UNIVERSIDAD DE MURCIA

ESCUELA INTERNACIONAL DE DOCTORADO

TESIS DOCTORAL

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Influencia de la luz y la temperatura en los ritmos de reproducción, termotolerancia y comportamiento del pez cebra (*Danio rerio*) y tilapia del Nilo (*Oreochromis niloticus*): Efecto del espectro de luz y termociclos diarios.

D. Gonzalo de Alba Costa

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doctorando del Programa de Doctorado en

Integración y modulación de señales en Biomedicina

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Influence of light and temperature on the rhythms of reproduction, thermotolerance and behavior of zebrafish (Danio rerio) and Nile tilapia (Oreochromis niloticus): Effect of light spectrum and daily thermocycles.

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Introduction

1. INTRODUCTION

1.1. Environmental synchronizers

The Earth is constantly affected by periodic and predictable environmental changes caused by geophysical cycles. The Earth's translational movements around the Sun (translation) and on its own axis (rotation) as well as the Moon rotation around the Earth and the Sun, generate environmental cycles with different periodicities (daily, seasonal, lunar and tidal cycles, respectively) (Kulczykowska et al., 2010). These predictable changes have exerted a selective pressure on organisms favoring the development of biological clocks that allow them to track time and anticipate a response to predictable events (Reiter et al., 2010). Therefore, this synchronization mechanism has fostered the development of a wide range of rhythmic adaptive strategies, such as, feeding and reproduction, to occur at a specific time of day and/or year, thereby increasing success of the species. On the other hand, these biological clocks generate internal order in physiological, biochemical and behavioral processes, optimizing the efficiency of these processes and minimizing energy expenditure (Cymborowski et al., 2010).

In order to consider environmental factors as true synchronizers (Zeitgeber) of biological rhythms, they must fulfill a series of conditions (Aschoff et al., 1981):

- Before and after exposure to the synchronizer, the biological rhythm must be free running with a certain period.
- A stable phase relationship will be established between the biological rhythm and the synchronizer.
- The period of the biological rhythm will coincide with the period of the synchronizer.

Environmental synchronizers allow endogenous clocks to become on track (synchronized) with environmental time, resulting in a stable phase relationship between the biological rhythm and the environmental factor (Kulczykowska et al., 2010). However, this stability can be impaired when the synchronizer is removed. In the absence of these environmental cycles, the biological rhythms of animals enter in a free running condition, revealing their natural periodicity (free running rhythms) which is always close to 24 hours (Aschoff, 1960, 1981). In addition, a masking effect can sometimes occur in which the synchronizer does not exert its direct effect on the endogenous pacemaker but modulates the expression of the biological rhythm (Aschoff, 1960).

External environmental conditions such as light and temperature are considered as the signals of abiotic nature. While, biotic signals such as food availability can act as powerful synchronizer of the biological rhythms of animals (Sánchez-Vázquez et al., 2019). In addition to these, other synchronizers such as salinity, dissolved gas levels of O2 and CO2, food characteristics and social factors, among others, have been described as important zeitgebers in the aquatic ambient (Volpato and Trajano, 2005; Kulczykowska and Sánchez-Vázquez, 2010).

1.1.1. Light

Light has been considered the most powerful abiotic synchronizing factor on biological rhythms in animals (Albrecht, 2012), from cellular (Dekens and Whitmore, 2008) and molecular to physiological and behavioral levels (López-Olmeda and Sánchez-Vázquez, 2010). Photoperiod and wavelengths influence the circadian rhythms of a multitude of biological processes throughout the life cycle of fish (Schibler et al., 2015).

Changes in the photoperiod caused by the alternation between day and night and between the duration of the light and dark phase provide daily and seasonal temporal information. Light is absorbed through the photosensitive cells of the pineal gland and the

photoreceptor cells of the retina (rods and cones) of fish, which are involved in processes related to visual acuity and color discrimination and brightness/intensity detection (Kusmic and Gualtieri, 2000).

In this way, fish can perceive light and temporal information and translate it into a humoral signal through the periodic secretion of melatonin whose function is to modulate and synchronize biological rhythms, mainly in peripheral tissues (Falcón et al., 2010). In most vertebrates, the phototransducer role of melatonin lies in its main secretion at night and inhibition during the day. Therefore, the photoperiod and light-dark (LO) cycle is able to promote the establishment of rhythms, acting from embryonic development marking the onset of locomotor (Hurd et al., 1998), feeding (Puvanendran and Brown, 2002) and retinal photoreceptor (Falcón et al., 2003) activity to the synchronization of adult physiology and behavior. Thus, light influences the locomotor activity of the animal, showing a diurnal, nocturnal or crepuscular pattern depending on whether the time of greatest activity occurs during the day, night or during dawn/dusk, respectively (Herrero et al., 2003; Oliveira et al., 2013). However, sometimes, due to the plasticity of the circadian system, fish can exhibit dualistic behavior, changing their behavior depending on the developmental stage of the animal as well as the conditions of the external environment (Reebs, 2002).

In addition, light is electromagnetic radiation characterized in terms of its wavelength (λ), composition, irradiance and direction of polarization (Collin et al., 2005). After passing through the water column, sunlight is absorbed and filtered modifying the above variables depending on the characteristics of the water (Kusmic and Gualtieri, 2000). In this way, water acts as a chromatic filter modulating intensity and wavelength, so that long wavelengths such as blue ($\lambda \sim 450$ nm) penetrate deeper, while in short wavelengths located below violet ($\lambda < 390$ nm) and beyond red ($\lambda > 600$ nm), light particles are absorbed faster reaching less depth in the water column (Kusmic and Gualtieri, 2000) (Figure 1).

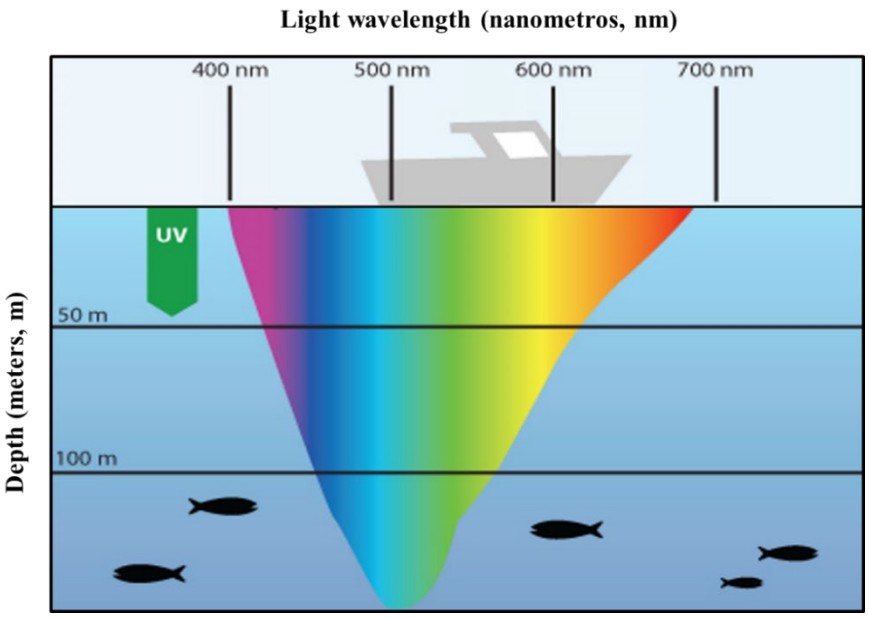

Fig. 1. Graphic representation of the penetration of visible colors of light into the deep ocean (Harrington et al., 2015).

In recent years, research has focused on simulating the light spectrum of the natural environment of fish to observe its influence on embryonic and larval development, growth and reproduction (Ruchin, 2020). From very early stages, visual and non-visual photopigments of photoreceptor structures have been described in fish, the ratio of which characterizes the response of fish to different wavelengths (Allison et al., 2010; Villamizar et al., 2014). In general, the effects during early development appear to depend on both fish species and wavelength. Thus, for example, in zebrafish, long wavelengths enhance larval survival, growth and feeding activity and decrease the number of malformations, whereas long wavelengths have a negative impact on development (Villamizar et al., 2014). However, the opposite happens for other species such as rainbow trout (*Oncorhynchus mykiss*) and European sea bass (*Dicentrarchus labrax*) whose development, growth and feeding activity are favored by red wavelengths (Karakatsouli et al., 2007, 2008). In recent years, it has been studied that these physiological responses could be determined by the effects of wavelengths on factors involved in Hypothalamus-Pituitary-Interrenal axis (HPI) influencing the stress processes, growth and feeding activity (orexigenic/anorexigenic neuropeptides) among others (Shin et al., 2012, 2014; Villamizar et al., 2014; Ruchin, 2020).

1.1.2.Temperature

Especially in ectotherm animals such as fish, temperature plays a fundamental role in the physiology of organisms and it is also a powerful synchronizer of the biological rhythm (Schibler et al., 2015). In nature, light intensity/photoperiod and temperature are subject to seasonal cycles simultaneously. In addition, solar radiation variation during the day caused by alternation between day and night generates daily temperature variations: high temperatures (thermophase) are observed during the day during the day, while lower temperatures are recorded during the night (cryophase), generating a daily thermocycle (Sánchez-Vázquez et al., 2018) (Fig. 2).

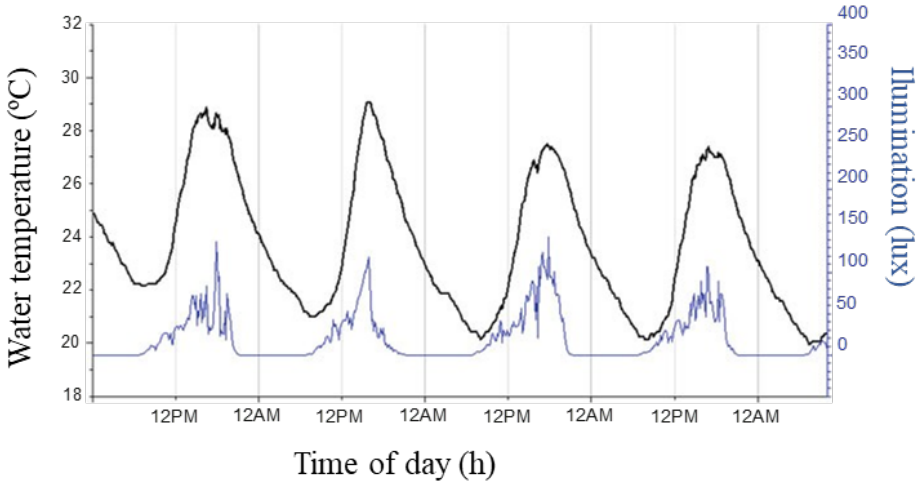

Fig. 2. Daily cycles of water temperature and illumination at the Santa Pola salt marsh marine park (Alicante, SE Spain: 38° 11′ 16″N, 0° 36′ 52″W). Data were recorded in May 2013 every 10 min for 4 consecutive days using a HOBO Pendant data logger (Onset Computer Corporation, Massachusetts, USA) (Sánchez-Vázquez et al., 2018).

These cyclic temperature changes act as true zeitgebers setting the phase of the biological clock rhythm (Sweeney and Hastings, 1960), both at the molecular (Lahiri et al., 2005) and physiological and behavioral levels (López-Olmeda et al., 2006, 2009). In addition, in the absence of light as a synchronizer, temperature cycles are able to steer activity rhythms, in which transitions between thermophase and cryophase and vice versa are interpreted by fish as the same as LO cycle phase transitions (López-Olmeda et al., 2006). Not only to activity rhythms, but the influence of thermocycles during early developmental

stages has been described in Senegalese sole and zebrafish increasing larval growth and decreasing larval mortality and malformations (Blanco-Vives et al., 2010; Villamizar et al., 2012), affecting also the thermal biology and sexual differentiation of the animal (Blanco-Vives et al., 2010, 2011; Villamizar et al., 2012).

However, in addition to daily cycles, environmental temperature is also subject to seasonal cycles influencing biological rhythms of seasonal periodicity such as those associated with reproductive processes (Oliveira et al., 2010). On the other hand, another adaptive response of circadian rhythms is the temperature compensation that allows the biological clock to show a stable phase at different temperature thresholds (Pittendrigh and Caldarola, 1973).

1.1.3.Availability of food.

In the natural environment, food is not constant, but rather its availability is restricted to a specific time of day, driving the development of adaptive feeding strategies to predict and anticipate feeding time through behavioral and physiological processes and optimize feeding efficiency (Patton and Mistlberger 2013). Thus, fish select their preferred feeding schedule throughout the day, characterizing themselves as diurnal or nocturnal feeders, if they prefer to feed during the day or at night (López-Olmeda and Sánchez-Vázquez, 2010). The feeding pattern can change throughout the life cycle of fish, a phenomenon known as diurnalism, which reveals the plasticity of the circadian system (Eriksson, 1978). In addition, when food is available (offered) at the same time every day, the rhythms of locomotor and feeding activity are co-synchronized and fish exhibit Food Anticipatory Activity (FAA) through increased locomotor activity several hours before the next meal allowing fish to maximize food intake and improve digestive processes (Aranda et al., 2001).

Food availability also modifies other behavioral patterns such as migrations in the

ocean's vertical temperature gradient (DVM) as well as food bioavailability, in which some fish select deep layers of the ocean for feeding and shallower layers while not feeding (Piet and Guruge, 1997). In turn, these feeding habits are also linked to the different variations in temperature and light observed in the marine ecosystem, establishing a complex network of interactions that establish the feeding behavior of fish.

1.2. The circadian system.

The circadian system is responsible for the temporal organization of physiological and behavioral processes allowing animals to track time and anticipate a response to predictable events of environmental variables (zeitgebers) (Kulczykowska et al., 2010). In general, the circadian system consists of three main components: the input pathways, the biological clock and the output pathways (Fig. 3).

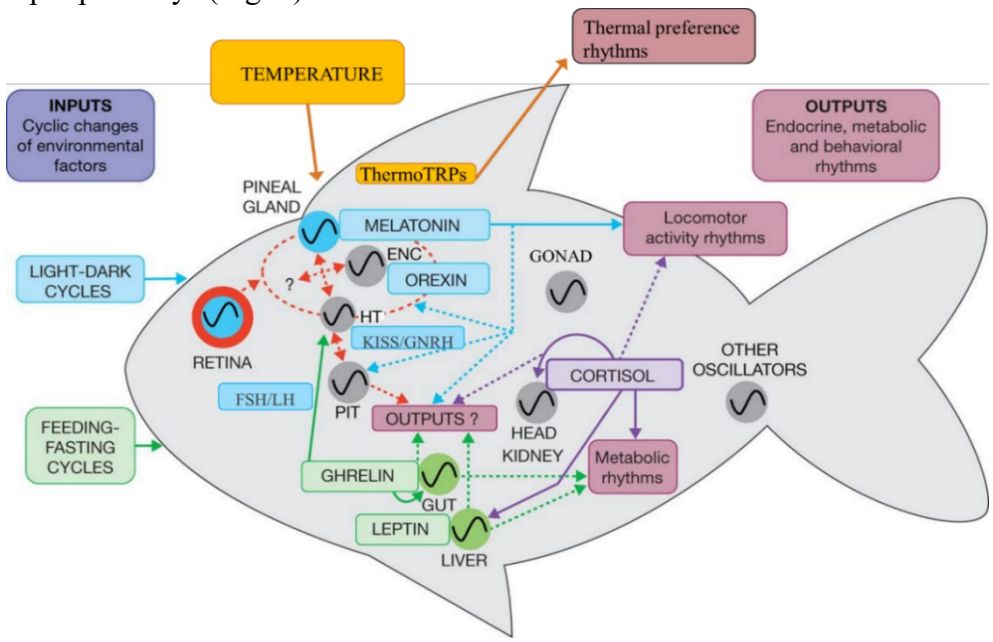

Fig. 3. The circadian system of fish. Biological oscillators or clocks are driven by input pathways, such as light-dark cycles, temperature, and food availability, and must be linked to generate biological rhythms (such as locomotor, reproductive, behavioral, and metabolic rhythms) in a coordinated manner. Endocrine organs (pineal gland, pituitary gland, intestine, liver, kidney and gonad) release hormones (melatonin, pituitary hormones, ghrelin, leptin and cortisol) provide temporal information to specific hormone receptors. ENC, encephalon; HT, hypothalamus; PIT, pituitary gland; INT, intestine (modified from Isorna et al., (2017).

On the one hand, the input pathways are responsible for receiving and sending

environmental information to the biological clock, so that it can synchronize its rhythm with the environmental synchronizers (Isorna et al., 2017). On the other hand, the output pathways are responsible for transmitting the information from the clock to other areas of the organism to drive overt rhythms such as locomotor activity, metabolic rhythms or reproductive rhythms (Kulczykowska et al., 2010). Evidence seems to indicate that, as in mammals, the organization of the circadian system in fish consists of a network of central and peripheral oscillators to process environmental information and execute biological rhythms in a synchronous and coordinated manner (Maemura et al., 2007). In turn, rhythms are self-regulated by molecular clocks, which are formed by negative (Per and Cry genes) and positive (Clock and Bmal genes) feedback loops. The positive loop genes are transcription factors that heterodimerize in the cytosol and translocate to the nucleus where they activate transcription of the negative loop genes and other genes involved in various physiological processes. Conversely, negative loop genes translocate and repress positive core genes (Saha et al., 2019).

1.3. Time-dependent responses

Biological rhythms synchronized with cyclical and predictable variations of environmental factors are present in most physiological processes mainly related to the main axis of the endocrine system (Nicolaides et al., 2014). This axis is also known as the hypothalamus-pituitary axis, which is responsible for regulating numerous physiological processes and hormones involved in such processes such as reproduction, stress response, growth and metabolism, and thermo/osmo regulation. Due to the presence of daily rhythms in various physiological processes, it is reasonable to think that the physiological response of these variables could depend on the time of day (Cowan et al., 2017).

For example, the existence of daily rhythms related to reproduction has triggered different physiological responses depending on the time of day in which the treatment is applied, influencing certain reproductive parameters in both breeders (ovulation, spawning)

and their progeny (fertilization and larval hatching) (Mylonas et al., 2010; Rasines et al., 2013). The influence of time of day on the stress response has been extensively studied by modulating certain variables such as, for example, the sensitivity of the adrenal gland to ACTH (adrenocorticotropic hormone) (Son et al., 2011) and the production and induction of glucocorticoids (Cowan et al., 2017). Likewise, different time-dependent responses to stress appear to be conditioned by daily behavioral patterns, resulting in a greater response if stress occurs in the resting phase compared to if it occurs in the activity phase. Thus, diurnal or nocturnal species show a greater response to stress if it occurs during the dark phase or light phase (respectively) as indicated in several species such as Senegalese sole (*Solea senegalensis*), sea bream (*Sparus aurata*), green sturgeon (*Acipenser medirostris*), and African sharptooth catfish (*Clarias gariepinus*) (Lankford et al., 2003; López-Olmeda et al., 2013; Vera et al., 2014). However, the influence of the time of day would not only be related to physical or chemical stress, as in previous studies, but could also condition the response to thermal stress by varying the thermotolerance of the animals.

From a point of view applied to aquaculture, the time of day at which the various protocols in the productive, sanitary and reproductive areas are applied must be considered in order to maximize the effectiveness of the physiological response of these protocols and minimize the negative effects derived from their application.

1.4. Reproduction.

The reproduction process is an essential biological function of the life cycle of organisms. Sexual determination and differentiation establish the reproductive system allowing living beings to produce new offspring with more or less similar genotypic and phenotypic characteristics, thus perpetuating the species and guaranteeing its survival (Billard and Breton, 1978). In addition, within reproduction, the influence of environmental factors on the reproductive physiology of fish must be considered in order to perform this vital function under the best environmental conditions and achieve the reproductive success of the species

(For more detailed information of the following sections related to reproduction, see experimental chapter 1 and 2).

1.4.1. Sex determination and sexual differentiation.

Sex determination is a physiological process that corresponds to any factor that initiates the establishment of the sex of an individual (male and female). This process can be defined at fertilization being determined by genotypic factors (Genotypic sex determination, GSD) or after fertilization being influenced by environmental factors (Environmental sex determination, ESD). GSD is the most frequent mechanism in fish species and is determined by the primary and secondary sex factors contained in the sex chromosomes (Yamamoto et al., 2019). Some fish possess some phenotypic plasticity (of the same genotype) in early stages of development as a function of environmental variables such as temperature and pH (Baroiller and D'Cotta, 2001), and to a lesser extent photoperiod and salinity (Abucay et al., 1999) establishing the ESD. Between these two extremes, the sex of some fish species is dependent on both genetic and environmental factors in different proportions (Ospina-Alvarez and Piferrer, 2008). Although it has been analyzed in depth during the last few years, current research is still focused on the determination of the fundamental genes related to sex determination and differentiation. Furthermore, these sex-determining genes are in turn involved in the synthesis of estrogens and androgens that impact sexual differentiation resulting in the feminization or masculinization of the individual, respectively. On the one hand, the key enzyme aromatase (Cyp19) is considered as a promoter of feminization as it catalyzes the conversion of androgens to 17β-estradiol determining the ratio of androgens to estrogens (Baroiller et al., 2009). Other factors that promote ovarian differentiation and inhibit testicular differentiation have been considered as Foxl2 (forkhead transcriptional factor L2), Dax1 (X chromosome gene 1 of congenital adrenal hypoplasia) and Ad4BP/SF-1 (Wang et al., 2016, 2019). Meanwhile, among the main determinants of testicular differentiation we find: Dmrt1 (Doublesex and mab-3-related transcription factor 1) which suppresses cyp19a expression and thus estrogen synthesis in *O. niloticus* (Rather et al., 2019; Wei et al., 2019).

Other factors, such as Amh (antimüllerian hormone), Sox9 (the Sry-related HMG box protein 9 gene) and Igf3 (insulin- like growth factor 3), are involved in the regulation of Dmrt1 and also play a critical role in testicular differentiation in fish (Wei et al., 2019; Liu et al., 2022).

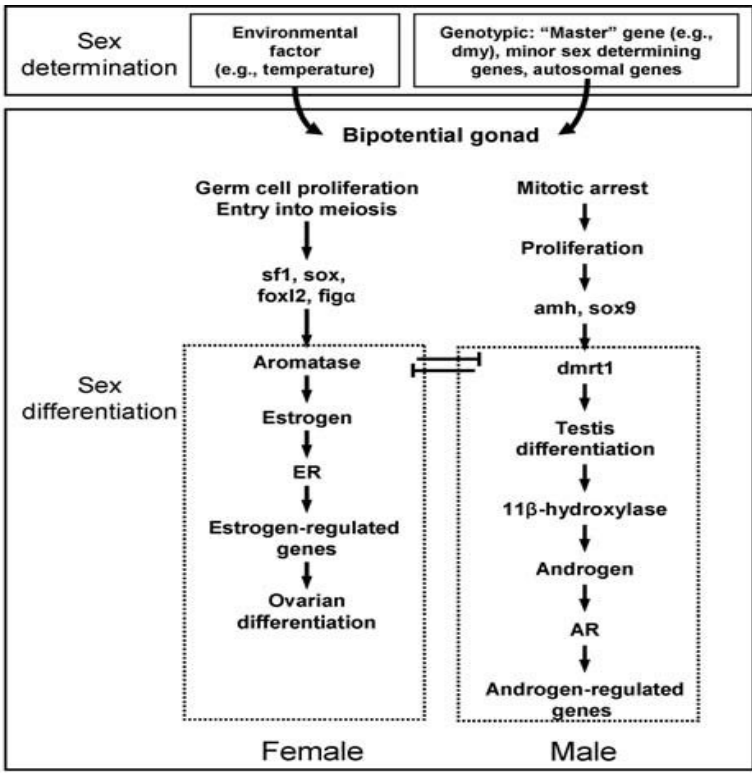

Fig. 4. Diagram showing fish sex determination and differentiation. Both aromatase and dmrt1 occupy a central position in sex differentiation in fish, which seems to be conserved across fish species, and they may antagonize each other (denoted by the-| symbols). Notice that in females, estrogen is essential for ovarian differentiation, whereas in males, androgens may be a product of testis differentiation. Thus, by no means, two events placed side by side in males and females necessarily occur at the same time during development (Piferrer and Guiguen, 2008).

Although the processes of sexual differentiation are fairly conserved among different fish species, sexual differentiation systems exhibit some environmental plasticity. In the differentiation process several stages are involved, starting from undifferentiated tissues and progressing to mature reproductive systems for males (testis) or females (ovary), thus establishing internal and external sexual characters and structures (anus, genital pore, urinary

pore) In all vertebrates, gonads are formed from developing primordial germ cells (PGCs) and mesodermal somatic tissue (Bhatta et al., 2012) which around the hindgut are located at this site after the formation of the coelomic cavity (Kobayashi et al., 2000, 2002, 2008). The migration of PGCs leads to the formation of gonads through successive mitotic divisions. During the last of these divisions, gonias become oocytes or spermatocytes through meiosis, establishing the cells that will give rise to the sexual gametes that will participate in the fertilization process.

1.4.2. Influence of temperature on sexual differentiation: Application in the aquaculture industry.

In most fish, the environmental factor with the greatest influence on sex determination and differentiation is temperature (Baroiller et al., 2009). During early stages of gonadal development, fish have windows of time in which they are sensitive to changes in temperature that can have a considerable effect on sex establishment. In general, high temperature treatments during temperature sensitive periods increase the proportion of males while cold temperature treatments increase the proportion of females (Honeycutt et al., 2019). However, as described above, in nature, water temperature exhibits both seasonal and daily cyclical variations. Regarding the application of daily thermocycles in fish during early developmental stages, several studies have observed that they considerably influence the sex ratio by increasing the number of females compared to larvae reared at constant temperatures as described in zebrafish and Senegalese sole (Villamizar et al., 2012; Blanco-Vives et al., 2011). This effect of thermocycling on sex ratio seems to depend on the fish species studied since in other species such as Mosambica tilapia (*Oreochromis mossambicus*), the proportion of females was increased although less considerably (Baras et al., 2000). In addition, the results obtained by the application of thermocycling suggest that the thermal sensitivity of the sex determination mechanisms varies depending on the time of day at which high and low temperatures are applied. The effect of temperature on sex ratio is determined by changes in sex differentiation genes: induction and upregulation of genes involved in testicular

development and inhibition and suppression of genes related to ovarian differentiation (Ospina-Alvarez and Piferrer, 2008).

The application of high temperatures during early stages of larval development in fish allows control of the sex ratio of culture populations (Baroiller et al., 2009). Thus, the establishment of single-sex populations has improved the efficiency of culture systems, especially hyperintensive cultures (Martinez et al., 2014). In monosex-sex populations of males, in addition to population heterogeneity, the absence of reproductive behavior allows animals to use the energy needed for reproduction in other processes such as development and growth, and thus, individuals reach marketable market size more quickly and at earlier ages (Martinez et al., 2014). However, the application of these methods leads to several negative effects on fish such as reduced larval survival, cellular stress, incomplete masculinization processes and malformations during development (Ribas et al., 2017; Yang et al., 2020; Biswas et al., 2021). To avoid these undesirable effects that compromise the thermal and reproductive biology of the animal, further research is needed to increase the efficiency of culture systems as well as the animal welfare of fish (Pandit and Nakamura, 2010; Jin et al., 2019; Mahmoud et al., 2020).

1.4.3. Brain-pituitary-gonadal axis

The brain-pituitary-gonadal-gonadal axis (BPG axis) is responsible for controlling reproduction in fish (Zohar et al., 2010; Cowan et al., 2017). It is responsible for perceiving environmental information (photoperiod, temperature, social interactions, chemical properties of water) and processing it through a complex neuroendocrine system which triggers a neurohormonal cascade that directs and drives the reproductive process from gamete formation to the release of high-quality gametes ready for fertilization (Zohar et al., 2010; Yaron, 2011). Each level of the BPG axis, is connected to the next so that there is both positive feedback with the next level structures and negative feedback on the previous levels (Fig. 5) (Yaron, 2011). Photoreceptors of the olfactory epithelium of the retina and pineal gland sense environmental light information (photoperiod duration, intensity and day/night

alternation) and transduce it into humoral and electrochemical signals by periodically modulating melatonin secretion. Melatonin is a hormone responsible for synchronizing biological clocks and rhythms to environmental factors (Falcon et al. 2007). When information reaches the hypothalamus via sensory neurons, hypothalamic neurosecretory cells release gonadotropin- releasing hormone (Gnrh). Gnrh stimulates the adenohypophysis of the pituitary gland which in turn induces the synthesis and release of gonadotropins into blood pathways. The main gonadotropins are FSH (Follicle Stimulating Hormone) and LH (Luteinizing Hormone), which stimulate the synthesis of steroid hormones at the gonadal level triggering the growth, development and release of sexual gametes (Yaron, 2011).

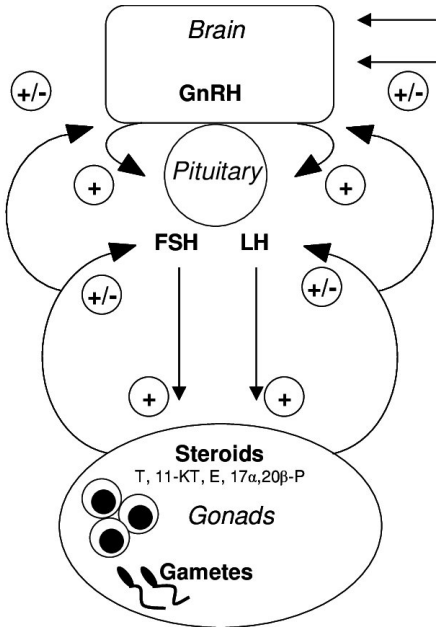

Fig. 5. Schematic representation of Brain-Pituitary-Gonadal of fish (Maugars, 2017).

In addition to the BPG system, other neuroendocrine systems have been considered for their fundamental role in reproduction such as the dopaminergic system and the Kisspeptin and gonadotropin inhibitory hormone (GnIH) system (Zohar et al., 2010). While dopamine has been described to have an inhibitory effect on gonadortrophins in fish, the kisspeptinergic system induces their release (Colledge, 2009; Shin et al., 2014). On the other hand, other

factors act directly in the regulation of reproductive cycles such as melatonin and cortisol (Falcón et al., 2007).

1.4.4. Influence of light and temperature cycles on the rhythms of reproduction

In most fish, reproduction is highly influenced by daily and seasonal changes in environmental factors, such as changes in photoperiod, temperature, weather conditions and food availability (Oliveira et al., 2010). In addition to these, other environmental factors have shown some influence on the synchronization of reproduction such as ocean currents, salinity (Baroiller et al., 1997, Abucay et al., 1999), and social aspects (Ridha and Cruz, 1999; Little et al., 1993; Ridha and Cruz, 2003).

The influence of photoperiod on fish reproduction is more marked in certain fish species that inhabit areas subject to seasonal variations in photoperiod, such as those belonging to temperate latitudes, compared to species that inhabit tropical areas (Migaud et al., 2010). Thus, the reproduction process occurs during increasing or decreasing photoperiods, thus maximizing the success and survival of the species (Migaud et al., 2010). In the synchronization of reproduction to the photoperiod, the pineal gland, as well as the daily and seasonal rhythms of melatonin production, are considered crucial factors in the transmission of environmental information and in the regulation of the daily and seasonal rhythms of reproduction (Falcon et al., 2010). In this way, in species of fish from temperate zones that reproduce in long-day photoperiods, their reproduction will be stimulated or inhibited by melatonin production in winter or summer, respectively. While in long-day breeders the opposite happens (Migaud et al., 2010). This fact allows fish to present seasonal reproduction rhythms influenced by photoperiod, as has been widely described in several fish species such as Nile tilapia (*Oreochromis niloticus*) (Campos-Mendoza et al., 2004), gilthead bream (*Sparus aurata*) (Zohar et al., 1995) and Senegalese sole (*Solea senegalensis*) (Oliveira et al., 2009 a,b), among others. However, the daily reproduction rhythms have been described in a reduced number of fish species such as zebrafish, Nile tilapia, sea bream, sea bass and Senegalese sole (Blanco-Vives and Sánchez-Vázquez, 2009; Bayarri et al., 2004; Meseguer et al., 2008; Oliveira et al., 2009a, b; de Alba et al., 2019). Both seasonal and daily rhythms

could indicate that all components and levels of the neuroendocrine system are synchronized by the influence of light (Cowan et al., 2017). Thus, most reproductive processes such as steriedogenesis, gonad maturation, gamete formation, as well as their release and participation in fertilization will be orchestrated by light information from the environment, establishing a rhythmic reproductive strategy dependent on the species of fish (Mañanós et al., 2008; Blanco-Vives and Sánchez-Vázquez, 2009).

However, in other species that inhabit tropical and subtropical zones, where the changes in the photoperiod are not as pronounced as in other latitudes, temperature seems to play a more important role in the reproduction process (Zachmann et al., 1992). Thus, temperature influences the reproductive physiology of fish, being able to stimulate or inhibit the production of melatonin and other key components in the reproductive system of fish and, consequently, reproduction in certain species (Vera et al., 2007). In most fish, reproduction is successful when they are in an environment with optimum reproductive temperature (Luksiene, and Svedang, 1997; Pankhurst, 1997; Beitinger, and Fitzpatrick, 1979). However, higher or lower temperatures than their optimal temperature can influence the reproductive physiology of fish (Pankhurst, 1997). In most fishes, increases of 1-2ºC to the optimum temperature simulate the thermal conditions close to the breeding season favoring reproduction while the use of cold temperatures usually negatively influences the reproductive process (Srisakultiew and Wee, 1988; Pankhurst, 1997; Baroiller et al., 1997). In addition, the use of cold or warm temperatures during intermittent periods of long or short duration are capable of inducing effects that maximize the reproductive efficiency of fish, such as courtship stimulation, reproductive synchronization, fertilization and spawning (Srisakultiew and Wee, 1988; Baroiller et al., 1997). However, these conditions do not reflect the thermal conditions of the natural environment. Thus, in the aquatic environment, fish are exposed to temperature variations that present both seasonally and daily oscillations, also known as daily and seasonal thermocycles (Villamizar et al., 2012). As it has been observed that the thermocycles have a considerable influence on the early development of fish, the latest advances showed that they had a huge impact on the mechanisms of sex determination and sexual differentiation of fish (Blanco-Vives et al., 2011, Villamizar et al., 2012).

In the last decades, climate change has increased the temperature of the ocean water, which can negatively compromise the development of the reproductive process of fish, causing gonadal malformations, permanent loss of germ cells and reduced quality of gametes, leading to complete inhibition of reproduction (Pandit et al., 2015; Servilli et al., 2020). This temperature rise scenario will not only affect the reproductive development of the species, but will also compromise the thermal biology of the fish and consequently, the survival and success of the different fish species.

1.5. Thermal tolerance.

Thermal tolerance in fish is the ability to tolerate different temperature ranges and cope with sudden changes in temperature, which generate a heat stress response in the fish (Donaldson et al., 2008). Heat stress is perceived by brain neurons and other sensory cells that produce a series of neuroendocrine signals that trigger a cellular and behavioral response as well as changes in osmoregulation, hematological and metabolic pathways (Van Den Burg et al., 2005; Flik et al., 2006).

Regarding the behavioral response, heat stress can compromise the survival and development of organisms (Morash et al., 2018). The factor with the greatest influence on thermal tolerance is the acclimation temperature during the development of individuals, which can modify the physiology and thermal tolerance of the individual irreversibly (Podrabsky and Somero, 2004). For example, the same fish species or cogeneric species reared in different thermal conditions may show a different tolerance to a thermal shock if it has been reared in warmer or colder environments (Campos et al., 2017). On the other hand, temperature variations in the aquatic environment also influence the development of thermotolerance depending on the fish species studied (Schaefer and Ryan, 2006). Certain fish species living in thermally very heterogeneous environments show higher tolerance to thermal shocks than those living in constant conditions (Healy and Schulte, 2012). Other species on the other hand, their response to thermal stress is not affected by the thermal

variability of the environment (Corey et al., 2017). On the other hand, very few studies show how thermotolerance varies as a function of daily and seasonal cycles of light and temperature. Studies developed in some fish such as *Clinostomus elongatus*, showed that their thermotolerance was influenced by the season of the year, presenting higher thermotolerance in summer months compared to winter months (Turko et al., 2020). In relation to the influence of light cycles on thermal tolerance, very few studies have also investigated how it varies as a function of time of day. Studies in killifish (*Fundulus heteroclitus*) under different photoperiods showed how it presented higher thermotolerance in the middle of the light phase compared to the beginning or end of the light phase (Kavaliers, 1980; Healy and Schulte, 2012). On the other hand, the effect of daily temperature variations on thermal tolerance and survival after heat shock has been studied in greater depth in some fish species compared to others, whose studies are scarce. While to date, very few investigations have been conducted in killifish (Fangue et al., 2006; Healy and Schutle, 2012) and zebrafish (Schaefer and Ryan 2006, Xia et al., 2016; Wang and Xia, 2019), in other species such as salmonids it has been studied in depth (Tunnah et al., 2017).

In addition to the behavioral response, heat stress generates a cellular response in which a series of proteins are involved to cope with the damage caused by temperature changes and allow cellular allostasis (Donaldson et al., 2008; Iwama et al., 1998). These proteins are known as heat shock proteins (HSPs) that together with their transcription factors (HSFs) are responsible for protecting and ensuring vital functions in all cells as they are involved in maintaining their conformation and function through protein assembly, folding and translocation (Fig. 6) (Donaldson et al., 2008). In addition, they are attributed an important role in the maintenance and regulation of the immune and neuroendocrine systems (Welch, 1993).

In heat stress there are several HSPs involved in cell protection against sudden changes in temperature. Among the most important in cell protection against temperature increases (hyperthermia) are HSP90, HSP27, HSP70 and HSP47, which differ mainly in their molecular weight and interactions with other non-protein molecules (Iwama et al., 1998). In

addition, other inducible proteins involved in sudden temperature decreases (hypothermia) have been described, one of which is encoded by the CIRBP (cold-inducible RNA-binding protein) gene (Verleih et al., 2015).

As with the thermotolerance response, the expression and induction of HSPs are influenced by acclimation temperature and other genetic factors (Dietz, 1994). Basal levels of HSP expression have been correlated with thermotolerance (Basu et al., 2002). However, upon abrupt changes in temperature, HSP transcription is activated and translated into new proteins that combat cellular damage by other proteins (Donaldson et al., 2008). The induction of expression varies depending on the tissue, fish species, maturity stage, type and duration of the stressor (Feder and Hofmann, 1999; Murtha and Keller, 2003). Thus, for example, studies in zebrafish showed a higher induction of HSPs expression by heat stress in liver, brain and gonads compared to anterior kidney and interrenal tissue, which showed lower levels of induction (Rabergh et al., 2000; Murtha and Keller, 2003). Furthermore, the expression of HSPs can vary as a function of season and time of day, showing the enormous influence of environmental cycles on the mechanisms involved in thermotolerance (Fader et al., 1994; Healy and Schutle, 2012).

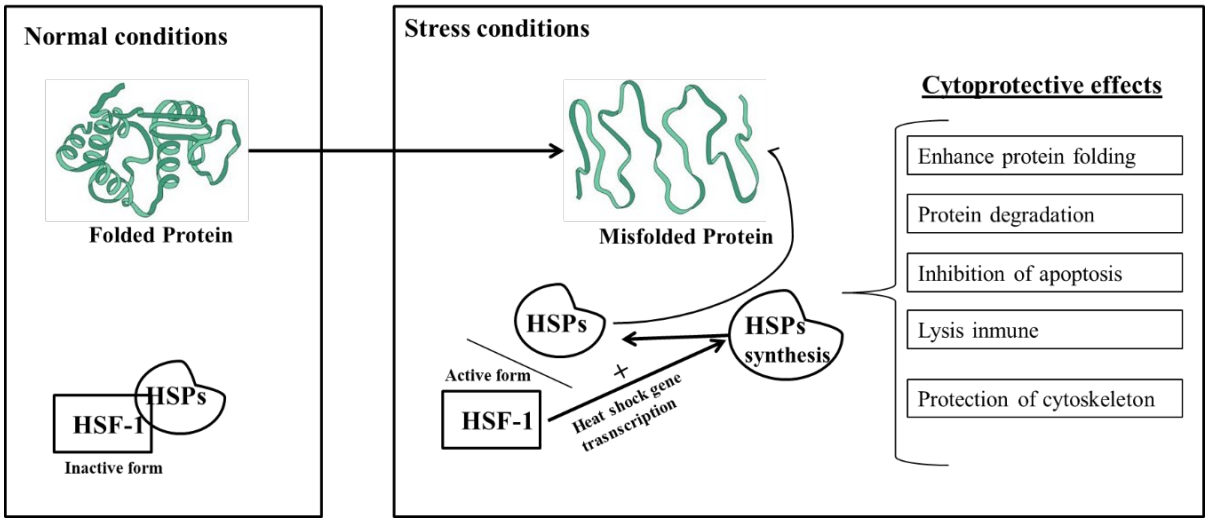

Fig. 6. Schematic representation of the heat shock mechanisms (HSP, Heat Shock Proteins; HSF, Transcription factor of HSP) under normal and stress conditions.

HSPs not only influence thermotolerance but in recent years studies have focused on determining the role of HSPs in temperature-dependent sex determination (Li et al., 2014). In high temperature heat treatments to induce masculination of Nile tilapia, the induction of HSPs expression correlates with changes occurring in sex determination genes at the gonadal level (Li et al., 2014). On the other hand, studies in the American alligator (*Alligator mississippiensis*) show how this induction of HSPs presents sexual dimorphism presenting higher HSP70 values in females while HSP27 expression is higher in males (Khono et al., 2010). Due to the scarcity of existing studies, the relationship between thermotolerance and temperature-dependent sex determination (TSD) should be further investigated.

On the other hand, it is required to study in detail the existence and role of daily rhythms of HSPs in thermotolerance in other fish such as zebrafish as well as to determine the influence of light and temperature cycles on their thermal tolerance and on the mechanisms of stimulus detection and transduction to maximize the efficiency of the response to a thermal stressor (Jeronimo et al., 2017; Germana et al., 2018).

1.6. Thermal detection

Most fish live in thermally very heterogeneous aquatic environments (Beitinger and Fitzpatrick, 1979). In this context, the temperature sensing system exerts a particularly relevant role in preventing physiological damage caused by abrupt temperature changes, which can irreversibly affect the performance of fish physiological functions (Fu et al., 2021; Li et al., 2014). An appropriate temperature sensing system will ensure that an adequate thermotolerance response is triggered to maintain the homeostasis of the organism (Morash et al., 2021). Thus, recent studies have focused on investigating the close relationship between the systems involved in thermotolerance and temperature sensing and thus, understanding the complex network of physiological and environmental interactions between them (Jeronimo et al., 2017; Germana et al., 2018).

The thermal information of the environmental temperature is perceived by animals

through the interaction between the environment and the sensory neurons of the trigeminal and dorsal root ganglia that innervate the skin (Germana et al., 2018). These neurons possess specialized structures in their cell membranes known as pores or membrane channels which are Transient Receptor Potential Channel (TRP) receptors (Germana et al., 2018). These structures are extremely sensitive to temperature variations and are also known as thermo TRPs channels (Pertusa et al., 2012).

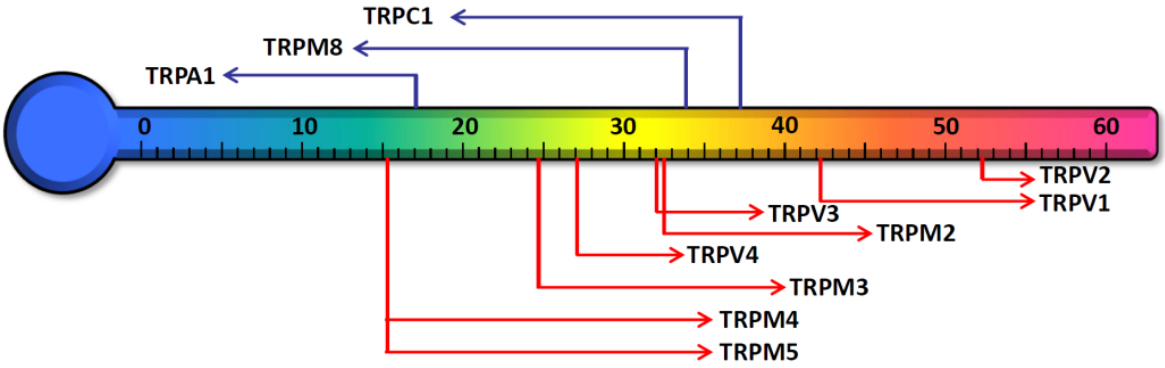

Fig. 7. Schematic representation of the temperature sensitivity of thermoTRPs in vertebrates (Pertusa et al., 2012).

In zebrafish, numerous thermo TRP subfamilies have been described that are activated by different temperature thresholds (York et al., 2022). Receptors belonging to the TRPA subfamily are activated by low temperatures (<17°C) (Oda et al., 2016). While others belonging to the TRPM or TRPV subfamily are activated in temperate or higher temperature ranges: TRPM4 and TRPM5 between 15-35ºC, TRPM2 between 35-42ºC, TRPV4 between 27-42ºC, and TRPV1/2 for temperatures above 42ºC (Saito and Shingai, 2006; Germana et al., 2018). Although there are no studies to date, it is critical to continue investigating the influence of the environment on the timing of thermal sensing mechanisms to ensure adequate temperature perception and trigger a behavioral response that maintains thermal homeostasis in fish.

1.7. Thermal preference

Temperature plays a very important role in the performance of biological processes, and more especially in fish, since being poikilothermic animals, their body temperature depends on water temperature (Smith, 1976; Coutant, 1976). The thermal optimum is defined as the temperature that maximizes the efficiency of energy metabolism and favors the physiological performance of fish (Kellogg and Gift, 1983). Thus, it is a limiting factor in the biological cycle of fish, where temperatures above or below the thermal optimum compromise their survival, development and success of the species, as well as condition the distribution of fish species in aquatic ecosystems (Neill, 1979). To regulate their internal body temperature and maintain it within a physiological range, most fish select their preferred temperature through behavioral processes, moving towards zones that guarantee their thermal optimum and establishing their final preferred temperature (Haesemeyer, 2020). The preferred temperature varies depending on the fish species and even its geographic distribution (Beitinger and Fitzpatrick,1979). In addition, other factors such as acclimatization temperature, health and nutritional status, as well as the developmental stage of the individual, have an enormous influence on the preferred temperature (Reynolds and Casterling, 1979; Golovanov, 2006; Wurtsbaugh and Neverman, 1988).

Seasonal and daily temperature variations also influence the behavioral regulation of internal temperature in fish. The preferred temperature varies throughout the day in a different pattern depending on the fish species studied. Thus, in some species such as *Amia calva* the lower temperatures were selected during the light phase while in other species such as *Salmo trutta* and *Carassius auratus* species, the lower temperatures were chosen in the dark phase (Reynolds et al., 1978a, 1978b; Reynolds and Casterling, 1979). So far, the cause of this different species-dependent response is unknown. Some hypotheses advocate the idea that the daily variation of preferred temperature is conditioned by the fluctuation of water temperature throughout the day in the aquatic environment, selecting higher or lower temperatures during the light or dark phase, respectively. Other hypotheses suggest that it could be due to the influence of light on the rhythms of locomotor activity conditioning the

choice of areas with higher temperature at times of increased activity. Other researches even claim that the daily rhythms of feeding behavior influence the selection of the desirable thermal space to optimize the metabolic needs of fish (Gleiss et al., 2017). Although there is a strong link between light and feeding cycles and fish thermal physiology (López-Olmeda et al., 2010), the different hypotheses cited above highlight the need to study the complex network of environmental interactions that determine the daily rhythms of fish thermal preference.

1.8. Species in focus

1.8.1.Nile tilapia (*Oreochromis niloticus,* Lineaus, 1758).

The Nile tilapia is also another cyprinid that inhabits tropical and subtropical environments although it is native to the northern half of Africa and western Asia (Trewavas, 1982). As reviewed by Beardmore et al. (2001), Nile tilapia presents a series of productive advantages such as: rapid growth and large commercial sizes; high resistance to disease; great colonization capacity in different marine ecosystems with very heterogeneous thermal and light conditions; sexual maturity at very early ages; high prolificacy; maternal care of the embryos until they have the capacity to feed and swim; and ease of cultivation in high intensity systems.

However, inefficient breeding leads to high costs in production and management of tilapia culture, with several consequences as: the limitation of growth due to the utilization of energy in reproductive activity instead of development, resulting in females reaching a lower marketable weight; aggressive behavior derived from courtship and reproduction; and asynchronous reproduction, obtaining spawning at irregular intervals (Biswas et al., 2005; Bombardelli, et al., 2017; Gonçalves-de-Freitas et al., 2019).

To limit or eliminate these negative reproductive characteristics, the Nile tilapia industry has focused on establishing monosex populations of individuals (especially male

populations), making tilapia the second most farmed fish species in the world after carp (FAO, 2022) and colonizing the aquaculture market. In addition, tilapia is a species of great interest as an animal model for ecotoxicological studies, evolution of sexual determination in fish and its genetic- environmental interaction, and behavioral and chronobiological studies (Vera et al., 2007; Baroiller and D' Cotta, 2019;).

Fig. 8. Picture of Nile tilapia (*Oreocrhomis niloticus*).

1.8.2. Zebrafish (*Danio rerio*, Hamilton, 1822)

Zebrafish is a cyprinid fish species whose natural habitat is composed of different marine ecosystems ranging from the waters of the Ganges River and Brahmaputra in India to other areas in the southeastern Himalayan region (Spence et al., 2008). It usually inhabits shallow, slow-moving waters dominated by aquatic vegetation (Engeszer et al., 2007). It is a eurythermal species that lives in highly heterogeneous thermal environments and whose temperature fluctuates both seasonally and daily on a daily basis. During the year, it can tolerate a wide range of temperatures from 38-40ºC during the summer to 4-6ºC in the winter season (Spence et al., 2008). In addition, it is exposed to daily thermocycles with high amplitudes (up to 6ºC difference between the minimum and maximum daily temperature values (Payne and Temple, 1996). On the other hand, in its natural environment, the zebrafish must face abrupt changes in water temperature caused by monsoon climates and climate change, which compromise the survival and development of this species. In the

laboratory, breeding and handling of zebrafish is described as relatively easy, due to the environmental conditions necessary for their development (Lawrence, 2007). The zebrafish is an excellent vertebrate animal model that during its rapid development presents optical accessibility during the early stages of embryonic development (Choi et al., 2021). The high conservation of genetic information and physiological processes, as well as the ease of genetic manipulation, allows the zebrafish to be used in various fields of research such as neuroscience, developmental biology, genetics, biomedicine, ecotoxicology, physiology, but also for behavioral and chronobiological studies (López-Olmeda and Sanchez-Vázquez, 2011; Sánchez-Vazquez and López-Olmeda, 2018; Guidi et al., 2022)

Fig. 9. Picture of zebrafish (*Danio rerio*).

Objectives

2. Objectives

The objective of this doctoral thesis is to elucidate the influence of light, temperature and feeding cycles at different life stages of zebrafish and Nile tilapia (Figure 10) on their reproduction, development, thermotolerance and behavioural rhythms. To this end, the following specific objectives were assessed:

1. Research the interaction between genetic and environmental components that determine the sex of Nile tilapia and describe the dynamics of the stages and endocrine mechanisms that comprise their sexual differentiation.
2. Review the role of environmental, physiological, and neuroendocrine factors in tilapia reproductive physiology to improve established breeding protocols in tilapia aquaculture.
3. Investigate the combined effects of light and temperature regimes (daily thermocycle *vs*. 24h-constant temperature) during early development, and the effect of a heat shock treatment during the period of sexual differentiation of Nile tilapia.
4. Determine the influence of the time of day of heat treatment on sexual differentiation and thermal tolerance in Nile tilapia.
5. Investigate the effect of two temperature regimes of rearing (thermocycle *vs*. constant temperature) and time of day on the rhythms of thermal detection and tolerance mechanisms.
6. Investigate the combined effect of daily thermocycles and light spectrum (blue vs red) during early development in zebrafish and understand the mechanisms driving the enhancement/deleterious effects of light.
7. Determine the existence of daily rhythms of thermal preference in zebrafish and Nile tilapia given free access to a temperature gradient tank.
8. Evaluate the effect of light and feeding (mealtime/starvation) on the daily rhythm of thermal preference in Nile tilapia.

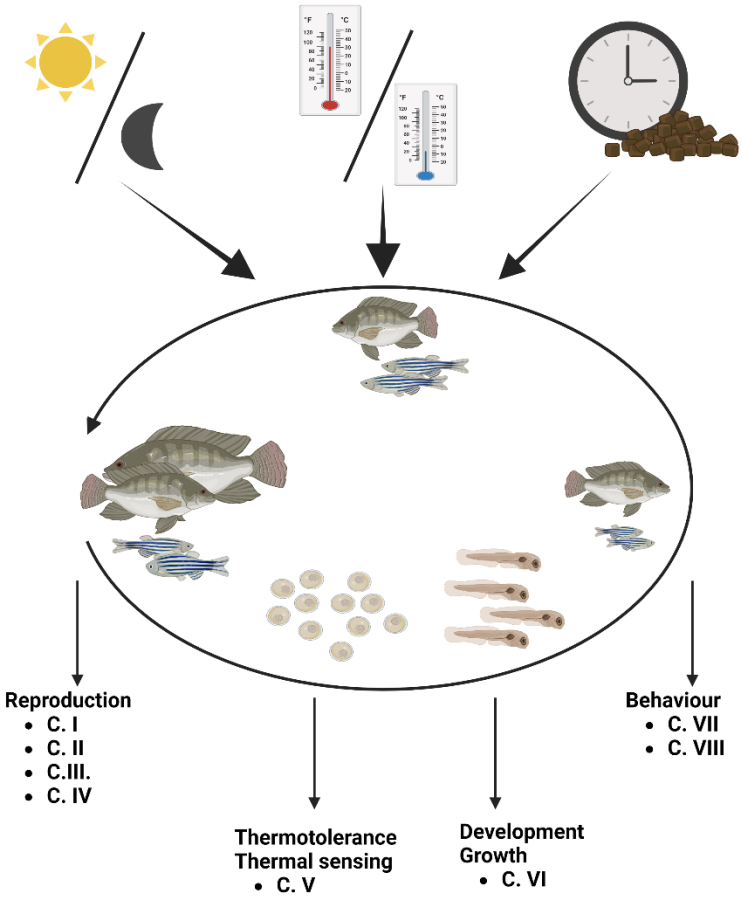

Fig. 10. Schematic overview of the thesis

Experimental Chapters

Experimental Chapter I

(Book chapter)

Sex determination and differentiation of Nile tilapia

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ABSTRACT

The high productive and reproductive capacities of tilapia have led this species to be considered one of the most important fish groups of commercial interest. However, certain reproductive aspects, such as early maturation, asynchronous gonadal development and low prolificacy make its management difficult and reduce tilapia aquaculture's productive intensity. To address these problems, industry has focused on the genetic and sexual control of tilapia to reduce genetic and phenotypic variability and to, thus, optimize its production. In the present chapter, we look in-depth at the interaction between the genetic and environmental components that establish sex determination. We also cover an extensive review on the main sexual differentiation stages, and the influence of endocrine and environmental mechanisms that intervene in the establishment of the individual's phenotype (sex).

INTRODUCTION

In aquaculture, tilapia has become one of the most popular genera of commercial fish species. The most cultivated tilapia species is *Oreochromis niloticus,* which accounts for 85% of the world's tilapia production because of its good availability, and its numerous productive and reproductive advantages. Tilapia's productive interest lies in its rapid growth and good disease resistance (Jalabert 1989, Lapeyre et al. 2008). In addition to these productive advantages, several other reproductive aspects make tilapia a great species for aquaculture. Firstly, females present high fecundity with successive breeding cycles every 2- to 4-week intervals all year round (Tacon et al. 2000, Lapeyre et al. 2008). Secondly, tilapia exhibits parental care, which means that after fertilization, females hold eggs inside their oral cavities (mouthbrooding) to safeguard offspring's survival against predators and other external hazards (Rana 1988). However, there are other reproductive aspects that compromise fish farming protocols and production. On the one hand, tilapia exhibits precocious sexual maturation, which may occur at around 3 months of age depending on both environmental conditions and genetic differences (De Silva and Radampola 1990, Suresh and Bhujel 2013). Hence especially in females, high breeding frequency involves using most energy reserves to fulfil reproduction needs, which limits muscle growth and productive yield (Beardmore et al. 2001). On the other hand, due to the asynchronicity of ovarian development, the female gonad involves different development stages that make it difficult to control not only her reproductive cycle but also, therefore, synchronization of breeding and the capacity of obtaining fertilised eggs at will. Moreover in Nile tilapia, fertility rates depend on both the weight (body size) of females and environmental conditions.

Due to high energy demands for reproduction in females, tilapia aquaculture has focused on the production of males-monosex populations (Omasakai et al. 2016), which provides a series of relevant advantages. For instance, monosex production allows the control of the reproduction process and both genetic and phenotypic variability. Furthermore, the use of monosex populations allows productive parameters to be intensified (males grow faster and bigger than females and they reach the market size earlier), which optimises the resources used in the tilapia industry (i.e. facilities and technical staff) (Beardmore et al. 2001, Pinto et al. 2018). The main techniques to obtain monosex populations are based on using thermal,

hormonal and genetic protocols to perform the sexual reversion of female to male during sensitive periods for sexual determinations (Baroiller and Jalabert 1989).

So as regards tilapia reproduction, knowing in-depth the interaction between the genetic and environmental components that establish sex determination is most important. In addition, it is essential to know the stages of sexual differentiation and the endocrine mechanisms which control the transformation of an undifferentiated gonadal tissue into ovaries or testicles, thus establishing the phenotype of the adult fish.

SEX DETERMINATION

Excellent fish flexibility is also reflected in the sexual determination mechanisms found among species of the same genus, and within populations of the same species (Trewavas 1983). Sex determination is a process that involves a series of mechanisms that establish an individual's gender (male/female). In fish, this process is influenced by genetic and/or environmental factors, which give rise to genotypic sex determination (GSD) and to environmental sex determination (ESD), respectively. The inheritance of sex is determined by sexual factors (related to GSD) and environmental effects (related to ESD), which are described in the next sections (Ospina-Alvarez and Piferrer 2008).

Genetic influence on sex determination

Genotypic sex determination is the most frequent mechanism in fish species. GSD is determined by the major and secondary sexual factors contained in sex chromosomes by establishing the main axis of chromosomal inheritance (Guinguen et al. 2019). This type of chromosomal inheritance occurs in fish species whose sex chromosomes are heteromorphic to the rest (they morphologically differ from other chromosomes). Chromosomal inheritance in fish comprises monofactorial systems as XX/XY or WZ/ZZ (formed by a single pair, or multiple pairs, of sex chromosomes) and multifactorial systems. The monofactorial system is the commonest in fish in which heterogametic sex (sex individual with a different type of sex

chromosomes) is only one of the sexes, either the male (in XX/XY system) or female (WZ/ZZ system). However in multifactorial systems, both sexes are heterogametic and sex is determined by three main factors or more (Devlin and Nagahama 2002).

The Nile tilapia is probably one of the best-documented sex determination systems in fish (Baroiller et al. 2009). Decades ago, the main genetic sex determination mechanisms in Nile tilapia were determined thanks to the use of chromosomal manipulation techniques (gynogenesis, androgenesis or triploidization) and progeny tests associated with employing hormonal therapies for sex reversion. It was then when it was established that *O. niloticus* presented a monofactorial XX/XY system with some particularities (Pruginin et al. 1975). In *O. niloticus*, as in *O. mossambicus*, heterogametic determination mechanisms are present in the male sex (XX-XY system). However in other species of the same genus such as *O. aureus*, it is the female sex that exhibits heterogametic chromosomes (WZ-ZZ system) (Carrasco et al. 1999, Campos-Ramos et al. 2001; Cnaani and Kocher 2008, McAndrew et al. 2016).

In recent years, research efforts have been made to identify the genes and region (locus) of the chromosomes that determine sex. To date, several genes have been described as master sex-determining genes in different teleost species: *doublesex- and mab-3-related transcription factor 1* (*dmrt1*), *SRY-box transcription factor 3* (*sox3*), *gonadal somatic cell-derived factor* (*gsdf*), *anti-müllerian hormone* (*amh*), *sexually dimorphic on the Y chromosome* (*sdY*) and *interferon regulatory factor 9* (*irf9*) (Guinguen et al. 2019). Although major sex-determining genes have not yet been found in tilapia, the use of whole-genome sequencing indicated strong evidence for *amh* as a major gene that controls sex differentiation in Nile tilapia (Caceres et al. 2019). Several studies have characterized 13 genes to be sex-linked markers (regions) in six different tilapia species (Lee et al. 2003, Shirak et al. 2007, Cnaai and Kocher 2008, Rahther et al. 2019). The identification of the candidate genes for sex determination was performed in most of these studies by using both linkage and physical mappings in the regions with different expression profiles from the first early development stages in order to associate the phenotypic sex (gender) with specific linkage groups (LGs). Thus, in the male heterogametic system of Nile tilapia (XY), the major sex-determining regions are described in LG1 and LG23 (Lee at al. 2003, Eshel et al. 2012, Li et al. 2015,

Conte et al. 2017). For blue tilapia, which presents a female heterogametic system (ZW), two sex-determining regions have been identified in LG1 and LG3 as sex-specific markers (Campos-Ramos et al. 2001, Lee et al. 2004, Bian et al. 2019). However, further studies in this field are needed to increase our knowledge so as to identify genomic regions to track sex-linked loci in breeding programmes to control the sex of the population's individuals.

As very high diversity in sex determination mechanisms and multiple sex-determining regions have been described for different tilapia species, they are a unique model to study not only the genes that determine sex in fish, but also the evolutionary origin of sex determination in vertebrates.

Environmental influence on sex determination

Regarding sex determination, some fish possess certain phenotypic plasticity (from the same genotype) in initial development stages depending on temperature. Nile tilapia's sex is determined by a system composed of three components. Two of them are, as described in the previous section, related to genetics and are key determining factors of tilapia sex. These include a major and determining locus and secondary genetic factors. In Nile tilapia, environmental variables can influence sex determination (Baroiller et al. 2009). The main environmental variables to impact the sex determination of tilapia are temperature and pH (Baroiller and D'Cotta 2001), while other environmental factors, such as photoperiod and salinity, may also have an influence, but have been investigated less (Abucay et al. 1999).

Temperature is the main environmental factor to influence sex determination in most teleost species. Out in the wild, warmer (27ºC) or colder (23ºC) water temperatures in different geographical locations induce a male or female sex ratio in Southern flounder (Honeycutt et al. 2019). In the sex determination of tilapia, the impact of temperature has been studied in several tilapia species (Baroiller et al. 1995a, b, Wang and Tsai 2000). Nile tilapia development involves several thermosensitive periods, which coincide with the periods during which gonadal tissue is more sensitive to hormonal stimuli (Rougeot et al. 2008, Baroiller and D'Cotta 2001, Nakamura 1975, Baroiller et al. 1999, Guiguen et al. 1999). Some

research works have aimed to determine this thermosensitivity window. Most studies generally agree that thermal treatments in the early larval development stages have the strongest effects on tilapia sex determination (Kwon et al. 2000, Ijiri et al. 2008, Rougeot et al. 2008, Baroiller et al. 2009). Exposure to high temperature (36ºC) in the critical sex determination stage (9-15 dpf, days post fertilization) for about 10-28 days in Nile tilapia induces sex reversal of genotypic XX-females to XX-pseudomales (D'Cotta et al. 2001a,b, Kwon et al. 2000, Ijiri et al. 2008, Baroiller et al. 2009). However, exposure to lower or higher temperatures prior to that sex determination stage has no effect on sex determination (Baroiller et al. 1995, Tessema et al. 2006, Sun et al. 2018, Zhao et al. 2019). Apart from following higher- or lower-temperature protocols, gonad development and sex ratio are also influenced by daily temperature fluctuations that fish experience in nature (higher temperatures in the daytime, cooler temperatures at night), which has been described in several fish species such as zebrafish (*Danio rerio*) (Villamizar et al. 2012) and Senegalese Sole (*Solea senegalensis*) (Blanco-Vives et al. 2011). In these research works, the fish that undergo daily thermocycles present higher percentages of females than when facing constant average temperature and reverse thermocycles (cooler temperatures in the daytime, higher temperatures at night). These results suggest that the thermal sensitivity of sex determination mechanisms varied throughout the day, with higher and lower masculinization levels after exposures to high temperature at night and in the daytime, respectively. Continuing to investigate the chronosensitivity of sex determination genes to heat shock could improve the efficiency of masculinization protocols and minimize the negative effects derived from high temperatures such as cellular stress, mortality, developmental malformations and incomplete masculinization process.

Although the effect of different temperature regimes on the sex ratio has been well studied, very few studies have focused on whether that sensitivity varies between breeding pairs (Baroiller and D'Cotta 2001, Tessema et al. 2006). Indeed, these studies show that maternal and paternal mating patterns strongly influence male proportions in temperature-treated progenies, which suggests some heritability in thermal sensitivity in Nile tilapia, as well as a significant parental effect on TSD.

Recently, some authors investigated how high-temperature treatments induce sex reversal in Nile tilapia females (Tao et al. 2020; Zhao et al. 2020). They also showed the effect of high temperature on the transcriptional changes of *dmrt1*, *gsdf* and other sex-determination mechanisms. In addition, high temperature effects on sex reversal can also be observed molecularly. The main molecular changes were found at the *vasa*, *cyp19a1* and *dmrt1* expression levels. Therefore, the sex reversal process has been described in morphological terms. Although very few morphological changes were observed in early gonad differentiation stages, the gonad of XX females acquired the morphological patterns of testis (sperm germ cells in different development stages) from 99 dpf.

GSD is the main sex determination process in Nile tilapia. Hence the phenotypic character of Nile tilapia (sex) is determined by a combination of genomic-environmental processes, although temperature can strongly impact the phenotypic sex ratio (i.e. temperature-influenced GSD). Temperature treatments can be helpful for inducing masculinization, which is actually the preferred method in tilapia aquaculture. Moreover, several hormonal agents and techniques have been applied to obtain monosex populations (Beardmore 2001). These topics are described in detail in Chapter II of the present book.

GONADAL DEVELOPMENT AND SEXUAL DIFFERENTIATION

In tilapia, and also in all vertebrates, gonads are formed from the development of primordial germ cells (PGCs) and mesodermal somatic tissue (Bhatta et al. 2012). On 3-4 dph (days post hatching), primordial germinative cells, which migrate to the developing gonads and lie around the posterior intestine, surround the outer lateral plate mesoderm layer and are located in this place after celomic cavity formation (Kobayashi et al. 2000, 2002, 2008). These primordial cells start to proliferate in females from 9 to 14 dph, whereas their numbers remain constant until day 14 dph in males (Ijiri et al. 2008). In this sexually undifferentiated stage, poor blood vessel development in the gonadal stroma takes place, along with a few steroid-producing cells (Nakamura et al. 1998). After early embryonic development, the migration of PGCs leads to gonia formation through successive mitotic divisions. During the last of these divisions, a differentiation process through meiosis begins, during which gonia

become oocytes or spermatocytes (in detail described in the next sections). Therefore, the sexual differentiation of tilapia gonads starts in relatively early larval stages (between approximately 15 and 30 dph), although complete sexual maturity is reached at 3-4 months of age (Yoshikawa and Oguri 1978, De Silva and Radampola 1990, Strüssmann and Nakamura 2003, Suresh and Bhujel 2013). Several stages are involved in the differentiation process, starting with undifferentiated tissues and moving to matured reproductive systems for males (testis) or females (ovary). Regarding external characters, Nile tilapia presents a clear sexual dimorphism in relation to their reproductive structures (Figure. 1). Thus males have only two ventral orifices (anus and genitourinary orifice), while females have three (anus, genital pore, urinary pore) (Rana 1988).

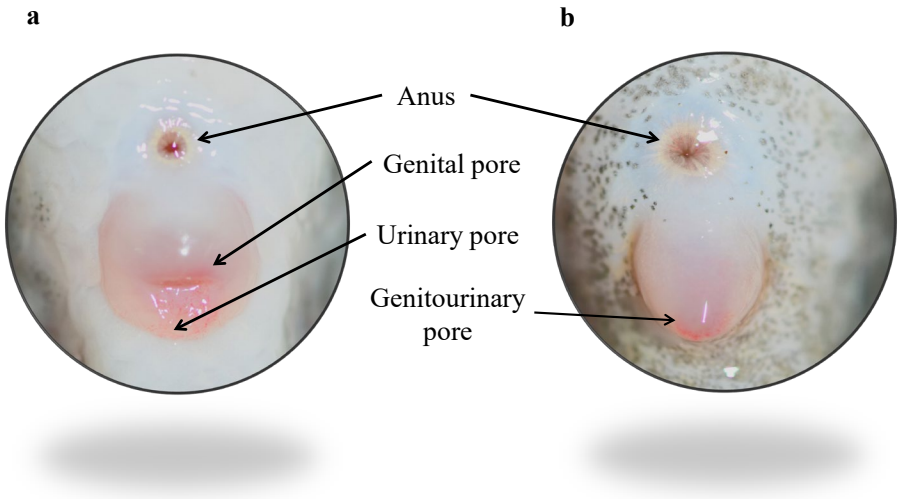

Figure 1. Sexual dimorphism in female (a) and male (b) Nile tilapia.

Testicular morphoanatomy and development

Testicles are responsible for producing sperm and sex hormones in males. In tilapia, testicles are elongated in shape, positioned in the dorsolateral situation and joined through the dorsal wall. Babiker and Ibrahim (1979) offered an extremely detailed description of male testes in different differentiation and maturation stages. In their studies, the colour and shape of testicles varied depending on the gonadal differentiation stage. In initial or immature

stages, male tilapia present flesh colours. However when males approach the maturity stages, testes displayed creamy colours and occupied the total visceral cavity length. In addition, the spermiduct leaves each testicle to reach its joint with the urogenital papilla. Studies performed in *O. mossambicus* (Bhatta et al. 2012) and *O. niloticus* (Babiker and Ibrahim 1979, Nakamura and Nagahama 1989, Melamed et al. 1997, de Graaf and Huisman 1999) showed that testicular development stages increase at the same time as body size and the gonadosomatic index (GSI) do. Tilapia has a lobular testicular structure-type composed of a set of lobes with several cysts. A cyst constitutes the functional unit of the testicle, and it is the place where spermatogenesis occurs. In this spermatic cyst, a few Sertoli cells and primary spermatogonia are found. After successive mitotic and meiotic divisions, these cells give rise to spermatocytes, spermatids and spermatozoa. Therefore in the testicular lobe, spermatocysts loaded with spermatozoa ready to be released to sperm can be found in the final stage (Grier and Fishelson 1995, Fishelson 2003).

Testicular development starts around 15 dph. At this point, the gonadal tissue that will give rise to the testicular apparatus is distributed along the enteric mesentery (Nakamura et al. 1998). Laxed-undifferentiated tissue is composed of PGCs and somatic tissue, which will be essential for the formation of the male reproductive system. Thus PGCs will be involved in the synthesis and differentiation of spermatozoa. Meanwhile, somatic tissue will be needed to bring about the structural and functional basis of germ cells by also forming the seminiferous tubes and connective tissue of the testicular apparatus. Between both germ and somatic tissues, specialised somatic tissue cells can be found: Sertoli and Leydig cells. While Leydig cells will be responsible for the production of sex steroids, Sertoli cells will be in charge of structural and nutritional functions, and of phagocytization of cytoplasmic residues and residual germ cells. Sertoli cells are involved in the production of the hormones and factors needed for the differentiation, development and survival of germ cells.

The histological changes that take place during testicular development are described in detail in *O. niloticus* (Babiker and Ibrahim 1979, Nakamura and Nagahama 1989, Fishelson 2003) and *O. mossambicus* (Bhatta et al. 2012). Most previous research works have divided the Nile tilapia testicular development process into seven phases: differentiation, renovation, mitotic proliferation, meiotic proliferation, spermiogenesis, spermiation, sperm maturation. In

the first phase (15 dph), the differentiation of PGCs to spermatogonial germ cells or spermatogonias A occurs (21-23 dph) (Melo et al. 2019). From 25 dph, a space begins to form in the cleft of testicular stroma that will form the efferent duct, accompanied by a proliferation of epithelial cells (23-26 dph) (Iriji et al. 2008). After differentiation, the renewal and multiplication of spermatogonia A occurs. These spermatogonia A will proliferate rapidly by successive mitosis divisions, which result in different types of spermatogonias B (30-50 dph). To characterise sexual differentiation in male tilapia, the presence of spermatogonias (before meiotic proliferation) and somatic cells has been identified as a marker sign (Nakamura and Nagahama 1989, Vilela et al. 2003). Following the beginning of meiotic divisions, the transformation of spermatogonias into spermatocytes (first meiotic division) and spermatids (second meiotic division) occurs. Spermatids undergo morpho-functional maturation during spermatogenesis, which gives rise to spermatozoa (Nakamura and Nagahama 1989). During spermatogonial proliferation, the increase in Leydig cells is slow. However, Leydig cell numbers start increasing rapidly after 70dph and appear between spermatogonia cysts in interstitial tissue (Gier and Fishelson 1995, Nakamura and Nagahama 1989). The breakdown of Sertoli cells causes cyst rupture and spermatozoa are released into the lobular lumen and spermiducts. On its way through spermiducts, sperm undergoes a capacitating process in which it acquires the ability to move and fertilise. From this stage (70 dph), smaller sized males can reach sexual maturity (14-20 cm). The maturity stage is characterised by the marked presence of sperm germ cells in different development stages that line sperm ducts as well as Leydig cells groups in interstitial tissue to mark the beginning of active spermatogenesis, the production of sex steroids in the testicle and, hence, the onset of male fertility.

Ovarian morphoanatomy and development

The ovary is the last effector organ of the female reproductive axis and is responsible for regulating the production of viable eggs and sex hormones needed for the ovarian development and differentiation process. The ovary of tilapia presents similar morphological characteristics to the cystic ovaries of other teleosts (Nagahama 1983). A detailed visual inspection (appearance, size, shape, colour) during the maturity stages of tilapia ovaries has been made by several authors (Babiker and Ibrahim 1979, Shoko et al. 2015). In their

description, ovaries are small in size with a flesh creamy colour in the first immature stages. As maturation progresses, ovaries become yellowish coloration, are oval-shaped, and occupy about one third (depending on both size and individuals) of this animal's coelom cavity (Babiker and Ibrahim 1979).

Ovarian development constitutes a highly complex process which is timely regulated by both environmental and endocrine pathways. On the first days of development, the peritoneum extension leads to the formation of the germinative epithelium, from which ovarian follicles form. The tunica albuginea can be found below the germinal epithelium, which is characterized as a dense connective tissue composed of muscle fibres and blood vessels. The tunica albuginea presents several folds (ovigerous lamellae) that are the structural and functional support of follicular development. Germ and follicular cells are located in ovarian lamellae, which is where oogenesis occurs. After oocytes form in ovarian stroma, they are released to the lumen of the ovary (Nakamura et al. 1998).

Histological ovarian morphology changes have been studied in tilapines (Ibrahim and Babiker 1979, Nakamura et al. 1998, Coward and Bromage 2000, 2002). The interest in ovarian structure, morphology and development is reflected in the different studies carried out in mouthbreeders, such as *O. niloticus* (Babiker and Ibrahhim 1979, Alves et al. 1983, Tacon et al. 2000), *O. mossambicus* (Dadzie 1974, Bhatta et al. 2012) and *O. aureus* (Garcia and Philip 1986). In most of these studies, the authors describe recrudescence in seven development stages depending on the the histological morphology characteristics (nucleus, cytoplasm and follicular layer) and biochemical properties: oogonic proliferation, oogeneis, folliculogenesis, alveolar cortical formation, vitellogenesis, final maturation; ovulation (Fig. 2). Around the first day of ovary development, which occurs between 8-15 dph, proliferative germ cells give rise to oogonias. The first sign of morphological differentiation in females is the ovarian cavity formation (22-26 dph) (Iriji et al. 2008). Oogonias are divided through mitotic phases and give rise to primary oocytes, which are surrounded by granulosa cells. These cells are, in turn, surrounded by a cellular connective tissue monolayer, also known as the Teca cell layer. Hence the granulosa and Teca layers, together with the oocyte, form the functional complex of the ovary, namely the ovarian follicle.

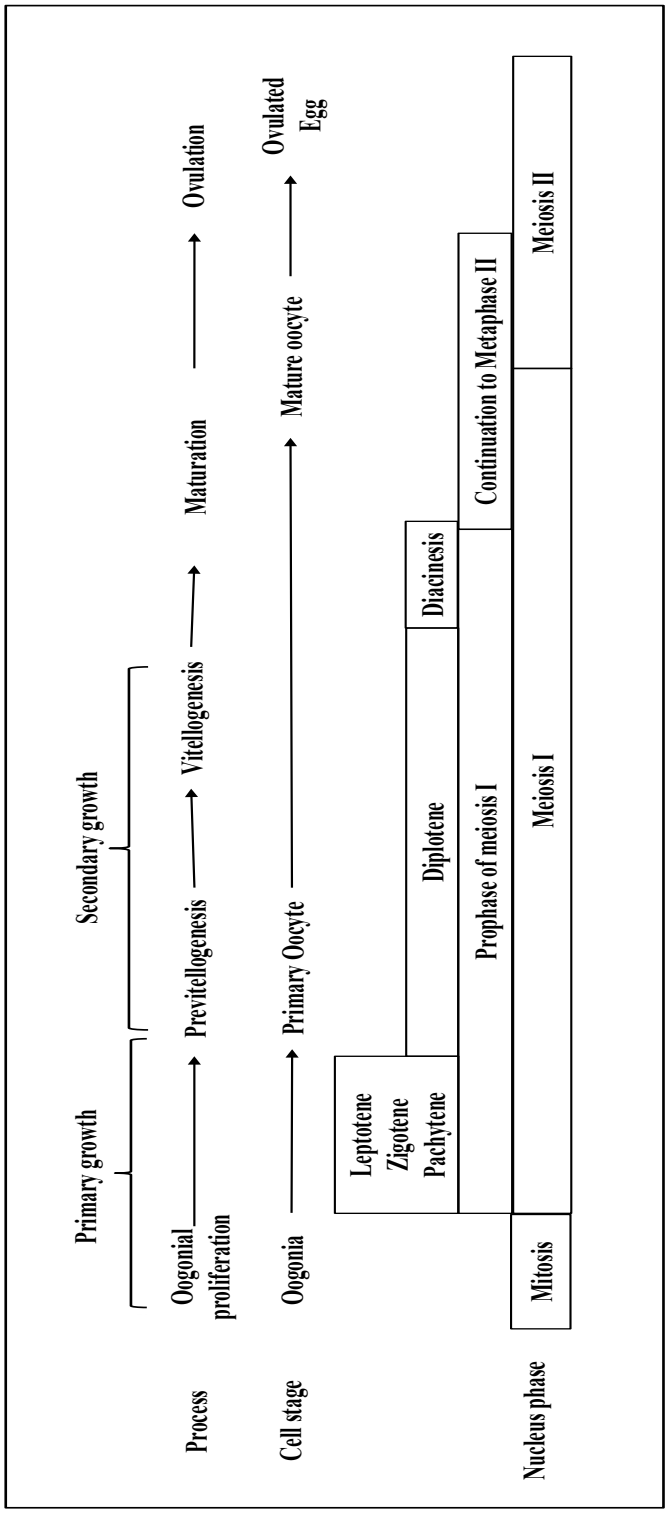

Fig 2. Diagram of the oogenesis process correlated with nucleus mitosis and meiosis in fish. From left to right: from oogonia to primary oocytes, oocytes in the previtellogenic and vitellogenic stages, mature oocytes and ovulated eggs. See the text for a detailed description.

After mitotic divisions, two series of meiotic cell divisions occur in the ovarian cycle (around 30 dph). In the prophase of the first meiotic division, the oocyte nucleus is subjected to five successive phases (leptotene, zigotene, pachytene, diplotene, diacinesis) with meiotic arrest. In the first three phases, primary follicle growth takes place. After this primary growth, follicle growth is arrested in the diplotene stage, in which the follicle undergoes secondary growth. This secondary growth is composed of two phases: previtellogenic (early, middle, late) and vitellogenic. Previtellogenic stages take place in the first secondary growth phase. In these stages, the oocyte increases in size and accumulates mRNAs, nutritional reserves and other necessary components for subsequent vitellogenesis, and also for the embryonic and larval fertilization and development processes (Nakamura et al. 1998). In early previtellogenic stages, the growing oocyte undergoes a series of transformations in its nucleus, in which numerous producing ribosomomas nucleoli appear in its periphery (perinuclear phase). In this phase, the oocyte begins to synthesise ribosomal RNAs, which are transported to the ooplasm to encode the lipids, proteins and enzymes involved in vitellogenesis. Following middle and late previtellogenesis, vitello particles assemble the cortical alveoli in the periphery, which incorporate glycoproteins as follicular development progresses (cortical alveolar stage). Previtellogenesis generally constitutes the first secondary growth stage, when all the necessary material is prepared for the second secondary growth stage: vitellogenesis. In this stage, the incorporation of plasma proteins of vitello precursor (vitellogenin) and lipoproteins occurs. After vitellogenesis, hormonal changes take place that trigger the resumption of meiotic arrest in the prophase of the cell cycle to continue to final oocyte maturation. It is in this phase when the oocyte nucleus moves towards the animal pole of the egg and arrests in the metaphase of meiosis II. In this stage, the hydration and proteolysis of yolk proteins stimulate oocyte growth. Then a hormonal change (mainly peaks in the luteinizing and maturation-inducing hormones) allows the cycle to resume by extruding the oocyte away from the follicle complex to the lumen of the ovary. The oocyte development cycle finishes with fertilization (Nakamura et al. 1998, Devlin and Nagahama 2002).

When ovarian development ends, the presence of oocytes in all development stages marks the beginning of the sexual maturity stage, in which females are able to perform the

reproduction process and reach first maturity with a small size, and age depends on tilapia strains and environmental conditions (Babiker and Ibrahim 1979, Trewavas 1983). With Nile tilapia, females are sexual mature at a size of 20-30 cm and a weight of 150-250 g under natural conditions. However under intensive aquaculture conditions, females mature more quickly and reach first maturity when they are lighter (30-50 g) (De Silva and Radampola 1990, de Graaf and Huisman 1999). During the reproductive period, tilapia ovaries go through an ovarian recrudescence period during which the ovary increases in size in the reproductive cycle. Hence the ovarian cycle begins vitellogenesis from previtellogenic stages. Eight days after the ovarian cycle starts, the ovary is mostly occupied (60-70%) by oocytes in the last development stage (late vitellogenesis/maturing oocytes) (Coward and Bromage 1998). In Nile tilapia, the increase in mature oocytes correlates with a higher gonadomatic index (GSI) (Babiker and Ibrahim 1979, de Graaf and Huisman 1999, Melamed et al. 2000). The GSI reaches its highest levels (5.5%) on day 14 after spawning, which indicates its availability to begin new ovulation and, therefore, another spawning event (see the next chapter). After ovulation, persistence of ovarian follicles (POF) has been described in some tilapia species, although more studies are needed to describe their possible steroidogenic functionality regarding mainly progestins (Coward and Bromage 2000). The reabsorption of both the oocyte and ovarian follicle (atresia) is a frequent phenomenon in tilapia species and an essential one to maintain ovarian homeostasis. Although the morphological changes of oocyte atresia have been studied in tilapia, the endocrine mechanisms involved in regulating the degeneration and reabsorption of ovarian follicles have been less investigated (Srisakultiew 1993).

Influence of temperature on testicular and ovarian development

The gonadal cycle is influenced by different factors like temperature, salinity (Viera et al. 2019) and feeding (Sales et al. 2020). Of these, water temperature is the variable that most affects gonadal development and sex determination (Baroiller and D'Cotta 2001, 2009). The temperature effect may differ depending on the species, strains or age of tilapia. For instance, high temperatures (37ºC) for long periods (45-60 days) lead to the permanent loss of germ cells in ovaries and infertility stages in *O. niloticus* (Pandit et al. 2015). In addition to

germ cell thermosensitivity, the effect of temperature has also been observed on vitellogenesis. *In vitro* studies into *O. mossambicus* hepatocytes have shown greater vitellogenin synthesis at 28ºC compared to 23ºC and 33ºC (Kim and Takemura 2003). The effect of temperature on the testicular function and spermatogenesis has been investigated by several research works. The use of low temperature (20ºC) in male Nile tilapia seems to positively affect the primary spermatogonia generation by increasing Leydig and Sertoli cell proliferation, and conserving the reservoir and renovation of germ cells (Alvarenga and França 2009, Melo et al. 2016). Other studies have shown that applying high temperatures (30-35ºC) causes faster germ cell differentiation and spermatogenesis (Vilela et al. 2003, Alvarenga and França 2009, Lacerda et al. 2018). However as observed in tilapia females, the application of very high temperatures (36-37ºC) provokes deleterious effects on testicular germ and somatic cells, characterised by loss of spermatogenic cells in testes (Jin et al. 2019a).

Endocrine control of sexual differentiation

In the last decade, research into Nile tilapia reproduction has focused on identifying the factors involved in sex determination and differentiation. Gonadal transcriptome and microRNAs analyses have indicated that the main sexual steroids and expression patterns of the genes that encode stereidogenic enzymes play an important role in processes such as gonadal development and sexual differentiation (Fig. 3) (Yoshiura et al. 2003, Ijiri et al. 2008, Tao et al. 2013, 2018, Eshel et al. 2014, Wang et al. 2016).

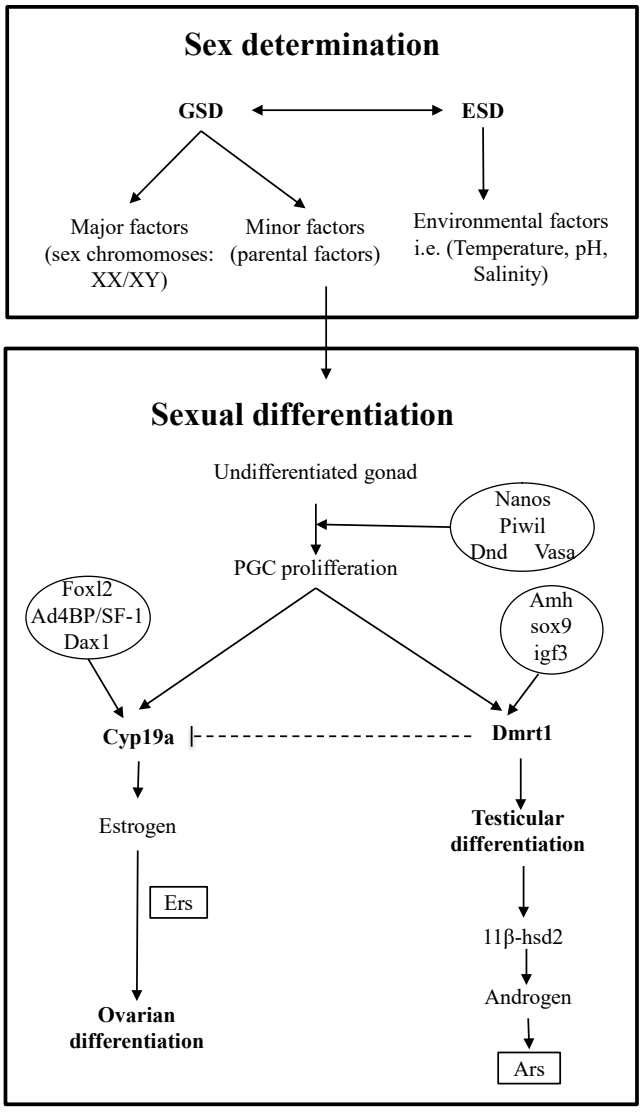

Figure 3. Diagram of the factors involved in the sex determination and differentiation of Nile tilapia. Sex determination causes the differentiation of an undifferentiated gonad. Following PGCs proliferation, certain transcription factors and stereidogenic enzymes participate in gonadal differentiation. In tilapia, the main factors of ovarian and testicular differentiation are Cyp19a and Dmrt1, respectively. The presence of oestrogens in females triggers differentiation in the ovary. In contrast, the presence of androgens is the result of testicular differentiation. The solid lines indicate stimulation, while the dashed lines indicate inhibition or suppression. The transcription factors that participate in the differentiation pathways are surrounded by circles. GSD, Genotipic Sex Determination; ESD, Environmental Sex Determination; PGC, Primordial Germ Cell; Ers, Oestrogen receptors; Ars, Androgen receptors. Modified from Piferrer and Guinguen (2008), and adapted to Nile tilapia.

Regardless of an individual's genotype, the involvement of androgens and oestrogens in the sexual differentiation process results in the individual's masculinization or feminization, respectively (Yamamoto 1969). The proportion of androgens and oestrogens

during stereidogenesis is determined by the key enzyme aromatase (Cyp19), which catalyses the conversion of androgens into 17β-Estradiol. Two genes (gonadal aromatase, *cyp19a* and brain aromatase, *cyp19b*), which encode this enzyme, have been described in many fish species, including Nile tilapia (Chang et al. 2005, Piferrer and Guinguen 2008). In ovaries, c*yp19a* is expressed from the first days of life (5 dph), and its expression continues to exponentially increase during ovarian differentiation (9-19 dpf) (Tao et al. 2013, 2018). Moreover, an increase in *cyp19a* levels has been linked with fish feminization (D'Cotta et al. 2001a, b). Blocking this enzyme during sensitive periods to temperature or aromatase inhibitors leads to the inhibition of oestrogen synthesis, which results in masculinization (male sex reversal) (Baroiller et al. 1995, 2009, Kwon et al. 2000, D'Cotta et al. 2001a, Tessema et al. 2006, Sun et al. 2014, 2018). In addition to Cyp19, other transcription factors have been identified to play a role in tilapia ovarian differentiation. To date, the latest studies have also considered Foxl2 (forkhead transcriptional factor L2) to be a marker of female sexual differentiation in undifferentiated stages of fish (Kobayashi et al. 2004, Wang et al. 2007). As observed for *cyp19a*, *foxl2* expression is higher in females than in males on 9 dpf. After this day, *foxl2* levels begin to sharply rise in XX females, which correlates with *cyp19a* levels (Wang et al. 2007, Iriji et al. 2008). Foxl2 seems to induce *cyp19a* expression. This might explain why the levels of both genes increase in parallel. In addition to *foxl2*, the role of other transcription factors like Dax1 (dose-sensitive sex reversal, adrenal hypoplasia, a critical region in the X chromosome) and Ad4BP/SF-1 has been studied. Dax1 has been suggested to suppress testicular differentiation which would, in turn, stimulate ovarian differentiation (Wang et al. 2002, Baroiller at al. 2009). A role of Ad4BP/SF-1 in the regulation of stereidogenic enzymes and the stimulation of aromatase transcription has been suggested, but more research is necessary (Yoshiura et al. 2003).

Oestrogens play a leading role as inductors of ovarian differentiation. However, androgens are a product of testicular differentiation (Tao et al. 2013). Thus the increase in androgen levels (11-ketotestoterone, 11-KT) and P450c 11β-hydroxylase enzyme (responsible for synthesizing the 11-KT precursor) are the result, and not the cause, of testicular differentiation (Baroiller and D'Cotta 2001, Sudhakumari et al. 2005, Ijiri et al. 2008, Zhang et al. 2010). Several transcription factors have been suggested to be involved in the pathway of differentiation to tilapia testis. Dmrt1 (Doublesex and mab-3-related transcription factor 1)

may be the main factor involved in testicular differentiation as it seems to suppress *cyp19a* expression and oestrogen synthesis in *O. niloticus* (Shirak et al. 2006, Ijiri et al. 2008, Kobayashi and Nakamura 2009, Wang et al. 2010, Tao et al. 2013, 2018, Rather et al. 2019). Other factors, such as Amh (antimüllerian hormone), Sox9 (the HMG-box protein 9 gene related to Sry) and Igf3 (insulin-like growth factor 3), are involved in Dmrt1 regulation and may also play an important role in testicular differentiation. For instance, Dmrt1 stimulates the expression of Sox family members such as *sox30* and *sox9,* which are involved in both the development of testis efferent ducts and the spermiogenesis process (Tang et al. 2019). In relation to Amh, it also plays a central role in gonad development and spermatogenesis. Several studies also indicate a role of *sox9* in the transcription of *amh* in tilapia as both expression levels increase in parallel for 10-15 dpf (days post-fertilization), with the highest levels in XY males on 20-25 dpf (Da Cotta et al. 2001, Iriji et al. 2008, Kobayashi et al. 2008). Igf3 has also been reported as a decisive factor in tilapia late spermatogenesis as it is regulated by androgens and intervenes in regulating the spermatocyte to spermatid transition (Li et al. 2020).

Finally, sex steroid receptors may also play an important role in the signalling pathway of sexual differentiation. Oestrogen (Esr1, Esr2a, and Esr2b), androgen (Ar1 and Ar2) and progestin receptors (Pgr) have even been detected in first development stages in tilapia (Gale 1996, Chang et al. 1999, Sudhakumariet al. 2005, Wang et al. 2005, Tao et al. 2013). Recently, other markers and genes involved in developmental processes, such as *nanos, piwil, dnd, vasa* and *pum,* appear to be implicated in the specification and maintenance of PGCs during Nile tilapia's ontogenic development (Kobayahashi et al. 2002, Jin et al. 2019b).

The previous factors involved in controlling steroidogenesis and gonadal function presented seasonal and daily changes in the gene expression synchronized to light:dark cycles, as reported in several teleost species like zebrafish (Di Rosa et al. 2016; Paredes et al. 2019a), Senegalese Sole (Oliveira et al. 2009) and Nile tilapia (De Alba et al. 2019). In addition to light cycles, studies performed in zebrafish revealed the influence of daily and seasonal temperature changes on the stereidogenesis pathway and the reproduction process (Villamizar et al. 2012). These recent findings highlight the rhythmic nature of the mechanisms that

intervene in the sexual differentiation of fish, as well as the influence of light and temperature cycles on their expression patterns.

CONCLUDING REMARKS

Sex-determination genes hare less conserved in fish compared to other vertebrates like mammals. Molecular markers and whole sequencing genomes are effective in identifying and isolating the loci related to GSD. Thanks to the genetic conservation of sex determination in tilapia, this species has been characterized as a relevant model for identifying the genes involved in the morphological, molecular and biochemical aspects of gonadal development and differentiation. Transcriptomic and microarrays analyses are very useful tools for studying the impact of hormonal and temperature protocols on the reproductive physiology of tilapia.

The expansion of aquaculture and the introduction of new species highlight the need to continue to strive to know the mechanisms that control the sex determination and differentiation of fish species. Tilapia aquaculture also requires better knowledge about techniques based on controlling the genotype and sex ratio of populations. Selecting productive characteristics of both sexual phenotypes will guarantee improvements in the tilapia industry's productive and reproductive efficiency.

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Experimental Chapter II

(Book chapter)

Reproductive Physiology of Tilapia

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ABSTRACT

In addition to its productive advantages, tilapia involves important aspects related to its reproduction that characterize it as a species of marked commercial interest worldwide. To optimize this species' reproductive management, it is necessary to increase our knowledge about the endocrine and environmental factors that regulate reproduction process in tilapia. In this chapter, a meticulous review is carried out on the main neuroendrocrine system that controls the reproductive axis of Nile tilapia (*Oreochromis niloticus*): the BPG axis. Therefore, the hormonal dynamics in the reproductive cycle are described by highlighting the influence of the environmental and hormonal factors that affect Nile tilapia reproduction. This information can be useful for optimizing the environmental conditions for reproduction in captivity and for improving the hormonal therapies that boost the genetic selection and reproduction efficiency processes with tilapia.

INTRODUCTION

The input of environmental information finely tunes the activation of the brain neuroendocrine machinery that triggers the hormonal cascade, which leads to the reproduction process (Yaron and Levavi-Sivan 2011). Although different neuroendocrine systems have been identified in fish reproduction, the Brain-Pituitary-Gonadal-Liver axis (BPGL axis) is the main mechanism to control fish reproduction (Figure 1). At each level of this system, a series of key factors intervene from gametogenesis to the release of high quality gametes ready for fertilization. Environmental information is collected by a series of chemical receptors located in the olfactory epithelium, which are photoreceptors in the retina and in the pineal organ. These structures receive light signals (day/night cycle and day length) and transduce them into electrochemical and humoral (melatonin) signals. Specifically, the pineal gland acts as an intrinsic main clock that intervenes in the periodic regulation of melatonin secretion (higher production at night), whose function is to modulate and synchronize biological rhythms, mainly in peripheral tissues (Falcon et al. 2007). Thus environmental stimuli arrive at the hypothalamus through sensory neuron pathways. In this region, stimulus-sensitive neurosecretory cells are responsible for releasing a neuropeptide that plays a primary role in reproduction: the gonadotropin-releasing hormone (Gnrh). This hormone acts primarily on the glandular pituitary gland (adenohypophysis) by stimulating the synthesis and release of gonadotropins into the bloodstream. These gonadotropins stimulate the production of steroid hormones at the gonadal level by acting as last effectors of gamete development and release. An important aspect to consider in this hormonal cascade is the negative feedback that certain key factors play at different levels of the BPGL axis (Yaron et al. 2001, 2011, Weltzien et al. 2004) (Fig. 1).

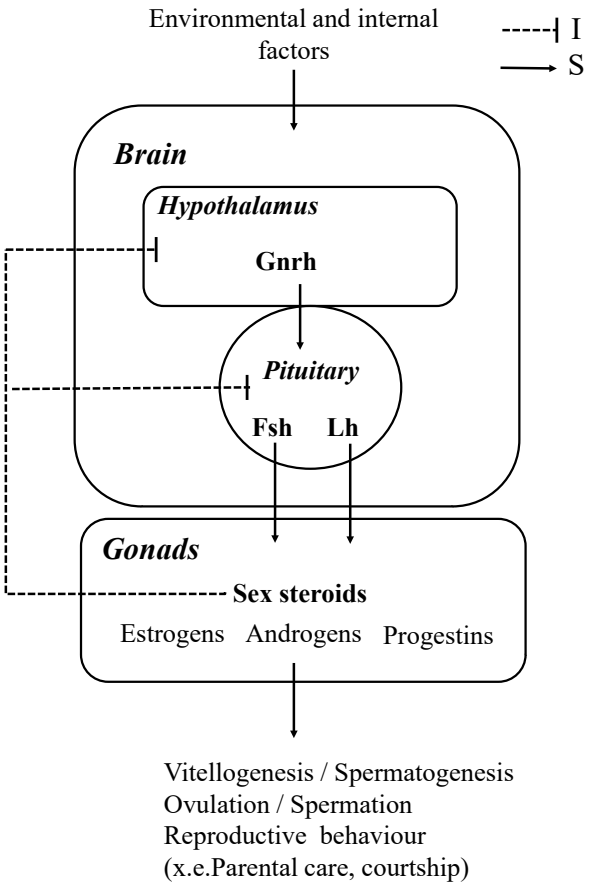

Figure 1. Schematic representation of the Brain-Pituitary-Gonadal (BPG) axis of fish. Gnrh, Gonadotropin releasing-hormone; Fsh, follicle-stimulating hormone; Lh, luteinizing hormone.

Better and integrative knowledge of the environmental, physiological and neuroendocrine factors that regulate the reproductive physiology of tilapia is needed to improve the breeding protocols established in tilapia aquaculture.

THE BRAIN-PITUITARY-GONADAL AXIS

Brain neurohormones

The brain plays its role at the highest level of the reproductive axis in fish (Fig. 2). It is responsible for receiving, modulating and transducing environmental stimuli into

neurohormonal signals that act at the following levels involved in the reproduction process. Other neuroendocrine systems of the BPGL axis considerably influence fish reproduction, such as the dopaminergic system, kisspeptins and the gonadotropin inhibitory hormone (Gnih) (Cowan et al. 2017). Moreover in recent years, other neuropeptides, such as Neurokinin B, have become important in tilapia reproduction (Biran et al. 2014).

Gnrh is a decapeptide that belongs to a family with high structural conservation on the phylogenetic scale. The influence of this neurohormone on stimulating the secretion and release of gonadotropins in the pituitary gland is well-known (Yaron et al. 2001). Three Gnrh isoforms have been characterized, which present different stimulation levels of the pituitary gland. Gnrh effects are elicited through binding to the specific Gnrh receptors present in the membrane of adenohypophyseal gonadotropic cells (Chen and Fernald 2008). These receptors are distributed throughout the body, but can be found mainly in gonadotropic and somatotropic cells of the pituitary gland and in extrahypophyseal tissues. In addition, the number of receptors is not constant, but shows variations depending on the reproductive cycle phase (Parhar et al. 2002). With tilapia, several studies have characterized the main Gnrh isoforms and their respective receptors (Parhar et al. 1997, Weber et al. 1997, Soga et al. 2005). On the one hand, in the tilapia brain, Gnrh1 neurons (seabream Gnrh, sbGnrh) have been described in the preoptic area of the hypothalamus and in the pituitary (Parhar et al. 1998). On the other hand, Gnrh2 (chicken Gnrh-II) and Gnrh3 (salmon Gnrh-III) neurons have been detected in the midbrain tegumentum and in the terminal nerve, respectively, and both areas are related to the modulation of reproduction, feeding and behavioral processes (Weber et al. 1997). Different studies have revealed the main role of sbGnrh on the regulation of gonadotropin production by describing the direct effect of sbGnrh on LHβ expression in cultured pituitary cells of tilapia hybrids (Melamed et al. 1996). Other functions attributed to Gnrh forms are mating and nesting, and stimulating the secretion of prolactin and somatolactin in *Oreochromis mossambicus* (Weber et al. 1997). The expression of Gnrh-receptors in tilapia has been described for the Gnrh-1, Gnrh-2 and Gnrh-3 neurons (Levavi-Sivan et al. 2004, Soga et al. 2005). In addition, their distribution and expression depend on the sexual maturity state and sex, with a higher expression in mature females than in males (Parhar et al. 2002, Levavi-Sivan et al. 2004, Martínez-Chávez et al. 2008).

Another key factor that plays an important role in the reproduction axis of vertebrates is the Kisspeptin system. Its stimulatory influence on the neuroendocrine regulation of reproduction has been described in various species, whose function and potency depend on the species. The teleost kisspeptin system was first described in Nile tilapia (Parhar et al. 2004). Although there are two kiss isoforms in most teleost fish (Servili et al. 2011), in tilapia Kiss2 is the only isoform to have been identified (Parhar et al. 2004). *Kiss2* expression has been detected in the brain, pituitary and gonad in both tilapia sexes, where it seems to perform an important function in early gonadal maturation (Park et al. 2012). In addition, its expression depends mainly on the ovarian cycle stage of female tilapia, with the highest levels in immature stages compared to mature and post-ovulatory stages. Park et al. (2016) proved its stimulatory effects at all levels of the tilapia reproductive axis, its participation in the synthesis and release of Gnrh, follicle-stimulating hormone (Fsh), Lh and sex steroids.

The stimulatory influence of Neurokinin B (Nkb) on tilapia reproduction was discovered in the last decade (Biran et al. 2012, 2014, Jin et al. 2016). Nkb is located in different neuronal hypothalamus complexes although the expression of its receptors, and appears in Lh cells during the Lh surge prior to ovulation, which suggests a role of this neuropeptide in the final maturation step of oocytes (Biran et al. 2014). In addition, the intraperiotoneal Nkb administration has stimulatory effects on pituitary gonadotropins (direct effects) and all the Gnrh variants (indirect effects due to Gnrh neuron activation) in the brain (Biran el al. 2014). Mizrhai et al. (2019) has recently described how receptors of Nkb are co-expressed in GnrH neurons.

In addition to the previous stimulatory systems, there are other neuroendocrine systems, such as dopamine and Gnih, which act as negative effectors of the BPGL axis. The dopaminergic system acts as the major antagonist of the Gnrh system by inhibiting the secretion of adenohypophyseal hormones and blocking reproduction processes (Beaulieu and Gainetdinov 2011). This inhibitory effect has been described in several freshwater fish species, but not in marine species, for which no negative effects have been clearly defined (Prat et al. 2001). With tilapia, dopamine agonists show an inhibitory *in vivo* and *in vitro* effect on the synthesis of Gnrh receptors as well as on Fsh and Lh release (Levavi-Sivan et al. 2006, Biran et al. 2014). The functional role that dopamine exerts on the regulation of

gonadotropins in tilapia is explained by not only the anatomical location of its dopamine fibers, but also by the high specificity of dopamine through its D2-like receptors present in the Lh cells of the adenohypophysis (Jiang et al. 2016).

The existence of the dodecapeptide GnIH was discovered for the first time in birds (Tsutsui et al. 2000), but it has also been found in vertebrates and invertebrates, which suggests a high degree of phylogenetic conservation. The wide distribution of GnIH cells and fibers throughout the brain and pituitary, as well as their proximity to GnRH cells, suggest that this peptide plays a key role as a neuromodulator at the brain level (Muñoz-Cueto et al. 2017). In some vertebrates, especially birds and mammals, GnIH seems to present an inhibitory effect on the synthesis and release of hypothalamic GnRH and pituitary gonadotropins (Muñoz-Cueto et al. 2017). However in fish, as the role of Gnih remains unclear, more studies in this line are necessary. In tilapia, Gnih orthologs have been described in females (Ogawa et al. 2016). It has also been observed that the effects that Gnih has on the pituitary seem to be species-dependent by stimulating or inhibiting the release of gonadotropins. Unlike what happens with other species, intraperitoneal Gnih administration to tilapia stimulates Fsh and Lh production (Biran et al. 2014). Hence further studies are required to clearly elucidate the role of Gnih in the tilapia reproduction system.

The few above-mentioned studies reflect the role of the brain as a system capable of integrating sensory and neuroendocrine information to trigger stimulatory or inhibitory responses to neurohormones of the following BPGL axis levels that lead to reproduction. Hence the importance of continuing to investigate other factors involved at the brain level and regulate reproduction in tilapia and, thus, improve its reproductive management, thanks to the development of hormonal protocols that guarantee optimal reproduction control of tilapia in captivity, should be noted.

Pituitary hormones: gonadotropins and their receptors

The second BPG axis level involves the neuroendocrine system of the brain, mainly Gnrh, by regulating the stimulations of adenohypophysis cells. These gonadotropic cells are

responsible for the synthesis and release of gonadotropins that will participate in different reproduction processes, such as GthI and GthII, which were first described in salmon (Yaron et al. 2003) (Fig. 2). Later they were called follicle-stimulating, Fsh, and Luteinizing Hormone, Lh, because they shared their structural and functional characteristics with tetrapod gonadotropins (Levavi-Sivan et al. 2010). These gonadotropins are formed by two subunits: a common subunit, named the glycoprotein hormone α subunit (Gpα), and two β subunits that are specific to each hormone (Fshβ and Lhβ), which are those with specific biological activity (Levavi-Sivan et al. 2010). The role of Gths and their receptors focuses mainly on stereidogenesis stimulation and gonadal development in testis and ovary (Yan et al. 2012). In fish, the action of gonadotropins is mediated through the binding with their receptors, known as Lhr and Fshr. The expression of both receptors has been observed at the gonadal level, mainly in Sertoli cells. However, only *fshr* expression appears in Leydig cells, and this expression presents a differential pattern depending on the gonadal development phase. Thus *fshr* expression increases during vitellogenesis and spermatogenesis, whereas *lhr* expression increases during maturation and gamete release (ovulation/spermiation) (Kwok et al. 2005, So et al. 2005).

In Cyprinids, a high degree of conservation in the Gths sequence has been observed. Specifically in tilapia, the existence of *fshβ* (Rosenfeld et al. 1997, 2001, Yaron et al. 2001) and *lhβ* genes (Rosenfeld et al. 1997, Elizur et al. 2000) has been reported. Parhar et al. (2002) and Kasper et al. (2006) have described β subunits of both gonadotropins, which are specially located in the *pars distalis proximalis* and peripheral regions of the *pars intermedia* of the pituitary gland of Nile tilapia, as with most teleosts (Weltzien et al. 2004). These subunits have also been determined in plasma thanks to the development of specific enzyme-linked immunoabsorbent assays (Aizen et al. 2007a). *In vivo* and *in vitro* studies conducted with tilapia have shown a direct effect of Gnrh on the increased secretion of pituitary Lhβ and Fshβ, and a time-dependent increase in the mRNA levels of these hormones (Levavi-Sivan and Yaron 1993, Melamed et al. 1996, Gur et al. 2000, Aizen et al. 2007b). In Nile tilapia, as in many teleosts, two gonadotropin receptors have been identified, with *fshr* expression found in Sertoli cells and oocyte granulose cells during vitellogenesis, while *lhr* is present in Leyding cells and mature oocytes (Oba et al. 2001). The location of gonadotropins and their receptors indicates the functionality of Fsh and Lh in the reproductive physiology of tilapia. In

females, gonadotropins regulate processes related to vitellogenesis, oocyte maturation, and ovulation and steroid secretion, while they regulate spermatogenesis, spermiation and testicular steridogenesis in males (Yaron et al. 2001, 2003) (described in the sections below).

Gonadal hormones

The gonadal level is the last fish reproductive BPG axis step. At this third level, gonadotropins regulate the expression of the gonadal genes involved in the synthesis of sex steroids (androgens, estrogens, progestins) (Fig. 2), and in the production of other growth-related factors (Tokarz et al. 2015). These factors are necessary for the differentiation and cell proliferation of gonads, and they also establish negative feedback by regulating the brain and pituitary levels of the reproductive axis (Yaron and Levavi-Sivan 2011). All steroid hormones derive from a common precursor, cholesterol, which is assimilated by gonadal cells thanks to the action of the steroidogenic acute regulatory protein (StAR), and is converted into pregnenolone via cytochrome p450 (Cyp11a). In Nile tilapia, two StAR isoforms (StAR1 and StAR 2) have been described to play different roles during gonadal development (Yu et al. 2014). On the steroidogenesis pathway of teleosts, numerous enzymes participate that gives rise to three types of steroids with 18, 19 and 20 carbon atoms, named estrogens (β-estradiol or E2), androgens (11-ketotestosterone or 11-KT and Testosterone) and progestins (17, 20, 21-trihydroxy-4-pregnen-3-one or 20B-S and 17α, 20β -dihydroxy-4-pregnen-3-one or DHP) (Fig. 2) (Tokarz et al. 2015). According to Wang et al. (2016), the key stereidogenic enzymes are Hsd3β2 (participates in the conversion of pregnenolone into progesterone) and Cyp17 (converts progesterone into 17OH-Progesterone). In addition, enzymes 20βHsd, 11βHsd and Cyp19 are essential for the synthesis of the main male and female sex steroids, respectively (Fig. 2). In females, E2 is the main estrogen, while 11-KT is the chief androgen in males (Ijiri et al. 2008). In addition, both sex steroids (T and E2) can be synthesized from androstenedione, which also exerts its own functions on reproduction. Other hormones like progestins have been related to processes associated with gonadal maturation (MIS, maturation-inducing steroid) and socio-reproductive factors (Chattoraj et al. 2009). Finally, sex steroids bind to their corresponding receptors in the gonad and liver to stimulate the synthesis and release of yolk proteins (Vitellogenin, Vtg, and Egg Yolk-Proteins) required for

egg development. In tilapia, sex steroids exert these effects through binding different types of androgen (Ar1 and Ar2), estrogens (Er1, Er2a and Er2b) and progestin receptors (Pgr) (Wang et al. 2005, Tao et al. 2013) (Fig. 2).

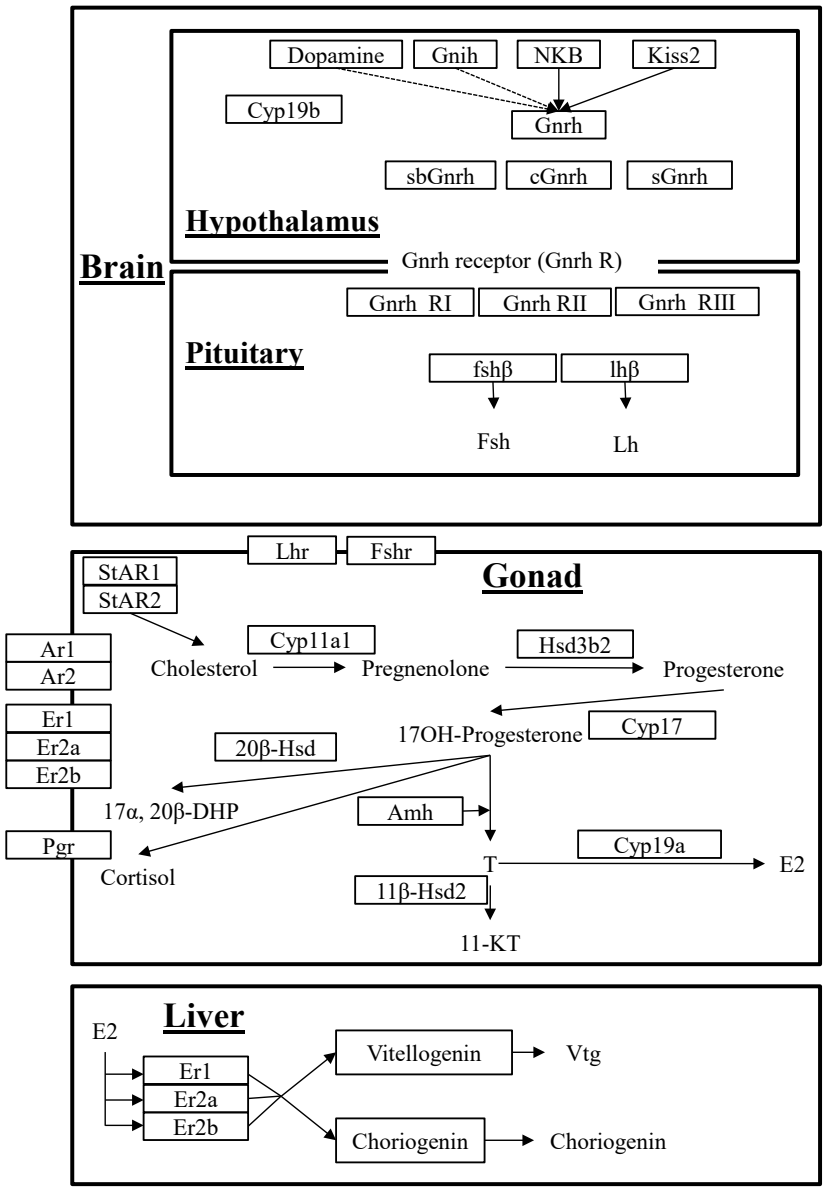

Figure. 2. Factors involved in the Brain-Pituitary-Gonadal-Liver (BPGL) axis of tilapia, including the synthesis pathways of sex steroids in gonads. Continuous and dashed arrows indicate stimulation or inhibition, respectively. Gnrh, gonadotropin releasing-hormone; Gnih, gonadotropin inhibitory hormone; Kiss2, Kisspeptin2; Fsh, follicle-stimulating hormone; Lh, luteinizing hormone; StAR, steroidogenic acute regulatory protein; E2, 17α-estradiol; T, testosterone; 11-KT, 11-ketotestosterone; Amh, Anti-Müllerian Hormone; Ar, androgen receptor; Er, estrogen receptor; Pgr, progestin receptor. For the abbreviations of other enzymes from the sex steroid pathway, please check the text. Scheme adapted to tilapia (see references in the text) from the medaka BPGL axis depicted by Saunders et al. (2015).

NEUROENDOCRINE REGULATION OF REPRODUCTIVE CYCLES

The control of both gametogenesis and gamete release processes involves a set of neuroendocrine processes located at four different levels (brain-pituitary-gonad-liver) of the reproductive axis in tilapia. As described in previous sections, the biological activity of hypothalamic systems play a direct (gonadal) and indirect (pituitary) role in the development of tilapia gametes, and ultimately in spermiation and ovulation.

Endocrine regulation of spermatogenesis and spermiation

As in most teleost speciess, the endocrine control of testis development is essential during the spermatogenesis and spermiation processes. Thus both gonadotropins and sex steroids play a critical role in the stimulation of each testicular development stage: germ cell differentiation to spermatogonia, spermatocytogenesis (spermatogonial renewal and proliferation), meiosis (from spermatogonia to spermatocytes and spermatid), spermiogenesis (from spermatid to spermatozoa), spermiation and sperm maturation. As sperm production in *Oreochromis niloticus* and *Oreochromis aureus* seems to continue throughout the year under natural environmental conditions (Hyder 1972), the research aim has barely focused on studying the endocrine mechanisms that regulate spermatogenesis and spermiation. Thus studies on male plasma sex steroids and gonadotropins in *O. niloticus* are still scarce compared to other fish species, which means that whether they present variations during the reproductive cycle has not yet been confirmed (Shawky et al. 2018, De Alba et al. 2019). Nevertheless, plasma sex steroids change throughout the reproductive cycle in males of *O. mossambicus* (Cornish 1998). So it is necessary to further investigate the dynamics of the hormonal profiles that regulate spermatogenesis and spermiation to improve our knowledge of male reproduction.

In early development stages, E2 and its receptors appear to enhance the renewal and proliferation of primary spermatogonia in testis through mitotic divisions (Schulz and Miura 2002). After spermatogonial proliferation, the secretion of both gonadotropins Fsh and Lh

triggers the onset of meiotic division, which leads to the beginning of spermatogenesis (gonadotropic stimulation). Lack of such gonadotropic stimulation can inhibit spermatogonial divisions and, consequently, spermatogenesis (Nakamura and Nagahama 1989, Kobayashi and Nakamura 2009). In most teleost species, spermatogenesis is regulated mainly by Fsh and androgens (11-KT), while the spermiation and sperm maturation processes are regulated by Lh and progestins (DHP). These premises coincide with a research work performed in tilapia, which showed increased tilapia expression levels for *fshβ*, *lhβ* and their receptors during spermatogenesis and spermiation (Yan et al. 2012). The main role of Fsh in tilapia spermatogenesis has been described as Fsh acts through its receptors (Fshr) on Sertoli cells to produce growth factors (Activin B, Igf-1), and also on the Leydig cells responsible for producing sex steroids (11-KT and DHP) (Oba et al. 2001, Vilela et al. 2003, Kobayashi and Nakamura 2009). The synthesis of growth factors has also been described to be stimulated by the role of 11-KT in Sertoli cells (androgenic stimulation). In this way, 11-KT has been characterized from the spermatogonial proliferation period to spermiogenesis (Nakamura and Nagahama 1989, Wang et al. 2016).

On the male gonadal stereidogenic pathway, the participation of Lh in sperm maturation and release should also be considered. Plasma Lh were found at basal levels during early tilapia spermatogenesis, which began to increase during spermiation. In addition, the male *lh* expression in male tilapia pituitary presented parallel fluctuations to *fsh* expression levels (De Alba et al. 2019). Moreover, the stimulatory effect of Lh and 11-KT on Leyding cells has been reported to trigger DHP production (Oba et al. 2001). *In vivo* studies in Nile tilapia show the essential role of DHP in spermatogonial cell proliferation (meiotic divisions) and spermatogenesis by intervening in spermiation, final sperm maturation (morpho-functional changes) and sperm motility enhancement (Oba et al. 2001, Fishelson 2003, Liu et al. 2014). In the pre-spermiation stage, tilapia males present swollen, reddish and prominent genital papilla and a reddish coloration pattern, which indicate their readiness to mate (Rana 1988) (Fig. 3A, B, E). Fsh and Lh levels in plasma increase in correlation with testicular growth from early testicular development stages (early spermatogenesis) to spermiation (Melamed et al. 2000). After spermiation, gonadotropins levels drop considerably and rise again as the reproductive cycle progresses toward spermatogenesis and spermiation.

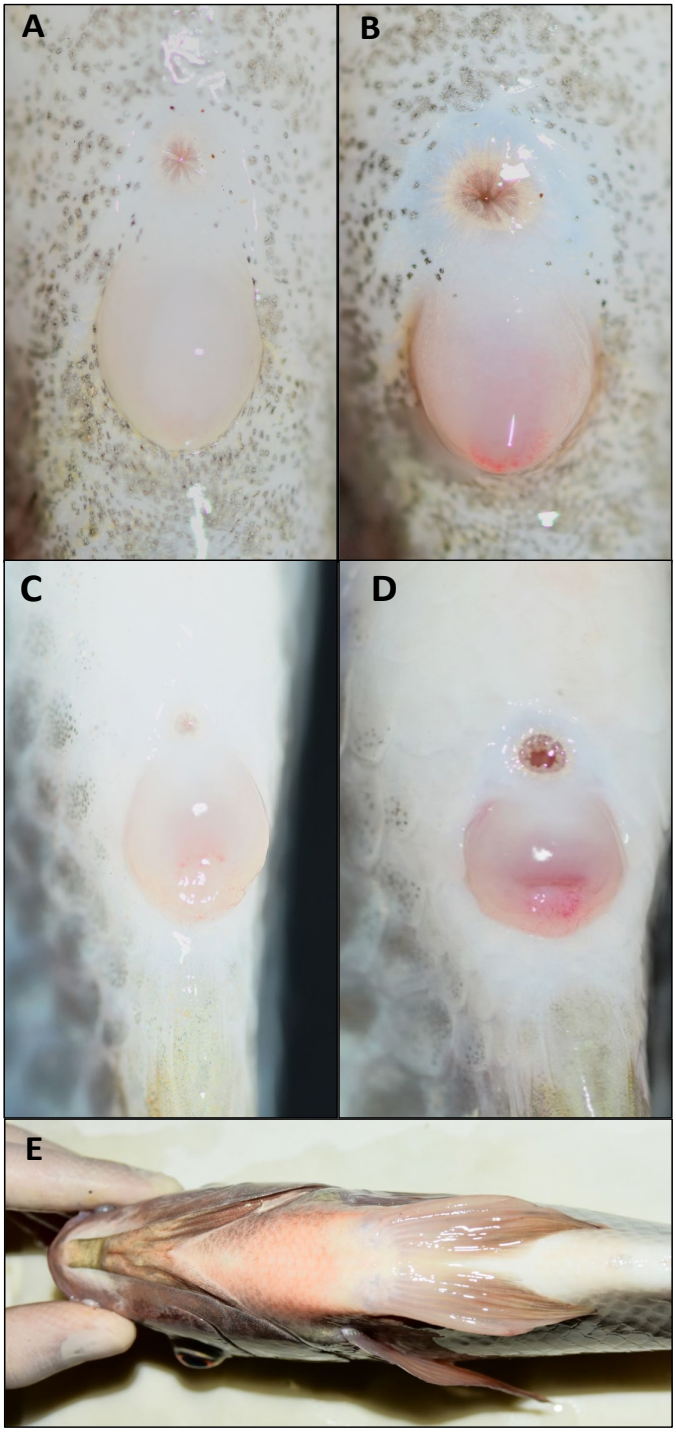

Figure 3. Macroscopic differences of genital papillas of Nile tilapia during non-breeding and breeding periods. Male (A) and female (C) papillas during non-reproduction periods. Male (B) and female (C) papillas during breeding periods. The reddish coloration pattern can also be observed on most of the body's ventral part length (E).

Endocrine regulation of ovarian development and spawning

In females, neuroendocrine coordination is established mainly by gonadotropins, estrogens and progestins, which regulate the dynamics of different ovarian development stages, which trigger ovulation and the release of eggs. The hormonal profiles of reproductive factors of female tilapia have been reported in detail for each ovarian development stage in *O. niloticus* (Srisakultiew 1993, Rothbard et al. 1991, Tacon et al. 2000, Biswas et al. 2005, Lapeyre et al. 2008, Shawky et al. 2018, Ortiz et al. 2017) and *O. mossambicus* (Cornish 1998, Smith and Haley 1988) (Fig. 4). Endocrine factors exert their role in specific ovarian development stages: oogonial mitotic and meiotic divisions, previtellogenesis (early, middle, late), vitellogenesis, oocyte maturation and ovulation.

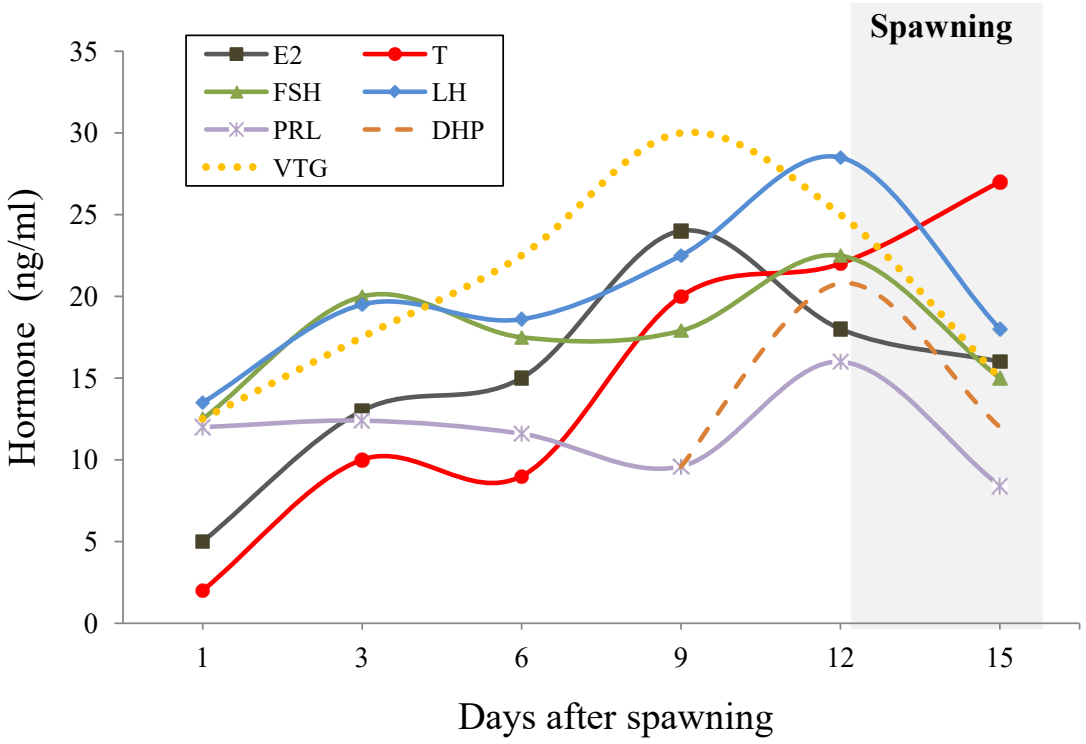

Fig. 4. Representative fluctuations of the plasma levels of the hormones involved in the reproductive cycle of Nile tilapia females in a 12:12LD cycle. Hormonal levels (ng/ml) were corrected by adjusting the Y axis: E2 (x1), T (x2), Fsh (x5), Lh (x3), PRL (x2), DHP (x8) and Vtg (x5). The gray area indicates the natural spawning period of Nile tilapia. Hormone levels are as obtained from several research works performed with Nile tilapia (Tacon et al. 2000, Melamed et al. 2000, Biswas et al. 2005, Aizen 2007a, Ortiz et al. 2017, Shawky et al. 2018).

Although very little is known about the endocrine control of the first previtellogenic phases, E2 and DHP have been described to be the main regulators of the mitotic and meiotic divisions of oogonies. However, androgens (T) acquired an additional role during oocyte development (Miura 2007). A rapid increase in both E2 and T on the first cycle days is associated with an accelerated vitellogenic process. In tilapia, as in most teleost species, vitellogenesis is regulated mainly by estrogens and Fsh. An increase in the plasma levels of E2 induces the hepatic synthesis of Vtg and the oocyte incorporation of vitello protein precursors from plasma (Van Bohemen et al. 1982). Thus plasmatic E2 and VTG levels run in parallel during previtellogenesis and vitellogenesis. In addition, the stimulatory effect of Gths on E2 synthesis has been suggested as higher levels of *gnrhs* and *gths* mRNA coincide with higher estradiol plasmatic levels in females upon vitellogeneis (Bogomolnaya et al. 1989, Melamed et al. 2000, Levavi-Sivan et al. 2004). In addition, the possible influence of tilapia testosterone as a precursor to E2 (Tacon et al. 2000, Ortiz et al. 2017) and the gonadotropin stimulator (Melamed et al. 1997) on ovarian development have been suggested.

Although the role of Fsh during vitellogenesis in stimulating the follicular production of E2 has been well characterized in tilapia, it would seem that Lh could also play an important role in early oogenesis (primary and secondary vitellogenesis phases) as the parallel fluctuations of both *fshβ* and *lhβ* expressions and plasmatic levels in each sex have recently been described in *O. niloticus* (Melamed et al. 2000, Biswas et al. 2005, Aizen et al. 2007a, b, Ortiz et al. 2017, De Alba et al. 2019). According to Aizen (2007a), the increase in Fsh occurs on the first few days after a new cycle starts, although Lh peaks have also been reported on ovarian cycle days 11-13 (Bogomolnaya et al. 1989, Rothbard et al. 1991). In short, both Lh and Fsh appear to have a stimulatory effect on the oocycte incorporation of plasmatic E2 and Vtg levels (Ortiz et al. 2017). These results suggest that gonadotropins are good indicators of spawning and recovery periods in Nile tilapia.

After vitellogenic growth, Lh and progestins (DHP) have been characterized as being the main regulators of oocyte final maturation and ovulation in tilapia (Tacon et al. 2000, Aizen et al. 2007a). In Nile tilapia, the highest Lh levels in plasma have been observed immediately before ovulation by stimulating the follicle to produce several progestins that induce oocyte final maturation (Levavi-Sivan et al. 2006, Aizen et al. 2010). The peaks in the

plasma levels of DHP and MIH (Maturation-inducing hormone) have been described immediately before spawning, which suggests their influence on the control of oocyte maturation and ovulation (Tacon et al 2000, De Alba et al. 2019). During the pre-spawning period, females present a swollen belly and genital papilla (Fig. 3C, D), which indicate imminent spawning, which is a good time for stripping by gently massaging the abdominal cavity (1 h after ovulation) to obtain fertilized eggs. According to several authors, on day 14 of natural reproduction in Nile tilapia, the highest plasma values of sex steroids precede the ovulation period (Tacon 2000, Baroiller and Toguyeni 2004, De Alba et al. 2019). At these time points before ovulation, plasma prolactin (PRL) levels also rise, which suggests an endocrine effect on maternal behavior (Weber et al. 1997, Tacon et al. 2000). After ovulation, plasmatic E2, Vtg and gonadotropins levels dramatically drop and return to basal levels (Tacon et al. 2000, Poleo et al. 2005, Fujimura and Okada 2007).

The spawning of most genus *Oreochromis* species occurs in the afternoon (Baroiller and Toguyeni 2004). Fertilization takes place directly on female genital papilla, after which females collect the eggs in their oral cavity. If mouth breeding occurs, the female lodges the eggs in her oral cavity to protect them against external agents for approximately the first 12 days after fertilization. While this maternal care lasts, circulating levels of sex steroids remain high, which induces the persistence of postovulatory follicles (POF) and prolongs females' ovarian cycle (Srisakultiew 1993, Tacon et al 2000, Smith and Haley, 1988). There are also reports that low Gnrh1 and Gnrh3 levels during mouth breeding can be involved in suppressing the ovarian cycle and sexual behavior (Levavi-Sivan et al. 2006, Das et al. 2018). After spawning and mouth breeding (if it occurs), the ovary's structural and endocrine system prepares to begin a new ovarian cycle. In most studies into Nile tilapia, an interspawning interval (ISI) lasting 14 days has been described under adequate environmental conditions (12 h Light:12 h Dark, 12:12 LD, and 27-29ºC). However, the tilapia ISI depends on factors like maternal behavior (egg deprivation), photoperiod, temperature and food composition (De Silva and Radampola 1990, Ridha and Cruz 2000, Tacon et al. 2000, Biswas et al. 2005, Lapeyre et al. 2008) (see the following sections).

Egg characteristics are species-specific. In Nile tilapia, females spawn large batches with up to 2000 eggs that are oval-shaped with yellowish ocher coloration (depending on diet

carotenoids). Egg size (1.0-2.0 x 1.5-3.0 mm) and weight (5-9.5 mg) have been correlated with females' size and age (Rana et al. 1988, Baroiller and Toguyeni 2004, Fujimura and Okada 2007). After fertilization, the embryo presents a series of morphogenetic differentiations throughout this period and larval and juvenile stages (Fujimura and Okada 2007).

ENVIRONMENTAL INFLUENCE ON REPRODUCTION

In nature, fish are constantly exposed to cyclic changes in environmental factors, such as day/night alternations or seasonal changes in day length or water temperature. Fish can anticipate these predictable changes thanks to biological clocks used to perform important functions under the best environmental conditions. During fish reproduction, the existence of rhythms in the neuroendocrine factors present all along the BPGL axis ensures harmonizing the reproductive system with the most favorable environmental conditions for progeny to be successful (Cowan et al. 2017) (see Chapter XII of the present book for more details). Actually, external environmental factors strongly influence the reproductive axis by triggering or changing the course of the reproduction timing process. The next sections deal with the influence of light and temperature cycles as the main environmental synchronizers that control the reproductive physiology of tilapia. In addition to these two factors, other external factors have been reported to influence the endocrine axis of tilapia, such as salinity (Baroiller et al. 1997, Abucay et al. 1999), food composition (El-Sayed et al. 2005), stocking density (Ridha and Cruz 1999, Lapeyre 2008) and social aspects (Little et al. 1993, Tacon et al. 2000, Ridha and Cruz 2003).

Influence of photoperiod on reproduction

The effect of daylight duration (photoperiod) on reproduction has been extensively investigated in fish, and it plays a primary role in species from temperate and arctic latitudes as they are subjected to wide variations during the photoperiod all year long (Migaud et al. 2010). Climate change and foul environmental cues appear to disrupt the fine-tune of the BPG

axis and fish reproduction (Servilli et al. 2020). In tilapia, this is a tropical species and, hence, the photoperiod does not seem to strongly impact its reproductive physiology, with tilapia breeding taking place in a wide variety of photoperiods. Nevertheless, reproduction tilapia has been described to have certain seasonal nature (Cornish 1998). Tilapia can apparently reproduce under very different photoperiods, which considerably influence certain reproductive parameters, such as number of spawning, number of eggs, synchronization of spawning, fecundity, survival and larval development (Ridha and Cruz 2000, El-Sayed and Kawanna 2007). Recent studies have shown the molecular mechanisms of transcriptional Nile tilapia ovarian development in different photoperiod regimes (Tang et al. 2019).

Most studies carried out in Nile tilapia indicate a general trend of higher egg production, gonadal development and spawning frequency (ISI) associated with prolonged photoperiod length. Indeed the studies carried out using long (18:6 light:dark, LD) or equinox (12:12 LD) photoperiods reveal a significant increase in number of spawning and fertility, and also better synchronization and positive stimulation of gonadotropins (Biswas et al. 2005, Ridha and Cruz 2000, Campos-Mendoza et al. 2004, Lapeyre 2008, El-Sayed and Kawanna 2007). On the contrary, in experiments performed during short photoperiods (e.g. 6:18 LD), reproductive behavior is inhibited after 3-4 spawning cycles, along with inhibited gonadotropic Fsh and Lh (Biswas et al. 2005). These results agree with El-Sayed and Kawanna (2007) and Lapeyre (2008), who have reported that a 6:18 LD photoperiod negatively affects gonadal development compared to longer photoperiods. In addition to the photoperiod, the effect of light intensity on the BPG axis has also been observed. Ridha and Cruz et al. (2000) used photoperiod combinations of different durations and light intensities to find that most reproductive parameters improved in tilapia with an 18:6 LD photoperiod and 2500 lux. There is also evidence for light wavelength (color) influencing Nile tilapia reproduction (Volpato et al. 2004). Blue light enhances reproduction by leading to a higher proportion of reproducing fish with active nest constructions.

To summarize, light manipulation (photoperiod, light intensity, color) has proven useful for controlling tilapia reproduction. Therefore, appropriate lighting protocols should be used to improve tilapia’s reproductive efficiency in aquaculture.

Influence of temperature on reproduction

The plasticity of endocrine mechanisms is a characteristic of poikilothermic animals such as fish, and is a highly influential technique for their aquaculture. In recent years, different temperature protocols have been established to control tilapia's reproductive physiology. This species is capable of surviving within a wide thermal range. However, temperature is also a limiting factor for its natural reproduction. Thus Nile tilapia needs warm water temperatures to breed in. Some authors set the limit at 22-25°C, with tilapia breeding the whole year if water temperature remains above these temperatures (Stickney 2000). Other studies have suggested an optimal temperature of 25-30ºC for spawning, below which spawning frequency decreases and stops below 20ºC (Bhujel 2000). In addition to lower temperature limits, very high temperatures can negatively affect tilapia breeding. For instance, the use of high temperatures (37ºC) over long periods (45-60 days) means the permanent loss of germ cells in ovaries by inducing infertility in tilapia females (Pandit et al. 2015).

The intensification of reproductive activity related to seasonal changes, such as rainy seasons, has also been described (Baroiller et al. 1997). One of the most widely used techniques to stimulate tilapia reproduction implies cooling down water (22ºC) (Srisakultiew and Wee 1988, Lapeyre et al. 2009). In these studies, females were exposed to cool water baths over long or short periods, and returned to an ambient temperature for 4 weeks to evaluate how cold temperatures induce effects on spawning. The results did not show any differences in using long cold exposures periods compared to the control group (maintained at ambient temperature: 29-30ºC). However, when females were exposed to shorts cool temperature periods (6-24 h), a significant increased in spawning synchronicity was described.

HORMONAL TREATMENTS FOR BREEDING STIMULATION

As described above, temperature and photoperiod are limiting factors for tilapia reproduction. Hence the manipulation of these environmental factors can be used to induce or inhibit tilapia breeding. In addition, the use of hormonal therapies has been described to have

considerable effects on the physiological mechanisms of tilapia reproduction, and protocols involving this technique can be applied. These protocols intend to improve the use of resources (personal, infrastructure, economic) of aquaculture production and to minimize the negative aspects of tilapia's reproductive biology.

Environmental conditions can be used to improve the control of tilapia reproduction in captivity. However, the inability to reproduce natural spawning conditions or to control fish reproductive cycles prevent us from mastering the dynamics of endocrine mechanisms (Lapeyre, 2008). With tilapia reproduction management, there are two main hormonal manipulation objectives: A) to induce spermiation/ovulation processes of gametes and stimulate the spawning process; B) to increase efficiency in spawning synchronization to improve the production of gametes and sperm.

During tilapia breeding, females generally present more problems than males, which are usually the limiting factor. For this reason, studies generally focus on females and protocols to induce spermiation are lacking. However, sperm may sometimes be a limiting factor in fertilization. So in order to obtain high mature sperm concentrations, hormonal stimulation processes can be performed with tilapia males. The use of LH releasing hormone (LHRHa) seems to increase sperm count on the day after injection (Garcia-Abiado et al. 1996). Other studies reflect on human Chorionic Gonadotropin (hCG) injections inducing the expressions of both *star1* and *star2* in male tilapia testis, which would contribute to MIH production during sperm maturation and spermiation (Yu et al. 2014).

Female tilapias are very prolific animals capable of spawning all year long with asynchronous gonadal development and maternal behavior (allocate eggs from different females). Thus efforts of hormonal therapies focus on not only improving spawning synchrony, but also on controlling final oocyte maturation for subsequent artificial fertilization. For these purposes, a wide variety of hormones has been tested in Nile tilapia, such as GnRH and its agonists (Piamsomboon et al. 2019), pituitary extracts (Fernandes et al. 2013) and hCG (Coward and Bromage 2002, El-Gamal and El-Greisy 2005, Owusu-Frimpong 2008). A comparative study into the effectiveness of these hormones has been performed by Fernandes et al. (2013), who determined that hCG was the most potent and effective treatment for inducing synchronicity and gamete collection in Nile tilapia. However,

the effects of hCG administration on gamete collection and fertilization quality varied depending on hCG concentration and number of doses (Fernandes et al. 2013). Piamsomboon et al. (2019) showed that a combination of two GnRHa injections (15 and 30 μg/kg, with an 8-hour interval) and oral dopamine antagonist administration (5 mg/kg) was also effective in successfully inducing spawning in tilapia females. In hormonal induction protocols, induction time has become increasingly more important as several studies have shown time-dependent responses of the hypothalamic-pituitary axis. The research by Rasines et al. (2013) into Senegalese sole demonstrated an effect of the time of day at which it is hormonally induced (with a GnRH analog) with the production of the obtained larvae. They carried out three inductions at three different times of the day (6 am, 12 am and 7 pm), and reported the induction of the highest values during larval production at 6 am compared to the groups induced at other times. These results highlight the importance of considering daytime responses when establishing hormonal protocols. Nevertheless, current research on hormonal treatments for tilapia reproduction is still scarce, hence the need to further investigate and develop new protocols that consider innovative factors like light color and stimulation timing. In this way, the development of improved therapies will help to minimize the difficulties found in relation to artificial fertilization, and to boost the genetic selection and reproductive efficiency processes of tilapia.

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Experimental Chapter III

Combined effects of rearing temperature regime (thermocycle vs. Constant temperature) during early development and thermal treatment on Nile tilapia (*Oreochromis niloticus*) sex differentiation.

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ABSTRACT

The purpose of the current study was to examine the combined effects of rearing temperature regimes (thermocycle (TC) *vs.* constant temperature (CTE)) on Nile tilapia's early development and posterior thermal shock throughout the period of sex differentiation (*Oreochromis niloticus*). To this end, embryos and larvae were kept under two temperature regimes: TC of 31ºC:25ºC day:night *vs.* CTE of 28ºC from 0 to 11 days post-fertilization (dpf). After this period, the larvae from each group were subjected to either heat treatment (HT, 36ºC for 12 days) or kept under the same rearing temperatures until 23 dpf (Control, C). Then all the groups remained at constant temperature until 270 dpf, when blood and gonad lobules were collected from 100 fish per group. Whole larval (11 and 24 dpf) samples were used to examine the expression of numerous genes related to male (*amh*, *ara*, *sox9a*, *dmrt1a*) and female (*cyp19a1a*, *foxl2*, *era*) sexual differentiation. In juveniles, individuals' sex was characterized by a histological analysis, the gonadal expression of the genes involved in the sex steroid synthesis was analyzed by qPCR, and plasma testosterone (T) and estradiol (E_2) levels were analyzed by ELISA. On 11 and 24 dpf larvae, daily TCs increased the survival rate against HT and up-regulated the expression of ovarian differentiation genes. HT induced changes in the CTE group by up-regulating testicular differentiation genes and down-regulating female promoter genes, which did not occur in the TC group. Juveniles reared in TC presented a higher proportion of females with higher plasma E_2 and *cyp19a1a* expression levels than the CTE+HT group. The fish from the CTE+HT group showed a higher percentage of males with highest T and *amh* expressions. These findings indicate that daily TCs during larval development promote ovarian differentiation, and diminish the masculinizing effects of HT.

Key Words: *Oreochromis niloticus* · Heat treatment· Sex reversal · Daily thermocycles · Sex ratio · Temperature-Sex Determination.

INTRODUCTION

Nile tilapia (*Oreochromis niloticus*) fish production is the second largest worldwide and is farmed in more than 80 countries, hence the keen global commercial interest and importance of this species for protein production for human consumption (FAO, 2020). Its rapid growth, good resistance to diseases, high prolificacy and early sexual maturation show great tilapia adaptability to colonize highly heterogeneous environments (Baroiller and Jalabert, 1989). Under farming conditions however, females' reproductive efficiency (asynchronous ovarian development, precocious sexual maturation, mouthbrooding behavior, high energy demand for reproduction) limits growth rates and makes the management and control of tilapia populations difficult. To avoid these female reproduction problems, the tilapia aquaculture industry has made efforts to investigate sex determination by establishing male-monosex populations with the associated economic and management benefits (higher growth rate and size/weight homogeneity) (Beardmore et al., 2001).

The Nile tilapia Sex Determination System is one of the best-documented in fish. Sex is determined by the interaction between genetic (genotypic sex determination, GSD) and environmental (environmental sex determination, ESD) factors that compose the monofactorial heterogametic determination XX/XY system of Nile tilapia (De Alba et al., 2021b). In this species, several genes have been identified as master sex-determining genes that exhibit sexually dimorphic expression patterns before and after testis or ovary differentiation (Iriji et al., 2008; Tao et al., 2018). Tilapia ovarian differentiation is particularly encoded by factors related to *cyp19a1* (P450 aromatase enzyme), which catalyzes the conversion of androgens into estrogens. In addition, *foxl2* (*forkhead transcriptional factor L2*) is considered a master sex-determining gene of female sex differentiation in tilapia (Kobayashi et al., 2004). Testis differentiation is encoded by genes related to *doublesex- and mab-3-related transcription factor 1* (*dmrt1*) and *anti-müllerian hormone* (*amh*) (Caceres et al., 2019; Melo et al., 2019). Dmrt1 activates the expression of *SRY-box transcription factor 9* (*sox9*) that, in turn, acts on *amh* expression (Kobayashi et al., 2008). Then Amh stimulates the production of a transforming growth factor b (TGF-b) glycoprotein that blocks *cyp19a1* expression in testes. Male sex differentiation is ensured because blocking *cyp19a1* expression prevents androgens from being converted into estrogens (Melo et al. 2019). Ultimately, the production of estrogens like 17β-estradiol (E_2) results in female sex differentiation.

Androgens like testosterone (T) and 11-ketotestosterone (11-KT) are the result of testis differentiation (Yamamoto, 1969). To complete the signaling pathway of sexual differentiation, sex steroid hormones exert their actions through estrogen or androgen receptors (Ers and Ars, respectively), which contributes to ovary or testis development, respectively (de Alba et al., 2021b).

Besides the gene control of the sex determination process in Nile tilapia, sex is also influenced by environmental conditions (ESD) (Ospina and Piferrer, 2008), of which temperature is the most pervasive factor because it impacts tilapia sex determination (Baroiller et al., 2009). From very early development stages, undifferentiated gonads present sensitive and susceptible periods to temperature in which exposure to temperature variations can induce changes in individuals' phenotypic character by shifting the population's sex proportion (Ospina and Piferrer, 2008). However, employing high or low temperature treatments before or after the sex differentiation period has been reported as being neffective (Baroiller et al., 1995, 2009). In Nile tilapia, the critical sex differentiation period seems to range from 9 to 25 days post fertilization (dpf) (Baroiller et al., 2009). Thus high temperature treatments above 32ºC that last at least 10 days during this period can result in a higher percentage of functional male phenotypes (sex reversal by high temperature) (Kwon et al., 2000; D'Cotta et al., 2001a, b; Baroiller et al., 1995, 2009). At the molecular level, high temperatures activate both *dmrt1* and *amh by* repressing ovarian sex differentiation genes (*cyp19a* and *foxl2*) and blocking the conversion of androgens into estradiol to, thus, cause female-to-male sex reversal (Wang et al., 2017).

In the natural environment, water temperature varies through the day because solar radiation generates daily thermocycles (TCs; temperature increases during the day and drops at night) (Patterson and Wilson, 1995). In the last decade, TCs have been shown to be very relevant for several fish behavior and physiology aspects by increasing growth, feeding, thermal tolerance and survival, and by decreasing the number of malformations (Blanco Vives et al., 2011; Villamizar et al., 2012; Espirito Santo et al., 2020; de Alba et al., 2021). However, very little attention has been paid to date to their effects on sex determination and differentiation processes in fish (Blanco-Vives et al., 2011, Villamizar et al., 2012). In these studies, daily TCs have been reported to influence sex determination mechanisms and to modify the sex ratio in zebrafish (*Danio rerio*) and Senegalese sole (*Solea senegalensis*) with higher proportion of females in the

progenies reared in TCs than at constant temperatures. In Nile tilapia, although TCs are present in their natural habitat, their importance in both sex determination and differentiation from early development stages is still unknown, as their influence on the sex reversal process by high temperature treatment.

The aim of the present research was to investigate the combined effects of rearing temperature regimes (TCs *vs.* constant temperature) during early development and a posterior heat treatment (HT) during the sexual differentiation period (from 11 to 23 dpf) on sex determination/differentiation processes and sex ratio with Nile tilapia.

MATERIAL AND METHODS

The current study was carried out at the Department of Physiology facilities of the University of Murcia (EU, Spain). The European Union's rules (2010/63/EU) and Spanish law (RD 53/2013, RD 1386/2018, and Law 32/2007) were followed for every procedure carried out during the experiment. The Committee of the University of Murcia on Ethics and Animal Welfare authorized the experimental protocols.

Animals and housing

Male and female Nile tilapia (*O. niloticus*) juveniles (aged 1 month) were obtained from a global aquaculture company (Fishgen, Swansea, Wales, UK). Animals were placed in individual 300-liter tanks that were connected to a recirculation system that had mechanical, biological, and aeration filters. A water heater and cooler (AB Aqua Medic, Gewerbepark, Germany) were used to keep the water at 28±0.5ºC. Weekly measurements were made of the water quality indicators (pH, ammonia, nitrate, nitrite, and dissolved oxygen). The photoperiod was set as a 12:12 h light/dark (LD) cycle with lights on at 09:00h. The feeding regime was set using automatic feeders with three daily meals (11:00h, 15:00h and 19:00h) at a feeding rate of 2% of the total biomass per day using commercial feed (D-4 Alterna Basic 2P, Skretting, Spain) with 36% crude protein (CP).

Juveniles were reared under the previous conditions for 8 months until they reached maturity. Then male and female tilapia individuals were separated for 2 weeks according to their sex by visual inspection of genital papilla (Hussain, 2004). After this period, fish were subjected to hormonal treatment with human Chorionic Gonadotropin

hormone (hCG, Sigma Aldrich, St. Louis, USA) as described elsewhere (Fernandes et al., 2013; Espirito Santo et al., 2020). After hCG administration, animals were placed together at the male:female ratio of 3:1 for natural reproduction in the afternoon (Fernandes et al., 2013). At the beginning of the next light phase, the fertilized eggs were removed from the mouth of females. This procedure allowed us to obtain fertilized eggs of less than 12 h post fertilization (hpf), which were used in the experiments.

Experimental design

The fertilized eggs were obtained from eight different tilapia broodstocks. Four progenies and 2400 fertilized eggs (N) were used in the experiments. Each progeny came from a spawning event during which the eight broodstocks were stimulated as described above. Each progeny consisted of 600 fertilized eggs, which were obtained in the blastula stage (4-12 hpf) (Fujimura and Okada, 2007). Eggs were pooled together and distributed in incubators for Cichlid eggs (Alimar SA, Murcia, Spain) (100 eggs per incubator, six incubators per progeny used) in two systems with different temperature regimes: a TC (thermophase:cryophase) of 31ºC:25ºC day:night *vs.* a constant temperature of 28ºC (CTE). The thermophase coincided with the light phase (09:00-21:00h) and the cryophase coincided with the dark phase (21:00-09:00h) (Fig. 1 and Suppl. Fig. A1). Electronic heaters (Askoll, Povolaro, Italy) and water coolers (Aqua Medic Titan 1500 GmbH, Bissendorf, Germany) were used to alter the water's temperature at a rate of 1.5°C per hour, mimicking the daily temperature differences that tilapia experience in the wild (Patterson and Wilson, 1995). To ensure that thermocycles (TCs) were correctly performed and water temperature was still controlled in the CTE group, an electronic timer (Bachmann GmbH & Co, Stuttgart, Germany) was used to control temperature variations, and an underwater data logger was used to record them every 15 minutes (HOBO PENDANT Onset Computer Corporation, Massachusetts, USA). The photoperiod was set as a 12:12h light/dark cycle with lights on and off at 09:00h and 21:00h, respectively. Up until 7 days after fertilization, embryos and larvae were raised in incubators. Then, they were moved to 9-liter tanks that were connected to the same temperature system as the larvae. At the same time (7 dpf), larvae were administered exogenous feed (Gemma, 42% CP, 0.5-0.8 micrograms, Skretting) as four daily meals (09:00h, 11:00h, 15:00h and 19:00h) until satiety. Larvae were reared under these temperature regimes until 11 dpf, when the survival rate was calculated as the percentage of live larvae on 11 dpf of the total number of embryos

originally placed inside incubators. On 11 dpf, the larvae from TC and CTE were subjected to either HT or maintained at the same rearing temperatures regime (Control group, C) from 11 to 23 dpf (Fig. 1). HT consisted of 12 days of being exposed to 36ºC with a temperature change rate on the first and last exposure days at 1ºC/h according to the treatments followed for tilapia masculinization (Baroiller et al., 1995). At the end of HT, the survival rate was calculated as the percentage of live larvae on 24 dpf of the total number of larvae on 11 dpf. Then all groups were left at constant temperature (28ºC) until 270 dpf (Fig. 1).

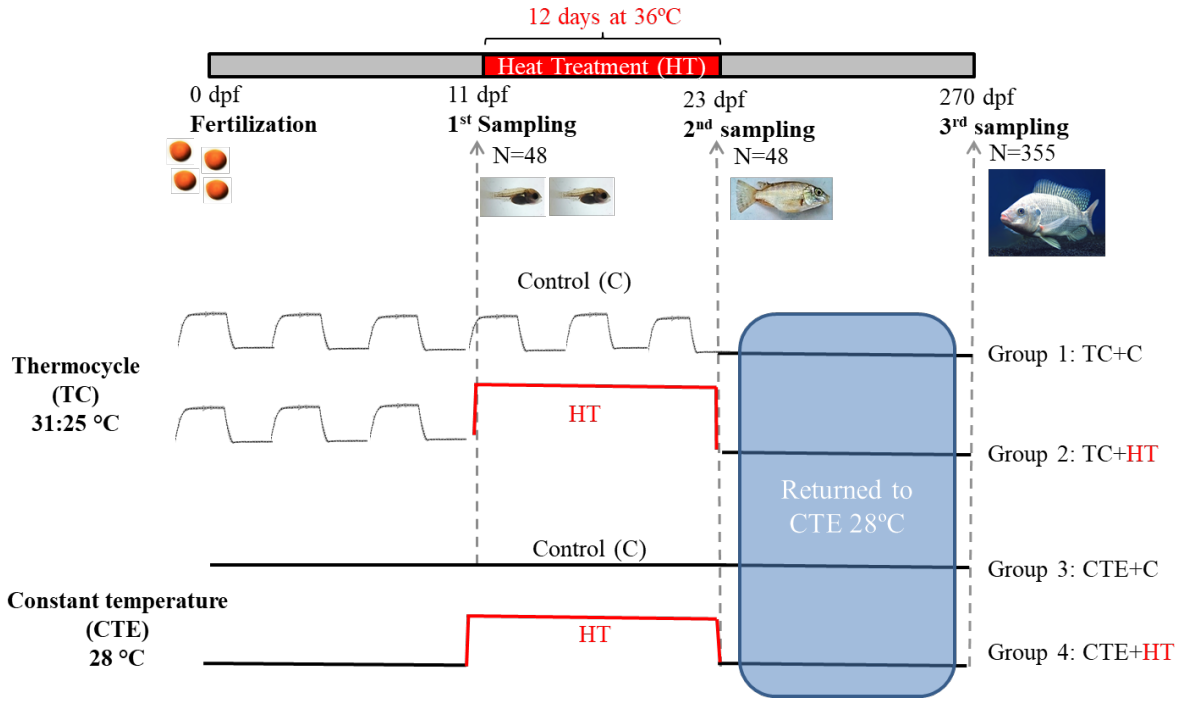

Fig. 1 Schematic representation of the experimental design. Fertilized eggs (stage 1) were divided into two groups reared in different thermal regimes: thermocycle (TC) of 31ºC:25ºC day:night *vs.* a constant temperature of 28ºC (CTE). Embryos and larvae were left nder these conditions from 0 to 11 days post fertilization (dpf). At the end of this period, the larvae from each temperature regime were either subjected to heat treatment (HT, 36ºC for 12 days) or left at the same rearing temperatures from 11 to 23 dpf (Control, C). Then after this period, all the groups remained at constant temperature (28ºC) until 270 dpf.

In addition, complete larval samples were taken on days 11 and 24 dpf (one day after HT ceased) for the mRNA expression study of the genes implicated in Nile tilapia sex differentiation and sex determination processes. According to the protocols established for the euthanasia of fish, larvae were instantly put to death by anesthetic overdose for these gene expression investigations, and their death was verified under a microscope. Then, each replicate was combined with a pool of larvae to obtain three

replicates for each group or progeny, resulting in a total of 12 replicates (n=12) from the four independent progenies. Depending on the larval stage, different numbers of larvae were employed in each pool replicate: two larvae/pool on 11 dpf and one larvae/pool on 24 dpf. Until the gene expression analysis, larvae were placed directly in 1.5 ml sterile tubes and frozen -80ºC. Finally at the end of the experiment, up to 25 fish from each group/progeny (up to 100 fish/group) were randomly selected and anesthetized with 50 µL/L of clove oil essence (eugenol, Guinama, Spain). In the groups of each progeny with fewer than 25 fish, all the animals from the group were used. The total number of animals sampled from each group was 92 for TC+C, 95 for TC+HT, 88 for CTE+C and 80 for CTE+HT. Before euthanasia, blood was collected by caudal puncture and whole body weight was measured. Blood was centrifuged for 15 min at 3000 rpm and 4ºC. After centrifugation, plasma was separated and frozen at –80ºC until analyzed. In addition, one lobule of the gonads from each fish were collected, transferred to sterile 1.5 ml RNase- and DNase-free Eppendorf tubes which were frozen rapidly in dry ice and stored at -80ºC until the analysis of mRNA gene expression. The other gonad lobule was collected, immersed in 10% formaldehyde fixative solution (Leica Biosystems, Spain) and stored until histological analyses.

Sex steroid ELISA analysis

With commercial ELISA kits (Tecan, Hamburg, Germany) that had previously been validated for tilapia plasma samples, the E_2 and T concentrations were determined in all of the adult fish plasma samples (270 dpf) (de Alba et al., 2019).

Histological analyses

Gonad samples were fixed for 24 h in 10% formaldehyde solution before transferring to 70% ethanol and after dehydration, embedding in paraplast (Leyca Bioystems, Spain). Hematoxylin and eosin (HE) was used to stain sections of around 5 µm. A light microscope with a camera (Axiolab, Zeiss, Oberkochen, Germany) was used to examine the slides (Photometrics Coolsnap, Roper Scientific, Buckinghamshire, UK). Female and male sex was determined by the identification of oocytes or spermatocytes, respectively (Fig. 2).

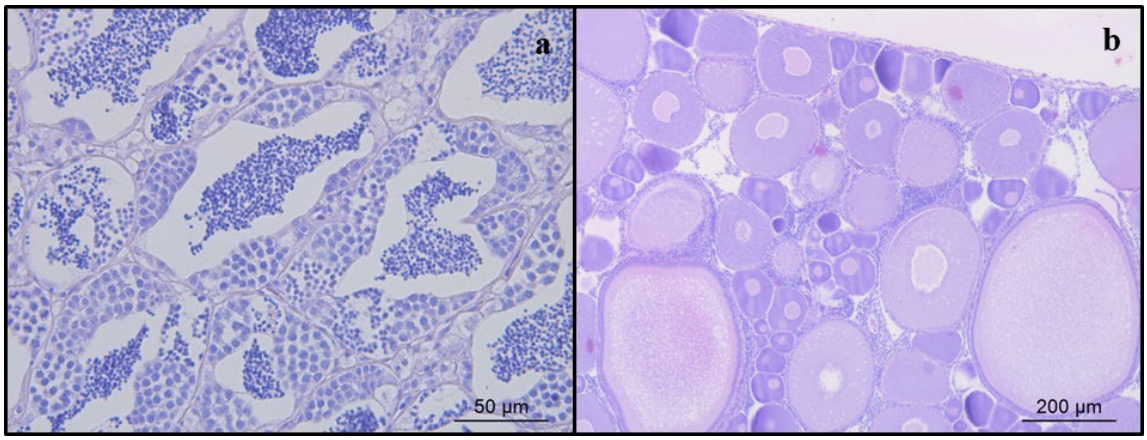

Fig. 2 Representative histological section of the testis (A) and ovary (B) of Nile tilapia juveniles in the reproductive stage. Longitudinal cut. Staining: H-E. Scale bar: (A) x 50 and (B) x 200.

Real-time RT-PCR analysis

With the use of a tissue homogenizer (TissueLyser LT, Qiagen, Hilden, Germany), the pool of larvae and gonad samples was homogenized in Trizol reagent (Ambion, Thermo Fisher Scientific, Waltham, USA). After dissolving the RNA in sterile DEPC water (Invitrogen, CA, USA), the RNA concentration and purity were determined by spectrometry (Nanodrop ND-1000, Thermo Fisher Scientific). Prior to retrotranscription, genomic DNA contamination was removed from RNA (1 g) by treating it with 1 U of DNase I (Thermo Fisher). A thermocycler (MiniAmp Plus, Applied Biosystems, Foster City, USA) and commercial reverse transcriptase kit (QSCRIPT cDNA Synthesis Kit, Quantabio, Beverly, USA) were used to synthesize cDNA. Before the expression analysis, all of the cDNa samples were diluted (1:10) in nuclease-free water (Thermo Fisher Scientific). Using Perfecta® SYBR® Green Fastmix (Quantabio) in a final volume of 20 µl for each qPCR experiment, quantitative PCR (qPCR) reactions were performed. With the use of a light thermocycler (7500 RT-PCR system, Applied Biosystems, Foster City, USA), all the samples were run in duplicate with the following steps: 15 min at 95ºC, followed by 40 cycles of 15 s at 95ºC and 1 min at 60ºC. The thermocycler ran melting curves right away after the amplification phase to ensure that the reaction was selective and that just one DNA species was amplified. Primer 3 Plus software was used to create primers (Table 1). (Untergasser et al., 2012). Dilution curves were used to determine the primer concentration and confirm that all of the primers had relative amplification efficiencies between 90% and 110%. (Table 1). A 500 nM final concentration of each primer was

used. Because *Beta-Actin (βactin)* was consistent and unaffected by temperature (coefficient of variation 5%), it was used as a reference gene (Vanhauwaert et al., 2014). Then, using the 2–ΔΔCt technique, the relative expression of all the genes was determined (Livak and Schmittgen, 2001).

Table 1. Primer sequences used for the quantitative PCR analyses.

Gene	F/R	Sequence (5'-3')	Ensembl/GenBank Accession number
amh	F	ACGACGCGCAAAGAAAACTG	ENSONIG00000004781
	R	TAATGTTTGCGCTGCTTGGG	
cyp19a1a	F	TTGCACAAAACCACGGTGAG	NM_001279586.1
	R	ACGTGCGGGTTTTGTTTGAG	
ara	F	CAGCCCTATGTCCTTGCTTACCAG	XM_005467840.4
	R	CGCTGGTCATTGAAAATCAGGTCT	
era	F	TATGTGCCCAGCGACAAATC	NM_001279770.1
	R	CGTTTTTCACGCCGCAAAAC	
sox9a	F	GTGTTGAAGGGTTACGACTGGACG	XM_003450119.4
	R	CCGTTCTTGACAGACTTTCTCCGC	
foxl2	F	AGCATTTCACCGATCGAGAC	NM_001279778.1
	R	CATTGCGCACACACCAAAAC	
dmrt1a	F	CGGATTGCAGCGGACCGA	XM_013270912.3
	R	GGACAGAGACACAGGACTAC	
βactin	F	TGGTGGGTATGGGTCAGAAAG	ENSONIG00000008505
	R	CTGTTGGCTTTGGGGTTCA	

Data analysis

The results are expressed as mean±SEM. For all the test, the significance threshold was set at P= 0.05. To perform the statistical analyses, the SPSS software (v. 19.0, IBM, Armonk, NY, USA) was used. The Kolmogrov-Sminorv test and Levene's test were used to determine and confirm the homoscedasticity and normality of the data, respectively. The data from the 11 dpf larvae (survival rate and gene expression) were subjected to a Student's t-test. The data from the 24 dpf larvae (survival rate and gene expression) and juveniles (sex ratio, weight, plasma concentrations of E_2 and T, gonadal gene expression) were subjected to: one-way ANOVA to examine whether there are any statistically significant differences between the four experimental groups; a two-way ANOVA to analyze the effects of rearing temperature regime (R), HT and their interaction. Following the one-way ANOVA, Duncan's post hoc analysis was used to determine whether there were any significant differences between the experimental groups (P 0.05).

RESULTS

Effects of the rearing temperature regime on the survival rate and gene expression on 11 dpf.

The Nile tilapia larvae survival rate from 0 to 11 dpf did not show any statistically significant differences between rearing temperature regimes (one-way ANOVA, P= 0.517, Table A1), with values of 51.6±3.9 and 45.3±4.4 % for the TC and CTE groups, respectively. In the gene expression analysis, significant differences were detected in the mRNA expression of some of the multiple factors from the analyzed sex determination and differentiation processes (Fig. 3) (one-way ANOVA, $P< 0.05$; Table A1). The larvae reared in CTE showed a higher mRNA expression of *ara* than those reared in the TC (Fig. 3c) (one-way ANOVA, $P= 0.028$; Table A1). On the contrary, the expressions of *cyp19a1a*, *era* and *foxl2* were higher in the larvae reared in the TC than in CTE (Fig. 3b, d, f) (one-way ANOVA, $P< 0.05$; Table A1). Finally, no statistically significant differences between both groups were detected for *amh*, *sox9a* and *dmrt1a* (Fig. 3a, e, g) ($P> 0.05$; Table A1).

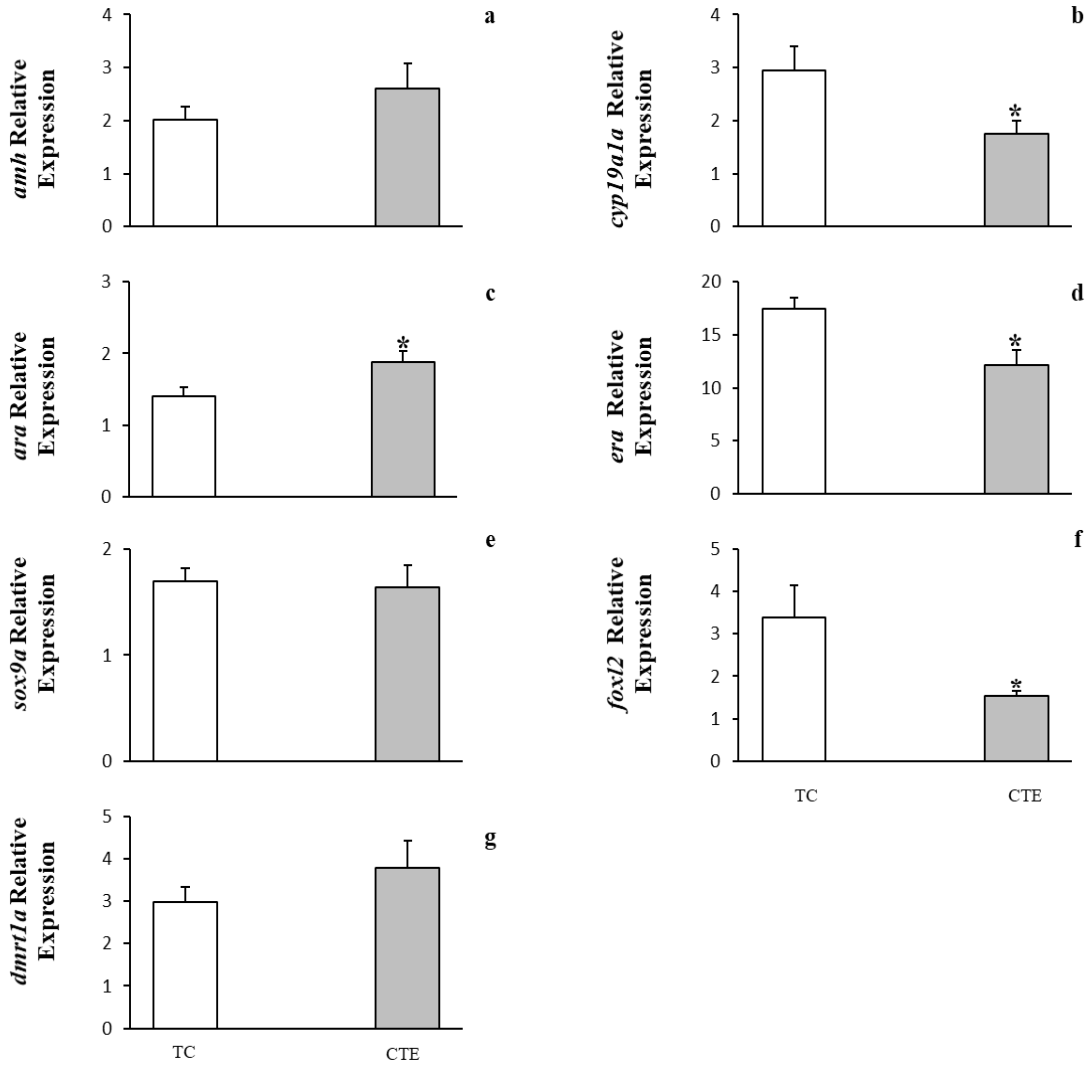

Fig. 3 Relative mRNA expression of *amh* (A), *cyp19a1a* (B), *ara* (C), *era* (D), *sox9a* (E), *foxl2* (F) and *dmrt1a* (G) in Nile tilapia larvae on 11 days post fertilization (dpf) and reared in two temperature regimes: thermocycle (TC, thermophase:cryophase 31:25±0.2ºC) (white bars) *vs.* constant temperature (CTE, 28±0.2ºC) (gray bars). Asterisks denote significant differences between rearing temperature regimes (Student's t-test, $P< 0.05$). Data (n=12) are represented as mean±SEM.

Effects of the rearing temperature regime and heat treatment on the survival rate and gene expression on 24 dpf.

The Nile tilapia larvae's survival rate after HT showed statistically significant differences between the experimental groups (Fig. 4) (one-way ANOVA, $P< 0.004$; Table A1). The lowest survival rate was observed in the larvae from the CTE+HT group (49.4±3.5%), while the highest survival rate appeared in the C groups under both temperature conditions (91.8±1.8% and 81.1±4.3% for TC and CTE, respectively) and in the TC group exposed to HT (72.2±5.9%). In addition, survival was influenced by not only HT because it was higher in the C than in the HT group (two-way ANOVA, $P=$

0.001), and by the rearing temperature regime for being higher in the TC groups (two-way ANOVA, *P*= 0.047) (Figure 4 and Table A1).

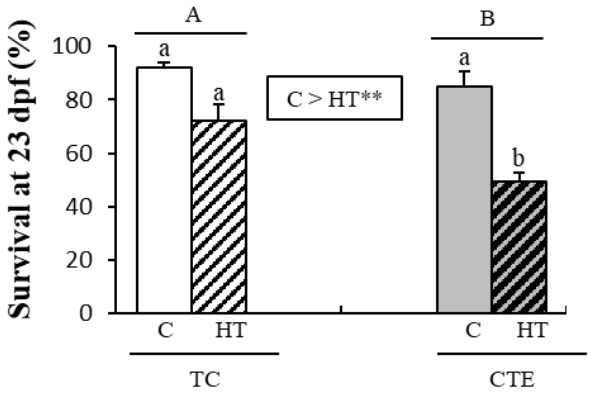

Fig. 4 Effect of heat treatment on the Nile tilapia larvae survival rate. Larvae were reared from 0 to 11 days post fertilization (dpf) in two temperature regimes: thermocycle (TC, thermophase:cryophase 31:25±0.2ºC) (white bars) *vs.* constant temperature (CTE, 28±0.2ºC) (gray bars). From 11 to 23 dpf, the larvae from each group were either exposed to a heat treatment of 36ºC (HT, stripped bars) or maintained in the same rearing regime as before (control group, C; solid bars). Survival was evaluated on 23 dpf. Different lower case letters indicate significant differences between experimental groups (one-way ANOVA, *P*< 0.05). Different upper case letters and asterisks denote significant differences between rearing temperature regimes and heat treatment effects, respectively (two-way ANOVA, *P*< 0.05). Data (n=4) are represented as mean±SEM.

On 24 dpf, all the genes showed significant intergroup differences (Fig. 5) (one-way ANOVA, *P*< 0.05; Table A1). The expressions of *ara, sox9a*, and *dmrt1a* were higher in the CTE+HT group (Fig. 5c, e, g) (one-way ANOVA, *P*< 0.05; Table A1). While the expressions of *ara* and *dmrt1a* were influenced by HT (higher in HT *vs*. C), the *sox9a* expression was influenced by temperature regime (higher in the animals reared in CTE *vs*. TC, regardless of them being subjected or not to HT) (Fig. 5c, e, g) (two-way ANOVA, *P*< 0.05; Table A1). A significant interaction between temperature regime and HT was found in the expressions of genes *ara* and *sox9a* (two-way ANOVA, *P*<0.05; Table 1). The *amh* expression was significantly higher in the CTE+C group (Fig. 5a) (one-way ANOVA, P= 0.042; Table A1), while the *cyp19a1* expression was higher in the TC+C group (Fig. 5b) (one-way ANOVA, *P*= 0.004; Table A1) and was influenced by HT (higher in the Control groups *vs.* HT) (two-way ANOVA, *P*= 0.009; Table A1). Finally, the expressions of *era* and *foxl2* presented similar patterns to one another, with a higher expression in TC+C than in the CTE+HT group (Fig. 5d, f) (one-way ANOVA, *P*< 0.05; Table A1). The statistical analyses also revealed a significant effect of temperature regime because the larvae reared at TC in early

development stages had higher *era* and *foxl2* expressions than those reared in CTE (two-way ANOVA, P <0.05; Table A1).

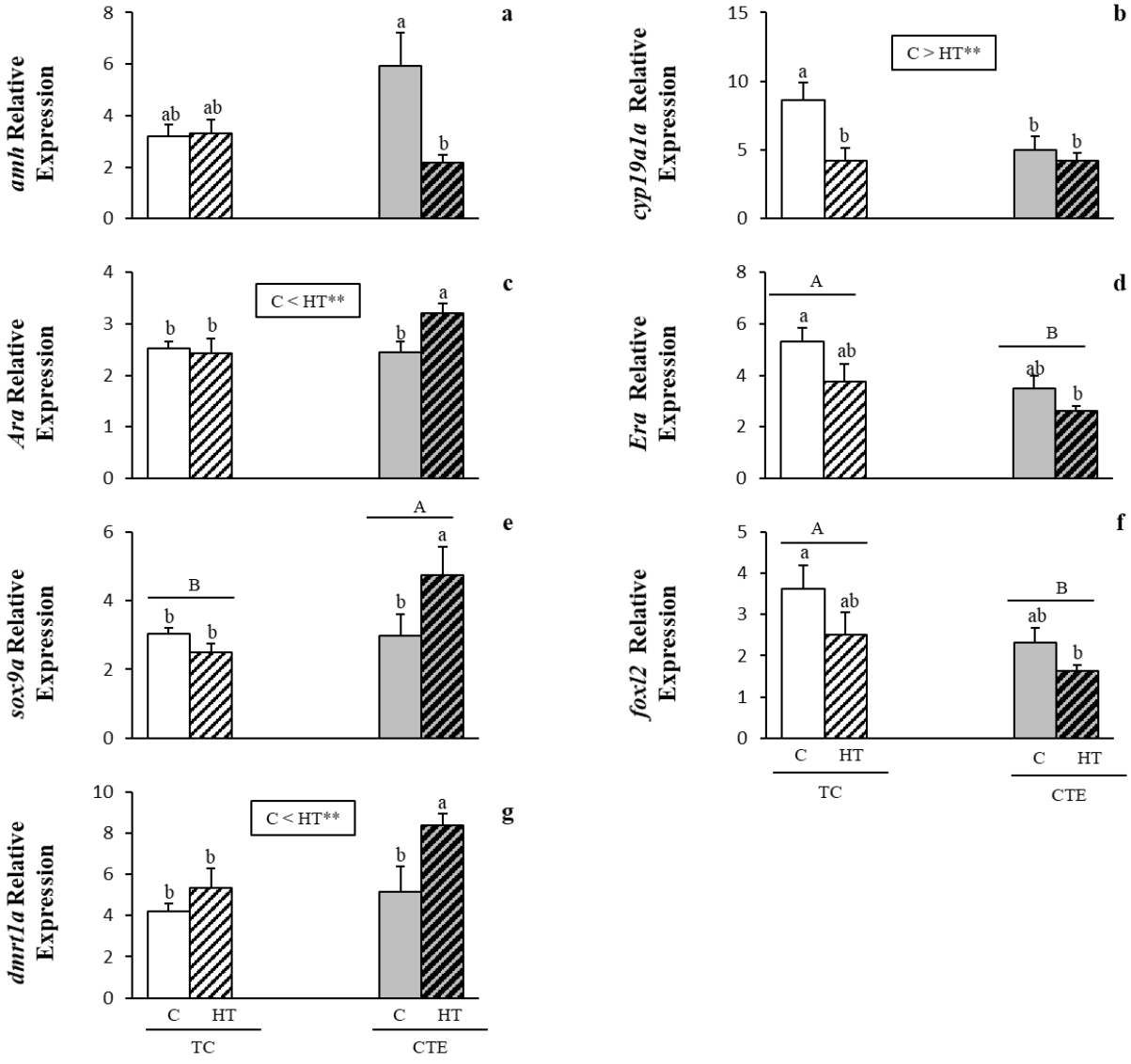

Fig. 5 Effect of heat treatment on the relative mRNA expression of *amh* (A), *cyp19a1a* (B), *ara* (C), *era* (D), *sox9a* (E), *foxl2* (F) and *dmrt1a* (G) on 24 days post fertilization (dpf) in Nile tilapia larvae. Larvae were reared from 0 to 11 dpf in two temperature regimes: thermocycle (TC, thermophase:cryophase 31:25±0.2ºC) (white bars) *vs.* constant temperature (CTE, 28±0.2ºC) (gray bars). From 11 to 23 dpf, the larvae from each group were either exposed to a heat treatment of 36ºC (HT, stripped bars) or maintained in the same rearing regime as before (control group, C; solid bars). Different lower case letters indicate significant differences between experimental groups (one-way ANOVA, P< 0.05). Different upper case letters and asterisks denote significant differences between rearing temperature regimes and heat treatment effects, respectively (two-way ANOVA, P< 0.05). Data (n=12) are represented as mean±SEM.

Effects of the rearing temperature regime and heat treatment on sex ratio, weight, plasma sex steroids and gonadal gene expression in the juvenile stage.

Tilapia embryo and larvae were reared under the four experimental conditions until the juvenile stage (270 dpf) when the sex ratio, whole body weight, plasma levels of sex steroids and mRNA expression of the genes involved in the synthesis of sex steroids in gonads were measured (Fig. 6 and 7).

For the sex ratio, the statistical analysis showed differences between experimental groups (Fig. 6) (one-way ANOVA, P= 0.003; Table A1). The fish reared in TC+C had a higher proportion of females (56.2±4.1%) than both the groups reared in CTE (38.1±7.7 and 22.2±2.7% for CTE+C and CTE+HT, respectively). However, no differences in the sex ratio were found between both groups from the TC: TC+C and TC+HT (42.1±3.3%). The statistical analysis also showed that rearing tilapia larvae in the TC on the first days of development led to a higher proportion of females than in CTE (two-way ANOVA, P= 0.002; Table A1). A significant effect of HT was also noted with a higher proportion of males in the groups exposed to HT than those maintained in rearing temperature regimes (C) (two-way ANOVA, P= 0.01; Table A1).

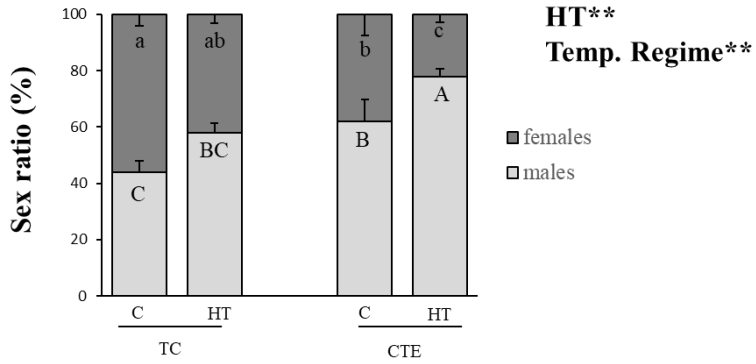

Fig. 6 Effect of heat treatment on the sex ratio (%) of the Nile tilapia juveniles (270 days post fertilization, dpf) reared from 0 to 11 dpf in two temperature regimes: thermocycle (TC, thermophase:cryophase 31:25±0.2ºC) *vs.* constant temperature (CTE, 28±0.2ºC). From 11 to 23 dpf, the larvae from each group were either exposed to a heat treatment of 36ºC (HT) or maintained in the same rearing regime as before (control group, C). Different lower and upper case letters indicate significant differences between experimental groups of males (light bars) and females (dark bars), respectively (one-way ANOVA, P< 0.05). Different asterisks denote significant differences between rearing temperature regimes and heat treatment effects, respectively (two-way ANOVA, P< 0.05). Data (n=4) are represented as mean±SEM.

About tilapia juveniles' weight, the statistical analysis detected significant differences between experimental groups with a similar pattern in both sexes (Fig. 7a-b) (one-way ANOVA, P< 0.05, Table A1). The juveniles from CTE+C and CTE+HT were

heavier than those from TC+C, and for both males (Fig. 7a) and females (Fig. 7b). No significant differences were detected between fish weight from both TC groups (one-way ANOVA, $P > 0.05$, Table A1). In both sexes, weight was influenced by the temperature regime with higher values in the fish reared under CTE than in the TC (two-way ANOVA, $P < 0.001$, Table A1). The statistical analyses also revealed a significant effect of HT ($P = 0.005$) on females' weight, with higher values for the fish exposed to HT than the C groups (Fig. 7b) (two-way ANOVA, $P < 0.05$, Table A1).

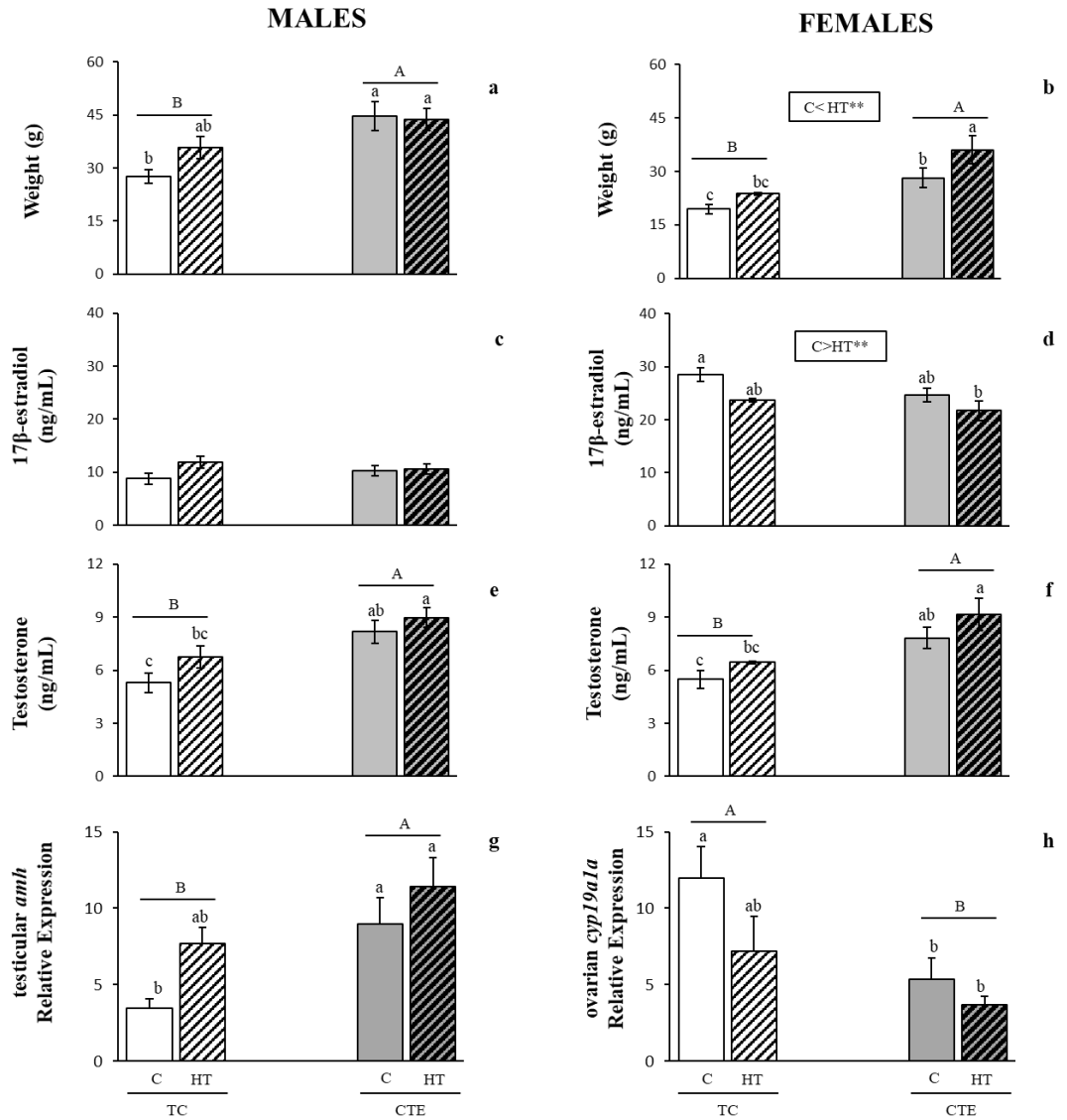

Fig. 7 Effect of heat treatment on weight (A-B), plasma 17β-estradiol (C-D), plasma testosterone (E-F), testicular *amh* (G) and *cyp19a1a* (H) relative mRNA expression in male (left panels) and female (right panels) Nile tilapia juveniles (270 days post fertilization, dpf). Larvae were reared from 0 to 11 dpf in two temperature regimes: thermocycle (TC, thermophase:cryophase 31:25±0.2ºC) (white bars) *vs.* constant temperature (CTE, 28±0.2ºC) (gray bars). From 11 to 23 dpf, the larvae from each group were either exposed to a heat treatment of 36ºC (HT, stripped bars) or maintained in the same rearing regime as before (control group, C; solid bars). Different lower case letters indicate significant differences between experimental groups (one-way ANOVA, $P < 0.05$). Different upper case letters and asterisks denote significant differences between rearing temperature regimes and heat treatment effects, respectively (two-way ANOVA, $P < 0.05$). Data (n=12) are represented as mean±SEM.

Plasma levels of sex steroids were measured in all the individuals (Figs. 7c-f). Regarding E_2, the analysis detected statistical differences between the experimental groups in females, but not in males (Fig. 7c-d) (one-way ANOVA, $P< 0.05$; Table A1). The females from TC+C showed higher plasma levels of E_2 (28.5±1.2 ng/ml) than the fish reared in CTE+HT (21.7±1.8 ng/ml) (Fig. 7d) (one-way ANOVA, $P= 0.019$, Table 1). As regards T, the plasma levels displayed similar statistical differences between groups for both sexes (Fig. 7e-f) (one-way ANOVA, $P< 0.05$; Table 1). The tilapia from the CTE+HT group obtained the highest T values (9.0±0.5 and 9.15±0.9 ng/ml for males and females, respectively), while the fish reared in TC+C presented the lowest levels (5.2±0.5 and 5.5±0.5 ng/ml for males and females, respectively) (one-way ANOVA, $P< 0.05$; Table A1). However, no significant differences in T were detected between both groups for each temperature regime (TC or CTE) in males or females (Figs. 7c-f). Regardless of the rearing temperature regime, an effect of HT was observed on females' E_2 production, with HT lowering its plasma levels (two-way ANOVA, $P= 0.02$; Table 1). Moreover, an effect of the rearing temperature regime was observed in T production because the fish of both sexes reared in CTE had higher levels than those reared in the TC (two-way ANOVA, $P< 0.001$; Table A1).

Finally, the analysis also revealed statistically significant differences in the relative mRNA expressions of *amh* and *cyp19a1a* in tilapia juveniles' testes and ovaries, respectively (Fig. 7g-h) (one- and two-way ANOVA, $P< 0.05$; Table A1). The highest *amh* expression was detected in the males reared in CTE, with the lowest one in the males reared in the TC and subjected to HT (Fig. 7g) (one-way ANOVA, $P= 0.039$; Table A1). The highest *cyp19a1a* expression for females was for the TC+C group, with the lowest in the females reared in CTE (Fig. 7h) (one-way ANOVA, $P< 0.05$; Table A1). The two-way ANOVA revealed that the males reared in CTE had a higher *amh* expression than those in the TC regardless of HT (Fig. 7g). The opposite effect on *cyp19a1a* expression occurred in females, with higher levels in the fish reared in the TC *vs.* CTE (Fig. 7h) (two-way ANOVA, $P< 0.05$; Table A1).

DISCUSSION

The present research work reveals the strong effect of daily temperature cycles on Nile tilapia sex determination and differentiation, and also their influence on the

female-male sex reversal process by high temperature treatment. TCs increased the survival rate against HT and also up-regulated the expression of the genes related to female sexual differentiation on both 11 and 24 dpf. HT modified the expression patterns of the sex differentiation genes only in the larvae reared in CTE by increasing the expression of the genes involved in testicular differentiation and decreasing the expression of the genes related to ovarian differentiation. This modification was not observed in the larvae reared in the TC, which showed less susceptibility to HT. The differences observed during larval sex differentiation between both rearing temperature regimes were maintained in the juvenile fish. In this stage, daily TCs increased the female proportion, which presented higher ovarian *cyp19a1a* expression and plasma E_2 levels. In addition, HT induced a more marked presence of males with higher plasma T and testicular *amh* expression levels only in the CTE group, but not in the TC group, which presented lower sensitivity to high temperature sex reversal.

In Nile tilapia aquaculture, high temperature treatments in the larval stage are one of the commonest methods for obtaining a higher proportion of males (Baroiller et al., 2009, de Alba et al., 2021b). However, this process entails some disadvantages, such as increased mortality (de Alba et al., 2021b, c). In the present study, a higher larval survival rate after HT was observed when larvae were reared in the TC compared to those reared in CTE. To date, the effect of TCs on thermal tolerance and survival after thermal shock has been studied in other fish species with different results. In Atlantic salmon (*Salmo salar*), very mild effects of TCs were observed on their thermal tolerance response (Corey et al., 2017; Gallant et al., 2017). However in zebrafish, diurnal TCs increased fish thermal tolerance compared to those reared at constant temperatures (Cortemeglia and Beitinger, 2005; Schaefer and Ryan, 2006; Xia et al., 2016; Wang and Xia, 2019; de Alba et al., 2022), as herein observed in tilapia. Such heat hardening seems to be related to acclimation to cyclical thermal environments, especially when fish are subjected to temperatures peaks higher than the temperatures faced by fish reared in stable or constant thermal environments, and even when the average temperature is the same in all groups (Schaefer and Ryan, 2006).

The two rearing temperature regimes herein applied not only impacted survival during larval development, but also influenced the expression of several genes. The analyzed genes have previously been identified as molecular markers involved in ovarian and testicular differentiation in Nile tilapia (Lu et al., 2022). In our study, on 11

and 24 dpf we observed that the tilapia larvae reared in TCs presented higher values in the expression of the genes related to ovarian differentiation, such as *cyp19a1a*, *foxl2* and *era*. In contrast, constant temperature promoted the expression of testicular differentiation factors like *ara*, *sox9a* and *dmrt1*. These results agree with those obtained by Villamizar et al. (2012), who showed that zebrafish larvae reared at constant temperature presented lower *cyp19a* expression and higher *amh* expression levels than the larvae reared in TCs. In other research works conducted in Nile tilapia, the effect of HT on these molecular markers has been observed from the first exposure days and in very early development stages (from 10 dpf) (Li et al., 2014; Lu et al., 2022). In tilapia, the *dmrt1* and *amh* expressions are up-regulated by HT that, in turn, brings about an increase in the expression of the other genes involved in testicular development, such as *sox9* and *11β-hydroxylase*. The result of the activation of testicular pathway genes is the inhibition of ovarian differentiating genes, such as *cyp19a* and *foxl2* (Lu et al., 2022). This agrees with our results because the larvae reared in CTE and exposed to high temperature had higher *sox9a*, *ara* and *dmrt1a* expressions. In addition, the up-regulation of the above genes was followed by a consequent suppression of gene expression for the factors involved in the ovarian pathway (*foxl2* and *era*), but only for the CTE-reared larvae. These changes in the up-regulation of male sex differentiation genes and the suppression of female sex differentiation genes were not observed in the TC group, except for *cyp19a1a* expression, which was inhibited by HT in the TC and CTE groups. Therefore in general, the larvae reared in the TC were less susceptible to the effects of HT on the expression of the genes involved in sexual differentiation pathways. This may be related to the effects of TCs on survival, which point out greater thermotolerance for the fish reared under these conditions. Despite some studies supporting this hypothesis (Li et al., 2014), further research is needed.

Therefore, the combined effect of rearing temperature regime and HT led to differences in the expression of sex differentiation genes. These differences ultimately impacted the sex ratio of the tilapia juveniles from the four tested groups. Although Nile tilapia are exposed to the daily TCs caused by alternations between day and night in their natural environment (Lopes and Henry-Silva, 2014), the effect of TCs on gonadal differentiation and sex ratio have not yet been studied in depth. In previous studies, similar feminizing effects of TCs on sex ratio have been observed in other fish species,

such as Senegalese sole (Blanco Vives et al., 2011) and zebrafish (Villamizar et al., 2012). With tilapia, the first study of the influence of daily TCs on phenotypic differentiation was performed in blue tilapia (*Oreochromis aureus*) by Baras et al. (2000). In this previous research work, TCs led to a slightly lower proportion of males compared to constant temperatures. In our study, TCs more considerably increased the proportion of females. This clearer feminizing response of TCs herein observed may be due to differences in the environmental response of the sex determination system among tilapia species (Prugirin et al., 1975). This could also be explained by the age of the larvae exposed to TCs. According to the research by Baras et al. (2000), larvae were exposed from 10 dpf. In our study, they were exposed from the first hours of fertilization when still in embryo stages. Thus our results could indicate that early development stages are also sensitive to temperature changes by producing irreversible effects on sexual determination and differentiation mechanisms. This hypothesis agrees with previous research performed in Nile tilapia, which describes an earlier thermal sensitivity window during embryogenesis from 12 hpf to hatching (Rougeot et al., 2008). In the present study we also observed an effect of rearing temperatures (TC *vs*. CTE) on the response to HT. In Nile tilapia, as in other fish species, exposure to high temperatures during the sexual differentiation period can induce profound effects on individuals' phenotype by shifting from females to functional phenotypic males (Baroiller et al., 2009). Although the effect of TCs in early developmental stages has been observed in the sexual differentiation of some fish, their influence on the female-male sex reversal process by high temperature has gone unnoticed. The present study shows, for the first time, that tilapia reared on the first days of life in daily TCs are less sensitive to the masculinizing effect of HT than those reared at constant temperature. Previous studies have suggested that the degree of sensitivity to sex reversal by high temperature (thermosensitivity) is considerably influenced by the rearing temperature regime during development (Baras et al., 2000; Baroiller and D'Cotta 2001; Tessema et al. 2006). For example, different Nile tilapia populations adapted to high or low temperature environments have shown greater or lesser variability, respectively, in their response to HT in sex ratio terms (Bezault, et al., 2007). According to our study results, further research is needed to investigate in depth the molecular mechanisms involved in the changes in thermosensitivity caused by implementing TCs during development.

Finally in the present research, the rearing temperature regimes implemented during development also impacted juveniles' sex steroid production. The females reared in TCs showed higher *cyp19a1a* expression and E_2 plasma levels, while the females reared in CTE had higher T levels. The males reared in CTE presented higher *amh* expression and plasma T levels than those reared in TCs. These results are similar to those found for Senegalese sole, in which the fish reared in TCs presented higher E_2 and *cyp19a1a* expression levels than those left at constant temperatures that, in turn, showed higher T levels (Blanco-Vives et al., 2011). However, that study was unable to differentiate according to individuals' gender. Our study observed that the T levels in the males and females from the different experimental groups presented a similar pattern, which coincides with the research carried out in other fish species (Borg, 1994; D'Cotta et al., 2001; Lokman et al., 2002). Our results also showed that the effect of HT on sex determination mechanisms in early developmental stages remained in the juvenile stage. From the first hours of fertilization, Nile tilapia presents sensitivity to temperature, and exposure to high temperatures can lead to the inhibition of the cyp19a1a enzyme (Baroiller et al. 1995, Li et al., 2014). This inhibition blocks the conversion of T into E_2 by lowering E_2 levels and, thus, inducing testicular differentiation (Baroiller et al., 2009; Mahmoud et al., 2020). So we hypothesize that TCs can reduce the suppressive effects of aromatase activity caused by HT during the sex differentiation period by limiting the female-to-male sex reversal process. Studies carried out on Nile tilapia have shown that the plasma level of sex steroids considerably impacts the animal's physiology and plays a role in the sexual dimorphism of growth by regulating the expression of growth factors (Yue at al., 2016). This could explain the results herein obtained because the females and males reared at constant temperature and exposed to HT had higher T levels and, thus, a heavier body weight than those reared in TCs and not exposed to HT.

CONCLUSIONS

To conclude, the present research demonstrates that implementing TCs from early development stages acts as a female-promoter inducing factor. TCs increase the expression of the genes involved in ovarian differentiation and, consequently, plasma E_2 levels and the proportion of females are higher. In addition, daily temperature cycles can reduce sensitivity to the masculinizing effect of HT by reducing the up-regulation of the genes involved in testicular differentiation and, thus, the plasma T levels and

proportion of males lower. These results suggest that the mechanisms involved in sensitivity to the female-to-male sex reversal process may be related to certain factors associated with individuals' heat tolerance. These findings should be considered when discussing the influence of environmental temperature on Nile tilapia sex determination and sexual differentiation.

DATA ACCESSIBILITY

The data that support the findings of this study are available from the corresponding author upon reasonable request.

COMPETING INTERESTS

The authors declare no competing or financial interests.

AUTHOR CONTRIBUTIONS

GA, JFLO and FJSV conceived and designed the experiments, and wrote the manuscript; GA and MC performed the experiments; GA and JFLO analyzed the data; FJSV, JFLO and MAE provided funding.

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SUPPLEMENTARY INFORMATION

Table A1. Statistic values obtained in the one- and two-way ANOVAs performed for all the variables measured in the experiments. The p values (*P*) and F-Statistic (F) are reported. Gray highlights the statistically significant results ($P < 0.05$).

	Variable			One-way ANOVA		Two-way ANOVA					
						Temperature regime		Heat treatment		Interaction	
				F	P	F	P	F	P	F	P
11 DPF	**Survival rate (%)**			0.517	0.499						
	Relative expression		*amh*	0.636	0.438						
			cyp19a1a	5.322	0.032						
			ara	5.685	0.028						
			era	8.489	0.008						
			sox9a	0.037	0.849						
			foxl2	5.264	0.033						
			dmrt1a	1.208	0.285						
24 DPF	**Survival rate (%)**			7.821	0.004	4.909	0.047	17.099	0.001	1.453	0.251
	Relative expression		*amh*	3.029	0.042	0.769	0.387	3.993	0.053	4.498	0.041
			cyp19a1a	5.316	0.004	2.776	0.105	7.597	0.009	2.609	0.116
			ara	3.234	0.034	0.760	0.389	4.212	0.048	6.300	0.017
			era	3.433	0.028	5.108	0.031	3.419	0.073	0.254	0.618
			foxl2	3.278	0.032	5.349	0.027	3.610	0.066	0.196	0.661
			sox9a	3.912	0.017	5.229	0.029	1.607	0.213	5.691	0.023
			dmrt1a	2.952	0.047	3.618	0.066	4.339	0.044	0.998	0.325
JUVENILES	**Sex ratio (%)**			8.209	0.003	15.228	0.002	9.370	0.010	0.030	0.864
	Weight		Male	5.047	0.002	13.668	0.000	1.080	0.300	1.868	0.173
			Female	10.118	0.000	24.647	0.000	8.143	0.005	0.685	0.409
	Sex steroids	T	Male	5.704	0.000	14.895	0.000	2.920	0.089	0.234	0.629
			Female	5.376	0.002	13.832	0.001	1.704	0.195	0.075	0.785
		E_2	Male	1.053	0.371	0.017	0.898	2.240	0.136	1.518	0.220
			Female	3.458	0.019	3.120	0.080	5.520	0.020	0.311	0.578
	Relative expression		*amh*	3.149	0.039	6.659	0.015	3.414	0.074	0.242	0.626
			cyp19a1a	3.860	0.017	7.487	0.009	3.008	0.091	0.692	0.411

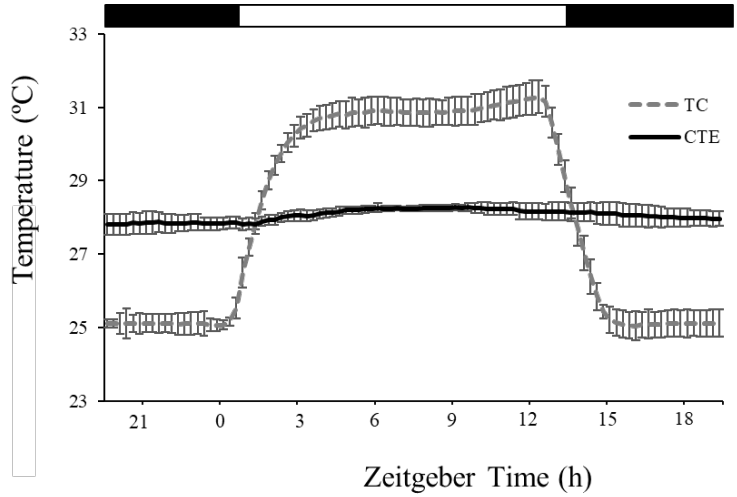

Fig. A1 Daily average water temperature (mean±S.D.) throughout the experiments in the two temperature regimes herein tested: a thermocycle (TC) of 31ºC:25ºC (dashed line) or a constant temperature (CTE) of 28ºC (continuous line). The time scale is expressed as Zeitgeber Time (ZT), where ZT0 h corresponds to light onset.

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Experimental Chapter IV

Effect of period of day of thermal treatment on the thermal tolerance and sex differentiation of Nile tilapia (*Oreochromis niloticus*).

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ABSTRACT

The aim of the present research was to determine the influence of the period of day of heat treatment on sexual differentiation and thermal tolerance in Nile tilapia (*Oreochromis niloticus*). To this end, larvae were subjected to a thermal treatment (HT, 36ºC for 12 days) at different time of day during the thermosensitive period (11-23dpf): light period (L-HT); dark period (D-HT); both light and dark period (LD-HT); and another group not exposed to heat treatment but maintained at rearing temperature (Control group, CTRL). At 25 dpf, thermal tolerance (survival) was measured and whole larvae samples were collected for mRNA expression analysis. Then, all groups were kept under constant temperature until 270 dpf when 100 fish from each group were randomly selected, anesthetized and weighted. The gonadal lobules of each individual were collected for histological and RNA expression analysis. At the same time, each experimental group were subjected to heat shock (HS; 36ºC for 2 hours) at ZT6 h (ML, middle of the light phase) or ZT18 h (MD, middle of the dark phase). In addition, at each time point, one control groups were not exposed to thermal stress (NS). When heat shock exposure finished, brains from juveniles were extracted to determine the heat shock response by mRNA analysis. We studied the expression of genes involved in female (*cyp19a1a*, *foxl2*, *Era*) and male (*amh*, A*ra, sox9a, dmrt1a*) sexual differentiation and thermal tolerance (*hsp70*, *hsp90a*, *hsp27*) in 25 dpf larvae and juveniles. The results revealed that all HT-treated larvae presented an upregulation of testicular differentiation gene expression and inhibition of ovarian differentiation gene expression. In addition, the time of the day of the thermal treatment during development influenced the thermotolerance at larval and juvenile stage with the lowest survival and highest cellular stress (higher expression of HSPs) in the larvae of the D-HT and LD-HT groups and different time-dependent cellular responses at the juvenile brain level. These findings reveal the need to further investigate the link between sex differentiation and thermotolerance as well as to consider time-dependent responses in thermal biology research. In addition, these results may be useful to optimize masculinization protocols in the tilapia industry that guarantee animal welfare and minimize the negative effects of thermal treatment on the physiology of the fish.

Key Words: *Oreochromis niloticus* · Heat treatment· Heat Shock · Sex differentiation · Time of day

INTRODUCTION

In fish as in other poikilothermic animals, temperature seems to be the most prevalent environmental factor which have a significant impact on biological activity and vital processes such as reproduction, development and growth through which living beings perpetuate themselves (Angiletta et al. 2010). The success of reproduction is to produce a new offspring thanks to the establishment and interaction of the reproductive systems of the breeders (Zohar et al., 2010). With regards to sex determination, although sex is mainly determined by genotypic characters (Genotypic Sex Determination, GSD), other environmental factors such as temperature exert a strong influence on sex determination (Environmental Sex Determination; ESD) (Baroiller et al., 2009). In the case of Nile tilapia (*Oreochromis niloticus*), the second worldwide farmed fish species (FAO, 2020), the effect of ambient temperature (Temperature Sex Determination, TSD) on its phenotypic sex has been thoroughly researched in order to optimize the efficiency of hyper-intensive systems of tilapia farming by establishing all-male populations (Baroiller et al., 2009). These male-monosex populations have productive advantages such us lower heterogeneity in weight, absence of reproductive behaviour and the faster reach of a marketable size (Baroiller and D' Cotta, 2018). In countries where hormonal treatments for fish are banned, most techniques for controlling the Nile tilapia sex ratio are based on the application of temperature protocols during temperature-sensitive periods of sexual differentiation (especially during the early stages of development) with different effects depending on the temperature range applied (Baroiller et al., 1995; 2009, Baras et al., 2001). For example, exposure to high temperatures can cause masculinization of progeny while cold temperatures (21.5 °C-25.5) induce an increase in the proportion of females (Lazaro-Velasco et al., 2019; Baroiller et al., 1995; Desprez & Mélard, 1998; Abucay et al., 1999). However, the application of these thermal methods affects larval survival, compromising the efficiency of the standards for their large-scale culture and may negatively influence reproductive and thermal biology, as well as the animal welfare of the fish (Angienda et al., 2010; Jin et al., 2019; Mahmoud et al., 2020; Pandit and Nakamura, 2010).

From the first days of larval development, the ovarian differentiation in Nile tilapia is encoded mainly by *cyp19a1a* (enzyme aromatase), which catalyzes the conversion of T to E_2 (Kwon et al., 2000; Zhou et al., 2021). The *cyp19a1a* expression continues to increase exponentially as the ovary progresses (Baroiller et al., 2009; Wang et al., 2016). At the same time, the Ers (estrogen receptors) and foxl2 (Forkhead Box L2) participate as inducers of *cyp19a* expression since its deficiency causes a decrease in aromatase leading to masculinization of the fish (Li et al. 2014; Wang et al., 2005, 2007). On the contrary, the Nile tilapia testicular differentiation is encoded by Dmrt1 (Doublesex and mab-3 related transcription factor 1) whose expression appeared from early stages of development (from 8 days post fertilization, dpf) (Baroiller et al., 2009). Its role lies in the inducing effect of sox9 (SRY-Box Transcription Factor 9) which stimulates the induction of amh (anti-mullerian hormone) and consequently the inhibition of cyp19a (Eshel et al., 2014; Wei et al., 2019; Wang et al., 2016). Furthermore, according to in vitro studies, dmrt1 is also able to induce the activation of gsdf (Gonadal soma-derived factor), which is responsible for suppressing aromatase activity (Eshel et al., 2014). The expression of both *dmrt1* and *amh*, begins to increase in parallel from day 10 dpf, finding the highest values in testis XY at day 20-25 dpf (Eshel et al., 2014; Liu et al., 2022; Wang et al., 2022). Thus, the increase in the expression of *dmrt1* will lead to the suppression of aromatase activity and consequently to a decrease in E_2 levels and an increase in androgens (Devlin and Nagahama, 2002). These androgens will act on target cells through androgen receptors (Ara) promoting testicular differentiation (Devlin and Nagahama, 2002; Wang et al., 2019). Environmental temperature influences both the dynamics and the synthesis of sexual differentiation mechanisms (Baroiller et al., 2009). Thus, exposure to high temperatures during the critical sexual differentiation period (9 to 25 dpf) induces the expression of testicular differentiation factors and inhibition of ovarian development factors, triggering an increase in androgen levels and a decrease in estrogen levels, increasing the proportion of males (Devlin and Nagahama, 2002; D' Cotta et al., 2008; Li et al., 2014).

When fish are faced with acute temperature changes derived from exposure to thermal treatments, a stress response is activated in the fish and certain mechanisms necessary to maintain the individual's internal homeostasis and recover allostasis appear in order to prevent cell damage (Basu et al., 2002). In fish, the of heat shock proteins

(HSPs) as HSP90a, HSP27 and HSP70 play a crucial role in maintaining the functional structure of proteins under thermal stress (Demeke and Tassew, 2016). HSP expression patterns are influenced by thermal acclimation as has been observed in several fish species, including killifish (*Fundulus heteroclitus*) and redband trout (*Oncorhynchus mykiss*) (Fangue et al., 2006; Narum et al., 2013). In addition, it has been studied that the influence of thermal conditions during development can modify the thermal tolerance response in the adult phenotype (Schaefer and Ryan, 2006). However, the influence of HSPs on the temperature-sex determination of fish has been little investigated (Li et al., 2014; Wang et al., 2019, 2022). In general, high temperature treatments for masculinization of Nile Tilapia induce an increase in the expression of HSPs in testis and ovary (Li et al, 2014). However, the increase in the gonadal expression of HSPs may present sexual dimorphism with higher values of HSP27 in males and HSP70a in females, as it has been observed in the American Alligator (*Alligator mississippiensis*) (Khono et al., 2010). Although these studies are very scarce, the important role that thermotolerance and HSPs might play in TSD should be investigated.

The mechanisms of sexual differentiation (Di Rosa et al., 2016; Villamizar et al., 2012) as well as of HSPs (Fadder et al., 1994; De Alba et al., 2022) present daily and seasonal variations synchronized with the light-dark cycle, which influence both the time-dependent physiological responses of the fish, in relation to their reproduction and thermotolerance. For example, the response of cortisol to acute stress is time dependent, being higher if the stress stimulus occurs during the activity phase (Kynard et al., 2005) or at resting phase (López-Olmeda et al., 2013; Vera et al., 2009). In addition, other investigations have shown these day time-dependent effects in hormones involved in reproduction such as GnRH analogs (Rasines et al., 2013). However, very few studies to date have evaluated the influence of the time of day of heat treatment on thermotolerance and sex differentiation in Nile tilapia. Assuming the close relationship between thermotolerance and sexual differentiation mechanisms, the aim of the present article was to determine the influence of the time of day of heat treatment on sexual differentiation and thermal tolerance in Nile tilapia.

MATERIAL AND METHODS

The present research was conducted at the facilities of the Department of Physiology of the University of Murcia. All the Nile tilapia husbandry and experimental procedures followed were approved by the European Union's directives (2010/63/EU) and Spanish law (RD 1386/2018 and Law 32/2007). The experimental protocols were approved by the University of Murcia's Ethics and Animal Welfare Committees.

Animals and housing

A commercial aquaculture company (Fishgen, Swansea, U.K.) supplied juvenile male and female Nile tilapia (1-month-old). Fish were kept in a recirculation system connected to individual tanks of 300 l with aeration system, biological and mechanical filters. Water temperature was kept at 28±0.5ºC by using a water heater and a cooler (AB Aqua Medic, Gewerbepark, Germany). The water quality (pH, ammonia, nitrate, nitrite, and dissolved oxygen) was assessed weekly. The light-dark cycle (LD) photoperiod was set at 12:12 h, with lights on at 9:00 h and off at 21:00h, respectively. Fish were fed with three daily meals (at 11:00 a.m., 15:00 p.m., and 19:00 p.m.) at a feeding rate of 2% of the daily biomass (D-4 Alterna Basic 2P, Skretting, Spain).

For eight months, juveniles were raised in the previous environment until they reached maturity. Fertilized eggs with less than 12 hours after fertilization were collected by the method described in Espirito Santo et al., (2020).

Experimental design

Experiment 1: Effect of the period of the day in which the heat treatment is applied on the sex determination and sexual differentiation of 24 dpf and juvenile Nile tilapia.

In the first experiment, the effect of the period of day and heat treatment was evaluated in the Nile tilapia larvae. In order to do this, fertilized eggs were collected from six different tilapia broodstocks. A total of 600 fertilized eggs (N) from 3

progenies were used in the experiments. Each progeny was compound of 200 fertilized eggs from a spawning event in which the broodstocks were stimulated as previously mentioned. Then, eggs from each progeny were pooled and distributed in incubators for Cichlid eggs (Alimar SA, Murcia, Spain) (50 eggs per incubator) in a system with constant temperature of 28±0.5ºC. Embryos were kept in the incubators until 7dpf, moment in which they were transferred to 9-liter tanks placed in the same temperature system. At the 7 dpf, larvae started with the exogenous feeding until satiety (Gemma, 42% CP, Skretting, Spain) by establishing 4 daily meals (at 9:00h, 11:00h, 15:00h and 19:00h). At 11 dpf, larvae were divided into 4 experimental groups and subjected to a heat treatment (HT) during different period of the day (light phase, L-HT; dark phase, D-HT; light and dark phases LD-HT) or maintained at the same rearing temperature (28±0.5ºC, Control group, C) from 11 to 24 dpf (Fig. 1).

The heat treatment consisted of 12 days of exposure at 36ºC according to the treatments used for tilapia masculinization (Baroiller et al., 1995). The water temperatures changes were performed by the use of electronic heaters (Askoll, Povolaro, Italy) and water coolers (Aqua Medic Titan 1500 GmbH, Bissendorf, Germany). In addition, an underwater data logger (HOBO PENDANT Onset Computer Corporation, Massachusetts, USA) was used to measure the water temperature changes every 15 min to ensure that the temperature changes into the thermal treatments were carried out correctly. Before the first feeding point, complete larval samples were taken at 25 dpf (one day after the end of thermal treatment) for mRNA expression analysis of genes involved in Nile tilapia sex determination and differentiation. For the studies of gene expression, larvae were immediately euthanized by anesthetic overdose and death confirmed under the microscope according to the guidelines established for fish euthanasia. Then, one larva was taken for each replicate obtaining 2 replicates for each group/progeny giving a total of 6 replicates (n=6) during the 3 independent progenies. Larvae were stored in 1.5 ml sterile tubes and immediately frozen at -80ºC until gene expression analysis.

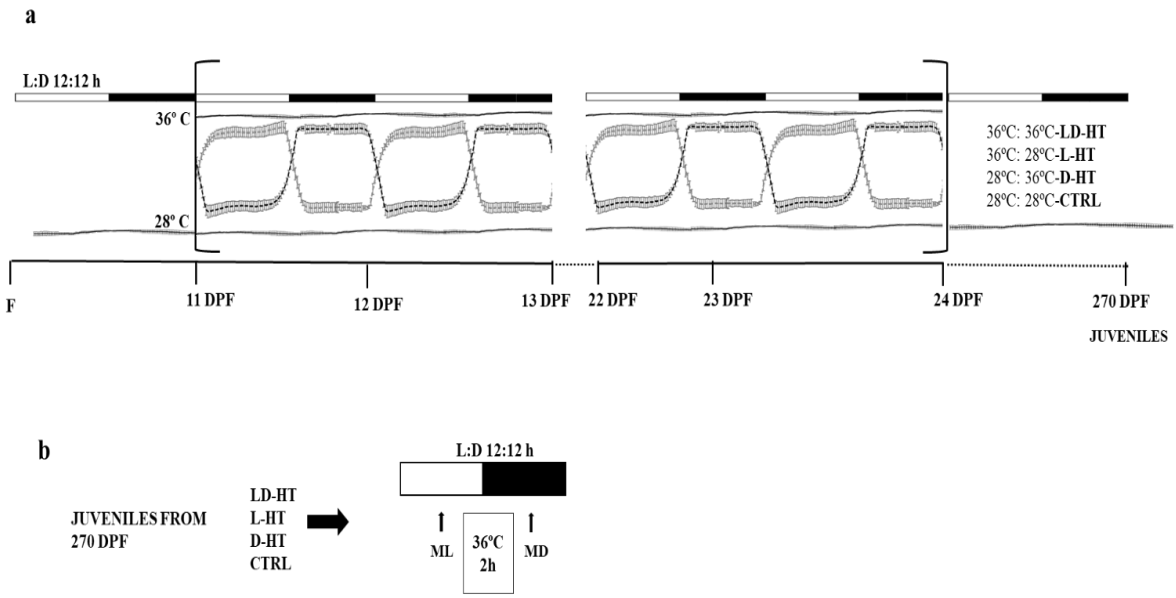

Fig. 1 Schematic representation of the experimental design. Experiment 1 (**a)** Fertilized eggs in stage 1 (F) were kept at constant temperature until 11 dpf, moment in which they were divided into 4 experimental groups and subjected to a heat treatment (HT, 36ºC for 12 days) during different period of the day (light phase, L-HT; dark phase, D-HT; light and dark phases LD-HT) or maintained at the same rearing temperature (28±0.5ºC, Control group, CTRL) from 11 to 24 dpf. Then, all groups were kept under constant temperature until 270 dpf. Experiment 2 (b): Nile tilapia juveniles previously subjected to a heat treatment (HT, 36ºC for 12 days) during different period of the day (L-HT, D-HT; LD-HT) or maintained at the same rearing temperature (CTRL) from 11 to 24 dpf. Then, they were kept at constant temperatures until 270 dpf. At this moment, juveniles were exposed to a heat shock (36ºC for 2 h) at two different time points: middle of the light phase (ML); middle of the dark phase (MD). Brain samples were collected to perform the gene expression analysis.

After thermal treatment, all groups were maintained under constant temperature (28±0.5ºC) until 270 dpf. From each experimental group/progeny, 35 fish (100 fish/group) were randomly selected and anesthetized with a concentration of 50 µL/L of eugenol (clove oil essence, Guinama, Valencia, Spain). In the experimental groups of each progeny with less than 35 fish, all animals form the group were used. Total number of animals sampled from each group were 83 for LD-HT, 105 for L-HT, 85 for D-HT and 61 for C. After euthanasia by decapitation, one lobule of the gonads from each fish were collected and transferred to sterile RNase- and DNase-free 1.5 ml Eppendorf tubes and they were immediately frozen in dry ice and stored at -80ºC until mRNA expression analysis. To determine the sex of the individual, the other gonad lobule was collected and immersed in 10% formaldehyde fixative solution (Leica Biosystems, Spain) and stored until histological analyses.

Experiment 2: Effect of the period of the day in which the heat treatment is applied on the thermal tolerance of 24 dpf and juvenile Nile tilapia.

In the first part of the second experiment, the effect of the period of day and heat treatment on thermal tolerance was evaluated in the Nile tilapia larvae. Lighting conditions and heat temperature treatments were those described in Experiment 1. After thermal treatments, the survival rate to the different periods of heat treatment was calculated as the percentage of alive larvae at 25 dpf from the total number of larvae at 11 dpf. The experiment was performed in triplicate to obtain 3 independent replicates (n=3) for each experimental group and progeny.

Then, the second part of the experiment was designed to determine the effect of the time of day and acute heat shock on the resistance (HSPs) mechanisms in the Nile tilapia juveniles (N=144) previously submitted to heat treatments at different periods of day during larval development (from 11 to 24 dpf). To this end, at the same time the first experiment was running, Nile tilapia juveniles (270 dpf) from each experimental group were randomly selected and sampled prior to (control; Ctrl) or after (heat shocked; HS) a thermal shock at two different time points: in the middle of the light phase (ML, ZT6 h) or in the middle of the dark phase (MD, ZT18 h). Then, each experimental group contained the following four groups: fish subjected to heat shock at ZT6 h (ML, middle of the light phase); fish subjected to heat shock at ZT18 h (MD, middle of the dark phase); two control groups for each time point not exposed to heat shock, but maintained at constant temperature (28ºC).The heat treatment challenge consisted of subjecting fish for 2 h to a bath previously heated at 36ºC according to previous research on the acute thermal tolerance in Nile tilapia in order to induce significant effects on the expression of HSPs, avoiding mortality (Kwon and Kim, 2000). Lighting conditions were those described in Experiment 1. Immediately after thermal exposure, 3 fish/progeny per group (n=9) were anesthetized with a concentration of 50 µL/L of eugenol (clove oil essence, Guinama, Valencia, Spain) and euthanized by decapitation. Brain samples were collected and transferred to sterile RNase- and DNase-free 1.5 ml Eppendorf tubes. After being instantly frozen on dry ice, the tubes were kept at -80°C until the HSP gene expression in the brain was analyzed.

Histological analyses

Gonad samples were fixed in 10% formaldehyde solution for 24 h before transferring to 70% ethanol and embedding in paraplast (Leyca Bioystems, Spain) after dehydration. Sections of approximately 5 μm were cut and stained with hematoxylin and eosin (HE). The slides were examined under a light microscope (Axiolab, Zeiss, Oberkochen, Germany) equipped with a camera (Photometrics Coolsnap, Roper Scientific, Buckinghamshire, UK). Female and male sex was determined by the identification of oocytes or spermatocytes, respectively.

Real-time RT-PCR analysis

With the use of a tissue homogenizer (TissueLyser LT, Qiagen, Hilden, Germany), the larvae, gonad, and brain samples were homogenized in Trizol reagent (Ambion, Thermo Fisher Scientific). RNA was dissolved in DEPC water (Invitrogen, CA, USA) and RNA concentration and purity were determined by spectrometry (Nanodrop ND-1000, Thermo Fisher). Prior to the retrotranscription, RNA (1 g) was processed with 1 U of DNase I (Thermo Fisher) to remove any DNA genomic contamination. A thermocycler and the Reverse Transcriptase commercial kit (QSCRIPT cDNA Synthesis Kit, Quantabio, USA) were used to synthesize the cDNA. Prior to expression analysis, all of the cDNa samples were diluted (1:10) in nuclease-free water (Thermo Fisher) and kept at – 20 ºC until expression analysis. The quantitative PCR reactions were performed using Perfecta® SYBR® Green Fastmix (Quantabio) in a final volume of 20 μl for each qPCR reaction. All the samples were run in duplicate in a light thermocycler with the following steps: 15 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. The thermocycler performed out melting curves right away following the amplification stage to regulate the reaction's specificity and confirm that just one DNA species was amplified. Primer 3 Plus software was used to design the primers (Table 1). (Untergasser et al., 2012). Dilution curves were used to determine the primer concentration and to confirm that all primers exhibited relative amplification efficiencies between 90 and 110 % (Table 1). All primers were added at a final concentration of 500 nM. *βactin* were used as reference genes since its expression was stable and not affected by temperature (coefficient of variation lower than 5%) (Vanhauwaert et al., 2014). Then, the relative expression of all the genes was calculated by the 2–ΔΔCt method (Livak and Schmittgen, 2001).

Table 1 Primer sequences used for the quantitative PCR analyses.

Gene	F/R	Sequence (5'-3')	Ensembl/GenBank Accession number
amh	F	ACGACGCGCAAAGAAAACTG	ENSONIG00000004781
	R	TAATGTTTGCGCTGCTTGGG	
cyp19a1a	F	TTGCACAAAACCACGGTGAG	NM_001279586.1
	R	ACGTGCGGGTTTTGTTTGAG	
sox9a	F	GTGTTGAAGGGTTACGACTGGACG	XM_003450119.4
	R	CCGTTCTTGACAGACTTTCTCCGC	
Era	F	TATGTGCCCAGCGACAAATC	NM_001279770.1
	R	CGTTTTTCACGCCGCAAAAC	
dmrt1a	F	CGGATTGCAGCGGACCGA	XM_013270912.3
	R	GGACAGAGACACAGGACTAC	
foxl2	F	AGCATTTCACCGATCGAGAC	NM_001279778.1
	R	CATTGCGCACACACCAAAAC	
Ara	F	CAGCCCTATGTCCTTGCTTACCAG	XM_005467840.4
	R	CGCTGGTCATTGAAAATCAGGTCT	
hsp70	F	CCTGATCAAACGCAACACCA	FJ207463.1
	R	GTTGTCGGAGTAGGTGGTGA	
hsp90a	F	TTGGCAGAGGCACAAAAGTG	XM_003440645.5
	R	AGCCAATGAACTGTGAGTGC	
hsp27	F	AACTAATGACACCGCATGCC	NM_001279530.1
	R	TGTCTTGGGTCTGCTTGATCTC	
βactin	F	TGGTGGGTATGGGTCAGAAAG	ENSONIG00000008505
	R	CTGTTGGCTTTGGGGTTCA	

Data analysis

The results are expressed as mean ± SEM. The significance threshold was set at P= 0.05 for all the tests. To perform the statistical analyses, the SPSS software (v. 19.0, IBM, Armonk, NY, USA) was used. Data from 24 dpf larvae (survival rate, gene expression) and juveniles (sex ratio, gonadal gene expression) were subjected to a one-way ANOVA to observe the statistically significant differences between the experimental groups. Data from the brain gene expression of thermotolerance mechanisms (HSPs) in the Nile tilapia juveniles (previously exposed to different periods of day of heat treatments during larval development) submitted to a heat shock (Experiment 2) at two different time points were subjected to a three-way ANOVA to compare the main effects and the interaction effect of the following independent variables: time period when thermal treatment was applied (TP), heat shock (HS) and time of day (T) when the heat shock was applied (ML/MD). In case of a significant interaction between independent variables, three-way ANOVA was used followed by Duncan's *post hoc* test to detect the existence of significant differences within experimental groups ($P< 0.05$).

RESULTS

Experiment 1: Effect of the period of the day in which the heat treatment is applied on the sex determination and sexual differentiation of 24 dpf and juvenile Nile tilapia.

At 25 dpf, the expression of the analyzed genes involved in the sex determination and differentiation process of Nile tilapia showed significant differences (Fig. 2) (one-way ANOVA, P< 0.05; Table A1). The expression of *amh*, *sox9a*, *Ara* presented the highest expression in the LD-HT group while the lowest expression was observed in the CTRL group. However, no differences were found between different heat-treated groups (Fig. 2a, c, g) (one-way ANOVA, P> 0.05, Table A1). In addition, the expression of *dmrt1a* was highest in larvae from LD-HT group in compare to the expression observed in CTRL and L-HT group (Fig. 2e) (one-way ANOVA, P= 0.002, Table A1). On the contrary, the heat treatment decreased the expression of *cyp19a1a*, *Era* and *foxl2* and in all heat-treated groups, except in the D-HT group whose *cyp19a1a* expression did not present significant differences (Fig. 2 b, d, f) (one-way ANOVA, P< 0.05, Table A1).

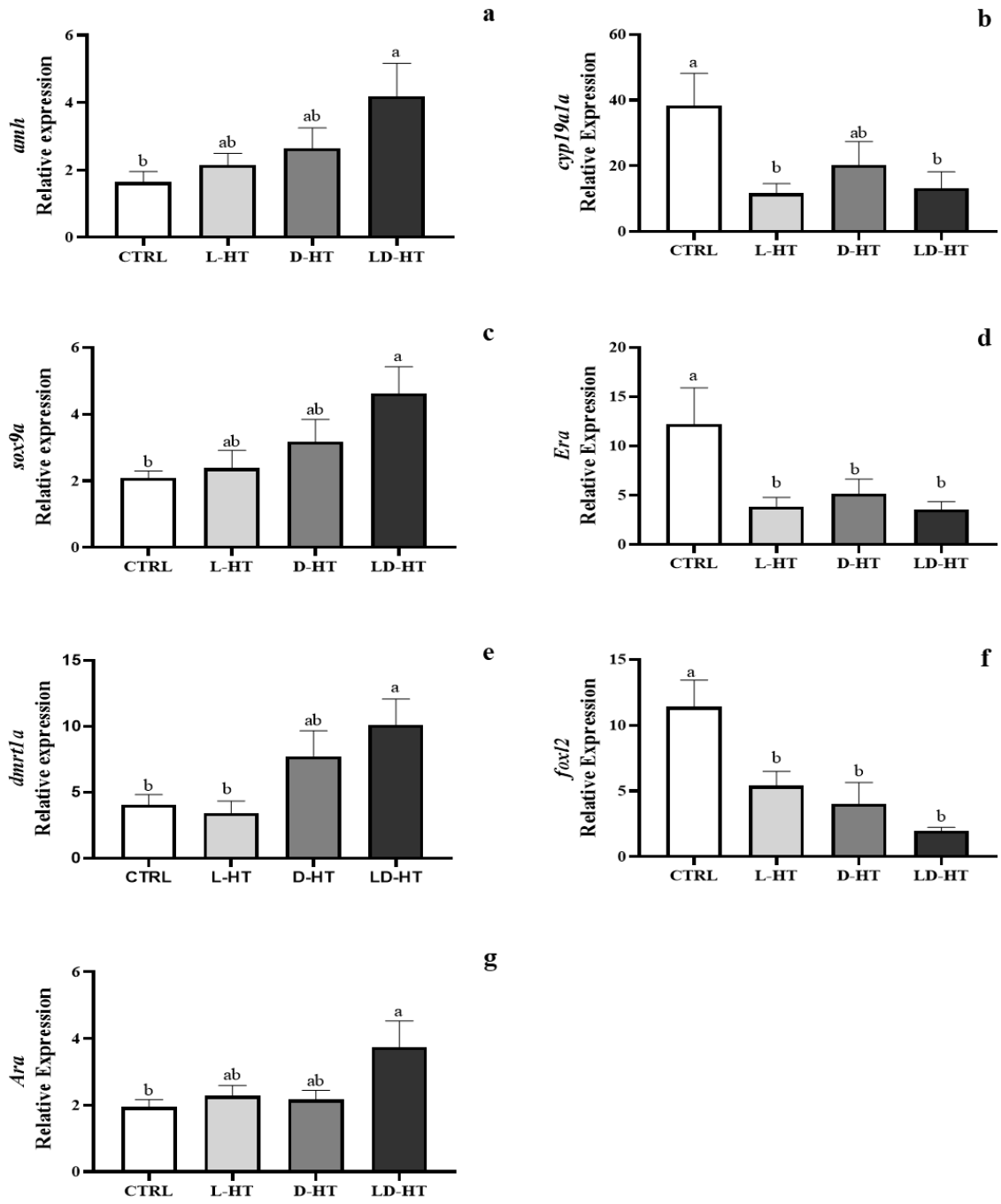

Fig. 2 Effect of different periods of heat treatment (36ºC for 12 days) on the relative mRNA expression of *amh* (a), *cyp19a1a* (b), *sox9* (c), *Era* (d), *dmrt1a* (e), *foxl2* (f) and *Ara* (g) of the 25 days post fertilization (dpf) Nile tilapia larvae. Larvae were subjected to a heat treatment (HT, 36ºC for 12 days) during different period of the day (light phase, L-HT; dark phase, D-HT; light and dark phases LD-HT) or maintained at the same rearing temperature (28±0.5ºC, Control group, CTRL) from 11 to 24 dpf. Different lower case letters indicate significant differences between the experimental groups (one-way ANOVA, $P< 0.05$). Data (n=6) are represented as mean±SEM.

At the juvenile stage, the sex ratio and gonadal relative expression were measured (Fig. 3). The statistical analysis detected significant differences in the sex ratio between experimental groups which were influenced by the applied heat

treatments (Fig. 3a) (one-way ANOVA, P= 0.016, Table A1). The highest number of males was observed in the LD-HT groups (73.5 ± 4.3%), followed by the D-HT (62.3 ± 4.6%), compared to the lowest proportion of males observed in the CTRL group (39.1 ± 9.1%). Furthermore, there were no significant differences in the percentage of males between the different heat-treated groups (one-way ANOVA, P> 0.05). Regarding the relative expression of key enzymes involved in the synthesis of sex steroids, the analysis also showed statistically significant differences in the mRNA relative expression of *amh* in the testis (Fig. 3b) (one-way ANOVA, P= 0.046, Table A1). The LD-HT group showed significantly higher *amh* expression than the CTRL and D-CT groups. Furthermore, no significant differences were observed between the LD-HT and D-HT groups (one-way ANOVA, P> 0.05). Regarding the ovarian expression of *cyp19a1a*, the statistical analysis did not detect any statistically significant differences between the different experimental groups (Fig. 3c) (one-way ANOVA, P> 0.05, Table A1).

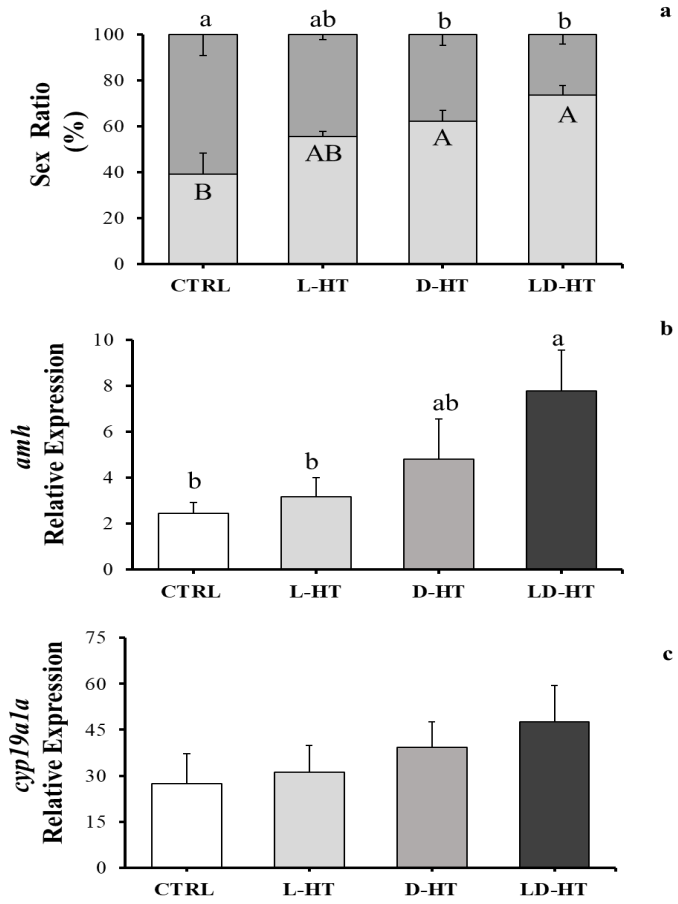

Fig. 3 Effect of different periods of heat treatment (36ºC for 12 days) on the sex ratio (a), testicular *amh* expression (b) and ovarian *cyp19a1a* expression (c) of the Nile tilapia juveniles previously subjected to a heat treatment (HT, 36ºC for 12 days) during different period of the day (light phase, L-HT; dark phase, D-HT; light and dark phases LD-HT) or maintained at the same rearing temperature (28±0.5ºC, Control group, CTRL) from 11 to 24 dpf. Different lower and upper case letters indicate significant differences between the experimental groups of males (light bars) and females (dark bars), respectively (one-way ANOVA, P< 0.05). Data (n=3) are represented as mean±SEM.

Experiment 2: Effect of the period of the day in which the heat treatment is applied on the thermal tolerance of 24 dpf and juvenile Nile tilapia.

The survival rate of Nile tilapia larvae was significantly influenced by the different heat treatments applied (Fig. 4) (one-way ANOVA, P= 0.012, Table A1). The highest survival rate was observed in the CTRL group (92.2 ± 0.7%) compared to the D-HT and LD-HT groups, which presented the lowest survival rate (53.7 ± 11.7% and 70.8 ± 1.0 %, respectively). In addition, the period of the day in which the heat treatment was applied influenced larval survival, with higher thermotolerance of larvae exposed to heat treatment during the light phase than those exposed during the dark phase (one-way ANOVA, P< 0.05, Table A1). However, the statistical analysis did not observe statistically significant differences between the CTRL and L-HT groups, nor between the LD-HT and D-HT groups (one-way ANOVA, P> 0.05, Table A1).

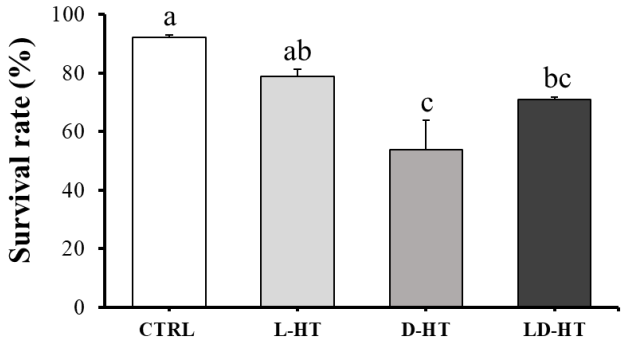

Fig. 4 Effect of different periods of heat treatment (36ºC for 12 days) on the survival rate in the 25 dpf Nile tilapia larvae subjected to a heat treatment (HT, 36ºC for 12 days) during different period of the day (light phase, L-HT; dark phase, D-HT; light and dark phases LD-HT) or maintained at the same rearing temperature (28±0.5ºC, Control group, CTRL) from 11 to 24 dpf. Different lower case letters indicate significant differences between the experimental groups (one-way ANOVA, P< 0.05). Data (n=3) are represented as mean±SEM.

With regards to the HSP genes analyzed in Nile tilapia larvae, the analysis pointed to striking statistical differences between experimental groups in the gene expression of *hsp70*, *hsp90a* and *hspb1* with the highest expression observed in the LD-HT group (Fig. 5) (one-way ANOVA, P< 0.05, Table A1). However, no differences were detected between CTRL and L- and D-HT (one-way ANOVA, P> 0.05, Table A1).

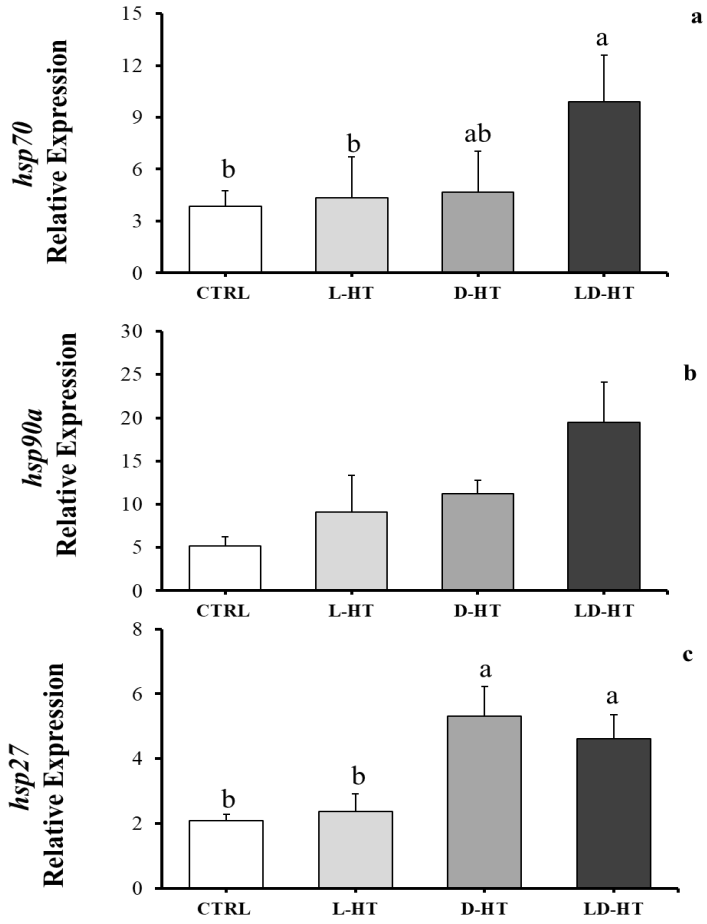

Fig. 5 Effect of different periods of heat treatment (36ºC for 12 days) on the relative mRNA expression of *hsp70* (a), *hsp90* (b) and, *hsp27* (c) of 25 dpf Nile tilapia larvae subjected to a heat treatment (HT, 36ºC for 12 days) during different period of the day (light phase, L-HT; dark phase, D-HT; light and dark phases LD-HT) or maintained at the same rearing temperature (28±0.5ºC, Control group, CTRL) from 11 to 24 dpf. Different lower case letters indicate significant differences between the experimental groups (one-way ANOVA, P< 0.05). Data (n=3) are represented as mean±SEM.

At juvenile stage, the results showed marked differences in the response of fish (previously exposed to different period of thermal treatment during early development) to a thermal shock (36ºC for 2 h) at two different time of day: ML (ZT 6h) and MD (ZT 18h) in the HSPs analyzed (Fig. 6) (three-way ANOVA, P< 0.001, Table A1). The three-way ANOVA showed that the expression of all HSPs were influenced by period of time of heat treatment (P< 0.005), heat shock (P< 0.001) and time of day (P< 0.05) with a significant interaction between both factors (P< 0.005 for PT*HS, P< 0.05 for T*HS) except for *hsp90a* expression whose expression was not influenced by the time of day and the interaction between time of day and heat shock (three-way ANOVA, P> 0.05, Table A1). With regards to *hsp70* expression, the CTRL and D-HT groups presented the highest levels of *hsp70* expression (three-way ANOVA, P< 0.05). In addition, higher *hsp70* expression in the L-HT and LD-HT groups was observed at MD

than at ML (three-way ANOVA, P< 0.05) (Fig. 6a). When fish were subjected to thermal shock at ML, the highest *hsp70* expression were found in the CTRL group while the lowest was found in the L-HT group, followed by the LD-HT group. However, when juveniles were exposed to heat shock at MD, the lowest *hsp70* expression was observed in the L-HT group (three-way ANOVA, P< 0.05) (Fig. 6a). The highest *hsp90a* expression was found in the CTRL group. Previous thermal treatments during the larval stage decreased the brain expression of *hsp90a* in juveniles after heat shock in ML with maximum values in the CTRL group. Similarly, the lowest expression of *hsp90a* in MD challenge was observed in L-HT and LD-HT (Fig. 6b and Table A1). Furthermore, the expression of *hsp90a* in the D-HT group was higher in MD than in ML (three-way ANOVA, P< 0.05) (Fig. 6b). Both in heat shock in ML and MD, the highest expression of *hsp27* was found in the D-HT group followed by CTRL group while the lowest was found in LD- and L-HT groups (three-way ANOVA, P > 0.05) (Fig. 6c and Table A1). In addition, all groups except CTRL group presented higher expression at MD than in ML (three-way ANOVA, P < 0.05) (Fig. 6c and Table A1).

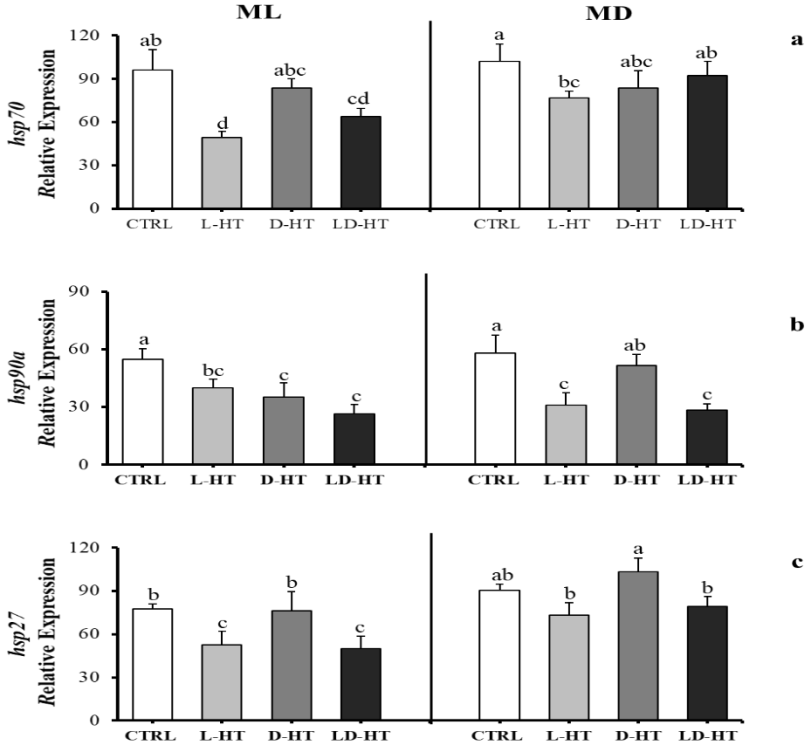

Fig. 6 Brain relative expression of the HSP genes: *hsp70* (a), *hsp90a* (b) and, *hsp27* (c). Fish were previously subjected to a heat treatment (HT, 36ºC for 12 days) during different period of the day (light phase, L-HT; dark phase, D-HT; light and dark phases LD-HT) or maintained at the same rearing temperature (Control group, CTRL) from 11 to 24 dpf. Then, they were kept at constant temperatures until 270 dpf. At this moment, juveniles were exposed to a heat shock (36ºC; 2 h) at two different time points: middle of the light phase (ML, white bars); middle of the dark phase (MD, dark bars). Different letters indicate significant differences between experimental groups (three-way ANOVA, P< 0.05).

DISCUSSION

The results of the present research revealed that both sexual differentiation and thermotolerance were influenced by the period of the day in which the thermal treatment was applied. The expression of the genes involved in testicular differentiation was upregulated in the LD-HT group, without showing significant differences with the expression of the other heat-treated groups (L-HT and D-HT). On the contrary, the expression of the genes involved in ovarian differentiation decreased in the different thermal treatments applied. These profound effects in the expression of sexual differentiation genes of larvae were consistent with the results obtained in the sex ratio and in the gonadal expression of juvenile tilapia, where highest male ratios and tesis *amh* expression were found in the D-HT and LD-HT. On the other hand, the period of the day of the thermal treatment influenced larval survival with lower survival and higher cellular stress (higher expression of HSPs) in the larvae from D-HT and LD-HT groups. In addition, exposure to different thermal treatments previously applied during the larval stage affected the thermotolerance of tilapia juveniles with different time-dependent cellular responses at the brain level.

Although the sex of Nile tilapia is determined by genotypic factors before fertilization, temperature is a relevant factor in sexual differentiation, which modifies the transcriptional expression of genes related to gonadal development (Baroiller et al., 2009). In general, treatments with temperatures above the thermal optimum during thermosensitive periods (9-25 dpf) of Nile tilapia produce a masculinizing effect caused by changes in sexual differentiation genes: upregulation of genes involved in testicular development and inhibition of genes related to ovarian differentiation (Ospina-Álvarez and Piferrer, 2008; Li et al., 2014; Baroiller et al., 2009). Our results showed that, applying the thermal treatment during a single phase of the light-dark cycle (light or dark phase), the expression of the mechanisms of sexual differentiation was modified with changes similar to those observed in the group that received the treatment during both phases of the day. The expression of the testicular differentiation genes increased in the LD-HT group, without significant differences with the other treated groups (L- and D-HT), except for *dmrt1*, whose expression was not less induced in L-HT group. On the contrary, the expression of the ovarian differentiation genes (*cyp19a1a*, *foxl2* and *era*) decreased in all heat-treated groups, except for *cyp19a1a*, whose sensitivity was higher during the light phase treatment, presenting a greater inhibition in its

expression. On the one hand, these differences in the sensitivity of the mechanisms involved in testicular and ovarian differentiation to thermal treatments during different periods of the day could be related to the daily rhythms of the components of the Hypothalamus-Pituitary-Gonadal (HPG) reproductive axis (De Alba et al., 2019; Cowan et al, 2017). According to studies carried out in zebrafish, the expression of aromatase (*cyp19a*) and antimüllerian hormone (*amh*) are rhythmic, with opposite acrophases: *cyp19a* presents its acrophase in the middle of the light phase, while *amh* is expressed mainly in the middle of the dark phase (Di Rosa et al., 2016). In the case of adult tilapia, it was observed that the expression of *cyp19a1a* and plasma estradiol (E_2) reached their maximum values in the first hours of light while the expression of *amh* and plasma testosterone (T) increased their values during the night (de Alba et al., 2019). Although the daily variations of the factors of the HPG axis in zebrafish and Nile tilapia are similarly influenced by the light-dark cycle, it would be necessary to carry out studies focused on the daily expression rhythms of genes responsible for sexual differentiation in tilapia larvae to check the daily pattern of masculinizing and feminizing genes during this period of development. In this way, the results that we observed in this article could indicate that circadian rhythms are capable of driving daily changes in the sensitivity of tissues to hormones, modifying the daily sensitivity of the components of the sexual differentiation pathway depending on the expression levels, being more sensitive to their inhibition/stimulation at the time that higher levels of gene expression were present (Sen and Hoffman, 2020). Thus, the male differentiation genes, which have maximum expression at night, could be more stimulated by nocturnal thermal treatments while the ovarian differentiation genes which present higher expression during the day would be more sensitive to their inhibition by diurnal thermal treatment. However, more studies are needed to test this hypothesis.

The expression changes in the mechanisms involved in male and female sexual differentiation will enhance the sex steroids synthesis and the gonadal development, favouring the establishment of the individual's sex (Baroiller et al., 2009). Thus, the upregulation of masculinization genes and inhibition of feminizing genes that we observed in our results due to heat treatments may have repercussions on the increase in androgen synthesis and the decrease in oestrogen synthesis, which influence considerably the gonadal development (Devlin and Nagahama, 2002). Sexual differentiation and gonadal development in Nile tilapia depend on the balance between

androgens and estrogens: higher levels of T and 11-KT induce masculinization by increasing the proportion of males while higher levels of E_2 induce feminization of the individual by increasing the female proportion (Devlin and Nagahama, 2002). This balance can be modified by exposure to high temperatures, mainly inhibiting aromatase, which catalyzes the conversion of T to E_2 during steroidogenesis (Zhou et al., 2021). In this way, most of the fish exposed to high temperatures would present higher levels of androgens and, consequently, a greater differentiation to males (Dussenne et al., 2020; Zhou et al., 2021). Although the sexual steroids levels have not been evaluated in the present research, numerous studies carried out in Nile tilapia obtained similar results on the effect of high temperature treatments on the sex ratio, varying the proportion of males after treatment from 60% to 100% (Baroiller et al., 1995; Abucay et al., 1999; Zhao et al. 2019; Tessema et al., 2006; Rougeout et al., 2008; Méndez Alboraleda, 2013; Wang et al., 2022). However, very few studies have considered that the masculinizing effect on the sex ratio could be influenced by the period of the day in which the heat treatment is applied. In our results, the thermal treatments applied at different periods of the day increased the proportion of males, although more considerably during the night phase or during both day phases (D-HT and LD-HT groups). This fact is consistent with previous research where it was observed that the proportion of males and androgen levels in Senegalese sole (*Solea senegalensis*) was higher in animals kept during their development under high temperatures (22ºC) during the dark phase and lower (19 °C) during the light phase, compared to the constant temperature group (20.5ºC). On the contrary, they observed a lower proportion of males in the group that received higher temperatures during the light phase and lower temperatures during the dark phase, which also coincides with our results (Blanco-Vives et al., 2011). However, the effect of heat treatments at different periods of the day on the sexual ratio seems to depend on the fish species and its sexual determination system, since other investigations in zebrafish or tilapia Aurea (*Oreochromis aureus*) showed that animals that receive higher temperatures in different phases of the day have a lower proportion of males than fish exposed to constant temperatures (Baras et al. 2000; Villamizar et al., 2012). Likewise, we observed that the LD-HT group presented the highest proportion of males in accordance with other research performed in Nile tilapia that highlights that the effect of heat treatment on the proportion of males was proportional to the temperature increase and the application period (Baroiller et al., 1995; Khater et al., 2017; Panda et al., 2022). These results highlight the phenotypic

plasticity of the tilapia sex determination and differentiation system, as well as the daily variation in sensitivity of these systems in their response to temperature.

The thermal treatments not only influence the sexual differentiation of fish, but the latest research focuses on knowing their effects on thermotolerance as well as the link between the thermotolerance and sexual differentiation mechanisms (Li et al., 2014; Tao et al., 2013; Wang et al.; 2019). Several researches showed that larval survival in Nile tilapia decreased significantly with high temperature exposures (Baroiller et al., 1995; Baras et al., 2001; Rougeot et al., 2008; Soltan et al., 2013; Villafuerte, 2014; Pandit and Nakamura., 2010). However, very few studies show the influence of the phase of the light-dark cycle in which the heat treatment is applied on larval survival. In our study, the lower survival was observed in the larvae of the D-HT group (53.8%) followed by LD-HT (70.9%) compared to the larvae from L-HT (78.9%) and CTRL (92.3%) groups which presented the highest survival. The higher thermotolerance to heat stress during the day than at night has also been described in other fish species such as zebrafish and killifish (De Alba et al., 2022; Healy and Schulte, 2012). On the one hand, this time-dependent response could be due to the different activity patterns between the fish species, presenting a greater thermotolerance at times of higher locomotor activity. According to this hypothesis, diurnal active fish such as zebrafish (Hurd et al., 1998), killifish (Žák et al., 2019), and Nile tilapia (Fortes-Silva et al., 2010; Guerra-Santos et al. al., 2017) would be more thermotolerant during the light phase than during the night. On the other hand, this response could be explained by the temperatures that ectotherms animals such as fish experience in nature, with higher temperatures during the day and cooler at night. Although more research is needed to know the cause of this greater thermotolerance, recent studies related it to the daily rhythms of HSPs expression (De Alba et al., 2021).

Under non-stressful situations, the expression of HSPs is present at basal levels while, under heat stress situations, its concentration increases to cope with cellular stress (mainly at the protein level) caused by the increase in temperature (Basu et al., 2002). Although the levels of HSPs are mainly influenced by the acclimatization temperature and the thermal history of the fish, several studies showed that thermotolerance correlated with the basal levels of HSPs, which presented daily rhythms in their expression that in turn influenced the induction of HSPs expression (Basu et al., 2002, Feder and Hoffman, 1999; De Alba et al., 2021). Thus, at the time of

day when the fish present higher levels of basal expression of HSPs, they will present a lower induction of HSPs under thermal stress and, therefore, less cellular stress and greater thermotolerance and survival. On the contrary, when the basal levels of HSPs expression are lower, the fish could present a greater induction of the expression of HSPs, consequently, greater cellular stress and mortality (De Alba et al., 2021). Our larval survival results agree with the levels of HSPs expression in the larvae, in which the LD-HT group presented higher expression (cellular stress) and therefore higher mortality. However, the effect of temperature fluctuation between light and dark phases on survival against heat stress should not go unnoticed, since recent studies have shown that fish living in fluctuating temperatures have greater thermotolerance as described in tidepool sculpin (*Oligocottus maculosus*) (Nanako and Iwama 2002, Todgham et al., 2006) and zebrafish (Schaefer and Ryan 2006, Xia et al., 2016; Wang and Xia, 2019) or with less significant effect on thermotolerance in other species such as killifish (Healy and Schulte, 2012), Atlantic salmon (*Salmo salar*) (Corey et al., 2017), and green sturgeon (*Acipenser mediostris*) (Rodgers et al., 2018). According to the previous research, the greater thermotolerance of zebrafish and Nile tilapia during day were related to higher basal levels of HSPs and, therefore, a lower HSPs expression induction under thermal stress (De Alba et al., 2021).

The effects of heat treatment during the sensitive period of early development of fish, can induce epigenetic and irreversible effects in the response of adults and offspring to an acute change in temperature (Schaefer and Ryan, 2006; Podrabsky and Somero, 2004; Fischer et al. al., 2012). In the present article, we observed that the different thermal treatments applied previously during the stage of sexual differentiation modified the response of HSPs to a subsequent thermal shock in juvenile tilapia. The fish not exposed to temperature variations during their larval stage (CTRL group) presented the highest levels of induction of HSPs expression after the heat shock. Meanwhile, the juveniles that were exposed to high temperature treatments during their larval stage, presented lower levels of induction of HSPs expression. These results coincide with what was observed in zebrafish, where the fish maintained at constant temperatures during their development presented the highest values of induction of brain HSPs expression under thermal stress (De Alba et al., 2021). In all experimental groups, a greater induction of HSPs was observed in juveniles exposed at night, coinciding with the greater sensitivity during the dark period observed in other species

such as zebrafish (De Alba et al., 2021). However, it was shown for the first time in fish that this time-dependent response was influenced by the period of heat treatment during the larval stage. Juveniles from L-HT group presented a lower induction of HSPs expression (lower cellular stress) both in the heat shock during the day and at night, compared to the juveniles of the D-HT group, which presented higher levels of expression (higher cellular stress) at both points of the day. These different time-dependent cellular responses at the brain level in the juvenile stage suggest that the environmental plasticity of thermotolerance in fish is determined by the thermal conditions during early development, as well as by the light-dark cycles and their relationship with the rhythms of HSP expression.

The role of HSPs in sex determination and differentiation has been little investigated to date. The function of HSPs in TSD has been described mainly in the maintenance of the native state of sex steroid receptors. Furthermore, it has been shown that the effect on sexual differentiation mechanisms may vary depending on the family of HSPs involved. For example, HSP70a is highly expressed in the testis during tesicular differentiation of Nile tilapia (Tao et al., 2018; Wang et al., 2019) and enhance the transcriptional activity of the androgen receptor (Knee et al., 2001). Meanwhile HSP27, HSP90 and HSP23 can act as co-repressors of estrogen signalling participating in the regulation of Era (Al-Madhoun et al., 2007, Khono et al., 2010; Oberman, 2018). In recent years, the induction of HSPs is being indirectly correlated with expression changes of sexual differentiation genes after heat treatments in fish (Li et al., 2014; Wang et al., 2019). For example, Li et al (2014), showed that after heat treatment in Nile tilapia, the induction of HSPs increased as the expression of *cyp19a1a* decreased. This agrees with our results, in which the maximum inhibition of the genes involved in feminization (*cyp19a1a*, *foxl2* and *Era*) was observed in the LD-HT and D-HT groups, which presented a greater induction of HSPs. On the contrary, the CTROL group presented the highest expression levels of the feminizing genes and the lowest HSPs expression. The present results could indicate a possible link between thermal tolerance and sexual differentiation by high temperature treatment. Although there are some studies supporting this hypothesis, further research is needed to demonstrate this link and the role of HSPs in TSD (Li et al., 2014; Wang et al., 2019).

CONCLUSION

The results of the present article revealed that the period of the day in which the masculinization thermal protocols are applied considerably influenced the thermal tolerance and the sexual differentiation of Nile tilapia. We observed that larvae exposed to thermal treatment at night or during both phases of the light-dark cycle show ed greater sensitivity to masculinizing treatments of high temperatures with higher upregulation of genes related to testicular differentiation and inhibition of genes involved in ovarian development, and consequently to a higher proportion of males. The larvae of this two experimental groups also presented the lowest levels of survival and higher cellular stress, which may indicate the existence of a possible link between the mechanisms of sexual differentiation and thermotolerance (HSPs). In addition, the effect of the thermal treatment applied at different times of the day during the sexual differentiation stages modified the time-dependent cellular response in juvenile animals. These findings reveal the need to further investigate the influence of the rhythms of the components of the BPG axis and HSPs on TSD as well as to consider time-dependent responses in studies of thermotolerance and thermal biology of fish. In addition, these results may be useful to optimize masculinization protocols in the tilapia industry that guarantee animal welfare and minimize the negative effects of thermal treatment both in the short and long term on the thermal and reproductive physiology of the fish.

COMPETING INTERESTS

The authors declare no competing or financial interests.

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SUPPLEMENTARY INFORMATION

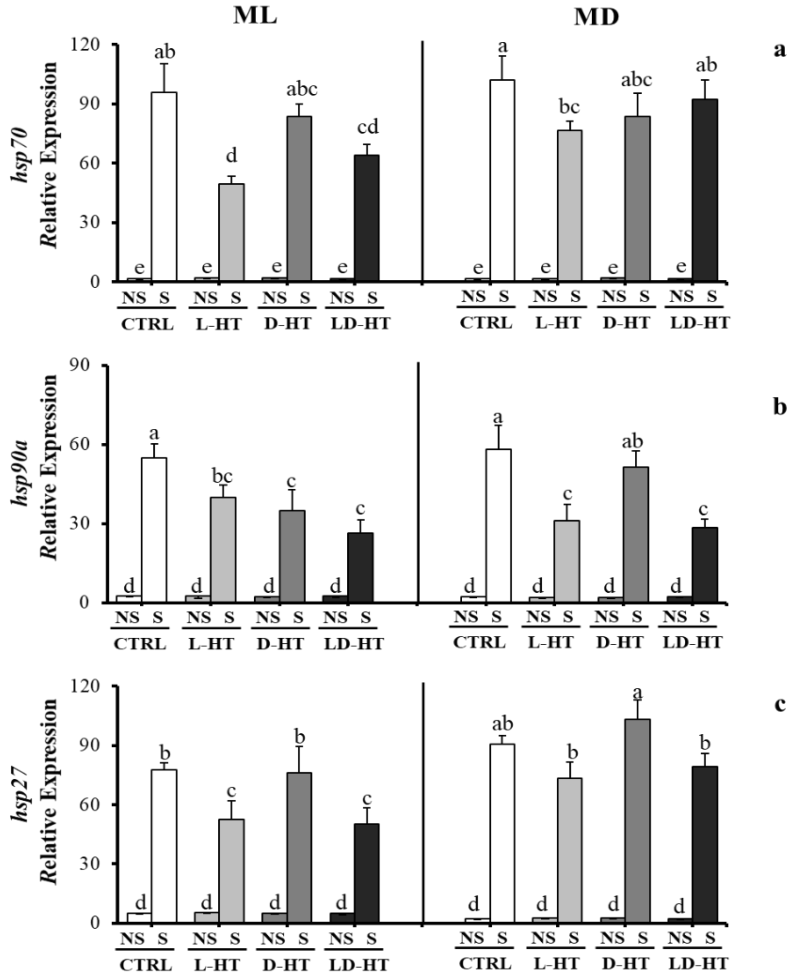

Fig. A1 Brain relative expression of the HSP genes: *hsp70* (a), *hsp90a* (b) and, *hsp27* (c). Fish were previously subjected to a heat treatment (HT, 36ºC for 12 days) during different period of the day (light phase, L-HT; dark phase, D-HT; light and dark phases LD-HT) or maintained at the same rearing temperature (28±0.5ºC, Control group, CTRL) from 11 to 24 dpf. Then, they were kept at constant temperatures until 270 dpf. At this moment, juveniles were exposed to a heat shock (36ºC for 2 h) at two different time points: middle of the light phase (ML, white bars); middle of the dark phase (MD, dark bars). For each group exposed to thermal shock (heat-stressed, S), its control group (non stressed; NS) was sampled at the same time. Different letters indicate significant differences between experimental groups (three-way ANOVA, $P< 0.05$). In addition, different asterisks denote significant interaction effect between different heat treatments periods heat shock, and time of day of heat shock (three-way ANOVA, $P< 0.05$). Data are represented as mean±SEM (n=6).

Table A1 Statistic values obtained in the one- and three-way ANOVAs performed for all the variables measured in the experiments. The p values (p) and F-Statistic (F) are reported. Gray highlights the statistically significant results ($P< 0.05$).

	Variable		One-way ANOVA		Three-way ANOVA											
					Period of day of Heat treatment (PT)		Heat Shock (HS)		Time of day (T)		PT*T		PT*HS		T*HS		PT*T*HS	
			F	P	F	P	F	P	F	P	F	P	F	P	F	P	F	P
25 DPF	**Survival rate (%)**		7.071	0.012														
	Relative expression	*amh*	6.618	0.001														
		cyp19a1a	5.372	0.005														
		sox9a	7.183	0.001														
		Era	5.235	0.005														
		dmrt1a	6.653	0.002														
		foxl2	7.404	0.002														
		Ara	6.297	0.002														
		hsp70	1.486	0.250														
		hsp90a	3.147	0.049														
		hsp27	5.901	0.005														
JUVENILES	**Sex ratio (%)**		6.453	0.016														
	Gonadal Relative expression	*amh*	3.088	0.046														
		cyp19a1a	0.823	0.492														
	Brain Relative expression	*hsp70*	39.847	0.000	5.007	0.003	550.444	0.000	4.638	0.034	1.078	0.363	5.028	0.003	4.820	0.031	1.090	0.358
		hsp90a	21.638	0.000	6.139	0.001	272.340	0.000	0.807	0.372	1.410	0.246	6.159	0.001	1.048	0.309	1.331	0.271
		hsp27	40.288	0.000	4.639	0.005	539.691	0.000	10.235	0.002	0.343	0.794	4.844	0.004	16.368	0.000	0.326	0.806

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Experimental Chapter V

Rearing temperature conditions (constant vs. thermocycle) affect daily rhythms of thermal tolerance and sensing in zebrafish

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ABSTRACT

In the wild, the environment does not remain constant, but periodically oscillates so that temperature rises in the daytime and drops at night, which generates a daily thermocycle. The effects of thermocycles on thermal tolerance have been previously described in fish. However, the impact of thermocycles on daytime-dependent thermal responses and daily rhythms of temperature tolerance and sensing expression mechanisms remain poorly understood. This study investigates the effects of two rearing conditions: constant (26ºC, C) *versus* a daily thermocycle (28ºC in the daytime; 24ºC at night, T) on the thermal tolerance response in zebrafish. Thermal tolerance (mortality) was assessed in 4dpf (days post fertilization) zebrafish larvae after acute heat shock (39ºC for 1 h) at two time points: middle of the light phase (ML) or middle of the dark phase (MD). Thermal stress responses were evaluated in adult zebrafish after a 37ºC challenge for 1 h at ML or MD to examine the expression of the heat-shock protein (HSP) (*hsp70*, *hsp90ab1*, *grp94*, *hsp90aa1, hspb1*, *hsp47, cirbp)* and transient receptor potential (TRP) channels (*trpv4*, *trpm4a*, *trpm2*, *trpa1b*) in the brain. Finally, the daily rhythms of gene expression of HSPs and TRPs were measured every 4 h for 24 h. The results revealed the larval mortality rates and the expression induction of most HSPs in adult zebrafish brain reached the highest values in fish reared under constant temperature and subjected to thermal shock at MD. The expression of most HSPs and TRPs was mainly synchronized to the light/dark (LD) cycle, regardless of the temperature regime. Most HSPs involved in hyperthermic challenges displayed diurnal rhythms with their acrophases in phase with warm-sensing thermoTRPs acrophases. The cold-sensing *trpa1b* peaked in the second half of the light period and slightly shifted toward the dark phase anticipating the acrophase of *cirpb,* which is involved in hypothermic challenges. These findings indicated that: a) thermal shocks are best tolerated in the daytime; b) the implementation of daily thermocycles during larval development reduces mortality and stress-cellular expression of HSPs to an acute thermal stress at MD; c) daily rhythms need to be considered when discussing physiological responses of thermal sensing and thermotolerance in zebrafish.

Key words: Thermal shock, thermocycles, biological rhythms, zebrafish, Heat Shock Proteins, Thermo-Transient Receptor Potential Channels.

INTRODUCTION

The cyclic variations in the environment have favored the natural selection and the appearance of biological clocks in most organisms. Biological rhythms allow organisms to keep track of time and anticipate periodic environmental changes to adapt and optimize their physiological and behavioral responses guaranteeing their survival and success (Kriegsfeld and Silver, 2006; Cowan et al., 2017). Light and temperature cycles are considered as the main synchronizers of biological rhythms. In the wild, daily and seasonal changes in light and temperature appear in a dynamic and simultaneously way (Angilletta et al., 2002). Light is a pervasive factor which influences in all stages of development in fish. The pineal gland and retina play a key role as main light receptor organs which transduce light information (day/night cycle and day length) into electrochemical and humoral (melatonin) signals (Falcon et al., 2010). Thus, melatonin modulates and synchronizes biological rhythms which influence on the physiological responses of organisms (i.e. feeding, reproduction, locomotor activity) (Cowan at al. 2017). In addition to light, water temperature determines profound effects on the biochemical, physiological and life history activities characterizing the phenotypic response to water temperature (acclimation) of ectothermic animals like fish (Angilletta et al., 2002; Donaldson et al., 2008; Morash et al., 2018). The ability of fish to tolerate different ranges of water temperature constitutes its thermal tolerance which can be permanently changed in response to the acclimation temperature during development (phenotypic plasticity) (Podrabsky and Somero, 2004; Fischer et al., 2012).

In the aquatic environment, water temperature is not constant, but oscillates: it warms up in the daytime and cools down at night generating a thermocycle (Schulte et al., 2014). The beneficial effect of thermocycles during early developmental stages has been described in Senegalese sole (*Solea senegalensis*) and zebrafish (*Danio rerio*) increasing the larval growth and decreasing the mortality and larval malformations (Blanco-Vives et al., 2010; Villamizar et al., 2012). In addition, the effect of thermocycles on acute thermal tolerance and survival after thermal shock have been extensively studied in fish. Most researches have been performed in salmonids (Threader and Houston, 1983; Wherly et al., 2007; Chadwick and McCormick, 2017; Corey et al., 2017; Gallant et al., 2017; Tunnah et al., 2017) and to a lesser extent in other eurythermal fish species such as killifish (*Fundulus heteroclitus*) (Fangue et

al., 2006; Healy and Schutle, 2012), tidepool sculpin (*Oligocottus maculosus*) (Nanako and Iwama 2002, Todgham et al., 2006, Fangue et al., 2011) and zebrafish (Schaefer and Ryan 2006, Xia et al., 2016; Wang and Xia, 2019). However, the influence of both light and temperature cycles on daily rhythms in thermal tolerance response has received little attention to date.

When water temperature increases/decreases suddenly, it can go above or below the temperature limit that fish can tolerate. This thermal stress response in the brain produces neuroendocrine signals that trigger changes in protein expression, osmoregulation, and hematological and metabolic pathways (Van Den Burg et al., 2005; Flik et al., 2006). When focusing on the cellular response to thermal stress, heat shock proteins (HSPs) and their transcription factors (HSF; i.e. HSF1) protect vital functions in all cells. HSPs intervene in the assembly, folding and translocation of proteins and also in the regulation of hormone-immune interactions (Welch, 1993; Donaldson et al., 2008). There are several HSPs that specifically intervene in cellular protection against hyperthermia such as a) HSP90, composed by HSP90β constitutive form (encoded by cytoplasmatic *hsp90ab1* and endoplasmic reticulum *grp94*) and HSP90α inducible form (encoded by cytosolic *hsp90aa1*), b) HSPB1 which is a low molecular weight HSP (also known as HSP27), c) inducible HSP70, and d) collagen-related HSP47 (Feder and Hoffman, 1999). In addition, other inducible proteins such as CIRBP (cold-inducible RNA binding protein) have been described to participate in thermal tolerance protecting cells against hypothermic challenges in fish (Grace et al., 2004; Verleih et al., 2015). The HSP expression is influenced by the animal's acclimation thermal history and genetic differences (Cortemeglia and Beitinger, 2006). The link between HSP expression and thermotolerance is well documented in fish. Under unstressed conditions, higher and lower basal levels of HSPs have been correlated with greater and lower thermotolerance, respectively (for review see Feder and Hoffman 1999; Basu et al., 2002). However, if a thermal stressor is present, the transcription of HSPs is activated and rapidly translated into new proteins that intervene in native cytoprotective mechanisms (Morimoto et al., 1992, Pirkkala et al., 2001; Donaldson et al., 2008). The dynamics (temporal pattern and abundance) of the induction of HSP expression vary according to the individual's age, fish species, tissue, type of stressor and stressor intensity (Feder and Hoffman, 1999; Iwama et al., 1998). For example, in adult zebrafish the higher induction by heat exposure was described in the brain,

liver and gonad, while the induction was lower in skeletal muscle, anterior kidney and gills (Rabergh et al., 2000; Airaksinen et al., 2003; Murtha and Keller, 2003; Marvin et al., 2008). In addition, the magnitude of the expression induction correlates with the stressor intensity and protein damage (duration and gradient) (Didomenico et al., 1982; Dyer et al., 1990; Feder and Hofmann, 1999; Murtha and Keller, 2003; Buckley et al., 2006). Although daily and seasonal rhythms of HSP expression have been acknowledged previously in fish and other organisms, their existence and role in thermal tolerance response patterns remains poorly understood to date in zebrafish (Fadder et al., 1994; Rensing and Monnerjahn, 1996; Podrabsky and Somero, 2004; Fangue et al., 2006; Todgham et al., 2006; Healy and Schutle, 2012).

The transient receptor potential (TRP) channels of the Central Nervous System (CNS) play a key role in perceiving environmental information. Here the main functions are sensory perception, including thermal, mechanical, chemical, nociceptive and light sensation, and intervening in the maintenance of homeostasis and osmotic regulation (Voets et al., 2005). Temperature sensing is achieved in cells by specialized membrane pores or channels known as thermoTRP channels. In zebrafish, the following subfamilies of thermoTRP channels have been described: TRPM (TRPM2, TRPM4, TRPM5), TRPV (TRPV1/2, TRPV4, TRPV6) and TRPA (TRPA1) (Saito and Shingai, 2006). All the above subfamilies of thermoTRPs are activated by diverse temperature thresholds. Thus, TRPM2 is activated between 35-42ºC, TRPM4 and TRPM5 between 15-35ºC, TRPV1/2 for temperatures above 42ºC, TRPV4 between 27-42ºC and TRPA1 for temperatures below 17ºC (Saito and Shingai, 2006; Oda et al., 2017). The thermoTRP channels are distributed through several tissues of the fish body with higher proportion in the CNS and the peripheral nervous system (e.g. the brain) (Saito and Shingai, 2006; Mangos et al., 2007; Kastenhuber et al., 2013; Song et al., 2016). Although, the essential role of thermoTRPs in the detection and transduction of thermal stimuli has been established in zebrafish, their role in thermal tolerance responses has been poorly investigated (Jeronimo et al., 2017; Germana et al., 2018). Besides, the existence of daily rhythms in thermo-sensing mechanisms has not been studied to any great extent in any organism.

Zebrafish are eurythermal cyprinids that live in heterogenic and fluctuating thermal environments with seasonal temperature ranging from 6 ºC in winter to 38 ºC in summer (Spence et al., 2006, 2008; Engescer et al., 2007). In one of the zebrafish habitats, the River Ganges, water temperature can oscillate daily up to 5.6 ºC along the day, generating often daily water temperature variations of several degrees (i.e. 24-28 ºC min-max) (Payne and Temple, 1996; Jindal and Takhur, 2013). Moreover, zebrafish must face large fluctuations in temperature caused by monsoon weather and climate change, which are close to their thermal tolerance limits (39.2 ºC and 41.7 ºC for zebrafish acclimated to 20 and 30 ºC, respectively) (Cortemeglia and Beitinger, 2005; López-Olmeda and Sánchez-Vázquez, 2011). To date, very few studies performed on zebrafish considered the possibility that the mechanisms involved in thermal tolerance and sensing could vary depending on the time of day and the temperature regime established during early development, which in turn affect response to acute thermal challenges.

The aim of the present research was to investigate the effect of two rearing temperature regimes with constant (C) *versus* daily thermocycle (T) and time of day (middle of the light phase, ML or middle of the dark phase, MD) on the rhythms of thermal tolerance and detection mechanisms in zebrafish. To this end, three experiments were performed with the following specific objectives: 1) describe the effect of the time of the day and rearing temperature on thermal tolerance response in zebrafish larvae; 2) describe the effect of the time of the day and rearing temperature on thermal tolerance response in zebrafish adults; 3) elucidate the daily rhythms in temperature sensing (TRPs) and resistance (HSPs) mechanisms in adult zebrafish reared under different temperature regimes.

MATERIALS AND METHODS

Animals and housing

The experiments were performed in the Chronolaboratory at the Faculty of Biology of the University of Murcia (Spain). Zebrafish (*Danio rerio*) of a wild-type line stock (aged 2 months) were obtained from a local commercial distributor (Alimar Pets, S.L., Murcia, Spain). They were acclimated in 54-liter glass tanks divided into six compartments (9 L each)

and placed inside a Chronobiology chamber under controlled lighting and temperature conditions. The photoperiod was set at 12:12 light:dark (LD) cycle, with light onset at 10:00h (ZT 0 h) and light offset at 22:00h (ZT 12 h). Light was provided by LED strips (SOLBRIGHT®, LED Flex Strip 1043-W, Rayte, S.L., Murcia, Spain), with a light intensity on the water surface of 0.84 W·m-2 (~200 lx). Water temperature remained constant at 28±0.3 ºC. The aquaria contained dechlorinated fresh water in recirculation, which was constantly filtered by biological and mechanical filters. The feeding regime was set using automatic feeders in each tank for experiments performed in adult zebrafish (Eheim GmbH & Co. KG, model 3581, Deizisau, Germany), establishing three daily meals (at 11:00h, 15:00h and 19:00h) at a feeding rate of 1 % of the total biomass per day using a commercial feed (Tropical fish flakes, Casone, Parma, Italy).

Experimental design

The experiments were designed to fulfill European Union guidelines (2010/63/EU) and Spanish legislation (RD 1201/2005 and Law 32/2007). The animal protocols (license number A13191003) were previously authorized by the National Committee and the Committee of University of Murcia on Ethics and Animal Welfare.

Experiment 1: Effect of the time of day and rearing temperature (T/C) on the thermal tolerance of ZF larvae

In the first experiment, the effect of the time of day and rearing temperature on thermal tolerance was evaluated in the 4dpf (days post-fertilization) zebrafish larvae submitted to thermal shock. To this end, the adult zebrafish from the stock were bred according to Westerfield (2000). Fertilized eggs were collected during the first hour after fertilization to be immediately placed in sterile Petri dishes (30 embryos per Petri dish) with embryo medium, and were distributed into the different temperature regimes. Zebrafish embryos and larvae were reared from 0 to 96 h post-fertilization (hpf) at two temperature regimes: 26 ± 0.3 ºC (Constant, C) and a thermocycle of 28:24 ± 0.3 ºC (thermophase:cryophase, T). The thermophase comprised the light phase (ZT0 h - ZT12 h) and the cryophase coincided with the dark phase (ZT12 - ZT0 h) (Fig. A1). Water temperature variations were generated by electronic heaters (Askoll, Povolaro, Italy) and water cooler

units (Aqua Medic Titan 1500 GmbH, Bissendorf, Germany). Temperature changes were controlled by an electronic timer and recorded every 15 minutes by an underwater data logger (HOBO PENDANT Onset Computer Corporation, Massachusetts, USA) to ensure that thermocycles were correctly performed. Then, the 4dpf zebrafish larvae (N=480) were divided into eight different groups (composed of two Petri dishes with 30 larvae each; n=2) (Fig. 1A). Each temperature regime (T or C) contained the following four groups: larvae subjected to thermal shock at ZT6 h (ML, middle of the light phase); larvae subjected to thermal shock at ZT18 h (MD, middle of the dark phase); two control groups not exposed to thermal shock, but maintained at rearing temperature. A dim red light was used for the experiments carried out in the dark phase (Takemura et al., 2006). The thermal stress challenge consisted of a 1-hour exposure at 39ºC. This temperature was chosen according to previous research on the acute thermal tolerance for wild-type zebrafish (Cortemeglia and Beitinger, 2005). Thermal shock was performed in the separate and previously conditioned aquaria. After the shock challenge, larvae were immediately returned to their rearing temperature regime. Mortality rates were measured continuously for 24 h after thermal shock, which ended by confirming the absence of heart activity using a binocular microscope (Leica MZ6, Heerburgg, Switzerland) (Villamizar et al., 2014). The experiment was performed in triplicate to obtain six independent replicates (n=6) for each experimental group.

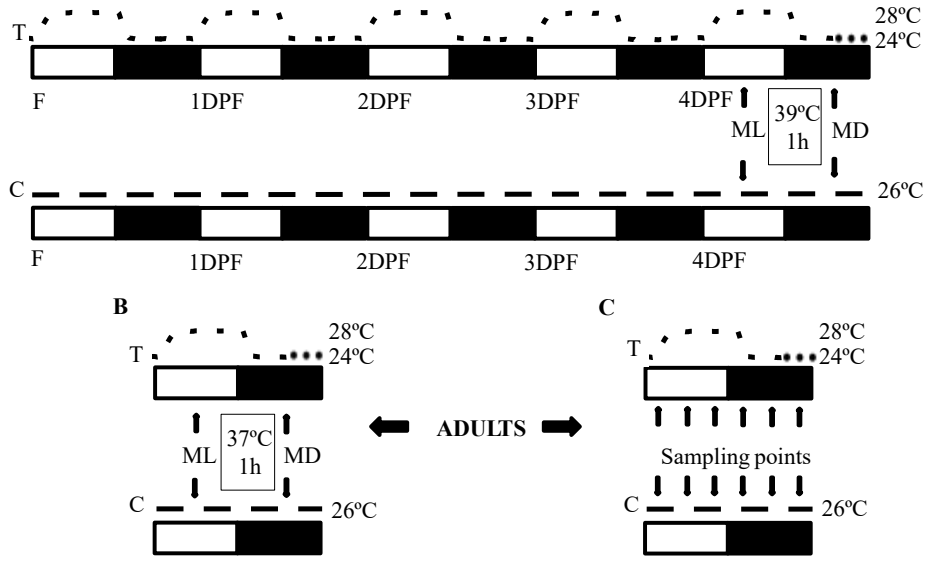

Fig. 1 Schematic representation of the experimental design. Experiment 1 (A): zebrafish embryos were maintained at T (thermophase:cryophase; 28:24±0.3ºC) or C (constant temperature; 26±0.3ºC) from fertilization (F) to 4 days post-fertilization (dpf). The larvae at 4dpf were exposed to thermal shock (39ºC for 1 h) at two

different time points (ML, middle of the light phase; MD, middle of the dark phase) and mortality rates were measured. The control groups were performed for each group subjected to thermal shock. Experiment 2 (B): zebrafish were maintained at T or C during the 3-month acclimation and were exposed to sublethal thermal shock (37ºC for 1 h) at two different time points (ML and MD). Brain samples were collected to perform the gene expression analysis. Experiment 3 (C): zebrafish maintained under T and C and sampled every 4 h for 24 h in non-stressed situations. Brain samples were collected to perform the gene expression analysis. The control groups were sampled at the same time and temperature regime for each experimental group sampled in Experiments 1 and 2.

Experiment 2: Effect of the time of day and rearing temperature on temperature-sensing (TRPs) and resistance (HSPs) mechanisms in adult zebrafish

The second experiment was designed to determine the effect of the time of day and rearing temperature on temperature-sensing (TRPs) and resistance (HSPs) mechanisms in the adult zebrafish submitted to sublethal thermal shock. For this purpose, male zebrafish aged 2 months (N=48) from the stock were divided into two groups with two temperature regimes (T or C). Then, fish from each temperature regime were distributed into 4 compartments of 9 L each (n=6 fish per compartment). Previous research reported thermal tolerance in zebrafish was affected by the age but not by the gender (Cortemeglia and Beitinger, 2005, 2006; Murtha and Keller, 2013; Xia et al., 2019). The experimental temperature and lighting conditions were those described in Experiment 1. After 3 months under T and C, zebrafish were sampled prior to (control; Ctrl) or after (heat exposed; H) a thermal shock at two different time points: in the middle of the light phase (ML) or in the middle of the dark phase (MD). A total of eight different experimental groups were used similarly to Experiment 1 (Fig. 1B). The thermal shock challenge consisted of subjecting fish for 1 h to a bath previously heated at 37 ºC. This combination of exposure duration and temperature was chosen in accordance with previous articles in order to induce significant effects on the expression of HSPs, avoiding mortality (Råberghet al., 2000, Airaksinen et al., 2003, Murtha and Keller, 2003). Immediately after thermal exposure, six male fish (n=6) per experimental group were anesthetized by immersion in icy water (5 parts ice/1 part water, 0-4 °C) and euthanized by decapitation according to the guidelines established for fish euthanasia (R. D. 1386/2018). Brain samples were collected within 2 minutes after heat exposure and transferred to sterile RNase- and DNase-free 1.5 ml

Eppendorf tubes. The tubes were frozen immediately in dry ice and stored at -80ºC until analysis of TRP and HSP gene expression in the brain.

Experiment 3: Daily rhythms in temperature-sensing (TRPs) and resistance (HSPs) mechanisms in the adult zebrafish reared in different temperature regimes (T/C)

To elucidate the daily rhythms of HSP and TRP gene expression in the brain, the 2-month-old male zebrafish (N=72) from the stock were acclimated for 3 months in each rearing temperature regime (T/C). The experimental temperature and lighting conditions were those described in Experiment 1. During acclimation, the fish from each group were distributed into six compartments (n=6 fish per compartment) to sample only one tank at each sampling point. After the 3-month period, zebrafish were sampled every 4 h for 24 h cycle at the following six time points: ZT2, ZT6, ZT10, ZT14, ZT18, ZT22 h (Fig. 1C). At each time point, six males (n=6) from each temperature regime were submerged in icy water (5 parts ice/1 part water, 0-4°C) (Close et al, 1996). Brain samples were collected and transferred to sterile RNase- and DNase-free 1.5 ml Eppendorf tubes. Immediately after collecting brain samples, the tubes were frozen in dry ice and stored at -80ºC until analysis of brain TRP and HSP gene expression.

RNA extraction and cDNA synthesis

Each brain sample was homogenized in Trizol reagent (Ambion, Thermo Fisher Scientific, Waltham, USA) following the manufacturer's instructions in a tissue homogenizer for mechanical homogenization (TissueLyser LT, Qiagen, Hilden, Germany). RNA was dissolved in 15 µl of sterile DEPC water (Invitrogen, CA, USA). The concentration and purity of RNA were determined by spectrometry (Nanodrop ND-1000, Thermo Fisher Scientific). Prior to the retrotranscription of 1 µg of RNA, 1 U of DNase I (Thermo Fisher) was added to eliminate any genomic DNA contamination. The Reverse Transcriptase commercial kit (QSCRIPT cDNA Synthesis Kit, Quantabio, Beverly, USA) and a thermocycler were employed to obtain cDNA. All the cDNa samples were diluted (1:10) in nuclease-free water (Thermo Fisher Scientific) and stored at -20 ºC for the subsequent analyses.

Real-time RT-PCR analysis

The quantitative PCR (qPCR) reactions were performed using Perfecta® SYBR® Green Fastmix (Quantabio). Each qPCR reaction was carried out in a final volume of 20 µl. All the samples were run in duplicate in a light thermocycler (7500 RT-PCR system, Applied Biosystems, Foster City, USA) with the following steps: 15 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. In order to check the specificity of the reaction, melting curves were performed by the thermocycler immediately after the amplification step. The curves were performed by increasing the temperature from 60 to 95º in 0.3º steps and measuring the fluorescence after each increase. We checked in these curves the presence of a single peak, belonging to a single amplicon to ensure that only one DNA species was amplified. The Primer 3 plus software was employed to design the forward and reverse primers which were described in a table (Table 1) (Untergasser et al., 2012). The primer concentration for the qPCR reactions was calculated using the primer dilution curves. The primers for *hsp70*, *hsp90ab1*, *hsp90aa1, hspb1, cirbp* and *hsp47* were added at a final concentration of 200 nM. The primers for *trpv4, trpm2, trpm4a* and *trpa1b* were added at a final concentration of 300 nM (Jeronimo et al., 2017). The primers for *grp94* were added at a final concentration of 400 nM. The amplification efficiency of all primers was between 97.5-101.5% (Table 1). The reference gene, *loopern4*, was selected after verifying that its coefficient of variation (CV) was lower than 5% in experiment 2 and 3, which indicated that its expression was stable and not affected by temperature or time of day among treatments (Vanhauwaert et al., 2014). The value of the reference gene was used in the first normalization and the sample with the lowest expression value within each gene and group was used as the reference for the second normalization according to the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Table 1 The primer sequences used for the quantitative PCR analyses.

Gene	F/R	Sequence (5'-3')	Amplification Efficiency (%)	Ensembl/GenBank/Repbase Accession number
hsp70	F	TCAAGCGCAACACAACCATC	100.14	AF210640.1
	R	ATTTGCCCAGCAGGTTGTTG		
hsp90ab1	F	TGTCAGAACTGCTGCGTTAC	100.67	ENSDART00000020084.9
	R	ACAAATGCAGAGTGGGCAAC		
grp94	F	AAAAAGCTGGTGCGCAAGAC	98.72	NM_198210.2
	R	TCACGCCCAGCTTAATGTTG		
hsp90aa1	F	AGCTGGCGGATCGTTCACTGTC	99.58	AF068773.1
	R	AAAACTCGCCGTACTCCTCATTGG		
hspb1	F	AACACGGCTTCATTTCCAGAT	97.79	BC097148
	R	GCGGAGCTTCAACAGTCAGG		
cirbp	F	GCTTTGGCTTTGTGACCTTTG	101.31	NM_200017
	R	AAACCACCAGACCGTCCACC		
hsp47	F	ACGGGGCTATGATCGTCAA	99.50	NM_131204
	R	AGAGCGGGTCACCAGGAAA		
trpv4	F	AATCGGCATGAGATGTTGGC	99.12	NM_001042730.1
	R	TCACCGCTGCAAATTTCTGC		
trpm4a	F	GGGAGGAGAGAGAAAAGCCC	98.29	NM_001288815.1
	R	GGAGGGGTGTCGAATGACAG		
trpm2	F	GACCCTTGTGCAAACACTCG	98.62	NM_001288817.1
	R	TTTGATGGCCTCATCGCAGT		
trpa1b	F	GGGAACACAGCCCTTCATATAG	99.64	EU826642.1
	R	GTCCACTTCATCTCCAGGTATTT		
loopern4	F	TGAGCTGAAACTTTACAGACACAT	100.27	LOOPERN4_DR
	R	AGACTTTGGTGTCTCCAGAATG		

Data collection and analysis

Statistical analyses were performed with the SPSS program for Windows (v. 19.0, IBM, Armonk, NY, USA). Data normality and homoscedasticity were checked by the Kolmogrov-Sminorv test and the Levene's test, respectively. The mortality rates of the thermal tolerance of zebrafish larvae (Experiment 1) and the gene expression levels of temperature-sensing and tolerance mechanisms in the adult zebrafish submitted to subthermal shock (Experiment 2) were subjected to a three-way ANOVA to compare the main effects and

the interaction effect of the following independent variables: rearing temperature regime (R), heat shock (H) and time of day (T) when the shock was applied (ML/MD). The data of the daily variations in gene expression in the adult fish brains (Experiment 3) were subjected to a two-way ANOVA to analyze the effects of the rearing temperature regime groups (R) and sampling time points (T) and its interaction. In case of a significant interaction between independent variables, three-way ANOVA was used followed by Duncan's *post hoc* test to detect the existence of significant differences within experimental groups ($P< 0.05$).

The existence of significant rhythmicity was tested for all the genes by the Cosinor analysis with the "EL TEMPS" software (v.1.179, Prof. Dıez-Noguera, University of Barcelona, Spain). The Cosinor analysis is based on the least squares approach of time series data with a cosine function of a known period of type Y = Mesor + Amplitude * cos (2π(t-Acrophase)/Period). This analysis provides statistical significance, as indicated by the *p* valuefor null hypothesis of zero amplitude to either accept the null hypothesis (Cosinor, $P> 0.05$), or reject it and accept the alternative hypothesis (Cosinor, $P< 0.05$). In all cases, values were expressed as mean ± SEM.

RESULTS

Effect of the time of day and rearing temperature (T/C) on the thermal tolerance of ZF larvae

The mortality rate of the 4dpf zebrafish larvae showed significant differences between experimental groups (three-way ANOVA, $F = 35.007$, $P< 0.001$) (Fig. 2 and Table A1). Actually, the mortality rate was influenced by the heat shock ($F = 221.56$, $P< 0.001$), the time of day ($F = 8.083$, $P = 0.007$) and the interaction between these two factors ($F = 8.083$, $P = 0.007$). However, no differences were found between the two rearing temperature regimes (three-way ANOVA, $F = 2.144$, $P = 0.151$). The highest mortality rate was observed at midnight (MD) for the C group (62.7 ± 3.3%). The lowest mortality rates appeared when thermal shock took place in the middle of the light phase (ML) under both rearing conditions (36.2 ± 2.7% and 37.7 ± 2.9% for T and C, respectively). No day/night differences in mortality were detected in the larvae reared in T (three-way ANOVA, $P> 0.05$).

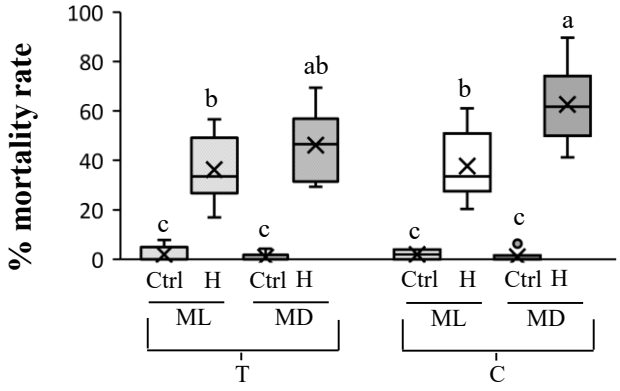

Fig. 2 Effect of thermal shock (39ºC for 1h) on the mortality rate in the zebrafish larvae reared in two temperature regimes: T (thermophase:cryophase; 28:24±0.3ºC) (striped boxes) *vs.*C (constant temperature; 26±0.3ºC) (solid boxes). Fish were subjected to thermal shock at two different time points: middle of the light phase (ML, white bars); middle of the dark phase (MD, dark bars). For each group exposed to thermal shock (heat-stressed, H), its control group (non stressed; Ctrl) was sampled at the same time. The data from each variable were subjected to a three-way ANOVA, followed by Duncan's *post hoc* test. Boxes represent the 25th and 75th percentile. Whiskers represent the minimum and maximum values and horizontal line within the box present the median. Small circles outside the diagram represent outliers. Different letters indicate significant differences between experimental groups (three-way ANOVA, $p< 0.05$). Data are represented as mean±SEM (n=6).

Effect of the time of day and rearing temperature on temperature-sensing (TRPs) and resistance (HSPs) mechanisms in adult zebrafish

In Experiment 2, the results showed marked differences in the response of adult zebrafish to thermal shock (37ºC for 1 h) influenced by the effects and interaction of the independent variables analyzed (three-way ANOVA, $P< 0.05$, Fig. 3 and Table A1). In most HSPs analyzed (except *hsp47* and *cirbp*), the effect of time of day when thermal shock was applied only affected to the induction of HSP expression in the C group (three-way ANOVA, $P< 0.05$, Fig. 3 and Table A1).

The expression of *hsp70* was influenced by the time ($F = 11.369$, $P = 0.020$) and heat shock ($F = 230.361$, $P< 0.001$), and the interaction between these two factors ($F = 11.452$, $P = 0.020$) (Fig. 3A and Table A1).

The expression of *hsp90ab1* was affected by the heat shock ($F = 4.568$, $P = 0.039$) and the interactions between heat shock and time ($F = 10.378$, $P = 0.030$), and between heat shock, time and rearing temperature ($F = 4.950$, $P = 0.032$) (Fig. 3B and Table A1).

Regarding *grp94* expression, the three-way ANOVA analysis showed the effects of the heat shock (F = 162.664, P< 0.001) and the three interaction effects between rearing temperature and time (F = 7.840, P = 0.008), heat shock and time (F = 4.789, P< 0.035) and heat shock, rearing temperature and time (F = 5.539, P = 0.024) (Fig. 3C and Table A1).

The expression of *hsp90aa1* was influenced by the time (F = 4.591, P = 0.039) or heat shock (F = 61.950, P< 0.001), and by the interaction between heat shock and time (F = 4.708, P = 0.037) (Fig. 3D and Table A1).

With regards to *hspb1* expression, the statistical analysis showed significant effects for time of day (F = 5.488, P = 0.025) or heat shock (F = 34.290, P< 0.001), with two interactions between rearing temperature and time (F = 4.239, P = 0.047), and between heat shock and time (F = 5.581, P = 0.024) (Fig. 3E and Table A1). Finally, the expression of *hsp47* and *cirbp* was influenced only by the effects of heat shock (F = 99.704, P< 0.001 and F = 51.057, P< 0.001, respectively) (Fig. 3F, G and Table A1).

Statistical differences were observed within experimental groups for *hsp70* (F = 37.008, P< 0.001), *hsp90ab1* (F = 4.008, P = 0.002), *grp94* (F = 26.395, P< 0.001), *hsp90aa1* (F = 11.359, P< 0.001), *hspb1* (F = 8.599, P< 0.001), *hsp47* (F = 14.863, P< 0.001) and *cirbp* (F = 7.793, P< 0.001) (Fig. 3 and Table A1). For the zebrafish reared in the C group, the analysis pointed striking statistical differences between experimental groups in the gene expressions of *hsp70, hsp90ab1, grp94*, *hsp90aa1* and *hspb1,* with a higher expression appearing when thermal shock was performed at MD (three-way ANOVA, P< 0.05) (Fig. 3A, B, C, D, E). However, no statistical differences were found in the thermotolerance response of HSPs between the time points for the zebrafish reared in the T group (three-way ANOVA, P > 0.05) (Fig. 3 and Table A1).

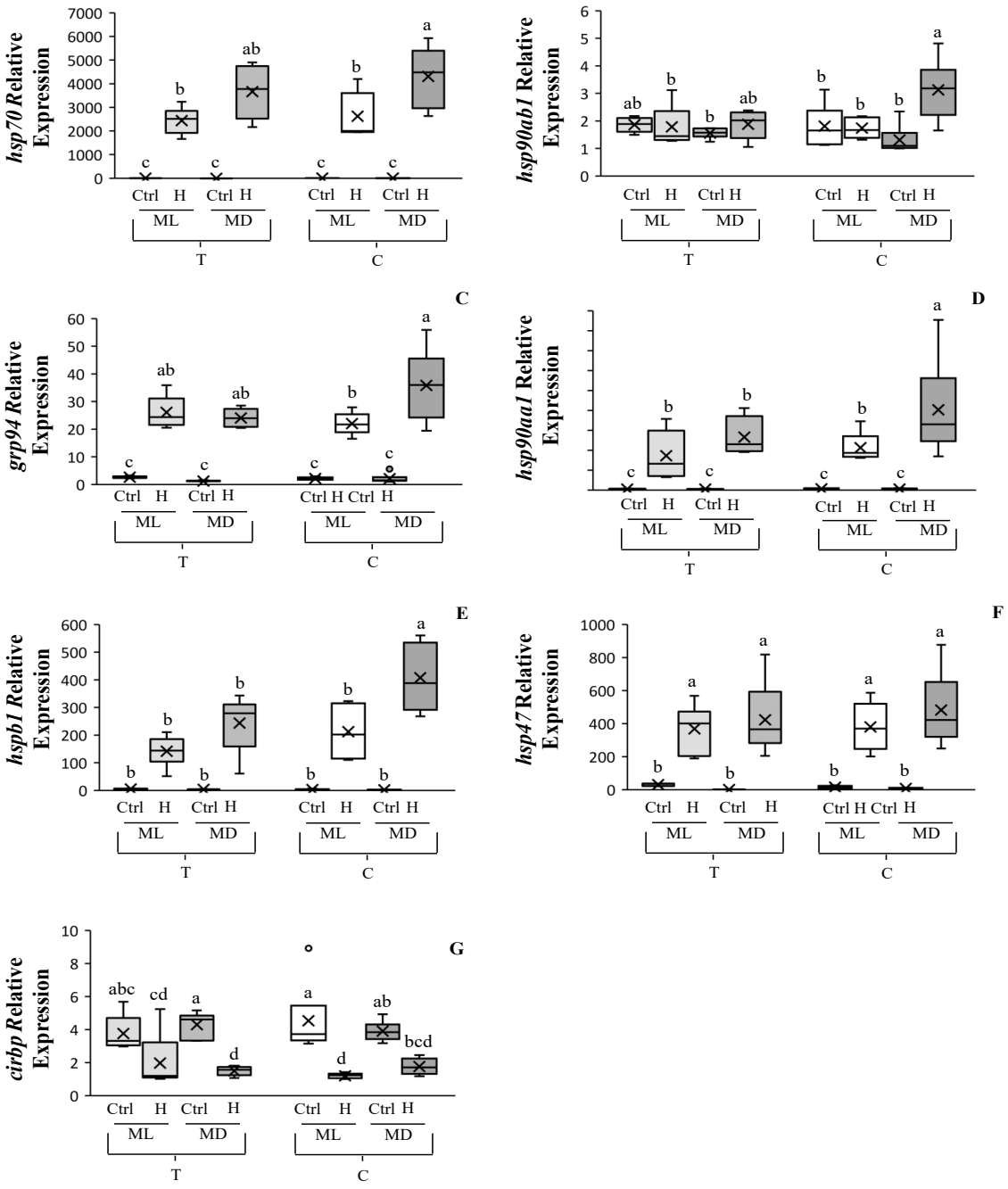

Fig. 3 Brain relative expression of the HSP genes: *hsp70* (A), *hsp90ab1* (B), *grp94* (C), *hsp90aa1* (D), *hspb1* (E), *cirbp* (G), *hsp47* (F). Fish were reared in two temperature regimes: T (thermophase:cryophase; 28:24±0.3ºC) (striped bars) *vs.* C (constant temperature; 26±0.3ºC) (solid bars). Zebrafish were exposed to sublethal thermal shock (37ºC for 1 h) at two different time points: middle of the light phase (ML, white bars); middle of the dark phase (MD, dark bars). For each group exposed to thermal shock (heat-stressed, H), its control group (non stressed; Ctrl) was sampled at the same time. The data from each variable were subjected to a three-way ANOVA, followed by Duncan's *post hoc* test. Boxplots show the first and third quartiles (boxes). Whiskers represent the minimum and maximum values and horizontal line within the box present the median. Small circles outside the diagram represent outliers. Different letters indicate significant differences between experimental groups (three-way ANOVA, $P< 0.05$). Data are represented as mean±SEM (n=6).

For the thermal-sensing mechanisms (TRPs), the statistical analysis showed only the effect of the heat shock on the expression of *trpm4a* (F = 33.799, P< 0.001) and *trpa1b* (F =4.857, P = 0.035) (Table A1 and Table A2). In addition, significant differences between experimental groups were found for *trpm4a* (F = 5.522, P< 0.001) (Table A1 and Table A2). However, the three-way ANOVA failed to detect any significant differences for *trpv4* (F = 0.622, P = 0.734), *trpm2* (F = 0.602, P = 0.750) and *trpa1b* (F = 1.014, P = 0.441) in the response to thermal shock compared to the control groups and time points/rearing conditions (three-way ANOVA, p > 0.05) (Table A1 and Table A2).

Daily rhythms in temperature-sensing (TRPs) and resistance (HSPs) mechanisms in the adult zebrafish reared in the different temperature regimes (T/C)

Under both rearing temperature regimes, most of the analyzed genes involved in the thermotolerance mechanisms presented significant daily rhythms (Cosinor, P < 0.05) (Fig. 4).

The relative expression of *hsp70* displayed significant daily rhythms under T (Cosinor, P = 0.0004) and C (Cosinor, P = 0.00007) with their acrophase located around ZT4 h (ZT3.88 h and ZT4.15 h, respectively). Statistically significant differences in *hsp70* relative expression between time points (two-way ANOVA, F = 9.916, P< 0.001) were also observed (Fig. 4A and Table A1).

Hsp90ab1 only displayed significant daily rhythms in the C group (Cosinor, P = 0.007), with its acrophase located at ZT4.58 h. In addition, significant differences were found for *hsp90ab1* expression between time points (two-way ANOVA, F = 3.022, P = 0.017) (Fig. 4B and Table A1).

Significant daily rhythms of *grp94* expression were found in both T and C groups (Cosinor, P = 0.001 and P = 0.0002, respectively), peaking around the middle of the light phase (ZT5.38 h and ZT5.03 h, respectively). As to *grp94*, statistical differences were found in relative expression between time points (two-way ANOVA, F = 7.657, P< 0.001) (Fig. 4C and Table A1).

Hsp90aa1 only displayed significant daily rhythms in the T group (Cosinor, P = 0.0006) with its acrophase located at ZT8.05 h. The expression of *hsp90aa1* also showed

differences between time points (two-way ANOVA, $F = 2.622$, $P = 0.036$) and an interaction between rearing temperature regime and time points was also found (two-way ANOVA, $F = 2.570$, $P = 0.039$) (Fig. 4D and Table A1).

Significant daily rhythms were found for the *hspb1* expression under T and C (Cosinor, $P = 0.027$, $P = 0.026$) with their acrophases located at around the mid-light phase (ZT6.96 h for T, ZT3.53 h for C). Moreover, statistical differences were found in *hspb1* expression depending on the time point (two-way ANOVA, $F = 3.254$, $P = 0.012$) (Fig. 4E and Table A1).

Hsp47 relative expression displayed significant daily rhythms in the T and C groups (Cosinor, $P = 0.00001$, $P = 0.00001$, respectively) whose expression peaked at ZT4.18 h for T and ZT3.88 h for C group. Significant differences in *hsp47* expression between rearing temperature regime (two-way ANOVA, $F = 70.798$, $P< 0.001$) were found at ZT2 h and ZT6 h. In addition, significant time points differences (two-way ANOVA, $F = 20.452$, $P< 0.001$) and an interaction between both factors were observed (two-way ANOVA, $F = 12.071$, $P< 0.001$) (Fig. 4F and Table A1).

The relative expression of *cirpb* displayed significant daily rhythms in the T and C groups (Cosinor, $P = 0.021$, $P = 0.039$, respectively) with their nocturnal acrophases located around at the end (ZT22.75 h) and at the beginning (ZT13.3 h) of the dark phase, respectively. *Cirbp* expression was influenced by the temperature regime at ZT2 h, ZT6 h, ZT18 h and ZT22 h (two-way ANOVA, $F = 16.075$, $P< 0.001$). Moreover, a significant interaction in the *cirbp* expression between both factors was observed (two-way ANOVA, $F = 5.679$, $P< 0.001$) (Fig. 4G and Table A1).

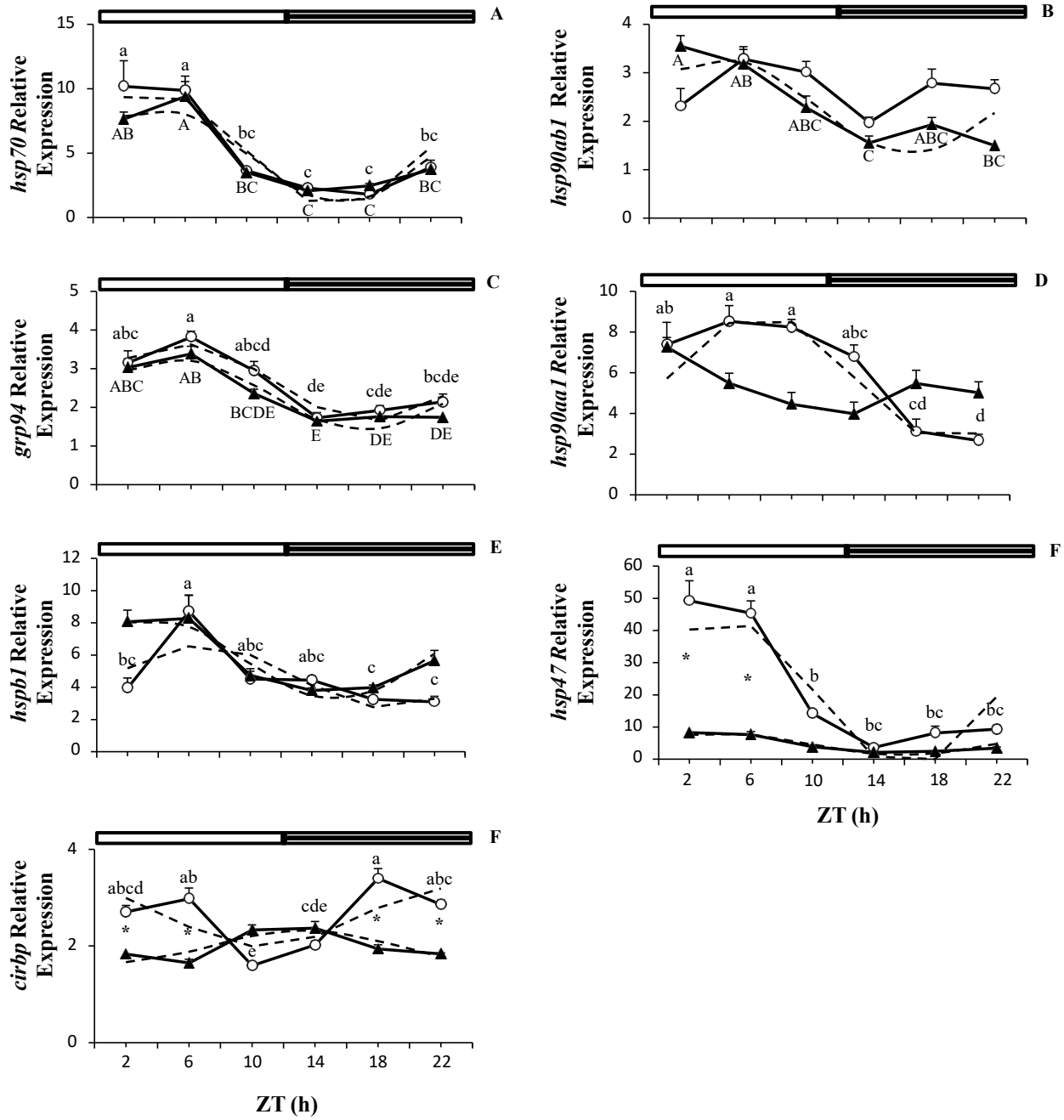

Fig. 4 Daily variations in the relative expression of the HSP genes in zebrafish brains: *hsp70* (A), *hsp90ab1* (B), *grp94* (C), *hsp90aa1* (D), *hspb1* (E), *cirbp* (G), *hsp47* (F).Fish were maintained in a 12:12LD cycle and two temperature regimes: T (thermophase: cryophase; 28:24±0.3ºC, white circles) and C (constant temperature, 26±0.3ºC, black triangles). The dashed line represents the adjustment to sinusoidal rhythm (Cosinor, $P< 0.05$). Different lower and uppercase letters indicate statistical differences between the time points in the same group for T and C, respectively (two-way ANOVA, $P< 0.05$). The asterisks indicate significant differences between groups in the same time point (two-way ANOVA).The white and black bars above the graphs represent the light and dark phases, respectively. The time scale (x-axis) is expressed as *Zeitgeber* Time (ZT), in which ZT0 h corresponds to light onset and ZT12h corresponds to lights off. Data are represented as mean ± SEM; n=6 replicates per point.

When the expression of the genes of TRPs was subjected to the Cosinor analysis, the results showed that the daily expression of most genes fit to the cosine function (Cosinor, $P< 0.05$) (Fig. 5).

The relative expression of *trpv4* displayed significant daily rhythms in the zebrafish maintained under the T and C conditions (Cosinor, $P = 0.049$, $P = 0.026$, respectively) peaking at ZT4.75 h and ZT2.18 h, respectively. Statistical differences were found for *trpv4* between rearing temperature regime at ZT6 h (two-way ANOVA, $F = 19.007$, $P< 0.001$) and between time points (two-way ANOVA, $F = 5.718$, $P< 0.001$). Moreover, an interaction between both factors was observed in *trpv4* relative expression (two-way ANOVA, $F = 4.253$, $P = 0.002$) (Fig. 5A).

Trpm4a only displayed significant daily rhythms in the C group (Cosinor, $P = 0.036$) with its acrophase located at ZT3.75 h. No significant differences were found in the *trpm4a* expression between time points (two-way ANOVA, $F = 0.980$, $P = 0.438$) and rearing temperature regime (two-way ANOVA, $F = 0.555$, $P = 0.460$) (Fig. 5B).

Significant daily rhythms of *trpm2* were found in both T (Cosinor, $P = 0.043$) and C (Cosinor, $P = 0.049$) groups peaking at ZT1.71 h and ZT3.36 h for T and C, respectively. Statistical differences were also found in *trpm2* expression with time points effects (two-way ANOVA, $F = 4.603$, $P = 0.001$) and an interaction between temperature regime and time point (two-way ANOVA, $F = 4.348$, $P = 0.002$) (Fig. 5C).

Lastly, significant daily rhythms were found for *trpa1b* expression under T and C (Cosinor, $P = 0.030$, $P = 0.033$, respectively) with their acrophases located at around the second half of the light phase (ZT6.55 h for T, ZT 8.91 h for C). Statistical differences were found in *trpa1b* expression depending on the time points (two-way ANOVA, $F = 3.365$, $P = 0.011$) (Fig. 5D).

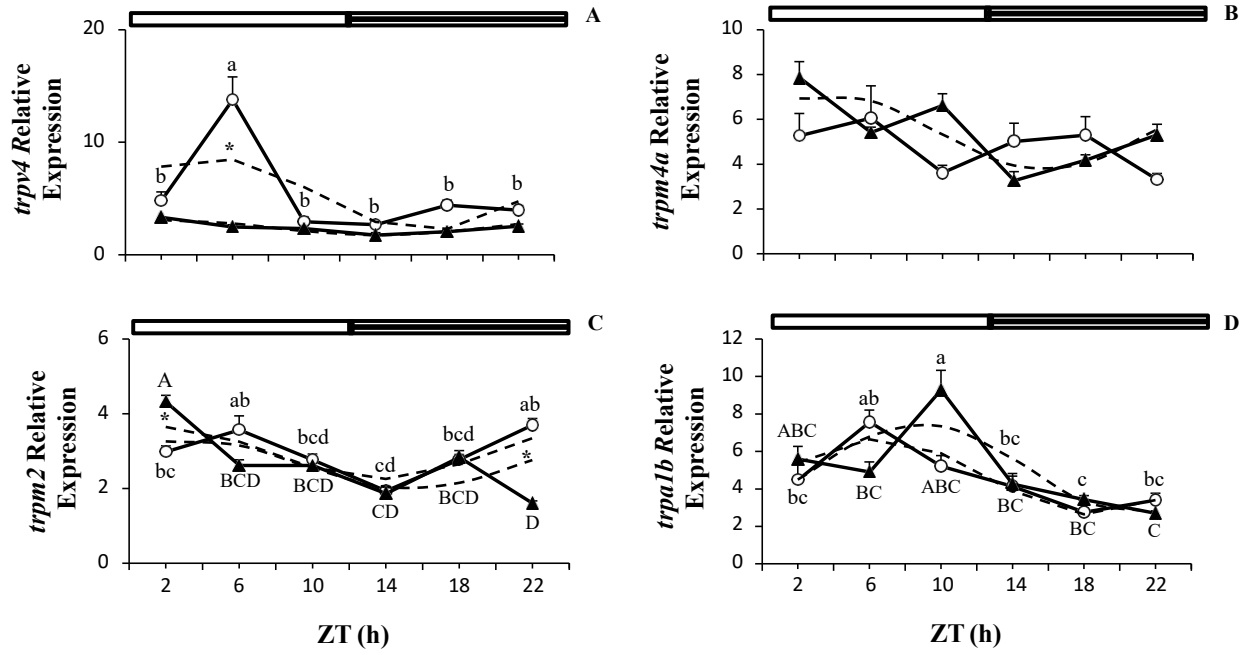

Fig. 5 Daily variations in the relative expression of the TRP genes in the zebrafish brains: *trpv4* (A), *trpm4a* (B), *trpm2* (C) and *trpa1b* (D). Fish were maintained in a 12:12LD cycle and two temperature regimes: T (thermophase: cryophase; 28:24±0.3ºC, white circles) and C (constant temperature, 26±0.3ºC, black triangles). The dashed line represents the adjustment to sinusoidal rhythm (Cosinor, $P< 0.05$). Different letters indicate statistical differences between the time points on the same graph (two-way ANOVA, $P< 0.05$). The asterisks indicate significant differences between groups in the same time point (two-way ANOVA).The white and black bars above the graphs represent the light and dark phases, respectively. The time scale (x-axis) is expressed as *Zeitgeber* Time (ZT), in which ZT0 h corresponds to light onset and ZT12h corresponds to lights off. Data are represented as mean ± SEM; n=6 replicates per point.

The sequence and time coordination of the above results are represented on a map of acrophases (Fig. A2) and in a table (Table A3) to help visualize the acrophases and Cosinor results of all the significant rhythms.

DISCUSSION

In this research, we assessed the influence of the rearing temperature regimes (T vs. C) on the daily rhythms of thermal tolerance. We found that the 4dpf zebrafish larvae obtained the highest mortality rates when reared in C and submitted to 39 ºC thermal shock at night (MD). The response of HSPs also differed depending on the time of day at which sublethal thermal shock was applied, with the highest gene expression occurring for *hsp70*, *hsp90ab1*, *hsp90aa1*, *gprp94* and *hspb1* in the zebrafish under C and subjected to thermal stress at MD. No significant daytime and rearing temperature regime differences were found for the

expression of *hsp47*, *cirbp* and TRPs. The daily rhythms in temperature-sensing (TRPs) and resistance (HSPs) expression appeared in the adult zebrafish brain mainly synchronized to the LD cycle, regardless of the rearing temperature regime. These findings may suggest a possible relation between TRP and HSP system in the thermal tolerance response of zebrafish.

The most critical period for temperature shock (39 ºC for 1 h) appeared at night, since significant differences in the mortality rates of the zebrafish larvae according to the time of day when thermal shock was applied. Under both the T and C conditions, similar mortality rates were detected at ML (middle of the light phase) (36-37% mortality). However, when thermal shock was applied at MD, the mortality rate significantly increased up to 62% in the larvae under the C conditions. This time-dependent response was not observed in the T group. On the one hand, previous studies performed in killifish (Podrabsky and Somero, 2004), rainbow trout (*Oncorhynchus mykiss*) (Threader and Houston, 1983) and zebrafish (Cortemeglia and Beitinger, 2006; Schaefer and Ryan, 2006, Xia et al., 2016, Wang and Xia, 2019), have described how fish reared in environments with variable temperatures display higher thermal tolerance and changes in behavioral responses than those reared at constant temperatures. Contrary to our results, other studies suggested that diel thermal cycling had little effect on acute thermal tolerance as has been described in other species such as Atlantic salmon (*Salmo salar*) (Corey et al., 2017, Gallant et al., 2017), killifish (Healy et al., 2012) and green sturgeon (*Acipenser medirostris*) (Rodgers et al., 2018). These studies also highlighted the influence of temperature amplitude and rate of temperature change in a diel thermal cycle which can considerably affect the acute thermal tolerance and other physiological functions (Schaefer and Ryan, 2006; Xia et al., 2016, Gallant et al., 2017). Although the rate of temperature change in a thermocycle did not affect acute thermal tolerance in zebrafish, our findings should be interpreted with care because zebrafish may experience a different rate of temperature change in the wild (Xia et al., 2016). Moreover, our findings suggested that the rearing temperature regime played an important role in the first development stages (from fertilization to 4dpf), compromising the daytime differences of animals' thermal tolerance. On the other hand, differences in the developmental age (between 102 and 114hpf) have to be considered, although previous experience in our laboratory suggested that such developmental differences may be ruled out (for instance when investigating daily rhythms in acute toxicity of ethanol in 5dpf zebrafish larvae, Vera et al.,

2018). The beneficial effect of daily thermocycles in early development has also been reported by Blanco-Vives et al. (2010) and Villamizar et al. (2012) in zebrafish and Senegalese sole, respectively. In their studies, the use of daily thermocycles rather than constant temperatures increased the growth and survival of larvae, and lowered the number of malformations suggesting that daily thermal cycles can affect metabolism, feeding efficiency and food intake (Morash et al., 2018). It is noteworthy that under farming conditions, fish have to cope with constant unnatural temperature regimes designed to optimize fish production without considering that these artificial protocols could compromise their physiological performance and, consequently, their welfare and survival (Basu et al., 2002; Morash et al., 2018; Villamizar et al., 2012).

Research in the last decade has revealed differences in the physiological responses of fish depending on the time of day when acute stress response (López-Olmeda et al., 2013), toxicity tests (Vera et al., 2018) or hormonal treatments (Costa et al., 2016) were performed. Regarding stress, the response to acute stressors (i.e. handling, air exposure, confinement) seems to be species-dependent in relation to daily behavior patterns, and this type of acute stress is better tolerated in the species' active phase. Thus diurnal species, such as gilthead seabream (*Sparus aurata*), show a more marked stress response at night, whereas nocturnal species like Senegalese sole display a more marked response in the daytime (López-Olmeda et al., 2013; Vera et al., 2014). However, in poikilothermic animals, body temperature oscillates, and depends mainly of environmental temperature regardless of the species' diurnal or nocturnal behavioral pattern. Hence all fish species will experience high temperatures in the light phase and low temperatures in the dark phase. Accordingly, we would expect the greatest thermotolerance to occur in the daytime and it would coincide with the natural thermophase. This hypothesis supported our findings, in which the zebrafish larvae reared under C conditions showed higher mortality rate in response to thermal stress at MD than at ML. Our results are also consistent with previous research which has shown that the killifish (*Fundulus heteroclitus*), a fish species with diurnal activity rhythms, presents higher thermotolerance at around the mid-photophase in a variety of photoperiod combinations (16:8, 14:10 and 8:16 LD and 14:10 DL) (Kavaliers, 1980; Healy and Schulte, 2012). However, more experiments are necessary to test this hypothesis with nocturnal fish.

HSPs constitute a highly conserved family of proteins that facilitate cellular recovery by allowing organisms to survive at temperatures that could be lethal for them (Fader et al., 1994; Iwama et al., 1998). Thus, basal levels of HSPs have been correlated with higher thermotolerance in several studies (for review see Basu et al., 2002, Feder and Hoffman, 1999). In most cases, changes in the HSP expression levels are correlated with parallel changes of HSP protein levels in fish (Todgham et al., 2006), suggesting the rapid translation of mRNA into protein within minutes after thermal exposure (Morimoto et al., 1992). Particularly in zebrafish brain, it has been described HSP expression increased significantly from 30 minutes to 4 hours after heat shock (Murtha and Keller, 2003). Our findings showed that the expressions of HSPs in the brain increased after sublethal thermal shock in zebrafish (37 ºC for 1 h) which highlights the importance of this tissue in the rapid response to acute thermal stress (Benítez-Dorta et al., 2017). Interestingly, our results also revealed remarkable time-dependent induction differences in the expressions of most HSPs (*hsp70*, *hsp90ab1*, *hsp90aa1, grp94, hspb1*) for the C group, but not for the T group. The effect of light in the induction of HSP expression has been poorly investigated in fish. A research in killifish showed that the induction of *hsp70* mRNA after a mild heat shock occurred only in the evening and at night, but not during the day (Healy and Schutle, 2012). Other studies performed in zebrafish showed a higher induction of *hsp90aa1* under constant darkness than under a LD cycle (Jeronimo et al., 2017). In addition to the effect of light, the existence of differences between temperature regimes suggested that the cycling temperature regime confers greater thermal tolerance by reinforcing cellular stress expression responses in zebrafish at MD, when fish are more sensitive to thermal challenges (cryophase) (Schaefer and Ryan, 2006; Healy and Schutle, 2012). Consequently, the acclimation temperature has been reported to influence the reduction of heat shock response in different fish species such as redband trout (*O. mykiss*) (Narum et al., 2013) and killifish (Fangue et al., 2006), suggesting a link between survival and thermal stress response. In our findings, we observed a lower induction in the expression levels of HSPs under T conditions at night compared with C conditions. This fact agrees with the idea that fish reared under T may developed an adaptive heat shock response mechanism in which the reduction of the high physiological costs and energy demands of producing HSPs, improving stress resistance, energy efficiency and consequently, survival and wellness (Sørensen et al., 2003). The high adult HSP expression

induction and high larval mortality rate in the C group subjected to thermal shock at MD could be explained by the costly energy demands of HSPs synthesis and the proteotoxicity effects of the accumulation of misfolded protein in the cytosol (Ananthan et al., 1986; Feder and Hoffman, 1999; Basu et al., 2002, Narum et al., 2013). Nevertheless, our results did not show any significant daytime differences in the expression of *hsp47* and *cirbp* in zebrafish brain under T or C. The absence of daytime differences in *hsp47* (collagen-related HSP) could be due to its induction pattern, which is tissue and stress-specific, differing from the expression patterns of other HSPs such as HSP70 as it has been described in zebrafish embryos (Lele et al., 1997). Hence the heat shock response observed in our experiments might have strongly induced the *hsp47* expression in the brain, decreasing and minimizing the daytime differences observed in the C group. As regards *cirbp*, its expression decreased and no differences between daytime sampling points were found, indicating that the role of CIRBP under hyperthermic challenges is limited and reinforcing its functional role against cold challenges (Grace et al., 2004).

ThermoTRP channels are related to perception of thermal stress in most organisms, although their role in the thermal shock has been little explored in fish (Voets et al., 2005). Our results showed that only the expression of *trpm4a* and *trpa1b* was affected by the heat shock, so that no significant changes were found in *trpv4* and *trpm2* expression. In addition, no TRPs presented daytime and rearing temperature regime expression differences, which indicates that sensing mechanisms are not daytime shock-inducible. Similar results about the absence of the thermal shock effect (42ºC for 3 h) on the induction of TRPV4 expression have been described (Bromberg et al., 2013). The relation of thermoTRP channels and heat sock proteins in thermal stress response has been little investigated. In mammals and insects, it was suggested that protection against thermal stress was coordinated by thermoTRP channels and HSPs (Hsu et al., 2015; Xiang et al., 2020). However in fish, this hypothesis is unclear. Jeronimo et al. (2017) research showed that a partially blockade of TRPV1 (using TRPV1 antagonist) decreased *per2* expression (clock gene period) and it inhibited indirectly the heat-induced increased expression of *hsp90aa1*. In agreement with our findings, heat shock induction may be exerted through a different pathway in which the biological clock machinery and its external inputs (light and temperature) play an important role.

We found that most HSPs and TRPs of zebrafish brains presented daily variations in the expression pattern synchronized mainly to LD cycle and to a lesser extent to the rearing temperature regime. In the last decade, considerable attention has been paid to the effect of environmental factors on HSP levels. Diurnal and seasonal changes of HSPs have been described in several organisms, such as plants (Merquiol et al., 2002) or mammals (Bitting et al., 1999). In addition, very few studies have reflected the functional role of daily and seasonal HSP rhythms in fish thermotolerance. For example, Fader et al. (1994) indicated that the production of HSPs shows seasonal variations in relation to water temperature, with the lowest levels appearing in winter. Regarding daily rhythms, studies performed in killifish found daily patterns of *hsp70* mRNA levels with higher values appearing at midday, which is consistent with our results in zebrafish (Healy and Schulte, 2012). Our findings indicated a nocturnal acrophase for the *cirbp* expression rhythm, which suggests greater cellular protection against apoptosis with cold temperature challenges at night. These results agree with studies performed in rodents, which are nocturnal animals, as they showed a daily rhythm of *cirbp* expression with a diurnal acrophase related to high body temperatures at night and low temperatures in the daytime (Nishiyama et al., 1998; Morf et al., 2012). Only *hsp47*, *cirpb* and *trpv4* daily rhythms were influenced by temperature regime, indicating that light was the predominant synchronizer of the brain compared to other environmental cues such as temperature. In addition, we may consider the influence of feeding on the synchronization of HSP and TRP expression rhythms in the brain of zebrafish, since zebrafish were fed during daytime to match the diurnal behavior of this species. The role of feeding as a synchronizer for peripheral oscillators like the liver has been described in many fish species (Lopez-Olmeda, 2017). However, regarding the brain, food is a synchronizer that exerts little effect on the circadian clock in this tissue, minimally affecting certain specific regions of the brain that would go unnoticed when analyzing the whole brain (Sanchez and Sanchez-Vazquez, 2009; Lopez-Olmeda et al., 2010). Although the effect of feeding time on thermotolerance was out of the scope in this research, this issue deserves further consideration as it may have metabolic effects (Morash et al. 2018). In general, the daily variations in HSPs fluctuated parallel to the temperature cycle that fish experience in nature, with higher expressions in the daytime when higher temperatures occur, and lower ones at night coinciding with lower temperatures. The influence of temperature of the thermal cycle on

daily variation of HSPs has been described in killifish (Podrabsky and Somero, 2004, Healy and Schutle, 2012), Atlantic salmon (Corey et al., 2017; Tunnah et al., 2017), rainbow trout (Callaghan et al., 2016) and tidepool sculpin (Todgham et al., 2006). However, when temperature rose at night after thermal shock (conversely to what happens in nature), we observed that the response to thermal stress was greater, as observed in the C group. This response was attenuated in the T group, which suggests that daily thermocycles could confer more tolerance to thermal shock at night. One possible hypothesis of the attenuated response of the T group exposed at MD could be the influence of the Fulton's condition factor on thermal tolerance (Gallant et al., 2017). Thus, several species of fish reared under thermocycles may present greater thermotolerance at night because they present higher growth rate and energy reserves (Blanco-Vives et al., 2010, Villamizar et al., 2012, Morash et al., 2018, do Espirito Santo et al., 2020). The energy reserves of fish reared under thermocycles may be necessary to supply the high energy costs of producing HSPs and minimize the negative impact of heat shock induction on fish physiology (proteotoxicity effects of high induction of HSPs) (Ananthan et al., 1986, Feder and Hoffman, 1999, Narum et al., 2013).

The relation between TRPs and HSPs and their role in the thermal shock has been discussed in few studies (Bromberg et al., 2013; Hsu and Yoshioka, 2015; Liu et al., 2020). TRP channels in fish brains are essential for accomplishing temperature sensitivity to maintain thermal homeostasis when thermal changes occur in the aquatic environment (Voets et al., 2005). Therefore, TRPs have been described to synchronize temperature cycles and are able to respond to light activation in *Drosophila melanogaster* (Niemeyer et al., 1996). In recent years, the link between circadian rhythms and TRPs has been investigated (Lee, 2013, Jeronimo et al., 2017). Our results describe the existence of daily expression patterns of TRPs for the first time in fish. These TRPs rhythms synchronized mainly to the LD cycle, regardless of the applied temperature regime. In addition, TRP rhythms took place in a phase with rhythms in the different analyzed HSPs. These genes, which are involved in warm thermal sensitivity (*trpv4*, *trpm2* and *trpm4a*), presented the highest expression in the daytime which coincided with the highest expression of HSPs involved in hyperthermic challenges (*hsp70*, *hsp90ab1*, *grp94*, *hsp90aa1*, *hspb1*, *hsp47*). Curiously, the cold-sensing *trpa1b* displayed a higher expression in the second half of the light period and slightly shifted toward the dark

phase, anticipating the increased expression of HSPs involved in hypothermic challenges (*cirbp*). Thus, our findings suggested the hypothetic existence of a coordinated daily time window when zebrafish brains present more sensitivity to detect temperature changes and quickly translate them to a complex system of cellular protection mechanisms. The synchronization of TRP and HSP system may perform a more effective and adaptive response that would minimize cellular damage.

CONCLUSIONS

Our results revealed the existence of daily rhythms in the mechanisms responsible for temperature sensing and protection synchronized mainly to the LD cycle. The time of day when an acute thermal challenge is applied, and the rearing temperature during early development, both considerably influence larvae mortality rates and induce the expression of thermotolerance mechanisms. We observed that the highest larvae mortality rates and the highest HSP expression were obtained for the zebrafish reared at a constant temperature and subjected to heat shock at midnight. Likewise, acclimation to thermocycles during development lowered daytime heat shock expression induction and larval mortality, which suggests a positive effect of thermocycles on the thermal tolerance of zebrafish. These findings reveal the necessity to consider time-dependent differences and daily rhythms when discussing physiological responses of thermal sensing and thermotolerance in zebrafish.

COMPETING INTERESTS

The authors declare no competing or financial interests.

AUTHOR CONTRIBUTIONS

GA, JFL and FJS conceived and designed the experiments, and wrote the manuscript; GA performed the experiments; GA and JFL analyzed the data; FJS provided funding.

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SUPPLEMENTARY DATA

Table A1 P values obtained in the three-way ANOVAs (experiment 1 and 2) and two-way ANOVA (experiment 3). The P-values (P) and F-Statistic (F) are reported for the Corrected model, rearing temperature regime (R), time of day (T), heat shock (H), and the interactions between factors (RxT, HxR, HxT, HxRxT). The grey color highlights the significant results ($P< 0.05$).

	Gene	Corrected model		R		T		H		RxT		HxR		HxT		HxRxT	
		F	P	F	P	F	P	F	P	F	P	F	P	F	P	F	P
Experiment 1	Mortality	35.007	0.000	2.144	0.151	8.083	0.007	221.56	0.000	1.519	0.225	2.144	0.151	8.083	0.007	1.519	0.225
Experiment 2	*hsp70*	37.080	0.000	0.922	0.343	11.369	0.020	230.361	0.000	0.286	0.596	0.920	0.344	11.452	0.020	0.280	0.600
	hsp90ab1	4.008	0.002	0.579	0.451	1.462	0.234	4.568	0.039	3.016	0.091	2.305	0.137	10.378	0.030	4.950	0.032
	grp94	26.395	0.000	0.286	0.596	3.597	0.065	162.664	0.000	7.840	0.008	0.283	0.598	4.789	0.035	5.539	0.024
	hsp90aa1	11.359	0.000	1.831	0.184	4.591	0.039	61.950	0.000	0.550	0.463	1.787	0.190	4.708	0.037	0.561	0.459
	hspb1	8.599	0.000	4.062	0.051	5.488	0.025	34.290	0.000	1.752	0.194	4.239	0.047	5.581	0.024	1.726	0.197
	hsp47	14.863	0.000	0.145	0.705	0.552	0.462	99.704	0.000	0.180	0.674	0.251	0.620	1.446	0.237	0.024	0.878
	cirbp	7.793	0.000	0.009	0.925	0.000	0.995	51.057	0.000	0.019	0.891	0.394	0.534	0.019	0.891	0.011	0.917
	trpv4	0.622	0.734	0.160	0.901	0.184	0.670	0.002	0.967	0.002	0.961	0.190	0.666	3.181	0.084	0.353	0.557
	trpm4a	5.522	0.000	1.189	0.283	3.764	0.061	33.799	0.000	0.279	0.601	0.125	0.726	0.639	0.430	0.087	0.769
	trpm2	0.602	0.750	0.621	0.436	0.021	0.885	0.544	0.465	2.431	0.127	0.086	0.771	0.650	0.425	0.003	0.960
	trpa1b	1.014	0.441	0.407	0.528	0.075	0.787	4.857	0.035	0.004	0.947	0.003	0.959	0.200	0.890	1.136	0.295
Experiment 3	*hsp70*	4.588	0.000	0.300	0.586	9.916	0.000			0.248	0.939						
	hsp90ab1	2.249	0.024	2.238	0.140	3.022	0.017			1.503	0.203						
	grp94	3.722	0.001	1.714	0.196	7.657	0.000			0.130	0.985						
	hsp90aa1	2.613	0.011	1.357	0.250	2.622	0.036			2.570	0.039						
	hspb1	2.070	0.037	1.854	0.179	3.254	0.012			0.881	0.500						
	hsp47	17.300	0.000	70.798	0.000	20.452	0.000			12.071	0.000						
	cirbp	4.553	0.000	16.075	0.000	1.466	0.216			5.679	0.000						
	trpv4	5.745	0.000	19.007	0.000	5.718	0.000			4.253	0.002						
	trpm4a	1.144	0.348	0.555	0.460	0.980	0.438			1.372	0.249						
	trpm2	4.596	0.000	1.943	0.169	4.603	0.001			4.348	0.002						
	trpa1b	2.150	0.036	0.303	0.585	3.365	0.011			1.510	0.206						

Table A2 Brain relative expression of the TRP genes: *trpv4*, *trpm4a*, *trpm2*, *trpa1b*. Fish were reared in two temperature regimes: T (thermophase:cryophase; 28:24±0.3ºC) *vs.* C (constant temperature; 26±0.3ºC). Zebrafish were exposed to sublethal thermal shock (37ºC for 1 h) at two different time points: middle of the light phase (ML, white bars); middle of the dark phase (MD, dark bars). For each group exposed to thermal shock (heat-stressed, H), its control group (non stressed; Ctrl) was sampled at the same time. The data from each variable were subjected to a three-way ANOVA, followed by Duncan's *post hoc* test. Data are represented as mean±SEM (n=6).

		trpv4		***trpm4a***		***trpm2***		***trpa1b***	
		Ctrl	**H**	**Ctrl**	**H**	**Ctrl**	**H**	**Ctrl**	**H**
T	**ML**	5.76 ± 0.94	3.91 ± 0.63	2.07 ± 0.14	4.62 ± 0.48	2.36 ± 0.11	2.32 ± 0.13	4.02 ± 0.41	3.21 ± 0.21
	MD	4.02 ± 0.29	4.81 ± 0.91	2.54 ± 0.18	5.60 ± 0.80	2.16 ± 0.09	1.72 ± 0.09	4.46 ± 0.38	3.15 ± 0.18
C	**ML**	5.86 ± 1.01	3.65 ± 0.62	1.21 ± 0.05	3.82 ± 0.39	2.12 ± 0.10	2.20 ± 0.13	4.29 ± 0.31	2.81 ± 0.33
	MD	2.68 ± 0.14	5.76 ± 0.45	1.92 ± 0.09	5.65 ± 0.31	2.64 ± 0.20	2.35 ± 0.21	3.35 ± 0.31	2.77 ± 0.37

Table A3 The Cosinor analysis results for the expression of HSPs and TRPs analyzed in the brains of the male zebrafish reared in a 12:12LD cycle and two temperature regimes: T (thermophase: cryophase; 28:24±0.3ºC) and C (constant; 26±0.3ºC). The mesor, amplitude and acrophase are indicated for each gene. Mesor and amplitude are indicated as relative expressions; acrophase is indicated in *zeitgeber* time (h).

Gene	Temperature Regime	Mesor (fold change)	Amplitude (fold change)	Acrophase (ZT hours)
hsp70	T	5.31 ± 1.47	4.57 ± 2.6	3.88 ± 2.3
	C	4.82 ± 1.02	3.61 ± 1.81	4.15 ± 2.00
hsp90ab1	T	-	-	-
	C	2.32 ± 0.4	0.97 ± 0.73	4.58 ± 3.17
grp94	T	2.64 ± 0.36	1.00 ± 0.64	5.38 ± 2.57
	C	2.33 ± 0.28	0.9 ± 0.49	5.03 ± 2.17
hsp90aa1	T	5.75 ± 1.05	3.14 ± 1.85	8.05 ± 2.34
	C	-	-	-
hspb1	T	4.65 ± 0.99	1.95 ± 1.76	6.96 ± 4.21
	C	5.77 ± 1.26	2.49 ± 2.24	3.53 ± 4.28
hsp47	T	20.54 ± 5.29	23.44 ± 10.07	4.18 ± 1.55
	C	4.71 ± 0.98	3.34 ± 1.74	3.88 ± 2.08
cirbp	T	2.59 ± 0.3	0.61 ± 0.53	22.75 ± 4.1
	C	1.99 ± 0.18	0.33 ± 0.32	13.3 ± 4.86
trpv4	T	5.37 ± 1.8	3.27 ± 3.25	4.75 ± 5.75
	C	2.42 ± 2.33	0.66 ± 0.59	2.18 ± 4.47
trpm4a	T	-	-	-
	C	5.44 ± 0.88	1.67 ± 1.58	3.75 ± 4.78
trpm2	T	2.95 ± 0.38	0.7 ± 0.68	1.71 ± 4.97
	C	2.65 ± 0.37	0.65 ± 0.64	3.36 ± 5.72
trpa1b	T	4.65 ± 1.04	2.02 ± 1.85	6.55 ± 4.12
	C	5.06 ± 1.25	2.38 ± 2.21	8.91 ± 4.22

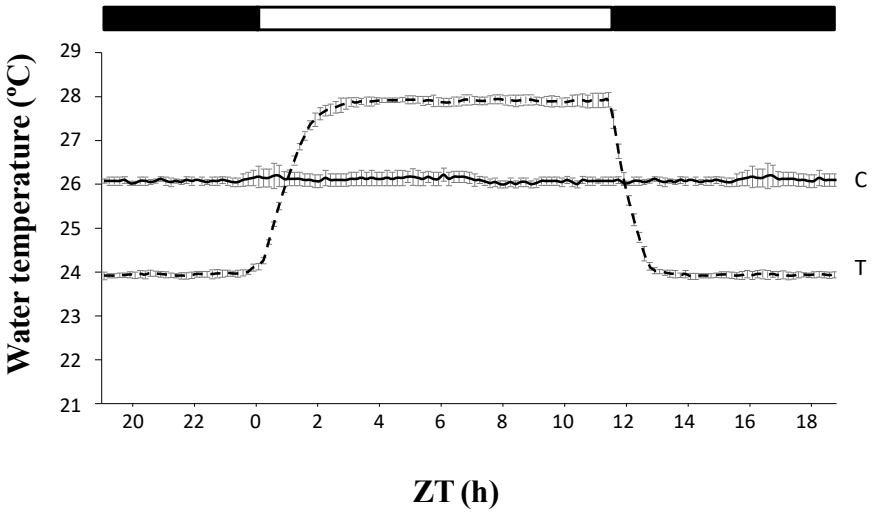

Fig. A1 Daily average water temperature throughout the experiments in the two temperature regimes herein tested: a thermocycle (T) of 28ºC:24ºC (dashed line) or a constant temperature (C) of 26ºC (continuous line). The presented data are expressed as mean±S.D.

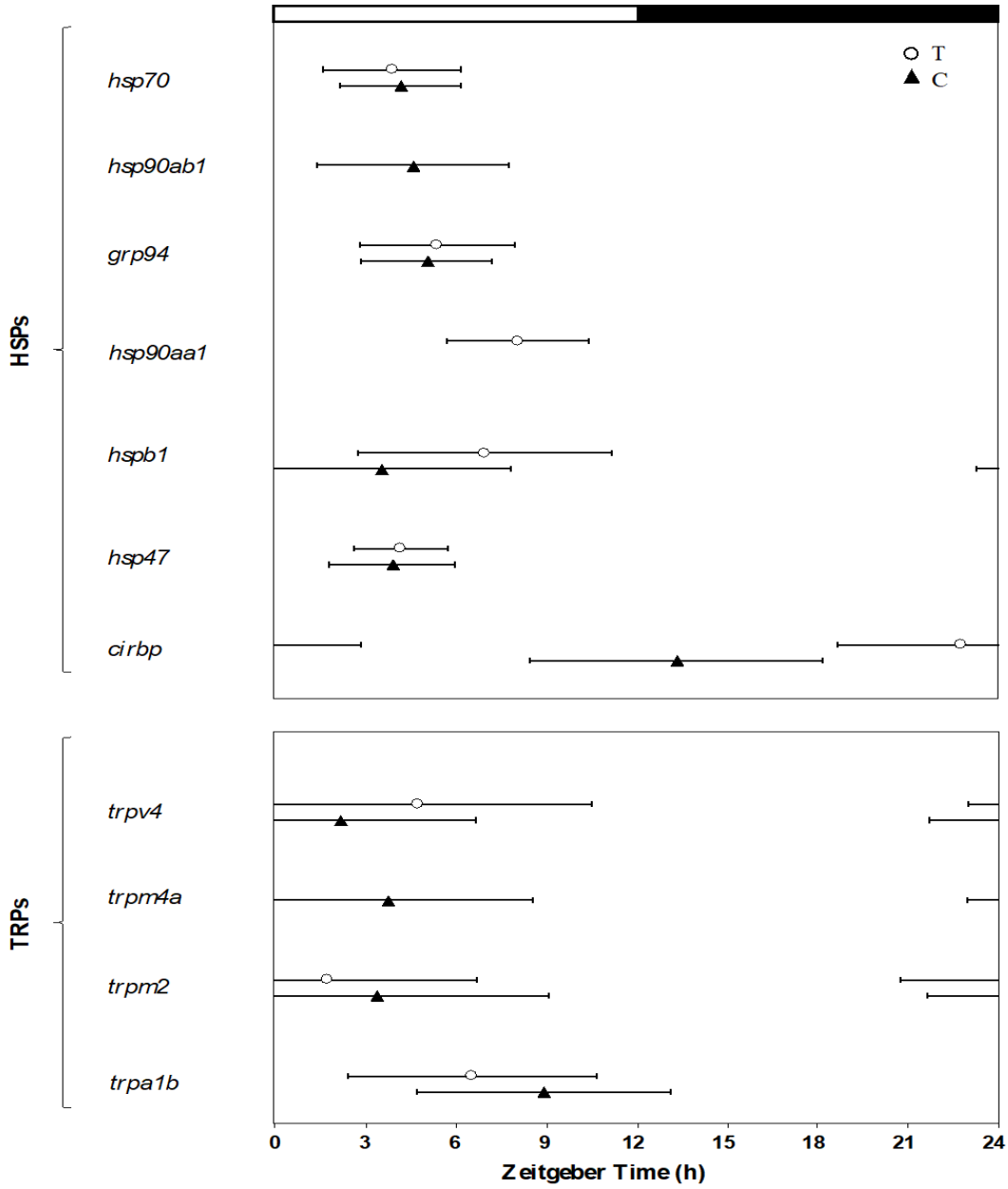

Fig. A2 Map of the acrophases of the factors involved in the thermal tolerance (HSPs) and sensing (TRPs) mechanisms analyzed in the brains of the male zebrafish reared in a 12:12LD cycle and two temperature regimes: T (thermophase: cryophase; 28:24±0.3ºC) (white circles) and C (constant; 26±0.3ºC) (black triangles). The acrophase is indicated only for the significant rhythms (Cosinor, $P< 0.05$). The name of each represented factor is indicated on the left. The light and dark phases are represented with white and black bars, respectively. The time scale (x-axis) is expressed as *Zeitgeber* Time (ZT), in which ZT0 h corresponds to light onset and ZT12h corresponds to light off.

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Experimental Chapter VI

Combined blue light and daily thermocycles enhance zebrafish growth and development

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ABSTRACT

In the wild, the light/temperature environment cyclically oscillates insofar as the temperature rises after dawn and drops after dusk. In the underwater photo-environment, light is filtered through the water column so that blue photons reach greater depths. This paper investigates the combined effects of both factors with two temperature regimes (constant temperature = 26ºC, CTE *vs.* daily thermocycle = 28ºC day:24ºC night, TC) and three light wavelengths (white-W, blue-B, red-R) on *Danio rerio* embryos and larvae from fertilization to 30 days post-fertilization (dpf). It studied hatching rate, larval survival, growth, and food intake (gut content). It analyzed the expression of the genes involved in stress (*crh*), somatic growth (*gh*, *ifg1a*, *igf2a*), and food intake control (*npy*, *agrp*, *ghrelin*, *orexin*, *mch1*, *mch2*, *grp*, *cck8)* at 10 and 30 dpf. The results revealed that the lowest hatching rate was in R regardless of the temperature regime. The highest growth rate was for the larvae reared with B+TC, which was consistent with the highest expression values of the growth factors. The highest feeding and expression levels of the genes involved in food intake were for the larvae in B (regardless of the temperature regime) and W+TC. Conversely, the R+CTE combination obtained the worst growth and feeding results. These findings indicate that the best larval performance can be achieved with combinations of blue wavelengths and cyclic temperature regimes that come closer to those in the natural environment. These results should be considered when optimizing rearing protocols to improve the growth and welfare of the fish larvae.

Keywords: Zebrafish, Light spectrum, Daily thermocycle, Larvae development.

INTRODUCTION

The Earth is submitted to environmental cyclic variations caused by predictable geophysical cycles, such as the Earth's axial rotation. Thus, daily temperature and light cycles became a selective pressure and favored the appearance of biological clocks, which animals use to keep track of time and to anticipate periodic events to optimize physiological processes and survival success (Aschoff, 1981).

Although not all species are subjected to them (e.g., deep-sea fish living below the thermocline or fish living in caves), light and temperature in most aquatic environments oscillate daily in a linked dynamic way: water temperature rises after sunrise and drops after sunset (i.e. daily thermocycle). Daily thermocycles can synchronize the daily rhythms of most organisms, ranging from unicellular algae to vertebrates (Rensing and Ruoff, 2002). In zebrafish (*Danio rerio*), the thermophase (high temperature) produces a similar physiological response to the light phase, while the cryophase (low temperature) is associated with nocturnal responses (López-Olmeda and Sánchez-Vázquez, 2011). Indeed, daily temperature cycles affect a wide range of fish larval activities, including survival, development, sex differentiation, behavioral rhythms, and the molecular clock as it has been described in Senegalese Sole (*Solea senegalensis*) and zebrafish (Lahiri et al., 2005; López-Olmeda et al., 2006; Blanco-Vives et al., 2010, 2011; Villamizar et al., 2012). In addition to water temperature, photoperiod, the light spectrum, and intensity strongly influence fish physiology in all life stages from fertilization to sexual maturation (Mangor-Jensen and Waiwood, 1995; Villamizar et al., 2011, 2014; Ikegami et al. 2014). The water column acts as a potent chromatic filter by modifying the spectral profile of sunlight so that the wavelengths below violet ($\lambda<390$nm) and beyond red ($\lambda>600$nm) are selectively absorbed. Therefore, blue wavelengths reach greater depths (McFarland, 1986). Phototransduction in zebrafish is mediated mainly by the pineal gland and the retina (Falcon et al., 2003; Vatine et al., 2011). In the teleost retina, light is absorbed by photopigments of photoreceptor cells (rod and cones), which are involved mainly in brightness/intensity detection, and also in visual acuity and color discrimination, respectively (Kusmic and Gualtieri, 2000). Furthermore, non-visual photoreceptors are present in every zebrafish cell (Whitmore et al., 2000). A wide variety of

these non-visual photoreceptors, which cover sensitivity to the entire visible light spectrum, has been described in zebrafish (Hankins et al., 2014).

The influence of light spectrum and diel thermocycles during early fish development has been separately investigated and shows different physiological responses depending on fish species as it has been reviewed in some aquacultured teleost species (Villamizar et al., 2011). The light wavelength and intensity to which fish embryos and larvae are exposed can have profound effects on fish survival, malformations, growth, and performance (for a review see Villamizar et al., 2011, Ruchin, 2020, 2021). Diel thermocycles can influence the metabolic rate, food intake control and growth efficiency in some wild fish species such as yellow perch (*Perca flavescens*) and green sturgeon (*Acipenser medirostris*) (Konstantinov et al., 2005, Coulter et al., 2016; Rodgers et al., 2018). On the other hand, other authors suggested that daily thermocycles may impact negatively or not significantly on fish metabolism and growth (Morash et al., 2018). Given the scarcity of studies the metabolic effects of thermocycles in fish, there is a need for more research on this topic.

Physiologically, the light wavelength and temperature can both stimulate and suppress fish nutrition influencing the development, growth, and other physiological processes of fish (Ruchin, 2021). Biochemically, fish growth development can be explained by changes in the neuropeptide expression involved in the endocrine regulation of survival, somatic growth, food intake control, and digestion process (Rønnestad et al., 2017; Bertucci et al., 2019). Regarding survival, corticotropin-releasing hormone (Crh) is the main stress response-regulating factor in fish and is also related to anxiogenic, anxiolytic and appetite-inhibiting roles (Volkoff et al., 2005; Matsuda et al., 2013). The most important neuropeptides involved in somatic growth regulation are the growth hormone (Gh) and insulin-like growth factors (Igf) as a decrease in the previous factors can inhibit growth and other physiological functions in fish (i.e. food intake) (Reinecke et al., 2005; Saera-Vila et al., 2009; Bertucci et al., 2019; Canosa and Bertucci, 2020). Other hormones are considerably implicated in the endocrine control of food intake and digestion. These factors act as appetite-stimulating factors (orexigenic peptides): neuropeptide Y (Npy), agouti-related peptide (Agrp), ghrelin, orexin, melanin-concentrating hormones 1 and 2 (Mch1 and Mch2); appetite-inhibiting factors (anorexigenic peptides): gastrin-releasing peptide (Grp) and cholecystokinin-8 (Cck8) (Koven

and Schutle, 2012; Matsuda et al., 2012a,b; Takahashi et al., 2016; Rønnestad et al., 2017; Bertucci et al., 2019).

Zebrafish is a widespread fish model used by biomedical and aquaculture research (Ribas and Piferrer, 2014). It is a eurythermal species that live in environments subjected to light and thermal fluctuations. For instance, in one of its natural environments, the River Ganges, both solar radiation and air thermal variation cause daily water temperature changes that can oscillate several degrees (between 0.1 and 5.6ºC) in the daytime (Payne and Temple, 1996). In zebrafish larvae, the effects of the light spectrum (from violet to red) and temperature regimes (constant vs. cycling) have been separately studied (Villamizar et al., 2012, 2014; Sánchez-Vázquez and López-Olmeda, 2018; Wang and Xia, 2019). In zebrafish, blue light elicited the best larval performance in terms of hatching, growth, survival and occurrence of malformations while red lights presented the worst results, causing the death of all larvae during development (Villamizar et al., 2014). In addition, beneficial effects on early larval development were found in zebrafish reared under daily thermocycles compared to constant temperatures (Villamizar et al., 2012). Although these studies showed separately the profound effects of light spectrum and temperature regimes on zebrafish development, no study to date has evaluated the effect of the combination of both factors. Moreover, these previous studies did not delve into the underlying physiological mechanisms that generate such effects, neither the ones that cause the improvement observed under blue light or thermocycles nor the ones that cause the lethal effects of red light applied to zebrafish during development.

Therefore, this research aimed to investigate the combined effect of daily thermocycles and light spectrum during early development in zebrafish, especially on understanding which mechanisms drive the enhancement under blue light or thermocycles and the deleterious effects of red wavelengths. For this purpose, the following developmental markers and physiological parameters were evaluated: hatching rate, survival, larval growth, food intake, and the mRNA expression of genes related to stress, growth, and food intake control (Figure 1).

MATERIALS AND METHODS

Animals and housing

The present research was conducted at the facilities of the Department of Physiology of the University of Murcia (Spain). Fish were reared following Spanish legislation on Animal Welfare and Laboratory Practices. Experimental protocols were performed following the Guidelines of the European Union (2010/63/UE) and Spanish legislation (RD 53/2013 and Law 32/2007) for the use of laboratory animals. They were approved by the National Committee and the Committee of the University of Murcia on Ethics and Animal Welfare.

Zebrafish adults and larvae were raised at the Fish Chronobiology laboratory in the Faculty of Biology at the University of Murcia (Spain). Broodstocks were obtained from a local supplier (Alimar Pets, Murcia, Spain) and acclimatized to laboratory conditions for 2 months according to standard methods (Nüsslein-Volhard and Dahm, 2002). Six groups of sexually mature zebrafish were used, with a 1:2 (female: male) sex ratio per group. The broodstock groups were set in the afternoon and kept overnight, and fertilized eggs were collected within the first 2 h after lights on. Then all the fertilized eggs were pooled and sorted into groups of 30 eggs in floating Petri dishes (85×10 mm) with embryo medium (E3 medium, Westerfield, 2000).

Experimental design

The complete experimental procedure was repeated 3 times using different broodstock groups, which means that the presented results came from three independent experiments. In all the experiments, 1728 (N) embryos and larvae were employed. In a single independent experiment, 576 fertilized embryos were distributed into six different experimental treatments using a given combination of three different wavelengths (white, W, blue, B, and red, R) and two distinct temperature regimes (constant temperature *vs.* daily thermocycles) (Supplementary Fig. S1). For each experimental group, four Petri dishes (n=96 embryos, 24 embryos per Petri dish) were utilized. Spectral analysis of lights was performed with a

spectroradiometer (FieldSpec® Hand-Held spectroradiometer UV/VNIR, ASD Colorado, USA) and a lux meter (MX Elektronik Minilux, Germany). This gave the spectral composition of each LED light expressed as the percentage of irradiance (white λ_{peaks}=466 nm and 668 nm; blue λ_{peak}=472 nm and red λ_{peak}=665 nm) (Supplementary Fig. S1a). The photoperiod was set at 12L:12D h (Light: Dark cycle) (lights on at 09:00h) in all the groups. The two tested temperature regimes were: constant temperature (CTE) of 26ºC (26.1±0.1ºC) or daily thermocycle (TC) with a thermophase of 28ºC (27.9±0.1ºC) occurring in the daytime and the cryophase of 24ºC (24.1±0.1ºC) occurring at nighttime (Supplementary Fig. S1b). Water temperature was modified by water heaters (Askoll, Povolaro, Italy) and cooler units (Aqua Medic Titan 1500 GmbH, Bissendorf, Germany) controlled by an electronic timer (Bachmann GmbH & Co, Stuttgart, Germany). Water temperature was recorded continuously throughout the experiment by underwater data recorders (HOBO PENDANT, Onset Computer Corporation, Massachusetts, USA). Zebrafish embryos and larvae were reared under these conditions from 0 to 30 days post-fertilization (dpf). At 5 dpf, larvae were transferred from the Petri dish to 2.5-liter nursery net cages (SERA GmbH, Heinsberg, Germany). Larvae mouth opening occurred 2 days after hatching (5 dpf). From that time, they were fed powder feed (Vipan Baby, SERA, Germany) to satiety twice a day at 11:00h and 16:00h (Westerfield, 2000).

To evaluate the performance of both the embryo and larvae reared under the six different experimental conditions, the following variables were analyzed: hatching rate, survival, growth, feeding activity. Whole larvae samples were collected before the first feeding point at 10 and 30 dpf for the mRNA expression analysis of the genes involved in the stress response, growth, and food intake control. For the gene expression studies, larvae were immediately euthanized by anesthetic overdose and death was confirmed under the microscope according to the guidelines for fish euthanasia (RD 1386/2018). Then larvae were pooled for each replicate to obtain two replicates per group/experiment, which gave six replicates (n=6) during the three independent experiments. The number of larvae used in each pool replicates differed depending on the stage: 12 larvae/pool at 10 dpf and 3 larvae/pool at 30 dpf. Larvae were stored in 1.5 ml sterile tubes and immediately frozen at -80ºC until the gene expression analysis.

Hatching and survival rates

The hatching rate was calculated as the percentage of embryos hatched at 3 dpf from the total number of embryos. Survival was measured every 2 days from 4 to 30 dpf (Figure 1) and was calculated as the percentage of live larvae from the total of larvae at 4 dpf. To measure larvae mortality, a binocular microscope (Leica EZ4 HD, Leica Microsystems GmbH, Wetzlar, Germany) was used to observe the cessation of heartbeat and blood circulation, which was set as an endpoint (Villamizar et al., 2014).

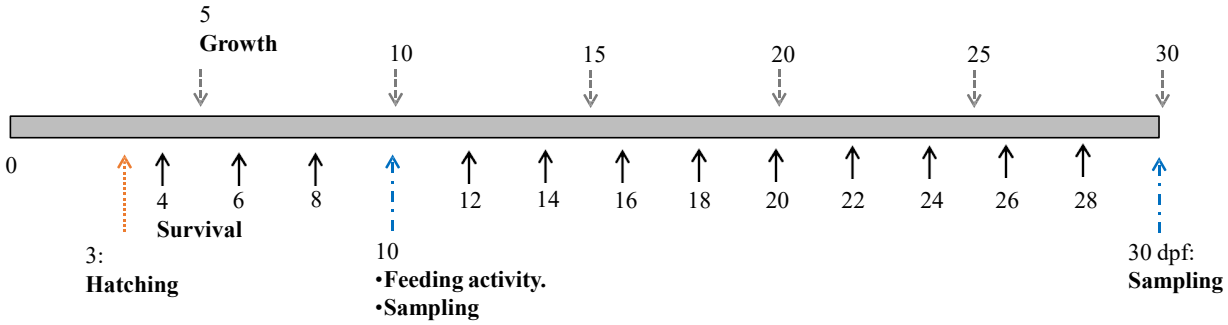

Fig. 1 Schematic representation of the experimental design indicating the variables analyzed from day 0 to 30 post fertilization (dpf). The orange dotted arrow indicates the hatching rate measurement at 3 dpf. The continuous black arrows denote the mortality measurement, taken every 2 days from 4 dpf. The grey continuous arrows represent the growth measurement, taken every 5 days from 5 dpf. The blue dotted arrows indicate the sampling points for analyzing gene expression. In addition, at 10 dpf, food intake was measured.

Growth rate and food intake (gut content)

The growth rate was assessed by longitudinally measuring the total lengths (TL) of 10 larvae per experimental group every 5 days from 5 to 30 dpf (Figure 1). Larval measurements were taken from living animals by a digital camera mounted on a binocular microscope and using the "ImageJ" image processing software (v. 1.8.0_112, Wayne Rasband, National Institute of Mental Health, Bethesda, USA) (Abramoff et al., 2004). At 10 dpf, the food intake of six larvae per experimental group was determined by calculating the proportion of larvae's digestive tube (DT) filled with food 1 h after being fed in relation to its TL (Villamizar et al., 2009).

Gene expression analysis: real-time RT-PCR analysis

Larvae pool samples were individually homogenized in Trizol reagent (Ambion, Thermo Fisher Scientific, Waltham, USA) using a tissue homogenizer for mechanical homogenization (TissueLyser LT, Qiagen, Hilden, Germany). RNA was extracted according to the manufacturer's instructions. The RNA pellet from the larvae samples of 10 and 30 dpf was dissolved in 15 and 50 µl of sterile DEPC water (Invitrogen, CA, USA), respectively. The RNA concentration was determined by spectrometry (Nanodrop ND-1000, Thermo Fisher Scientific). Then RNA (1 µg) was first treated with 1U of DNase I (Thermo Fisher), followed by retrotranscription with a commercial kit (QSCRIPT cDNA Synthesis Kit, Quantabio, Beverly, USA). All the cDNA samples were diluted (1:10) in nuclease-free water (Thermo Fisher Scientific) and stored at -20ºC for subsequent analyses. The quantitative PCR (qPCR) reactions were performed using Perfecta® SYBR® Green Fastmix (Quantabio). All the samples were run in duplicate and qPCR reactions were performed in a final volume of 20 µl. The quantitative PCR (qPCR) analyses were run in a light thermocycler (7500 RT-PCR system, Applied Biosystems) following this protocol: 15 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Melting curves were run after amplification to ensure that only one DNA species was amplified. Twelve genes were selected and divided according to their physiological functions in embryo and larvae performance: stress and survival (*crh*), growth (*gh*, *igf1a*, *igf2a*), and food intake control (*npy*, *agrp*, *ghrelin*, *orexin*, *mch1*, *mch2*, *grp*, *cck8*). All the primer sequences (Table 1) were designed with the Primer 3 plus software (Untergasser et al., 2012). The relative amplification efficiencies of all the genes were analyzed by cDNA dilution curves. The primer concentrations were determined by a primer dilution curve. The primers of *crh*, *igf2a*, *agrp*, *ghrelin*, *orexin*, *mch1, grp*, and *cck8* were added at a final concentration of 200 nM. The primers of *gh*, *igf1a*, *npy*, and *mch2* were added at a final concentration of 400 nM. The relative expression of all the genes was calculated by the $2^{-\Delta\Delta Ct}$ method, and using zebrafish *ef1a* as the housekeeping gene after assessing that its coefficient of variation (CV) was lower than 5% between the experimental groups.

Table 1 The primer sequences used for the quantitative PCR analyses**.**

Gene	F/R	Sequence (5'-3')	GenBank Accession number
ef1a	F	CTGGAGGCCAGCTCAAACAT	NM_131263.1
	R	ATCAAGAAGAGTAGTACCGCTAGCATTAC	
crh	F	GCCGCGCAAAGTTCAAAA	BC085458.1
	R	GCGAGGAGAATCTGTGCGTAA	
gh	F	AAGATCAGTGTTCAAAGGGTTCACT	NM_001020492.2
	R	TTAAGGCAAGAATCTATCAGACAGACA	
igf1a	F	CAGGCAAATCTCCACGATCTC	AH010825.2
	R	CTTTGGTGTCCTGGAATATCTC	
igf2a	F	GTGAAGTCGGAGCGAGATTGTT	NM_131433.1
	R	GAGCCTGTGACACTG GGAAGA	
npy	F	GACTCTCACAGAAGG GTATCC	BC162071.1
	R	GGTTGATGTAGTGTCTTAGTGCTG	
agrp	F	TCGCACAGAGAATCCACAGAG	NM_001328012.1
	R	TAAAACCGCAGCCAATGGTG	
ghrelin	F	CAGCTTCCTCAGTCCGACTCA	EU908735.1
	R	TTCTCTTCTGCCCACTCTTGGT	
orexin	F	GTCGCCAGACATTTAGTGCATC	NM_001077392.2
	R	TTCGCCCACTTTACGTTTGC	
mch1	F	CAAACCGCTAAAGCAAACGC	NM_001162488.1
	R	AAAGTGCAACGGTGATGAGG	
mch2	F	GCTGGCAAGCTTGAAAATGG	FJ204828.1
	R	TTGCAAGATCAAGGGATGGC	
grp	F	GACAACACAGAGGTCAACGCTTT	NM_001161350
	R	ACTGGCGTCCCTTTTCGAT	
cck8	F	CAAAGGCTCATACCGCAGAAG	XM_001346104
	R	TCTGTGAGATGCACCCATGGT	

Statistical analysis

All the results are expressed as the mean±SEM. The SPSS software (v. 19.0, IBM Analytics, Armonk, USA) performed the statistical analysis. The normality of data distribution was checked by the Kolmogorov-Smirnov test and homogeneity of variance by

Levene's test. The data from each gene and day were subjected to a one-way ANOVA to check for statistically significant differences between the six experimental groups, and to a two-way ANOVA to analyze the effects of light wavelength (W, B, R), temperature regimes (TC, CTE) and their interaction. The one-way ANOVA was followed by Duncan's *post hoc* test to determine statistically significant differences between the experimental groups. The significance threshold was set at p=0.05 in all statistical tests.

RESULTS

Effects of light wavelength and daily thermocycles on hatching, survival, and growth

At 3 dpf, all the larvae had either hatched or died during embryonic development, and the hatching rate was calculated on this day. The analysis detected statistically significant differences between lighting conditions (p=0.044), but not between temperature regimes (p=0.809) (two-way ANOVA, Supplementary Table S1, Fig. 2). The embryos left in B and W light obtained higher hatching rates (70.4±5.1% and 69.2±3.5%, respectively) than those reared in R light (59.7±5.9%) (two-way ANOVA, p<0.05) (Fig. 2).

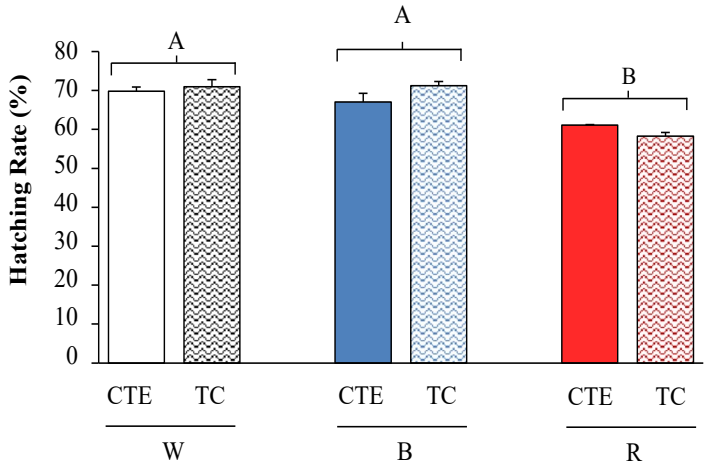

Fig. 2 The hatching rates (%) of the zebrafish exposed to the combination of three different light wavelengths (white, W; blue, B; red, R) and two temperature regimes (a thermocycle of 28:24ºC thermophase:cryophase, TC; and a constant temperature of 26.1±0.1ºC, CTE). Data (n=6) are expressed as mean±SEM of the percentage of hatched eggs, calculated at 3 days post fertilization (dpf). Different upper case letters indicate significant differences between light treatments (two-way ANOVA, p<0.05).

From 4 to 30 dpf, survival was measured every 2 days in all the groups (Fig. 1). Zebrafish larvae survival presented striking differences, which depended mainly on the light wavelength, although an effect of temperature regime was also detected (Fig. 3) (one- and two-way ANOVA, $p<0.05$, Supplementary Table S1). The highest survival rates were for the larvae reared in either W or B, with a final survival (at 30 dpf) of 78.9±4.8% and 75.5±3.2%, respectively (Fig. 3). In contrast, R obtained 100% mortality, although some differences were observed depending on the temperature regime (Fig. 3) (two-way ANOVA, $p<0.001$). In R+CTE, survival was significantly lower right from the start (6 dpf), while the survival of the larvae reared in R+TC was significantly lower as of 14 dpf. A significant effect of temperature regime was detected from 6 to 14 dpf, with the groups reared in TC surviving better than in CTE (two-way ANOVA, $p<0.01$). No significant differences in survival rates were observed between the larvae reared in W and B regardless of the temperature regime (two-way ANOVA, $p>0.05$, Supplementary Table S1).

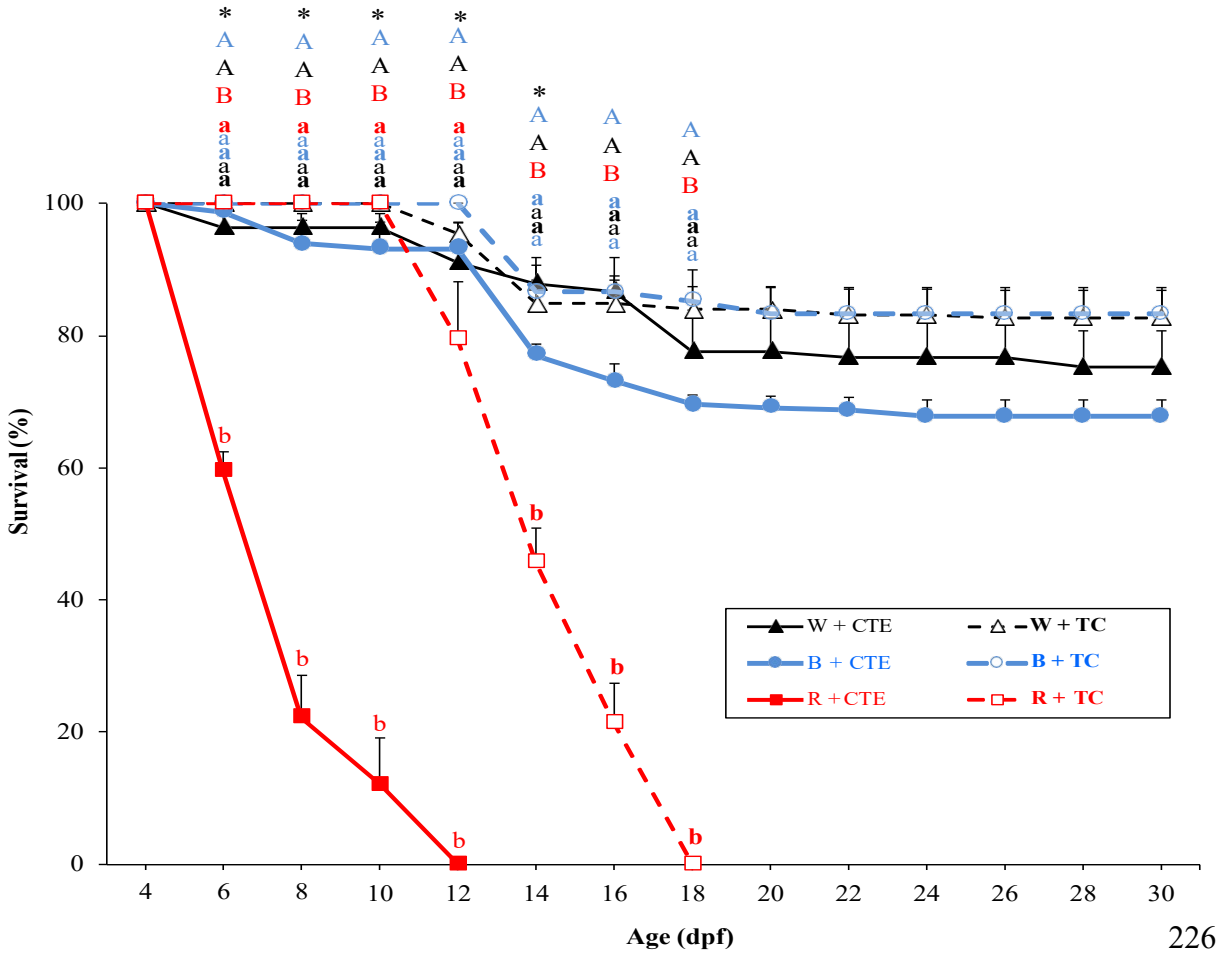

Fig. 3 Effect of three different light wavelengths (white, W; blue, B; and red, R) and two temperature regimes (a thermocycle of 28:24ºC thermophase:cryophase, TC; and constant temperature of 26.1±0.1ºC, CTE) on zebrafish larval survival from 4 to 30 days post fertilization (dpf). Triangles, circles and squares indicate the groups in the W, B and R light spectra, respectively. Dashed and continuous lines denote the groups kept at the TC and CTE temperatures, respectively. Different lower case letters indicate significant differences between the experimental groups within the same dpf (one-way ANOVA, $p<0.05$). Different upper case letters and asterisks indicate significant differences between light treatments and rearing temperature regimes, respectively, at the same dpf (two-way ANOVA, $p<0.05$). Data (n=4) are represented as mean±SEM.

The larval length was measured every 5 days from 5 to 30 dpf to evaluate the effects of the different experimental conditions on growth. Statistically significant differences in length were observed from 10 dpf onward (Fig. 4) (one-way ANOVA, $p<0.05$; Supplementary Table S1). At 10 dpf, statistically significant differences due to the lighting conditions, but regardless of temperature, were noted (two-way ANOVA, $p<0.05$; Supplementary Table S1). From 15 to 30 dpf, significant differences in growth were due to not only lighting but also to the temperature conditions (two-way ANOVA, $p<0.05$; Supplementary Table S1). In general, the larvae that obtained the highest growth rates were those reared in B+TC (Fig. 4). This effect was evidenced from 20 dpf onward, when the larvae from B+TC were longer (9.2±0.1 mm) than those in the other groups (6.9-7.3 mm) (one-way ANOVA, $p<0.05$). When examining the lighting conditions, the growth rate for the fish maintained in B was higher than for those reared in W from 10 to 30 dpf, and then those larvae reared in R, which were shorter than those in B and W (Fig. 4) (two-way ANOVA, $p<0.01$). Moreover from 15 dpf to the end of the experiment (30 dpf), the larvae reared in TC had a higher growth rate than those reared in CTE (Fig. 2) (two-way ANOVA, $p<0.01$).

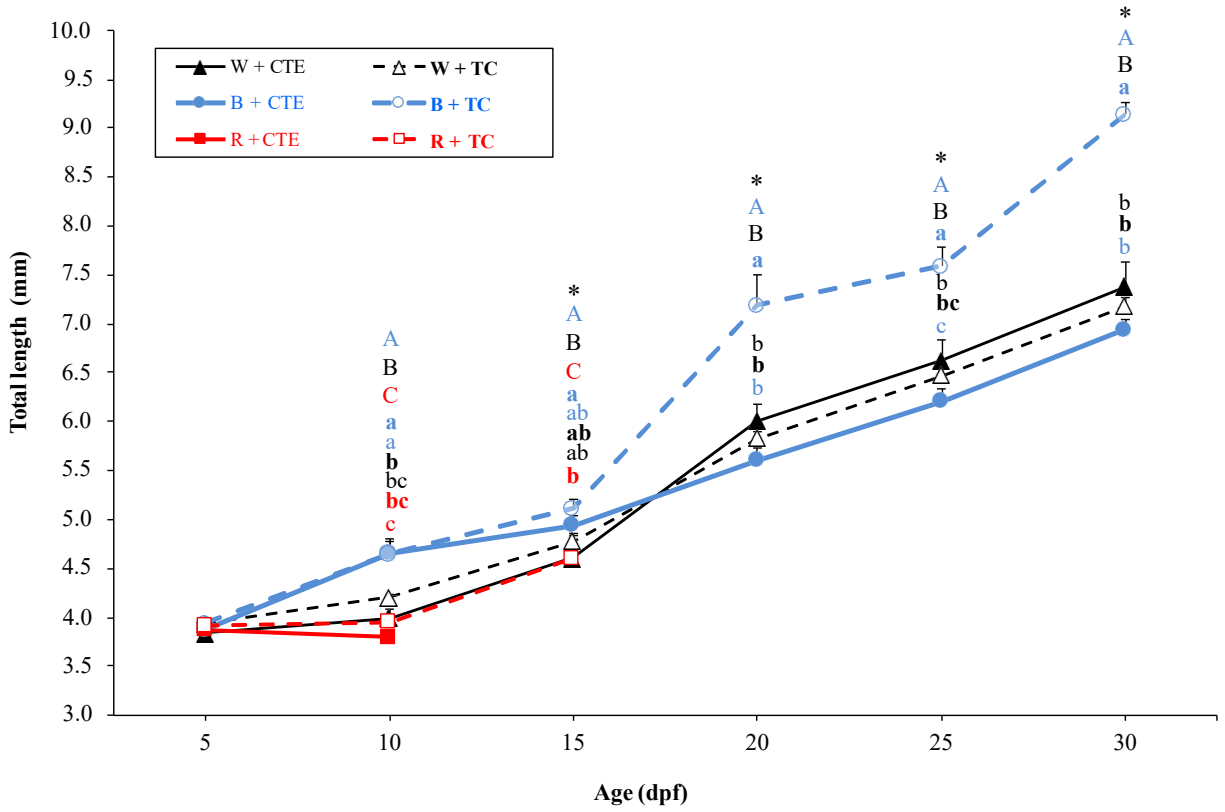

Fig. 4 Effect of three different light wavelengths (white, W; blue, B; and red, R) and two temperature regimes (a thermocycle of 28:24ºC thermophase:cryophase, TC; and constant temperature of 26.1±0.1ºC, CTE) on the total length (mm) of zebrafish larvae from 5 to 30 days post fertilization (dpf). Triangles, circles and squares indicate the groups in the W, B and R light spectra, respectively. Dashed and continuous lines denote the groups kept at the TC and CTE temperatures, respectively. Different lower case letters represent significant differences between the experimental groups on the same dpf (one-way ANOVA, $p<0.05$). Different upper case letters and asterisks indicate significant differences between the light treatments and rearing temperature regimes, respectively, on the same dpf (two-way ANOVA, $p<0.05$). Data (n=10) are represented as mean±SEM.

Effects of light wavelength and thermocycles on food intake (gut content)

To test whether the survival and growth results were related to food intake, we analyzed the presence of food in the digestive tract of the 10 dpf larvae 1 h after mealtime. Statistically significant differences were observed in the experimental groups (Fig. 5) (one-way ANOVA, $p<0.001$; Supplementary Table S1). The highest food intake levels were for those in B+TC (67.0±1.1%), W+TC (54.3±1.8%) and B+CTE (53.1±2.2%), whereas the lowest food intakes were found in the larvae in R (0.8±0.2 and 8.4±1.0% in R+CTE and R+TC, respectively). In general, the larvae reared in B and W presented higher food intake

than those reared in R light (Fig. 5) (two-way ANOVA, $p<0.001$). On the effects of temperature regime, the larvae reared in TC had a higher food intake compared to those in CTE (two-way ANOVA, $p<0.001$).

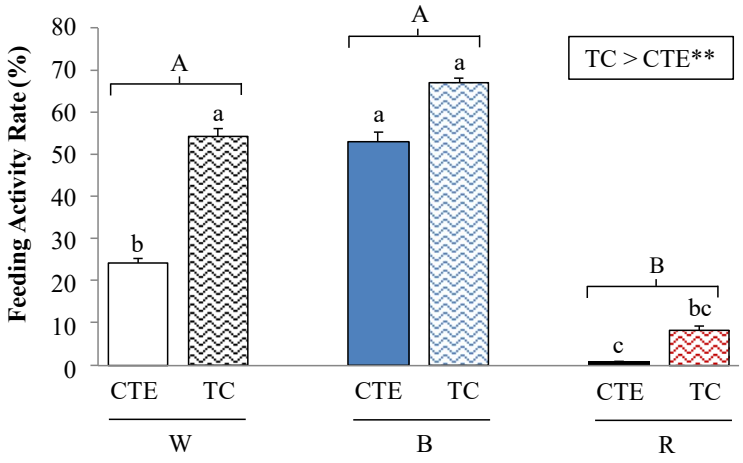

Fig. 5 Effect of three different light wavelengths (white, W; blue, B; red, R) and two temperature regimes (a thermocycle of 28:24ºC thermophase:cryophase, TC; and constant temperature of 26.1±0.1ºC, CTE) on the food intake (gut content) in the zebrafish larvae at 10 days post fertilization (dpf). Different lower case letters indicate significant differences between the experimental groups on the same dpf (one-way ANOVA, $p<0.05$). Different upper case letters and asterisks denote significant differences between the light treatments and rearing temperature regimes, respectively, on the same dpf (two-way ANOVA, $p<0.05$). Data (n=10) are represented as mean±SEM.

Effects of light wavelength and thermocycles on gene expression

As we observed marked differences in survival, growth, and food intake, especially between larvae reared in R compared with the rest of groups, we went on to elucidate the underlying molecular mechanisms that could lead to these effects. For this purpose, we analyzed the mRNA expression of the multiple factors involved in stress, growth, and food intake control in the 10 and 30 dpf larvae of all the experimental groups (Fig. 1).

Genes involved in stress response

One of the processes that can be affected by light and temperature conditions is the stress response mediated by Crh, which is related to survival. The expression of *crh* at 10 dpf showed statistically significant intergroup differences (Fig. 6, left panel) (one-way ANOVA, $p<0.05$). The larvae reared in R+CTE presented a higher *crh* expression than the other groups. Significant differences depending on temperature regime were also detected (two-way

ANOVA, p=0.023; Supplementary Table S1), with the larvae reared in CTE displaying a higher *crh* expression than those in TC (Fig. 6, left panel). No significant differences in *crh* expression was found at 30 dpf (Fig. 6, right panel) (Supplementary Table S1).

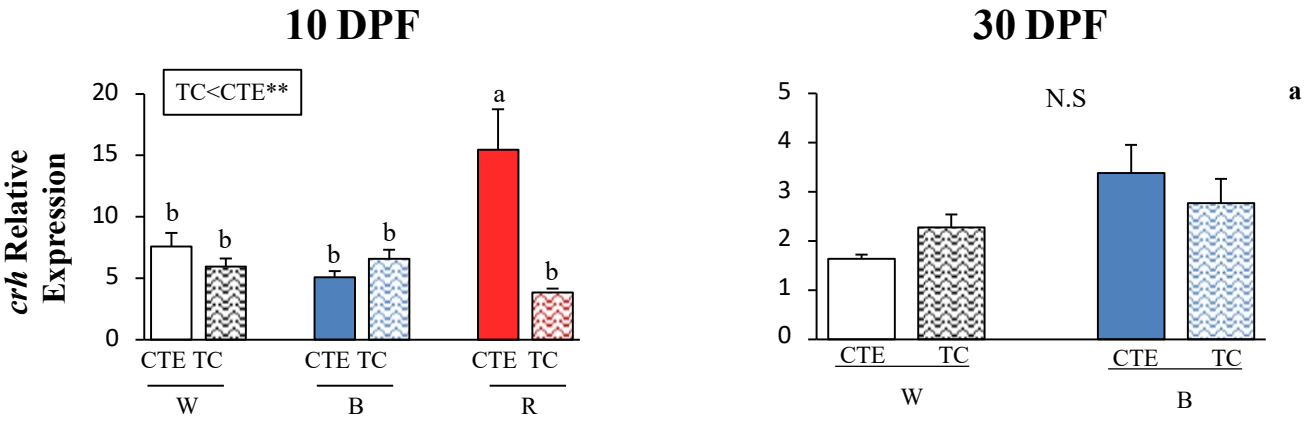

Fig. 6 Relative mRNA expression of *crh* of the 10 (left panel) and 30 (right panel) days post fertilization (dpf) zebrafish larvae reared in three different light wavelengths (white, W; blue, B; red, R) and in two temperature regimes (a thermocycle of 28:24ºC thermophase:cryophase, TC; and constant temperature of 26.1±0.1ºC, CTE). Different lower case letters indicate significant differences between the experimental groups on the same dpf (one-way ANOVA, $p<0.05$). Different upper case letters and asterisks denote significant differences between the light treatments and rearing temperature regimes, respectively, on the same dpf (two-way ANOVA, $p<0.05$). Data (n=6) are represented as mean±SEM.

Genes involved in growth

We also analyzed the expression of the main endocrine factors involved in somatic growth regulation: *gh*, *igf1a*, and *igf2a*. Statistically significant differences were observed in groups for *gh* expression at both 10 and 30 dpf (Fig. 7) (one-way ANOVA, $p<0.05$; Supplementary Table S1). In both developmental stages, the highest *gh* expression was detected in the larvae reared in B+TC. A significant effect of temperature regime was observed at both 10 and 30 dpf, with a higher *gh* expression in TC than in CTE (Fig. 7a) (two-way ANOVA, $p<0.05$; Supplementary Table S1). At 30 dpf, *gh* expression also differed between light treatments, with B inducing a higher expression than W (Fig. 7a, right panel) (two-way ANOVA, $p<0.05$).

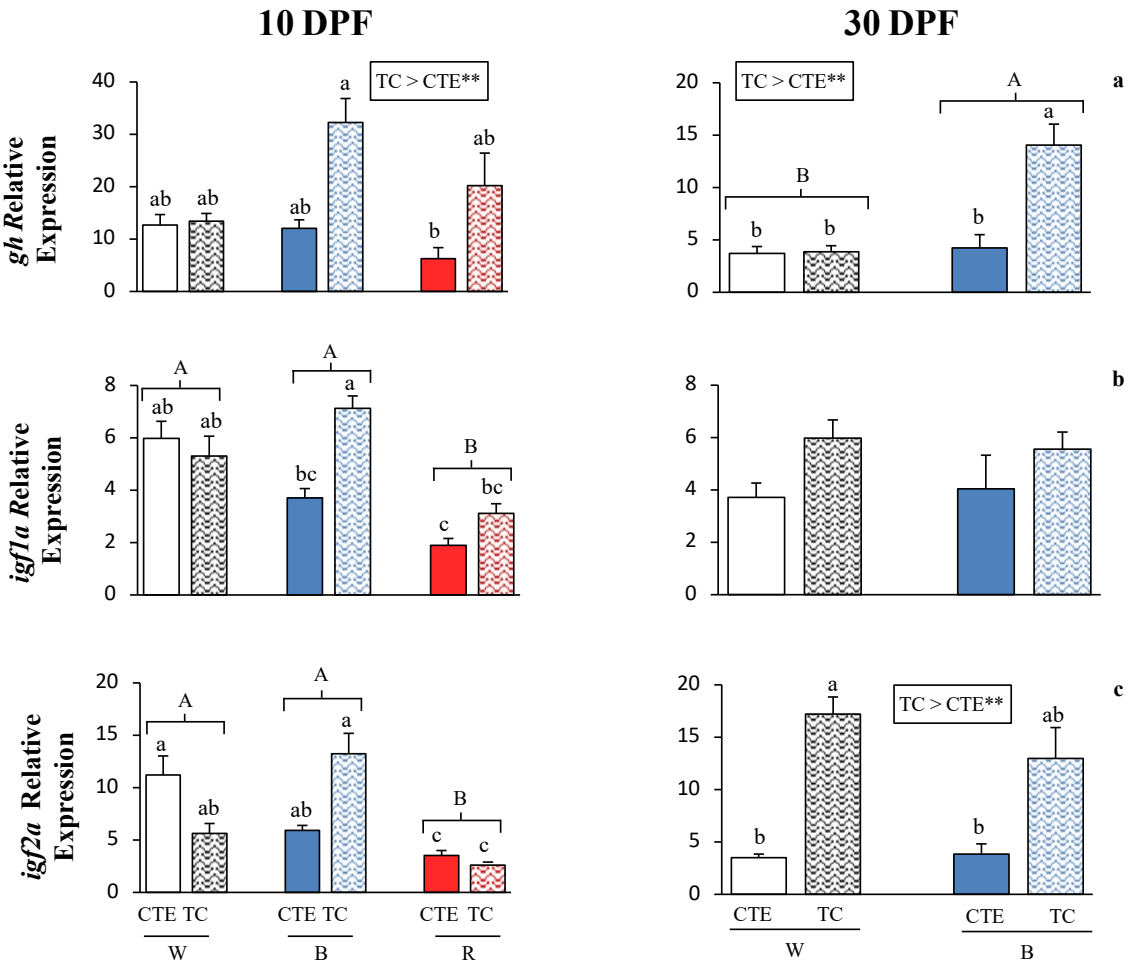

Fig. 7 Relative mRNA expression of *gh* (a), *igf1a* (b) and *igf2a* (c) of the 10 (left panels) and 30 (right panels) days post fertilization (dpf) zebrafish larvae reared in three different light wavelengths (white, W; blue, B; red, R) and in two temperature regimes (a thermocycle of 28:24ºC thermophase:cryophase, TC; and constant temperature of 26.1±0.1ºC, CTE). Different lower case letters indicate significant differences between the experimental groups on the same dpf (one-way ANOVA, $p<0.05$). Different upper case letters and asterisks denote significant differences between the light treatments and rearing temperature regimes, respectively, on the same dpf (two-way ANOVA, $p<0.05$). Data (n=6) are represented as mean±SEM.

The highest expression for *igf1a* at 10 dpf was observed in the larvae reared in B+TC and the lowest for the larvae left in R+CTE (Fig. 7b, left panel) (one-way ANOVA, $p<0.05$; Supplementary Table S1). The light wavelength also influenced *igf1a* expression, with higher values for the larvae reared in W and B than in R (two-way ANOVA, $p<0.05$). No statistically significant differences in *igf1a* appeared at 30 dpf (Fig. 7b, right panel) (one- and two-way ANOVAs, $p>0.05$).

Finally, *igf2a* expression at 10 dpf was significantly higher in all the groups reared in W and B than in the groups reared in R (Fig. 7c, left panel) (one- and two-way ANOVAs,

p<0.05). For the 30 dpf larvae, *igf2a* was higher in the larvae in W+TC than in the W+CTE and B+CTE larvae (Fig. 7c, right panel) (one-way ANOVA, p<0.05). The statistical analyses also revealed a significant effect of temperature regime because the larvae in TC showed higher *igf2a* expression than those in CTE (two-way ANOVA, p<0.05).

Genes involved in food intake control

As the different treatments affected food intake, we evaluated the mRNA expression of several hormones involved in the endocrine control of food intake and digestion. These factors intervene as either appetite-stimulating factors (orexigenic peptides: *npy*, *agrp*, *ghrelin*, *orexin*, *mch1,* and *mch2*) or appetite-inhibiting factors (anorexigenic peptides: *grp* and *cck8*).

At 10 dpf, significant intergroup differences were observed in all the genes (Fig. 8, left panels) (one-way ANOVA, p<0.05; Supplementary Table S1), except for *mch1* (one-way ANOVA, p>0.05). The highest expression levels were generally detected in the larvae reared in B+TC, whereas the lowest expression was detected in the group reared in R+CTE (Fig. 8, left panels). The expression of *ghrelin*, *mch2,* and *grp* was also higher in B+TC than in R+TC (Fig. 8c, f, g). The expression of *orexin*, *mch2,* and *grp* was higher in B+TC than in B+CTE (Fig. 8d, f, g). The two-way ANOVA revealed significant differences depending on lighting conditions in the expression of *npy*, *ghrelin*, *mch2*, *grp,* and *cck8* (Fig. 8a, c, f, g, h) (two-way ANOVA, p<0.05; Supplementary Table S1). In these genes, the expression levels of the larvae reared in W or B lights were higher than the larvae in R. Significant differences were also reported according to the temperature regime in *agrp*, *orexin*, *mch2* and *grp* (Fig. 8b, d, f, g) (two-way ANOVA, p<0.05). In these genes, the larvae in TC presented higher expression levels than those in CTE.

At 30 dpf, the expression of the analyzed genes involved in food intake control followed similar patterns. All the genes showed significant intergroup differences (Fig. 8, right panels) (one-way ANOVA, p<0.05; Supplementary Table S1). The B+TC-reared larvae showed a higher expression than the other groups (B+CTE, W+TC, and W+CTE) in all the analyzed genes, except for *npy,* for which differences were only found between B+TC vs. W+CTE and B+CTE (Fig. 8a). The two-way ANOVA also revealed a significant effect of lighting conditions on *ghrelin*, *grp* and *cck8* (Fig. 8c, g, h) (p<0.05; Supplementary Table S1).

The expression levels of these genes were higher in the larvae in B light than in W. All the genes except for *agrp* showed a significant effect of temperature regime, with the groups in TC displaying higher expression levels than in CTE (Fig. 8, right panels) (two-way ANOVA, $p<0.05$).

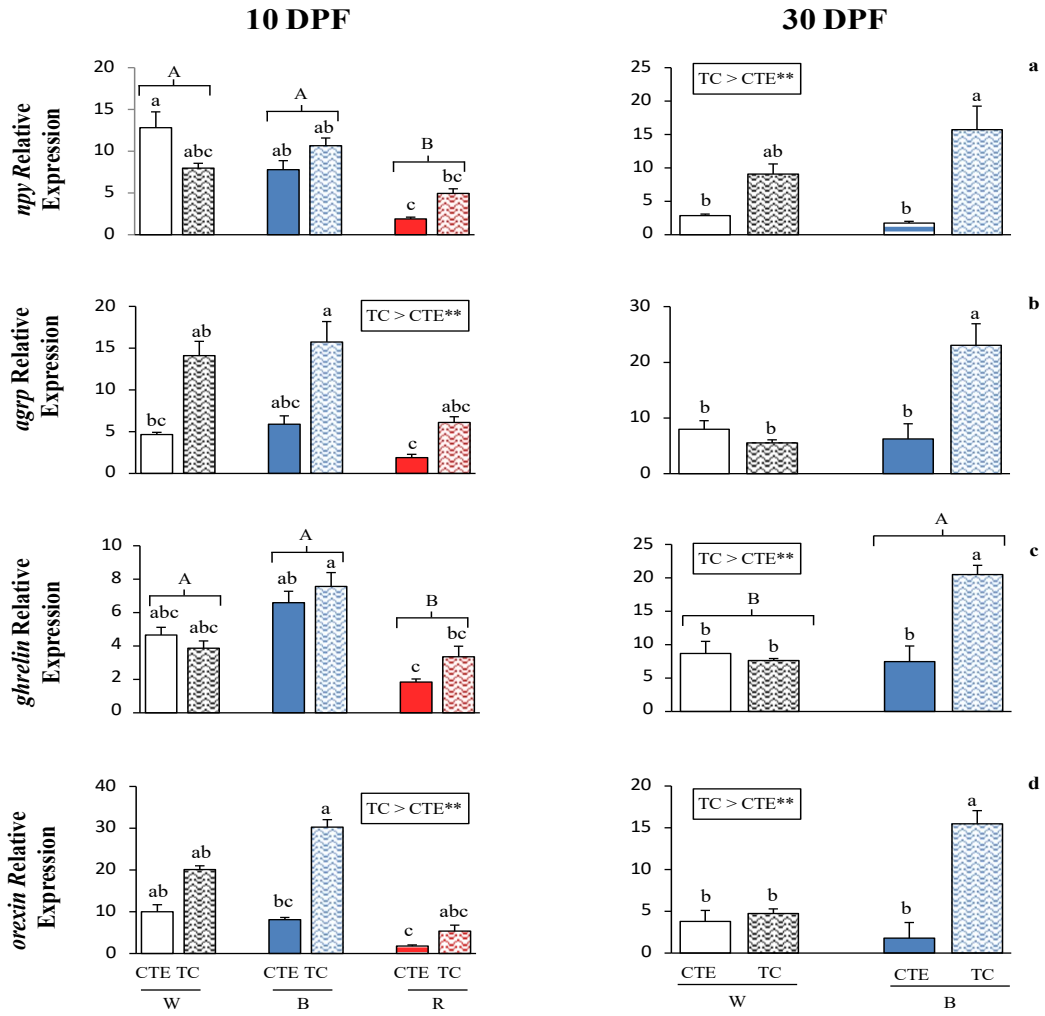

Fig. 8 Relative mRNA expression of *npy* (a), *agrp* (b), *ghrelin* (c), *orexin* (d), *mch1* (e), *mch2* (f), *grp* (g) and *cck8* (h) of the 10 (left panels) and 30 (right panels) days post fertilization (dpf) zebrafish larvae reared in three different light wavelengths (white, W; blue, B; red, R) and in two temperature regimes (a thermocycle of 28:24ºC thermophase:cryophase, TC; and constant temperature of 26.1±0.1ºC, CTE). Different lower case letters indicate significant differences between the experimental groups on the same dpf (one-way ANOVA, $p<0.05$). Different upper case letters and asterisks denote significant differences between the light treatments and rearing temperature regimes, respectively, on the same dpf (two-way ANOVA, $p<0.05$). Data (n=6) are represented as mean±SEM.

DISCUSSION

The present research findings revealed that the combined effects of the light spectrum and daily temperature conditions (cycling vs. constant) considerably influenced zebrafish development from very early stages. The most favorable combination was blue light with daily thermocycles (B+TC), which positively affected hatching and survival, and stimulated growth and food intake. On the contrary, the combination of red light and constant temperature (R+CTE) obtained the worst results. These profound effects were consistent with the results obtained in the gene expression analysis performed under the different environmental conditions.

The effect of different light intensities and spectrums has been reviewed in fish (Villamizar et al., 2011). In zebrafish, the use of short wavelengths enhanced early larval development whereas long wavelengths caused the worst results (Villamizar et al., 2014). In addition to light wavelength, the use of thermocycles *versus* constant temperatures showed similar beneficial effects in zebrafish larval performance (Villamizar et al., 2012). However, the combined effects of light spectrum and daily thermocycles on fish larval development, as well as on the underlying molecular mechanisms that lead to these effects, had not been studied to date in zebrafish. Our results showed that the zebrafish larvae reared in lights W and B had a higher hatching rate than those kept in light R. This finding suggests that zebrafish embryos can detect light wavelength from very early development stages. In agreement with our findings, other studies have revealed that zebrafish hatching is affected by light spectrum, and ranges from 90.1% in violet light to 79.4% in red light (Villamizar et al., 2014). However, in other species, such as turbot (*Scophtalmus maximus*), the use of different light spectra led to no substantial differences in hatching rates (Song et al., 2019). About the temperature regime effect, the lack of differences in the hatching rate between CTE and TC herein observed agrees with other studies performed in zebrafish and Nile tilapia (*Oreochromis niloticus*) (Villamizar et al., 2012; Espirito Santo et al., 2020).

The present paper revealed that zebrafish larvae showed a higher survival rate for the B and W light spectra than for R. Indeed, all the larvae reared in light R died while

developing. This implies that light wavelength is one of the main factors for survival rates. The light spectrum effect during early fish development seems species-specific. In Senegalese sole and Russian Sturgeon (*Acipenser baerii*), short wavelengths increase survival as in zebrafish (Blanco-Vives et al., 2010; Villamizar et al., 2014; Ruchin, 2016). In contrast, European eel (*Anguilla anguilla)* survival is enhanced by long wavelengths (Politis et al., 2014). Regarding the temperature regime, the effects of daily thermocycles on fish survival have been poorly studied to date. In the present research work, daily thermocycles were relevant on the first days of larval development. Thermocycles enhanced the survival of the larvae reared in R light because those reared with daily thermocycles died later than those maintained at a constant temperature. However, thermocycles were not enough to counteract the deleterious effects of red light. In Senegalese sole and brook trout (*Salvelinus fontinalis*), beneficial effects of thermocycles in early developmental stages have been described: they increased survival rate and lowered the incidence of malformations (Blanco-Vives et al., 2010).

As we observed marked differences in survival, growth, and feeding between experimental groups, we went on to elucidate the underlying molecular mechanisms that could lead to these effects. The present study analyzed the expression of the genes involved in processes like survival, growth, and food intake control to investigate whether the differences in biometric parameters can be explained by the differences in the expression of these factors. Of them, *crh* expression was evaluated to explain survival differences. Higher *crh* expression levels are related to the stress response in zebrafish and can limit important physiological processes like food intake, survival, and larval development (Alderman and Bernier, 2009; Ruchin, 2020; 2021). In our experiments, the larvae reared in R and CTE obtained the highest *crh* expression levels, which coincides with previous studies performed in zebrafish and European sea bass (Villamizar et al., 2014; Yan et al., 2019). However, the use of thermocycles lowered both the *crh* expression levels and survival rate differences with the remaining groups. This scenario suggests that zebrafish larvae under red light and constant temperature may be under a stressed state, which could be partly responsible for the deleterious effects of these environmental conditions. Daily thermocycles may improve zebrafish larval survival by minimizing such stressing effects.

The B light and TC temperature combination led to the highest growth rate. In agreement with our results, the use of daily thermocycles has also shown better early development for zebrafish (Villamizar et al., 2012), Nile tilapia (Espirito Santo et al., 2020), Senegalese sole (Blanco-Vives et al., 2010), green sturgeon (Rodgers et al., 2018) and perch (Coulter et al., 2016). Nevertheless, the effect of daily thermocycles on the growth rate depends on the fish species. Non-significant effects have been found for juvenile Nile tilapia (Azaza et al., 2010) and Chinese bream (*Parabramis pekinensis*) (Peng et al., 2014), while a lower growth rate has been reported for rainbow trout (*Oncorhynchus mykiss*) (Flodmark et al., 2004), Atlantic Salmon (*Salmo salar)* (Imholt et al., 2011; Morissette et al., 2021) and brook trout (Chadwick and McCornick, 2017). Not only can daily thermocycles have major effects on growth, but so can the light spectrum. In general, higher growth rates with shorter than longer wavelengths have been reported in zebrafish (Villamizar et al., 2014), European sea bass (*Dicentrarchus labrax*) (Villamizar et al., 2009), Senegalese sole (Blanco-Vives et al., 2010), Atlantic cod (*Gadus morhua*) (Migaud et al., 2009; Sierra-Flores et al., 2016), gilthead seabream (*Sparus aurata*) (Karakatsouli et al., 2007), turbot larvae (Sierra-Flores et al., 2016), goldfish (*Carassius carassius*), Chinese sleeper (*Perccottus glenii*) and guppy (*Poecilia reticulata*) juveniles (Ruchin, 2004, 2016, 2020). However, other species like rainbow trout, perch, and common carp (*Cyprinus carpio*) have presented higher growth, weight gain and feed efficiency rates with red light (Karakatsouli et al., 2008, 2010; Head and Malison, 2000).

Very little information is available about how the combination of different light spectra and thermal regimes can act on fish growth. In the present study, short wavelengths stimulated the mRNA expression levels of somatic growth factors (*gh*, *igf1a* and *igf2a*), which correlated with a higher growth rate, while long wavelengths reduced the expression of growth factors and negatively influenced the growth rate, and probably other physiological functions (Reinecke et al., 2005; Saera-Vila et al., 2009; Canosa and Bertucci, 2020). The stimulatory effect of short wavelength on growth factors has also been recently described in yellowtail clownfish (*Amphiprion clarkia*) (Shin et al., 2012), goldfish (*Carassius auratus*) (Shin et al., 2014), barfin flounder (*Verasper moseri*) (Takahashi et al., 2004, 2016; Yamanome et al., 2009) and zebrafish (Villamizar et al., 2014). In other studies performed in rainbow trout and European sea bass, the somatic growth factors were stimulated in red light

(Karakatsouli et al., 2007, 2008; Yan et al. 2019). Apart from the light wavelength, water temperature is also a determining factor that directly affects embryonic and fish larval growth. This environmental cue can modify the plasma levels and mRNA expression of GH and IGFs (Gabillard et al., 2005). In most fish species, higher temperatures increase the expression of GH and IGF1, which fluctuates in parallel to the growth rate. However, cold temperatures decrease the expression profiles of growth factors (for a review, see Deane and Woo, 2009). In the natural environment, seasonal water temperature changes affect the activities of metabolic enzymes and expression profiles by increasing the growth rate and the mRNA expression at the GH level, and in warmer months compared to colder months (for a review, see Deane and Woo, 2009). In addition to seasonal water temperature changes, many fish in nature undergo daily temperature fluctuations. The results of the present research work suggest that thermocycles can enhance the growth rate by up-regulating the expression of several growth factors, but more studies are necessary to test this hypothesis.

The findings observed in the growth and survival rates can be partly explained by the differences in the food intake levels between the combinations of light wavelengths and temperature regimes. In several fish species, light wavelength influences food consumption and the efficiency of its utilization for fish growth (for a review see Ruchin 2020). The zebrafish larvae reared in the W and B light wavelengths presented a higher food intake than those in R light in correlation to survival and growth parameters. Similar findings have been found in haddock larvae with a higher feeding rate in blue light than either full-spectrum (white) or green light (Downing and Litvak, 2001, 2002). This more efficient adaptive response of zebrafish to shorter wavelengths can be mediated by visual and non-visual photopigments (Villamizar et al., 2014). With blue wavelengths, the retina of zebrafish larvae presents a large amount of UV and blue cones of zebrafish larvae retina (Allison et al., 2010). Thus the marked presence of these visual pigments may have helped the zebrafish reared in blue light to acquire greater visual acuity for capturing food and enhancing the feeding rate (Ruchin 2020).

Temperature also played an important role in food intake because, for instance, the larvae reared in W+TC obtained higher food intake than those in W+CTE. These results agree with previous research works performed in Nile tilapia, in which the larvae reared in TC presented higher growth rates than those reared in CTE. These results suggest that

thermocycles can enhance food intake and digestion process synchronization by improving feeding efficacy and, consequently, growth rates (Espirito Santo et al., 2020). Both short wavelengths and thermocycles directly affect the intake control system. For this reason, we analyzed the mRNA expression of orexigenic (*npy*, *agrp*, *ghrelin*, *orexin*, *mch1*, *mch2*) and anorexigenic (*grp* and *cck*) neuropeptides. Npy acts as a potent orexigenic neuropeptide involved in feeding regulation and Gh stimulation in fish (Matsuda et al., 2012a; Yokobori et al., 2012; Delgado et al., 2017). Gh can act in AgRP production, a stimulator of appetite and feeding. Ghrelin is a peptide that exerts a synergic effect with orexin, and both stimulate Gh secretion and appetite via Npy/AgRP (Kaiya et al., 2008; Yokobori et al., 2011, Matsuda et al., 2012b). Recently, Mch has been considered to be a potent orexigenic regulator of fish appetite because it participates in the transduction of photic conditions by modulating feeding behavior (Takahasi et al., 2004, 2014). Cck and Grp act as satiety (anorexigenic) signals in fish and participate in the secretion of pancreatic enzymes (Volkoff et al. 2005; Koven and Schutle, 2012). In the present study, the different light and temperature treatments induced differences in the expression of most of the analyzed genes. Regarding lighting conditions, all the genes involved in food intake control and digestion, except for *agrp*, *orexin,* and *mch1,* presented higher expression levels in B and W than in R. Similar stimulatory effects of short wavelengths on expressions *mch1* and *mch2* in association with *npy* and *orexin* have been described in spotted halibut (*Verasper variegatus*) (Shimizu et al., 2019), barfin flounder (Yamanome et al., 2009; Takahashi et al., 2004, 2016, 2018), yellowtail clownfish (Shin et al., 2012) and goldfish (Shin et al., 2014). In these studies, the stimulatory effects of short wavelengths on the food intake system correlated with enhanced food intake and subsequently higher levels of somatic growth factors and growth rate, which also occurred in our study. Regarding temperature, the effect of diel thermal cycles on the endocrine control of feeding and appetite has been less investigated than light. Konstatinov et al., (2005) showed that young sturgeons reared under daily and seasonal water changes presented higher feeding and food energy to growth than those kept at a constant temperature. Here we observed that TC increased the expression of several of the peptides involved in food intake control (*agrp*, *orexin*, *mch2*) and digestion (*grp*) in very early development stages, such as 10 dpf. Moreover, the stimulatory effects of thermocycles were more pronounced at 30 dpf as the expression of all the genes was higher in TC than in CTE (except for *agrp*). One hypothesis to

explain this effect is that circadian clocks can be entrained by temperature cycles (Lahiri et al., 2005). It has been suggested in Nile tilapia that thermocycles induce a better synchronization of the rhythms of food intake factors to, thus, improve the timing of digestion processes, metabolism efficiency, and, consequently, growth (do Espirito Santo et al., 2020). However, more research is necessary to test this hypothesis.

CONCLUSION

As far as we know, the present research is the first to show that the combination of light wavelengths and different temperature regimes considerably affects fish larval development by generating changes in both biometric parameters (hatching, survival, growth, food intake) and the mRNA expression of the genes related to them. The combined effects of both shorter wavelengths and thermocycles, which come closer to the natural underwater environment, positively influenced most of the analyzed parameters. In addition, the present study identified several physiological factors that are affected by long wavelengths and whose changes may be responsible for the deadly effects observed when fish are reared under red light.

From a practical point of view, our findings suggest that using similar environmental conditions that fish experience in their aquatic environment may have a beneficial impact on both their development and survival. Therefore, these findings should be considered to optimize the growth and welfare of the fish reared in a laboratory and fish farming.

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AUTHOR CONTRIBUTIONS

GA, SC, JFL and FJSV conceived and designed the experiments, and wrote the manuscript; GA and SC performed the experiments; GA, SC and JFL analyzed the data; FJSV and JFLO provided funding.

COMPETING INTERESTS

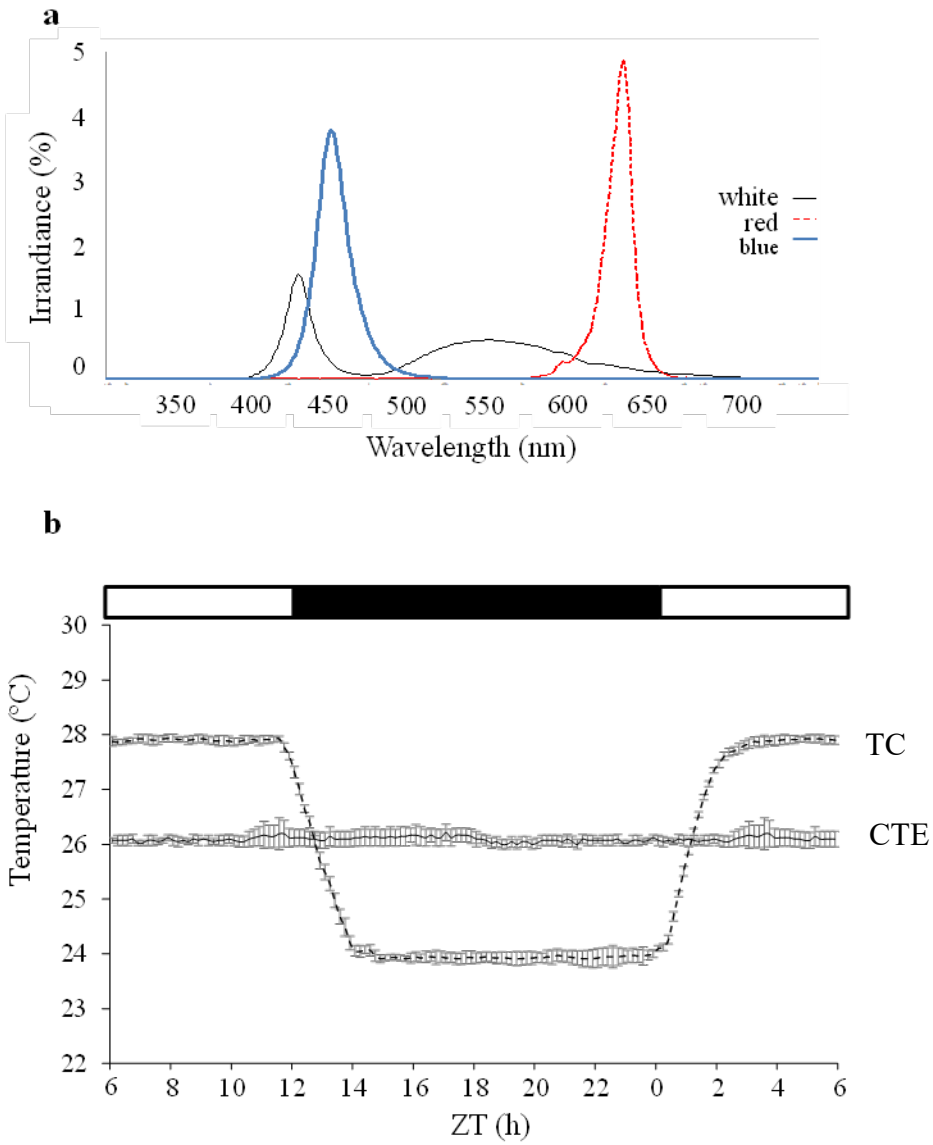
The authors declare no competing or financial interests.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jtherbio.2021.102880



Supplementary Fig. S1 a. Spectral composition of each experimental light emitting diode (LED) lamp (blue, red, and white) expressed as percentage of irradiance. **b.** Representation of the two temperature regimes performed in the experiment: a daily thermocycle of 28:24ºC thermophase:cryophase (TC, dotted line) and constant temperature (26.1±0.1ºC, continuous line). The upper white and black bars represent the light and dark periods, respectively (12:12 LD).

Variable			One-way ANOVA		Two-way ANOVA					
					Light		Temperature regime		Interaction	
			F	P	F	P	F	P	F	P
Hatching rate (%)			1.802	0.187	4.103	0.044	0.061	0.809	0.371	0.698
Survival (%)		4 dpf	1.000	0.458	1.000	0.397	1.000	0.397	1.000	0.397
		6 dpf	36.549	0.000	33.622	0.000	48.259	0.000	33.622	0.000
		8 dpf	32.660	0.000	29.996	0.000	43.316	0.000	29.996	0.000
		10 dpf	35.596	0.000	32.857	0.000	46.549	0.000	32.857	0.000
		12 dpf	29.908	0.000	41.997	0.000	28.058	0.000	18.765	0.000
		14 dpf	31.024	0.000	63.715	0.000	11.538	0.005	8.077	0.006
		16 dpf	32.462	0.000	77.582	0.000	4.024	0.068	1.561	0.250
		18 dpf	46.091	0.000	113.281	0.000	2.194	0.164	0.850	0.451
		20 dpf	0.915	0.476	0.413	0.538	2.032	0.192	0.301	0.599
		22 dpf	0.910	0.478	0.293	0.603	2.113	0.184	0.323	0.585
		24 dpf	0.985	0.447	0.351	0.570	2.221	0.174	0.383	0.553
		26 dpf	0.931	0.469	0.308	0.594	2.975	0.188	0.410	0.540
		28 dpf	1.015	0.435	0.220	0.651	2.514	0.151	0.310	0.593
		30 dpf	1.015	0.435	0.220	0.651	2.514	0.151	0.310	0.593
Growth (mm)		5 dpf	0.900	0.484	0.116	0.891	3.890	0.051	0.189	0.828
		10 dpf	16.146	0.000	38.932	0.000	2.488	0.118	0.795	0.454
		15 dpf	207.304	0.000	163.139	0.000	152.691	0.000	98.270	0.000
		20 dpf	449.049	0.000	1095.347	0.000	14.050	0.000	20.250	0.000
		25 dpf	681.933	0.000	1688.305	0.000	6.887	0.010	11.508	0.000
		30 dpf	743.132	0.000	1827.564	0.000	7.602	0.007	12.975	0.000
Feeding activity (%)			31.937	0.000	67.477	0.000	18.991	0.000	2.870	0.063
10 DPF Relative expression	Survival	*crh*	3.022	0.028	1.767	0.191	5.881	0.023	5.281	0.012
	Growth	*gh*	3.014	0.032	2.164	0.139	7.909	0.010	1.925	0.170
		igf1a	3.399	0.018	5.497	0.011	2.484	0.128	2.093	0.145
		igf2a	3.309	0.018	5.377	0.011	0.423	0.521	2.653	0.088
	Appetite	*npy*	3.392	0.017	6.392	0.006	0.048	0.829	2.599	0.094
		agrp	3.100	0.027	2.595	0.095	8.540	0.007	0.332	0.720
		ghrelin	2.803	0.037	6.415	0.005	0.310	0.582	0.489	0.619
		orexin	2.926	0.033	2.178	0.134	7.239	0.013	1.520	0.238
		mch1	1.822	0.142	2.883	0.074	0.504	0.484	1.745	0.195
		mch2	5.414	0.002	5.719	0.009	8.923	0.006	2.068	0.148
		grp	4.872	0.003	4.699	0.019	8.353	0.008	2.388	0.112
		cck8	2.747	0.044	3.722	0.040	0.068	0.796	3.266	0.056

30 DPF Relative expression	Survival	*crh*	1.330	0.300	3.040	0.100	0.000	0.986	0.948	0.345
	Growth	*gh*	5.170	0.013	5.106	0.040	4.438	0.049	4.143	0.061
		igf1a	0.732	0.549	0.001	0.972	2.058	0.172	0.081	0.780
		igf2a	3.966	0.031	0.328	0.576	11.397	0.005	0.462	0.508
	Appetite	*npy*	5.004	0.015	0.985	0.338	12.030	0.003	1.942	0.185
		agrp	3.853	0.032	3.339	0.088	2.770	0.117	4.995	0.041
		ghrelin	5.979	0.007	4.933	0.042	5.188	0.038	7.154	0.017
		orexin	4.564	0.018	0.429	0.522	7.659	0.014	5.884	0.028
		mch1	3.645	0.042	1.540	0.237	4.618	0.049	4.729	0.076
		mch2	5.107	0.012	2.539	0.132	7.097	0.018	5.391	0.035
		grp	25.342	0.000	15.038	0.001	40.495	0.000	18.441	0.001
		cck8	9.293	0.001	6.039	0.028	16.060	0.001	8.034	0.013

Supplementary Table S1 Statistic values obtained in the one- and two-way ANOVAs performed for all the variables measured in the experiments. The p values (p) and F-Statistic (F) are reported. Gray highlights the statistically significant results ($p<0.05$).

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Experimental Chapter VII

Circadian rhythm of preferred temperature in fish: behavioural thermoregulation linked to daily photocycles in zebrafish and Nile tilapia

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ABSTRACT

Ectothermic vertebrates, *e.g*. fish, maintain their body temperature within a specific physiological range mainly through behavioural thermoregulation. Here, we characterise the presence of daily rhythms of thermal preference in two phylogenetically distant and well-studied fish species: the zebrafish (*Danio rerio*), an experimental model, and the Nile tilapia (*Oreochromis niloticus*), an aquaculture species. We created a non-continuous temperature gradient using multichambered tanks according to the natural environmental range for each species. Each species was allowed to freely choose their preferred temperature during the 24h cycle over a long-term period. Both species displayed strikingly consistent temporal daily rhythms of thermal preference with higher temperatures being selected during the second half of the light phase and lower temperatures at the end of the dark phase, with their acrophases at Zeitgeber Time (ZT) 5.55 h (zebrafish) and ZT 12.7 h (tilapia). Interestingly, when moved to the experimental tank, only tilapia displayed consistent preference for higher temperatures and took longer time to establish the thermal rhythms. Our findings highlight the importance of integrating both light driven-daily rhythm and thermal choice to refine our understanding of fish biology and improve the management and welfare of the diversity of fish species used in research and food production.

Keywords: Zebrafish; Nile Tilapia; Temperature preference; Daily rhythms; Thermal ecology; Stress Induced Hyperthermia

INTRODUCTION

Both light and temperature are considered the main abiotic factors influencing ectotherms behaviour, physiology, and habitat distribution (Brett, 1971). Behavioural regulation of internal temperature is the main thermoregulatory mechanism to keep body temperature within a physiological range in ectotherms (Crawshaw, 1979; Hutchinson and Maness, 1979; Angilletta et al., 2002; Gordon, 2005; Haesemeyer 2020), which usually select their preferred temperature in a thermal gradient. When initially placed in a wide thermal gradient, the acute thermal preference is strongly influenced by previous thermal acclimation but after some time they move to a preferred temperature known as the final thermal preferendum (Reynolds and Casterlin, 1979; Johnson and Kelsch, 1998; Fangue et al., 2009). This final preferendum is species-specific and displays little variation even in fish species with a broad geographic distribution (Beitinger and Fitzpatrick, 1979). In addition to the acclimation temperature, the preferred temperature is also influenced by various factors such as the season of the year (Sauter et al., 2001; Mortensen et al., 2007), feeding and nutritional state (Wallman and Bennett, 2006), developmental state (McCauley and Huggins, 1979), health status (Golovanov, 2006, Boltana et al 2013, Rakus et al., 2017), and stress levels (Rey et al 2015a).

The circadian system has evolved as an internal timekeeper mechanism that synchronizes biological processes with environmental cycles, allowing the organisms to predict and anticipate events, thereby adjusting their behaviour and physiology according to the time of the day (Wilkins, 1960; Ding et al., 1994; Villamizar et al., 2013). In all vertebrates the molecular mechanism controlling circadian rhythmicity involves interlocked transcriptional-translational feedback loops of circadian clock genes and proteins (Vatine et al., 2011) which is ultimately entrained by external cues that show daily cycles, also called zeitgebers (ZT; Zeitgeber Time), such as photoperiod (Panda et al., 2002), thermal cycles (Rensing & Ruoff, 2002) or food availability (Mistlberger, 2009). Among these, the light-dark (LD) cycle is considered the main zeitgeber, synchronising many functions in living organisms (DeCoursey, 2014; Metcalfe et al., 1999). However, in nature, there is a direct relationship between the light-dark cycles and the daily variations in temperature: temperature peaks during the day, whereas the lowest temperatures are registered at night. Moreover, biological rhythms are temperature-compensated, so their periodicity remains

constant in a wide range of temperatures, which adds complexity to the relationship between circadian rhythms and temperature (Pittendrigh and Caldarola, 1973).

Increasing temperatures due to climate change are impacting the aquatic ecosystems, affecting the organisms' ecophysiology. Recently, predictive models have revealed that fish inhabiting tropical regions might be particularly affected by global warming, potentially limiting the performance of these species (Hasting et al; 2020; Lavender et al. 2021). Zebrafish (*Danio rerio*) and Nile tilapia (*Oreochromis niloticus*) are both tropical fish species that can tolerate a wide range of water temperatures, ranging from 24-32 ºC (Cortemeglia and Beitinger, 2005; Spence et al. 2008) and 22-34 ºC (Trewavas, 1983), respectively. However, in the wild, both species are subjected to strong daily and seasonal thermal variations, being able to survive under more extreme temperatures: between 6-38 ºC in the case of zebrafish (Spence et al., 2008) and between 17-40 ºC in tilapia (Bezault et al., 2007).

The main objective of the present research was to characterise in detail the daily rhythm of thermal preference for zebrafish and Nile tilapia, what we have called 'thermal rhythms', using a thermal gradient tank model similar that used in previous thermal preference experiments (Boltana et al 2013, Rey et al 2015a, b). For this, fish were kept under a 12 h L: 12 h D photoperiod and allowed to freely choose between a wide range of water temperatures, in line with those found in their natural environments. Ultimately, understanding how temperature preference of fish species varies during the day will provide new data about the thermal biology of ectotherms and shed light upon the evolution of thermal adaptation and its coupling to light-driven circadian rhythm in vertebrates, a theme of increasing urgency in view of biodiversity and global climate change. Additionally, our study directly impacts upon core ideas of animal welfare and improved husbandry by using two extensively exploited fish species that are critical for research (zebrafish) and global food security (tilapia). Likewise, we consider it necessary to highlight the importance of developing biological models to promote optimal animal welfare that provides increased resolution in downstream biological analyses and therefore value in research outcomes.

MATERIAL AND METHODS

The use of Zebrafish and Nile Tilapia as a model species

Zebrafish (*Danio rerio* Hamilton, 1822) and Nile tilapia (*Oreochormis niloticus* Linnaeus, 1758) are both tropical freshwater fish species. Zebrafish can be found in the Ganges and Brahmaputra rivers in India and in the south-eastern Himalayan region (Engeszer et al., 2007; Spence et al., 2008), Nile tilapia is native to the northern half of Africa and western of Asia (Patterson and Wilson, 1995). Both inhabit shallow and slow-moving waters rich in aquatic vegetation (Patterson and Wilson, 1995; McClure et al., 2006; Spence et al., 2006; Engeszer et al., 2007). Zebrafish and Nile tilapia can easily be raised and maintained in captivity, showing several features that make them interesting research model species (Choi et al., 2021). Thus, they are used in various fields of research such as: neurosciences, developmental biology, genetics, biomedicine, ecotoxicology, physiology (Vascotto et al., 1997; Fishman, 2001; Grunwald and Eisen, 2002) but also for behavioural (Engeszer et al., 2007; Moretz et al., 2007; Oliveira et al., 2011; Rey et al., 2013, 2015b; Cerqueria et al., 2016) and chronobiology studies (Dekens and Whitmore, 2008; Vatine et al., 2011). Zebrafish is the most used ectothermic vertebrate for biomedical research (Choi et al., 2021; Paredes et al., 2019). Tilapia is the second most farmed species in the world after carps and crucial for global food security across the globe (Zhang et al., 2022; FAO, 2022).

Fish breeding and holding conditions

All zebrafish (n=72) (*Danio rerio*, wild-type strain) were bred at the Institute of Aquaculture of the University of Stirling (Scotland, UK) and raised in a ZS660 stand-alone system (Aquaneering, Inc. USA). Nile tilapia (*Oreochromis niloticus*) juveniles were bred at the facilities of the University of Murcia (Spain) and kept in 300-liter separated tanks connected to a recirculation system, equipped with an aeration system and biological and mechanical filters. Water temperature was controlled using a water heater and a refrigerator (AB Aqua Medic, Gewerbepark, Germany) and it was established at 26±0.5ºC and 30±0.5ºC for zebrafish and Nile tilapia, respectively. Fish were fed twice daily to satiation during the holding period with a commercial diet specific for each fish species (Skretting® Gemma Micro and Skretting® Gemma Wean for zebrafish and Nile tilapia, respectively). Zebrafish diet was supplemented with Artemia (Zebrafish Management Ltd, UK). The water quality parameters (pH, ammonia, nitrate, nitrite, and dissolved oxygen) were measured daily. For

both fish species, the lighting conditions were set to a 12:12 h Light: Dark (LD) cycle, with lights on at 08:00 h (Zeitgeber Time 0 h, ZT0 h) and lights off at 20:00 h (ZT12 h).

Experimental setup and procedures

Gradient tank

Thermal preference was assessed using a custom-built multichambered tank (Rey et al., 2015) (Figure 1a). The 126 L tank (140 x 30 x 30 cm) was divided into seven interconnected chambers divided by six opaque PVC screens, with the two extreme lateral chambers used only for cooling and heating the water, for which a cooler, a water bath and two pumps were installed (AB Aqua Medic, Gewerbepark, Germany). Connection between chambers, allowing movement of fish and water, was enabled by a circular hole on each glass screen (10 cm diameter; 20 cm from the bottom), creating a continuous thermal gradient of 24 ºC to 31 ºC and 26 ºC to 34 ºC for zebrafish and Nile tilapia, respectively (Table A1). Access to the cooler and heater chambers was sealed using a mesh screen to prevent any intrusion of fish in them during the experiments. Mechanical filters were placed in the five central chambers and a picture of gravel substrate, used as environmental enrichment, was fitted under each chamber.

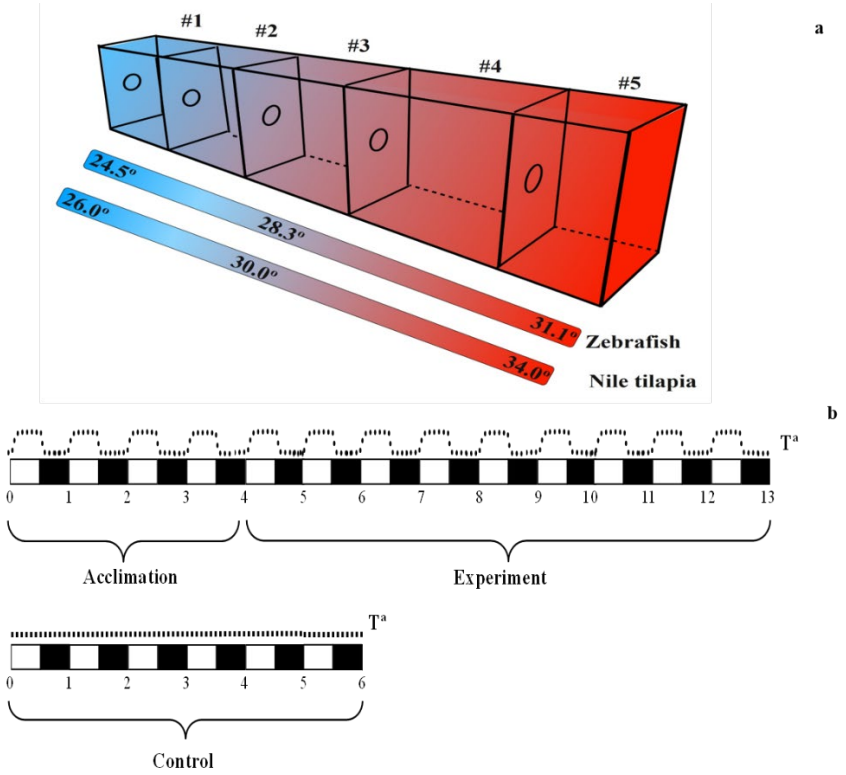

Fig. 1. Schematic representation of the multichambered temperature gradient tank (a) and experimental design (b). Fish were exposed to a temperature gradient during a 3-day acclimation period followed by a 10-day period of the experimental phase. The control group was maintained to a constant temperature during a 6-day period.

Environmental parameters

Water quality was monitored daily during the experiments. Oxygen (Handy Polaris l OxyGuard International, Farum, Denmark), pH (Mettler Toledo FiveEasy^{TM} FE20, Columbus, USA), TAN and NO_2 (Palin test, Gateshead, UK) levels were recorded once a day in the morning before feeding. Water temperature of each chamber was recorded continuously throughout the experiment by a Thermochron iButton (Maxim integrated, Rio Robles, San Jose, CA, USA).

Experimental design

For each fish species, three independent groups of fish (n=12/group) were placed in the central chamber of the gradient tank and allowed to freely distribute throughout the chambers for 13 days and their behaviour was video recorded to assess temperature preference during the whole period. The first 3 days were considered as the acclimation phase prior to the 10-day experimental period. In addition, to rule out that chamber preference was affected by other factors other than temperature, three additional independent fish groups (n=12/group) were placed in the same multichambered tank but exposed to a constant water temperature during a 6-day period (control) (Fig. 1b). Prior to the start of each trial, fish were placed in the central chamber of the gradient tank, and recordings immediately started. All animals were fed *ad libitum* a commercial diet delivered evenly in all chambers (Skretting® Gemma Micro and Gemma Wean for zebrafish and Nile tilapia, respectively) to avoid feed chamber preference, twice a day during the duration of the experiments and the behaviour and welfare of the animals was carefully monitored, with no deaths or negative welfare indicators observed (external appearance and normal behaviour monitored). At the end of each trial, fish were returned to their stock tank and the water was completely replaced.

Video recording and data acquisition

For each experiment, video recording started on the first day at zeitgeber time (ZT)=0 and was restarted daily at that time throughout the 13-day experimental period. During daytime, light was provided by LED strips (SOLBRIGHT®, LED Flex Strip 1043-W, Rayte, S.L., Murcia, Spain), with a light intensity on the water surface of 0.84 W·m-2 (~200 lx). At night, five infrared LED lamps (BW® 48 LED Infrared Illuminator) were installed behind the experimental tank to allow camera nocturnal vision. A translucent acrylic white sheet (Falken Design WT2447-1-8/2436 Acrylic White Sheet, Translucent 55%, 100 x 30 x 0.3 cm) was

fitted on the back wall of the tank to diffuse the infrared light and improve the image quality at night. All experimental videos were recorded using a video camera (Logitech Webcam C300-1.3MP, Switzerland) and the Multiviewer software (Computer System Department, University of Murcia, Spain), which stored 60 images (1 frame/s) every minute. The Multiviewer software had already been validated in zebrafish (Di Rosa et al., 2015). All video recordings were analysed using Fish Counter software (Dr. Ginés García Mateos, University of Murcia, Spain, Version 3.0). The software creates a fixed background model (static number of frames) and compares the analysing frames with the background model to detect the fish giving a value that corresponds to the number of fish per chamber in the gradient tank every minute (block of 60 frames) and recorded into a Microsoft Excel spreadsheet.

Data analysis

To determine how temperature preference (preferred temperature) changed during the day in the thermal gradient experiment, the mean temperature selected during each hour of the day in each experimental replicate was calculated as:

$$preferred\ temperature\ = \frac{(n_1T_1 + n_2T_2 + n_3T_3 + n_4T_4 + n_5T_5)}{\sum_{i=1}^{5} n_i}$$

Where n is the number of fish in chamber 1 to 5, and T is the temperature of the corresponding chamber.

We used hierarchical Bayesian models to analyse the daily rhythms of preferred temperature and the distribution of fish across chambers in each treatment group for each species, performing multi-level Cosinor analyses (Refinetti et al. 2007), such that:

$$y_{ijk} = M_{jk} + A_{jk}\cos(\theta_{ijk} + \phi_{jk}) + \sigma_k$$
$$M_{jk} \sim mvNorm(\mu_k, \Sigma_{Mk})$$
$$A_{jk} \sim mvNorm(\alpha_k, \Sigma_{Ak})$$
$$\phi_{jk} \sim mvNorm(\Phi_k, \Sigma_{\phi k})$$

where y_{ijk} is the preferred temperature at hour i in tank j and treatment group k, M_{jk} is mesor, A_{jk} is amplitude of the oscillation, θ_{ijk} is the hour in radians (2π * ZT/24), ϕ_{jk} is the acrophase or phase of the maximum, and σ_k is residual error. Tank-specific parameters (M, A, ϕ)

followed population-level distributions (μ, α, Φ) which represent the overall estimates and were used for inference. The model with hourly number of fish per chamber as the response further included chamber (1–5) as a predictor of each parameter along with an interaction between chamber and treatment group, with the response variable log-transformed. Finally, we evaluated acclimation using a similar model, but with each component (M_{ij}, A_{ij}, ϕ_{ij}) as a smooth function of elapsed time using splines.

Models were fit in R 4.1.3 using the package *brms* 2.16.3 (Bürkner 2017), which fits hierarchical Bayesian models using Markov Chain Monte Carlo with the statistical platform Stan (Stan Development Team, 2022). For each model, we used four chains of 4,000 iterations, with the first 3,000 iterations as warmup, and the final 1,000 iterations as samples from the posterior distribution. Diffuse prior distributions were used for each parameter to constrain sampling to plausible ranges (Table A2). To assess model fit, we performed posterior predictive checks and confirmed that all R-hat < 1.01 and that no divergences occurred during sampling.

RESULTS

Acclimation and daily rhythm of thermal preference

Fish were placed in a multi-chamber tank with a thermal gradient and the first three days were considered as the acclimation phase (Fig. 1b). Acclimation was defined as time needed to show stable circadian rhythms. The preferred temperature of zebrafish varied rhythmically throughout the day (Fig. 2, Fig. 5b) during the acclimation and experimental periods. The highest preferred temperature (Fig. 5c, Table 1) occurred in the middle of the light phase in both the acclimation (acrophase: ZT 6.99 h [6.43–7.56], mean [95% credible interval]; maximum: 28.7ºC [28.3–29.1]) and experimental phases (acrophase: ZT 5.55 h [5.13–6.06]; maximum: 28.8ºC [28.5–29.2]). The minimum preferred temperatures occurred during the middle of the dark phase (~ 27.3 ºC in both phases). Nile tilapia also showed daily rhythms in preferred temperature (Fig. 2, Fig. 5b, Table 1) during both the acclimation and experimental periods. The highest preferred temperature (Fig. 5c) occurred at the end of the light phase in the acclimation (acrophase: ZT11.4 h [10.5–12.0]; maximum: 32.0ºC [31.8–32.3]) and experimental phases (acrophase: ZT 12.7 h [12.0–13.7]; maximum: 30.7ºC [30.4–31.0]). The minimum preferred temperatures occurred at the end of the dark phase (acclimation: 31.2ºC [30.9–31.5]; experimental: 29.6ºC [29.3–30.0]).

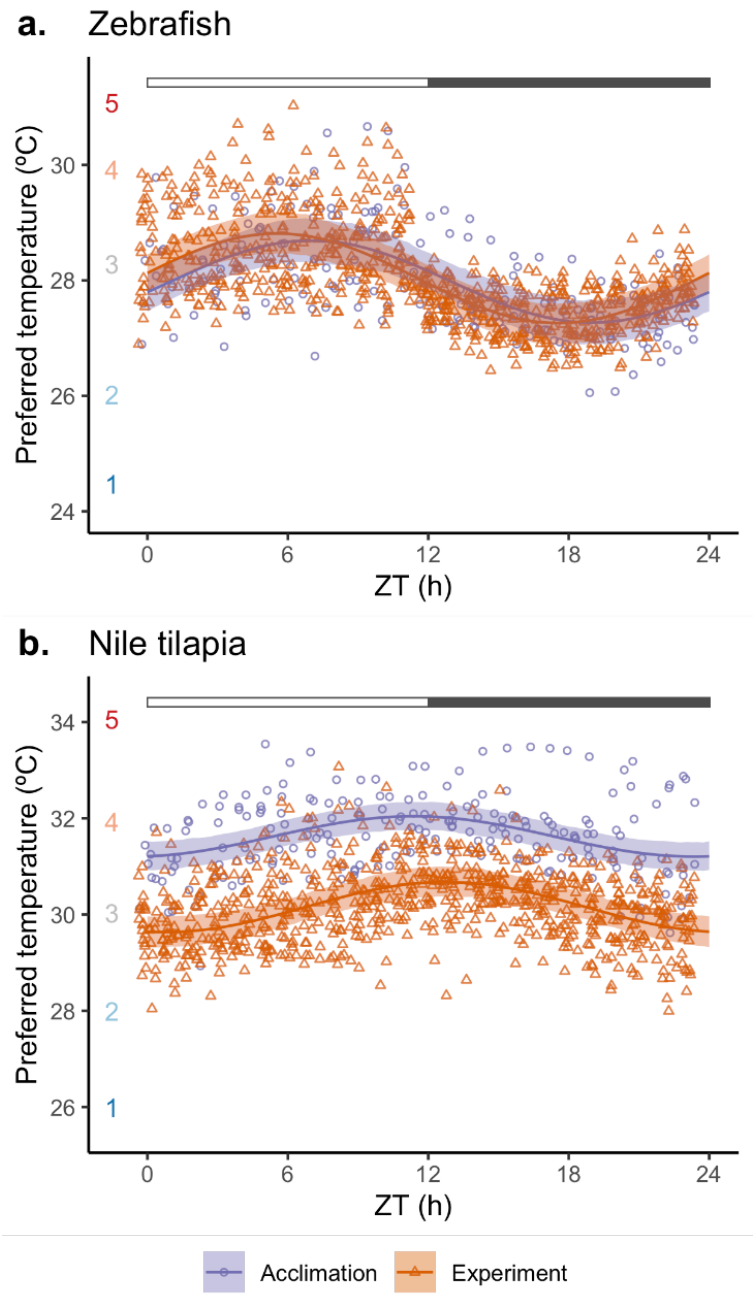

Fig. 2. Preferred temperature of *Danio rerio* (a) and *Oreochromis niloticus* (b) exposed to a temperature gradient during a 3-day acclimation period (purple, circles) and subsequent 10-day experimental period (orange, triangles). Data from all 3 experiments are shown, with lines and ribbons showing the population-level posterior mean temperature (°C) and 95% credible intervals. The white and black bars above the graphs represent the light and dark phases, respectively. The time scale (x-axis) is expressed as *Zeitgeber* Time (ZT), in which ZT 0 h corresponds to light onset and ZT 12 h corresponds to lights off. The chamber temperatures are indicated by the numbers to the right of the y-axis.

Table 1. Cosinor analysis results for the preferred temperature for the acclimation and experimental phases of zebrafish (*Danio rerio*) and Nile Tilapia (*Oreochromis niloticus*). Clear daily rhythms were detected during each phase for each species (Amplitude > 0, >99% confidence)

		Cosinor	**Preferred Temperature (ºC)**	
			Mean [95% credible interval]	
			Zebrafish	**Nile Tilapia**
Thermal gradient	**Acclimation**	Mesor (fold change)	28.0 [27.7–28.3]	31.6 [31.4–31.9]
		Amplitude (fold change)	0.713 [0.486–0.959]	0.422 [0.275–0.577]
		Acrophase (ZT hours)	6.99 [6.43–7.56]	11.4 [10.5–12.0]
	Experiment	Mesor (fold change)	28.0 [27.7–28.3]	30.2 [29.9–30.4]
		Amplitude (fold change)	0.776 [0.588–0.984]	0.524 [0.300–0.730]
		Acrophase (ZT hours)	5.55 [5.13–6.06]	12.7 [12.0–13.7]

Zebrafish and Nile tilapia showed differences in acclimation and this variability also resulted in different temperature preferences. Zebrafish showed a largely stable daily pattern of preferred temperature throughout the acclimation phase (Fig. A2, blue), with similar mean preferred temperatures at the start (28.4ºC [28.0–28.9]) and end (27.9ºC [27.6–28.2]) of acclimation, as well as similar amplitudes, whereas acrophases showed a slight decrease (from ZT 8.96 h to ZT 6.46 h). There was no difference between the preferred temperature of zebrafish during the acclimation (~27.9ºC) and experimental (~28.0ºC) periods (Fig. 2, Fig. 5a). In contrast, Nile tilapia showed a strong preference for higher temperatures displaying a stress induced hyperthermia (SIH) response during the acclimation phase (Fig. A2, green). Mean preferred temperature declined 1.5ºC from the start to the end of acclimation, while amplitude increased somewhat and acrophase remained stable. The mean preferred temperature of Nile tilapia was higher during the acclimation (31.6ºC [31.4–31.9]) period than during the experimental (30.2ºC [29.9–30.4]) period (Fig. 2, 4).

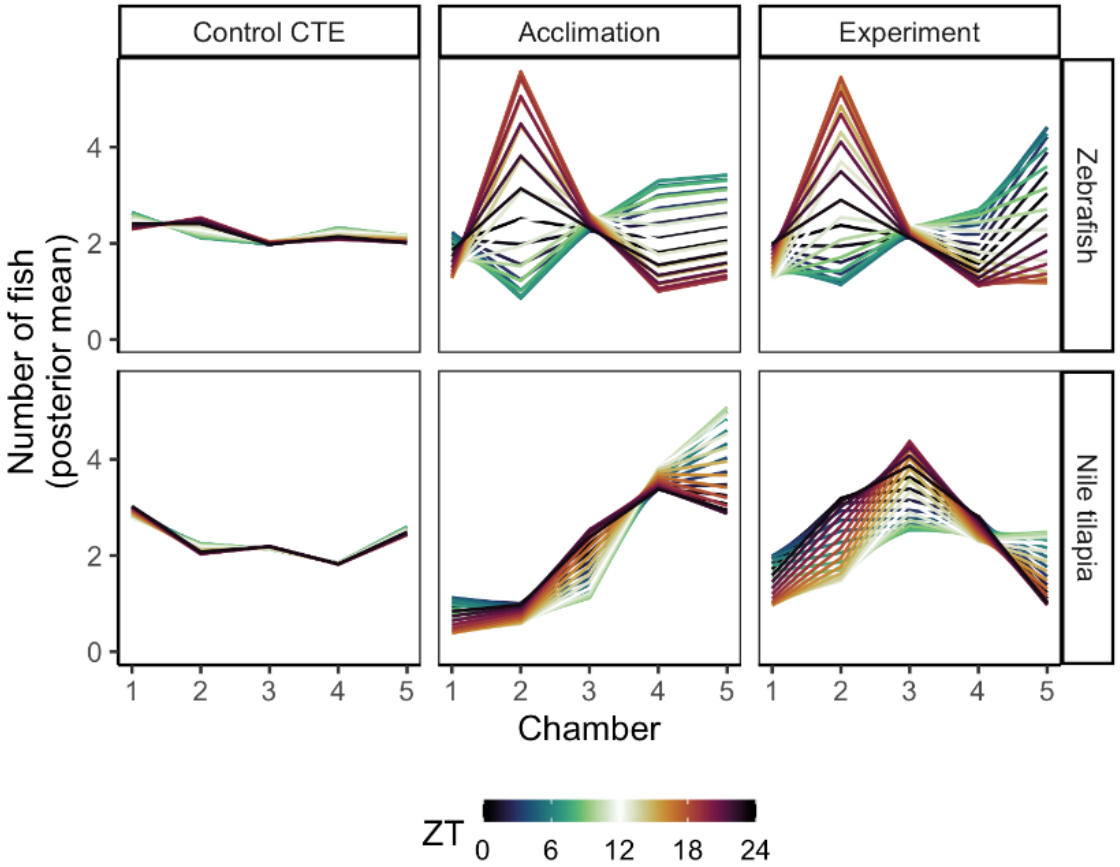

Fig. 3. Line plots of the mean predicted fish count in each chamber for Zebrafish (*Danio rerio*) and Nile tilapia (*Oreochromis niloticus*) by hour during the 3-day acclimation period, subsequent 10-day experimental period, and 6-day constant temperature control period. Lines show the posterior mean for each hour, expressed as *Zeitgeber* Time (ZT), in which ZT 0 h corresponds to light onset and ZT 12 h corresponds to lights off. Chambers were equivalent temperatures in the Control, but contained a gradient in the Acclimation and Experimental periods from colder (chamber 1) to warmer (chamber 5).

Accordingly, the distribution of zebrafish among chambers showed strong patterns of behavioural thermoregulation during the acclimation and experimental phases (Fig. 3, Fig. 4, Table A3), with clear thermal preferences for the warmest chambers near the middle of the light phase (Chamber 5: ZT 6.42 h [5.58–7.27] (Acc.), ZT 4.63 h [4.12–5.09] (Exp.)) and for the cooler chambers during the middle of the dark phase (Chamber 2: ZT 18.0 [17.5–18.7] (Acc.), ZT 17.3 [17–17.9] (Exp.); Fig. 5d, Fig. A1a).

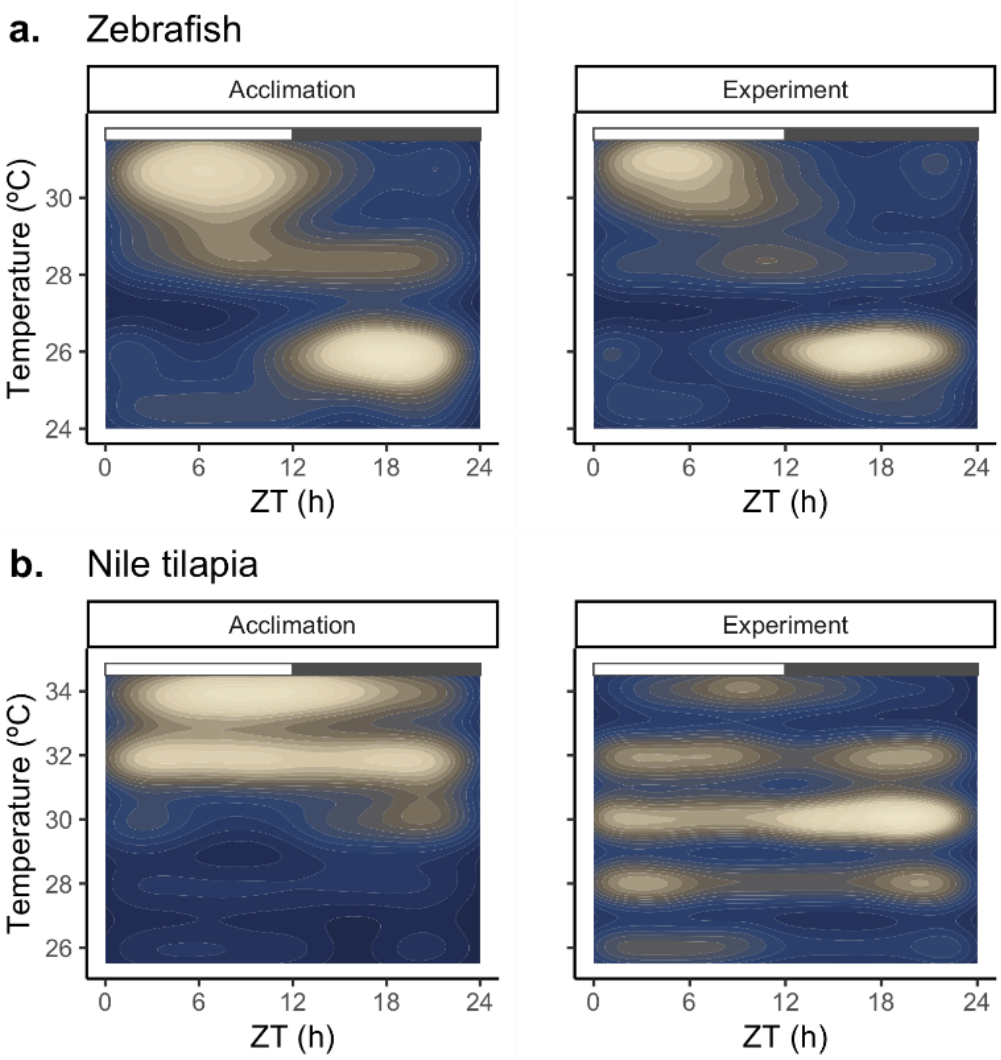

Fig. 4. Heat maps of the preferred temperature of *Danio rerio* (a) and *Oreochromis niloticus* (b) exposed to a temperature gradient during a 3-day acclimation period (Acclimation) and a subsequent 10-day experimental period (Experiment). Values represent population-level posterior distributions, with lighter colours indicating a higher relative density of fish. The time scale (x-axis) is expressed as *Zeitgeber* Time (ZT), in which ZT 0 h corresponds to light onset and ZT 12 h corresponds to lights off, indicated by the photoperiod bars above each panel.

The distribution of Nile tilapia among chambers also showed strong daily patterns during the acclimation and experimental phases (Fig. 3, Fig. 4, Table A3). The warmest chamber was favoured near the end of the light phase (Chamber 5: ZT 9.35 h [8.00–10.9] (Acc.), ZT 9.28 h [8.48–10.2] (Exp.)), and intermediate to cool chambers (1-3) during the end of the dark phase and beginning of the light phase (Chamber 1: ZT 3.76 h [2.42–4.85] (Acc.), ZT 3.82 h [2.91–4.85] (Exp.); Chamber 2: ns (Acc.), ZT 0.281 h [22.8–1.94] (Exp.); Chamber 3: ZT 20.7 h [19.6–21.8] (Acc.), ZT 19.1 h [18.4–19.9] (Exp.); Fig. 5d, Fig. A1b, Table A3).

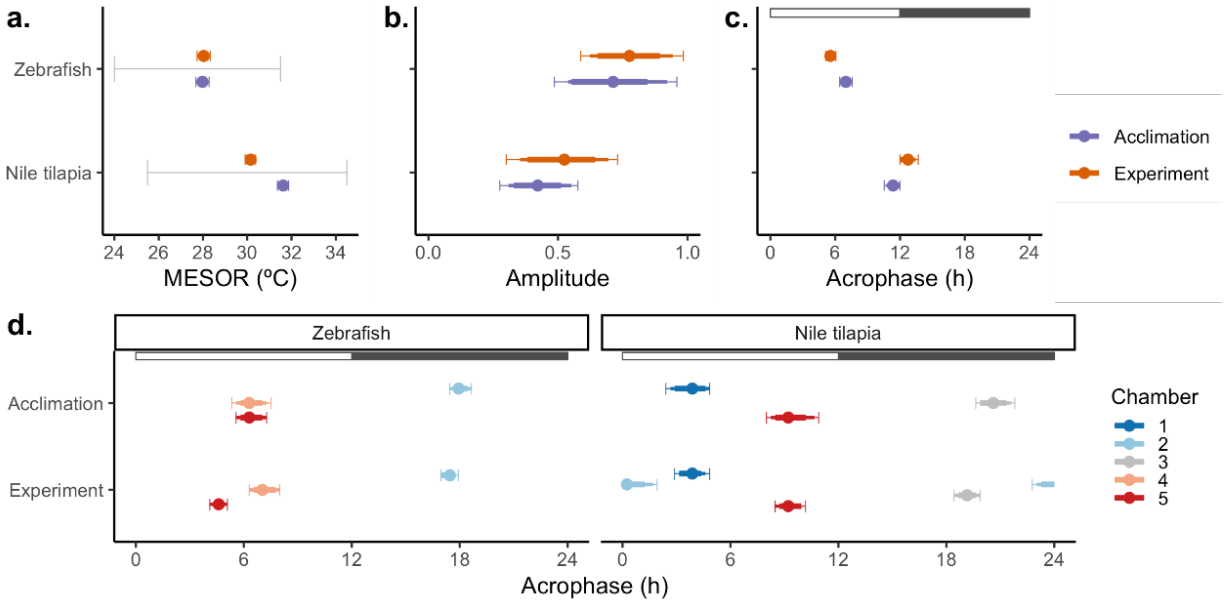

Fig. 5. Posterior summaries of daily cycle components for *Danio rerio* and *Oreochromis niloticus* during the acclimation (purple) and experimental (orange) phases. Points show posterior medians, with 80% (thick line), 90% (medium line), and 95% (thin line) credible intervals. (a) Mesor estimates for each species represent the average preferred temperature. Thin grey lines indicate the range of available temperatures. (b) Amplitude represents the range of the preferred temperature throughout the day. (c) Acrophase shows the hour with the highest preferred temperature. (d) Acrophase for the number of fish in each chamber (1–5, blue–red, cold–warm), representing the hour with the highest fish counts, with open points indicating amplitudes for daily rhythms indistinguishable from 0. Only acrophases with non-zero (95% confidence) amplitudes are shown. No chambers showed non-zero amplitudes during the control.

Control

In the absence of a thermal gradient, neither zebrafish nor tilapia presented clear daily rhythms in preferred chamber (Table A3). However, tilapia showed preference (>95% confidence) for chamber 1 over chambers 2–4 and for chamber 5 over chamber 4 (Fig. 3), indicating a preference for edge chambers (Fig. 1).

DISCUSSION

Our study reveals the existence of a robust daily rhythm of temperature preference in both the zebrafish and the Nile tilapia. When fish were exposed to a temperature gradient, both fish species selected higher temperatures during the second half of the light phase and colder temperatures at the end of the dark phase. Light-dark cycles have been described as the environmental variable that has the most influence on physiological functions in fish, being capable of synchronizing circadian rhythms during development, locomotor activity, reproductive behaviour, feeding and stress response (Kulczykowska et al., 2010; Cowan et al., 2017; Sánchez-Vázquez et al., 2018, 2019). In addition, the effect of temperature on the

circadian biology of fish has also been explored, with most studies focusing on variations of activity when both day length and temperature change (Reebs, 2002), on the molecular effects of temperature cycles on circadian rhythms (Lahiri et al., 2005) and the relative importance of light and temperature cycles as synchronisers of behavioural rhythms (López-Olmeda et al., 2006; López-Olmeda et al., 2009). Daily thermal cycles can set the phase of the clock rhythm (Sweeney & Hastings, 1960) and they are also able to entrain biological rhythms in fish, as reported for zebrafish activity rhythms (López-Olmeda et al., 2006). However, when conflicting LD and temperature cycles are imposed, zebrafish display their activity mostly during the day, irrespective of the temperature, suggesting that light is a stronger zeitgeber than temperature (López-Olmeda et al., 2006; López-Olmeda et al., 2009). Many investigations have determined the preferred temperature of marine and freshwater fish using theoretical and experimental models (Beitinger and Fitzpatrick 1979; Golovanov 2013), showing that thermal preference varies widely across fish species but the strategies of behavioural regulation of the internal temperature are conserved (Beitinger and Fitzpatrick 1979). However, few studies have considered the effect of the daily photocycle on the rhythm of temperature selection by fish. Early investigations by Reynolds and Casterlin (1978a,1978b; 1978c; 1979) showed daily variations in the preferred temperature of fish, which were species-specific. For example, *Amia calva* showed a diurnal peak of preferred temperature while *Salmo trutta* and *Carassius auratus* preferred maximum temperatures at night (Reynolds and Casterlin 1978a, 1978b, 1979). In fact, maximum values of preferred temperature were opposite in congeneric species, suggesting that different thermal rhythms might reflect niche segregation (Reynolds and Casterlin 1978c).

In our study, fish were allowed to freely choose between a thermal gradient with a wide range of temperatures (24-32 ºC and 26-34ºC for zebrafish and Nile tilapia, respectively), allowing us to characterise the daily rhythm in thermal preference, which was species-specific and related to the daily behavioural patterns. Zebrafish is a diurnal species which presents higher activity during the first hours of the light period and lower activity at night (Lopez-Olmeda et al., 2006). In the present investigation, zebrafish preferred the highest temperature (28.7 ºC) during the active phase whereas the minimum value (27.2 ºC) was selected during the resting phase (at night). Likewise, similar results have been found in terrestrial animals and humans regarding the effect of temperature on the sleep pattern (Murphy and Campbell, 1997, Harding et al 2019). In the case of Nile tilapia, the daily pattern of activity can differ between individuals, being diurnal or nocturnal (Vera et al.,

2006). Although this species may present dualism in its locomotor activity rhythms, other studies suggest that maximum swimming levels are displayed towards the end of the light period, when reproductive behaviour and spawning events also occur (Baroiller and Toguyeni 2004). When fish were allowed to choose between a thermal gradient, Nile tilapia selected the highest temperature during the last hours of the light phase whereas coldest temperatures were preferred at the end of the dark phase. Our results in zebrafish and tilapia agree with the behavioural thermoregulation hypothesis known as "hunt-warm-rest-cool", which proposes that fish would select cold waters during periods of inactivity to reduce metabolic rates (Sims et al., 2006; Gleiss et al., 2017). However, contrary to this hypothesis, the results of Macnaughton et al. (2018) in cutthroat trout (*Oncorhynchus clarkia lewisi*) showed that the preferred temperature at night increased while swimming activity decreased. Also, other studies observed that when zebrafish were allowed to choose between two temperatures (24 ºC *versus* 20 ºC), fish mostly chose the higher temperature, although their selection was influenced by their daily behavioural rhythms, displaying a diurnal pattern when choosing the higher temperature and a nocturnal pattern when selecting the compartment with the lower temperature (López- Olmeda et al., 2009). Altogether, these data suggest a complex relationship between behavioural thermoregulation and circadian activity rhythms in fish.

In the wild, the internal temperature of fish fluctuates throughout the day, which mainly depends on the environmental temperature. In fact, aquatic animals are subjected to natural thermocycles with higher temperatures being experienced during the light phase and lower temperatures during the dark phase. Accordingly, we might expect that fish selected higher temperatures during daytime, coinciding with the natural thermophase, and the lowest at night, coinciding with the natural cryophase. However, in addition to the thermocycles, the water temperature presents a vertical gradient, with temperatures being higher at the water surface and decreasing with depth. Thus, in the habitats of freshwater fish species, Diel Vertical Migrations (DVM) are commonly observed phenomena (Rudstam and Magnuson 1985). The vertical distribution is influenced by several factors, including temperature, light, dissolved oxygen, prey/food availability and predator risk (Tarling 2000, Afonso et al 2014). In the case of Nile tilapia, DMV rhythms are influenced by photoperiod and feeding behaviour. Thus, tilapias are usually found in the deepest (coldest) layers at dawn and move to the surface (warmest) when it gets dark, coinciding with the peak of active feeding in this species, at the end of the night (Piet Guruge, 1997). This fact suggests that fish may move towards warmer temperature to enhance digestion and growth rates, indicating a

link between feeding state and thermoregulatory behaviour, as described in other fish species (Reynolds and Casterlin, 1979; Wurtsbaugh and Neverman, 1988).

Interestingly, when tilapias were firstly placed into the experimental tank (during the acclimation phase), they selected higher temperatures than during the rest of the experimental phase. This behavioural response has been previously observed in this species, as well as in zebrafish, subjected to a stressful agent, and it is known as Stress Induced Hyperthermia (SIH) or emotional fever (Rey et al, 2015a, Boltana et al 2013, Rakus et al 2017). Most studies related to emotional and pathological fever have been carried out in endothermic animals (Bicego et al 2017) including humans (Briese 1995, Oka 2015). In terrestrial endothermic animals, exposure to continuous stress conditions causes an increase in body temperature, leading to emotional fever (Stress Induced hyperthermia, SIH) which is endogenously generated (Bouwknecht et al., 2007, Bhatnagar et al., 2006, Vinkers et al., 2008) by the same neural mechanism as infectious fever (Oka et al 2018). Seemingly, in ectothermic animals such as fish, when exposed to situations that cause immunological (e.g. pyrogens) or social stress (e.g. anxiety), the animals move to higher temperature areas to increase their body temperature and cope with the possible stressor to maintain the homeostasis (stress-induced hyperthermia) (Rey et al., 2015a, Rakus et al 2017, Key et al., 2017, Rey et al., 2017). On the contrary, when fish are in constant temperatures (without thermal gradient), they cannot perform this behavioural response, limiting the effectiveness of the immune response to the stressor (Rey et al., 2015a, Huntingford et al., 2020). The fact that our study offered the animals a gradient of temperatures allowed us to describe this SIH response related to the daily rhythm of thermal preference. In the case of zebrafish, they did not show a noticeable SIH response possibly due to the quick transfer from the holding tanks to the novel gradient tanks that did not elicit a stress response. Also, both zebrafish and tilapia exhibited a different temporal pattern of stabilization of thermal preference rhythm. From being moved to the novel experimental tank, the daily rhythm of thermal preference appeared from the first day of acclimation in zebrafish, while tilapia needed more than 24 h to establish the daily rhythm, selecting higher temperatures during this phase. This could indicate that tilapia, compared to zebrafish, is a more sensitive species in which, in addition to the stress caused by the movement of animals to the multichamber tank (handling stress), exposure to a new environment might generate environmental and social stress as in the case of the establishment of the hierarchy in a new group and environment (e.g. fights, food hierarchy, aggressiveness) (Rey et al., 2015a; Cunningham et al., 2017). Consequently, in thermal

experiments this species specific SIH effect needs to be accounted for by allowing the animals to acclimatize and recover from the stressor event before determining final thermal preferences.

The mechanisms involved in behavioural thermoregulation in fish are still unknown. However, the temperature detection system must play a fundamental role in the behavioural response, to prevent physiological damage caused by acute increases/decreases in temperature (Morash et al. 2021). Thermal environmental information is perceived through membrane pores or channels that are extremely sensitive to temperature variations, known as thermoTRP channels (transient receptor potential channels) (Saito and Shingai, 2006; Patapoutian et al. 2005). These thermoreceptor mechanisms are distributed in various fish tissues, but mainly in neurons of the trigeminal and dorsal root ganglia that innervate the skin (Germana et al., 2018). In zebrafish, numerous subfamilies of thermo TRPs have been described which are activated by different temperature thresholds. In addition, the expression of these thermoreceptors showed daily rhythms that are synchronised by light-dark cycles in zebrafish (Jerônimo et al., 2017; de Alba et al., 2021). Thus, in previous studies the genes involved in warm thermal sensitivity presented the highest expression during daytime, coinciding with the highest preferred temperature in our study. However, the cold-sensing gene displayed higher expression levels during the dark phase, when zebrafish chose the lower temperature (de Alba et al., 2021). The harmonization of the thermoTRPs with the environment is essential to achieve an appropriate temperature perception and trigger a behavioural response that maintains the thermal homeostasis of the fish. However, the link between the daily rhythms of gene expression of thermoTRPs and the temperature selection rhythms should be further explored, as well as the underlying mechanisms of how SIH modifies the thermal preference after a stress challenge.

In the absence of the thermal gradient, tilapia showed a tendency to prefer certain chambers over others. This effect was observed mainly in the chambers located at both ends of the tank and it could be attributed to the fact that in new environments the fish respond behaviourally by moving to the corners or edges of the tanks displaying a thigmotaxis behaviour or scototaxis if the corners are darker and less exposed (Blaser and Rosenberg 2012). However, when fish are exposed to a thermal gradient, they show a completely different chamber preference with a daily occupation rhythm. This fact demonstrates how temperature is an important environmental factor which impacts on the selection of space and the daily behavioural pattern of fish (Krylov et al., 2021).

The fact that fish showed higher temperature preferences when first introduced to the gradient tank and daily rhythms of preferred temperature suggests that behavioural regulation of internal temperature is an evolutionarily conserved adaptive response in fish. Some speculations have indicated coadaptation between thermoregulatory behaviour and thermal physiology of the animal, highlighting that the role of the preferred temperature lies in its direct relationship with physiological performance (Beitinger and Fitzpatrick 1979). Thus, fish that choose a preferred temperature that maximizes their physiological processes will be able to enjoy a better physical condition. Consequently, the preferred temperature might present phenotypic plasticity in which a change in the thermal physiology of the animal (thermal sensitivity) will lead to a change in the thermoregulatory response and vice versa (Anglietta et al., 2002). During the last decades, the increase in temperature of aquatic ecosystems due to climate change has altered the abundance and composition of species, modifying the structure and function of ecosystems (Pörtner et al., 2010). However, the effect of the temperature increase on the thermal physiology of fish has been little studied. Recent studies have shown the response of fish populations to the general warming of the oceans during the last century. Marine fish populations decreased in abundance in tropical areas near the equator and were more abundantly distributed in areas closer to the poles (Hastings et al., 2020). This phenomenon could extend to coastal subsistence species in the coming years. Latitudinal changes in the abundance of fish populations will mean that species must face large temperature fluctuations close to their thermal tolerance limits. Therefore, their thermal physiology could be compromised, which could have a considerable impact with profound effects on the biochemical, physiological and life cycle activities of the fish (Alfonso et al, 2020).

CONCLUSION

The present paper revealed the existence of daily rhythms in temperature preference of zebrafish and Nile tilapia, mainly synchronized to the LD cycle. Furthermore, during the acclimation period, Tilapia presented a stress induced hyperthermia response by choosing higher temperatures. These findings reveal the need to consider daily rhythms when discussing thermal preferences of fish. Studies related to thermoregulation and thermal ecology need to consider the circadian rhythms to clearly establish a real diurnal pattern of thermal preference and the optimal ranges of temperature. If, due to climate change, ranges are narrower and waters do not cool down at night or thermoclines change, the implications

could be dramatic and multiply exponentially due to the changes in natural thermal cycles. This work shows the adaptive nature of the behavioural regulation of the internal temperature in which fish species with broad geographic ranges present different daily patterns of preferred temperature. Our results may be useful in understanding the ecological and evolutionary aspects of thermoregulatory behaviour and in discussing the effect of climate change on the biochemical, physiological and life cycle activities of fish. In addition, our results have clear implications for the health and welfare of fish under confined environments including research, farming or as pets, and suggest that husbandry procedures should incorporate thermal cycles where possible or at least provide animals with a range of temperatures from which to choose.

ETHICAL STATEMENT

The experimental procedure complied with the Guidelines of the European Union (2010/63/UE) and the Animal (Scientific Procedures) Act 1986 UK under the approval of the Animal Welfare and Ethical Review Body (AWERB) of the University of Stirling (ref number: AWERB/1819/065/New Non ASPA) and Murcia (RD 1201/2005 and Law 32/2007; ref number A13191003).

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DATA ACCESSIBILITY

The datasets supporting this article have been uploaded as part of the electronic supplementary material.

CONFLICT OF INTEREST DECLARATION

The authors declare no competing or financial interests.

AUTHOR CONTRIBUTIONS

LMV, GA, SM, SS, FJSV and SRP conceived and designed the experiments; LMV, GA, TS, SS, FJSV and SRP analyzed the data; All authors contributed on the writing of the manuscript; LMV, FJSV and SRP provided funding.

SUPPLEMENTARY DATA

		DAY	Chamber 1		Chamber 2		Chamber 3		Chamber 4		Chamber 5	
			Zebrafish	Nile Tilapia	Zebrafish	Nile Tilapia	Zebrafish	Nile Tilapia	Zebrafish	Nile Tilapia	Zebrafish	Nile Tilapia
Temperature gradient	Acclimation	1	24.27± 0.17	25.96± 0.09	26.05± 0.34	27.90± 0.06	28.50± 0.12	29.88± 0.06	30.36± 0.21	31.73± 0.19	31.44± 0.07	33.80± 0.06
		2	24.46± 0.26	25.93± 0.18	26.06± 0.46	27.94± 0.18	28.41± 0.15	29.91± 0.12	30.19± 0.22	31.76± 0.22	31.20± 0.19	33.90± 0.15
		3	24.40± 0.35	25.83± 0.17	25.72± 0.72	27.90± 0.21	28.06± 0.41	30.06± 0.12	29.70± 0.37	32.10± 0.21	30.78± 0.42	34.30± 0.21
	Experiment	4	24.53± 0.22	26.10± 0.06	25.64± 0.71	28.05± 0.03	28.017± 0.27	30.13± 0.13	29.60± 0.17	32.08± 0.25	30.48± 0.32	34.16± 0.23
		5	24.85± 0.14	26.00± 0.06	26.02± 0.49	27.97±0.06	28.52± 0.30	29.90± 0.08	29.85± 0.39	31.70± 0.21	30.78± 0.24	33.80± 0.12
		6	24.95± 0.05	26.10± 0.06	26.11± 0.49	28.07± 0.06	28.64± 0.09	30.15± 0.03	30.21± 0.27	32.10± 0.10	31.13± 0.04	34.20± 0.10
		7	24.73± 0.05	26.06± 0.12	26.19± 0.24	28.07± 0.12	28.65± 0.11	29.93± 0.03	29.89± 0.14	31.66± 0.23	31.04± 0.03	33.80± 0.17
		8	24.51± 0.48	26.03± 0.03	26.10± 0.10	28.14± 0.09	28.21± 0.17	30.23± 0.09	29.76± 0.15	32.20± 0.15	31.12± 0.09	34.43± 0.19
		9	24.58± 0.42	25.93± 0.07	26.17± 0.04	27.99± 0.11	28.04± 0.45	30.10± 0.06	29.74± 0.25	32.08± 0.14	31.06± 0.08	34.26± 0.12
		10	24.48± 0.07	25.96± 0.03	26.20± 0.22	28.05± 0.06	28.17± 0.03	30.13± 0.09	30.02± 0.10	32.08± 0.14	31.38± 0.04	34.30± 0.15
		11	24.29± 0.21	25.96± 0.09	25.98± 0.52	27.87± 0.12	28.03± 0.16	29.96± 0.07	29.83± 0.27	31.93± 0.03	31.13± 0.23	33.96± 0.19
		12	24.03± 0.46	25.96± 0.07	26.02± 0.48	27.90± 0.01	28.11± 0.17	29.90± 0.09	29.73± 0.20	31.76± 0.18	30.98± 0.21	33.83± 0.07
		13	24.09± 0.36	26.06± 0.07	26.01± 0.47	28.00± 0.01	28.24± 0.22	30.03± 0.07	29.96± 0.35	31.93± 0.18	31.39± 0.28	34.00± 0.12
		Mean	**24.47± 0.07**	**25.99± 0.02**	**26.02± 0.05**	**27.99± 0.02**	**28.28± 0.07**	**30.02± 0.03**	**29.91± 0.06**	**31.93± 0.05**	**31.07± 0.08**	**34.05± 0.06**

	DAY	Chamber 1		Chamber 2		Chamber 3		Chamber 4		Chamber 5	
		Zebrafish	Nile Tilapia	Zebrafish	Nile Tilapia	Zebrafish	Nile Tilapia	Zebrafish	Nile Tilapia	Zebrafish	Nile Tilapia
Control	1	26.36± 0.12	30.09± 0.07	26.65± 0.05	30.01± 0.05	26.34± 0.03	29.91± 0.02	26.47± 0.06	29.86± 0.12	26.07± 0.11	30.10 ± 0.08
	2	26.60± 0.24	30.12 ± 0.13	26.80± 0.12	30.30 ± 0.11	26.47± 0.11	29.91± 0.21	26.76± 0.12	30.06± 0.17	26.30± 0.10	30.06± 0.03
	3	26.20± 0.03	30.27 ± 0.13	26.62± 0.01	30.17± 0.10	26.35± 0.08	29.91± 0.32	26.64± 0.05	29.91± 0.15	26.22± 0.04	30.17± 0.10
	4	26.00± 0.00	30.20 ± 0.13	26.44± 0.03	30.20 ± 0.10	26.25± 0.05	30.31± 0.21	26.52± 0.03	30.06± 0.21	26.15± 0.04	29.86± 0.12
	5	26.00± 0.00	29.97 ± 0.09	26.38± 0.06	30.04 ± 0.087	26.10± 0.08	29.71± 0.12	26.48±0.01	30.06± 0.17	26.02± 0.02	29.76± 0.14
	6	26.00± 0.00	30.22 ± 0.02	26.43± 0.06	29.97 ± 0.07	26.11± 0.09	30.16± 0.05	26.50± 0.00	29.76± 0.14	26.03± 0.03	30.31± 0.21
	Mean	**26.19± 0.10**	**30.14± 0.04**	**26.55± 0.07**	**30.11± 0.05**	**26.27± 0.06**	**29.98± 0.08**	**26.56± 0.05**	**29.95± 0.05**	**26.13± 0.05**	**30.04± 0.08**

Table A1. Mean temperature of the multichambered tank for the experimental and control groups of zebrafish (*Danio rerio*) and Nile Tilapia (*Oreochromis niloticus)*. The data of temperature from each chamber/day of each independent experiment was pooled together and is shown as mean temperature (°C) ± SEM for each hour of the day and night.

Model	Species	Prior distribution
Preferred temperature	Nile tilapia	μ_k ~ Normal(μ=30, σ=2)
		α_k ~ Normal(μ=0, σ=1) T(0, ∞)
		Φ_k ~ vonMises(μ=0, κ=0.001) T(-π, π)
		σ_k ~ Student-t(μ=0, σ=2.5, ν=3) T(0, ∞)
		Σ ~ lkj(η=0)
	Zebrafish	μ_k ~ Normal(μ=27.5, σ=2)
		α_k ~ Normal(μ=0, σ=1) T(0, ∞)
		Φ_k ~ vonMises(μ=0, κ=0.001) T(-π, π)
		σ_k ~ Student-t(μ=0, σ=2.5, ν=3) T(0, ∞)
		Σ ~ lkj(η=0)
Fish count per chamber	Nile tilapia & Zebrafish	β_μ ~ Normal(μ=0, σ=1)
		β_α ~ Normal(μ=0, σ=1)
		β_Φ ~ vonMises(μ=0, κ=0.001) T(-π, π)
		σ_k ~ Student-t(μ=0, σ=2.5, ν=3) T(0, ∞)
		Σ ~ lkj(η=0)

Table A2. Multi-level Cosinor analysis prior distributions.

Species	Group	Chamber	MESOR mean [95% CI]	Amplitude mean [95% CI]	Acrophase (ZT h) mean [95% CI]
Zebrafish	Control CTE	1	2.47 [2.07–2.90]	0.0733 [0–0.135]	---
		2	2.32 [1.94–2.73]	0.0651 [0–0.167]	---
		3	2.01 [1.45–2.60]	0.0378 [0–0.110]	---
		4	2.20 [1.88–2.56]	0.0386 [0–0.122]	---
		5	2.10 [1.50–2.80]	0.0505 [0–0.133]	---
	Acclimation	1	1.72 [1.20–2.33]	0.187 [0.043–0.363]	---
		2	2.50 [1.92–3.13]	0.635 [0.508–0.789]	18.0 [17.5–18.7]
		3	2.45 [1.63–3.29]	0.097 [0.003–0.205]	---
		4	1.95 [1.33–2.58]	0.389 [0.225–0.548]	6.42 [5.33–7.52]
		5	2.19 [1.44–3.00]	0.338 [0.204–0.495]	6.42 [5.58–7.27]
	Experiment	1	1.61 [0.984–2.20]	0.152 [0.042–0.289]	---
		2	2.73 [2.14–3.31]	0.556 [0.377–0.761]	17.3 [17.0–17.9]
		3	2.26 [1.46–3.06]	0.137 [0.007–0.305]	---
		4	1.81 [1.29–2.38]	0.283 [0.171–0.412]	7.14 [6.30–8.00]
		5	2.46 [1.62–3.32]	0.46 [0.258–0.677]	4.63 [4.12–5.09]
Nile tilapia	Control CTE	1	2.91 [2.36–3.52]	0.0403 [0–0.107]	---
		2	2.14 [1.78–2.50]	0.0455 [0–0.114]	---
		3	2.16 [1.95–2.40]	0.0308 [0–0.087]	---
		4	1.84 [1.48–2.20]	0.0298 [0–0.090]	---
		5	2.52 [1.98–3.08]	0.0341 [0–0.087]	---
	Acclimation	1	0.724 [0.372–1.08]	0.218 [0.030–0.383]	3.76 [2.42–4.85]
		2	0.789 [0.448–1.16]	0.128 [0.024–0.241]	---
		3	1.74 [1.44–2.03]	0.260 [0.110–0.398]	20.7 [19.6–21.8]
		4	3.62 [2.71–4.59]	0.085 [0.003–0.188]	---
		5	3.87 [2.89–4.78]	0.231 [0.047–0.412]	9.35 [8.00–10.9]
	Experiment	1	1.44 [0.897–2.08]	0.213 [0.073–0.366]	3.82 [2.91–4.85]
		2	2.22 [1.74–2.69]	0.272 [0.096–0.437]	0.281 [22.8–1.94]
		3	3.37 [2.67–4.01]	0.212 [0.061–0.361]	19.1 [18.4–19.9]
		4	2.55 [2.08–3.04]	0.075 [0.001–0.183]	---
		5	1.63 [1.18–2.17]	0.289 [0.077–0.475]	9.28 [8.48–10.2]

Table A3. Cosinor results for chamber preference. Acrophase means and 95% credible intervals are shown for chambers with a non-zero amplitude for a daily cycle. During the Acclimation and Experiment phases, temperature increased from chamber 1 to chamber 5.

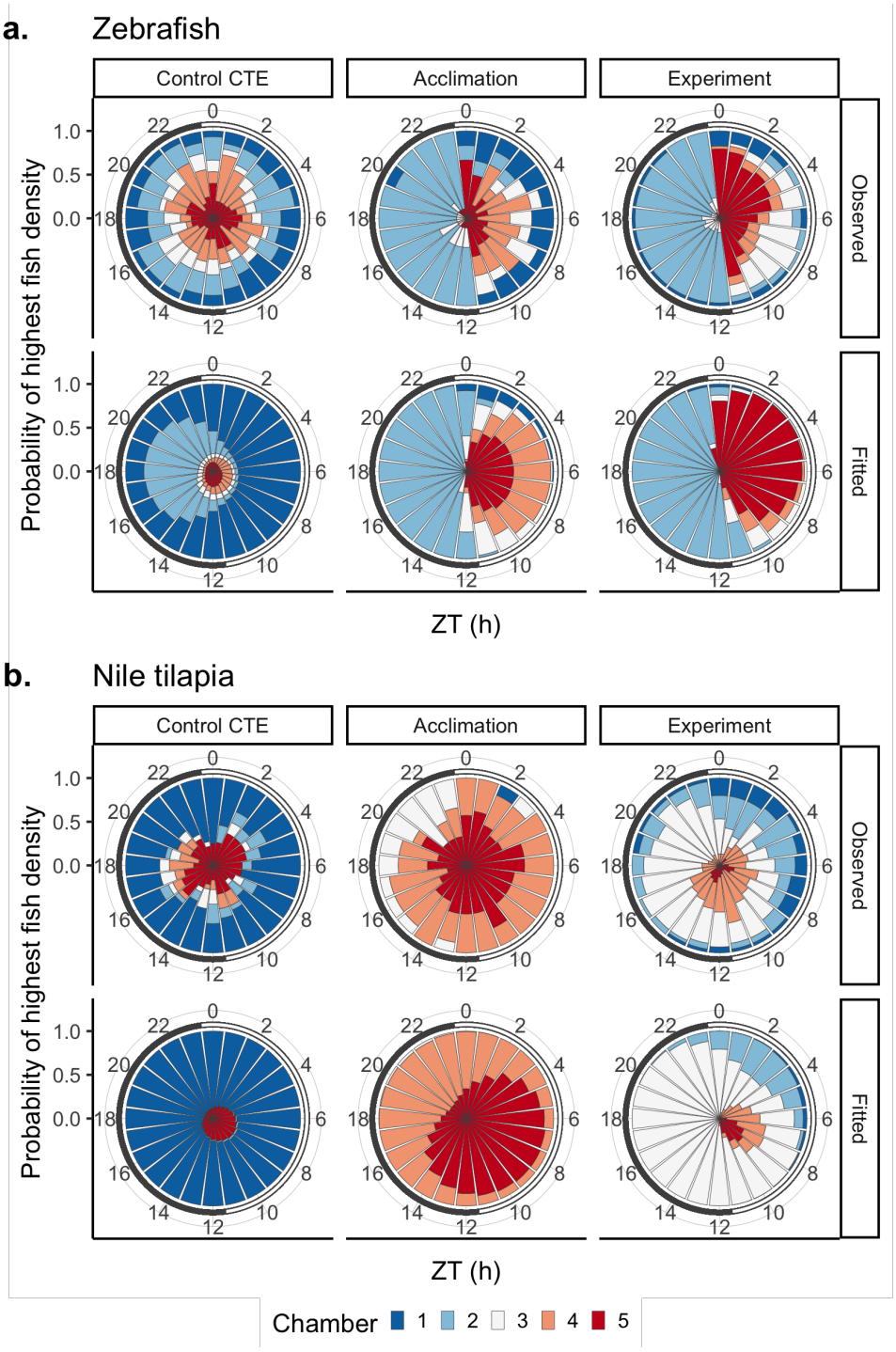

Figure A1. Daily pattern in preferred chamber. For each hour (0-24), the height of the bar shows the probability that each chamber (1-5, blue-red) is the chamber with the highest density of fish. During the acclimation and experimental phases, the thermal gradient ran from cool (chamber 1) to warm (chamber 5), with all chambers identical temperatures in the control. White and gray rings show photoperiods. Zebrafish (a) preferred warmer chambers (4-5) overall in the acclimation and experiment periods, with strongest affinity for warmth near in the middle of the light phase. Tilapia (b) showed clear preference for warmer chambers (4-5) overall in the acclimation period, with strongest affinity for warmth near the end of the light phase.

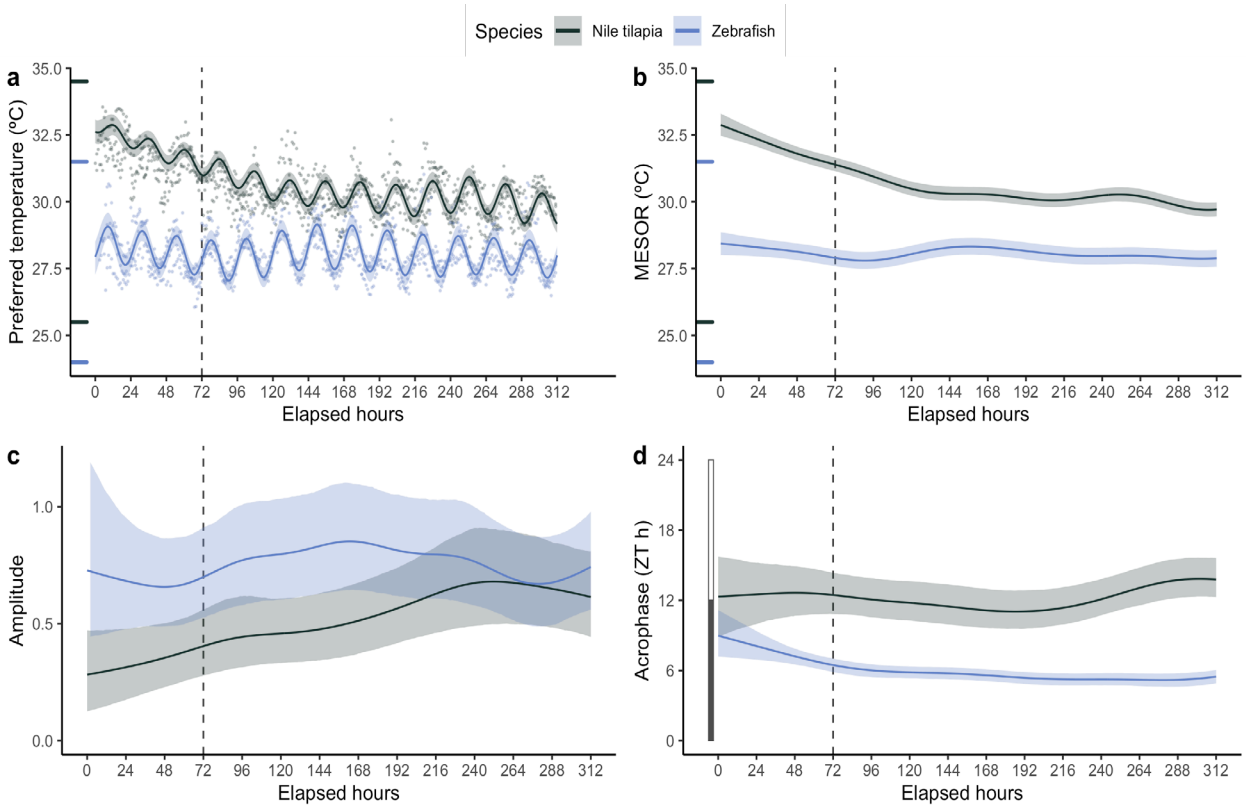

Figure A2. Patterns in preferred temperature using smoothed predictors. Each cosinor parameter was fitted as a smoothed function of elapsed time for Nile tilapia (dark green) and zebrafish (light blue). Lines show population-level posterior mean and ribbons show 95% credible intervals for (a) preferred temperature, (b) mesor, (c) amplitude, and (d) acrophase. Vertical dashed lines indicate the threshold between acclimation and experimental periods. The rug along the y-axis (a, b) shows the range of temperatures available to each species, and the white and black bar (d) shows the light and dark phases.

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Capítulo Experimental VIII

Effect of light and feeding regimes on the daily rhythm of thermal preference in Nile tilapia (*Oreochromis niloticus*)

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ABSTRACT

Thermal preference in fish varies through day, allowing them to develop their biological processes in the most suitable thermal conditions. The aim of this study was to determine the effect of photoperiod and feeding regimes on the daily rhythm of thermal preference in Nile tilapia (*Oreochromis niloticus*). For this purpose, using a multichamber tank with a thermal gradient of 26 ºC to 34 ºC or with a constant temperature of 30 ºC (control), the fish were allowed to freely choose their preferred temperature under different photoperiods and feeding schedules. Fish were exposed to 3 different photoperiod combinations [Light:Dark (LD) cycle, inverted LD cycle (DL) and constant conditions (DD)] and 4 different feeding schedules [random (RD); feeding in the middle of the light phase (mid-light, ML); feeding in the middle of the dark phase (mid-dark, MD) and starvation]. Regardless of the feeding/starvation protocol, a daily rhythm of temperature preference was observed, suggesting the role of LD cycles as the main synchronizer. In all experimental phases tilapia chose higher temperatures at dusk and cooler temperatures at dawn. In addition, the persistence of the rhythm under DD and fasting conditions indicated that an endogenous pacemaker may be driven this rhythm. Also, during acclimation, fish exhibited a stress induced hyperthermia (SIH) response, characterised by the selection of higher temperatures. On the contrary, fish fed at MD selected cooler temperatures at night than fish fed at ML or RD. Finally, starvation resulted in the selection of temperatures below the thermal optimum. These findings provide new insights into ecological and evolutionary aspects of fish thermoregulation and might be useful when designing feeding protocols, which could impact on fish welfare and thereby optimise fish production.

Keywords: Nile tilapia, Light, Mealtime, Starvation, Thermal preference, Daily rhythms.

INTRODUCTION

The predictable changes in the environment caused by the geophysical cycles have promoted natural selection and the emergence of biological clocks (Kulczykowska et al., 2010). Biological rhythms allow the organisms to keep track of time which confer them adaptive advantages, by displaying their physiological and behavioral responses at the most suitable times of the day and year (DeCoursey, 2004). In addition, biological clocks have evolved in such a way that they are able to maintain the circadian rhythmicity endogenously (Mulder et al., 2013). In the absence of environmental cues (e.g. constant lighting conditions), the biological clock loses its external entrainment, and the circadian rhythms can run freely on their own for some days with a period (τ) of approximately 24 h, thus indicating their endogenous control (Aschoff, 1960, 1981). In most cases, this adaptive response is synchronized with environmental time cues, or zeitgebers, causing a stable phase link between the rhythm and the zeitgeber, allowing endogenous clocks to synchronize with environmental cycles and resulting in a biological rhythm (Villamizar et al., 2013; Prokkola et al., 2018; Sánchez-Vázquez and López-Olmeda, 2018).

In the aquatic environment, light/dark cycles are considered the strongest of these zeitgebers and, as a result, influence numerous physiological rhythms in fish (Villamizar et al., 2011; 2013; Sánchez-Vázquez and López-Olmeda, 2018). In addition, other factors, such as temperature cycles and food availability, may alter the phase of intrinsic clocks and, it might even be more significant than light cues in some fish species. While light affects biological rhythms through variations in photoperiod (seasonal fluctuations) and light intensity (day/night variations), temperature affects biological rhythms through daily and seasonal thermocycles (Villamizar et al., 2012). Indeed, in the absence of light, thermocycles can synchronise biological rhythms (Rensing and Ruoff, 2002). The synchronizing effect of thermocycles has been reported for a wide range of biological processes, from the expression of clock genes to behavioral rhythms (Lahiri et al.,2005; López-Olmeda et al., 2006; López Olmeda and Sánchez-Vázquez, 2009). Furthermore, temperature compensation is another adaptive response of circadian rhythms that enables the biological clock to display a stable phase at different temperature thresholds (Sweeney and Hastings, 1960).

The role of light and temperature cycles on daily feeding rhythms has also been extensively studied in several fish species (Boujard and Leatherland., 1992; López-

Olmeda and Sánchez-Vázquez, 2010; Pickel and Sung, 2020). Thus, fish prefer to feed at certain times of the day, and different species can be classified as diurnal or nocturnal feeders, if they prefer to feed during the day or at night, respectively (López-Olmeda and Sánchez-Vázquez, 2010). In addition, when food is offered at the same time every day, fish increase their locomotor activity levels several hours before meal time, showing food anticipatory activity (FAA), which enables them to maximize food intake and enhance the digestive processes (Aranda et al., 2001). On the other hand, temperature sets the dynamics of biochemical reactions, affecting ectotherms metabolic rate and thus their energy balance which impact on the physiological processes involved in metabolism, feeding, digestion, locomotor and feeding behavior (Volkoff and Rønnestad, 2020). In turn, feeding habits of fish are also determined by other factors, such as diel vertical migration (DVM) patterns, in which they are able to choose the space with the optimum light intensity and temperature to optimize their digestive and metabolic processes (Mehner, 2012; Neverman and Wurtsbaugh, 1994). However, despite the strong link between light and feeding cycles with the thermal physiology of fish (López-Olmeda and Sánchez-Vázquez, 2010), their effect on the daily rhythms of thermal preference has not been clarified yet.

In ectothermic animals like fish, water temperature plays an important role on their physiology, since temperatures above or below the optimal temperature have a considerable impact on their performance, compromising their survival and therefore largely dictating species distribution (Brett, 1971; Hasting et al., 2020). In most fish, the main mechanism for maintaining the internal temperature within a physiological range is the behavioral regulation through movement toward optimal temperature zones (Haesemyer, 2020). Thermal preference is species-specific and it is determined primarily by the individual's thermal history and acclimation temperature (Haesemyer, 2020). Other factors also influence thermal preference such as age (Kwain and McCauley, 1978), size (Morita et al., 2010), maturity stage (Golovanov, 2006), feeding behavior (Wurtsbaugh and Neverman, 1988), nutritional status (Reynolds and Casterlin, 1979), immunity and stress state (Rakus et al., 2017). In addition to these factors, it has been observed that the preferred temperature can vary during the season of the year (McCauley and Huggins, 1979) and even throughout the day, showing a specific pattern depending on the fish species (Vera et al., 2022). Thus, temperature selection allows fish to

synchronize their biological processes with their daily thermal needs to optimize their performance and ensure their survival.

Nile tilapia (*Oreochromis niloticus*) is a fish species of high interest in aquaculture worldwide. In nature, tilapia is exposed to very heterogeneous thermal environments (Omondi et al., 2014; Ndiwa et al., 2016) and daily variations in temperature (22-34 ºC) (Trewavas, 1983; Culberson and Piedrahita, 1996). However, in aquaculture, fish are very often exposed to artificial photoperiod (Ridha and Cruz, 2000; Hui et al., 2019) and feeding (Sousa et al., 2012) protocols and are reared under constant temperatures which may alter the thermal physiology of the individual (Angiletta et al., 2002). Given the role of light-dark cycles and feeding regimes as the most important synchronizers of biological rhythms in fish, the aim of this study was to evaluate the effect of photoperiod and mealtime/starvation on the daily rhythm of thermal preference in Nile tilapia.

MATERIALS AND METHODS

The present research was conducted at the facilities of the Department of Physiology of the University of Murcia (Spain). Fish were reared following Spanish legislation on Animal Welfare and Laboratory Practices. Experimental protocols were performed following the Guidelines of the European Union (2010/63/UE) and Spanish legislation (RD 53/2013 and Law 32/2007) for the use of laboratory animals and were also approved by the National Committee and the Committee of the University of Murcia on Ethics and Animal Welfare (CEEA-OH 764/2022).

Animals and housing

One-month old male and female Nile tilapia were obtained from a major aquaculture company (Fishgen, Swansea, U.K.). The recirculation system was connected to 300-liter individual tanks for the animals, each of which was provided with an aeration system, biological and mechanical filters. Using a water heater and a refrigerator, the water temperature was maintained at 30.1 ± 0.5°C (AB Aqua Medic, Gewerbepark, Germany). Weekly measurements of the water quality indicators (pH, ammonia, nitrate, nitrite, and dissolved oxygen) were performed. The photoperiod was set at 12:12 h light:dark (LD), with lights on at 9:00 h (ZT 0h) and light off at 21:00 h (ZT 12 h). The

feeding schedule was established using automatic feeders (Eheim GmbH & Co. KG, model 3581, Deizisau, Germany) with three meals per day (at 11:00 a.m., 15:00 p.m., and 19:00 p.m.) at a feeding rate of 2% of the total biomass each day (D-4 Alterna Basic 2P, Skretting, Spain) with 36% crude protein (CP).

Juveniles were reared under the previous conditions for 8 months until they reached sexual maturity. After acclimation, animals were sexed by visual inspection of the genital papilla (Hussain, 2004) and males and females separated for 2 weeks. Then, tilapia breeders were subjected to hormonal treatment with human Chorionic Gonadotropin hormone (hCG, Sigma Aldrich, St. Louis, USA) as described elsewhere (Fernandes et al., 2013; Espirito Santo et al., 2020). After hCG administration, animals were placed together in a male:female ratio of 2:1 for natural reproduction during the afternoon (Fernandes et al., 2013). At the beginning of the next light phase, fertilized eggs were removed from the mouth of the female. This procedure allowed us to obtain fertilized eggs of less than 12 hours post fertilization, which were used for the experiments. Fertilized eggs were obtained from more than 10 different groups of tilapia breeders. Embryos and larvae were reared in incubators until 7 days post fertilization (dpf), when they were transferred to 9-liter tanks connected to the same temperature system larvae. At the same time (7 dpf), larvae started exogenous feeding (Gemma, 42% crude protein, 0.5-0.8 micrograms). Thus, four daily meals were provided until satiety (at 9:00h, 11:00h, 15:00h and 19:00h). Embryos and larvae were kept at a constant temperature of 30.0 ± 0.2 ºC until the weight of each individual was 2.05 ± 0.10 g

Experimental design

Thermal preference was assessed using a custom-built multichambered tank (Rey et al., 2015; Vera et al., 2022). A total of 108 Nile tilapia juveniles (older than 40 days post fertilization) were used in two independent experiments to evaluate the effects of (1) light and (2) mealtime/starvation on Nile tilapia thermal preference rhythm (Figure 1). In both experiments, fish were placed in the tank with a continuous thermal gradient between 26-34 ºC (Table A1 and A2a) during the different experimental phases and their behavior was video recorded to assess their temperature preference during the whole period. To establish the thermal gradient, the range of temperatures were chosen according to previous studies evaluating Nile tilapia thermal preferences (Cerqueria et al., 2016; Vera et al., 2022). Prior to the start of each trial, fish were placed in the central chamber

(chamber 3) of the gradient tank, and recordings immediately started. The first 3 days of each experiment were considered as the acclimation phase to the tank prior to the start of the experiments. In the acclimation phases, fish were exposed to a 12L:12D photoperiod cycle and fed at ramdon times. In both experiments, feeding was provided using automatic feeders at a feeding rate of 2% of the total biomass each day, with 42% crude protein (CP) (Gemma wean, Skretting, Spain). LED strips (SOLBRIGHT®, LED Flex Strip 1043-W, Rayte, S.L., Murcia, Spain) were used at a height of 30 cm from the water to illuminate the tank, producing 0.84 Wm-2 (around 200 lx) of light irradiance on the surface of the water. Five infrared LED lights (BW® 48 LED Infrared Illuminator) were placed behind the tank to enable video recording at night. To disperse the infrared light and enhance the image clarity at night, a translucent acrylic white sheet (Falken Design WT2447-1-8/2436 Acrylic White Sheet, Translucent 55%, 100 x 30 x 0.3 cm) was installed on the back wall of the tank.

Effects of light on daily rhythms of thermal preference

In order to determine the effect of photoperiod on the daily rhythm of thermal preference in Nile tilapia, three independent groups of fish (n=12/group) were subjected to different lighting regimes (Fig. 1a). After acclimation, the fish were subjected to a 12:12 h Light: Dark (LD) cycle and a random feeding regime (LD + RD) to determine the daily rhythm of thermal preference. Then, the LD cycle was inverted (DL) to evaluate the synchronizing effect of light on the rhythm of thermal preference. After this phase, to examine the effect of feed intake on thermal preference, fish were kept under a DL photocycle but starved for 4 days (DL+ST), followed by a 10 days phase in which feeding was resumed at random times (DL+RD) . Finally, the endogenous control of thermal preference rhythmicity, fish were subjected to constant darkness (DD) and starvation for 7 days. All phases lasted 10 days. Feeding was supplied at random times during all phases and it was removed to rule out between the DL and DD phases to avoid synchronization to food (Sánchez-Vázquez et al., 1997).

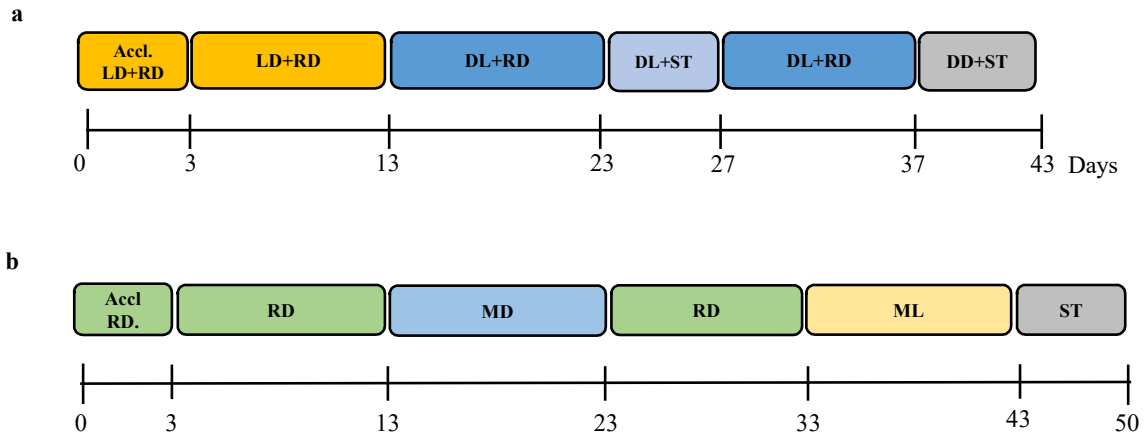

Fig. 1. Schematic representation of the experimental design. **A** Fish were exposed to a temperature gradient and subjected to different consecutive photoperiod conditions: acclimation under 12:12 h Light: Dark (LD) cycle (Aclimation); LD cycle; inverted LD cycle (DL); DL and Starvation (DL+ST); DL+Refeeding (DL+R); and constant darkness (DD). **B** Fish were exposed to a temperature gradient or constant temperature (control) and subjected to different consecutive feeding schedules: acclimation under random feeding (acclimation); ramdom feeding (RD); feeding at midnight (mid-dark, MD = ZT 18 h); feeding at the middle of light phase (mid-light, ML = ZT 6 h); 2nd Random (RD2), and prolonged starvation (ST).

Effects of feeding regimes on the daily rhythms of thermal preference

In order to determine the effect of feeding regimes on the daily rhythm of thermal preference, tilapia were subjected to consecutive experimental phases in which the photoperiod was set at 12 h:12 h LD in all cases but different meal times were imposed: (1) Acclimation during 3 days: LD + RD feeding (ACCL), (2) LD + RD feeding for 10 days, (3) LD + feeding at mid-dark (MD = ZT 18h) for 10 days, (4) LD + RD feeding for 10 days, (5) LD + feeding at mid-light (ML = ZT 6h) for 10 days and (6) LD + starvation (ST) for 7 days (Fig. 1b). Feeding was provided once a day by means of automatic feeders, at a feeding rate of 2% of the total biomass. Special care was taken to allow a uniform distribution of feed between all the tank chambers. After the MD phase, the animals were fed randomly for 10 days to desynchronize the effect of the mealtime (Sánchez-Vázquez et al., 1997). For the random feeding phases, the feeding interval was calculated between 12 and 36 h (Interval function = 12 + Random*24) so that, on average, fish received the same amount of food every 24 h than in the ML and MD phases. In addition, to rule out that chamber preference was affected by other factors different than temperature, three additional independent replicated experimental tanks (n=12 fish/tank)

were kept a constant water temperature of 30.0 ± 0.2 ºC and fish subjected to the same photoperiod and feeding phases indicated above (Table A2b) (control experiment).

Video recording and data acquisition

For each experiment, video recording started on the first day at zeitgeber time (ZT) 0 h and was carried out during all experimental phases. All experimental videos were recorded at one frame per second using a video camera (Logitech Webcam C300-1.3MP, Switzerland) and the Multiviewer software (Computer System Department, University of Murcia, Spain). All video recordings were analysed using Fish Counter software (Dr. Ginés García Mateos, University of Murcia, Spain, Version 3.0). The results were recorded into a Microsoft Excel spreadsheet.

Statistical analysis

In the thermal gradient experiments, the mean temperature chosen at each time of the day (ZT) in all of the experimental replicates was calculated using the formula below, to determine how temperature preference changed throughout the day.

$$preferred\ temperature\ = \frac{(n_1T_1 + n_2T_2 + n_3T_3 + n_4T_4 + n_5T_5)}{N}$$

Where N is the total number of fish, and n is the number of fish in compartments 1 through 5. T is the temperature of the corresponding compartment.

The existence of circadian rhythmicity of thermal preference in both experiments was tested by the Cosinor analysis, performed with the chronobiology software "El Temps" (v.1.291, Prof. DíezNoguera, University of Barcelona, Spain) in order to determine whether daily changes of thermal preference fitted the cosine function: $Y = M + A * [Cos (\Omega t + \Phi)]$, where M is mesor, A is amplitude, Ω is angular frequency (360°/24h for the circadian rhythms) and Φ is acrophase. In addition, this software was also used to determine the length (tau) of the circadian rhythm in the absence of environmental synchronizers (DD + ST) by chi-square periodogram analysis.

A GLM repeated measures was performed to study differences in temperature preference during the day, as well as the effects of light and feeding regimes: the within-

subjects factor was “time of the day (ZT)” and the between-subjects factors were “light regime” or “feeding regime”. Also, a GLM repeated measures was performed to study differences in tank chamber preference during the day between control (constant temperature) and experimental (temperature gradient) conditions. For all tests, the significant threshold was $p < 0.05$. All analyses were performed using IBM SPPS statistics v17 for Windows 10 (IBM, Armonk, NY). Graphs were plotted with GraphPad PRISM v8 for Windows.

RESULTS

Effects of photoperiod on daily rhythms of thermal preference

A significant daily rhythm of preferred temperature was found in all the experimental phases, with the highest temperature being selected at the end of the light phase (ZT 10.83 h = acrophase) during the acclimation period (Figure 2a and A1; Table A3) (Cosinor $p<0.001$), whereas during the LD and DL phases were located at the beginning of the night (at ZT 13.10 h and ZT 14.76 h, respectively) (Figure 2b, c and A1; Table A3) (Cosinor $p<0.001$).

While the maximum preferred temperatures during acclimation, LD and DL occurred in the first hours of the night (32.04 ± 0.04 ºC; 31.14 ± 0.20 ºC; 30.58 ± 0.01 ºC, respectively), the minimum values (30.50 ± 0.29 ºC; 29.21 ± 0.53 ºC; 27.79 ± 0.09 ºC, respectively) occurred from the late night to the early morning (Figure 2a, b, c) (GLM repeated measures, $p < 0.05$). In addition, different feeding regimes (starvation *versus* random feeding) did not affect the rhythm of thermal preference. Thus, the acrophases during the DL+ST and the DL+RD phases were located at ZT 14.46 h and ZT 14.58 h, respectively (Cosinor $p < 0.001$) (Figure 2d, e and A1; Table A3). Thus, during acclimation, tilapia chose a higher mean temperature (31.52 ± 1.10 ºC) than in the LD (30.06 ± 0.08 ºC) and DL (29.08 ± 0.08 ºC) phases (Fig. 2a) (GLM repeated measures, $p < 0.05$). A significant difference in the mean preferred temperature was observed between the 3-days acclimation phase and the subsequent ones. Thus, during acclimation, tilapia chose a higher mean temperature (31.52 ± 1.10 ºC) than in the LD (30.06 ± 0.08 ºC) and DL (29.08 ± 0.08 ºC) phases (Fig. 2) (GLM repeated measures, $p < 0.05$).

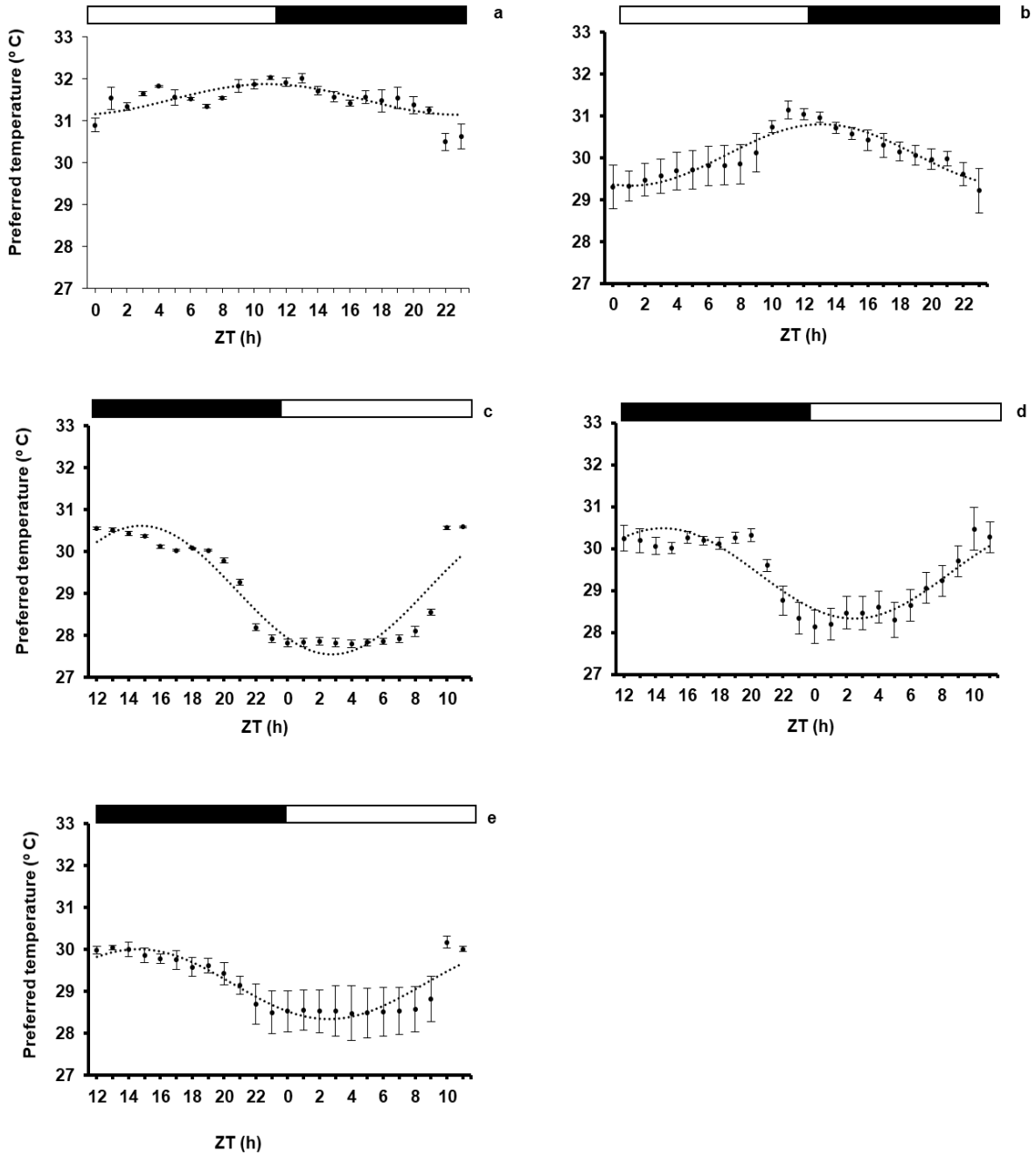

Fig. 2. Preferred temperature of *Oreochromis niloticus* exposed to a temperature gradient during acclimation under 12:12 h Light: Dark (LD) cycle (Aclimation, a); LD cycle (LD, b); inverted LD cycle (DL, c); DL and Starvation (DL+ST, d); DL+Refeeding (DL+R, e); and constant darkness (DD, f). Data from all 3 experiments was pooled together and is shown as mean temperature (°C) ± SEM for preferred temperature. The discontinuous line represents the adjustment to sinusoidal rhythm (Cosinor, $P< 0.05$). The white and black bars above the graphs represent the light and dark phases, respectively. The time scale (x-axis) is expressed as *Zeitgeber* Time (ZT), in which ZT0 h corresponds to light onset and ZT12h corresponds to lights off.

Finally, in all the experimental replicates, the daily rhythm of thermal preference persisted for 7 days under DD conditions with a significant mean period (tau, τ) of 22.55 ± 0.41 h (Fig. 3) (chi-square periodogram, confidence level of 95%).

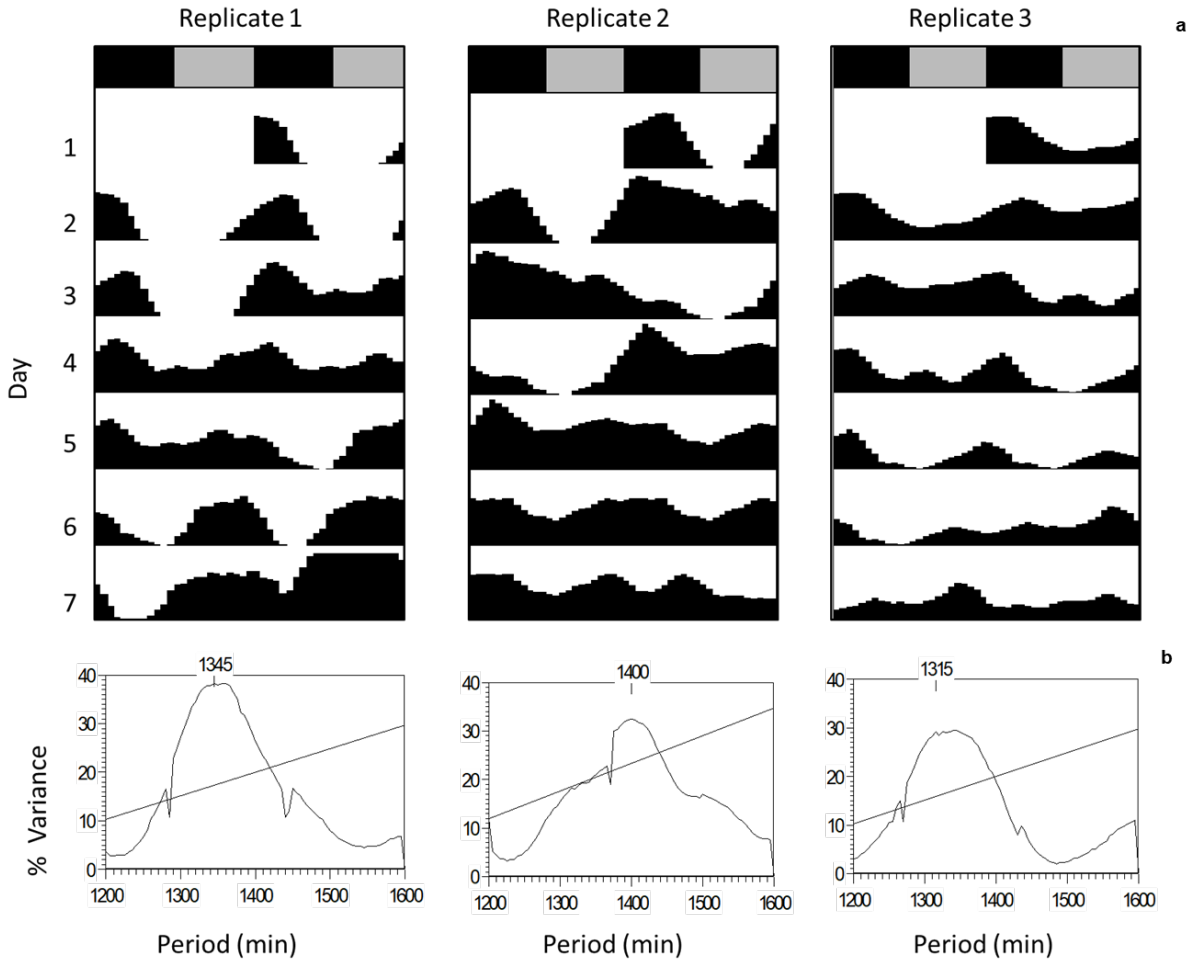

Fig. 3. **A.** Free-running daily rhythms of preferred temperature of the three groups of Nile tilapia (*Oreochormis niloticus*) under a DD cycle and deprived of food (DD+ Starvation). For convenient visualization, the data have been double plotted (48 h); the y-axis progresses in single days, with each day being plotted twice (day 1 on the right side is repeated on day 2 on the left side). The preferred temperature was binned every 1 hour, the height of each point representing the value of preferred temperature. The grey and black bars represent the supposed light and dark period, respectively, indicating the continuous darkness conditions during the DD phase. **B**. Chi-square periodogram analysis (confidence level, 95%) for each replicate during DD phase are also shown. The periodogram indicates the percentage of variance of the rhythm explained by each analyzed period within a range of 20 to 26.6 h. The highest percentage is associated with the real value of the period (τ). The significant τ (in minutes) is indicated at the top of each plot. The horizontal line represents the threshold of significance, set at p = 0.05.

The distribution of Nile tilapia among chambers showed daily patterns of preference during the acclimation and experimental phases (Fig. 4) (Cosinor $p < 0.001$). During the acclimation, higher number of fish selected the warmest chambers (Fig. 4a). In addition, during the acclimation, LD, DL, DL+ST and DL+R phases, the warmest chamber (4-5) was selected near the end of the light phase and intermediate to cool chambers (2-3) during the end of the dark phase and beginning of the light phase (Fig. 4) (Cosinor $p < 0.001$).

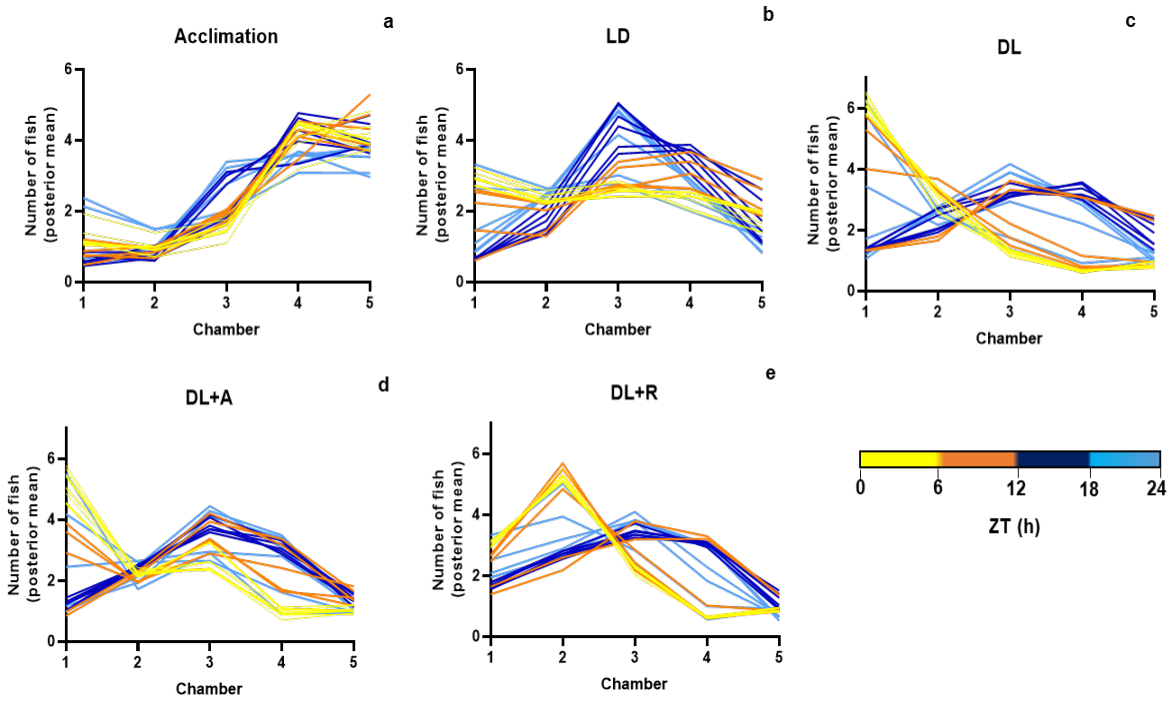

Fig. 4. Line plots of the mean fish count in each chamber for Nile tilapia (*Oreochromis niloticus*) by hour exposed to a thermal gradient during the different light photoperiod schedules: acclimation under 12:12 h Light: Dark (LD) cycle (Aclimation, a); LD cycle (LD, b); inverted LD cycle (DL, c); DL and Starvation (DL+ST, d); DL+Refeeding (DL+R, e); and constant darkness (DD, f). Lines show the posterior mean for each hour, expressed as *Zeitgeber* Time (ZT), in which ZT 0 h corresponds to light onset and ZT 12 h corresponds to lights off.

Effects of feeding regimes on the daily rhythms of thermal preference

The statistical analysis showed that the daily rhythm of preferred temperature was affected by different feeding regimes and thermal treatments (thermal gradient vs. constant temperature) (GLM repeated measures, $p<0.05$). As observed in the previous experiment, during the acclimation phase, fish selected a higher mean temperature (31.93 ± 0.10 °C) than during the rest of the experiment (30.71 ± 0.08 °C in RD, 30.06°C ± 0.07

in MD, 30.34 ± 0 .07 °C in RD2; 30.32 ± 0 .12 ºC in ML) (Fig. 5 and A2a; Table A4) (GLM repeated measures, $p < 0.001$). In addition, when compared to the values observed in fish fed at MD, a higher preferred temperature was selected during the first phase in which they were fed at RD times (Fig. 5b, c and A2a; Table A4) (GLM repeated measures, $p < 0.001$). Likewise, when subjected to starvation, tilapia preferred a lower daily mean temperature (28.82 °C ± 0.07) than during the previous phases (Fig. 5e and A2a; Table A4) (GLM repeated measures, $p < 0.001$).

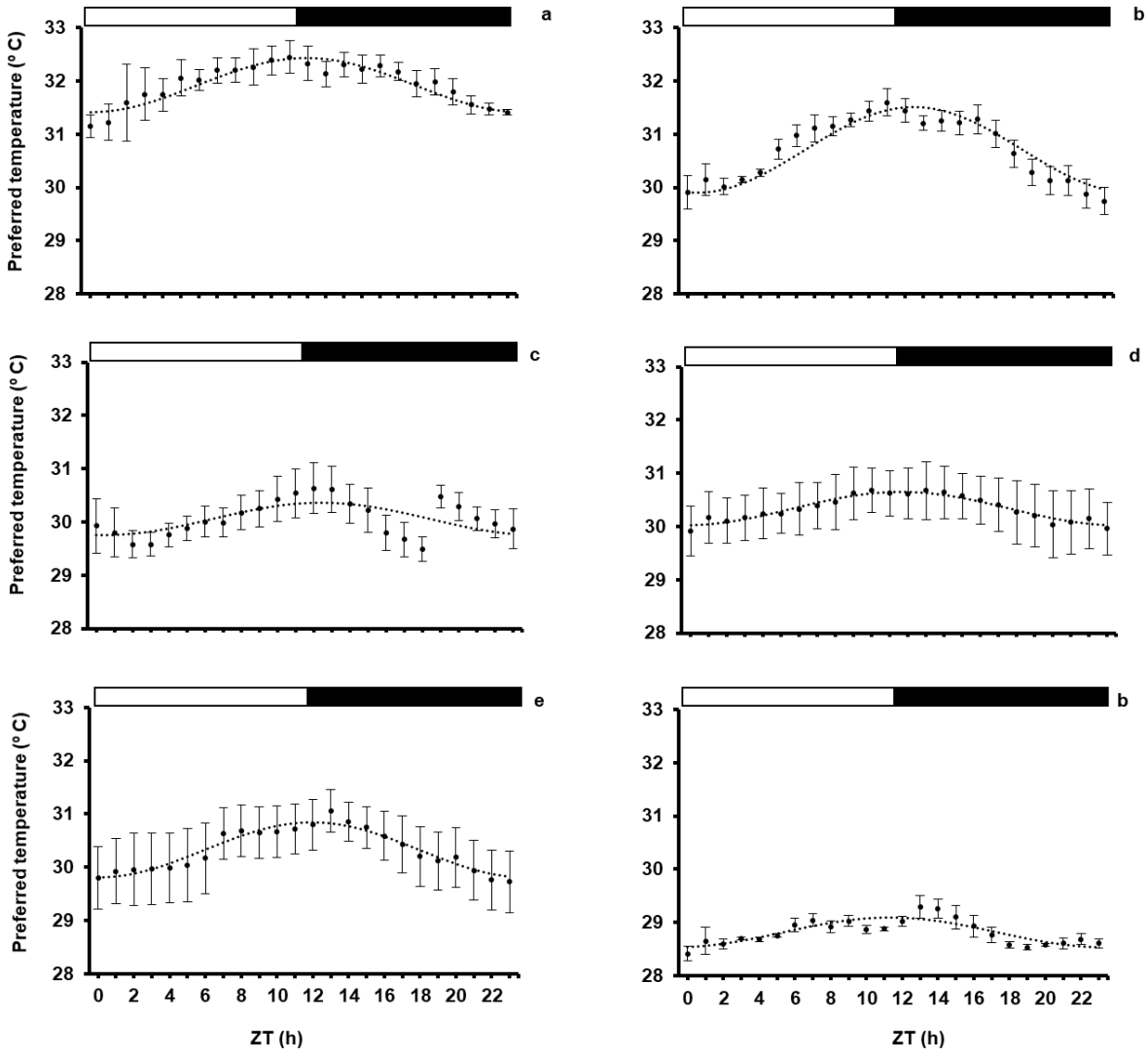

Fig. 5. Preferred temperature of *Oreochromis niloticus* (b) exposed to a temperature gradient during feeding/starvation schedules: acclimation under random feeding (acclimation, a); ramdom feeding (RD, b); feeding at midnight (mid-dark, MD = ZT 18 h, c); feeding at the middle of light phase (mid-light, ML = ZT 6 h, d); 2nd Random (RD2, e), and prolonged starvation (ST, f). Data from all 3 experiments was pooled together and is shown as mean temperature (°C) ± SEM for preferred temperature. The discontinuous line represents the adjustment to sinusoidal rhythm (Cosinor, $P< 0.05$). The white and black bars above the graphs represent the light and dark phases, respectively. The time scale (x-axis) is expressed as *Zeitgeber* Time (ZT), in which ZT0 h corresponds to light onset and ZT12h corresponds to lights off.

Regardless of the feeding regime, a daily rhythm of temperature preference was observed in all experimental phases, in which tilapias selected higher temperatures around the light-dark transition (around ZT 12 h) and cooler temperatures at end of the dark phase and beginning of the light phase (Fig. 5) (Cosinor, $p<0.001$). However, the difference between the maximum and minimum values (Amplitude) differed depending on the feeding schedule (Fig. A2 and Table A4) (GLM repeated measures, $p < 0.001$). The highest amplitude was observed in the first phase in which tilapia received food at random times while the lowest amplitude was during the starvation phase (Fig. A2 and Table A4) (Cosinor, $p<0.05$).

During acclimation, the fish showed a significant daily rhythm of temperature preference, with the acrophase observed at ZT 11.90 h. In addition, the maximum value selected by the fish was 32.45 °C (at ZT 11 h) and the minimum was 31.15 °C (at ZT 0 h) (Fig. 5a) (Cosinor, $p < 0.001$). The results of the first phase of random feeding confirmed the existence of a daily rhythm of preferred temperature with the acrophase found at ZT 12.48 h, while the maximum and minimum selected were lower than those observed during acclimatization phase: 31.60 °C (at ZT 11 h) and 29.73 °C (at ZT 23 h), respectively (Fig. 5b) (Cosinor, $p < 0.001$). When tilapias were fed at MD (ZT 18 h), the significant daily rhythm of temperature preference was maintained peaking at ZT 12.40 h, with the maximum temperature (30.64 °C) at ZT 12 h and the minimum (29.49 °C) at ZT 18 h (Fig. 5c) (Cosinor, $p < 0.001$). However, in this phase a change in thermal preference pattern was observed during the dark phase, compared to the rest of the experimental phases. Fish selected lower temperatures in the hours prior to feeding time (ZT 18h) and higher temperatures after mealtime (ZT 19h). For the desynchronization of the animals from the previous feeding schedule, a second phase of random feeding was carried out where the rhythm of temperature selection was again observed with results very similar to those obtained in the first random feeding phase (acrophase at ZT 11.61 h) (Fig. 5d and A4, Table A4) (Cosinor, $p < 0.001$). In this phase, the maximum and minimum temperatures selected were 30.67 °C (at ZT 13 h) and 29.92 °C (at ZT 0 h), respectively. In the ML phase, the daily thermal preference rhythm was observed with the acrophase located at ZT 12.05 h (Fig. 5e) (Cosinor, $p < 0.001$). As for the maximum and minimum temperatures selected, they were at ZT 13 h with a temperature of 31.05 °C and at ZT 23 h with a temperature of 29.73 °C, respectively. In ML phase, as happened in the MD phase lower temperatures were selected in the hours prior to feeding at ML

(ZT 6h) and higher temperatures afterwards (Fig. 5e). In the starvation phase, the rhythm of temperature preference was also maintained with the acrophase located at ZT 11.31 h and the maximum and minimum temperatures selected were 29.29 °C (at ZT 13 h) and 28.41 °C (at ZT 0 h), respectively (Fig. 5f) (Cosinor, $p < 0.001$).

According to the chamber distributions, the mean number of fish followed daily rhythms during all experimental feeding phases (Fig. 6) (Cosinor, $p < 0.05$). During acclimatization, a higher number of fish chose warmer temperatures (Fig. 6a) (GLM repeated mesaures, $p<0.05$). However, in the following phases, the fish preferred the hot chambers (4 and 5) during the end of the light phase and the beginning of the night while the cold compartments were chosen during the late night and early morning hours (1 and 2) (Fig. 6) (Cosinor, $p < 0.05$). In addition, during the prolonged starvation phase, a greater number of fish selected the cold chambers (1 and 2) (Fig. 6f) (GLM repeated measures, $p < 0.05$).

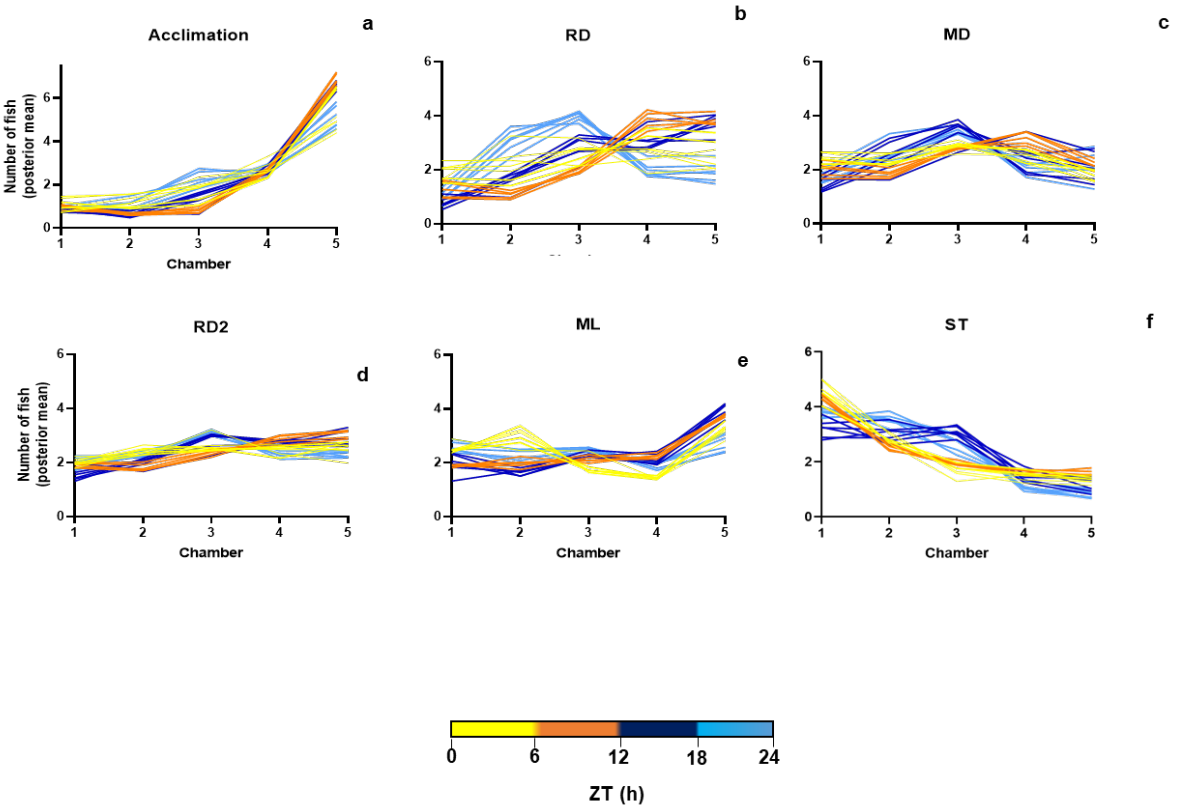

Fig. 6 Line plots of the mean fish count in each chamber for Nile tilapia (*Oreochromis niloticus*) by hour exposed to a thermal gradient and subjected to different feeding/starvation schedules: acclimation under random feeding (acclimation, a); ramdom feeding (RD, b); feeding at midnight (mid-dark, MD = ZT 18 h, c); feeding at the middle of light phase (mid-light, ML = ZT 6 h, d); 2nd Random (RD2, e), and prolonged starvation (ST, f). Lines show the posterior mean for each hour, expressed as *Zeitgeber* Time (ZT), in which ZT 0 h corresponds to light onset and ZT 12 h corresponds to lights off.

To determine if chamber preference was affected by other factors different than temperature, fish were placed in the same multichambered tank but exposed to a constant water temperature during the same feeding schedule (control experiment). In the absence of a thermal gradient, Nile tilapia did not present daily rhythms of preferred temperature in any experimental phase (Fig. 7) (Cosinor, $p < 0.05$). According to the chamber distributions, the number of fish did not follow a daily pattern (Fig. 7) (Cosinor, $p < 0.05$). However, higher number of fish selected the chambers that were at the ends of the tank.

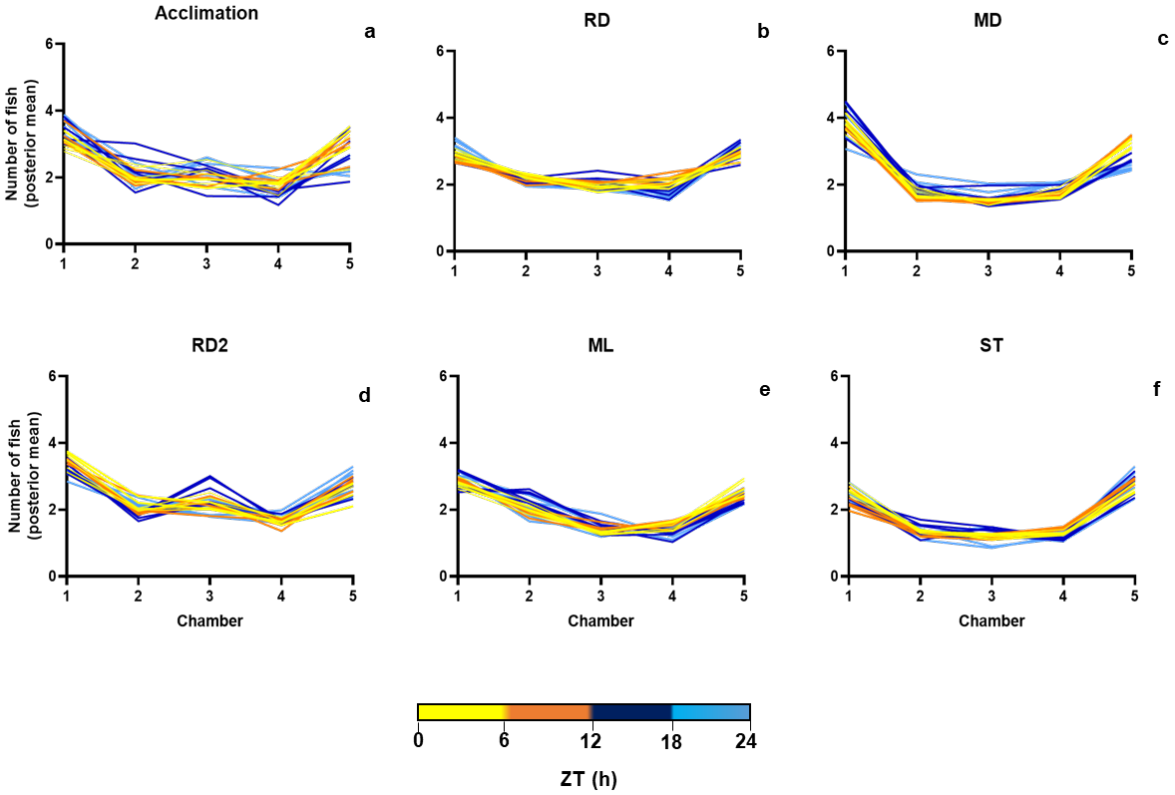

Fig. 7 Line plots of the mean fish count in each chamber for Nile tilapia (*Oreochromis niloticus*) by hour exposed to a constant temperature and subjected to different feeding/starvation schedules: acclimation under random feeding (acclimation, a); ramdom feeding (RD, b); feeding at midnight (mid-dark, MD = ZT 18 h, c); feeding at the middle of light phase (mid-light, ML = ZT 6 h, d); 2nd Random (RD2, e), and prolonged starvation (ST, f). Lines show the posterior mean for each hour, expressed as *Zeitgeber* Time (ZT), in which ZT 0 h corresponds to light onset and ZT 12 h corresponds to lights off.

DISCUSSION

Our results showed that Nile tilapia exhibited a strong daily rhythm of thermal preference under all light and feeding regimes investigated in this study. The role of light as the main synchronizer was supported by the selection of higher temperatures at dusk and lower temperatures at dawn in all the experimental phases from both independent experiments, regardless of the feeding schedule. Furthermore, the persistence of the rhythm in the absence of environmental synchronizers (in constant darkness and starvation) suggests its control by an endogenous pacemaker. However, several parameters and features of this thermal preference rhythm in tilapia were influenced by both mealtime and starvation. Thus, when subjected to cyclic mealtimes (at either ML or MD), the fish selected the lowest temperatures in the hours prior to feeding, whereas higher temperatures were preferred post-feeding. In addition, when tilapia was fasted, temperatures below the thermal optimum were selected.

The daily light and dark cycle is the main abiotic factor that affects the synchronization of a large number of biological rhythms in fish, modulating the circadian rhythms of locomotor activity (Hurd et al., 1998), development (Villamizar et al., 2013), feeding (López-Olmeda and Sánchez-Vázquez, 2010) and reproduction (Oliveira and Sánchez-Vázquez, 2010). Regarding thermal preference rhythms, our results agree with those reported in previous studies in other fish species, also showing that temperature selection could be influenced by light-dark cycles since the preferred temperature varied throughout the day, although in a species-dependent manner. Thus, for example, *Salmo trutta* and *Carassius auratus* presented the lowest preferred temperature during the day, while *Amia calva* and *Danio rerio* preferred lower temperatures at night (Reynolds et al., 1978a, 1978b; Reynolds and Casterling, 1979; Vera et al., 2022). A hypothesis that might explain such differences would be that different patterns of temperature selection throughout the day could be related to different locomotor activity patterns, so fish would choose higher temperatures when activity levels peaked. Consequently, nocturnal fish such as *Salmo trutta* or diurnal fish such as *Danio rerio* would select higher temperatures during the night or the day, respectively (Reynolds and Casterlin 1979; Vera et al., 2022). In the case of Nile tilapia, their activity pattern has been described to be flexible. While several authors describe it as a strictly diurnal species (Toguyeni et al., 1997; Fortes-Silva et al., 2010; Guerra-Santos et al., 2017), other studies have reported that tilapia can

display a diurnal, nocturnal and, in some cases, an arrhythmic (Vera et al., 2009) or even a crepuscular behavior (Fortes-Silva et al., 2010). More recent research by our group has also found a daily rhythm of preferred temperature in this species, with higher temperatures being selected in the evening and lower temperatures around sunrise (Vera et al., 2022). In nature, daily light-dark cycles cause the increase in water temperature by solar radiation during the day (thermophase) while at night temperature values drop (cryophase), thus establishing a daily thermocycle that directly influences the internal body temperature of ectotherm organisms, such as fish (Villamizar et al., 2012).Therefore, it would be reasonable to think that fish would have evolved to choose higher temperatures during the day (thermophase) and cooler temperatures at night (cryophase). However, the aquatic environments also present a vertical distribution of temperature, with higher temperatures on the water surface where sunlight mostly radiates and lower temperatures as darkness and depth progress. Therefore, the daily rhythm of thermal preference could be also influenced by Diel Vertical Migration (DVM) patterns (Gleiss et al., 2016). For example, in tropical environments where Nile tilapia is found, thermal gradients of up to 5-6 ºC difference between the surface and deeper layers have been recorded (Ling et al., 2018). Actually, earlier studies carried out in their natural habitat have reported that tilapia preferred cooler and dimmer areas (deeper) during the beginning of the light phase but moved to shallower (warmer) areas in the transition between the light and dark phases (Piet and Guruge, 1997) coincing with our results. In the present research, a rapid resynchronization of the thermal preference rhythm was found following reversal of the light-dark cycle (from LD to DL), suggesting a direct behavioral response to light. However, when all environmental cues were removed and fish were kept in constant darkness and starvation, rhythmicity free-ran with a periodicity that is either slightly less than 24 hours, indicating that thermal preference rhythms in tilapia would be controlled by an internal circadian clock.

Temperature is a factor of special relevance in fish metabolism, influencing the dynamics of metabolic reactions both in basal metabolism and in the energy required to perform functions such as digestion, locomotion, growth and reproduction (Volkoff and Rønnestad, 2020). Although light-dark cycles are the main synchronizer of biological rhythms, food availability and regular feeding cycles are among the most important biotic factors that affect the molecular clock and physiological rhythms, resulting in a synchronization of behavior and metabolic processes and thereby the optimization of

nutrients utilization (López-Olmeda and Sánchez-Vázquez, 2010). In our results, regardless of the feeding schedule, a similar daily rhythm of temperature preference was observed, characterized by the selection of higher temperatures at dusk and cooler temperatures at dawn, indicating that photoperiod was a stronger synchronizer that feeding time of this rhythm in Nile tilapia. These results coincide with the daily thermal variations that tilapia experience in their natural environment, marked mainly by their daily feeding pattern and vertical migration phenomena. In their habitat, Nile tilapia are found near the bottom (characterized by cooler temperatures) during their active feeding phase, while they are distributed throughout the water column or in shallower layers (where temperatures are warmer) when they are not feeding, i.e. at the end of the light phase (Piet and Guruge, 1997). In addition, tilapia migrate to the water surface in the evening, when temperature is higher, coinciding with the time of the day in which tilapia present relatively high intestinal fullness (Trewavas 1983; Moriarty and Moriarty 1973; Piet and Guruge 1997; Di Santo and Bennett, 2011) and highest protease activity in the mid gut (Guerra-Santos et al., 2017), thus favoring digestion and energy expenditure (Volkoff and Rønnestad, 2020). Actually, in the present study, when fish were fasted a decrease in the preferred temperature was observed, which could be advantageous for the animals, since this would allow them to reduce their metabolic rate and save energy that otherwise would be invested in digestion and nutrients assimilation (Miegel et al., 2010; Volkoff and Rønnestad, 2020). However, this effect is species-specific and while in some fish species such as *Leuresthes sardina* and *Rutilus rutilus* it appears after 4 days (Reynolds and Thomson, 1974; Van Dijk et al., 2002), in other species, such as *Ambloplites rupestris,* does not appear even after 7 days of fasting (Reynolds et al., 1978). However, this starvation effect can be reversed by feeding (Reynolds and Casterling, 1979; Van Dijk et al., 2002). In our research, after several cycles of periodic feeding (at either ML or MD), the fish selected lower temperatures a few hours prior to feeding, followed by the selection of higher temperatures just after food ingestion. Furthermore, this response was more marked when fish were fed at night (MD). This effect might be a compensatory response related to the food-anticipatory activity that tilapia shows, which is characterized by an increase in locomotor activity levels and an activation of the digestive physiology (Guerra-Santos et al., 2017). Interestingly, we observed a postprandial effect on temperature preference when tilapia was fed every day at the same time, characterized by the selection of higher temperatures after feeding, an effect that

was more marked when mealtime was set at night (MD). Previous studies in other fish species had also observed this phenomenon, which is known as post-feeding thermotaxis (Wurtsbaugh, and Neverman, 1988; Reynolds and Casterlin, 1979; Di Santo and Bennett, 2011). Water temperature impacts digestion rates and nutrient digestibility in fish, as well as the activity of digestive enzymes (Volkoff and Ronnestad, 2020). Thus, within the physiological temperature range, higher temperatures increase the speed of digestive processes and induce further increases in metabolic rates, which has a considerable impact on fish performance (Miegel et al., 2010).

Temperature exerts a huge influence on fish spatial distribution and daily behavioral patterns (Krylov et al., 2021). In our study, when fish were placed in the experimental tank in a constant temperature (no thermal gradient), they did not show daily patterns of chamber occupancy. However, we observed a preference of fish for the chambers on both ends of the experimental tank. This spatial preference could be explained by certain behaviors known as scototaxis or thigmotaxis, which are characterized by the preference for darker areas or less exposed to social interactions (Blaser and Rosenberg 2012). However, this phenomenon was not observed when the thermal gradient was imposed and tilapia showed daily rhythms of thermal preference, indicating that temperature was a stronger factor affecting chamber occupation.

During the acclimatization phase of both experiments, when fish were placed for the first time in the experimental tank under a thermal gradient, the animals selected higher mean temperatures than during the rest of the experimental phases. This behavioral response is known as stress-induced hyperthermia (SIH), also called emotional or behavioral fever, and it is produced as a result of exposure to a stressful agent, either biological (e.g. pyrogen) or social (e.g. anxiety) (Rey et al., 2015; Boltana et al., 2013; Rakus et al., 2017). Both physiological fever in endotherms and behavioral fever in ectotherms seem to be mediated by similar pathways of the autonomic nervous system (Bouwknecht et al., 2007). However, unlike what happens in endotherms, the increase in internal temperature in ectotherms is produced by a thermoregulatory behavioral response to elevate their body temperature by moving to warmer zones and thus maintain the internal homeostasis of the animal (Huntingford et al., 2020). Although there is considerable controversy as to whether fish are capable of showing SIH (Jones et al., 2019; Key et al., 2017), our results coincide with other studies carried out on Nile tilapia

and zebrafish, where fish presented a similar behavioral response selecting higher temperatures during the first days of acclimatization in response to either environmental or immune stress (Rey et al., 2015; Boltana et al., 2013; Vera et al., 2022). This emotional fever response that we observed in tilapia could be due to environmental stress caused by interactions with the new environment or with the establishment of social hierarchies (Rey et al., 2015; Cunningham et al., 2017; Vera et al., 2022). However, when fish are not exposed to an environment with temperature variation (constant temperature) and they encounter a stressful situation, they do not have the possibility of carrying out behavioral thermoregulation, so the effectiveness of their physiological response to stress might be limited (Huntingford et al., 2020). Although more studies are needed to determine the effect of environmental stressors on the thermal preference of fish, the behavioural fever response that we observed in our study should be considered in future research where the thermal preference of fish were evaluated.

Ectotherms, including fish, must perform navigational strategies to maintain their internal temperature near the metabolic optimum, suggesting that temperature preferences and the animal's thermal physiology are co-adapted (Beitinger and Fitzpatrick, 1979). When an animal is exposed to a thermal gradient, the thermal preference values most likely correspond to the temperature conditions experienced by the fish in their regions of origin (Golovanov, 2006). However, as adaptation proceeds, fish shift from selecting their acclimatization temperature to actively choosing their final thermal preferendum (Golovanov, 2006). This co-adaptation hypothesis is supported by the adaptive plasticity of thermal preference in relation to environmental influence, adapting its physiological processes (e.g. metabolic strategies) to environmental conditions (e.g. availability of food, light). Thus, in our study, we observe that the plasticity of thermoregulatory behavior may be co-adapted to the thermal sensitivity of digestive functioning since the preferred temperature increased during digestion and decreased when digestion was completed, thus maximizing food intake and metabolism efficacy (Volkoff and Rønnestad, 2020).

CONCLUSION

In the present study, both the light and dark cycles and the feeding/starvation schedule considerably influenced the daily thermal preference patterns of Nile tilapia. The light-dark cycles play a powerful synchronizing role, revealing the endogenous nature of the rhythm. The effect of the feeding/starvation protocol on rhythm showed the environmental plasticity of thermal preference. Knowing the daily rhythms of thermal preference of fish is essential to maintain fish at a daily temperature close to optimal levels to improve their adaptation and welfare in fish farming (Basu et al., 2002; Morash et al., 2018; Villamizar et al., 2012). These observations can be of great relevance in some aquaculture farming conditions where fish must cope with constant and unnatural lighting and temperature conditions designed to maximize fish production without considering the possibility of that these artificial protocols can damage the thermal biology of fish and numerous biological processes linked to it. In addition, our findings could be implemented in the development of feeding protocols for fish in aquaculture facilities, considering the daily rhythm of preferred temperature, improving the efficiency and environmental adaptation of metabolic processes to optimize their production.

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AUTHOR CONTRIBUTIONS

GA, FJSV, JFLO and LMV conceived and designed the experiments, and wrote the manuscript; GA, FC and JS performed the experiments; GA, FC, JS, FJSV, JFLO and LMV analyzed the data; FJSV, JFLO and LMV provided funding.

COMPETING INTERESTS

The authors declare no competing or financial interests.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

SUPPLEMENTARY INFORMATION

Table A1 Mean temperature of the multichambered tank for the different light photoperiods of Nile Tilapia (*Oreochromis niloticus).* The data of temperature from each chamber/day of each independent experiment was pooled together and is shown as mean temperature (°C) ± SEM for each hour of the day and night.

	chamber 1	chamber 2	chamber 3	chamber 4	chamber 5
Acclimation	25.99 ± 0.07	27.97 ± 0.03	30.07 ± 0.09	32.23 ± 0.10	34.07 ± 0.20
LD	26.04 ± 0.02	28.06 ± 0.09	30.06 ± 0.04	32.13 ± 0.01	34.07 ± 0.10
DL	26.06 ± 0.03	27.97 ± 0.06	30.07 ± 0.04	32.01 ± 0.07	34.08 ± 0.06
DL+A	26.04 ± 0.04	27.97 ± 0.08	30.08 ± 0.08	32.01 ± 0.08	34.11 ± 0.12
DL+R	26.11 ± 0.02	27.98 ± 0.05	30.01 ± 0.07	32.08 ± 0.05	33.92 ± 0.15
DD+A	26.06 ± 0.04	28.09 ± 0.06	30.03 ± 0.03	32.19 ± 0.06	34.00 ± 0.06
Mean	**26.06 ± 0.01**	**28.03 ± 0.02**	**30.05 ± 0.01**	**32.11 ± 0.03**	**34.04 ± 0.02**

Table A2 Mean temperature of the multichambered tank for the feeding phases of Nile Tilapia (*Oreochromis niloticus)* under a thermal gradient (a) and control temperature (b). The data of temperature from each chamber/day of each independent experiment was pooled together and is shown as mean temperature (°C) ± SEM for each hour of the day and night.

THERMAL GRADIENT	chamber 1	chamber 2	chamber 3	chamber 4	chamber 5
Acclimation	26.08 ± 0.02	28.09 ± 0.12	30.07 ± 0.09	32.23 ± 0.10	34.07 ± 0.20
RD	26.04 ± 0.02	28.06 ± 0.09	30.06 ± 0.04	32.13 ± 0.01	34.07 ± 0.10
MD	26.06 ± 0.03	27.97 ± 0.06	30.07 ± 0.04	32.01 ± 0.07	34.08 ± 0.06
RD2	26.04 ± 0.04	27.97 ± 0.08	30.08 ± 0.08	32.01 ± 0.08	34.11 ± 0.12
ML	26.11 ± 0.02	27.98 ± 0.05	30.01 ± 0.07	32.08 ± 0.05	33.92 ± 0.15
Starvation	26.06 ± 0.04	28.09 ± 0.06	30.03 ± 0.03	32.19 ± 0.06	34.00 ± 0.06
Mean	**26.06 ± 0.01**	**28.03 ± 0.02**	**30.05 ± 0.01**	**32.11 ± 0.03**	**34.04 ± 0.02**

CONTROL	chamber 1	chamber 2	chamber 3	chamber 4	chamber 5
Aclimatation	30.02± 0.01	30.05 ± 0.01	30.01 ± 0.04	30.06 ± 0.00	30.04 ± 0.00
RD1	30.01 ± 0.02	30.06 ± 0.01	30.03 ± 0.03	30.07 ± 0.01	30.09 ± 0.02
MD	30.08 ± 0.05	30.01 ± 0.05	30.05 ± 0.03	29.99 ± 0.03	30.03 ± 0.04
RD2	29.97 ± 0.06	29.98 ± 0.08	30.05 ± 0.07	29.98 ± 0.08	29.95 ± 0.05
ML	30.10 ± 0.04	30.01 ± 0.04	30.00 ± 0.07	30.02 ± 0.07	29.97 ± 0.04
Starvation	29.97 ± 0.05	30.06 ± 0.03	30.04 ± 0.03	30.16 ± 0.03	30.09 ± 0.08
Mean	**30.02 ± 0.02**	**30.03 ± 0.01**	**30.03 ± 0.01**	**30.05 ± 0.03**	**30,03 ± 0.02**

Table A3 Cosinor analysis results for the preferred temperature for the different light photoperiods of Nile Tilapia (*Oreochromis niloticus*).

	MESOR (° C)	AMPLITUD	ACROPHASE (ZT H)
ACCLIMATION	31.52 ± 0.10	0.36 ± 0.18	10.83 ± 1.93
LD	30.06 ± 0.08	0.73 ± 0.14	13.10 ± 0.77
DL	29.08 ± 0.08	1.54 ± 0.13	14.76 ± 0.33
DL+A	29.41 ± 0.11	1.08 ± 0.20	14.46 ± 0.71
DL+R	29.18 ± 0.05	0.84 ± 0.08	14.58 ± 0.40
DD+ST	29.42 ± 0.07	0.14 ± 0.13	

Table A4 Cosinor analysis results for the preferred temperature for the different feeding/starvation phases of Nile Tilapia (*Oreochromis niloticus*).

	MESOR (° C)	AMPLITUD	ACROPHASE (ZT H)
ACCLIMATION	31.93 ± 0.10	0.51 ± 0.18	11.9 ± 1.38
RD1	30.71 ± 0.08	0.81 ± 0.13	12.48 ± 0.62
MD	30.06 ± 0.07	0.30 ± 0.12	12.4 ± 1.52
RD2	30.34 ± 0.07	0.32 ± 0.12	11.61 ± 1.50
ML	30.32 ± 0.12	0.54 ± 0.22	12.05 ± 1.57
STARVATION	28.82 ± 0.07	0.28 ± 0.11	11.31 ± 1.56

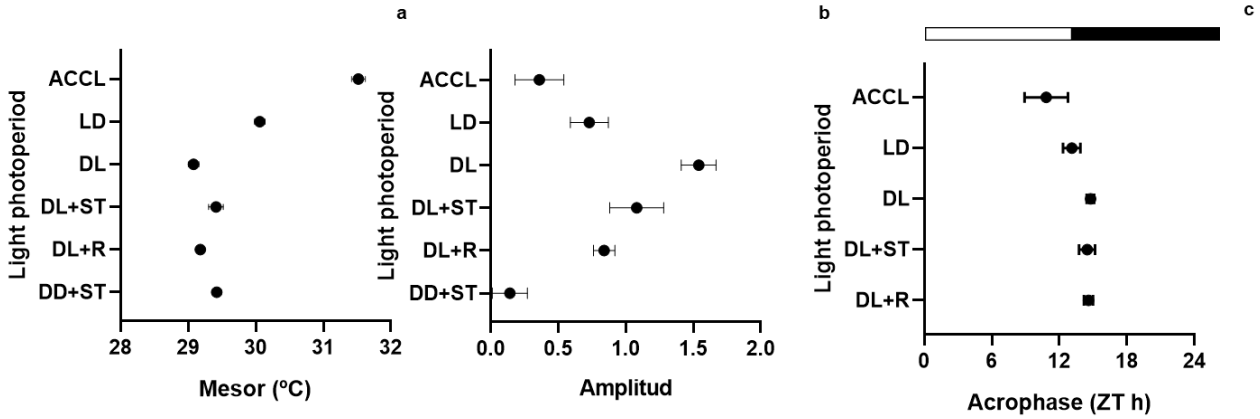

Fig. A1. Cosinor analysis results for the preferred temperature for the different light photoperiods.

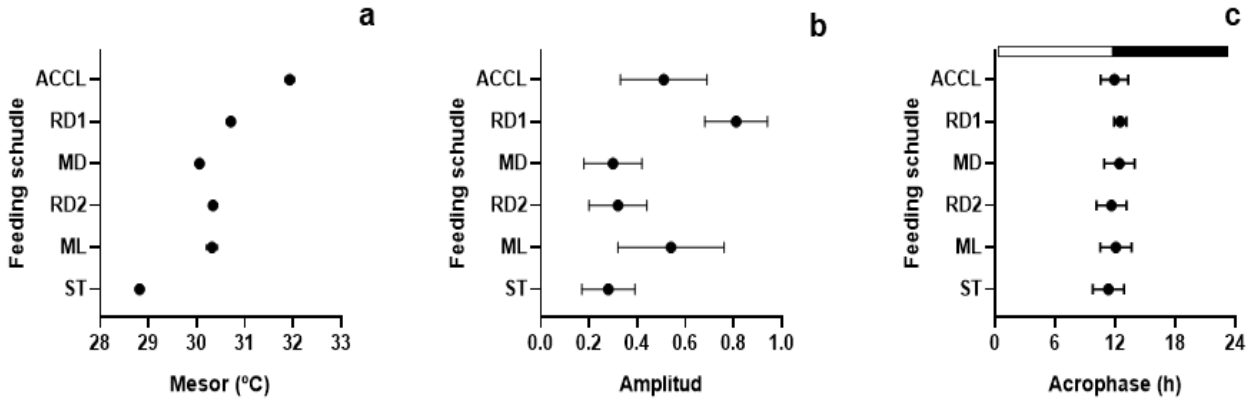

Fig. A2 Cosinor analysis results for the preferred temperature for the different feeding/starvation phases.

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General Discussion

4. General discussion

The present PhD thesis reveals the influence of light and temperature on different physiological processes in zebrafish and Nile tilapia. In addition, certain abiotic factors such as temperature cycles or light cycles/spectrum were examined to deepen the knowledge of the biological rhythms of reproduction, development, thermotolerance and behavior.

Reproduction is a vital process that ranges from the development of reproductive systems to the production of new progeny. In this process, a complex network of environmental, physiological and neuroendocrine factors is involved that regulate both sex determination and sexual differentiation processes (Baroiller and D'Cotta, 2019; Yamamoto et al., 2019) as well as the reproductive physiology of fish (Chapter 1 and 2). The mechanisms of the BPG axis mainly control reproduction in teleost fish so their environmental synchronization leads to reproductive success (Cowan et al., 2017). In a previous article from my personal research, we revealed the rhythmic nature of the reproduction control axis in the brain, pituitary, gonad, plasma and eggs of Nile tilapia (de Alba et al., 2019). In the brain, genes peaked at different LD cycle times. However, at the following BPG axis levels, rhythms appeared to be harmoniously timed in such a way that the peak values in the pituitary occurred at night, while the acrophases in the gonads and plasma sex steroids shifted toward the daytime. Thus, the environmental synchronization of all the factors that make up the neuroendocrine reproductive machinery culminates in the release of high-quality gametes for fertilization, ensuring reproductive success (de Alba et al., 2019; Paredes et al, 2019).

Light is not the only abiotic factor that influences the reproduction of fish. Thus, in fish species that inhabit zones with small changes in the photoperiod (tropical and subtropical zones), water rearing temperature plays a determining role in the reproductive process of fish from sexual determination and differentiation to gamete release and fertilization (Migaud et al., 2009). In the chapter 3, we revealed that the implementation of thermocycles (higher temperature during day and lower temperature at night) from early stages of development of Nile tilapia act as female-promoter inducing an increase in the expression of genes involved in ovarian differentiation and consequently, higher levels of plasma E_2 and proportion of females. These effects were also observed in some fish species such as Senegalese sole and zebrafish (Blanco-Vives et al., 2011, Villamizar et al., 2012). Once we observed the daily

variations of the BPG axis factors (de Alba et al., 2019) and the effects of thermocycles on sexual differentiation (Chapter 3), in Chapter 4 we wanted to determine whether the period of the day in which the water temperature was elevated by heat treatment could influence the sexual differentiation of Nile tilapia. The results revealed that larvae exposed to thermal treatment at night or during both phases of the light-dark cycle showed greater sensitivity to masculinizing treatments of high temperatures with higher upregulation of genes related to testicular differentiation and inhibition of genes involved in ovarian development, and consequently to a higher proportion of males. These results were consistent with previous research where it was observed that the proportion of males in Senegalese sole was higher in fish kept during their development under high temperatures during the dark phase and lower during the light phase, compared to the constant temperature group (Blanco-Vives et al., 2011).

Water temperature not only affects reproduction, but is a factor that limits physiological performance and compromises the survival of animals (Angiletta et al., 2002; Donaldson et al., 2008). Especially in poikilothermic animals, changes in water temperature during early development can have a considerable impact on the thermal biology of fish and on their ability to tolerate different temperature ranges, constituting the individual's thermotolerance (Schaefer and Ryan, 2006). In the chapter 3, thermocycles increased the thermal tolerance of tilapia by increasing the survival rate against the heat treatment, compared to fish kept at constant temperatures. In addition to the Nile tilapia as an experimental model with great productive interest, in chapter 4 we wanted to observe the influence of thermocycles on the thermotolerance of another experimental model with great importance in biomedical research, such as the case of zebrafish (Choi et al., 2021). Although in both species studied, the thermocycles had a similar influence on the increase in the thermotolerance of the fish, the effects of thermocycles on acute thermal tolerance seem to be fish species specific. Thus, in species such as killifish (Healy and Schutle, 2012), tidepool sculpin (Nakano and Iwama 2002; Todgham et al., 2006; Fangue et al., 2011) and zebrafish (Schaefer and Ryan 2006; Xia et al., 2016; Wang and Xia, 2019), thermocycles increased the thermotolerance while in other fish species such as salmonids no significant effects of thermocycles were observed (Chadwick and McCormick, 2017; Corey et al., 2017). This greater thermotolerance in fish reared in thermocycles against heat shock could be due to the

influence of thermocycles on the time-dependent response to induction of mechanisms linked to thermotolerance (HSPs). In both Nile tilapia and zebrafish (Chapters 4 and 5), we observed that higher thermotolerance (higher survival rate and less HSP induction) to heat stress during the day than at night has also been described in other fish species such as killifish (Healy and Schulte, 2012). On the one hand, this time-dependent response could be due to the different activity patterns between the fish species, presenting a greater thermotolerance at times of higher locomotor activity. According to this hypothesis, diurnal active fish such as zebrafish (Hurd et al., 1998), killifish (Žák et al., 2019), and Nile tilapia (Fortes-Silva et al., 2010; Guerra-Santos et al., 2017) would be more thermotolerant during the light phase than during the night. On the other hand, this response could be explained by the temperatures that ectotherms animals such as fish experience in nature, with higher temperatures during the day and cooler at night. This greater survival or resistance of the thermocycles could be the influence of the Fulton's condition factor on thermal tolerance (Gallant et al., 2017) in which fish reared under thermocycles may present greater thermotolerance at night because they present higher growth rate and energy reserves (Blanco-Vives et al., 2010, Villamizar et al., 2012, Morash et al., 2018, do Espirito Santo et al., 2020) necessary to supply the high energy costs of producing HSPs and minimize proteotoxicity effects of high induction of HSPs (Ananthan et al., 1986, Feder and Hoffman, 1999, Narum et al., 2013).

The time-dependent induction of HSPs and TRPs, as well as the different survival between day and night, could be triggered by a complex biological clock machinery in which its external inputs (light and temperature) play an important role in the thermal shock response (Jerônimo et al., 2017). In chapter 5, we found that most HSPs and TRPs of zebrafish brains presented daily variations in the expression pattern synchronized mainly to the LD cycle and to a lesser extent to the rearing temperature regime. TRP rhythms took place in a phase with rhythms in the different analyzed HSPs. These genes, which are involved in warm thermal sensitivity (*trpv4*, *trpm2* and *trpm4a*), presented the highest expression in the daytime which coincided with the highest expression of HSPs involved in hyperthermic challenges (*hsp70*, *hsp90ab1*, *grp94*, *hsp90aa1*, *hspb1*, *hsp47*). Curiously, the cold-sensing trpa1b displayed a higher expression in the second half of the light period and slightly shifted toward the dark phase, anticipating the increased expression of HSPs involved in hypothermic challenges (*cirbp*). Thus, our findings suggested the hypothetic existence of a coordinated

daily time window when zebrafish brains present more sensitivity to detect temperature changes and quickly translate them to a complex system of cellular protection mechanisms. The synchronization of TRP and HSP system may perform a more effective and adaptive response that would minimize cellular damage.

Reproduction and thermotolerance are not two isolated physiological processes, rather the role of HSPs in sex determination and differentiation has recently been investigated (Li et al., 2014). In recent years, the induction of HSPs is being indirectly correlated with expression changes of sexual differentiation genes after heat treatments in fish (Li et al., 2014; Wang et al., 2019). For example, Li et al (2014), showed that after heat treatment in Nile tilapia, the induction of HSPs increased as the expression of *cyp19a1a* decreased. This agrees with our results from chapter 4, in which the maximum inhibition of the genes involved in feminization (*cyp19a1a*, *foxl2* and *Era*) was observed in the larvae exposed to heat treatment during dark phase and both light and dark phases, which presented a greater induction of HSPs. On the contrary, the group kept at constant temperature (Control group) presented the highest expression levels of the feminizing genes and the lowest HSPs expression. The present results could indicate a possible link between thermal tolerance and sexual differentiation by high temperature treatment. Although there are some studies supporting this hypothesis, further research is needed to demonstrate this link and the effect of HSPs on TSD (Li et al., 2014; Wang et al., 2019).

During the first experimental chapters (chapter 3-5) we observed that simulating thermal conditions similar to those that the fish experience in their aquatic environment, a beneficial impact on both their thermotolerance and reproduction was observed. But along with thermocycles, the light spectrum strongly influences fish physiology in all life stages regulating vital function such as survival, development, growth, reproduction and behavior (Ruchin, 2020). Using zebrafish as experimental model due to easy handling, rapid development and optical accessibility during the early stages of embryonic development, in the chapter 6 we investigated the combined effects of both factors with two temperature regimes (constant temperature = 26ºC, CTE *vs.* daily thermocycle = 28ºC day:24ºC night, TC) and three light wavelengths (white, blue, red) on zebrafish embryos and larvae from fertilization to 30 days post-fertilization (dpf). Coinding with previous research (Villamizar et

al., 2012, 2014), our findings revealed that the most favorable combination for zebrafish early development was blue light with daily thermocycles, which positively affected hatching and survival, and stimulated growth and food intake. On the contrary, the combination of red light and constant temperature obtained the worst results affecting to several physiological factors and whose changes may be responsible for the deadly effects observed when fish are reared under red light.

In previous chapters (chapters 3-6), we imposed thermal conditions similar to what fish experience in their aquatic environment and observed its positive effect from early development to reproduction. In chapter 7, fish were allowed to freely choose between a wide range of water temperatures, in line with those found in their natural environments (Patterson and Wilson, 1995; Spence et al., 2006). Both zebrafish and Nile tilapia displayed strikingly consistent temporal daily rhythms of thermal preference which was species-specific and related to the daily behavioral patterns. Zebrafish preferred the highest temperature during the active phase (acrophase at ZT 5.55 h) whereas the minimum value was selected during the resting phase (at night). Nile tilapia selected the highest temperature during the last hours of the light phase (acrophase at ZT 12.7 h) whereas coldest temperatures were preferred at the end of the dark phase. Interestingly, when tilapias were first placed into the experimental tank (during the acclimation phase), they selected higher temperatures than during the rest of the experimental phase. This behavioral response has been previously observed in this species, as well as in zebrafish, subjected to a stressful agent, and it is known as Stress Induced Hyperthermia (SIH) or emotional fever (Boltana et al., 2013; Rey et al., 2015; Rakus et al., 2017).

Given the role of light-dark cycles and feeding as the most important synchronizers of biological rhythms in fish, in chapter 8 we evaluate the effect of light photoperiod and mealtime/starvation on the daily rhythm of thermal preference in Nile tilapia. Although the persistence of the rhythm in constant darkness indicated the existence of an endogenous pacemaker, the main role of light as a synchronizer was evident as the fish selected higher temperatures at dusk and lower temperatures at dawn in all experimental phases from both independent experiments. This rate of thermal preference could be influenced by activity patterns (Lopez-Olmeda et al., 2006) or by thermocycles that tilapia experience in the wild

(Patterson and Wilson, 1995). However, the results seem to indicate that these rhythms of thermal preference are influenced by feeding habits and Diel Vertical Migration (DVM) patterns in the aquatic environment (Piet and Guruge, 1997). In their habitat, Nile tilapia are found near the bottom (with cooler temperatures) during active feeding while they are distributed throughout the water column or in shallower layers (with warmer temperatures) when they do not feed during the end of the light phase (Piet and Guruge, 1997). In addition, tilapia migrate to higher surface temperatures in the evening, coinciding with the time when tilapia present highest digestive content, relatively high intestinal fullness (Moriarty and Moriarty 1973; Piet and Guruge 1997; Di Santo and Bennett, 2011) and highest protease activity in the midgud (Guerra-Santos et al., 2017), thus favoring digestion (Volkoff and Rønnestad, 2020). When the mealtime was periodic in ML and MD phases, the fish selected lower temperatures in the hours prior to feeding, and higher temperatures after eating predisposing the optimal thermal conditions that optimize metabolism and nutritional utilization (Mistlberger, 1994) and showing a post-feeding thermotaxis response (Di Santo and Bennett, 2011). However, when the food was deprived in the starvation phase, a decrease in the preferred temperature was observed, which could be advantageous for organisms to save energy by reducing their metabolic rates with lower rates of digestion and, consequently, digestibility of the nutrients (Miegel et al., 2010; Volkoff and Rønnestad, 2020). These results show the enormous influence of light, temperature and food availability cycles on the synchronization of behavior and metabolic processes to optimize the utilization of the nutrients (López-Olmeda et al., 2010).

Conclusions

5. Conclusions.

1. The genetic conservation of sex determination and the physiology of Nile tilapia has rendered this species as a model for identifying the genes involved in the morphological, molecular, and biochemical aspects of gonadal development and differentiation as well the influence of environmental, physiological, and neuroendocrine factors in them.

2. The implementation of daily thermocycles at early development stages acts as a female-promoter inducing factors that increase the expression of the genes involved in ovarian differentiation and, consequently, leading to higher proportion of females. In addition, daily temperature cycles reduce the masculinizing effect of heat treatment by reducing the up-regulation of the genes involved in testicular differentiation and, thus, leading to higher plasma testosterone levels and ultimately a lower proportion of males.

3. Larvae exposed to thermal treatment at night or continuously (day and night) showed greater sensitivity to masculinizing treatments of high temperatures, with higher upregulation of testicular differentiation genes and inhibition of ovarian differentiation genes, and consequently leading to a higher proportion of males. The effect of the thermal treatment applied at different times of the day during the sexual differentiation stages modified the time-dependent stress response in larvae and juvenile fish.

4. Exposure to thermocycles during early development lowered daytime induction of heat shock protein expression and larval mortality, which suggests a positive effect of thermocycles vs. constant temperature on the thermal tolerance of zebrafish.

5. The combined effects of both shorter wavelengths (blue) and daily thermocycles positively influenced survival, growth and feeding rate. On the contrary, when fish were reared under red light, the changes in several physiological factors explained its deadly effects.

6. Zebrafish and Nile tilapia exhibited species-specific daily rhythms in temperature preference. Zebrafish chose higher and lower temperature during day and night, respectively. The rhythm of thermal preference of Nile tilapia was slightly shifted and

peaked at the transition from light to dark phase. During the acclimation period, tilapia presented a stress induced hyperthermia response by choosing higher temperatures.

7. Although light is a powerful synchronizer of daily rhythm of temperature preferences, the persistence of the rhythm in constant darkness indicated the existence of an endogenous pacemaker. Regardless of the feeding/starvation protocol, a daily rhythm of temperature preferences was observed in all experimental phases, choosing higher temperatures at dusk and cooler temperatures at dawn. Prolonged starvation resulted in the selection of lower temperatures, which may be explained as a compensatory response to save energy.

General Bibliography

6. General Bibliography

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Annexes

7. Annexes

Scientific publications

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Resumen en castellano

8. Resumen en castellano

El objetivo de esta tesis doctoral fue dilucidar la influencia de la luz y la temperatura en diferentes fases del ciclo de vida del pez cebra y la tilapia del Nilo: reproducción, desarrollo, termotolerancia y comportamiento. Para ello, se diseñaron los siguientes objetivos específicos:

1. Resumir la interacción entre los componentes genéticos y ambientales que determinan el sexo de la tilapia del Nilo y describir la dinámica de las etapas y mecanismos endocrinos que comprenden su diferenciación sexual.

2. Revisar el papel de los factores ambientales, fisiológicos y neuroendocrinos en la fisiología reproductiva de la tilapia para mejorar los protocolos de reproducción establecidos en la acuicultura de tilapia.

3. Revelar la existencia de ritmos diarios en la expresión de genes clave en el eje BPG de tilapia del Nilo en ambos sexos.

4. Investigar los efectos combinados de los regímenes de temperatura (termociclo *vs*. temperatura constante) durante el desarrollo temprano y un choque térmico posterior durante el período de diferenciación sexual de la tilapia del Nilo.

5. Determinar la influencia de la hora del día del tratamiento térmico en la diferenciación sexual y la tolerancia térmica en la tilapia del Nilo.

6. Investigar el efecto de dos regímenes de temperatura de crianza (termociclo *versus* temperatura constante) y la hora del día en los ritmos de detección térmica y mecanismos de tolerancia.

7. Investigar el efecto combinado de los termociclos diarios y el espectro de luz durante el desarrollo temprano en el pez cebra y comprender qué mecanismos impulsan la mejora bajo la luz azul y los termociclos y los efectos nocivos de las longitudes de onda rojas.

8. Determinar la existencia de ritmos diarios de preferencia térmica en pez cebra y tilapia del Nilo.

9. Evaluar el efecto de la luz y la hora de la alimentación/ayuno en el ritmo diario de preferencia térmica en la tilapia del Nilo.

Para cumplir estos objetivos se desarrollaron diversos experimentos que aparecen organizados en 9 capítulos experimentales, los cuales aparecen resumidos a continuación.

Capítulo experimental 1: Determinación del sexo y diferenciación de la tilapia del Nilo.

Las altas capacidades productivas y reproductivas de la tilapia han llevado a que esta especie sea considerada uno de los grupos de peces de mayor interés comercial. Sin embargo, ciertos aspectos reproductivos, como la maduración temprana, el desarrollo gonadal asincrónico y la baja prolificidad dificultan su manejo y reducen la intensidad productiva de la acuicultura de tilapia. Para abordar estos problemas, la industria se ha enfocado en el control genético y sexual de la tilapia para reducir la variabilidad genética y fenotípica y así optimizar su producción. En el presente capítulo, analizamos en profundidad la interacción entre los componentes genéticos y ambientales que establecen la determinación del sexo. Realizamos también una extensa revisión sobre las principales etapas de diferenciación sexual, y la influencia de los mecanismos endocrinos y ambientales que intervienen en el establecimiento del fenotipo (sexo) del individuo.

Capítulo experimental 2: Fisiología reproductiva de la tilapia.

Para optimizar el manejo reproductivo de esta especie, es necesario aumentar nuestro conocimiento sobre los factores endocrinos y ambientales que regulan el proceso de reproducción en la tilapia. En este capítulo se realiza una minuciosa revisión del principal sistema neuroendocrino que controla el eje reproductivo de la tilapia del Nilo (Oreochromis niloticus): el eje BPG. Por lo tanto, la dinámica hormonal en el ciclo reproductivo se describe destacando la influencia de los factores ambientales y hormonales que afectan la reproducción de la tilapia del Nilo. Esta información puede ser útil para optimizar las condiciones ambientales para la reproducción en cautiverio y para mejorar las terapias hormonales que potencien los procesos de selección genética y eficiencia reproductiva con tilapia.

<u>Capítulo experimental 3</u>: Efectos combinados del régimen de temperatura de crianza (termociclo *versus* temperatura constante) y el tratamiento térmico en el proceso de diferenciación sexual de la tilapia del Nilo (*Oreochromis niloticus*).

El propósito del estudio actual fue examinar los efectos combinados de los regímenes de temperatura de crianza (termociclo (TC) *versus* temperatura constante (CTE) en el desarrollo temprano de la tilapia del Nilo y el choque térmico posterior durante el período de diferenciación sexual de la tilapia del Nilo (*Oreochromis niloticus*). Para ello, los embriones y larvas se mantuvieron bajo dos regímenes de temperatura: TC de 31ºC:25ºC día:noche *vs.* CTE de 28ºC de 0 a 11 días postfecundación (en inglés days post-fertilization o dpf). Después de este período, las larvas de cada grupo fueron sometidas a tratamiento térmico (HT, 36ºC durante 12 días) o mantenidas a las mismas temperaturas de crianza hasta los 23 dpf (Control, C). Luego todos los grupos permanecieron a temperatura constante hasta los 270 dpf, cuando se recolectaron sangre y lóbulos gonadales de 100 peces por grupo. Se utilizaron muestras de larvas enteras (11 y 24 dpf) para examinar la expresión de numerosos genes relacionados con la diferenciación sexual masculina (*amh*, *ara*, *sox9a*, *dmrt1a*) y femenina (*cyp19a1a*, *foxl2*, *era*). En los juveniles se caracterizó el sexo de los individuos mediante análisis histológico, la expresión gonadal de los genes implicados en la síntesis de esteroides sexuales mediante qPCR y los niveles plasmáticos de testosterona (T) y estradiol (E_2) mediante ELISA. En larvas de 11 y 24 dpf, los TC diarios aumentaron la tasa de supervivencia contra HT y aumentaron la expresión de genes de diferenciación ovárica. HT indujo cambios en el grupo CTE mediante la regulación positiva de los genes de diferenciación testicular y la regulación negativa de los genes promotores femeninos, lo que no ocurrió en el grupo TC. Los juveniles criados en TC presentaron una mayor proporción de hembras con mayor expresión plasmática de E_2 y *cyp19a1a* que el grupo CTE+HT. Los peces del grupo CTE+HT mostraron un mayor porcentaje de machos con mayor expresión de T y *amh*. Estos hallazgos indican que los TC diarios durante el desarrollo larvario promueven la diferenciación ovárica y disminuyen los efectos masculinizantes de la TH.

<u>Capítulo experimental 4</u>: Efecto de la hora del día del tratamiento térmico sobre la tolerancia térmica y el proceso de diferenciación sexual de la tilapia del Nilo (*Oreochromis niloticus*).

El objetivo de la presente investigación fue determinar la influencia del período del día del tratamiento térmico en la diferenciación sexual y tolerancia térmica en tilapia del Nilo (*Oreochromis niloticus*). Para ello, las larvas se sometieron a un tratamiento térmico (HT, 36ºC durante 12 días) en diferentes momentos del día durante el periodo termosensible (11-23dpf): periodo de luz (L-HT); periodo oscuro (D-HT); período de luz y oscuridad (LD-HT); y otro grupo no expuesto a tratamiento térmico pero mantenido a temperatura de crianza (Grupo Control, CTRL). A los 25 dpf, se midió la tolerancia térmica (supervivencia) y se recolectaron muestras de larvas completas para el análisis de expresión de ARNm. Luego, todos los grupos se mantuvieron a temperatura constante hasta los 270 dpf cuando se seleccionaron al azar, anestesiaron y pesaron 100 peces de cada grupo. Los lobulillos gonadales de cada individuo se recolectaron para análisis histológicos y de expresión de ARNm. Al mismo tiempo, cada grupo experimental fue sometido a choque térmico (HS; 36ºC durante 2 horas) a ZT6 h (ML, mitad de la fase de luz) o ZT18 h (MD, mitad de la fase de oscuridad). Además, en cada punto de tiempo, un grupo de control no estuvo expuesto al estrés térmico (NS). Cuando finalizó la exposición al choque térmico, se extrajeron los cerebros de los juveniles para determinar la respuesta al choque térmico mediante análisis de ARNm. Estudiamos la expresión de genes implicados en la diferenciación sexual y tolerancia térmica (*hsp70*, *hsp90a*, *hsp27*) de hembras (*cyp19a1a*, *foxl2*, *era*) y machos (*amh*, *ara*, *sox9a*, *dmrt1a*) en larvas y juveniles de 25 dpf. Los resultados revelaron que todas las larvas tratadas con HT presentaron una regulación positiva de la expresión génica de la diferenciación testicular y una inhibición de la expresión génica de la diferenciación ovárica. Además, la hora del día del tratamiento térmico durante el desarrollo influyó en la termotolerancia en estadio larvario y juvenil con la menor supervivencia y el mayor estrés celular (mayor expresión de HSPs) en las larvas de los grupos D-HT y LD-HT y diferentes respuestas celulares dependientes del tiempo a nivel del cerebro juvenil. Estos resultados pueden ser de utilidad para optimizar protocolos de masculinización en la industria de la tilapia que garanticen el bienestar animal y minimicen los efectos negativos del tratamiento térmico sobre la fisiología de los peces.

<u>Capítulo experimental 5</u>: Las condiciones de temperatura de cría (constante frente a termociclo) afectan los ritmos diarios de tolerancia térmica y detección en el pez cebra.

En la naturaleza, el medio ambiente no permanece constante, sino que oscila periódicamente de modo que la temperatura sube durante el día y desciende durante la noche, lo que genera un termociclo diario. Los efectos de los termociclos sobre la tolerancia térmica se han descrito previamente en peces. Sin embargo, el impacto de los termociclos en las respuestas térmicas dependientes del día y los ritmos diarios de tolerancia a la temperatura y los mecanismos de expresión de detección siguen siendo poco conocidos. Este estudio investiga los efectos de dos condiciones de crianza: constante (26ºC, C) *versus* un termociclo diario (28ºC durante el día; 24ºC por la noche, TC) en la respuesta de tolerancia térmica en el pez cebra. La tolerancia térmica (mortalidad) se evaluó en larvas de pez cebra 4dpf (días posteriores a la fertilización) después de un choque térmico agudo (39ºC durante 1 h) en dos puntos de tiempo: en la mitad de la fase de luz (ML) o en la mitad de la fase oscura (MD). Las respuestas al estrés térmico se evaluaron en peces cebra adultos después de un desafío a 37 °C durante 1 h en ML o MD para examinar la expresión de la proteína de choque térmico (HSP) (*hsp70*, *hsp90ab1*, *grp94*, *hsp90aa1*, *hspb1*, *hsp47*, *cirbp*) y los canales de receptor potencial transitorio (TRP) (*trpv4*, *trpm4a*, *trpm2*, *trpa1b*) en el cerebro. Finalmente, se midieron los ritmos diarios de expresión génica de HSPs y TRPs cada 4 h durante 24 h. Los resultados revelaron que las tasas de mortalidad de las larvas y la inducción de la expresión de la mayoría de las HSP en el cerebro del pez cebra adulto alcanzaron los valores más altos en peces criados a temperatura constante y sometidos a choque térmico en MD. La expresión de la mayoría de las HSP y TRP se sincronizó principalmente con el ciclo de luz/oscuridad (LD), independientemente del régimen de temperatura. La mayoría de las HSPs involucradas en desafíos hipertérmicos o hipotérmicos mostraron ritmos diurnos con sus acrofases en fase con las acrofases de termoTRP sensibles al calor o al frío, respectivamente. Estos hallazgos indicaron que: a) los choques térmicos se toleran mejor durante el día; b) la implementación de termociclos diarios durante el desarrollo larvario reduce la mortalidad y la expresión celular de estrés de las HSP a un estrés térmico agudo en MD; c) los ritmos diarios deben tenerse en cuenta al analizar las respuestas fisiológicas de la detección térmica y la termotolerancia en el pez cebra.

<u>Capítulo experimental 6</u>: La luz azul combinada y los termociclos diarios mejoran el crecimiento y desarrollo del pez cebra.

En la naturaleza, el entorno de luz/temperatura oscila cíclicamente en la medida en que la temperatura sube después del amanecer y desciende después del anochecer. En el fotoambiente submarino, la luz se filtra a través de la columna de agua para que los fotones azules alcancen mayores profundidades. Este trabajo investiga los efectos combinados de ambos factores con dos regímenes de temperatura (temperatura constante = 26ºC, CTE *vs*. termociclo diario = 28ºC día:24ºC noche, TC) y tres longitudes de onda de luz (blanco-W, azul-B, rojo-R) sobre embriones y larvas de *Danio rerio* desde la fecundación hasta los 30 días posfecundación (dpf). Se estudió la tasa de eclosión, la supervivencia de las larvas, el crecimiento y la ingesta de alimentos (contenido intestinal). Se analizó la expresión de los genes implicados en el estrés (crh*),* el crecimiento somático (*gh*, *ifg1a*, *igf2a*) y el control de la ingesta de alimentos (*npy*, *agrp*, *ghrelin*, *orexin*, *mch1*, *mch2*, *grp*, *cck8*) a los 10 y 30 dpf. Los resultados revelaron que la tasa de eclosión más baja estaba en R independientemente del régimen de temperatura. La tasa de crecimiento más alta fue para las larvas criadas con B+TC, lo que fue consistente con los valores de expresión más altos de los factores de crecimiento. Los niveles más altos de alimentación y expresión de los genes involucrados en la ingesta de alimentos fueron para las larvas en B (independientemente del régimen de temperatura) y W+TC. Por el contrario, la combinación R+CTE obtuvo los peores resultados de crecimiento y alimentación. Estos hallazgos indican que se puede lograr el mejor rendimiento de las larvas con combinaciones de longitudes de onda azules y regímenes de temperatura cíclica que se acerquen más a los del entorno natural. Estos resultados deben tenerse en cuenta al optimizar los protocolos de cría para mejorar el crecimiento y el bienestar de las larvas de peces.

<u>Capítulo experimental 7</u>: Ritmo circadiano de la temperatura corporal en peces: termorregulación conductual vinculada a fotociclos diarios en pez cebra y tilapia del Nilo.

Los vertebrados ectotérmicos como los peces mantienen su temperatura corporal dentro de un rango fisiológico específico principalmente a través de la termorregulación conductual. Aquí caracterizamos la presencia de ritmos diarios de preferencia térmica en dos especies de peces filogenéticamente distantes y bien estudiadas: el pez cebra (*Danio rerio*), un modelo experimental, y la tilapia del Nilo (*Oreochromis niloticus*), una especie acuícola. Creamos un gradiente de temperatura continuo usando tanques multicámara de acuerdo con el rango ambiental natural de cada especie. A cada especie se le permitió elegir libremente su temperatura preferida durante el ciclo de 24 horas durante un período a largo plazo. Ambas especies mostraron ritmos diarios temporales sorprendentemente consistentes de preferencia térmica con temperaturas más altas seleccionadas durante la segunda mitad de la fase de luz y temperaturas más bajas al final de la fase de oscuridad, con sus acrofases en Zeitgeber Time (ZT) 5.55 h (pez cebra) y ZT 12,7 h (tilapia). Curiosamente, cuando se trasladaron al tanque experimental, solo la tilapia mostró una preferencia constante por temperaturas más altas y tomó más tiempo para establecer los ritmos térmicos. Nuestros hallazgos resaltan la importancia de integrar tanto el ritmo diario impulsado por la luz como la elección térmica para refinar nuestra comprensión de la biología de los peces y mejorar la gestión y el bienestar de la diversidad de especies de peces utilizadas en la investigación y la producción de alimentos.

<u>Capítulo experimental 8</u>: Efecto del fotoperíodo de luz y la hora de alimentación/ayuno sobre el ritmo diario de preferencia térmica en la tilapia del Nilo (*Oreochromis niloticus).*

La preferencia térmica permite a los peces elegir el momento del día con las mejores condiciones térmicas para desarrollar sus procesos biológicos. El objetivo de este estudio fue determinar el efecto del fotoperíodo de luz y la hora de alimentación/ayuno sobre el ritmo diario de preferencia térmica en la tilapia del Nilo (*Oreochromis niloticus*). Para ello, utilizando un tanque multicámara con un gradiente térmico de 26 ºC a 34 ºC o con una temperatura constante de 30 ºC (control), se permitió a los peces elegir libremente la temperatura preferida al ser sometidos de forma independiente a diferentes fotoperiodos de luz y horarios de alimentación. Los peces fueron expuestos a 3 combinaciones diferentes de fotoperíodos: ciclo de luz:oscuridad (LD); ciclo LD invertido (DL) y condiciones constantes (DD); y 4 horarios de alimentación diferentes: aleatorio (RD); alimentación en el medio de la fase de luz (media luz, ML; ZT 6 h); alimentación en medio de la fase oscura (media oscuridad, MD; ZT 18 h) y ayuno. La temperatura preferida se determinó mediante análisis de video del comportamiento de los peces. Independientemente del horario de alimentación/ayuno, se observó el papel principal de la luz como sincronizador ya que se observó un patrón similar de ritmo diario de preferencias de temperatura en todas las fases experimentales eligiendo temperaturas más altas al atardecer y temperaturas más frías al amanecer. Además, la persistencia del ritmo bajo DD, indicó la existencia de un marcapasos endógeno. Durante la aclimatación, los peces exhibieron una respuesta de fiebre emocional caracterizada por la selección de temperaturas más altas. Por el contrario, los peces alimentados en MD seleccionaron temperaturas más frías durante la noche que los peces alimentados en ML o RD. Además, la inanición prolongada resultó en la selección de temperaturas por debajo del óptimo térmico. Estos resultados podrían ser útiles para comprender los aspectos ecológicos y evolutivos de la termorregulación y la adaptación ecológica de los peces. Estos hallazgos revelan la necesidad de considerar los ritmos diarios de temperatura preferida al diseñar protocolos de alimentación, lo que podría mejorar el bienestar de los peces y optimizar la producción de peces.