The attractive effects of amino acids and some classical substances on grass carp (Ctenopharyngodon idellus)

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Abstract Grass carp (Ctenopharyngodon idellus) is one of the most essential fishing species in China. The bait for this fish is rapidly developing. However, the study on the attractants in the bait for this fish lacks. This study was designed to systematically investigate the effects of 16 kinds of test substances on the perspective of behaviour and physiology of grass carp by using different kinds of methods, including behavioral tests (maze test and biting-balls test) and electro-olfactogram (EOG). Our experiment’s idea is mainly to imitate: in addition to vision, fish in nature also use smell to find food and finally swallow under the action of olfaction, taste, and other sensory systems. Firstly, the behavioral maze test was used to screen the attractive or suppressive effect of 16 test substances on grass carp, and the electronic olfactory recording method was used to further evaluate the olfactory response of grass carp to the eight stimuli selected from the maze test. Then, the best concentrations of these eight stimuli and their combination were investigated by the biting-balls test to compound a formula with the strongest appetite for grass carp. The results of behavioral maze test showed that dimethyl-β-propiothetin (DMPT), dimethylthetin (DMT), glycine, taurine, l-glutamic, l-alanine, l-proline, and l-arginine have different degrees of usefulness in attracting grass carp. The electro-olfactogram recoding showed that the EOG response of grass carp to the stimuli is a transient biphasic potential change and all of the eight stimuli could induce the EOG response of grass carp. The biting-balls test showed that glycine, l-glutamic, and l-arginine at $10^{-2}$ mol/L had significant feeding stimulation and DMT at $10^{-1}$ mol/L had significant feeding stimulation than the other groups. Finally, formula 9 composed of DMT, glycine, l-glutamic acid, and l-arginine has the greatest attraction for grass carp. The results of this study verified the attractive effect of some amino acids and other chemicals on grass carp fishing, and would provide support for the production of specific grass carp attractants.

Keywords Behavioral test · Electro-olfactogram · Feeding attractant · Ctenopharyngodon idellus

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Introduction

China has a long history of fishing with bait. With the continuous development of the economy and the improvement of people’s living conditions, the sport of fishing has flourished in China. Grass carp (Ctenopharyngodon idellus) is one of the essential fishing species in China. Most anglers use some natural baits, such as corn and aquatic plants, which are not effective in attracting the grass carp. The commercial bait is composed of food-based basic materials and attractants, among which the attractants play a decisive role in the entire bait (Løkkeborg and Johannessen 1992). The feeding activity of grass carp was improved by adding amino acids and other stimuli to the bait made from food-based basic materials so that the angler can better enjoy the fun of fishing.

In previous studies, the olfactory systems of various fishes have been shown to be highly sensitive to chemical stimuli (Hara 1975; Franklyn et al. 2017; Kasumyan and Mikhailova 2017; Hu et al. 2021). The olfactory epithelium of fish is similar in structure to that of other vertebrates. However, because olfaction of fish takes place in an aquatic medium, a wide variety of compound classes are sensitive stimuli (Ozorio et al. 2010). Among these soluble compounds are amino acids, which have been demonstrated to be feeding cues and function as potent olfactory stimuli. L-Type amino acids are recognized as effective phagosomes in fish (Franklyn et al. 2017). L-Cysteine, L-alanine, L-lysine, and L-proline induce bottom searching in rainbow trout (Oncorhynchus mykiss), and L-arginine, L-glutamate, and L-proline cause a pecking behavior in goldfish (Carassius auratus Linnaeus) (Hara 2010). These findings have been clarified from the electric physiological experiment results for rainbow trout (Hara 1994) and channel catfish (Ictalurus punctatus). Studies on substances other than amino acids have shown that dimethylthetin (DMT) could act as an effective feeding stimuli in high plant-based diets for juvenile GIFT tilapia (Oreochromis sp.) (Zou et al. 2017). Dimethyl-β-propiothetin (DMPT) is not only a strong olfactory stimulant but also a (CH₃)₂S group donor, which is a crucial group that activates animal taste receptors (Olsén et al. 2018). It shows a robust promoting effect on feeding and growth in a variety of fish species, and its feeding stimulating impact is 2.5 times that of glutamine (Kenji et al. 1989). It has been confirmed that trimethylamine N-oxide (TMAO) can be used as a feeding attractant in the feed for Penaeus vannamei (Zhang et al. 2005). In previous studies, the amino acid mixture was found to function more efficiently than single amino acids as a stimulus (Lim et al. 2015).

The current research on fish attractions is mainly based on the behavioral test (Miyasaki et al. 1999) and electrophysiological test (Jiesheng and John 1991). Previous studies mostly used a single method to study the effect of attractants. The experimental results of the fish behavioral test are different from the electrophysiological test results, and the electrophysiological results alone are difficult to distinguish which stimulus is a useful attractant substance.

At present, there are relatively few studies on attractants for grass carp diet and bait. Besides, the attraction effect of commercial grass carp bait is not apparent for fishing. It is deserved to develop a special attractant for grass carp. The present study is the first time to study the attractants of grass carp using different methods systematically. The aim of the present study was to screen several substances that have an attractive effect on grass carp from 16 kinds of test substances through the behavioral maze test, then to explain the stimuli effect of the selected substances on grass carp from a physiological point of view using electro-olfactogram (EOG) test, and finally to determine the optimal concentration of each stimulus and the optimized formula of compound attractants through an orthogonal design using the behavioral biting-balls test.

Materials and methods

Grass carp maintenance

Young grass carp (Ctenopharyngodon idellus, body weight: 30 ± 5 g) were purchased from Honghu Fisheries Co., Ltd. The fish were cultured in a tank (300 L) with circulating filtered and aerated (> 24 h) tap water (pH 7.0–7.4) in a temperature-controlled room (25 ± 1 °C) before the experiment. Water quality parameters, including pH, temperature, and dissolved oxygen (> 6 mg/L), were measured weekly. The fish were fed with a commercial grass carp diet twice a
day, at 9:00 am and 4:00 pm, respectively. The feces and debris in the bottom were cleared daily.

The behavioral maze test

An experimental aquarium (Fig. 1), designed according to K Harada (Harada 1982), was made of tempered glass, composed of two resident compartments (1 and 2 in Fig. 1) and four test compartments to conduct a duplicate test. In order to avoid the fish from following the dominant fish to choose the source of chemical substances due to the visually guided behavior, an elliptical hole (a = 5 cm, b = 8 cm) in the partition between the test compartments and the resident compartments was designed. The entrance (elliptical hole) allows at most 2 experimental fish to enter or leave the test compartment at the same time. The device effectively suppresses the colony behavior of experimental fish in actual tests, so that the experimental fish can randomly select to enter the dummy or experimental area. The experimental aquarium with a water depth of 35 cm was set in an air-conditioned room (water temperature: 25 ± 0.5 °C). Thirty individuals of fish in each resident compartment were used in the test.

The maze test was conducted as follows: 16 kinds of test substances (food grade chemicals) including $\text{l}$-alanine, $\text{l}$-arginine, $\text{l}$-cysteine, $\text{l}$-glutamic acid, glycine, $\text{l}$-histidine, $\text{l}$-lysine, $\text{l}$-methionine, $\text{l}$-phenylalanine, $\text{l}$-proline, $\text{l}$-threonine, $\text{l}$-valine and taurine (Hebei Wubai Technology Co., Ltd), dimethyl-$\beta$-propiothetin (DMPT), dimethylthetin (DMT), and trimethylamine N-oxide (TMAO) (Beijing Green Hengxing Biotechnology Co., Ltd) were tested for the estimation of repellence or attraction. For each test substance, $10^{-1}$ mol/L solution was prepared, and then 16 g of wheat flour and 8 mL of prepared solution were thoroughly mixed and made into a test bait ball. The control ball (wheat flour bait without test substances addition) was used as a dummy. The test and control ball were wrapped in two layers of gauze (8 cm × 8 cm). The wrapped gauze was quietly hung on the S position of the test compartments. Freshwater was introduced continuously into the aquarium through each inlet of the delivery tube at the rate of 500 mL per minute. During the test, a control and a test bait ball wrapped with gauze were randomly put into the two test compartments corresponding to the same resident compartment simultaneously, with one ball in one of the test compartment at the S-position. After the inlet water rinsed the wrapped gauze to deliver the test substance into the entire test compartment for 1 min, the partition P was removed. The fish in the resident compartment were allowed to enter and left the two test compartments freely. A camera (TL-IPC42A-4, TP-LINK) connecting to a computer was placed above the test aquarium.

*Fig. 1* The test aquarium designed in the study. 1 and 2, resident compartments; A, test compartments; D, the water regulating valve; E, water storage bucket; h, elliptical holes (a = 5 cm, b = 8 cm); P, partition; S, the position of gauze ball containing test sample or dummy (control)
to record the activity of fish. After the partition P was removed, the recording began, and the fish entering and leaving the test compartments was recorded for 10 min. After each test, the video was reviewed manually to count per minute. The number of fish entering the test compartment from the resident compartment through the elliptical hole P and the number of fish leaving the test compartment into the resident compartment through the elliptical hole P were counted during a 1-min interval, represented by \( y_{E, i} \) and \( y_{L, i} \) (\( i \) means the 1st, 2nd, ..., 10th minute during the recording time), respectively. The number of fish remaining in the test compartment every minute was calculated as \( y_{R, i} = y_{E, i} - y_{L, i} \). In order to show the actual observation curve more intuitively, the numbers of fish entering, leaving, and remaining counted per minute were successively integrated with passing the time to represent \( Y_{E, j} = \sum_{i=1}^{j} y_{E, i} \), \( Y_{L, j} = \sum_{i=1}^{j} y_{L, i} \), and \( Y_{R, j} = \sum_{i=1}^{j} y_{R, i} \) in the time courses, respectively. The relationship between the cumulative number of each behavior \( Y_{f, j} \) (where \( I \) represents the three behaviors: E, entering; L, leaving; or R, remaining) and the time course (x, minute) was fitted by a logistic curve \( Y = g / \{1 + \exp[-r(x - a)]\} \), where the coefficients \( g, r, \) and \( a \) are constants. The coefficients were calculated according to the following procedures: Firstly, the parameter \( r \) was estimated from the regression coefficient \( b_1 \) in the following equation: \( 1/Y_{f, j} = b_0 + b_1/Y_{f, j-1} \), and \( r = -1 \times \log(b_1) \). Then, parameters of \( g \) and \( a \) were estimated by using the estimated \( r \) and the following regression equation: \( 1/Y_{f, j} = b_0 + b_1 \times \exp(-r \times x) \), then \( g = 1 / b_0 \), \( a = \log(b_1 \times g) / r \). The parameter \( g \) shows the level of asymptote of a fitting logistic curve, \( a \) shows the delay in the number of individuals showing the respective behavior types, and \( r \) represents the growth slope of the logistic curve. The fitness was estimated by the chi-square test. By comparing the \( gr \) value of the test group and the dummy group, the attractiveness/repellance of the test substances can be intuitively compared. The larger the \( gr \) value of the test group relative to the dummy group, the stronger the attraction/repellance of the test substance to grass carp (Harada et al. 2010). In order to have a better match between the regression line and the observation curve, several points in the first few minutes were appropriately deleted. Finally, by comparing the numerical \( gr \) of the experimental bait ball and the control bait ball, it is determined which of the 16 kinds of test substances species has an attractive effect on grass carp.

When testing the same test substance, the experimental compartments were randomly assigned to the test substance and the control in the duplicate experiments, which also effectively reduced the systematic error caused by the following behavior. In addition, before the start of this experiment, a pre-experiment under dark conditions was also carried out. At the moment the light source was turned on to terminate the pre-experiment, it was found that the law of entering of the experimental fish was consistent with that under light conditions. However, due to the dark conditions, the camera shot was blurred, and it was inconvenient for the researcher to calculate the fish entering or leaving the experimental compartments. So we used experimental conditions with light to conduct the test. All the test substances were tested in the same aquarium, and the experiments were carried out twice a day, at 10:00 am and 3:00 pm. At the end of each experiment, the water at the same temperature was changed in the aquarium, and the experimental fish were fed to satiation only once at 4:00 pm daily. Each experiment was repeated at least triplicate times with the random assignment of the test substances and the control to the test compartments.

The electro-olfactogram test

The electronic olfactory recording test further detected the olfactory responses of grass carp to the stimuli selected by the behavioral maze test. A capillary glass tube (80 \( \mu \)m tip diameter) was filled with gelatin (8% in 0.6% KCl solution). Then the fine platinum wire of the microelectrode (ME-1 microelectrode with amplifier, Chengdu Taimeng Software Co., Ltd) was inserted into the processed glass capillary to form a microelectrode system according to a previous study (Murphy et al. 2001). Grass carp were held in laboratory tanks supplied with dechlorinated water at (25.0 ± 0.5 °C). Larger grass carp (body weight 40 ± 0.5 g) was chosen because it was too difficult to obtain a recording from the smaller fish as the incident pore was too small for the size of electrodes used (Murphy et al. 2001). Immediately before recording, fish were anaesthetized by orally perfusing the gills with dechlorinated tap water containing...
3-aminobenzoic acid ethyl ester methanesulfonate (MS-222) (1:8000) (Hill et al. 2002) and an injection of gallamine triethiodide (0.004 mg/g BW) into the dorsal muscle, wrapped in a wet towel, and secured to a stand placed in an electrically grounded water bath. Throughout the recording process, a polyethylene tube was used to inject dechlorinated water into the grass carp’s mouth to assist its passive breathing. The water bath temperature and the anaesthetic water (25.0 ± 0.5 °C) during the test approximated that of the holding aquaria to reduce the stress of grass carp. In an attempt to facilitate recording, the overlying skin, cartilage, and dorsal aspect of the olfactory sac were removed to expose the olfactory receptor cells. And then, the recording electrode (ME-1 microelectrode with amplifier, Chengdu Taimeng Software Co., Ltd) was placed lightly against, generally perpendicular to the surface of the mucosa, and the reference electrode is clamped on the mouth of the fish. The naris was continuously perfused with dechlorinated tap water to keep the nostrils in a water environment and the stimulating solution was continuously dropped into the dechlorinated tap water injected into the nostril at a rate of 10 μL every 10 s. The electrical signal is displayed on the computer through BL-420A biological function experiment system (Chengdu Taimeng Software Co., Ltd). The EOG test system was designed as in Fig. 2. The response was recorded for 5 s from the odor pulse initiation (Tine and Aleš 2000). The response potential amplitude measurement is the difference between the lowest point of the negative wave and the highest point of the positive wave. Besides, to determine the experiment’s accuracy, recording began by determining the olfactory response to 10^{-3} mol/L DMPT. If the EOG response to DMPT was normal, testing with stimuli was initiated. If the response was abnormal, the recording electrode and odor tube were repositioned until the normal response was obtained. Each stimulus solution was continuously observed more than five similar EOG (amplitude difference ≤5%), and three grass carp were used in the experiment. Throughout the experiment, when its EOG amplitude changed more than 5%, the experimental data was discarded (Ma et al. 2003). After the recording was completed, the gills were perfused with tap water until the fish began ventilating, and then fish was put back into its aquarium.

The biting-balls test

The behavioral biting-balls test was used to determine the best attractant concentration of 8 stimuli screened. Sixty young grass carp body weight (45 ± 5 g) were held in an aquarium (length, 100 cm; width, 60 cm; height, 60 cm) supplied with dechlorinated water for the test. The water depth was kept at 40 cm, and the tank’s water temperature was controlled at (25.0 ± 0.5 °C) using constant temperature apparatus (Kawabata et al. 1992; Carlberg et al. 2015). The
experiments were carried out twice a day, at 10:00 am and 3:00 pm. Stock solutions at the concentration of $1 \times 10^{-1}$ mol/L for each stimulus were prepared, stored at 4 °C. One day before the test, the working solutions at $10^{-2}$ and $10^{-3}$ mol/L were obtained by diluting the stock solution. Three different concentrations of stimuli (6 ml) were added into flour (10 g) respectively and stirred, and then they were made into bait balls. An 8×8 cm² mesh then wrapped the bait balls with a mesh size of 0.5-cm diameter. Bait balls made from distilled water without stimulus were used as a control group. Three experimental balls and a control ball were randomly suspended in the test tank with a distance of 15 cm apart from each other. Each ball was 20 cm from the bottom of the fish tank. The number of bites for each bait ball in 6 min was recorded with the previously described camera. The optimal concentration of the stimuli was determined by the biting-balls test from the three different concentrations designed.

In order to determine a formula of compound attractants that is the most attractive to grass carp, the L₉(3⁴) orthogonal table was used to design the compound stimuli. The biting-balls test was conducted again to determine which combination had the greatest attraction for grass carp. During the test, three randomly selected formulas and a dummy were arranged in a test, and then the three groups with the best performance from each test were further compared. Grass carp were fed with commercial feed at the end of the experiment every afternoon and the fish tank was cleaned and replaced with new water. Each experiment was repeated at least triplicate times.

Statistics
In the behavioral maze test, a chi-square test (STATISTICA-7.0) was adopted to test the fitness of the estimated series of logistic curve $y = g / \{1 + \exp[-r(x-a)]\}$ to the observed one. $P > 0.05$ was adopted as the criterion.

In the electro-olfactogram (EOG) and the biting-balls test, the obtained data were analyzed by one-way ANOVA. In addition, two-way ANOVA was used to determine the main effects of concentration levels, substance kinds, and their interactions on the response amplitude of the EOG test. Then, Tukey’s multiple-range tests were used to compare the difference in group means among the experimental groups and the control group at the same time point. Data were expressed as mean±SE (standard error), and $P < 0.05$ was considered statistically significant. All the data were analyzed with SPSS (Version 19.0, SPSS Inc).

Results
Evaluation of repellance and attraction behavior in the maze test
Figure 3 shows the time courses of three behaviors in the maze test of 16 kinds of test substances and the respective dummy using 30 individuals of grass carp. The number in Fig. 3 indicated the number of each behavior for ten min. Of two systems composed of different test substances and dummy, the slope (r) of the entered time courses reflects the locomotion activity. The slope of each stimulus can roughly reflect the attraction and avoidance of the stimuli to grass carp. For example, the enter slope of l-lysine decreased obviously in comparison with that of the dummy. It can be judged that l-lysine showed a repellence activity on grass carp. To the opposite, the enter slope of DMPT was roughly bigger than that in the opposite dummy. It can be judged that DMPT showed an attraction behavior on grass carp. Figure 3 also shows that the number of grass carp staying in the test compartments is small and the gap between them is small, so we will not discuss it. After a long time of observation, the grass carp became active after entering the test area and then left after a short stay. Therefore, the entering or leaving time courses can best reflect the attraction and repellence effects of various stimuli on grass carp. The cumulative curves of entering or leaving would be analyzed in the following studies by fitting to a logistic curve of time-course represented by $Y = g / \{1 + \exp[-r(x-a)]\}$, where the coefficients, g, r, and a are constant.

Fig. 3 Time course of the number of grass carp respondent to the stimuli in the maze test using 30 individuals. Values are the means of the triplicate observed values. Circle, triangle, and square indicate the number of grass carp which entered, left, and remained in the test compartment, respectively. The numbers in the circle, triangle, and square indicate the cumulative numbers of grass carp which entered, left, and remained in the test compartment at the end of the test
Fig. 3 (continued)
Table 1 Estimation of the logistic curve from the results of observation with using 30 individuals

| Group | $Y_{E,x}$ | Compartment 1 | $\chi^2$ | df | $Y_{L,x}$ | Compartment 2 | $\chi^2$ | df |
|-------|----------|---------------|--------|----|----------|---------------|--------|----|
| 1     | Dummy    | $g \ r \ a \ \chi^2$ | $g \ r \ a \ \chi^2$ | 8   | Dummy    | $g \ r \ a \ \chi^2$ | $g \ r \ a \ \chi^2$ | 8   |
|       |          | 32 0.5 2.1 5.4t | 34 0.6 1.8 6.5t | 3   |          | 38 0.4 2.3 5.7t | 5 22 0.3 3.6 5.9t | 5   |
|       |          | 27 0.3 5.0 5.6t | 30 0.3 4.5 8.2t | 5   |          | 19 0.2 4.8 8.8t | 4 20 0.1 6.1 2.6t | 3   |
| 2     | Dummy    | $g \ r \ a \ \chi^2$ | $g \ r \ a \ \chi^2$ | 7   | Dummy    | $g \ r \ a \ \chi^2$ | $g \ r \ a \ \chi^2$ | 7   |
|       |          | 50 0.4 4.3 3.9t | 60 0.7 2.1 3.9t | 2   |          | 28 0.6 1.9 8.4t | 6 36 0.6 1.7 6.7t | 4   |
|       |          | 27 0.7 1.6 3.5t | 43 1.0 1.7 5.1t | 3   |          | 22 0.7 1.6 7.5t | 6 27 1.0 1.5 9.1t | 5   |
| 3     | Dummy    | $g \ r \ a \ \chi^2$ | $g \ r \ a \ \chi^2$ | 6   | Dummy    | $g \ r \ a \ \chi^2$ | $g \ r \ a \ \chi^2$ | 6   |
|       |          | 60 0.5 3.8 3.5 | 59 0.4 2.5 8.9t | 8   |          | 48 0.4 2.1 3.1t | 3 58 0.5 2.4 3.9t | 5   |
|       |          | 65 0.3 3.5 5.2t | 58 0.4 2.9 7.7t | 6   |          | 41 0.5 1.8 3.4t | 4 43 1.0 1.4 7.7t | 5   |
| 4     | Dummy    | $g \ r \ a \ \chi^2$ | $g \ r \ a \ \chi^2$ | 5   | Dummy    | $g \ r \ a \ \chi^2$ | $g \ r \ a \ \chi^2$ | 5   |
|       |          | 38 0.4 4.7 6.5t | 40 0.6 2.8 6.1t | 4   |          | 36 0.2 5.5 5.5t | 4 40 0.4 2.3 7.2t | 4   |
|       |          | 14 0.7 1.9 5.8t | 28 0.4 2.0 6.4t | 4   |          | 26 0.5 2.7 28t | 2 75 0.2 6.2 36t | 3   |
| 5     | Dummy    | $g \ r \ a \ \chi^2$ | $g \ r \ a \ \chi^2$ | 4   | Dummy    | $g \ r \ a \ \chi^2$ | $g \ r \ a \ \chi^2$ | 4   |
|       |          | 26 0.4 2.9 7.8 | 25 0.3 2.7 7.4t | 4   |          | 54 0.3 2.1 5.4t | 3 60 0.5 3.5 8.8t | 7   |
|       |          | 60 0.2 4.4 6.4t | 14 0.7 1.5 5.6t | 7   |          | 47 0.4 1.8 7.3t | 5 67 0.5 2.3 7.0t | 3   |
| 6     | Dummy    | $g \ r \ a \ \chi^2$ | $g \ r \ a \ \chi^2$ | 3   | Dummy    | $g \ r \ a \ \chi^2$ | $g \ r \ a \ \chi^2$ | 3   |
|       |          | 65 0.5 3.0 5.6t | 70 0.2 4.9 7.8t | 3   |          | 112 0.4 3.5 7.4 | 3 65 0.5 2.7 0.8t | 4   |
|       |          | 58 0.5 3.4 8.9 | 92 0.4 3.5 12.0 | 3   |          | 102 0.4 2.3 3.2t | 2 76 0.2 5.5 1.7t | 5   |
| 7     | Dummy    | $g \ r \ a \ \chi^2$ | $g \ r \ a \ \chi^2$ | 2   | Dummy    | $g \ r \ a \ \chi^2$ | $g \ r \ a \ \chi^2$ | 2   |
|       |          | 11 0.2 5.8 3.6t | 10 0.1 5.7 10.4t | 6   |          | 28 0.5 2.7 4.6t | 7 42 0.5 2.6 4.8t | 4   |
|       |          | 13 0.2 5.7 5.9t | 5 0.1 5.1 0.8t | 7   |          | 28 0.3 3.4 1.5t | 7 42 0.3 3.2 6.5t | 6   |
| 8     | Dummy    | $g \ r \ a \ \chi^2$ | $g \ r \ a \ \chi^2$ | 1   | Dummy    | $g \ r \ a \ \chi^2$ | $g \ r \ a \ \chi^2$ | 1   |
|       |          | 36 0.7 4.7 3.0t | 28 0.7 5.4 6.3t | 9   |          | 32 0.6 2.1 3.4t | 3 28 0.4 2.9 7.3t | 5   |
|       |          | 36 0.3 3.8 2.7t | 32 0.2 5.0 6.1t | 3   |          | 38 0.2 5.4 6.5t | 4 30 0.2 4.8 2.1t | 5   |

$^t P(\chi^2 > X^2_{0.05}) > 0.05$. $Y_{E,x}$ and $Y_{L,x}$ show the numbers of grass carp which entered and left the test compartment, respectively.

Table 2 General evaluation of the 16 kinds of test substances as grass carp attractants through the observations of exploratory and feeding

| Stimuli     | Behavior               | Entering times | Exploratory and feeding | General evaluation |
|-------------|------------------------|----------------|-------------------------|--------------------|
| Dummy       | Little                 | None           |                         | None               |
| Taurine     | Strong                 | Strong and ceaseless | High                  |
| Dummy       | Little                 | None           |                         | None               |
| Glycine     | Moderate               | Strong         |                        | Moderate           |
Through a series of calculations on the data in Fig. 3, Table 1 shows the fitness and the coefficients of logistic curves to the two behavioral time-courses. The chi-square value of the entering logic curve is relatively small, which indicates that the time course of grass carp entering the compartments was more consistent with the fitting curve than that of fish leaving. Behaviorally, taking taurine and glycine as examples, Table 2 describes some specific behaviors of grass carp after checking the video of the experimental fish entering the test compartment. Grass carp entered the experimental area more times than the control group and showed a certain feeding reaction. Meanwhile, when further multiplying \( g \) by \( r \) of the entering time course for the two stimuli, the product of \( g \) and \( r \) values of each stimulus was higher than that of the entering time-course in the dummy group (Table 3). This means that the coefficient \( gr \) of the entering time course can be used as a single attractive index for the evaluation of the attractive effect. So we will evaluate the attraction effect of attractants by the entering time courses using the product of \( gr \).

Through a series of calculations on the \( g \) and \( r \) of the entering course in Table 1, Table 3 can be obtained and shows the coefficient values (the product of \( gr \) in the entering time course) of sixteen different substances and the respective dummy. By comparing the coefficient \( gr \) between each test substance and the dummy, the attractive effect of the test substance was obtained.

It is shown in Table 3, among these 16 stimulating species, that DMPT and glycine have a strong attractive effect (\( gr_{stimulant} / gr_{dummy} \geq 2 \)), and DMT, l-proline, l-alanine, l-arginine, taurine, and l-glutamic acid have an attractive effect on grass carp slightly weaker (\( 1.2 < gr_{stimulant} / gr_{dummy} < 2 \)), while TMAO, l-cysteine, and l-threonine do not attract or inhibit fish (\( 0.8 < gr_{stimulant} / gr_{dummy} < 1.2 \)), and l-methionine, phenylalanine, l-lysine, l-valine, and l-histidine show an inhibitory effect (\( 0 < gr_{stimulant} / gr_{dummy} < 0.8 \)) on grass carp.

Response amplitude of EOG

The stimulating effect of eight substances selected by the maze experiment on the olfactory sense of grass carp was tested through the microelectrode system. The EOG response of grass carp to the stimuli is a transient biphasic potential change (take taurine as an example, shown in Fig. 4). Upon termination of the stimuli of amino acids, the EOG response returns quickly to baseline.

Table 4 shows the EOG response potential amplitude of grass carp to the eight stimuli. At the same time, it can be proven that the substances selected by...
the maze experiment have a stimulating effect on the smell of grass carp, and it can be seen that the EOG response potential amplitude increases with the concentration of each stimuli. What is interesting is that the sorting of EOG response potential amplitude of the 8 stimuli is inconsistent in each concentration. Tukey’s multiple-range test indicated that when the response intensity is $10^{-3}$ mol/L, the order of stimuli intensity from low to high was as follows: taurine, L-proline, glycine, DMPT, L-glutamic acid, L-alanine, L-arginine, and DMT. When the response intensity is $10^{-2}$ mol/L, the order of stimuli intensity from low to high was as follows: taurine, glycine, L-glutamic acid, L-proline, L-arginine, DMPT, L-alanine, and DMT. When the response intensity is $10^{-1}$ mol/L, the order of stimuli intensity from

Table 4 The comparison of EOG responses of grass carp to eight kinds of stimuli at different concentration (μV)

| Concentration | 10$^{-3}$ mol/L | 10$^{-2}$ mol/L | 10$^{-1}$ mol/L |
|---------------|-----------------|-----------------|-----------------|
| L-Alanine     | 117.7 ± 6.2Ac   | 457.9 ± 19.2Bd  | 567.0 ± 15.6Cde |
| Taurine       | 49.3 ± 2.9Aa    | 80.2 ± 2.7Ba    | 161.9 ± 5.6Ca   |
| L-Proline     | 62.4 ± 1.8Aab   | 252.0 ± 8.0Bb   | 380.9 ± 15.0Cb  |
| Glycine       | 85.1 ± 6.9Aabc  | 226.3 ± 13.3Bb  | 485.5 ± 15.8Ccd |
| DMPT          | 94.6 ± 9.2Abc   | 455.1 ± 17.9Bd  | 612.1 ± 18.4Cef |
| DMT           | 231.7 ± 16.2Ae  | 573.7 ± 8.5Be   | 659.9 ± 20.4Cf  |
| L-Glutamic acid| 105.6 ± 10.3Ac  | 227.9 ± 17.9Bb  | 347.7 ± 25.9Cb  |
| L-Arginine    | 166.2 ± 8.9Ad   | 346.7 ± 19.4Bc  | 416.1 ± 21.0Bbc |

Two-way ANOVA

| Factors        | F value | P > F |
|----------------|---------|-------|
| Concentration  | 1145.4  | 0.000 |
| Stimuli source | 212.2   | 0.000 |
| Concentration × stimuli source | 29.3 | 0.000 |

All data were expressed as means ± SE (n = 3). Different lowercase letters after the same row data indicate a significant difference between different stimuli (P < 0.05). Different capital letters after the data in the same column indicate significant difference between different concentrations of each stimulus (P < 0.05).
low to high was as follows: taurine, L-glutamic acid, L-proline, L-arginine, glycine, L-alanine, DMPT, and DMT. Two-way ANOVA confirmed that concentration × stimuli have a significant synergetic effect on olfactory electrical response of grass carp.

The biting-balls test

For each substance, three experimental groups (concentrations) and a dummy group were set to choose the optimal concentration by comparing the biting times. It is shown in Fig. 5 that the biting times of the eight substances selected in the maze test showed different degrees of attraction and repulsion to grass carp. Generally, the biting frequency of DMPT, DMT, glycine, L-arginine, L-glutamic acid, and L-alanine was significantly higher than that of the dummy group, and grass carp showed a strong desire to eat these bait balls. In contrast, L-proline showed food rejection as indicated by that the biting frequency of the L-proline bait ball was significantly lower than that of the dummy bait ball. When reviewing the video, it could be seen that most of the experimental fish gathered near the dummy bait ball. There were relatively few fish baiting the ball added with L-proline. In fact, the experimental fish biting the L-proline bait ball did not leave immediately, but continued to hover between the two bait balls and to choose a ball to eat. In the biting test, taurine showed no significant effect on grass carp. Specifically, at $10^{-1}$ mol/L, the number of biting times of DMT is significantly higher than that of the

![Fig. 5](https://example.com/fig5.png)

**Fig. 5** The biting-balls test result of grass carp responding to the 8 stimuli at 3 different concentrations. All data were expressed as means ± SE (n = 3). Different letters indicate the significant difference in the biting-balls times between the 4 concentrations of the same substance by Tukey’s test after one-way ANOVA (P < 0.05)

| Formula | DMT (mol/L) | Glycine (mol/L) | L-Glutamic acid (mol/L) | L-Arginine (mol/L) |
|---------|-------------|-----------------|------------------------|-------------------|
| 1       | $2 \times 10^{-1}$ | $2 \times 10^{-2}$ | $2 \times 10^{-2}$ | $2 \times 10^{-2}$ |
| 2       | $2 \times 10^{-1}$ | $1 \times 10^{-2}$ | $1 \times 10^{-2}$ | $1 \times 10^{-2}$ |
| 3       | $2 \times 10^{-1}$ | $5 \times 10^{-3}$ | $5 \times 10^{-3}$ | $5 \times 10^{-3}$ |
| 4       | $1 \times 10^{-1}$ | $2 \times 10^{-2}$ | $1 \times 10^{-2}$ | $5 \times 10^{-3}$ |
| 5       | $1 \times 10^{-1}$ | $1 \times 10^{-2}$ | $5 \times 10^{-3}$ | $2 \times 10^{-2}$ |
| 6       | $1 \times 10^{-1}$ | $5 \times 10^{-3}$ | $2 \times 10^{-2}$ | $1 \times 10^{-2}$ |
| 7       | $5 \times 10^{-2}$ | $2 \times 10^{-2}$ | $5 \times 10^{-3}$ | $1 \times 10^{-2}$ |
| 8       | $5 \times 10^{-2}$ | $1 \times 10^{-2}$ | $2 \times 10^{-2}$ | $5 \times 10^{-3}$ |
| 9       | $5 \times 10^{-2}$ | $5 \times 10^{-3}$ | $1 \times 10^{-2}$ | $2 \times 10^{-2}$ |
other three concentrations. The biting times of glycine, L-glutamic acid, and L-arginine at 10^{-2} \text{ mol/L} are significantly different from that of the control group.

Taking into account the cost issues such as the price and addition amount of stimuli, four substances, DMT, glycine, L-glutamic acid, and L-arginine, were selected for further investigation of the attractive effect of their compounds. The best concentration determined and two more concentrations which were half or twice of the optimal attractant concentration, respectively, of each stimulus were set, and the \text{L}_{9}(3^{4}) orthogonal table was used to design 9 formulas (Table 5) for further comparing the number of biting times.

Fig. 6 shows that formulas 2, 5, and 9 have the highest number of pecks, all of which are significantly different from the control group. When comparing formula 2, 5 and 9, formula 9 is the most attractive to grass carp (Fig. 6d).

**Discussion**

The fishing bait needs to be based on an odor source that releases feeding attractants to elicit the food search behavior of the target species (Løkkeborg et al. 2014). The olfactory system of fish can receive signals from a long distance, which makes it very important to obtain information about food supply and existence and to choose the most suitable search direction (Kasumyan 2004). Most fish completely lose their foraging performance after losing their sense of smell and cannot find the odor source even at high concentrations of food substances (Hara 1975; Kasumyan 1997). In contrast, the taste system
determines whether the fish will accept the food (Ishida and Kobayashi 1992; Kasumyan and Sidorov 2012). Based on these theories, this is the first study to explore the substances that may stimulate grass carp’s sense of smell (do not exclude other systems that play a role in foraging behavior) by the behavioral maze test and EOG test. The maze test results showed that DMPT and glycine have a strong attractive effect on grass carp. DMT, L-proline, L-alanine, L-arginine, taurine, and L-glutamic acid have a slightly weaker attractive effect on grass carp. At the same time, TMAO, L-cysteine, and L-threonine do not affect (attract or repel) grass carp, and L-methionine, phenylalanine, L-lysine, L-valine, and L-histidine even showed inhibitory effects on grass carp (Table 3). The results indicate that there were significant differences in the attractiveness of different substances to grass carp. This is consistent with the conclusions of other fish studies (Harada et al. 1988, 1995; Harada and Miyasaki 1993). An interesting phenomenon was discovered in the labyrinth experiment. For example, DMPT, DMT, L-proline, glycine, and L-alanine have an obvious attraction for grass carp; after entering the test area, the grass carp slowly enter the bottom of the test compartment and gather at the bottom, showing obvious feeding behaviors such as pecking at the glass wall. This phenomenon is the same as that of the experiment by Harada et al. who investigated the attraction effect of sugars to the yellowtail (Seriola lalandi) (Harada et al. 1995). The behavioral biting-ball test also showed that these substances can stimulate the feeding response of grass carp. The attractive olfactory effect of these substances on fish has also been reported in other fish species.

It was observed that DMPT has a strong attraction to carp (Cyprinus carpio) (Kenji et al. 1989). L-Alanine has a significant effect on carp attraction and exploration (Saglio et al. 1990). It has also been proven that glycine has an attractant effect on the crucian carp (Carassius auratus auratus) (Wu et al. 1993). This may also explain that the bait of grass carp added with DMPT and glycine also attracts the most common carp and crucian carp in Chinese waters (personal observations during fishing). The results of the present study showed that arginine could attract grass carp, but Kasumyan and Moris found that L-arginine can repel common carp (Kasumyan and Morsi 1996). This result also has theoretical support for the production of special grass carp bait. For example, in Wuhan, China, the citizens like grass carp but not common carp. The bait of grass carp added with L-arginine can repel the common carp, reduce their chance of hooking, thus to focus on grass carp fishing.

The results of the EOG test confirmed the stimulating effect of eight substances screened by the behavioral maze test on grass carp olfactory. All of the eight substances had different degrees of stimulation on the olfactory function of grass carp. In terms of the waveform, the olfactory response of the grass carp recorded in this experiment is a transient biphasic potential change (Fig. 4). The waveform of this experiment is the same as that of gibel carp (Carassius auratus gibelio) to amino acids (Zhao 2007). In contrast, the EOG responses recorded during electrophysiological studies on channel catfish (Ictalurus punctatus), white catfish (Ictalurus catus), and sea catfish (Arius felis) are all transient single-phase negative potentials (Caprio 1980; Yamada and Nakatani 2001). It is also worth noting that Johnsen et al. used aocalm electrode and a Grass P-18 preamplifier to study the EOG response of grass carp to amino acids and the recorded waveform was single-phase wave, and the unit of amplitude was mV (Johnsen et al. 1988), which was different from our experimental results. The reason may be that different electrodes can be used to obtain EOG signals and the characteristic of these signals change depending on the structure and placement of the electrodes (López et al. 2016). The ME-1 microelectrode amplifier produced by Taimeng company is used in this experiment; the ME-1 microelectrode amplifier is composed of electrode and amplifier. It has a simple structure and limited amplification. This leads to different units of EOG response amplitude of grass carp. On the other hand, the EOG signal is the result of many individual receptor cell currents producing a recordable voltage drop over the extracellular pathway completed through the mucus and water (Getchell 1974). The relative position of the recording and reference electrodes, their internal resistance, and the conductivity of the water flowing over the epithelium will affect the signal amplitude; the relative position of the recording and reference electrodes, their internal resistance, and the conductivity of the water flowing over the epithelium will affect the signal amplitude (Zhao 2007).

In the present study, the EOG response amplitude of 8 kinds of stimuli was different at the same
It should be pointed out that in terms of the response amplitude of EOG, the order of stimuli at $10^{-3}$ mol/L concentration are $\text{l-arginine} > \text{l-alanine} > \text{l-glutamic acid} > \text{glycine} > \text{l-proline}$ (Table 4). The results support the conclusion of a previous study on grass carp olfaction (Johnsen et al. 1988). Previous studies have shown that among the 19 amino acid stimuli at the tested concentrations, l-alanine all had the strongest response to the rabbitfish ($Siganus fuscescens$ Houttuyn) (Ishida and Kobayashi 1992), and young yellowtail (Kohbara et al. 2000). But, under the stimuli of $10^{-3}$ mol/L amino acids, l-lysine had the largest response to carp (Goh and Tamura 1978). In this experiment, the response amplitude of grass carp to DMT was the largest at the three different concentrations. It can be concluded that although the response of fish to amino acids in fish olfactory electrophysiological experiments is not species-specific, the response intensity of different fish species to different amino acids varies greatly.

In this study, the response of grass carp to DMT, l-arginine, l-glutamic acid, and glycine in general was strong. The biting-ball test results showed that the behavior of grass carp to DMT was the strongest in this experiment, which was the same as that of the olfactory electrophysiology experiment. It can be concluded that DMT is an effective attractive monomer for grass carp. However, it should be noted that the results of biting behavioral responses induced by stimulus were not completely the same as those of olfactory electrophysiology. Our results showed that l-arginine had a great effect on the olfactory function of grass carp and stimulated the biting response at higher concentrations. However, l-arginine at a concentration of $10^{-3}$ mol/L has a strong response in electrophysiological experiments but showed a slight inhibitory effect on the feeding of grass carp in the behavioral biting-balls test. The same phenomenon was found in the experiment of crucian carp (Zhao 2007), which indicated that the olfactory electricity experiment could not totally distinguish the substances that could attract fish.

In the biting-balls test, grass carp eats small pieces of dough into the mouth. This process is a comprehensive function of smell and taste and other sensory systems. The taste system plays a major role in this process because evaluation by fish of taste characteristics of the food and its agreement with the food requirements significantly depends on the functioning of the taste system (Chaudhari et al. 2009). The biting frequency of DMPT, DMT, glycine, l-arginine, l-glutamic acid, and l-alanine was significantly higher than that of the dummy group, and grass carp showed a strong desire to eat the bait. But l-proline showed food rejection, and taurine showed no significant difference (Fig. 5). The behavioral biting responses of the eight substances screened by the maze test were not completely consistent with those of the maze test, which all had an attractive effect on grass carp. This may be the fact that the feeding mechanism of grass carp is affected by olfactory, gustatory, and other sensory mechanisms. As a result, l-proline and taurine are not effective to improve swallowing. In fact, it was found that l-proline could attract grass carp’s sense of smell (Table 3), but when grass carp really ate it, it showed a repellant response. Similarly, Kasumyan et al. reported that the oral taste system of grass carp rejected l-proline and that glycine and arginine promote food intake (Kasumyan and Döving 2003). This is consistent with our experimental results. Interestingly, he also found that l-cysteine can promote feeding activity in the oral gustatory system of grass carp, but we removed this substance because it cannot attract the smell of grass carp in the maze test (Table 3).

The orthogonal experiment showed that formula 9 containing DMT, glycine, glutamic acid, and l-arginine showed stronger phagocytosis than the control group and other formulas. In the experimental video, it was observed that grass carp kept not only biting at the bait ball containing these stimuli, but also shaking their head to tear off small pieces of dough after biting it. In the actual fishing, the author used the bait added with formula 9 and achieved to catch many grass carp of different sizes, in association with crucian carp and common carp.

To conclude, this study combined behavioral and physiological tests to systematically elaborate the feeding promoting effects of 16 kinds of stimuli that can promote the feeding activity of different kinds of fish on grass carp, and finally came to a formula composed of DMT, glycine, l-glutamic acid, and l-arginine that can stimulate the smell and taste systems of grass carp, lure the fish close to the bait, and promote the feeding activity of grass carp. With the rise of the leisure fishing industry, adding the compound attractants to fishing bait can provide...
anglers with fishing pleasure and it also has good economic prospects. Furthermore, the results of the current study might also provide some guideline to the feed formula design for the grass carp culture.

Author contribution All authors contributed to the study conception and design, material preparation, data collection, and analysis. The first draft of the manuscript was written by Haojie Yu, while XiaoYu Wang, Fanshuang Kong, and Qingsong Tan commented on previous versions of the manuscript. Qingsong Tan received the funding. All the authors read and approved the final manuscript.

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Data availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Code availability Not applicable

Declarations

Ethics approval With the submission of this manuscript, I would like to confirm that the abovementioned manuscript has not been published elsewhere, accepted for publication elsewhere, or under editorial review for publication elsewhere and that my Institute’s Key Laboratory of Freshwater Animal Breeding, Ministry of Agriculture of China/Hubei Provincial Engineering Laboratory for Pond Aquaculture/College of Fisheries, Huazhong Agricultural University, Wuhan 430070, China representative is fully aware of this submission. All procedures were approved by the Institutional Animal Care and use Committee (IACUC) of Huazhong Agricultural University (Wuhan, China) for laboratory animal use. Culturing of fish was performed according to the common Organization for Economic Cooperation and Development (OECD) protocol for fishes.

Consent to participate Agree

Consent for publication Agree

Conflict of interest The authors declare no competing interests.

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