Nematologica 24 (1978): 384-392. E. J. Brill, Leiden

OSMOTIC STRESS AND THE HATCHING OF *GLOBODERA ROSTOCHIENSIS*

BY

A. J. CLARKE, R. N. PERRY and J. HENNESSY
Nematology Dept., Rothamsted Experimental Station, Harpenden, Herts., AL5 2JQ, England

When hatched juveniles are transferred from distilled water to 0.4 M sucrose or trehalose solutions their water content falls from 72% to 67%, the value observed for unhatched juveniles in eggs equilibrated with water. Juveniles move little after 6 hr immersion in 0.4 M or more concentrated sugar solutions, but dilution with water to give a sugar concentration ⩽0.1 M restores many to active movement. Few juveniles emerge from cysts in solutions of potato-root diffusate or of an artificial hatching agent (0.4 mM picrolonic acid) which contain sucrose at concentrations ⩾0.4 M. The evidence suggests that loss of solutes from the egg fluid permits hatching and supports the belief that changes in permeability of the egg shell precede other steps in the hatching process. Prolonged storage of second-stage juveniles is possible in ⩾0.4 M solutions of sugars.

Ellenby & Perry (1976) showed that the water content of *Globodera rostochiensis* (Woll.) juveniles increases immediately before hatching. Other work (Clarke & Hennessy, 1976) indicated that the fluid within the egg which surrounds the unhatched juvenile contained the sugar, trehalose (6.4%, on a dry weight basis, of whole eggs containing juveniles) at a calculated concentration of 0.34 M. In this paper we relate the above findings to each other and to a proposed mechanism of hatching (Clarke & Perry, 1977). The effects of osmotic stress on cyst-nematodes and related species were studied by Dropkin *et al.* (1958), Kämpfe (1962), Wallace (1957, 1963 & 1966), Reversat (1975) and Wright & Newall (1976).

MATERIALS AND METHODS

Cysts of *G. rostochiensis*, Ro1, were raised on pot-grown potato plants and extracted by the usual methods (Shepherd, 1970). Second-stage juveniles were obtained from cysts soaked for one week in distilled water and then immersed in 0.4 mM picrolonic acid. The juveniles were collected 3 days after adding picrolonic acid, washed three times with distilled water and used after storage for 24 hr in distilled water. Potato root diffusate, obtained as described by Shepherd (1970) was used for experiments after dilution 1 : 64.

Test solutions were prepared by adding the appropriate amounts of sugar (sucrose, trehalose) or triethylene glycol to a volumetric flask and making up to the mark with distilled water, 0.4 mM picrolonic acid or diluted potato root diffusate (1 : 64). In applying test solutions to juveniles, distilled water was added to sugar or triethylene glycol in a volumetric flask until it filled to within about 0.5 ml of the mark. After a thorough mixing, a suspension (0.2-0.4 ml) containing a known number of juveniles was added, the solution was made up to the mark and again mixed.

For experiments on the effect of osmotic stress on movement the freshly prepared suspensions (25 ml) of juveniles (about 50-60,000) were transferred to large tubes (17.5 × 3 cm) and closed with Parafilm sealing tissue (Gallenkamp). The concentration of sugars was checked with a Bellingham & Stanley pocket refractometer. Samples containing 200-400 juveniles were removed at intervals and the number moving and not moving were counted. At the end of the experiment samples of the suspension were removed, diluted ten-fold with distilled water and, after 6 and 24 hr respectively, further counts were made.

The emergence of juveniles from cysts immersed in sugar or triethylene glycol solutions containing a hatching agent was examined in diluted potato root diffusate and in 0.4 mM picrolonic acid. Triethylene glycol was used to produce osmotic stress because it combined a similarity to sugars with resistance to microbial attack. Batches of 100 cysts in threefold replication were placed in solid watch glasses. The cysts were soaked for one week in tap water, after which the bulk of the tap water was removed and replaced by a test solution of an appropriate concentration but without the hatching agent to replace the water inside the cysts by the solution. After 24 hr the solution was removed and replaced by a comparable test solution containing the hatching agent. The number of hatched juveniles was counted (Shepherd, 1970) after 10 days.

The water content of juveniles when transferred from water to various sugar solutions was determined at intervals (see Fig. 1) by interference microscopy (Ellenby, 1968a). Sugar solutions containing juveniles were placed in solid watch glasses, sealed with Parafilm under a glass lid and kept at 20°. At each time interval juveniles in the test solutions were transferred rapidly in a 25 μl micropipette to slides and sealed under a coverslip to avoid contact with the atmosphere and the water content of twenty individuals in each sucrose solution and ten in each trehalose solution was determined. The water content of unhatched juveniles was determined (Ellenby & Perry, 1976) using juveniles from eggs obtained from cysts soaked for 7 days in distilled water.

RESULTS

Fig. 1 shows the change in water content of *G. rostochiensis* juveniles after transferring from distilled water to sucrose and trehalose solutions respectively. The water content reaches a minimum after 4 to 6 hr, then increases slightly to a more constant value after 24 hr. The effects of sucrose and trehalose solutions are similar; water loss increases with the concentration of the solute. The mean water content for twenty unhatched juveniles from cysts soaked in water for 7 days is 67.2% ± 0.3. This is identical with that determined by Ellenby & Perry (1976) for unhatched juveniles from a different cyst population and is similar to the water content of free juveniles in sugar solutions of about 0.4 M.

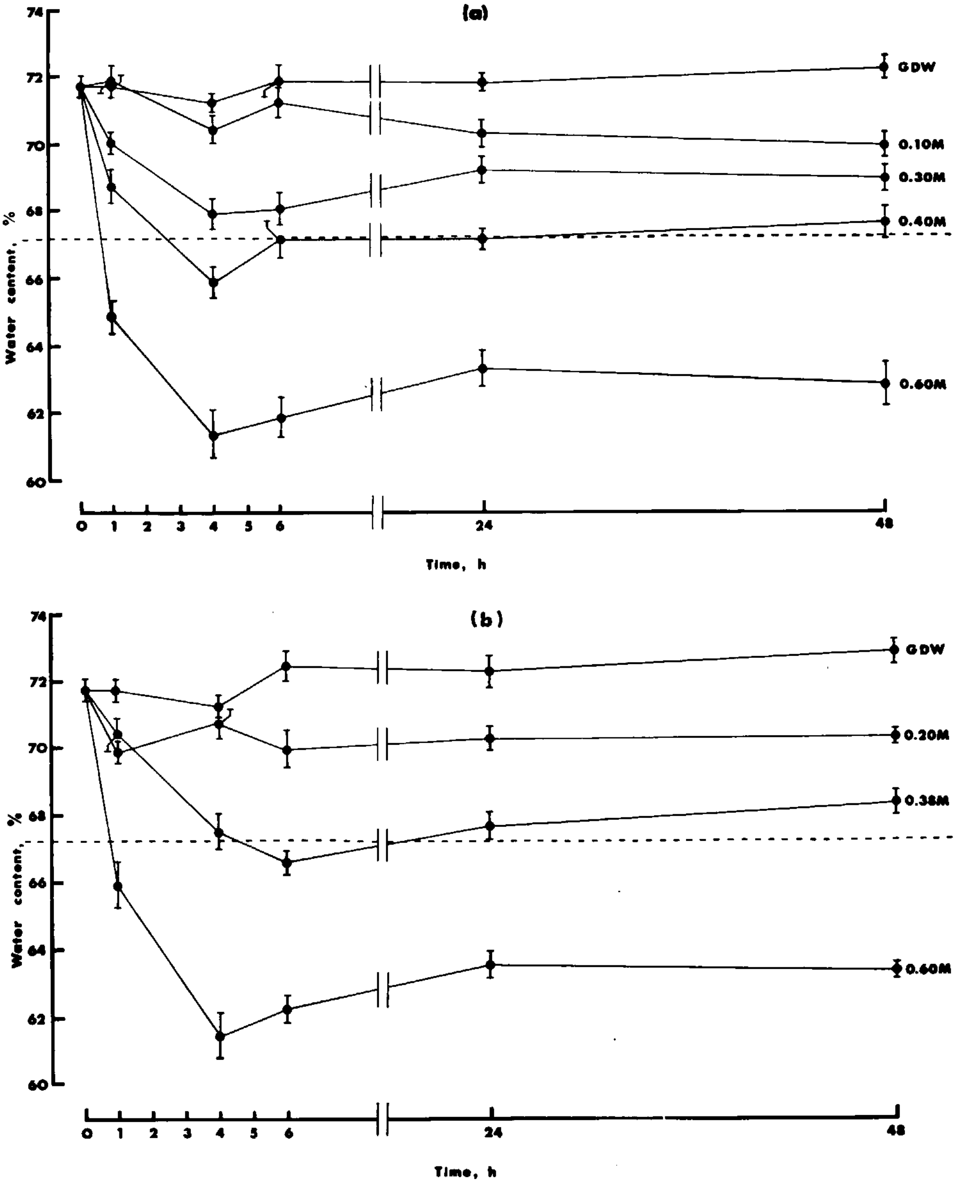

Fig. 1. Changes in water content of second-stage juveniles of *G. rostochiensis* in glass distilled water (GDW) and after removal from distilled water to various sucrose (a) and trehalose (b) solutions. Limits of standard error of mean Φ.

The movement of the juveniles in sugar and triethylene glycol solutions was observed before and after adding distilled water. Preliminary tests showed that one hour (cf. Kämpfe, 1962) was too short for juveniles to recover from osmotic stress and that 6 hr was necessary. Fig. 2 shows the percentage of juveniles moving

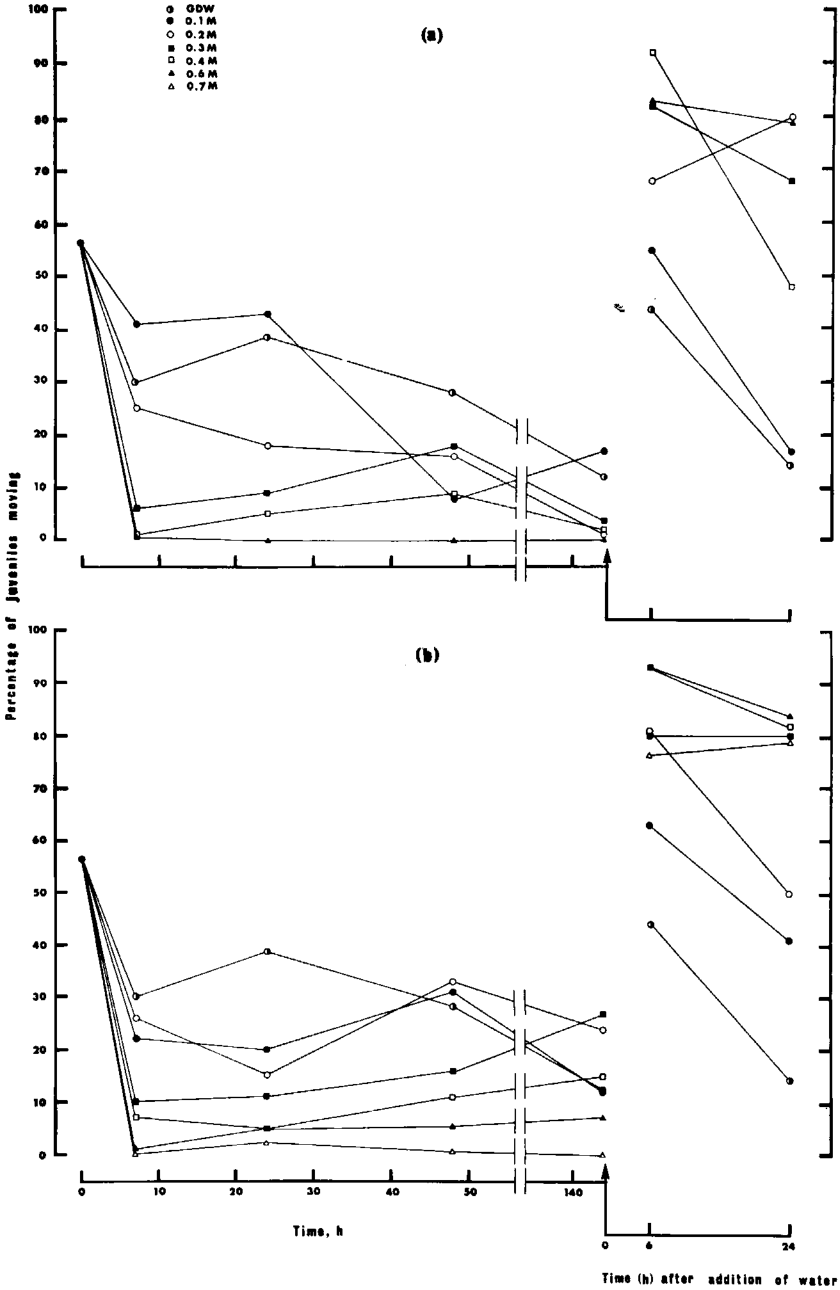

Fig. 2. Percentage of second-stage juveniles of *G. rostochiensis* moving in glass distilled water (GWD) and 0.1-0.6 M sucrose solutions (a) and 0.1-0.7 M triethylene glycol solutions (b) for periods up to 144h and then at 6h and 24h after water addition.

after 0 to 144 hr in water and in 0.1-0.6 M sucrose and 0.1-0.7 M triethylene glycol.

None of the juveniles moved after 24 hr in 0.6 M sucrose or 0.7 M triethylene glycol. In 0.4 M sucrose and 0.6 M triethylene glycol, 9% or fewer of the juveniles were observed moving during the course of the experiment. However, many juveniles moved vigorously after the solute concentration was reduced to ⩽0.1 M. The proportion of juveniles moving after adding distilled water tended to increase with increasing concentration (up to 0.4 M) of the initial solution. In similar experiments, suspensions of juveniles in 0.4 M glycerol, 0.4 M sucrose and 0.4 M trehalose stored at 20° for 4 weeks and then diluted, contained 41, 40 and 37% moving juveniles respectively. A distilled water suspension of juveniles similarly treated contained 19% moving juveniles compared with 45% at the start of the experiment. Fungi and bacteria began to grow when tests with the sugars and glycerol were prolonged and may be toxic to juveniles.

The number of juveniles moving declined irregularly when stored in water. The percentage moving increased during the early days of storage when more water was added (Fig. 2) but when a solution of ⩾0.3 M sucrose was added to a suspension of juveniles previously kept for 24 hr in a solution at the same concentration, the change in percentage moving was 1% or less.

We tested 0.1-0.7 M solutions of sucrose and triethylene glycol, which con-

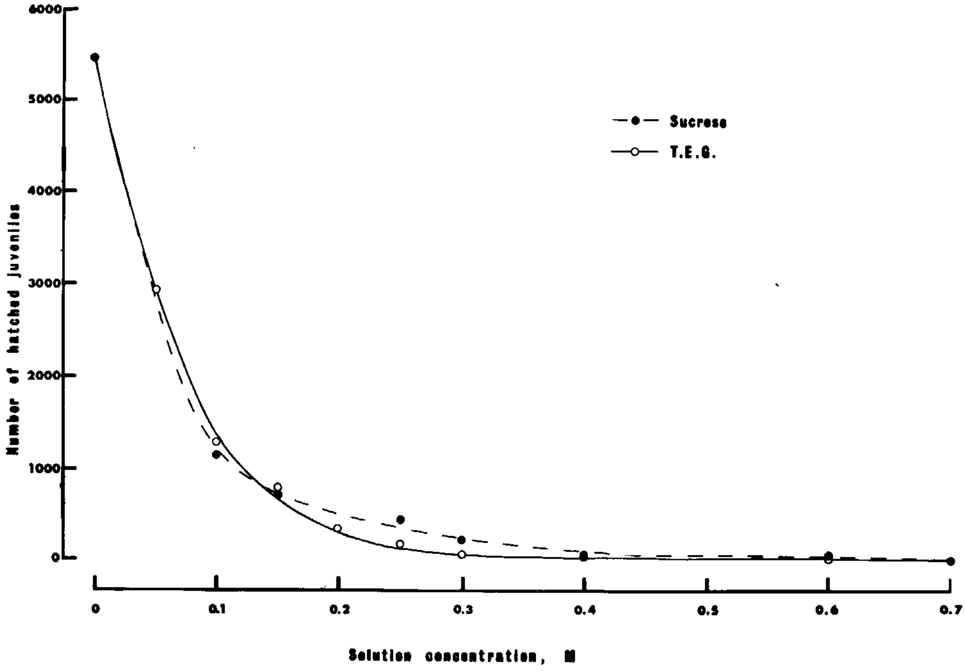

Fig. 3. Emergence of second-stage juveniles from cysts of *G. rostochiensis* immersed in 0.05-0.7 M solutions of sucrose and triethylene glycol (T.E.G.) containing potato root diffusate. Hatch in diffusate alone is initial point of curves.

tained either diluted potato-root diffusate or 0.4 mM picrolonic acid for their effect on the hatch from *G. rostochiensis* cysts. The test was improved (cf. Dropkin *et al.*, 1958) (a) by pre-treating the cysts for 24 hr with the test solution in the absence of the hatching agent, (b) by the use of dilute solutions of hatching agent and (c) by using many eggs (in cysts). Inhibition of hatching was similar for both compounds and both hatching agents. The hatches in $\leqslant$0.2 M sucrose were variable because of microbial growth in the test solutions. 8-Hydroxyquinoline (4 ppm) added to the test solutions limited microbial growth; mercuric chloride at 10 ppm inhibited hatching. Few fungi or bacteria grew in glycol solutions.

Fig. 3 shows the hatches in 0.1-0.7 M sucrose (with 8-hydroxyquinoline, 4 ppm) and triethylene glycol solutions containing diluted potato-root diffusate. Hatching decreased sharply with increasing solute concentration and was reduced to about 13% of the hatch in the potato-root diffusate in 0.15 M sucrose, 4% in 0.3 M and zero in 0.7 M. A similar response was obtained in the triethylene glycol solutions. The inhibition of hatching was reversible for experiments with sucrose, but less completely reversible for those with triethylene glycol solutions.

DISCUSSION

Wallace (1956, 1957, 1966) reported the effects of solutions of electrolytes and non-electrolytes on the hatching of *Heterodera schachtii* and *Meloidogyne javanica*. Dropkin *et al.*, (1958) studied similar effects on *G. rostochiensis*, *M. arenaria* and *M. javanica*. With *H. schachtii*, for example, osmotic inhibition of hatching was observed with solutions $>$0.01 M sodium chloride and was reversible for solutions up to and including 1 M. Reversat (1975) also studied the inhibition of hatching and its reversal in *H. oryzae* with many inorganic salts and some organic compounds. Experiments by Newall (Wright & Newall, 1976) indicated that *G. rostochiensis* juveniles had an internal osmotic pressure equivalent to about 0.1 M sodium chloride and that juveniles lost internal sodium ions rapidly in solutions containing less than 16 mM sodium chloride. Our experiments showed that the water content of juveniles decreased in 0.2 M sucrose but was little altered in 0.1 M sucrose; 0.1 and 0.2 M sucrose are osmotically equivalent to 0.05 and 0.10 M sodium chloride respectively (Wolf *et al.*, 1971). Dropkin *et al.*, (1958) suggested that potato-root diffusate might alter the egg or larval permeability of *G. rostochiensis* to permit the net influx of water, lack of which prevented hatching. However, Ellenby (1968b) showed that the egg shell was permeable to water in both directions when wet, and Ellenby (1974) and Ellenby & Perry (1976) found that juveniles in eggs treated with potato-root diffusate took up water before and immediately after hatching. They suggested that the water content of the unhatched juvenile was constrained by the egg shell.

The two barriers, egg shell and nematode body wall, which may regulate entry of water and solutes into the unhatched *G. rostochiensis* second-stage juvenile, both appear to act as semi-permeable membranes towards aqueous sugar solutions.

Thus trehalose is retained within the egg (Clarke & Hennessy, 1976) despite washing in the soil and during the isolation of cysts. The juvenile body wall is permeable to water (Ellenby, 1968b) but not to sugar solutions (Kämpfe, 1962). Marks *et al.* (1968) and Castro & Thomason (1973), using several non-cyst forming plant parasitic nematodes, found an extremely slow rate of permeability to glucose.

Our experiments showed that the water content of unhatched juveniles in eggs equilibrated with water and that of hatched juveniles in 0.4 M sugar solutions is 67% compared with 72% when fully hydrated. Furthermore, only a small proportion of juveniles move or eggs hatch in 0.4 M sugar solutions. This suggests that an essential step in the hatching mechanism is the diffusion or transport of egg fluid solutes such as trehalose out of the egg, thereby increasing hydration of the juvenile and its activity. The egg shell provides an upper limit to the water uptake of the unhatched juvenile and the egg fluid solutes limit water concentration within the egg. This hypothesis assumes that the permeability of the egg shell is changed by hatching agents, which has yet to be established. Our results suggest that a water content of about 69% is needed for freed *G. rostochiensis* juveniles to become fully mobile, and that the quiescence of unhatched juveniles may be due to water stress maintained by solutes in the egg fluid.

Ellenby's (1946, 1956) observations that hatching occurs sooner in half or punctured cysts than in whole cysts and sooner still in isolated eggs, may be attributable to accumulation of solutes from hatched and hatching eggs which may temporarily delay hatching until the concentration is reduced by diffusion. Similar effects may account for differences in the rate of water uptake of unhatched juveniles from whole and halved cysts in root diffusate (Ellenby & Perry, 1976) and the more rapid hatching of eggs near the cyst wall than nearer the centre of the egg mass (Onions, 1955; Hesling, 1959). Okada (1972) reported that aqueous extracts of crushed *H. glycines* cysts inhibited the hatching of eggs of this species but the inhibition appears to occur with too dilute a solution to be due to osmotic stress.

There are indications that the hatching of other nematodes may be governed in a similar way. The unhatched juveniles of *H. schachtii* contain more water (69%, Perry, 1977) than those of *G. rostochiensis*, and the former hatches more readily in water, although the fully hydrated nematodes of both species contain ⩾72% water. *M. incognita* eggs which hatch abundantly in water, contain juveniles with a water content of about 80% (Ellenby, 1974) and do not take up water after eclosion.

A few *G. rostochiensis* juveniles in 0.4-0.6 M sugar solutions i.e. subject to an osmotic stress similar to or greater than that of the unhatched nematode, did move; this may be related to the small spontaneous hatch which occurs in water in the laboratory.

Long-term resistance to desiccation is likely to be favoured by a relatively high concentration of solutes in the egg fluid, loss of water from the egg being opposed by the increasing concentration of egg fluid solutes. Examples of the prolonged survival of juveniles of various species in eggs under osmotic stress have been given by Dropkin *et al.* (1958), Wallace (1956) and Reversat (1975). Kämpfe (1962) studied the effects of osmotic stress induced by sucrose and sodium chloride solutions on hatched juveniles of *G. rostochiensis* and *H. schachtii.* Our results showed that after 6 hrs (cf. Kämpfe, 1962) in water the osmotic stress caused by ⩽0.6 M sucrose was reversed and many juveniles moved; juveniles stored in 0.4 M sucrose for 30 days moved after diluting the solution to ⩽0.1 M. This suggests that the hatched second-stage juveniles of *G. rostochiensis* may be stored for prolonged periods in a quiescent state in solutions which cause osmotic stress provided that microbial attack can be prevented.

ZUSAMMENFASSUNG

Osmotische Belastung und das Schlüpfen von Globodera rostochiensis

Beim Überführen geschlüpfter Larven aus destilliertem Wasser in 0,4 M Saccharose- oder Trehaloselösungen geht ihr Wassergehalt von 72% auf 67% zurück, also auf den Wert, der bei ungeschlüpften, mit Wasser äquilibirierten Larven und Eiern festgestellt wurde. Larven bewegen sich nach 6-stündigem Aufenthalt in 0,4 M oder höher konzentrierten Zuckerlösungen nur wenig, doch brachte eine Verdünnung mit Wasser auf Konzentrationen ⩽0,1 M viele zu aktiver Beweglichkeit zurück. In Lösungen von Kartoffelwurzelsekreten oder 0,4 mM Pikrolonsäure mit Zucker in Konzentrationen von ⩾0,4 M schlüpften nur wenige Larven. Die Ergebnisse lassen vermuten, daß der Verlust von gelösten Stoffen aus der Eiflüssigkeit das Schüpfen ermöglicht und unterstützen die Annahme, daß Permeabilitätsänderungen der Eischale allen anderen Schritten im Schlüpfvorgang vorausgehen.

REFERENCES

CASTRO, C. E. & THOMASON, I. J. (1973). Permeation dynamics and osmoregulation in *Aphelenchus avenae. Nematologica* **19**, 100-108.

CLARKE, A. J. & HENNESSY, J. (1976). The distribution of carbohydrates in cysts of *Heterodera rostochiensis. Nematologica* **22**, 190-195.

CLARKE, A. J. & PERRY, R. N. (1977). Hatching of cyst-nematodes. *Nematologica* **23**, 350-368.

DROPKIN, V. H., MARTIN, G. C. & JOHNSON, R. W. (1958). Effect of osmotic concentration on hatching of some plant parasitic nematodes. *Nematologica* **3**, 115-126.

ELLENBY, C. (1946). Ecology of the eelworm cyst. *Nature, Lond.* **157**, 451-452.

—— (1956). The cyst of the potato-root eelworm (*Heterodera rostochiensis* Wollenweber) as a hatching unit. *Ann. appl. Biol.* **44**, 1-15.

—— (1968a). Determination of the water content of nematode worms by interference microscopy. *Experientia* **24**, 84.

—— (1968b). Desiccation survival in the plant parasitic nematodes, *Heterodera rostochiensis* Wollenweber and *Ditylenchus dipsaci* (Kühn) Filipjev. *Proc. R. Soc.* B. **169**, 203-213.

—— (1974). Water uptake and hatching in the potato cyst nematode *Heterodera rostochiensis* and the root knot nematode *Meloidogyne incognita. J. Exp. Biol.* **61**, 773-779.

ELLENBY, C. & PERRY, R. N. (1976). The influence of the hatching factor on the water uptake of the second-stage larva of the potato cyst-nematode *Heterodera rostochiensis. J. Exp. Biol.* **64**, 141-147.

HESLING, J. J. (1959). The emergence of larvae of *Heterodera rostochiensis* Woll. from single cysts. *Nematologica* **4**, 126-131.

KÄMPFE, L. (1962). Vergleichende Untersuchungen zur Autökologie von *Heterodera rostochiensis* Wollenweber und *Heterodera schachtii* Schmidt. *Parasitologische Schriftenreihe* **14**, 142-170.

MARKS, C. F., THOMASON, I. J. & CASTRO, C. E. (1968). Dynamics of the permeation of nematodes by water, nematicides and other substances. *Exptl. Parasitol* **22**, 321-337.

OKADA, T. (1972). Hatching inhibitory factor in the cyst contents of the soybean cyst nematode *Heterodera glycines. Appl. Entomol. Zool.* **7**, 99-102.

ONIONS, T. G. (1955). Distribution of hatching within the cyst of the potato root eelworm *Heterodera rostochiensis*. *Quart. J. micro. Sci.* **96**, 495-513.

PERRY, R. N. (1977). Water content of the second-stage larva of *Heterodera schachtii* during the hatching process. *Nematologica* **23**, 431-437.

REVERSAT, G. (1975). Influence d'une inhibition préalable par la pression osmotique sur l'éclosion des masses d'oeufs du nématode *Heterodera oryzae*. *Cah. ORSTOM, ser. Biol.*, **10**, 189-206.

SHEPHERD, A. M. (1970). In: (SOUTHEY, J. F., Ed.) *Laboratory Methods for Work with Plant & Soil Nematodes* pp. 23-33, HMSO, London.

WALLACE, H. R. (1956). The emergence of larvae from cysts of the beet eelworm *Heterodera schachtii* Schmidt, in aqueous solutions of organic and inorganic substances. *Ann. appl. Biol.* **44**, 274-282.

—— (1957). The stimulating properties of some organic substances on cysts of the beet eelworm *Heterodera schachtii* Schmidt. *Ann. appl. Biol.* **45**, 251-255.

—— (1963). Influence of the environment. In: *The Biology of Plant Parasitic Nematodes*. pp. 69-74. E. Arnold, London.

—— (1966). The influence of moisture stress on the development, hatch and survival of eggs of *Meloidogyne javanica*. *Nematologica* **12**, 57-69.

WOLF, A. V., BROWN, M. G. & PRENTISS, P. G. (1971). In WEAST, R. C. (ed): *Handbook of Chemistry & Physics*, 52nd Ed. The Chemical Rubber Co., Cleveland, Ohio, U.S.A.

WRIGHT, D. J. & NEWALL, D. R. (1976). Nitrogen excretion, osmotic and ionic regulation in nematodes. In CROLL, N. A. (ed): *Organization of Nematodes* pp. 163-210. Acad. Press, London & New York.