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VITAL STAINING OF BIVALVE MOLLUSK SHELLS WITH ALIZARIN SODIUM MONOSULFONATE

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ABSTRACT

Alizarin sodium monosulfonate was used for vital staining of shells of larval and post-metamorphic hard- and soft-shelled clams, Mercenaria mercenaria *and* Mya arenaria. *In post-metamorphic clams a week-long immersion in 5 to 20 ppm alizarin resulted in peripheral and medial deposition of red shell material. Larval* M. mercenaria, *when subjected to 0.25 to 0.75 ppm alizarin for 48 hours, produced distinctive check marks on shells which may have value in marking. The long persistence (at least 18 months in the present study) of metabolically induced red shell coloration gives this method an advantage over more superficial marking techniques used in ecological research.*

INTRODUCTION

Shells of living mollusks have been marked for later identification in various scientific studies by painting (Carriker, 1955), tagging (Posgay, 1961), notching (Rounsefell, 1963), and application of fluorescent material (Tufts, 1967). Larvae of pelecypods have been marked by staining the soft tissue with neutral red (Loosanoff and Davis, 1947). These methods have been successful for their purpose to greater or lesser degree but all have disadvantages that limit their general applicability. Most require the handling of individuals, which precludes marking the great number of animals which are often necessary to obtain meaningful ecological data. A method such as painting may produce ephemeral marks or may cause mortality through injury, limiting its usefulness in studies requiring identification of marked individuals after a time lapse.

Alizarin sodium monosulfonate has been used in vital staining of vertebrate bone (Lillie, 1952). Also, Turner[2] used alizarin dye in marking the calcareous tubes of the marine polychaete *Hydroides dianthus*. These applications suggested that alizarin might also be used as a vital stain for the shells of bivalve mollusks.

The experiments reported here demonstrate the incorporation of alizarin red coloration in the deposition of new shell material in post-metamorphic hard- and soft-shelled clams, *Mercenaria mercenaria* and *Mya arenaria*. Also, alizarin has produced distinctive check marks on shells of larval hard-shelled clams.

MARKING POST-LARVAL BIVALVES

In the initial trials, post-metamorphic *M. mercenaria* were successfully marked by holding them in a solution of alizarin in sea water. Clams 2 to 3 mm in width across the shell were produced in the hatchery by spawning and rearing techniques summarized by Loosanoff and Davis (1963). One hundred clams were placed in each of several glass 1-liter beakers containing concentrations of alizarin of 0.25 to 20 ppm. Other beakers contained untreated animals as a control. All were reared for 7 days at 24° C in sea water that had been filtered and treated with ultra-violet light; the water and the alizarin concentrations were renewed at 2-day intervals. A mixture of live flagellates, *Isochrysis galbana, Dunaliella euchlora, Mono-*

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chrysis lutheri, and *Chlorella* 580, was fed every day at an approximate rate of 0.01 ml of packed cell volume per liter culture per day (Davis and Guillard, 1958).

Clams were active and feeding at all test concentrations of alizarin during the treatments. Shell exteriors soon became stained uniformly dark red.

After treatment, clams were held in screened cages in Milford Harbor for later observation. Under outdoor summer conditions all clams grew rapidly and soon lost the general dark red coloration of alizarin. The red margins of the shells, however, representing areas of growth during alizarin treatment were retained as red bands and had faded very little up to 18 months after treatment (Figures 1 and 2 show alizarin marks 6 months after treatment). The red bands were most distinctive at the higher dosage rates of alizarin, between 5 and 20 ppm, and appeared to be excellent marks.

In an attempt to place more than one red band on a single clam, the clams of the original experiment were subjected to a second 7-day exposure of alizarin (8 ppm in this trial) after a month's post-marking growth in the natural environment. The clams again acquired general dark red coloration. When returned to natural conditions the red color again faded, but a second red band was formed representing shell growth during exposure to the second alizarin dosage (Figs. 1 and 2).

Results of trials with *M. arenaria* were equally good. Juvenille *M. arenaria* were subjected to a 7-day treatment with 5 ppm of alizarin under conditions described above and then held under natural conditions for observation. Six months after treatment these clams had added new shell and had also retained red bands on the shells (Fig. 4). In addition to the persistent red bands, the general red coloration of the shell had not faded to normal coloration, but has remained light pink (Fig. 4). The pink shell, contrasting with the white color of shell deposited later also produced a distinctive marking effect. Similar results were obtained with the coot clam, *Mulinia lateralis.*

Cross-sections were made of shells to determine the site of alizarin deposition. Sections of *M. mercenaria* shells marked with a single red band revealed a red striation running with the laminae and extending through the complete thickness of the shell (Fig. 3). Sections of *M. arenaria* shells showed that the red coloration was deposited on the entire inner surface of the shell and extended to the outer surface in the area which was peripheral at the time of treatment (Fig. 5). Colorless shell beneath the red band represents additional medial shell that was deposited when clams were returned to natural conditions.

MARKING LARVAL BIVALVES

Larval *M. mercenaria,* when exposed to alizarin, produced a distinctive check mark on the shell. The larvae, like the post-larval animals, were initially treated for 7-day periods. Concentrations of alizarin below 0.10 ppm produced no ill effects or distinctive marking; concentrations higher than 0.25 ppm stopped growth and produced high mortality. If, however, larvae were subjected to 0.25 to 0.75 ppm alizarin for 48 hours and then returned to untreated sea water, growth of larvae

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FIG. 1 and 2: *External and internal shell surfaces of alizarin-marked juvenile* M. mercenaria, *6 months after marking. The smaller clam at right shows single red band after one 7-day alizarin immersion (5 ppm) whereas the larger individual at left shows two bands that represent two separate marking treatments; size bears no relationship to treatment. Shell length parallel to hinge line of largest individual — 6 mm.*

FIG. 3: *Cross-section of shell perpendicular to hinge line of juvenile* M. mercenaria *which received single alizarin treatment 6 months before sacrifice. Red striation reveals site of shell deposition during term of alizarin immersion. Shell length parallel to hinge line — 11 mm.*

FIG. 4: *External shell surface of alizarin-marked juvenile* M. arenaria *6 months after marking. Visible is red band and general red discoloration of the shell representing alizarin deposition with peripheral and medial shell growth during the term of alizarin treatment. Shell length parallel to hinge line — 2 cm.*

FIG. 5: *Cross-section perpendicular to hinge line of juvenile* M. arenaria *which received single alizarin treatment 6 months prior to sacrifice. Red striation reveals site of shell deposition during term of alizarin immersion. Shell length parallel to hinge line — 2 cm.*

FIG. 6: *Late stage larval* M. mercenaria *showing check marks produced by 48-hour immersion in 0.40 ppm alizarin. These shell marks are distinct from a later demarcation line between the prodissoconch shell of the larva and the dissoconch shell of the metamorphosed individual. Shell length parallel to hinge line — about 200 μ.*

FIG. 1

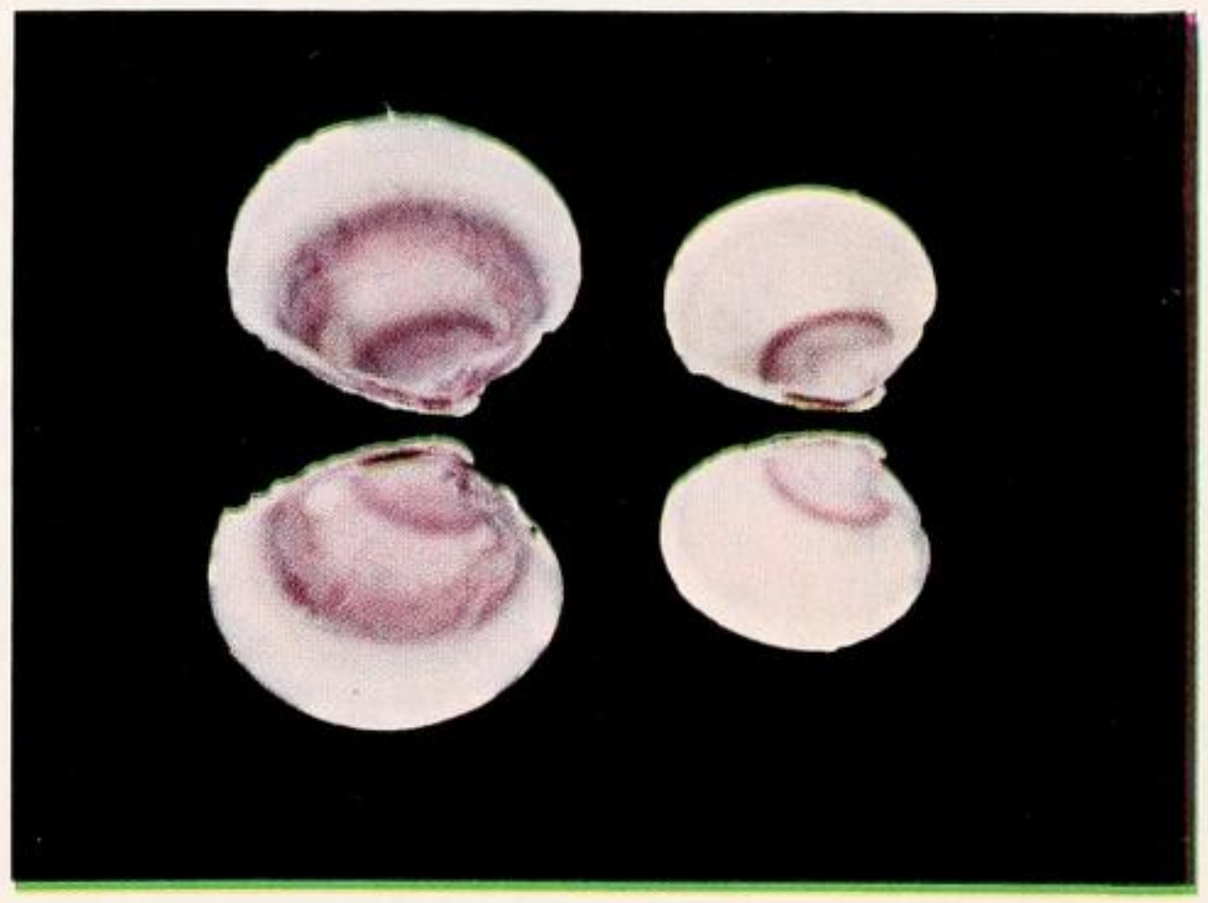

FIG. 2

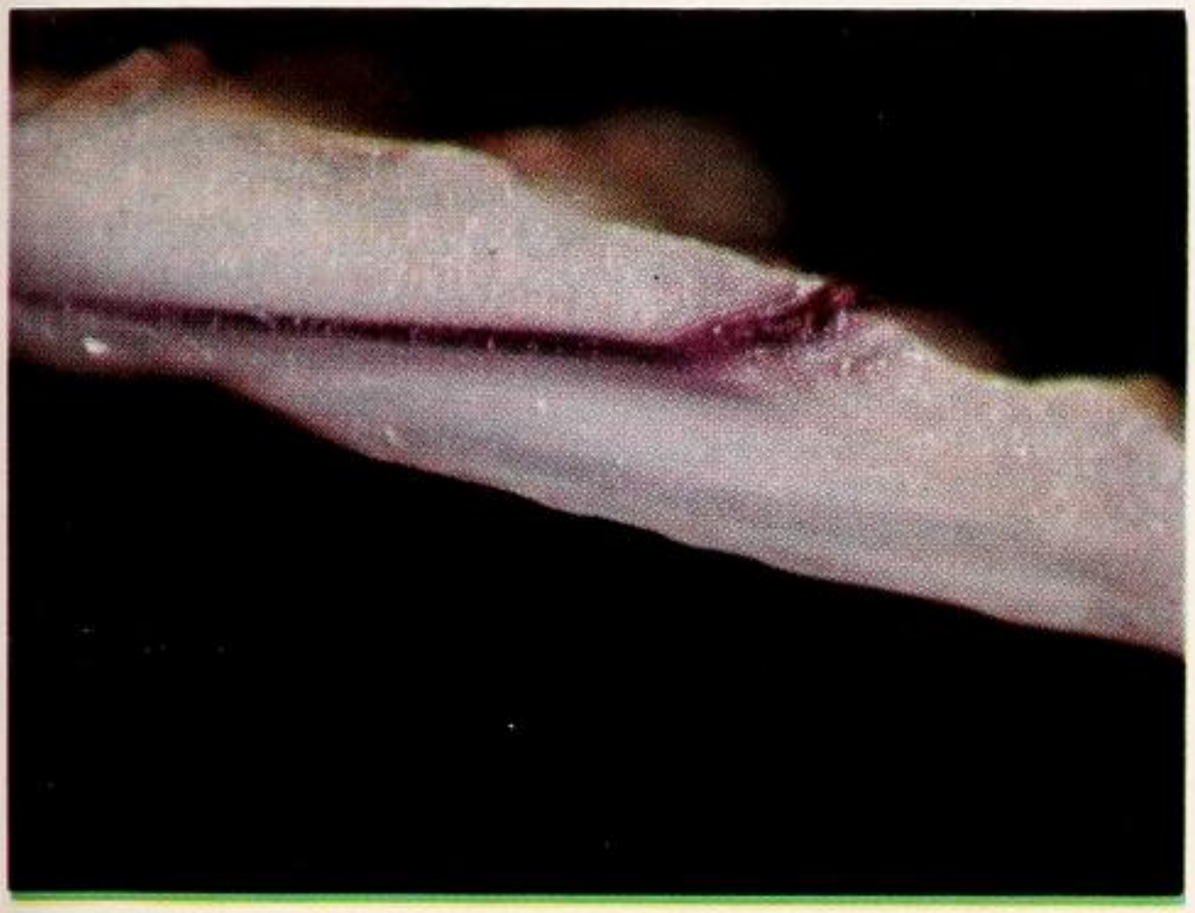

FIG. 3

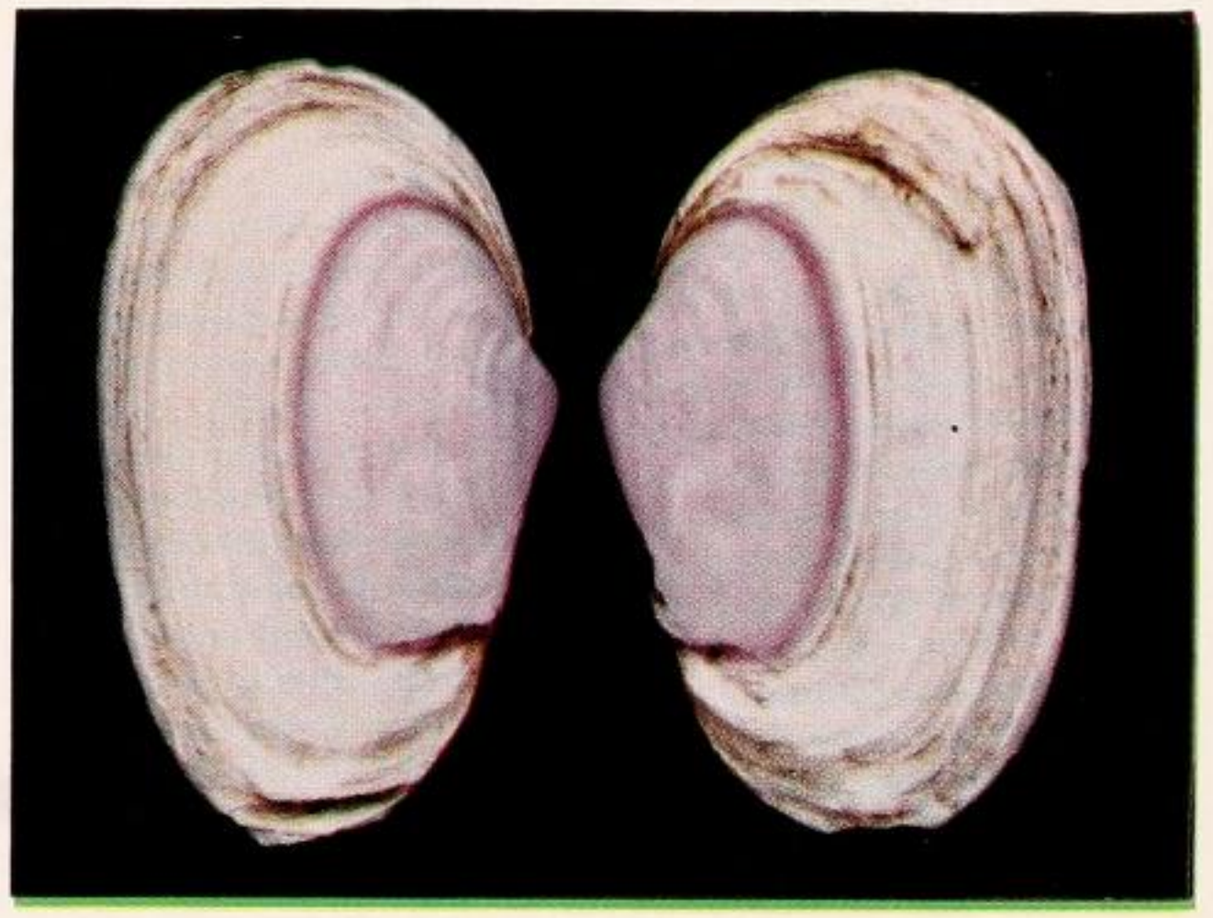

FIG. 4

FIG. 5

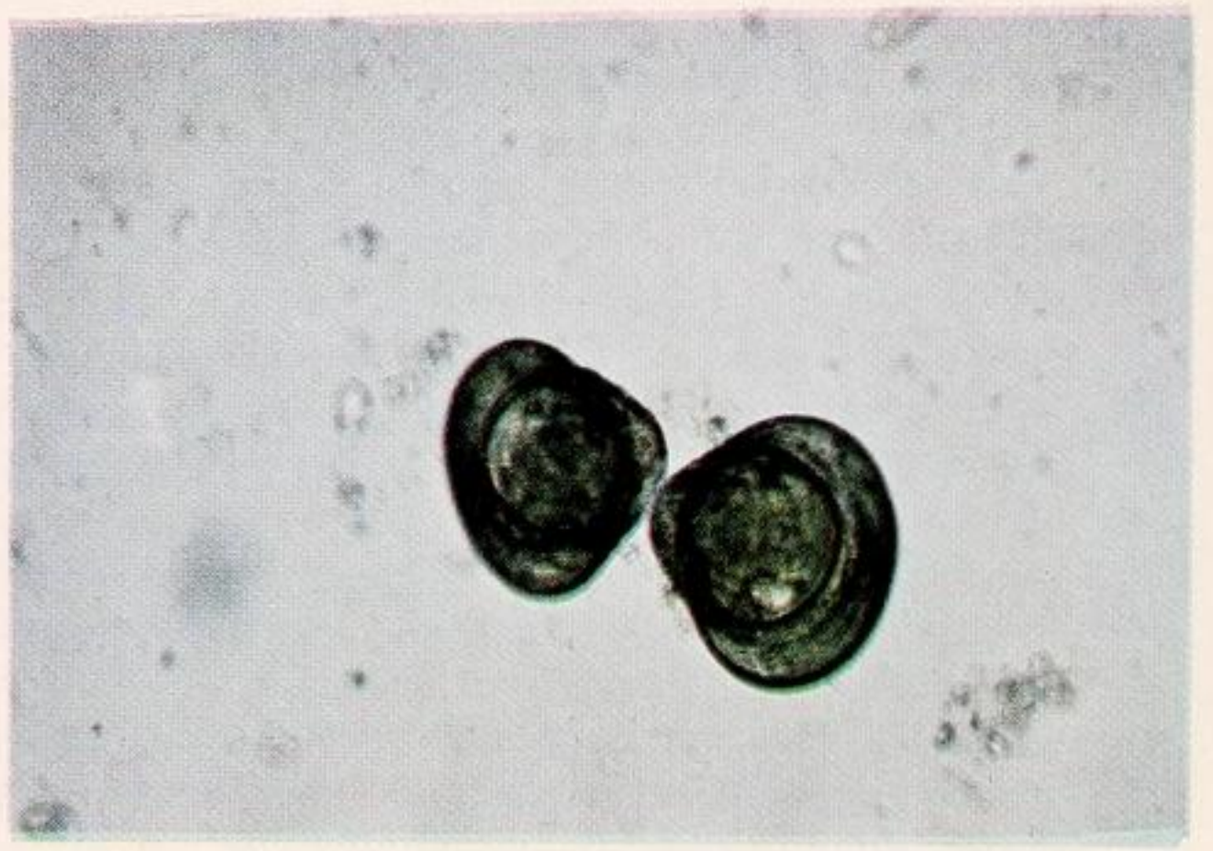

FIG. 6

resumed and a very distinctive check mark resulted (Fig. 6). Immersion in 0.30 to 0.40 ppm for 48 hours produced a light check mark recognizable in all individuals. With increasing concentrations check marks became more pronounced, until at 0.75 ppm larvae greatly indented the shell before normal growth was resumed. Larvae subjected for 48 hours to concentrations of alizarin above 0.75 ppm were unable to resume normal growth.

M. mercenaria larvae of all sizes from 115 μ straight-hinge veligers up to 225 μ pediveligers were marked in this manner, although success was somewhat more difficult to achieve than in the technique used for post-larval clams. Many cultures of marked clam larvae were reared past metamorphosis; the check marks could be readily identified as long as the larval shell itself was recognizable in the juveniles. Considerable work remains in testing the general value of this method of marking, but it probably could be used for marking other species as well.

DISCUSSION

The red bands in post-larval shells of *M. mercenaria* and the bands and pink discoloration of *M. arenaria* appear to result from the deposition of alizarin in the new shell added during treatment. The deposition of red shell was first suggested by the rapid fading of red coloration in all areas of the shell except in the area which was peripheral during the exposure to alizarin. Later, cross-sections of shells (Figs. 3 and 5) revealed sharp alizarin bands only on shell surfaces which were medial and peripheral during time of treatment.

An adequate food supply favoring shell deposition during alizarin treatment would appear to be the determining factor in the laying down of a distinctive mark, although present experiments have not conclusively demonstrated this. Adequate food may be particulary important in marking smaller juvenile animals because their growth rates may depend more on immediate food supply than on a stored food reserve (which might be more important for larger, more mature mollusks).

The alizarin marking method will be useful in ecological research requiring the marking of a large number of shellfish with a highly durable mark. The only limitations on the number of individuals that can be marked are the ability to keep them under such conditions that adequate shell deposit is formed while the animals are being treated and the number of animals that can be procured either through collection of wild stock or through hatchery rearing. Marks appear to be stable and are expected to persist for the life of the animal. After 18 months the marks had faded very little.

Certain difficulties might be expected in the field use of the alizarin technique. Marked animals released in natural water undoubtedly will become dark or discolored, thus obscuring the mark on the shell exterior. It may be necessary to hold the captured animals in sea water away from a substrate for several days to bleach shells sufficiently to distinguish marked individuals. Alternatively, shells of live mollusks could be filed lightly for examination of subsurface shell for traces of red marking. If the animals were to be sacrificed, the interior margins or complete cross-sections of shells could be examined.

M. mercenaria shells on occasion contain natural purple striae which might be mistaken for an alizarin mark. Nearly all natural striations of this type instead of running the complete thickness of the shell are confined to the medial third of the shell. The natural striae are probably variations of natural purple pigmentation of certain areas of the inner translucent region (Shuster, 1957), and seem to be associated with less than optimum growth conditions in *M. mercenaria*. Such coloration has not been observed in *M. arenaria*.

Trials are being continued to determine the general applicability and limitations of the method in molluscan research. No doubt other species of pelecypods and also gastropods can be similarly marked.

ACKNOWLEDGMENT

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