

# A photographic technique to evaluate the consumption of food reserves in individual starved second stage juveniles of *Meloidogyne javanica*

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## SUMMARY

Individual nematodes, 1, 8, and 15-days-old, were fixed, stained and photographed. Amounts of silver fixed on the prints were determined by atomic absorption. Results compared favorably with colorimetric determination of lipids, carbohydrates, proteins and dry weight utilizing  $\pm 30,000$  individuals. This technique may be useful to measure depletion of metabolic reserves in small organisms in which large quantities of specimens are difficult to obtain.

## RÉSUMÉ

*Évaluation individuelle de la consommation des réserves nutritives chez les juvéniles du second stade de Meloidogyne javanica par une technique photographique.*

Des nématodes âgés de 1, 8 et 15 jours sont fixés, colorés et photographiés individuellement. Sur des épreuves agrandies, la quantité d'argent fixée est dissoute chimiquement puis dosée par absorption atomique. Les résultats correspondent bien aux mesures du poids sec et aux dosages colorimétriques de lipides, glucides et protéines, réalisés sur des échantillons d'environ 30.000 individus. Cette technique pourrait s'appliquer à l'évaluation de la consommation des réserves métabolisables chez de petits organismes pour lesquels les quantités d'individus du même stade, nécessaires aux dosages colorimétriques, ne peuvent être obtenues.

Many free-living nematodes must withstand more or less prolonged periods of starvation during which endogenous food reserves, mainly lipids, are consumed (Cooper & Van Gundy, 1971). Chemical techniques for quantitative determination of such reserves often require several thousands of individuals per determination (Reversat, 1976). Consumption of food reserves in a few individuals has been estimated using camera lucida drawings of nematodes treated with a lipid staining compound (osmium tetroxide) after which all the stained portions were cut out and weighed (Van Gundy, Bird & Wallace, 1967). In nematodes of increasing age there was a progressive reduction in stained areas which was attributed to the consumption of endogenous lipid reserves. The photographic

technique described in this paper avoids individual errors in drawing and cutting and provides a more precise analysis of density of the stained area. On a photographic print of a stained nematode, opaqueness of stained portions is represented by the darkening of the paper, which corresponds to immobilization of metallic silver. Thus, opaqueness can be measured by determining the quantity of fixed silver in the photographic image.

To demonstrate this principle infective juveniles of *Meloidogyne javanica* were chosen because they can be obtained in large quantities required for chemical determination and because a relationship has been demonstrated between their infectivity and the amount of their food reserves (Van Gundy, Bird & Wallace, 1967).

## Materials and methods

### JUVENILES

The juveniles used were from a single egg-mass population of *M. javanica*, maintained on the roots of kenaf (*Hibiscus cannabinus*). Ten seeds of kenaf were sowed in 2.5 liters plastic pots, containing steam sterilized sandy soil, and watered daily. After four weeks, each pot was inoculated with 20,000 freshly hatched juveniles, then pots were kept in a greenhouse with an average temperature of 26-28°. Five weeks after inoculation, infected roots were washed to remove soil and debris and placed over a funnel collector in a misting chamber, where juveniles hatched from egg-masses. Nematodes obtained during the first 24 hours were discarded and nematodes for experiment were collected 24 hours later. Nematodes were placed on four layers of tissue paper which remove debris and inactive nematodes and allowed the active ones to migrate through. One day later clean and active nematodes were collected as one day old juveniles. Juveniles were stored in Petri dishes, in phosphate buffer pH 7 at a concentration of 4 mM, at 28°, in the dark. After one and two weeks of storage active juveniles were selected by the tissue paper method described above. At the time 1, 8 and 15 days the sample of active juveniles was divided in two parts: the first of several hundred individuals for the photographic method, the second of several hundred thousand individuals for chemical determinations.

### PHOTOGRAPHIC METHOD

Juveniles were fixed (Netscher & Seinhorst, 1969) and then stored in 4 % formalin for observation. Fixed juveniles were transferred to a lipid staining solution, Sudan Black (Merck ref. 1387) saturated in 60 % ethanol (Gabe, 1968) for 72 hours at 28°. The stained nematodes were mounted in glycerine on microslides (Seinhorst, 1959). Ten juveniles of each age were photographed individually with a Leitz Orthoplan microscope equipped with an automatic Orthomat camera and Kodak Panatomic X film of 16 Din sensibility. Three prints on paper Kodabrom G5, (X600) were made of each of

the juveniles with the same exposure and development times and at the same temperature. Each of the 90 images was cut into pieces approximately 1 cm long and placed in a 250 ml erlenmeyer flask containing 25 ml of 6.5 % nitric acid. Flasks were heated until the disappearance of the image by chemical dissolution of the silver. Each of the 90 solutions was adjusted to 250 ml by weighing with addition of distilled water, filtered, and measured for silver content using an Atomic Absorption Spectrophotometer (Instrumentation Laboratory Type IL 151) and reference solutions of silver nitrate in 0.65 % nitric acid.

### CHEMICAL DETERMINATIONS

At each harvest time, juveniles were divided into eight equivalent samples of  $\pm 30,000$  individuals (Reversat, 1980). For dry weight determinations juveniles were transferred with a very small amount of water in preweighed aluminium foil cups. After two hours of desiccation at 110° cups were weighed again with an accuracy of  $\pm 0,005$  mg. For chemical determinations, juveniles were first digested in an appropriate chemical (Reversat, 1976) and then colorimetric reactions were used: with the anthrone reagent for total carbohydrates (Seifter *et al.*, 1950), with the sulfophosphovanillic reagent for total lipids (Chabrol & Charonnat, 1937) and with the Folin phenol reagent for total proteins (Lowry *et al.*, 1951).

## Results

The progressive clearing of the stained nematodes with age, observed on prints (Fig. 1), is correlated with a decrease of the silver content of the nematodes prints (Tabl. 1). There was a significant difference at the 0.1 % level for the quantities of silver measured between means of juveniles 1 and 8 days old and between means of juveniles 8 and 15 days old.

Two facts are noted with respect to the reliability of the method. First, statistical study within the results of each age group (Tab. 1) showed that variability between the three replicate prints of the same individual was very slight compared to the variability between the

Table 1

Evaluation of food reserve consumption by *Meloidogyne javanica* juveniles during *in vitro* storage by silver method and by chemical determination. Dates are expressed as means  $\pm$  standard deviation. Silver method : results in  $10^{-6}$  g of silver per print. Ten juveniles for each of the three ages and three prints for each juvenile. Chemical determination : results in  $10^{-9}$  g of substance per juveniles. For each age, two replications for each of the four determinations and  $\pm$  30,000 juveniles for each replication.

Determination	1 day	8 days	15 days
Silver method juv n° 1	847 $\pm$ 14	452 $\pm$ 14	282 $\pm$ 8
juv n° 2	860 $\pm$ 28	626 $\pm$ 34	307 $\pm$ 2
juv n° 3	904 $\pm$ 18	538 $\pm$ 55	283 $\pm$ 7
juv n° 4	741 $\pm$ 52	498 $\pm$ 45	368 $\pm$ 11
juv n° 5	740 $\pm$ 50	308 $\pm$ 20	359 $\pm$ 29
juv n° 6	876 $\pm$ 34	581 $\pm$ 16	169 $\pm$ 12
juv n° 7	856 $\pm$ 76	524 $\pm$ 9	132 $\pm$ 15
juv n° 8	977 $\pm$ 35	473 $\pm$ 30	244 $\pm$ 12
juv n° 9	802 $\pm$ 18	572 $\pm$ 20	160 $\pm$ 9
juv n° 10	669 $\pm$ 49	553 $\pm$ 26	134 $\pm$ 32
mean $\pm$ SD	827 $\pm$ 90	512 $\pm$ 89	244 $\pm$ 90
Chemical determination			
Dry weight	23.9 $\pm$ 0.2	16.7 $\pm$ 0.6	14.3 $\pm$ 0.2
Total carbohydrates	1.7 $\pm$ 0.2	1.5 $\pm$ 0	1.1 $\pm$ 0
Total lipids	9.2 $\pm$ 0.2	3.4 $\pm$ 0.2	1.4 $\pm$ 0.2
Total proteins	9.0 $\pm$ 0.2	7.3 $\pm$ 0.4	7.5 $\pm$ 0.3

Analysis of variance for silver method :

Within each age : F = 15 for 1 day, F = 22.5 for 8 days, F = 79 for 15 days with a F limit of 5.24 at the 1 % level.

Between ages : t = 7.6 between 1 and 8 days, t = 6.7 between 8 and 15 days with a t limit of 3.92 at the 1 % level.

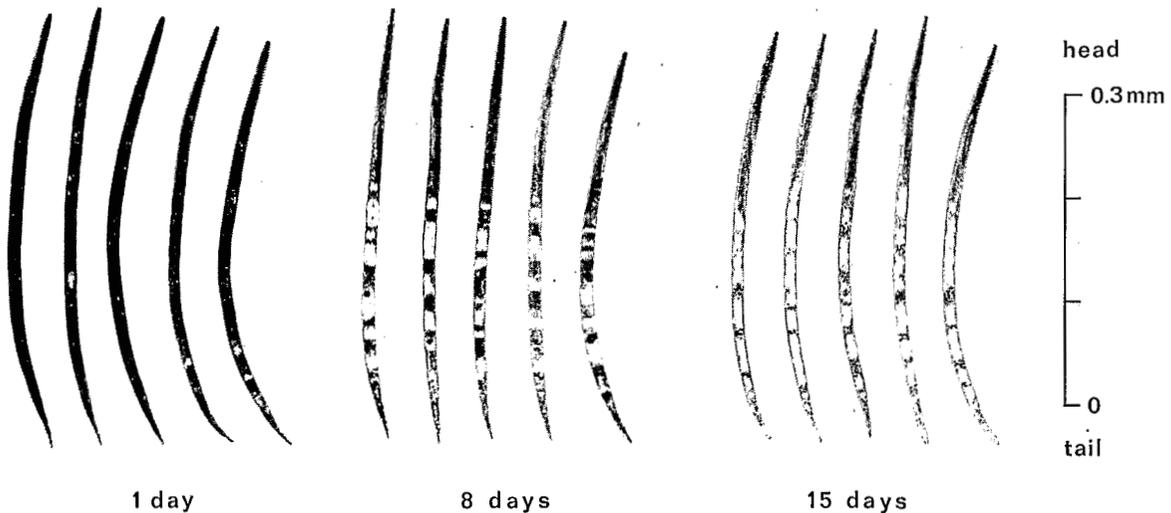


Fig. 1 : Photographic prints of stained *Meloidogyne javanica* juveniles of three different ages. (Only five of the ten juveniles used in the study, are represented. Note reduction of the overall black area with increasing age, i.e. fixed silver on the photographic paper).

ten individuals of the same age. Moreover, the increasing value of F with age demonstrated that the rate of the food reserves consumption varied widely between individuals, as had been previously observed (Van Gundy, Bird & Wallace, 1967). Secondly, the photographic image of a one-day-old juvenile is saturated to only 1/4 of the maximum saturation of silver content. As a matter of fact solar exposed and developed photographic paper Kodabrom G5 exhibit a metallic silver content of  $156 \gamma/\text{cm}^2$  (100 % of saturation). The corresponding value for the one-day-old juvenile image was  $37.6 \gamma/\text{cm}^2$  (24 % of saturation), for the background of the prints  $1.6 \gamma/\text{cm}^2$  (1 % of saturation) and for unexposed and developed paper  $1.2 \gamma/\text{cm}^2$  (0.8 % of saturation). Therefore, although one-day-old juveniles appear to be very opaque (Fig. 1), it is possible to detect individual variations (Tab. 1).

Chemical determinations confirmed that lipids are the main food reserves consumed during starvation, and carbohydrates and proteins were less consumed (Tab. 1).

## Discussion

In *Caenorhabditis briggsae*, ageing is characterized by the progressive development of dark colored granules of lipofuscine, called age pigment, and located in the intestinal epithelium (Epstein, Himmelhoch & Gershon, 1972). On a photographic print, this darkening could compensate the progressive disappearance of stained food reserves. If such age pigment was present in the intestine of *M. javanica* juveniles, its contribution to the opaqueness was very reduced, since unstained areas in 8 and 15 days old juveniles, located in the gut region, were very similar to the background of the prints (Fig. 1).

When plotted as percentage of initial values on the same graph, dry weight, lipids and silver content of *M. javanica* juveniles decreased with related rates (Fig. 2). In conclusion, it appears possible to quantify in a reproducible manner nematode opaqueness by silver determination (Tab. 1) and that there is a direct relationship between the nematode opaqueness and its reserve content (Fig. 2). Thus, this photographic

technique can actually evaluate consumption of food reserves.

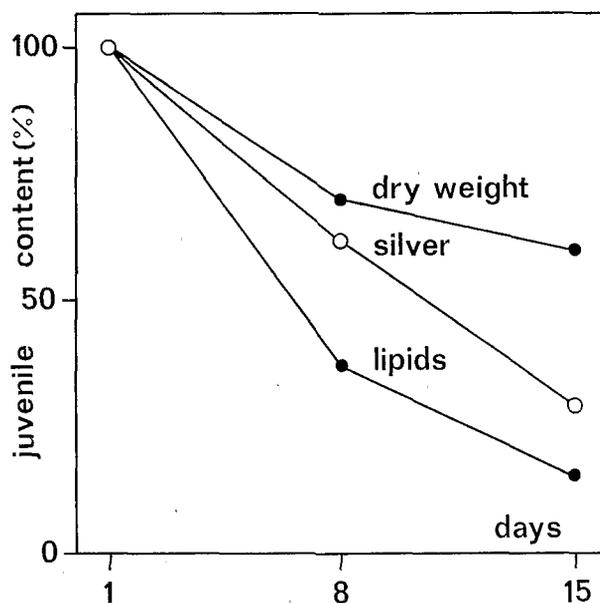


Fig. 2. Effect of storage time on *Meloidogyne javanica* juveniles : decrease of dry weight, silver content of photographic prints and lipid content as percentage of initial values.

Since this technique requires only ten individuals of *Meloidogyne javanica* juveniles whereas chemical determinations are made upon samples of 30,000, it could be applied to the study of nematode species in which large numbers of individuals of the same stage are difficult to obtain. Further, we suggest that this technique can be applied to the study of other small Invertebrata exhibiting, as nematodes, soft and transparent integuments, as rotifers and enchytraeids; application to others groups, as tardigrades, collembola and acarid, would probably require particular adaptation due to the thickness of their integument.

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