# ANDROGENETIC *PANGASIUS HYPOPHTHALMUS* PRODUCED BY UV IRRADIATION OF EGGS OF *CLARIAS GARIEPINUS* AND TEMPERATURE SHOCKS

**By**

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**ABSTRACT :-** *Clarias gariepinus* eggs were fertilized with sperm of *Pangasius hypophthalmus* (sutchi). Paraffin sections were made at different times after fertilization to determine the timing of the first mitotic division. The UV- irradiated (240 mJ/cm$^2$) eggs were also used to produce androgenetic offspring. To induce diploidization the temperature shocks (7°C, 15°C, 38°C and 40°C) with different shocking durations (10-25 min for cold shocks or 15-60 sec for heat shocks) were applied at different times post-insemination (20-45 min) The results showed that the highest number (a cluster of 25-30%) of fertilized eggs synchronized development 20-30 min after incubation. At this time a high percentage (25-30%) of early metaphase and metaphase chromosomes could be observed. Shocking the eggs at 7°C or 40°C with longer durations resulted in decreased fertilization and 0% normal fry (putative androgenetic *P. hypophthalmus*). Variation in number of normal fry at hatching ranged from 0 to 4% in fertilized eggs exposed to 15°C or 38°C temperature shocks. Ten specimens of normal fry were examined by polymerase chain reaction using primers targeting a portion of the gene encoding the immunoglobulin heavy chain. Eight of these specimens exhibited the specific DNA band (~300 bp) of *P. hypophthalmus*.

**Key words :** androgenesis, CH4 primers, temperature shock, *Pangasius hypophthalmus*

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## INTRODUCTION

The natural process of gametogenesis in animals usually leads to the production of two kinds of haploid cell types, sperm and egg. Fertilization, combines these into a zygote re-establishing the diploid chromosome number of the somatic cell. Attempts have been made to selectively manipulate the contribution of genetic materials from male or female gametes through the mechanisms of androgenesis or gynogenesis. Androgenetic offspring in fish have been produced using genetically inactivated eggs and normal sperm (Bongers et al. 1994; Thorgaard et al. 1990). Duplication of the male genome is accomplished by suppressing the first mitotic cleavage. These androgenetic fish not only provide useful resources for monosex populations but also help preserve genotypes of endangered fish species through the use of cryopreserved sperm of *Pangasium gigas* (Mongkonpunya et al. 1995), for example.

Using percent hatch of haploid androgenic offspring of intraspecific crosses of *Clarias gariepinus* to determine the effective UV dose, complete inactivation of the egg genome (100% haploid) could be achieved at a dose of $\geq$240 mJ/cm$^2$ (Mongkonpunya et al. in preparation). The optimum dose of radiation is specific for particular species depending on egg size and the experimental procedure. Thus efforts should be made to optimize the UV dose and to standardize the whole procedure. During irradiation, eggs of common carp were stirred in a synthetic ovarian fluid without activation the eggs (Bongers et al. 1994) to assure homogenous irradiation of all pronuclei. Diploidy was restored by heat shocks (40°C, 2 min) applied at different times after fertilization (26, 28 or 30 min) and the yield of putative diploid androgens ranged from 7 to 18% at the time of hatching. Komen et al. (1991) stated that heat shocking of eggs at metaphase of the first mitotic division resulted in replication of the genome in gynogenetic common carp. However, when applied earlier (prometaphase) the efficiency of the heat shock was reduced resulting in high hatching percentages of haploid larvae. Thus, suppression of first mitosis must take place at one specific point of time after fertilization. The precise actions of the physical shocks in the suppression of the first mitotic cleavage are poorly understood. Given the narrow induction windows for effective suppression, there is a need to: 1) characterize the stages of karyogenesis, in relation to incubation time and water temperature, of developing zygotes, and 2) develop the mechanisms of restoration of diploidization by manipulation of temperature or pressure.

In this study, fresh eggs of *C. gariepinus* were fertilized with sperm of *P. hypophthalmus* and the fertilized eggs were sampled at different times for paraffin sectioning to evaluate timing of first mitotic division. The UV-irradiated eggs were used in another trial and subjected to temperature shocks at different times post-insemination to suppress the first mitotic division to induce androgenesis.

Finally, the identity of the putative androgenetic offspring were examined using DNA primers specific for the CH4 exon of the gene encoding the immunoglobulin M heavy chain (Thongpan et al. 1997).

## MATERIALS AND METHODS

### Metaphase of first mitotic division

Eggs of *C. gariepinus* were inseminated with sperm of *P. hypophthalmus* and the eggs were incubated in 26-27°C hatchery water. Immediately after insemination (0 min), and after 10-35 min of incubation, egg samples (~100 eggs) were collected at 5 min intervals. The samples were placed into 0.05% colchicine solution for 15 min and fixed in cold Bouin's. The details of treatment periods are shown in Table 1. The fixed samples were subjected to paraffin sectioning (~8 μ and stained using Spicer's method for nucleic acid as described by Luna (1968) which is highly specific for chromatin materials.

**Table 1.** Survey of egg treatment procedures for evaluating the first mitotic metaphase.

| I. Incubation period (min) | II. Colchicine treatment period (min) | Fixation (I + II) |
|---|---|---|
| 0 | 15 | 15 |
| 10 | 15 | 25 |
| 15 | 15 | 30 |
| 20 | 15 | 35 |
| 25 | 15 | 40 |
| 30 | 15 | 45 |
| 35 | 15 | 50 |
| 15 | 50 | 65 |

### Suppression of the first mitotic karyogenesis

Eggs of *C. gariepinus* were UV-irradiated (240 mJ/cm$^2$) before fertilization with fresh sperm of *P. hypophthalmus*. The inseminated eggs were incubated in 26-27°C hatchery water for 20 to 45 min before being subjected to the first mitotic suppression by shocking in cold water (7°C or 15°C for 10 to 25 min) or warm water (38°C or 42°C for 15 to 60 sec). Immediately after shocking, the eggs were incubated in 26-27°C hatchery water. Percent fertilization and number of normal fry (putative diploid androgenetic offspring of *P. hypophthalmus*) were recorded at 24 and 48 h after insemination. Ten normal fry were frozen in liquid nitrogen for

further analysis by polymerase chain reaction (PCR) using primers specific for CH4 exon of the channel catfish (*Ictalurus punctatus*) gene encoding the immunoglobulin M heavy chain as described by Thongpan et al. (1997).

**Statistical analysis**

The experimental design was factorial (4 x 6 x 4) in complete randomized design with unequal replication (n = 3 to 19) and the mean yield of percent fertilization and percent normal fry were compared among treatment combinations and the deviation from the control using Duncan's multiple range test ($P$ <0.05).

## RESULTS

**First mitotic karyogenesis**

From microscopic observation, about 50% of fertilized eggs of pangasiids and clariids completed the first mitotic cleavage showing two-cell stage within 90 min of incubation in 20°C hatchery water. At higher incubation temperature (26-27°C) duration of this process was shortened to ~50 min. In addition, the chromosome cross-sections at various stages of mitotic division were also characterized (Figure 1). Though the fish chromosomes were small, cross sections of metaphase chromosome were distinguishable from those of the other stages. Pairing of condensed sister chromatids was the distinctive feature of the metaphase stage. Lesser condensation without paired chromatids was classified as early metaphase stage. Chromatin in other stages (anaphase and telophase) were not found due to the colchicine treatment. Data shown in Figure 2 demonstrated the rate (%) of development at different stages of mitosis of *C. gariepinus* eggs after insemination with *P. hypophthalmus* sperm. The developing zygotes (fertilized eggs) arrested the first mitotic cleavage at the metaphase stage due to the colchicine blockage. Karyogenic mitotic metaphase (~3%) began after incubation for 35 min (20 min in water plus 15 min in colchicine). The number of metaphase mitotic cells accumulated and amounted to about 35% at 40 min of incubation and ~65% at 45 min of incubation and remained constant thereafter. After 25-50 min of incubation, only ~5% of incubated eggs remained without karyogenesis. These eggs were considered to be unfertilized. The differences among incubation batches in unfertilized eggs (1-9%) were not significantly different.

**Induction of androgenetic *P. hypophthalmus***

Percent fertilization and percent normal (putative androgenetic) fry resulting from this experiment are shown in Figures 3 and 4. There was no significant difference due to the effect of shocking time in percent fertilization except for the 42°C heat shock (for 60 sec) which resulted in 0% fertilization for all post-insemination times. In addition, there was a decrease in percent fertilization with increasing duration of shock treatments across the ages of the zygote (Figure 3). Considerably low percent

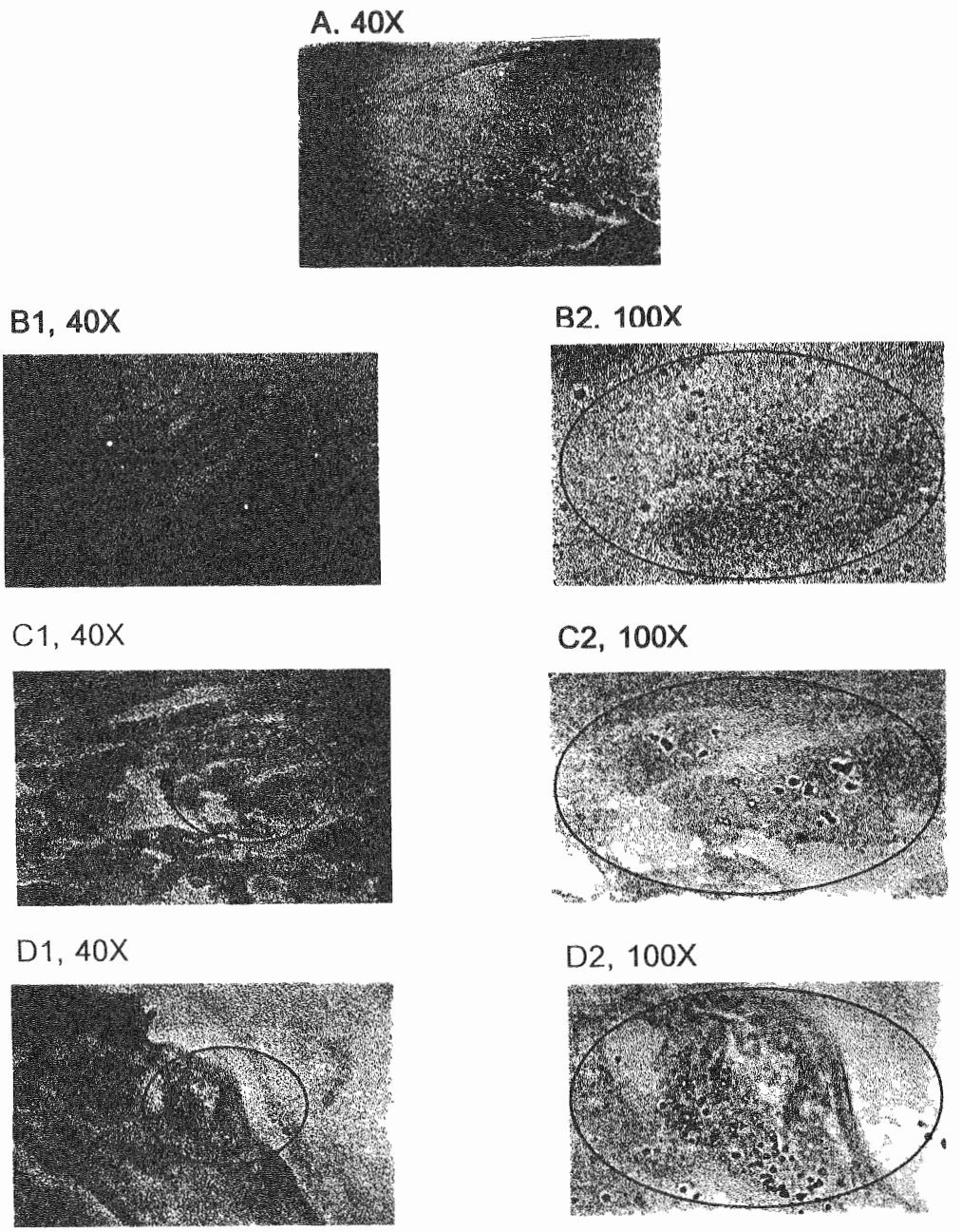


**Figure 1.** Cross-sections of *Clarias gariepinus* fertilized eggs showed characterization of the chromosome at various stages of mitotic division : without karyogenesis in unfertilized eggs (A), Prophase ($B_1$, $B_2$), early Metaphase ($C_1$, $C_2$) and Metaphase ($D_1$, $D_2$). Small red granules are cross-section of the chromosome. For further explanation see text.

fertilization suggested a need of a suitable time to begin shocking and the optimum temperature. Data from this experiment indicated the highest percent fertilization where shocking (15°C and 30°C) began at 30-35 min post fertilization and a number of 2-day normal fry (putative androgenetic) could be obtaind (Figure 4) with high variation (0-4%). The amplification of genomic DNA of 10 individuals of the putative androgenetic offspring using the specific primers (CH4) indicated that 8 of them were androgenetic *P. hypophthalmus* having a single band of ~ 300 bp while the rest (2 specimens) having two DNA bands (~300 and 340 bp) of the maternal DNA pattern (Figure 5), indicating hybrid (*C. gariepinus x P. hypophthalmus*) identity.

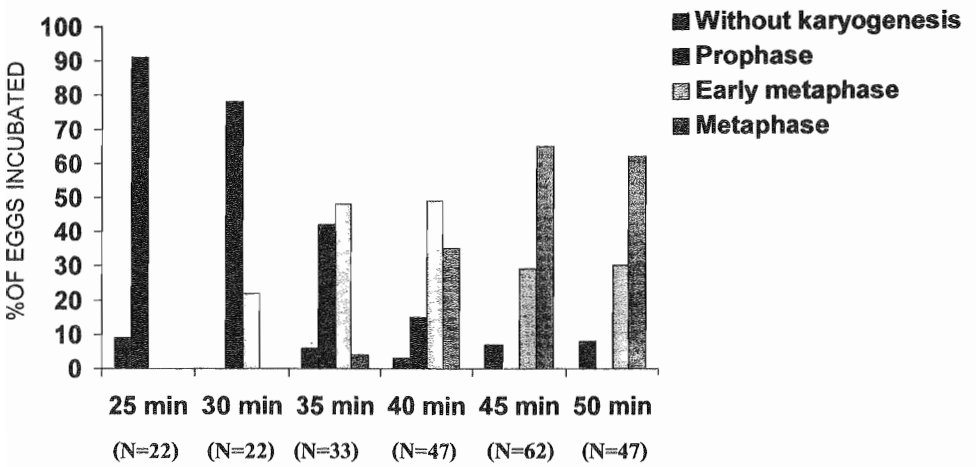


**Figure 2.** First mitotic division in relation to time (min) after fertilization of Duk Yuk eggs (*Clarias gariepinus*). The inseminated eggs (Duk Yuk x Duk Yuk) were incubated for different time periods (10 to 35 min) before transferring into 0.05% colchicine solution for 15 min and then fixed in cold Bouin's to suppress mitotic division. Thereafter, the specimens were subjected to paraffin section, dehydration and stained in accordance with Spicer's method for nucleic acid staining (Luna 1968).

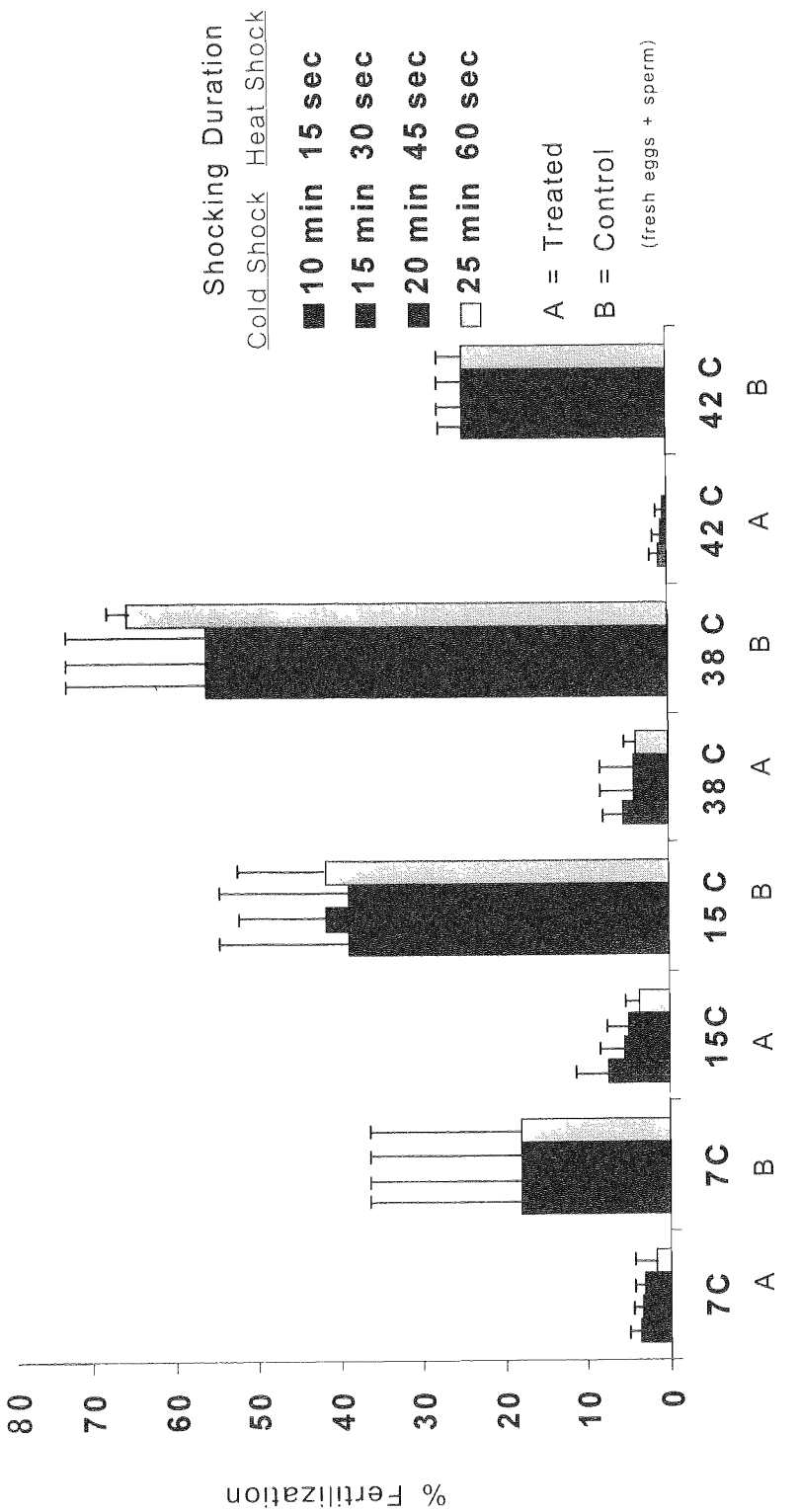


**Figure 3.** Percent fertilization (mean ± SD) of UV-irradiated (240 mJ/cm$^2$) eggs of *Clarias gariepinus* after insemination with fresh sperm of *Pangasius hypophthalmus* and shocked in cold water (7°C or 15°C for 10 to 25 min) or warm water (38°C or 42°C for 15 to 60 sec). Shocking began between 20 to 45 min after insemination. Fertilization (developing embryo) percentages were determined 24 h after insemination.

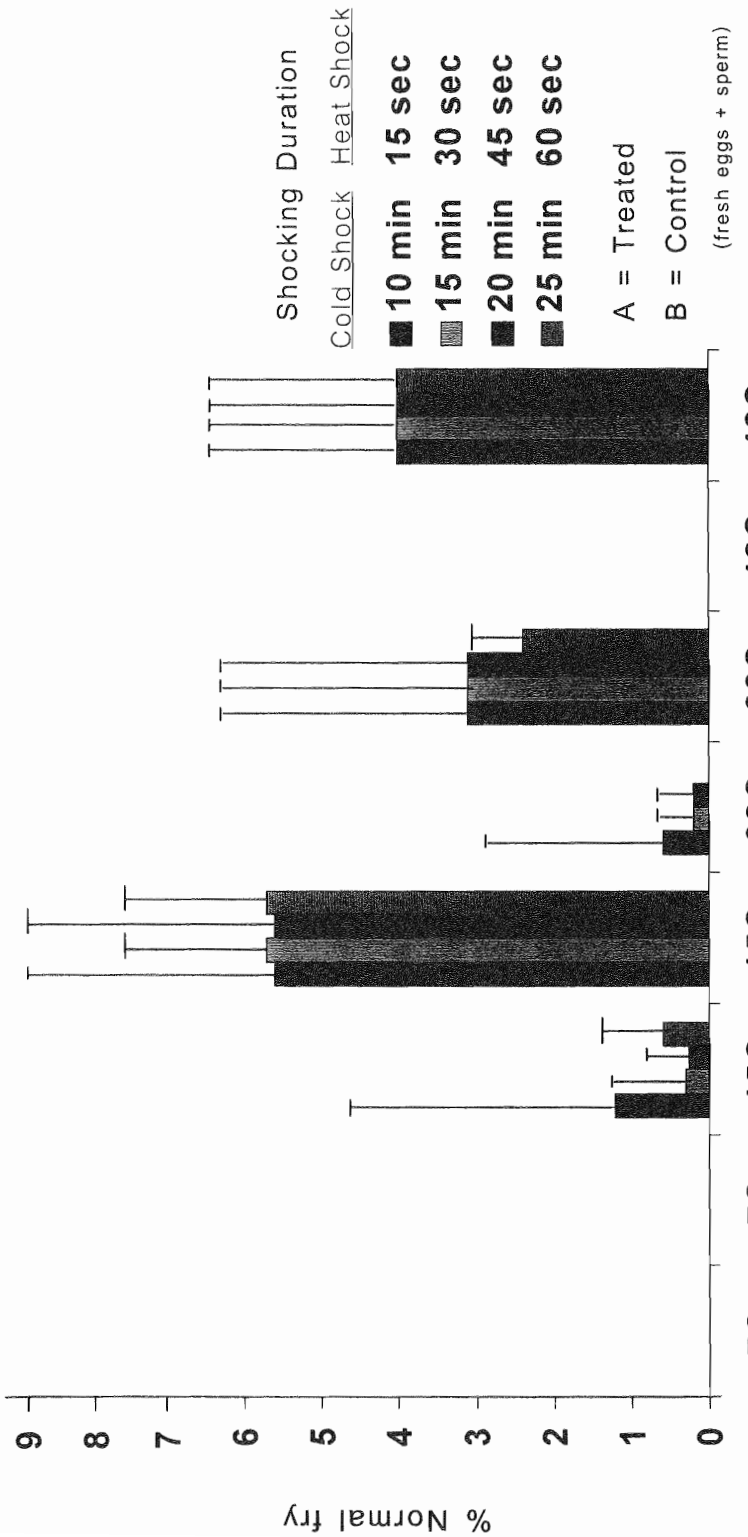


**Figure 4.** Percent (mean ± SD) normal fry (putative androgenetic *Pangasius hypophthalmus*) hatched from fertilized eggs shown in Figure 1. The data shown in this table are percentages of fertilized eggs.

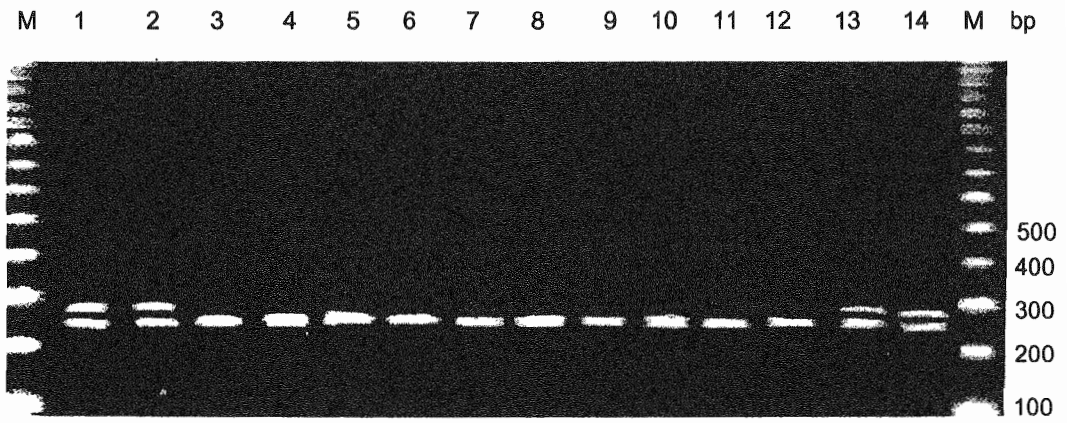


Figure 5

**Figure 5.** Representatives (n = 10) of amplification of genomic DNA from individual intact fry of putative androgenetic Sawai (*Pangasius hypophthalmus* by PCR. Primer sequences targeted a 300-bp fragments or two DNA fragmants of about 300 and 340 bp of CH4 exon of the gene encoding the immunoglobulin M heavy chain of Sawai or Duk Yuk (*Clarias gariepinus*), respectively. Lanes 1-10, putative androgenetic offspring; Lanes 11-12, putative haploid Sawai (UV irradiated Yuk egg x Sawai fresh sperm) and Lanes 13-14 (diploid Yuk), standard reference. This result indicated that there were 8 androgenetic Sawai (Lanes 3 to 10) out of 10 specimens.

## DISCUSSION

Colchicine blockage is often used to demonstrate chromosome characterization at various mitotic stages. In this study, the developing zygotes were incubated in water (26-27°C, 10 to 35 min) followed by colchicine treatment (0.05%, 15 min) before fixation. The paraffin sections revealed no telophase (Figures 1 and 2) indicating the effectiveness of the colchicine blockage. It is likely that we would observe cells in telophase after 50 min of incubation, as shown by microscopic observation of untreated zygotes, if colchicine were not applied.

Assuming that colchicine blockage in cells occurred equally through time, the data could be interpreted that 25-30% of fertilized eggs had synchronized their development. This was clearly seen by the differences in number of eggs found in metaphase during 35-45 min (Figure 2). Thus, shocking to suppress the first mitotic cleavage could be done at some time between 25 to 30 min of incubation in hatchery

water (26-27°C). Early metaphase stage, however, began about 5 min earlier than metaphase and reached the maximum at about 20 to 25 min of incubation, and then decreased. It is also noteworthy that decrease in the number of eggs in prophase stage was opposed by an increase in the number of eggs in metaphase.

Kleinsmith and Kish (1995) presented the behavior of the sperm nucleus after fertilization in sea urchins. Shortly after fertilization the egg cytoplasm moves up around the entering sperm nucleus, creating a fertilization cone. As the sperm head moves through the egg cytoplasm, the chromatin begins to disperse and the surrounding nuclear envelope disappears. Uncoiling of the sperm chromatin continues as a new nuclear envelope begins to form. At this stage only a small region of the sperm chromatin remains condensed. After a new nuclear envelope has formed around the sperm chromatin, it is called the male pronucleus. As the male pronucleus and egg cell nucleus (female pronucleus) approach one another, projections appear on the surface of the female pronucleus. Fusion of the female and male pronuclei leads to a mixing of their chromatin. In androgenesis, however, only the sperm chromatin is active in the irradiated egg. There was no report on the behavior of the two pronuclei. Thus, behavior of the sperm pronucleus is still unknown in androgenesis.

Preliminary trials were performed to titer the UV dosage. Eggs of *C. gariepinus* were UV-irradiated (0-360 mJ/cm$^2$) before fertilizing with *C. gariepinus* sperm. After ~48 h of incubation the number of normal and abnormal (putative haploid) fry were recorded (data not shown). The abnormal fry were characterized by the haploid syndrome : short head and tail with large cavity between yolk mass and the heart (Mongkonpunya et al. in preparation). The UV treatment ≥240 mJ/cm$^2$) resulting in 100% abnormal fry was chosen as the effective dose. The minimal effective dose was used in this experiment because higher doses resulted in lower percent fertilization and lesser number of hatching fry. Bongers et al. (1994) reported an effective dose of 250 mJ/cm$^2$ for genomic inactivation of common carp eggs. They obtained putative androgenic diploids (7-18%) by heat shocks (40°C, 2 min) applied at different points of time after fertilization (26, 28 and 30 min).

It is known that the retention of second polar body in gynogenesis could result in heterozygosity at recombinant loci located distal to the centromere. But androgenetic offspring induced by suppression of first mitosis are completely homozygous. They therefore can express recessive deleterious and lethal alleles. Consequently, application of shocks to disrupt the first mitosis resulted in low survival rates with an average of less than 5% hatching normal androgenetic fry (Figure 4). It is likely that the rate of diploidization in androgenesis is actually higher than the observed survival rates. This may be due to mortality brought about by complete homozygosity, and the physical shocks which are applied to the eggs.

The egg quality alone seems to be the major determining factor in the success of androgenesis. Great differences exist between egg batches in their susceptibility to the UV irradiation and the temperature shocks. We found that only a small number of egg batches were resistant to the negative side effects of the effective dose of UV irradiation and the temperature shock. In general, percent fertilization and percent hatch were decreased by 50-100% at each step of the treatments. This is not surprising, because only a few reports have recorded successful suppression of first mitosis in fish by the application of temperature or pressure shocks during first cleavage. Parsons and Thorgaard (1985) and May et al. (1988) used high pressure shocks and obtained androgenetic diploid rainbow trout and brook trout, respectively. The most successful induction of polyploids in rainbow trout was accomplished by using pressure shock, which resulted in 100% tetraploid (Chourrout 1984).

For warmwater species with smaller eggs (~1 mm) such as *C. gariepinus* and *P. hypophthalmus,* the shocks must be applied earlier after fertilization (20-35 min) than those of coldwater fish. We have found that only 25-30% of the zygotes synchronized their development during that particular period of time mentioned above. In addition, the eggs subjected to UV irradiation (240-300 mJ/cm$^2$) and inseminated with fresh sperm resulted in 100% abnormal fry with haploid syndrome. Thus, if the temperature shocks (15°C or 38°C) with various durations were effective, the normal fry obtained from this study could be androgenetic *P. hypophthalmus.*

The putative androgenetic *P. hypophthalmus* fry resulted from our study were further examined by PCR analysis using the specific primers (CH4) as described by Thongpan et al. (1997). Nuclear DNA amplified with this primers yielded a single band of ~300 bp for *P. hypophthalmus* while the same primers yielded two DNA bands of about 300 and 340 bp in *C. gariepinus.* We have found that 80% of the fry (n = 10) subjected to PCR analysis exhibited a single band of 300 bp of *P. hypophthalmus.* Still, it is not clear whether the genetic contribution of the female *C. gariepinus* is fully eliminated, since maternal (mitochondrial) DNA is present in eggs (Gardner et al. 1991) as well as possible maternal nuclear DNA fragments not amplified by the primers (CH4) used in this experiment. However, the results from this study indicate the possibility of recovering genotypes from cryopreserved sperm of Mekong giant catfish *(Pangasius gigas)* which are available in our laboratory. This is of increasing importance as wild populations of *P. gigas* come under greater threat of extinction, and the establishment of sperm banks, as well as development of successful androgenesis procedures become more vital.

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