

# Effect of heat-shock duration and zygote age on tetraploid induction for triploid broodstock production in platinum koi (*Cyprinus rubrofuscus*)

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**Abstract.** The koi fish (*Cyprinus rubrofuscus*) is an ornamental freshwater species valued for its vibrant body patterns, colors, and high market price. Despite extensive breeding efforts, the demand for koi fry remains unmet. Advances in biotechnology, such as chromosome manipulation, offer a promising solution to increase production yields. This study evaluated the effectiveness of tetraploid induction in koi through heat shock treatment, testing different zygote ages and shock durations. The highest proportion of tetraploids (86.6%) was achieved with a heat shock of 39 °C for 2.5 minutes. When considering zygote age, heat shock applied at 31 minutes post fertilization (mpf) produced 66.7% tetraploids, while treatment at 27 mpf yielded a comparatively higher proportion than other age groups. Tetraploidy was confirmed through cytogenetic analysis, ensuring accurate identification of induced individuals. These findings provide a practical protocol for koi breeders to produce tetraploid broodstock, which can be used for triploid production or to enhance genetic resources in ornamental fish aquaculture.

## 1 Introduction

Koi fish (*Cyprinus rubrofuscus*) is one of the introduced species of freshwater ornamental fish that is highly favored by ornamental fish enthusiasts and has high economic value. The beautiful coloration of koi fish makes it one of the most popular freshwater ornamental fish. From 2015 to 2018, the average production of koi fish reached 11.6%, in line with the increasing demand each year. In fact, in 2018, koi fish ranked first in ornamental fish production, with a total of 476,345 individuals produced [2]. In 2022, Japan's ornamental fish exports reached around 48.6 million USD more than doubling over the past 14 years. Other leading exporters included Indonesia, with a trade value of 36.4 million USD, and Singapore, at 35.1 million USD. Among these, Koi fish stands out as one of the most traded species. The high trade volume demonstrates the global popularity of Koi among hobbyists, highlighting its potential as a thriving and expanding business sector. Although koi fish breeding efforts have been ongoing for a long time, the demand for seedlings has still not been fully met.

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A significant increase in koi fish productivity needs to be achieved through chromosome manipulation. Chromosome manipulation is a method used to modify genetic material in order to produce individuals with superior qualities. This technique can be applied at the level of genes, chromosome sets, or populations. Genetic modification represents another biotechnological method that can be applied to enhance fish breeding [8].

Several models of chromosome manipulation that have been tested include androgenesis, gynogenesis, triploidization, and tetraploidization. These chromosome manipulation techniques can be applied in the production of tetraploid fish within the teleostei group [11]. The sterility of triploid fish results in the redirection of energy that would normally support gonadal development and gametogenesis toward somatic growth instead. During gonadal formation, triploid fish are unable to properly align their chromosome sets, preventing normal gonad development and leading to sterility. Typically, the testes and ovaries of triploid fish are smaller and show abnormal structures compared to those of diploid fish. In many cases of triploid induction, chromosomes appear damaged or incomplete. Sterility in triploid fish arises from the presence of an odd number of chromosome sets, which disrupts meiosis and leads to abnormal gonadal development or the formation of aneuploid gametes aneuploidy being the primary cause of their sterility. Triploid fish or sterile fish have faster growth compared to diploid fish [10].

Polyploidization can be carried out using shock methods, such as cold or heat treatment, hydrostatic pressure, or chemical treatments, aimed at preventing the release of the second polar body or the first mitotic division in fertilized eggs [5]. Polyploidy induction in fish can be achieved through the application of physical shock treatments that disrupt specific cellular events during early embryogenesis. Inhibition of the second polar body extrusion results in the formation of triploid individuals, whereas suppression of the first mitotic cleavage leads to the development of tetraploid embryos. Induction of polyploidy through heat shock is a method applied immediately after the first mitotic division (for tetraploid induction) at a lethal temperature. This technique has several advantages, including relatively low cost and effectiveness on a large scale. Temperature shock treatment, with variations in temperature, is the most commonly used physical method for producing polyploid fish.

One of the physical methods used in tetraploidization techniques is heat shock. Tetraploid organisms typically exhibit greater heterozygosity owing to the presence of four chromosome sets, which can contribute to the development of larger body size [3]. Moreover, tetraploids play a significant role in evolutionary processes because of their elevated genetic variability [8]. In artificially induced tetraploids, all chromosome sets are derived from diploid parental individuals [15]. The success of tetraploid induction is influenced by several factors, such as the age of the zygote at the time of treatment (initial time), the duration of the shock, and the intensity of the shock temperature. These factors need to be adjusted according to the characteristics of the specific fish species, especially when determining the age of the zygote, because each species has a different timing for the first mitotic division. To optimize the determination of zygote age before tetraploid induction, observations of the cell division process can be conducted beforehand.

Ploidy induction is highly time-sensitive, with even minute differences affecting outcomes, and the precise developmental timing specific to koi fish has not been thoroughly investigated. This study aims to determine the optimal zygote age and heat shock duration for inducing tetraploidy in platinum koi (*C. rubrofasciatus*), providing a basis for developing tetraploid broodstock for mass production.

## **2 Materials and methods**

### **2.1 Test materials**

The platinum variety of koi fish (*C. rubrofasciatus*) has an average weight of  $0.72\pm 0.04$  kg for males and  $0.9\pm 0.15$  kg for females. The mature gonad broodstock used came from breeders in the Bogor area, consisting of two pairs. Broodstock were acclimatized before the experiment and under what water quality conditions. The material used consisted of koi fish eggs resulting from artificial spawning of broodstock using ovaprim (GnRH + Anti Dopamine). Other materials used included ethanol solution, Carnoy's solution, 50% acetic acid, glacial acetic acid, gelatin, glycerin, and formic acid as fixatives and stains for observing the number of nucleoli.

### **2.2 Experimental design**

The study was designed using an experimental method in the form of a completely randomized design. The treatments in the study related to heat shock duration included: K1 (control for duration), A1 (heat shock at 39°C for 1.5 minutes with zygote age of 31 minutes after fertilization), A2 (heat shock at 39°C for 2 minutes with zygote age of 31 minutes after fertilization), and A3 (heat shock at 39°C for 2.5 minutes with zygote age of 31 minutes after fertilization). Meanwhile, the treatments in the study comparing zygote age were: K2 (control for zygote age), B1 (heat shock at 39°C for 1.5 minutes with zygote age of 27 minutes after fertilization), B2 (heat shock at 39°C for 1.5 minutes with zygote age of 29 minutes after fertilization), and B3 (heat shock at 39°C for 1.5 minutes with zygote age of 31 minutes after fertilization). This study applied a heat shock treatment at 39°C, based on conditions determined during preliminary trials

### **2.3 Spawning**

Spawning of the broodstock was carried out in a concrete tank measuring  $3\times 1.2\times 0.7$  m. The broodstock was induced using ovaprim (GnRH + Antidopamine). The ovaprim dosage administered was 0.2 ml/kg of body weight for males and 0.4 ml/kg for females. Eight hours after hormone induction, the broodstock were checked for ovulation and then stripped with dry method. The eggs and sperm obtained through stripping were fertilized artificially by mixing them in a container and adding sufficient water.

### **2.4 Heatshock treatments**

Before determining the appropriate zygote age for the heat shock treatment, observations of the first mitotic process were conducted using a microscope. This observation was carried out prior to the heat shock treatment by fertilizing the eggs with sperm, then recording the time required from fertilization to the first cell division from one cell to two cells. In platinum koi fish, the first cell division was observed to occur at 31 minutes after fertilization.

The fertilized eggs were then spread onto a glass which served as a substrate for the eggs to adhere to. Each treatment used approximately 656 fertilized eggs. After that, the eggs were placed on plates measuring  $10\text{ cm} \times 23\text{ cm}$ , and were placed into an incubation container in the form of a styrofoam box measuring  $75\text{ cm} \times 42\text{ cm} \times 32\text{ cm}$ , with a water volume of 78 liters. During the first cell division phase, the water temperature in the medium was maintained at 27°C. After this stage, the eggs were transferred to a styrofoam container filled with water at 39°C

to receive the heat shock treatment. The duration of the heat shock was precisely controlled using a stopwatch.

## 2.5 Hatching and larval rearing

Egg hatching was carried out in 12 tray-shaped containers measuring  $33 \times 25 \times 10$  cm, with a water depth of 8 cm, each equipped with aeration. The hatching process lasted for 24–48 hours at a water temperature ranging from 26–30°C. To prevent fungal infections, methylene blue was added to each hatching container at a dose of 0.3 mg/L. The stocking density of each treatment varies because it is adjusted to the eggs produced by the parents during spawning in each treatment.

Observations were conducted at several stages after fertilization. At the 6th hour, the fertilization rate was calculated by comparing the number of fertilized eggs to the total number of eggs observed. At the 24th hour, the embryo survival rate was determined by counting the number of surviving embryos relative to the total number of embryos observed. Then, at the 48th hour or after all the eggs had hatched, the hatching rate was calculated by comparing the number of hatched larvae to the total number of fertilized eggs.

The hatched larvae were then reared for 14 days. After the yolk sac was depleted on the third day, the larvae were fed *Artemia nauplii* for six days, with a feeding frequency of once every three hours. At nine days old, the larvae began receiving powdered feed (Feng-Li 0) from PT. Matahari Sakti company with *ad libitum* method. Siphoning was carried out every two days to remove 20–25% of the water, cleaning waste and uneaten feed.

## 2.6 Tetraploid identification

Tetraploid identification was carried out by counting the maximum number of nucleoli per cell. The process began with fixation of 2-week-old larval samples using Carnoy's solution at a volume ten times that of the sample, for a duration of 2 hours, with the solution being replaced with a fresh one at the 30-minute mark. After fixation, the larval samples were placed on tissue paper to remove any remaining fixative solution before being transferred to a new microtube. Cell dissociation was then performed by adding 1–2 drops of 50% acetic acid solution, followed by homogenization with a dropper pipette until a cell suspension was formed. The suspension was then dropped onto microscope slides that had been soaked in 70% alcohol for two hours and placed on a hotplate at 45–50 °C. Once a ring with a diameter of 1–1.5 cm formed, the suspension was aspirated back, and 2–3 rings were made on each slide, which were then air-dried.

The slide was then stained using solution A (10 g of  $\text{AgNO}_3$  dissolved in 20 mL of distilled water) and solution B (2 g of gelatin dissolved in 50 mL of water mixed with 50 mL of glycerin solution and added with 2 drops of formic acid per 10 mL). Two drops of solution A and one drop of solution B were placed on the slide, mixed, and spread using a toothpick. The slide was then placed in a staining box at a temperature of 40–45 °C for 16 minutes until it turned yellowish-brown. Afterward, the slide was rinsed with distilled water, air-dried, and observed under a microscope at 100× magnification to identify 2n and 4n chromosomes in the cells.

## 2.7 Analysis

All test data were collected and initially processed using Microsoft Excel 2016. Statistical analysis was performed using IBM SPSS Statistics 25.0, with differences among treatments evaluated by one-way ANOVA.

### 3 Result

#### 3.2 Growth performance

Based on the observations, the parameters of total eggs, fertilization rate, 24-hour embryo survival, hatching rate and 2-weeks larval survival are presented in Table 1 and Table 2.

**Table 1.** Parameters of total eggs, fertilization rate, 24-hour embryo survival, hatching rate and 2-weeks larval survival in the heat shock duration treatment

Treatments	Eggs	Survival rate (24 hours) (%)	Hatching rate (%)	Survival rate (2 weeks) (%)
K1	385	61.80	8.27	80
A1	656	71.63	17.69	86
A2	762	70.19	10.90	80
A3	747	71.67	11.47	81

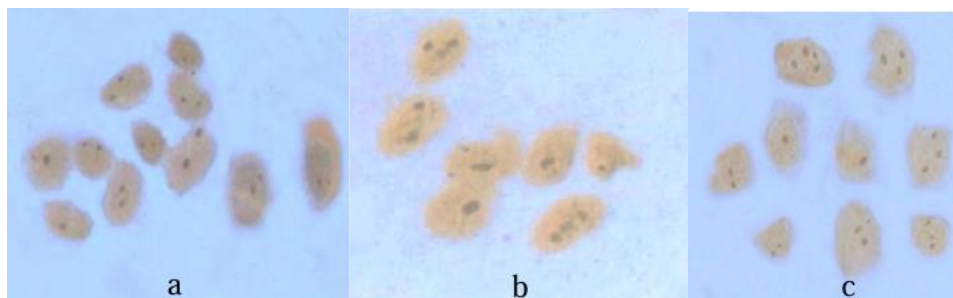
Control (K1): treatment without heat shock, A1: heat shock at 39°C for 1.5 minutes with zygote age of 31 minutes after fertilization, A2: heat shock at 39°C for 2 minutes with zygote age of 31 minutes after fertilization, A3: heat shock at 39°C for 2.5 minutes with zygote age of 31 minutes after fertilization.

**Table 2.** Parameters for total eggs, fertilization rate, 24-hour embryo survival, hatching rate, and 2- week larval survival in the zygote age treatment.

Treatments	Eggs	Survival rate (24 hours) (%)	Hatching rate (%)	Survival rate (2 weeks) (%)
K2	1870	79.82	24.44	78.51
B1	2303	78.37	39.71	77.32
B2	1718	77.97	32.99	78.64
B3	1699	76.40	32.91	81.00

Control (K): treatment without heat shock. B1: heat shock at 39 °C for 1.5 minutes with a zygote age of 27 minutes after fertilization; B2: heat shock at 39 °C for 1.5 minutes with a zygote age of 29 minutes after fertilization; B3: heat shock at 39 °C for 1.5 minutes with a zygote age of 31 minutes after fertilization.

The number of nucleoli per cell in koi fish fry resulting from tetraploidization treatment using the heat shock method is presented in Table 3 and Table 4, with images of the nucleoli preparations shown in Figure 1. Observations for each treatment were conducted by examining a sample of 120 cells. Cells observed to have 3-4 nucleoli were assumed to be tetraploid cells, while those with 1-2 nucleoli were assumed to be diploid cells.

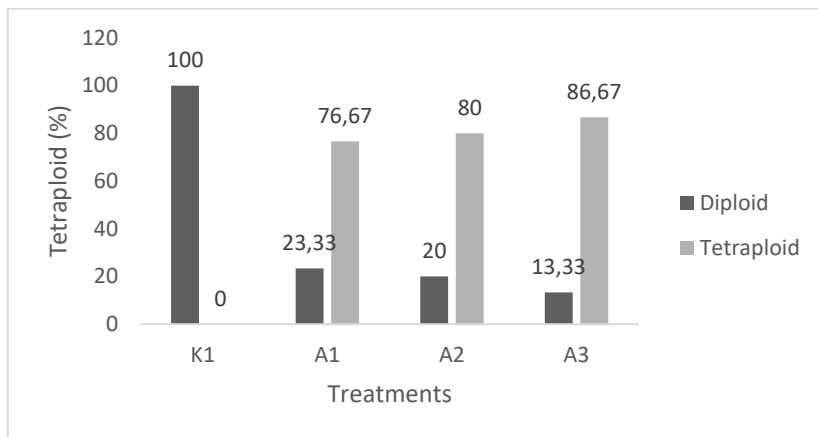


**Fig 1.** Diploid koi fish cells (*C. rubrofasciatus*) with a maximum of 2 stained nucleoli per cell (a), and tetraploid cells with a maximum of 3 (b) and 4 stained nucleoli per cell (c).

**Table 3.** Distribution of the number of nucleoli per cell in diploid and tetraploid koi fish (*C. rubrofuscus*) from 30 random samples in the heat shock duration treatment

Sample	Number of Nucleoli per Cell (%)				Ploidy Level
	1	2	3	4	
1	44 (36.7)	60 (50)	16 (13.3)	0 (0)	Tetraploid
2	38 (31.7)	72 (60)	10 (8.3)	0 (0)	Tetraploid
3	45 (38)	60 (50)	13 (10.3)	2 (1.7)	Tetraploid
4	39 (32)	71 (59.7)	10(8.3)	0 (0)	Tetraploid
5	50 (41.7)	65 (55)	5 (3.3)	0 (0)	Tetraploid
6	40 (33.3)	70 (58.4)	10 (8.3)	0 (0)	Tetraploid
7	55 (45.3)	65 (54.7)	0 (0)	0 (0)	Diploid
8	46 (38.3)	62 (51.7)	12 (10)	0 (0)	Tetraploid
9	35 (29.7)	70 (58.3)	15 (12)	0 (0)	Tetraploid
10	60 (50)	60 (50)	0 (0)	0 (0)	Diploid
11	42 (35)	62 (62)	16 (13)	0 (0)	Tetraploid
12	50 (41.7)	60 (50)	10 (8.3)	0 (0)	Tetraploid
13	38 (31.7)	65 (54)	17 (14.3)	0 (0)	Tetraploid
14	50 (41.7)	70 (58.3)	0 (0)	0 (0)	Diploid
15	45 (37)	65 (54.7)	10 (8.3)	0 (0)	Tetraploid
16	35 (29.7)	60 (50)	25 (20.3)	0 (0)	Tetraploid
17	53 (44.7)	58 (48.3)	9 (7.2)	0 (0)	Tetraploid
18	42 (35)	65 (54.7)	13 (10.3)	0 (0)	Tetraploid
19	40 (33.3)	64 (53.3)	14 (11.7)	2 (1.7)	Tetraploid
20	40 (33.3)	64 (53.3)	16 (13.4)	0 (0)	Tetraploid
21	55 (45.3)	60 (50)	5 (41.7)	0 (0)	Tetraploid
22	32 (26.7)	71 (60)	16 (13)	1 (0.8)	Tetraploid
23	35 (29.7)	70 (58.3)	15 (12)	0 (0)	Tetraploid
24	54 (45)	60(50)	6 (5)	0 (0)	Tetraploid
25	50 (41.7)	55 (45.3)	15 (13)	0 (0)	Tetraploid
26	45 (38)	68 (56.7)	7 (5.3)	0 (0)	Tetraploid
27	35 (29.7)	60 (50)	25 (20.3)	0 (0)	Tetraploid
28	47 (39.7)	63(52)	10 (8.3)	0 (0)	Tetraploid
29	50 (41.7)	60 (50)	10 (8.3)	0 (0)	Tetraploid
30	39 (33)	55 (45.3)	26 (21.7)	0 (0)	Tetraploid

The percentage of diploid and tetraploid larvae originating from eggs treated with different heat shock durations is shown in Figure 2. The highest number of tetraploid larvae was obtained in treatment A3, with a value of 86.66%, followed by treatment A2 at 80%, and treatment A1 at 76.67%.

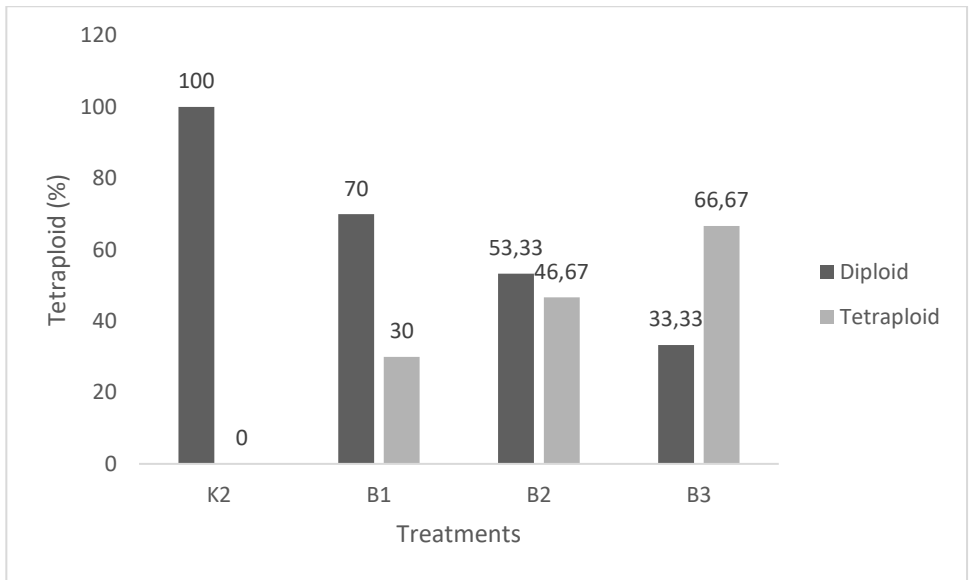


**Fig. 2.** Percentage of diploid and tetraploid koi larvae resulting from heat shock treatments with different durations.

**Table 4.** Distribution of the number of nucleoli per cell in diploid and tetraploid koi fish (*C. rubrofascus*) from 30 random samples in the zygote age treatment

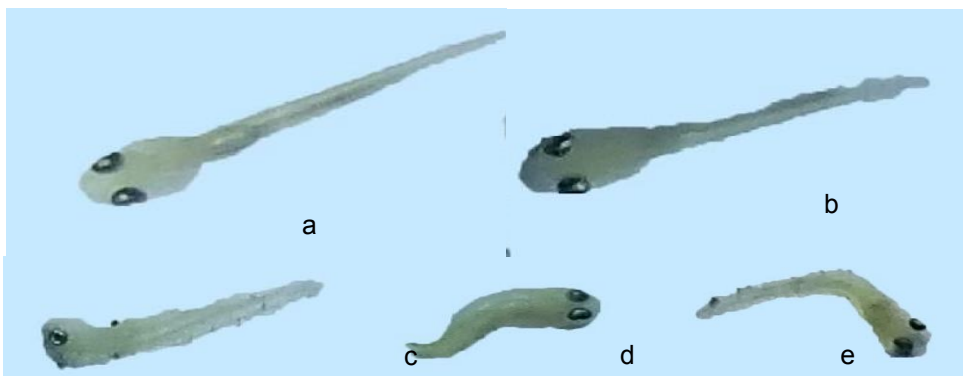
Sample	Number of Nucleoli per Cell (%)				Ploidy Level
	1	2	3	4	
1	38 (31.7)	82 (68.3)	0 (0)	0 (0)	Diploid
2	46 (38.3)	74 (61.7)	0 (0)	0 (0)	Diploid
3	12 (10)	86 (71.7)	22 (18.3)	0 (0)	Tetraploid
4	46 (38.3)	74 (61.7)	0 (0)	0 (0)	Diploid
5	13 (10.8)	78 (65)	29 (24.2)	1 (0.8)	Tetraploid
6	15 (12.5)	87 (72.5)	18 (15)	0 (0)	Tetraploid
7	26 (21.7)	82 (68.3)	10 (8.3)	2 (1.7)	Tetraploid
8	49 (40.8)	71 (59.2)	0 (0)	0 (0)	Diploid
9	41 (34.2)	69 (57.5)	10 (8.3)	0 (0)	Tetraploid
10	32 (26.7)	73 (60.8)	15 (12.5)	0 (0)	Tetraploid
11	43 (35.8)	77 (64.2)	0 (0)	0 (0)	Diploid
12	37 (30.8)	75 (62.5)	7 (5.8)	1 (0.8)	Tetraploid
13	27 (22.5)	81 (67.5)	12 (10)	0 (0)	Tetraploid
14	47 (39.2)	73 (60.8)	0 (0)	0 (0)	Diploid
15	37 (30.8)	71 (59.2)	12 (10)	0 (0)	Tetraploid
16	38 (31.7)	82 (68.3)	0 (0)	0 (0)	Diploid
17	34 (28.3)	69 (57.5)	17 (14.2)	0 (0)	Tetraploid
18	44 (36.7)	76 (63.3)	0 (0)	0 (0)	Diploid
19	50 (41.7)	70 (58.3)	0 (0)	0 (0)	Diploid
20	37 (30.8)	83 (69.2)	0 (0)	0 (0)	Diploid
21	46 (38.3)	74 (61.7)	0 (0)	0 (0)	Diploid
22	40 (33.3)	80 (66.7)	0 (0)	0 (0)	Diploid
23	37 (30.8)	65 (54.2)	18 (15)	0 (0)	Tetraploid
24	49 (40.8)	71 (59.2)	0 (0)	0 (0)	Diploid
25	45 (37.5)	75 (62.5)	0 (0)	0 (0)	Diploid
26	63 (52.5)	48 (4)	0 (0)	0 (0)	Diploid
27	39 (32.5)	81 (67.5)	0 (0)	0 (0)	Diploid
28	38 (31.7)	69 (57.5)	13 (10.8)	0 (0)	Tetraploid
29	26 (21.7)	82 (68.3)	12 (10)	0 (0)	Tetraploid
30	36 (30)	84 (70)	0 (0)	0 (0)	Diploid

The percentage of diploid and tetraploid larvae originating from eggs treated with heat shock at different zygote ages is shown in Figure 3. The highest number of tetraploid larvae was obtained in treatment B2 with a value of 6.08%, followed by treatment B3 at 5.37%, and treatment B1 at 3.69%.



**Fig 3.** Percentage of diploid and tetraploid koi larvae resulting from heat shock treatment at different zygote ages

Abnormal larvae found in the heat shock duration treatment were characterized by inactive movement, smaller size compared to normal fish, a bent growth shape resembling the letter L, and abnormal swimming behavior. In the zygote age treatment, spinal deformities such as scoliosis and kyphosis, as well as bent neck bones, were observed. The percentage of abnormalities in koi fish (*C. rubrofascus*) larvae is presented in Figures 5 and 6.



**Fig 4.** Normal larvae (a and b), larvae with abnormal curved neck bones (c), scoliosis (d), and kyphosis (e) resulting from the heat shock treatment.

## 4 Discussion

Based on Table 1, the highest total number of eggs was found in treatment A2 at 762, while the lowest was in treatment K1 at 385.33. Meanwhile, Table 2 shows that the highest total

number of eggs was observed in treatment B1 with 2303 eggs, and the lowest in treatment B3 with 1699 eggs. Fecundity is related to metabolism, which responds to changes in food availability and, in turn, affects egg development, including age, size, number of eggs, and spawning cycles [10].

The highest fertilization rate in the heat shock duration treatment was observed in treatment A3, at 62.15%, while the lowest was in treatment K1, at 42.96%. In the zygote age treatment, the fertilization rate was consistent across treatments K2 to B3, at 46.7%. The main factor influencing the fertilization success rate in these results is believed to be related to egg quality. This assumption is based on the fact that immature eggs can hinder the fertilization process, and egg maturation itself is influenced by hormonal activity. Fertilization rate can also be affected by broodstock factors such as the quality of male and female gametes, as well as handling factors like sperm mixing. Low fertilization rates may be caused by various factors, including the quality of eggs and sperm, broodstock maturity, and water quality [14].

Embryo survival during 24 hours in the heat shock duration treatments showed the highest survival rate in treatment A3 at 71.67%, while the lowest survival rate was found in treatment K1 at 61.80%. In the zygote age treatments, the highest survival rate was observed in treatment K2 at 79.82%, and the lowest in treatment B3 at 76.40%. The embryo mortality rate was higher in the heat shock treatments compared to the control. The high temperature during heat shock affected larval abnormalities, causing egg membrane hardening and inhibiting hatching enzyme activity [2]. The timing of cell division post-fertilization varies among fish species depending on light, yolk amount diversity, and incubation temperature [4]. Embryos that survived the heat shock process did so due to sub-lethal factors. Egg mortality was caused by various factors, including incomplete fertilization and eggs sticking or piling up during spreading, which can hinder oxygen circulation and ultimately lead to egg death [7].

The highest hatching rate for the heat shock duration treatment was found in treatment A1 at 17.69%, while the lowest was in treatment K1 at 8.27%. For the zygote age treatment, the highest hatching rate was in treatment B1 at 39.71%, and the lowest was in treatment K2 at 24.44%. The hatching rate in the control group was lower than all treatment groups, which was suspected to be due to technical errors during egg spreading, causing the eggs to be too dense and clumped (the eggs were first spread in the control, resulting in uneven distribution on the glass substrate). Factors affecting the hatching rate include the quality of the sperm and broodstock eggs, as well as the environmental conditions of the medium. Prolonged heat shock can damage the embryo membrane, causing issues in cell division such as loss of genetic information, spindle fiber damage, and disrupted enzyme activity [6]. This is consistent with the results showing that treatment A1 had a higher hatching rate compared to treatments A2 and A3.

Larval survival data over two weeks for the heat shock duration treatment showed the highest survival rate in treatment A1 at  $86 \pm 1.00\%$  and the lowest in treatment A2 at 80%. For the zygote age treatment, the highest two-week larval survival was observed in treatment B3 at 81.00%, while the lowest survival was in treatment B1 at 77.32%. Larval survival refers to the percentage of larvae that survive until harvest out of the total larvae reared. This survival rate is influenced by the quality of the eggs produced. Additionally, environmental factors also play a role in determining larval survival success, with temperature being one of the most influential aspects.

Based on Tables 3 and 4, the highest number of four nucleoli was found in fewer tetraploid larvae (0.1%), while the highest number of three nucleoli was found more frequently in tetraploid larvae (8%). However, the maximum number of three nucleoli can be considered tetraploid rather than triploid. This is because the treatment could not have produced triploids, as tetraploid induction disrupts the first cell division, whereas triploids result from the

retention of the second polar body. This is relates to anomalies observed in the variation of the maximum number of nucleoli [13].

Additionally, anomalies in the maximum number of nucleoli were also found in tetraploid catfish [11]. The probability of the maximum number of nucleoli per cell is suspected to be due to differences in ribosome synthesis activity responsible for protein production. Variation in the maximum number of nucleoli in each cell nucleus can be influenced by differences in cellular activity. The count of the maximum number of nucleoli was determined by the number of black dots (nucleoli) excluding impurities in each cell nucleus, which appeared yellowish-brown due to the staining process. Impurities can resemble nucleoli as black dots, so careful observation is needed to accurately count the number of nucleoli.

Based on the results of the tetraploid induction obtained, there is an indication that in fish resulting from tetraploid induction, embryos that received heat shock at a later zygote age tend to have lower tetraploid induction success. Each fish species has different timing for cell division after fertilization, which can be influenced by incubation temperature, species, and the maturity level of the broodstock gonads [9]. The timing of the first mitotic division is a key determinant for the success of heat shock, especially in producing tetraploid fish. The results from the heat shock duration treatment showed that the treatment with a heat shock duration of 2.5 minutes (A3) produced more tetraploids compared to durations of 1.5 and 2 minutes. Meanwhile, in the zygote age treatment, heat shock applied at 31 minutes after fertilization (B3) produced the highest number of tetraploid fish. This is consistent with observations showing that cell division in koi embryos occurs at 31 minutes post-fertilization.

Abnormalities in larvae with different heat shock durations were highest in treatment A3, followed by treatments A1, A2, and the control. The highest abnormality rate was observed in the heat shock treatment with the longest duration, at 10.33%. The lowest abnormality rate was in the control group at 4.33%. Meanwhile, in the zygote age treatment, the highest abnormal larvae rate was found in treatment B2 at 6.08%, and the lowest in treatment B1 at 3.69%. Heat shock applied during the embryonic development phase can damage the embryo membrane, cause the outer layer of the egg to harden, and inhibit the activity of hatching enzymes. This can lead to abnormalities in the larvae. Another form of larval abnormality caused by heat shock treatment is a short and curved tail.

## 5 Conclusion

Tetraploid induction in koi fish (*C. rubrofasciatus*) was influenced by both heat shock duration and the timing of treatment relative to zygote age. The highest proportion of tetraploids (86.6%) was achieved with a heat shock of 39 °C for 2.5 minutes, while treatment at 39 °C for 90 seconds also successfully induced tetraploidy, albeit at a lower rate. Regarding zygote age, applying heat shock at 31 minutes post-fertilization (mpf) resulted in 66.7% tetraploids, which was higher than other age treatments, indicating that the timing of first cleavage plays a critical role in inhibiting cytokinesis and promoting tetraploidy.

This study demonstrates that different experimental factors specifically heat shock duration and zygote age had distinct effects on the success of tetraploid induction. While variations in heat shock duration primarily determined the overall efficiency of chromosome doubling, differences in zygote age significantly influenced the developmental stage responsiveness to treatment. Thus, each factor contributed differently to the outcome, highlighting the need to optimize both duration and timing to maximize tetraploid yield.

These findings provide a practical reference for koi breeders aiming to produce tetraploid broodstock for triploid production or to enhance genetic resources in ornamental fish. However, some treatments showed larval abnormalities and reduced hatching rates, suggesting that further optimization and confirmation are necessary to refine the protocol.

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