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Research Article

Salinity tolerance response of ornamental black molly, *Poecilia sphenops* exposed to inland saline water

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Abstract

Freshwater black molly (*Poecilia sphenops*) is considered one of the most important commercial ornamental fish due to its high popularity among aquaculturists, hobbyists, and breeders across the globe. Inland saline groundwater, akin to brackish water, with salinities ranging from 3 to 15 ppt, is abundant in India's South Western Punjab, which is now considered as the thrust area for aquaculture. The laboratory study has been designed to investigate the tolerance, survivability and behavior of black molly at different salinities i.e., 0 (control), 2, 4, 6, 8, 10, 12, 14 and 16 ppt for 10 days. Significant differences were observed in water quality variables, ionic compositions, and the survival rate of fish in all the treatments. Though the survival rate was 100% in 0 to 8 ppt, it gradually decreased from 10-16 ppt (99.29-89.72%) and drastically decreased on fourth day onwards in 12 to 16 ppt. Low swimming activity was observed from the third to fifth day onwards in 6-16 ppt. Feeding response was high to moderate in 0 to 8 ppt and moderate to less in 10-16 ppt. No threat response was observed in 0 to 8 ppt, whereas modest and weak response was noticed in 10-16 ppt. The findings of the study suggest that black molly can tolerate salinity up to 14 ppt (≥ 90% survival rate) and the optimum salinity range can be up to 8 ppt to achieve good swimming activity, feeding response and without any threat response.

Keywords: Black molly, Fish behaviour, Inland saline water, Ionic composition, Salinity tolerance

Highlights

- Ornamental black molly can be reared in inland saline water.
- Higher survival rate of black molly achieved in 0-8 ppt.
- Low swimming activity at higher salinities.
- High appetite was observed up to 8 ppt.
- No threat response was observed up to 8 ppt salinity.

INTRODUCTION

The global ornamental fish trade is estimated to be worth roughly US$ 18-20 billion, with the participation of over 100 million hobbyists (Biondo and Burki, 2020), which is significant for livelihood generation and economic development in developing countries including India (Khairnar and Kaur, 2021). The domestic ornamental fish trade is projected to be worth around 500 crores, with an export value of around US$ 1.4 million (Raja *et al.*, 2019).

Further, the industry is currently growing at an annual rate of 11-12% (Parappurathu *et al.*, 2021). Ornamental fish have a long history of popularity in both domestic and foreign markets (Gupta *et al.*, 2016). Around 80% of ornamental fishes from India are sold to the worldwide market, with most of them coming from all regions of the country, which are rich in natural water bodies and ornamental fishes. Moreover, India has a huge untapped potential for producing indigenous ornamental fish which can easily contribute towards economic upliftment (Kumari *et al.*, 2017).

Salinity in inland regions is generally caused by naturally salty soils or underground saline water or by waterlogging of land caused by intensive irrigation. The south-west districts of Punjab (Sri Mukatsar Sahib, Ferozepur, Fazilka, Faridkot, Mansa and Bathinda) are also facing the dual problems of underground salinity and water logging (1.51 lakh ha), which has dramatically reduced agricultural productivity (Dhawan *et al.*, 2010). In these districts, the salinity of underground water ranged between 10-15 ppt, while that of waterlogged areas varied from 1-47 ppt (Kumari *et al.*, 2017). Ornamental fish culture can be a very good alternative for small-scale or marginal farmers in Punjab by utilizing inland saline water resources of the aforementioned districts. Moreover, it could be a boon for Punjab's ornamental fish industry, which is heavily reliant on purchasing freshwater fish from coastal regions. There

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are reports on successful koi carp and goldfish culture trials in inland saline waters of Punjab (Sharma *et al.*, 2017; Bhatt *et al.*, 2018), in which salinity related physiological changes were studied. Salt-tolerant live-bearer ornamental fish like black molly (*Poecilia sphenops*) can also be appropriate for this region. Considering the high growth rate, birth size, reproduction, appearance and wide range of food preferences, this is one of the most popular ornamental fish. Because of its hardiness, black molly is the most popular ornamental fish in the world. It is also commonly considered the first species reared by many ornamental fish hobbyists (Dasgupta *et al.*, 2017).

In view of the above background, the present study was conducted to determine how black molly adapts to inland saline water and the effect of salinity on survival and behavioral responses.

MATERIALS AND METHODS

The experiment was carried out at the Instructional cum Research Farm, College of Fisheries (30°54'21.5" N and 75°48'04.7" E), Guru Angad Dev Veterinary and Animal Sciences University in Ludhiana, Punjab for 10 days in April 2021. Inland saline water (stock water; 16 ppt) was collected from salt-affected/water-logged areas of Birawala (29°86'77.8" N and 75°24'95.9" E), District Mansa, Punjab for the tests. The stock water was continuously aerated for 5 days and filtered with a clean muslin cloth (20 µm) and the filtrate water was diluted with borewell freshwater to prepare different saline water of 2, 4, 6, 8, 10, 12, 14 and 16 ppt salinity. A total number of one hundred eight fishes were randomly stocked in a clean glass aquarium (each 50 liters capacity) in nine treatments in triplicates with respect to eight salinity regimes (2- 16 ppt). Fish were procured from the backyard ornamental fish seed production unit of the college and acclimatized for 2 hours in different salinities before stocking. All the aquaria were supported with 24 x 7 hours continuous aeration supplied by a diaphragm air blower (RS-15000; 220-240 V/50Hz; 20 Watt; 0.024 MPa). All the aquariums were placed by following the completely randomized design (CRD) method (Table 1).

Fish were fed *ad libitum* with commercial feed (OPTIMUM: 28% crude protein, 4% crude fibre, 3% crude fat and 10% moisture) twice (9-10 AM; 3-4 PM) a day. Both faecal matter and unutilized feed waste were cleaned out by siphoning before feeding. Water samples were collected daily in the morning at 9.00 AM-10.00 AM for the analyses of physico-chemical parameters like temperature, pH, electrical conductivity (E_C), dissolved oxygen (DO), total hardness (TH), total alkalinity (TA), ammonia -nitrogen (NH_3-N), nitrite-nitrogen (NO_2-N), nitrate-nitrogen (NO_3-N), phosphate-phosphorus (PO_4-P) and ionic composition (Na^+, K^+, Ca^{2+}, Mg^{2+}, Cl^- and SO_4^{2-}) by following standard methods (APHA, 2005). Fish survival and behavior were also recorded daily in each treatment. The survival and different responses of black molly including swimming (very active, less active, erratic swimming), feeding (high, moderate, low appetite) and threat (normal, modest, weak response) were observed throughout 10 days of salinity response. The survival rate was calculated according to the following formula (Sultana *et al.*, 2018): Survival rate (%) = (No. of fish alive/ No. of fish stocked) x 100

The swimming response was determined by observing the fish's swimming patterns in the water column, and the feeding response was determined by observing the amount of leftover feed at the tank's bottom (Lawson and Alake, 2011). At various time periods, behavioral stress reactions were detected at all salinity levels.

The data was statistically analyzed using statistical software (SPSS 20.0 Richmond, CA, USA). To determine differences between salinity treatments ($P \leq 0.05$), one-way ANOVA and Duncan's multiple range test were used to determine the effect of different salinities on physico-chemical, ionic parameters and survival of experimental fish.

RESULTS

The mean values for water quality parameters and ionic composition of inland saline water (stock water) are presented in Table 2. The relative abundance of Na^+ and Cl^- were greater than that of other cations and anions

Table 1. Experimental design

	Treatment and replication*								
	I	II	III	IV	V	VI	VII	VIII	IX
A	S0R1	S8R3	S2R2	S4R3	S2R3	S10R2	S16R1	S12R1	S12R2
B	S14R1	S4R2	S14R3	S16R2	S0R3	S8 R2	S0R2	S2R1	S16R3
C	S6R1	S10R3	S8R1	S12R3	S6R3	S14R2	S4R1	S6R2	S10R1

* S0 = 0 ppt, S2 = 2ppt, S4 = 4 ppt, S6 = 6ppt, S8 = 8 ppt, S10= 10ppt, S12 = 12ppt, S14 = 14 ppt, S16 = 16ppt.; R1, R2, R3 refer to the replicates

Table 2. Water quality parameters and ionic composition of stock water

Parameters	Mean ± SE	Parameters	Mean ± SE
Salinity (ppt)	16.00±0.05	NO_3-N (mgL^{-1})	0.008±0.001
Temperature (^0C)	23.00±0.28	O-PO_4^{-3} (mgL^{-1})	0.011±0.01
pH	08.02±0.13	Ca^{2+} (mgL^{-1})	252.30±62.06
EC ($mScm^{-1}$)	19.78±0.33	Mg^{2+} (mgL^{-1})	510.42±3.60
TA ($CaCO_3$ mgL^{-1})	316.00±0.00	Na^+ (mgL^{-1})	1014.00±1.24
TH ($CaCO_3$ mgL^{-1})	3160.00±60.09	K^+ (mgL^{-1})	63.06±2.33
NH_3-N (mgL^{-1})	0.031±0.001	Cl^- (mgL^{-1})	3135.36±2.08
NO_2-N (mgL^{-1})	0.001±0.001	SO_4^{2-} (mgL^{-1})	150.22±5.25

($Na^+ > Mg^{2+} > Ca^{2+} > K^+$ and $Cl^- > SO_4^{2-}$), thus indicating Na^+ and Cl^- to be the most important cation and anion.

There was a significant difference ($P<0.05$) in water quality parameters and ionic composition (Table 3). Other than the temperature, all the parameters and ion concentration increased with increasing salinity in all treatments, with the highest and lowest values found in S16 and S0 (control), respectively.

The overall mean survival rate of black molly differed ($P<0.05$) among the treatments. In spite of the identical stocking densities in all treatments, the survival rates were 100% for S0 to S8 and 89.72-99.52% for S10 to S16 (Fig. 1A). As shown in Fig. 1B, mortality was observed on the second day in S14 and S16, whereas in S12 and S10 it occurred on the third and seventh day.

The behavioral responses of black mollies concerning swimming, feeding, and threat responses are summarized in Table 4. During the study period, molly exhibited very active swimming in S0 and S2 and an active response was observed in all the treatments until after a certain point in time. The swimming response became less active from the third day onwards in treatments S12 to S16, and from the fourth to fifth day onwards in treatments S6 to S10. Erratic swimming response was noticed only from S12 to S16.

Throughout the study, molly exhibited a high appetite from S0 to S6. Moderate appetite was noticed within the initial two days in the rest of the treatments. High appetite was also noticed in S8 followed by moderate appetite. Moreover, less appetite was observed in S12 to S16 followed by moderate appetite during the study period. Molly showed normal response up to the 3rd day in all the treatments, whereas, the response continued to the end of the study period in S0 to S8. A moderate response was noticed from the fourth day onwards in S10. Moreover, a weak response was also observed in S12 to S16 followed by a moderate response.

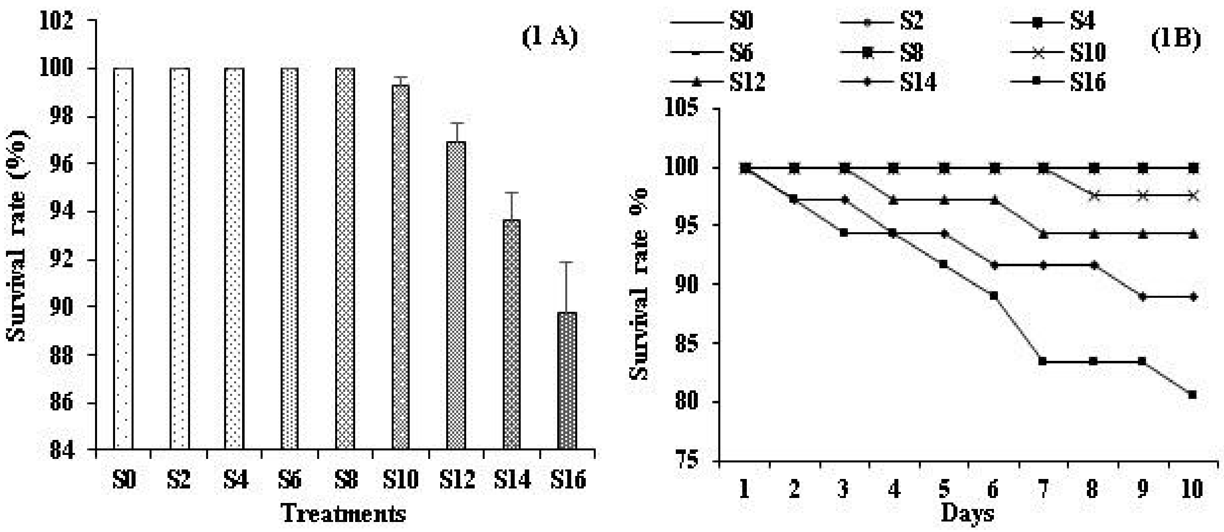

Fig. 1. Survival percentage (A) and survival curves of black molly during the study (B) in different treatments

Table 3. Mean physico-chemical parameters of water in different treatments during salinity tolerance test

Parameters	Treatments* (Mean ± SE)									P value
	S0	S2	S4	S6	S8	S10	S12	S14	S16	
T (^{O}C)	29.68±0.14	29.59±0.12	29.68±0.08	29.78±0.04	29.66±0.12	29.58±0.12	29.66±0.12	29.66±0.12	29.58±0.12	P<0.001
pH	7.69±0.06^{h}	7.84±0.03^{g}	7.95±0.10^{f}	8.09±0.03^{e}	8.13±0.04^{e}	8.24±0.01^{d}	8.37±0.01^{c}	8.41±0.02^{b}	8.52±0.02^{a}	P<0.001
EC (mS cm^{-1})	0.60±0.02^{f}	2.34±0.01^{e}	6.21±0.03^{d}	7.24±0.01^{c}	11.53±0.26^{b}	13.23±0.49^{a}	11.53±0.26^{b}	11.53±0.26^{b}	13.23±0.49^{a}	P<0.001
TA (mgL^{-1})	278.8±8.53^{e}	310.3±4.50^{d}	348.6±3.46^{c}	375.0±1.96^{b}	400.3±1.92^{a}	405.3±5.61^{a}	400.3±1.92^{a}	400.3±1.92^{a}	405.3±5.61^{a}	P<0.001
TH (mgL^{-1})	289 ±1.63^{f}	505 ±5.68^{e}	625.3±3.61^{d}	945.3±4.28^{c}	1030 ±8.62^{b}	1260 ±13.06^{a}	1030.0±8.62^{b}	1030.0±8.62^{b}	1260 ±13.06^{a}	P<0.001
NH_3-N (mgL^{-1})	0.16±0.01^{g}	0.17±0.01^{g}	0.15±0.02^{g}	0.28±0.01^{f}	0.56±0.01^{e}	0.87±0.02^{d}	1.01±0.01^{c}	1.18±0.01^{b}	1.36±0.02^{a}	P<0.001
NO_2-N (mgL^{-1})	0.002±0.001^{e}	0.003±0.00^{e}	0.003±0.00^{e}	0.004±0.00^{d}	0.005±0.001^{d}	0.009±0.001^{c}	0.012±0.001^{b}	0.013±0.001^{b}	0.017±0.001^{a}	P<0.001
NO_3-N (mgL^{-1})	0.013±0.001^{e}	0.014±0.001^{e}	0.017±0.001^{d}	0.02±0.001^{c}	0.023±0.001^{ab}	0.023±0.001^{ab}	0.025±0.001^{a}	0.027±0.001^{a}	0.028±0.001^{a}	P<0.001
PO_4-P (mgL^{-1})	0.007±0.001^{b}	0.008±0.001^{b}	0.01±0.001^{ab}	0.011±0.001^{ab}	0.013±0.001^{a}	0.015±0.001^{a}	0.016±0.001^{a}	0.017±0.001^{a}	0.019±0.001^{a}	P<0.001
Ca^{2+} (mgL^{-1})	51.33±1.76^{f}	95.87±4.04^{e}	126.20±2.4^{d}	160.70±4.09^{c}	206.10±3.30^{b}	224.70±1.91^{a}	206.10±3.30^{b}	206.10±3.30^{b}	224.70±1.91^{a}	P<0.001
Mg^{2+} (mgL^{-1})	60.3±0.36^{f}	109.30±1.36^{e}	135.40±0.80^{d}	208.20±1.02^{c}	227.40±2.04^{b}	282.85±2.95^{a}	227.40±2.04^{b}	227.40±2.04^{b}	282.85±2.95^{a}	P<0.001
Na^{+} (mgL^{-1})	57.93±0.88^{f}	162.10±2.65^{e}	199.50±2.19^{d}	320.20±1.83^{c}	472.10±4.35^{b}	759.70±1.83^{a}	472.10±4.35^{b}	472.10±4.35^{b}	759.70±1.83^{a}	P<0.001
K^{+} (mgL^{-1})	7.56±0.25^{e}	9.41±0.11^{d}	10.18±0.22^{d}	19.18±0.78^{c}	36.56±0.67^{b}	42.30±0.62^{a}	36.56±0.67^{b}	36.56±0.67^{b}	42.30±0.62^{a}	P<0.001
Cl^{-} (mgL^{-1})	67.1±0.94^{f}	268.8±15.32^{e}	666.6±8.87^{d}	957.0±6.43^{c}	1048.0±7.82^{b}	1230 ±13.58^{a}	1048±7.82^{b}	1048 ±7.82^{b}	1230±13.58^{a}	P<0.001
SO_4^{2-} (mgL^{-1})	9.24±0.28^{f}	64.46±1.23^{e}	79.37±2.27^{d}	93.96^{c}±3.19^{c}	117.2±2.82^{b}	140.80±1.29^{a}	117.2±2.82^{b}	117.2±2.82^{b}	140.80±1.29^{a}	P<0.001

* S0 = 0 ppt, S2 = 2ppt, S4 = 4 ppt, S6 = 6ppt, S8 = 8 ppt, S10= 10ppt, S12 = 12ppt, S14 = 14 ppt, S16= 16ppt
Values with same superscripts (a,b,c…..f) in a row do not differ significantly ($p \leq 0.05$)

DISCUSSION

The present study demonstrated the effects of salinity on the survival and behaviour of *P. sphenops*. According to some of the previous reports, freshwater fish can tolerate 8 ppt salinity in inland saline waters (Sawant *et al.*, 2001; Kucuk, 2013; Sharma *et al.*, 2017). The results of the present study suggested salinity tolerance of black molly up to 14 ppt; however, better survival and behaviour responses were recorded up to 8 ppt.

Throughout the study, the mean value of water temperature was below 30ºC, which is optimum for aquaculture (Boyd, 1982). However, increase of pH level with increasing salinity established a positive relationship with NH_3-N which could be a probable reason for increasing mortality in higher salinity treatments (Fig. 2). The toxic form of ammonia (free ammonia) can become more abundant at high pH values and also affect osmoregulation and reduce oxygen carrying capacity in blood (Boyd, 1990; Lawson, 1995).

In the present experiment, conductivity and hardness increased with increasing salinity (S2-S16). The findings agree with De Moura *et al.* (2021). Except NH_3^-, rest of the water quality parameters and ionic compositions established positive correlation and polynomial relationship with increasing salinity (Table 4).

These results are in agreement with earlier studies which stated that increased concentration of salts and the ability to conduct electric current can significantly influence the ionic compositions and other water quality parameters (Sharma *et al.*, 2017; Bhatt *et al.*, 2018).

Increase of Cl^- with increasing salinity in the treatments established positive relationship with NO_2^- (Fig. 3A), which could be a reason for increasing mortality in S12, S14 and S16 as NO_2^- also showed positive polynomial relationship with mortality rate of black molly (Fig. 3B). NO_2^- must have hampered the homeostasis of the fish due to its active absorption in gills (De Moura *et al.*, 2021). Moreover, the reduction in survival rate of black molly in high saline treatments (S10 to S16) could be due to negative effect of fish. The survival, metabolism, and distribution of fish species are influenced by salinity (Suresh and Lin, 1992). Moreover, the ability of bodily fluid to endure fluctuations in osmolality and ion concentrations determines the influence of salinity on survival rate (Stickney, 1979). Therefore, decline in the overall survival rate of black molly was observed beyond 8 ppt (Fig. 4).

Table 4. Swimming activity and feeding response of black molly, *P. sphenops* during the salinity tolerance test

Fish behaviour	Day	Treatments*								
		S0	S2	S4	S6	S8	S10	S12	S14	S16
Swimming activity	1	+	+	+	+	+	+	+	+	+
	2	+	+	+	+	+	+	+	+	+
	3	+	+	+	+	+	+	–	–	–
	4	+	+	+	+	+	+	–	–	–
	5	+	+	+	–	–	–	–	–	–
	6	+	+	+	–	–	–	–	–	–
	7	+	+	+	–	–	–	–	–	–
	8	+	+	–	–	–	–	–	∞	∞
	9	+	+	–	–	–	–	–	∞	∞
	10	+	+	–	–	–	–	∞	∞	∞
Feeding response	1	≥	≥	≥	≠	≠	≠	≠	≠	≠
	2	≥	≥	≥	≥	≠	≠	≠	≠	≠
	3	≥	≥	≥	≥	≥	≥	≠	≠	≠
	4	≥	≥	≥	≥	≥	≥	≠	≠	≠
	5	≥	≥	≥	≥	≥	≥	≠	≠	≠
	6	≥	≥	≥	≥	≥	≠	≠	≥	≥
	7	≥	≥	≥	≥	≥	≠	≠	≥	≥
	8	≥	≥	≥	≥	≥	≠	≥	≥	≥
	9	≥	≥	≥	≥	≥	≠	≥	≥	≥
	10	≥	≥	≥	≥	≥	≠	≥	≥	≥
Threat response	1	*	*	*	*	*	*	*	=	=
	2	*	*	*	*	*	*	*	=	=
	3	*	*	*	*	*	*	*	=	=
	4	*	*	*	*	*	=	=	=	€
	5	*	*	*	*	*	=	=	=	€
	6	*	*	*	*	*	=	=	€	€
	7	*	*	*	*	*	=	=	€	€
	8	*	*	*	*	*	=	=	€	€
	9	*	*	*	*	*	=	=	€	€
	10	*	*	*	*	*	=	=	€	€

* Swimming Activity: '+' Very Active; '—' Less Active, '∞' Erratic Swimming; Feeding Response: '≥' High Appetite, '≠' Moderate Appetite, '≤' Low Appetite; Threat Response: '*' Normal Response, '=' Modest Response, '€' Weak Response

Table 5. Correlation and relationship between salinity Vs water quality parameters and ionic compositions

Variables	Correlation (r)	Polynomial regression relationship
pH	0.858	$y = -0.0032x^2 + 0.0765x + 7.7015$; $R^2 = 0.974$
EC	0.913	$y = -0.0639x^2 + 1.8118x - 0.0959$; $R^2 = 0.945$
TA	0.885	$y = -0.8478x^2 + 21.145x + 277.06$; $R^2 = 0.984$
TH	0.888	$y = -4.6579x^2 + 129.39x + 273.3$; $R^2 = 0.906$
NO_2^-	0.984	$y = -5E\text{-}06x^2 + 0.0004x + 0.0019$; $R^2 = 0.973$
NO_3	0.986	$y = -2E\text{-}05x^2 + 0.0014x + 0.0123$; $R^2 = 0.984$
PO_4	0.997	$y = -2E\text{-}06x^2 + 0.0008x + 0.0068$; $R^2 = 0.994$
Ca^{2+}	0.909	$y = -0.9441x^2 + 25.506x + 48.42$; $R^2 = 0.966$
Mg^{2+}	0.888	$y = -1.0371x^2 + 29.12x + 56.749$; $R^2 = 0.898$
Na^+	0.867	$y = -1.5801x^2 + 64.63x + 34.605$; $R^2 = 0.777$
K^+	0.908	$y = -0.117x^2 + 4.341x + 2.614$; $R^2 = 0.863$
Cl^-	0.875	$y = -7.121x^2 + 180.81x + 39.536$; $R^2 = 0.944$
SO_4^{2-}	0.876	$y = -0.6588x^2 + 17.265x + 19.413$; $R^2 = 0.919$

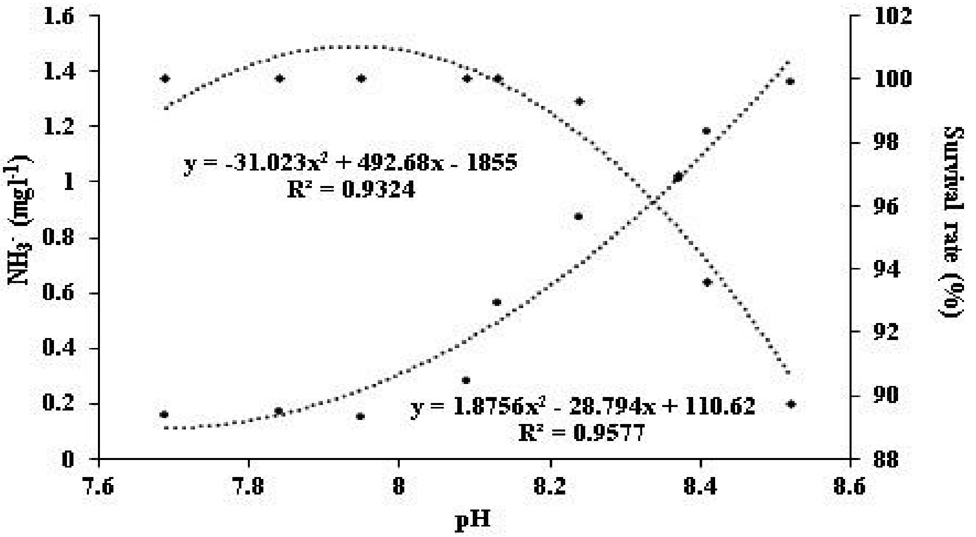

Fig. 2. Effect of pH on NH_3^- and survival rate of black molly in different treatments

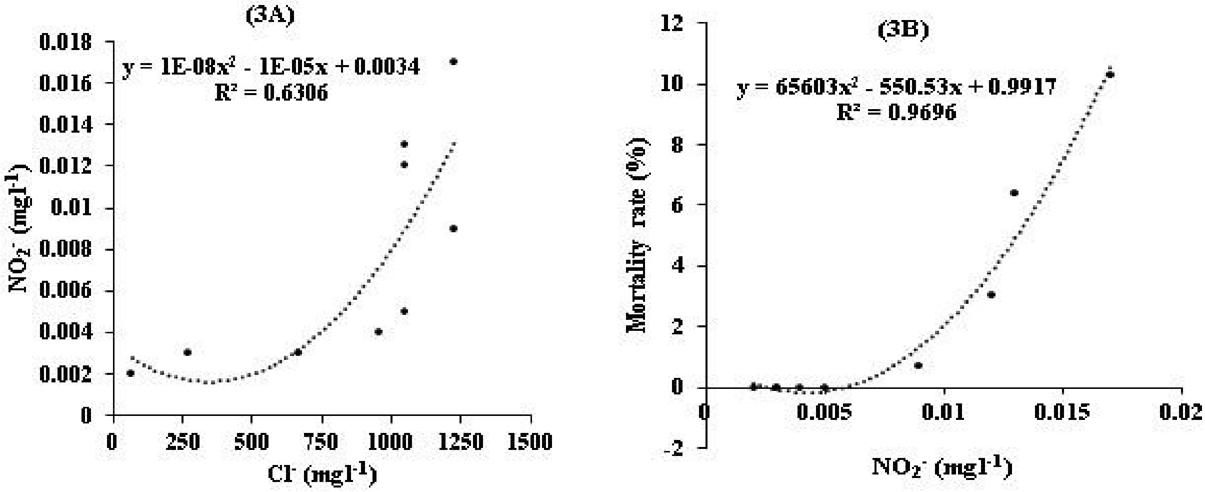

Fig. 3. Relationship between Cl^- *vs* NO_2^- and NO_2^- *vs* mortality rate of black molly

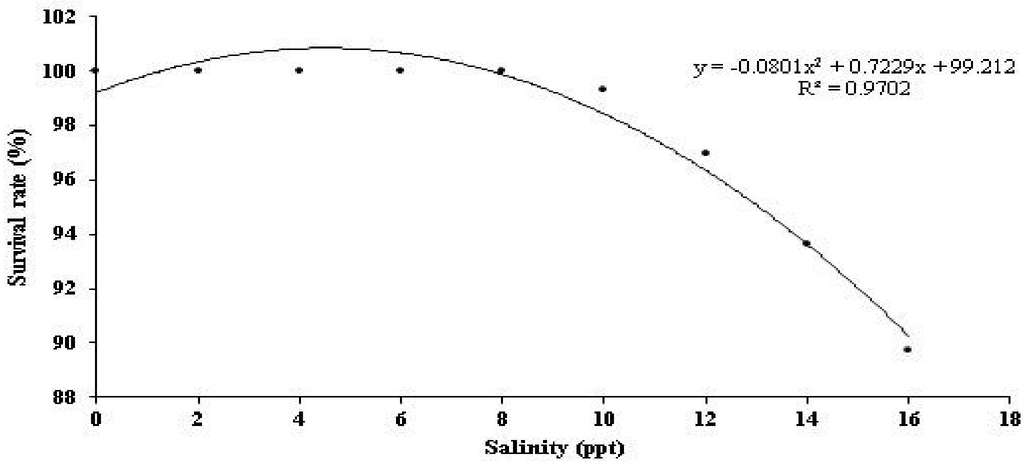

Fig. 4. Changes in survival rate of black molly with respect to changes in salinity

Changes in swimming activity, feeding, and threat reaction are clearly suggested as an adaptive approach in response to changing environmental conditions, like salinity which was noticed in different treatments (Bhatt *et al.*, 2018). Fish swim to a specific spot on a salinity gradient indicating their salinity preferences in a favourable situation (Baggerman, 1959). Salinities were approaching towards fish's tolerance limits, or above as evidenced by such fluctuations with increasing salinity (Khairnar *et al.*, 2015; Nahar *et al.*, 2016). In the present experiment high active swimming response, high appetite and normal threat response of black molly were observed in S0 to S8, after which all the responses became moderate to low in the rest of the treatments with increasing salinity and exposure period. Environmental salinity fluctuations can function as a stressor, causing behavioral and physiological responses. Behavioral endpoints are useful tools to analyze and identify the effects of environmental stresses on fish and can serve as indicators for ecosystem evaluation (Kane *et al.,* 2005). Further, osmoregulation and salinity tolerance in fish can be simple or complex (Khairnar *et al.*, 2016; Cao *et al*., 2018; Bonzi *et al.*, 2021) depending upon various factors. Adult black mollies can tolerate salinity of up to 16 ppt, therefore they can be reared in both fresh and saltwater. Molly cannot keep an osmotic equilibrium between the salt in her body fluids and the salt concentration in the surroundings (Sirimanna and Dissanayake, 2019). Kane *et al.* (2005) reported that fish behavioral changes can provide essential indices for eco-system evaluation, and behavioral endpoints are useful instruments for distinguishing and evaluating the impacts of exposure to environmental stressors. The swimming, feeding, and normal reactions of the fish were good throughout the study period, but when exposed to greater salinity, they were poor (12 to 16 ppt). The findings are in agreement with Amin *et al.* (2016) who also reported that impatience or agitated activeness or erratic movements in Java barb (*Barbonymus gonionotus*) was noticed when exposed to higher salinity level (14-16 ppt).

The findings of the present study indicated that although molly can tolerate salinity level up to 14 ppt (>90% survival rate), however, it can be reared up to 6 to 8 ppt, in order to achieve good survival rate and behavioral responses. Further, studies are required to evaluate the effect of salinity on other physiological responses, including colouration of fish under field conditions.

Conflict of interest: Authors have no conflict of interest in this study.

Authors contribution: LS: Performed the experiment and analyze the data; SOK: Conceived and designed the study, statistical analysis and manuscript writing; VIK: Helped in designing the experiment and manuscript writing; AM: Experimental set-up, statistical analysis and manuscript writing.

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