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Diploid Gynogenesis in Lampam Jawa *Puntius gonionotus* Using UV Irradiated Sperm of *Puntius schwanenfeldii* Followed by Temperature Shock

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The effects of genetic manipulation of eggs and sperm on the yields of gynogenetic fry of *Puntius gonionotus* were investigated. Gynogenesis was achieved by cold-and heat-shocking eggs fertilized with ultraviolet irradiated sperm of *P. schwanenfeldii* at various times after fertilization and at different duration intervals. 66.6% viable, gynogenetic fry were obtained when eggs were inseminated with irradiated sperm and cold shocked at 2°C for 5 minutes duration 1 minute after fertilization. At warm water temperature shocks the fertilized eggs performed best at 42°C, with percent survival rates of 20.0% 1 minute after fertilization for 1.0 minute duration and 17.2% and 14.7% 13 and 23 minutes after fertilization respectively for a duration of 1.5 minutes.

Puntius gonionotus, commonly known as lampam jawa, is a highly popular freshwater food fish and constitutes an important aspect of inland fisheries in Malaysia. The fish was introduced into the country in the early 1950's from Indonesia[1] for pond culture. Since then it has been cultivated in most parts of the country. The fish breeds easily in ponds during the onset of the rainy season, and its propagation through induced spawning by pituitary extract has been successful as an alternative to ensure a constant supply of seed. However, for the development and improvement of the lampam jawa seed, chromosome manipulation needs to be investigated and performed.

Chromosome manipulation by retaining the second polar body or suppressing the first cell cleavage or diploid gynogenesis involves fertilization of the eggs without genetic contribution from the sperm followed by shock treatment. Gynogenesis has been known as a rapid method for producing inbred lines compared with the traditional method, which takes a long time. It may be useful for rapid improvement of genetic characters in fish species. Successful diploid gynogenesis using various methods of sperm irradiation and temperature or pressure shock treatment has been reported by various authors[2-8] in many fish species such as zebra fish,[9] pacific salmon,[10] rainbow trout,[3] medaka,[11,12] European catfish,[13] carp,[14,15] ayu,[16] and tilapia,[17] but not in lampam jawa for a breeding program.

The objective of the study is to examine the conditions required to produce diploid gynogenesis in lampam jawa *Puntius gonionotus* using ultraviolet (UV) irradiated sperm of *P. schwanenfeldii* (sperm donor) so as to ensure that the paternal genes are not transmitted,[5,8,9] followed by temperature (heat and cold) shock.

Materials and Methods

Hatchery stock mature female and male lampam jawa *Puntius gonionotus* obtained from the Hatchery and Pond Complex Unit, Faculty of Fisheries and Marine Science, University of Agriculture of Malaysia were used. The females, averaging in total length and weight of 28.8 cm and 457 g, respectively, were each given an intramuscular injection of powdered carp pituitary extract homogenised in distilled water at a dose of 6 mg/kg body weight of female. The male was

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injected at half the female dose.[18] After 5–6 hours the eggs and milt were obtained by stripping. A *P. schwanenfeldii* male obtained from the wild (Kenyir Lake, in Kuala Trengganu) was used as a source of sperm.

The sperm was diluted 100 times by physiological saline solution containing 7.98 g NaCl and 0.02 g $NaHCO_3/l$ distilled water. The sperm solution was evenly spread out on a petri dish in a 1 mm layer and exposed to ultraviolet (UV) irradiation.[8] Based on the preliminary experiment the intensity of irradiation was fixed at 2337 erg/mm²/s as a result of the high survival rates obtained. During irradiation treatment, each petri dish was spun on a shaker at 110 rpm. The irradiated sperm was then used to inseminate the eggs. Samples of 100 eggs were mixed with the irradiated (UV control) and non-irradiated *P. schwanenfeldii* sperm (hybrid control) or *P. gonionotus* sperm (normal control). The moment of water addition to the mixture of eggs and milt was taken as the fertilization time. The inseminated eggs were incubated at 27°C for further experiment. Cold and heat shocking were carried out at different times after the fertilization for various lengths of time.

The first experiment examined the effects of cold shock at 0 and 2°C at various times after fertilization, 1, 3, 5, 9, 11, 13, 15, 20, 23, 25, 30, 40, and 60 minutes for durations of 2.5, 5, 10, 15, and 30 minutes.

In the second experiment the effects of heat shock at 35, 38, 40 and 42°C 1, 3, 5, 7, 9, 11, 13, 15, 20, 23, 25, and 30 minutes after fertilization for durations of 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 min were carried out. Both experiment 1 and 2 were carried out on different days using different female and male broodfish, as well as being performed in triplicate.

The survival of viable normal larvae after 16 hours was recorded. The hatching of normal larvae was the primary criterion for estimating the success of induced diploidization, with all percentages expressed in relation to the initial egg number. Data were analysed with ANOVA and Tukey multiple range test ($P=0.05$) using a statographic computer programme.

Result

The survival rates of normal control larvae from eggs inseminated with normal sperm of *P. gonionotus* varied from 72.5 to 81.6%, depending on the experiments, the average being 77.9%. The one-day-old larva presented an elongated yolk sac and a tapered posterior end (Fig. 1A). On the other hand, the survival rates of the hybrid control (*P. schwanenfeldii* sperm) varied between 70.0 and 93.7%, with an average of 78.5%. This one-day-old larva had an oblonged-shaped yolk sac (Fig. 1B) which can be used as an indicator of paternal genetic contribution in the case of gynogenetic larva. The average survival rates resulting from insemination with UV-irradiated sperm without shock treatment was 66.0% and all embryos showed the haploid syndrome. This non-viable larva was characterized by short caudal vertebrae and a distorted yolk sac (Fig. 1C).

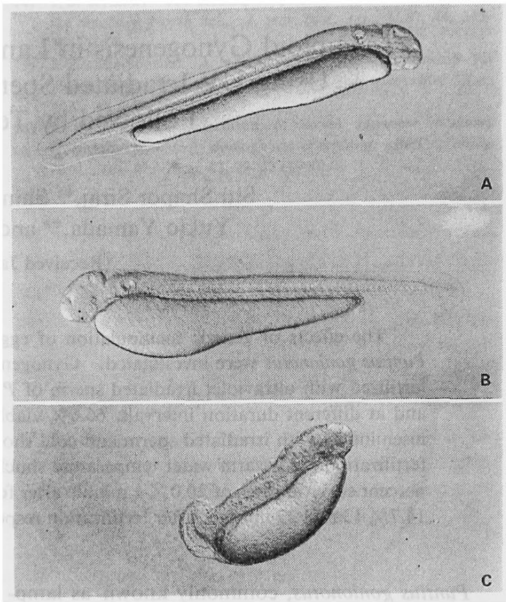

Fig. 1A: One-day-old normal larva of *Puntius gonionotus*.
1B: One-day-old hybrid larva of *Puntius gonionotus* fertilized with *Puntius schwanenfeldii* male.
1C: Newly-hatched larva with short curved caudal vertebrae and distorted yolk sac or haploid syndrome.

Cold Shock after Fertilization with UV-irradiated Sperm

Significantly ($P<0.05$) higher yields of normal diploid larvae were obtained in cold shock treatment at 2°C as compared to cold shock at 0°C. Fig. 2 shows a clear peak of normal diploid larvae at intervals of 1–3 min. Cold shock applied after 7–14 min did not produce any normal diploid

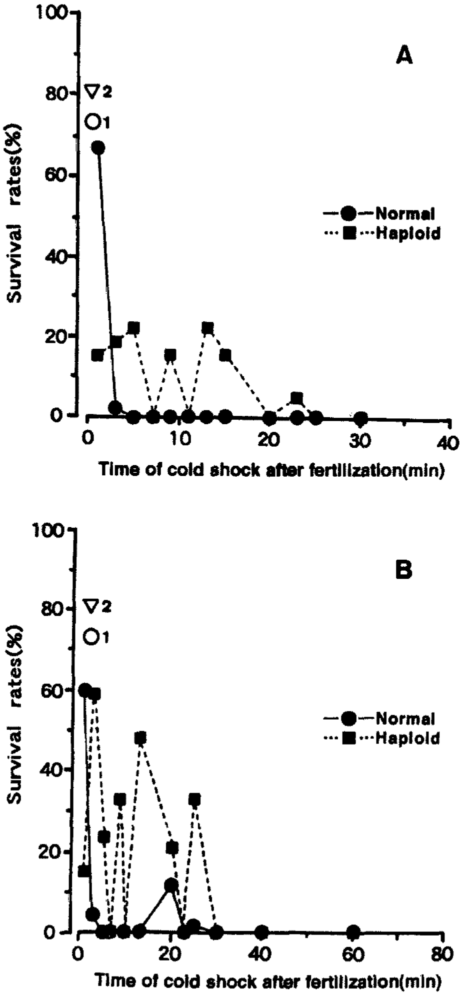

Fig. 2. Survival rates of eggs fertilized with UV-irradiated sperm and subjected to cold shock (2°C) started at various minutes after fertilization.
a) Cold shock with 5 min durations, b) Cold shock with 2.5 min durations (1: Normal control; 2: UV-control).

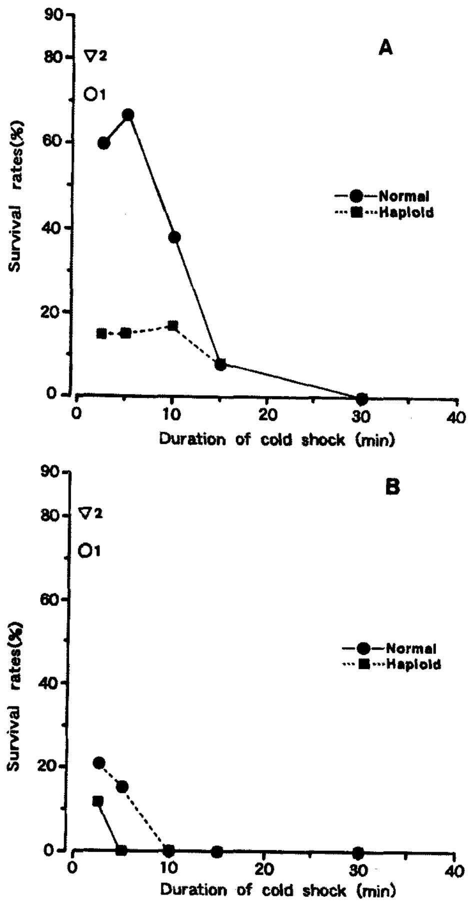

Fig. 3. Effect of various shock durations on survival rates of eggs fertilized with UV-irradiation and shocks at various minutes after fertilization.
a) Cold shock, 2°C, 1 min after fertilization, b) Cold shock, 2°C, at 20 min after fertilization. (1: Normal control; 2: UV-control).

larvae. There were two other time intervals with normal diploid appearance, between 20–60 min after fertilization cold shock at 2°C. Normal diploid appeared at 20 and 25 min after fertilization and cold shock for 2.5 min durations with survival rates of 11.7% and 1.8% respectively (Fig. 2B). Shocks applied beyond 5 min showed a high occurence of haploid larvae.

Time intervals with a 2.5–10 min duration were the best period for 2°C cold shock treatment at 1 min after fertilization (Fig. 3A). However, a 5 min duration time produced significantly ($P < 0.05$) high survival rates of 66.6% normal diploid. For 2°C cold shock treatment 20 min after fertilization, a 2.5 min duration time was the best point. Cold shock treatment over a 2.5 min duration showed low survival rates (Fig. 3B).

Heat Shock after Fertilization with UV-irradiated Sperm

The effect of heat shock treatment applied at various times after fertilization, to eggs inseminated with UV-irradiated sperm, was observed. (Heat shock treatment at 35°C did not produce any normal diploid larvae.) The time interval

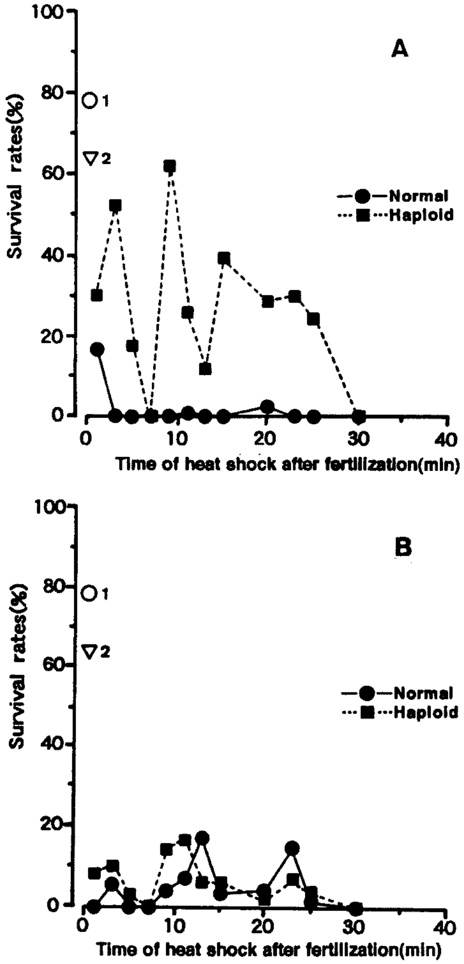

Fig. 4. Survival rates of eggs fertilized with UV-irradiated sperm and subjected to heat shocks at various minutes after fertilization.
a) Heat shock, 42°C, with 0.5 min duration, b) Heat shock, 42°C, with 1.5 min duration. (1: Normal control; 2: UV-control).

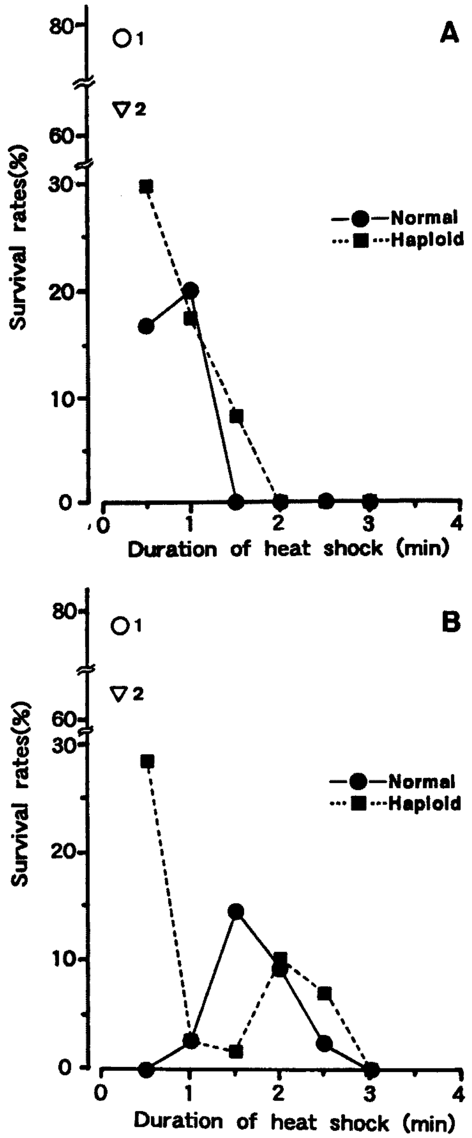

Fig. 5. Effects of various shock durations on survival rates of eggs fertilized with UV-irradiation and heat shocks at various minutes after fertilization.
a) Heat shock, 42°C, 1 min after fertilization b) heat shock, 42°C, at 23 min after fertilization. (1: Normal control; 2: UV-control).

of 1.0–1.5 min was the best for heat shock of 38°C, 4 min after fertilization with a highest survival rate of 10.1%. Very low survival rates of normal diploid larvae were observed using heat shock treatment of 40°C, the highest being 7.5% for 1.5 and 2.0 min duration, at 1 and 12 min after fertilization, respectively.

Heat shock treatment of 42°C given 1 min after fertilization over 1 min resulted in 1 peak in the survival rate of normal diploid larvae (Fig. 4A), while giving a shock over 1.5 min 1, 13, and 23 min after fertilization produced 3 peaks of survival rate (Fig. 4B). The best survival rates for shock given over 1.5 min, 1, 13 and 23 min after fertilization, were 20.0% (Fig. 5A), 17.2% (Fig. 4B) and 14.7% (Fig. 5B), respectively.

Discussion

All groups inseminated with irradiated sperm but not subjected to cold and heat shock developed abnormal embryo characteristics of haploid syndrome described by Taniguchi *et al.*[16] and Gervai *et al.*[19] The appearance of 100% elongated yolk sac, a diploid larvae characteristic of the *P. gonionotus* maternal parent, reveals the complete efficiency of UV-irradiation of sperm as indicated by Chourrout.[20]

This study shows that a short duration of cold and heat shocks is equally successful in restoring the inhibition of meiotic and mitotic processes. The best time after insemination for retaining the second polar body to produce gynogenetic diploid was 1 and 3 min, which is close to the findings by most authors in common carp, zebrafish, and salmonids.[4,9,21-23] The success of the faster initial time to shock application could be due to the higher incubation temperature 27°C,[4] and better results are observed with a higher temperature of 42°C as suggested by Hollebecq *et al.*[22]

A bimodal response to cold and heat shock application was observed. We found bimodal response in the first mitotic division 19 and 25 min after fertilization in cold (2°C) shock treatment, and in retaining the second polar body at 1 min and a high recovery of survival rates at 13 min (with 1.5 min duration) in heat shock (42°C) or cold shock treatment. This is in agreement with studies on carps.[4,8,24] The bimodal response could possibly be due to various factors such as husbandry of broodstock and strain,[24] interfemale variation,[23] differences in aging or development of fish before and after ovulation,[4] absorption of the second polar body by the ovaplasma caused by shocks applied at a later stage of second polar body formation,[25,26] and dissociation of microtubules during meiosis II.[27]

Puntius schwanenfeldii, being in the same species as *P. gonionotus*, produced a true hybrid when used as a source of sperm. However, the hybrid was confirmed morphologically at the larval stage. Sperm donors have been used for the induction of gynogenesis in cyprinid loach,[28] rainbow trout,[29] red sea bream,[30] carp,[4] and ayu.[31] Those gynogenetic offsprings were confirmed by observing their morphology, karyology, isozyme, and DNA marker.

The diploid gynogenetic fish obtained in this study will be kept for further use to verify their mitotic and meiotic efficiency. Streisinger *et al.*[9] and Taniguchi *et al.*[16] reported that treatment suppressing the first mitotic cell division would be more promising for the development of a gynogenetic inbred broodstock line. Hence, future study will be aimed at achieving this direction.

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