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THE EFFECT OF CYTOCHALASIN B DOSAGE ON THE SURVIVAL AND PLOIDY OF *CRASSOSTREA VIRGINICA* (GMELIN) LARVAE

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ABSTRACT   Survival and ploidy of D-stage oyster larvae (*Crassostrea virginica*) were determined following the rearing of embryos exposed to CB dosages of 0.5 mg/L, 0.25 mg/L, and 0.125 mg/L for 10 minutes, with 0.05% DMSO and ambient seawater as controls. The experiment was replicated three times on the same day with the same procedures and partially stripping the same male oysters; different females were used for each replicate. CB dosage treatments began when 50% of the eggs reached PBI (24–31 min). Embryos were reared for 48 h at ambient temperature and salinity. Resulting triploid percentages were 13% ± 6.7% (0.125 mgCB/L), 61.8% ± 6.2% (0.25 mgCB/L), and 68.2% ± 14.1% (0.5 mgCB/L). No significant difference ($P \leq 0.05$) in mean survival was found between the three CB treatments. Significant differences in mean survival between the three replicates implies variability because of different sources of eggs.

KEY WORDS: *Crassostrea virginica*, oyster, triploid, cytochalasin B, dosage

INTRODUCTION

Cytochalasin B (CB), a cytokinetic inhibitor, was first used to produce triploid *Crassostrea virginica* and *Crassostrea gigas* (Thunberg) over a decade ago (Allen 1986, Stanley et al. 1981). Optimal treatments; that is, those yielding high proportions of triploids, have been reported for *C. gigas*, based on temperature, dosage, time of application, and duration; namely, 0.5 mgCB/1mL dimethyl sulfoxide (DMSO)/L of seawater for 20 min at 25 °C, when 50% of the eggs were at meiosis I (Allen et al. 1989, Downing and Allen 1987). Because *C. virginica* is less fecund than *C. gigas* (Galtsoff 1964), there is more concern for egg survival. Lower dosages and treatment times of 0.5 mgCB/L for 15 min at 25 °C (Shatkin and Allen 1990) and 0.25 mgCB/L for 10 to 15 min, at 27 to 29 °C (Barber et al. 1992) were suggested to increase the survival of embryos while maintaining high yields of triploids.

We tested the feasibility of triploid *C. virginica* production in Louisiana, based on the premise that higher summertime meat yields resulting from triploidy could be profitable for the oyster industry. Triploid induction, using 0.5 mg/L CB, was variable with commercial size broods (≥ 4 million eyed larvae). During the first summer of commercial-scale production, survival of CB-treated embryos was ≤ 5% compared to ≤ 21% for diploid controls using stripped gametes. Differences between the salinity at our hatchery and salinities at sites where broodstock were obtained affected development time, in particular meiotic synchrony, and have been identified as major causes of this variation (Supan 1995).

The objective of this study was to investigate the effect of CB dosage ($H_0: \mu_{0.5mgCB} = \mu_{0.25mgCB} = \mu_{0.125mgCB} = \mu_{0mgCB};$ $H_a: \neq$) on survival and triploidy induction in *C. virginica*, and to determine what component of the variability was attributable to females, held in identical environments.

METHODS AND MATERIALS

Survival and ploidy of oyster larvae were estimated after exposing embryos to CB dosages of 0.5 mg/L, 0.25 mg/L, and 0.125 mg/L for 10 min, with 0.05% DMSO and ambient seawater as controls. The experiment was replicated three times on the same day with the same procedures by partially stripping the same male oysters; different females were used.

Preparation of Gametes

Gametes were obtained for each replicate in a fashion similar to the method described by Allen and Bushek (1992). Oysters were collected from nearshore containers, opened, and their gender was determined microscopically using gonadal smears. Ripeness was visually recognized by the presence of prominent genital canals. Female and male oysters were placed in separate areas to avoid cross contamination.

Eggs were obtained from three ripe females, randomly chosen for each replicate. Females were individually dry-stripped (i.e., without using seawater) to ensure equivalent periods of hydration (defined as the length of time eggs are exposed to seawater after stripping) and simultaneous fertilization. The resulting eggs were pooled and washed of gonadal debris with filtered (1 μm) ambient (24‰) seawater (FAS) by passing them through a 75 μm Nytex screen onto a 15 μm screen. They were then resuspended for enumeration and brought to a volume of approximately 8 million in 1 L FAS. The eggs were allowed to hydrate for 60 min at 28 °C before fertilization and treatment.

Three male oysters were partially stripped for each replicate by scraping away only a portion of the gonad into a beaker and then covering the oyster with plastic wrap to prevent desiccation. Sperm from the three males was pooled in a beaker after being washed of gonadal debris by passage through a 15 μm screen.
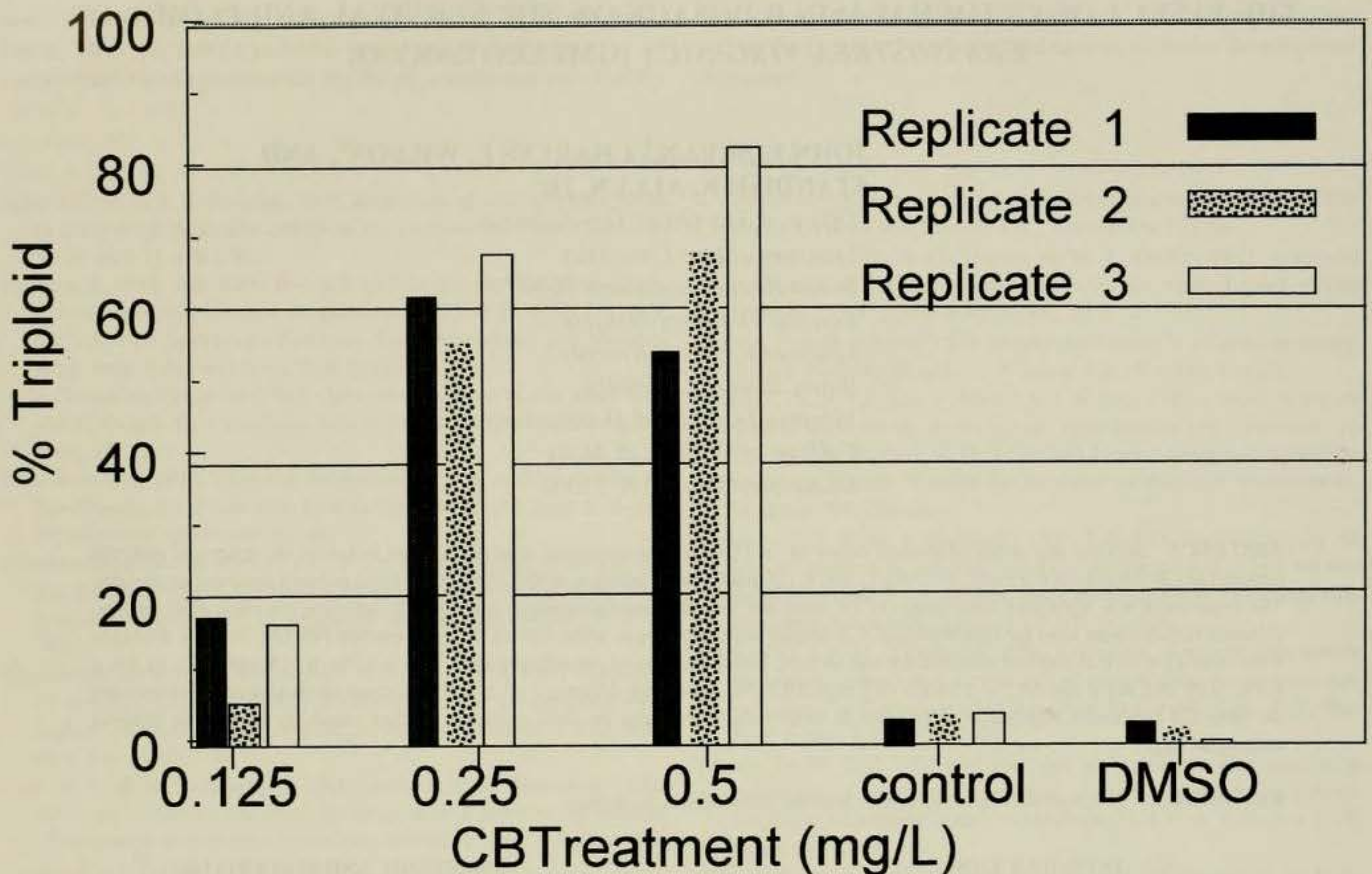
Figure 1. Percentage triploidy in *C. virginica* D-stage oyster larvae after treatment with cytochalasin B, by replicate.

Fertilization and Treatment

Pooled eggs were fertilized with approximately 10 sperm/egg and stirred regularly. After fertilization of the 8 million eggs, they were divided into five treatment beakers each containing 800 mL of FAS, bringing the eggs per treatment to about 1.5 M eggs/L. Eggs from individual beakers were examined microscopically for polar body formation at appropriate intervals. Treatments began when approximately 50% of the eggs reached PBI (24 to 31 min among replicates).

Treatments consisted of adding the appropriate aliquot of 1 mg CB/1 ml DMSO to the beakers of developing eggs to obtain dosages of 0.5 mg/L, 0.25 mg/L, and 0.125 mg/L. Our control consisted of 0.05% DMSO (*v/v*) dissolved in FAS and FAS alone served as a normal. Treatments lasted for 10 min. Afterward, each CB-treatment group of embryos was rinsed of CB with FAS over a 15 μm screen then placed in separate beakers containing 0.05%

DMSO-FAS solution for 15 min. The embryos from each beaker were then rinsed of the DMSO solution and put into separate, labeled culture vessels containing 15 L of FAS for a final culture density of 15 embryos/L. Culture vessels were aerated and equal volumes *Isochrysis* aff. *galbana* clone CISO added. Embryos were incubated for 48 h at ambient temperature and salinity until they reached D-stage. All counts were obtained using triplicate 1 mL subsamples from each culture vessel. At 48 h, each vessel was individually drained onto a 40 μm screen, and subsamples were placed into 1.5 mL centrifuge tubes and shipped overnight to Rutgers University's Haskin Shellfish Research Laboratory for ploidy determination using flow cytometry.

TABLE 1.

Results of analysis of variance (ANOVA): Effect of cytochalasin B treatment and experimental replication on the percentage triploidy of *C. virginica* oyster larvae.

Sources of Variation	DF	F-ratio	Prob > F
Treatment	4	49.70	0.0001
Replicate (error)	2	0.69	0.5291

$R^2 = 0.9616$.

TABLE 2.

Results of ANOVA: *Post hoc* comparisons of mean percentage triploidy of *C. virginica* larvae by treatment.

Treatment	Triploidy*		Comparisons**
	Mean	SD	
0.125 mg CB	0.3594	0.110	A
0.25 mg CB	0.9052	0.063	B
0.5 mg CB	0.9783	0.156	B
Control w/o DMSO	0.1862	0.009	A
Control w/DMSO	0.0949	0.084	A

* Triploidy = arcsin ($\sqrt{(\%Triploid)}$ (0.01)).
** Tukey's honestly significant difference ($\propto = 0.05$).
SD = Standard deviation.

Data Analyses

Differences among treatment means for survival and percentage triploidy were determined using analysis of variance (SAS 1991). Percentage triploidy was determined as a proportion of triploid cells among the total number analyzed by the curve-fitting program ModFit (Verity Software House, Topsham, ME) (Allen and Bushek 1992). Survival and percentage triploidy met the assumptions of normality and variance homogeneity after angular transformation (Dowdy and Wearden 1991). The models used survival and percentage triploidy as separate dependent variables and treatments and experimental replicates as independent variables. Tukey's Honestly Significant Difference Procedure was used to test the difference among the treatments and replicates ($\alpha = 0.05$).

RESULTS

Percentage triploidy and survival were not different between 0.5 mg/L and 0.25 mg/L CB treatments.

Percentage Triploidy

In treatments, mean percent triploidy was 13% ± 6.7% for 0.125 mgCB/L, 61.8% ± 6.2% for 0.25 mgCB/L, and 68.2% ± 14.1% for 0.5 mgCB/L. In controls, 1.4% ± 1.3% of the 0.05% DMSO treatment and 3.4% ± 0.3% of the FAS normal larvae were triploid. Figure 1 depicts percentage triploidy by treatment and replicate. Variation seems high among the three replicates; however, transformed data revealed no significant difference ($P < 0.05$).

The model (% triploidy = treatments, replicates) defined the relationship between the treatment effects and percentage triploidy and explained most of the variability ($R^2 = 0.9616$). Treatment was highly significant ($P < 0.0001$), and the replicate effect was not significant ($P = 0.5291$) (Table 1). Post hoc comparisons of mean percentage triploidy found neither significant differences between the 0.125 mgCB/L and the two controls, nor between the 0.25 mgCB/L and 0.5 mgCB/L dosages (Table 2).

Survival

Figure 2 shows survival by treatment and replicate. On average, the results demonstrate an inverse relationship between survival and CB dosage and a lack of effect (slight enhancement) with DMSO exposure. Although there was moderate variability among the replicates, overall, they all demonstrated the same trends across treatments.

The model explained a reasonable amount of variation in survival ($R^2 = 0.7172$). Both replicate and treatment were highly significant ($P < 0.0001$, Table 3). For treatments, both control and normal were the same, and all CB groups were the same (Table 4). Overall, CB groups had about 20% lower survival than did the controls.

DISCUSSION

These results support previously reported findings that 0.25 mgCB/L (Barber et al. 1992) and 0.5 mgCB/L (Shatkin and Allen 1990) are appropriate dosages for inducing triploidy in C. virginica. However, results are variable depending upon egg or sperm quality or some other factor (Allen and Bushek 1992).

Treatment Recommendations

We found no statistical difference in percentage triploidy or survival between the two higher CB dosages. Considering the cost
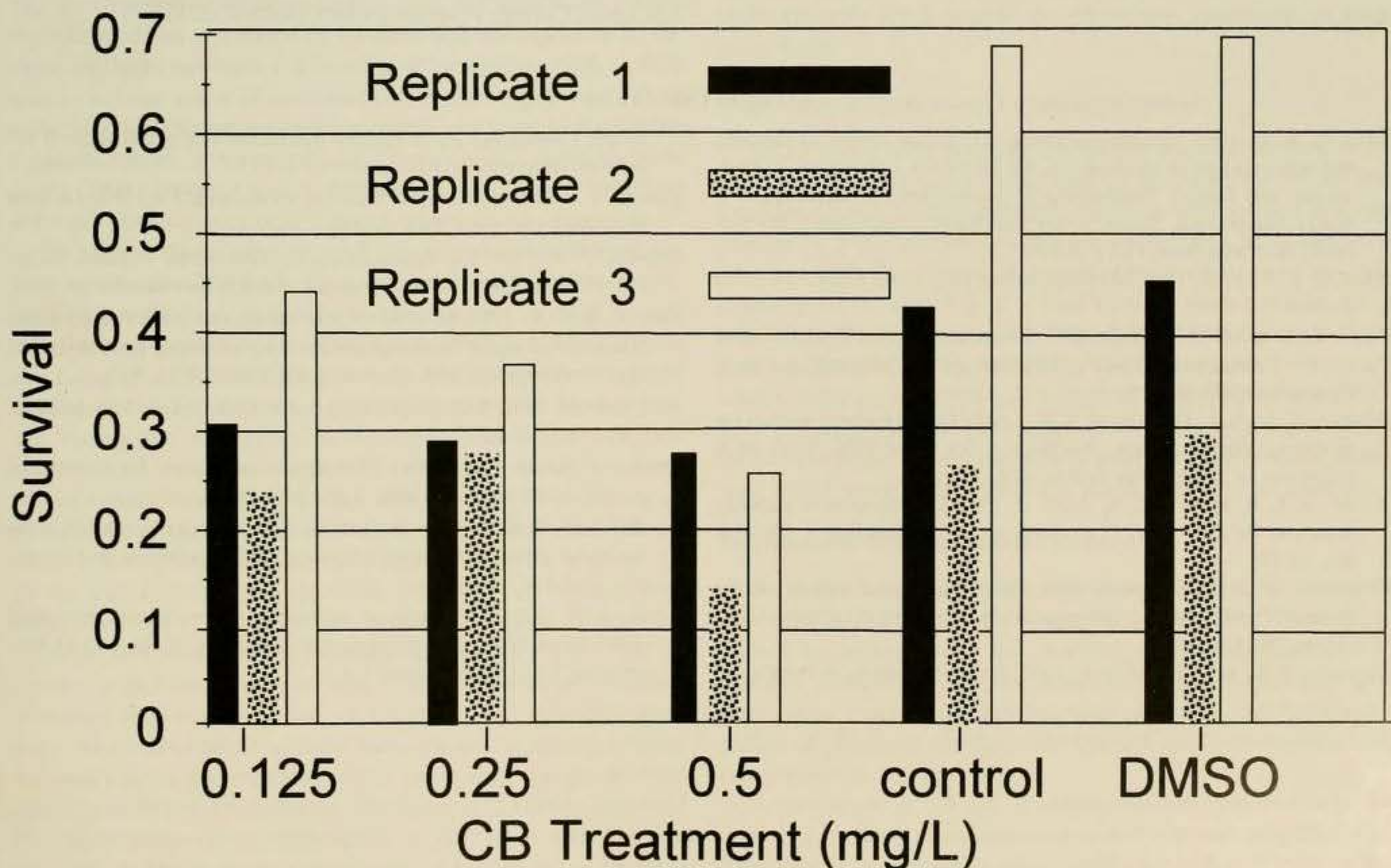

Figure. 2. Survival of C. virginica embryos to D-stage larvae after cytochalasin B treatment, by replicate.

TABLE 3.

Results of ANOVA: Effect of cytochalasin B treatment and experimental replication on survival of *C. virginica* larvae.

Sources of Variation	DF	F-ratio	Prob > F
Treatment	4	11.54	0.0001
Replicate (error)	2	25.11	0.0001

$R^2 = 0.7172$.

TABLE 4.

Results of ANOVA: Means and standard deviations (SD) of survival of *C. virginica* by treatment with comparisons.

Treatment	Survival*		Comparisons**
	Mean	SD	
0.125 mg cb	0.5961	0.1204	A
0.25 mg cb	0.5795	0.0860	A
0.5 mb cb	0.4748	0.0913	A
Control w/o dmso	0.7334	0.2035	B
Control w/dmso	0.7585	0.2126	B

* Survival = arcsin ($\sqrt{}$(Normal larvae/embryos)).
** Tukey's honestly significant difference ($\propto = 0.05$).

of CB ($10/mg in the U.S.), economics suggest that the lower effective dosage is preferable, at 28 °C for 10 min. However, with a range of 54 to 82% triploidy (0.5 mgCB/L) versus 55 to 67% (0.25 mgCB/L), one is inclined to use the higher dosage. Greater triploidy might have resulted from a longer (15 min) treatment time, at the sacrifice of lower survival. For maximum triploid production, embryos should be exposed to CB for a period of time long enough to have a high proportion captured at PBI extrusion but short enough to minimize mortality (Barber et al. 1992). Allen and Bushek (1992) attributed low variance in triploid production to using meiosis I as a benchmark to begin treatment, claiming to have effectively removed meiotic rate as a factor. Although the time of initiation is determined by an appropriate developmental milestone (i.e., 50% PBI), the duration is fixed and does not accommodate varying meiotic rates. We suggest that appropriate duration of treatment be addressed by using a developmental benchmark to cease treatment as it is used for beginning it. Observation of a subsample of eggs, held at the same temperature but without treatment, could provide such a cue. Although this cue must be determined empirically, we suggest 2–5% cleavage might

be appropriate. This benchmark could be used for *C. virginica* or any bivalve species.

The real solution to improving efficiency of triploid production is the development and use of tetraploid broodstock. Tetraploid male oysters produce diploid sperm; when used to fertilize eggs from diploid females, 100% triploid offspring result (Guo and Allen 1994).

LITERATURE CITED

Allen, S. K. Jr. 1986. Genetic manipulations: critical review of methods and performances in shellfish. *In:* K. Tiews (ed.). Selection, Hybridization, and Genetic Engineering in Aquaculture., Proceedings of a World Symposium, Schriften der Bundesforschungsanstalt für Fischerei Hamburg Band 18/19, Berlin.

Allen, S. K. Jr. 1988. Triploid oysters ensure year-round supply. *Oceanus* 31:58–63.

Allen, S. K. Jr. & D. Bushek. 1992. Large-scale production of triploid oysters *Crassostrea virginica* (Gmelin), using "stripped" gametes. *Aquaculture* 103:241–251.

Allen, S. K. Jr., S. L. Downing & K. K. Chew. 1989. Hatchery manual for producing triploid oysters. Washington Sea Grant Publ. WSG-89-3, University of Washington, Seattle, WA. 27 pp.

Barber, B. J., R. Mann & S. K. Allen Jr. 1992. Optimization of triploidy induction for the oyster, *Crassostrea virginica* (Gmelin). *J. Shellfish Res.* 11:189.

Chaiton, J. A. & S. K. Allen Jr. 1985. Early detection of triploidy in the larvae of Pacific oysters, *Crassostrea gigas*, by flow cytometry. *Aquaculture* 48:35–43.

Downing, S. L. & S. K. Allen Jr. 1987. Induced triploidy in the Pacific

oyster, *Crassostrea gigas*: optimal treatments with cytochalasin B depend on temperature. *Aquaculture* 61:1–15.

Dowdy, S. & S. Wearden. 1991. Statistics for research. John Wiley & Sons Publishing Co., New York. 629 pp.

Galtsoff, P. A. 1964. The American oyster, *Crassostrea virginica* [Gmelin]. Fisheries Bulletin, U.S. Fish and Wildlife Service. 480 pp.

Guo, X. & S. K. Allen Jr. 1994. Viable tetraploids in the Pacific oyster (*Crassostrea gigas* Thunberg) produced by inhibiting polar body I in eggs from triploids. *Mol. Mar. Biol. Biotechnol.* 3:42–50.

SAS Institute. 1991. SAS system for linear models. 3rd ed. SAS Institute, Cary, NC. 327 pp.

Shatkin, G. M. & S. K. Allen. 1990. Recommendations for commercial production of triploid oysters. *J. Shellfish Res.* 8:449.

Stanley, J. G., S. K. Allen Jr. & H. Hidu. 1981. Polyploidy induced in the American oyster, *Crassostrea virginica*, with cytochalasin B. *Aquaculture* 23:1–10.

Supan, J. E. 1995. The effects of salinity on the production of triploid oyster larvae (*Crassostrea virginica* Gmelin) in Louisiana. Ph.D. Dissertation, LSU, Baton Rouge, LA.