Effect of daily thermo- and photo-cycles of different light spectrum on the development of Senegal sole (*Solea senegalensis*) larvae.

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Abstract.

This paper investigates the impact of different thermo- and photo-cycles of distinct wavelengths on *Solea senegalensis* larvae from day 1 to 30 post-hatching. In experiment 1, larvae were exposed to 12 h light:12 h dark (12L:12D) cycle and (A) constant temperature (20.7ºC), (B) thermocycle of 12h thermophase: 12h cryophase, 22.1ºC day: 19.0ºC night (referred to as TC) or (C) 12h cryophase: 12h thermophase, 19.2ºC day: 22.0ºC night (referred to as CT). In experiment 2, larvae were kept under constant temperature (20.8ºC) and exposed to (A) continuous light (LL), (B) continuous darkness (DD), and LD 12L:12D cycles of (C) white light (LD_W), (D) blue light (LD_B) or (E) red light (LD_R). The sole larvae achieved the best performance, and showed fastest development and lowest degree of deformity under natural thermocycle conditions (TC) with a deformity percentage of 31.1% and LD_B cycles with 27.7% of malformation, conditions which were nearest their natural aquatic environment. Larvae reared under TC started eye migration at 9 day post-hatching (DPH), while larvae exposed to CT started eye migration at 11 DPH. In larvae under the LD_B treatment the migration of the eye started earlier than in the other treatments (9 DPH), while larvae reared under LL and DD photoperiods died before metamorphosis. These findings highlight the importance of light and temperature cycles during the early development of *Solea senegalensis* larvae, which should be taken into consideration in experimental or rearing protocols.

Keywords: Temperature, Light spectrum, Metamorphosis, Senegal sole larvae.

1. Introduction

In the natural environment, daily temperature and light cycles are key synchronising factors for fish. The Earth's rotation imposes a day/night light cycle which fish have to cope with in order to survive. Moreover, the cyclic infrared radiation from the Sun generates a thermocycle: during the day the temperature rises (the thermophase, or phase of higher temperature), while during the night the temperature drops (the cryophase, or phase of lower temperature). Thus, transitions from cold to warm temperature are roughly associated with dawn, and transitions from warm to cold temperature with dusk (Johnson et al., 2004). However, under artificial rearing conditions, the environmental conditions are set by fish farmers to try to ensure fish survival and growth, which has been documented in many aquaculture species (Barahona-Fernandes, 1979; Tandler and Helps, 1985; Batty, 1987; Downing and Litvak, 1999). Little attention has been paid to the influence of these "unnatural" environmental conditions (constant lighting and temperature) and light characteristics of artificial globes (incandescent and halogen globes) in shallow tanks, which would have more red/yellow in comparison to light conditions underwater lit by natural sunlight, although they have recently been reported to affect the rhythmic development of larvae of fish models such as zebrafish (Vallone et al., 2007).

The water column acts as a chromatic filter, so that the aquatic environment changes the spectral composition of incident light. There is rapid attenuation with depth, so blue wavelengths become predominant in all but the most shallow or turbid waters (Jerlov, 1968). However, standard lighting systems (bulbs and fluorescent lamps) commonly used in hatcheries are shifted towards the red wavelengths and create bright points, light sources that are not environment-specific and could compromise fish

welfare. Besides, inappropriate light intensity can affect fish by provoking skeletal abnormalities (Villamizar et al., 2009).

The metamorphosis in flatfish is related to the change from the pelagic to benthic habitat, and it implies important changes in fish physiology (Fernandez-Díaz et al., 2001). The transformation occurs at a wide range of sizes depending on species and environmental circumstances (Policansky, 1982; Ottesen and Bolla, 1998). Whether age or size are key factors in starting metamorphosis is a question that has been considered in several studies with other species, such as Atlantic halibut larvae (Ottesen and Bolla, 1998). In addition, this question is important in laboratory populations in which growth and therefore, the size of larvae and successful metamorphosis depend on the rearing conditions (Fernandez-Díaz et al., 2001).

Temperature has long been reported to influence the growth and development of fish larvae both in the wild and laboratory (Methot and Kramer, 1979; Pepin, 1991; Green and Fisher, 2004; Johnston et al., 2004), and there are reports that have studied thermal effects on embryonic development in flatfish larvae and juveniles, although without temperature control (Buckley, 1982; Daniels et al., 1996). Moreover, we are not aware of any published information about the effect of daily thermocycles on fish larvae.

The Senegal sole *Solea senegalensis* Kaup (1858) is a flatfish adapted to temperate waters of around 20-21ºC (Drake et al., 1984). It is extensively exploited in aquaculture, mostly in Spain and Portugal (Dinis et al., 1999). Substantial progress has been achieved in its domestication, though suitable reproduction and zootechnical optimal conditions are still to be determined (Porta et al., 2007). Due to the interest in this species for aquaculture and its characteristic embryonic development

(metamorphosis process), this species was selected as our experimental model to investigate the effects that temperature and light cycles may induce in larval survival, growth, development and on the onset/offset of metamorphosis. To this end, sole larvae were subjected to different temperature cycles and they were exposed to different photoperiods and light spectra.

2. Material and methods

2.1. Animals and housing

Fertilised eggs of Senegal sole were provided by the Spanish Oceanographic Institute (IEO) at Santander (Cantabria) and sent to the Aquaculture Institute in Torre la Sal (IATS-CSIC, Castellón) and the University of Murcia (UMU, Fish Chronolab, Murcia). Wild broodstock were distributed into 14 m^3 tanks, in a female:male proportion of 1:1. The tanks were covered with a shadow net that provided 0.21 W m^{-2} (50 lux) on the surface of water. The fish were fed five days a week, three days with mussels and two days with small cuttlefish; one of the days on which they were fed with mussels, frozen polychaetes were added (Sebait Ltd., UK). Water temperature varied between 16 and 19ºC (imitating nature fluctuations of temperature) and the photoperiod was 12L:12D. An egg collector was placed at the water outflow of the tank. Once fertilised eggs were collected, they were incubated in 70 L incubators under continuous darkness until hatching. On 1 DPH they were transported under darkness conditions, and the transport had duration of 12 hours approximately.

To feed the larvae, *Brachionus picatilis* rotifers were cultured and enriched with commercially available freeze-dried green algae *Nannochloropsis* sp. (Phytobloom Prof® Necton, Portugal) in a proportion of 300,000 cells/ml/day from day 3 to day 7.

These enriched rotifers were added to tanks daily as an early live food at a density of 20 individuals mL^{-1} from 3 to 7 DPH. *Artemia* sp. nauplii at a density of 2–3 nauplii mL^{-1} day^{-1} were introduced from 8 to 30 DPH. Before being fed to the larvae, the nauplii were enriched with a mixture (INVE DC DHA Selco®). Three to five metanauplii mL^{-1} day^{-1} were added from 27 to 30 DPH. Before being fed to the larvae, the metanauplii were enriched with a mixture (ORI-GO, ORI-PRO®, Skretting AS, Spain) of phytoproteins and highly unsaturated fatty acids (HUFA) during 24 hours.

2.2. Experimental design

The experiments performed in the present research followed the Spanish legislation on Animal Welfare and Laboratory Practices. The experimental protocol was approved by both the National Committee on Animal Welfare and the Committee of the University of Murcia.

2.2.1. Experiment 1. Effect of temperature.

This trial was performed at IATS-CSIC. Spawned egg volume was 120 mL, with a fertilization rate of 73% and hatching rate of 78.1%. The larvae were distributed into six 500 L cylinder-conical tanks at 1 DPH and at a density of 50 larvae L^{-1} to study the effect of temperature. The water system was semi-closed with a flow of 50 L/h exchanged seawater in each tank per day. Thermal cycles had a duration of 12 hours, the photoperiod was 12L:12D and light intensity was 0.84 W m^{-2} (200 lux) supplied by a lamp (PHILIPS, HPL N 250W). The spectral analysis of the lights was performed using a spectroradiometer (FieldSpec® Hand Held spectroradiometer UV/VNIR from ASD Colorado, USA, with a wavelength range of 325 nm to 1075 nm at 1.6 nm

intervals) and a lux meter (MX Elektronik Minilux, Germany, with 6 measuring ranges from 2 lux to 200 klux,).

From 1 to 30 DPH, three temperature regimes with two replicates were applied: TC cycle (22.1±0.6ºC day/19.0±0.4ºC night, mean±S.D, n=60, here and throughout); CT cycle (19.2±0.5ºC day/22.0±0.3ºC night) and constant temperature (20.7±0.4ºC,). Water temperature was modified by means of water heaters (Themal Compact 100 W, Askoll, Italy) or coolers (Cubigel, E-500, Spain). Temperature was continuously recorded with an underwater sensor and data logger (HOBO PENDANT® Onset Computer Corporation, Massachusetts, USA) placed in each of the tanks (Fig. 1TC, 1CT).

2.2.2. Experiment 2. Effect of photoperiod and light spectrum.

This experiment was carried out at UMU. The fertilised eggs were collected in complete darkness to ensure fish larvae did not receive any light before treatment. Spawned egg volume was 160 mL, with a fertilization rate of 98.1% and hatching rate of 77.6%. The larvae were distributed into ten 80 L aquaria with flat bottoms at 1 DPH and at a density of 18 larvae L^{-1} to study the effects of different light spectra. The larvae were kept under constant temperature (20.8±1.3ºC). The static closed water system used filtered artificial seawater by means of a biological filter EHEIM 2227 and using a bio-balls filter system, filtering surface ratio being approximately 1 L bio-balls / 10 L of water.

From 1 to 30 DPH, five lighting regimes with three replicates were applied: 12L:12D cycle with red (LD_R, half-peak bandwidth 592-668 nm), blue (LD_B, half-peak bandwidth 435–500 nm) and white (LD_W), 24L:0D white (LL), and 0L:24D (DD). The

163 white light had a broad spectrum, with 95% irradiance within the range of 367–757 nm.
164 The spectral analysis of the lights was performed using a spectroradiometer
165 (FieldSpec®, ASD, Colorado, USA) (Fig. 1A, 1B, 1C). To avoid the effects of any
166 background light on the experiments, the experimental tanks were covered with a light-
167 proof, black screen. For the different spectral trials, lamps were constructed using light
168 emitting diodes (LEDs) mounted on fibreglass plaques (160×232 mm). Each red and
169 blue lamp had 17 homogeneously distributed LEDs. White lamps had 18 white and 4
170 red LEDs to produce a broader range wavelength. Each lamp was encased in a
171 waterproof container which hung approximately 50 cm above the water surface. The
172 lamps were powered by a 3 V DC supply connected to a variable resistor (0–2 kΩ) that
173 allowed the light intensity to be adjusted to 0.42 W m^{-2}, which is low but well above the
174 light threshold (0.053 W m^{-2}) required to modify melatonin contents in both the eye and
175 plasma in Senegal sole (Oliveira et al., 2007). In addition, previous observations have
176 shown that sole are capable of feeding in the dark from the very early hatching stage
177 (Blaxter, 1969).

178 ***2.3. Data collection and analysis***

179 Every two days from 1 to 30 DPH, 60 larvae from each treatment were
180 arbitrarily chosen to measure only total length (making a straight measurement from the
181 tip of the head to the tip of the longest lobe of caudal fin) and evaluate metamorphosis
182 (the criteria for defining the beginning and the finishing of metamorphosis was the eye
183 migration) and to detect physical abnormalities focusing on jaw elongation (jaw
184 malformations). In addition, at 3, 5, 7, 9 and 11 DPH, yolk sac dimension were
185 measured in these larvae. Yolk volume was calculated using the formula $V=\pi/6\ l\ h^2$,
186 where l is yolk sac length and h is yolk sac height (Bagarinao, 1986). Larvae were killed

on ice and fixed with 5% formaldehyde and were measured using a digital camera mounted on a stereoscopic microscope EZ4D. Samples were photographed and calibrated measurements were made using image processing and analysis software (Leica Microsystems Imaging Solutions Ltd, Cambridge, UK.).

To find statistical differences in growth, development and jaw malformation among treatments, all data were tested for normality (Student's *t*-test) by examining residual values and all data were found to be normal. Then, a one-way ANOVA was used to analyse all treatments. Yolk sac volumes were analysed by one-way ANOVA to establish statistical differences among treatments and time. Significant ANOVA analyses were followed by the Duncan's post hoc test to determine differences between means, with $P<0.05$ taken as the statistically significant threshold (SPSS 15.0 for Windows). Data are expressed as mean±S.D. values.

3. Results.

3.1. Experiment 1. Effect of temperature.

3.1.1. Growth.

Initially, there were no significant differences in growth in larvae from different treatments until the day on which metamorphosis started. At 13 DPH however, larvae under CT (19ºC day/22ºC night) showed lower growth than those under the other treatments. Towards the end of the metamorphosis (23 DPH) and in first few days post-metamorphosis, larvae under TC showed significantly higher than those under constant temperature ($F=5.37$, df= 2, $p=0.033$) and turn significantly higher ($F=4.55$, df= 2, $p=0.026$) than those under CT ($F=4.67$, df= 2, $p=0.031$). At 30 DPH, TC larvae reached a size of 8.5±0.5 mm, significantly higher than those under constant temperature at

7.7±0.2 mm which were in turn significantly than those under CT 7.2±0.2 mm (F=4.35, df= 2, p=0.023, Fig. 2).

3.1.2. Yolk sac.

The larvae which most quickly absorbed the yolk sac were those exposed to TC cycles and constant temperature. By 5 DPH they had already absorbed large part of the yolk sac, unlike the CT (F=5.36, df=2, p=0.036, Fig. 3). On 9 DPH larvae exposed to TC and those under constant temperature had already completely absorbed the sac. In contrast, larvae kept under a CT cycle showed slower yolk sac absorption, completed at 11 DPH.

3.1.3. Metamorphosis.

Significant differences in the onset and finish of metamorphosis were detected among treatments. Larvae reared under TC started eye migration at 9 DPH and 100% finished the process at 17 DPH, larvae under constant temperature started at 11 DPH and 100% finished at 17 DPH, while larvae exposed to CT started eye migration at 11 DPH and 100% finished at 19 DPH (F=5.4, df=2, p=0.022, Fig. 4A).

Larvae from the TC group which finished eye migration earliest (23 DPH) (F=6.28 df=2, p=0.028, Fig. 4B). In the other two treatments the process did not conclude until 25 DPH (F=9.51, df=2, p=0.035, Fig. 4B).

3.1.4. Jaw malformation.

From day 9 DPH jaw malformation was significantly higher in CT than in the other treatments. The larvae with the lowest percentage of malformation at 30 DPH were those exposed to TC (45.2±3.2%) and larvae submitted to constant temperature

(48±3.3%), but those under CT had a malformation percentage of 67±2.8% (F=4.55, df=2, p=0.026, Fig. 5).

3.2. Experiment 2. Effect of photoperiod and light spectrum.

3.2.1. Growth.

Larvae exposed to different lights showed differences in size before metamorphosis began, those exposed to blue light (LD_B) being longer than the larvae from the other two light colour treatments (LD_W and LD_R) from 9 DPH onwards, while the larvae under complete darkness showed a lower growth during the experiment (from 2 mm at 3 DPH to about 3.5 mm at 13 DPH). The larvae reaching the largest size at 30 DPH were those under blue light (LD_B), which reached 9.9±0.4 mm long, followed by those exposed to white light (LD_W) which reached 8.3±0.6 mm, and finally the larvae under red light (LD_R) that reached 7.2±0.4 mm (F=3.72, df= 2, p=0.028, Fig. 6).

3.2.2. Yolk sac.

The larvae which most quickly absorbed the yolk sac were those exposed to LD_B, which showed significantly lower volume than LL and DD, but not other light colour treatments. By 7 DPH they had already absorbed a large part of the yolk sac, unlike the other treatments (F=4.05, df=4, p=0.041, Fig. 7). Larvae exposed to LL and DD absorbed the sac more slowly, and the yolk sac had not disappeared by 9 DPH.

3.2.3. Eye migration and completed metamorphosis.

In larvae under the LD_B treatment the migration of the eye started earlier than in the other treatments, beginning at 9 DPH, while in the others groups, the migration started at 11 DPH. (F=5.12, df=4, p=0.015, Fig. 8A).

254 The groups of larvae under continuous darkness (DD) and continuous light (LL)
255 died 15 and 17 DPH respectively at the beginning of metamorphosis. At 25 DPH, 100%
256 of larvae in the LD_B group had completed metamorphosis (F=5.03, df=2, p=0.039, Fig.
257 8B), earlier than the other groups, where metamorphosis was not complete until 27 DPH
258 (F=4.23, df=2, p=0.021, Fig. 8B).

259 ***3.2.4. Jaw malformation.***

260 Jaw malformation in larvae at 30 DPH from LD_B (27.7±2.9%) was significantly
261 lower than LD_W (45.6±3%), in turn significantly lower than LD_R (70.6±2.3%) (F=4.68,
262 df=2, p=0.031, Fig. 9). Larvae under LL and DD treatments had 43.3±3.3% and
263 26.7±2.3% of malformation respectively from early in the experiment (5 DPH),
264 reaching 57.8±3.9% and 66.6±2.8% a few days before they died.

265 ## 4. Discussion

266 Different conditions of temperature, photoperiod and light spectrum affected the
267 development of Senegal sole larvae, driving growth performance and probably
268 provoking malformations. Larvae exposed to TC (experiment 1) and LD_B (experiment
269 2) cycles provided the best results in terms of survival, growth and malformations.
270 These experimental conditions closely approached natural environmental conditions: in
271 the open sea, the water column acts as a chromatic filter, quickly absorbing red
272 wavelengths, so that, temperature decreases and blue wavelengths become predominant
273 as depth increases (Jerlov, 1968). In flatfish larvae the environmental factors may be
274 crucial in determining recruitment to nursery grounds via their effects on growth and
275 mortality during the metamorphosis-settlement period (Yamashita et al., 2001).

Some studies have demonstrated the existence of diel rhythms in temperature selection in fish maintained in wild conditions. In these studies, fish showed diel migrations as they searched for a preferred temperature for physiological activity and growth (Gibson et al., 1998; Sims et al., 2006). The present paper revealed that Senegal sole larvae showed higher growth, lower jaw malformation and faster absorption of the yolk sac under TC, and worse development under CT or constant temperature. Ottesen and Bolla (1998) reported that some physical parameters, including inappropriate temperature or salinity, may be associated with jaw malformation due to mechanical damage in cultured larvae. Under TC metamorphosis started earlier when the migration of the eye began, and were first to finish. In the other two treatments, the process of metamorphosis was slower, specially in larvae exposed to CT. Most studies about biological development and temperature in Senegal sole have used a constant temperature of 20ºC (Martínez et al., 1999; Parra and Yúfera, 1999; Ribeiro et al., 1999; Yúfera et al., 1999; Cañavate et al., 2006), but little attention has been paid to the effects that temperature fluctuations in natural environment may cause in *Solea senegalensis*. The first study on temperature selection in fish was conducted in goldfish, in which the animals showed a diel pattern of temperature selection (Reynolds et al., 1978), which seemed to be related to body weight gain and gonadal growth (Spieler et al., 1977).

Through the rhythmic secretion of its hormone melatonin, the pineal organ of fish transducers photoperiod information (Ekström and Meissl, 1997). However, different light spectra do not affect melatonin production equally. As first evidenced in sea bass, the application of blue light during the dark phase inhibits nocturnal melatonin, while red light does not (Bayarri et al., 2002). Later, other studies have

300 reported that different light thresholds were required for sea bass (between $3.8x10^{-3}\mu W/cm^2$ and $3.8x10^{-4}\mu W/cm^2$) and Atlantic salmon (between $3.8x10^{-2}\mu W/cm^2$ and $3.8x10^{-3}\mu W/cm^2$) to be perceived as night, measured by a significant increase in melatonin concentrations during the dark phases (Migaud et al., 2006) and it was demonstrated that light sensibility varies with the species. In Senegal sole, a recent paper reported a different effect of the light spectrum (red, violet and white lights) on plasma melatonin, with shorter wavelengths having the biggest impact (Oliveira et al., 2007). These observations agree with our results that larvae responded best to blue light (LD_B), which appeared to be the most efficient light spectrum on the development on Senegal sole larvae.

Several studies have found a correlation between extended photoperiod and increased growth in teleost fish larvae such as *Sparus aurata* (Tandler and Helps, 1985), *Gadus morhua* (Puvanendran and Brown, 2002), *Latris lineata* (Trotter et al., 2003) and *Solea senegalensis* (Cañavate et al., 2006), and the same occurred in our experiment. Other studies, such as those carried out by Villamizar et al. (2009) with European sea bass, found no significant differences in total length between larvae exposed to either LL or LD_B cycles. Contrarily, we observed significant differences in size in our study. However, our results are supported by those obtained by Barahona- Fernandes (1979), which showed that continuous light in sea bass larvae did not induce the best growth. Downing (2002) found no relationship between growth and spectral composition of light in haddock larvae, while our experiment showed the highest growth in terms of length in those larvae reared under LD_B treatment.

The relationship between light conditions and malformations has been studied in recent experiments. For instance, Cobcroft and Battaglene (2009) found a relationship

between the colour of the rearing tank and the appearance of jaw malformations in *Latris lineata*, the larvae reared in a red tank showing the highest incidence of severe jaw malformations. Walling behaviour has been reported in Atlantic halibut larvae, where high light conditions are associated with walling and abrasion of larval jaw tissues on the tank wall (Morrison and MacDonald, 1995). This finding is in agreement with of our results in which LD_R, together with LL and DD, showed the highest proportion of jaw malformation. Moreover, Villamizar et al. (2009) also reported jaw malformation and swim bladder hypertrophy in sea bass larvae under LL. The exact mechanism by which light affects malformations is unknown, but there are links between larval behavioural changes in response to light and other factors that implicate contact with walls (Morrison and MacDonald, 1995; Cobcroft and Battaglene, 2009).

Studies carried out by Geay et al. (2009) reported that an excess of retinoic acid increases craniofacial malformation in fish during development and Villeneuve et al. (2006) observed skeletal deformities in larvae fed high levels of vitamin A, which has an essential role in retinal function, together with carotenoids. Other study in Senegal sole related light intensity and diets supplemented with β-carotene, observing effects in antioxidant biomarkers (Cañavate et al., 2007). In addition, light intensity may provoke high proportion of malformations due to the mainly visual feeding activity of marine fish larvae (Blaxter, 1986). Recently, Villamizar et al. (2009) reported that light spectrum and photoperiod influenced feeding behaviour of sea bass larvae. For this reason, the fact that LD_R, LL and DD treatments led to a higher level of malformations in our experiment may therefore have been due to impaired photoperiod and light spectrum. Further studies, however, are necessary to confirm whether a single culture

parameter (including subnutrition) or an interaction of physical parameters can contribute to the appearance of jaw malformation.

Since there seems to be a relationship between light and the exhaustion of endogenous sources, commercial hatcheries apply continuous darkness or dim light conditions to decrease the use of endogenous reserves and delay the start of exogenous feeding (Downing and Litvak, 1999). This agree with our results: larvae exposed to DD and LD_R exhibited a delay in growth and thus the yolk sac was visible, while in the LD_B, LD_W and LL treatments the yolk sac was completely absorbed. These results are also consistent with those obtained by Villamizar et al. (2009) using a similar experimental design in sea bass larvae, in which the yolk sac in DD and LD_R was still visible up to 48 h after its complete absorption in the LL, LD_W and LD_B groups. Parameters such as stocking density, salinity, feeding or light have been observed to affect the development and metamorphosis process in flatfish (Bolla and Holmefjord, 1988; Daniels et al., 1996). In the present study, the LD_B treatment provoked faster development in larvae than the other treatments, and these larvae completed the metamorphosis process several days before those under different light spectra.

The circadian system of fish develops quickly during the first stages of larval development, so the pineal organ becomes light sensitive before the lateral eyes and participates in photoperiod-controlled events such as hatching (Kazimi and Cahill, 1999). In zebrafish, the key enzyme controlling melatonin production, AA-NAT, is expressed at the blastula stage from the first day post-fertilisation onwards (Ziv and Gothilf, 2006). Furthermore, in zebrafish larvae, lighting conditions were seen to influence the development of circadian clock gene expression, the clock starting ticking under LD cycles but not under constant darkness (Kaneko and Cahill, 2005). In short,

light and temperature cycles are required so that the rhythmic circadian clock works properly. In fish, the circadian clock mature extremely early during larval development and are thought to regulate temporal co-ordination of many physiological processes (Vallone et al., 2007). Differences in the development of the circadian system of sole in our experimental conditions may explain the differences found in the experimental treatments. Ongoing research aims to characterise clock gene expression in sole larvae, which seems to appear also very early (Muñoz-Cueto, personal communication) and is most likely influenced by light and temperature cycles during sole ontogeny.

In conclusion, our experiments revealed that the early development of Senegal sole larvae is affected by both, temperature and lighting conditions, larvae under TC and LD_B cycles showing the best survival and performance, and the lowest incidence of jaw malformations. "Unnatural" or inappropriate light and temperature conditions seriously compromised the welfare of sole larvae and negatively affected their development. Thus, natural temperature and light cycles of blue wavelength appeared optimal conditions for the culture of this species, since they were nearest those of the natural underwater environment; such conditions seem to be a prerequisite for the proper maturation of the biological clock and the physiology of the larvae of this flatfish species. The potential benefits of TC and LD_B during larval development, however, remain to be tested in other fish species.

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547 Figure Captions.

548 Figure 1. Treatment conditions in experiment to assess performance of Senegal sole
549 larvae. Daily mean temperature of each treatment during the experiment: TC)
550 thermocycle of 12h thermophase: 12h cryophase; CT) 12h cryophase: 12h thermophase.
551 Spectral composition of each LED lamp expressed as the percentage of irradiance: A)
552 LD_B (λ=435–500 nm); B) LD_R (λ=592– 668 nm); C) LD_W (λ=384– 728 nm).

553

554 Figure 2. Total length (mm) of Senegal sole larvae with age under different
555 thermocycles. Data are expressed as mean±SD. Different letters indicate means within
556 age significantly different from each other.

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558 Figure 3. Yolk sac absorption in Senegal sole larvae reared under different temperatures
559 from 3 DPH. Data are expressed as mean±S.D. Different letters indicate statistically
560 significant differences from each other.

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562 Figure 4. Evolution of the metamorphosis process in Senegal sole larvae exposed to
563 different thermocycles. A) Percentage of larvae that started metamorphosis between 9
564 DPH and 19 DPH. Inset: larva premetamorphosis. B) Percentage of larvae that
565 concluded metamorphosis from 17 DPH to 30 DPH. Inset: metamorphosed larva. The

scale bar corresponds to 1 cm. Data are expressed as mean±SD. Different letters indicate means within age significantly different from each other.

Figure 5. Effect of temperature on jaw malformation of Senegal sole larvae with larval age. Black arrow in the picture indicates jaw malformation. Data are expressed as mean±S.D. Different letters indicate statistically significant differences from each other. The scale bar corresponds to 0.5 cm.

Figure 6. Total length (mm) of Senegal sole larvae with age under different light spectra and photoperiods. Data are expressed as mean±SD. Different letters indicate statistically significant differences from each other.

Figure 7. Yolk sac absorption in Senegal sole larvae with age reared under different light spectra and photoperiods from 3 DPH. Data is expressed as mean±S.D. Different letters indicate statistically significant differences from each other.

Figure 8. Evolution of the metamorphosis process in Senegal sole larvae with age exposed to different photoperiods and light spectra. A) Percentage of larvae that started metamorphosis between 9 DPH and 19 DPH. Inset: larva premetamorphosis. B) Percentage of larvae that concluded metamorphosis from 17 DPH to 30 DPH. Inset: metamorphosed larva. The scale bar corresponds to 1 cm. Data are expressed as mean±SD. Different letters indicate means within age significantly different from each other.

589 Figure 9. Effect of light spectrum and photoperiod on malformation of Senegal sole
590 larvae during the experiment. Black arrow in the picture indicates jaw malformation.
591 Data are expressed as mean±S.D. Different letters indicate statistically significant
592 differences from each other. The scale bar corresponds to 0.5 cm.

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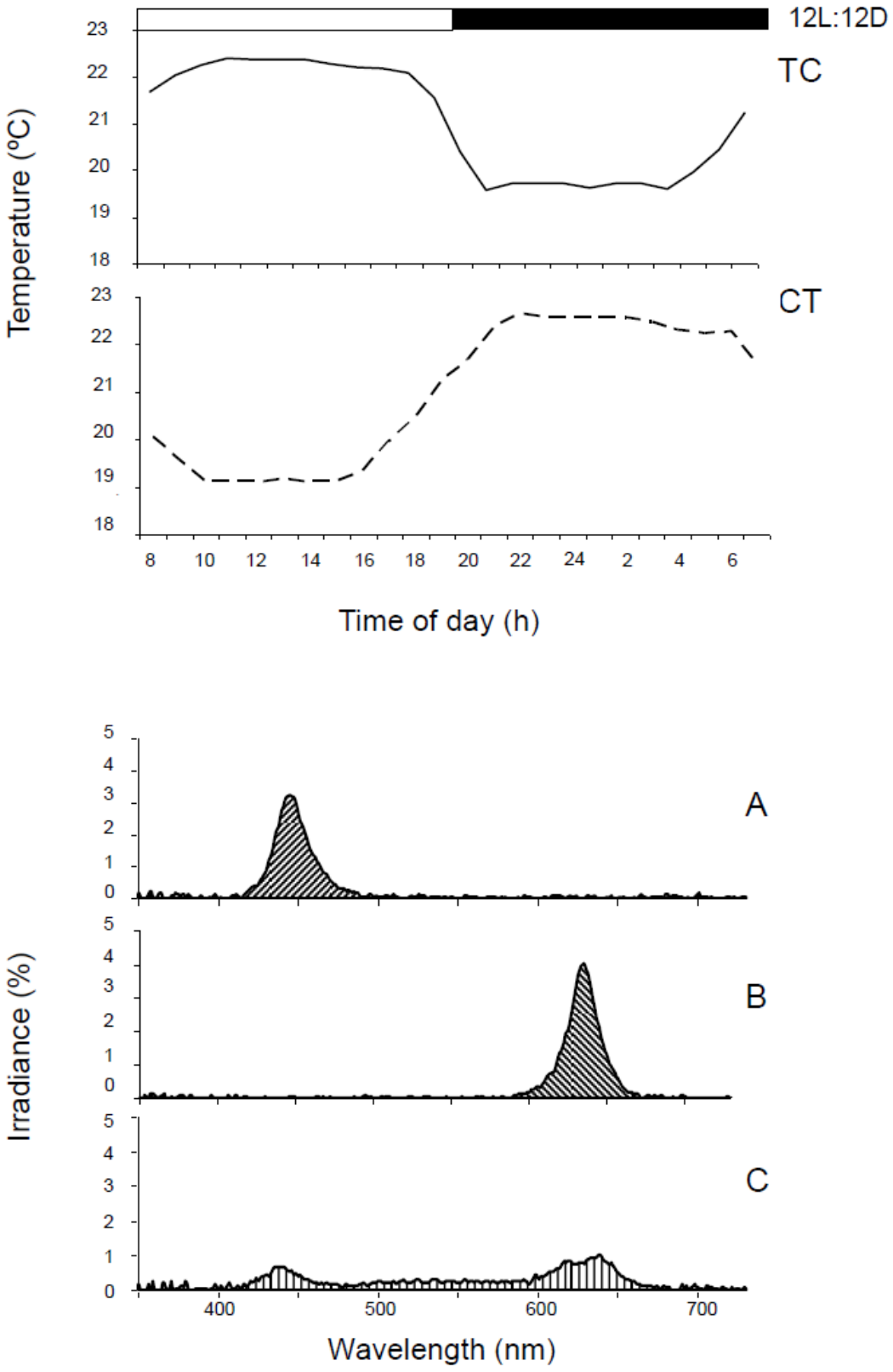
608 Figure 1.



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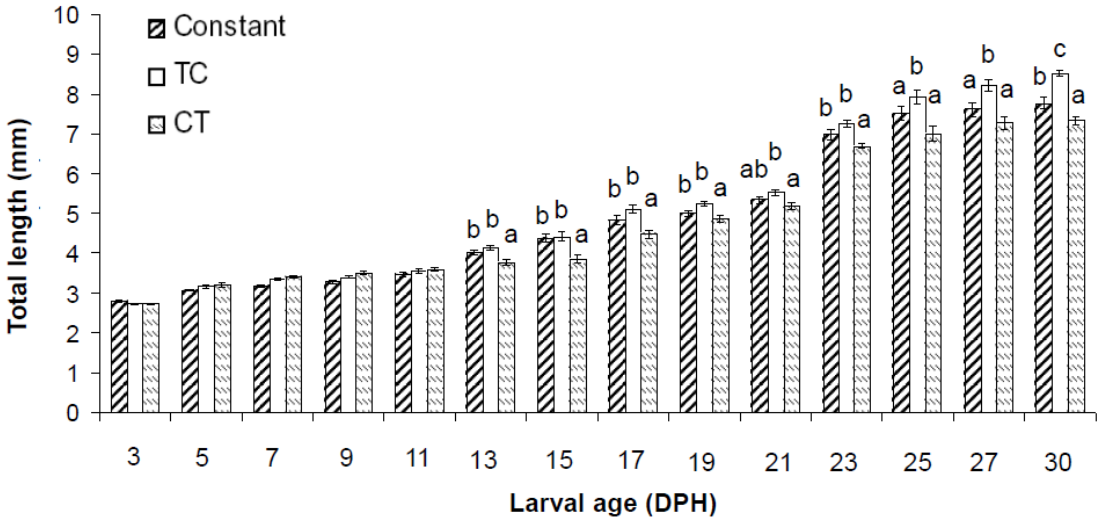
610 Figure 2.



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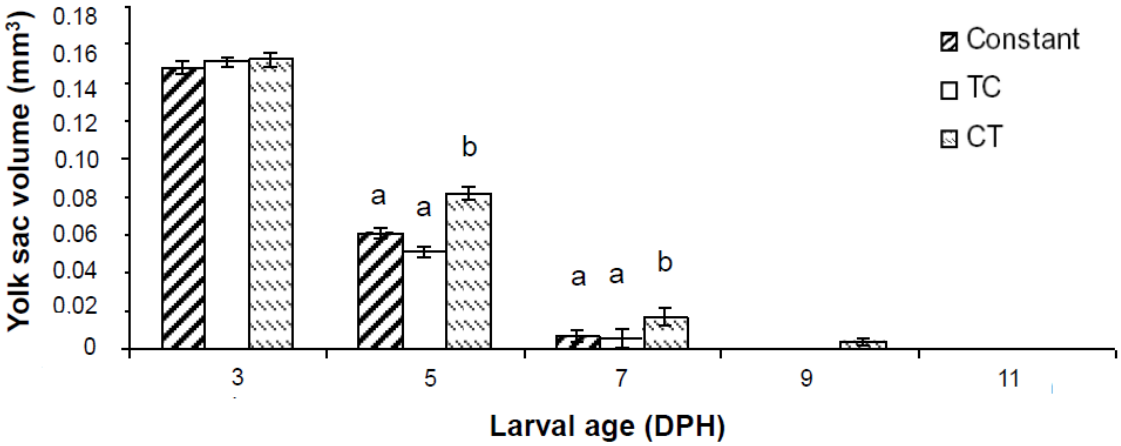
623 Figure 3.



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637 Figure 4.

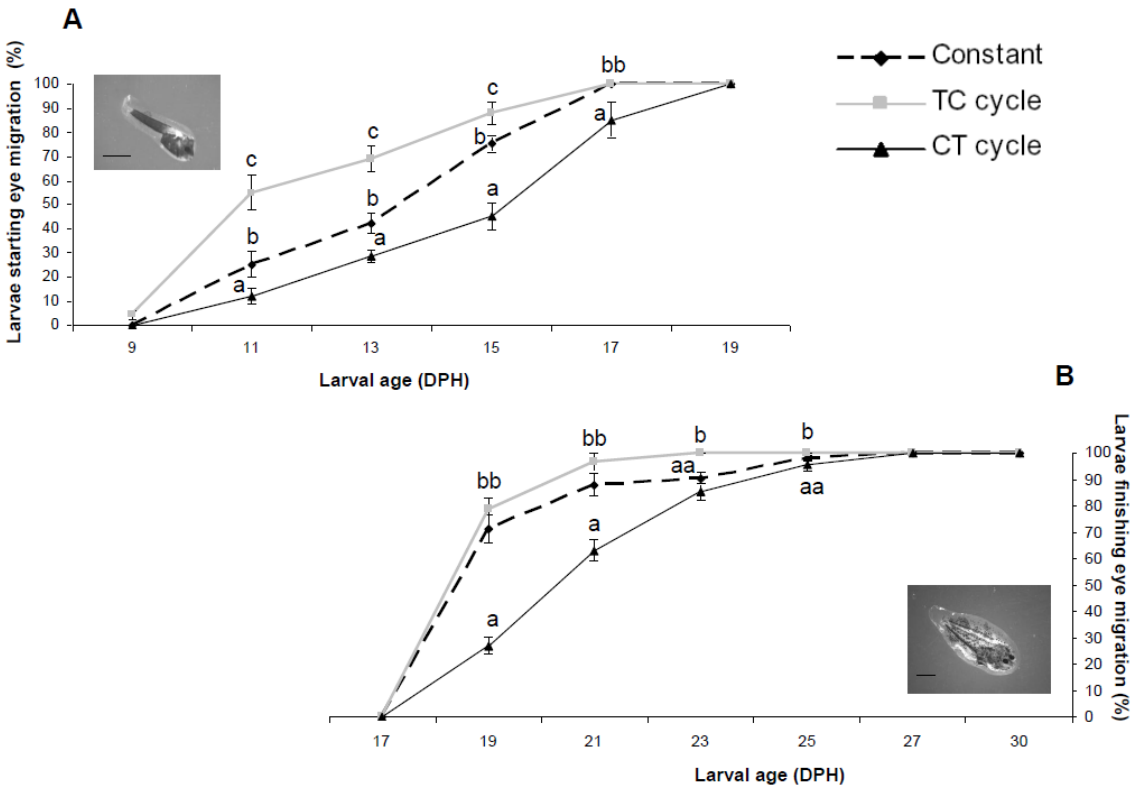

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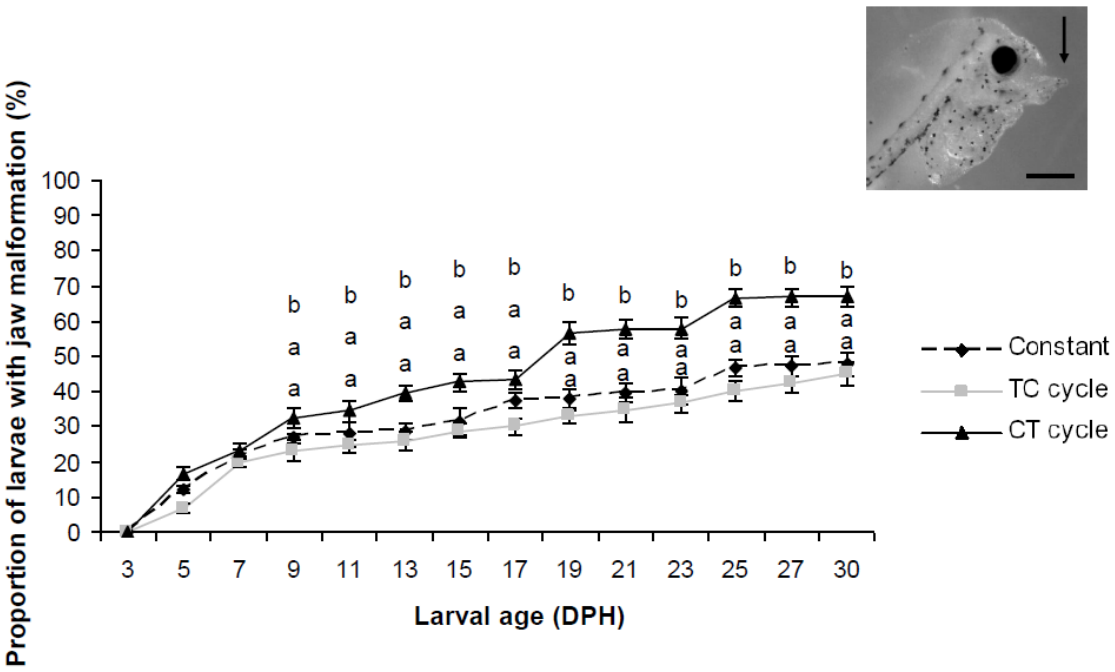
Figure 5.


Proportion of larvae with jaw malformation (%)
100
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60
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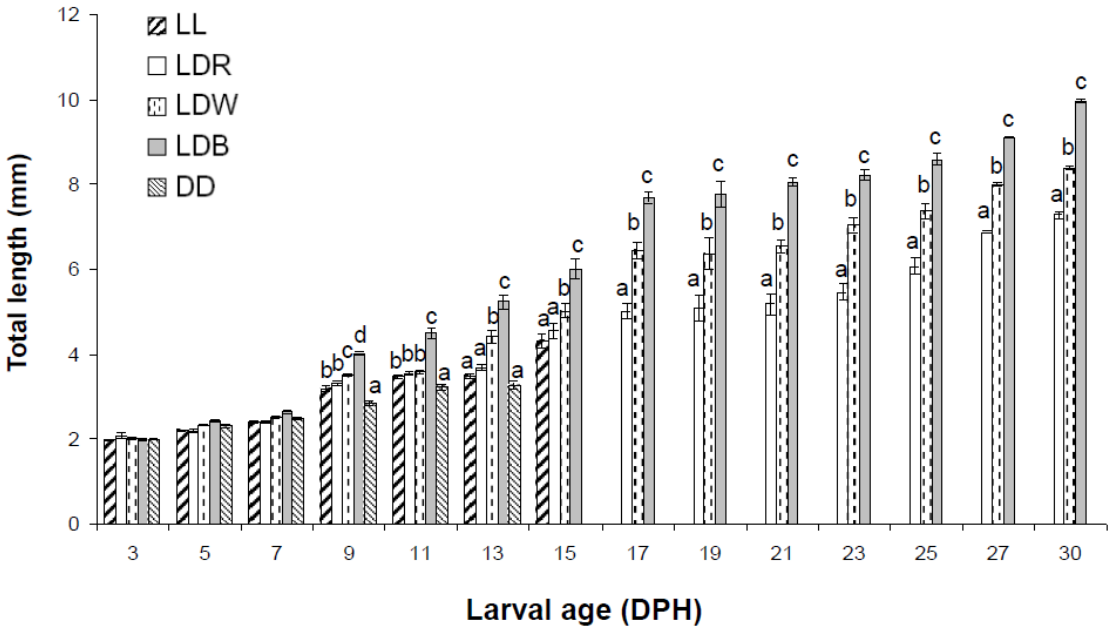
Larval age (DPH)
Constant
TC cycle
CT cycle

Figure 6.


LL
LDR
LDW
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Total length (mm)
Larval age (DPH)
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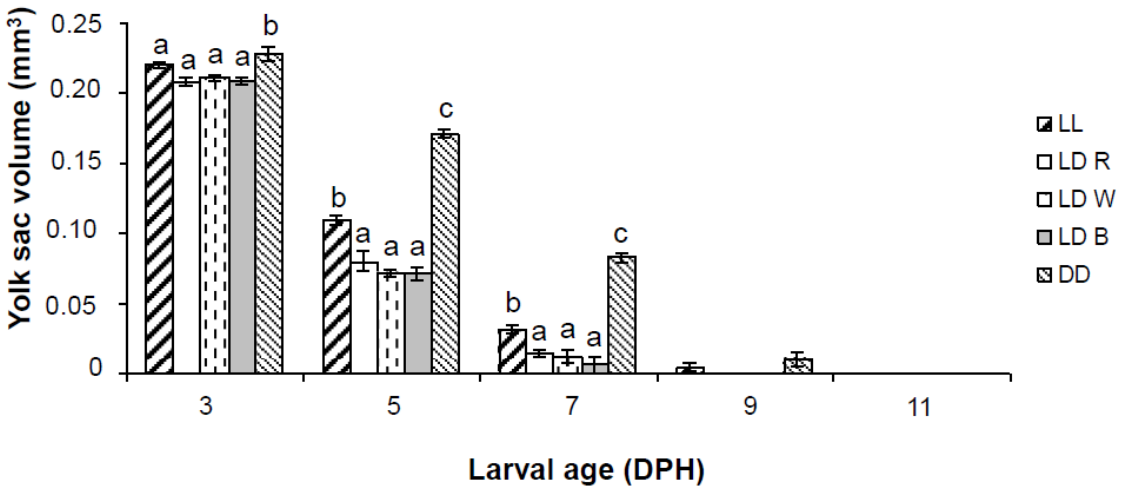
672 Figure 7.



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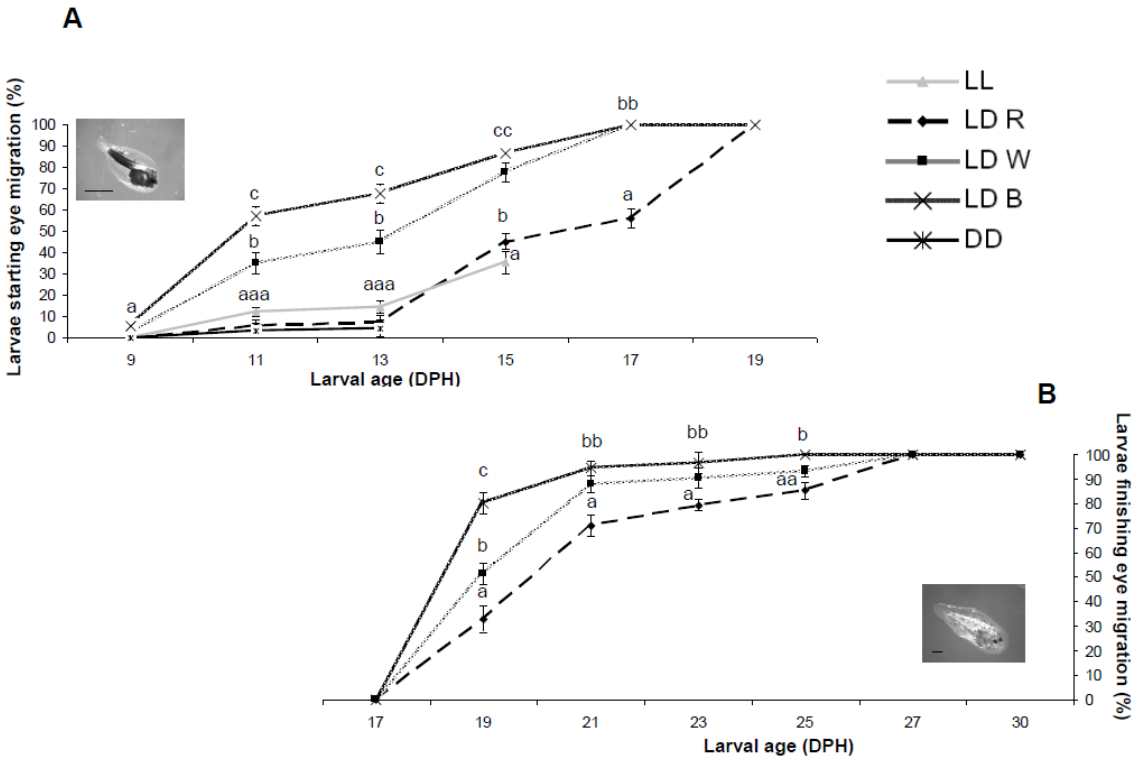
686 Figure 8.



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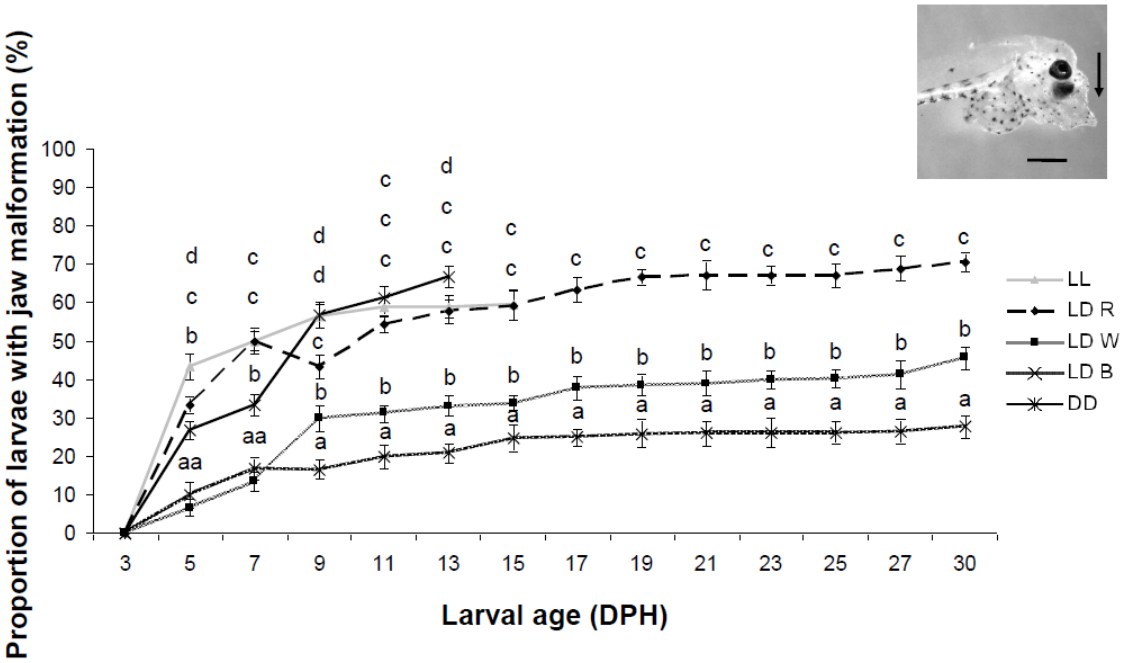
697 Figure 9.



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