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Induction of triploidy in *Melicertus kerathurus* (Forskal, 1775) with temperature shock

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Abstract

Triploidy in fertilized eggs of *Melicertus kerathurus* was induced by cold (8, 10, 12°C) and heat (34, 36, 38°C) shock for different duration times (2, 4 and 8 min) after 10 min of post spawning. The best individual treatment produced 64.5% triploid nauplii in cold shock application at a temperature of 10°C for a duration of 8 min. Temperature did not have significant effect ($P > 0.05$) on triploid rate but duration time had a significant effect ($P < 0.05$) for individual cold or heat shock. This study demonstrates that because of a wide variety of effective parameters, it is essential to optimize shock conditions for each species strain at each location.

Keywords: triploid, *Melicertus kerathurus*, temperature shock

Introduction

Triploid induction is considered to be one of the most important techniques to achieve genetic improvement in aquatic animals. In comparison with mollusks and fish, research on shrimp triploid induction has developed slowly due to their special reproductive characteristics, such as spawning at night and asynchronous embryo development (Li, Xiang, Zhou, Wu & Zhang 2003; Li, Zhang, Yu, Liu, Zhang, Zhou & Xiang 2006). However, very important research on some shrimp species to obtain triploid individuals has been carried out so far around the world. The most studied species are *Fenneropenaeus chinensis*, *Litopenaeus vannamei*, *Marsupenaeus japonicus* and *Penaeus monodon* (Sellars, Li, Preston & Xiang 2010). These penaeid shrimp species which have been studied to date are generally distributed in the Eastern Pacific and Indo-West Pacific (Bailey-Brock & Moss 1992).

Melicertus kerathurus, a commercially valuable penaeid shrimp, unlike the previously studied species, is endemic to the Mediterranean Sea and Eastern Atlantic Ocean (Pellerito, Arculeo & Bonhomme 2009). As well as being an important resource for fisheries, it has been considered and used for aquaculture since the beginning of the 1970s (Arculeo, Pellerito & Bonhomme 2010; Lumare 1979). There have been no reported attempts to induce triploidy in *M. kerathurus*.

Two categories of triploidy have been studied in penaeid shrimps: Meiosis I and Meiosis II triploidy. In these categories, triploid shrimps are induced by the inhibition of polar body (PB I or PB II; PB I and PB II) extrusion during meiosis (Li *et al.* 2003, 2006; Sellars, Degnan & Preston 2006; Sellars *et al.* 2010). Thermal hydrostatic pressure and chemical shocks are applied to inhibit the extrusion of polar bodies in newly fertilized eggs (Sellars *et al.* 2010).

In this study, triploids in *M. kerathurus* were induced using thermal shock induction to stop second meiotic division (PB II extrusion). The study was undertaken to determine the effectiveness of shock temperature and duration time on triploidy.

Materials and methods

Broodstock, spawning and embryo collection

The broodstock shrimps required for spawning in the study were obtained from a population of the coast of the Aegean Sea and transferred to the fish farm of the Kılıç Holding near Bafa Lake in Turkey.

The Broodstock were kept in maturation tanks (1000 L) at 28°C sea water and fed squid and mussels twice daily. The gravid shrimps were then individually put into 30-L circular spawning tanks. The tanks contained a manipulation basket (500-μm mesh size) placed inside another net (100-μm mesh size), which served as egg collectors. The temperature and salinity of the water in the tanks were 28°C and 36 mg L^{-1} respectively. To determine the time of spawning, the shrimp in the spawning tank was consistently observed by night vision camera connected a computer. After the shrimp had spawned, the manipulation basket was suspended to remove spermatophores and excrement debris.

Triploid induction by thermal shock

Prior to the triploid induction, microscopic observations were carried out to determine the timing of polar body (PB I and PB II) extrusions (Chavez, Murofushi, Aida & Hanyu 1991; Garnica-Rivera, Arredondo-Figueroa & Barriga-Sosa 2004). A total of 108 triploid induction treatments with a control were carried out within three spawnings. The treatments were conducted using cold (8, 10, 12°C) and heat (34, 36, 38°C) shock temperatures with three duration times (2, 4 and 8 min). All experimental treatments were replicated twice with a control (28°C). After spawning, the fertilized eggs were distributed into 200-mL plastic shock containers (PSC). Each PSC contained about 500 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 to perform the thermal shock. The treatment began 10 min post spawning. After thermal shock, the PSCs with eggs were then returned directly to the initial water temperature of 28°C.

Determining of hatching rate

About 15 h after spawning, six 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 non-hatched eggs in the distinct thermal shock treatments.

Assessment of triploidy

The naupli samples were disintegrated in 1 mL marine phosphate buffered solution (MPBS) containing 0.1% Triton X-100 (Amresco 0694-1L, Solon, OH, USA) (11 g L^{-1} NaCl, 0.2 g L^{-1} KCl, 1.15 g L^{-1} Na_2HPO_4 $2H_2O$) and then homogenized by passing through a 25-gauge needle. Samples pushed against the wall of tube firmly for eight times and cell suspension passed through 80-μm mesh to obtain single cells (Sellars *et al.* 2006). Rnase A of 100 μL (200 μg mL^{-1}) was added for each sample. After 30-min incubation at 37°C, 100 μL propidiun iodide (PI) (1 mg/mL) stain was added and incubated 15 min at room temperature in dark. The ploidy assessment of the samples was performed by fluorescence activated cell sorting (FACS) technique using a flow cytometer (Beckton Dickinson Immunocytometry Systems, San Jose, CA, USA). To detect PI, green solid state 488 laser was used for excitation, 556/LP and 585/40 filter 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 effects of temperature and duration time on triploid and hatching rates were analysed by analysis of variance using SPSS 15.0. Regression analyses were subsequently performed to determine the relationship between duration time and triploid rates using the Microsoft Excel program.

Results and discussion

The extrusion of PB I and PB II were observed in eggs of *M. kerathurus* within 6–8 min and 14–16 min post spawning at 28°C respectively. Higher frequency of extrusion occurred at 6 and 14 min. These results are slightly different from those reported in previous studies for *L. vannamei* (Dumas & Ramos 1999), *M. japonicus* (Norris, Coman, Sellars & Preston 2005) and *P. monodon* (Pongtippatee-Taweepreda, Chavadej, Plodpai, Pratoomchart, Sobhon, Weerachatyanukul & Withyachumnarnkul 2004). It is noticed that PB I and PB II extrusion happens 3–5 and 10–15 min in eggs of *P. monodon* respectively (Pongtippatee-Taweepreda *et al.* 2004). Sellars, Wood, Murphy, McCulloch and Preston (2012) treated chemical

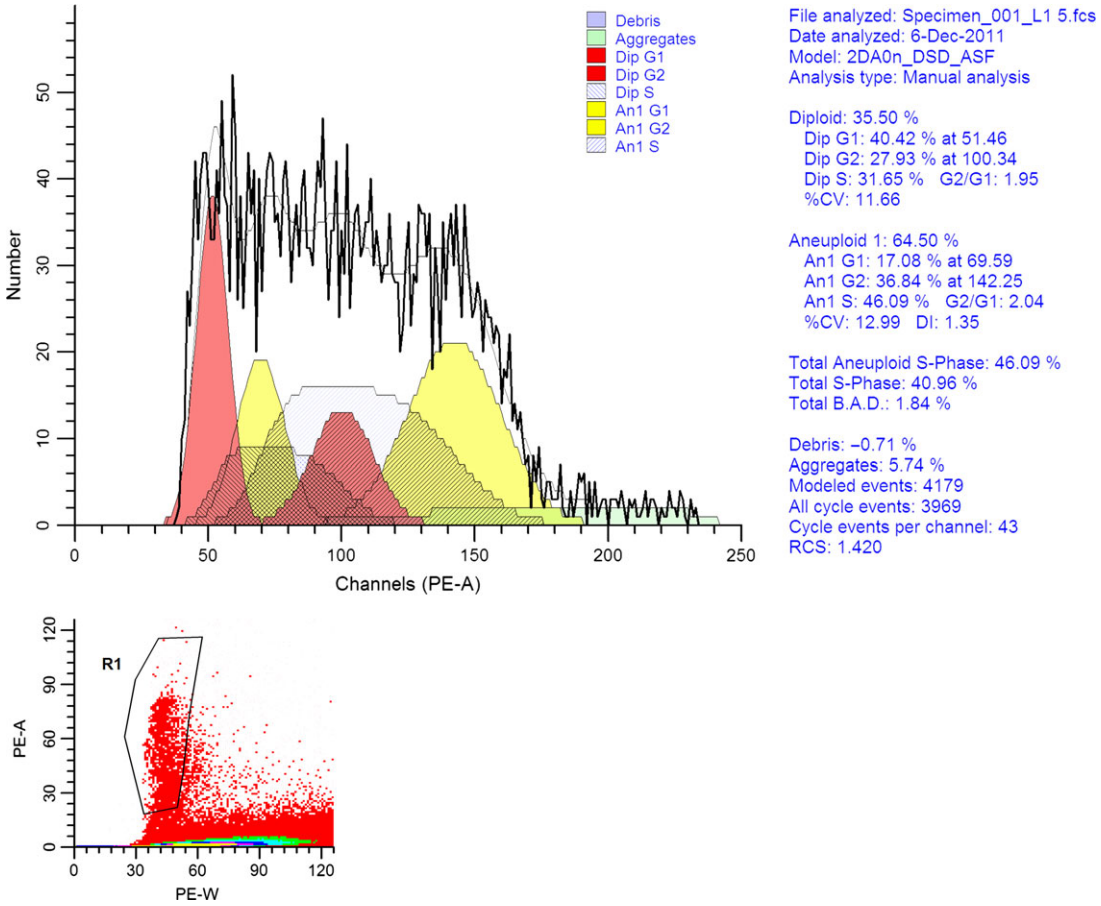

Figure 1 The FACS data output to detect the highest triploid rate from cold shock application at a temperature of 10°C for a duration of 8 min for spawning 3.

shock to eggs of *P. monodon* after 6 min and 40 s spawning to get PB II triploid. Pongtippatee, Laburee, Thaweethamsewee, Hiranphan, Asuvapongpatana, Weerachatyanukul, Srisawat and Withyachumnarnku (2012) reported that PB II triploids (61.4%) of *P. monodon* were induced when cold shock was applied at 10 min post spawning at 8°C for 10-min duration. In this study, temperature shocks were applied at 10 min after spawning. Since the spawning duration of the gravid shrimp usually lasts several minutes, triploids induced in the present study might be PB I and PB II triploids.

The best individual treatment produced 64.5% triploid nauplii in spawning 3 at a temperature of 10°C for an 8-min duration in the present study. The FACS data output of the highest triploid rate is illustrated in Fig. 1. Dumas and Ramos (1999) and Aloise, Maia-Lima, Oliveira, Cabral and Molina (2011) have reported triploid inductions of up to 100% in another penaeid shrimp, *L. vannamei*, using a cold shock temperature of 10°C which was obtained from the most successful individual induction rate in this study. This induction temperature is similar to the most successful induction (76.7%) at a temperature of 9°C for a 6-min duration in *P. monodon* (Wood, Coman, Foote & Sellars 2011). Regression analyses for individual cold or heat shock applications for all spawnings in this study determined that temperature did not have a significant effect ($P > 0.05$) on triploid induction rate (Fig. 2). Triploid nauplii induction rates ranged between 0–64.5% and 0–13.3% for cold and heat shock respectively across all duration times.

Multiple regression analyses for individual spawning determined that the duration of shock application had a significant impact ($P < 0.05$) on induction rates in spawning 2 and 3, with longer shock durations producing a higher proportion of triploid nauplii. Duration of shock application had

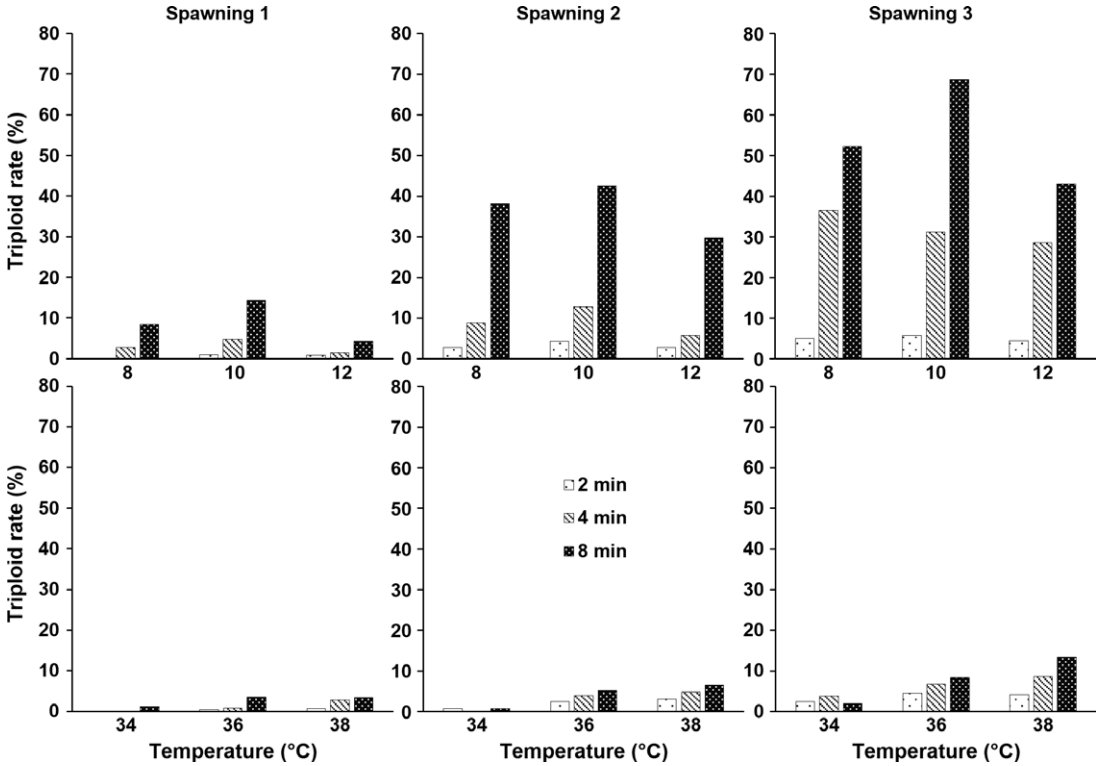

Figure 2 Triploid induction rates (%) of *Melicertus kerathurus* nauplii from three spawnings when subjected to different shock temperatures (°C) at durations of 2, 4 and 8 min.

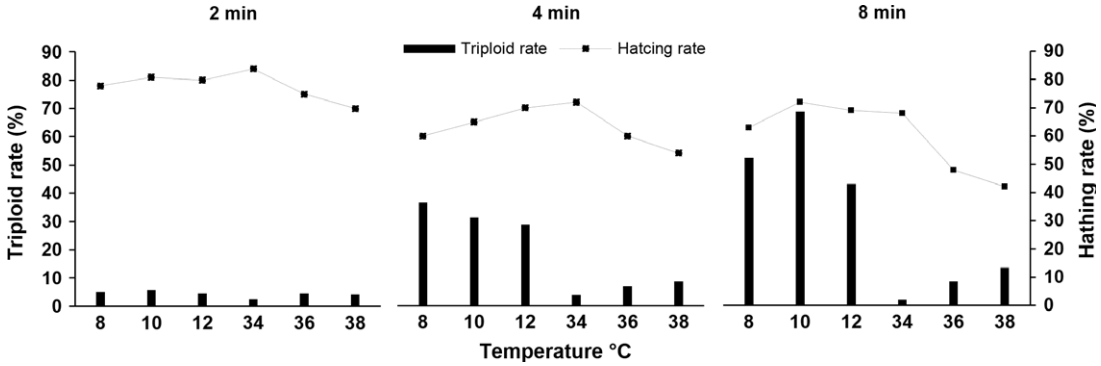

Figure 3 Relationship between the induction and hatching rates (%) from spawning 3 at different shock temperatures (°C) for durations of 2, 4 and 8 min.

no significant impact ($P > 0.05$) on triploid induction rates in spawning 1. It is well accepted that the percentage of triploids induced in any spawning is dependent on several variables including the timing and duration of the shock. Percentage induction also differs with asynchronous embryo development, water temperature and water chemistry (Sellars *et al.* 2010).

A mean hatching rate of 72% was observed when the highest triploid rate (64.5%) was obtained in this study. The hatching rate of fertilized eggs after thermal shock was directly related to the duration time of the shock application. Statistical analysis indicated that duration time had a significant ($P < 0.05$) effect on the hatching rate. Although longer shock durations of cold and heat

application increased the rates of triploid induction, they generally had an adverse effect on hatching rates in the study (Fig. 3). Hatching rates ranged between 55–81% and 42–84% for cold and heat shock respectively across all duration times.

The results of this study show that, like other penaeid shrimps, high triploid induction rates are possible in *M. kerathurus*, although the optimum conditions for this species were found to be relatively different from those defined for other well-known penaeid shrimps such as *P. monodon, F. chinensis, L. vannamei and M. japonicus*. (Wood *et al.* 2011; Xiang, Li, Zhang, Zhang, Yu, Zhou & Wu 2006; Sellars *et al.* 2006). This study demonstrates that because of a wide variety of effective parameters, it is essential to optimize shock conditions for each species strain at each location. The findings of this research can help researchers and producers improve the aquaculture industry by producing triploid *M. kerathurus*.

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