Combined blue light and daily thermocycles enhance zebrafish growth and development

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

Keywords
daily thermocycle, larvae development, light spectrum, Zebrafish

1 | INTRODUCTION

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

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

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

Physiologically, the light wavelength and temperature can both stimulate and suppress fish nutrition influencing the development, growth, and other physiological processes of fish (Ruchin, 2021). Biochemically, fish growth development can be explained by changes in the neuropeptide expression involved in the endocrine regulation of survival, somatic growth, food intake control, and digestion process (Bertucci et al., 2019; Rønnestad et al., 2017). Regarding survival, corticortin-releasing hormone (Crh) is the main stress response-regulating factor in fish and is also related to anxiogenic, anxiolytic, and appetite-inhibiting roles (Matsuda, 2013; Volkoff et al., 2005). The most important neuropeptides involved in somatic growth regulation are the growth hormone (Gh) and insulin-like growth factors (Igf) as a decrease in the previous factors can inhibit growth and other physiological functions in fish (i.e., food intake) (Bertucci et al., 2019; Canosa & Bertucci, 2020; Reinecke et al., 2005; Saera-Vila et al., 2009). Other hormones are considerably implicated in the endocrine control of food intake and digestion. These factors act as appetite-stimulating factors (orexigenic peptides): neuropeptide Y (Npy), agouti-related peptide (Aggrp), ghrelin, orexin, melatonin-concentrating hormones 1 and 2 (Mch1 and Mch2); appetite-inhibiting factors (anorexigenic peptides): gastrin-releasing peptide (Grp) and cholecystokinin-8 (Cck8) (Bertucci et al., 2019; Koven & Schulte, 2012; Matsuda, Azuma, et al., 2012; Matsuda, Sakashita, et al., 2012; Rønnestad et al., 2017; Takahashi et al., 2016). Zebrafish is a widespread fish model used by biomedical and aquaculture research (Ribas & Piferrer, 2014). It is a eurythermal species that live in environments subjected to light and thermal fluctuations. For instance, in one of its natural environments, the River Ganges, both solar radiation and air thermal variation cause daily water temperature changes that can oscillate several degrees (between 0.1°C and 5.6°C) in the daytime (Payne et al., 1996). In zebrafish larvae, the effects of the light spectrum (from violet to red) and temperature regimes (constant vs. cycling) have been separately studied (Sánchez-Vázquez & López-Olmeda, 2018; Villamizar et al., 2012, 2014; Wang & Xia, 2019). In zebrafish, blue light elicited the best larval performance in terms of hatching, growth, survival, and occurrence of malformations while red lights presented the worst results, causing the death of all larvae during development (Villamizar et al., 2014). In addition, beneficial effects on early larval development were found in zebrafish reared under daily thermocycles compared to constant temperatures (Villamizar et al., 2012). Although these studies showed separately the profound effects of light spectrum and temperature regimes on zebrafish development, no study to date has evaluated the effect of the combination of both factors. Moreover, these previous studies did not delve into the underlying physiological mechanisms that generate such effects, neither the ones that cause the improvement observed under blue light or thermocycles nor the ones that cause the lethal effects of red light applied to zebrafish during development.

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

2.1 | Animals and housing

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

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

2.2 | Experimental design

The complete experimental procedure was repeated three times using different broodstock groups, which means that the presented results came from three independent experiments. In all the experiments, 1728 (N) embryos and larvae were employed. In a single independent experiment, 576 fertilized embryos were distributed into six different experimental treatments using a given combination of three different wavelengths (white, W, blue, B, and red, R) and two distinct temperature regimes (constant temperature vs. daily thermocycles) (Figure S1). For each experimental group, four Petri dishes (n = 96 embryos, 24 embryos per Petri dish) were utilized. Spectral analysis of lights was performed with a spectroradiometer (FieldSpec® Hand-Held spectroradiometer UV/VNIR; ASD Colorado) and a lux meter (MX Elektronik). This gave the spectral composition of each LED light expressed as the percentage of irradiance (white \(\lambda_{peak}=466\,\text{nm}\) and 668 nm; blue \(\lambda_{peak}=472\,\text{nm}\), and red \(\lambda_{peak}=665\,\text{nm}\) (Figure S1a). The photoperiod was set at 12 L:12D h (light:dark cycle) (lights on at 09:00 h) in all the groups. The two tested temperature regimes were: constant temperature (CTE) of 26°C (26.1 ± 0.1°C) or daily thermocycle (TC) with a thermophase of 28°C (27.9 ± 0.1°C) occurring in the daytime and the cryophase of 24°C (24.1 ± 0.1°C) occurring at nighttime (Figure S1b). Water temperature was modified by water heaters (Askoll) and cooler units (Aqua Medic Titan 1500 GmbH) controlled by an electronic timer (Bachmann GmbH & Co.). Water temperature was recorded continuously throughout the experiment by underwater data recorders (HOBO PENDANT; Onset Computer Corporation). Zebrafish embryos and larvae were reared under these conditions from 0 to 30 days post-fertilization (dpf). At 5 dpf, larvae were transferred from the Petri dish to 2.5-liter nursery net cages (SERA GmbH; Heinsberg). Larvae mouth opening occurred 2 days after hatching (5 dpf). From that time, they were fed powder feed (Vipan Baby; SERA) to satiety twice a day at 11:00 h and 16:00 h (Westerfield, 2000).

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

2.3 | Hatching and survival rates

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

2.4 | Growth rate and food intake (gut content)

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

2.5 | Gene expression analysis: Real-time reverse-transcription polymerase chain reaction (RT-PCR) analysis

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

2.6 | Statistical analysis

All the results are expressed as the mean ± SEM. The SPSS software (v. 19.0; IBM Analytics) performed the statistical analysis. The normality of data distribution was checked by the Kolmogorov–Smirnov test and homogeneity of variance by Levene’s test. The data from each gene and day were subjected to a one-way analysis of variance (ANOVA) to check for statistically significant differences between the six experimental groups, and to a two-way ANOVA to analyze the effects of light wavelength (W, B, R), temperature regimes (TC, CTE) and their interaction. The one-way ANOVA was followed by Duncan’s post hoc test to determine statistically significant differences between the experimental groups. The significance threshold was set at p = 0.05 in all statistical tests.

3 | RESULTS

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

At 3 dpf, all the larvae had either hatched or died during embryonic development, and the hatching rate was calculated on this day. The analysis detected statistically significant differences between lighting conditions (p = 0.044), but not between temperature regimes (p = 0.809) (two-way ANOVA, Table S1, Figure 2). The embryos left in B and W light obtained higher hatching rates (70.4 ± 5.1% and 69.2 ± 3.5%, respectively) than those reared in R light (57.9 ± 5.9% (two-way ANOVA, p < 0.05) (Figure 2).

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

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

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

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

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

| Gene  | F/R | Sequence (5′–3′)                      | GenBank Accession number |
|-------|-----|--------------------------------------|--------------------------|
| ef1a  | F   | CTGGAGGCCAGCTCAACCAT                 | NM_131263.1              |
|       | R   | ATCAAGAAGTAGTACGGCTTAGCATTAC         |                         |
| crh   | F   | GCCGGCGAAGTTCAAAA                    | BC085458.1               |
|       | R   | GCGAGGAGATCTGTGGCTAA                 |                         |
| gh    | F   | AAGATCAGGTTCGAAAGGTTGACTCA          | NM_001020492.2           |
|       | R   | TTAAGGCAAGTAATCTCATCAGACAGA          |                         |
| igf1a | F   | CAGGCAAATCTCAGCAGCTC                | AH010825.2               |
|       | R   | CTTTGTTGGCTGGAATATCTC               |                         |
| igf2a | F   | GTGAAGTCGGAGCACATTGTTG              | NM_131433.1              |
|       | R   | GAGCCTGTGACACTGGAAGA                |                         |
| npy   | F   | GACTTCACAGAAGGATATC                 | BC162071.1               |
|       | R   | GTTTGATGATGTGTCATTGCTG              |                         |
| agrp  | F   | TCGCACAGAAGATCAGACAAGAG             | NM_001328012.1           |
|       | R   | TAAACCCCGAGCCAATGTTG               |                         |
| ghrelin| F  | CAGCTTCCAGTCCGACTCA                | EU908735.1               |
|       | R   | TTTCTTCTGCCACTTTGTC                 |                         |
| orexin| F  | GTCGCCAGACATTAAGTGCATC              | NM_001077392.2           |
|       | R   | TTTCGCCCCATCTTAGTTGCC              |                         |
| mch1  | F  | CAAACCGCTAAAGCACAAGGC              | NM_001162488.1           |
|       | R   | AAAGTGCAACGGTATGAGAG               |                         |
| mch2  | F  | GCTGGCAAGGCTTGAATAGG              | FJ204828.1               |
|       | R   | TTGCAAGATCAGGGAGTGGGC             |                         |
| grp   | F  | GACAACACAGAGGTCACAAGCCTTT          | NM_001161350             |
|       | R   | ACTGGCGGCCTCTTTGCGAT               |                         |
| cck8  | F  | CAAAGGCTCATACGCCAGAAG             | XM_001346104             |
|       | R   | TCTGTGAGATGCACCACACATG             |                         |

**Abbreviation:** PCR, polymerase chain reaction.
3.3 Effects of light wavelength and thermocycles on gene expression

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

3.3.1 Genes involved in stress response

One of the processes that can be affected by light and temperature conditions is the stress response mediated by Crh, which is related to survival. The expression of \textit{crh} at 10 dpf showed statistically significant intergroup differences (Figure 6, left panel) (one-way ANOVA, \( p < 0.05 \)). The larvae reared in R + CTE presented a higher \textit{crh} expression than the...
other groups. Significant differences depending on temperature regime were also detected (two-way ANOVA, $p = 0.023$; Table S1), with the larvae reared in CTE displaying a higher crh expression than those in TC (Figure 6, left panel). No significant differences in crh expression were found at 30 dpf (Figure 6, right panel) (Table S1).

### 3.3.2 | Genes involved in growth

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

At 30 dpf, gh expression also differed between light treatments, with B inducing a higher expression than W (Figure 7a, right panel) (two-way ANOVA, $p < 0.05$). The statistical analyses also revealed a significant effect of temperature regime because the larvae in TC showed higher gh expression than those in CTE (two-way ANOVA, $p < 0.05$).

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

Finally, igf2a expression at 10 dpf was significantly higher in all the groups reared in W and B than in the groups reared in R (Figure 7c, left panel) (one- and two-way ANOVAs, $p < 0.05$). For the 30 dpf larvae, igf2a was higher in the larvae in W + TC than in the W + CTE and B + CTE larvae (Figure 7c, right panel) (one-way ANOVA, $p < 0.05$). The statistical analyses also revealed a significant effect of temperature regime because the larvae in TC showed higher igf2a expression than those in CTE (two-way ANOVA, $p < 0.05$).
FIGURE 6  Relative mRNA expression of crh of the 10 (left panel) and 30 (right panel) days post fertilization (dpf) zebrafish larvae reared in three different light wavelengths (white, W; blue, B; red, R) and in two temperature regimes (a thermocycle of 28:24°C thermophase:cryophase, TC; and constant temperature of 26.1 ± 0.1°C, CTE). Different lower case letters indicate significant differences between the experimental groups on the same dpf (one-way ANOVA, p < 0.05). Different upper case letters and asterisks denote significant differences between the light treatments and rearing temperature regimes, respectively, on the same dpf (two-way ANOVA, p < 0.05). Data (n = 6) are represented as mean ± SEM. ANOVA, analysis of variance; mRNA, messenger RNA

FIGURE 7  Relative mRNA expression of gh (a), igf1a (b), and igf2a (c) of the 10 (left panels) and 30 (right panels) days post fertilization (dpf) zebrafish larvae reared in three different light wavelengths (white, W; blue, B; red, R) and in two temperature regimes (a thermocycle of 28:24°C thermophase:cryophase, TC; and constant temperature of 26.1 ± 0.1°C, CTE). Different lower case letters indicate significant differences between the experimental groups on the same dpf (one-way ANOVA, p < 0.05). Different upper case letters and asterisks denote significant differences between the light treatments and rearing temperature regimes, respectively, on the same dpf (two-way ANOVA, p < 0.05). Data (n = 6) are represented as mean ± SEM. ANOVA, analysis of variance; mRNA, messenger RNA
As the different treatments affected food intake, we evaluated the mRNA expression of several hormones involved in the endocrine control of food intake and digestion. These factors intervene as either appetite-stimulating factors (orexigenic peptides: npy, agrp, ghrelin, orexin, mch1, and mch2) or appetite-inhibiting factors (anorexigenic peptides: grp and cck8).

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

At 30 dpf, the expression of the analyzed genes involved in food intake control followed similar patterns. All the genes showed significant intergroup differences (Figure 8, right panels) (one-way ANOVA, p < 0.05; Table S1). The B + TC-reared larvae showed a higher expression than the other groups (B + CTE, W + TC, and W + CTE) in all the analyzed genes, except for npy, for which differences were only found between B + TC versus W + CTE and B + CTE (Figure 8a).

The two-way ANOVA also revealed a significant effect of lighting conditions on ghrelin, grp, and cck8 (Figure 8c,g,h) (p < 0.05; Table S1). The expression levels of these genes were higher in the larvae in B light than in W. All the genes except for agrp showed a significant effect of temperature regime, with the groups in TC displaying higher expression levels than in CTE (Figure 8, right panels) (two-way ANOVA, p < 0.05).

4 | DISCUSSION

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

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

The present paper revealed that zebrafish larvae showed a higher survival rate for the B and W light spectra than for R. Indeed, all the larvae reared in light R died while developing. This implies that light wavelength is one of the main factors for survival rates. The light spectrum effect during early fish development seems species-specific. In Senegalese sole and Russian Sturgeon (Acipenser baerii), short wavelengths increase survival as in zebrafish (Blanco-Vives et al., 2010; Ruchin, 2016; Villamizar et al., 2014). In contrast, European eel (Anguilla anguilla) survival is enhanced by long wavelengths (Politis et al., 2014). Regarding the temperature regime, the effects of daily thermocycles on fish survival have been poorly studied to date. In the present research work, daily thermocycles were relevant on the first days of larval development. Thermocycles enhanced the survival of the larvae reared in R light because those reared with daily thermocycles died later than those maintained at a constant temperature. However, thermocycles were not enough to counteract the deleterious effects of red light. In Senegalese sole and brook trout (Salvelinus fontinalis), beneficial effects of thermocycles in early developmental stages have been described: they increased survival rate and lowered the incidence of malformations (Blanco-Vives et al., 2010; Pisano et al., 2019).

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

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

Very little information is available about how the combination of different light spectra and thermal regimes can act on fish growth. In the present study, short wavelengths stimulated the mRNA expression levels of somatic growth factors (gh, igf1a, and igf2a), which correlated with a higher growth rate, while long wavelengths reduced the expression of growth factors and negatively influenced the growth rate, and probably other physiological functions (Canosa & Bertucci, 2020; Reinecke et al., 2005; Saera-Vila et al., 2009).
The stimulatory effect of short wavelength on growth factors has also been recently described in yellowtail clownfish (Amphiprion clarkii) (Shin et al., 2012), goldfish (Carassius auratus) (Shin & Choi, 2014), barfin flounder (Verasper moseni) (Takahashi et al., 2004, 2016; Yamanome et al., 2009), and zebrafish (Villamizar et al., 2014). In other studies performed in rainbow trout and European sea bass, the somatic growth factors were stimulated in red light (Karakatsouli et al., 2007, 2008; Yan et al., 2019). Apart from the light wavelength, water temperature is also a determining factor that directly affects embryonic and fish larval growth. This environmental cue can modify the plasma levels and mRNA expression of GH and IGFs (Gabillard et al., 2005). In most fish species, higher temperatures increase the expression of GH and IGF1, which fluctuates in parallel to the growth rate. However, cold temperatures decrease the expression profiles of growth factors (for a review, see Deane & Woo, 2009). In the natural environment, seasonal water temperature changes affect the activities of metabolic enzymes and expression profiles by increasing the growth rate and the mRNA expression at the GH level, and in warmer months compared to colder months (for a review, see Deane & Woo, 2009). In addition to seasonal water temperature changes, many fish in nature undergo daily temperature fluctuations. The results of the present research work suggest that thermocycles can enhance the growth rate by upregulating the expression of several growth factors, but more studies are necessary to test this hypothesis.

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

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

5 | CONCLUSION

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

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

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CONFLICT OF INTERESTS
The authors declare that there are no conflict of interests.

AUTHOR CONTRIBUTIONS
Gonzalo de Alba, Sherezade Carrillo, José Fernando López-Olmeda, and Francisco Javier Sánchez-Vázquez conceived and designed the experiments, and wrote the manuscript; Gonzalo de Alba and Sherezade Carrillo analyzed the data; Francisco Javier Sánchez-Vázquez and Francisco Javier Sánchez analyzed the data; Gonzalo de Alba and Sherezade Carrillo performed the experiments; Gonzalo de Alba, Sherezade Carrillo, and José Fernando López-Olmeda analyzed the data; Francisco Javier Sánchez-Vázquez and José Fernando López-Olmeda provided funding.

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

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