

Article

# Efficient Artificial Fertilization and Ovulated Egg Preservation in Kawakawa *Euthynnus affinis*

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**Abstract:** Artificial fertilization of cultured fish is essential for seed production using breeding techniques. However, in tuna species, the success rate of artificial fertilization is tremendously low. In this study, it was reported that the adequate procedure for ovulated egg collection and storage for artificial fertilization in kawakawa *Euthynnus affinis*. The collection of ovulated eggs was attempted using new techniques that disrupt only spawning activity without discontinuing ovulation. The available time to use ovulated eggs was also examined by assessing the optimal preservation process and temperature. As a result, artificial fertilization was effectively executed by assessing spawning time and thoroughly extracting ovulated eggs immediately after ovulation, with a success rate of 70% and an ovulation rate of 51.7%. Ovulated eggs could be stored with small quantities of ovarian fluid to sustain fertility. However, fertility was better preserved with Hanks' solution. Ovulated eggs with high productivity were achieved 3 h after egg extraction when maintained in Hanks' solution at 20 °C, leading to a supply of one-cell stage embryo for microinjection treatment constantly by continuously executing artificial fertilization. This systematic procedure permitted selective breeding by 1:1 mating between top-quality parental fish and applying several developmental engineering techniques to kawakawa breeding.

**Keywords:** artificial insemination; breeding; eastern little tuna; egg storage; fertility; microinjection; ovulation cycle; spawning activity; tuna; unfertilized egg



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## 1. Introduction

Kawakawa *Euthynnus affinis* (Scombridae, Thunnini) is a marine fish which is distributed extensively in the tropical and subtropical waters of the Indo-Pacific region [1]. Kawakawa, a related species of the bluefin tuna *Thunnus orientalis*, is studied not only as an investigative model for the bluefin tuna due to its small size, but because of its top-quality meat as a tuna. Breeding exploration is now being performed to cultivate fish of excellent quality with quick growth due to their economic value. Recently, the databank of chromosome-level genome assemblage [2] and technology of genome editing using TALEN [3] has been used to innovate kawakawa breeding. After gathering genomic data and genetically superior lines, crosses between specific parental fish are facilitated by artificial fertilization.

Artificial fertilization is a technique in which the eggs and sperm are inseminated in vitro. One significant merit of artificial fertilization is that it permits 1:1 mating between

superior individuals so that selective breeding can proceed effectively. Additionally, cryopreserved sperm can also be used for seed production, resulting in the effective utilization of genetic resources [4]. The second benefit is that a large number of embryos can be produced with harmonized developmental stages. This implies that embryos can be made available for microinjection treatment immediately after fertilization, which involves injecting regents by the one-cell stage. Microinjection is now a critical technology for genome editing, management of gene expression by morpholino antisense oligo (MO), and cell visualization [5].

Furthermore, breeding techniques such as the chromosome (ploidy) manipulation and interspecific hybridization have become feasible through successful artificial fertilization [6–8]. With the microinjection of MO against germ-cell-related genes, triploidization by chromosome manipulation and interspecific hybridization are efficient tools for sterilization [9]. The sterile individual possesses a double status of being the cultured fish itself [10] and the recipient for surrogate propagation [9]. As explained above, the creation of the artificial fertilization technology will be a profitable endeavor in the area of selective breeding and developmental engineering techniques in kawakawa breeding. However, there are no reports of a method for artificial fertilization in kawakawa. In addition, the success rate of artificial fertilization in bluefin tuna, which is the same tuna species as kawakawa, is tremendously low. This is because artificial fertilization was performed using ovulated eggs from commercially harvested females that happen to be ovulating [11].

Efficient artificial fertilization involves rearing of the fish to sexual maturation and collecting ovulated eggs and sperm while they are fertile. The timing of ovulation and the quality of the eggs must be given special consideration to sustain the fertility of ovulated eggs. In most fish, the eggs are released into the ovarian cavity or body cavity at the instance of ovulation and are kept in the ovarian or coelomic fluid, respectively. Numerous studies have examined the association between the time after release of eggs and developmental capability through constant artificial fertilization using ovulated eggs collected from the same individual. Earlier studies have reported that fertility decreases with time after ovulation when ovulated eggs are kept in the ovarian (body) cavity [12–21]. It was suggested that the productiveness of the ovulated eggs is affected by the immediate surroundings in the ovarian cavity or body cavity [22]. The immediate environment is a function of the quality of ovarian fluid or coelomic fluid. In the barfin flounder *Verasper moseri* [12], the spotted halibut *Verasper variegatus* [19], and the coho salmon *Oncorhynchus kisutch* [23], the fertility of ovulated eggs decreased because of over-ripening or broken eggs in the ovarian cavity or body cavity. It was also reported that time after ovulation is not only influenced on the fertility also for presence of larvae with abnormal appearance [24]. To avert the reduction of egg quality after ovulation, several studies have examined the external storage of ovulated eggs in an artificial medium [22,25–28]. In salmonid species, the temporary maintenance of ovulated eggs with Cortland medium enhanced the fertilization capacity when compared with eggs surrounded by the natural ovarian fluid [22,28]. Therefore, replacing the ovarian (coelomic) fluid with an artificial medium is demonstrated to be an alternative to avert the decline in egg quality. The benefit of washing or preserving eggs in an artificial medium before artificial fertilization has been reported to mitigate constraints such as cytoplasm from broken eggs in the ovarian (coelomic) fluids upon fertilization [22].

Currently, there is no information regarding the use of artificial fertilization and external storage of ovulated eggs of kawakawa, although a complete life cycle culture comprising spawning induction by hormonal techniques and cultivation in a land-based tank has been designed for kawakawa [8,29]. Notwithstanding, spawning induction by hormonal treatments and natural spawning confers numerous problems to be addressed to efficiently proceed with kawakawa breeding. The first drawback of hormonal treatment is that damage from hormone usage can destroy the parental fish before spawning [8]. Moreover, identifying the parental fish involved in spawning from a population of spawning candidate fish is expensive and laborious because 1:1 mating is not feasible. It is time-consuming to collect the fertilized eggs that are released into the tank or sea cages, making

it strenuous to provide opportunity for the microinjection procedures. The introduction of artificial fertilization into kawakawa seed production could successfully mitigate these problems. Furthermore, it has been published that ovulated eggs for artificial fertilization are collected based on the ovulation cycle in the bluefin tuna [11], barfin flounder [12], and zebrafish *Danio rerio* [25]. If a similar method is developed in kawakawa, ovulated eggs could be collected without damage to the parent fish because there was no hormonal injection, resulting in systematic artificial fertilization.

Furthermore, when investigating the relationship between time after ovulation and fertilization rate in kawakawa, the method used in previous studies, which involved repeated confirmation of ovulation and ovulated egg collection, is thought to be not suitable due to the extreme sensitivity of kawakawa to handling. Therefore, it is critical to destroy the fish after ovulation and collect ovulated eggs with excellent quality and use the ovulated eggs for an extended duration. Furthermore, in a preliminary experiment with kawakawa in 2020, the fertilization rate of the ovulated eggs when soaked and preserved in the Hanks' solution for 5 h was more efficient than that of the control preserved without any solution. Therefore, it is expedient to investigate the preservation constraints of the ovulated eggs including the efficiency of the artificial medium. The artificial fertilization procedure reported in the present study was according to the ovarian cycle without hormonal treatment in the kawakawa *Euthynnus affinis*. The ovulated eggs with excellent egg quality were methodically obtained by a new technique that disrupts spawning activity without interrupting ovulation. Furthermore, we examined the optimal solution and temperature situation that permitted extended preservation of kawakawa ovulated eggs with high fertilization reproductive capacity. Additionally, we demonstrated allowable preservation time for ovulated eggs, which can offer viable progeny after ovulated egg preservation. This research is expected to permit the usage of different breeding techniques for kawakawa seed production.

## 2. Materials and Methods

### 2.1. Ethics

This research was executed following the regulations of the South Ehime Fisheries Research Center, Ehime University for the use and care of experimental animals.

### 2.2. Fish and Sampling Information

Kawakawa, which were cultivated in a floating sea cage (10 m wide × 10 m long × 8 m deep) at the South Ehime Fisheries Research Center, Ehime University, were utilized in this research. The fish comprise 45 specimens of 2-year-old fish produced by artificial seed production in 2019 and two specimens of 3-year-old fish and one specimen of 4-year-old fish caught from a wild population in 2018 and 2017, respectively. Ten artificial fertilization assessments were performed between 4 August 2021, and 14 September 2021. Thirteen ovulated females were 2-year-old fish with a 49.7–56.4 cm fork length and a body weight of 2117–3164 g (Table S1). Fifteen males were 2- or 3-year-old fish with a fork length of 50.6–56.9 cm and body weight of 2147–3846 g (Table S2). During the experiment period, the range of water temperature at 1 m, 5 m, and 10 m depth was 24.6–29.3 °C, 25.1–28.1 °C, and 24.4–27.6 °C, respectively (Figure S1) [30].

### 2.3. Gamete Collection and Artificial Fertilization

Spawning activity was monitored in the sea cage to comprehend natural spawning time. After that, the fertilized eggs were collected from the sea cage using a soft net as described previously [5]. The eggs were observed using a stereo microscope (SZ61, Olympus, Tokyo, Japan). The natural spawning time in the sea cage was evaluated by the developmental stage of the embryos according to the stage of embryonic development at varying water temperatures in kawakawa [31]. Since it was empirically known that not only kawakawa female individuals which were induced by hormonal treatment [8], but also those which were reared in the sea cages spawn in the same time periods on successive

days, it is necessary to examine the spawning time on the day before the experiment. When multiple females spawned in the same sea cage at different times, the spawning time of the main spawners was identified by the number of collected fertilized eggs and adopted as the target spawning group for our experiment. On the day of the artificial fertilization test, the fish were caught and transferred from the rearing sea cage (10 m wide × 10 m long × 8 m deep) to a small sea cage (5 m wide × 5 m long × 4 m deep) about 2 h before the envisaged spawning time of the main spawners. The spawning activity in the smaller sea cage was disrupted due to the stress associated with catching and the small sea cage environment. Therefore, the females who were expected to spawn on that day had completed their final oocyte maturation and ovulation by the predicted spawning time in the small sea cage. However, the ovulated eggs were still held in the ovarian cavity because there was spawning activity. About 30 min after the envisaged spawning time, the fish were caught with a landing net, sacrificed by vertebral amputation, allowed to bleed in seawater, and kept on slurry ice for 15 min during relocation from the sea cage to the laboratory. The fish were dissected immediately. The gonads were carefully isolated from the abdominal cavity. Ovary removal was performed with a holding clamp in front of the genital pore to avert the exit of ovulated eggs. The exterior of the ovary was extensively cleaned with a paper towel to prevent contamination of the cavity fluid and blood. The ovulated eggs were flowed out spontaneously into a dry plastic vessel by unclamping the genital pore. The surface of the testis was thoroughly wiped out while the posterior end of the sperm duct was excised. After that, the semen was collected using Microman E (Gilson, France) by pressing the testis gently. Motility, concentration, and velocity of spermatozoa were not measured.

Then, after mixing the same proportion of semen obtained from 2–3 individuals, the sperm were diluted 100-fold with Hanks' Balanced Salt Solution (+) (Hanks' solution; Nacalai Tesque, Inc., Kyoto, Japan) and preserved at 10 °C. Finally, artificial fertilization was executed using the dry method of fertilization. First, 0.15–0.2 g (approximately 350 to 500) of each fish's ovulated eggs with its ovarian fluid was smeared on the bottom of a 420 mL plastic cup and then inseminated with 100 µL of diluted sperm. Next, about 50 mL of filtered seawater (25 °C) was added to activate the sperm. After 5 min of activation, 200 mL of filtered sea water was introduced for incubation. Then, cross combinations for artificial fertilization were performed, as shown in Table S2.

#### 2.4. Evaluation of Optimum Preservation Solution

The Hanks' solution (Nacalai Tesque), Leibovitz's L-15 medium (L-15 medium; Life Technologies, Carlsbad, CA, USA), and Ringer's solution for marine teleost (Ringer's solution) [32] were used to preserve the ovulated eggs. Ringer's solution is a similar composition to Hanks' solution, which was found to be capable of maintaining ovulated eggs in our preliminary experiment, expect for not containing glucose. L-15 medium, like Hanks' solution, is a medium used for cell culture and contains galactose and L-glutamine instead of glucose. Approximately 25 mL of each preservative was introduced into 5 mL (approximately 12,500) of the ovulated eggs and kept in a 50 mL conical tube at 20 °C. For the control group, the ovulated eggs were kept in 50 mL tubes without preservatives at 20 °C. The preservation time was started from the moment each solution was added to the ovulated eggs. The artificial fertilization was carried out immediately after introducing each preservation solution (0 h preservation) and after 1 h, 3 h, 5 h, and 12 h preservation. After that, the fertilized eggs from all test groups were incubated at 25 °C. To ascertain the developmental capacity of each experimental category, the fertilization rate, hatching rate, and normal developed larvae rate (normal rate) were calculated. The fertilization rate was calculated as the number of cleaved eggs comparative with the number of total buoyant eggs at the 8–512 cell stage. A fraction of the fertilized eggs were relocated from the plastic cup to a 6-well plate (AGC TECHNO GLASS Co., Ltd., Shizuoka, Japan) and incubated until the hatching stage. At 24–30 h post-fertilization, the hatching rate and normal rate were calculated as the number of hatching larvae or normal appearance larvae,

respectively, compared with the number of cleaved eggs that were incubated in the 6-well plate. Hatched larvae that did not demonstrate aberrations, such as a curved body axis or edema, were referred to as demonstrating normal appearance. The ovulated eggs were monitored in different solutions at different preservation times using a stereo microscope (SZ61, Olympus). The observed eggs were categorized as “clear egg” if transparent and unclouded, “over-ripened egg” if partially stained with alterations in internal structure, and “broken egg” if possessing distorted internal structure and clouding to ascertain the preservation ability of each solution.

### 2.5. Evaluation of Optimum Preservation Temperature

The preservation efficiency of the ovulated eggs was ascertained at six different temperatures (5, 10, 15, 20, 25, and 30 °C). The lower limit of the temperature test was set at 5°C to determine if the ovulated eggs could be maintained refrigerated. The higher temperature was set to 30 °C, which is above the water temperature during the spawning period. Additionally, the preservation efficacy of the ovulated eggs containing Hanks’ solution was compared with the control group (no additional egg preservation solution). Artificial fertilization was executed immediately after introducing Hanks’ solution (0 h preservation) and after 1 h, 3 h, and 5 h preservation. After artificial fertilization, the fertilized eggs from all preservation condition groups were cultured in a plastic cup at 25 °C until the hatching stage. The fertilization rate was calculated as the number of cleaved eggs compared with the total number of eggs with no distinction between buoyant and non-buoyant eggs at the 8–512 cell stage. Buoyant eggs are generally floating and still living, while non-buoyant eggs are sinking and already dead. The hatching rate was calculated as the number of hatching larvae compared with the number of total eggs, including unfertilized eggs and non-buoyant eggs.

### 2.6. Statistical Analysis

The data for developmental capacity in evaluating optimum preservation solution and temperature were analyzed by the Steel–Dwass test ( $p < 0.05$ ).

## 3. Results

### 3.1. Success of Artificial Fertilization

The ovulated eggs were obtained from 13 females in seven out of ten trials (Table S1). The number of fish utilized and the proportion of ovulated females for each experimental date are presented in Table 1. On 4 August, 6 August, 2 September, and 7 September, all-female samples were ovulated, and the ovulation rate was 100%. In contrast, the trials from 11 August to 25 August resulted in low ovulation rates. In the Misho area, where the sea cages of kawakawa were found, precipitation of 40 mm or more per day was recorded seven times between 8 August and 25 August (Table S3) [33]. On 14 September, the ovulation rate was low, while 13 mm of precipitation per day and zero daylight hours were recorded.

**Table 1.** The ovulation rate of females in a series of artificial fertilization trials.

Trial	Date	No. of Fish Used			Ovulation Female	Ovulation Rate * (%)
		Total	Male	Female		
1	4 August 2021	6	3	3	3	100.0
2	6 August 2021	5	2	3	3	100.0
3	11 August 2021	5	3	2	0	0.0 **
4	16 August 2021	5	2	3	0	0.0 **
5	23 August 2021	6	3	3	1	33.3 **
6	25 August 2021	3	1	2	0	0.0 **
7	2 September 2021	5	3	2	2	100.0

**Table 1.** *Cont.*

Trial	Date	No. of Fish Used			Ovulation Female	Ovulation Rate * (%)
		Total	Male	Female		
8	7 September 2021	4	2	2	2	100.0
9	10 September 2021	4	2	2	1	50.0
10	14 September 2021	5	2	3	1	33.3
	Total	48	23	25	13	51.7

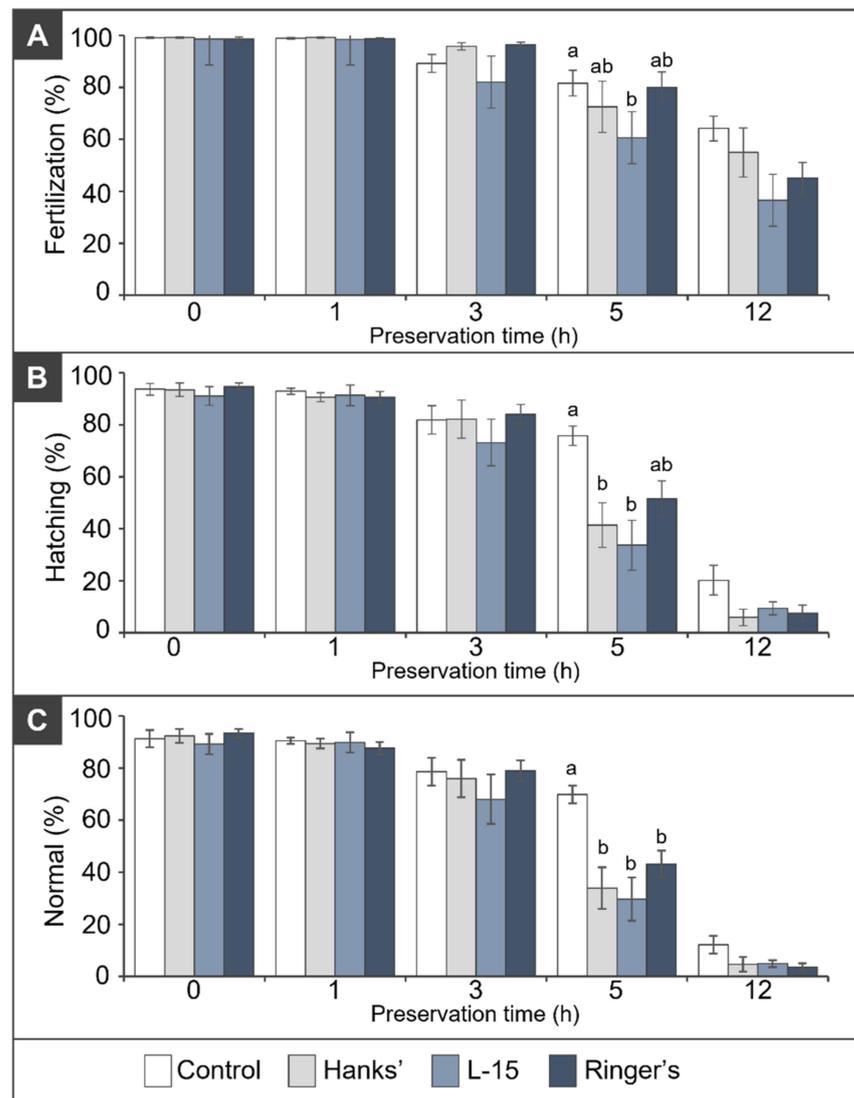
\* The ovulation rate was calculated as the number of ovulating females compared to the total number of females used. \*\* The period from 11 August to 25 August, when the ovulation rate was low, was marked by frequent days of heavy rainfall. Precipitation of more than 40 mm per day was recorded on 8 August, 11 August to 13 August, and 18 August to 20 August.

### 3.2. Evaluation of Optimum Preservation Solution

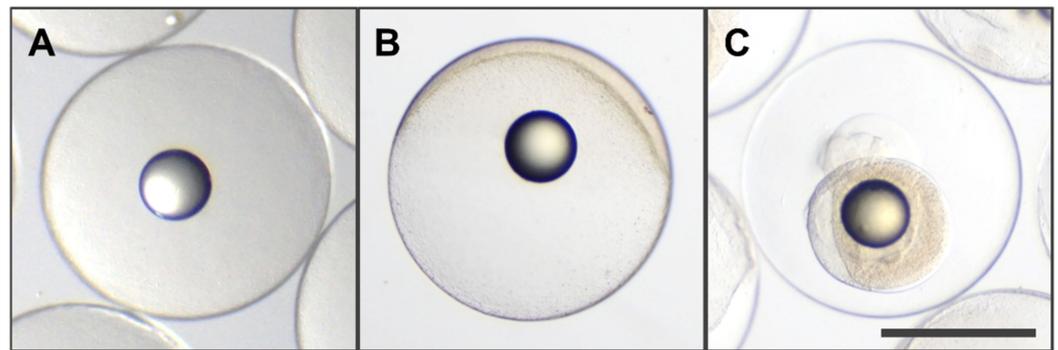
To examine the optimal egg preservation solution, comparisons between preservation efficacy in the three different solutions and the control group were performed 12 times using ovulated eggs from six females. The developmental efficiency after preservation in the various solutions was presented in Figure 1 and Table S4. The developmental capacity of all test groups was comparable to the control until 1 h of preservation. At 3 h of preservation, the fertilization rate and hatching rate of the eggs preserved in Hanks' and Ringer's solutions were higher than in the control and L-15 medium (Figure 1A,B). At 5 h preservation, the fertilization rate in the L-15 medium preservation group was significantly lower than that of the control group. In contrast, the ovulated eggs preserved in Hanks' and Ringer's solutions as well as those in the control group had a sustained fertilization ability. The hatching rates of the eggs preserved in Hanks' solution and L-15 medium were significantly lower than those in control and Ringer's solution. The normal rates of eggs kept in the Hanks' solution, L-15 medium, and Ringer's solution groups significantly decreased compared to that of the control group (Figure 1C). After 12 h of egg preservation for artificial fertilization, fertilized eggs and normal hatching larvae were slightly derived in all test groups. Since there was no significant difference between the preservation group of Hanks' and Ringer's solution compared to developmental capacity, we eventually compared the condition of the ovulated eggs during preservation.

Furthermore, the ovulated eggs after 1, 3, 5, and 12 h preservation were categorized into three groups: clear egg, which retained the ability to fertilize (Figure 2A); over-ripened egg (Figure 2B); and broken egg (Figure 2C). The latter two lost their fertility.

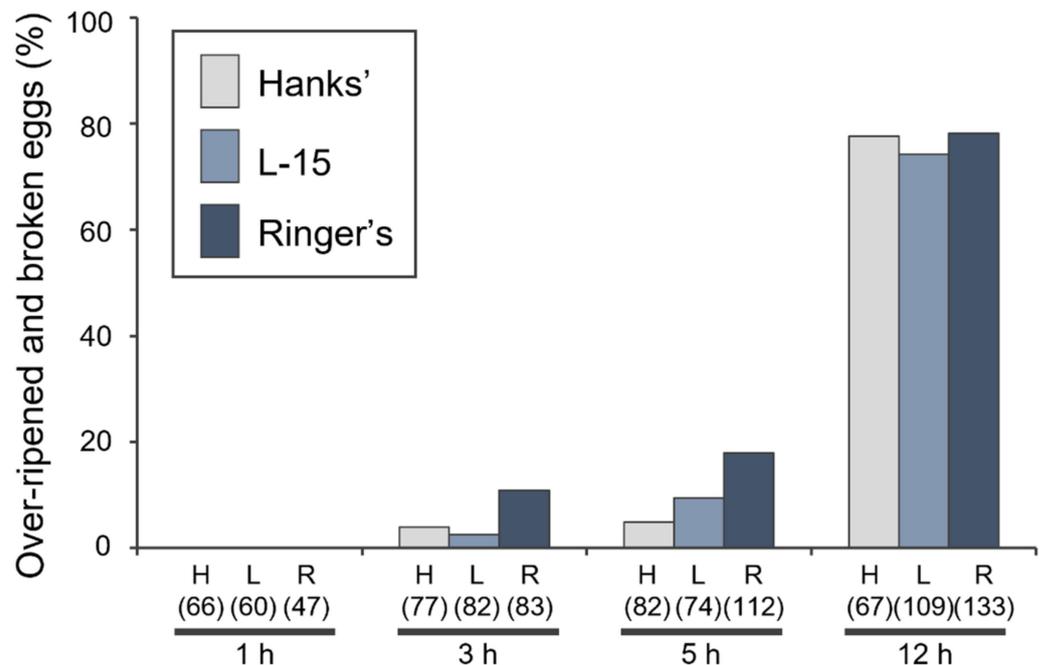
The rates of over-ripened and broken eggs during preservation were estimated and presented in Figure 3. We could not estimate over-ripened and broken eggs in the control group because observing the ovulated eggs of the control group under the same conditions as in each solution was cumbersome. No over-ripened and broken eggs were noticed until 1 h preservation in all categories (Figure 3 and Figure S2A1–C1). The number of over-ripened and broken eggs rose from 3 h of preservation in each solution group (Figure S2A2–C2). The over-ripened and broken egg rates in the Ringer's solution at 3 h and 5 h preservation (10.8% and 17.9%, respectively) were more significant than the other solution groups (Figure 3). We also noticed clear eggs in all test groups after 12 h of preservation. From observation of the ovulated eggs condition, we can conclude that preservation in Hanks' solution was more appropriate than in Ringer's solution because the growth of over-ripened and broken eggs began from an early preservation time in the Ringer's solution group.



**Figure 1.** Developmental efficiency after preservation of ovulated eggs of kawakawa *Euthynnus affinis* in different solutions. (A) Fertilization rate, (B) hatching rate and (C) normal developed larvae rate (normal rate) after preservation of ovulated eggs in Hanks' solution, L-15 medium and Ringer's solution, and without artificial medium (control) for 0, 1, 3, 5, and 12 h at 20 °C. Different superscript letters indicate significant differences determined by the Steel–Dwass test ( $p < 0.05$ ). There is a significant difference between “a” and “b”. There is no significant difference between “a” and “ab” or “b” and “ab”.



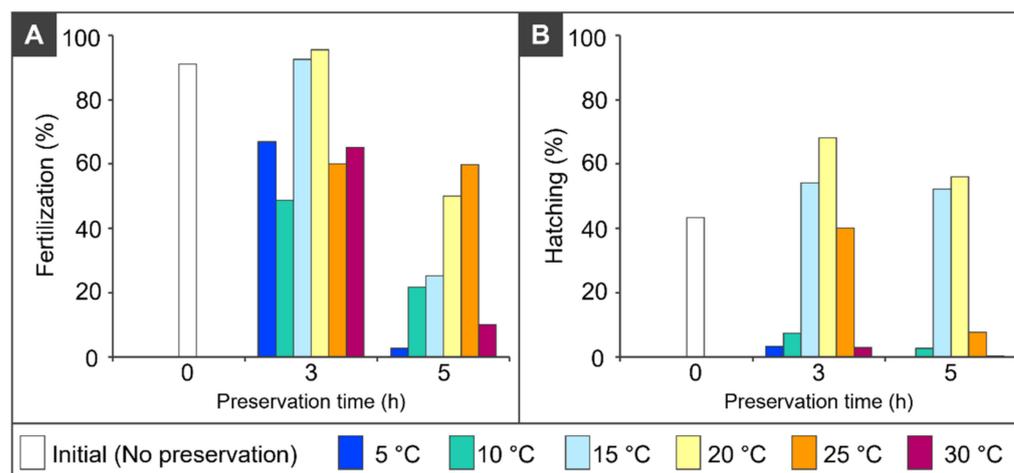
**Figure 2.** Ovulated eggs of kawakawa *Euthynnus affinis* are classified into three types by their condition after preservation. (A) “Clear egg” was transparent and unclouded, which was thought to retain the ability to fertilize; (B) “over-ripened egg” had partial discoloration and alterations in internal structure; and (C) “broken egg” had distorted internal structure and was clouded. “Over-ripened egg” and “broken egg” were thought to have lost their fertility. Scale bar denotes 500  $\mu\text{m}$ .



**Figure 3.** The rates of over-ripened and broken eggs of kawakawa *Euthynnus affinis* after preservation in different solutions. The rates of over-ripened and broken eggs during preservation were calculated after preservation of ovulated eggs in Hanks’ solution, L-15 medium, and Ringer’s solution for 0, 1, 3, 5, and 12 h at 20 °C. The number of analyzed ovulated eggs is in brackets. Statistical analysis could not be carried out because each value is the total number of ovulated eggs from one female.

### 3.3. Evaluation of Optimum Preservation Temperature

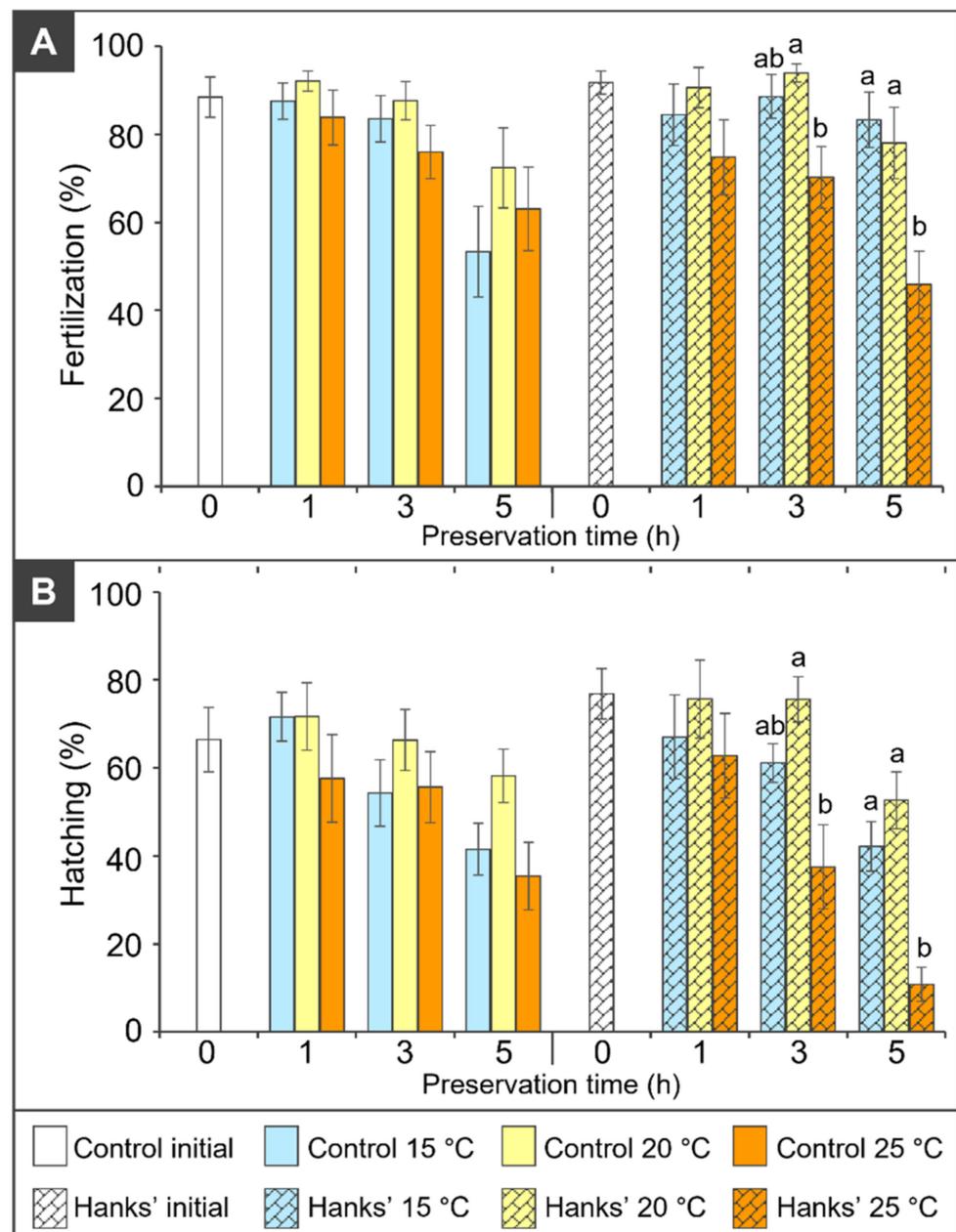
First, we compare the developmental capacities between preservation temperatures at 5 °C to 30 °C. The fertilization rate was less than 10% after 5 h of preservation at 5 °C and 30 °C, although it was more than 60% after 3 h of preservation (Figure 4A). On the other hand, the hatching rate was tremendously low in the preservation groups of more than 3 h at 5 °C, 10 °C, and 30 °C (Figure 4B). Therefore, it was decided to reproduce the experimental groups at 15 °C, 20 °C, and 25 °C eleven times using the ovulated eggs from eleven females to examine the optimum temperature for ovulated egg preservation.



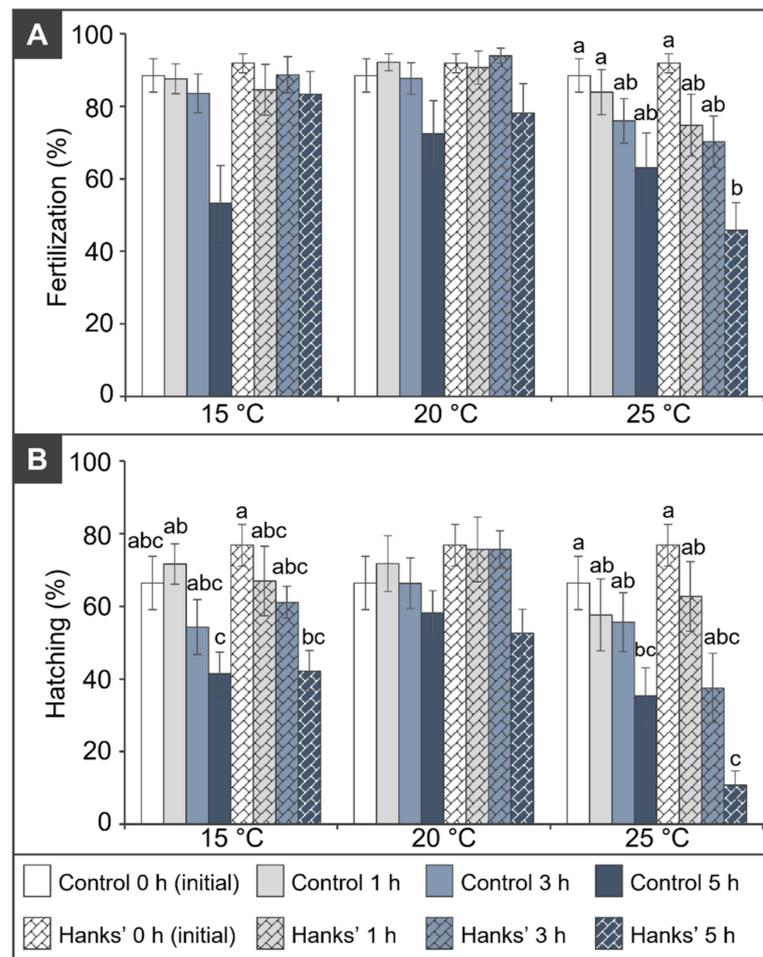
**Figure 4.** Developmental capacity after preservation of ovulated eggs of kawakawa *Euthynnus affinis* in different temperatures. (A) Fertilization rate and (B) hatching rate after the preservation of ovulated eggs without artificial medium for 0, 3, and 5 h at 5 °C, 10 °C, 15 °C, 20 °C, 25 °C, and 30 °C. Statistical analysis could not be carried out because each value is the mean of the duplicate using ovulated eggs from two females.

The developmental capacities after preservation in different temperatures as shown in Table S5. To analyze which preservation temperature was best, we first compared the developmental capacity between the varying preservation temperature categories at each preservation time point. The fertilization and hatching rates of the preservation at 20 °C groups were higher than those of the other preservation temperature categories, except for the fertilization rate of the 5 h preservation in Hanks' solution (Figure 5A,B). The fertilization and hatching rates of the preservation in Hanks' solution at 25 °C were significantly less than those of the preservation at 15 °C or 20 °C. The temperature range of 15 °C to 20 °C was discovered to be the suitable temperature for preserving kawakawa ovulated eggs.

To analyze whether preservation with the Hanks' solution was better than the control, we compared the developmental capacities between the different preservation times (0 h to 5 h) of each group at each preservation temperature. High fertilization rates were sustained up to 3 h after preservation at 15 °C and 20 °C, regardless of the control and the Hanks' solution groups (Figure 6A). Following 3 h and 5 h of preservation in Hanks' solution at 15 °C and 20 °C, the fertilization rates were greater than those of the control group. In contrast, the fertilization rate in the control groups progressively dwindled from 1 h to 5 h preservation. After 5 h of preservation in Hanks' solution at 25 °C, the fertilization rate was significantly lower than those of the initial (no preservation) control and Hanks' solution. In the 20 °C preservation group, there was no significant variance between the initials and all preservation categories in hatching rates of the control and Hanks' solution (Figure 6B). The hatching rates after 1 h and 3 h of preservation in Hanks' solution at 20 °C were greater than those of the control group. In the groups preserved at 15 °C and 25 °C, the hatching rate of both the control and Hanks' solution after 5 h preservation was significantly lower than that of the Hanks' initial. These outcomes signified that preserving kawakawa ovulated eggs in Hanks' solution was more suitable than the control, and the analysis further reinforced that preservation at 20 °C was appropriate.



**Figure 5.** Developmental capacity after preservation of ovulated eggs of kawakawa *Euthynnus affinis* in different temperatures when compared between 15 °C, 20 °C, and 25 °C. (A) Fertilization rate and (B) hatching rate after the preservation of ovulated eggs in Hanks' solution and without artificial medium (control) for 0, 1, 3, and 5 h at 15 °C, 20 °C, and 25 °C. Different superscript letters indicate significant differences determined by the Steel–Dwass test ( $p < 0.05$ ).



**Figure 6.** Developmental capacity after preservation of ovulated eggs of kawakawa *Euthynnus affinis* in different temperatures when compared between Hanks’ solution and control groups. (A) Fertilization rate and (B) hatching rate after the preservation of ovulated eggs in Hanks’ solution and without artificial medium (control) for 0, 1, 3, and 5 h at 15 °C, 20 °C, and 25 °C. Different superscript letters indicate significant differences determined by the Steel–Dwass test ( $p < 0.05$ ).

#### 4. Discussion

##### 4.1. Success of Systematic Artificial Fertilization

In this research, we have recognized two techniques that can (1) systematically obtain ovulated eggs with excellent quality and (2) preserve ovulated eggs with optimal conditions in kawakawa *Euthynnus affinis*. Ovulated eggs were effectively obtained by relocating parent fish to a smaller sea cage before examining spawning time and disrupting only spawning activity without interrupting ovulation. Seven out of ten trials of adequate artificial fertilization, including four trials with a 100% ovulation rate, were performed. In earlier reports regarding the pacific bluefin tuna, the frequency of acquirement in ovulated females was 0.59% (15 of 2554 fish examined) and 1.57% (60 of 3817 fish examined) in 2011 and 2012, respectively [11]. This frequency was calculated as the number of ovulated females relative to the total number of fish sampled without consideration between males and females. In this research, the frequency of ovulated females in the kawakawa, calculated using the same formula, was 27.08% (13 of the total 48 used). Our planned procedure for acquiring ovulated eggs was more efficient than the previous study in pacific bluefin tuna. The spawning induction of kawakawa by hormonal treatment was reported as seed production in a land-based tank [8,34]. To the best of our knowledge, this study is the first report of systematic artificial fertilization in kawakawa belonging to the tribe *Thunnini*.

In many fish species which have a seasonal spawning reproductive type, maturation and spawning are regulated by natural environmental constraints such as photoperiod and water temperature [35,36]. Therefore, much research has been conducted on spawning control by manipulating rearing conditions in cultured fish to establish effective seed production. For example, photoperiod manipulation has been used to advance the spawning season in the yellowtail *Seriola quinqueradiata* [37] and delay sexual maturation in the European seabass *Dicentrarchus labrax* [38]. Additionally, both water temperature manipulation and short-day treatment repressed spawning even after entering the spawning period in the Japanese parrotfish *Oplegnathus fasciatus* [39]. In this research, kawakawa spawned energetically in sea cages during the experimental period from 4 August to 14 September, when water temperature rose beyond 25 °C. On the other hand, there were several periods when no ovulated eggs for artificial fertilization were obtained after dissection and gonad extraction. These moments coincided with the period of heavy precipitation in the region, where the sea cages of kawakawa were located, so that the temporal disruption of the ovulation cycle after heavy rainfall was observed, resulting in no spawning. Furthermore, there was a sharp drop in water temperature around sea cages with increasing precipitation (Figure S1). This could have caused disruption of the ovulation cycle and spawning activity. A similar phenomenon was found in the asp *Aspius aspius* [40], the common bream *Abramis brama* [41], and the dace *Leuciscus leuciscus* [42] after rapid fluctuations in water temperature. Incongruent fluctuations in water temperature during the spawning season were considered to inhibit the expression of genes responsible for hormone secretion that influence final gamete maturation and ovulation [43,44]. The relationship between environmental factors (e.g., precipitation, water temperature and salinity) and interruption of spawning in sea cages should be elucidated in the prospective studies because heavy rainfall may impart stress on cultured kawakawa. However, for practical use, we could improve the effectiveness of systematic artificial fertilization by confirming spawning activity and spawning time daily before artificial fertilization trials.

#### 4.2. Evaluation of Optimum Preservation Solution and Temperature

The preservation of ovulated eggs in different culture conditions was conducted in the present study to ascertain the optimal temperature and culture medium for extending the fertility of ovulated eggs and to increase the number of embryos for microinjection techniques, which was possible for one-cell stage embryos. In earlier studies, the fertilization rate decreased with time after ovulation [12–21]. Even if the fertilization rate is high, the survival of embryos or the hatching rates are greatly influenced by the time of preservation of ovulated eggs. The hatching rate dropped to about 10% in the yellowtail, although the fertilization rate 12 h post-ovulation was kept above 70% [18]. In the present research, the fertilization and hatching rates after 3 h of preservation of ovulated eggs were sustained at 70% or higher when incubated in Hanks' solution at 20 °C. This indicates that artificial fertilization can obtain viable offspring after 3 h of preservation of the ovulated eggs of the kawakawa.

The optimal temperature for storing the kawakawa ovulated eggs was concluded to be 20 °C. On the other hand, for ovulated eggs preserved at 5 °C, 10 °C, and 30 °C, the hatching abilities were quickly reduced until 3 h of preservation. We have evaluated the temperature of the ovary surface of some ovulated females just after ovary removal and discovered that the ovary temperature was 22.2–26.3 °C. Since the developmental efficiency of the preservation groups at 15–20 °C performed better than that at 25 °C, it might be better to set the preservation temperature of ovulated eggs somewhat lower than that of the ovary. Holding ovulated eggs in the ovarian cavity for an extended period after ovulation has been thought to stimulate over-ripening in earlier studies [12,19,23,24]. By comparison of the spawning (rearing) temperatures of spotted halibut *Verasper variegatus* and barfin flounder *Verasper moseri*, the over-ripening progressed more rapidly depending on water temperature [19]. In the chum salmon *Oncorhynchus keta*, the temporal preservation of

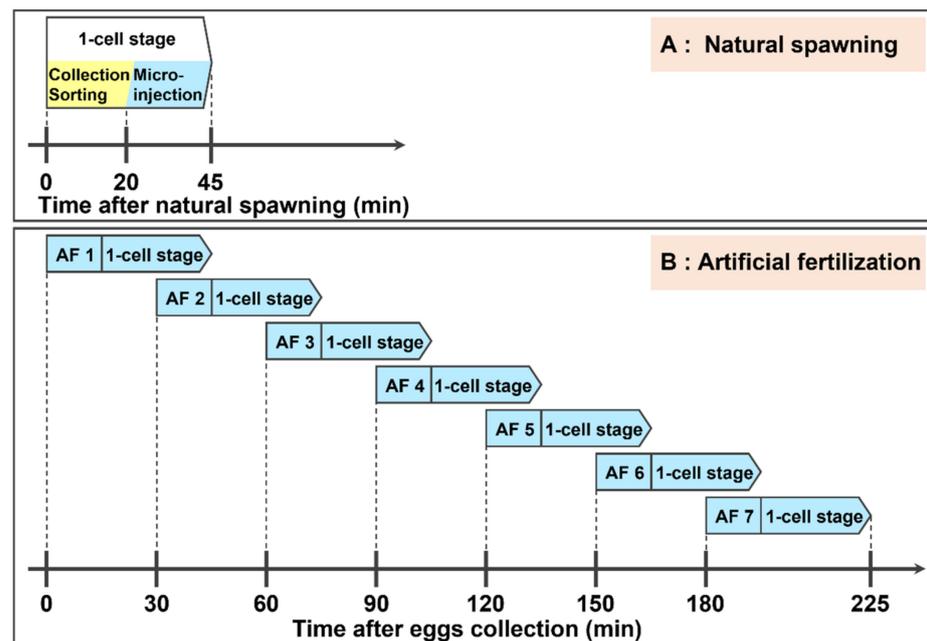
ovulated eggs at 3 °C was better than that at 15 °C [45]. As with the kawakawa, the ovulated eggs' preservation at a higher temperature (25 °C) may have facilitated the over-ripening.

Also, effective preservation of ovulated eggs was executed in kawakawa with Hanks' solution, L-15 medium and Ringer's solution without a decline in fertility. Furthermore, preservation in Hanks' solution had a tendency to exhibit improved fertilization ability after temporal preservation up to 3 h. Therefore, it is possible that substituting the ovarian fluid that surrounded the kawakawa eggs with Hanks' solution enhanced the fertilization environment for artificial fertilization. Alternatively, the volume of fluid that surrounded eggs during the preservation period was enhanced by adding Hanks' solution, which may have functioned as an oxygen supply and buffering agent.

#### 4.3. Application for Kawakawa Breeding

First, the establishment of an artificial fertilization method in kawakawa contributed to excellent selective breeding by 1:1 mating between the genetically superior fish. Artificial fertilization explored the use of genetic resources such as cryopreservation of sperm [4] and interspecific hybridization using sperm from different species [46]. Ovulated egg preservation and artificial fertilization methods efficiently involve microinjection techniques. The time available for microinjection treatment was 45 min because the first cleavage occurred 45 min after fertilization in kawakawa embryos incubated at 24 °C, then embryos reached the two-cell stage [31]. When fertilized eggs for microinjection treatment were obtained from natural spawning, fertilized eggs spread in the land-tank or sea cages had to be collected immediately [5]. Even if numerous eggs were spawned, the number of eggs that could be collected was unstable. Moreover, it was necessary to sort the eggs immediately after fertilization (one-cell stage) from the mixture of fertilized eggs of different developmental stages since multiple females spawned on the same day. For this reason, we could not spend more than 20 min on the microinjection technique out of the 45 min one-cell stage, excluding the time required for egg collection and sorting (Figure 7A). In contrast, artificial fertilization eliminated the need for transporting fertilized eggs from sea cages to the laboratory and allowed us to produce large amounts of embryos at the same developmental stage. Since we could keep ovulated eggs until 3 h after collection with high developmental capacity, it is feasible to provide many embryos for microinjection techniques by replicating artificial fertilization at least seven times every 30 min. As elucidated above, we were able to remarkably increase the time available for microinjection treatment from 20 min to 225 min (Figure 7B).

This study also permitted the induction of several polyploids by chromosome manipulation. The chromosome manipulation in kawakawa was restricted to triploid induction by using collected fertilized eggs from natural spawning in a cultivation tank [47]. In the future, it will be possible to generate androgenetic or gynogenetic haploids that necessitate genetic inactivation of ovulated eggs or sperm before fertilization. Parthenogenesis such as androgenesis and gynogenesis is the dominant tool for sex control, establishing a genetically identical (clone) strain, and fixation of superior traits in fish [48]. This study established the introduction of several breeding techniques into seed production in kawakawa aquaculture.



**Figure 7.** Schematic illustration of the available time of embryos from (A) natural spawning and (B) artificial fertilization for microinjection treatment in kawakawa *Euthynnus affinis*. In natural spawning (A), it is necessary to collect fertilized eggs which are spread in the land-tank or sea cages, and to sort out the eggs just after fertilization (one-cell stage) from the mixture of fertilized eggs of various developmental stages since multiple females spawn on the same day. Therefore, the microinjection treatment process remained only about 20 min out of the 45 min one-cell stage, excluding the time required for egg collection and sorting. On the other hand, when using embryos from artificial fertilization (B), it is feasible to provide many embryos for microinjection treatment by repeating artificial fertilization at least seven times (AF1 to AF7) every 30 min because we could preserve ovulated eggs until 3 h after collection with high developmental capacity.

**Supplementary Materials:** The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/jmse10050599/s1>. Table S1. Fish information of all females for the series of artificial fertilization; Table S2. Fish information of males used for artificial fertilization; Table S3. Precipitation and daylight hours of the Misho area [33] from 3 August 2021 to 15 September 2021; Table S4. Developmental efficiency after preservation of ovulated eggs in different solution; Table S5. Developmental capacity after preservation of ovulated eggs in different temperatures; Figure S1. Water temperature at the depth of 1 m, 5 m, and 10 m in the experiment period from 3 August 2021 to 15 September at the rearing site [30]. White arrows indicate the days which recorded more than 40 mm of the precipitation per day; Figure S2. Ovulated eggs after preservation in different solutions. (A) Preservation in the Hanks' solution, (B) in the L-15 medium, and (C) in the Ringer's solution for marine teleost. Column 1: 1 h preservation, Column 2: 3 h preservation, Column 3: 5 h preservation, Column 4: 12 h preservation. Scale bars denote 1 mm.

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