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Repeatability of the enrichment procedure for triploid C. ariakensis larvae

Standish K. Allen Jr.
Virginia Institute of Marine Science

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Stan Allen
Aquaculture Genetics and Breeding Technology Center
Virginia Institute of Marine Science

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Introduction

The principal concern for an industry based on aquaculture of triploid *C. ariakensis* is biosecurity, that is, the assurance against unwanted introduction of the species into the Bay during aquaculture practices. Essentially, this boils down to keeping as many diploids as possible out of the triploid population.

We set about addressing some questions concerning reducing or eliminating the number of diploids (which on average occur at a rate of 0.1%).

- Can diploids be purged from 4n x 2n batches by size grading?
- Is there a difference in the number of diploids eliminated between first and last harvests of eyed larvae?
- Can we quantify the degree to which we have "purified" the triploids?

Materials and Methods

We produced spawns of triploid *C. ariakensis* on three occasions during the 2008 spawning season: March 31st, May 1st, and July 17th. Triploids were made by crossing diploid females with two male tetraploids. Tetraploidy was confirmed for the male via flow cytometry by sampling gill tissue and sperm. In all cases, gill tissue was tetraploid and sperm was di-haploid. At the same time, a normal diploid spawn was produced within a day or two of each triploid spawn date.

<u>50:50 cultures</u>

A third, composite larvae culture was established using approximate equal numbers of diploids and triploids: a so-called 50:50 group. The idea was to set up a culture with vastly more diploids among the triploids than we would ever seen with typical 4n x 2n crosses, and see to what degree we could purify it.

During the culture period and before eyed stage, we sampled the 50:50 group periodically to confirm the continued presence of diploids (or not). At the end of the larval cycle, when larvae were eyed, we screened the 50:50 culture on 275 µm and (often) 250 µm screens every two to three days. Samples of these two larval populations were evaluated by flow cytometry. For larvae samples, about 1,000 – 3,000 larvae (depending on size) were put in a single sample tube, aspirated with a 20G syringe needle to break the shells, and frozen at -80°C in DAPI solution for at least 15 minutes. The sample was then run on the flow cytometer, which distinguished diploid from triploid cells. The proportion of each type of cell (diploid and triploid) was calculated as a ratio to the total cells examined. Generally, if we could not detect a diploid signal in the larvae by flow cytometry, we set the batch so that, later, we could run spat individually to look for diploids. This is because flow cytometry has a resolution of ~1%, whereas we need resolution of 0.1% or greater.

To test spat, we sampled them individually, although we pool three spat in a tube in order to cut down on the total number of samples run on the flow cytometer. Sampling individuals allows us higher resolution by sampling 1000 or more. If the flow cytometry sample of three spat has only a triploid peak (only triploid cells), we infer that all three spat were triploid. If there is a composite of diploid and triploid cells, we infer that one of the three spat is diploid. Because the possibility of having *two* diploid spat of the three sampled is so improbable, we do not consider that possibility, although it is quantifiable as an error term. For example, the probability of randomly sampling two diploids out of three chosen when the percentage of diploids is about 1/100, is about 1/10,000. One percent represents the limit of flow cytometry, as mentioned above, so that any sample with >1% diploids, we do not sample as individuals.

"100%" cultures

We also extensively sampled "pure" triploid spawns. The period of larval culture in which we are harvesting larvae lasts for about a week. That is, we will start harvesting on (e.g.) day 18 and finish harvesting on (e.g.) day 26. Each of these harvests represents a different stage in the ontogeny of the larval population as a whole. The first larvae growers harvested are the fast growers and the last – slower. In a mixed culture (even with very small proportions of diploids), there may also be a difference in when diploids are obtained. This we tracked by sampling each "set" – representing different harvests of eyed larvae.

We sample sets proportionally. We count the larvae from each harvest and set them, until the last eyed larvae are obtained. Each set (harvest) represents a proportion of the total eyed larvae obtained from the culture. For example, set 1 may have had 100,000 eyed larvae that were set out of a total of 1,000,000 overall. Set 1 is then 10% of the total. Table 1 below shows how the overall sample sizes are obtained given that the total sample size is 10,000.

Table 1: Example of how sampling is accomplished when there are multiple sets. The proportion of each set is calculated from the final number of eyed larvae from a given batch of larvae. That proportion is then used to calculate the appropriate number of samples to be taken from each set. Examples of n = 10,000 and n = 1,000 are shown below.

Set	Number eyed Larvae	Proportion of Total (%)	Number of spat sampled for	
			10,000	1000
1	100,000	10	1000	100
2	200,000	20	2000	200
3	450,000	45	4500	450
4	150,000	15	1500	150
5	100,000	10	1000	100
TOTALS	**1,000,000**	**100**	**10,000**	**1,000**

Results

Spawn 1 (used for Virginia Seafood Council)

50:50 culture

We mixed diploid and triploid larvae (the latter being the spawn for the 2008 VSC trials) at the beginning of the culture in approximately 1:1 ratio. The triploids were two days older than the diploids. At 11 days, 56% of the larvae were diploid but this percentage dropped to around 22% after the first harvest (Table 2) on day 20. About 25,000 larvae were harvested on the 275 µm screen, and all were triploid as determined by flow cytometry; about 25,000 larvae came off on the 250 µm screen – 91% were triploid.

On day 22, another 3,000 larvae were harvested on the 275 µm screen, and again, they were all triploid by flow cytometry. About 50,000 larvae were harvested on the 250 µm screen and 87% were triploid. After day 22, increasing proportions of diploids were obtained on both the 250 µm and 275 µm screens (Table 2, Figure 1).

It's important to note that larvae from both the 250 µm and 275 µm screen were removed from culture. The implication of this is discussed in Discussion.

Table 2: Spawn 1 – 50:50 mix. Percent diploid and triploid **larvae** from samples taken either on a <250 µm, 250 µm, or 275 µm screen. 1,000 – 3,000 larvae were batch sampled as described above for flow cytometry. Any samples where 2n > 1% were not set. Only the first two batches caught on a 275 µm screen were set (shaded boxes). (–) = no sample taken.

	Screen size for harvesting					
	<250µm		**>250µm**		**>275µm**	
Day	**2n**	**3n**	**2n**	**3n**	**2n**	**3n**
11	56	44	--	--	--	--
20	22	78	9	91	0	100
22	--	--	13	87	0	100
24	--	--	38	62	6	94
27	--	--	74	26	48	52
29	--	--	86	14	44	56
31	84	16	92	8	67	33

The triploids harvested on a 275 µm screen on days 20 and 22 were set, because no diploids were detected in the batch sample of larvae. After setting, we sampled 3,072 spat between 0.5 and 1.0 mm, and detected 0 (zero) diploids. We know there was a significant proportion of diploids in the system, though, because 9% of the larvae on the 250 µm screen were diploid, and 22% were diploid in larvae <250 µm.

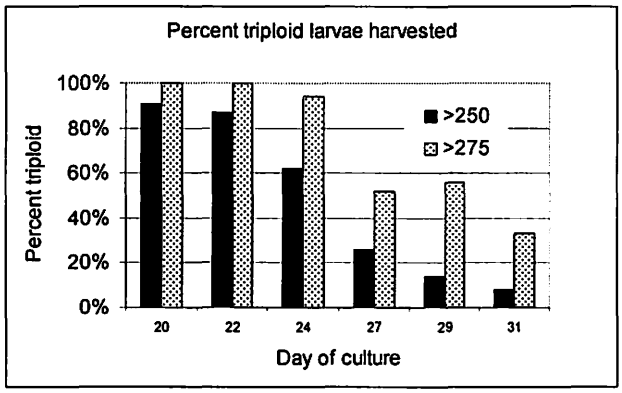

Figure 1: Results of flow cytometry from larvae of 50:50 mix experiment shows decreasing proportions of triploids (on both 250 µm and 275 µm screens) as more and more triploids are removed from the system. This results in a concomitant enrichment of remaining culture for diploids, and increased chance of harvesting diploid larvae.

"100%" culture

A pure 2n x 4n cross was reared for the 2008 VSC trials. The work described in this section was not related to Project CF 08-01, but data are instructive for our objectives. We sampled larvae by flow cytometry on days 2, 4, 9 and 11. Surprisingly, we got relatively high levels of diploid contamination (Table 3). On average, across the four days of sampling, there were 13.2% diploid, about 132 times more diploids than allowable for the trials. Admittedly, we gambled that we could use our screening technique to clean up this spawn. Thus, this exercise is useful to our overall goal. When we started harvesting eyed larvae on day 22, flow cytometry failed to detect diploids on either the 250 µm or 275 µm screens. So all but the 250 µm harvested on day 23 were set, separately and sampled later individually. That diploids were still present in the culture was indicated by the sample on the <250 µm screen from day 32 (but not day 35) (Table 3).

Table 3: Percent diploid and triploid **larvae** from samples taken either on a <250 µm, 250 µm, or 275 µm screen. 1,000 – 3,000 larvae were batch sampled as described above for flow cytometry. Any samples where 2n > 1% were not set. Larvae in the shaded boxes were set. (–) = no sample taken.

	Screen size for harvesting					
	<250µm		**>250µm**		**>275µm**	
Day	**2n**	**3n**	**2n**	**3n**	**2n**	**3n**
2	9	91	–	–	–	–
4	19	81	–	–	–	–
9	8	91	–	–	–	–
11	17	83	–	–	–	–
16	–	–	–	–	–	–
22	–	–	–	–	0	100
23	–	–	0	100	0	100
25	–	–	–	–	0	100
32	4	96	0	100	0	100

35	0	100	0	100	0	100
37	–	–	0	100	0	100

We ended up with a total of 9 separate sets: 6 – 275 µm harvests and 3 – 250 µm harvests. A total of 11,130 spat were sampled in the proportions indicated in Table 4.

Table 4: Number of diploid and triploid **spat** from sets taken either on a 250 µm or 275 µm screen determined by flow cytometry (three per tube).

	Screen size for harvesting					
	>250µm			**>275µm**		
Set	**sampled**	**2n**	**3n**	**sampled**	**2n**	**3n**
1	–	–	–	1707	2	1705
2	–	–	–	537	1	536
3	–	–	–	384	0	384
4	2391	1	2390	960	0	960
5	3552	8	3544	111	1	110
6	480	3	477	144	0	144

Diploids occurred in the 250 µm harvests at about 80% higher frequency than the 275 µm harvests. For 250 µm, 12 diploids were found among 6423 spat (0.19%); for 275 µm, 4 diploids were found among 3842 spat (0.10%). Assuming that there was about 10% diploid contamination (as indicated by earlier FCM) of the culture when harvesting began, and the final percentage of diploids was 0.10%, then the culture was enriched by 100 times (10% v. 0.1%).

The frequency of diploids harvested on both screen sizes went up as a function of the age of the culture, also (Figure 2). The increase in frequency of diploids on the 275 µm screen is troublesome. A hypothesis for this is presented in the discussion below.

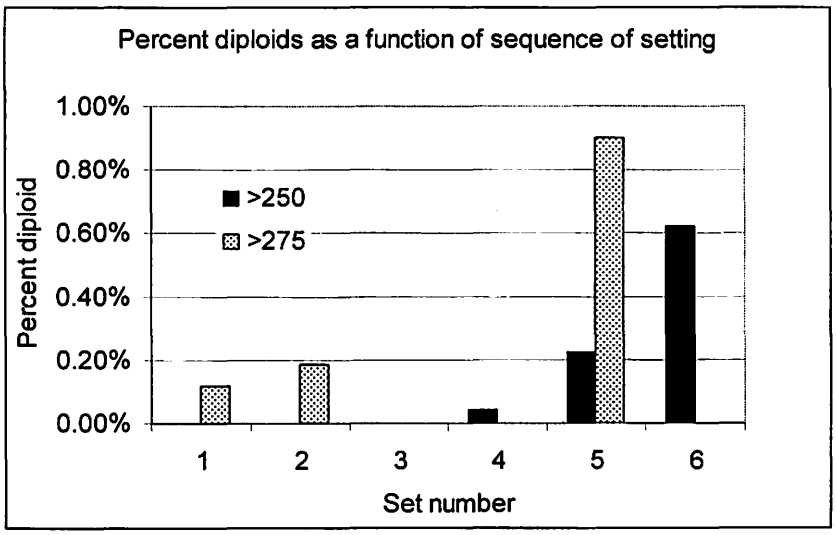

Figure 2: The percent of diploids found in samples of individual spat are plotted against the set number, for harvests on either a 250 μm or 275 μm screen. Set numbers represent harvests of larvae on days 22, 23, 25, 32, 35, and 37.

Spawn 2

Our second triploid *C. ariakensis* spawn was accomplished on March 31, 2008, as a back up spawn for VSC and to run another round of enrichment trials. Both cultures exhibited retarded development and harvesting for the "pure" triploid batch only began at 40 days. The mixed culture, grown in a different tank system – began to crash just as some larvae started to eye-up, around day 24. All harvest of eyed larvae for spawn 2 was done on a 275 μm screen.

50:50 culture

Only one harvest of eyed larvae from the 50:50 mixed culture was obtained on day 24. Up to that point, we periodically tested the culture to verify the proportion of diploids and triploids. These data are shown in Table 5.

Table 5: Percent diploid and triploid **larvae** from samples taken from either the 50:50 mix or "pure" triploid cultures, by day of culture. Shaded boxes were taken on a 275 μm screen. 1,000 – 3,000 larvae were batch sampled as described above for flow cytometry. Any samples where 2n > 1% were not set. Larvae in the shaded boxes were set. (–) = no sample taken. Data in parentheses are inferred.

	Type of culture			
	50:50 mix		**"Pure" triploid**	
Day	**2n**	**3n**	**2n**	**3n**
4	56	44	0	100
6	54	44	–	–
8	56	44	0	100
12	60	40	–	–
13	61	39	–	–
18	59	41	0	100
24	(50)	(50)	–	–
32	–	–	0	100
34	–	–	0	100
40	–	–	0	100
43	–	–	0	100

The mixed culture maintained high proportions of diploid larvae until at least day 18 (Table 5). On day 24, the culture was starting to crash (i.e., high larval mortalities were apparent) so we screened all eyed larvae on a 275 μm screen. We can only infer that the proportion of diploids and triploids was (still) around 50:50 at that point, as remaining larvae were not examined.

The larvae harvested from the 50:50 mix were set, and we examined 3,004 of them via flow cytometry. Among the total we found 6 diploids (0.2%). Although there were still unacceptable levels of diploids in the spat (i.e., >1/1000), the culture started with about 50% diploids. Therefore, the screening step – at least in this culture – seemed to have enriched the proportion of triploids by about 250-fold (50%/0.2% = 250.3).

"100%" culture

A pure 2n x 4n cross was reared as a backup spawn for the 2008 VSC trials. We sampled larvae by flow cytometry on days 4, 8 and 18 before harvesting eyed larvae and found no diploids in the larvae flow cytometry sample, nor were any detected from samples taken from larvae harvested on a 275 μm screen on days 32, 34, 40 and 43 (Table 4), so all larvae harvested were set. Spat were then examined individually as described above. Overall, we examined 10,146 spat all harvested on the "selective" 275 μm screen. Of the total, 13 spat were diploid (0.128%) – over the acceptable limit of 0.1%. Thus, in this culture, the screening seemed to be ineffective. Possibly this has to do with the length of time for the culture to reach eyed, which was extraordinarily long (almost twice).

The trend to find increased percentages of diploid with time of harvest (i.e., set number) was consistent with what we found in spawn 1 (Figure 3). Between set 1 and 8, the proportion of diploids increased from 0.05% to 0.6%, about 12-fold.

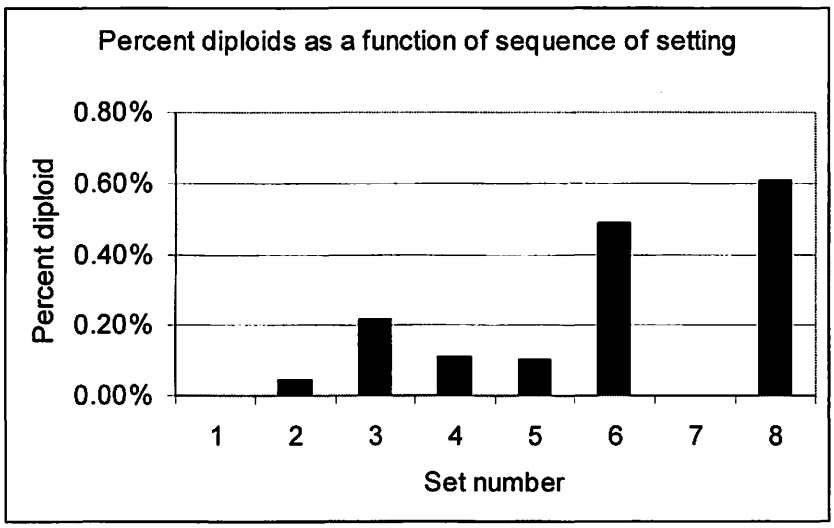

Figure 3: The percent of diploids found in samples of individual spat are plotted against the set number, in "pure" triploid culture. All harvests were done on a 275 μm screen. Set numbers represent harvests of larvae on days 26/27, 29, 32, 34, 36, 39, 41, 43, and 46.

Spawn 3

A third spawn was accomplished but we did it at University of Maryland's Horn Point Environmental Lab (HPL) in Cambridge, Md. on July 17, 2008. The salinity at the time of the spawn and subsequent larval rearing was 9 ppt. We used broodstock from HPL to avoid salinity shock to the eggs. We produced both a "pure" triploid spawn and a mixed group. Diploid and triploid larvae were combined to make a 50:50 mix on July 28th.

50:50 culture

The results for proportions of diploids and triploids in the mixed ploidy culture are shown in Table 6. The starting percentage of triploids, before screening, was 71%. Larvae separated on the 275 μm screen on days 19 and 21 were 100% triploid by flow cytometry. These two groups were set. For the last two harvests on 275 μm, proportions of triploids were higher than 71% -- 91% and 89% respectively. (The last two harvests were not set.) Results on the 250 μm screen are a little hard to interpret, because besides the first harvest, they were virtually the same as the results from harvesting on the 275 μm.

The spat produced from harvests one and two (harvested on a 275 μm screen) were sampled individually (n = 3,039) and flow cytometry results indicated that 0.53% (16 of 3,039) were diploid. That is, the culture went from about 89% triploid (FCM of larvae on day 28) to 99.5% triploid after harvesting on the larger screen – the diploids went from 29% to 0.53%, a 55-fold reduction in diploids.

Table 6: Spawn 3 – 50:50 mix. Percent diploid and triploid **larvae** from samples taken either on a 250 μm or 275 μm screen. In the table, "all" means a random sample from the population was taken before screening. 1,000 – 3,000 larvae were batch sampled as described above for flow cytometry. Larvae from shaded cells were set. (–) = no sample taken.

	Screen size for harvesting					
	all		**>250μm**		**>275μm**	
Day	**2n**	**3n**	**2n**	**3n**	**2n**	**3n**
19	29	71	26	74	0	100
21	–	–	0	100	0	100
25	–	–	10	90	9	91
28	–	–	11	89	11	89

"100%" culture

We started harvesting eyed larvae on day 19, and continued on days 21, 25, and 28. On each harvest day, except the first (day 19), both 250 μm and 275 μm screens were used. On day 19, only a 275 μm was used. Flow cytometry of larvae results indicated that all harvests were 100% triploid. All four harvests on the 275 μm screen were set: sets 1-4.

The spat produced from these sets were sampled (n = 10,272) and evaluated individually by flow cytometry. Results for sets 1 through 4 are 99.88% (3347 of 3351), 99.80% (1524 of 1527), 98.88% (2646 of 2676), and 99.56% (2482 of 2493) triploid. These results suggest that there was no enrichment for triploids in this culture from harvesting on a 275 μm screen, since the overall percentage of triploids was the highest of the three test spawns and greater than the 3/3000 standard typically applied to experimental trials with triploid *C. ariakensis*.

The trend of increased percentages of diploid with time of harvest (i.e., set number) was also apparent and consistent with what we found in spawn 1 and 2 (Figure 4).

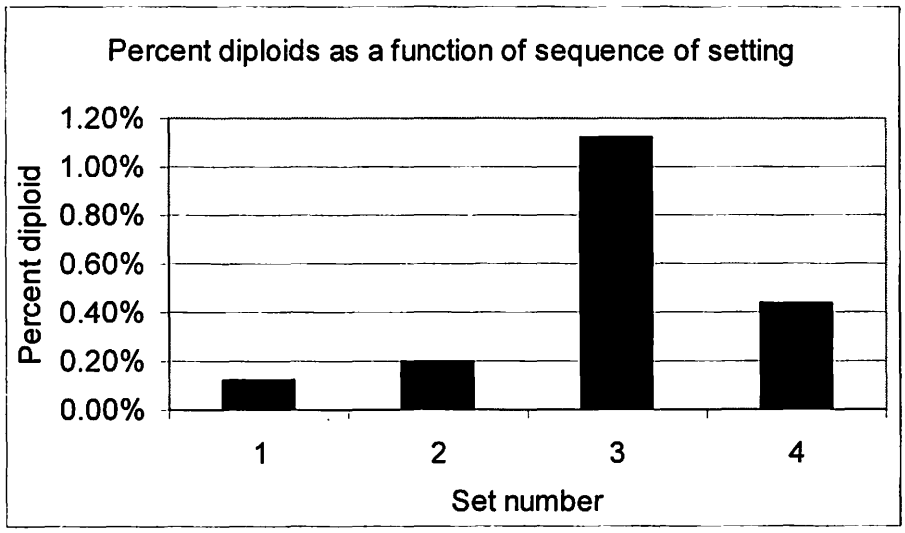

Figure 4: The percent of diploids found in samples of individual spat are plotted against the set number, in "pure" triploid culture. All harvests were done on a 275 μm screen. Set numbers represent harvests of larvae on days 19, 21, 25, and 28.

Reflections on experimental design

One aspect of experimental design that that was problematic was the issue of removing larvae from the system that are too small. For example, to obtain data on where the diploids and triploids were, we harvested on 250 μm and 275 μm screens. However, the larvae on the 250 μm screen were not returned to culture. We did this for both spawns 1 and 2, but not 3. This allows us to compare the two methods. Figure 5 shows the harvest of larvae on 250 μm and 275 μm screens (where the 250 μm were not returned to culture) for the first mix experiment, compared to the harvest of larvae from the mix spawn at HPL where the 250 μm larvae *were* returned to culture.

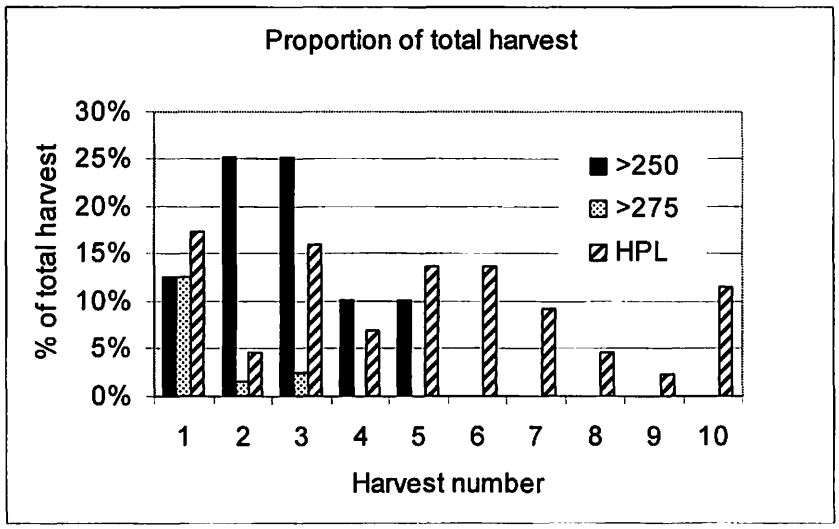

Figure 5: Comparison of yield of larvae on a 275 μm screen (stippled bars) when smaller eyed larvae (250 μm screen – black bars) are removed from culture compared to *not* removing the 250 μm sized larvae (HPL).

When larvae from both 250 μm and 275 μm screen are removed, the harvests of 275 μm larvae decreases drastically (stippled bars, Fig. 5), while harvests of 250 μm larvae (black bars – Fig. 5) increase for days afterward. In contrast, when *only* 275 μm larvae are removed (striped bars – Fig. 5), harvests of eyed larvae are sustained over many days.

What we are doing when we remove the smaller larvae is cropping the average size of the population so that larvae that are almost eyed are all about the same size, diploid and triploid. In order for triploid larvae to get large enough to be retained on a 275 μm screen at the next harvest, it has a long way to grow. Cropping off the smaller larvae like this, may affect our ability to enrich for triploids. We will test this premise in experiments next year.

Discussion

In our exploratory experiments previously and the ones above, we have learned that enriching the proportion of triploids is indeed possible and can, on occasions, enrich the proportion of triploids 200-300 fold. Table 7 summarizes the "enrichment" calculated from the mix experiments for spawns 1, 2 and 3. The corollary selectively harvesting the triploids, which wasn't immediately apparent originally, is that we are also enriching the culture for greater proportions of diploids, therefore over time, enrichment of triploids by using a larger screen gets increasingly intense.

Table 7: Approximate improvement (reduction) of triploid percentage by aggressive screening on a 275 μm screen for experiment spawns described above.

	Calculated reduction of diploids
Spawn	**x**
1	100
2	250
3	55–

At the same time, leaving eyed diploid larvae behind in the culture probably delays their setting, during which time they may be getting bigger than diploid larvae that are removed immediately. This hypothesis is supported by the increase in the proportion of diploid spat in all spawns (Figs. 2, 3, and 4).

It seems that environmental conditions may also have something to do with the effectiveness of enrichment, as results varied when cultures took a long time (spawn 2) or at low salinity (spawn 3).

The bottom line for the enrichment procedure seems to be that we need to have both a size and time cutoff for larval populations. That is, the 275 μm screen is useful for enriching triploid proportions provided it is used for the initial 2-3 harvests. Afterward, diploids seem to creep in. At that time, harvests might cease and the rest of the larvae discarded. The implication for hatchery production of triploid *C. ariakensis* populations for aquaculture is that higher (maybe *double*) production is needed for the same amount of seed. That is, half the larvae (the late ones) may have to be discarded to avoid diploids. The upside is that, if indeed we can enrich triploids, for example, 100-fold, then the percentage of diploids may drop from 1/1000 to 1/100,000. Proving 1/100,000 is difficult, but this is why the 50:50 mixed cultures are so valuable – to demonstrate exclusion of large numbers of diploids.