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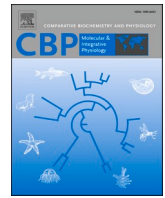
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Warm, but not hypoxic acclimation, prolongs ventricular diastole and decreases the protein level of Na⁺/Ca²⁺ exchanger to enhance cardiac thermal tolerance in European sea bass

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ABSTRACT

One of the physiological mechanisms that can limit the fish's ability to face hypoxia or elevated temperature, is maximal cardiac performance. Yet, few studies have measured how cardiac electrical activity and associated calcium cycling proteins change with acclimation to those environmental stressors. To examine this, we acclimated European sea bass for 6 weeks to three experimental conditions: a seasonal average temperature in normoxia (16  C; 100% air sat.), an elevated temperature in normoxia (25  C; 100% air sat.) and a seasonal average temperature in hypoxia (16  C; 50% air sat.). Following each acclimation, the electrocardiogram was measured to assess how acclimation affected the different phases of cardiac cycle, the maximal heart rate (fH_{max}) and cardiac thermal performance during an acute increase of temperature. Whereas warm acclimation prolonged especially the diastolic phase of the ventricular contraction, reduced the fH_{max} and increased the cardiac arrhythmia temperature (T_{ARR}), hypoxic acclimation was without effect on these functional indices. We measured the level of two key proteins involved with cellular relaxation of cardiomyocytes, i.e. sarco(endo)plasmic reticulum Ca²⁺-ATPase (SERCA) and Na⁺/Ca²⁺ exchanger (NCX). Warm acclimation reduced protein level of both NCX and SERCA and hypoxic acclimation reduced SERCA protein levels without affecting NCX. The changes in ventricular NCX level correlated with the observed changes in diastole duration and fH_{max} as well as T_{ARR} . Our results shed new light on mechanisms of cardiac plasticity to environmental stressors and suggest that NCX might be involved with the observed functional changes, yet future studies should also measure its electrophysiological activity.

1. Introduction

High temperature and low oxygenation level represent major environmental challenges for ectothermic aquatic animals such as fish (Diaz and Rosenberg, 2008; Doney et al., 2012; Schulte, 2015; Somero, 2012). Moreover, the severity and the frequency of these two environmental occurrences is increasing in the current era of global change (Breitburg et al., 2018; Oliver et al., 2018; Russo et al., 2014). In its natural habitats, our model species, the European sea bass (*Dicentrarchus labrax*), is often exposed to episodes of warming and hypoxia (S anchez V azquez et al., 2014), making this species an appropriate model to better understand how phenotypic plasticity contributes to coping with these

environmental stressors.

Modulation of heart rate is one of the first physiological mechanisms in the line of defence used by fish to respond to new environmental conditions. In most of the fishes, acute warming is initially associated with an increased heart rate, while warm acclimation can be accompanied with species-specific responses that range from a progressive partial return, and sometimes even a complete return, of heart rate to its initial value (see review in Eliason and Anttila, 2017). Acute exposure to hypoxia usually decreases the resting heart rate of fish (see reviews in Gamperl and Driedzic, 2009; Stecyk, 2017), while hypoxic acclimation sometimes increases the heart rate (Petersen and Gamperl, 2010; Bursleson and Silva, 2011) or shows no effect (Motyka et al., 2017; Stecyk

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et al., 2020), indicating species specific responses to hypoxia acclimation. High temperature and hypoxia commonly co-occur in nature, since the O₂ solubility decreases when the water temperature rises (Keeling et al., 2010). Both hypoxia and high temperature challenge the aerobic capacity of ectotherms, and therefore are liable to raise the demands on the cardiac performance (Casselmann et al., 2012; Steinhausen et al., 2008). Previous studies suggested that acclimation to one stressor, i.e. hypoxia, may induce a shared physiological mechanisms (e.g. improved oxygen uptake or carrying capacity or optimized cardiorespiratory function) that could increase the tolerance to another environmental stressor, i.e. high temperature (Anttila et al., 2013b; McBryan et al., 2013). This phenomenon is known as cross-tolerance (Rodgers and Gomez Isaza, 2021). However, there are few studies that investigate how acclimation to hypoxia will change the capacity of the cardiac system to respond to acute warming and the results are contradictory showing

both enhancements (Burluson and Silva, 2011) and impairments (Leeuwis et al., 2021; Motyka et al., 2017). Therefore, more mechanistic studies about the cross-tolerance are needed to elucidate the possible protective mechanisms shared between hypoxia and high temperature acclimation during acute warming.

Investigating the changes affecting the cardiac cycle in fish (Fig. 1) is one way to reveal if there are any shared cardiovascular mechanisms between temperature and hypoxia, which may explain the cross-tolerance. To the best of our knowledge, so far only Badr et al. (2016) has investigated how warm and cold temperature acclimation affects the cardiac cycle during acute warming. They showed that the reduction of heart rate in warm acclimated roach (*Rutilus rutilus*) was due to prolongation of the atrial repolarisation. Hypoxia, on the other hand, can shorten the duration of the ventricular action potential (QT interval) without changing the heart rate of the Alaska blackfish under winter

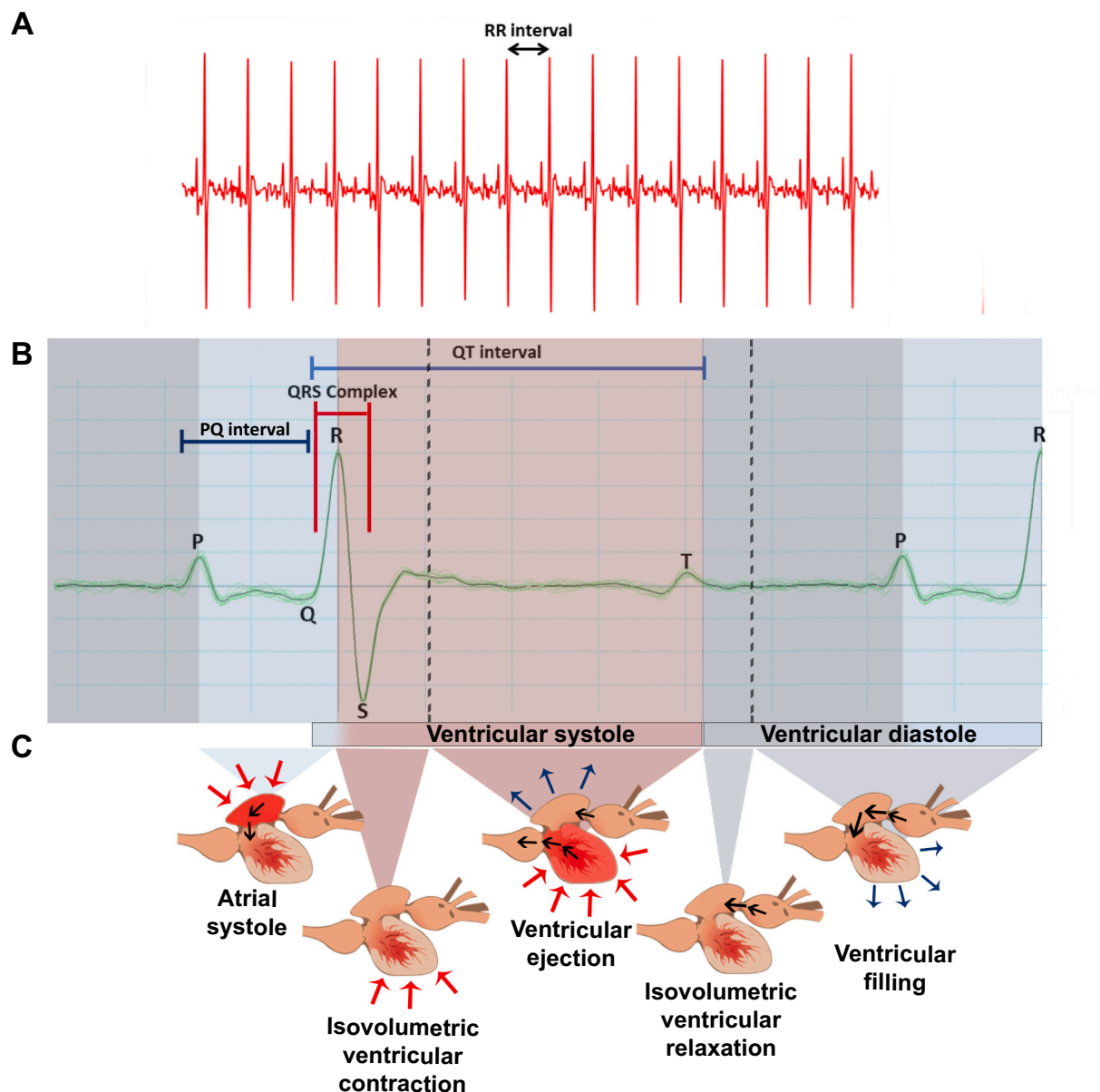


Fig. 1. Representative electrocardiogram (ECG) recording and details of the different cardiac phases. (A) Representative raw ECG segment of 15 heartbeats from European sea bass (*Dicentrarchus labrax*). (B) Single ECG waveform averaged from the raw representative ECG. Note that the P, Q, R, S and T waves of the ECG are clearly visible. The PQ interval, QRS complex and QT interval are delimited. (C) Schematic representation of the different phases - contraction-relaxation - of the cardiac activity. Black arrows represent the blood movement between the cardiac chambers, red arrows represent contraction, and blue arrows represent relaxation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

conditions (Stecyk et al., 2020). However, it is unknown if hypoxia acclimation influences the fH_{max} and the electrocardiogram (ECG) parameters of fish when they are exposed acutely to high temperature. Furthermore, very little is known about how other temperature acclimated species than roach modulate the cardiac cycle during acute warming. Also, it is unclear if hypoxia and temperature acclimation modulate similarly the ECG of fH_{max} .

The fH_{max} represents the maximal beat frequency that heart can achieve at a given temperature, i.e., the maximal attainable rate to pump blood through the gills and transport oxygen to tissues. In the natural environment, fishes may reach fH_{max} in several energy demanding situations such as swimming maximally against a current, to escape a predator or to catch a prey (Iversen et al., 2010; Steinhausen et al., 2008). The ecological relevance of the fH_{max} lies in the fact that it determines, at a given temperature, the physiological limit beyond which the heart cannot beat faster. In nature, fish may apply central vagal inhibition, which is considered to be cardioprotective against high temperature (Eliason et al., 2013; Farrell, 2007). However, slowing heart rate might then limit cardiac output and therefore aerobic scope (Leeuwis et al., 2021). There are different ways to explore how acclimation to elevated temperature or hypoxia may lead to higher cardiac thermal tolerance. For instance, it can be assessed by measuring thermal performance curves (TPC) for fH_{max} and/or aerobic scope (McKenzie et al., 2021), with the first being performed more easily and within a shorter timeframe than the latter. The fH_{max} can be measured from slightly anesthetised fish if the vagal control is removed by using atropine and stimulating the β -receptors with isoproterenol, as it has been done in wide variety of fish species (Anttila et al., 2017; Anttila et al., 2013a; Casselman et al., 2012; Chen et al., 2015; Ferreira et al., 2014; Gilbert et al., 2019; Gilbert and Farrell, 2021; Hardison et al., 2021; Safi et al., 2019). The fH_{max} values using the above-mentioned method are similar to fH_{max} values measured from fish chased to exhaustion (Casselman et al., 2012). The fH_{max} measurements during acute warming can also define the threshold temperatures such as the arrhythmia temperature (T_{ARR}), i.e., the temperature at which the heart shows signs of arrhythmias, which characterizes the upper thermal tolerance of heart and which is typically 1–2 °C lower than the critical thermal maximum (CT_{MAX}) of the whole organism. Based on this context, the first aim of our study was to investigate how acclimation to high temperature or hypoxia affect the cardiac electrical activity during the cardiac cycle (i.e. ECG parameters), the maximal heart rate (fH_{max}) during acute warming and the T_{ARR} . We hypothesized, based on cross-tolerance phenomenon, that acclimation to hypoxia alters the thermal performance curve of fH_{max} and ECG traits, leading to an improve of T_{ARR} similarly as high temperature acclimation.

An overlooked aspect of cross-tolerance between hypoxia and temperature is the existence of shared molecular mechanisms underpinning the changes in cardiac cycle related to those stressors. In this context we investigate the changes in the protein levels of key calcium cycling proteins involved in cardiac excitation-contraction coupling (EC-coupling) (Fig. 1). Therefore, our second aim was to compare if hypoxia and temperature acclimation lead to similar changes of calcium cycling proteins. We hypothesized that modifications to the expression of these EC-coupling proteins would partially explain the cardiac functional plasticity during acclimation to hypoxia and temperature (Anttila et al., 2019; Vornanen, 2017), something not previously studied. By measuring EC-coupling protein we could also associate at a mechanistic level any acclimatory changes in cardiac cycle and T_{ARR} with changes in molecular level. In terms of EC-coupling, calcium cycling proteins are crucial in setting the frequency and amplitude of the changes in cytosolic $[Ca^{2+}]$ during a cardiomyocyte contraction (Hove-Madsen et al., 2000; Hove-Madsen and Tort, 2001). The sarco(endo)plasmic reticulum Ca^{2+} -ATPase (SERCA) and the Na^+/Ca^{2+} exchanger (NCX) are calcium cycling proteins mainly involved in the relaxation phase of the EC-coupling (Shiels, 2017; Shiels et al., 2002a; Vornanen, 2017), and so during isovolumetric ventricular relaxation, i.e., diastole (Fig. 1). Warm

acclimation or acclimatization are known to reduce the expression of SERCA and NCX which might be connected to the reduced heart rate observed with warm acclimation (Keen et al., 2017; Korajoki and Vornanen, 2012; Shiels et al., 2002b; Tikkanen et al., 2016). Hypoxia acclimation, on the other hand, has been shown to increase the mRNA levels of the EC-coupling proteins in Alaska blackfish (Stecyk et al., 2020), the intracellular Ca^{2+} transient ($\Delta[Ca^{2+}]_i$) (Shiels et al., 2022) and alters the cardiac K_{ATP} channels (Cameron et al., 2013), which could mechanistically explain how fish enhanced heart rate under hypoxic conditions. Therefore, aim of the current study was also to link the specific functional changes in the cardiac contraction cycle (ECG i.e. contraction/relaxation of heart) to possible changes in the protein levels of those EC-coupling proteins that are involved in their specific phases of cardiac contraction. By doing so, we intended to provide more insight to the cross-tolerance hypothesis by revealing if hypoxia and warming lead to similar modulations at molecular and functional level.

2. Materials and methods

2.1. Animals

Juvenile European sea bass ($n = 28$; fork length = 18.3 ± 0.3 cm; body mass = 76.4 ± 4.6 g) were reared in indoors tanks (2000 l) under natural temperature and photoperiod conditions at Ifremer aquaculture facility in Brest, France in summer 2018. A month before the experiments started, fish were anaesthetized (MS-222; 100 ppm) and subcutaneously implanted with an individual identification tag (RFID; Biolog-id, France). Fish were fed daily ad libitum with a commercial diet (Neo Start Coul 2, Le Gouessant, France), but fasted for at least 24 h before tagging or use in experiments. The current experiment complied with French laws and ethical guidelines regarding animal welfare (APAFIS Number 2018040916374437).

2.2. Acclimation groups

Fish were randomly assigned to one of three acclimation conditions and were maintained under these conditions for 6 weeks in 500 l tanks (Fig. S1): a control using the seasonal average temperature with normoxia, an elevated temperature with normoxia, and a seasonal average temperature with moderate hypoxia. The seasonal average summer temperature of the Bay of Brest was 16 ± 1 °C for normoxic control group (labelled 16 N; $n = 9$). The elevated temperature was 25 ± 1 °C for the other normoxic group (labelled 25 N; $n = 9$). For the hypoxia-acclimated group, 50% air saturation at 16 ± 1 °C was used (labelled 16 H; $n = 10$). Experimental hypoxia was set at 50% air saturation (sat.) because it corresponds to the oxygenation level below which sea bass progressively display reduced food ingestion and growth (Thetmeyer et al., 1999; Zambonino-Infante et al., 2017). Fish were gradually acclimated to the experimental conditions with a rate of 0.3 °C \cdot day $^{-1}$ for the high temperature group and a decreasing rate of 10% air sat. \cdot h $^{-1}$ for the hypoxic group. The fish tanks were supplied with continuously flowing, aerated, bio-filtered seawater pumped from the Bay of Brest. A titanium heat exchanger (3 kW, Galvatek, Bonnières-sur-Seine, France) heated the seawater to 25 ± 1 °C for the 25 N test group. Oxygen saturation and temperature were regularly checked (WTW Oxi 340, Xylem Analytics Germany Sales GmbH & Co, Weilheim, Germany) in all the tanks. Oxygen level was regulated manually by modulating the nitrogen flow in the column via a manual flow meter unit. The effects of warm and hypoxic acclimation were investigated six weeks after the acclimation started by measuring the ECG and fH_{max} during acute warming and by measuring the protein levels of two Ca^{2+} cycling proteins.

2.3. Maximum heart rate (fH_{max}) measurement using ECG

Maximum heart rate (fH_{max}) was defined as the heartbeat frequency

recorded at a given temperature during an acute warming protocol conducted on a fish pharmacologically treated with atropine to prevent vagal cardiac inhibition and while being provided with maximum beta-adrenergic cardiac stimulation with isoproterenol, according to Casselman et al. (2012). The strengths of this methodology are that 1) fH_{\max} TPC can be performed easily and faster compared to the TPC based on AS, 2) the pharmacological stimulation of the fH_{\max} avoids the confounding factors of behaviour and autonomic control on the fH_{\max} , 3) the pharmacological stimulation of the fH_{\max} provides the maximum limit of heart rate beyond which the fish heart is not able to beat, 4) provides ecologically relevant threshold temperatures (T_{ARR} , T_{peak}) and fH_{\max} values, that are similar to the fH values of un-anesthetised fish during acute warming (Adams et al., 2022; Casselman et al., 2012; Ekström et al., 2016; Eliason et al., 2013; Gilbert et al., 2022; Gilbert et al., 2020; Penney et al., 2014).

To do so each fish was initially anesthetised with first dose of 100 ppm MS-222, for biometry measurements, then the fish was let to quickly recover from the high dose of anaesthetic in un-treated water. After the biometry measurement the fish were quickly transferred to the heart rate setup where they were immersed belly-up in a test apparatus for the duration of the experiment having low dose of MS-222 (60 ppm). The apparatus simultaneously measured fH_{\max} in three individual fish held in separate chambers that were connected to a common water sump containing aerated seawater. The MS-222 anaesthesia during measurements was used to stop skeletal muscle movements to prevent electrical artefacts in the ECG signal. However, high dose of MS-222 anaesthesia (100 ppm) could sometimes also stop the natural gill ventilation movements. In the absence of spontaneous gill ventilation, hypoxemia could develop, to which a reduction in heart rate mediated by vagal inhibition is a common response in fish (Carter et al., 2011; Casselman et al., 2012; Farrell, 2007; Randall, 1962). However, during measurements we used a concentration of MS-222 low enough to avoid complete paralysis of the gill musculature, as previously reported (Anttila et al., 2014a; Casselman et al., 2012; Safi et al., 2019).

In the chambers the fish received continuous gill irrigation with 100% air saturated seawater from the sump tank via customized mouthpieces to avoid hypoxemia. All the fish were measured under normoxia to allow comparison in a common measurement condition. The water was thermoregulated to the experimental starting temperature of 16 °C. Two silver needle-electrodes were placed cranially and caudally of the heart of each fish to detect the ECG signal, which was amplified using a differential amplifier (band pass: 5-50 Hz; Bioelectric amplifier, Gould & Nicolet, 91,942 Courtaboeuf, France). The output signal from the device was digitalized at 1000 Hz and data was recorded using PowerLab 4/30 data acquisition system (ADInstruments, Oxford, England) and LabChart Pro software (v.7.0; ADInstruments, Oxford, England).

After placing the fish to chambers, the ECG signal was allowed to stabilize for 30 min before an intraperitoneal injection of 3 mg kg⁻¹ of atropine sulphate (Alfa Aesar™, Fisher Scientific Oy, Vantaa, Finland) dissolved with 0.9% NaCl solution. Fifteen minutes later an intraperitoneal injection of isoproterenol (3.2 µg kg⁻¹, Sigma-Aldrich, Darmstadt, Germany) followed to induce fH_{\max} by maximally stimulating cardiac beta-adrenergic receptors. The acute warming protocol began 15 min later. Water temperature was increased from 16 °C by 1 °C stepwise increments every 6 min until a cardiac arrhythmia (absent of QRS complex in the ECG signal) was first observed (T_{ARR}) (see Supplementary Fig. S2). T_{ARR} was regarded as the upper critical thermal limit for cardiac function (Anttila et al., 2017; Anttila et al., 2013a; Casselman et al., 2012).

The fH_{\max} at each temperature was calculated from the ECG recording using 15 continuous heartbeats near the end of each 6-min period (Casselman et al., 2012). The electrical intervals associated with a cardiac cycle, i.e., PQ, PR, RR, QT, PQ, and QRS (Fig. 1B), were determined by processing the signals off-line using 'ECG Averaging View' software in the PowerLab library following a visual inspection of

individual ECGs for quality control to ensure accurate automatic placement of markers. Four fish (2 fish from 16 N and 2 from 16H) were omitted from the analysis because at least one wave was unrecognizable in a noisy ECG signal. For each fish and at each temperature, the length of a single cardiac cycle was obtained from the RR-interval. The QT-interval indicated the duration of ventricular systole. The numerical difference between the RR-interval and the QT-interval indicated the duration of ventricular diastole, which then allowed calculation of the ratio of diastolic duration/systolic duration: (difference between the RR-interval and the QT-interval) / (duration of QT interval) (Badr et al., 2016). The ECG traces was also used to calculate the highest value of fH_{\max} (peak fH_{\max}) and the temperature where it was achieved (T_{peak}). After cardiac arrhythmia was first observed, define as clear and abrupt skipping of ventricle beat (absent of QRS complex in the ECG signal; see Supplementary Fig. 2), the fish was considered to have reached the experimental end point and the temperature was recorded as T_{ARR} . Then the fish was immediately euthanized using an overdose of MS-222 (200 ppm). Since the vagal regulation of the heart rate is blocked by atropine and the maximal heart rate is induced by the isoproterenol, the onset of sudden missing of QRS complex is due to intrinsic impairments of the heart contraction or action potential conduction. The ventricle was then quickly excised, weighed, frozen in liquid nitrogen and storage at -80 °C until molecular analysis.

2.4. Ventricular Ca²⁺ cycling protein measurement

We semi-quantified with Western blotting two proteins involved with ventricular relaxation (NCX and SERCA) because warm acclimation altered ventricular diastole more than systole (see Results section). For these analyses, ventricles were homogenised in six volumes of ice-cold homogenization buffer (62.5 mmol l⁻¹ Tris-HCl, pH 6.8 containing 1 mM PMSF, 1 µg ml⁻¹ leupeptin and pepstatin) using Tissuelyser (Qiagen, Hilden, Germany) with a frequency of 30 s⁻¹ for 2 min. After homogenization the samples were centrifuged at +4 °C with 5000 g. We took 7 µl of the supernatant from each sample for the total protein concentration analyses that were performed with BCA kit (Pierce BCA Protein assay kit, Thermo scientific, Rockford, USA) in order to load equal amount of protein into each well (one sample per well). The rest of the supernatants were denatured in 1:1 v/v of Laemmli buffer by heating the samples for 7 min at 95 °C. We loaded 30 µg of denatured protein into each well of the TGX Stain-Free gels (Bio-Rad, Hercules, CA, USA). The gel electrophoresis lasted 35 min using 200 V. Gels were imaged with ChemicDoc MP Imaging system (Bio-Rad) and the relative total protein amounts per sample were calculated by measuring the intensity of all the protein bands in each well-column. Thereafter the proteins were transferred onto a nitrocellulose membrane (wet-transfer at 4 °C, 1 h, 150 V) and non-specific binding was blocked by soaking the membrane in a solution of Tris-buffered-saline (TBS) and 5% fat-free skimmed milk for 1 h. SERCA and NCX on the membranes were detected by incubating the membranes overnight in primary antibody solutions (in TBS + 0.1% Tween +5% fat-free skimmed milk) at 4 °C. As reported in previous fish studies (Korajoki and Vornanen, 2012; Monteiro et al., 2016), the antibodies used were Anti-SERCA2 ATPase (produced in rabbit, ab 91,032, Abcam, Cambridge, UK) at concentration of 1:2000 and Anti-NCX1 (produced in mouse, ab2869, Abcam) at concentration of 1:1000, respectively. After the primary antibody incubation, the membranes were exposed to the secondary antibodies: Goat anti-rabbit IgG StartBright Blue 700 (Lot No. #12004162, Bio-Rad) (1:5000) for SERCA and Goat anti-mouse IRDye 800cw (Lot No. C80306-03, Licor, Lincoln, NE, USA) (1:5000) for NCX. Images of the membranes were taken again (as above) and the intensities of the NCX and SERCA bands were measured (Image Lab 6, Bio-Rad). The band intensities were related to the total protein band intensities in each sample giving the relative protein level. The gel-to-gel variation was controlled and normalized by pipetting to each gel a control sample (mix of samples of three individuals). Images of representative membranes are shown in

Supplementary Fig. 3 (Fig. S3) showing the molecular weights of the detected bands and the specificity of the antibodies.

2.5. Statistical analyses

Throughout the manuscript, values are presented as means and s.e. m. Statistical significance ($p < 0.05$) was assessed using SPSS ver. 26 (IBM Corp. Released 2019, IBM SPSS Statistics for Windows, Version 26.0. Armonk, NY, USA) and SigmaPlot ver. 14 (Systat Software, San Jose, CA, USA). Before conducting our statistical analysis, we checked (one-way ANOVA followed by a Holm-Sidak *post-hoc* test) whether acclimation to high temperature and hypoxia affect sea bass morphology: body mass (g), fish fork length (cm), condition factor [(body mass * (fish fork length)⁻³]*100) and relative ventricle mass [RVM = (ventricular mass (g) * body mass (g)⁻¹)*100]. Since the mass of the fish varied between the acclimation groups (see Results section), a Pearson correlation was used to evaluate the influence of the body mass on the fH_{max} at 16 °C and on T_{ARR} within the acclimation groups, but neither fH_{max} at 16 °C (p -value between 0.228 and 0.999) nor T_{ARR} (p -value between 0.182 and 0.980) correlate with body mass and so, body mass was not used as a covariate in the statistical analyses. Equal variances and normal distribution of data were also tested before the statistical analyses with Levene Median and Kolmogorov-Smirnov tests, respectively. If the data for one-way ANOVA analyses did not follow the assumptions, non-parametric tests were used (Friedman Test and Mann-Whitney test).

Two-way repeated measures of ANOVA (*group* and *temperature* as factors) tested for the effects of warm and hypoxic acclimation on fH_{max} and cardiac cycle indices that were measured during acute warming. The effects of the atropine and isoproterenol injections on the fH was tested with a two-way repeated measures ANOVA (*groups* and *injections* as factors). One-way ANOVA was used to reveal if the acclimation conditions affected the highest fH_{max} (peak fH_{max}), the temperature where it was achieved (T_{peak}) and the thermal tolerance (T_{ARR}). One-way ANOVA was also used to evaluate differences between acclimation groups in NCX and SERCA levels. Spearman correlations examined for a correlation of the relative levels of either SERCA or NCX with either the electrical activity (ECG intervals) or the fH_{max} measured at each temperature increment during the acute warming among all the groups to reveal how acclimatory changes at functional level are connected to changes at molecular level. Linear regression analyses evaluated the relationship between the fH_{max} at 16 °C and T_{ARR} . A multiple linear regression analysed the correlation between the relative levels of SERCA and NCX (independent variables) and the T_{ARR} (dependent variable). The multiple linear regression analysis between protein levels and T_{ARR} revealed the presence of one influential outlier with a high discrepancy (Leverage = 0.338; Cook's distance = 1.357), which was removed from the final analysis. We discriminated the separate effects of SERCA and NCX on the T_{ARR} by referring the regression statistics (standard beta coefficients, t value and p -value) for each independent variable.

Table 1

Effects of acclimation conditions on biometric parameters of fish, on the fH_{max} threshold temperatures (T_{peak} , T_{ARR}) and on relative band intensities of sarco(endo)plasmic reticulum Ca^{2+} -ATPase (SERCA) and Na^{+}/Ca^{2+} exchanger (NCX).

	16 N			16 H			25 N		
	Mean	s.e.m.	<i>n</i>	Mean	s.e.m.	<i>n</i>	Mean	s.e.m.	<i>n</i>
Mass (g)	66.5 ^a	7.8	9	64.4 ^a	4.9	10	99.6 ^b	5.3	9
Fork length (cm)	17.5 ^a	0.6	9	17.8 ^a	0.4	10	19.5 ^b	0.3	9
Condition factor	1.2 ^a	0.03	9	1.1 ^a	0.02	10	1.3 ^b	0.05	9
Relative ventricle mass (%)	0.060 ^a	0.002	9	0.065 ^a	0.002	10	0.059 ^a	0.002	9
peak fH_{max} (bpm)	123.9	8.07	7	115.62	6.37	8	118.33	5.4	9
T_{peak} (°C)	22.2 ^{ab}	0.9	7	21.5 ^a	0.7	8	24.9 ^b	0.7	9
T_{ARR} (°C)	22.7 ^a	0.8	7	22.0 ^a	0.6	8	25.4 ^b	0.7	9
SERCA	0.89 ^a	0.10	9	0.57 ^b	0.08	10	0.59 ^b	0.05	9
NCX	0.97 ^a	0.05	9	1.03 ^a	0.04	9	0.71 ^b	0.05	8

Different letters indicate significant differences ($p < 0.05$) among the experimental groups.

3. Results

3.1. Biometry

The 16 N and 16H groups did not differ in their body mass or fork length (Table 1). Warm-acclimated fish, however, had a significantly higher body mass ($F_{2,25} = 10.48$; $p < 0.001$; 25 N vs 16 H Sidak $p < 0.001$; 25 N vs 16 N Sidak $p = 0.003$), fork length ($F_{2,25} = 10.85$; $p = 0.011$; 25 N vs 16 H Sidak $p = 0.033$; 25 N vs 16 N Sidak $p = 0.018$) and condition factor ($F_{2,25} = 8.62$; $p < 0.001$; 25 N vs 16 H Sidak $p < 0.001$; 25 N vs 16 N Sidak $p = 0.034$) than both groups 16 °C-acclimated (16 N and 16 H). Relative ventricle mass was similar among the three acclimation groups ($F_{2,25} = 2.06$; $p = 0.146$).

3.2. Maximum heart rate (fH_{max}) measurement using ECG

Before the start of the warming ramp (16 °C), the heart rate of the 25 N group was always significantly lower than the compare to 16 N and 16 H (groups $F = 36.393$, $p < 0.001$; groups*injections $F = 0.697$, $p = 0.598$), both before and after the pharmacological stimulation with atropine and with isoproterenol. Pharmacological stimulation significantly affected the fH of all the acclimations groups (Injections $F = 44.940$, $p < 0.001$) without any significant interaction between groups and injections types (Supplementary Table 1). The fH increased significantly after atropine injection while isoproterenol did not induce further increase. In the range 16 °C to 23 °C, warm-acclimated fish (25 N) displayed a significantly lower fH_{max} than 16 °C-acclimated fish (16 N and 16 H). This difference was independent of hypoxia vs normoxia acclimation condition (groups, $F = 47.64$, $p < 0.001$) (Fig. 2A) because 16 °C-acclimated fish (16 N and 16 H) had a similar fH_{max} at all test temperatures (Fig. 2A). Statistical comparisons of fH_{max} above 23 °C were not possible because n values progressively decreased as an increasing number of individuals displayed cardiac arrhythmia and therefore reached the endpoint of the fH_{max} measurement (see inset in Fig. 2A). Individuals with an initially low fH_{max} at 16 °C had significantly higher T_{ARR} as compared to fish having an initially high fH_{max} (Fig. 3B; $R^2 = 0.167$, Adj $R^2 = 0.13$) and, as a result, a significant negative correlation existed between fH_{max} at 16 °C and T_{ARR} (standard Beta coefficient = -0.409 ; $t = -2.103$; $p = 0.047$). The highest fH_{max} values recorded (peak fH_{max}) were similar between the groups ($F = 0.397$, $p = 0.677$) although warm acclimated fish reached peak fH_{max} at significantly higher temperature (T_{peak}) as compared to the 16 H acclimated group ($F = 6.072$, $p = 0.008$; 25 N vs 16 H Sidak $p = 0.011$; 25 N vs 16 N Sidak $p = 0.057$, 16 N vs 16 H Sidak $p = 0.907$) (Table 1). Warm acclimated fish displayed a higher T_{ARR} than both 16 °C-acclimation groups ($F_{2,21} = 7.20$, $p = 0.004$; 25 N vs 16 H Sidak $p = 0.005$; 25 N vs 16 N Sidak $p = 0.036$) (Figs. 2A, 3A).

The RR-interval was significantly longer at all test temperatures in the 25 N group when compared to both 16 °C-acclimation groups (16 N and 16 H) ($F = 52.006$, $p < 0.001$; 25 N vs 16 N Sidak $p < 0.001$; 25 N vs 16 H Sidak $p < 0.001$), while the 16 °C-acclimation groups did not differ

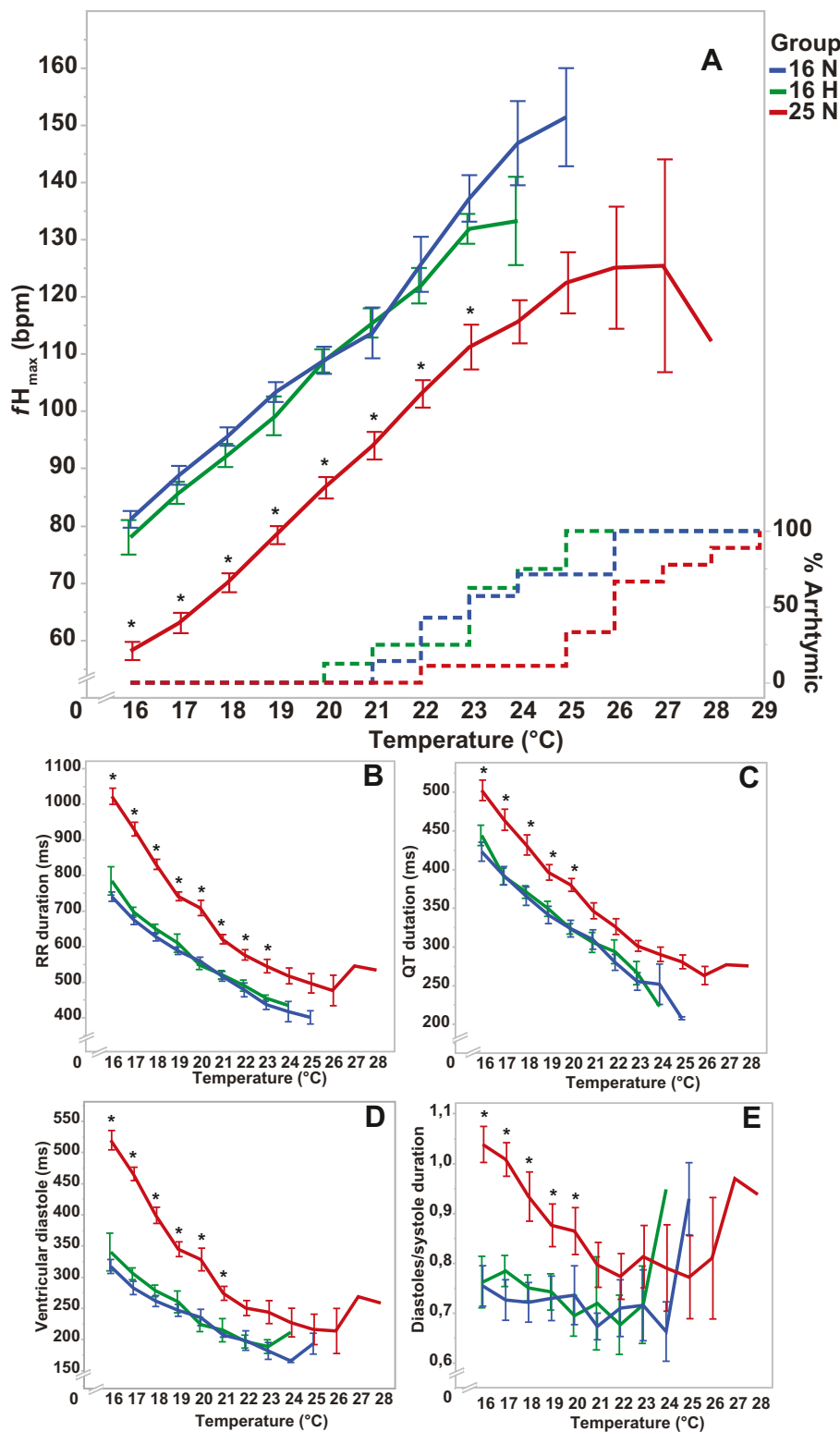


Fig. 2. Effect of acclimation conditions on maximal heart rate (fH_{max}) and ECG parameters during the thermal challenge. (A) fH_{max} (\pm s.e.m.) of the fish acclimated to 16 °C and normoxia (16 N; $n = 7$); 16 °C and hypoxia (16 H; $n = 8$); 25 °C and normoxia (25 N; $n = 9$) during acute warming. Dashed lines represent the % of fish that got cardiac arrhythmias during each temperature step of the warming challenge. (B) RR duration (\pm s.e.m.), (C) QT duration (\pm s.e.m.), (D) Ventricular diastolic duration (\pm s.e.m.) and (E) Diastole/systole ratio (\pm s.e.m.) among the groups during the acute warming. * indicated significant difference between 25 N vs 16 N and between 25 N vs 16 H.

significantly from each other (16 N vs 16 H Sidak $p = 0.340$) (Fig. 2B). This increase in the RR-interval was associated with a prolonged QT-interval (ventricular systole; $F = 12.780$, $p < 0.001$) and a greater relative prolongation of the diastole duration (RR minus QT) ($F = 39.46$, $p < 0.001$) (Fig. 2C, D) because warm acclimation significantly increased the ventricular diastole/systole ratio ($F = 8.075$, $p = 0.002$) (Fig. 2E). Other ECG intervals (i.e., PQ, PR and QRS) were not

significantly different among acclimation groups at any temperature increment (Fig. S4). Similar ECG intervals for the 16 N and 16 H groups again indicated that hypoxic acclimation at 16 °C did not influence the cardiac cycle.

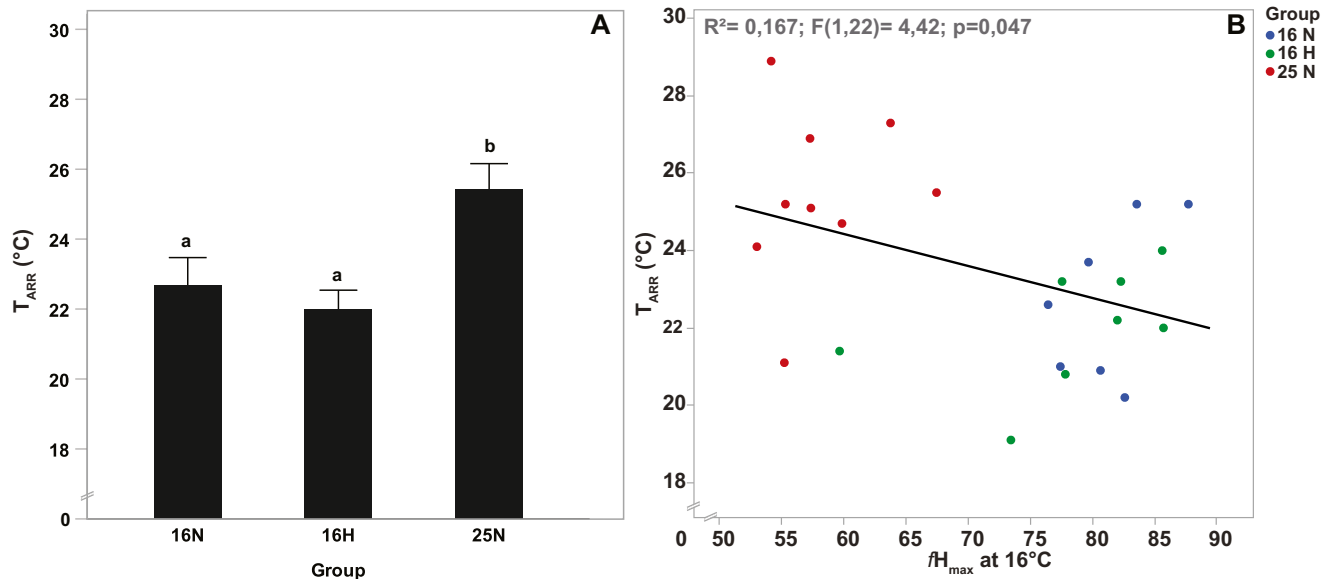


Fig. 3. Comparison of the cardiac thermal tolerance between the acclimation groups. (A) Average (\pm s.e.m.) temperature where cardiac arrhythmias (missing QRS peaks) were observed (T_{ARR}). Different letters indicate significant differences ($p < 0.05$) among the groups. Fish were acclimated either 16 °C and normoxia (16 N; $n = 7$); 16 °C and hypoxia (16 H; $n = 8$) or 25 °C and normoxia (25 N; $n = 9$). (B) Scatterplot representing the relationship between the initial fH_{max} at 16 °C and T_{ARR} among the experimental groups (R^2 , F value and p value for the linear regression are insert in the chart; linear regression equation $T_{ARR} = 29.4 - 0.08276 \times$).

3.3. Ca^{2+} cycling protein levels in the ventricle

Warm and hypoxic acclimation changed the relative levels of the SERCA ($F_{2, 25} = 5.10$, $p = 0.014$) and NCX (Fig. 4; $F_{2, 23} = 12.751$ $p < 0.001$) proteins. Both warm (25 N) and hypoxic (16 H) acclimation significantly reduced the relative level of SERCA compared with the control normoxic group at 16 °C (25 N vs 16 N Sidak $p = 0.046$; 16 H vs 16 N Sidak $p = 0.021$) and to the same extend (25 N vs 16 H; $p > 0.05$). Also, the relative level of NCX was significantly lower for 25 N than both 16 °C-acclimation groups (25 N vs 16 N Sidak $p = 0.002$; 25 N vs 16 H Sidak $p < 0.001$). However, hypoxic acclimation did not change NCX protein level (16 H vs 16 N Sidak $p > 0.05$). Thus, warm acclimation reduced the relative levels of two Ca^{2+} -handling proteins involved in cellular relaxation, whereas acclimation to hypoxia only reduced SERCA and not NCX.

3.4. Correlations between the protein levels and cardiac function

Correlations of NCX and SERCA with fH_{max} and the ECG intervals were analysed at each temperature increment of acute warming (Supplementary Tables 2 & 3). In general, a significant positive correlation existed between NCX and fH_{max} (Fig. 5A) whereas a significant negative correlation existed between NCX and the RR-interval (Supplementary Table 2). Relative NCX level was also negatively correlated with diastole duration (Fig. 5C) and with the diastole/systole ratio (Supplementary Table 2). However, NCX and the QT-interval were negatively correlated only at two test temperatures (Fig. 5B, Supplementary Table 2). After outlier exclusion, the relative level of SERCA and NCX proteins together explained 46% of the total variability (Adj $R^2 = 0.46$; $p = 0.001$) in the T_{ARR} . A high relative level of NCX (standard Beta coefficient = -0.641 ; $t = -3.910$; $p = 0.001$), but not SERCA (standard Beta coefficient =

-0.315 ; $t = -1.924$; $p = 0.070$), was associated with low T_{ARR} (Fig. 6). The relative level of SERCA was not correlated with either fH_{max} or any ECG interval (Supplementary Table 3).

4. Discussion

The objective of the current study was to investigate cardiac function (electrical activity of cardiac cycle, fH_{max} and T_{ARR}) and the ventricular protein levels of NCX and SERCA of sea bass after both warm and hypoxic acclimation. By doing so, we associated functional cardiac changes with molecular-level adjustments and examined if the hypoxia and temperature acclimation induced similar changes to provide mechanistic support for cross-tolerance. The warm acclimation effect noted here for sea bass confirmed what shown in other fish species. Warm acclimation enhanced the upper thermal limit of cardiac performance as revealed by the significantly increased T_{ARR} and decreased fH_{max} . Additionally, increased ventricular diastolic duration and, to a lesser extent, systolic duration, were associated with a lower fH_{max} after warm acclimation. These changes were reflected at the molecular level, with warm acclimation decreasing the relative protein levels of both SERCA and NCX. Moreover, NCX protein level was strongly associated with T_{ARR} , diastolic duration and fH_{max} . We found no support for the hypothesis that hypoxia and temperature acclimations would induce common adjustments in cardiac function. Indeed, unlike warm acclimation, we found that hypoxia acclimation had no effect on fH_{max} and T_{ARR} . Furthermore, hypoxic acclimation significantly decreased SERCA protein level without changing NCX level or the cardiac function indices. Results at molecular level suggest that the relaxation phase of sea bass cardiac cycle is functionally more dependent upon cycling Ca^{2+} to the extracellular space via NCX than to sarcoplasmic storage via SERCA when modulating its maximum heart rate. This is in line with what has

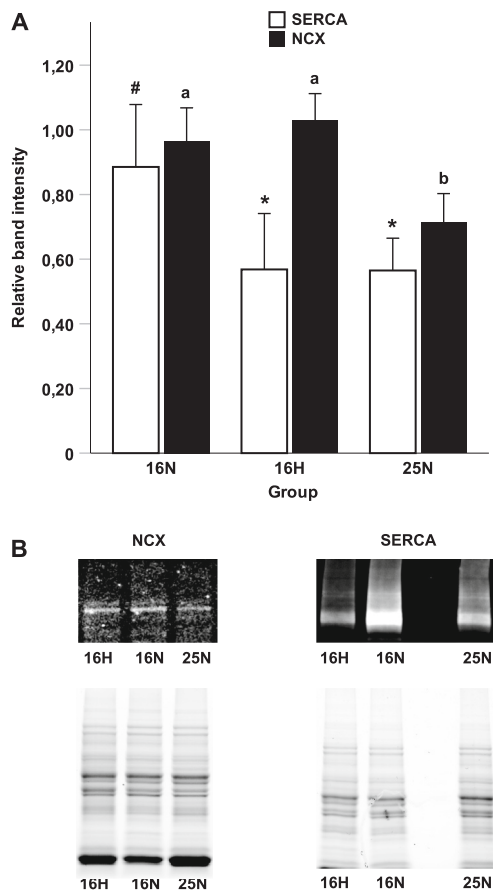


Fig. 4. Ca^{2+} cycling proteins levels. (A) Average (\pm s.e.m.) relative band intensity of the sarco(endo)plasmic reticulum Ca^{2+} -ATPase (SERCA) (white bar; $n = 28$) and $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCX) (black bar; $n = 26$) in the three experimental groups. Different letters indicate significant difference ($p < 0.05$) in relative NCX level between the 25 N group and the 16 N and 16 H groups. Different symbols * and # indicate significant difference ($p < 0.05$) in relative SERCA level between the 16 N group and 16 H and 25 N groups. (B) Representative Western blot membranes and total protein gels of NCX and SERCA (see Supplementary Fig. 2 for full membrane).

been shown in previous studies (Imbert-Auvray et al., 2013; Joyce et al., 2016; Ollivier et al., 2015). However, as the NCX is working bidirectionally we cannot exclude its involvement also in the initiation of the contraction, as well L-type Ca^{2+} channels and ryanodine receptors. Therefore, further studies about electrophysiological activities of channels are needed.

4.1. Warm acclimation, but not hypoxic acclimation, modulated fH_{\max} and electrical activity of cardiac cycle

Consistent with previous observations in several fishes (Aho and Vornanen, 2001; Farrell et al., 2007; Anttila et al., 2014b; Badr et al., 2016; Ekström et al., 2016; Sutcliffe et al., 2020) warm acclimation reset fH_{\max} to a lower rate, with fH_{\max} in 25 °C-acclimated fish being consistently lower than in 16 °C-acclimated fish at all test temperatures. Our ECG intervals analysis revealed that the reduction of fH_{\max} with warm acclimation in sea bass was associated with a clear prolongation of the diastolic periods and to a lesser extent the ventricular systolic. In roach, the reduction of heart rate with warm acclimation was associated

with a longer PQ-interval, i.e., a longer atrial systole and slower conduction of action potential from atrium to the ventricle (Badr et al., 2016). This finding introduces the possibility that different fish species may vary the strategies to regulate the heart rate (Ma et al., 2019) and a slower heart rate can be associated to differential changes in the ECG components durations among fish species. These changes could be partly due to changes in ion channel function.

Hypoxic acclimation (50% air sat.) did not affect fH_{\max} of sea bass. This refractoriness of fH_{\max} to hypoxic acclimation could reflect the species lifestyle as European sea bass inhabits coastal and estuarine areas that may be susceptible to hypoxia, shaping their phenotype (Clairiaux et al., 2013; Dambrine et al., 2021; Sánchez Vázquez et al., 2014; Thetmeyer et al., 1999). In previous study with steelhead trout (*Oncorhynchus mykiss*), chronic hypoxia acclimation (> 3 months at 40% air sat.) did not affect the heart rate during acute warming similarly as in our study (Motyka et al., 2017), but T_{ARR} decreased significantly when compared to normoxia-acclimated trout, unlike in sea bass. The responses to hypoxia seem to be species-specific since, during winter conditions i.e. hypoxia at +5 °C, Alaska blackfish has been shown to be active and able to maintain normal heart rate even though having shorter QT interval as compared to conspecifics kept at +5° and normoxia (Stecyk et al., 2020). On the other hand, crucian carp (*Carassius carassius*) acclimated to anoxia for 5 weeks had a slower heart when measured in anoxia as compared to control normoxic fish, and this lower heart rate was associated with prolonged RT- and QRS-intervals (Tikkanen et al., 2016). Furthermore, hypoxia-acclimated (50% air sat.) channel catfish (*Ictalurus punctatus*) had a significantly higher routine heart rate than the normoxic group, but only between 26 and 28 °C (Burlinson and Silva, 2011). Apart from possible interspecies differences, these contrasting results could also reflect methodological differences, such as the level of hypoxia tested and/or to the heart rate analysed (e.g., anaesthesia, routine heart rate with the possibility of increased vagal inhibition vs. maximal heart rate without possibility of vagal inhibition).

4.2. Warm, but not hypoxic acclimation, improved cardiovascular thermal tolerance

Warm acclimation increased T_{ARR} in 25 °C-acclimated sea bass by 4 °C compared with fish acclimated to a 9 °C cooler temperature. This observed shift is in accordance with previous studies showing that warm acclimation can improve cardiac upper thermal tolerance (Anttila et al., 2014a; Badr et al., 2016; Safi et al., 2019). Contrary to cross-tolerance hypothesis, hypoxic and temperature acclimation did not induce similar effects on T_{ARR} . Thus, even though these two environmental stressors sometimes co-occur and their tolerances can be correlated in some fish species (Anttila et al., 2013b; McBryan et al., 2013; Zhang et al., 2018), our results are consistent with other studies that have shown no cross-tolerance between hypoxia acclimation and thermal tolerance (Leeuwis et al., 2021; Motyka et al., 2017). For example, while channel catfish acclimated to moderate hypoxia (40% air sat.) for one week achieved a marginally higher CT_{MAX} compared to normoxic ones (Burlinson and Silva, 2011), rainbow trout (*Oncorhynchus mykiss*) acclimated to hypoxia for 3 months had significantly lower T_{ARR} compared to the normoxic group (Motyka et al., 2017). The reasons for these contrasting results are unclear. Again, species differences, heart rate measurements methodologies and the severity of the hypoxia could all play roles. We cannot, for example, be certain whether or not the hypoxic treatment was too mild to significantly induce functional changes in European sea bass, especially since growth was not affected by acclimation to 50% air sat. Furthermore, it has been shown that sea bass can tolerate 40% air sat. without affecting the food conversion efficiency (Thetmeyer et al., 1999) and the escape response latency (Lefrançois and Domenici, 2006). Future studies, therefore, could acclimate sea bass to more severe hypoxic conditions and also investigate the interactive effects of the stressors by acclimating fish to high temperature combined with hypoxia.

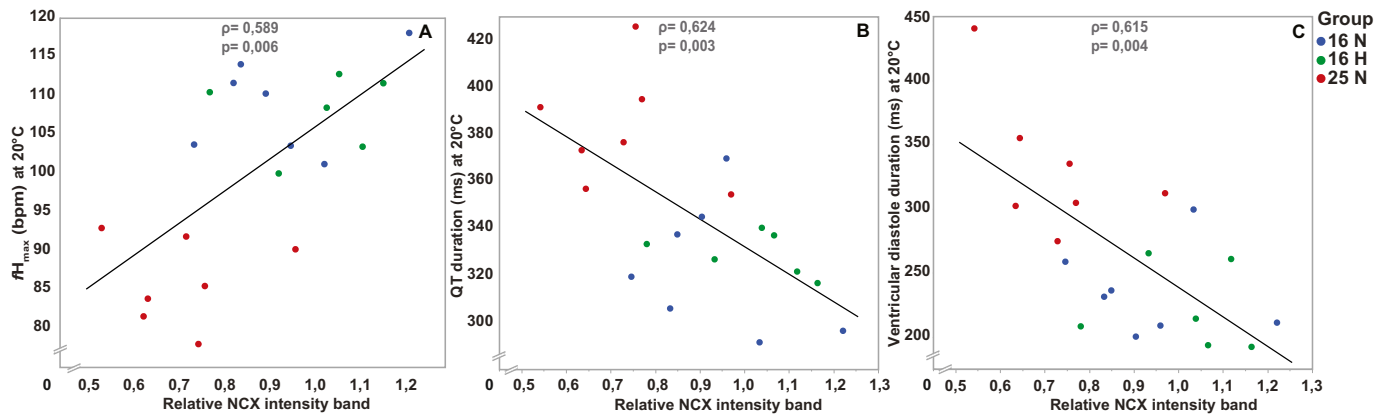


Fig. 5. Correlation analyses between the relative level of $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCX) and ECG parameters: (A) fH_{\max} , (B) QT duration, (C) Ventricular diastole at 20 °C. See Tables 1 & 2 in supplementary material for the Spearman correlations values at the other temperatures. Spearman correlation coefficients (ρ) and p values are insert in the charts.

4.3. Changes in cardiac function are associated to NCX remodelling

Warm acclimation significantly reduced protein levels of both NCX and SERCA, a result consistent with earlier studies (Aho, 1999; Keen et al., 2017; Keen et al., 1994). On the other hand, hypoxia acclimation significantly reduced relative level of SERCA, but not NCX. The reduction of SERCA in both stressors is logical response since SERCA has been shown to be one of the highest ATP demanding ion pump and therefore a potentially important energy saving target (Schramm et al., 1994). The reduction of NCX in warm acclimated group could be connected to general reduction of cardiac pumping activity while the heart rate of the hypoxia acclimated fish did not change nor the NCX level.

The observed association between cardiac remodelling of protein levels and functional cardiac remodelling due to the acclimation brings insights in the mechanisms of fish response to environmental stressors. NCX protein level was negatively correlated with the RR-interval and the ventricular diastolic duration and positively with fH_{\max} at many temperatures. Yet, SERCA was not correlated with any of the cardiac

function variables (ECG intervals and fH_{\max}) that we measured. Changes in the level of NCX could possibly influence the refractory phase of the cardiomyocyte. Shortening the refractory phase could imply an earlier onset of repolarization, so the next action potential could occur sooner. In sea bass, the observed correlation between NCX and ECG intervals might be due to the larger role of NCX as compared to SERCA in the regulation of the excitation-contraction coupling, as reported also in human (Tse, 2016). However, NCX being bidirectional, it is also involved in the initiation of the cardiac contraction making it difficult to conclude about its full role in regulating the contraction cycle. Furthermore, these are not the only Ca^{2+} cycling proteins that may influence the heart rate and the ECG. Therefore, future studies should adopt a more holistic approach, i.e., 1) analysing other proteins involved with regulation of the cardiac cycle (i.e. RyR, L-type Ca^{2+} channel, tropomyosin, calmodulin etc.), 2) analysing the activity of proteins and 3) verifying if the connections from molecular to functional level are also detected when resting/routine heart rate is measured without anaesthetics/drugs. In future studies when analysing the activity of

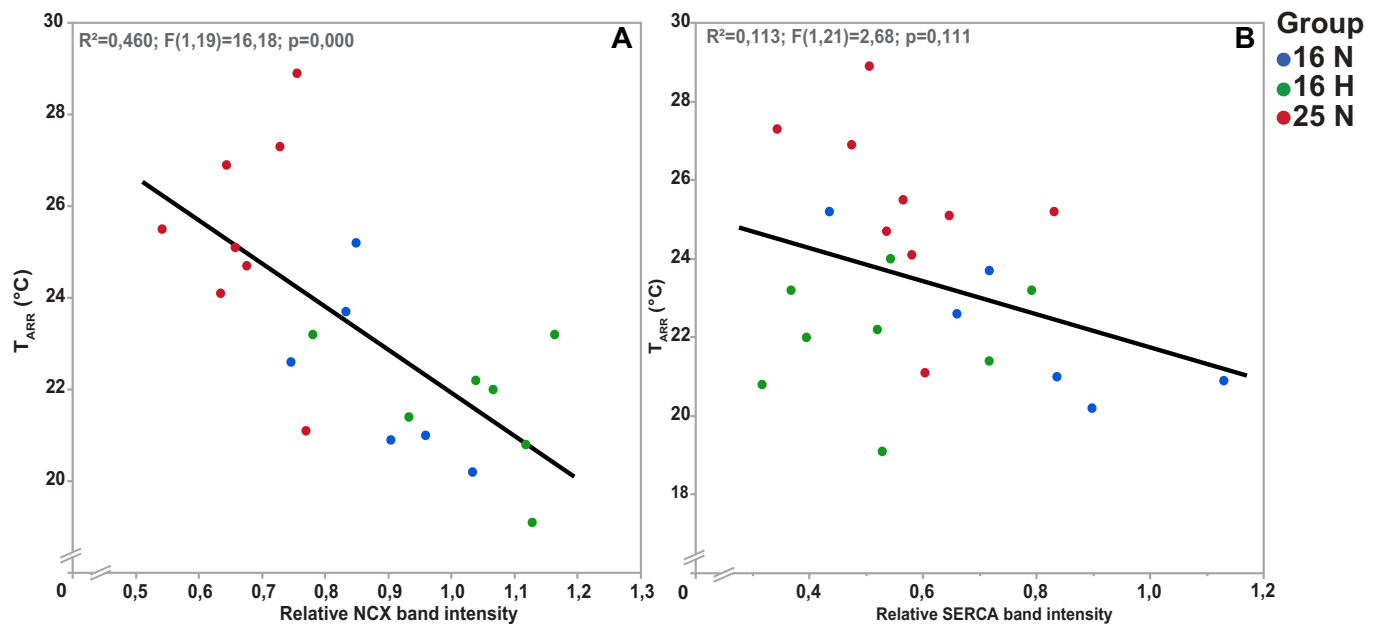


Fig. 6. The relationship between the temperature at which fish experienced cardiac arrhythmias (T_{ARR}) and the relative levels of the calcium channel proteins: (A) $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCX) and (B) sarco(endo)plasmic reticulum Ca^{2+} -ATPase (SERCA). R^2 , F value and p value in charts refer to the linear regressions that fit respectively (A) NCX and T_{ARR} (equation $T_{\text{ARR}} = 31.33 - 9.408 \times$) and (B) SERCA and T_{ARR} (equation $T_{\text{ARR}} = 25.96 - 4.212 \times$). The multiple linear regression examined the combined effects of NCX and SERCA on the T_{ARR} .

calcium cycling proteins, we suggest that care be taken with adrenalin/ isoproterenol treatments as they could modulate the activity of channels (Kumari et al., 2018).

Beyond the correlation between the level of Ca^{2+} cycling proteins and ECG intervals and fH_{\max} , we investigated the association between Ca^{2+} cycling proteins and T_{ARR} . Since NCX, but not SERCA level, was negatively associated with T_{ARR} , we think that NCX might play a prominent contributory role in setting the upper thermal cardiac tolerance in sea bass. The correlation between NCX and T_{ARR} supports the temperature-dependent deterioration of electrical excitability (TDEE) hypothesis, also known as “Source-sink mismatch hypothesis” (Vornanen, 2020; Vornanen, 2016). TDEE hypothesis suggests that Na^+ ion current is the weak link in cardiac upper thermal tolerance since at high temperatures the Na^+ current starts to decline and prevents the cardiac depolarisation (Haverinen and Vornanen, 2020). The functioning of NCX directly depends from Na^+ ion gradient and current, since NCX moves Na^+ ions down its gradient across the sarcolemma in exchange for the counter-transport of Ca^{2+} ions (Shiels, 2017). Therefore, an impaired Na^+ ion current due to high temperature (Vornanen, 2016) could also affect NCX function. High level of NCX might increase the vulnerability to dysfunction of Na^+ ion current and the consequent temperature-induced arrhythmias, as shown in our results. Support to this idea comes also from the mammalian studies since high protein levels of NCX has been correlated with heart failure and arrhythmias propensity (Hobai and O'Rourke, 2000; Schillinger et al., 2003) which suggests that NCX plays significant role in genesis of cardiac arrhythmias. However, despite these promising results, questions remain. The upper thermal tolerance of cardiac system is likely defined by many other mechanisms. Enhanced mitochondrial efficiency could be one of many mechanisms to improve cardiac performance of warm-acclimated fish (Gerber et al., 2020; Howald et al., 2019; Voituron et al., 2022). Further mechanistic studies focusing on the role of NCX in cardiac plasticity are also needed. In particular, this study should be completed by an analysis of NCX activity because it is clear that the protein level does not necessarily reflect the functional output of the proteins. Although not conclusive on this point, our study nevertheless provides interesting insights into the mechanisms underlying cardiac thermal tolerance and the functional changes induced by warm and/or hypoxia acclimation.

5. Conclusion

Marine habitats are notoriously heterogeneous in term of temperature and oxygen level through both space and time. The magnitude and frequency of extreme temperature and hypoxia events are predicted to increase with climate change, thereby challenging fishes' physiology. Capacity for phenotypic plasticity will clearly be a determining factor of fish performance in future environment with elevated temperatures and low oxygen levels. Indeed, European sea bass showed high cardiac phenotypic plasticity to adjust to warming and hypoxia at both functional and molecular levels, which could give to this species an edge to face predicted future environmental conditions. However, these results did not provide evidence for shared acclimation mechanism supporting the cross-tolerance. From a mechanistic point of view, the cardiac plasticity in response to warm acclimation was achieved mainly by increasing diastole duration, lowering the fH_{\max} and increasing T_{ARR} , beneficial functional changes that were associated with reducing the level of NCX involved in cellular relaxation of the cardiomyocytes. However, hypoxia induced merely molecular adjustments with SERCA which were not reflected at functional level. The novel correlation between NCX and diastole duration and T_{ARR} needs, however, further mechanistic study.

Authors' contributions

H.O., F.M. L.P., Y.F., F.L., A.P.F., G.C. and K.A. conceived and designed the experiments.

H.O., F.L., F.M., Y.F. and G.C. were responsible for the fish rearing and acclimation.

L.P. and F.L. conducted the fH_{\max} and ECG experiments and data analyses.

L.P. and K.A. analysed the molecular level changes.

L.P. and K.A. wrote the original draft of the manuscript.

All the authors collaborated in reviewing and editing the manuscript.

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Data accessibility

All data are provided in the manuscript and in the electronic supplementary material.

Declaration of Competing Interest

The authors declare no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.cbpa.2022.111266>.

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