CHAPTER: 1

INTRODUCTION

Aquaculture, which refers to the cultivation of aquatic organisms, is the most dynamic and highly promising sector of agriculture. The productivity and profitability of aquaculture relies on the growth performance of cultivable species. Utilizing modern technology and methods, intensification increases fish production while simultaneously reducing costs is the prime goal of aquaculture. However, it can potentially harm to the aquatic environment and the health status of fish. For instance, fish raised under stress in intensive cultivation may lead to lower growth and reduced disease resistance (Yarahmadi et al., 2015; Yousefi et al., 2016). Furthermore, temperature is just one of the several environmental factors that have an impact on the complexity of aquaculture growth and production. Temperature is a key regulator of metabolic activity which exert an influential role in the ability to survive and physiological processes of all living beings (Portner and Farrell, 2008). Previous studies demonstrated that temperature-related stress has a significant impact on the biochemical processes, energy metabolism, and growth in fish (Islam et al., 2019, 2020a). Hence, an understanding related to the physiological responses to temperature is important to get better aquaculture output in the modern aquaculture system.

In recent years, an intense level of climate change susceptibility has been shown by the significant interaction between global aquaculture and the surrounding ecosystem (Reid et al., 2019). The effects of global warming and subsequent climate change on the ecosystem integrity, as well as the rise in water temperature brought on by the changing environment could lead to a potential drop in fish abundance (Portner and Peck, 2010; Cheng et al., 2013; IPCC, 2014; Shahjahan et al., 2013, 2017). Nevertheless, rising temperatures do not influence all aquaculture species and fish physiological functions, particularly growth, reproduction, and metabolism, may be variably regulated by temperature depending on the species and habitat (Shahjahan et al., 2017; Rahman et al., 2019; Islam et al., 2019, 2020b; Kuhn et al., 2023). For example, during a production cycle within an optimal temperature, a slight rise in the average temperature may have a favorable effect on the growth of certain cultivable species of fish (Reid et al., 2015; Islam et al., 2019; Ashaf-Ud-Doulah et al., 2020; Islam et al., 2020a). Moreover, some species can be able to compensate for their

growth, and those cultured in lower temperatures may eventually grow more quickly than those maintained in environments with higher temperatures (Sfakianakis et al., 2013). Exposure to stressful conditions leads to a multitude of alterations in the gene expressions, growth, blood physiology, gill morphology, and metabolism of fish (Islam et al., 2019, 2020a, 2020c; Shahjahan et al., 2021). An appropriate temperature at any step of growth processes within an aquaculture system can therefore be identified by using molecular research, which offers a feasible option in modern aquaculture for a specific species. Hence, it is crucial to comprehend the fundamental mechanisms by which environmental temperature (in response to climate change effect) regulates the growth, physiological mechanism, and reproduction in an aquaculture system.

Heat shock proteins (HSPs) comprises a wide variety of proteins that are crucial for maintaining protein balance within cells, safeguarding cellular integrity, and promoting cell viability under different physiological and metabolic stresses (Lanneau et al., 2008). HSP play a vital role in responding to stressful conditions, highlighting their importance in fish physiology (Han et al., 2017; Junprung et al., 2019). The usual balance between physiological functions can be disrupted by thermal stress in organisms, which could lead to potential death (Aleng et al., 2015). To counteract with this stress, the metabolic processes are frequently altered in order to restore metabolic balance quickly and efficiently and HSP proteins play an important role in coordinating these metabolic alterations (Brocchieri et al., 2008). HSPs, which are expressed by a family of several genes, subdivided on the basis of their functions, amino acid sequence homologies, and molecular weight into five different families, such as- (a) small HSPs family consisting of HSP27 (also known as HSPB), (b) HSP60 (HSPD), (c) HSP70 (HSPA), (d) HSP90 (HSPC), and (e) HSP110 (also known as HSPH) (Dubrez et al., 2019). These HSP families are widely distributed and expressed in various cellular compartments. Under normal circumstances, these proteins are synthesized when fish experience temperatures exceeding their typical ranges. However, their specific functions within the cell can vary based on the particular HSP and the physiological context (Yan et al., 2017; Dubrez et al., 2019). Fish are poikilothermic and serve as optimal models for the regulation of HSPs due to their natural exposure to thermal variations as a consequence of climate change and other

intricate stress factors within their native habitat. Thus, they provide a valuable vertebrate framework for exploring the functions and expressions of heat shock proteins.

The HSP70, being the extensively researched and most conserved HSP compared to other HSPs includes proteins with molecular weight spanning from 66–78 kDa. The HSP70 proteins are encrypted by a multi-gene family comprising a minimum of 11 genes (Shi and Thomas, 1992). Within the HSP70 family, there are a total of 13 members. Apart from their function in facilitating the proper folding of recently synthesized proteins, they possess the capability to refold and disassemble misfolded substances, as well as dissociate protein complexes using ATP as an energy source (Clerico et al., 2019; Luengo et al., 2019). A variety of stressors, including alterations in temperatures have the potential to trigger the activation of the *hsp70* in diverse fish species (Blair and Glover, 2019; Zhou et al., 2020). On the other hand, HSP90, the 90 kDa protein found abundantly in eukaryotic cells. It plays a substantial impact on multiple cellular processes, such as protein degradation and folding, signal transduction, and morphological evolution. The notable representatives of the HSP90 are HSP90α and HSP90β which are vital for ensuring the survival of the eukaryotic cells. These proteins are consistently present in significant amounts, constituting around 1−2% of cellular proteins in the absence of stress. Additionally, their expression levels can be further enhanced in response to stressful conditions (Sreedhar et al., 2004). Fluctuations in temperature result in the initiation of *hsp70* and *hsp90* genes in a number of fish (Wu et al., 2012; Shahjahan et al., 2021). Due to their high conservation across species and their rapid upregulation in reaction to adverse environmental circumstances, HSP70 and HSP90 act as appropriate molecular indicators for assessing temperature-related stress (Luft et al., 1996). In order to anticipate production and development of fish particularly in a changing climate, it is crucial to understand the relationship between high temperature, the potency of stress response, the involvement of HSPs in cellular processes, and growth in an aquaculture system.

In a changing climatic environment, controlling the consumption of food and managing the energy balance is crucial for animals to ensure an adequate amount of energy to sustain their well-being and proper development. Both the brain and peripheral tissues produce hormones that participate in the regulation of food ingestion by interacting with feeding centers in the brain, either stimulating or inhibiting the act of feeding (Volkoff, 2019).

Ghrelin, a peptide hormone, has a notable function in the endocrine regulation of energy homeostasis. It stimulates the secretion of growth hormones by interacting with the GH secretagogue receptor (Kojima et al., 1999). Recently, the genetic and peptide composition of ghrelin have been documented in various fish species (Unniappan et al., 2002; Parhar et al., 2003; Kaiya et al., 2003a, b). In fish, *ghrelin* mRNA is expressed predominantly in the intestinal tract with lower amount detected in the brain, kidney and gills. Ghrelin plays a pivotal role in governing multiple physiological functions in fish, including the triggering of food intake and the regulation of pituitary secretion of hormones. It may also have neurological effects preventing eating and drinking behavior (Unniappan and Peter, 2005). An increase in temperature can lead to changes in the expression of the ghrelin gene, causing it to be elevated or reduced. This alteration in gene expression is ultimately accountable for the decline in appetite and the impaired growth observed in fish (Hevroy et al., 2012; Kuhn et al., 2023).

The Nile tilapia (*Oreochromis niloticus*), belonging to the family Cichlidae, is a euryhaline species that can be found in a diverse habitat including fresh and brackish water. It is one of the most significant commercially important fish species that can be grown worldwide due to its faster growth and excellent nutritional value. The global tilapia farming industry is undergoing a dynamic phase of expansion to satisfy the needs of both domestic and international markets. Following carp, tilapia have now become the second most widely cultivated group of fish worldwide (Wang and Lu, 2016). This fish is a significant source of sustenance, employment, and income-generation in Bangladesh. Due to its hardiness, adaptability as a cultural subject, lack of muscular bone, and greater marketability, this fish is rising in popularity day by day. Additionally, this type of fish can be reared in high stocking densities and adapts to a wide range of culture techniques. Recent studies showed that increase in temperature influences growth, hemato-biochemical parameters, cellular and morphological abnormalities of erythrocytes in this species (Islam et al., 2020a). A few studies have also included the expression of heat shock proteins under stressful conditions (Delaney and Klesius, 2004). However, there is a scarcity of research regarding the impact of elevated temperatures on the expression of *hsp70, hsp90,* and *ghrelin* genes and their influence on the growth process in *O. niloticus*. Determination of the impact of acclimation temperature through the expression of *hsp70, hsp90* and *ghrelin* genes will help to

comprehend the molecular mechanisms to facilitate higher growth which will ultimately improve productivity and contribute to the economy of Bangladesh.

1.1 Objectives of the study:

- To investigate the expression patterns of *hsp70*, *hsp90*, and *ghrelin* genes in *O. niloticus* under varied temperature conditions;
- To ascertain the level of stress response at different acclimation temperatures in *O. niloticus*; and
- To understand how increased temperature can alter the growth of *O. niloticus*.

CHAPTER: 2

REVIEW OF LITERATURE

Global warming is a leading concern among global climate-changing trends, which provoke a significant challenge in the 21st century. As fish are poikilothermic animals, they are unable to circulate warm blood across their bodies and are consequently extremely vulnerable to thermal stress (Gabillard et al., 2006; Purohit et al., 2014). The stimulation from the brain and the production of stress hormones such as cortisol and catecholamine result in an explosion of the neuroendocrine system and a subsequent cascade for biochemical and physiological changes in fish (Zahangir et al., 2015). Here, in this review, studies related to the molecular physiological responses to temperatures in relation to the heat shock proteins (HSP) and ghrelin system in fish are discussed below.

2.1 Heat shock proteins (HSPs):

2.1.1 History of HSP discovery

Heat shock proteins (HSPs) constitute a cluster of remarkably conserved cellular proteins that are present in both prokaryotic and eukaryotic cells and participate in essential cellular mechanisms (Kiang and Tsokos, 1998). As implied by their name, cells subjected to sub-lethal heat shock experience the stimulation for the release of HSPs. The initial documentation of HSPs date back to 1962 when an experiment involving *Drosophila* salivary gland cells (Ritossa, 1962) exposed to a temperature of 37°C for 30 minutes before being returned to their regular temperature of 25°C to assist recovery. As a result of this treatment, there was an observable activation of certain genes known as “gene puffing” in the chromosomes of the recovering cells (Ritossa, 1962). The distinct attributes of HSPs became apparent during the decade following Ritossa's initial observations, as different researchers outlined unique features of this protein category. Berendes et al. (1965), Ashburner (1970), and Tissieres et al. (1974) demonstrated that the heat-triggered segments were also found in *Drosophila* salivary gland chromosomes. Tissieres et al. (1974) showcased that these proteins were not restricted exclusively to the salivary glands but also recorded the existence in other regions of the fruit fly (*Drosophila*), including the brain, Malpighian tubules, and wing marginal discs. Definitive confirmation that specific HSPs have their origins in genes located within the chromosome puffs became evident in the late

1970's. The discovery that isolated mRNA has the ability to attach to heat shock puffs when introduced into in vivo systems resulted in the development of several HSPs (McKenzie et al., 1975; Spradling et al., 1977; Mirault et al., 1978). Through the utilization of these purified fractions of heat shock mRNA, the cloning of HSP genes marked one of the early accomplishments in the realm of eukaryotic genetic research (Iwama et al., 1998).

2.1.2 Nomenclature and functions of HSPs

The nomenclature of HSPs typically relies on their molecular weight (measured in kilo Daltons, kDa), as established through sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Comprehensive research conducted on model organisms has unveiled three primary categories of heat shock proteins: HSP90 (size range 85–90 kDa), HSP70 (ranging from 68 to 73 kDa), and heat shock proteins with lower molecular weights (measuring between 16 and 47 kDa). Additionally, heat shock proteins are categorized depending on their roles, DNA sequences, and cross-reactivity with antibodies (Morimoto et al., 1994; Hightower, 1991). These proteins serve fundamental functions that are essential to different types of protein metabolism in the non-stressed cell. HSP70 is recognized for aiding in the proper folding of newly formed polypeptide chains, functioning as a molecular chaperone, and playing a role in the restoration and breakdown of modified or unfolded proteins (Kiang and Tsokos, 1998). HSP90 is involved in assisting multiple elements of the cytoskeleton and receptors for steroid hormones (Csermely et al., 1998; Pearl and Prodromou, 2000). The heat shock proteins with lower molecular weights possess a variety of functions that vary depending on the species. In contrast to other HSPs, these proteins lack any recognized inherent function and are exclusively activated under stressful conditions (Ciocca et al., 1993). In fish, the activation of families of HSPs has been documented in cell lines, initial cell cultures, and various tissues extracted from whole organisms (Iwama et al., 1998). These results suggest that the intracellular physiological reaction to stress undoubtedly supports the survival and well-being of the fish under stressed condition.

2.2 Molecular mechanisms related to of HSP expression:

The active heat shock transcription factor (HSF), which binds to promoter called heat shock elements (HSE) that are situated in front of the heat shock genes, is primarily responsible

for the transcriptional level initiation of HSPs. Notably, the absence of introns in the key heat shock genes permits fast translation of messenger RNA into new proteins right after contact to the stressful event (Morimoto et al., 1992).

Experimental evidence has provided support to a conceptual framework proposing the control of HSP expression through proteotoxicity model (Morimoto et al., 1996). This model is grounded in the observation that denatured or non-native proteins serve as potent stimuli for inducing HSPs. Heat shock factor (HSF1) exists either as a monomer (Westwood et al., 1991) or as part of a heterodimer (Baler et al., 1992) combined with HSP70 within the cytoplasm of cells that are not under stress. The presence of a stress-inducing factor attracts HSP70 and other chaperone proteins which facilitating repair or removal of impaired or misfolded proteins.

2.3 Isolation of *hsp70* and *hsp 90* genes from fish:

Genes encoding HSPs in fish have been successfully characterized from very few species. For instance, *hsp70* has been cloned from various fish species (Kothary et al., 1984; Arai et al., 1995; Lele et al., 1997; Lim and Brenner, 1999; Molina et al., 2000). Elevated levels of mRNA corresponding to these genes have been observed as a reaction to heat stress for several of these fish species. In the red blood cell of rainbow trout, it was found that a segment of HSP70 is linked with the HSP90 (Currie and Tufts, 1997). Mammals possess two closely linked *hsp90* genes (alpha and beta), both have been sequenced from zebrafish, and has been demonstrated that they experience distinct patterns of regulation during embryonic development (Krone and Sass, 1994). The total genetic sequence of a *hsp90a* gene has been successfully isolated from the chinook salmon (*Onchorhynchus tshawytscha*) species. Researchers examined the activity of this gene in a cell line derived from chinook salmon embryos and found that it is responsive to heat, being induced under elevated temperature conditions (Palmisano et al., 2000).

2.4 Temperature induced gene expression of *hsp70* and *hsp90* in fish:

Comprehensive studies have revealed that fluctuations in temperature can trigger the activation of *hsp70* and *hsp90* in different fish species. Following the application of a heat shock treatment (37°C), the mRNA levels of *hsp70* exhibited a remarkable and statistically significant increase in varied type of tissues including the liver, kidney, spleen, and brain

of *Channa argus* from Jialing Rivers System of China (Zhou et al., 2020). Another study has revealed that peripheral blood leukocytes of channel catfish display a typical heat shock response, wherein the heat shock genes that remain dormant under normothermia (23°C) is induced upon exposure to heat shock conditions (37°C). Moreover, it was noted that the pre-existing synthesis of specific molecules was arrested under this condition (Luft et al., 1996).

Labeo rohita exhibited distinct variations in the expression of two heat shock protein genes (*hsp70* and *hsp90*) under different acclimation temperatures. Specifically, the mRNA level of *hsp70* was significantly elevated solely at the highest acclimation temperature of 36°C compared to the fish raised at 30°C and 33°C. Conversely, the *hsp90* mRNA showed a significantly higher level both at 33°C and 36°C in comparison to 30°C (Shahjahan et al., 2021). In the case of grass carp (*Ctenopharyngodon idella*), quantitative analysis through RT- PCR revealed that *hsp90* was expressed at relatively decreased level across various exposed tissues and displayed an up-regulation in response to both cold shock (4°C) and heat shock (34°C) (Wu et al., 2012).

Another experiment on the expression of HSP from the liver tissue of *Channa striatus* showed that, fish inhabiting the runoff of hot springs demonstrate a continuous expression of heat shock proteins, which can be simulated in farmed fish through prolonged induction of HSPs when their environmental temperature is elevated to 36°C (Purohit et al., 2014).

Elevated expression of *hsp70* was also observed in Nile tilapia (*Oreochromis niloticus*) and *O. mossambicus* where these fish were subjected to a heat stress period lasting 5.5 hours. This study reveals that subjecting tilapia fish muscles to stress, specifically through temperature variations either above or below the optimal conditions within a specific timeframe, results in the production of the HSP70 in a distinct manner (Joseph and Sujatha, 2010). El-Sappah et al. (2017) also observed the elevated expression was exhibited in the liver during cold shock treatments in both male and female fish when *O. niloticus* fish were subjected to various temperature conditions for a brief duration. The reaction of *hsp* genes varies depending on the tissue, specific families of HSPs, and types of the stressors (Kayhan and Duman, 2010). However, there is a paucity of research examining the

influence of higher acclimation temperatures on fish growth in relation to the expression of *hsp70* and *hsp90* where the mRNA is collected from brain tissue.

2.5 Ghrelin:

The term "ghrelin" originates from the root word "ghre", which signifies "growth", highlighting its capacity to trigger the release of growth hormone (Kojima and Kangawa, 2005). Ghrelin was first identified as an organic compound that binds to the growth hormone secretagogue (GHS) receptor in the stomachs of both rats and humans (Kojima et al., 1999). Ghrelin is primarily a peptide comprising 28 amino acids, with a modification involving the addition of a fatty acid (Kojima and Kangawa, 2005). Ghrelin mRNA shows higher levels of presence in the stomach and intestine whereas lower levels are observed in various remaining organs including the pituitary, brain (primarily the diencephalon), head, kidney, lung, pancreas, and placenta (Kojima et al., 1999; Mori al., 2000; Date et al., 2002). Ghrelin is currently recognized as a versatile peptide engaged in the modulation of food consumption and maintenance of energy balance in organisms (Ueno et al., 2005). Studies have indicated that *ghrelin* is comprised of four exons and three introns in human (Wajnrajch et al., 2000) and in rats and mice *ghrelin* possess an extra non-coding exon of 19 base pairs (Tanaka et al., 2001).

2.6 Ghrelin in fish:

Among fishes, the arrangement of the *ghrelin* gene sequence and the structure of exons and introns have been documented in a number of fish species (Unniappan et al., 2002; Kaiya et al., 2003c; Parhar et al., 2003). The *ghrelin* in seabream, tilapia and goldfish consist of four exons and three introns. Conversely, in rainbow trout the *ghrelin* gene encompasses five exons and four introns, mirroring the pattern identified in rats, mice and human.

A growing study evidenced that *ghrelin* plays a remarkable role in the feeding, metabolic processes, and reproductive functions of fish. It triggers food consumption, which in turn supplies crucial nutrients for development of fish along with the necessary energy required for metabolic activities and reproduction (Unniappan et al., 2004). In goldfish, a study was commenced to investigate the variations in preproghrelin mRNA expression and serum ghrelin levels during pre-meal, post-meal, and starvation conditions. Additionally, it explored the impact of administering ghrelin through intracerebroventricular and

intraperitoneal routes on goldfish food intake (Unniappan et al., 2004). Ghrelin could potentially contribute the alterations in appetite and metabolic functions that align with the body's response to stress such as an increase in temperature above the optimal range.

2.7 Temperature induced expression of *ghrelin* gene in fish:

Previous study found that temperature influences species-specific changes in the expression of appetite regulators and feeding behavior in Characidae fish (Kuhn et al., 2023). In accordance with another study, ghrelin portrays a role in the occurrence of self-imposed reduced appetite (anorexia) in Atlantic salmon reared in an environment where sea temperature was higher (Hevroy et al., 2012). Atlantic salmon were kept at various temperatures for up to three months, and those subjected to the highest temperature displayed inferior growth and reduced consumption of food. A decline in ghrelin signaling might be responsible for the observed drop in hypothalamic growth hormone secretagogue receptor 1a- like receptor (GHSR 1a- LR) mRNA expression, along with diminished levels of factors that regulate appetite, ultimately resulting in decreased appetite. This finding implies a connection between *ghrelin* and the stress response mechanism. Elevated temperatures that aren't ideal can increase the metabolic rate in fish, potentially inducing stress and a corresponding decrease in feeding and growth. These reactions might be strategies to conserve energy (Hevroy et al., 2012).

2.8 Ghrelin in Nile Tilapia:

Identification of single nucleotide polymorphisms (SNPs) in the *ghrelin* gene in Nile tilapia (*O. niloticus*) indicates that a specific polymorphism at the ghrelin locus (C-226T) is connected to improved growth in this species. This finding showed that the specific locus could be used to identify and select breeding stock in the future to improve the development rate and quality of farmed fish (Chen et al., 2020). However, the underlying mechanisms through which changes in temperature affect appetite in this fish are still not fully understood. Therefore, our study will be helpful in unveiling the effects of high temperature-induced expression of *ghrelin* gene as a stress indicator during the growth process of Nile tilapia.

Though there are few studies related to the HSPs and ghrelin system in some fish species, very little is known regarding the relationships between temperatures and the HSP and

ghrelin system in Nile tilapia. Therefore, this study is undertaken to know the effects of acclimation temperature through the expression of *hsp70, hsp90* and *ghrelin* genes to understand the molecular mechanisms that will facilitate higher growth in this commercially important fish species.

CHAPTER:3

MATERIALS AND METHODS

3.1 Experimental fish:

Juvenile *O. niloticus* (9.0 ± 0.5 cm and 5.8 ± 1.34 gm) of both sexes were obtained from the Bangladesh Fisheries Research Institute (BFRI) and acclimated in the fish ecophysiology laboratory of the Faculty of Fisheries, Bangladesh Agricultural University (BAU), Mymensingh. They were placed in a 500L tank with flowing fresh water for a duration of 30 days, maintaining the natural photoperiod of L:D at 14:10 hours in the laboratory conditions. Continuous aeration was provided to ensure optimal oxygen levels. A commercial diet with 35% crude protein content was fed to the fish twice daily up to the apparent satiation.

Figure 1: Experimental fish (*O. niloticus*)

3.2 Experimental design:

In this study, *O. niloticus* juveniles (n = 20) were reared in the nine glass aquaria (with a volume of 150 L and dimensions of 75cm ×45cm × 45cm) which were filled with 100L of freshwater. The *O. niloticus* juveniles were acclimatized and exposed to three different acclimation temperatures (31°C, 34°C and 37°C) for a period of 30 days. The aquarium water temperature was raised gradually (Δ1°C/12h) from the control temperature (31°C) to the target temperatures (34°C and 37°C). The stocked *O. niloticus* were fed a commercial diet which contains a protein content of 35% twice in a day, at 9:00 and 17:00, until they reached apparent satiation.

Aquarium for 31°C Aquarium for 34°C Aquarium for 37°C

Figure 2: Aquarium set up for different experimental temperature

3.3 Sample collection:

Fish were anesthetized with clove oil (5mg/L) on the 15th and 30th days of the temperature exposure period and fish were weighed (BW in gm) individually to measure their growth performance under three different temperature conditions.

Figure 3: Measurement of body weight

The whole brain of *O. niloticus* was collected (n = 12, males-6 and females-6) and soaked in RNAlater (Ambion, Austin, TX) and stored at 4°C for 24hr. After 24hr, collected brain samples were transferred to the Molecular Biology and Biotechnology laboratory of the Chattogram Veterinary and Animal Sciences University (CVASU), Chattogram and stored at −80°C for further analysis.

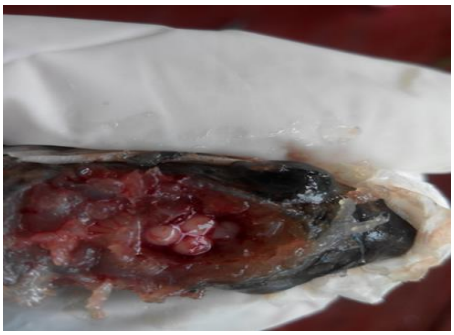


Figure 4: Collection of brain sample from fish

3.4 Growth performance analysis:

The growth performance of fish including weight gain (g), percent weight gain (%) and specific growth rate (%/day) were estimated by utilizing the following equations:

Weight gain = final weight (g) − initial weight (g)

$$\text{Weight gain (\%)} = \frac{\text{final weight(g)} - \text{initial weight (g)}}{\text{initial weight(g)}} \times 100$$

$$\text{Specific growth rate (SGR, \% per day)} = \frac{\ln \text{final weight(g)} - \ln \text{initial weight(g)}}{\text{number of days}} \times 100$$

3.5 Blood glucose measurement:

After an exposure period of 15 days at different temperatures, blood samples were collected from the caudal peduncle of fish. Blood glucose level was assessed instantly after collection of blood with the help of glucose strips (Digital blood glucose monitoring system, EasyMate® GHb).

Figure 5: Measurement of blood glucose level

3.6 Water quality parameters measurement:

Different water quality parameters like pH, free CO_2, dissolved oxygen (DO), ammonia (NH_3) and total alkalinity were recorded at day 15. Free CO_2 was estimated by employing titrimetric method with the help of NaOH titrant and phenolphthalein indicator. DO and pH was measured by using a portable DO meter (DO 5509, Taiwan) and pH meter (RI02895, HANNA Co.) respectively. Total alkalinity was measured by titrimetric method, using H_2SO_4 titrant and methyl orange indicator. NH_3 was measured by test kit solution (Sera ammonia test kit, Germany) and the color was compared with a standard color chart for ammonia (mg/L) as supplied by the manufacturer.

Figure 6: Measurement of water quality parameters

3.7 Extraction and quantification of RNA:

Total RNA was extracted from the entire brain of *O. niloticus* using TRIzol™ reagent (Invitrogen™, Thermo Fisher, USA) as per manufacturer's guidelines. In this process, 1ml TRIzol™ reagent was used for individual brain samples to precipitate RNA. After homogenization of the individual sample by using a homogenizer (Stuart, SHM15130, USA), chloroform was added and the homogenate was allowed to separate by centrifugation into a transparent upper level containing RNA. Then, the RNA was

precipitated at the bottom of the tube by adding isopropanol and centrifugation. The precipitated RNA was washed to remove impurities using 75% ethanol. The sample was then vortexed and centrifuged for 5 minutes at 7500xg at 4°C and the supernatant was discarded and finally, the RNA pellet was air dried by using a desiccator. Later, the pellet was resuspended with 0.5M EDTA pH 8.0 and incubated at 55°C and it was then stored at −80°C until further use. RNA integrity and quality were checked by a ratio of 260/280 using a nanodrop spectrophotometer (Nanophotometer NP80, Germany) and values ~2.0 used for the preparation of cDNA. The list of equipment and reagents required for RNA isolation is presented in Table 1.

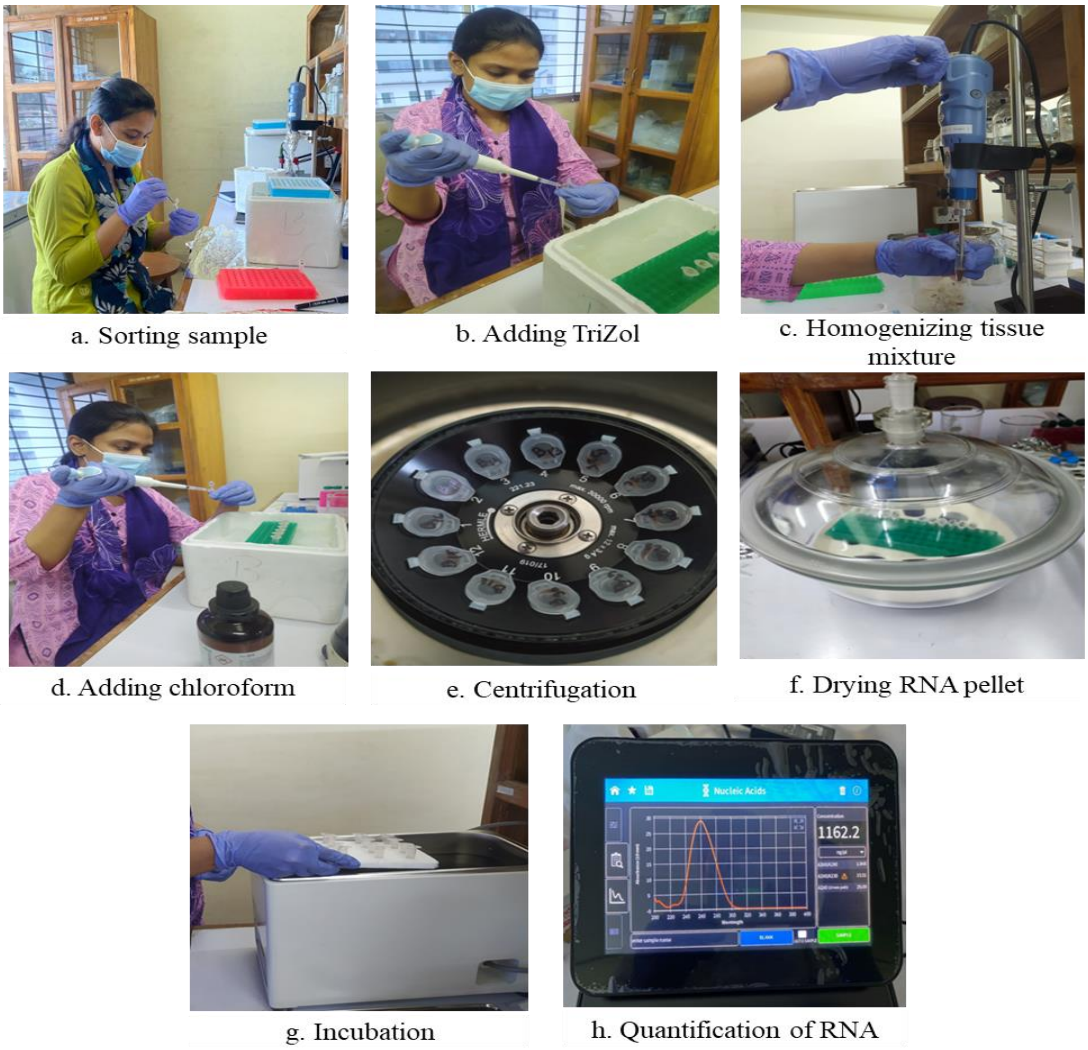


Figure 7: Extraction and quantification of RNA from *O. niloticus* brain sample

Table 1: Equipment and reagents required for RNA isolation

Type	Item
Equipment	Homogenizer
	Centrifuge and rotor capable to reach 12,000 xg and 4ºC
	Polypropylene microcentrifuge tubes
	Water bath at 55−60ºC
	Desiccator
	Nanodrop Spectrophotometer
Reagents	TRIzol^{TM} reagent
	Chloroform
	Isopropanol
	75%Ethanol
	RNAse free water
	0.5M EDTA pH 8.0

3.8 Preparation of cDNA:

Total RNAs (500–1000 mg) were used to synthesize the first strand of cDNA by using a cDNA synthesis kit (PrimeScript 1st strand cDNA synthesis kit, TaKaRa Bio, Japan) as per the instructions of the manufacturer. For cDNA synthesis, at first a mixture was prepared in a microtube by adding oligo dT primer, dNTP mixture, template RNA, and RNase free water which was incubated at 65ºC for 5 min and then allowed to be cooled on ice immediately. The denaturing step of template RNA is needed to improve the efficiency of the reverse transcription. After that, a reaction mixture was prepared containing template RNA-primer mixture, 5X primescript buffer, RNase inhibitor, primescript RTase and RNase free water and then incubated at 45ºC for 30 min. Later, the enzyme was inactivated by incubating at 95ºC for 5 min and then the prepared cDNA samples were stored at −20ºC until next use.

a. cDNA synthesis kit

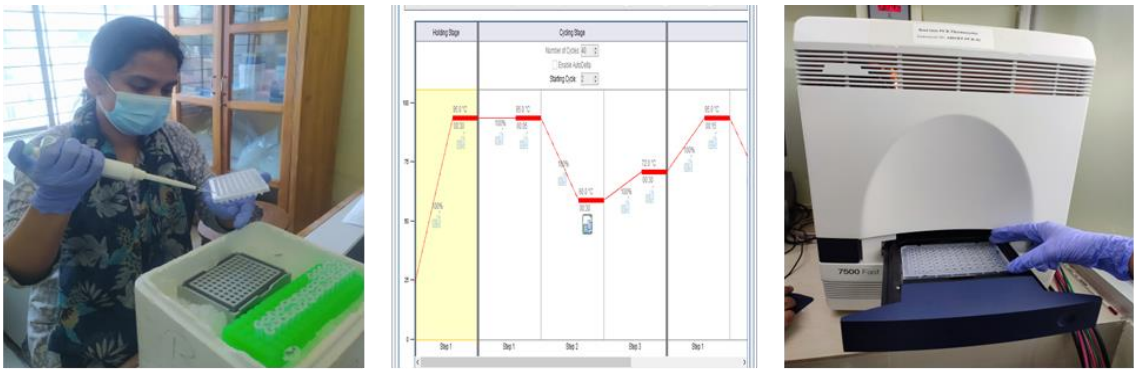
b. Incubating reaction mixture

c. Cooling on ice

Figure 8: Preparation of cDNA

3.9 Real time PCR assays of *hsp70, hsp90,* and *ghrelin* mRNAs:

Real-time quantitative PCR was carried out with a 7500 Fast Real-Time PCR System (Applied Biosystem, USA). Primers used for the measurement of *hsp70*, *hsp90*, and *ghrelin* were chosen from the prior studies are listed in Table 2. The reaction mixture (10 µl) for PCR consists of standard cDNA or sample (1 µl), forward and reverse primer (0.4 µl each), and SYBR TB GREEN premix (5µl) (TaKaRa, Japan). Amplification was performed at 95°C for 30s, followed by 40 cycles at 95°C for 5s, 60°C for 30s and 72°C for 30s. Melting curve analysis of the product was utilized to verify the specific amplification of each cDNA. Expression level was normalized by employing the ΔΔCt method and *β- actin* was used as a housekeeping gene. All qPCR experiments were conducted in duplicate.



a. Plate preparation for RT PCR reaction

b. Temperature set up

c. Placing plate in RT PCR

Figure 9: Procedure for real time PCR assay

Table 2- Primer for *O. niloticus*, temperature induced experiment

Gene	Primer sequence	Reference
hsp70	F- 5′ CATCGCCTACGGTCTGGACAA 3′	Magouz et al., 2021
	R- 5′ TGCCGTCTTCAATGGTCAGGAT 3′	
hsp90	F- 5′ TTTGCTGAACTGGGTGAGGA 3′	Kwon and Kim, 2010
	R- 5′ AGAGACCAGGGTCTTGCCAT 3′	
ghrelin	F- 5′ GTGGTGCAAGTCAACCAGTG 3′	Amin et al., 2019
	R- 5′ CATGGCTTGGCGACCAATTC 3′	
β- actin	F- 5′ CGAGCTGTCTTCCCATCCA 3′	Wei et al., 2013
	R- 5′ TCACCAACGTAGCTGCTTTCTG 3′	

3.10 Statistical analysis:

Data sets for the gene expression and other studied measured variables are displayed as mean ± standard deviation (SD) of the mean. Data were subjected to a one-way analysis of variance (ANOVA) and then means were compared by Tukey's HSD post hoc test using SPSS 26.0 (SPSS Inc., Chicago, IL) to determine statistically significant differences among the three different temperature treatments. Statistically significant differences were indicated by a *p*-value of 0.05 unless otherwise stated in the text.

CHAPTER: 4

RESULTS

4.1 Variations in the expression of *hsp70* in the brain of Nile tilapia at different acclimation temperature:

In the present study, the relative expression level of the genes for heat shock proteins at different acclimation temperatures was measured from the whole brain of Nile tilapia. Results found a noticeable change in the mRNA level for *hsp70* when exposed to different acclimation temperatures in *O. niloticus* (Figure 10). The relative expression level of *hsp70* was significantly ($p < 0.05$) higher at the 37°C acclimation temperature compared to the fish acclimated at 31°C and 34°C. However, no significant differences were observed between 31°C (control) and 34°C in the expression level of *hsp70* in Nile tilapia (Figure 10).

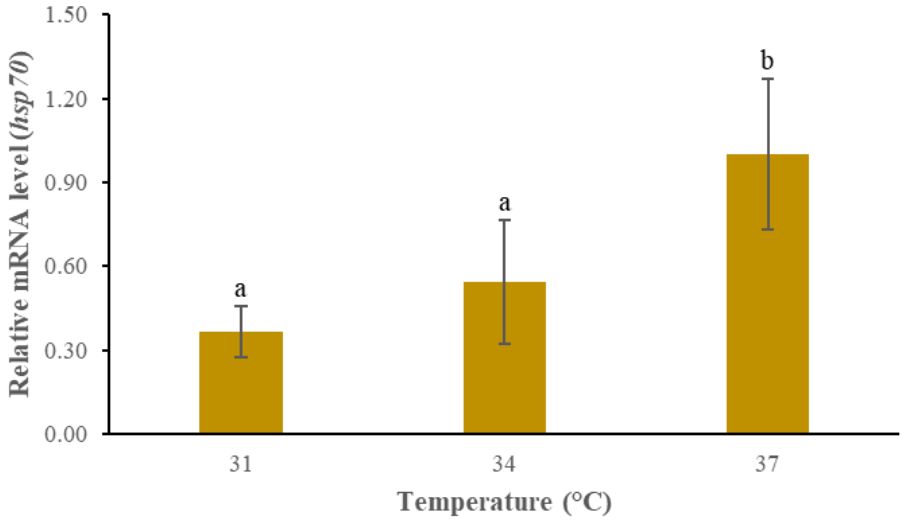


Figure 10: Variations in the mRNA levels of *hsp70* in the brain of *O. niloticus* subjected to different acclimation temperature (31, 34 and 37°C) for a duration of 15 days. The relative abundance of mRNAs was analyzed against *β-actin* mRNA and expressed as the relative values. Values are presented as mean ± standard deviation of the mean (SD) (n = 12). Different subscripts of alphabets are statistically significant at $p < 0.05$ according to the Tukeys's HSD post-hoc test.

4.2 Variations in the expression of *hsp90* in the brain of Nile tilapia at different acclimation temperature:

The relative expression of *hsp90* mRNA in the brain of Nile tilapia was higher at 34°C and 37°C compared to the 31°C acclimation temperature (Figure 11). However, no statistically significant differences were found in the expression of *hsp90* among these three acclimation temperatures in the brain of *O. niloticus* (Figure 11).

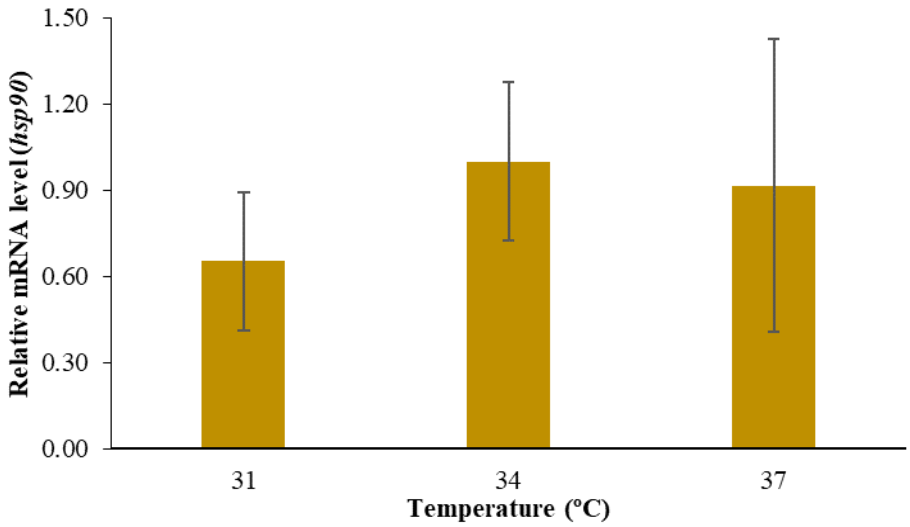


Figure 11: Variations in the mRNA levels of *hsp90* in the brain of *O. niloticus* subjected to different acclimation temperature (31, 34 and 37°C) for a duration of 15 days. The relative abundance of mRNAs was analyzed against *β-actin* mRNA and expressed as the relative values. Values are presented as mean ± standard deviation of the mean (SD) (n = 12).

4.3 Variations in the blood glucose level of Nile tilapia at different acclimation temperature:

In the present study, blood glucose level was measured to understand the level of stress response in Nile tilapia. Results found that fluctuations in temperature give rise to altered blood glucose (mg/dl) levels (Figure 12). At the end of 15 days, blood glucose level (mg/dl) was significantly ($p < 0.05$) higher (154.14 ± 37.91) at 37°C compared to 31°C and 34°C acclimation temperatures. However, blood glucose level was 71.57 ± 9.11 and 81.33 ± 22.66 at 31°C (control) and 34°C acclimation temperatures respectively and no

significant differences were observed between these two acclimation temperatures (Figure 12).

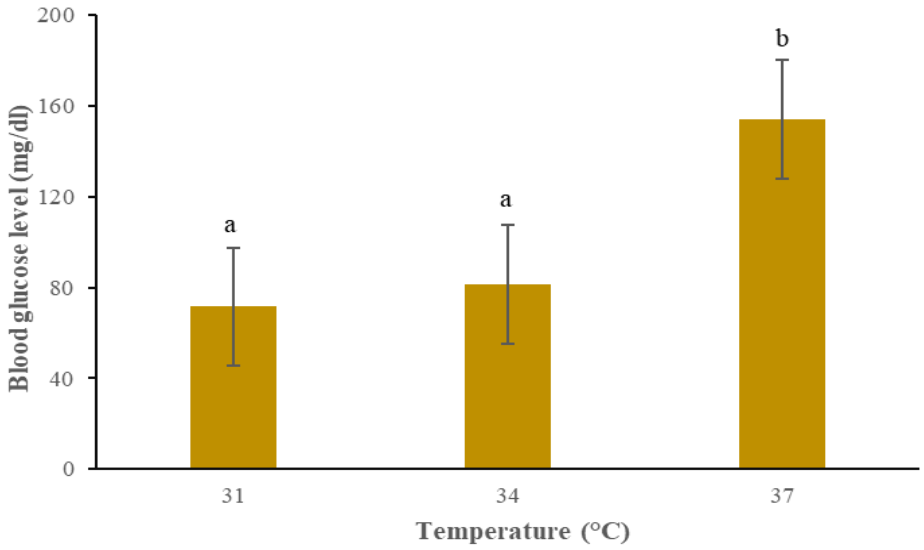


Figure 12: Variations in the blood glucose level (mg/dl) of *O. niloticus* exposed to different acclimation temperature (31, 34 and 37°C) for a duration of 15 days. Values are presented as mean ± standard deviation of the mean (SD) (n = 12). Different subscripts of alphabets are statistically significant at $p < 0.05$ according to the Tukey's HSD post-hoc test.

4.4 Variations in the expression of *ghrelin* in the brain of Nile tilapia at different acclimation temperature:

In the brain of *O. niloticus*, distinct changes in the relative mRNA level for the *ghrelin* gene was observed when exposed to different acclimation temperature (Figure 13). The expression of *ghrelin* mRNA was relatively lower at the higher acclimation temperature (37°C) compared to 31°C and 34°C. Though the relative expression of *ghrelin* was 2-fold higher at 31°C and 34°C acclimation temperatures, no statistically significant differences were found among the three acclimation temperatures in the brain of Nile tilapia (Figure 13).

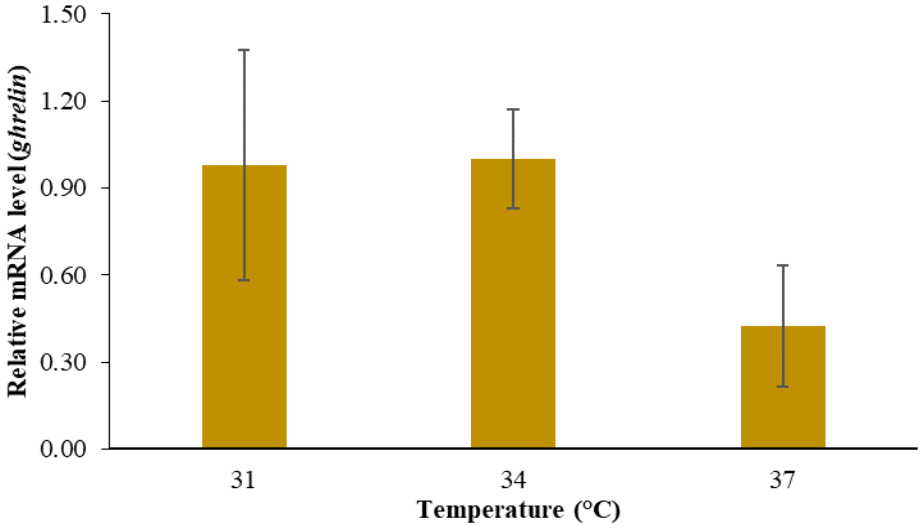


Figure 13: Variations in the mRNA levels of *ghrelin* in the brain of *O. niloticus* exposed to different acclimation temperature (31, 34 and 37°C) for a duration of 15 days. The relative abundance of mRNAs was analyzed against *β- actin* mRNA and expressed as the relative values. Values are presented as mean ± standard deviation of the mean (SD) (n = 12).

4.5 Growth performance of *O. niloticus* at different acclimation temperature:

The data presented in Table 3 illustrates the growth performance of *O. niloticus* at different acclimation temperatures. Fish that were acclimatized to the highest temperature of 37°C exhibited relatively reduced weight gain and specific growth rate (SGR) compared to the fish acclimatized at control temperature of 31°C for the experimental duration of 30 days. Yet, an increase in weight gain and SGR was observed in fish raised at 34°C acclimation temperatures in contrast to fish kept at 31°C (Table 3). However, no statistically significant differences were found in these growth parameters among the three acclimation temperatures.

Table 3: Growth response of *O. niloticus* at three different acclimation temperature for a duration of 30 days (n=12)

Growth parameters	**Acclimation temperature (°C)**		
	31	**34**	**37**
Initial BW (g)	5.80 ± 1.34	5.78 ± 1.54	5.84 ± 1.41

Final BW (g)	19.01 ± 3.62	19.23 ± 1.97	14.25 ± 3.30
Weight gain (g)	12.56 ± 3.86	13.29 ± 2.27	8.78 ± 3.35
(%) Weight gain	207.93 ± 82.35	242.57 ± 93.57	169.70 ± 80.81
SGR (% /day)	1.63 ± 0.37	1.74 ± 0.39	1.31 ± 0.46

4.6 Variations in the water quality parameters of rearing tank at different temperature exposure:

The water quality parameters of the rearing tank such as dissolved oxygen (DO) (mg/L), pH, free CO_2 (mg/L), total alkalinity (mg/L), and ammonia (NH_3) (mg/L) at different acclimation temperatures are depicted in Table 4. As the water temperature increased over time, the DO (mg/L) was significantly decreased. Free CO_2 (mg/L) level was increased with the increase of water temperature over time but no significant difference was observed. pH and total alkalinity remained relatively consistent regardless of temperature. Moreover, there was no variation in ammonia (mg/L) level among these three experimental temperatures (Table 4).

Table 4: Water quality parameters (mean ± SD) of rearing tank at three different temperatures during the study period of 15 days (n=3)

Water quality parameters	**Acclimation temperature (°C)**		
	31	**34**	**37**
Dissolved oxygen (mg/L)	5.9 ± 0.12^{a}	5.25 ± 0.06^{b}	5.15 ± 0.06^{b}
pH	8.7 ± 0.12	8.6 ± 0.12	8.35 ± 0.06
Free CO_2 (mg/L)	5 ± 1.15	9 ± 1.15	8 ± 2.31
Total alkalinity (mg/L)	209.5 ± 0.58	241 ± 35.80	213 ± 1.15
Ammonia (mg/L)	0.25 ± 0	0.25 ± 0	0.25 ± 0

Values are presented as mean ± SD. Different alphabetical superscripts indicate the values of a single water quality parameter in a column are significantly ($p < 0.05$) different.

CHAPTER: 5

DISCUSSION

The temperature of the water surrounding fish plays a significant role in their physiological process, stress response, and growth performance. Each species of fish has a specific temperature range that allows them to tolerate their environment optimally. Any deviations from this range can induce stress, which disrupts their usual physiological and growth processes (Beitinger et al., 2000). This study examined how elevated temperatures modulates growth-related gene expression, stress response, and growth efficiency in juvenile *Oreochromis niloticus*. The findings align with previous research, indicating that elevated temperatures trigger stress response and affect the growth processes of *O. niloticus*. This occurs by elevating the levels of heat shock proteins (HSP70 and HSP90) and suppressing the production of ghrelin in *O. niloticus*.

HSPs serve as potential indicators for understanding how fish respond to the alteration in their surrounding temperature. HSPs shields internal cellular structure and coordinate cellular functions during time of stress (Richter et al., 2010; Sharma et al., 2017). Additionally, it provides protection to the protein substrates by preventing structural damage, enhancing protein functionality, and hindering the formation of detrimental inclusion bodies (Tutar and Tutar, 2010). In the present study, fish acclimatized at 37°C exhibited significantly higher expression of the *hsp70* in the brain of juvenile *O. niloticus*. Elevated level of *hsp70* in the brain of *O. niloticus* connotes potential protein impairment in thermally stressed fish. Increase in *hsp70* expression in the brain of Nile tilapia also indicates the initiation of a defensive mechanism against structural impairments in the cellular components at the higher acclimation temperatures (37°C) in this species. The finding of the present experiment corresponds with previous research conducted on various fish species including *Ictalurus punctatus* (Luft et al.,1996), *Channa argus* (Zhou et al., 2020), *Channa striatus* (Purohit et al., 2014), *Labeo rohita* (Shahjahan et al., 2021) and *Catla catla* (Sharma et al., 2017) where fish exhibited increased expression of *hsp70* in association with increased acclimation temperatures.

In the case of HSP90, previous experiments depicted the overexpression of *hsp90* from liver tissue when fish were raised in elevated temperatures from their optimum level and

subjected to thermal stress in *Labeo rohita* (Shahjahan et al., 2021), *Ctenopharyngodon idella* (Wu et al., 2012) and *Puntius sophore* (Mahanty et al., 2017). However, elevated temperatures did not alter the expression of *rkhsp90α* and *rkhsp90β* in the brain at different time intervals in the fat minnow, *Rhynchocypris kumgangensis* (Kwon and Ghil, 2019). In the present study, expression of *hsp90* was measured from the brain and no statistically significant differences were observed among different acclimation temperatures with elevated expression at 34ºC and 37ºC compared to the control (31ºC). The reaction of heat shock protein genes differs depending on tissues and types of families (Kayhan and Duman, 2010). HSP90, like HSP70, responds to a variety of stressors such as temperature change, disease, salinity, low oxygen levels, hydrogen concentration, and environmental contaminants. Heat shock factor, which binds to the HSP90 promoter and initiates gene transcription, induces *hsp90* expression (Roberts et al., 2010; Wang et al., 2011). Additional investigation is needed to determine the involvement of a heat shock factor in the expression of *hsp90* under thermal stress in different tissues from Nile tilapia.

In the context of physiological stress in fish, blood parameters serve as crucial indicators for both internal and external alterations (Cataldi et al., 1998). In our present study, significantly higher blood glucose level was observed when fish was acclimatized at a higher acclimation temperature (37ºC) compared to the 31ºC and 34ºC. This may occur due to increased glycogenolysis which helps to meet the higher energy requirements caused by stressful conditions (Zahangir et al., 2015; Shahjahan et al., 2018; Islam et al., 2020a). The finding of the present study is supported by previous research where *Pangasianodon hypopthalmus* reared at a high temperature (36°C), an elevated level of glucose was observed when compared to the control temperature (28°C) (Shahjahan et al., 2018). Moreover, temperature-induced increases in blood glucose levels were also noticed in *Labeo rohita* (Alexander et al., 2011) and *Tor putitora* (Akhtar et al., 2013) as a response to stress. Therefore, the elevation of glucose level found at 37°C in juvenile *O. niloticus* is clearly an indicator of thermal stress in fish raised at higher acclamation temperatures.

Temperature has a considerable impact on the growth and development, as well as regulating the biochemical reaction rate in fish and promotes feeding, digestion and absorption within the optimum temperature range (Volkoff and Ronnestad, 2020). The functions of metabolic enzymes are also influenced by temperature, and active metabolites

in the bloodstream stimulate the basal metabolism of fish (Kuzmina et al., 2008). In addition, elevated metabolite levels induce hunger in fish, initiating appetite and ultimately enhancing both feed utilization and fish growth performance (Kuzmina et al., 2008). Ghrelin is a potent appetite stimulant, leading to an increase in body weight and also triggers the release of growth hormone that is essential for the proper growth of an organism (Nakazato et al., 2001; Choi et al., 2003). In present study, elevated expression of *ghrelin* was observed at 31ºC and 34°C compared to the 37°C in the brain of Nile tilapia. Increased *ghrelin* expression at 31ºC and 34°C temperature may upregulate the food intake and secretion of growth hormone and ultimately result in higher growth in Nile tilapia juveniles. Reduction of *ghrelin* expression in fish brains at higher acclimation temperature (37ºC) reveals the decreased appetite and lower growth in juvenile *O. niloticus*. Prior experiments on Atlantic salmon (Hevroy et al., 2012) also revealed the impact of increased temperature on *ghrelin* gene confers with the similarity of the present study. In addition, the outcome of the current study corresponds with the findings of Das et al. (2005) and Ashaf-Ud-Doulah et al. (2020). Both the authors revealed the reduced growth performance at higher acclimation temperatures in *Labeo rohita*. Therefore, the association between optimum environmental temperature and fish growth demonstrates a positive correlation, substantiated by the influence of the thermal growth coefficient (Schulte et al., 2011). So, this study may have concluded that high elevation in temperature induces stress and reduces feed intake and growth in the juvenile Nile tilapia.

CHAPTER: 6
CONCLUSIONS

The growth and development of cultured species is considered the prime concern in conducting aquaculture and fisheries research. Temperature is a fundamental factor that directly influences the physiological and metabolic processes in fish, affecting their growth responses. It is a critical parameter in aquaculture and fisheries management. Both the natural aquatic environment and aquaculture are experiencing greater fluctuations in water temperature which are trending towards higher levels due to climate change. It is imperative to ascertain how fish react to these temperature shifts, as this knowledge is vital for predicting consequences of global climate change on fish population, aquaculture production, and dynamics of ecosystems. The present study emphasizes the intricate relationship among gene expression, thermal stress, and growth performance in Nile tilapia. This study reveals a remarkable impact of elevated temperature on the expression of heat shock proteins (*hsp70* and *hsp90*) and *ghrelin* genes. Heat shock protein genes showcase an upregulation and *ghrelin* showcases a reduction and results in lower feed intake in response to thermal stress. These findings will contribute to our broader understanding regarding the mechanism of impact of environmental temperature on gene expression and growth in aquatic organisms with potential implications for sustainable aquaculture practice in the context of climate change.

RECOMMENDATIONS AND FUTURE PERSPECTIVES

This study was conducted to understand the impact of increased temperature on the growth process by analyzing the expression of *hsp70*, *hsp90,* and *ghrelin* genes in the brain of Nile tilapia. The result of the study will be beneficial for the determination of optimum thermal range and impactful for sustainable fisheries management. Although we tried to explore the objective of the research, yet, it is not free from limitations. There are ample scopes for enhancing the outcomes by employing the following approaches-

- Exploring a greater number of genes (growth hormone, insulin-like growth factors, etc.) from different organs such as the pituitary, liver, stomach and intestine will provide a better understanding of thermal stress response and growth in Nile tilapia.
- A broader spectrum of temperature would offer a more in-depth insight into the stress response of *O. niloticus* under different temperature conditions.
- Moreover, analyzing samples from diverse acclimation periods will yield a more comprehensive understanding of the impact of elevated temperature on the growth process.
- Incorporating samples from various life stages of fish will offer more precise knowledge of the temperature-dependent growth processes of Nile tilapia.

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