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Short Communication

Effect of temperature on the incubation, growth, survival, and presence of skeletal deformities in larvae of the clownfish *Amphiprion ocellaris* (Pomacentridae) under rearing conditions

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ABSTRACT. The results of the effect of five different incubation temperatures on the development of clownfish (*Amphiprion ocellaris*) larvae are presented. This ornamental species is widely marketed worldwide and is reared in captivity at CIAD to meet the demand in the domestic market. The experimental work was carried out over 30 days post-hatching to observe the temperature's effect on the development of the larvae's vertebral column and caudal complex. The eggs were incubated at 26, 28, 30, 32, and 34°C. The percentage of hatching, survival, growth, and the presence of skeletal malformations were evaluated. Hatching and survival percentages did not show significant differences between treatments. Regarding growth, there were significant differences between treatments, with organisms showing slower growth at 26°C and larger sizes at 34°C. The highest percentage of malformations occurred in larvae cultured at 34°C, with lordosis being the most frequently observed deformity among the treatments. Therefore, it can be concluded that the optimal temperature range for cultivating and harvesting quality *A. ocellaris* organisms is between 28 and 30°C.

Keywords: *Amphiprion ocellaris*; clownfish larvae; abiotic factor; skeletogenesis; malformations; clearing technique

In recent years, climate change has led to a significant decrease in the population of coral reef ecosystems, which harbor a wide variety of ornamental fish. In addition to the threat of overfishing, efforts have been considered to replenish natural populations through captive breeding (Ajith-Kumar & Balasubramanian 2009, Abduh et al. 2011, Madeira et al. 2016).

Water temperature is an influential and variable parameter in aquaculture, a crucial factor in developing fish eggs and larvae. This variable affects hatching, growth, survival, and metabolic and digestive processes vital to successful juvenile production (Abdel et al. 2004, Dionísio et al. 2012, Boglione et al. 2013b, Yang et al. 2016).

One of the main challenges in intensive aquaculture is the presence of skeletal malformations, such as lordosis, kyphosis, scoliosis, and vertebral fusion. These malformations can be caused by various factors, such as nutritional aspects, environmental conditions, farming practices, and genetic factors (Fernández et al. 2008, Georgakopoulou et al. 2010).

Fish deformities can be detected as early as the embryonic stages and in the differentiation and development stages of the skeletal structures. Deformed fish are discarded during harvesting as they are less attractive to consumers due to their external morphology (phenotype), leading to significant economic losses in fish hatcheries (Gavaia et al. 2002, Boglioni et al. 2013a, Berillis 2015, Fragkoulis et al. 2017).

The clownfish, *Amphiprion ocellaris* (Cuvier, 1830), is one of 10 highly sought species and holds

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high-value potential in the aquarium trade due to its vibrant colors and adaptability to captivity (Ajith-Kumar & Balasubramanian 2009, Ye et al. 2011, Lango-Reynoso et al. 2012, Khoo et al. 2018, 2019). Currently, at the Centro de Investigación en Alimentación y Desarrollo, A.C. (CIAD), Mazatlán, a constant supply of juveniles is maintained. However, during each production cycle, deformed organisms have been observed and discarded at harvest time since they are not considered for commercialization (Nagano et al. 2007, Fernández et al. 2008, Boglione et al. 2013a). The optimization and the application of rearing protocols for this species (Abdo de la Parra et al. 2013, Velasco-Blanco et al. 2016, 2019, Rodríguez-Ibarra et al. 2017) have contributed by increasing hatching and survival rates in each reproductive cycle. However, due to the frequent presence of skeletal malformations, it is necessary to evaluate the effect of temperature on skeletogenesis from the egg incubation stage to larval rearing, which will help determine the impact of this abiotic factor on the occurrence of these deformities. Therefore, the objective of this study was to evaluate the effect of four different temperatures during eggs incubation and the larval rearing period of *A. ocellaris*, as well as the presence of skeletal malformations in the vertebral column, dorsal and anal fins, and caudal complex.

The clownfish eggs were obtained from the same breeding pair at CIAD, Mazatlán, from May to July 2019. Each batch of spawned eggs (343 ± 32.7) was incubated at five different temperatures: 26, 28, 30, 32, and 34 (± 0.5°C). The fertilized eggs from each spawning event were incubated in 100-L fiberglass tanks and, after hatching, were harvested and separated into three tanks per treatment. Larviculture was conducted in the same incubation tanks using the rearing protocol described by Abdo-de la Parra et al. (2013) and Rodríguez-Ibarra et al. (2017). Each mentioned temperature was controlled using an electric thermostat heater (Termal, 300 W). The average salinity was 34 ± 1.0, and the oxygen concentration was 6.2 mg L^{-1}.

The hatching and survival percentages were calculated using the following formulas:

$$H = \frac{L}{L+E} \times 100$$

where: *H*: hatching percentage, *L*: number of larvae, *E*: number of eggs.

$$S = \frac{No.FL}{No.IL} \times 100$$

where: *S*: survival percentage, *No. FL*: number of final larvae, *No. IL*: number of initial larvae.

From each treatment, larvae samples were collected from hatching up to day 30 (five organisms per day). These larvae were euthanized using an overdose of clove oil (Vázquez et al. 2013, Fernandes et al. 2017). The organisms' total length (TL) was measured using a digital vernier caliper with a 0.01 ± 0.03 mm resolution. The specimens were processed using the clearing technique and differential staining of bone and cartilage (double staining), following the method described by Potthoff (1984) with some modifications.

After the clearing and bone and cartilage staining process, the vertebral column, dorsal and anal fins, and caudal complex of the larvae were observed using a stereoscopic microscope (Olympus® SZ) equipped with a camera (Olympus® SP-350). Deformed specimens were photographed based on the normal osteological phenotype of the species (Rodríguez-Ibarra et al. 2017), and references from other studies reporting skeletal malformations in larvae of other marine fish species (Georgakopoulou et al. 2010, Boglione et al. 2013b) were also used for support.

The hatching and survival rates results and skeletal malformations obtained in each treatment were transformed using arcsine. Normality (Bartlett's test) and variance homogeneity (Levene's test) were tested for all results, and as the data were normal, they were analyzed using one-way analysis of variance (ANOVA) ($P < 0.05$). Significant differences between treatments were determined using Tukey's multiple comparison tests ($P < 0.05$) through the Statgraphics Plus 5.1 software, Statgraphics™ net.

The hatching rate results from this study did not show significant differences between treatments. The highest hatching percentage was 98.2 ± 1.03% at a temperature of 30°C, and the lowest was 95.6 ± 1.08% at 34°C. However, the hatching time varied among treatments at different temperatures. Larvae hatched at 168 h after fertilization (HAF) at 26, 28, and 30°C, at 156 HAF at 32°C, and 144 HAF at 34°C.

Regarding growth, the newly hatched larvae recorded an average initial TL of 4.4 ± 0.29 mm with no significant differences ($P = 0.05$) between the treatments. At the end of the experiment, significant differences were observed among the treatments, with the larger sizes (final TL) being recorded in organisms cultured at 34°C and the smaller sizes at 26°C (Fig. 1).

Rearing temperature did not affect the survival of the organisms, with the highest percentage (96%) recorded at 30°C and the lowest at 34°C (94%).

The highest percentage of skeletal malformations was recorded in organisms reared at 34°C (13.3%). In comparison, the lowest was observed at 28°C (1.2%),

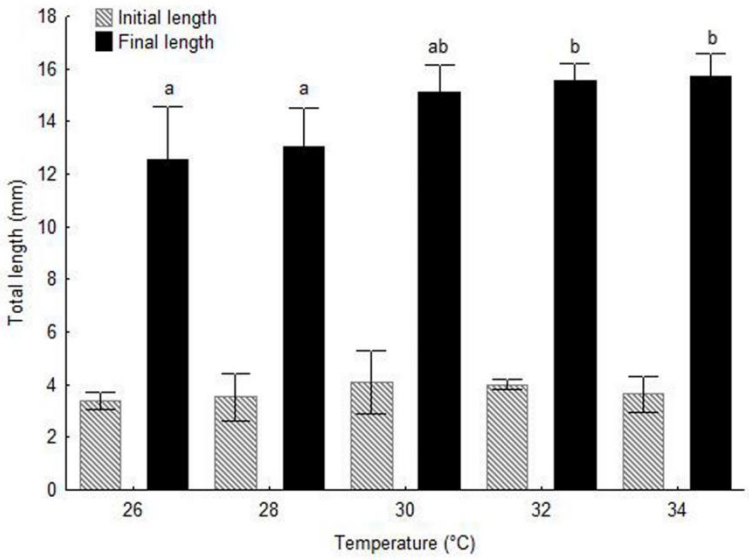

Figure 1. Mean total length between clownfish larvae's initial and final size in each treatment. Different letters on the bars indicate significant differences between treatments ($P < 0.05$).

showing significant differences ($P < 0.05$) among the treatments (Fig. 2). The deformities that occurred most frequently among the different treatments were lordosis (11%), kyphosis (7.6%), scoliosis (6.4%), and malformations of the pterygiophores in the dorsal and anal fins (9.4%). Figure 3 displays some organisms that exhibited deformities at different culture temperatures.

The optimal temperature for egg incubation and larval rearing under captive conditions of *A. ocellaris*, according to various studies (Madhu et al. 2012, Madeira et al. 2016, Rodríguez-Ibarra et al. 2017, Velasco-Blanco et al. 2019, Raheem et al. 2021, Soman et al. 2021), is within a range of 26 to 30°C. However, in the case of the species under study, it has been observed that it can be reared at temperatures of up to 32°C. Therefore, in this research, we decided to evaluate hatching, growth, survival, and the incidence of deformities within a range of temperatures, using 26°C as the minimum temperature since below this temperature, no embryonic development occurs, and 34°C as the maximum temperature to explore its effect on organism development, considering this extreme temperature.

Regarding the hatching results of the clownfish, the outcomes obtained in the present study at different incubation temperatures are very close to those reported in species of the same genus, such as black-finned anemonefish *A. nigripes* (94-95%) cultured at 27°C, and in *Amphiprion* sp. where they recorded a 99% hatching rate at a rearing temperature of between 26 and 29°C (Anil et al. 2012, Sahuisilawane et al. 2020). Likewise, Han et al. (2020) reported high hatching percentages in golden pompano *Trachinotus ovatus* at 26, 29, and 32°C, which coincides with the results obtained in this study. Soman et al. (2021) examined the impact of temperature (26 to 30°C) on the embryonic development and hatching of *A. ocellaris*. At temperatures of 29 and 30°C, hatching percentages of 95 and 98%, respectively, were observed. These findings align closely with the results reported in our study, especially at the higher incubation temperatures. On the other hand, Ignatius et al. (2001) reported a low hatching percentage (70%) in tropical clownfish *A. sebae* reared at temperatures of between 28 and 30°C, indicating that this is the first time that reproduction and rearing of this species have been carried out in captivity in India, which could have been the cause of these results, and, as in all such research, will require optimization of methods to increase hatching percentages.

The effect of temperature on larval growth (TL) was more evident in organisms reared above 30°C, but the largest sizes were recorded at 34°C. This premise is that as the rearing temperature increases, organisms grow faster. On the one hand, this can be beneficial as they can reach commercial sizes sooner. However, this muscular acceleration caused by the increase in the water temperature above optimal levels can exert mechanical pressure on the vertebrae of developing larvae, leading to osteological deformities in the organisms (Gisbert et al. 2008, Balbuena-Pecino et al. 2019). This effect could cause a higher percentage of

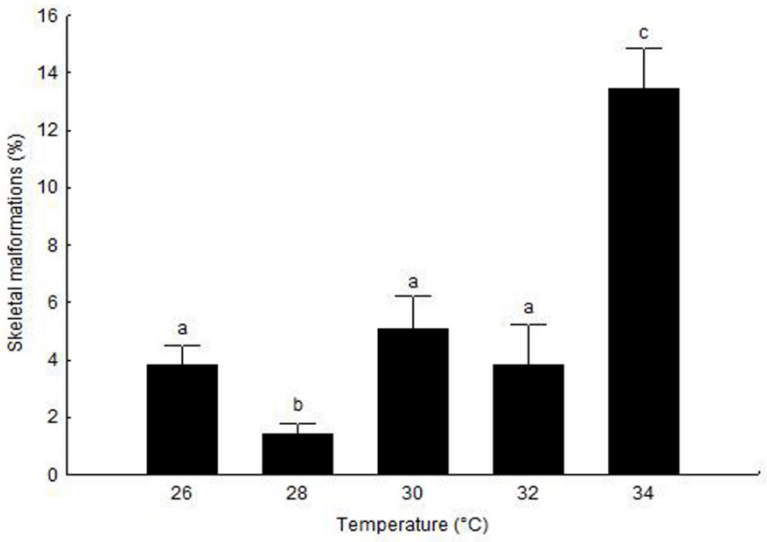

Figure 2. Percentage of malformations present in different thermal treatments. Different letters on the bars indicate significant differences ($P < 0.05$).

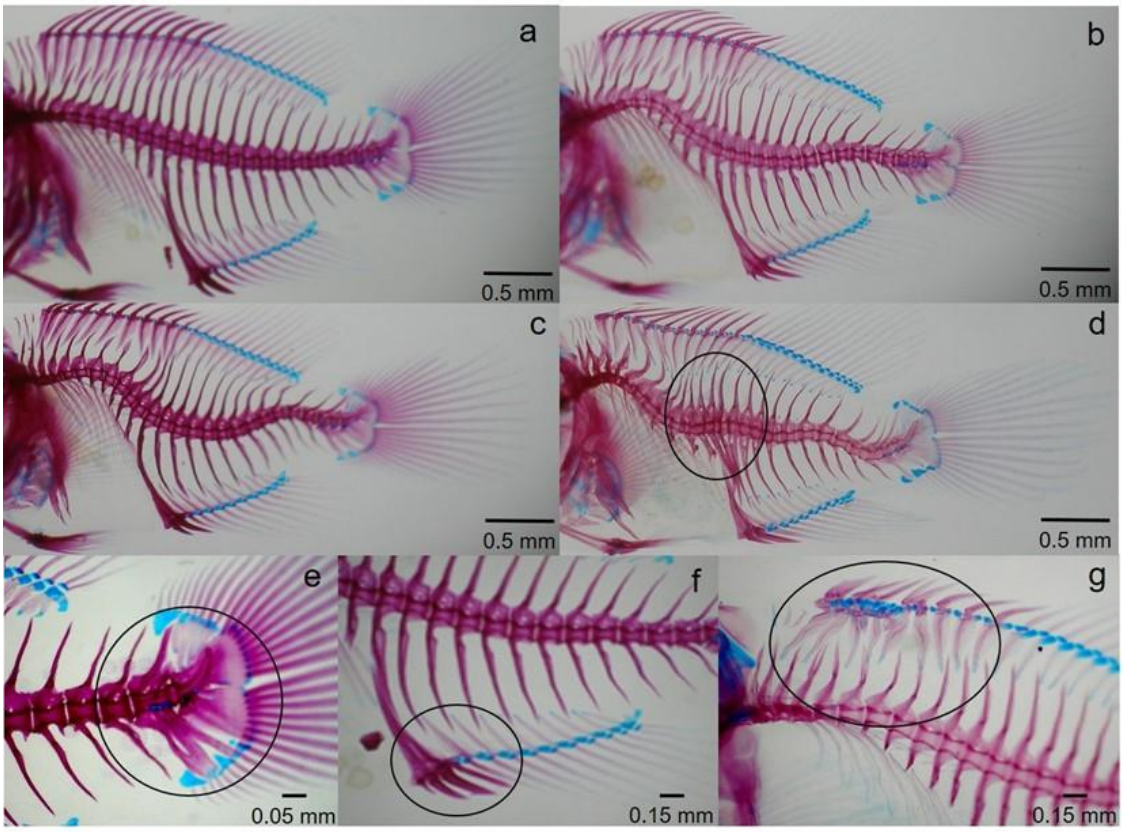

Figure 3. Clownfish larvae (22 to 28 days after hatching) with normal osteological development and different types of malformations. a) Normal individual at 28°C, b) with kyphosis at 32°C, c) with kyphosis, lordosis, and scoliosis at 34°C, d) with vertebral fusion at 34°C, e) with malformations in the caudal complex at 30°C, f) with supernumerary structures at 32°C, and g) with pterygiophore malformations in the dorsal fin at 34°C.

lordotic vertebral columns in clownfish larvae (13.3%), mainly observed at high temperatures, due to the asynchrony between bone and muscle growth.

In this study, it was observed that temperature did not significantly impact survival rates, as the percentages ranged between 94% at 34°C and 96% at 30°C, which differs from the results of Ye et al. (2011), where temperatures of 23, 26, and 29°C had a significant effect on the survival of yellowtail clownfish *A. clarkii* larvae as their recorded highest survival rate was 83.7% at 29°C, which is lower than what we report. Furthermore, the results of our study differ from those obtained by Rao et al. (2014), who compared culture temperatures for juvenile *A. ocellaris* in a range of 23 to 37°C and observed low survival rates at all temperatures, ranging from 51 to 75%.

Skeletal abnormalities in fish can be attributed to various factors (biotic, abiotic, and xenobiotic). Changes in water temperature (increase or decrease) have been reported as a major physical factor that can induce phenotypic changes in reared species, affecting skeletogenesis and leading to morphological malformations (Seikai et al. 1986, Klimogianni et al. 2004, Fraser et al. 2015). This study observed a higher incidence of deformities in organisms as rearing temperatures increased. This result is supported by Han et al. (2020), who found a higher percentage of deformities at 32°C in *T. ovatus*. Among the observed deformities, lordosis in the vertebral column of the caudal region of the organisms was the most predominant (11%), which is consistent with the findings of Andrades et al. (1996), who noted that lordosis frequently occurs in gilthead bream.

Regarding other skeletal deformities such as kyphosis (7.6%), observed in the abdominal region of the vertebral column, along with scoliosis (6.4%) and lordosis, these are the most notable external deformities in fish and can be detected as early as 15 days post-hatching, enabling an external evaluation of the larvae during rearing, and if necessary, cull them before they reach market size. Regarding other malformations observed in the clownfish, such as deformities in the pterygiophores of the dorsal and anal fins, vertebral fusion, and abnormalities in the caudal complex, which, although present, are deformities that do not affect the external shape of the fish (Boglione et al. 2013b). Some of these deformities, as mentioned earlier, have been observed in certain reared fish species, and they can affect growth, swimming, feeding, resistance, speed, and survival (Gavaia et al. 2002, Lewis-McCrea & Lall 2006, Boglione et al. 2013b, Berillis 2015, 2017, Fraser et al. 2015). However, such issues have not been reported in the species under study, with the most relevant impact being on external appearance. Since clownfish are ornamental fish, the emphasis is on producing aesthetically normal organisms.

This study provided significant insights into the effects of temperature on the growth, survival, and presence of skeletal deformities in clownfish *A. ocellaris*. While temperature did not influence the hatching rate or larval survival, it did affect TL and the incidence of malformations as the rearing water temperature increased. Therefore, it is suggested that rearing this species should be between 28 and 30°C. These findings contribute to optimizing aspects of clownfish aquaculture, facilitating proper development and growth of organisms, and consequently yielding high-quality harvests to meet the demands of the important ornamental fish market industry.

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