



Polyploid induction by heat shock-induced meiosis and mitosis inhibition in the dwarf surfclam, *Mulinia lateralis* Say

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Abstract

Heat shock is an effective and widely used method for polyploid induction in fish, but has not been well studied in molluscs. In this study, heat shock was tested for polyploid induction through meiosis or mitosis inhibition in the dwarf surfclam, *Mulinia lateralis* Say. To determine the most effective temperature, different heat shocks (32, 35 and 38 °C) were applied to newly fertilized eggs at 8–11 min post-fertilization (MPF) to inhibit the extrusion of polar body 1 (PB1), and ended when the second polar body began to form in control groups. Ploidy of resultant larvae was determined by flow cytometry (FCM). Both 35 °C and 38 °C were effective in inhibiting PB1, but 38 °C led to complete mortality of larvae. Heat shock of 32 °C only accelerated embryonic development without inhibiting PB1, as all resultant larvae were diploid. High percentages of triploids (86.3–98.5%) were produced by the 35 °C treatment as detected at the juvenile stage. Different durations (10, 15 and 20 min) of the 35 °C heat shock were used for PB1-inhibition to test effects on polyploid formation, which produced primarily triploids (10 min) and pentaploids (20 min), but virtually no tetraploids. To inhibit mitosis I (M1) and mitosis II (M2) for tetraploid induction, a heat shock of 35 °C was applied to fertilized eggs at 35–37 and 50–57 MPF for about 20 min, producing up to 82.8% and 44.4% tetraploid larvae, respectively. Most tetraploid larvae were abnormal and did not develop to the D-stage, and no tetraploids were observed at the juvenile stage. This study shows that heat shock is a highly effective method for polyploid induction in the dwarf surfclam and possibly in other molluscs also.

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1. Introduction

Polyploid fish and shellfish have useful applications in aquaculture. Triploids are useful because of their potential sterility and fast growth, and tetraploids are desired for their ability to produce 100% triploid

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by mating with normal diploids. Even with intensive efforts, tetraploid induction remains a challenge in both fish and shellfish. So far, tetraploid fish have been successfully produced in two stains of rainbow trout by inhibiting mitosis I (Chourrout, 1984; Myers, 1986; Hershberger and Hostuttler, 2005). In shellfish, the most successful way to obtain viable tetraploids is using eggs from triploids fertilized with sperm from diploids, followed by inhibiting polar body 1 (PB1). This method was first developed in the Pacific oyster, *Crassostrea gigas*, producing a high percentage of tetraploids (Guo and Allen, 1994). Using this novel approach, tetraploids were also produced in other bivalve species, such as the pearl oyster, *Pinctada martensii* Dunker (He et al., 2000), the eastern oyster, *Crassostrea virginica* (Guo et al., 2002), Suminoe oyster, *Crassostrea ariakensis* (Allen et al., 2003) and Catarina scallop, *Argopecten ventricosus* (Maldonado et al., 2003).

Unfortunately, most triploid molluscs, such as soft-shell clam, *Mya arenaria* (Allen et al., 1986), noble scallop, *Chlamys nobilis* (Komaru et al., 1988), Sydney rock oyster, *Saccostrea commercialis* (Cox et al., 1996) and blue mussel, *Mytilus edulis* (Brake et al., 2004), show highly retarded gametogenesis and do not produce or spawn mature eggs. Therefore, tetraploid induction using triploid eggs is not feasible in these species, and other approaches are still needed for tetraploid induction.

Theoretically, direct induction of tetraploids using normal zygotes is possible through PB1 inhibition, mitotic inhibition, cell fusion or gynogenesis combined with meiosis inhibition (Guo, 1991). There have been extensive research in this field, but most reports show that the induced tetraploids cannot survive beyond metamorphosis. Tetraploid juveniles were produced by direct induction with PB1 inhibition in four shellfish species: Mediterranean mussel, *Mytilus galloprovincialis* (Scarpa et al., 1993), Manila clam, *Tapes philippinarum* (Allen et al., 1994), zhi-kong scallop, *Chlamys farreri* (Yang et al., 2000b) and dwarf surclam, *Mulinia lateralis* (Peruzzi and Guo, 2002; Yang and Guo, 2004). However, only a few individuals were obtained in each study, and breeding populations were not established.

Mitosis inhibition is a widely used method for tetraploid induction and has led to the successful production of tetraploid fish (Chourrout, 1984;

Myers, 1986; Hershberger and Hostuttler, 2005). In shellfish, however, tetraploid induction by this approach has not been successful (Guo et al., 1994; Jiang et al., 1998; Yang et al., 1997, 1999; Chang et al., 2002). In most studies, the induction efficiency was low, and the induced tetraploids were only detected in embryonic or larval stages. More investigations are needed to evaluate and improve mitosis inhibition as a method for tetraploid induction.

Mitosis can be inhibited by chemical (cytochalasin B or colchicine) or physical agents (heat shock or high pressure). In fish, heat shock and high pressure are widely used and have proven to be effective in the production of both triploids and tetraploids (Thorgaard, 1983; Chourrout, 1984; Myers, 1986; Hershberger and Hostuttler, 2005). In shellfish, chemicals such as cytochalasin B (CB) and 6-dimethylamino-purine are widely used and have been shown to be effective (Desrosiers et al., 1993; Scarpa et al., 1994). However, heat shock, which is used effectively in fish, has not been fully evaluated in molluscan polyploid induction, especially for mitosis inhibition. In this study, we investigated the effectiveness of heat shock in polyploid induction by inhibiting either meiosis or mitosis in fertilized eggs of dwarf surfclam, a model species with small body size and short life cycle (about 3 to 4 months) to search for optimal methods for tetraploid production.

2. Materials and methods

2.1. Gametes collection and larvae culture

The dwarf surfclams used in this study were collected from Rhode Island, United States. The clams were conditioned at 21–22 °C and under intensive feeding for 3–4 weeks. Gametes were obtained by natural spawning with thermal stimulation or by dissection. Eggs were passed through a 60- μ m screen to remove debris, collected on a 25- μ m screen, and then suspended in fresh seawater, ready for fertilization. Sperm were filtered through a 25- μ m screen to remove tissue and debris.

The eggs were fertilized by adding sperm into the egg suspension to a density of 5–7 sperm per egg. This time point was recorded as the fertilization time, or 0 min post-fertilization (MPF). All experiments and

larval culture were conducted at 23–24 °C. Embryos were cultured at a density of 10–50 embryos/ml. Beginning at 24-h PF, larvae were cultured at 10–20 larva/ml and fed daily with *Isochrysis galbana* at a density of 50,000 cells/ml. Culture seawater was completely changed every 2–3 days with larvae being collected and washed gently on screens of proper size. At each water change, larvae were counted and measured using a microscope eyepiece micrometer. After metamorphosis, spat were cultured in up-wellers in a well-aerated re-circulating system at 19–20 °C. Changes of culture seawater (approximately 1/5 of total volume) were performed twice a week.

2.2. Ploidy determination

The ploidy of larvae and juveniles was determined using flow cytometry (FCM) with DAPI (4,6-Diamidino-2-phenylindole) staining (10 mg/ml, dissolved in Tris buffer: 10 mM Tris, 146 mM NaCl, 2 mM CaCl₂, 22 mM MgCl₂, 0.005% bovine serum albumin, 0.1% Triton-X and 10% DMSO). Larvae (200–300) were pooled for FCM analysis, while juvenile clams were analyzed individually. Before running on FCM, the samples in DAPI staining solution were vortexed for 10 s and syringed with 25 gauge needle three times to dissociate cells.

2.3. Experiment I: block polar body 1 with 32, 35 and 38 °C heat shocks

Fertilized eggs from the same parents were separated into four groups before applying treatments: one group was used as control, and the other three groups were used for different heat shock treatments. Heat shocks (32, 35, 38 °C) were applied to the fertilized eggs by sharply raising the temperature within 10 s from 24 °C at 8–11 MPF. When PB2 began to form in the control group, the heat shock treatment was ended and the treated eggs were returned to 23–24 °C seawater for culture. The duration of heat shock was 21–30 min depending on the development speed of each batch of eggs. The larvae were sampled to determine ploidy at day 1, day 6 and day 11 in both control and treated groups. After metamorphosis, the juveniles were harvested and sampled individually for ploidy analysis by FCM. Four replicates were produced using different pairs of parents.

2.4. Experiment II: block PB1 with 35 °C for different durations

Based on the results obtained in Experiment I, heat shock of 35 °C was used in the second experiment to block PB1 for different durations of time. Newly fertilized eggs were treated with a 35 °C heat shock at 10–15 MPF, and returned to 23–24 °C after 10, 15 and 20 min of the treatments. The larvae were sampled at days 1, 6 and 12 for ploidy analysis by FCM, and at day 6 the trochophores and D-stage larvae were sampled and analyzed separately using the proper size screen. Fertilization levels and survival to different stages were recorded. In this experiment, two replicates were produced by using different pairs of parents.

2.5. Experiment III: inhibition of mitosis I or mitosis II with 35 °C heat shock

In this experiment, heat shock of 35 °C was used to block mitosis I or mitosis II for tetraploid induction. For mitosis I inhibition, the heat shock treatment started when approximately 70% of the fertilized eggs had released both polar bodies (35–37 MPF at 23–24 °C) and ended when approximately 70% fertilized eggs had developed to the 2-cell stage in the control group. The duration of heat shock was around 21–23 min. For mitosis II inhibition, the heat shock (35 °C) started when 70% of the fertilized eggs developed to 2-cell stage (around 50–57 MPF), and ended when 70% of the fertilized eggs developed to 4-cell stage in the control group. The duration of heat shock was about 18–20 min. Three replicates were produced for each treatment using different pairs of parents. Larvae were sampled at days 1, 6 and 12 for ploidy analysis and at day 6, D-stage larvae were separated from trochophores by using a screen of proper size and analyzed separately.

2.6. Data collection and analysis

The percentage of divided eggs was recorded at 2-h PF. Survival was calculated as the number of survivors over the initial number of divided eggs. Length (the longest dimension) of larvae was measured under a microscope. Data were analyzed using SYSTAT 11. Effects of treatment (or group) were tested by

ANOVA. Percentage data were arcsine-transformed before analysis. Significance level was set at $P < 0.050$ unless otherwise noted.

3. Results

3.1. Effects of different heat shocks (32, 35 and 38 °C) on PB1 inhibition

Treatment time, number of eggs used and divided, and survival to different stages in all control and treated groups are presented in Table 1. Heat shock (32, 35 and 38 °C) treatments were applied to the fertilized eggs at around 10 MPF, but lasted for different durations, ranging from 21 to 30 min. The standard for ending the treatment was when about 70% of the fertilized eggs in the control group released PB1. Because the fertilized eggs from different parents developed at different speeds, the duration of treatment varied among replicates.

During treatments, fertilized eggs reacted differently to the three different heat shock temperatures. With the 32 °C treatment, fertilized eggs developed faster than those in control groups, rather than being inhibited. With the 35 °C treatment, fertilized eggs stopped development, and the membrane of fertilized eggs shrank. When returned to 23–24 °C seawater after treatment, the shrunken membrane returned to normal, and development resumed. With the 38 °C treatment, fertilized eggs also stopped development, and membrane shrank. But when returned to 23–24 °C seawater, most fertilized eggs developed abnormally. These abnormally developed embryos were degraded by day 1 and lost through the screen during the first water change.

The three heat shock temperatures had different effects on larval development and survival. Compared with the control group, the 32 °C heat shock did not significantly affect either egg development or larval survival ($P > 0.082$). The 35 °C heat shock significantly decreased the number of eggs that divided and

Table 1

The timing, duration, number of eggs used, percentage of eggs divided after treatment, and cumulative survival of divided eggs to days 1, 3, 6, 8 and 11 in four replicates of three heat shock treatments (32, 35, and 38 °C) in the dwarf surfclam, *Mulinia lateralis* Say

Group	Beginning (MPF ^a)	Duration (min)	Total eggs (×1000)	Divided eggs (%)	Survival (%)				
					Day 1	Day 3	Day 6	Day 8	Day 11
Control 1			40	100.0	60.0	44.0	44.0	20.0	13.8
Control 2			67	97.3	–	–	–	–	–
Control 3			109	98.5	82.7	62.0	55.6	48.7	48.7
Control 4			188	97.8	86.0	58.0	41.4	41.4	35.7
Average				98.4	76.2	54.7	47.0	36.7	32.7
32 °C-1	8	28	295	99.0	64.4	44.7	32.8	29.8	20.1
32 °C-2	11	30	241	97.9	33.2	33.2	23.2	–	13.7
32 °C-3	9	23	282	97.6	44.0	30.5	21.6	–	15.1
32 °C-4	9	21	226	98.1	47.8	43.4	21.2	–	21.2
Average				98.2	47.4	38.0	24.7	29.8	17.5
<i>P</i> -value				0.082	0.707	0.933	0.536	0.207	0.704
35 °C-1	9	26	224	95.5	9.8	5.8	3.8	1.8	1.8
35 °C-2	10	30	489	95.9	0.2	0.02	0.02	–	0.02
35 °C-3	10	23	748	82.0	0.03	0.03	0.007	–	0.003
35 °C-4	10	21	636	91.8	1.4	1.1	0.2	0.08	0.08
Average				91.3	2.9	1.7	1.0	0.9	0.5
<i>P</i> -value				0.024	0.030	0.024	0.021	0.081	0.064
38 °C-1	10	23	240	0.8	0				
38 °C-2	10	29	161	10.8	0				
38 °C-3	11	23	282	1.7	0				
38 °C-4	11	21	80	2.7	0				
Average				4.0					

P-values were from ANOVA comparing treated with control groups.

^a MPF: min post-fertilization.

cumulative survival to days 1, 3 and 6 ($P < 0.030$), but not to days 8 and 11. The 38 °C heat shock was the harshest treatment, with only about 4% of the treated eggs dividing. No normal D-stage larvae were

obtained at day 1, and all surviving larvae were apparently abnormal, with no or abnormal shells.

The ploidy of larvae (about 500–1000 in a pool) from each heat shock treatment was analyzed by FCM

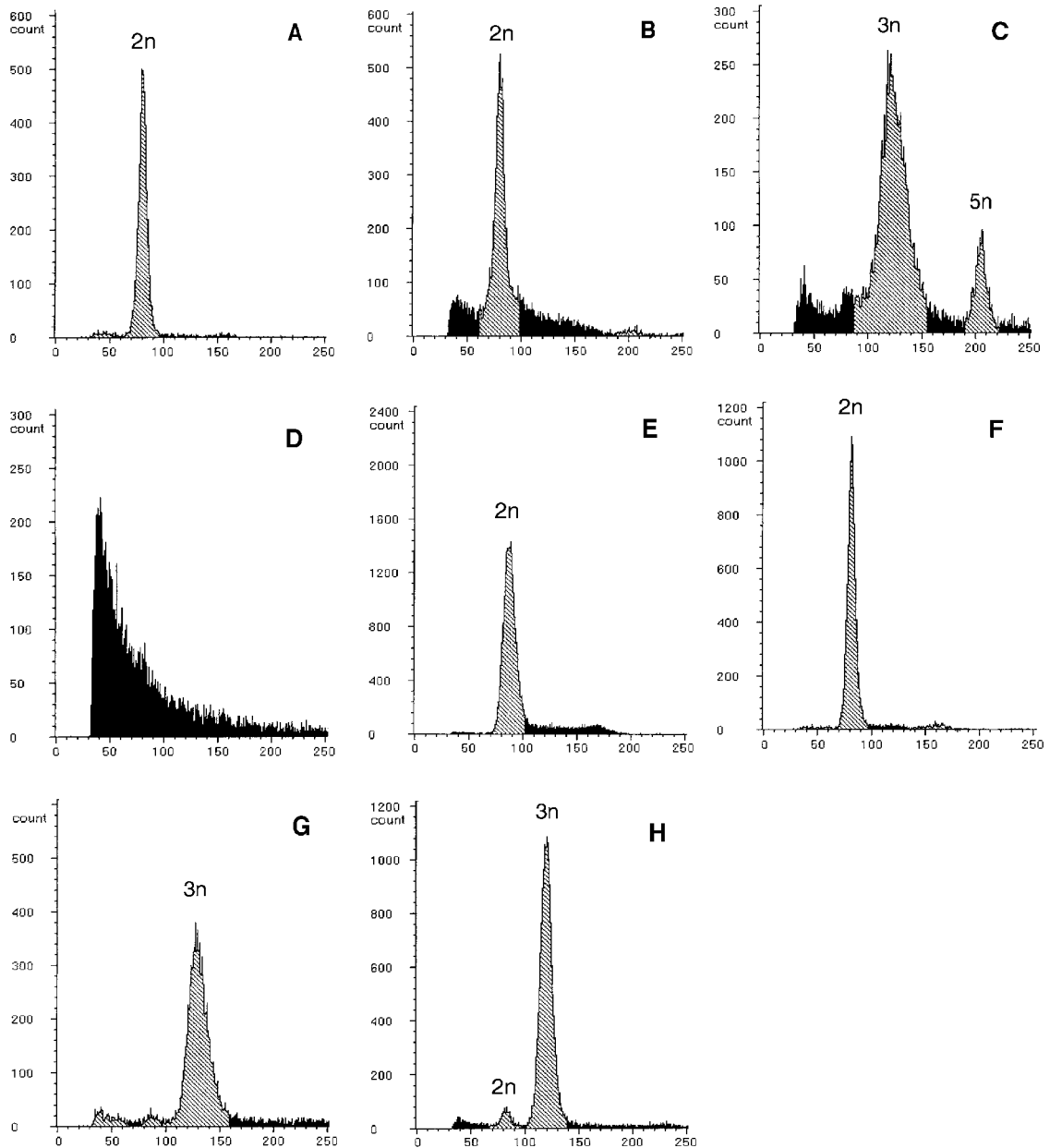


Fig. 1. Flow cytometry analysis of larvae resulting from blocking polar body 1 with different heat shocks (32, 35 and 38 °C) in the dwarf surfclam, *M. lateralis*: (A–D) 1-day-old larvae from the control, 32 °C, 35 °C and 38 °C-treated groups, respectively; (E–H) 6-day-old larvae from the control, 32 °C, 35 °C and 38 °C-treated groups, respectively. The X-axis represents the channel number (or DNA content), and the Y-axis represents cell count.

Table 2

The application of different durations (10, 15 and 20 min) of a 35 °C heat shock to block polar body 1 in the dwarf surfclam, *Mulinia lateralis* Say: number of parents and eggs used, percentage of eggs divided after treatment and cumulative survival of divided eggs to days 2 and 6

Replicate	Parents (F × M) ^a	Beginning time (MPF ^b)	Duration (min)	Total eggs (× 1000)	Divided eggs (%)	Survival (%)	
						Day 2	Day 6
1	10 × 3		Control	108	86.5	89.9	48.2
			10	320	77.4	13.3	7.7
			10	810	72.7	3.7	1.2
			20	620	66.7	0.7	0.4
2	12 × 3		Control	124	90.3	100.0	73.2
			10	522	65.6	18.1	1.3
			15	514	63.4	2.6	0.4
			20	418	73.2	1.0	0.2

^a F × M: Female × Male.

^b MPF: min post-fertilization.

(Fig. 1). In the control group, all larvae were diploid at days 1 and 6 (Fig. 1A and E). In the 32 °C treatment, the larvae were primarily diploid at days 1 and 6 (Fig. 1B and F). In the 35 °C treatment, however, the larvae

were represented by diploid, triploid and pentaploid peaks at day 1 (Fig. 1C), and by only a triploid peak at day 6 (Fig. 1G). In the 38 °C treatment, the larvae sampled at day 1 did not give a clear FCM peak due to

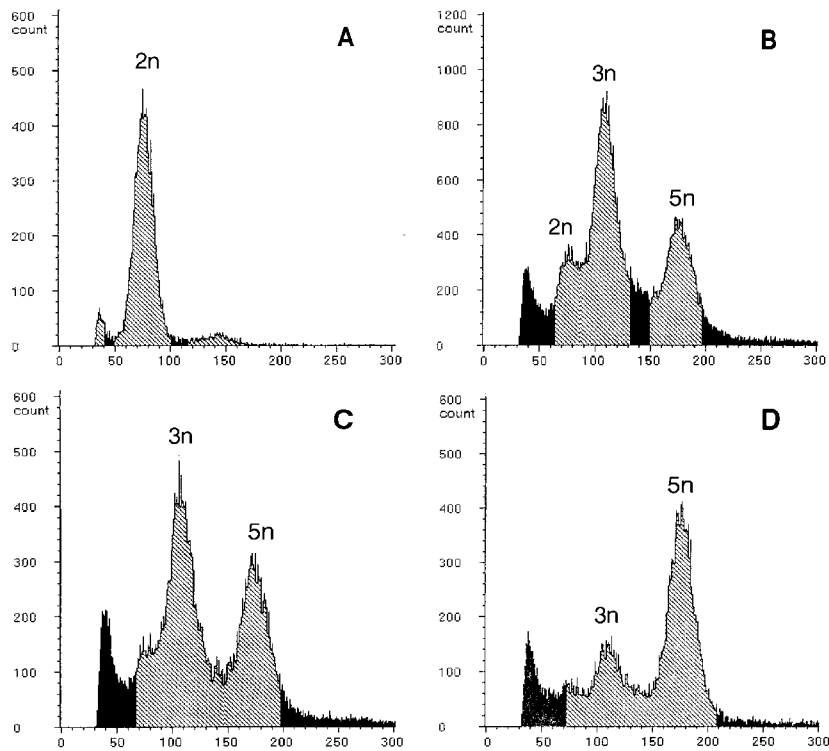


Fig. 2. FCM analysis of 2-day-old larvae resulting from different durations of a 35 °C heat shock intended to inhibit the first polar body in the *M. lateralis* Say: (A) control; (B) 10-min duration; (C) 15-min duration; and (D) 20-min duration. The X-axis represents the channel number (or DNA content) and the Y-axis represents cell count.

degeneration of eggs or embryos damaged by heat shock. At day 6, the surviving abnormal larvae were screened and sampled for FCM analysis. The result showed primarily a triploid peak (Fig. 1H).

Larval size in all control and treated groups was recorded at each water change, and there was no significant difference in size among all groups at all sampling dates.

Between 20 and 29 days post-fertilization, the juveniles were harvested in the control, 32 °C and 35 °C treated groups, while no juveniles survived in the 38 °C treated groups. The ploidy of the juveniles was individually determined by FCM. In the control and 32 °C treated groups, the juveniles were 100% diploid ($n=50$ in each replicates). In the 35 °C treated groups, high percentages of triploids were observed. In replicate 1, 19 out of 23 individuals (82.6%) were triploid. In replicate 4, 199 out of 202 individuals (98.5%) were triploid. Replicates 2 and 3 produced seven and three juveniles, respectively, which are too few for any meaningful analysis. No tetraploids were observed in any of the replicates.

3.2. Effect of heat shock (35 °C) duration on PB1 inhibition

In this experiment, heat shock of 35 °C was applied to fertilized eggs to block PB1 for 10, 15 and 20 min. The results are presented in Table 2. There was no significant difference among the three heat shock durations tested in the percentage of eggs that divided ($P>0.934$) and survival to day 2 ($P>0.796$) and day 6 ($P>0.981$). Compared to the control group, however, the heat shock treatments significantly affected survival to day 2 ($P<0.006$) and day 6 ($P<0.020$), but not the number of eggs that divided ($P>0.058$).

FCM analysis of larvae from the control and treated groups are shown in Fig. 2. Different treatment duration produced different proportions of diploids, triploids and pentaploids. As a general pattern, short treatment (10 min, Fig. 2B) produced more triploids, while longer treatment (20 min, Fig. 2D) produced more pentaploids. There was no clear evidence that significant numbers of tetraploids were produced in any groups.

Table 3

Mitosis I or II inhibition (M1 or M2) with a 35 °C heat shock in the dwarf surfclam, *Mulinia lateralis* Say: number of parents and eggs used, starting time and duration, percentage of divided eggs and cumulative survival to days 2 and 6

Group	Parents (F × M ^a)	Beginning time (MPF ^b)	Duration (min)	Total egg (× 1000)	Divided eggs (%)	Survival rate (%)	
						Day 2	Day 6
<i>Mitosis I inhibition</i>							
Control-1	5 × 1			65	98.4	77.2	46.3
Control-2	5 × 1			102	98.8	79.4	57.2
Control-3	7 × 2			142	100.0	93.7	81.4
M1-1		35	22	700	98.2	0.07	0.06
M1-2		38	21	960	98.6	0.06	0.01
M1-3		37	23	1155	96.2	0.13	0.03
<i>P</i> -value					0.200	0.001	0.008
<i>Mitosis II inhibition</i>							
Control-1	7 × 1			63	98.4	74.6	54.0
Control-2	8 × 2			80	100.0	75.0	53.8
Control-3	10 × 2			130	97.2	55.8	56.9
M2-1		50	20	1196	99.8	0.29	0.05
M2-2		55	21	970	99.4	0.43	0.25
M2-3		57	18	1055	95.6	0.08	0.05
<i>P</i> -value					0.867	0.001	0.008

P-values were from *t*-test comparisons of treated with control groups.

^a F × M: Female × Male.

^b MPF: min post-fertilization.

3.3. Effects of heat shock (35 °C) on mitosis I or II inhibition

Heat shock (35 °C) treatment was applied to the fertilized eggs around 35–38 MPF to block mitosis I, and the treatment lasted for 21–23 min (Table 3). After the treatment, treated eggs suffered heavy mortalities, and cumulative survival to days 2 and 6 in treated groups was only 0.09% and 0.03%, respectively, a significant decrease compared to that in control groups: 83.4% to day 2 ($P=0.001$) and 61.6% to day 6, ($P=0.008$). FCM analysis at day 1 showed, on average, 62.4% tetraploid larvae were produced (Fig. 3B; Table 4). Tetraploid larvae were also detected by FCM at high percentages at day 2, ranging from 64.6% to 82.8% (Table 4). At day 6, separate FCM analysis of D-stage larvae and trochophores showed all D-stage larvae were diploid (Fig. 3C), and the trochophores were mostly diploid and tetraploid

with some being triploid (Fig. 3D, Table 4). During culture, the trochophores died out around day 6 or 7. At day 12, the surviving larvae metamorphosed, and FCM analysis showed that all larvae from the treated groups were diploid (Fig. 3F), the same as in the control groups (Fig. 3E).

To block mitosis II, the 35 °C heat shock was applied to the 2-cell stage embryos at 50–57 MPF, and lasted for 18–21 min (Table 3). The survival after treatment was significantly lower than that in control groups ($P\leq 0.008$): only 0.26% to day 2 and 0.12% to day 6. FCM analysis of resultant larvae showed patterns of ploidy composition similar to that for mitosis I inhibition (FCM graphs not shown). Larvae in M2-inhibited groups were tetraploid (average 41.5%) and diploid (Table 4). No triploids were observed. The number of tetraploid larvae remained at approximately 40% at day 2. At day 6, separate FCM analyses of D-stage larvae and trochophores showed that the D-stage

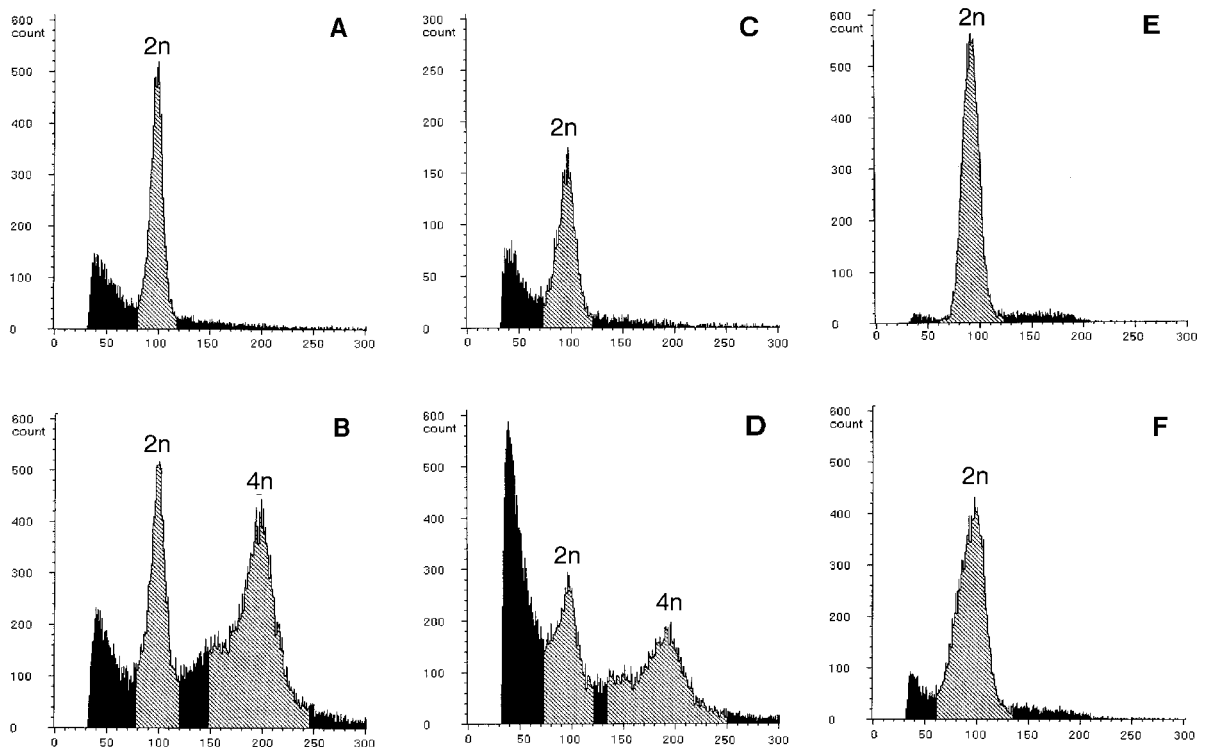


Fig. 3. Flow cytometry analysis of larvae resulting from a 35 °C heat shock intended to inhibit mitosis I in fertilized eggs of dwarf surfclam, *M. lateralis* Say: (A) 1-day-old from control; (B) 1-day-old from treated; (C) D-stage larvae from treated at day 6; (D) abnormal trochophores from treated at day 6; (E) metamorphosed larvae from control at day 12; (F) metamorphosed larvae from treated groups at day 12. The X-axis represents the channel number (or DNA content) and the Y-axis represents cell count.

Table 4

Ploidy composition determined by flow cytometry analysis of dwarf surfclam, *Mulinia lateralis* Say larvae resulting from a 35 °C heat shock intended to inhibit mitosis I (M1) or mitosis II (M2)

Group	24 h (%)			Day 2 (%)		Day 6 (%)		
	All larvae			All larvae		Trochophores		D larvae
	2n	3n	4n	2n	4n	2n	4n	2n
M1-1	41.0	21.9	37.1	35.4	64.6	43.3	56.7	100
M1-2	23.5		76.5	21.4	78.6	45.0	55.0	100
M1-3	26.3		73.7	17.2	82.8	40.0	60.0	100
M2-1	58.5		41.5	59.0	41.0	64.1	35.9	100
M2-2	50.1		49.9	58.1	41.9	67.8	32.2	100
M2-3	67.0		33.0	55.6	44.4	60.0	40.0	100

larvae were all diploid, and the trochophores were diploid and tetraploid (Table 4). From days 6 to 12, the trochophores died gradually, and all surviving larvae metamorphosed at day 12, which were all diploid.

In both mitosis I- and II-inhibited groups, trochophores at day 6 were morphologically abnormal, usually without velum, and with cilia over the entire body. Some larvae developed shells, but the two halves did not fit and could not close.

4. Discussion

4.1. Effects of heat shock on meiosis and mitosis inhibition

Heat shock is a widely used method for polyploid induction in fish. So far, at least 25 publications (not listed here) have reported the use of heat shock to produce triploid, tetraploid or gynogenic fish by blocking either meiosis or mitosis. The heat shock temperature and duration varied among species. Similarly, heat shock has been shown to be effective for both triploid and tetraploid induction by inhibiting meiosis and mitosis in shrimp (Li et al., 2003a,b). In molluscs, heat shock has also been used for polyploid induction, especially triploid induction by meiosis II inhibition. The induced triploid percentages range from 20% to 97.4% at larval or juvenile stage, and the species studied included oysters (Zeng et al., 1994; Yu et al., 2001), mussels (Yamamoto and Sugawara, 1988; Desrosiers et al., 1993; Toro and Sastre, 1995; Davis, 1997), scallops (Wang et al., 1990; Toro

et al., 1995) and clams (Gosling and Nolan, 1989). In some reports, heat shock was used in combination with caffeine (Durand et al., 1990; Yu et al., 2001) or 6-dimethylaminopurine (Davis, 1997) to increase the efficiency of polyploid induction. Only in one study in the Pacific oyster was heat shock used to inhibit mitosis I for tetraploid induction (Guo et al., 1994). The effective temperature for heat shock varies among different species of fish, shrimp and shellfish.

In our experiment, the heat shock temperature for *M. lateralis* was chosen based on the sub-lethal temperature of 38 °C for egg development. The other two temperatures were determined by lowering 38 °C by 3 °C increments (35 °C and 32 °C). These three temperatures had distinctive effects: 38 °C was effective in inhibiting meiosis I, but resulted in almost no survival to D-stage; 35 °C was also effective, resulted in high percentages of triploids, and allowed some to survive to D-stage and even beyond metamorphosis. Instead of inhibiting meiosis I, the 32 °C heat shock actually accelerated the speed of meiotic development. These results indicate that in *M. lateralis*, among the temperatures tested, 35 °C is the most efficient temperature for polyploid induction through meiosis inhibition. The 35 °C treatment produced as high as 98.5% triploid juveniles in one of the replicates. Triploid induction by inhibiting PB1 rarely produces high percentages of triploidy in molluscs, especially at the juvenile stage.

4.2. Duration of heat shock and polyploid production

In treatments for polyploid induction, two crucial factors are the starting time and duration of treatment. The standard is usually set by observing the development of fertilized eggs in control groups. The development of fertilized eggs is often unsynchronized and varies greatly among different females and under different incubation temperatures and conditions. The use of polar body release as a milestone has proven to be effective and dependable in other molluscan species for polyploid induction (Yang et al., 2000b; Yang and Guo, 2004). In this study, the starting time was set within 5 min before the target events (meiosis I, mitosis I and mitosis II) began. The duration of treatment was determined based on the development speed of fertilized eggs in control groups. Under our experimental conditions, treatment dura-

tions were around 21–30 min for the PB1 inhibition, 18–21 min for mitosis I and 21–23 min for mitosis II inhibition.

Inhibition of PB1 in this study produced triploid and pentaploid larvae, but not tetraploids. At the juvenile stage, only triploid individuals were observed. This result differs from an early study where inhibition of PB1 with CB produced tetraploids (Yang and Guo, 2004). The duration of treatment cannot explain the lack of tetraploid induction, as different durations of heat shock in experiment II did not produce any tetraploids. The increase in heat shock duration led to an increase in the percentage of pentaploid larvae. PB1 inhibition with CB seriously alters chromosome segregation in meiosis II and results in the formation of diploids, triploids, tetraploids and aneuploids (Guo et al., 1992; Yang et al., 2000a). Both triploid and tetraploid juveniles were obtained in zhikong scallop and dwarf surfclam by PB1 inhibition (Yang et al., 2000b; Peruzzi and Guo, 2002; Yang and Guo, 2004). However, PB1 inhibition by heat shock did not produce obvious tetraploid peaks at the larval stage, and no tetraploids at the juvenile stage. This difference is probably due to different mechanisms by which heat shock and CB inhibit meiosis I and affect chromosome segregation, differences that are largely unknown at this time.

4.3. Mitosis I inhibition as a method for tetraploid induction

Theoretically, mitosis I inhibition is an effective way to induce tetraploidy. This approach has been used successfully for tetraploid induction in fish (Chourrout, 1984; Myers, 1986; Hershberger and Hostuttler, 2005). In molluscs, tetraploid induction by mitosis I inhibition has not been extensively studied. Only a few studies reported the production of tetraploid embryos by blocking mitosis I (Guo et al., 1994; Yang et al., 1997, 1999; Chang et al., 2002). In this study, the effective temperature for blocking meiosis I (35 °C) was also effective for blocking mitosis I and II. High percentages of tetraploid larvae were produced by mitosis I inhibition (up to 82.8% at day 2), and by mitosis II inhibition (up to 44.4% at day 2). The percentage of tetraploids induced in this study is considerably higher than previous studies. However, all tetraploid larvae were morphologically

abnormal, and died out around day 7 or 8. Similarly, no viable tetraploids were obtained in all previous studies. In both the mitosis I- and mitosis II-inhibited groups, the abnormal larvae were analyzed by FCM at day 6, the results showed these abnormal larvae included not only tetraploids, but also a large percentage of diploids. The presence of diploid individuals among abnormal larvae shows that heat shock disrupted normal development when inhibiting the first two mitotic divisions. Consequently, survival was very low (Table 3), and no tetraploid individuals survived to the juvenile stage. Further studies are needed to determine whether the inability of tetraploids to survive beyond metamorphosis is caused by lethal effects of heat shock or tetraploidy. It has been suggested that tetraploids produced by direct induction using eggs from diploids have limited viability because of a cell number or cytoplasm deficiency (Guo et al., 1994). Tetraploids produced using large eggs from triploids have improved survival (Guo and Allen, 1994). On the other hand, it has been shown in several species that some tetraploids produced from direct induction, albeit very few, can survive beyond metamorphosis (Scarpa et al., 1993; Allen et al., 1994; Yang et al., 2000a,b; Peruzzi and Guo, 2002; Yang and Guo, 2004).

In summary, this study shows that heat shock is highly effective for polyploid induction by inhibiting either meiosis I, mitosis I or II in *M. lateralis*. Heat shock produces high percentages of triploids (up to 98.5%) by inhibiting meiosis I and high percentages of tetraploid larvae (up to 82.8%) by inhibiting mitosis I. There is no direct comparison between heat shock and other induction methods in this study, but the high percentages of triploids and tetraploids produced here clearly place heat shock among the most effective methods for polyploid induction in molluscs, especially for tetraploid induction by mitosis I inhibition. Although *M. lateralis* is not cultured commercially, findings in this model species may be applied to other molluscan species that are of aquaculture importance.

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