

Chemical Cues which Include Amino Acids Mediate Species-Specific Feeding Behavior in Invasive Filter-Feeding Bigheaded Carps

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Abstract This study tested whether and how dissolved chemicals might assist food recognition in two filter-feeding fishes, the silver (*Hypophthalmichthys molitrix*) and the big-head carp (*H. nobilis*). These species evolved in Asia, are now invasive in the Mississippi River, and feed voraciously on microparticles including plankton. The food habits and biology of these carps are broadly similar to many filter-feeding fish, none of whose chemical ecology has been examined. We conducted five experiments. First, we demonstrated that buccal-pharyngeal pumping (BPP), a behavior in which fish pump water into their buccal cavities, is responsible for sampling food: BPP activity in both silver and bighead carps was low and increased nearly 25-fold after exposure to a filtrate of a planktonic food mixture ($P < 0.01$) and over 35-fold when planktonic food was added ($P < 0.001$). Next, we showed that of nine food filtrates, the one containing chemicals released by spirulina, a type of cyanobacterium, was the most potent planktonic component for both species. The potency of filtrates varied between species in ways that reflected their different chemical compositions. While L-amino acids could explain about half of the activity of food filtrate, other unknown chemical stimuli were also implicated. Finally, occlusion experiments showed the olfactory sense has a very important, but not exclusive, role in bigheaded carp feeding behaviors and this might be exploited in both their control and culture.

Keywords Asian carp · Microphagy · Filter-feeding · Amino acids · Olfaction · Introduced species

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Introduction

Although hundreds of species of adult fish feed on small planktonic and detrital food by filtering these tiny food items through and across their gills (Gee 1989; Lazzaro 1987; Moyle and Cech 2004), the role that chemical cues might play in food recognition by these fishes has not yet been elucidated. Nevertheless, a role for chemical cues in filter-feeding seems likely because the extremely small size of their planktonic food and the turbidity of many waters limit the utility of visual cues in food finding. Indeed, many filter-feeding fishes appear to possess well-developed olfactory systems as well as specialized internal taste organs, or epibranchial organs (Bauchot et al. 1993; Hansen et al. 2014; Hansen and Zielinski 2005; Lazzaro 1987; Sinha 1987). The diets of many filter-feeding fishes are also broad and include many forms of fine particulate food in addition to plankton which seemingly puts these fish at risk of eating inedible or even toxic water-borne particulates unless these can be identified in advance. The present study investigated how chemical food cues might be discerned by two species of filter-feeding fishes from the genus *Hypophthalmichthys* (also known as bigheaded or sometimes “Asian” carps), one of the world’s most important genera of fish as it has both been widely cultured for consumption by humans but has also escaped to become one of our most invasive fishes.

Silver (*Hypophthalmichthys molitrix*) and bighead (*H. nobilis*) carp are native to large rivers in east Asia (Kolar et al. 2005; Tang et al. 2013; Wilson 2014) where they exist as voracious filter feeders that have specialized, fine gill rakers and well-developed epibranchial organs that they seem to use to taste and package particulates in mucous for ingestion (Hansen et al. 2014). Feeding studies in laboratory tanks and ponds have shown that both species of carp consume a wide array of plankton and particulates including zooplankton, phytoplankton, bacteria (including many types of cyanobacteria),

and detritus, with silver carp consuming slightly smaller food items (down to 10 μm) than bighead carp (Cremer and Smitherman 1980; Dong and Li 1994; Pongruktham et al. 2010; Radke and Kahl 2002; Spataru and Gophen 1985; Wilamowski 1972; Williamson and Garvey 2005; Xie 2001). This broad variety of items contains, and presumably releases, a wide variety of chemicals, some of which are toxic (ex. the cyanotoxins). While several studies of bigheaded carp feeding behavior in ponds and aquaria suggest that these carps may select specific food items, the findings of these studies are inconsistent, perhaps because fish may have been limited by their ability to make clear choices in small arenas (Cremer and Smitherman 1980; Dong and Li 1994). In contrast, in natural systems (large rivers), food selection by filter feeding fish might be better informed and easier to evaluate on both a large scale (e.g. identifying and locating food patches in unproductive water), and/or on a smaller scale e.g. identifying and selecting food items within patches. A role for chemoreception in food selection has not been directly tested in any species of marine or freshwater filter-feeding fish.

Filter-feeding can take many forms in fishes and can be energetically expensive because of the high density of water (Jobling 1995; Lazzaro 1987; Sanderson and Cech 1992). One form of filter-feeding uses buccal-pharyngeal pumping (BPP) in which fish actively open their mouths and expand their buccal cavities, thereby pumping large volumes of water that are expelled after passing across the gills. Several hundred species of filter-feeding microphagous fishes, including the big-headed carps use BPP (also termed pump-suspension-feeding [Moyle and Cech 2004]) to both respire and feed (Lazzaro 1987; Smith 1989). These filter-feeding fishes maintain basal pumping rates to acquire oxygen. The pumping rate increases when food is present, presumably increasing the flow of food-laden water across the gill rakers and thence to the epibranchial organs which then package particulates in mucous to be swallowed, perhaps after being crushed by pharyngeal teeth (Dong and Li 1994; Hansen et al. 2014; Kolar et al. 2005; Lazzaro 1987; Smith 1989). However, whether and how dissolved chemicals might increase BPP rates at appropriate times has not been determined. It is possible that in addition to maintaining water flow to acquire oxygen for respiration, BPP activity may perform a role in olfactory sampling (sniffing) as it presumably causes the olfactory pits to open, as noted in both Pleuronectid flounders and gobies (Murphy et al. 2001; Nevitt 1991). The olfactory sampling rate might increase if food is detected providing location information. Enhanced BPP activity might also enable gustatory sampling and swallowing by pumping water into the tubular epibranchial organs, which are rich in taste buds (Hansen et al. 2014). Both solid food and the chemicals it releases could thus be detected by internal taste and tactile receptors. However, whether and how water-borne food chemicals mediate BPP activity and its role(s) in feeding has not to the best of our knowledge been examined in any fish.

This study addressed several questions about the role of chemical cues in filter-feeding in the bigheaded carps. These included: 1) Is food recognition in these carps mediated by BPP activity and food chemicals? 2) Do these bigheaded carps have chemically-mediated feeding preferences and, if so, do the preferences of these species differ from each other and vary by food-type? 3) Does the composition of chemical cues mediate sampling? 4) Do L-amino acids, which have been implicated in food recognition in other fishes (Sorensen and Caprio 1998), have a role in food selection? 5) What role does the sense of smell (vs. taste) play in feeding behavior?

Methods

Experimental Animals

Laboratory reared juvenile silver and bighead carp were obtained from the USGS Columbia Environmental Research Center (Columbia, MO, USA) and held in 200-l circular holding tanks supplied with well-water (1 l/min). Photoperiod was held constant at (16 h:8 h) and water temperature was maintained at 21–23 °C. The silver carp were 16–25 cm in total length and the bighead carp were 5–18 cm in total length. All carp were sexually immature. Once daily, carp were fed a well-established multi-component algal diet comprised of: 19.8 g/L dried spirulina (a type of blue-green cyanobacteria or algae) (www.bulkfoods.com), 11.4 g/L dried *Chlorella* algae (a genus of green algae) (www.bulkfoods.com), 8.7 g/L Oncor FW™ trout pellet crumble (www.skretting.us), 1.1 g/L tropical flake food (www.aquaticceco.com), 1.6 g/L Otohime C1™ marine larval food (www.reed-mariculture.com), 0.7 g/L nannochloropsis 3600 condensed micro-algal culture, 0.7 g/L shellfish 1800 condensed micro-algal culture (www.reed-mariculture.com), 0.6 g/L Cyclopeeze© freeze-dried decapod crustaceans (www.argent-labs.com), and 0.6 g/L soluble vitamin mixture (www.aquaticceco.com) in well water (Robin Calfee, USGS, MO). This complex diet was developed over several years by testing many commercially available foods in various mixtures and formulations (Robin Calfee, USGS, Columbia, MO, USA; personal communication). While it included some items naturally found in rivers (e.g. spirulina), it was not intended to reflect a natural diet. The diet used food items already known to be eaten by cultured fish (e.g. Otohime). When needed for experiments, pairs of carp were transferred into 70-l glass test aquaria (see below).

Behavioral Assay

A feeding behavior assay was developed to quantify BPP activity. In this assay, pairs of either silver or bighead carp (carp are social and thus were kept as pairs) were held in 70-

1 glass aquaria supplied with 21 °C well water (0.5 l per min). Carp were held for 30 days prior to being used for experiments and fed the multi-component food mixture daily to acclimate them to testing conditions. Aquaria were lit by overhead 40-watt incandescent bulbs on a 16 h:8 h photoperiod and isolated from each other by opaque polycarbonate dividers. Each aquarium was also equipped with an air-stone in the upper back corner, below which a 3-ml stimulus delivery syringe was hung from tubing. These syringes were filled with stimuli (food, food filtrates, or chemical stimuli) 30-min in advance of each trial and their contents injected over 1–2 s period during each test period (see below) using a pneumatic tube system which was remotely controlled from behind an opaque observation curtain where observers monitored carp behavior through small holes cut into the curtain. Dye tests using fluorescein dye showed that convection currents produced by the bubbles lead to complete dilution of test stimuli throughout aquaria within 1-min.

One test was conducted per day on each pair of carp. Each test included a pre-test observation period and a test period. The order in which fish was tested with specific stimuli was determined by a Latin square design blocked by day and tank with the order of stimulus presentation being randomized. For tests, the BPP activity of one fish of each pair (selected by random at the start of each experiment) was monitored visually by an observer during an 8-min pre-test, then 3-ml of the appropriate test (or control) stimulus was injected into their aquarium using the stimulus syringe over 2-s, and their BPP activity monitored again for another 8-min. BPP activity was scored as any discernible opening of a carp's buccal cavity. A Noldus Observer XT system (Leesburg, VA, USA) was used to score BPP. For analysis, BPP events for each fish were summed over 30-s intervals. All data were evaluated for normality, transformed to meet assumptions of parametric analyses, and then tested using t-tests or analysis of variance (see below). With the exception of the first experiment which evaluated the time course of responses, data from 30-s time intervals were then summed again to obtain averages over 4-min (Experiment 4) or 8-min (Experiments 1, 2 and 3) blocks of time. Fish were fed with the food mixture at the end of each day after experiments were complete.

Chemical Stimuli

Chemical test stimuli were prepared from the food mixture or its components. They were added to 250-ml of well water, stirred, allowed to sit at room temperature for 1-h, centrifuged at 7000 rpm for 25 min, and then decanted through paper filter to remove particulates greater in size than 10 µm (Whatman Grade 3, Kent, UK) into 250 ml Nalgene flasks (Nalgene, Rochester, NY). Food items were added in ways to mimic the relative concentrations in their food (i.e. one quarter as much as that used to make 1-l of food was added to 250 ml).

Well water control was handled in the same manner. Amino acids were tested by dissolving them in well water, again in the same ratios and concentrations measured in food filtrate (Sigma, St. Louis, MO). Food, food filtrates and amino acids were refrigerated (4 °C) and used within 4 days of preparation.

Experiment 1: Is Food Recognition and Feeding Associated with BPP Activity?

Our first experiment tested the role of chemicals in food recognition in both species of carp, whether the potencies of the different food items were different, and how each species discerned those food items. It had five sub-objectives: 1) establishing the role of BPP behavior in food sampling and consumption; 2) testing the role of water-borne chemicals in eliciting BPP behavior (food sampling); 3) determining how long BPP responses might endure; 4) determining whether some food filtrates (chemicals) might be more potent than others; and 5) determining whether the carp species have different food preferences. All sub-objectives were addressed at the same time in a single large experiment. We tested the responses of 12 pairs of silver and 12 pairs of bighead carp to 24 stimuli that included blank control, the food mixture, and the chemicals (filtrates) found in each of the nine components of the food mix. Each test started with an 8-min pre-test period and was followed by an 8-min test period based on an initial pilot study that indicated that BPP activity of both species to the components of the food mix filtrate had declined to basal levels within that time. Test stimuli included: well water blank control, food (3 ml of the food mixture), food filtrate, and filtrates of each of the 9 items found in their food. Individual filtrates were tested at the same concentrations and proportions (by mass relative to water volume) found in the food mixture. Each test stimulus was tested 11 times for both silver carp and bighead carp.

This experiment was analyzed in two steps: an initial analysis was performed to characterize the temporal nature of the behavioral responses we were observing, while a second analysis directly tested our objectives using summed data and statistical tests. In the initial analysis, the BPP data were binned by 30-s intervals, plotted, and data for basal and peak activity levels evaluated by two ANOVAs (one for each species). The second analysis proceeded in several steps. First, we calculated summed and average BPP activity per min during each of the 8-min pre-test and 8-min test intervals. Quantile-quantile diagnostic plots (R version 3.1.2, CRAN, r-project.org) of the summed values showed they were not normal so we transformed using log base 10. Pre-test control basal BPP values of the two species were compared using an *a priori* Welch comparison. Next, we tested (and confirmed) that adding a blank stimulus control had no effect by comparing pre-test BPP activity of each species with the BPP activity seen after addition of well-water control for that species using

paired t-tests. No effects ($P > 0.01$) were noted so we were then able to simply focus on testing the effects of adding stimuli. We then tested the effects of the 12 stimulus types using two one-way ANOVAs, which examined the effects of stimulus type, species, and their interaction with blocks for day, pair of fish (tank), and order of testing (time of day; R version 3.1.2, CRAN.r-project.org). We did not explore other possible interactions because they were not related to our *a priori* hypotheses about chemically mediated feeding behavior. Lastly, to examine differences within species, Tukey's honest significant difference (HSD) post hoc comparisons were made between average BPP responses to different stimulus types within each species.

Experiment 2: Does the Chemical Composition of Food Have a Role in Feeding Preferences?

Although experiment 1 found that filtrate of spirulina was the most potent planktonic food component for both species and more potent than *Chlorella* for silver carp, this result could have been confounded by the fact that the concentration of spirulina we tested was greater than *Chlorella* in the food mixture (19.7 g/L vs. 11.4 g/L). To directly test the hypothesis that spirulina was more potent because of its chemical composition and not because of its higher concentration, we tested the effects of spirulina and *Chlorella* filtrates at a range of (overlapping) concentrations (0.099 g/L, 0.985 g/L, 9.85 g/L, and 19.7 g/L for spirulina and 0.114 g/L, 1.14 g/L, 11.4 g/L, and 22.8 g/L for *Chlorella*) on 12 pairs of silver carp using the same methods used in experiment 1. This experiment employed a balanced incomplete blocked design and silver carp because they were more robust test subjects. We hypothesized that responses to filtrates of these foods should have similar dose-response relationships if the same chemical constituents were involved in each food. For analysis, data were summed across 8-min intervals and log transformed after normal quantile-quantile diagnostic plots suggested they followed the log-normal distribution. A linear mixed model with separate slopes for *Chlorella* and spirulina was fit to the data, with log of g/L as the predictor and random slopes and intercepts for each tank. To test for an overall food type effect, this model was compared using the likelihood ratio test to a model with only one slope. The main effects and interaction terms were tested using Type II ANOVA, and least squares means and differences were reported after back-transforming to the original scale.

Experiment 3: Do Amino Acids have a role in Bigheaded Carp Feeding Behavior?

L-amino acids are commonly hypothesized to play key roles in the feeding behavior of fishes (Sorensen and Caprio, 1998). To test this, the BPP activities of silver carp and bighead carps

were recorded while they were exposed to either well-water blank control, food filtrate, or the concentration of 18 common L-amino acids (0.62 mM L-glutamic acid, 0.62 mM L-glutamine, 0.01 mM L-aspartic acid, 0.12 mM L-asparagine, 0.16 mM L-serine, 0.10 mM L-histidine, 0.39 mM L-glycine, 0.12 mM L-threonine, 1.01 mM L-alanine, 0.49 mM L-arginine, 0.17 mM L-tyrosine, 0.19 mM L-valine, 0.09 mM L-methionine, 0.19 mM L-phenylalanine, 0.16 mM L-isoleucine, 0.30 mM L-leucine, 0.26 mM L-lysine and mM 0.14 L-proline) previously measured in the food mixture filtrate by HPLC equipped with a fluorescence detector (see Hansen et al. 2014). Twelve pairs of fish of each species were used in a repeated Latin square design. For analysis, BPP activity was summed across 8-min and log transformed before testing for effects of design factors (stimulus type, species, pair of fish, day, order of testing, and the interaction between species and treatment) by ANOVA. The Tukey's honest significant difference method was used for post-hoc comparison of stimulus types.

Experiment 4: What Role Does the Olfactory Sense Play in Bigheaded Carp Feeding Behavior

In this experiment, we tested the role of olfactory sense in food recognition by temporarily occluding the olfactory systems of both fish in three pairs of silver carp and three pairs of bighead carp (to evaluate possible species differences). We tested food filtrate followed by food (tests compared odor responses to those for whole food) for 4-min intervals (Step 1 of experiment 1 showed that responses peaked after 1-min so 4-min intervals could be used to save time) and observed. The olfactory systems of both carp were occluded by removing each fish from its aquarium and inserting about 5 μ l of an inert polymer (3 M Express vinyl polysiloxane, St Paul, MN) into both of its olfactory nares following established procedures known to have minimal effects on fish health (Levesque et al. 2011; Vrieze et al. 2010). This experiment lasted 9 days and included a 3-day control period, followed by an assessment of occluded carps for 3-days and then an assessment of carp response after the occlusions were removed. Polymer occlusions were carefully removed using forceps in fish that we captured in a net. Each day, we first observed carp for a 4-min pre-test interval, added food filtrate and observed for a 4-min interval, and finally added food while observing for a 4-min interval. We did not test a blank control because we had few fish and experiments #1–3 had already demonstrated the lack of an effect of a blank well-water stimulus. This experiment also recorded fish behavior in high-definition video and used three observers, one of whom was blind to treatments and scored BPP activity later to confirm that the observers were not biased (they were not; average scores were within 10% of each other). For analysis, data were summed over 4-min intervals and log transformed after normal quantile-

quantile diagnostic plots revealed that data likely followed a log-normal distribution. Initial ANOVA showed no effect of species so data were combined for the final analysis, which asked three specific questions using three separate ANOVAs (a multiple-level, dependent ANOVA was also evaluated but deemed unnecessary, overly complex, and low in power) Were basal BPP rates of these fish affected by olfactory occlusion? 2) Were BPP responses to food chemicals (filtrate) affected by olfactory occlusion?, and 3) Were BPP responses to food affected by olfactory occlusion? ANOVAs were blocked to test for effect of olfactory tract state (occlusion) and species with blocks for pair of fish (tank) and order of testing (time of day) (R version 3.1.2, CRAN.r-project.org). Tukey's HSD method was then used for post-hoc comparisons for each of the three ANOVAs.

Results

Experiment 1. Chemically-Mediated Feeding Behavior and Preferences

Analysis of BPP activity summed over 30-s intervals during the pre-test period showed that both silver and bighead carps exhibited very slow, extremely constant levels of basal BPP activity (5.4 ± 2.4 [mean \pm S.D.] BPP/min; 1.8 ± 1.2 BPP/min, respectively) that differed from each other ($F(1151) = 8.433$, $p < 0.05$; Fig. 1). This analysis also showed that when the food mixture was added to the aquaria, BPP rates of each species increased over 35-fold within 1-min to nearly 180 BPP/min for each species ($F(1410) = 549.096$, $p < 0.01$) and then declined slightly while remaining above 120 BPP/min for the entire 8-min experimental period for each species. Addition of the food filtrate also caused an initial response that was about two thirds as strong as food itself for both silver carp (121.2 ± 37.1 BPP/min) and bighead carp (116.9 ± 65.5 BPP/min) but these rates steadily decreased with time to about 25–50 BPP/min after 8-min. Similar, but smaller, increases in BPP activity were also elicited by exposure to filtrates of spirulina or *Chlorella* in both carp species with responses to spirulina statistically greater in silver carp ($P < 0.05$) but not bighead carp. In both cases, BPP activity returned to near baseline (about 10–25 BPP/min) after 8 min (Fig. 1).

A second analysis explicitly examined the relative tendencies of the different filtrates to stimulate BPP activity in each species of carp by averaging data over the 8-min test period. A *priori* analysis of the effects of adding well-water blank to aquaria containing either silver or bighead carps showed no evidence for an effect of well-water control (carrier) in either species ($p > 0.05$ for control pre-test values compared to well water control). Addition of food filtrate alone was highly stimulatory ($P < 0.05$ vs well water control) in both species suggesting a role for water-borne chemicals in food recognition.

Analysis of all stimuli and species found differences in the potency of stimulus type ($F(11151) = 41.2885$, $p < 0.05$), an effect of species ($F(1151) = 4.4642$, $p < 0.05$), and an interaction between stimulus type and species ($F(11151) = 4.1679$, $p < 0.0001$), as well as significant effects of blocking factors pair of fish (tank; $F(11151) = 3.5796$, $p < 0.05$) and order of testing (time of day; $F(24,151) = 2.3845$, $p < 0.05$). Different food stimuli had different relative potencies in each species, although spirulina filtrate was the most potent ingredient (by rank) of the food mixture for both species (Fig. 2). Note that the statistical significances varied by species. Groupings of stimulus potencies by Tukey's HSD comparisons were complex; 4 overlapping groups were evident in silver carp while 7 were measured in bighead carp. Briefly, in silver carp, when analyzed in this fashion, the whole filtrate was as potent as spirulina, Cyclopeeze©, pellet crumble or Otohime ($p > 0.05$). In contrast, in bighead carp, whole food filtrate was as potent as spirulina, pellet crumble, or *Chlorella* ($p > 0.05$), which in silver carp was the least stimulatory and no more potent than blank ($p > 0.05$). Responses of silver carp differed from those of bighead carp ($p < 0.05$).

Experiment 2. Effects of Food Composition on BPP Activity

Summed data from experiment 2 followed a log-normal distribution and were normally distributed after transformation. While BPP activity in silver carp stimulated by spirulina and *Chlorella* filtrates were similar at low concentrations (Fig. 3), responses to the *Chlorella* filtrate only increased slightly with concentration before reaching a plateau of about 30 BPP/min at 11.4 g/L. Responses to spirulina increased more sharply and did not appear to reach a plateau. Overall, BPP responses to the two food filtrates were significantly different ($p < 0.0001$), with significantly different main effects ($p = 0.005$) and slopes ($p = 0.01$). At 10 g/L, the least squares mean for spirulina was 53.7 (95% CI: 41.0, 70.4) and for *Chlorella* was 21.9 (95% CI: 16.0, 30.1); spirulina was 2.45 times larger (95% CI: 1.66, 3.61).

Experiment 3: Testing the Effects of Amino Acids on chemically-Mediated Feeding Activity

Summed data from experiment 3 followed a log-normal distribution and after transformation were normal. ANOVA found an effect of stimulus ($p < 0.05$) and blocking factor day ($p < 0.05$) but not species ($p > 0.05$). Species was thus not considered further. Tukey's post hoc comparisons showed that the mixture of 18 amino acids produced a BPP response (24.6 ± 20.3 BPP/min) that was greater than well-water blank control (3.2 ± 2.6 BPP/min, $p < 0.05$) but only about half the response to the food filtrate (71.2 ± 34.3 BPP/min, $p < 0.05$) (Fig. 4).

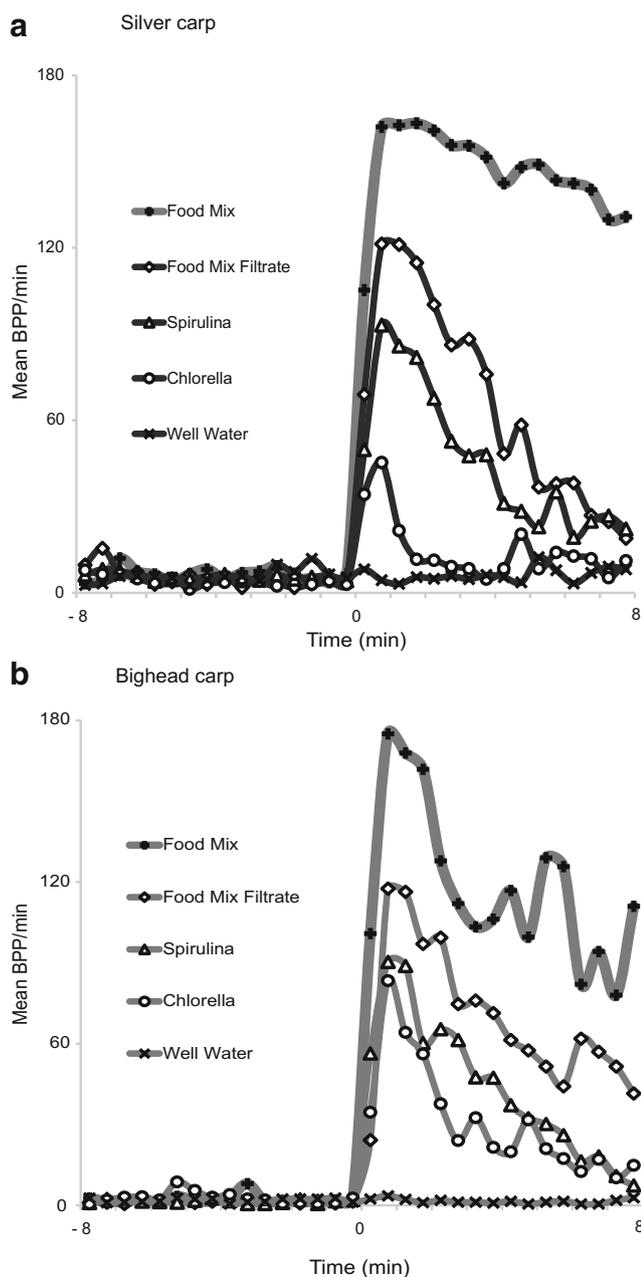


Fig. 1 Mean buccal-pharyngeal pumping (BPP) rates of silver (A, $N = 11$) and bighead carp (B, $N = 12$) to food and representative food filtrates over 30-s intervals in 80-l aquaria. Food stimuli (3 ml) were added during a 1–2 s period after 8-min of pre-test observations at time 0

Experiment 4: Testing the Role of Olfaction on Chemically-Mediated Feeding Activity

Summed data from experiment 4 followed a log-normal distribution and were normally distributed after transformation. No differences between species were noted so species data were combined for further analysis. Responses to both food filtrate and food were strongly suppressed by olfactory occlusion but recovered after occlusion. Briefly, we found no effect of either order of testing or olfactory occlusion on basal BPP

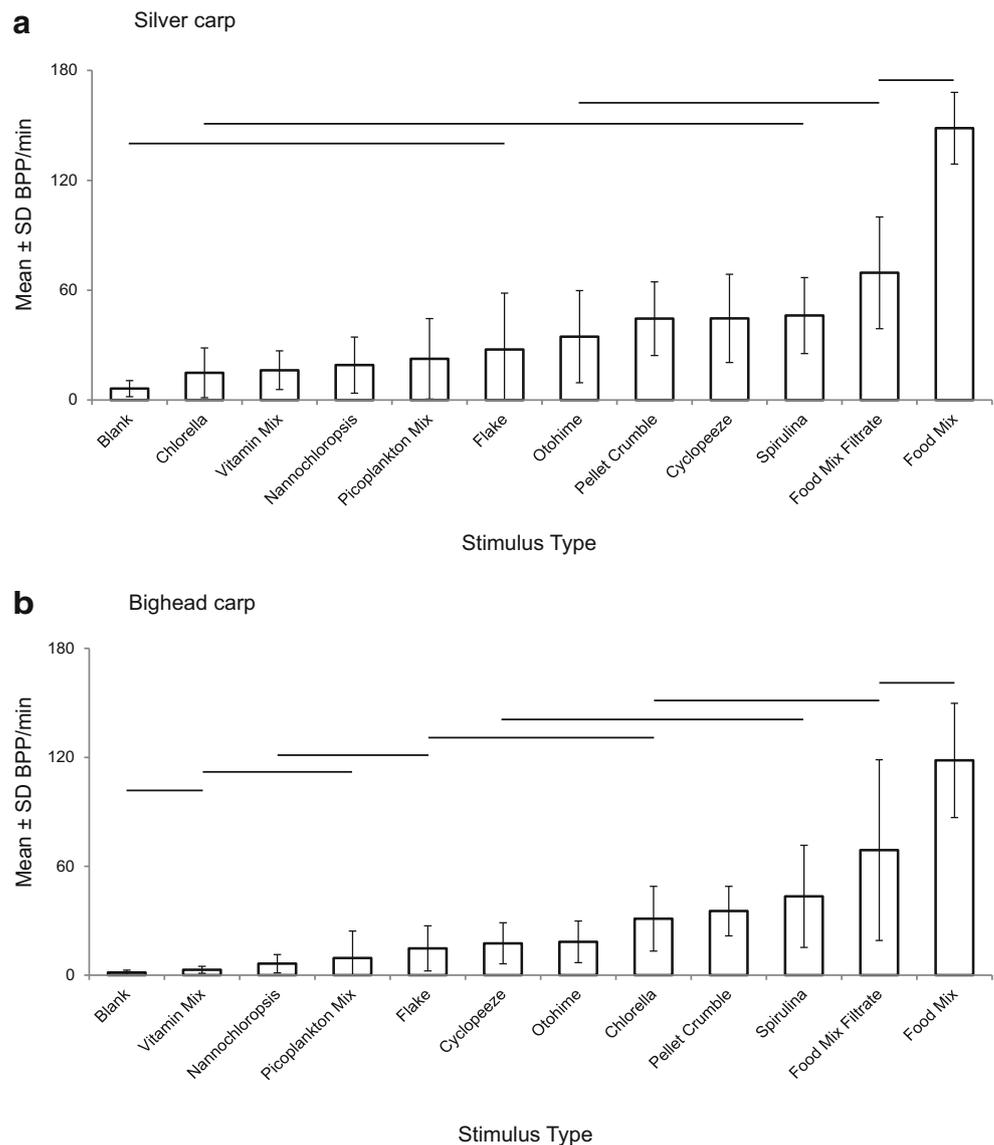
activity ($F(2,45) = 4.3$, $p > 0.05$) although a small nonsignificant decline after occlusion was suggested (4.7 ± 3.1 BPP/min)(Fig. 5). In contrast, our analysis of whether BPP activity (question 2) was stimulated by food filtrate showed a strong overall response to this stimulus ($F(2,45) = 23.2$, $p < 0.05$) with follow-up paired Tukey's HSD describing a large decrease between the mean pre-test value (66 ± 30.6 BPP/min) and the test value for occluded carp (14.9 ± 3.1 BPP/min; $p < 0.05$) along with a subsequent robust recovery to the mean pre-test value after the occlusions had been removed (i.e. mean post-occlusion BPP rate was not statistically different from mean pre-occlusion value; 43.1 ± 28.9 BPP/min; $p > 0.05$) (Fig. 5). ANOVA also showed both species responded to food alone ($F(2,45) = 3$, $p < 0.05$) (Fig. 5) with BPP responses to food (162 ± 19.9 BPP/min) suppressed after olfactory occlusion (87.5 ± 56.9 BPP/min; $p < 0.05$) but recovering to a level that did not statistically differ from the pre-test level after the occlusions had been removed (128.7 ± 29.5 BPP/min; $p > 0.05$) (Fig. 5). No carp exhibited diminished swimming or feeding activity although some inflammation of the olfactory epithelia of occluded carp was noted during the first day after removal of the occlusions.

Discussion

This study is the first we know of to demonstrate that chemical cues play an important role in regulating filter feeding activity of microphagous filter-feeding fishes including the bigheaded carps. We found that a complex mixture of planktonic and detrital foods was detected by its chemical components, with those chemicals released by a cyanobacterium (spirulina) having an especially strong ability to stimulate buccal pumping. There were small, but clearly measurable differences in the preferences of the closely related silver and bigheaded carps. The olfactory system appears to be an important mediator of chemically controlled BPP activity and sampling, although the internal taste system may also have a secondary role. Despite being tested at laboratory scale, we believe our results likely reflect the feeding behaviors of bigheaded carp in the open river. At the scale and habit complexity of the river, the differences in odor profiles may be easier to discern, these fish to locate food patches and perhaps select food within them. It is likely that chemically-mediated filter-feeding behavior increases the feeding efficiency of these species and thus contributes to the high growth rates of these carps. Other filter feeders may employ chemical cues in similar ways although this has yet to be investigated. Chemical feeding stimulants could be extremely useful in both the culture and control of bigheaded carps, especially if they are unique to bigheaded carps.

Although we had expected chemical cues to play a role in food identification in the bigheaded carps, we had not

Fig. 2 Mean buccal-pharyngeal pumping (BPP) rates \pm S.D. of silver (A, $N = 11$) and bighead carp (B, $N = 12$) to food and food filtrates over 8-min intervals. Responses are ranked by mean. Values that do not differ from each other (Tukey's HSD $p = 0.05$) are connected by lines

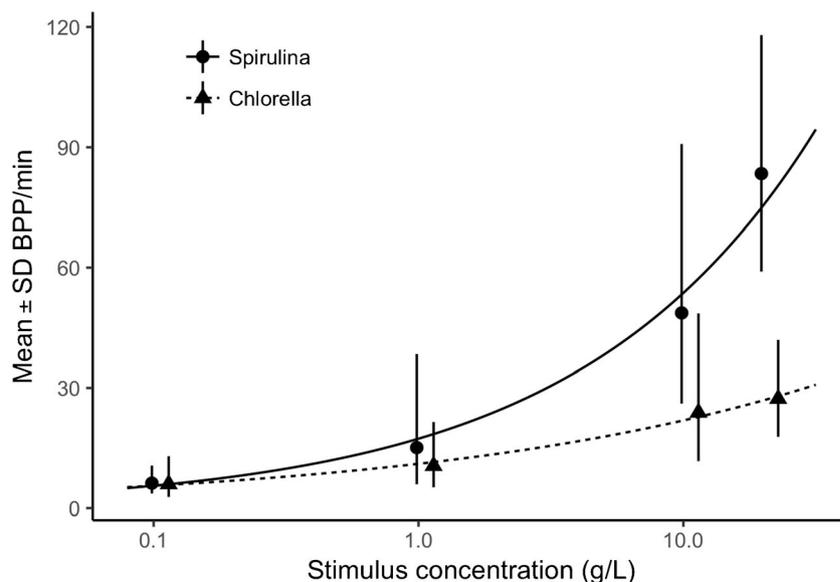


expected this role to be as prominent or as complex as our results indicate. Food filtrate was nearly as active as the food for many minutes after full dilution, strongly suggesting that dissolved chemical cues play a prominent role in initial stages of food recognition. Prolonged BPP activity to food might also reflect a role for taste in consumption. The sophisticated and important nature of chemically-mediated feeding responses in the bigheaded carps was also demonstrated by our dose-response study which showed that spirulina filtrate, the most potent component, was both specifically discerned and active at extremely low concentrations (1 g/L) for several minutes even after complete 20,000-fold dilution which we estimate takes less than a minute, at least for hydrophilic chemicals.

While chemical food preferences appeared relatively broad for both carp species, differences between both food items and species were also evident. An ability to recognize particulate

planktonic food makes sense in large rivers where planktonic food can be hard to see, patchy, and presumably varies in quality (Baker and Baker 1981; Wehr and Descy 1998). The small but significant differences between silver and bighead carp also make biological sense because the latter appears to consume more zooplankton than the former (Kolar et al. 2005). Because we did not test zooplankton filtrates, we cannot directly address the ecological relevance. Extrapolating our findings to natural settings is also rather hypothetical because the relationship between bigheaded carps and plankton composition in rivers has not been formally studied. It is known that carp densities are higher in highly productive tributaries such as the Illinois River, which must have high densities of plankton. It is especially intriguing that spirulina, a type of cyanobacteria, was a preferred food item for both silver and bighead carp, as reports from the field and aquaculture show that bigheaded carps readily consume cyanobacteria

Fig. 3 Mean buccal-pharyngeal pumping (BPP) rates \pm S.D. of silver ($N = 11$) carp to increasing concentrations of spirulina filtrate and *Chlorella* filtrate. Lines that describe these data were generated using linear mixed models

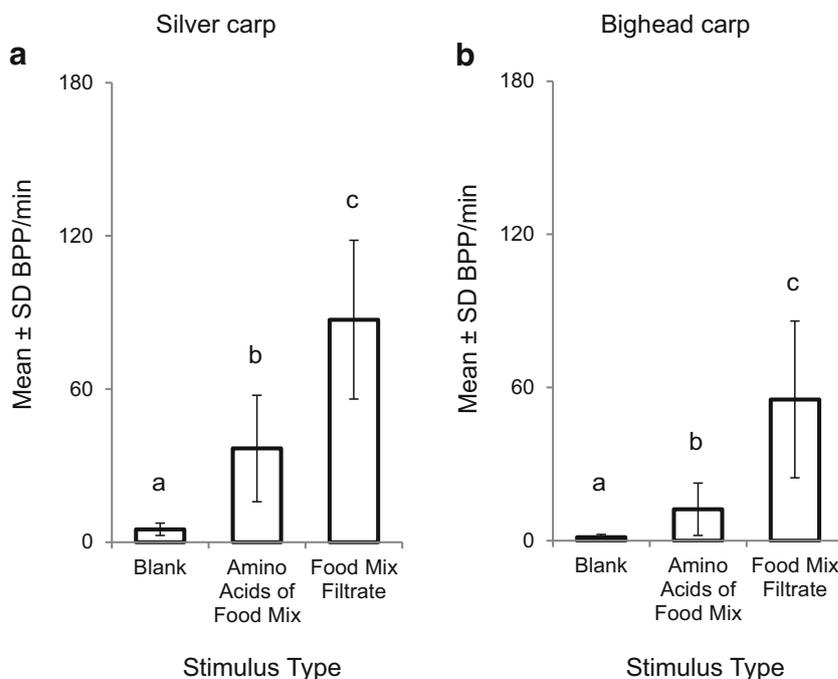


(Beveridge 1993; Ye et al. 2013). Notably, spirulina is extraordinarily high in nutritional content and contains many fatty acids (Ötles and Pire, 2001), which may be chemostimulatory in fishes (Silver 1982). The possibility that foods that stimulate feeding activity carp more actively than spirulina exist in rivers should now be studied, as should the possibility that the chemical cues they release are innately discerned. It will also be fascinating to determine whether these chemical cues are close range and/or long range attractants and how carp might employ them naturally to locate and select tiny food particles in large, flowing environments. Release rates of chemical by plankton, odor gradients in flowing waters, and chemical

detection mechanisms presumably all play roles in how food chemicals are naturally used by carps in rivers.

Our study clearly shows that the olfactory sense has a role triggering food sampling and BPP activity. Food filtrates alone triggered almost as much activity as food itself and for many minutes in both carp species. The effects of filtrates were also immediate which seemingly rules out a role for taste in arousal because taste systems in these species are entirely internal (Hansen et al. 2014). Further, behavioral responses to the food filtrate disappeared when the olfactory system was occluded and returned fully when the nasal plugs were removed. While it is possible that stress associated with

Fig. 4 Mean buccal-pharyngeal pumping (BPP) rates \pm S.D. of silver (A, $N = 11$) and bighead carp to control blank, a mixture of 18 amino acids found in the food filtrate plotted over 8 min-time intervals, and the food filtrate. Letters designate Tukey's HSD means that were not different from each at the $p = 0.05$ level



occlusion may have had a confounding role in the occlusion experiments (there was some apparent, albeit nonsignificant, suppression of basal BPP activity in occluded carps), it was likely not a major factor as occluded carp continued to feed when food was added to their tanks and carp olfactory pits are small so only very small amounts inert material (μs) were injected. Further, several studies using this occlusion technique in other fishes have not shown any discernable side effects and have clearly shown the olfactory system to be effectively blocked (Levesque et al. 2011; Vrieze et al. 2010). The olfactory system is well-developed in bigheaded carps (Sinha 1987) although its sensitivity has not yet been studied. Olfaction is also well known to play key role discerning complex food mixtures in other fishes (Derby and Sorensen 2008; Stacey and Kyle 1983).

In addition to the olfactory system in food sampling, it seems likely that taste-driven responses associated with the epibranchial organ may have an important secondary role with food processing and consumption in the bigheaded carps.

Prolonged BPP activity was evoked by food itself, even when the nose was occluded. The epibranchial organ is located deep in the buccal cavity and has many taste buds. This organ packages ingested food in its mucous-cell-rich tubular structure so it can be swallowed (Hansen et al. 2014). It detects amino acids and contract when exposed to them (Hansen et al. 2014). Cues detected by the olfactory sense might thus alert carp to the presence of food, which they might then locate as they sample and filter by buccal pumping. After detection, cues that stimulate taste buds on the epibranchial organ could stimulate more pumping and perhaps grinding by the pharyngeal teeth, followed by swallowing if the food is edible. The relative role of olfactory cues is likely much greater in large rivers where fish roam greater distances, plankton blooms can be restricted to productive tributaries (Wehr and Descy 1998) and the costs of swimming and sampling are presumably greater. Tactile cues might be also be associated with chemically-mediated buccal pumping, with this stimuli serving a sophisticated multi-modal role as noted in many species

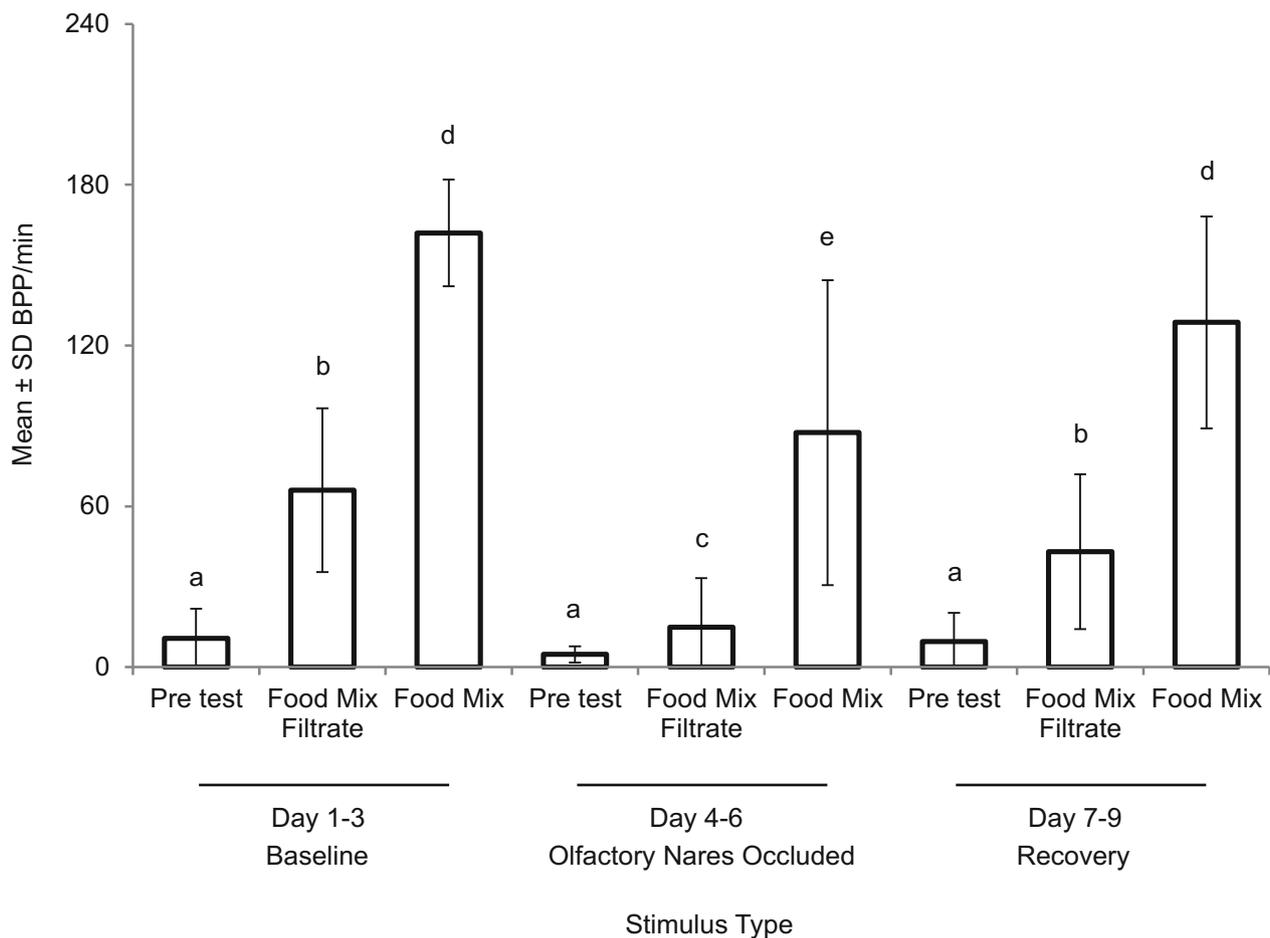


Fig. 5 Mean buccal-pharyngeal pumping (BPP) rates \pm S.D. of 3 silver and 3 bighead carp to a control blank, followed by food filtrate and then food (data from the two species did not differ so are combined). Fish were tested three times over three days, then subjected to olfactory occlusion,

tested three times again, and then their occlusions were removed and tested again ($N = 6$ for each mean). Letters designate Tukey's HSD means that were not different from each other at the $p = 0.05$ level

including humans (e.g. Filingeri et al. 2014). Neural recording from olfactory, taste and tactile systems would provide insights. Whether carps also discern distasteful or noxious olfactory chemicals released by cyanobacteria (Falconer 2003) is not known but warrants study. In sum, food recognition in bigheaded carps clearly appears to be a highly sophisticated chemically mediated process, with some similarities to the Eurasian carps including the goldfish, *Carassius auratus*, which locate, sort and then swallow benthic food particles using a combination of smell and internal taste systems (Finger 2008; Hara 2006). It is very likely that other filter-feeding fish also use olfactory cues to trigger searches for food and/or consumption and we speculate that different types of filter-feeding fish (e.g. ram-feeders that lack epibranchial organs) that eat different foods likely use chemical cues in different manners.

While it is interesting that filter-feeding carps employ amino acids as feeding stimuli, it is especially intriguing that additional, unknown, compounds explain approximately half the activity of the planktonic food we tested. A similar finding emerged from our earlier studies of the epibranchial organ, which detects the same amino acid mixture tested here using neural recording (Hansen et al. 2014). Free amino acids are well known to be important odorants and tastants for many species of fishes and often account for much of the behavioral or electrophysiological activity of a preferred food in other fishes although this work has focused on carnivorous species (reviewed by Derby and Sorensen 2008). Bacteria and algae, and cyanobacteria in particular, are undoubtedly releasing many novel compounds in addition to amino acids. In our study, and in almost all studies of natural chemical cues used by aquatic organisms, the feeding stimuli discerned by carps are mixtures (Carr and Chaney 1976; Derby and Sorensen 2008). Often chemical sensitivity to cues is linked to dietary needs; nutritional demands of filter-feeding planktivorous fishes may also be very different than most carnivores and new types of chemosensory cues may be involved. Fatty acids found in cyanobacteria should be considered. Bioassay guided fractionation could be used to identify the full complexity of algal food as has been used for fish pheromones (Fine and Sorensen 2008; Sorensen and Hoye 2007).

If identified, bighead and/or silver- carp specific chemical feeding attractant/stimulant(s) would be very useful in both invasive carp control and culture. For instance, if the cues that stimulate BPP activity are also attractive, as our pilot data now suggest (Sorensen, University of Minnesota, unpublished results), they might be used to attract these invasive carps, which are typically very difficult to locate in rivers. Fisheries bycatch (e.g. the accidental and incidental capture of fishes by commercial fishers), a complication with riverine removal strategies, could be small if these cues were highly specific or novel. Similarly, the distribution of spirulina or its exudates in large rivers including the Mississippi River might be used to

predict bigheaded carp distribution. Chemical feeding cues could also be used in selective poisoning schemes now being developed that use toxin-infused microparticles (personal communication, Jon Amberg, USGS, LaCrosse, WI). Of course, feeding stimulants would be useful in aquaculture (Michielsens and Lorenzen 2002). More broadly, bigheaded carps can now serve as a model for understanding the physiological basis of feeding behavior in many other filter-feeding, microphagous, planktivorous fish.

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