Chapter 1: Introduction

1.1 Background

Climate change driven by human activity has created noticeable changes in the patterns of temperature and precipitation around the world, and it is anticipated that these changes will continue to grow in the future (Meehl et al., 2007). Furthermore, climate change models forecast that more extreme events (such as flooding and prolonged droughts) will increase the seasonal and multi-annual amplitude of water level fluctuations, becoming hydrological stresses in lakes, rivers, and oceans such as extended hydraulic retention time (IPCC, 2014). Climate change also contributes to the rise of sea levels and gradual expansion of salinity in the shoreline areas resulting in salinity incursion in the coastal lands, freshwater rivers, and ponds. The construction of dams in the upstream rivers for hydroelectric power generation and irrigation of farmland enhances the salinity level in the coastal habitat. The agricultural productivity of the land, the viability of agriculture, and long-term food security are all being impacted by salinity intrusion into the coastal areas (Islam et al., 2006). Increased salinity results in an ionic imbalance in salt-sensitive taxa, leading to osmotic stress, which is connected to high mortality or decreased reproduction and growth rates in coastal water environment (Nielsen et al., 2003; Jeppesen et al., 2007; Bezirci et al., 2012). Therefore, it is necessary to evaluate the impact of salinity on the physiological and behavioral responses of coastal aquatic organisms, especially those living in fluctuating salinities.

Salinity is one of the key environmental elements in brackish water environments that affect metabolism and osmotic pressure (Francis et al., 2007; Mustafayev and Mekhtiev 2008; Fazio et al., 2013). Most of the estuarine and marine fish species have an ideal salinity level where development rates are the highest and osmoregulation costs are the lowest which may have an impact on the dispersion of fish in the wild (Blaber, 1997). Several species can withstand varying salinities, and a select few can endure prolonged exposure in such water (Nordlie and Haney, 1998). In addition, the energy required for osmoregulation is not available for growth, and salinity frequently inhibits the growth and physiology of euryhaline organisms (Brett, 1979; Wootton, 2012). Since the circulatory system of fish is closely linked to the outside environment, the hematological profile serves as an excellent predictor of physiological dysfunctions (Elahee and Bhagwant, 2007). Blood physiology also provides information on the physical and chemical characteristics of the water, and the health status of fish, and also helps researchers to evaluate the relationship between environmental variables and determine how sensitive an organism is to changes in the environment (Debala Devi and Usha Anandhi 2010; Ayoola et al., 2011). Toxic impacts of various contaminants can cause physiological stress, resulting in the induction of various genotoxic anomalies and can cause cellular and nuclear abnormalities in different fish species (Gaffar et al., 2021). Therefore, understanding the hematological profile, salinity tolerance, and associated behavioral and physiological responses is critically important to evaluate the optimum growth performance of coastal species.

It is well understood that greater amounts of energy are required in the hypo or hyperosmotic conditions in fish, which might impede growth by altering osmoregulatory and physiological processes (Suzuki et al., 2009; Ostrovski et al., 2011). Previous studies have shown that salinity-induced physiological alterations change the rate of metabolism and patterns of gene expression and are correlated with changes in plasma/serum ion concentrations and osmotic pressure in a number of fish species (Niu et al., 2008; Zhou et al., 2022). Reactive oxygen species (ROS) are produced in significant quantities as a result of the disruption of metabolic equilibrium in the cell membrane caused by salinity stress (Chen et al., 2011). Various antioxidant enzymes, including enzymatic and non-enzymatic, play an important role in regulating redox status and oxidative stress equilibrium by providing defense against free radicals (Gaffar et al., 2021; Li et al., 2022). Additionally, various studies have shown that a higher salinity level causes problems with oxygen intake, irregular respiration, and oxidative stress, all of which can lower antioxidant defense (Paital and Chainy, 2010). Therefore, understanding the physiological mechanisms regulating growth and metabolism in changing salinity conditions is important for estuarine fish species.

Anesthesia can be defined as a state caused by an applied external agent resulting in a loss of sensation through depression of the nervous system. Anesthesia can be a physical or chemical agent that helps to calm the fish down, minimizing stress (Ross and Ross, 2009). During blood sampling, surgical procedures, vaccination purposes, transportation, and spawning procedures fish might get stressed. In aquaculture, routine procedures have been performed in the fish farms and handling must be done during experimental works. If these works are performed incorrectly severe problems may arise in the function of the fish body (Gholipourkanani and Ahadizadeh, 2013; Bowker et al., 2019) which may increase susceptibility to infectious diseases and even cause death (Gimbo et al., 2008). Proper anesthetization could be a possible solution to minimize the stress during aquaculture operations, experimental works, and easy and safe transportation (Mylonas et al., 2005; Ross and Ross, 2009). Sedating fish at the right dose of anesthetic reduces the chance of stress, epithelial damage, or other injuries and makes the process simpler and safer for the handler (Trushenski et al., 2013). Previous studies demonstrate that salinity does not alter the effectiveness of menthol in transportations of juvenile fat snook (Sepulchro et al., 2016). Depending on the fish's habitat, anesthetics can have varied degrees of efficacy during the adaption stages. The relationships between salinity, anesthetic induction, and recovery times in different fish species are not well understood. Therefore, appropriate handling and transportation methods for fish breeding in various salinities that require anesthetics must be established.

Mudskippers are members of the family Gobiidae and subfamily Oxudercinae, and are often referred to as "Gobi fishes or Walking Gobies” Murdy (1989). The Gobi fish can crawl on land using their pectoral fin and are totally amphibious (Swanson and Gibb, 2004). Since they are amphibious animals that have been specifically adapted to intertidal habitats by virtue of their ecology, physiology, and biochemistry (Graham, 1997). The mangrove environments and mudflats of Africa, Madagascar, Bangladesh, India, Southeast Asia, Northern Australia, and Southeast China, Polynesia, Hoga Island in Indonesia, the Bay of Kuwait region of the Arabian Gulf, southern Japan, Samoa and the Tonga Islands, Saudi Arabia are home to the mudskippers. The coastal regions of Australia, New Guinea, and South-East Asia have the highest documented species richness. Due to their burrowing lifestyle, they are found actively jumping, feeding, and associating with other members of the same group and other individuals on mudflats (Randall, 1995). Mudskippers are considered to be economically significant since they are consumed in China, Taiwan (Liao et al., 1973), India (Siddiqi, 1974), and Bombay, they offer an alternative fishery during the monsoon season. Some Malaysians think that the raw flesh of these fishes has aphrodisiac properties (Johnstone, 1903) and they are commonly grown in Taiwan (Chen, 1976). However, mudskippers have only ever been considered intriguing aquarium fish among Europeans (Murdy, 1986).

Mudskippers have evolved both anatomical and behavioral adaptations that enable them to survive both on land and in the water. They often spend the majority of their time out of the water, and therefore possess a wide range of amphibious-specific physiologic, morphological, and behavioral characteristics (Lee et al., 2002; Bucholtz et al., 2009). However, to date, very little is known about the salinity tolerance, and physiological and behavioral responses at different salinity of mudskipper especially from the coastal region of Bangladesh. In addition, relationships between salinity and anesthesia have not been yet studied in any mudskipper species. Therefore, the aim of this study is to know the salinity tolerance, associated physiological responses and effect of salinity in the induction and recovery in a common anesthetic solution (dygenol) in intertidal mudskipper (*Apocryptes bato*).

1.2 Aims and objectives of the study

The aim of this study is to know about the-

1. Salinity tolerance limit of mudskipper (*Apocryptes bato*) at the indoor level;
2. Behavioral and hematological alterations of mudskipper at different salinity; and
3. Relationship between salinity and anesthesia induction and recovery time in mudskipper.

Chapter 2: Review of Literature

2.1 Mudskipper

Apocryptes bato also known as "Chiring" or "Dali Chewa," is one of the most common coastal fishes of Bangladesh that is present all the year-round, primarily in the dry season (Islam et al., 2006). According to Murdy (1989), it is found in the shallow coastal waters of the Bay of Bengal from the east coast of India to Myanmar and the Malay Archipelago. Almost 23 recognized species of amphibious fish from the goby (family: Oxudercidae and subfamily: Oxudercinae) are mudskippers (Nelson et al., 2016). In seven genera—*Periophthalmus, Periphthalmodon, Boleophthalmus, Scartelaos, Pseudapocryptes, Zappa, Apocryptes*—the group currently consists of 34 described species (Larson and Takita, 2004; Jaafar and Larson, 2008). The most varied and extensive genus of mudskippers is *Periophthalmus*. They are distinguished by their peculiar body forms, affinity for semi-aquatic habitats, limited capacity for jumping and mobility on land, and capacity for long-term survival both in and outside of water. The majority of mudskippers are a brownish-green color that ranges from dark to light and can reach lengths of up to 30 cm (12 in). Males acquire vividly colored spots during mating seasons to entice females. These spots might be red, green, or blue.

Figure 1: Mudskipper (*Apocryptes bato*)

Scientific classification

Kingdom: Animalia
Phylum: Chordata
Class: Actinopterygii
Order: Gobiiformes
Family: Oxudercidae
Subfamily: Oxudercinae
Genus: *Apocryptes*
Species: *Apocryptes bato*

2.2 Morphology of mudskipper

2.2.1 Eyes

The dorsally protruding eyes of mudskippers are unique structures among vertebrates. Mudskipper eye muscles are comparable to other fish (Karsten, 1923) but are lengthier because their length exceeds the diameter of their eyeball. They are longer compared to deep-sea fishes.

2.2.2 Skin

The ratio between gill and skin area is a rudimentary way to measuring the contribution of skin to overall respiration (Rauther, 1910; Schottle, 1931). The ability for gaseous exchange can be found in many places and is reliant on proper skin vascularization. Starting with *Periophthalmus argentilineatus*, the species with the highest level of skin vascularization, in the top of the skin bloods flow is greatest. Head placed in the front side and the gill, is diminished in the tail and the body, and is almost nonexistent on the under surfaces (Schottle, 1931).

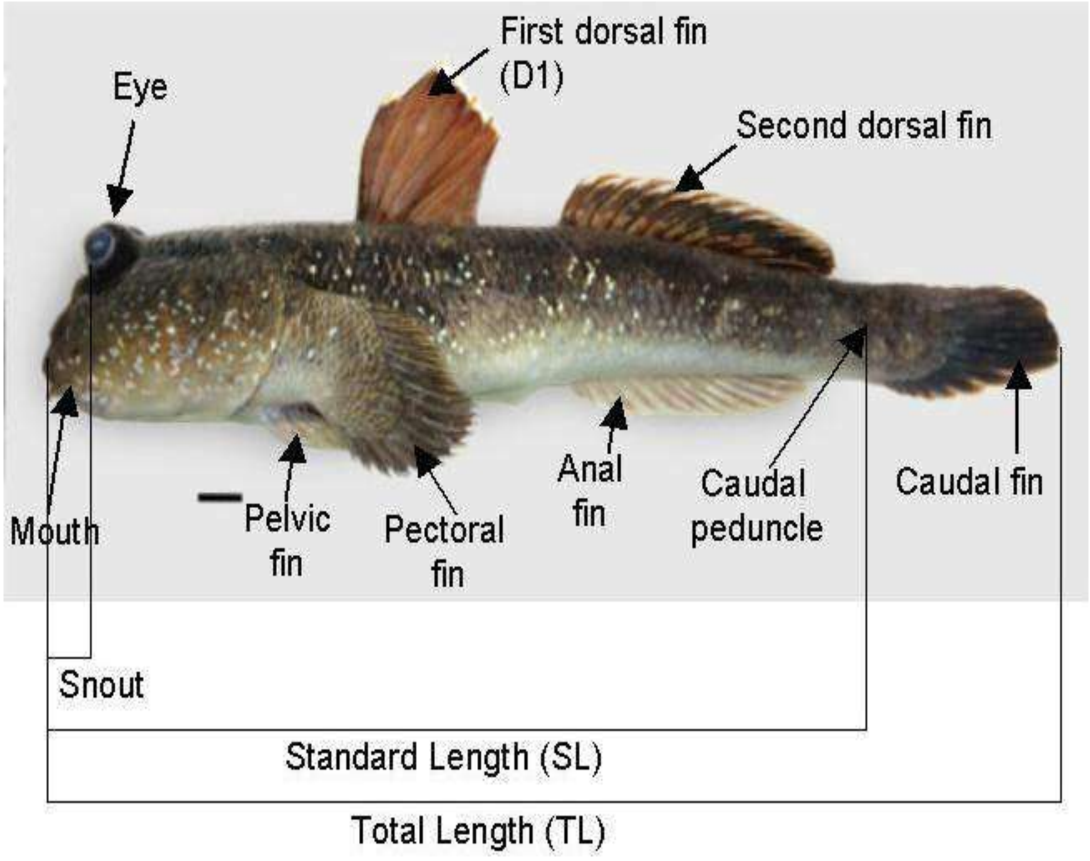

Figure 2: External morphology of mudskipper

2.3 Chromosomes

Examinations of some karyo mitosis chromosomal have been conducted as a supplement to the excessively common morphological technique for the analysis of gobioid fishes; few of these results are described by Nishikawa et al. (1974). There is evidence that *Periophthalmus cantonensis* has a diploid chromosome number of 46 (Nishikawa et al., 1974).

2.4 Sensory and nervous systems

2.4.1 Brain

The basic anatomy of the nervous system of mudskippers has mostly remained to be explore. The dorsal brain regions of *Boleophthalmus boddarti* are depicted (Lim, 1967; Datta and Das, 1980). *Periophthalmus* brain is bigger than that of the *Boleophthalmus*. Patel and Desai (1976) provide microscopic views of the hypothalamic hypophyseal area of the brain which is part of the investigation into frequent alterations in *B. dentatus*.

2.4.2 Alimentary tract or gastrointestinal tract

For *Boleophthalmus boddarti, Periophthalmodon schlosseri* (Lim, 1967), *Periophthalmus vulgaris* (Lim, 1971; Milward, 1974), *Periphthalmodon schlosseri* (Lim, 1971*)*, *Scartelaos histophorus* (Milward, 1974), gastrointestinal tract morphology has been histologically documented. Large symphysial teeth are present in *Scartelaos* and *Oxuderce*s, which are likely utilized in agonistic interactions (Lim, 1967; Milward, 1974; Murdy, 1989). Because canine-like teeth are seen in carnivores such as *Periophthalmus barbarous* (Sponder and Lauder, 1981) and in *Boleophthalmus* (Lim, 1967), pharyngeal plates also reflect nutrition.

2.5 Kidney

The single nephron of the anterior lobes and the renal capsules are paired in the head kidney of the body of *P. waltoni* and are structurally identical to other kidney tubules. The nephronic tubule is divided into three segments by its ultrastructure: two segments of proxima, a distal segment and a collecting duct. Ciliated cells support filtrate migration in the proximal segments. Both here and in the proximal tubules of the body kidney may be phagocytic when acting as a wandering cell (El-Sayed and Safer, 1986). In euryhaline teleost, the distal tubule is occasionally present but typically lacking.

2.6 Habit and habitat

Mudskipper utilizes its pectoral fins to move on land. The fish have intertidal environmental adaptations (both low tide and high tide) and they are quite active when out of water for food and interactions with another mudskipper. They also construct their own profound burrows as a form of protection to keep out any disturbances in their homes. Some common mudskippers developed the majority of terrestrial behaviors and spend 90% of their time in the open environment (Aligaen and Mangao, 2011). Some species only come out at night to rub on algae, evade predators, and avoid hypoxic conditions that might arise in pools at extremely low tides. The mudskippers that live in mangrove swamps are directly impacted by tide changes, and when threatened, they can leap into the open water or swim quickly across muddy land utilizing their powerful pectoral fins (Bob-Manuel, 2012). The streaky rock skipper (*Istiblennius dussumieri*) is more common in rock pools along the coastal regions and mangrove zones than the common mudskipper (*Periophthalmus kalolo*) and barred mudskipper (*Periophthalmus argentilineatus*). The adults live in a variety of sympatric assemblages in habitats ranging from subtidal to high intertidal zones, along with tidal portions of rivers, supratidal ecotones, and freshwater swamps (Polgar and Crosa, 2009).

2.7 Reproduction

The most remarkable behavior is observed in the reproduction of mudskippers which are the storage and maintenance of air within the egg chambers. During the time of active maturation mudskipper spawn once yearly from July to September (gonadal stages IV—VI), which lasts from February to May for males and March to June for females. However, in Korangi Creek (25°N), *Boleophthalmus dentatus* spawns twice in a year, first in April to May and secondly in July to September (Hoda and Akhtar, 2011). The mechanism for the induction of embryonic hatching by actively flooding the chambers by the parental fish, the development of the embryo, and the induction of embryonic hatching therein are all described by egg-guarding parental fish (Ishimatsu et al., 2007). Due to their sexual monomorphism, the morphologies of male and female mudskippers are similar. The male-only exhibits and advertises his sexual activity during breeding season. The mudskippers make lengthy burrows in which they rear their young. The male cares for and protects the eggs by fertilizing and oxygenating them (Ishimatsu et al., 1998). The larvae swim away and float alongside other kinds of plankton after hatching. When they become mature and are able to establish a new territory, the young little mudskippers emerge from hiding and dwell there. Only a handful of the mudskipper's larvae survive out of hundreds of eggs since they are the preferred prey of many predators including crabs. The larvae spend 30 to 50 days at sea before settling in the intertidal zone. The average lifespan of a mudskipper is thought to be five years (Etim et al., 1996).

2.8 Behavior

Numerous brief descriptions of general behavior, including locomotion (De and Nandi, 1984), courting and nesting (Magnus, 1972), aggression, and territoriality (Clayton and Vaughan, 1982), are available. *Periophthalmus sobrin*, an amphibious blenny that lives on rocky coastlines in Madagascar, and *Lophalticus kirkii* share a lot of behavioral traits (Brillet, 1986). There are also significant differences, but if mudskippers from a similar rocky habitat had been investigated (Gordon et al., 1968), these differences might not have been as obvious.

2.8.1 Rhythmic behavior

Gordon et al. (1968) discovered that *Periophthalmus sobrinus* was active at all hours. The latter observations frequently receive support from research on activity rhythms. *P. waltoni* was discovered to be highly diurnal (Al Naqi, 1977). It displayed two activity peaks in the light period under a 12L: 12D regime in the lab, with the greater peak beginning of the period and the lesser peak coming toward the termination of light. A comparable regularity of activity was maintained under both continuous light and darkness, though it was much less pronounced (Eissa et al., 1978). *Boleophthalmus pectinirostris* behavioral span have also been studied (Ishibashi and Nishikawa, 1973).

2.8.2 Burrowing and territoriality

Although mudskippers have been known to dig burrows for a long time (Champeau, 1951), the frequent observations of fish migrating across the intertidal zone in response to shifting tidal levels (Gordon et al., 1968; MacNae, 1969; Gibson, 1982). *P. sobrinus* have two territorial types in Madagascar comprising fish swimming between tall beach burrows on the bank, as well as low coast feeding habitats (Brillet, 1975). In locations away from banking canals, permanent resident territories are more common. The number of tunnel systems within a territory varies depending on the density of fish within it (Brillet, 1975), while *P. waltoni* made many burrow systems within a single zone (Clayton, 1987).

2.8.3 Social behavior

Mudskippers exhibit social behavior that is comparable to other substratum-bound fishes, including the erecting of fins in various configurations and leaping into the air (Gibson, 1982). *Periophthalmus* (Magnus, 1972) and *Boleophthalmus* (Clayton and Vaughan, 1987) both provide descriptions of courtship.

2.9 Adaptations

2.9.1 Behavioral changes

Previously, mudskippers resided in areas that they had created. They continue to offer polygonal, mud-walled territories, which are regarded as a prime illustration of territories. Mudskippers also build mud barriers on all their grounds to prevent adjacent animals from attacking one another. Because diatoms are a major source of food for mudskippers, the mud walls indirect role in preserving populations inside the zones (Al-Behbehani et al., 2010).

2.9.2 Morphological changes

The pectoral fins of the mudskipper are used for skip movement. They have a strong body that they can flip to throw themselves up to two feet into the air. The mouths face downward as they eat on the mud surface and have large, moveable, and protruded eyes. Only the eyeballs protrude from the murky water while they are lying in wait for their prey since they are highly adapted to seeing in the air.

2.9.3 Physiological changes

Mudskippers have a variety of breathing techniques. They can breathe through their skin, the pharynx (the back of their throat), and the mucous lining in their mouth. However, they require constant hydration because they can only take in oxygen through the diffusion mechanism. The mudskipper population is restricted to damp environments exclusively as a result of this behavior, which is identical to the cutaneous air-breathing habit adopted by frogs. Their larger gill chambers, which let them to hold an air bubble, are a significant alteration to their breathing system. While mud fishes have to keep the gills moist so they can work and give oxygen for breathing while they are on land, the huge gill chambers protect tightly (Ishimatsu et al., 1998).

2.10 Environmental influences on behavior

For several species of mudskippers, aggressive intraspecific and interspecific behaviors have been observed. Various degrees of stances, fin displays, fighting, and avoidance are used to express agonism (Nursall, 1981; Brillet, 1986). Instead of using physical barriers, *Scartelaos histophorus* seems to use a complicated set of behaviors to minimize agonistic confrontations. Two elements, physiological needs (such as evaporation, heat stress, energy requirements, and reproduction) and time restrictions (such as tide state, time of day, and predator activity), affect the types and frequency of behaviors displayed by *S. histophorus.* According to Milward (1974), *S. histophorus* was restricted to surface pools at low tide and temperatures between 15 and 35 °C. According to this study, there was no discernible variation in pool time spent in relation to temperature or weather. Instead, rolling around in the mud seems to be the animal's primary means of avoiding dehydration. The locomotion abilities of *Scartelaos histophorus* included crawling, swimming, and skipping. When the creatures were exposed, crawling predominated and was comparable to the "crutching" described for *Periophthalmus koelreuteri* (Harris, 1960). In *S. histophorus*, skipping was the second most often observed style of locomotion. It was utilized to advance quickly, frequently at the conclusion of an agonistic interaction. According to Milward (1974), this behavior has also been seen in other species of mudskippers including *Periophthalmus koelreuteri*, *P. gracilis* and *P. vulgaris.* Three physical factors—tide, time of day, and temperature—were discovered to have an impact on skipping behavior. Skipping likely uses a lot of energy, and the amount of time spent engaging in this behavior might be constrained by physiological as well as external variables.

2.11 Behavioral responses in mudskipper (*Periopthalmus papilio)* exposed to sodium bromide under laboratory conditions

P. pappillis, displayed increased skin pigmentation after being subjected to acute sodium bromide concentrations. According to Anderson et al. (1988) in addition to scales in scaly fish, a layer of mucus (glycoproteins, proteoglycans, and proteins) creates the contact between the skin of the fish and the outside environment. Mucus-secreting cells continuously replace the layer, and the rate might go up in reaction to an illness, certain chemicals, or physical irritants. In this experiment, exposed fish had more mucus coating their bodies and gills than the control group did. The creation of mucus is a reaction to an irritant like fish using sodium bromide as one of their defense mechanisms against harm, however, this has serious consequences, namely for the gills since this reduces oxygen absorption. On exposure to petroleum compounds, *C. gariepinus* was shown to produce more mucus due to the mucus cells' enhanced activity (Gabriel et al., 2007).

2.12 Salinity tolerance of mudskipper

The simplest and most basic way to gauge how mudskippers react to variations in salinity is to look at percent mortality. According to (Mansuri and Bhan, 1978), *Periophthalmus dipe* mortality is high in seawater concentrations of 50% and 70% but low in 100% and 0% seawater. In contrast, the highest mortality in *P. chrysospilos and Boleophthalmus boddarii* at salinities of 0% and 100% and 8% mortality in 50% and 80% seawater (Chew and Ip, 1990). Despite the fact that *B. dussumieri* had a higher survival rate than *Periophthalmus dipes* at all salinities. Mansuri et al. (1982) found that mortality in both species was highest in 50% and 70% seawater. Furthermore, both species fared better in winter (12–16°C) than during the summer season (22–25°C). The significant disparity between the two data sets must be balanced for *Periophthalmus* (Mansuri and Bhan, 1978). Mansuri et al. (1982) provided that all fish perished within the dry and winter seasons, contrary (Mansuri and Bhan, 1978). The gap is even more startling results in summer and winter survival shows because the fish rambled better at the temperature is high (27–28°C), (Mansuri and Bhan, 1978).

Apocryptes bato is one of the mudskipper species found in the tropical rivers, estuaries and coastal waters of the Indian Ocean. To date, several scientists have worked on the length-weight relationships and food and feeding habits (Rahman et al., 2016), reproductive biology (Ahamed et al., 2018), effects of sub-lethal doses of DDT on the liver (Islam et al., 2006). The effects of the organophosphorus pesticide of *Apocryptes bato* on behavioral pattern and mortality were studied by Islam et al. (2001). However, to date, very little is known about the salinity tolerance and physiological modulation and appropriate doses of anesthesia of this species. Therefore, this study was undertaken to know the behavior, physiology, survival and response to anesthetic at different salinity of a mudskipper species *(Apocryptes bato*) from the Chattogram coast of Bangladesh.

Chapter 3: Materials and Method

3.1 Experimental fish and sample collection

Mud skipper (*Apocryptes bato*) of both sexes (5.36 ± 1.12) were collected from the local fisherman from the Foillatoli Bazar, Halishahar, Chattogram. Apparently healthy, disease-free, and similar-sized mudskippers were chosen for this study. The study was conducted at the wet laboratory of the Department of Fish Biology and Biotechnology, Chattogram Veterinary and Animal Sciences University (CVASU), Chattogram.

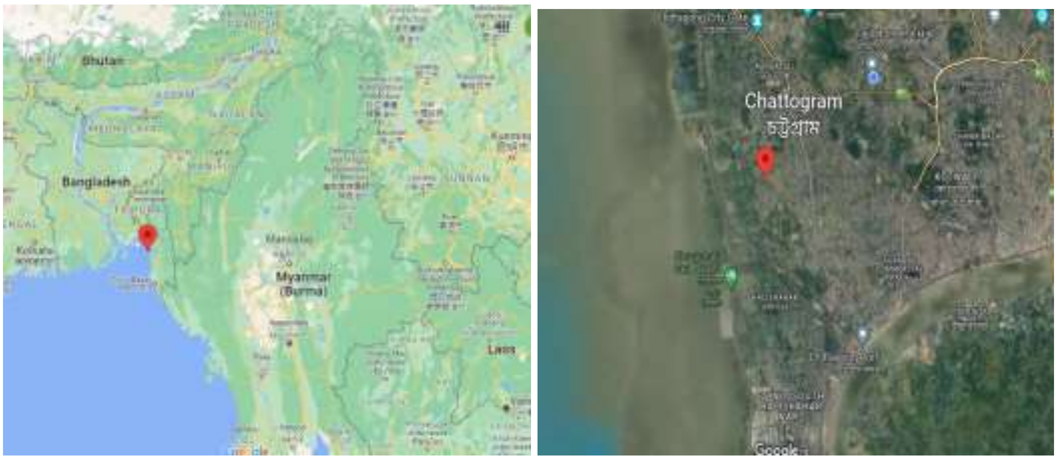

Figure 3: Sampling sites of mudskipper

3.2 Acclimation of experimental fish

Collected live fish were brought to the wet laboratory in plastic buckets troughs containing only seawater and maintaining aeration. They were acclimatized under laboratory conditions in small aquaria with aeration at room temperature for 1 day and providing appropriate water (at 10 ppt salinity) . During acclimation in each aquarium, 20 fish were stocked. Fishes were fed with frozen shrimp once a day *ad libitum*. Unused feeds were regularly cleaned to maintain good water quality in the aquarium. Each aquarium was also monitored with an individual camera (DVR-M01D, China) to observe their behavior in the aquarium.

3.3 Experimental design for studying the induction and recovery pattern of mud skipper after anesthesia at different salinities

In this experiment, dygenol solution (100ppm) was used as an anesthetic agent in mud skipper. The induction and recovery pattern of mud skipper in dygenol solution was studied at five different salinities such as 5ppt, 10ppt, 12ppt, 15ppt, and 18ppt. The required amount of dygenol solution was added by using a micropipette in the following seawater to get the desired concentration of anesthesia (100 ppm). The dygenol solutions were mixed by stirring with a spoon until they were completely dissolved in different saline water to get the different concentrations of the anesthesia-saline solution. After the preparation of different anesthesia-salinity water and then ten (10) fishes were transferred one by one in the following solutions. The induction and recovery times were recorded for each individual fish at different salinities.

Figure 4: Experimental design for anesthesia in different salinity level. A) Taking water by using measuring cylinder, B) taking anesthesia solution (dygenol) using micropipette, C and D) mixing the anesthesia with the water properly by using a spoon, E) taking out the fish from the anesthesia solution, F) measuring length, G) measuring weight, H) keeping the fish in the recovery tank.

3.4 Behavioral observation of mudskipper at different salinities in the aquarium conditions

To observe the land-use pattern at different salinities in the aquarium, healthy and apparently active mudskippers were collected from the Chattogram coast of Bangladesh. Mudskippers were acclimatized for 7 days in the laboratory conditions. Four different aquariua were prepared with the following salinities 5ppt, 10ppt, 12ppt and 15ppt. In addition, a layer of mud was also provided maintaining a slope of 30-degree angle to facilitate the mudskipper staying on both land and water (Figure 5). In each aquarium, 10 mudskippers were stocked and a video camera (DVR-M01D, China) was installed to observe their land-use pattern, locomotion, jumping, and feeding behavior was recorded at different salinities. The fish were fed with small shrimps once a day about 2% of their body weight.

Figure 5: Experimental design for behavioral observation of mudskipper

3.5 Growth performance study

After twenty-one days of rearing in the aquarium conditions, weight gain, % weight gain, specific growth rate (SGR %/day), feed conversion ratio (FCR), feed conversion efficiency (FCE) and survival (%) were calculated on the basis of the following formulae:

- Weight gain = Final weight − Initial weight
- Percent weight gain = (Final weight − Initial weight) ×100
- (SGR%/day) = 100 × (In W_n-In W_{n-1})/t
- FCR = Amount of feed fed/ Total weight gain
- FCE = Total weight gain/ Amount of feed fed
- Survival (%) = 100*(final no. of shrimp / initial no. of shrimp)

3.6 Measurement of hematological parameters

In this study, several hematological parameters like red blood cell (RBC), white blood cell (WBC), glucose, hemoglobin (Hb), and cholesterol were measured from the mudskipper at the end of 21 days of rearing period from different salinities (5, 10, 12, and 15 ppt) (Figure 6). Blood glucose was measured immediately after collecting blood using glucose strips through a digital blood glucose meter (eB-P01, Taiwan). Hemoglobin and cholesterol were measured immediately after blood collection using strips of a portable digital hemoglobin and cholesterol assay machine (ET-321 & ET-

321 BT, Taiwan). A hemocytometer was used to calculate the RBC and WBC numbers under a light microscope (ISH500) (Schaperclaus et al., 1991).

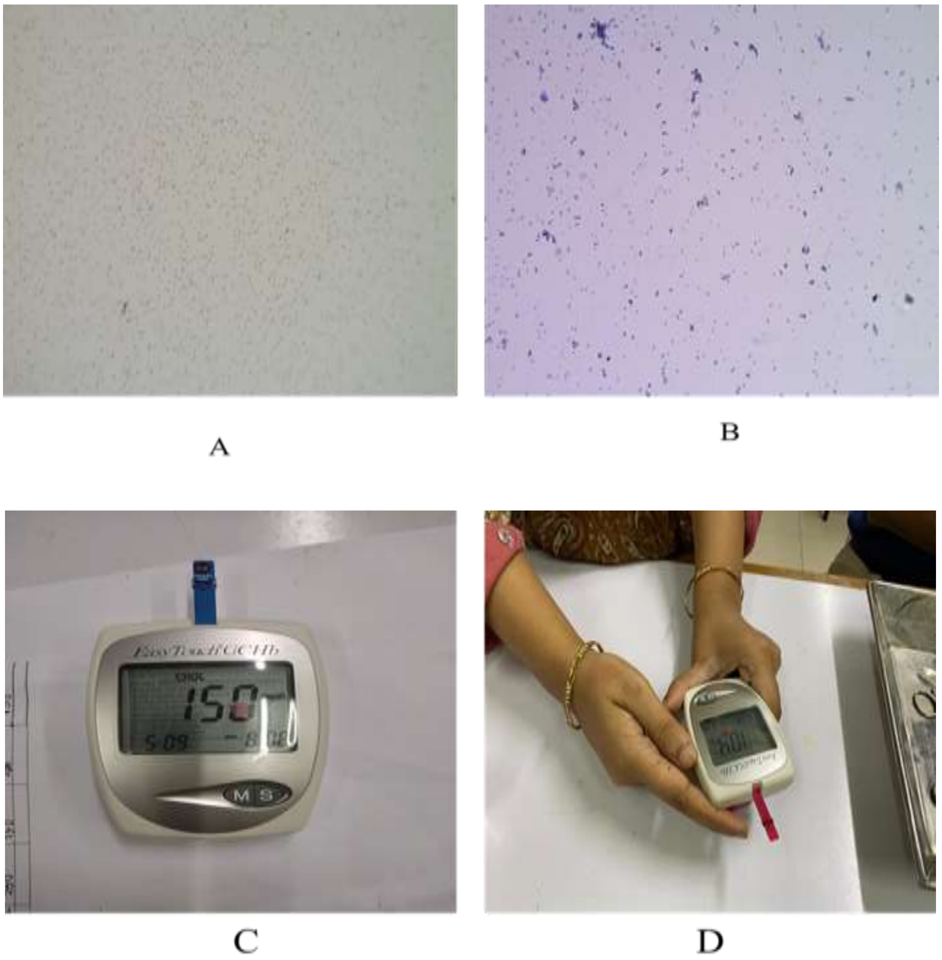

Figure 6: Measurement of hematological parameters a) RBC, b) WBC, C) Cholesterol d) Glucose

3.7 Analysis of cellular and nuclear abnormalities of erythrocytes

Different cellular and nuclear abnormalities like twin cells, tear-dropped cells, binuclear cells and tri-nuclear cells were observed under a light microscope attached to a camera (DVR-M01D, China). At first, a drop of fresh blood was smeared in a clean and grease-free slide immediately after collection. The slide containing blood was then air dried for 10 min, fixed using methanol solution and finally, stained using 5% Giemsa solution. The slides were then washed using running tap water and air dried overnight and finally mounted using dibutylphatalate polystyrene xylene (DPX) until further analysis of erythrocytes (Steinhagen et al., 1990). A total of 1000 cells were counted from each slide different cellular and nuclear abnormalities were classified as per

Carrasco et al. (1990) and a score was provided for each of the abnormalities by following the criteria adopted by Al-Sabti and Metcalfe (1995).

3.8 Statistical analysis:

Values are presented as mean ± standard deviation of the mean (SD). The one-way analysis of variance (ANOVA) followed by Tukey's HSD post-hoc test was performed to observe the statistical difference among the different salinities. Statistically significant values were set $p < 0.05$ unless described anywhere in the text. All statistical analysis was performed using Microsoft excel and SPSS v. 23.

Chapter 4: Results

4.1 Anesthesia induction time of *Apocryptes bato* at different salinities

In the present study, the anesthesia induction time was measured in the similarly sized (15.27 ± 1.13 cm) *Apocryptes bato* at 200pm concentration of dygenol (Eugenol solutions) at different salinities (5 ppt, 10 ppt, 12ppt, 15ppt, and 18 ppt). Results showed that an increase in salinity also increases the anesthesia induction time in mudskippers. It was found that the longest induction time (8.11 ± 0.69 min) was needed to anesthetize the fish at 18ppt salinity using 200 ppm of dygenol concentrations (Figure 7). On the other hand, the shortest induction time (1.61 ± 0.73 min) was recorded for the 5 ppt saline water at the same doses of dygenol concentration. The anesthesia induction time was 4.5 ± 0.92, 4.08 ± 1.12, and 3.44 ± 1.12 min for 10, 12, and 15ppt salinity respectively (Figure 7). Statistically significant differences were found in the anesthesia induction time among different concentrations of saline waters in the mudskipper (Figure 7).

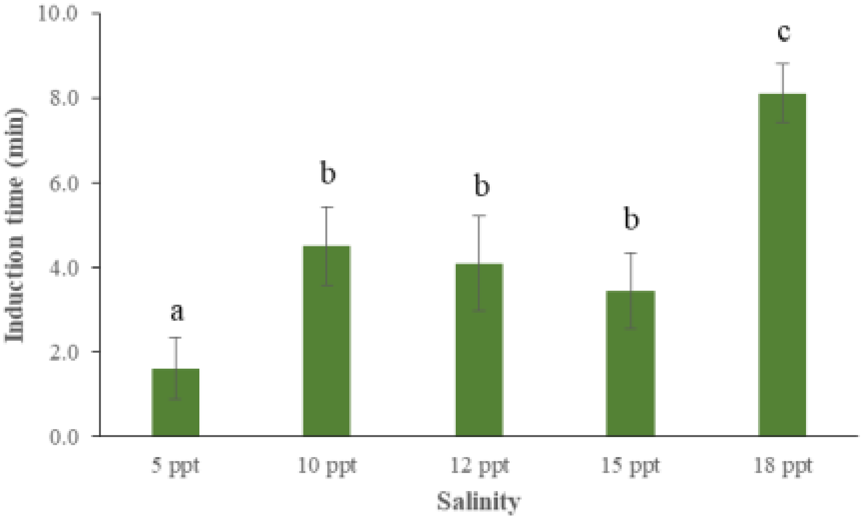

Figure 7: Anesthesia induction time of dygenol in *Apocryptes bato* at different salinities (5, 10, 12, 15, and 18 ppt). Values are presented as mean ± standard deviation (SD) of the mean (n = 10 for each treatment group). Different letters of the alphabet indicate the statistically significant differences among groups (Tukey's HSD post-hoc test, $p < 0.05$).

4.2 Anesthesia recovery time of *Apocryptes bato* at different salinities

The anesthesia recovery time followed the pattern of anesthesia induction in mudskipper. The highest recovery time (12.86 ± 2.64 min) was needed for *Apocryptes bato* at 18ppt salinity and the lowest recovery (6.63 ± 1.21 min) was recorded at 5ppt salinity at 200ppm dygenol concentration. Besides, for 10ppt, 12ppt, and 15ppt salinity, the recovery time was 7.52 ± 1.77, 6.94 ± 1.83, and 10.41 ± 2.22 min respectively at 200 ppm dygenol solution (Figure 8). Statistically significant differences were found in the anesthesia recovery time among different concentrations of saline waters in the mudskipper (Figure 8)

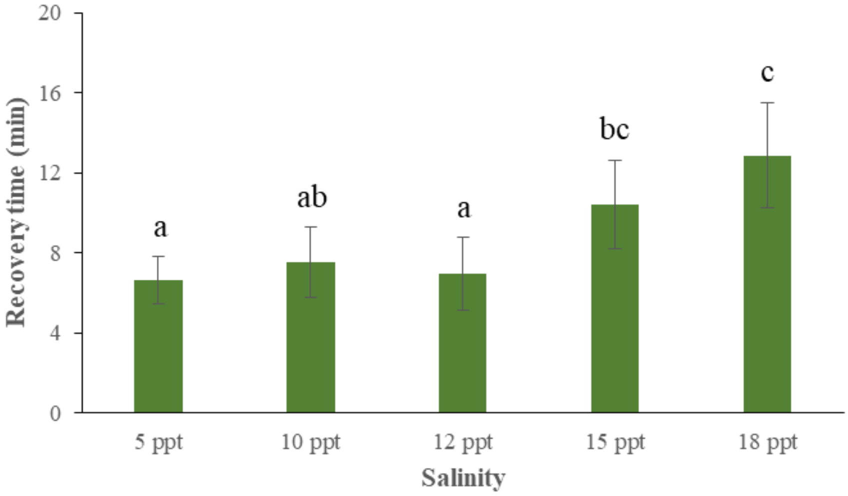

Figure 8: Anesthesia recovery time of dygenol in *Apocryptes bato* at different salinities (5, 10, 12, 15, and 18 ppt). Values are presented as mean ± standard deviation (SD) of the mean (n = 10 for each treatment groups). Different letters of the alphabet indicate the statistically significant differences among groups (Tukey's HSD post-hoc test, $p < 0.05$).

4.3 Behavioral response of *Apocrptes bato* at different salinities

The land-use pattern, locomotion, jumping, and feeding behavior of mudskipper was studied at different salinities (5, 10, 12 and 15 ppt) for a period of 72 hrs. in the aquarium conditions. It was observed that mudskippers stayed on the land during night time and in the daytime, they stayed in the saline waters. The time spent in the land and saline waters did not vary significantly according to the salinity of the aquarium. While staying in the mud, they make holes and channels. Sometimes, mudskipper jumped in to escape from the aquarium, however, the number of jumps did not vary significantly depending on the salinities. In addition, lower feed intake was observed at salinity 5 and 15 ppt by the mudskipper in compared to the fish from salinity 10 and 12ppt.

4.4 Growth performance of mud skippers reared at different salinity

In the present study, mudskipper was reared at different salinities (5, 10, 12 and 15 ppt) for a period of 21 days. Results showed that the higher weight gain, % weight gain, specific growth rate (SGR) and feed conversion ratio (FCR) was found in fishes reared at 10 and 12ppt salinity compared to the fish reared at 5 and 15 ppt (Table 4.1). A statistically significant difference ($p < 0.05$) in weight gain, SGR, and FCR was observed among different salinities (Table 4.1)

On the other hand, the relatively higher and similar feed conversion efficiency (FCE) was recorded in mudskipper reared at 5 and 15 ppt salinity of (0.85 ± 0.20 and 0.86 ± 0.36 respectively). The FCE was 0.54 ± 0.11 and 0.53 ± 0.11 for fishes reared at 10 and 12 ppt respectively. Statistically significant differences were observed in the FCE values for mudskipper reared at different salinities (Table 4.1).

Table 4.1: Growth performance of mud skippers reared at different salinity.

Growth parameters	**Salinities**			
	5 ppt	**10 ppt**	**12 ppt**	**15 ppt**
Initial weight (g)	5.26 ± 1.47	5.62 ± 0.47	4.98 ± 1.18	5.56 ± 1.36
Final weight (g)	5.69 ± 1.49	6.34 ± 0.49	5.76 ± 1.10	5.88 ± 1.40
Weight gain (g)	0.43 ± 0.09^{a}	0.72 ± 0.12^{b}	0.78 ± .017^{b}	0.32 ± 0.11^{a}
% weight gain	6.91 ± 3.69^{a}	12.80 ± 0.44^{ab}	16.94 ± 6.37^{b}	5.57 ± 2.35^{a}
Specific growth rate (%/day)	0.14 ± 0.07^{ab}	0.25 ± 0.04^{bc}	0.32 ± 0.11^{c}	0.12 ± 0.05^{a}
Feed conversion ratio (FCR)	1.23 ± 0.26^{ab}	1.79 ± 0.30^{bc}	1.96 ± 0.42^{c}	1.14 ± 0.39^{a}
Feed conversion efficiency (FCE)	0.85 ± 0.20^{ab}	0.54 ± 0.11^{a}	0.53 ± 0.11^{a}	0.86 ± 0.36^{b}

Values of a single anomalies in a row with different alphabetical superscripts are significantly ($p < 0.05$) different. All values expressed as mean ± SD.

4.5 Blood physiology of mud skipper reared at different salinity

Changes in the hemato-biochemical parameters were recorded from the mudskipper under different salinity conditions (Table 4.2). Blood samples were collected to measure red blood cells (RBC), white blood cells (WBC), hemoglobin (Hb), glucose, and cholesterol after 21 days of rearing at different salinities. The blood glucose level was significantly higher (4.1 ± 0.60 mg/dL) in fishes reared at 15 ppt salinity followed by 5 ppt (3.07 ± 0.64 mg/dL), 10 ppt (2.73 ± 1.26 mg/dL) and 12 ppt (2.68 ± 0.73 mg/dL) indicating a stressful condition of fish reared at 15 and 5 ppt salinity (Table 4.2). A relatively higher but statistically non-significant increase in RBC and WBC was observed in fishes reared at 5 and 15 ppt compared to the 10 and 12 ppt. In addition, there were no statistically significant values found in the case of Hb and cholesterol under different salinity conditions.

Table 4.2: Hemato-biochemical parameters of mud skipper reared at different salinities

Blood parameters	**Salinities**			
	5 ppt	**10 ppt**	**12 ppt**	**15 ppt**
Red blood cell (RBC) (cells/mm^3) (×10^6)	2.2 ± 0.87	1.78 ± 0.43	1.79 ± 0.53	2.21 ± 0.62
White blood cell (WBC) (cells/mm^3) (×10^4)	3.8 ± 0.28	3.4 ± 0.52	3.4 ± 0.10	4.0 ± 0.25
Glucose (mg/dL)	3.07 ± 0.64^{ab}	2.73 ± 1.26^{a}	2.68 ± 0.73^{a}	4.1 ± 0.60^{b}
Haemoglobin (Hb) (g/dL)	11.9 ± 1.34	11.6 ± 0.83	11.10 ± 0.66	11.70 ± 1.13
Cholesterol (mg/dL)	185.0 ± 3.61	200.3 ± 38.6	223.7 ± 83. 2	164.3 ± 20.7

Values of a single anomalies in a row with different alphabetical superscripts are significantly ($p < 0.05$) different. All values expressed as mean ± SD.

4.6 Cellular and nuclear abnormalities of erythrocytes of mudskipper reared at different salinities

In this study, the cellular and nuclear abnormalities of erythrocytes were observed from mudskipper reared at different salinities. A number of cellular and nuclear abnormalities including a) normal erythrocytes, b) twin cell, c) nuclear budding, d) micronucleus, e) binucleus, f) triplet cell, g) tear- dropped cell, h) multiple nucleus, i) dead cells were found in the erythrocytes of mud skipper reared at 5 and 15 ppt salinities indicating cellular and metabolic dysfunction in very high or very low salinity.

However, the frequencies of abnormalities in the erythrocytes were absent or lowered in fishes reared at 10 and 12 ppt (Figure 9)

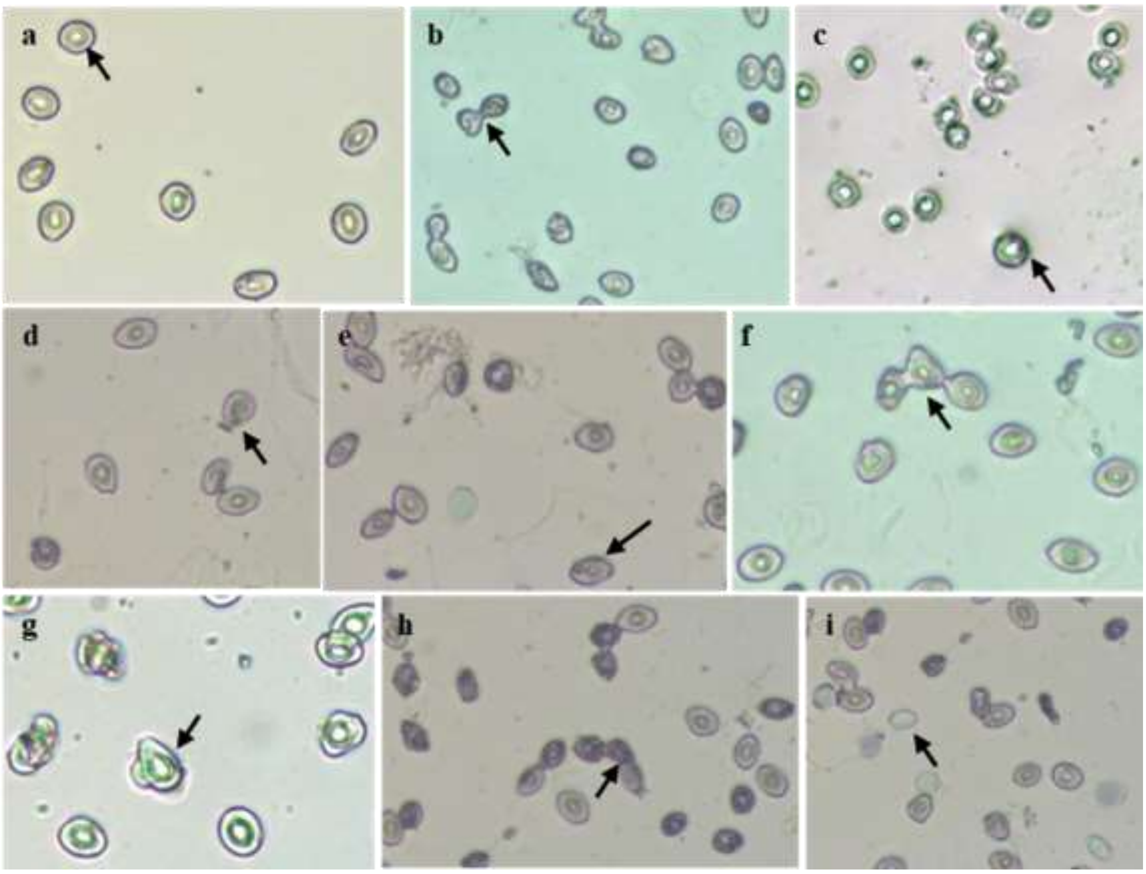

Figure 9: Several cellular and nuclear abnormalities in the erythrocytes of mudskipper under different salinity condition; a) normal erythrocytes, b) twin cell, c) nuclear budding, d) micronucleus, e) binucleus, f) triplet cell, g) tear- dropped cell, h) multiple nucleus, i) dead cells

Chapter 5: Discussion

In the present study, behavioral, physiological, and hematological analysis was conducted in *Apocryptes bato* at different salinity conditions. The findings of the present study indicate that mudskipper differentially responds to their physiology and growth at varying salinities. The findings of the present study may be helpful in understanding the salinity tolerance and the regulation of growth of this intertidal mudskipper species.

5.1 Anesthesia induction and recovery time of *Apocryptes bato* at different salinity

In this study, the anesthesia induction and recovery time was determined for the *Apocryptes bato* by using dygenol at 200ppm doses. It was observed that the same doses of anesthesia at different salinity condition appeared to have a statistically significant difference on the induction and recovery pattern of this species. In addition, there was no mortality found in this study, indicating that all fish were used in this study, successfully recovered after anesthetization which further confirms the efficacy of the dygenol as potential anesthetics. Though there are several studies on anesthesia on different fishes in different countries, no studies have been done on mudskipper (*Apocryptes bato*) in Bangladesh. So far, this study is the first record of anesthesia induction and recovery which may insights an appropriate method to get the mudskipper (*Apocryptes bato*) tranquilized using dygenol (eugenol solutions) as anesthetics during laboratory handling and other routine works.

There are some basic requirements for being a good anesthetic in aquaculture use. The ideal anesthetic should take less than 3 min to anesthetized and less than 5 minutes to recover, non-toxic for fish and hatchery operators, no residual effects, and be inexpensive (Mylonas et al., 2005). In this study, only 100 ppm dose of dygenol solution has been tested and the average induction time is about 4 minutes and average recovery time is about 7 minwhich is very much optimum for an ideal anesthetic. Further studies with an increment of doses needs to be tested to confirm the best suited doses for dygenol in this species. Furthermore, environmental (e.g., temperature, pH, hardness, and salinity) and biological parameters (e.g., size, weight, fat content, and fish species) may influence anesthetic effectiveness of anesthesia (Ross and Ross, 2008; Weber et al., 2009). In goldfish, salinity does not have any impact on the induction and recovery pattern at tricaine methanesulfonate (MS-222) anesthetic (Küçük and Çoban, 2016). However, Ghazilou et al. (2010) discovered higher induction times at higher water salinities in Caspian salmon (*Salmo trutta caspius*) utilizing clove oil-induced anesthesia, implying that the increased times were due to the "salting out" phenomena which is similar to the findings of the present study.

5.2 Behavioral response of *Apocrptes bato* at different salinities

In the present study, behavioral activities were observed in the aquarium conditions at four different salinities such as 5ppt, 10ppt, 12ppt and 15 ppt. Results showed that mudskippers stayed on the land during nighttime and stayed in the saline waters during the daytime indicating nocturnal feeding and locomotion behavior of *Apocryptes bato*. Under a 12L:12D regime in the lab, *Periophthalmus waltoni* showed two activity peaks in the light period, with the greater peak arriving at the onset of the period and the smaller peak occurring toward the close of the light (Al Naqi, 1977). Mudskippers build their own deep tunnels to keep away any disruptions in their homes and spend 90% of their time out in the open (Aligaen and Mangao, 2011). In the aquarium, *Apocryptes bato* also burrow in the mud and make holes and channels that facilitates them to hide during the daytime. However, the number of holes and tunnels did not vary significantly in each aquarium. Brillet (1975) described that that the number of tunnel systems within a territory varies depending on the density of fish within it. In this study, similar number of fish was stocked in each aquarium which might be the possible reasons of parallel number of holes and tunnels in this study. In addition, lower feed intake was observed at salinity 5 and 15 ppt by the mudskipper in compared to the fish from salinity 10 and 12ppt. Sutton et al., (2018) investigated that mangrove fish were exposed to an acute change in salinity significantly decreased the oxygen consumption in response to a 10% decrease in salinity and increased when salinity was elevated by 30%. Since mudskipper are euryhaline species they can tolerate higher range of salinity. No abnormal behavior was seen in the medium during the entire period of acclimation to different salinity.

5.3 Growth performance of mud skippers reared at different salinity

Because of increased osmotic pressure at higher salinities, stenohaline fishes have lower survival rates with increasing salinity (Kilambi and Zdinak, 1980). It is commonly understood that fish demand for greater amounts of energy in hypo or hyperosmotic circumstances, which could inhibit growth (Tsuzuki et al., 2007; Herrera et al., 2009). Due to possessing high alkalinity, hardness, and electrical conductivity, saline water is a rich source of monovalent and divalent ions. Fish may need to expend more energy to maintain osmotic balance in environments with low or extremely high salinities to maintain an internal equilibrium level of salt-water in fish body. It has been found that salinity ≥ 9 ‰ has a negative impact on goldfish growth (Altinok and Grizzle, 2001). Salinity levels > 6 ‰ resulted in higher mortality and a lower development rate in *Heteropneustes fossilis* fingerlings (Ahmed et al., 2016). In the present study, higher growth performance was found in mudskipper reared at 10 and 12 ppt compared to the fish at 5 and 15 ppt. The highest possible percentage of survival was recorded in 10‰ seawater environment for mudskipper (Huong et al., 2009) which is similar to the findings of the present study.

5.4 Blood physiology of mud skipper reared at different salinity

Blood parameters are frequently used to evaluate the functional states of blood oxygen-carrying capacity, influenced by environmental conditions and are the most crucial ecological indicators in exogenous and endogenous alterations in fish under physiological stress (Cataldi et al., 1998; Shah and Altindag, 2004). In the present study, significantly higher level of glucose was detected in fishes reared at 5 and 15 ppt salinity. Glucose is the primary source of energy for the central nervous system, which is controlled by hormones. A rise in glucose levels may be related to increased gluconeogenesis in stressed fish as they try to meet their new energy demands (Winkaler et al., 2007). In addition, elevated level of RBC and WBC was also recorded in mudskipper at 5 and 15 ppt salinity. Whereas no significant changes were observed in case of Hb and cholesterol in this study. Variations in haematological profile may be linked to osmoregulatory dysregulation caused by salinity changes (Fazio et al., 2013). The effect of salinity on hematobiochemical parameters varies depending on the salinity ranges, period of exposure, and adaption capability of each particular species (Jahan et al., 2018). Furthermore, increases in WBCs are associated with leucocytosis, which is thought to be of adaptation value for tissues that are under biochemical stress and can be associated with an increase in antibody generation, which aids in the survival and recuperation of the stressed fish (Joshi et al., 2002). Previous studies on Nile tilapia, silver barb and rainbow trout also displayed similar alterations in the haematological profiles (Sahafi et al., 2013; Amin et al., 2016).

5.5 Cellular and nuclear abnormalities of erythrocytes of mudskipper at different salinities

This study showed the cellular and nuclear abnormalities of erythrocytes when mudskippers exposed to different salinities. Islam et al., (2020) investigated that erythrocyte cellular and nuclear abnormalities during the high temperature tolerance under normoxic and hypoxic conditions in Nile tilapia. Ghaffar et al., (2015) reported that the failure of the hematopoietic system may be the cause of the production of micronuclei, cellular abnormalities, and nuclear abnormalities at the CTmax under hypoxia and normoxia. It is also widely known that hazardous compounds alter cell metabolism, ion permeability, and transmembrane structure, which results in architectural damage to erythrocytes. These structural alterations could be producing more lipid peroxidation chemicals in erythrocytes (Bai et al., 2014; Gaffar et al., 2015; Sadiqul et al., 2016). In this study, greater number of cellular and nuclear abnormalities at 5 and 15 ppt might be due to the lipid peroxidation and alterations in the cell membrane structure.

Chapter 6: Conclusions

This study on mudskippers exposed to various salinity conditions reveals a significant correlation between salinity levels and both behavioral patterns and hematological indices. The observed changes in behavior, such as how many drains and holes they make, how many species were in the water and land, how long time in the land and water, swimming patterns, food intake, and jumping activities, along with the hematological alterations in blood includes different abnormalities faced by mudskippers under these conditions. This study also includes anesthetic concentrations of dygenol solution (100 mg/L) which can be used for anesthetization of *Apocrptes bato*. This study also found that, with the increase of the salinity, the anesthesia induction and recovery time were increasing. These findings emphasize the significance of maintaining stable salinity environments to ensure the habitat and health of mudskipper populations. Further research is warranted to better comprehend the underlying mechanisms driving these responses and their potential implications for the broader aquatic ecosystem. It should now be clear that mudskippers might be a valuable resource for comparative studies on adaptation to the littoral ecosystem.

Chapter 7: Recommendations and future perspectives

Recommendations

- This experiment was conducted in a range of salinity levels to understand the threshold of tolerance and behavioral changes in mudskippers. Diverse salinity ranges including freshwater, brackish water, and marine water should also be tested.
- Mudskipper behavior under different salinity conditions, focusing on activities like swimming, feeding, burrowing, and territorial behavior should also have monitored.
- Anesthetizing with different other anesthesia agents should also be tested.
- Histopathological examinations on key organs such as gills, liver, and kidneys to identify any cellular or tissue-level changes in response to varying salinity levels need to be examined.

Future perspectives

- ✓ To explore the genetic basis of mudskipper's adaptability to various salinity levels.
- ✓ To compare the findings with other amphibious species to uncover evolutionary trends in adaptations to changing salinity environments.
- ✓ To investigate how mudskipper behavior and health impact the broader ecosystem, including their role in nutrient cycling.
- ✓ To use the gained knowledge to develop conservation strategies for mudskipper populations in areas prone to salinity fluctuations due to climate change

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