n°3, Juill.

Nematologica 23 (1977): 369-381. E. J. Brill, Leiden

INFLUENCE OF SOME EXTERNAL FACTORS ON THE RATE OF OXYGEN UPTAKE BY SECOND-STAGE JUVENILES OF *HETERODERA ORYZAE*

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The rate of oxygen uptake of second-stage juveniles of *Heterodera oryzae* was measured relative to osmotic pressure, pH, oxygen tension, nutrients and temperature using the cartesian diver technique. Osmotic pressure affected the rate of oxygen uptake in relation to the nature and the concentration of the osmotic agent used. Rate of oxygen uptake was weakly affected by pH within the range 3-8. Citrate depressed the rate of oxygen uptake whereas glucose, fructose and sucrose had no effect. The effect of oxygen tension on the rate of oxygen uptake demonstrated that this species is oxygen dependent. Postanaerobic overshoot occurred after 12 h. and 24 h. of anaerobiosis. Rate of oxygen uptake increased with temperature within the range 20-36°.

The topic of respiration in plant-parasitic nematodes has recently been reviewed by Rohde (1971), who pointed out that only a small amount of information was available on this subject. Rohde (1971) discussed several factors known to influence respiration including oxygen, reduced oxygen levels, carbon dioxide, temperature, osmotic pressure, metabolic cycles and others.

Although Sembdner *et al.* (1961) measured respiration rates of juveniles and cysts of *Globodera rostochiensis* (Woll.), little is known about respiration in the genus *Heterodera*. Since *H. oryzae* Luc & Berdon, 1961 is an important parasite of inundated rice in the Ivory Coast (Merny, 1970), a study was made of the effects of osmotic pressure, pH, certain nutrients, oxygen tension, postanaerobic respiration and temperature on the rate of oxygen uptake of second-stage juveniles of this species.

MATERIALS AND METHODS

Egg masses of *H. oryzae* were obtained from roots of rice plants five weeks after inoculation with infective juveniles. These were kept 4 days in a 0.3 M sodium chloride solution known to inhibit hatching and then transferred to demineralized water in which hatching occurred (Dropkin *et al.*, 1958; Reversat, 1975a). After 3 days, juveniles were collected and surface-sterilized with antibiotics (Reversat, 1975b).

The rate of oxygen uptake was measured exclusively by the cartesian diver technique. The diver, the apparatus, the manipulations and calculations have been previously described (Reversat, 1975b). Results are expressed as picoliters (10^{-12} l)

of oxygen consumed per juvenile per hour (pL/J2/hr). Twenty identical divers were charged with 10 μl of ambient air (21% V/V oxygen) and 80 surface sterilized juveniles suspended in standard buffer (pH 7, 50 mM phosphate buffer sterilized by filtration). For calculations, two measurements of the equilibrium pressure of the diver were made 20 h apart. Measurements were made at $28 \pm 0.01°$. The flotation medium of the diver, 0.1 N sodium hydroxide, maintained a zero tension of carbon dioxide in the diver. All measurements have been carried out in these conditions except when otherwise noted.

A replication was considered the result obtained with one diver. A separate experiment was the set of replications made with juveniles hatched at the same time.

Materials and methods specific to the study of a particular factor are described below under the appropriate headings.

RESULTS AND DISCUSSION

Osmotic Pressure

Three separate experiments were made. For each, four osmotic pressure values and five replications for each value were used. For two experiments, pH 7 phosphate buffer was used as the osmotic agent at different concentrations: 5, 10, 20, and 40 mM for the first experiment and 40, 80, 160, and 320 mM for the

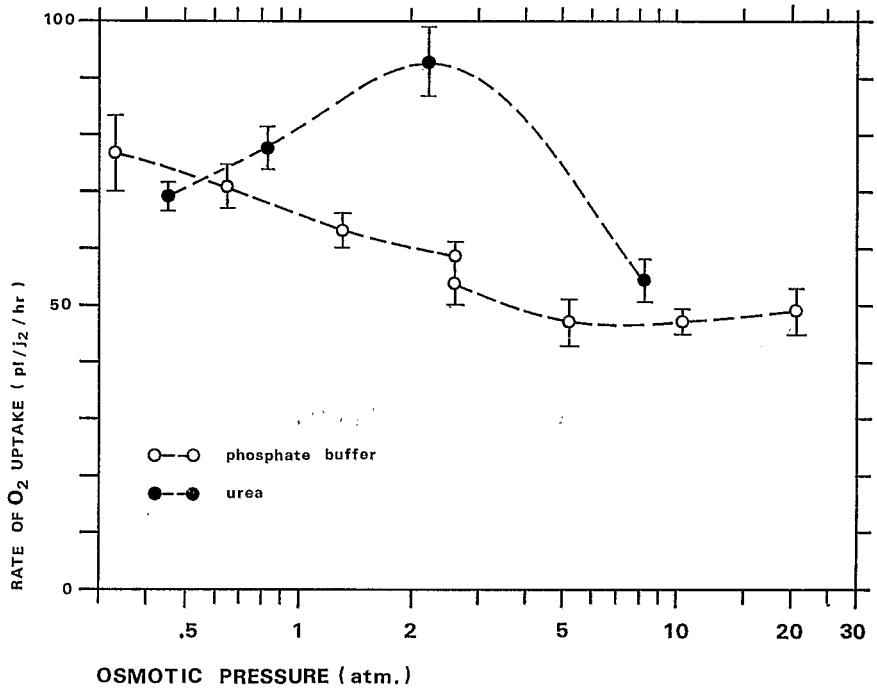

Fig. 1. Influence of osmotic pressure on the rate of oxygen uptake of second-stage juveniles of *Heterodera oryzae* (The vertical line equals the confidence interval at 95%).

second. In the third experiment, urea at 5, 20, 80, and 320 mM in pH 7, 5 mM phosphate buffer was used to produce osmotic pressure. Osmotic pressures are expressed in atmospheres and were calculated from the concentration according to Collis-George & Sands (1962). Juveniles were suspended in the solution 6 h before the first measurement of the equilibrium pressure of the diver.

The results of the three experiments are shown in Fig. 1. Analysis of variance showed a significant effect of osmotic pressure ($p<1\%$) for the three experiments. With urea a maximum rate of oxygen uptake was observed at 2 atm., whereas the rate of oxygen uptake decreased regularly with increasing osmotic pressure when phosphate buffers were used.

The influence of increasing concentrations of urea on the rate of oxygen uptake by some plant-parasitic nematodes has been reported by Wallace & Greet (1964) and Bhatt & Rohde (1970). They observed that the relations between the rate of O_2 uptake and osmotic pressure curve presented a characteristic pattern, with a maximal rate of oxygen uptake for an optimal concentration. The same pattern was obtained for juveniles of *H. oryzae* in this study. The optimal value of 2 atm observed with *H. oryzae* is similar to the value of 2.24 atm observed for *Anguina tritici, Ditylenchus dipsaci,* and *Tylenchorhynchus icarus* (Bhatt & Rohde, 1970; Wallace & Greet, 1964).

Some nematodes have exhibited a decreasing rate of oxygen uptake with increasing concentration of mineral salts as osmotic agents (Barrett, 1969b; Bhatt & Rohde, 1970; von Brand, 1942; Fernando, 1963; Schwabe, 1957). The same effect was observed with juveniles of *H. oryzae.*

If both solutes, urea and phosphate, are considered only as osmotic agents, their different effects on oxygen uptake at the same osmotic pressure is difficult to explain. Different responses to osmotic stress in nematodes, related to the electrolyte or non-electrolyte nature of the solute, has been demonstrated by Viglierchio *et al.* (1969). They suggested that the solute is not a perfect osmotic agent and it can penetrate the nematode. Data of Myers (1966) also supports this view. Nematodes are permeable to water as shown by use of tritiated water (Marks *et al.*, 1968), or by changes in body volume after changes of tonicity of the surrounding medium (Stephenson, 1942; Myers, 1966; Viglierchio *et al.*, 1969). Such water movement is attributed to an active osmoregulation process, which involves energy expenditure (Stephenson, 1942; von Brand 1960b). The respiratory response of *H. oryzae* juveniles to osmotic stress can be explained as follows. Urea functioned as a real osmotic agent causing removal of water from the nematode's body. The osmoregulatory mechanism of the nematode opposed this drying effect, and the metabolic expence involved, as measured by increased oxygen uptake, increased with the concentration of urea. But above the optimal concentration (2 atm), the osmoregulatory mechanism became inefficient and the resultant drying of the nematode depressed metabolism. In the case of phosphate buffer, one or more of the ions from the buffer may have penetrated the nematode and upset the normal osmoregulatory mechanism.

pH

Two separate experiments were made, each with four pH values replicated five times. The first experiment utilized citrate buffer (50 mM; pH: 3, 4, 5, and 6) and the second experiment citrate-phosphate buffer (50 mM; pH: 5, 6, 7, and 8). Juveniles were suspended in the solutions six hours prior to making the first measurement of the equilibrium pressure of the diver.

Results of the two experiments are shown in Fig. 2. Analysis of variance showed a significant effect of pH ($p<1\%$) in each of the two experiments. With citrate buffer, the rate of oxygen uptake decreased (10%) when the pH value increased

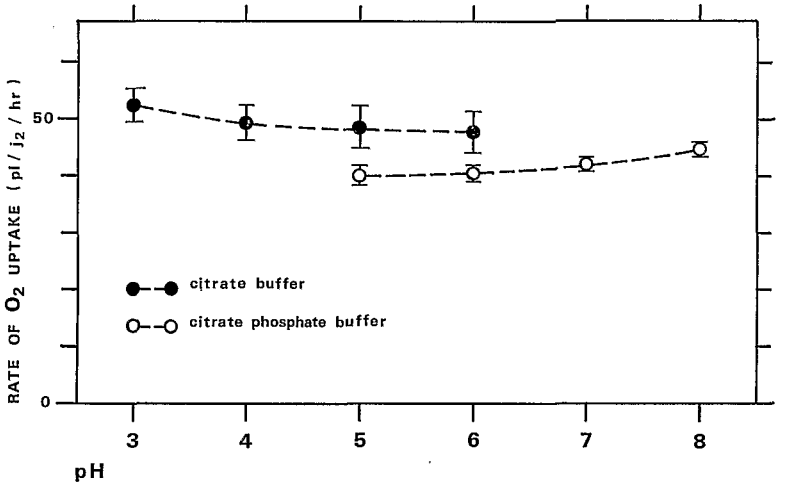

Fig. 2. Influence of pH on the rate of oxygen uptake of second-stage juveniles of *Heterodera oryzae*. (The vertical line equals the confidence interval at 95%).

from 3 to 6. With citrate-phosphate buffer, the rate of oxygen uptake increased (12%) when the pH value increased from 5 to 8. It should be noted that the latter buffer produced an especially low variability among the five replications compared to the variability obtained with citrate buffer or phosphate buffer (see Fig. 1).

The range of pH, within which the respiration of *H. oryzae* juveniles was studied, is close to the normal range of pH in flooded rice fields (Angladette, 1966). Usually in flooded soil the pH varies within a small range even in the presence of decaying organic material (Bell, 1969). Thus, in natural conditions, pH might exhibit only a minor stress on the respiratory rate of *H. oryzae* juveniles. Similar results were obtained with juveniles of free-living and zooparasitic nematodes (de Cooman, 1950; Barrett, 1969b; Schwabe, 1957; von Brand, 1943).

Influence of certain nutrients

Two separate experiments were made. In the first, sugars at a concentration of 50 mM were added to the standard phosphate buffer. There were four treatments

with five replications each: glucose, fructose, sucrose, and a control containing no sugar. In all treatments 2 ethoxy ethyl mercuric chloride was added at 5 ppm as an antibiotic. The second experiment consisted of two treatments with five replications each. The first treatment consisted of pH 7, 50 mM phosphate buffer; the second of pH 7, 50 mM citrate-phophate buffer as the suspension medium. In both experiments, juveniles were suspended in the medium 6 h prior to the first measurement of the equilibrium pressure of the diver.

Results of the two experiments are presented in Table I. In the first experiment, the oxygen uptake rates of the three sugars did not differ significantly from the control. In the second, citrate-phosphate buffer significantly depressed the rate of oxygen uptake. Variability of measurements was reduced when using this buffer.

TABLE I

Effect of some nutrients on the rate of oxygen uptake of second-stage juveniles of Heterodera oryzae

Experiment number	Nutrient	Rate of O_2 uptake (pl/j2/hr) mean of 5 replications	Standard deviation
1	Control	53.6	5.5
	Glucose 50 mM	52.3	2.4
	Fructose 50 mM	61.6	7.6
	Sucrose 50 mM	48.8	2.2
2	Phosphate buffer pH 7-50 mM	53.8	5.2
	Citrate-phosphate buffer pH 7-50 mM	47.2	1.1

Endogenous food reserves seem to limit survival time of nematodes (van Gundy, 1965; van Gundy *et al.*, 1967). In rice fields, sugars and amino acids are exuded into the soil solution by rice roots (MacRae & Castro, 1966). If these could be metabolized by *H. oryzae* juveniles, their survival time might be increased. Although Sembdner, *et al.* (1961) reported that glucose stimulated the respiratory rate of second-stage juveniles of *G. rostochiensis*, other workers (Bhatt & Rohde, 1970; Barett; 1969a; Stannard *et al.*, 1938; Fernando, 1963) failed to detect any effect of several nutrients, including glucose, on the respiratory rate of selected nematode species. Marks *et al.* (1968) reported that some nematodes are incapable of absorbing glucose from a liquid medium. Results of the first experiment indicate that sugars were not absorbed by *H. oryzae* juveniles or that they were not metabolized so as to affect oxygen uptake.

Karpiak *et al.* (1965), studying *Trichinella spiralis*, reported the depression of rate of oxygen uptake by citrate as observed in the second experiment. This observation has not been resolved, one hypothesis is that the citrate and phosphate penetrated the *H. oryzae* juveniles where they saturated a metabolic pathway. This would explain the reduction in oxygen uptake and the low variability. Additional studies are necessary to elucidate this phenomenon.

Oxygen tension

One experiment was made using four values of oxygen tension replicated five times. Mixtures of compressed air (21% V/V oxygen) and compressed industrial nitrogen (3% V/V oxygen) were made in four cylinders. The oxygen tensions of these mixtures were verified with a Beckman process oxygen analyzer: 156; 111.4; 66.8; and 22.3 mm Hg. The pipette used to deliver air into the diver was filled with the desired oxygen-nitrogen mixture in a small chamber into which air from the appropriate cylinder was flushed continuously with a slight overpressure. Juveniles were first in contact with the gaseous mixtures three hours before the first measurement of the equilibrium pressure of the diver.

Results of this experiment are given in Fig. 3. Analysis of variance showed a significant effect of oxygen tension on the rate of oxygen uptake ($p<0.1\%$). In Fig. 4, the ratio oxygen tension/rate of oxygen uptake, was plotted against

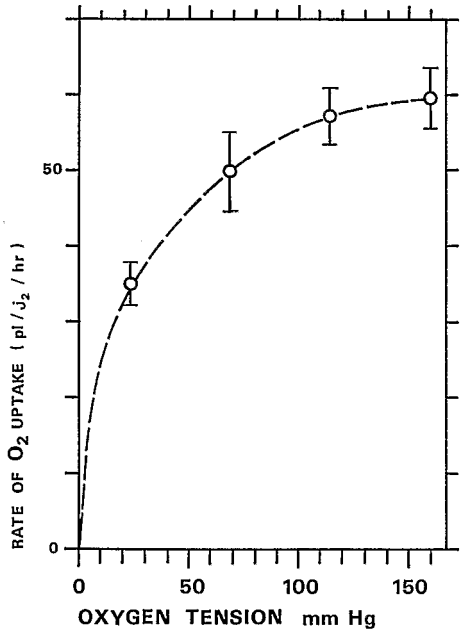

Fig. 3

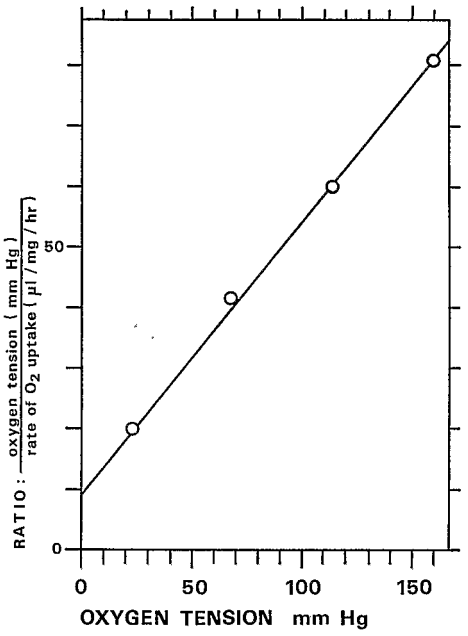

Fig. 4

Fig. 3. Influence of oxygen tension on the rate of oxygen uptake of second stage-juveniles of *Heterodera oryzae.* (The vertical line equals the confidence interval at 95%).

Fig. 4. Influence of oxygen tension on the ratio: O_2 tension/rate of O_2 uptake in the second-stage juveniles of *Heterodera oryzae.* (Data of fig. 3 transformed according to the formula of Rogers (1949). The rate of O_2 uptake was calculated in µL/mg of dry weight on the basis of a dry weight of 30 ng/juvenile (Reversat 1975 b)).

oxygen tension (Rogers, 1949). The results show a linear relationship corresponding to the formula:

$$QO_2 = \frac{[O_2]}{K_1 + K_2 [O_2]}$$

where QO_2 is the rate of oxygen uptake and $[O_2]$ the oxygen tension. This formula is characteristic of a reaction between oxygen and an enzyme. The values of K_1 and K_2 were determined from Fig. 4 respectively, by the intersect ($K_1 = 9.5$) and the slope ($K_2 = 0.44$). These values lie within the range for nematodes (Rogers, 1949).

Rate of oxygen uptake in nematodes may be dependent on or independent of oxygen tension (von Brand, 1960a; Bair, 1955; Barrett, 1969b; Stannard *et al.*, 1938). In this experiment, juveniles of *H. oryzae* were shown to be oxygen dependent. Oxygen tension in the soil fluctuates widely (von Brand, 1960a). Oxygen is supplied to flooded rice fields by diffusion from the atmosphere and excretion from rice roots (Hollis, 1967; Naphade, 1971). Oxygen tension is presumably high near the roots, but probably decreases as the distance from the roots increases. At the beginning of rice growth, juveniles may be far from the roots and may have to withstand oxygen tensions between zero and that near the roots. During active rice growth, juveniles from egg masses and cysts on the roots probably have no limitation of oxygen supply.

Postanaerobic oxygen uptake

This experiment consisted of three treatments with five replications each: control with no period of anaerobiosis, a 12-hour period of anaerobiosis and a 24-hour-period of anaerobiosis. To reduce oxygen tension the sodium sulphite solution method was used (Feldmesser & Feder, 1954; Feder & Feldmesser, 1955). The respiratory chamber of the diver, containing the juveniles, was introduced vertically into a test tube filled with a 400 ppm sodium suphite solution in standard buffer, prepared immediately before use. The tube was then hermetically sealed without trapping any air bubble. The sulphite, in excess, was oxidized to sulphate by dissolved oxygen (Reinders & Vles, 1925). Kolthoff & Laitinen (1940) demonstrated that under these conditions no trace of oxygen was polarographically detectable in the solution after 10 min. Thus juveniles of *H. oryzae* were considered in anaerobiosis. After the required time intervals the respiratory chamber was removed from the tubes and carefully rinsed with standard buffer without disturbing the pellet of juveniles at the bottom of the chamber. Then the standard procedures were followed, with the introduction of an air bubble into the chamber.

For the three treatments, the measurements of the equilibrium pressure of the diver were carried out every four hours and the rate of oxygen uptake was calculated for the middle of each time interval.

This experiment has been briefly described elsewhere (Reversat, 1975c). The results of the three treatments are shown in Fig. 5. For the control the initial rate of oxygen uptake was 65 pl/J2/hr and afterwards the rate decreased steadily. After anaerobisis, juveniles exhibited a higher rate of oxygen uptake: 91 pl/J2/hr after 12 hours of anaerobiosis and 103 pl/J2/hr after 24 hours of anaerobiosis. Afterwards the rate decreased steadily until it reached the level of the control after 30 hours. It was observed that during anaerobiosis all juveniles were motionless

with a straight body form. However, several hours after air was re-admitted, all juveniles resumed motility.

General effects of anaerobiosis on nematodes have been summarized by von Brand (1960a). Some nematodes, when returned to air after anaerobiosis, exhibit a temporarily increased respiratory rate. This overshoot, called the repayment of the oxygen debt, is attributed to a rapid oxidation of fermentative waste products accumulated during anaerobiosis. Second-stage juveniles of *H. oryzae* typically

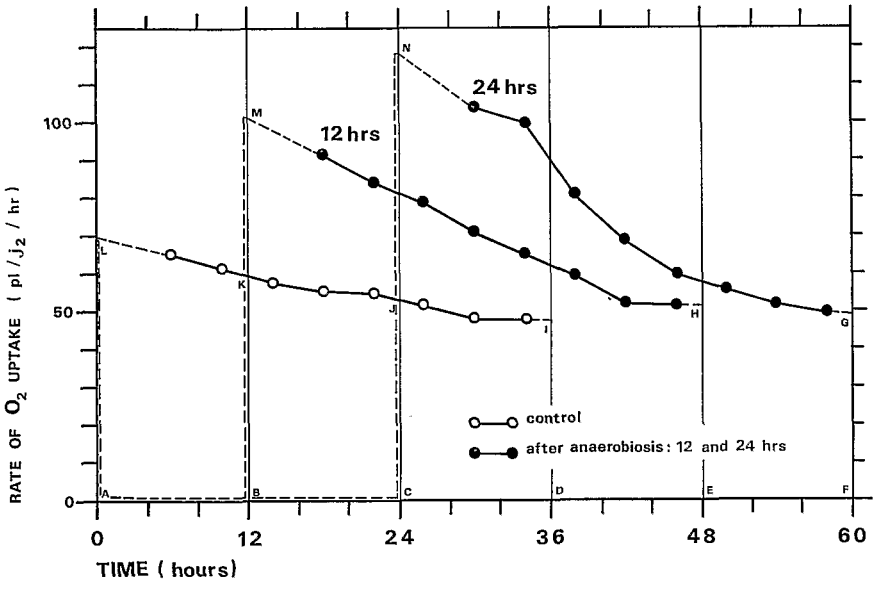

Fig. 5. Postanaerobic rate of oxygen uptake in second-stage juveniles of *Heterodera oryzae*. (The dotted lines indicate extrapolated values of rates of oxygen uptake and the capital letters A, B, ..., M, N, are related to the calculations involved in Table II).

exhibit a post-anaerobic overshoot (Fig. 5) when compared with the patterns obtained with zooparasitic forms (Barrett, 1969b; von Brand, 1943).

The extent to which the oxygen overconsumption compensated the oxygen deficit accumulated during anaerobiosis was calculated from Fig. 5. First, values of the rate of oxygen uptake were extrapolated as dotted lines. Then, the cumulative quantities of oxygen debt or oxygen consumption were calculated as surfaces. Limits of the surfaces involved in the calculations were designated by capital letters A, B, ... M, N. Results are expressed in Table II. After 12 hours of anaerobiosis the rate of repayment was 72% but only 47% after 24 hours of anaerobiosis. This result can be explained either by a partial excretion of the waste products of fermentation or by a reduced metabolism of the juveniles in anaerobiosis. Loss of motility during anaerobiosis is generally attributed to a toxic effect of accumulated fermentative waste products (von Brand, 1960a).

TABLE II

Calculation of the rate of the oxygen debt repayment in second-stage juveniles of Heterodera oryzae *

Parameters		Duration of anaerobiosis		
		0	12 hours	24 hours
O_2 debt	Reference letters	—	A B K L	A C J L
	Symbol	—	S_{12}	S_{24}
	Numerical value	—	782.6	1449.6
O_2 consumption	Reference letters	A D I L	B E H M	C F G N
	Symbol	R_0	R_{12}	R_{24}
	Numerical value	2 036.9	2 602.5	2 718.8
O_2 repayment	Symbol	—	$(R_{12}-R_0)$	$(R_{24}-R_0)$
	Numerical value	—	565.6	681.9
Rate of O_2 debt	Symbol	—	$\frac{(R_{12}-R_0)}{S_{12}}$	$\frac{(R_{24}-R_0)}{S_{24}}$
repayment	Numerical value	—	0.72	0.47

* Calculated from data from Fig. 5. See explanation in text.

Temperature

Five separate experiments were made, each at a different temperature. Each experiment included eight replications and two control divers without nematodes. Two measurement apparatuses were used. The temperature of the water bath of the first was maintained at 28°. The second one was maintained at one of the following temperatures: 20, 24, 28, 32, and 36°. In each experiment, the ten divers were placed in the first apparatus at 28°, where two measurements of the equilibrium pressure were taken 2 h apart. They were then transferred to the second apparatus, where the equilibrium pressure was measured every 4 h. The rate of oxygen uptake was calculated for the middle of each time interval. Final calculations were made after two corrections. The first correction was made by subtracting the value of the variation of the equilibrium pressure of the control divers from the variation of the equilibrium pressure of the divers with nematodes, for every time interval between two measurements. This took into consideration the purely physical effect of temperature change on the equilibrium of the diver. The second correction was related to the calculation of the diver constant (Reversat, 1975b), where the value of absolute temperature is involved.

The results of the experiments are presented in Fig. 6. Rates of oxygen uptake were calculated as per cent of the rate measured at 28°. For the standard temperature, the rate of oxygen uptake decreased steadily with time. At 32° and 36°, the rate of oxygen uptake increased after transferring the divers, reaching a maximum rate after 4 h, after which there was a decrease in the rate of oxygen uptake. At 20° and 24°, the rate of oxygen uptake decreased after transferring the divers. After 8-10 h, the rate of oxygen uptake seemed to stabilize.

The standard temperature of 28° was chosen arbitrarily but it is within the range of temperature occurring in the pots in the green-house in which *H. oryzae* has been reared. It is also within the range of temperatures recorded at a depth of 20 cm in flooded rice fields near the laboratory.

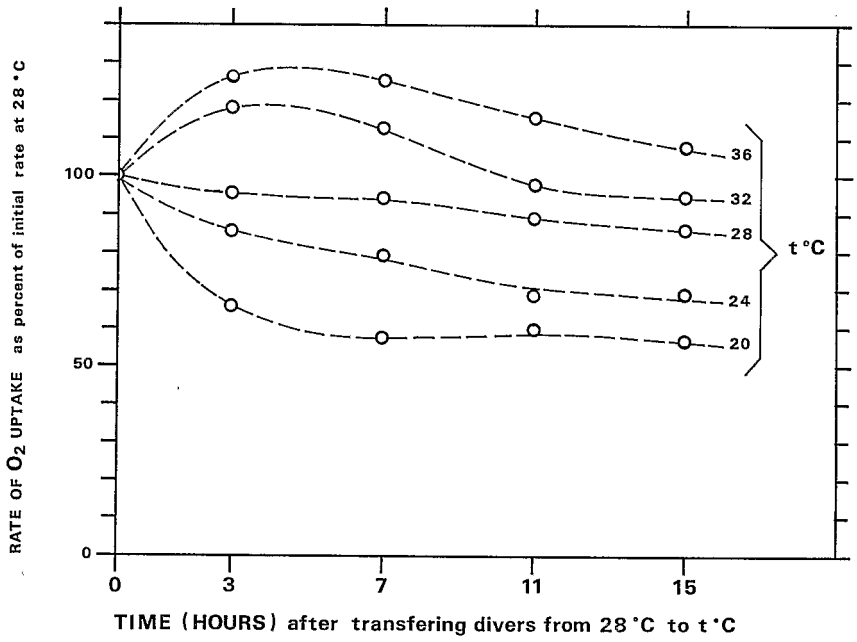

Fig. 6. Influence of the change of temperature on the rate of oxygen uptake of second-stage juveniles of *Heterodera oryzae*.

These results can be interpreted in one of two ways. The more obvious interpretation considers *H. oryzae* as passively withstanding the temperature changes. According to Van 't Hoff's principle, a constant level of metabolism exists for each temperature, although it may be reached only after a period of adaptation. The apparently abnormal decreasing rate of oxygen uptake at 32° and 36° could be explained on the basis of aging (van Gundy *et al.*, 1967). In these experiments it is considered that the true relationship between the rate of oxygen uptake and temperature was observed only at the time of 7 h (Fig. 6) at which time adaptation had occurred and the decrease in rate of oxygen uptake was not yet pronounced. Results for this time are plotted in Fig. 7A in which a linear relationship is evident between rate of oxygen uptake and temperature. Similar results have been reported for *Anguina tritici* (Bhatt & Rohde, 1970) and for *Panagrellus redivivus* (Santmyer, 1956). In addition the Q_{10} (von Brand, 1960c) of *H. oryzae* between 20° and 36° gives a value of 1.63 which is similar to the values calculated from the data of Bhatt & Rhode (1970) and Santmyer (1956). The data expressed according to the Arrhénius formula (von Brand, 1960c) are given in Fig. 7B: two straight lines are intersecting at 28°, the standard temperature.

A more probable interpretation considers that *H. oryzae* was able to react against the primary effect of temperature. The ability of poikilotherms to compensate against temperature was reviewed by Bullock (1955). In these experiments such a compensation can be visualized when the values for 15 h are plotted (Fig. 7A). From 1.63 at 7 h, the Q_{10} between 20° and 36° reached a value of 1.49 at 15 h.

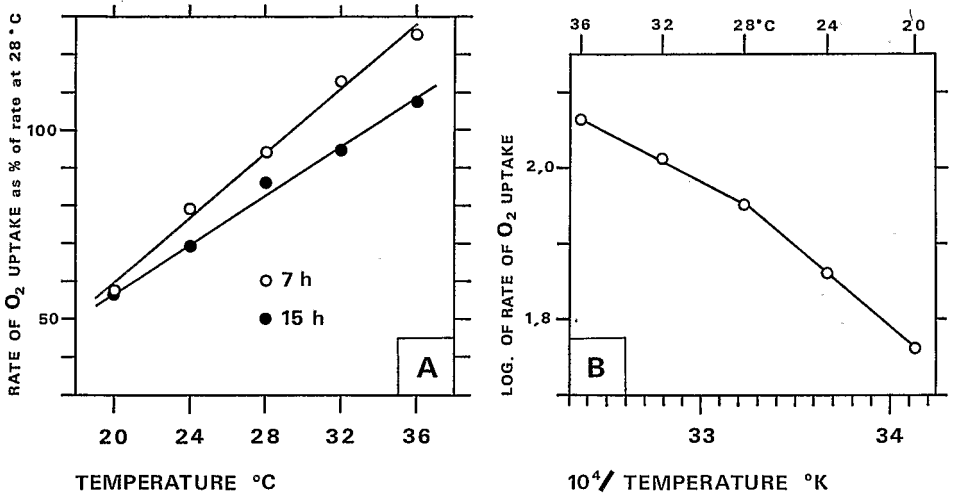

Fig. 7. Influence of temperature on the rate of oxygen uptake of second stage juveniles of *Heterodera oryzae*. (Data from fig. 6).

A. Untransformed data: rate of O_2 uptake at the time 7 hr. and 15 hr. B. Data expressed according to the Arrhenius formula: mean of the rates of O_2 uptake at the time 7 hr, 11 hr and 15 hr.

From Fig. 6 however, it is obvious that compensation would occur rapidly for higher temperatures (32° and 36°) as has been reported by Wilson (1965) for juveniles of *Nippostrongylus*, but for lower temperatures (24° and 20°) compensation would not have been reached within the time limits of the study. In fact for some nematodes, this adaptation to lower temperatures is stabilized only after several days (Cooper & Ferguson, 1973).

The author thanks Mr M. Gnahore Gohi for his technical assistance and is fully indebted to Dr D. P. Taylor for writing the English text.

RÉSUMÉ

*Influence de quelques facteurs externes sur l'intensité de la consommation d'oxygène des juvéniles du second stade d'*Heterodera oryzae

On a mesuré à l'aide de la technique du ludion l'influence qu'exercent certains facteurs externes sur l'intensité respiratoire (consommation d'oxygène) des juvéniles du second stade d'*Heterodera oryzae*, nématode parasite du riz. Ces facteurs étaient la pression osmotique, le pH, la pression partielle d'oxygène, l'effet d'une anaérobiose préalable, des métabolites et la température.

La pression osmotique agit sur l'intensité respiratoire en relation avec la nature et la concentration de l'agent osmotique utilisé. L'intensité respiratoire se montre relativement peu affectée par le pH

variant de 3 à 8. Le citrate déprime l'intensité respiratoire alors que le glucose, le fructose et le saccharose ne révèlent aucun effet. L'intensité respiratoire diminue avec la pression partielle d'oxygène. Après une anaérobiose, les juvéniles présentent une intensité respiratoire temporairement plus importante que la normale révélant ainsi l'acquisition d'une dette d'oxygène en anaerobiose. L'intensité respiratoire augmente avec la température entre 20° et 36°.

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