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THE INFLUENCE OF THE HATCHING FACTOR ON THE WATER UPTAKE OF THE SECOND STAGE LARVA OF THE POTATO CYST NEMATODE *HETERODERA ROSTOCHIENSIS*

BY C. ELLENBY AND R. N. PERRY*
Department of Zoology, The University, Newcastle upon Tyne, Great Britain

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SUMMARY

1. Larvae-containing eggs of the potato cyst nematode were soaked in root diffusate in artificial tap water or in artificial tap water alone. In one experiment, the eggs were still contained in intact cysts; in another experiment they were in a halved cyst.

2. Eggs were ruptured after 0–5 days treatment and the water content of the liberated larvae immediately estimated by interference microscopy.

3. Larvae from treated eggs from both the halved and intact cysts had a higher water content than controls.

4. Larvae liberated from treated eggs from the halved cyst reached their maximum water content after 24 h treatment; those from intact cysts attained the same value after 2 days. The delay, for the intact cyst, is in keeping with the hatching response for intact cysts and supports the view that the results for water content are due to the activity of the hatching factor.

5. Reasons are advanced for the view that the hatching factor may work via a neurosecretory mechanism.

INTRODUCTION

The second stage larva of the potato cyst nematode is stimulated to hatch by a diffusate given off by the roots of the potato plant (Triffitt, 1930). The nature of the so-called 'hatching factor' and its physiological action are unknown; it is still not clear whether, like the cardiac glycosides, it contains an unsaturated lactone ring (Marrian *et al.* 1949) though diffusates and extract concentrates undoubtedly possess cardiotonic activity (Ellenby & Gilbert, 1957). It was suggested in 1957 (Ellenby) that water movement might be involved in the hatching mechanism of the potato cyst nematode. More recently (Ellenby & Smith, 1969; Ellenby, 1974), estimation of the water content of larvae has shown that they take up additional water as soon as the egg-shell is ruptured in hatching. On the other hand, an attempt to show that the hatching factor increased the water content of the larva was unsuccessful – the slight difference in water content was not statistically significant. The present paper reports another attempt, with an improved experimental design, to examine the effect of the hatching factor on the water content of the second stage larva.

* Present address: Department of Biology, The University of Keele, Staffs, Great Britain.

MATERIALS AND METHODS

Eggs of *Heterodera rostochiensis* were derived from cysts obtained from Golden Wonder plants especially grown for the purpose in infested soil in 1971; since then the cysts had been stored in dry soil at room temperature. Hatching tests with cysts showed that they gave over 80% emergence when stimulated. After a 14-day 'pre-soak' at 20 °C in artificial Newcastle tap water (Greenaway, 1970), a number of cysts of similar size were selected. Two experiments were carried out with them; in both they were incubated at 20 °C using the cells of a single cyst hatching set-up (Ellenby, 1943). In one experiment, cysts were paired; one member of each pair was stimulated with root diffusate in artificial tap water, the other was maintained in artificial tap water alone. In the second experiment, a single cyst was halved by a cut through neck and vulval regions. One half, with its several hundred eggs still intact, was stimulated as before, the other half was kept in artificial tap water as a control. Artificial tap water or root diffusate in the cells containing the half or intact cysts was replenished each day from stock solutions stored at 4 °C. Root diffusate was prepared as described (Ellenby & Gilbert, 1958) except that artificial tap water was used instead of glass distilled water. Tests carried out subsequently with a Fiske Osmometer, showed that both the artificial tap water and root diffusate solution had identical osmotic pressure, viz. 5 mOsm.

The water content of individual living larvae was estimated by means of interference microscopy using techniques already described (Ellenby, 1968*a*, *b*). Individual eggs were transferred to artificial tap water on a slide and carefully covered with a cover glass. Slight pressure on the cover glass ruptured the egg-shell; almost immediately it was freed the larva was photographed through the fringe-field eyepiece of the interference microscope, using monochromatic green light. The interval between rupturing an egg and photographing the freed larva was as brief as possible and in no case exceeded 50 s. The anterior end of the larva was always photographed for in this region the interference fringes are particularly clear; in the intestinal region, food reserves often distort the fringes. From enlarged photographic prints the centre of the selected interference fringe could be pin-pointed. Nematodes are, truly, 'round-worms', so specimen thickness is easily determined from the diameter at the point of optical displacement.

In the whole-cyst experiment, immediately before the cysts were transferred to root diffusate, 10 eggs were taken from one of them and the larvae immediately photographed; a similar lot of 10 larvae were also photographed from a cyst in the control group in artificial tap water. These 20 constituted day 0. Twenty-four hours later (day 1) 10 eggs were taken from a cyst in the root diffusate and the larvae photographed; 10 eggs from a paired control cyst were similarly treated. The same procedure was followed in days 2, 3 and 5. Different cysts were, of course, used each day. This experiment, therefore, gave estimates of the water content of 100 larvae; 10 from each of four cysts in root diffusate for 1–5 days, and a total of 60 from six similar cysts in artificial tap water.

The procedure was similar for the half-cyst experiment. Five eggs were taken from each half-cyst; five from the half in root diffusate, and five from the half in tap water, each day of the experiment. For day 0, five eggs were taken from each half-cyst just

prior to the transfer of one of the half cysts to diffusate. The eggs were ruptured and the liberated larvae photographed as before. In this experiment, therefore, the water content of 50 larvae was estimated, 20 in root diffusate, and 30 in artificial tap water.

As the difference, if any, in the water content of the treated and untreated larvae is likely to be small, some aspects of the technique need to be stressed. The interference microscope is used to determine the refractive index of the specimen. This measurement is very accurate indeed. For example, in an essentially similar experiment (Ellenby, 1974), a typical mean value for larval refractive index was 1·3836; the standard error was ± 0·00054, or only 0·04% Values for cell solids, per cent, however are *estimates* based on assumptions about the specific refractive increment, α. Most cell solids have the same value for α of 0·0017, and the value generally used, 0·0018, makes some allowance for the presence of other substances. Estimates of water content from cell solids, in turn, involves assumptions about the volume occupied by unit weight of the solids. For convenience, it is assumed that unit weight of solids occupies unit volume; an organism would therefore be assumed to have a specific gravity of 1. Water content is then given by subtraction from 100 of the value for cell solids, per cent. Estimates of solids are generally considered to have an accuracy of ± 5%; with knowledge of the actual specific gravity of a nematode, this level of accuracy could be equalled for water content. But, clearly, with the assumptions involved, estimates of solids and of water content cannot have anything like the precision of the refractive index measurements. The present work, however, is essentially of a comparative nature. The measurements are based on animals of the same type and of the same age; they differ only in the treatment to which they are subjected. Clearly, the errors involved in the conversion factors used to convert refractive index into cell solids or water content will only affect the absolute validity of the values for the estimates, not their relative relationship. As far as the comparison is concerned, the accuracy will be that of the refractive index measurements.

RESULTS

Results are presented in Figs. 1 and 2 for the half- and whole-cyst experiments respectively. For the estimates of water content, it is assumed that the specific gravity of the cell solids and therefore of the nematode, is 1. As pointed out above, this is not strictly true; absolute values will be somewhat higher, as discussed elsewhere (Ellenby, 1968*c*). Statistical analyses were carried out using the values for refractive index, rather than the estimated values for water content derived from them.

The results, for both experiments, are essentially similar; the hatching factor treated larvae have a higher water content than the controls. In both experiments, for the eggs in artificial tap water, the water content of the newly liberated larva shows no statistically significant change over the 5-day period. On the other hand, for the eggs stimulated by root diffusate, the water content of the newly liberated larva increases over this time.

An analysis of variance for the results for the whole-cyst experiment was carried out with the data in lots of 10, using the values for refractive index; zero time, of course, included 20 items. After appropriate apportioning of the variance, the error mean

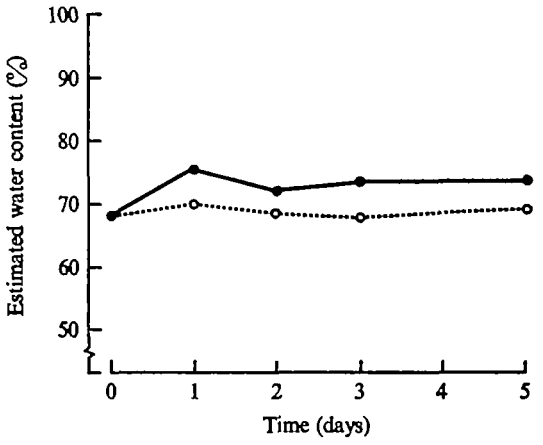

Fig. 1

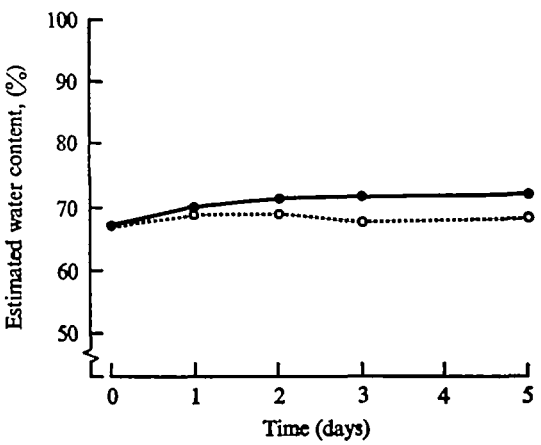

Fig. 2

Fig. 1. Half-cyst experiment. Each point is a mean of estimations on 5 larvae liberated from eggs immediately before immersion in root diffusate (day 0) and on subsequent days (●); controls were maintained in artificial tap water (○).

Fig. 2. Whole cyst experiment. Each point is a mean of estimations on 10 larvae liberated from eggs immediately before immersion in root diffusate (day 0) and on subsequent days (●); controls were maintained in artificial tap water (○).

square, derived from the residual variance, gave the standard error. This was partitioned for the planned comparisons.

The mean value for the refractive index of larvae from the stimulated eggs was 1·3853, and that for the control 1·3910; with a standard error of ±0·00086, the difference between them is highly significant ($P < 0·01$). Corresponding values for the half-cyst experiment were 1·3811 and 1·3902 for the larvae from stimulated eggs and control respectively; with S.E. of 0·00144, the difference between them, also, is highly significant ($P < 0·01$).

There is an important difference between the results for the two experiments. As Fig. 1 shows, for the half-cyst series, all the increase in water content of the stimulated larva occurs during the first 24 h; values for the water content of the larvae liberated on subsequent days are not significantly greater. Analysis of the results for the whole-cyst experiment, however (Fig. 2), show that the water content of the stimulated larvae liberated on day 2 is significantly higher than that of the larvae liberated on day 1 ($P < 0·05$); after that there is no further increase.

DISCUSSION

Root diffusate was obtained by standing the well washed roots of an actively growing potato plant in artificial tap water for 2 h. Our experience shows that the washing process is efficient, and that, under these conditions, the roots make no ionic contribution to the surrounding solution (Ellenby & Gilbert, 1958). Unfortunately, they may make other contributions to the solution, in addition to the hatching factor. It is well known that the hatching factor will work at a very great dilution; even impure

concentrates may have an optimum concentration of about $1:10^6$ (Ellenby & Gilbert, 1960). It is not surprising, therefore, that our measurements show that there is no detectable difference in the osmotic pressure of the hatching factor solution and the artificial tap water used to prepare it. But it is possible that other very active substances in great dilution may have been given off into the solution by the roots. Moreover, although the washed roots may not add ions to the solution, it is not impossible that they may absorb ions from it, and perhaps, therefore, alter the ionic balance in some important and unknown way. Clearly, it is not absolutely certain that the results of this work can be attributed solely to the action of the hatching factor; confirmatory tests will have to be made if and when the factor is isolated. Until that day, we can perhaps be forgiven if, with the reservations outlined above, we discuss our results on the assumption that they are, in fact, due to the action of the hatching factor. There are grounds for believing that the factor is, indeed, responsible for the increase in the water content of the stimulated larvae; the interesting difference between the results for the whole- and half-cyst experiments.

It is well known that response to the stimulus is not immediate and that some days may elapse before hatching begins. It has been shown, however, that hatching begins sooner in half cysts and sooner still in isolated eggs (Ellenby, 1956). It is therefore particularly interesting that the water content of newly liberated larvae reaches its maximum after the eggs have been in root diffusate for 24 h in the eggs from the halved cyst, and only after 2 days in the eggs from the whole cysts. The reason for the delay in hatching is unknown, but since it has been shown that movement of dissolved substances into and out of the cyst is rapid (Ellenby, 1958), it is unlikely that a delayed entry of the hatching factor is responsible.

If the water content of a larva in an egg in tap water is limited by the constraints of the egg shell (Ellenby, 1974), how is it that the larva in the stimulated egg has a higher water content? There seem to be a number of possibilities: (1) perhaps the hatching factor has a softening effect on the egg-shell so that the larva is less constricted inside it; (2) although the stimulated and control eggs were treated similarly, and, in particular, were photographed within the same time limits, perhaps the former took up water more rapidly on liberation; (3) the hatching factor may produce some change in the larva which increases the tendency for it to take up water; under such conditions it might exert greater pressure on the egg shell than the control larva.

The first of these possibilities seems the most unlikely. If the hatching factor influenced the physical nature of the egg-shell, all eggs would be affected if they were immersed in root diffusate for a sufficient time and the effect would be permanent. It is well known, however, that a characteristic feature of the hatching pattern of this organism is that not all eggs in a cyst hatch when stimulated, and that when hatching ceases there are still large numbers of eggs present, apparently identical with those which have already hatched; and almost the same pattern is repeated if the cysts are stimulated again some weeks later. Indeed, it was this phenomenon which was central to the concept of the cyst as a hatching unit (Ellenby, 1956).

That the stimulated larvae take up water more rapidly than the controls, once they are liberated, also seems unlikely; and it could not account for the difference in the results for the whole- and half-cyst experiments, unless some additional concept is involved. On the other hand, this difference between the experiments is not only

consistent with the third hypothesis, it is almost a requisite; stimulated eggs in a half-cyst respond sooner than eggs in a whole cyst, and, apparently, they take up water sooner, also.

The larva of the potato-cyst nematode fits into the egg-shell very tightly and, while there, its movement is limited; and so, too, is its water content, for it takes up water immediately it is liberated from the shell (Ellenby, 1974). But if the larva fits the shell so tightly, the constraint on it is a reflexion of the pressure it itself is exerting on the shell; and it is not impossible that it could exert more pressure without the shell being ruptured. The results of the present experiments suggest that that must be so; that the stimulated larva takes up more water than the unstimulated larva, while still inside the egg-shell, and that it must therefore exert more pressure on the shell. It should be noted that the amount of extra water is not large, and not impossible to envisage.

The results strongly suggest, then, that the hatching factor induces some change in the unhatched larva which leads to an increase in its water content, and that, inside the cyst, the effect is delayed. But there is also a delay in the response of the individual larva. Doncaster & Shepherd (1967) in their classic 'time-lapse' studies of hatching, comment on the delay in the response of the individual larva to the hatching stimulus. It varies, but 'Usually at least 3 days elapse between application of root diffusate to the eggs and the onset of vigorous movement within them'. They also point out that the movement begins sooner in a metavanadate solution; as they think that the metavanadate solution softens the eggs, this observation is consistent with the 'constraint' theory. Moreover, they point out that the three pharyngeal glands are active before active movement begins; clearly, the unhatched larva responds to the hatching factor before movement begins.

The response to the hatching factor is obviously a complex one, and the act of hatching is by no means the beginning of the response. Movement of the larva inside the shell and the act of hatching itself are the overt responses, and these are delayed by intermediate processes. One is tempted to suggest that a neurosecretory link is involved for it is the sort of situation in which it may be expected; and the delay in the response is very suggestive. Moreover, the present results which show that the hatching factor affects the water content also suggest that there may be a neurosecretory link. Davey (unpublished observations) and Davey & Sommerville (1974) have shown that water enters the activated *Phocanema*; and a neurosecretory link in the moulting response has been demonstrated for that organism (Davey & Kan, 1968).

Various aspects of the hatching response could be readily accommodated in a theory which envisaged that the reception of the stimulus triggered neurosecretory activity which, in turn led to a number of activities including hatching. For example, not all larvae may be sensitive to the stimulus at the same time and those which responded initially, could well be inhibited at a later stage along the chain of the reaction, as postulated in the view of the cyst as a hatching unit (Ellenby, 1956). Moreover, the well-known specificity of the different species could be due to differences in the receptor and not in the fundamentals of the whole response.

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