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ORIGINAL ARTICLE

Effect of acclimation temperature on thermoregulatory behaviour, thermal tolerance and respiratory metabolism of *Lutjanus guttatus* and the response of heat shock protein 70 (*Hsp70*) and lactate dehydrogenase (*Ldh-a*) genes

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Abstract

This study evaluated the effect of different environmental temperatures in the physiology of *Lutjanus guttatus* juveniles by analysing their thermoregulatory behaviour, thermal tolerance, oxygen consumption rates and thermal metabolic scope. Jointly, the effect of acclimation and critical temperatures on heat shock protein 70 (*Hsp70*) and lactate dehydrogenase (*Ldh-a*) gene expressions were also analysed using acclimation temperatures of 20, 23, 26, 29 and 32°C. The results showed that the final preferred temperature in juvenile snapper was 26°C with a thermal window of 336.5°C^2, which was related to an optimal temperature for their physiology determined by the routine metabolic rate and thermal metabolic scope. At temperatures from 20 to 26°C, the routine metabolic rate and *Hsp70* and *Ldh-a* genes had the lowest values related to a basal expression level. At acclimation temperatures from 29 to 32°C and after critical thermal maximum (CT_{max}) limit, the relative expression of *Hsp70* and *Ldh-a* genes increased significantly, but the main response at CT_{max} was the upregulation of *Hsp70* gene.

KEYWORDS

Hsp70 and *Ldh-a* genes, *Lutjanus guttatus*, preferred temperature, routine metabolic rate, thermal metabolic scope, thermal tolerance

1 | INTRODUCTION

Temperature is considered a controlling factor because it affects distribution, abundance and interactions of all organisms (Angilletta, 2009; Somero, 2005). Consequently, climate warming represents an important challenge for ectotherms, increasing the extinction risk of many species that already live close to their upper thermal limits (Huey et al., 2012). Despite these repercussion, ectotherms

can avoid these thermal challenges through different mechanisms, such as behavioural thermoregulation, which might mitigate the effects of environmental thermal fluctuations to maintain their body temperatures close to the thermal optimum and thus maximize their fitness (Gunderson & Stillman, 2017; Huey, Hertz, & Sinervo, 2003; Reynolds & Casterlin, 1979).

Imminently, ocean warming significantly alters the performance and fitness of many marine organisms where some of them may not acclimate at high heat wave temperatures. In this case, thermal limits have been considered one of the approaches to compare the physiological adaptation mechanisms among species and extensively applied to investigate thermal biology in species inhabiting thermal gradients (Campos et al., 2017; Campos, Val, & Almeida-Val, 2018). For example, critical thermal limits (e.g. critical thermal maximum and critical thermal minimum; CT_{max} and CT_{min}) are determined by raising or lowering temperature progressively from the acclimation temperature until muscle disorganization occurs in response to the thermal stressor (Lutterschmidt & Hutchison, 1997). Both responses identify the temperature at which the first stress symptoms appear, and they are a direct reflection of how acclimation temperature modulates thermal tolerance in aquatic ectotherms (Becker & Genoway, 1979; Beitinger & Bennett, 2000; Paladino, Spotila, Schubauer, & Kowalski, 1980).

The specific thermal sensitivity of ectothermal species can be analysed by a molecular approach, which can help us determine and expand our knowledge of the different response mechanisms. For example, when temperatures reach their critical values, it is possible to observe structural damages in the cell that prevent the organism from escaping from these conditions. In this case, inflammatory processes, sub-regulation of the tricarboxylic acid cycle, protein denaturation and toxic effect of reactive oxygen species have been observed (Basu et al., 2002; Katschinski et al., 2002; Logan & Buckley, 2015; Madeira et al., 2017). Temperature regulation of gene expression is thought to be critical for growth and survival of ectotherms that live in variable thermal environments (Iwama, Vijayan, Forsyth, & Ackerman, 1999). The temperature range to which fish can adapt differs among species, and temperature above the range induces cellular stress responses in many cells and tissues (Wendelaar Bonga, 1997). Currently, different molecular indicators could contribute to understanding the effects of environmental factors on fish physiology. The use of molecular markers, such as *Hsp70* gene as an indicator of cell damage and the *Ldh-a* gene as an indicator of anaerobic metabolism activation, could contribute to understanding the effect of temperature increase on fish physiology.

Thermal limits may be related to restrictions in the organism physiology associated with the metabolic scope available for performance at higher temperatures. The direct effect of acclimation temperatures on aerobic metabolism can be analysed by assessing the aerobic scope, calculated as the difference in standard and maximum metabolic rates (Chabot, Koenker, & Farrell, 2016; Farrell, 2016; Schulte, 2015). The aerobic scope represents the excess oxygen available to support biological fitness, and, as any other physiological function, it has an optimum temperature range (Top). Beyond this optimum point, temperature causes energy to be channelled into protecting (*pejus* interval of temperature) or repairing fish cellular integrity (Pörtner, 2010; Pörtner & Knust, 2007).

Paschke et al. (2018) stated that the temperature-induced metabolic rate method may be used to estimate the aerobic power budget of aquatic organisms; it can be obtained through stimulating the organism activity using high or low non-lethal temperatures to obtain high and low metabolic rates. Based on this result, this study hypothesises that a thermal metabolic scope, obtained by temperature-induced metabolic stimulation, could be used as a new indicator of the aerobic power budget for sluggish or sedentary organisms.

Lutjanus guttatus is a fish that lives in marine waters in the eastern Pacific Ocean in tropical and subtropical coastal areas. Currently, its distribution includes from the Gulf of California in Mexico to Peru (Amezcua, Soto-Avila, & Green-Ruiz, 2006; Soto, Mejía-Arana, Palacios, & Hiramatsu, 2009). In Mazatlan, Mexico, this fish is successfully farmed in outdoor tanks and floating cages where temperature can fluctuate from 23.6 to 29.8°C (Hernández et al., 2015). The thermal conditions in Mexico permit not only farming this species but also this thermal area seems to be an adequate thermal environment to find *L. guttatus* specimens in their natural environment. Although global warming affects not only sea surface temperature but also the ecology and aquaculture of marine species (FAO, 2012), the response mechanism of organisms, such as *L. guttatus*, to these temperatures is still unknown.

Therefore, the purpose of this study was to determine the preferred temperature, thermal tolerance, thermal window width and the thermal metabolic scope in *Lutjanus guttatus* juveniles acclimated at different temperatures to optimize their culture conditions and also determine the *Hsp70* and *Ldh-a* gene expressions as molecular indicators of cellular damage and activation of anaerobic metabolism. Our results are discussed around the physiological and metabolic responses that may contribute to the knowledge of the thermal biology of subtropical organisms.

2 | MATERIALS AND METHODS

2.1 | Acclimation of juveniles

Juveniles (N = 450 of 2 ± 1 g wet weight) used in this study derived from a cross between six adult females with an average weight and body length of 2.5 kg and 45.5 cm, respectively, and six males with an average weight and body length of 2.6 kg and 46.3 cm in length respectively. Breeders were selected according to the protocols of animal care established by the Centro de Investigación en Alimentación y Desarrollo A. C. (CIAD). The juvenile organisms were transferred in plastic bags with seawater at 24 ± 1°C and saturated oxygen concentrations by air to the Centro de Investigación Científica y de Educación Superior de Ensenada, Baja California, México (CICESE). During fish transfer, the plastic bags with juveniles were introduced in coolers to maintain adequate temperature and oxygen conditions as long as possible to avoid stress.

Once in CICESE facilities, the organisms were placed in two fibreglass tanks of 2,000 L each with seawater recirculation and constant

aeration. The juveniles were kept for three months at 28 ± 2°C with an 800-watt titanium heater (Finnex; TH-800 POLUS) connected to a digital temperature control system (Finnex; HC-0810M). The dissolved oxygen in the water was maintained at an average concentration of 5 mg O_2/L with aeration supply through air stones. Juveniles were fed with commercial food (Otohime brand Chita, JP) (1.7 mm) three times a day at satiation.

After 3 months, 400 fish of an average size of 20 ± 1 g were distributed at random in five fibreglass tanks of 500 L each (80 juveniles each); fish were kept inside a laboratory where the environmental temperature was at 23°C, which allowed maintaining greater control on temperature variability of the water recirculation systems. Water temperature of the different tanks increased or decreased according to a rate of 1°C per day to acclimate the juveniles to the temperatures of 20, 23, 26, 29 and 32°C. Once the experimental temperature had been reached, the organisms remained under these conditions for 21 days.

2.2 | Thermoregulatory behaviour

When the acclimation period ended, 20 juveniles per each acclimation temperature were tagged with the objective of carrying out the individual follow-up in the subsequent trials. For this purpose, the fish were anaesthetized with phenoxyethanol (0.8/1,000), and surgical clamps were used to place plastic sequins in the area where the dorsal fin begins (Ruiz-Campos & Villalobos-Ramírez, 1991). After being tagged, the fish were returned to their acclimation tank for 48 hr for recovery. Each plastic sequin was of a different colour and marked with a different number (1–5) for the accurate individual monitoring each time. In each replicate (five fish each time), colours and numbers were not repeated. The behaviour from each individual was followed into the gradient and recorded for the biological and statistical perspectives.

A horizontal thermal gradient was used (Díaz et al., 2006) to determine the preferred behaviour of *L. guttatus* juveniles at different acclimation temperatures. A linear thermal gradient was created inside a 400-cm polyvinyl chloride (PVC) pipe with a cooler (Neslab, model HX 150) and a 1,000-watt heater, each one placed at opposite ends of the pipe. The thermal gradient was from 8 to 36°C ($y = 6.20 + 1.60\,x$; $r^2 = 0.98$; where x = segments of the gradient; y = temperature of the segments). A porous aeration hose was placed along the pipe to prevent stratification of the water column. The rate of seawater change in the gradient was 180–200 ml/min.

The acute thermal preference of juveniles was determined by placing five fish from the same acclimation temperature within the temperature gradient (Díaz et al., 2006, 2007). The acute method described by Reynolds and Casterlin (1979) was used, which consisted of recording the individual location of each fish along the gradient every 10 min for 120 min. The temperature of each segment where the fish were found was measured with a digital infrared thermometer (Steren HER-425 Atzcapozalco MX). The acute thermal preference was determined with 20 fish by acclimation temperature. The preferred temperature was determined by establishing the equality line between the acclimation temperature and final preferred acute temperatures; for this purpose, a graphical method was used, consisting of drawing a 45° line of equality on the graph of thermal preferences.

2.3 | Thermal tolerance

The dynamic method (Lutterschmidt & Hutchison, 1997) was used to determine the thermal tolerance of juveniles, which consisted of increasing water temperature from the acclimation temperature to observe the point of loss of equilibrium in each fish. Thus, five fish from the same acclimation temperature were randomly selected and placed in a 50-L aquarium to determine the CT_{max}. The water temperature in the aquarium depended on the experimental acclimation temperature from which the fish came. The tagged fish remained in the aquarium 30 min before the CT_{max} assays to avoid manipulation effects (Pérez, Díaz, & Espina, 2003). After 30 min, the water temperature was increased at 1°C/min (Lutterschmidt & Hutchison, 1997) with an 800-watt heater (Finnex; TH-800 POLUS). The critical temperature was established when the fish showed accelerated swimming (increased activity) followed by a state of disorientation and finally loss of equilibrium (Becker & Genoway, 1979; Bennett & Beitinger, 1997; Mora & Ospína, 2001). The temperature was recorded with a mercury thermometer (±0.01), and the juveniles were quickly removed from the aquarium and returned to their acclimation reservoir, and survival was monitored for 96 hr.

Critical thermal minimum (CT_{min}) was assessed using the methodology described by Noyola et al. (2015). A stainless steel coil was connected to a chiller (PolyScience IP-35) inside the 40-L aquarium to allow temperature decline at a rate of 1°C/min. Five fish were placed into an aquarium filled with water of the same acclimation temperature from which they came. An air-stone was used to avoid thermal stratification. The criterion for determining the end point of CT_{min} was the same used for CT_{max}, where an animal was used for the CT_{max} or CT_{min} tests. Four repetitions of the dynamic method were performed to determine the CT_{max} and the CT_{min} of 20 fish per acclimation temperature. The thermal window area was obtained with the CT_{max} and CT_{min} data following Bennett and Beitinger (1997) and expressed in $°C^2$.

2.4 | Relative expression of the *Ldh-a* and *Hsp70* genes

The relative expression of *Hsp70* and *Ldh-a* genes was evaluated in the gill and muscle of *L. guttatus* through real-time polymerase chain reaction (qPCR). For this purpose, five organisms of each acclimation temperature were euthanized through an overdose of phenoxyethanol (2:1,000), and samples of muscle and gill were taken. On the other hand, from the organisms used to determine critical temperatures, ten of them were sacrificed after determining their critical temperature, and gill and muscle samples were taken. The tissues were placed in 1.5 ml Eppendorf tubes with 1.0 ml of NAP buffer

(Camacho-Sanchez, Burraco, Gomez-Mestre, & Leonard, 2013) and stored at −80°C for further analysis.

For tissue homogenization (cell lysis), 100 mg of each tissue was weighed and individually placed in 1.5 ml Eppendorf tubes with 1 ml of Tri-Reagent (Sigma-Aldrich) and 100 mg of zirconia beads. Each tissue was homogenized with the aid of a FastPrep®-24 (MP Biomedicals) twice for 30 s at 5.0 m/s. Subsequently, RNA extraction was carried out by phase separation using 200 µl of chloroform. After a 5 min incubation period, it was centrifuged at 10 000 *g* for 15 min, and ~500–600 µl of the organic phase was recovered and placed in a new tube. Once the RNA was isolated from the cell debris produced by lysis, the nucleic acid was precipitated by adding 500 µl of 95% ethanol and centrifuged at 10 000 *g* at 4°C for 10 min. The RNA pellets were washed with 75% ethanol and centrifuged at 11 000 *g* at 4°C for 5 min. Finally, the RNA pellets were eluted in 100 µl of nuclease-free water and stored at −80°C. The concentration of RNA for each sample was measured with a NanoDrop Lite spectrophotometer (Thermo Scientific), and the RNA quality was evaluated by electrophoresis in 1% agarose gel.

The RNA samples were purified with RQI RNase-Free, DNase (Promega) to ensure the elimination of the genomic DNA (according to the manufacturer's protocol). DNA absence was further confirmed by PCR amplification. The concentration and quality of RNA after the DNAse treatment were evaluated as described above.

The cDNA was synthesized with ImProm-II™ Reverse Transcription System (Promega) with 1.0 µg of Total RNA previously purified in a total reaction volume of 20 µl following the manufacturer's protocol. The obtained cDNA was stored at −20°C until relative expression analysis.

The amplification of target genes was performed with IQ™ SYBR® Green Supermix (Bio-Rad) in a CFX96 thermal cycler (Bio-Rad). Specific primers for target and housekeeping genes were designed with PerlPrimer software and Primer3 (Table 1). The qPCR reactions were prepared with 3 µl of cDNA (Diluted 1:5 or equivalent to 30 ng), 0.2 µM of forward and reverse primers, 5 µl of 2× IQ ™ SYBR® Green (Sigma-Aldrich) and 1.6 µl of nuclease-free water, for a final volume of 10 µl. The qPCR reactions were carried out in triplicate. The qPCR cycling conditions were as follows: 95°C for three min, followed by 40 cycles at 95°C for 10 s, 56°C for 30 s. A melt curve analysis was added (95°C for 10 s, 65°C to 95°C for 5 s, with increments of 0.5°C to corroborate PCR product specificity). The efficiency of qPCR (90%–110%) and the correlation coefficient (R^2: 0.90–0.99) were determined by the standard curves generated using 1:5 serial dilutions of cDNA.

The expression stability of housekeeping genes (*GAPDH* and *Fe-1α*, Table 1) was analysed with geNorm and NormFinder software. Finally, a RetFinder tool was used from the cotton EST software database (http://www.leonxie.com/referencegene.php) to corroborate the expression stability results. The different outcomes of quantitative relative gene expression were analysed in the software qBase-PLUS v2.0 software (Biogazelle).

2.5 | Oxygen consumption

An intermittent respirometry system was used to assess the effect of acclimation temperature on the routine metabolic rate (RMR). This system consisted of six acrylic chambers of 1,500 ml placed inside a 100-L thermal regulated reservoir. The water temperature in the respirometry system was controlled with a titanium 800-watt heater (Finnex; TH-800 POLUS) connected to a digital temperature control system (Finnex; HC-0810M) to adjust the temperature as required. Each chamber was closed with an acrylic lid that allowed it to be hermetically sealed using rubber bands and metal clamps placed around the perimeter. In the upper part of the cover, three 1-cm holes in diameter were designed to be the independent water inlets and outlets that worked when closing or opening the replacement channels through a 250-watt pump. The third hole was where the fibre optic sensor was placed (accuracy ± 0.005% O_2, detection limit 0.03% O_2) for the dissolved oxygen measurements. An OXY-10 mini-amplifier (PreSens GmbH, PreSens©) was used to measure dissolved oxygen every 30 s for 300 s.

Five fish from the same acclimation temperature were individually placed inside the acrylic chambers to measure oxygen consumption at the acclimation temperatures from which they came (different fish from those used for the previous essays). In each case, the fish remained in the chamber for 30 min with the open water recirculation system (inlet and outlet) to avoid stress effects caused by handling. After this time, the water recirculation system was closed, and the dissolved oxygen in each chamber was recorded every 30 s for five min. This time period was previously calculated to avoid reducing 25%–30% of dissolved oxygen in the chamber and generating collateral stress. Simultaneously, the dissolved oxygen values were recorded in a chamber without any fish, which were used as control to make any correction for the presence of the microorganism

TABLE 1 Primers used in real-time qPCR in *Lutjanus guttatus* juveniles acclimated at 20, 23, 26, 29 and 32°C

Gene	Forward primer	Reverse primer	pb
GAPDH	5′-caccctggaggtgagaagag-3′	5′-ccgttaccatggtgaggtct-3′	182
Fe-1α	5′-gcttcgaggaaatcaccaac-3′	5′-caaccttccatcccttgaac-3′	153
Hsp70	5′-gcggagaggactttgacaac-3′	5′-cgatgccctcaaacagaga-3′	187
Ldh-a	5′-gcatcctgatggtggtttca-3′	5′-tgctttccgatgatccaac-3′	187

Abbreviations: *Fe-1α*, elongation factor-1alpha; *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase; *Hsp70*; heat shock protein 70; *Ldh-a*, lactate dehydrogenase-a.

in the system. Finally, the fish were removed from the chambers to be weighed and placed in their corresponding acclimation tank. The fish remained fasting for 48 hr before the trials to avoid the effect of nutrient assimilation in the oxygen consumption values. Oxygen consumption replicates were performed twice to determine the metabolic rate of 10 fish/ acclimation temperature.

Oxygen consumption rates (OCR) of *L. guttatus* juveniles were determined according to the equation of Cerezo, Martínez, and García (2006).

$$\mathrm{OCR} = (Ct - C0)\, V/\,(W \times T) \quad (1)$$

where *Ct* is the change in dissolved oxygen in the chambers before and after the test; *C*0 is the difference in the oxygen of the target (control); *V* is the volume of the chamber; *W* is the weight of the juveniles in kg and *T* is the time in hours. With the values of oxygen consumption, the Q_{10} was calculated:

$$Q_{10} = (\mathrm{rate2}/\mathrm{rate1})^{(10/\mathrm{tem2}-\mathrm{temp1})} \quad (2)$$

2.6 | Thermal metabolic scope

The temperature-induced metabolic rate (TIMR) methodology described by Paschke et al. (2018) was used to determine the thermal metabolic scope using a temperature range at which locomotive activity was enhanced, and temperature provoked high metabolic rates (HMR). Based on this fact, this study proposed that the weight-specific oxygen consumption upon stimulation of organism activity caused high metabolic rates when exposed to 95% of the CT_{max} ($TIMR_{Max}$). Similarly, a minimum metabolic rate (LMR) could be obtained from the CT_{min} when the metabolic activity was depressed to 105% of the CT_{min} ($TIMR_{Min}$), assuming that a thermal metabolic scope induced by temperature could be obtained as high metabolic rates minus low metabolic rate.

For each acclimation temperature, ten animals were used to calculate $TIMR_{Max}$ and $TIMR_{Min}$ according to the novel methodology proposed by Paschke et al. (2018). Organisms were placed individually in a 40-L aquarium equipped with an 800-W heater and constant aeration at their respective acclimation temperature to determine HMR. The animals remained in these conditions for 30 min to recover from stress caused by handling; later, the temperature was increased 1°C/min until it reached $TIMR_{Max}$, and then, they were immediately transferred to an intermittent respirometric system (Díaz et al., 2007) at the same $TIMR_{Max}$ temperature. Oxygen consumption was measured every 30 s for 5 min; this time was chosen considering that more exposure time could provoke fatigue and anaerobic metabolism (Norin & Clark, 2016) although it was related to the high metabolic rate. Oxygen concentrations were measured with dipping probe oxygen mini-sensors (Loligo Systems) connected to a PC-controlled fibre optic trace oxygen transmitter (OXY-10 trace transmitter, PreSens Precision Sensing GmbH).

For the determination of low metabolic rate, fish were exposed to temperature reduction at a rate of 1°C/min, similarly to that described to obtain CT_{min}. When organisms reached $TIMR_{Min}$, they were immediately transferred to an intermittent respirometric system (Díaz et al., 2007) at the same $TIMR_{Min}$. Oxygen consumption was measured every 30 s for five min with dipping probe oxygen mini-sensors (Loligo Systems) connected to a PC-controlled fibre optic trace oxygen transmitter (OXY-10 trace transmitter; PreSens Precision Sensing GmbH).

Records of three chambers filled with water without fish were used as control to account for microbial oxygen consumption in the filtered seawater to make the necessary corrections. Respiration rate was calculated as shown in this equation:

$$\mathrm{MO2} = (O_{2(A)} - O_{2(B)}) \cdot (V/t)\,/M \quad (3)$$

where MO2 is respiration rate (mg O_2 hr^{-1} g^{-1} w.w.); $O_{2(A)}$ is the initial oxygen concentration in the chamber (mg O_2/L); $O_{2(B)}$ is the final oxygen concentration in the chamber (mg O_2/L); V is water volume in the chamber minus volume of water displaced by the animal; t is the time elapsed during measurement (hr); and *M* is the body mass of the experimental animal (w.w.).

The thermal metabolic scope was calculated according to the novel methodology in this study as the difference between the high and low metabolic rates (both as mg O_2 hr^{-1} kg^{-1} w.w.).

2.7 | Statistics

A Kruskal–Wallis (nonparametric test) was performed with all data according to the normality of the values obtained. Finally, multiple comparisons of each treatment group were conducted to show the groups with significant differences using Dunn's method. Descriptive statistics, graphs, a nonparametric test and multiple comparison tests were performed in the Sigma Plot 12.0 and Excel program.

3 | RESULTS

The thermal preference of fish acclimated at 20, 23 and 26°C was 26.2 ± 1.8, 26.5 ± 2.3 and 26.7 ± 3.3°C (median ± standard deviation [*SD*]), respectively, while the thermal preference of the acclimated fish at 29 and 32°C was 27.2 ± 1.9 and 29.2 ± 3.9°C (Figure 1). Regardless of the previous acclimation temperatures, the main response of the juveniles within the thermal gradient was the congregation at temperatures from 26.2 to 29.2°C. Only did the fish acclimated at 29 and 32°C showed statistically significant difference (p < 0,05) compared with the thermal preference of the fish acclimated to the other temperatures. Furthermore, the final preferred temperature was 26°C using the graphical method (Figure 1, black circles).

Both the CT_{max} and the CT_{min} values (mean ± *SD*) showed a proportional increase with respect to the increase of the acclimation temperatures. A statistically significant difference ($p \leq .05$) was found between the organisms acclimated at 20, 26 and 32°C exposed to CT_{max} and CT_{min}. The CT_{max} values were 35.9 to 40.4°C in the acclimation range from 20 to 32°C while the CT_{min} values were

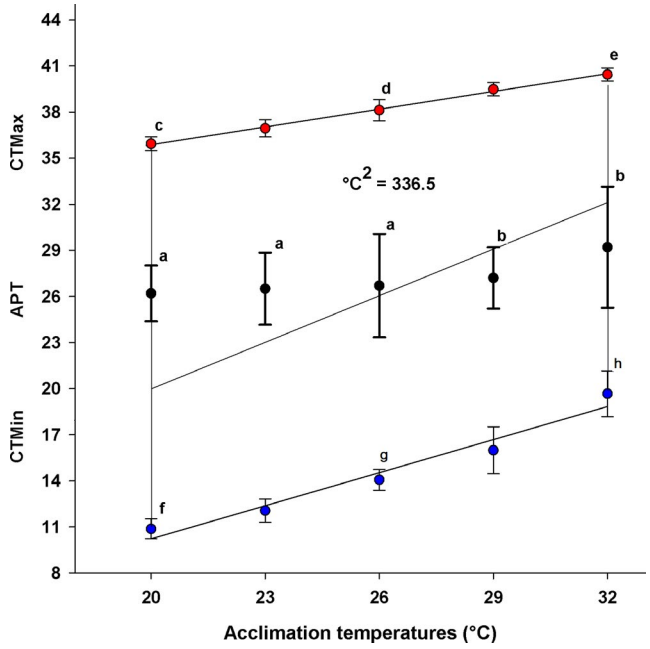

FIGURE 1 Acute preferred temperature and Critical temperatures of *Lutjanus guttatus* juveniles acclimated at 20, 23, 26, 29 and 32°C. Acute preferred temperature (black circles; median ± standard deviation (*SD*) with a statistically significant difference ($p \leq .05$), all pairwise multiple comparison procedures performed with Dunn's method. Critical thermal maximum (red circles; mean ± *SD*) with a statistically significant difference ($p \leq .05$). Critical thermal minimum (blue circles; mean ± *SD*) with a statistically significant difference ($p \leq .001$). In CT_{max} and CT_{min} multiple comparison procedures with Dunn's method. Significant differences are represented by lowercase letters

10.8 to 19.6°C respectively (Figure 1, red and blue circles). The area of the thermal window was calculated at 336.5°C^2.

The molecular responses of fish acclimated at different acclimation temperatures and after CT_{max} were evaluated through the gene expression analysis of *Hsp70* and *Ldh-a* genes. The *Hsp70* transcript values in gill and muscle increased as the acclimation temperatures increased ($p = .05$). The *Hsp70* transcripts obtained from gill and muscle were significantly lower at the acclimation temperature treatments than those observed after the organisms were exposed to CT_{max} ($p \leq .05$; Figure 2).

The relative expression of *Ldh-a* in juvenile gills was higher ($p \leq .001$) at acclimation temperatures of 20 and 23°C than the organism exposed to CT_{max}. After the organisms were exposed to CT_{max}, the *Ldh-a* transcripts increased significantly at 26, 29 and 32°C (Figure 3). In muscles, the *Ldh-a* transcripts were significantly higher in the organisms acclimated from 20 to 32°C after being exposed to CT_{max} ($p \leq .001$) than the *Ldh-a* values in the organisms not exposed to CT_{max}.

When the oxygen consumption of fish acclimated at the different temperatures was analysed, an increase was observed in the RMR values (mean ± standard error [*SE*]) as the acclimation temperature increased. The organisms acclimated at 20, 23 and 26°C had lower RMR (256.6–260.5 mg O_2 hr^{-1} kg^{-1} w.w.), and they were significantly different ($p \leq .05$) compared to those obtained in organisms acclimated at 29 and 32°C (374.7 and 419.4 mg O_2 hr^{-1} kg^{-1} w.w. respectively) (Figure 4). The Q_{10} values were determined to explain the effect of the acclimation temperature in the metabolism (Table 2).

The HMR values (mean ± *SD*) of the fish acclimated from 20 to 29°C increased from 375.3 to 442 mg O_2 hr^{-1} kg^{-1} w.w. (Figure 5) after

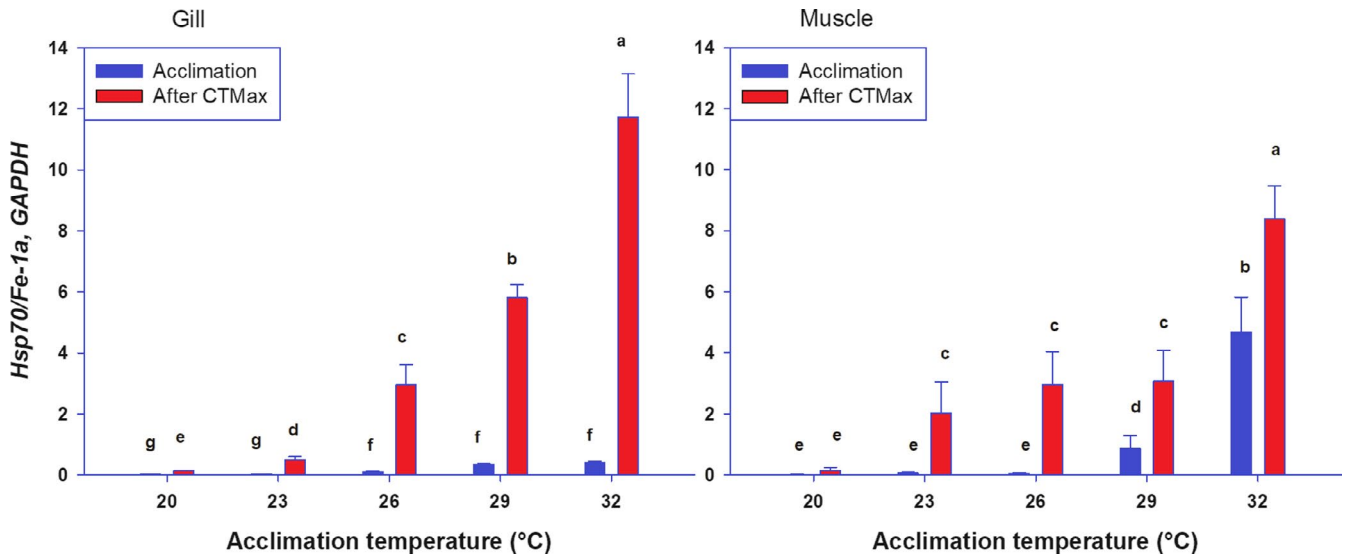

FIGURE 2 Expression of *Hsp70* in gill and muscle of *Lutjanus guttatus* under different thermal conditions. The blue columns represent the mRNA levels of *Hsp70* in controls (acclimation temperatures 20, 23, 26, 29 and 32°C); red columns represent the *Hsp70* values after CT_{max} (36, 37, 38.3, 39.5 and 40.5°C). The statistical differences were estimated with Kruskal–Wallis ($p \leq .50$). The mean transcript values between the controls and CT_{max} were estimated with a *t* test ($p \leq .05$). *Hsp70*; heat shock protein 70, *Fe-1α*; elongation factor 1-alpha, *GAPDH*; glyceraldehyde 3-phosphate dehydrogenase, CT_{max}; critical thermal maximum. Significant differences are represented by lowercase letters

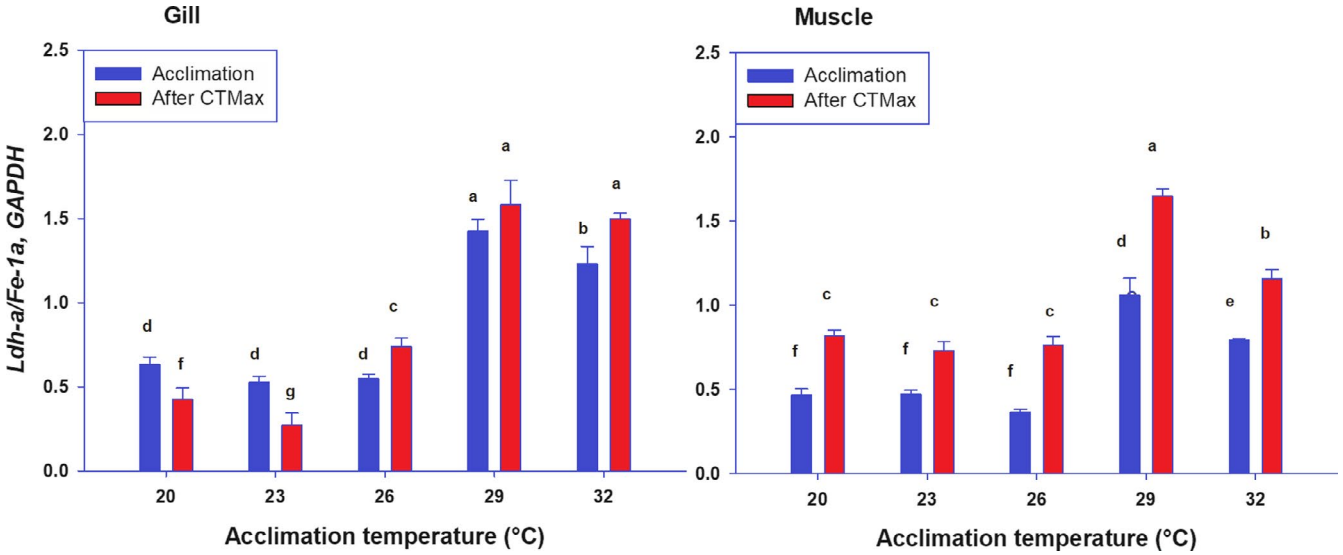

FIGURE 3 Expression of *Ldh-a* in gill and muscle of *Lutjanus guttatus* under different thermal conditions. The blue columns represent the mRNA levels of the controls (acclimation temperatures 20, 23, 26, 29 and 32°C); red columns represent the *Ldh-a* values after CT_{max} (36, 37, 38.3, 39.5 and 40.5°C). Statistical differences at the acclimation temperature and after CT_{max} were estimated with ANOVA = $p \leq .001$. The mean transcript values between the controls and CT_{max} were assessed with a *t* test ($p \leq .001$). CT_{max}, critical thermal maximum; *Fe-1α*, elongation factor-1alpha; *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase; *Ldh-a*, lactate dehydrogenase-a. Significant differences are represented by lowercase letters

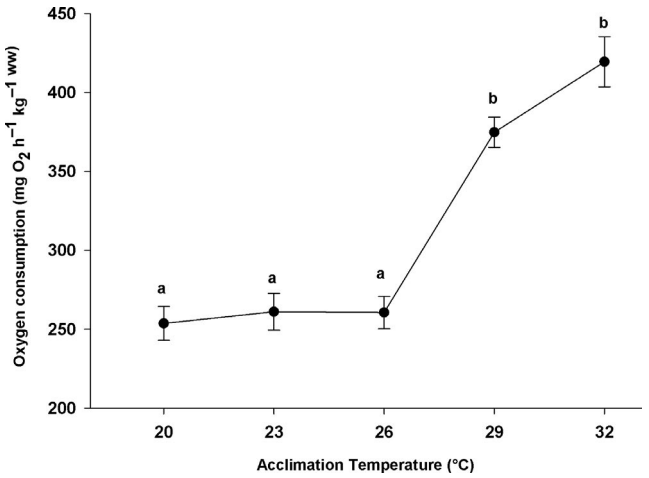

FIGURE 4 Routine metabolic rate (RMR) of *Lutjanus guttatus* juveniles acclimated at different temperatures. RMR = mean ± standard error (*SE*) with a statistically significant difference ($p \leq .001$). Multiple comparison procedures with Dunn's Method. Significant differences are represented by lowercase letters

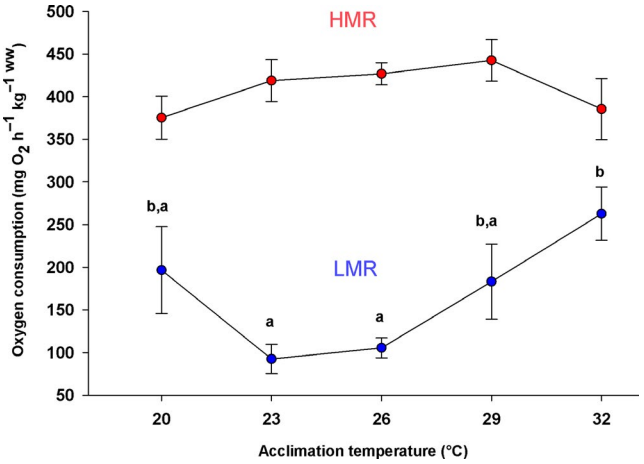

FIGURE 5 High metabolic rate (HMR) and low metabolic rate (LMR) of *Lutjanus guttatus* acclimated at five temperatures. HMR = mean ± standard deviation; no statistically significant difference determined with Kruskal–Wallis method ($p = .230$). LMR = mean ± *SD*; a statistically significant difference determined with Dunn's method ($p = .012$). Significant differences are represented by lowercase letters

TABLE 2 Q_{10} of *Lutjanus guttatus* acclimated at five temperatures

Rate2/rate1 (°C)	Q_{10}
20–23	1.10
23–26	0.99
26–29	3.35
29–32	1.45

they were exposed to $TIMR_{Max}$ (32.3–35.5°C; Table 3). The fish acclimated at 32°C showed a decrease of HMR to 385 mg O_2 hr^{-1} kg^{-1} w.w. after they were exposed to $TIMR_{Max}$ compared with the fish acclimated at 29°C. The HMR determined from the juveniles did not show significant statistical differences ($p = .230$). On the other hand, the LMR values (mean ± *SD*) increased significantly from 92.4 to 262.7 mg O_2 hr^{-1} kg^{-1} w.w. after exposed fish acclimated from 23 to 32°C to the $TIMR_{Min}$ (Table 3). The fish acclimated at 20°C showed

TABLE 3 Temperature induces metabolic rate (TIMR) on *Lutjanus guttatus* acclimated at different temperatures

AT (°C)	$TIMR_{Max}$	$TIMR_{Min}$
20	32.3	11.9
23	33.2	13.2
26	34.3	15.4
29	35.5	17.5
32	36.4	21.6

Note: $TIMR_{Max}$ and $TIMR_{min}$ were calculated for 90% of CT_{max} and 110% of CT_{min} respectively.

an LMR of 196.6 mg O_2 hr^{-1} kg^{-1} w.w. after $TIMR_{Min}$, which was significantly higher than the LMR determined in the fish acclimated at 23°C ($p < .05$; Figure 5).

The thermal metabolic scope was calculated as the difference between HMR and LMR. The highest thermal metabolic scope value obtained was from 23 to 26°C with an interval from 326.3 to 320.5 mg O_2 kg^{-1} hr^{-1} w.w. On the other hand, at the extreme acclimation temperatures (20 and 32°C) the lowest values were observed, from 178.7 to 122.3 mg O_2 kg^{-1} hr^{-1} w.w. respectively (Figure 6).

4 | DISCUSSION

The effects of temperature changes due to global warming, specifically in relation to physiological responses, such as thermoregulatory behaviour, thermal tolerance, metabolic and gene expression of marine organisms could help us understand the possible consequences of climate change. This information will aid in taking actions for aquaculture to develop and maintain successful projects. Thus, the reason why different approaches of thermal biology of an economically important fish for aquaculture in Mexico, such as the Spotted rose snapper *Lutjanus guttatus*, should be studied.

The observed behaviour of *L. guttatus* juveniles in the thermal horizontal gradient indicated that the fish used thermoregulatory behaviour to maintain their body temperature close to 26°C despite their acclimation temperatures. Although the determination of the optimal temperature for growth was not an objective in this study, 26°C corresponded to the final preferred temperature of *L. guttatus*, which according to Jobling, (1981), Kellogg and Gift, (1983), Schram et al. (2013), this final preferred temperature can be related to the optimal temperature for growth. The link between thermoregulatory behaviour and optimum growth temperature was demonstrated in fish where they could use specific temperature information as a guide for selecting rearing temperatures that favour maximum growth (McCauley & Casselman, 1981); therefore, 26°C is a temperature that may be related to the presence of *L. guttatus* in their natural habitat or better growth performance in farming areas when the temperature is close to 26°C.

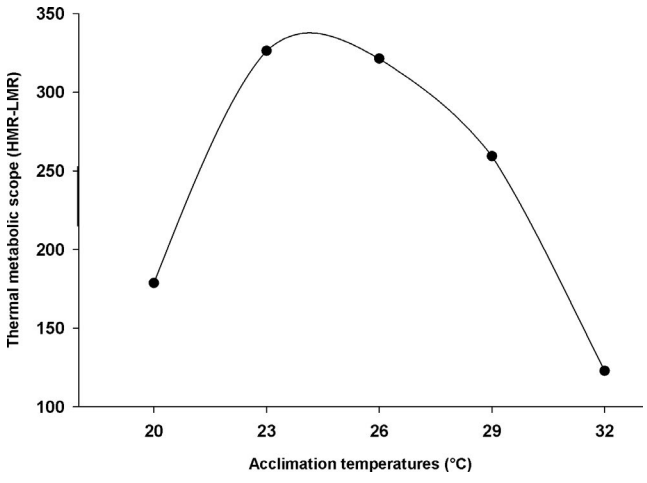

FIGURE 6 Thermal metabolic scope of *Lutjanus guttatus*

Furthermore, *L. guttatus* thermotolerance was analysed due to acute thermal increase when juveniles showed an increase in thermal tolerance directly proportional to the increase in acclimation temperature. With the CT_{max} and CT_{min} values obtained at each acclimation temperature, a thermal polygon of *L. guttatus* showed a total area of the thermal window smaller (336°C^2) than that of tropical species, such as *Apogon novemfascitus* (408°C^2), *Dascyllus aruanus* (442°C^2; Eme & Bennett, 2009) but higher than *Centropomus undecimalis* (288.6°C^2) (Noyola et al., 2015) and *Seriola lalandi* (258°C^2; Larios-Soriano, 2018). Because *L. guttatus* inhabits the demersal zone, they should have greater adaptability to thermal variations compared to species as *C. undecimalis* and *S. lalandi* that inhabit near- and offshore waters (Larios-Soriano, 2018; Pachske et al 2018). Scenarios, such as coastal zones have high and low tides, limited food areas that could allow a greater displacement ability on organisms, modifying their thermoregulatory behaviour. With this information, we may point out that *L. guttatus* is a subtropical species that avoids temperatures above 26°C, and however, it may tolerate temperatures above 32°C at least 21 days and above 35°C for a short period of time depending on the recent thermal history or the need to swim at sub-optimal temperatures in search for food.

The expressions of *Hsp70* and *Ldh-a* genes were analysed in gills and muscle of *L. guttatus* at the acclimation temperatures and after CT_{max}. This study observed the lowest expression values of *Ldh-a* and *Hsp70* genes at the acclimation temperatures from 20 to 26°C, which could refer to their regulatory functions as maintaining cellular integrity and energetic homoeostasis (Iwama et al., 1999; Podrabsky & Somero, 2004). *Ldh-a* has been reported to have a determinant role in the regulation of energy processes through anaerobic metabolism during active muscular contraction (Nelson, 2016). In addition, gills are tissues where high energy requirements are needed for physiological processes as osmoregulation; thus, lactate metabolism in gills is even required as previously reported (Tseng & Hwang, 2008; Tseng et al., 2008). On the other hand, HSP70 proteins perform essential cell maintenance protecting them from thermal stress (Boone & Vijayan, 2002; Dietz & Somero, 1993). In this study, *L. guttatus* showed the lowest *Hsp70* levels at the thermal range from 20 to 26°C, thus determining that

these thermal ranges did not represent a structural danger for cells.

When the fish were exposed to a CT_{max}, a significant response of the *Hsp70* gene was observed. In almost all acclimation temperatures (from 23 to 32°C), the fish showed an overexpression of *Hsp70* in both tissues after CT_{max}, which evidenced the importance of HSPs in cell structure maintenance in facing thermal shock. Heat shock proteins are currently one of the most studied cell protection mechanisms. The function of HSP70 is to help the folding of the nascent polypeptide chains to act as a molecular chaperone and replenish the thermally damaged proteins, as well as their cytotoxic aggregation (Basu et al., 2002). This result suggested that temperature increase above 29°C (acclimation temperature where the first response in the expression of *Hsp70* gene was observed) and after the CT_{max} considerably affected cellular structure and stability. As a reduction in the *Ldh-a* transcript levels was detected at 20 and 23°C after CT_{max}, this result could be explained as a heat shock effect in the gene transcripts after CT_{max} in *L. guttatus* acclimated at low temperatures.

One of the main physiological functions associated with the stress-induced accumulation of inducible HSP70 is acquired thermotolerance, which is defined as the ability of a cell or organism to become resistant to heat stress after previous sub-lethal heat exposure (Mizzen & Welch, 1988). The phenomenon of acquired thermotolerance is of a transitory nature and depends primarily on the severity of the initial heat stress. The heat shock proteins (HSPs) play essential roles in normal conditions and situations involving both systemic and cellular stress (Kregel, 2002); thus, the closest approximation to the acquired thermotolerance in *L. guttatus* could be observed in the acclimation temperatures of 29 and 32°C, where, in addition to the increased RMR, other compensatory mechanisms were observed, such as an *Ldh-a* upregulation as a molecular index to high energy requirements and *Hsp70* upregulation as a molecular index to cellular damages.

When the oxygen consumption values were analysed, two main responses were observed: (a) tendency to maintain a stable metabolic rate at the acclimation temperatures from 20 to 26°C and (b) significant increment of the respiratory metabolism at 29 and 32°C. Since the lowest values of Q_{10} were determined in the thermal range 20–26°C, this could be a favourable thermal condition for metabolic performance. The lowest values in Q_{10} have been related to an optimum metabolic state (Díaz et al., 2007; Dowd, Brill, Bushnell, & Musick, 2006). According to Sokolova, Frederich, Bagwe, Lannig, and Sukhotin (2012), in the energy state called 'optimum', organisms also have the possibility of allocating energy to generate reserves and grow besides covering the essential energy requirements. Das et al. (2005) hypothesized that the point at which Q_{10} diminished related to acclimation temperatures corresponded to the optimal temperature for growth since the decrease in Q_{10} indicated that fish metabolism had decreased and that more energy for growth was available. Thus, the lowest Q_{10} values in *L. guttatus* indicated that the temperatures from 20 to 26°C were the temperatures where an advantageous energy state was present, and the metabolic processes were efficiently carried out to maintain homoeostasis. On the other hand, in the acclimation temperatures at 29 and 32°C, the highest Q_{10} values were found, which suggested a higher metabolic requirement (Pirozzi & Booth, 2009) and represented the sub-optimal conditions. This state is known as 'pejus' (Sokolova, 2013; Sokolova et al., 2012), where different mechanisms of metabolic compensation exist to maintain the different essential and non-essential energy requirements. The increase in the RMR at 29 and 32°C could be a metabolic compensation in the effort to recover and maintain the energetic homoeostasis as it was observed in other fish (Madeira et al., 2017) where the glycolytic potential had to be upregulated to maintain locomotion and recover homoeostasis at high temperatures.

The relationship between temperature and available aerobic energy to cover the energy demand of fish physiological processes is linked with the concept of aerobic scope (Fry, 1947). The highest thermal metabolic scope obtained for *L. guttatus* was observed at the interval 23–26°C that corresponded to the preferred temperature determined and the lowest values of Q_{10} (23–26°C). In this temperature interval, *L. guttatus* juveniles had the highest amount of available energy that could be channelled to different functions, such as activity, growth, and survival or other physiological processes that the organism demanded (Jobling, 1981). All these data supported that under these thermal intervals, juveniles were effectively found in better metabolic conditions. On the other hand, the thermal metabolic scope at the temperatures at 20 and 32°C were the lowest, which means that these temperatures exerted a metabolic pressure causing the use of alternative mechanisms to maintain homoeostasis and cellular integrity (Chabot et al., 2016; Clark & Seymour, 2006; Nelson, 2016; Pörtner, 2010; Rosewarne, Wilson, & Svendsen, 2016).

5 | CONCLUSIONS

The results obtained in this study supported the information that *L. guttatus* is a species with different physiological responses to live and support different thermal fluctuations in this habitat, which make this species an interesting organism for aquaculture projects. However, if global climate change continues, this species could be severely affected. This study identified that the temperatures from 23 to 26°C were the conditions where a series of responses were observed, such as the preferred temperature, a stable RMR, the greatest thermal metabolic scope range and different molecular parameters that could indicate a state of equilibrium or homoeostasis. These results could indicate an optimal physiological and metabolic state of *L. guttatus* juveniles. In response to the increase in acclimation temperatures at 29 and 32°C, the organisms had to increase the rates of oxygen consumption and the upregulation of *Hsp70 and Ldh-a* genes in gill and muscle. Moreover, when the organisms were exposed to CT_{max}, the induction of anaerobic metabolism was observed through the increase of *Ldh-a* gene expression levels. Finally, in the face of thermal stress, such as exposure to temperatures from 29 to 32°C for 21 days and acute stress, such as CT_{max}, an increment of *Hsp70* gene expression level was obtained in these trials.

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CONFLICT OF INTEREST

All authors have no conflict of interest to disclose.

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