Dissociation of behavioral and endocrine stress responses by arecoline in adult zebrafish

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Abstract
Behavioral and endocrine stress responses represent two fundamental and interlinked pathways in vertebrate stress biology. Although both responses can respond to pharmacological modulation but their relationship remains poorly understood across species and comparative models. Arecoline, the key alkaloid of Areca catechu and a partial muscarinic acetylcholine receptor agonist, has been shown to induce behavioral anxiolysis in vertebrates. However, it is unclear whether this behavioral anxiolysis reduces cortisol-mediated physiological stress or, paradoxically, enhances it. This knowledge gap in comparative stress literature served as the foundation for this study by exposing adult Zebrafish (Danio rerio) to arecoline (0.5 µM, 5 µM, and 50 µM) under acute (30 minutes) and chronic (7 days) paradigms. Anxiety-like behavior in fish was assessed using vertical exploratory parameters in the novel tank test (NTT), and whole-body cortisol levels were measured using enzyme-linked immunosorbent assay (ELISA). Arecoline demonstrated duration-dependent behavioral anxiolysis by increasing top zone exploration metrices in maximum of acute and chronic parameters, yet not all reached the statistical significance but the cortisol value was significantly raised in both the exposure scenarios. Collectively, these findings indicate that arecoline alters behavioural and endocrine stress response components in adult zebrafish. The coexistence of anxiolytic-like behaviour and higher cortisol levels suggests that cholinergic modulation does not always cause behavioural and physiological stress responses to shift concurrently. These findings emphasise the need of integrating behavioural and endocrine measures in comparative stress research, as well as laying the groundwork for future study into the neuroendocrine mechanisms driving alkaloid-mediated stress regulation.

Keyword: Arecoline, Zebrafish, Novel tank test, Behavioral anxiolysis, Neuroendocrine stress, Cortisol

Introduction

Stress is a necessary physiologic response, conserved across all living beings, which is monitored by the Hypothalamic-Pituitary-Adrenal (HPA) axis in mammals and the Hypothalamic-Pituitary-Interrenal (HPI) axis in non-mammalian species. However, prolonged exposure to stress is able to disrupt the physiological homeostasis of any organism, causing stress-related disorders, including anxiety, depression, and many more [1,2]. In this ongoing era, stress-related disorders are emerging as a new global burden for public health sectors, causing a socio-economic strain for the world population [3]. Moreover, chronic stress can be a common major factor for almost all the comorbid patients, majorly Type 2 diabetes, cardiovascular diseases, as well as Major Depressive Disorder (MDD), and many more. Therefore, these crucial factors undoubtedly highlight the systemic significance of stress in clinical settings. Research into novel or more effective stress-relieving drugs is therefore urgently needed for the benefit of individual and public health [3,4,5].

The fruit (nut) of Areca catechu is one of the most popular psychoactive plant products, with an estimated 600 million regular users across Southeast Asia, the Pacific Islands, and migratory tribes [6]. Chewing areca nuts, sometimes as part of betel quid, is a long-standing sociocultural habit in these areas, associated with personal bonds and ritual significance. Consumption of areca nut can elicit stimulation, improved alertness, mild euphoria, mood relaxation, and short-term relief from mental stress. However, chewing this nut can be addictive, resulting in withdrawal symptoms [6,7]. Therefore, a comprehensive study of areca nuts or their alkaloids is necessary to clarify both their therapeutic potential and the associated risk of dependency [8].

Arecoline, the primary alkaloid found in Areca catechu, is a partial agonist of the muscarinic acetylcholine receptor (mAChR) [8]. Activation of mAChRs can change neuronal excitability, synaptic plasticity, and neurotransmitter release, which influences cognitive and affective activities like attention, learning, memory, and emotional modulation. Therefore, arecoline may have the potential to cause and regulate the behavioral anxiolytic-related pathways, which is especially significant to anxiety-related disorders [8,9]. Recent studies on rodent models of chronic unpredictable mild stress (CUMS) suggest that arecoline may have the potential to be a functional neuroactive compound for treating stress-induced anxiety disorders [10]. However, in some cases, cortisol and corticosterone levels rise simultaneously, activating the endocrine stress axis. Although experimental data across animals on cortisol elevation remain inconsistent. Therefore, this contradictory relationship between arecoline's behavioral effects and cortisol's neuroendocrine output needs to be addressed to fully comprehend arecoline's stress axis dynamics [10,11].

Zebrafish (Danio rerio) is a useful translational model for studying behavior and neurobiology [12]. Like the rodent model system, cortisol in Zebrafish acts as the principal stress hormone, utilizing its Hypothalamic Pituitary Interrenal (HPI) axis, which is homologous to the Hypothalamic Pituitary Adrenal (HPA) axis [13]. Furthermore, the Novel Tank Test (NTT) is regarded as one of the most robust behavioral techniques for distinguishing behavioral anxiety based on fish's vertical exploration. Complementary assessment of Whole Body Cortisol by Enzyme-Linked-Immunosorbent Assay (ELISA) refers to the endocrine output of the study and the status of HPI axis activation [13,14]. Therefore, the present study was designed using acute (30 minutes) and chronic (7 days) arecoline exposure paradigms. Through this exposure and integrated work through NTT and Cortisol study, this experiment intends to solve the long-time conflict between arecoline's behavioral modulation and its cortisol-based stress dynamics [15,16]. Addressing this conflicted status not only reconciles the existing literature but also helps to understand in detail the action and mechanism of muscarinic-based receptors and their role in stress axis activation, if any.

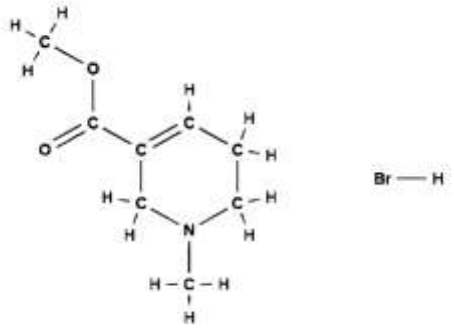


Fig 1. Structure of Arecoline Hydrobromide

Materials & Methods

1.1. Animal & housing

Five to seven-month-old adult zebrafish were purchased from a local fish distributor and housed in a standardized aquatic environment in the lab. Before beginning any experimental treatments, fish were acclimated for 15 days following arrival to minimize the effects of handling and transit stress. The housing system maintained a constant temperature of 27±1 °C, and the water pH was maintained between 6.8-7.55. Systems of overtop circulation and continuous aeration were used to sustain steady dissolved oxygen levels. To replicate natural day-night cycles, the lighting was adjusted to a 14-hour light and 10-hour dark cycle [17,18]. Fish were fed a commercial meal (Optimum Mini pellet, Thailand) twice a day (morning and afternoon), with the quantity controlled to prevent overfeeding and water quality degradation. Zebrafish used in the trials were experimentally naive, having never been exposed to drugs, tested behaviorally, or undergone any invasive procedures.

1.1 Drug and dosage

Arecoline hydrobromide (CAS-300-08-3, Thermo Scientific, USA) was employed as the muscarinic receptor agonist. A pilot study was performed to determine the lethal dose and optimal working concentration range of arecoline. Based on this data, three concentrations, 50 µM, 5 µM, and 0.5 µM, were selected for further experiments. Acute exposure, involving a single 30-minute treatment, and chronic exposure, involving ongoing exposure for 7 days, were finalized and planned after that. Standardized tank parameters were used for both acute and chronic exposures, and dosing solutions were changed daily to maintain constant drug bioavailability.

1.2. Novel Tank Test

The novel tank test (NTT) assesses anxiety-like behavior in zebrafish as per their natural diving response to a new or novel environment. Initially, the fish remain at the bottom of the tank before gradually moving to the upper sections, indicating habituation and reduced anxiety. The NTT is a reliable substitute for fish anxiety tests and is sensitive to environmental, genetic, and pharmacological factors [19,20]. The novel tank apparatus used was a trapezoidal transparent glass tank (height × length × width: 20 × 15 × 6 cm^3) that was filled to approximately 75% capacity with system water maintained at 26±1 °C. Ambient illumination was controlled at 300 lux, with gradual transitions to reduce stress [21].

During the experiment, fish were pre-exposed to the testing environment (pre-exposure tank) for at least 1 hour prior to behavioral assessment to minimize stress. After that, individual fish were carefully moved from the pre-exposure tank to the holding tank, using system water, for 10 minutes of acclimatization. Before being introduced to the novel tank, the fish were immersed in a drug tank containing dissolved drug at the previously mentioned doses, or in water for controls, for either 30 minutes (acute groups) or 7 days (chronic groups). Finally, the fish were transferred individually to the experimental novel tank, where their vertical swimming behavior was captured for 6 minutes using a camera connected to automated tracking software from the point of entry [21].

Freezing bout, freezing duration, latency to enter the top, time spent in the top and bottom, and average entry duration were among the behavioral parameters of NTT measured. Utilizing statistical software [Graph Pad, Boston, MA,

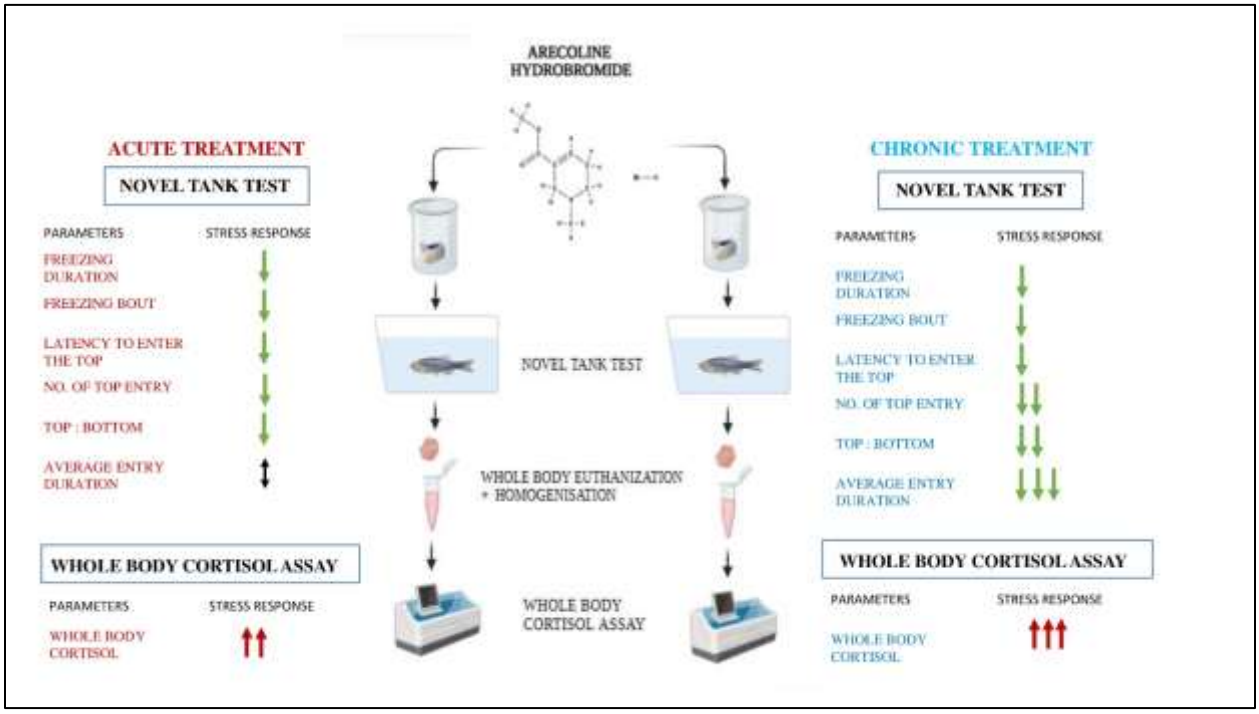
USA], data analysis was carried out with significance set at $p<0.05$. Each treatment group had 5 fish ($n = 5$). The experiment was technically replicated three times, with individual fish serving as biological replicates for statistical analysis. Following the NTT evaluation, the same fish was promptly collected and processed for whole-body cortisol measurement.

1.3. Whole Body Cortisol Assay

In accordance with accepted guidelines for teleost euthanasia, zebrafish were put to death after completing the novel tank test by submersion in ice-cold water until their opercular movements stopped and their loss of reflexes was verified. To prevent cortisol degradation, the entire fish was collected, and the tissue was quickly processed on dry ice. Using a handheld motorized homogenizer, the entire fish was precisely weighed and homogenized in ice-cold phosphate-buffered saline (PBS, pH 7.4) at a ratio of 1 ml PBS per 100 mg tissue. The remaining assay was carried out using Biomatik EKC31067 ELISA Kit in accordance with M Ramsay et al. (2011) [22].

1.4. Statistical analysis

The non-parametric Kruskal-Wallis test and Dunn's multiple comparisons correction were used to examine the behavioral data from the novel tank test maximally; however, one-way ANOVA and the brown-forsythe-westernach ANOVA test were also employed when necessary to account for group differences. The results of the whole body cortisol assay were studied through one-way analysis of variance (ANOVA) and Tukey's multiple comparison test to determine whether the mean ± standard error of mean (SEM) was significant. The statistical significance level was set at $p<0.05$, and all data are presented as group means ± SEM. Statistical software [Graphpad Prism, USA, version 8.4.2] was used to conduct the analyse



Results

1.5. Freezing duration

Acute arecoline exposure was associated with a reduction in mean freezing duration across all treatment groups relative to the control group (Figure 2A). The greatest decreases were observed at 0.5 µM and 5 µM arecoline, whereas fish exposed to 50 µM also exhibited lower freezing durations than controls. Despite this consistent reduction in freezing behavior, the differences among treatment groups were not statistically significant (Kruskal–Wallis test, $P = 0.1348$). A similar pattern was seen after chronic exposure (Figure 2B). Control fish had the longest freezing length and the greatest degree of variability, whereas all arecoline-treated groups had comparatively shorter freezing periods. The 50 µM and 5 µM treatment groups showed the most notable reductions, but the 0.5 µM group also had a shorter freezing duration than controls. Although the general trend indicated a decrease in freezing behavior after chronic arecoline treatment, the difference did not reach statistical significance.

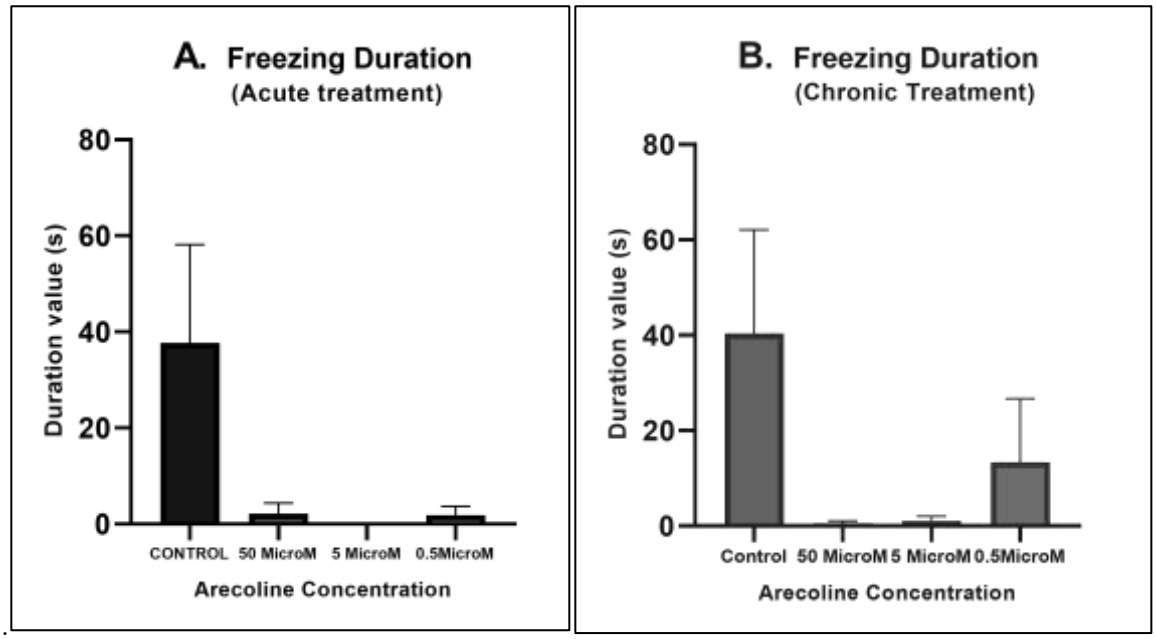


Figure 2. Effect of arecoline exposure on adult zebrafish freezing duration (NTT). **(A)** After acute arecoline treatment at the indicated concentrations (Control, 50 µM, 5 µM, and 0.5 µM), the freezing time (seconds) was recorded during the novel tank test. **(B)** After long-term exposure to arecoline, the freezing time was measured. Mean±SEM is shown by bars. Although groups treated with arecoline tended to exhibit shorter freezing times than controls, the Kruskal-Wallis test did not find statistical significance for these reductions across doses ($p>0.05$).

1.6. Freezing bout

Under both acute and chronic settings, arecoline therapy reduced the mean freezing bout frequency (Figures 3A and 3B). In both experimental conditions, the control group had the highest mean freezing bout frequency, while the arecoline-treated groups had lower values, with a progressive decrease as concentrations increased.

In case of acute arecoline exposure, it did not significantly impact freezing bout frequency, despite a persistent trend (one-way ANOVA: $F = 1.631$, $P = 0.2138$, $R^2 = 0.1966$). Variance homogeneity was assessed using the Brown-Forsythe and Bartlett tests, and no significant variations in variance were found among treatment groups. A similar pattern was observed after chronic treatment, with freezing bout frequency remaining lower in arecoline-treated fish than in controls. However, there were no statistically significant variations in group means (one-way ANOVA: $F = 1.263$, $P = 0.3140$, $R^2 = 0.1592$). The Brown-Forsythe test found comparable variances among groups; however, Bartlett's test revealed considerable heterogeneity in variance ($P = 0.0001$), indicating differential variability among the chronic treatment groups.

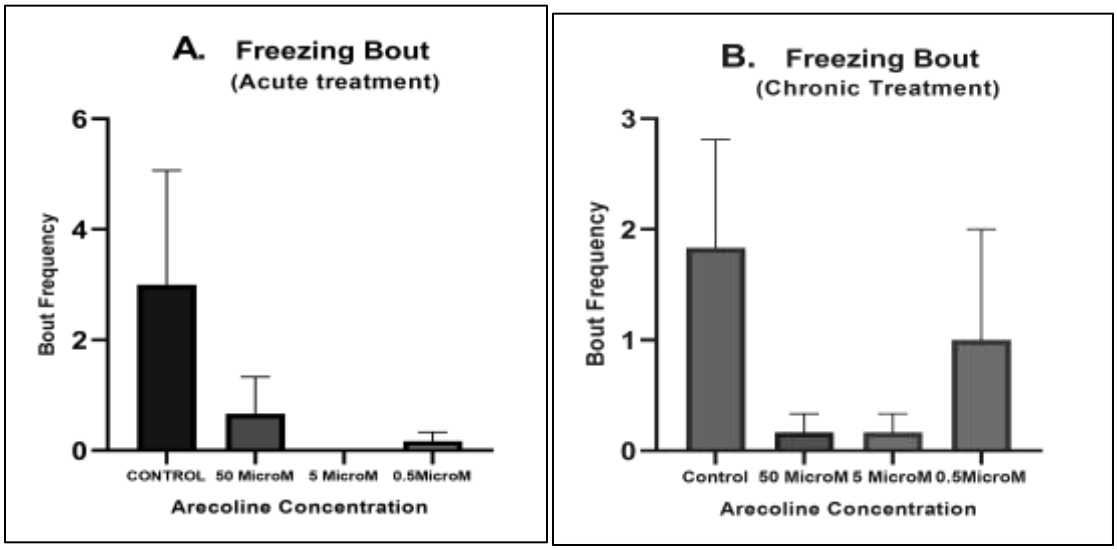


Figure 3. Effect of arecoline exposure on freezing bout (NTT) in adult zebrafish. **(A)** Freezing bout (seconds) was measured during the novel tank test following acute arecoline treatment at indicated concentrations - control, 50 µM, 5 µM, 0.5 µM. **(B)** Freezing duration was assessed after chronic arecoline exposure. Bars represent mean ± SEM. While arecoline-treated groups tended to show reduced freezing durations compared to controls, these reductions did not reach statistical significance throughout doses in the one-way ANOVA ($p > 0.05$).

1.7. Latency to enter the top

There were no significant changes in latency to enter the top between the arecoline treatment groups in either acute or chronic exposure scenarios. In both acute ($F = 1.494$, $P = 0.2465$, $R^2 = 0.1831$) and chronic ($F = 2.230$, $P = 0.1161$, $R^2 = 0.2507$) situations, ordinary one-way ANOVA revealed no significant effects. The Brown-Forsythe and Bartlett tests both failed to reject the null hypothesis of variance homogeneity (all $P > 0.05$). A bar graph visualization revealed a tendency toward lower latency values in arecoline-treated groups compared to the control, with the most apparent reduction at the lowest dose. However, neither of these increases reached statistical significance under acute or chronic settings.

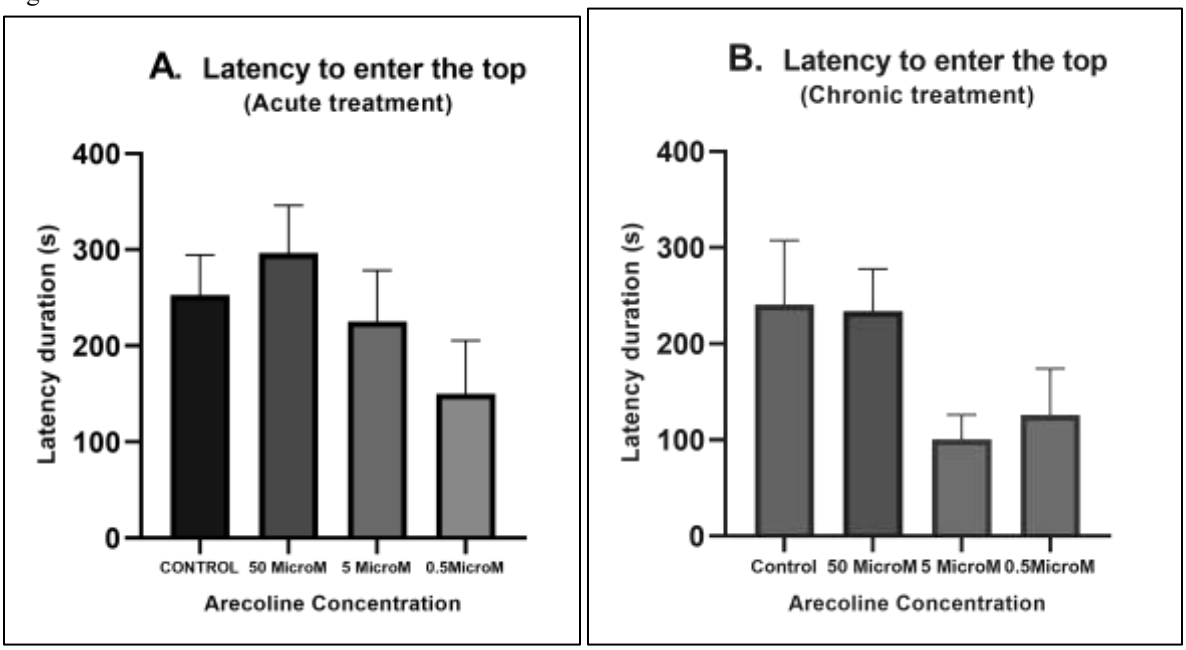


Figure 4. Effect of arecoline exposure on latency to enter the top during the novel tank test in adult zebrafish. **(A)** Latency duration (seconds) was recorded during the novel tank test after acute arecoline treatment at control, 50 µM, 5 µM, and 0.5 µM concentrations. **(B)** Latency to enter the top was assessed post-chronic arecoline exposure at the same doses. Bars represent mean ±SEM. Statistical analysis using ordinary one-way ANOVA showed no significant differences between the control and arecoline-treated groups for both acute & chronic paradigms.

1.8. Number of top entries

The effect of arecoline treatment on the number of top entries was assessed using Kruskal-Wallis tests for both acute and chronic exposure paradigms. Acute treatment showed a trend toward increased top entries at 0.5 µM arecoline, but differences among group medians did not reach statistical significance [$P = 0.0740$, Kruskal-Wallis statistic = 6.934]. On the other hand, chronic exposure to arecoline is associated with a significant rise in top entries [$P = 0.0140$, Kruskal-Wallis statistic = 10.61]. Both the 5 µM and 0.5 µM groups showed significantly more top entries than the control group [$P = 0.0473$], according to post hoc Dunn's multiple comparisons. Therefore, a dose-dependent increase in vertical exploration was brought about by persistent arecoline administration, with significant variations seen at higher dosages. In comparison to controls during chronic exposure, bar graphs show noticeably greater top entries at 5 µM and 0.5 µM. Although exhibiting a similar pattern, the acute data was not statistically significant. All of these results suggest that prolonged exposure to arecoline has a significant impact on vertical exploration behavior.

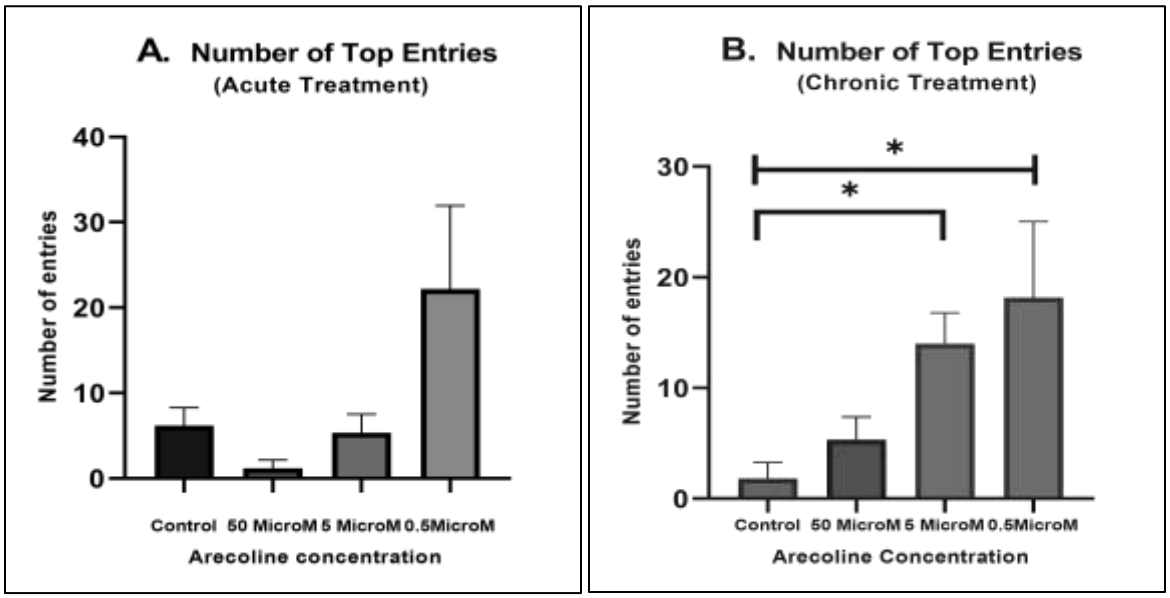


Figure 5. Effect of arecoline exposure on the number of top entries in adult zebrafish during the novel tank test. **(A)** The number of top entries was measured following acute arecoline exposure at control, 50 µM, 5 µM, and 0.5 µM. Acute treatment showed a trend toward increased top entries at 0.5 µM arecoline, but differences among group medians did not reach statistical significance (P = 0.07; Kruskal-Wallis statistic = 6.93). **(B)** Chronic arecoline exposure significantly increased top entries in a dose-dependent manner (P = 0.001, Kruskal-Wallis statistic = 10.61). Post hoc dunn's test showed both 5 µM and 0.5 µM groups had significantly (*P<0.05) more top entries than the control (P = 0.04).

1.9. Top to bottom ratio

Acute arecoline administration resulted in an increasing trend in the top-to-bottom ratio with increasing concentration, with the greatest ratio observed at 0.5 µM arecoline. Statistical analysis using the Kruskal-Wallis test did not disclose significant differences among group medians [P = 0.2379; Kruskal-Wallis statistic = 4.228]. Chronic arecoline exposure produced a statistically significant increase in the top: bottom ratio among the groups [P = 0.0140; Kruskal-Wallis statistic = 10.61]. Post hoc multiple comparisons indicated that both 5 µM and 0.5 µM concentrations were significantly higher than the control. Longer-term exposure to arecoline has a significant impact on vertical exploration, according to these studies, with higher dosages significantly raising the top-to-bottom ratio. With the chronic 5 µM and 0.5 µM arecoline groups exhibiting the largest top-to-bottom ratios in comparison to the control, bar graph visualisation validates the statistical results.

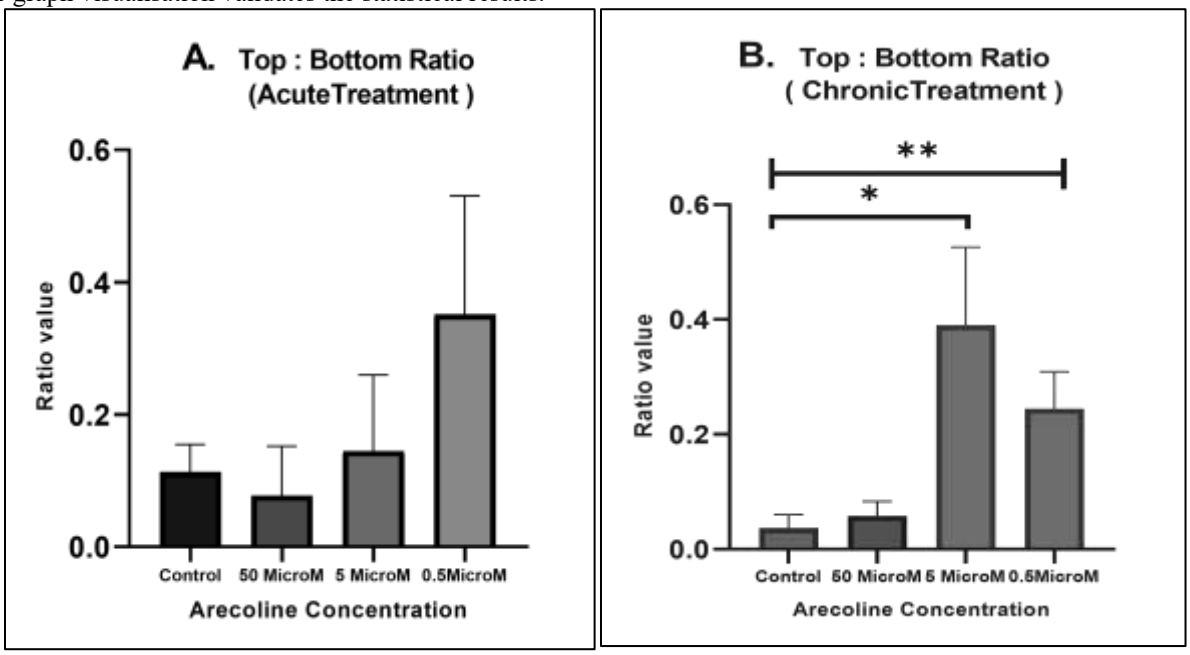


Figure 6. Effect of arecoline exposure on the top : bottom ratio in adult zebrafish during the novel tank test. **(A)** Acute exposure produced a non-significant increasing trend, with the highest ratio at 0.5 µM; However, differences did not reach statistical significance (P = 0.23, Kruskal-Wallis statistic = 4.22). **(B)** Chronic arecoline treatment resulted in a statistically significant, dose-dependent increase in the top: bottom ratio (P = 0.01, Kruskal-Wallis statistic = 10.61).

Post hoc multiple comparisons revealed that both the 5 µM and 0.5 µM groups exhibited significantly higher ratios than the control (**P≤0.01; *P≤0.05).

1.10. Average entry duration

Acute arecoline exposure had no significant effect on average entry duration in the Novel Tank Test (Figure 7A and 7B). Fish from all treatment groups had similar average entrance durations, and no significant differences were found between group medians (Kruskal-Wallis test: H = 1.112, P=0.7741).

In contrast, chronic arecoline administration had a substantial effect on average entry duration (Figure 7B). The Kruskal-Wallis test revealed a significant difference between treatment groups (H = 12.09, P = 0.0071). Fish treated with 5 µM and 0.5 µM arecoline had considerably longer average entry durations than control fish, according to post hoc analysis. Furthermore, treated groups had longer entry lengths than controls, with the effect most pronounced at lower treatment concentrations. These findings show that persistent arecoline exposure alters exploratory behavior in adult zebrafish.

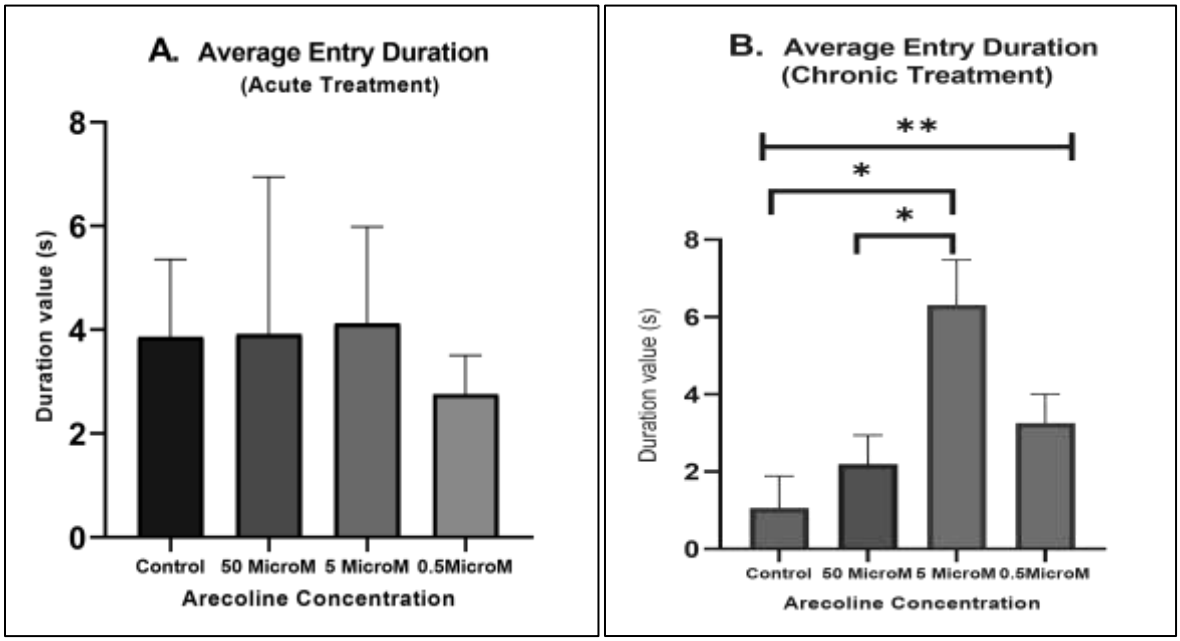


Figure 7. Effect of arecoline exposure on average entry duration in adult zebrafish during the novel tank test. **(A)** Acute arecoline administration did not produce significant differences in average entry duration across experimental groups, with Kruskal-Wallis analysis confirming the lack of statistically significant variation among medians (P = 0.77, Kruskal-Wallis statistic = 1.11). **(B)** In contrast, chronic arecoline exposure produced a dose-dependent increase in average entry duration. Statistical analysis demonstrated significant group differences (P = 0.0071, kruskal-wallis statistic = 12.09), with the 5 µM and 0.5 µM groups showing significantly longer durations than the control (**P≤0.01; *P≤0.05).

1.11. Whole body cortisol assay

Both acute and chronic arecoline exposure had a substantial dose-dependent effect on whole-body cortisol levels. Cortisol levels in the acute treatment group increased significantly from 1.7ng/g in controls to 4.75 ng/g after exposure to 50 µM arecoline ($p<0.05$). Lower dosages (5 µM and 1.25 µM) showed modest increases equivalent to the control. Cortisol levels in chronically treated fish rose from 3.0 ng/g in controls to 7.2 ng/g in the 50 µM group ($p<0.01$). Cortisol levels were substantially higher at intermediate dosages (5 µM) compared to lower doses and controls (3.8 ng/g) ($p<0.01$). Lower chronic dosages (0.5 µM and 0.05 µM) resulted in cortisol levels similar to those in the control group, showing a threshold effect. The results show that both acute and chronic arecoline treatments cause considerable increases in stress hormone release, with chronic exposure resulting in larger cortisol elevation.

Both acute and chronic arecoline exposure significantly increased whole-body cortisol levels in a dose-dependent manner, as shown by one-way ANOVA [acute: (P = 0.0170); chronic: (P = 0.0012)] and post hoc analyses that confirmed significant differences between means. In the acute treatment group, 50 µM of arecoline considerably elevated cortisol concentrations relative to controls, while lower dosages resulted in moderate elevations. Chronic treatment resulted in significantly higher cortisol levels than controls at 50 µM and 5 µM. Post-hoc analysis confirmed the group-wise significance. These outcomes were not due to differences in variance (all Bartlett's and brown-forsythe tests [P>0.05]), confirming hormonal stress axis activation as a strong effect of arecoline exposure in the two paradigms.

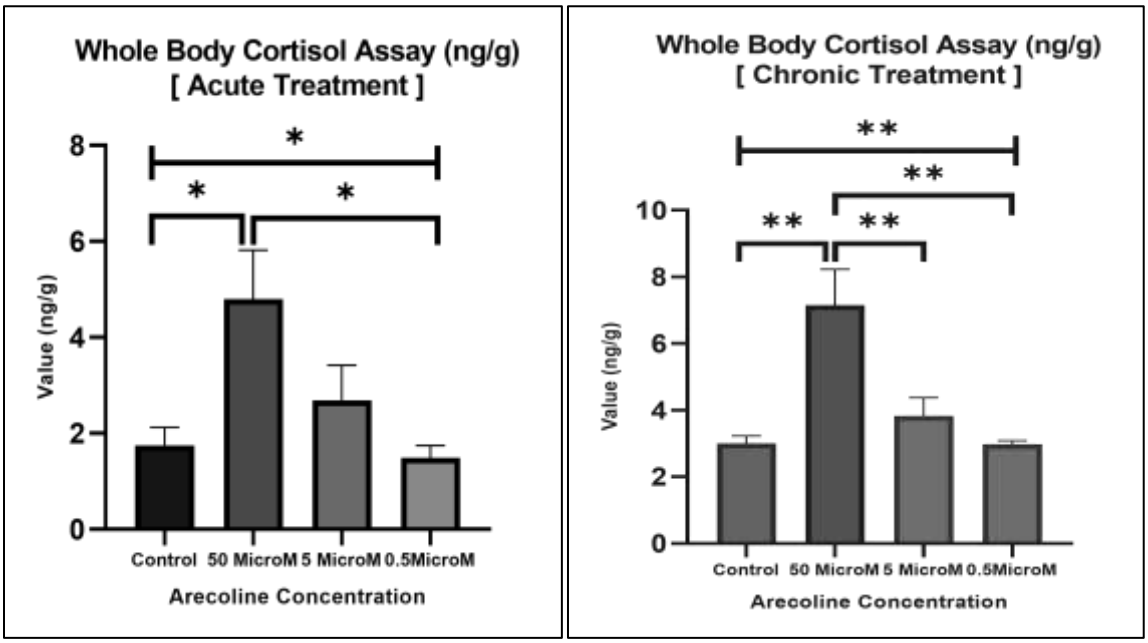


Figure 8. Effect of arecoline exposure on whole body cortisol assay in adult zebrafish. **(A**) Acute arecoline exposure produced a significant and dose-dependent increase in whole body cortisol, with 50 µM resulting in a marked increase from baseline control. It also showed significant dose dependency with a 0.5 µM dosage. Further, all the treated doses collectively showed a significant difference from the control. **(B)** Chronic arecoline exposure treatment elicited a dose-dependent response of even greater significance. The 50 µM dose showed the greatest significant elevation compared to control, while all treated doses collectively differed significantly from control. Dose-dependent significance is also found between the treated doses (**P≤0.01; *P≤0.05).

Discussion

The current work demonstrates that arecoline exposure can impact both behavioral and endocrine stress responses in adult zebrafish. In the novel tank test, arecoline administration generated anxiolytic-like behavioral effects, while whole-body cortisol concentrations increased after both acute and chronic exposures. These findings imply that behavioral and endocrine stress responses may not necessarily change simultaneously, raising the potential of a partial dissociation between these two components of the vertebrate stress response system.

As per the experimental study, arecoline was administered at concentrations of 50, 5, and 0.5 µM under acute (30 minutes) and chronic (7 days) dosing paradigms, following pilot trials. In both acute and chronic scenarios, arecoline reduced stress or anxiety-like metrics such as freezing duration, freezing bouts, latency-to-enter-the-top, number of top entries, top-to-bottom ratio, and average entry time via the Novel Tank Test. Among these, the number of top entries, the top-to-bottom ratio, and the average entry duration were shown to be statistically significant under chronic exposure. These experimental effects aligned with a previous rodent-based study in which arecoline's anxiolytic potential was evaluated at the chronic unpredictable mild stress (CUMS) model [10]. The engagement of muscarinic receptors, especially the partial mAChR agonist potential of arecoline (M1, M2 receptors), was concluded as the possible mechanistic reason for that outcome.

In contrast to the behavioral findings, whole-body cortisol concentrations were significantly elevated following both acute and chronic arecoline exposure. Because cortisol is the principal glucocorticoid hormone regulated by the hypothalamic–pituitary–interrenal (HPI) axis in teleosts, the observed increase is consistent with activation of endocrine stress pathways. The simultaneous occurrence of anxiolytic-like behavior and elevated cortisol represents the most notable finding of the present study and suggests that behavioral and endocrine components of the stress response may not always change in parallel.

One of probable explanations for this paradoxical consequence is cholinergic modulation of the HPI axis itself. Acetylcholine and muscarinic receptors are reported to be functional at hypothalamic regions, where they can modulate the secretion of corticotropin-releasing factor (CRF) [24]. Therefore, partial agonism of mAChR in the hypothalamus reduces limbic anxiogenic circuitry while activating CRF-driven adrenocorticotropic (ACTH) hormone release from the pituitary, resulting in cortisol biosynthesis in interrenal tissues [25]. Although the present study did not investigate CRF, ACTH, or receptor-specific mechanisms, but the differential distribution of mAChR from the limbic system to hypothalamic regions can be a reason of the real-time co-occurrence of behavioral relaxation and cortisol spiking [26]. Therefore, the explanation states that the behavioral and endocrine stress pathways are distinct, and a previous study on mammalian systems also supported this conclusion. In that study, the dissociation of behavioral and endocrine stress parameters, especially the glucocorticoid dysregulation, was reported in the case of certain anxiolytic as well as antidepressant treatments [27]. The present study expands on this concept in a non-mammalian animal model using an alkaloid exposure system while also widening the scope to compare the vertebrate

behavioral endocrine system. Arecoline has also been shown to interact with nicotinic acetylcholine receptors (nAChRs) at higher concentrations, and these receptors are also a potent HPA / HPI axis activator across all vertebrate species [28]. Therefore, there is an important mechanistic possibility that the utilized dose range or the highest experimental dose (50 μM) may stimulate nAChRs and contribute to the ultimate cortisol rise. It is also noteworthy that the cortisol elevation reported in this study differs from previous rodent-based studies, in which arecoline was associated with glucocorticoid stability, suggesting species-specific differences in mAChR/nAChR distribution, interrenal versus adrenal sensitivity, or arecoline metabolism across vertebrate taxa [10].

This dissociated variation of behavioral and endocrine pathways has major translational implications beyond the zebrafish model, too. Apart from the endocrine context, Arecoline is also documented as pro-cognitive, antidepressant, anti-inflammatory, antioxidant, and anti-helmintic in human and non-human experimental settings [29,30,31]. Aside from its biological significance, areca nut, the fruit from which arecoline is extracted, has gained cultural, ritualistic, and social significance among Southeast Asian communities, where its use is deeply embedded in ceremonial identification, communal practice, and traditional medicine. However, cortisol escalation via possible mAChR- or nAChR-mediated mechanisms can't be dismissed, but in practice, it causes a silent co-occurrence with subjective behavioral relaxation, leaving users physiologically vulnerable to immunosuppression, carcinogenic threat, cardiovascular burden, metabolic disruption, or hippocampal damage without any symptomatic awareness [32,33,34]. The main challenge, therefore, is not only to determine the harm-versus-benefit ratio but also to determine exposure thresholds, delivery matrices, and preparation methods. These measures may allow the beneficial effects to be retained while reducing the neuroendocrine dysregulation, including the cortisol elevation reported in this study as well as associated risk of carcinogenicity with chronic oral areca nut consumption. Therefore, sound translational research on areca alkaloids and their applications is important, as it can lead to effective public health policies rather than existing narratives that have failed to prevent overconsumption in high-prevalence countries.

However, some limitations must be acknowledged when interpreting the current findings. Only whole-body cortisol concentrations were measured, and upstream regulators of the HPI axis such as CRF and ACTH were not evaluated. Furthermore, receptor-specific mechanisms were not explored with muscarinic or nicotinic antagonists, and molecular markers linked with stress-axis activation were not assessed. As a result, the specific mechanisms behind the simultaneous occurrence of anxiolytic-like behaviour and high cortisol levels remain unexplained. Future research combining receptor-selective pharmacological approaches, gene expression analyses, neuroanatomical mapping, and post-exposure cortisol recovery assessments will be useful in clarifying the pathways responsible for the observed behavioral-endocrine dissociation and improving the understanding of cholinergic regulation of vertebrate stress physiology.

Conclusion

In conclusion, arecoline exposure produced anxiolytic-like behavioral effects while also elevating whole-body cortisol levels in adult zebrafish. The presence of behavioral anxiolysis as well as endocrine stress activation shows that cholinergic modulation can influence the behavioral and physiological components of the stress response differently. These findings emphasize the necessity to integrate behavioral and endocrine endpoints when evaluating neuroactive drugs and provide additional evidence that stress-related behavioral and hormonal responses do not always change in parallel. The present study contributes to comparative stress physiology by demonstrating a potential dissociation between behavioral and endocrine stress responses in a non-mammalian vertebrate model and also provides a foundation for future mechanistic investigations of cholinergic regulation of the stress axis.

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Data Availability Statement

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Author contributions statement

Koushik Mondal: Conceptualization, Methodology, Investigation, Data curation, Formal analysis, Visualization, Writing of original draft.

Dr. Praveen Kumar Sharma: Supervision, Resources, Project administration, Review & editing, Validation.

All authors contributed to the manuscript and approved the submitted version.

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Competing interests policy

The authors declare no competing interests.

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