

## Odor Stimulation and Relation to Taste Stimuli in the Blind Cave Fish *Astyanax fasciatus*

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**Abstract**—The influence of odor stimulation with solutions of the amino acids L-glutamine and L-phenylalanine on the response of the cave fish *Astyanax fasciatus* (blind form) to the taste of agar pellets with L-glutamine and L-phenylalanine has been studied. It has been established that the odors used cause a food search behavior in fish, but do not affect the orosensory testing of the pellets. It is noted that the taste characteristics of both amino acids differ in the evaluation of the extraoral and oral taste systems, which indicates food selectivity in the blind cave fish, the food of which in its habitats is extremely monotonous. The attitude to the taste of amino acids is stable with a different smell background and a changing combination of odor and taste stimuli.

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### INTRODUCTION

All sensory organs of fish participate in the regulation of feeding behavior. At the phase of obtaining a signal of food presence and determination of the search direction, the main role is played by sensory systems with the maximum remoteness capacity, first of all, the sense of smell. Then, during the local search and evaluation of the external properties of the detected prey, sensory organs with limited remoteness capacity, such as vision, lateral line, and electroreception, play a leading role. The final control of the food quality occurs after its grasping by the fish. It is based on the function of contact sensory systems—the sense of taste and some other systems, the receptors of which are represented in the oral cavity (Atema, 1980; Pavlov and Kasumyan, 1998).

It is known that fish responses to various stimuli, including food signals, depend on many factors. Since the taste reception entailed a chain of sensory systems that are consistently involved in the regulation of feeding behavior, the solution formed by the taste buds (swallow or reject the object) may depend on the information obtained earlier through other sensory channels. Olfactory signals capable of having a primer effect on fish, i.e., changing the motivation and physiological state of the recipient, are of particular interest. Sex pheromones, for example, change the hormonal status of fish and accelerate the maturation of the gonads (Olsén et al., 2006; Stacey et al., 2012), the physiological stress (Rehnberg and Schreck, 1987; Lebedeva et al., 2000), and food smells dramatically

increase nutritional motivation (Tandler et al., 1982; Kasumyan and Marusov, 2016).

The available literature information indicates that the taste preferences in fish are stable and vary slightly against the background of the action of natural olfactory signals (smell of food, alarm pheromone). However, fish behavior while testing the orosensory quality of food is influenced much more by these stimuli. Changes induced by the smell of food are most noticeable in the testing behavior of those objects the taste properties of which have a different or opposite signal value in relation to the acted odor (Kasumyan et al., 2009). It is believed that olfactory images of the food objects are formed in fish (Atema et al., 1980), and food odors are associated with the taste, shape, and color of the specific food (Kasumyan and Marusov, 2016). Therefore, the discrepancy between expectation evoked by food odour and information about the object quality obtained in subsequent stages of sensory assessment may be, as expected, the main cause of changes in the behavior of food testing and the intensity of food consumption (Kasumyan and Marusov, 2016).

The aim of this study is to obtain new information on the effects of odor stimulation preceding the grasping of the food object to the fish's attitude to its taste quality and to the behavior during the orosensory testing of food. To exclude the possible impact of information about the object entering the visual canal on the processes under consideration, this study was performed using the blind cave form of *Astyanax fasciatus*.

The use of free amino acids which are adequate stimuli for chemosensory systems in fish made it possible to create combinations with different degrees of conformity of olfactory and taste properties of food objects.

## MATERIALS AND METHODS

Experiments were performed on six mature adult blind cave fish (total length (TL) 58–68 mm and weight 3.1–4.6 g). Fish were purchased at a pet store (Moscow); their population origin is unknown. After delivery to the laboratory, the fish were kept in an aquarium (80 L) equipped with an internal filter for several months, then were put individually in experimental aquariums (30.5 × 20.5 × 14.5 cm, water level 12 cm) with closed circulation of water through a coal-gravel biofilter. The water from the biofilter was continuously fed into the aquarium at a rate of 0.075 L/min through a tube (inner diameter 4 mm), the outlet of which was directed downwards and was located at a height of 5 cm from the bottom in the front part of the aquarium. Water passing along the long axis of the aquarium was again directed by the airlift to the biofilter. The complete water exchange time was ~1.5 hours. During the experiment, clean water taken from the biofilter outlet (control) or odor solution was injected at a rate of 0.025 L/min for three minutes into the flow channel from the biofilter to the aquarium. The flow rate was constant (0.075 L/min) before and during the experiment. There was no bottom-ground in the experimental aquariums. The water temperature was kept constant (24°C) with electric heaters. The aquariums were cleaned, the tubes were washed, and the charcoal in the biofilter was totally or partly replaced once every five to seven days. On the day of the experiment, some of the water in the aquariums was replaced with fresh water. Fish were fed live mosquito larvae (family Chironomidae) once a day (evening). The food was added in amount that could be completely consumed by the fish in 10–15 minutes. Experiments began after acclimation of fish to the conditions of their maintenance in experimental aquariums (four weeks).

The odor solutions were a water extract of living chironomid larvae ( $10^{-3}$  g/L) and solutions of L-glutamine and L-phenylalanine amino acids ( $10^{-5}$  M). To prepare the extract, the larvae were homogenized in a porcelain mortar. The homogenate was diluted (1 g of biomaterial per one liter of water), extracted for 15 minutes, and then filtered through filter paper and diluted to the desired concentration. The extract and amino acid solutions were used not later than three hours after preparation. All odor solutions were prepared using water passed through the biofilter.

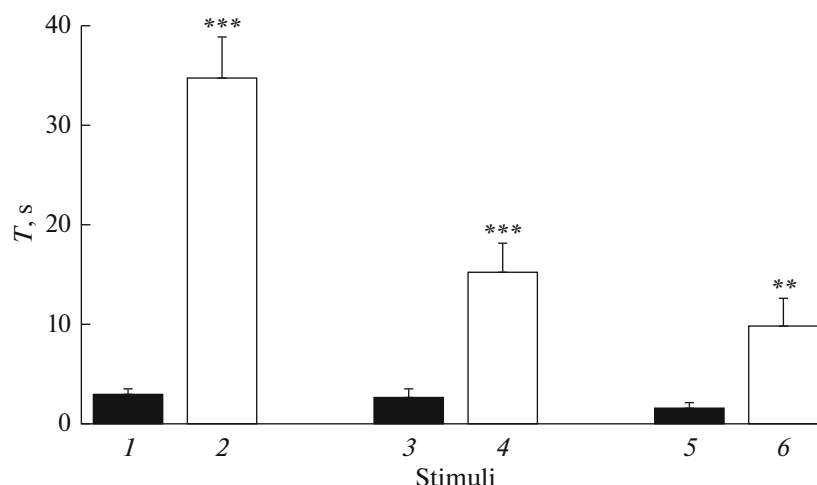
In the first set of experiments, a control stimulus (clean water) was first applied to each aquarium, and after 20 minutes the next experiments was carried out, in which one of the odor solutions was introduced into

the aquariums. After three hours, the test sets were repeated, but after another control, another odor solution was used. Within one day, not more than two sessions with the control and two sessions with odor solutions were conducted. The changes in fish behavior in response to stimulation were visually assessed; the total time of the characteristic fish food search reaction—swimming in an inclined position near the chemical stimulus source (1/3 of the aquarium) with the bottom touching by the head—was recorded using a hand-held stopwatch. Twelve control sessions and four sessions with each of the odor solutions were performed; the total number of experiments ( $n$ ) is 144.

In the second set of experiments, agar-agar pellets with chironomid larvae extract (175 g wet weight/L) were added to the aquarium near the tube outlet, along which clean water from the biofilter constantly entered. Pellets were added one by one, each subsequent pellet was added immediately after consumption of the previous one, or (if a fish refused to consume the grasped pellet within one minute) immediately after its removal from the aquarium. The pellet was also removed if within one minute the fish did not find it and did not grasp it. The duration of the experiment was three minutes: the time during which the pellets were added. The latent reaction time (the interval from the moment of pellet dropping into water to grasping it) was recorded with the help of a stopwatch, the number of repeated grasps of the pellet, the ingestion or refusing by the fish were recorded too. Four sessions were held ( $n = 24$ ).

In the third set of experiments, pellets with L-glutamine or L-phenylalanine (0.1 M) with different palatability for the blind cave fish (Mikhailova et al., 2014) were added into the aquarium. Pellets were introduced 10 s after the start of the entry of clean water or one of the odor solutions of amino acids, L-glutamine or L-phenylalanine ( $10^{-5}$  M). The duration of the injection of solutions into the aquarium was three minutes. Control experiments (in clean water) were conducted before the experiments with amino acid odor solutions. In each subsequent session, which was conducted no earlier than three hours later, the odor background was created by a solution of an alternative amino acid. After the first three sessions, three more sessions were performed, in which the sequence of introduction of pellets with amino acids into the aquarium was reversed. Clean water and odor solutions were added in the same way as in the first set of experiments. The introduction and removal of pellets and registration of the fish response to pellets were carried out in the same way as in the second set of experiments. Six sessions of experiments were performed against the background of clean water and odor solutions ( $n = 108$ ).

The pellets were made from agar-agar gel (2%). An amino acid or extract of chironomid larvae was added into the hot agar-agar solution together with the Pon-



**Fig. 1.** Duration of food search ( $T$ , s, for 3 min of stimulation; mean value and its error) of blind cave fish *Astyanax fasciatus* in clean water (1, 3, 5); in an extract of chironomid larvae ( $10^{-3}$  g/L) (2); in L-phenylalanine solution ( $10^{-5}$  M) (4); in L-glutamine solution ( $10^{-5}$  M) (6). (\*\*) and (\*\*\*), the differences are significant for  $p < 0.01$  and  $p < 0.001$  for the Wilcoxon matched pairs test.

ceau 4R dye ( $5 \mu\text{M}$ ) (Aldrich, Germany), giving the solution a red color. After mixing, the solution was poured into a Petri dish, where a gel disc was formed after cooling. Cylindrical pellets (length 4 mm, diameter 1.35 mm) were cut using a stainless steel tube just before the experiments.

Statistical processing of the data was performed using a nonparametric  $\chi^2$  test, the Mann–Whitney  $U$  test, and the Wilcoxon matched pairs test ( $T$ ).

## RESULTS

### *Reaction to Odor Solutions*

Single blind cave fish move constantly. They move around the aquarium, more often at the bottom and along the lower half of the walls. At the moments of sporadic food search, the fish swim forward and occasionally touch the bottom with their heads; they can make short series of several frequent bites. The introduction into the aquarium of live chironomid larvae causes an increase in search swimming, if the fish, as a result of nondirected movements, accidentally turn out to be 3–5 cm from the food lying on the bottom. Near the bottom they take a characteristic slope at an angle of  $30^\circ$ – $40^\circ$ ; they begin to move quickly, make sharp turns, and move in circles and loops, with a radius not exceeding the body length of the fish. Grasping occurs only after touching the food with the lips; it activates the search at the location of the food. If the fish swim near the chironomid larvae or touch the larvae with the head, body, or fins, but not with the lips, grasping does not occur. Upon missing the food, the fish continue their local swimming, successively narrowing the search zone.

If the fish occur in the area of the smell after the addition of an extract or solution of an amino acid into

the aquarium, they stay in this zone. The smell causes the typical food search behavior: increased motor activity, swimming in an inclined position with touching the bottom, sharp turns and zigzags, and moving in circles and loops. Sometimes fish can make rapid spontaneous grasping movements with the mouth, not provoked by touching. Food search may be interrupted if fish leave the odor spreading area. Repeated visits to this zone stimulate a food reaction during the entire time the odor enters the aquarium (three minutes). The total duration of the food search behavior for the extract and amino acid solutions is always significantly greater than in the control experiments, and these values for the responses to the extract are two and three times higher than for L-phenylalanine and L-glutamine, respectively (Fig. 1). The durations of food search for solutions of these amino acids do not differ ( $p > 0.05$ ).

### *Reaction to Pellets with Chironomid Larvae Extract*

After falling into the water pellets drop down to the bottom of the aquarium in 10 seconds. Accidental passage of the blind cave fish near the pellet with the chironomid larvae extract lying on the bottom causes the fish to have a typical food search behavior: an indirect approach to it and an instant grasping after touching it with the lips. This can happen even 7–12 seconds after the pellet has fallen to the bottom. Most of the introduced pellets are quickly found by fish, grasped, and immediately swallowed; fish do not try to reject and regasp the pellets (Table 1). Rapid detection and consumption of the offered pellets allows us to consistently present up to six pellets to the fish in a three-minute period. Water streams caused by vigorous swimming of fish can move a pellet having almost neutral buoyancy, far to the side. In such situations the

**Table 1.** Reaction of the blind cave fish *Astyanax fasciatus* to agar-agar pellets with chironomid larvae extract (175 g /L), L-glutamine (0.1 M), and L-phenylalanine (0.1 M) in clean water, in L-glutamine solution ( $10^{-5}$  M), and in L-phenylalanine solution ( $10^{-5}$  M)

Pellet	Introduced pellets, items	Latent response time ( $M \pm m$ ), s	Grasped pellets, % of introduced	Consumed pellets, % of grasped	Average number of grasps for one pellet in the experiment ( $M \pm m$ )
Clean water					
With chironomid extract	98	$29.6 \pm 1.6$	85.7	100	$1 \pm 0$
With glutamine	70	$29.4 \pm 2.1$	78.3	100	$1 \pm 0$
With phenylalanine	63	$27.5 \pm 2.1$	53.7 /-/*	27.8 /***/***	$1.19 \pm 0.1$ /-/*
L-glutamine solution					
With glutamine	68	$23 \pm 2.6$ */-	69.1	100	$1 \pm 0$
With phenylalanine	68	$24.5 \pm 2.1$	58.8	20 -/***	$1.08 \pm 0.04$ -/*
L-phenylalanine solution					
With glutamine	70	$23 \pm 1.9$ **/*	75.7	96.2	$1 \pm 0$
With phenylalanine	70	$29.6 \pm 2.5$ -/*	54.3	23.7 -/***	$1.13 \pm 0.07$ -/*

( $M \pm m$ ) is the average value of the indicator and its error; (\*), (\*\*), and (\*\*\*) are the differences, significant for  $p < 0.05$ ,  $p < 0.01$ , and  $p < 0.001$ , respectively; (-), there are no differences; before the first oblique line, for pellets of the same type with respect to the reaction in clean water; after the first oblique line, for pellets with amino acids in the same background solution; after the second oblique line, in clean water with respect to the pellets with chironomid extract. A nonparametric criterion  $\chi^2$  was used to estimate the reliability of the differences in pellet consumption and the proportion of grasped pellets relative to the introduced pellets; the Mann–Whitney  $U$  test was used in order to assess the reliability of differences in the latent response time and the number of grasps of one pellet in the experiment.

fish continue their local search at the site of the initial location of the pellet and make test bites of the bottom. But usually it is not possible for them to find and grasp the pellet within one minute after its introduction.

#### Reaction to Pellets with Amino Acids

Pellets with L-glutamine and L-phenylalanine stimulate the food search in blind cave fish in clean water and against the background of solutions of the same amino acids. But the number of L-glutamine-containing pellets found and grasped by fish was always higher in comparison to L-phenylalanine by 1.46, 1.18, and 1.39 times in clean water and against the background of L-glutamine and L-phenylalanine, respectively. The pellet consumption differs more noticeably. Pellets with chironomid extract or L-glutamine were eaten in 100% of cases or slightly less, whereas the consumption levels for the pellets with L-phenylalanine were several times less: from 20 to 27.8%. Pellets with L-phenylalanine, unlike other types of pellets, can be rejected and regasped by fish during the experiment. Statistically significant differences between the reaction of fish to pellets of the same type against the background of L-glutamine and L-phenylalanine were not detected in any of the response parameters recorded (Table 1).

In clean water, fish spend practically the same time for search and grasping of pellets of any type. The latent reaction time for pellets with L-glutamine in clean water is always significantly higher in compari-

son with the presence of the smell of any of the amino acids. Against the background of amino acids, pellets with L-glutamine were found for the same time, 23 s. Fish spent less time for searching pellets with L-phenylalanine only against the background of the alternative amino acid, L-glutamine, whereas against the background of L-phenylalanine, the search for these pellets requires even slightly more time than in clean water, although these differences were not significant ( $p > 0.05$ ) (Table 1).

## DISCUSSION

#### Reaction to the Odors

Food odor is a strong stimulus that causes in the blind cave fish an increase in locomotor activity, attraction to the source of odor, and a characteristic search for a food object (local movements along a complex trajectory, swimming along the bottom in an inclined position, and frequent bites). This response is typical for the blind (cave) form of *A. fasciatus* (Schemmel, 1980; Wilkens, 1988; Bibliowicz et al., 2013; Kasumyan and Marusov, 2015); the reaction is completely controlled by the olfactory system (Kasumyan and Marusov, 2015). The sensitivity to food odors in blind *A. fasciatus* is comparable to the sensitivity of many benthophagous fishes (Kasumyan and Marusov, 2015); the threshold concentrations of amino acids that cause an attractive effect for the blind form are several orders of magnitude lower than for the eyed form (Hinaux et al., 2016).

Both amino acids (L-glutamine and L-phenylalanine) have an attractive odor for the blind cave fish. Their solutions keep fish in the odor zone and cause a characteristic food search for these fish. The amino acids used are greatly inferior to the extract of chironomids by the odor efficiency; but L-glutamine and L-phenylalanine do not differ statistically in the duration of the induced search behavior ( $p = 0.14$ ). It is known that the amino acids alanine and serine are also attractive for early larvae of blind *A. fasciatus* (Hinaux et al., 2016) (the isomerism of amino acids was not mentioned in this publication); there are no data on the olfactory efficacy of other amino acids in the literature.

#### *Search and Detection of Pellets*

High olfactory sensitivity allows the blind *A. fasciatus* to detect the pellets lying on the bottom of the aquarium, despite their small size (volume 5.72 mm<sup>3</sup>), short exposure (minimum 7–12 s), and, accordingly, insignificant concentration of the odor in the water and the limited size of the odor area. However, a focused search begins only if a fish, swimming along the aquarium, turned out to be in close proximity to the pellet, i.e., in the odor area of the “point” source, which a pellet is.

Fish occurred near the pellets accidentally during continual and nondirectional movements along the aquarium. This is indicated by the coincidence of the latent reaction time in clean water for all types of pellets, despite the fact that the odors contained in the extract or amino acids differ significantly by the strength of the effect they cause, i.e., the duration of food search behavior (Fig. 1). Thus, the smell area created around the pellet is relatively small in size and cannot promote in fish a remote search for its source.

In most cases (three out of four), fish detection of pellets occurs faster against the background of the amino acid solution entering the aquarium than in clean water; in half of the cases this difference is statistically significant (for pellets with L-glutamine) (Table 1). The faster detection of pellets could be explained by the fact that the odor addition to the aquarium activates swimming and stimulates the food search, which increases the probability of accidental contact of the fish with the pellets or an approach to a distance sufficient for directional search.

#### *Pellet Grasping*

The grasping of pellets by the blind cave fish occurs only after accidentally touching it with the lips, in the epidermis of which there are numerous taste buds. External taste buds are also present in these fish on the lower surface of the head and gular region, but the density of taste buds is lower here. The density and wideness of the external taste bud distribution in blind *A. fasciatus* is much higher than in the sighted form of

this species (Schemmel, 1967; Bensonilah and Denizot, 1991); the innervation of these sensory structures is more developed (Boudriot and Reutter, 2001). Together with the increased development of other sense organs (lateral line, hearing, and tactile reception), this allows the blind cave fish to compensate for its total loss of vision and successfully exist in cave water bodies (Popper, 1970; Windsor et al., 2008; Patton et al., 2010; Yoshizawa et al., 2010; Tan et al., 2011).

#### *External and Oral Taste*

Our research allowed us to determine for the first time the functional characteristics of the external taste buds for the blind form of *A. fasciatus*. The proportion of the grasped pellets with L-phenylalanine under all conditions of the experiment was always the lowest; difference between grasping the pellets with this amino acid and with the chironomid extract in clean water was statistically significant. This indicates that the extraoral taste of L-phenylalanine is low. Perhaps this amino acid has the properties of a suppressant, i.e., a substance that reduces the likelihood of grasping food objects (Kasumyan and Døving, 2003).

This conclusion is fully confirmed by data on the consumption of the pellets by the blind cave fish, a characteristic that reflects the function of another taste subsystem—oral one. Extraoral and oral taste subsystems differ in the wideness of spectra, sensitivity, and some other characteristics, but are interrelated functionally. The oral subsystem, which carries out the final control of the quality of the grasped food, has a wider range of deterrent (aversive) stimuli (Kasumyan and Døving, 2003). This principle, which is manifested in many fish, is also true for the blind cave fish: fish grasp pellets with L-phenylalanine just as well as other types of pellets, but swallow them always extremely reluctantly: 4–5 times less in comparison to the pellets with chironomid extract or with L-glutamine.

The lack of experiments with control pellets (placebo) containing just agar-agar and dye does not accurately determine palatability of the amino acids used for blind cave fish (attractive, aversive, or indifferent). The absence of significant differences between the consumption of pellets with chironomid extract and L-glutamine is very likely indicative of the high orosensory attraction of this amino acid for the blind cave fish, whereas L-phenylalanine most likely has an aversive or indifferent taste. This coincides with preliminary estimates of the taste qualities of these amino acids for the blind form of *A. fasciatus* (Mikhailova et al., 2014).

#### *Influence of the Odor Background*

The attitude towards the taste of pellets with amino acids in the blind cave fish does not change after stim-

ulation with solutions of the same amino acids. Olfactory information received in the pre-taste testing phase does not affect the results of this assessment, regardless of the differences between these sensory flows. The preferences of amino acids, manifested by the function of the external and oral taste subsystems in the blind cave fish, coincide with different combinations of odor and taste stimuli. Stimulation by food odors does not cause changes in taste preferences in other fish species, but it modifies the food behavior manifested during the orosensory tasting of food (Kasumyan et al., 2009).

The influence of odors on the food behavior of the blind cave fish has not been revealed in our work. Perhaps this is due to the high stereotype of this behavior in this fish species: the almost complete lack of inclination to reject and regasp food objects during testing. This behavior is typical for fish with poorly developed vision (Kasumyan, 1997). In blind cave fish, repeated grasps occur, but extremely rarely, only in experiments with pellets containing L-phenylalanine, the palatability of which is low. This suggests that for the blind cave fish the orosensory testing of food objects with opposite taste properties can be different in stereotypes, as is the case with other fish (Mikhailova and Kasumyan, 2016).

The blind form of *A. fasciatus* can be found in small cave water bodies of Mexico, in which the density of these fish can be high (Espinasa et al., 2017). The usual and quite stable food for this species is the guano of bats that spend the bright time of day in caves. Other potential food can enter cave water bodies only occasionally with flood waters (Wilkens, 1972; Mitchell et al., 1977; Espinasa et al., 2017). The sensory equipment for the feeding behavior of blind *A. fasciatus* attracted researchers' attention almost immediately after the discovery and description of these fish in 1936 (Breder and Rasquin, 1943, 1947; Schemmel, 1967, 1980; Wilkens, 1988). In recent years, *A. fasciatus*, both its blind and sighted forms, has been widely used as a model for studying various aspects of evolution, genetics, and developmental biology (Schemmel, 1980; Wilkens, 1988; Jeffery, 2001; Franz-Odenaal and Hall, 2006; Protas et al., 2008; Varatharasan et al., 2009; Yoshizawa et al., 2010; Hinaux et al., 2016). All this predetermines great interest in new information about the lifestyle of these fish and the peculiarities of their reproduction and embryonic development, physiology, and behavior.

It has been established that blind *A. fasciatus* has a well-developed sense of smell, allowing it to find sources of food odor quickly, even such weak ones as small artificial pellets. Blind *A. fasciatus* uses an external (extraoral) taste reception for preliminary assessment of the taste qualities of objects found during continuous nondirectional moving or targeted search. Information obtained with the help of the external

taste buds determines the choice of objects for grasping, i.e., potentially suitable for nutrition.

The data show that, despite the extremely limited nutritional resources and dominance of bat guano in the diet of the blind cave fish, these fish already at the stage of preliminary assessment of the taste qualities of objects are capable of food selection, preferring one (pellets with L-glutamine) and less likely grasping others (pellets with L-phenylalanine). Even more strict selection of the food takes place in the oral cavity. After it, most of the captured objects with inappropriate taste qualities are rejected by fish (>70% for pellets with L-phenylalanine). A characteristic feature of the blind cave fish is the decision to swallow or refuse to consume the food object after a single orosensory testing. This study did not reveal the influence of the odor effect on the selection of food by the fish at the stages of its preliminary and final taste evaluations with a background corresponding to varying degrees to the taste of the food. Obtaining new information about the chemosensory regulation of feeding behavior in blind cave fish, including comparison of its blind and sighted forms, seems to be a promising direction for further studies of the biology of this species.

## COMPLIANCE WITH ETHICAL STANDARDS

*Conflict of interests.* The authors declare that they have no conflict of interest.

*Statement on the welfare of animals.* All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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