

Hatching agents as stimulants of movement of *Globodera rostochiensis* juveniles

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SUMMARY

When *Globodera rostochiensis* juveniles inactivated by storage in distilled water for eight to ten days were treated with millimolar solutions of organic and inorganic (Ba^{2+} , Zn^{2+} , La^{3+}) hatching agents, many juveniles were stimulated to move. Inactivated juveniles were not stimulated by treatment with solutions of nicotinic acid, Ca^{2+} , K^+ , Mg^{2+} or Na^+ , which have little hatching activity. In general, more juveniles migrated through sand columns moistened with solutions of hatching agents than through columns treated with non-hatching agents. The stimulation of movement appears to be a property of both natural and artificial hatching agents. Movement was induced in inactivated juveniles by treatment with decationised potato-root exudate or 0.3 mM picrolonic acid in 4 mM sodium 1, 2-di (2-aminoethoxy) ethane-N, N', N', N' -tetra-acetate (EGTA), suggesting that external Ca^{2+} is not essential for the stimulation. The stimulants may act by binding to, or displacing internal Ca^{2+} of the juvenile. Juveniles inactivated by storage in 0.4 M trehalose moved sluggishly when potato-root exudate was added, but removal of the osmotic stress by dilution caused most juveniles to move vigorously. The results support the hatching mechanism suggested by Clarke and Perry (1977).

RÉSUMÉ

Les agents d'éclosion considérés comme stimulants des mouvements des juvéniles de Globodera rostochiensis

Lorsque des juvéniles de *Globodera rostochiensis* rendus inertes par stockage dans l'eau distillée pendant 8 à 10 jours sont traités avec des solutions millimolaires d'agents d'éclosion, organiques ou inorganiques (Ba^{2+} , Zn^{2+} , La^{3+}), une partie d'entre eux sont incités à se mouvoir. En revanche, les juvéniles inertes ne sont pas stimulés par des solutions d'acide nicotinique, de Ca^{2+} , K^+ , Mg^{2+} ou Na^+ lesquelles n'ont que peu d'action sur l'éclosion. D'une manière générale, un plus grand nombre de juvéniles migre à travers des colonnes de sable humidifiées par des agents d'éclosion qu'à travers celles traitées avec des produits n'ayant pas d'influence sur l'éclosion. La stimulation du mouvement paraît donc être une caractéristique des agents d'éclosion, tant naturels qu'artificiels. Chez des juvéniles inertes, le mouvement a été induit par traitement avec un exsudat radiculaire décationisé de pomme de terre ou par une dose de 0,3 mM d'acide picrolinique dans 4 mM de 1, 2-di (2-aminoéthoxy) éthane-N, N', N', tétra-acétate de sodium, suggérant ainsi que le Ca^{2+} externe n'est pas indispensable pour cette stimulation. Les stimulants pourraient agir en bloquant ou déplaçant le Ca^{2+} interne du juvénile. Les juvéniles rendus inertes par stockage dans du tréhalose 0,4 M ont des mouvements très lents lorsque de l'exsudat radiculaire est ajouté, mais la diminution de la pression osmotique, par dilution, amène ces juvéniles à se mouvoir plus activement. Ces résultats sont en conformité avec le mécanisme de l'éclosion suggéré par Clarke et Hennessy (1977).

Potato-root exudate (PRE) appears to have a dual role in the hatching of eggs of the potato-cyst nematode, *Globodera rostochiensis*, acting both on the egg-shell (Clarke, Perry & Hennessy, 1978; Atkinson, Taylor & Ballantyne, 1980; Atkinson & Taylor, 1983; Clarke & Perry, 1985) and on the juvenile. We confirmed (Clarke & Hennessy, 1983a, 1984) that PRE stimulates the active movement and migration of the free second stage juveniles (Weischer, 1959) and showed that juveniles treated with PRE contained less glycogen and lipid than juveniles not exposed to PRE. Robinson, Atkinson and Perry (1986) also found increased lipid consumption by juveniles in the presence of PRE; furthermore they observed an increase in the lipid consumption of the unhatched juvenile in eggs treated with PRE. In the latter case as

with other experiments demonstrating increased metabolic activity of juveniles in eggs treated with PRE (Atkinson & Ballantyne, 1977a, b), the increased activity cannot be dissociated from that which may arise from changes in egg-shell permeability and the removal of osmotic stress by the loss of egg-fluid solutes (Clarke, Perry & Hennessy, 1978).

The stimulation of movement of *G. rostochiensis* juveniles by PRE may be an additional role for the hatching factor which is also common to certain metal cations and artificial hatching agents. Therefore, we tested the effect of decationised potato-root exudate (DPRE), a purified hatching factor isolated from PRE, and various artificial hatching agents on the motility of *G. rostochiensis* juveniles.

Materials and methods

The materials and methods used were similar to those of Clarke and Hennessy (1984). A population of *G. rostochiensis* (Rol) was raised on potato plants grown under glass; the cysts were extracted by standard methods (Shepherd, 1970). To obtain second-stage juveniles, cysts were kept in glass distilled water (GDW) for one week, and then in a 1:4 dilution of PRE. The free juveniles were harvested three to seven days after emergence, washed five times with distilled water by decantation, and the bulk of the water removed by filtration.

For experiments on movement, the juveniles were inactivated by storage in GDW (2 to 3 mm deep) in Petri dishes at 25°. Samples containing about 200 juveniles were removed at intervals and those moving and not moving were counted. When only a small proportion of juveniles was moving, usually after storage for seven to ten days, a suspension of the juveniles was transferred to a volumetric flask together with a solution of the test material, and the suspension made up to volume. After mixing, the suspensions were transferred to sample jars which were kept at 25°. Samples were removed at intervals and the juveniles moving and not moving were counted.

The procedure for experiments on nematode migration was based on that of Evans and Wright (1982). Sand columns (20 mm high) were prepared by adding a suspension of sand (particle size 250-600 µm). The columns were equilibrated with the inorganic salt solutions by passage of 200 ml of solution through each tube before use. Aliquots (1 ml) of each solution containing about 200 juveniles were carefully added to the columns. Five columns were used for each test solution. The number of juveniles added to the columns was determined by counting the juveniles in five samples; a sample for counting and a sample to be added to the column being removed alternately. Each column was placed in a sample jar (20 ml) containing the test solution (5 ml). After 24 h at 25° the columns were removed and the number of juveniles that passed through the columns was counted.

Suspensions of juveniles in 0.4 M trehalose containing PRE or DPPE were prepared as described by Clarke, Perry and Hennessy (1978). Samples containing about 200 juveniles were removed at intervals and the number moving and not moving counted. After two days, the suspensions were diluted ten-fold with the appropriate solution; samples were removed at intervals for further counts.

PRE and DPPE were prepared as described by Shepherd (1970) and Clarke and Hennessy (1983b) respectively. A sample of the major hatching factor was isolated from PRE after extensive purification (Clarke, unpubl. data). The test solution contained 50 µg/ml. Solutions of EGTA and EGTA/picrolonic acid were adjusted to pH 7.8 by the addition of NaOH; the EGTA

solutions to contain DPPE were brought to about pH 8 before the addition of the hatching agent to give a final pH of 7.8. The pH of the EGTA solutions was checked at the beginning and end of the experiment. Artificial tap water (ATW) was prepared as described by Greenaway (1971).

Hatch ratings (Clarke & Shepherd, 1964; 1966) were calculated from $(H_s - H_w/H_E - H_w) \times 100$, where H_s is the hatch obtained with the test compound, and H_w and H_E are the hatches obtained with distilled water and PRE at its optimum concentration, respectively. The significance of results was established by analysis of variance.

Results

Figure 1 shows the percentage of juveniles moving after juveniles inactivated by storage in GDW were treated with GDW, a 1:4 dilution of PRE, a solution of purified hatching factor, and four organic acids. The solutions of purified hatching factor, 0.3 mM picrolonic acid and 0.3 mM picric acid, all caused many juveniles to move, the proportion of juveniles moving being comparable to that observed for juveniles in a 1:4 dilution of PRE. Mechanical stimulation or increased oxygenation were not responsible for the increased movement of juveniles in the test solutions because the addition of further volumes of ATW or GDW to suspensions of juveniles immobilised by storage in ATW or GDW caused few juveniles to move (Fig. 1, and Clarke & Hennessy, 1984). When tested at their optimum hatching concentrations for *G. rostochiensis* (Clarke & Shepherd, 1968) the effectiveness of the organic acids in promoting the movement of juveniles after 24 h treatment was 0.3 mM picrolonic acid > 3 mM cinchononic acid > GDW > 10 mM nicotinic acid > 2 mM picric acid. Juveniles in 2 mM picric became tightly coiled and few moved, whereas in a 0.3 mM solution, most moving and non-moving were serpentine in form. Figure 2 shows the percentage of juveniles moving after the addition of various cations to suspensions of juveniles inactivated by storage in GDW. Few juveniles moved in 0.6 mM Ca^{2+} , K^+ , Mg^{2+} , or Na^+ . The proportion moving was comparable to that observed for juveniles in GDW or ATW. In 0.6 mM solutions of Ba^{2+} , Zn^{2+} , or La^{3+} , however, there were more juveniles moving than in the GDW or ATW controls. The proportion moving in the Zn^{2+} or La^{3+} solutions was comparable to that found for juveniles in a 1:4 dilution of PRE. The order of effectiveness of the ions in promoting movement was $La^{3+} > Zn^{2+} > Ba^{2+} > GDW$. When $BaCl_2$ was tested at its optimum hatching concentration of 10 mM (Clarke & Shepherd, 1966), the percentage of juveniles moving was only slightly greater than that observed for juveniles in a 0.6 mM solution. In both solutions the number of juveniles moving decreased rapidly.

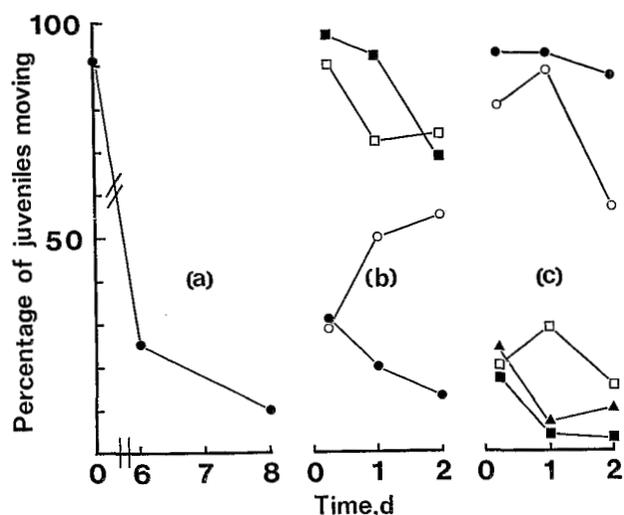


Fig. 1. (a) Percentage of juveniles of *G. rostochiensis* moving in glass-distilled water (GDW) (black circle) for periods up to 8 days; (b) and thereafter at 6 h, one and two days in GDW (black circle), 3 mM cinchomeric acid (white circle), 0.3 mM picrolonic acid (black square), a 1 : 4 dilution of potato-root exudate (white square); (c) a solution of purified hatching factor (black circle) 0.3 mM (white circle) and 2.0 mM (black square) picric acid, 0.3 mM (white square) and 10 mM (black triangle) nicotinic acid, respectively.

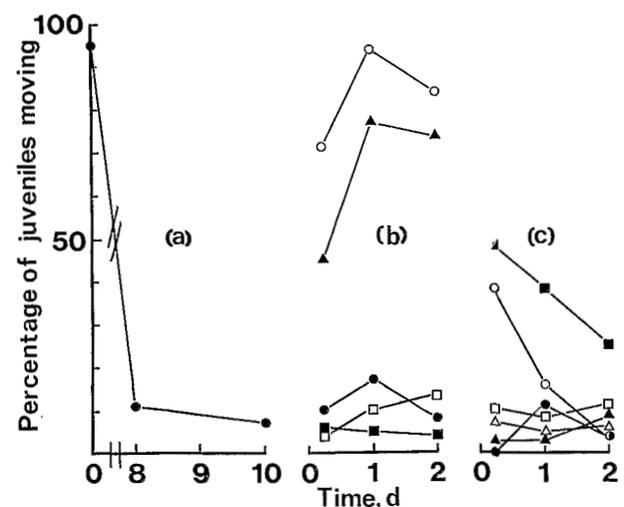


Fig. 2 (a) Percentage of juveniles of *G. rostochiensis* moving in glass-distilled water (GDW) (black circle) for periods up to 10 days; (b) and thereafter at 6 h, one and two days in GDW (black circle), 0.6 mM LaCl₃ (white circle), 0.6 mM MgCl₂ (black square), 0.6 mM MgSO₄ (white square), 0.6 mM ZnSO₄ (black triangle); (c) artificial tap water (black circle) 0.6 mM (white circle) and 10 mM (black square) BaCl₂, 0.6 mM CaCl₂ (white square), 0.6 mM KCl (black triangle), 0.6 mM NaCl (white triangle), respectively.

In general, juveniles inactivated by storage in GDW or ATW tended to adopt a straight or slightly curved posture, but in the presence of active stimulants even the non-moving juveniles assumed a serpentine form. The onset of curvature and movement rapidly followed the addition of an active stimulant to a suspension of juveniles. Table 1 shows that 2 h after the addition of DPRE to inactivated juveniles, 89 % were moving compared with only 22 % moving in the GDW control.

Table 1

Percentage of juveniles of *G. rostochiensis* moving after eight days in glass distilled water (GDW) and then in GDW or a 1 : 4 dilution of decationised potato-root exudate (DPRE) for a further 0, 2 or 4 h.

Time (hours)	Percentage of juveniles moving in GDW	Percentage of juveniles moving in DPRE
0	15	—
2	22	89
4	26	93

Table 2

Number of juveniles (mean of five replicates with standard error of mean) that passed in 24 h through sand columns moistened with artificial tap water (ATW), glass-distilled water (GDW) 10 mM BaCl₂, 0.6 mM MgSO₄, 10 mM CaCl₂, 0.6 mM ZnSO₄, 0.6 mM LaCl₃, 0.3 mM picrolonic acid, 1 : 4 dilution of potato-root exudate (PRE), and of potato-root exudate (PRE), and a solution of purified hatching factor (HF), respectively, after 197 ± 3 juveniles were added to each column. Numbers followed by a * were significantly greater (P > 0.01) than those unmarked.

Test solution	Number of migrated juveniles (Mean ± SE)
ATW	3 ± 1
GDW	5 ± 1
10 mM BaCl ₂	6 ± 1
0.6 mM MgSO ₄	20 ± 1
10 mM CaCl ₂	24 ± 1
0.6 mM ZnSO ₄	71 ± 3*
0.6 mM LaCl ₃	127 ± 5*
0.3 mM picrolonic acid	138 ± 6*
PRE	160 ± 12*
HF	194 ± 2*

Table 2 shows that significantly more juveniles ($p < 0.01$) migrated through sand columns moistened with 0.6 mM LaCl_3 , 0.6 mM ZnSO_4 , a solution of purified hatching factor, or 0.3 mM picrolonic acid, than through columns moistened with GDW, ATW, 10 mM BaCl_2 , 10 mM CaCl_2 , or 0.6 mM MgSO_4 . Picrolonic acid and LaCl_3 caused almost as many juveniles to migrate as a 1 : 4 dilution of PRE.

DPRE and picrolonic acid induced movement in GDW-inactivated juveniles when the test solutions also contained 4 mM EGTA (Fig. 3). The reagents also induced movement in GDW-inactivated juveniles after their prolonged treatment (48 h) with 4 mM EGTA, whereas the juveniles in 4 mM EGTA remained largely inert.

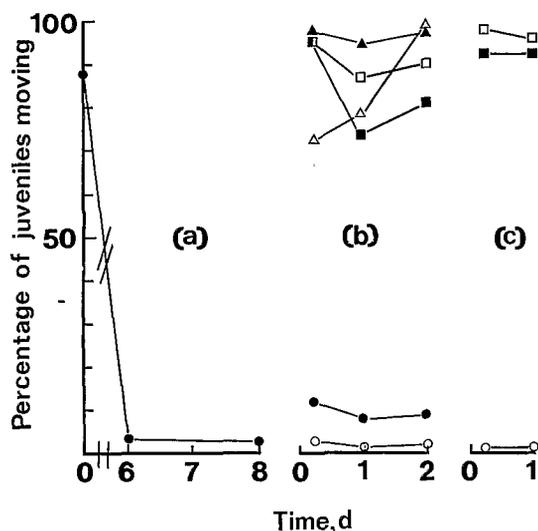


Fig. 3. (a) Percentage of juveniles of *G. rostochiensis* moving in glass-distilled water (GDW) (black circle) for periods of up to 8 days; (b) and thereafter in GDW (black circle), 4 mM 1, 2-di (2-aminoethoxy) ethane-N, N, N', N'-tetra-acetate (EGTA) (white circle), 0.3 mM picrolonic acid in 4 mM EGTA (black square), 0.3 mM picrolonic acid (white square), a 1 : 4 dilution of decationised potato-root exudate (DPRE) (black triangle), and a 1 : 4 dilution of DPRE in 4 mM EGTA (white triangle) respectively for periods up to two days; (c) and in 4 mM EGTA for 2 days and then in 4 mM EGTA (white circle), 0.3 mM picrolonic acid in 4 mM EGTA (black square) and a 1 : 4 dilution of DPRE in 4 mM EGTA (white square), respectively, for 6 and 24 h.

Figure 4 shows that when juveniles inactivated by osmotic stress through immersion in 0.4 M trehalose were treated with a 1 : 4 dilution of DPRE in 0.4 M trehalose, or a 1 : 16 dilution of PRE in 0.4 M trehalose, many of the juveniles were stimulated into movement. A 1 : 4 dilution of PRE in 0.4 M trehalose was less

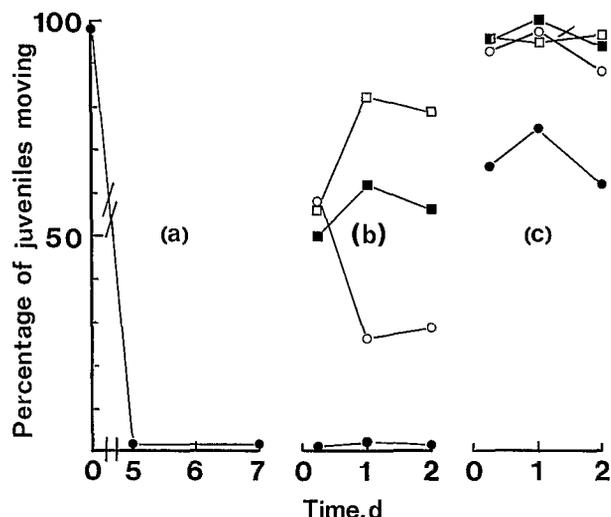


Fig. 4. (a) Percentage of juveniles of *G. rostochiensis* moving in 0.4 M trehalose (black circle) for periods up to seven days; (b) and thereafter at 6 h, one and two days in 0.4 M trehalose (black circle), 0.4 M trehalose containing a 1 : 4 (white circle) or 1 : 16 (black square) dilution of potato-root exudate (PRE), and 0.4 M trehalose containing a 1 : 4 dilution of decationised potato-root exudate (DPRE) (white square); (c) and at 6 h, one and two days after a ten-fold dilution of the solution with glass-distilled water (black circle), a 1 : 4 (white circle) or a 1 : 16 (black square) dilution of PRE, or a 1 : 4 dilution of DPRE (white square), respectively.

effective than the 1 : 16 dilution, perhaps because of the additional osmotic stress. The movement of the stimulated juveniles was, however, slow and irregular, and in marked contrast to similarly stimulated juveniles not under osmotic stress. The sluggish behaviour was not because of the viscosity of the medium as active juveniles freshly immersed in 0.4 M trehalose continued to move vigorously for several hours (e. g. Fig. 4, > 60 % of the juveniles were actively moving after 1 h immersion).

Many of the juveniles kept in 0.4 M trehalose, moved when the solute concentration was diluted ten-fold with the appropriate concentrations of DPRE or PRE. In the absence of the osmotic stress the juveniles moved vigorously.

Discussion

Clarke and Hennessy (1984) showed that *G. rostochiensis* juveniles inactivated by storage in GDW were stimulated into active movement by the addition of PRE. We now report that the inactivated juveniles are similarly stimulated by treatment with DPRE or solutions of a hatching factor isolated from PRE. The stimulatory action of PRE thus seems related to its hatching factor content.

Tests of four organic acids showed that when assayed at their optimum hatching concentrations, the order of their effectiveness (picrolonic acid > cinchomeric acid > nicotinic acid = picric acid = GDW) in stimulating movement conformed with their hatch ratings, 102, 59, 4 and 4, respectively, PRE = 100 (Clarke & Shepherd, 1968). Picric acid, however, was found to be an effective stimulant of movement when tested as a more dilute solution (0.3 mM). Although inactive as hatching agents for *G. rostochiensis*, picric acid and nicotinic acid are excellent hatching agents for *Heterodera schachtii* (Clarke & Shepherd, 1964).

Figure 2 shows that of the seven metal cations tested, only those with hatching activity, i. e. Ba^{2+} , La^{3+} and Zn^{2+} , stimulated an appreciably greater proportion of juveniles into movement, than did the ATW or GDW controls. The order of effectiveness of the ions was $La^{3+} > Zn^{2+} > Ba^{2+}$ but the hatch ratings of the ions do not differ greatly, $LaCl_3$ 50, $ZnSO_4$ 49, $BaCl_2$ 59 (Clarke & Shepherd, 1966; Clarke & Hennessy, 1981). The different order of response to the ions may reflect the somewhat different requirements of the sites of action in the egg-shell and juvenile.

In additional experiments (Tab. 2), solutions of $LaCl_3$, $ZnSO_4$, picrolonic acid, and of purified hatching factor facilitated the migration of juveniles through sand columns, although $BaCl_2$ solutions did not. Ba^{2+} , however, was the least effective of the cations in stimulating movement (Fig. 2), and the proportion of juveniles moving in the $BaCl_2$ solutions decreased rapidly. Moreover, simple counts of the number of juveniles moving, make no allowance for any differences in the vigour of movement.

Overall, the evidence suggests that the stimulation of the movement of *G. rostochiensis* juveniles is a property of both inorganic and organic hatching agents. Some compounds, e. g. picric acid, may be more effective as stimulants of movement than as hatching agents, and *vice versa* for compounds such as $BaCl_2$. The stimulation of inactivated juveniles may be of use as an alternative form of bioassay for the hatching agents of *G. rostochiensis* and perhaps for those of other cyst nematode species.

Clarke, Perry and Hennessy (1978), suggested that unhatched *G. rostochiensis* juveniles were immobilised because of the osmotic stress, equivalent to 0.4 M trehalose, of the egg-fluid solutes. We showed that eggs did not hatch in solutions of PRE containing ≥ 0.4 M sucrose i. e. that the inactivating effect of osmotic stress overrode any stimulation of the unhatched juvenile by PRE. As an extension of the hatching experiments we examined the effect of PRE and DPRE on free juveniles immobilised by storage in 0.4 M trehalose (Fig. 4). We found that stimulation of juvenile movement was observable. The juveniles showed similar lethargic and limited body movements to juveniles in eggs treated with PRE for one to two days (Doncaster & Shepherd, 1967). The

low intensity of activity is in marked contrast with the vigorous movement of free juveniles in PRE, or juveniles in eggs about to hatch, i. e. after three or more days in PRE. The results support the belief that a change in egg-shell permeability and the removal of osmotic stress by the release of solutes is an essential preliminary for the initiation of hatching. It is also probable that the stimulation of the unhatched juvenile occurs as the hatching factor reaches it, and that juvenile movement will increase as the osmotic stress decreases.

Solutions of $CaCl_2$ or of ATW (containing about 2 mM Ca^{2+}) did not initiate movement in inactivated juveniles and the hatching agents, DPRE and picrolonic acid, stimulated juvenile movement both in the presence and absence of the Ca^{2+} -chelating agent, EGTA. The evidence suggests that the stimulation of the juvenile is not closely dependent on the concentration of external free Ca^{2+} , and the results reinforce previous evidence (Clarke & Hennessy, 1983b) which showed that *G. rostochiensis* eggs hatch when there is little free Ca^{2+} in the medium. Furthermore, La^{3+} which, because of its role as a Ca^{2+} -mimic is an inhibitor of active Ca^{2+} -transport, is effective as a stimulant of both movement and of hatching (Clarke & Hennessy, 1981).

Our evidence suggests that Ca^{2+} uptake by egg-shell and juvenile is not essential for the initiation of hatching but, although internal free or bound Ca^{2+} may be involved in the stimulation of juveniles, the role of hatching agents stimulants may be to displace, or bind Ca^{2+} , perhaps initiating a permeability change and influencing Ca^{2+} flow.

We suggested (Clarke & Perry, 1977; Clarke, Perry & Hennessy, 1978) that hatching agents induce a permeability change in the egg-shell. Evidence for such a change is the uptake of water (Ellenby & Perry, 1976) and of Ca^{2+} (Atkinson, Taylor & Ballantyne, 1980) by the unhatched juveniles when eggs are treated with PRE. Other evidence (Clarke & Hennessy, unpubl. data) indicates that the egg-fluid trehalose is released from PRE-stimulated eggs in the lag period prior to the emergence of the juveniles. Ca^{2+} may have a structural role in maintaining egg-shell impermeability. Clarke and Perry (1985) showed that the hatching agents, DPRE, La^{3+} , and Zn^{2+} displaced bound Ca^{2+} from the lipoprotein layer of the egg-shell.

Maintenance of an intracellular Ca^{2+} -concentration of 0.01-1.0 μM appears to be essential for the normal working of some cellular systems. The low concentrations are achieved by the active transport of Ca^{2+} from the cell, and are opposed by extra cellular concentrations of about 1 mM. Muscle action in particular is dependent on Ca^{2+} flow. It is noteworthy that the GDW-inactivated juveniles tend to adopt a linear form and that they respond relatively rapidly to stimulants (Tab. 1) by curving their bodies, even when this is not followed by more active motion. The presence of hatching factors may be essential for the vigorous movement of *G.*

rostochiensis juveniles and for their attraction to the host plant roots (Clarke & Hennessy, 1984).

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