ZEBRAFISH
Volume 00, Number 00, 2013

DOI: 10.1089/zeb.2013.0926

Original Article

Effect of Lighting Conditions on Zebrafish Growth and Development

Natalia Villamizar,[1] Luisa María Vera,[1] Nicholas Simon Foulkes,[2] and Francisco Javier Sánchez-Vázquez[1]

Abstract

In the underwater environment, the properties of light (intensity and spectrum) change rapidly with depth and water quality. In this article, we have described how and to what extent lighting conditions can influence the development, growth, and survival of zebrafish. Fertilized eggs and the corresponding larvae were exposed to different visible light wavelengths (violet, blue, green, yellow, red, and white) in a 12-h light–12-h dark (LD) cycle until 30 days posthatching (dph), when the expression of morphometric parameters and growth (*igf1a*, *igf2a*)- and stress-related (*crh* and *pomca*) genes were examined. Another group of larvae was raised under constant darkness (DD) until 5 or 10 dph, after which they were transferred to a LD of white light. A third group remained under DD to investigate the effects of light deprivation upon zebrafish development. The results revealed that the hatching rate was highest under blue and violet light, while total length at 30 dph was greatest under blue, white, and violet light. Red light led to reduced feeding activity and poor survival (100% mortality). Larvae raised under constant white light (LL) showed a higher proportion of malformations, as did larvae raised under LD violet light. The expression of growth and stress factors was upregulated in the violet (*igf1a*, *igf2a*, *pomca*, and *chr*) and blue (*igf2a*) groups, which is consistent with the higher growth recorded and the higher proportion of malformations detected under the violet light. All larvae kept under DD died before 18 dph, but the survival rates improved in larvae transferred to LD at 5 dph and at 10 dph. In summary, these findings revealed that lighting conditions are crucial factors influencing zebrafish larval development and growth.

Introduction

Throughout evolution, animals have developed biological clocks for keeping track of time and directing complex, coordinate behavioral and physiological rhythms that enabled them to thrive under natural daily environmental cycles. The so-called circadian clock is a highly conserved timing mechanism that is located in specific neural structures (pineal, retina, and brain areas) as well as in most peripheral tissues of vertebrates.[1,2] This autonomous mechanism is constituted by clock genes, which are organized in regulatory circuits of interlocking transcription–translation feedback loops, thereby generating a characteristic circa 24-h cycle. External environmental signals such as the light–dark (LD) cycle reset the clock on a daily basis to ensure it remains synchronized with the environmental cycle of 24 h.

In the case of fish, the aquatic environment poses particular challenges for the photoreceptive mechanisms, which entrain the circadian clock. The water column acts as a potent chromatic filter, modifying the sun's spectral profile since wavelengths below violet ($\lambda < 390$ nm) and beyond red ($\lambda > 600$ nm) are selectively absorbed. However, blue wavelengths ($\lambda \sim 450$ nm) penetrate deeper, reaching depths of up to 150 m in the clearest ocean waters.[3] It has been suggested that fish living in different underwater photoenvironments have adapted their maximum photoreceptor sensitivity accordingly.[4] The signaling pathways that couple photoreception to the circadian timing system have yet to be deciphered; however, many reports have highlighted the importance of LD cycles in influencing the function of several key elements of the circadian system, such as the hormone melatonin,[5–7] arylalkylamine *N*-acetyltransferase, the rate limiting enzyme in melatonin synthesis,[8] and light-induced clock genes.[9,10] Interestingly, many reports have also documented light-induced expression of many nonclock genes. Taken together, these findings suggest a complex interplay between light and various aspects of fish physiology.

The effect of light on fish biology appears to be species specific.[11] For example, European sea bass larvae exposed to LD cycles of blue light (463 nm) show increased feeding activity, earlier weaning, and better spatial distribution, which coincides with the distribution of their live prey (*Artemia*

[1]Department of Physiology, Faculty of Biology, University of Murcia, Murcia, Spain.
[2]Institute of Toxicology and Genetics, Karlsruhe Institute of Technology, Karlsruhe, Germany.

sp.).[12] Although the functions of visual and nonvisual photoreceptive structures and mechanisms have been studied in detail in adult fish, the response of embryos and larvae to specific wavelengths and photoperiods, and the implications thereof for their development, remain unclear. As regard the ontogeny of their photoreceptive capacity, zebrafish larvae adapt their visual system to their photic environment by altering the abundance of cones and opsin expression in the retina.[13,14] Whereas in the larval stages, there is an abundance of UV and blue cones (which mediate short-wavelength sensitivity), a more complex mosaic develops in juveniles, including green- and red-sensitive opsins.[15,16]

By virtue of its transparent, rapidly developing embryos, the zebrafish has become a powerful model system for the genetic analysis and *in vivo* imaging of early embryonic development as well as the study of mechanisms that control growth and survival.[17,18] Furthermore, the species has become established as a novel model for exploring molecular clocks.[19] Direct exposure of both central and peripheral tissues to light entrains the cellular clock in this species. Thus, the zebrafish represents a potentially attractive model for studying more generally, the influence of photoperiod and light spectrum during the first stages of development.

The insulin-like growth factors (*igf1a* and *igf2a*) act through a conserved signaling pathway that regulates growth, development, metabolism, and longevity in a wide variety of animals.[20] In the zebrafish, mRNA expression of these two genes is first detected during embryogenesis (*igf2a* in the zygote stage and *igf1a* in later stages),[21] when they have been shown to mediate notochord formation and nephron development.[22,23] However, the influence of lighting conditions on the function of these growth factors has not been investigated. Another key aspect of the effect of light exposure during early development is the potential for stress. The elements of the hypothalamus–pituitary–interrenal axis play a key role during ontogeny in the response to environmental stressors. The production and release of corticotropin-releasing hormone (CRH) from the hypothalamus is induced in response to exposure to stress and, in turn, CRH stimulates the secretion of the adrenocorticotropic hormone (ACTH) from the pituitary.[24] ACTH is generated by the proteolytic cleavage of proopiomelanocortin (POMC) and activates the signaling pathway leading to corticosteroid biosynthesis.[25] The effect of exposure to different light wavelengths and photoperiods on CRH and POMC function has not been studied.

In this article, innovative light emitting diode (LED) technology was applied to explore the effect of exposure to different wavelengths of light on the development and growth of zebrafish embryos and larvae. We specifically tested the impact of different wavelengths on the expression of growth (*igf1* and *igf2*)- and stress-related genes (*crh* and *pomca*). Furthermore, given that zebrafish embryos raised under constant darkness lack circadian rhythmicity,[10,26] we also studied the effects of constant darkness on the development, growth, and survival of zebrafish during larval and postlarval stages.

Materials and Methods

Ethics statement

All the zebrafish husbandry and experimental procedures followed 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 housing

Adult zebrafish (*Danio rerio*), larvae, and postlarvae of heterogeneous wild-type stock (standard short-fin phenotype) were raised according to standard methods[27] at the Chronobiology Laboratory of the Faculty of Biology, University of Murcia (Spain). Eight groups of sexually mature zebrafish (two females and four males per group) were used as broodstock. Fertilized eggs from spontaneous spawnings were collected and pooled within 2 h of spawning. Aliquots of 30 eggs were maintained into sterile Petri dishes (85 × 10 mm) filled with an embryo medium[27] for 5 days postfertilization (dpf). After this time, the hatched larvae were transferred into 2.5-L nursery net cages (2 Petri dishes per cage, 30 larvae per dish) (SERA GmbH). The Petri dishes and cages were incubated in 9-L thermostat-controlled (100 W; Askoll Water Heater) glass aquaria at a temperature of 28.5°C. From 7 dpf onward, larvae were fed to satiation, twice a day, with a powdered feed (JBL Novo Tom; JBL GmbH & Co. KG). From 15 dpf onward, artemia nauplii (JBL 151 GmbH & Co. KG) were also supplied (once a day) and from 20 dpf until 30 dpf, the powdered feed was replaced by granulated food (twice a day until satiation) (Biogran Small; PRODAC).

Experimental procedures

Experiment 1: For the different spectral trials, lamps were built using 10 LEDs (Kopa Electronica) mounted on a fiberglass plate (160 × 100 mm) and powered by a 3-V DC supply. The effects of six different wavelengths delivered as a 12-h light–12-h dark (12L:12D) photocycle were tested: violet ($\lambda_{peak} = 416$ nm), blue ($\lambda_{peak} = 472$ nm), green ($\lambda_{peak} = 436$ nm), yellow ($\lambda_{peak} = 603$ nm), red ($\lambda_{peak} = 665$ nm), or white (control) (Fig. 1). In addition, constant white lighting conditions were also applied (LL). Since the energy per photon differs depending on the wavelength (the shorter the wavelength, the higher the energy content) and biological photoreceptors are basically photon counters, the photon irradiance of all lamps was adjusted to $1.57 \pm 0.03\ \mu\text{moles} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$. The spectral analysis was performed using a spectroradiometer (FieldSpec®; ASD). For each experimental group, two aquaria were used (one cage per aquarium, $n = 2$). Embryos, larvae, and

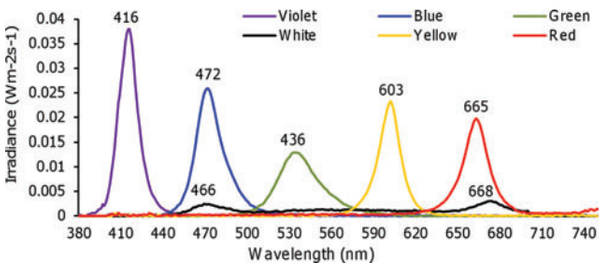

FIG. 1. Spectral composition of each experimental light emitting diode (LED) lamp (*violet*, *blue*, *green*, *yellow*, *red*, and *white*) expressed as the percentage of irradiance (Analytical Spectral Devices FieldSpec® Handheld). Color images available online at www.liebertpub.com/zeb

postlarvae were reared from 2 hours postfertilization (hpf) until 30 days posthatching (dph) under each one of the light regimes mentioned above. To evaluate the existence of growth compensation, all groups were maintained under the same light conditions of 12L:12D of neutral white light (flexible LED stripes; Superlight Technology Co., Ltd) from 30 to 80 dph.

Experiment 2: Embryos, larvae, and postlarvae were reared either under constant darkness (DD) or white LD cycles (control group). For DD, Petri dishes (30 larvae per dish) were wrapped in adhesive black tape and the aquaria were covered with thick black plastic to avoid any light contamination. At 5 dph, one-third of the larvae reared under DD were transferred to LD (LD5). At 10 dph, another third were transferred to LD (LD10), while the rest of the larvae remained under DD. Fish were kept under these conditions until 30 dph. For each experimental group (DD, LD, LD5, and LD10), two aquaria were used (one cage with two Petri dishes per aquarium, $n=2$).

Data collection

Hatching, growth, feeding, malformations, and survival were used as evaluation criteria to assess the observable effects of light. The hatching rate was calculated as the number of embryos hatched by 48 hpf divided by the total number of embryos. Growth in terms of total length (TL) was recorded every 5 days in each treatment by measuring all the animals. Larvae and postlarvae were measured live using a digital camera mounted on a stereo microscope; digital images were analyzed using image processing software (Leica Microsystems Imaging Solutions Ltd).

Growth was assessed in terms of wet weight (WW) at 80 dph. At 10 dph, the feeding activity was determined in all postlarvae. To this end, 1 h after being fed, the proportion of the larvae's digestive tube (DT) filled with food was measured and analyzed in relation to its TL. Malformations were recorded in all groups every day from 0 to 10 dpf by reference to the gross changes described for the species as evaluation criteria.[28–30] Mortality was calculated as the number of dead embryos found every other day, the cessation of heartbeat and circulation being used as endpoints for mortality. Overall survival was calculated at 30 dpf.

Gene expression analysis

At 8 dph, 15 postlarvae per treatment (2 replicates) were anesthetized and euthanized on ice. Then, they were pooled and homogenized in Trizol reagent (Invitrogen) using a tissue homogenizer (Polytron®, PT1200; Kinematica). Total RNA concentration was determined by spectrometry (Nanodrop® ND-1000; Thermo Fisher Scientific, Inc.), and 1 μg was treated with amplification grade DNase I (1 unit/μg RNA; Invitrogen) to prevent genomic DNA contamination. cDNA synthesis was carried out with Superscript III Reverse Transcriptase (Invitrogen) and Oligo (dT)18 (Invitrogen) in a 20-μL reaction volume. Quantitative polymerase chain reaction (qPCR) was performed using the StepOnePlus Real-Time PCR System (Applied Biosystems) with SYBR green primer master mix according to the manufacturer's recommendations. Amplification followed the PCR cycle conditions: 15 min 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.

Table 1. Primer Oligonucleotide Sequences Used for qPCR Analysis

Gene	*Sequence (5′→3′)*
Igf1a	(F): CAG GCAAATCTCCACGATCTC
	(R): CTTTGGTGTCCTGGAATATCTC
Igf2a	(F): GTGAAGTCGGAGCGAGATTGTT
	(R): GAGCCTGTGACACTG GGAAGA
pomca	(F): CGCAGACCCATCAAGGTGTGTA
	(R): CGTTTCGGC GGATTCCT
crh	(F): GCCGCGCAAAGTTCAAAA
	(R): GCGAGGAGA ATCTGTGCGTAA
ef1α	(F): CTGGAGGCCAGCTCAAACAT
	(R): ATCAAGAAGAGTAGTACCGCTAGCATTAC

qPCR, quantitative polymerase chain reaction.

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), and the primer sequences for *crh* were taken from Alderman and Bernier.[31] The primer oligonucleotide sequences are shown in Table 1. The amplification efficiency, specificity of primers, and the 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,[32] using *D. rerio ef1α* (ENSDART00000023156) as the endogenous reference.[33]

For comparison purposes, the final levels of gene 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 are expressed as mean ± SEM. Data were analyzed by one-way analysis of variance (ANOVA I) followed by the Tukey's test to determine significant differences. All statistical analyses were carried out using the software SPSS 15.0 (SPSS, Inc.). $p<0.05$ was considered statistically significant.

Results

Experiment 1: Light spectrum

We first tested the effects of light and dark cycles of different wavelengths on larval and postlarval growth and development. At 48 hpf, the highest level of hatching was observed in the group maintained under constant white light (LL) (29.2% ± 2.1%), followed by the LD cycle groups involving violet (24.3% ± 1.4%), white (23.1% ± 0.7%), and blue (20.7% ± 0.8%) light. Statistically significant differences in the hatching rate were confirmed between the above two groups and for green (14.2% ± 1%), red (13.2% ± 1%), and yellow (10.5% ± 2%) LD cycle groups. At 72 hpf, the highest proportion of hatched larvae was observed in the violet, followed by the blue and white LD groups, while the lowest level of hatching was observed under the red and yellow LD cycles (Table 2). Concerning growth (as measured by TL), statistically significant differences between the various groups were first detected at 5 dph. TL was higher in the larvae reared

Table 2. Effects of Lighting Conditions on Zebrafish Larvae

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

Observed parameters are expressed as mean ± SEM. Malformation data represent all observed abnormalities. Different superscript letters indicate statistical differences among treatments (ANOVA, Tukey's test, $p < 0.05$).

DT, digestive tube.

under LD violet light than in the constant white light (LL), green, and red groups (Fig. 2B). Such differences were maintained until 30 dpf, with the highest TL being observed in the blue, white, and violet groups (Fig. 2A). The WW was also measured at 80 dpf, when there was no evidence of compensatory growth (catch up), since the highest was observed in the white, blue, and violet groups and the lowest WW was observed in the LL larvae (Table 2). Interestingly, the feeding activity of postlarvae under blue and violet light cycles was higher than in larvae raised under red light cycles, as measured by the higher proportion of these larvae showing a digestive tract filled with food (Table 2).

When we also investigated the effect of lighting conditions on developmental abnormalities, the most common malformations found were those related with the spinal cord, cranium, pericardium, and yolk sac. Such malformations were first detected under green and red lights around 52 hpf. In these groups, embryos were already observed with severe abnormalities (pericardial and yolk sac edema and craniofacial malformations) before hatching. Morphological abnormalities were visible at 5 dpf under LL (highest percentage of malformations) violet, green, and red lighting, where larvae typically showed jaw malformations and spinal bending. The lowest levels of malformations were detected among the groups exposed to yellow, white, and blue lighting conditions (Table 2). Mortality was highest in postlarvae under red light.

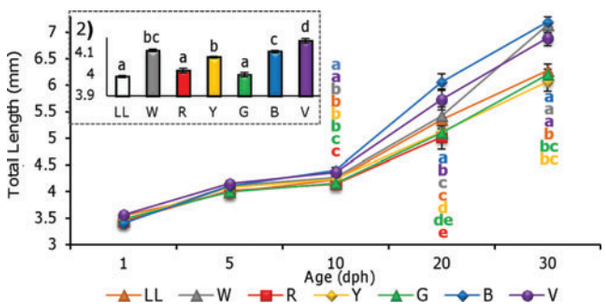

FIG. 2. The effect of light spectrum and constant light on zebrafish larval growth from 1 to 30 days posthatching (dph) (1) and when significant differences were first observed at 5 dph (2). Constant *white light* (LL) and *light–dark* (LD) cycles of *white* (W), *red* (R), *yellow* (Y), *green* (G), *blue* (B), and *violet* (V) light. Data are expressed as mean of the total length (TL) (mm) ± SEM. Different lower case letters indicate statistically significant differences between each treatment with each colored letter representing the corresponding wavelength (blue letters for blue light treatment, red letters for red light treatment, etc.) (ANOVA, $p < 0.05$, $n = 2$). Color images available online at www.liebertpub.com/zeb

In this group, survival decreased rapidly between 10 and 20 dph, at which point, 100% mortality was recorded. Within the remaining groups, larvae under LL, violet, green, and yellow lights showed significantly higher mortality compared with postlarvae under blue and white lighting conditions (Fig. 3). By 30 dph, survival was significantly higher under white and blue lights than under LL, where the lowest survival was encountered.

We finally tested the expression of growth- and stress-related genes under the various lighting conditions. *Igf1a* expression was significantly higher in postlarvae exposed to violet light, while the lowest expression was observed in the green group (Fig. 4A). Significant differences were also found in the expression of *igf2a,* the highest levels being observed under violet and blue light (Fig. 4B). *Pomca* expression was also significantly higher in postlarvae reared under violet and blue light, while postlarvae reared under green light showed the lowest levels of such expression (Fig. 4C). In contrast, *crh* was highly expressed under all lighting conditions except for green light (Fig. 4D).

Experiment 2: DD

We next investigated the effect of raising larvae under constant darkness (DD). Significant differences were found when comparing the percentage of hatched larvae in the DD groups compared with controls (LD) at 72 hpf, with 64.4% ± 9.1% hatched in DD conditions and 87.3% ± 1.4% hatched under LD. Under DD, reduced growth was already evident at 5 and 10 dph compared with the control LD group. We wondered whether transfer from DD to LD cycle conditions would subsequently rescue normal growth and

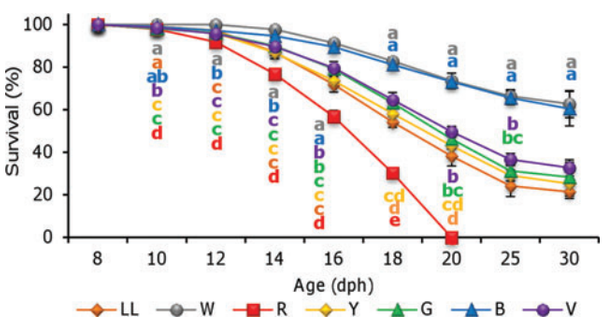

FIG. 3. Survival (%) of zebrafish larvae reared under different light spectra until 30 dph. Values are mean ± SEM. Statistically significant differences are represented as explained in Figure 2 (ANOVA, $p < 0.05$, $n = 2$). Color images available online at www.liebertpub.com/zeb

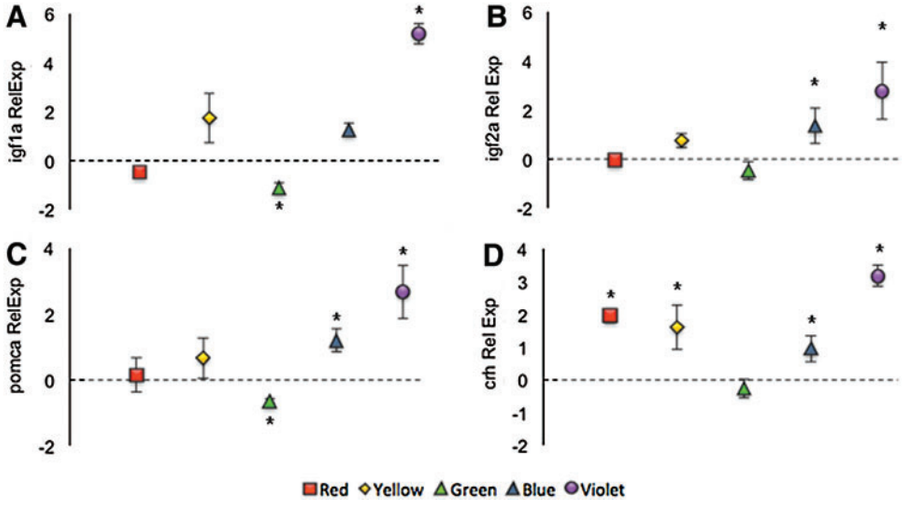

FIG. 4. Expression levels of *igf1a* **(A)**, *igf2a* **(B)**, *pomca* **(C)**, and *crh* **(D)** in 8 dph zebrafish reared under *red*, *yellow*, *green*, *blue*, and *violet* light. *Asterisks* represent statistically significant differences compared with the control group (white LD). Data are expressed as mean ± SEM. (ANOVA, $p < 0.05$, $n = 2$). Color images available online at www.liebertpub.com/zeb

development. For this, larvae were raised for the first 5 or 10 days under DD conditions and then transferred to LD cycles (LD5 and LD10 larvae, respectively). Whereas at 30 dph, LD10 postlarvae started to grow more actively following transfer to LD conditions, LD5 postlarvae showed no significant difference compared with a control group raised from fertilization under LD cycles (Fig. 5). By 15 dph, the feeding activity was very low under DD conditions, with only 12.1% ± 3.5% of the larval digestive tracts filled with food. At this point, LD5 and LD10 postlarvae were found to have similar levels of prey ingestion (26.2% ± 2.3% and 28.5% ± 3.1% of the DT filled, respectively), while the control LD group registered the highest level of ingestion (43.2% ± 4.1%).

Malformations were observed in a higher number of postlarvae kept under LD5 (5.3% ± 0.6%), LD10 (6.4% ± 1.3%), and DD (5.3% ± 0.7%) conditions, compared with the control LD group (1.3% ± 0.3%). The anomalies found under LD were characterized by dorsal curvatures, whereas in the other three groups, pericardial and yolk sac edemas, as well as cranial deformities, were more common. Survival was clearly affected by lighting conditions, and was higher in the LD5 group compared with the control group. After transferring the LD10 larvae to LD conditions, survival improved, but remained significantly lower for the rest of the experiment. However, in DD larvae, 100% mortality was recorded by 18 dph (Fig. 6).

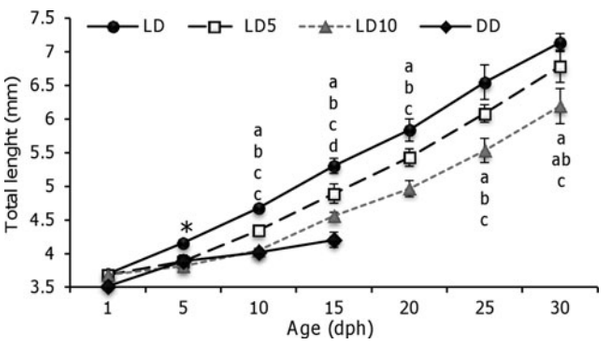

FIG. 5. Increase in length (mm) of zebrafish larvae reared either under white LD light (LD, control), constant darkness until 5 dph and then under LD (LD5), constant darkness until 10 dph and then under LD (LD10), or constant darkness (DD). Values are mean ± SEM. Different letters or an asterisk indicates statistically significant differences between treatments (ANOVA, $p < 0.05$, $n = 2$).

Discussion

Our findings confirm the significant influence that ambient lighting has on embryogenesis and subsequent larval growth and development in zebrafish. The light spectrum influenced the hatching rate, with blue light LD leading to the highest hatching frequency. However, since zebrafish inhabit a wide range of continental water environments (from clear streams to stagnant waters),[34] the optimal response might have been predicted to occur in the spectrum profile found in these habitats (blue-greenish wavelengths). However, surprisingly, green light did not have a positive effect on zebrafish embryogenesis and larval and postlarval development, resulting instead, in low feeding activity levels, poor growth, a high proportion of malformations, and low survival rates. In

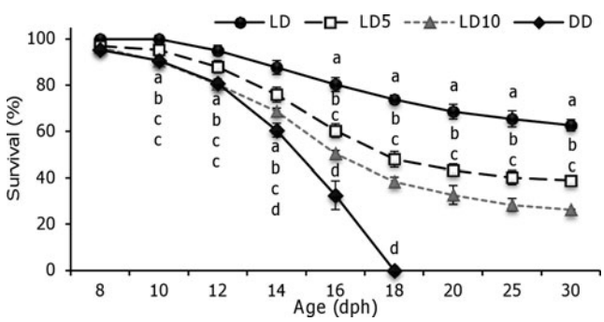

FIG. 6. Mean percent survival (± SEM) over time for larval zebrafish reared under LD (control), LD5, LD10, or DD. Values not sharing a common letter differ significantly (ANOVA, $p < 0.05$, $n = 2$).

contrast, exposure to white light (ranging from 400 to 700 nm wavelengths) improved many aspects of zebrafish development and growth.

At the larval and postlarval stages, light sensing mechanisms, such as the retina and peripheral photoreceptors, are already functional. The retina structure during these stages shows abundance of UV and blue cones, which is typical of planktivorous teleosts that inhabit shallow waters.[35,36] In our trials, these visual pigments may have aided zebrafish under blue and violet lights to detect prey by increasing contrast against the background, whereas the red, yellow, and green light groups showed the lowest feeding activity, probably because of the reduced number of red and green cones in the postlarval retina.[15] However, research into light and its effects on prey detection is not an easy task due to the complex relationship among several factors. Among these, is the fact that down-welling light is absorbed or scattered in the water column; moreover, light is reflected and enters the retina of the postlarvae, which have a short visual range that increases as the fish develops.[37] Previous studies reported an optimal wavelength of 550 nm in species such as *Gobiusculus flavescens*,[38] 470 nm in *Melanogrammus aeglefinus*,[39] and 450–500 nm in *Salmo salar* and *Dicentrarchus labrax*,[40,41] highlighting the importance of short wavelengths in fish.

Other studies also revealed that nonvisual photopigments (pinopsin, melanopsin, and exorhodopsin) are expressed in a wide variety of zebrafish tissues, where they have been implicated in nonvisual light-dependent processes such as synchronizing the circadian clock.[42,43] Indeed, studies on zebrafish photoreception showed that blue light is involved in the transcriptional response of clock gene *zper2* in zebrafish cells, which is necessary for the early development of the pineal clock during embryogenesis.[44] External factors, such as handling,[28] temperature,[45] and light,[1] have been extensively documented as affecting embryonic development. As regard the ontogeny of light detection, it has been suggested that most of the cells of embryonic zebrafish are capable of light detection as early as 5 hpf (gastrula stage),[46] while the pineal organ detects light by 24 hpf[47] and retinal photoreception develops at 2–3 dpf.[48] This early ability of zebrafish to detect light has a significant impact on embryo and larvae survival as light induces the expression of key genes such as those involved in DNA repair[46] and clock genes involved in cell proliferation and growth regulation.[49] Light-dependent differences in larval growth were already evident by 5 dph, before exogenous feeding had started. At this point, the groups under white, blue, and violet lights exhibited a longer total body length, which was maintained throughout the experiment. Despite the lack of information on the influence of different wavelengths on the early stages of zebrafish development and growth, studies in marine fish species such as European sea bass, Senegal sole and Atlantic cod,[11] haddock,[50] and the two-spotted goby[38] showed that larval growth and development were enhanced under blue-yellow wavelengths (ranging from 470 to 550 nm). This suggests that the response to light could be species specific, with photoreceptor mechanisms, such as photopigments, matching the spectral distribution of the natural environment of the fish in question.[36]

In our study, larvae exposed to constant white light exhibited the lowest growth rate at 5 dph and growth remained low until the end of the experiment. Different results in this respect have been found for marine species such as European sea bass and Senegal sole,[51,52] in which constant light enhanced growth. The presence of light/dark cycles is known to be important for the normal onset of circadian clock function[53] as well as for establishing clock-controlled rhythms of processes such as cell proliferation[45] and affecting brain and eye development.[54] These findings may explain the high percentage of malformations and poor survival of the constant light (LL) group.

To obtain a more complete picture of how the light spectrum may affect zebrafish larval and postlarval development in zebrafish, the differential expression patterns of growth (*igf1a*, *igf2a*)- and stress (*pomca* and *crh*)-related genes were compared with the biometric parameters investigated in the experimental groups. In the case of the growth-related genes, *igf1a* and *igf2a* were overexpressed in the violet light group, which also showed the highest growth rate. Moreover, the expression of *igf1a* was downregulated in the green group, where the body length was shorter at 5–10 dph. The green and violet light groups showed a high incidence of abnormal development of the spinal cord. Recent studies have found that both the overexpression and knockdown of *igf1a* and *igf2a* are associated with several developmental processes, such as midline formation and notochord development.[21,23] The expression levels of *igf1a* and *igf2a* in the red and yellow light groups did not show significant differences from those observed in the control white light group. However, these findings were not reflected in growth, which was higher under white light conditions, pointing to the existence of additional pathways controlling zebrafish larval and postlarval growth.

Differences in growth and, particularly, malformations, may be linked with a differential stress response. Recent studies have reported that low *pomca* expression serves as an indicator of both chronic and acute stress in zebrafish larvae and that the knockdown of this gene results in the impairment of behavioral and physiological responses such as camouflage.[55] Whereas *pomca* expression was downregulated under green light and overexpressed under violet light, *crh* expression was significantly higher in most of the experimental groups (red, yellow, blue, and violet light) compared with the control white light group. Recent studies in zebrafish and rainbow trout report an increase of *crh* mRNA after severe and repeated stress.[56,57] However, under acute stress, *crh* mRNA expression decreased considerably suggesting that the regulation of this gene expression is controlled by increased glucocorticoid levels through a genomic feedback mechanism which, in turn, suggests that *crh* and *pomca* may follow a common pathway.[58,59] Overall, our results documenting upregulated stress- and growth-related genes under the violet light suggest a strong effect of this particular wavelength on zebrafish development. Furthermore, this group presented the highest incidence of malformations and low survival, which suggests that the violet light may act as a stressor for embryos and larvae. Interestingly, the range of wavelengths for the violet light used in this experiment (half-peak bandwidth 390–440 nm) is close to that of UVA light (315–400 nm), which is known to induce lesions to the brain and retina,[60] impaired larval development,[61] and oxidative stress in adult fish.[62]

We have mentioned the impaired development and strikingly low survival rate of zebrafish larvae raised under

constant darkness. Indeed, 100% mortality was observed by 18 dph, with both hatching and feeding activity being negatively affected. These results agree with the key role reported for light and melatonin in early development, meaning that keeping zebrafish embryos in constant darkness causes late differentiation of the neurons in the brain, while adding melatonin restores neurogenesis.[63] Upon transfer from DD to LD conditions, the negative effect on growth seemed to be permanent in the LD10 group despite the fact that food intake increased, as no evidence of growth compensation was observed during the rest of the experimental period. However, LD5 postlarvae were capable of reaching the total body length of the normal LD control group by 30 dph. This suggests that the critical developmental time point, whereby transfer to LD conditions can reverse the negative effects of exposure to constant darkness, is around 5 dph. Constant darkness has been observed to impair the normal development of the molecular clock of fish larvae, which requires the input of an LD cycle to synchronize individual cell clock.[19] The general importance of exposure to light/dark cycles has been documented in many organs and cell culture lines of zebrafish, in which ~117 light inducible/repressible genes have been identified (including genes involved in circadian rhythms, growth, stress response, and DNA repair), suggesting a broad range of mechanisms linking light with physiology.[53]

In summary, our findings reveal the strong effect of light upon key morphological and functional elements of zebrafish. Exposure to white or blue LD cycles provided the optimal outcome in terms of rapid development and low mortality, accompanied by the lowest frequency of malformations. Moreover, our results point to the involvement of the *igf1a*, *igf2a*, *pomca*, and *crh* genes in the stress response of zebrafish to specific wavelengths. Thus, the complex picture highlights the need for special attention to be paid to the lighting conditions used for developing fish larvae.

Acknowledgments

This research was funded by the Spanish Ministry of Economic Affairs and Competitiveness (MINECO) by projects "Circasole" (AGL2010-22139-C03-01), "Aquagenomics" (CSD2007-0004), and SENECA (05690/PI/07) granted to F.J.S.V. Thanks are also due to COST ACTION FA0801 (Larvanet), in whose meetings the results were partially presented. N.S.F. was supported by the Karlsruhe Institute of Technology (KIT, Germany) through the Helmholtz funding program: BioInterfaces.

Disclosure Statement

The authors confirm that no competing financial interests exist.

References

1. Whitmore D, Foulkes NS, Sassone-Corsi P. Light acts directly on organs and cells in culture to set the vertebrae circadian clock. Nature 2000;404:87–91.
2. Pando MP, Pinchak AB, Cermakian N, Sassone-Corsi P. A cell-based system that recapitulates the dynamic light dependent regulation of the vertebrate clock. Proc Natl Acad Sci U S A 98 2001;10178–10183.
3. Lalli CM, Parsons TR. Biological Oceanography: An Introduction. Oxford, England: Butterworth-Heinemann Ltd., 1995.
4. Kusmic C, Gualtieri, P. Morphology and spectral sensitivities of retinal and extraretinal photoreceptors in freshwater teleosts. Micron 2000;31:183–200.
5. Vera LM, Davie A, Taylor JF, Migaud H. Differential light intensity and spectral sensitivities of Atlantic salmon, European sea bass and Atlantic cod pineal glands *ex vivo*. Gen Comp Endocrinol 2010;165:25–33.
6. Falcon J, Migaud H, Munoz-Cueto JA, Carrillo M. Current knowledge on the melatonin system in teleost fish. Gen Comp Endocrinol 2010;165:469–482.
7. Amano M, Iigo M, Ikuta K, Kitamura S, Yamamori K. Characterization and maturational differences of melatonin binding sites in the masu salmon brain. Gen Comp Endocrinol 2003;131:338–344.
8. Falcon J. Celular circadian clocks in the pineal. Prog Neurobiol 1999;58:121–162.
9. Kobayashi Y, Ishikawa T, Hirayama J, Daiyasu H, Kanai S, Toh H, *et al.* Molecular analysis of zebrafish photolyase/cryptochrome family: two types of cryptochromes present in zebrafish. Genes Cells 2000;5:725–738.
10. Vatine G, Vallone D, Gothilf Y, Foulkes NS. It's time to swim! Zebrafish and the circadian clock. FEBS Lett 2011;585:1485–1494.
11. Villamizar N, Blanco-Vives B, Migaud H, Davie A, Carboni S, Sánchez-Vázquez FJ. Effects of light during early larval development of some aquacultured teleosts: a review. Aquaculture 2011;315:86–94.
12. Villamizar N, García Mateos G, Sánchez-Vázquez FJ. Behavioral responses of European sea bass (*Dicentrarchus labrax*) larvae and *Artemia sp*. exposed to constant light or darkness vs. light/dark cycles of white, red or blue wavelengths. Aquaculture 2011;317:197–202.
13. Shand J, Davies WL, Thomas N, Balmer L, Cowing JA, Pointer M, *et al.* The influence of ontogeny and light environment environment on the expression of visual pigment opsins in the retina of the black bream, *Acanthopagrus butcheri*. J Exp Biol 2008;211:1495–1503.
14. Carleton KL, Spady TC, Streelman JT, Kidd MR, McFarland WN, Loew ER. Visual sensitivities tuned by heterochronic shifts in opsin gene expression. BMC Biol 2008;6:22.
15. Allison WT, Barthel WT, Skebo KM, Takechi SK, Raymond PA. Ontogeny of cone photoreceptor mosaics in zebrafish. J Comp Neurol 2010;518:4182–4195.
16. Takechi M, Kawamura S. Temporal and spatial changes in the expression pattern of multiple red and green subtype opsin genes during zebrafish development. J Exp Biol 2005;208:1337–1345.
17. Biga PR, Goetz FW. Zebrafish and giant Danio as models for muscle growth: determinate versus indeterminate growth as determined by morphometric analysis. Am J Physiol: Reg Integ Comp Physiol 2006;291:R1327–R1337.
18. Brittijn SA, Duivesteijn SJ, Belmamoune M, Bertens LF, Bitter W, Debruijn JD, *et al.* Zebrafish development and regeneration: new tools for biomedical research. Int J Dev Biol 2009;53:835–850.
19. Idda ML, Kage E, López-Olmeda JF, Phillipp M, Foulkes NS, Vallone D. Circadian timing of injury-induced cell proliferation in zebrafish. PLoS One 2012;7:e34203.
20. Wood AW, Duan C, Bern HA. Insulin-like growth factor signaling in fish. Int Rev Cytol 2005;243:215–285.
21. Zou S, Kamei H, Modi Z, Duan C. Zebrafish IGF genes: gene duplication, conservation and divergence, and novel roles in

midline and notochord development. PLoS One 2009; 4:e7026.
22. Eivers E, McCarthy K, Glynn C, Nolan CM, Byrnes L. Insulin-like growth factor (IGF) signalling is required for early dorso-anterior development of the zebrafish embryo. Int J Dev Biol 2004;48:1131–1140.
23. Brown YAR, Kyle JT, Wood AW. Targeted gene knockdown in zebrafish reveals distinct intraembryonic functions for insulin-like growth factor 2 (IGF-II) signaling. Endocrinology 2009;150:4366–4375.
24. Alsop D, Vijayan MM. Molecular programming of the corticosteroid stress axis during zebrafish development. Comp Biochem Physiol A Mol Integr Physiol 2009;153:49–54.
25. Aluru N, Vijayan MM. Molecular characterization, tissue-specific expression and regulation of melanocortin 2 receptor in rainbow trout. Endocrinology 2008;149:4577–4588.
26. Ziv L, Gothilf Y. Circadian time-keeping during early stages of development. Proc Natl Acad Sci U S A 2006;103:4146–4151.
27. Nüsslein-Volhard C, Dahm R: Zebrafish: A Practical Approach. England: Oxford University Press, 2002.
28. Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, Schilling TF. Stages of embryonic development of the zebrafish. Dev Dyn 1995;203:253–310.
29. Dong Q, Svoboda K, Tiersch TR, Monroe WT. Photobiological effects of UVA and UVB light in zebrafish embryos: evidence for a competent photorepair system. J Photochem Photobiol B 2007;88:137–146.
30. Verreijdt L, Vandervennet E, Sire JY, Huysseune A. Developmental differences between cranial bones in the zebrafish (*Danio rerio*): some preliminary light and TEM observations. Connect Tissue Res 2002;43:109–112.
31. Alderman SL, Bernier NJ. Ontogeny of the corticotropin-release factor system in zebrafish. Gen Comp Endocrinol 2009;164:61–69.
32. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001;25:402–408.
33. Tang R, Dodd A, Lai D, McNabb W, Love DR. Validation of zebrafish (*Danio rerio*) reference genes for quantitative real time RT-PCR normalization. Acta Bioch Bioph Sin 2007;39: 384–390.
34. Spence R, Gerlach G, Lawrence C, Smith C. The behaviour and ecology of the zebrafish, *Danio rerio*. Biol Rev Camb Philos Soc 2008;83:13–34.
35. Browman HI, Novales Flamarique I, Hawryshyn CW. Ultraviolet photoreception contributes to the foraging performance of two species of zooplanktivorous fishes. J Exp Biol 1994;186:187–198.
36. Bowmaker JK. The visual pigments of fish. Prog Retin Eye Res 1995;15:1–31.
37. Huse G, Fiksen Ø. Modelling encounter rates and distribution of mobile predators and prey. Prog Oceanogr 2010;84: 93–104.
38. Utne-Palm AC, Bowmaker JK. Spectral sensitivity of the two-spotted goby *Gobiusculus flavescens* (Fabricius): a physiological and behavioural study. J Exp Biol 2006;209:2034–2041.
39. Downing G, Litvak MK. The effect of light intensity and spectrum on the incidence of first feeding by larval haddock. J Fish Biol 2001;59:1566–1578.
40. Max M, Menaker M. Regulation of melatonin production by light, darkness, and temperature in the trout pineal. J Comp Physiol A 1992;170:479–489.
41. Bayarri MJ, Madrid JA, Sánchez-Vázquez FJ. Influence of light intensity, spectrum and orientation on sea bass plasma and ocular melatonin. J Pineal Res 2002;32: 34–40.
42. Pierce LX, Noche RR, Ponomareva O, Chang C, Liang JO. Novel functions for Period 3 and Exo-rhodopsin in rhythmic transcription and melatonin biosynthesis within the zebrafish pineal organ. Brain Res 2008;1223:11–24.
43. Peirson SN, Halford S, Foster RG. The evolution of irradiance detection: Melanopsin and the non-visual opsins. Philos Trans R Soc Lond B Biol Sci 2009;364:2849–2865.
44. Levkovitz Y, Gil-Ad I, Zeldich E, Dayag M, Weizman A. Differential induction of apoptosis by antidepressants in glioma and neuroblastoma cell lines: evidence for p-c-Jun, cytochrome c, and caspase-3 involvement. J Mol Neurosci 2005;27:29–42.
45. Dekens MPS, Santoriello C, Vallone D, Grassi G, Whitmore D, Foulkes NS. Light regulates the cell cycle in zebrafish. Curr Biol 2003;13:2051–2057.
46. Tamai TK, Vardhanabhuti V, Foulkes NS, Whitmore D. Early embryonic light detection improves survival. Curr Biol 2004;14:R104–R105.
47. Kazimi N, Cahill GM. Development of a circadian melatonin rhythm in embryonic zebrafish. Brain Res Dev Brain Res 1999;117:47–52.
48. Burrill JD, Easter SS Jr. Development of the retinofugal projections in the embryonic and larval zebrafish (*Brachydanio rerio*). J Comp Neurol 1994;346:583–600.
49. Dekens MPS, Whitmore D. Autonomous onset of the circadian clock in the zebrafish embryo. EMBO J 2008;27:2757–2765.
50. Downing G. Impact of spectral composition on larval haddock, *Melanogrammus aeglefinus* L., growth and survival. Aqua Res 2002;33:251–259.
51. Villamizar N, García-Alcazar A, Sánchez-Vázquez FJ. Effect of light spectrum and photoperiod on the growth, development and survival of European sea bass (*Dicentrarchus labrax*) larvae. Aquaculture 2009;292:80–86.
52. Blanco-Vives B, Villamizar N, Ramos J, Bayarri MJ, Chereguini O, Sánchez-Vázquez FJ. Effect of daily thermo- and photo-cycles of different light spectrum on the development of Senegal sole (*Solea senegalensis*) larvae. Aquaculture 2010;306:137–145.
53. Weger BD, Sahinbas M, Otto GW, Mracek P, Armant O, Mracek P, *et al.* The light responsive transcriptome of the zebrafish: function and regulation. PLoS One 2011;6: e17080.
54. Chapman GB, Tarboush R, Connaughton VP. The effects of rearing light level and duration differences on the optic nerve, brain and associated structures in developing zebrafish larvae: a light and transmission electron microscope study. Anat Rec 2012;295:515–531.
55. Wagle M, Mathur P, Guo S. Corticotropin-releasing factor critical for zebrafish camouflage behavior is regulated by light and sensitive to ethanol. J Neurosci 2011;31:214–224.
56. Ghisleni G, Capiotti KM, Da Silva R, Oses JP, Piato AL, Soares V, *et al.* The role of CRH in behavioral responses to acute restraint stress in zebrafish. Prog Neuropsychopharmacol Biol Psychiatry;36:176–182.
57. Doyon C, Trudeau VL, Moon TW. Stress elevates corticotrophin-releasing factor (CRF) and CRF-binding protein mRNA levels in rainbow trout (*Oncorhynchus mykiss*). J Endocrinol 2005;186:123–130.

58. Dallman MF, Akana SF, Levin N, Walker CD, Bradbury, MJ, Suemaru, *et al.* Corticosteroids and the control of function in the hypothalamo-pituitary-adrenal (HPA) axis. Ann NY Acad Sci 1994;746:22–31.
59. Bernier NJ, Lin X, Peter RE. Differential expression of corticotrophin-releasing factor (CRF) and urotensin I precursor genes, and evidence of CRF gene expression regulated by cortisol in goldfish brain. Gen Comp Endocrinol 1999;116: 461–477.
60. Hunter JR, Taylor JH, Moser HG. Effect of ultraviolet-irradiation on eggs and larvae of the northern anchovy, *Engraulis mordax* and the Pacific mackerel, *Scomber japonicus* during the embryonic stage. Photochem Photobiol 1979;29: 325–338.
61. Browman HI, Vetter RD, Alonso-Rodríguez C, Cullen JJ, Davis RF, Lynn E, *et al.* Ultraviolet (280–400 nm) induced DNA damage in the eggs and larvae of *Calanus finmarchicus* G. (Copepoda) and atlantic cod (*Gadus morhua*). Photochem Photobiol 2003;77:2–29.
62. Zagarese HE, Williamson CE. The implications of solar UV radiation exposure for fish and fisheries. Fish Fisheries 2001; 2:250–260.
63. Borsetti NH, Dean B, Bain EJ, Clanton JA, Taylor RW, Gamse JT. Light and melatonin schedule neuronal differentiation in the habenular nuclei. Dev Biol 2011;358: 251–261.

Address correspondence to:
Prof. Francisco Javier Sánchez-Vázquez
Department of Physiology
Faculty of Biology
Regional Campus of International Excellence
"Campus Mare Nostrum"
University of Murcia
Murcia 30100
Spain

E-mail: javisan@um.es