SALINITY TOLERANCE OF CATFISH (*Mystus gulio*) OF INDIAN SUNDARBANS: PERSPECTIVES OF DISTRIBUTION IN COASTAL SOUTH-EAST ASIA

Sourav Paul

Estuarine and Coastal Studies Foundation, Howrah, West Bengal

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ABSTRACT

Salinity gradient of an estuary is the osmotic barrier of fish distribution between river and sea. Eco-physiology *e.g.* salt tolerance of most catfish of South-East Asia (SEA) is unknown. Catfish *Mystus gulio* inhabits coastal-freshwaters and seldom enters the sea despite that it enjoys a large spatial distribution in the coasts of Indian Ocean. This led us to study its salt tolerance in increasing salinities (*e.g.* 5, 10, 15 and 20). Salt tolerance was evaluated separately at 20, 24, 28, and 32ºC which approximates the seasonal conditions of the Indian Sundarbans. This study may hint if catfish like *M. gulio* has the ability to use estuaries as saline bridges to invade adjacent rivers and expand their spatial distribution in SEA. The result shows 80-100% survival records of *M. gulio* in salinity 5 and 10. Mortality significantly increased in salinity 15 and 20 when the temperature is 28ºC. Hsp70 expressions of *M. gulio* is not significantly different between salinity 5 and 10 if the temperature is between 20 and 24ºC. During monsoon estuaries in the region remain relatively warm and oligohaline because they receive excessive rains. During the monsoon catfish similar to *M. gulio* may survive and use estuaries as saline bridges to invade adjacent rivers so their spatial distribution may expand. Increasing monsoon breaks of SEA and any significant rise of salinity-temperature amplitudes of coastal waters may force them to seek refuge in the cooler oligohaline sections of estuaries.

Keywords: *Survival, Invasion, Estuary, Hsp70.*

1. INTRODUCTION

'When considering coastal freshwaters, next to estuarine waters—waters that join inland freshwater bodies with the sea—the relevant abiotic factor is certainly water salinity (*e.g.* Langston *et al.*, 2010; Vitule *et al.*, 2013)' (Gutierre *et al*. 2014). Salinity tolerance is one of several important physiological attributes that determines the pattern fish species distribution in fresh to brackish water habitats (Capps *et al.*, 2011). Based on salt tolerance, different degrees of euryhalinity exist in nature (Myers, 1949; Griffith, 1974; Freire *et al*., 2008). Many fish are arbitrarily classified (without the knowledge of evolutionary origin and salt tolerance physiology) as 'freshwater fish' based on their current habitat preference. We often conclude that such species cannot adapt to saline environments, but in reality, if it tolerates (even for a short time) higher salinities, then it may explore a wide variety of brackish habitats (Capps *et al*., 2011; Gutierre *et al*., 2014). Arbitrary classification of a fish based on its current habitat (*e.g*. freshwater/brackish) is very typical in South-East Asia (SEA). Eco-physiological literature of coastal-marine fish of SEA hardly exists so we have very little eco-physiological perspectives of their distribution.

Experiments of salt tolerance often predict the survival and distribution chances of a fish under specific salinity gradients of an estuary (Bringolf *et al*., 2005; Gutierre *et al*., 2014). A freshwater fish may even die from osmotic stress because its proteins generally denature in higher salinities (Marshall and Grosell, 2006). Higher expression of molecular chaperones such as heat-shock proteins (Hsp) (which aid in maintaining homeostasis) is expected when a freshwater fish faces osmotic stress from higher salinities (Yang *et al*., 2009). When a fish is exposed to higher gradients of salinity and does not show high Hsp expression, then it is either truly eurihaline or partly salt tolerant; for such a fish a statistically significant relationship between Hsp expression and increasing salinity is not expected (Luft *et al*., 1996; Schofield *et al*., 2011; Gutierre *et al*., 2014). Such a fish may invade new to newer saline habitats and explore a large spatial distribution by virtue of its greater molecular flexibility to counter any stress that may arise from exposure to higher gradients of salinity (Bringolf *et al*., 2005; Kültz, 2005; Gutierre *et al*., 2014). There are more than 40 species in the genus *Mystus* only few are coastal species *e.g. Mystus gulio, M. wolffii* (Jayaram and Sanayal, 2003). Salt tolerance literature of fish are often species/genus specific but currently there is not a single salt tolerance literature exists of *Mystus* spp. Such literature is essential for better understanding the survival ability and distribution of a fish such as *Mystus gulio* which enjoys wide distribution in coastal-freshwaters of SEA (Mondal and Mitra, 2016).

The Bagrid catfish *Mystus gulio* inhabits coastal-aquatic habitats of the Pakistan to the Indonesian islands (Mondal and Mitra, 2016) (see Figure 1). The species earns major revenue from food and ornamental fish markets (Begum *et al*., 2008). The species was originally described from the higher parts of the Gangetic estuary (Hamilton, 1822) and it maintains a large population in the lower Gangetic delta (Begum *et al*., 2009). In the breeding season (*i.e*. monsoon), *M. gulio* even ascends freshwater zones and occasionally descends towards the sea (Pandian, 1966; Talwar and Jhingran, 1991). No information is available on the evolutionary origin of this species. Mukherjee *et al*., (2012), while reporting the sudden high density of *M. gulio* in the Matla estuary of the Indian Sundarbans in 2009 when the said estuary maintained low salinity for long time, the authors commented that the species may have a freshwater origin without any evolutionary and or physiological evidences. Previous literature which dealt with aquaculture or general biology of the species considered it as a fresh to brackish water fish without any experimental evidence (Mondal and Mitra, 2016; Begum *et al*., 2009; Pandian, 1966; Talwar and Jhingran, 1991). The eco-physiological work of Paul *et al*., (2019) on the sub-adults of *M. gulio* in Indian Sundarbans showed that the respiration of the individuals of the species significantly varies with the varying salinity-temperature

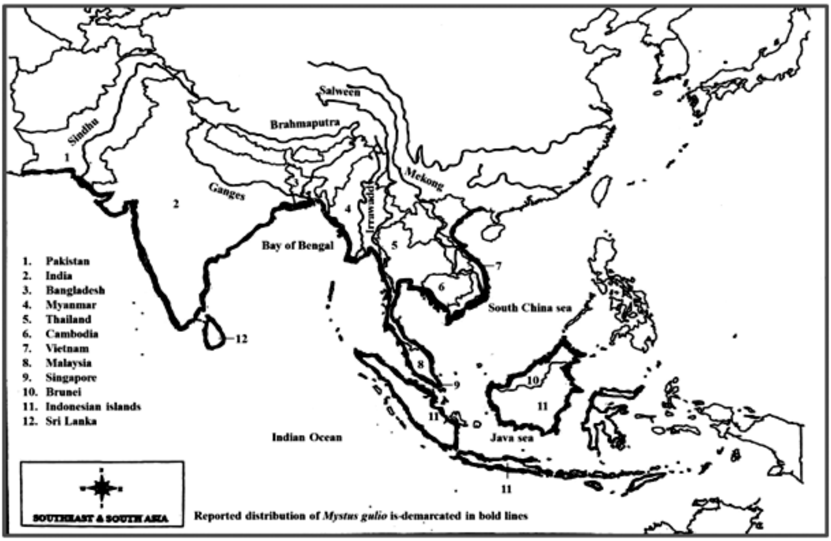

Fig. 1. *Currently reported distribution of Mystus gulio along the coasts of South-East Asia.*

amplitudes of their habitats. According to Paul *et al*., (2019), variability in respiratory response may lead the early life stages of this species to move towards the oligohaline sections of an estuary to avoid death from respiratory stress. Considering the literature gap related to salt tolerance of *M. gulio* authors have decided to study the salinity tolerance of the species in various salinity conditions that are common (in various seasons) in the estuaries of Indian Sundarbans. It is hypothesized that *Mystus gulio* is a moderately salt tolerant fish that has the potential to survive and maintain a steady constituent protein expression in oligohaline conditions. This work may help to understand if *M. gulio* and similar catfish could use estuaries as saline bridges to invade adjacent rivers and expand their distribution along the coasts of SEA.

2. MATERIAL AND METHODS

2.1. Sample Collection

Adults of *Mystus gulio* (body weight 8.55 ± 3.762 gm, 9.182 ± 0.374 cm) were caught from a sub-estuary (24º46′ 00″N, 88º14′ 00″E) of the Hooghly Estuary of Indian Sundarbans. All fish (~500 adults) were collected after dark in the second week of July 2017 from a non-mechanically powered boat using drag nets of 2.5 mm mesh size. The salinity at the sampling site ranged from salinity 5.2 to 6.8 and the water temperature was 24.7 ± 1.2ºC during the course of sampling. Fish, once caught in numbers (30-35 individuals per bucket), were quickly transferred into buckets filled with 30 L of water from the sampling site and aerated by battery-operated oxygen pumps. In total 16 buckets full of fish were carried back to the laboratory within 1.5 hours from end of sampling time. No mortality was observed during the sampling and transfer phase.

2.2. Acclimation Phase

Fish were acclimated in four environmental rooms (rooms were alike in shape, size and facilities) inside the animal house facility at the University of Calcutta. These rooms were preset at desired temperature [*i.e*. 20ºC / 24ºC / 28ºC / 32ºC (± 0.5ºC)] required for acclimation and later for experiments. Inside each environmental room a 400L glass aquarium was filled with water from the sampling site and it housed 125 adults of *Mystus gulio*. The water temperature of 400L aquarium was either gradually warmed or cooled with the help of thermostats (SOBO, Japan) so as to maintain the desired temperature [*i.e*. 20ºC / 24ºC / 28ºC, or 32ºC (± 0.5°C)]. Then desired salinities were made by diluting artificial sea salt (SEA SALT, AQUAFOREST, India) with distilled water and kept at ± 0.2 (checked by EUTECH Salt 6+ salinity meter) and filled in several 100L aquaria. These aquaria were placed on the bench tops each filled with salinities of 5, 7, 10, 13, 15 and 20 respectively. Fish were first exposed to salinity of 5, then sequentially transferred to salinity 7, 10, 13, 15, and finally to salinity 20. At each salinity, fish were left for 3 hours before they were moved to a higher salinity. Finally, equal number of fish (*i.e*. 30+ individuals) were kept at four 100L aquarium each having salinity of 5, 10, 15, 20 respectively. In each 100L aquarium fish (*i.e*. 30+ individuals) were left undisturbed till the start of survival experiments.

2.3. Water Quality Maintenance

During acclimation and in the later experimentation phases fish were fed fresh Tubifex (regularly around 4 pm) to their satiation. Each aquarium was cleaned of any excess food, feces, and dead individuals to avoid fouling. pH (measured by Hanna Instruments, India) was kept to ± 0.2 of the field sites (*i.e*. 7.4 to 7.9). NH_3 levels were checked at 12-hour intervals with Test Kits (API, India) and did not change significantly from the initial condition (*i.e.* 0.04 ± 0.01 mg/L). Light-dark conditions of each room were set either at 13 : 11 hrs or 12 : 12 hrs or 11 : 13 hrs to approximate various seasonal conditions.

2.4. Survival Experiments

Survival experiments were conducted at salinities of 5, 10, 15 and 20 at 20ºC, 24ºC, 28ºC, and 32ºC for 72 hours or until 100% mortality (if that occurred earlier than 72 hours). Experiments began ~15 minutes after the end of the acclimation phase. Within an environmental room, 4 glass aquaria (each of 100L volume) at the desired salinities (5, 10, 15, and 20) were pre-placed on the bench tops. 30 fish individuals were housed within each 100L aquarium. Water temperature of all 100L aquaria was gradually brought to the desired level and maintained at that level by placing thermostats inside the aquarium. In summary, the factorial design of the experiment was Salinity Levels [4] $*$ Temperature Levels [4] $*$ Fish Individuals [30] per aquarium; in total 480 adult fish individuals (males and females not separated). At T0 each 100L aquarium (having 30 adult fish) was subjected to salinity tolerance experiments. After 24, 48 and 72 hours from T0 the fish in each aquarium were observed and those ‘Alive’ or ‘Dead’ were counted. At the end of 72 hours of survival experiments, a variable number of fish were alive in the different salinity levels (*i.e.* within each 100L aquarium). Thus, we randomly picked 10 individuals from each salinity level (*i.e.* from each 100L aquarium) and sacrificed them by overdosing Benzocaine (Benzocaine is a powder when dissolved in ethanol and used at a concentration of 80 ppm is a quick and smooth anaesthetic for fish) for Hsp70 assays. These assays were conducted to quantify the constitutive expression of Hsp70 under a given salinity.

2.5. Tissue Collection and Hsp70 Assays

Brain is the central organ that controls and integrates different physiological responses such as osmoregulation which in turn affects stasis and ecological distribution of an organism in a given habitat (Barton, 2001; Weis *et al*., 2001). Fish brain is minimally protected by the blood-brain barrier, therefore, a slight deviation in plasma osmolality is rapidly reflected through changes in volume of the extracellular fluid that surrounds brain cells and affects the physiological response (Gardell *et al*., 2013). Thus, brain protein levels are the crucial indicators of water quality changes (*e.g.* salinity, temperature) that a fish may face in the habitat (Rakhi *et al*., 2013). The brain tissues of fish (10 individuals from each salinity at a given temperature) were rapidly dissected out and freed from extraneous tissues. The collected tissues were washed thoroughly in ice-cold phosphate-buffered saline (PBS), homogenized (20% w/v) with Tris buffered saline (10 mM Tris–HCl, 0.1 mM EDTA-2Na, 10 mM sucrose, 0.8% NaCl, pH 7.4), centrifuged (12000g for 10 min, at 4ºC) and the supernatant was collected. The collected supernatant was stored at –80ºC for future measurements of heat shock protein (Hsp70) and expression of housekeeping genes (â-actin) as control. The protein expression of the samples was measured according to Bradford (1976). For equal loading 100 μg protein from each treatment group was added to sample buffer (under reducing conditions), submitted to 12.5% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotted on the Poly-vinylidene difluoride (PVDF) membrane following a wet electroblotting method (Kinoshita *et al*., 2009). The primary antibody (dilution 1:1000; Anti-Hsp70 antibody; ab2787) was raised against a peptide corresponding to Hsp70 and incubated overnight. The membrane was washed three times in TBST buffer, then incubated with the respective secondary antibody (dilution 1 : 500; Goat Anti-Rabbit IgG Alkaline Phosphatase conjugate; ab6722) for 2 hours. The same membrane was then washed three times in TBST buffer and incubated for 15 min with Premixed BCIP (5-bromo-4-chloro-3-indolyl phosphate)–NBT (nitro blue tetrazolium) solution (Sigma Aldrich) to reveal alkaline phosphatase activity in western Blotting (Seth and Maitra, 2011). Densitometric scanning was performed to quantify individual band intensity of each immunoblot using Image J Software (Gassmann *et al*., 2009).

2.6. Statistical analysis

We evaluated if a salinity level (*i.e*. 5, 10, 15 and 20) after 72 hours of exposure had affected the survival and Hsp70 expressions of adults of *M. gulio* (Fig. 2). Temperature in this exercise was considered as only a proxy to seasonal conditions. We assumed that given a specific

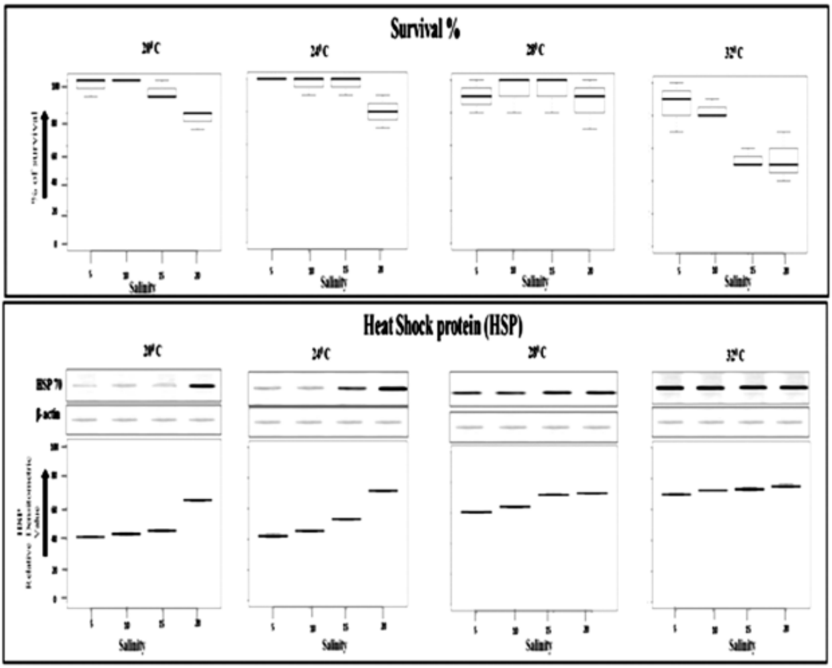

Fig. 2. Percentage of survival and heat shock protein (Hsp70) expression of *Mystus gulio* adults in response to various levels of salinity (5, 10, 15 and 20) stress. Experiments were conducted separately at 20ºC, 24ºC, 28ºC, and 32ºC. For survival experiments n = 30 individuals/salinity treatments and for Hsp70 experiments n = 10 individuals (brain tissue)/salinity-temperature treatments were considered.

temperature, the sole stress factor is salinity so we did not compare the results of different temperature levels to each other. Survival data were not normally distributed (Shapiro-Wilk test: $W = 0.8$, p-value = 0.009) nor were the Hsp70 (Shapiro-Wilk test: $W = 0.69$, $p < 0.001$) data. Thus, Kruskal-Wallis tests were conducted. If the Kruskal-Wallis tests were found to be significant (when $\alpha \leq 0.05$), then further post hoc tests (pairwise Nemenyi tests) were conducted. After post-hoc tests, results were reported relative to the results of salinity 5 (because that approximates *in situ* salinity at the time of fish sample collections). Confidence intervals were set at 95%, the K-W chi-squared values and the P-values were reported along with the Degrees of Freedom (DF) for the statistical significance. For the analysis, PMCMR package of CRAN R 3.4.1 (R Core Development Team, 2018) of Platform: x86_64-apple-darwin15.6.0 (64-bit) was utilised.

3. RESULTS

3.1. Experiments at 20ºC

Salinity affected survival response (DF = 3, K-W chi-squared = 12.89, $P = 0.002$) and Hsp70 (K-W chi-squared = 10.38, DF = 3, $p = 0.01556$) expressions of the adults of *M. gulio*. In specific, experiments run at salinity 20 found significant changes in survival and Hsp70 expressions of *M. gulio*, but not those experiments that were conducted in salinity 5, 10 and 15 (Fig. 2).

3.2. Experiments at 24ºC

High salinity affected survival (DF = 3, K-W chi-squared = 5.86, $P = 0.02$) and Hsp70 (K-W chi-squared = 10.42, DF = 3, $p = 0.01$) levels. Survival was significantly less in salinity 20 than other salinities (*i.e.* 5, 10, and 15). Hsp70 expressions in 15 and 20 PSU differed considerably from each other whereas those for salinity 5 and salinity 10 did not (Fig. 2).

3.3. Experiments at 28ºC

Increasing salinity did not significantly (DF = 3, K-W chi-squared = 0.204, $P = 0.89$) affect survival responses but it did affect the Hsp70 (K-W chi-squared = 10.3, DF = 3, $p = 0.015$) levels of *M. gulio*. Hsp70 expressions for salinity 15 and 20 were considerably higher than those for salinity 5 and 10 (Fig. 2).

3.4. Experiments at 32ºC

Increasing salinity levels affected survival (DF = 3, K-W chi-squared = 7.56, P = 0.01) and Hsp70 (K-W chi-squared = 9.46, DF = 3, p = 0.023) levels. Survival in salinity 5 and 10 were higher than in salinity 15 and 20 (Fig. 2). Hsp70 expressions in salinity 20 was significantly different (P = 0.012) than those in salinity 5, but the remaining salinity treatments did not vary significantly from each other (Fig. 2).

4. DISCUSSION

Adults of *Mystus gulio* demonstrated better survival in the oligohaline to mesohaline conditions. Results indicated that the species thrives (quantified through its survival response and Hsp70 expressions) well in salinity 5 and 10, but struggles to survive beyond salinity 15. The catfish *M. gulio* is known to inhabit tidal lakes and paddy fields near the coasts where salinity could be less than 1 and the species is also known to enter sea during the rainy season in the Indian sub-continent (Pandian, 1966; Mondal and Mitra, 2016). Our results demonstrate the osmoregulation ability of *M. gulio* under various saline conditions and support their occupancy in different coastal-aquatic systems where salinity could vary significantly. Sub-adults of *M. gulio* could manage their respiratory stress well in the oligohaline to the mesohaline conditions (Paul *et al*., 2019). We found less mortality under salinity 5 and 10 that supports the work of Paul *et al*., (2019). Sub-adults of *M. gulio* thrive mostly in autumn when the temperature of estuaries of Indian Sundarban barely exceeds 25ºC (Chaudhuri *et al*., 2013; Mukherjee *et al*., 2012, 2013) and the respiration rates of adult and sub-adult *M. gulio* decreases sharply ≥ 28ºC because such high temperature falls beyond their aerobic scope (Murugaian *et al*., 2008; Paul *et al*., 2019). Our results suggest that the species suffers high mortality at 32ºC which supports the work of previous authors. In the estuaries of SEA high salinity-temperature conditions prevail in summer. Summer may not be a suitable time for the survival and dispersal of *M. gulio* because populations would move towards the upper sections of an estuary and they would be more bottom dwelling to find refuge from hot and saline condition of estuaries (Paul *et al*., 2019). During monsoon, estuaries in the region become more of a homogeneous habitat because the excessive rain reduces salinity gradients so river-sea continuum maintains oligohaline or mesohaline conditions for few days to weeks (Chaudhuri *et al*., 2013;

Mukherjee *et al*., 2013). Mukherjee *et al*., (2012), reported sudden high density of *M. gulio* in the Matla estuary of the Indian Sundarban in 2009 when the said estuary maintained low salinity for long time. Present results and the earlier work of Paul *et al*., (2019) suggest that such conditions are less stressful survival and respiration of *M. gulio*. The catfish *Pylodictis olivaris* had limited salt tolerant ability but it exploits the depressed salinity of its habitat and use estuaries as saline bridges to colonise adjacent coastal-fresh waters in North America (Scot *et al*., 2008). The monsoon in SEA possibly offers *M. gulio* a similar opportunity to invade in the adjacent rivers and find new to newer habitats.

Exposure of a freshwater fish to higher salinity may lead to an increase in plasma-osmolality and result in cellular dehydration (Marshall and Grosell, 2006; Freire *et al*., 2008). Cellular dehydration may result in improper folding or damage intracellular proteins which in consequence may lead to high induction of Hsp70 (Kültz, 2005; Iwama *et al*., 2006; Yang *et al*., 2009). Results show that Hsp70 expressions had no statistically significant relationship in salinity 5 to 10 when temperature ranged from 20ºC to 24ºC. Gutierre *et al*., (2014) found no significant change in Hsp70 expression in the muscles of *Oreochromis niloticus* and *Ictalurus punctatus* with rising salinity levels. These species are known for their large spatial distribution and invasiveness due to their ability to maintain a high constitutive expression of Hsp70 proteins in the face of rising salinity stress (Luft *et al*., 1996; Schofield *et al*., 2011; Gutierre *et al*., 2014). Results also show that Hsp70 expression was particularly high in salinity 20, especially when the temperature was ≥ 28°C, which indicates a fatal physiological consequence for adult *M. gulio*. These results have similarities to those for the catfish *Rhamdia quelen* and *Clarias gariepinus* which show an increase in Hsp70 expression in their muscles when exposed to higher salinities (Gutierre *et al*., 2014). Similarly, a temperature beyond optimal range (*i.e*. 27- to 32ºC) induces higher expression of Hsp70 in *I. punctatus* and *I. natalis* (Fader *et al*., 1994; Luft *et al*., 1996; Weber and Bosworth, 2005). Dispersal of *M. gulio* between adjacent estuaries/rivers may be difficult in warmer seasons when the temperature is ≥ 30ºC and poly-halinity prevails in most estuaries of the region. The salinity of the estuaries of the Mekong delta generally remains between 0-5 ppt in the main rainy season (Le Vay *et al*., 2001). Similarly during monsoon, the lower reaches of the Ganges river and its tributaries stay oligohaline for weeks (Mukherjee *et al.*, 2012, 2013; Chaudhury *et al.*, 2013) and many fish species are reportedly washed down to the Indian Ocean and then re-enter estuaries of the region (Pandian, 1966; Mukherjee *et al.*, 2012, 2013; Chaudhury *et al.*, 2013). During monsoon, *M. gulio* may expand its spatial distribution by invading adjacent rivers by virtue of its better survival chances under less warm and oligohaline conditions of estuaries. In recent years climate change has led to frequent monsoon breaks in SEA and the salinity-temperature amplitudes of the coastal waters are rising in the region (Vecchi and Harrison, 2002). Such may reduce the spatial distribution of *M. gulio* and the species could seek refuge in the relatively cooler upper reaches of estuaries in the region.

ACKNOWLEDGEMENTS

Thanks to Dr. Mohammed Moniruzzaman, Mr. Samya Karan and Miss Saheli Kumar for helping in the various stages of the sampling and laboratory works. Thanks to the Estuarine and Coastal Studies Foundation for providing research facilities.

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