Effect of stunning and freezing on carp (Cyprinus carpio L) survival rate

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Abstract: Carp is freshwater fish which popular in Indonesia. The transportation handling system is needed to keep its survival rate (SR) of fish still high until the destination place. This research aims to determine 1) effect of freezing on the consciousness time (CT) and its SR which treated of stunning using clove oil (Eugenia aromatic); 2) influence of 125 ppm clove oil solution for fainting process and the freezing process to two groups of carp (biggerA, smallerB). The best concentration of clove oil was 125 ppm, where the fish fainting time was 1.43 minutes and the revitalisation 3.70 minutes. The freezer temperatures used were -11, -13, and -15 °C and the freezing time (FT) were 20, 40, and 60 minutes. The best temperature was -13 °C. SmallerB indicated more sensitive to clove oil than the biggerA (duration to unconscious 3.58 minutes compared to 5.03 minutes). While duration to conscious was 15.15 minutes for the smallerB and 14.09 for the biggerA. After being frozen for 60, 90, and 120 minutes, the biggerA’s SR was 89% while the smallerB was 66% for 120 minutes frozen. The clove oil fainted fish has better SR compare to the direct freezing. The FT was 60, 90 and 120 minutes at -13 °C. The fastest CT is 4.83 minutes obtained at fish with fainting treatment at 60 minutes freezing. The slowest CT is 26.20 minutes obtained at fish without fainting treatment at 120 minutes freezing. This study can be concluded that the highest SR of 100% using a fainting treatment and 90 minutes of FT. Histology analysis found damage to fish tissue at freezing 120 minutes which gill filaments are bleeding.

Keywords: anaesthesia; carp; Eugenia aromatic; eugenol; histology.

1. Introduction
In 2017, Indonesia produced more than 16.16 million ton of fish through culture activities (mainly from freshwater pond culture, brackish water culture, biofloc cultures system of catfish, rice-fish combine farming, and mariculture) [1]. The report stated that during five years (2013-2017), the annual growth of fish culture production was 5.11%. One of quite popular of fish is common carp (Cyprinus carpio) which the production in 2018 reached 1.81 million ton, increase more than double from 2017 (as many as 841.75 thousand tons) [2].
As commonly known, the price of live fish is higher than the dead fish. For example, the price of live groupers is more than double compared to the dead one. The consumers know that the prime quality of fish is guaranteed as long as the fish alive. Keeping fish alive after harvesting need special treatment. During transportation for distributing of fish to either fish market or growth culture pond, the fish will always get stress which leads to decreasing the quality and sometimes lead to the death of fish. Several factors affected to the fish survival rate during transportation are distances, time of transportation, water quality, size of fish, type/species of fish, environmental temperature, densities, oxygen availability, etc. Fainting using chemical (such as ether, propoxate, quinaldine sulfate, and tricaine methanesulfonate-MS-222 [3]), or natural (such as clove oil, cinnamon [4], aromatic ginger [5], nutmeg leaf [6], betelnut leaf [7] substances) [8, 9] of fish prior to transportation is sometime applied to increase the survival rate.

Several discoveries have shown that the frozen fish is possible to come alive,1,2 not only after being frozen in days or weeks, even months. However, when we try to browse several scientific journals on this subject, we almost find nothing. This condition is very strange why nobody/institution does not want to do research such a topic which will give enormous benefit to all humankind.

The research was conducted to observe 1) the effect of freezing on the level of carp consciousness and its SR which treated of stunning using clove oil (Eugenia aromatica); 2) the influence of 125 ppm clove oil solution for fainting process and the freezing process to two groups of carp, bigger and smaller.

2. Materials and method
The fish sample is a common carp (C. carpio) with average weight was 100 gram (for the first work) and 200 gram (for the second work) bought from the local market and clove oil (E. aromatica). Equipment/tools were used were an aquarium, aerator, freezer Sharp FRV-200, digital scale, and digital thermocouple.

2.1. The first work
The step of first work (A) of research was started to observe the suitable concentration of clove oil for the fainting of fish before being frozen and determining the freezer temperature. All carps have been acclimatised and fasted for 24 hours after being bought from the market. Every step of research used three carps, replicated three times, and repeated two times. The same size three glass of aquariums (A1, A2, A3) (50x40x40 cm) were prepared and filled with 4 L water. The clove oil “solution” was then set up to 125, 188 and 250 ppm). The fainted fish were then put into an aquarium contain room temperature (28 °C) and aerated’s water, the period time of consciousness of fish were observed. The work was repeated three times.

The second step of the first work (B) was done with the freezing process for the carps sample (B1 = direct freezing for live carp; B2 = direct freezing for fainted carp). The freezer was set to -11 °C, and nine live fishes are put directly into the freezer, three fishes are taken after 20 minutes and directly moved to an aquarium contain room temperature and aerated’s water, the period time of consciousness of fish, were observed. The same treatment was done to three other fishes which taken after 40 minutes, and three remain fishes taken after 60 minutes. The work was repeated three times. All step of works then were done using -13 °C and -15 °C freezer temperature.

1 Telegraph.co.uk 2019 Frozen fish comes back to life after being defrosted https://www.telegraph.co.uk/news/newstopics/howaboutthat/12117658/Frozen-fish-comes-back-to-life-after-being-defrosted.html. 5th August 2019
2 Dailymail.co.uk, 2019. Incredible moment frozen fish brought life defrosted warm water. https://www.thesun.co.uk/news/7866889/frozen-fish-alive-defrosted-warm-water/. 5th August 2019
2.2. The second work

2.2.1. The first phase of the second work
Groups of unconscious carp fishes (fainted with chosen clove oil concentration-125 ppm) were directly put into the freezer at -13 °C (the chosen freezing temperature). The observation was done after 20, 40, and 60 minutes of freezing and being consciousness process in an aquarium with aerated water (as the above procedures). The work was repeated three times.

2.2.2. The second phase of the second work
Groups of fresh live carp fishes (without fainting process) were directly put into the freezer at -13 °C (the chosen freezing temperature). The treatment was done as above.

2.3. The third of work
A group of fishes were put in -13 °C freezer for 60, 90, and 120 minutes after being fainted by clove oil “solution” 125 ppm. Series of observation were done to get information concerning the most suitable freezing time viewed from the time period before unconscious, conscious, and SR. Histological analyses were carried out using the fish gills. The sample preparation was started using paraffin method (slicing the gills, fixation, dehydration, clearing, impregnation, embedding, blocking, trimming, colouring, and sticking the samples on the glass object using a mounting agent. The “preparat” then observed under optical microscope DP21 Olympus ex 41; the pictures were optimised using software image analyser.

2.4. Data Analyses
All data were analysed using Microsoft Excel 2016. After being analysed using a completely randomised design (RAL) with triple replication. Data were presented the mean and its standard deviation with descriptive discussion with graphs.

3. Result of the first work

3.1. The best concentration of clove oil solution
The result is shown in Figure 1. The result indicated that the 125 ppm clove oil “solution” could make fish fainted in 1.43 minutes and needed 3.70 minutes to regain their consciousness. Higher concentration of clove oil “solution” made the fish fainted faster and longer regain their consciousness. The work of Hajek et al. [10] using 40 ppm clove oil “solution” caused the carps fainted in less than 3 minutes and got consciousness approximately in four minutes. Pramono [11] stated that the ideal of anesthetic substances is if could faint fish under three minutes and period time-consciousness less than five minutes.

Generally stated that higher the concentration of clove oil ‘solution” will make faster the fainting of fish and longer the period to consciousness; this is due to much eugenol will be absorbed by fish gill. Lawless [12], there are three types of clove oil: 1) Bud oil is derived from the flower-buds of Syzygium aromaticum. It consists of 60-90% eugenol, acetyl eugenol, caryophyllene and other minor constituents; 2). Leaf oil is derived from the leaves of S. aromaticum. It consists of 82-88% eugenol with little or no eugenyl acetate, and minor constituents; and 3) Stem oil is derived from the twigs of S. aromaticum. It consists of 90-95% eugenol, with other minor constituents.

Imanpoor et al. [13] stated that clove oil could be an effective anaesthetic substance (through the eugenol content) used in aquaculture. The active compound will be absorbed by the blood flowing in the gills [14].

Anaesthetic substances have characteristic a high solubility and easy to diffuse into the blood through fish gills [15]. The anaesthetic compound will reach the brain and suppresses the nerve
system. In the long term of using the anaesthetic substances, there will damage the fish gills, nerves, kidney, and brain [16]. Neiffer and Stamper [17] reported that during the regaining consciousness, the fish mouth would be a ventilation system for cleaning/leaching the anaesthetic compound through the mouth and the gills, till the fishes get consciousness fully.

Figure 1. The influence of different concentration clove “solution” to carps time period toward unconscious (■) and toward conscious (□).

In Indonesia, the clove oil used in this research is mostly made from leaf and stem of *S. aromaticum*. Because the only bud of *S. aromaticum* could be sold in the market (the price is about US$65/kg of dry bud), while the stem/twig and leaf are by-product of *S. aromaticum* trees which extracted the oil content. The price of clove oil varies from US$ 2-3/25 mL.

3.2. The temperature of freezer and survival rate (SR)

From three different freezer temperature (-11°C, -13°C and -15°C) combined with freezing duration (20, 40, and 60 minutes), the result is shown in Figure 2. As a decreasing temperature media of fishes will suppress oxygen consumption and metabolism processes (which produce CO2, ammonia NH3, and faeces) [18], freezing will entirely stop all live activities such as breath, swimming, and others metabolism process. The fishes whom directly put in the freezer, seem to flutter for the first minute before totally being stiff.

Figure 2. Time period to consciousness at -11°C (■), -13°C (■), -15°C (■).

After being frozen for 20 minutes in -11 °C freezer, the fishes still alive after 6.38 minutes regaining processes in a room temperature aerated waters. After 40 and 60 minutes freezing in the same temperatures, the fishes come to alive after 23.19 and 54.48 minutes of regaining process. In the -13°C freezer and after 20/40/60 minutes being frozen, the fishes come to alive after 9.32, 20.64, 44.93 minutes regaining processes. In the -15°C freezer, only fishes which store for 20 and 40 minutes come
to alive after 54.48 and 46.32 minutes regaining processes — the death fish after being frozen for 60 minutes in -15 °C freezer.

The survival rate of fish after being frozen is shown in Figure 3. From the Figure 3 shown that only freezing process at -11 °C all treated fishes were 100% alive, while at the -13 °C freezer only 20 and 40 minutes freezing were 100% alive, and after 60 minutes only 67%. In the -15 freezer, only 40 minutes freezing the SR reach 67%.

Figure 3. Survival rate of carp after being frozen at -11°C ( ), -13°C ( ), -15°C ( ).

Storey and Storey [3] after exploring frogs and turtles, reported that while frozen, all these animals show no movement, respiration, heart-beat or blood circulation, and our latest experiments show barely detectable neurological activity. Ice accumulates in all extracellular fluid compartments and fills the abdominal cavity and the bladder; crystals run under the skin and in between muscles. These animals have mastered the tricks of organ cryopreservation the freezing of live tissue for storage and the subsequent use and they studies of frozen frogs and turtles are revealing the molecular mechanisms essential to life in a frozen state.

According to Storey and Storey [3], there are two alternatives exist strategies in the freezing temperature for the animal. The first-and most familiar-strategy is to avoid exposure to temperatures below the freezing point of body fluids. Animals simply "choose" relatively warm hibernation sites underwater or deep underground. Numerous insect species overwinter as aquatic larvae, and many types of frogs and turtles hibernate at the bottom of ponds, where they are safe unless the body of water freezes completely. On land, toads may dig into the earth to remain below the frost line, and snakes may congregate in underground communal dens. The second alternative to freezing is to use specific adaptations that stabilise the liquid state at subzero temperatures. All water solutions, including body fluids, have an equilibrium freezing point, or the temperature at which an ice crystal placed in the solution will begin to grow. However, all water solutions can also be supercooled; that is, they can be chilled well below the equilibrium freezing point before the water crystallises spontaneously into ice.

In this research, the fish carp have no time to implement both strategies, besides they have no experience (habit) facing any freezing situation, and have no time for adaptation process (they were directly put into the freezer). It must be there is some mechanism process in the fish body which protect them from death.
4. Result of the second work

4.1. The comparison of the direct freezing process from alive and previously fainted fish (125 ppm of clove oil “solution”) before freezing at -13°C.

The result is shown in Figure 4. From Figure 4 can be seen that there is an indication the fainted fish have less period to consciousness compare to direct freezing of live fish. All treated fishes have SR 100%.

![Figure 4](image)

Figure 4. The period to consciousness between fainting (■) and no fainting process (□).

During the regaining consciousness process, shortly can be described briefly as follow (for the fishes which frozen 20 minutes in -13 °C freezer):
1) Minutes 2-3: the operculum start moving slowly;
2) Minutes 4-6: the fish body start moving from laydown position to unsteady swimming;
3) Minutes 6-9: the fish start to more active moving but still unsteady, seem to vomit something;
4) Minutes 9-10: the fish gain full consciousness.

For 40 and 60 minutes freezing, the recovery process of fish nearly the same with the above description, except the first movement of operculum was occurred starting at minutes 4-6 (need more time for each step of regaining process).

5. Result of the third work

Data is shown in Figure 5 and Figure 6. The mortality rate of unfainted fish (direct freezing of live fish) was higher than the fainted fish. This was probably caused by less shock suffered by the unfainted fish during the initial freezing process. The fishes had been floundering for several seconds before coming to motionless. Short floundering of fishes prior to being frozen surely will have physiological effect, as mention by Barton [19], physiological responses to stress are grouped as primary, which includes endocrine changes such as in measurable levels of circulating catecholamines and corticosteroids, and secondary, which include changes in features related to metabolism, hydromineral balance, and cardiovascular, respiratory and immune functions. van den Burg et al. [20] researched the influence of temperature to carp concluded that acute cold exposure did not influence blood plasma alpha-MSH concentrations. Acclimation to 15, 22 or 29 °C led to a temperature-dependent increase of both alpha-MSH and NAc beta-END plasma concentrations. Moreover, the in vitro sensitivity to TRH of melanotrope cells (that synthesise these peptides) also correlated positively with ambient temperature. In accordance with the result, Sumahiradewi [4] stated in the transportation process of Oreochromis niloticus found that the fainted fish have higher survival rate compare to unfainted fish.

Freezing for 120 minutes in -13 °C caused 11% (fainted fish) and 33% (unfainted fish) mortality of carp. Based on simple economic calculation, the result could be implemented in live fish trading if the
mortality rate less than 25%, with considering: the price of live fish and the dead one, the distance between fish farm to the market, and cost of fish fainting and freezing process. Inevitably, more extended periods of keeping live fish in freeze condition and higher the survival rate will result in more advantages to living fish business.

Figure 5. The period to the consciousness of previously fainted and unfainted fishes. The superscript indicated a 95% significant data.

Figure 6. Mortality of carps after being frozen for 60, 90, 120 minutes. The superscript indicated a 95% significant data.

6. Histology of gills carp
The photos of fresh carp gills filament are shown in Figure 7. Commonly, mostly there was not any damage to the fresh carp gills lamellas. This was indicated that the carp was normal and healthy.

After being frozen for 60 minutes, the carp gills ware shown in Figure 8. From Figure 8 appear that there was damage on the lamellas (some of the secondary lamellas were detached and broken from primer lamellas. This was quite visible, and bleeding could be seen from the fish gills. With broken lamellas, the fish will be difficult to catch oxygen from the water.
Figure 7. Photos of fainted carp gills filament before freezing (magnification 10x10). Note: a) cartilage; b) primer lamella; c) secondary lamella.

Figure 8 Photos of fainted carp gills filament after 60 minutes freezing (A = magnification 10x10; B = magnification 40x10). Note: a) cartilage; b) primer lamellas; c) secondary lamellas

Figure 9 Photos of fainted carp gills filament after 120 minutes freezing (A = magnification 10x10; B = magnification 40x10). Note: a) cartilage; b) primer lamellas; c) secondary lamellas

After being frozen for 120 minutes, the carp gills were shown in Figure 9. From Figure 9 appear that the gills damage were spread out nearly to the whole part of gills filaments. The blood clot appeared nearly on the surface of the gills. Sukarni et al. [21] stated that the fish blood would be piled up on the gills blood vessel. Necrosis (a form of cell injury which results in the premature death of cells in living tissue by autolysis, Proskuryako et al. [22]) of gills filament presumably caused by the cell membrane of lamellas were swollen (oedema) and broken due to increasing the cytoplasm volume during freezing. When the fish was revitalised, the blood then spills out of the gills [23]. The high mortality of carp caused this.
7. Conclusions
1) Clove oil solution 125 ppm is sufficient as anaesthesia substances for the fainting of carps;
2) Freezer temperature -13 °C is the most suitable freezing condition for carps compare to -11 °C and -15°C;
3) Carps could still survive after being frozen for 90 minutes;
4) Fainted carps have better endurance compare to unfainted carps after freezing;
5) Gills filament of carps were damaged after being frozen for 60 minutes and caused high mortality after 120 freezing.

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