UNIVERSIDAD DE MURCIA

ESCUELA INTERNACIONAL DE DOCTORADO

TESIS DOCTORAL

Effects of environmental cycles (light and temperature) on daily rhythms and its application to fish aquaculture

Efectos de los ciclos ambientales (luz y temperatura) en los ritmos diarios y su aplicación a la acuicultura de peces

Dª. Francesca Conti

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Introduction

1. Introduction

1.1. Biological clock and circadian rhythms

The geophysical characteristics of the planet Earth leads to predictable environmental changes, such as the alternation of day and night or temperature variations (Panda et al., 2002). These fluctuations, throughout geological time, are responsible for most evolutionary changes and adaptations of living beings (Shweiki, 2001). Indeed, most biochemical, physiological and behavioural parameters and patterns exhibited by almost all organisms show daily fluctuations (Menaker et al., 1997) resulting in daily biological rhythms that can be entrained by external stimuli called *Zeitgebers* ("time givers" in German; Monk, 2010). These rhythms are generated and regulated by the "biological clock" or pacemaker, which can be defined as an intrinsic time tracking system that constitutes an adaptive advantage by enabling organisms to anticipate rhythmic environmental changes and modify their internal state or response accordingly (Junko et al., 2019). In this way, organisms can implement biological functions at the right time of the day resulting in increasing their well-being and survival (Vitaterna et al., 2001).

The biological rhythms that persist under constant conditions and in absence of external cues display a free-running period (tau, τ) close to 24-hours and are defined circadian rhythms driven by an endogenous circadian pacemaker (Aschoff, 1967).

The circadian system can be simply described as in Figure 1: input signals or *Zeitgebers*, central oscillator or pacemaker that generates rhythmicity and output pathways in response to the external stimuli (Harmer et al., 2001; Pando & Sassone-Corsi, 2002). A pacemaker is a functional anatomical region that can sustain its own oscillations and to

synchronise others. In addition, several circadian oscillators within the same individual could respond and be entrained independently by different environmental inputs and they could also interact with each others (López-Olmeda, 2017). In mammals, the main oscillator that generates and sustains circadian rhythms resides in the suprachiasmatic nuclei (SCN) of the anterior hypothalamus and it entrains the peripheral oscillators. At the molecular level the clock consists of positive and negative feedback loops on the transcription and translation of the "clock genes" (Shearman et al., 2000; Honma, 2018).

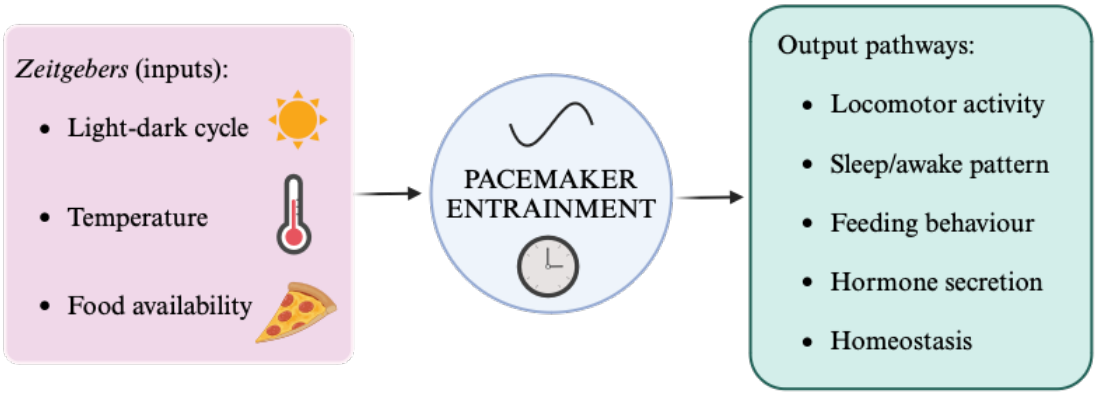

Figure 1. Schematic representation of a circadian timekeeping system composed by three main components: inputs signals such as environmental variations, the endogenous pacemaker that sustains its own oscillations and synchronise others and output pathways that can result in rhythmic physiological and behavioural patterns. Created with BioRender.com.

Circadian rhythmcities are characterised by certain mathematically fixed parameters (Cornelissen, 2014; Figure 2):

- MESOR (M): defined as the rhythm-adjusted mean;
- AMPLITUDE (A): defined as half the extent of rhythmic change in a cycle;

- ACROPHASE (ϕ): measure of the timing of overall high values recurring in each cycle and defined as the time of the rhythm peak value.
- PERIOD (τ): duration of one cycle.

The study of circadian and other biological rhythms is addressed by chronobiology.

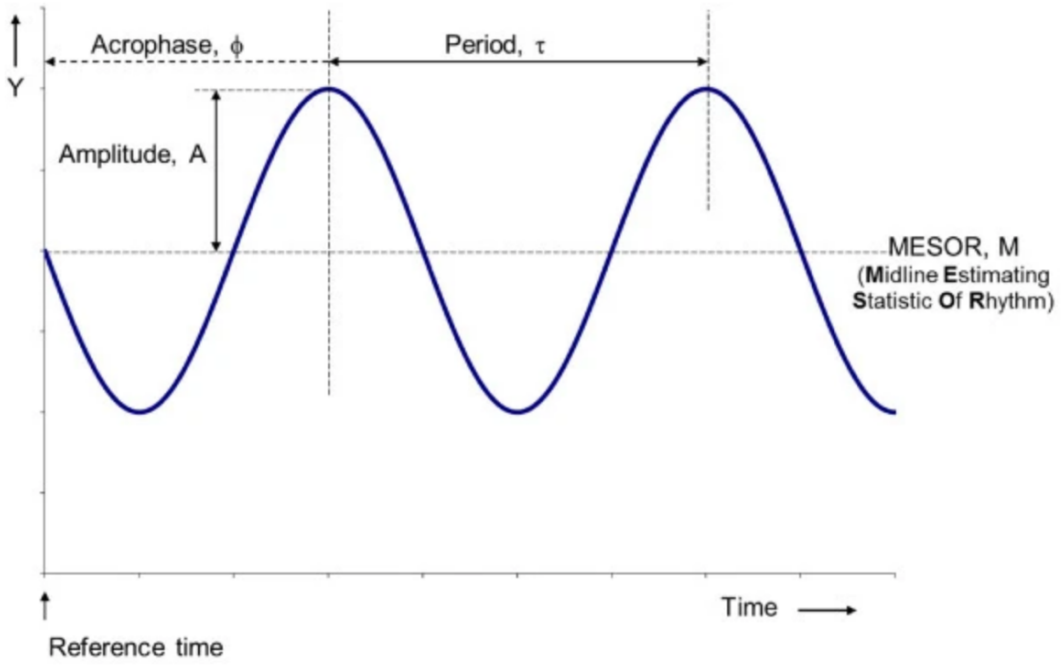

Figure 2. Definition of rhythm parameters: Mesor (M), Amplitude (A), Acrophase (ϕ) and Period (τ). © Halberg Chronobiology Center.

1.1.1 Circadian rhythms in fish

Aquatic organisms inhabit highly dynamic ecosystems that are exposed to cyclic fluctuations with different periodicities: tides, day-night alternations, lunar phases and seasons (Sánchez-Vázquez & López-Olmeda, 2018). Within this context, fish represent one of the most successful groups among vertebrates, showing diversified adaptations to a wide range of habitats. Therefore, studying these animals provides a great chance for further knowledge on the circadian clocks and biological

rhythms (Idda et al., 2012). Indeed, fish exhibit circadian rhythms of activity, food intake and some physiological patterns.

The existence of a multilevel circadian system in fish composed of a network of independent and coordinated central and peripheral oscillators and many clock genes can explain the plasticity and flexibility of this animals in spontaneously switching their behaviour from diurnal to nocturnal, or vice versa, depending on the season or during ontogeny (Zhdanova & Reebs, 2005; Idda et al., 2012).

In fish, the photosensitive central pacemakers are the pineal gland and the retina which are also responsible to produce melatonin, an important circadian hormone secreted exclusively during the night phase (Falcon et al., 2010). However, in some fish species, pinealectomy does not disrupt circadian rhythmicities suggesting the absence of an intrinsic clock in the pineal and melatonin production, in these species, is under exclusive light control (Falcon, 1999; Sanchez-Vazquez et al., 2000; Masuda et al., 2003). Moreover, fish have multiple autonomous peripheral oscillators in a variety of tissues directly entrainable by light (Whitmore et al., 2000) and by other environmental stimuli as temperature. In this way, fish circadian machinery is well coordinated and able to provide an adaptive advantage to a periodically changing environment to enhance their fitness and survival.

1.2. Environmental cycles

In accordance with seasonal changes or geophysical cycles there are certain biological oscillations, occurring rhythmically with tidal (~12 h), daily (~24 h), semilunar (~14 days), lunar (~29 days), or annual (~a year) periods in organisms (DeCoursey, 2004). In this dynamic context, the biological circadian rhythms need to be set in time by external

environmental *Zeitgebers* to achieve an effective daily use of energy and resources.

Daily day/night alternation and temperature are considered the two most powerful external signals entraining the biological clocks. Moreover, the feeding-fasting cycle is also an important input for the clocks, especially in food-entrainable oscillators (FEOs; Stephan, 2002; Gómez-Boronat et al., 2018). In addition to these, other environmental and social parameters such as dissolved gas levels of O2 and CO2, water salinity, predation risk, intra- and inter-species competition have been described as important *Zeitgebers* in aquatic ecosystems. (Sharma & Chandrashekaran, 2005).

Fish can be classified as ‘‘diurnal’’, ‘‘nocturnal’’ or their mixed types (Iigo & Tabata, 1996), depending on the phase of the daily cycle when they perform most of their activities such as exploratory behaviour, feeding intake and social interactions. The differences in activity phasing promote the organization of fish communities and their spatial distribution, allowing their ecological separation (Volpato & Trajano, 2005; Krittika & Yadav, 2020).

1.2.1 Light

Light is considered the main environmental factor to entrain circadian rhythms via the light-entrainable oscillators (LEOs; Reppert & Weaver, 2002; Isorna et al., 2017). Light comprises multiple factors as colour spectrum, intensity and photoperiod that provide daily and seasonal temporal information to aquatic organisms throughout their life cycle. For example, nocturnal melatonin production plays a key role in the entrainment of daily and annual physiological rhythms in vertebrates

impacting on time regulated functions such as feeding, growth, reproduction and immunity (Falcón et al., 2010).

Light is essential for proper development in many teleost species, and the lack of this factor is associated with high mortality rates and deformities (Tamai et al., 2004; Ben-Moshe et al., 2014). Zebrafish and other teleosts, such as the Mexican cavefish (*Astyanax mexicanus*) and Medaka (*Oryzias latipes*), have tissues that are directly light responsive (Whitmore et al., 2000; Beale et al., 2013; Cuesta et al., 2014). In addition, teleost embryos hatch at a specific time during the day and the absence of light-dark cycle can disrupt this timed process (Steindal et al., 2018). Fish can display their activity during the photophase (during the day) or scotophase (during the night), being classified as diurnal, nocturnal or crepuscular (when activity happens at dawn and dusk; Madrid et al., 2001). Some fishes can show a dualism behaviour, shifting their activity phase throughout their life, due to high plasticity of the circadian system (Del Pozo et al., 2014).

From the physical point of view, light is an electromagnetic radiation characterised by its wavelength (λ), polarization degree, irradiance and direction. As light propagates through water column, it is absorbed and scattered importantly affecting the intensity and the spectral quality of light at different depths (Collin & Hart, 2015). Indeed, the shortest and longest wavelengths are absorbed quite near the surface including the below violet ($\lambda < 390$ nm) and beyond red ($\lambda > 600$ nm) while blue wavelengths can penetrate deeper ($\lambda \sim 450$ nm), reaching about 200 m in the ocean (Dickey et al., 2011; Figure 3). Thus, fish adapted their photopigment sensitivity and the biological response to light depending on the species-specific ecology (e.g., in deep sea fish have a maximised

visual sensitivity in the blue band, while coastal fish species have maximum sensitivity in the green band; Villamizar et al., 2011).

Visual light detection by cones and rods is clearly very important in most vertebrates, but non-visual photoreception and the involvement of non-visual opsins are also essential in many biological processes (Frøland Steindal & Whitmore, 2019).

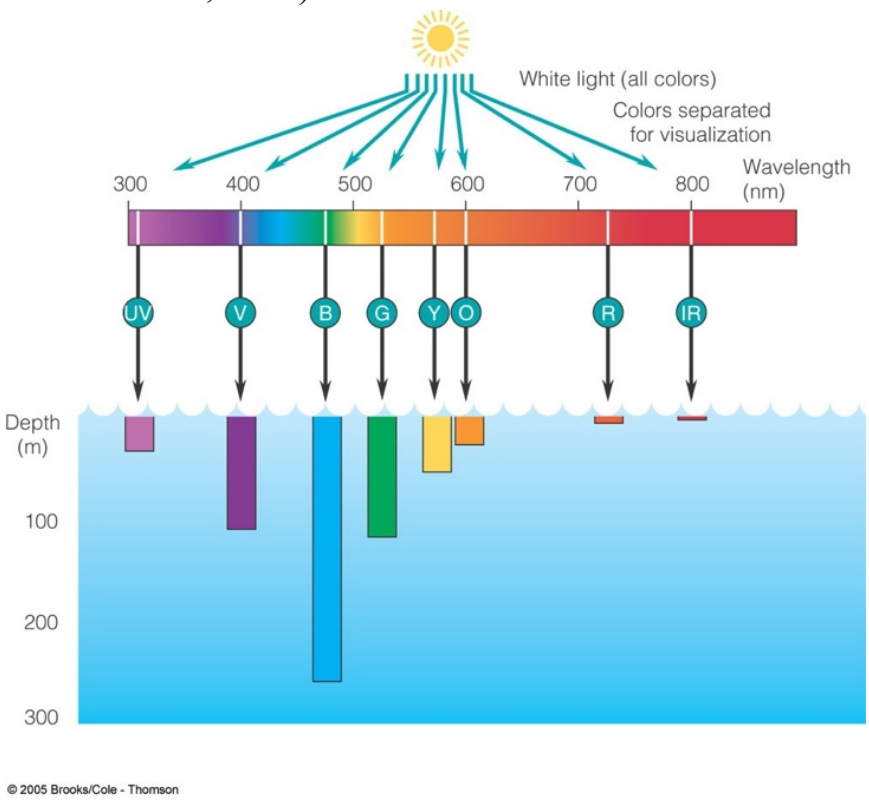

Figure 3. Spectral profile of light propagating through the water column.

1.2.2 Temperature

Most aquatic organisms, as fish, are ectotherms as they are unable to internally regulate their body temperature. Therefore, water temperature influences many biological processes and spatial distribution of these organisms that display optimal temperature ranges below which the chance of survival decreases (Jain et al., 2013). As a result, they evolved strategies to regulate their body temperature (T_b) by actively choose a thermal environment that favours high physiological performances (Reynolds & Casterlin, 1979; Angilletta et al., 2002; Christensen et al., 2021).

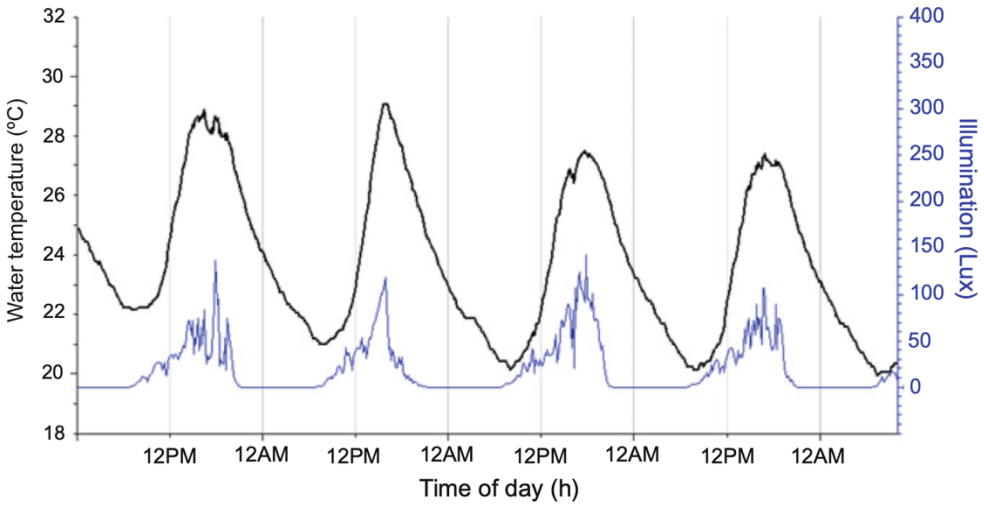

Figure 4. Daily water temperature and illumination cycles in the marine park of Santa Pola salt flats (Alicante, SE of Spain: 38° 11′ 16″N, 0° 36′ 52″W). From Sánchez-Vázquez & López-Olmeda, 2018.

In the natural environment, light and temperature are strictly linked (i.e., higher water temperature during the day and cooler water temperature during the night) creating daily thermocycles in accordance with light-dark cycles (Figure 4). Thus, the transition from cold to warm temperature and vice versa is associated with dawn and the dusk

respectively (Johnson et al., 2004). Daily temperature variations can serve as a powerful time cue to entrain biological clocks suggesting the existence of weakly coupled light and temperature-entrainable oscillators (López-Olmeda et al., 2006; López-Olmeda & Sánchez-Vázquez 2009). Indeed, different investigations proved the importance of daily thermocycles from early development stages in fish, affecting embryo development, hatching rhythms, sex differentiation and improving growth and survival rates (Blanco-Vives et al., 2010; Villamizar et al., 2012; de Alba et al., 2022).

1.3. Non-visual photoreception

Most vertebrates process visual or irradiance information using their lateral eyes. However, other extraocular tissues, as the pineal and the deep brain photoreceptors, can detect and respond to photic stimuli (Bertolucci & Foà, 2004; Fernandes et al., 2012; Figure 5). Photoreception is mediated by specific photopigments called opsins which are expressed in photoreceptor structures. Opsins are a family of seven-transmembrane-domain G-protein-coupled receptors (GPCRs) that are mainly divided into visual and non-visual opsins expressed in ocular and extraocular tissues respectively (Terakita et al., 2012).

Much research in zebrafish have highlighted that tissues and cells can be entrained directly to photic stimuli displaying circadian oscillations, without the involvement of retinal photoreceptors (Whitmore et al., 2000). In addition, 42 different opsin genes (10 corresponding to visual opsins and 32 to non-visual opsins) are expressed in diverse extraocular zebrafish tissues (i.e., brain, liver, heart, gut, muscle, pineal, skin, and testis; Davies et al., 2015).

The pineal is involved in the rhythmic production of melatonin under light-dark cycle condition coordinating many different animals' rhythmic patterns. However, it has been proved that pinealectomized fish, in certain species, are still able to display photic entrainment (Bertolucci & Foà, 2004; Simon et al., 2019). These results highlight the involvement of deep brain photoreceptors and extra ocular non-visual opsins in the biological rhythms entrainment to light signal. Indeed, non-visual photoreception plays a crucial role in various biological processes, including seasonality, photoperiodism, circadian entrainment and DNA repair (Benoit, 1935; Goldman, 2001; Foster et al., 2003; Bertolucci & Foà, 2004; Frøland-Steindal & Whitmore, 2019).

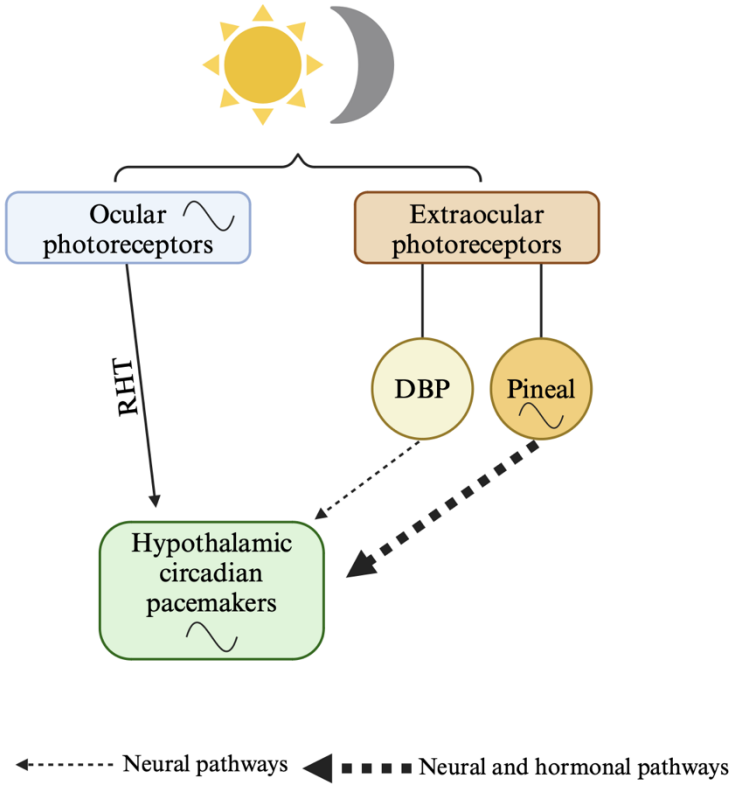

Figure 5. Schematic diagram illustrating ocular and extraocular photoreceptors involved in the vertebrate circadian system. Arrows indicate pathways between circadian oscillators and photoreceptors. DBP: deep brain photoreceptors; RHT: retino- hypothalamic tract. Created with BioRender.com; modified from Bertolucci & Foà, 2004.

1.4. Thermal preference

Environmental temperature plays a key role in affecting and regulating many animal biological processes (i.e., reproduction, growth and metabolic rates, spatial distribution of species, locomotor and feeding behaviour; Volkoff & Rønnestad, 2020; Gomez-Maldonado & Camacho-Cervantes, 2022). This is true especially in ectotherms as fish, so-called cold-blooded animals, whose regulation of body temperature depends on external sources. Thus, fish implement behavioural thermoregulation by navigating water thermal gradients to attain a preferred thermal optimum to enhance their performances (Pawson et al., 2000; Angilletta et al., 2002; Cerqueira et al., 2016). Given the dynamic nature of aquatic habitats, where water temperature can range from near freezing to more than 30 °C, preferred temperatures differ widely across fish species and could depend also on other environmental cues and internal states of the animals (Coutant, 1977; Engeszer et al., 2007). In addition, importantly, water temperatures fluctuate in both space and time because of wind direction, vertical thermal gradient or inflows of cold or warm streams and due to daily and seasonal cycles (Haesemeyer, 2020).

So, fish evolved strategies to actively select and seek out the thermal optimum in their habitat that promote better functioning of their metabolism. Thermal optimum is the temperature that boosts physiological performance with lower energy expenditure (Beitinger & Fitzpatrick, 1979). The thermal optimum for an individual is defined as the final thermal preferendum and that would be the temperature in which an animal in a thermal gradient will finally tend to stay regardless of its prior thermal experience (Reynolds & Casterlin, 1979).

Like other vertebrates, fish perceive water temperature by means of thermoreceptors in trigeminal and dorsal root ganglia neurons that

innervate their skin. Subsequently, this thermal sensation is processed through neural circuits implanting a thermoregulatory behavioural response (Haesemeyer et al., 2015; Germanà et al., 2018; Haesemeyer, 2020).

1.5. Food intake brain control mechanisms

Food intake is highly regulated and this regulation is essential for the development and survival of organisms. Indeed, these complex mechanisms ensure an efficient allocation and utilization of energy resources in order to support metabolism, immune system, growth, reproduction and the cost of daily activities (Schwartz et al., 2000). Feed intake is influenced by both external (e.g., photoperiod, temperature, predation risk, food availability) and internal (e.g., life stage, genetics) conditions (Rønnestad et al., 2017). The regulation of feed intake is very complex and it is driven by two complementary mechanisms: the homeostatic and hedonic pathways (Figure 6).

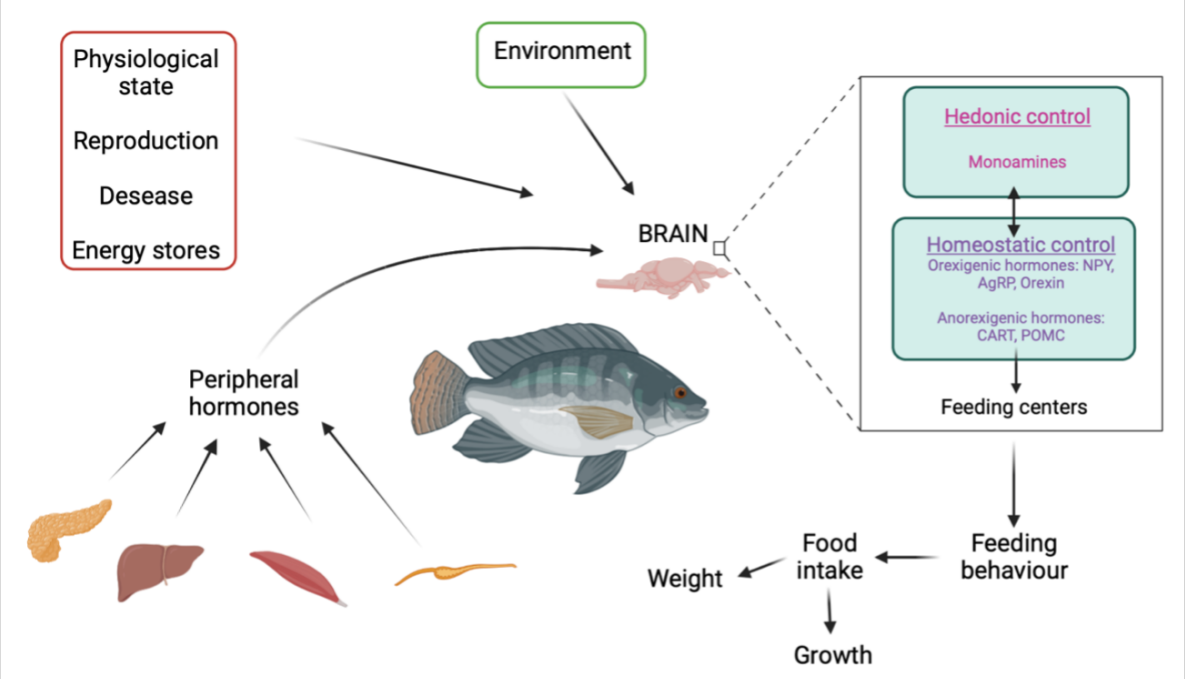

Figure 6. Diagram showing major structures and signals involved in the regulation of food intake in fish and how they can be affected by internal and external factors. Created with BioRender.com.

1.5.1 Homeostatic pathway

The homeostatic pathway maintains normal energy balance homeostasis taking over in response to nutritional demands and metabolic needs. In vertebrates, including fish, the hypothalamus is where this complex regulation happens being the main feeding centre, controlling appetite and integrating peripheral signals (Naslund & Hellstrom 2007; Volkoff, 2019). Many of the neuropeptides and hormones that regulate appetite in mammals are also found in fish (Volkoff et al., 2005; Kulczykowska & Sánchez Vázquez, 2010). However, due to the different feeding habits of teleost species, differences in appetite-controlling systems can be found. In general, the neuroendocrine factors that originate from the hypothalamus either stimulate or inhibit feed intake in fish and are defined as orexigenic and anorexigenic signals, respectively.

Neuropeptide Y (NPY) and agouti-related peptide (AgRP) are the strongest orexigenic signals and principal feeding stimulators in mammals (Kohno & Yada, 2012). In teleosts, the role of these peptides can vary among the species; in most of them the orexigenic role is confirmed. Indeed, it has been found that NPY injections increase feeding behaviour and food deprivation increases brain *npy* and *agrp* expression in several species (Lopez-Patino et al., 1999; Kiris et al., 2007; Yokobori et al., 2012; Tian et al., 2015). In some cases, brain expression of these signals can be modulated by diet as they contain neurons sensing the metabolic status (Narnaware et al., 2002; Riley et al., 2009; Bonacic et al., 2016).

The peptide cocaine-amphetamine-related transcript (CART) was firstly isolated from rat brain as a transcript regulated by acute administration of cocaine or amphetamine (Zhang et al., 2012). CART is considered a potent anorexigenic signal inhibiting food intake (Volkoff &

Peter, 2000). Indeed, fasting has been shown to decrease *cart* brain expression in several fish species (Murashita & Kurokawa, 2011; Nishio et al., 2012; Bonacic et al., 2015) and it is also involved in detecting metabolism status (Conde-Sieira et al., 2015). The corticotropin-releasing hormone (CRH) stimulate the hypothalamic–pituitary–interrenal axis in fish to induce release of glucocorticoids as cortisol and it act as anorexigenic factors (Bernier & Peter, 2001; Volkoff et al., 2005).

Feeding centers also receive information about nutritional status from the periphery (e.g., gastrointestinal tract or GIT, liver, pancreas) (Rønnestad et al., 2017; Volkoff, 2019).

1.5.2 Hedonic pathway

The hedonic mechanism involved in the food intake is related to the brain reward system. This circuitry is activated by the taste and consumption of palatable food and thus is driven by sensory perception or pleasure (Díaz-Rúa et al., 2022). This pathway leads to a feeding behaviour independent of energy requirements (Rossi & Stuber, 2018). Hedonic feeding is regulated by central monoamine neurotransmitters as dopamine (DA), noradrenaline (NE, norepinephrine), serotonin, opioids (e.g., beta-endorphin), and endocannabinoids (Bojanowska & Ciosek, 2016; Díaz-Rúa et al., 2021).

The knowledge about hedonic regulation of food intake in fish is still lacking as it is mostly restricted to investigation of homeostatic mechanisms.

1.6. Microalgae and cell regeneration

Microalgae generally include both eukaryotic (microalgae) and prokaryotic (cyanobacteria) microorganisms that perform oxygenic photosynthesis (Miguel et al., 2021). These organisms can be typically

found in aquatic (freshwater, estuarine, and marine environment) and terrestrial habitats (Mobin & Alan, 2017). It has been estimated that microalgae include about 200,000 to 800,000 species of which around 50,000 have been described (Richmond, 2004). As they have adapted to live in so many different environmental conditions, they present metabolic plasticity. For this, these microorganisms are able to synthesise several complex compounds with interesting applications in various biotechnology sectors (i.e., food, energy, pharmaceutical and biomaterials; Romano et al., 2017; Mobin et al., 2019). Indeed, pigments (carotenoids and phycobilins), peptides, fatty acids and polysaccharides are active biomolecules of microalgae cell metabolism that have high potential for pharmaceutical and cosmeceutical purposes, due to their antioxidant, antibacterial, antiviral, skin regenerative, immunomodulatory and immune-stimulatory effects (Michalak & Chojnacka, 2015; Miguel et al., 2021).

The skin constitutes a protective barrier from external environment and it is subjected to various potential injuries; thus it has to regenerate as quickly as possible through the wound healing. In mammalian adults, this process consists of multiple coordinated steps: hemostasis, inflammation, proliferation, and remodeling. These physiological phases should occur in the proper sequence and different biological components (e.g., cells, growth factors, cytokines) take action for successful wound healing (Richardson et al., 2013; Simões et al., 2018; Edirisinghe et al., 2020). The inflammatory phase involves the recruitment of immune cells to maintain the wound clean and then cytokines and growth factors are secreted for the proliferation phase. In turn, the migration and proliferative phases are characterized by the fibroblast migration to the wound site and differentiation into myofibroblasts to produce

extracellular matrix components. Lastly, in the remodelling phase, all processes end (Miguel et al., 2021).

Some vertebrates as fish and amphibians can perfectly regenerate skin. The zebrafish regenerated skin can also recover its striped pigmentation pattern and it has a minimal scar formation, making it almost indistinguishable from the uninjured one. This ability, together with the conservation of the wound healing process among mammals and zebrafish, makes this teleost a valuable model organism for regenerative studies in vertebrates (Richardson et al. 2013). Currently, also other teleosts such as Atlantic salmon (Sveen et al., 2019), rainbow trout (Schmidt et al., 2016), common carp (Przybylska-Diaz et al., 2013) and gilthead seabream (Ceballos-Francisco et al., 2017) have been used for tissue regeneration and wound healing studies.

In conclusion, microalgal extracts contain bioactive compounds with high-interest biological properties for wound healing applications, creating a favourable environment for cell adhesion and proliferation (Yarkent et al., 2020).

1.7. Species in focus

Many different species of fish were used in the realisation of the following thesis. From the experimental model species zebrafish (*Danio rerio*) to high commercial interest species, mainly freshwater (*Ameiurus melas*, *Tinca tinca*, *Micropterus salmoides* and *Oreochromis niloticus*) but also marine (*Sparus aurata*), to cave species (blind *Astyanax mexicanus* and *Phreatichthys andruzzii*). From a chronobiological point of view, it was of high interest for us to compare diurnal with nocturnal species and to use blind species that have evolved under constant

conditions in which the circadian clock may no longer be functioning in order to obtain a complete characterisation.

1.7.1 Diurnal species

Zebrafish *Danio rerio*

Zebrafish (*Danio rerio*) is a small freshwater fish that belongs to the family of Cyprinidae, the most species-rich vertebrate family (Nelson, 1994). Its scientific name Danio derives from the Bengali name "*dhani*", meaning "of the rice field" (Talwar & Jhingran, 1991) however, its common name represents its distinctive colour pattern based on alternating dark and light horizontal stripes. It was first described by Fracis Hamilton at the beginning of the 19th century (1822).

This teleost in the wild habits the Ganges and Brahmaputra river basins in north-eastern India, Bangladesh and Nepal (Talwar & Jhingran, 1991). These regions include big artificial lakes, ponds and irrigation channels, constructed for fish and rice cultivation and zebrafish tend to occupy the shallow waters. This geographical area is subject to monsoons and seasonal variations thus, zebrafish experience a wide range of temperatures, from 6 °C in winter to over 38 °C in summer. They feed on terrestrial and aquatic insects, zooplankton and inorganic material (Spence et al., 2008).

Zebrafish is one of the vertebrates most used as an in vivo model in various research fields. And this is because it is a small and robust fish that can be easily bred and maintained in laboratory and reproduces all year around. Under spawning conditions, males are easily distinguishable from females (Figure 7) by their slimmer body shape. Females can be recognized by their swollen bellies (Braunbeck & Lammer, 2006); indeed they can spawn hundreds of eggs very frequently (every 2-3 days). Eggs

are transparent and fertilisation is external making embryos accessible to manipulation and can be monitored through all developmental stages under a microscope (Kimmel et al., 1995). In addition, hatching and development are rapid.

The zebrafish is increasingly important in pre-clinical trials of biomedical research (Shin & Fishman, 2002), particularly as in vivo and in vitro model for investigating human diseases (Berghmans et al., 2005) and for the screening of therapeutic drugs (Rubinstein, 2006). The greatest advantage of the zebrafish is its well-characterised genetics, the large number of both molecular and behavioural techniques and protocols that can be used and the availability of various mutants.

Figure 7. Adult zebrafish (*Danio rerio*) female (above) can easily be discerned from male (below) by their bellies full of eggs and the lack of reddish tint along the silver longitudinal lines. From Braunbeck & Lammer, 2006.

Largemouth bass *Micropterus salmoides*

The largemouth bass (*Micropterus salmoides*) is one of the most widely distributed fish in the world and it belongs to the Centrarchidae family. This fish is native to eastern North America, however currently it is possible to find it in South Africa, Europe, Japan, Lebanon, New Zealand, and Philippines. Its popularity as a sport fish has led to its

introduction in so many areas where it has become invasive (Costantini et al., 2018). The scientific name for largemouth bass derives from the Greek *micropterus*, "small fin" and the Latin *salmoides*, "trout-like." The term "small fin" is probably a misnomer. The common name describes its most evident physical characteristic: a very large mouth that extends past the eye and the pharyngeal jaws are well developed, with fine teeth (Scott, 1973; Figure 8).

The largemouth bass lives in almost all types of freshwaters, including swamps, ponds, lakes, reservoirs, creeks, large rivers and estuaries. In general, they prefer habitats with little current, moderate water clarity and density of aquatic vegetation (Brown et al., 2009). Largemouth bass may consume small fish, adult and larval insects, worms, small molluscs and crustaceans depending on their development stage. In general, they are diurnal and most often feed in the early morning or late in the day (Brown et al., 2009).

Figure 8. Picture of largemouth bass *Micropeterus salmoides*.

Optimal spawning water temperature for this species is around 18°-20°C. The male starts to select the nest site generally when water temperatures reach 15.6°C (McPhail, 2007). The nests are built on the bottom substrate composed of sand or gravel of shallow waters. Depending on the female body size, she may release from 2,000-145,000

eggs. Shortly after spawning, the female leaves the nest and the male is left to larval care and development. Bass fry hatch in 3-7 days and they are transparent and they remain in the nest until the yolk absorption, that happens generally in 10 days, then they leave the nest (Moyle, 1976).

M. salmoides has always been an important species in recreational fisheries in North America since the 1880s (Cooke & Philipp, 2009) and became the most widely distributed and popular gamefish in the United States. In addition, The United Nations Food and Agriculture Organization (FAO 1996) reported for the first time the largemouth bass culture for food in 1994. Indeed, it has become one of the most economically important aquaculture species in China since its first introduction in 1980s (Tidwell et al., 2018; Hussein et al., 2020).

Nile tilapia *Oreochromis niloticus*

The Nile tilapia (*Oreochromis niloticus*) belongs to the family of Cyprinidae and it is a freshwater fish that inhabits tropical and subtropical environments although it is native to large parts of north-central Africa but it has been widely introduced on other continents, including Asia, Europe, North America and South America. In these places it often becomes highly invasive, threatening ecosystems and native species. (Trewavas, 1982; El-Sayed & Fitzsimmons, 2023). Nile tilapia are mainly herbivores and with omnivorous tendencies; in fact they feed mainly on phytoplankton and algae but also on insect larvae (Snoeks et al., 2018).

Banks of Nile tilapia establish social hierarchies in which the dominant males can feed and mate first (Barki & Volpato, 1998). Circular nests are built by males to become future egg-laying sites and these nests will become sites of courtship rituals and parental care (Castro et al., 2009). Nile tilapia females incubate the fertilized eggs in their mouth at

27-29°C (Figure 9) and hatching happens at 70-90 hours of incubation. In addition, they hold the hatched larvae and gives parental care until the swim-up stage which might need up to 6-10 days.

Figure 9. Female of Nile tilapia (*Oreochromis niloticus*) incubates an advanced stage of embryonic development eggs.

Nile tilapia presents a series of productive advantages for their culture as: rapid growth and large commercial size, high resistance to disease and to a wide range of water temperatures, early sexual maturation and ease of cultivation in high intensity systems. As a result, aquaculture of Nile tilapia is currently practiced in more than 80 countries and is the third one among the top farmed freshwater fish species, behind grass carp and silver carp (El-Sayed & Fitzsimmons, 2023).

Gilthead seabream *Sparus aurata*

The gilthead seabream (*Sparus aurata*) is a subtropical Sparidae that occurs naturally in the Mediterranean and in the Eastern Atlantic. It is characterized by a silvery grey body that remembers the shape of the glittering metallic tip of a spear, giving the name of the genus *Sparus* (Pavlidis & Mylonas, 2011; Figure 10). This fish inhabits seagrass beds, sandy bottoms as well as the surf zone. It can tolerate a wide range of salinities and perform a trophic migration towards the coastal area in early spring then it returns to the open sea for breeding purposes during late

autumn. The seabream is mainly carnivorous feeding on shellfish, including mussels and oysters but it can be accessorily herbivorous (Hutchings & Baum, 2005).

This species is a protandrous hermaphrodite, so it is a functional male in the first two years and when it is over 30 cm in length it turns female. Females are batch spawners that can lay 20,000-80,000 eggs per day for a period of up to 3 months (Sola et al., 2007).

Figure 10. Gilthead seabream (*Sparus aurata*)

The gilthead seabream ranks 33rd among the most reared fish, with an estimated annual production volume of 258,754 T/year (Mhalhel et al., 2023). It is commonly cultivated in the Mediterranean sea in marine cages and recirculating aquaculture systems (RAS) and the main producers are Greece, Turkey, Spain and Italy (Sola et al., 2007; Seginers, 2016). Thus, its economic potential is quite evident and it has become an important target research species over the years. In fact, a better understanding of its biological aspects and molecular pathways has significantly increased its aquacultural aspects such as its reproductive success, survival, and growth (Sadek et al., 2004; Kir, 2020). On the other hand, hatchery conditions are still far from ideal, resulting in frequent challenges and economic losses (Mhalhel et al., 2023).

1.7.2 Nocturnal species

Tench *Tinca tinca*

Tench (*Tinca tinca*) is a cyprinid fresh and brackish water species found throughout Eurasia from Western Europe into Asia (Kottelat & Freyhof, 2007). It typically inhabits shallow, densely vegetated and with moody substrate still waters and it tolerates low oxygen concentrations. Tench has a carp-like shape body and dark green almost black skin, darker above and almost golden below (Figure 11). Tench feeds on detritus, benthic animals plant materials and adults prey mainly on molluscs (Allen et al., 2002). This fish shows a nocturnal activity pattern, with higher locomotor activity and feeding behaviour during the night (Herrero et al., 2003).

Figure 11. Tench (*Tinca tinca*)

Spawning takes place in shallow water usually among aquatic plants where the sticky green eggs can be deposited between May and September, depending on the latitude (Kottelat & Freyhof, 2007).

This species is sold on the European and Asian market as food, for recreational fishing and for ornamental purposes. It has been cultured for centuries as a secondary fish in polyculture with carp ponds in many European countries (Arlinghaus et al., 2003).

Black bullhead catfish *Ameiurus melas*

The black bullhead *Ameiurus melas* is a native to North America freshwater species belonging to the Ictaluridae family and it was first introduced in Europe around the late 1800s for aquaculture purposes (Holčík, 1991). The body shape is siluriform and it has black chin barbels and its tail fin is notched (Figure 12). The black bullhead occurs in a variety of habitats but is most abundant in those with turbid water without current or strong flow as backwaters and ponds. In general, this fish can tolerate really harsh conditions as pollution, low oxygen concentration, elevated temperatures that characterize small and isolated aquatic systems (Braig & Johnson, 2003; Novomeská et al., 2013). The black bullhead, like other catfishes, relies on an acute sense of smell to find its food and they are omnivorous. They feed from the bottom on a diversity of plant and animal material, both live and dead. This species shows a nocturnal behavioural pattern (Jaćimović et al., 2021).

Black bullhead matures at the age of 2-3 years and the spawning season may be from May to August, depending on local temperature water conditions (Scott, 1973). Spawning happens on the shallow muddy bottoms and females excavate a nest where to deposit approximately 200 adhesive eggs. Until embryos hatching, the eggs are guarded by both parents (Novomeská & Kováč, 2009).

Ictalurid catfish have been an important commercial, aquaculture, and sport fish for several decades in the United States and they were cultured on a large scale in the late 1800s for sport fish application, especially. Channel and blue catfish (*Ictalurus punctatus* and *Ictalurus furcatus*) and their hybrids became the major aquaculture species in the United States but other catfish species such as *A. melas* have been propagated by government or private hatcheries and currently they are worldwide spread

and they have a quite high invasive potential (Novomeská & Kováč, 2009; Dunham & Elaswad, 2018).

Figure 12. Black bullhead catfish (*Ameiurus melas*)

1.7.3 Cavefish species

Blind Mexican cavefish *Astyanax mexicanus*

The blind Mexican tetra, *Astyanax mexicanus* is a member of the Characidae family and this name includes 30 cavefish populations that inhabit river caves in the Northeast of Mexico and Southern Texas and they descend from the same ancestral river strain (Gross, 2012). However, the cave or hypogean populations exhibit degenerate eyes sunken into the orbits with loss of its function, depigmentation and expanded gustatory system; these are typical troglomorphic traits (Borowsky, 2008). Indeed, 1 day post fertilization (dpf), a “normal” eye has formed and after the lens enters apoptosis process (Alumni et al., 2007); despite the lack of functional eyes, juveniles and adults still show a light-dependent basal locomotor activity (Beale et al., 2013; Simon et al., 2019).

The cave strain and the surface-dwelling strain (Figure 13) have not fully speciated from each other and can still be crossed in the laboratory to produce multiple hybrid F1 generations. In fact, this species adapts

really well to laboratory conditions making it a powerful and interesting model to study adaptive and regressive evolution (Gross et al., 2015).

Figure 13. Photo of *Astyanax mexicanus*, surface strain with functional eyes (top) and cave strain without eyes and depigmented (bottom).

Somalian cavefish *Phreatichthys andruzzii*

Phreatichthys andruzzii is cave species Cyprinid, that inhabits the subterranean waters under the central Somalian desert (Ercolini et al., 1982). The wild population, sampled in the Bud-Bud region of Somalia in a sub-Saharan horizontal limestone formation, has been isolated in a subterranean environment for approximately two million years (Stemmer et al., 2015). For this, it exhibits extreme troglomorphic features such as completely loss of pigment, scales and functional eyes (Figure 14), slow metabolism but an increase in neuromast sensory cells, as in the blind Mexican tetra. However, this species shows negative phototaxis or photophobic behaviour (Tarttelin et al., 2012).

Figure 14. Somalian cavefish *Phreatichthys andruzzii.*

Objectives

2. Objectives

The aim of the present doctoral thesis was to deepen the effect and influence of the most important cyclical environmental stimuli, which are light and temperature on many different species of fish. Specifically, for this purpose, it was decided to characterise species of commercial interest with diurnal and nocturnal activity patterns. The results could be used for improving husbandry protocols according to the species and to refine breeding, since most are reared under constant conditions. From a chronobiological point of view, cave species that have evolved for millions of years in a constant environment were used to investigate whether these stimuli are still able to entrain their biological clocks or not (Figure 15).

Furthermore, as this was an industrial doctoral thesis, I spent half of my PhD (18 months) at companies that were part of the project: Sparos Lda, a Portuguese company that develop new feed technologies mainly in the field of aquaculture, and Alga&Zyme, an Italian company specialised in the development of biotechnologies to produce microalgae. The objectives (objectives 8, 9 and 10) set during these secondments were mainly of commercial interest, differing somewhat from the main topic of the thesis (Figure 15).

Therefore, the specific objectives listed below were designed:

1. To design and prove an automated-recording, low-cost and user-friendly-interface solution for studying thermal preferences and locomotor activity in fish.
2. To ascertain and describe the daily thermal preference of commercially interesting diurnal and nocturnal fish to describe thermal preference according to their daily activity patterns.

3. To investigate if this daily thermal preference is driven by endogenous circadian clocks or not.
4. To evaluate the possible daily thermal preference in blind cavefish species pointing out temperature variations as a feasible *Zeitgeber* for these animals that have evolved in constant conditions environments.
5. To delineate whether fish can exhibit daily rhythms of light selection and determine whether these rhythms are endogenous, testing different wavelengths (white and red) on the experimental zebrafish model.
6. To ascertain and characterise the long-term photic entrainment of locomotor activity and circadian endogenous rhythmicity in the blind Mexican tetra.
7. To investigate a possible daily relative expression of selected non-visual opsin genes in the brain of the blind Mexican tetra that allow this species to entrain their activity to the light-dark cycles pointing out the importance of non-visual photoreception in fish.
8. To describe the possible effects on fish physiology of emergent ingredient diets focusing on the central mechanisms that regulate food intake.
9. To determine a potential application of microalgal extract treatments in captive fish breeding, by investigating in vitro zebrafish cell proliferation and migration activity, involved in the healing process, treated with microalgae.
10. To essay the possible efficiency in the healing processes using time-dependent (day or night) microalgal treatments, being that fish cells are photosensitive and directly entrainable by light.

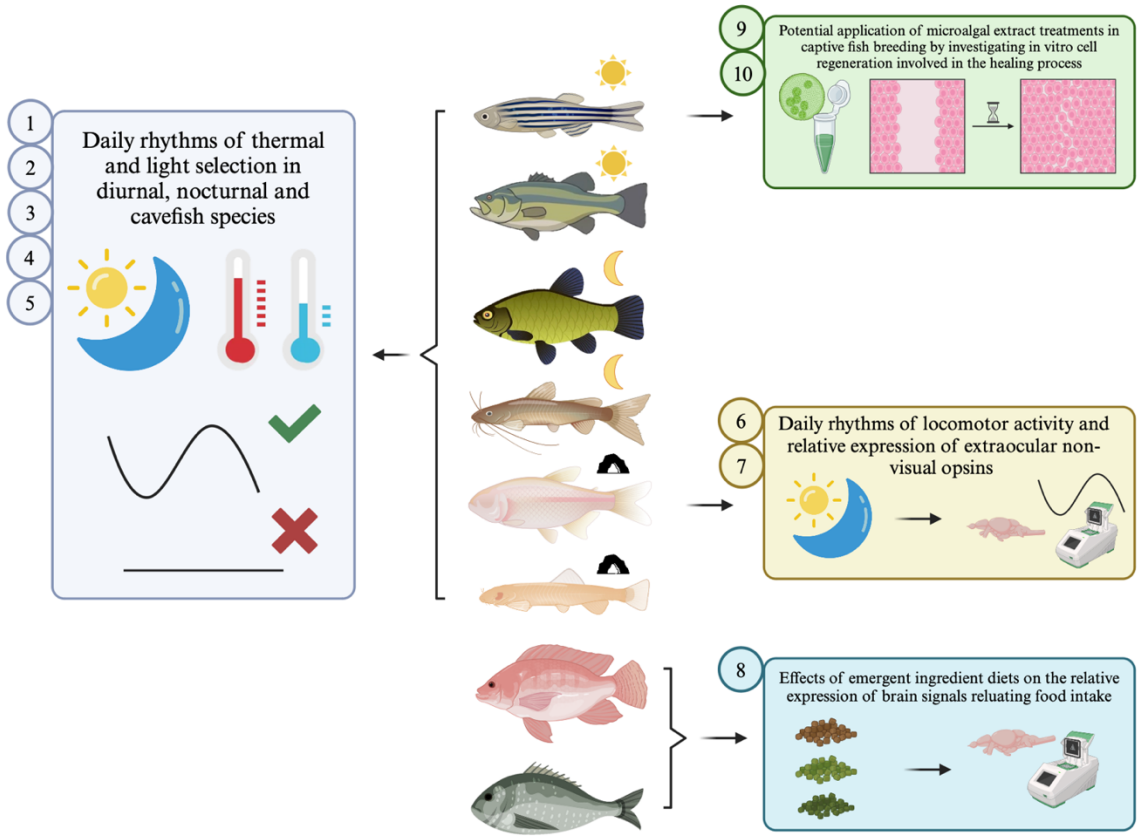

Figure 15. Schematic overview of the objectives of the present doctoral thesis. Created with BioRender.com.

Experimental chapters

Chapter I. Temperature

Chapter 1.1

Daily rhythms of thermal preference in zebrafish: an automated solution to generate a horizontal thermal gradient and long-term recording fish behaviour.

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Manuscript submitted to Zebrafish

Abstract

Photoperiod and temperature are two of the most powerful environmental cues that entrain circadian clocks. Being ectothermic, fish must keep their body temperature within a physiological range to optimize biological processes exclusively applying behavioural strategies. Here, we developed a low-cost, automated system that allow to create a horizontal thermal gradient and videorecord fish behaviour for long-term periods. To validate the system, we assessed daily thermal preference and locomotor activity in the teleost Danio rerio. Because captive conditions in fish often failed to provide the possibility to perform behavioural thermoregulation, our results highlight the importance for considering this behaviour when designing husbandry protocols.

Keywords: circadian rhythms, daily activity, thermal preference, welfare, zebrafish

Main Text

Circadian clocks have evolved to allow almost all organisms to entrain their physiological functions and behaviour to cyclic environmental fluctuations.[1-5] Among these, light and temperature cycles are strictly correlated in the wild, and their daily variations strongly affect biological processes of organisms, such as reproduction, metabolism, spatial distribution, locomotor and feeding behaviour.[6-10] Ectotherm species exclusively rely on behaviour to actively choose a thermal environment that favours high physiological performance (i.e., behavioural thermoregulation).[11-15] However, breeding animals are commonly reared at constant environmental temperature, an unnatural condition in which animal can not exploit their natural behaviour. Indeed, recent studies pointed several advantages of the thermoregulation in captive breeding (i.e., decreased cortisol levels, reduced aggressive behaviours, decrease in mortality, oxidative stress and DNA damage) supporting how a thermoregulatory environment can promote a potential strategy to allow organisms to avoid stress-induced effects improving their welfare.[16-18]

Over the last 20 years, several systems have been developed to study the thermal preference in fish such as the shuttlebox[19,13] and the annular chamber,[20,21] consisting in two/four cylindrical tanks differed in water temperature and connected by a narrow tunnel through which fish can move throughout them and choose the preferred temperature. Given their limited size and/or number of connected tanks, these systems are used to investigate thermal preferences of individual or small group of fish for short periods.[22-24] Recent studies have developed horizontal thermal gradient systems, which allow long-term studies of thermal preference in breeding conditions. However, these systems require daily intervention of the experimenter to maintain a constant gradient, thus potentially arousing fish stress level.[25,15,26]

Here, we describe an automated-recording, low-cost and user-friendly-interface solution for studying thermal preferences and locomotor activity in fish (Fig. 1). Briefly, two separate automatic systems were used i) to regulate water temperature by heater-cooler systems and ii) to analyse fish behaviour for long period by producing high-quality video recordings. The horizontal thermal gradient is maintained and controlled automatically by an Arduino microcontroller (Fig. 1C); an open-source platform that can be easily programmed to control water temperatures using dedicated probes.[27] The video recording system is based on the raspberry pi (Fig. 1B); a small, single-board computer highly customisable programming capabilities, that provides an effective, low-cost solution.[28]

To validate the system, daily thermal preference and locomotor activity of the model teleost *Danio rerio* were assessed by recording continuously the fish behaviour for at least 4 complete days (N = 12 fish/system; three systems in total; see details in Supplementary Materials). The horizontal temperature gradient was set at 24 - 32 °C according to the physiology of the species.[29] Cosinor analisys[30,31] revealed the presence of daily rhythmicity in the thermal preference and distance covered across days in each system (Table S1). Repeated measures ANOVA analysis on the pooled dataset found a significant differences in both thermal preferences ($F_{1,108}$ = 203.450, $P < 0.001$) and distance covered ($F_{1,108}$ = 109.090, $P < 0.001$) across *Zeitgeber* Time (ZT). Fish showed a significant daily rhythm in their preferred temperatures (zero-amplitude test: $F_{2,111}$ = 48.726, $P < 0.001$; Fig. 1D) by selecting higher temperatures during the day and lower temperatures during the night with a diurnal acrophase at ZT 6.10 ± 0.54 (Table S1). Being a diurnal species, zebrafish also displayed a significant daily rhythm of activity ($F_{2,111}$ = 267.7951, $P < 0.001$; Fig. 1E; Table S1), with more than 80% of locomotor activity (85.17 ± 1.50 %, mean ± SD)

during the light phase and a mean acrophase at the middle of the light phase (ZT 6.38 ± 0.17; Table S1).

Our system might provide an automatic low-cost solution for characterizing thermal preferences as well as assessing behavioural parameters of activity as possible indicators of welfare in laboratory and farm fish. These behavioural and physiological parameters could be used for improving husbandry protocols to refine breeding conditions.[32] Furthermore, the investigation of daily thermoregulation in fish will provide new accurate and consistent data about the thermal biology of ectotherms and how temperature linked to photoperiod can entrain circadian clocks in fish in a dynamically changing world. Climate change and the consequent global warming impacts fish ecophysiology limiting their performance and fitness.[33,34] Being able to replicate such extreme conditions in the laboratory by manipulating water temperature and studying the derived behavioural thermoregulation could be significant to understand the future impacts that global warming will have on ecosystems.

Data availability statement

The Practical Guide to realize the system are available in the Supplementary Material.

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Authorship contribution statement

F. Conti: Conceptualization, Methodology, Investigation, Data Curation, Writing – Original Draft, Writing - Review & Editing, Visualization; **G. de Alba**: Conceptualization, Methodology, Writing - Review & Editing; **J.F. López-Olmeda**: Conceptualization, Writing - Review & Editing; **L. M. Vera**: Conceptualization, Writing - Review & Editing; **G. Lucon-Xiccato**: Software, Writing- Review & Editing; **E. Mainardi**: Methodology, Writing - Review & Editing, Visualization; **S. Cesari**: Methodology, Writing - Review & Editing, Visualization; **M. Bottarelli**: Methodology, Writing - Review & Editing, Visualization; **C. Bertolucci**: Conceptualization, Methodology, Writing - Original Draft, Writing - Review & Editing, Visualization, Funding acquisition; **F.J. Sánchez-Vázquez**: Conceptualization, Writing - Review & Editing, Funding acquisition; **E. Gatto**: Conceptualization, Methodology, Software, Formal analysis, Investigation, Data Curation, Writing - Original Draft, Writing - Review & Editing, Visualization, Funding acquisition.

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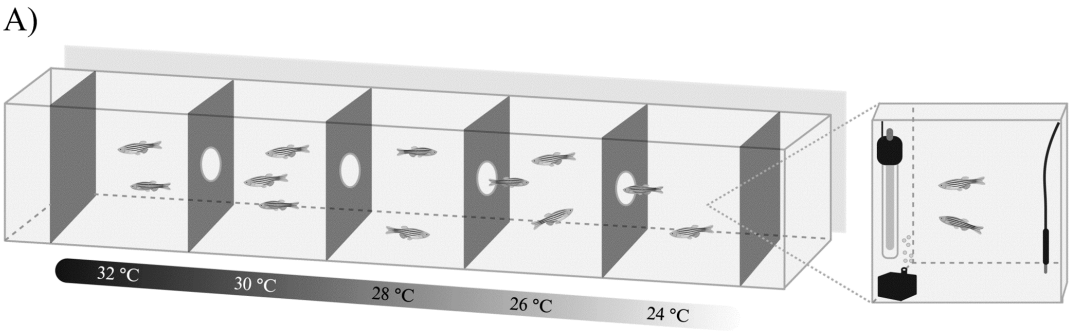
Figure caption.

Figure 1: (**A**) Representation of the thermal gradient multi-chambered tank to investigate daily variation of thermal preferences in fish. (**B**) Custom-written application used to remotely video record fish behaviour. (**C**) Flowchart of the algorithm developed for creating and controlling the thermal gradient in the multi-chambered tank. (**D**) Average daily thermal preference and (**E**) distance covered of *Danio rerio* exposed to a horizontal thermal gradient of the three systems (N= 12 fish/systems) across five days of videorecording. Data points represented mean ± standard deviation of parameters measured each day. Dotted lines represented the 95% confidential interval predicted from the least squares linear regression of Cosinor model. The white and dark grey bars above graphs represent the light and dark phases, respectively. The time scale (x-axis) is expressed as *Zeitgeber* Time (ZT), in which ZT0 corresponds to lights on and ZT14 corresponds to lights off. Created with BioRender.com.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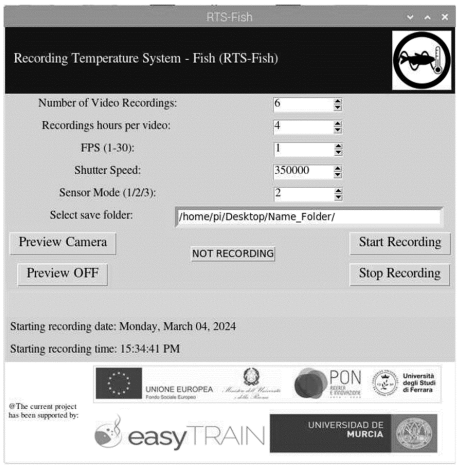
B)
RTS-Fish
Recording Temperature System - Fish (RTS-Fish)
Number of Video Recordings:
Recordings hours per video:
FPS (1-30):
Shutter Speed:
Sensor Mode (1/2/3):
Select save folder:
/home/pi/Desktop/Name_Folder/
Preview Camera
NOT RECORDING
Start Recording
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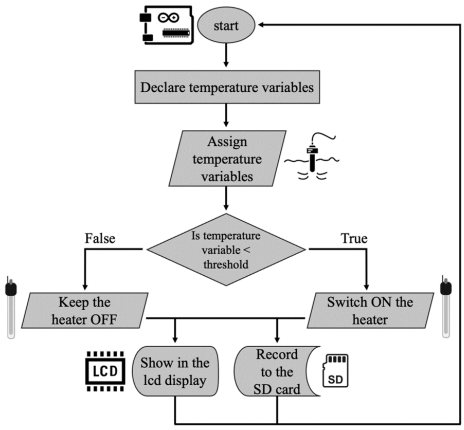
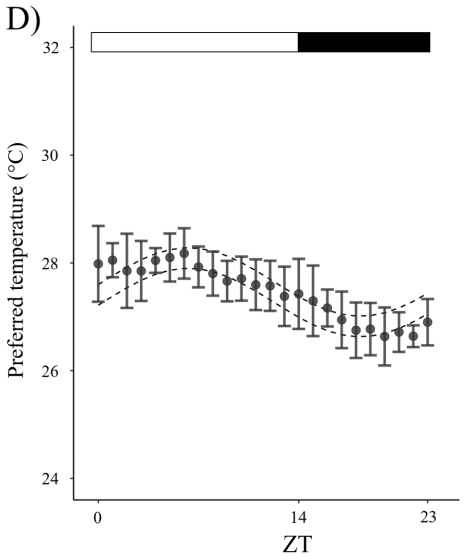
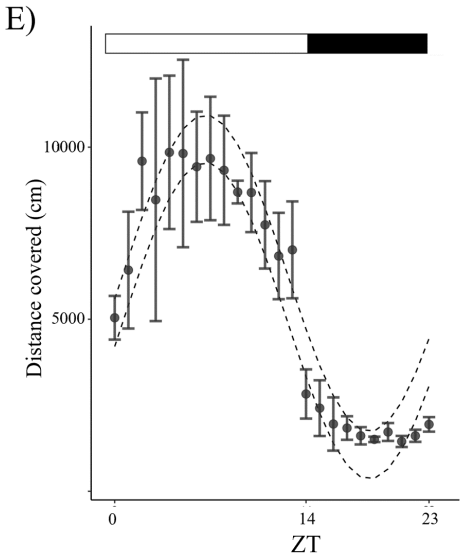

C)
start
Declare temperature variables
Assign temperature variables
Is temperature variable < threshold
False
True
Keep the heater OFF
Switch ON the heater
LCD
Show in the lcd display
Record to the SD card
SD


D)
Preferred temperature (°C)
32
30
28
26
24
0
14
23
ZT


E)
Distance covered (cm)
10000
5000
0
14
23
ZT

Supplementary Information

Temperature gradient multi-chambered tank setup

We built three 108 L multi-chamber glass tanks (180 × 30 × 20 cm) internally divided into seven interconnected chambers. The two lateral chambers are used only for filtering the water (Magic filter 50, Prodac, Italy), while the remaining five housed the fish (Fig. S1). Each chamber (30 × 30 × 20 cm) is separated from the others by black non-toxic polyvinyl chloride (PVC) panels which has a hole (Ø 5 cm; heigh 12 cm from the bottom of the tank) positioned in the middle to allow fish passage. This set up resembled a semi-natural condition in which fish can freely move and choose among different thermal environments across the day (for similar system).[1-4] The system was placed in constant darkness room maintained at a temperature of 28 ± 1 °C by air condition system (Vitoclima 300-S, Viessmann, Germany).

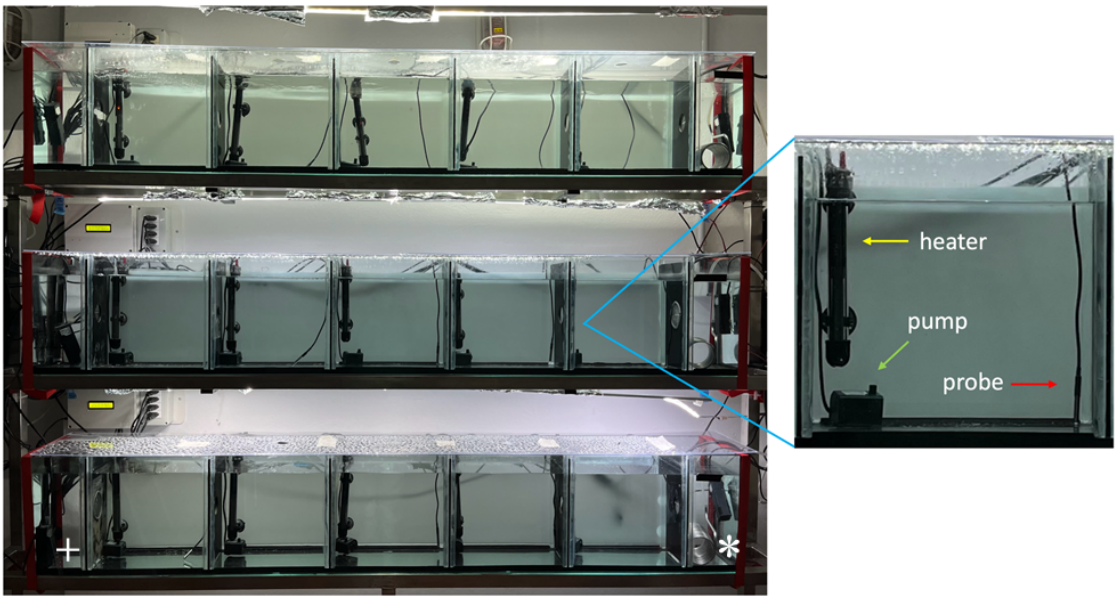


Figure S1. Photo of the three multi-chamber glass tanks built for investigating daily variation of thermal preference in fish. Each chamber is equipped with a mini water pump (green arrow), a thermostat resistance heater (yellow arrow) and a temperature probe (red arrow). In the right-extreme chambers of each multi-chamber tank (white asterisk), there

is a steel coils through which cooled water flow, while a filter was positioned in the left-extreme chambers (white cross).

Each chamber was equipped with i) a water pump (SunSun HJ-311, China), which prevent the creation of a vertical temperature gradient and to oxygenate the water; ii) a thermostat resistance heater (AquaHeat 50, Juwel Aquarium, Germany) and iii) a water-proof probe (Ds18b20, AZ-Delivery Vertriebs GmbH, Germany) which constantly measure the temperature (Figure S1). The lowest temperature of 24 °C was guaranteed by the thermal exchange of cooled water passing through a steel coil immersed in the lateral compartment of the multi-chamber tank. The water inside the steel coil was maintained within a closed circuit connected to an external chiller (Tk 500, Teco Srl, Italy). An infrared lamp (141.5 ×18 × 4 cm; Fig. S2) covered with translucent acrylic white sheet (Falken Design WT2447-1-8/2436 Acrylic White Sheet, Translucent 55%, 152 × 26 × 0.2 cm) was placed on the back of each multi-hamber tank for videorecording in the dark condition.

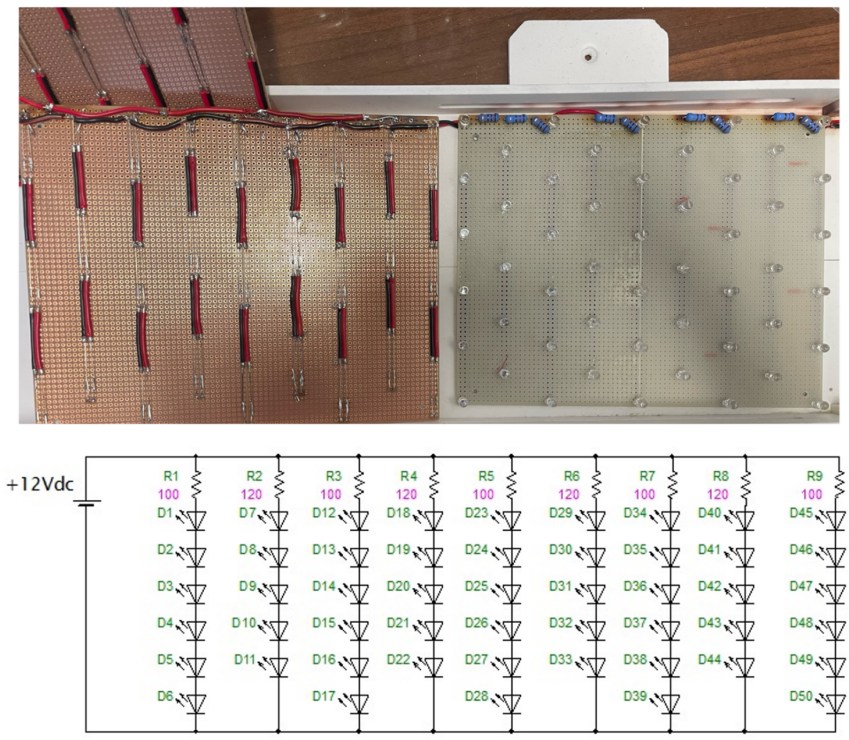


Figure S2. Photo and representative diagram of the circuit used to create infrared LED lamp. Each lamp presented 5 panel connected in series, where each panel consist in 50

Infrared LED (938-942 λ; Forward Voltage 1.15-1.20 Superlight, Elcart Distribution S.p.A., Italy; see the list of components). Each panel consisted of 5 lines of 6-leds in parallel wiring and 4 lines of 5-leds in parallel wiring. The 9 lines were connected in series wiring.

Automated temperature controller and videorecording system

A microcontroller board compatible with Arduino IDE (Elegoo Mega R3, Elegoo, China) was used to automatically create and maintain the horizontal thermal gradient in the multi-chamber tank (Fig. S3).

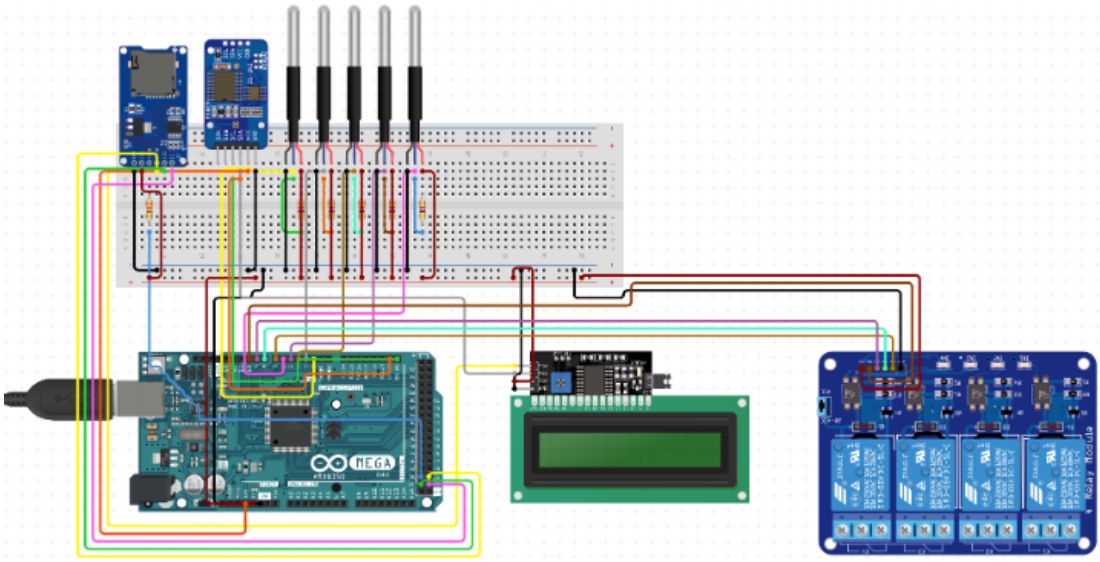


Figure S3. Arduino-based microcontroller circuit built to create the horizontal thermal gradient in the multi-chamber tank.

The microcontroller continuously red data from the temperature probe every 5 mins and recorded the information in a microSD memory (SanDisk Ultra 32 GB, Milpitas, USA). The time of recording was provided from a RTC Clock module (DS3231, AZ-Delivery Vertriebs GmbH, Germany) and stored in the microSD (Fig. S4).

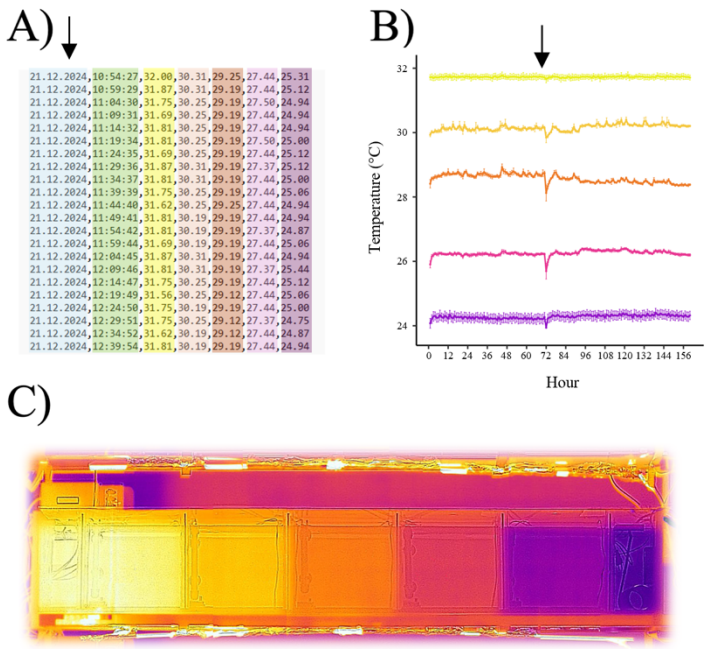


Figure S4. (A) Example of recorded temperature saved in .txt file. The first column (light blue) reported the date, the second column (green) the time of recording, the remaining columns represented the temperature from the hotter chamber (3rd column, 32 °C, yellow column) to the cooler chamber (7th column, 24 °C, purple column). Each column is separated by coma. (B) Hourly temperature stability for each chamber recorded across 6 days. Data points re presentative mean ± standard error of hourly temperatures recorded every 5 minutes. The arrow indicated the moment in which the recording was interrupted, and the multi-chamber tank was refilled. After few hours, the temperature returned to the pre-set range for each chamber. (C) Thermal images of the multi-chamber tank using a FLIR E54 Thermal Imaging Camera (Teledyne FLIR LLC, Oregon, USA). Image showed the horizontal thermal gradient from 32 °C (yellow) to 24 °C (purple) generated and maintained by the microcontroller. The vertical temperature was equal between the bottom and the top of each tank.

Once the microcontroller recorded the temperature from each temperature probe, the information was projected from a 16 × 2 LCD Display (HD44780, AZ-Delivery Vertriebs GmbH, Germany), then the microcontroller switched on/off the single heater in each chamber depending on the settled temperature threshold as described in the following flowchart (Fig. 1 in the main text).

Since the thermostat resistance heater required high-voltage electric current (220v), the relay circuit was designed to be always open/disconnected, i.e. sending power to the heaters is constrained to the activation of microcontroller and whether acquired temperature from the probe is below the threshold.

The microcontroller is programmed using Arduino IDE (Integrated Development Environment), a friendly open-source software development platform. We created a custom program where it was possible to define several parameters, such as the temperature in each chamber and the range of temperature tolerance for switch on/off the heater as well as the latency between recording time point. The Arduino code and the practical guidance to assemble the board can be downloaded from https://github.com/CristianoBertolucci/FishTemperatureGradientSystem.

To assess daily thermal preference, fish behaviour was recorded by using three different camera modules, placed at 170 cm from the tanks, connected each to a Raspberry Pi (Raspberry Pi 4 model B, Raspberry Pi, UK). Videorecording is performed by a custom written Python script (Fig. S6) based on picamera package (ver. 1.13), which provided a flexible solution for the collection of long-term recording of good quality (i.e., 2 hours of continuously recording are 240 MB). We used Tkinter to create a user-friendly GUI for the application.

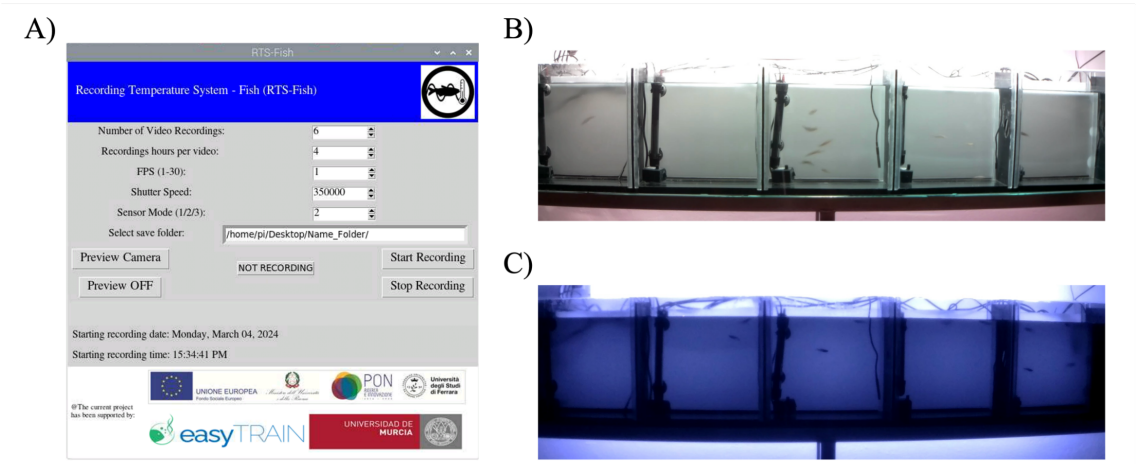


Figure S6. (A) The video recording software used to prolong record fish behaviour. (B) Photo of video recording in the condition of light on and (C) darkness during the night.

We reported the parameters that the users can modify to increase image quality according to the light condition and settings:

- *Number of Video Recordings*: user defined the number of video recording to be generated according to the duration of the experiment.
- *Recordings hours per video*: users defined the number of hours per each video recording.
- *FPS* (framerate per second): defined the number of frames the camera could capture per second. Because the experiments required longer recordings, we suggested to set up to 1 frame per seconds.
- *Shutter Speed* ("shutter_speed" parameter): define the exposure attribute (length of time) that the camera is exposed to light (e.g., the time at which point the captured scene would become blurry). The time was expressed in microseconds. We suggested to increase this parameter when observing high-active fish and/or low light intensity condition.

- *Sensor Mode* (“sensor_mode” parameter): defined the resolutions of video recordings. According to the FPS (1) and shutter speed (35-50 ms) parameters and the position camera from the tank (180 cm), we usually set to 2, corresponding to 3280 × 2464 resolution and 4:3 aspect ratio.
- *Select save folder*: defined the path where the video recordings would be saved.

To increase the final quality of recordings, experimenters might consider acquiring multiple images rather than a continuously recording because more parameters might be implemented, such as brightness and light contrast. We suggested to look at the documentation of the library for setting the parameters according to the experimental condition (https://picamera.readthedocs.io/en/release-1.13/fov.html).

Once recording started, the application generated a short video called “trash” (duration 15-sec). This was necessary because the camera module required a short loading-time to set up the recording parameters defined by the user. Then, the application generated a user-defined number of videos of the chosen duration, which were identified by a sequential number, e.g. "0Video", "1Video". The format of the videos was H.264, which could be covert to other type of format code. To further reduce the disturbance and limiting the entrance to the experimental room, we remotely controlled the Raspberry Pi by using Virtual Network Computing (VNC) app, such as RealVNC® server (https://www.realvnc.com/en/). The source code for video recording and the information to execute the code can be downloaded from https://github.com/CristianoBertolucci/FishTemperatureGradientSystem.

We designed the in the optic of acquiring automatically long-term data with the minimum of components, which are low cost and easily reachable from common marketplaces. Although there is several commercial

equipment that allows researchers to record the behavior of fish, we preferred to build our system by assembling separated components that might be easily adjusted according to experimental conditions and quickly substitute in case of necessity/maintenance.

The list of components is reported below:

Quantity	**Component**	**Description**
Automated temperature controller		
1	Elegoo Mega R3 (ATmega2560 micro-controller board)	
1	Real Time Clock (RTC) module compatible with DS3231	Maintain accurate timekeeping
1	MicroSD Card Reader Module Adapter SPI Interface	Store the red temperature and time for further checking of the thermal gradient stability across days
1	LCD I2C 16x2	Display the temperature read from each probe
2	400-pins Breadboard	Build the circuit (see scheme Fig. S2)
	jumper and solid wire	
1	4-channer relay 5V	The relay shield provided an easy way to control high-voltage devices, e.g. the 200-V heater.
5	Water-Proof temperature probe (Ds18b20)	Read the water temperature in a specific compartment.

4	thermostat resistance heater	Once read the temperature, the micro-controller board switch ON/OFF the heater via the relay according to the desired temperature threshold (Fig. S5). By changing the type of heater, the system might be potential applied for creating a horizontal thermal gradient in terrestrial cage.
1	USB A to USB B Cable	Connect the micro-controller board to a PC for uploading the source code.
1	30W AC-to-DC adapter	External power supply for the micro-controller board setting up 9V.
7	Resistor ¼ W	N = 5 resistor 4.7k Ω ± 5%, one resistor connected to the input cable of each temperature probe. N = 1 resistor 330 Ω ± 5% wired to the Micro SD adapter.
Videorecording system		
1	Raspberry Pi 4 Model b + 32GB MicroSD for mounting the system	A custom-made code based on picamera library was used to long-exposition record fish behaviour by running on a Raspberry Pi 4 Module b
1	Power Suply (5.1V – 3A)	
1	5-MP RPi IR-CUT module Camera for supporting night vision.	
1	Flat Camera Cable (1 m)	

Experimental protocol

To validate our system, we investigated thermal preference of the teleost model zebrafish *Danio rerio*. Adult zebrafish (n= 36), derived from a wild-type strain descendant bought from a local shop and maintained at the facility of University of Ferrara (Italy) since 2011, were kept in 230-liter glass tanks composed of mixed-sex groups. The aquaria were provided with mechanical, chemical, and biological filters and water temperature was set at 27 ± 1 °C; water conductivity was maintained at < 500 µS/cm, NO_2^- at < 0.1 mg/L and NO_3^- at < 25 mg/L. The fish were maintained under a LD 14:10 h (light/dark cycle) and were daily fed three times with live *Artemia salina* nauplii (Artemia Salina Premium GLS, Belgium) and commercial dry flake food (Vipan Nature, Sera, Germany). The sample size was chosen according to the UE recommendations to guarantee animal welfare (2010/63/UE), establishing a maximum density of 1 fish/L. In the present experiment fish density was 12 fish/18 L (1.5L per fish), a threshold kept below the UE recommendations to avoid stress and negative effects on fish behaviour as in previous studies.[2,3]

The experimental subjects were randomly selected from breeding stock. Each subject was tested only once and released at the end of experiment for breeding and reproduction purpose; therefore, the data of the different experiments and multi-chamber tanks were independent. Three groups of zebrafish (n=12/group) were used. A continuous thermal gradient, from 24 °C to 32 °C, was designed and create according to the physiology of the species.[5] During the experiment, fish were kept under a LD 14:10 h that was provided by a LED strip (light intensity 730 lux, TMR, ELCART, Italy) placed 15 cm above each tank. Fish were fed *ad libitum* with *Artemia salina* live nauplii once per day. Food was randomly administered during the day to avoid feeding entrainment.[6-8] Excluding the first day of recording for

acclimatization, the video recording of each group lasted at least four complete days.

Video Analysis

Processing of videos in situ motion detection is time and memory consuming for the limited hardware of Raspberry Pi, consequently the analysis of the videos was successively performed using the EthoVision XT tracking software (Noldus, The Netherlands). The software can track fish across days to obtain i) the cumulative time spent for each chamber from a single tank and ii) the average quantification of locomotor activity of fish in the group. To increase the performance of the software, tracked position of fish were weighted for 5 frames/second.

To verify the quality of the tracking, a blind experimenter performed a manual count of the fish. By using a free multimedia player (VLC, VideoLAN, France), the experimenter extracted 1 frame every 5 minutes of real time recording. The frame ratio may vary depending on the operating system, so we recommend checking the parameters to be reported for frame extraction. For example, using Windows and VLC media player, the videos lasted 4 minutes (corresponding to 2-hour recordings), we set 1 frame every 300 frames and obtained a total of 12 images/h. Then, the blind experimenter counted manually the number of fish in each chamber of the system to calculate the inter-rater reliability of the measure of thermal preference (total number of counted hours: 70). The reliability test showed a strong correlation between the manual scores and the output from the tracking software (Pearson's $r = 0.822$, $t68 = 11.918$, $P < 0.001$), highly supporting the use of automated tracking system for quantifying average thermal preference and distance covered across tank of the fish groups.

Data and statistical analysis

The statistical analysis for daily thermal preference and locomotor activity were performed in RStudio version 2022.02.3 Build 492 (Integrated Development for R. RStudio, PBC, Boston, MA, USA; http://www.rstudio.com/). The statistical tests were two-tailed, and significance threshold was set at P = 0.05.

The possible existence of daily and circadian periodicity was determined by means of cosinor method (reviewed in [9,10]). Briefly, the method assumed that circadian rhythms can defined as smooth rhythms defined by parameters that could be estimate by fitting a least squares model consisting of cosine curves with known periods (24 h):

$$y = M + Acos\left(\frac{2\pi t}{\tau} + \phi\right) + e(t)$$

Where M is the MESOR (Midline Estimating Statistic of Rhythm), A is the amplitude of the oscillation (a measure of the extent between the Mesor and the peak of predictable variation within a cycle), *t* is standardizing time points expressed in *Zeitgeber* (synchronizer) time (ZT)[11], ϕ is the Acrophase (a measure of the time in a cycle that the predictable variation reached the peak), τ is the period (duration of one cycle), and *e*(t) is the error term. Assuming that the τ is known (24-hours), the equation could be arithmetically estimating by least squares linear regression:

$$y \sim intercept + sin\left(\frac{2\pi t}{24}\right) + cos\left(\frac{2\pi t}{24}\right) + e(t)$$

The intercept of linear regression equals to the Mesor, i.e. the arithmetic means of all data points, while the Amplitude and the Acrophase can be calculated as following:

$$A = \sqrt{{\beta_{sin}}^2 + {\beta_{cos}}^2}$$

$$\phi = -\tan\frac{\beta_{sin}}{\beta_{cos}} \; when \;\; \beta_{sin} > 0 \; and \; \beta_{cos} \;\geq 0 \;\; or$$

$$= -\frac{\pi}{2} - \tan\frac{\beta_{sin}}{\beta_{cos}} \; when \;\; \beta_{sin} \geq 0 \; and \; \beta_{cos} < 0 \;\; or$$

$$= -\pi - \tan\frac{\beta_{sin}}{\beta_{cos}} \; when \;\; \beta_{sin} < 0 \; and \; \beta_{cos} \leq 0 \;\; or$$

$$= -\frac{3}{2}\pi - \tan\frac{\beta_{sin}}{\beta_{cos}} \; when \;\; \beta_{sin} \leq 0 \; and \; \beta_{cos} > 0 \;\; or$$

The negative sign. "-" identified the clockwise direction of vector in the circular analysis. The existence of rhythmicity within the circle, i.e. the probability that the Amplitude is significantly different from zero, can be assessed by calculating the F distribution with 2 and N-3 degrees of freedom from the predicting value of linear regression. As reported in the Table S1, each group showed a daily rhythmicity on the preferred temperature and distance covered across the multi-tank system.

	Parameters	**Mesor (r.e.)**	**Amplitude (r.e.)**	**Acrophase (ZT)**	**Rhythm detection (zero-amplitude test)**
System #1	Preferred temperature	27.64±0.41	0.61±0.45	10.03±4.98	$F_{2,85} = 9.638$ $p < 0.001$
	Distance moved	5724.42±591.96	4837.69±1158.64	6.36±0.22	$F_{2,85} = 121.861$ $p < 0.001$
System #2	Preferred temperature	27.42±0.21	0.82±0.08	8.47±4.44	$F_{2,95} = 24.433$ $p < 0.001$
	Distance moved	4131.796±1172.84	3558.22±1768.841	8.21±2.17	$F_{2,95} = 117.700$ $p < 0.001$
System #3	Preferred temperature	27.03±0.69	0.82±0.09	8.13±5.26	$F_{2,103} = 25.800$ $p < 0.001$
	Distance moved	5883.974±528.86	4387.11±551.18	6.59±1.04	$F_{2,103} = 102.433$ $p < 0.001$

Table S1. Cosinor parameters of preferred temperature and distance moved of the three system in which a group of 12 *D. rerio* maintained under LD 14:10 h photoperiod condition in the horizontal temperature gradient system during five complete days of recording. Table shows numeric values of Mesor, Amplitude, Acrophase (± standard deviation) across the 5 videorecording days and the significance of the rhythmicity (P-value) reported by the Cosinor analysis (zero-amplitude test). Mesor and Amplitude are given as relative expression values (r.e.) and Acrophase as *Zeitgeber* time (ZT).

Due to the presence of significant rhythmicity in all 3 systems, we pooled the data (i.e, average parameters from the three systems for each ZT across the five days) and performed the Cosinor analysis on the new datasets as previously described. Additionally, we performed a repeated measures analysis for evaluating differences on the thermal preference and distance covered across the ZT. We analysed each behavioural parameter via Liner Mixed Model ('*lmer*' function from '*lme4*' R packaged) fitted with ZT as fixed factor, Day as random factor to account for the repeated measures structures of data. Significance of the models' parameters was assessed via Satterhwaite's degrees of freedom ('*anova'* function from the '*lmerTest*' R packaged).

Ethical Approval

Husbandry and experimental procedures were performed in accordance with European Legislation for the Protection of Animals used for Scientific Purposes (Directive 2010/63/EU) and the Italian (D.L. 26/2014) animal protection standards. Research was also approved by the University of Ferrara Institutional Animal Care and Use Committee and the Italian Ministry of Health (auth. num. CB/01-2019). General license for fish maintenance and breeding at the University of Ferrara: 29/2023-UT. No physical invasive manipulations were performed on the fish during the

experiments and no fish showed sign of distress. At the end of the experiments, all subjects were released into stock tanks.

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Chapter 1.2

Daily rhythms of thermal preference in diurnal, nocturnal and cavefish species.

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Manuscript in preparation

Introduction

Almost all organisms, from unicellular to vertebrates, are exposed to predictable environmental fluctuations with a constant periodicity of 24 hours on our planet. To anticipate these rhythmic changes, organisms evolved an endogenous clock that confers an adaptive survival advantage, allowing them to entrain their physiological and behavioural patterns to the cyclical environmental stimuli called *Zeitgebers* ('time givers') generating biological rhythms (Menaker et al., 1997; Ouyang et al., 1998; Harmer et al., 2001; Panda et al., 2002; Bell-Pedersen et al., 2005; Junko et al., 2019). Rhythms that persist under aperiodic conditions (e.g., constant lighting conditions), occurring with a free-running periodicity close to 24 hours (tau, τ) are defined circadian rhythms (Menaker et al., 1997; Herrero et al., 2003; Junko et al., 2019).

Several cyclic environmental factors, such as light-dark, temperature cycles and food availability; rhythmic activities of prey, predators, intra- and inter-species competition can entrain circadian rhythmicities (Sharma & Chandrashekaran, 2005). Light-dark (LD) cycles are considered the main environmental factor synchronising circadian rhythms via the light-entrainable oscillator (Reppert & Weaver, 2002) affecting many biological processes and consequently directly contributing to the organisms' survival performance (Frøland Steindal &Whitmore, 2019). Besides LD cycle, temperature variations are a powerful *Zeitgeber* to entrain circadian clocks in plants and ectotherm animals even in absence of photoperiod (Rensing & Ruoff, 2002; Lahiri et al., 2005; Sharma & Chandrashekaran, 2005). Daily cycles of light and temperature are directly linked in the wild: warmer temperatures during the day (thermophase) and cooler temperatures during the night (cryophase) (Sánchez-Vázquez & López-Olmeda, 2018). In this way, temperature influences biological rhythms through daily thermocycles

affecting mainly ectotherm's behavioural and physiological patterns (López-Olmeda & Sánchez-Vázquez, 2009; Villamizar et al., 2012; Vera et al., 2023).

In the aquatic environment, temperature changes have important effects on various biological processes, such as reproduction, growth and metabolic rates, spatial distribution of species, locomotor and feeding behaviour (Volkoff & Rønnestad, 2020; Gomez-Maldonado & Camacho-Cervantes, 2022). While temperatures below critical values limit cellular metabolism and nervous system function, high temperatures can cause protein instability and cell death (Haesemeyer, 2020). Mobile aquatic ectotherms, like fish, therefore evolved strategies to regulate their body temperature, which is strictly correlated with that of the water they occupy (Angilletta et al., 2002). Indeed, they can actively choose a thermal environment that favours their performances by implementing the behavioural thermoregulation (Reynolds & Casterlin, 1979; Christensen et al., 2021). So, in a challenging ecosystem as the aquatic one, temperature entrainment during daily and seasonal changes is an important adaptation in order to avoid stress and to increase the fitness (Schaefer & Ryan, 2006). However, under artificial breeding conditions (e.g., fish farms), the natural environmental fluctuations are rarely considered, and the fish are reared under conditions of constant temperature, standardised feeding protocols and artificial photoperiods (Hui et al., 2019; de Alba et al., 2024).

The species studied in this investigation are: the diurnal largemouth bass (*Micropterus salmoides*), the nocturnal tench (*Tinca tinca*), the nocturnal black bullhead catfish (*Ameiurus melas*), the Mexican (*Astyanax mexicanus*) and Somalian (*Phreatichthys andruzzii*) blind cavefish.

The diurnal, *M. salmoides* has always been an important species in recreational fisheries in North America (Cooke & Philipp, 2009) and it has

become one of the most economically important aquaculture species in China and water temperature plays a key role in the spawning season of this species (Hussein et al., 2020).

Tench is a cyprinid species sold on the European market as food, for recreational fishing and for ornamental purposes (Arlinghaus et al., 2003). This fish shows a nocturnal behaviour (Herrero et al., 2003) and it has been observed a deficit in growth and quality under controlled rearing conditions (i.e., feeding protocols, diets and constant temperature; Wolnicki & Myszkowski, 1998; Kamiński et al., 2017; Wolnicki et al., 2017).

The black bullhead *A. melas* is a native to North America species and it was first introduced in Europe around the late 1800s for aquaculture purposes (Holčík, 1991). This catfish shows a nocturnal behaviour (Declerck et al., 2002; Preiszner et al., 2020; Jaćimović et al., 2021) and, in the wild, is tolerant to water pollution, turbidity, low oxygen concentration, elevated temperatures and a range of pH values (Novomeská et al., 2013). However, in rearing conditions, the water quality becomes important to guarantee the fish productivity and water temperature is essential to avoid virus spreading (Roncarati et al., 2014) and for establishing fecundity parameters and spawning season (Novomeská & Kováč, 2009).

The blind Mexican cavefish *A. mexicanus* and the blind Somalian cavefish *P. andruzzii* represent two important model systems for the investigation of adaptive and regressive evolutionary changes to life underground (Beale et al., 2013; Pavlova & Krylov, 2023). Indeed, both are cave-dwelling vertebrates that inhabit arrhythmic environments (i.e., constant darkness, stable temperature and limited food sources) that exhibit extreme troglomorphic traits such as loss of functional eyes, complete depigmentation and reduced metabolic rate (Berti et al., 2001; Borowsky, 2008; Tarttelin et al., 2012). *A. mexicanus* exhibits daily activity rhythms in

laboratories, with a diurnal pattern, suggesting that it still possess a light-entrainable clock (Beale et al., 2013; Simon et al., 2019; Conti et al., 2024 in Experimental Chapter 2.2); while *P. andruzzii* exhibits a significant food-entrainable rhythm (Cavallari et al., 2011). A previous study showed that different populations of *A. mexicanus* displayed differences in temperature preference and strength of preference (Tabin et al., 2018) meaning that temperature could be an important environmental stimulus also for that animals which have evolved under constant conditions. In addition, a reduced temperature compensation in the Somalian cavefish clock was highlighted (Cavallari et al., 2011) suggesting a possible susceptibility to temperature variations in this species.

We are already aware of the fact that animals are commonly reared at constant temperature conditions, whilst recent studies pointed several advantages of the thermoregulation in captive breeding (i.e., decreased cortisol levels, reduced aggressive behaviours, decrease in mortality, oxidative stress and DNA damage) (Sanhueza et al., 2018; Huntingford et al., 2020; Sanhueza et al., 2023). Thus, the aim of the present study was to investigate the daily thermal preference of commercially interesting diurnal and nocturnal fish (*M. salmoides*, *T. tinca* and *A. melas*) and if this preference is driven by and endogenous clock. In addition, we also wanted to evaluate the possible daily thermal preference in two blind cavefish (*A. mexicanus* and *P. andruzzii*) pointing out temperature variations as a feasible *Zeitgeber* for these species. The resulting behavioural and physiological parameters could be used for improving husbandry protocols to refine breeding conditions and to deepen knowledge about the coupled light and temperature-entrainable oscillators in fish (López-Olmeda et al & Sánchez-Vázquez, 2009).

Materials and Methods

Ethic Statement

Part of the present research (experiments on tench and Mexican blind cavefish) was conducted in the Department of Physiology facilities of the University of Murcia (Spain) while the other part the investigation (experiments on largemouth bass, black bullhead catfish and Somalian cavefish) was conducted in the Department of Life Sciences and Biotechnology facility of the University of Ferrara (Italy). Husbandry and experimental procedures were performed in accordance with European Legislation for the Protection of Animals used for Scientific Purposes (Directive 2010/63/EU), Spanish (RD 53/2013 and Law 32/2007) and Italian (D.L. 26/2014) animal protection standards. Research was also approved by the Committee of the University of Murcia on Ethics and Animal Welfare (A13220605), by the University of Ferrara Institutional Animal Care and Use Committee and the Italian Ministry of Health (auth. num. CB/01-2019). No physical invasive manipulations were performed on the fish during the experiments and no fish showed sign of distress. At the end of the experiments, all subjects were released into stock tanks.

Animals Housing

For the present study, Mexican blind cavefish (*A. mexicanus*) and tench (*T. tinca*) juveniles of both sexes were obtained from a local commercial supplier (Alimar Pets, S.L., Murcia, Spain) and from a Spanish fish farm (Tencas Atanasio, Badajoz, Spain) respectively. Fish were acclimated in a 54-liter glass tank divided into six compartments (9 L each; 10-11 animals/tank) placed inside the animal facility of the University of Murcia. The tank contained dechlorinated fresh water in recirculation, which was constantly filtered by biological and mechanical filters. Animals were

maintained under controlled lighting and constant temperature conditions (24 ± 0.5 °C for Astyanax and 22 ± 0.5 °C for tench). LED strips (SOLBRIGHT®, LED Flex Strip 1043-W, Rayte, S.L., Murcia, Spain) provided light, with a photoperiod set at 12h light:12h dark (LD 12:12) cycle. In chronobiology, the time of light onset is defined as *Zeitgeber* Time 0 h (ZT0), to standardize time points regardless local time (Guerra-Santos et al., 2017; Santo et al., 2020). Therefore, light onset at 09:00 h corresponds to ZT0 and light offset at 21:00 h corresponds to ZT12. *A. mexicanus* were fed randomly once per day (Interval function = 12 + Random*24) with freeze-dried *Artemia* (Prodac International, Padova, Italy) and tench were randomly fed once a day during the night phase (Interval function = 12 + Random*12; Gemma Wean, Skretting®) by means of automatic feeders (Eheim GmbH & Co. KG, model 3581, Deizisau, Germany).

Juveniles of largemouth bass *M. salmoides* and black bullhead catfish *A. melas* of both sexes were supplied by a local fish farm pond (Soc. Agr. I persici Srl., Finale Emilia, Italy) and kept in 50 L tanks (13-14 fish/tank) at the University of Ferrara facility. The dechlorinated fresh water was constantly filtrated and oxygenated using mechanical filters and air pumps. Animals were kept under controlled lighting and temperature conditions (22 ± 0.5 °C). The natural photoperiod of LD 14:10 cycle (ZT0 = 06:00 h; ZT14 = 20:00 h) was provided by means of LED strips placed above each tank (TMR, Elcart, Milano, Italy). Largemouth bass were randomly fed once per day (Interval function = 12 + Random*24) and catfish were randomly fed once per day during the night phase (Interval function = 14 + Random*10; Caviar, BernAqua NV, Olen, Belgium) by means of automatic feeders (Eheim GmbH & Co. KG, model 3581, Deizisau, Germany).

Somalian cavefish (*P. andruzzii*) were originally collected around the locality of Bud-Bud (04°11’19”N–46°28’27”E, central Somalia) during

several expeditions (1968-1982) and then reproduced in the laboratory using standard methods (Tarttelin et al., 2012; Sokolowska et al., 1984). Adult *P. andruzzii* born in 2012 from the colony maintained at the University of Ferrara facility, were used for the present study. Fish were kept in 160 L tanks dechlorinated fresh water constantly filtered and oxygenate by means of mechanical, biological filter, aerators and at constant temperature of 29 ± 1°C. They were maintained in total darkness and fed twice per week with frozen *Chironomidae* larvae (Amtra Pro Nature, Germany).

Experimental protocol

The entire experiment was carried out at the University of Murcia (testing *T. tinca* and *A. mexicanus*) and at the University of Ferrara (testing *M. salmoides*, *A. melas* and *P. andruzzii*) using two slightly different experimental setups (Vera et al., 2023; de Alba et al., 2024; Conti et al., 2024 described in Experimental Chapter 1.1).

Thermal preference of the interested species was investigated by means of a custom-multichambered tank built in triplicate (Rey et al., 2015; Vera et al., 2023; de Alba et al., 2024; Conti et al., 2024 described in Experimental Chapter 1.1). The sample size was chosen according to the UE recommendations to guarantee animal welfare (2010/63/UE), establishing a maximum density of 1 fish/L. In the present study fish density in each chamber was kept below the threshold to avoid stress and negative effects on fish behaviour (a maximum of 12 fish in 18 L). Specifically, we used three independent groups of each species: Mexican blind cavefish (n= 10/group), tench (n= 12/group), largemouth bass (n= 12/group), catfish (n= 8/group) and Somalian cavefish (n= 8/group) according to the size of the subjects. A continuous and controlled thermal gradient was designed and create according to the biology of these species (from 20 °C to 28 °C for blind *A. mexicanus*, from 18 °C to 26 °C for *T. tinca*, *M. salmoides*, *A. melas* and

from 24 °C to 32 °C for *P. andruzzii*). In particular, the temperature of the central chamber of the tank corresponds to the rearing temperature.

In order to evaluate the daily thermal preference, across the 24-hours, and the presence of a possible circadian rhythmicity driving this preference, fish behaviour was long-term recorded for approximately one full month (27 days). During this experimental time, the animals were subjected to a LD cycle for 10 days to determine the daily rhythm of thermal preference. This cycle was subsequently inverted (DL cycle) for another 10 days to assess the resynchronizing effect of light on the rhythm of thermal preference. Finally, to evaluate the endogenous character of this possible rhythm, fish were kept in constant darkness (DD) for the last 7 days. The photoperiod was provided by LED strips placed 15 cm above each tank and connected to electronic timers. As for *P. andruzzii*, the LEDs were covered with tin foil except for one LED per chamber as this species exhibits a photophobic response to light (Ercolini & Berti, 1975). During the LD and DL cycle phases (20 days) fish were fed: once per day in blind *A. mexicanus*, once per day during the light phase for the diurnal largemouth bass, once per day during the night phase for the nocturnal tench and *A. melas* and finally twice per week in *P. andruzzii*. Food was randomly administrated (using interval function for every species already described in Animals housing section) to avoid feeding entrainment (Boujard & Leatherland, 1992) and was provided using automatic feeders, placed above each chamber, at a feeding rate of 2% of the total biomass with the specific rearing diets. In addition, during the DD phase, fish were fasted in order to remove any possible synchronising stimulus of the clock (Menaker et al., 1997; Herrero et al., 2003).

Therefore, three independent replicated groups of the different investigated species (largemouth bass: 2.34 ± 0.28 g; black bullhead catfish: 6.57 ± 0.36 g; tench: 6.29 ± 0.69 g; Mexican blind cavefish: 0.71 ± 0.16 g

and Somalian cavefish: 2.67 ± 0.64 g) were placed in the central chamber of each gradient tank and immediately videorecorded.

Video recording and Video analysis

Video recordings were carried out using a webcam (Logitech Webcam C300–1.3MP, Switzerland) at the University of Murcia and three different camera modules connected each to a Raspberry Pi (Raspberry Pi 4 model B, Raspberry Pi, UK) at the University of Ferrara. Videorecording was performed by Multiviewer software (Computer System Department, University of Murcia, Spain; Vera et al., 2023; de Alba et al., 2024) at the University of Murcia and a custom written Python script (Conti et al., 2024 described in Experimental Chapter 1.1) at the University of Ferrara. Video recordings started on the first day at Zeitgeber time ZT0 for all the experimental phases and the videos were recorded at one frame per second. To allow video recording in the dark, five infrared LED lights (BW® 48 LED Infrared Illuminator; University of Murcia; de Alba et al., 2024) and an infrared lamp (141.5 ×18 × 4 cm; University of Ferrara; Conti et al., 2024 described in Experimental Chapter 1.1) were placed on the back of each multi-chamber tank. To disperse infrared light and to maximise image clarity at night, a translucent acrylic white sheet (Falken Design WT2447-1-8/2436 Acrylic White Sheet, Translucent 55%; de Alba et al., 2024; Conti et al., 2024 described in Experimental Chapter 1.1) was installed on the back wall of the tank.

All the behavioural video recordings performed at the University of Murcia were analysed with the Fish Counter software (Dr. Ginés García Mateos, University of Murcia, Spain, Version 3.0; Vera et al., 2023; de Alba et al., 2023) that counts the number of fish per chamber in the gradient tank every minute. All the videos collected at the University of Ferrara were analysed by using the EthoVision XT tracking software (Noldus, The

Netherlands) obtaining the cumulative time spent by fish in each chamber of all experimental tanks and the average quantification of locomotor activity of fish in the group (Conti et al., 2024 described in Experimental Chapter 1.1). All the results were recorded Microsoft Excel spreadsheets.

Statistical analysis

The mean temperature chosen at each time of the day (ZT) in all the experimental replicates was calculated using two formulas depending on the data calculated (de Alba et al., 2024 for tench and Mexican cavefish; Conti et al., 2024 described in Experimental Chapter 1.1 for largemouth bass, black bullhead catfish and Somalian cavefish). Daily distance covered was calculated as already described in in Experimental Chapter 1.1 for the species tested at the University of Ferrara.

The existence of circadian rhythmicity of thermal preference and locomotor activity was tested by the Cosinor analysis to determine whether daily changes in thermal preference and distance covered fitted the cosine function (Marler et al., 2006). The chronobiology software "El Temps" (v.1.313, Prof. Díez Noguera, University of Barcelona, Spain) was used to determine the length (tau) of the circadian rhythm in the absence of environmental synchronizers (DD and fasting) by a Lomb-Scargle periodgrams analysis and to plot the actograms and thermograms. ANOVA repeated measures were performed using RStudio software (version 2022.02.3 Build 492; Integrated Development for R. RStudio, PBC, Boston, MA, USA; http://www.rstudio.com/) to study the effect of ZT on thermal preference and distance covered. For all the tests, the significant threshold was $p < 0.05$.

Results

During the first 10 days of LD cycle, all species display significative daily rhythms of thermal preference (Table 1) and locomotor activity (Table 2), in those assessed. Specifically, the largemouth bass show a significative diurnal locomotor activity (diurnal acrophase at ZT 6.20; ANOVA $F_{1,638}$= 375.051 p <0.001; Cosinor p <0.001; Figures 1B and 6A; Table 2) and they significantly prefer lower temperatures during the light phase and higher temperatures during the dark phase (nocturnal acrophase at ZT 19.25; ANOVA $F_{1,636}$= 73.081 p <0.001; Cosinor p <0.001; Figures 1A and 6B; Table 1). The black bullhead catfish confirm a significant nocturnal activity pattern (nocturnal acrophase at ZT 17.50; ANOVA $F_{1,610}$= 259.373 p <0.001; Cosinor p <0.001; Figures 2B and 7A; Table 2) and they significantly select higher temperatures during the day and lower temperatures during the night (diurnal acrophase at ZT 7.20; ANOVA $F_{1,610}$= 79.415 p <0.001; Cosinor p <0.001; Figures 2A and 7B; Table 1), which is the same daily thermal preference pattern of the nocturnal tench (diurnal acrophase at ZT 5.47; ANOVA $F_{1,707}$= 306.245 p <0.001; Cosinor p <0.001; Figures 3A and 8A; Table 1). Regarding the blind cavefish species investigated, they both display a significant preference for warmer temperatures during the light phase and cooler temperatures during the dark phase (*A. mexicanus*: diurnal acrophase at ZT 5.12; ANOVA $F_{1,707}$= 93.687 p <0.001, Cosinor p <0.001, Figures 4A and 9A; *P. andruzzii*: diurnal acrophase at ZT 5.45; ANOVA $F_{1,310}$= 47.962 p <0.001; Cosinor p <0.001, Figures 5A; Table 1). In addition, *P. andruzzii* exhibit a significant diurnal activity pattern (diurnal acrophase at ZT 6.19; ANOVA $F_{1,310}$= 21.900 p <0.001; Cosinor p <0.05, Figure 5B; Table 2).

When the LD cycle is reversed (DL), all the species keep displaying both behavioural rhythms of thermal preference, except the Somalian cavefish *P. andruzzii*, (largemouth bass: ANOVA $F_{1,656}$= 288.851 p <0.001,

Cosinor p <0.001, Figures 1C and 6B; black bullhead catfish: ANOVA $F_{1,647}$= 937.499 p <0.001, Cosinor p <0.001, Figures 2C and 7B; tench: ANOVA $F_{1,707}$= 109.800 p <0.001, Cosinor p <0.001, Figures 3B and 8A; *A. mexicanus*: ANOVA $F_{1,707}$= 182.344 p <0.001, Cosinor p <0.001, Figures 4B and 9A; Table 1) and locomotor activity (largemouth bass: ANOVA $F_{1,656}$=141.211 p <0.001, Cosinor p <0.001, Figures 1D and 6A; black bullhead catfish: ANOVA $F_{1,647}$= 763.327 p <0.001, Cosinor p <0.001, Figures 2D and 7A; *P. andruzzii*: ANOVA $F_{1,310}$= 27.913 p <0.001, Cosinor p <0.001, Figure 5D; Table 2) confirming the almost all the rhythms entrainment to the new cycle.

During the las 7 days in constant conditions (DD and fasting) not all the species tested maintained the exhibition of the daily behavioural rhythms displayed during the previous phases. Specifically, the largemouth bass, the black bullhead catfish and tench keep displaying both significant daily thermal preference (largemouth bass: ANOVA $F_{1,438}$= 0.220 p =0.639, Cosinor p <0.05, Figures 1E and 6B; black bullhead catfish: ANOVA $F_{1,458}$= 11.479 p <0.001, Cosinor p <0.001, Figures 2E and 7B; tench: ANOVA $F_{1,494}$= 16.155 p <0.001, Cosinor p <0.001, Figures 3C and 8A; Table 1) and activity pattern (largemouth bass: ANOVA $F_{1,438}$= 5.906 p =0.015, Cosinor p <0.001, Figures 1F and 6A, black bullhead catfish: ANOVA $F_{1,457}$= 73.716 p <0.001, Cosinor p <0.001, Figures 2F and 7A; Table 2) rhythms. Moreover, the circadian nature of both behavioural rhythms is confirmed by the LS analysis (thermal preference: largemouth bass tau= 25.2 h in Figure 6D, black bullhead catfish tau= 24 h in Figure 7D and tench tau= 22.5 h in Figure 8B; distanced moved: largemouth bass tau= 24.2 h in Figure 6C and black bullhead catfish tau= 23.5 h in Figure 7C). On the other hand, both cavefish species become arrhythmic under these conditions, losing the entrainment to the previous phases (Figures 4C, 9A-B and 5E-F; Tables 1 and 2).

Species	Photoperiod	Mesor (r.e.)	Amplitude (r.e.)	Achrophase (ZT)	Significance (p-value)
Micropterus salmoides	LD 14:10	24.34	0.60	19.25	***
	DL 10:14	23.69	0.51	4.51	***
	DD	24.14	0.21	0.49	*
Ameiurus melas	LD 14:10	24.65	0.55	7.20	***
	DL 10:14	24.98	0.88	16.25	***
	DD	24.27	0.32	14.39	***
Tinca tinca	LD 12:12	23.78	0.80	5.47	***
	DL 12:12	23.47	0.43	16.45	***
	DD	23.27	0.18	17.22	***
Astyanax mexicanus	LD 12:12	23.96	0.18	5.12	***
	DL 12:12	24.22	0.36	17.33	***
	DD	-	-	-	n.s.
Phreatichthys andruzzii	LD 12:12	30.44	0.48	5.45	***
	DL 12:12	-	-	-	n.s.
	DD	-	-	-	n.s.

Table 1. Cosinor parameters of daily thermal preference of all the species tested. Table shows thermal preference numeric values of mesor, amplitude, acrophase and significance of the rhythms (p-value) reported by the cosinor analysis. Mesor and amplitude are given as relative expression values (r.e.) and acrophase as *Zeitgeber* time (ZT). Asterisks indicate statistically significant rhythms: *p <0.05, **p <0.01, ***p <0.001 and non-significant rhythms are indicated as n.s.

Species	Photoperiod	Mesor (r.e.)	Amplitude (r.e.)	Achrophase (ZT)	Significance (p-value)
Micropterus salmoides	LD 14:10	106.28	97.51	6.20	***
	DL 10:14	125.56	93.72	15.15	***
	DD	60.58	13.92	14.52	***
Ameiurus melas	LD 14:10	3014.87	1907.81	17.50	***
	DL 10:14	3487.27	2620.61	3.38	***
	DD	2949.80	1173.17	3.06	***
Phreatichthys andruzzii	LD 12:12	422.55	52.20	6.19	*
	DL 12:12	355.50	58.29	16.20	***
	DD	-	-	-	n.s.

Table 2. Cosinor parameters of daily amount of distance moved of the species tested for this parameter. Table shows distance covered numeric values of mesor, amplitude, acrophase and significance of the rhythms (p-value) reported by the cosinor analysis. Mesor and amplitude are given as relative expression values (r.e.) and acrophase as *Zeitgeber* time (ZT). Asterisks indicate statistically significant rhythms: *p <0.05, **p <0.01, ***p <0.001 and non-significant rhythms are indicated as n.s.

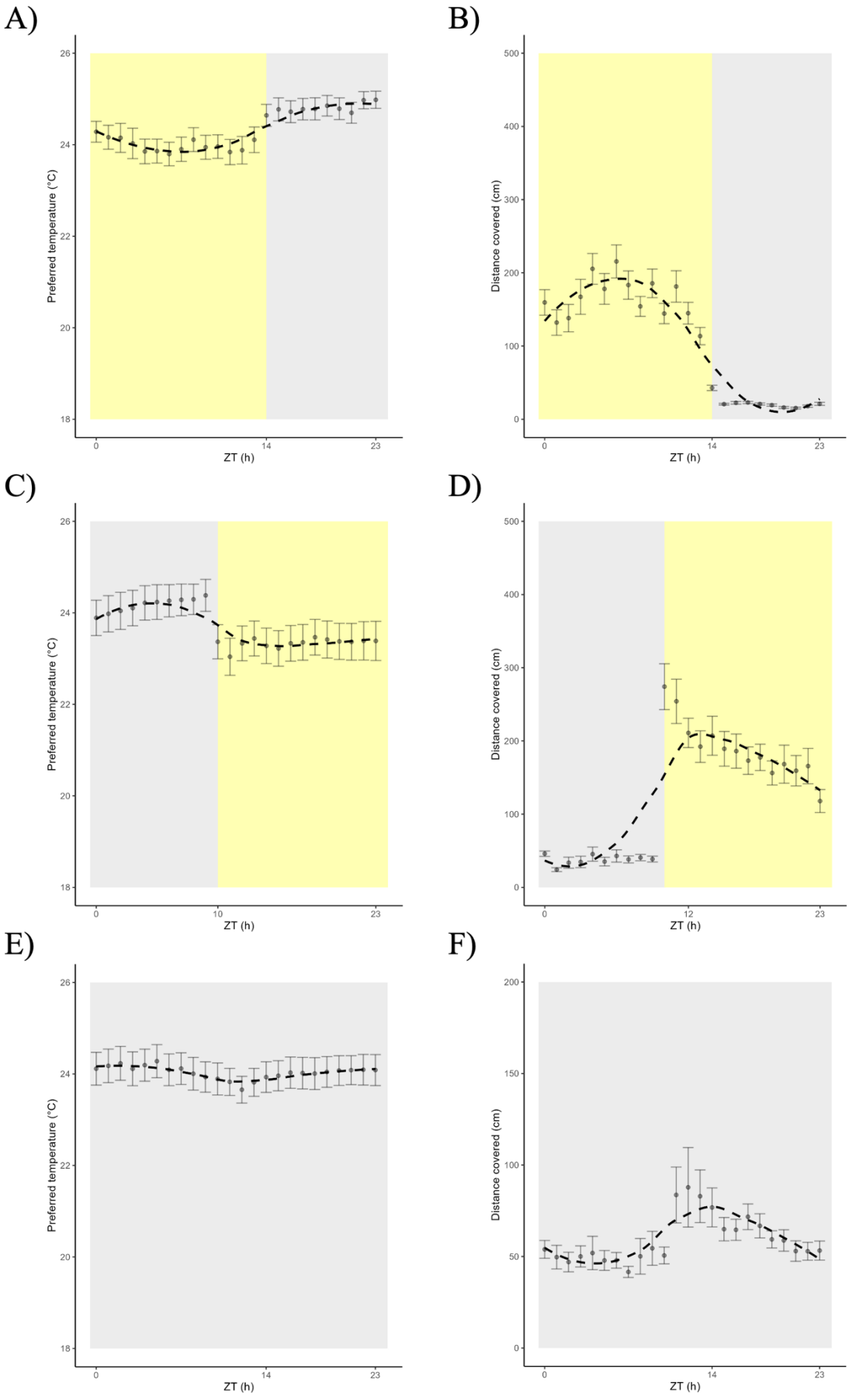
A)
B)
C)
D)
E)
F)
Preferred temperature (°C)
Distance covered (cm)
ZT (h)

Figure 1. Daily rhythms of thermal preference and distance moved in the diurnal largemouth bass *Micropterus salmoides* exposed to a temperature gradient in a 14h light:10h dark cycle for 10 days (LD, **A-B**); reversed LD cycle for 10 days (DL, **C-D**); and constant darkness condition and fasting for 7 days (DD, **E-F**). Data from all three independent experimental replicated groups (n =12 fish/group) were pooled together and are shown as mean temperature (°C) ± SD for preferred temperature and mean distance covered (cm) ± SD for locomotor activity. The dashed line represents the adjustment to sinusoidal rhythm (Cosinor, $p < 0.05$). The time scale (x-axis) is expressed as Zeitgeber Time (ZT).

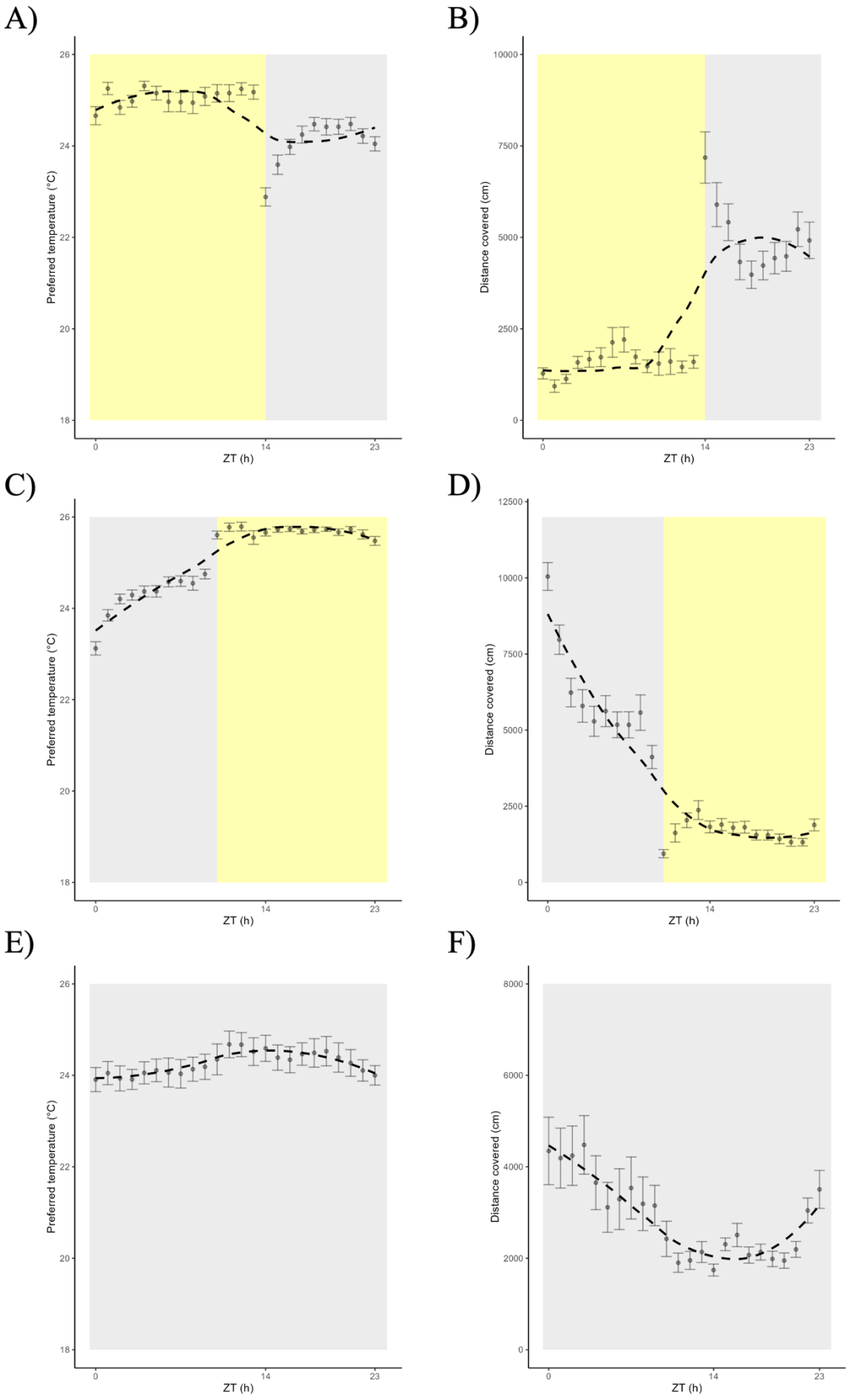
A)
B)
C)
D)
E)
F)
Preferred temperature (°C)
Distance covered (cm)
ZT (h)
0
14
23
18
20
22
24
26
2500
5000
7500
10000
12500
4000
6000
8000

Figure 2. Daily rhythms of thermal preference and distance moved in the nocturnal black bullhead catfish *Ameiurus melas* exposed to a temperature gradient in a 14h light:10h dark cycle for 10 days (LD, **A-B**); reversed LD cycle for 10 days (DL, **C-D**); and constant darkness condition and fasting for 7 days (DD, **E-F**). Data from all three independent experimental replicated groups (n =8 fish/group) were pooled together and are shown as mean temperature (°C) ± SD for preferred temperature and mean distance covered (cm) ± SD for locomotor activity. The dashed line represents the adjustment to sinusoidal rhythm (Cosinor, $p < 0.05$). The time scale (x-axis) is expressed as Zeitgeber Time (ZT).

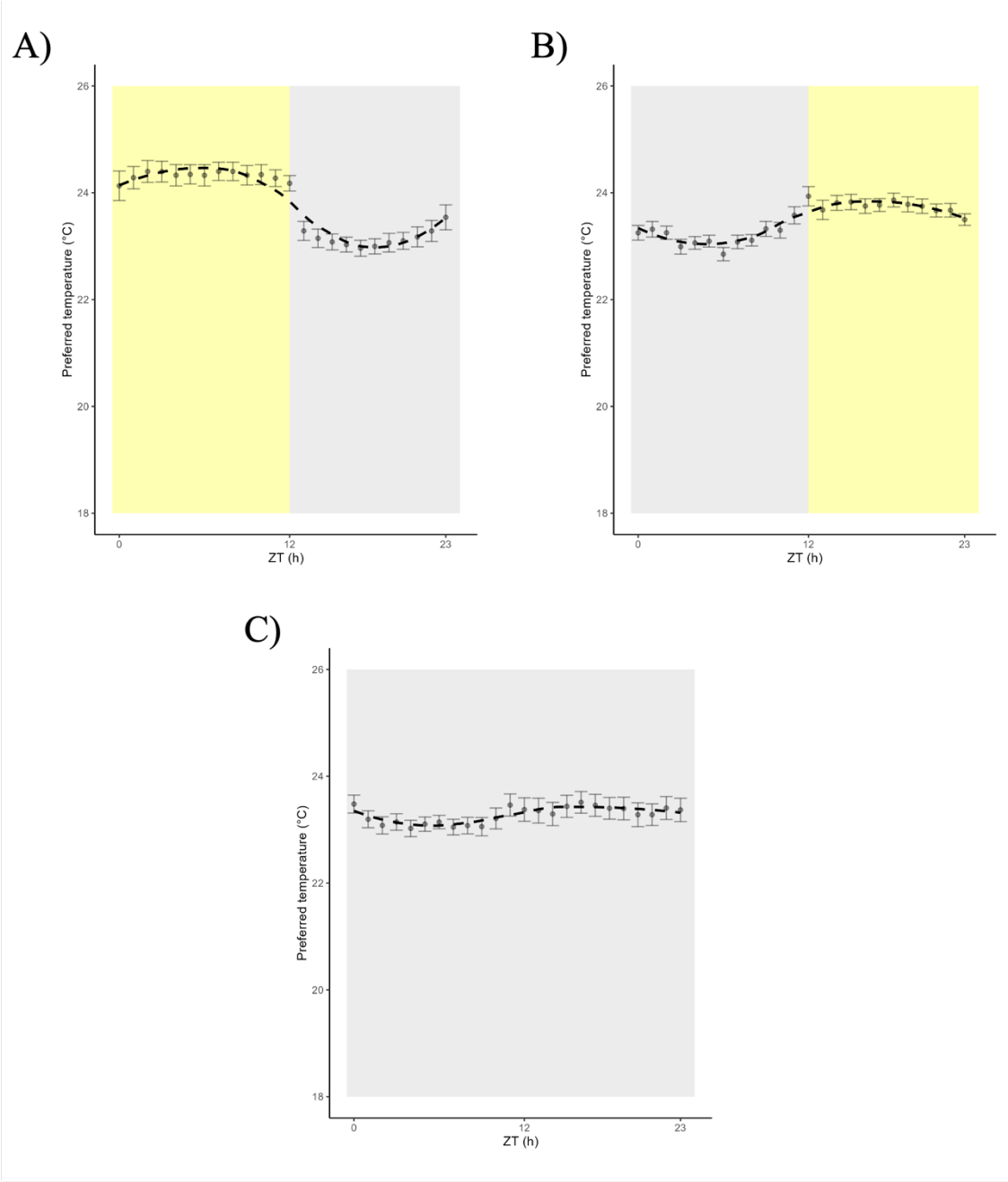

Figure 3. Daily rhythms of thermal preference in the nocturnal tench *Tinca tinca* exposed to a temperature gradient in a 12h light:12h dark cycle for 10 days (LD, **A**); reversed LD cycle for 10 days (DL, **B**); and constant darkness condition and fasting for 7 days (DD, **C**). Data from all three independent experimental replicated groups (n =12 fish/group) were pooled together and are shown as mean temperature (°C) ± SD for preferred temperature and mean distance covered (cm) ± SD for locomotor activity. The dashed line represents the adjustment to sinusoidal rhythm (Cosinor, $p < 0.05$). The time scale (x-axis) is expressed as Zeitgeber Time (ZT).

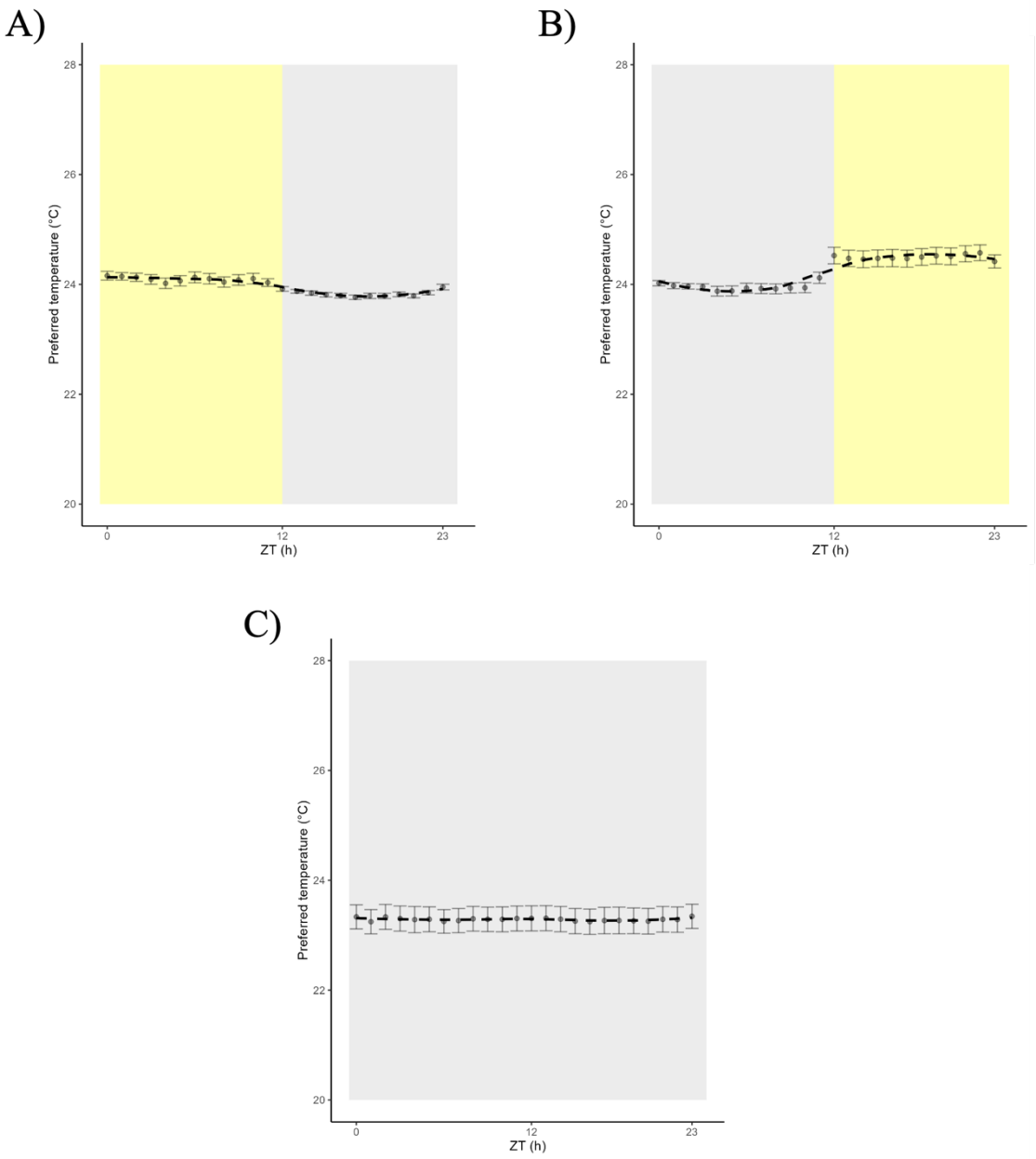

Figure 4. Daily rhythms of thermal preference in the Mexican blind cavefish *Astyanax mexicanus* exposed to a temperature gradient in a 12h light:12h dark cycle for 10 days (LD, **A**); reversed LD cycle for 10 days (DL, **B**); and constant darkness condition and fasting for 7 days (DD, **C**). Data from all three independent experimental replicated groups (n =10 fish/group) were pooled together and are shown as mean temperature (°C) ± SD for preferred temperature and mean distance covered (cm) ± SD for locomotor activity. The dashed line represents the adjustment to sinusoidal rhythm (Cosinor, $p < 0.05$). The time scale (x-axis) is expressed as Zeitgeber Time (ZT).

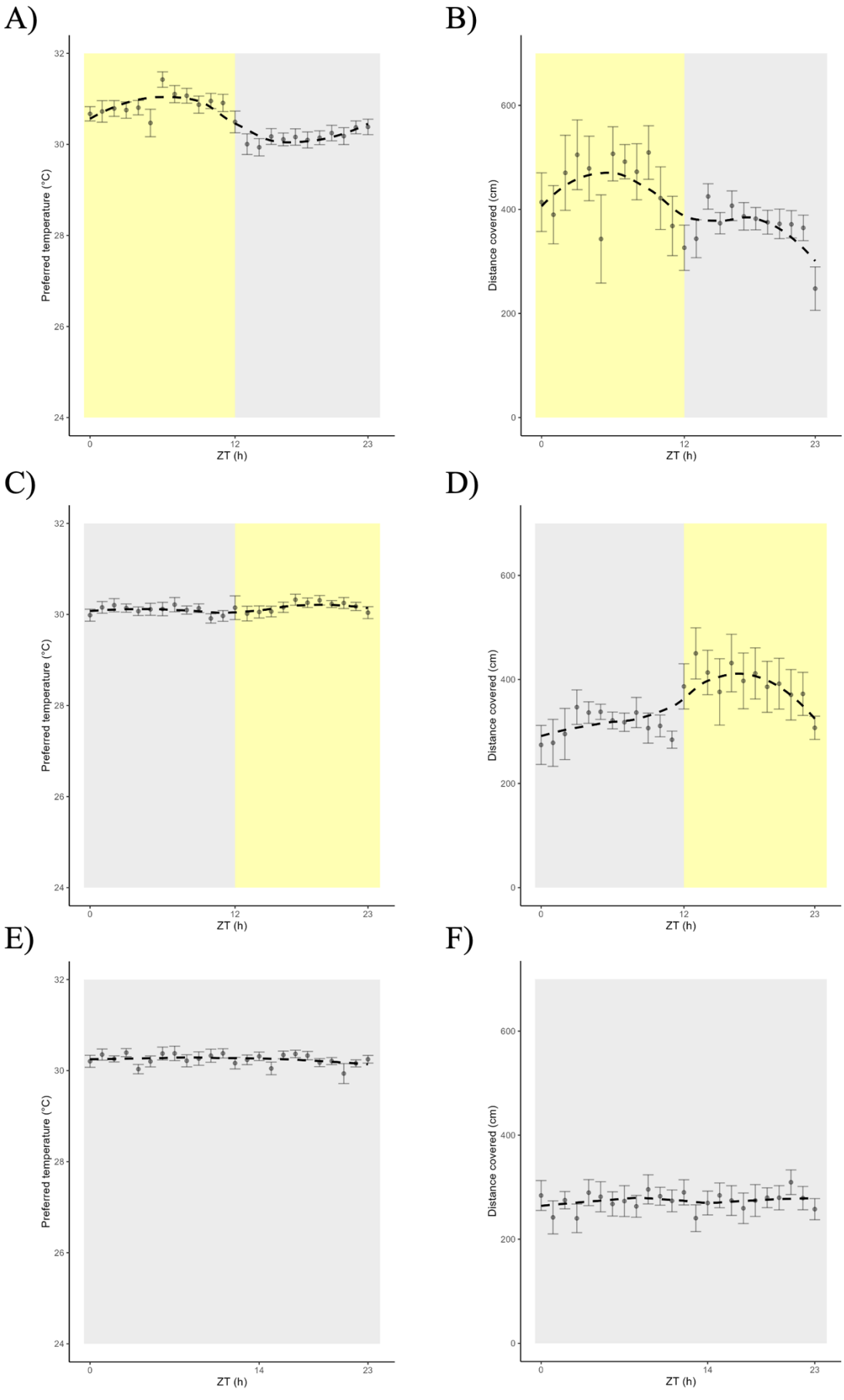
A)
B)
C)
D)
E)
F)
Preferred temperature (°C)
Distance covered (cm)
ZT (h)
0
12
14
23
24
26
28
30
32
200
400
600

Figure 5. Daily rhythms of thermal preference and distance moved in the Somalian cavefish *Phreatichthys andruzzii* exposed to a temperature gradient in a 12h light:12h dark cycle for 10 days (LD, **A-B**); reversed LD cycle for 10 days (DL, **C-D**); and constant darkness condition and fasting for 7 days (DD, **E-F**). Data from all three independent experimental replicated groups (n =8 fish/group) were pooled together and are shown as mean temperature (°C) ± SD for preferred temperature and mean distance covered (cm) ± SD for locomotor activity. The dashed line represents the adjustment to sinusoidal rhythm (Cosinor, $p < 0.05$). The time scale (x-axis) is expressed as Zeitgeber Time (ZT).

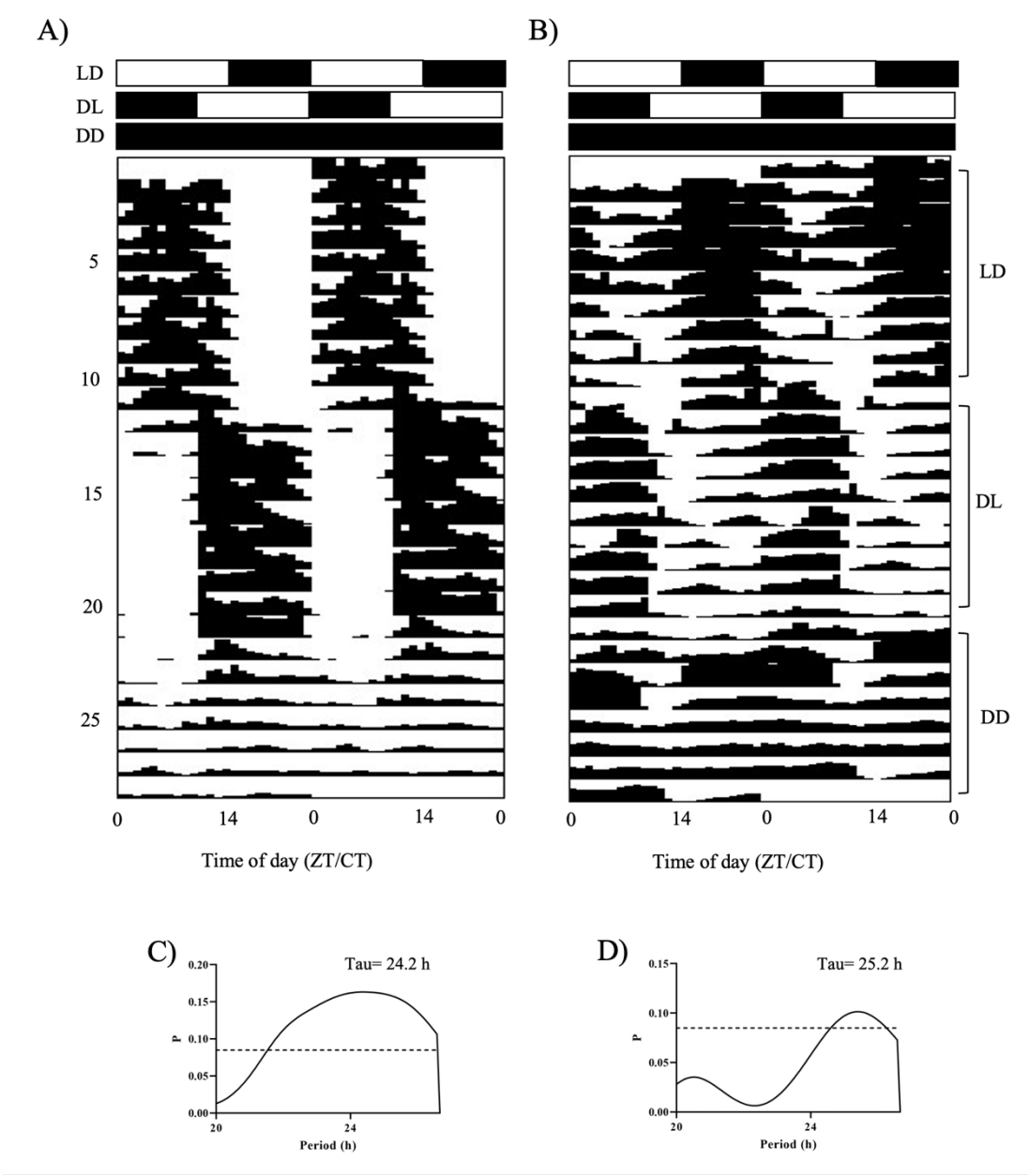

Figure 6. Double plot (48 h) actogram representing the amount of daily locomotor activity (**A**) and thermogram representing the daily thermal preference (**B**) of the mean of the three replicates in the largemouth bass *Micropterus salmoides* (n= 12 fish/replicate) throughout the duration of the experiment (27 days, y- axis). The Lomb-Scargle periodgrams indicates the length of the rhythm (tau) during the 7 days in DD condition for both distance moved (**C**) and thermal preference (**D**) parameters. Significant tau (in hours) is indicated at the top of each plot. The horizontal line represents the threshold of significance, set at p =0.05.

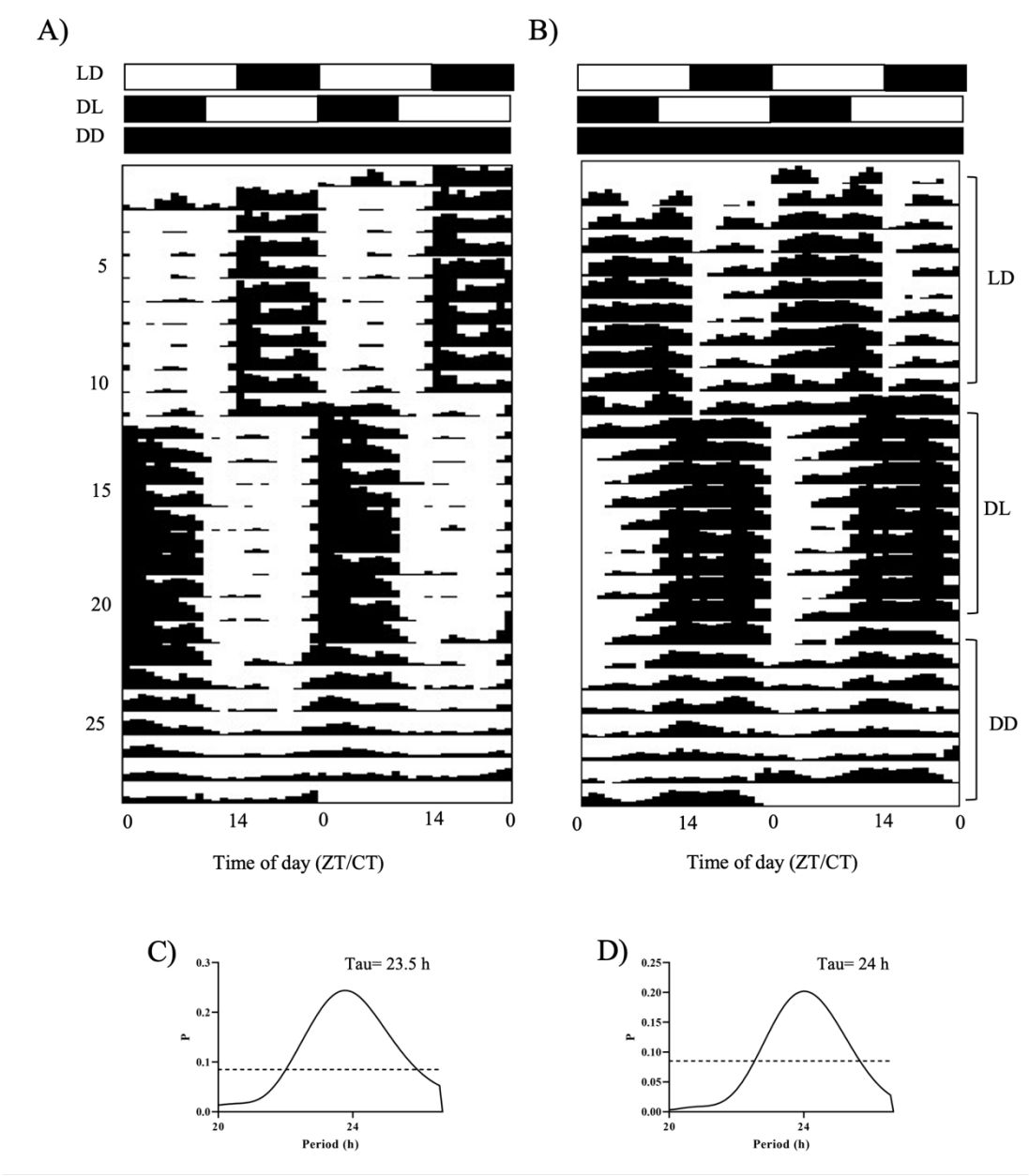

Figure 7. Double plot (48 h) actogram representing the amount of daily locomotor activity (**A**) and thermogram representing the daily thermal preference (**B**) of the mean of the three replicates in the black bullhead catfish *Ameiurus melas* (n= 8 fish/replicate) throughout the duration of the experiment (27 days, y- axis). The Lomb-Scargle periodgrams indicates the length of the rhythm (tau) during the 7 days in DD condition for both distance moved (**C**) and thermal preference (**D**) parameters. Significant tau (in hours) is indicated at the top of each plot. The horizontal line represents the threshold of significance, set at p =0.05.

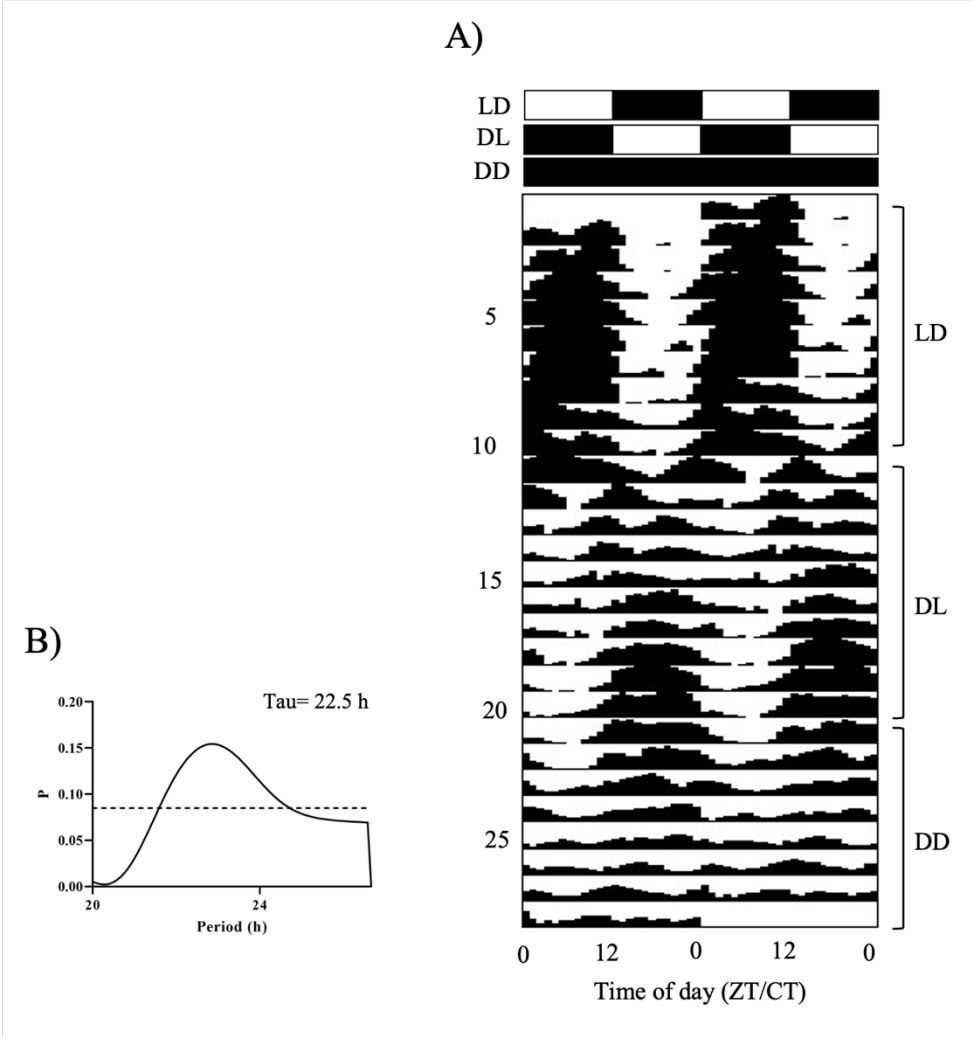

Figure 8. A) Double plot (48 h) thermogram representing the daily thermal preference of the mean of the three replicates in the tench *Tinca tinca* (n= 12 fish/replicate) throughout the duration of the experiment (27 days, y- axis). The Lomb-Scargle periodgrams indicates the length of the rhythm (tau) during the 7 days in DD condition for thermal preference (**B**) parameter. Significant tau (in hours) is indicated at the top of each plot. The horizontal line represents the threshold of significance, set at p =0.05.

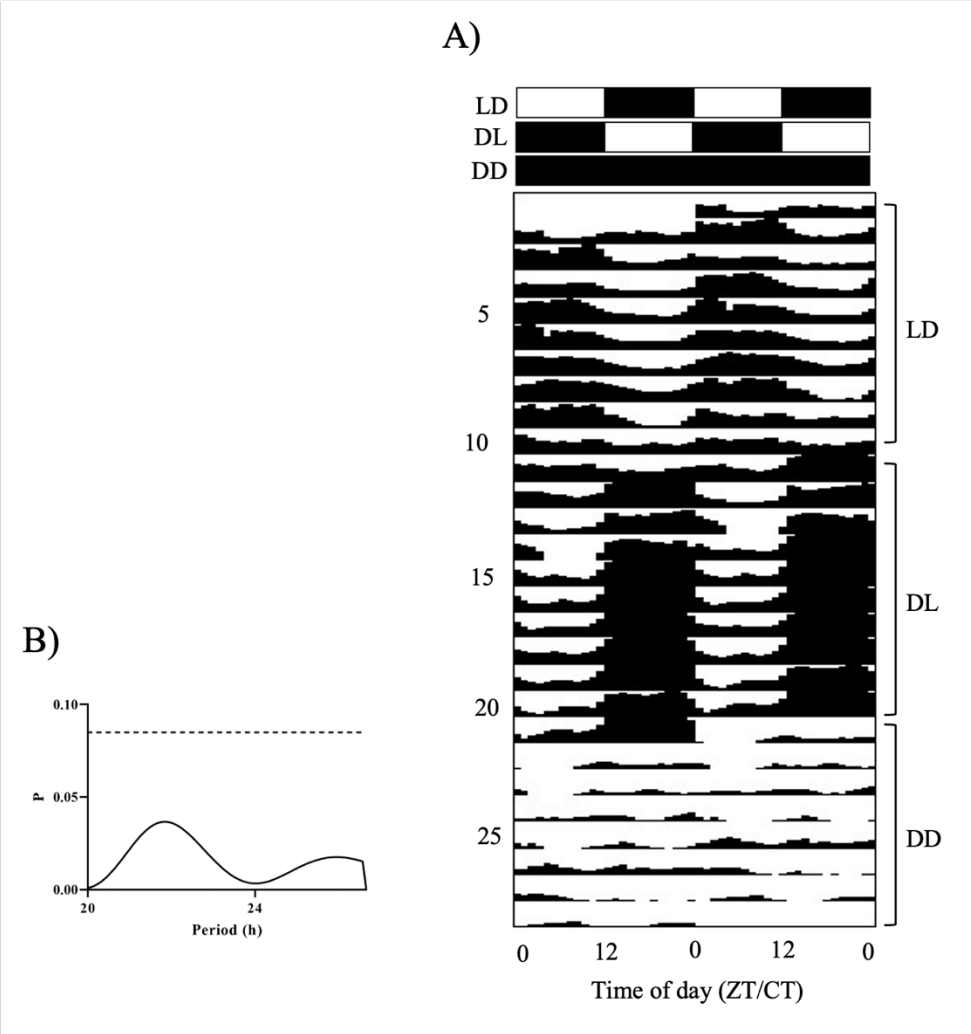

Figure 9. A) Double plot (48 h) thermogram representing the daily thermal preference of the mean of the three replicates in the blind Mexican cavefish *Astyanax mexicanus* (n= 10 fish/replicate) throughout the duration of the experiment (27 days, y- axis). The Lomb-Scargle periodgrams indicates the length of the rhythm (tau) during the 7 days in DD condition for thermal preference (**B**) parameter. Significant tau (in hours) is indicated at the top of each plot. The horizontal line represents the threshold of significance, set at p =0.05.

Discussion

Our findings showed that all the species tested exhibited a daily rhythm of thermal preference when kept in LD cycle and when this is reversed (DL), with the exception for the Somalian cavefish in DL. The role of light as the main synchronizer was supported by the selection of lower temperatures during the day and higher temperatures during the night in the largemouth bass trial, while all the other species tested display an opposite daily temperature selection pattern. In addition, our results confirm the daily activity pattern of the diurnal largemouth (Binder et al., 2012) and nocturnal black bullhead catfish (Roncarati et al., 2014) and the endogenous nature of this behaviour.

Light-dark cycles are a powerful environmental signal able to entrain circadian clocks, playing an important role in a lot of biological rhythms such as locomotor and feeding activity (Zhdanova & Reebs, 2005; López-Olmeda & Sánchez-Vázquez, 2010), reproduction (Oliveira & Sánchez-Vázquez, 2010; Cowan et al., 2017), hatching and development (Villamizar et al., 2013). Our study agrees with previous one (Reynolds et al., 1978a; Reynolds et al., 1978b; Vera et al., 2023; de Alba et al., 2024), as we found that thermal preference is related to LD cycles daily temperature fluctuations happen in the wild. Therefore, it would be reasonable to assume that fish would have evolved to choose higher temperatures in the day (thermophase) and cooler ones at night (cryophase). This agrees with the results that we found investigating the nocturnal and cavefish species; but the diurnal largemouth bass exhibited the opposite pattern of temperature selection in disagree with previous findings of diurnal species as *Danio rerio* and *Oreochromis Niloticus* (Vera et al., 2023; de Alba et al., 2024; Conti et al., 2024 in Experimental Chapter 1.1); meaning that thermal preference can be species-specific and also not strictly related to the activity pattern. Indeed, a previous

study on *M. salmoides* indicated that thermal experiences and depth preferences of this species are dependent on a specific set of factors (i.e., total length, metabolism and season; Peat et al., 2016). However, it is possible to point out that, for both diurnal and nocturnal, fish were selecting lower temperatures during their "active phase". This may be associated with an exploratory behaviour (Kotrschal & Essler, 1995), so they visit all the chambers of the experimental system more, being more active and thus choosing a lower average temperature in comparison with their "resting phase" (Reeve et al., 2022).

Regarding the cavefish species tested, our results proved that both exhibited significant daily rhythms of thermal preference with the same pattern of selection. So, they still can show preference for temperature changes if they are given the opportunity to thermoregulate and this behaviour is directly related to light, despite having evolved for millions of years in a constant environment. However, regarding *P. andruzzii*, both daily locomotor activity and thermal preference rhythms could also be associated to a photophobic response. Even though we covered most of the LEDs, this species has proven to show extremely photophobic behaviour (Tarttelin et al., 2012; Calderoni et al., 2016), so the increased activity and the thermal preference during the day could be associated with continuous movement to 'escape' the light. For this reason, it would be interesting to repeat the experiment in the future by keeping the fish in constant darkness and using feeding time as a synchroniser (Cavallari et al., 2011).

In other taxa, the endogenous molecular clock incorporated into the dorsal ganglion and central nervous system neurons controls both preferred temperature and locomotor activity rhythms (Kräuchi, 2007; Kaneko et al., 2012). In the present study, a rapid resynchronization of the thermal preference and locomotor activity rhythms was observed after the reversal

of the light-dark cycle (from LD to DL) in almost all fish species tested, which can suggest a direct behavioural response to light.

However, when all the *Zeitgebers* were removed and fish were kept in constant darkness and fasting (DD), these rhythmicities ran free with periodicity around or equal to 24 h, within the circadian interval, except for both cavefish species. The persistence of both behavioural rhythms for 7 days in aperiodic conditions would indicate and confirm that thermal preference and activity rhythms in both diurnal and nocturnal species, are driven by the internal circadian clock. However, in *A. mexicanus* and *P. andruzzii*, we could not find any significant endogenous rhythm as both became arrhythmic in DD. As this species have evolved to life underground in constant conditions for millions of years is really fascinating to investigate and deepen whether they have retained a functional circadian clock. Previous studies proved that the Somalian cavefish exhibits a significant food-entrainable rhythm, and it could be potentially susceptible to temperature changes (Cavallari et al., 2011). Our study pointed out the absence of endogenous and clock-driven thermal selection and locomotor activity rhythms; meaning that for this species the most important environmental signal is food availability, that can lack in cave environment, to enhance their survival. On the other hand, *A. mexicanus*, in laboratory conditions, is already proved to retain an endogenous clock that is light-entrainable (Conti et al., 2024 in Experimental Chapter 2.2). Our study proved, that the daily significant rhythm of thermal preference displayed by this species is totally driven by the photic stimulus and not endogenously controlled.

Our findings can be used to improve husbandry protocols in fish captivity conditions in order to allow them to exploit the behavioural thermoregulation that they proved to exhibit. However, as thermal preference seems to be species-specific, it would be interesting to investigate

as many species as possible and to integrate behavioural data with molecular analysis in order to associate behaviour with the physiological status of the fish.

Conclusions

Our findings confirmed daily rhythms of locomotor activity and their endogenous nature in both diurnal and nocturnal species. In addition, we prove the existence of daily rhythms of thermal preference, with different patterns, for all the tested species. Except for cavefish species, the temperature selection across the 24 h has proved to be endogenous and circadian clock-driven. This study highlights the importance of thermoregulation in fish, and it is the first one investigating the circadian pattern of thermal preference in nocturnal and cavefish species.

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Chapter II. Light

Chapter 2.1

Daily behavioural rhythms of light/dark selection in zebrafish

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Manuscript in preparation

Introduction

The Earth's rotational motion lasts 24 hours and allows the day and night alternation. These daily light-dark cycles are the most powerful environmental cue, also called *Zeitgeber* ("time givers" in German; Sharma & Chandrashekaran, 2005; Monk, 2010), capable of entraining almost all organisms circadian clocks via the light-entrainable oscillators (Reppert & Weaver, 2002; Isorna et al., 2017) generating biological rhythms. These are essential to confer an evolutionary advantage in terms of fitness and survival. Thus, light plays a key role on the behavioural modulation, regulation of physiological processes and proper development of living beings.

Light-dark (LD) cycles have been extensively studied in fish, pointing out their important effect on fish biology as they have tissues that are directly light responsive (Whitmore et al., 2000; Hurd & Cahill, 2002). Indeed, LD cycles are essential for proper development, growth and survival, for the onset of hatching and behavioural rhythmicity and for determining the pattern of daily activity. Indeed, teleost embryos of diurnal (zebrafish *Danio rerio*), nocturnal (Senegalese sole *Solea senegalensis*) and cavefish (*Phreatichthys andruzzii*) species, hatch at a specific time during the day and the absence of LD cycle can disrupt this process (Villamizar et al., 2013). Moreover, zebrafish larvae raised in constant conditions as constant darkness (DD) and continuous light (LL) displayed hight deformity and mortality rates (Villamizar et al., 2014).

In aquatic ecosystems, light is absorbed and scattered by particles in the water column that acts as chromatic filter and so affecting spectral and intensity quality of light at different depths (Collin & Hart, 2015; Carleton et al., 2020). Specifically, shortest and longest wavelengths are absorbed near the surface (ultraviolet $\lambda < 390$ nm and red $\lambda > 600$ nm) while blue wavelengths ($\lambda \sim 450$ nm) can penetrate deeper (Dickey et al., 2011).

Therefore, fish have adapted their photopigment sensitivity according to the ecology of the environment they inhabit (Villamizar et al., 2011; Carleton et al., 2020). This variety in the visual systems means that different wavelengths have different effects on reproduction, growth and survival, locomotor and feeding activity, behaviour, stress and circadian system of fish (Villamizar et al., 2009; del Pozo Cano & Sánchez Vázquez, 2015; Di Rosa et al., 2015; Güller et al., 2020; Noureldin et al., 2021). Many studies conducted on European sea bass larvae *Dicentrarchus labrax* (Villamizar et al., 2009), zebrafish *Danio rerio* (Villamizar et al., 2014; Adatto et al., 2016; de Alba et al., 2022), gilthead seabream *Sparus aurata* (Karakatsouli et al., 2007), gold fish *Carassius auratus* (Noureldin et al., 2021) and Senegalese sole *Solea senegalensis* (Blanco-Vives et al., 2010 and 2011) has shown how certain wavelengths, such as blue, benefit growth and survival while others, such as red, reduce spawning, cause malformations, poor feeding activity, lower growth and subsequent high mortality rate (Tsutsumi et al., 2014). On the other hand, these negative effects can be species-specific as rainbow trout *Oncorhynchus mykiss*, yellow perch *Perca flavescens* and common carp *Cyprinus carpio* have presented higher growth and weight gain under red light (Head & Malison, 2000; Karakatsouli et al., 2008, 2010).

Furthermore, light is also very important from a behavioural point of view, as exposure to light/dark environments is crucial for animal's survival. In this way, they can easier find food and hide from predators. A study proved that, as a diurnal species, zebrafish prefers the light area during the daytime and the dark area during nighttime, and this preference is mediated by the circadian system (Wang et al., 2014, 2015). However, to prove the involvement of the circadian clock and the endogenous nature of this light/dark preference, the LD cycle signal must be removed, so that the

possible biological rhythm manifests with a free-running period (tau, τ) of around 24 hours (Aschoff, 1967).

The present study aims to verify whether fish exhibit daily rhythms of light/dark preference, to determine whether these are driven by an endogenous circadian system and how different wavelengths could affect this behavioural rhythmicity. To do so, we used the model species zebrafish *Danio rerio* and we tested two different coloured lights, the white (400 nm < λ < 700 nm) and the red (λ > 600 nm) one. In zebrafish husbandry, white light is the one that is normally used; whereas red light is the one that has been shown to have the most negative effects (Villamizar et al., 2009; Villamizar et al., 2014; Adatto et al., 2016; Hartmann et al., 2018; de Alba et al., 2022). The results may highlight the importance of rearing fish under LD cycles to improve animal welfare and better designed captive husbandry protocols. Indeed, in some farms or captive facilities, fish are kept under constant light/dark (LL or DD) conditions, affecting negatively their endogenous biological patterns and increasing the mortality and malformations rate (Villamizar et al., 2009; Blanco-Vives et al., 2010; Johnsson et al., 2014; Villamizar et al., 2014).

Materials and Methods

Ethics statement

This investigation was conducted at the Department of Physiology, Faculty of Biology, University of Murcia, Spain. Fish were raised in accordance with Spanish legislation on Animal Welfare and Laboratory Practices. Experimental protocols adhered to 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.

Animals and housing

Zebrafish adults (*Danio rerio*) of a wild-type line stock used for the present study were obtained from a local supplier (Alimar Pets, S.L., Murcia, Spain). They were kept in 54-liter glass tanks divided into six compartments (9 L/ compartment) placed inside a closed chamber under controlled lighting and temperature conditions. The photoperiod provided was set at 12h light:12h dark (LD 12:12 cycle), with light onset at 03:00 h (ZT 0) and light offset at 15:00 h (ZT 12). Light was provided by LED strips (SOLBRIGHT®, LED Flex Strip 1043-W, Rayte, S.L., Murcia, Spain) placed above each tank. The tanks contained recirculated dechlorinated fresh water, constantly filtered by biological and mechanical filters and water temperature was maintained at 28 ± 0.3 °C. Fish were fed three times per day commercial feed (Tropical fish flakes, Casone, Parma, Italy) by means of automatic feeders (Eheim GmbH & Co. KG, model 3581, Deizisau, Germany). For the following experiment, a total of 36 zebrafish adults (n= 12/replication) with random sex ratio were used for each wavelength tested (white and red).

Experimental set up and protocol

The experimental set up consisted of a 108 L multi-chambered glass tank (180 x 30 x 20 cm; already described in Chapter 1). The different chambers (30 × 30 × 20 cm each) were separated by black opaque non-toxic PVC panels which have circular holes (Ø 5 cm; 12 cm from the bottom) with grey PVC tubes inserted to allow fish passage and to reduce possible light pollution. To filter and oxygenate water, five cascade filters (SEA STAR, model HX-003) were placed in each chamber and water temperature was maintained constant at 28 ± 0.02 ºC by controlling the room temperature.

Fish only had access to three out of five compartments which, depending on the experimental phase, have different lighting conditions. A

PVC panel without hole was used to prevent passage into the other chambers not in use. Each experimental replicate (three replications; n= 12 fish/replication per wavelength) consisted of four phases: acclimation, experiment 1, resynchronisation and experiment 2 (Figure 1A and B).

First acclimation (LDW) to the experimental tank lasted 3 days. During this phase, fish were kept at the same rearing photoperiod LD 12:12 white light (ZT0= 03:00 h; ZT12= 15:00 h). Lighting was provided by a 180 cm white light (W) LED strip (SOLBRIGHT®, LED Flex Strip 1043-W, Rayte, S.L., Murcia, Spain) attached to a stand and placed above the experimental tank at 4 cm from the water surface (with a surface intensity of 0.59 klx; Figure 2A). During this phase, fish were allowed to occupy section 3, 4 and 5 of the tank (Figure 2A). During acclimation fish get used to the new environment and recover from any stress caused by the change of environmental conditions.

During the first experimental phase (Experiment 1) the photoperiod provided was removed and the three sections in which the fish were free to move presented three different lighting conditions. Specifically, section 3 and 4 were in constant darkness (DD; Figure 2B) while section 5 had continuous lighting (LL; Figure 2B). This condition was provided by mean of a 21 cm LED strip (SOLBRIGHT®, LED Flex Strip 1043-W, Rayte, S.L., Murcia, Spain) placed only above section 5 (intensity at surface level of 0.59 klx; Figure 2B). Intensity at surface level for section 3 and 4 was 0.001 klx and 0.056 klx, respectively. Being next to the continuous light, section 4 will be called transition, while section 3 darkness. During this phase, fish were free to select their preferred lighting condition. This first experimental phase lasted 7 days and we tested two different wavelengths that were white (LLW; 400 nm < λ < 700 nm) and red light (LLR; λ > 600 nm; Figure 2C).

During the resynchronization phase (LDW) the initial white light photoperiod LD 12:12 was restored for 7 days. During these, fish were allowed to occupy section 1, 2 and 3 of the tank (Figure 2D). This phase is used to entrain again fish activity to the photoperiod and to change sections previously used.

Throughout the second experimental phase (Experiment 2) the same methodology as in the first one (Experiment 1) was used but inverting the sections: section 1 for continuous light (LL; Figure 2E), section 2 as transition (DD; Figure 2E) and finally section 3 as darkness (DD; Figure 2E). Reversing sections is done to check there is no preference for one section of the tank over the others by the fish. The second experimental phase also lasted 7 days and we tested again the two different wavelengths (LLW and LLR; Figure 2F).

In addition, during experiment 1 and 2, carton boxes were attached to the sections with continuous lighting (LL) with black tape to avoid light pollution in the other sections (DD). Fish has been fed once a day randomly (Interval function = 12 + Random*24) and food was distributed to all sections equally.

Video recording and analysis

Throughout the course of the experiment, fish behaviour was video recorded by means of two video cameras (Logitech Webcam C300–1.3MP, Switzerland), one recording sections 2,3 and 4 and the other recording section 1 or 5, depending on the experimental phase. The video cameras were connected to the Multiviewer software (Computer System Department, University of Murcia, Spain) which stored 60 images (1 frame/s) every minute and it had already been validated in zebrafish (Di Rosa et al., 2015). To allow video recording in the dark, infrared LED lamps (BW® 48 LED Infrared Illuminator) were installed behind the experimental tank and a

translucent acrylic white sheet (Falken Design WT2447-1-8/2436 Acrylic White Sheet, Translucent 55%, 100 × 30 × 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 video recordings were analysed using the Fish Counter software (Dr. Ginés García Mateos, University of Murcia, Spain, Version 3.0) that permits to count the number of fish in each section every minute (block of 60 frames) and to record the results on a Microsoft Excel spreadsheet.

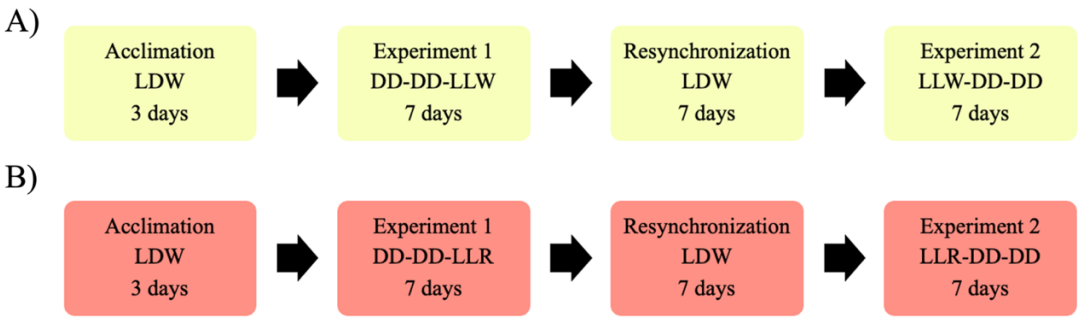

Figure 1. Experimental protocol diagram of the present study and their duration. As described in the text the experiment was carried out testing two different wavelengths: **A)** white light (400 nm < λ < 700 nm) and **B)** red light (λ > 600 nm).

Statistical analysis

Data were processed using chronobiological software (El Temps, version 1.313, Prof. A. Dìez-Noguera, University of Barcelona) to perform Cosinor analysis, to calculate Lomb-Scargle periodograms (LS) and acrophases, corresponding to the peak of the rhythm. The endogenous free-running circadian period length (tau, τ) of the behavioural rhythm was determined by LS periodograms analysis at a confidence level of 95% (Van Dongen et al., 1999).

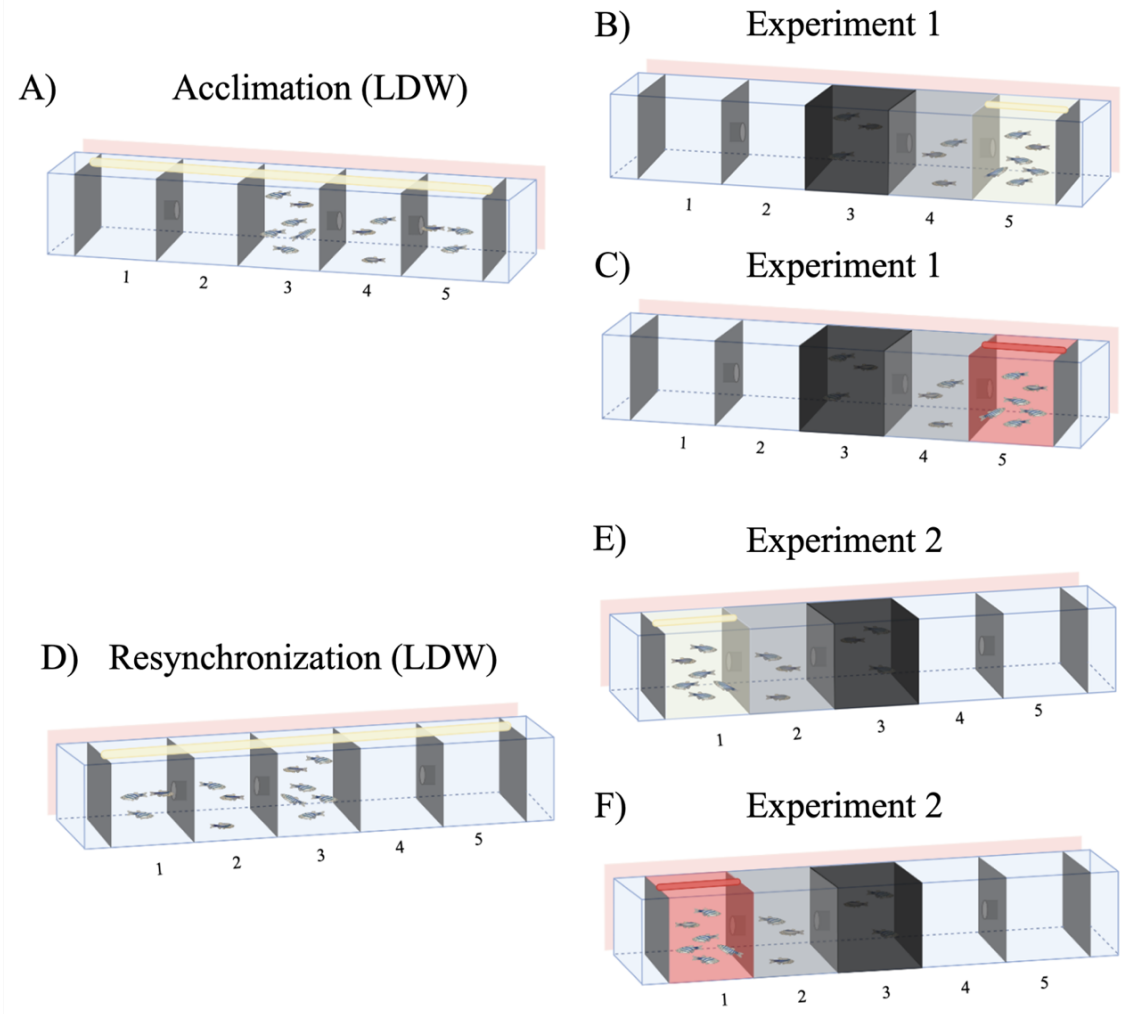

Figure 2. Diagrams representing the experimental phases and set up, in which it is possible to see which sections the fish can occupy depending on the phase. **A)** Acclimation phase during which fish are subjected to 12h light: 12h dark photoperiod in white light (LDW 12:12) and they can occupy sections 3,4 and 5. During the first experimental phase (Experiment 1), fish are free to swim between section 3 that is in constant darkness (DD), section 4 as transition (DD) and section 5 that is continuously lit (LL) **B)** with white light (LLW) or **C)** with red light. **D)** During the resynchronization phase the initial LDW 12:12 is restored but the order of the sections is inverted and fish can occupy sections 1,2 and 3. During the second experimental phase (Experiment 2) fish are free to move between section 3 that is in constant darkness (DD), section 2 as transition (DD) and section 1 that is continuously lit (LL) **E)** with white light (LLW) or **F)** with red light.

Results

During the first three days of acclimation phase, fish show a preference for section 5 during the night phase (Figure 3A and 4A); although during this phase the photoperiod is equally distributed among the sections.

Throughout the first experimental phase (experiment 1) of the trial conducted in white light, the results show a daily rhythm of light/dark preference driven by an endogenous clock. Specifically, the LS periodogram analysis reveal a significant self-sustained circadian rhythm in all the three sections during the 7 days, with an average of the circadian rhythm period of $\tau \pm$ SEM of 22.6 ± 0.9 h for the dark section (Figure 3E), 22.3 ± 0.9 h for the transition section (Figure 3F) and 22.4 ± 0.4 h for the light section (Figure 3G). In addition, the fish prefer to visit the dark and transition sections during the subjective night phase with a nocturnal acrophase of the selection rhythm (ZT 18 h; Cosinor $p < 0.001$; Table 1), while they prefer to stay in the light section during the subjective day with a diurnal acrophase (ZT 6; Cosinor $p < 0.001$; Table 1).

During the resynchronisation phase, the sections to which the animals had access were inverted and the initial photoperiod restored. The behaviour observed during this time is similar to that of the acclimation phase. Indeed, fish exhibit a preference for section 1 during the night phase (Figure 3C and 4C), the one covered with the carton box.

Throughout the second experimental phase (experiment 2) of the trial conducted in white light, fish still exhibit an endogenous daily rhythm of light/dark preference. Indeed, the LS periodogram analysis report a significant self-sustained circadian rhythm in all the three sections during the 7 days, with an average of the circadian rhythm period of $\tau \pm$ SEM of 22.8 ± 1.6 h for the dark section, 24.7 ± 1.6 h for the transition section and 23.5 h

for the light section. In addition, the fish still select the dark and transition sections during the subjective night phase with a nocturnal acrophase (ZT 16.8 and ZT 12.6 respectively; Cosinor $p < 0.001$; Table 1) while they select the light section during the subjective day with a diurnal acrophase (ZT 2.7; Cosinor $p < 0.001$; Table 1).

Experimental phase	Section	Mesor (r.e.)	Amplitude (r.e.)	Acrophase (ZT)	Significance (*p-value*)
EXP 1 (LLW)	Dark	2.02	0.45	18.1	***
EXP 1 (LLW)	Transition	3.57	0.45	18.2	***
EXP 1 (LLW)	Light	6.42	0.90	6.2	***
EXP 2 (LLW)	Dark	2.29	0.42	16.8	***
EXP 2 (LLW)	Transition	3.56	0.34	12.6	***
EXP 2 (LLW)	Light	6.12	0.61	2.7	***
EXP 1 (LLR)	Dark	-	-	-	n.s.
EXP 1 (LLR)	Transition	-	-	-	n.s.
EXP 1 (LLR)	Light	-	-	-	n.s.
EXP 2 (LLR)	Dark	-	-	-	n.s.
EXP 2 (LLR)	Transition	-	-	-	n.s.
EXP 2 (LLR)	Light	-	-	-	n.s.

Table 1. Cosinor parameters of light/dark selection experimental (1 and 2) phases of the two wavelengths tested. The fish could choose the section were to stay along the 24 hours. Table shows numeric values of mesor, amplitude, acrophase and significance circadian rhythms (*p-value*) reported by the cosinor analysis. Mesor and amplitude are given as relative expression values (r.e.) and acrophase as zeitgeber time (ZT). Asteriks indicate statistically significant rhythms: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and non-significant rhythms are indicated with n.s.

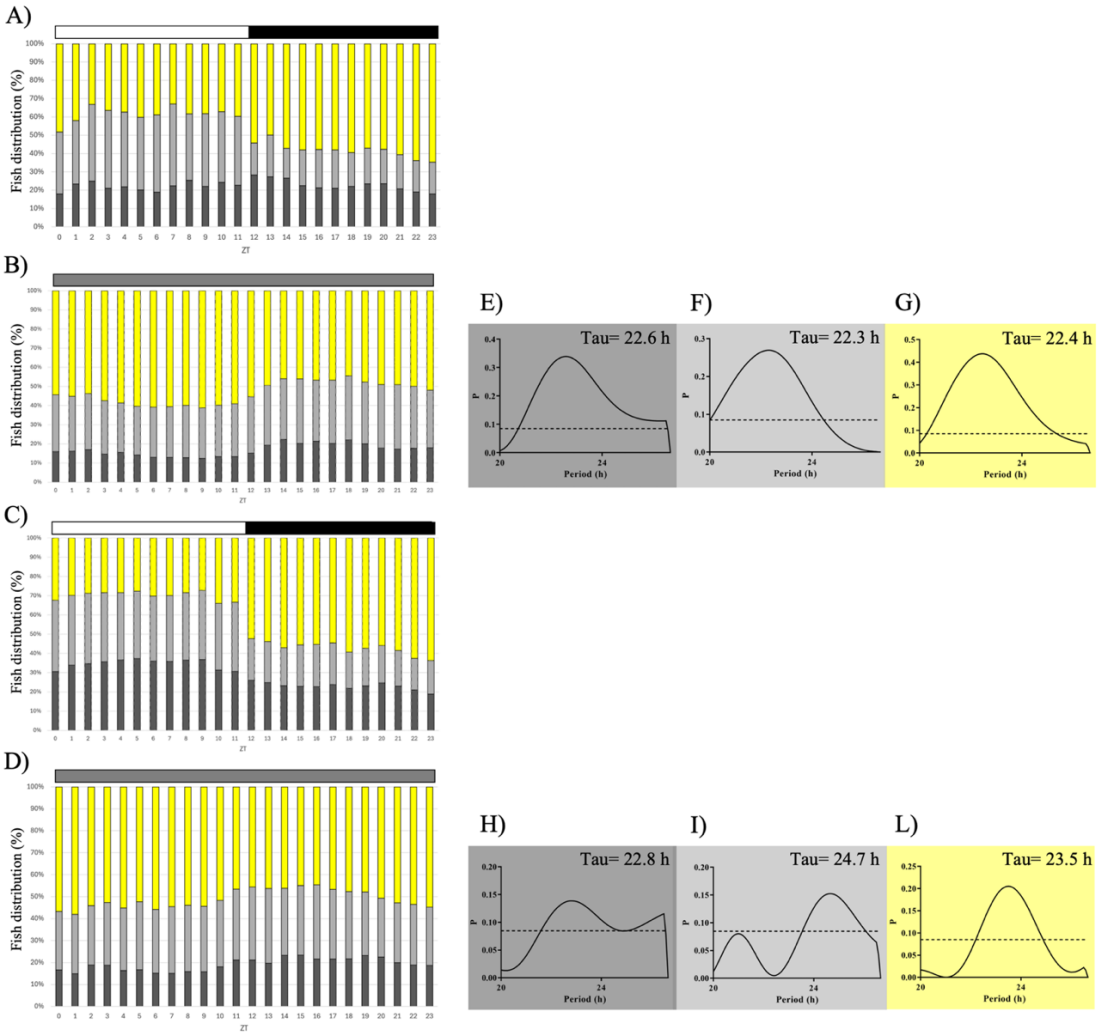

Figure 3. Bar diagrams of the average distribution of fish (n=36) in each section during the acclimation (**A**), experiment 1 phase (**B**), resynchronization (**C**) and experiment 2 phase (**D**) of the white light trial. The X-axis shows the ZT, while the Y-axis shows the fish distribution among the sections in percentage (%). The white and black bars above diagrams **A** and **C** represent the light phase and the dark phase, respectively (LD 12:12; ZT0= 03:00 h and ZT12= 15:00 h). Grey bars above diagrams **B** and **D** show the continuous white lighting condition during the experimental phases. In addition, LS periodogram analysis related to 7 days of the first and second experimental phase for the dark (**E**, **H**), transition (**F**, **I**) and light (**G**, **L**) sections are shown. In each LS periodogram the period of the rhythm (tau, τ) is indicated.

Regarding the first experimental phase (experiment 1) of the trial conducted in red light, fish do not show a daily rhythm of light/dark preference driven by an endogenous clock under this wavelength (Figure 4B). In fact, the results of LS periodogram analysis do not reveal a significant self-sustained circadian rhythm in any of the three sections during the 7 days. Also, during the second experimental phase (experiment 2) fish do not exhibit a significant daily rhythm of light/dark preference under red light condition (Figure 4D). The results are also confirmed by LS periodograms analysis.

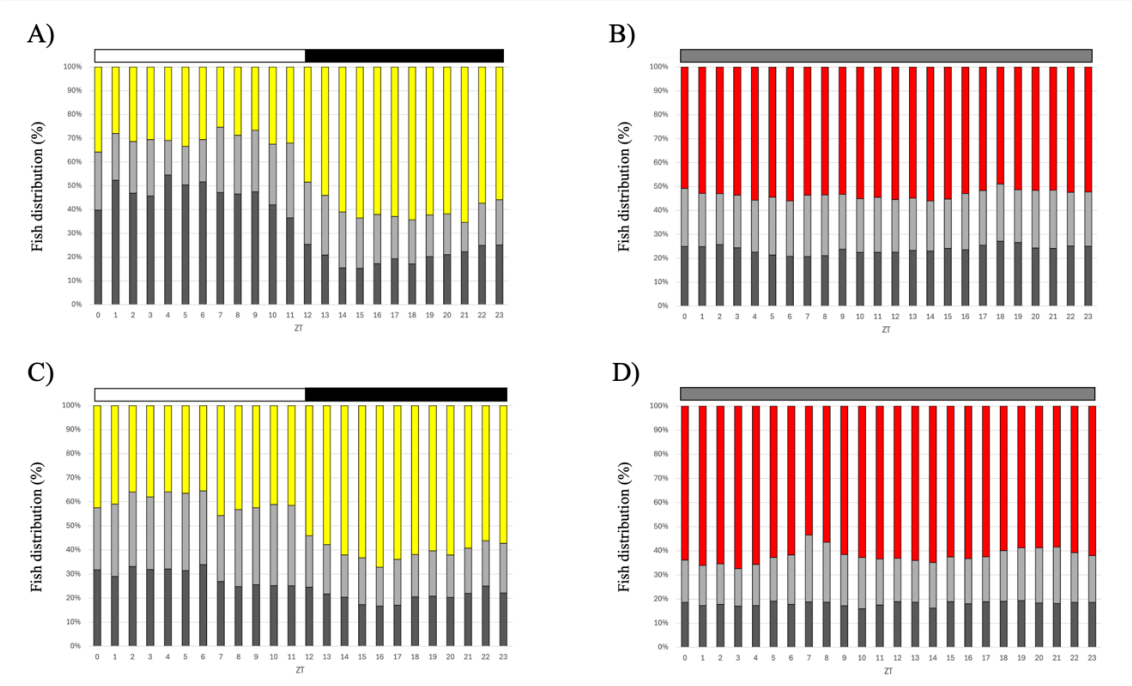

Figure 4. Bar diagrams of the average distribution of fish (n=36) in each section during the acclimation (**A**), experiment 1 phase (**B**), resynchronization (**C**) and experiment 2 phase (**D**) of the red light trial. The X-axis shows the ZT, while the Y-axis shows the fish distribution among the sections in percentage (%). The white and black bars above diagrams **A** and **C** represent the light phase and the dark phase, respectively (LD 12:12; ZT 0= 03:00 h and ZT 12= 15:00 h). Grey bars above diagrams **B** and **D** show the continuous red lighting condition during the experimental phases.

Discussion

The present study findings revealed that zebrafish exhibited a significant circadian rhythm of light/dark selection under white light condition. Indeed, this self-sustained behavioural rhythm is maintained for several days in absence of external signals emphasising its endogenous character. On the other hand, zebrafish does not display any significant light/dark preference rhythm under red light.

Light/dark preference in fish has been widely investigated by many groups, especially when it comes to behavioural tests that are used to measure and assess anxiety-like behaviours as the scototaxis test (Serra et al., 1999; Maximino et al., 2010; Araujo et al., 2012; Lucon-Xiccato et al., 2022). Zebrafish and other species of interest in neurosciences and neuroethology studies (e.g., goldfish, guppy *Poecilia reticulata* and Nile tilapia *Oreochromis niloticus*) have been shown to exhibit a marked preference for the dark sector (Gouveia Jr. et al., 2005; Maximino et al., 2007). This response is attributed to seeking refuge and hide from predators while increased activity in white sector should reflect antianxiety behaviour (Maximino et al., 2010; Lucon-Xiccato et al., 2022). However, this is a short test that allows each individual fish to explore the experimental tank for 15 minutes that normally is performed during the day. Though, the intraday variation of the light/dark preference should be taken into account as previous studies proved that the light/dark preference of zebrafish exhibited a clear circadian-like pattern (Wang et al., 2014 and 2015). The results that we achieved confirm the ones obtained before, showing that zebrafish displayed a circadian behavioural rhythm when free to choose lit or dark environments, under white light condition. Specifically, zebrafish selected the lit section during the subjective days and dark section during the subjective nights. Indeed, the peaks of these rhythms occurred in the middle

of the subjective day and night phase, respectively (ZT 6 and ZT 18). Our findings agree with previous ones confirming the diurnal behavioural pattern of this species (Hurd et al., 1998; López-Olmeda et al., 2006; Krylov et al., 2021).

Most studies (Richardson et al., 1974; López-Olmeda & Sánchez-Vázquez, 2010; Montoya et al., 2010; Morbiato et al., 2019; Vanecek, 2019) test whether biological rhythms are driven and controlled by an endogenous circadian clock by subjecting experimental subjects to aperiodic conditions such as constant darkness (DD) and continuous light (LL). In our study, we use both conditions simultaneously, giving the fish the option to select which lighting condition they prefer and when during the 24-hour period for 7 days. Under white light, zebrafish displayed a significant circadian rhythm of light/dark preference across the 7 days with an average of the rhythm period (τ) within the circadian interval (22.6 ± 0.9 h for the dark section, 22.3 ± 0.9 h for the transition section and 22.4 ± 0.4 h for the light section). These results confirm ones achieved before testing constant conditions (Li et al., 1998; López-Olmeda et al., 2006; del Pozo et al., 2011; Wang et al., 2014; Sacksteder & Kimmey, 2022) and they underline even more the importance of LD cycles as the most powerful synchronizer of circadian rhythms in almost all organisms (Vitaterna et al., 2001; Steindal & Whitmore, 2020). Indeed, there are a lot of light-dependent circadian behaviours in zebrafish (e.g., spawning, feeding, locomotor activity, shoaling and stress-related behaviours; Spence et al., 2007; del Pozo et al., 2011; Paciorek & Mcrobert, 2012; Pintos et al., 2023). Since we maintained a constant temperature throughout the experiment and fed them randomly (Zhdanova & Reebs, 2005; Frøland Steindal & Whitmore, 2019), we can state that the behavioural endogenous circadian rhythm exhibited is exclusively driven by the light-dark cycle. When the order of the sections is inverted, during the second

experimental phase, fish still displayed a significant circadian rhythm of light/dark preference driven by the endogenous clock during white light test. Indeed, the duration of the free-running period of the present rhythm is within the circadian interval in all the three sections (22.8 ± 1.6 h for the dark section, 24.7 ± 1.6 h for the transition section and 23.5 h for the light section). Zebrafish continue to display a significant light preference during the subjective days and dark preference during the subjective nights with diurnal and nocturnal acrophases, respectively (ZT 16.8 and ZT 2.7), confirming its diurnal activity pattern. Circadian rhythms can be observed as daily changes in behavioural endpoints; and so a relatively long-term period of registration is required to detect these changes (Krylov et al., 2021). Thus, our findings proved the persistence of the present endogenous rhythmicity for 7 days.

It has been widely proved as different light wavelengths can affect positively or negatively various aspects of fish biology. Specifically, red light produces the most negative effects on zebrafish and other species survival and growth (Ruchin, 2004; Villamizar et al., 2014; Yuan et al., 2017). For this reason, we wanted to test whether different wavelengths could affect the endogenous rhythm of light/dark selection. Our study proved that zebrafish exhibited no significant behavioural circadian rhythms under this coloured light. Although zebrafish expresses two red (LWS-1 and LWS-2) opsin genes (Takechi & Kawamura, 2005) by which it can exhibit daily rhythmic locomotor activity and clock genes expression under a red light-dark cycle (Di Rosa et al., 2015), when they are free to choose which section to be in during the day, they preferred to occupy all the sections evenly, becoming arrhythmic and avoiding red light as seen for other species (grey mullet *Mugil cephalus* and striped bream *Lithognathus mormyrus*, Marchesan et al., 2005; largemouth bass *Micropterus salmoides*, Sullivan et al., 2016). This can be explained as zebrafish in the wild inhabits a wide range of continental

water environments characterised by a blue-greenish wavelengths spectrum profile (Spence et al., 2008).

The maintenance of the presence (under white light) or absence (under red light) of the light/dark selection pattern, even during the inversion of the sections leads us to exclude the involvement of a possible spatial preference (Volpato et al., 2007).

During the acclimation and resynchronisation phases, fish distribution is homogeneous across the sections when the light is on while they tend to stay in the outermost sections (1 and 5), where the cardboard box is, during the night phase. This behaviour could be related to a sense of major protection during the night phase, when they are less active, to minimize their visibility (Gerlai, 2010). Indeed, in the wild, zebrafish hide among the aquatic vegetation as a refuge from predators (Lawrence, 2007).

Finally, the results achieved with this study may be taken as a reference for improving the welfare of farm-raised and captive-bred fish, which are often kept in unnatural and constant conditions affecting negatively their circadian clock and potentially causing stress.

Conclusions

In the present study, we proved that zebrafish exhibit a significant circadian rhythm of light/dark preference driven by and endogenous clock under white light condition providing a greater understanding of the light-dependent circadian rhythms of fish. Indeed, this rhythm is maintained for several days in absence of external signals. In contrast, this rhythm is not present when testing red light. For future research it might be interesting to investigate other wavelengths such as blue, which in literature is the most beneficial in this species, but also other species such as nocturnal to

investigate whether the light/dark preference pattern is reversed and still endogenous.

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Chapter 2.2

Daily rhythms of locomotor activity and transcript levels of non-visual opsins in the brain of the blind Mexican cavefish (*Astyanax mexicanus*).

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Introduction

Most organisms inhabiting our planet possess endogenous circadian clocks that synchronise their physiological and behavioural activities with environmental cycles (Bell-Pedersen et al., 2005; Idda et al., 2012; Kuhlman et al., 2018; Menaker et al., 1997; Panda et al., 2002; Rivkees, 2007). Light serves as the primary environmental time cue for synchronising circadian rhythms via a light-entrainable oscillator (Androulakis, 2021; Pittendrigh and Minis, 1964; Reppert and Weaver, 2002). Numerous studies conducted in fish cell cultures, tissues and whole organisms have demonstrated this direct synchronisation of molecular circadian clocks by light (Beale et al., 2013; Cuesta et al., 2014; Frøland-Steindal and Whitmore, 2019; Martín-Robles et al., 2012; Pando et al., 2001; Vergès-Castillo et al., 2021, 2024; Whitmore et al., 2000). How cells, tissues and organisms are able to capture light and transduce it into downstream biochemical signals that ultimately entrain rhythmic functions represents a complex and fascinating process. This process is sustained by photoreceptor structures and their corresponding photopigments, with opsins being the most studied. Opsins are a family of seven-transmembrane-domain G-protein-coupled receptors (GPCRs) that form photosensitive complexes and have evolved to satisfy the particular lighting niches of the organisms that express them (Shichida and Matsuyama, 2009; Terakita, 2005; Terakita et al., 2012; Upton et al., 2021). These photoreceptor molecules can be divided into two groups: visual opsins and non-visual opsins, mainly expressed in ocular and extraocular tissues, respectively. Research in zebrafish (*Danio rerio*) has revealed the existence of 42 different opsin genes, with 10 corresponding to visual opsins and 32 to non-visual opsins expressed in extraocular tissues such as the brain, liver, heart, gut, muscle, pineal, skin and testis (Davies et al., 2015). Non-visual photoreception has been shown to play a crucial role in a number of

biological processes, such as seasonality, photoperiodism, circadian entrainment and DNA repair (Benoit, 1935; Bertolucci and Foà, 2004; Foster et al., 2003; Frøland-Steindal and Whitmore, 2019; Goldman, 2001).

Cavefish species provide an intriguing model for understanding vertebrate circadian clocks and light detection mechanisms, having adapted to life underground, in constant darkness. These species exhibit troglomorphic phenotypes, including degenerated eyes from early developmental stages and loss of pigmentation (Behrmann-Godel et al., 2024). Studies on Somalian cavefish *Phreatichthys andruzzii* have highlighted a loss of the ability to entrain activity to light-dark cycles in laboratory conditions, possibly due to the expression of two truncated non-visual opsins: *mammalian-like melanopsin2* (*opn4m2*) and *teleost multiple tissue opsin3a* (*tmt3a*) (Calderoni et al., 2016; Cavallari et al., 2011). However, there is limited research on entrainment to daily light-dark cycles in blind cavefish. Therefore, this study focuses on the blind Mexican cavefish, *Astyanax mexicanus*, encompassing 30 cavefish populations in Northeast Mexico that descend from the same ancestral river strain (Gross, 2012). Their excellent adaptation to laboratory conditions and non-speciation from the surface-dwelling strain make them an ideal model for studying adaptive and regressive evolution. Eye degeneration in A. mexicanus occurs through lens apoptosis after 1-day post-fertilization (dpf), with adults exhibiting only residual cysts in the orbits covered by skin (Alunni et al., 2007; Yamamoto and Jeffery, 2000). Despite the absence of functional eyes, both juveniles and adults exhibit light-dependent basal locomotor activity, increasing their activity in enlightened environments (Beale et al., 2013; Duboué et al., 2011; Simon et al., 2019). Furthermore, they still express numerous non-visual opsin genes under a light-dark cycle (Simon et al., 2019). Moreover, light-dependent locomotor activity may be

regulated by non-visual extraocular/extrapineal tissues, as both pinealectomized surface and cavefish still display light-dependent locomotor behaviour with a diurnal activity pattern (Simon et al., 2019).

In this context, our research aims to provide further insights into the long-term photic entrainment of locomotor activity and circadian endogenous rhythmicity in this eyeless hypogean species, subjecting it to various photic regimes: light-dark (LD) and shifted LD cycles, constant darkness (DD) and continuous dim light (LLdim). Additionally, in order to gain information on photopigments that might be involved in light synchronisation of locomotor activity, we intend to characterise daily variations and rhythms in transcript levels of selected non-visual extraocular opsins, *exo-rhodopsin* (*exo-rhod*), *encephalopsin* (*opn3*), *retinal G-protein receptor a* (*rgra*) and *b* (*rgrb*), and *teleost multiple tissue opsin 1a* (*tmt1a*) and *1b* (*tmt1b*), from the brain of *A. mexicanus* kept in 24-h light-dark cycles using RT-qPCR.

Materials and Methods

Animal housing

For this study, a total of 110 juvenile blind Mexican cavefish (*A. mexicanus*) of both sexes were obtained from a local commercial supplier (Alimar Pets, S.L., Murcia, Spain). Fish were acclimated in a 54-liter glass tank divided into six compartments (9 L each) and maintained in the animal facilities of the University of Murcia under controlled lighting and constant temperature conditions (24 ± 0.5 °C). Light was provided by LED strips (SOLBRIGHT®, LED Flex Strip 1043-W, Rayte, S.L., Murcia, Spain), and the photoperiod was set to a 12 h light:12 h dark (LD 12:12) cycle, with light on at 09:00 h and light off at 21:00 h. Therefore, light onset corresponded to Zeitgeber Time (ZT) 0 and the light switch-off to ZT12. Blind Mexican

cavefish were fed once daily with freeze-dried Artemia (Prodac International, Cittadella, Padova, Italy). Fish were handled and sacrificed following the guidelines for experimental procedures of the Animal Welfare Committee of the University of Murcia, in accordance with Spanish legislation on Animal Welfare and Laboratory Practices. Experimental protocols adhered to 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.

Long-term recording of fish locomotor activity

The locomotor activity of *A. mexicanus* was recorded under different photoregimes to characterize light entrainment and possible endogenous circadian rhythmicity. For this purpose, fish were divided into two groups and two different behavioural experiments were performed. First group consisted of six aquaria (n= 6 fish/aquarium) subjected to LD 12:12 (LD1) for 26 days, followed by constant darkness (DD) for 8 days, and then returned to the initial LD1 condition for 7 days (Figure 1A). The second group comprised five aquaria (n = 5 fish/aquarium) exposed to LD 12:12 (LD1) for 23 days, followed by a 6-h delayed photoperiod (LD2) for 13 days, and then returned to LD1 for 13 days. Finally, they were kept under continuous dim light (LLdim) condition for 12 days (Figure 1B). The locomotor activity was recorded using an automated system based on infrared photocells (E3S-AD62, Omron, Kyoto, Japan) placed at the front side of each compartment of the tank. Light-beam interruptions were counted and stored every 10 minutes by means of a computer connected to the photocells using the "Contador de Eventos" software (DIO98USB, University of Murcia, Spain).

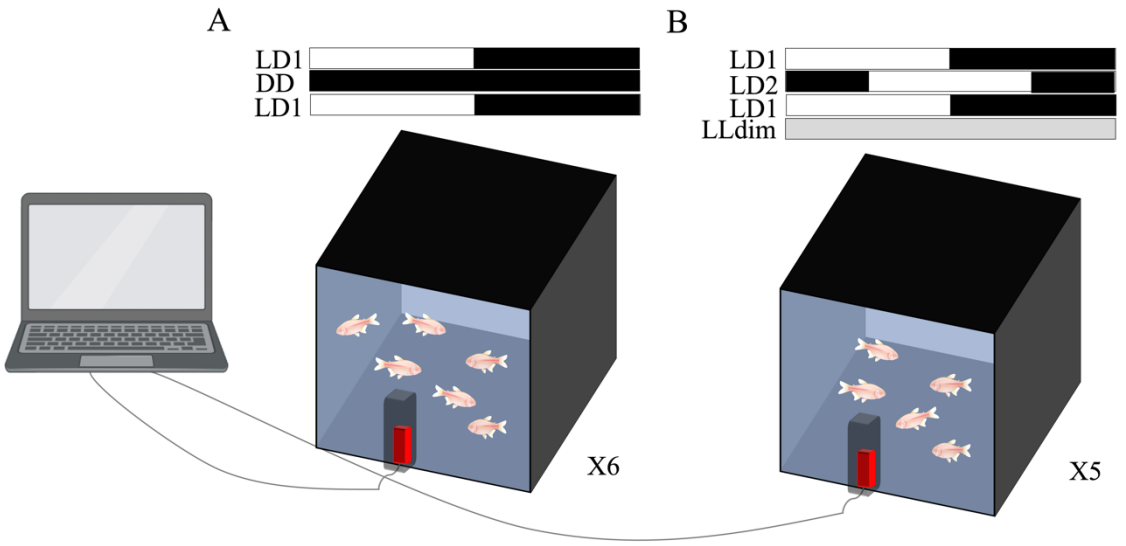

Figure 1. Schematic representation of the set up used for recording the locomotor activity of the juveniles of *Astyanax mexicanus*. Figure A represents the different lighting regimes used for the first experimental group (six aquaria in total): 12h light:12h dark photoperiod (light switches on at ZT0 [09:00 h] and off at ZT12 [21:00 h], LD1) and constant darkness (DD). Figure B shows lighting regimes used for the second experimental group (five aquaria in total), 12h light:12h dark photoperiod (light switches on at ZT0 [09:00 h] and off at ZT12 [21:00 h], LD1), 6-hours shift photocycle (light switches on at 15:00 h and off at 03:00 h, LD2) and continuous dim light (LLdim). The white and black bars above the aquaria represent the light phase and the dark phase, respectively. Grey bar show constant light phase (LLdim) condition.

Experimental set-up and sampling for the analysis of gene expression

To analyse the daily gene expression and rhythms of non-visual extraocular opsins of A. mexicanus, 49 juvenile specimens were acclimated to LD 12:12 for two months. After acclimatization, they were sampled every 4 h during a 24 h daily cycle, at seven time points (ZT2, ZT6, ZT10, ZT14, ZT18, ZT22, ZT2). At each time point, 7 fish were euthanized with an overdose of MS-222 anaesthetic (0.5 mg/ml, pH 7.0; Millipore-Sigma, Burlington, MA, USA). Brain samples were collected, immediately frozen in dry ice, and stored at −80 °C until use.

RNA extraction and cDNA synthesis

Total RNA was isolated from brains by TRIsure reagent (Bioline, London, UK), according to the manufacturer's protocol. Tissues were homogenized in a mixer mill MM400 (Retsch GmbH, Haan, Germany) and total RNA concentration and quality were measured on a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Total RNA (1mg) was retro-transcripted and DNA removed using a Primer ScripTM RT Reagent Kit with gDNA Eraser (Takara Bio Inc., Shiga, Japan). The cDNA samples were stored at −20 °C for further analyses.

Gene expression analysis

Non-visual opsins (*exo-rhodopsin, opn3, rgra, rgrb, tmt1a* and *tmt1b*) were selected according to the expression levels previously reported in the brain of blind Mexican cavefish by Simon et al. (2019). To analyse the daily expression and rhythms of the non-visual opsins, RT-qPCR was conducted using a Bio-Rad CFX96 Touch detection system (Bio-Rad, Hercules, CA, USA). Primers were designed according to Simon et al. (2019) and purchased from STAB Vida (Caparica, Portugal). Sequences of the primers, amplicon sizes and GenBank accession numbers of the non-visual opsins are shown in Table 1. Reactions were performed by duplicate with 5 µl iTaq Universal SYBR® Green SuperMix (Bio-Rad), 0.5 µl of 10 µM forward and reverse primers and 4 µl of cDNA in a final volume of 10 µl per reaction, with the following amplification conditions: 95°C for 30 s, 30 cycles of 95°C for 5 s, annealing at 60°C for 30 s and elongation at 72°C for 5 s. Relative expression of target genes was calculated using the ΔΔCt method (Livak and Schmittgen, 2001) with *elongation factor 2a.1* (*eef2a.1*) and *beta-2-microglobulin* (*b2m*) as reference genes (Table 1).

Name	Accession number	Sequence (5'-3')	Amplicon size (bp)
am_*exo-rod*_F am_*exo-rod*_R	ENSAMXG00000017182	CTCATGGTCACCTCATTTCCT TGTGCGGAGCTTCTTGTG	71
am_*rgra*_F am_*rgra*_R	ENSAMXG00000012172	TGTTATGTCCTGCTACAAATCC GTGCTACAGCTGAACTTATCC	72
am_*rgrb*_F am_*rgrb*_R	ENSAMXG00000004323	GATCCGAATGGTTCTTCCAG CCTCTTGTCTGTCTGGCT	133
am_*opn3*_F am_*opn3*_R	ENSAMXG00000020951	TCGCCATTATCCCGTCCT CAACGACGAAACTTTCTGCTC	88
am_*tmt1a*_F am_*tmt1a*_R	ENSAMXT00000017410	GCAATCAAACAGGTCAGTGGA AGCAGAGCAGGTAGCAGAC	103
am_*tmt1b*_F am_*tmt1b*_R	ENSAMXG00000008135	CATCAAGCAGGTGAGCAG CCAGCACAACAGGTAACAC	103
am_*b2m*_F am_*b2m*_R	ENSAMXG00000011344	TTCACACCTCAGAAGAACGA ACTGCATTCTCCATCTGGT	122
am *eef2a.1*_F am *eef2a.1*_R	ENSAMXG00000018020	TATCATTGAGGAGTCTGGAGAG TGGGTCGGATTTCTTAATTGG	118

Table 1. Sequences of primers used for the characterization of *Astyanax mexicanus* non-visual opsins in brain. PCR amplicon sizes (bp) are also indicated as well as accession number.

Statistical and rhythm's analyses

GraphPad Prism Version 9.4.1 software (San Diego, California, USA) was used for statistical analyses. Normality and homoscedasticity assumptions were tested before the comparison of mean values. When required, expression values were transformed to obtain normality and homogeneity of variances. One-way ANOVA or Kruskal-Wallis (when normality and homoscedasticity were not accomplished) tests followed by Dunn's post-hoc comparison test were performed, and statistical significance was accepted when $p < 0.05$. All data were presented as mean ± standard error of the mean (SEM).

For the analysis of locomotor activity, data were processed using a chronobiological software (El Temps, version 1.313, Prof. A. Díez-Noguera,

University of Barcelona, Spain) to generate actograms, mean waveforms, and Lomb-Scargle periodograms (LS). The endogenous free-running circadian period length (tau, τ) of the behavioural rhythm was determined by LS periodogram analysis at a confidence level of 95% (Van Dongen et al., 1999).

To evaluate the rhythmic expression pattern of non-visual opsins, rhythm parameters (mesor, amplitude, acrophase and significance) were determined by Cosinor analysis. Statistically significant rhythms were considered when $p < 0.05$.

Results

Daily and circadian rhythms of locomotor activity in blind Mexican cavefish

In the first experiment, our results revealed the entrainment of A. *mexicanus* to the LD 12:12 cycle (LD1), manifesting a clear diurnal activity pattern (Figs. 2A-C). Specifically, the fish exhibited increased locomotor activity during daytime, averaging 78 ± 0.03 % (mean percentage ± SD), with decreased activity during nighttime. When exposed to DD condition, fish demonstrated self-sustained circadian rhythm in locomotor activity, with an average of the circadian rhythm period of 24.1 ± 0.2 h (mean τ ± SEM), as confirmed by Lomb-Scargle (LS) periodogram analysis (Fig. 2D). Upon restoration of LD1, animals resynchronized to the initial cycle, displaying an average diurnal locomotor activity of 65 ± 0.05 % (mean percentage ± SD), as shown in the representative locomotor actogram, mean waveform graph and LS periodogram (Figs. 2A, E, F).

In the second experiment, fish exhibited daily behavioural rhythmicity, with locomotor activity entrained to the LD 12:12 cycle (LD1),

also showing a diurnal activity pattern (Figs. 3A-C). As illustrated in the representative locomotor actogram (Fig. 3A) and respective mean waveform (Fig. 3B), animals increased their activity during the light phase, averaging 83.6 ± 0.03 % (mean percentage ± SD). Daily behavioural rhythmicity fell within the circadian interval, with a 24-h period (Fig. 3C). When the light cycle was modified, causing a 6-h shift in the photoperiod (LD2), fish immediately entrained to the new photocycle, maintaining a diurnal activity pattern that averages 79.4 ± 0.07 % (mean percentage ± SD), as shown in the representative locomotor actogram (Fig. 3A) and the mean wave graph (Fig. 3D). LS periodogram analysis confirmed the daily behavioural rhythmicity, with a period of 24 h (Fig. 3E). Upon restoration of the initial LD 12:12 cycle (LD1), animals resynchronized their locomotor activity, always maintaining a diurnal pattern, which averages 82.3 ± 0.08 % (Figs. 3A, F-G). Finally, under continuous dim light condition (LLdim), blind Mexican cavefish exhibited self-sustained circadian rhythm of locomotor activity, within the circadian interval, with an average period of 22.3 ± 0.5 h (mean τ ± SEM, Fig. 3H).

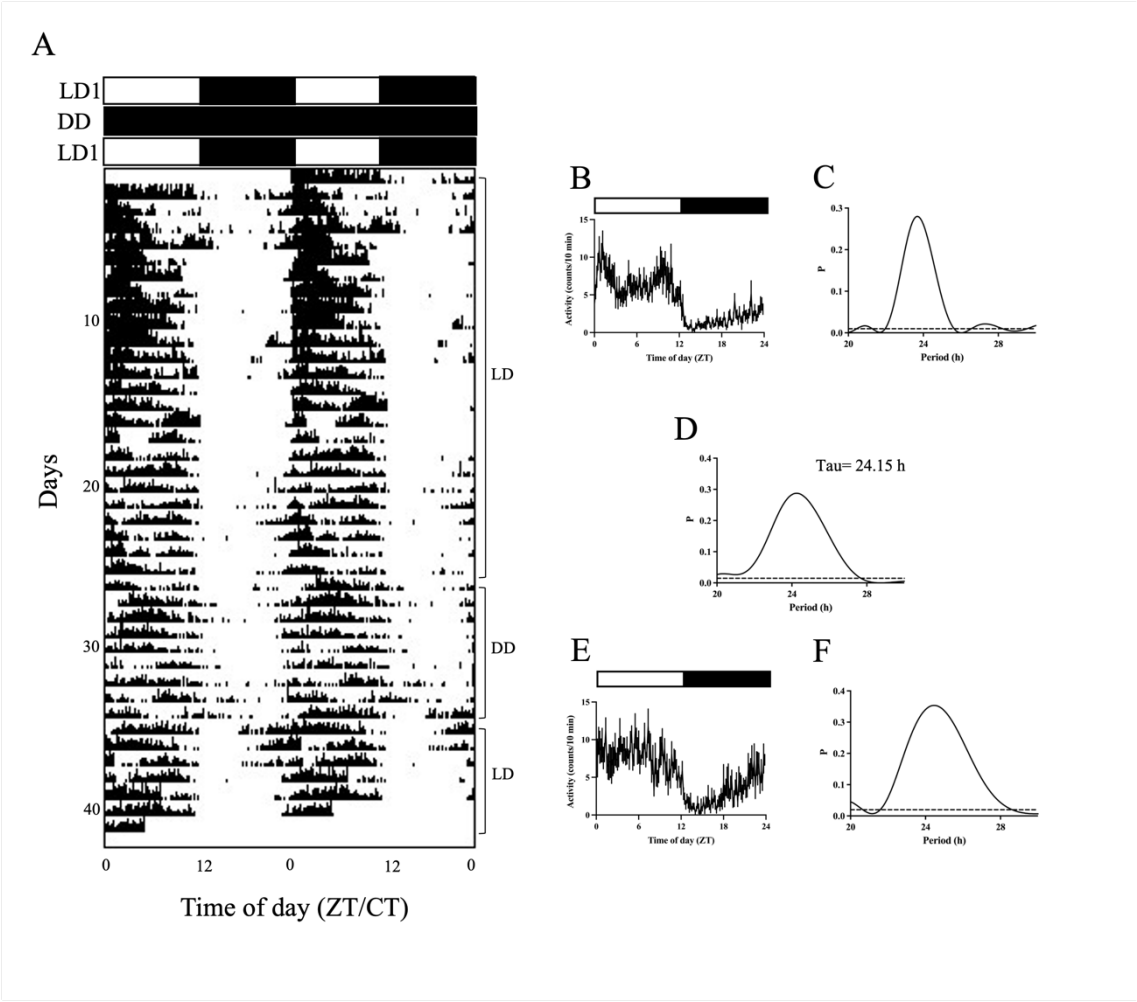

Figure 2. A) Representative double plot actogram of the recording of the locomotor activity of one aquarium (n=6 fish) of the first experimental group. Each horizontal line indicates daily locomotor activity. The height of each point representing the number of infrared light-beam interruptions per 10 min. The aquarium was maintained 26 days under a 12h light:12h dark cycle (light switches on at ZT0 [09:00 h] and off at ZT12 [21:00 h], LD1), 8 days under constant darkness condition (DD) and finally, the initial 12h light: 12h dark cycle (LD1) was restored for 7 days until the end of the experiment. **B)** Mean waveform graph relating to the last 7 days of the LD1. **C)** LS periodogram analysis relating to the last 7 days of LD1. **D)** LS periodogram analysis relating to the DD condition, indicating the period of the rhythm (τ). **E)** Mean waveform graph relating to the last 7 days of the LD1 restoration. **F)** LS periodogram analysis relating to the last 7 days of LD1 restoration. The white and black bars above graphs represent the light and dark phases, respectively.

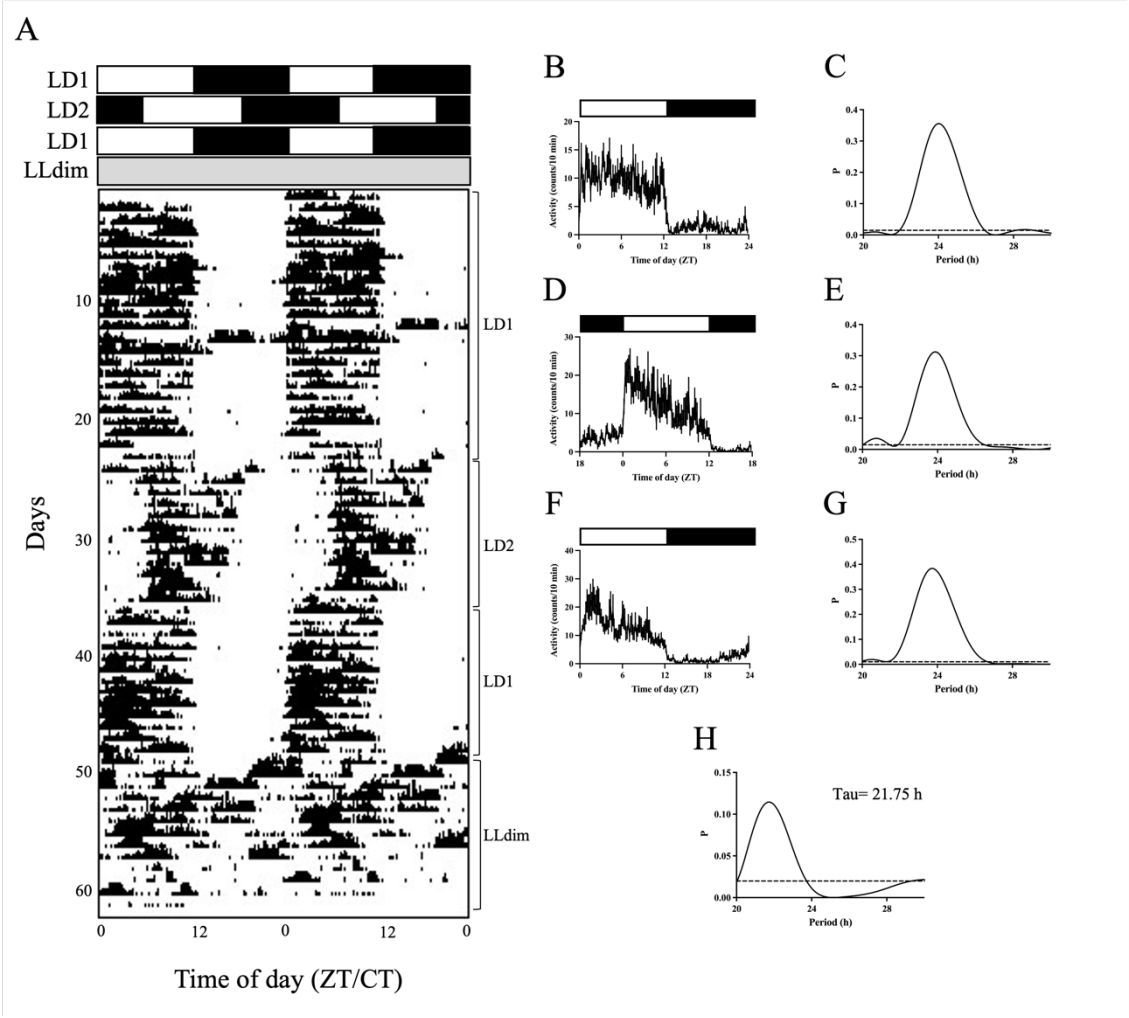

Figure 3. A) Representative double plot actogram of the recording of the locomotor activity of one aquarium (n=5 fish) of the second experimental group. Each horizontal line indicates daily locomotor activity. The height of each point representing the number of infrared light-beam interruptions per 10 min. The aquarium was maintained 23 days under a 12h light:12h dark cycle (light switches on at ZT0 [09:00 h] and off at ZT12 [21:00 h], LD1), 13 days under 6-hours shifted 12h light:12h dark cycle (light switches on at 15:00 h and switches off at 03:00 h; LD2), 13 days under the initial restored 12h light:12h dark cycle (LD1) and finally, under continuous dim light condition (LLdim) until the end of the experiment. **B)** Mean waveform graph relating to the last 7 days of the LD1. **C)** LS periodogram analysis relating to the last 7 days of LD1. **D)** Mean waveform graph relating to the last 7 days of the LD2. **E)** LS periodogram analysis relating to the last 7 days of LD2. **F)** Mean waveform graph relating to the last 7 days of the LD1 restoration. **G)** LS periodogram analysis relating to the last 7 days of LD1 restoration. **H)** LS periodogram analysis relating to the LLdim condition indicating the

period of the rhythm (τ). The white and black bars above graphs represent the light and dark phases, respectively. The grey bar represents the LLdim condition.

Daily variations and rhythms of non-visual opsin gene expression in the brain of blind Mexican cavefish

In the brains of *A. mexicanus* kept under an LD 12:12 cycle, three out of the six non-visual opsin genes analysed (opn3, rgra, and tmt1b) showed significant daily rhythms in transcript levels (Cosinor analysis, $p<0.05$), but statistically significant daily differences in expression were only evident for opn3 (Kruskal-Wallis test, $K = 18.03$, $p = 0.0061$) and rgra (Kruskal-Wallis test, $K = 13.85$, $p = 0.0314$) (Fig. 4, Table 2).

Overall, *opn3, rgra* and *tmt1b* exhibited similar daily expression profiles, with low gene expression levels during the day, peaks/acrophases during the first half of the nocturnal phase (ZT13.48 for *opn3*, ZT15.15 for *rgra*, and ZT16.34 for *tmt1b*, Table 2) followed by a sharp decline in transcript levels during the second half of the night and into early morning (Figs. 4B, C, F). However, more robust daily rhythms were observed for *opn3*, with higher mesor, amplitude and significance when compared to *rgra* and *tmt1b*, as revealed by Cosinor analysis (Table 2).

No significant daily variations or rhythms were detected for *exo-rhod, rgrb*, and *tmt1a* (Figs. 4A, D, E, Table 2).

Gene name	Mesor (r.e.)	Amplitude (r.e.)	Acrophase (ZT)	Significance (*p-value*)
exo-rod	-	-	-	n.s.
opn3	2.19	0.71	13.48	**
rgra	1.08	0.26	15.15	*
rgrb	-	-	-	n.s.
tmt1a	-	-	-	n.s.
tmt1b	1.22	0.32	16.34	*

Table 2. Cosinor parameters of extraocular non-visual opsins in the brain of *Astyanax mexicanus* maintained under 12h light:12h darkness photoperiod (LD) photoperiod conditions during one complete 24 hours cycle. Table shows non-visual opsin genes numeric values of mesor, amplitude, acrophase and significance circadian rhythms (*p-value*) reported by the cosinor analysis. Mesor and amplitude are given as relative expression values (r.e.) and acrophase as zeitgeber time (ZT). Aesthetics indicate statistically significant rhythms: *$p < 0.05$, **$p < 0.01$ and non-significant rhythms were indicated with n.s.

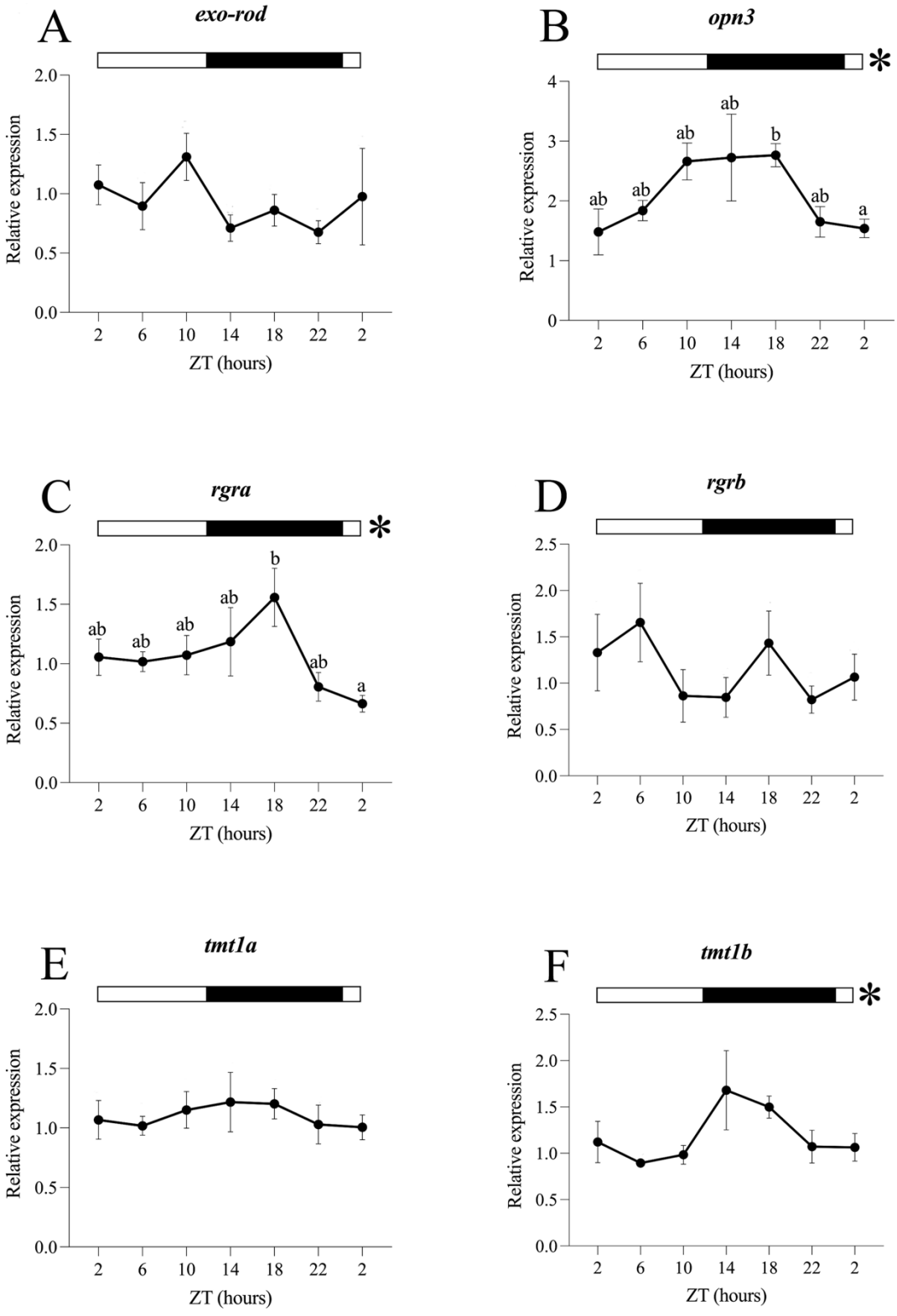

Figure 4. Daily relative expression of non-visual extraocular opsin genes (*exo-rod* **(A)**, *opn3* **(B)**, *rgra* **(C)**, *rgrb* **(D)**, *tmt1a* **(E)** and *tmt1b* **(F)**) in the brain of the blind *Astyanax mexicanus*. Seven animals per point (n=7/time point) were collected every 2 hours during

one complete cycle (24 hours) and mRNA relative expression was analysed by RT-qPCR. X-axis shows time in hours (*Zeitgeber* time, ZT). Letters above graphs indicate statistically significant differences between mean values (p-value < 0.05). Asterisks located next to the photoperiod bars indicated statistically significant daily rhythms (p-value < 0.05). White and black bars above graphs represent the light and dark phases, respectively.

Discussion

The most common and powerful synchronising cyclic environmental signal is the photoperiod or light-dark cycle. Consequently, it can be assumed that animals that have evolved in caves, in constant dark conditions, no longer possess a functional light-entrainable circadian clock as it happens in the blind Somalian cavefish *P. andruzzii* (Cavallari et al., 2011). The blind Mexican cavefish *A. mexicanus* represents another interesting experimental model to investigate and broaden knowledge on circadian clocks and light detection systems. Although due to the environment in which they evolved, blind Mexican cavefish have also lost their functional eyes, this troglomorphic species expresses a number of non-visual opsins in the retina, pineal and brain, and maintains a diurnal activity pattern even after pineal removal (Simon et al., 2019).

The first objective of the present research was to investigate the photic entrainment and a possible circadian endogenous rhythmicity of the eyeless hypogean A. mexicanus. To do so, we performed long-term locomotor activity recording subjecting the fish to different photic regimes (LD, shifted LD, DD and LLdim). The animals displayed a long-term entrainment to the LD 12:12 cycle (LD1) with a diurnal activity pattern, i.e., increased locomotor activity during daytime and lower locomotor activity during nighttime. These results are in agreement with those obtained in previous studies in the same species (Beale et al., 2013; Carlson and Gross, 2018;

Erckens and Martin, 1892; Menna-Barreto and Trajano; 2014; Simon et al., 2019). When the LD cycle was shifted by 6 h (LD2), fish were able to resynchronise immediately to the new cycle without any transient period, further confirming the strong behavioural entrainment of *A. mexicanus* to the photic stimulus. Moreover, when the initial photocycle was restored (LD1) the initial behavioural rhythmic entrainment was also re-established. To prove the endogenous character of circadian rhythms, animals must be isolated from any external temporal signals using constant darkness (DD) or continuous dim light (LLdim) regimes (Herrero et al., 2003; Kuhlman et al., 2018), and we therefore tested both aperiodic conditions. When experimental fish were subjected to DD and LLdim, they continued to show behavioural rhythmicity, manifesting a free-running period within the circadian interval (τ of 24.1 ± 0.2 h in DD and τ of 22.3 ± 0.5 h in LLdim). Interestingly, although the circadian rhythm persists under both constant conditions, the free-running period vary between DD and LLdim. This could be explained by the "Aschoff's First Rule", stating that the endogenous free-running circadian period (tau, τ) observed in complete darkness will shorten for diurnal animals when they are exposed to constant light (Aschoff, 1960). As *A. mexicanus* entrained to a LD 12:12 photocycle showed a diurnal activity pattern, we could expect that $\tau_{LL} < \tau_{DD}$, as was indeed the case in our study and Aschoff's First Rule predicts for diurnal animals (Beaulé, 2009). Moreover, according to the Aschoff's Third Rule, the free-running period in DD was slightly longer than 24 h in this diurnal species (τ of 24.1 ± 0.2 h in DD in the present study). The free-running period could also vary depending on the light history that the organisms have previously experienced (Scheer et al., 2007). In summary, the diurnal activity pattern reported here and the persistence of daily behavioural rhythmicity under constant conditions, supported by chronobiological and statistical analyses, confirms the

existence of an endogenous circadian clock that could entrain the locomotor activity even in the absence of light-dark cycles in this blind cavefish species.

The opsin family is composed of visual opsins, mainly expressed in retinal photoreceptors, and non-visual opsins expressed in both ocular and extraocular tissues. It has been shown that, in addition to visual photoreception, non-visual photoreception also plays a key role in some important biological processes in vertebrates (Cronin and Johnsen, 2016). In this context, many non-visual opsin genes are expressed in extraocular tissues of teleost fish, which might be employed in the detection of the light and in the circadian rhythmicity (Davies et al., 2015; Frøland-Steindal and Whitmore, 2019). According to previous studies and our present results, the blind Mexican cavefish *A. mexicanus* has retained the ability to sense light and to entrain its locomotor activity to the photic stimulus (Beale et al., 2013; Carlson and Gross; 2018; Simon et al., 2019). Which photosensitive organ(s) and/or cell populations may be involved in this response to light still represents an enigma. Previous research has demonstrated that blind cavefish do not show any significant changes in their behavioural response to light after induction of functional eyes, suggesting that extraocular photoreceptors play a relevant role in the photic response shown by this species (Romero et al., 2003). The pineal is a directly photosensitive extraocular organ that contains opsins-expressing photoreceptor cells and plays an important role in the circadian regulation, being involved in the production of the nocturnal time-keeping hormone melatonin (Bertolucci and Foà, 2004; Falcón et al., 2010; Pierce et al., 2008). Melatonin represents a key hormone for the entrainment of daily locomotor activity rhythms in different fish species (Falcón et al., 2010). In zebrafish, it has been reported that the *exo-rhodopsin* gene exhibits a daily rhythmic expression (with a nocturnal acrophase) in the pineal and is involved in the synthesis of melatonin in response to light in

this organ (Pierce et al., 2008). Exo-rhod pigments have also been implicated in the origin of the phototactic behaviour in the Somalian blind cavefish *Phreatichthys andruzzii* (Tarttelin et al., 2013). The blind cavefish *A. mexicanus,* despite the evolution and adaptation to a complete dark environment, has retained a functional pineal able to detect light signal and with high transcript levels of the non-visual opsin *exo-rhodopsin* (Simon et al, 2019; Yoshizawa and Jeffery, 2008). Therefore, a tentative hypothesis was that the pineal may be involved in regulating the shift in locomotor activity between day and night in this species. However, this failed to be supported by research performed by Simon and coworkers (2019), as both pinealectomised and control blind Mexican cavefish showed the same diurnal pattern of activity, indicating that the photosensitive organ(s) responsible for entraining locomotor activity to the photic stimulus should be extraocular and extrapineal. In our research, we did not find any significant daily variation or rhythm in the expression of *exo-rhodopsin* in the brain of *A. mexicanus*, suggesting that this non-visual opsin is not involved in the photic entrainment in this species.

In contrast, we found significant daily variations and/or rhythms in transcript levels of three genes encoding non-visual opsins (opn3, rgra and tmt1b), all of them exhibiting nocturnal acrophases. In previous studies in teleost fish, encephalopsin (Opn3) and Tmt-opsins have been found to play an active and functional role as light sensors and Tmt-opsins could work as upstream elements of the peripheral clock light input pathway (Cavallari et al., 2011; Fischer et al., 2013; Koyanagi et al., 2013). Based on the expression pattern of the opn3/tmt-opsins in the retina and brain of medaka revealed by in situ hybridization, it has been suggested that these blue light sensors present in the fish retina and brain may be involved in the integration of visual inputs, vestibular function, somatosensation, motor outputs, and

pituitary endocrine regulation (Sato et al., 2021). RGR-opsins are known to act as photoisomerases and they were originally described in the retinal pigment epithelium (RPE) of mammals (Jiang et al, 1993). In the flatfish *Paralichthys olivaceus*, both *rgra* and *rgrb* were highly expressed in the eye but weakly expressed in the brain, heart, testis and fin. But in contrast to mammals, retinal *rgra* and *rgrb* mRNA-positive signals were detected in the ganglion cell layer but were absent in the intracellular compartment of RPE of this flatfish species (Liu et al., 2020). A study on *A. mexicanus* found that *rgra* expression was upregulated in blind fish embryos compared to surface specimens at the same development stage, and it has been suggested a possible role of this non-visual opsin in light-induced DNA repair processes and in other physiological processes (Carlson et al., 2018). Further comprehensive studies need to be conducted to elucidate the functions of rhythmically-expressed *opn3*, *rgra* and *tmt1b* in *A. mexicanus*

In conclusion, our research has revealed the existence of endogenous circadian rhythms in the locomotor activity of *A. mexicanus* and suggested that daily rhythms in extraretinal non-visual opsins present in the brain are able to transduce daily photic cycles and could be sustaining this behavioural and other light-entrained rhythms in this species. This research may represent one more step towards an ever more complete understanding of the complicated brain photoreception system and the relationship of this with synchronising circadian clocks. Future research should be directed to elucidate precisely which brain areas and cells masses express these rhythmic non-visual opsins, to determine their G protein coupling and signalling mechanisms in these tissues and cells and to characterise the effects of light intensity and spectrum on the expression of non-visual opsins and on the entraining of locomotor activity rhythms and other behavioural and physiological rhythms in blind Mexican cavefish.

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CRediT authorship contribution statement

Francesca Conti: Conceptualization, Investigation, Formal analysis, Data curation, Writing – original draft; **Alba Vergès-Castillo**: Investigation, Formal analysis, Data curation, Writing – original draft; **Francisco J. Sánchez-Vázquez**: Conceptualization, Formal analysis, Writing – review and editing, Funding acquisition; **José F. López-Olmeda**: Conceptualization, Formal analysis, Writing – review and editing; **Cristiano Bertolucci**: Writing – review and editing; **José A. Muñoz-Cueto**: Conceptualization, Formal analysis, Writing – review and editing, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data that support the findings of this study will be made available on request.

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Chapter III.

Food intake regulation mechanisms of alternative feeds in Nile tilapia and Gilthead Seabream

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Introduction

Feeding is a complex behaviour, essential to living beings, which can be affected by both external (e.g., environment, season, time of day, availability of food, stress) and internal aspects (e.g., circulating levels of nutrients such as glucose, or hormones such as insulin; Volkoff et al., 2009). Indeed, the body needs to monitor energy stores and expenditure to control homeostasis by regulating food intake. Energy balance is maintained by the secretion of neurohormones from the brain feeding centres that promote (orexigenic neuropeptides, e.g., neuropeptide Y, Agouti-related neuropeptide and orexins) or inhibit (anorexigenic neuropeptides, e.g., cocaine- and amphetamine-regulated transcript) appetite and subsequent feeding behaviour, depending on the nutritional status and metabolic needs of the individual (Näslund & Hellström, 2007; Barrios, 2014; Conde-Sieira & Soengas, 2016; Volkoff, 2016; Delgado et al., 2017). In addition, feeding centres are also strongly affected by information (chemical, mechanical and endocrine) received from the periphery (e.g., gastrointestinal tract GIT and adipose tissue; Kuz'mina, 2019; Volkoff, 2019).

Due to the large numbers of fish species, habitats and feeding habits the food intake regulation mechanisms can be species-specific, involving many different hormones and tissues (Hoskins & Volkoff, 2012; Soengas et al., 2018). Indeed, unlike mammals, fish feeding centres are not limited only to the hypothalamus but may be more widespread in their brains (Cerda-Reverter & Canosa, 2009).

Moreover, as mammals, fish can experience feeding behaviour solely driven by sensory perception of palatable food (Saper et al., 2002; Rossi & Stuber, 2018; Soengas et al., 2018). This brain reward circuitry is part of the hedonic pathway involved in the food intake independent of energy requirements and only based on previous learned experiences

(Kulczykowska & Sánchez Vázquez, 2010). Hedonic feeding is regulated by central monoamine neurotransmitters (e.g., dopamine, noradrenaline and serotonin), opioids (e.g., beta-endorphin), and endocannabinoids (Le Merrer et al., 2009; Bojanowska & Ciosek, 2016; Díaz-Rúa et al., 2022).

Aquaculture is one of the fastest growing sectors in terms of providing food for the world's population. In recent years, fish production has become subject of attention from an environmental and economic sustainability perspective. Indeed, the focus of consumers is mainly on buying and consuming affordable and healthy products (Mancuso et al., 2016; Boyd et al., 2020). In this scenario, a major attention has been paid to alternative ingredient fish feeds to replace sources that negatively impact the environment (e.g., soy, fish meal and oil; Ghamkhar, & Hicks 2020; Hilmarsdóttir et al. 2022). Thus, research field has focused on testing emergent ingredients (e.g., cyanobacteria, microalgae, yeast, sunflower, quinoa, insect meal) and their effects on fish, to meet consumer interests and promote sustainability in this sector (Trosvik et al., 2012: Van Huis, 2013; Barroso et al., 2014; Molina-Poveda et al., 2017; Glencross et al., 2020; Li et al., 2022; Mendes et al., 2024).

Thus, the present work aims to investigate the potential effects of emergent ingredient feeds on molecular brain mechanisms that regulate feed intake (orexigenic, anorexigenic and reward signals) of two commercially important fish species, freshwater red Nile tilapia *Oreochromis niloticus* and marine gilthead seabream *Sparus aurata*, by using the Real-Time qPCR technique.

Materials and Methods

Ethic Statement

The Nile tilapia trial was carried out at the University of Trás-os-Montes e Alto Douro (UTAD, Vila Real, Portugal), by trained scientists (following category B FELASA recommendations) according to the European Parliament and European Union Council guidelines on the protection of animals used for scientific purposes (Directive 2010/63/EU, 2010). The Gilthead seabream trial was carried out at the Ramalhete Experimental Research Station of the Centre of Marine Sciences of Algarve (CCMAR, Faro, Portugal). Trained scientists performed the trial, following the European Directive 2010/63/EU of European Parliament and of the Council of European Union on the protection of animals used for scientific purposes, being approved by the Committee of Ethic and Animal Experimentation of CCMAR.

Animals and housing

A total of 396 juveniles of red Nile tilapia (*Oreochromis niloticus*) supplied from a commercial hatchery (Til-aqua, Someren, the Netherlands) were transferred to the closed recirculating system of the UTAD experimental facilities and placed in 12 fiberglass tanks of 300 L (n= 33 fish/tank; average weight of 30.78 ± 0.11 g). The RAS system was equipped with a mechanical filter, a biological filter, UV sterilizer and mechanical aeration for oxygenation on each tank. Dissolved oxygen in water was 4.30 ± 1.2 mg/l. Water temperature was maintained constant at 24.4 ± 1.2 °C and a 12h light: 12h dark photoperiod (light switches on at 08:00 h and off at 20:00 h). During the acclimation period fish were fed a control diet (Standard 4 Orange, Portugal) twice per day, by hand *ad libitum*.

A total of 810 juveniles of Gilthead seabream (*Sparus aurata*) were supplied from a commercial company (Atlantik Fish Lda; Castro Marim, Portugal) and carried to the Ramalhete Experimental Research Station. Nine homogenous groups (n= 90 fish/tank; average weight of 14.1 ± 0.02g) were formed in outdoor cylinder fiberglass tanks of 500 L. The acclimation period lasted three weeks, during which fish were fed a commercial diet (Standard 4 Orange, Sorgal, Portugal) twice per day. Tanks were supplied with flow-through, gravel-filtered, aerated seawater and subjected to natural photoperiod changes through summer conditions (from April to August). Water temperature ranged from 18.8 °C to 27.3 °C (mean ± SD 23.5 ± 2.1 °C), salinity was 37.8 ± 0.4 ‰ and oxygen saturation was 96.3 ± 1.4 %.

Experimental protocol and sampling

Regarding the Nile tilapia trial, the three experimental diets of interest, practical (PD), organic (ORG) and eco-efficient (ECO) were formulated and produced by SPAROS Lda. (Olhão, Portugal). The formulation concept and ingredient selection (Table 1A) was chosen within an eco-efficient and organic framework, on market availability and nutritional composition. The control diet (PD) includes ingredients that mimic commercial formulations. The alternative two diets (ORG and ECO) were formulated to include functional ingredients (e.g., spirulina and quinoa), emergent and low carbon-footprint alternatives replacing those ingredients that may raise environmental concerns. The three diet treatments were randomly assigned to replicate tanks (n = 4 tanks/ diet). Fish were fed the three experimental diets by hand *ad libitum* twice per day, at 10:00 h and 15:00 h for 60 days. On day 3 and 60 of the trial, three fish per tank (n=36 fish) were fasted for 6 hours and euthanized by 2-phenoxyethanol (900mg/L; Sigma-Aldrich, Spain) to dissect brains, after being individually weighed. Brain samples (n=

12/diet) were preserved in 500-600 µl of RNA later (Sigma-Aldrich, Spain) at -20 °C for further molecular analysis

Regarding the seabream trial, the three experimental diets of interest, control (CTRL), organic (ORG) and eco-efficient (ECO) were also formulated and produced by SPAROS Lda. (Olhão, Portugal) within a circular economy or organic framework, on market availability and nutritional composition (Table 1B). The control feed (CTRL) mimics a commercial formulation, being soy-free and with medium levels of land animal by-products (LAPs). The organic (ORG) feed was designed to include ingredients compatible with organic certification (microalgae, yeast) and plant sources (e.g., pea protein, potato protein, rapeseed meal and oil) to replace fishmeal and LAPs. The eco-efficient (ECO) feed was formulated with similar ingredients to those used in the CTRL one, but with higher inclusion levels of LAPs (feathermeal hydrolysate, poultry meal and poultry blood meal) limiting fishmeal.

The three diet treatments were randomly assigned to replicate tanks (n = 3 tanks/ diet). Fish were fed the three experimental diets by hand *ad libitum* three times per day from Monday to Saturday, at 09:45 h, 11:45 h and 16:00, and twice per day on Sundays, at 09:45 h and 11:45 h, for 65 days. On day 65 of the trial, four fish per tank (n=36 fish) were fasted for 6 hours, individually weighed and euthanized by 2-phenoxyethanol (1000 mg/L; Sigma-Aldrich, Spain) to collect brains. Tissue samples (n= 12/diet) were placed in 500-600 µl of RNA later (Sigma-Aldrich, Spain) and stored at -20 °C for further molecular analysis.

A)

Ingredients (% inclusion levels)	PD	ORG	ECO
Poultry meal	5.00		2.50
Porcine blood meal			5.00
Feathermeal hydrolysate			5.00
Insect meal			7.50
Microbial biomass			5.50
Brewer's yeast		10.00	5.00
Spirulina		10.00	2.50
Soy protein concentrate	5.00		
Pea protein concentrate		5.00	
Corn gluten meal	12.00		
Soybean meal	25.00	12.50	
Rapeseed meal	13.00	26.00	13.00
Sunflower meal	7.50	15.00	15.00
Wheat (whole)	13.90		15.61
Rice bran	9.78	9.78	
Quinoa		5.00	2.50
Whole peas			11.00
Vitamin and mineral premix	1.00	1.00	1.00
Choline chloride	0.20	0.20	0.20
Antioxidant powder	0.20	0.20	0.20
Mono-calcium phosphate	2.55	2.00	2.75
L-Lysine	0.30		0.30
DL-Methionine	0.15		0.22
Yttrium oxide	0.02	0.02	0.02
Salmon oil	2.00	2.00	2.00
Rapeseed oil	2.40	1.30	3.20

B)

Ingredients (% inclusion levels)	CTRL	ORG	ECO
Fishmeal Super Prime	15.00	15.00	
Fishmeal	5.00		5.00
Fish protein hydrolysate	3.00		3.00
Poultry meal	15.00		20.00
Poultry blood meal	3.00		5.00
Feathermeal hydrolysate	5.00		10.00
Microbial meal	4.00		4.00
Brewer's yeast		5.00	
Arthrospira platensis		5.00	
Potato protein concentrate		8.90	
Pea protein concentrate		11.00	
Wheat gluten		11.00	
Corn gluten meal	8.00		5.70
Guar korma	5.00	9.50	5.00
Rapeseed meal		4.50	
Sunflower meal	3.00		6.00
Wheat meal	13.48	7.58	12.63
Whole peas	5.50	5.50	5.50
Vitamin and mineral premix	1.00	1.00	1.00
Choline chloride	0.20	0.20	0.20
Antioxidant	0.20	0.20	0.20
Mono-calcium phosphate		1.10	1.65
L-Lysine[23]			0.50
DL-Methionine			0.05
Yttrium oxide	0.02	0.02	0.02
Algae meal (*Schyzochytrium* spp)			1.30
Rapeseed lecithin	0.50	0.50	0.50
Fish oil	4.45	7.00	4.45
Salmon oil	8.65		8.30
Rapeseed oil		7.00	

Table 1. Diet formulation (% inclusion levels) of A) the experimental diets (PD, ORG and ECO) for Nile tilapia (*Oreochromis niloticus*) and of B) the experimental diets (CTRL, ORG and ECO) for Gilthead seabream (*Sparus aurata*).

Gene expression analysis

Total RNA was isolated from brain tissues using Trizol reagent (Thermo Fisher Scientific, USA) following the manufacturer's instructions. The amount, quality and composition of isolated RNA were analysed by BioSpec-nano (Shimadzu, Japan). cDNA was synthesized from 1 mg of Dnase-treated RNA using iScript™ cDNA Synthesis Kit (Bio-Rad, USA) and Real-Time qPCR was carried out using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, USA) in triplicate on a CFX Connect Real-Time PCR Detection System (Bio-Rad, USA) instrument. Relative expression of target genes (Table 2 and 3) was calculated using the ΔΔCt method (Livak & Schmittgen, 2001) with elongation factor 1a (*ef1a*) and glyceraldehyde-3-phosphate dehydrogenase (*gadph*) as reference genes for Nile tilapia (Table 2) and ribosomal protein S18 (*rps18*) and beta-actin (*bactin*) as reference genes for Gilthead seabream (Table 3).

	Name	Accession number	Sequence (5'-3')
Orexigenic	**nt_*npy*_F** **nt_*npy*_R**	KJ778894.1	TGAAGGAAAGCACAGACACG GAATATACGTGGCGGCTCAT
Orexigenic	**nt_*agrp*_F** **nt_*agrp*_R**	MT740813.1	GACACGTCTGCAGCCTTACA CAGTAGCAGATGGCGTTGAA
Orexigenic	**nt_*hcrt*_F** **nt_*hcrt*_R**	ENSONIG00000001128	CGCCTTTATGTGTTGCTGTG TCGTCCTCTTTTCGTTTGCC
Orexigenic	**nt_*mch*_F** **nt_*mch*_R**	AF534542	CTGACAGTGGCATTACCCAT GTCCCTCCACAGGTTTGCAT
Orexigenic	**nt_*apln*_F** **nt_*apln*_R**	XM_013271331	CTGGTGATTGTGCTCTTGGTGT GCCCCTTGCAGAGTTTTGCT
Anorexigenic	**nt_*cart*_F** **nt_*cart*_R**	XM_003455220.5	CTCGGACTCACAACCAGTGA AGTAGCAGCGCAGGAAGAAG
Anorexigenic	**nt_*crh*_F** **nt_*crh*_R**	XM_003443615	AACGGGGACTCGAACTCTTT TTTGCCCTGTAAAAGACGCC
Reward	**nt_*drd2*_F** **nt_*drd2*_R**	ENSONIG00000006162	AGTGGCGCTTTAGCAAGATC AGCATTGGCATTGCAACTGC
Reward	**nt_*tph1*_F** **nt_*tph1*_R**	ENSONIG00000008252	AAGATGTCTCACGCTTCCTCAG AAAACGCGGAATGCCAAACC
Reward	**nt_*htr1a*_F** **nt_*htr1a*_R**	ENSONIG00000021056	TGCGCAATATTTGGGAACGC ATTGGCCACATTCTGGAGAGAG
Reference	**nt_*gadph*_F** **nt_*gadph*_R**	JN381952	GATAATGGCAAACTTGTCGTCG ACATTGGAGCATCGGGTGAG
Reference	**nt_*ef1a*_F** **nt_*ef1a*_R**	AB075952	AGACAACATGCTGGAGACCA CTCCTTGCGCTCAATCTTCC

Table 2. Sequences of primers used for the characterization of Nile tilapia brain food intake regulation signals. Accession number is also indicated.

	Name	Accession number	Sequence (5'-3')
Orexigenic	**sb_*npy*_F** **sb_*npy*_R**	XM_030411288.1	GGAGCTGGCCAAGTACTACTCA GAGACCAGCGTGTCCAGAAT
Orexigenic	**sb_*agrp*_F** **sb_*agrp*_R**	MG570185	CCAACAGTCCTGTCTGGGTTA CAGTAGCAGATGGCGTTGAA
Orexigenic	**sb_*ghrl*_F** **sb_*ghrl*_R**	MG570187	CCCGTCACAAAAACCTCAGAAC TTCAAAGGGGGCGCTTATTG
Anorexigenic	**sb_*cart*_F** **sb_*cart*_R**	MG570186	CTGAGGAGCAAAGAGATGCCCTTA GAGAAA GCGTCACACGAAGGCAGCCA
Anorexigenic	**sb_*crh*_F** **sb_*crh*_R**	KC195964	ATGGAGAGGGGAAGGAGGT ATCTTTGGCGGACTGGAAA
Reference	**sb_*rps18*_F** **sb_*rps18*_R**	AM490061.1	GGGTGTTGGCAGACGTTAC CTTCTGCCTGTTGAGGAACCA
Reference	**sb_*bactin*_F** **sb_*bactin*_R**	X89920	TCCTGCGGAATCCATGAGA GACGTCGCACTTCATGATGCT

Table 3. Sequences of primers used for the characterization of Gilthead seabream brain food intake regulation signals. Accession number is also indicated.

Statistical analysis

Prism 9.3 software (GraphPad, Dotmatics, USA) was used for statistical analyses. One-way ANOVA was performed to analyse relative gene expression. When the assumptions of the parametric analysis were violated, the non-parametric Kruskal-Wallis test followed by the Dunn's post-hoc tests corrected for multiple comparison was performed. Statistical significances were accepted when $p < 0.05$.

Results

During the tilapia trial, there are major differences across the dietary treatments for the growth performance. Specifically, diet PD was well accepted, but diet ECO and especially ORG received a negative response from the fish. Thus, fish fed the PD diet almost quadruplicate their initial body weight (107.80 ± 5.32 g), fish fed the ECO diet duplicated their weight (62.73 ± 4.64 g), while fish fed the ORG one maintained their weight and almost no growth occurred (32.74 ± 1.14 g). Therefore, in terms of growth performance the supplementation with spirulina and quinoa (ORG and ECO diets) did not have a positive significative impact in fish fed diets with these ingredients. Brain gene expression analysis, on day 3 after the start of the trial, show significative differences between the three diets administrated regarding signals involved in the regulation of food intake. For the orexigenic signals, there is a significant effect of the diets on gene expression levels for the Agouti-related peptide AgRP (ANOVA, $F_{2,29}= 3.742$, $p <0.05$; Figure 1C), for the Hypocretin neuropeptide precursor HCRT (ANOVA, $F_{2,27}= 5.743$, $p <0.001$; Figure 1E), for the preprotein generating the Pro-Melanin Concentrating Hormone MCH (ANOVA, $F_{2,30}= 3.554$, $p <0.05$; Figure 1G) and for the Apelin (ANOVA, $F_{2,29}= 3.474$, $p <0.05$; Figure 1I). Specifically, for the first three genes, the ORG diet and the control PD diet differ significantly, while for *apln* gene, ECO diet and the PD one differ

significantly (Figures 1C, E, G and I). Regarding the two anorexigenic factors investigated, both the neuropeptide Cocaine and Amphetamine Regulated Transcript CART and the Corticotropin Releasing Hormone CRH show significative difference in their expression among diets (Kruskall-Wallis; *cart*: K= 10.33, p <0.001; *crh*: K= 19.42, p <0.0001). Specifically, in *cart* expression, the ORG diet differs significantly from the PD diet; while in *crh* expression, both ORG and ECO differ significantly from the PD diet (Figures 1K and M). Finally, regarding the brain reward signals, none of the investigated genes exhibit a significant difference in their expression between diets (Figures 1O,Q and S). After 60 days of trial, brain gene expression analysis show significative differences in the signals involved in the regulation of feed intake between the three dietary treatments. Regarding the orexigenic factors, there is a significant effect of the diets on gene expression levels for the Neuropeptide Y NPY (ANOVA, $F_{2,32}$= 5.057, p <0.05), for AgRP (ANOVA, $F_{2,32}$= 14.88, p <0.0001) and for the Apelin (ANOVA, $F_{2,32}$= 7.955, p <0.001). Specifically, for *npy* gene the ORG and ECO diets differs significantly (Figure 1B), for *agrp* gene all three diets differ significantly (Figure 1D) and for *apln* gene, ECO diet differs from both ORG and PD significantly (Figures 1J). Regarding the anorexigenic factors, in *cart* expression, both ECO and ORG diets differ significantly from PD diet, while in *crh* expression ECO and ORG diets differ significantly (ANOVA; *cart*: $F_{2,31}$= 9.077, p <0.001; *crh*: $F_{2,31}$= 4.945, p < 0.05; Figures 1L and N). Finally, only the gene expression for the Dopamine receptor D2 displays a significant difference among the diets, with ECO that differs from ORG and this differs from PD diet (ANOVA, $F_{2,32}$= 15.00, p <0.001; Figure 1P).

During the seabream trial, fish responded positively to the three dietary treatments, tripling or more their initial body weight with a better

result observed in the ECO diet (47.8 ± 1.4 g for the CTRL diet; 42.6 ± 0.3 g for the ORG diet and 48.8 ± 1.6 for the ECO diet). Brain gene expression analysis exhibit no significative differences among the three diets administrated regarding signals involved in the central regulation of food intake that we analysed (Figures 2A-E).

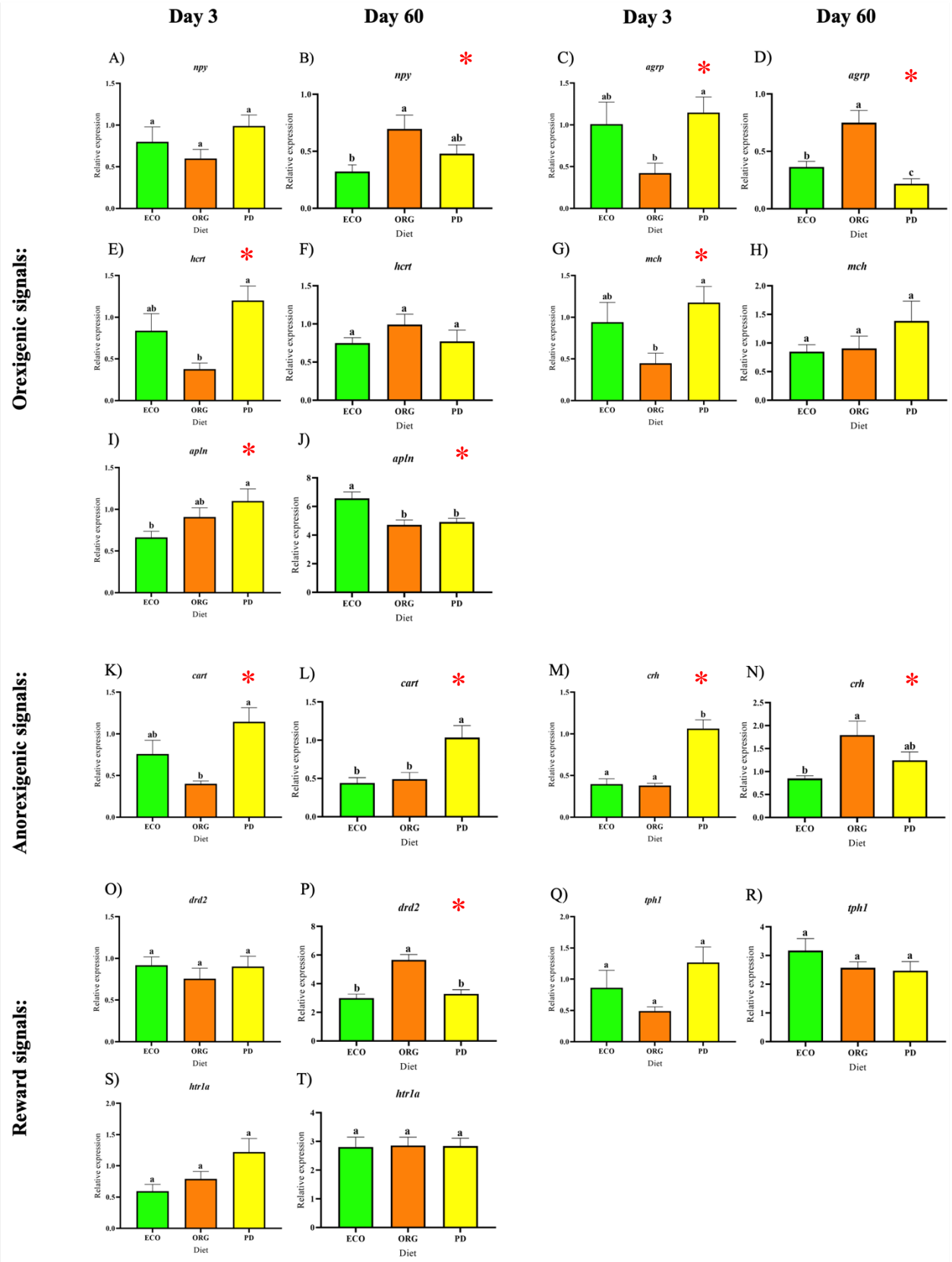

Figure 1. Relative expression of orexigenic (**A-J**), anorexigenic (**K-N**) and reward (**O-T**) signals genes in the brain of the red Nile tilapia (*Oreochromis niloticus*) fed three different diets (ECO, ORG and PD) at day 3 and at day 60 of the trial. Bars represent the

mean ± SEM (n= 12/diet). Different letters indicate significant differences (one-way ANOVA or Kruskal-Wallis; p <0.05). Red asterisks indicate genes that present statistical difference among the diets.

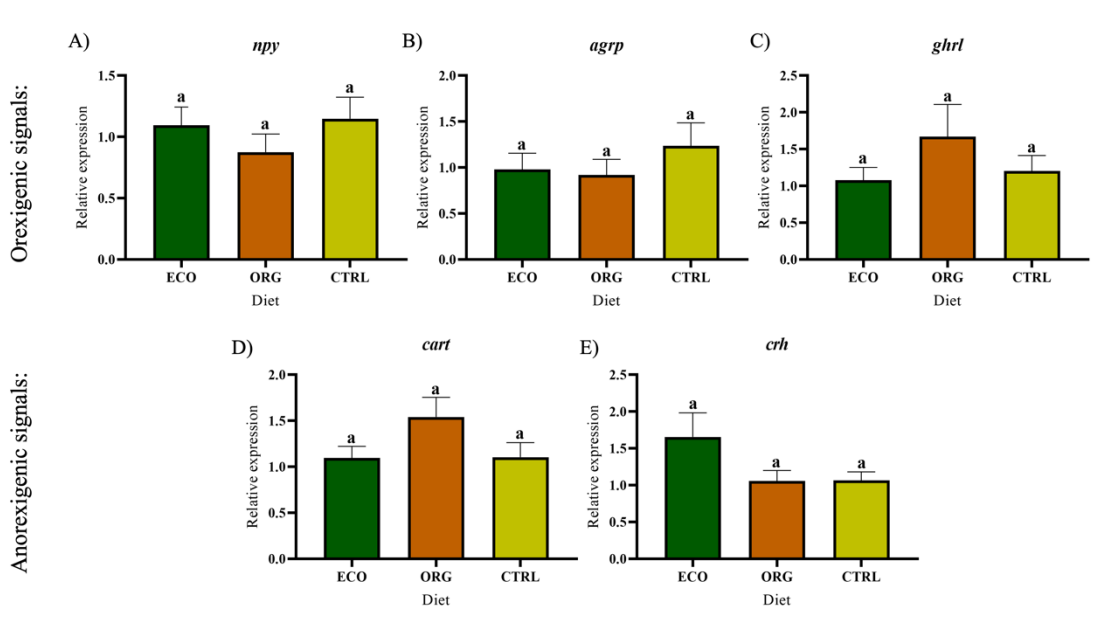

Figure 2. Relative expression of orexigenic (**A-C**) and anorexigenic (**D-E**) signals genes in the brain of the Gilthead seabream (*Sparus aurata*) fed three different diets (ECO, ORG and CTRL) at day 65 of the trial. Bars represent the mean ± SEM (n= 12/diet). Different letters indicate significant differences (one-way ANOVA or Kruskal-Wallis; p <0.05).

Discussion

The research field has been investigating different alternatives to replace marine ingredients with protein-rich plant derivatives and emergent sources to be applied in the aquaculture sector, in a more sustainable vision to impact less negatively on the environment. Spirulina represents a nutritionally balanced ingredient to be included in aquafeeds, replacing fish oil (Hanel et al., 2007; Mosha, 2019). However, our study on Nile tilapia showed that the diet with higher inclusion of spirulina (ORG) was the one fish responded most negatively in terms of growth performance and feed intake. Indeed, after 60 days of trial fish fed to maintain their weight resulting in a very low weight gain (0.08 %). Our results disagree with previous

findings on dietary self-selection where tilapia exhibited a preference for the diet with higher inclusion of spirulina (Mendes et al. 2024). However, it has to be stated that experiments on dietary selection do not necessarily correlate the most selected diet with optimal performance (Fortes-Silva et al., 2012).

Gene expression analysis of the brain signals regulating food intake reflect the results of the growth performance. On day 3 of the trial, a lower expression of the orexigenic genes (*agrp*, *hcrt* and *mch*) can be observed in fish feeding the ORG diet, differing significantly only from the PD control diet. After 60 days of trial, the expression pattern is almost the opposite, with a higher level of expression of orexigenic signals (*npy* and *agrp*) and a decreased level of expression for the anorexigenic *cart* gene in fish brains fed the ORG diet. The *npy* and *agrp* genes have been identified and studied in several fish species, including goldfish (*Carassius auratus*; Narnaware & Peter, 2001; Cerdá-Reverter & Peter, 2003), zebrafish (*Danio rerio*; Song et al., 2003; Yokobori et al., 2012), sea bass (*Dicentrarchus labrax*; Leal et al., 2013; Agulleiro et al., 2014), Nile tilapia (*Oreochromis niloticus*; Yan et al., 2017; Liu et al., 2020) and Gilthead seabream (*Sparus aurata*; Babaei et al., 2017) pointing out their appetite-stimulator role and consequent increased feeding behaviour. Indeed, food deprivation and fasting cause an increase in the expression of hypothalamic *npy* and *agrp* and their intracerebroventricular (ICV) administration stimulates food intake (Assan et al., 2021). Our results confirm the orexigenic role of these peptides, as fish fed the ORG diet at day 60 displayed higher levels of orexigenic signals expression because they were eating little just to maintain their weight. On the other hand, the fish fed PD diet showed lower levels of orexigenic factors expression reflecting the growth performance, indeed the almost quadruplicate their weight after 60 days. Fish fed ECO diet showed an intermediate situation not differing significantly from PD for *npy* but

differing significantly from PD for *agrp*; also reflecting the growth obtained. Consequently, gene expression of the anorexigenic *cart* is lower in fish fed ORG and ECO, whereas it is higher in fish fed PD. CART injection decreases food intake in fish (Volkoff & Peter, 2000) and fasting/food deprivation causes a lower *cart* brain expression confirming its anorexigenic role as appetite suppressant (Volkoff & Peter, 2001; Nishio et al., 2012; Wan et al., 2012). Apelin is a peptide that was first identified in bovine stomach (Habata et al., 1999) and it is known to control cardiovascular functions in mammals (Assan et al., 2021). In fish, this peptide seems to act as orexigenic. Indeed, apelin injections increase food intake in fish (Volkoff & Wyatt, 2009; Penney & Volkoff, 2014) and fasting increases apelin mRNA expression in their brain (Lin et al., 2014; Volkoff, 2014). However, in our research, apelin (*apln*) expression pattern in the different dietary treatments does not resemble that of the other orexigenic genes; with the highest level of expression in fish fed with ECO. This could be explained as apelin may interact with others food intake regulating signals, both orexigenic and anorexigenic, possibly altering its expression profile (Volkoff, 2014; Yan et al., 2020). In addition, this brain signal has still been little studied in fish and therefore further studies are needed to investigate its role and whether it may also be species specific. At day 3 of the trial, both *hcrt* and *mch* exhibited an expression profile as *agrp*, with high levels in PD fed fish differing significantly from ORG. However, at day 60, there is no longer any significant difference between the three dietary treatments in the expression of these orexigenic genes (Novak et al., 2005; Nakamachi et al., 2006; Volkoff, 2016). Regarding the other anorexigenic signal analysed *crh*, at day 3 its expression is significantly higher in fish fed PD diet while at day 60 is higher in fish fed ORG diet differing significantly from fish fed ECO. It has been proved that , in fish, CRH peptide impacts on food intake, as well as locomotor and psychomotor activities, consequently regulating both feeding

and psychophysiological activity such as anxiogenic- or anxiolytic-like behaviours (Bernier, 2006; Matsuda et al., 2008; Matsuda , 2013). Thus, the *crh* relative expression could be also strongly affected by anxiety and stress and this might make it difficult to link its expression profile exclusively to dietary treatments.

In addition, feeding behaviour can be also driven by the hedonic pathway through central monoamine neurotransmitters. Thus, we investigated some genes involved in the brain reward circuitry (Dopamine Receptor D2 *drd2*, Tryptophan hydroxylase 1*tph1* and 5-hydroxytryptamine receptor 1A *htr1a*). We found statistically significant difference between the three diets only in the expression of the *drd2* gene at day 60; with higher expression in fish fed ORG differing from fish fed PD and ORG. Dopamine is the major transmitter in the brain reward networks, and important for motivational processes and stress coping (Scornaiencki et al., 2009; Koob & Volkow, 2016). The effects of dopaminergic system on feeding behaviour have been less explored in fish, except for some studies that proved that dopamine inhibits food intake in goldfish, sea bass and Chinese perch *Siniperca chuatsi* (De Pedro et al., 2001; Leal et al. 2013; He et al., 2018). However, De Pedro et al. (2003) proved that fasted tenches (*Tinca tinca*) exhibited higher levels of hypothalamic dopamine that could be related to a stress-induced response to starvation. In the present study, as fish fed ORG diet displayed higher levels of *drd2* gene expression, this finding could be associated to a stress-related effect given that fish did not like this diet.

In the sea bream trial, an almost opposite situation occurred. Indeed, fish positively accepted the alternative diets (ORG and ECO), tripling or more their initial body weight with a better result observed in the ECO diet after 65 days of trial. This result could relate with previous findings where sea bream did not show a preference for any feed (Mendes et al., 2024).

Gene expression analysis confirm growth performance results as we did not find any statistically significant difference among the dietary treatments in the expression levels of both orexigenic and anorexigenic genes. Thus, it possible to state that the proposed alternative diets (ORG and ECO), which both have limited inclusion of fishmeal replaced with single cell microorganisms as cyanobacteria, microalgae and yeast and plant sources (ORG) and a higher inclusion levels of land animal by-products (ECO), did not influence the central food intake control mechanisms. Thus, these alternative feeds could be a good and viable alternative to the diets rich in marine ingredients used in the aquaculture industry, in this commercially interesting species. Our results agree with previous studies that proved that a partial inclusion of *Arthrospira platensis* replacing fish oil in aquafeeds can improve the growth performance, increase antioxidant responses and does not affect the survival of the fish (Macias-sancho, 2014; Rosas et al., 2019; Galafat et al., 2022).

In addition, there might be differences in appetite-regulating genes in herbivorous as Nile tilapia and carnivorous fish as Gilthead seabream since they have distinct appetite and feeding behaviours. Indeed, one study in grass carp *Ctenopharyngodon idella* proved gene expression changes in the brain between the two contrasting feeding habits (He et al., 2015; Ahi et al., 2020). Furthermore, it may be triky to consider diet alone in the expression pattern of these genes because they are influenced by many internal and external components as stress, sexual maturity, life stage, environmental conditions of the individuals (Volkoff, 2016).

Conclusions

Our study proved that it is best to avoid using a diet with high levels of spirulina inclusion in Nile tilapia (ORG). In fact, fish did not positively accept this diet after a 60-day trial probably due to poor palatability and taste.

Gene expression analyses of brain signals controlling feeding confirmed this. On the other hand, alternative diets are positively accepted by the Gilthead seabream without any difference with the control diet in final weight or gene expression of both orexigenic and anorexigenic signals. Further studies should investigate and characterise the brain mechanisms of feeding control in these species and optimise organic and eco-efficient formulations before they are commercially deployed.

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Chapter IV.

Effects of microalgal extract on zebrafish caudal fin cell regeneration

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Manuscript in preparation

Introduction

Microalgae include both eukaryotic (microalgae) and prokaryotic (cyanobacteria) microorganisms that perform oxygenic photosynthesis (Miguel et al., 2021). These organisms present metabolic plasticity synthesising several active biomolecules (e.g., pigments, peptides, fatty acids and polysaccharides) with high potential in the biomedical, pharmaceutical and cosmetics fields due to their antioxidant, antiviral, antibacterial, skin regenerative, immunomodulatory and immune-stimulatory effects (Michalak & Chojnacka, 2015; Liang et al., 2019; Miguel et al., 2021). Indeed, microalgal extracts contain bioactive compounds that promote cell adhesion and proliferation and they can be used in wound-healing applications treating skin lesions (Yarkent et al., 2020).

In aquaculture intensive production systems, skin injuries are frequent in farmed fish due to poor management practices, improper handling, aggressive behaviour and stress (Tørud & Håstein, 2008; Schmidt et al., 2018; Sveen et al., 2020). Skin wounds can cause high mortality rates, developing into bigger injuries and pathologies. Thus, skin must regenerate quickly through a multiphases and coordinated process that is the wound healing. The different stages include re-epithelialization, inflammation, proliferation and remodelling; and these should occur in the right sequence to succeed (Cañedo-Dorantes & Cañedo-Ayala, 2019: Edirisinghe et al., 2020). Teleost fish have exceptional regeneration ability in a variety of tissues and organs (Nakatani et al., 2007) and, in the last years, different studies have been carried out on wound healing testing the model species zebrafish *Danio rerio* (Gemberling et al., 2013; Richardson et al., 2013) and farmed fish species as common carp *Cyprinus carpio* (Przybylska-Diaz et al., 2013), rainbow trout *Oncorhynchus mykiss* (Schmidt et al., 2016) and Gilthead sea bream *Sparus aurata* (Ceballos-Francisco et al., 2017)

investigating different molecules such as *Spirulina maxima* pectin (Edirisinghe et al., 2020; Rajapaksha et al., 2020).

The objective of the present study was to investigate the possible effects of the green microalga *Neochloris oleoabundans* extract, as immunomodulatory and antiviral properties have been highlighted in this species (Baldisserotto et al., 2022), on the in vitro cell proliferation and migration activity of zebrafish caudal fin fibroblast cell line AB9 (Bhadra et al., 2015; Kalaiselvi Sivalingam et al., 2019) by means of the wound healing technique. In addition, as zebrafish cells are photosensitive and directly light-entrainable (Dekens et al., 2003; Tamai et al., 2005), we subjected the cells to a 12 h light:12h dark photoperiod and perform the algae extract treatment in the middle of the light and in the middle of dark phase to verify different efficiency in the wound healing process between different phases of the day. The findings can be useful to investigate a possible treatment for injured farmed fish of commercial and ornamental interest.

Materials and Methods

Microalgal culture and microalgal whole extract preparation

The present research study was performed using the green microalga *Neochloris oleoabundans* UTEX-1185 (Chlorophyta, Sphaeropleales) cultivated in BG11 medium (www.utex.org). The culture was grown and maintained in a 100 L photobioreactor at room temperature under 80 μmol photons m−2 s−1 of photosynthetically active radiation (PAR) with a 16h light: 8h dark photoperiod, intermittent bubbling and without CO_2 addition in Alga&Zyme facility (Ferrara, Italy). Culture was morpho-physiologically and biochemically characterized up to the stationary phase of growth in order to assess the best time for the extract preparation. Specifically, algae growth

was monitored as cell density and dry biomass yield (Baldisserotto et al., 2022).

The aqueous whole extract was prepared using the algal biomass that was washed with distilled sterile water and harvested by centrifugation. Then, cells were mechanically disrupted in a cold pre-sterilized mortar in the presence of liquid N2 and used to prepare aqueous extracts at a final concentration of 25 gDW L−1 using Leibovitz's L-15 complete medium suitable for the cultivation of zebrafish caudal fin fibroblast cell line AB9. The extract was stored at 4 ◦C until the wound healing assay.

Cell cultures and cell viability test

AB9 zebrafish caudal fin fibroblast cells were grown in monolayers in T25 and then T75 flasks (Thermo Fisher Scientific, USA) and maintained in Leibovitz's L-15 growth medium (Sigma-Aldrich, USA) supplemented with 15% heat-inactivated fetal bovine serum (FBS; Sigma-Aldrich, USA) and Pen-Strep penicillin streptomycin (1%; Sigma-Aldrich, USA), gentamicin (0,1%; Sigma-Aldrich, USA), L-glutamine (0,75%; Sigma-Aldrich, USA), at 28° C set incubator without CO_2. Flasks were checked daily, when the cells reached confluency, they were sub-cultured at different ratio following the standard trypsinization method washing with 1% Pen-Strep phosphate-buffered saline 1x (PBS) and trypsin-EDTA solution (trypsin 0.25%, EDTA 0.2%).

Non-toxic concentration of *N. oleoabundans* extract was determined by MTT assay. AB9 cells were seeded at a density of 30,000 cells per well into 96- well plates and incubated overnight. The cells were treated with 2-fold serial dilutions of microalgal extract (from 1:4 to 1:64; Baldisserotto et al., 2022) and incubated for 24 hours. The culture medium was replaced with fresh medium, followed by addition of 110 µL of MTT 1x to each well; the plate was then incubated for 4 hours. The resulting formazan crystals were

dissolved in 100 µL of dimethyl sulfoxide (DMSO) (Sigma-Aldrich, USA) and absorbance was measured at 570 nm using a microplate spectrophotometer (Bio-Rad Laboratories, Inc., USA). Triton-X100 (Sigma-Aldrich, USA) 0.1% was used as positive control for cell death, and untreated cells were used as negative control for viability. MTT assay revealed that 1:16 and 1:32 microalgal extract dilutions were not cytotoxic and consequently the ones that we tested.

In vitro wound healing assay

The in vitro wound healing activity was performed according to a previously described method (Valacchi et al., 2009). Specifically, cell suspensions at a density of 2×10^5 cells/mL were seeded into each well (65 µL/well) of two 12-wells plates and incubated for 24 hours. When the cells were attached and spread to form a confluent monolayer the wound was performed by using a P10 tip in each well. The growth medium was replaced with 1:16 and 1:32 dilutions (2 mL/well) of microalgal extract. As a control, untreated cells were used. The two plates were incubated for 30 minutes and 2 hours, after which the L-15 medium with (treated) and without (control) algal extract was totally replaced with fresh growth medium and cells incubated.

Regarding the day phase-dependant treatment trial, cell suspensions at a density of 2×10^5 cells/mL were seeded into each well (75 µL/well) of two 12-wells plates and incubated for at least 72 hours. One plate was subjected to a 12h light: 12h dark cycle (LD 12:12; lights on at 09:00 h and off at 21:00 h) and one plate was subjected to a 12h dark: 12h light cycle (DL 12:12; lights off at 09:00 h and off at 09:00 h). When the cells formed a confluent monolayer, the wound was performed in the middle of the light phase (15:00 h; ML plate) and in the middle of the dark phase (15:00 h; MD). The growth medium was replaced with 1:16 and 1:32 dilutions (2 mL/well) of microalgal

extract. As a control, untreated cells were used. The plates were incubated for 30 minutes treatment, after which the L-15 medium with (treated) and without (control) algal extract was totally replaced with fresh growth medium and cells incubated again.

Images of the cell free-gap were captured at 0, 8, 16, 20, 24, 32 and 40 hours (until the wound closes) using an inverted light microscope (Motic® AE31E, China), to determine the time taken to fill the cell-free gap by cell migration. The experiments were performed in triplicate (n= 3 wells/treatment).

Data and statistical analysis

Cell-free gaps were analysed using ImageJ software (ImageJ, ver. 1.53k, USA) by normalizing to the 0 h cell-free gap. Open wound area (%) compared to T0 (0 h) = (At/ A0) ×100; where A0 is the open wound area at 0 h, and At is open wound area at respective time point. Statistical analysis were performed using GraphPad Prism 9.3 software (GraphPad, Dotmatics, USA) using two-way ANOVA analysis to determine the overall significance between the treated groups and/or time points. Moreover, Bonferroni's post hoc test was conducted to compare the controls and treatments. Significant differences were considered when $p < 0.05$.

Results

Based on the cell migration performance of monolayer AB9s, wound healing activity was estimated by measuring the open wound area after 30 min and 2 h of *N. oleoabundans* extract treatment (1:16 and 1:32 dilutions) at 0, 8, 16, 20, 24 h. The findings indicate that microalgal whole extract treatment accelerates the migration of zebrafish fibroblasts, decreasing significantly the cell free gap (open wound area) when cells are treated with a higher dilution of extract for 30 min (Figure 1A). Specifically, two-way

ANOVA revealed a significant effect of the treatment at different time points ($F_{10,36}$= 3.22, p= 0.0047), a significant effect of the treatment on the results ($F_{10,36}$= 18.99, p < 0.0001) and a significant effect of the time on the results ($F_{5,36}$= 267.48, p < 0.0001). Therefore, quantitative analysis of the open wound area% confirmed a significant reduction in the cells-free gap in 30 min 1:32 extract treatment at 8 h (control vs 1:32 p < 0.001; 1:16 vs 1:32 p < 0.01), 16 h (control vs 1:32 p < 0.001) and 20 h (control vs 1:32 p < 0.01) (Figure 2A). Regarding the 2 h treatment, only a significant effect of the time on the results was found ($F_{5,30}$= 70.12, p < 0.0001; Figure 2B).

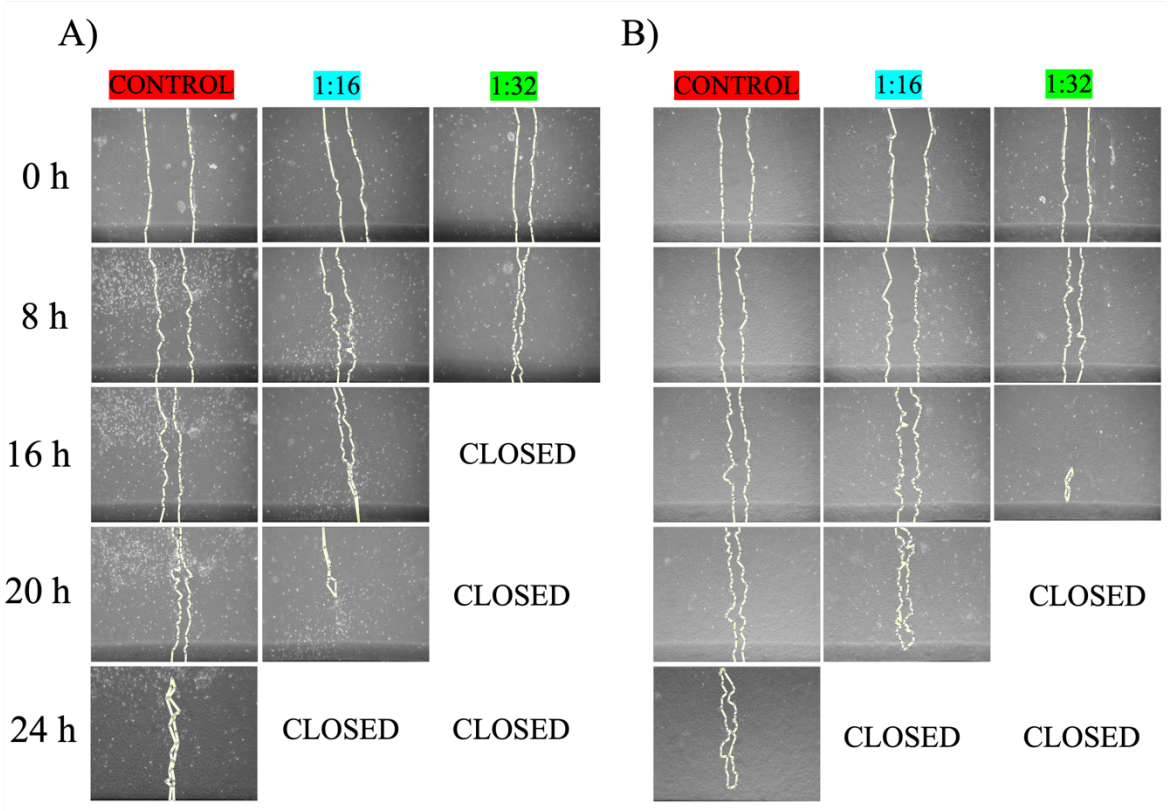

Figure 1. Images of the wound assay. The scratch was performed to determine the in vitro wound healing activity of zebrafish caudal fin fibroblasts (AB9 cell line) by measuring the cell-free area treated with 1:16 and 1:32 dilutions of *N. oleoabundans* whole extract at 8, 16, 20 and 24 hours after performing the scratch. **A)** 30 minutes treatment and **B)** 2 hours treatment.

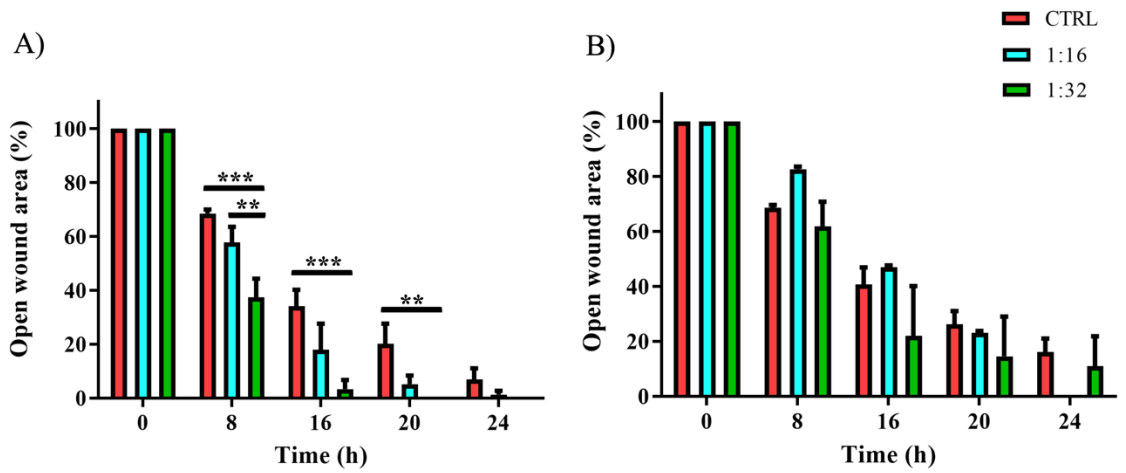

Figure 2. Effect of 1:16 and 1:32 *N. oleoabundans* whole extract on in vitro zebrafish caudal fin fibroblasts (AB9 cell line) migration at 8, 16, 20 and 24 hours. **A)** Graph representing the effect after 30 min of the microalgal extract treatment on cell wound healing activity (n= 3/treatment). **B)** Graph representing the effect after 2 hours of the microalgal extract treatment on cell wound healing activity (n= 3/treatment). Quantitative analysis of the open wound area (%). The open wound area% was analysed based on the 0 h time point. Two-way ANOVA was performed to find statistical significance. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Regarding the day phase-dependant treatment trial, wound healing activity in ML and MD was estimated by measuring the open wound area after 30 min of *N. oleoabundans* extract treatment (1:16 and 1:32 dilutions) at 0, 8, 16, 24, 32 and 40 h. When performing the wound and the microalgal treatment in the middle of the dark phase (MD), any significant differences were found (Figures 3A and 4A). However, when the wound and the treatment are carried out in the middle of the light phase (ML), the microalgal extract seems to produce a negative effect on wound healing activity of AB9 fibroblasts, as the wounds in control cells closes before (Figure 3B). Specifically, two-way ANOVA revealed a significant effect of the treatment on the results ($F_{2,32}$= 22.54, $p < 0.0001$) and a significant effect of the time on the results ($F_{6,36}$= 106.01, $p < 0.0001$). Any significant effect of the treatment at different time points was found. Therefore, quantitative analysis of the open wound area% revealed a significant reduction in the cells-free

gap in the untreated control cells compared to the treated ones at 16 h (control vs 1:16 $p < 0.05$; control vs 1:32 $p < 0.05$), 24 h (control vs 1:16 $p < 0.001$; control vs 1:32 $p < 0.01$) and 32 h (control vs 1:32 $p < 0.01$) (Figure 4B).

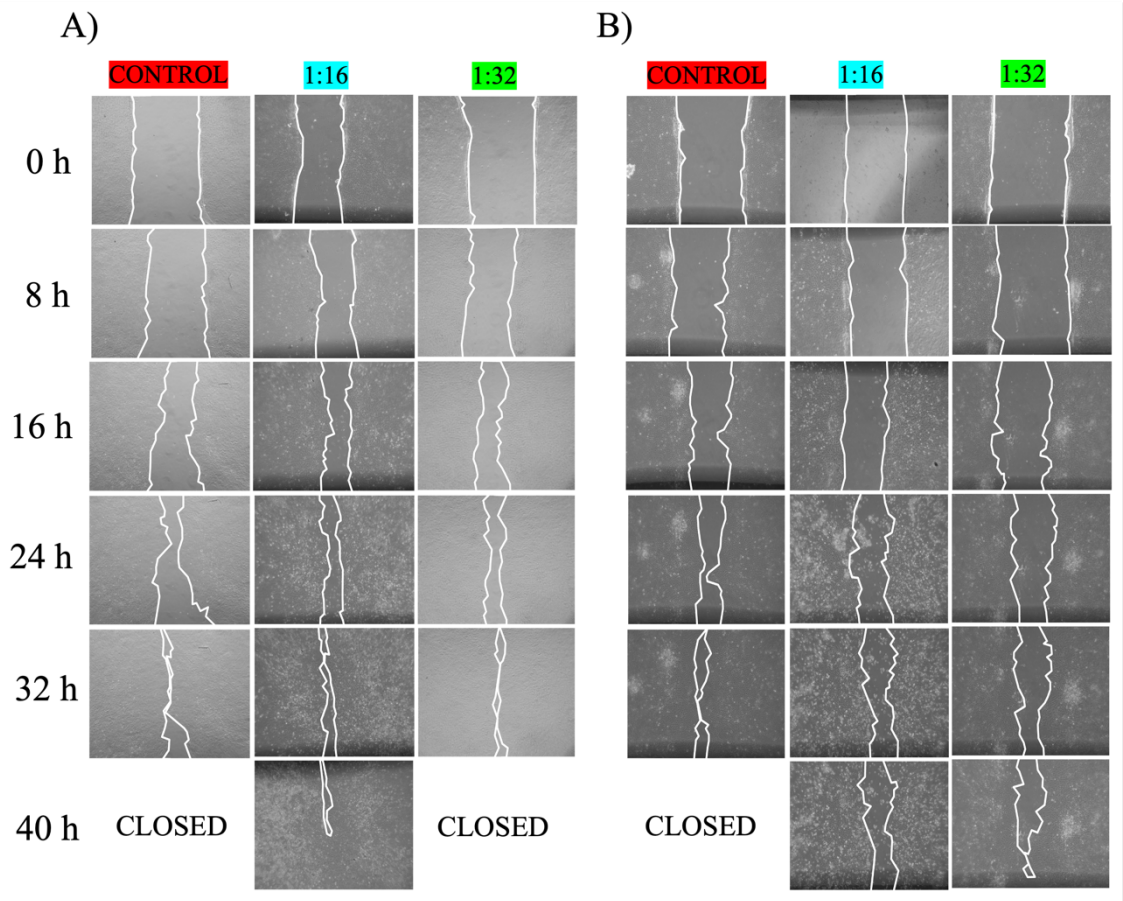

Figure 3. Images of the wound assay. The scratch was performed to determine the in vitro wound healing activity of zebrafish caudal fin fibroblasts (AB9 cell line) by measuring the cell-free area treated with 1:16 and 1:32 dilutions of *N. oleoabundans* whole extract for 30 minutes at 8, 16, 24, 32 and 40 hours after performing the scratch. **A)** Scratch and treatment performed during the middle of the dark phase (MD) in cells subjected to DL 12:12, **B)** scratch and treatment performed during the middle of the light phase (ML) in cells subjected to LD 12:12.

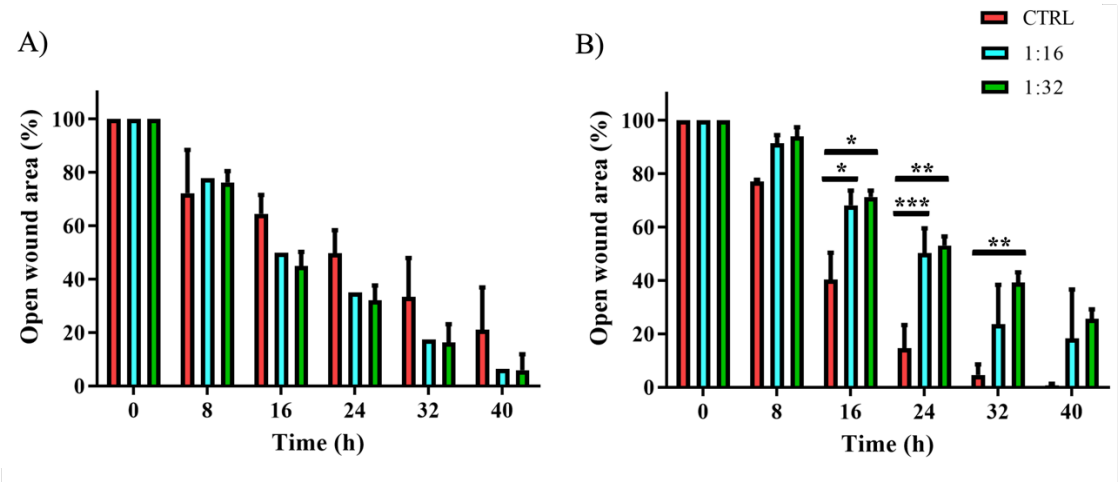

Figure 4. Effect of 1:16 and 1:32 *N. oleoabundans* whole extract on in vitro zebrafish caudal fin fibroblasts (AB9 cell line) migration, subjected to a LD 12:12, at 8, 16, 24, 32 and 40 hours. **A)** Graph representing the effect after 30 min of the microalgal extract treatment on cell proliferation and migration activity performing the wound and the algal treatment in the middle of the dark phase (MD; n= 3/treatment). **B)** Graph representing the effect after 30 min of the microalgal extract treatment on cell proliferation and migration activity performing the wound and the algal treatment in the middle of the light phase (ML; n= 3/treatment). Quantitative analysis of the open wound area (%). The open wound area% was analysed based on the 0 h time point. Two-way ANOVA was performed to find statistical significance. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Discussion

High-density stocks, poor welfare and handling of fish in captive breeding farms leads to the occurrence of skin injuries that, if not treated, can lead to more bad consequences such as the onset of infections and diseases leading to contagions and increased mortality rates. In this scenario, microalgal biotechnology has proven to be very interesting and numerous studies have been conducted on the application of photosynthetic organisms to promote fish health under different aspects (e.g., immunomodulation, stress tolerance, gut health, wound healing; Nagappan et al., 2021; Ma et al., 2022; Bahi et al., 2023; Sheikhzadeh et al., 2024). Most of the studies so far focused on the benefits observed using *Spirulina maxima*, which promotes

an accelerated cell proliferation and migration (Edirisinghe et al., 2020; Rajapaksha et al., 2020; Ragusa et al., 2021; Pham et al., 2023). The present research aimed to investigate the potential effect of *N. oleoabundans* whole extract to treat skin injuries in fish. Indeed, this green microalga has been mainly studied for bio-energetic sector purposes (Baldisserotto et al., 2016; Karthikeyan et al., 2018); however, some studies highlighted positive immunomodulatory, antiviral and anti-inflammatory properties (Castro-Puyana et al., 2017; Baldisserotto et al., 2022; Morocho-Jácome et al., 2022). When we performed the scratch on zebrafish caudal fin fibroblasts and then treated it with the algal extract, a faster wound healing activity was observed compared to the control. Specifically, a 30-minutes treatment with 1:32 extract dilution, resulted in a significant positive effect, stimulating cell proliferation and migration and accelerating wound closure. Indeed, the wound closed within 24 hours after the treatment. Microalgae produce a wide range of bioactive compounds (e.g., pigments, peptides, fatty acids, polysaccharides) that have positive effects on cellular mechanisms favoring skin regeneration (Miguel et al., 2021). The n-3-polyunsaturated fatty acids (PUFAs) are important elements of cell membranes, affecting their permeability and integral membrane proteins and they are also involved in the inflammatory response (Shetty et al., 2022). Application of PUFAs has been proved to aid wound repair and burns (Hidalgo-Lucas et al., 2014; Seth et al., 2022; El-Sheekh et al., 2024). In addition, others bioactive compounds as carotenoids, vitamins and chlorophylls can decrease the oxidative stress processes, reduces inflammatory cell infiltration and secretion of pro-inflammatory cytokines, and avoids the microorganism's colonization at the wound site, creating a favourable environment for cell proliferation and wound regeneration. *N. oleoabundans* is known for the ability to increase its fatty acid and carotenoids content when grown under nitrogen limitation and depletion (Bona et al., 2014; Urreta et al., 2014; Castro-Puyana et al., 2017).

Thus, using a whole aqueous extract in our study, we can potentially address the beneficial effects on in vitro cell regeneration to all cited above biocomponents. However, in the 2-hours treatment trial, we could not find any significant effects in the treated AB9 cells compared to the control ones. So, this could mean that a potentially better outcome is found in a treatment of shorter time (30 min) and lower extract concentration (1:32 dilution), implying a dose- and time-dependent effect in the use of this species extract.

The circadian clock coordinates biochemical, physiological and behavioural processes to make them occur an optimal time of day. As, zebrafish cells and tissues are proven to be directly light-entrainable (Cahill, 2002; Moore & Whitmore, 2014), previous studies showed that the circadian clock regulates the timing of mitosis in a light-responsive, clock-containing zebrafish cell line (Dekens et al., 2003; Tamai et al., 2012). This mitotic circadian rhythm is maintained in constant darkness (DD) while it is disrupted in continuous light (LL) leading to slower cell proliferation (Tamai et al., 2007; Tamai et al., 2012). Moreover, as the timing of epidermal cell proliferation in response to injury seems to be driven by the circadian clock (Idda et al., 2012), our research aimed also to investigate a potential different efficiency of the microalgal treatment in the wound healing process between different phases of the day. However, on one hand we could not find any significant effect of the extract treatment on the wound closure when performed in the middle of the dark phase (MD); on the other hand, when the scratch and the treatment are carried out in the middle of the light phase (ML), the control cells closed significantly before. As we used a whole extract, it is rather difficult to address the negative effect of the treatment to one possible single compound produced by the algal culture. However, chlorophyll derivatives, when excited by light, undergo a series of biochemical reactions to return to their ground state resulting in the

formation of ROS and $^1O_2$. These radicals are very reactive and can activate apoptotic and necrotic mechanisms in cells and tissues (Mansoori et al., 2019; Pucci et al., 2021). This process could cause an arrest or slowing of cell proliferation in the ML wound assay in the AB9 cells treated with the microalgal extract which is rich in chlorophylls. In addition, it could be also possible that the cells were not kept in LD cycle for enough days and were not completely entrained by the photoperiod. Moreover, as the mitosis rhythm peak in zebrafish cell line occurs in late night/early morning (Tamai et al., 2012), it might be decisive to perform the scratch and the treatment with the extract nearest this phase of LD cycle.

Finally, it should be noted that there are few studies of wound healing assays on a fish cell line as most focus on human cells. Therefore, this study represents a promising starting point for the study of in vitro cell regeneration and skin injuries recovery on fish using microalgae treatments, which would be interesting to investigate further with in vivo studies as well.

Conclusion

The present research findings suggest that *N.oleoabundans* extract promotes cell survival, proliferation, migration and wound healing in AB9 cells maintained in constant darkness and that the effect of the treatment could be dose- and time-dependent. Accordingly, these results suggest a potential application of microalgae extract in the welfare of fish husbandry field.

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General discussion

4. General discussion

The present PhD thesis highlights the importance of light and temperature as *Zeitgeber* in different fish species with different activity patterns: diurnal, nocturnal and blind cavefish species and that these preferences (thermal and active phase) should be considered when designing husbandry protocols to breed fish in captivity. In addition, the utilization of alternative "ingredients" as microalgae in fish diets and treatments can produce some positive effects, although with some kinds of limitations.

In the natural environment, light and temperature are strictly correlated (i.e., higher water temperature during the day and cooler water temperature during the night) resulting in daily thermocycles coordinated to light-dark cycles (Johnson et al., 2004) that can synchronise and influence circadian organism's biological patterns (López-Olmeda et al., 2006; López-Olmeda & Sánchez-Vázquez 2009). In addition, water temperature represents an important abiotic factor for ectotherm animals as fish, which had to evolve strategies to regulate their body temperature (Angilletta et al., 2002; Christensen et al., 2021). Indeed, they can implement a behavioural thermoregulation actively moving to seek their preferred temperature to perform metabolic functions, feeding activity etc (Haesemeyer, 2020; Volkoff & Rønnestad, 2020). This thermoregulation has been proven to follow a circadian pattern and to be endogenous in previous studies on some species (Reynolds & Casterlin, 1980; Vera et al., 2023; de Alba et al., 2024). Despite this, under captive breeding conditions (e.g., indoor, fish farms, laboratories), the natural environmental cycles are little considered, and fish are often reared under constant conditions (i.e., constant temperature, standardised feeding protocols, artificial photoperiods or continuous illumination; Villamizar et al., 2014; Hui et al., 2019; de Alba et al., 2024). Therefore, investigating temperature fluctuation in indoor environments is

not always easy as temperature is a sensitive parameter. Many different set ups have been used to investigate it; however, they present some limitations as limited size and/or number of connected tanks and they allow small group of fish for short period tests (Cocherell et al., 2014; Elsner & Shrimpton, 2019; Harman et al., 2021). For the realisation of the present thesis (Chapters 1.1 and 1.2) we used a custom horizontal thermal gradient tank that allowed us to investigate the daily rhythms of thermal preference in many different fish species for a long period (Rey et al., 2015; Vera et al., 2023; de Alba et al., 2024). In Chapter 1.1 we improved this set up by making it an automated-recording, low-cost and user-friendly-interface solution to investigate thermal preferences and locomotor activity in fish. This set up allowed us to observe for a long-term period (27 days) the exhibition of daily rhythms of thermal preference in different species of fish; from diurnal to nocturnal and also in cavefish species. The pattern of thermal selection was species-specific. Specifically, the diurnal largemouth bass significantly preferred cooler temperatures during the day and warmer temperatures during the night, differing from the pattern previously observed in other diurnal species such as zebrafish and Nile tilapia (Vera et al., 2023; de Alba et al., 2024; Conti et al., 2024 in Experimental Chapter 1.1). The nocturnal tench and black bullhead catfish displayed the same significant pattern of daily temperature selection going to warmer temperatures during the day and moving to cooler temperatures during the dark phase. This research represents the first study investigating daily thermal selection in nocturnal species. When the light-dark cycle is inverted all the species cited kept displaying the previous thermal pattern. When the fish were subjected to constant dark condition, removing any possible synchronizer (i.e., light and food), they kept displaying a self-sustained circadian rhythm of thermal preference for 7 days proving its endogenous nature (Allan & Czeisler, 1994).

Investigating temperature in cave species can be interesting from an evolutionary point of view as they adapted to life underground where temperature and other environmental signals (i.e., light and food availability) are constant. However, they are still able to entrain to light-dark cycle (blind *Astyanax mexicanus*, Beale et al., 2013) or to food administration (*Phreatichthys andruzzii,* Cavallari et al., 2011); for these reasons we decided to study both this species. Blind *A. mexicanus* and *P. andruzzii* exhibited a significant daily thermal preference that was preserved also when the light-dark cycle was reversed; favouring hight temperatures during the light phase and lower temperatures during the night. However, both this species lost the rhythmic pattern when kept under constant darkness and also when the cycle is reversed in *P. andruzzii*, highlighting that this preference could be mainly driven by the light and it is not endogenously controlled. Therefore, at the University of Ferrara, by using the software Ethovision XT we could also calculate the daily amount of distance travelled for the fish species tested there. The largemouth bass confirmed to show a diurnal activity pattern, while the black bullhead catfish confirmed to display a nocturnal activity pattern. Thus, the locomotor activity seems to exhibit a pattern opposite to that of temperature; suggesting that they could tend to preferer lower temperatures during their active phase to consequently reduce their energy expenditure through the metabolism. The Somalian cavefish displayed daily rhythms of locomotor activity with a diurnal pattern that disappear under DD condition. Thus, this observed behaviour could be related to the strong photophobic response that they showed (Calderoni et al., 2016). Our research represents a complete and important starting point to better understand thermal preference in fish and the correlation with circadian rhythms. Moreover, further studies must be carried out, investigating more fish species and correlating also physiological parameters to give a complete picture of fish thermal biology.

Light comprises multiple factors as colour spectrum, intensity and photoperiod that provide daily and seasonal inputs to aquatic organisms throughout their life cycle (Millar, 2004). Indeed, light is the main environmental factor to entrain circadian rhythms via the light-entrainable oscillators (LEOs; Reppert & Weaver, 2002; Isorna et al., 2017). Light-dark cycles have proven to be essentials for proper development and survival in many teleost species (Tamai et al., 2004; Ben-Moshe et al., 2014; Villamizar et al., 2014). The results that we achieved in Chapter 2.1 testing the diurnal model species zebrafish *Danio rerio*, showed an endogenous rhythm of light/dark preference under white light in this species. Specifically, zebrafish kept manifesting its diurnal pattern of activity preferring lit sector during the subjective days and dark sector during the subjective nights. This behavioural rhythm was self-sustained, with a period within the circadian interval, and maintained for 7 days. Thus, Chapter 2.1 highlights the importance of providing light-dark cycles in captive breeding conditions as, when given the possibility to choose, fish select to stay in both environments with a circadian-like pattern. However, in some cases fish farms prefer to rear animals under artificial photoperiod or under continuous lighting to improve the growth performance (Boeuf & Le Bail, 1999; Taranger et al., 2006) although it has a very negative impact leading to high rates of malformations and mortality (Villamizar et al., 2014). Different light wavelengths can affect positively or negatively fish biology. Specifically, red light produces the most negative effects on zebrafish and other species survival and growth (Ruchin, 2004; Villamizar et al., 2014). Our study in Chapter 2.1 proved that zebrafish exhibited no significant behavioural circadian rhythms under this coloured light advising against its use for this species rearing and potentially others as well (Villamizar et al., 2009; Adatto et al., 2016; Noureldin et al., 2021; de Alba et al., 2022).

Visual light detection by cones and rods is very important in most vertebrates, but non-visual photoreception and the involvement of non-visual opsins are also essential in many biological processes (Frøland Steindal & Whitmore, 2019). To better investigate this system, it is interesting to test blind cavefish species as they completely lost their functional eyes. Our study described in Chapter 2.2 proved that the Mexican blind cavefish *Astyanax mexicanus* held the capacity to entrain their activity to a light-dark cycle by exhibiting daily rhythms of locomotor activity with a diurnal pattern for a long-term period. In addition, these rhythms have been shown to be endogenous and controlled by the circadian clock as they persist even in aperiodic conditions (DD and LLdim). As this species lost their functional eyes but they retained the ability to sense light and to entrain their locomotor activity to the photic stimulus (Beale et al., 2013; Carlson & Gross; 2018; Simon et al., 2019) suggesting that extraocular photoreceptors play a role in the light response. Chapter 2.2 describes the daily rhythmic expression of three non-visual opsin genes in the brain of this species that can be involved in detecting light (Cavallari et al., 2011; Fischer et al., 2013; Carlson et al., 2018) and consequently entrain the activity to the photic stimulus. Our study proved the importance of the non-visual photoreception system and the deep brain photoreceptors in entraining circadian rhythms in fish.

Microalgae biotechnology is an increasingly expanding field in recent years, including in research, as microalgae are photosynthetic organisms capable of producing many bioactive compounds with many beneficial properties (Michalak & Chojnacka, 2015; Miguel et al., 2021). Microalgae are increasingly being incorporated into fish feeds in the aquaculture sector to partly replace the use of fish oils and meals that concern the costumers and can negatively impact the environment (Mosha, 2019). However, these alternative ingredients are not always positive accepted by fish, depending

on the species. Our study in Chapter 3 proved that Nile tilapia did not accept positively the diet mainly composed of spirulina and quinoa, as in a 60-days trial they did not grow but they maintained their weight. Gene expression analysis of the brain circuitries (both homeostatic and hedonic pathways) reflected the feed intake as fish fed with organic diet (ORG) upregulated orexigenic signal genes and downregulated anorexigenic genes. Indeed, starvation causes an increased level of mRNA expression of orexigenic genes (Volkoff, 2016). However, the other alternative diet tested (ECO) that includes lower levels of spirulina and quinoa, did not produce significant effects on the expression of some genes. On the other hand, the Gilthead sea bream positively accepted the alternative diets, showing a better growth performance and no significant differences in orexigenic and anorexigenic gene expression analysis. This research highlights the importance of studying the right inclusion levels of emergent and alternative ingredients in fish feeds (Galafat et al., 2022), depending on the species.

Microalgal extracts can be used to treat skin injuries as the bioactive compounds promote cell adhesion and proliferation (Yarkent et al., 2020). The research findings explained in Chapter 4 suggest that *N.oleoabundans* extract promotes cell survival, proliferation, migration and wound healing in AB9 fibroblast zebrafish cell line maintained in constant darkness and that the effect of the treatment could be dose- and time-dependent. Indeed, compounds as PUFAs, chlorophylls, carotenoids and carbohydrates are involved in in cell membrane mechanisms and reduce oxidative stress that can be caused by wound (Hidalgo-Lucas et al., 2014; Seth et al., 2022; El-Sheekh et al., 2024). Accordingly, these results suggest a potential application of microalgae extract in the welfare of fish husbandry field as in the aquaculture sector wounds and skin injuries due to poor and bad maintenance can lead to disease emergence (Sveen et al., 2020).

Conclusions

5. Conclusions

1. We designed and evaluated an automated-recording, efficient, low-cost and user-friendly-interface solution for studying thermal preferences and locomotor activity in fish.
2. We described for both diurnal and nocturnal commercial interest species significant daily rhythms of thermal preference with opposite patterns. Specifically, the diurnal largemouth bass selected lower temperatures during the day and higher temperatures during the night, while the nocturnal black bullhead catfish and tench preferred higher temperatures during the day and cooler temperatures during the night.
3. We proved that the daily rhythms of thermal preference exhibited are endogenous and driven by the circadian clock.
4. We found daily rhythms of thermal preference in cavefish species that were proven to be driven exclusively by the photic stimulus. Indeed, these behavioural rhythms were not self-sustained during the DD phase.
5. We found daily rhythm of locomotor activity for the Somalian cavefish *P. andruzzii* when kept in light-dark cycle but it is not endogenous and probably it is related to a photophobic response.
6. We confirmed the endogenous daily rhythms of locomotor activity for diurnal (largemouth bass) and nocturnal (black bullhead catfish) species.
7. We described that fish exhibit daily rhythms of light/dark preference and proved that these rhythms are endogenous under white light.

8. We ascertained and characterised the long-term photic entrainment of locomotor activity and circadian endogenous rhythmicity in the blind Mexican tetra *A. mexicanus*.
9. We found daily relative expression of selected non-visual opsin genes (*opn3, rgra, tmt1b*) in the brain of the blind Mexican tetra that might allow this fish to entrain their activity to the light-dark cycles.
10. We described the effects on fish orexigenic and anorexigenic brain signals expression of emergent ingredient diets (i.e., spirulina, quinoa, insect meal). Specifically, Nile tilapia did not accept positively the ORG diet with higher inclusion levels of spirulina and quinoa while Gilthead seabream accepted positively all the alternative diets. These results are reflected in brain food intake control mechanisms gene expression.
11. We proved that microalgal extract treatment can accelerate wound healing and closure in zebrafish fibroblasts cell line kept in constant darkness. Although, this treatment could be time and concentration-dependent.

6. Conclusiones

1. Diseñamos y evaluamos una solución de grabación automatizado, eficiente, de bajo coste y con una interfaz fácil de usar para estudiar las preferencias térmicas y la actividad locomotora en peces.
2. Describimos, tanto para especies diurnas como nocturnas de interés comercial, ritmos diarios significativos de preferencia térmica con patrones opuestos. En concreto, el diurno *M. salmoides* seleccionó temperaturas más bajas durante el día y más altas durante la noche, mientras que el pez gato y la tenca nocturnos prefirieron temperaturas más altas durante el día y más frías durante la noche.
3. Demostramos que los ritmos diarios de preferencia térmica exhibidos son endógenos y controlados por el reloj circadiano.
4. Encontramos ritmos diarios de preferencia térmica en especies de peces cavernícolas que estaban dirigidos exclusivamente por el estímulo fótico. De hecho, estos ritmos comportamentales no persistieron durante la fase DD.
5. Encontramos ritmo diario de actividad locomotora en el pez de cueva somalí *P. andruzzii* cuando se mantiene en ciclo luz-oscuridad pero no es endógeno y probablemente esté relacionado con una respuesta fotofóbica.
6. Confirmamos los ritmos diarios endógenos de la actividad locomotora para especies diurnas (*M. salmoides*) y nocturnas (*A. melas*).
7. Describimos que los peces exhiben ritmos diarios de preferencia luz/oscuridad y demostramos que estos ritmos son endógenos bajo luz blanca.

8. Determinamos y caracterizamos la sincronización fótica a largo plazo de la actividad locomotora y la ritmicidad circadiana endógena en el tetra ciego mexicano *A. mexicanus*.
9. Encontramos la expresión relativa diaria de genes de opsinas no visuales seleccionados (*opn3, rgra, tmt1b*) en el cerebro del tetra mexicano ciego que podría permitir a este pez sincronizar su actividad a los ciclos luz-oscuridad.
10. Describimos los efectos sobre la expresión de señales cerebrales orexigénas y anorexigénas de peces con dietas formuladas con ingredientes emergentes (espirulina, quinoa, harina de insectos). En concreto, la tilapia del Nilo no aceptó positivamente la dieta ORG con mayores niveles de inclusión de espirulina y quinoa, mientras que la dorada aceptó positivamente todas las dietas alternativas. Estos resultados se reflejan en la expresión génica de los mecanismos de control de la ingesta de alimentos en el cerebro.
11. Demostramos que el tratamiento con extracto de microalgas puede acelerar la cicatrización y el cierre de heridas en la línea celular de fibroblastos de pez cebra mantenidos en oscuridad constante. Aunque, este tratamiento podría ser dependiente del tiempo y la concentración.

7. Conclusioni

1. Abbiamo progettato e valutato una soluzione di registrazione automatica, efficiente, a basso costo e con un'interfaccia facile da usare per studiare le preferenze termiche e l'attività locomotoria nei pesci.
2. Abbiamo descritto per specie di interesse commerciale, diurne e notturne, ritmi giornalieri significativi di preferenza termica con pattern opposti. In particolare, il persico trota diurno, sceglie temperature più basse durante il giorno e più alte durante la notte, mentre il pesce gatto e la tinca notturni preferiscono temperature più alte durante il giorno e più fresche durante la notte.
3. Abbiamo dimostrato che i ritmi giornalieri di preferenza termica esibiti sono endogeni e guidati dall'orologio circadiano.
4. Abbiamo trovato ritmi giornalieri di preferenza termica in specie di pesci di caverna che si sono dimostrati controllati esclusivamente dallo stimolo fotico. In effetti, questi ritmi comportamentali non si sono autosostenuti durante la fase DD.
5. Abbiamo riscontrato un ritmo giornaliero dell'attività locomotoria per il pesce di grotta somalo *P. andruzzii* quando è tenuto in un ciclo luce-buio, ma non è endogeno e probabilmente è legato a una risposta fotofobica.
6. Abbiamo confermato i ritmi giornalieri endogeni dell'attività locomotoria per le specie diurne (persico trota) e notturne (pesce gatto).
7. Abbiamo descritto che i pesci mostrano ritmi giornalieri di preferenza tra luce e buio e abbiamo dimostrato che questi ritmi sono endogeni in presenza di luce bianca.

8. Abbiamo accertato e caratterizzato la sincronizzazione fotica a lungo termine dell'attività locomotoria e la ritmicità endogena circadiana nel tetra messicano di grotta *A. mexicanus*.
9. Abbiamo trovato l'espressione relativa giornaliera di geni di opsine non visive selezionati (*opn3, rgra, tmt1b*) nel cervello del tetra messicano di grotta, che potrebbe consentire a questi pesci di sincronizzare la loro attività ai cicli luce-buio.
10. Abbiamo descritto gli effetti sull'espressione dei segnali cerebrali oressigeni e anoressigeni di diete formulate con ingredienti emergenti (ad esempio, spirulina, quinoa, farina di insetti). In particolare, la tilapia del Nilo non ha accettato positivamente la dieta ORG con livelli di inclusione più elevati di spirulina e quinoa, mentre l'orata ha accettato positivamente tutte le diete alternative. Questi risultati si riflettono sull'espressione genica dei meccanismi di controllo dell'assunzione di cibo nel cervello.
11. Abbiamo dimostrato che il trattamento con estratti microalgali può accelerare la guarigione e la chiusura delle ferite in una linea cellulare di fibroblasti di zebrafish tenuti in condizioni di buio costante. Tuttavia, questo trattamento potrebbe essere dipendente dal tempo e dalla concentrazione.

General bibliography

8. General bibliography

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Annexes

9. Annexes

9.1 Scientific publication and publications in progress

de Alba, G., **Conti, F.**, Sánchez, J., Godoy, L. M., Sánchez-Vázquez, F. J., López-Olmeda, J. F., & Vera, L. M. (2024). Effect of light and feeding regimes on the daily rhythm of thermal preference in Nile tilapia (*Oreochromis niloticus)*. *Aquaculture*, *578*, 740122.

Conti, F., de Alba, G., López-Olmeda, J.F., Vera, L.M., Lucon-Xiccato, G., Mainardi, E., Cesari, S., Bottarelli, M., Bertolucci, C., Sánchez-Vázquez, F.J., Gatto, E. (2024). Daily rhythms of thermal preference in zebrafish: an automated solution to generate a horizontal thermal gradient and long-term recording fish behaviour. Sent to Zebrafish, under review.

Conti, F., Vergès-Castillo, A., Sánchez-Vázquez, F. J., López-Olmeda, J. F., Bertolucci, C., Muñoz-Cueto, J. A. (2024). Daily rhythms of locomotor activity and transcript levels of non-visual opsins in the brain of the blind Mexican cavefish (*Astyanax mexicanus*). Manuscript ready to be sent to Comparative Biochemistry and Physiology Part A.

Conti, F., Gatto, E., de Alba, G., Pintos, S., López-Olmeda, J.F., Vera, L.M., Bertolucci, C., Sánchez-Vázquez, F.J. Daily rhythms of thermal preference in diurnal, nocturnal and cavefish species. In preparation.

Conti, F., de Alba, G., García-Hernández, M., Vera, L.M., López-Olmeda, J.F., Sánchez-Vázquez, F.J. Daily behavioural rhythms of light/dark selection in zebrafish. In preparation.

Conti, F., Mendes, R., López-Olmeda, J.F., Bertolucci, C., Conceição, L., Sánchez-Vázquez, F.J. Food intake regulation mechanisms of alterative feeds in Nile tilapia and Gilthead Seabream. In preparation.

Conti, F., Frigato, E., Ruohisto, E., Bertolucci, C., Baldisserotto, C., López-Olmeda, J.F., Sánchez-Vázquez, F.J., Pancaldi, S. Effects of microalgal extract on zebrafish caudal fin cell regeneration. In preparation.

9.2 Oral and poster communications at congresses

Oral communication

World Aquaculture Society (AQUA2024), Copenhagen 26-30/8/2024. Daily rhythms of thermal preference in commercial interest species: optimizing rearing and welfare in diurnal and nocturnal fish. **F. Conti**, E. Gatto, G. de Alba, J.F. López-Olmeda, L. M. Vera, C. Bertolucci, F.J. Sánchez-Vázquez.

Poster communications

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XIV Congress of the Iberian Association of Comparative Endocrinology (AIEC), Bilbao 11-13/9/2023. Daily rhythms of locomotor activity and expression of non-visual opsins in the blind Mexican cavefish. **F. Conti**, A. Vergès-Castillo, F.J. Sánchez-Vázquez, J.A. Muñoz-Cueto.

XVIII Congreso Nacional de Acuicultura (CNA 2022), Cadiz 21-24/11/2022. Efecto de la luz en los ritmos diarios de preferencia térmica en *Astyanax Mexicanus*. **F. Conti**, G. de Alba, J.F. López-Olmeda, C. Bertolucci, L. M. Vera, F.J. Sánchez-Vázquez.

European Aquaculture Society (EAS) 2022, Rimini 27-20/9/2022. Effects of feeding time on daily rhythms of temperature selection in the blind Mexican cavefish (*Astyanax mexicanus*). **F. Conti**, G. de Alba, J.F. López-Olmeda, C. Bertolucci, L. M. Vera, F.J. Sánchez-Vázquez.

Summary in Italian

10. Summary in Italian

Lo scopo di questa tesi è stato principalmente quello di studiare l'effetto di due importanti variabili ambientali, quali la luce e la temperatura, sui ritmi comportamentali giornalieri in diverse specie di pesci con pattern diversi di attività: pesci diurni, notturni e di caverna. Nello specifico, sono stati studiati, utilizzando un sistema messo a punto durante il mio dottorato, i ritmi giornalieri di preferenza termica nelle diverse specie di pesci confermandone anche i pattern di attività giornalieri. Inoltre, è stato valutato se questi ritmi giornalieri comportamentali (legati alla luce e alla temperatura) sono di origine endogena e quindi controllati e regolati dall'orologio circadiano, testando anche diverse lunghezze d'onda. È stata inoltre evidenziata l'importanza della fotorecezione non visiva (in cui sono coinvolte le opsine non visive), studiando una specie di pesce cavernicola che ha mantenuto la capacità di sincronizzare la propria attività locomotoria ai cicli luce-buio.

Durante i miei periodi all'estero in aziende (Sparos, Portogallo e Alga&Zyme, Italia), ho avuto la possibilità di studiare e approfondire i possibili effetti di diete alternative (con ingredienti emergenti come le microalghe) sull'espressione di geni centrali responsabili di regolare l'alimentazione nella tilapia del Nilo e nell'orata. Infine, ho investigato i possibili effetti di estratti contenenti microalghe sulla rigenerazione cellulare, utilizzando una linea cellulare di zebrafish.

Gli obbiettivi prefissati per la realizzazione della seguente tesi sono stati i seguenti:

1. Progettare e testare una soluzione di registrazione automatica, a basso costo e con un'interfaccia facile da usare, per studiare le preferenze termiche e l'attività locomotoria dei pesci.

2. Valutare e descrivere i potenziali ritmi di preferenza termica giornaliera in pesci diurni e notturni di interesse commerciale.

3. Indagare se questi ritmi di preferenza termica giornaliera sono regolati o meno dagli orologi circadiani endogeni.

4. Valutare la potenziale preferenza termica giornaliera in specie di pesci cavernicoli ciechi, indicando le variazioni di temperatura come un possibile *Zeitgeber* per questi animali che si sono evoluti in ambienti son condizioni costanti.

5. Delineare se i pesci possono mostrare ritmi giornalieri di selezione della luce e determinare se questi ritmi sono endogeni, testando diverse lunghezze d'onda (bianco e rosso) utilizzando il modello sperimentale zebrafish.

6. Caratterizzare la sincronizzazione a lungo termine dell'attività locomotoria alla luce e la possibile ritmicità endogena circadiana nel pesce di caverna messicano (*Astyanax mexicanus*).

7. Indagare la possibile espressione genica giornaliera di opsine non visive selezionate, nel cervello del pesce di caverna messicano (*Astyanax mexicanus*), che consentono a questa specie di sincronizzarsi ai cicli luce-buio.

8. Descrivere i possibili effetti sulla fisiologia dei pesci delle diete con ingredienti emergenti, concentrandosi sui meccanismi centrali che regolano l'assunzione di cibo.

9. Determinare una potenziale applicazione dei trattamenti con estratti microalgali nell'allevamento di pesci in cattività, studiando in vitro la proliferazione e l'attività migratoria delle cellule di zebrafish, trattate con microalghe.

10. Saggiare la possibile efficienza di trattamenti a base di microalghe nei processi di guarigione, in funzione del tempo (giorno o notte), dato che le cellule dei pesci sono fotosensibili.

Capitolo 1.1 - Ritmi giornalieri di preferenza termica in zebrafish: una soluzione automatizzata per generare un gradiente termico orizzontale e registrare a lungo termine il comportamento dei pesci.

Il fotoperiodo e la temperatura sono due dei più potenti segnali ambientali che sincronizzano gli orologi circadiani. Essendo ectotermi, i pesci devono mantenere regolare la loro temperatura corporea in modo tale da ottimizzare tutti i diversi processi biologici mettendo in atto strategie comportamentali. Durante la realizzazione di questa tesi, abbiamo sviluppato un sistema automatizzato e a basso costo che consente di creare un gradiente termico orizzontale e di videoregistrare il comportamento dei pesci, liberi di muoversi tra i diversi compartimenti, per periodi prolungati per studiarne la preferenza termica giornaliera. Per convalidare il sistema, abbiamo valutato la preferenza termica giornaliera e l'attività locomotoria nel teleosteo *Danio rerio*. Nello specifico, abbiamo confermato l'attività diurna di questa specie ed evidenziato una preferenza per temperature più alte durante la fase luce e di temperature più basse durante la fase di buio, in maniera significativa. I nostri risultati hanno evidenziato l'importanza di considerare questo comportamento di termoregolazione nella progettazione di protocolli di allevamento di pesci in cattività.

Capitolo 1.2 - Ritmi giornalieri di preferenza termica in specie di pesci diurni, notturni e di caverna.

Come evidenziato nel capitolo precedente, luce e temperatura sono variabili ambientali molto potenti nella sincronizzazione degli orologi

circadiani e in natura sono collegate tra loro (temperature più calde durante il giorno e temperature più basse durante la notte). Essendo i pesci ectotermi, hanno bisogno di mettere in atto strategie comportamentali che consentano loro di mantenere la temperatura corporea entro un range ottimale per ottimizzare la loro performance e sopravvivenza. In questo capitolo, è stata valutata la possibile preferenza termica giornaliera e confermato il pattern di attività locomotoria in diverse specie di pesci: persico trota (diurno), pesce gatto nero (notturno), tinca (notturno), pesce di grotta messicano e pesce di grotta della Somalia (entrambi privi di occhi funzionali). Sono stati evidenziato ritmi giornalieri di preferenza termica per tutte le specie testate, anche se con pattern diversi. Il persico trota è stato confermato essere diurno, il pesce gatto notturno ed il pesce di grotta della Somalia ha esibito una tendenza diurna di attività locomotoria. Entrambi i ritmi comportamentali saggiati (preferenza di temperatura e attività locomotoria) si sono mantenuti in condizione di buio costante e digiuno provandone la natura endogena, tranne che per i due pesci di caverna. I nostri risultati sono importanti per caratterizzare la biologia termiche di diverse specie con diversi pattern di attività, evidenziando come questi comportamenti siano specie-specifici e quindi sia importante ampliare lo studio a più specie possibili. Infine, questo studio è il primo che coinvolge specie notturne e di caverna nell'ambito dello studio della preferenza termica.

Capitolo 2.1 - Ritmi comportamentali giornalieri di selezione luce/buio in zebrafish.

Il moto di rotazione causa l'alternanza del giorno e della notte, nel corso delle 24 ore, consentendo alla maggior parte degli organismi di sincronizzare i propri ritmi circadiani al fotoperiodo. Nei pesci, la luce è un fattore abiotico molto importante poiché ne influenza la riproduzione, il corretto sviluppo e la conseguente sopravvivenza. Inoltre, le cellule dei pesci

sono direttamente fotosensibili. Nel presente capitolo abbiamo dimostrato che zebrafish mostra un significativo ritmo circadiano di preferenza luce/buio guidato da un orologio endogeno in condizioni di luce bianca. Nello specifico, i pesci si spostano attivamente nel compartimento illuminato durante le ore del giorno soggettivo e si muovono nel compartimento buio durante la notte soggettiva. Inoltre, questo ritmo si mantiene per diversi giorni in assenza di segnali esterni. Al contrario, questo ritmo non è presente quando viene testata la luce rossa. Per ricerche future potrebbe essere interessante studiare altre lunghezze d'onda come il blu, che apporta benefici in questa specie, come spiegato nella letteratura.

Capitolo 2.2 - Ritmi giornalieri dell'attività locomotoria e livelli di trascrizione di opsine non visive nel cervello del pesce di grotta messicano (*Astyanax mexicanus*).

Come già precedentemente evidenziato, la maggior parte degli organismi possiede orologi circadiani endogeni che sincronizzano la loro fisiologia e il loro comportamento con i cicli ambientali; il ciclo luce-buio (LD) è il segnale di sincronizzazione più potente. Di conseguenza, si può ipotizzare che gli animali che si sono evoluti nelle grotte non possiedano più un orologio biologico funzionale legato alla luce. In questo studio, *Astyanax mexicanus* è stato scelto come organismo modello per studiare i potenziali effetti delle variazioni di luce giornaliere su questa specie di caverna. In primo luogo, ci siamo concentrati sulla descrizione della sincronizzazione fotica e sulla possibile presenza di una ritmicità endogena circadiana nell'attività locomotoria di questa specie, registrando questo comportamento utilizzando diversi regimi di illuminazione: LD 12:12, uno spostamento di 6 ore di LD, buio costante (DD) e luce debole continua (LLdim). In secondo luogo, abbiamo cercato di caratterizzare i meccanismi di fotorecezione investigando le variazioni giornaliere e i possibili ritmi nell’espressione di

alcune opsine extraoculari non visive selezionate (*exo-rhod, opn3, rgra, rgrb, tmt1a e tmt1b*) nel cervello di questa specie mediante Real Time-qPCR. I nostri risultati hanno rivelato che l'attività di questo pesce è legata al ciclo LD, con un pattern di attività diurno che persiste in condizioni di illuminazione costante. Inoltre, sono state osservate variazioni e/o ritmi giornalieri statisticamente significativi in tre dei sei geni di opsine non visive analizzati (*opn3, rgra* e t*mt1b*), tutti caratterizzati da acrofasi notturne. Questi risultati suggeriscono che i ritmi giornalieri delle opsine extra retiniche non visive possono trasdurre i cicli fotici giornalieri e contribuire alla sincronizzazione dell'attività locomotoria con la luce nelle specie di pesci cavernicoli privi di occhi funzionali.

Capitolo 3. - Meccanismi di regolazione dell'assunzione di cibo nella tilapia del Nilo e nell'orata, utilizzando mangimi con ingredienti alternativi.

Negli ultimi anni, la ricerca sta studiato diverse alternative per sostituire ingredienti di origine marina che hanno un impatto negativo a livello ambientale (olio di pesce e farina di pesce) con ingredienti di derivazione vegetale (quinoa, Spirulina…) che sono ricchi di proteine da utilizzare nel settore dell'acquacoltura, in una visione più sostenibile. La ricerca svolta per questo capitolo, si è proposta di indagare i potenziali effetti dei mangimi con ingredienti emergenti sull'espressione di geni cerebrali che regolano l'assunzione di cibo (segnali oressigeni, anoressigeni e di ricompensa) di due specie ittiche di importanza commerciale, la tilapia rossa del Nilo *Oreochromis niloticus* e l'orata *Sparus aurata*. Tuttavia, il nostro studio sulla tilapia del Nilo ha dimostrato che la dieta con una maggiore inclusione di spirulina e quinoa è stata quella meno preferita dai pesci che hanno risposto più negativamente in termini di performance di crescita e di assunzione di cibo; probabilmente a causa della scarsa appetibilità e del

gusto. Le analisi di espressione genica dei segnali cerebrali che controllano l'alimentazione hanno confermato questo risultato. D'altra parte, le diete alternative sono accettate positivamente dall'orata senza alcuna differenza con la dieta di controllo per quanto riguarda il peso finale o l'espressione genica dei segnali oressigeni e anoressigeni. Ulteriori studi dovrebbero indagare e caratterizzare i meccanismi cerebrali di controllo dell'alimentazione in queste specie e ottimizzare le formulazioni biologiche ed eco-efficienti prima di utilizzarle a livello commerciale.

Capitolo 4. - Effetti dell'estratto a base di microalghe sulla rigenerazione delle cellule della pinna caudale di zebrafish.

Le microalghe sintetizzano diverse biomolecole attive, come risultato del loro metabolismo (come pigmenti, peptidi, acidi grassi e polisaccaridi) con un elevato potenziale in campo biomedico, farmaceutico e cosmetico grazie ai loro effetti antiossidanti, antivirali, antibatterici, rigenerativi della pelle, immunomodulatori e immunostimolanti. Infatti, gli estratti a base di microalghe contengono composti bioattivi che promuovono l'adesione e la proliferazione cellulare e possono essere utilizzati nelle applicazioni di guarigione delle ferite per il trattamento delle lesioni cutanee. Questo studio si è concentrato sui potenziali effetti sulla rigenerazione cellulare in una linea cellulare di fibroblasti di pinna caudale di zebrafish (AB9) trattatati con estratto *Neochloris oleoabundans*. Abbiamo provato che questo promuove la sopravvivenza, la proliferazione, la migrazione e la guarigione delle ferite in cellule AB9 mantenute in condizioni di buio costante e che l'effetto del trattamento potrebbe essere dose e tempo dipendente. Di conseguenza, questi risultati costituiscono un punto di partenza e suggeriscono una potenziale applicazione di estratti a base di microalghe nel campo della piscicoltura, per curare ferite e lesioni cutanee prima che conducano e conseguenze molto negative.

Summary in Spanish

11. Summary in Spanish

El objetivo de esta tesis fue principalmente estudiar el efecto de dos importantes variables ambientales, la luz y la temperatura, sobre los ritmos diarios de comportamiento en diferentes especies de peces con distintos patrones de actividad: diurnos, nocturnos y cavernícolas. En concreto, se estudiaron los ritmos diarios de preferencia térmica en diferentes especies de peces mediante un sistema desarrollado durante mi doctorado, confirmando sus patrones diarios de actividad. Además, se evaluó si estos ritmos diarios de comportamiento (vinculados a la luz y la temperatura) son endógenos y, por tanto, controlados y regulados por el reloj circadiano, probando también diferentes longitudes de onda. Se estudió la importancia de la fotorrecepción no visual (en la que intervienen opsinas no visuales) investigando una especie de pez de cueva que conserva la capacidad de sincronizar su actividad locomotora con los ciclos de luz-oscuridad.

Durante mi estancia en empresas (Sparos, Portugal y Alga&Zyme, Italia), tuve la oportunidad de estudiar e investigar los posibles efectos de dietas alternativas (con ingredientes emergentes como las microalgas) sobre la expresión de genes cerebrales responsables de regular la ingesta en la tilapia del Nilo y la dorada. Por último, investigué los posibles efectos de los extractos que contienen microalgas en la regeneración celular utilizando una línea celular de pez cebra.

Los objetivos fijados para la realización de la siguiente tesis fueron los siguientes:

1. Diseñar y probar una solución de grabación automática de bajo coste con una interfaz de fácil manejo para estudiar las preferencias térmicas y la actividad locomotora de los peces.

2. Evaluar y describir posibles ritmos diarios de preferencias térmicas en peces diurnos y nocturnos de interés comercial.

3. Investigar si estos ritmos diarios de preferencia térmica están regulados por relojes circadianos endógenos.

4. Evaluar la potencial preferencia térmica diaria en especies de peces de cueva ciegos, indicando las variaciones de temperatura como un posible *Zeitgeber* para estos animales que evolucionaron en ambientes con condiciones constantes.

5. Estudiar si los peces pueden mostrar ritmos diarios de selección de luz y determinar si estos ritmos son endógenos probando diferentes longitudes de onda (blanca y roja) utilizando el modelo experimental del pez cebra.

6. Caracterizar la sincronización a largo plazo de la actividad locomotora a la luz y la posible ritmicidad circadiana endógena en el pez de cueva mexicano (*Astyanax mexicanus*).

7. Investigar la posible expresión génica diaria de opsinas no visuales seleccionadas en el cerebro del pez de cueva mexicano (*Astyanax mexicanus*), que permiten a esta especie sincronizarse con los ciclos luz-oscuridad.

8. Describir los posibles efectos sobre la fisiología de los peces de las dietas con ingredientes emergentes, centrándose en los mecanismos cerebrales que regulan la ingesta de alimentos.

9. Determinar una posible aplicación de los tratamientos con extractos de microalgas en la cría de peces en cautividad mediante el estudio de la proliferación y actividad migratoria de células de pez cebra tratadas con microalgas in vitro.

10. Comprobar la posible eficacia de los tratamientos con microalgas en los procesos de cicatrización, en función de la hora (día o noche), ya que las células de los peces son fotosensibles.

Capítulo 1.1 - Ritmos diarios de preferencia térmica en el pez cebra: una solución automatizada para generar un gradiente térmico horizontal y registrar el comportamiento de los peces a largo plazo.

El fotoperiodo y la temperatura son dos de las señales ambientales más potentes que sincronizan los relojes circadianos. Al ser ectotermos, los peces deben mantener regulada su temperatura corporal para optimizar los diferentes procesos biológicos mediante la aplicación de estrategias de comportamiento. Durante la realización de esta tesis, desarrollamos un sistema automatizado de bajo coste para crear un gradiente térmico horizontal y vídeo grabar el comportamiento de los peces, libres de moverse entre diferentes compartimentos, durante largos periodos para estudiar su preferencia térmica diaria. Para validar el sistema, evaluamos la preferencia térmica diaria y la actividad locomotora en el teleósteo *Danio rerio*. En concreto, confirmamos la actividad diurna de esta especie y mostramos una preferencia por temperaturas más altas durante la fase de luz y temperaturas significativamente más bajas durante la fase de oscuridad. Nuestros resultados subrayaron la importancia de tener en cuenta este comportamiento termorregulador a la hora de diseñar protocolos de cría de peces en cautividad.

Capítulo 1.2 - Ritmos diarios de preferencia térmica en especies de peces diurnos, nocturnos y cavernícolas.

Como se ha destacado en el capítulo anterior, la luz y la temperatura son variables ambientales muy importantes en la sincronización de los

relojes circadianos, y en la naturaleza están interconectadas (temperaturas más cálidas durante el día y más bajas por la noche). Al ser peces ectotermos, necesitan actuar estrategias de comportamiento que les permitan mantener la temperatura corporal dentro de un rango óptimo para optimizar su rendimiento y supervivencia. En este capítulo se evaluó la posible preferencia diaria por la temperatura y se confirmaron los patrones de actividad locomotora en varias especies de peces: perca trucha (diurna), pez gato negro (nocturna), tenca (nocturna), pez de cueva mexicano y pez de cueva somalí (ambos sin ojos funcionales). Se evidenciaron ritmos diarios de preferencia térmica en todas las especies analizadas, aunque con patrones diferentes. Se confirmó que la perca trucha era diurna, el pez gato negro nocturno y el pez cueva somalí mostraba una tendencia diurna de la actividad locomotora. Ambos ritmos de comportamiento ensayados (preferencia de temperatura y actividad locomotora) se mantuvieron en condiciones de oscuridad y ayuno constantes, lo que demuestra su naturaleza endógena, excepto en el caso de los dos peces cavernícolas. Nuestros resultados son importantes para caracterizar la biología térmica de diferentes especies con diferentes patrones de actividad, destacando cómo estos comportamientos son específicos de cada especie y, por lo tanto, es importante ampliar el estudio a tantas especies como sea posible. Por último, este estudio es el primero que incluye especies nocturnas y cavernícolas en el estudio de la preferencia térmica.

Capítulo 2.1 - Ritmos diarios de comportamiento de la selección luz/oscuridad en el pez cebra.

El movimiento rotatorio provoca la alternancia del día y la noche a lo largo de 24 horas, lo que permite a la mayoría de los organismos sincronizar sus ritmos circadianos con el fotoperiodo. En los peces, la luz es un factor abiótico muy importante, ya que influye en su reproducción, correcto

desarrollo y posterior supervivencia. Además, las células de los peces son directamente fotosensibles. En el presente capítulo, demostramos que los peces cebra muestran un importante ritmo circadiano de preferencia luz/oscuridad controlada por un reloj endógeno en condiciones de luz blanca. En concreto, los peces se mueven activamente hacia el compartimento iluminado durante las horas subjetivas de luz y se mueven hacia el compartimento oscuro durante la noche subjetiva. Además, este ritmo se mantiene durante varios días en ausencia de señales externas. En cambio, este ritmo no está presente cuando se ensaya la luz roja. Para futuras investigaciones, podría ser interesante estudiar otras longitudes de onda, como el azul, que es beneficioso en esta especie, como se explica en la bibliografía.

Capítulo 2.2 - Ritmos diarios de la actividad locomotora y niveles de transcripción de opsinas no visuales en el cerebro del pez de las cavernas mexicano (*Astyanax mexicanus*).

Como se ha señalado anteriormente, la mayoría de los organismos poseen relojes circadianos endógenos que sincronizan su fisiología y comportamiento con los ciclos ambientales, siendo el ciclo luz-oscuridad (LD) la señal de sincronización más potente. Por consiguiente, cabe suponer que los animales que evolucionaron en cuevas ya no poseen un reloj biológico funcional vinculado a la luz. En este estudio, se eligió *Astyanax mexicanus* como organismo modelo para investigar los efectos potenciales de las variaciones diarias de luz en esta especie cavernícola. En primer lugar, nos centramos en la descripción de la sincronización fótica y la posible presencia de una ritmicidad circadiana endógena en la actividad locomotora de esta especie, registrando este comportamiento utilizando diferentes regímenes de iluminación: LD 12:12, un cambio de 6 horas en LD, oscuridad constante (DD) y luz débil continua (LLdim). En segundo lugar, intentamos

caracterizar los mecanismos de fotorrecepción investigando las variaciones diarias y los posibles ritmos en la expresión de opsinas extraoculares no visuales seleccionadas (*exo-rhod, opn3, rgra, rgrb, tmt1a* y *tmt1b*) en el cerebro de esta especie mediante PCR-q en tiempo real. Nuestros resultados revelaron que la actividad de este pez está conectada al ciclo LD, con un patrón de actividad diurna que persiste en condiciones de iluminación constante. Además, se observaron cambios estadísticamente significativos y/o ritmos diarios en tres de los seis genes de opsinas no visuales analizados (*opn3, rgra* y *tmt1b*), todos caracterizados por acrofases nocturnas. Estos resultados sugieren que los ritmos diarios de las opsinas no visuales extrarretinas pueden transducir los ciclos fóticos diarios y contribuir a la sincronización de la actividad locomotora con la luz en especies de peces cavernícolas que carecen de ojos funcionales.

Capítulo 3. - Mecanismos de regulación de la ingesta alimentaria en tilapia del Nilo y dorada utilizando piensos con ingredientes alternativos.

En los últimos años, se están investigando diferentes alternativas para sustituir ingredientes de origen marino que tienen un impacto ambiental negativo (aceite y harina de pescado) por ingredientes de origen vegetal (quinoa, espirulina...) ricos en proteínas para su uso en acuicultura, con una visión más sostenible. La investigación llevada a cabo para este capítulo tenía como objetivo investigar los efectos potenciales de los piensos con ingredientes emergentes sobre la expresión de los genes cerebrales que regulan la ingesta de alimentos (señales orexigénicas, anorexigénicas y de recompensa) de dos especies de peces de importancia comercial, la tilapia roja del Nilo *Oreochromis niloticus* y la dorada *Sparus aurata*. Sin embargo, nuestro estudio sobre la tilapia del Nilo demostró que la dieta con una mayor inclusión de espirulina y quinoa era la menos preferida por los peces, que

respondieron más negativamente en términos de rendimiento de crecimiento e ingesta de alimentos; probablemente debido a su escasa palatabilidad y sabor. Los análisis de expresión génica de las señales cerebrales que controlan la ingesta confirmaron este resultado. Por otro lado, las dietas alternativas son aceptadas positivamente por la dorada sin diferencias con la dieta control en cuanto al peso final o la expresión génica de señales orexigénicas y anorexigénicas. Futuros estudios deberán investigar y caracterizar los mecanismos cerebrales que controlan la ingesta en estas especies y optimizar las formulaciones biológicas y ecoeficientes antes de su uso comercial.

Capítulo 4. - Efectos del extracto de microalgas en la regeneración de las células de la aleta caudal del pez cebra.

Las microalgas sintetizan diversas biomoléculas activas como resultado de su metabolismo (como pigmentos, péptidos, ácidos grasos y polisacáridos) con un alto potencial en los campos biomédico, farmacéutico y cosmético debido a sus efectos antioxidantes, antivirales, antibacterianos, regeneradores de la piel, inmunomoduladores e inmunoestimulantes. De hecho, los extractos de microalgas contienen compuestos bioactivos que favorecen la adhesión y proliferación celular y pueden utilizarse en aplicaciones de cicatrización de heridas para el tratamiento de lesiones cutáneas. Este estudio se centró en los efectos potenciales sobre la regeneración celular en una línea celular de fibroblastos de aleta caudal de pez cebra (AB9) tratada con extracto de *Neochloris oleoabundans*. Demostramos que éste promueve la supervivencia, la proliferación, la migración y la cicatrización de heridas en células AB9 mantenidas en condiciones de oscuridad constante y que el efecto del tratamiento podría depender de la dosis y del tiempo. Por consiguiente, estos resultados constituyen un punto de partida y sugieren una posible aplicación de los

extractos de microalgas en el ámbito de la acuicultura, para curar heridas y lesiones cutáneas antes de que acarreen consecuencias más negativas.

Summary in English

12. Summary in English

The aim of this doctoral thesis was mainly to study the effect of two important environmental variables, light and temperature, on daily behavioural rhythms in different fish species with different activity patterns: diurnal, nocturnal and cavefish. Specifically, the daily rhythms of thermal preference in different fish species were studied using a system developed during my PhD, confirming their daily activity patterns. Furthermore, it was assessed whether these daily behavioural rhythms (linked to light and temperature) are endogenous and therefore controlled and regulated by the circadian clock, also testing different wavelengths. The importance of non-visual photoreception (in which non-visual opsins are involved) was also highlighted by studying a cavefish species that retained the ability to entrain its locomotor activity to light-dark cycles.

During my secondments in companies (Sparos, Portugal and Alga&Zyme, Italy), I had the opportunity to study and investigate the possible effects of alternative diets (with emerging ingredients such as microalgae) on the expression of brain genes responsible for regulating feed intake in Nile tilapia and Gilthead sea bream. Finally, I investigated the possible effects of microalgae-containing extracts on cell regeneration using a zebrafish cell line.

The objectives set for the realisation of the following thesis were as follows:

1. To design and test an automatic, low-cost recording solution with a user-friendly interface to study the thermal preferences and locomotor activity of fish.

2. Assess and describe potential daily thermal preference rhythms in diurnal and nocturnal fish of commercial interest.

3. Investigate whether or not these daily thermal preference rhythms are regulated by endogenous circadian clocks.

4. To evaluate potential daily thermal preference in blind cavefish species, indicating temperature variations as a possible *Zeitgeber* for these animals that evolved in environments with constant conditions.

5. To delineate whether fish can show daily rhythms of light/dark selection and determine whether these rhythms are endogenous by testing different wavelengths (white and red) using the experimental zebrafish model.

6. To characterise the long-term entrainment of locomotor activity to light and possible endogenous circadian rhythmicity in the Mexican blind cavefish (*Astyanax mexicanus*).

7. To investigate the possible daily gene expression of selected non-visual opsins in the brain of the blind Mexican cavefish (*Astyanax mexicanus*), which allow this species to entrain to light-dark cycles.

8. Describe the possible effects on fish physiology of diets with emerging ingredients, focusing on the central mechanisms regulating food intake.

9. To determine a potential application of microalgae extract treatments in captive fish breeding by studying the proliferation and migratory activity of zebrafish cells treated with microalgae in vitro.

10. To test the possible efficiency of microalgae treatments in healing processes, depending on time (day or night), as fish cells are photosensitive.

Chapter 1.1 - Daily rhythms of thermal preference in zebrafish: an automated solution to generate a horizontal thermal gradient and record long-term fish behaviour.

Photoperiod and temperature are two of the most powerful environmental signals that synchronise circadian clocks. Being ectotherms, fish must keep their body temperature regulated in order to optimise all the different biological processes by implementing behavioural strategies. During the realisation of this thesis, we developed an automated, low-cost system to create a horizontal thermal gradient and video-record the behaviour of fish, free to move between different compartments, for extended periods to study their daily thermal preference. To validate the system, we assessed daily thermal preference and locomotor activity in the teleost *Danio rerio*. Specifically, we confirmed the diurnal activity of this species and showed a preference for higher temperatures during the light phase and significantly lower temperatures during the dark phase. Our results highlighted the importance of considering this thermoregulatory behaviour when designing captive fish rearing protocols.

Chapter 1.2 - Daily rhythms of thermal preference in diurnal, nocturnal and cavefish species.

As highlighted in the previous chapter, light and temperature are very powerful environmental cues in the synchronisation of circadian clocks, and in the wild they are linked (warmer temperatures during the day and lower temperatures at night). As fish are ectothermics, they need to implement behavioural strategies that allow them to maintain body temperature within an optimal range to optimise their performance and survival. In this chapter, possible daily temperature preference was assessed and locomotor activity patterns were confirmed in several fish species: largemouth bass (diurnal), black bullhead catfish (nocturnal), tench (nocturnal), Mexican blind cavefish

and Somalian cavefish (both without functional eyes). Daily rhythms of thermal preference were evidenced for all species tested, although with different patterns. The largemouth bass was confirmed to be diurnal, the catfish nocturnal and the Somalian cavefish exhibited a diurnal trend of locomotor activity. Both behavioural rhythms tested (temperature preference and locomotor activity) were maintained under constant darkness and fasting conditions proving their endogenous nature, except for the two cavefish species. Our results are important for characterising the thermal biology of different species with different activity patterns, highlighting how these behaviours are species-specific and therefore it is important to extend the study to as many species as possible. Finally, this study is the first to involve nocturnal and cave species in the study of thermal preference.

Chapter 2.1 - Daily behavioural rhythms of light/dark selection in zebrafish.

Rotational motion causes day and night to alternate over the 24-hour period, allowing most organisms to synchronise their circadian rhythms to the photoperiod. In fish, light is a very important abiotic factor as it influences their reproduction, proper development and subsequent survival. Furthermore, fish cells are directly photosensitive. In the present chapter, we demonstrated that zebrafish show a significant circadian rhythm of light/dark preference driven by an endogenous clock under white light conditions. Specifically, fish actively move into the illuminated compartment during subjective daylight hours and move into the dark compartment during subjective night. Furthermore, this rhythm is maintained for several days in the absence of external signals. In contrast, this rhythm is not present when red light is tested. For future research, it may be interesting to study other wavelengths such as blue, which is beneficial in this species, as explained in the literature.

Chapter 2.2 - Daily rhythms of locomotor activity and transcription levels of non-visual opsins in the brain of the blind Mexican cavefish (*Astyanax mexicanus*).

As previously pointed out, most organisms possess endogenous circadian clocks that synchronise their physiology and behaviour with environmental cycles, with the light-dark (LD) cycle being the most powerful synchronisation signal. Consequently, it can be assumed that animals that evolved in caves no longer possess a functional biological clock linked to light. In this study, *Astyanax mexicanus* was chosen as a model organism to investigate the potential effects of daily light variations on this cave species. Firstly, we focused on the description of photic synchronisation and the possible presence of an endogenous circadian rhythmicity in the locomotor activity of this species, recording this behaviour using different illumination regimes: LD 12:12, a 6-hour shift in LD, constant darkness (DD) and continuous dim light (LLdim). Secondly, we sought to characterise the mechanisms of photoreception by investigating the daily variations and possible rhythms in the expression of selected non-visual extraocular opsins (*exo-rhod, opn3, rgra, rgrb, tmt1a* and *tmt1b*) in the brain of this species by Real Time-qPCR. Our results revealed that the activity of this fish is linked to the LD cycle, with a diurnal activity pattern persisting under constant illumination conditions. Furthermore, statistically significant changes and/or daily rhythms were observed in three of the six non-visual opsin genes analysed (*opn3, rgra* and *tmt1b*), all of which are characterised by nocturnal acrophases. These results suggest that the daily rhythms of non-visual extra-retinal opsins may transduce daily photic cycles and contribute to the synchronisation of locomotor activity to light in cavefish species lacking functional eyes.

Chapter 3. - Food intake regulation mechanisms of alternative feeds in Nile tilapia and Gilthead Seabream.

In recent years, research has been investigating different alternatives to replace marine ingredients that have a negative environmental impact (fish oil and fishmeal) with plant-derived ingredients (quinoa, spirulina...) that are rich in protein for use in aquaculture, in a more sustainable view. The research carried out for this chapter, aimed to investigate the potential effects of feeds with emerging ingredients on the expression of brain genes regulating food intake (orexigenic, anorexigenic and reward signals) of two commercially important fish species, the Nile red tilapia *Oreochromis niloticus* and the Gilthead sea bream *Sparus aurata*. However, our study on Nile tilapia showed that the diet with a higher inclusion of spirulina and quinoa was the least preferred by the fish, which responded most negatively in terms of growth performance and food intake; probably due to poor palatability and taste. Gene expression analyses of brain signals confirmed these results. On the other hand, alternative diets are positively accepted by Gilthead sea bream with no difference to the control diet with regard to final weight or gene expression of orexigenic and anorexigenic signals. Further studies should investigate and characterise the brain mechanisms controlling feed intake in these species and optimise biological and eco-efficient formulations before commercial use.

Chapter 4. - Effects of microalgae extract on the regeneration of zebrafish caudal fin cells.

Microalgae synthesise several active biomolecules as a result of their metabolism (such as pigments, peptides, fatty acids and polysaccharides) with high potential in the biomedical, pharmaceutical and cosmetic fields due to their antioxidant, antiviral, antibacterial, skin regenerative, immunomodulatory and immunostimulant effects. Indeed, microalgae

extracts contain bioactive compounds that promote cell adhesion and proliferation and can be used in wound healing applications for the treatment of skin lesions. This study focused on potential effects on cell regeneration in a zebrafish caudal fin fibroblast cell line (AB9) treated with *Neochloris oleoabundans* extract. We proved that this promotes survival, proliferation, migration and wound healing in AB9 cells maintained under constant dark conditions and that the treatment effect could be dose and time dependent. Consequently, these results constitute a starting point and suggest a potential application of microalgae extracts in the field of aquaculture, to heal wounds and skin injuries before they lead to very negative consequences.