Florida Sea Grant Project Synopsis

Title:

Enhancing Stress Resistance/Production of Cultured Hard Clams in Florida by Triploidy

Investigators:

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Attainment of Technical Objectives:

The goal of this research was to examine triploidy, a basic breeding technique, to improve survival and growth of the hard clam *Mercenaria mercenaria*, which is the basis of a still growing shellfish aquaculture industry in Florida. We hypothesized triploid clams, as compared to diploid clams, would exhibit superior survival during the summer environmental stressors experienced in the subtropical waters of Florida. We hypothesized that reduced gametogenesis, typical of induced triploid bivalves, would allow for greater allocation of somatic growth and glycogen storage that would be reflected in enhanced survival and production.

OBJECTIVE 1. To grow triploid and diploid families under commercial conditions in natural bodies of water.

Three separate families of clams were produced in late 2003; each family contained sibling triploid and diploid clams. Triploids were produced by inhibiting polar body I or II formation with cytochalasin B. Chemical treatment (cytochalasin B, 1.0 mg/L) for the production of triploids was not 100% effective, therefore, we aimed to have triploid groups with >75% triploids; the remaining clams were diploid. The first set of triploid groups produced by inhibiting PB I or II were destroyed in the hurricanes of 2004 (see problems encountered section). Only PBII triploids were produced in 2005 to replace the lost groups and continue the research. Triploid proportion was >90% for two triploid groups and ~65% for the remaining triploid group.

Production and early culture of triploid and diploid groups occurred at Harbor Branch Oceanographic Institution. Clams were reared until they held up on a 1.2-2.0 mm sieve at which

time seed clams were dispersed to participating farmers in SW Florida and Cedar Key for further nursery culture. Dispersal of seed clams to the different farmers was completed in stages as done in most hatcheries. The first farmer received the fastest growing clams that were sieved from the total population of each group, followed by the next farmer receiving the next fastest growing clams as the clams reached size. These seed clams were grown by farmers in either a FLUPSY (i.e., floating upweller system) or land-based system until they reached a size appropriate for field-based planting (>9mm screen size). Project personnel and farmers did not notice any performance differences in the clam groups during hatchery or nursery stage as compared to their previous experience. As clam seed reached grow-out planting size, farmers planted the families in the field with the assistance of Associate Investigator Leslie Sturmer. Clams were planted in soft-bags and linked together. Each "row" of clam bags contained all six groups of clams (i.e., three triploid and three diploid) that were randomly assigned the order in the row. Multiple rows of clams were planted at three locations (two in SW Florida and one in Cedar Key) as sampling of clams over the year would disturb a group. At each sampling period an entire row was removed to count total number of clams and take samples of clams for size, weight, ploidy, condition index and reproductive potential.

OBJECTIVE 2. To compare production characteristics (e.g., growth and survival) between the triploid and diploid families grown commercially in natural bodies of water.

Before seed clams were dispersed to the farmers, a lab-based experiment was conducted to detect if there would be any noticeable growth difference between diploid and triploid clams at such an early life-stage. Growth was examined in approximately 14 week-old (Exp I) and 15-18 week-old (Exp II) triploid juvenile hard clams in two 3-week experiments. Triploidy was induced chemically (cytochalasin B, 1.0 mg/L) by inhibiting polar body I (PBI) or polar body II (PBII). Growth, as a percentage change in live weight (LW), of triploids was significantly (P= 0.000) less compared to diploids in both experiments. In Exp I, LW increased 250% and 269% for PBI and PBII triploids (initial avg size 93.6 ± 19.0 and 59.5 ± 11.7 mg/clam), respectively, and 341% for diploids (initial avg. size 72.0 ± 16.7). In experiment II, LW increased 422% for PBII triploids (initial avg. size 11.8 ± 1.6 mg/clam) and 549% for diploids (initial avg. size 11.7 ± 1.9 mg/clam). While early juvenile triploid clams did not exhibit better growth than diploids in these laboratory trials, these results do not preclude a growth advantage as adults during the normal reproductive stage; triploids are virtually sterile, which would allow for somatic growth during a time when diploids are spawning and losing mass. We did note an unusually low proportion of triploids in the PBII triploid group of Exp I (18%), which earlier analysis had indicated 86% triploids. During selection of clams for the experiment, we selected for similar sized clams, but all were the largest from each group. This may explain the skewed proportions, but this also leads to the question of why a greater portion of diploids grew better than triploids at such an early stage; we expect no difference in growth according to the energy allocation hypothesis.

In 2004, we were unable to get initial field-planting samples from the first farmer in SW FL. Initial field-planting samples from the second farmer in SW Florida indicated that in triploid groups, triploid clams were in the smaller size fraction after culture in the FLUPSY and sieving. There was almost a complete dichotomy of diploids in the larger size fraction and triploids in the

lower size fraction of each triploid group (4 of 5 triploid groups; other triploid group had 40% triploids in the larger size fraction as compared to 90% in the smaller fraction). The samples from the farmer in Cedar Key, who received the third round of clam seed, did not exhibit this dichotomy after their land-based culture and sieving, but the average size difference between the groups was not as apparent. We are unaware of any literature discussing such drastic size difference of triploids and diploids within a triploid cohort at such a young age.

Field growth studies at the three different sites on the west coast of Florida were interrupted during the severe 2004 hurricane season, with loss of most groups. The Cedar Key site is the only site that we were able to obtain any growth data after field planting as hurricanes obliterated the plantings in SW Florida (see problems encountered section). After moving clams at 9-months and sampling them at 10-months, we found the proportions of triploid clams in each group had decreased 14-26%, but that proportions were still high enough (38-77%) for comparisons. Comparing only verified triploid clams from each triploid group to the diploid control group, it was found that triploids were not significantly different than diploids in shell length (ant.-post. measure: 12.5-13.0 vs 13.6-14.0mm; P=0.067) or whole-weight (0.61-0.75 vs 0.75-0.86 g; P=0.076).

At the Cedar Key site, all deployed experimental clams were moved at 9-months (see above), but "extra" clams (4 of 5 groups) were left in place. After hurricane season ended and salinities increased (12-months), the extra clams were sampled. Survival ranged from 18.6-42.5%. Only the three groups (control diploid, PBI triploid and PBII triploid) in Family 2 were compared. The proportion of triploids had dramatically decreased, leaving only 4 and 29% triploids in the PBI and PBII triploid groups, respectively. Verified triploid clams from the PBII group (the 50 clams sampled did not contain enough triploids in the PBI group) were then compared with diploids within the PBII triploid treatment group. It was found that the triploids were significantly smaller in shell length (P=0.002; 19.7 vs 23.4 mm); shell width (P<0.001; 9.3 vs 11.3 mm); whole-weight (P=0.001; 1.97 vs 3.33 g); and dry weight (P=0.034; 0.07 vs 0.10 g). However, triploids had a significantly higher condition index (P<0.001; 6.6 vs 5.6). Once again we found that diploids were larger.

Gonad formation was assessed by histology in the 12-month old (December 2004) Cedar Key extra clam samples. Fifty percent of the diploid control clams had completed spawning and 40% had no gonad. In contrast, 100% of the verified triploids had no gonad and diploids found within the triploid group had gonad stages similar in proportion to the control group. This data indicates that gonad development is inhibited in triploids as has been seen in other bivalve species. It was this observation upon which we based our major hypothesis of increased survival during stress post-spawning.

The process of growing and comparing production characteristics of a new set of triploid and diploid clams was repeated for 2006/2007 as the data from 2004/2005 was not complete (i.e., no replication) and may have been skewed from mortalities caused by excessive burial due to the hurricanes. Production of replicate triploid and diploid families was successful as noted in Objective 1. Clam seed was again dispersed successfully to farmers in SW FL and Cedar Key. Farmers performed nursery and grow-out culture, which was overseen by Associate Investigator Sturmer. At the end of field culture for this new set of triploid clams, differences were found

between triploids and diploids. At Cedar Key, triploids were similar in length (46mm) and weight (30g), but had a higher condition index (5.3 vs 4.8) and lower survival (69 vs 80%) compared to diploids. In SW Florida, triploids were similar in length (48-50mm) and condition index (4.6), but lower in weight (34 vs 44 g) and survival (43 vs 49%) compared to diploids. The data from this second set of triploid clams cultured to market size, and those clam affected by the hurricanes, does not support consistent superior survival or growth of triploid clams in Florida waters, which would have led to higher production. Reduced gametogenesis was evident and resulted in similar or higher condition index.

OBJECTIVE 3. To compare responses of these clams subjected to laboratory challenges of salinity, temperature, and oxygen.

Triploid and sibling diploid clams (45 mm shell length) were exposed to salinities of 10, 25, and 40 ppt and dissolved oxygen levels of <2 $mg \cdot L^{-1}$ (hypoxia) and >5 $mg \cdot L^{-1}$ (normoxia) with temperature constant at 32°C for 21 days. Observations of clam mortality, siphon extension, and burial were conducted at 12 hour intervals to assess lethal and sublethal treatment effects. Burial rates decreased over the duration of the experiment in the 25 ppt hypoxia and 40 ppt hypoxia treatments, indicating a sublethal effect of hypoxia. Survival analysis was performed to test the difference in survival between triploids and diploids in each treatment. Triploids had significantly greater survival than diploids in the 25 ppt hypoxia treatment (P=0.0159), while diploids had significantly greater survival than triploids in the 10 ppt hypoxia treatment (P=0.0387). In a second challenge of smaller clams (20 mm SL), triploids and sibling diploids were exposed to salinities of 15 or 25 ppt and dissolved oxygen levels of <2 $mg \cdot L^{-1}$ (hypoxia) and >5 $mg \cdot L^{-1}$ (normoxia) at temperatures of 27°C or 33°C. Diploids consistently performed better than triploids; diploid clams had significantly greater survival than triploid clams in the 15 ppt hypoxia (P<0.0001) and normoxia treatments (P<0.0001) at 33°C and in the 25 ppt hypoxia treatment at 33°C (P<0.0001). Taken together, results of these two experiments suggest that triploidy does not confer an advantage for survival of *M. mercenaria* during environmental extremes.

OBJECTIVE 4. To characterize and compare the metabolic efficiency of diploids and triploids.

This study characterized and compared the metabolic efficiency of diploid and triploid clams at typical water temperatures in Florida. Oxygen uptake rates were determined at 20, 25, 27, 30, and 32°C at 25 ppt for clams of approximately 55 mm shell length. Attempted acclimation to 35°C at 25 ppt resulted in 100% mortality. Oxygen uptake rate varied significantly as a function of temperature (P<0.000). Oxygen uptake rate increased with temperature to 27°C. However, oxygen uptake rate did not increase with further increases in temperature above 27°C. These results suggest that 27°C is a critical temperature threshold, and represents the onset of partial anaerobic metabolism. Oxygen uptake rates of triploid and diploid clams were not statistically different (P=0.694). Therefore, triploid clams may not offer any significant physiological advantage over diploids. Oxygen uptake rates were also determined at 27°C at 25 ppt and 15 ppt. Salinity had no affect on diploid oxygen consumption rate (P> 0.05). Triploid clams, however, had significantly lower oxygen consumption rates (P=0.012) at 15 ppt than at 25 ppt.

Whether these differences offer triploids any significant physiological advantage over diploids during fluctuating and sometimes extreme environmental conditions in the field is unknown.

OBJECTIVE 5. To compare and characterize economics of triploid seed production and the financial considerations of nursery and grow-out of triploid hard clams.

The original objective for this effort was to focus on contrasting the financial characteristics associated with diploid and triploid hard clam seed production. Completion of this objective, as written, would have required that a survey of existing diploid hard clam seed producers in Florida be conducted. At the time of the proposal development, no current information existed on the financial characteristics of a "typical" hard clam hatchery in Florida. The hurricane seasons of 2004 and 2005 resulted in many hardships for these existing producers, especially those located along the east central and south west coasts of Florida. Several of these hatchery operations were rendered inoperable for lengthy periods of time and some eventually ceased operation. The remaining hatcheries were considered much too harried by the storm events to ask for a detailed cost and earnings survey.

As an alternative, the project team decided to conduct an abbreviated telephone survey of 8-10 of the remaining operating hard clam hatcheries in Florida. The objective of the survey was to determine what percentage of the current market price for a unit of seed (1000 1-mm seed) was represented by total costs. This information would allow a determination of the basic cost and profit margin components of market price. Thus, if the triploid hatchery process was found to be more costly than the diploid process, those marginal costs associated with triploid production could simply be added onto the total cost component. The resulting margin decrease would then have to be offset by increased revenues obtained during grow-out (e.g., increased survival).

Of the 8-10 hatcheries contacted only 5 provided responses. Of those five, none of the responses concerning the cost component of the current market price were consistent. Thus, no "typical" cost structure could be determined from the responses. As a result, the objective associated with contrasting the financial characteristics associated with diploid and triploid hard clam seed production could not be completed as originally proposed.

In lieu of utilizing a survey approach to gather hatchery cost data, the decision was made to obtain marginal costs estimates from the trial triploid hatchery runs. The hatchery process employed for triploid hard clam seed production was similar to the process utilized for diploid seed production. Similar costs are incurred with respect to broodstock acquisition, energy to move and heat water, labor, algae, various supplies, capital cost of space for broodstock maintenance and spawning, and other expenses. These types of costs are common to both the diploid and triploid production process. However, several additional steps are utilized in the production of triploid larvae. These include the chemical treatment that induces triploidy (i.e., inhibition of polar body II formation), verification of triploidy, and chemical waste disposal. These marginal, or additional, costs categories were recognized and estimated based on several trial runs of triploid egg and seed production. The costs are the best estimates based on the project team's knowledge of the costs associated with producing diploid and triploid hard clam seed in a typical commercial hatchery in Florida.

The anticipated marginal costs associated with the triploid production process are summarized below in Table 1. Note that the only operating cost changes are associated with the triploidy treatment, waste disposal, and triploidy verification. Other costs associated with additional capital investment were not estimated. These costs may be significantly minimized by utilizing pre-existing materials. Costs for producing triploid seed were estimated to be only 5-10% more than diploids from these factors.

Table 1. Anticipated Triploid Production Operating Costs

Cost Category	Per 5 million eggs	10% seed set (per 1,000)	20% seed set (per 1,000)
Broodstock conditioning*	--	--	--
Spawning*	--	--	--
Treatment	$20.45	$0.041	$0.021
Waste disposal	$71.20	$0.028	$0.014
Triploidy verification	$100.00	$0.200	$0.100
Larvae culture	--	--	--
Setting	--	--	--
TOTAL	$191.65	$0.269	$0.135
* Additional capital investment may be required. Not measured.			

Cost Recovery at Grow-out: The total additional cost associated with triploid production, as compared to diploid seed production would be $191.65 per 5 million eggs. This cost increase would amount to an increase per 1000 1-mm seed of $0.269 with a 10% seed production rate (500K seed), and $0.135 with a 20% seed production rate (1 million seed). Given these cost estimates, the assumption was held that a typical commercial hatchery would simply add that additional cost onto the current market price for 1-mm hard clam seed. If the demand for triploid seed was sufficient, then existing seed producers may find this to be a successful cost recovery strategy. Since the current market price of diploid seed should theoretically cover both the cost and any profit margin, an additional profit margin was not included for the triploid example.

If the total cost of the triploid seed production cost is passed on to the seed buyer, then the seed buyer will need to recover that cost in the grow-out process to make the purchase of triploid seed a rational business decision. In that case, the grow-out revenue will need to increase due to the benefits purportedly associated with the use of triploid seed (i.e., faster growth, higher survival) in order to offset the higher seed cost.

Assuming the current market price for diploid hard clam seed is $3 per 1000 1-mm seed, the additional costs for triploid seed would, if simply added to the current market price for seed, result in approximately a 9% increase in seed price (assuming a 10% seed production rate). If cost of seed represents approximately 50% of the total operating expenses for small-scale hard clam growers, then a 9% increase in seed cost will result in a 4.5% increase in total costs. This cost increase would need to be offset by an increase in total revenue of at least 4.5%. These percentages would change if the share of operating expenses represented by the seed cost is significantly different than used in this example. The overall conclusion is that the additional cost associated with triploid seed, if passed on to the seed buyer by the hatchery, will need to be

offset with an increase in grow-out revenue. As seen in the field trials, triploids did not increase production by higher survival or growth. Therefore, triploid seed would not be adopted by growers if the decision is based on current financial information.

Advancement of the Field:

Triploid clams were able to be produced, using chemical methods, with high proportions in a population. Clam farmers did not notice any substantial differences between groups during nursery or grow-out culture. Therefore, clam farmers would not need to change methods already known and utilized by them. Unfortunately, triploid clams were similar or smaller than diploid clams at most times. This is contrary to published material for other bivalves. However, triploid clams are sterile, which would limit recruitment of farmed or "domesticated" clam species into natural assemblages.

The laboratory challenge studies were one of the first to examine the effects of multiple stressors of salinity and dissolved oxygen on *M. mercenaria* survival when combined with the high temperatures reached during Florida summers. We found that even at these higher temperatures, mortality does not occur unless there is at least one additional stressor present, such as low salinity or low dissolved oxygen levels. In addition, this is one of the first laboratory studies to examine the effects of high temperatures on oxygen uptake rates by *M. mercenaria*. Most studies of *M. mercenaria* metabolic rates have been conducted over temperature ranges of 10-27°C. One highly cited dissertation (Hamwi, 1969), in which oxygen uptake rates were measured at temperatures up to 32°C, suggests that rates peak at 25°C and fall to zero at 32°C. Our data show a very different pattern; one that is more consistent with known physiological processes.

Although the basic breeding technique of triploidy did not show consistent superior improvement, ancillary research of heat shock proteins indicated that alternative parameters may be selected for through breeding to improve survival and production.

Benefits:

Discovery and Application of New Products and Processes Induction of triploidy in hard clams was successful, but this basic breeding technique tested on hard clams did not have the anticipated effect of increased survival. Triploid hard clams were found to be sterile that resulted in a clam with a higher meat to shell ratio at certain times of the year (i.e., post-reproductive in diploids). However, the market does not categorize clams on meat yield, therefore the increased cost of triploid induction can not be captured by the seed producer or farmer from the buyer.

Tools, Technologies and Information for Improved Ecosystem Management As triploid clams were found to be sterile, this breeding technique could be used to limit recruitment of farmed clam species into natural assemblages, but would come at an increased economic cost that is currently not able to be captured.

The results highlight the importance of investigating the effects of multiple stressors on survival and physiology to more accurately determine tolerance of estuarine invertebrates to abiotic factors.