THERMOSENSITIVITY DURING EMBRYONIC DEVELOPMENT OF *LYMNAEA STAGNALIS* (MOLLUSCA)

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Abstract—1. The percentage of survival after 1 hr at 40.0°C is lowest at the larval trochophore stage and at hatching of the young snail.
2. Heat resistance depends on the stage of development.
3. From the early cleavage stage onwards a higher percentage of embryos can withstand high temperature after a previous heat treatment than without it.
4. The pattern of thermosensitivity is discussed in relation to the organizational level of the stage of development.
5. It is concluded that the developing *Lymnaea* is a suitable system to study heat resistance and thermotolerance at the level of cells, organs and organism.

Key Word Index—Embryonic development; heat resistance; thermotolerance; *Lymnaea stagnalis*; Mollusca.

INTRODUCTION

The development of the pond snail *Lymnaea stagnalis* is of the mosaic type, like that of other spiralian embryos. The cleavages are according to a well-defined scheme and lead to an early differentiation of the different cell lines (Costello and Henley, 1976; Raven, 1976).

The effect of a short heat treatment during the cleavage stage on later development of *Lymnaea* has been studied extensively (Visschedijk, 1953; Raven *et al.*, 1955; Geilenkirchen, 1966; Verdonk and de Groot, 1970; Boon-Niermeijer, 1976). Specific malformations arise after treatment at well-defined developmental stages. Apart from the effect on morphogenesis heat shock also induces extension of the cell cycle of the blastomeres according to a characteristic, periodic sensitivity pattern (Geilenkirchen, 1966; Boon-Niermeijer, 1976). Mitotic abnormalities arise in those blastomeres and their descendants which are heat treated during late meta- to telophase. They are regarded as being responsible for the specific morphogenetic malformations of those organs, which normally arise from the disturbed cell lines. Little is known about the effect of heat on later developmental stages of *Lymnaea* from gastrulation onwards.

In developing embryos of the sea urchin hatching is a critical point in the thermosensitivity (Roccheri *et al.*, 1981). Before this stage the embryos are both unable to withstand a heat shock of 1 h at 31°C and to synthesize heat-shock proteins. After hatching the same heat treatment does not affect morphogenesis any more, and at the same time it evokes the synthesis of heat-shock proteins.

The development of *Lymnaea* offers the opportunity to study the effect of heat both at the cellular level, in relation to the cell cycle, and at the level of the organism as a whole, in relation to the successive stages of differentiation. This makes it a suitable system for the study of thermosensitivity.

Exploring the possibilities of the development of *Lymnaea* as a model system, this study describes the thermosensitivity from egg to young snail. This sensitivity shows a very characteristic pattern. In addition, it appears that embryos can be protected against heat by means of a previous heat treatment, i.e. they can become thermotolerant. Possible explanations of the thermosensitivity pattern are discussed.

MATERIALS AND METHODS

The egg material of *Lymnaea stagnalis* has been described by Geilenkirchen (1961). The snails are the first generation of wild specimens kept in ditches near Utrecht. They are cultured in glass aquaria (about 20 snails/20 l.) at a constant temperature of 20°C and fed with lattice and algae. The eggs are deposited in egg masses, each containing about 100–150 egg capsules. As a rule a capsule surrounds one egg. In each experiment a single egg mass was used, since the variability in heat sensitivity within an egg mass is smaller than that between egg masses. The egg capsules were freed from the egg mass and transferred into tapwater. Eggs were left within their capsules during the experiment. All experiments were carried out at 25°C. When heat shock was applied at 1 day after oviposition or later the capsules were laid down on a bottom of 1.75% agar–agar in Petri dishes.

Heat treatment was applied by transfer of the capsules from tapwater at 25°C to tapwater at the elevated temperature, i.e. 37.0–41.0°C (±0.1°C). The temperature transition is abrupt because of the excess amount of water used. The effect was observed immediately after treatment and thereafter by daily inspection.

RESULTS

Normal development and the effect of heat treatment during 1 h at 40.0°C on survival and morphogenesis

During normal development at a culture temperature of 25°C the embryo flattens in an animal–vegetative direction about 24 h after first
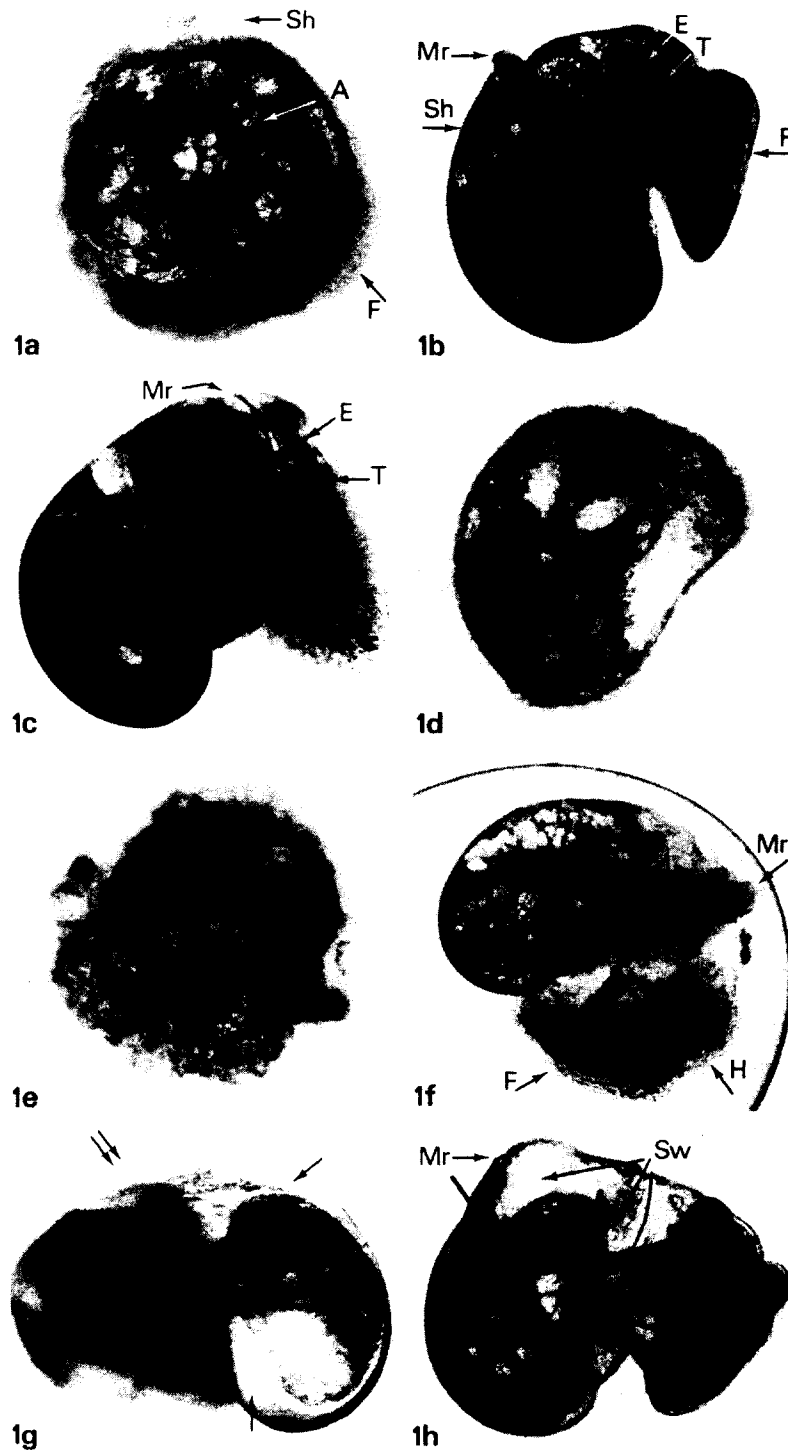
Fig. 1. Larval stages of *Lymnaea*. A = large endodermal cells with vacuoles of capsule fluid in the process of digestion, E = eye, F = foot, H = head, Mr = mantle ridge, Sh = shell, T = tentacle. (a)–(c) Normal larvae 3, 5 and 7 days old. (d)–(h) After treatment (1 hr, 40.0°C) at the stages indicated. (d) Abnormal larva 7 days old, treated at 24 h. Development is very much retarded and abnormal (compare with Fig. 1c). (e) 3-Day-old trochophore shortly after heat shock, falling apart into loose, dying cells. (f) 6-Day-old larva shortly after heat shock. Dead cells extruding at foot, head and mantle ridge (→). (g) 8-Day-old larva 1 day after treatment. The visceral sac is shrivelled and the large endoderm cells have lost their light-refraction. The contact of the visceral sac with the shell has disappeared (→). The close contact of the shell with the mantle ridge has been lost too (⇉). (h) Hydrobic larva with shell malformation, 6 days old, treated 1 day before (Sw = swollen area).

cleavage and a wide blastopore is formed vegetatively. Later on the blastopore narrows to a slit and is displaced ventrally. At 30 h after first cleavage the embryo leaves the vitelline membrane and moves freely within the capsule by means of cilia. From 2 days onwards large endoderm cells, containing light-refracting vacuoles, filled with capsule fluid in the process of digestion, can be distinguished. In the 3-day-old trochophore larva (Fig. 1a) the shell gland has been developed. In the following days the shell grows at the edge by the activity of the mantle fold with which it is tightly connected (Timmermans, 1969). Eventually the shell covers the visceral sac which is still characterized by the large vacuolized cells (Fig. 1b). The shell assumes a cone-shape asymmetric growth. Typical large-cell larval structures, such as apical plate, head vesicle, prototroch and nuchal cells form an extensive part of the body besides the adult structures, such as foot, cephalic plates with tentacles and eyes etc. When the larva is 5 days old there is a regularly beating heart and a functional larval kidney, the protonephridia. The larval structures degenerate gradually in the following 2 days. At the age of about 8 days the young snail hatches (Fig. 1c). An extensive description of normal development has been published previously (Verdonk, 1965; van den Biggelaar, 1971; Raven, 1975).

The effect of heating during the 7 days of development was studied by dividing the eggs of one egg mass into eight groups and by transferring these at 0-, 1-, 2-, 3-, ..., or 7-day-old stages to water at 40.0°C for 1 h. The time of treatment on the first day was carefully determined, starting 30 min after second cleavage, to avoid the immediate disruption of the mitotic process and the consequent abnormal development (Boon-Niermeijer, 1976). The blastomeres did not divide during heating. About every 24 h subsequently the next group of embryos of the same egg mass was heated. The effect on the embryos has been scored at successive days after treatment. If they died either instantly or within 2 days without any sign of further development they were considered to be killed by the heat treatment directly. Apart from the direct lethal effect further development could be highly disturbed. The abnormalities were characteristic for the stages at which treatment was carried out. All embryos which developed further after treatment, normally or abnormally, were regarded as survivors. In Fig. 2 the percentages of survivors at the successive stages are shown. The standard deviation is rather high because the data of the different experiments may be displaced with regard to each other either horizontally (a slower or faster development) or vertically (greater or less sensitive egg mass). Also an all or none effect causes a high variability. In each experiment, however, the characteristic features of the graph are observed.

At the four-cell stage the variability in sensitivity is the most pronounced: in some experiments most embryos die at this stage, in others one or more of the four blastomeres of the embryo may stay alive and go on to divide. This gives rise to abnormal embryos: a clump of cells, a vesicle-like structure and occasionally a well-defined exogastrula. Normal gastrulation has never been observed.
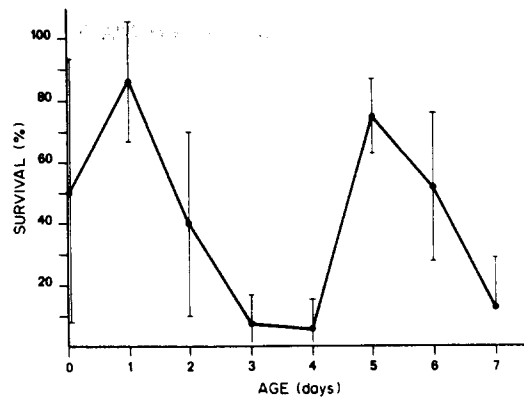

Fig. 2. Sensitivity to 1 h treatment at 40.0°C at 0–7 days of development expressed as percentage survival at the successive stages. Mean of 15 experiments. Vertical bars represent standard deviation.

At 1 day of age the embryos are relatively unsusceptible to heat. Most embryos survive. However, they never develop normally, and they are highly retarded in development (Fig. 1d). Many become hydrobic, i.e. they look normal except for a, sometimes extremely, swollen appearance.

The percentage survival of embryos treated at the age of 2, 3 and 4 days drops sharply. The embryos are either killed immediately (Fig. 1e), or unimpaired development is observed with 1 day of retardation.

The 5- and 6-day-old larvae show a higher percentage of survival, but many of them become abnormal. The direct effects of the heating are cessation of the heart beat and of ciliary movement. Dead cells are extruded from the small-celled adult foot and head structures, and from the mantle ridge (Fig. 1f). The close contact between mantle ridge and shell edge disappears (Fig. 1g). In some cases head and foot recover while the visceral sac dies and loses contact with the shell completely (Fig. 1g). Depending on recovery from these injuries, the embryos either die or survive. The survivors show abnormalities which are relics of the direct effect of heat: foot and shell malformations, absence of the heart beat, the swollen appearance called hydrobia caused by a water imbalance etc. (Fig. 1h).

Heat shock administered to 7-day-old larvae evokes the same phenomena as those displayed by 5- and 6-day-old larvae, such as extrusion of small dead cells at head, foot and mantle, a dead visceral sac, no heart beat etc. At this stage, however, the larvae apparently are less able to recover from these losses.

The heat resistance of 3- to 6-day-old larvae

The percentage of survival depends on the severity of the treatment, i.e. the combination of temperature and duration of heating. In view of the change in heat resistance during the development from trochophore to young snail (Fig. 2) a temperature range from 39.0 to 41.0°C was applied to determine the critical temperature for killing at these stages. An egg mass was subdivided into groups which were treated for 1 h at the 3-, 4-, 5- or 6-day-old stage (Fig. 3). When 3-day-old larvae were subjected to a temperature of 39.5°C, this treatment resulted in 40% lethality. Increasing the temperature to 40.0°C resulted in 88%
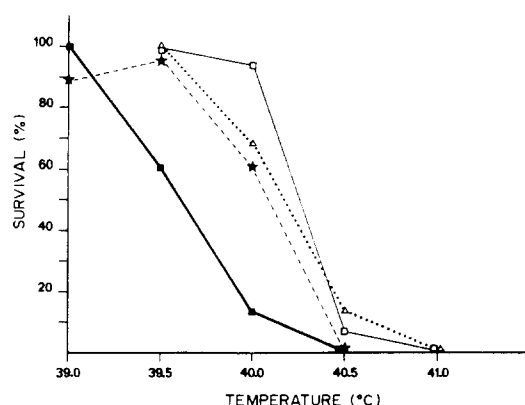
Fig. 3. Sensitivity to 1 h treatment at 39.0–41.0°C at successive stages of development expressed as percentage survival. Mean of 4 experiments. ■, 3 days old; ★, 4 days old; □, 5 days old; △, 6 days old.

lethality, while at 40.5°C none of the larvae survived treatment. Treatment of 4-day-old stages at 39.5°C resulted in only 4% lethality, while 40% lethality was observed after incubation at 40.0°C; 5-day-old stages completely survived a 40.0°C treatment. In contrast, 6-day-old larvae showed increased lethality again.

These results agree with those of Fig. 2. They suggest that the lethal temperature for 3- to 5-/6-day-old stages shifts by approx. 0.7°C.

Thermoresistance after heat treatment: thermotolerance

A well-known phenomenon in a variety of organisms and cell types is thermotolerance, i.e. the raised thermoresistance after a previous heat treatment with sublethal temperatures (Bauer and Henle, 1979; Henle and Dethlefsen, 1978; Li and Hahn, 1980; McAlister and Finkelstein, 1980; Nielsen and Overgaard, 1982). The question can be asked whether the differences in thermosensitivity during development are based on differences in the ability to become thermotolerant.

For three stages (0, 3 and 5 days) the protection against heat by a previous heat shock was investigated. To show a maximal thermotolerance requires an optimal combination of pretreatment (or conditioning treatment = CT) and test treatment (TT). This combination is different for the three stages, but for the sake of comparison we aimed at as much uniformity as possible with respect to the treatments used. Table 1 shows the survival after a TT of 1 h at 40.0 or 40.5 C with and without a CT of 30 min at 37 C. All stages exhibit a dramatic rise

Table 1. Thermotolerance after a conditioning treatment with heat shock

Age (days)	TT[a]	No CT[a]	CT (30 min, 37 C)
		Percentage survival	
0	1 h. 40.0 C	0–88	100
	1 h. 40.5 C	4	96
3	1 h. 40.0 C	25	72
	1 h. 40.5 C	3	38
5	1 h. 40.5 C	13	100

[a]CT = conditioning treatment; TT = test treatment. The two groups without and with CT are always from the same egg masses.

in survival after the CT. The variation at 0 days is due to the great differences in sensitivity to heat between the different egg masses.

DISCUSSION

The results presented in this paper show that the sensitivity to heat treatment of embryos of *L. stagnalis* has a characteristic pattern during development (Fig. 2). The 3- and 4-day-old stages are more sensitive than the four-cell stage provided that treatment is avoided during mitosis. The young snail at 7 days is again much more susceptible than the 5- and 6-day-old stages.

Moreover, our data show that differential thermosensitivity is not dependent on the ability of the embryo to trigger a resistance which would then counteract the lethal effect of heat. Not only 3- and 5-day-old larvae can protect themselves against subsequent heat, embryos at the cleavage stage also can. This situation is different from that found for sea urchin embryos, which are more sensitive to heat before hatching than afterwards (Roccheri et al., 1981). These authors also demonstrated that heat shock promotes the synthesis of heat-shock proteins (hsp) after hatching, but not before. This strengthens the suggestion that before hatching the inability to synthesize hsp makes the embryos less resistant to subsequent heat. After hatching, newly-synthesized hsp should protect in some way the free-swimming larvae against heat. For *Lymnaea*, hsp synthesis has been demonstrated at the trochophore stage, but, under certain circumstances trochophores may become thermotolerant without the synthesis of these hsp (manuscript in preparation). Such a mechanism of defence without specific protein synthesis might be active at the cleavage stage too. Anyhow, it is obvious that the thermosensitivity pattern during development cannot be explained merely by the development of thermotolerance caused by a previous heat shock.

In order to understand the varying thermosensitivity at the different developmental stages, we have to consider the effect of heat at these stages in relation to the characteristic developmental events at each stage. At the four-cell stage the blastomeres are quite individual. When one or more of the blastomeres die the embryo may develop abnormally, but it survives (Boon-Niermeijer, 1976). This has been observed in our experiments and it explains why some embryos survive although the cells are very sensitive. At this stage the all or none effect operates at the level of the cell and not for the embryo as a whole.

The 24 h stage is characterized by the gastrulation process and the formation of the different organ 'Anlagen'. Apparently gastrulation as such is not disturbed by heat, but the organ Anlagen are, because organ formation does not take place normally.

Three- and four-day-old larvae are fully engaged with expansion of the areas of the Anlagen of the adult organs, while the larval organs are just starting their functional activities. The high mitotic rate in the organ Anlagen makes them relatively sensitive to heat. Mitotic cells are highly thermosensitive (Boon-Niermeijer, 1976) and will be killed in any case. A high percentage of dead cells within an Anlagen may

cause death within the whole area and ultimately of the whole organism. Thus the all or none effect at this developmental stage operates at the level of large parts of the embryo (the organ Analgen) and consequently for the embryo as a whole.

At 5 and 6 days the larvae have well-functioning larval organs at their disposal, while the adult organs are in the final phase of differentiation. Heat shock apparently disturbs the functioning of the larval organs, which leads to characteristic abnormalities, but the function may be partly taken over by the almost completed adult organs. The increased thermosensitivity at the 7th day may be explained by the absence of this double safe of larval organs which are still functioning and adult organs which are already able to function.

It is evident that spatial relations and the effect at the organ level play a part in the effect of heating. Our findings justify the choice of the developing *Lymnaea* as a useful object for the study of the differential damage caused by high temperature at the various levels of organization.

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