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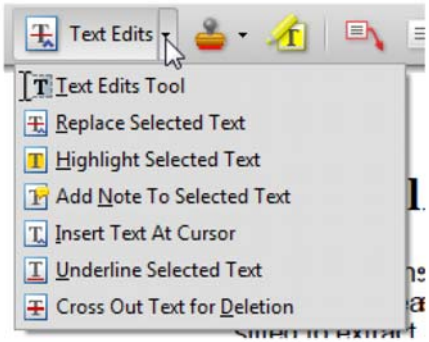

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Triploidy induction by heat shock treatment in red tilapia

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The possible use of sterile triploid red tilapia is an interesting option for culture due to their proliferating breeding activities. The aim of the present study was to investigate and optimize the time of heat shock treatment to prevent second polar body extrusion from the newly fertilized eggs of red tilapia, to produce a maximum number of triploid individuals. Heat-shock treatment was applied at a temperature of 41°C for a total duration of 3.5 minutes after 2, 3, 4, 4.5, 5 and 6 minutes of fertilization. The best survival rate (67.0%) and triploid percentage (89.7%) was observed for the treatment at 4 minutes after fertilization. It is thus successfully demonstrated that 4 minutes after fertilization was the most suitable timing of heat shock treatment for second polar body retention in newly fertilized eggs of red tilapia.

Keywords: polar body; red tilapia; time after fertilization; triploidy

Introduction

As a technique of chromosome engineering, there has been special interest in triploidy induction for producing sterile populations of fishes (Piferrer et al. 2009). Sterilization can overcome the detrimental effects of sexual maturation in cultured fishes. Sterility in fish can be induced either by exogenous hormone treatments (Hunter and Donaldson 1983) or by triploidy induction (Hussain et al. 1991; Hussain 1996; Pradeep et al. 2012). Since hormone treatments have known detrimental effects on consumers, triploidy induction offers the most promising mechanism for sterility. The sterility offered by triploids has been known to benefit the fishes by protecting their somatic growth, survival and flesh quality from the unpredictable effects of sexual maturation (Maxime 2008). In nature, the degeneration of three of four meiotic products is a very common process in the female gender of oogamous eukaryotes, both in animals and plants (Papini et al. 2011). This phenomenon occurs during female gamete development in animals (diplontic life cycle) by extrusion of two polar bodies. Triploidy induction can be artificially induced in fishes by suppressing the second meiotic division or by preventing the escape of the second polar body from recently fertilized eggs by applying various shock treatments, e.g. temperature, pressure or chemicals (Tiwary et al. 2004). Another method for producing triploidy is by crossing a tetraploid individual with a normal diploid individual (Maxime 2008).

The red tilapia, *Oreochromis niloticus* (Linnaeus, 1758) x *O. mossambicus* (Peters, 1852) is a fertile hybrid used in semi-intensive culture systems, which shows an antagonistic relationship between its reproductive process and its bodily growth. Previously, various researchers have suggested that production of triploid tilapias is one of the best possible solutions for negotiating the problem of precocious sexual maturity and unwanted reproduction in culture (Mair 1993). Moreover, a recent study has also shown the possibility of skewing the red tilapia into phenotypic males using heat-shock induction of triploidy (Pradeep et al. 2012), which could be a possible alternative for replacing the steroidal usage in tilapia farming in the future.

Although 100% triploidy has been reported in several species of tilapia, the results were not always consistent. AQ1 A suitable time after fertilization remains to be determined, as well as the time when the second polar body is expelled for triploidy induction. This timing is critically important for the success of induction, and is linked to species specificity. Optimization of this parameter seems to be essential. Hence an attempt has been made to determine the exact time when the second polar body is extruded from the newly fertilized eggs of red tilapia, to optimize the timing of heat shock treatment and to maximize triploid production in this species.

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Materials and methods

Red tilapias were maintained at the University Malaysia Terengganu. The broodstock maintenance and artificial breeding methods were followed as described by Pradeep (2011). In order to find out the time after fertilization (TAF) in red tilapia, the optimized values of heat shock in Nile tilapia, *O. niloticus* (Hussain et al. 1991) were used: a temperature of 41°C and a shock duration of 3.5 minutes were kept constant for this experiment. The timings of the different shock treatments were 2, 3, 4, 4.5, 5 and 6 minutes. The aim was to determine the appropriate timing for preventing extrusion of the second polar body from the egg. In the control group, no shock treatment was given and fertilization was at 28°C. For the TAF study, a total of four females and 12 males ranging in size from 200 to 250 g were used, and each female was considered as a replicate. As TAF is crucially important for optimizing the other treatment protocols of triploidy induction, the results of all three replicates were reevaluated by conducting a confirmation experiment using one more female to ensure the accuracy of timing.

Evaluation of time after fertilization for different treatments

For each experiment, eggs from only one female and concentrated sperms from three males (0.6–1 ml) were used. This is intended to avoid any bias in the experiments, because there are always chances of variation in sperm quality if used individually, which ultimately influences the fertilization rate. Prior to the experiment eggs were collected in Petri dishes. Previously collected sperms were then spread over eggs. Immediately afterwards, 20 ml of fresh water (28 ± 1°C) was directly added to initiate activation of sperms. The time of water addition was considered as the time of fertilization (t_o). The Petri dish was gently shaken for thorough mixing of sperms with eggs. The eggs were then left undisturbed for proper fertilization before initiation of different treatments. After a time gap of 1 min 30 s, the fertilized eggs from the Petri dishes were siphoned using a small plastic pipette, and eggs were distributed to different plastic strainers assigned for respective treatments. These strainers were kept inside a plastic tub containing water with a temperature of 28 ± 1°C. The eggs (100–300 in number) were placed in each strainer, where five strainers were designated for the heat shock treatment and one strainer for the control group. The heat shock was applied to fertilized eggs by transferring the respective strainers to a temperature controlled (41°C) water bath (45 l), precisely 2, 3, 4, 5 and 6 minutes after fertilization. Heat shock of 3.5 min duration was applied to the eggs in each of the five treatment strainers. The control group was left undisturbed in the plastic tub where a temperature of 28°C was maintained. In the confirmation trial experiment, an additional 4.5 minutes after fertilization was also included along with 4 and 5 minutes for the triploidy induction, to determine precisely the timing of second polar body extrusion. The treated and untreated eggs after counting were identically incubated in round-bottom glass jars (250 ml) connected to a recirculatory incubation system (Pradeep, Srijaya, Mithun et al. 2011). Fertilized eggs were counted at the blastula stage, 10 h after fertilization (AF), hatching rate at 80–90 h AF and survival rate on the fifth day (120 h AF). Hatching and survival rates in both control and treatment groups were calculated on the basis of the following formula:

$$\text{Hatching rate}(\%) = \frac{\text{Number of hatched larvae}}{\text{Number of fertilized eggs}} \times 100 \quad (1)$$

$$\text{Survival rate}(\%) = \frac{\text{Number of survived larvae}}{\text{Number of hatched larvae}} \times 100 \quad (2)$$

Triploidy induced in each shock treatment was identified using well-spread metaphase chromosomes prepared from 1–2-day-old larvae (20–30 larvae) following the protocol of Pradeep, Srijaya, Zain et al. (2011) and the percentage of the triploids was calculated. Triploid yield in each treatment calculated as follows:

$$\text{Triploid yield} = \frac{\%\ \text{triploid survival related to control} \times \text{triploid percentage}}{100} \quad (3)$$

Results

A shock at 41°C for 3.5 minutes at TAF of 4 minutes yielded 89.7% triploids (Table 1). This treatment showed

Table 1. Assessment of time after fertilization (TAF) to get the maximum triploid percentage. Values are ± standard deviation.

TAF	Hatching rate (%)	Survival rate (%)	Percentage of survival in relation to control (%)	Triploid rate (%)	Triploid yield (%)
2	36.0 ± 2.1	54.5 ± 4.2	63.4 ± 2.7	54.4 ± 2.0	34.4 ± 0.6
3	48.7 ± 3.7	60.9 ± 1.6	70.9 ± 0.6	73.6 ± 1.5	52.2 ± 0.9
4	62.7 ± 5.7	67.0 ± 4.1	77.9 ± 2.2	89.7 ± 2.5	69.8 ± 0.5
5	66.9 ± 4.0	69.0 ± 2.6	80.3 ± 2.6	62.9 ± 2.7	50.5 ± 3.5
6	69.8 ± 1.9	72.7 ± 1.4	84.7 ± 2.3	37.2 ± 2.1	31.5 ± 2.1
Control	77.9 ± 2.8	85.8 ± 3.0	100	0	0

Table 2. Confirmation trial for time after fertilization (TAF) to get maximum triploid percentage. Values are ± standard deviation.

TAF	Hatching rate (%)	Survival rate (%)	Percentage of survival in relation to control (%)	Triploid rate (%)	Triploid yield (%)
4	64.2 ± 2.6	70.1 ± 1.5	82.7 ± 1.8	88.0 ± 1.0	72.8 ± 0.7
4.3	72.4 ± 3.5	73.3 ± 1.3	86.5 ± 1.6	70.8 ± 0.0	61.2 ± 1.2
5	74.1 ± 0.6	71.3 ± 1.2	84.1 ± 1.5	62.5 ± 0.5	52.5 ± 1.3
Control	88.6	84.7	100	0	0

relatively satisfactory survival of 67% with a maximum triploid yield of 69.8% ($p < 0.001$). The appropriate time of heat shock treatment to prevent the extrusion of the second polar body, thereby inhibiting the second meiotic division, was thus experimentally arrived at 4 minutes in red tilapia. Heat-shock initiated at 2 and 3 minutes after fertilization indicated hatching below 50%. However, hatching percentage was relatively higher at 4, 5 and 6 minutes post fertilization by 62.7%, 66.9% and 69.8%, respectively. It was observed that when the timing of shock initiation after fertilization was increased, the hatching percentage also increased considerably. Similarly as the time of initiation was increased gradually, it considerably affected the survival of the hatched larvae. An increasing trend in survival of the larvae was observed when the shock timing was increased from 2 to 6 minutes. Maximum survival was observed at 6 minutes after fertilization (72.7%) whereas 2 minutes after fertilization resulted in only 54.5% survival. The treatment which showed maximum triploid percentage of 89.7% (i.e. 4 minutes after fertilization) accounted for a percentage survival of 67%. It was observed that all treatments showed triploid percentage rate of more than 35%. At 2, 3 and 4 minutes after fertilization, the heat-shock application showed an increasing trend of triploid rate ($p < 0.001$). Maximum triploid percentage was at 4 minutes after the fertilization trial experiment, where a comparatively higher triploid rate of 89% was obtained. However, shock initiated after 4 minutes showed a decreasing trend in triploid rate, where 5 minutes after the fertilization resulted in 62.9% triploids followed by 6 minutes with only 37.2% triploids ($p < 0.001$).

The confirmation trial experiment done on a single female showed a similar result, i.e. 4 minutes after the fertilization resulted in 88% triploids (Table 2). Hence it was confirmed that the results obtained from the previous trial experiments were accurate and acceptable. In the confirmation trial experiment also, a maximum triploid rate of 88% was achieved at 4 minutes after fertilization, with triploid yield attaining 72.8% (Table 2). Like previous results, a decreasing trend in triploidy percentage was yielded after 4 minutes of initiation of shock.

Discussion

Retention of the second polar body by shock treatments for triploidy induction in a variety of species has shown different timings for initiation of the shock treatments, suggesting precise timings are required for each species. Previous studies have shown that triploidy induction could be made possible within a wide range of zygotic ages (0–15 minutes) using various induction methods in different species of tilapia (Chang and Liao 1996). TAF values of 0.5–9 min for cold-shock treatment (7–11°C) of duration 30–60 minutes; 3–5 min for heat-shock treatment (39.5–42°C) of duration 3–4.5 min; and 2.5–9 min for pressure shock treatment (7500–9000 psi) of duration 2–7 min have been successfully applied for triploidy induction in all the three commercially important species of tilapia (Mair 1993).

Peruzzi et al. (2007) found that a temperature of 20°C at 20 minutes after fertilization resulted in 66–100% triploids in Atlantic salmon at the time of hatching, with survival ranging from 10 to 20% as compared to the controls. Lower heat-shock temperatures or delayed shocks increased survival but decreased the number of triploids, providing no net gain in triploid yield (range 1–9%). The above-mentioned study thus showed that fishes preferring lower temperature for better survival require slightly longer gaps between the TAF and application of heat-shock treatments for inducing triploidy. However, the present study showed that in red tilapia, an early timing of only 4 minutes was adequate to block the second meiotic division. Peruzzi et al. (2007) reported that among the different times after fertilization, 20 minutes post-fertilization resulted in less survival, and survival rate increased with delayed shock applications at 30 and 40 minutes. On the other hand, the percentage of triploidy was the highest ($p < 0.001$) when shock treatments were administered at 20 minutes post fertilization (92–100%); and declined to 13–46% and 2–13% with shock treatments applied at 30 and 40 minutes post fertilization; respectively. A similar pattern was found in the present study, where the survival rate showed an increasing pattern when shock treatment was initiated from 2 minutes after the fertilization (54.5%) to 6 minutes (72.7%). The triploid percentage in the present research reached its highest value (89.7%) at 4 minutes after fertilization; however, the percentage reduced slowly and dropped to 37.2% when it was initiated at 6 minutes post fertilization.

An opposite observation to the present research and the findings of Peruzzi et al. (2007) was reported by Linhart et al. (2001). There triploidization was done in European catfish (*Silurus glanis* L.). It was seen that the hatching percentage was affected considerably when the time of initiation of shock was increased above 3 minutes, using hydrostatic pressure shock (600 kg cm^{-2}) treatment for 4 minutes. The hatching percentage was decreased

from 34.1% at 3 minutes initiation time to 0.9% at 9 minutes post fertilization. It is clear from this finding that the treatment method for triploid induction can show variability in hatching, survival and even triploid percentage. It was further observed from the experiment by Linhart et al. (2001) that the second polar body was completely expelled after 6 minutes of fertilization using 4 minutes hydrostatic pressure shock treatments of 600 kg cm^{-2}. During 6 minutes of initiation of shock treatment, the percentage of triploidy was 100%. However, the percentage dropped to zero when it reached 7.5 minutes after fertilization. Linhart et al. (2001) further reported that a heat shock of 41°C for a duration of 1 minute initiated after 7 and 9 minutes after fertilization produced 100% triploidy. They observed that when the time after fertilization increased from 5 to 9 minutes, the triploid percentage increased gradually, reaching a maximum of 100% at 9 minutes after fertilization. This suggested that the second polar body was completely blocked by heat shock at 9 minutes after the fertilization. It was further observed that cold shock after fertilization of 9 minutes produced 100% triploidy when treatment was applied at 6°C for 20 minutes duration. This shows that the time required for initiation of shock treatment to achieve maximum triploidy is lower when using pressure shock (3 minutes) compared to heat shock (9 minutes), in the case of the European catfish. However, this phenomenon was not acceptable in Nile tilapia, where Hussain et al. (1991) found that to achieve 100% triploidy induction by pressure shock treatment, a longer initiation time of 9 minutes was required, whereas for cold shock the initiation time was 7 minutes and the lowest time was with heat-shock, at 5 minutes. The present study also supports Hussain et al. (1991) as the best result was shown at 4 minutes after the fertilization using heat shock.

In the present study, the percentage of triploidy increased gradually from 54.4% when shock treatment was initiated 2 minutes after fertilization, to 89.7% at 4 minutes and then decreased to 37.2% at 6 minutes. A similar trend was observed in the common carp where the percentage of triploidy was 15.26% for shock treatments at 0 minutes to 94.96% at 5 minutes after fertilization. This then decreased to 6.45% when the initiation time reached 15 minutes and dropped to 0% at 25 minutes (Linhart et al. 1991). Chang and Liao (1996), in their experiment determining the zygotic stages for triploidy induction in *O. aureus*, showed an almost similar pattern. In their experiment, triploid percentage success was ∼93% at one minute after fertilization, reaching a maximum of 100% at 3 minutes and then decreasing to 80% at 5 minutes. There were no triploid individuals encountered when the initiation timing had reached 7 minutes. Thus it was evident from the study of Chang and Liao (1996) that the second polar body was completely released within 7 minutes after the fertilization.

Experimental conditions similar to the present research on red tilapia (a constant temperature of 41°C and duration of 3.5 minutes) were used in a study conducted in Nile tilapia by Hussain et al. (1991). The% survival in the control group under various TAF values of 2, 3, 4, 5 and 6 minutes showed increasing rates of 60, 60.7, 69.6, 82.1 and 85.7%, respectively (Hussain et al. 1991). In the present research the same TAF values as in Hussain et al. (1991) showed ratios of% survival in the control with values of 63.4, 70.9, 77.9, 80.3 and 84.7%, respectively. Although a similar pattern in triploid percentage to the present research was reported by Hussain et al. (1991), the maximum number of triploid individuals was found at 5 minutes of initiated shock treatment compared to shocks initiated at other TAF. However, in the present research, a maximum triploid rate was observed at 4 minutes post fertilization. Hussain et al. (1991) reported that triploid rate increased from treatment at 2 minutes after fertilization to 7 minutes of fertilization. Two minutes initiation timing resulted in 54.2% triploidy in Hussain et al. (1991). This rate slowly increased from 67.1 to 92.7 and reached a maximum of 100% at 3, 4 and 5 minutes after fertilization, respectively. This was followed by a decreasing trend, dropping to 72.6% and 23.1% at 6 and 7 minutes after fertilization, respectively. The present research on red tilapia showed optimal results at 4 minutes after fertilization with shock treatment, like those reported by Hussain et al. (1991) where triploid rate was 89.7% in the preliminary trial. The confirmation trial also showed values close to the preliminary trial at 88% triploid production.

Hussain et al. (1991) further reported that the time of the second meiotic division in Nile tilapia varied widely, from 2 to 15 minutes after fertilization, under various shock-inducing agents with altered durations. However, the meiotic spindle apparatus was disturbed effectively in a very narrow window of time in all the three induction protocols. Hence it was proved from the study of Hussain et al. (1991) that any variation from the optimized value of time after fertilization considerably affected either survival or triploidy yield in the embryo. Pandian and Varadaraj (1988) achieved 100% triploidy in *O. mossambicus* with a TAF of 2.5 minutes, when a temperature of 42°C was applied for 3 minutes. Hussain et al. (1991) obtained 100% triploidy in *O. niloticus* with a TAF of 5 minutes, when a temperature of 41°C was applied for 3.5 minutes. In red tilapia the TAF fell between the timings of the parent species: 4 minutes duration proved to be the best timing for inhibiting the second polar body. Puckhaber and Schwark (1996) showed that for retaining the second polar body in newly fertilized eggs of Nile tilapia, a temperature shock of 41°C for 4 minutes of initiation time for duration of 4.5 minutes was also effective for producing 100% triploidy. Don and Avtalion (1988) suggested that triploidy induction by heat-shock treatment could be possible only within a narrow range of zygotic ages of 2.5–3.5 and 3.5–4.5 minutes for O. aureus and O. niloticus, respectively. A wider range of zygotic ages between 0 and 15 minutes was possible with cold-shock treatment in O. niloticus and O. aureus. In another study by Don and Avtalion (1986), a TAF of 3 minutes was found to

produce 100% triploid fish when applied for 3.5–4 minutes in *O. aureus*.

Further attempts to optimize the treatment protocols for triploidy induction in red tilapia using thermal shocks revealed that a TAF of 4 minutes was appropriate for producing high percentages of triploids (91.8% and 98.7%) using heat shock (41°C applied for 5 minutes; Pradeep et al. 2010) and cold shock (9°C applied for 30 minutes; Pradeep, Srijaya, Mithun et al. 2011), respectively.

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