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Original Paper

Effect of incremental salinity on survival and behaviour of zebrafish (*Danio rerio*)

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Abstract

Freshwater ecosystems in coastal regions are occasionally flooded with saline water due to tidal, seasonal and catastrophic water movements, thereby affecting physiology and behaviour of freshwater fishes. The aim of the present study was to determine the tolerance limit of salinity in zebrafish as a freshwater fish model, to characterize and quantify their behavioural response under mild to high incremental salinity exposure. Wild-type zebrafish (*Danio rerio*) individuals were maintained and exposed from fresh water to increasing NaCl concentration up to 20 g L^{-1} with a repeated exposure schedule for 96 h. Observation was made per tank for their mortality and for cumulative behavioural patterns under defined time frames. The estimated LC_{50} value was 14.122 g L^{-1} NaCl. Abrupt shifts of behavioural expression were found from 10 g L^{-1} of NaCl, with predominant stress movements followed by aggression and fighting as well as diminishing feeding and playing movements. The incremental salinity concentration and exposure time both were found to control the key behavioural indicators in a defined pattern.

Key words: behaviour, LC_{50}, NaCl, salinity, stress, zebrafish.
Abbreviations: CNI, cumulative number of individuals.

Introduction

Coastal freshwater ecosystems are fragile due to the risk of salinity contamination in several ways, which is a serious threat to their sustainability. Three possible factors contribute to the increasing salinity in inland freshwater ecosystems and one of them is the increasing sea level (Sherin et al. 2020). The most severe threat in South Bengal is increasing salinisation due to sea level rise with a rate 4.5 mm per year over the period of 2013 to 2021 (Dasgupta et al. 2017). Successive rise of sea level is causing slow compounding intrusion of saline water in the inland freshwater ecosystems (Mitra et al. 2009; Banerjee 2013; Shammi et al. 2017), which is expected to increase further in the next decade (Brown et al. 2018). Due to the risk of increasing salinisation of freshwater habitats around the globe (Kantamaneni et al. 2022), different studies have examined the effects of increasing salinity on freshwater organisms. These include reports of mortality of different organisms (Beatty et al. 2011; Struewing et al. 2014), of life cycle traits like reduced growth and reproduction rate (Ghazy et al. 2009; Simmon et al. 2012), metabolic costs (Tyree et al. 2016) or even effects on survivability of eggs and embryogenesis (Farhana et al. 2019). However, there have been few studies on the effect of increasing salinity on the behaviour of fishes. Effect of salinity on fish may be described by the diffraction of responses shown by fish exposed with different concentrations of salinity (Peterson, Meader 1994).

Several definite behavioural patterns can be observed among fish, which have been previously studied and recorded (Sih et al. 2015; Audira et al., 2020). Behaviours like shoaling, playing, courtship, resting, feeding, aggression and fighting, and stress movement etc. are primarily behavioural patterns, which mostly can be characterized and monitored in zebrafish (Kalueff et al. 2013). Behavioural patterns of zebrafish, like shoaling, i.e., movement of five and more fish towards same direction, aggression or stress movements have been observed and analysed under variable experimental and environmental conditions (Pitcher et al. 1998; Lopez et al. 2018; Leite et al. 2022; Zidan et al. 2022). However, the effect of salinity on freshwater fish behaviour is a novel area to investigate under mild salinity to brackish to marine salinity conditions, along with estimation of physiological tolerance limits.

The present work was aimed to determine the effect of

increase in salinity by NaCl with time on the behaviour and physiology of the zebrafish as a freshwater fish model and to provide an understanding on the gradual shift of behaviour during the exposure period of salinity stress. This study can provide understanding of the effect of natural to catastrophic contamination or flooding of coastal freshwater ecosystems with brackish or marine water and subsequent effects on fish populations.

Materials and methods

Animals

The wild-type zebrafish (*Danio rerio* Hamilton) for this experiment was sourced from the West Bengal Livestock Development Corporation Limited, India. In the given experiment, all of the fish were 3 to 4 months old with an average size of 3 cm and the total number of male and female fish in the stocking tank were 100 and 50, respectively, of which 10 were randomly chosen and placed in each of the six experimental tanks for observation.

Experimental system

The aquarium tanks used in the experiment were made of glass, rectangular in shape with stocking tank size of 76.20 × 30.48 × 60.96 cm and experimental tank size of 30.48 × 20.32 × 20.32 cm. The whole experiment was kept under ideal laboratory conditions according to animal care guidelines, in a natural day/night condition with aeration, and were conducted in six tanks filled with a volume of 10 L water each and room temperature of 28 ± 1 °C. The temperature of water used was 27 ± 1 °C with pH 7.8, concentration of dissolved oxygen in water of 3.6 mg L^{-1} and total hardness of 278.22 mg L^{-1}. Each tank was maintained with a supplied oxygen pump and subjected to a natural day/night cycle. Fish were fed with commercial food Tetra-bit Complete (Tetra, Germany) at dosage 2 to 3 mg per g body weight daily at 10:00. Fish were allowed to acclimatize in a stock tank for 10 days and further for two days in experimental tanks to nullify the effect of handling stress and other factors due to transfer, taking note of the gradual acclimatizing trait of zebrafish to a new environment (Spence et al. 2008; Audira et al. 2020).

Salinity exposure

Along with a control tank, five tanks were exposed to NaCl solutions at 9:00 daily for consecutive four days with a gradually increasing concentration of 50, 500, 2500, 3750 and 5000 mg L^{-1} NaCl. After four days of repeated exposure, the final concentration of the five experimental tanks reached 0.2, 2.0, 10.0, 15.0 and 20.0 g L^{-1} NaCl (Fig. 1). According to accepted criteria, salinity exposure 50 to 200 mg L^{-1} represents freshwater, 500 to 2000 mg L^{-1} is mild brackish water, 2500 to 10 000 mg L^{-1} is brackish to mild marine water, 3750 to 15 000 mg L^{-1} is moderately brackish to moderately marine salinity, and 5000 to 20 000 mg L^{-1} is highly brackish to highly marine salinity (Nitonye, Uyi 2017; Sherin et al. 2020). During the whole process of administration of NaCl, the fish were observed carefully in all the six tanks including the control tank to note behaviour change and reaction of fish to the stress.

LC_{50} test

The LC_{50} or lethal concentration for 50% of individuals indicates the concentration of any compound or molecule at which 50% of the test animals face mortality within a restricted period of experiment, generally, within 96 h. In the present study where NaCl was used as the exposure chemical, the time considered for the LC_{50} test was about

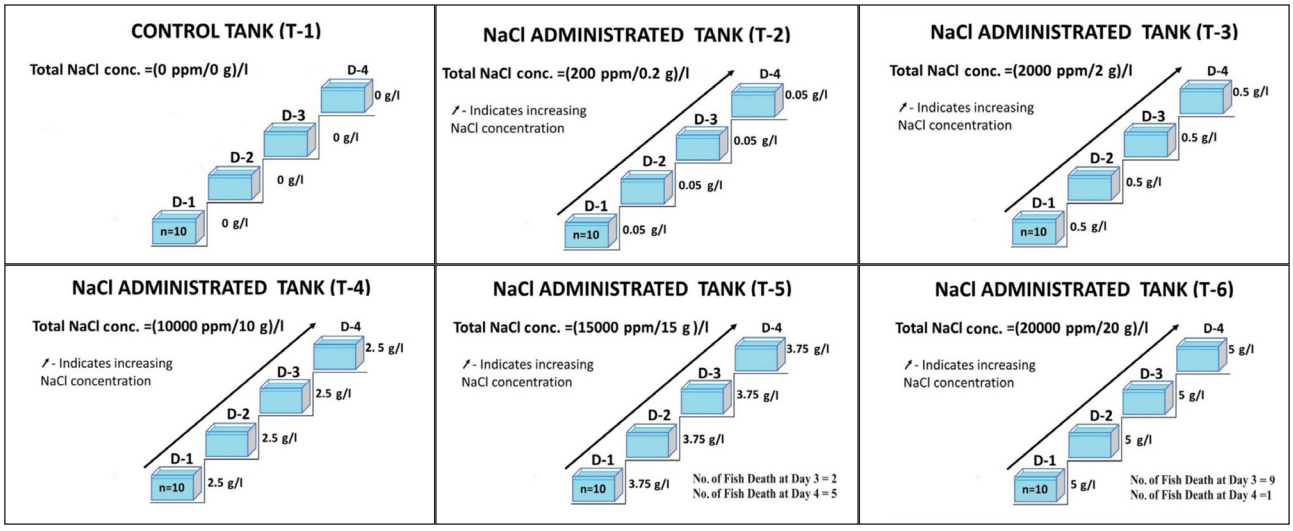

Fig. 1. Pictographic representation of experimental study design for the LC_{50} test of NaCl and behavioural manifestation. First tank (T1) specifies as control, while rest five tanks (T2 – T6) are exposed with different concentrations of NaCl repeatedly in consecutive 4 days at 24 h interval. Initial zebrafish number was 10 in each tank. Initial NaCl concentration and day-wise increment, final concentration, number of fish death with days are depicted in the diagram.

96 h. The LC_{50} value was determined by using the probit analysis tool in SPSS software, while the recoded death and survival of individuals in each 4-h time segment was estimated by a Kaplan-Maier survival curve (Fig. 2). In Kaplan-Maier survival curve, cumulative survival indicates the number of living fish at different times of the experiment, while censored data indicate the number of living fish in each 4 h of the time period.

Study of behavioural parameters

The behavioural study of zebrafish was conducted in control and experimental tanks for four consecutive days in morning, early afternoon, late afternoon and evening, for 1.5 h at each time. Within each 1.5-h period, 18 time segments were considered with a duration of 5 min each. The times periods of observation were: morning between 9:00 and 10:30, early afternoon between 12:00 and 13:30, late afternoon between 15:00 and 16:30, and evening between 18:00 and 19:30. During these time periods, the zebrafish were exposed to NaCl at 9:05 and fed at 10:00. The behaviour of fish was observed and noted for each 5-min time segment accompanied by digital capture. In the present study, shoaling, playing, courtship, resting, feeding, aggression and fighting, and stress movement were noted (Spence et al. 2008; Leite et al. 2022; Zidan et al. 2022). Aggregation of a group of fish consisting of four to 10 individuals, which moved gradually from one corner to another corner or zone of the tank, was considered as shoaling. Freely moving condition of fish in any zone of the tank, without showing any hyper activity, was noted as playing behaviour. A key feature was courtship behaviour, seen as reduced distance among individual fish and circular movement or chasing of each other non-aggressively. Staying at the bottom of the tank without any movement was recorded as resting behaviour. Engulfing or nibbling of food particles as well as foraging and chewing at the bottom in search of food were recorded as feeding behaviour. Hyperactive movement towards each other, chasing and biting were recorded as a signature feature of aggression and fighting, while hyper movement in a zig zag manner without any direction or target as well as static phase of fish observed at the top surface of water were recorded as stress movement (Pitcher et al. 1998; Barreto et al. 2009; Kalueff et al. 2013; Lopez et al. 2018; Eisenbeiser et al. 2022). Death of fish was also recorded, and fish death was considered as the highest level of stress in data interpretation.

The cumulative number of individuals (CNI) was used for behavioural quantification where CNI designated the sum of number of individuals with a behaviour in the observable time unit of 5 min over a 90 min period slot.

Data analysis

Behavioural observations were represented graphically by CNI values using SPSS (Version-22.0.0.0), Stanford, USA, to portray daily sequences under control and experimental conditions. The non-parametric Mann-Whitney U Test was used to determine significant differences between concentrations of NaCl and control conditions for the observed behaviour (Audira et al. 2018). Effect of NaCl concentration on behaviour of individuals in five-minute intervals of each 90-min time period were displayed graphically as CNI using dot-plots in GraphPad Prism and level of significance was determined by the pairwise comparisons tool (for significance $p < 0.001$ or $p < 0.05$).

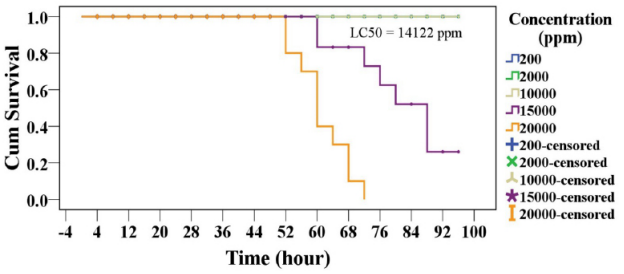

Fig. 2. The Kaplan-Meier Survival analysis curve of *Danio rerio* under the administration of different concentration of NaCl. Presence of total number of fish in a tank is represented as cumulative survival on the Y axis. On the X axis the whole-time frame of the experiment is presented, while the count of fish was recorded in each 4th h. The censored data indicates the number of fish survived at least for 4 h of observation.

Results

LC_{50}

Among the five NaCl treatments, death of fish was observed only in the two that had 15 000 mg L^{-1} and 20 000 mg L^{-1} final concentration. In the 15 000 mg L^{-1} NaCl treatment, two fish were found dead on Day 3 (when the concentration of NaCl was 11 250 mg L^{-1}), and five fish were found dead at Day 4 of observation (during which the concentration of NaCl was 15 000 mg L^{-1}) leaving only three zebrafish alive at Day 4. In the treatment with 20 000 mg L^{-1} final concentration of NaCl, nine fish were found dead within 48 to 68 h (concentration of NaCl was 15 000 mg L^{-1}) and the last fish was found dead at 72 h as soon as the NaCl concentration reached 20 000 mg L^{-1} (Fig. 2). The LC_{50} value of NaCl on zebrafish was calculated to be 14 122 mg L^{-1}, and the mortality of fish represented by Kaplan-Meier survival curve is shown in Fig. 2.

Behavioural changes

Significant behavioural changes were observed within and between consecutive days. Mortality was very high and abrupt after 48 h in the extreme salinity treatment; hence, the behavioural observation was discontinued. Therefore behaviour was analysed for the other treatments: 50 to 200, 500 to 2000, 2500 to 10 000 and 3750 to 15 000 mg L^{-1} NaCl.

In all four days of each observation period, control and 50 to 200 mg L^{-1} NaCl exposed fish maintained almost

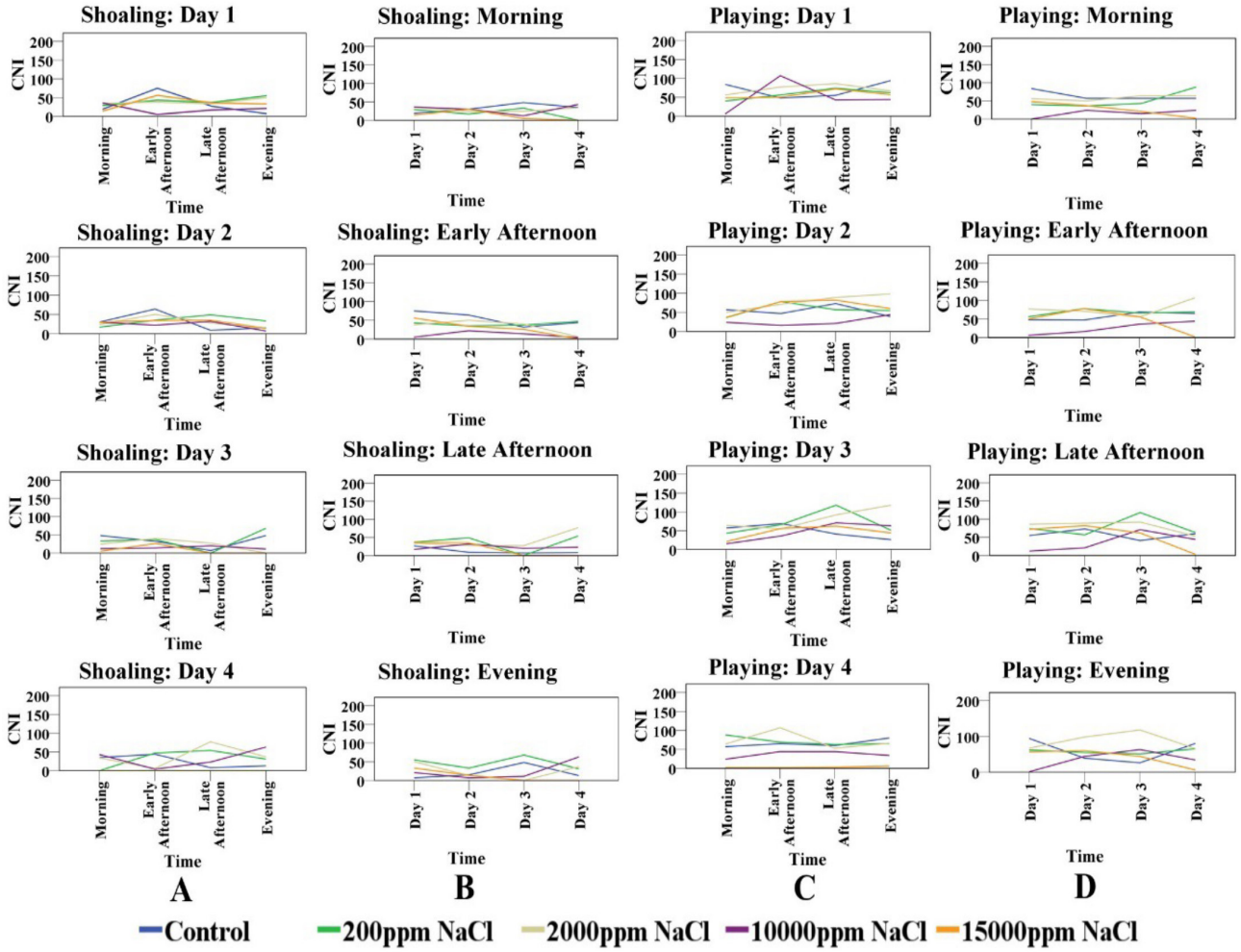

Fig. 3. Pattern of shoaling and playing behaviourof *Danio rerio* in different experimental condition under different exposure concentration. A and C indicates day-wise fluctuation while B and D represents diurnal fluctuation of shoaling and playing behaviour, respectively.

similar trends of shoaling behaviour, but those exposed to 3750 to 15 000 mg L^{-1} NaCl showed a decreasing trend from Day 1 to Day 4 of the experiment, with a sudden spike in the evening of Day 4 in fish exposed to 2000 to 10 000 mg L^{-1} NaCl (Fig. 3). In early afternoon and late afternoon, fish exposed to higher concentration showed a decreasing trend of shoaling behaviour with passing each day. During late afternoon, at lower NaCl concentration, fish showed increasing shoaling behaviour from Day 3. Moreover, a significant decreasing trend of shoaling was observed in 15 000 mg L^{-1} during the evening period of each day (Fig. 3). Fish in control conditions tended to show high shoaling activity during the early afternoon period and a slow decrease in shoaling towards the evening throughout the four days of observation. On Day 1, fish in 2000 to 10 000 mg L^{-1} NaCl exhibited the lowest shoaling activity during early afternoon and in other concentrations there were no significant differences throughout the day. Similarly, on Day 2, fish in all of the three higher concentrations showed decreasing shoaling behaviour towards the evening period. On Day 3, fish at 50 to 200 mg L^{-1} NaCl showed a sudden spike in shoaling behaviour in the evening, as in the control, but at other concentrations there was a decreasing trend towards evening. Furthermore, on Day 4, there was an absence of shoaling throughout the day in the 3750 to 15000 mg L^{-1} treatment (Fig. 3).

Playing was a prominently observed behaviour in the whole experimental period, while the variation of playing in different concentrations of exposure in each day of experiment was also visible. At lower exposure, playing showed a similar trend from the beginning to end of the experiments to that for the control. Additionally, playing at lower NaCl concentration increased from morning to evening. In higher concentrations of NaCl, exposed fish behaved differently from the others; on the last day of experiment both of the groups of fish exposed to higher concentration showed a reduce trend of playing, and remarkably in 15 000 mg L^{-1} NaCl when playing nearly ceased (Fig. 3).

In the control, playing behaviour was maximum in

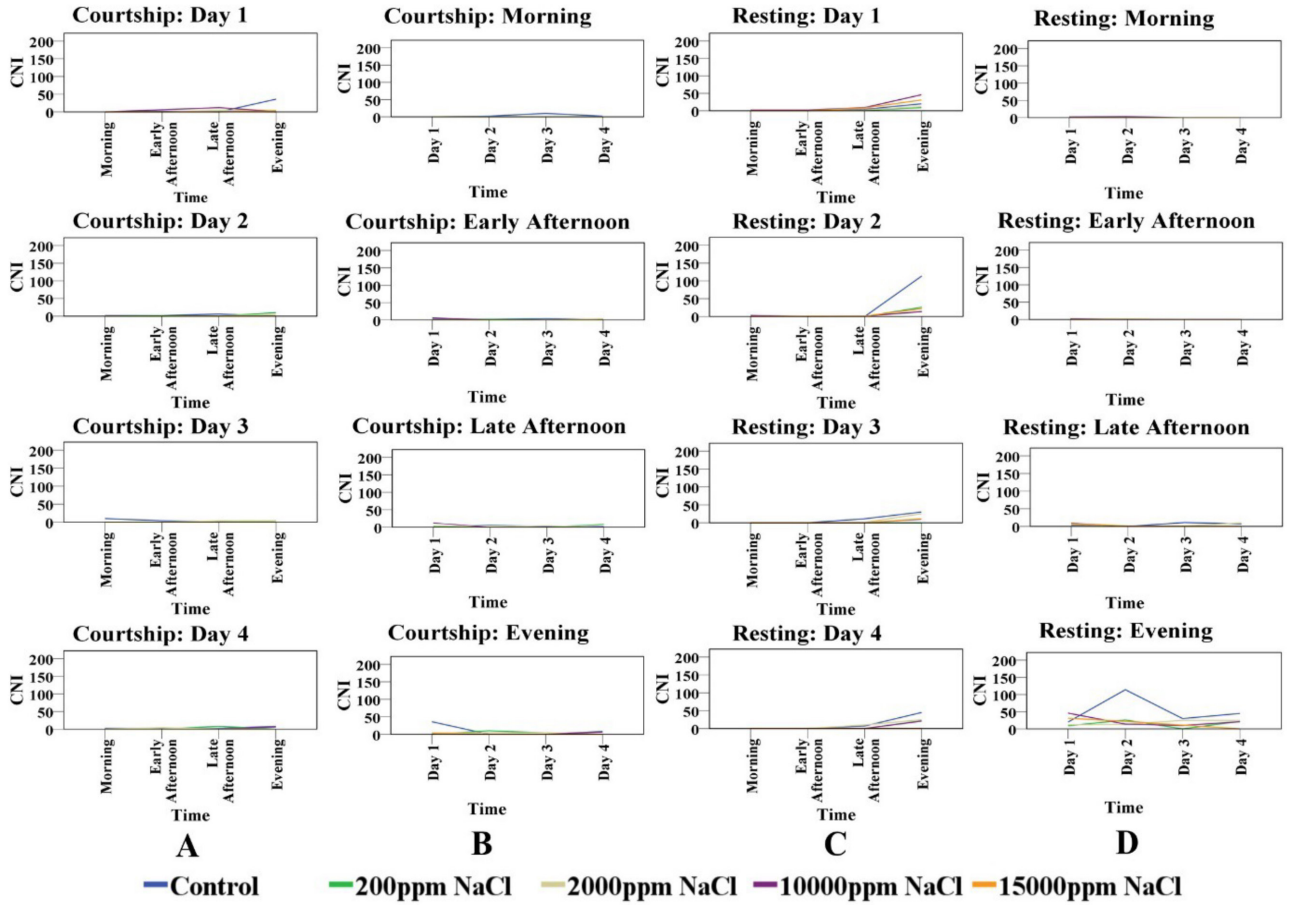

Fig. 4. Courtship and resting behavioural patterns of *Danio rerio* under different NaCl concentration compared among groups. A, day-wise fluctuation of courtship pattern; B, cumulative diurnal fluctuations of courtship; C, day-wise fluctuation of resting pattern; D, cumulative diurnal fluctuation of resting pattern.

morning and evening on Day 1, but for other groups playing was prominent in morning and decreased towards the end of the day. On Day 2, fish in 500 to 2000 and 2500 to 10 0000 mg L^{-1} treatments showed significant increase of playing behaviour in late afternoon and the others showed average playing movements. A similar pattern was observed on Day 3, except in the 3750 to 15 000 mg L^{-1} treatment, where a further decrease was occurred. Very low and almost negligible playing was observed for both 2500 to 10 000 and 3750 to 15 000 mg L^{-1} treatments on Day 4, while in the control and lower concentrations average playing behaviour was observed (Fig. 3).

In morning, excepting in the control, all salinity exposed groups showed no courtship throughout the experimental days (Fig. 4). In the evening, fish in the control tank showed several courtship behaviour events, but the salinity exposed groups showed very little or negligible courtship. Although courtship was observed as the least prominent behaviour in this study, it was also the most affected under mild to high salinity condition for zebrafish.

Almost complete absence of resting was found in both morning and early afternoon for four days with a marginal increase at higher concentrations for the first two days. During late afternoon, fish in the control and 50 to 200 mg L^{-1} treatment exhibited resting on almost every day, and 500 to 2000, 2500 to 10 000 and 3750 to 15 000 mg L^{-1} exposed groups also showed resting in evening throughout the period (Fig. 4). Therefore, prominent resting behaviour was found only in the evening from Day 1 to Day 4. However, the exposed groups showed a gradual decrease of resting with increasing days, which was most prominent in the 2500 to 10 000 and 3570 to 15 000 mg L^{-1} treatment, and there was no resting in Day 4 in the 15 000 mg L^{-1} treatment, indicating a prominent shift of this behaviour (Fig. 4).

A general trend of feeding was observed throughout the day. Between days, mild to moderate fluctuations in control and exposed groups up to 2500 to 10 000 mg L^{-1} were observed (Fig. 5). However, a prominent reduction of food intake was observed in Day 1 and Day 2 for 3750 to 15 000 mg L^{-1} exposed zebrafish and they completely ceased to feed on Day 4. Comparing feeding behaviour in a diurnal cycle from Day 1 to Day 4, feeding throughout the day with related movements were common. However,

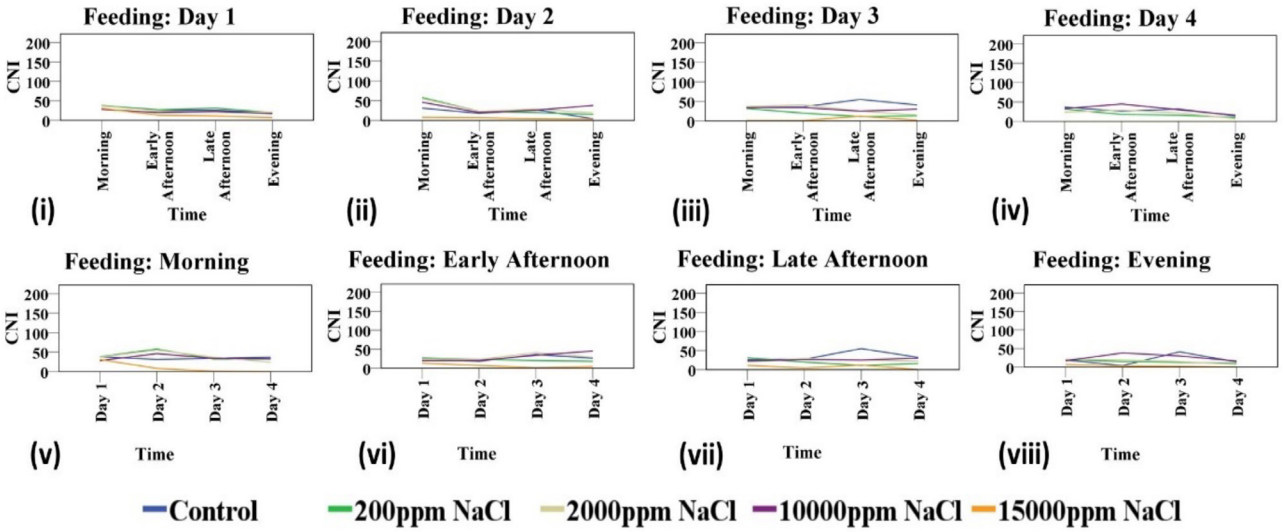

Fig. 5. Feeding behaviourof *Danio rerio* under different NaCl concentration compared among groups i – iv that represents day-wise fluctuation of feeding pattern, and v – viii that represents diurnal fluctuations of feeding.

the magnitude of feeding behaviour gradually decreased with days for the salinity exposed groups. We observed a prominent reduction and avoidance of feeding for the 2000 to 10 000 and 3750 to 15 000 mg L^{-1} treatments (Fig. 5).

Aggression and fighting behaviour were common in the control group, with an increase in the late afternoon from Day 1 to Day 4 followed by reduction in the evening (Fig. 6). However, the fish in the 50 to 200 and 500 to 2000 mg L^{-1} NaCl treatments showed moderate aggression or fighting throughout the periods and days. Contrasting changes occurred in the 2500 to 10 000 mg L^{-1} group, which showed increasing aggressive behaviour with days irrespective of time, while for the 3750 to 15 000 mg L^{-1} exposure group, aggressive behaviour shown in Day 1 and Day 2 was completely lost by Day 4. Considerable aggressive behaviour was observed in morning to late afternoon in all the groups for Day 1 to Day 4, with the exception of the 3750 to 15 000 mg L^{-1} group. Moderate aggression and fighting was seen for control and exposed groups, but particularly for higher salinity groups, aggression and fighting behaviour significantly reduced from Day 3 and was minimal in next day, which indicated that continued high salinity disrupted this social behaviour (Fig. 6).

Stress behaviour and movements were almost absent in the control group from Day 1 to Day 4 at all times, over time higher exposure caused increased signs of stress (Fig. 6). In Day 1, stress was observed in all exposed groups initially after salinity exposure and continued mostly in 2500 to 10 000 and 3750 to 15 000 mg L^{-1} NaCl exposed groups. Starting from Day 3, high stress including mortality was observed in the higher salinity treatments. Control fish showed negligible stress, while the salinity exposed groups showed stress movement especially in the morning in all four days of observation, which continued throughout the day with varied magnitude. For the 3750 to 15 000 mg L^{-1} group of fish, the stress movement including immobility and mortality increased with time (Fig. 6).

Statistical analysis of behavioural changes

Significant changes in behaviour (CNI units) in the control and treatments at the end of the experiment, i.e., at Day 4, were determined using the non-parametric Mann-Whitney U Test. There were no significant difference in the control and lower exposure groups for shoaling behaviour, expect the 3750 to 15 000 mg L^{-1} exposure group, where found almost no shoaling movement was found ($p < 0.001$) (Fig. 7). Playing pattern also did not significantly differ between the control and up to the 2000 mg L^{-1} NaCl exposed groups, but showed significant changes when salinity reached 10 000 mg L^{-1} and above ($p < 0.001$). Similarly, significant difference observed in resting pattern was found between the control and 10 000 mg L^{-1} ($p < 0.05$), and 15 000 mg L^{-1} treatments ($p < 0.001$). The unrest movement throughout Day 4 at this high exposure also very significantly differed from other exposed groups. Feeding did not differ significantly between control and 2000 mg L^{-1} groups, but it was significantly different for 2500 to 10 000 mg L^{-1} ($p < 0.05$) and 3750 to 15 000 mg L^{-1} groups ($p < 0.001$) where lower feeding behaviour was observed. Clear and significant variation was observed for aggressive behaviour between all the groups from control to lower and higher salinity exposed groups. Along with aggressive and fighting movements, prominent changes occurred for stress movement where all exposed groups showed significant differences with the control. However, courtship behaviour was very low even with mild salinity, and found highly sensitive to salinity as no such behaviour was observed from 10 000 mg L^{-1} NaCl concentration ($p < 0.05$).

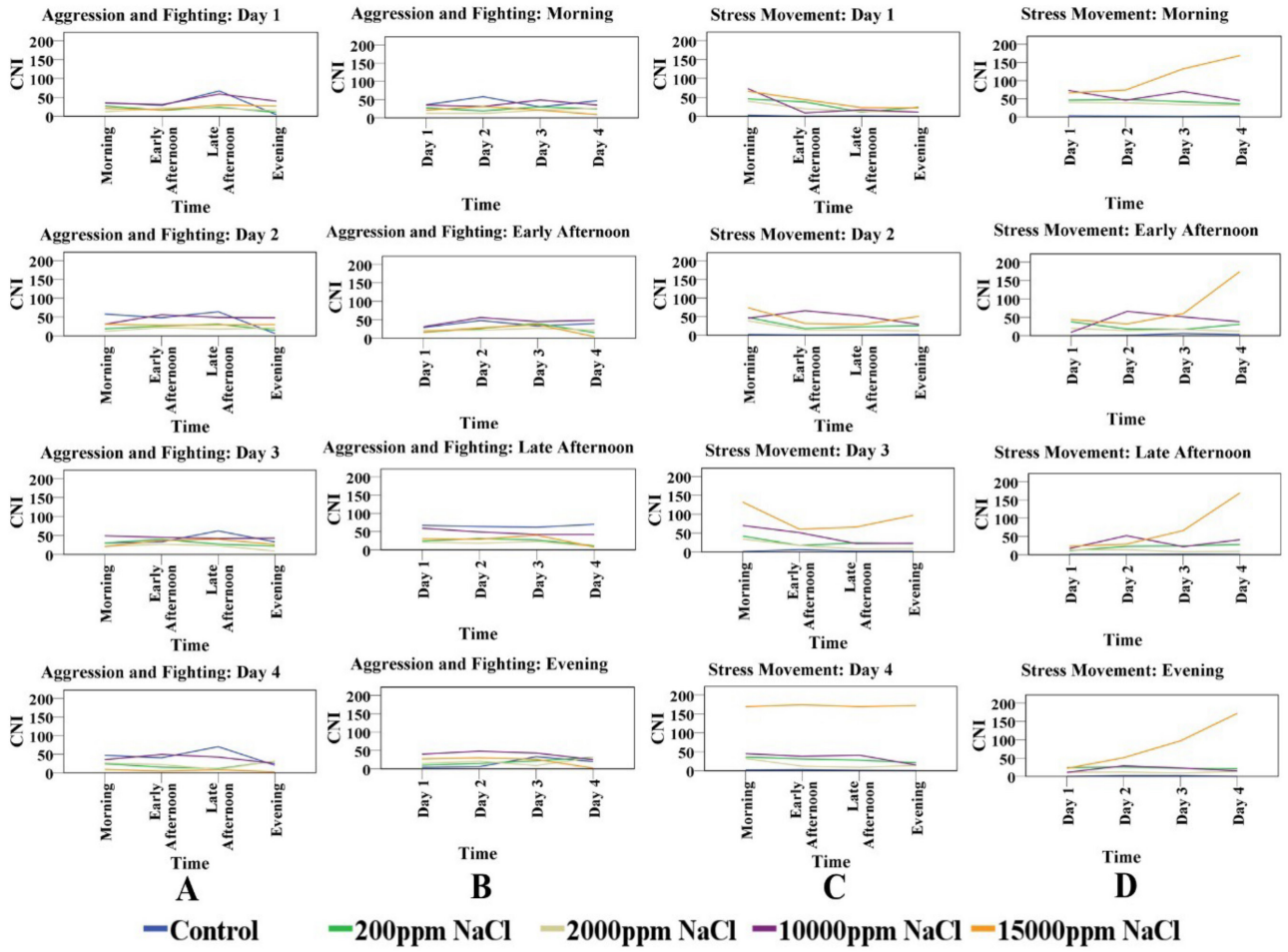

Fig. 6. Aggression-fighting and stress movement behavioural patterns of *Danio rerio* under different NaCl conc. compared among groups. A and C, day-wise fluctuation of aggression-fighting and Stress movement behaviour, respectively; B and D, diurnal fluctuations of aggression-fighting and stress movement.

Discussion

The present study was aimed to delineate the effect of overall salinity on a freshwater fish species in experimental conditions. The experimental design grossly simulated a condition when sudden coastal disaster hits the nearby inland freshwater ecosystem and fisheries. This is a common scenario of coastal regions of tropical and semi-tropical parts of the world, when global warming and oceanic cyclones have been frequently hitting these regions of the world in recent years (Saha et al. 2015; Gayathri et al.,2016, Kantamaneni et al. 2022; Shivanna 2022). Due to natural catastrophe when coastal embankments breaks, a sudden inflow of brackish or estuarine water (salinity range 2000 to 8000 mg L^{-1}) or marine water (salinity range 10 000 to 35 000 mg L^{-1}) enters into the land adjacent to coast. This initial contamination of salt water is followed by the subsequent inflows of salt water. Normally, tropical depression and stormy weather continue for next one or two days and this contamination may continue further due to the broken embankment. Therefore, in the experimental setup, NaCl exposure at first time was analogous to the first contamination of freshwater ecosystem with estuarine or marine water, and the following exposures resembled contamination of subsequent days by increasing the salinity further, thus, simulating a natural coastal flooding.

In this study zebrafish (*Danio rerio*) individuals, the common fish model for experimental studies, were exposed to different concentrations of common salt i.e., NaCl in experimental tanks to determine their tolerance limit to salinity and behavioural responses to salinity stress. Behavioural attributes were observed and recorded to determine the key behavioural changes indicating the salinity stress (Spence et al. 2008; Farhana et al. 2019). This simple and unique study provided important information on effects of general salinity increase induced by common salt, and the results could be validated in natural coastal ecosystems in disasters with salinity contamination.

It was found that zebrafish can tolerate a slight to moderate range of brackish water salinity with range 1000 to 10 000 mg L^{-1}, particularly for acute exposure as the experimental system reached 10 000 mg L^{-1} with

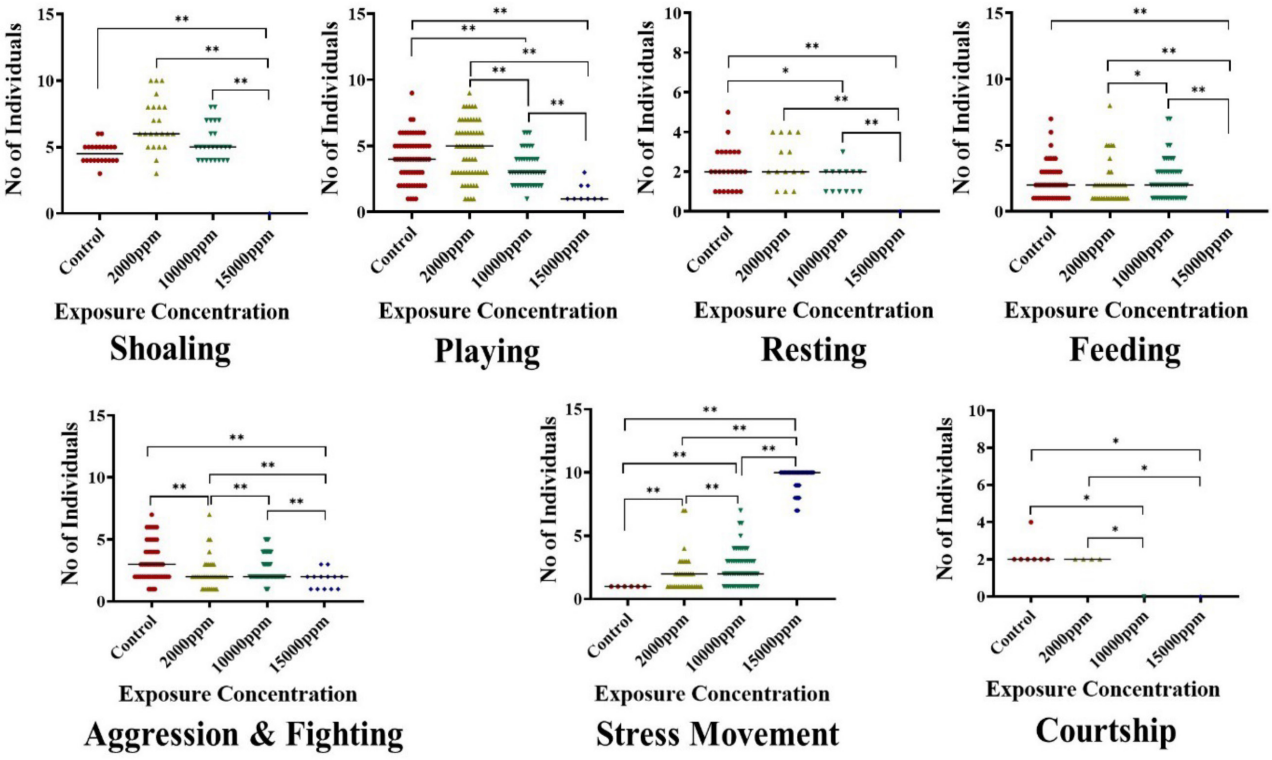

Fig. 7. Variations of behavioural manifestations of *Danio rerio* due to the exposure of different concentration of NaCl during experiment recorded as number of individuals for each 5 min time unit of observation of each 90 min diurnal time slots against the days. Number of participants observed in each 5 min slot of the four diurnal time slots of a day for a behavioural pattern were plotted in Y axis while exposure conditions were plotted in X axis. The dot-plots are showing the weightages of behaviours among the individuals and their shift under changing conditions. In the graphs (*) indicates the p value < 0.05 and (**) indicates the p value < 0.001.

fractionated dose exposure for consecutive four days. This was done to simulate natural conditions when a breakage of a estuarine guard-wall in any natural disaster in Sundarban region of West Bengal (a prominent estuarine and coastal region of south-east Asia frequently facing tropical cyclonic storm and part of global biosphere reserve and largest mangrove forest of the world) took several days to weeks to repair, thus contaminating and flooding the freshwater inland fishery farms for the period. The Sundarban estuary possesses three different salinity zones; the zones of higher human settlements and economic activities including fisheries have an average salinity range of 5000 to 7000 mg L^{-1} with wide spatiotemporal variations (Sherin 2020). Therefore, the values of 2500 and ~3750 mg L^{-1} of average contamination per day were set for four consecutive days in experimental simulation, expecting a mixing of fresh and saline water in a disastrous condition. The experiment clearly demonstrated that above 2500 mg L^{-1} exposure per day or cumulative salinity exposure over 10 000 mg L^{-1} in a period of 96 h causeds behavioural and physiological stress and over 3500 mg L^{-1} per day with repeated exposure was found fatal with LC_{50} value ~14 000 mg L^{-1} for salinity within a period of 96 h (Fig. 2).

In the present behavioural study, it was found that of the seven studied behaviours some showed striking changes in response to increasing salinity both diurnally and daily. While lower concentration did not have much effect on zebrafish, higher concentration of NaCl proved fatal to zebrafish, leading to unrestful stress movements. During the stress period of zebrafish, sharp changes in behaviour was noted. In the initial days of exposure the zebrafish expressed their stress conditions through erratic, zig-zag and hyper-movement, and in the last days of exposure in reduced swimming movement, excessive surfacing and lethargy. Similar observations were found in a study of *Anabas testudineus* exposed to triclosan stress (Priyatha, Chitra 2018). Also, when *Gambusia holbrooki* was exposed to pharmaceuticals it exhibited lethargy in movements (Nunes et al. 2008). In the time period between 72 and 96 h, when salinity reached above 10 000 mg L^{-1}, stress immobilized the fish accompanied with sudden jerk movements and followed by death (Fig. 6). This result is similar to the study where zebrafish during the transformation from two cell to larvae stage did not survive under high concentration of NaCl and showed developmental abnormalities with genetic expression aberrations (Seli et al. 2024). In that study 250 mM (≈14 610 mg L^{-1}) and higher NaCl concentration caused developmental restriction in embryonic stage. In comparison, the present study showed a very similar range

of NaCl concentration as LC_{50} value for the adult zebrafish.

It was observed that all seven behaviours did not necessarily display a similar pattern of diurnal and daily changes. While behaviours like shoaling and feeding reduced both diurnally and every passing day of increasing concentration, playing, stress movement and courtship did not follow the same path as that of shoaling and feeding. In a previous studies it was found that increasing temperature and hypoxic conditions reduced shoaling behaviour in freshwater fish (Domenici et al. 2007; Bartolini et al. 2015). In our study it was found that the shoaling pattern significantly deviated when salinity reached 15 000 mg L^{-1} level. Decrease in shoaling behaviour under stress might be explained by the fact of fish becoming bolder to break away from a shoal and explore a safer area in an environment to refuge (Sih et al. 2015).

In this study, playing and courtship altered diurnally, where courtship behaviour was prominently aligned with the day and night cycle showing prominence in evening hours. However, courtship behaviour showed high sensitivity to salinity change and under successive exposure courtship ceased when the salinity level as a single exposure or cumulative exposure reached 2000 mg L^{-1} , indicating that courtship can only continue in fresh water to very mildly saline water (Fig. 4 and 7). The finding of reduced playing in response to stress was confirmed in several previous studies conducted by other groups (Klein et al. 2010; Saxena et al. 2021; Eraslan et al. 2023). Resting behaviour, as defined earlier, occurred in evening and not in the day. Increasing salinity led to increase of unrest among zebrafish, where generally control and lower concentrations were associated with more resting in the evening, and fish exposed to higher concentration displayed comparably higher unrest (Fig. 4).

Reduced feeding in increasing salinity was in agreement with a study where exposure of aquatic snails to toxic cadmium caused significant decrease in feeding activity (Alonso, Valle-Torres 2018). In the present study, withdrawal from feeding when salinity reached above 10 000 mg L^{-1} had been marked as prominent behavioural change and prominently demonstrated stress in zebrafish (Fig. 5 and 7). In previous studies, it was found that aggressive behaviour in Nile tilapia indicated physiological stress induced through confinement (Barreto et al. 2009). Similarly, in our study, zebrafish also displayed significant aggressive behaviour in the afternoon compared to control, although the intensity of aggression and fighting was decreasing on a day-by-day basis and reached a minimum on the last day of observation (Fig. 6 and 7). The results showed contrasting aggression patterns in concentrations between 10 000 and 15 000 mg L^{-1} salinity, where the first was marked with high aggression, but a drastic reversal of behaviour was found in the next. This switching of behaviour after a particular salinity threshold is indicative of the salinity stress tolerance limit of zebrafish.

Conclusions

Increase of salinity stress in freshwater aquatic ecosystems can cause habitat destruction as well as life threats to aquatic organisms. At the same time, where livelihood is mainly dependent on aquatic ecosystems, inland or freshwater fish farming in coastal regions is also hampered. The present study emphasized the effect of raising low to high salinity on a fish species, but other organisms may also be affected. It is found that the freshwater fish model zebrafish cannot withstand a threshold of moderate salinity level of 15 000 mg L^{-1} and this level is sufficient for saline toxicity induced death. However, sensitive behaviour like courtship showed indicative changes above 2000 mg L^{-1} salinity levels and day-wise changes were observed with drastic shift of shoaling, feeding and aggression patterns around and above 10 000 mg L^{-1} salinity. Behavioural screening pointed out the key indicative behavioural changes associated with salinity intolerance, showing diminished feeding activity, increased aggression and erratic stress movements. Further increase in salinity showed sudden immobilization and surfacing of the fish followed by death. Thus, behavioural changes may act as clear indicators of tolerable to intolerable salinity stress and the present study showed the physiological and behavioural thresholds to this stress.

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