Observations on the response of the dorsal and subventral oesophageal glands of *Globodera rostochiensis* to hatching stimulation

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SUMMARY

Using video-enhanced contrast microscopy, the timing and nature of the response of the oesophageal glands of unhatched *Globodera rostochiensis* to hydration and hatch stimulation were studied. Secretory granule accumulation in subventral glands was solely a response to hydration. Accumulation of granules and increase in size of the gland cell nucleolus both occurred in dorsal glands in response to hydration and were further significantly affected by exposure to potato root difusate (PRD). No secretory material was observed to be voided into the oesophagus or intestine and it is concluded that the oesophageal glands are not involved in the process of eclosion and the limited response to PRD was part of the preparation of the juveniles for a feeding phase after hatching.

RÉSUMÉ

Observations sur la réaction des glandes œsophagiennes dorsales et subventrales de Globodera rostochiensis *aux stimuli d'éclosion*

Le déroulement et la nature de la réaction des glandes œsophagiennes de juvéniles non encore éclos de *Globodera rostochiensis* à l'hydratation et aux stimuli d'éclosion ont pu être étudiés, grâce à l'utilisation de la vidéo-microscopie à contraste renforcé. Une accumulation de granules secrétoires dans les glandes subventrales, est la seule réaction à l'hydratation. L'accumulation de granules et l'augmentation de la taille du nucléole des cellules glandulaires se produisent dans la glande dorsale en réaction à l'hydratation; cette réaction est significativement modifiée par l'action de diffusat de racines de pomme de terre (PRD). Comme il n'a pas été observé de transit de matériel secrétoire vers l'œsophage ou l'intestin, il est supposé que les glandes œsophagiennes ne prennent pas part au processus d'éclosion et que la réaction, limitée, au PRD participe à la préparation des juvéniles pour la phase nutritionnelle qui suit l'éclosion.

Recent research on the hatching mechanism of cyst nematodes has concentrated on the sequence of events during the period between application of the hatching stimulus and eclosion (Perry, 1986). However, studies on the hatching process of the potato cyst nematode, *Globodera rostochiensis*, have not examined the response of the three oesophageal glands in detail. Cinematographic studies by Doncaster (1974) demonstrated that these glands became active soon after stimulation by potato root diffusate (PRD) and were swollen with secretory material at eclosion; however, oral emission of secretions was not seen and Doncaster and Shepherd (1967) considered them unimportant in egg hatch. Observations have not been made on *G. rostochiensis* to determine whether secretory material is voided through the anus, but during hatching of the animal parasitic nematode, *Necator americanus*, Croll (1974) considered that " feeding " movements may be instrumental in flushing enzymes backwards down the intestine and into the egg via the anus.

Using high resolution, video-enhanced contrast microscopy we have examined the timing and nature of the response of the dorsal and subventral oesophageal glands of *G. rostochiensis* to hatch stimulation.

Materials and methods

Cysts of *G. rostochiensis* Ro 1, grown on potato cv. Arran Banner in pots, were taken from a single generation harvested in 1985 and stored dry at room temperature (20°) for six months after extraction from the soil. Cysts were soaked for one week in glass distilled water (GDW) prior to experimentation unless otherwise stated. PRD was obtained (Fenwick, 1949) from pot cultures (cv. Désirée) and used after dilution with

GDW 1 in 4 by volume; in tests of four weeks duration, the PRD elicited > 85 % hatch.

Batches of soaked cysts were transferred to PRD for various periods between 2 to 48 h (see Fig. 2); control cysts were retained in GDW. Cysts were then cut open and the freed eggs mixed before an aliquot was transferred to GDW on a microscope slide. Eggs were viewed under a Reichert differential interference contrast microscope. The responses to PRD stimulation of the dorsal and subventral glands of unhatched, second stage juveniles and the process of eclosion were recorded at high magnification on 2.5 cm video tapes using video-contrast enhancement (Wyss & Zunke, 1986 *a*) and were analysed where required by single frame evaluation (Wyss & Zunke, 1986 *b*). Hatched second stage juveniles were also examined. An attempt to quantify secretory activity was made by recording, at each period of exposure to PRD, the extent of granule formation in the glands. Four categories were used : a gland was termed " full " when numerous secretory granules were present in all parts of the gland; " half-full " if only about 50 % of the gland contained granules, usually concentrated at the anterior end; " filling " if only a few granules were present and " empty " if granules were rare or absent.

Changes in the diameter of the dorsal and subventral gland cell nucleoli were examined in unhatched juveniles from batches of soaked cysts in PRD and GDW. The diameter of each nucleolus was measured using an eyepiece graticule (calibrated with a slide graticule) fitted on a Zeiss Nomarsky interference contrast microscope. For each period of PRD or GDW exposure (Tab. 1), the gland cell nucleoli of at least twenty unhatched juveniles were measured; results were subjected to two way analysis of variance.

Initial observations indicated that changes in the diameter of the nucleoli and in the state of the glands may have been, in part, a response to hydration of previously dry cysts. To check this aspect, dry cysts were placed in GDW and the changes in dorsal and subventral glands of unhatched juveniles were monitored at intervals over a period of fourteen days. The shrunken state of unhatched juveniles from dry cysts, causing marked enhancement of cuticular annulations (Fig. 1 A), prevented detailed examination of the oesophageal glands until the cysts had been soaked for at least 4 h. The unhatched juveniles had then become sufficiently hydrated for observations to be made.

Results

Accumulation of secretory material in the dorsal oesophageal gland

The dorsal glands of most of the unhatched juveniles examined after 4 h in GDW were empty of secretory granules (Fig. 1 B); the few exceptions contained only a small number of granules. During the hydration period, the glands showed marked secretory activity and granules accumulated. After seven days soak, about 35 % of the nematodes had dorsal glands which were full of granules and less than one third remained empty; however, there was little additional accumulation of granules during the second week in GDW.

Further secretory activity was observed when unhatched juveniles were transferred to PRD. Secretory material accumulated in the dorsal gland of unhatched juveniles after increasing periods in PRD (Fig. 2 a); this was most marked after 4 h in PRD when the majority of juveniles had full dorsal glands (Fig. 1 C). By 48 h, 80 % of the juveniles had dorsal glands packed full of granules.

Where juveniles were about to commence eclosion, the dorsal gland was full and granules were also present in the distended gland duct (Fig. 1 D). Movement of granules was frequently visible within the gland duct, which opens about 5 µm behind the stylet, and occasionally seen in the anterior portion of the gland itself, but there were no indications of granules disintegrating in the ampulla at the end of the duct or of any material passing through the outlet into the oesophagus.

The dorsal gland of every hatched second stage juvenile examined was full of secretory granules (Fig. 1 E). The granules were packed closely together in the anterior portion of the gland and more loosely packed in the posterior portion. Granules were present in the dorsal gland duct and were frequently moving backwards and forwards within the duct.

Response of the dorsal gland nucleolus

During hydration, the nucleolus of the dorsal oesophageal gland increased significantly ($P < 0.01$) in diameter from a mean of 2.38 ± 0.09 µm after 4 h in GDW to 3.35 ± 0.13 µm after seven days; there was no increase in diameter during the following seven days (Tab. 1). A further increase in the diameter of the nucleolus was induced by exposure to PRD. Unhatched juveniles from soaked cysts placed in PRD had larger ($P < 0.01$) nucleoli compared with juveniles from cysts in GDW whose gland cell nucleolus showed no significant change.

Accumulation of secretory material in the two subventral glands

After 4 h soak in GDW the subventral glands contained no secretory granules. However, during subsequent hydration granules began to accumulate until by seven days 87 % of the unhatched juveniles had subventral glands full of secretory granules. There was no further increase in the accumulation of subventral gland secretions with exposure to PRD (Fig. 2 b). Thus,

Table 1

The diameter (μm) of the nucleolus of the dorsal œsophageal gland of unhatched second stage juveniles of *Globodera rostochiensis* (Twenty values for each time period; S. E. = standard error of mean)

A. Changes in diameter of the nucleolus of unhatched juveniles from dry cysts after transfer to glass distilled water (GDW)

	Period in GDW					
	4 h	*6 h*	*24 h*	*7 d*	*11 d*	*14 d*
Mean	2.38	2.37	2.39	3.35	3.31	3.33
S.E.	0.09	0.10	0.08	0.13	0.13	0.15

B. Changes in the diameter of the nucleolus of unhatched juveniles from batches of cysts soaked for 1 wk in GDW and transferred to either potato root diffusate (PRD) or fresh GDW for a further 1 wk

	Period in GDW									
	0	*1 h*	*2 h*	*3 h*	*6 h*	*24 h*	*48 h*	*72 h*	*96 h*	*7 d*
Mean	3.44	3.44	3.36	3.22	3.29	3.44	3.58	3.58	3.58	3.58
S.E.	0.10	0.14	0.15	0.12	0.12	0.14	0.15	0.15	0.19	0.19
	Period in PRD									
	0	*1 h*	*2 h*	*3 h*	*6 h*	*24 h*	*48 h*	*72 h*	*96 h*	*7 d*
Mean	3.44	3.72	3.65	3.65	3.79	3.94	3.86	3.79	4.01	4.01
S.E.	0.10	0.14	0.17	0.13	0.15	0.12	0.12	0.11	0.12	0.12

by contrast to the dorsal gland, the secretory activity of the subventral glands appears to be solely a response to hydration rather than PRD stimulation.

Movement of granules within the glands was not seen and, although there is an indication from observations at 1 and 2 h (Fig. 2 b) of a reduction in secretory content of the glands, there was no evidence of disintegration of granules and granules were rarely seen in the gland ducts. Similarly, no feeding movements were observed in unhatched juveniles or in hatching juveniles indicating that enzymes were not flushed from the glands backwards down the intestine.

In all hatched second stage juveniles examined, both subventral glands were full of secretory granules which were evenly distributed throughout the glands (Fig. 1 E).

RESPONSE OF THE SUBVENTRAL GLAND NUCLEOLUS

There was no change in the diameter of the nucleolus of either subventral gland at any time during or after eclosion, with or without PRD stimulation. The mean diameter (30 values) was 1.92 ± 0.08 μm.

Discussion

Eggshells of *G. rostochiensis* have an inner lipoprotein layer (Perry, Wharton & Clarke, 1982) controlling permeability. A Ca^{2+} - dependent change in permeability of the eggshell is one of the initial stages of the hatching sequence (Clarke, Perry & Hennessy, 1978; Clarke & Perry, 1985). The involvement of enzymes in eggshell changes prior to nematode hatching has been postulated

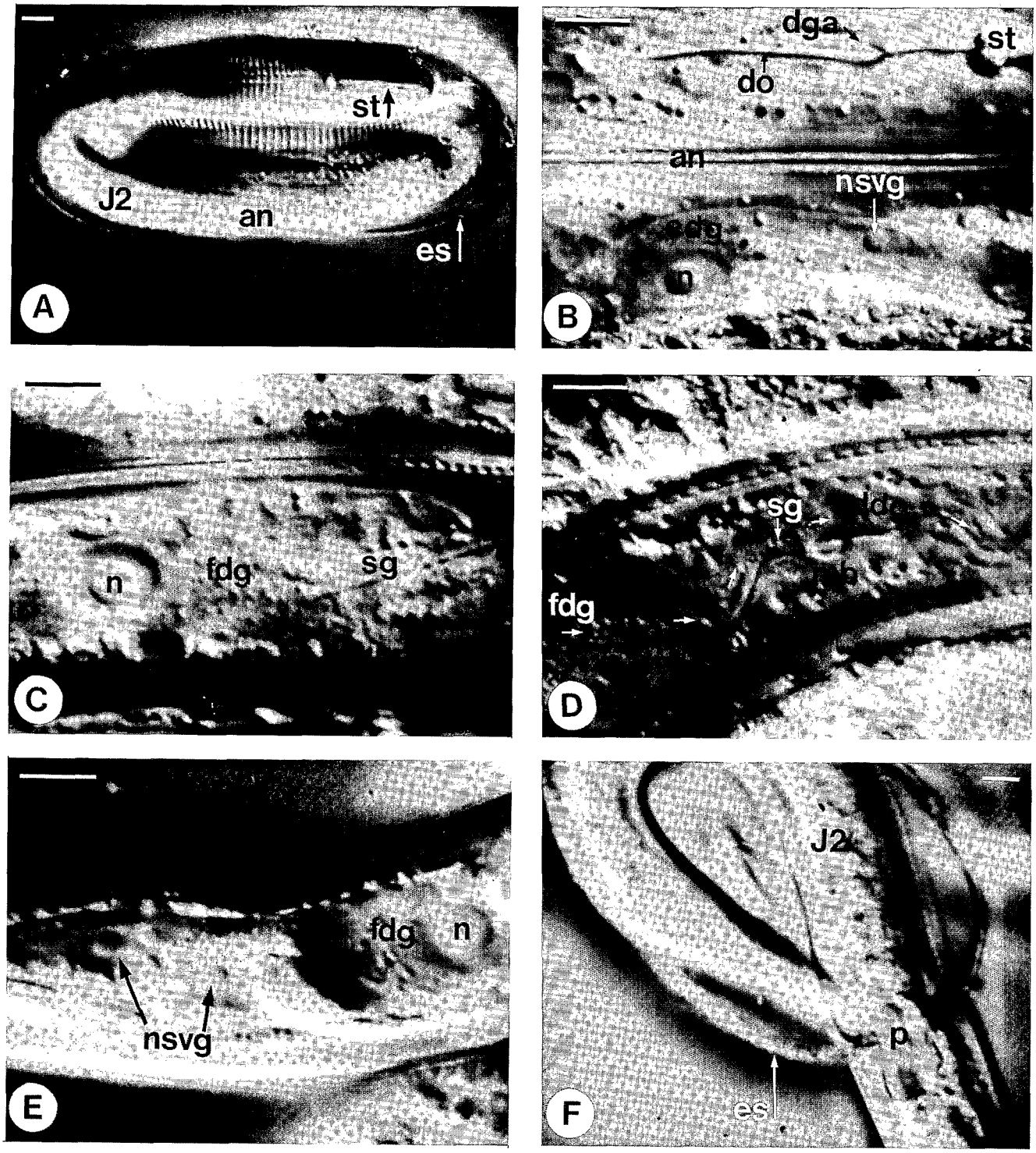

Fig. 1. The shrunken state of unhatched juveniles of *Globodera rostochiensis* from dry cysts (A) prevented examination of the oesophageal glands until after 4 h (B) when the dorsal gland was empty of secretions. Stimulation by potato root diffusate resulted in the majority of juveniles having full dorsal glands (C) often with some secretory granules present in the gland duct (D); the glands remained full after hatching occurred (E) with granules distributed evenly throughout the glands. There were no indications of softening or flexibility in eggshells prior to hatching of the juveniles and the eggshell clearly remained rigid during eclosion (F).

Key to abbreviations; an : annulations. ddg : duct of the dorsal gland. dga : dorsal gland ampulla. do : duct of the oesophagus. edg : empty dorsal gland. es : eggshell. fdg : full dorsal gland. J 2 : second stage juvenile. n : dorsal gland nucleus, showing typical " fried egg " appearance with central nucleolus clearly defined. nsvg : subventral gland nucleus with distinct nucleolus. mb : median bulb. p : J 2 hatching through inextensible opening in the eggshell. sg : secretory granules. st : stylet. Scale bars = 5 μm.

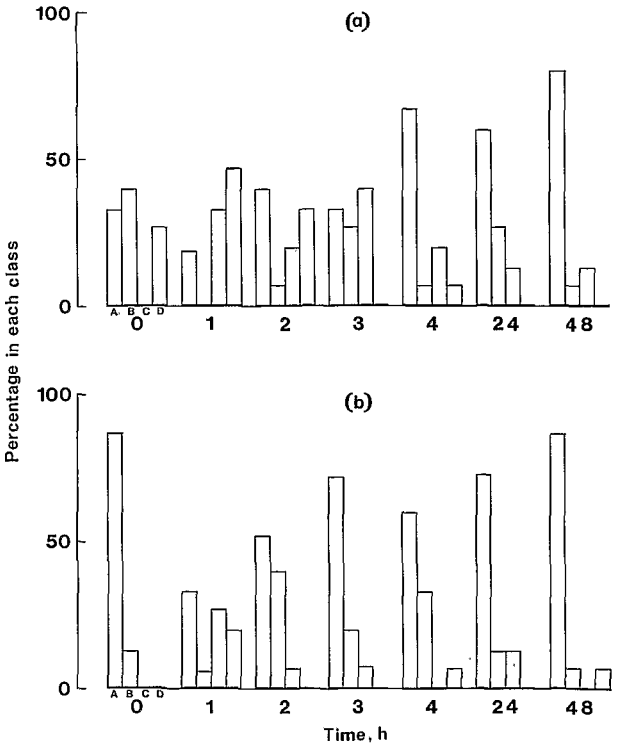

Fig. 2. Changes in the amount of secretory material accumulating in (a) the dorsal oesophageal gland and (b) the subventral oesophageal glands of unhatched second stage juveniles of *Globodera rostochiensis* from soaked cysts during a 48 h period after transfer to potato root diffusate. A : glands full of secretory granules; B : glands half-full; C : glands filling; D : glands empty (see text).

frequently (Perry & Clarke, 1981) and the oesophageal glands are often suggested as a source for enzyme secretions which may be involved in hatching.

In juveniles of *Meloidogyne javanica*, Bird (1968) considered that the hemizonid may function as a receptor for hatching stimuli and trigger enzyme synthesis in the subventral oesophageal glands causing hydrolysis of the eggshell lipid layer before hatching. However, the overt responses of *M. javanica* and *G. rostochiensis* to hatching stimuli differ markedly. In *M. javanica*, the eggshell becomes flexible and distorts in response to juvenile head movements (Wallace, 1968) indicating enzyme involvement in eclosion while, in *G. rostochiensis*, the eggshell remains inflexible and the unusual behavioural sequence of coordinated stylet thrusts leading to eclosion (Doncaster & Seymour, 1973) is probably dictated by the rigid eggshell and indicates absence of enzymic activity (Perry, 1987). This is supported in the present work, for there were no indications of any softening or flexibility in eggshells prior to hatching of the juveniles (Fig. 1 F).

Observations on the response of the dorsal and subventral oesophageal glands of unhatched juveniles of *G. rostochiensis* to hydration and hatch stimulation indicate that the glands are unlikely to be involved in the process of eclosion. The response of the subventral glands was solely to hydration and involved only accumulation of secretory material without any changes in nucleolus size; PRD did not elicit any further response. There was no observable passage of secretions of the subventral glands into the intestine, and there were no metacorpus pulsations or indications of fluid passage backwards through the intestine. Thus, it seems improbable that secretory material is voided through the anus during the hatching process in the manner suggested by Croll (1974) for *Necator americanus*. The role of the subventral glanc secretions seems entirely related to feeding and specifically to food digestion within the intestine as observed for *Heterodera schachtii* (Wyss & Zunke, 1986 *b*).

Accumulation of secretory granules in the dorsal gland and an increase in the diameter of the dorsal gland cell nucleolus were primarily a response to hydration of previously dry cyst contents. An additional response to PRD stimulation, found only in the dorsal gland, was manifest in two ways. Firstly, there was a further increase in the diameter of the nucleolus. This has also been observed in juveniles of *G. rostochiensis* exposed to PRD and then artificially hatched before measurement (Atkinson, Taylor & Fowler, 1987). The second response of the dorsal gland was an increase in number and activity of the secretory granules in the gland and, especially, the gland duct. The activity was restricted to movement of granules backwards and forwards in the duct and there was no evidence of disruption of granules, or of passage of secretory material from the ampulla of the gland duct into the oesophagus, or of any discharge through the stylet orifice. This supports the conclusions of Doncaster and Shepherd (1967) that gland secretions were unimportant for eclosion.

During the feeding cycle of *H. schachtii*, the glands empty secretory material into the oesophagus and intestine and then accumulate further secretory material (Wyss & Zunke, 1986 *b*). The dorsal and subventral glands of hatched juveniles of *G. rostochiensis* were packed full of secretions, and at no stage in the hatching sequence were the glands observed to void their contents. It seems probable that the role of the dorsal and subventral glands relates solely to the sequence of events involved in feeding. The response of the dorsal gland to PRD is not in the context of a hatching role but more likely to be a preparation of the juvenile for an active feeding phase soon after eclosion. As the water hatch of this population of *G. rostochiensis* was less than 10 %, the large number of unhatched juveniles showing gland responses to hydration cannot represent the few that would hatch without PRD stimulation. In the field, cysts are likely to remain hydrated.

Physiological changes in the unhatched juvenile are known to occur rapidly after hatch stimulation (Perry, 1986) and only 5 min exposure to PRD is required to trigger the hatching response in *G. rostochiensis* (Perry & Beane, 1982). Although the oesophageal glands are involved in events subsequent to hatching, the dorsal glands respond rapidly to PRD stimulation. Thus, by 4 h in PRD, accumulation of secretory granules resulted in a majority of unhatched juveniles with full dorsal glands with a concomitant significant increase in the size of the gland cell nucleoli. This supports and extends the nature of the bimodal action of PRD (Perry, 1986) to alter eggshell permeability and to stimulate juvenile activity and metabolism; part of the latter role now appears to include a preparation for the invasion and feeding phases following hatching and host location.

Although oesophageal gland secretions are unlikely to play a role in hatching, this does not necessarily preclude enzymic involvement. Enzymes need not emanate from the juvenile; they could be located in the egg fluid or eggshell (Perry, 1987). These possibilities are currently being investigated.

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