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Triploid Induction of Green Tiger Shrimp, *Penaeus semisulcatus* (De Haan, 1844) Using Temperature and Chemical Shock

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Abstract

Triploidy in fertilized eggs of *Penaeus semisulcatus* was induced by temperature and chemical shocks. The eggs, which were obtained from the shrimp broodstock maintained at 29 C, were exposed to cold temperature (8, 10, 12, and 14 C) and 6-dimetiloaminopurine (6-DMAP) concentrations (100, 150, 200, and 250 µM) for different durations (4, 6, and 8 min) 9 min after spawning was detected. While the highest triploidy rate of 49.7 ± 4.5% was obtained with a 200 µM 6-DMAP concentration for a duration of 8 min, the best mean triploidy rate of 45.5 ± 2.8% for cold shock was obtained at a temperature of 10 C for a duration of 8 min. Temperature and 6-DMAP concentration did not have significant effect on triploidy rate ($P > 0.05$) but shock duration had significant effect on triploidy rate for individual cold temperature shock or 6-DMAP chemical shock ($P < 0.05$). Although longer durations of shock agent increased the rates of triploid induction, they generally had an adverse effect on hatching rates in the study.

Induction of triploidy is the most commonly used technique that has been investigated in shrimp that has the potential to protect breeders' rights through conferring reproductive sterility (Sellars et al. 2012b). Triploid inductions have been successful in penaeid shrimp using a variety of shock agents including temperature (heat and cold) (Dumas and Ramos 1999; Li et al. 2003; Aloise et al. 2011; Kır et al. 2014) and chemicals (6-dimetiloaminopurine [6-DMAP] and cytochalasin B) (Bao et al. 1994; Norris et al. 2005; Sellars et al. 2006a). Triploidy induction methods are made up of a combination of a shock agent, magnitude of shock, timing of the shock, and duration of the shock (Sellars et al. 2010). Two categories of triploidy have been studied in penaeid shrimps: meiosis I and meiosis II triploidy. In these categories, triploid shrimps are induced by inhibiting the extrusion of the first polar body (PBI), or the second polar body (PBII) during meiosis. Chemical or temperature shock is commonly used to produce meiosis I or II triploids in penaeid shrimp (Li et al. 2003, 2006; Sellars et al. 2006a, 2010).

Important research on triploid induction of several shrimp species has been conducted so far around the world. The most studied species are *Fenneropenaeus chinensis*, *Litopenaeus vannamei*, *Marsupenaeus japonicus*, and *Penaeus monodon* (see Sellars et al. 2010). Triploid induction has also been successful but less intensively studied in *Fenneropenaeus indicus* (Morelli and Aquacop 2003), *Metapenaeus ensis* (Zhou et al. 1999; Zhang 2001) and *Melicertus kerathurus* (Kır et al. 2014).

Meiotic I triploid *F. chinensis* are reliably produced by applying a temperature shock at 4–10 min after spawning for a 10-min duration (Li et al. 2006). In comparison, meiotic I triploid *M. japonicus* are reliably produced by applying a shock at 1–2 min after spawning for a 6-min duration (Sellars et al. 2006b). While meiotic II triploids are reliably produced in *F. chinensis* by applying the shock 20–30 min after spawning

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for a duration of 10 min (Li et al. 2003). Meiotic II triploids are reliably produced by applying the shock 7–8 min after spawning for a duration 2–15 min in *M. japonicus* (Norris et al. 2005; Coman et al. 2008), *P. monodon* (Wood et al. 2011; Pongtippatee et al. 2012; Sellars et al. 2012a) and *L. vannamei* (Sellars et al. 2012b).

Penaeus semisulcatus is an Indo-Pacific shrimp species distributed along the coast of the Eastern Mediterranean Sea. It is one of the most important and appropriate shrimp species for aquaculture in subtropical regions (Kır and Kumlu 2006). Triploid induction of *P. semisulcatus* has not been studied to date. In this study, triploidy was induced in *P. semisulcatus* embryos using cold temperature shock and 6-DMAP chemical shock induction to stop first or second meiotic division (PBI or PBII extrusion). The study was undertaken to determine the effectiveness of shock agent and shock duration on triploidy induction in *P. semisulcatus*.

Materials and Methods

Broodstock, Spawning, and Embryo Collection

The broodstock shrimp required for spawning in the study were obtained from a wild population off the North Eastern Mediterranean coast of Turkey (36°43′N, 35°46′E). The broodstock were kept in 1500 L maturation tanks supplied with sea water maintained at 29 C. Twice daily the shrimp were fed crab and mussels. Gravid females were individually put into 50 L circular spawning tanks. The tanks contained a manipulation basket (500 µm mesh size) placed inside egg collectors (100 µm mesh size). The temperature and salinity of the water in the spawning tanks were 29 C and 36 ppt, respectively. In order to determine the time of spawning, the shrimp in the spawning tank was consistently observed by a night vision camera connected to computer. After the shrimp had spawned, the manipulation basket was suspended to remove spermatophores and excrement debris.

Triploidy Induction

Prior to the triploid induction, microscopic observations were carried out to determine the timing of PBI and PBII extrusions (Chavez et al. 1991; Garnica-Rivera et al. 2004). A total of 216 triploid induction treatments with a control were carried out with embryos from four separate spawnings at a salinity of 36 ppt. The treatments were conducted using cold temperature (8, 10, 12 and, 14 C) and 6-DMAP (100, 150, 200, and 250 µM) shock with three shock durations (4, 6, and 8 min). All experimental treatments were replicated twice with a control (29 C). After spawning, the fertilized eggs were distributed into 200 mL plastic shock containers (PSC). Each PSC contained about 650 eggs. The bottom and top of the PSC were covered with a 100-µm mesh screen. In accordance with the experimental design, the PSCs that held shrimp eggs were submerged in the water baths that contained different temperatures of water and different concentrations of 6-DMAP to perform the cold temperature shocks and chemical shocks. The treatment began 9 min after spawning. After shock, the PSCs with eggs were then returned directly to clean sea water (29 C).

Determining of Hatching Rate

About 15 h after spawning, five egg aliquots (10 mL) from each treatment (each PSC) were removed. The rate of hatched eggs was calculated by establishing the total number of eggs in the PSC and the relative frequencies of hatched eggs and nonhatched eggs in the distinct thermal and chemical shock treatments.

Assessment of Triploidy

The nauplii samples were disintegrated in 1 mL marine phosphate buffered solution (MPBS) containing 0.1% Triton X-100 (11 g/L NaCl, 0.2 g/L KCl, and 1.15 g/L Na_2HPO_4 $2H_2O$) and then homogenized by passing through a 25-gauge needle. Samples were pushed against the wall of the tube firmly eight times and the cell suspension passed through and 80-µm mesh to obtain single cells (Sellars et al. 2006a). Hundred microliters RNase A (200 µg/mL) was added for each sample. After 30 min of incubation at 37 C 100 µL propidium iodide (PI) (1 mg/mL) stain was added and incubated for 15

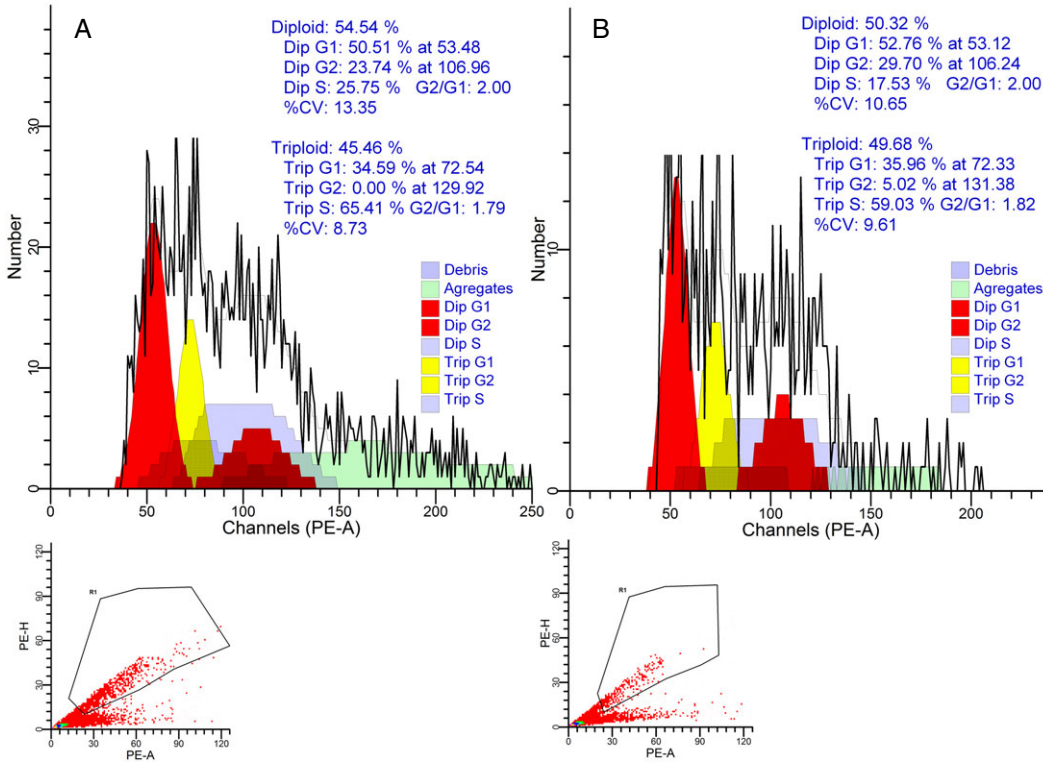

FIGURE 1. *The fluorescence-activated cell sorting (FACS) data output to detect the highest triploid rate from (A) cold temperature of 10 C and (B) 6-dimetiloaminopurine (6-DMAP) shock of 200 µM for a duration of 8 min for spawning 4.*

min at room temperature in the dark. The ploidy assessment of the samples was performed by fluorescence-activated cell sorting (FACS) using a flow cytometer (Beckton Dickinson Immunocytometry Systems, San Jose, CA, USA). To detect PI, a green solid state 488 laser was used for excitation, and a 556/LP and 585/40 configuration was used for detection. The level of triploidy in each sample was then determined using ModFit LT software (Verity Software House, Topsham, ME, USA).

Statistical Analysis

The effect of temperature, 6-DMAP concentration and shock duration on triploid and hatch rates were analyzed by ANOVA using SPSS 15.0. Regression analyses were subsequently performed to determine the relationship between shock duration and triploid rates using the Microsoft Excel program.

Results and Discussion

In this study, triploidy induction was performed on *P. semisulcatus* embryos using cold temperature shocks or 6-DMAP chemical shocks in a progressive factorial experiment. Prior to the experiment, the extrusion of PBI and PBII in eggs of *P. semisulcatus* at 29 C were observed within 4–6 and 10–16 min post spawning, respectively. Higher frequency of extrusion occurred at 5 and 15 min. These results are slightly different from those reported in previous studies for *M. kerathurus* (4–16 min) (Kır et al. 2014) and *L. vannamei* (8–15 min) (Dumas and Ramos 1999) but, similar to those reported for *M. japonicus* (4–15 min) (Norris et al. 2005) and *P. monodon* (5–15 min) (Pongtippatee-Taweepreda et al. 2004). Sellars et al. (2012a) got meiosis I or meiosis II triploid of *L. vannameii* using 200µM 6-DMAP shock at 1 and 7 min 30 s post spawning, respectively. Sellars et al. (2012b) applied chemical shock

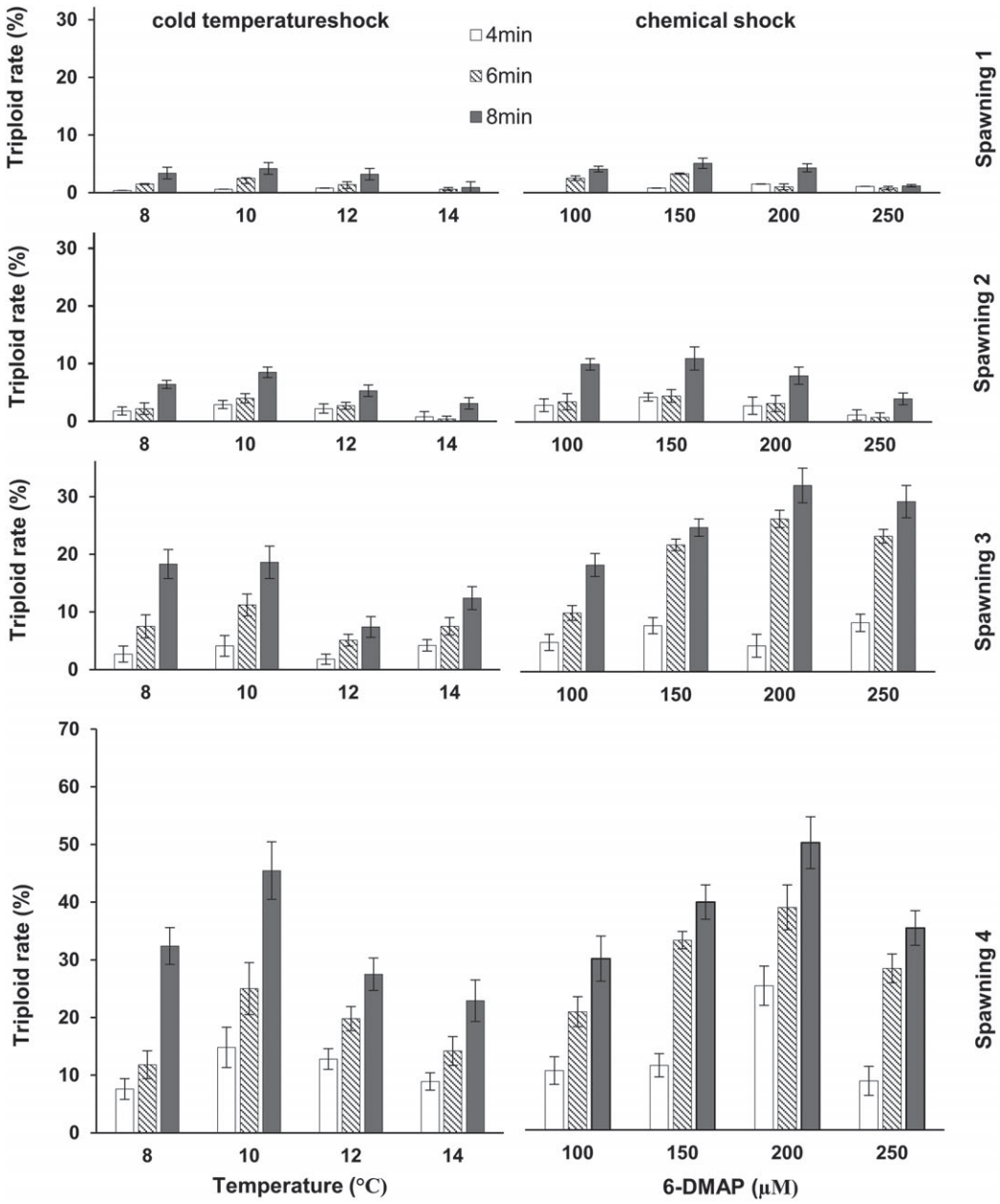

FIGURE 2. *Triploid induction rates [mean ± standard error (SE)%] of* Penaeus semisulcatus *nauplii from four spawning when subjected to different cold temperatures shock (C) and different 6-dimetiloaminopurine (6-DMAP) shock (µM) at durations of 4, 6, and 8 min.*

(6-DMAP) to eggs of *P. monodon* after 6 min and 40 s spawning to get meiosis I and meiosis II triploid. Pongtippatee et al. (2012) reported that meiosis II triploids (61.4%) of *P. monodon* were induced when cold shock at 8 C was applied at 10 min post spawning for duration of 10 min. Kır et al. (2014) produced meiosis I and meiosis II triploids of *M. kerathurus* using cold shock at 10 min post spawning at 10 C for a duration of 8 min. In this study cold temperature shocks

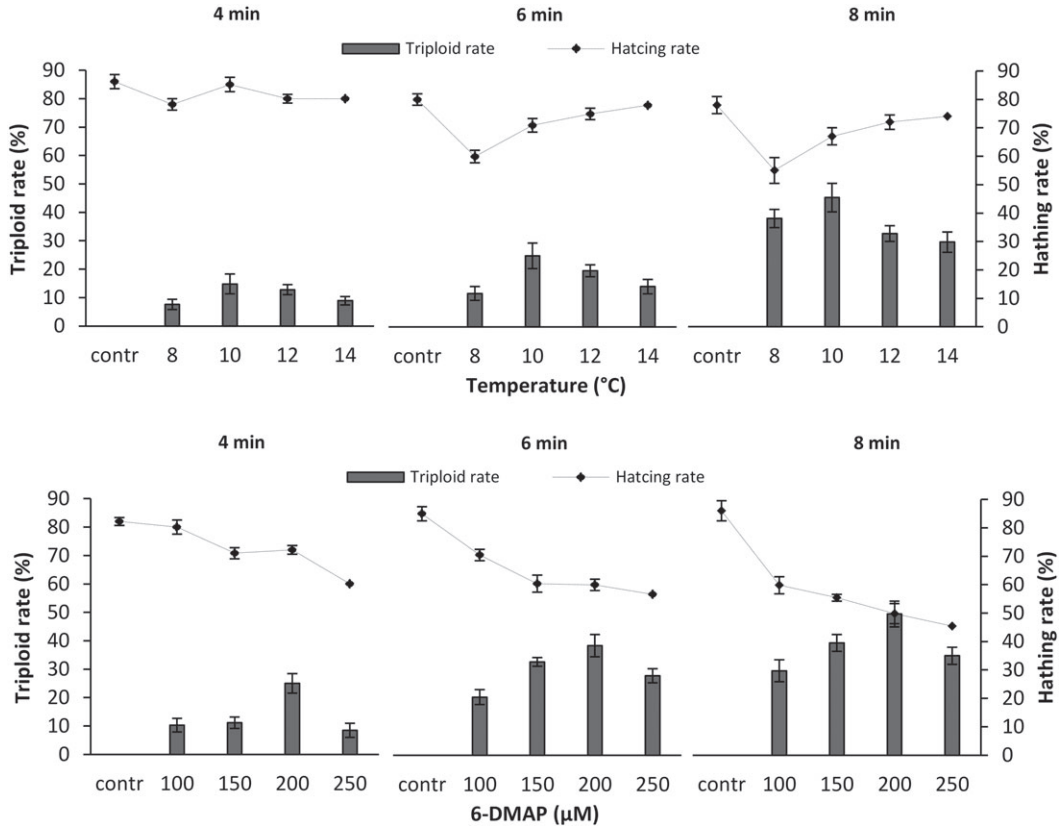

FIGURE 3. *Relationship between the induction and hatching rates (mean ± standard error (SE)%) from spawning four at different cold temperatures shock (C) and 6-dimetiloaminopurine (6-DMAP) shock (μM) for durations of 4, 6, and 8 min.*

and 6-DMAP shocks were applied 9 min after spawning. Because the spawning duration of the gravid shrimp is several minutes, triploids induced using shock agents in this study might be meiosis I and mostly meiosis II triploids.

The best individual cold shock treatment, in this study, produced 45.5% triploid nauplii in spawning 4, using a temperature of 10 C for an 8-min duration (Fig. 1A). Dumas and Ramos (1999) and Aloise et al. (2011) have reported triploid inductions of up to 100% in another penaeid shrimp, *L. vannamei*, using a cold shock temperature of 10 C, the same as the most successful individual induction rate in this study. This induction temperature is similar to those reported in previous studies for *P. monodon* (76.7%; Wood et al. 2011) and for *M. kerathurus* (64.5%; Kır et al. 2014). The highest individual treatment produced 49.67% triploid nauplii in spawning 4 using 6-DMAP (200 μM) for an 8-min duration in the current study (Fig. 1B). 6-DMAP has also been successfully used to produce viable meiosis I and II triploids in *M. japonicus* (Sellars et al. 2006a), *P. monodon* (Sellars et al. 2012b) and *L. vannamei* (Sellars et al. 2012a) with similar optimal induction parameters. Regression analyses for individual cold temperature or 6-DMAP applications for all spawning in the current study determined that the level of shock agent did not have a significant effect ($P > 0.05$) on triploid induction rate. However, triploidy rates obtained from 6-DMAP shock were significantly higher ($P < 0.05$) than those obtained from cold temperature shock (Fig. 2). Across all shock durations, triploid nauplii induction rates ranged between 0–49.7% and 0–45.5% for 6-DMAP shock and cold temperature shock, respectively.

Induction rate increased for each longer shock duration in the study. While duration of shock application had a significant impact ($P < 0.05$) on induction rates in spawning 3 and 4, duration of shock application had no significant impact ($P > 0.05$) on triploid induction rates in spawning 1 and spawning 2 (Fig. 2). It is well accepted that the percentage of triploids induced in any spawning is dependent on several variables including the shock agent, timing, and duration of the shock. Percentage induction also differs because spawning lasts several minutes, so embryo development is asynchronous and water temperature and chemistry may change over the duration of the spawning (Sellars et al. 2010).

In this study, the mean hatching rates, for the most successful induction treatments, were 85% for 6-DMAP shock and 80% for cold temperature shock. The hatching rate of fertilized eggs after chemical shock or temperature shock was directly related to the duration time of the shock application. Statistical analysis indicated that duration had a significant ($P < 0.05$) effect on the hatching rate at all treatment levels. Although longer shock durations of cold temperature and 6-DMAP application increased the rates of triploid induction, they generally had an adverse effect on hatching rates in the study (Fig. 3). Across all shock durations hatching rates ranged between 62 and 85% for cold temperature shock, 45 and 80% for 6-DMAP shock and 78 and 86% for control.

The results of this study indicate that, similar to other penaeid shrimps, production of triploid individuals of *P. semisulcatus* is possible. This study demonstrates that, owing to a wide variety of effective parameters, it is essential to optimize shock conditions for each species. The findings of this research can help researchers and producers improve the aquaculture industry by producing triploid *P. semisulcatus*.

Acknowledgments

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