonic acid), each of them was administered 1 μl of physiological saline solution containing 0.5 μCi of [methyl-^{3}H]thymidine (sp. act. 10.9 Ci/mM, Radiochemical Centre, Amersham, Buckinghamshire) by intraperitoneal injection through tail muscles using a micro-syringe (Hamilton microliter syringe 710) equipped with a repeating dispenser (Hamilton Pb600-1, Hamilton Co., Reno, Nevada).

We removed intestines from the larvae administered [methyl-^{3}H]thymidine (abbreviated [^{3}H]Tdr hereafter) after the lapse of the desired time. The intestine removed was measured for its total length, and one piece 10 mm in length was extirpated from near the turning point for autoradiography, as was described above. The remaining intestine was used for determination of both epithelial cell number and [^{3}H]Tdr incorporation into DNA of the cell nucleus.

[^{3}H]Tdr incorporation was expressed as cpm of tritium per 10^6 epithelial cells, and the radioactivity was measured by the filter disk method as follows: A 100-μl aliquot of the cell homogenate was dripped by a micropipet (Gilson Pipetman P-200) onto a filter disk (Whatman 3MM) 24 mm in diameter. The filter disks were air-dried, treated sequentially in a beaker with ice-chilled 10% and 5% TCA for 30 min each, washed at room temperature in absolute ethanol 2–3 times for about 20 min in total, and finally rinsed in ethyl ether for 5 min. After the ethyl ether had completely evaporated, the filter disk was placed in a vial with 2.5 ml of scintillation mixture (PCS: Amersham Japan, Tokyo), and the radioactivity was measured in a liquid scintillation counter (Beckman LS8000, Beckman Japan, Tokyo). Two filter disks were prepared for radioactivity measurement from each cell homogenate.

For autoradiography, we used the dipping method to determine labeled mitotic index of intestinal epithelial cells in larvae administered [^{3}H]Tdr. Paraplast sections of the intestine, 5 μm thick, prepared as mentioned above, were deparaplasted, hydrated, and finally dipped into autoradiographic emulsion, Sakura NR-M2 (Konika, the former Konishiroku Photo Industry, Tokyo). Being exposed for 3 weeks below 5°C, the emulsion was developed with Kodak D19b developer. A diluted Ehrlich's hematoxylin or Mayer's carmalum staining solution was applied for cytological examination after photographic development.

RESULTS AND DISCUSSION

Larval development

Larvae of *Rhacophorus arboreus* obtained from one egg-mass were divided into two groups. The

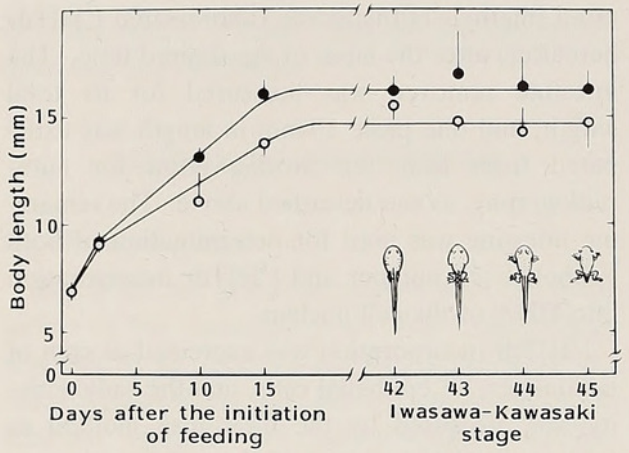


FIG. 1. Time courses of body lengthening in herbivorous (●) and carnivorous larvae (○). Bars show standard deviation.

larvae of one group were fed on the boiled spinach (herbivorous larvae, for short, hereafter); and those of the other group, the scrambled eggs (carnivorous larvae, hereafter). Figure 1 shows the change in body length with development in both herbivorous and carnivorous larvae. The trunk elongated in both larvae for about 2 weeks after the initiation of feeding, then hardly underwent further elongation. Both larvae grew similarly and metamorphosed into froglets in the laboratory, as would be expected in the field. It was convenient to show larval ages by days after hatching or after the initiation of feeding in the early larval phase. However, the expression by Iwasawa-Kawasaki stage [8] was convenient in the late larval phase, because the larvae became less synchronized in their development after the stage 42 (metamorphic climax). From these preliminary surveys, we analyzed diet effects on the intestinal epithelium.

Intestine and diets

Figure 2 shows the change in intestinal length with development in both herbivorous and carnivorous larvae. Three phases were distinguished in the curves of Figure 2: the lengthening phase, in which the intestine elongated rapidly for about 2 weeks after the initiation of feeding; the plateau phase, in which the intestine practically ceased to elongate and which followed the lengthening phase and lasted for more than 2 weeks; and the shorten-

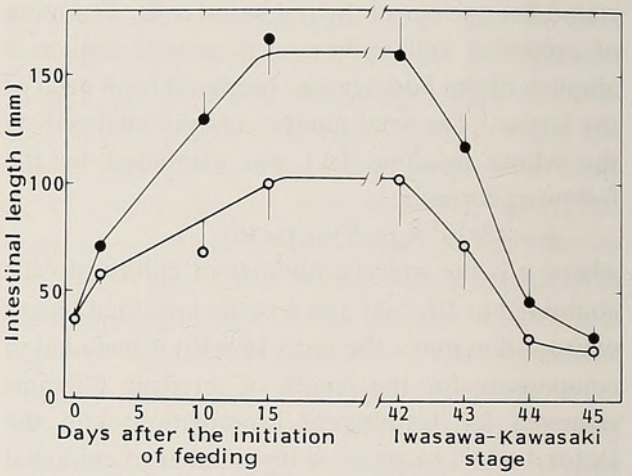


FIG. 2. Time course of intestinal lengthening in herbivorous (●) and carnivorous larvae (○). Bars show standard deviation.

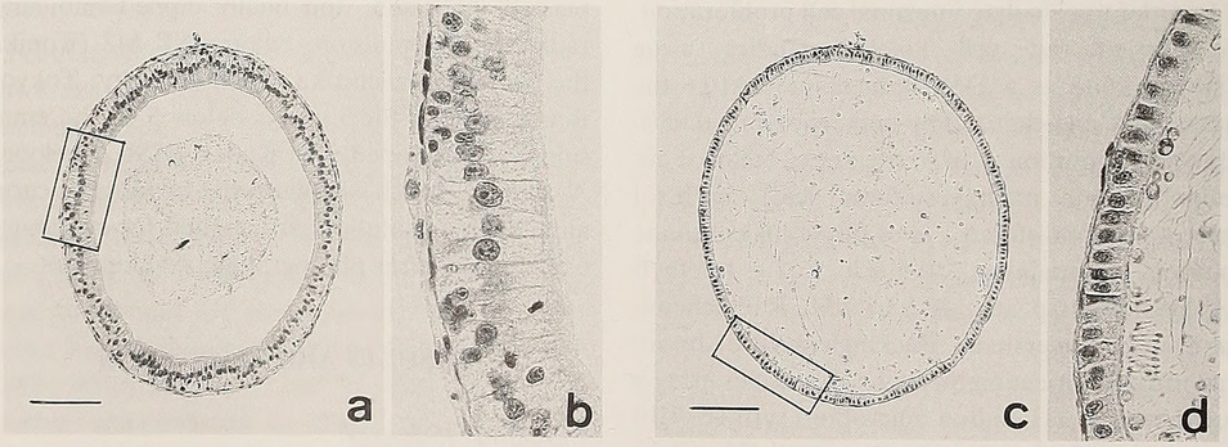


FIG. 3. Transverse-section of intestine. a: a carnivorous larva 20 days after the initiation of feeding. Scale bar represents 100 μm. b: High-power view of the area outlined in a. c: a herbivorous larva 20 days after the initiation of feeding. Scale bar represents 100 μm. d: High-power view of the area outlined in c.

ing phase, in which the intestine shortened and which began from the metamorphic climax. The herbivorous larvae had a longer intestine than the carnivorous larvae, and the average intestinal length of the former larvae reached a value more than 1.5 times greater as that of the latter larvae at the plateau phase.

As depicted in Figure 3, no significant difference in diameter of intestine was found between herbivorous and carnivorous larvae at the plateau phase. In both larvae the intestine was tube-like in the one cell-thick epithelium and having neither villi nor outfoldings of intestinal lining were observed. The epithelium was composed of columnar cells in carnivorous larvae (Fig. 3a and b), but cuboidal cells in herbivorous ones (Fig. 3c and d); although the reason for this difference of cell type was unsolved. A considerable amount of indigestible material remained in the feces of herbivorous larvae, while few undigested remnants were found in feces of carnivorous ones. We concluded that boiled spinach was more difficult to be digested than the scrambled eggs and that cellulose may be the main indigestible substance in the herbivorous diet.

As intestinal epithelium is the most responsible tissue for digestion and absorption of the food, the total number of epithelial cells in it may be regarded as an index of these functions. Figure 4 shows how the total epithelial cell number of intestine changed with development in both herbivorous and carnivorous larvae. These intestines were the same ones measured for Figure 2. The patterns of curves for herbivorous larvae depicted in Figures 2 and 4 were similar to each other.

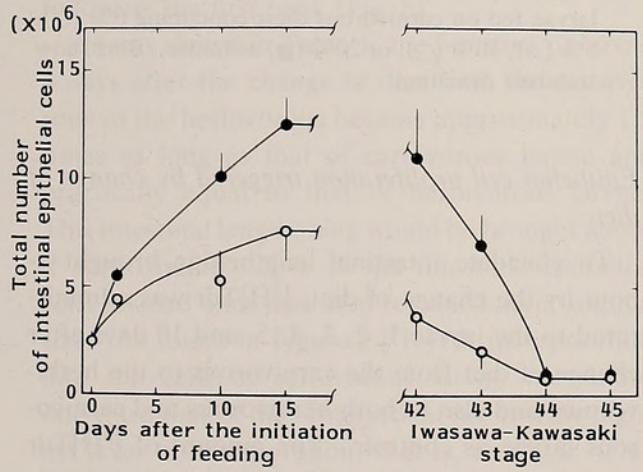


FIG. 4. Time courses of total number of intestinal epithelial cells in herbivorous (●) and carnivorous larvae (○). Bars show standard deviation.

As shown in Figure 5, in both herbivorous and carnivorous larvae, the ratio of epithelial cell number to length of intestine, increasing very slowly for several days after the initiation of feeding, became practically constant at least for about 2 weeks (constant phase), and then increased drastically after the metamorphic climax. Before the metamorphic climax, the intestine of the larvae was tube-like with a uniform diameter; and, accordingly, an increase in this ratio was regarded as a decrease in cell size. However, after outfoldings of epithelial lining began to develop, accompanied with other intestinal changes that occur during the transformation from larva to adult, an increase in this ratio could be regarded as a decrease in the cell size and/or an increase in the cell number. Thus, we carried out diet-change experiments with the larvae in this constant phase in which intestinal lengthening was ascribed to an increase in epithelial cell number.

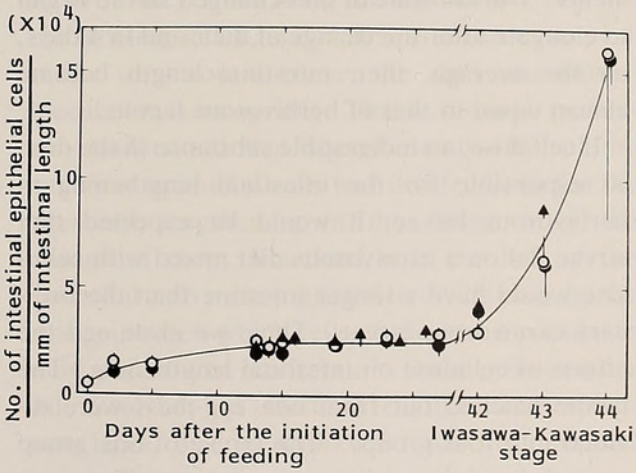
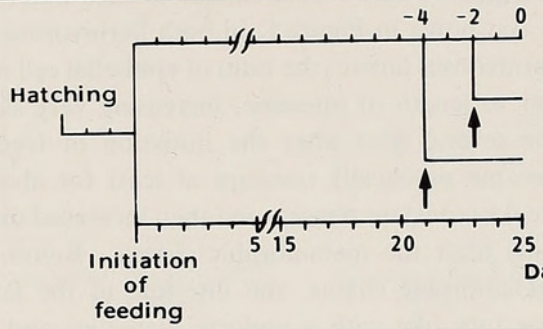


FIG. 5. Time courses of intestinal epithelial cell number per unit length of intestine in herbivorous (●), carnivorous (○) and diet-changed larvae (▲). Bars show standard deviation.

Intestinal lengthening by change of diet

The larvae obtained from one egg-mass were divided into three groups. Larvae of the first group were fed on the herbivorous diet; and larvae of the second group, on the carnivorous diet. But larvae

TABLE 1. comparison of intestinal length in larvae under various feeding conditions

Experimental schedules of feeding	Larva	Intestinal length (mm) at day 25
	Carnivorous larvae	58.5 ± 14.3
	Diet-changed larvae	75.8 ± 14.7
	Diet-changed larvae	91.0 ± 11.3
	Herbivorous larvae	93.8 ± 4.3

Arrows show change of diet from the carnivorous to the herbivorous.

of the third group were fed on the carnivorous diet for 12 days at least until they had grown to the constant phase shown in Figure 5, and then the diet was changed from the carnivorous to the herbivorous (diet-changed larvae, for short, hereafter). In Table 1, data are given of intestinal length in larvae obtained from a series of experiments. The intestine of diet-changed larvae began to elongate after the change of diet; and in 4 days, on the average, their intestinal length became almost equal to that of herbivorous larvae.

If cellulose, an indigestible substance in the diet, is responsible for the intestinal lengthening in herbivorous larvae, it would be expected that larvae fed on a carnivorous diet mixed with cellulose would have a longer intestine than the ordinary carnivorous larvae. Thus, we examined the effects of cellulose on intestinal lengthening. The larvae hatched out from one egg-mass were divided into four groups. The larvae of one group were fed on the ordinary carnivorous diet, i.e., scrambled eggs only; while the larvae of the other three groups were fed on scrambled eggs containing 5%, 10%, and 20% cellulose, respectively. Results shown in Figure 6 were as was to be expected. The larvae fed on the ordinary carnivorous diet had the shortest intestine; and the intestines were longer with the amount of cellulose in their diets, although the ratio was not directly proportional.

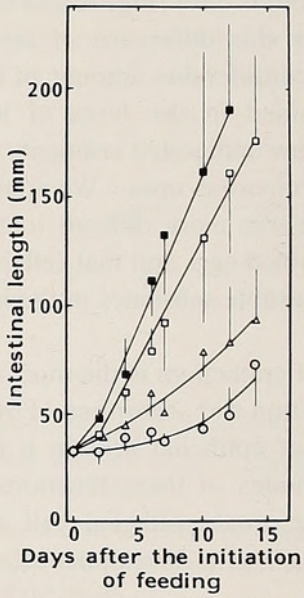


FIG. 6. Comparison of intestinal lengthening among the larvae fed on carnivorous diets containing 0% (○), 5% (△), 10% (□), or 20% (■) cellulose. Bars show standard deviation.

Epithelial cell proliferation triggered by change of diet

To elucidate intestinal lengthening brought about by the change of diet, [^{3}H]Tdr was administered to the larvae 1, 2, 3, 4, 5, and 10 days after change of diet from the carnivorous to the herbivorous, and also to both herbivorous and carnivorous larvae as controls. The amount of [^{3}H]Tdr incorporated into DNA of intestinal epithelial cells 1 hr after administration was the highest in the larvae 2 days after the change of diet (Fig. 7),

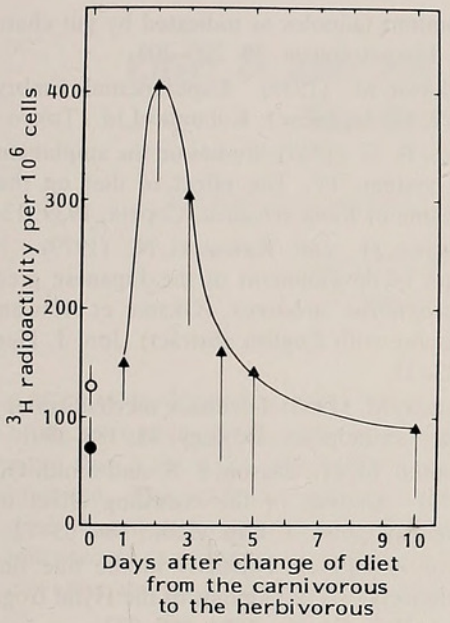


FIG. 7. [^{3}H]Tdr incorporation into DNA of intestinal epithelial cells 1 hr after administration in herbivorous (●), carnivorous (○), and diet-changed larvae (▲). Bars show standard deviation.

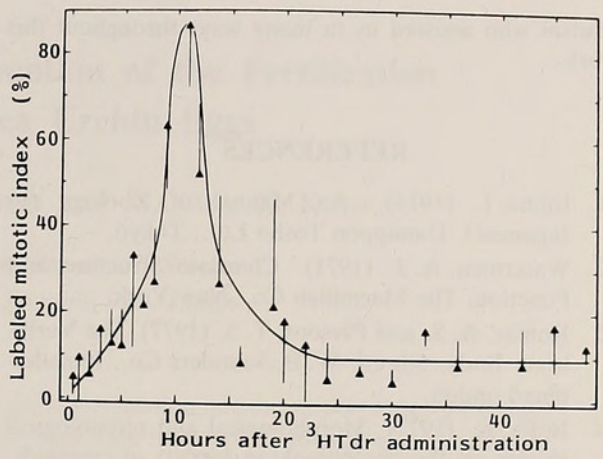


FIG. 8. Time course of labeled mitotic index of intestinal epithelial cells in the larvae injected with [^{3}H]Tdr 48 hr after change of diet from the carnivorous to the herbivorous. Bars show standard deviation.

apparently indicating that the number of cells entering the S-phase of the cell cycle was the highest in the intestine 2 days after change of diet from the carnivorous to the herbivorous.

Next, the labeled mitotic index was determined for the intestinal epithelial cells in larvae that had been administered [^{3}H]Tdr at 48 hr after the change of diet. A peak of labeled mitotic index was seen at about 12 hr after [^{3}H]Tdr administration, and no second peak was found for 36 hr following the first peak (Fig. 8).

As was shown in Table 1, the intestine of larvae 4 days after the change of diet from the carnivorous to the herbivorous became approximately 1.5 times as long as that of carnivorous larvae and practically equal to that of herbivorous larvae. This intestinal lengthening would be brought about if approximately 50% of the intestinal epithelial cells divided once and then regained their original size. As found in Figure 7 [^{3}H]Tdr incorporation into the cells of carnivorous larvae was about one-third that of diet-changed larvae at their highest level of incorporation, that is, at day 2. This may mean that the labeling index of intestinal epithelial cells in the diet-changed larvae at day 2 was about 3 times as high as that of carnivorous larvae. The labeling index of the latter larvae measured about 7%, so 20% may be reasonably regarded as the estimated value of the labeling index for the former larvae. If the total number of intestinal epithelial cells which multiplied during the 4 days after the change of diet is expressed as a term of integration along the time course curve of the labeling index, and if 20% is taken as its peak in the time course curve, the above-mentioned speculation is acceptable. That is, it can be reasonably understood that approximately 50% of the intestinal epithelial cells had divided once for the intestinal lengthening in the larvae after the change of diet. If indeed only the half of the epithelial cells were triggered to divide once by the change of diet, which cells of the entire populaion were destined to proliferate? These problems are still unsolved, but intestinal length of the herbivorous larva, that is, about 1.5 times the intestinal length of the carnivorous larva, may be the maximal length capable of being held within the abdominal cavity of the diet-changed larva.

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