THE ESCAPE OF VELIGERS FROM THE EGG CAPSULES OF NASSARIUS OBSOLETUS AND NASSARIUS TRIVITTATUS (GASTROPODA, PROSOBRANCHIA)

by

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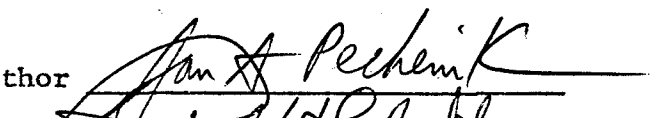
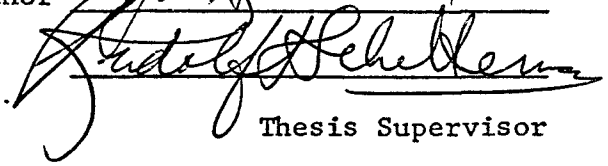
Signature of Author 

Certified by 

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ABSTRACT

Many species of prosobranch gastropod deposit their eggs in tough capsules affixed to hard substrates. Generally, there is a small opening near the top of such capsules, occluded by a firm plug (operculum) which must be removed before the veligers can escape. Although the removal of the operculum is generally attributed to embryonic secretion of enzymes, there is little experimental support for this suggestion. In the limited experiments which have been reported, all dealing with species that emerge as juvenile snails, no attempt was made to determine the properties of the hatching substance, or the timing of its production.

My research has dealt with the escape of veligers from the egg capsules of two related species, Nassarius obsoletus and N. trivittatus. Their egg capsules are quite similar in size, number of eggs contained, general morphology, and the thickness of the material plugging the opening at the top. Both hatch as swimming veligers, after about one week of encapsulated development.

By adding fresh plug material to small volumes of sea-water containing veligers obtained prior to, or at known times after their normal hatching, I have demonstrated conclusively the essentially chemical nature of operculum removal for these two species. In addition, the hatching substance was found to be produced in a short pulse, to be functionally short-lived, and to be species-specific in its action for the two species considered. There is no evidence that the secretion of the hatching substance is stimulated by short pulses of light or increased temperature; the capsules of N. obsoletus contain many more embryos than are needed to successfully remove the plug, so that complete synchrony of hatching

substance production by all individuals within a capsule is probably not necessary.

Lastly, the observed rates at which N. obsoletus veligers leave their egg capsules were compared with those predicted from an equation assuming random movement of individuals. A close agreement was found, the capsules losing 98% of their residents within 45 to 55 minutes of the first escape. Thus, the location of the exit by an individual is probably by chance.

INTRODUCTION

Many species of prosobranch gastropod deposit their eggs in tough capsules affixed to hard substrates. Generally, there is a small opening near the top of such capsules, occluded by a firm plug (operculum) which must be removed before the embryos can escape. The sizeable oothecan literature deals almost exclusively with basic descriptions - size, shape, number of eggs or embryos contained, where and when the capsules are found in the field (e.g., Anderson, 1966; Bandell, 1974; D'Asaro, 1969, 1970a, 1970b; Franc, 1941; Golikov, 1961; Graham, 1941; Kohn, 1961; Knudsen, 1950; Ponder, 1973; Radwin and Chamberlin, 1973; Thorson, 1946). The remaining studies deal primarily with the structure and formation of the capsules, rather than with how the young escape.

Studies conducted to determine the fine-structure of prosobranch egg capsules have dealt with only a few species. Although Ankel (1937), Fretter (1941), and Fretter and Graham, 1962 (pp 337-338) thought that the material plugging the top of the capsule of Nucella lapillus was produced apart from the rest of the capsule and simply cemented in, it is apparently continuous with the thin innermost layer (Kostitzine, 1940) and seems to be composed of the same histochemically distinctive material (Bayne, 1968). Fretter (1941) asserted that the plug was composed of mucous, and Hyman (1967, p. 305) claimed that all prosobranch capsule opercula are protein, although she cites no reference. Recently, however, the plug of Nucella lapillus has been shown to contain carbohydrate as well as protein (Bayne, 1968). Additional fine-structural (Flower et al., 1969) and biochemical (Hunt, 1966, 1971) studies have been conducted on the capsules of Buccinum undatum, but no attention was devoted specifically

to the operculum. The sectioned capsule of Urosalpinx cinerea, including the plug, has been figured (Hancock, 1956) and photographed (Tamarin and Carriker, 1968); the operculum appears as a separate entity, cemented into the top of the capsule. Appearances of oothecae in section have also been reported for Colus stimpsoni (West, 1973), Murex trunculus (Dulzetto, 1950), Nassarius reticulatus (Ankel, 1929), Nassarius mutabilis (Ankel, 1929), and Ocenebra erinacea (Fretter, 1941), but no special attention is drawn to the opercular plug.

It required over one-hundred years from the time attention was first drawn to egg capsule formation by Cuvier (1817) (as discussed by Dulzetto, 1950) until workers agreed on the basics of the process for any species. Although it is now accepted that the capsules are formed within the capsule gland before leaving the oviduct, the details of the process are unknown. Fretter (1941) gave detailed descriptions for Buccinum undatum, Nassarius reticulatus, and Nucella lapillus. However, these are based entirely on the orientation of fibers in the different capsule layers and on the position and orientation of the capsule within the gland; capsules have never been sectioned at different stages of formation, so that Fretter's scheme is quite speculative. Moreover, Fretter's hypothesis does not explain the structure of all prosobranch egg capsules equally well, as revealed by the ultrastructural studies of Tamarin and Carriker (1971) on the capsules of Urosalpinx cinerea.

In a review paper on the hatching of aquatic invertebrates, Davis (1968) suggested that the removal of the plug is commonly attributable to embryonic secretion of enzymes (p. 336). However, most of the ideas about how this first step in the hatching process is accomplished are without experimental support, deriving solely from descriptions of the

process (e.g., Bandell, 1974; Chess and Rosenthal, 1971; Davis, 1967; Houbrick, 1974; Kohn, 1961; Murray and Goldsmith, 1963; Portman, 1955). The limited experiments which have been reported, all dealing with species that complete their development within the egg capsule, did suggest that a hatching substance is produced by advanced embryos, but no attempt was made to determine the nature or properties of the substance, or the timing of its production.

Ankel (1937) demonstrated that the plug material of Nucella lapillus capsules was unaffected by sea-water, pancreatin and pepsin, potash lye, and mineral acids; plugs were also unaffected by trypsin (Kostitzine, 1940). Opercula were, however, degraded after emersion in the juices of crushed, prehatched embryos, and in the presence of living embryos (Ankel, 1937). Capsule opercula remained firmly held in the mouths of Urosalpinx cinerea egg capsules from which all embryos had been removed, even after five months in sea-water (Hancock, 1956). De Mahieu et al. (1974) followed changes in salinity, pH, and protein and free amino acid concentrations in the intracapsular fluid of Adelomelon brasiliana during development. They observed slight, but erratic changes in salinity, and only a small change in pH, from 7.2 initially to 6.8 - 6.9 in the last stages of development. They did find a slight increase in amino acid concentration, and a more substantial increase in total protein concentration shortly before hatching, which they claim indicates the essentially chemical nature of the process. However, the same graph illustrates considerable increases in protein and free amino acid concentrations much earlier in development as well, which did not result in release of juveniles. Moreover, they detected no change in the protein composition of the intracapsular fluid during development.

Regarding the liberation of larvae from the sand-mucous collars of Polinices heros, Giglioli (1955) appeals to a hatching substance released by the embryos, whereas Hanks (1960) claims that the embryos have no active role in the process, the disintegration of the collar being an inevitable consequence of prolonged exposure to sea-water. Ziegelmeier (1961) has apparently come to a similar conclusion of embryonic passivity during hatching in Lunatia nitida (cited in Davis, 1968). Thus the belief in a chemically-mediated release of young is not universal. West (1973) has suggested that the capsule plug of Colus stimpsoni is degraded by "external factors such as bacteria and fungi"; this idea lacks any experimental support.

The egg capsules of Nassarius obsoletus and N. trivittatus are quite similar in size (approximately 1.5 mm high), number of eggs contained (fifty to several hundred), and general morphology (Scheltema and Scheltema, 1964), and both have openings occluded by plugs of approximately 100 μ in thickness. Hatching takes place at the veliger stage for both species, after about one week of encapsulated development (Scheltema and Scheltema, 1964; Scheltema, 1967). ~~The precise method of escape from the capsule is unknown (Scheltema, 1967).~~

~~This thesis~~ demonstrates that a specific hatching substance is produced by encapsulated embryos of both species and examines some of its properties and the timing of its production, primarily for N. obsoletus, through laboratory experimentation.

In addition, the actual escape of veligers from the egg capsules of N. obsoletus was examined. Due to the large size of the egg capsule

relative to the size of an individual veliger, the small size of the opening of the capsule, and the jelly-like nature of the intracapsular fluid (Costello and Henley, 1971), one might expect that many hours would be required for the capsules to become empty if the veligers are swimming about aimlessly. However, if movements within the egg capsule are directed towards the opening of the capsule, much less time would be required. This directed motion would not require a sophisticated physiological mechanism. Since the intracapsular fluid is soluble in sea water (Costello and Henley, 1971), a viscosity gradient might be set up in the capsule soon after hatching began, with the intracapsular medium becoming decreasingly viscous near the opening. Under such conditions, the veligers would be directed towards the capsule opening simply because they would move more quickly when facing that direction as they rotated about within the capsular fluid. Evidence is presented here for a random escape of veligers.

I use the terminology of Giese and Pearse (1974). "Embryo " refers to an individual which has not yet taken up a free-living existence, whereas the term "larva" refers to a free-swimming individual which has entered the plankton to complete its development.

METHODS

Egg capsules were obtained from snails held in the laboratory. N. obsoletus were collected from the mudflats at Barnstable Harbor, Massachusetts, and N. trivittatus were collected by dredging in Buzzard's Bay, Massachusetts. All animals were fed on shredded clam meat.

The experiments fall into two main categories - those designed to examine the production of a hatching substance by embryos and its functional longevity, and those designed to detect its continued production by hatched larvae. Shelled, pigmented embryos were obtained from intact capsules by slicing into the capsule wall with a razor blade and expelling the embryos with gentle squeezing of the capsule using forceps. Other capsules were monitored hourly until hatching occurred; larvae from these capsules were then used at known times from the onset of hatching.

To assay for the production of hatching substance, a known number of embryos or larvae were first pipetted into the bottom of a small glass chamber, whose empty weight had been determined on a Roller-Smith Precision balance. The chamber was then reweighed and the weight difference before and after filling used to estimate the volume of water in the chamber. A freshly-deposited egg capsule was then sliced in half parallel to its long axis so that each half contained one part of the plug firmly held in the sectioned neck (fig. 1). One half of this capsule was placed at the bottom of the chamber with the animals, while the other half of the same capsule was placed in a similar chamber with sea-water but no embryos or larvae, as a control. Chambers were examined daily for three to six days, or until the plug was observed missing from the neck of the capsule.

Chambers were held in air-tight containers at room temperature (20 - 23 °C), at a relative humidity of 100%. Embryos and larvae remained active under these conditions for at least six days. Five-micron filtered sea-water was used in all experiments.

The functional longevity of the hatching substance was assessed by placing intact egg capsules into individual glass chambers in a small volume of water (approximately 10 µl), and monitoring until hatching began. The actual volume of water in each chamber was determined by weighing, as above. One hour after hatching had begun, the chamber fluid was removed to a new glass chamber. Fresh plug material was then added to the hatching fluid from 0 to 3 hours later, and its integrity examined several days later. Capsule opercula were held in sea-water as controls.

Specific details for each experiment are given in the tables. In experiments testing the species specificity of hatching substance, plugs of both species were included with the advanced embryos of one species; the control plug was produced by the species whose embryos were being tested.

Additional controls were set up to examine the possibility of eventual spontaneous plug detachment. Capsules containing osmotically-killed eggs and embryos were held for two months in five-micron filtered sea-water at room temperature; periodically these were gently squeezed to assess plug integrity. Freshly deposited egg capsules were emptied of their contents and sliced in half as described above, and their opercula examined after two months in sea-water at room temperature.

To compare experimentally-determined densities of embryos required to initiate plug release with the actual density of embryos in egg capsules, the volumes of four N. obsoletus capsules which had hatched out known num-

bers of larvae were estimated with a microsyringe.

Finally, a few preliminary experiments were conducted to see whether light and/ or increased temperature might stimulate the release of the hatching substance by embryos. N. obsoletus egg capsules were harvested within 24 hours of their deposition in the laboratory, and placed individually in plastic "Beem" capsules normally used for SEM work, in approximately 0.1 ml of five-micron filtered sea-water. The trays of "Beem" capsules were then kept in the dark. Each night, in the dark, the water in each container was agitated with a pipette for aeration. After 10 days, some of the trays were placed under the light from two "Wild" microscope lamps for two hours. No attempt was made to control temperature. After the light/ heat pulse, the trays were returned to the dark. Five to 10 hours later the extent of hatching was determined in the experimental trays relative to the unexposed controls.

An equation predicting the rate of collisions of confined gas molecules with the walls of their container was used to predict the rate at which veligers would leave an egg capsule if their escape were a purely chance process. The equation is (Sears, 1959):

$$\text{Number of collisions per unit area per unit time} = \tfrac{1}{4}\, n\, \bar{v},$$

where "n" is the number of molecules per unit volume, and "$\bar{v}$" is the average speed of the molecules in the container. The equation assumes that the gas molecules are uniformly distributed in the container. By treating the encapsulated veligers as gas molecules, and assuming that the collision of a veliger with the opening at the top of the capsule results in the escape of the individual from the capsule, the rate at which veligers should leave is predicted by multiplying the above expression by the

surface area of the opening of the egg capsule, 0.08 mm^2 (N = 2). The average speed of encapsulated veligers was determined by analyzing movie footage of the movements inside an emptying egg capsule when only 8 veligers remained, by which time individual movements could be followed. The model egg capsule was assumed to initially contain 50 veligers. The value of "n" in the equation was changed after each hypothetical 5-minute interval by subtracting the number of escapees in the interval from the number present at the beginning of the interval, and dividing by the hypothetical egg capsule's volume, 0.6 µl. Predicted rates of escape were compared with those actually observed in the laboratory.

RESULTS and DISCUSSION

Before hatching, the capsule plug has very distinct outlines in both species, and is firmly held in the neck of the egg capsule (fig. 1). The plug is not actually dissolved in the hatching process, but becomes amorphous, greatly softened, and is easily dislodged from the capsule wall. The plugs of freshly-deposited capsules appeared as distinct in outline, and as securely held as those from capsules containing veligers close to hatching. Plug degredation seems to be a sudden event, rather than a gradual process. Hancock (1956) and Kostitzine (1940) implied that the loosening of the plug is a gradual process in Urosalpinx cinerea and Purpura (=Nucella) lapillus capsules, respectively.

No control plugs became softened, or loose in the necks of their egg capsules over the two-month observation period or in the course of any particular experiment. Those plugs destined to be degraded generally fell away from the necks of the sliced egg capsules by the day following the start of the experiment. This supports the contention that degredation of the plug does not involve a gradual build-up of hatching substance within the capsule.

Density of N. obsoletus embryos necessary to free the plug

The results of 14 experiments in which different numbers of embryos were confined in volumes of sea-water ranging from 2.4 µl to 230.0 µl are presented in order of increasing embryonic density (Table I). Plug release was scored in all chambers holding more than 0.4 embryos per µl, regardless of the absolute number of individuals contained.

The mean number of developing eggs enclosed by a single capsule is highly variable between species, even within the same genus. N. vibex capsules, for example, generally hold 20 eggs (Scheltema, 1962), N. pygmaeus capsules hold 40 to 50 eggs (Lebour, 1937), and N. obsoletus capsules hold as many as 300 eggs (Costello and Henley, 1971), although Scheltema (1962) gives a range of 40 to 100. If removal of the capsule operculum is generally a chemical process, one might expect the parcelling of eggs into capsules to be related to the minimum number of individuals required to produce sufficient hatching substance to degrade the plug. However, the actual densities of embryos in N. obsoletus capsules are over one hundred times the minimum density required for successful hatching (Table II). For these egg capsule volumes (Table II), a single individual should be able to remove the operculum and escape.

Continued production after hatching

Although no clear cut-off time is revealed, the secretion of hatching substance is no longer detectable 4 hours after hatching for N. obsoletus, and by 24 hours after hatching for N. trivittatus (Table III). Indeed, production by N. obsoletus larvae seems to decline within one hour of hatching, since plugs were degraded only at the highest densities of one-hour old larvae. Note that all larval densities used in these experiments were well above those previously found adequate for plug dissolution by embryos (Table I). The hatching substance is thus no ordinary metabolite, but rather a substance produced specifically to loosen the

capsule plug prior to escape of the veligers. Larvae which have apparently ceased production of hatching substance do not degrade capsule opercula, indicating that physical manipulation plays a minor role in removing the plug.

As in N. obsoletus, embryos of the urchin Strongylocentrotus pulcherrimus seem to produce a hatching substance, which dissolves the fertilization membrane about the egg prior to gastrulation, in a short pulse. There is evidence of hatching substance production between 11 and 14 hours after fertilization, but not before or after this period (Sugawara, 1943). There is no other comparable data for any other marine invertebrate.

Longevity and species-specificity of the substance

The hatching substance produced by N. obsoletus embryos loses its potency within 3 hours, in sea-water at room temperature (Table IV).

Despite the similarity between the capsules of the two species, the substance produced by the embryos of one species is incapable of weakening the plugs of the other (Table V).

The only comparable experimental work done on marine invertebrate hatching is that investigating the dissolution of sea-urchin fertilization membranes prior to gastrulation, and studies on the dissolution of the chorion by ascidian embryos. In contrast to the hatching substance produced by N. obsoletus, that produced by the urchin Arbacia punctulata is said to remain active for several weeks in sea-water, although the conditions of storage are not given (Kopac, 1941). Functional longevity of the hatching substance has not been specifically considered for other

species studied, although the substance produced by the urchins Hemicentrotus pulcherinus and Anthacidaris crassispina was concentrated for experiments by evaporating the hatching water for 3 to 10 hours at room temperature, after which time it was quite potent (Yasumasa, 1960).

The species specificity of the hatching substance produced by the two Nassarius species in my study contrasts sharply with the known action of other marine invertebrate hatching substances. Those produced by the urchins Strongylocentrotus purpuratus and S. franciscanus will dissolve the fertilization membranes of either species (Barrett and Angelo, 1969). In the Ascidiacea, there is no evidence of species specificity of the hatching substance in the 5 species studied. In fact, the substance produced by Ascidia conchilega acts more quickly on the chorion of Phallusia mammillata eggs than on the chorion of its own eggs (Berrill, 1929).

Effect of light and increased temperature on hatching in N. obsoletus

The production of hatching substance in a short pulse, and the apparently short life-span of the substance suggests that synchronized production by all residents within a capsule would be desirable. Light is known to stimulate the release of gametes and larvae by a number of invertebrate adults (Giese and Pearse, 1974, pp 31-32). Recently, light has been shown to accelerate hatching of Artemia eggs (Sorgeloose, 1973). In the case of N. obsoletus, increased temperature could also be a trigger for hatching due to the intertidal nature of the capsules. However, neither light nor heat pulses affected the release of veligers in my experiments (Table VII). This is not surprising. N. obsoletus needs no environmental coordinating stimulus, as all individuals within a capsule seem to develop at approximately the same

rate, at least initially (Clement, 1971), and each capsule contains many more embryos than the minimum number necessary to achieve successful hatching (Tables I and II).

The rate of escape of veligers from egg capsules of N. obsoletus

The average speed of veligers within the capsule when only a few remained was 1.8 mm per minute (N = 11 measurements on 8 individuals). Using this value of $\bar{v}$, the equation predicts that 98% of the veligers will leave the capsule within only 55 minutes. Both of the capsules actually monitored, containing 45 and 51 individuals, lost 98% of their veligers within 45 to 50 minutes after hatching commenced, and the shape of the escape curve is similar to that predicted for a chance escape (fig. 2). Thus, movements with the capsule are probably undirected. However, the observed rate of escape is initially in better agreement with that predicted for a slower swimming speed instead of the speed actually determined. This suggests that the intracapsular fluid does become less viscous during hatching, although there is no evidence that the hypothesized gradient is established.

SUMMARY

1) The loosening of the egg capsule plug prior to escape of the veligers is shown to be chemically mediated in Nassarius obsoletus and N. trivittatus.

2) The hatching substance is not produced continuously during development, but rather in a short pulse beginning just prior to hatching and ending within hours of escaping from the capsule.

3) The hatching substance produced by the embryos of one species is effective only on the capsule plugs of that species, for the two species studied.

4) The substance is functionally short-lived, at room temperature in sea-water, losing its potency within 3 hours after its secretion.

5) There is no evidence that the secretion of the hatching substance by N. obsoletus veligers is stimulated by 2-hour pulses of light or increased temperature. The egg capsules of N. obsoletus contain many more embryos than are needed to effect an escape from the capsule, so that complete synchrony of hatching substance production by all individuals within a capsule is not necessary.

6) The observed rate at which N. obsoletus veligers leave their egg capsules is shown to be in close agreement with the rates predicted from an equation assuming random movement of individuals within the capsule.

TABLE I. Production of hatching substance by advanced embryos of *Nassarius obsoletus*.

+: Release of plug from capsule neck

-: Plug remaining firm in capsule neck

Number Embryos	Number Embryos per µl	Outcome
45	0.196	-
55	0.239	-
2	0.400	-
4	0.408	+
210	0.913	+
6	1.000	+
16	1.176	+
45	1.364	+
16	1.454	+
329	1.645	+
16	1.951	+
13	2.241	+
13	2.500	+
16	4.473	+

TABLE II. Embryos per µl in egg capsules of *N. obsoletus*.

Capsule Height mm	Number Embryos Contained	Approximate Capsule Volume (µl)	Approximate Number Embryos per µl
1.25	47	0.6	78
1.4	51	0.6	85
1.8	152	1.8	84
1.3	17	0.5	34

TABLE III. Production of hatching substance by larvae of N. obsoletus and N. trivittatus (*) at various times after the initiation of hatching

+: Release of plug from capsule neck

-: Plug remaining firm in capsule neck

Hours After Initiation of Hatching	Number Larvae	Number Larvae per µl	Outcome
1	14	2.641	-
1	15	3.750	-
1	14	3.784	+
1	20	4.545	+
2	19	3.518	-
2	31	5.000	-
2.5 - 3	24	3.750	+
3.5 - 4	14	2.258	-
4	93	1.390	-
4	17	3.696	-
5	19	2.714	-
8	17	7.083	-
8	14	3.500	-
11	8	3.077	-
16	19	4.750	-
24 - 48	177	1.264	-
* within 24	194	1.492	-
* 24 - 48	217	1.447	-

TABLE IV. Functional longevity of N. obsoletus hatching substance.

+: Release of plug from capsule neck

-: Plug remaining firm in capsule neck

Number Larvae Hatched	Number Larvae per ul	Hours Between Removal of Hatching Water and Addition of Plug	Outcome
45	6.618	0	+
49	7.000	0	+
25	4.630	0	+
38	4.130	0	+
34	3.840	1.50	+
41	4.254	2.25	-
44	4.889	2.50	+
51	6.892	3.0	-
47	5.014	3.0	-

TABLE V. Species specificity of hatching substance produced by veligers of N. obsoletus and N. trivittatus (*)
+: Release of plug from capsule neck
-: Plug remaining firm in capsule neck

Number Embryos	Number Embryos per ul	Outcome
291	1.455	N. obsoletus : +
		N. trivittatus: -
264	1.600	N. obsoletus : +
		N. trivittatus: -
19	3.800	N. obsoletus : +
		N. trivittatus: -
23	4.600	N. obsoletus : +
		N. trivittatus: -
208 (*)	3.480	N. obsoletus : -
		N. trivittatus: +
224 (*)	1.600	N. obsoletus : -
		N. trivittatus: +

Table VI. The effect of light and increased temperature on the timing of veliger escape. Entries are the number of capsules empty / total number of capsules on the tray.

	Before Light/Temperature Pulse	After Light/Temperature Pulse
Experimental	0/8	3/8
Control	---	2/8
Experimental	3/6	3/6
Control	---	3/6
Experimental	1/13	5/13
Control	---	4/14

Figure 1. Diagramatic illustration of a Nassarius obsoletus egg capsule which has been sliced parallel to its long axis to expose the plug in the mouth of the capsule. Drawn from a photograph. The base of the capsule has been cut away. Cut surfaces are stippled.

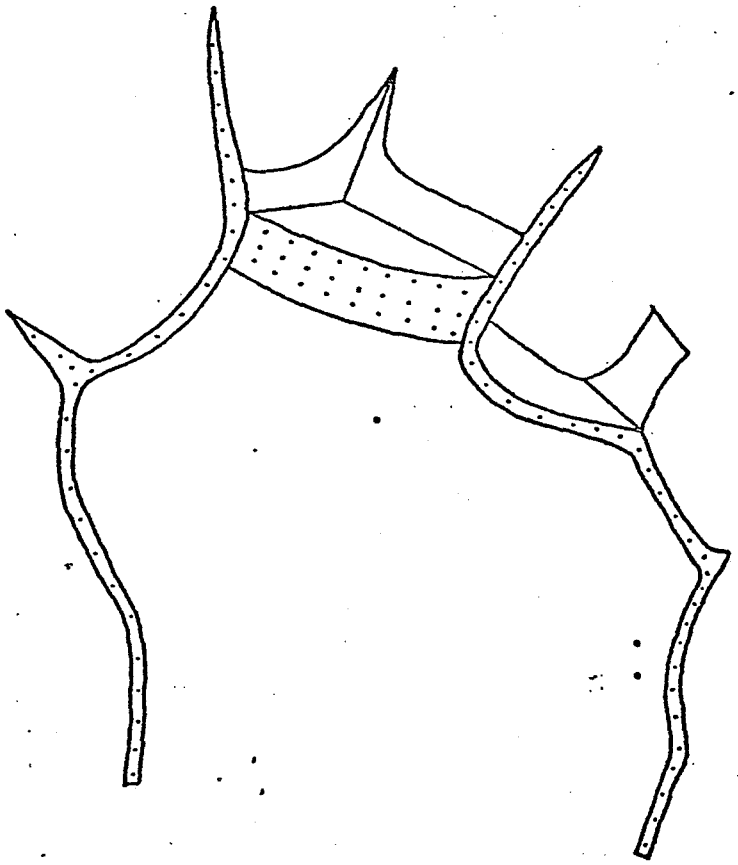
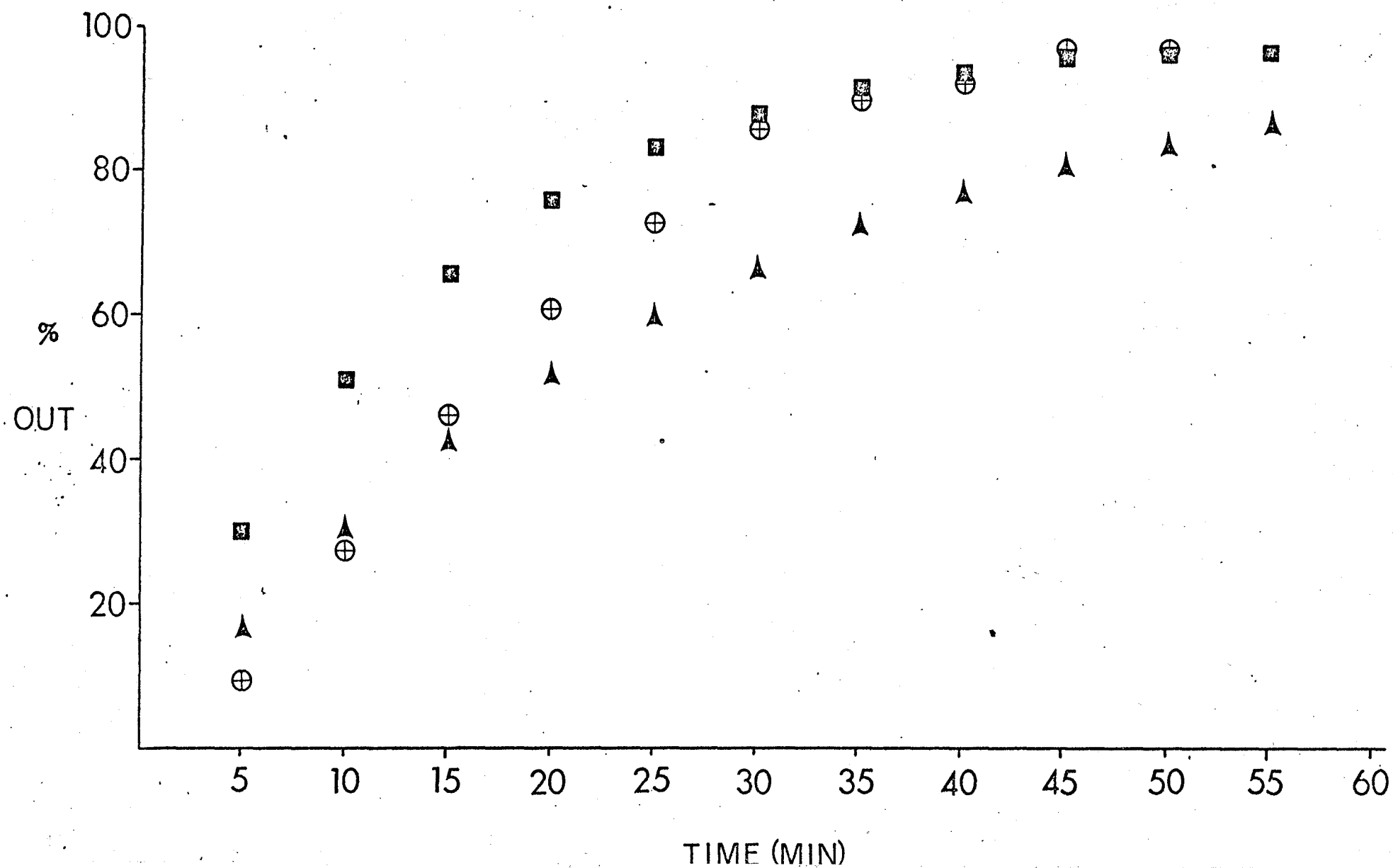


Fig. 2. The observed and predicted rates of escape of veligers from egg capsules of N. obsoletus. Squares trace the predicted rate of escape based on the average swimming speed measured, 1.8 mm per minute. Triangles trace the predicted rate of escape based on a swimming speed of 1.0 mm per minute. Circles trace the average observed rate of escape from two capsules.


100
80
60
40
20
% OUT
5
10
15
20
25
30
35
40
45
50
55
60
TIME (MIN)

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