UNIVERSIDAD DE MURCIA

FACULTAD DE BIOLOGÍA

Influencia de la luz y temperatura sobre la ontogenia del reloj biológico y ritmos de actividad de la lubina (*Dicentrarchus labrax*).

Dña. Natalia Villamizar Villamizar

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TESIS DOCTORAL

Natalia Villamizar Villamizar

Murcia, 2012

Memoria de la Tesis Doctoral presentada por Dña. Natalia Villamizar Villamizar para optar al grado de Doctor en Biología por la Universidad de Murcia.

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Animal Physiology Unit

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DOCTORAL THESIS

Natalia Villamizar Villamizar

Murcia, 2012

Thesis submitted by Natalia Villamizar Villamizar to obtain the PhD Degree by the University of Murcia

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To my Family

A mi Familia

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1. Introduction

1.1. The biological clock in fish

1.1.1. The circadian system

Earth´s orbital motion around the sun and on its own axis creates a dynamic environment of cycling events that occur with annual or daily periodicity. Annual (seasons) and daily (day and night) fluctuations are characterized by predictable changes of the environmental light and temperature. Through the curse of evolution, long term adaptations have enabled living organisms to orientate in time as well as in space by developing an internal clock mechanism which allows animals and plants to keep track of time in order to anticipate environmental changes, by physiological and behavioural adaptations. Important events such as reproduction, spawning and feeding are scheduled to happen under the best environmental conditions in order to ensure the survival of the specie (DeCoursey, 2004). In vertebrates, the endogenous oscillator is synchronized or entrained on a daily basis by external cues (zeitgebers) of biotic (food availability) and abiotic nature (light and temperature). The zeitgebers signals are essential to avoid the progressive drift out of phase (free-run) of the endogenous clock with respect to the environment (Vallone *et al*., 2007).

In fish, all the necessary tools for the biological clock as well as for its input and output pathways, seem to be concentrated in the retina and the pineal organ which are respectively responsible for visual and non-visual photoreception (Falcon, 1999). **The pineal** complex is located dorsal to the forebrain of fish and immediately below or within the skull roof (Ekström and Meissl, 2003). In some fish species the dorsal surface of the cranium has a translucid pineal covering (the pineal window) that allows the entrance of light to the pineal (McNulty and Nafpaktitis, 1997). Among the types of cells present in the pineal organ, the photoreceptor cells have been the focus of investigation as they respond to the changes in the environmental photoperiod through morphological modifications and synthesis of proteins (McNulty, 1982) (Figure 1). Perhaps the most important of these proteins is the so called "time-keeping" hormone, the melatonin (*N*-acetyl-5-methoxytryptamine), which is produced in the retina and the pineal organ in high amounts during the night and immediately secreted to the bloodstream (Falcón *et al*., 2007). Through melatonin secretion levels, animals are aware not only of daily environmental changes (circadian rhythms) but also of seasonal photoperiodic variations (circannual rhythms) as the duration of the melatonin rhythm depends on the duration of the night (Reiter, 1993). If the photoperiod determines the length of the melatonin elevation, the temperature determines the amplitude of the rhythm, creating a specific melatonin profile according to the seasons (Falcón *et al*., 2007). Furthermore, the

melatonin hormone is linked with important process in fish such as reproduction timing (Amano *et al*., 2000; Bayarri *et al*., 2004) and cell proliferation in embryos (Danilova *et al*., 2004). It has been shown that the development of light-entrainable circadian oscillations in melatonin occurs in fish as early as 26 hours post-fertilization (hpf) suggesting that the initial light-responsive rhythms are generated by the pineal gland and not by the retina which functionality starts by 68 hpf (Easter and Nicola, 1996; Gothilf, *et al*., 1999). There is scarce information as to the photosensitive pigments of pineal organs and, in teleosts, it limits to a small number of species. In the pike *Esox lucius*, there are potentially three photopigments with λmax at around 380, 530 and 620 nm (Falcón and Meissl, 1981). However, the list of photopigments found in fish has grown considerably in the last years and at present it includes the rhodopsin, vertebrate ancient opsin, exo-rhodopsin, UV opsin, parapinopsin, melanopsin and parietopsin (Blackshaw and Snyder, 1997; Mano *et al*., 1999; Philp *et al*., 2000; Kojima *et al*., 2000; Forsell *et al*., 2001 and 2002; Peirson *et al*., 2009). In sea bass, both rod opsin-like and cone opsin-like cells have been detected in the pineal complex which in turn, coincides with the photopigments found in the retina of developing brown trout (Herrera-Pérez *et al*., 2011; Candal *et al*., 2005).

The retinal phototransduction mechanism of fish comprehends rods and cones of distinct sensitivities and response kinetics. There are five groups of visual opsins including rod rhodopsin (*rh1*) and four cone opsins: rhodopsin-like (*rh2*), short-wavelength sensitive 1 (*sws1*), short-wavelenght sensitive 2 (*sws2*) and log-wavelength sensitive (*lws*) (Vihtelic *et al*., 1999; Bilotta and Saszik, 2001; Allison *et al*., 2010). The visual opsins constitute the first step in the sensory transduction cascades of the visual process at low and high levels of specialization. Regarding cone photoreceptors, which are similar to those of other vertebrates, fish have multiple subtypes varying in their spectral sensitivity as a result of the differential expression of opsin genes. However, the spatial arrangement of teleost cones is unique, as they form regular heterotypic mosaics which organizational pattern may vary during ontogeny (Allison *et al*., 2010). Interestingly, retinal counterparts of homologous function, the nonvisual opsins (pinopsin, melanopsin and exorhodopsin) have been found expressed outside the retina and the pineal gland (Pierce *et al*., 2008; Peirson *et al*., 2009). Indeed, in most fish organs, tissues and cells, researchers have discovered a functional circadian clock which comprises sufficient circadian oscillators and photoreceptive mechanisms for light entrainment without the involvement of the retina or the pineal gland (Cahill, 2002; Ziv and Gothilf, 2006). This **peripheral clock** becomes functional in fish larvae far before the

differentiation of specialised light-receptive structures is completed (Dekens and Whitmore, 2008).

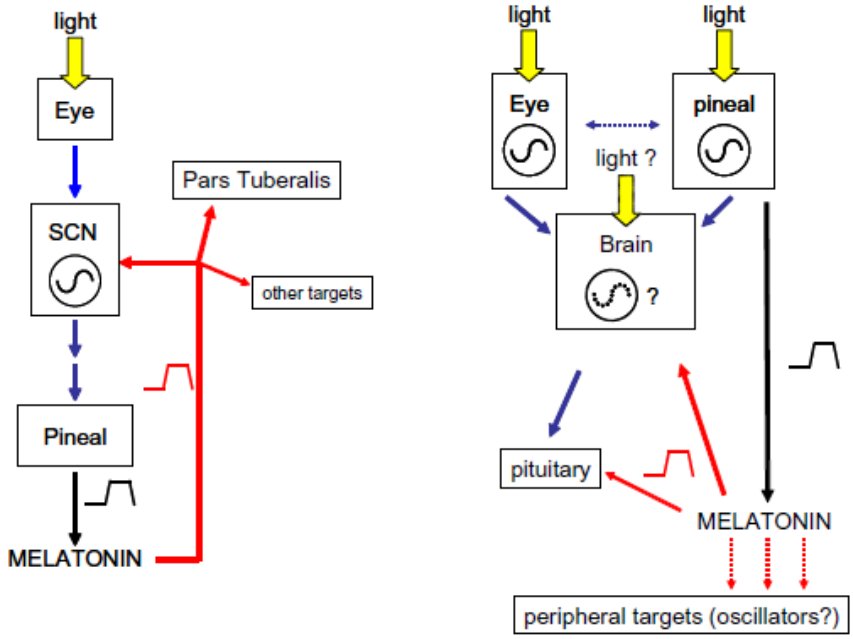


Figure 1. Photoperiodic and circadian control of neuroendocrine functions in mammals (A) and fish (B). In the first, a linear flow leads to the rhythmic production of melatonin. In the second, the photoneuroendocrine system seems to be organized as a network of independent and interconnected light-sensitive oscillatory units in the retina, the pineal and perhaps, in the brain. The dashed blue arrow indicates a hypothetical connection. '?' in the brain indicates the hypothetical presence of brain circadian oscillators (Modified from Falcón *et al*., 2010).

At the **core of the biological clock** is a transcription-translation feedback loop of circadian rhythmicity that consists of positive elements (Clock and Bmal) that drive the expression of negative elements (Period (Per) and Cryptochrome (Cry)) that in turn, feedback to down-regulate their own expression, allowing the start of a new cycle of the feedback loop. These proteins are encoded by multiple clock genes which identification and description have been a matter of profound study. For instance in the fish model, the zebrafish, three *clock* genes (*clock1*, *2* and *3*), three bmal genes (*bmal1a, 1b* and *2),* fourth per genes (*per1a, 1b, 2* and *3*) and six *cry* genes (*cry1a, 1b, 2a, 2b, 3* and *4*) have been identified (Vatine *et al*., 2011). However, despite recent important advances, the entire mechanism for the photoreception and entrainment of the biological clock in fish remains unclear.

The **ontogeny of the biological clock** in vertebrates is a matter of investigation and most of the existing knowledge relies on studies performed in the zebrafish. Firstly, it is yet

to be established the functional role of maternally inherited relevant components on the establishment of clock rhythmicity (Delaunay *et al*., 2003). The earliest detectable circadian rhythms in the developing embryo are rhythms of clock gene expression (observed in the entire embryo), melatonin synthesis and *aanat2* expression in the pineal gland (Gothilf *et al*., 1999; Kazimi and Cahill, 1999; Dekens and Whitmore, 2008). This circadian mechanism clearly requires the signals of environmental zeitgebers such as the oscillations of light and temperature, for the establishment of clock outputs as embryos raised under constant conditions of temperature and darkness lack of circadian rhythmicity (Vatine *et al*., 2011). Furthermore, the exposure of only one LD cycle is sufficient to establish and set the phase of rhythmic *aanat2* expression and melatonin synthesis (Ziv *et al*., 2005; Vuilleumier *et al*., 2006). When zebrafish embryos are exposed to light pulses as early has blastula stage (4–16 hpf, before any photoreceptor organ is formed), the signal seems sufficient to set the phase of central oscillator in the pineal gland, 2–3 day later (Ziv and Gothilf, 2006). Indeed *per2* and *6–4 DNA photolyase* mRNA expression levels have been detected in embryos at blastula and gastrula stages as a consequence of light signals (Tamai *et al*., 2004). Zebrafish cell cultures are also influenced by light as they display circadian rhythms of proliferation when exposed to LD cyles however, this cell activity persists after applying constant conditions, which implies that the zebrafish clock has also an autonomous regulation (Dekens *et al*., 2003).

1.1.2. Underwater photo- and thermo-environments

Light and temperature cycles are considered as the main synchronizers of seasonal (i.e. reproduction, migration) and daily (i.e. feeding, spawning) rhythms in fish (Sánchez-Vázquez and Madrid, 2001; Bromage *et al*., 2001). Light may act through daylength seasonal patterns, while temperature influences fish by the natural oscillations of the thermo-cycle. The aquatic **photo-environment** is highly dynamic as the radiant energy from the sun is selectively absorbed and scattered by particles present in the water column which thus affect the magnitude (square of the electric field vector), polarisation (direction of oscillation of the electric field vector), wavelength (frequency of oscillation), direction and propagation of the light (Smith, 1974). The water column acts as a potent chromatic filter with wavelengths below violet ($\lambda < 390$ nm) and beyond red ($\lambda > 600$ nm) being quickly absorbed. Blue wavelengths ($\lambda \sim 450$ nm) however, penetrate deeper in the underwater environment, reaching depths of up to 150 m in the clearest ocean waters (Lalli and Parsons, 1995). In coastal and continental water, particles in suspension and substances originated from the decay of organic matter affect water transparency and spectral absorbance, which in turn

shifts the perceived spectral profile (Figure 2). Fish living in different underwater photo-environments appear to have adapted their maximum photopigment sensitivity accordingly (Kusmic and Gualtieri, 2000). This would suggest that the biological response to light depends on the species-specific ecology however, while fish retinas have been well characterized at the ultrastructure level, the sensitivity to light remains unclear in most fish species.

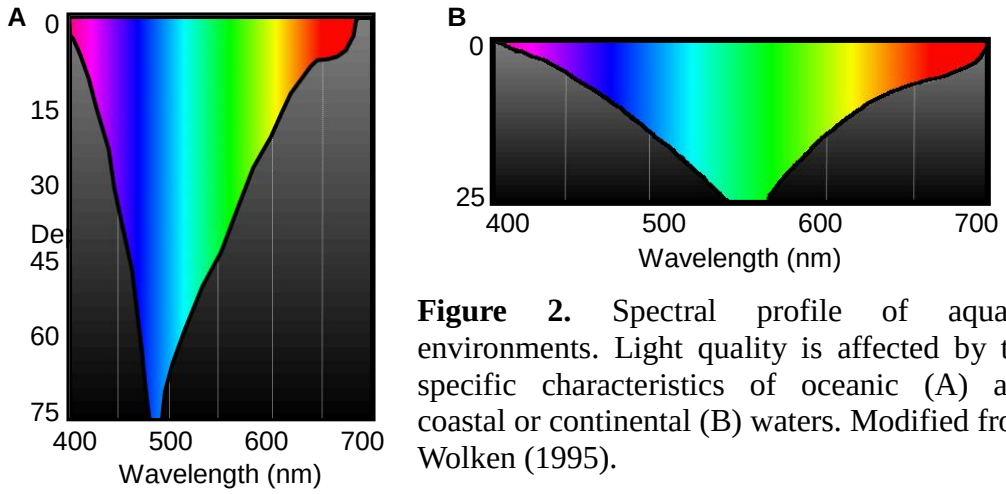


Figure 2. Spectral profile of aquatic environments. Light quality is affected by the specific characteristics of oceanic (A) and coastal or continental (B) waters. Modified from Wolken (1995).

The **thermo-environment** is generated by the cyclic infrared radiation from the Sun within a year (annual rise of temperature during the spring and summer seasons and a drop of temperature during the autumn and winter months) or during the day (rise of temperature during the light phase and a drop in temperature during the dark phase) (Johnson *et al*., 2004). These basic natural oscillations of light and temperature are, in most of the cases, not being considered in the aquaculture industry. For fish rearing, all environmental parameters are meticulously controlled to avoid possible fluctuations, as they are thought to compromise fish survival rates. Regarding the experimental field, scarce studies have being dedicated to the natural oscillations of environmental parameters on fish performance. Furthermore as occurs in the natural environment, the oscillations of light and temperature are strongly related which caused that in several occasions, researches fail to differentiate photoperiodic from temperature effects. For example, there is continuous controversy about whether the effects of temperature are direct or if temperature acts as a modulating factor in important aspects of fish such as reproduction timing and sexual maturation (Bromage *et al*., 2001).

1.2. Lighting conditions

1.2.1. Photoperiod

As seen previously, fish exhibit circadian and circannual cycles in their activities which are synchronized to the natural environmental fluctuations. Annual clocks regulate daily and seasonal rhythmic physiological processes, including smoltification and reproduction by the periodical synthesis and release of gonadotropins, sex steroids and gonadal growth factors (Amano *et al*., 2000; Bayarri *et al*., 2004; Bromage *et al*., 2001; Falcón *et al*., 2010). Several fish species reproduce once a year during a specific season, which provides the offspring with the most suitable environmental conditions for survival (Oliveira and Sánchez-Vázquez, 2010). For this annual event, daily physiological modifications are needed, which in most cases involve the secretion of sexual steroids or gonadotropins and the maturation of oocytes (Prat *et. al*, 1999; Oliveira *et al*., 2009; Blanco-Vives *et al*., 2011). Furthermore, daily rhythms of reproductive hormones (sbGnRh and LH) have been seen to be synchronized to the dark phase of the photocycle in caged European sea bass (*Dicentrarchus labrax*) (Bayarri *et al*., 2004). However, in this study, the daily rhythm of LH release was observed to be affected by a constant long photoperiod (18 h of light : 6 h of darkness, 18L:6D) which also suppressed circulating melatonin. Indeed it has been suggested that melatonin is one of the factors that mediate the transduction of photoperiodic information to the hypothalamic-pituitary-gonad axis (Amano *et al*., 2000).

In fish species such as the European sea bass and Senegalese sole, which reproductive and spawning cycles are lengthy (more than 1 year), long and short-term cues are likely to control the timing of egg and sperm release. Indeed, in captive European sea bass, which naturally spawns during the winter and early spring (when daylight is reduced), the application of constant short days (9L:15D) are capable of bring forward ovulation and spawning (Carrillo *et al*., 1989). However, the manipulation of the photoperiod in order to control fish reproduction is rather complicated as in many fish species, a decreased in egg quality has been observed in out of season spawnings (Breton *et al*., 1983; Devauchelle *et al*., 1988; Davies and Bromage, 2002; Van der Meeren and Ivannikov, 2006). The direct reason for this remains unclear, although alterations in ovulation timing after photoperiod-advance have been suggested as possible causes (Bonnet *et al*., 2007).

The time of reproduction seems to coincide with the specific rhythms of behaviour; however it is not clear if the spawning event is the causative or the consequent factor in the behavioural responses of some fish species. In zebrafish maintained under artificial LD conditions, both spawning and locomotor activities rhythms were observed to be synchronized to the light phase. Under constant light, the **daily spawning rhythm** of zebrafish persisted, suggesting a strong control of the endogenous clock on both activities

(Blanco-Vives *et al*., 2009). In a study with Senegal sole, a daily spawning rhythm was observed taking place during the dark phase which coincided with the nocturnal locomotor activity registered from the broodstock (Oliveira *et al*., 2011). For sea bream, a clear daily spawning rhythm has been observed taking place at sunset, which agrees with the daily activity rhythm reported for this species (Meseguer *et al*., 2008; Velázquez *et al*., 2004). European sea bass juveniles show an interesting phase shift of activity rhythms which coincide with the reproductive cycle. Thus, during the resting season (spring-summer) the feeding activity takes place during the day and during the spawning season (winter), the European sea bass feeds during the night (Sánchez-Vázquez *et al*., 1998). However, little is known about the existence of this shift in the behaviour of adults or if daily spawning rhythms take place in this species.

After fertilization, the specific lighting conditions for the initiation of rhythmic clock outputs have been extensively explored in the zebrafish as it has been suggested that 4 LD cycles are needed to establish a cell cycle rhythm, while just one LD cycle is required for melatonin synthesis and rhythmic expression of clock gene *per1b* (Dekens *et al*., 2003; Dekens and Whitmore, 2008; Vuilleumier *et al*., 2006). Light starts to be detected by many of the **embryo cells** as early as gastrula stage (5 hours post fertilization, hpf), even though cells are still undifferentiated and actively dividing. This early detection takes place ahead of any neural or classical light detecting form such as the pineal gland (24 hpf) and the retina (2-3 days post fertilization, dpf) (Kazimi and Cahill, 1999; Tamai *et. al*., 2004). Therefore, LD cycles influence the cell proliferation (and consequently, growth) process by mitogenic stimulation, which remarkably in some species is timed to occur at the end of the day or during the night (Nikaido and Cahill, 1999; Dekens *et al*., 2003). Although **embryogenesis** in fish has been classically considered as a succession of developmental stages controlled by temperature, recent findings point to the key role played by the entrainment of the biological clock on early fish developmental rhythms (Gorodilov, 2010). Indeed the embryos internal clock is so strong that has allowed researchers to create predictable time models for several specific periods within embryogenesis such as cell division in the cleavage stage (Kirschner et al., 1985), transition to the middle blastula stage (Newport and Kischner, 1982), start of gastrulation (Satoh, 1982) and somitogenesis (Schröter *et al*., 2008). To present, light synchronization and the different phasing (diurnal/nocturnal) of embryo rhythmicity, and most importantly its circadian nature, remains unknown.

Early **larvae development** and growth also respond to light and dark (LD) cycles as observed in a remarkably great variety of species. Although light signals have been suggested

as necessary cues for the early normal growth and development of fish, the intensive rearing protocol applied in the aquaculture industry is in many cases, characterized by the application of periods of either constant light (LL) or darkness (DD) (Coves *et al*., 1991; Shields, 2001). On an experimental level, the numerous contrasting results found among the great variety of studied fish leads to the hypothesis that the response to a particular light regime is species specific (Downing and Litvak 2001, Saka *et al*. 2001, Monk *et al*. 2006, Blanco-Vives *et al*. 2010; Partridge *et al*., 2011). In some cases long photoperiods and even LL conditions seem to favoured larval growth through a better feeding rate (Moustakas *et al*., 2004; Puvanendran and Brown, 2002). In visual feeders, larvae relies on ambient light for prey detection and this is even more critical during the early stages due to their short visual range that increases as larvae develop (Huse and Fiksen, 2010). However, LL as well as DD have been reported to act as a detrimental factor in species like the Western Atlantic sea bream larvae, *Archosargus rhomboidales* (Dowd and Houde, 1980) and the snapper *Pagrus auratus* (Fielder *et al*., 2002). Apart from growth, other important parameters have been observed to be influenced by the application of LL and DD conditions. Most physostomic fish larvae, including sea bass, initiate swim bladder inflation after the onset of darkness, possibly as an adaptation for predator avoidance since larvae have to migrate to the water surface to gulp air (Battaglene and Talbot 1990). Furthermore, as reported by Ronzani Cerqueira *et al*. (1991) sea bass larvae have a negative phototactic behaviour, which makes the application of dark periods a mandatory condition for the natural daily migrations of the larvae and consequently, the development of the swim bladder. Strongly related to the abnormal development of the swim bladder is the high frequency of vertebral deformities such as lordosis and kyphosis (Koumoundouros *et al*., 2002a) which in turn, increase fry mortality rate. Another consequence of swim bladder anomalies or the lack of it is the poor growth performance resulting from the high energy cost for the larvae to maintain their buoyancy (Chatain and Ounais-Guschemann, 1990). A recent study in European sea bass reared under an intensive system (DD during the first days), reported an increase in ossification problems that leads to skeletal malformations such as the loss of vertebra (Zouiten *et al*. 2011).

Continuous illumination or dark conditions are evaluated by the culturists as a balance of benefits and losses in order to obtain the best outcome. However, this is often a complicated issue especially when the natural physiology and behaviour of the species is not taken into account. In a great variety of fish the exposition to light (including UV radiation), occurs very early during embryogenesis which have triggered the development of photoprotective strategies such as the synthesis of pigments (melanin and carotenoids) or the

synchronization of sensitive process to the night phase in order to prevent DNA damage. Indeed, zebrafish embryos (7 hpf) reared under LD cycles survived significantly higher (85%) when exposed to short pulses of UV than embryos raised under DD or LL, which survival decreased to 61% and 20% respectively (Tamai *et al*., 2004). Regarding sea bass, an investigation performed by Roncarati *et al*. (2001) compared their performance when reared intensively (DD, clear water, high stocking density) and extensively (natural photoperiod, mesocosms, low density) and found no differences regarding specific growth rate (SGR), feed conversion ratio (FCR) and weight. Furthermore, in this study, sea bass reared under a mesocosm system had a better survival rate and better body shape, ideal conditions for commercially producing a top quality product. The results in growth agree with those obtained in early studies where sea bass reared under natural conditions grew better when compared with intensively cultured groups (Melotti *et al*., 1993 and 1994). Apart from the specificity of the response of each fish species to the photoperiod, some studies have found that there may also be diverse light requirements for different populations of the same species as reported for two different populations of Atlantic cod, where the best results were obtained when the natural specific photoperiod was applied to each population (Puvanendran and Brown 1998, Van der Meeren and Jorstad 2001). Based on the above mentioned results and many others, there is enough information that suggest that under artificial conditions, fish larvae performed better under the photoperiod that best resemble their natural environment.

1.2.2. Light spectrum

In fish, the physiological and behavioural responses to light spectrum are the result of long term adaptations to their surrounding environment, which signals are rather complex and can display large variability over short periods of time during both daily and annual cycles. Throughout the different stages of their life, most fish species are exposed to waters of very different spectral characteristics that may lead to a change on the function, composition and cellular organization of their visual system (Evans and Fernald, 1990). In some salmonids, **newly hatched larvae** have ultraviolet-sensitive (UVS) cones that mostly disappear during the smoltification process and reappear in the adult stage (Cheng *et al*., 2006). These retinal morphological changes match the spectral sensitivity of salmonids as they prepare to the oceanic life (Migaud *et al*., 2007; Novales Flamarique, 2000). In another species, the winter flounder (*Pseudopleuronectes americanus*), one single cone pigment have been identified during its pelagic stage which expression completely disappears during the benthic stage while three new cone opsins appear together with a new mosaic formation (Evans *et al*.,

1993). Interestingly, in both salmonids and flatfish, the changes related to colour vision are not induced by a difference in the **visual environment**, but rather precede the change in habitat. Such endogenous control of the visual system is different from active developmental optimisation of colour vision, which would involve information about the spectral environment to feed back on the differentiation and organisation at different stages of visual information processing (Wagner and Kröger, 2005).

Fish visual plasticity allows morphological adjustments to the spectral characteristics of their surrounding environment. As observed in an experiment performed by Wagner and colleagues (2005), in analogy to photostasis (rods compensatory mechanism to different light intensities), the blue acara (*Aequidens pulcher*) presented spectral selective modifications in its cones as a response to specific wavelength stimulation. Thus, the term "chromostasis" was suggested to describe the morphological adaptation of fish to new light profiles of different spectral composition. However, in the natural environment fish colour vision is optimised by regulatory mechanisms acting on various levels within the visual system. For example, the variation in opsins and rhodopsin protein components that leads to individual differences in the peak sensitivities of the absorption spectra in the different types of photoreceptors (Archer *et al*., 1987; Archer and Lythgoe, 1990).

Thus, the understanding of the physiology and behaviour of a specific fish species together with knowledge of its natural environment are mandatory in order to elucidate the complete mechanism behind the **photoresponse behaviour**. For example, the response to light of deep sea fish species relies on photoreceptors that maximize visual contrast in the blue band, while coastal fish species have maximum sensitivity in the green band (Lythgoe, 1979). The zebrafish, an inhabitant of shallow margins of freshwater lakes and rivers, seemed to have undergone a shift of photoreceptor λmax (maximal absorbance) towards the short wavelengths in order to enhance the luminance contrast of food and predators dark profile against the relative bright background of the down-welling light (Nawrocki *et al.*, 1985; Cameron, 2002). Larval milkfish (*Chanos chanos*) have rod cells and red, green, blue and violet cone cells, while juvenile loss the violet cone cells and shift the blue cones to shorter wavelengths (Chang *et al*., 2009). Taking the existing information of fish larvae visual system together, there is the possibility that they could be predisposed to perform best under the spectral conditions most frequently encountered in their particular ecological niche. In Senegal sole, plasma melatonin of adults was significantly reduced when exposed to short wavelengths of light during the night, suggesting a high sensitivity of the species towards this particular light spectrum (Oliveira *et al*., 2007). Interestingly, sole embryos and larvae have

been observed to performed better (i.e. highest growth, earlier metamorphosis and lower jaw malformations) under blue light (Blanco-Vives *et al*., 2010). These findings agree with similar studies on other larvae marine species such as haddock (*Melanogrammus aeglefinus*) and the Atlantic cod (*Gadus morhua*) were short wavelengths enhanced growth, myotome development and feeding (Downing and Litvak, 2001; Migaud *et al*., 2009).

Although the morphology of visual photoreception is well studied in some fish species, conclusive studies about the their **spectral sensitivity** are scarce, perhaps due to the lack of standardisation of experimental components such as the units of light measurement (i.e. lux, watts, photon flux), the light sources of very different spectral compositions (i.e. halogen, tungsten, light emitting diodes LED), the rearing system (i.e. tank dimensions) and husbandry protocols (green vs clear water). In the **aquaculture field**, the lighting system and illumination protocol of a hatchery are generally based on larval performance (growth and survival) and the economic costs of the light source. Thus the particular ecology of a given fish is rarely taken into account. In most of the rearing tanks of a hatchery, the incident light is highly directional as the shallow and often clear water used has little absorption and scattering ability in comparison to natural habitats. Therefore, the light environment experienced by larval fish under culture conditions is substantially different from the natural environment that fish have been selectively programmed to develop under (Naas *et al*., 1996). At present, many hatcheries use either 'true-light' tubes, tungsten filament, fluorescent or metal halide lights, which create bright point light sources with spectral emissions of unnatural wavelengths that may not be detected by fish and/or could potentially compromise their welfare (Boeuf and Le Bail, 1999; Migaud *et al*., 2007). These lights are characterized by lower colour temperatures (2700–3000 K; yellowish-white through red) rather than the higher colour temperatures or cool colours (5000 K or more; bluish white) found in the natural photo-environment (Wolfgang, 1992) (Figure 3). In the aquaculture industry new lighting technologies such as light emitting diodes (LED) and cathode lights (CL) are currently being investigated to support the development of species and stage of development specific lighting systems (Migaud *et al*., 2007). One reason for exploring these technologies is that such systems can be tuned to match the target species sensitivities through narrow bandwidth outputs.

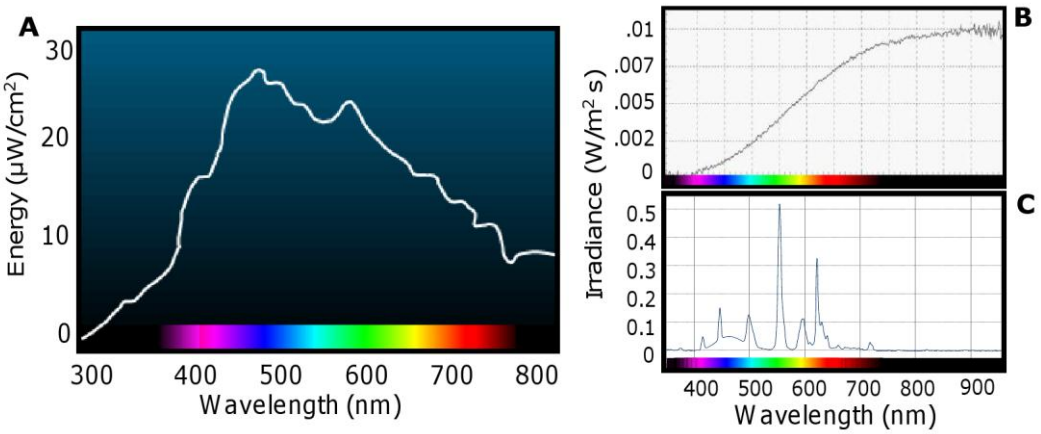


Figure 3. Natural sun spectrum differs from artificial lights. Spectral profile at earth's surface (A) (Modified from Wolken, 1995), contrasted with the profile of a standard electric bulb (B) and fluorescent light (C).

As stated before, from a very early stage fish are exposed to UV wavelengths (280–400 nm) for which specific sensitive pigments have been developed (Chang *et al*., 2009). Indeed UV vision is more common in fish than in any other vertebrate group as it complements visual communication, camouflage, colour vision and protection against UV overexposure (Losey *et al*., 1999; Jokinen *et al*., 2001). The fact that zooplankton UV-protective pigments enhance their contrast under UV light, may serve as an adaptive explanation for the importance of these pigments in planktivorous fish larvae (Flamarique, 2005; Britt *et al*., 2001). Indeed, one of the bottlenecks for larvae rearing is the start of **exogenous feeding** as it is, in most of the times, composed of live prey. Many studies on larvae foraging behaviour considered only one or two critical factors that influence prey detection and ingestion, due to the complex relationship among the several elements involved. For example, down welling light is absorbed or scattered and is reflected from the prey, becoming an image-forming light that enters the retina of the larvae, which has a short visual range that increases as larvae develop (Huse and Fiksen, 2010). In previous studies, this range was found to be different among species, so it peaks at 550 nm in the two-spotted goby *Gobiusculus flavescens* (Utne-Palm and Bowmaker, 2006), 470 nm in the haddock *Melanogrammus aeglefinus* (Downing and Litvak 2001) and 450–500 nm in the salmon *Salmo salar* and the European sea bass (Max and Menaker 1992). Another important aspect to bear in mind when studying larval feeding is actually the **prey behaviour** towards light stimuli (i.e. phototaxis, feeding, reproduction). For example, *Artemia sp*. is known to gather in monoespecific groups (swarming behaviour) of densities usually 100–1000 times greater than in surrounding waters (Omori and Hamner, 1982). There are several factors involved in swarming that have been

studied in the past and are still a matter of investigation. Among the most studied ones is light, as artemia swarming behaviour has been observed both under open sunlight as well as under concentrated light. The formation of swarms has been reported to affect larval feeding as the swimming activity of artemia increases within the swarm, making them less likely to be captured (Gulbrandsen 2001).

Although the mechanism by which light spectrum affects larval physiology and behaviour during early development remains unclear, the possible long-term consequences of early exposure to unsuitable lighting environments is becoming more evident, as proved by the ongoing results of recent research. The development of flexible lighting platforms that allow spectral specific adjustments for fish larvae requirements would certainly improve the knowledge of their visual system.

1.3. Temperature regime

1.3.1. Constant vs Cycles

From all environmental factors influencing fish, the temperature is considered the "abiotic master factor" as it influences the behaviour, physiology and distribution of aquatic ectotherms (Fry 1947). Over the last few decades, research has been focused on the fish response to different temperature regimes as they display a variety of ecological responses (eurythermal or stenothermal species) depending of the specific tolerance level given by the species genetics (Lutterschmidt and Hutchinson 1998, Beitinger *et al*., 2000). Thermal tolerance is also strongly related with the capacity of a developing fish to permanently change its phenotype in order to adapt to the variable temperatures of the water environments (developmental plasticity) (Kinne, 1962). Despite fish being able to adapt to a rather wide range of temperatures, they have a preference for a particular temperature which is closely related with the **optimal temperature** for many biochemical and physiological process such as growth rate, food conversion, immune response and reproduction (Hutchinson and Maness 1979, Johnson and Kelsch 1998). This preferred temperature may change during fish's life time since its selection could be influenced by the season of the year (Sauter *et al*., 2001; Mortensen *et al*., 2007), feeding and nutritional state (Van Dijk *et al*., 2002), health (Golovanov, 2006), developmental state and seasonal variations (McCauley and Huggins, 1979). Moreover, it has been reported that the response to environmental factors varies in different strains of a particular species. In European sea bass for example, juveniles from the Mediterranean Sea are reported to grow quickly under the optimal range reported for the

species, but juveniles from British waters grow better at lower temperatures such as 18°C (Russell *et al*. 1996).

In the **early stages of fish** development, temperature is known to have a major impact as suboptimal temperatures can lower fertilization (Brown *et al*. 1995), egg quality (King *et al*. 2003, Bobe and Labbé, 2010), hatching rate (Hokanson *et al*. 1973), as well as increase embryo deformities (Aegerter and Jalabert, 2004). The rearing techniques for commercial fish species involved, in most of the cases, constant temperature conditions that are far from the natural environment where seasonal and daily oscillations of temperature influence several (if not all) aspects of the developing process. Surprisingly, the experimental research has also focused on the effects of constant temperature conditions. In some teleost, different responses have been obtained when exposing embryos and larvae to different temperatures within a narrow range (~ 2°C). In their study, Saka and colleagues (2001) investigated just the upper limit of the optimal temperature recommended for sea bass incubation and found that **hatching** was brought forward 20 h under 17°C when compared to 15°C. As reported for lighting conditions a given temperature can bring both positive and negative results. Indeed, early studies have found that the rapid growth as a result of high rearing temperatures could be associated with muscle malformations such as fibre hyperplasia (Gibson and Johnston 1995, Johnston and McLay 1997). In another research, sea bass growth was found to be improved at high temperatures (18°C and 21°C) but the normal development of the swim bladder was obtained at low temperature (12.5°C) which in turn, coincided with the **natural ambient temperature** (Johnson and Katavic, 1984). Ayala and colleagues (2001) applied the same temperatures that Saka et al. (2001) during the egg incubation of sea bass (15 and 17°C) but after hatching, reared both groups under the same natural temperature and found that at the end of metamorphosis, muscle growth was higher in larvae whose incubation was performed under 17°C. This suggests that temperature influences sea bass at a very early stage during embryo ontogeny and its effects may modify the latter development of the larvae and juveniles. As with the lighting conditions, the temperature protocol for fish rearing during their early stages is at present the result of a delicate balance between growth, survival and normal development without considering the natural thermo-environment of the fish.

In the wild, water warms up after sunrise (thermophase) and cools down after sunset (cryophase). This dynamic of the water creates a challenging ecosystem to which the organism must adapt in order to survive. Currently, fish are reared under **constant thermal environments** which are thought to maximize growth, but at the cost of reducing thermal tolerance and phenotypic diversity. Indeed, animals reared under cyclic conditions are more

likely to survive environmental changes as temperature oscillations maximized thermal resistance (Woiwode and Adelman 1992, Schaefer and Ryan 2006). Under controlled conditions, in the absence of light cues, when thermocycles are applied the thermophase usually elicit the same physiological response as the light phase and the cryophase brings out the response corresponding to the dark phase (Rensing and Ruoff, 2002). When light/dark and high/low temperature cycles are combined, a stabile phase and maximum amplitude can be observed in the rhythms, but when the two synchronizers are phase-shifted, the phase of the rhythm is determined by either zeitgeber or by both, depending on the relative strength of each one and the sensitivity of the species (Rensing and Ruoff, 2002). Thus "**optimal temperature cycles**", rather than optimal constant temperatures, should be investigated in order to maximise growth performance, survival and minimise malformations in a particular fish species. The application of thermocycles have been performed in some species such as the largemouth bass *Micropterus salmoides*, whose growth performance was better when maintained at high-low cycles of temperature and were fed at the onset of high temperature (Diana 1984). In zebrafish, when a daily thermocycle was applied under constant light, the activity rhythm was synchronized to the thermophase (López-Olmeda *et al*. 2006). This suggests that as well as photocycles, the daily oscillations of temperature can also synchronize the activity patterns of fish. A more recent research with Senegal sole (*Solea senegalensis*) shows that larvae growth and development was best when reared under natural thermocycles (TC, 22°C during the day and 19°C during the night) rather than under an inverted thermocycle (CT, opposite to TC) or constant temperature (20°C). In this study, the eye migration of larvae under TC occurred earlier than in the rest of treatments (Blanco-Vives *et al*. 2010).

1.3.2. Temperature sex determination (TSD)

Other important aspect of the influence of the temperature in fish is the **sex determination** as it is influenced by environmental or genetic factors. Fish show a large variety of mechanisms of sexual determination and patterns of sexual differentiation. Among the first, monogenic and polygenic systems have been discovered in autosomes or chromosomes (Devlin and Nagahama, 2002). The sexual differentiation of teleost fish is characterized by a high diversity of reproductive strategies such as synchronous hermaphroditism, protandrous and protogynous hermaphroditism and gonochorism (Yamamoto, 1969). Both determination and differentiation processes are highly plastic as even on top of a given genetic background, environmental factors such as temperature may

wield a strong influence (Baroiller *et al*., 1999; Munday *et al*., 2006). Temperature fluctuations inherent of the aquatic environment alter biochemical pathways of sexual determination and act upon an individual to induce male or female development (Devlin and Nagahama, 2002).

In gonochoristic vertebrates, two main sex-determination processes can be defined: genotypic or chromosomal sex determination (GSD) and environmental sex determination (ESD) where no genetic differences between sexes are present and sex is determined after fertilization by environmental factors (Penman and Piferrer, 2008). In ESD fish, the most important environmental factor is the temperature, and this process is termed **temperature sex determination** (**TSD**). According to data available in the bibliography, around 60 fish species seem to present TSD, in which most of them, the proportion of males increases with temperature (Ospina-Álvarez and Piferrer, 2008) (Figure 4). This may be the case of some species of the genus *Apistogramma*, *Atherinid*, and *Oreochromis* (*O. mossambimbicus* and *O. aureus*) (Baroiller *et al*., 1999; Römer and Beisenherz, 1996; Strüssmann *et al*., 1997; Desprez and Melard, 1998; Wang and Tsai, 2000). However, this is not a general rule for teleost fish since low temperatures may also increase the proportion of males (Cyprinodontiform *Rivulus marmoratus*) (Harrington and Crossman 1976) or temperature may not affect sexual proportion (*Fundulus heteroclitus*, *Cyprinodon variegatus*, *Coregonus hoyi*) (Conover and Daemond 1991). It has been suggested that in the case of the gonochoristic European sea bass, sex determination can be controlled by both genetic and temperature effects for which it has been classified as a GSD+TE species (Vandeputte *et al*., 2007; Navarro-Martín, 2009). Furthermore, in TSD fish (including sea bass) the temperature experienced during a particular time during early development (or thermosensitive period), irreversibly determines gonadal sex (Navarro-Martín, 2011). Regardless of the sex determining system, in non-mammalian vertebrates the androgen-to-estrogen ratio determines whether a sexually undifferentiated gonad sexually differentiates into a testis or ovary. This sex steroid ratio depends of the activity of the **enzyme aromatase**, Cyp19a, the product of the ***cyp19a*** gene, which irreversibly converts androgens into estrogens, thus the masculinizing effect of high temperatures are invariably caused by an inhibition of *cyp19a* expression and enzymatic activity (Van Nes and Andersen, 2006; D'Cotta *et al*., 2001). In turn, *cyp19a* negatively regulate the levels of the *amh* (Anti-Müllerian hormone) gene (and visceversa) which has been shown to have a dimorphic expression as it plays a key role in the proliferation and differentiation of spermatogonia of fish species such as the Nile tilapia *O. niloticus*, the rainbow trout *Oncorhynchus mykiss*, the Japanese flounder *Paralichthys*

olivaceus and the zebrafish (Ijiri *et al*., 2008; Vizziano *et al*., 2007; Yoshinaga *et al*., 2004; Rodríguez-Marí *et al*., 2005).

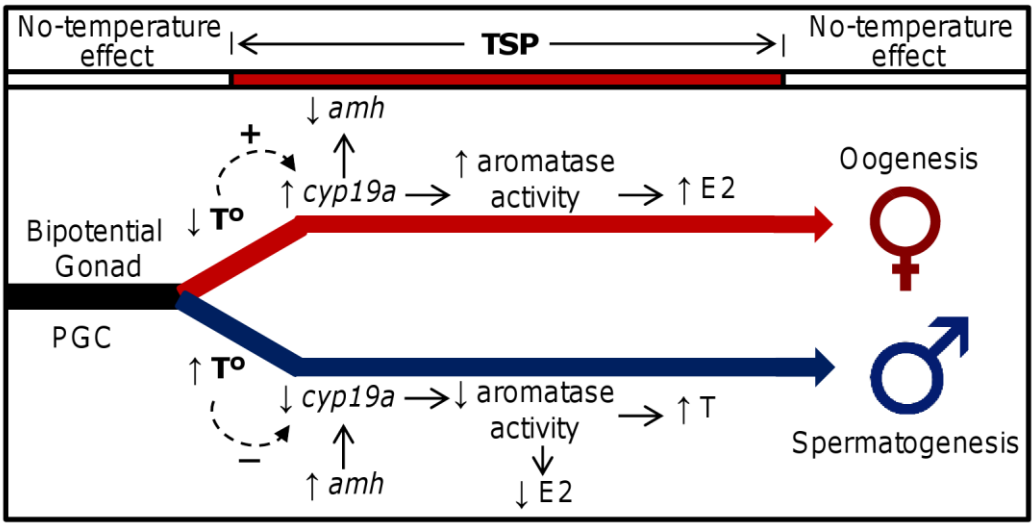


Figure 4. Role of aromatase (*cyp19a)* and anti-müllerian hormone (*amh*) on temperature sex determination in fish (TSD). The bipotential gonad is sensitive to ambient temperature during a specific critical period (thermo-sensitive period, TSD). Thus, high temperatures act as male-promoter by suppression of *cyp19a* gene expression which lowers the aromatase activity and in turn, the levels of estrogen (E2). *amh* is involved in spermatogonia proliferation and differentiation and act as a negative regulator of *cyp19a* and visceversa. On the other hand, low rearing temperatures result in female differentiation by an increase of *cyp19a* gene expression. The direct mechanisms in which temperature acts on cyp19a are unclear (dashed arrows). PGC: primordial germ cells, T: testosterone. (Based on: Leet *et al*., 2011; Tong *et al*., 2010; Siegfried 2010; Guiguen *et al*., 2010; Rodríguez-Marí *et al*., 2005).

For the aquaculture industry, the control of the sex ratio means an important improvement of economic benefits. In some species such the European sea bass, females are larger than males and reach puberty at 3 years of age, long after attaining marketable size. In contrast, puberty in males usually appears in the second year of life, essentially coinciding with the time of marketing. In males, puberty is associated with growth retardation and results in an important limitation in the commercial production of sea bass since males must be either harvested at a smaller size or reared for longer than females (Zanuy *et al*., 2001). As a result, numerous studies have been dedicated to obtain an optimal male:female ratio in a variety of cultured fish by means of temperature manipulation but so far, contrasting results have been found even when studying the same species (Blázquez *et al*. 1998, Koumoundouros *et al*. 2002b, Saillant *et al*. 2002). The temperature turns out to be a complex element which manipulation in favour of female sex determination may affect other aspects

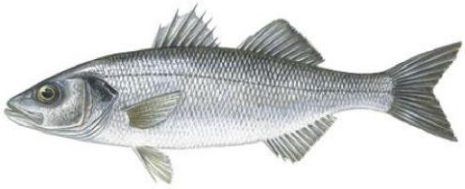
such as growth, as fish reared at low temperatures also attained lower weights. Interestingly, this problem seems to be solved when fish are exposed to low temperatures only during the larval stages as compensatory growth is observed at the end of the first year (Mylonas *et al*., 2005). Based on the important amount of existing results, it is clear that constant temperatures cannot induce ovarian differentiation as stated by the equivocal **sex ratios** results across different studies. In contrast, the influence of thermocycles has been studied in species such as the tilapia *Oreochromis aureus*, where a TC (35°C during the day and 27°C during the night) not only beneficiated growth rate, survival, and size heterogeneity but also increased the number of females (Baras *et al*. 2000). In this case the application of the temperature regimens started at the onset of exogenous feeding (mean body weight: 12 mg) after incubating embryos and larvae under constant temperature (27°C) which could have influenced the results as temperature affects fish right from spawning and fertilization. In a more recent investigation, eggs and larvae of Senegal sole were reared under TC (22°C during the day and 19°C at night), CT (opposite to TC) and constant 20°C. As a result, fish exposed to TC had a better performance (growth and gonadal development), higher proportion of females (70.8 ± 2.6%) and higher concentration of estradiol (E_2) in contrast with the CT and constant temperature groups where the proportion of females was significantly low (17.5 ± 7.6% and 38.3 ± 4.3% respectively) (Blanco-Vives *et al*. 2011). The recent advances reveal the strong impact of **daily thermocycles** when applied during the early development of fish and therefore they open an interesting research path for the application of the natural thermocycles on an experimental level to those species of commercial interest.

1.4. Species in focus

1.4.1. European sea bass *Dicentrarchus labrax* (Linnaeus, 1758)

This marine teleost fish which genus name derives from the presence or two dorsal fins has the following taxonomic hierarchy (Van der Land et al., 2001):

Phylum: Chordata
Subphylum: Vertebrata
Superclass: Osteichthyes
Class: Actinopterygii
Order: Perciformes
Family: Moronidae
Genus: Dicentrarchus



Species: *Dicentrarchus labrax* (Linnaeus, 1758)

This species is found in European waters of the Atlantic Ocean (from Norway to Senegal), the Mediterranean Sea and the Black Sea (Wheeler, 1975). It has been classified as euryhaline as well as eurythermal as inhabits both coastal and brackish waters (occasionally can be found in rivers), and it can survive within a wide range of temperature (2–32°C) (Smith, 1990). The Mediterranean sea bass becomes sexually matured between 2–4 years of age and reproduces seasonally (February to June) by external fertilization with females releasing between a quarter to half a million eggs per kilogram of their body weight. Reproduction, spawning and hatching occur in sea water, while eggs and pre-larvae passively drift towards coastal zones and larvae actively search nursery sites in low-salinity environments (Barnabé, 1989). Hatching takes place between 4 and 9 days after fertilization, depending of the environmental conditions. Juveniles seek shelter in estuaries until reaching sexual maturation, when they adopt the migratory movements of adults (inshore during spring and summer, moving to deeper waters up to 70 m in late autumn). At an early age, sea bass can be seen forming shoals that may vary from a few dozen individuals to many thousands depending on the year class and local conditions (Pickett and Pawson, 1994). The species is also characterized by its powerful swimming that increases with size and allows it to overcome strong tides and sustain a high average speed while migrating. The behaviour of adult sea bass seems to match the predator type, being particularly sensitive to short wavelengths (Marchesan *et al*., 2005). Among its preys are small pelagic fish (i.e. sardins, sprats and sand smelts), crustaceans and squids which sea bass voraciously capture by means of a wide range of tactics such as attacking the prey from below at a steep angle and high speed (Wheeler, 1975).

For most European fisheries and Mediterranean aquaculture, the European sea bass is one of the most important species. Its zootechnical handling is well mastered which allows year round fingerling availability through the manipulation of environmental elements. Mass production of juveniles for aquaculture relies on a large variety of hatchery techniques ranging from highly intensive systems (standardized operating procedures) to extensive systems (Zouiten *et al*., 2011). Among the most controlled parameters in the culture of sea bass are the illumination and the temperature. For the broodstock, a strict illumination protocol is followed to mimic the natural photoperiod as for instance, from September to January the gametogenesis process takes place, when the hours of light range from 14–8.5. For larvae and postlarvae the recommended illumination when the “French method” is not

applied (DD from fertilization to 5–7 days), is of 500 lux at water surface and the photoperiod should be of 16L:8D at 21°C increasing to 20L:4L at 21°C (Moretti *et al*., 1999). While light intensity and photoperiod have been fairly studied (Ronzani Cerqueira *et al*., 1991; Roncarati *et al*., 2001) there is a lack of information on the spectral visual system of this species especially during the first developmental stages. Regarding temperature, it has been suggested to maintain sea bass juveniles and adults at 25°C all year round (Dülger *et al*. 2012). However, for incubation and larvae rearing, the same temperature of spawning and fertilization should be applied (13-15°C), after which and following yolk sac resorption, temperature should be increased 0.5°C/day until reaching 18°C, when the inflation of the swim bladder is completed. Then temperature should be increased to 20°C by the 15th day (Moretti *et al*., 1999). At the present, this protocol is widely applied in commercial hatcheries bringing out good larval growth, development and survival. However, it also causes an important imbalance in sex ratio as 75-95% of fish (sometimes reach 99%) develop as males (Navarro-Martín, 2009). Although this problem has been deeply studied, the application of daily thermocycles which in the natural environment influence the sex ratio proportionally, have been mostly ignored.

1.4.2. Zebrafish *Danio rerio* (Hamilton 1822)

This freshwater fish common name originates from the five uniform, pigmented and horizontal blue stripes which extend to the end of the caudal fin. The name *Danio* derives from the Bengali name "dhani" meaning "of the rice field" (Talwar and Jhingran, 1991) and its taxonomic hierarchy is as follows (Fang, 2003):

Phylum: Chordata
Subphylum: Vertebrata
Superclass: Osteichthyes
Class: Actinopterygii
Order: Cypriniformes
Family: Cyprinidae
Genus: Danio
Species: *Danio rerio* (Hamilton, 1822)

The zebrafish distribution has been a matter of long dispute. On the basis of confirmed occurrences, the species may be widely distributed over the Indian subcontinent (Barman, 1991). Its habitats range from slow-moving or standing bodies and ditches adjacent to rice fields (Jayaram, 1999) to rivers and hill streams (Daniels, 2002). Field studies have found the zebrafish inhabiting shallow water bodies with a visibility to a depth of ~30 cm, in unshaded locations with aquatic vegetation (Spence *et al*., 2008; Engeszer *et al*., 2007). Its diet consists primarily of zooplankton and insects as well as phytoplankton and other biological material found in its habitat (Spence *et al*., 2007). While under laboratory conditions spawning takes place all year round, in nature zebrafish tends to reproduce seasonally depending perhaps, on food availability. Sexual maturity is reach depending on the temperature of the water which in turn, influence the growth rate and fertility, as bigger females produce more eggs (mean of 185 eggs per female) (Spence *et al*., 2008). Spawning is influenced by photoperiod as the reproductive behaviour starts within the first minute of light exposure and continues for approximately an hour (Darrow and Harris, 2004). Egg release and external fertilization take place within few hours of lights on (Selman *et al*., 1993). Fertilized eggs are demersal and hatching takes place within 48–72 hours depending on temperature, chorion thickness and muscular activity of the embryo (Kimmel *et al*., 1995). Development is rapid, with precursors to all major organs developing within 36 h and larvae displaying food seeking and active avoidance behaviours within five days post fertilisation, i.e. 2-3 days after hatching (Kimmel *et al.*, 1995). The species has been described as diurnal and even during larval development, it has been observed that a minimum number of LD cycles are essential for the establishment of its clock rhythmicity (Dekens and Whitmore, 2008; Cavallari *et al*., 2011).

Over the past thirty years, the zebrafish has emerged as a pre-eminent vertebrate model for studying genetics and development (Parichy *et al*., 2009), and more recently, for human disease and the screening of therapeutic drugs (Penberthy *et al*., 2002; Sumanasa and Lin, 2004). The greatest advantage of the zebrafish as a model system comes from its well-characterised genetics, genetic and developmental techniques and tools, and the availability of well-characterised mutants. Zebrafish are also a tractable species for behavioural experiments, readily acclimatising to new environments and being little disturbed by the presence of observers (Spence *et al*., 2008). Furthermore, its embryonic and postembryonic stages have been very well described and at present, are used as guideline for other teleost developmental process (Parichy *et al*., 2009).

Objectives

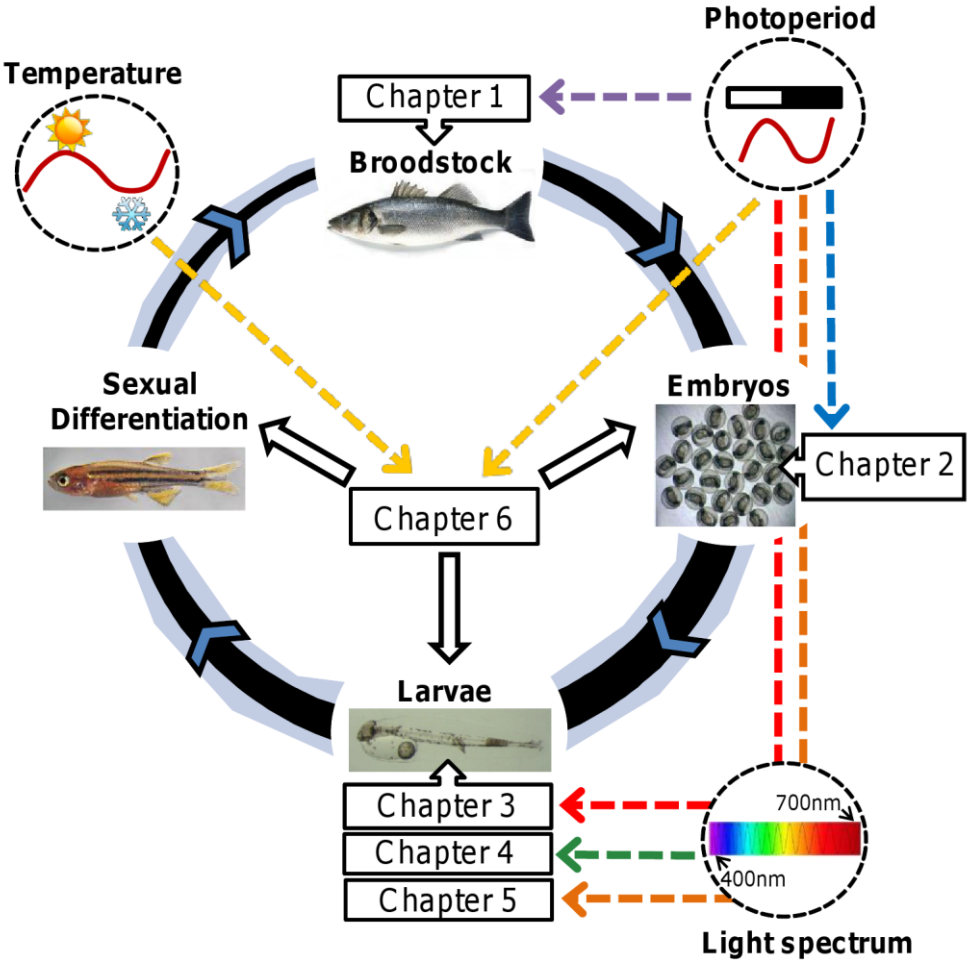
2. Objectives

The main objective of this doctoral thesis was to study the influence of environmental conditions on reproduction rhythms, early development and later performance of fish. In particular, we have studied the biological response to light and temperature cues of broodstock, embryos and larvae of fish, by evaluating their circadian behaviour and physiology, as well as important molecular and morphological changes.

For that purpose, the following specific objectives were established:

1. To determine the daily rhythms of spawning and their relation to the activity patterns (diurnal/nocturnal) in broodstock of European sea bass.
2. To describe the existence of a biological clock in fish embryos and their synchronisation to different light and temperature cycles in species with different behavioural patterns: diurnal (zebrafish), nocturnal (solea) and blind (cavefish).
3. To investigate the effect of different lighting conditions (constant vs photocycles) during the incubation, and larval development of European sea bass.
4. To evaluate the influence of light spectrum during the early development of sea bass and zebrafish on larvae behaviour and performance.
5. To study the effects of light on fish prey (*Artemia sp.*) hatching rate and spatial distribution.
6. To investigate the effect of water temperature (constant vs daily thermocycles) on embryo development, hatching rhythms, larvae performance and sex differentiation in zebrafish.

The scientific questions raised in the objectives have been addressed in the 6 experimental chapters depicted in the next figure:



Experimental Chapters

Experimental Chapter 1:

DAILY SPAWNING AND LOCOMOTOR ACTIVITY RHYTHMS OF EUROPEAN SEA BASS BROODSTOCK (*Dicentrarchus labrax*)

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Abstract

Fish have an internal biological clock that drives their daily rhythms, including those of feeding and reproduction. The European sea bass has been reported to exhibit a phase inversion of its diel feeding pattern: nocturnal in winter (coinciding with the spawning period) and diurnal during the remainder of the year. In this study, we investigated the locomotor activity of sea bass during the spawning and resting periods as well as the possible existence of a daily spawning rhythm by calculating the number of eggs (and their viability) released every hour. The results showed that sea bass locomotor activity was clearly nocturnal (68 ± 4.3% of the daily activity displayed at night) during the spawning season and mostly diurnal (65.5 ± 7.5% of the activity displayed during the day) out of the reproductive period. Spawning occurred during the dark phase with two peaks, 6 and 11 hours after lights off. Egg viability was highest in the batch released near the spawning peaks. These findings could be useful for optimizing egg production and to improve hatchery practices, which should take into account the reproduction rhythms of sea bass.

Introduction

Environmental cycles of annual and daily periodicity (including seasonal and day/night changes of light and temperature) synchronize reproductive rhythms in fish. The manipulation of photoperiod and temperature in finfish culture is widely used to control maturation and spawning time to produce year-round supplies of eggs and fingerlings (Bromage *et al*., 2001). Both photoperiod and temperature oscillations are transduced by the retina and the pineal organ into melatonin rhythms (high levels during the night and low levels during the day) which provides the animal with information about the time of day, the season and the lunar phase (Falcón *et al*., 2010). Annual clocks regulate daily and seasonal rhythmic physiological processes, including smoltification and reproduction, by means of gonadotropins, sex steroids and gonadal growth factors production (Amano *et al*., 2000; Bromage *et al*., 2001; Bayarri *et al*., 2004; Falcon *et al*., 2010). Several fish species reproduce once a year during a specific season, which provides the offspring with the most suitable environmental conditions for survival (Oliveira and Sánchez-Vázquez, 2010). Futhermore, previous studies found that European sea bass kept under constant photoperiod

conditions maintained the annual spawning rhythm, which suggests a strong endogenous mechanism that regulates the reproductive process (Prat *et al*., 1999)

Although in most fish species reproduction is an annual event, daily modifications in oocyte maturation and the secretion of sexual steroids or gonadotropins have been described in species like red seabream (*Pagrus major;* Matsuyama *et al*., 1988), kisu (*Sillago japonica;* Kobayashi *et al*., 1988), snapper (*Pagrus auratus;* Carragher and Pankhusrt, 1993), European sea bass (*Dicentrarchus labrax;* Bayarri *et al*., 2004) and Senegal sole (*Solea senegalensis;* Oliveira *et al*., 2009). Furthermore, recent studies have revealed daily reproduction rhythms in the red snapper *Lutjanus campechanus* (Jackson *et al*., 2006), gilthead seabream *Sparus aurata* (Meseguer *et al*., 2008) and the zebrafish *Danio rerio* (Blanco-Vives and Sánchez-Vázquez, 2009).

European sea bass *Dicentrarchus labrax* (Linnaeus, 1758) is one of the most intensively cultured species in the Mediterranean, recent research being focused on its domestication and behaviour (Millot *et al*., 2010), and reproduction control (Carrillo *et al*., 2010). European sea bass is a synchronous gonochoric species which reproduces in winter. Gonadal maturation starts in September/October, post-vitellogenic oocytes are observed in December and ovulation lasts from January to mid-March (Asturiano *et al*., 2000). Under artificial environment, egg release can be obtained throughout the year by manipulating both photoperiod and temperature (Carrillo *et al*., 1993). Although many efforts have been made to study the environmental and neuroendocrine regulation of reproduction in this species (Zanuy *et al*., 2001), little is known about daily rhythms in the broodstock and about egg release. Previous work investigating the feeding behaviour of sea bass revealed a phase shift of feeding rhythms, which became nocturnal during winter (coinciding with the spawning season) and returned to diurnal in spring-summer (resting season) (Sánchez-Vázquez *et al*., 1998). However, that research on feeding rhythms was performed in juveniles (immature), so that little is known about the behavioural patterns of adults.

The aim of this paper was to investigate (1) the daily locomotor activity rhythm of sea bass broodstock during the reproductive (winter) and resting (spring) seasons, and (2) the possible existence of daily spawning and egg viability rhythms.

Materials and Methods

The present research was performed in the facilities of a commercial hatchery (Aquicultura Balear, S.A.U., Culmarex S.A.), using three groups of pit-tagged sea bass

broodstock (4.2±0.32 kg body weight). The groups (1 female: 1 male) were kept in $20m^3$ tanks at an average density of 16 kg/m^3 (n=74 individuals per tank, n=222 in total). Controlled temperature (average 15^oC in winter, 18^oC in spring) and natural photoperiod (ranging from 9h of light in winter to 14h of light in summer) were applied throughout the experiment. The tanks were supplied with natural sea water from a well in an open system.

Experimental Design

To study sea bass locomotor activity, one infrared photocell (OMRON E3S-AD62, Japan) was placed in each tank at 22 cm of the water surface. Every time a fish swam within 20 cm from the photocell, the infrared light beam was interrupted and the signal was sent to a computer for locomotor activity recording. Locomotor activity observations were made during the spawning (January to March, 2010) and resting season (April to June, 2010). To determine the existence of daily spawning rhythms in European sea bass, the eggs released by broodstock were monitored by means of an autonomous egg collector built by the Research and Development Support Centre of the University of Murcia, C.A.I.D. (Meseguer *et al*., 2008). The collector was placed at the water outflow of the tank and was programmed to rotate every hour, so batches of eggs fell into a different compartment every time. During the first hour of light, the eggs collected in each compartment were placed in a transparent 2 l beaker with sea water, and they were left for 15 minutes without aeration in order to separate the floating (viable) from the sinking (non-viable) eggs (Bromage, 1995). Wet weight was measured for both groups and the viability rate calculated. The time elapsed from spawning to eggs collection was assessed by releasing 100 g of eggs at different points of the tanks. This procedure was performed four times in which similar results were obtained (eggs took about 20 min to reach the egg collector). Feeding activity and growth performance (SGR) were recorded during one year in the three above mentioned tanks. For the first six months, food was provided *ad libitum* following the normal schedule of the company (i.e. 6 hours after lights on). During the following six months, food was provided 1 hour after lights on. The weight of each fish was measured at the start and the end of each trial, as well as the amount of food given.

Statistical Analysis

Sea bass activity data were analysed by means of the chronobiology software *El Temps*© (version 1.231; Prof. Díez-Noguera, University of Barcelona) for the representation

of locomotor activity records. The quantity and viability of eggs for the different spawning times were subjected to one-way ANOVA and Tukey's test. To find a relationship between the locomotor activity and the amount of eggs released per hour, a linear regression was applied using the SPSS 15.0 software (SPSS Inc.). The results are given as mean±SD with $P< 0.05$ taken as the statistically significant threshold. Growth performance (SGR) was calculated at the end of each tank using the formula SGR= (ln (Wf)- ln (Wo)/T)*100, where Wf and Wo are the final and initial wet weight of the fish, respectively, and T is the duration of the experiment in days. The daily amount of food was also weighed to calculate the corresponding daily percentage of the total biomass of fish in each tank.

Results and Discussion

Dual phasing of activity rhythms

During winter and early spring, European sea bass showed nocturnal locomotor activity with 68 ± 4.3% of the total daily activity being displayed at night (Figure 1A). This nocturnal pattern of behaviour became less evident during the resting season (April to June), as diurnal activity increased to 65.5 ± 7.5% (Figure 1B). These results are in accordance with the observations made by Sánchez-Vázquez *et al*. (1998), who found that feeding activity of sea bass juveniles was nocturnal during winter and shifted to diurnal during summer.

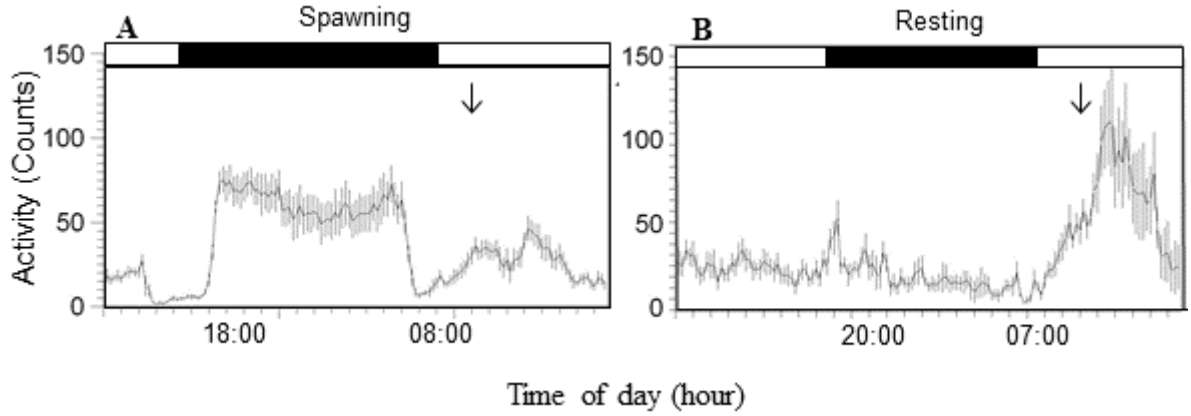

Figure 1. Daily locomotor activity of European sea bass during the (A) reproductive (January to March) and (B) resting (April to June) periods. The figures represent the mean activity profile observed. Arrows indicate the feeding time.

In our study, statistical differences were found in SGR and the amount of food supplied to fish between the two groups. When feeding time was set 1 hour after lights on (closer to the night) during the spawning season, SGR and the amount of food supplied were higher

(0.4 ± 0.04% and 0.3 ± 0.01 % of total biomass % respectively) in contrast with the results obtained when feeding time was set 6 hours after lights on (0.2 ± 0.02% and 0.22 ± 0.03% respectively). In this respect, Azzaydi *et al*. (1999) observed a better growth performance when food was provided during the dark phase in winter. Moreover, in that study when sea bass were given the opportunity to self-feed, they chose to feed during the dark phase.

Daily Spawning Rhythms

The change in daily patterns of locomotor activity (nocturnal/diurnal) suggested that sea bass broodstock seek the best time of day to spawn, as reproduction rhythms in fish are tuned to ensure the offspring meets the best environmental conditions for best survival (Oliveira and Sánchez-Vázquez, 2010). Furthermore, the existence of an endogenous reproductive mechanism in sea bass was suggested in early studies by Pratt et al. (1999), who found that under constant photoperiod conditions, the annual spawning rhythm was maintained for several cycles in accordance with rhythmic changes of gonadal steroids. Indeed, in our study sea bass showed a marked daily spawning rhythm during the dark phase (Fig. 2). These findings are in accordance with other nocturnal species like *Solea senegalensis* (Oliveira *et al*., 2009), *Sparus aurata* (Messeguer *et al*., 2008) and *Pleuronectes platessa* (Nichols, 1989), in which spawning took place near or during the night.

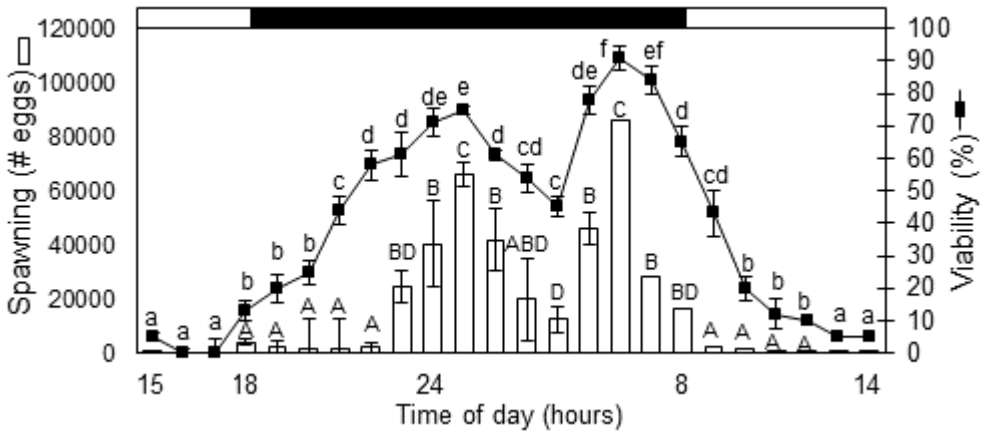

Figure 2. Daily spawning rhythm and egg viability of European sea bass. Collected number of eggs per hour is represented by bars and percentage of viable eggs by a line. Light and dark hours are represented by white and black bars at the top of the figure. Data are expressed as mean ± S.D. Different letters indicate statistically significant differences from each other (ANOVA, Tukey's test, $P<0.05$).

In diurnal species such as zebrafish, spawning takes place during the day, around two hours after lights on (Blanco-Vives and Sánchez-Vázquez, 2009). In our study, spawning started 4 hours after lights off and was positively correlated with the locomotor activity of the broodstock (Table 1, Figure 3). Furthermore, egg viability was highest in the batches released when spawning peaked (75 ± 4.3% at 1 am; 91 ± 5.4 % at 6 am, Table 1). These results contrast with those obtained in sole, sea bream and zebrafish, where differences in egg viability were not detected between day and night (Oliveira *et al*., 2009; Blanco-Vives and Sánchez-Vázquez, 2009; Meseguer *et al*., 2008).

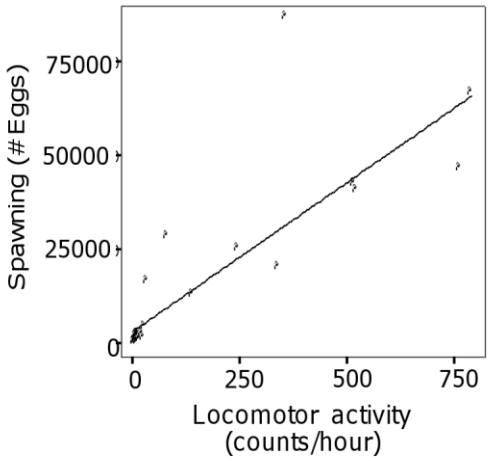

Table 1. Correlation (r and P values) for spawning time/egg viability and spawning time/locomotor activity.

Correlation	Egg viability (%)	Locomotor Activity
Spawning (eggs quantity)	r = 0.802 $P < 0.012$	r = 0.69 $P < 0.001$

Figure 3. Daily locomotor activity and spawning rhythm correlation analysis. The results are given as mean±S.D. with $P < 0.05$ taken as the statistically significant threshold (SPSS 15.0).

In short, the daily pattern of locomotor activity and the timing of spawning should be considered to maximize broodstock performance in commercial hatcheries. Actually, the correlation between spawning size and egg viability suggests that only the more abundant spawning should be collected, while the less abundant (with poor quality) should discarded.

Acknowledgments

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Experimental Chapter 2:

CIRCADIAN RHYTHMS OF EMBRYONIC DEVELOPMENT AND HATCHING IN FISH: A COMPARATIVE STUDY ON ZEBRAFISH (DIURNAL), SENEGALESE SOLE (NOCTURNAL) AND SOMALIAN CAVEFISH (BLIND)

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ABSTRACT

During early development, most organisms display rhythmic physiological processes that are shaped by the daily changes of their surrounding environment (i.e. light and temperature cycles). In fish, the effects of daily photocycles and its interaction with temperature during their first developmental stages remain largely unexplored. Here, we investigated the existence of circadian rhythms in embryos and larvae of three teleost species with opposite behaviour: zebrafish (diurnal), sole (nocturnal) and cavefish (blind, not entrained by light). To this end, fertilized eggs were exposed to three light regimes: 12 hours of light : 12 hours of darkness cycle (LD), continuous light (LL), or continuous darkness (DD); and three appropriate temperature treatments: 24ºC, 28ºC or 32ºC for zebrafish and cavefish, and 18ºC, 21ºC or 24ºC for sole. The results showed the existence of daily rhythms of embryonic development and hatching synchronized to the LD cycle as embryos developed faster and hatched during the light phase in the zebrafish and during the dark phase in sole. Under LL and DD conditions, circadian rhythms in embryogenesis and hatching persisted in both species. Zebrafish embryos completed the three stages of development in less time under constant conditions (9.7±0.9 h under LL and 10.8±1.4 h for DD) than under LD (29.3±1.7 h). Cavefish embryogenesis and hatching time was not entrained by light under LD, although DD seemed to improve both processes. Hatching rate was best under LD and the reported optimal temperature for the three species (95.2±2.7% of the zebrafish and 83.3±0.1% of the cavefish embryos hatched at 28°C, while 93.1±2.9% of the sole embryos hatched at 21°C). These results revealed for the first time that early developmental rhythms in fish are endogenously driven, modulated by the temperature and strongly synchronized to light, with the phasing being determined by the diurnal/nocturnal behaviour of the species.

INTRODUCTION

Fish inhabit a highly dynamic environment characterized by daily, lunar and annual light cycles driven by the rotation and translation movements of the Earth and the moon around the Sun (López-Olmeda and Sánchez-Vázquez, 2009). Furthermore, the infrared radiation from the

Sun determines the temperature of the water, which can influence individual fitness and survival (Magnuson *et al*., 1979; Johnson and Belk, 2004). Actually, inappropriate light and temperature act as stress factors by impairing physiological and behavioural activities in fish (Barahona-Fernandes, 1979; Downing and Litvak, 1999; Beyers and Rice, 2002; Donaldson *et al*., 2008) and particularly, during larvae development (Villamizar *et al*., 2011; Blanco-Vives *et al*., 2011)

Light and temperature cycles affect several if not all aspects of fish by entraining their circadian activity rhythms (Reebs, 2002). The fish biological clock has been linked with the daily rhythms of cell division as most cell types are not only influenced by the response of the fish central and peripheral pacemakers to environmental signals, but also by the cells own circadian clock (Schibler and Sassone-Corsi, 2002). Interestingly, it has been found that even fish embryonic cells are capable of light detection as early as gastrula stage (5 hours post fertilization, hpf), well ahead of non-visual (pineal organ develops by 24 hpf) and visual (retina by 2-3 days post fertilization, dpf) photoreception (Burrill and Easter, 1994; Kazimi and Cahill, 1999; Tamai *et al*., 2004). Cell proliferation, and consequently growth, responds to light and dark (LD) cycles by mitogenic stimulation which remarkably in some species is timed to occur at the end of the day or during the night (Nikaido and Johnson, 2000; Dekens *et al*., 2003a). Regarding temperature, this has been found to affect fish embryo somitogenesis (Schröter *et al*., 2008), growth (by increasing the metabolic rate at high temperatures) (Schmidt and Starck, 2009; Kamler, 2008), oxygen consumption (Clarke and Johnston, 1999), hormone production (Deane and Woo, 2009) and heart rate (Denvir *et al*., 2008). Although embryogenesis in fish has been classically considered as a succession of developmental stages which are mainly controlled by temperature, recent findings point to the key role played by the biological clock (Gorodilov, 2010). However, light synchronization and the different phasing (diurnal/nocturnal) of embryo rhythmicity, and most importantly its circadian nature, remains unknown to date.

The zebrafish (*Danio rerio*) is a model species for developmental research due to important characteristics such as high fertility, early light responsiveness, fast embryogenesis and embryo transparency. Under controlled conditions, the zebrafish circadian rhythm (i.e. locomotor activity) can respond differently to temperature regimes within a range of only 1 or 2°C (López-Olmeda and Sánchez-Vázquez, 2011). Regarding light, the zebrafish embryo's tissues and cells are directly light sensitive as early as 5 hpf and their exposure to LD cycles causes a rise in cellular proliferation and expression of clock genes (i.e. *per*2) which are synchronized to the light phase (Tamai *et al*., 2004). The Senegalese sole (*Solea senegalensis* Kaup, 1858) is a flatfish of great scientific interest that is being extensively cultured in the

Mediterranean region, mostly in Spain and Portugal (Dinis *et al*., 1999). In juvenile and adults the environmental cues (i.e. photoperiod, temperature, lunar cycles) seem to synchronize the most important daily activities such as locomotion, feeding, spawning and hatching, which have been observed to occur during the dark phase (Gibson, 2005; Oliveira *et al*., 2009, 2011, Blanco-Vives *et al*., 2010, 2011). Since this species inhabits estuarine and coastal nurseries of widely differing temperatures, its thermal tolerance is rather high (Anguis and Cañavate, 2005). The Somalian cavefish, *Phreatichthys andruzzii* Vinciguerra, 1924 (Family: Cyprinidae) is an endemic tropical freshwater species with strong physiological and behavioural adaptations to underground water environments. During the course of evolution, the Somalian cavefish had undergone important changes such as eye degeneration during early development (Berti *et al*., 2001). Apart from the lack of light, the underground aquatic habitat is also characterized by little oscillations of temperature (28–31°C) that have influenced the cavefish during millions of years (Ercolini *et al*., 1982). The Somalian cavefish biological clock is not entrainable by light, but it is synchronized by periodic food availability (Cavallari *et al*., 2011). Interestingly, cell line derived from adult cavefish fins transiently treated with dexamethasone displays a long infradian period (about 47 hours), and lacks temperature compensation (Cavallari *et al*., 2011). Despite the cavefish been catalogued as a functional "truly blind" species, its eyes develop in the embryo in a morphologically normal way, reaching its maximal development by 36 hpf after which, rapid degeneration starts as a result of a decrease in neuroblastic cell proliferation (Berti *et al*., 2001). Also, a recent study with adults observed that the Somalian cavefish is photophobic, which implies that somehow, light is detected by the fish (Tarttelin *et al*., 2012). The prolonged isolation of the cavefish from environmental cues offers an interesting path for comparative studies of the development of the biological clock in vertebrates.

The objective of this research was to investigate the existence of circadian rhythms in fish under different light and temperature cycles, during the early development of three species with different activity pattern: zebrafish (diurnal), Senegalese sole (nocturnal) and Somalian cavefish (blind, without a light-entrainable and poor temperature-compensated clock)

MATERIALS AND METHODS

Ethics Statement

All husbandry and experimental procedures were approved by the European Legislation for the Protection of Animals used for Scientific Purposes (Directive 2010/63/EU). The experimental protocol in zebrafish was previously authorized by the Spanish National

Committee on Animal Welfare (Law 32/2007) and the Bioethical Committee of the University of Murcia. Senegalese sole experimental protocol was also approved by the Portuguese Law for the use of vertebrated animals for scientific and experimental purposes (86/609/CEE) and Somalian cavefish research was approved by the University of Ferrara (Italy) Institutional Animal Care and Use Committees.

Animal Rearing Conditions

The present research was carried out in the following laboratories: the Chronobiology Laboratory (Faculty of Biology, University of Murcia, Spain) for zebrafish; the Centro de Ciências do Mar do Algarve (CCMAR, Faro, Portugal) and at A. Coelho e Castro fish farm (ACC, Póvoa de Varzim, Portugal) for Senegal sole and the Department of Biology and Evolution of the University of Ferrara (Italy) for cavefish.

Adult zebrafish (*Danio rerio*) from heterogeneous wild-type stock (standard short-fin phenotype) were obtained from a local commercial distributor and housed for six months in 9 L glass aquaria (1 fish L^{-1}) according to standard methods (Nüsslein-Volhard and Dahm, 2002). For spontaneous spawning, ten groups of sexually mature zebrafish consisting of six fish (2 females: 4 males) were separately transferred into 2.5 L breeding net cages (SERA GmbH, Germany) during the afternoon. Spawning took place the next morning within 2 h after lights on (11:00 h). Soon after being fertilized, eggs were collected, pooled together and distributed in sterile 85x10 mm Petri-dishes (20 eggs per Petri-dish) filled with embryo medium (Nüsslein-Volhard and Dahm, 2002).

Senegal sole broodstock (1female:1male) was obtained from the Aquaculture Research Center of Olhão (IPIMAR) and housed in cylindrical 18.000 L tanks at a density of 10 g of biomass L^{-1}. The broodstock was kept in natural hyper-filtered seawater in an open circulation system. The photocycle was of 12 hours of light:12 hours of darkness (LD) and temperature of 18.3±0.5 °C. Naturally spawnings occurred at midnight (0:00 h), so eggs were collected soon after fertilization under dark conditions. Eggs were pooled together and distributed into sterilized 12-well cell culture plates (CELLSAR®, Greiner Bio-One GmbH, Germany) at a density of 15 eggs / well. Each well contained 35 ml of filtered sea water.

Somalian blind cavefish broodstock was housed in 150 L glass aquaria (0,1fish L^{-1}) filled with filtered fresh water at constant temperature (28±0.5°C) and darkness (DD). Fertilized eggs were obtained by means of hormonal induction of the broodstock. For this, 3 males and 3 females were anesthetised with MS222 (30 mgL^{-1}; Sigma, Milan, Italy), for 30 min and injected

with LH-RH (0,05 µg per g of body weight; Sigma, Milan, Italy) and Pimozide (2,5 µg per g of body weight; Sigma, Milan, Italy). Spawning took place the next morning 12 h after hormonal induction (09:00 h). Soon after, fertilized eggs were collected, pooled together and individually distributed in 24-well cell culture plates (Sarsted Inc., Newton, NC, USA) filled with 1 ml of zebrafish embryo medium (Nüsslein-Volhard and Dahm, 2002).

Experimental Procedure

The lighting treatments applied in the experiment were the same for the three species. In each temperature group the lighting conditions were of 12 hours of light: 12 hours of darkness (LD), 24 h of constant light (LL) or 24 h of constant darkness (DD).

Experiment 1. Zebrafish.

From 0 to 75 hpf, three petri dishes were carefully placed at the water surface (water bath) of 9 L thermostat-controlled glass aquaria and incubated at three constant temperatures: high of 32.2±1.1ºC, mild of 28.3 ±1.1ºC or low of 24.1±1.5 ºC. Thus the experiment consisted of nine experimental groups: LD32, LL32, DD32, LD28, LL28, DD28, LD24, LL24 and DD24. For each experimental group, three 9 L aquaria were used (N=180). Water temperature was thermostat-controlled and set up by means of water heaters (100 W, Askoll, Italy) or chillers (Aqua Medic Titan 500 GmbH, Germany). Temperature was recorded every 10 minutes by means of an underwater data logger (HOBO PENDANT® Onset Computer Corporation, Massachusetts, USA). Illumination was provided by means of neutral white LED light (flexible LED stripes, Superlight Technology Co., Ltd, China) and was measured with a spectroradiometer (1.7 ± 0.03 photons m^{-2} s^{-1}) (FieldSpec® ASD, Colorado, USA).

Experiment 2. Senegal sole.

Sole eggs were incubated in 15 L tanks (two per treatment) filled with natural sea water from 0 to 72 hpf by placing two 12-well plates at the water surface (N=360). Temperature regimes were: 24.4±1.4ºC, 21.2±1.1 ºC or 18.2±1.6 ºC. Thus, the experimental groups for this species consisted of: LD24, LL24, DD24, LD21, LL21, DD21, LD18, LL18 and DD18. Temperature was obtained by means of water heaters (Themal Compact 100 W, Askoll, Italy) or coolers (Cubigel, E-500, Spain) and was continuously recorded with an underwater sensor and data logger (HOBO PENDANT® Onset Computer Corporation, Massachusetts, USA) placed in

each tank. Illumination was supplied by standard mercury lamps (PHILIPS, HPL-N 250 W) which provided a light intensity of 2.8 photons m^{-2} s^{-1} (FieldSpec® ASD, Colorado, USA).

Experiment 3. Cavefish.

From 0 to 37 hpf cavefish eggs (N=48) were incubated by placing two 24-well plates at the water surface of 18 L aquaria. As the cavefish is also a tropical species, the same temperature treatments applied to the zebrafish were used. Therefore the experiment consisted of the same groups: LD32, LL32, DD32, LD28, LL28, DD28, LD24, LL24 and DD24. The temperature was held constant by means of water heaters (100 W, Sera GmbH, Germany) and recorded with data logger (TFA Dual-Temp-Pro, Germany). Illumination was provided by neutral white LED light (Superlight Technology Co., Ltd, China) and was measured with a spectroradiometer (1.7 ± 0.03 photons m^{-2} s^{-1}) (FieldSpec ASD, Colorado, USA).

Data collection

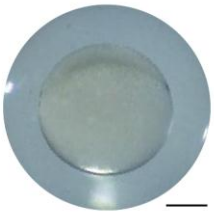
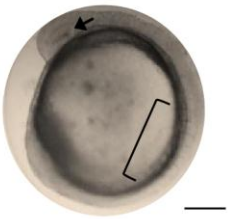
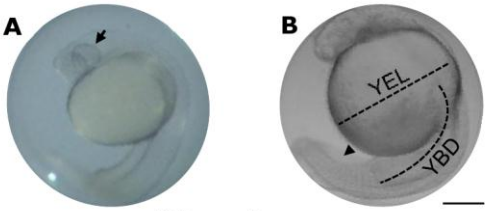
The influence of temperature, light and their combined effect on embryo development of the three species was evaluated by observing (1) embryogenesis, (2) hatching times (rhythms) and (3) hatching rate (final percentage of hatched larvae with respect to the initial number of fertilized eggs of each treatment). Embryogenesis was observed every two hours in 50 fertilized eggs from 0 to 60 hpf in zebrafish, 0 to 54 hpf in Senegalese sole and in all embryos from 0 to 25 hpf in the cavefish. For this, all Petri-dishes (zebrafish) and well-plates (sole and cavefish) were collected from each aquaria/tank and placed under a microscope. Embryos were quickly photographed using a digital camera mounted on the microscope and image processing and analysis software for standard measurements (Leica Microsystems Imaging Solutions Ltd, Cambridge, UK). A dim red light was used whenever sampling took place in the dark.Three different stages were established as the evaluating parameters of embryo development (Kimmel *et al*., 1995; Table 1).

To evaluate the existence of hatching rhythms and the influence of the experimental treatments, every two hours the number of newly hatched larvae was registered until 75, 65 and 37 hpf in zebrafish, sole and cavefish respectively. At the end of the experiment, the total number of hatched larvae was recorded in order to calculate the hatching rate for the three species.

Data analysis

All data were first tested for normality with the Kolmogorov-Smirnov's test. After normality confirmation, data were arcsine transformed before being examined by a one-way analysis of variance (ANOVA) to determine significant differences among the effect of the temperature regimes and light regimes separately. A two-way ANOVA analysis was also applied in order to find statistical differences among the influence of the combined effect of temperature and light on fish embryos. Both ANOVA analyses were followed by Tukey's multiple range tests. The differences in pace of zebrafish and sole embryo development during the light and dark phases of the LD cycle were analysed by separately calculate the slope of each phase (from all temperature groups) and analysed them by a one-way ANOVA. All statistical analyses were carried out using the software SPSS 15.0 (SPSS Inc.). *P* values < 0.05 were considered statistically significant. All data are expressed as mean ± S.E.M.

Table 1. Stages within fish embryo development used to evaluate the effect of temperature and light conditioning on the zebrafish, Senegalese sole and Somalian cavefish. The amount of embryos undergoing any of the stages was recorded every two hours. The features for each stage are as described by Kimmel *et al*. (1995) for the zebrafish, but were easily found in the other two species. (*) Approximate time for each stage based on current knowledge (Kimmel *et al*., 1995). Scale bars = 250 µm.

Stage1	Stage2	Stage3
		
Dome stage. Epibolia starts. ~ 4 hpf*. Photo: zebrafish	8 somites. Optic primodium (black arrow). ~ 13 hpf*. Photo: cavefish	30 somites. Pigmentation begins (black arrow). Median finfold (black arrow head). Yolk extension length (YEL)=Yolk ball diameter (YBD). ~ 24 – 25 hpf*. Photos: **A** zebrafish, **B** cavefish

RESULTS

Embryo Development

In zebrafish, the embryonic development was affected by both light and temperature regimens. Embryos reached the three stages earlier under LL in the three experimental temperatures, followed by embryos under LD and DD, which development was delayed. Among temperature groups, 32ºC favoured embryo development, with all larvae reaching the three developmental stages 4.0 ± 1.2 h earlier than the second best group, LL28 (P=0.01). The low temperature groups (24ºC) brought the slower embryo development (P=0.03) (Figure 1A, B, C). Interestingly, under LD24, LD28 and LD32 embryo development did not occurred regularly during the light and dark phases as it was faster during the first and slower during the last (Figure 4A).This difference in developmental frequency influenced the general pattern of embryo ontogeny as under LD conditions all embryos took 19.0 ± 0.8 h longer in reaching each stage than the LL and DD groups, regardless the temperature regimen (P=0.01).

Senegalese sole embryos raised under LL did not showed the same developmental performance as seen in the zebrafish, as similar developmental patterns were observed under LL and LD and in some occasions, embryos under LD were seen in a higher developmental stage than LL embryos (P=0.02) (Figure 2A, B). DD conditions caused a delay in embryogenesis when compared with the LL and the LD groups. Higher temperatures led to faster embryo development although in some sampling points no significant differences were found between 24°C and 21°C (Figure 2A, B, C). The frequency of development of sole embryos was different during the light and dark phases, although contrasting with the developmental pattern of the zebrafish, sole embryos developed faster during the dark phase (P=0.04) (Figure 4B).

The embryogenesis of cavefish occurred more rapidly than in the other two species, as the third developmental stage was reached in all experimental groups by 25 hpf, in contrast with zebrafish (60 hpf) and sole (54 hpf). Cavefish embryos development occurred faster under 32°C than under 28°C and 24°C, where the slowest ontogenesis was observed (P<0.05) (Figure 3). As embryogenesis happened so fast in the cavefish, the impact of the LD cycle could not be clearly established, and the results were similar to those obtained under LL (P=0.07). However, contrasting with the zebrafish and the Senegalese sole, the cavefish development occurred faster under the DD regimen than under the LL and LD groups regardless the temperature regimen (P<0.05).

Hatching Rhythms

In zebrafish, hatching was affected by temperature regardless of the light regimen as under 32°C, larvae embryos hatched first, followed by 28°C and 24°C (P<0.05) (Figure 5). A

rhythmic pattern of hatching was clearly observed in this species as under LD, the hatching was synchronized to the light phase starting in the LD32 group by 25 hpf (peak at 28 hpf) and LD28 by 29 hpf (peak at 32 hpf). Embryos that did not hatch during the first light phase (1 dpf) hatched during the next one (2 dpf) when all embryos from LD32 were observed out of the chorion. LD24 embryos started to hatch at this time (peak at 58 hpf) and finished this stage by the next light phase (3 dpf) together with the LD28 group (Figure 5A).

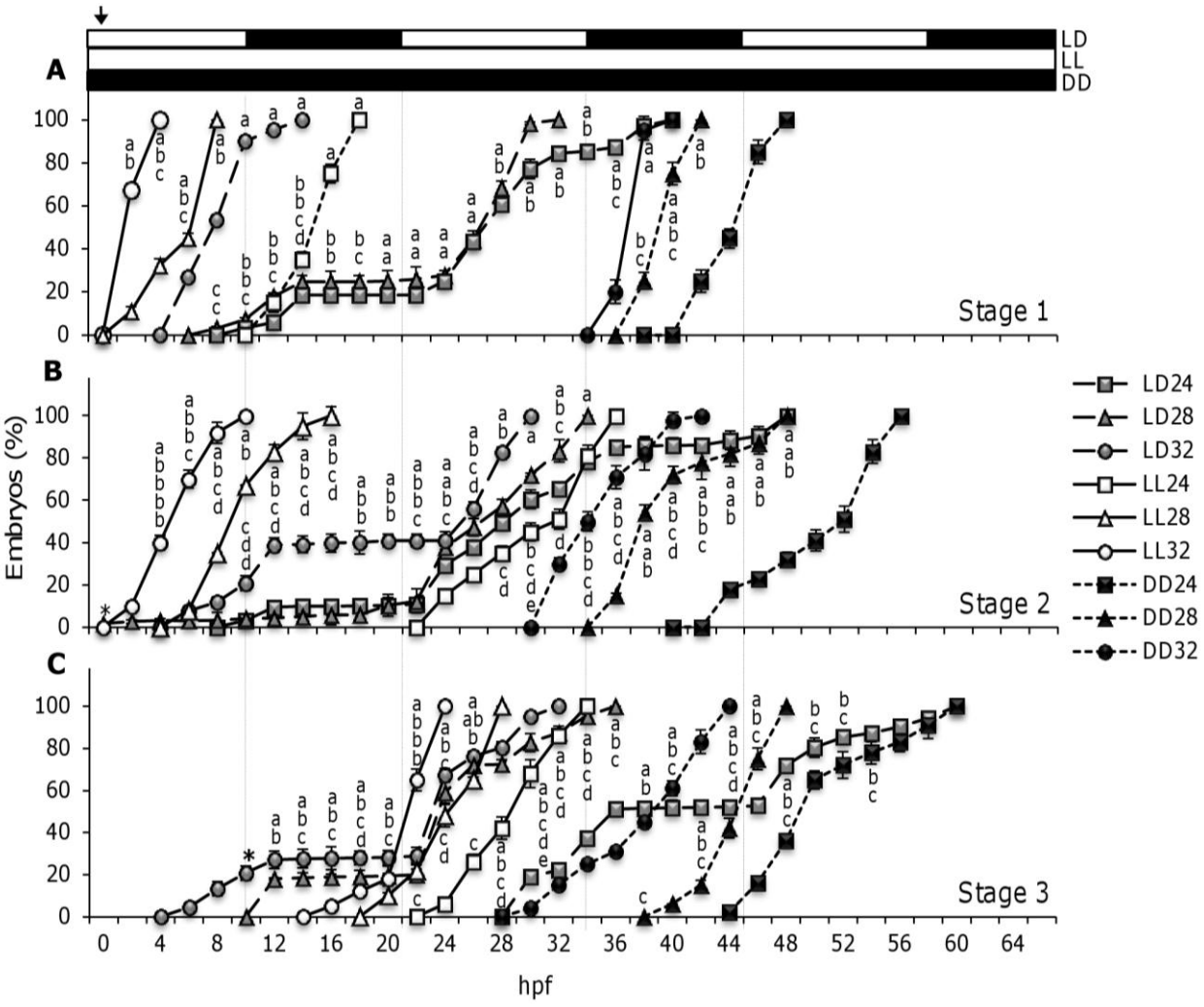


Figure 1. Zebrafish Embryogenesis as a function of temperature and light. Values are expressed as the percentage (mean±SEM) of embryos undergoing the first (**A**), second (**B**) and third (**C**) developmental stage. Horizontal black/white bars represent the night and day periods of the 12:12LD cycle and the white and black bars represent the constant light (LL) and constant darkness (DD) regimes. The figure legend corresponds to each experimental group of light (LD, LL and DD) combined with the temperature regimes (24°C, 28°C and 32°C). Vertical dotted lines show the switch from light to dark or dark to light phase. An arrow indicates the moment of fertilization. Different letters indicate statistical differences among groups (ANOVA, $P<0.05$, N=50 per experimental group).

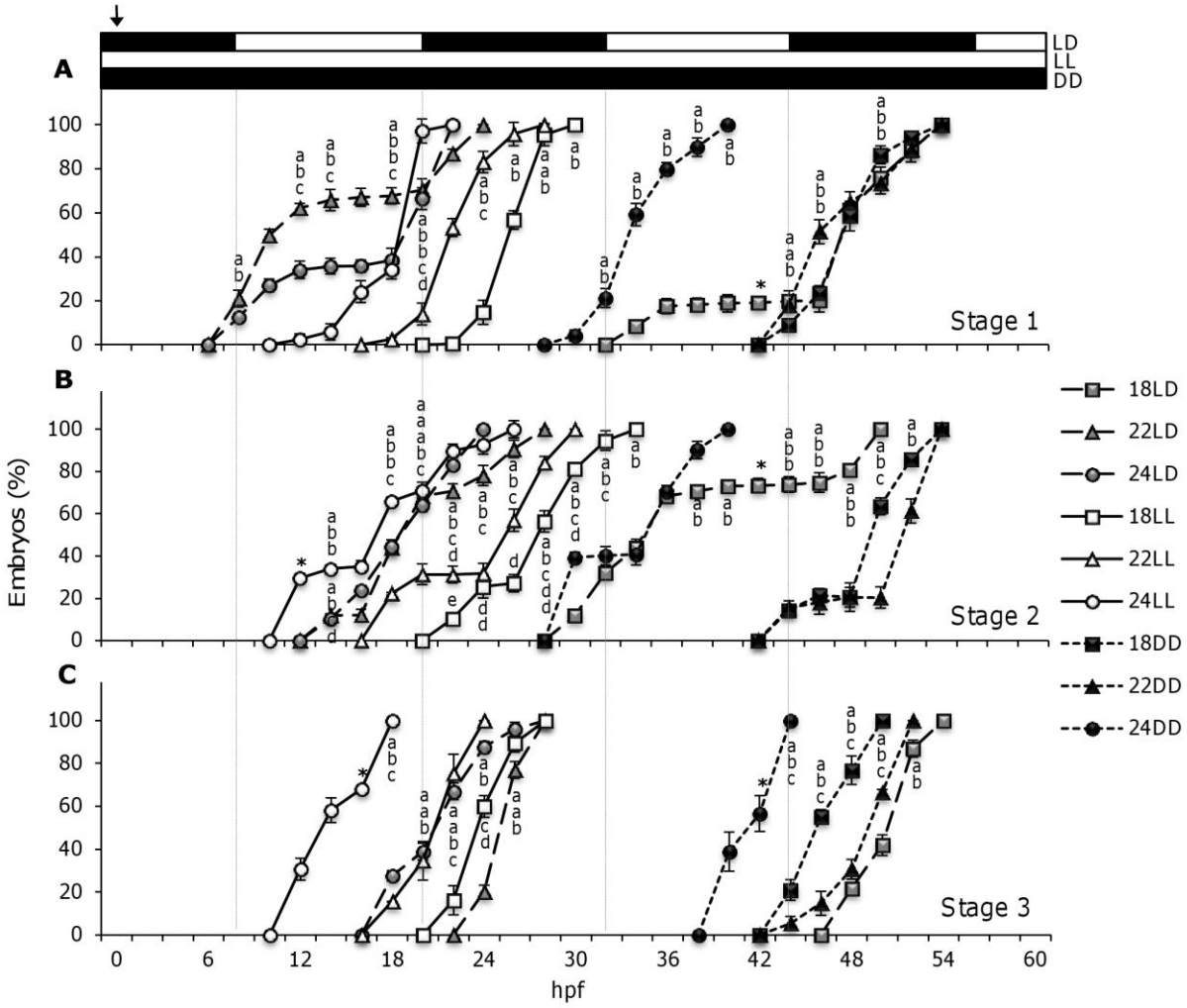


Figure 2. Effect of the temperature and light on Senegalese sole embryogenesis. Values are expressed as the percentage (mean±SEM) of embryos undergoing the first (**A**), second (**B**) and third (**C**) developmental stage. The figure legend corresponds to each experimental group of light (LD, LL and DD) combined with the temperature regimes (18°C, 21°C and 24°C). Different letters indicate statistical differences among groups (ANOVA, $P<0.05$, N=50 per experimental group). Arrow, bars and abbreviations as in Figure 1.

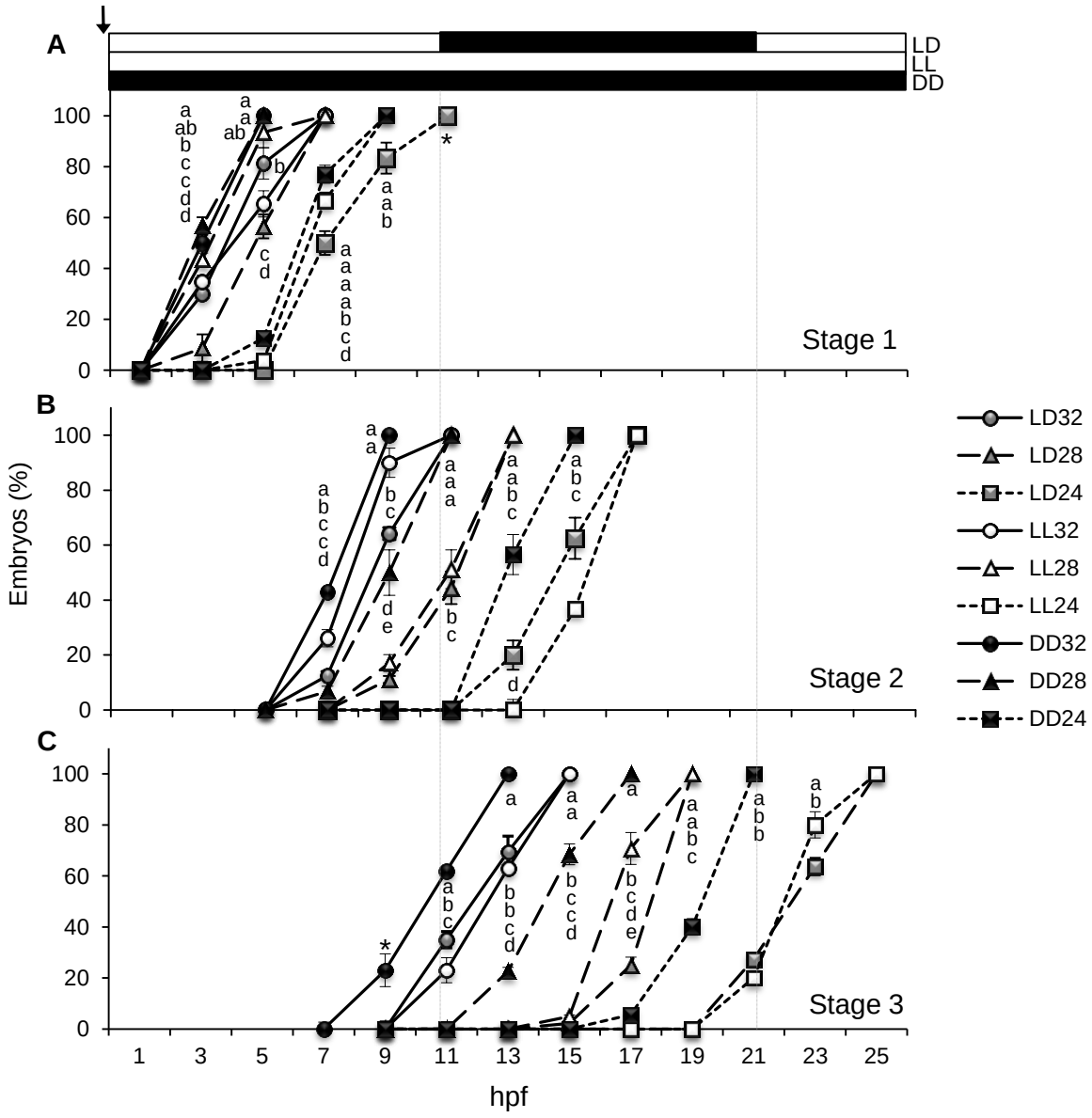


Figure 3. Influence of light and temperature on Somalian cave fish embryo development. Values are expressed as the percentage (mean±SEM) of embryos undergoing the first (**A**), second (**B**) and third (**C**) developmental stage. The figure legend corresponds to each experimental group of light (LD, LL and DD) combined with the temperature regimes (24°C, 28°C and 32°C). Different letters indicate statistical differences among groups (ANOVA, $P<0.05$, N=48 per experimental group). Arrow, bars and abbreviations as in Figure 1.

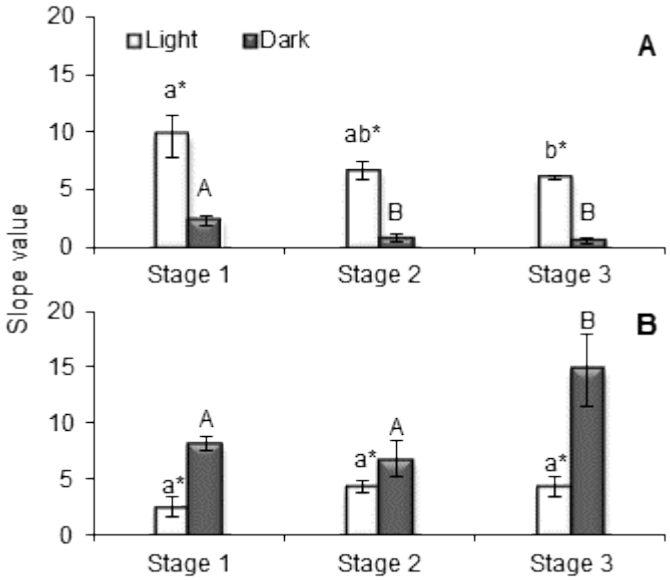


Figure 4. Effect of daily photocycles on fish somitogenesis pace. Slope values of zebrafish (**A**) and sole (**B**) embryo development when raised under 12L:12D. Data represent the mean slope value of all temperature groups (24°C, 28°C and 32°C for zebrafish and 18°C, 21°C and 24°C for sole) during the light and the dark phases. Upper case letters: differences among the stages during the dark phase. Asterisks: slope differences between light and dark phases in each developmental stage. (ANOVA, $P< 0.05$)

Under LL and DD conditions, a hatching rhythm was still observed although its period was shorter in contrast with the LD groups ($P<0.05$). Both LL32 and DD32 embryos started to hatched on the first subjective light phase (peaks at 27 and 28 hpf, respectively), however DD32 mayor hatching rate was observed 10 hours later (peak at 38 hpf) (Figure 5B, C). LL28, LL24, DD28 and DD24 hatching peaks occurred within no more than 1 hours of difference and the time between one hatching peak and the other was also of ~ 10 hours for each experimental group

In the Senegalese sole, hatching was also affected by temperature in all experimental groups as it was observed occurring earlier in 24°C, followed by 22°C and 18°C ($P<0.05$) (Figure 6). However, the end of the hatching period was reached by all groups earlier than in the zebrafish (10 hours of difference) and in one or two hatching peaks. Under LD a rhythmic pattern of hatching was also observed in this species but in this case, it was synchronized to the dark phase with a single hatching peak per temperature group. During the first dark phase (1 dpf) the hatching peaks were by 28 hpf and 30 hpf in LD24 and LD22 respectively. LD18 hatched 23 hours after (peak at 55 hpf), during the second dark phase (2 dpf) (Figure 6A). Under LL hatching in the LL24 group also occurred under the first subjective night (peak at 29 hpf). However, it was delayed in the LL22 group (peak at 34 hpf) when compared to the LD22 group.

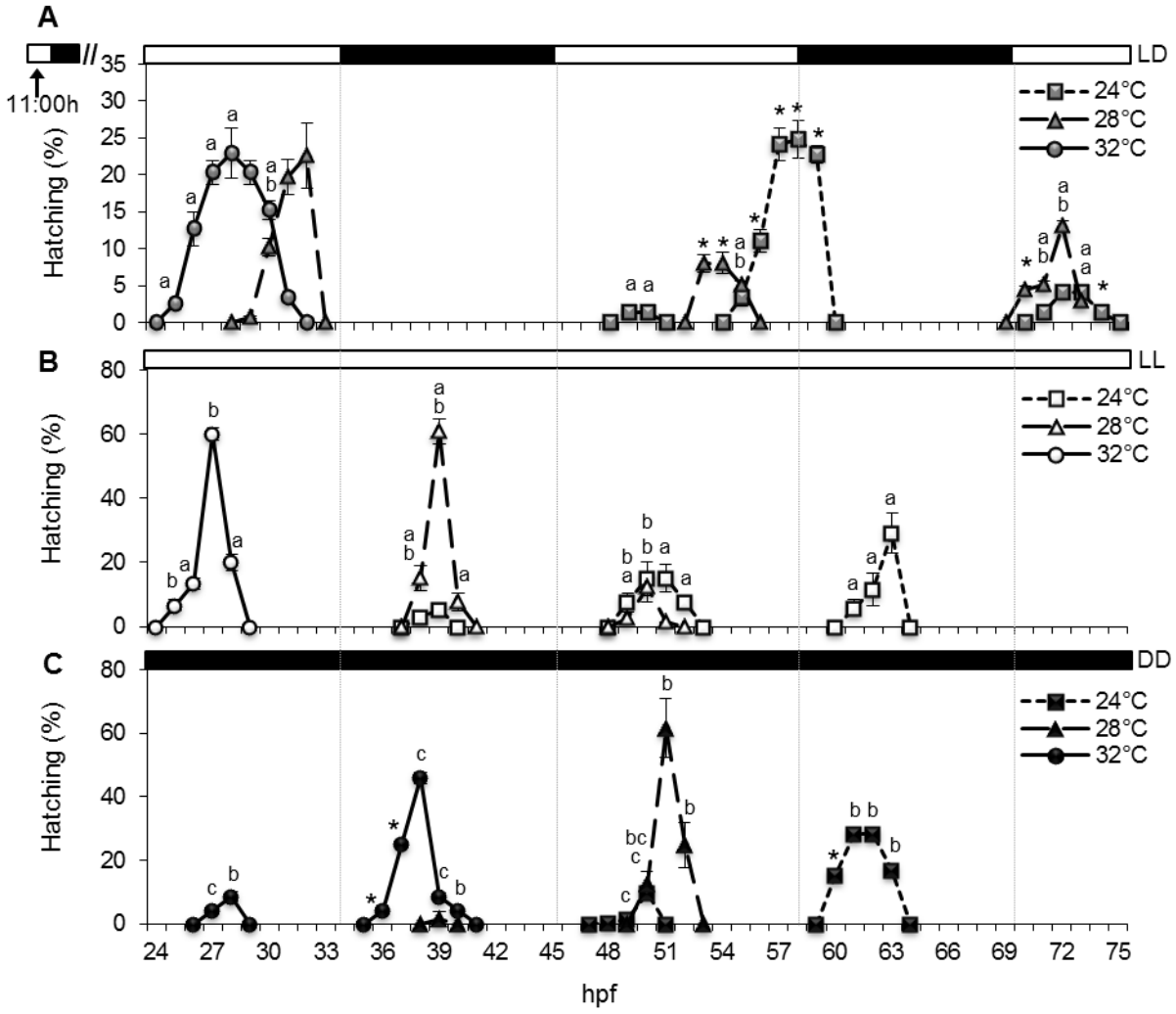


Figure 5. Hatching rhythms of zebrafish when reared under different temperature and light conditions. Values are expressed as percentage of newly hatched embryos every 2 h ± SEM. Different letters: Significant differences found at each sampling time among treatments (T° and Light) (ANOVA, $P<0.05$). Arrow, bars and abbreviations as in Figure 1.

The hatching time under LL18 was advanced in 17 h, presenting a peak at 38 hpf (1 dpf) (Figure 6B).In contrast, DD conditions delayed the hatching time in all three experimental groups as the first hatched larvae was observed in DD24 by 45 hpf (21 and 20 h after LD24 and DD24, respectively) ($P<0.05$). Under DD22 and DD18 embryos hatched similarly as hatching peak occurred by 57 hpf in the first and by 58 hpf in the latest during 2 dpf (Figure 6C).

Cavefish hatching was also clearly affected by temperature as it was advanced under the highest temperature regimen and delayed under the lowest regardless the light conditioning (Figure 7).

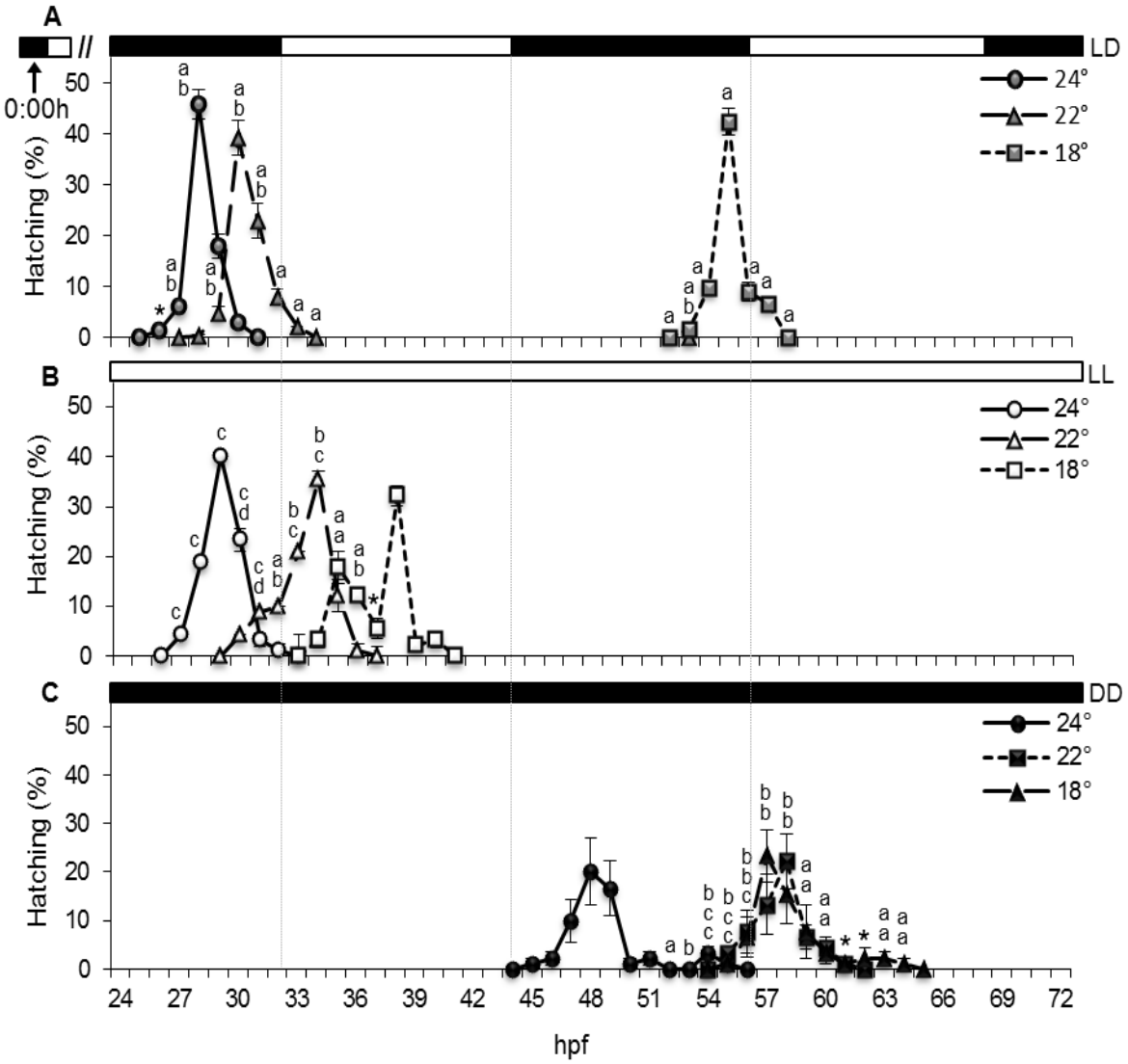


Figure 6. Hatching rhythms of sole when reared under different temperature and light conditions. Values are expressed as percentage of newly hatched embryos every 2 h ± SEM. Different letters: Significant differences found at each sampling time among treatments (T° and Light) (ANOVA, $P<0.05$). Arrow, bars and abbreviations as in Figure 1.

Despite no evidence of hatching rhythms, under LD32 and LL32 cavefish embryos hatched continuously for a rather long period (14 h; peak at 17 hpf) which was not seen in any other experimental group (Figure 7A, B). In contrast, under DD32 all embryos hatched within 2 h at 15 hpf ($P<0.05$) (Figure 7C). The hatching peak observed in the DD28 group was also more distinct and advanced (85.7 ± 5.7% of embryos hatched by 19 hpf) than LD28 and LL28 were two hatching peaks were observed (LD28; 19 hpf: 47.5 ± 3.5%, 21 hpf: 52.5 ± 3.5%; LL28, 19 hpf: 42.5 ± 3.3%, 21 hpf: 47.5%; P>0.1).

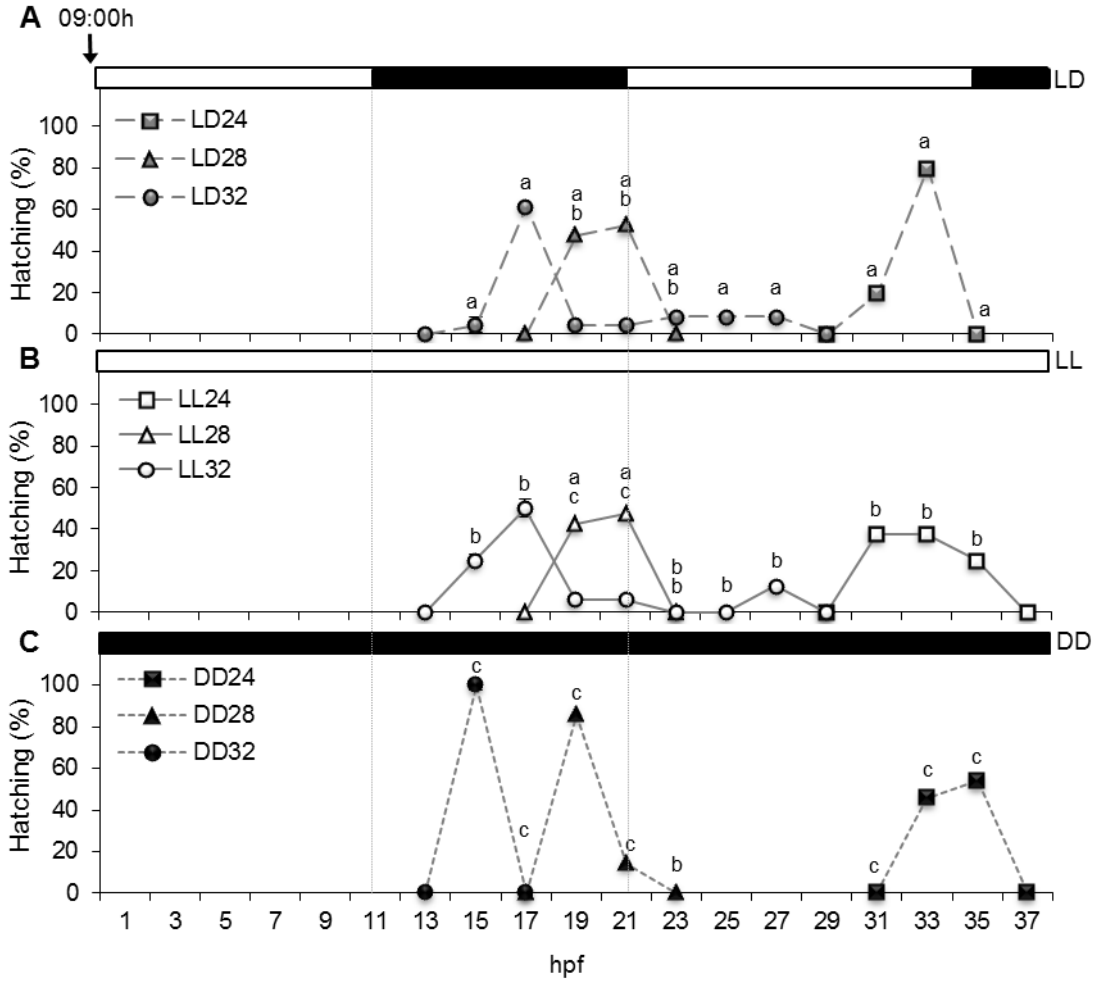


Figure 7. Somalian cavefish hatching times when exposed to different environmental conditions of light and temperature. Data is expressed as the percentage of newly hatched larvae (mean±SEM) observed every two hours. Different letters: Significant differences found at each sampling time among treatments (T° and light) (ANOVA, $P<0.05$). Arrow, bars and abbreviations as in Figure 1.

Hatching rate

In zebrafish, the influence of the different temperature regimens did not affected the hatching rate as no significant differences were found among temperature groups ($P=0.23$). On the other hand light significantly influenced zebrafish hatching rate as LD and LL regimes brought out the best hatching rates in contrast with the DD group ($P<0.05$). By 75 hpf the group with the highest hatching rate was LD28 with 95.2 ± 2.7% of embryos swimming free out of the chorion. On the contrary, at this time just 44.4 ± 1.5% of the embryos hatched under DD24 ($P<0.05$) (Figure 8A).

In Senegal sole, 18°C brought out the lowest hatching rate (57.4 ± 2.4% hatched embryos) when compared with 24°C and 21°C (70.1 ± 4.5% and 77.1 ± 3.3% respectively) ($P<0.05$, Figure 8B). Light also affected the hatching rates of this species as it was lower in DD than in

LD and LL regardless the temperature regime. Significant differences were found among LD and LL under 21°C and 18°C temperature groups as hatching rate was highest under LD when compared to LL ($P<0.05$). Overall the group with the best result at the end of the experiment was LD21 with 93.1 ± 2.9% of hatched larvae ($P<0.05$).

Regarding the cavefish, its hatching rate was clearly affected by the temperature regimes as the best outcome was clearly observed under 28°C regardless the lightning conditions (Figure 8C). A total of 73.6 ± 6.0% of the embryos raised under 28°C hatched in contrast with 28.7 ± 6.1% of embryos under 32°C and just 16.1 ± 2.4% of embryos under 24°C ($P<0.05$). Lighting conditions within each temperature group affected the hatching rate. At 32°C the percentage of hatched larvae was higher under LD and LL with no significant differences between them ($P=0.63$). However, under 28°C, the hatching rate was higher in LD followed by DD ($P=0.072$) and LL where the number hatched larvae was the lowest in comparison with the LD group ($P=0.11$). At 24°C no significant differences in the hatching rate were found among the light regimes ($P=0.16$).

DISCUSSION

In this study, the existence of a daily rhythmic process occurring at different stages of embryogenesis was observed in zebrafish and Senegalese sole. Under LD (regardless the temperature) the embryogenesis of both species did not follow a regular sequential pattern along the day but rather presented a marked daily rhythm of development synchronized to the light phase (faster development) in the case of the zebrafish and to the dark phase in the case of the Senegalese sole. These embryogenesis rhythms coincided with the specific daily activity rhythm reported for each species (diurnal in zebrafish and nocturnal in sole) (Cavallari *et al*., 2011; Gibson 2005). Furthermore, the persistence of rhythmicity indicates the existence of a basic time-keeping system in the cells of the developing embryo, before organs appear or become functional. In cavefish embryos, daily developmental rhythms could not be clearly observed due to their very fast embryogenesis (less than 24 h). However, an interesting pattern of development (also regardless the temperature regime) was observed among the experimental light groups as embryos raised under DD reached all three developmental stages ahead of embryos under LD and LL. The reasons for this is unclear, so we can only hypothesized that embryos under DD were exposed to those lightning conditions that best mimic their natural environment and to which they have adapted during the curse of evolution.

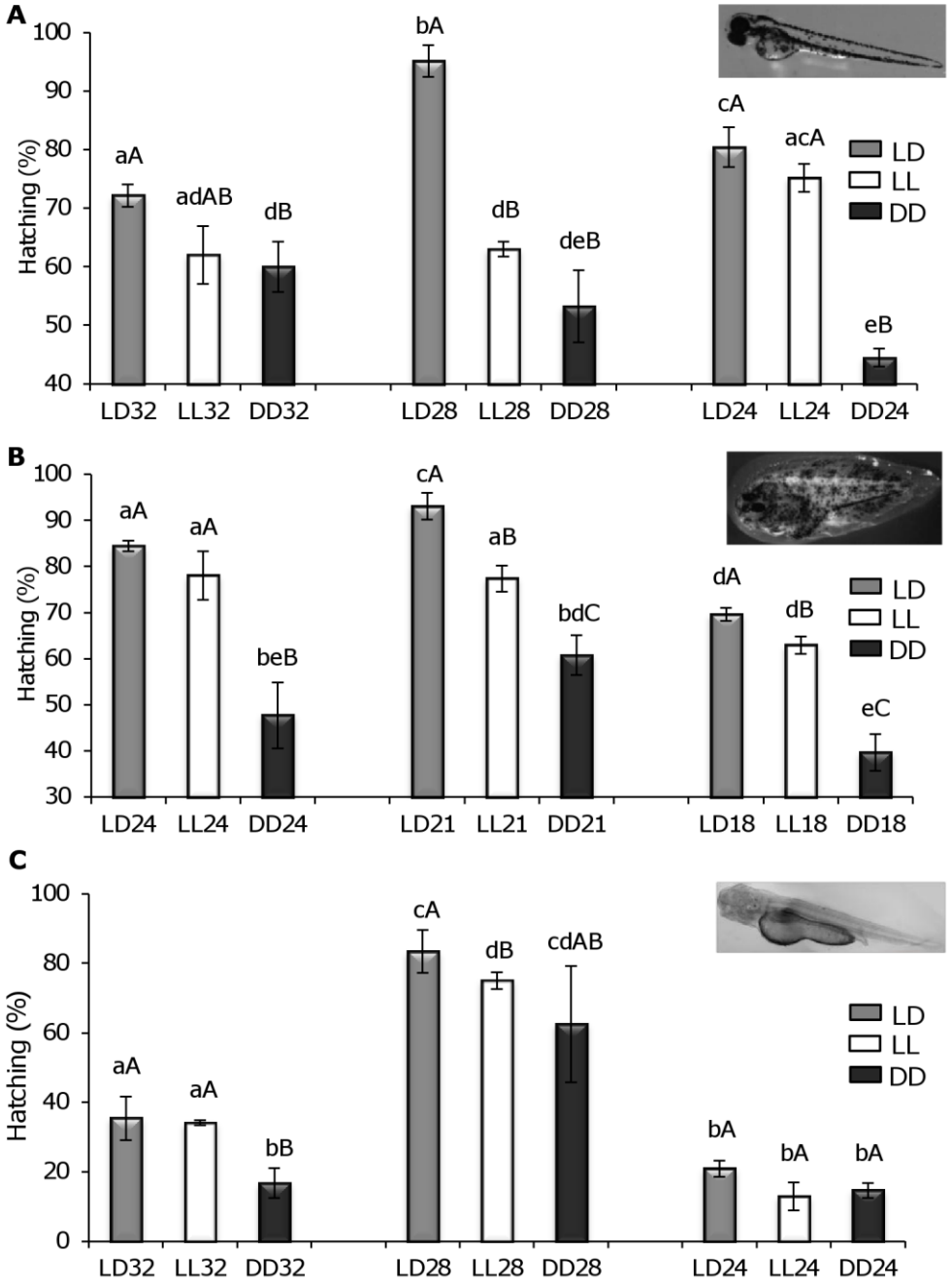


Figure 8. Effect of light and temperature on the hatching rate of fish. Values represent the percentage of zebrafish (**A**), sole (**B**) and cavefish (**C**) hatched embryos found during and until the end of the experimental period (75 hpf in zebrafish, 72 hpf in the sole and 37 hpf in the cavefish). Data is expressed as mean ± SEM. Lower case letters: Significant differences among treatments (T° and Light). Upper case letters: Significant differences within each temperature group (ANOVA, $P<0.05$). Arrow, bars and abbreviations as in Figure 1.

Temperature played a modulating role on the embryogenesis of the three species by altering the pace of the process (highest temperatures elicit faster development), as mentioned in

many studies were fish growth and development have been studied under different constant temperatures (Guerreiro *et al*., 2012; Schröter *et al*., 2008; Delaunay *et al*., 2000). However, the circadian system of the zebrafish although light- and temperature-sensitive is also temperature-compensated, so the circadian oscillations of a particular process remain stable over a rather wide range of temperatures (Lahiri *et al*., 2005). Therefore, a key question to be solved is when the biological clock of fish becomes functional. In the zebrafish, a light-entrainable biological clock analogous to the "peripheral clock" is present in early embryonic cells prior to the development of known specialized light-receptor structures (Dekens and Whitmore, 2008). Furthermore, it has been suggested that the time-control mechanism in embryos, apparently begins as early as the fusion of the pronuclei process (14–20 minutes pf) (Dekens *et al*., 2003) and continues up to the end of embryogenesis (Gorodilov, 2010).

Hatching rhythms were observed in both zebrafish and Senegalese sole and as described for embryogenesis, LD conditions brought out a clear rhythmic activity synchronized by light. In the case of zebrafish, hatching occurred during the light phase and in Senegalese sole hatching occurred during the dark phase. However, under constant conditions (LL and DD) the hatching rhythm persisted, although with lower amplitude which suggests that time in embryos is kept according to an endogenous periodic oscillation mechanism. This internal clock has allowed researchers to create predictable time models for several specific periods within embryogenesis such as cell division in the cleavage stage (Kirschner *et al*., 1985), transition to the middle blastula stage (Newport and Kischner, 1982), start of gastrulation (Satoh, 1982) and somitogenesis (Schröter *et al*., 2008). The presence of an endogenous clock in the zebrafish has been also observed to control the spawning rhythm as under LD, spawning takes place within 2 h of lights on (Selman *et al*., 1993). However, pulses of darkness applied during the light phase caused a delay in the spawning peaks (Blanco-Vives and Sánchez-Vázquez, 2009). In the present study, the hatching rhythms of sole coincided with those previously found when the hatching rhythm of this species was firstly described (Blanco-Vives *et al*., 2011). However, in this case, the effect of the temperature was not taking in to account so the modulating effect of the temperature and its combined influence with light could not be established. Indeed, hatching rhythms were temperature sensitive as hatching was advance under the highest temperature regimen (32°C for zebrafish, 24°C for sole) and delayed under the lowest temperatures (24°C for zebrafish, 18°C for sole).

Contrasting with the results found in the zebrafish and Senegalese sole, no hatching rhythms were observed in the Somalian cavefish as hatching times were similar among the

different lighting regimes. However, hatching was advance under DD32 and DD28, which could be explained by the earliest of embryo development under these two groups. As the species has been previously reported as a photophobic, it might be possible that some structures (central and/or peripheral) are still able to perceive light stimuli, but this hypothesis remains unanswered at the moment. However, a recent investigation found that the lack of responsiveness of the cavefish is not only due to the eye loss but also to mutations of non-visual opsin photoreceptors such as melanopsin and TMT (teleost multiple tissue) opsin, (Cavallari *et al*., 2011). The temperature effect on the cavefish hatching times was similar to the zebrafish and sole, of modulating influenced as hatching occurred first under the higher temperature (32°C) and delayed under the coolest (24°C).

The hatching rate for the three species was highest under the intermediate temperature regimen (28°C for zebrafish and cavefish and 21°C for sole) under LD cycle (although in cavefish no significant differences were found between LD and DD). In zebrafish and sole, the hatching rate under DD, regardless the temperature regimen was markedly low, which suggests the importance of light cues on the early development of both species. Constant light or darkness have been suggested as impair the normal development of embryos and larvae of several teleost fish (Blanco-Vives *et al*., 2010; Villamizar *et al*., 2009; Liu et al., 1994). On the other hand, LD cycles are thought to influence a broad range of functional and morphological aspects during the early stages of fish, such as the regulation of cell proliferation, the activation of UV protective systems (DNA repair enzymes), pigmentation through visual and non-visual photoreceptors and the expression of diverse light responsive genes (Dekens *et al*., 2003; Tamai *et al*., 2004; Shiraki *et al*., 2010; Vatine *et al*., 2011).

In conclusion, our findings reveal for the first time the existence of circadian hatching rhythms in embryos incubated under different light/temperature conditions, and confirm that rhythms during early development are strongly synchronized to light and temperature in a specific way.

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Experimental Chapter 3:

EFFECT OF LIGHT SPECTRUM AND PHOTOPERIOD ON THE GROWTH, DEVELOPMENT AND SURVIVAL OF EUROPEAN SEA BASS (*Dicentrarchus labrax*) LARVAE

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Abstract

This study investigates how the characteristics (spectrum and photoperiod) of artificial light affect European sea bass eggs and larvae from −1 to 40 days post-hatching. Fertilised eggs and larvae were reared under five different light treatments: 12L:12D red light (LDR; half-peak bandwidth = 641–718 nm), 12L:12D blue light (LDB; half-peak bandwidth = 435–500 nm), 12L:12D broad-spectrum white light (LDW; 367< λ <1057 nm), 24L:0D broad-spectrum white light (LL) and 0L:24D (DD). The results showed that total length at day post-hatching 40 was significantly larger in larvae reared under LDB (15.4 ± 0.6 mm) and LL (15.2 ± 0.6 mm) than in larvae reared under LDR (11.7 ± 0.7 mm). Overall wet weight was highest under LDB (21.6 ± 2.02 mgr) and lowest in LDR larvae (13.6 ± 1.48 mgr). Yolk sac and oil globule absorption occurred more slowly in LDR and DD larvae, while LDB larvae developed their fin, teeth and swim bladder significantly earlier than the rest of the groups. DD larvae were unable to capture food and mortality was 100% by day post-hatching 18, while LDR larvae did not feed on rotifers, but fed on Artemia from day post-hatching 16 onwards. The best survival was obtained with the LL treatment, although significantly more problems with swim bladder development and lower jaw malformations were also identified in this group. In summary, these results highlight the key role of the light spectrum and photoperiod for European sea bass larvae, the best performance being achieved under the light conditions that best approached those of their natural aquatic environment (LDB).These findings should be considered when designing rearing protocols for larvae in aquaculture.

Introduction

The day and night light cycle is one of the main environmental challenges that every organism has to cope with in order to survive in nature. To this end, light-sensitive circadian clocks have evolved in most animals, including fish. Light cycles provide internal synchronization for the rhythmic synthesis and release of time-keeping hormones (i.e. melatonin), whose signal affects rhythmic physiological functions in fish (Bromage *et al.*, 2001). Under artificial rearing conditions, however, lighting regimes are established by fish

farmers according to the benefits in terms of survival and growth, as long documented in many cultured species (Barahona-Fernandes, 1979; Tandler and Helps, 1985; Batty, 1987; Downing and Litvak, 1999). Nevertheless, little attention has been paid to the influence of "unnatural" light conditions (constant light or darkness) and light characteristics (yellow/reddish spectral composition), even though they are known to affect the development of fish larvae and their circadian clock (Vallone *et al.*, 2007).

The water column acts as a chromatic filter, and so the aquatic environment dramatically changes the spectral composition of incident light. There is rapid attenuation with depth, so blue wavelengths become predominant in all but the most shallow or turbid waters (Jerlov, 1968). In contrast, standard lighting systems commonly used in hatcheries create bright point light sources that are neither environment-specific nor species-specific and could potentially compromise fish welfare. Moreover, much of their light energy is wasted in the form of unsuitable wavelengths (e.g. longer wavelengths, yellow-red light) which are rapidly absorbed by water molecules and therefore cannot be detected by fish (Loew and McFarland, 1990; Migaud *et al.*, 2006). Light emitting diodes (LEDs) are currently being developed for the fish farming industry since they can be tuned to environmental and species sensitivities through narrow bandwidth outputs. It has been suggested that LEDs focusing on the blue-green spectrum may be most suitable, as these wavelengths generally penetrate seawater more efficiently (Migaud *et al.*, 2007). However, prior to implementing these new alternatives, any potentially adverse effects must be determined in fish larvae.

Several studies have investigated the impact of light on cultured finfish larvae and showed that light affects larval foraging and subsequently their growth and survival (Puvanendran and Brown, 2002; Monk *et al*., 2006; Yoseda *et al*., 2008). Moreover, inappropriate light intensity and spectra during the early development of teleost fish are linked with a number of skeletal abnormalities (Battaglene and Talbot, 1990; Liu *et al*., 1994.) and the absence of a functional swimbladder (Cerqueira and Chatain, 1991; Battaglene *et al*., 1994; Trotter *et al*., 2003).

European sea bass is one of the most intensively cultured species and recent advances in the field have allowed the setting of several abiotic conditions, although the response to certain light intensities and photoperiods have shown that they may not only have a positive but also a negative influence in terms of early development and survival (Barahona-Fernandes, 1979; Ronzani Cerqueira *et al*., 1991). Despite the evidence of the impact of light quantity, no model to date has incorporated the influence of light quality on European sea

bass larvae or its interaction with important aspects like growth, development, prey ingestion and survival.

The aim of this study was to investigate the effects of different light spectra and photoperiods on European sea bass eggs and larvae exposed to white, blue or red light in 12L:12D cycles, constant light or constant darkness from -1 day to 40 days post-hatching (DPH).

Materials and Methods

Eggs and Larval rearing

The experiment was conducted at the facilities of the Spanish Oceanographic Institute (IEO) at Mazarrón (Murcia). Fertilized eggs of European sea bass were obtained from spontaneous spawning by captive broodstock and distributed into fifteen 500 L cylindrical tanks at a density of 40 eggs L^{-1}. The water volume was maintained at 250 L by slowly flowing seawater with gentle, but continuous, aeration. Rotifers *Brachionus picatilis* were cultured and enriched with commercially available freeze-dried green algae *Nannochloropsis sp.* (Phytobloom Prof®. Necton, Portugal), and added to tanks daily as an early live food at a density of 20 individuals ml^{-1} from 8 to 20 DPH. Artemia sp. nauplii at a density of 2-3 nauplii $day^{-1}ml^{-1}$ were introduced from 16 to 30 DPH. 3-5 metanauplii $day^{-1}ml^{-1}$ were added from 27 to 35 DPH. One day before being fed to the larvae, the metanauplii were enriched with a mixture (ORI-GO, ORI-PRO®, Skretting AS, Spain) of phytoproteins and highly unsaturated fatty acids (HUFA).

Experimental Design

From -1 to 40 DPH, five lighting regimes with three replicates were applied: 12L:12D cycle with red (LDR, half-peak bandwidth 641-718 nm), blue (LDB, half-peak bandwidth 435-500 nm) and white (LDW), 24L:0D white (LL), and 0L:24D (DD). The white light used had a broad-spectrum, with 95% irradiance within the range of 367-1057 nm. The spectral analysis of the lights was performed using a spectroradiometer (FieldSpec®, ASD, Colorado, USA) (Figure 1). To avoid the effects of any background light on the experiments, the experimental tanks were covered with a light-tight, black screen. For the different spectral trials, lamps were constructed using light emitting diodes (LEDs) mounted on a fibreglass plaque (160x232 mm). Each red and blue lamp had 17 homogeneously distributed LEDs.

White lamps had 18 white and 4 red LEDs to produce a broader range wavelength. Each lamp was encased in a waterproof container which hung approximately 50 cm above the water surface. The lamps were powered by a 3V DC supply connected to a variable resistor (0-2KΩ) that allowed the light intensity to be adjusted at 0.42 $Wm^{-2}sec^{-1}$, which is low but well above the light threshold (0.06 $Wm^{-2}sec^{-1}$) required to modify melatonin contents in both the eye and plasma of European sea bass (Bayarri *et al*., 2002).

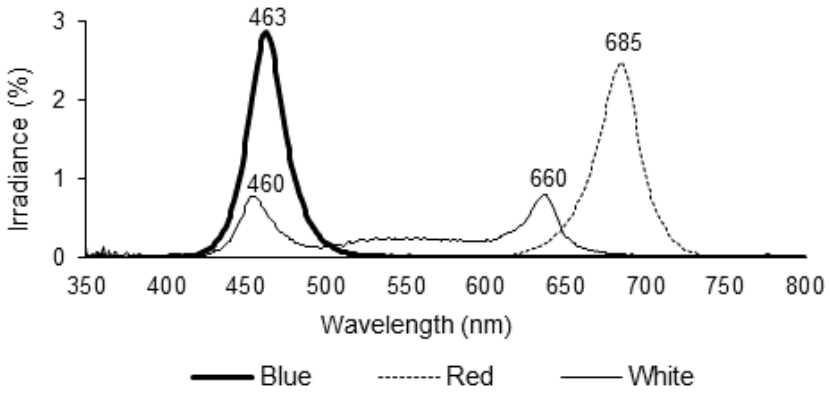

Figure 1. Spectral composition of each LED lamp (blue, red and white) expressed as the percentage of irradiance (Analytical Spectral Devices FieldSpec Handheld).

Data Collection

Every third day from 1 to 36 DPH and 40 DPH, ten larvae from each tank (thirty per treatment) were arbitrarily chosen for total length measurements. On DPH 1, 3, 5 and 7 and on DPH 1, 3, 7, 9, 11 and 15, the same number of samples was taken for yolk sac and oil globule diameter measurements. Yolk volume was calculated using the formula V=π/6 l h2, where l is yolk sac length and h is yolk sac height. The oil globule volume was estimated from the formula V=π/6 d3, where d is the oil globule diameter (Bagarinao, 1986). Larvae were measured alive using a digital camera mounted on a dissecting microscope. Samples were photographed and the digital images were analysed to obtain standard measurements using image processing and analysis software (Leica Microsystems Imaging Solutions Ltd, Cambridge, UK.).

Initial development of the pectoral fins was evaluated on DPH 3 in ten larvae randomly taken from each tank (n=30), killed on ice and fixed with 25% glutaraldehyde. The same sampling method was used on DPH 30 and on DPH 32 to observe the initiation of dorsal fin and anal fin, respectively. The development (number of rays and flexion of the urostyle) of the caudal fin was evaluated on DPH 40. The presence of the swim bladder was

evaluated on DPH 15 and the appearance of teeth was examined on DPH 30 and DPH 33. The sampling days for fin, swim bladder and initial tooth development were chosen following the general ontogeny described for the European sea bass (Moretti *et al*., 1999). On DPH 17, 40 samples were randomly collected from each tank (n=120) to detect physical abnormalities. On DPH 40, forty larvae were arbitrarily taken from each tank for wet weight measurements using an analytical scale and all the remaining larvae were counted to calculate total survival (taking into account the number of fish sampled).

In the present study, first feeding and later food intake were evaluated by counting the number of prey in the gut of ten larvae from each tank (n=30). Rotifer´s undigested mastax were counted once they were expelled from the gut, while Artemia and copepods were identified and quantified in the digestive organ using a microscope. The sampling took place one hour after adding live food to the tanks on DPH 8, 11, 13, 24, 30 and 36.

Statistical Analysis

To establish statistical differences in growth, development, survival and food intake among treatments, all data were tested for normality by examining residual values and all data were found to be normal. Then, a one-way ANOVA was used to analyse all treatments. Yolk sac and oil globule volumes were analysed by two-way ANOVA to establish statistical differences among treatments and time. Both ANOVA analyses were followed by the Duncan´s Test with $P < 0.05$ taken as the statistically significant threshold (SPSS 15.0 for Windows). Data are expressed as mean ± S.D. values. To find statistical differences among treatments in malformations, swim bladder and fin development proportions were arcsine transformed before being analysed using one-way ANOVA.

Results

Survival

Overall survival was significantly higher in LL, LDB and LDR larvae than in LDW larvae (11.6 ± 0.59%) (Table 1). Complete mortality of DD larvae was recorded on DPH 18.

Growth

Significant differences in total length between treatments were observed from 3 DPH (Figure 2). Such differences were more noticeable after the addition of live prey (DPH 8) and

remained until the end of the experiment, with the mean total length of larvae reared under LDB and LL being significantly greater than in the rest of the groups (Figure 3A).

Table 1. Effect of light spectrum and photoperiod on the development of swim bladder and teeth and overall survival of European sea bass larvae.

Percentage (%)	DPH	DD	LDR	LDW	LDB	LL
Larvae with SB	15	92.4 ± 7.1^a	87.6 ± 4.7^a	73.5 ± 12.4^a	77.1 ± 4.7^a	42.2 ± 8.1^b
Larvae with teeth	30	0	0	0	6.0 ± 0.1^a	56.7 ± 0.3^a
	33	0	40.0 ± 0.1^a	63.0 ± 0.1^b	100^b	100^b
Survival	40	0	19.2 ± 2.7^a	11.6 ± 0.5^b	21.6 ± 2.6^a	24.2 ± 4.5^a

At the end of the experiment (DPH 40), LDB and LL larvae had the greatest total length (15.4 ± 0.6 and 15.2 ± 0.6 mm, respectively), while LDR larvae had the shortest total length (11.7 ± 0.7 mm) (Fig. 3A). Total wet weight was also affected by the light spectrum and photoperiod. By DPH 40, LDB larvae had significantly higher total wet weight (21.6 ± 2.02 mg), while the lowest (13.6 ± 1.48 mg) was observed in LDR larvae (Figure 3B).

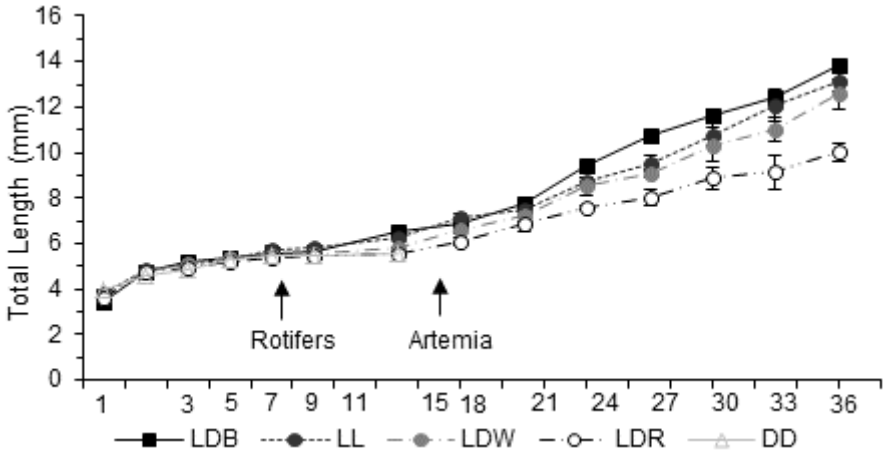

Figure 2. Growth in total length (mm) of European sea bass larvae under different photoperiods (DD and LL) and light spectra from 1 to 36 DPH. (LDW: white light cycle, LDR: red light cycle and LDB: blue light cycle). Data are expressed as mean±S.D. Arrows indicate the day when live prey was added to the rearing tanks.

Yolk sac and oil globule Volumes

Absorption of the yolk sac was clearly affected by the light regimes, as significant differences were observed among treatments during the experiment (Figure 4A). The greatest absorption of yolk reserves was seen in LL and LDB larvae, whose yolk sac volume were the smallest of the experiment. Complete yolk sac absorption was observed by DPH 9 in larvae

reared with LDB, LDW and LL treatments, in contrast to DD and LDR treatments, where yolk reserves (too low to be measured) remained until DPH 11. In contrast with the yolk sac, no significant differences in the oil globule volume were found by DPH 1 among the treatments (ANOVA, P = 0.13). However, from 3 to 15 DPH, significant differences appeared (Figure 4B). The largest oil globule was recorded in the DD and LDR larvae. By DPH 16 the oil globule had been completely reabsorbed in all groups.

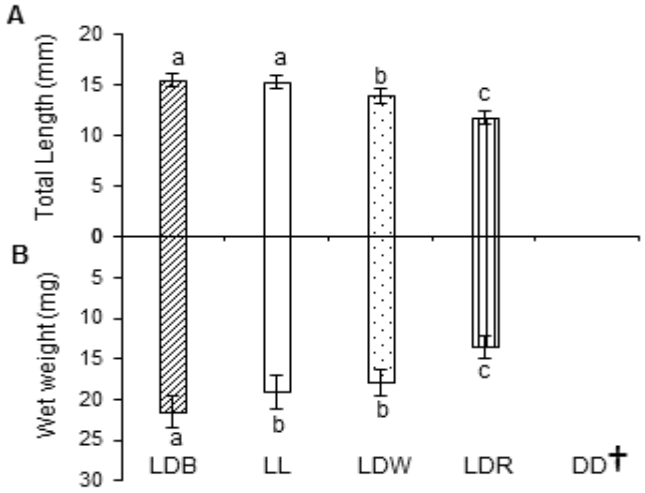

Figure 3. Total length (mm) (A) and wet weight (mg) (B) of larvae reared under different light spectra and photoperiods by 40 DPH. No data from DD treatment was registered as complete mortality was observed on 18 DPH (represented by a cross). Data is expressed as mean ± S.D. Different letters indicate statistically significant differences from each other (ANOVA, Duncan's test, $P < 0.05$).

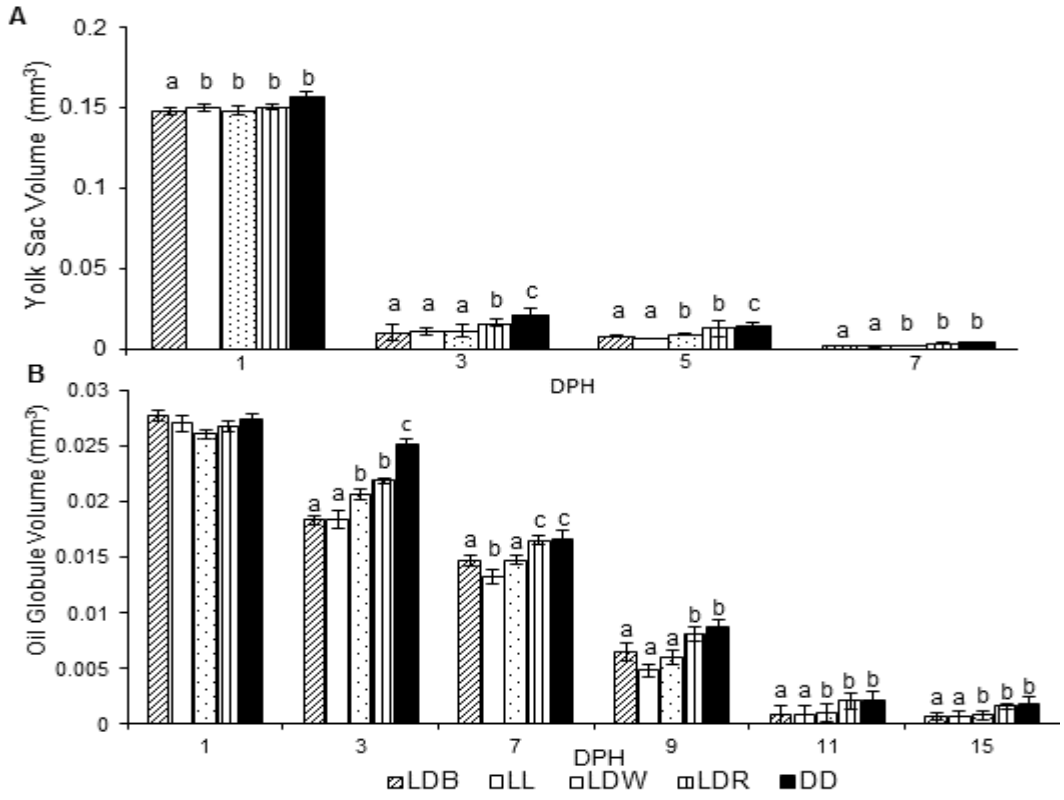

Figure 4. Effect of light spectrum and photoperiod on the absorption of the yolk sac (A) and oil globule (B) reserves. Data is expressed as mean of the volumes ± S.D. Different letters indicate statistically significant differences from each other (ANOVA, Duncan's test, P < 0.05).

Fin Development

Development of the pectoral, dorsal, anal and caudal fins was significantly affected by the light regimes (Figure 5A). Pectoral fins were clearly distinguishable by DPH 3 in all groups, but with significantly different proportions: in the LDR group 46% of larvae showed PF, while 100% of the LDB group did. By DPH 30, the anal fin was observed in all LDB larvae, but in none of the LDR larvae.

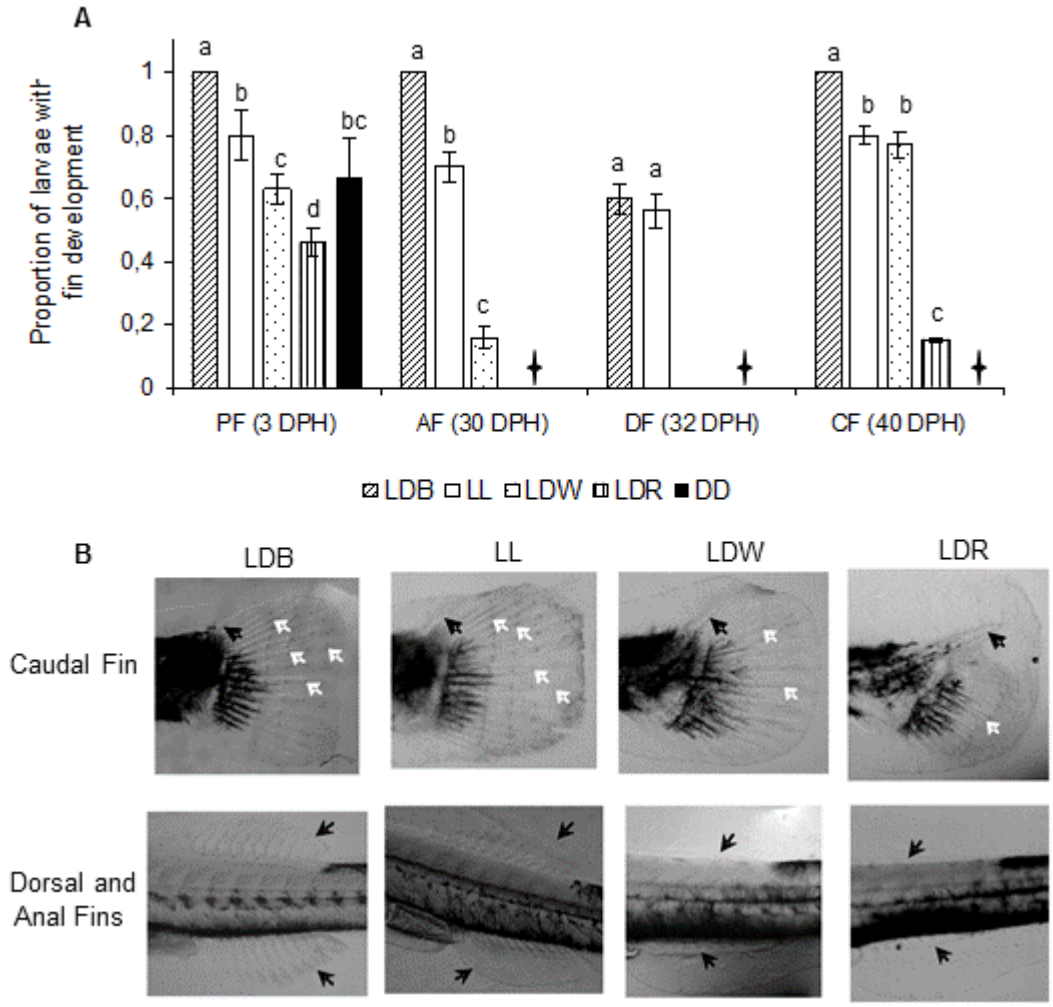

Figure 5. Influence of light spectrum and photoperiod on European sea bass larvae development. (A) Proportion of larvae with pectoral, anal, dorsal and caudal fins on DPH 3, 30, 32 and 40, respectively DD complete mortality was observed on 18DPH (cross). Different letters indicate statistically significant differences from each other (ANOVA, Duncan's test, $P < 0.05$). (B) Development of larvae pectoral (PF), caudal (CF), dorsal (DF) and anal (AF) fins by 40DPH Pictures show the representative development stage of each group. Black arrows indicate the urostyle migration as well as dorsal and anal fins development. White arrows/lines indicate caudal fin ray segmentations (B).

Swim Bladder

This structure was first noticed DPH 8 in larvae reared under LDB and LL regimes, while in the rest of the treatments the swim bladder began to appear on DPH 9. By DPH 15 the proportion of larvae with swim bladder was significantly lower in the LL (Table 1).

Teeth

By DPH 30, significant differences were found among treatments as teeth developed first in LDB and LL larvae, while none of the larvae reared under LDR and LDW showed teeth at this time (Table 1). By DPH 33, teeth development was complete in LL and LDB larvae, while the LDR and LDW larvae were just beginning to show teeth.

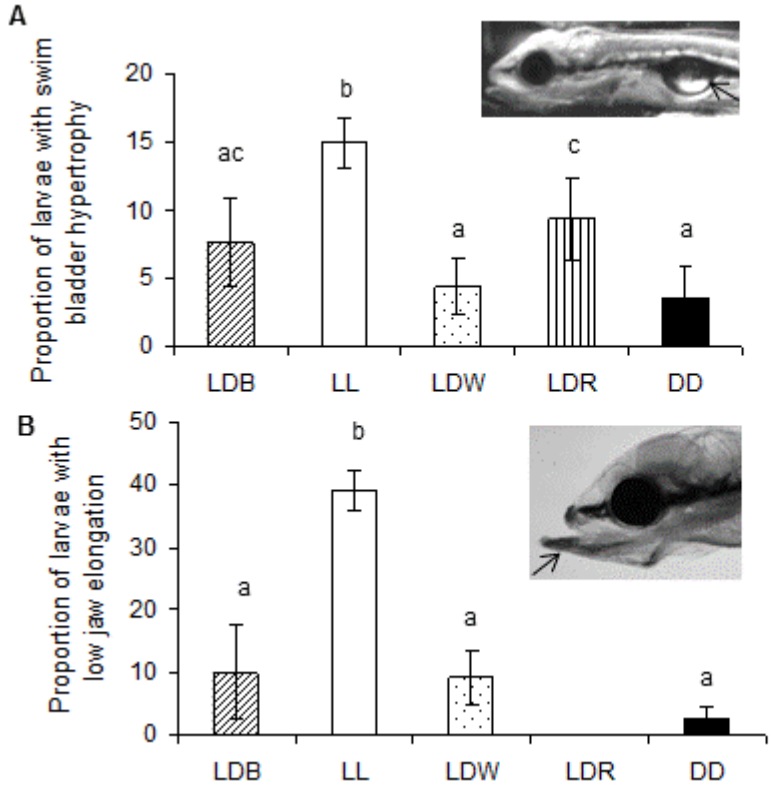

Figure 6. Effect of light spectrum and photoperiod on malformation frequency of European sea bass larvae by DPH 17. Proportion of swim bladder hypertrophy (A) and low jaw elongation (B). Black arrows indicate each malformation. Different letters indicate statistically significant differences from each other (ANOVA, Duncan's test, $P < 0.05$).

Malformations

One of the most common abnormalities registered in this experiment was the over-inflation (hypertrophy) of the swim bladder. By DPH 17, significant differences were found among treatments, LL having a higher proportion of hypertrophy (15.0 ± 1.4 %) than the DD and LDW (3.5 ± 1.8% and 4.3 ± 1.6%, respectively) (Figure 6A). Another malformation observed in the different treatments was the elongation of the lower jaw. This abnormality

was particularly frequent in the LL larvae (38.0 ± 0.2%) (Figure 6B). Lower jaw elongation was scarce (2.5%) in the DD group and absent in the LDR larvae.

Food intake

On DPH 8 live prey (rotifers) were added for the first time to the tanks. Prey capture increased rapidly with time. DD larvae did not consume live prey at any time of the experiment. The number of rotifers ingested on DPH 11 and 13 was significantly higher in LL larvae and lower in LDB and LDW larvae while LDR larvae almost starved and ingested very fewer rotifers (Figure 7A).

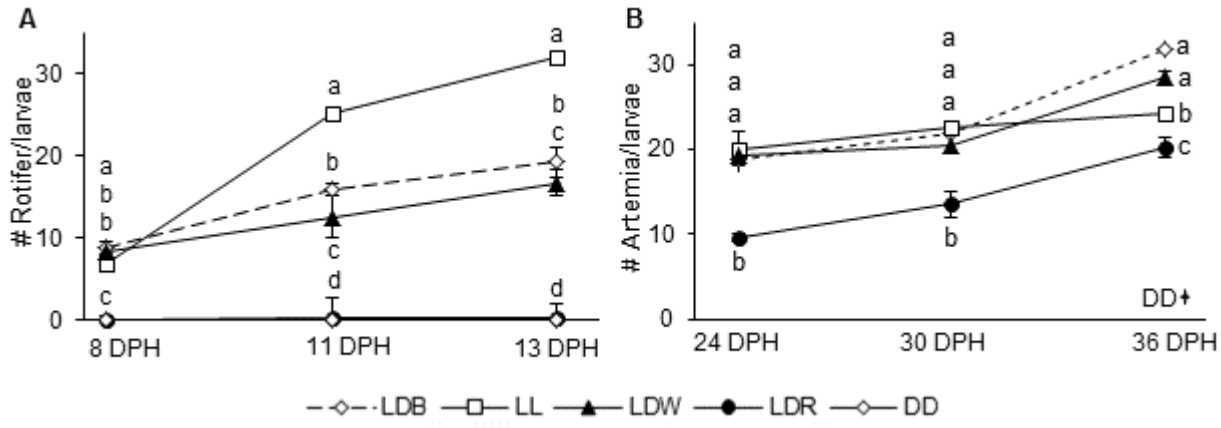

Figure 7. Gut content with rotifers (A) and Artemia (B) of European sea bass larvae under different light spectra and photoperiods. Live prey was added for the first time on DPH 8. No data of artemia ingestion was registered on DD treatment as complete mortality was observed on DPH 18 (represented by the cross). Data are expressed as mean ± S.D. of prey found in larval guts. Different letters indicate statistically significant differences from each other (ANOVA, Duncan's test, $P < 0.05$).

On DPH 16, *Artemia nauplii* were added to the tanks in addition to rotifers. By DPH 18, the artemia found in the larvae guts accounted for 64, 48 and 44% of their content in LDW, LDB and LL larvae, respectively. LDR larvae actively consumed Artemia, showing a great preference for this prey (86.6 ± 0.02 % of the total gut content). From DPH 21 onwards, only Artemia nauplii and metanauplii were added to the tanks. Prey capture greatly increased in LDR larvae, although their food intake remained significantly lower compared with the other groups. By the end of the experiment, feeding activity was highest in the LDW and LDB larvae (Figure 7B).

Discussion

European sea bass larvae were clearly affected by the different rearing conditions of photoperiod and light spectrum, displaying better growth (wet weight), development and fewer malformations under an LD cycle of blue light (LDB), which was close to the natural environmental conditions that larvae encounter in the wild. Indeed, light and dark (or temperature) cycles are required for the normal appearance of rhythmic circadian clock outputs, which, in fish, mature extremely early during larval development and are thought to regulate the temporal co-ordination of many physiological processes (Vallone *et al.*, 2007). Besides, the water column acts as a potent chromatic filter with blue wavelengths becoming predominant as depth increases (Nicol, 1989). In other words, photoperiod manipulation (e.g. constant light or darkness) and unusual spectra (e.g. red light) towards "unnatural" conditions, negatively affect important aspects of larval development and performance.

Aquatic organisms inhabit a highly dynamic photo-environment and their photopigments respond optimally at certain wavelengths (λmax), facilitating object detection underwater; depending on a species' ecology, λmax may maximize visual sensitivity, as in the case of deep-sea fish (Bowmaker, 1990), or maximize visual contrast, as in the case of fish in shallow or coastal waters (Lythgoe, 1979). Therefore, fish should respond optimally under the spectral conditions most frequently encountered in their particular ecological niche (Downing and Litvak, 2001). In the present experiment, larvae reared under LDB performed better in terms of total length (together with LL larvae), wet weight, fin and teeth development and also captured more prey upon first feeding than other treatments. These results are supported by the experiment carried out by Bayarri *et al.* (2002) who found that melatonin, which mediates light effects, was strongly affected by the "blue-appearing" light with half-peak bandwidth 434-477 nm, suggesting the importance of considering not only the number of light hours (photoperiod) but also the power and colour of light when designing an effective artificial lighting system for fish.

Several studies have found a correlation between extendend photoperiod and growth in teleost fish larvae (Tandler and Helps, 1985; Puvanendran and Brown, 2002; Trotter *et al*., 2003), however, our results showed no significant differences in total length between larvae reared under LL and LDB cycles. These results are supported by those of Barahona-Fernades (1979), who found that in the case of larval sea bass, continuous light did not induce the best growth. Moreover, the highest growth in terms of wet weight was observed in LDB larvae,

contrasting with the results reported by Downing (2002), who found no relationship between growth and the spectral composition of light in haddock larvae.

LL larvae showed the highest proportion of malformations (lower jaw elongation and swim bladder hypertrophy) and the lowest proportion of swim bladder development. Several physical parameters have been associated with jaw malformation in cultured larvae including inappropriate incubation temperature/salinity (Ottesen and Bolla, 1998), bacterial invasion of physically damaged oral membranes (Morrison and MacDonald, 1995), unspecified pollutants and intensive rearing conditions (Koumoundouros *et al*., 1997), nutritional factors (Cobcroft, *et al*., 2001) and inappropriate light intensity and spectra (Battaglene and Talbot, 1990; Liu *et al*., 1994). Our results agree with those reported early by Barahona-Fernandes (1979), who confirmed that strong light intensities can be lethal to newly hatched sea bass larvae, and those of Ronzani Cerqueira *et al.* (1991), who concluded that continuous illumination, although good for growth, is not suitable for the normal development of the larvae. The relation between the light condition and the appearance of malformations has begun to be addressed in recent experiments. For example, Cobcroft and Battaglene (2009) found a link between the colour of the rearing tank and the appearance of jaw malformations and walling behaviour (larvae vigorously swim into the walls of the tank) of *Latris lineata:* a high number of severe jaw malformations and walling behaviour was observed in the red tanks and the lowest number in the black tanks, both parameters being positively correlated. Thus, it is apparent that a further systematic multi-factor experimental approach will be needed to determine whether a single culture parameter, or indeed an interaction of physical culture parameters contribute to the prevalence of jaw deformity in cultured teleost species.

Unnatural light conditions may cause other malformations, as found by Johnson and Katavic (1984), who concluded that high light intensities induce swim bladder hypertrophy in sea bass. In nature, the swim bladder is mostly inflated at night when larvae reach the water surface to gulp air avoiding potential predators. Therefore, a nocturnal phase (LD cycle) is important during the early development of this species (Büke *et al.,* 2005) in order to prevent hypertrophy of the swim bladder, which causes larvae to float near the water surface, stop feeding and die due to starvation (Planas and Cunha 1999; Chatain, 1994).

The relation between light and the exhaustion of endogenous reserves by European sea bass larvae is used to their benefit by some commercial hatcheries since the application of total darkness or dim light conditions decreases the use of endogenous sources, so that the start of exogenous feeding can be delayed. This relation was confirmed in our results, as the

yolk sac of larvae under DD and LDR treatments was still visible up to 48h after its complete absorption in the LL, LDW and LDB groups. However as mentioned above, DD larvae, together with LDR showed the lowest growth rate up to DPH 18 (when DD larvae died of starvation). LL and LDB larvae showed the greatest total length, wet weight and development of body structures (fins and teeth). Indeed, in their experiment with *D. labrax* larvae, Kailasam *et al*., (2007) obtained better growth and survival when live prey was available in the rearing system 48h after hatching. Although endogenous sources in European sea bass larvae remain available for several more hours, it is possible that the energy from the yolk and oil globule alone might not be sufficient and exogenous feeding is necessary to meet the nutritional requirements.

The onset of exogenous feeding is also associated with massive mortality, both in nature and in laboratory populations (Yúfera and Darias, 2007). In our experiment, the poor feeding performance under DD and LDR treatments was probably due, to some extent, to a lack of appropriate photons that stimulate the larval visual system which, in marine species, are supported by some rostral free neuromasts and olfactory ciliate receptor cells that, together with eye pigmentation, are present at mouth opening (Hall *et al.*, 2004; Baglione *et al*., 2003). The present research found that LDR larvae did not feed on rotifers but, interestingly, actively captured Artemia nauplii and metanauplii; however, their growth and development were compromised from the beginning of the experiment and prevailed until the end. These results suggest that other physiological processes, apart from the vision-feeding-growth relationship, are affected by light at a very early stage, perhaps even during the incubation period and could be involved in the development of the diverse structures needed for the larvae to capture, ingest and digest their food. In their experiment, Kazimi and Kahill (1999) found that the circadian oscillator that regulates melatonin synthesis in zebrafish embryos becomes functional and responsive to light between 20 and 26 h post-fertilization; at this stage, pineal photoreceptors have begun to differentiate, but retinal photoreceptors have not (48-60 h post-fertilization), suggesting that the first circadian melatonin rhythms are of pineal origin.

In addition to larvae feeding behaviour, different light regimes could affect the behaviour of the live prey. In fact, we noticed that artemia nauplii and metanauplii gathered together on the tank walls of the LDR, LDW and LL groups, while they spread homogenously through the LDB tanks. As regards rotifer phototaxis, Cornillac *et al*., (1983) investigated the effect of light spectrum and only found a positive response between 500 and

650 nm, which suggests that light colour may affect larvae and prey distribution, feeding encounters and, ultimately, their development and growth.

Conclusions

The early development of European sea bass was strongly affected by light. Natural light and dark cycles and a blue spectrum (LDB) provided the best rearing conditions for larvae and produced the best performance. Inappropriate light conditions (LL, DD and LDR) at this early stage resulted in a delay on development that compromised fish welfare and survival, since larvae did not initiate exogenous feeding and a number of malformations occurred. Thus, artificial light conditions, such as the DD used to prolong yolk sac reserves or LL used to stimulate feeding, create an unnatural environment and should be avoided because of their potentially negative consequences.

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Experimental Chapter 4:

EARLY DEVELOPMENT OF ZEBRAFISH: EFFECT OF LIGHT SPECTRUM AND LIGHT:DARK CYCLES

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Abstract

Light plays a key role on multiple biological and physiological processes taking place in living organisms. Although previous research has investigated the influence of light in adult fish, studies on the effect of photoperiod and light spectrum on their early life stages remain scarce. In the current study we investigated how and at what extent, light can influence zebrafish growth, development and survival. For this, fertilized eggs were exposed to (1) pure spectral colours included in the range of visible light (white, violet, blue, green, yellow and red) until 30 dph and (2) constant darkness for 5 and 10 days after which larvae were transferred to 12 hours of light: 12 hours (LD) of darkness. The later was performed in order to investigate the point of no return for larvae reared under light absence. Growth and stress related genes *(igf1a*, *igf2a*, *pomca* and *crh*) were also examined. This study provides novel evidence about how light characteristics influence the very beginning of zebrafish life as all the investigated parameters were influenced by light spectrum. Hatching was higher under short wavelengths (hatching: 90.1 ± 1.4% under violet light and 87.1±1.4% under blue light). Growth was best under blue light (7.2 ± 0.1 mm), white light (7.1 ± 0.1 mm) and violet light (6.9 ± 0.1 mm). Feeding activity was also highest under blue and violet lights (88.2 ± 3.1% and 78.3 ± 4.2% of the digestive tube filled with food, respectively), while red light led to low feeding (25.6 ± 2.5 % DT) and poor survival (100% mortality by 20 dpf). Despite the optimal results observed under violet light, larvae of this group presented high number of malformations (5.2 ± 0.4 %) together with the green (5.4 ± 0.9%) and red (5.2 ± 1.1%) groups. Both growth and stress-related genes were found overexpressed in the violet group, which suggests that this specific wavelength might stimulate a broad range of physiological processes, some of which could have led to both positive and negative consequences.while yellow, green and red lights led to low performance and poor survival. The critical period for zebrafish larvae kept under DD seemed to be close to 10 dph as after being transferred to LD, survival by 30 dpf drastically dropped (26.6 ± 0.2%) in contrast with the control (62.7 ± 2.5%). In summary, the present investigation contributes to increase understanding of the mechanisms involved in the interactions of light exposure and fish ontogeny, which is crucial to elucidate the genetic and physiological pathways used by living organisms to thrive in their natural photo-environment.

Introduction

In living organisms, the natural cyclic conditions of our surrounding environment have led to the evolution of adaptive processes such as the development of a biological clock. By keeping track of time, organisms are able to control complex behavioural and physiological mechanisms in order to thrive in its natural surroundings. External signals like

natural light-dark cycles (LD) regulate the daily adjustments of this circadian clock, which is present in specific neural structures (pineal gland, retina) and peripheral tissues (perhaps all cells in general) (Whitmore *et al*., 2000; Pando *et al*., 2001). Although the signalling pathways that couple light photoreception to the ontogeny and survival of fish have yet to be deciphered, recent studies remark the importance of LD cycles on the expression of several key elements such as the melatonin hormone (Vera *et al*., 2010; Falcon *et al*., 2010; Amano *et al*., 2003), the arylalkylamine N-acetyltransferase (AA-NAT) enzyme (Falcon, 1999) and diverse light responsive genes, such as those composing the molecular mechanism of circadian clocks (Kobayashi *et al*., 2000; Vatine *et al*., 2011).

The response to light in fish appears to be species specific as the physiology and behaviour of each species seem to have evolved in order to adapt to their surrounding environment (Villamizar *et al*., 2011). Thus, the photic sensitivity of a particular fish will depend on the adaptations of its photoreceptor systems to the properties of the underwater light, which profile is rather complex as it is influenced by elements such as depth, organic suspended matter and chemical composition (Smith, 1974). Although visual and non-visual photoreceptive structures and mechanisms have been fairly studied in adults, the response of fish embryo and larvae to specific wavelengths and photoperiod and its implications on their ontogeny and survival are unclear. Regarding photoreception ontogeny, some research teams have found that zebrafish larvae adapt their visual system to the natural changes in the photic environment by altering cones and opsin abundances (Shand *et al.*, 2008; Carleton *et al.*, 2008). This photoreceptor pattern develops from an excess of UV and blue cones (which mediate short-wavelength sensitivity) present at larval stage to a more diverse row mosaic in juveniles which includes the green and red-sensitive opsins (Allison *et al*., 2010; Takechi and Kawamura, 2005).

Zebrafish has become a major model system for genetic approach thanks to the development of technologies and resources required to determine gene expression. Moreover, this fish species is a premier genetic and developmental model for understanding the ontogeny of important mechanisms such as growth, development and survival. Therefore, the early light responsiveness of central and peripheral structures of zebrafish makes this species a perfect candidate to study the influence of photoperiod and light spectrum on vertebrate early development. Embryonic growth and development signalling pathway has been highly conserved in animal evolution with molecular elements such as the insulin-like growth factors (*igf1a* and *igf2a*) playing a key role. Although the expression of both genes starts at different times during zebrafish ontogeny (*igf2a* at zygote stage while *igf1a* starts at later

stages of zebrafish embryogenesis) (Zou *et al.*, 2009), the product of these genes (IGF-1 and IGF-2) seem to mediate in the notochord formation and nephron development of zebrafish embryos (Eivers, *et al*., 2004; Brown *et al.*, 2009). However, the influence of light conditions on the expression of these growth factors has been scarcely investigated.

The components of the hypothalamus-pituitary-interrenal (HPI) also seem to play a key role during ontogeny, since they are involved in the response of fish to environmental stressors. The sequence of events in the functioning of the hypothalamus-pituitary-interrenal (HPI) axis includes the increase of corticotropin-releasing hormone (CRH) in response to the stressor exposure, which stimulates the secretion of the adrenocorticotropic hormone (ACTH) from the pituitary (Alsop and Vijayan, 2009). ACTH is produced from the cleavage of pro-opiomelanocortin (POMC) and activates the signalling pathway leading to corticosteroid biosynthesis (Aluru and Vijayan, 2008). Although it seems that the HPI axis is mature at hatch, the stressor-mediated cortisol stress axis is not active in vivo until 97 hpf (Alsop and Vijayan, 2009). This early functionality of POMC and CRH makes them potential candidates to study the stress status during early development of zebrafish.

In this study, we used innovative LED technology in order to apply pure distinct wavelengths to describe the influence of light spectrum and photoperiod on zebrafish embryos and larvae from a macrostructural point of view. In addition, we discuss the possibility of a correlation between the obtained results with growth (*igf1* and *igf2*) and stress related genes (*crh* and *pomca*) in order to find an explanation from a functional point of view.

Material and Methods

Ethics Statement

All zebrafish husbandry and experimental procedures were approved by the European Convention for the Protection of Animals used for Experimental and Scientific Purposes (ETS N° 123, 01/01/91). The experimental protocol was previously authorized by the Spanish National Committee on Animal Welfare (Law 32/2007) and the Bioethical Committee of the University of Murcia.

Raising adult and larval zebrafish

Adult zebrafish (*Danio rerio*) and larvae of heterogeneous wild-type stock (standard short-fin phenotype) were raised according to standard methods (Nüsslein-Volhard and Dahm, 2002) at the Chronobiology laboratory located at the Faculty of Biology, University of

Murcia (Spain). Eight groups of sexually mature zebrafish (2 females: 4 males per group) were used as broodstock. Fertilized eggs from spontaneous spawnings were collected and pooled together within 2 h of laying. Aliquots of 30 eggs were distributed into sterile Petri-dishes (85x10mm) filled with embryo medium (Nüsslein-Volhard and Dahm, 2002) for 5 days post fertilization (dpf) after which, larvae were transferred into 2.5 L nursery net cages (2 Petri dishes per cage, N=60) (SERA GmbH, Germany). Petri dishes and cages were incubated until 30dph in 9 L thermostat-controlled (100 W, Askoll water heather, Italy) glass aquaria at a temperature of 28.5 °C. For each experimental group two aquaria were used (one cage per aquarium, N=120). From 7 dpf onwards, larvae were fed to satiation twice a day with powder food (JBL Novo Tom, JBL GmbH & Co. KG, Germany). From 15dpf onwards, artemia nauplii (JBL 151 GmbH & Co. KG, Germany) were also supplied (once a day) and from 20dpf until 30 dpf, powder food was replace for granulated food (twice a day until satiation) (Biogran Small, PRODAC, Italy).

Experimental Procedure

Experiment 1: All spectra. For the different spectral trials, lamps were constructed using 10 light emitting diodes (LED, Kopa Electronica; Barcelona, Spain) mounted on a fiberglass plate (160×100 mm) and powered by a 3 V DC supply. Six different wavelengths in a 12 h light: 12 h dark (12L: 12D) photocycle were used: violet, blue, green, yellow, red or white (control) (Fig.1). Constant white lighting conditions were also applied (LL). The photon irradiance was adjusted to 1.57 ± 0.03 m^{-2}. s^{-1}. The spectral analysis was performed using a spectroradiometer (FieldSpec®, ASD, Colorado, USA). Embryos and larvae were reared from 2 hours post fertilization (hpf) until 30 dph under each one of the light regimes mentioned above (with two replicates).

Experiment 2: Embryos and larvae were reared either under constant darkness (DD, N=180) or white LD (control group, N=60). For DD, petri dishes were wrapped in adhesive black tape and the aquaria were covered with thick black plastic to avoid any light contamination. By 5 dph, 60 larvae reared under DD were transferred to LD (LD5) and until 30 dph. The same protocol was performed by 10 dph: 60 larvae from DD were transferred to LD (LD10). The remained larvae under DD were kept under this treatment.

Effects of lighting conditions on growth and development

Hatching, growth, feeding, malformations and survival were used as evaluation criteria for the assessment of light observable effects. Hatching was calculated as the number of embryos hatched within 48 dpf after fertilization divided by the total number of embryos.

By 72 hpf all hatched larvae were counted. Growth in terms of total length (TL) was recorded every five days in each treatment by measuring all larvae (N = 120).

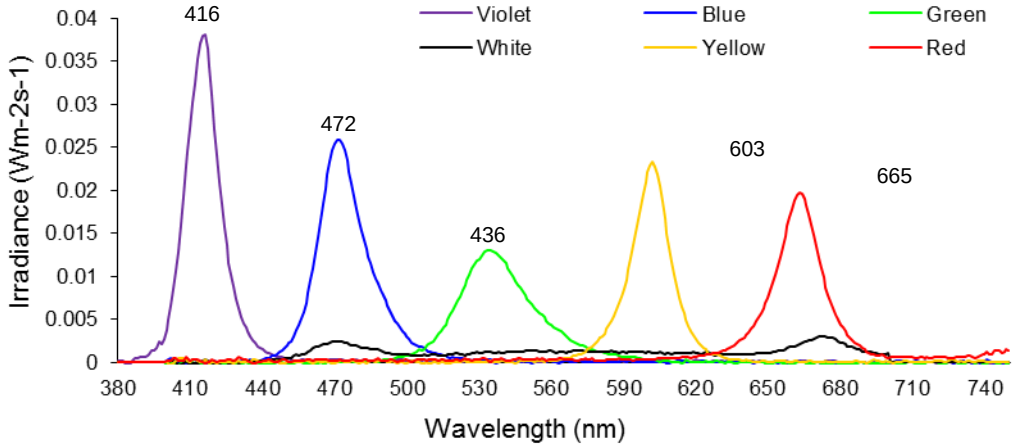

Figure 1. Spectral composition of each experimental LED lamp (violet, blue, green, yellow, red and white) expressed as the percentage of irradiance (Analytical Spectral Devices FieldSpec® Handheld).

Larvae were measured alive using a digital camera mounted on a stereo microscope; digital images were analysed using image processing software (Leica Microsystems Imaging Solutions Ltd, Cambridge, UK.). To evaluate the existence of growth compensation in Experiment 1, all groups were maintained under the same light conditions of 12L:12D of neutral white light (flexible LED stripes, Superlight Technology Co., Ltd, China) from 30 dph to 80 dph. Growth was accounted in terms of wet weight by 80dph. By 10 dph, feeding activity was determined in all larvae. For this, 1 hour after being fed, the portion of the larvae's digestive tube (DT) filled with food was measured and analysed in relation with its total length. Malformations were recorded in all groups every day from 0dpf to 10dpf by using the gross changes early described for the species as the evaluation criteria (Kimmel *et al.*, 1995; Dong, *et al.*, 2007; Verreijdt *et al.*, 2002). Mortality was calculated as the number of dead embryos found every other day. Cessation of heartbeat and circulation were used as end points for mortality. Overall survival was calculated by 30dpf.

Genes expression analysis

By 8dph, 15 larvae per treatment (two replicates) were anesthetized and euthanized on ice. Larvae were pooled and homogenized in Trizol reagent (Invitrogen, Madrid, Spain) using a tissue homogenizer (POLYTRON®, PT1200, Kinematica, Switzerland). Total RNA concentration was determined by spectrometry (Nanodrop® ND-1000, Thermo Fisher Scientific Inc., Wilmington, DE, USA), and 1 µg was treated with DNase I amplification

grade (1 unit/µg RNA, Invitrogen, Madrid, Spain) to prevent genomic DNA contamination. cDNA synthesis was carried out with Superscript III Reverse Transcriptase (Invitrogen, Madrid, Spain) and Oligo (dT)18 (Invitrogen, Madrid, Spain) in a 20 µl reaction volume. Quantitative PCR was performed using the StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with SYBR-green-primer-master mix according to the manufacturer's recommendations. Amplification followed the cycle conditions of: 15 minutes at 95°C, then 40 cycles of 15 s at 95°C and 30 s at 60°C. The final volume of the PCR reaction was 20 µl: 5 µl of cDNA, 10 µl of the qPCR Master Mix and 5 µl of forward and reverse primers. All samples were run in triplicate.

Four target genes (*igf1a*, *igf2a*, *pomca* and *crh*) were investigated. The primers for *igf1a*, *igf2a* and *pomca* genes were designed with Primer Express Software (Applied Biosystems, Foster City, CA, USA) whereas the primers sequences for *crh* were retrieved from Alderman and Bernier (2009). The primers used were (F): 5-CAG GCAAATCTCCACGATCTC-3; (R): 5- CTTTGGTGTCCTGGAATATCTC-3 for *igf1a*; (F): 5-GTGAAGTCGGAGCGAGATTGTT-3; (R): 5-GAGCCTGTGACACTG GGAAGA-3 for *igf2a*; (F): 5-CGCAGACCCATCAAGGTGTGTA-3; (R): 5-CGTTTCGGC GGATTCCT-3 for *pomca* and (F): 5-GCCGCGCAAAGTTCAAAA-3; (R): 5-GCGAGGAGA ATCTGTGCGTAA-3 for *crh.* The amplification efficiency, specificity of primers and amount of cDNA/sample were tested by the standard-curve method. The relative expression of all genes was calculated by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001), using *D. rerio ef1α* (F): 5-CTGGAGGCCAGCTCAAACAT-3; (R): 5-ATCAAGAAGAGTAGTACCGCTAGCATTAC-3 (ENSDART00000023156) as the endogenous reference (Tang *et al.*, 2007). For comparison purposes, the final levels of genes expression (*igf1a*, *igf2a*, *pomca* and *crh*) in larvae exposed to red, yellow, green, violet and blue lights were referred to levels observed under white light (control conditions).

Data Analysis.

All the results were expressed as means ± SEM. Data were analysed by one-way analysis of variance (ANOVA I) followed by Tukey's test to determine significant differences. All statistical analyses were carried out using the software SPSS 15.0 (SPSS Inc.). *P* values <0.05 were considered statistically significant.

Results

Experiment 1

Hatching

By 48 hpf, hatching was highest in the LL group (29.2 ± 2.1%), followed by violet (24.3 ± 1.4%), white (23.1 ± 0.7%) and blue (20.7 ± 0.8%) groups. Statistical differences were found among these groups and hatching values under green (14.2 ± 1%), red (13.2 ± 1%) and yellow (10.5 ± 2%) lights. At 72 hpf, the largest number of hatched larvae was observed in the violet and blue groups in contrast with the red and yellow treatments, in which hatching was lower (Table. 1).

Table 1. Effects of lighting conditions on zebrafish larvae. Observed parameters are expressed as means ± S.E.M. DT: digestive tube. Different superscript letters indicate statistical differences among treatments. (ANOVA, Tukey's test, $P < 0.05$).

Parameter	Age	LL	LD	R	Y	G	B	V
Hatching (%)	72hpf	83.1±1.1^{a}	85.5±1.7^{ac}	79.4±1.5^{b}	81.7±0.6^{b}	84.8±1.1^{a}	87.1±1.4^{c}	90.1±1.4^{d}
Malformations (%)	10dph	8.6±0.6^{a}	1.3±0.2^{b}	5.2±1.1^{c}	1.3±0.1^{b}	5.4±0.9^{c}	1.1±0.1^{b}	5.2±0.4^{c}
Filled DT (%)	10dph	55.3±0.5^{a}	58.2±1.5^{a}	25.6±2.5^{b}	40.5±2.5^{c}	45.6±5.5^{c}	88.2±3.1^{d}	78.3±4.2^{d}
Wet weight (mg)	80dph	76.1±7.7^{a}	130.2±11.2^{b}	N/A	55.6±10.1^{c}	50.4±4.1^{c}	102.6±7.2^{d}	112.4±11.6^{d}

Growth and development

Regarding growth, on 5dph the first statistical differences among groups were found. The lowest TL was observed in the LL, green and red groups and the highest data was recorded in the larvae reared under violet light (Fig. 2B). These differences were maintained with few changes until 30 dpf with the highest growth being observed in the blue, white and violet groups (Figure 2A). In this experiment we did not observe a compensatory growth, since the larvae were reared under the same lighting conditions from 30 dpf, the wet weight (mgr) by 80dpf was statistically different among groups: lowest in LL larvae and highest on white, blue and violet treatments (Table 1).

Feeding activity of larvae under blue and violet lights was higher, as larvae had their digestive tube almost filled with food. In contrast, low feeding was recorded in larvae under red light (Table 1). The most common malformations found in this study were those related to the spinal cord, cranium, pericardium and yolk sac.

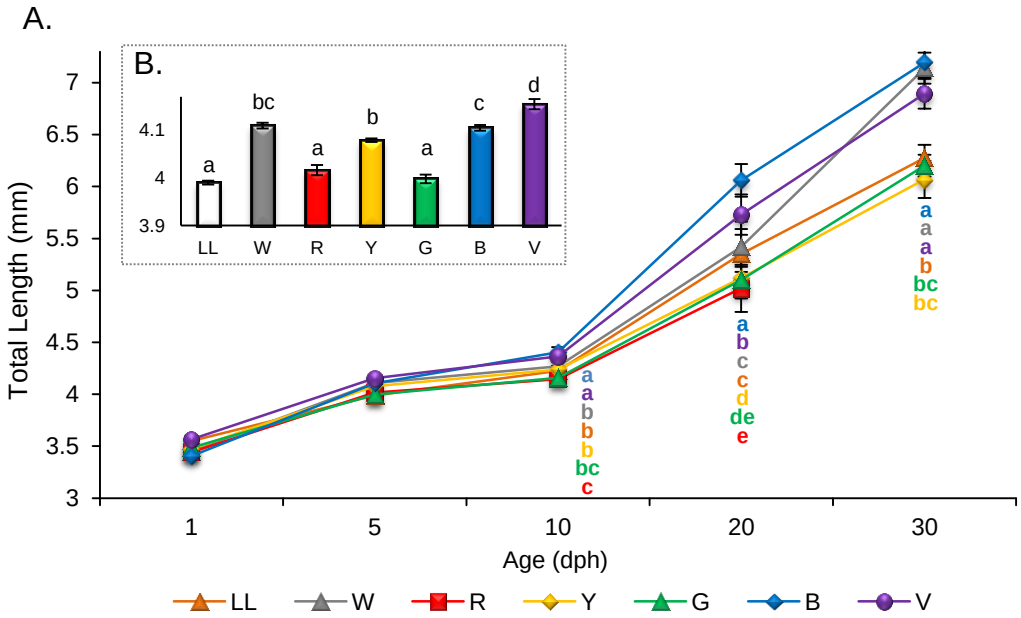

Figure 2. Effect of light spectrum and constant light on zebrafish larval growth from 1 to 30 dph (A) and when significative differences were first observed on 5 dph (B). Constant white light (LL) and LD of white (W), red (R), yellow (Y), green (G), blue (B) and violet (V). Data is expressed as mean of the total length (mm) ± S.E.M. Different letters indicate statistically significant differences from each treatment.

Malformations were first detected under green and red lights by 52hpf. In these groups, embryos were observed with deep abnormalities (pericardial and yolk sac edema and cranial-facial malformations) inside the corion. By 5 dph morphological abnormalities started to be observed under LL (highest percentage of malformations) violet, green and red lights, where by general rule larvae presented jaw malformations and spinal bending. No significant differences among the groups with yellow, white and blue lights were found; these groups presented the lowest malformations occurrence (Table 1).

Mortality was always higher under red light, starting to be more acute from 10 dph to 20 dph, when total mortality was observed. From the remaining groups, larvae under LL, violet, green and yellow lights presented a significative higher mortality compared with blue and white lights. Overall, a critical period of larvae survival started from 10dph and lasted until approximately 25 dph when mortality decreased in all experimental groups (Figure 3). Survival by 30 dph was significative higher under white and blue lights in contrast with the LL group where the lowest survival was found.

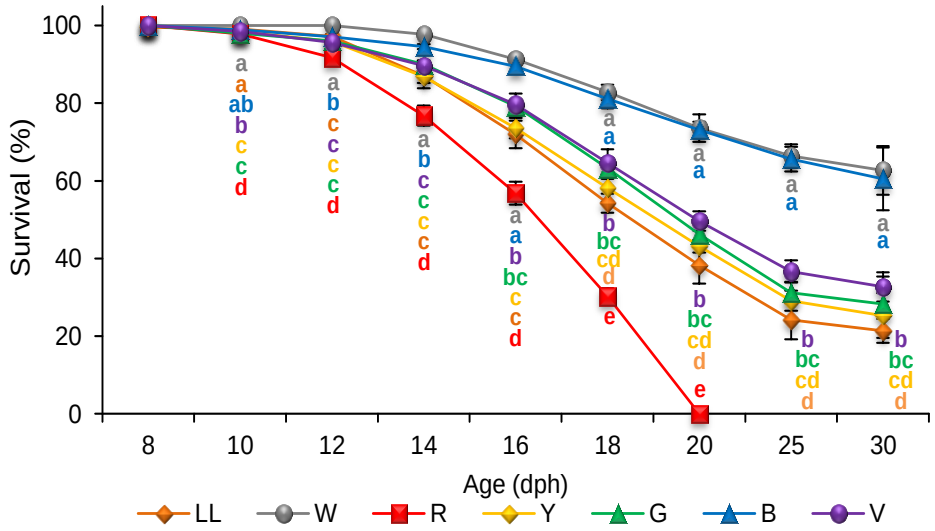

Figure 3. Survival (%) of zebrafish larvae reared under different light spectra until 30 dph. Values are mean ± S.E.M. Means not sharing a common letter differ significantly.

Growth Genes

Under violet and blue light, the *igf1a* expression was significative higher than in larvae exposed to white light (control group). In contrast, the lowest expression was found in the green group, (Figure 4A). Significative differences were also found in the expression of *igf2a*, with the highest levels also being observed under violet light (Figure 4B).

Stress response genes

Pomca expression was significantly higher in larvae reared under violet and blue light than under control conditions (white light) and lower in larvae reared under green light. Similar levels of *pomca* expression were observed in the rest of the groups when comparing with the control (Figure 4C). As for *crh*, it was overexpressed in the red, yellow, blue and violet groups, compared with the control, whereas the expression levels of this gene in larvae exposed to green light did not show differences with those observed in the white group (Figure 4D).

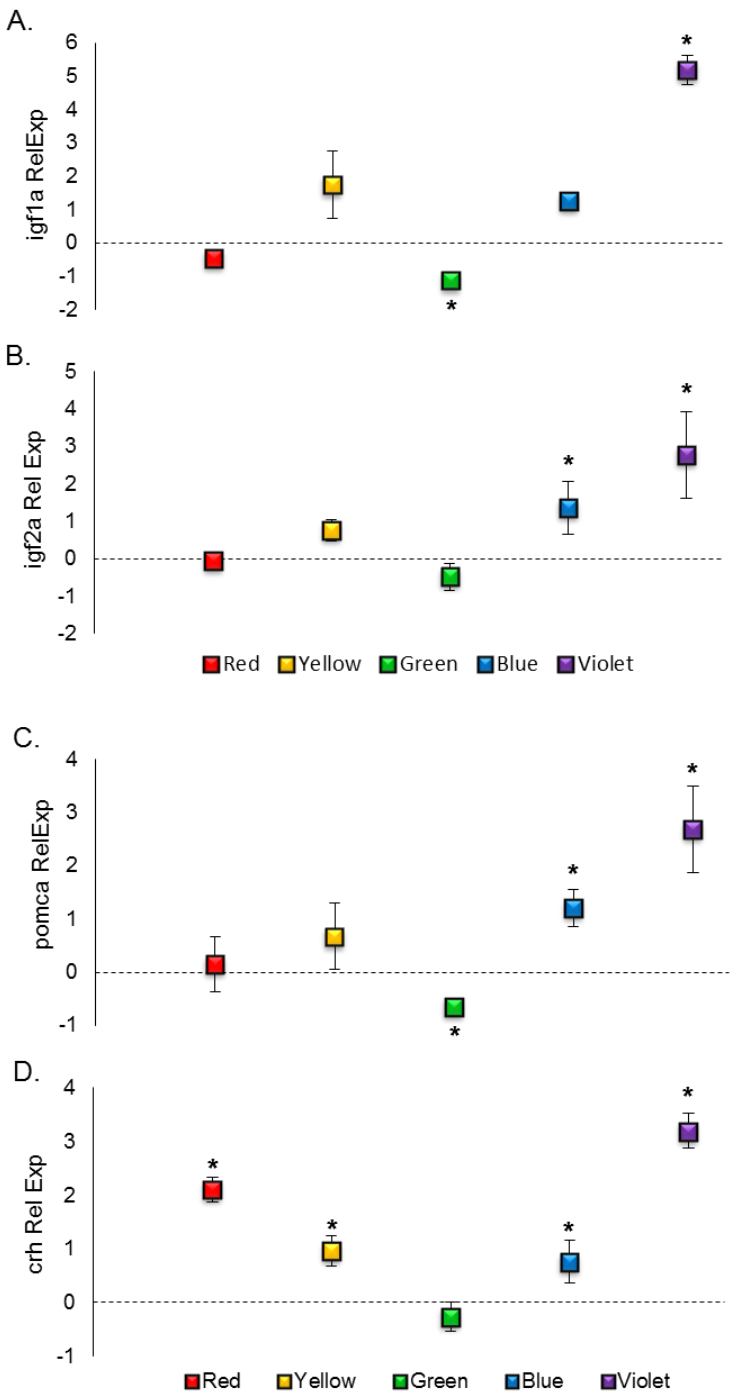

Figure 4. Expression values of *igf1a* (A), *igf2a* (B), *pomca* (C) and *crh* (D) in 8 dph zebrafish reared under red, yellow, green, blue and violet light. Asterisks represent statistical differences compared to the control group (white LD). Data are expressed as mean ± S.E.M.

Experiment 2

By 72hpf, significative differences were found when comparing the percentage of hatched larvae of the control (LD) and DD treatment, since 87.3± 1.4% of free larvae was found in the first and 64.4 ± 9.1% in the latter.

Even after being transferred to LD conditions, growth was always lower on the DD and LD10 larvae, followed by LD5, whereas the control group showed the highest values. However, once larvae were exposed to LD (LD5 and LD10 groups), they started to feed and growth significantly increased in comparison with larvae kept on DD conditions during the whole experiment. No compensatory growth was observed in the LD10 group whereas in the LD5, at 30dph no statistical differences were found with the control group (Figure 5). By 15dph, feeding activity was very low under DD with only 12.1 ± 3.5% of DT filled with food. At this point LD5 larvae and LD10 were found to have similar prey ingestion (26.2 ± 2.3% and 28.5 ± 3.1% of DT filled) while the control group registered the highest (43.2 ± 4.1%). Malformations were observed in a higher number on larvae kept under LD5 (5.3 ± 0.6%), LD10 (6.4 ± 1.3%) and DD (5.3 ± 0.7%) in contrast with the control group (1.3 ± 0.3%). The anomalies found in LD were related with dorsal curvatures, whereas in the other three groups pericardial and yolk sac edemas as well as cranial deformities were more common.

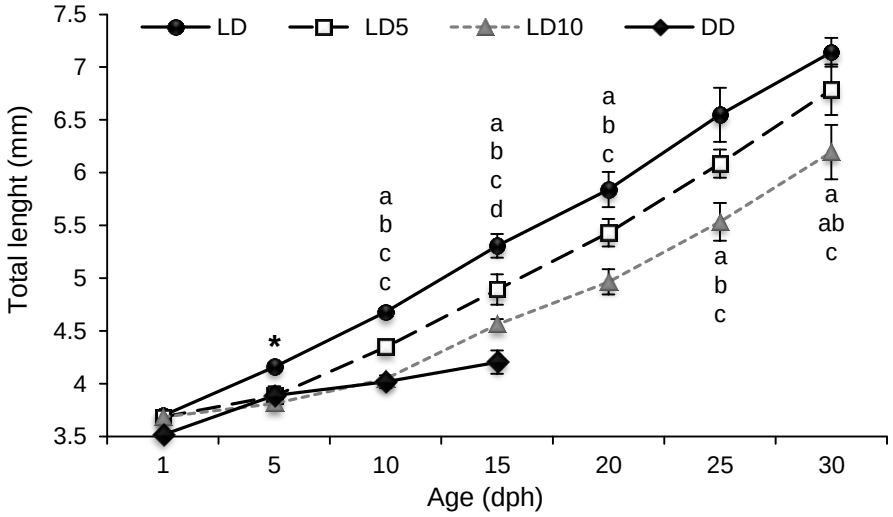

Figure 5. Increase in length (mm) of zebrafish larvae reared either under white LD light (LD, control), constant darkness until 5dph and then under LD (LD5), constant darkness until 10dph and then under LD (LD10) or constant darkness (DD). Values are mean ± S.E.M. Different letters or an asterisk indicates statistically significant differences among treatments.

Survival was clearly affected by the lighting conditions as it was higher under LD5 when compared with the control group. After transferring the DD larvae to LD survival in LD10 improved but remained significantly lower throughout the experiment. DD larvae total mortality was recorded on 18 dph (Figure 6).

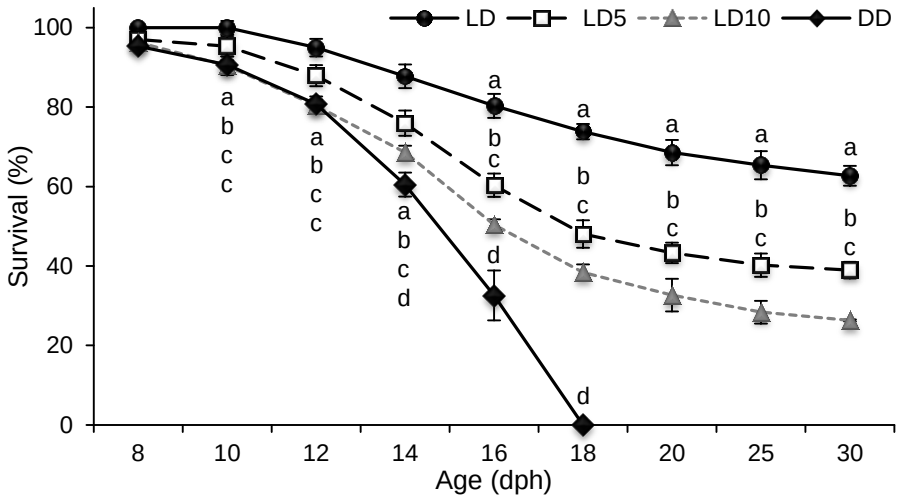

Figure 6. Mean per cent survival (± S.E.M.) over time for larval zebrafish reared under LD (control), LD5, LD10 or DD. Values not sharing a common letter differ significantly.

Discussion

In this study, we have described the influence of light spectrum and photoperiod on several morphological and physiological parameters of zebrafish embryo and larvae. Light spectrum effects were observed at a very early stage during embryogenesis which in turn, influenced the time of hatching. This was the case of larvae reared under red and green lights were the highest percentage of strong malformed embryos were found (pericardial and yolk sac edemas and cranial malformations). In these cases, the malformed embryos had a strong difficulty to break the chorion to hatch and those who succeeded died at an early stage of the larval period. Asynchrony in zebrafish embryo development and hatching is well known and early studies had shown that not even genetic uniformity can eliminate this problem (Streisinger *et al*., 1981). External factors such as handling practices (Kimmel *et al*., 1995), temperature (Dekens *et al*., 2003) and light (Whitmore *et al*., 2000) have being described to affect embryo development. Regarding the ontogeny of light detection, it has been suggested that most of the embryonic cells of zebrafish are capable of light detection as early as 5 hpf (gastrula stage) (Tamai *et. al*., 2004); the pineal gland detects light by 24 hpf (Kazimi and Cahill, 1999) and retinal photoreception develops at 2-3 dpf (Burrill and Easter, 1994). This early ability of zebrafish to detect light has also a dramatic impact on embryo and larvae survival as light mediates in the rise of important transcript genes such as those implicated in DNA repair (Tamai *et al*., 2004) and clock genes involved in cell proliferation and consequently, growth regulation (Dekens and Whitmore, 2008).

In all the evaluated parameters in the present study, a positive response was observed under blue light, which is in accordance with the fact that zebrafish naturally inhabits a great variety of continental water environments (from clear streams to stagnant waters) (Spence *et al*., 2008), and therefore an optimal response would be expected under green-bluish wavelengths. However, green light did not seem to have such positive effects during zebrafish embryogenesis and larval development, since low feeding activity, poor growth, high malformations and low survival were observed in this experiment. Instead, our results showed that LD white light beneficiated all aspects of zebrafish performance. In the larval stage, this may be explained by the existence of four types of cone cells (with ultraviolet supplementing the red, green and blue) bestowing them with the ability to observe a vast spectrum of colours, necessary for the different habitats where this species is found (Allison *et al*., 2010). Then, it is possible that larvae beneficiated of the broad spectrum profile of the white light used in the present study (from 400 to 700 nm). Apart from the visual characteristics of the species, recent studies have found that non-visual photopigments (pinopsin, melanopsin and exorhodopsin) are also present in a wide variety of the zebrafish tissues and are related with nonvisual light-dependent processes such as the circadian system (Pierce *et. al*., 2008; Peirson *et. al*., 2009). Indeed, a study on zebrafish photoreception had showed that blue light is involved in the transcriptional response of clock gene *zper2* which is necessary for the early development of the pineal clock during embryogenesis (Levkovitz *et al*., 2005) through the cryptochromes *cry1b* and *cry3* (Cermakian *et al*., 2002).

We observed significant differences on growth by 5 dph, before exogenous feeding had started. At this point, the groups with higher TL were those reared under white, blue and violet lights, which also maintained the highest TL throughout the experiment. Despite the lack of information about the influence of different wavelengths on the early stages of zebrafish life, some studies have been performed in marine fish species such as European sea bass, Senegal sole and Atlantic cod (Villamizar *et al*., 2011), haddock (Downing, 2002) and the two-spotted goby (Utne-Palm and Bowmaker, 2006). In these works, authors found better larvae performance under different wavelengths (ranging from 470 to 550 nm), suggesting that the response to light could be species specific; with photoreceptor pigments matching the spectral distribution of the fish natural environment (Bowmaker, 1995).

In the present study, LL larvae presented one of the lowest TL by 5 dph and growth stayed low until the end of the experiment. Different results have been found in marine species such as European sea bass and Senegal sole (Villamizar *et. al*., 2009; Blanco-Vives *et. al*., 2010), in which constant light beneficiated growth. However, light/dark cycles are known

to be important for early key processes such as cell proliferation (Dekens *et al*., 2003), brain and eye development (Chapman *et. al.*, 2012), and the onset of the circadian clock of zebrafish (Weger *et. al*., 2011). This might explain the high percentage of malformations and poor survival of the LL group.

To obtain a global picture of how light affected the early development of zebrafish, the expression levels of growth *(igf1a*, *igf2a*) and stress related (*pomca* and *crh*) genes were compared with the biometric parameters investigated in the experimental groups. Regarding growth genes, *igf1a* and *igf2a* were overexpressed in the violet group, which TL was also among the highest in this study. Moreover, the expression of *igf1a* was downregulated in the green group, which TL was one of the lowest by 5-10 dph. Together with the green, the violet group presented high incidence of malformations corresponding to the abnormal development of the spinal cord. Recent studies have found that both overexpression and knockdown of *igf1a* and *igf2a* were linked with several developmental processes such as the midline formation and notochord development (Zou *et. al*., 2009; Brown *et al*., 2009). However, the expression levels of *igf1a* and *igf2a* found in the control group did not show significant differences with those observed in the red and yellow groups. This finding was not reflected in larval growth, since the TL of larvae under white light was higher, suggesting the existence of different pathways controlling zebrafish growth at early stages of development. However, further research will be needed to clarify this point.

Regarding the expression of stress response genes, our results showed that the *pomca* expression was downregulated under green light and overexpressed under violet light. Recent studies have reported that *pomca* low expression could be an indicator of both chronic and acute stress; thus, in zebrafish larvae, the knockdown of this gene resulted in impaired camouflage (Wagle *et al*., 2011), and in Senegal sole and channel catfish, high stock density and low-water stress resulted in a downregulation of *pomc* expression (Wunderink *et. al.*, 2012; Karsi *et. al*., 2005). Liu and collegues (2003) found that the expression of *pomc* in 5 dpf zebrafish larvae was down-regulated after being treated with dexamethasone, a glucocorticoid analogous to cortisol. Interestingly, high doses of this synthetic hormone led to abnormal embryonic development, including pericardial sac enlargement, and death before 72 hpf. These results are in accordance with the low hatching percentage and the type of malformation found in the green group of the present study. Our results on *crh* expression were significantly higher in most of the experimental groups (red, yellow, blue and violet lights) when comparing with the control group (white light). Recent studies in zebrafish and rainbow trout have found an increase of CRH mRNA after severe and repeated stress

(Ghisleni *et. al*., 2012; Doyon *et. al*., 2005). However, under acute estress, CRH mRNA expression decreased drastically suggesting that the regulation of this gene expression could be controlled by the increased glucocorticoid levels by a genomic feedback, which in turn suggests that *crh* and *pomc* may act in a common pathway (Dallman *et. al*., 1994; Bernier *et. al.,* 1999).

Overall, the overexpression observed under the violet light for all the evaluated genes, suggests a strong effect of this particular wavelength on the early stages of zebrafish development. As stated before, this group presented high incidence of malformations and low survival which suggests that the violet light may act as a stress inducer in embryo and larvae. Apart from playing a key role in fish early development, several studies have also linked the overexpression of IGF genes with stress elements such as food deprivation (Maures and Cunming, 2002) and hypoxic conditions (Lo *et al.,* 2011). Moreover, the proximity of wavelength range of the violet light used in this experiment (half-peak bandwidth 390-440 nm) to the UVA light (315-400 nm) might bring some light to this matter as the effect of UVA radiation in fish includes lesions to the brain and retina (Hunter *et. al*., 1979), larval impaired development (Browman *et. al*., 2003) and oxidative stress in adults (Zagarese and WIlliamson, 2001). Furthermore, UVA radiation has been linked with changes in the concentration and activity of enzymes used as stress biomarkers (DNA damage and lipid peroxidation, LPO) and stress response genes (CYP 1) (Mekkawy *et. al*., 2010; Behrendt *et. al*., 2010).

In experiment 2, we further investigated the very low survival in larvae reared under DD (total mortality registered by 18 dph). The results showed that not only hatching was affected but also feeding activity (the lowest among the experimental groups). Despite the increase in food intake soon after larvae were transferred to LD conditions, the negative effect on growth seemed to be permanent in the group LD10 as we did not observed growth compensation throughout the experimental period. However, LD5 larvae were capable of reaching the TL of the control group, which suggests that the critical point for growth compensation in larvae reared under DD is close to 5 dph. The importance of light/dark cycles have been observed in many organs and culture cell lines of zebrafish and to the present, the approximately 117 light inducible/repressible genes identified in this species, suggest the broad range of functional mechanisms (including circadian rhythms, growth, stress response and DNA repair) influenced by light (Weger *et. al.,* 2011).

Conclusions

In summary, the present study reveals the strong effect of light quality and quantity on several important morphological and functional elements of zebrafish ontogeny. White and blue wavelengths and LD cycles provide the best outcome for zebrafish early development and survival. Moreover, our results suggest an implication of *igf1a*, *igf2a*, *pomca* and *crh* genes in the growth, development and stress response of zebrafish to light, although the mechanisms regulating this expression needs to be further investigated. Altogether, the present study shows that zebrafish possess early light sensitivity, which affects several physiological processes during this species ontogeny creating a complex picture which calls for further research in order to explain the effects caused by light in living organisms.

Acknowledgements

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Experimental Chapter 5:

BEHAVIOURAL RESPONSES OF EUROPEAN SEA BASS (*DICENTRARCHUS LABRAX*) LARVAE AND *ARTEMIA* SP. EXPOSED TO WHITE, RED OR BLUE LIGHT AND LIGHT DARK CYCLES VS. CONSTANT LIGHT OR DARKNESS

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Abstract

Light plays a key role on multiple biological and physiological processes taking place in living organisms. Although previous research has investigated the influence of light in adult fish, studies on the effect of photoperiod and light spectrum on their early life stages remain scarce. In the current study we investigated how and at what extent, light can influence zebrafish growth, development and survival. For this, fertilized eggs were exposed to (1) pure spectral colours included in the range of visible light (white, violet, blue, green, yellow and red) until 30 dph and (2) constant darkness for 5 and 10 days after which larvae were transferred to 12 hours of light: 12 hours (LD) of darkness. The later was performed in order to investigate the point of no return for larvae reared under light absence. Growth and stress related genes *(igf1a, igf2a, pomca* and *crh*) were also examined. This study provides novel evidence about how light characteristics influence the very beginning of zebrafish life as all the investigated parameters were influenced by light spectrum. Hatching was higher under short wavelengths (hatching: 90.1 ± 1.4% under violet light and 87.1±1.4% under blue light). Growth was best under blue light (7.2 ± 0.1 mm), white light (7.1 ± 0.1 mm) and violet light (6.9 ± 0.1 mm). Feeding activity was also highest under blue and violet lights (88.2 ± 3.1% and 78.3 ± 4.2% of the digestive tube filled with food, respectively), while red light led to low feeding (25.6 ± 2.5 % DT) and poor survival (100% mortality by 20 dpf). Despite the optimal results observed under violet light, larvae of this group presented high number of malformations (5.2 ± 0.4 %) together with the green (5.4 ± 0.9%) and red (5.2 ± 1.1%) groups. Both growth and stress-related genes were found overexpressed in the violet group, which suggests that this specific wavelength might stimulate a broad range of physiological processes, some of which could have led to both positive and negative consequences.while yellow, green and red lights led to low performance and poor survival. The critical period for zebrafish larvae kept under DD seemed to be close to 10 dph as after being transferred to LD, survival by 30 dpf drastically dropped (26.6 ± 0.2%) in contrast with the control (62.7 ± 2.5%). In summary, the present investigation contributes to increase understanding of the mechanisms involved in the interactions of light exposure and fish ontogeny, which is crucial to elucidate the genetic and physiological pathways used by living organisms to thrive in their natural photo-environment.

Introduction

Although organism responses to light have often been ascribed to one basic mechanism (i.e. visual processing), the regulation of circadian rhythms by light led to the discovery of non-visual photoreceptors that are involved in several physiological processes related to the task of extracting seasonal and time-of-day information (Foster *et al*., 2007). In fish, the photosensory systems and extraretinal photoreceptors are complex and far from fully understood, as the response to light is often species-specific, depending on phylogenetic and ecological factors (Marchesan *et al*., 2005). Scarce information about the features of the environmental light and the photoresponse behaviour of fish, particularly at the larval stage, makes the interpretation and correlation of existing data a difficult task.

In nature, the spectral characteristics of underwater light are determined by a combination of the ambient skylight and the optical properties of the water. Light becomes increasingly monochromatic with depth, because the spectral profile is selectively attenuated as light passes into deep water (Jerlov, 1968). Thus, clear ocean waters transmit maximally at blue wavelengths (470 nm), while coastal waters transmit better at blue-green wavelengths (500 nm) and estuarine waters at green wavelengths (580 nm) (Cohen and Forward, 2002). Furthermore, fish have adapted their photopigment sensitivity according to their surrounding environment (Kusmic and Gualtieri, 2000).

European sea bass (*Dicentrarchus labrax*) is one of the most widely cultured fish species of the Mediterranean sea and large numbers of larvae are produced at industrial level. However, the impact of environmental factors such as lighting conditions is poorly understood, although recent work reviewed the response to certain light spectra, intensities and photoperiod, which were seen to have a great influence in terms of larval growth, development and survival (Villamizar *et al*., 2010). Nevertheless, the behavioural responses of larvae and their prey under different lighting conditions remain unexplored.

The aim of this research was to investigate the Modal Action Patterns (swimming duration, orientation, capture, miss and pass frequency) and distribution of sea bass larvae (from 1 DPH to 30 DPH) exposed to different light spectra and photoperiod. In addition, live prey (*Artemia*) hatching and distribution was observed in order to investigate daily patterns and predatory-prey relationships.

Materials and Methods

Eggs and larval rearing

The experiments were conducted at the Laboratory of Aquaculture at the Naval Base of Algameca (ENA, Murcia, Spain) and at the Laboratory of Fish Chronobiology at the University of Murcia (Spain). Fertilized eggs of European sea bass were obtained from the Spanish Oceanographic Institute (IEO) at Mazarrón (Murcia) from spontaneous spawning by captive broodstock. Newly hatched larvae (0 DPH) were transported in total darkness and distributed into (exp. 1) fifteen glass-reinforced polyester (GRP) tanks of 150-L capacity with an open seawater circulation system and (exp. 2) fifteen 80 L glass aquaria at a density of 30 larvae L^{-1}. Aeration was provided by means of continuous slow seawater flow. Rotifers *Brachionus picatilis* were cultured and enriched with commercially available freeze-dried green algae *Nannochloropsis sp.* (Phytobloom Prof®. Necton, Portugal), and added to the tanks/aquaria daily (09:00 h) as an early live food at a density of 20 individuals ml^{-1} from 8 to 20 DPH. *Artemia* nauplii at a density of 2-3 nauplii $day^{-1}ml^{-1}$ were introduced twice a day (09:00 and 13:00) from 18 to 30 DPH. From 26 DPH to 30 DPH , 2 gr l^{-1} of dry food (Gemma wean 0.2, Skretting AS, Spain) were fed to the larvae.

Experimental Design

Two experiments were performed, submitting animals to (exp. 1) continuous or (exp. 2) acute exposure to different lighting conditions. For experiment 1, sea bass larvae were reared from 1 to 30 DPH under five lighting regimes with three replicates: 12L:12D cycle with red (LD_R, peak at 685 nm), blue (LD_B, peak at 463 nm) or white (LD_W), 24L:0D white (LL), and 0L:24D (DD). The white light used had a broad-spectrum, with 95% irradiance within the range of 367 – 700 nm. The spectral analysis was performed using a spectroradiometer (FieldSpec®, ASD, Colorado, USA). To avoid the effects of any background light on the experiments, the experimental tanks were covered with a light-tight black screen. For the different spectral trials, lamps were constructed using 17 light emitting diodes (LED) powered by a 3V DC supply connected to a variable resistor (0–2KΩ) that allowed the light intensity to be adjusted to 0.42 $Wm^{-2}sec^{-1}$, which is low but well above the light threshold (0.06 $Wm^{-2}sec^{-1}$) required to modify the melatonin content in both the eye and plasma of European sea bass (Bayarri *et al*., 2002).

The effect of the light spectrum and photoperiod on vertical and horizontal distribution was observed from 1 DPH to 18 DPH. Sampling was performed every other day in all tanks, one hour after live prey addition to allow larvae to return to 'normal'

behaviour following the disturbance of feeding. Horizontal distribution was described by counting the larvae over a white secchi disc placed in three positions within the GRP tanks (wall, intermediate and centre) at 12 cm depth. Vertical distribution and the shift from live prey to dry food (weaning) were observed in 6L experimental glass aquaria (25 x 17 x 14 cm) filled with the same water of the rearing tanks and illuminated using the spectral LED lamps. For vertical distribution, one hour after feeding, larvae were randomly sampled and introduced to the experimental aquaria at the same density as in the rearing tanks. Observations were made after a 30 minute acclimatization period when larvae had recovered from the stress associated with the transfer. The larvae were video recorded for one hour (Sony Handicam SR55), after which, those present in the three levels (upper, intermediate and bottom) of the glass aquaria were counted. This sampling method was repeated three times in all the replicates and treatments.Weaning was observed from 26 DPH, when *Artemia* nauplii and dry food were added to the experimental aquaria. The focal animal technique (Altman, 1974) was applied to collect information from one randomly chosen larva during one minute, 20 larvae per sampling point (60 per treatment). Prey item captures and dry food ingestion were counted by means of 20 minute video recordings.

For experiment 2, fish larvae were first reared under a 12L:12D photoperiod of white light (LED lamp) from 1 DPH to 30 DPH and then exposed for one hour to total darkness and blue and red LED lights in order to observe their behavioural responses after such an acute light treatment. For all video recordings, IR lights (λ = 880 nm) were used as the principal illumination source. Sea bass larvae and *Artemia* behavioural observations were performed on alternated days two hours after lights on, in three 6L glass aquaria (25x17x14cm for fish larvae and 25x17x7cm for *Artemia*) and illuminated using the LED lamps (red, blue or white),. The occurrence of five Modal Action Patterns (MAP) including swimming duration, orient, capture, miss and pass frequency (Table 1; Puvanendran and Brown, 1998) were video recorded after 30 min of light exposure, during a 30 minute period (Sony Handicam SR55) and quantified using the focal animal technique, either for frequency (events/minute) or duration (s). The resulting data for each MAP, larva and replicate were averaged to obtain a single value of a given MAP in each treatment. *Artemia* were incubated separately under the LED lamps (red, blue or white) in LL or under total darkness for 24 hours to observe hatching time differences among treatments as well as distribution patterns. The distribution of newly hatched *Artemia* nauplii was observed from one hour video recordings (1 frame per second; Sony Handicam SR55) performed three times per treatment and 30 minutes after

being introduced into the aquaria. Image analysis was performed by means of specialized software designed by the Computer Vision Research Group of the University of Murcia, which offers an in-house alternative to other methods. The program transforms each image (I) of the recordings into an 8 bit gray scale image, where a pixel with a value of intensity I(x,y) = 0 is completely black (total absence of *Artemia* in the location (x,y)) and a pixel with a value of I(x,y) = 255 is completely white (representing maximum occupation by *Artemia*). To process the video recordings, the software divided the image of each aquarium into nine equal sections prior to the analysis. A parameter of *Artemia* occupation (Occ) was defined as:

$$Occ = 100/255 \cdot \overline{I(\cdot,\cdot)}; \qquad \overline{I(\cdot,\cdot)} = 1/N \cdot \sum_{\forall \text{ pixel } (x,y)} I(x,y)$$

where $\overline{I(\cdot,\cdot)}$ represents the average intensity value of the *N* pixels in the chosen region of the aquarium; to better interpret the results, Occ was scaled by 100/255, so it ranges from 0 to 100. The image analysis software also provided a second parameter defined as aggregation tendency (AgT), calculated as follows:

$$AgT = \overline{100/255 \cdot \sqrt{\sigma^2(I(\cdot,\cdot))}}; \qquad \sigma^2(I(\cdot,\cdot)) = 1/N \cdot \sum_{\forall \text{ pixel } (x,y)} \overline{\{I(x,y) - I(\cdot,\cdot)\}^2}$$

where $\sigma^2\{I(\cdot,\cdot)\}$ is the variance of the intensity values; after scaling its square root by 100/255, AgT will be in the range 0 to 100. Thus, the higher the AgT values, the lower the homogeneous distribution of *Artemia.*

Table 1. Modal action patterns (MAP) for European sea bass larvae adapted from Puvanendran and Brown (1998).

MAP	Description
Swim	Forward movement of larvae accomplished by caudal fin action.
Orient	Larvae is stationary and aligns itself towards a prey item.
Capture	Prey item is ingested by larvae.
Miss	An attempt is made but larvae fail to capture the prey item.
Pass	Larvae orients towards prey item but instead of make a capture attempt, swims in a different direction.

The present study was conducted according to the Spanish legislation on Animal welfare and laboratory practices. The experimental protocol was previously authorized by the National Committee on Animal welfare and the Committee of the University of Murcia.

Statistical Analysis

Sea bass larvae and *Artemia* behaviour and distribution data for each tank/aquarium were analysed individually, using a two way analysis of variance to determine statistical differences among treatments. If no differences were found, data was pooled together and assessed for differences among the treatments using one-way analysis of variance ANOVA followed by the Duncan´s Test. All data were tested for normality by examining residual values and all data were found to be normal. All analyses were performed with SPSS 16.0 (SPSS Inc.). Results are reported as mean ± SD and significance accepted as $P < 0.05$.

Results

Experiment 1: Continuous exposure

Lighting conditions influenced the horizontal distribution of sea bass larvae differently, depending on treatments. Under LD_W and LL, the highest density of larvae were seen on the tank walls (51.6 ± 5.4% and 52.6 ± 5.8% in LD_W and LL larvae, respectively) and the lowest density in the centre of the tank. In contrast, larvae under LD_B showed no significant differences in their distribution around different tank regions (Figure 1A). The light spectrum and photoperiod also affected vertical distribution as larvae exposed to LD_B were hardly seen in the bottom area, in contrast to larvae from DD and LD_R which were predominantly distributed in the bottom of the aquaria, while larvae under LL and LD_W showed a preference for the upper layer of the aquaria (Figure 1B).

The weaning process was also affected by light exposure. In general, at the start of artificial feeding, larvae reached the water surface after approximately 10 min of food supply, when they approached the pellets slowly and made contact with their mouths. All groups except LD_R and DD had started to feed on dry pellets by 26 DPH. Larvae under DD hardly fed on *Artemia* nauplii and by 20 DPH, 100% of mortality was recorded in this treatment. Larvae under the LD_R regimen did not start feeding on artificial food until 30 DPH, when significant differences were found among treatments: 62.0 ± 0.9 % of larvae under LL were feeding on dry food in contrast with 15.0 ± 0.2 % of larvae under LD_R (Figure 2A; $P < 0.05$).

Experiment 2: Acute response

Sea bass larvae reared under an LD cycle of white light strongly changed their behavioural patterns when exposed to different wavelengths. Under white light and blue light, larvae showed longer swimming duration than larvae exposed to red light or total darkness, which showed significantly less locomotor activity from 15 DPH onwards (Figure 2B; $P < 0.05$).

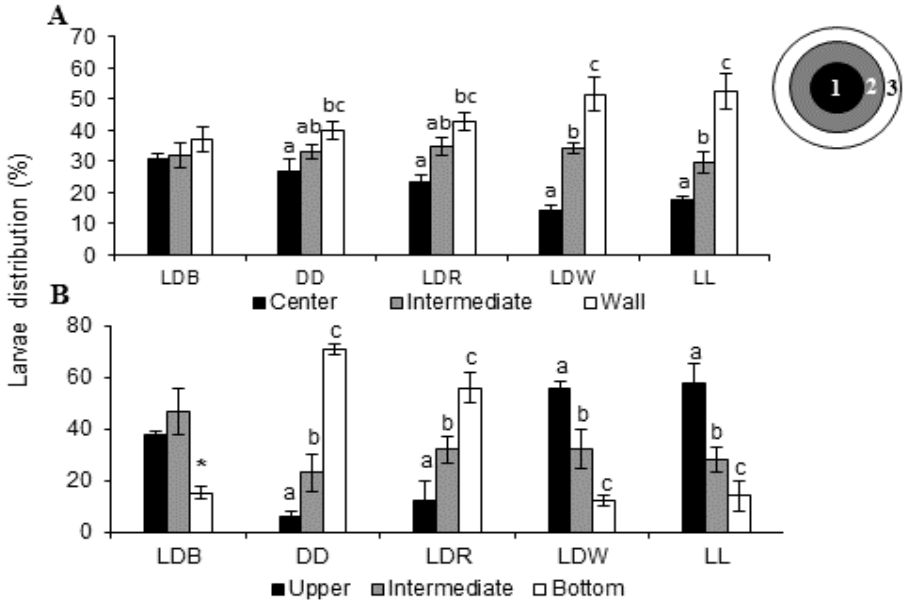

Figure 1. European sea bass horizontal (A) and vertical (B) distributions when reared under different light spectra and photoperiod (12L:12D of blue-LDB; red-LDR; white-LDW and constant light-LL and darkness-DD). Circles represent the areas of the tank in which horizontal distribution was observed (1: center; 2: intermediate; and 3: wall). Data is expressed as mean ± S.D. of larvae percentage from 1 to 18 DPH. Different letters indicate statistically significant differences from each other (ANOVA, Duncan's test, $P < 0.05$).

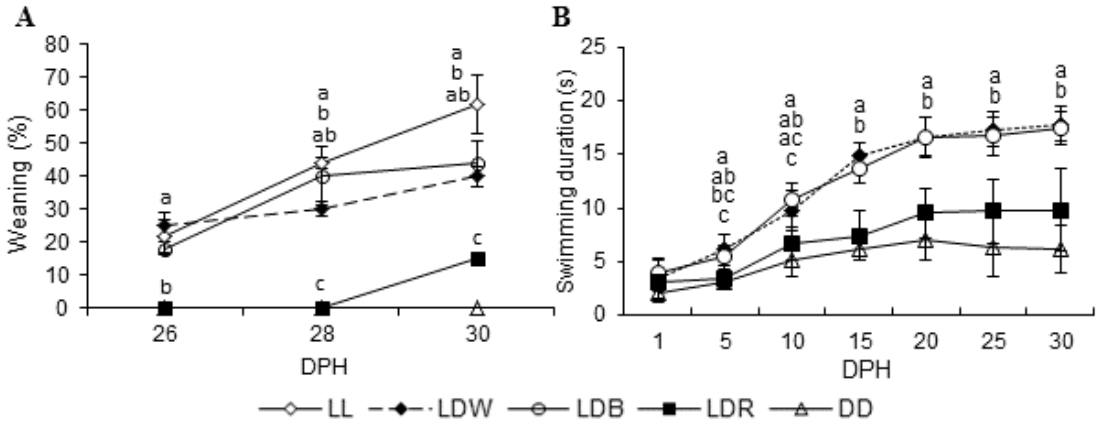

Figure 2. Effect of light spectrum and photoperiod on the time of weaning (A) and swimming duration (B) of sea bass larvae. Data is expressed as mean ± S.D. Different letters indicate statistically significant differences from each other (ANOVA, Duncan's test, $P < 0.05$).

Feeding behaviour was also affected by the light spectrum as larvae orientated themselves towards ("orient") prey to a much lesser extent under red light or in total darkness (Figure 3A; $P = 0.006$). Throughout the experiment, these two groups also captured ("capture") less prey (darkness 0.20 ± 0.07 and red light 1.40 ± 0.79 prey min^{-1} by 30 DPH) than the larvae exposed to blue light, which was the group that significantly captured more prey on almost all experimental days (10.02 ± 1.03 prey min^{-1} by 30 DPH) (Figure 3B; $P = 0.016$). Under blue and white light, captures occurred mostly in the intermediate area (between the water surface and bottom) of the aquaria. Once live prey in the intermediate area had run out, larvae preferred to move to the bottom to feed rather than to the surface. A higher frequency of misses ("miss") was seen in total darkness, although this decreased with age, regardless of lighting conditions (Figure 3C; $P < 0.05$). Overall, larvae under white and blue light ignored ("pass") prey more frequently than the rest of the groups (Figure 3D). Expel" patterns were observed under blue and white lights and to a lesser degree, under red light. These larvae were seen capturing prey and expelled the same (alive in 90% of the cases) in less than 2 seconds.

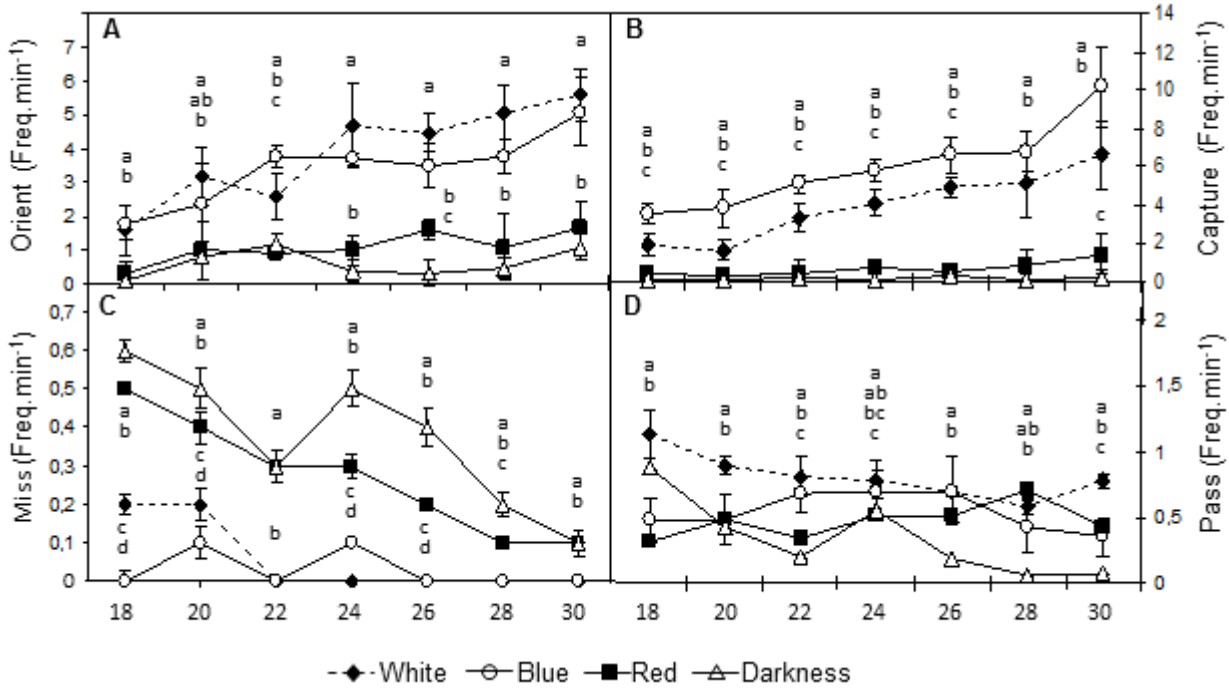

Figure 3. Orientation (A), capture (B), miss (C) and pass frequencies (D) of European sea bass larvae exposed to different light spectra. Data is expressed as # min−1 (mean ± S.D). Different letters indicate statistically significant differences from each other (ANOVA, Duncan's test, $P < 0.05$).

Significant differences were observed in larvae exposed to white light, which showed a higher expulsion frequency (two out of three captures) than those under red light and in total darkness (Figure 4; P = 0.047). Expulsion behaviour was seen also with dry pellets during the weaning process.

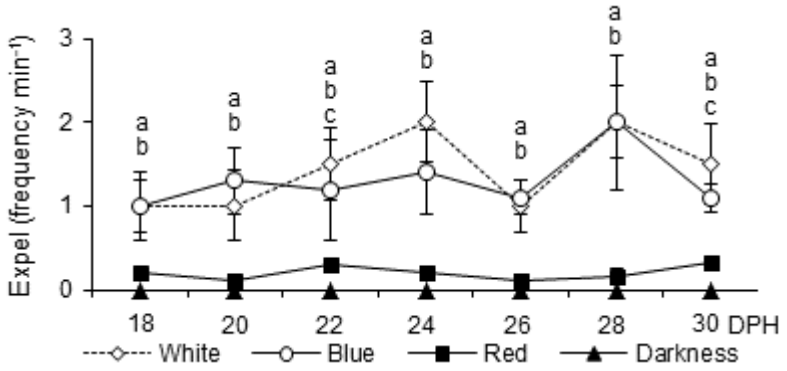

Figure 4. Expel frequency (#min-1) of European sea bass larvae exposed to different light spectra. Data is expressed as mean ± S.D. Different letters indicate statistically significant differences from each other (ANOVA, Duncan's test, P < 0.05).

Significant differences among light treatments were found in *Artemia* hatching rate. Under blue light, after the 24 hour incubation period, 56.5 ± 2.9 % of eclosion was observed in contrast with the other treatments of white light (42.0 ± 2. 8 %), red light (26.3 ± 1.4 %) and total darkness (27.9 ± 3.9) (Figure 5).

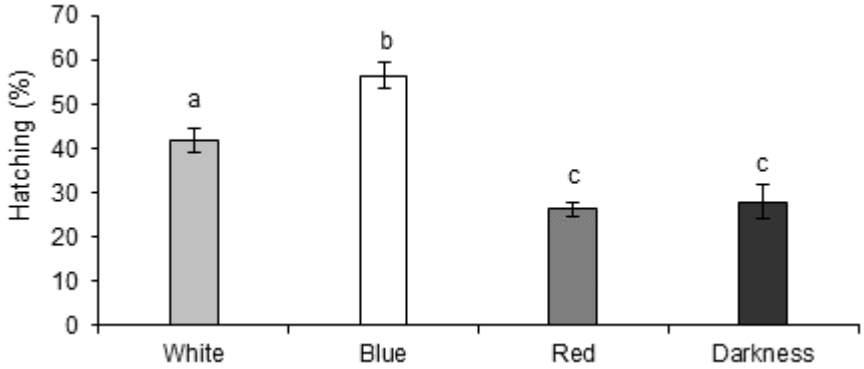

Figure 5. Effect of light spectrum on Artemia sp. hatching time. Data is expressed as mean ± S.D. Different letters indicate statistically significant differences from each other (ANOVA, Duncan's test, pb0.05).

Artemia sp. showed a more even horizontal distribution under blue light in contrast with the rest of the groups where they preferred to occupy the corners of the aquaria (under white and red light; Figure 6A and B) or the bottoms (under total darkness; Figure 6C). Under blue light

Artemia was evenly distributed (Figure 6D). Under white and red light, *Artemia* showed a marked swarming behaviour with strong cyclical (clock and anticlockwise respectively) movements around the aquarium that prevailed throughout the sampling.

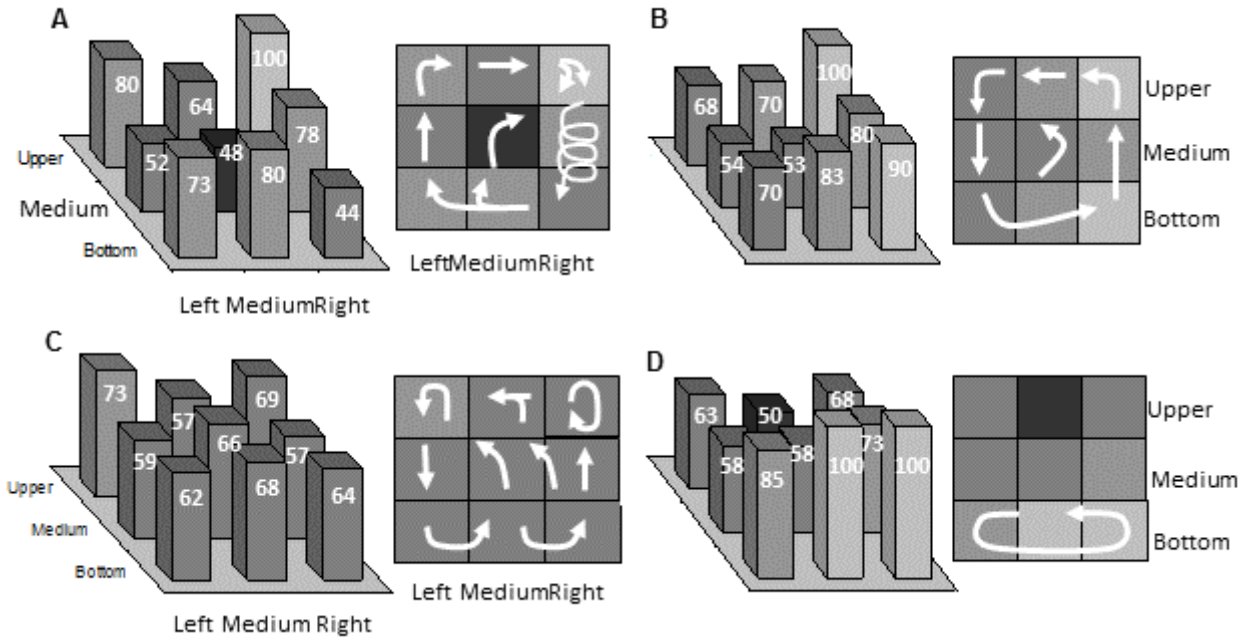

Figure 6. Artemia sp. nauplii distribution preference (Occ) and aggregation tendency (AgT) when exposed to white (A), red (B), blue (C) light and total darkness (D). Data is expressed as mean±S.D. of Artemia sp. percentage from 1 to 18 DPH. Different letters indicate statistically significant differences from each other (ANOVA, Duncan's test, $P < 0.05$).

Discussion

Our behavioural observations revealed that lighting conditions brought out different responses in European sea bass larvae, blue light leading to a more homogeneous distribution, better feeding and higher swimming activity. These results confirm previous studies in other fish species, where lighting conditions were seen to have a strong effect on the performance of fish larvae such as haddock (*Melanogrammus aeglefinus;* Downing and Litvak, 2001), Atlantic cod (*Gadus morhua*; Puvanendran and Brown, 2002), striped trumpeter (*Latris lineata*; Trotter *et al*., 2003) and Senegal sole (*Solea senegalensis*; Blanco-Vives *et al*., 2010). Remarkably, differences in growth and development due to the application of diverse light regimens are observed even before mouth opening, suggesting that other mechanisms apart from visual feeding are being influenced by light (Villamizar *et al*., 2010).

Depending on the studied species, contrasting outcomes have been found, suggesting that the responses to light depend on the species and phylogenetic and ecological factors (Downing, 2002; Marchesan *et al*., 2005). If a given lighting regime enhances the growth and development of fish larvae, it will, in turn, improve the capacity to swim and to detect and capture food. A previous study with sea bass larvae showed that total length and development of some structures were greater in larvae reared under constant light, or light dark cycles of blue and white light (Villamizar *et al*., 2009) and our present results confirmed that the behaviour of larvae was also affected by light. Indeed, larvae under LD_B and LD_W were the most active (highest swimming duration) in contrast with larvae reared in DD or LD_R conditions.

In nature, the most common foraging mode used by planktivorous larvae is visual detection, and so the encounter rate between fish and prey seems to depend on factors such as the contrast of the prey against the background, the visual capabilities of the predator and the ambient light (Aksnes and Utne, 1997; Utne-Palm, 1999; Huse and Fiksen, 2010). In the present study, the poor larvae foraging behaviour displayed under red light (λ_{peak}=685 nm) suggested that larvae needed better illumination in order to find and succesfully capture prey. Many studies on larvae foraging behaviour considered only one or two critical factors that influence prey detection and ingestion, due to the complex relationship among the several elements involved. For example, down welling light is absorbed or scattered and is reflected from the prey, becoming image-forming light that enters the retina of the larvae, which has a short visual range that increases as larvae develop (Huse and Fiksen, 2010). In previous studies, this range was found to peak at 550 nm in *Gobiusculus flavescens* (Utne-Palm and Bowmaker, 2006), 470 nm in *Melanogrammus aeglefinus* (Downing and Litvak 2001) and 450-500 nm in *Salmo salar* and *Dicentrarchus labrax* (Max and Menaker, 1992; Bayarri *et al*., 2002).

In addition to the predator, it is necessary to consider the biology and behaviour of its prey. In general, zooplankton become more visible to predators like sea bass, which is sensitive to short wavelengths of light (Marchesan, *et al*., 2005), when the background illumination contains this particular spectrum profile, as occurs in nature (Novales Flamarique and Browman, 2001). Perhaps as a result of this enhanced contrast, sea bass larvae under blue light could detect and capture more prey than larvae from the rest of the treatments. Of particular importance in hatcheries is the period of weaning, since the transition from live prey to dry diet is generally size-dependent (Kestemont *et al*., 2003), and

so it was expected that fish larvae from the LL, LD_W and LD_B groups were start the weaning process earlier than LD_R larvae.

In the present study, we have found that different wavelengths bring out different responses on *Artemia*. Under total darkness, the locomotor activity decreased and the distribution pattern was limited to the bottom of the aquaria. In contrast, in the presence of light, *Artemia* became very active, facilitating the observation of its swimming and distribution patterns. Overall, *Artemia* distribution was more homogeneous under blue light than under red or white light, which led them to gather in the upper corners of the aquaria. However, in all treatments swarming behaviour was observed, the causes of which have been studied in the past and are still a matter of investigation. In our study, *Artemia* also formed a marked swarm pattern of swimming activity with individuals entering and leaving the swarm in a repetitive circulating pattern.

There are several factors that seem to be involve in swarming, including the response to predation (Miliniski, 1979), light and food availability (Johnsen and Jakobsen, 1987), density and age (Gulbrandsen, 2001) and reproduction (Ambler, 2002). The feeding factor is unlikely in *Artemia* at the nauplii stage because they do not need exogenous nutrients other than their own reserves. The fact that immature newly hatched *Artemia* were used in our trials also excludes the formation of swarms as a mechanism for reproduction. Gulbransen (2001) discussed *Artemia* swarming mechanisms and concludes that swarming may reduce its availability for fish larvae in a hatchery as swimming activity within the swarm increases. This observation is in accordance with the present results as larvae were seen feeding on the *Artemia* located in mid-water, which were entering and leaving the swarms. When prey in the mid area drastically decreased, the larvae then started to feed from prey located in the bottom of the aquarium. Larvae avoided feeding at the water surface where *Artemia* swarms were strongly consolidated and dynamics among the swarm were quite high. Under white light, a vertical swirling swarm was observed, which increased the dynamics of the *Artemia.* in the aquarium making it harder for larvae to orient and capture them. This behaviour has been seen naturally in the presence of open sunlight as well as in concentrated light (light shafts created by vegetation) (Gulbransen, 2001). We cannot confirm that the swarming formation of *Artemia* decreases the foraging rate of fish larvae in general, as early studies with other species have found that zooplankton swarms of *Acartia sp.* were the predominant prey item of sea bream (*Pagrus major*) juveniles (Tanaka *et al.*, 1987). Moreover, there may be more than one trigger of swarming formation (e.g. phototaxis, self defense, chemical or mechanical

elements) and these might, in turn, interact not only with each other but also with the specific adaptations of each species to its environment.

In this study larvae increased their capture success in accordance with their developmental stage, and larger larvae seemed to identify and ingest prey items easily as a result of better visual acuity. Previous studies have reported that, despite the clear advantages of locating patches of concentrated prey, small larvae appear to have limited ability to detect the presence of such patches through vision or olfaction, the reaction distances of larvae to individual prey generally being less than 0.5 body lengths in this stage of development (Browman and O' Brien, 1992; Hunt von Herbing *et al*., 2001). Colton and Hurst (2010) found that small larvae of two gadids (*Gadus macrocephalus* and *Theragra chalcogramma*) were unable to respond to the cumulative signal of a prey aggregation at ranges of 10 – 50 cm.

Conclusions

Sea bass larvae and *Artemia* phototactic behaviour was influenced by lighting regimes and as a result, larvae foraging and performance was affected. The highest feeding activity and most even distribution of sea bass larvae and *Artemia* were observed under the blue spectrum. *Artemia* hatching rate was also highest under blue light. The widespread use of *Artemia* in the fish aquaculture calls for a closer investigation of the phototactic responses and swarming mechanisms of larvae under different light spectra during their early development.

Acknowledgements

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Experimental Chapter 6:

EFFECT OF DAILY THERMOCYCLES ON EMBRYONIC AND LARVAL DEVELOPMENT, SURVIVAL, BEHAVIOUR AND SEX DIFFERENTIATION OF ZEBRAFISH (*DANIO RERIO*)

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Abstract

In the wild, water temperature cycles daily: it warms up after sunrise, and cools rapidly after sunset. Surprisingly, the impact of such daily thermocycles during the early development of fish remains neglected. We investigated the influence of constant *vs* daily thermocycles in zebrafish, from embryo development to sexual differentiation, by applying four temperature regimens: two constant (24ºC and 28ºC) and two daily thermocycles: 28:24ºC, TC (thermophase coinciding with daytime, and cryophase coinciding with night-time) and 24:28ºC, CT (opposite to TC) in a 12:12h light:dark cycle (LD). Embryo development was temperature-dependent but enhanced at 28ºC and TC. Hatching rhythms were diurnal, but temperature- and cycle-sensitive, since hatching occurred sooner at 28ºC (48 hours post fertilization; hpf) while it was delayed at 24ºC (96 hpf). Under TC, hatching occurred at 72 hpf, while under CT hatching displayed two peaks (at 70 hpf and 94 hpf). Under 28ºC or TC, larvae showed the best performance (high growth and survival, and low malformations). Three days post fertilization (dpf) larvae showed diurnal behaviour under 28ºC and TC, in contrast with CT, where the activity was distinctly nocturnal. By 10 dpf, all groups displayed diurnal activity. The sex ratio was strongly influenced by temperature, as the proportion of females was higher in CT and TC (79 and 83% respectively), contrasting with 28ºC and 24ºC, which led to more males (83 and 76%). Ovarian aromatase (*cyp19a*) expression was highest in TC and CT females (6.5 and 4.6 fold higher than at 28°C, respectively); while anti-müllerian hormone (*amh*) expression in testis increased in males at 24ºC (3.6 fold higher compared to TC) and particularly at 28°C (14.3 fold increase). Taken together, these findings highlight the key role of environmental cycles during early development, which shaped the daily rhythms in fish embryo and larvae, and ultimately influenced sex differentiation.

INTRODUCTION

Seasonal and daily environmental changes caused by geophysical cycles (i.e., solar and Earth´s rotation) have shaped the evolution of rhythmic physiological and behavioural processes in animals, which have developed biological clocks to anticipate cyclic events, such as alternations between night and day, and the subsequent changes in temperature [1]. In aquatic ectotherms, temperature entrainment has a crucial adaptive significance as the physical dynamic of the water creates a challenging ecosystem to which the organism must adapt in order to avoid thermal stress [2]. In the wild, water warms up after sunrise (thermophase) and cools down after sunset (cryophase). However, under artificial rearing conditions (e.g., fish farms), the natural environmental fluctuations are rarely considered. Currently, constant thermal environments are thought to maximize growth, but at the cost of reducing thermal tolerance and phenotypic diversity in a population (animals reared under cyclic conditions are more likely to survive environmental changes) [2].

Temperature cycles may act as the main entrainment factor even when light oscillations are also applied, as reported for the circadian rhythms of hatching and clock gene expression in *Drosophila* [1]. In fish, temperature affects virtually all aspects of their behaviour and physiology, including (but not limited to) locomotion [3], foraging ability [4], growth [5] and sex differentiation [6]. However, the effect of daily thermocycles on embryo and larvae development has been neglected, although there is evidence indicating that temperature cycles play an important role in the onset of the fish molecular clock. In fact, in zebrafish (*Danio rerio*) embryos reared in the absence of light, the temperature cycles entrained their circadian rhythms of clock gene expression [7].

Fish account for more than half of all known vertebrate species and zebrafish is a well-known model system for the study of the circadian clock [8]. Zebrafish has small, transparent embryos with a fast development process (24 h from one fertilized cell to a recognizable vertebrae embryo) that can be observed non-invasively under a microscope [9]. As in most ectotherms, temperature is such a strong synchronizer that even daily thermocycles of narrow range (1–2°C) are capable of entraining their circadian rhythms of locomotor activity [10]. Adult zebrafish have a marked activity rhythm, which starts to oscillate robustly early in larvae [11]. Although zebrafish has been described as a diurnal species [12], recent studies have found that it is capable of displaying either diurnal or

nocturnal behavioural rhythms depending on the rearing conditions (i.e., feeding and temperature cycles) [13, 14].

Water temperature may have irreversible effects during sensitive periods of early development in fish. For instance, sex determination can be temperature-dependent, so that temperature determines whether an individual will develop as a male or as a female [15]. Recently, an epigenetic mechanism linking high temperature and masculinization through hypermethylation of the promoter of the gonadal aromatase gene (*cyp19a*) has been proposed in the European sea bass, a species with polygenic sex determination with environmental influences [16]. The zebrafish is a gonochoristic species following what is known as the undifferentiated type, whereby all individuals start ovarian differentiation around 10 dpf. Sometime near 20 dpf, about half of them stop ovarian differentiation and engage in male differentiation while the other half continue with ovarian differentiation [17]. In addition, thermal effects on sex differentiation are superimposed on an otherwise essential genetic sex determination system [6]. However, although it is agreed that zebrafish has no heterochromosomes, their sex determining mechanism is far from clear, except that the genetic component (as opposed to the environmental one) is of some relevance. Sex in zebrafish has been proposed to be determined by female-dominant genetic factors compatible with a ZZ/ZW chromosomal system [18]. However, a different study using three different zebrafish strains and a single nucleotide polymorphism (SNP) -based linkage map, found two chromosomes governing sex as a complex trait rather than a XX/XY or a ZZ/ZW system [19]. Later, a study based on high throughput sequencing of more than 30.000 SNPs revealed that sex-associated locus in zebrafish was found in two other different chromosomes [20]. Furthermore, a recent research using classical breeding experiments and selection demonstrated that zebrafish sex has a genetic basis and proposed a polygenic sex determining mechanism [21]. Likewise, the effects of temperature on zebrafish sex differentiation remain unclear as just few studies have been carried out [22,23], although a masculinizing effect of high temperature is suspected [6]. As in other teleosts, gonadal aromatase (*cyp19a*) and the anti-müllerian hormone (*amh*) have been found to play a key role during sex differentiation. On the one hand, *cyp19a* controls sex differentiation in teleosts by regulating estrogen synthesis and so ovarian development [24,25]. On the other hand, zebrafish *amh* has a sexually dimorphic expression pattern as it has been detected in testis but not in ovaries, being antagonistic of *cyp19a* [26]. Thus, the effects of temperature on zebrafish sex ratios are

far from clear. Furthermore, the effects of daily thermocycles on sex differentiation remain completely unexplored.

Therefore, the aim of this study was to investigate the effect of water temperature (constant *vs* daily thermocycles) on embryo development, daily hatching rhythms, larval growth, behaviour and sex differentiation of zebrafish.

MATERIALS AND METHODS

Ethics Statement

All zebrafish husbandry and experimental procedures were approved by the European Convention for the Protection of Animals used for Experimental and Scientific Purposes (ETS N° 123, 01/01/91). The experimental protocol was previously authorized by the Spanish National Committee on Animal Welfare (Law 32/2007) and the Bioethical Committee of the University of Murcia.

Animal Rearing Conditions

The present research was conducted at the Chronobiology laboratory located at the Faculty of Biology, University of Murcia (Spain). Adult zebrafish (*Danio rerio*) of heterogeneous wild-type stock (standard short-fin phenotype) were obtained from a local commercial distributor and housed for 6 months at the Chronobiology laboratory according to standard methods [27]. Six groups of sexually mature zebrafish (2 females: 4 males per group) were used as broodstock. Fertilized eggs from spontaneous spawnings were collected and pooled together in the morning, within 2 h of laying and were distributed into 85 x 10 mm sterile Petri-dishes filled with embryo medium for 5 days (35 embryos per Petri dish). After this period, larvae were transferred into 2.5 L nursery net cages (3 Petri dishes per cage, N=105 larvae) (SERA GmbH, Germany). Petri-dishes and cages were both kept in 9 L thermostat-controlled glass aquaria. For each experimental group two aquaria were used (N=210). The photoperiod was 12 h of light:12 h of darkness (LD) of neutral white light (1.7±0.03 photons. m^{-2}. s^{-1}; Flexible LED stripes, Superlight Technology Co., Ltd, China). From 7 days post fertilization (dpf) onwards, larvae were fed to satiation twice a day with powdered food (JBL Novo Tom, JBL GmbH & Co. KG, Germany). From 15 dpf onwards, artemia nauplii (JBL GmbH & Co. KG, Germany) were also supplied (once a day), and from

20 to 90 dpf powdered food was replaced by granulated food (also twice a day) (Biogran Small, PRODAC, Italy).

The aforementioned procedure was repeated in order to obtain two independent biological replicates and to confirm the sex ratio results. Therefore, two different batches of eggs from the same broodstock groups were used.

Experimental Procedure

From 0 to 42 dpf, embryos and larvae were reared in one of the following four temperature regimes (Figure 1): low constant temperature of 24.3±0.1ºC, high constant temperature of 28.1±0.1ºC, a thermocycle of 28.3:24.1±0.1ºC (thermophase:cryophase, TC), where the thermophase coincided with the light period (lights on: 09:00 h, lights off: 21:00 h) and the cryophase coincided with the dark period and an inverted thermocycle of 24.2:28.3±0.1ºC (CT), where the thermophase coincided with the dark period and the cryophase coincided with the previous light period. Water temperature was modified by means of water heaters (100 W, Askoll, Italy) or chillers (Aqua Medic Titan 500 GmbH, Germany), controlled by an electronic timer (Bachmann GmbH & Co, Germany). Temperature was recorded every 10 minutes by means of an underwater data logger (HOBO PENDANT® Onset Computer Corporation, Massachusetts, USA) placed in each aquaria. From 43 to 150 dpf, all fish were separately kept under 28.0± 0.5°C.

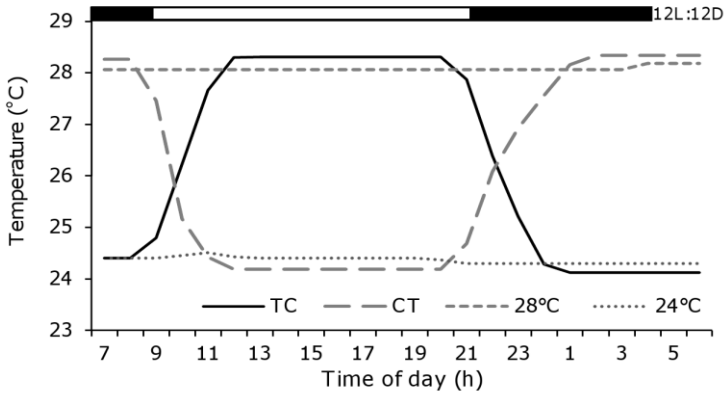

Figure 1. Experimental thermocycles and constant temperatures. 24:28°C thermophase:chryophase (TC) and 28:24°C (CT). Grey horizontal dotted lines represent the constant treatments of 24°C and 28°C. The bar at the top represents the photocycle (12:12 LD).

Data Collection

Growth performance and development

All observations were made in live embryos and larvae and no mortalities were registered during the sampling procedures. Embryo development was assessed every two hours by counting the number of somites from 10 to 30 h post-fertilization (hpf, 60 embryos per temperature group) [28]. Hatching was determined by counting the number of newly hatched larvae from 48 to 100 hpf, also every two hours. Growth was recorded by measuring the total length of 60 larvae per temperature group from 2 dpf and every fifth day until 42 dpf. The number of larvae with physical malformations was recorded throughout the experiment. Embryos and larvae were observed and measured using a digital camera mounted on a dissecting microscope. To ensure the survival of the embryos and larvae, the samples were collected (30 per aquarium) and placed into a new Petri dish filled with the same water of their corresponding temperature group. Samples were quickly photographed and placed back into each aquarium. The digital images were analysed to obtain standard measurements using image processing and analysis software (Leica Microsystems Imaging Solutions Ltd, Cambridge, UK). Survival was determined at 42 dpf, by counting the number of remaining larvae with respect to the initial number of hatched larvae of each temperature group.

Behavioural recording

At 3 and 10 dpf, 60 larvae from each per temperature group were video recorded for 1 h (Sony Handicam, DCR-SR55E) during the middle of the dark phase (MD, 03:00 h) and the middle of the light phase (ML, 15:00 h). For all video recordings, infrared lights (λ=880 nm) were used as the principal illumination source. Videos were analysed to count the number of active/inactive larvae (swimming/motionless) and to calculate the swimming activity of each temperature group during the MD and ML phases and total activity in each temperature group. Swimming activity analysis was performed by means of specialised "Fishtracker" software designed and validated by the Computer Vision Research Group of the University of Murcia [29]. The software tracked and updated the position (X, Y) of each fish every second, creating a data worksheet from which mean activities were calculated and compared (diurnal vs nocturnal activity within each group and among temperatures groups).

Histological analysis

To assess sex differentiation at 42 dpf, 70 post-larvae per temperature group were killed in iced water and fixed in Bouin's fixative for 48 h. Specimens were dehydrated in alcohol, cleared in xylol, and infiltrated and embedded in paraffin. Serial sections (6-7 μm)

were stained with haematoxylin-eosin (H&E) and examined by light microscope. Gonadal cell stages were classified according to previous morphological studies on gametogenesis in female and male zebrafish [30,31].

Gene expression analysis

At 90 dpf, 30 fish were randomly collected from each temperature group, anesthetized and euthanized on ice and gonads were removed for sex ratio assessment. Then gonads of 10 males and 10 females per experimental group were individually homogenized in Trizol reagent (Invitrogen, Madrid, Spain) using a tissue homogenizer (POLYTRON®, PT1200, Kinematica, Switzerland). Total RNA concentration was determined by spectrometry (Nanodrop® ND-1000, Thermo Fisher Scientific Inc., Wilmington, DE, USA), and 1 µg was treated with DNA Wipeout (Qiagen, USA) to prevent genomic DNA contamination. cDNA synthesis was carried out with Quantiscript Reverse Transcriptase and Primer Mix (Qiagen, USA) in a 20 µl reaction volume. Quantitative PCR was performed using the StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with SYBR-green-primer-master mix according to the manufacturer's recommendations. Amplification was carried out with the following cycle conditions: 15 minutes at 95°C, then 40 cycles of 15 s at 95°C and 30 s at 60°C. The final volume of the PCR reaction was 20 µl: 5 µl of cDNA, 10 µl of the qPCR Master Mix and 5 µl of forward and reverse primers.

The primers for *cyp19a* were designed with Primer Express Software (Applied Biosystems, Foster City, CA, USA), (F) 5-TGCTGGCCATCAGACACCAT-3 and (R) 5-CAGATGAACCGACAGTAGGAGACAA-3. The primer sequences for *amh* were retrieved from the literature, (F) or 5- GGGTGTGCATGCTACAGAAGAT- 3 and (R) 5-CTCAGAAATGCAAACAGTCTGTGT-3 [32]. The amplification efficiency, specificity of primers and amount of cDNA/sample were tested by the standard-curve method. The relative expression of all genes was calculated by the $2^{-\Delta\Delta CT}$ method [33], using *D. rerio ef1α* (F): 5-CTGGAGGCCAGCTCAAACAT-3 and (R): 5-ATCAAGAAGAGTAGTACCGCTAGCATTAC-3(ENSDART00000023156) as the endogenous reference [34].

Sex ratio assessment in mature fish

By 150 dpf, the remaining fish (N for 24ºC=60, for 28ºC=78, for TC=69 and for CT=36 fish) were externally identified as males or females following the dimorphic features

described for this species [35]. This sampling was used to confirm the final sex ratio among treatment groups.

Data Analysis

To test for statistical differences among treatments, all data were first tested for normality with the Kolmogorov–Smirnov's test and all were found to be normal. Then, data were analysed by one-way analysis of variance (ANOVA) followed by Tukey's multiple range test to determine significant differences. The Student's *t*-test was performed on data of larval activity to compare the number of active larvae and swimming activity of each group in ML and MD. To find statistical differences among treatments in the proportion of malformations and in sex ratios, data were arcsine transformed before being analysed using one-way ANOVA. Statistical analyses were carried out using the software SPSS 15.0 (SPSS Inc.). *P* values <0.05 were considered statistically significant. All data are expressed as mean±S.E.M.

RESULTS

Embryo development and hatching rhythms.

The effect of temperature was clearly observed during zebrafish embryogenesis as somitogenesis occurred first in larvae under 28ºC, with a regular/constant formation of somites during the dark phase, ending at 22 hpf. In contrast, in the CT and 24ºC groups, somitogenesis took 4 and 6 hours longer to be completed (until 26 and 28 hpf, respectively) while in the TC group it took place at 24 hpf. Somite formation was not regular in the TC, CT or 24ºC larvae since it was slower during the dark phase (with a mean of 2.1±0.3 somites being formed every two hours) than during the light phase (6.2±0.5 somites 2 h^{-1}, $P<0.05$) (Figure 2).

A rhythmic pattern of hatching synchronized to the light phase was observed in all treatments regardless of temperature conditioning. Embryos that did not hatch at the beginning of the light phase at 2 dpf hatched 24 hours later, coinciding with the start of the next light cycle. Temperature marked the start of hatching since by 50 hpf 79.2±4.4% of larvae reared under constant 28ºC were swimming free out of their chorion. At this time, 12.5±4.3% of larvae reared under TC had hatched, in contrast with only 5.6±3.6% and 5.3±0.4% of the CT and 24ºC groups, respectively. By 3 dpf (76 hpf), all larvae from the

28ºC group hatched as well as most of the TC larvae. In contrast, larvae under 24ºC showed the lowest hatching rate (45.3±2.3%, P<0.05). The time of the first hatching peak was the same for all treatments by 48 hpf. However, by 3 and 4 dpf, the hatching peak of larvae under CT occurred 2 h before the rest of the groups (70 and 94 hpf) (Figure 3).

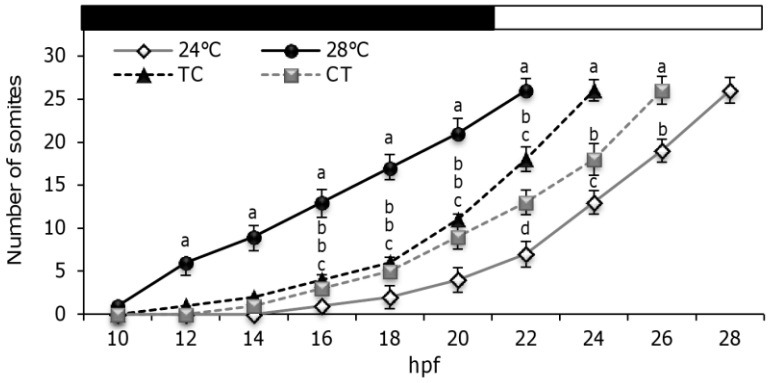

Figure 2. Zebrafish somitogenesis as a function of constant and cycling rearing temperature regimens. Black and white bars represent the night and day periods of the 12:12LD cycle, respectively. Different letters indicate statistical differences among groups (ANOVA, P< 0.05, N=30 per temperature group) (mean±SEM). Abbreviations as in Figure 1.

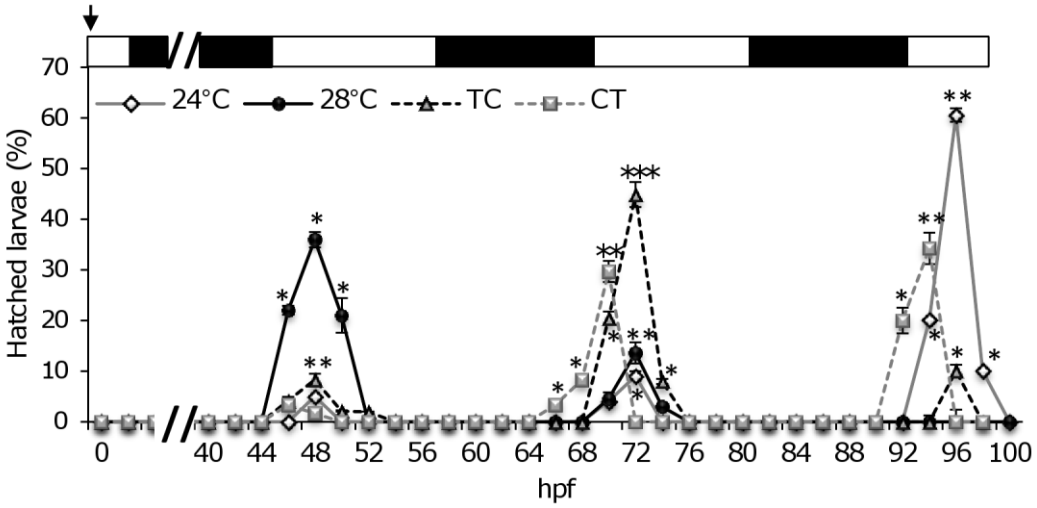

Figure 3. Effect of temperature on zebrafish hatching rhythms. Values are expressed as the percentage of newly hatched embryos observed every two hours, during three consecutive days (2 - 5 days post fertilization, dpf; 44 - 100 hours pf). White and black bars represent the day and night periods (12:12LD) respectively, and the arrow represents the time of fertilization. Data are expressed as mean±SEM. Asterisks indicate statistical differences among groups at the specific sampling time (ANOVA, P<0.05, N=30 per temperature group). Abbreviations as in Figure 1.

Larval performance

Significant differences in growth were found among treatments from the beginning of the experiment. The greatest larval length was found in the 28ºC group, followed by TC, while the lowest size was observed in larvae from the 24ºC regimen (P<0.05). These differences were present throughout the experiment until 42 dpf (Figure 4).

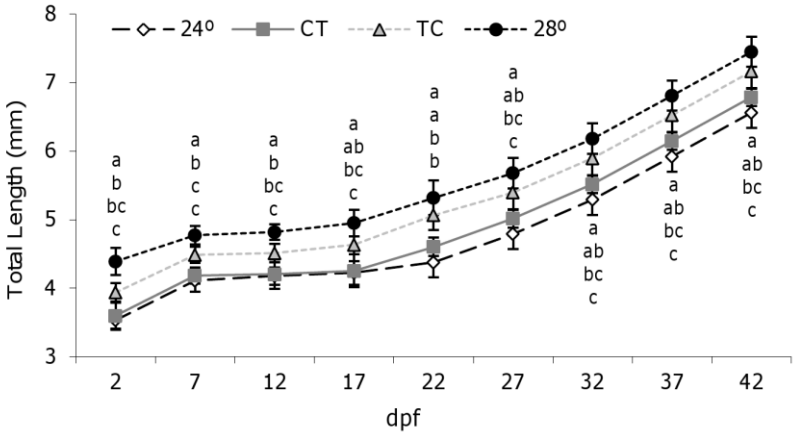

Figure 4. Influence of temperature on zebrafish larval development. Data are expressed as total length (TL, mm) mean±SEM from 2 to 42 days post fertilization (dpf). Different letters indicate statistical differences among groups (ANOVA, P<0.05, N=30 per temperature group). Abbreviations as in Figure 1.

The main physical alterations observed in this study were bent and hook-like tails, spinal column curving, pericardial edema, retarded yolk sac resorption, and shorter body length. Malformations were found in all groups in 16.4±3.1% of the individuals (mean value of the four temperature groups) but they were significantly lower in the TC group, where malformations were only present in 8.5±1.3% of the larvae (P<0.05; Figure 5). By 42 dpf, the highest survival was also found in the TC group, with no statistical differences compared with the 28ºC group (82.8±6.7% and 76.5±6.8%, respectively; P=0.18). The lowest survival was found in larvae reared under the CT regimen (40.9±5.8%) (Figure 5).

Activity rhythms

By 3 dpf, the locomotor activity rhythm was clearly diurnal in the TC larvae (96.5±6.1% of the daily activity occurring during the daytime), in contrast to the nocturnal activity found in the CT group (97.6±5.2%, P<0.05) (Table 1). Swimming activity was highest in the 28ºC group by 3 dpf (Figure 6), with all larvae being active (swimming) during ML, in contrast with the low number of active larvae found in the 24ºC group (1.3±03%,

P<0.05) (Table 1). By 10 dpf, the swimming events of larvae under all treatments were significantly higher when compared to 3dpf (Figure 6), with the CT group displaying the lowest percentage of diurnalism (61.1±4.9%, *P*<0.05), which was also reflected in the number of active larvae counted at ML and MD as no significant differences were found (*P*=0.23) (Table 1).

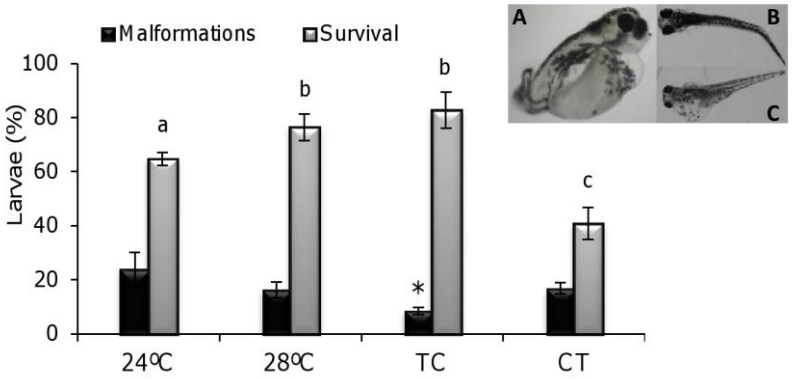

Figure 5. Malformations and survival of zebrafish larvae reared under constant and cycling temperatures. Observed malformations (0 to 42 dpf, N=30 per temperature group) included delayed yolk sac resorption (A), spinal column curving (B) and pericardial edema (**C**). Asterisk (malformations) or different letters (survival by 42 dpf) indicate statistical differences among groups (ANOVA, *P*<0.05). Abbreviations as in Figure 1.

Table 1. Influence of water temperature (constant vs daily thermocycles) on locomotor activity of zebrafish larvae.

	3 dpf				10 dpf			
	24°C	**28°C**	**TC**	**CT**	**24°C**	**28°C**	**TC**	**CT**
1. ML	46.3±2.6^{a}	60.4±2.3^{*b}	96.5±6.1^{*c}	2.4±0.6^{*d}	74.9±3.6^{*a}	88.9±5.3^{*b}	90.2±6.6^{*b}	61.1±4.9^{*c}
MD	53.7±3.4^{a}	39.6±4.1^{b}	3.5±5.3^{c}	97.6±5.2^{d}	25.1±1.6^{a}	11.1±2.1^{b}	9.8±3.1^{b}	38.9±4.3^{c}
2. ML	1.3±0.3^{*a}	100^{*b}	61.3±1.4^{*c}	30.6±4.0^{*d}	100*	100*	100*	100
MD	32.3±3.1^{a}	85.1±3.2^{b}	2.5±0.1^{c}	75.6±6.2^{b}	63.2±4.1^{a}	36.5±2.0^{b}	22.1±2.1^{c}	94.3±6.1^{d}

1. Daily distribution of swimming activity. Percentage of the activity observed in the middle of the light (ML) and dark (MD) phases.
2. Active larvae. Percentage of the number of larvae in motion (swimming) during ML and MD (mean±SEM, n=60) in each group.

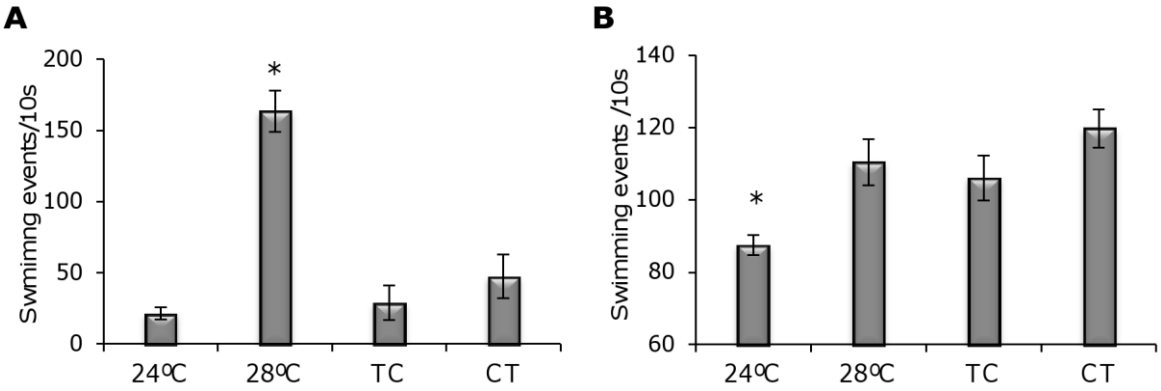

Figure 6. Locomotor behaviour of zebrafish larvae reared under different temperature groups. Swimming activity (number of events recorded every 10 seconds) was observed in 3 and 10 days post fertilization (dpf) larvae. Data is expressed as mean±SEM. Different lower (3 dpf) and uppercase (10 dpf) letters indicate statistical differences among temperature groups (Student's t-test and ANOVA, $P<0.05$, N=30 per temperature group). Abbreviations as in Figure 1.

Sex ratio and sex differentiation

The sex ratios were determined from two biological replicates at 42 dpf by histological analysis, by observation of the gonads upon dissection at 90 dpf, or by examination of the external dimorphic sex phenotype at 150 dpf. The total number of fish used for each group was: 24ºC=160, 28ºC=178, TC=169 and CT=136.

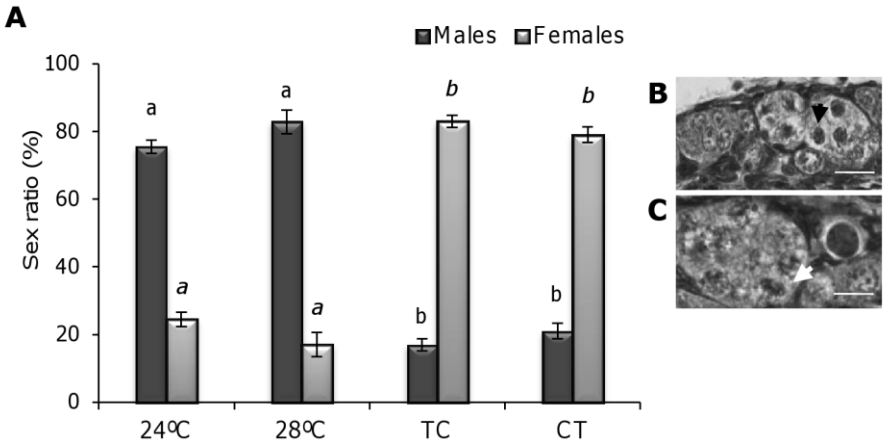

Figure 7. Sex ratio of zebrafish exposed to constant and cycling temperatures for 42 dpf. Values are expressed as mean percentage of males or females obtained at 42, 90 and 150 dpf (**A**) (mean±SEM). Different letters indicate statistical differences in the percentage of males and females (italic letters) respectively, found among groups ($P<0.05$). Photos: developing male gonad (**B**) with spermatogenic cells (black arrowhead) and female gonad (**C**) with clusters of oogonia (white arrowhead). Scale bar: 20 µm. (N of each group: 24ºC=160, 28ºC=178, TC=169 and CT=136). Abbreviations as in Figure 1.

In zebrafish larvae reared under 28°C and TC gonadal development by 42 dpf occurred earlier than in the other groups, as sex could be determined in most of the sampled fish (92%), in contrast with the CT and 24°C groups, where only 80% and 74% of the fish, respectively, could be histologically sexed. Sex ratio results revealed that fish under the thermocycles showed a significantly higher proportion of females (79.0±2.3% in CT and 83.0 ±1.8% in TC), whereas females accounted for just 24.4±2.1% and 17.0±3.5% of larvae exposed to the constant temperatures of 24°C and 28°C respectively (P<0.05) (Figure 7).

Gene expression of *cyp19a* and *amh* was also affected by the temperature regimens, as *cyp19a* was overexpressed in females reared under the thermocycles (TC and CT), in contrast with the low levels found in the constant temperature groups (24°C and 28°C). In addition, *cyp19a* expression was significantly higher in the TC than in the CT groups (6.5 and 4.6 fold higher than in the 28°C group, respectively, P<0.05) (Figure 8A). In contrast, *amh* was overexpressed in males reared under 28°C followed by males kept under 24°C (14.3 fold increase and 3.6 fold higher compared to TC, respectively) (Figure 8B) but lower expression was found in the CT and TC groups (P<0.05).

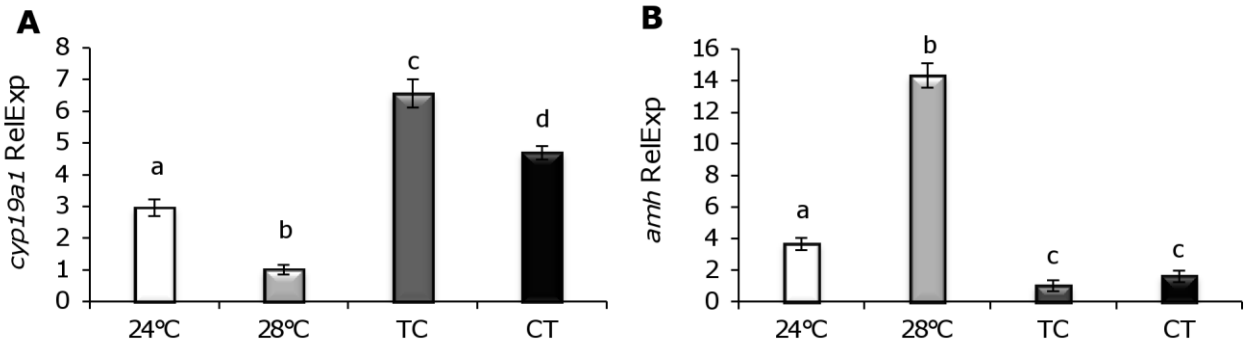

Figure 8. Effect of constant and cycling temperatures on gonadal sex determination genes. Relative expression of *cyp19a* (**A**) and *amh* (**B**) measured in the gonads of 90 days post fertilization zebrafish. Different letters indicate statistical differences among groups (P<0.05). Data are expressed as mean±S.E.M. (n=10 each sex). Abbreviations as in Figure 1.

DISCUSSION

Daily temperature cycles elicit clock-independent but temperature-driven responses and clock-dependent, circadian responses. On the one hand, temperature is a key factor affecting the behaviour and physiology of ectotherms in which body temperature depends on the thermal environment [36]. Our findings provide evidence for such exogenous control in

accordance with a previous study, where 28.5°C was established as the optimal temperature for zebrafish embryo development and hatching rate [28]. On the other hand, endogenous mechanisms also play a role, as the fish circadian system comprises a multioscillatory system entrained by light (LEO), food (FEO) and temperature (TEO) cycles, although the coupling between them remains unknown [10]. In addition, the zebrafish circadian system is light- and temperature-sensitive, but temperature-compensated, so the period of the circadian oscillations remains stable over a wide range of temperatures [7]. Somitogenesis is a rhythmically repeated process determined by a genetic oscillator known as the segmentation clock [37]. This sequential pattern of embryo development has been studied by many authors and its periodicity calculated in several species under a wide range of temperatures, and it has been found that somitogenesis frequency depends linearly on constant temperature [38,39,40]. Although the delay in larvae segmentation due to low temperatures is well known in zebrafish, the effect of daily thermocycles and their interaction with the photocycle had never being investigated. Our data revealed that segmentation is slower in CT than in TC, even though the mean temperature in both thermocycles was similar (26ºC). Thus, the ultimate effect on somitogenesis is not only the temperature itself, but also the relationship between the daily rhythm of cell division and the thermo-/light-phase. Since light regulates the zebrafish cell cycle, so that cells enter the S phase during daytime [41], we hypothesise that the combined effect of high temperatures during the day (TC) enhances cell proliferation, while the reverse condition (lower temperature during daytime, CT) delays normal development.

Daily reproduction rhythms have recently been reported in some teleost fish in which, depending on the daily pattern of behaviour of the species (i.e., diurnal or nocturnal), spawning takes place during the light phase (e.g., zebrafish [42]), during the dark phase (e.g., Senegalese sole, *Solea senegalensis* [43] and European sea bass, *Dicentrarchus labrax* [44]), or in the transition between the two (e.g., gilthead seabream, *Sparus aurata* [45]). It appears that the diurnal/nocturnal behaviour of the species sets not only the phase of spawning rhythm, but also the phase of embryogenesis and hatching rhythms. Our results in zebrafish support this hypothesis as both spawning and hatching occurred exclusively during daytime. Nevertheless, although hatching rhythms were strongly light-entrained, they were also temperature-sensitive, as hatching was advanced to 48 hpf in the 28ºC group and delayed to 96 hpf in the 24ºC group, while hatching occurred at 72 hpf in TC and was split into two peaks taking place at 70 hpf and 94 hpf in the CT group (Figure 2). Interestingly, hatching at

night did not occur, which suggests that zebrafish embryos are light-sensitive. Indeed, light detection has been observed in zebrafish embryonic cells as early as 5 hpf (gastrula stage) and this early ability to detect light has been linked with the rise of clock genes and transcripts implicated in DNA repair [46,47].

Studies on growth and development under cyclic temperatures are scarce, so current knowledge is mostly based on results obtained under constant temperature regimens, where sub-optimal temperature conditions were found to increase the occurrence of fatal deformities and mortalities due to the stress caused by altered developmental timing [48]. In the present study, the total length of larvae in the 28ºC group was always significantly higher than in the rest of the groups, while larvae kept at 24ºC showed the slowest growth (Figure 4). It is interesting to note that the growth of larvae under TC and CT differed during the course of the study despite being exposed to the same mean temperature. As hypothesised for embryos, such differences in larvae development may be linked to the existence of daily rhythms in the endocrine somatotropic axis and the relationship between daily cycles of light and temperature. The expression of growth factors (GH, IGF-I, IGF-II) in fish has a daily rhythm synchronised to light [49]. Furthermore, an early study with the largemouth bass (*Micropterus salmoides*), found that fish grew faster when maintained at high-low cycles of temperature and were fed at the onset of high temperature [50]. These results point to "optimal temperature cycles", rather than optimal constant temperatures, to maximise growth performance and minimise malformations. In the present study, malformations were lowest while survival was highest under the TC regime (although there was no significant difference in survival compared to 28ºC), in accordance with a previous study in which zebrafish maintained under a variable temperature displayed a higher temperature tolerance than fish maintained under a constant temperature [2].

Light environmental conditions during early development have drastic effects on fish larvae performance and behaviour [51]. Recently, a light-dependent switch from diurnal to nocturnal behaviour has been reported in flatfish larvae undergoing metamorphosis [52]. In zebrafish we observed high diurnal activity in 3 dpf larvae of the 28°C and TC groups, while in CT larvae locomotor activity was higher during the night. It has been suggested that LD cycles set the phase of the clock that controls zebrafish larval behaviour, with rhythmic activity synchronized to the light phase as early as 2 dpf [53]. These results suggest that at this stage the daily thermocycle could be the main synchroniser, in agreement with a previous study where most zebrafish under constant light synchronised their activity rhythms to daily

thermocycles (26:20ºC [54]). Moreover, when light and temperature cycles with different periods (25 h and 23 h period for the light and temperature cycles, respectively, i.e., conflicting zeitgebers) were applied, relative coordination between light and temperature could be observed. It appears that when light/dark and high/low temperature cycles are combined (TC), a stable phase and maximum amplitude can be observed in the rhythms, while when the two synchronizers are phase-shifted (CT), the phase of the rhythm is determined by either zeitgeber or by both, depending on the relative strength of each and the sensitivity of the species [55].

In fish, temperature-dependent sex determination (TSD) is not as common as was previously thought. Thus, many instances of sex ratio shifts observed at high temperatures are, most likely, the consequence of thermal effects on an otherwise predominantly system of genotypic sex determination (GSD) rather than proof of the existence of TSD [6]. In some species, the shift in sex ratios under laboratory conditions may occur at extreme temperatures, which are not normally found in the wild [15]. The natural areas where zebrafish live (the Indian subcontinent and adjacent areas) have a monsoon climate with wide variations in temperature, from 16 to 38ºC, and daily oscillations of up to 5.6ºC [10]. In our trial, we used 24°C and 28°C as constant experimental temperatures, the latter one being the standard for rearing zebrafish, but we did not observe differences in sex ratio between them, as at both constant temperatures we obtained a major proportion of males. Slightly male-biased sex ratios in cultured zebrafish appear to be common since they have been observed in many facilities around the world. In contrast, both daily thermocycles resulted in a significantly higher proportion of females, which indicates this species is more sensitive to temperature changes than constant temperatures during early development. This finding is supported by the striking differences found in the expression of *cyp19a* and *amh* (Figure 8). Thermocycles acted as a female-promoter by increasing *cyp19a* expression (leading presumably to more estrogen formation [56]) and suppressing *amh* expression in the gonads, resulting in feminization. Proper female sex differentiation in the zebrafish not only depends on germ cells [57] but also on the presence of a certain number of premeiotic germ cells before meiosis can start. In females this process takes place earlier than in males, thus if light regulates the zebrafish cell cycle [41], it could be argued that TC increases or facilitates germ cell proliferation leading to the observed higher proportion of females. However, this would not explain the higher number of females also observed under CT. In any case, it seems clear that cycling temperatures rather than constant ones promote female development. The effects

of thermocycles on zebrafish sex ratios presented in this study is consistent with recent finding in Senegalese sole larvae exposed to daily thermocycles (19:22ºC, TC), which also produced a larger proportion of females than the constant temperature (20.5ºC [58]). In that report, however, reversed thermocycles (22:19ºC, CT) led to a larger proportion of males, indicating that temperature effects may be species-dependent. Understanding of the generalities and species-specific differences in relation to sex determination/differentiation in fish is still incomplete. However, current research findings point to the need to integrate not just the influence of environmental factors but also of *cycling* environmental factors and their relationship with the development of the biological clock. Actually, in mammals, clock genes have recently been found to regulate steroid production and aromatase expression in ovarian granulosa cells [59].

In summary, our findings revealed the high sensitivity of zebrafish to temperature and light cycles during early development and for the first time in this species it is shown that daily thermocycles have multiple and irreversible effects in developing embryos and larvae that ultimately shape the adult phenotype including the sexual phenotype.

Acknowledgements

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General Discussion

4. General Discussion

Based on the outcome of this PhD thesis, our results not only confirm the importance of the environmental cues on physiological and behavioural processes of the European sea bass, but also suggest new pathways for light and temperature which influence all life stages of the fish. The discovery of daily spawning rhythms in the European sea bass brought out the hypothesis of the existence of circadian rhythmicity in other stages during the life of the fish. Since the incubation, larval and postlarval period are probably (as well as interesting), the most critical breakpoints in marine aquaculture, we decided to focus on these stages in an attempt to improve hatchery practices through the implementation of those environmental conditions that best suit the natural requirements of the species.

Under natural environmental conditions, European sea bass feeding rhythms have been described as nocturnal in winter and as diurnal the rest of the year (Sánchez-Vázquez *et al*., 1998; Azzaydi *et al*., 2007). Such interesting phase inversion was further studied under artificial conditions by manipulating the photoperiod and, the results founded that photoperiod itself could not have caused the behavioural shift from nocturnal to diurnal, suggesting other factors (i.e. temperature cycles) involved in the control of the annual rhythm of behaviour (Aranda *et al*., 1999). Although the biological meaning for sea bass feeding phase inversion remains unclear, the shift of broodstock locomotor activity (diurnal during the resting season and nocturnal during the spawning period) found in our research (Figure 1, Chapter 1) coincides with the feeding annual behaviour early reported for the species, which in turn, match with its spawning period (Barnabé, 1989). Taking all the information together, it seems that the reproductive and behavioural adaptations of European sea bass could be strongly related, being part of a circannual rhythm of metabolic activities synchronized to the natural seasonal oscillations of light and temperature. Furthermore, the importance of considering the natural behaviour of the cultured species has been proved in both juvenile and adults as well as at experimental and industrial levels: feeding sea bass by night during winter, when they show nocturnal behaviour, improves their growth and feeding efficiency (Azzaydi *et al*., 2000; Villamizar *et al*., 2012). From the beginning of European sea bass culture in the 80's, the commercial production procedures have greatly improved thanks to the existing knowledge on the biology of the species that has allow among others, to controlled its reproduction cycle, so spawning can be advanced or delayed by the manipulation of the photoperiod and temperature (Carrillo *et al*., 1989; Mañanós *et al*., 1997).

Another important aspect that may improve aquaculture practices is the time of egg collection. Most Mediterranean hatcheries collect the spawned eggs with a mesh located at the water out-flow of the broodstock tank. Sea bass produces a large amount of eggs in a very short time, which increases the risk of clogging the collectors causing mechanical and thermal stress to the eggs. As spawning takes place during the night, the batches of eggs remain over 8 hours floating in the collector until the hatchery operator starts his/her working shift. Based on our results, spawning occurs at the end of the dark phase in two peaks, the latest at approximately 2 h before lights on after which, egg quality drastically decreases (Figure 2, Chapter 1). The setup of an earlier egg collection schedule may results in acquiring high quality eggs, avoiding the unviable ones as well as many possible deleterious factors.

During embryogenesis, temperature is known to have a major impact as it affects great variety of physiological process. Depending on the species, under artificial conditions the temperature is set in order to avoid a negative effect on hatching rate and incidence of embryonic deformities (Bobe and Labbé, 2010). The existing rearing protocols are based on research mostly performed under constant temperatures and the effects on embryogenesis have been evaluated as a linear succession of developmental events that can be modified (and therefore, predicted) by increasing or decreasing the temperature of incubation (Pelosi *et al*., 1993; Saka *et al*., 2001; Alami-Durante *et al*., 2006). However, the role of temperature during embryogenesis is starting to change, as it is no longer regarded as the primary factor affecting the synchronicity of the early stages of development. Instead, the embryonic period is nowadays considered as a relative rapid but rhythmical process of differentiation and morphogenesis, driven by a biological clock right from the start, during the cleavage stage. Therefore, this endogenic rhythm is responsible for the coordination and integration of multilevel processes in order to compensate the lack of a formed nervous system (Gorodilov, 2010). Indeed, as stated in Chapter 2, our research confirms this affirmation as temperature played a modulating role on the embryogenesis of the three different species (zebrafish, Senegalese sole and cavefish). Further on in Chapter 3, the expected effect of constant low and high temperatures was observed respectively speeding or delaying the somitogenesis process. Daily thermocycles in turn, were seen interacting with the LD cycles, as the segmentation stage was slower in CT than in TC even though the mean temperature in both thermocycles was similar.

Regarding light, it has been observed that fish can be light-responsible from a very early stage as zebrafish embryo's tissues and cells have been found to be directly light sensitive as early as 5 hpf and their exposure to LD cycles causes the synchronization to the

light phase of physiological functions such as cellular proliferation and expression of clock genes (Tamai *et al*., 2004). In Chapter 2 and the subsequent experimental research described in Chapter 3, agrees with this early response to light as both in zebrafish and Senegalese sole a daily rhythm of development was observed, which synchronicity depended of the behaviour of the fish (diurnal in zebrafish and nocturnal in sole). On the other hand, due to the Somalian cavefish fast embryogenesis, the evaluation of light and temperature could not be clearly established. However, the results suggest that embryogenesis could be improved by DD conditions which, as with the other two studied species, agree with the natural behaviour reported for the species (Figures 2 and 3, chapter 2 and Figure 2, chapter 3). Ongoing research is focused on finding when and how the clock is established during the development of vertebrae. Although clock gene transcripts are components of the maternally inherited pool, its functional significance is still unclear. In the vertebrae model, the zebrafish, the earliest detectable circadian activity is the rhythmical expression of clock genes (*per1b* and *per 2* within the first post fertilization day) as well as melatonin synthesis and *aanat2* expression in the pineal gland (after the first LD-DL cycle) (Dekens and Whitmore, 2008; Ziv *et al*., 2005; Vuilleumier *et al*., 2006). Embryos expose to constant conditions of temperature and light have fail to show circadian rhythmicity (Vatine *et al.*, 2011), which coincides with our results on developmental stages rhythmicity under LL and DD.

Further on in development, and following the same pattern observed during the embryogenesis, hatching was observed occurring with circadian rhythmicity. Also in this stage, light acted as the entrainment factor and temperature was seen to modulate the speed of the process. Under constant (high, intermediate and low) and cycling (TC and CT) temperatures, hatching was synchronized to the light phase in zebrafish and to the dark phase in the sole of the LD cycle (Figures 6 and 7, Chapter 2 and Figure 3, Chapter 3). Hatching rhythmicity was maintained under LL and DD although with lower amplitude, which suggests the importance of the endogenous clock of fish during the early stages of development. Regarding hatching rate, this was clearly improved in the zebrafish and the sole by the application of LD cycles (Figure 9, Chapter 2 and Chapter 3) and by the application of the optimal temperatures reported for the species. The blind cave fish hatching rates did not differed among the light regimes but they were improved by the application of the intermediate temperature (28°C). Importantly, zebrafish hatching rate was also high when embryos were raised under TC. Despite the wide variety of studies reporting the negative effects of non-optimal constant temperatures, few studies have applied daily temperature oscillations in order to elucidate their influence on fish early life, even though daily

thermocycles are a key factor in the natural environment. In the aquaculture field, this is an important factor leading to significant production losses, as on the one hand, sub optimal temperature regimes could cause asynchronous hatching, which leads to intracohort cannibalism due to larval size heterogeneity (Puvanendran *et al*., 2008; Baras and Jobling, 2002). On the other hand, temperature affects important metabolic functions in the embryo such as fat yolk deposition, hatching rates and growth, which in turn leads to higher deformities and poor survival (Brown *et al*., 2008; Jordaan *et al*., 2006; Wiegand, 1996). Based on our results, daily thermocycles not only beneficiated zebrafish development by reducing the incidence of malformations, but also influence survival and growth on the same way that the optimal reported temperature for the specie (Figures 4 and 5, Chapter 3). This suggests that thermocycles may be applied in the culture of fish in order to improve some aspects of the fish without affecting other aspects such as growth and survival. Another possible factor in the rearing of fish larvae that could be positively influenced by the application of daily thermocycles is the setting of optimal environmental conditions that correspond with the natural diel behaviour of the fish, as zebrafish has a marked activity rhythm that starts to oscillate very early in larvae (Dunlap *et al*., 2004). In our study, the daily activity patters of 3 dpf zebrafish larvae reared under TC and CT differed significantly (almost completely diurnal in the first, and nocturnal in the second) which suggests that the temperature regimen was the main synchronizer of the daily patterns of activity of zebrafish at this stage (Table 1, Chapter 3). At present, the scarce existing information regarding the influence of daily thermocycles on fish larvae, calls for further research in order to find an optimal oscillation range rather than constant values of temperature for the improvement of fish production.

Over the course of our research, special attention has been paid to larval growth, development and survival and the way they could be influenced by temperature and especially, by light. Regarding the last, the effect of photocycles on the early stages of both zebrafish and European sea bass was clear, as it affected crucial aspects of their development (Chapters 4 and 5). Firstly, it needs to be emphasized the significant deleterious effect that continuous darkness (i.e. “french method” for sea bass rearing (Coves *et al*., 1991)) causes in fish embryo and larvae. An extended DD condition impairs growth and survival of sea bass larvae as a consequence (among others) of poor feeding performance (Chapter 4). Indeed, the results of our studies are in accordance with similar experimental research were the application of those environmental conditions closer to the fish natural environment (LD cycles, mesocosm, natural photoperiod) improved many if not all aspects of the larvae and

postlarvae (growth, digestive system maturation, normal development and survival) (Downing and Livak, 2000; Fielder *et al*., 2002; Zouiten *et al*., 2011;). However, despite these consistent results, the application of close-to natural conditions at industrial level has been poor or none. In contrast, the intensive rearing protocols that includes extreme lighting conditions, not only comprises larval welfare (i.e. chronic stress, malformations, immunosuppression) but also results in clear physical differences between farmed and wild fish which lowers the product quality resulting in economic losses for the industry (Roncarati *et al*., 2001; Leonardi and Klempau, 2003; Valenzuela *et al*., 2006). Using the zebrafish as a vertebrate model, the importance of the LD cycle signals is even more evident. When applying DD conditions to zebrafish larvae, we observed 100% mortality by 18 dph and although larvae was able to compensate their growth when transfer to LD conditions by 5 dph, that compensation was no reached when larvae was reared under DD until 10 dph, resulting in impaired growth and survival (Figure 5 and 6, Chapter 5). These results suggests that the critical point of no return for optimal growth and survival of the zebrafish reared under DD conditions is around 5 dph, even though larvae is still able to tolerate 10 days in DD.

At the opposite side and despite LL conditions have been suggested as optimal for the rearing of some teleost species (Partridge *et al*., 2011; Blanco-Vives *et al*., 2010; Moustakas *et al*., 2004), the benefits of continuous light on growth cannot be taken as a general rule for all fish as some species do not respond to LL conditions in the same way. In the case of sea bass, rearing larvae under LL caused high proportion of malformations such as low jaw elongation and swim bladder anomalies (Villamizar *et al*., 2009) (Table 1 and Figure 6, Chapter 4). A recent study of the sea bass pineal complex, under natural LD conditions significant higher nocturnal levels in a melatonin precursor, the Aanat2 mRNA expression, were found (Herrera *et al*., 2011). In turn, the rhythmic expression of Aanat2 in fish depends on the correct expression of a pineal protein, the Exo-rhodopsin (Exorh) and *per2*, a light-induced gen (Pierce *et al*., 2008; Ziv and Gothilf, 2006). These functional studies provide a functional basis in the understanding of how ambient light cues are perceived and transduced into hormonal signals to regulate physiological and behavioural rhythms in fish. Regarding the zebrafish, larvae reared under LL had surprisingly, one of the lowest growth rates and survival as well as high proportion of malformations (Table 1 and Figures 2 and 3, Chapter 5). Therefore, the application of continuous light should be deeply and specifically investigated before its application to a particular fish species. As research in this field progress, the importance of the LD cycles is by no means casual, as approximately 117 light inducible genes have been identified in a great variety of the zebrafish organs and cells which suggests

the broad range of functional mechanisms (i.e. circadian rhythms, growth, stress response, DNA repair) influenced by light. This ongoing research is essential for the discovery of the complete pathway that couples light photoreception with fish ontogeny and survival (Valenzuela *et al*., 2012; Weger *et al*., 2011; Falcon *et al*., 2010).

Together with the photocycles, the quality of light has proved to be crucial even though it has been much less studied. From the available information regarding the European sea bass, it seems that the species have adapted to brightness variations and dim light conditions as their retina has a well-developed pigmentary epithelium, abundant rods and large cones, which mosaic layout (single and twin cones) contribute to the perception of movements, hence the capture of fast prey (Mani-Ponset *et al*. 1993). In an earlier experiment where the levels of circulating and ocular melatonin were measured in adult sea bass exposed to different light spectra, the blue wavelength (454 nm) was the most effective for melatonin suppression (Bayarri *et al*., 2002). Indeed the behaviour of adult sea bass seems to match the predator type, being particularly sensitive to short wavelengths hence indicating a tendency to scotopic vision which in turn is produced by rod cells, sensitive to blue-green wavelengths (Marchesan et al. 2005). Our results in sea bass larvae (Chapter 4), confirm these proposals as larvae grew better, developed faster (fins and swim bladder) and fed more actively under the blue wavelength (LD cycle) than in the rest of the groups (Villamizar *et al*. 2009). There is, therefore, the possibility that larvae's visual systems could be predisposed to perform best under the spectral conditions most frequently encountered in its particular ecological niche (blue wavelengths and daily photocycles). An early study performed by Mani-Ponset and colleagues (1993), the retinal organogenesis during sea bass early stages was observed and the results indicated that light detection may appear with photoreceptor differentiation by 3 days after hatching and from day 5, larvae sight improves as the retinal structure starts to resemble the adult retina in a simpler way. This stage coincides with the start of the exogenous feeding when larvae is only able to detect their prey at 1 mm distance, but rapidly develops and increases the distance to 3 mm by day 13 and to 5-6 mm by day 20. In this period, light plays a crucial role, since the retina of larvae is formed only by cones, limiting their vision to day sight. However, the results found in our research suggests that sea bass may become light sensitive from an earlier stage as growth differences among light spectra (blue, red and white) were observed right from 3 dph, before exogenous feeding had started (Chapter 4). Indeed, when we reared zebrafish embryos and larvae under different light spectrum (Chapter 5), the experimental lights affected the fish right from the start, as a significant high percentage of strong malformed embryos were found under the red and green

wavelengths. These malformed embryos had a strong difficulty to break the chorion to hatch, which in turn impaired hatching timing and those who succeeded, died at an early stage of the larval period. The early effect of light on zebrafish embryos is by no means surprising if we take into account that they can detect light as early as 5 hpf as demonstrated by Tamai and colleagues (2004).

In sea bass larvae as well as in zebrafish larvae all the evaluated parameters included in our studies were beneficiated by the blue light as larvae not only grew better, developed faster and had better survival rates, but also presented a better feeding activity. This coincides with the spectral profile described by earlier researches for both species which λmax values correspond to short wavelengths of light (Raymond *et al*., 1993; Robinson *et al*., 1993; Mani-Ponset *et al*. 1993). However, care should be taken with this spectral group since light of short wavelengths have been also found to be harmful as they may over-stimulate cones photoreceptors (Wagner and Kröger, 2005). This may explain the overexpression of stress- and growth-related genes (*pomca, crh, igf1a* and *igf2a*) observed in larvae reared under violet light. However, the results on gene expression were quite complex and could not be explained by a simple extrapolation of the morphological results as different pathways controlling zebrafish growth at early stages of development may be involved.

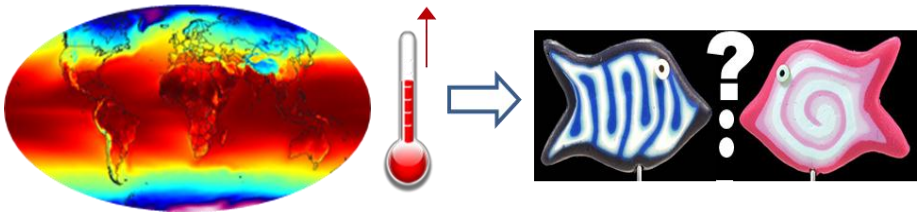
To further study the benefits of blue light on European sea bass, we wanted to investigate the feeding behaviour and swimming activity of fish larvae as well as the distribution patterns and hatching rate of *Artemia sp*., the most common live prey used in sea bass larvae rearing (Chapter 6). As result, larvae feeding activity as well as the distribution of both fish larvae and artemia nauplii were improved by blue light in contrast with the observations obtained under red, white or DD conditions (Villamizar *et al*. 2011). Morphologically, European sea bass seems to have a well-developed pigmentary epithelium, abundant rods and large cones, which mosaic layout (single and twin cones) contribute to the perception of movements, hence the capture of fast prey (Mani-Ponset *et al*. 1993). Perhaps as a result of the enhanced contrast, sea bass larvae under blue light could detect and capture more prey than larvae from the red light treatment, suggesting that optimal illumination is crucial during this stage for larvae to find and successfully capture prey. Also, the behaviour of artemia nauplii may have improved the feeding success of the larvae under blue light, as they were evenly distributed through the tank. Although our findings in sea bass and zebrafish larvae suggests the benefits when rearing them under blue light, further studies are needed in order to establish the optimal blue range (and light intensity) for other stages of fish ontogeny such as embryogenesis as well as for the juvenile and adult periods. As fish migrate

during their lifetime, they are exposed to diverse visual environments to which they adapt by functional and morphological adjustments. Another important aspect is to study the UV vision capacity as it has been suggested to be used by fish in order to detect their planktonic prey (Britt *et al*., 2001; Siebeck and Marshall, 2001).

Using the zebrafish as model and based on earlier studies were the sex determination of this species was found to be influenced by the temperature, we wanted to investigate if daily oscillations of temperature had an effect on the sex ratio of the zebrafish. By general rule, this species is reared under constant temperature of approximately 28°C as it has been reported to be the optimal temperature for this specie (Kimmel *et al*., 1995). Under this conditions, slightly male-biased sex ratios appear to be common for the specie since this have been observed in many facilities around the world. However, in our study both TC and CT daily thermocycles resulted in a significantly higher proportion of females, which indicates that the species is highly sensitive to daily temperature oscillations during early development (Figure 7, Chapter 3). These results were further supported by the levels of sex-determine genes as daily thermocycles increased the expression of *cyp19a* which in turn raised the estrogen formation and suppressed of the expression of *amh* in gonads, resulting in feminization (Figure 8, Chapter 3). These results point to the importance of cyclic temperature as well as suggested for the light regimens. The application of thermocycles as the solution for male-biased populations of cultured fish has been long ignored, even though it mimics the natural environment where a sex ratio balance is key for the survival of a population. In European sea bass the high proportion of males was thought to be solved by estrogen treatments, but as the species is cultured for human consumption, this raised many concerns (Blázquez *et al*., 1998), instead, temperature regimens have been extensively studied as sea bass sex determination comprehends a polyfactorial mechanism that depends on both genetic and environmental (temperature) effects (Vandeputte *et al*. 2007). One of the latest researches in which temperature has been used in order to obtain a better sex ratio without impairing growth was also based on constant temperatures: 17°C from spawning until approximately 53 days post fertilization for the majority of population being female and acceptable growth (Navarro-Martín, 2009). However, the application of constant conditions of temperature leads to a loss in phenotypic plasticity, reducing the thermal tolerance of the fish and therefore its capacity to overcome possible fluctuations of temperature (Schaefer and Ryan, 2006). Future research should focus on finding the optimal thermocycle (daily as well as annual) for a particular species which will elicit the best response from the fish. For this, the starting point should have a good knowledge of the species biology, natural habitat and

history. Current knowledge on European sea bass natural habitat and biology is fairly good although there are many fields to be covered such as behavioural studies (especially at larval stage) and the ontogeny of diverse structures. Regarding the zebrafish, a species used in over 400 laboratories around the world (www.zfin.org), it is shocking that so little is known about its ecology and behaviour. For example, basic questions on its sexual behaviour and mating are just being addressed (Owen *et al.*, 2012) and the information on its geographical distribution is questionable.

To conclude, the results presented in this research strongly suggest the importance of the cyclic photo- and thermo-environments as crucial factors influencing all stages of fish. **For the aquaculture field**, the innovation of existing rearing practices towards more natural environmental protocols, will not only beneficiate fish performance trough optimal growth rates, development and survival but also will improve its welfare and phenotypic diversity. **For the ecological field**, the effects of lifelong exposure to greater or more variable thermal regimes on fish could open a pathway in the understanding of global warming consequences.



Conclusions

5. Conclusions

5.1. Light plays a key role in synchronising European sea bass spawning rhythms, which presents a spawning peak during the dark phase, egg viability being strongly correlated with spawning size. Most interestingly, such spawning rhythm coincided with the seasonal (winter) switch from diurnal to nocturnal behaviour of the broodstock.

5.2. Zebrafish and Senegalese sole embryogenesis and hatching show a daily rhythm synchronised to the light/dark cycle, thought the phase of these rhythms depended on the species: diurnal in the zebrafish, while nocturnal in sole. The pace of embryo development and hatching of the blind Somalian cavefish is very fast and light-independent, embryogenesis occurring faster under constant darkness regardless the temperature conditions, which in the other two species produce an advance/delay of the light-synchronised rhythms.

5.4. Extreme lighting conditions such as constant light or darkness and inappropriate light spectrum, compromise not only the normal development of fish larvae, but also the natural behaviour of its planktonic prey (*Artemia sp.*). Light and dark cycles of blue wavelengths appear to bring about the best performance for European sea bass and zebrafish larvae, producing higher growth, faster development, increased feeding behaviour, best survival and lower malformations.

5.5. Daily thermocycles, together with light cycles, act as the main synchronizers of the daily behavioural patterns in zebrafish larvae. Activity rhythms occur with maximum amplitude and stable phase when the two synchronizers are in phase (TC/LD).

5.6. Our findings reveal the crucial influence of daily thermocycles on developing fish embryos and larvae, which ultimately shape the adult sexual phenotype. Thermocycles act as female promoter elements by directly or indirectly increasing the expression levels of ovarian aromatase, which in turn, downregulates the expression of the spermatogoinian proliferation factor, the anti-müllerian hormone.

General Bibliography

6. General Bibliography

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Web citations

Zebrafish Information Network (ZFIN), The Zebrafish International Resource Center, University of Oregon: http://zfin.org/

Anexes

7. Annexes

7.1. Annex I: Scientific production resulting from the present PhD thesis

- Scientific Publications:

Villamizar, N., García-Alcázar, A., Sánchez-Vázquez, F.J. 2009. Effect of light spectrum and photoperiod on the growth, development and survival of European sea bass (*Dicentrarchus labrax)* larvae. Aquaculture 292, 80–86.

Villamizar, N., Blanco-Vives, B., Migaud H., Davie, A., Carboni S., Sánchez-Vázquez, F.J. 2011. Effects of light during early larval development of some aquacultured teleosts: A review. Aquaculture 315, 86–94.

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Villamizar, N., Blanco-Vives, B., Oliveira, C., Dinis, M.T., Bertolucci, C., di Rosa, V. Sánchez-Vázquez, F. J. Circadian rhythms of embryonic development and hatching in fish: A comparative study on Zebrafish (diurnal), Senegalese sole (nocturnal) and Somalian cavefish (blind). In preparation.

Villamizar, N., Vera, L.M., Sánchez-Vázquez, F.J., Early development of zebrafish: Effect of light spectrum and light:dark cycles. In preparation.

- Congress Contributions:

Villamizar, N., Davie, A., Migaud H., Treasurer J. and Sánchez-Vázquez, F.J., 2009. Effect of light spectrum and photoperiod on the growth, development and survival of European sea bass (*Dicentrarchus labrax*) and Atlantic cod (*Gadus morhua*) larvae. Oral communication in the "Larvi 2009. 5th Fish and shellfish larviculture symposium" Ghent University, Belgium.

Villamizar, N., Sánchez-Vázquez, F.J. 2009. Ritmos de puesta y ontogenia de la lubina sometida a diferentes condiciones de iluminación (*Dicentrarchus labrax*). Oral communication in the "I Workshop Hispano-Brasileño de Acuicultura" Murcia, Spain.
Blanco-Vives, B., **Villamizar, N.,** Sánchez-Vázquez, F.J. 2009. Efecto del espectro de luz y fotoperiodo en el crecimiento desarrollo y supervivencia en el lenguado *Solea senegalensis.* Oral Communication in the "XII Congreso Nacional de Acuicultura" Madrid, Spain.

Villamizar, N., Sánchez-Vázquez, F.J. 2010. Effect of lighting conditions (photoperiod and spectrum) on the development, performance and behaviour of fish larvae. Oral Communication in the "I Meeting of the fish chronobiology network" Murcia, Spain.

Villamizar, N., García-Mateos, G., Blanco-Vives, B., Sanchez-Vazquez, F.J. 2010. Behavioural (activity and feeding) responses of sea bass (*Dicentrarchus labrax* L., 1758) larvae and prey reared under different ligthing conditions. Poster in "Aquaculture Europe, EAS". Porto, Portugal.

Villamizar, N., García-Mateos, G., Sánchez Vázquez, F.J. 2011. Impact of light on behavioural responses of European seabass (*Dicentrarchus labrax*) larvae and *Artemia* sp. Oral communication in the "Larvanet Meeting, Aquaculture Europe" Rhodes, Greece.

Villamizar, N., Blanco-Vives, B., Aliaga-Guerrero, M., Cañavate, J.P., Muñoz-Cueto, J.A., Sánchez Vázquez, F.J. 2011. Light effects on locomotor activity rhythms and behavioural patterns of Senegal sole (*Solea senegalensis*) larvae. Poster in the "Larvanet Meeting, Aquaculture Europe" Rhodes, Greece.

Villamizar, N., Blanco-Vives, B., Vera, L.M., Mañanós, E., Ramos, J., Bayarri, M.J., Sánchez Vázquez, F.J. 2011. Effect of daily thermocycles on sex ratio, sexual steroids and development in Senegal sole (*Solea senegalensis*) and zebrafish (*Danio rerio*). Poster in the "Larvanet Meeting, Aquaculture Europe" Rhodes, Greece.

Villamizar, N., García-Mateos, G., Sánchez-Vázquez, F.J. 2011. Efecto de los termociclos diarios durante el desarrollo temprano del pez cebra (*Danio rerio*): Análisis del crecimiento y comportamiento. Poster in the "XIII Congreso Nacional de Acuicultura" Barcelona, Spain.

7.2. Annex II: Scientific production resulting from collaborations with colleagues

Blanco-Vives, B., **Villamizar, N**., Ramos, J., Bayarri, M.J., Chereguini, O., Sánchez-Vázquez, F.J. 2010. Effect of daily thermo- and photo-cycles of different light spectrum on the development of Senegal sole (*Solea senegalensis*) larvae. Aquaculture 306, 137–145.

- Congress Contributions:

Oliveira C., Blanco-Vives, B., **Villamizar**, **N.,** Sánchez-Vázquez, F.J., 2009. Spawning rhythms in fish: is the time of day important? Poster in the "XI Congress of the European Biological Rhythms Society (EBRS)" Strasbourg, France.

Blanco-Vives, B., **Villamizar, N.**, Sánchez-Vázquez, F.J., 2009. Influencia del espectro de luz y el fotoperiodo en los ritmos de actividad de juveniles de lubina (*Dicentrarchus labrax*). Poster in the "XII Congreso Nacional de Acuicultura" Madrid, Spain.

Oliveira, C., **Villamizar, N.,** Blanco Vives, B., Santos, C., Mañanos, E., Cabrita, E., Soares, F., Dinis, M.T., Sanchez Vazquez, F.J. 2011. Chronobiology applied to spawning and gamete withdrawal: Importance of daily rhythms. Poster in the "3rd International Workshop on the Biology of Fish Gametes" Budapest, Hungary.

7.3. Annex III: Projects supporting the present PhD thesis

- Project Title: Aquagenomics: mejora de la producción en acuicultura mediante herramientas de biotecnología

Financer Organization: MEC (Programa Consolider-Ingenio 2010, proy. Nº 28502)
Realization period: 2007 – 2012.
Coordinator: Dr. A. Figueras

- Project Title: Ontogenia del reloj biológico de la lubina: papel sincronizador de la luz durante el desarrollo temprano e impacto sobre los ritmos de alimentación diurnos/nocturnos.
  Financer Organization: Fundación SENECA (08743/PI/08)
  Realization period: 2009 – 2010.
  Coordinator: Dr. F.J. Sánchez Vázquez.

- Project Title: Fish Chronobiology Network: Ontogeny of the biological clock and reproduction rhythms.
  Financer Organization: MEC (Acción Integrada DE 2009-0045).
  Realization period: 2010 – 2012.
  Investigador principal: Dr. J. Sánchez Vázquez.

- Project Title: Ritmos de reproducción en el lenguado senegalés: regulación neuroendocrina y papel de los termo-/foto-ciclos durante del desarrollo temprano en su establecimiento y maduración
  Financer Organization: MINECO (AGL2010-22139-C03-01)
  Realization period: 2011-2013.
  Coordinator: Dr. F.J. Sánchez Vázquez

Resumen en Castellano

8. Resumen en castellano

El objetivo general de esta tesis doctoral fue estudiar la influencia de la luz y la temperatura sobre el desarrollo temprano de los peces. En particular, hemos investigado la respuesta biológica a las señales ambientales a través de la evaluación el sistema circadiano del pez así como también de importantes parámetros morfológicos y comportamentales.

Con este propósito, se plantearon los siguientes objetivos específicos:

2.1. Determinar la existencia de ritmos diarios de puesta y su relación con los patrones de comportamiento (diurnos/nocturnos) de reproductores de lubina Europea.

2.2. Describir la existencia del reloj biológico en embriones de peces y su sincronización a diferentes condiciones de luz y temperatura en especies con diferentes patrones comportamentales: diurno (pez cebra), nocturno (lenguado) y ciego (pez troglobio).

2.3. Investigar el efecto de diferentes condiciones de luz (constante vs fotociclos) durante la incubación y los periodos larvarios y post-larvarios de la lubina Europea.

2.4. Evaluar la influencia del espectro de luz durante el desarrollo temprano de la lubina y del pez cebra a través de la observación de parámetros morfológicos y comportamentales.

2.5. Estudiar los efectos de la luz sobre la tase de eclosión y distribución espacial de el alimento vivo de larvas de pez (*Artemia salina*).

2.6. Investigar el efecto de la temperatura (constante vs termociclos diarios) sobre el desarrollo embrionario, ritmos de eclosión eclosión, desarrollo larvario y diferenciación sexual del pez cebra.

<u>Capitulo Experimental 1</u>. Ritmos diarios de puesta y actividad locomotora de reproductores de lubina Europea (Dicentrarchus labrax)

El objetivo de este capítulo fue estudiar la existencia de un ritmo diario de puesta en reproductores de lubina mantenidos en cautividad, así como también su ritmo diario de actividad locomotora durante el periodo natural de puesta y reposo. Para ello, en las instalaciones de Aquicultura Balear S.A.U (Culmarex S.A.), se utilizaron tres grupos de 74 reproductores (1 macho: 1 hembra) mantenidos en tanques de 20 m^3 bajo condiciones naturales de luz y temperatura. Para el registro y posterior análisis de comportamiento, una fotocélula infrarroja fue colocada dentro de cada tanque a 22 cm de la superficie del agua. Cada interrupción del haz de luz de la fotocélula por parte de los peces, enviaba una señal a un ordenador, el cual almacenaba los registros de actividad. Estas observaciones se realizaron durante la temporada de puesta (Enero a Marzo) y reposo (Abril a Junio). Para establecer el ritmo diario de puesta de la lubina se colocó un colector automático de huevos diseñado en la UMU en la salida de agua del tanque de los reproductores. En cada día de puesta se contaron los huevos de cada grupo temporal. Se realizaron tres muestreos, suficientes para establecer el patrón diario de puesta. Como resultado se obtuvo que la lubina presenta un ritmo diario de actividad marcadamente nocturno durante el periodo de puesta (68±4.3% de la actividad diaria se registró durante la noche) y diurno durante el periodo de reposo (65.5±7.5%). Teniendo éste resultado como base, se procedió a adelantar el horario de alimentación para que se acercara más a la fase de oscuridad y se observó un aumento apreciable (27% de la biomasa diaria en gr.) en el consumo de pienso. El momento de la puesta coincide con un aumento de actividad locomotora, es decir se presenta durante la fase de oscuridad en dos picos principales de puesta de puesta (a las 6 y 11 h después del apagado de las luces). La viabilidad de los huevos fue mayor en los grupos de puestas correspondientes a los picos de liberación de huevos. Los anteriores resultados pueden ser utilizados por las empresas de cultivo de lubina, para el mejoramiento de las prácticas de mantenimiento de reproductores y recogida de huevos.

<u>Capítulo Experimental 2.</u> Ritmos circadianos de desarrollo embrionario y eclosión de peces: Un estudio comparativo entre el pez cebra (diurno), el lenguado Senegalés (nocturno) y el pez troglobio Somalí (ciego).

El objetivo del presente estudio fue investigar la existencia de ritmos circadianos de desarrollo y eclosión en embriones de peces mantenidos bajo diferentes temperaturas y fotociclos. Para ello, huevos recién fertilizados de pez cebra (*Danio rerio*), lenguado (*Solea senegalensis*) y pez troglobio (*Phreatichthys andruzzii*) fueron expuestos a tres diferentes tratamientos de temperatura constante (24ºC, 28ºC o 32ºC para el pez cebra y pez troglobio; 18ºC, 21ºC o 24ºC para el lenguado) e iluminación (LD: 12 h de luz: 12 h de oscuridad; LL: iluminación constante blanca, y DD: oscuridad constante). Como resultado, se observó un ritmo diario de desarrollo embrionario sincronizado al ciclo LD y estimulado por la luz en el caso del pez cebra y por la oscuridad en el caso del lenguado. Los ritmos de desarrollo diurnos y nocturnos de ambas especies coincidieron con sus respectivos ritmos de eclosión. Ambos ritmos persistieron bajo LL y DD. En cuanto al pez troglobio, la luz no influyó en sus ritmos de desarrollo y eclosión pero el tratamiento DD parece haber sido positivo para ambos procesos. Para las tres especies, la temperatura jugó un papel modulador, acelerando (alta temperatura) o retrasando (baja temperatura) el desarrollo y eclosión. En cuanto a la tasa de eclosión, ésta fue mayor bajo LD en el pez cebra y el lenguado y sin diferencias significativas en el pez troglobio. Las temperaturas cercanas a las óptimas para cada especie beneficiaron la

tasa de eclosión: 28°C para el pez cebra y el troglobio Somalí (95.2±2.7% y 83.3±0.1% larvas eclosionadas respectivamente) y 21°C para el lenguado (93.1±2.9% larvas eclosionadas).

<u>Capítulo Experimental 3.</u> Efecto del espectro de luz y fotoperiodo sobre el crecimiento, desarrollo y supervivencia de larvas de lubina Europea (*Dicentrarchus labrax*).

El propósito de éste estudio fue investigar los efectos de diferentes espectros de luz y fotoperiodo sobre el desempeño de larvas de lubina Europea. Para ello, huevos fertilizados naturalmente fueron expuestos a luz blanca, roja o azul en fotociclo LD, LL (blanca) o DD hasta el día 40 post-eclosión (dpe). El efecto de las condiciones de iluminación se evaluó a partir de la observación de diferentes parámetros morfológicos (crecimiento, desarrollo de estructuras, posibles malformaciones) y comportamentales (actividad alimentaria), además de observar la tasa de supervivencia. Los resultados obtenidos mostraron un mejor crecimiento final (longitud total) bajo condiciones de luz azul (15.4±0.6 mm) y LL (15.2±0.6 mm), en comparación con larvas expuestas a luz roja (11.7±0.7 mm). Sin embargo, el peso húmedo fue más alto (21.6±2.02 mgr) y el desarrollo de aletas, dientes y vejiga natatoria se presentó con anterioridad en el grupo de luz azul. DD afectó el inicio de la alimentación exógena ya que la actividad alimentaria de las larvas fue casi nula desencadenando el 100% de mortalidad para el 18 dpe. La menor supervivencia para el 40 dpe se observó en el grupo de luz blanca (11.6±0.59%) y la mayor incidencia de malformaciones (anomalías de la vejiga natatoria y elongación de la mandíbula inferior) se presentó bajo LL (15.0±1.4% and 38.0±0.2% respectivamente). Los resultados obtenidos sugieren el importante efecto de las condiciones de luz sobre el desarrollo temprano de la lubina. Aquellas condiciones que más asemejaron el ambiente natural de la especie (fotociclos diarios y espectro de luz azul) provocaron una mejor respuesta en todos los parámetros evaluados. Para el campo de la acuicultura, aquellos protocolos que impliquen condiciones drásticas (antinaturales) de iluminación, deben ser tomados con extrema precaución debido al potencial detrimento de importantes procesos de desarrollo que se llevan a cabo durante el periodo larvario (y muy posiblemente, embrionario). Mejoras en los sistemas de iluminación incluyen longitudes de onda, intensidad y fotociclos óptimos y particulares para cada especie, que se verán reflejados en un mejor desempeño del pez y por tanto, en mayores beneficios para la empresa.

<u>Capítulo Experimental 4.</u> Desarrollo temprano del pez cebra: Efecto del espectro de luz y ciclos de luz:oscuridad.

En éste capítulo utilizamos al pez cebra como modelo de investigación para intentar establecer el momento en el cual el pez empieza a ser susceptible a las condiciones de luz. Para ello, aplicamos seis longitudes de onda (blanca, violeta, azul, verde, amarilla o roja) en fotociclo LD a huevos recién fertilizados y los mantuvimos hasta el día 32 post-fecundación (dpf). La tasa de eclosión fue mayor bajo la luz violeta (90.1 ± 1.4%) y menor bajo luz amarilla (81.7 ± 0.6%). El crecimiento al 32 dpf fue mayor en los grupos de luz azul y violeta (7.2 ± 0.1 mm y 6.9 ± 0.1 mm, respectivamente) al igual que la actividad alimentaria (88.2 ± 3.1% y 78.3 ± 4.2% del tubo digestivo se observó con presencia de alimento respectivamente). A pesar de los beneficios descritos, el grupo de luz violeta también presentó alta incidencia de malformaciones (5.2 ± 0.4 %) y sobre-expresión de genes relacionados con le estrés (*pomca y crh*). Además se encontraron altos niveles de expresión de genes relacionados con el crecimiento (*igf1a, igf2a*). Bajo la luz roja, los peces presentaron una baja actividad alimetaria (25.6 ± 2.5 %) y el 100% de mortalidad se observó para el 20 dpf. Al finalizar el tratamiento de espectros de luz (32 dpf), los peces fueron

expuestos a luz blanca LD hasta el 80dpf cuando fue registrado su peso húmedo. Los resultados obtenidos sugieren la importancia del espectro de luz durante las primeras etapas de vida del pez cebra, ya que al día 80 no se presentó ninguna compensación de crecimiento. Teniendo como base la mortalidad presentada con anterioridad de larvas de peces expuestas a DD (observaciones previas), decidimos estudiar el punto de no retorno después del cual las larvas de pez no pueden sobrevivir si continúan bajo DD. Para ello, dos grupos de huevos recién fertilizados fueron expuestos a DD por 5 y 10 días después de los cuales se trasladaron a LD. Teniendo un grupo LD como control, se obtuvo que las larvas del día 10 no lograron alcanzar el crecimiento del grupo control incluso después de 30 días de estar bajo LD. Por el contrario, las larvas trasferidas a LD al día 5 no presentaron diferencias significativas en crecimiento con el grupo control. Lo que sugiere que el punto de no retorno para el pez cebra expuesto a DD está alrededor de los 5 dpf. En conclusión, el pez cebra es afectado por el espectro de luz desde su ontogenia. Longitudes de onda blanca y azul y ciclos LD promueven un mejor desempeño y supervivencia.

Capítulo Experimental 5. Respuestas comportamentales de larvas de lubina Europea (*Dicentrarchus labrax*) y *Artemia sp*. expuestas a luz blanca, roja o azul e iluminación constante vs ciclos de luz y oscuridad.

Este capítulo tuvo como objetivo el investigar el comportamiento (locomotor y alimentario) y distribución espacial de larvas de lubina Europea expuestas a espectros de luz blanca, azul o roja en ciclo LD, así como también LL (blanca) y DD. La eclosión y distribución (agregación) de los nauplios de *Artemia sp*. utilizados como alimento vivo también fueron investigados. Para ello, huevos fertilizados de lubina fueron mantenidos bajo los diferentes tratamientos desde 1 a 30 dph para observar su distribución vertical y horizontal dentro del tanque, además de Patrones Modales de Comportamiento (tiempo de desplazamiento, orientación hacia la presa, frecuencia de éxito en la captura, fallo de captura y abandono de ataque). Como resultados obtuvimos que las larvas de lubina presentan un comportamiento fototáctico positivo el cual bajo luz azul, ocasiona una mejor distribución de las larvas dentro del tanque, mientras que en los tratamientos de luz blanca y DD el 52% de las larvas se concentraban en las paredes de los tanques. La actividad locomotora y el inicio de la alimentación exógena fueron mejores (más desplazamientos y mayor frecuencia de captura) en los grupos de luz azul y blanca. Por el contrario, bajo luz roja y DD, los desplazamientos y capturas de alimento fueron significativamente menores. Bajo luz azul, el porcentaje de eclosión de la artemia fue mayor (56.5±2.9%) que los encontrados en los tratamientos de los roja (26.3±1.4%) y DD (27.9±3.9%). Además, la luz blanca y LL ocasionaron una mayor tendencia de agregación por parte de la artemia, la cual formaba grupos de tamaño importante junto a las paredes del tanque. Como conclusión, tenemos que la respuesta comportamental de la lubina así como también de su alimento vivo, la artemia está fuertemente afectada por la condiciones de iluminación, por lo que cabría considerar la aplicación de mejores sistemas de iluminación que permita una distribución homogénea de ambas y así mejorar las condiciones de cultivo de la lubina.

Capítulo Experimental 6. Efecto de termociclos diarios sobre el desarrollo, supervivencia, comportamiento y diferenciación sexual de embriones y larvas de pez cebra (*Danio rerio*).

El propósito de éste último capítulo fue el investigar el efecto de la temperatura (constante y termociclos diarios) sobre el desarrollo embrionario, eclosión, crecimiento, comportamiento y diferenciación sexual del pez cebra. Para ello, huevos recién fertilizados se

distribuyeron en los siguientes tratamientos: temperatura constante alta (28°C), temperatura constante baja (24°C), termociclo diario natural o TC (28:24°C, donde la temperatura alta coincide con el día y la baja con la noche) y termociclo invertido o CT (24: 28°C, inverso a TC). Ciclos diarios de LD blanca se aplicaron como protocolo iluminación. Los resultados obtenidos mostraron que el desarrollo del embrión dependió de la temperatura ya que fue acelerado bajo 28°C. Los ritmos diarios de eclosión fueron influenciados tanto por la temperatura como por los ciclos de luz/oscuridad ya que la eclosión ocurrió durante la fase de luz y con antelación en el grupo 28°C (48 hpf) mientras que en el grupo 24°C ésta ocurrió mucho más tarde (96 hpf). La eclosión en el grupo TC sucedió a las 72 hpf y en CT hubo dos picos de eclosión, a las 70 hpf y a las 94 hpf. Un mayor crecimiento y supervivencia y un menor índice de malformaciones se presentaron en los grupos 28°C y TC. En cuanto al comportamiento, para el día 3 pf (post-eclosión) éste fue diurno bajo 28°C y TC y claramente nocturno en CT. En el 10 dpf todos los grupos mostraron una actividad diurna. La proporción de sexos fue significativamente influenciada por la temperatura ya que un mayor porcentaje de hembras con respecto a machos fueron encontrados en CT y TC (79 and 83% respectivamente), por el contrario, las temperaturas constantes 28°C y 24°C ocasionaron un mayor número de machos (83 and 76%). Para tratar de comprender los anteriores resultados desde un punto de vista funcional, se evaluaron los niveles de expresión del gen aromatasa ovárico (*cyp19a*) en las hembras de los cuatro grupos experimentales y la hormona anti-mülleriana (*amh*) en los machos. La expresión de *cyp19a* fue mayor en las hembras de los grupos TC y CT (6.5 y 4.6 veces mayor que en 28°C) mientras que la expresión de *amh* fue mayor en los grupos 24°C y 28° (3.6 y 14.3 veces mayor que en TC). Como conclusión, tomando en cuenta todos los resultados obtenidos en éste estudio, hemos comprobado la importancia de los ciclos diarios ambientales durante las primeras etapas de vida del pez, los cuales dan forma a ritmos diarios de desarrollo y comportamiento en el embrión y la larva, influenciando además la determinación sexual.