Chapter 1
Introduction

INTRODUCTION

Global climate change all around the world especially in the wild environment has become a great concern (Mohanty and Mohanty, 2009). About 93% of additional heat on earth has been absorbed by the planet's oceans, which regulate the temperature on a global scale through constant exchanges with the atmosphere, serving as the major cause to rise in seawater temperature since 1970's, (Church et al., 2013; Bopp et al., 2013). Climate change exerts a direct impact on freshwater ecosystems as shift in temperature and precipitation patterns alter flow dynamics and elevated water temperature (Fussel et al., 2012; Field et al., 2014). Being a basic regulatory factor, water temperature, exerts a direct impact on the physiological activities of ectothermic animals like fish. As ectotherms are unable to regulate their body temperatures in response to their surroundings (Moyle and Cech, 2004), they need to adapt to the thermal conditions of their habitat, which eventually alters their physiology affecting growth, survival, reproduction and so on (Shahjahan et al. 2013; Shahjahan et al., 2017; Islam et al., 2019). Moreover, successful reproduction with increased survival and higher growth is the baseline for aquaculture. So, it is important to address the impacts of rising temperatures in aquatic habitats to maintain good aquaculture production. Although Bangladesh stands in 5th position in world aquaculture having a total production of around 47.58 lakh MT (DoF, 2022), the increased water temperature has become a great threat to its aquaculture. Therefore, dealing with and overcoming the impacts of global warming for higher reproductive success and growth of different commercially important species is now critically important for better aquaculture output.

Fish reproduction is the basic and fundamental biological process that follows the seasonal rhythm of environmental cues (Takemura et al., 2004; Wang et al., 2005). Water temperature, among all environmental factors, significantly influence the regulation of various reproductive events in teleost. However, depending on reproductive seasonality, both high and low temperatures can affect reproductive events in fish like gonadal development, maturation, spawning, and reproductive success (Wang et al., 2010; Pankhurst and King, 2010; Okuzawa and Gen, 2013; Shahjahan et al., 2017). Previous studies found that anomalous temperature proscribes the fish reproductive activities in

fish (reviewed by Zahangir et al., 2022). For instance, increased temperature reduces fertility and steroid hormones in Atlantic salmon, *Salmo salar* (King et al., 2003), while low temperature induces better fertility and survivability (King et al., 2007). In European eel, *Anguilla anguilla*, high-temperature delays vitellogenesis, and low temperature increase the level of plasma estrogen (Pérez et al., 2011), but in rare minnow, *Gobiocypris rarus* and grass puffer, *Takifugu alboplumbeus*, both high and low-temperature results in decreased reproductive events (Luo et al., 2017) and lower gonadosomatic index (Rahman et al., 2019) respectively. So, anomalous temperatures beyond the optimum level can inhibit fish reproduction, leading to lower abundance and eventually leading to the extinction of a particular species (Soria et al., 2008; Portner and Peck, 2010). Thus, it is crucial to comprehend the impact of anomalous temperature and subsequent physiological and reproductive alterations for successful reproductive events in a particular fish species.

The hypothalamus-pituitary-gonadal (HPG) axis is the key neuroendocrine axis governing reproduction in vertebrates (Zohar et al., 2010; Das et al., 2019; Zahangir et al., 2021). The HPG axis controls the reproductive processes through the release of hormones originating from the brain, pituitary gland and gonads. Among distinct neurohormones in the brain, the kisspeptin, gonadotropin-releasing hormone (GnRH) and gonadotropin-inhibitory hormone (GnIH) are proven to be vital, especially, in regulating HPG axis during reproduction. In fish, like all other vertebrates, these neuropeptides are synthesized in the hypothalamus and initiates the release of gonadotropin hormones, like follicle-stimulating hormone (FSH), luteinizing hormone (LH) from pituitary gland. These hormones, in turn, impact the gonadal maturation by promoting sex steroids (androgen and estrogen) (Yaron et al., 2003; Shahjahan et al. 2010a; Zohar et al., 2010). The gonadotropins also control other reproductive activities such as vitellogenesis, oocyte maturation, spermatogenesis, spermiation etc. (Alba et al., 2019). Previous studies showed the modulation of HPG axis by temperature in different fish species like red seabream (*Pagrus major*), blue gourami (*Trichogaster trichopterus*), grass puffer (*Takifugu niphobles*), sheephead minnow (*Cyprinodon variegatus*) (Levy et al., 2011; David and Degani, 2011; Okuzawa and Gen, 2013; Shahjahan et al., 2017; Bock et al.,

2021). Therefore, understanding how anomalous temperature affects the HPG axis is crucial for ensuring fish reproduction success.

In vertebrates, there are eighteen distinct variations of GnRH neurons with multiple types of receptors (Roch et al., 2011; Roch et al., 2014; Chang and Pemberton, 2018). In teleost, a single species typically exhibits a maximum of three distinct varieties of gonadotropin-releasing hormone (*gnrh1, gnrh2, gnrh3*). Of these, *gnrh1* neurons, localized in preoptic area of brain, play a central role in the secretion of gonadotropins (Das et al., 2019). Whereas, GnRH2 and GnRH3 neurons are found in mid-brain tegmentum and terminal nerve and plays modulatory roles in feeding and socio-reproductive behavior respectively (Kauffman and Rissman, 2004; Ogawa et al.2006; Mastuda et al., 2008). Past studies have shown that temperature can influence the expression of *gnrh1* in grass puffer (*Takifugu alboplumbeus),* red seabream (*Pagrus major*) and pejerrey (*Odontesthes bonariensis*) and expression of *gnrh3* in blue gourami (*Trichogaster trichopterus*) and zebrafish (*Danio rerio*) (Levy et al., 2011; David and Degani, 2011; Elisio et al., 2012; Shahjahan et al., 2013; Okuzawa and Gen, 2013; Shahjahan et al., 2017). Hence, it is crucial to identify the effect of anomalous temperature on GnRH in different fish to mitigate the repercussion of global warming.

Kisspeptin is inevitable for HPG axis regulation in many animal species (De Roux et al., 2003; Roa et al., 2008; Tena-Sempere et al., 2012). It is understood that Kisspeptin regulates mammalian reproduction via interacting with GnRH (Okley et al., 2009; Kanda et al., 2013). In the case of teleost, genes associated with kisspeptin and its receptor exert significant influence on both puberty and reproduction (Zohar et al., 2010). Two forms of kisspeptin (*kiss1, kiss2*) and their respective receptors (*kiss1r; kiss2r*) have been identified from medaka (*Oryzias latipesi*) and zebrafish (*Danio rerio*) (Van Aerle et al., 2008; Kitahashi et al., 2009). However, some fish like grass puffer (*Takifugu alboplumbeus)* and Nile tilapia (*Oreochromis niloticus*) have single type of kisspeptin (*kiss2*) and kisspeptin receptor (*gpr54*) (Akazome et al., 2010; Ogawa et al., 2013; Gopurappilly et al., 2013). For many species, it is observed that *kiss2* plays more vital role in reproduction compared to *kiss1* (Felip et al., 2009; Shahajahan et al., 2010b). Previous studies demonstrate that both increased and decreased temperatures from control one decreased *kiss2/kiss2r* expressions in grass puffer, *Takifugu niphobles* (Shahjahan et al.,

2017). Elevated temperature also suppressed *kiss2/kiss2r* gene expressions in zebra fish (*Danio rerio*), but low temperatures was found to improve the *kiss1/kiss1r* expression than control temperature (Shahjahan et al., 2013). Thus, understanding the thermal influence on kisspeptin and its receptor (*kiss2-gpr54*) is now essential to ensure successful reproduction in both culture and wild fisheries.

Nile tilapia (*Oreochromis niloticus*), is recognized as one of the commercially important species in aquaculture all over the world. *O. niloticus* is a highly tolerant fish with a higher growth rate, which triggers its suitability for being cultured on a large scale. To keep pace with this large-scale culture, we need to overcome the problem of rising temperatures for a higher rate of reproductive success. Therefore, it is necessary to understand how the extreme temperature regime affects gene transcription and subsequent secretion of hormones during reproduction. Although there are few studies regarding thermal influence on kisspeptin and GnRH gene expressions in fish species, there are no relevant studies about the thermal effects on the expression of kisspeptin, GnRH, and their receptors in Nile tilapia. So, this study was undertaken to understand the effect of higher acclimation temperature on the expression of gondoropin-releasing hormone (*gnrh1*), GnRH1 receptor (*gnrh1r*), kisspeptin (*kiss2*) and kisspeptin receptor (*gpr54*) in regulation of HPG axis in *Oreochromis niloticus.*

Objectives:

1. To assess the effect of elevated temperature on the expression of kisspeptin, GnRH and their receptor genes in the regulation of hypothalamus-pituitary-gonadal axis in Nile tilapia; and
2. To understand the level of stress response in the reproductive performance at different acclimation temperatures in Nile tilapia.

Chapter 2

Review of Literature

REVIEW OF LITERATURE

2.1. Climate change and reproduction

At the present time, climate change is triggering severe weather conditions, which have a harmful impact on all living beings, particularly those in the wild. Climate change also has a dreadful impact on reproductive events, the fundamental biological process in every living organism. Temperature in aquatic ecosystem, considered as a crucial factor in the existence of fish, with an impact on all developmental and reproductive processes (Alix et al., 2020). Issues with high-temperature induction primarily result in decreased fecundity, oocyte maturation, altered follicular cell structures, altered reproductive events in several species. For instance, Gillet et al. (2011) found inhibition of ovulation in cold water arctic char, *Salvelinus alpinus,* at high-temperature induction (10°C) which was 5°C higher than normal. Similar results were also found for Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*) while treated in warm conditions (Taranger and Hansen, 1993; Pankhurst and Thomas, 1998). In common wolffish (*Anarhicas lupus*), delayed and decreased ovulation with increased ovulation time were found during high-temperature treatment (Tveiten and Johnson, 1999; Tveiten et al., 2001). In many species, induction of high temperature reduces the plasma sex steroids like 17β-estradiol (E_2), which is secreted by the regulation of *gnrh1*, controls vitellogenesis and oocyte development during reproduction (Pankhurst et al., 1996; Watts et al., 2004; King et al., 2007; Soria et al., 2008; Gillet et al., 2011; Elisio et al., 2012; Anderson et al., 2012; Miller et al., 2015; Mahmod et al., 2020).

In a study using red seabream (*Pagurs rarus*) by Okuzawa and Gen (2013) found that warm acclimation temperature (24°C) affects the oocyte development, failure of ovulation and reduced E_2 and GSI at 10 days of exposure. Likewise, Lim et al. (2003) also found inhibited oocyte development at 20°C and 25°C in red seabream (*Pagrus rarus*) at 4 and 8 weeks of exposure. Authors studied pejerrey (*Odontesthes bonariensis*) against high-temperature treatments and found reduced plasma T, E_2, fewer spermatozoa and the presence of vitellogenic oocytes at high (27°C) temperature exposure for 8days and reduced E_2 and advanced spawning but lower fertility at 9 and 12 hours of exposure at 27°C respectively (Soria et al., 2008 and Miranda et al., 2013). King et al. (2007)

studied the effect of warm conditions (22°C) on Atlantic salmon (*Salmo salar*) and found reduced fertility, egg survival, and lower plasma E_2 than normal temperature (14°C). Decreased GSI was also found in grass puffer (*Takifugu alboplumbeus*) while exposed to elevated temperature (28°C) for 7days (Rahman et al., 2019). Exposure of coho salmon (*Oncorhynchus kisutch*) to high temperatures (18°C) showed similar results of lower E_2 and reduced GSI for both male and female individuals (Little et al., 2020).

2.2. Endocrine control and HPG axis

The HPG axis of fish coordinates reproduction through the action of several endocrine and neuroendocrine components (Zahangir et al., 2022). Several neurohormones like kisspeptin, gonadotropin-releasing hormone (GnRH), and gonadotropin inhibitory hormones (GnIH) are essential in the regulation of the HPG axis. Among them, GnRH is the principle regulator of the pituitary for releasing gonadotropins (FSH, LH) for gonad maturation (Ando and Urano, 2005; Zohar et al., 2010; Munoz-Cueto et al., 2020). GnIH has been expressed to have both inhibitory and stimulatory roles on gonadotropins secretion in fish depending on fish species, sex and gonadal stage (Muñoz-Cueto et al., 2017; Di Yorio et al., 2019). Several fish species like pejerrey (*Odontesthes bonariensis*), red seabream (*Pagrus rarus*), and grass puffer (*Takifugu alboplumbeus*) showed decreased expression of sex steroids (FSH, LH), which are primarily controlled by *gnrh1* (Elisio et al., 2012; Okuzawa and Gen, 2013; Shahjahan et al., 2017). Both European seabass (*Dicentrarchus labrax*) and grass puffer (*Takifugu alboplumbeus*) showed lower expression of *gnih* and *gnihr* at high temperatures of 21°C and 28ºC, respectively (Paullada-Salmerón et al., 2017; Rahman et al., 2019). In sheephead minnow, GnIH is considered to have an inhibitory effect on reproduction as it showed increased expression at high temperatures while FSH and LH had lower expression levels at the same time (Bock et al., 2021).

2.3. Gonadotropin releasing hormone (GnRH)

GnRH, previously known as luteinizing hormone-releasing hormone (LHRH), was first reported by Baba et al. and Amoss et al. in 1971. Later on, it was found that it is also responsible for releasing follicle-stimulating hormone (FSH) and now it is known as GnRH. In the same year, Breton et al. (1971) first found that gonadotropin release is

stimulated by a hypothalamic factor in carp. After this pioneering study, GnRH was first discovered in salmon fish (Sherwood, 1983). Afterward, the receptor for GnRH was first discovered in goldfish (Habibi et al., 1987) and then in catfish (Tensen et al., 1997). Until 2002, around 8 variants of GnRH were found in teleosts, and they were named after their origin species (Lethimonier et al., 2004). Among them, two variants of mammal (mGnRH) (Matsuo et al., 1971) and chicken (cGnRH-II) (Miyamoto et al., 1984), were found first in goldfish (Yu et al., 1988) and eel (King et al., 1990). Other six forms were from specific fish lineage: catfish GnRH (cfGnRH) (Bogerd et al., 1992; Ngamyongchon et al., 1992), sea bream GnRH (sbGnRH) (Powell et al., 1994), herring GnRH (hgGnRH) (Carolsfeld et al., 2000), pejerrey GnRH (pjGnRH) (Montaner et al., 2001) and whitefish GnRH (wfGnRH) (Adams et al., 2002).

With time, multiple variants of GnRH was discovered (Tsai, 2006; Okubo and Nagahama, 2008; Roch et al., 2011; Kochman, 2012; Roch et al., 2014). Based on structural and phylogenetic analysis, GnRH genes are classified into three types (White and Fernald, 1998). But most species of vertebrates possess two or three types of GnRH (Sherwood, 1987; Sherwood et al., 1993; Sealfon et al., 1997; Millar, 2003). GnRH type 1 (*gnrh1*) is species-specific and it regulates the pituitary to release gonadotropins (Parhar et al., 2002) and is found in almost all fish (Okubo and Nagahama, 2008). The most abundantly found form of GnRH in teleost is *gnrh2*, formerly known as cGnRH-II (Miyamoto et al., 1984) regulating the feeding behavior. The third type of GnRH (*gnrh3*) is the salmon GnRH-III, exhibited only in the teleost forebrain (Gopurappily et al., 2013; Chang and Pemberton, 2018) and involved in neuromodulation. It is found that fish brain contains at least two types of GnRH, some may also have three forms (Karigo and Oka, 2013). Multiple forms of GnRH receptors are also found against each type of GnRH and all of them are 7 transmembrane domain having class A superfamily of G-protein-coupled receptors (GPCRs) (Chang and Pemberton, 2018). GnRH is widely known to play significant role in controlling reproductive activities by secreting gonadotropins from anterior part of pituitary in all vertebrates, including teleost (Kah et al., 2007; Munoz-Cueto et al., 2020). Several teleost species have demonstrated to have physiological significance of GnRH1 in the GTH secretion (Andersson et al., 2001; Holland et al., 2001; Amano et al., 2004, Amano et al., 2008). However, GnRH2 and

GnRH3 appear to serve as a hypophysiotropic in some fishes, including goldfish (Lethimonier et al., 2004). Multiple species have demonstrated *gnrh3* as a regulatory factor of reproductive activity. For instance, nest building activity in male dwarf gourami (Yamamoto et al., 1997), homing and migration in sockeye salmon (Kitahashi et al., 1998) and nest-building activity in cichlids (Ogawa et al., 2006) are regulated by GnRH3.

Authors have studied the thermal effect on the GnRH gene expressions in several species. Levy et al. (2011) and David and Degani (2011) studied blue gourami (*Trichogaster trichopterus*) in male and female individuals. In both studies, fish exposed to 23°C (low), 27°C (control) and 31°C (high) temperatures for 9 days found significantly lower *gnrh3* expression in high temperature (31ºC) than control (27ºC) during both reproductive condition (nest building) and non-reproductive condition in male (David and Degani, 2011), whereas females showed lower *gnrh3* expression while acclimatized at high temperature (31ºC) during non-reproductive stage and low temperature (23ºC) in reproductive condition (Levy et al., 2011).

Elision et al. (2012) studied the effect of high temperatures on the endocrine axis of pejerrey fish (*Odontesthes bonariensis*). Where the fish were exposed to 8ºC higher acclimation temperature (27ºC) than their control temperature (19ºC) for 8 days. The authors found the suppressed level of *gnrh1* expression at 27°C after the short-term warm temperature exposure. Similarly, Okuzawa and Gen (2013) studied female red seabream (*Pagrus* major), where red seabream was exposed to high temperature (24°C) for 5days and 10days to examine HPG axis regulation and ovarian activity. In this study, both exposure (24ºC) caused a decreased level of *gnrh1* expression in the brain and *gnrhr* in the pituitary of red seabream than the optimum temperature (17ºC).

Shahjahan et al. (2013) studied the temperature-regulated GnRH systems in zebrafish (*Danio rerio)*, where authors examined the expressions of *gnrh2* and *gnrh3* against high (35°C) and low (15°C) water temperature than control (27°C). After 7 days of exposure, lower levels of *gnrh3* mRNA were found at both low and high temperatures, while *gnrh2* showed no significant changes. Another study by Shahjahan et al. (2017) on grass puffer (*Takifugu alboplumbeus*) showed a decreased level of *gnrh1* mRNA at high temperature

(28°C), while *gnrh3* expression was decreased at low (14°C) temperature than optimum (21°C) after 7days exposure period.

Bock et al. (2021) examined the impact of elevated temperature on the HPG axis of sheephead minnow (*Cyprinodon variegatus*) by exposing them at 27°C (control) and 37°C for 14 days. Authors found a significantly higher concentration of *gnrh3* mRNA in both male and female individuals at high temperatures (Bock et al., 2021), while *gnrh1* showed no variation at high temperatures.

2.4. Kisspeptin

Initially, *kiss1* gene was identified in the perspective of cancer, especially melanoma, where it is found to have an inhibitory role in metastasis (Nash et al., 2007; Beck and Welch, 2010). During that time, numerous vertebrates have been given exogenous kisspeptin, which stimulated the secretion of gonadotropins such as FSH, LH and testosterone (Dhilo et al., 2005; Cho et al., 2009; George et al., 2011). In a non-mammal vertebrate, kisspeptin receptor (*gpr54*) was first discovered in a teleost, Nile tilapia (Parhar et al., 2004). After that, Kiss1/Kiss1r system was first studied in zebrafish (van Aerle et al., 2008). Later on, a paralogous gene of *kiss1*, named *kiss2*, have been discovered in zebrafish and medaka (Kitahashi et al., 2009). Authors have found two forms of kisspeptin in several fish species including goldfish (Li et al., 2009), medaka, seabass and zebrafish (Felip et al., 2009), club mackerel (Selvarej et al., 2010) and stripped bass (Zmora et al., 2012) along with these two paralogous gene (*kiss1, kiss2*). Teleost have several genes expressing as kisspeptin receptors including *kiss1r, kiss2r, kiss3r, kiss4r* in their kisspeptin system (Ogawa and Parhar, 2018; Dofour et al., 2020). The *kiss1* neurons are localized in habenula of the brain (Servili et al., 2011; Kanda et al., 2012; Ogawa and Parhar, 2013), and the *kiss2* neurons are only expressed in the preoptic area and hypothalamus (Dunham et al., 2009; Kanda et al., 2012; Ogawa and Parhar, 2013). Later on, the roles of kisspeptin and its receptor were also studied in teleost. Kisspeptin has been demonstrated to be vital for fish reproduction, although depending on species, different authors have found kisspeptin as stimulating, inhibiting, or having no impact at all (Kitahashi et al., 2009; Felip et al., 2009; Pasquier et al., 2011; Kanda et al., 2013; Ohga et al., 2018; Nakajo et al., 2018).

Parhar et al. (2004) first reported the co-localization of kisspeptin receptor and GnRH neuron in tilapia, which then developed the idea of direct regulation of GnRH neurons through kisspeptin. In mullets, it is found that gene expression of the kisspeptin receptor (*kissr*) has a positive correlation with *gnrh2* and *gnrh3* (Nocillado et al., 2007). A study in zebrafish showed higher expression of *gnrh2, gnrh3, kiss1* and *kiss2* in the initiation of puberty (Kitahashi et al., 2009). Another research on zebrafish demonstrated a strong relationship between *kiss2r* and GnRH neuron (Servili et al., 2011). Therefore, it is evident that kisspeptin/kisspeptin receptor system have significant role in regulating HPG axis (Park et al., 2016; Das et al., 2019). However, the influence of rising temperature on the kisspeptin system yet to be explored more.

Shahjahan et al. (2013) studied thermal effect on kisspeptin systems (*kiss1/kiss1r, kiss2/kiss2r*) in zebrafish (*Danio rerio*). Fish were exposed to temperatures including 15°C, 27°C (control) and 35°C for 7days. After this exposure, the *kiss1* mRNA levels were found 2.9-fold increased at a lower temperature (15°C) than the normal temperature, whereas, high temperature didn't have an impact on *kiss1* mRNA level. Likewise, *kiss1r* mRNA levels were found upregulated by 1.5−2.2-folds at low temperatures. On the contrary, both high (35°C) and low (15°C) temperatures downregulated (0.5-fold) the *kiss2* and its receptor *kiss2r* expressions in the brain of zebrafish.

Another study regarding thermal regulation of the kisspeptin and kisspetin receptor system (*kiss2/kiss2r*) in grass puffer (*Takifugu niphobles*) was done by Shahjahan et al. (2017).   The grass puffers were exposed to 14°C (low), 21°C (control), and 28°C (high) for 7 days. After completion of 7days of exposure, *kiss2* and its receptor *kiss2r* expressions were found to be decreased at both high (28ºC) and low (14ºC) temperatures than optimum (21ºC) (Shahjahan et al., 2017).

2.5. Nile tilapia (*Oreochromis niloticus*)

For Nile tilapia, it is evident that high temperature exceeding the optimum limit reduces the growth rate and has an negative impact on blood profile including lower specific growth rate (SGR) and survivability with a higher feed conversion ratio (FCR) (El-Sayed et al., 2008; Islam et al., 2020). Concerning reproduction, Fares (2005) found increased GSI and oocyte maturation in high temperature (32ºC). But authors also have

found lower survivability and altered sex differentiation (Rougeot et al., 2008; Azaza et al., 2008), testis structure (Alvarenga and Franca, 2009) and complete sterility in all life stages (Pandit et al., 2015) in *O. niloticus*. In addition, Qiang et al. (2022) have found a notable amount of atretic follicles in the ovary and decreased GSI while incorporated with higher temperature (34.5ºC).

Although there are few relevant studies concerning the effect of anomalous temperature on the expression of GnRH, kisspeptin and their receptor systems in some species of fish, no relevant study is found for Nile tilapia (*Oreochromis niloticus*). Therefore, this study was undertaken to examine the impact of higher acclimation temperature on the expression of *gnrh1/gnrh1r* and *kiss2/gpr54* in Nile tilapia.

Chapter 3

Materials and methods

MATERIALS AND METHODS

3.1. Experimental fish

Juvenile *o. niloticus* (6.54 ± 1.2g) were collected from Bangladesh Fisheries Research Institute (BFRI) and acclimatized in the fish ecophysiology laboratory of the Faculty of Fisheries, Bangladesh Agricultural University (BAU), Mymensingh. Fish were kept in 500L tank with flowing freshwater for a period of 15 days, under the maintenance of natural photoperiod of 14:10 (Light:Dark) hours. To ensure optimum oxygen level, continuous aeration was provided and the fish were fed (twice in a day) *ad libitum* with a commercial diet (35% crude protein).

Plate 1. Experimental Fish (Nile tilapia, *Oreochromis niloticus*).

3.2. Experimental design

Juvenile *O. niloticus* were stocked (n = 20/aquarium) in nine glass aquaria (150 L, 75cm × 45 cm × 45 cm) filled with 100L freshwater. Then fish were exposed to three experimental temperatures (31°C, 34°C and 37°C) for a duration of 14 days. The temperature was increased steadily (Δ1°C/12 hr) from 31°C (control) to target (34°C, 37°C) temperatures by using thermos-controller. *O. niloticus* were fed with a commercial diet (35% crude protein) up to satiation, at 9:00am and 5:00pm daily.

Plate 2. Experimental tank setup.

3.3. Sample collection

At the end of 14 days rearing period at different temperatures, fish were anesthetized (clove oil, 5mg/L) and then body weight (BW) for all individuals were recorded. Brain of *O. niloticus* were collected (n = 6) after scarifying fish and soaked in RNAlater (Ambion, Austin, TX) and kept at 4°C for 24hr. In next day, the brain samples were transferred to −80°C with RNAlater. The samples were then transferred to Molecular Biology and Biotechnology Laboratory, Faculty of Fisheries, Chattogram Veterinary and Animal Sciences University (CVASU), Chattogram for further analysis. For gonad samples, gonads of both males and females were collected, weighed and preserved in Bouin's fixative and transferred in 70% ethanol after 24h for further analysis of gonad structure.
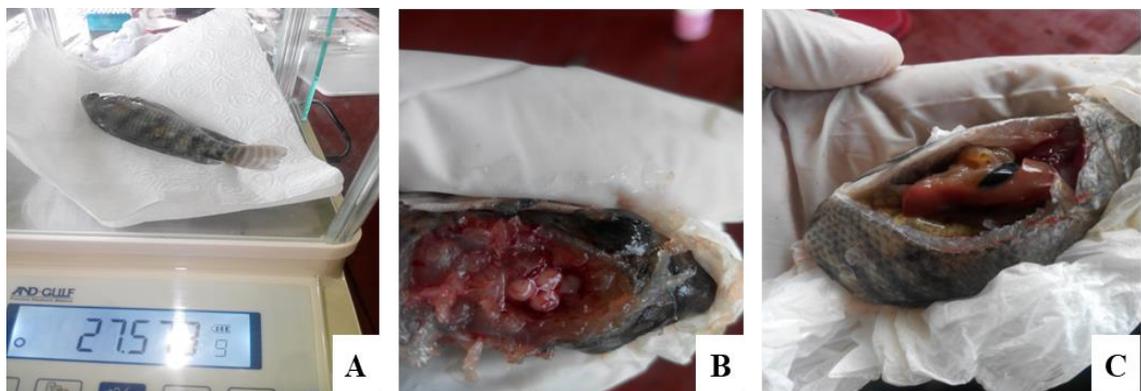
Plate 3. Final Sampling. **A.** Weighing whole fish; **B.** Collecting brain sample; **C.** Collecting gonad.

3.4. RNA extraction

Total RNA from the whole brain was extracted using TRIzol™ reagent (Invitrogen™, Thermo Fisher, USA) as per manufacturer's instructions. For each sample, 1ml TRIzol™ reagent was added with the samples and homogenized with TRIzol™ reagent for precipitating RNA using a homogenizer (Stuart, SHM1 5130, USA). After adding chloroform, homogenate was allowed to partitioned into a clear uppermost aqueous layer containing RNA by centrifugation. The RNA were then precipitated from the supernatant with isopropanol as a white gel like pellet was found at the bottom of the tube. The RNA was washed to remove impurities using 75% ethanol, vortexed and centrifuged for 5 minutes at 7500xg at 4°C. RNA pellet was then air dried by using desiccator. Later, the pellet was re-suspended with 0.5M EDTA pH 8.0 and incubated at 42°C for 30 minutes as per manufacturer protocol and stored at -80°C until further use. RNA integrity and quality were checked by a 260/280 ratio using Nanodrop spectrophotometer (NanoPhotometer® NP80, Germany) and values ~ 2.0 were used for cDNA preparation.

Table 1: Equipment and reagents required for RNA isolation.

Type	Item
Equipment	Homogenizer (Stuart, SHM1 5130, USA)
	Polypropylene microcentrifuge tubes
	Centrifuge and rotor (12000xg and 4°C) (HERMLE Labortechnik GmbH, Germany)
	Water bath at 55−60ºC
	Desiccator
	Nanodrop Spectrophotometer (NanoPhotometer® NP80, Germany)
Reagents	TRIzol™ reagent
	Chloroform (≥99%)
	Isopropanol
	75% Ethanol
	RNAse free water
	0.5M EDTA pH 8.0

Plate 4. Isolation and quantification of RNA; **A.** Sorting samples; **B.** Adding TRIzol; **C.** Homogenizing; **D.** Centrifugation; **E.** Transferring supernatant; **F.** Incubating samples; **G.** Drying total RNA; **H.** Isolated RNA samples; **I.** Checking RNA quality and integrity.

3.5. cDNA synthesis

Total RNAs (500-1000ng) were used for the synthesis of first strand of cDNA using kit (PrimeScript™ 1st strand cDNA Synthesis Kit, Japan) as per manufacturer's instructions. At first, a mixture was prepared in a micro tube by adding dNTP mix, oligo dT primer, template RNA and RNAase free water, incubated at 65°C for 5min and then cooled immediately on ice. The denaturation of RNA is essential to improve the

efficiency of reverse transcription. After that, a reaction mixture was prepared containing template RNA-primer mixture, RNase inhibitor, 5X prime script buffer, primescript RTase free water and then incubated at 45°C for 30min. the enzyme was inactivated by incubating at 95°C for 5min in hot water bath and cooled immediately on ice. Finally, cDNA was stored at −20°C until further use.

3.6. Real-time PCR assays for *kiss2, gpr54, gnrh1* and *gnrh1r*

Real-time quantitative PCR was carried out with a 7500 Fast real time PCR System (Applied Biosystem, USA). Primers used for measuring the gene expression of *kiss2*, *gpr54*, *gnrh1* and *gnrh1r* were collected from previous studies (Table 2). 10µL of PCR reaction mixture (1µL of standard cDNA sample, 0.4µL of forward primer, 0.4µL of reverse primer and 5µL of TB Green™ premix Dimereraser™ (Takara Bio, Japan) were used for gene assay. Amplification was done at 95°C for 30s, then 40cycles of 95°C for 5s, 60°C for 30s and 72°C for 30s. Melting curve analysis and gel electrophoresis of the final product were used to confirm the precise amplification of each cDNA. *β*-actin was used as housekeeping gene and expression levels were normalized by $2^{-\Delta\Delta Ct}$ method. All qPCR experiments were done in duplicate.

Table 2: List of primers used in the present study.

| Primers		Nucleotide sequences	References
gnrh1	F	5´ GCACCTTGCAGTGTTTTTGG 3´	Alba et al., 2019
	R	5´ TTTGCCTGCCTTTTGACAGC 3´	
gnrh1r	F	5´ GTGGCTTGCCGGAGACTTTG 3´	Martinez-Chavez, 2008
	R	5´ AGAGGGTTGAGGATGGCTGACT 3´	
kiss2	F	5´ CTACTGTTGGCTGTGGT 3´	Park et al., 2016
	R	5´ CTGCTCCTGTTGCATGTGTT 3´	
gpr54	F	5´ ATGCCTGGCTGGTCCCTCTGTTCT 3´	Martinez-Chavez, 2008
	R	5´ GGCGGCCAGGTTTGCTATGTA 3´	
β-actin	F	5´ CGAGCTGTCTTCCCATCCA 3´	Wei et al., 2013
	R	5´ TCACCAACGTAGCTGCTTTCTG 3´	
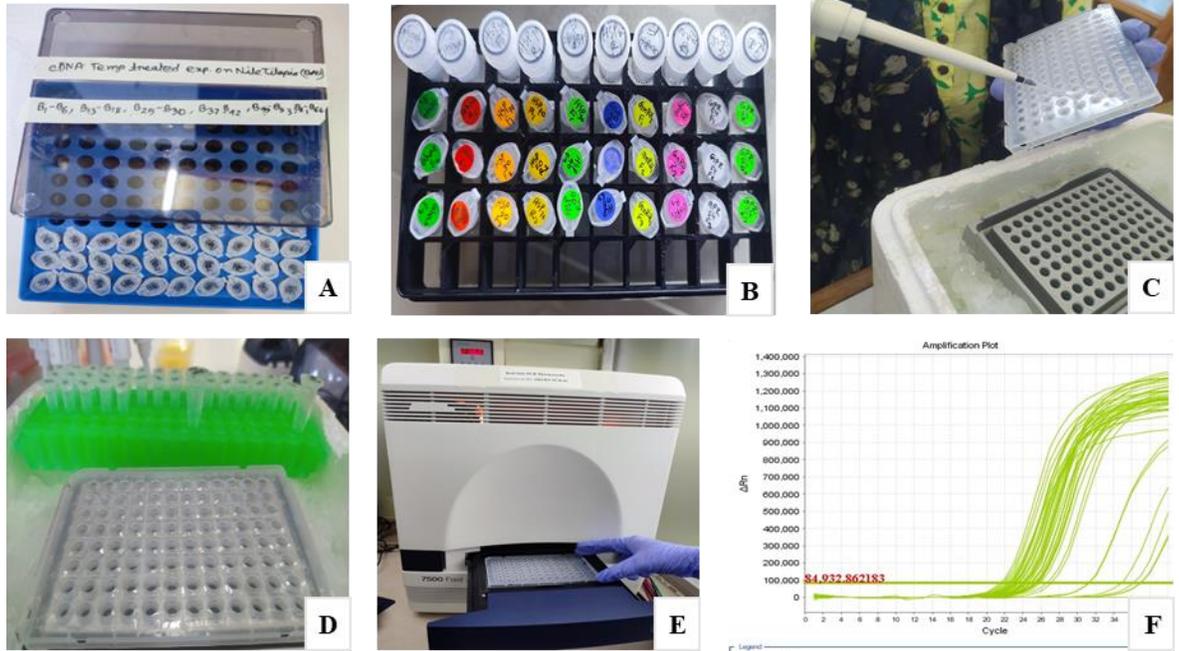
Plate 5. RT-PCR analysis. **A.** Prepared cDNA; **B.** Prepared primer; **C.** Plate preparation **D.** Prepared plate; **E.** Loading sample; **F.** Amplification plot.

3.7. Calculation of gonadosomatic index (GSI) and gonad histology

To know the gonadal condition, gonadosomatic index was calculated and gonad histology was done through the following formula and procedure respectively,

$$\text{Gonadosomatic index (GSI) (\%)} = (\text{Weight of gonad/Total weight of body}) \times 100$$

For histological analysis of gonad, the samples were first preserved in Bouin's fixative. After 24 hours the samples were transferred to 70% ethanol. Then samples were first dehydrated through different graded series of alcohol (70, 80, 90, 95, 100 and 100%). After dehydration, samples were embedded in paraffin wax, which was then sectioned by microtome machine (KD 2258, China). Afterwards the sections were stained by using hematoxylene and eosin. The stained sections were then dried and mounted with required amount of DPX (Qualikems Fine Chemical PVT. Ltd., India). Cover slips were used to protect the mounted samples and after hardening, the sectioned samples were examined under microscope (Carl ZEISS Microscope Gmblt, Optica B-190 series, USA).
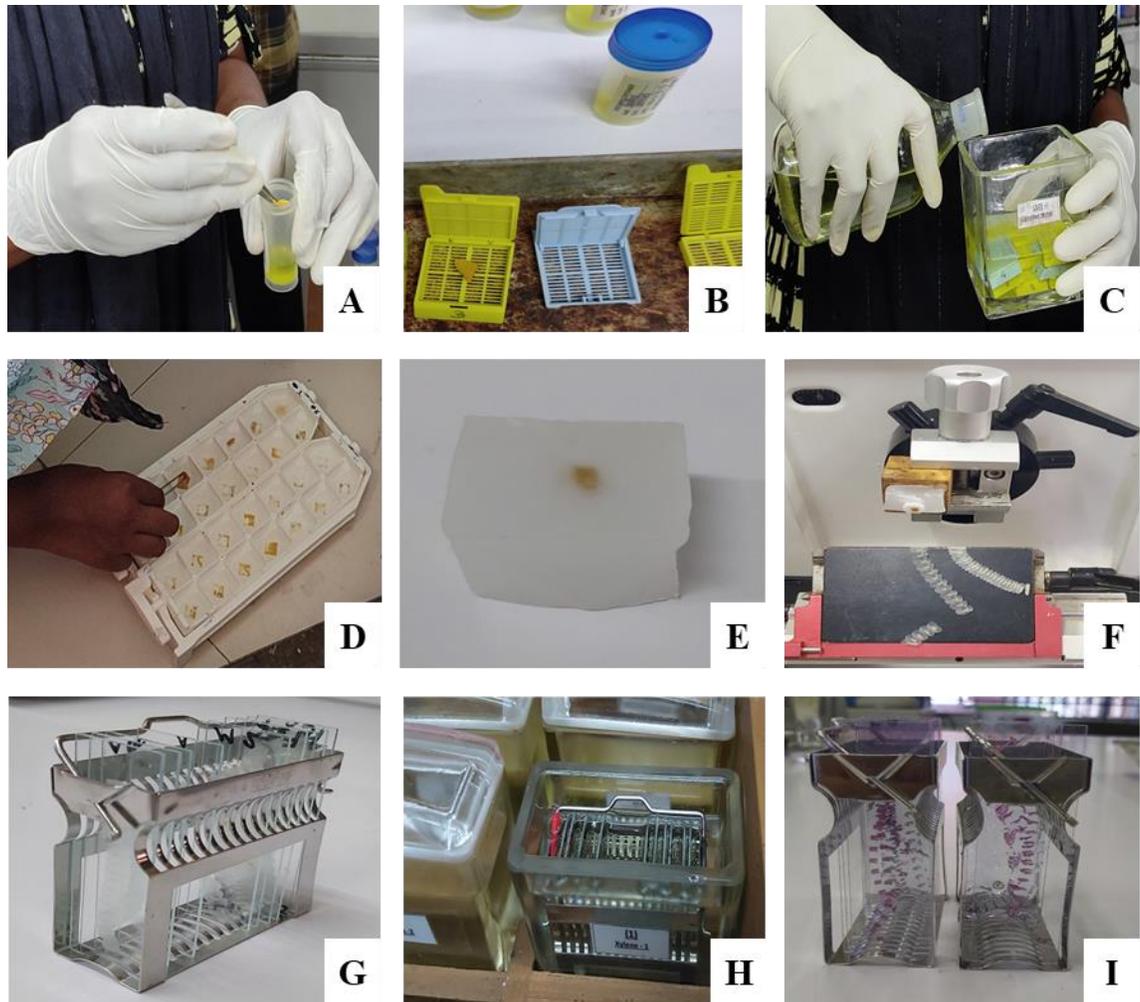
Plate 6. Gonad histology. **A.** Preserved sample; **B.** Sample taken for dehydration; **C.** Adding alcohol; **D.** Embedding in paraffin; **E.** Prepared block; **F.** Trimming; **G.** Slides prepared for staining; **H.** Staining; **I.** Stained samples.

3.8. Water quality parameters

Various water quality parameters like pH, dissolved oxygen (DO), ammonia (NH_3), free CO_2 and total alkalinity were studied in this study. pH and DO were measured by using portable pH meter (RI02895, HANNA Co., Romania) and DO meter (DO 5509, Lutron, Taiwan) respectively. Methyl orange indicator and H_2SO_4 were used as titrant for total alkalinity measuring. NH_3 test kit solution (Sera ammonia test kit, GmbH D 52518, Germany) were used to determine the level of ammonia (NH_3) in each tank. For the test, 5ml of sample water was poured into the test vial, followed by 5 drops of the solution-1

from the NH$_3$ test kit, which was then thoroughly mixed in. Once more, kit solution 3 was added in 5 drops, stirred and allowed to sit for 3 to 5 minutes. It was then matched to a standard color chart for measuring NH$_3$ (mg/L).
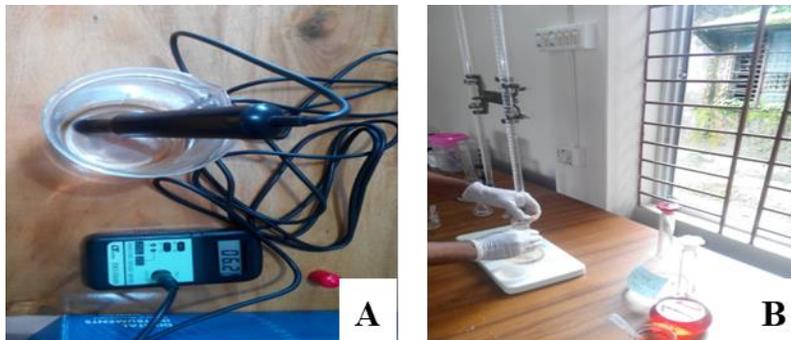
Plate 7. Measuring water quality parameters. **A.** DO measurement; **B.** Titrimetric analysis.

3.9. Statistical analysis

All measured variables are expressed as mean ± standard deviation (SD) of the mean. To assess the significance of these variations between three temperature treatments, the collected data were first analyzed using one-way analysis of variance (ANOVA), then the means were compared using Tukey's HSD post-hoc test in SPSS V.26.0 (SPSS Inc., Chicago, IL). Significant differences were denoted by $p < 0.05$ unless otherwise specified in the text.

Chapter 4

Results

RESULTS

## 4.1. Effect of higher acclimation temperatures on the expression of *kiss2* in the brain of *O. niloticus*

In the present study, the expression level of the genes for *kiss2, gpr54, gnrh1* and *gnrh1r* were measured from the brain of Nile tilapia at different acclimation temperatures (31°C, 34°C, 37°C) after 14days. Results showed that mRNA levels of *kiss2* were relatively higher at 34°C compared to 31°C and 37°C in the brain of male Nile tilapia. However, the mRNA levels of *kiss2* showed no statistically significant differences among three temperatures in males (Figure 1A). In females, statistically significant differences were observed in the expression of *kiss2* with a higher mRNA level at 34°C compared to 31°C and 37°C (Figure 1B).
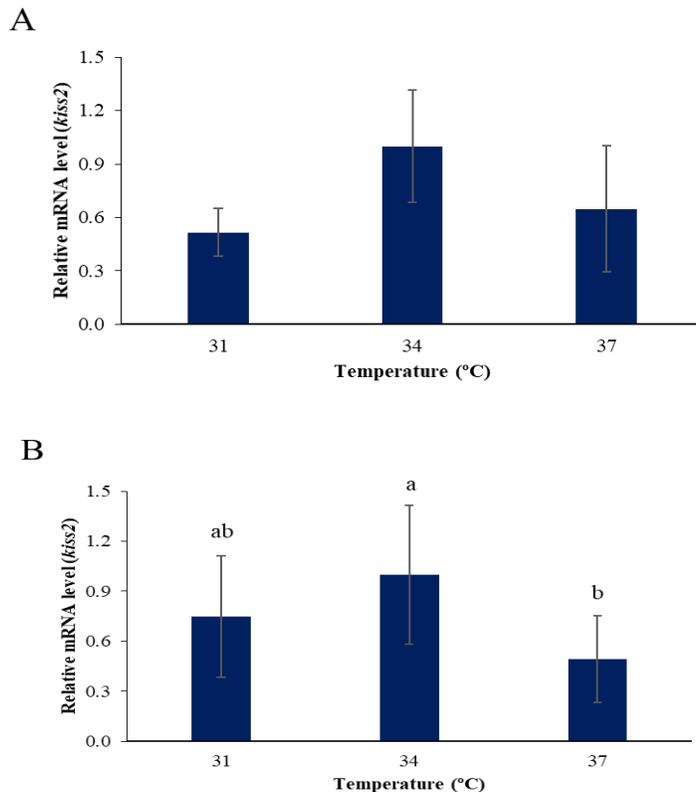
Figure 1. Changes in mRNA levels of *kiss2* in the brain of *O. niloticus* at different acclimation temperatures (31°C, 34°C and 37°C) in **(A)** male and **(B)** female. Different letters of alphabets represent significant differences among temperatures ($p < 0.01$). Values are presented as mean ± standard deviation of the mean (SD) (n = 6, for each treatment group).

4.2. Effect of higher acclimation temperatures on the expression of *gpr54* in the brain of *O. niloticus*

The relative expression of *gpr54* was also examined and found a similar expression pattern to *kiss2* in the brain of Nile tilapia. In male *O. niloticus,* the *gpr54* mRNA levels were relatively lower at 37°C than the other two acclimation temperatures and found statistically significant differences among three acclimation temperatures (Figure 2A). In females, the mRNA levels of *gpr54* were significantly ($p < 0.05$) lower at 37°C than the control temperature (31°C) (Figure 2B).
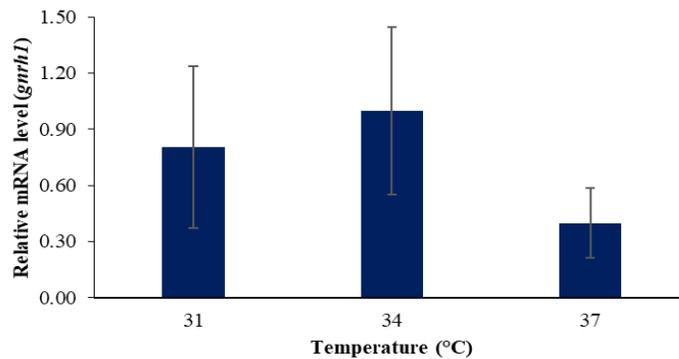
A

B
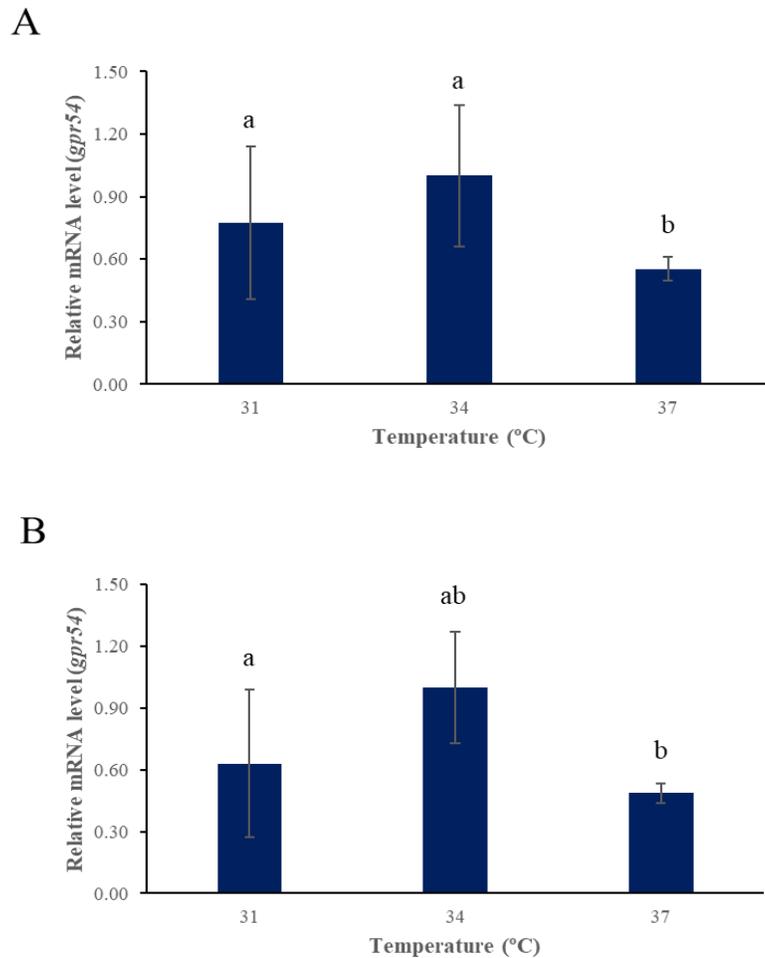
Figure 2. Changes in mRNA levels of *gpr54* in the brain of *O. niloticus* at different acclimation temperatures (31°C, 34°C and 37°C) in **(A)** male and **(B)** Female. Different letters of alphabets represent significant differences between temperatures ($p < 0.01$). Values are presented as mean ± standard deviation of the mean (SD) (n = 6, for each treatment group).

4.3. Effect of higher acclimation temperatures on the expression of *gnrh1* in the brain of *O. niloticus*

The mRNA levels of *gnrh1* were relatively lower at high temperature (37°C) than 31°C and 34°C in male Nile tilapia (Figure 3A). However, no significant statistical differences were observed among three treatment groups.

On the other hand, the expression level of *gnrh1* in females were significantly ($p <$ 0.01) lower at high temperature (37°C) compared to 31°C and 34°C (Figure 3B). However, no notable differences were observed between *gnrh1* expression at 31°C and 34°C in both sexes (Figure 3).
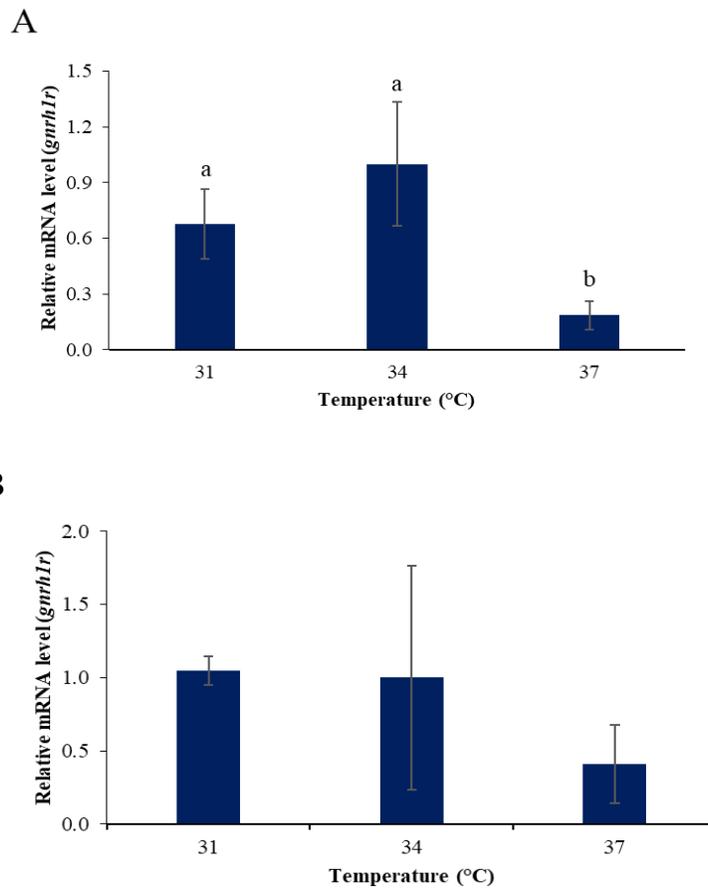
A

B
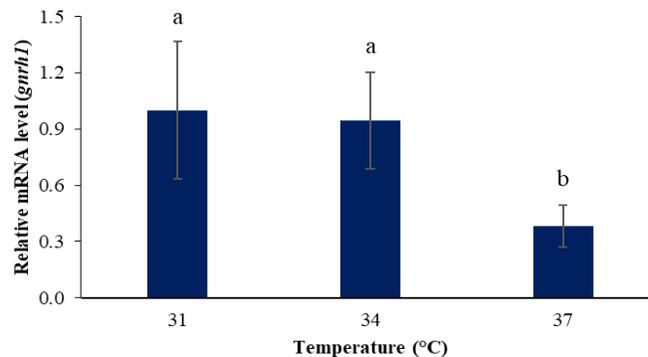
Figure 3. Changes in mRNA levels of *gnrh1* in the brain of *O. niloticus* at different acclimation temperatures (31°C, 34°C and 37°C) in **(A)** males and **(B)** females. Different letters of alphabets represent significant differences between temperatures ($p <$ 0.01). Values are presented as mean ± standard deviation of the mean (SD) (n = 6, for each treatment group).

4.4. Effect of higher acclimation temperatures on the expression of *gnrh1r* in the brain of *O. niloticus*

The expression level of *gnrh1r* was also measured from the brain of Nile tilapia. The relative mRNA levels *gnrh1r* in male were significantly ($p < 0.01$) lower at 37°C than the other two acclimation temperatures (31°C and 34°C), while no significant differences were found between 31°C and 34°C (Figure 4A). In females, the *gnrh1r* expression pattern was similar to the *gnrh1r* expression in males (Figure 4). However, no statistically significant differences were observed among different acclimation temperatures in the expression of *gnrh1r* for females (Figure 4B).

Figure 4. Changes in mRNA levels of *gnrh1r* in the brain of *O. niloticus* at different acclimation temperatures (31°C, 34°C and 37°C) in **(A)** male and **(B)** female. Different letters of alphabets represent significant differences between temperatures ($p < 0.01$). Values are presented as mean ± standard deviation of the mean (SD) (n = 6, for each treatment group).

4.5. Effect of acclimation temperatures on the gonadal development in *O. niloticus*

At commencement of the experiment, only primary spermatogonia (psg) and lumen pf lobule (L) were present in the male gonad in control group (31°C). Afterward, on day 14, the testis of the control (31°C) fish had spermatogonia, spermatid and spermatozoa. While the spermatogonia and primary spermatocyte were observed in fishes acclimatized at 34°C and 37°C. The secondary spermatocyte was only observed in the testis of 34°C treated fish (Plate 8).
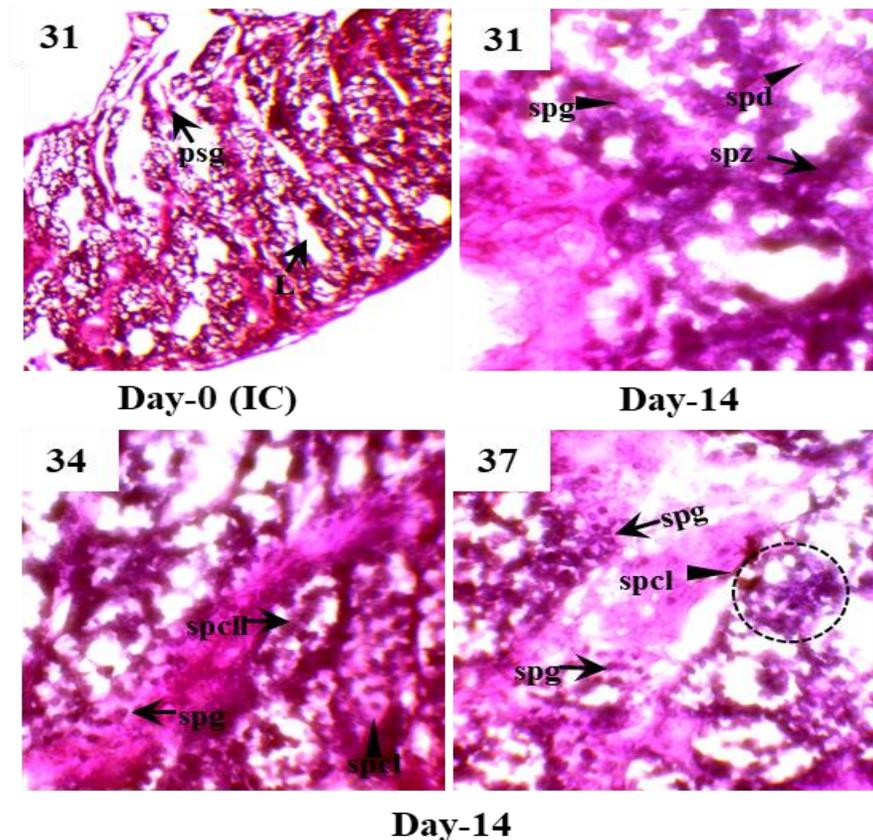
Plate 8. Changes in gonadal developmental stages at different acclimation temperatures (31°C, 34°C, 37°C) in male *O. Niloticus*. Primary Spermatogonia (psg); lumen of lobule (L); Spermatogonia (spg); spermatid (spd); spermatozoa (spz); primary spermatocyte (spcI); secondary spermatocyte (spcII).

In females, only primary growth oocytes were observed at day 0 in the control group (31°C). After 14 days of temperature acclimation, mature oocytes were prominent in ovaries of female fish reared at both 31°C and 34°C, but previtellogenic, vitellogenic and

atretic oocytes were observed in ovaries of high temperature (37°C) treated females (Plate 9).
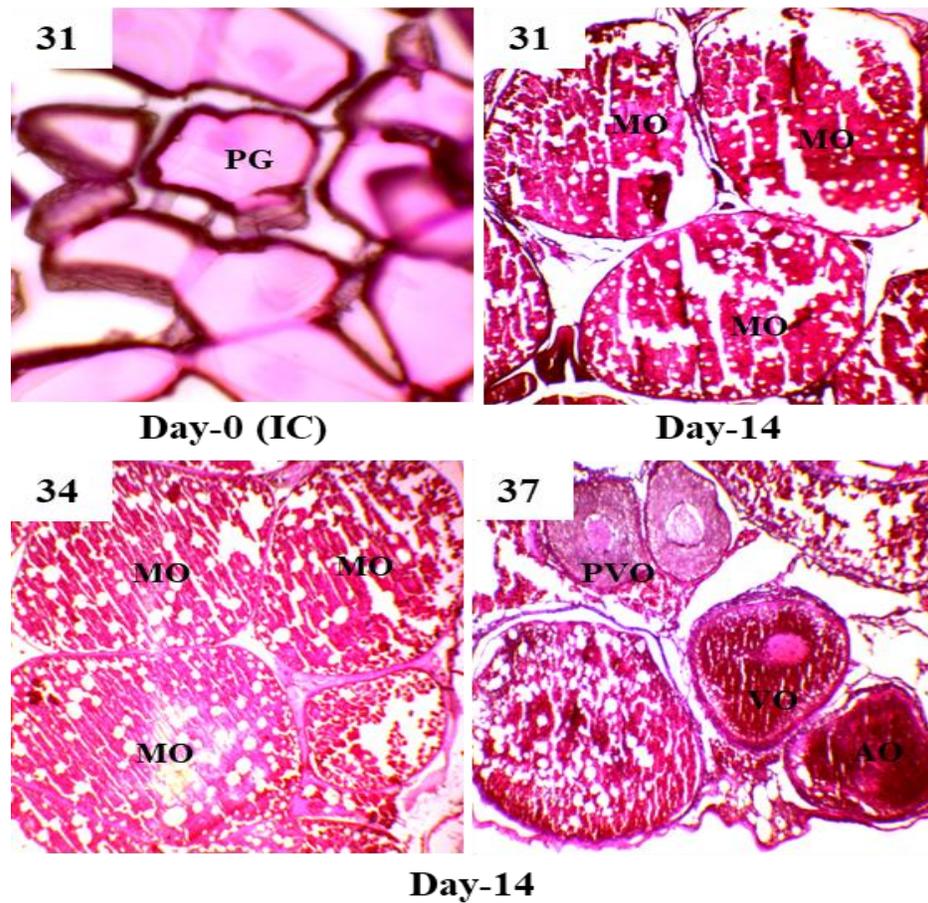
Plate 9. Changes in gonadal developmental stages at different acclimation temperatures (31°C, 34°C, 37°C) in female *O. Niloticus*. Primary growth oocytes (PG); Mature oocytes (MO); Previtellogenic oocytes (PVO); Vitellogenic oocytes (VO); Atretic oocytes (AO).

4.6. Effect of higher acclimation temperatures on GSI of *O. niloticus*

The gonadosomatic index (GSI) for both males and females was also calculated in the present study and changes in the GSI value were recorded for each temperature group. In both males and females, statistically higher GSI values were recorded in fishes reared at 31°C and 34°C compared to 37°C (Figure 5).
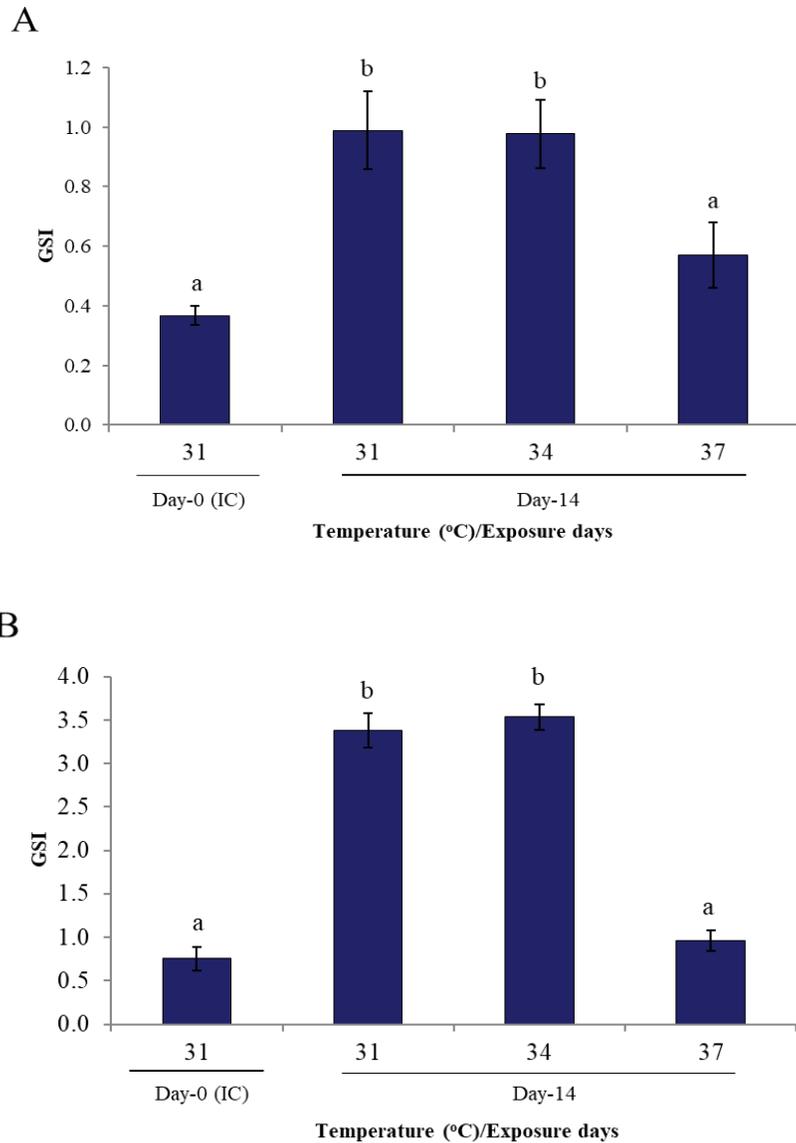
Figure 5. Changes in gonadosomatic index (GSI) of *O. niloticus* at different acclimation temperatures (31°C 34°C, 37°C) in **(A)** males and **(B)** females. Different letters of alphabets represent significant differences between temperatures ($p < 0.01$). Values are presented as mean ± standard deviation of the mean (SD) (n = 6, for each treatment group).

4.7. Effect of temperature on water quality parameters of rearing tanks

The water quality parameters recorded during the study are presented in table 3. With the elevated temperatures, DO (mg/L) was significantly decreased ($p < 0.01$) (Table 3).

While, free CO_2, total alkalinity, pH and ammonia (NH_3) showed no distinct changes among temperatures (Table 3).

Table 3. Water quality parameters recorded during the experiment

Parameters	Temperature (°C)	14 days
Dissolved oxygen (mg/L)	31	5.9 ± 0.12^a
	34	5.25 ± 0.06^b
	37	5.15 ± 0.06^b
pH	31	8.7 ± 0.12
	34	8.6 ± 0.12
	37	8.35 ± 0.06
Free CO_2 (mg/L)	31	5 ± 1.15
	34	9 ± 1.15
	37	8 ± 2.31
Total alkalinity (mg/L)	31	209.5 ± 0.58
	34	241 ± 35.80
	37	213 ± 1.15
Ammonia (mg/L)	31	0.25 ± 0
	34	0.25 ± 0
	37	0.25 ± 0

**Different letters represent significant differences between temperatures ($p < 0.01$).

Chapter 5

Discussion

DISCUSSION

In the present study, it was observed that higher acclimation temperature (37°C) suppresses the expression of *kiss2, gpr54, gnrh1* and *gnrh1r* in the brain of *O. niloticus* juvenile. In addition, slightly elevated temperature (34°C) induces the *kiss2, gpr54, gnrh1* and *gnrh1r* expressions in the brain of studied species in both males and females. Gonadal histology also suggests the likewise impact of elevated temperatures on the hypothalamus-pituitary-gonadal (HPG) axis. The findings of the study imply that temperature have a profound impact on the regulation of reproductive events in this commercially important fish species.

Kisspeptin is an important neurohormone for regulating GnRH secretion and also essential for the maintenance of reproductive activities (Gopurappilly et al., 2013; Elakkanai, 2015). In the present study, expression of *kiss2* was significantly decreased at 37°C compared to 31°C and 34°C in both sexes of juvenile Nile tilapia. The expression pattern of the kisspeptin receptor (*gpr54*) also followed the same pattern in both sexes indicating that *kiss2/gpr54* system is highly inducible by higher acclimation temperature in Nile tilapia. Concomitant results also found in grass puffer and zebrafish. In zebrafish (*Danio rerio*), the *kiss2/kiss2r* expression were notably decreased at elevated temperature (Shahjahan et al., 2013). Moreover, grass puffer (*Takifugu niphobles*) also gave similar results, where *kiss2/kiss2r* expressions were decreased at higher acclimation temperature than normal (Shahajaan et al., 2017). In our study, reduced levels of *gnrh1* and *gnrh1r* mRNA were also observed in fishes acclimatized to higher temperature 37°C compared to 31°C and 34°C in both sexes of *O. niloticus.* Suppressed levels of *gnrh1* mRNA in both sexes at high temperature were also reported in pejerrey (Elisio et al., 2012). In male grass puffers, increased temperature also suggested a negative impact on *gnrh1* and *kiss2* expression (Shahjahan et al., 2017). Moreover, the expression levels of *gnrh1r* in female red seabream (*Pagrus major*) were also reduced at high temperatures (Okuzawa and Gen, 2013) which is similar to the results reported for at high-temperature exposure in the present study.

In tilapia, it was first reported that kisspeptin fibers innervate GnRH neurons, suggesting a close connection between these two neurohormones in regulating fish

reproductive activities (Parhar et al., 2004). During the spawning season, a positive association in the *kiss2* and *gnrh1* expressions were observed in grass puffer (Shahjahan et al., 2010b). Temperature is the primary environmental element that governs seasonal reproduction-related physiological activity in fish. Both cold and warmer sensing neurons have been localized in the hypothalamus containing center of temperature regulation in mammalian body (Tabarean et al., 2010), the mechanism underlying how temperature affecting kisspeptin-GnRH neurons in the hypothalamus of fish remains obscure. Very little is known about the neural pathways concerning the temperature dependent control of HPG axis. Temperature is mainly recognized by thermo-sensitive transient receptor potential (TRP) ion channel, which play a significant part in conveying temperature signals to the neural circuits (Patapoutian et al., 2003; Diaz-Franaulic et al., 2016). Therefore, higher acclimation temperature may have been perceived by the TRPs and inhibit the reproductive neuroendocrine activity of kisspeptin and GnRH system in Nile tilapia.

The hypothalamus *gnrh1* triggers the cells in pituitary to release gonadotropin that eventually control the gamete growth for gonadal maturation (Counis et al., 2005). Gamete formation in males undergoes some sequential steps including the formation of primary spermatocyte from spermatogonia, then secondary spermatocyte, and later on spermatid. In this study, male Nile tilapia that are acclimatized at normal temperature (31°C) had spermatogonia, spermatozoa and spermatid in their testis, which suggests proper growth of gamete in the testis. Nile tilapia males acclimatized at 34°C in this experiment, gonads develop until the secondary spermatogonia stage. However, at high temperatures (37°C), males only had spermatogonia and primary spermatocytes, even secondary spermatocytes didn't form in this group of fish. These results suggest the slower spermatogenesis in *O. niloticus* at high acclimation temperatures. In male pejerrey, a decreased number of spermatozoa was reported at 23°C and fewer spermatocytes at 27°C (Soria et al., 2008). Almost identical results were found in the same species of pejerrey fish denoting reduced spematogonia and spermatocytes in testis at high acclimation temperature (Elisio et al., 2012). A lower number of spermatozoa was also reported in coral reef fish (*Acanthochromis polyacanthus*), while treated with elevated temperatures (Donelson et al., 2010). Higher acclimation temperatures may

result in reduced testosterone (T) and gonadal steroids (11-Ketotestosterone, 11-KT) that could not maintain the reproductive functions in Nile tilapia. Further studies are required to elucidate the hormonal control mechanisms (level of sex steroids) of reproductive functions at different acclimation temperatures in Nile tilapia.

For females, the oogonia are the first cell where the oocyte development starts to form a primary growth oocyte and finally an ovum through oogenesis. Afterward, the ovum undergoes previtellogenic and vitellogenic phases and then forms a mature oocyte. In this study, when female *O. niloticus was* treated with three different acclimation temperatures, the control group (31°C) and 34°C group had oocytes that had passed the maturation stage. But in the high temperature group (37°C), the oocytes were in pre-vitellogenic and vitellogenic stages. In addition, atretic oocytes were also found in the 37°C treated group, which specifies the inhibition of oocyte growth and maturation. Qiang et al. (2022) also reported the presence of atretic oocytes in Nile tilapia while exposed to high temperatures than normal. A higher number of atretic oocytes and absence of vitellogenic oocytes were also demonstrated in pejerrey at high temperature acclimation (Elisio et al., 2012). In concomitant with these results, Okuzawa and Gen (2013) also marked gonadal regression and increased number of atretic oocytes as an impact of high temperature in red seabream (*Pagrus major*). This may be associated with the lower levels of estrogen and vitellogenin in the thermally stressed fish. Determination of female sex steroids and vitellogenin warrants further investigation at different acclimation temperatures in Nile tilapia.

The GSI serves as a significant indicator of gonadal maturation and spawning period (Yeldan and Avsar, 2000). An increase in GSI signifies mature gonads and the impending onset of the spawning season, while a decrease in GSI indicates regressed or immature gonads. The present study demonstrated higher GSI in control (31°C) temperature and 34°C treated group, whereas the high temperature (37°C) group had significantly lower GSI in both male and female *O. niloticus.* Similar results were also found in female Nile tilapia (Qiang et al., 2022), male grass puffer (Shahjahan et al., 2017), female seabream (Okuzawa and Gen, 2013) and male pejerrey (Elisio et al., 2012). These results suggest the slower or inhibited gonadal maturation as a consequence of high-temperature exposure, which also aligns with the results of *gnrh1* expression and gonadal structures in

both males and females. Since we found higher *gnrh1* expression at 31°C and 34°C, it might have stimulated enough secretion of gonadotropins from the pituitary which caused mature oocytes in females of 31°C and 34°C group, indicating matured ovaries and resulting in increased GSI. Subsequently, the *gnrh1* expression was lower in 37°C group females with no mature oocytes and having degenerated follicles (atretic oocytes) caused reduced GSI in that group. Likewise, the male *O. niloticus* had a better level of *gnrh1* expression and proper growth of spermatid while treated at control temperature but the growth seemed to be reduced with increased temperatures, which also resulted in lower GSI at high temperatures (37°C) than the other two groups. Therefore. our study suggests high temperature adversely regulates the HPG axis and can inhibit reproduction in Nile tilapia (*Oreochromis niloticus*).

Chapter 6
Conclusions

CONCLUSIONS

Being ectotherm, fish are susceptible to emerging climatic conditions like increasing temperature. To address this issue, it is critical to understand how and to what extent temperature affects the life events of a fish species. Moreover, fish reproduction requires a certain range of temperature for their optimum gonadal maturation and spawning. Thus, reproduction is a crucial one to be impacted by temperature. Since, the neurohormones from the HPG axis regulate the reproductive events, understanding the thermal effects on neurohormone expressions is also essential to evaluate and address the upcoming issues that might show up due to rising temperatures in the globe. In the present study, we evaluated the impacts of temperature on two neurohormones (kisspeptin and gonadotropin-releasing hormone) subunits from the HPG axis and gonadal maturation in *O. niloticus*. Through this study, it is evident that high temperature, after a certain limit, has a negative role in fish reproduction and might even inhibit reproduction. However, different species might have varied impacts of thermal conditions and there is a paucity of research on the thermal regulation of the HPG axis in fish reproduction. Therefore, future studies in this regard are indisputable not only to maintain the fish stocks in open waters but also in aquaculture.

Chapter 7
Recommendations
and
Future Prospects

RECOMMENDATIONS AND FUTURE PROSPECTS

Fish reproduction is a critical aspect in the maintenance of fish stock and successful aquaculture, as it ensures recruitment in natural stock and continuity of aquaculture. We tried to explore the effect of high temperatures on the expression of gonadotropin-releasing hormone, kisspeptin and their receptors to understand how rising temperature might impact the HPG axis in *O. niloticus.* Taking the findings of the study altogether, it is plausible that climatic conditions of increased water might disturb the regular reproduction of Nile tilapia in the near future. As *O. niloticus* is a widely cultured species around the world, this study will help to maintain sustainable culture production by maintaining proper temperature during reproduction. Additionally, this research will also help to understand thermal stress response from a molecular aspect, which will help in further research regarding this issue. Although a qualitative approach was followed to explore the objective of the study, there are some limitations and aspects that can be addressed through the following recommendations:

- Higher experimental temperature could be used to identify the temperature that completely ceases the gonadal maturation and reproduction.
- Using sample fishes from different life stages could help to understand the thermal effect in a broader aspect.
- Identifying the thermal impact on the expression of gonadotropins (FSH, LH) could have helped to understand the whole HPG axis regulation in fish reproduction.
- Further research including different environmental aspects could be undertaken to identify the interactive effects on fish reproduction more precisely.

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