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Feeding stimulants in an omnivorous species, crucian carp *Carassius carassius* (Linnaeus 1758)

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ABSTRACT

Many fish are during feeding dependent on both an olfactory and gustatory sense. Olfaction that acts as the distance sense induces arousal, food search behaviour and attraction to the source, followed by examination of food items by the gustatory sense. During buccal handling the fish decide if the feed will be rejected or swallowed. Amino acids are often stimulatory to the gustatory sense and can act as feeding stimulants. There are, however, inter-species differences concerning what kinds of amino acids act as feeding stimulants or deterrents. The species differences are probably dependent on the natural food choice. As feeding stimulating molecules increase feeding and growth, but deterrents have the reverse effect, it is important to know what kind of molecules have either effect. In the present study we record mouth handling time in the omnivorous crucian carp, *Carassius carassius*, of agar pellets containing water extracts of meal consisting of ordinary food pellets, blue mussels or a commercial carp attractant. These tests were followed by testing with agar pellets with synthetic amino acids, based on the content of the water extracts of the food pellets that was the only feeding stimulant. Neither extracts of mussel meal or of commercial carp attractants had a stimulating effect, i.e. no significant difference in handling time compared to agar pellets with only water. A mixture of five of the major amino acids in the food pellet extract (40 mM alanine, 20 mM glycine, 20 mM arginine, 8 mM serine, 8 mM leucin) gave a significant longer handling time compared to agar pellets with only water. The handling time was also longer for the three amino acids that had the highest concentrations (40 mM Ala, 20 mM Gly, 20 mM Arg) and finally with only alanine (128 mM). Agar pellets with only Ala gave, however, a significant shorter handling time compared to agar pellets with food pellet extract. The mussel meal extract had the same content of free amino acids and their ranking order was the same as in extracts of food pellets, but at much higher concentrations. Based on the free amino acid content, the mussel extract should have stimulated feeding. This indicates that the mussel extract contained compounds that acted as feeding deterrents in omnivorous crucian carp that do not feed on blue mussels in their natural environment. Previous studies have shown that blue mussel extracts act as feeding stimulants in several bottom feeding carnivorous fish. We finally tested betaine (100 mM) but the molecule had no significant stimulating effect that has been observed in some other fish species.

1. Introduction

There is great diversity between fish species concerning the kinds of molecules that are detected by taste buds, that are accepted and stimulate foraging, the natural food choice being probably important (e.g. Carr, 1982; Mackie, 1982; Jones 1992; Kasumyan and Døving, 2003). Feed that does not taste right, does not taste at all or that contains bitter compounds is rejected after handling in the mouth (Mackie, 1982). Plant materials can contain bitter-tasting compounds which may reduce the palatability. Reduced palatability results in lower growth rates whereas feeding stimulants (stimulus that promotes ingestion and continuation of feeding, Mackie, 1982) increase growth rate (e.g. Takeda and Takii, 1992; Papatryphon and Soares Jr., 2000; Pratoomyot et al., 2010). It is also important to use feeding stimulants to initiate feeding behaviour in young macrosmatic fish given artificial feed (e.g. Cadena-Roa et al., 1982; Métailler et al., 1983; Kamstra and Heinsbroek, 1991; Yacoob and Browman, 2007a). In several of these studies synthetic amino acids were used. Extracts of mussels acted as strong feeding stimulants in some bottom feeding carnivorous

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fish species as Dover sole (*Solea solea*), increasing their growth rate when added to artificial feed (Mackie, 1982). Fish species with a more vegetarian or omnivorous diet would probably have other preferences than carnivorous fish (Kasumyan and Nikolaeva, 2002). As palatable feed results in good growth rates it is important to know the preference of fish (Nagel et al., 2014; Carlberg et al., 2015). It has been suggested that Baltic Sea blue mussels should be an important feed component in aquaculture (e.g. Langeland et al., 2014; Carlberg et al., 2015; Vidakovic et al., 2015), increasing the palatability of artificial feed.

In common carp (*Cyprinus carpio*) 6 out of 21 tested free amino acids stimulated consumption when added to agar pellets (Kasumyan and Morsi, 1996), but there were also amino acids that were deterrent, at least when they were tested individually. Fish that were made anosmic showed the same behaviour which indicates that the stimulation of feeding was dependent on the gustatory sense, with the olfactory sense not being included at that stage. Neutral amino acids with few carbon atoms, unbranched and uncharged side chains are often highly stimulatory to the gustatory sense (reviewed in Kasumyan and Døving, 2003). The molecule betaine (trimethylglycine), present in high concentrations in mussels and other marine invertebrates such as crustaceans (e.g. Carr et al., 1996), can by itself stimulate foraging in some species of fish (Kasumyan and Døving, 2003) but in other species only in mixtures with amino acids (Mackie, 1982). Betaine is an important stimulatory compound in the commercial product Finnstim™ (Finnish Sugar co. Ltd; Helsinki, Finland) used by sport fishermen interested in common carp. Sucrose (present in plants) has been shown to stimulate feeding in some omnivorous fish species, including roach (*Rutilus rutilus*) (Kasumyan and Nikolaeva, 2002; Kasumyan personal. comm.). Some di- and tricarboxylic acids such as citric acid and α-ketoglutaric acid stimulate feeding of agar pellets in common carp, tench and roach (Kasumyan and Morsi, 1996; Kasumyan and Nikolavea, 2002; Kasumyan and Døving, 2003). All compounds known to be stimulatory are small, water soluble and non-volatile.

Crucian carp (*Carassius carassius*) is a freshwater omnivorous species that feeds on organic detritus, filamentous algae, zooplankton, small benthic animals and pieces of aquatic weeds (Lelek, 1987; Kullander et al., 2012). The species has a strong disease and parasite resistance, and highly hypoxia and heat tolerant (Piironen and Holopainen, 1986; Laurila et al., 1987; Karvonen et al., 2005). The optimal temperature for larvae and juvenile growth is as high as 25–28 °C. The species is also present in coastal waters in the Baltic Sea where it commonly grows to 2 kg (personal observations) and individuals up to 4 kg have been caught. Historically crucian carp was one of the most common fish used in aquaculture in Sweden (Svanberg et al., 2012; Janson et al., 2014). The species has recently experienced sharp declines in the number and size of populations through its native range by water draining and hybridization with non-native species such as gibel carp (*Carassius gibelio/Carassius auratus gibelio*) and goldfish (*Carassius auratus*) (e.g. Copp et al., 2010; Wouters et al., 2012; Rylková et al., 2013; Jeffries et al., 2016).

Goldfish and common carp that are closely related to crucian carp show a complex pattern of mouth handling of big food particles which is a post-capture selection process between food and non-food particles (Sibbing and Uribe, 1985; Sibbing et al., 1986; Lamb and Finger, 1995). In goldfish the handling time is completed in 3–5 s with unflavored pellets and 30–60 s with moderately aversive stimuli in pellets (Lamb and Finger, 1995). In the present study with crucian carp we used handling time as an observation variable to be an indicator of palatability of agar pellets flavoured with extracts and free amino acids. If the fish likes the taste of an agar pellet the handling time will increase and likely result in the fish swallowing the pellet or parts of it. The aim of this study was to investigate if extracts of blue mussel meal stimulate feeding and if free amino acids can act as feeding stimulants in the omnivorous crucian carp.

2. Materials and methods

2.1. Fish and experimental set up

One hundred and fifty crucian carp (*Carassius carassius*) (total weight range 25–55 g) from a local pond (Olsén et al., 2006) were caught in the autumn half a year before the experiments were performed during spring 2014. The fish were transported to the fish facility at Department of Animal Nutrition and Management, Swedish University of Agricultural Sciences, Uppsala, and placed in an 800 L fish tank continuously supplied with aerated tap water. Artificial plastic plants were placed in the tank to give the fish shelter. They were fed daily with ®Hikari Sinking Wheat-Germ pellets (for information see http://www.hikariusa.com/diets/koi/hikari-sinking-wheat-germ/) (herafter referred to as Hikari pellets or only Hikari). Two fish died during the period. Two months before the start of the experiments all fish were divided into equal sized groups and placed in four different aquaria with the same size as used in the experiments described below. Enrichments of holding tanks and aquaria consisted of artificial plants.

At least two days before an experiment started, two fish were placed together in a glass aquarium, 95 (L) × 41 (W) × 43 (D) cm, with a water depth of 37 cm. There were six test aquaria running at the same time. To reduce the risk of accumulating food odours the aquaria had a continuous supply of tap water. The water temperature was 24 °C. Light was given by fluorescent lights placed 25 cm over the water surface. All aquaria windows were covered with black plastic sheeting. On the front window a 7 cm high (from the bottom) and 95 cm wide split was arranged for behaviour observations. The fish were alerted by the sinking pellet and caught it just over or on the bottom. Two fish were placed together to reduce stress as they naturally keep together in groups or shoals. To distinguish from each other the fish were anaesthetized with 2-phenoxy ethanol (0.05%, Sigma-Aldrich), measured and a small incision was made in the upper or the lower part of the caudal fin. The observations started when the pair had started to eat Hikari pellets. The incisions made it also possible to, in the communal aquaria, distinguish between fish that had been used in the experiment and those that had not.

The testing procedure of palatable molecules with agar pellets were adopted from the method described by Kasumyan (Kasumyan and Morsi, 1996; Kasumyan, 2012; see also Adams and Johnsen, 1986). In studies with common carp Kasumyan and Morsi (1996) observed that the handling time, the length of time the agar pellet is handled in the mouth, before being rejected or swallowed, was longer if the pellet contains molecules stimulating feeding. We observed in a pre-study that crucian carp handle Hikari pellets one to three minutes before swallowing, but reject non-flavoured agar pellets after some seconds. Tests were performed before noon with agar pellets containing different taste molecules in random order, only one kind of agar pellet each day. Each fish was given only one agar pellet per day and tested only once with the same extract or compound or mixture. In almost all occasions the most bold fish took the agar pellet. Control pellets were used during each trial (each day). In total eight groups of fish were run with two or three agar pellets. Fish were fed Hakari pellets daily after the test runs.

We recorded the handling time in seconds with a stop watch. The behaviour is easy to observe as crucian carp perform a vigorous chewing to be able to swallow the pellet. If they do not like the taste the pellet is rejected immediately or after a few seconds. We recorded only the handling time of the first grasp of the pellets as very few fish rejected the pellets and grasped them again.

2.2. Preparation of agar pellets

Water extracts of Hikari pellet meal, blue mussel meat meal and the commercial cyprinid attractant Swedbait (Uppsala, Sweden) (www.Swedbait.se) were included in agar pellets of similar size as the Hikari pellets (4–4.5 mm long, diameter 4.5–5 mm). The commercial attractant Swedbait strongly increases the efficiency of traps to crucian carp, tench (*Tinca tinca*) and rudd (*Scardinius erythropthalmus*) and the paste added is eaten by the fish (Stacey et al., 2012).The water extract should include free amino acids and other small water soluble molecules. If water soluble molecules are important as feeding stimulants, agar pellets with extracts of Hikari pellets should be stimulatory and function as a positive control. The content of free amino acids (FAA) were analyzed in dried matter and water extracts. The major amino acids were tested to investigate their ability to stimulate feeding.

Boiled and deshelled blue mussel (*Mytilus edulis*) of human food grade, was bought from Royal Frysk Muscheln (GmbH, Emmelsbüll-Hornsbül, Germany) were dried at 55 °C for 24 h. Thereafter were the blue mussels milled (Novital Davide,Novital S.r.l., Lonate Pozzolo Italy) through a 1-mm screen, whereas Hikari pellet and Swedbait attractant were grinded with coffee grinder. The mussels were caught in the North-east Atlantic Ocean. The ground ingredients were kept frozen at −25°C until extraction and used in agar pellets. The Twenty five grammes of each component were suspended in 300 ml tap water. After vortexing the suspensions were centrifuged for 15 min at 6000 rpm. The supernatant was collected and filtered through a 0.45 μm filter. The resulting filtrate was collected and used in agar pellets or analyzed to determine the content of free amino acids (FAAs). The filtrates were put on ice and after 30 min 40 ml was withdrawn and mixed with 60 ml of a solution of the food dye Brown HT (E155; CAS number 4553-89-3; water solubility 150 g/l; donated by National Food Agency, Sweden), and 3 g agar powder (Sigma-Aldrich® A1296). The dye was used to give the agar pellet a brownish color resembling the Hikari pellets. Brown HT solution was prepared by dissolving 29 mg in 1000 ml tap water. New Brown HT solution was prepared at least once a week and kept in a refrigerator. The filtrate-dye-agar mixture was poured in to a small stainless steel pan and heated under mixing up to 75 °C. The mixture was immediately poured in to 2–3 petri dishes and put in a refrigerator. Just before a test agar pellets were punched out with an aluminum tube with an inner diameter of 5 mm. The agar pellet was collected with a stainless steel needle and carefully put into a test aquarium. Control agar pellets contained only tap water and Brown HT solution. In test with FAAs or betaine, solutions of mixtures or single molecules were included.

2.3. Chemical analyses

Samples of 200 mg of each of the three meals were collected and analyzed concerning concentrations of different FAAs. 1000 μl 0.1N HCl was added to each sample (200 mg / ml).Water extract of Hikari pellet meal (25 g/300 ml) and of mussel meal (25 g/300 ml) were also analyzed. As the total content of FAA was very high in the mussel solution, a second analysis was done with 5.5 g/300 ml. First, the water extracts were deprotonated by adding sulphosalicilic acid and then centrifuged at 2500*g* for 30 min. Thereafter samples were diluted in a borate buffer with the addition of internal standard (Reverter et al., 1997). The samples were derivatised by using ACCQTag™ Ultra ® (Waters Corp, Milford, USA). The amino acid analyses were performed by using Waters UPLC® amino acid analysis solution protocol, and an UPLC system consisted of Dionex, Ultimate 3000 binary rapid separation LC system with a variable UV-detector (Thermo Fisher, Sweden, Stockholm). Empower 2 (Waters) software was used for system control and data acquisition.

2.4. Amino acids tested

As there are no published data on stimulatory effects of FAAs in crucian carp we used data from studies of common carp and goldfish (Marui and Caprio, 1992; Kasumyan and Nikoleva, 2002; Kasumyan and Døving, 2003). Both goldfish and common carp are closely related to crucian carp and they eat both invertebrates and plant materials. We chose five L-amino acids to compose an amino acid mixture. Alanine is highly stimulatory to common carp and in 12 out of 14 species tested (Kasumyan and Døving, 2003). Arginine, glycine, leucine and serine are highly stimulatory to goldfish (Kasumyan and Døving, 2003). Glycine is present in high concentrations in crustaceans and molluscs (Carr et al., 1996). Betaine was tested as it is a feeding stimulant in several species. All amino acids were purchased from Sigma-Aldrich® (purity ≥ 98%)

2.5. Statistical treatment

Each fish was offered each agar pellet once. A paired *t*-test was used when two stimulants were compared. Repeated measures ANOVA was applied and followed by Dunnett's multiple comparisons test when there were three different stimulants. In the comparisons between the different stimulants that gave a significant handling time data were log transformed before the one-way ANOVA followed by Tukey's multiple comparisons test. The difference in time between the stimulant and the control for each individual was used as an observation. The concentration ranking order of 19 amino acids in water extracts of Hikari meal was compared with water extracts of mussel meal (5.5 g/300 ml) with Spearman's rank correlation coefficient. All calculations, except Spearman's ranking correlation that was done manually, were done on GraphPad Prism™ software (Graph Pad Inc., San Diego, CA).

3. Results

3.1. Analyses of free amino acids

Free amino acid contents in meal of different sources and their water extracts are given in Table 1. In addition to 18 free amino acids and the nonprotein amino acid ornithine, high concentrations of another physiological amino acid, taurine, were detected in mussel meal and extracts. No taurine was detected in Hikari or Swedbait, but gamma-aminobutyric acid (GABA) was found in the latter two. The total concentration of amino acids was very high in mussel meal and its water extracts. Alanine dominated the Hikari

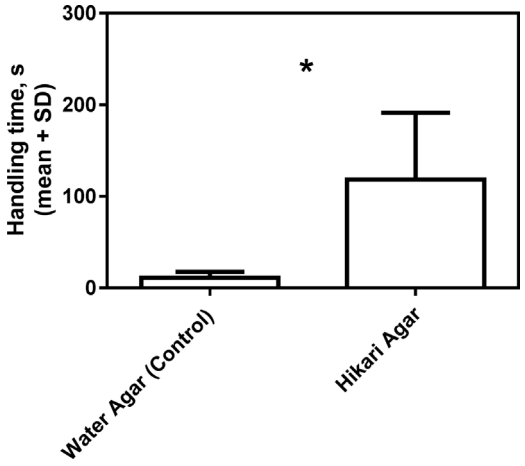

Fig. 1. Agar pellet handling time in crucian carp. The control agar contained only water and dye. Hikari agar contained water extract of feed pellets and dye. Paired *t*-test, $P < 0.05$, $N = 6$.

Table 1
Free amino acid composition in liquid extracts of commercial carp feed, carp attractant and mussel powder. The concentrations of gamma amino butyric acid (GABA) are also stated. Concentrations are expressed as pmol/μl injected sample. For calculation of pmol/μl sample multiply with factor 13.75 for samples 1, 3 and 4, and dilution factor 12.5 for samples 2, 5 and 6.

AA pmol/μL	Hikari 200 mg/ml	Hikari 25 g/300 mL	Swed bait 200 mg/ml	Mussel 200 mg/ml	Mussel 5.5 g/300 mL	Mussel 25 g/300 mL
His	484.4	193.3	22.5	475.1	48.2	252.6
Tau	–	–	–	9736.2	994.6	5115.8
Ser	238.6	101.6	53.5	897.9	96.1	497.8
Arg	612.7	235.1	136.1	851.5	76.8	393.8
Gly	473.7	194.6	70.2	8440.5	865.3	4426.6
Asx*	347.4	182.9	140.8	1805.8	138.8	979.8
Met	67.9	29.1	–	101.9	–	–
Glx**	449.3	237.1	170.4	2542.2	274.3	1457
Thr	172.3	74.5	39.5	68.6	57.1	299.8
Ala	1045.7	517.7	204.9	3733.7	386.3	1990.2
GABA	332.8	188.9	105.1	–	–	–
Pro	275.4	10.5	340.0	773.1	85.9	430.4
Orn	135.3	57.0	17.2	1979.2	145.0	749.6
Cys	7.3	4.2	–	–	1.9	5.0
Lys	204.5	89.4	41.1	1661.0	167.8	878.2
Tyr	91.0	63.9	21.3	272.3	35.1	176.8
Met	73.8	30.6	19.5	93.2	12.8	57.6
Val	263.1	117.3	43.9	246.5	27.2	138.6
Ile	133.0	61.7	19.8	168.9	18.6	100.4
Leu	246.9	109.8	30.6	311.2	28.4	152.4
Phe	102.6	45.3	20.9	140.1	17.0	91.6
Σ	5757.7	2544.5	1497.3	34298.9	3527.8	18266.0

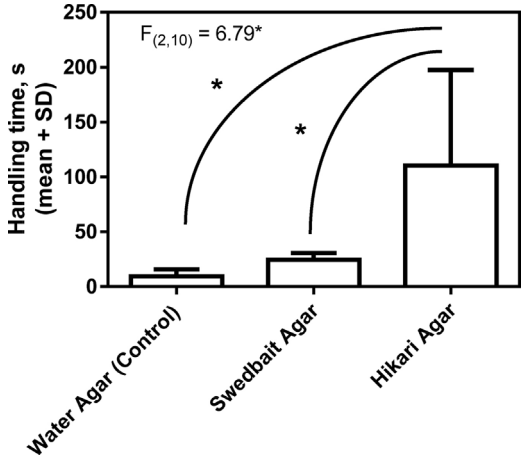

Fig. 2. Comparisons in crucian carp handling time with agar pellets containing only water, extract (control), extracts of Swedbait or Hikari pellet meal. Repeated measures ANOVA revealed statistical significant differences between the agar pellets ($P < 0.05$), and Dunnett's multiple comparisons test revealed that the handling time was significant longer time with Hikari compared to control agar pellets and Swedbait agar pellets, both $P < 0.05$, $N = 6$.

extract, its concentration was 20% of the total amount. Arginine had the second highest concentration, 9% of the total amount in the water extract. Glycine had the highest concentration in the mussel meal extracts (5.5 or 25 g/300 ml), 24% of the total amount and alanine was the second most abundant with 11%. Spearman's rank correlation analysis gave a significant correlation between the concentration ranking order of amino acids in the Hikari water extract and the water extract of mussel meal (5.5 g/300 ml)($\rho = 0.6579$; $t = 3.602$; $df = 17$; $P < 0.01$).

3.2. Stimulating effect of crude extracts

Crucian carp handling time was significantly longer with Hikari meal extract compared to agar pellets with only tap water (Fig. 1). The mean handling time with Hikari was 105 s. Swedbait agar pellets were included in the second test. The repeated measures ANOVA revealed that there were significant differences between the three agar pellets (Fig. 2; $P < 0.05$) and Dunnett's test showed that there were significant differences between Hikari (mean 110 s) and Swedbait (mean 24 s) ($P < 0.05$), and between controls (mean 10 s) and Hikari ($P < 0.05$). Agar pellets with Swedbait were not significantly different from the control (Fig. 2). The handling time was, however, somewhat longer for all five fish with Swedbait compared to the water controls. The effect was close to significant ($P = 0.06$, two tailed). In the third test mussel meal extract was compared with control pellets. We tested two different concentrations of mussel extracts (5.5 g/300 ml; 25 g/300 ml). Neither of these two mussel extracts were significantly different from the control, mean 15 and 32 s, respectively. Two fish handled the agar pellet for 93 and 95 s, respectively, when it contained the higher concentration (Fig. 3A and B). Pooling of the results gave no significant effect ($P > 0.05$; $N = 10$).

3.3. Stimulating effects of amino acids

Amino acids were tested in the following four tests. As only agar pellets with Hikari extract gave a clear significant effect the following tests were based on the FAA composition shown in analyses of that water extract. Statistical analyses of the ranking order of FAA showed, however, that there were no differences between extracts of mussels and Hikari, respectively.

Two different amino acid solutions were tested; a low and a high concentration mixture. The final nominal concentrations in the pellets were in the low concentration group 2.0 mM alanine, 1.0 mM glycine, 1.0 mM arginine, 0.4 mM serine and 0.4 mM leucine. The concentrations in the high concentration group were 20 times higher, alanine 40 mM, glycine 20 mM, arginine 20 mM, serine 8 mM and leucine 8 mM.

The low concentration AA mixture (LAA) did not given any significant difference in handling time compare to the agar pellet with only water (Fig. 4A). There were three fish that increased their handling time with amino acids (two from 10 to 24 s, and the third from 15 to 44 s). The high concentration AA mix (HAA) gave a significant increase in handling time compare to the controls (Fig. 4B; $P < 0.05$).

As the mixture of five amino acids gave an increase in handling time the amino acids were divided into two groups with three (3AA; Ala, Gly, Arg; total conc. 80 mM) and two (2AA; Ser, Leu; total conc. 16 mM) amino acids, respectively. The repeated measures ANOVA gave significant differences between the treatments ($F_{(2,8)} = 4.81$,

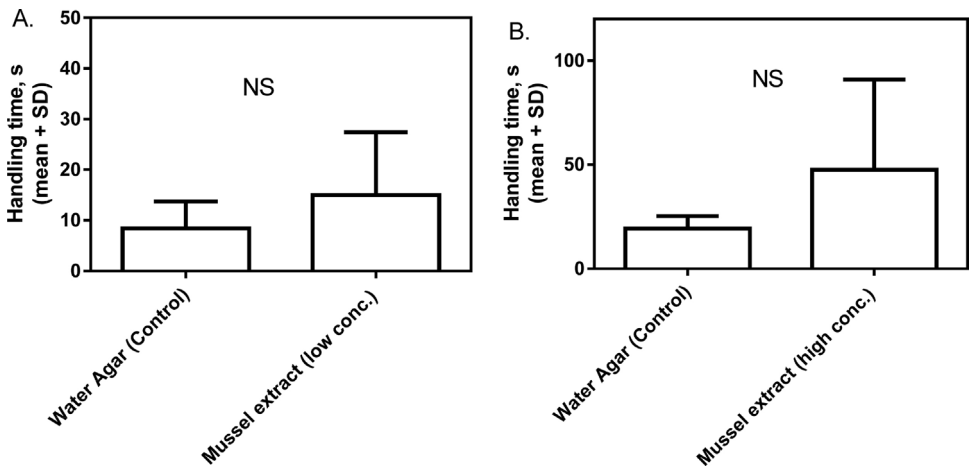

Fig. 3. Crucian carp handling time with agar pellets with two concentrations of mussel meal extracts, 5.5 g/300 ml water (a) and 25 g/300 ml water (b). There were no statistical significant differences between control pellets and pellets with mussel extracts. Both N = 5.

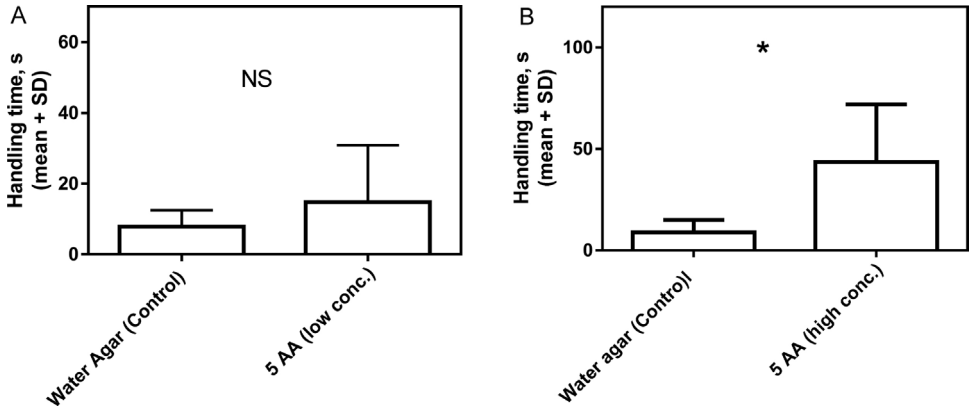

Fig. 4. Crucian carp handling time with agar pellets containing a mixture of five amino acids (Ala, Arg, Gly, Leu, Ser) at two total concentrations, 4.8 and 96 mM, respectively. There was no significant difference between control agar pellets and pellets with the low concentration of amino acids (a), but there was a statistical significant difference with the high concentration, $P < 0.05$ (paired *t*-test) (b). Low concentration N = 7, high concentration N = 6. The same individuals were used with both concentrations but on different days. The high concentration mixture was tested first.

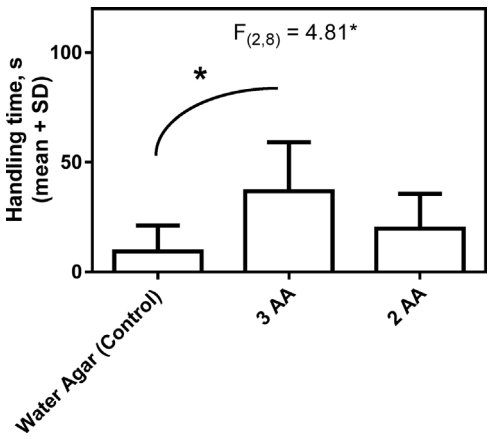

Fig. 5. Crucian carp handling time with agar pellets containing two different amino acid mixtures, one mixture with three amino acis (Ala, Arg, Gly, total 80 mM) and with two amino acids (Ser, Leu, total 16 mM). A repeated measure ANOVA revealed that there were differences in handling time between the three pellets ($p < 0.05$). The three amino acids gave significant longer handling time compared to the control pellets, $P < 0.05$ (Dunnett's multiple comparisons test), N = 5.

$P < 0.05$). The 3AA solution gave a significant effect ($P < 0.05$, one tailed; we predicted that the AA solution should give longer handling time compare to controls) but this effect was not shown with 2AA (Fig. 5). Finally we included only alanine or glycine in the agar pellet. Alanine (128 mM) gave a significantly longer handling time compare to the control pellets ($P < 0.05$, one tailed) (Fig. 6A). This was not the case with glycine (151 mM) (Fig. 6B). The final test was done with betaine (100 mM). No significant effect was observed with betaine (Fig. 7).

3.4. Comparisons between all stimulating solutions

In the final analyses all solutions that gave a significant increase in handling times were compared. The difference between the agar pellets with stimulants and the control water agar for each individual was used as an observation. The one way ANOVA with log transformed values revealed that there were significant differences between the stimulants ($P < 0.05$; Fig. 8). The following Tukey's multiple comparisons test showed that the handling time with alanine pellets was significant shorter than with pellets with Hikari extract ($P < 0.05$; Hikari 1: $q = 4.935$; Hikari 2: $q = 4.160$).

4. Discussion

The water extract of Hikari pellets stimulated mouth handling in crucian carp whereas no stimulating effect was observed with extract of blue mussel meal or the commercial cyprinid attractant. That blue mussel did not have a stimulating effect in crucian carp

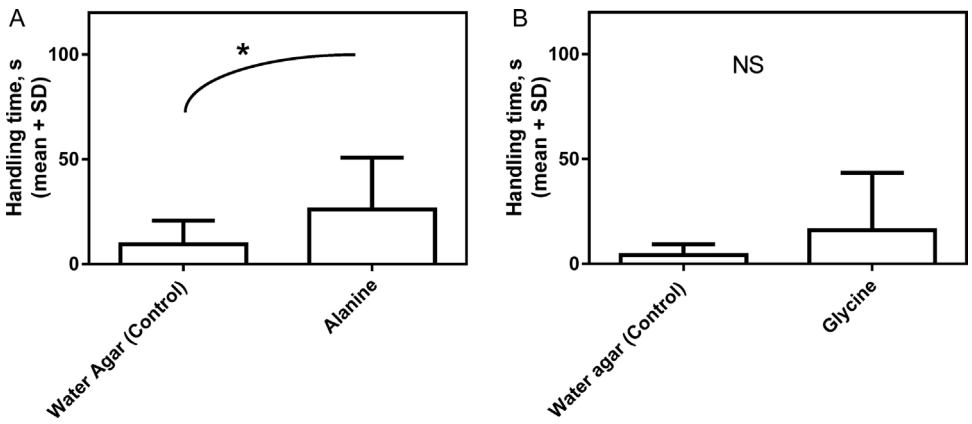

Fig. 6. Crucian carp handling time with agar pellets containing either L-alanine (128 mM) (a) or glycine (151 mM) (b). Only L-alanine gave a longer handling time compared to controls, paired *t*-test one-tailed, $P < 0.05$. The same individuals were tested with both amino acids on different days. Alanine N = 9, glycine N = 8.

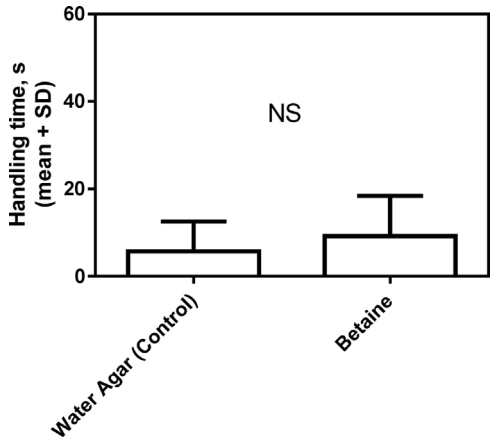

Fig. 7. Crucian carp handling time with agar pellets with betaine (100 mM). Paired *t*-test gave no significant different in handling time between betaine and control pellets, N = 6.

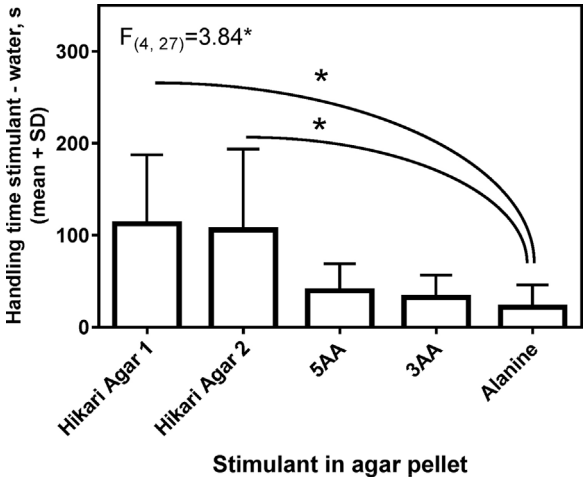

Fig. 8. Comparisons between the stimulants that gave an increase handling time compared to controls. The difference in handling time between the agar pellets with stimulants and the control agar for each individual was used as an observation. The one way ANOVA revealed that there were differences among the stimulants, $P < 0.05$. The Tukey's multi comparisons test showed that alanine gave a significant shorter handling time than with Hikari extracts, $P < 0.05$. Hikari agar 1, N = 6; Hikari agar 2, N = 6; 5 AA, N = 6; 3AA, N = 5; Alanine, N = 9.

can probably be explained by the fact that blue mussel is not a natural feed for crucian carp. A mixture of five amino acids (alanine, glycine, arginine, serine and leucine), that made up approx. 40% of the total concentration of the amino acids analyzed in Hikari and mussel extracts, gave a significantly longer handling time compared to agar pellets with only water. This was also the case with a mixture of the three with the highest concentrations (Ala, Gly, Arg) and alanine alone, but not with glycine. The glycine concentration tested was in the range found in marine crustaceans (Carr et al., 1996). These results show that amino acids act as feeding stimulants in the omnivorous crucian carp. Previous studies found neither feeding stimulants nor deterrents in crucian carp when testing 21 l-amino acids (Kasumyan and Døving, 2003). The comparisons of agar pellets with Hikari extract reveal that there are more than the amino acids tested that are important. This may have been some other amino acid that was not included in the mixture or some other water soluble compound. The taste preferences in fish are highly species-specific (e.g. Yacoob and Browman, 2007b) and the compounds important as feeding stimulants differ even between closely related species (Kasumyan and Døving, 2003). Electro-physiological studies of the gustatory sense in fishes have shown species differences in the number of amino acids that gave a response, from 3 to 5 amino acids in some species ("limited response range") to several in other species ("wide response range")(Hara, 1994). Goldfish and common carp were included in the former group sharing three of the five amino acids, e.g. alanine, proline and betaine.

The amino acids detected by both the gustatory and the olfactory senses are neutral with few carbon atoms, unbranched and uncharged side chains (e.g. Hara, 1994, 2006). In addition to being highly stimulatory to the gustatory sense (reviewed in Kasumyan and Døving, 2003) amino acids can induce various olfactory mediated behaviours connected to foraging (e.g. Olsén et al., 1986; Jones, 1992; Hara, 2006). The concentrations of free amino acids in the commercial cyprinid attractant were low and may have been too low to stimulate the gustatory sense, but may be stimulatory to the olfactory sense, as a long distance sense is often much more sensitive (e.g. Kohbara et al., 2000), but there are exceptions in species with facially innervated taste bud barbels (Marui and Caprio, 1992).

The mussel extract did not act as feeding stimulant, though the concentrations of amino acids in the water extract were much higher than in the Hikari extract. Further, there were no significant differences in concentration ranking order of FAA between the extracts. A possible explanation could be that the mussel extract contained molecules that act as feeding deterrents in crucian carp. Compounds acting as feeding stimulants and deterrents vary between species due to natural food choice (Kasumyan and

Døving, 2003). As earlier mentioned there were high concentrations of taurine in the mussel meal and its water extract. Taurine is important in marine molluscs to increase their osmolarity (e.g. Sherwood et al., 2005) and the compound is essential in several marine fish species, e.g. Japanese flounder (*Paralichthys olivaceus*), but not in the freshwater species carp and rainbow trout (Takeuchi, 2014). It is probably important to species that need taurine to find food that is a good source of the compound. In fact taurine acts, in combination with amino acids, as a feeding stimulant for plaice (*Pleuronectens platessa*) and dab (*Limanda limanda*)(Mackie, 1982) feeding on molluscs and polychaets. Further the author found that in rainbow trout (*Onchorhynchus mykiss*) taurine in combinations with alanine and arginine were feeding deterrents (prevent or terminate feeding; Mackie, 1982).

Betaine also acts as an osmolyte in marine molluscs (e.g. Sherwood et al., 2005). Recently, blue mussel meal has been used to increase the palatability of rapeseed protein-based diets in turbot (*Psetta maxima*) (Nagel et al., 2014). In three *Salvelinus* species no electrophysiological taste response was shown with betaine (Hara et al., 1993; Hara, 1994). The only amino acid taste receptor was sensitive to L-proline that is also highly stimulatory to the rainbow trout gustatory facial nerve (Yamashita et al., 2006) but no responses were observed in olfactory receptor cells (Hara, 1994). Rainbow trout also, in contrast to the charr species, has a taste receptor to betaine (Hara, 1994; Yamashita et al., 2006). In the present study with crucian carp, betaine had no significant effect on handling time. In a study with the closely related gibel carp betaine had a slightly stimulating effect on feeding, but L-phenylalanine, glycine, L-methionine and L-lysine individually had much stronger effects (Xue and Cui, 2001).

The present study shows that measures of mouth handling time can be a method of obtaining information about gustatory preferences in benthic cyprinid fish. It is important to know what kind of compounds make feed more palatable and stimulate ingestion; the final step in foraging. As the olfactory sense is important to induce arousal and foraging behaviour in many fish (e.g. Holland, 1978; Atema et al., 1980; Olsén et al., 1986; Døving and Knutsen, 1993; Hara, 2006), it is also important to study these parts of the feeding process. Amino acids are often important to induce food search behaviour and the active molecules can be different between species. It has been shown in goldfish that two behaviours connected to foraging, increased swimming activity and gravel pecking, are induced by different amino acids (Hara, 2006). The next step in the present project will be to study the importance of free amino acids and other small molecules as foraging stimulating compounds via the olfactory sense. The stimulating odours should induce arousal with increased swimming behaviour, food searching behaviour and attraction to the source followed by feeding stimulation via the gustatory sense. As plant materials are part of the diet of several cyprinids, crucian carp included, it would be worhtwhile to investigate the importance of water soluble plant related compounds as attractants and feeding stimulants. The water plant and alga related sulphur-containing compound dimethyl-ß-propiotethin (DMTP) induced snapping behaviour in goldfish, common carp and Japanese crucian carp (*Carassius auratus cuvieri*) and the response was much stronger than with glutamine (Nakajima et al., 1989a, b). Organic acids and acidic amino acids such as glutamic acid have been shown to act as feeding stimulants in herbivorous fish (Adams et al., 1988; Ishida and Kobayashi, 1992).

Statement of relevance

During buccal handling fish decide if the feed will be rejected or swallowed. Feed that does not taste correctly is rejected. In the present study buccal handling in crucian carp was significant longer when agar pellets contained extracts of feed pellets or synthetic amino acids. Blue mussel meal extract had no stimulating effect though high concentrations of free amino acids.

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References

Adams, A.M., Johnsen, P.B., 1986. A solid matrix bioassay for determining chemical feeding stimulants. Prog. Fish Cult. 48, 147–149.
Adams, M.A., Johnsen, P.B., Hong-Q.I, Z., 1988. Chemical enhancement of feeding for the herbivorous fish *Tilapia zilli*. Aquaculture 72, 95–107.
Atema, J., Holland, K., Ikehara, W., 1980. Chemical search image: olfactory responses of yellow fin tuna (*Thunnus albacares*) to prey odors. J. Chem. Ecol. 6, 457–465.
Cadena-Roa, M., Huelvan, C., Le Borgne, Y., Métailler, R., 1982. Use of rehydratable extruded pellets and attractive substances for the weaning of sole (*Solea vulgaris*). J. World Maricul. Soc. 13, 246–253.
Carlberg, H., Cheng, K., Lundh, T., Brännäs, E., 2015. Using self-selection to evaluate the acceptance of new diet formulation by farmed fish. Appl. Anim. Behav. Sci. 171, 226–232.
Carr, W.E.S., Nertherton, J.C., Gleeson, R.A., Derby, C.D., 1996. Stimulants of feeding behavior in fish: analyses of tissue of diverse marine organisms. Biol. Bull. 190, 149–160.
Carr, W.E.S., 1982. Chemical stimulation of feeding behavior. In: Hara, T.J. (Ed.), Chemoreception in Fishes. Developments in Aquaculture and Fisheries Science, Vol. 8. Elsevier, Amsterdam, pp. 259–273.
Copp, G., Tarkan, S., Godard, M., Edmonds, N., Wesley, K., 2010. Preliminary assessment of feral goldfish impacts on ponds: with particular reference to native crucian carp. Aquat. invasions 5, 413–422.
Døving, K.B., Knutsen, J.A., 1993. Feeding responses and chemotaxis in marine fish larvae. In: Kanshik, S.J., Luquet, P. (Eds.), Fish Nutrition in Practice. Institut National de la Recherche Agronomique, Paris (France), pp. 579–583.
Hara, T.J., Sveinsson, T., Evans, R.E., Klaprat, D.A., 1993. Morphological and functional characteristics of the olfactory and gustatory organs of three *Salvelinus* species. Can. J. Zool. 71, 414–423.
Hara, T.J., 1994. The diversity of chemical stimulation in fish olfaction and gustation. Rev. Fish Biol. Fish. 4, 1–35.
Hara, T.J., 2006. Feeding behaviour in some teleosts it triggered by single amino acids primarily by olfaction. J. Fish Biol. 68, 810–825.
Holland, K., 1978. Chemosensory orientation to food by a Hawaiian goatfish (*Parupeneus prophyreus*, Mullidae). J. Chem. Ecol. 4, 173–186.
Ishida, Y., Kobayashi, H., 1992. Stimulatory effectiveness of amino acids on the olfactory response in an algivorous marine teleost, the rabbitfish *Siganus fuscescens* Huottuyn. J. Fish Biol. 41, 737–748.
Janson, S., Wouters, J., Bonow, M., Svanberg, I., Olsén, K.H., 2014. Population genetic structure of crucian carp (*Carassius carassius*) in man-made ponds and wild populations in Sweden. Aquacult. Int., http://dx.doi.org/10.1007/s10499-014-9820-4.
Jeffries, D.L., Copp, G.H., Lawson Handley, L., Olsén, K.H., Sayer, C.D., Hänfling, B., 2016. Comparing RADseq and microsatellites to infer complex phylogeographic patterns, an empirical perspective in the crucian carp *Carassius carassius*, L. Mol. Ecol., http://dx.doi.org/10.1111/mec.13613.
Jones, K.A., 1992. Food search behaviour in fish and the use of chemical lures in commercial and sports fishing. In: Hara, T.J. (Ed.), Fish Chemoreception. Fish and Fisheries Series 6. Chapman & Hall, London, pp. 288–320.
Kamstra, A., Heinsbroek, L.T.N., 1991. Effects of attractants on start feeding of glass eel, *Anguilla anguilla* L. Aquacult. Fish. Manag. 22, 47–56.
Karvonen, A., Bagge, A.M., Valtonen, E.T., 2005. Parasite assemblages of crucian carp (*Carassius carassius*) –is depauperate composition explained by lack of parasites exchange, extreme environmental conditions or host unsuitability? Parasitology 131, 273–278.
Kasumyan, A.O., Døving, K.B., 2003. Taste preferences in fishes. Fish 4, 289–347.
Kasumyan, A.O., Morsi, A.M. Kh., 1996. Taste sensitivity of common carp *Cyprinus carpio* to free amino acids and classical taste substances. J. Ichthyol. 36, 391–403.
Kasumyan, A.O., Nikolaeva, E.V., 2002. Comaprative analysis of taste preferences in fishes with different ecology and feeding. J. Ichthyol. 42, S203–S214.
Kasumyan, A.O., 2012. The intraoral tactile reception and its interaction with the gustatory system in fish. Dokl. Akad. Nauk 447, 579–581.
Kohbara, J., Hidaka, I., Morishita, T., Miyajima, T., 2000. Gustatory and olfactory sensitivity to extracts of jack mackerel in young yellowtail *Seriola quinqueradiata*. Aquaculture 181, 127–140.

Kullander, S.O., Nyman, L., Jilg, K., Delling, B., 2012. *Carassius carassius* ruda. In: Nationalnyckeln till Sveriges fauna och flora. Strålfeniga fiskar. Actinopterygii, Artdatabanken. SLU, Uppsala, pp. 126–127.

Lamb, C.F., Finger, T.E., 1995. Gustatory control of feeding behavior in goldfish. Physiol. Behav. 57, 483–488.

Langeland, M., Vidakovic, A., Vielma, J., Lindberg, J.E., Kiessling, A., Lundh, T., 2014. Digestibility of micobial and mussel meal for Arctic charr (*Salvelinus alpinus*) and Eurasian perch (*Perca fluviatilis*). Aquacult. Nutr., http://dx.doi.org/10.1111/anu.12268.

Laurila, S., Piironen, J., Holopainen, I.J., 1987. Notes on egg development and larval and juvenile growth of crucian carp (*Carassius carassius* (L.)). Ann. Zool. Fennici 24, 315–321.

Lelek, A., 1987. The freshwater fishes of Europe. Threaten Fishes of Europe. European Committee for the Conservation of Nature and Natural Resources, Vol. 9. Council of Europe Wiesbaden, A ula-Verlag GmbH, pp. 168–170.

Métailler, R., Cadena-Roa, M., Person-Le Ruyet, J., 1983. Attractive chemical substances for the weaning of Dover sole (*Solea vulgaris*): qualitative and quantitative approach. J. World Maricul. Soc. 14, 679–684.

Mackie, A.M., 1982. Identification of the gustatory feeding stimulants. In: Hara, T.J. (Ed.), Fish Chemoreception. Fish and Fisheries Series 6. Chapman & Hall, London, pp. 275–291.

Marui, T., Caprio, J., 1992. Teleost gustation. In: Hara, T.J. (Ed.), Fish Chemoreception. Fish and Fisheries Series 6. Chapman & Hall, London, pp. 171–198.

Nagel, F., von Danwitz, A., Schlachter, M., Kroeckel, S., Wagner, C., Schulz, C., 2014. Blue mussel meal as feed attractant in rapeseed protein- based diets for turbot (*Psetta maxima* L.). Aquacult. Res. 45, 1964–1978.

Nakajima, K., Uchida, A., Ishida, Y., 1989a. A new feeding attractant dimethyl-ß-propiothetin, for freshwater fish. Nippon Suisan Gakkashi 55, 689–695.

Nakajima, K., Uchida, A., Ishida, Y., 1989b. Effect of supplemental dietary feeding attractant, dimethyl-ß-propiothetin, on growth of goldfish. Nippon Suisan Gakkashi 55, 1291.

Olsén, K.H., Karlsson, L., Helander, A., 1986. Food search behavior in Arctic charr, *Salvelinus alpinus* (L.), induced by food extracts and amino acids. J. Chem. Ecol. 12, 1987–1998.

Olsén, K.H., Sawisky, G.R., Stacey, N.E., 2006. Endocrine and milt responses of male crucian carp (*Carassius carassius* L.) to periovulatory females under field conditions. Gen. Comp. Endocrinol. 149, 294–302.

Papatryphon, E., Soares Jr., J.H., 2000. The effect of dietary feeding stimulants on growth performance of striped bass, Morone saxatilis, fed-a-plant feedstuff-based diet. Aquaculture 185, 329–338.

Piironen, J., Holopainen, I.J., 1986. A note on seasonality in anoxia tolerance of crucian carp (*Carassius carassius* (L.)) in the laboratory. Ann. Zool. Fennici. 23, 335–338.

Pratoomyot, J., Bendiksen Å, E., Bell, J.G., Tocher, D.R., 2010. Effects of increasing replacement of dietary fishmeal with plant protein sources on growth performance and body lipid composition of Atlantic salmon (*Salmo salar* L.). Aquaculture 305, 124–132.

Reverter, M., Lundh, T., Lindberg, J.E., 1997. Determination of free amino acids in pig plasma by precolumn derivatization with 6-N-aminoquinyl-N-hydroxysuccinimidyl carbamate and high performance liquid chromatogrphy. J. Chrom. B 696, 1–8.

Rylková, K., Kalous, L., Bohlen, J., Lamatsch, D.K., Petrtýl, M., 2013. Phylogeny and biogeographic history of the cyprinid fish genus Carassius (Teleostei: Cyprinidae) with focus on natural and anthropogenic arrivals in Europe. Aquaculture, 13–20 (380–383).

Sherwood, L., Klandorf, H., Yancey, P.H., 2005. Animal physiology. In: From Genes to Organisms. Brooks/Cole. Thomson Learning, Belmont, USA.

Sibbing, F.A., Uribe, R., 1985. Regional specializations of the oro-pharyngeal wall and food processing in the carp (*Cyprinus carpio*). Neth. J. Zool. 35, 377–422.

Sibbing, F.A., Osse, J.W.M., Terlouw, A., 1986. Food handling in the carp (*Cyprinus carpio*): its movement patterns, mechanisms and limitations. J. Zool. London A 210, 161–203.

Stacey, N.E., Van Der Kraak, G.J., Olsén, K.H., 2012. Male primer endocrine responses to preovulatory female cyprinids under natural conditions in Sweden. J. Fish Biol. 80, 147–165.

Svanberg, I., Bonow, M., Olsén, H., 2012. Fish ponds in Scania: and Linnaeus's attempt to promote aquaculture in Sweden. Svenska Linnésällskapets Årsskrift 2012, 83–98.

Takeda, M., Takii, K., 1992. Gustation and nutrition in fishes: application to aquaculture. In: Hara, T.J. (Ed.), Fish Chemoreception. Fish and Fisheries Series 6. London, Chapman & Hall, pp. 271–287.

Takeuchi, T., 2014. Progress on larval and juvenile nutrition to improve the quality and health of seawater fish: a review. Fish. Sci., http://dx.doi.org/10.1007/s12562-0140744-8.

Vidakovic, A., Langeland, M., Sundh, H., Sundell, K., Olstorpe, M., Vielma, J., Kiessling, A., Lundh, T., 2015. Evaluation of growth performance and intestinal function in Arctic charr (*Salvelinus alpinus*) fed yeast (*Saccharomyces cerevisiae*), fungi (*Rhizopus oryzae*) and blue mussel (*Mytilus edulis*). Aquacult. Nutr., http://dx.doi.org/10.1111/anu.12344.

Wouters, J., Janson, S., Lusková, V., Olsén, K.H., 2012. Molecular identification of hybrids of the invasive gibel carp *Carassius auratus gibelio* and crucian carp *Carassius carassius* in Swedish waters. J. Fish Biol. 80, 2595–2604.

Xue Cui, M., Cui, Y., 2001. Effects of several feeding stimulants on diet preference by juvenile gibel carp (*Carassius auratus gibelio*), fed diets with or without partial replacement of fish meal with meat and bone meal. Aquaculture 198, 281–292.

Yacoob, S.Y., Browman, H.I., 2007a. Prey extracts evoke swimming behavior in juvenile Atlantic halibut (*Hippoglossus hippoglossus*). Aquaculture 270, 570–573.

Yacoob, S.Y., Browman, H.I., 2007b. Olfactory and gustatory sensitivity to some feed-related chemicals in the Atlantic halibut (*Hippoglossus hippoglossus*). Aquaculture 263, 303–309.

Yamashita, S., Yamada, T., Hara, T.J., 2006. Gustatory responses to feeding- and non-feeding-stimulant chemicals with an emphasis on amino acids, in rainbow trout. J. Fish Biol. 68, 783–800.