Effect of in vitro storage time on the physiology of second stage juveniles of *Heterodera oryzae*

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SUMMARY

Populations of freshly hatched juveniles of *Heterodera oryzae* were stored in the dark at 28° in aerated aqueous medium. At the zero time and then every week, samples of motile juveniles were selected from populations for measurement of certain physiological parameters.

The percentage of motile juveniles decreased steadily with time and reached zero after 6 weeks of storage. At the zero time, 52% of motile juveniles were infective ; this rate increased slightly to 62% after one week and then dropped rapidly to 1.6% after 5 weeks of storage. Rate of O_2 consumption was initially 58 pl per individual per hour and decreased to 26 pl/ind./hr at the end of 4 weeks.

Freshly hatched juveniles, with a dry weight of 31 ng per individual, contained 8.5 ng of total lipid, 3.9 ng of total carbohydrates, 13.5 ng of total protein and 2.5 ng of total nitrogen. Lipid content decreased regularly during storage to 2.4 ng per juvenile, a 70% loss, after 5 weeks. Carbohydrate content increased significantly from 3.9 ng to 4,4 ng per individual during the first week of storage, remained constant until the third week and then decreased to 2,9 ng per individual at the end of 5 weeks. Protein and nitrogen contents remained constant until the third week of storage and then decreased to respectively 11.3 ng and 2.2 ng per individual.

RÉSUMÉ

Effet de la durée du stockage in vitro *sur la physiologie des juvéniles d'*Heterodera oryzae

Des populations de juvéniles fraîchement éclos d'*Heterodera oryzae* sont placées à l'obscurité à 28° dans un milieu aqueux aéré. Au temps zéro puis à des intervalles de temps d'une semaine, des échantillons de juvéniles actifs sont sélectionnés à partir de ces populations pour la mesure de certains paramètres physiologiques.

La proportion des juvéniles actifs décroit régulièrement avec le temps et devient nulle à 6 semaines. Au temps zéro, 52% des juvéniles sont capables de pénétrer dans les racines de l'hôte ; cette proportion croît jusqu'à 62% après une semaine de stockage puis décroît rapidement jusqu'à 1,6% à 5 semaines. La respiration, dont la valeur initiale est de 58 pl O_2/juvénile/heure décroît avec l'âge pour atteindre 26 pl/juv./h. à 4 semaines.

Pour un poids sec individuel de 31 ng, les juvéniles fraîchement éclos contiennent 8,5 ng de lipides, 3.9 ng de glucides, 13,5 ng de protéines et 2,5 ng d'azote total. La quantité de lipides décroît régulièrement et atteint 2,4 ng à 5 semaines (perte totale de 70%). La quantité de glucides augmente au cours de la première semaine de stockage puis diminue à partir de la troisième semaine. Les quantités de protéines et d'azote total restent constantes jusqu'à la troisième semaine puis décroissent.

Infective forms of plant parasitic nematodes do not feed until they invade the host plant. Thus in the absence of the host, they undergo both ageing and starvation. Effects of these stresses on behaviour and metabolism have been reviewed by Van Gundy (1965) and Cooper and Van Gundy (1971).

Behavioural effects of ageing and starvation on nematodes have received more attention, probably because of their practical implications.

Studies were concerned with survival and with the progressive disappearance of motility and infectivity with age. Some important investigations include Dropkin (1957), Bergeson (1959), Golden and Shafer (1960), Thomason, Van Gundy and Kirkpatrick (1964), Merny (1966, 1972), Van Gundy, Bird and Wallace (1967), Slack, Riggs and Hamblen (1972), Davies and Fisher (1976) and Ogunfowora (1979).

Age related changes of O_2 consumption were studied by Rohde (1960), Sembdner, Osske and Schreiber (1961), Wallace and Greet (1964), Van Gundy, Bird and Wallace (1967) and Bhatt and Rohde (1970). Age related changes with body content have been demonstrated by Chitwood (1951), Wallace (1966), Van Gundy, Bird and Wallace (1967), Davies and Fisher (1976) and Ogunfowora (1979).

It appears that the most extensive study, conducted with the same biological material was that of Van Gundy, Bird and Wallace (1967) on *Meloidogyne javanica* juveniles.

The present study measured the relationship of motility, infectivity, O_2 consumption, dry weight and body content as lipids, carbohydrates, proteins and nitrogen, to age of *Heterodera oryzae* juveniles. This species parasitizes roots of inundated rice in Ivory Coast (Luc & Berdon, 1961) and Senegal (Fortuner & Merny, 1973) and roots of banana in Senegal (Taylor, 1978). Merny (1972) studied the effect of storage of second-stage juveniles of *H. oryzae* in wet soil on their subsequent ability to establish an infection of females on rice plants.

Materials and methods

Juveniles

Populations of *H. oryzae* were developed on rice cv. Morobérékan. Pots containing 2 liters of steam sterilized sandy soil, in which at least six plants were planted, were inoculated with about 2,000 freshly hatched juveniles and then grown in a greenhouse. Experiments on motility, infectivity and respiration were carried out in the ORSTOM laboratory of Adiopodoumé (Ivory Coast) in 1974, with a strain of *H. oryzae* which has been cultured since 1961 and egg-masses were collected five weeks after inoculation (first generation). Experiments on chemical composition were completed in the ORSTOM laboratory of Dakar, with a second strain (topotype) of *H. oryzae* maintained since 1976. In this case the large number of juveniles which were required made it necessary to collect the egg-masses after a second generation had been completed (11 weeks). Roots were harvested from the growing soil by gentle washing and were then exposed to a high pressure water spray using a 0.16 mm sieve to retain egg-masses, cysts and some root fragments. These materials were transferred to 0.3 M NaCl which allowed development to second stage juveniles but prevented hatch (Reversat, 1975*a*). After 2 weeks in 0.3 M NaCl the materials were transferred to deionized water for 3 days. The hatched juveniles were collected and allowed to migrate through four layers of tissue paper (Kleenex ®) in a modified Baerman extraction procedure (Merny & Luc, 1969). Inactive juveniles did not pass through this filter and motile juveniles collected at the end of 24 hours were considered at the zero time of the experiment whereas their actual mean age was 2.5 ± 1.5 days. In each experiment, all juveniles were submitted to this selection the day before 1, 2, 3, 4, 5 and 6 weeks and only the motile juveniles were used for experimentation. For experiments on motility, infectivity and respiration, juveniles were placed in Petri dishes in aliquots of up to 50,000 juveniles/25 ml of deionized water. For experiments on chemical composition, juveniles were placed in 1 liter Roux bottles in aliquots of 100 ml each with a maximum of 800,000 juveniles suspended in pH 7Na phosphate buffer 4 mM. Petri dishes and Roux bottles were stored in the dark at 28 °C.

Motility and infectivity

The motility vs time curve was determined on four fractions of the population, treated separately and with an initial population number of 8,000 juveniles. At the end of the 24 hours period of selection for motility, the sieve was placed again in fresh water and only a few juveniles were recovered one week later.

Juveniles were individually selected under the binocular with a micropipette and 100 motile juveniles were placed around the roots of five one-week-old rice seedlings growing in a glass test tube (18 × 180 mm) in sand and a mineral nutritive solution (Yoshida, Ohnishi & Kitagishi, 1959). The particle size of sand was less than 0.25 mm in order to maximize the invasion of roots by *H. oryzae* juveniles (Reversat & Merny, 1973). The roots were harvested at the end of 9 days, washed, fixed in boiling lactophenol and colored in cold cotton blue lactophenol (de Guiran, 1966). Later the roots were pressed firmly between two glass plates and the colored juveniles were counted. Twenty replications were made for each of the storage times : 0, 1, 2, 3, 4 and 5 weeks.

Dry weight determinations

A first determination was performed on the same population as that used for respiration measurements. A suspension of 30,000 juveniles in 1 liter of deionized water was mixed homogeneously by bubbling air. Its nematode content was determined by counting three subsamples of 5 ml which were eventually returned to the suspension. The juveniles were subsequently allowed to settle at 4° for 14 hours and the 900 ml of supernatant were put aside. The remaining 100 ml were filtered on a 8 µm dried and weighed Millipore ® filter. Nematodes and filter were dried over silica gel at ambient temperature (24°) and weighed to 0.01 mg. The remaining juveniles in supernatant and rinsings were counted to obtain the weight of individual nematodes. Each storage time was replicated twice.

A second determination was made on the same population as that used for chemical composition. Six equivalent numbered samples of juveniles were rinsed twice in bidistilled water using centrifugation to separate out the nematodes. The nematodes were killed by plunging the centrifuge tubes into boiling water and the pellet of nematodes from further centrifugation was transferred to a pre-weighed aluminium foil cup and nematodes in rinsings were counted. The nematodes were dried over silica gel under reduced pressure for 3 days and the cup was reweighed on a quartz fibre microbalance to 0.005 mg. This procedure was repeated after 0, 1, 2, 3, 4 and 5 weeks of storage.

Respiration

The rate of O_2 consumption was determined by the cartesian diver technique (Reversat, 1975*b*). Ten replications, i.e. divers, were charged with 80 surface sterilized juveniles (Reversat, 1975*c*) for each of the storage times : 0, 1, 2, 3 and 4 weeks. At five weeks of storage, the juveniles would not settle in the diver normally and further experiments were technically infeasible. Measurements were made over a 20 hours period and results are expressed as picoliters of O_2 per juvenile per hour (pl/juv./hr.).

Chemical composition

Reproducible aliquots of about 30,000 juveniles were obtained by a technique of known accuracy (Reversat, 1976, 1980). Counting was made on six aliquots and dry weight determinations on six other aliquots (see above). The remaining samples were divided into groups of four for total carbohydrate determination by the anthrone reagent (Seifter *et al.*, 1950), total lipid determination by the sulfo-phosphovanillic reagent (Drevon & Schmit, 1964), total protein determination by the Folin's reagent (Lowry *et al.*, 1951) and total nitrogen determination by the Nessler's reagent. Details of the use and adaptation of these techniques for nematodes have been given previously (Reversat, 1976). Determinations were made after 0, 1, 2, 3, 4 and 5 weeks of storage and results are expressed as nanograms per juvenile (ng/juv.)

Results

Motility

There was a steady decrease of motility throughout the experiment (Fig. 1). At the end of 5 weeks only 1.4% of the juveniles remained motile and after 6 weeks no juveniles were motile.

Infectivity

The percentage of invading juveniles was 52% at the zero time of the experiment (Fig. 2).

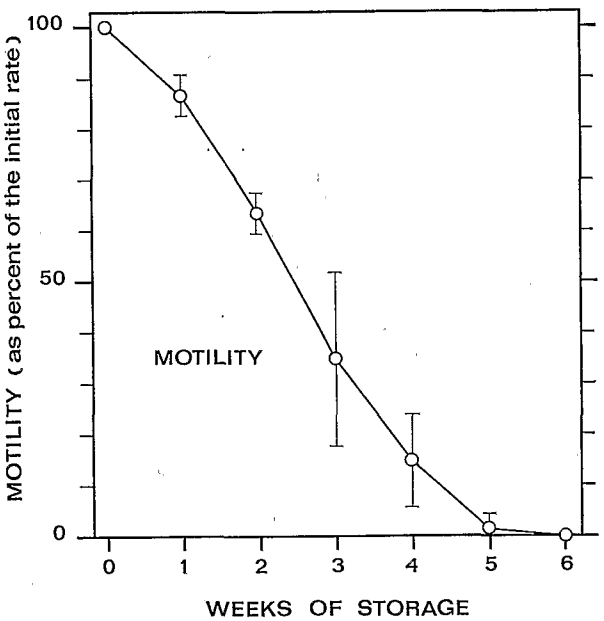

Fig. 1. The effect of storage time on the motility of juveniles of *Heterodera oryzae* (Each point is the mean of four replications and the vertical line equals the confidence interval at 95%).

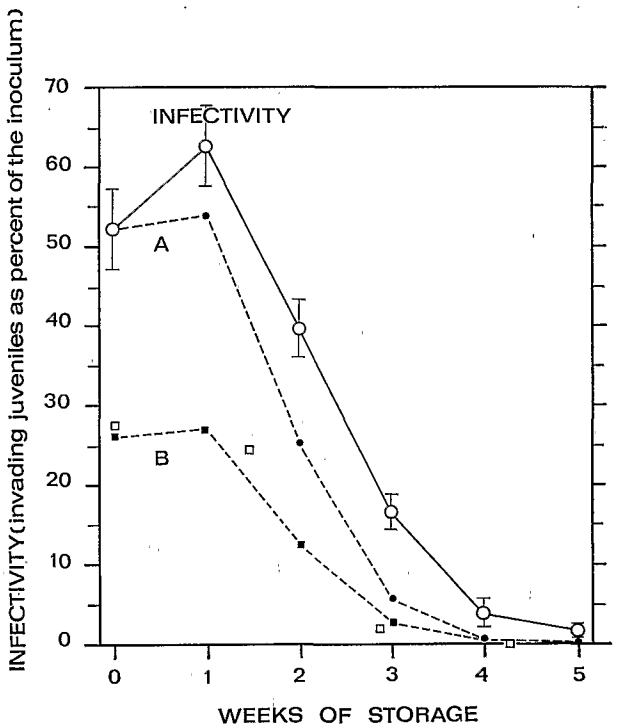

Fig. 2. The effect of storage time on the infectivity of juveniles of *Heterodera oryzae*. (Each point is the mean of 20 replications and the vertical line equals the confidence interval at 95%.) Curve A : product of infectivity by motility. Curve B : data of curve A halved to account for a sex ratio of 1 : 1 giving the theoretical percentage of females obtained. Open squares : the effect of storage time in wet soil on the ability of juveniles of *H. oryzae* to produce females (Data from Merny, 1972).

During the first week of storage, the infectivity significantly increased to 62% ($p < 0.01$). After that, the infectivity decreased sharply until it reached 1.6% at the end of 5 weeks of storage.

Respiration

The rate of O_2 consumption was initially 58 pl/juv./hr and decreased to 26 pl /juv./hr at the end of 4 weeks of storage (Fig. 3).

Dry weight

The dry weight of juveniles used in the respiration determination decreased steadily from 31.9 ng/juv. at the zero time of the experiment to 19.4 ng/juv. at the end of 4 weeks and regression analysis suggested a decrease of 3.22 ng/juv. per week (Tab. 1). In the second experiment accompanying measurements of chemical composition, the change in dry weight, calculated by regression analysis, was 2.11 ng/juv. per week and co-variance analysis showed there was a significantly lower rate of weight loss than in the previous experiment ($p < 0.001$; Fig. 4)

Freshly hatched juveniles of *H. oryzae* exhibited an opaque digestive tract. With increasing storage time, the digestive tract became progressively clearer and after 5 weeks of storage the posterior part of the juveniles was almost transparent.

Chemical determinations

The lipid content of juveniles (Fig. 5) decreased from 8.5 ng/juv. at the zero time of the experiment to 2.5 ng/juv. at the end of 5 weeks of storage, an overall loss of 70% of the initial content. The carbohydrate content of juveniles (Fig. 6) increased significantly ($p < 0.01$) during the first week of storage from 3.9 to 4.4 ng/juv. and then remained at the same value until the third week of storage. Beyond this date a subsequent fall occured to 2.9 ng/juv. after 5 weeks. The protein content and the total nitrogen

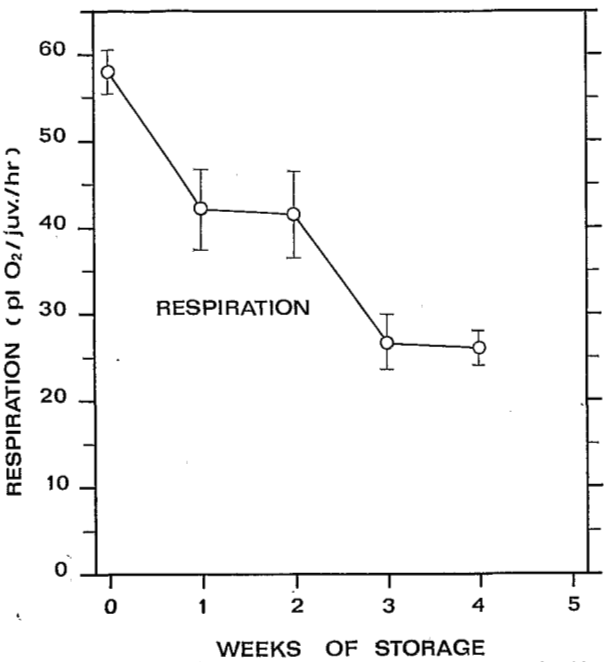

Fig. 3. The effect of storage time on the respiration of juveniles of *Heterodera oryzae* (Each point is the mean of ten replications and the vertical line equals the confidence interval at 95%).

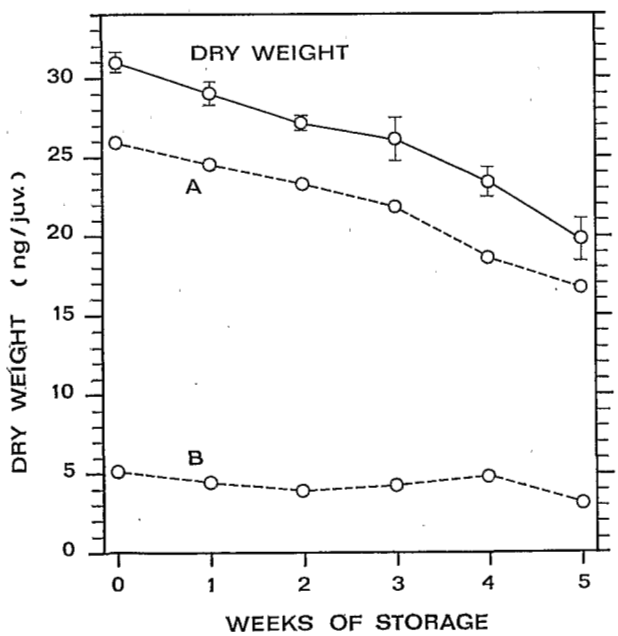

Fig. 4. The effect of storage time on the dry weight of juveniles of *Heterodera oryzae* (Each point is the mean of six replications and the vertical line equals the confidence interval at 95%). Curve A : sum of total lipids (Fig. 5), plus total carbohydrates (Fig. 6), plus total proteins (Fig. 7). Curve B : difference between dry weight and Curve A.

Table 1

The effect of storage time on the dry weight of juveniles of *Heterodera oryzae* (Determination made on the population used for respiration measurement, two replications for each time)

Weeks of storage	*Dry weight (ng/juv.) Mean ± S.E.*
0	31.9 ± 0.3
1	28.6 ± 1.2
2	23.5 ± 1.3
3	21.3 ± 1.2
4	19.4 ± 0.4

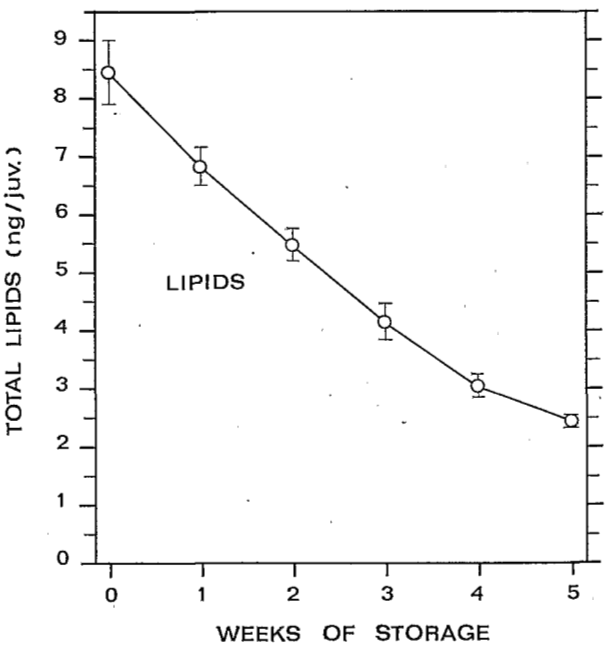

Fig. 5. The effect of storage time on the total lipid content of juveniles of *Heterodera oryzae* (Each point is the mean of four replications and the vertical line equals the confidence interval at 95%).

content of juveniles (Fig. 7) remained almost constant between 0-3 weeks of storage but underwent subsequently a similar fall giving a constant ratio of protein to nitrogen of 5.1 to 5.4 throughout the experiment.

Discussion

The total energy balance

The consumption of endogenous food reserves, which covered the metabolic expenses of starved

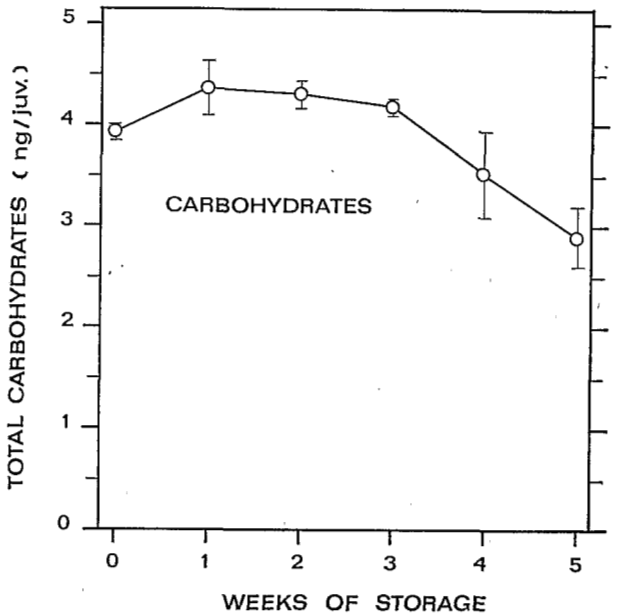

Fig. 6. The effect of storage time on the total carbohydrate content of juveniles of *Heterodera oryzae* (Each point is the mean of four replications and the vertical line equals the confidence interval at 95%).

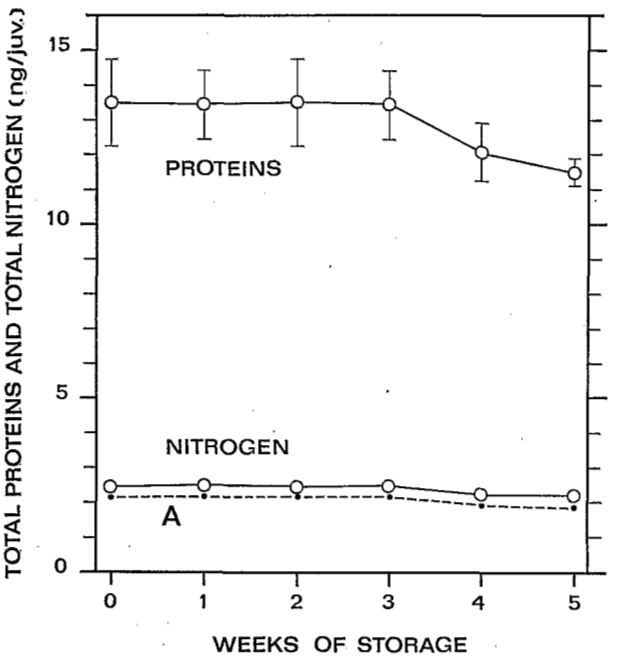

Fig. 7. The effect of storage time on the total protein content and the total nitrogen content of juveniles of *Heterodera oryzae* (Each point is the mean of four replications and the vertical line equals the confidence interval at 95%). Curve A : protein nitrogen (Data of protein curve divided by 6.25).

juveniles, was overall estimated by the loss of dry weight. There was a difference between the rate of loss of dry weight of the population used in the respirometry and the population used in the chemical analysis. This may be due to inherent differences between the populations or it may have occured because oxygen availability during storage at different densities and the dissimilar ionic composition of the two storage media influenced the level of metabolism (Reversat, 1977).

The total lipid, carbohydrate and protein content of the freshly hatched juveniles accounted for about 26 ng/juv. of the total dry weight of 31 ng/juv. The value of the difference between these two figures was maintained for all periods of storage (Fig. 4 A & B) and this shows that the chemical analyses were reliable. The rather high amount of this undetermined portion of the dry weight (15% of initial dry weight) suggests it was constituted not only by mineral salts, which amounted for 5% of the dry weight in zooparasitic nematodes (von Brand, 1966), but also by some organic substances involved in the structure and which were not metabolized during starvation.

The ratio of proteins to nitrogen ranged from 5.1 to 5.4 throughout the experiment whereas the expected value for animal tissues was 6.25 (Herbert, Phipps & Strange, 1971). This suggests that juveniles contained a high amount of non protein nitrogen (about 12% of total nitrogen). Since variations of total nitrogen and variations of the ratio of proteins to 6.25 agreed closely (Fig. 7), the consumption of proteins during starvation may be well estimated by the sole determination of total nitrogen, technically easier than the protein determination.

Much of the loss of weight in starved *H. oryzae* juveniles was due to the catabolism of lipids reserves and this agrees with previous work on many zoo-parasitic species (Elliot, 1954 ; Wilson, 1965 ; Barrett, 1969 ; Clark, 1969 ; Croll, 1972 ; Croll & Matthews, 1973), free living species (Cooper & Van Gundy, 1970 ; Barrett, Ward & Fairbairn, 1971) and in particular with work on *Meloidogyne javanica* (Van Gundy, Bird & Wallace, 1967) and *M. naasi* (Ogunfowora, 1979).

The significant increase of carbohydrate content during the first week of storage (Fig. 6),

when protein content remained constant, demonstrated that some lipids were used for synthetizing these carbohydrates. This conversion has been demonstrated by Passey and Fairbain (1957) in zoo-parasitic nematodes and by Barrett, Ward and Fairbairn (1963) and Cooper and Van Gundy (1970) in free living and mycophagous species.

Using the rate of O_2 consumption recorded each week as a basis for calculation (Fig. 3), it can be estimated, by integrating the curve, that each juvenile consumed 25,600 pl in 4 weeks for a corresponding dry weight loss of 12.5 ng, i.e. 2,047 ml/g. This value is very close to the value of oxygen consumed by lipid oxidation : 2,019 ml/g (Polonowski *et al.*, 1966). Since lipids were not the only material consumed during storage, the overall O_2 consumption seemed to be a little overestimated. Probably the disturbance of the animal during respirometry may stimulate its metabolism relative to that during storage. Overall, the results suggest that most of the food reserves utilized by the metabolism underwent complete oxidation. This agrees with the low amount of metabolizable organic substances excreted by some plant parasitic nematodes (Myers & Krusberg, 1965 ; Wang & Bergeson, 1978).

The physiological decline

There seems to be a broad correlation between the extent of lipid reserves (Fig. 5) and both motility (Fig. 1) and infectivity (Fig. 2) of the juveniles. However, a loss of infectivity is not solely due to effects on mobility as all the animals used were capable of activity. This effect has been observed previously for *M. javanica* (Thomason, Van Gundy & Kirkpatrick, 1964 ; Van Gundy, Bird & Wallace, 1967).

The clearing of the digestive tract represented an individual index for the consumption of food reserves. When one week old juveniles were observed before the motility test, none of them exhibited an appreciable clearing. The test, however, discarded 10% of them at this date (Fig. 1), while the consumption of food reserves was just at the beginning (Fig. 5, 6 & 7). Thus the loss of motility seemed not to be closely related to the loss of food reserves.

The results also show decrease in O_2 consumption during storage which has been reported for some other nematodes (Santmyer, 1956 ; Rohde, 1960 ; Sembdner, Osske & Schreiber, 1961 ; Van Gundy, Bird & Wallace, 1967 ; Bhatt & Rhode, 1970) although other species maintained a constant O_2 consumption (Nielsen, 1949 ; Wallace & Greet, 1964). Short term decrease in O_2 consumption may be due to stresses resulting from respirometry (Rohde, 1960 ; Reversat, 1975*b*). Long term decrease in O_2 consumption may result from the exhaustion of food reserves and from alteration of enzymes involved in energetic metabolism (Van Gundy, Bird & Wallace, 1967 ; Zeelon, Gershon & Gershon, 1973).

Thus, if there is an attempt to correlate the physiological decline of starved *Heteroderidae* juveniles with the sole exhaustion of food reserves, a real ageing process cannot be dismissed.

Storage in vitro and storage in soil

The data for infectivity in Fig. 2 give good agreement with the number of females developing on rice after storage in wet field soil for varying times (Merny, 1972) after the data in Fig. 2 is halved to account for a sex ratio of 1 : 1 in this species (Cadet, Merny & Reversat, 1975).

H. oryzae juveniles emerge from cysts and egg-masses even in the absence of the host (Merny, 1966, 1972) and may experience anoxia during flooding of the soil (Yoshida, 1971). Merny (1972) reported that the ability of stored *H. oryzae* juveniles to produce females decreased more rapidly in flooded soil than in wet soil, whereas Reversat (1975*d*) showed that *in vitro* anoxia reduced the loss of dry weight and increased the survival time of juveniles. This may be due either to a microbial development in flooded soil, providing organic acids and soluble sulphides (Yoshida, 1971), which are toxic to nematodes (Banage & Visser, 1965 ; Fortuner & Jacq, 1976) or to the loss of infectivity in juveniles surviving anoxia. More investigations are needed for solving this dicrepancy.

Roots of the rice plant supply oxygen to the soil (Yoshida, 1971) and ageing and starvation stress acts until the *H. oryzae* juveniles invade

the roots. In glass tubes, invasions occured during 7 days with a diameter of 18 mm (present study) and during 16 days with a diameter of 30 mm (Reversat & Merny, 1973). In the field where the juveniles move a greater distance, delays before invasion may be even more important.

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