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Determinants of submerged macrophytes palatability to grass carp *Ctenopharyngodon idellus*

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ABSTRACT

Submerged macrophytes reestablishment is one of key processes in restoration of water ecological system. However, adverse effect caused by grass carp on macrophyte communities is existed during the process of ecological restoration. At present, little is known about determinants of submerged macrophytes palatability to grass carp. In this study, we measured the relative consumption rates (RCR) of grass carp on four species of submerged macrophytes *Hydrilla verticillata*, *Vallisneria natans*, *Ceratophyllum demersum*, and *Myriophyllum spicatum*, analysed the correlation between macrophytes traits and RCR, then investigated the kinds of flavonoids in four species of submerged macrophytes and their effects on the palatability for grass carp. The results showed that RCR of grass carp on *H. verticillata* and *V. natans* were much higher than *C. demersum* and *M. spicatum*. And for each submerged macrophyte, RCR of grass carp declined in the order of July > October > May. Stepwise multiple liner regression analyses showed that water temperature and protein were positively correlated, and flavonoids were negatively correlated, with RCR of grass carp. Rutin, quercitrin, quercetin and kaempferol, as the typical kinds of flavonoids, were in different contents in four kinds of macrophytes. And rutin, quercetin and kaempferol can deter grass carp feeding. These results conducted that grass carp favoured macrophytes with high protein and ate less macrophytes with high flavonoids. And it is helpful to provide fundamental basis for administrators to select appropriate macrophytes in restoration of water ecological system.

1. Introduction

Herbivory plays a fundamental role in reducing primary producer abundance and species diversity by top-down regulation (Wood et al., 2017). As a result, herbivores alter the functioning of aquatic ecosystems such as nutrients transportion and cycling, habitat for other organisms, weakening the growth and reproductive capacity of macrophytes (Bakker et al., 2016). As one of the typical aquatic herbivores, grass carp *Ctenopharyngodon idellus*, famous for high feeding rate, can consume up to 27.6 kg of vegetation per kg of fish per year and cause macrophyte damage and uprooting by foraging activity (Kapuscinski et al., 2015; van der Lee et al., 2017). However, there exists different palatability of different macrophytes for grass carp (Parker and Hay, 2005).

Up to now, there have been many studies about the factors determining the palatability for aquatic herbivores. Structural determinants of plants play important roles in palatability for herbivores (Elger and Willby, 2003; Elger and Lemoine, 2005). The invasive freshwater snail *Pomacea canaliculata* avoided eating macrophytes with high dry matter content (DMC) (Wong et al., 2010) and amphipod *Orchestoidea tuberculata* preferred fresh *Macrocystis integrifolia* over *Lessonia nigrescens* since *L. nigrescens* has firmer physical features (Duarte et al., 2014). On the contrary, plants with high protein content are more vulnerable to herbivores since herbivores are normally nitrogen-limited (Cruz-Rivera and Hay, 2003; Sanchez and Trexler, 2016). Preference on plants may be related to the presence of deterrent secondary chemical compounds in plants. Kapuscinski et al. (2014) found that palatability for rudd *Scardinius erythrophthalmus* was negatively affected by soluble phenolic compounds. Similar to rudd, generalist isopod grazer *Idotea baltica* and isopod *Idotea chelipes* showed increasing resistance to alga with high chemical determinants (Nylund et al., 2011; Martínez-Crego et al., 2016). And the palatability for grass carp was intensively concerned with plant secondary metabolites (phenolic) and stoichiometry (C:N ratio, C:P ratio and N:P ratio) (Parker et al., 2006; Dorenbosch and Bakker, 2011). However, less is known about the flavonoids, a common kind of plant secondary metabolites, have a positive or negative effect

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on the palatability of plants for grass carp. Furthermore, flavonoids contain many compounds in plants and what kinds of compounds deter against grass carp feeding have not been studied.

Submerged macrophytes, the main primary producers in freshwater ecosystems, play vital roles in reestablishment of degraded water ecological system by maintaining ecological integrity and stability, purifying water quality, and increasing the diversity of the physical habitats (Wang et al., 2014; Bolduc et al., 2016). However, grass carp via feeding can cause large influences on submerged macrophytes such as weakening the growth and reproductive capacity of macrophytes and influencing macrophyte community succession (Dorenbosch and Bakker, 2012). Therefore, it is crucial to investigate the determinants of submerged macrophytes palatability to grass carp and to offer managers useful information to develop strategies for the restoration of ecosystems containing submerged macrophytes.

Here, we measured the relative consumption rate (RCR) of grass carp on four species of submerged macrophytes *Hydrilla verticillata*, *Vallisneria natans*, *Ceratophyllum demersum,* and *Myriophyllum spicatum*. And each submerged macrophyte was further analysed for physical, nutritional, and chemical traits to assess whether any of these parameters were correlated with the palatability for grass carp. Additionally, we investigated the contents and amounts of flavonoids in four species of submerged macrophytes.

2. Materials and methods

2.1. Collection of fish and macrophytes

Grass carp were obtained from Linghu fishery (Huzhou, Zhejiang Province, China) and were reared in laboratory conditions before experiments. Four species of fresh submerged macrophytes *H. verticillata*, *V. natans*, *C. demersum,* and *M. spicatum* were collected from West Lake (30°14′27″N, 120°7′11″E, Hangzhou, Zhejiang Province, China). Marl and periphyton coverage on macrophytes without roots were washed away under running water before feeding experiments and traits analysis. All applicable institutional and/or national guidelines for the care and use of animals were followed.

2.2. Assessment the palatability of macrophytes for grass carp

We did the experiments with the same method in three different months: May, July, and October, respectively. Feeding assays were done in aquaria (100 cm × 50 cm × 50 cm), in which the recirculating water flow rates were maintained at 25 L/min and the water was filtered through biofilters to remove fish feces and reduce the ammonia concentration. To avoid stressful conditions caused by low oxygen concentrations, aeration was maintained at a constant level in aquaria. In each aquarium, there were five grass carp (mean ± SD, weight: 80 ± 5 g; total length: 19 ± 0.5 cm) that had been starved for 24 h before feeding assays.

Each of four species of fresh submerged macrophytes (200 g) was added to six aquaria, with six replicates for each species for a total of 24 aquaria. During the experiments, there was no mortality of grass carp. And the physico-chemical parameters of water quality in each month were same except for the water temperature (mean ± SD, May: 21.66 ± 0.63 °C; July: 28.33 ± 0.92 °C; October: 19.27 ± 0.58 °C, d.f. = 2, $F = 190.494$, $P < 0.001$). Given autogenic mass change in the macrophytes, the paired control in other six aquaria contained water and macrophytes without grass carp. Grass carp were permitted to feed for 24 h. After removing excess water with a paper towel, we weighed macrophyte biomass at the beginning and the end of the experiments. RCR was calculated as $[H_0 \times C_f/C_0 - H_f]/W \times 100\%$, Where H_0 and H_f were the wet masses of macrophytes exposed to the grass carp before and after the experiment, respectively; C_0 and C_f were the wet masses of macrophytes from the paired control before and after experiment; W was the mass of grass carp (Wong et al., 2010).

2.3. Measurement the traits of submerged macrophytes

Three aspects of macrophyte traits were measured: physical properties (dry matter content (DMC)), nutritional contents (soluble protein and lysine) and chemical contents (tannins and flavonoids). All trait measurements were implemented on randomly chosen plant samples. DMC was determined following Elger and Lemoine (2005). Soluble protein content of macrophytes was determined at 595 nm after grinding the plant material and using coomassie brilliant blue G-250 to react. Lysine was measured at 500 nm after degreasing dried ground macrophytes with 60–90 °C petroleum ether for 8 h and addition of ninhydrin. The amount of tannins was estimated using permanganate titration methods, where solution turned to be bright golden yellow after slow addition of 0.1 mol/L^1 $KMnO_4$. The concentration of flavonoids was analysed at 510 nm after addition of 5% mass/volume $NaNO_2$, 10% mass/volume $Al(NO_3)_3$ and 4% mass/volume NaOH into the 70% ethanol extract. All analyses for three individuals of the same macrophytes in each month were performed.

2.4. HPLC–MS/MS analysis of flavonoids

2.4.1. Chemicals, plant materials and sample preparation

The reference standards of rutin, quercitrin, quercetin and kaempferol were purchased from Chengdu Pufei Biotech (Chengdu, China). Formic acid and methanol of HPLC grade were provided by TEDIA Company Inc. (Fairfield, OH, USA).

Four species of fresh submerged macrophytes *H. verticillata*, *V. natans*, *C. demersum* and *M. spicatum* were lyophilized at −70 °C for 24 h to a constant weight and then finely ground to a homogeneous consistency by sieving through a 149-μm mesh screen. First, 1 g powered sample of each submerged macrophyte were extracted 3 times with 20 mL 70% methanol in an ultrasonic bath (30 min each, ambient temperature). After centrifugation the exacts were combined and subjected to liquid-liquid partition with petroleum ether (b.p. 60–90 °C) to remove chlorophyll thrice. Next, the exacts were evaporated under reduced pressure at 40 °C to afford the crude residues. Later, the crude extracts were dispersed in 70% methanol (10 mL) and were filtered through membrane filter (0.22 μm). Finally, the filters were stored at −20 °C for the subsequent analysis. All analyses for three individuals of the same macrophytes collected on May were performed after stepwise multiple liner regression analysis between multiple traits and RCR.

2.4.2. Instrumental conditions

LC analyses were performed on a Waters Acquity ultraperformance liquid chromatography system with column oven temperature maintained at 40 °C, using an Acquity BEH C_{18} column (50 mm × 2.1 mm i.d., 1.7 μm particle size) (Waters, Milford, MA, USA). The mobile phase was constituted by solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in methanol). The injection volume was 10 μL. The flow rate was 0.2 mL/min with a linear gradient at the following conditions: 0–15 min, 10%–90% B; 15–16 min, 90%–100% B; 16–21 min, 100% B; 21–21.1 min, 100%–10% B; 21.1–25 min, 10% B.

The UHPLC system was coupled to a Micromass Xevo TQ triple quadrupole mass spectrometer (Waters, Manchester, UK) fitted with an electrospray ionization (ESI) source. Typical source conditions for maximum intensity of precursor ions were as follows: capillary voltage, 3.0 kV; source temperature, 150C; desolvation temperature, 350C; cone gas (N_2) flow rate, 0 L/h; desolvation gas (N_2) flow rate, 650 L/h. For all compounds, the MS instrument was operated in the ESI positive mode and the data was acquired in multiple reaction monitoring (MRM) mode. The collision gas flow was set at 0.16 mL/min. Optimized MS/MS transitions as well as specific cone voltages and collision energies are summarized in Table 1. Auto dwell time was applied to ensure that approximately 15 data points were acquired for each chromatographic peak.

Six different concentrations (1.0–333 μg/L) of each standard were