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Elucidating Behavioral Approach of Freshwater Teleost *L. Rohita* towards an Aberrant Odor Class of Amines

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Abstract

Problem: Multifarious odor detection through olfaction is one of the vital processes that regulates observable behaviors together with physiological functions in fish. The aquatic environment accommodates a vast range of olfactory cues, among which free amino acids are very common. Moreover, polyamines, which are products of amino acid decarboxylation, constitutes another important class of odors. Discernment of certain responses against these important odor molecules may enable the advancement of aquatic feed preparation facilitating fish growth. **Approach:** The behavioral response of *L. rohita* against L-amino acids of different nature was analyzed. These responses were compared with the responses against two biologically pertinent polyamines. The dose dependent responses were also discussed among four successive polyamine doses. This behavioral study was further followed by neuronal activity dependent labelling of olfactory epithelium using immune ofluorescence method. **Findings:** The findings suggest putrescine and spermidine as potent olfactory stimulator as well as attractant for this *L. rohita*. Among L -amino acids, arginine exhibited a well pronounced behavioral response, although a more positive behavioral approach was found for both the environmentally relevant biogenic amines relative to L-amino acids. This is supported by the concomitant number of c-FOS immune positive cells found on olfactory rosette upon application of these stimuli. **Conclusion:** The behavioral response of *L. rohita* was highest at the dose of 10^{-5} M for both putrescine and spermidine. Neuronal activity dependent labelling after exposure to differential polyamine doses revealed maximum p-ERK labelled cells in olfactory epithelium at the dose of 10^{-4} for both the diamines. Thus, *L. rohita* show attractive behavioral approach for both the polyamines. Future research on feeding behavior against these polyamines added diet will pave the path for sustainable aquaculture growth.

Keywords: Behavioral response, *L. rohita*, Olfactory epithelium, Putrescine, Spermidine

Introduction

Fish perceive multifarious odor molecules present in their aquatic environment in a diverse concentration range. The detection of vast range of molecules entails a highly developed olfactory organ, as well as specialized cells present over the olfactory epithelium (Igulu, 2014). Perception of these odoriferous molecules directly regulate the physiological activities along with the visible behavioral actions (Heraud et al., 2022). Essential physiological processes like, feeding, reproduction, migration and homing, social responses are extensively controlled through olfactory cues (Mukherjee & Saikia, 2024). Elaborative understandings on the molecules influencing olfactory-guided feeding, homing together with reproduction is essential, as human activities may reconfigure the odorant composition of streams, potentially interfering with fish physiological processes and restraining successful population recruitment.

As evident from the studies on salmon, important odorant molecules of parent habitat steer homing in them (Gardiner et al., 2015; Igulu, 2014). It is evident that, coral reef fish, *Lethrinus nebulosus* deals with environmental cues which regulate their habitat selection (Arvedlund & Takemura, 2006). In addition to habitat picking, olfactory input shapes feeding preference in non-migratory fish like grass carp, where it has been found that water solutions of free amino acids evoke food searching behavior in them (Yu et al., 2021).

Recent report on zebrafish reveals that the fish exhibit lysine addiction, which could alter their food choices (Lu et al., 2025). Behavioral experiments with other amino acids like, cysteine, alanine, serine in teleost like trout, lake charr, goldfish, and lake whitefish infers that aforementioned amino acids may stimulate feeding associated behaviors which are similar for food extracts (Hara, 2006). However, study on Nile tilapia *Oreochromis niloticus* discloses that free amino acid palatability differs among fish species validating taste preferences plasticity among fish species (Levina et al., 2021). Although, available literature provides enormous evidences for perplexing role of sensory cues modulating essential behavioral activities in fish, behavioral response of *Labeo rohita* towards L-amino acids has been hardly attempted. This led us to re-evaluate the effect of free amino acids on behavioral action of an economically important teleost, *L. rohita*.

Besides free amino acids, polyamines have been found to evoke predominant electro-olfactogram responses with greater magnitude (Sato & Sorensen, 2018). As evident from goldfish, polyamines like cadaverine, putrescine and spermine trigger olfactory stimulation at electrophysiological threshold of 10^{-8} to 10^{-7} molar. Also, these polyamines presents them as attractant for this teleost referring themselves as feeding stimulator (Rolen et al., 2003). Previously, cadaverine has also been described as feeding stimulant for rats (Heale et al., 1996). In contrast, in a study on zebrafish, Fuss & Korsching (2001) discloses the non-stimulatory properties of

putrescine, whereas, later, Hussain et al. (2013), reported strong aversive properties of cadaverine for zebrafish.

Apart from studies on goldfish and zebrafish, literatures demonstrating about the behavioral counteraction against these ecologically significant and environmentally relevant cues in any other teleost are still lacking until now. In this study, *L. rohita* has been attempted as a target fish to understand the stimulatory effect of different amines in fish under laboratory conditions. The primary aim is to unveil behavioral approaches as well as neuronal activity pattern advocating the overall response pattern of *L. rohita* against diamines like putrescine and spermidine in comparison with frequently studied L-amino acids. The fish *L. rohita* is highly cultivable fish across all freshwaters in the world and South East Asia in particular. The outcome from this study is expected to accelerate the feeding behavior exerting a positive effect on its growth in cultivable environment.

Materials and methods

Animal maintenance

A group of ~30 *L. rohita* adult fishes [Individual Total length (TL) 16 ± 2 cm and Weight 60 ± 5 gm] were collected from the local fish farm of Birbhum, West Bengal, India (23.6453° N, 87.6933° E). Primarily, fishes were acclimatized under 14/10-hour light/dark cycles and environmental temperature for two weeks in large cemented tanks. Prior to any behavioral experiments, an approval of the Institutional Animal Ethics Committee is obtained (Ref. IAEC/III-12/2020). Healthy fishes, irrespective of sexes were accounted for behavioral studies and they were habituated in a glass tank of length 3 feet, height 1 feet, width 1.5 feet for at least 72 hours before experimental commencement and kept unfed for 24 hours.

Stimulus preparation

Simultaneous to animal habituation, stimulus solutions of L-amino acids and polyamines were prepared for different experimental purposes. Stock solution of 10^{-2} molar (M) was prepared using Milli-Q water for three different amino acids (aa), neutral aa L-alanine, basic aa L-arginine and acidic aa L-glutamic acid. The pH of all stimulus solutions was adjusted to 7.6-7.8, to suit with the tank water. Stock solutions were kept frozen at -20°C until use. During each experiment a working concentration of standard 10^{-5} M was made for immediate use. This particular dose was opted based on previous behavioral studies conducted on teleost (Fuss & Korsching, 2001; Hubbard et al., 2011; Rolen et al., 2003). The experimental concentrations that were applied to the fish, invariably denotes the injected stimulus concentration. For polyamine stimulus preparation, two diamines, putrescine and spermidine were chosen and solutions were prepared for four different concentrations, i.e., 10^{-7} M, 10^{-6}

M, 10^{-5} M, 10^{-4} M. Unlike free amino acid stimulus preparation, the stimuli preparations were done immediately before the experiment onset.

Experimental set-up

All experiments were done in a constant stream of filtered tap water (30 Liter volume, pH- 7.6-7.8, DO- 8-9 mg/l). After acclimatization to the glass tank the initial movement pattern of the fish prior addition of the stimulus were utilized for comparing behavioral movement after stimulus exposure. Application of the stimulus was done in a single injection bolus in a corner opposite to the fish position at the initial period. All the testes were repeated multiple times. The movement pattern of *L. rohita* was video recorded using a Canon EOS 600D camera (666 frames= 3 minutes) for three minutes before (pre-stimulus) and after stimulus addition (post-stimulus) period, for each of the experiments. Following experiments, fishes were collected immediately and anaesthetized with MS-222 (Ethyl 3-aminobenzoate methanesulfonate, Sigma-Aldrich) in a dose range of 30mg/l-50 mg/l and olfactory organs were dissected out cautiously.

Behavioral analysis

The rectangular water tank was hypothetically compartmentalized in two opposite ends, stimulus source zone (S), the end where the stimulus is applied (as amine) and no stimulus neutral zone (N) opposite to S. In a rough dye diffusion experimental testing, it is seen that it takes more than 3 min for a solution to reach from N to S. Under normal condition and before adding stimulus, the fish is gently released and allowed to reside near N initially. Before and after application of stimulus, the movement of the fish was recorded carefully. Later, these recorded videos were subjected to analysis for behavioral patterns. For analyzing frequency of visits and determination of retention period at source zone, the videos were carefully monitored manually, calculations were done using MS Excel (Microsoft) and the values were converted into percentage for better comparison. Detailed analysis was performed using an open-source software employed for animal behavior analysis, named AnimalTA (version 3.2.2, vchiara.eu). Relative distance of the fish from the stimulus source zone was determine for every pre and post stimulus video and mean displacement from the source calculated and denoted as percentage of total tank length (TL). Close approach time is measured as number of video frames in which the fish approaches the stimulus application site closer than 10% of tank length. Normalized difference in close approach time is determined by a formula, i.e. [(time in close approach post stimulus) – (time in close approach pre stimulus)] / [time in close approach pre stimulus].

Dose dependent responses

The effective diamines stimulating the movements of *L. rohita* were further taken for experiments to see differential responses of these diamines with a concentration gradient on the fish behavior. To elucidate this, four different doses of concentrations were selected according to the preliminary investigative studies on polyamines and literature. The fishes were exposed separately against a concentration range of 10^{-7} – 10^{-4} M of Putrescine and Spermidine.

Immunofluorescence study

To visualize neuronal activity patterns the collected olfactory rosettes (n=47) were fixed in 4% paraformaldehyde at 4 °C, and kept overnight. Then cryoprotection was made through sequential incubation of fixed olfactory tissue in 15%, 20%, and 30% sucrose solutions, prepared in phosphate buffer saline (pH 7.4), at 4 °C until the tissues were fully infiltrated. Next, the tissues were embedded in cryo-embedding media (M-FREEZE™, MERCK) and processed for serial cryosections. Obtained cryosections were thoroughly washed with PBS, followed by permeabilization with 0.5% Triton X-100 in PBS (PBST) for 10 minutes. After that, the sections were washed with PBS repeatedly and then blocked using 3% bovine serum albumin (BSA) solubilized in PBST for a duration of 1 hour, to limit nonspecific binding.

Thereafter, the blocked sections were incubated with the primary antibodies against c-FOS (Invitrogen, PA1-830) and p-ERK (Santa Cruz Biotechnology, sc-7383) at 1:150 dilution, prepared in 1% BSA in PBST. Incubation was done for 2 hours at room temperature, then left overnight at 4 °C. This was followed by incubation in FITC (ABclonal, AS040) and Rhodamine (ABclonal, AS011) tagged goat anti-rabbit secondary antibodies at 1:250 dilution. Next, the sections were washed with PBS for three times and were mounted with polyvinyl alcohol (PVA) mounting medium and a coverslip. The immunofluorescent signal was visualized using a confocal microscope (Leica DMI8) equipped with a digital imaging system.

Statistical analysis

Levene's test was performed to check if variances maintain homogeneity. Student's t-test and one-way ANOVA were performed if the equal variances assumption was satisfied ($p > 0.05$). When Levene's test showed that variances are not equal ($p < 0.05$), Mann-Whitney U test and Kruskal-Wallis's test were opted. The significance level (α) for all statistical tests was maintained at ≤ 0.05 . The statistical analyses were done using SPSS version 16.0 software.

Results

Relative behavioral response *L. rohita* for L-amino acid and polyamines

Behavioral responses towards L-amino acids and polyamines were evaluated through frequency of visits to the stimulus source zone i.e. S (closer than 10% of TL), retention period at S and mean displacement from the S. For neutral amino acid L-alanine, there was insignificant change in visit frequency (Student's t test, $t_{18, 0.05} = -0.447$, $p > 0.05$, $n=10$) as well as retention period (Student's t test, $t_{8, 0.05} = -0.884$, $p > 0.05$, $n=5$) in comparison to pre stimulus period (Figure 1). Additionally, as demonstrated in Figure 2, no change was observed for mean displacement (Student's t test, $t_{4, 0.05} = 1.670$, $p < 0.05$, $n=3$) as well. Conversely, application of positively charged L-arginine triggered the frequency of visit (Student's t test, $t_{18, 0.05} = -1.750$, $p > 0.05$, $n=10$) and retention period (Student's t test, $t_{8, 0.05} = -2.374$, $p < 0.05$, $n=5$). Albeit, there was a reduction of mean displacement which was found to be insignificant (Mann-Whitney U test, $p > 0.05$, $n=3$) when L-arginine was the stimulus. Moreover, application of negatively charged L-glutamate did not affect the frequency of visit (Student's t test, $t_{18, 0.05} = 0.383$, $p > 0.05$, $n=10$), which is explained in Figure 3. In addition to this, the change in retention period (Mann-Whitney U test, $p > 0.05$, $n=5$) and mean displacement (Student's t test, $t_{4, 0.05} = -0.605$, $p > 0.05$, $n=3$) by negatively charged L-glutamate was not significant.

Interestingly both the diamines conjointly allowed positive movement of the fish in contrast to the L-amino acids. For putrescine exposure, *L. rohita* exhibited a higher frequency of visits (Student's t test, $t_{18, 0.05} = -3.607$, $p < 0.05$, $n=10$) following greater retention period (Student's t test, $t_{8, 0.05} = -2.982$, $p < 0.05$, $n=5$) at the S compared to peristimulus duration. Regarding this, a lesser mean displacement from S was also obtained from the experiments analyzed through Mann-Whitney U test ($p < 0.05$, $n=3$) and is shown in Figure 4. Likewise, an extended frequency of visits (Mann-Whitney U test, $p < 0.05$, $n=10$) was observed after spermidine application. This succeeded to an elevated retention period (Mann-Whitney U test, $p > 0.05$, $n=5$) at the S. The diminution of mean displacement after spermidine exposure is discussed in Figure 5 (Student's t test, $t_{4, 0.05} = 3.793$, $p < 0.05$, $n=3$).

Regarding the evaluation of track length ratio alteration, a total of 660 frames were considered for track length estimation for each stimulus and comparative analysis was done between pre and post stimulus intervals. Here, a significant change in track lengths in comparison to water (dH₂O) as negative control (mentioned in tabular format supplementary details) was not observed. This unaltered track length ratios justify that there was no change in velocity as well. Additionally, normalized close approach time analysis (Figure 6) reveals that after diamine application *L. rohita* spent significantly higher duration of time in close vicinity to the odor application site relative to the mentioned L-amino acids (Kruskal Wallis test; $\chi^2=13.234$, $df=4$, $p < 0.05$, $n=15$).

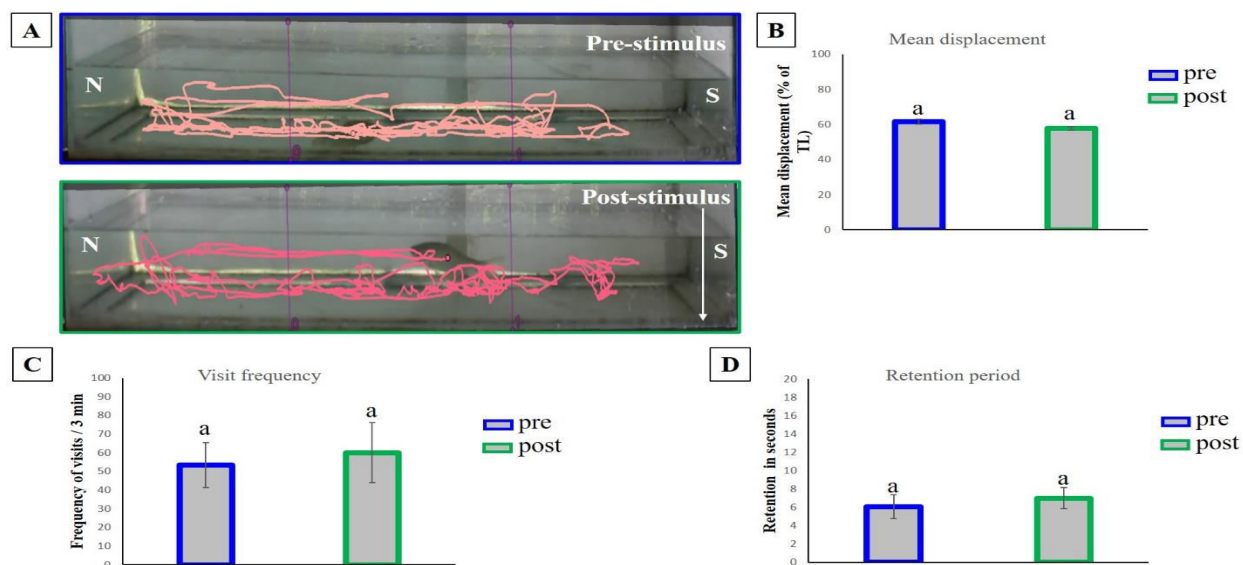


Figure. 1 Comparison of fish behavior during Pre-stimulus and post stimulus period with L-Alanine as stimulus. (a) Movement pattern in accordance with track length in pre and post stimulus periods. Behavioral analysis of *L. rohita* in terms of mean displacement (b), frequency of visit (c), retention period (d) for post-stimulus vs. pre-stimulus condition. Means ($\pm$ SE) were compared using independent sample t-test. Different lowercase alphabets indicate statistically significant difference at $p < 0.05$. S, Stimulus source zone; N, Stimulus neutral zone

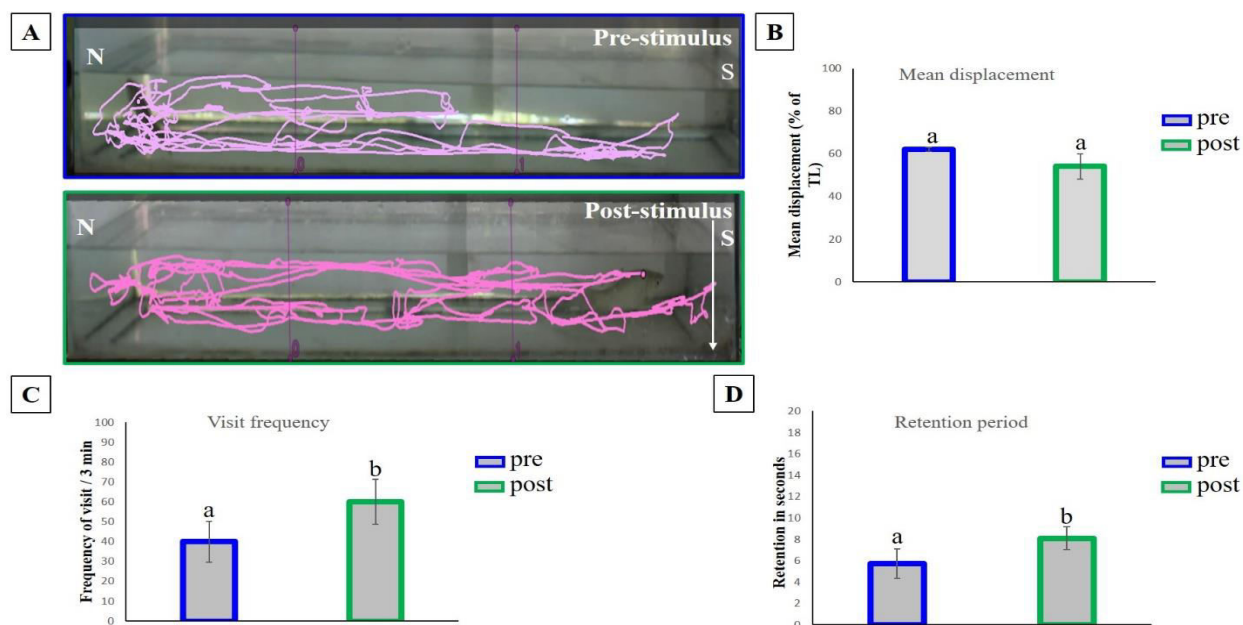


Figure. 2 Comparison of fish behavior during Pre-stimulus and post stimulus period with L-Arginine as stimulus. (a) Movement pattern in accordance with track length in pre and post stimulus periods. Behavioral analysis of *L. rohita* in terms of mean

displacement (b), frequency of visit (c), retention period (d) for post-stimulus vs. pre-stimulus condition. Means ($\pm$ SE) were compared using independent sample t-test and Mann-Whitney-U test, wherever applicable. Different lowercase alphabets indicate statistically significant difference at $p < 0.05$. S, Stimulus source zone; N, Stimulus neutral zone

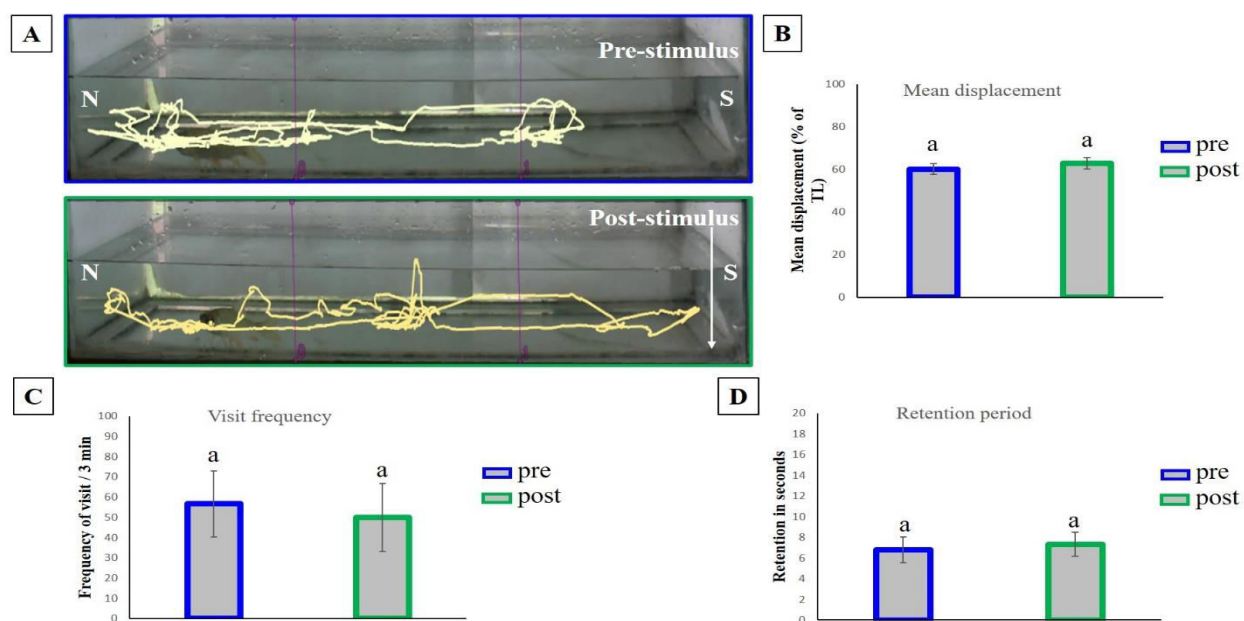


Figure. 3 Comparison of fish behavior during Pre-stimulus and post stimulus period with L-Glutamate as stimulus. (a) Movement pattern in accordance with track length in pre and post stimulus periods. Behavioral analysis of *L. rohita* in terms of mean displacement (b), frequency of visit (c), retention period (d) for post-stimulus vs. pre-stimulus condition. Means ($\pm$ SE) were compared using independent sample t-test and Mann-Whitney-U test, wherever applicable. Different lowercase alphabets indicate statistically significant difference at $p < 0.05$. S, Stimulus source zone; N, Stimulus neutral zone

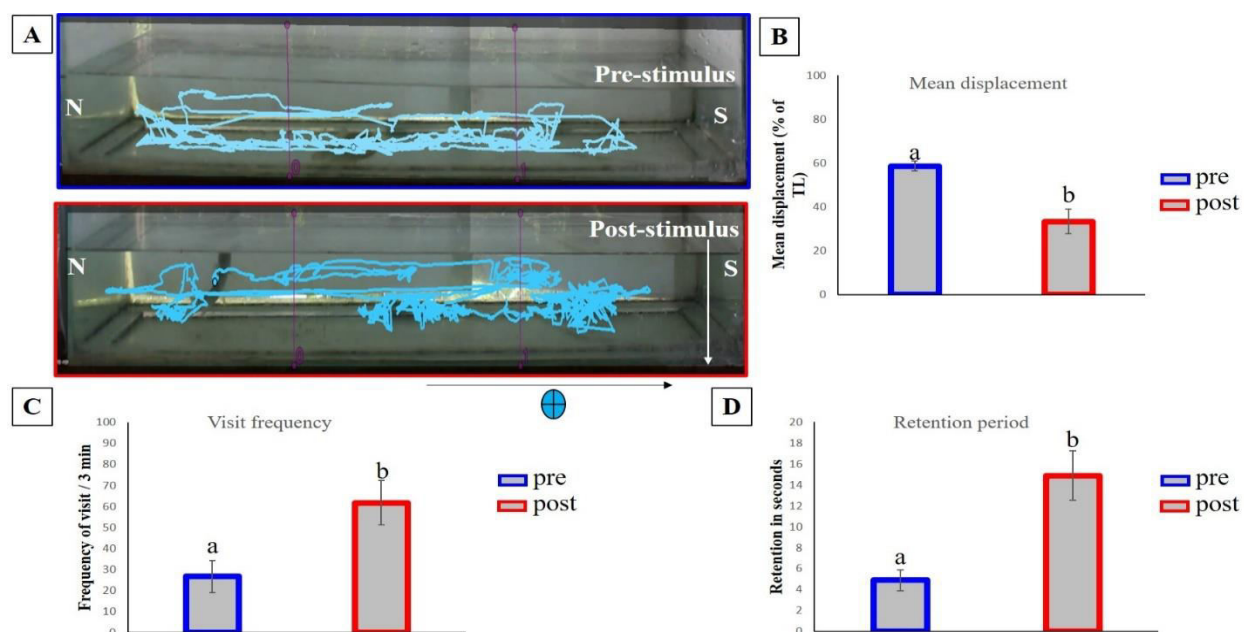


Figure. 4 Comparison of fish behavior during Pre-stimulus and post stimulus period with Putrescine as stimulus. (a) Movement pattern in accordance with track length in pre and post stimulus periods. Behavioral analysis of *L. rohita* in terms of mean displacement (b), frequency (c), retention period (d) for post-stimulus vs. pre-stimulus condition. Means ($\pm$ SE) were compared using independent sample t-test and Mann-Whitney-U test, wherever applicable. Different lowercase alphabets indicate statistically significant difference at $p < 0.05$. S, Stimulus source zone; N, Stimulus neutral zone

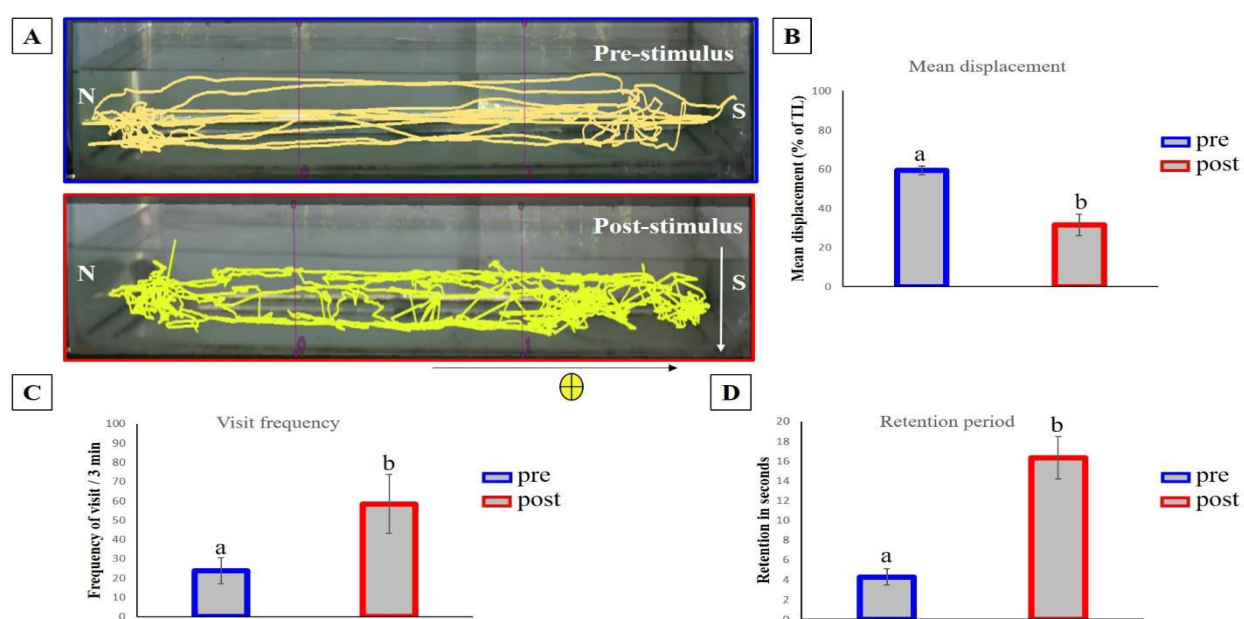


Figure. 5 Comparison of fish behavior during Pre-stimulus and post stimulus period with Spermidine as stimulus. (a) Movement pattern in accordance with track length

in pre and post stimulus periods. Behavioral analysis of *L. rohita* in terms of mean displacement (b), frequency of visit (c), retention period (d) for post-stimulus vs. pre-stimulus condition. Means ($\pm$ SE) were compared using independent sample t-test and Mann-Whitney-U test, wherever applicable. Different lowercase alphabets indicate statistically significant difference at $p < 0.05$. S, Stimulus source zone; N, Stimulus neutral zone

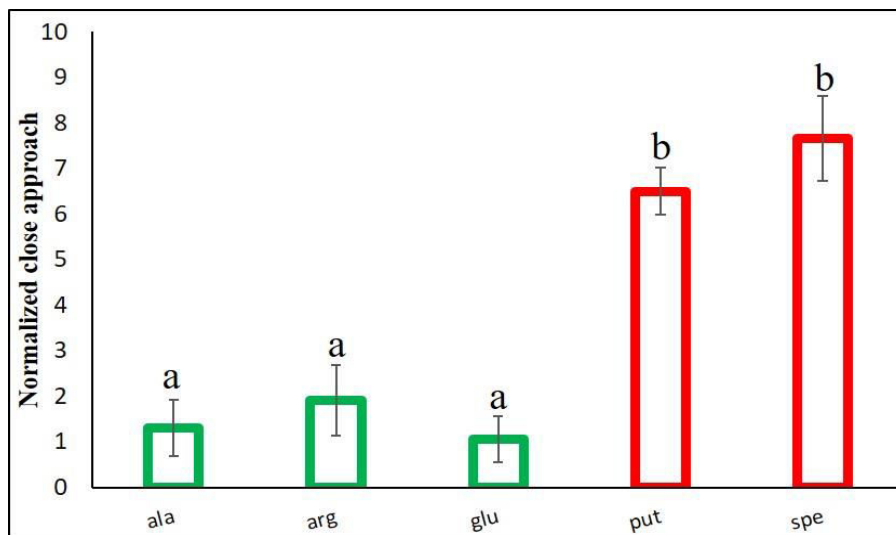


Figure. 6 Comparison of different odor induced close approach time for *L. rohita* upon various amino acids and polyamines application. Here, lesser values indicate least effective stimulus. Means ($\pm$ SE) were compared using independent sample t-test and Kruskal-Wallis test, wherever applicable. Different lowercase alphabets indicate statistically significant difference at $p < 0.05$. ala, L-Alanine; arg, L-Arginine; glu, L-Glutamine; put, Putrescine; spe, Spermidine

L-amine and polyamine stimulated labelling of ORNs

Various studies have been considered c-FOS as a reliable neuronal activity marker. Being an immediate early gene, this gets expeditiously induced as soon as there is an increased neural activity (Joo et al., 2016; Perrin-Terrin et al., 2016). Thus, to check neuronal activity in olfactory epithelium, after L-amino acid and polyamine applications, the olfactory rosettes were accounted for c-FOS labelled neuronal counting. This revealed that there was an insignificant change in c-FOS positive nuclei numbers for l-amines when compared to water as control. In contrary, the frequency of c-FOS positive nuclei was found to be higher in both the diamines putrescine and spermidine. The significant difference in c-FOS labelled cells (One way ANOVA, Levene's test; $p=0.342$, $F_{5,30}=18.348$, $p<0.05$, $n=6$) upon various stimulus

application is described in Figure 7. There were a few numbers of cells labelled with c-FOS counted for control groups, probably due to lingering molecules of tank water.

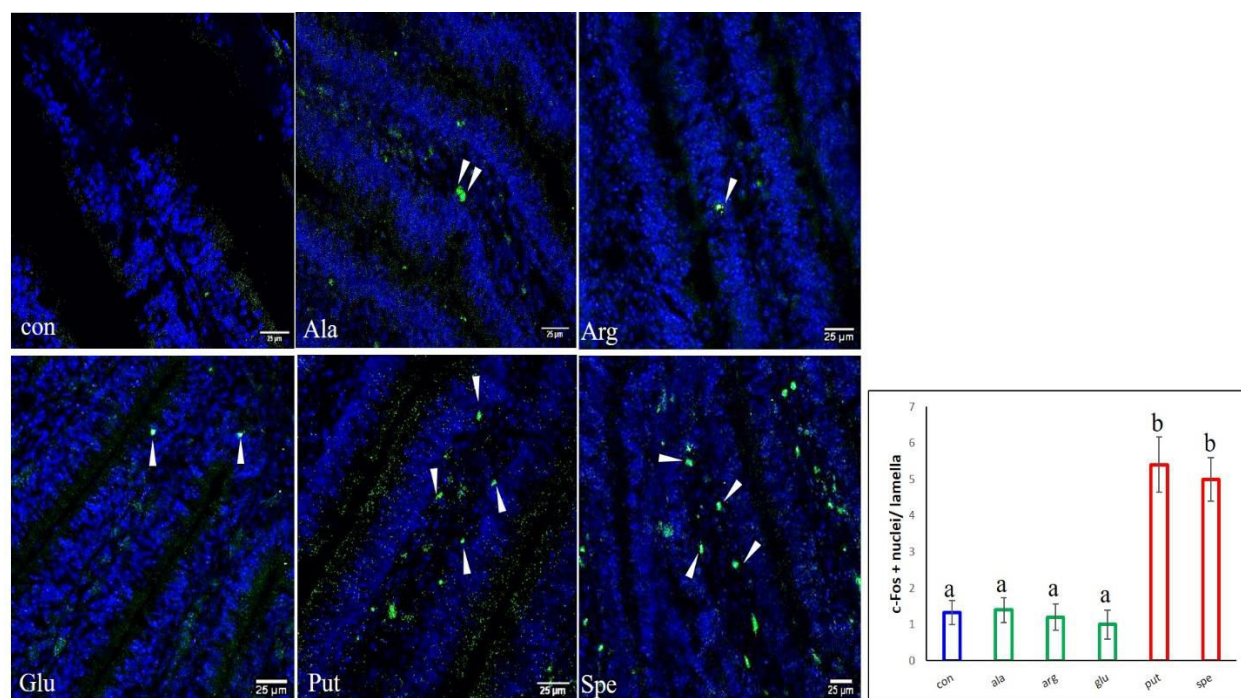


Figure. 7 Imaging of c-Fos signal after exposure to various amino acids and polyamines. Graphical representation indicates frequency of c-Fos positive nuclei after different amine exposure. Values are expressed as mean $\pm$ SE, means were compared using one-way ANOVA followed by Tukey's post-hoc test. Different lowercase alphabets in a group indicate statistically significant difference at $p < 0.05$. ala, L-Alanine; arg, L-Arginine; glu, L-Glutamine; put, Putrescine; spe, Spermidine

Dose dependent behavioral response against polyamines

In Figure 8, one can find concentrated movement at the stimulus source zone (S) in 10^{-5} M dose of putrescine. Consequently, highest visit frequency (One way ANOVA, Levene's test; $p=0.295$, $F_{3,12}=6.448$, $p<0.05$, $n=4$) was found for 10^{-5} M dose with the maximum retention period (One way ANOVA, Levene's test; $p=0.062$, $F_{3,8}=23.306$, $p<0.05$, $n=10$) involved for the same dose. In continuity, the lowest mean displacement from the S was evaluated for this particular dose (One way ANOVA, Levene's test; $p=0.122$, $F_{3,8}=7.150$, $p<0.05$, $n=3$). Similarly, spermidine also induced a dose dependent positive movement as explained in Figure 9. Here, a significant raise in frequency of visits were obtained for the dose 10^{-5} M (One way ANOVA, Levene's test; $p=0.399$, $F_{3,12}=2.858$, $p>0.05$, $n=4$). Subsequently, the highest retention period in vicinity of stimulus applied site (i.e. S) was evaluated for the similar dose, which is proved to be significant through One way ANOVA, (Levene's test; $p=0.268$, $F_{3,8}$

=33744, $p < 0.05$, $n = 3$). Also, the lowest mean displacement was assessed for this aforementioned dose, where significance was explained by One way ANOVA, Levene's test; $p = 0.216$, $F_{3,8} = 5.144$, $p < 0.05$, $n = 3$).

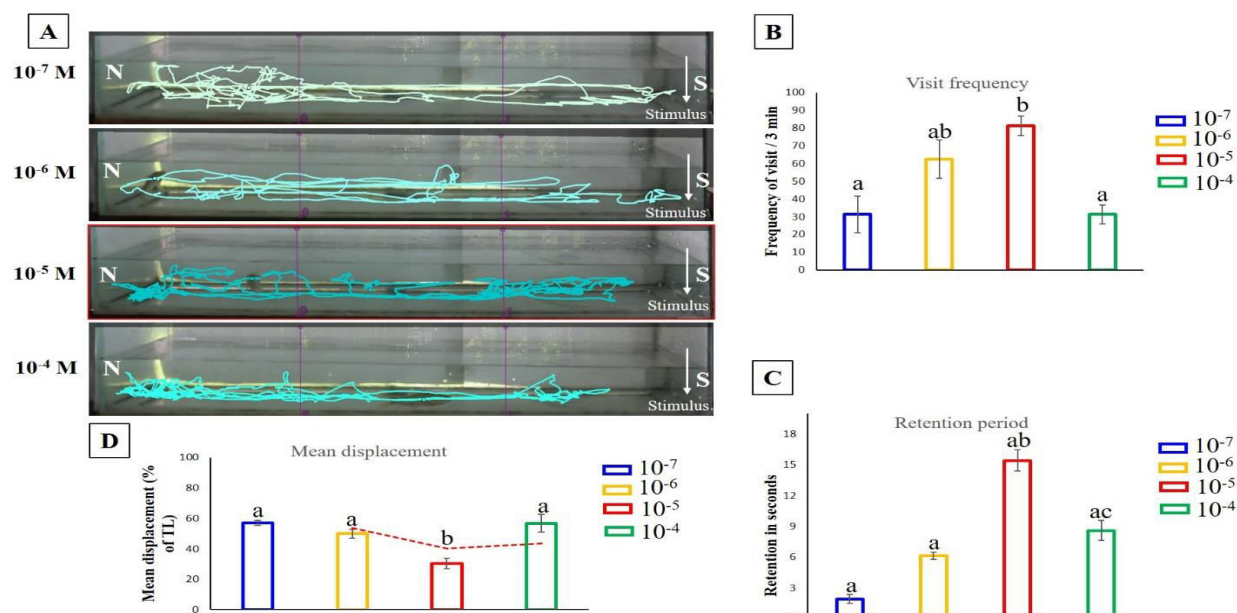


Figure. 8 (a) Behavior pattern against dose dependent response (positive movement) towards ascending molar doses of putrescine. Graphical explanations of dose dependent approaches towards ascending molar doses of putrescine showing (b) Frequency of visits (c) retention period and (d) mean displacement. Means ($\pm$ SE) were compared using one-way ANOVA followed by Tukey's post-hoc test. Different lowercase alphabets indicate statistically significant difference at $p < 0.05$. S, Stimulus source zone; N, Stimulus neutral zone

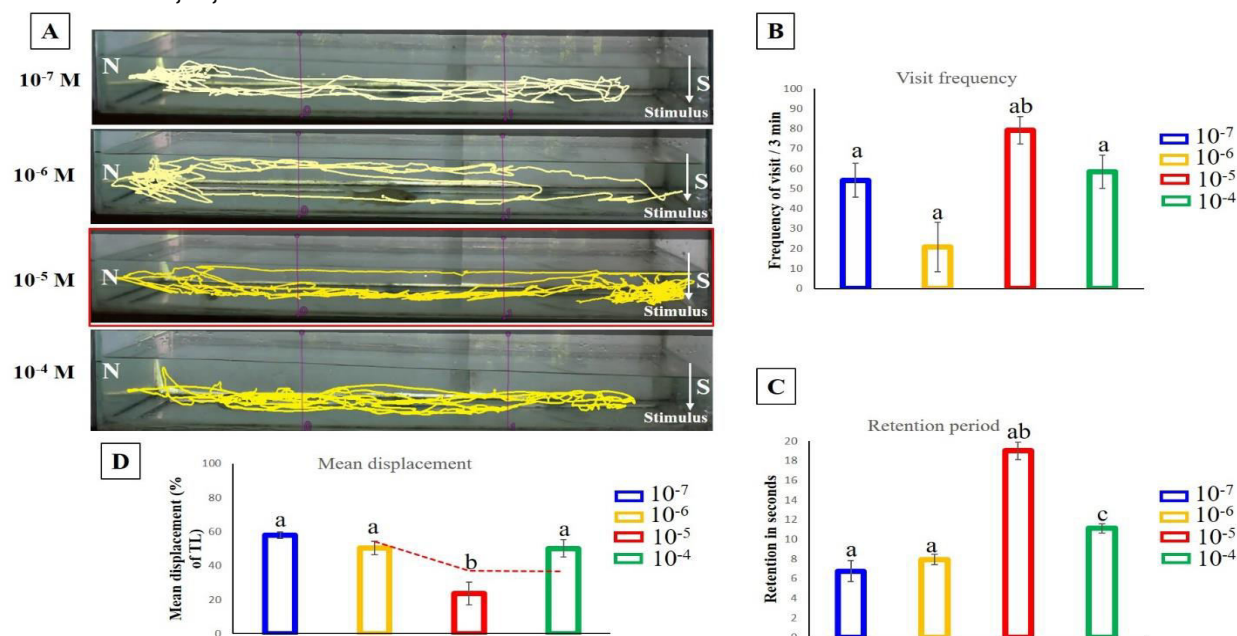


Fig. 9 (a) Behavior pattern against dose dependent response (positive movement) towards ascending molar doses of spermidine. Graphical explanations of dose dependent approaches towards ascending molar doses of putrescine showing (b) frequency of visits (c) retention period and (d) mean displacement. Means ($\pm$ SE) were compared using one-way ANOVA followed by Tukey's post-hoc test. Different lowercase alphabets indicate statistically significant difference at $p < 0.05$. S, Stimulus source zone; N, Stimulus neutral zone

Neuronal activity on olfactory epithelium upon application of various diamine doses

Phosphorylation of ERK has also been established to be synchronized with differences in neuronal activity previously. Both for ORNs and olfactory bulb mitral cells, induction of p-ERK has been found upon odor stimulation (Kim et al., 2015; Mirich et al., 2004). With reference to this, the frequency of p-ERK labelled cells upon application of the diamines (putrescine and spermidine) at the applied doses that were opted for behavioral study were assessed (Figure 10). For tissues exposed to putrescine, the number of p-ERK labelled cells were found to be increased concurrently with the dose applied. Although the highest number of p-ERK expressing cells were obtained for the dose of 10^{-4} M, roughly equivalent number of p-ERK immunoreactive cells were found at 10^{-5} M and 10^{-6} M dose of putrescine (One way ANOVA, Levene's test; $p=0.741$, $F_{3,20}=51.821$, $p<0.05$, $n=6$). Alike the results from putrescine application, different doses of spermidine exploitation revealed comparable scenario (Figure 11). The maximum number of cells were acquired from for the dose of 10^{-4} M and lowest number of p-ERK labelled cells found at the 10^{-7} M dose (One way ANOVA, Levene's test; $p=0.493$, $F_{3,20}=10.307$, $p<0.05$, $n=6$). The dose response verses neuronal activity analysis demonstrated threshold p-ERK activation at 10^{-5} M dose of putrescine and 10^{-6} M dose of spermidine.

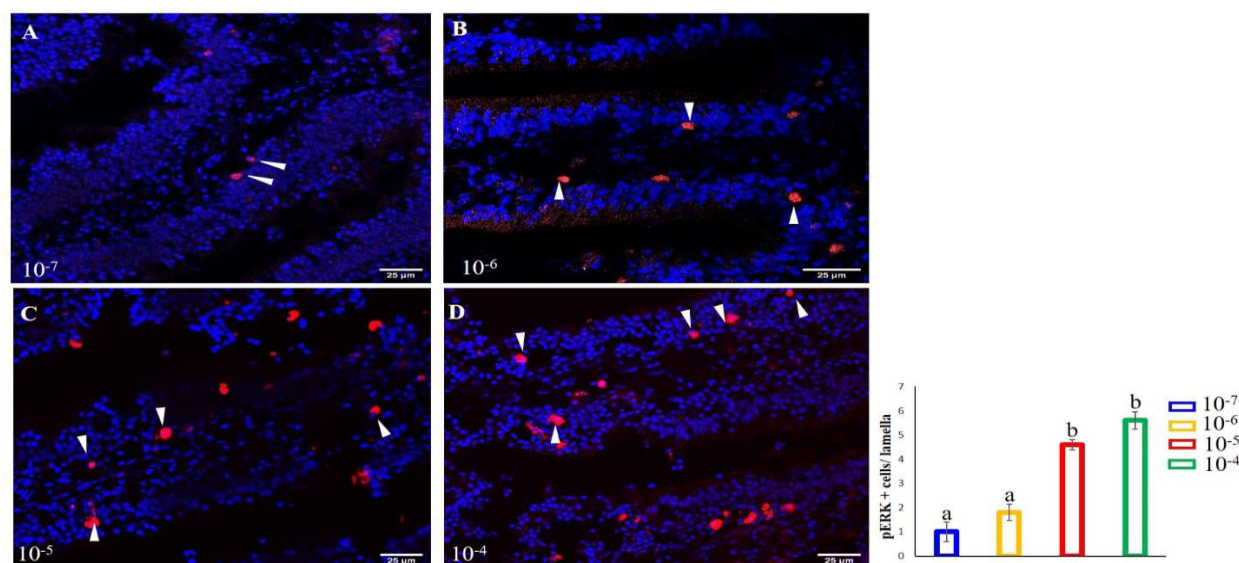


Figure. 10 Imaging of p-ERK Signal after exposure to ascending molar doses of putrescine. Graphical representation indicates frequency of p-ERK positive cells (shown with arrowheads) in different doses. Values are of mean $\pm$ SE, all the means were compared using one-way ANOVA followed by Tukey's post-hoc test. Different lowercase alphabets in a group indicate statistically significant difference at $p < 0.05$

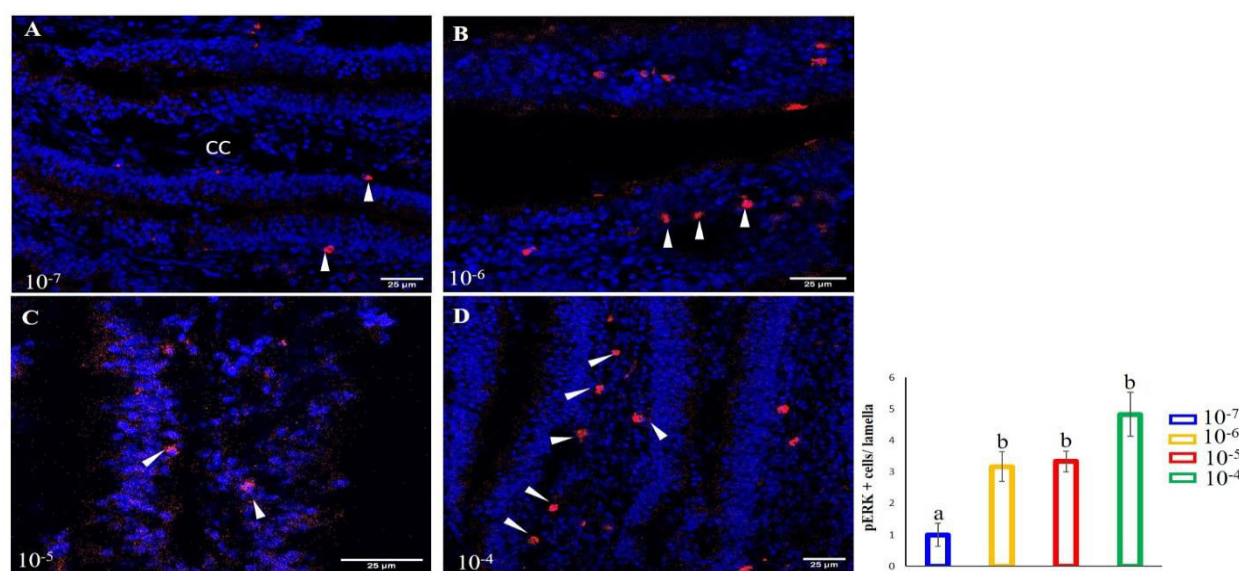


Figure. 11 Imaging of p-ERK Signal after exposure to ascending molar doses of spermidine. Graphical representation indicates frequency of p-ERK positive cells (shown with arrowheads) in different doses. Values are of mean $\pm$ SE, means were compared using one-way ANOVA followed by Tukey's post-hoc test. Different lowercase alphabets in a group indicate statistically significant difference at $p < 0.05$

Discussion

Olfactory odor coding involves odor identification, intensity break-down, as well as time and spatial assessment that parallelly activates receptor neuron activation at the level of olfactory epithelium (Olivares & Schmachtenberg, 2019). The present study demonstrates behavioral response pattern of *L. rohita* towards a distinct class of odorants, polyamines, which is abundantly present in our surroundings. The behavioral actions towards these groups of molecules were compared with another environmentally relevant odor class, i.e., L-amino acids.

Previously, L-amino acids have been extensively studied in teleost, like, channel catfish *Ictalurus punctatus*, where both L-alanine and L-arginine act as effective olfactory stimuli at a dose of 10^{-4} M, while arginine proved to be more potent in olfactory response generation (Valentinčič & Caprio, 1994). Comparative behavioral study on rainbow trout *Oncorhynchus mykiss*, lake charr *Salvelinus namaycush*, lake whitefish *Coregonus clupeaformis* and goldfish *Carassius auratus* revealed alanine may elicit partial feeding behavior in these fishes (Hara, 2006). However, present behavioral study on a less attempted freshwater teleost *L. rohita*, revealed that the same amino acid merely exerts any effect on the fish behavioral actions. In case of lake charr, lake whitefish L-arginine has been found to enhance feeding behaviors, while in case of rainbow trout this doesn't imply. Also, in case of goldfish, the arginine has been described to be most stimulatory amino acid and electrophysiological studies using amino acids on catfish, *Ameiurus melas*, shows arginine to evoke a large olfactory response when exposed (Dolensek & Valentincic, 2010; Hara, 2006; Rolen et al., 2003). Similar to these *L. rohita* also shows a positive but non-significant movement towards the stimulus source when L-arginine stimulus was applied. Behavioral experiments revealed that in rainbow trout glutamate suppress locomotor activities (Hara, 2006). In contrast to this, *L. rohita* shows a negligible behavioral response towards L-glutamate demonstrating it to be non-stimulatory for them. Earlier, Michel & Lubomudrov, (1995) stated that the stimulatory effectiveness of amino acids in zebrafish can be arranged in a most to least potency ranking series as neutral > basic > acidic > imino amino acids. However, present behavioral study in *L. rohita* with selected amino acids reveals that the most effective amino acid may be basic in nature with least effective being acidic.

Concentrations of diamines like putrescine, cadaverine and spermine is apparently correlated with the degree of decaying aquatic animals, thus advocating its relevance in the aquatic ecosystem (Mietz & Karmas, 1978). The current study provides behavioral evidences signifies this class of amines as potent olfactory stimulator. Being putrescine precursor, agmatine has been reported previously to exhibit effective olfactory stimulation in zebrafish (Michel et al., 2003). However, later, investigation on putrescine derived behavior in zebrafish revealed that this odorant exerts a negative behavioral response (Hussain et al., 2013). Conversely, in

goldfish, electro-olfactogram recordings for polyamines putrescine, cadaverine and spermine was found to be 4.2x, 4.3x and 10.3x the response of the 0.1mM L-arginine (Rolen et al., 2003). These contrasting results led us to reconsider the diamines putrescine and spermidine for investigation on important Indian Major Carp *L. rohita*. Behavioral response analysis in terms of frequency of visit, retention period and mean displacement discloses that putrescine and related compound spermidine are considerably more efficient molecules that influence positive behavioral movements in *L. rohita* in comparison to the L-amino acids. The maximum behavioral approach in terms of frequency of visit, retention period and mean displacement towards the source of diamine stimuli by the fish supports the hypothesis that diamines are more preferred odorant class for *L. rohita* relative to other free amino acids.

The observed behavioral outcomes in this study are further certified through immunological observations through c-FOS and pERK. Previously, study on odor stimulation and c-fos expression relationship revealed that c-fos mRNA expression is correlated with the increased neuronal activity in the olfactory bulb of mice (Jin et al., 1996). Pertaining to that, in the present study, analysis of frequency of c-FOS positive cell in the olfactory epithelium exposed to amino acid and polyamine indicates a higher number of c-FOS positive nuclei for both the diamines, sufficiently substantiating the hypothesis of maximum behavioral response for selected diamines. The dose-response relationship for the diamines in *L. rohita* revealed an integral behavioral response upon concentration increase where response is maximum at the dose of 10^{-5} M for both the diamines. However, at physiological level the maximum neuronal activation through p-ERK immunopositive cell positivity was obtained for the dose of 10^{-4} M, suggesting olfactory epithelium accommodates large number of receptor that receive higher dose of these diamine (Hussain et al., 2013). Thus, evident from the results, related polyamines may exert similar behavioral response in *L. rohita* which depends on the degree of concentration. Although, in comparison with goldfish, where molecules like putrescine, agmatine and cadaverine increase feeding associated pecking behaviors, the current study does not stretch to feeding associated behavioral assessment (Rolen et al., 2003). Future investigations on this area may pave a new path on food formulation for this economically important teleost. Moreover, it is obvious from the available literature and from results of the current study, the odor preference is definitely species specific, portraying diverse behavioral mannerism facilitating differential physiological processes for fish as well as other aquatic animals.

Conclusions

These current findings conclude that *L. rohita* show behavioral preferences for both even chained diamine like putrescine as well as odd chained polyamine spermidine, over L- amino acids. This scenario is advocated by the concomitant change in

frequency of c-FOS labelled sensory neurons on olfactory epithelium. Moreover, the degree of behavioral response largely contingent on the concentration of the biogenic amines. Also, this dose dependent response is reflected with the relative numbers of p-ERK expressing sensory cells on olfactory epithelium. Along with the current study on *L. rohita*, previous findings on zebrafish as well as goldfish behavioral experiments establish the behavioral plasticity against similar class of molecules coordinates in a species-specific manner.

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Compliance with ethical standards

All the experiments were conducted as per the guidelines of the Institutional Animal Ethical Committee (Proposal No. IAEC/VB/2022-II/8).

Conflict of interest

The authors declare no competing interests that are relevant to the content of this article.

Supplementary details

Track length ratio changes in *L. rohita* upon stimulus addition was calculated as pre- and post-approach time was compared to the results obtained from the experiments and compared with water (control). The total track length varied between experimental replicates, as well as among fishes, but the change in track length was not significant at $p < 0.05$ indicating an unaltered velocity and total distance travelled. Therefore, the positive movement towards the stimulus source was due to odor preference and not by change in motility.

1mM stimulus	Track length ratio (post-stimulus/pre-stimulus)	P value
L-Alanine	0.74243 ± 0.041	0.51
L-Arginine	1.025357 ± 0.073	0.39
L-Glutamine	0.557499 ± 0.070	0.17
Putrescine	1.08982 ± 0.036	0.26
Spermidine	0.851287 ± 0.030	0.18

Table 1. Track length ratio changes in *L. rohita* upon stimulus addition. P value was determined by student t-test when pre- and post-approach time was compared to the results obtained from the experiment with water (negative control). Means ($\pm$ SE) were compared using independent sample t-test where significant difference was determined at $p < 0.05$.

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