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CHANGES IN THYMIDINE INCORPORATION BY LARVAE OF THE AMERICAN OYSTER *CRASSOSTREA VIRGINICA* (GMELIN) AFTER CHALLENGE BY TWO SPECIES OF YEAST (*CANDIDA*)

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ABSTRACT Larval cultures of the American oyster *Crassostrea virginica* were challenged with the yeasts *Candida albicans* (Robin) and *C. tropicalis* (Castellani). After 48 hr, microscopic examination revealed abnormal shell development and reduced size for the oyster larvae. The results from autoradiographical studies using ^{3}H leucine indicate that *C. albicans* and *C. tropicalis* were ingested by the larvae. Oyster injury caused by the experimental yeasts was determined by measuring ^{3}H thymidine incorporation. During an 8-hr incubation, larvae challenged with various numbers of yeast cells exhibited a 16 to 78% decrease of ^{3}H thymidine incorporation compared to unchallenged control oysters. This test provides a simple and rapid measurement of metabolic injury.

KEY WORDS: *Candida*, *Crassostrea*, oyster, yeast, thymidine, shell development

INTRODUCTION

It has been reported that human-associated yeast cells are present in the waters of Long Island Sound (Combs et al. 1971). This finding was consistent with the report of Fell and Van Uden (1963) who noted that, in general, yeasts are found in littoral waters with high organic content. Furthermore, Buck et al. (1977) and Buck (1981) found a total of 28 species of human-associated yeasts in adult native populations of the American oyster *Crassostrea virginica* (Gmelin), the northern quahog *Mercenaria mercenaria* (Linné), and the blue mussel *Mytilus edulis* (Linné). The literature, however, reveals no studies that investigated a possible pathological association between yeast cells and bivalve molluscs. Such an association might be suspected as certain other fungal types are known to cause serious infections of commercially important bivalves, including *C. virginica* (Davis et al. 1954, Sindermann 1970).

In 1981, Combs et al. first reported significant alterations of oyster larvae due to challenge by *Candida* species. Radioactive-labeling techniques were also described in their report which became the impetus for the present investigation.

The purpose of this investigation was to determine whether two human-associated yeasts, *Candida albicans* (Robin) and *Candida tropicalis* (Castellani), were capable of interfering with the normal development of the larvae of *Crassostrea virginica* during an aquatic bioassay. The assay consists of exposing oyster larve to tritiated (^{3}H) thymidine in the presence of the appropriate yeast. By quantifying the rate of incorporation of an exogenously added, radioactive nucleoside, changes were observed in DNA synthesis. A similar technique was recently cited by Jackim and Nacci (1984) for embryos of the sea urchin *Arbacia punctulata* (Lamarck).

MATERIALS AND METHODS

Candida albicans and *Candida tropicalis*

Freshly transferred stock cultures of *Candida albicans* (ATCC 10231) and *Candida tropicalis* (ATCC 750) were grown at 37°C and maintained at 4°C on brain-heart infusion slants. In our laboratory, routine use of brain-heart infusion media extended the transfer time of these yeasts to 30 days versus 14 days for most other media. The inoculum for all experiments was obtained in the following manner: *C. albicans* or *C. tropicalis* were grown at 37°C for 18 hr on brain-heart infusion agar plates. Yeast cells were collected in 0.9% saline (wt/vol), centrifuged (3,000 x g, 4°C) for 10 min, washed twice, and then harvested and suspended in 0.9% saline. Yeast cells were counted using a hemocytometer.

Bioassays of *Crassostrea virginica*

The pathogenicity of each of the yeast isolates to freshly fertilized eggs of the American oyster was examined by bioassay of the resulting larvae. The initial step in the procedure was to inoculate yeasts on brain-heart infusion slants and incubate them overnight (18 to 24 hr) at 37°C. Broodstock oysters that were maintained at the NMFS

Milford Laboratory were induced to spawn by gradually raising the water temperature (Loosanoff and Davis 1963), and eggs were fertilized and counted. An inoculum of 15,000 freshly fertilized eggs was added to 1 *l* of Millipore-filtered, UV-treated seawater (MUVSW) in glass beakers kept in a constant-temperature incubator at 26°C as recommended by ASTM (1980). Subsequently, an appropriate inoculum of resuspended yeasts was added from the 0.9% saline solution to attain an initial concentration of 1.5 to 2.0 x 10^6 count-forming units per ml in the challenge beakers. Cultures were maintained for 48 hr, which allowed ample time for normal development to the straight-hinge, shelled veliger stage of *C. virginica*. Controls for the challenge beakers consisted of fertilized egg cultures without added yeast cells.

After 48 hr, cultures were sampled by screening the contents through a 36-μm nylon mesh screen, resuspending the trapped larvae in 200 ml of filtered seawater, then collecting a 2-ml aliquot, rinsing the pipet with 2 ml of seawater and preserving the sample with a few drops of 5% buffered formalin (Loosanoff and Davis 1963; Edwin Rhodes, NMFS Milford Laboratory, Milford, CT, pers. comm.). Samples were preserved as described above and examined with a compound microscope.

Larvae for challenge samples were counted and classified as normal or abnormal; normal larvae were "D"-shaped, which is typical for straight-hinge, 48-hr oyster larvae; abnormal larvae departed significantly from the normal "D" shape (Figure 1). Larvae were further classified as to whether they were alive or dead prior to fixing; live larvae have tissue with a darkened central gut and dead larvae are devoid of the tissue. The percent of normal development in experimental cultures was computed relative to that in control cultures and those isolates which caused greater than 80% mortality were considered suspect and examined further. Percent mortality was calculated as the number of surviving experimental larvae divided by number of live larvae in the control x 100.

Larvae were challenged with either yeast, *C. albicans* or *C. tropicalis*, in a larvae:yeast ratio of 1:100 for 48 hr of microscopically counted cells. Incorporation of ^{3}H leucine is a standard method of measuring protein synthesis (Bogóroch 1972). Using the larval oyster bioassay method described earlier, approximately 0.1 ml of each was viewed under light microscopy. Subsequently, 1 ml of each suspension (*C. albicans* or *C. tropicalis*, oyster larvae, *C. tropicalis* or *C. albicans* with oyster larvae) was placed on coverslips dipped in 1% bovine serum albumin and allowed to settle for 90 min (25°C). Tritiated (^{3}H) leucine (6 Ci/mmol - New England Nuclear, Boston, MA) was then added to each coverslip and incubated for an additional 30 min at 25°C. The incorporation of radioactive leucine was stopped with 95% ethanol. Each coverslip was dried and dipped into melted Ilford K-2 emulsion as described by Braun and Calderone (1978). Coverslips were exposed for 14 days (4°C) and then stained with 1% methylene blue.

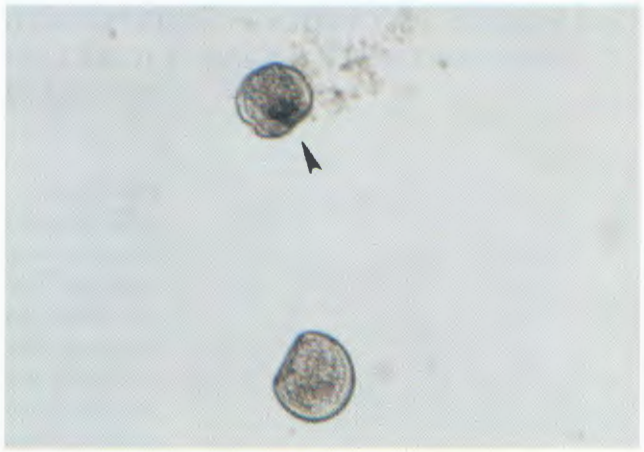

Figure 1. Photomicrograph of normal and abnormal (arrow) live larvae of *C. virginica* challenged with *C. albicans*. (x 100.)

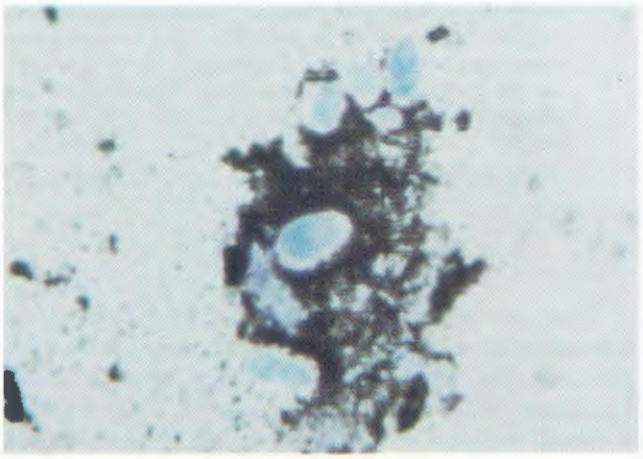

Figure 2a. Autoradiograph of TCA fixed, stained larvae with ingested *C. albicans* shown in pale blue. Leucine incorporation is shown as black spots in the larval tissue. (x 900.)

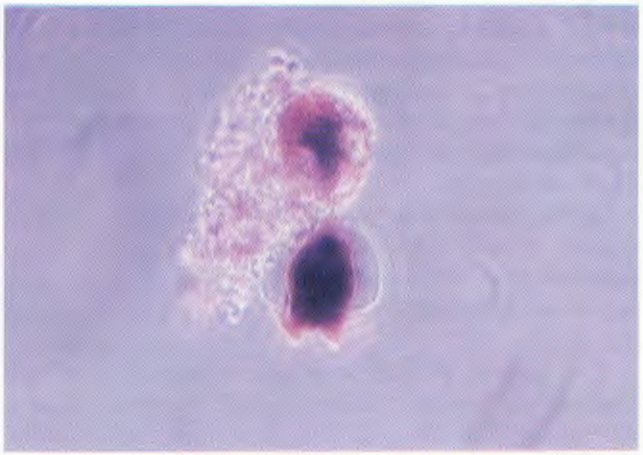

Figure 2b. Photomicrograph of two normal larvae of *C. virginica* treated with TCA. Note distorted shape and rupture of cellular content causing the spread of tissue shown in 2a. (x 400.)

Effect of *C. albicans* and *C. tropicalis* on Incorporation of ^{3}H Thymidine by *Crassostrea virginica*

Culture tubes contained a cell suspension of either *Crassostrea virginica* (1.2 x 10^3 cells), *Candida albicans*, *Candida tropicalis*, or both a yeast and larvae mixture in a total volume of 2 ml MUVSW. *Crassostrea virginica* was challenged with a yeast culture in 1:5, 1:100, and 1:1000 (larvae:yeast) ratio. All cultures were incubated with ^{3}H thymidine (101.0 Ci/mmol - New England Nuclear, Boston, MA) at 25°C. At designated intervals, duplicated cell suspensions were precipitated on ice with cold 15% trichloroacetic acid (TCA) and washed with absolute ethanol. Nonspecific trapping of label was determined by labeling cultures on ice and precipitating immediately with 15% TCA. Subsequently, all precipitated cultures were filtered onto glass fiber filters (Whatman GF/A) and washed with cold 95% ethanol.

All radioactive measurements were made using a Hewlett Packard Model 3380 liquid scintillation counter in scintillation liquid (0.1 g of p-bis [-5 phenyloxazolyl] benzene and 5 g of 2.5 diphenyloxazole dissolved in 1.0 *l* of scintillation grade toluene).

Calculations to determine the inhibitory effect of *Candida albicans* and *C. tropicalis* on incorporation of ^{3}H thymidine by *Crassostrea virginica* were as follows: the counts per minute (cpm) from the appropriate controlled yeast cultures were subtracted from the cpm obtained from the larval cultures and *C. albicans* or *C. tropicalis* incubated together. This figure representing *C. virginica* specific incorporation when incubated with a yeast was compared with the cpm obtained from cultures of *C. virginica* incubated without a yeast using the methods of Peterson and Calderone (1977).

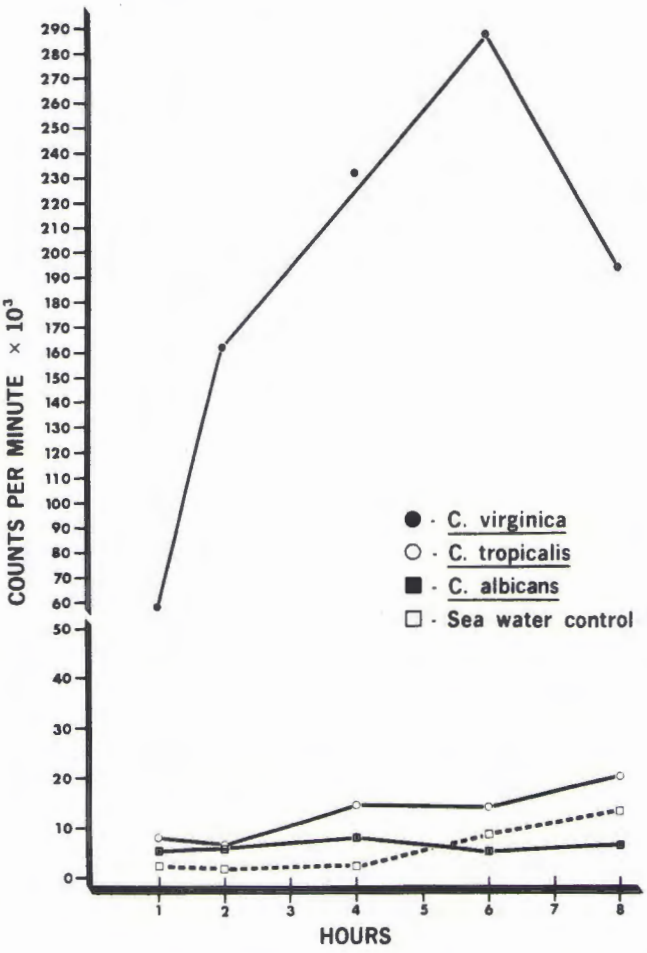

Figure 3. Incorporation of ^{3}H thymidine by larvae of *C. virginica*, *C. tropicalis*, *C. albicans* and seawater control.

RESULTS

After the 48-hr period, both challenged and unchallenged larvae were examined under light microscopy. Figure 1 represents the live normal unchallenged larva next to a larva that was challenged with *Candida albicans*. This live abnormal animal has impaired shell development and reduced size.

Autoradiographic studies of *Crassostrea virginica* showed that *Candida albicans* (Figure 2a) and *C. tropicalis* were ingested. Because of the TCA precipitation, the natural larval morphology was markedly altered (Figure 2b), and yeast cells were revealed in the gut area. It is evident from the heavy grain concentrations, which are indicative of leucine incorporation into tissue, that the challenged larvae continued to incorporate ^{3}H leucine after yeast ingestion. Comparisons between control larvae and challenged larvae for incorporation of this radioactive label indicated that protein synthesis by the challenged larvae did not appear to decrease. Both *Candida* species also incorporated ^{3}H leucine into their protein and after numerous slide examinations it was concluded that there was no decrease in grain development by the ingested yeasts when compared to controls.

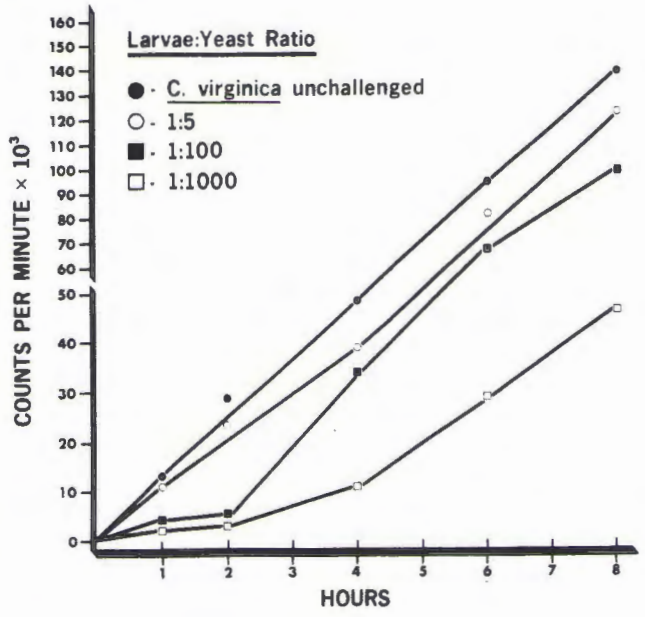

Figure 4. Incorporation of ^{3}H thymidine by *C. virginica* incubated with *C. albicans*.

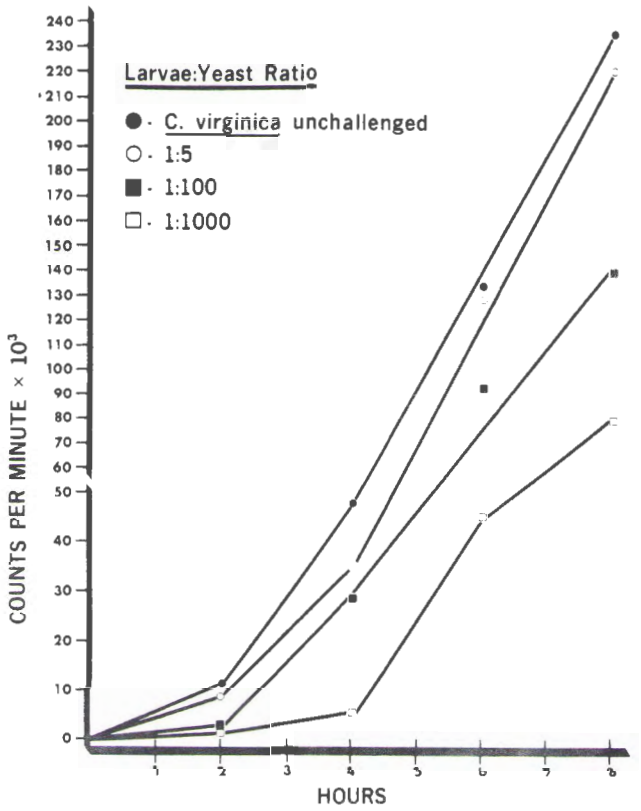

Figure 5. Incorporation of ^{3}H thymidine by *C. virginica* incubated with *C. tropicalis*.

These results suggest that ingestion of these yeasts by *C. virginica* caused no apparent injury to either *C. albicans* or *C. tropicalis*.

Tritiated Thymidine Incorporation

The results shown in Figures 3, 4, and 5 are the means of five separate trials. At individual time periods each trial was within ± 1000 cpm from the mean shown. Control studies were executed initially to determine the amount of ^{3}H thymidine incorporated by *Crassostrea virginica* and *Candida* species. An 8-hr study determined that the oyster larvae incorporated the majority of radioactive label, whereas approximately 5% of ^{3}H thymidine was incorporated into either of the yeasts (Figure 3). This small amount of radioactive label represents nonspecific binding of the ^{3}H thymidine to the surface of the yeasts. As shown in Figure 3, there was no significant incorporation of radioactive label by UV-filtered seawater alone.

The results shown in Figure 3 indicate that the *Candida* cells lacked a functional transport system for thymidine compared to the larvae. Therefore, change in thymidine incorporation by oyster larvae was considered to be an excellent method to measure yeast-induced injury. To quantify the effect of *Candida albicans* on *Crassostrea virginica*, various larvae:yeast ratios were used. As shown in Figure 4, significant decreases of nucleoside incorporation by *C. virginica* occurred. As the larvae:*C. albicans* ratio increases from 1:5, 1:100, and 1:1000, it reflects a mean decrease of ^{3}H thymidine incorporation of 16, 34, and 70%, respectively. After the 8-hr incubation, similar results were observed when *C. tropicalis* was employed as the challenge organism. Using the same larvae:yeast ratios, *C. tropicalis* inhibited the incorporation of radioactive thymidine by oyster larvae by means of 20, 40, and 78%, respectively (Figure 5).

DISCUSSION

The results presented herein indicate that the larvae-yeast association does produce abnormal development of larvae of *C. virginica*. We propose three possibilities that, at least partially, may account for the observed shell deformities. First, normal larval developmental mechanisms may have been altered by some byproduct of yeast metabolism. Second, since yeast cell walls, including *Candida* sp., contain highly polymerized glucans and mannans (Farkas 1979), it is possible that oyster larvae were unable to digest them (Tripp 1958, 1960) and, thus, abnormal shell development was simply one manifestation of a general pattern of starvation. Third, an invasion of larval tissue by yeast cells took place; in the case of *C. albicans* this explanation is unlikely because invasion is usually accompanied by germ tube development (Reynolds and Braude 1956). None was seen in this study.

The finding that incorporation of ^{3}H thymidine by larvae decreased with increases in yeast cell numbers strongly indicates that a larvae-yeast interaction did take place. Tritiated thymidine is an efficient and sensitive method to determine metabolic injury. This bioassay demonstrated a decrease in thymidine incorporation which is directly proportional to inhibition of DNA synthesis. It might also be emphasized that both *Candida* species produced similar decreases in the percentage of ^{3}H thymidine incorporated at comparable larvae:yeast ratios.

It is conceivable that metamorphosing larvae of *C. virginica* might be subject to the same type of injury as observed in this study since *C. albicans* and *C. tropicalis* gain entrance to marine waters through fecal pollution. This conclusion is underscored by the observation that as few as five yeast cells per larva—an attainable level found in contaminated waters—caused significant metabolic changes in the developing oyster.

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NOTE: Use of trade names in no way implies endorsement of commercial products by Fairfield University or the National Marine Fisheries Service.

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