



Cold temperature tolerance of the alien Indo-Pacific damsselfish *Neopomacentrus cyanomos* from the Southern Gulf of Mexico

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ABSTRACT

The abundance of the alien, Indo-Pacific damsselfish *Neopomacentrus cyanomos* on an oil-loading platform in the southwest Gulf of Mexico indicates that widely distributed platforms could facilitate the expansion of its geographic range across the western and northern fringes of the Gulf. From there it likely will spread to other areas of the Greater Caribbean. The lionfish example demonstrates that it eventually happens, and can do so rapidly. Reduced temperature effects on the physiology of this species were examined to better predict its survivability in the northern Gulf during winter, when sea surface temperatures fall as low as 15 °C along the coast. Overall, our results show that when the degree of experimental temperature decline was large and rapid, no compensation occurred and the stress response observed mostly reflected cellular processes that minimized damage. Integrated biomarker response values were significantly different between fish rapidly exposed to colder vs. warmer temperatures (declines of −4 °C each day, from 26 to 14 °C), reflected in higher values of blood metabolites and routine metabolic rates observed in fish exposed to 14 and 18 °C respectively, and lower activity of all enzymes, lower protein carbonylation, and higher oxidative damage to lipids in fish exposed to 14 °C. While the physiological proxies responded to minimize damage during the rapid-decrease experiment, the same proxies reflected the consequences of compensation when fish were thermally challenged after a 45 days acclimation at 18 °C. In this case, lower values of blood metabolites and high antioxidant levels and indicators of damages underpinned its pejus lower range. Based on the results of the present work, it seems clear that low winter SSTs in the northern Gulf will slow down the colonization of the inshore area of *N. cyanomos*. We suggest that the use of physiological cellular stress markers on specimens acquired at the beginning of an invasion should be implemented in new standardized experimental protocols, including both rapid increases/decreases of temperature and post-acclimation temperature challenges, to assess the invasiveness potential of aquatic species such as this.

1. Introduction

Neopomacentrus cyanomos, a species native to the Indo-west Pacific Ocean, was first reported in the southwest corner of the Gulf of Mexico

in 2013, on coastal reefs near Coatzacoalcos (González-Gándara and Cruz-Francisco, 2014). Since then, the species was found on other reefs to the east and northwest of that location (de la Cruz-Francisco et al., 2015; Robertson et al., 2016), and more recently on oil platform

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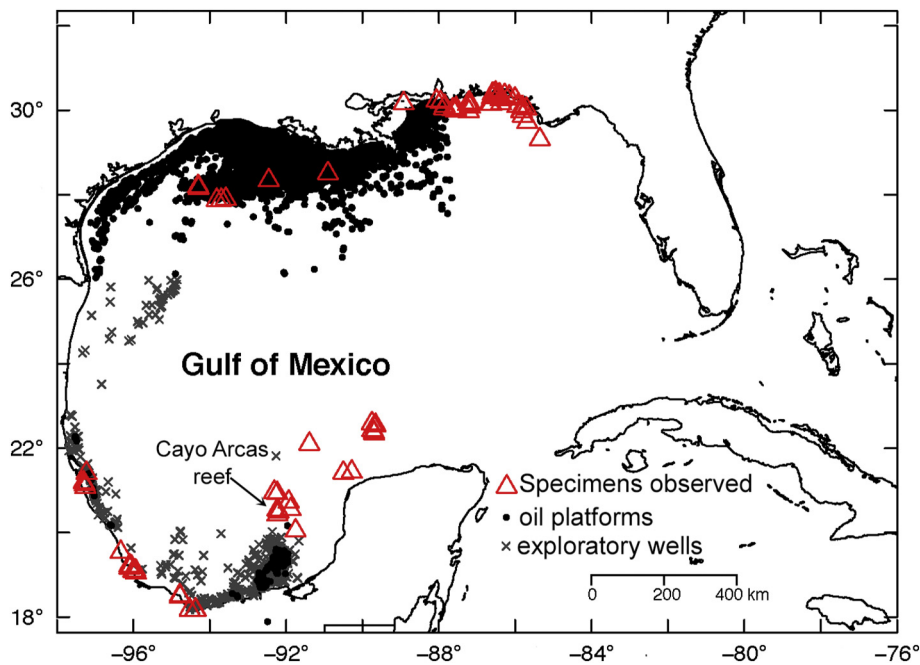


Fig. 1. Locations where *N. cyanomos* has been reported as of November 2019 (Nonindigenous Aquatic Species information resource for the United States Geological Survey; updated 25.11.2019; nas.er.usgs.gov/viewer/omap.aspx?SpeciesID=2936; red triangles), with Cayo Arcas reef indicated and distribution of oil platforms in the entire Gulf of Mexico (filled black circles) and of exploratory wells in the Mexican section of that gulf (grey cross marks). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

structures, during the warmer months of 2016 and 2017, off the coast between Texas in the west and Alabama and the Florida panhandle in the east (Fig. 1) (Bennett et al., 2019; Schofield and Neilson, 2019). In the south, a massive aggregation has been observed under an oil-loading platform adjacent to Cayo Arcas reef 350 km northeast of Coatzacoalcos (Simões and Robertson, 2016), between 0 and 45 m depths (DRR personal observations). Oil platforms host high fish biodiversity in many areas around the world (Claisse et al., 2014; Friedlander et al., 2014; Torquato et al., 2017), including the Gulf of Mexico (Hastings et al., 1976; Ajemian et al., 2015) and long-distance relocation of such platforms has been proposed as the vector of introduction of non-native fish species to the Canary Islands (Pajuelo et al., 2016) as well as the introduction of *Neopomacentrus cyanomos* to the southwest Gulf (Robertson et al., 2018). Given the ability of *N. cyanomos* to thrive on an oil platform, and its recent occurrence on platforms in the northern Gulf, it seems possible that this species has expanded its range permanently to that area. As, the lionfish (*Pterois volitans*) example has demonstrated, a spread of *N. cyanomos* to a much larger area is quite likely. In addition, this species was recently found in abundance at Trinidad (Robertson and Kingon, 2019) from where it could easily spread northwards throughout the Caribbean Sea.

Little information is available on the thermal tolerance to low temperatures of *N. cyanomos* that could indicate if this species can effectively colonize the Northern Gulf of Mexico, where low winter temperatures could limit its distribution. The present study examined physiological responses of *N. cyanomos* collected in the southwest Gulf of Mexico to low temperatures, with and without acclimation, to allow prediction of its capacity to survive through winter in the northern Gulf of Mexico. From an oceanographic point of view and working with pelagic larvae duration, Johnston and Akins (2016) highlighted the possibility that disconnection of surface currents between the southwest and northern zones of the Gulf likely would slow any spread northward of *N. cyanomos*. They did not include temperature or salinity in their model because the species was considered tolerant to a wide range of both parameters; e.g. it can breed in reduced-salinity conditions (Setu et al., 2010; Johnston and Akins, 2016).

Temperature has been shown to be the main drivers of the geographical distribution of marine species (Poloczanska, et al., 2016), and this is also true for alien species. The degree of thermal plasticity is crucial to defining where an organism can and cannot occur, while

acclimation capacity reflects the potential of an organism to express a wider thermal tolerance range (Pörtner and Knust, 2007; Pörtner, 2010; Sokolova et al., 2012). To date, research on thermal tolerance of tropical reef fishes, including damselfishes, has mostly focused on effects of high temperatures, in order to assess potential effects of global warming. Johansen and Jones (2011) studied responses to several weeks of acclimation to temperatures ~ 3 °C above prevailing summer conditions on the Australian Great Barrier Reef in *N. cyanomos* and nine other damselfishes in four genera. They observed a reduction of $\sim 2/3$ in the aerobic scope of these fishes, i.e. the energy available to maintain cellular processes and the potential capacity to convert energy in physical activity such as swimming. Also, significant mortality was observed in another damselfish (*Acanthochromis polyacanthus*) that was maintained at 3 °C above current average temperatures during ten months of acclimation (Rodgers et al., 2018).

A study of the effects of local winter sea surface temperatures (23 °C SST) on the Great Barrier Reef on *N. cyanomos*, two other *Neopomacentrus* species and seven other damselfish species from three genera demonstrated that swimming performance was reduced under such conditions (Johansen et al., 2015). The species *N. cyanomos*, in its native range (see www.gbif.org/species/2398535; accessed November 25th, 2019), is exposed to average winter SSTs as low as 18 °C at sites such as southeast Africa, southeast Australia, the Gulf of Eilat, the Gulf of Oman, and southern Japan. This suggests that winter SSTs below 15 °C in the inshore areas of the northern zone of the Gulf of Mexico could slow down the permanent establishment of *N. cyanomos*. To test *N. cyanomos*' cold tolerance, we combined the results of two independent experiments involving several tested characters in individuals collected adjacent to a coral reef in the southwest Gulf of Mexico: 1) fish exposed to rapidly declining temperature; and 2) fish acclimated to reduced temperatures for sufficient time to allow the expression of compensatory mechanisms. Measurements of RMR, metabolites (in blood), and cellular stress markers (in muscle) were used to assess the cold tolerance of this alien species.

2. Materials and methods

2.1. Animals

The damselfish *Neopomacentrus cyanomos* were collected twice

under an oil-loading platform 1.5 km from the Cayo Arcas reef (20.21°N, -91.98°W), in August 2016 ($n = 16$) and August 2017 ($n = 30$). Due to a lack of aquarium facilities at Cayo Arcas fish were collected only during the last two days of the diving campaign to preserve their physiological integrity until arrival to the experimental facilities. These logistic limitations explain the relatively small sample size. Divers captured specimens either by shaking fish out of tubular sponges they were using as hiding places into plastic bags that enveloped the sponges. Collecting methods were approved by the ACUC of the Smithsonian Tropical Research Institute (no. 2017-1107-2020). Fish were transported to the Unidad Multidisciplinaria de Docencia e Investigación de la Universidad Autónoma de México (UMDI-UNAM, Sisal, Mexico) and acclimatized in natural filtered (5 μm) seawater for two weeks (*in situ* temperature, at 26 ± 1 °C with salinity of 37), two to three individuals per aquarium (11L), with dissolved oxygen $> 5.5 \text{ mg}\cdot\text{L}^{-1}$, pH > 8 , and a 12 h photoperiod. Fish were fed twice a day with *Artemia nauplii* in 2016 and with tropical fish flake food (Wardley, United-States of America) in 2017.

2.1.1. Experiment 1: Acute decreasing temperature protocol

All fish sampled in August 2016 ($n = 16$; wet weight or WW = $2.67 \pm 0.99 \text{ g}$) were transferred to a 25 L tank that was maintained at 26 ± 1 °C and equipped with shelters and plastic grasses 72 h prior to the beginning of the decreasing temperature protocol. The acute-thermal-decline protocol was based on Norin et al. (2014) and consisted in exposing all fish simultaneously to decreasing temperature, by -4 °C over a period of 1 h, and maintaining that temperature for 23 h. This protocol allowed the evaluation of the cumulative effect of a rapid decline (in three steps over three days) in temperature from 26 °C to 14 °C. Each tank was placed in a quiet and semi-dark environment to obtain the routine metabolic rate (RMR) and has enough space to accommodate both respirometry chambers (on stilts) and free-swimming fish. Four fish were randomly selected from the tank and placed individually in respiration chambers at 26 °C (see Section 2.2) to assess their baseline physiological state at *in situ* temperature. After 24 h in the respirometers, the four fish were sacrificed (decapitation) for blood metabolites and biochemical stress indicators (see Section 2.3). Then, another four fish were randomly selected from the tank, placed in the respirometry chambers and the seawater temperature of the whole system (respiration chambers and tank) was reduced to 22 °C over a period of 1 h. Oxygen consumption of the fish within the respiration chambers was evaluated during the following 23 h. That procedure was repeated every 24 h to expose fish sequentially to 22 °C, 18 °C and 14 °C, with the individuals in the respirometers being sacrificed for biochemical analysis at the end of the 24 h exposure and replaced with other individuals. The replacement of the fish in the respiration chambers was intended to obtain independent respiration measurements of the cumulative effect of the rapid decline in temperature. Fish were fed twice a day with *Artemia nauplii*, except individuals inside the respiration chambers, which were not fed during the entire 24 h measurement. All 24 h measurements started at 10 am. As the exposure to 18 °C was lethal for one fish in the tank, only three fish were available for the 14 °C measurement.

2.1.2. Experiment 2: Acclimation

Fish collected from the Cayo Arcas platform in August 2017 ($n = 30$; WW = $2.35 \pm 1.80 \text{ g}$) were transferred individually to 3 L aquaria equipped with shelters at 26 ± 1 °C in a controlled multi-parameters system (Aquabiotech, Coaticook, Canada) at the laboratory of Applied Ecophysiology. The controlled multi-parameter system consists of four independent units with digital control of temperature and oxygen, each equipped with its own circulating and filtered seawater. Six (26°) to eight (22 °C, 20 °C, and 18 °C) aquariums were used per unit of the system. Fish were fed twice a day with tropical fish flake food (Wardley, United-States of America), except for 24 h prior to RMR measurement. To avoid mortality, the lowest temperature selected was

18 °C. The day after their transfer, the temperature of the three units containing each eight fish was decreased of 1 °C per day, at a rate of $0.1 \text{ }^{\circ}\text{C}\cdot\text{h}^{-1}$ (the decrease of 1 °C started at 9 h, ended at 19 h and the temperature was maintained until the next day) until 22, 20 and 18 °C were reached, respectively. Once the target acclimation temperature was reached, a period of 45 days acclimation began. After this period, all fish were transferred individually to the respiration chambers at their acclimation temperature for 24 h (see Section 2.2). Respirometry chambers were maintained in a quiet and semi-dark environment to obtain the RMR of fish at each acclimation temperature. Subsequently, half of the fish were sacrificed (decapitation) for analyses of blood metabolites and biochemical stress indicators (see Section 2.3).

The remaining fish in the respiration chambers were used for a high temperature challenge. This challenge was designed to assess the effect of cold thermal acclimation on heat tolerance scope. It consisted in increasing by 1 °C the water circulating within the respiration chambers at a rate of $0.2 \text{ }^{\circ}\text{C}\cdot\text{min}^{-1}$. The temperature was maintained for 5 min at each degree of increase to assess if there was a noticeably increase of swimming activity, which we considered equivalent to escape behaviour. If no escape behaviour was observed, the temperature was increased 1 °C further following the same rate. When the first escape behaviour was noted the fish was sacrificed (decapitation) for blood metabolites and biochemical stress indicators analysis (see Section 2.3). The escape behaviour was observed at 25 ± 3 , 28 ± 2 , 28 ± 3 and 30 ± 3 °C for the acclimation temperatures of 18, 20, 22, and 26 °C respectively.

2.2. Routine metabolic rates (RMR)

RMR was measured using a continuous flow respirometer, with the respirometry chambers connected to a well-aerated open re-circulating seawater system at the experimental temperature (Rosas et al., 2008). To obtain a steady state of the system, the water was well-mixed and the measurement lasted 23 to 24 h to ensure that any change in fish oxygen consumption was revealed accurately in the out-flowing water, following Steffensen (1989) and Svendsen et al. (2016) recommendations. The volumes of the respiration chambers were of 100 mL or 500 mL to ensure fish movement without restrictions into the chamber. The water flow was measured at the beginning and at the end of the measurement by filling a graduated cylinder for a duration of 15 s and converting this volume in $\text{L}\cdot\text{h}^{-1}$. Measurements of dissolved oxygen ($\text{mg}\cdot\text{L}^{-1}$) were recorded for each chamber (entrance and exit) every second using oxygen sensors attached to flow-cells that were connected by optical fiber to a witrox amplifier (Loligo Systems, Denmark). One empty chamber was used for each chamber size as a control to account for microbial oxygen consumption in the filtered seawater. The sensors were previously calibrated at each temperature using saturated seawater (100% air saturation) and a 5% sodium sulphate solution (0% air saturation) to obtain the phase values of each optical fibre coupled with its oxygen sensor. RMR was calculated as the difference in dissolved oxygen concentrations between the input and output of each chamber, with incorporation of the water-flow rate and the microbial oxygen consumption. The RMR values of fish exposed to acute decrease of temperature and of acclimated fish were selected using the R package 'fishMO2' created by Chabot (2016) following his recommendation (Chabot et al., 2016). Oxygen consumption data were expressed as $\text{mg O}_2\cdot\text{h}^{-1}\cdot\text{g WW}^{-1}$. The Q_{10} temperature coefficient was calculated with the respiration values of both experiments to give insights on the changing performance of the fish and their compensation capacity:

$$Q_{10} = \left(\frac{\text{mean RMR at } T_2}{\text{mean RMR at } T_1} \right)^{\frac{10}{(T_2 - T_1)}}$$

where T_2 and T_1 corresponded to the highest and lowest acclimation temperature (°C), respectively. The Q_{10} temperature coefficient can be calculated for intervals < 10 °C (Atkin and Tjoelker, 2003). Here we

calculated Q_{10} for a minimum interval of 4 °C.

2.3. Metabolites and biochemical stress indicators analysis

Metabolites and biochemical stress indicators were analysed in fish subject to three experimental treatments: 1) acute temperature reduction; 2) after RMR following acclimation to low temperature; 3) and after the high temperature challenge following acclimated fish RMR measurements. At the end of the respiration measurement, fish were collected one by one with an aquarium net, dried with tissue paper and weighted (± 0.01 g; Ohaus Scout pro, USA). After wet weights were taken, fish were immediately decapitated. Fresh blood from the head was directly applied to lactate [$\text{mM}\cdot\text{L}^{-1}$] (end-product of anaerobic metabolism) and glucose [$\text{mg}\cdot\text{dL}^{-1}$] (principal energetic fuel) testing strips manufactured for use by the Accutrend Plus instrument (Roche Diagnostics, Mannheim, Germany), making sure that each strip was completely covered with blood (approximately 15 μL). Lactate and glucose values under the level of detection of the Accutrend apparatus were assigned to half of the detection limit value for each metabolite (i.e. lactate: $0.35 \text{ mM}\cdot\text{L}^{-1}$, glucose: $10 \text{ mg}\cdot\text{dL}^{-1}$). This type of device has been widely used in applied studies to assess blood physiology parameters in teleost fish (Stoot et al., 2014).

Muscle tissue was separated on ice and snap frozen in liquid nitrogen, at the same time as the metabolite's measurement were made. Muscle samples were then transferred to an ultra-freezer at -80 °C for later analysis in the Coastal Environmental Science Laboratory at Facultad de Química de UMDI-UNAM. Samples were homogenized in cold buffer Tris pH 7.4 at $100 \text{ mg tissue}\cdot\text{mL}^{-1}$ using a Potter-Elvehjem homogenizer. Subsequently, the muscle homogenate was divided for triplicate assays of several biochemical stress markers: activities of acetylcholinesterase (AChE; Ellman et al., 1961 adapted by Rodríguez-Fuentes et al., 2008), carboxylesterase (CES; Hosokawa and Satoh, 2001 with modifications), catalase (CAT; Goth, 1991 modified by Hadwan and Abed, 2016), glutathione S-transferase (GST; Habig and Jakoby, 1981), superoxide dismutase (SOD; only in 2017; Sigma-Aldrich assay kit 19160), as well as quantification of carbonyls in oxidized proteins (CO; Mesquita et al., 2014), lipid peroxidation (LPO; Sigma-Aldrich PeroxiDetect Kit) levels, and total glutathione (GSH; only in 2017; Sigma-Aldrich Glutathione Assay Kit CS0260). All spectrophotometric measurements were made in a micro-plate reader and proteins were analysed in the supernatant following Bradford (1976) to standardize all enzyme activities in activity unit (U) $\cdot\text{mg protein}^{-1}$. For AChE, CES, SOD, CAT, and GST activity assays, muscle homogenates were centrifuged at $10,000 \times g$ for 5 min at 4 °C and the supernatant was separated for analysis.

Citrate synthase (CS; Sidell et al., 1987), and lactate dehydrogenase (LDH; Lactate dehydrogenase-SL assay, Diagnostic Chemicals Limited) were only assessed in acclimated fish from the 2017 experiments, and were done to complement the energetic indicators analysis. CS is frequently measured as an indicator of mitochondrial density to assess the aerobic capacity in a tissue, while LDH catalyses the final step in anaerobic glycolysis through the conversion of pyruvate to lactate via coupled oxidation of reduced nicotinamide adenine dinucleotide to its oxidized form. These enzymes were analysed separately in a second aliquot of the fish muscle. Muscle samples were weighed in a Precellys homogenization tube (Sartorius LA230S, Goettingen, Germany) and diluted 1:20 (w/v) with ice-cold Trizma™ hydrochloride (Tris-HCl) buffer [20 mM Tris-HCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.1% (v/v) Tween 20, pH 7.4]. Subsequently, tubes were placed in a homogenizer (Bertin Technologies - MINILYS, Montigny-le-Bretonneux, France) with the following cycle: 2:10 s, 5000 rotations. After centrifugation at $7400 \times g$ for 5 min at 4 °C, the supernatant of the homogenate was removed and used for measurement.

2.4. Statistical analysis

A complete set of original data from all experiments is provided in the open access online data repository PANGAEA (doi:<https://doi.org/10.1594/PANGAEA.891264>). All statistical analyses were done using R (R core team, 2018). The package 'vegan' (Oksanen et al., 2019) was used to assess the effects of temperature in the physiological responses of fish for each set of experiments with distance-based multivariate statistics. First, physiological variables were normalized (centred on their means), and the homogeneity of the multivariate dispersion in the Euclidean space was tested with a multivariate analogue to Levene's test prior to permutational analysis of variance (PERMANOVA) procedure, using distances to centroids and 9999 permutations (Anderson, 2006). Subsequently, one-way PERMANOVA tests were performed based on Euclidean distances, the probability associated with the *pseudo-F* statistic under a true null hypothesis being estimated with 9999 permutations (Anderson, 2017). If a significant difference was obtained, a pairwise multivariate *t*-test was done. For each experiment, the variables responsible for the differences between groups were then identified using decomposition of the Euclidean distance between each pair of samples and averaging the contributions of each variable to the differences between treatments (SIMPER analysis). Principal Component Analysis ordinations based on the correlation matrix revealed that, in neither case, the first two to three components accounted for > 50% of total variation. Consequently, non-metric multidimensional scaling (nMDS) plots were made for output visualization.

To support the PERMANOVA approach and visualize which variable influenced the stress responses, parameters were divided in two groups for an Integrated Biomarker Response (IBR) analysis (Beliaeff and Burgeot, 2002). IBR analysis have been employed in many eco-physiological studies to assess thermal acclimation of fishes and invertebrates (see Marigómez et al., 2013; Ferreira et al., 2015; Madeira et al., 2016, 2018, 2019). For this, energetic indicators (concentrations of lactate, glucose, WW, RMR, LDH and CS) and the biochemical stress indicators were analysed for each set of experiment (AChE, CES, SOD, GSH, CAT, GST, CO and LPO). The global response of both groups was summarized in one general "stress index" for each fish to assess the effect of low-temperature exposure or acclimation (following Li et al., 2016). Radar plots were done using the 'fmsb' package (Nakazawa, 2018) in R with mean scores.

The sample size (number of individuals per treatment), although small, was sufficient to allow establishment of large-magnitude effects using a power analysis of 0.8 and a significance level of Error Type I fixed at 95% ($\alpha = 0.05$). Differences between RMR and IBR following the rapid-decrease temperature protocol and the 45-days acclimation were tested separately with one-way analysis of variance (ANOVA) and a Tukey post-hoc comparison. Data were checked for outliers with Cleveland dot plots, transformed (square root or logarithmic) and tested for normality (Shapiro-Wilk test) and variance homogeneity (Levene's test) prior to ANOVA. If criteria were not met, Kruskal-Wallis test was used and post-hoc comparison were realized with the package 'pgirmess' (Giraudeau, 2018).

3. Results

3.1. Routine metabolic rates (RMR)

3.1.1. Acute temperature decrease protocol

Neopomacentrus cyanomos exposed to the acute-decrease temperature protocol had higher RMR at 18 °C compared to 22 °C (ANOVA, $F_{(3,11)} = 6.271$, $p = .010$; Table 1). Temperature coefficients of $Q_{10} < 1$ were observed when RMR of fish exposed to 14 °C ($Q_{10} = 0.5$) and 18 °C ($Q_{10} = 0.1$) were compared with fish exposed to 22 °C, and when fish exposed to 18 °C ($Q_{10} = 0.5$) were compared with fish maintained at 26 °C. Values of $Q_{10} > 1$ were observed when RMR of fish exposed to 14 °C ($Q_{10} = 1.1$) and 22 °C ($Q_{10} = 4.7$) were compared

Table 1

Routine metabolic rate (RMR; in $\text{mg O}_2\cdot\text{h}^{-1}\cdot\text{gWW}^{-1}$) of *Neopomacentrus cyanomos* exposed to an acute temperature decrease protocol (experiment 1) and acclimated 45 days (Experiment 2); (n) – number of individuals measured; Letters indicate significant differences; mean \pm standard error.

Temperature ($^{\circ}\text{C}$)	Experiment 1	Experiment 2
26	0.23 ± 0.09 (4) ^{ab}	0.75 ± 0.17 (6) ^a
22	0.13 ± 0.08 (4) ^a	0.86 ± 0.35 (8) ^a
20	–	0.44 ± 0.27 (8) ^b
18	0.43 ± 0.13 (4) ^b	0.23 ± 0.03 (8) ^b
14	0.20 ± 0.06 (3) ^{ab}	–

with fish maintained at 26°C . The highest Q_{10} value was obtained when fish exposed to 14°C were compared with fish exposed to 18°C ($Q_{10} = 6.5$).

3.1.2. Acclimation protocol

When acclimated for 45 days, RMR of fish acclimated at warmer temperatures (26° and 22°C) were higher than fish acclimated at cold temperatures (20° and 18°C ; ANOVA, $F_{(3,26)} = 15.49$, $p < .000$; Table 1). Fish acclimated to 18°C showed the highest Q_{10} values when their RMR was compared to that of fish acclimated to 22°C ($Q_{10} = 27.0$). Intermediate values of Q_{10} were obtained when RMR at 18°C -exposure ($Q_{10} = 4.4$) and 20°C -exposure ($Q_{10} = 2.4$) were compared to those at 26°C , while a low value ($Q_{10} = 0.7$) was obtained when the RMR at 22°C was compared with RMR at 26°C .

3.2. Physiological proxies: Metabolites and biochemical parameters

3.2.1. Experiment 1: Acute temperature decrease protocol

Rapidly decreasing temperature provoked significant changes in blood metabolites and antioxidant defence mechanisms in centroids of fish subject to declines down to 18 and 14°C (PERMANOVA, $pseudo-F_{(3,11)} = 1.912$, $p = .009$; Fig. 2A), compared to 26°C (paired-wise test). Energetical and biochemical stress IBR indices corroborated these changes with higher IBR values in the colder vs. warmer temperatures when integrating wet weight (WW), lactate (Lac), glucose (Glu), and RMR (ANOVA, $F_{(3,11)} = 6.249$, $p = .010$; Fig. 3A). This difference probably was due to higher values of blood metabolites and RMR observed in fish exposed to 14 and 18°C respectively (Fig. 3A). When integrating the biochemical stress variables, a lower IBR value at 14°C compared to 26°C (Kruskal-Wallis chi-squared $_{(3,11)} = 8.5167$, $p = .036$; Fig. 3B) was observed from lower activity of all enzymes, as well as lower protein carbonylation (CO), and a higher oxidative damage to lipids (LPO) in fish exposed to 14°C (Fig. 3B).

3.2.2. Experiment 2: Acclimation

The effect of cold temperature acclimation on physiological proxies was evaluated using fish sampled immediately after the measurement of RMR. No significant changes in blood metabolites and antioxidant defence mechanisms were observed between centroids of fish acclimated to 18°C and those maintained at 26°C (PERMANOVA, $pseudo-F_{(3,10)} = 1.509$, $p = .077$; Fig. 2B). However, energetical IBR indices showed significant differences with higher IBR values in the warmer vs. colder temperature when integrating WW, Lac, Glu, RMR, lactate dehydrogenase (LDH) and citrate synthase (CS) activities (ANOVA, $F_{(3,11)} = 9.219$, $p = .002$). These differences are probably due to lower values of RMR and CS activity observed in fish acclimated to 18 and 20°C , and to the lowest values of all parameters at 18°C (Fig. 3C). When integrating the biochemical stress variables, no significant changes were observed (Fig. 3D). However, a bell-shaped thermal curve underpinned lowest levels of parameters at 18 and 26°C : At 26°C , low acetylcholinesterase (AChE) and superoxide dismutase (SOD) activities were notable. At 18°C , fish maintained low levels of antioxidants, LPO and CO (Fig. 3D).

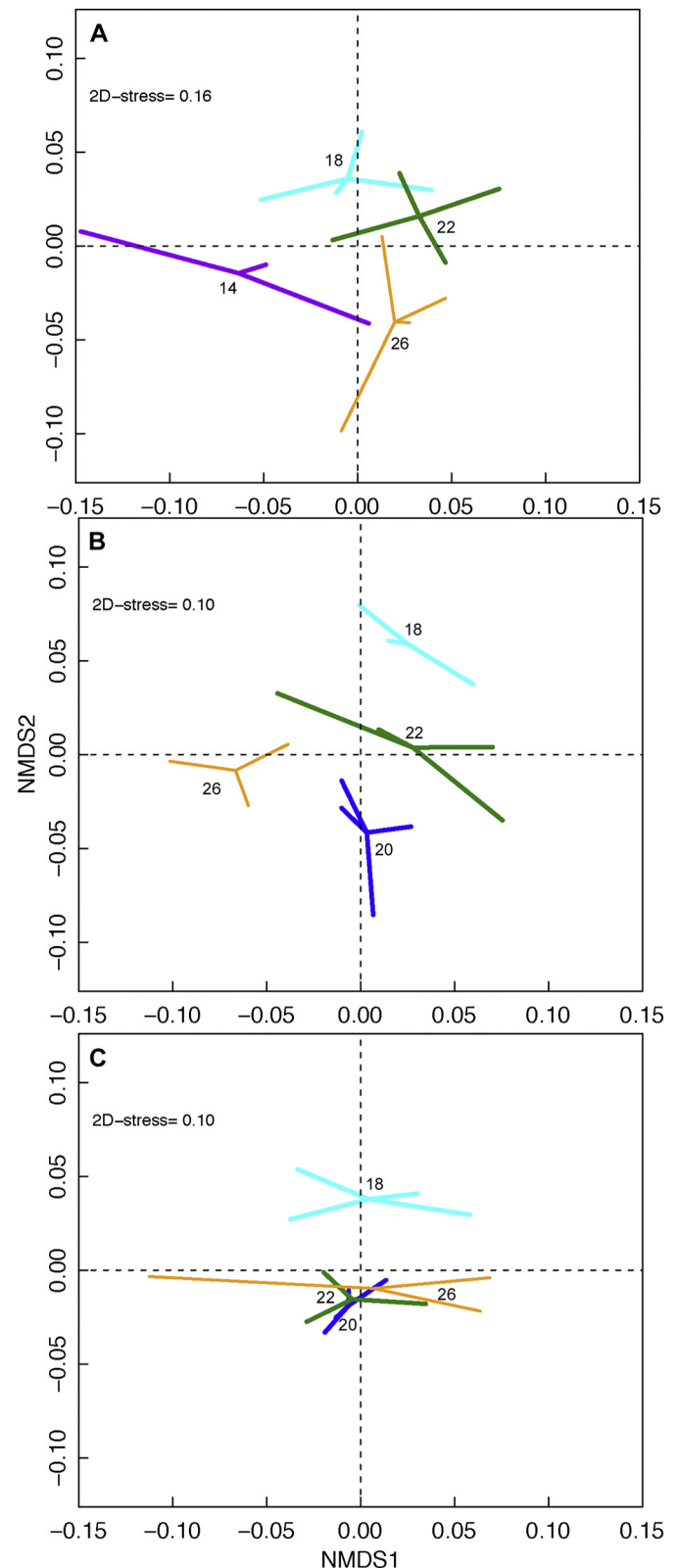


Fig. 2. Non-metric multidimensional scaling (nMDS) analyses showing *Neopomacentrus cyanomos* distance to centroids categorized by temperature (14°C = purple; 18°C = cyan; 20°C = blue; 22°C = green; 26°C = orange) in the physiological proxies (metabolites and biochemical parameters) space after (A) the acute decrease in temperature protocol (experiment 1), (B) the routine metabolic rate measurement post 45 days-acclimation (experiment 2, part 1), and (C) the high-temperature challenge post 45 days-acclimation (experiment 2, part 2). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

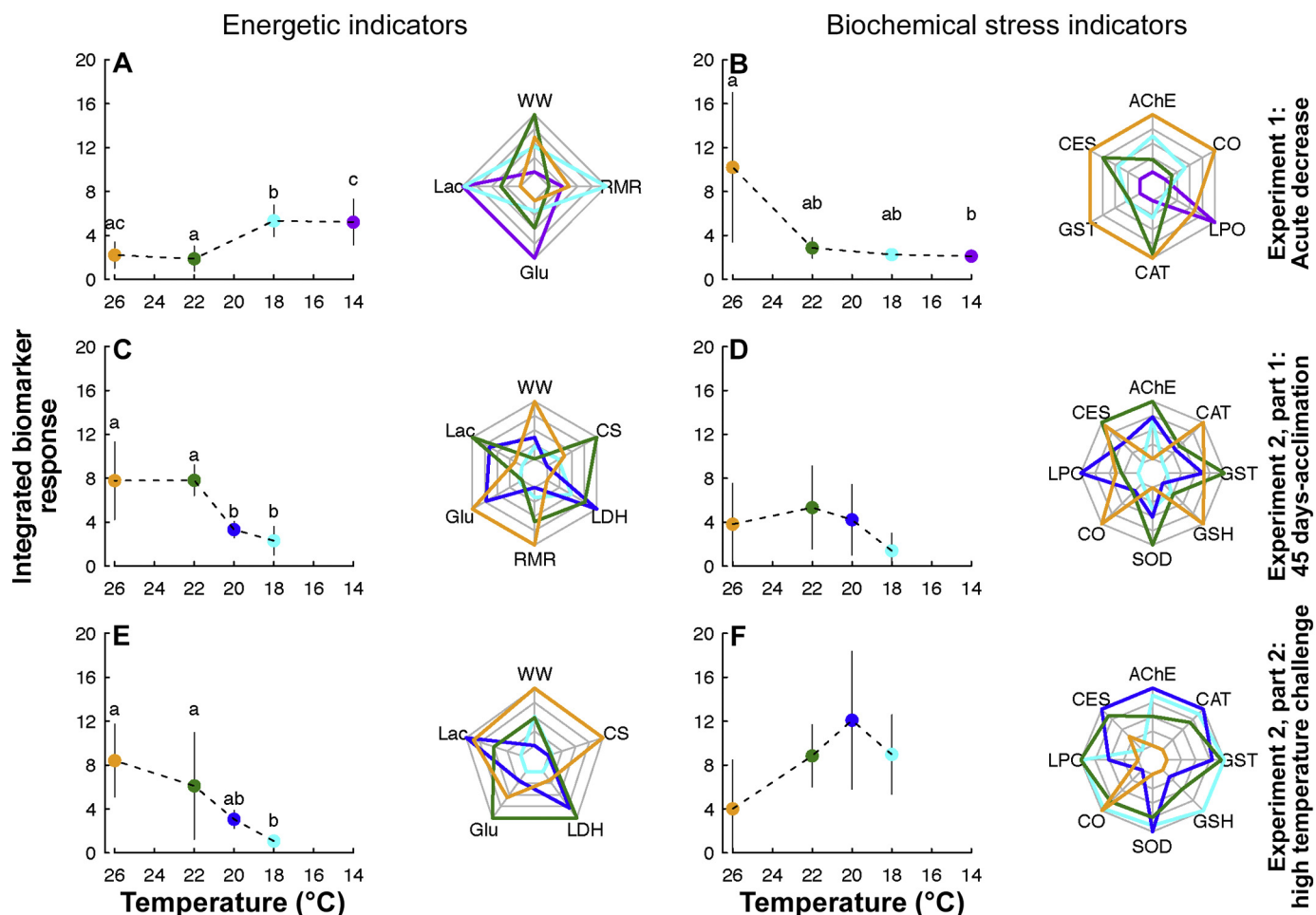


Fig. 3. Integrated biomarker response (IBR; mean \pm standard deviation) and radar plots of energetic and biochemical stress indicators in *Neopomacentrus cyanomos* categorized by temperature exposure (14 °C = purple; 18 °C = cyan; 20 °C = blue; 22 °C = green; 26 °C = orange) following (A and B) the acute decrease in temperature protocol (experiment 1), (C and D) the routine metabolic rate measurement post 45 days-acclimation (experiment 2, part 1), and (E) the high temperature challenge post 45 days-acclimation (experiment 2, part 2). Lower case letters indicate significant differences ($p < .05$) in IBR values among exposure or acclimation temperatures. Radar plots show normalized means for WW = wet weight, RMR = routine metabolic rate, Glu = [glucose], Lac = [lactate], LDH = lactate dehydrogenase activity, CS = citrate synthase activity, AChE = acetylcholinesterase activity, CES = carboxylesterase activity, CO = [carbonyls], LPO = [lipids peroxidation], CAT = catalase activity, GST = glutathione S-transferase activity, SOD = superoxide dismutase activity, and GSH = [reduced glutathione]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

When acclimated fish were thermally challenged, the acclimation temperature had a significant effect on the physiological proxies deployed at 20 °C compared to 18 °C (PERMANOVA, $pseudo-F_{(3,10)} = 1.831, p = .011$; pair-wise test; Fig. 2C). Energetical IBR indices showed significant differences with higher IBR values in the warmer (26 and 22 °C) vs. 18 °C when integrating WW, Lac, Glu, LDH and CS (ANOVA, $F_{(3,11)} = 6.635, p = .008$), probably due to comparably lower values of Lac and Glu observed in fish acclimated to 18 °C (Fig. 3E). Again, a non-significant bell curve trend was observed when integrating the biochemical stress variables, highlighting oxidative stress at 18 °C as all antioxidant levels and indicators of damages were increased (Fig. 3F). Oxidative damages were not observed in fish acclimated to 20 °C.

4. Discussion

Limited sample sizes likely constraint the inferences that can be drawn from the results presented here. However, any direct information about alien specimens in the newly colonized area reduces the need to use of information from secondary sources to predict invasion success, which will increase accuracy of models about such success (Lyons et al., 2019). Lennox et al. (2015) have highlighted the fact that very few

studies have made physiological assessments of invasive species. The present study was designed to obtain as much physiological information as possible we could get from a small sample of alien *Neopomacentrus cyanomos* individuals to offset the data deficiencies often reported outside of species native ranges.

The three approaches used here (respirometry, energy metabolites in blood, and biochemical indicators in muscle) in two different experiments involving rapidly and slowly decreasing temperatures show that fish maintained at warm temperatures (22 and 26 °C) differed from those that were rapidly reduced to 14 °C or were acclimated to 18 and 20 °C for 45-days. Overall, our results show that when the degree of temperature change was large and rapid, no compensation occurred and the stress response observed mostly reflected cellular processes that minimized damage (Clarke, 1991). However, while a set of physiological proxies responded to minimize damage during the rapid-decrease experiment, the same proxies reflected the consequences of compensation in the second, 45-days of acclimation, experiment.

4.1. No compensation when temperature decreases rapidly

Results obtained in the first experiment (acute temperature decline) showed that fish were unable to regulate their metabolic rate to cope

with a change towards low temperatures, as shown by the increased routine metabolic rate (RMR) when they reached 18 °C. In this experiment, effective compensation, *i.e.* a reduction on respiratory rates, was only observed in fish exposed to the first temperature decrease step (26° to 22 °C), which was strongly dependent on temperature ($Q_{10} = 4.7$). From 22 °C to 18 °C or 14 °C, Q_{10} values < 1 indicate overcompensation, *i.e.* extra energy was consumed (see Precht, 1958). In other words, metabolism did not decrease with decreasing temperature, perhaps related to higher swimming activity behaviour aimed at avoiding low temperatures (Habary et al., 2017). Accumulated thermal stress following declines of 12 °C (from 26 °C to 14 °C) provoked increases in blood lactate (Lac) and glucose (Glu) levels, indicating that fish used all their physiological resources to maintain a high metabolic rate that might eventually have allowed them to escape to warmer habitat if such was possible. Blood Glu changes observed in *N. cyanomos* followed similar trends as those seen in the red spotted grouper *Epinephelus akaara* (habitat temperature range 11.7°-26.3 °C) exposed to a temperature decrease from 10 °C to 4 °C at a rate of 1 °Cd⁻¹ (Park et al., 2016), and in the genetically modified farmed tilapia, *Oreochromis niloticus* (optimal temperature range of 26°-29 °C) after 24 h exposure to 13 °C (He et al., 2015). Umminger (1970) studied in detail the role of serum Glu in providing energetic fuel in the killifish *Fundulus heteroclitus* (habitat temperature range 6°-35 °C) exposed to extremely low temperature (-1.5 °C). He found that the increased serum Glu was accompanied by a progressive depletion of hepatic glycogen, and that fish survived only as long as Glu was available. Thus, at the lowest temperature of the present study (14 °C), regal damselfish probably had little energy left to maintain activity and repair cell damage caused by the accumulated stress of a rapid decrease in temperature. At this temperature, cell damage was evident from the accumulation of lipid peroxidation (LPO), there was a general decrease in antioxidant enzyme defence activities, and an inhibition of acetylcholinesterase (AChE) activity, indicating a lower efficiency in breaking down acetylcholine molecules essential for the communication between nervous and muscle cells. The high Q_{10} value of 6.5 obtained for a comparison of fish exposed to 14 °C vs. 18 °C indicates that fish were completely exhausted by the acute exposure to 14 °C.

Values of carbonyls in proteins (CO) were surprisingly high in *N. cyanomos* maintained at 26 °C compared to fish at all reduced temperatures. The opposite would be expected, as fish exposed to 14 °C demonstrated more signs of stress than did fish maintained at 26 °C. Since the temperature throughout the Gulf is at or above 26 °C during August, when the fish were collected, physiological responses at this temperature prior to the rapid-decrease temperature experiment correspond approximately to the state at which the habitat was successfully colonized by this species. While high CO levels at 26 °C may indicate some oxidative stress, they may also represent a mechanism of protein-quality control in the unstressed fish, by making proteins more easily targeted by proteolytic degradation (Dukan et al., 2000; Bota and Davies, 2002). Rapid exposure to reduced temperatures could have stimulated proteasome mechanisms, which could explain the lower concentrations of CO observed at 22 °C, 18 °C, and 14 °C. Protein degradation found in a mutant yeast deficient in proteasome substrates that was exposed to cold temperature suggest the activation of apoptosis (Isasa et al., 2016). In a tropical fish species (*Nothobranchius rachovii*), an extended lifespan was correlated with lower CO values during moderate (25 °C) and low (20 °C) acclimation temperatures (Hsu and Chiu, 2009) was also observed. The authors of that study suggested that cold temperatures enhanced proteolytic degradation efficiency, provoking a reduction in aging. Lamarre et al. (2010) also noted high 20S proteasome activity in juvenile spotted wolffish (*Anarhichas minor* Olafsen) acclimated to cold temperatures (4 °C), reaching levels 130% of that of fish acclimated to 8 °C when measured at a common temperature. Perhaps, a similar beneficial mechanism activated in response to cold temperatures is also present in *N. cyanomos* subject to rapid decreases of temperature.

4.2. Cost of compensation when temperature decreases slowly and cold persists

Acclimation, which represents short-term adaptation to a new environmental circumstance, is mostly physiologically or metabolically based. Results obtained in the second experiment described here (acclimation to reduced temperatures for 45 days) showed that fish were able to regulate their metabolic rate relative to colder thermal conditions. This was shown by the decrease of RMR at 20 and 18 °C, suggesting energy saving. The reduction of RMR at 18 °C and 20 °C coincides with the reduction of citrate synthase (CS) activity, which is the pacemaker of aerobic mitochondrial respiration. The decrease of RMR and CS activity would mean that mitochondrial biogenesis was not induced in response to cold temperature, as has often been observed in polar fishes (O'Brien, 2011). This translates into a lower concentration of aerobic metabolic enzymes per gram of tissue to maintain ATP production, a lower density of mitochondrial membrane phospholipids to enhance oxygen diffusion, and greater diffusion distance for oxygen and metabolites between capillaries and mitochondria. The strong effects of temperature on RMR of *N. cyanomos* were evidenced by the high Q_{10} values obtained when the RMR of damselfish acclimated at 18 °C were compared with those acclimated at 22 °C ($Q_{10} = 27.0$) and 26 °C ($Q_{10} = 4.4$). In other words, fish at 18 °C were outside their normal temperature range, and were obliged to invest greater energy to maintain their RMR. The slight decrease of performance ($Q_{10} = 0.7$) noted between fish acclimated at 22 °C and those maintained at 26 °C, and the typical Q_{10} value (between 2 and 3) obtained when comparing fish at 20 °C and 26 °C, provide evidence that between 20 °C and 26 °C (and probably higher temperatures) *N. cyanomos* has the physiological mechanisms to compensate for thermal decrements (Precht, 1958). In other damselfish species, typical Q_{10} values were observed when fish were exposed to 29–31 °C, suggesting that this thermal range is where physiological mechanisms are optimal for typical conditions in their native habitat (Nilsson et al., 2009; Rummer et al., 2014).

As 26 °C represents the summer habitat temperature for the experimental *N. cyanomos*, it is unlikely that low AChE activity at that temperature indicates failure between the nervous and locomotor systems. That reduction of AChE activity could be explained by the differential effects of temperature on the Michaelis constant (K_m) of acetylcholine between AChE isoforms. Baldwin and Hochachka (1970), in a study on the rainbow-trout *Oncorhynchus mykiss* (habitat temperature range of 10°-24 °C), detected a warm and a cold variant of AChE in the brain after acclimatization to warm (17 °C) and cold (2 °C) conditions, respectively, and the presence of both variants at an intermediate temperature (12 °C). Because the AChE assay used here measures the total activity of the enzyme without discriminating among any variants that may have been activated, we suggest that AChE generally operates more efficiently in *N. cyanomos* acclimated to colder temperatures (18 °C, 20 °C and 22 °C) compared to those acclimated to 26 °C. A similar decrease in AChE activity was observed in the digestive gland of the mussel *Modiolus barbatus* after 20 and 30 days of warm acclimation at 28 °C and 30 °C, compared to a shorter acclimation of 10 days (Dimitriadis et al., 2012).

N. cyanomos acclimated to 18 °C was observed to feed less than fish acclimated to all other temperatures (NT, personal observations), behaviour that may account for the reduced swimming performance of foraging planktivorous fishes during winter in the field study of Johansen et al. (2015). Below 20 °C, energetic reserves of *N. cyanomos* were depleted, as indicated by reduced concentrations of metabolic fuels (Glu), as well as reduced activity of carboxylesterase (CES) and lactate dehydrogenase (LDH). The lack of acquisition of energy by means of feeding and the high maintenance costs at low temperatures may have affected lipid and carbohydrate reserves of *N. cyanomos*, resulting in lower activity levels of both enzymes as both require lipids and carbohydrates to function. Whereas CES is involved in cell detoxification and lipid metabolism (Ross et al., 2010), LDH converts

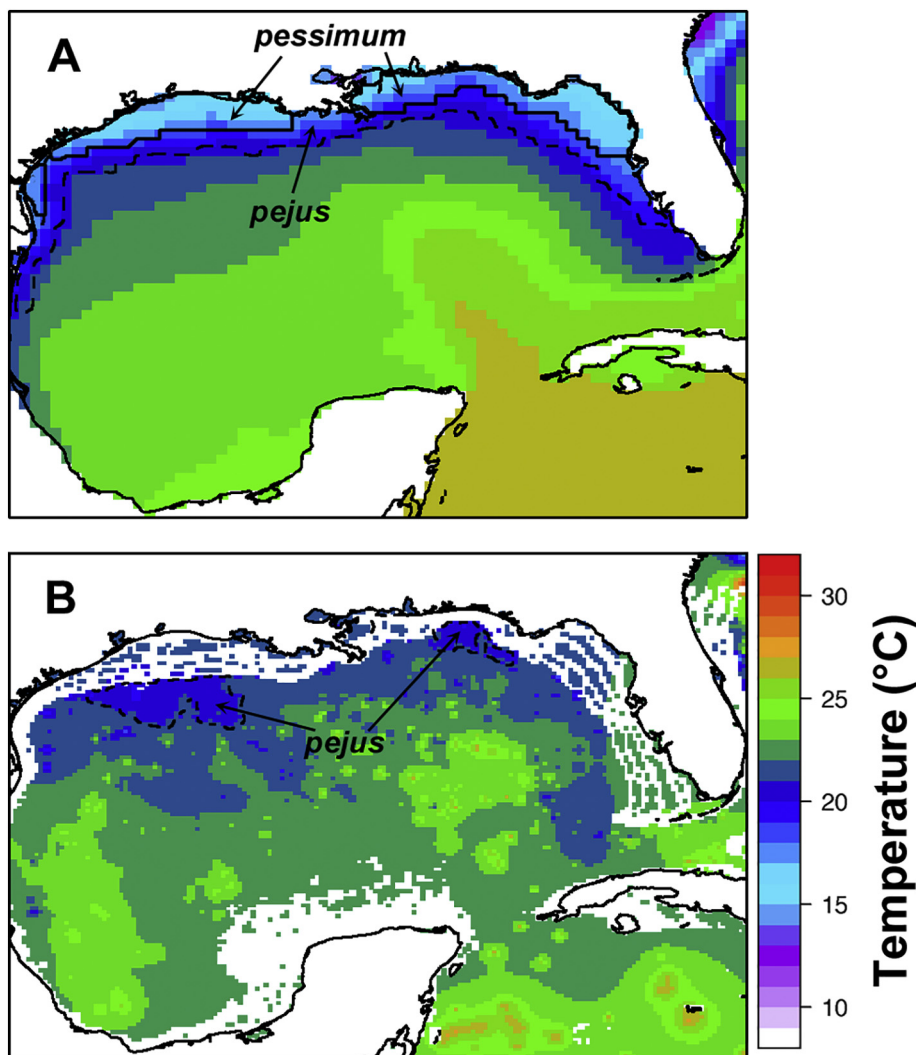


Fig. 4. *Pejus* and *pessimum* range thermal zones for *Neopomacentrus cyanomos*. Coldest mean sea surface temperature (A), and at 40–50 m (B) during 2000–2018, with *pejus* (20–18 °C) and *pessimum* (< 18 °C) range thermal stress zones for *N. cyanomos*. *Pejus* corresponds to a physiological state in which the maintenance cost increases and is partly covered by the use of anaerobic metabolism, with fluctuations in mechanisms that compensate for the excess of reactive oxygen species produced by aerobic metabolism. *Pessimum* range will be lethal under prolonged exposure as all energy will be likely directed to maintenance and repair. Sea surface temperatures for the Gulf of Mexico during 2000–2018 were downloaded from NOAA High-resolution Blended Analysis provided by the NOAA/OAR/ESRL PSD (Boulder, Colorado, USA, <http://www.esrl.noaa.gov/psd/>). R packages ‘ncdf4’ (Pierce, 2015) and ‘chron’ (James and Hornik, 2016) were used to extract the data, and daily data were averaged per month using ‘matrixStats’ (Bengtsson, 2016). The ‘Lattice’ (Sarkar, 2008) package was used to display the visualizations. Temperatures at 40–50 m were extracted and monthly averaged from the World Ocean Database (Boyer et al., 2013; www.nodc.noaa.gov/OC5/SELECT/dbsearch/dbsearch.html). The 40 m isobath (gcoos.tamu.edu/products/topography/SRTM30PLUS.html) and the package ‘tmap’ (Tennekes, 2017) was used for the interpolation.

pyruvate into lactate to produce energy in absence of oxygen. In *N. cyanomos* acclimated to 18 °C, some enzymatic and non-enzymatic antioxidants remained although such fish had lower levels of LPO and CO compared to fish maintained at 26 °C. Similarly to what occurred in the acute decreasing temperature protocol (experiment 1), cold temperature acclimation could have stimulated proteasome mechanisms, explaining the existence of lower concentrations of CO at 18 °C.

4.3. Thermal acclimation effects via high temperature challenge

To reach their new habitat, the exotic specimens often undergo strong gradients in physico-chemical conditions that require a phenotypic plasticity often not experienced in their native distribution range. The high temperature challenge described here was useful in assessing what physiological plasticity remained after a potentially costly acclimation to a temperature outside of the comfort zone. Reduced feeding and maintenance costs at low temperatures may have affected the lipid and carbohydrate reserves, which resulted in decrease of CS, CES and LDH activities, as was the case in acclimated *N. cyanomos* after RMR measurements. The high levels of all antioxidant enzymes and compound (reduced glutathione) and oxidative damages (LPO and CO) in the cold-acclimated *N. cyanomos* submitted to high temperature challenges indicate an imbalance in reactive oxygen species (ROS) production caused by over- and under-oxygenation of tissues. The high temperature challenge activated energetic indices, but did not increase any biochemical stress markers in fish acclimated at 26 °C, indicating

that these fish had the cellular capability to deal with this sudden temperature increase. Fish acclimated to colder temperatures increased their integrated biomarker response (IBR) when subject to increased temperature challenge, indicating reduced plasticity to respond to increased temperature.

4.4. Optimum, *pejus*, *pessimum*, and future conditions in the Gulf of Mexico

The oxygen- and capacity-limited thermal tolerance hypothesis (Pörtner and Knust, 2007; Pörtner, 2010) proposes that optimal capacity exists when aquatic ectotherms show maximal aerobic scope, a physiological state characterized by low lactate and antioxidant levels, and reflecting low levels of peroxidation. Beyond the optimum limits, maintenance cost increases, reducing the amount of ATP available to cover completely those physiological demands. The ranges of temperatures that provoke such conditions are called *pejus* ranges and reflect acclimation, a physiological state marked by increased anaerobic metabolism to satisfy the energy demands that the aerobic metabolism cannot support. Beyond the *pejus* range ectotherms enter the *pessimum* range, where all the ATP is theoretically directed to maintenance and repair mechanism (Pörtner, 2010; Sokolova et al., 2012). The combined results of both experiments indicate that the three states of acclimation to low temperatures described by the oxygen- and capacity-limited thermal tolerance hypothesis were observed in *N. cyanomos*: 1) it has an optimum range (26°–22 °C), when fish are able to compensate and use energy supply to cover maintenance costs and maintain maximal

physiological functions; 2) it has a *pejus* range (between 20 °C and 18 °C), in which the maintenance cost increases and is partly covered by anaerobic metabolism, with fluctuations in defence mechanisms to compensate for the excess of reactive oxygen species produced by aerobic metabolism; and 3) it has a *pessimum* range, the least favourable conditions under which an organism can survive (here between 18 °C and 14 °C), when all energy is theoretically directed to maintenance and repair mechanisms and when time of exposure to low temperatures becomes crucial. The agreement between the critical temperature range indicated by our experiments and the minimum temperatures at the limits of the native range provides evidence that *N. cyanomos* is unlikely to survive well in habitats that experience minimum temperatures below 20 °C, the acclimation temperature at which its respiration rate is significantly reduced.

The question arising from our experimental results and the minimum temperatures at the latitudinal limits of *N. cyanomos*' native range is whether physiological limitations due to low winter temperatures could prevent this species establishing a persistent resident population in the northern Gulf of Mexico? Exploratory oil platforms off northwest Mexico between 24 and 26°N (Fig. 1) could allow *N. cyanomos* to fill the gap in its distribution between the Mexican platform grid and the several thousand oil platforms located in northern, US section of the Gulf. Based on the physiological constraints to low temperatures and the mean SST during the coldest month (February) between 2000 and 2018 (Fig. 4A), we conclude that *N. cyanomos* will not survive near the surface during the coldest months at locations experiencing *pessimum* state temperatures. However, winter temperatures at 40–50 m depth in the northern Gulf are consistently above the *pessimum* upper limit (18 °C), and *pejus* conditions (18°–20 °C) are not present over the entire northern Gulf at that depth, particularly on the outer parts of the continental shelf and slope (Fig. 4B). The ability of *N. cyanomos* to live at depths between 40 and 50 m may well mean that parts of a population in the northern Gulf can avoid adverse temperature conditions in the surface layers. Fish may use this strategy during winter off Alabama, as the presence of many early-stage juvenile specimens along with large adults there in July 2017 suggests that the species likely survived the winter conditions of 2016 (Bennett et al., 2019). Use of deeper warmer waters during the night has been observed during periods of low SST in the Atlantic cod *Gadus morhua* (Freitas et al., 2015), and *N. cyanomos* potentially could respond by moving to deeper areas (if available in the immediate vicinity) during cold periods.

Hence for the time being, *N. cyanomos* is likely to be limited to year-round persistence at subsurface levels of platforms located on the outer shelf and deep water far from the coast. However, on-going global warming is resulting in tropicalization of many temperate regions, including the northern Gulf, where it is facilitating expansion of native tropical western Atlantic shore-fish species into near-shore habitats (e.g. Gericke et al., 2014). Continuation of this tropicalization process should facilitate the expansion and establishment of *N. cyanomos* on platforms in inshore areas as well as deeper offshore areas. Oil-platform habitat is already populated by a variety of tropical West Atlantic native fishes, including planktivorous damselfishes of genera other than *Neopomacentrus* (*Stegastes*, *Chromis* and *Abudefduf*; see Hastings et al., 1976, Ajemian et al., 2015), with which *N. cyanomos* could compete for food, shelter and nest sites.

5. Concluding remarks

Based on the results of the present work, it is now clear that winter sea surface temperatures in the northern Gulf will slow down *N. cyanomos* to colonize the inshore area. The use of physiological cellular stress markers to assess the invasiveness potential of aquatic species, particularly at the start of an invasion, should be implemented in new standardized experimental protocols, including rapid increases/decreases of temperature and post-acclimation high temperature

challenge. The output of experiments such as these could be integrated in replicable risk analysis that would provide clear tools to decision makers prior to the widespread establishment of invasive species in newly colonized habitat.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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