=== **ZOOLOGY** ===

Orosensory Food Testing in Fish: Chronology of Behavior

E. S. Mikhailova and A. O. Kasumyan

Faculty of Biology, Moscow State University, Moscow, 119234 Russia
e-mail: elena_mikhailova@mail.ru
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Abstract—The orosensory food testing behavior in fish was studied using the nine-spined stickleback *Pungitius pungitius* as an example. The patterns of the chronology of manipulations performed by fish in testing food objects were identified. The existence of two stereotypical patterns of feeding behavior was confirmed, and their new characteristics were obtained. The relationship between the responsiveness to food and the predisposition of fish to feeding was revealed.

INTRODUCTION

Many animals manipulate food objects when they learn hunting skills, study the properties of these objects, and prepare for ingestion. Food manipulations is also typical of fish that do not always ingest food immediately after grasping. Usually this is preceded by holding the object in the mouth, which is interrupted by throwing it outwards and grasping again. These actions occur for different reasons. They are required to reorient the object and are observed when fish feed on large prey or on prey with well-developed passive defense (spines, thorns, hard shell, etc.) (Kislalioglu and Gibson, 1976; Gill and Hart, 1994; L'Abe'e-Lund et al., 1996). Mollusk-eating fish in order to ingest the wholesome components and discard the indigestible fragments perform breaking down the shell of prey and expelling its fragments (Hoogerhoud, 1989). However, repeated grasping is primarily associated with food testing. The orosensory evaluation of a grasped food object by fish involves the gustatory and tactile systems, whose receptors are distributed in the epithelium of the oral cavity (Kapoor et al., 1975; Devitsina, 2005). The highest density of taste buds is observed on the lips and on the teeth-carrying structures (i.e., in the areas with the highest probability of contact with prey) (Hara et al., 1993; Linser et al., 1998). The textural qualities of food are evaluated through mechanosensory nerve endings, which are found throughout the epithelium and penetrate, in particular, into the perigemal region of the taste buds (Sakata et al., 2001; Kasumyan, 2011). The epithelium of the oral cavity also contains single chemosensory cells whose function remains obscure (Whitear, 1992).

Manipulation of a food object, as performed by fish, depends on the gustatory and texture properties of food, which emphasizes the relationship between the handling activity and orosensory evaluation (Kasumyan, 2012). However, the specificity of the testing process and its dependence on the fish state and feeding type and manner and on the properties of the food consumed remain unstudied. Special studies in this field have not been performed, and the available data are fragmentary and were obtained in studies of related problems—feeding ecology of fish, feeding behavior, and sensory regulation of the feeding behavior (Ibrahim and Huntingford, 1992; Gill and Hart, 1994; Lamb and Finger, 1995; Ellis and Gibson, 1997; Kasumyan and Marusov, 2015b). It is known, in particular, that repeated grasps of food objects are not typical or completely absent in fish with poor vision and fish living in streams (Shamushaki et al., 2011; Kasumyan, 2014). However, this behavior is well expressed in fish with well-developed vision or in those living in slowly flowing or stagnant water (Kasumyan and Mikhailova, 2014).

The number of repeated grasps of a food object before ingestion or rejection may reach 10 or more. The total time spent on the sensory evaluation of a grasped object differs in different species and depends on the state of the fish (Kasumyan and Prokopova, 2001; Kasumyan et al., 2009; Kasumyan and Marusov, 2015a). The duration of intermediate grasps and the time intervals between them were not determined. It is also unclear whether the chronology of these actions has certain consistent patterns and whether it depends on the properties of the test object. Clarification of these issues is important for understanding the mechanisms of feeding behavior of fish.

The aim of this study was to investigate the chronology of fish behavior during orosensory food testing.

MATERIALS AND METHODS

The study was performed with 22 adult nine-spined stickleback *Pungitius pungitius* (6−7 cm long) caught in Khimka Brook (Moscow). Fish were kept individually in aerated 5 L aquariums at a water temperature of

12−14°C. After 2−3 days of acclimation, the fish were taught to grasp individually supplied agar-agar (2%, Reanal) pellets containing an water extract (175 g/L) of larval Chironomidae.

In experiments we used both the control pellets containing the dye alone and pellets filled with 0.01 M L-aspartic acid, which at this concentration was shown earlier to be palatable to the nine-spined stickleback from the Moskva River population and, unlike other substances, did not reduce the frequency of grasps of pellets (Mikhailova and Kasumyan, 2015). All pellets (4 mm in length and 1.35 mm in diameter) were of a bright red color due to the presence of 5 μM Ponceau 4R dye (Chroma-Gesellschaft Schmid Gmbh, Germany).

The experiments were performed in two consecutive series. In series 1, the individual characteristics of the response of 22 fish to the pellets were determined. After adding one pellet to an aquarium, the number of grasps was counted and the duration of retention of each pellet at the first grasp and totally throughout the trial was determined with a stopwatch. At the end of the experiment, we recorded whether or not the pellet was consumed by the fish.

For series 2, which was performed 50 days later, two sticklebacks (fish nos. 12 and 16) were selected, which more often than others repeatedly grasped pellets and whose food response parameters (including consumption) were close to the average group values. After the introduction of one pellet containing aspartic acid into the aquarium, the latent response time (the time from the pellet fell into the water until the first grasp by fish), the duration of retention of pellets at each grasp (R_1, ..., R_n), and the duration of the intervals between retentions (I_1, ..., I_n) were recorded using the BH-Fish software with an accuracy of 0.1 s. The time that elapsed after the pellet fell into the water until its ingestion or final rejection by fish was taken as the duration of the entire trial. The trials with fish nos. 12 and 16 were performed alternately, with at least a 15-min interval between the tests. The total number of trials with one fish was 15−20 per day.

The moment of ingestion of the pellet was determined by the characteristic movements of jaws and gill covers of the fish, and the moment of ultimate rejection of the pellet was determined by the following behavior: the fish swam away from the pellet (often to the opposite side of the aquarium) and showed no interest in it for several minutes. If the fish did not grasp the pellet within 1 min, the trial was not taken into account. The experiments in which the fish destroyed but did not ingest the pellet or ingested less than half of the pellet were regarded as experiments in which consumption did not occur.

In total, we performed 671 experiments (264 in series 1 and 407 in series 2; 212 and 195 tests with fish nos. 12 and 16, respectively). Data were statistically processed using the Chi-square test, the Mann−Whit-

ney U test, and the Spearman rank correlation coefficient (r_S).

RESULTS

Fish occupied the standard position in the center of the aquarium at a distance of 3−5 cm from the water surface, with the head oriented towards the place where the pellets usually fell. When moving aside or during routine search activity at the bottom, they immediately returned to the center of the aquarium at the approach of the experimenter. When the experimenter placed a hand over the aquarium, the expectation and readiness of fish to grasp the pellet were always clearly expressed.

Individual features of fish (series 1). In total, we performed six trials with the control pellets and six trials with the pellets with aspartic acid with each fish. Out of the 22 fish, seven fish refused to consume the pellets with aspartic acid, four fish consumed the maximum number of pellets (five out of six), and the remaining fish occupied an intermediate position with respect to this parameter. In nine fish, the consumption of pellets with the amino acid significantly exceeded the consumption of the control pellets. The mean number of grasps of the pellets with the amino acid by fish varied from 1.2 to 8.2, and the total duration of the first and the total retention of pellet was 0.8−11.1 and 2.4−29.5 s, respectively; i.e., these parameters of fish response differed by 7, 12.5, and 13 times, respectively. The responses of fish to the control pellets also varied significantly (Table 1).

For the next stage of the study, we selected fish nos. 12 and 16, which more often than others repeatedly grasped the pellets with aspartic acid (8.2 and 4.8, respectively) and the control pellets (6.3 and 6.2, respectively) and differed significantly in this parameter from other fish ($p < 0.001$ and $p < 0.01$ for the pellets with aspartic acid and the control pellets, respectively, for the pooled sample of fish nos. 12 and 16 versus the sample including the remaining 20 fish). Fish nos. 12 and 16 were far ahead of other individuals in the total pellet retention time ($p < 0.01$ for both types of pellets). Consumption of pellets with aspartic acid and control pellets by fish nos. 12 and 16 was close to the group-average values ($p > 0.05$) (Table 1).

Duration of response components (series 2). Fish nos. 12 and 16 grasped the pellet that had fallen into the water, on average, in 2.2 s (latent time). In 157 out of 407 trials, fish ingested the pellet with aspartic acid (38.6%); i.e., the consumption did not differ from the group-average consumption in series 1 ($p > 0.05$). In 98 trials (24%), only one grasp was recorded. The greater the number of grasps in an experiment, the smaller the number of such trials. The maximum number of grasps (11) was recorded in only two trials (figure). The mean number of grasps was 3.3 ± 0.1, which is close to the group-average value in series 1 (3.2 ± 0.3). The duration of the first and total pellet

Table 1. Individual indices of the behavioral response of the nine-spined stickleback *Pungitius pungitius* to the pellets with 0.01 M L-aspartic acid and the control pellets (series 1) (data are expressed as the mean value and the standard error of the mean, $M \pm m$)

Fish no.	Pellet consumption, %		Number of grasps		Pellet retention time, s			
					after the first grasp		throughout the trial	
	aspartic acid	control	aspartic acid	control	aspartic acid	control	aspartic acid	control
1	0	0	4.5 ± 1.1	3.2 ± 1.1	1.1 ± 0.2	1 ± 0.1	3.9 ± 0.9	2.3 ± 0.8
2	0	0	2.2 ± 0.4	1.7 ± 0.3	1.2 ± 0.3	1.2 ± 0.3	2.4 ± 0.4	1.9 ± 0.4
3	0	0	3.2 ± 1.1	2.3 ± 0.6	1.2 ± 0.1	2.2 ± 0.5	4.2 ± 1.8	4 ± 1.1
4	0	0	2.8 ± 0.8	1.7 ± 0.3	1.3 ± 0.2	1.1 ± 0.1	3.3 ± 1.2	2 ± 0.5
5	0	0	2.7 ± 0.9*	1 ± 0	1.3 ± 0.2	1.4 ± 0.3	3.8 ± 1.5	1.4 ± 0.3
6	0	16.7 ± 16.7	3.5 ± 0.9	3.8 ± 0.5	0.8 ± 0.1	1.5 ± 0.3	4.3 ± 0.6	6.2 ± 1.4
7	0	16.7 ± 16.7	2 ± 0.5	1.8 ± 0.7	1.7 ± 0.4	3 ± 1.5	3.6 ± 1	6 ± 4
8	16.7 ± 16.7	0	4 ± 1.2	1.7 ± 0.3	4.9 ± 1.1*	1.2 ± 0.1	13.5 ± 3.4*	1.8 ± 0.3
9	33.3 ± 21.1	0	1.3 ± 0.3	1.8 ± 0.4	7.3 ± 1.6	3.5 ± 0.9	8.2 ± 1.3	5 ± 1.6
10	33.3 ± 21.1	0	4.8 ± 1.9	4.3 ± 1	11.1 ± 2.3**	1.7 ± 0.4	15.8 ± 2.4**	5.6 ± 1.1
11	33.3 ± 21.1	16.7 ± 16.7	4 ± 1*	1.3 ± 0.2	6.2 ± 1.4*	1.8 ± 0.2	12.4 ± 2.3*	3.1 ± 0.3
12	33.3 ± 21.1	50 ± 22.4	8.2 ± 2	6.3 ± 1.1	5.1 ± 1.3	6.9 ± 1.6	29.5 ± 5.6	21.3 ± 6.6
13	50 ± 22.4*	0	1.5 ± 0.3	2.3 ± 0.6	5 ± 1.9	2.4 ± 1.1	6.3 ± 2.2	4.3 ± 1.8
14	50 ± 22.4*	0	2.3 ± 1	3.7 ± 1	5.2 ± 2.2	1.9 ± 0.4	9.7 ± 3	5.7 ± 1.8
15	50 ± 22.4*	0	2.5 ± 0.6	3.2 ± 0.6	8.4 ± 2.4**	1.7 ± 0.4	12.1 ± 2.1*	5.2 ± 1.2
16	66.7 ± 21.1*	0	4.8 ± 1.7	6.2 ± 0.9	2.6 ± 0.5	1.6 ± 0.2	14.3 ± 3.9	8.6 ± 1.6
17	66.7 ± 21.1*	0	3 ± 1.3	6 ± 1.4	6.9 ± 1.5	2.6 ± 0.9	9.8 ± 0.8	10.5 ± 3.7
18	66.7 ± 21.1	50 ± 22.4	1.8 ± 0.5	3.3 ± 0.7	6.9 ± 2.6	7 ± 1.5	9.8 ± 2.8	13.1 ± 1.6
19	83.3 ± 16.7**	0	1.2 ± 0.2	2 ± 0.4	7.1 ± 1.2*	1.7 ± 0.4	7.3 ± 1*	3.5 ± 1.2
20	83.3 ± 16.7*	16.7 ± 16.7	4.2 ± 1.7	3.5 ± 0.8	8.4 ± 2.5	3.6 ± 1.4	18.9 ± 3.9**	9 ± 1.4
21	83.3 ± 16.7*	16.7 ± 16.7	3.5 ± 2	3.3 ± 0.3	10.8 ± 1.4*	4.3 ± 1	17.6 ± 3.5*	8.9 ± 1.2
22	83.3 ± 16.7*	16.7 ± 16.7	2.5 ± 0.7	3 ± 0.2	11 ± 3.2	6.1 ± 2.1	17.6 ± 3.2	9.6 ± 2.5
Mean	37.9 ± 04.2***	9.1 ± 2.5	3.2 ± 0.3	3.1 ± 0.2	5.3 ± 0.4***	2.7 ± 0.2	10.4 ± 0.8***	6.3 ± 0.6

*, **, *** Differences from control significant at $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively. In total, six trials were performed with each fish with each type of pellet.

retention in series 2 was 3.2 ± 0.2 and 12.8 ± 0.8, respectively, which was not statistically different from the group-average values in series 1 ($p > 0.05$) (Table 2). The comparison of the results of series 2 with the pooled data for fish nos. 12 and 16 in series 1 showed that only the number of grasps differed significantly (3.3 and 6.5, respectively; $p < 0.01$).

The pellet retention time with each successive grasp decreased from 3.2 s in R_1 to 2 s in R_6, but then increased again. The intervals between grasps varied insignificantly and usually were 0.8–1 s (Table 2). The more grasps performed by the fish, the longer the trial (the mean experiment duration was ~30 s) (Table 2).

Comparison of trials with pellet consumption and pellet rejection (series 2). The more grasps performed by a fish in a trial, the less frequently such experiments were observed. In the experiments in which fish eventually rejected the pellet (PR-trials), the reduction was

faster than in the experiments with the pellet consumption (PC-trials). In trials with 1–4 grasps, the pellet was usually rejected rather than consumed. The trials in which the number of grasps was greater than 5 usually ended with swallowing the pellet (Table 2).

The two compared experimental groups differed in many characteristics. The latent period duration in the PC-trials was 1 s, which was 3 times less than in the PR-trials. At 1–3 pellet grasps in experiments, this difference was 4–5 times ($p < 0.001$) (Table 2).

The pellet retention time in the PC-trials was always significantly greater than in the PR-trials (the comparative analysis was performed with samples of a size was not less than 6). The pellet retention time gradually decreased (3.9 times in R_1 versus 1.3 times in R_6 and R_7). In the PC-trials, the pellet retention time consistently decreased with each subsequent grasp
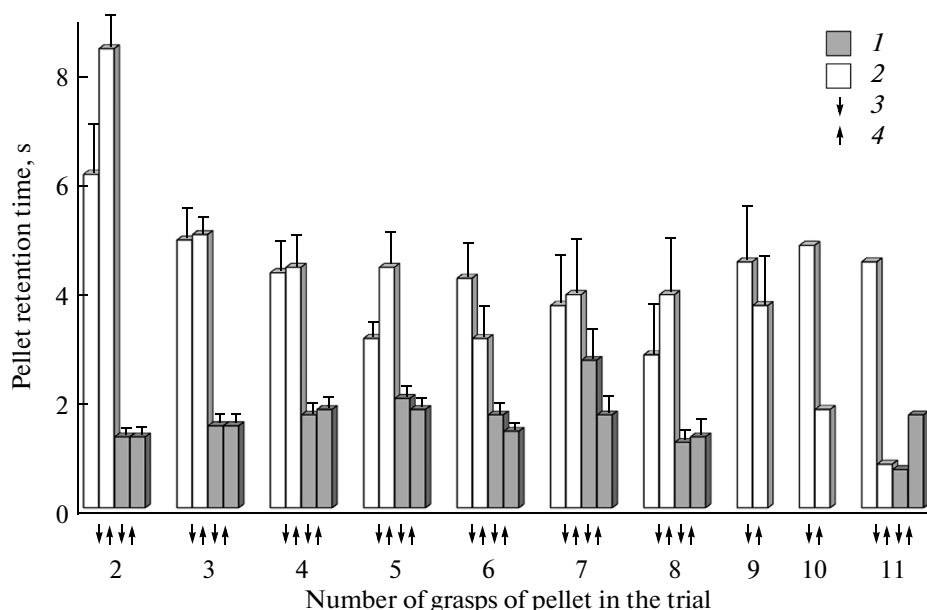
Duration of the (*3*) first and (*4*) last retentions of the pellet with 0.01 M L-aspartic acid by the nine-spined stickleback *Pungitius pungitius* in trials with different numbers of repeated grasps of the pellets: (*1*) trials with refusal to consume the pellet, (*2*) trials with pellet consumption. Data were expressed as $M \pm m$.

from R_1 to R_6, but then increased again from R_7 to R_9 (Table 2). In the PC-trials, the first and last retentions were usually longer regardless of the number of repeated grasps; the last retention, after which the pellet was ingested, was usually longer than the first one. In the PR-trials, the retention time was always several times smaller and less variable (1.4–1.7 s) and did not increase toward the end of the experiment (Table 2, figure).

The greater the number of grasps in the PC-trials, the shorter the pellet retention time, including the first and last retentions. In the PR-trials, this trend was absent (Table 2).

The intervals between the grasps were several times shorter than the pellet retention time, regardless of whether the trial ended with ingestion or rejection of the pellet. These intervals in the PC-trials were always shorter than in the PR-trials; the differences were small but sometimes significant (for I_1, I_2, and I_6). In both groups of trials, the duration of intervals varied slightly (0.6–1.3 and 0.6–1.8 s, respectively). In the PR-trials, the duration of intervals decreased as the number of grasps increased. In the PC-trials, this dependence was not observed.

Correlation analysis revealed no significant correlation between the pellet retention time and the subsequent interval between grasps for both PC-trials ($r_S = 0.1$, $p > 0.05$) and PR-trials ($r_S = 0.6$, $p > 0.05$).

At the same number of grasps, the PC-trials were, on average, always longer than the PR-trials. The difference is most pronounced in the trials with a small number of grasps: at one or two grasps, the duration of

response in the PC-trials was greater by 11 and 4 times, respectively ($p < 0.001$ in both cases), whereas at 6 and 7 grasps it was greater by only 1.6 ($p < 0.05$) and 1.2 ($p > 0.05$) times, respectively.

DISCUSSION

Orosensory food testing behavior. Fish perform various actions with the grasped food objects: destroy them and reject indigestible parts, selectively hold and wash out of the mouth the worthless collateral material, reorient large or morphologically protected prey for ingestion, form conglomerates of microscopically small objects with the use of oral mucus, etc. (Hoogerhoud, 1989; Frazer et al.,1991; Sibbing, 1991; Coyer, 1995; L'Abe'e-Lund et al., 1996; Sanderson et al., 1996; Osse et al., 1997).

Orosensory food testing is also accompanied by numerous grasps. In the nine-spined stickleback, similarly to other fish species, such behavior after grasping includes two main actions: retention of the object in the mouth and throwing it out. Testing is preceded by a short latent period required for preparing and making an accurate hunter's dart. Testing ends either with swallowing or with final refusal by the fish of the object after several retentions: up to 7–8 in the tench *Tinca tinca*, up to 10 in the carp *Cyprinus carpio* and cod *Gadus morhua*, and up to 16 in the bream *Abramis brama* (Ellis and Gibson, 1997; Kasumyan and Prokopova, 2001; Isaeva 2007; Kasumyan and Sidorov, 2010). The nine-spined stickleback repeatedly grasped the pellet a maximum of 11 times. Multiple grasps of an object, even if they are accompanied

Table 2. Duration of the latent period, successive retentions, and intervals between grasps of pellets with 0.01 M L–aspartic acid by the nine-spined stickleback *Pungitius pungitius* (series 2) (data are expressed as the mean value and the standard error of the mean, $M \pm m$)

Number of grasps of the pellet in the trial	Number of trials	L	R_1	I_1	R_2	I_2	R_3	I_3	R_4	I_4	R_5	I_5	R_6	I_6	R_7	I_7	R_8	I_8	R_9	I_9	R_{10}	I_{10}	R_{11}	Total retention	Total interval	Duration of trial, s
													All trials													
1	98	3.1	4.2																					4.2		4.2
2	74	2.6	3.1	1.4	3.9																			7	1.4	8.2
3	72	2.5	2.7	1.1	2.3	1.1	2.7																	7.7	2.2	9.5
4	69	1.1	2.8	0.9	2.3	0.8	1.9	0.9	2.8															9.8	2.6	12.2
5	36	1.5	2.5	0.6	2.5	0.8	2.3	0.9	2	1	3.1													12.4	3.3	15.5
6	28	2.1	3.1	0.6	1.9	0.8	1.2	0.7	2	1.1	1.7	1.3	3.8											13.7	4.5	16.8
7	14	2.5	3.2	0.7	1.9	0.7	2.1	0.7	1.9	0.8	2.2	1	1.5	1.1	2.8									15.6	5	20.6
8	8	1.1	2.2	0.6	1.5	0.9	1.7	1	1.4	0.7	1.8	0.7	1.7	0.8	2.1	1.2	2.9							15.3	5.9	20.4
9	5	0.5	4.5	0.5	2.1	0.6	2.2	0.6	1.8	0.6	2.1	0.6	1.9	0.7	1.7	0.9	1.4	0.6	3.7					17.7	5.1	26.6
10	1	0.3	4.8	1.1	1.5	0.7	0.6	0.5	1.2	0.9	0.8	0.8	1.5	0.5	1.5	0.8	7	0.6	0.7	0.7	1.8			21.4	6.6	27
11	2	0.5	2.6	0.5	3.3	0.6	0.6	0.6	3	0.6	1.6	0.6	1.6	0.5	1.1	0.7	1.2	0.6	1.4	0.6	1.2	0.5	1.3	18.9	5.8	26.3
Mean	407	2.2	3.2	1	2.6	0.9	2.3	0.8	2.3	1	2.3	1	2	1	2.3	1	2.5	0.6	3.5	0.6	1.4	0.5	1.3	25.7	8.4	15.9
											PC trials / PR trials															
1	24 / 74	0.9*** / 3.8	13.4*** / 1.2																					13.4 / 1.2		13.4*** / 1.2
2	27 / 47	1*** / 3.6	6.1*** / 1.3	0.7*** / 1.8	8.4*** / 1.3																			14.5 / 2.6	0.7 / 1.8	15.2*** / 4.1

Table 2. (Contd.)

Each cell is shown as *top value / bottom value* (PC-trial mean over PR-trial mean). The last three columns are summary (duration) columns.

#	n	L	R1	I1	R2	I2	R3	I3	R4	I4	R5	I5	R6	I6	R7	I7	R8	I8	R9	I9	R10	I10	R11			
3	25/47	0.7***/3.5	4.9***/1.5	0.6***/1.4	4***/1.5	0.6***/1.2	5***/1.5																		13.9/4.5	1.2/2.6	15***/6.6
4	28/41	1/1.2	4.3***/1.7	0.7*/1.1	3.7***/1.5	0.6/0.9	2.6*/1.4	0.7/1	4.4*/1.8															15/6.4	2/3	17.2***/8.8
5	18/18	0.7***/2.1	3.1*/2	0.6/0.6	3.2**/1.7	0.6/0.9	2.9*/1.7	0.8/1	2.2/1.8	0.7*/1.3	4.4*/1.8													15.7/9	2.7/3.8	18.7*/12.1
6	16/12	2/2.2	4.2**/1.7	0.6/0.6	2.3**/1.1	0.7/0.9	2.3*/1.6	0.7/0.6	2.3*/1.6	1.1/1.1	2*/1.3	0.7*/1.3	4.4*/1.8		3.1**/1.4									16.2/8.7	4.4/4.4	20.2*/12.6
7	7/7	1.4**/3.7	3.7/2.7	0.8/0.6	4.5***/1.5	0.7/0.6	2.3/2	0.7/0.7	2.3*/1.5	0.7/1	2.4/2	1.2/0.9	1.6/1.4	0.6*/1.7	3.9*/1.7									20.7/12.8	4.7/5.5	22.8/18.4
8	5/3	1.1/1.2	2.8/1.2	0.6/0.5	1.7/0.9	0.9/0.7	1.9/1.3	1.2/0.6	1.9/1	0.6/0.8	2/1.5	0.6/0.7	1.9/1.4	0.8/0.7	1.8/2.6	0.6/2.2	3.9/1.3							17.6/11.2	5.3/2.6	22.6/16.7
9	5/0	0.5	4.5	0.5	2.1	0.6	2.2	0.6	2.1	0.6	1.8	0.6	1.9	0.7	1.7	0.9	1.4	0.6	3.7					21.4	5.1	26.6
10	1/0	0.3	4.8	1.1	1.5	0.7	0.6	0.5	1.2	0.9	0.8	0.5	1.5	0.5	1.5	0.8	7	0.6	0.7	1.8	0.7			20.4	6.6	27
11	1/1	1/0.1	4.5/0.7	0.5/0.4	2.2/4.3	0.6/0.6	0.7/0.5	0.6/0.5	1.9/4.1	0.7/0.5	4.6/1.5	0.6/0.6	1/2.1	0.5/0.5	0.8/1.3	0.6/0.5	0.6/1.7	0.6/0.6	1/1.7	0.7/1.6	1.3/1.6	0.5/0.5	0.8/1.7	18.8/21.2	5.8/5.2	24.6/27.9
Mean	157/250	1***/3	5.9***/1.5	0.7***/1.3	4.2***/1.4	0.7**/1	3.1***/1.5	0.7/0.9	2.9***/1.7	0.8/1.2	2.8***/1.7	0.7/1.8	2.5/1.9	0.7**/1.3	2.3**/1.5	1/1	2.8/1.4	0.6/0.6	3.8/1.7	0.7/0.5	1.3/1.6	0.5/0.5	0.8/1.7	32.4/17.6	7.1/10.1	17.2/6.1

Designations: L—latent period (time from the introduction of the pellet into the aquarium until its first grasp by the fish); $R_1, \ldots, R_{11}$—the duration of pellet retention in the mouth of fish after the first (1) and eleventh (11) grasp, respectively; $I_1, \ldots, I_{10}$—the duration of the first (1) and tenth (10) intervals between the pellet retentions. The duration of the trial is specified without the latent period.

*, **, ***—Differences between PC trials (trials ended with pellet consumption) and PR trials (trials ended with pellet rejection) were significant at $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.

by its reorientation or any other actions, represent repeated evaluation of the sensory qualities of the object with the intraoral gustatory and tactile receptors. The chemical (gustatory) and mechanical (texture and hardness) characteristics are crucial for fish to make decisions about the food adequacy and consumption of an object (Kasumyan and Døving, 2003; Kasumyan, 2012).

It remains unclear why fish need multiple testing of the properties of the object. Most likely, repeated testing ensures better perception of taste or reduces the receptor adaptation rate (the gustatory system is a rapidly adapting sensory system) (Kassil', 1972). The factors that determine the duration of intervals between grasps are also obscure. Perhaps this time is required to restore the sensitivity of taste receptors.

The behavior of fish during orosensory evaluation of food is characterized by certain consistent patterns. The analysis of pooled data (PC- and PR-trials together) showed that, in the nine-spined stickleback and other fish species studied, the first retention (R_1) is the longest. Subsequent retentions with each grasp become shorter throughout the trial (carp, tench, and bream) or a substantial part of it (nine-spined stickleback). In the carp and tench, the retention time decreases monotonically, and in the bream it decreases sharply (in 3−4 times) after R_1 and then changes slightly. A characteristic feature of the nine-spined stickleback is that the retention time decreases during the first six grasps and then ($R_7−R_9$) reaches the baseline level. The intervals between retentions usually do not have characteristic dynamics and do not depend on the previous retention: the duration of intervals in the course of testing remains approximately the same in the nine-spined stickleback and bream, varies widely in the tench, and gradually decreases in the carp. The ratio of the retention time and the duration of the intervals between grasps in fish varies much more significantly: in the nine-spined stickleback, the retention time is many times longer than the duration of intervals; the difference between these parameters is slight in the carp and is not expressed in the bream; and in tench the retention time is shorter than the duration of intervals (Kasumyan and Prokopova, 2001; Isaeva, 2007; Kasumyan and Sidorov, 2010). Special studies are required to investigate the correlation of these characteristics with the feeding type, feeding behavior strategy, and lifestyle of fish.

Stereotypes. In recent years, it was established that the quantitative characteristics of fish behavior during orosensory testing differ drastically when the ingestion and rejection of food is compared. These differences manifest themselves regardless of the gustatory or mechanical qualities of the object grasped, which indicates the existence of two alternative stereotypes of feeding behavior in fish (Kasumyan and Sidorov, 2010a, 2010b, 2012; Kasumyan and Mikhailova, 2014). These behavioral stereotypes were also found in

the nine-spined stickleback: the ingestion of an object is preceded by a large number of intermediate retentions, and testing itself lasts longer than in the case of refusal from consumption (Mikhailova and Kasumyan, 2015).

Food testing stereotypes also differ in other characteristics. For example, in case of refusal to consume a pellet (PR-trials), pellet retentions are short and differ only slightly in duration (1.4−1.9 s), whereas pellet consumption (PC-trials) is preceded by a longer retention (2.3−5.9 s), changing the course of the trial (first decreasing and then rapidly increasing again). In these trials, the first and, most interestingly, the last pellet retention are always the most long-lasting, whereas in the trials with pellet rejection this pattern is not observed (figure). Assuming that the pellet retention time reflects, to some extent, the strictness, correctness, and reliability of the sensory evaluation of food, the first and last retentions in the PC-trials should be considered the most important for such evaluation.

The information obtained by fish at the first contact with the grasped object is, apparently, crucial for making a decision on its correspondence to nutritional needs. Despite the fact that this decision is preliminary, since it requires confirmation in intermediate testing in the majority of trials (repeated grasps were observed in 85% of PC-trials and in 70% of PR-trials), it is important for the further behavior of the fish. If, during the first retention, the fish makes a preliminary decision to consume the object (PC-trials), the food object will long be retained in the mouth of the fish at the first and all subsequent grasps. The shorter the first retention, the higher the probability of repeated tests; however, this correlation is nonsignificant ($r_S = −0.41$, $p > 0.05$) due to the small size of the compared data sets ($n = 7$). Possibly, the short time of the first retention determines the necessity to perform more grasps by the fish to increase the total retention time. The importance of intermediate retentions is emphasized by the fact that, the larger the number of intermediate retentions, the smaller the duration of the last retention, after which food is ingested ($r_S = −0.92$, $p < 0.001$). In the PR-trials, no significant consistent changes in the duration of pellet retentions have been detected; they were short and fluctuated in a narrow range.

The fact that the last retention in the PC-trials, after which the pellet was ingested, lasted much longer than any other retention, except for the first one, has not yet been observed in fish. During the last retention, the final control is performed, after which the food is ingested. These processes require not only involving the peripheral and central sensory mechanisms but also performing motor acts (Finger, 2008) and, hence, time expenditures. This new and important feature of orosensory food testing distinguishes the nine-spined stickleback from the previously studied carp, tench, and bream. Most likely, this difference is related to the

feeding type. Carp, tench, and bream are benthophages feeding primarily on infauna representatives, which are grasped together with the bottom substrate (Zhiteneva, 1980; Giles et al., 1990). A characteristic feature of such fish is that they separate food organisms from soil by forward and reverse oral cavity ventilation (rinsing and backwashing) (Sibbing, 1991; Osse et al., 1997). The diet of the nine-spined stickleback includes benthic organisms that belong to the epifauna (Wootton, 1976; Hart, 2003) and are grasped one at a time (Ibrahim and Huntingford, 1989; Gill and Hart, 1994; Hart, 2003). This feeding characteristic is reflected in the distribution of taste buds. In Cyprinidae, they form dense aggregations in the palatal and postlingual organs, where wholesome objects are retained under forced ventilation of the oral cavity with water flows (Osse et al., 1997). In sticklebacks, such regions in the oral cavity have not been found (Campos, 1969; Jakubowski and Whitear, 1990).

The latent period. The latent period in the response of the nine-spined stickleback varies widely, on average from 0.5—0.7 to 3—4 s. The cause of such fluctuations cannot be a different distance from the fish to the point of falling the pellet into water or the time required for reorientation. The latent period in the PC-trials was always 3—4 times shorter than in the PR-trials. This fact allows predicting on the basis of the latent period, as well as the first retention, whether the response will be ended with the ingestion or rejection of the pellet and which behavioral stereotype will be manifested. However, the latent period does not allow predicting the number of grasps.

The differences between the PC- and PR-trials in the speed of response of fish to a pellet that has fallen into the water cannot be random ($p < 0.001$). The same results were obtained for the bream (Isaeva, 2007). Apparently, the relationship between the speed of response to the pellet and the probability of it consumption reflects the state of experimental individuals and their food motivation (predisposition) to feeding: the faster the fish grasps the pellet, the more often the pellet is ingested; the slower the fish responds to the pellet, the more often it eventually refuses to consume it. The experimental fish were kept under the same conditions, the frequency of feeding and the diet were standard for a long time. Therefore, the degree of satiation, water temperature, or illumination can be excluded from the factors that might determine the heterogeneity of the responses of fish in our experiments. The testing procedure (size, shape, and color of pellets as well as the composition and concentration of the gel from which they were made) was standard. The individual characteristics of fish cannot be ruled out completely; however, the total characteristics of responses of fish nos. 12 and 16 to the pellets with aspartic acid and the control pellets (consumption and first retention time) did not differ from the group-average values (series 1 and 2; $p > 0.05$).

Possibly, the variation in the latent period in the PC- and PR-trials may be due to the minor fluctuations of food motivation, which in fish occur even under stable experimental conditions. They can be caused by various factors that are difficult to control, such as spontaneous (routine) activation of research and food searching behavior in fish, short-term alertness in response to the sudden approach of the experimenter and his other activities, as well as differences in atmospheric pressure and geomagnetic activity. The effect of fluctuations in the state of fish must be particularly noticeable in the responses to weak stimuli. The stronger the stimulus, the more stable the answer to it, and the more significant deviations in the motivational state or background factors will be able to change it (Kasumyan and Sidorov, 2010b).

In our experiments, we used pellets with 0.01 M aspartic acid, the consumption of which by the nine-spined stickleback was approximately 40%. This allows aspartic acid to be classified with gustatory substances of low palatability (Mikhailova and Kasumyan, 2015). It can be assumed that, if the supply of such pellets coincided with a short-term enhancement of food motivation of fish, they quickly grasped it and, as a consequence, consumed pellets more often. If the pellets were supplied during reduced motivation, fish grasped them with a greater delay and rejected more often.

Pellets with a more palatable gustatory substance (e.g., glutamine, alanine, or cysteine) were consumed by the nine-spined stickleback almost 2 times better (70%) than the pellets with aspartic acid (Mikhailova and Kasumyan, 2015). Undoubtedly, the latent period of the response of the nine-spined sticklebacks to these, as well as to any other, will be the same as in our experiments with aspartic acid. Therefore, the stronger the gustatory properties of a substance, the weaker the influence of background fluctuations in the state of fish on their behavior and the more stable the responses of fish. To a greater extent they are determined by the gustatory properties of the substance, and they depend to a lesser extent on external influences. Data on the effect of starvation on the gustatory preferences of fish confirm this conclusion (Kasumyan and Sidorov, 2010b). The proposed model is applicable not only to the gustatory but also, most likely, to other stimuli as well.

Duration of orosensory testing. Orosensory evaluation in food consumption apparently requires a certain time. This is indicated by a disproportionate change in the duration of the PC-trials (1.13, 1.13, 1.35, and 1.31 times) with a twofold increase in the number of grasps (from 1 to 2, from 2 to 4, from 3 to 6, and from 4 to 8, respectively). The mean duration of the experiment and the mean total pellet retention time in the PC-trials with 1 and 7 grasps were 13.4 and 22.8, 13.4 and 20.7 s, respectively (i.e., they differed only by a factor of 1.7 and 1.54, respectively). The relative constancy of the test time was achieved due to the balance

between the duration of the first and last (associated with the first) retention and the duration of intermediate retentions: the short first retention was compensated by the intermediate ones. This behavior is not typical for the PR-trials: the duration of these trials and the total pellet retention time were much smaller and increased in proportion to the increase in the number of grasps.

A longer total time of orosensory testing of food in the case of its consumption is typical for all fish species studied (Kasumyan and Sidorov, 2010a, 2012; Kasumyan and Mikhailova, 2014; Mikhailova and Kasumyan, 2015). This behavior provides a reliable and accurate identification of food properties by fish, which, in turn, reduces the probability of consuming inadequate food. A rapid completion of testing food with inappropriate sensory properties or at a low food motivation minimizes unproductive time costs spent on feeding.

Variability of orosensory food testing. Orosensory food testing, as well as other behavioral responses, is characterized by a high variability due to influences of various external and internal factors (Colgan, 1993) and individual differences in experimental animals (Magurran, 1993; Lee and Bereijikian, 2008). One of the main approaches that partially reduces the effect of variability and makes it possible to obtain the objective characteristics of behavior is to use a large number of experimental animals (Still, 1982). According to our data, replicating trials also make it possible to obtain such characteristics. The mean values of all parameters for fish nos. 12 and 16 in series 2 (407 trials) coincided with or were close to the group-average values in series 1, performed with 22 specimens (132 trials) but differing from the mean data for fish nos. 12 and 16 in series 1 (12 trials).

CONCLUSIONS

This is the first study to obtain objective characteristics of fish behavior manifested during orosensory food testing. This was largely achieved through a methodical approach that differed from the ones used previously. Firstly, in this study we intentionally used a substance that stimulated repeat grasps more often than other substances. Secondly, for experiments we selected the individuals that performed repeated grasps more often than other fish. Other parameters of their behavior were close to the group-average ones. Thirdly, a large number of trials were carried out, which allowed us to perform a statistical analysis of response variants with a large number of grasps.

Using this approach, we showed that the final stage of feeding behavior of fish, during which the sensory evaluation of the grasped object takes place, is characterized by certain consistent patterns. The first contact of the food object with the intraoral receptors has maximum duration, and the information obtained during this contact determines the subsequent behavior of fish. It was also shown for the first time that the final testing is not less important for fish in cases when it directly precedes the ingestion of food. The existence of two behavioral stereotypes of orosensory food testing in fish was confirmed, and their additional characteristics were determined. The stereotype leading to food intake not only requires more time and is accompanied by a larger number of tests but also is more complex in manifestation than the stereotype leading to the refusal of consumption. The analysis of the latent period of the response of fish to the food object allowed us to correlate for the first time the responsiveness of fish and their readiness for food consumption, as well as to make an assumption on the effect of low-amplitude fluctuations in the internal state of an individual and its motivation to feed on the behavior of fish.

The new data on the behavior of fish during orosensory testing of food objects change our notion of the final stage of feeding behavior as a simply organized and fast event. This stage has its own structure, characteristics, and dynamics. However, much of this behavior remains unclear (in particular, whether the identified characteristics and patterns are versatile, whether they concern the studied species or can be extrapolated to other species, the extent to which these features are determined by the lifestyle and feeding type of fish, and the development of sensory systems in them) and requires further studies. Their results can be interesting and important for understanding the biology of nutrition and feeding behavior of fish, for solving the applied problems of aquaculture (feeding) and fisheries (longline and recreational fishing).

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