Effects of anesthetic eugenol on respiration and excretion of Sinogastromyzon szechuanensis

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Abstract. In order to select a more suitable anesthetic for the breeding and transportation of Sinogastromyzon szechuanensis, we used different concentrations of anesthetic eugenol (0, 6, 12, 18, 24, 30 mg•L\(^{-1}\)) to test the effects of the respiratory and metabolic on Sinogastromyzon szechuanensis with an average mass of (2.029±0.26) g under the water temperature (18±0.5) ℃. Based on regression analysis method, the regression equations of oxygen consumption rate, ammonia removal rate and temperature were calculated. (R\(^2\)> 0.99). Oxygen consumption rate and ammonia removal rate were calculated and compared by One-Way ANOVA (Turkey method) using SPSS 25.0 software. The multiple comparison results revealed that when the mass concentration of eugenol reached 24 mg•L\(^{-1}\), it could significantly reduce the oxygen consumption rate and ammonia excretion rate (p<0.05). With the increase of eugenol concentration, the oxygen consumption rate and ammonia excretion rate of Sinogastromyzon szechuanensis first increased and then decreased. Under the influence of eugenol, the oxygen consumption rate and ammonia excretion rate of Sinogastromyzon szechuanensis were increased with the increase of temperature (p<0.01). When the water temperature is 14-26℃, the oxygen-nitrogen ratio is 8.0-19.4, and the temperature is positively correlated with the oxygen-nitrogen ratio. It is concluded that eugenol with a mass concentration of 24 mg•L\(^{-1}\) can make Sinogastromyzon szechuanensis enter the stage of anaesthesia II (sedation), so this concentration is recommended as the anesthetic dosage for transportation, and the anesthetic effect at low temperature is more obvious.

1. Introduction
There are many types of anesthetics used in the transportation and production of aquatic animals, of which MS-222 and eugenol are most commonly used. Its mechanism is: it passes through the gill or body surface into the fish body, inhibits the cerebellar cortex, then acts on the basal ganglia and cerebellum, and finally reaches the medulla spinalis [1]. Studies have shown that MS-222, eugenol and other anesthetics can reduce the oxygen consumption and ammonia nitrogen emissions of aquatic animals, resulting in changes in oxygen consumption and ammonia excretion rates of aquatic animals. The tolerance level of different aquatic animals to anesthetics is also different. [2].

The Sinogastromyzon szechuanensis live in the tributaries of the upper reaches of the Yangtze River in China, mainly distributed in the gravel beaches of the swift flowing river. They swim swirly, feeding on algae, chironomid larvae and other aquatic invertebrates [3]. The body color is beautiful and it is delicious, so it is popular. Human destruction of its habitat and overfishing have led to a sharp decline in its population resources. It has been listed as a local protected fish in some province in China. There published literatures related to Sinogastromyzon szechuanensis mainly focusing on resource investigation, early development,
mitochondrial genome and phylogenetic analysis [4]. In this study, the effects of eugenol on oxygen consumption rate, ammonia excretion rate and oxygen-nitrogen ratio of the Sinogastromyzon szechuanensis were studied for the purpose of selecting suitable anesthetics for reducing the damage in production and transportation and improving their survival rate. It also provides basic information for the study of respiratory physiology of the Sinogastromyzon szechuanensis.

2. Materials and methods

2.1. Materials
The experimental fish were collected from Neijiang city of Sichuan Tuojiang River System, the sampling point was in N 29° 36'41.93", E 105°01'47.67". After a month of indoor domestication culture, 135 healthy Sinogastromyzon szechuanensis with a uniform size, whose average body weight was (2.029±0.26) g and the average total length was (5.01±0.15) cm, were chosen. The experimental water was fully-aerated tap water. The initial dissolved oxygen amount was 11.18±0.1 mg•L⁻¹, and the water temperature was (18±0.5) ℃ (except for different temperature tests). And the pH is 7.6±0.1.

We choose a 2.5L glass triangle bottle as the respiration chamber, supplemented by plastic wrap and rubber band to make a sealed hydrostatic breathing apparatus. The anesthetic eugenol are purchased from the Jiangxi Jin Yuan Natural Perfume Co., Ltd.. Eugenol and ethanol are mixed by mass ratio 1:5 and then transferred to volume container and then add pure water to the scale line. After the mother liquor is prepared, it is refrigerated in the refrigerator, and prepare the liquid is temporarily before the experiment is carried out.

2.2. Methods

2.2.1. Effects of eugenol on oxygen consumption rate and ammonia excretion rate of Sinogastromyzon szechuanensis under the same temperature.
We set the mass concentration of eugenol to be 0, 6,12,18,24,30 mg•L⁻¹ on the basis of pre-experiment,. In each cone bottle, put 5 Sinogastromyzon szechuanensis, and set three parallel for each concentration, and add a blank control group (respiration chamber without fish), and the water temperature is (18±0.5) ℃.

Let the fish starve for 24 hours before the test. We place the respiratory chamber in a constant temperature water tank. The breathing experiment lasts for 2 hours (9:00 am-11:00 am). At the end of the experiment, the bottle was upside down and blended, then we siphon water sample from bottom of triangle bottle. We use Winkler iodometry to determine the dissolved oxygen and use nesslerization to determine ammonia (ammonium) nitrogen. After taking the water sample, anaesthetize the fish quickly then weigh the test fish without water (accurate to 0.01g), and measure the body length with foot gauge and ruler (accurate to 0.1cm), and then put the fish into the fresh water for recovery.

2.2.2. Effects of eugenol on oxygen consumption rate and ammonia excretion rate of Sinogastromyzon szechuanensis at different temperatures.
According to the anesthetic effect in 1.2.1, eugenol 24 mg•L⁻¹ was selected to carry out experiments on the effects of different temperatures on oxygen consumption rate and ammonia excretion rate of Sinogastromyzon szechuanensis. Set up tanks with temperature gradient of 14℃, 20℃, 26℃, Nine 2.5L conical flasks (respiratory chamber) are placed in each water tank, and among them, there are respectively three eugenol and no-anesthetic which are used as contrast. In addition, set a blank control (no fish, and it is used to measure initial dissolved oxygen at different temperatures). Place 5 Sinogastromyzon szechuanensis in each of them (Note: Transfer temperature difference is no more than 2 ℃), Start experiment immediately after adding anesthetic. The other methods are the same as 1.2.1.

2.2.3. Measurement and calculation of each index.

\[ R_{oc} = \frac{1000 \times (C_0 - C_1) \times V}{W \times T} \]

\[ R_{am} = \frac{1000 \times (C'_0 - C'_1) \times V}{W \times T} \]

\[ O/N = \frac{R_{oc}}{R_{am}} \]
Roc is the oxygen consumption rate \((\mu g \cdot g^{-1} \cdot h^{-1})\); Ram is the ammonia excretion rate \((\mu g \cdot g^{-1} \cdot h^{-1})\); \(C_0\) and \(C_1\) represent the dissolved oxygen concentration \((mg\cdot L^{-1})\) of the control group and the experimental group after the experiment, \(C'_0\) and \(C'_1\) represent the dissolved oxygen concentration \((mg\cdot L^{-1})\) of the control group and the experimental group after the experiment, \(V\) is the actual water volume \((L)\) of the respiratory chamber (conical bottle); \(W\) represent the quality of the test fish \((g)\); \(T\) represent the test time \((h)\). O/N represent the oxygen-nitrogen ratio.

2.2.4. Statistics and analysis of data.
The data were processed by Excel and SPSS Statistics 25 software. Duncan's was used to compare the differences of groups. If one of the above parameters is the same in the same column, there is no significant difference between the groups \((p > 0.05)\). The statistical values are expressed by average value± standard deviation. Regression analysis of oxygen consumption rate and ammonia discharge rate with temperature by Excel software.

3. Result and analysis

3.1. Effects of eugenol with different concentrations on oxygen consumption rate, ammonia excretion rate and oxygen-nitrogen ratio of Sinogastromyzon szechuanensis

The effects of eugenol on oxygen consumption rate, ammonia excretion rate and oxygen-nitrogen ratio of Sinogastromyzon szechuanensis are shown in Table 1. With the increase of eugenol concentration, the oxygen consumption rate and ammonia excretion rate increase first and then decrease. One-way ANOVA shows that when the concentration of eugenol is 12 mg•L\(^{-1}\), the oxygen consumption rate and ammonia excretion rate of the Sinogastromyzon szechuanensis are significantly higher than those of the control group \((p < 0.05)\). The sucker of fish is absorbed on the glass wall or bottom of the bottle, and the breathing rate is slightly higher than that of the control group, and the difference is significant \((p<0.05)\). When the concentration of eugenol reaches 24 mg•L\(^{-1}\), the oxygen consumption rate and ammonia excretion rate are significantly decreased, and the breathing rate ratio is significantly lower than that of the control group \((p<0.05)\), and when concentration continues to rise, the oxygen consumption rate and ammonia excretion rate have no significant difference. Observing their behavior, we find that abdomen of some fish is upward, facing upwards on the bottom of the bottle, and muscles are flabby. The oxygen-nitrogen ratio is between 11.30 and 13.00, and there is no significant difference among the groups \((p>0.05)\).

| Anesthetic | Concentrations \((mg\cdot L^{-1})\) | \(R_{oc}/(\mu g\cdot g^{-1}\cdot h^{-1})\) \(x\pm SD\) | \(R_{am}/(\mu g\cdot g^{-1}\cdot h^{-1})\) \(x\pm SD\) | O/N |
|------------|---------------------------------|---------------------------------|---------------------------------|-----|
|            | \((n=90,\ x\pm SD)\)           | \((n=90,\ x\pm SD)\)           |                                 |     |
| Eugenol    | 0                               | 457.8±28.7\(^b\)               | 38.3±4.9\(^b\)                 | 11.50 |
|            | 6                               | 485.3±31.2\(^{ab}\)            | 40.9±2.3\(^b\)                 | 12.10 |
|            | 12                              | 543.2±23.5\(^a\)               | 49.5±8.0\(^a\)                 | 11.30 |
|            | 18                              | 470.9±40.8\(^{ab}\)            | 37.3±2.0\(^b\)                 | 12.40 |
|            | 24                              | 387.4±40.1\(^c\)               | 33.8±4.4\(^c\)                 | 11.90 |
|            | 30                              | 383.3±35.5\(^{c}\)             | 30.0±5.9\(^{c}\)               | 13.00 |

3.2. Effects of eugenol on oxygen consumption rate, ammonia excretion rate and oxygen-nitrogen ratio of Sinogastromyzon szechuanensis at different temperatures

The control group and eugenol \((24\ mg\cdot L^{-1})\) group, the oxygen consumption rate and ammonia excretion rate of the Sinogastromyzon szechuanensis increase significantly with the increase of temperature, and there is a significant correlation \((Fig\ 1,\ R^2>0.99)\). One-way ANOVA analysis showed that there are significant differences in oxygen consumption rate and ammonia excretion rate among the three temperature gradients of different test groups \((p<0.01)\). The oxygen-nitrogen ratios of anesthetic group and blank control group increase with the
increase of temperature. At the same temperature, the oxygen-nitrogen ratio of anesthetic group is lower than that of control group (Table 2).

Table 2. Effects of eugenol on oxygen consumption rate, ammonia excretion rate and oxygen-nitrogen ratio of Sinogastromyzon szechuanensis at different temperatures

| Treatment group | Temperature °C | $\dot{R}_{oc}/(\mu g \cdot g^{-1} \cdot h^{-1})$ (n=45, $\bar{x} \pm SD$) | $\dot{R}_{am}/(\mu g \cdot g^{-1} \cdot h^{-1})$ (n=45, $\bar{x} \pm SD$) | O/N |
|-----------------|----------------|-------------------------------------------------|-------------------------------------------------|-----|
| Control group   | 14             | 135.9±20.0a                                      | 13.9±1.3a                                       | 9.8 |
|                 | 20             | 340.3±42.5b                                     | 19.5±2.2b                                       | 17.4|
|                 | 26             | 476.9±49.0c                                     | 24.6±3.7c                                       | 19.4|
| Eugenol 24 mg/L | 14             | 115.2±24.8a                                     | 11.3±0.4a                                       | 10.2|
|                 | 20             | 208.2±28.8b                                     | 15.7±1.8b                                       | 13.3|
|                 | 26             | 293.4±28.9c                                     | 20.1±1.7c                                       | 14.6|

Figure 1. Effects of eugenol on oxygen consumption rate and ammonia excretion rate of the Sinogastromyzon szechuanensis at different temperatures

4. Discussion
There are many factors affecting the anesthetic effect of anesthetics, including the anesthetics type, concentration, temperature, dissolved oxygen and other water quality indicators, as well as the tolerance of different varieties to anesthetics [5]. After the tested aquatic animals are anesthetized, we can use oxygen consumption rate, ammonia excretion rate and breathing rate to judge the depth of anesthesia. According to the behavioral characteristics of the Sinogastromyzon szechuanensis in the anesthetic solution, the anesthetic process was divided into five stages (Table 3):
Table 3. Stages of anaesthesia in the *Sinogastromyzon szechuanensis*

| Stages of anaesthesia | Behavior characteristics |
|-----------------------|--------------------------|
| I—Stress stage        | The response to stimulation was obvious, swimming was active, scurrying around, and breathing frequency increased. |
| II—Sedation stage     | The response to stimulus was reduced, adsorbed on the bottle wall, sometimes the head reached to the surface of the water, and the respiratory rate decreased slightly. |
| III—Mild anesthetic stage | The tactile response was slow, lying on the bottom of the bottle, and the respiratory rate was further reduced. |
| IV—Deep anesthetic stage | Complete loss of swimming ability, no response to touch, lying on the bottom of the bottle, breathing rate dropped sharply, gill moved slightly. It can be resuscitated about 1 minute after being transferred to clean water. |
| V—Death stage         | The gill completely stopped opening and it could not recover after it was transferred to fresh water. |

4.1. Effects of different concentrations of eugenol on oxygen consumption rate and ammonia excretion rate of *Sinogastromyzon szechuanensis*

The metabolism of aquatic animals usually takes oxygen and then releases carbon dioxide and nitrogen, so the oxygen consumption rate and ammonia excretion rate can directly or indirectly reflect the metabolic state and physiological condition. In production and transportation, the use of a certain number of anesthetics can reduce oxygen consumption, reduce carbon dioxide and ammonia nitrogen emissions, reduce stress injury, and help to improve survival in production and transportation. Different kinds of aquatic animals, and different individual sizes, water temperatures and dissolved oxygen all influence the anesthetic effect.

When *Sinogastromyzon szechuanensis* were anesthetized with eugenol at different concentrations, their oxygen consumption rate and ammonia excretion rate decreased significantly. The oxygen consumption rate and ammonia excretion rate of eugenol group were higher than those of control group when the concentration of eugenol was 12 mg•L⁻¹. The results of this study were consistent with that of Zhuang. The main reason was that the stress effect of anesthetics increased respiratory rate and metabolism. When concentration of eugenol was 24 mg•L⁻¹, it could lead to the stage II of anaesthesia, reduce the oxygen consumption rate and ammonia excretion rate significantly. Among them, eugenol reduced its oxygen consumption rate by 15.4%, ammonia excretion rate by 11.7%. The anesthetic effect of eugenol on *Procambarus clarkii* was similar to that of this study. With the increase of concentration, the oxygen consumption rate and ammonia excretion rate increased first and then decreased. When the concentration was 40 mg•L⁻¹, the oxygen consumption rate and ammonia excretion rate both decreased significantly[5]. When eugenol anesthetized the *Acipenser baeri* with an average body weight of 1.44g, the oxygen consumption rate of 2-4 mg•L⁻¹ group was significantly higher than that of the control group, and 8-30 mg•L⁻¹ was significantly lower than that of the control group [6]. Wang (2018) simulated the transport of 130g juvenile largemouth bass. When concentration of eugenol was 10 mg•L⁻¹, the juveniles remained in the second stage of anaesthesia.

4.2. Effects of eugenol on oxygen consumption rate, ammonia excretion rate and oxygen-nitrogen ratio of *Sinogastromyzon szechuanensis* at different temperatures

With the increase of temperature, the basal metabolism and active metabolism become stronger, resulting in the increase of oxygen consumption rate and ammonia excretion rate. Oxygen consumption rate and ammonia excretion rate of most shellfish increase with the increase of temperature. After exceeding the tolerance temperature range, the physiological function of shellfish is disturbed, and the oxygen consumption rate and ammonia excretion rate decrease[7].

Regardless of whether the *Sinogastromyzon szechuanensis* were anesthetized, the oxygen consumption rate and ammonia excretion rate increased with the increase of temperature at the temperature of 14-26°C (p<0.05). The results are similar to those of juvenile *Aci penser sinensis, Oplegnathus punctatus, juvenile Lucioperca lucioperca, Monopterus albus* eel [8-10]. From Figure 1, although the anesthetic agent can reduce the oxygen consumption and ammonia excretion rate of *Sinogastromyzon szechuanensis*, the effect of temperature on the oxygen consumption rate and ammonia excretion rate of *Sinogastromyzon szechuanensis* is extremely significant. The eugenol has better anesthetic effect at low temperature.
Oxygen-nitrogen ratio (O/N) is an important physiological index in the process of animal metabolism, which can predict the chemical nature of energy substances consumed in the metabolism of organisms. When energy is supplied entirely by protein, the oxygen-nitrogen ratio is about 7 [11]. When energy is supplied by protein and fat, the oxygen-nitrogen ratio is about 24 [12]. When energy is supplied by fat or carbohydrates, the oxygen-nitrogen ratio becomes infinite [13]. In this paper, the ratio of oxygen-nitrogen ratio of eugenol groups with different concentrations ranged from 11.30 to 13.00. However, there was no significant difference among the groups, which indicated that anesthetics eugenol did not significantly change the main energy-supplying substance—protein. Zhuang (2009) pointed out that the oxygen-nitrogen ratio decreased with the increase of anesthetic concentration in the anesthesia test of juvenile Acipenser sinensis, which was inconsistent with the results of ours experiment. Zhang (2010) considered that the oxygen-nitrogen ratio was not positively correlated with the concentration of anesthetic in the narcotic experiment of Nile tilapia, and the results are consistent with the viewpoints of this study. In the temperature range of 14-26°C, the oxygen-nitrogen ratio of Sinogastromyzon szechuanensis ranged from 8.0 to 19.4, and it increased with the increase of temperature. At the beginning, energy was mainly protein, then the protein utilization rate decreased, and the utilization of fat and carbohydrates increased.

5. Conclusion
The results showed that anaesthetic eugenol had significant effects on oxygen consumption rate and ammonia excretion rate of Sinogastromyzon szechuanensis (p<0.05). With the increase of anesthetic concentration, anaesthetic eugenol with a concentration of 24 mg•L⁻¹ can make the Sinogastromyzon szechuanensis enter the stage of anaesthesia II, and this concentration meets the requirements of live Sinogastromyzon szechuanensis transportation, so it can be used as the recommended anaesthetic dosage for transportation, and the anaesthetic effect at low temperature is more obvious.

Acknowledgment
This research was financially supported by “Qing Lan Project of Jiangsu Province”. This work was supported by “311 Talents Project in Taizhou”, Research project of the Jiangsu Agri-animal Husbandry Vocational College (Grant No. NSF201711).

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