Effect of ethanol immersion time on parthenogenesis

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Abstract. The purpose of this research was to investigate the probability of common carp
(Cyprinus carpio L.) egg parthenogenesis activated by ethanol. The method used was
experimental design, with 7 treatments and 3 replications. The treatment includes positive
control (using sperm) and negative control (without sperm). The immersion times in 7%
ethanol were 2; 2.5; 3; 3.5 and 4 minutes. The main parameter was percentage of activated and
development egg. The support parameters were temperature, DO, and pH values. The results
showed that sperm submerged in ethanol 7% for 2 minutes displayed very significant influence
(P<0.05) for activation and development egg with maximum embryo development
reached was blastula phase. Water quality data showed that the temperature ranged from 20.1-
22.7°C, DO 6.72-6.84, and pH in the range of 7.19-7.55.

1. Introduction
Carp is one of freshwater commodities with promising market [1]. Carp production in 2005 was
216,920 tons and increased in 2009 as much as 446,800 tons [2]. Demand for carp from year to year
tends to increase especially in major cities such as Jakarta, Surabaya and Bandung [3]. Nevertheless,
the demands of carp seeds still cannot be fulfilled by the fish seed producers due to limited production
[4]. Therefore, it is necessary to develop main technology in spawning. In fish spawning aquaculture,
there are two spawning systems, natural and artificial. There are three types of homemade spawning,
i.e. Gynogenesis, Androgenesis, and Parthenogenesis. Parthenogenesis is one of the artificial spawning
by activating egg cells using certain chemicals as its activator replacing sperm. In other word, the
parthenogenesis is an asexual reproduction process where eggs from female individuals become
embryos that develop without the role of sperm [5].

Materials that have been used for the activation of parthenogenesis include ethanol in carp eggs [6],
Ca-Ionopore and 6– dimethylaminopurine on carp eggs [7], protein kinase on carp eggs [8], enzymes
protease on Dumbo catfish eggs [5], and cytochalasin B on carp eggs [5]. Ethanol can be used as a
means of fish eggs activation due to its ability to facilitate increased calcium ions in the ooplasm
which is a sign that the oocyte has been activated [9]. The release of calcium into the ooplasm is
important to activate systemic reactions of the oocytes that are based on the forwarding of meiosis
division. Ethanol induction increases calcium ions as a result of extracellular calcium ions coming
from the intracellular storage place [10]. Activation with ethanol will cause a meiosis division on
activated oocytes and will develop into a haploid-like embryo [6]. Haploid embryos in a
morphological fish cause abnormalities (haploid syndrome) and die before or shortly after hatching.
In order to obtain a diploid genetically capable fish surviving, the haploid female chromosome should be duplicated. Female chromosomal doubling can be done in two ways, with the detention of a second meiosis cleavage in an egg or the first mitosis cleavage of the haploid embryo. One way for the detention (suppression) of meiosis or mitosis division is by giving physical treatment to the embryo. Most treatments used were low or high temperatures (heat shock/cold shock). Hot shock is the most common physical treatment technique used to produce polyploidy in fish [12]. The purpose of this study was to determine the effect of ethanol immersion time variations in activating the process of parthenogenesis.

2. Materials and methods

2.1. Materials

This research was conducted in the Fish Reproduction Laboratory of the Faculty of Fisheries and Marine Sciences, Universitas Brawijaya. Materials used include: carpfish eggs, carp fishes' sperm, 7% ethanol, distilled water, physiological sodium and ovaprim.

2.2. Methods

The study design was based on the research of Kurniawan [6] which got the best results, namely 7% ethanol immersion for 3 minutes and at a temperature of 40°C for 15 seconds. But the duration of the temperature shock refers to the reference gynogenesis (4 minutes) [13]. The design used was a Completely Randomized Design (CRD); consisting of 5 treatments, 1 negative control and 1 positive control with 3 replications, as follows:

Control (-) = eggs that are left alone without treatment
Control (+) = sperm-fertilized egg
Treatment A = egg immersed in 7% ethanol for 2 minutes and a temperature shock of 40°C for 4 minutes
Treatment B = eggs soaked with 7% ethanol for 2.5 minutes and a shock temperature of 40°C for 4 minutes
Treatment C = eggs soaked by 7% ethanol for 3 minutes and a temperature shock of 40°C for 4 minutes
Treatment D = eggs soaked by 7% ethanol for 3.5 minutes and a temperature shock of 40°C for 4 minutes
Treatment E = eggs soaked by 7% ethanol for 4 minutes and a shock temperature of 40°C for 4 minutes

2.3. Method

2.3.1. Broodstock preparation

Carp used as broodstocks were approximately 1-2 years old or after their weight reaches 1.5-2.0 kg. The broodstock must also have a mature gonad. The selection of the broodstock must be based on the health and physiological condition of the broodstock.

2.3.2. Preparation of activator solution (7% ethanol)

The making of 7% ethanol stock solution was done by putting 93 ml of distilled water into a bottle and then tightly closed. 7ml of absolute ethanol using a syringe, then injected it into a bottle that already contained distilled water and homogenized. This technique was aimed to avoid ethanol evaporation, so that the ethanol concentration in 100 ml was exactly 7%.

2.3.3. Fertilization (control)

Activation of eggs naturally was done by mixing eggs and sperm of carp. carp sperm was first diluted with physiological sodium in a ratio of 1:9 (1 ml of carp sperm plus 9 ml of physiological sodium) then homogenized. Parts of the carp stripping results were placed in a different egg collection basin.
Sperm that had been diluted with physiological sodium was then put into an egg-collecting basin while it was stirred using pigeon feathers to maximize the mixing of eggs with carp sperm. The eggs were then spread in an incubator using pigeon feathers and then incubated in a prepared aquarium.

2.3.4. Activation of carp fish (Ciprinus carpio) with 7% ethanol and giving heat shock
Artificial carp egg cell activation was done by immersion the eggs in 7% ethanol. 7% ethanol was poured in an aquarium measuring 20x20x30cm then the stripping eggs were spread in a tea filter using pigeon feathers. Fish eggs were immersed in 7% ethanol for 2; 2.5; 3; 3.5 and 4 minutes and given a hot shock by soaking them in hot water (40°C) for 4 minutes at. The spread of the eggs was carried out evenly and not stacked to facilitate the observation and so that the activation ran optimally. The process of natural egg activation (fertilization) was used as a comparison of the success of artificial egg activation using ethanol and hot shock. Temperature shock for 4 minutes is able to withstand polar discharge [13].

2.3.5. Egg incubation
Eggs were incubated in an 70x50x50cm aquarium filled with well water as an incubation medium. Aquarium conditions must be managed carefully to support the egg development process. Heater was provided to maintain the water temperature at 20-22°C [14]. In addition, an aerator or pump was also added to meet the oxygen needs of the egg. Water changes regularly for as much as 10% every 6 hours during incubation to reduce toxic compounds during egg development and avoid temperature fluctuations. The development of fish eggs also requires optimal and relatively stable environment i.e.: temperature (20-22°C) and consistent of dissolved oxygen (5-6 ppm). Plankton-free water is a basic requirement for hatcheries. Polluted water will cause various kinds of problems, such as the possibility of attacks from planktonic animals, especially cyclopid copepods. During its development, the egg secretes several dangerous compounds such as CO₂ and NH₃. These compounds when accumulated can be toxic to the eggs themselves [15].

2.4. Egg Observation
Observation of egg development started a 10 minutes after activation and was continued every 1 hour until the last embryo development. Observations were made under a light microscope. The activated egg will appear clear green, the perivitelin zone is expanding and the cytoplasm will move towards the anima pole, while the unactivated egg will be white. Eggs that develop into embryos are characterized by prominent formations in the anima poles. The eggs that are successfully activated and continue to develop their embryos and hatch are counted for each treatment. After 8 hours of fertilization, we will find white eggs that indicate that the fish eggs are dead or not fertilized [13].

2.5. Statistics analysis
This study used a Completely Randomized Design (CRD) with 3 replications. The treatments tested were 7% ethanol immersion with 2 variations of immersion duration; 2.5; 3; 3.5 and 4 minutes and continued with 40°C heat shock for 4 minutes. The parameters observed were egg activation and development at each treatment. The data obtained are converted in units of percentages (%) and analyzed quantitatively and qualitatively. Qualitative analysis was done descriptively, whereas quantitative analysis was carried out using the CRD method. Calculation formula for percentage of activated eggs:

\[
\text{Percentage Activated Egg} = \frac{\sum \text{activated egg}}{\sum \text{stocked egg}} \times 100
\]  

(1)

The formula for calculating the percentage of developed eggs:

\[
\text{Percentage Development Egg} = \frac{\sum \text{egg development}}{\sum \text{egg stocked}} \times 100\%
\]  

(2)
3. Results and Discussion

3.1. Activation of carp (Ciprinus carpio L) eggs

Data on the effect of variations in immersion time on egg cell activation in the formation of embryo parthenogenesis of carp (Ciprinus carpio L) is shown in Table 1.

Table 1. Percentage of activated carp (Ciprinus carpio) eggs.

| Treatment | Activated eggs (%) | Total | Average |
|-----------|---------------------|-------|---------|
| A         | 99.25 98.95 99.85   | 298.05| 99.35   |
| B         | 98.74 97.85 98.25   | 294.84| 98.28   |
| C         | 97.75 96.95 97.35   | 292.05| 97.35   |
| D         | 95.45 94.95 95.85   | 286.25| 95.42   |
| E         | 92.75 93.65 92.85   | 279.25| 93.08   |

Total 1450.44

Control (-) 0 0 0 0 0
Control (+) 99.89 99.68 99.46 299.03 99.68

Description:
A: immersion in ethanol 7% for 2 minutes
B: immersion in ethanol 7% for 2.5 minutes
C: immersion in ethanol 7% for 3 minutes
D: immersion in ethanol 7% for 3.5 minutes
E: immersion in ethanol 7% for 4 minutes

Table 1 and Figure 1 show that there is a decreasing percentage rate of egg activation. The percentage rate of activation of all ovum treatment was below positive control but is above negative control. Based on the analysis of variance calculation, it can be seen that the F count is greater than F 1%(P<0.05).

![Figure 1. Percentage of activated of carp (Ciprinus carpio) egg cells with](image-url)
Kurniawan [6], mentioned that there is a tendency for the percentage of activated egg cells to decrease in accordance with the length of ethanol immersion time and the duration of heat shock, which causes low egg viability. Figure 1 showed that the best treatment is in treatment A, which is close to the average value of positive control. 7% ethanol could be considered as the optimal concentration to be used as an activator.

A negative control egg it looks like an embryo is developing, but actually the egg doesn't develop because it's not activated (Figure 2.). If the carp eggs stay in the water for too long then the eggs will absorb a lot of water considering the concentration of the egg is more concentrated than water. Woynarovict and Horvart [15], stated that if carp eggs immersed in the water for 45 to 60 seconds, then the eggs will expand and the microphiles close. Hijriyati [16], mentioned that egg weight was one of factors that influence egg fertilization besides ovary fluid pH and protein concentration.
Activated egg (Figure 3) indicated the presence of perivitelline space or the distance between the egg nucleus and the egg cell wall. Murtidjo [13] explained that fertilization of fish eggs in the form of the entry of the head of the spermatozoa into the egg and the tail of the spermatozoa is left outside. If so, the cytoplasm and chorion dilate and a kind of plug immediately closes the micropyle to block the entry of other spermatozoa. According to Hasbi et al., [17] described that activated oocytes are characterized by several events including cortical reactions, pronucleus formation, and cell division. Likewise, Campbell et al., [18] explained that within a few seconds after the sperm bind to the egg, these vesicles, called cortical granules, fuse with the plasma membrane of the egg, initiating cortical reactions. These cortex granules contain deposits of molecules that are now secreted into the perivitelline space, which is located between the plasma membrane and the vitelin layer. The secreted enzymes and other macromolecules simultaneously push the vitelin layer away from the egg and harden the layer, forming a protective fertilization envelope that refuses the entry of additional sperm nuclei.

### 3.2. Development of eggs after activation

Research data can be seen in table 4 below.

**Table 2. Percentage of developed carp eggs (Ciprinus carpio).**

| Treatment | Developed eggs (%) | Total  | Average |
|-----------|--------------------|--------|---------|
|           | 1      | 2      | 3      |         |
| A         | 55.89  | 55.96  | 53.23  | 163.07  | 54.36  |
| B         | 23.23  | 22.86  | 21.56  | 67.65   | 22.55  |
| C         | 3.86   | 2.92   | 3.23   | 9.83    | 3.28   |
| D         | 0.17   | 0.76   | 0.46   | 1.39    | 0.46   |
| E         | 0.35   | 0.17   | 0.17   | 0.69    | 0.23   |
| Total     |        |        |        | 242.63  |

| Control (-) | 0 | 0 | 0 | 0 | 0 |
| Control (+) | 87.12 | 82.29 | 77.08 | 246.49 | 82.16 |

Note: (See table 1)

Table 1 showed that treatment A had an average value was similar to the average value of normal control and there was a tendency to decrease in percentage. Figure 4 shows the relationship between the immersion time of the egg in ethanol 7% and the temperature shock to activate the egg.
Figure 4 shows that the number of eggs that develops continues to decrease with immersion time in ethanol 7%, which can be caused by decreased systemic ability of cells due to too long. The activation process will affect the development of the egg. This is in accordance with the opinion of Kurniawan [6], damage to the components of the egg cell causes the egg cell cannot continue to divide and the cell eventually dies.

Treatment A had the best effect on egg cell development, namely blastula phase (54.36%). It was assumed that the appropriate time to soak the eggs in the 7% ethanol for 2 minutes and get enough temperature shock time so that it was able to divide and develop well. For treatment B had an influence on the development of egg cells in the morula phase by 22.55%, this was thought to be caused by the ethanol immersion time of 7% which was longer than treatment A, which was 2.5 minutes and the temperature shock time was less so the cleavage process cells and their development with a smaller amount than treatment A. While treatments C, D and E had a relatively small effect on egg cell development in the morula phase, respectively, 3.28%, 0.46% and 0.23%. This is thought to be due to the long immersion time of ethanol 7% which is quite long and the temperature shock time is not quite right, thus causing the cell's systemic ability to decrease which causes the number of cells that can divide and develop less. Other factors that influence the decrease in the percentage of embryonic development may be due to an incomplete activation process where the chromosome division process is still ongoing then given a temperature shock so that the development of the egg cell is disrupted or an abnormality in embryonic development can even result in the egg cell dying. This condition results in the cell experiencing a decrease in systemic action of the cell so the cell is unable to develop properly.

On the other hand temperature shock can also affect the blocking of the occurrence of polar body II or withstand the release of polar body II. If the temperature shock occurs in the egg which is still in the process of chromosome division, it will cause the egg to experience developmental abnormalities because the chromosome has not been perfectly split into two polar bodies. The time of polar body formation during the time of initiation takes place, which is estimated to occur right after activation until 3 minutes after activation. Thus, if an activated egg and polar body formation occur in less than 3 minutes and get a temperature shock with sufficient time, so that the polar body remains retained in the egg so that the egg will be able to develop properly and have a chromosome 2n (diploid) . And if the egg is activated for more than 3 minutes and gets a temperature shock, the polar body that is formed will come out of the egg, causing the egg to have an n (haploid) chromosome. The condition is
like the opinion of Rustidja [19], the purpose of this shock is to hold the polar body out because not all eggs are timed simultaneously in the polar body discharge for that it takes time to hold the polar body out of all the same eggs.

Figure 4 illustrated that the best treatment was treatment A, where in the treatment the number of eggs develops the most and has an average value below normal control but has an average value above negative control that is equal to 54.36%. Kurniawan's [6] previously explained that the highest average treatment value was in the immersion treatment of 7% ethanol for 3 minutes, with an average value of 0.007% and the maximum cell development that could be achieved was the 8 cell division stage.

The maximum embryonic development that can be achieved only reaches the blastula stage with the characteristics of a greater number of blastoderm, smaller in size than the previous phase and looks like they are fused (Figure 5, 6). This result was accordance with Woynarovich and Horvarth [15]. As the number of blastomers increases, the size becomes smaller. Within the cell a small space is formed between the egg yolk and the cell mass called segmentation cavity. Embryo in this stage is called blastula.

The maximum development in the blastula phase, where the time required by carp eggs varies in the activation process and temperature shock at the wrong time (the egg is still in the chromosome division stage) can be a factor that inhibits the development of the egg so the egg cannot develop optimally or even die. According to Woynarovich and Horvarth [15], the time required for egg development is different for each species. The time needed also depends on the temperature during incubation and oxygen supply at the beginning of its development. O2 deficiency during embryonic development can kill embryos. Grupen et al., [20] argued that artificial activation treatment (using ethanol) cannot precisely mimic calcium isolation patterns as induced by sperm fertilization. Increased calcium induced by ethanol has not been able to activate the cellular system that causes the egg cells to develop normally as occurs in fertilization by sperm [6]. The treatment of heat shock can reduce cell viability. Giving a heat shock that exceeds 44°C causes damage to the zygote [21]. In mammals there are two causes of not developing partenot embryos further. Firstly, during oocytes activation, several species was lacking centriole so that the centrosome does not function optimally and cause the cessation of the initial phases of division. Secondly, it was because of imprinting [22].

4. Conclusion
7% Ethanol with variation of immersion time was able to activate carp eggs (Ciprinus carpio). 2 minutes immersion time could activate 99.35% of eggs and 54.36% of embryo developed well. The maximum embryo development that can be achieved was the blastula phase. Water quality data during
the observation showed that the average water quality was in the normal range, i.e.: temperatures ranged from 20.1-22.7°C, pH ranged from 7.19-7.55 and DO ranged from 5.72-5.84 ppm.

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