



Cardiovascular oxygen transport and peripheral oxygen extraction capacity contribute to acute heat tolerance in European seabass

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ABSTRACT

This study evaluated whether different parameters describing cardiovascular function, energy metabolism, oxygen transport and oxidative stress were related to the critical thermal maximum (CT_{MAX}) of European seabass (*Dicentrarchus labrax*) and if there were differential changes in these parameters during and after heat shock in animals with different CT_{MAX} in order to characterize which physiological features make seabass vulnerable to heat waves. Seabass ($n = 621$) were tested for CT_{MAX} and the physiological parameters were measured in individuals with good or poor temperature tolerance before and after a heat shock (change in temperature from 15 °C to 28 °C in 1.5 h). Fish with good thermal tolerance had larger ventricles with higher maximal heart rate during the heat shock than individuals with poor tolerance. Furthermore, they initially had a high ventricular Ca²⁺-ATPase activity, which was reduced to a similar level as in fish with poor tolerance following heat shock. The activity of heart lactate dehydrogenase increased in fish with high tolerance, when they were exposed to heat shock, while the aerobic enzyme activity did not differ between groups. The tolerant individuals had smaller red muscle fibers with higher myoglobin content than the poorly tolerant ones. The poorly tolerant individuals had higher hematocrit, which increased with heat shock in both groups. The poorly tolerant individuals had also higher activity of enzymes related to oxidative stress especially after heat shock. In general, CT_{MAX} was not depending on merely one physiological factor but several organ and cellular parameters were related to the CT_{MAX} of seabass and when working in combination they might protect the highly tolerant seabass from future heat waves.

1. Introduction

Anthropogenic activities are changing the Earth's climate and this is seen especially at high latitudes. Predicted global temperature increase varies between 1.5 and 5.7 °C before 2100 depending on the modelled greenhouse gas accumulation (IPCC, 2021). In addition to the increase of average temperatures also the frequency and intensity of the heat waves are predicted to increase in future (IPCC, 2021). This will be challenging for animals since environmental temperature is one of the most pervasive abiotic factors governing the biology of ectothermic animals like fish. Temperature is a controlling factor of ectotherm's metabolism and, for that reason, it is projected that ongoing ocean warming will determine their survival, activities, life history trajectories

and evolution (Poloczanska et al., 2013; Pecl et al., 2017; Pörtner et al., 2017; Moyano et al., 2020). For example, mass mortalities of fish have already been associated with extreme heat waves (Till et al., 2019; Roberts et al., 2019; Garrabou et al., 2022).

The mechanisms underpinning the relationship between temperature, fish metabolism and distribution stand on the fact that as water temperature increases, it progressively shifts from being a driving factor of fish behavior to being a limiting factor of their activities (Pörtner et al., 2017). According to the oxygen and capacity limited thermal tolerance (OCLTT) hypothesis, at the upper limit of fish thermal range, life-sustaining activities fully mobilize their capacity for ATP synthesis, leaving no capacity for discretionary activities such as growth and reproduction. The OCLTT hypothesis further suggests that this limiting

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effect could result from a mismatch between the temperature-driven increase in oxygen demand and the capacity of the cardio-respiratory system to deliver oxygen to tissues, making the heart a key determinant of fish thermal tolerance (Farrell et al., 2009; Pörtner et al., 2017).

Despite significant research efforts, the physiological underpinnings of ectotherms' upper thermal tolerance are still not well understood and the most comprehensive hypothesis of thermal tolerance, i.e. OCLTT remains a debated issue (Pörtner et al., 2017; Jutfelt et al., 2018; Vornanen, 2020; Pörtner, 2021; Schauffele et al., 2021; Lefevre et al., 2021). Nevertheless, in salmonids, it has been shown that the temperature-related increase in maximum heart rate (f_{Hmax}) stops at a temperature where aerobic metabolic scope is maximal. Beyond that point, a reduction in f_{Hmax} coincides with a reduction in aerobic scope, supporting the central role of the heart in determining fish upper thermal tolerance (Steinhausen et al., 2008; Eliason et al., 2011). Ecologically the cardiovascular capacities and their thermal limits are, for example, connected to annual productivity in Atlantic herring (*Clupea harengus*): during warm springs when temperatures are above the cardiovascular thermal limits of the fish, the productivity is low (Moyano et al., 2020).

However, as temperature reaches a species' critical thermal maximum (CT_{MAX}), conflicting results have been reported, making it difficult to identify the determining factors of acute upper lethal temperature in fish. Parameters related to oxygen carrying capacity / oxygen availability to tissues have been suggested to play a role in determining CT_{MAX} but results are varying. For example, environmental oxygen level does not seem to have a marked influence on CT_{MAX} in red drum (*Sciaenops ocellatus*) or marine lumpfish (*Cyclopterus lumpus*) (Ern et al., 2016), suggesting that the limitation may not be linked to the oxygen diffusion capacity across gill epithelium in these species. In European perch (*Perca fluviatilis*) and zebrafish (*Danio rerio*) environmental hyperoxia increased the CT_{MAX} (Ekström et al., 2016; Andreasen et al., 2022), but reducing the hematocrit, i.e. reducing the oxygen carrying capacity of blood, had only a minor reducing effect on the CT_{MAX} in European seabass (*Dicentrarchus labrax*) in the study by Wang et al. (2014). Since the main task of heart is to generate blood (and oxygen) flow to tissues, heart might also be involved with CT_{MAX} of fish, if oxygen availability is linked to CT_{MAX} . Indeed, in about 300 g Atlantic salmon (*Salmo salar*), European seabass and Nile perch (*Lates niloticus*) it has been shown that larger ventricles (higher relative ventricle mass (RVM)) correlate with increased CT_{MAX} (Anttila et al., 2013; Ozolina et al., 2016; Nyboer and Chapman, 2018). In 100 g Atlantic salmon, on the other hand, highest CT_{MAX} was observed in fish with small ventricles (Bartlett et al., 2022). For the relationship between high CT_{MAX} and large ventricle size, it was hypothesized that since larger ventricles are associated with larger stroke volume and cardiac output (Farrell, 1991; Hillman and Hedrick, 2015), fish with high RVM might have higher oxygen flow to tissues which might benefit them at high temperatures, if CT_{MAX} is connected to oxygen availability (Ozolina et al., 2016). Along the same line, a positive correlation between CT_{MAX} and ventricular myoglobin level, which increases the tissue oxygen extraction capacity, has been observed in Atlantic salmon, reinforcing the hypothesis that cardiac function contributes to determining the thermal tolerance of fish (Anttila et al., 2013). Likewise, it has been shown in the Nile perch that RVM and the portion of compact myocardium correlated positively with aerobic scope (Nyboer and Chapman, 2018). It has also been shown that by restricting the coronary blood flow to ventricles, the CT_{MAX} of rainbow trout (*Oncorhynchus mykiss*) could be reduced indicating that supply of oxygenated blood to heart plays a role in acute upper thermal tolerance (Ekström et al., 2019). Nevertheless, the results seem to be quite variable between studies and more clear answers are needed. One of the factors causing the variability of the findings between studies could be the way how upper thermal tolerance is measured. There are recent evidence that acute and chronic thermal tolerance may depend on different physiological factors (Bartlett et al., 2022) and thus, the differences in the rate of temperature change during measurements might explain the varying results. Furthermore, the inter-individual

variability between animals may be an important component of thermal adaptations (Nikinmaa and Anttila, 2019).

Nevertheless, heart function seems to fail at high temperature and in a wide variety of fish species cardiac arrhythmias have been observed when fish have been acutely exposed to close to critical temperatures (for review see Eliason and Anttila, 2017). It has been proposed that this progressive dysfunction of the heart resulted from the gradual failure of oxidative phosphorylation in the heart mitochondria (Hilton et al., 2010; Iftikar et al., 2014, 2015; Penney et al., 2014; Rodnick et al., 2014; Ekström et al., 2017), ATP production capacity dropping even though oxygen was still largely available in the blood circulation (Iftikar and Hickey, 2013). The mitochondrial function at high temperatures can result in increased reactive oxygen species (ROS) level and the resulting oxidative stress may destroy essential structures of the cell (Heise et al., 2006; Madeira et al., 2013; Cheng et al., 2015) and lead to dysfunction. On the other hand, the cardiac failure at high temperatures might depend on the failure in the electrical excitation of the heart. At high temperatures the efficiency of the sodium influx channels is reduced while the outward potassium efflux channels keep working which induce a compromise in electrical excitability of cardiac cells and can induce missing heartbeats – i.e. arrhythmias (Vornanen et al., 2014; Vornanen, 2016; Badr et al., 2018a, 2018b; Vornanen, 2020; Haverinen and Vornanen, 2020).

To clarify what determines the acute upper thermal tolerance in fish and, more particularly, how animals with different CT_{MAX} respond to and recover from a heat stress, we capitalized on the strong and repeatable inter-individual variability of CT_{MAX} observed in the European seabass (Mauduit et al., 2016) to compare thermally sensitive and thermally tolerant individuals before and after exposure to heat shock. The aim was to find out which physiological pathways from molecular to functional level are connected with upper thermal tolerance of fish as measured by CT_{MAX} and how fish with different tolerances are reacting to heat stress since these are the parameters where natural selection could act upon when environmental temperature and the frequency of heat waves continue to increase. To reach these objectives, a population of 621 European seabass was screened for CT_{MAX} and the individuals situated at the both ends of the frequency distribution curve ($n = 30$ per experimental group) were selected to constitute two experimental groups (time for losing equilibrium 2.5 ± 0.03 h vs. 5.0 ± 0.03 h, i.e., temperatures of 31.1 ± 0.05 °C vs. 33.1 ± 0.04 °C). From these fish we measured 45 parameters related to cardiovascular function, energy metabolism, oxygen carrying and extraction capacity and oxidative stress as these could be key parameters related to the acute upper thermal tolerance of the fish.

2. Materials and methods

The seabass (age 1+; 17.8 ± 0.1 cm; 68.4 ± 1.2 g, $n = 621$) were reared at Unité de Physiologie Fonctionnelle des Organismes Marins, Ifremer, France in 1000 L, thermoregulated (15 °C) tanks. Upon their arrival in the laboratory, fish were anesthetized (MS-222; 100 mg L^{-1}) and individually implanted subcutaneously with an identification tag (RFID; Biolog-id, France). No pH buffering was used since the seawater buffered the potential pH changes of MS-222 and pH stayed constant. Fish were fed daily with commercial pellets (Le Guessant, Lamballe, France). Photoperiod followed seasonal conditions and salinity oscillated within the range 30–32 ppt. Fish were starved for 24 h before any manipulation or experiment. All the experiments were approved by Ministère Délégué à l'Enseignement Supérieur et à la Recherche.

2.1. Sorting fish on the basis of CT_{MAX}

To rank individual fish on the basis of their upper critical thermal tolerance (CT_{MAX}), a challenge test was conducted directly in the fish-rearing tank (Claireaux et al., 2013). This test consisted of increasing water temperature from 15.5 °C to 27 °C in 2.5 h and thereafter at a rate

of $0.5\text{ }^{\circ}\text{C h}^{-1}$ (JULABO F10, 2500 W heater, Seelbach, Germany). As fish lost their capacity to maintain equilibrium they were rapidly removed from the tank, identified (RFID tag reading) and placed in a recovery tank at acclimation temperature. The corresponding time and temperature were also noted. The experiment ended when the last fish was removed from the rearing tank. During the test, water oxygenation and homogenization were assured by bubbling air or oxygen into the tanks, keeping the oxygenation level above 80% of air saturation throughout the test. Only the 30 best and 30 poorest performers were involved in the experimental procedure that followed. Fish were allowed to recover for 3 weeks before further testing.

2.2. Heart rate measurements

To evaluate whether the maximal heart rate ($f_{H_{\max}}$) and its change during a heat stress differed between individuals with good and poor thermal tolerance as given by CT_{\max} , the $f_{H_{\max}}$ of fish from both experimental groups ($n = 8$ per experimental group) was measured following Casselman et al. (2012). We were particularly interested about maximal heart rate since its failure seems to connect to the reduction in aerobic scope when fish are exposed to high temperatures in some fish species (Steinhausen et al., 2008; Eliason et al., 2011) and therefore $f_{H_{\max}}$ could possibly be related to thermal tolerance. The fish were anesthetized in 80 mg L^{-1} MS-222 (Sigma-Aldrich Chemie GmbH, Steinheim, Germany), weighed and submerged in the experimental setup the temperature of which was controlled via a chilling/heating unit (F10, JULABO, Seelbach, Germany). The water contained a maintenance concentration of anesthetics (70 ppm of MS-222, no buffering as the measurements were performed in seawater). The fish were breathing during the measurements and low dose of anesthetics has not been shown to interfere the $f_{H_{\max}}$ of fish (Casselman et al., 2012). To detect fish electrocardiogram (ECG), two silver electrodes were placed caudally and cranially to the heart touching the skin of the fish. The signal from these electrodes was acquired with BioPac MP36R (BIOPAC Systems Inc., Essen, Germany) with a built-in amplification system. ECG analysis was performed with AcqKnowledge software (v. 4.2). Fish were equilibrated at the initial temperature ($15\text{ }^{\circ}\text{C}$) for 1 h, after which they were given an intraperitoneal injection of atropine sulphate (2.5 mg kg^{-1} , Sigma-Aldrich) and isoproterenol ($8\text{ }\mu\text{g kg}^{-1}$, Sigma-Aldrich) according to Casselman et al. (2012) to block vagal control and stimulate beta-receptors i.e. induce maximal beating frequency pharmacologically. This means that we were evaluating the absolute maximum beating frequencies of the fish and whether they relate to CT_{\max} . In nature the fish can control the cardiac beat frequency. Once the heart rate had stabilized, water temperature was increased in $1\text{ }^{\circ}\text{C}$ increments at a rate of $10\text{ }^{\circ}\text{C h}^{-1}$. After every temperature increment, the heart rate was allowed to stabilize before recording the corresponding value. Warming continued until cardiac arrhythmias (missing QRS complex) were noticed. At that time, water temperature was recorded and the fish rapidly removed from the apparatus and euthanized by cranial percussion.

2.3. Heat shock

Before the heat shock, 7 fish from both experimental groups were euthanized with an overdose of MS-222 (200 mg L^{-1}). Within 5 min, fish weight and fork length were recorded and a blood sample taken from the caudal vein using heparinized syringes. The first gill arch was then dissected and a sample of red and white muscle taken. These tissues were fixed in 4% formalin in phosphate-buffered saline (PBS) for histological analyses. The ventricles and livers were then removed, weighed, cut into pieces and frozen in liquid nitrogen for later Ca^{2+} -ATPase (SERCA), enzymatic and Western blotting analyses.

For the heat shock, the same heating element as used in CT_{\max} measurements was submerged in the fish rearing tank and water temperature was increased from $14.7\text{ }^{\circ}\text{C}$ (acclimation temperature) to $28\text{ }^{\circ}\text{C}$

in 1.5 h and then returned to acclimation temperature within 1.5 h. In order to assess how fish with different thermal tolerances responded and recovered from a heat shock seven fish from both experimental groups were euthanized with an overdose of MS-222 (200 mg L^{-1}) either four hours after the heat shock (immediate effects - this time was selected in order to see effects of shock at protein level as well) or seven days after the shock (recovery effects). A blood sample was taken from the caudal vein of the fish immediately after euthanizing using heparinized syringes and ventricle and liver were dissected, cut into pieces and frozen in liquid nitrogen for further analyses.

2.4. SERCA activity

SERCA activity in the ventricles was measured according to Aho and Vornanen (1998) with minor modifications. A piece of the ventricle was homogenized in 10 volumes of homogenization buffer with Tissue Lyser (Qiagen, Hilden, Germany) with two stainless steel beads (2^*1 min with frequency of 30 s^{-1}). The activity of SERCA was determined as the difference in ATP hydrolysis in the presence and absence of SERCA-inhibitor thapsigargin ($20\text{ }\mu\text{M}$), i.e., nmol PO_4 liberated $\text{mg protein}^{-1}\text{ min}^{-1}$. Homogenates' protein concentration was analyzed with the BCA protein assay kit (ThermoFisher, Waltham, MA, USA). Inorganic phosphate concentration was determined using ammonium molybdate assay (Bonting et al., 1961). Assays were performed in triplicates at three temperatures (15 , 23 and $27\text{ }^{\circ}\text{C}$) in randomized order. Reagents were purchased from Sigma-Aldrich.

2.5. Western blotting

A small piece of ventricle and liver was used for Western blot analyses. The tissue samples were homogenized in six volumes of ice-cold homogenization buffer containing protease inhibitors with TissueLyser (Qiagen) (2^*1 min with frequency of 30 s^{-1}). The protein concentration of samples was analyzed with the BCA protein assay kit (ThermoFisher). The samples were denatured and run according to Laemmli (1970). For SERCA (ventricle), myoglobin (ventricle) and VEGF (liver) determinations, 5, 15 and $40\text{ }\mu\text{g}$ of protein were used per lane, respectively. An identical control sample was included on each gel to control for gel-to-gel variation. 5% (w/v) non-fat powdered milk was used as blocking solution. The membranes were incubated overnight at $4\text{ }^{\circ}\text{C}$ in primary antibody (1:3000 polyclonal rabbit SERCA2 antibody, ab91032, Abcam, Cambridge, UK; 1:3000 polyclonal rabbit antimyoglobin antibody, M8648, Sigma-Aldrich; 1:2000 polyclonal rabbit VEGFA antibody, ab209835, Abcam). Thereafter, membranes were incubated in 1:2000 alkaline phosphatase conjugated secondary antibody (goat Anti-Rabbit IgG H&L, ab6722, Abcam) for 1 h. Proteins were visualized by incubating the membranes in a substrate solution containing 0.17 mg mL^{-1} bromo-4-chloro-3-indolyl phosphate mono-(–toluidinium) salt and 0.49 mg mL^{-1} Nitro Blue Tetrazolium (Sigma-Aldrich). Intensities of the detected bands were analyzed with a Gel Doc XR System (BioRad) using ImageLab software (v. 6.0.). Specificities of antibodies are shown in Supplementary Fig. 1. Protein amount was expressed relative to total protein loaded into each well and normalized to the control sample. We preferred to relate the expression relative to the total protein level loaded to the gel instead to regularly used β -actin level, since environmental temperature has been shown to influence the β -actin expression (Mottola et al., 2020). The samples were run in randomized order and each gel contained fish from both groups.

2.6. LDH and CS activity

Activity of the aerobic enzyme citrate synthase (CS, EC 2.3.3.1) was analyzed from ventricle and liver samples and the activity of anaerobic enzyme lactate dehydrogenase (LDH, EC 1.1.1.27) from ventricle samples. The tissue samples were homogenized in 19 vol. homogenization buffer and assays were performed according to Dalziel et al. (2012) at

room temperature. The measurements were done with the EnSpire 2300 Multilabel Reader (Perkin Elmer, Turku, Finland). Assays were performed in triplicate for each sample at randomized order and a background reaction rate was subtracted. The concentration of protein in homogenates was analyzed with the same kit as used in other assays. The activities of enzymes were calculated per g^{-1} protein.

2.7. Histological analyses

Histological analyses were done only from fish sampled before the heat shock since short term heat shock was not expected to induce changes at structural level. Formalin-fixed gill and muscle samples were prepared according to Anttila et al. (2015). To analyze capillary density and cross section areas of muscle fibers, muscle samples were stained with amylose-periodic acid-Schiff's reagent (Andersen, 1975). For analyzing the relative myoglobin intensity, immunohistochemistry was used. The unspecific antibody binding was blocked by incubating the sections in 3% bovine serum albumin in PBS containing 0.3% Triton-X for 30 min. Thereafter the sections were incubated in primary antibody solution (1:200, polyclonal rabbit antimyoglobin antibody, M8648, Sigma-Aldrich) for 45 min (negative control sections were incubated in PBS only), washed 3 times with Triton-X-PBS and incubated in the dark in secondary antibody solution (chicken anti-rabbit IgG (H + L), Alexa Fluor® 488, Invitrogen, Eugene, OR, USA) 60 min. Finally, the sections were washed 3 times with Triton-X-PBS, rinsed with water and cover glasses were mounted with Vectashield Mounting medium (Vector Laboratories, Inc., Burlingame, CA, USA). The sections were allowed to dry for 20 min before microscopy. The gill sections were prepared according to Anttila et al. (2015) and stained with Mayer's haematoxylin (RAL Diagnostics, Martillac, France). The sections were examined under a Leica DM RXA microscope (Leica Microsystems, Wetzlar, Germany). The capillary density was measured by counting the number of capillaries in an area of $\sim 10,000 \mu m^2$ (10 different areas from red and white muscles from each individual). The cross-sectional areas of 30 muscle fibers from both red and white muscle from each individual were measured. The fluorescence intensity of myoglobin was measured with ISCapture software (version 2.6, Xintu Photonics Co, Ltd., Fujian, China) from 15 different areas both from red and white muscle of each individual. The height of gill secondary lamellae and interstitial cell mass was measured from 20 lamellae for each individual and the height of the lamellae in contact with water was calculated by subtracting the height of the interstitial cell mass from the total height of the lamellae giving an estimate for the oxygen extraction capacity from water.

2.8. Blood sample analyses

Directly after withdrawing the blood from the caudal vein into a heparinized syringe, 10 μL of blood was pipetted to tubes containing 1 mL of cyanide solution for hemoglobin analyses (0.6 mM $K_3[Fe(CN)_6]$, 0.77 mM KCN, 1.2 mM KH_2PO_4). The hemoglobin concentration was measured according to Clark et al. (2008) with EnSpire 2300 Multilabel Reader (Perkin Elmer). A portion of the blood was also collected to hematocrit capillaries and centrifuged with a Hettich hematocrit (D-7200, Andreas Hettich GmbH & Co. KG, Tuttingen, Germany) centrifuge at 13,000 rpm for 3 min for measuring the hematocrit. The rest of the blood samples was collected to 1.5 mL tubes and frozen in liquid nitrogen for mRNA analyses.

For mRNA analyses blood samples were homogenized with a TissueLyser (Qiagen) with two stainless steel beads for 2×1 min at $30 s^{-1}$ and RNA was extracted using TriReagent (Molecular Research Center, Cincinnati, USA). RNA was quantified using a NanoDrop 2000 (Thermo Scientific, Bonn, Germany) and only samples with $OD_{260/280}$ and $OD_{260/230} > 1.8$ were used in the analyses. RNA integrity was checked by bleach gel analysis (Aranda et al., 2012). RNA was digested using DNase I (Promega, Madison, USA), cleaned up with the NucleoSpin® RNA kit (Macherey-Nagel, Germany) and 250 ng RNA was reverse transcribed

using the SensiFAST™ cDNA synthesis kit (Bioline, UK) and random hexamer primers according to the manufacturer's instructions.

Using red blood cell samples, we tested all 16 qPCR primers (14 Hb genes and 2 reference genes) published by Cadiz et al. (2017). For 7 Hb genes (LA-*hba2*, MN-*hba1*, MN-*hba2*, MN-*hbb1*, MN-*hbb2*, MN-*hbb3*, MN-*hbb4*) and both reference genes (*18S*, *ef1a*) amplifications were above background level. The expression stability of the reference genes *18S* and *ef1a* was tested using NormFinder (Andersen et al., 2004) and BestKeeper (Pfaffl et al., 2004). *18S* was found to be the most stable reference gene and was subsequently used to normalize transcript levels.

The cDNA products were amplified in triplicates using the KAPA-SYBR FAST qPCR Master Mix (KAPA Biosystems) on a QuantStudio™ 12 K Flex Real-Time PCR System (Applied Biosystems, Darmstadt, Germany). Each 10 μL reaction mixture contained cDNA template corresponding to 2.5 ng RNA and the cycling protocol consisted of 20 s at $95^\circ C$ followed by 40 cycles of 1 s at $95^\circ C$ and 20 s at $60^\circ C$. In a final step, specificity of primer and amplification was evaluated using dissociation curves with a temperature range from $60^\circ C$ to $95^\circ C$. Each qPCR plate contained non-template controls to detect potential contamination in reaction mixes. Data were analyzed with the QuantStudio 12 K Flex software version 1.2. All primer pairs gave a single peak in the dissociation curve and reaction efficiency for each gene was calculated using a standard curve generated from a 1:2 serial dilution of randomly chosen and pooled samples. PCR efficiency was within the range of 92 to 106%. Calculations of relative expression levels with the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) were done in Microsoft Office Excel and the expression of target genes was normalized against *18S* expression levels.

2.9. Oxidative stress enzyme and small molecule analyses

A piece of liver was homogenized in 9 vol. of cold 100 mM K-phosphate buffer with 150 mM KCl, pH 7.4 with a TissueLyser (Qiagen) with one stainless steel bead for 2×1 min at $30 s^{-1}$. After homogenization the samples were centrifuged at $4^\circ C$, 15 min, 10,000 g. The supernatant was aliquoted to several tubes for the measurement of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GP), glutathione reductase (GR), glutathione S-transferase (GST) and glucose-6-phosphate dehydrogenase (G6PDH) activities, protein carbonylation and total reactive oxygen species (ROS) content. The tubes were frozen in liquid nitrogen and stored at $-80^\circ C$. For the measurement of total and reduced glutathione (totGSH and GSH) content an 8 μL aliquot of the supernatant was taken into a new Eppendorf tube and 24 μL of 5% sulphosalicylic acid (SSA) was added to the sample to deproteinize it. For oxidized glutathione (GSSG) measurement the volumes were 18 μL of sample and 18 μL of SSA. The samples were incubated 10 min on ice and centrifuged at $4^\circ C$, 10 min, 10,000 g. For GSSG measurement, a 10 μL aliquot was taken into a new tube and 1.2 μL of 33 mM 1-methyl-2-vinylpyridiniumtrifluoromethanesulfonate (Sigma Aldrich) was added to prevent further oxidation of GSH. For GSH measurement, all the supernatant was taken. Both tubes were frozen in liquid nitrogen and stored at $-80^\circ C$. For the measurement of lipid hydroperoxides a pre-weighed piece of liver was homogenized in 200 μL of 100% methanol with a TissueLyser (Qiagen) with one stainless steel bead for 2×1 min at $30 s^{-1}$ and centrifuged at $4^\circ C$, 10 min, 5000 g. The supernatant was aliquoted in two Eppendorf tubes, 45 μL in each, frozen in liquid nitrogen and stored at $-80^\circ C$.

The inhibition percentage of SOD, and GR, GST and CAT activities were measured with Sigma kits (19160-1KT-F, GR-SA, CS0410 and CAT100, respectively, Sigma Aldrich). The GP activity was measured with Sigma kit (CGP1-1KT, Sigma Aldrich) using 2 mM H_2O_2 as the substrate. G6PDH measurement was done according to Noltmann et al. (1961). The totGSH and GSSG were measured with ThioStar glutathione detection reagent (Arbor Assays, Ann Arbor, MI, USA) using GSH and GSSG as the standards (Sigma Aldrich) as described in the protocol of Glutathione Fluorescent Detection Kit K006-F1D (Arbor Assays). The protein carbonylation measurement was done as described in Lilley et al.

(2014). Lipid hydroperoxides were measured using the FOX-II method as described in Vuori et al. (2015). The measurement of ROS was adapted from the methods described by Socci et al. (1999), Viarengo et al. (1999) and Ferreira-Cravo et al. (2007). The method is based on the oxidation of non-fluorescent 2',7'-Dichlorofluorescein diacetate (DCFEI) to the highly fluorescent 2',7'-dichlorofluorescein (DCF). DCFEI crosses the cell membrane. In the cytoplasm esterases cleave the molecule to impermeable and fluorescent DCF. The fluorescence is a function of ROS level (Socci et al., 1999). For the measurement, samples were diluted to 1 mg mL⁻¹ with 0.1 M potassium phosphate buffer containing 0.15 M KCl, pH 7.4. DCF (410,217, Sigma Aldrich) diluted in the same buffer was used as a standard. Fifty µL of sample or standard was pipetted on black 384-well plate in quadruplicates. For the detection of autofluorescence, 5 µL of dilution buffer alone was added to one of the four samples, and to all four standard wells of a given DCF level. Five µL of 0.55 µM DCFEI (D6883, Sigma Aldrich) dissolved in dilution buffer was added to the three remaining sample wells. The plate was incubated 10 min in room temperature in dark and the fluorescence was measured at excitation wavelength of 485 nm and emission wavelength of 535 nm. Protein concentration was measured with Pierce™ BCA Protein Assay kit (ThermoFisher) using BSA as standard.

All the measurements were done with EnVision plate reader (Perkin Elmer, Turku, Finland). All the samples, standards and blanks were pipetted in quadruplicates (intra sample CV % average 2.6–6.5) in randomized order and three control samples were used in every plate to correct inter assay differences.

2.10. Glycogen assay

The glycogen content in the liver was analyzed according to Zhang (2012) using glucose (HK) assay reagent (Sigma-Aldrich). The concentrations were measured in triplicates in randomized order with EnSpire 2300 Multilabel Reader (Perkin Elmer, Turku, Finland) at 340 nm subtracting the free glucose level from the glycogen level of samples. The glycogen concentration was calculated as mmol g⁻¹ tissue.

2.11. Statistical analyses

All data are expressed as means ± SEM. Initially, data were tested for equal variances (Brown-Forsythe test) and normality (Shapiro-Wilk test) before performing a two-way ANOVA to analyze the effects of heat shock and tolerance on the parameters measured. If the data were not normally distributed, they were log-transformed. If the log-transformation did not make the data normally distributed or the variances were not equal, the non-parametric Scheirer-Ray-Hare test was used (*hbb4* mRNA level). For the parameters that were measured only from control fish either student's *t*-test or *U* test was used depending on if the data were normally distributed or not, respectively. The maximal heart rate was analyzed with repeated measures of two-way ANOVA using tolerance experimental group and measuring temperature as factors. For this statistical test only temperatures between 15 and 23 °C were used since after 23 °C majority of the poorly tolerant individuals were already removed from the setup due to cardiac arrhythmias. When significant differences were identified, a post-hoc analysis was done using the Holm-Sidak method. When there were significant differences between poorly and highly tolerant groups, we also run a Pearson correlation analysis between the variable and the CT_{MAX} temperature. All statistical analyses were performed using SigmaPlot14 (SyStat Software, San Jose, CA, USA) or SPSS (v. 24, IBM, Armonk, NY, USA) and *p* < 0.05 was considered statistically significant.

3. Results

3.1. Cardiac performance and energy metabolism of the ventricle

The cardiac performance of the groups differed significantly. First,

the fish with elevated CT_{MAX} had significantly higher RVM than fish with low thermal tolerance (0.089 ± 0.003% versus 0.072 ± 0.002%; *t* = 5.4, *p* = 0.0003; correlation between RVM and CT_{MAX} R² = 0.73, *p* = 0.0004). Second, they also had higher cardiac contraction frequency, as indicated by their significantly higher maximum heart rate during acute warming (Fig. 1A; *F* = 5.9, *p* = 0.016 tolerance as factor, *F* = 35.7, *p* < 0.001 temperature as factor and *F* = 0.18, *p* = 0.99 in interaction). Furthermore, fish with higher tolerance to elevated temperatures seemed to display cardiac arrhythmias later during the test (i.e., at higher temperature) than the thermally less tolerant animals although the difference was not quite statistically significant (27.5 ± 3.5 °C versus 24.8 ± 2.4 °C; *t* = 2.0, *p* = 0.06).

The functional measurements were reflected at molecular level as well since ventricular Ca²⁺-ATPase (SERCA) activity was higher in fish with high temperature tolerance than in fish with low thermal tolerance when activity was measured at 27 °C before the heat shock (post-hoc test, *t* = 2.1, *p* = 0.04, correlation between SERCA and CT_{MAX} R² = 0.40, *p* = 0.021, Fig. 1B, Table S1). However, this difference in SERCA activity was not significant any more after the fish had been exposed to a heat shock (*F* = 0.03, *p* = 0.86 tolerance as factor, *F* = 2.2, *p* = 0.13 heat shock as factor and *F* = 4.0, *p* = 0.027 in interaction, Fig. 1B). This difference also faded when SERCA activity was measured at lower temperatures although the activities were nearly statistically different at 23 °C (*p* = 0.075). There were no differences between experimental groups in SERCA protein levels when measured with Western blotting (*p* > 0.19) (Table S1).

Lactate dehydrogenase (LDH) activity was significantly higher in thermally tolerant individuals as compared to fish with poor thermal tolerance (*F* = 6.3, *p* = 0.02 tolerance as factor, *F* = 0.77, *p* = 0.47 heat shock as factor and *F* = 1.3, *p* = 0.28 in interaction, correlation between LDH and CT_{MAX} R² = 0.35, *p* = 0.021; Fig. 1C, Table S1). The post-hoc test revealed that this difference between experimental groups was seen especially after the heat shock (at 4 h *t* = 2.1, *p* = 0.04; at 7 d *t* = 2.1, *p* = 0.04). There were no differences in citrate synthase activity or myoglobin levels in ventricles between experimental groups before or after the heat shock (Table S1).

3.2. Oxygen extraction and carrying capacity

The length of gill secondary lamellae in contact with water did not differ significantly between the experimental groups (the lengths being 112.3 ± 9.2 and 106.2 ± 4.6 µm for fish with good and poor tolerance, respectively, *t* = 0.6, *p* = 0.57). Regarding blood oxygen transport capacity, the fish with poor thermal tolerance had higher hematocrit values than the ones with good tolerance (*F* = 4.0, *p* = 0.05 tolerance as factor, correlation between hematocrit and CT_{MAX} R² = 0.39, *p* = 0.018). Furthermore, heat shock increased the hematocrit level 7 d after the shock (*F* = 3.6, *p* = 0.04 heat shock as factor and *F* = 0.7, *p* = 0.5 in interaction, Fig. 2A, Table S2). Similarly, hemoglobin concentration increased 7 days after the heat shock in both groups. However, no difference between experimental groups was found in hemoglobin levels (*F* = 0.4, *p* = 0.50 tolerance as factor, *F* = 13.9, *p* < 0.001 heat shock as factor and *F* = 1.6, *p* = 0.2 in interaction, Table S2).

Regarding hemoglobin isoforms, fish from the thermally tolerant groups displayed significantly higher levels of MN-*hba1* and MN-*hbb1* (*p* = 0.002 and *p* = 0.001, respectively) but significantly lower level of MN-*hbb3* and MN-*hbb4* (*p* = 0.001 and *p* = 0.016, respectively) than fish from the intolerant group (Table S2 and Fig. 2B). Exposure to the heat shock resulted in a significantly increased MN-*hbb3* level 4 h after the shock (*p* = 0.004) and a similar trend was observed with regard to MN-*hba2* (*p* = 0.058). There were significant interacting effects of tolerance and heat shock on the expression of MN-*hbb2* (*p* = 0.017) so that heat shock increased the expression only in poorly tolerant fish especially 4 h after the shock (Table S2, Fig. 2B).

Thermally less tolerant fish had bigger red muscle fibers (1160 ± 131 µm² versus 853 ± 31 µm², *U* = 4.0, *p* = 0.01, Fig. 3A-C) with lower

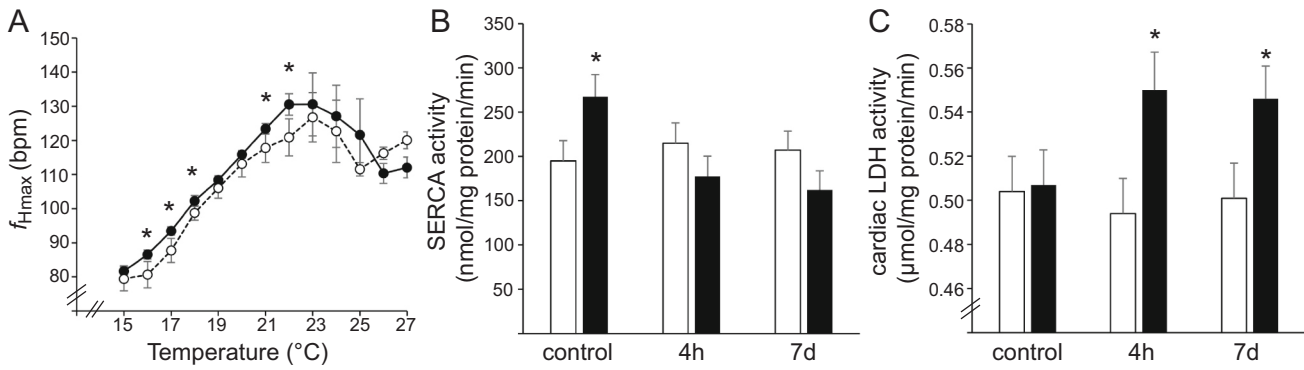


Fig. 1. The cardiac responses to temperature in individuals with poor (white symbols) and good (black symbols) temperature tolerance. A) The maximal heart rate (f_{Hmax}) of seabass to incremental warming, B) Ca^{2+} -ATPase (SERCA) activity in 27 °C assay temperature in seabass before (control) and 4 h and 7 days after a heat shock, C) lactate dehydrogenase (LDH) activity in seabass before (control) and 4 h and 7 days after a heat shock. * indicates significant differences between seabass with poor and good temperature tolerance at $p < 0.05$. For the f_{Hmax} measurements the n-value per group was 8 until 23 °C and thereafter in poorly tolerant group it was reduced to 3. In highly tolerant group the n-value was reduced to 5. The statistical comparison between groups were evaluated only until 23 °C. For the molecular analyses the n-value was 7 per group in each time-point.

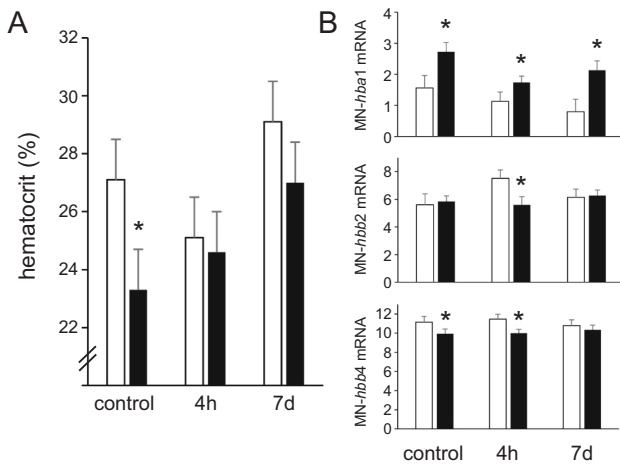


Fig. 2. The difference in blood parameters between seabass with poor (white bars) and good (black bars) temperature tolerance. A) hematocrit % in seabass before (control) and 4 h and 7 days after a heat shock and B) *MN-hba1*, *MN-hbb2* and *MN-hbb4* transcript levels in red blood cells of in seabass before (control) and 4 h and 7 days after a heat shock. The mRNA levels are relative levels normalized to *18S* mRNA. * indicates significant differences between in seabass with poor and good temperature tolerance at $p < 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

myoglobin level (127 ± 4 versus 137 ± 3 optical intensity units (oDu), $t = 2.2$, $p = 0.05$, Fig. 3D-F) than the tolerant ones. However, these variables did not correlate with individual CT_{MAX} values significantly (for fiber size $R^2 = 0.17$, $p = 0.16$ and for myoglobin $R^2 = 0.28$, $p = 0.06$). Furthermore, there were no differences in these parameters between experimental groups in white muscle (3636 ± 367 versus $3041 \pm 266 \mu m^2$, $t = 1.3$, $p = 0.21$ for size and 106 ± 4 versus 118 ± 7 oDu, $t = 1.6$, $p = 0.15$ for myoglobin in poorly versus highly tolerant fish, respectively). There were no differences in capillary-to-cell ratio (red muscle: 1.2 ± 0.1 versus 1.4 ± 0.07 capillaries per cell, $t = 1.7$, $p = 0.11$; white muscle 0.8 ± 0.06 versus 0.8 ± 0.06 capillaries per cell, $t = 0.5$, $p = 0.66$ in fish with good and poor tolerance, respectively) or in capillary density (red muscle: 14.4 ± 1.2 versus 12.9 ± 1.0 capillaries per $10,000 \mu m^2$, $t = 1.0$, $p = 0.34$; white muscle 2.7 ± 0.3 versus 2.7 ± 0.3 capillaries per $10,000 \mu m^2$, $t = 0.8$, $p = 0.45$ in fish with good and poor tolerance, respectively) between experimental groups.

Fish with poor tolerance tended to have lower level of vascular

endothelial growth factor (VEGF) in liver (group as factor $F = 3.3$, $p = 0.08$) than the more tolerant ones. The heat shock magnified the differences between experimental groups even further 4 h after the shock (Fig. 3G, heat shock as factor $F = 3.1$, $p = 0.06$, interaction $F = 0.87$, $p = 0.42$). However, the differences in VEGF were not quite statistically significant.

3.3. Oxidative stress indices and energy metabolism of liver

Thermally less tolerant fish had bigger livers than more tolerant ones ($F = 21.7$, $p < 0.001$, tolerance as factor, correlation between relative liver mass and CT_{MAX} $R^2 = 0.28$, $p < 0.001$). Furthermore, 7 days after the heat shock we observed an increase in liver mass especially in poorly tolerant individuals ($F = 3.5$, $p = 0.04$ heat shock as factor, Table S3). There were no differences between experimental groups in glycogen concentration of the liver (Table S3). However, the citrate synthase activity was higher in fish with poor temperature tolerance than in highly tolerant ones especially directly after the heat shock (Table S3, $F = 9.6$, $p = 0.004$ tolerance as factor, correlation between relative CS and CT_{MAX} $R^2 = 0.36$, $p = 0.024$).

The experimental groups had similar ROS levels, protein carbonylation and lipid peroxidation in livers (ROS, $p = 1.0$, protein carbonylation, $p = 0.57$, lipid peroxidation, $p = 0.89$). Similarly, the ratio between reduced and oxidized glutathione was similar in both experimental groups ($p = 0.54$) (Table S3). However, fish with poor temperature tolerance had higher liver glutathione peroxidase activity than fish with good temperature tolerance ($F = 23.2$, $p < 0.001$ tolerance as factor, correlation between GP and CT_{MAX} $R^2 = 0.24$, $p < 0.001$). Furthermore, the activities of the glutathione reductase ($F = 5.1$, $p = 0.031$) and glucose-6-phosphate-dehydrogenase ($F = 8.2$, $p = 0.007$) 4 h after heat shock were higher in the fish with poor than in those with good temperature tolerance (Fig. 4, Table S3). The reduced form of glutathione increased 7 days after the heat shock ($F = 6.8$, $p = 0.003$) while catalase activity was reduced 7 days after the shock ($F = 7.7$, $p = 0.002$). The group or heat shock did not affect the activities of superoxide dismutase or glutathione-S-transferase (Table S3).

4. Discussion

The main findings of this study support previous suggestions that the cardiac function plays a significant role in determining fishes' upper acute thermal tolerance (Farrell, 2009; Eliason and Anttila, 2017). The main point is the central role of heart in preserving the oxygen supply to tissues to meet the increased tissue oxygen demand caused by the temperature-driven increase in metabolic rate. Because fish are

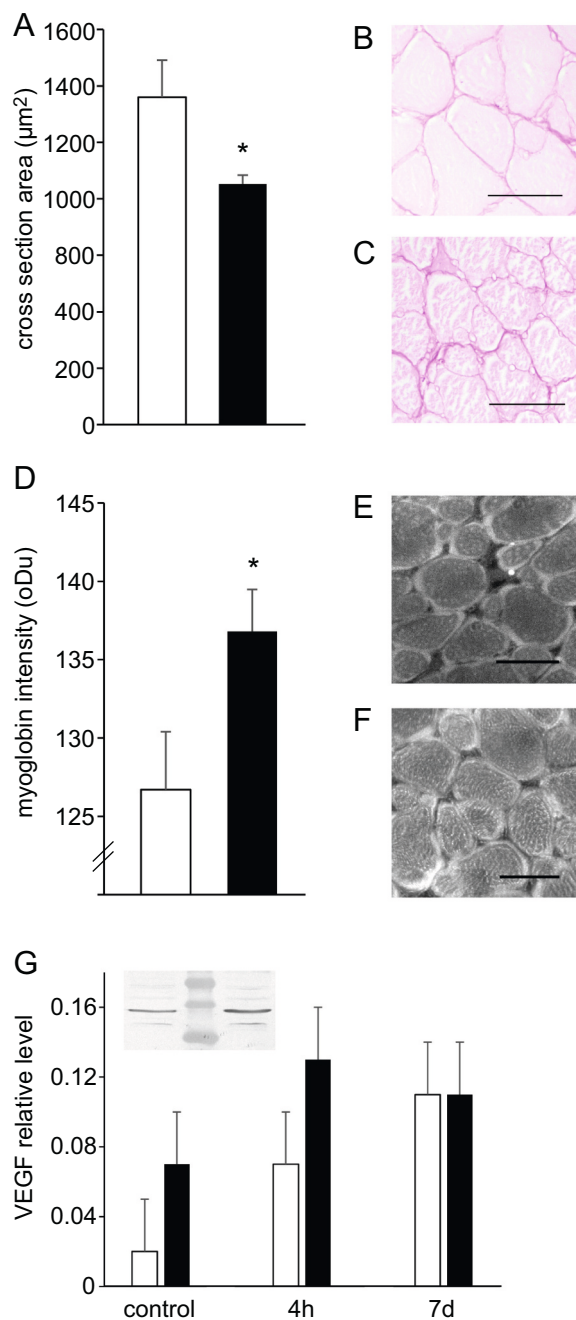


Fig. 3. A) The cross-sectional area of fibers in red muscle in seabass with poor (white bars) and good (black bars) temperature tolerance. The cross sections of the red muscle of fish tolerating high temperature B) poorly and C) well. D) The relative myoglobin level as analyzed with the fluorescence intensity in red muscle of in seabass with poor (white bars, cross section in E) and good (black bars, cross section in F) tolerance. G) the relative level of vascular endothelial growth factor (VEGF) in liver samples in seabass with poor (white bars) and good (black bars) temperature tolerance before (control) and 4 h and 7 days after a heat shock. The insert shows the Western blots of seabass with good tolerance before (first band) and 4 h after (second band) a heat shock. Between the bands is molecular weight marker with molecular weights of 50, 37 and 25 kDa. The length of the bar in microscope images is 50 μm. * indicates significant difference between seabass with poor and good tolerance at $p < 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

ectotherms, increasing water temperature increases their metabolic demand for oxygen leading to a gradual reduction of oxygen partial pressure in venous blood (Farrell, 2007; Ekström et al., 2016) with consequences on the oxygenation of all tissues, including the heart itself. Because the ventricles lack a coronary circulation (e.g. seabass), or have it only on the surface layer (e.g. salmonids), the performances of fish hearts are highly dependent upon the oxygen level in the venous blood (Axelsson, 1995; Davie and Farrell, 1991). The coronary blood supply to the ventricle seems to be especially important in high temperatures in salmonids (Ekström et al., 2019). At a temperature approaching a species' upper thermal limit, the amount of oxygen delivered to the different organs is expected to decrease, eventually leading to the death of the animal. In support of this heart-centered hypothesis of thermal tolerance, we observed that thermally tolerant fish had significantly bigger hearts (relative ventricle mass, RVM) than thermally less tolerant ones. It has been suggested that bigger heart is able to pump higher volume of blood in one stroke (Farrell, 1991; Hillman and Hedrick, 2015). This might lead to higher oxygen supply to tissues (cardiac output) in warm-tolerant individuals if the heart rate is similar between the groups. This is, however, speculative since in this study we did not measure the resting heart rate of the fish. Nevertheless, the connection between RVM and CT_{MAX} has also previously been shown in Atlantic salmon (Anttila et al., 2013), Nile perch (Nyboer and Chapman, 2018) and European seabass (Ozolina et al., 2016). However, the relationship between RVM and CT_{MAX} may depend on the size of fish since in small Atlantic salmon there was no positive correlation between RVM and CT_{MAX} (Bartlett et al., 2022). Therefore, RVM cannot be the only factor influencing CT_{MAX} .

Indeed, several other factors were connected to CT_{MAX} as well. The highly tolerant individuals displayed higher maximum heart rate values during acute warming than individuals with poor thermal tolerance. These results in general suggest that fish with both high RVM and f_{Hmax} have high upper critical thermal tolerance. Previously it has been shown that seabass with high CT_{MAX} also have high force generation capacity in their ventricles (Ozolina et al., 2016). Therefore, it seems that several aspects of the cardiac contraction are related to CT_{MAX} . At population level connections between environmental temperatures and cardiac thermal capacities have been shown in salmonids (Eliason et al., 2011; Chen et al., 2018) and at ecological level it appears that cardiac arrhythmias occur at lower temperature in higher acclimation temperatures, which can be the reason for reduced growth during warm springs in herring (Moyano et al., 2020). In the current study the seabass with high CT_{MAX} tended to have cardiac arrhythmias at higher temperatures than fish with poor thermal tolerance ($p = 0.06$). It needs to be, however, noted that in the current study the f_{Hmax} was induced pharmacologically i.e. we evaluated the absolute highest heart rate that the fish can achieve. In nature fish can control their heart rate and e.g. increasing the vagal tone at high temperatures seems to increase their thermal tolerance (Gilbert et al., 2019). This could be also the reason why there was no statistical significance between arrhythmia temperature and CT_{MAX} in the current study. Further studies should be conducted measuring both the resting heart rate and heart rate of fish swimming maximally, and how those relate to the thermal tolerance of fish.

Nevertheless, clearly the cardiac function fails when temperatures reach the upper limits of fish as seen also in current study i.e. reduction of heart rate and occurrence of cardiac arrhythmias at high environmental temperatures. There have been several suggestions why this happens from functional to biochemical level (e.g. Iftikar et al., 2014, 2015; Vornanen et al., 2014; Vornanen, 2016; Haverinen and Vornanen, 2020). To reveal which molecular mechanisms are related to CT_{MAX} and especially how these parameters change in animals with different tolerances when they are exposed to heat stress (the responses could reveal why some individuals survive heat waves and some not; natural selection could act upon these parameters) we measured parameters related to cardiac contraction (Ca^{2+} -ATPase, SERCA) and energy metabolism.

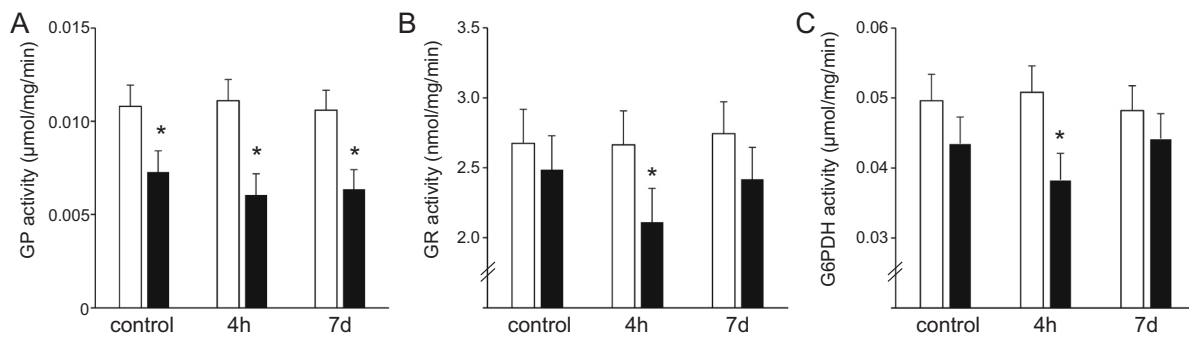


Fig. 4. The differences in the activities of enzymes involved in handling redox disturbances (oxidative stress) between seabass with poor (white bars) and good (black bars) temperature tolerance. A) glutathione peroxidase (GP), B) glutathione reductase (GR) and C) glucose-6-phosphate-dehydrogenase (G6PDH) activities in the liver samples of in seabass with poor and good tolerance. * indicates significant difference between in seabass with poor and good tolerance at $p < 0.05$.

The results showed that a temperature beyond the species' thermal optimum (24 °C; [Claireaux and Lagardère, 1999](#)) seabass with poor temperature tolerance had lower ventricular SERCA activity than thermally tolerant ones. The causal link between CT_{MAX} , f_{Hmax} and SERCA was strengthened by the observation that at assay temperatures at or below seabass optimal temperature, SERCA activity and protein level were similar in both experimental groups. SERCA is a crucial component of the machinery underlying cardiac contraction in European seabass ([Imbert-Auvray et al., 2013](#); [Ollivier et al., 2015](#); [Pettinau et al., 2022](#)). It is essential during the relaxation phase of the contraction cycle as it contributes to the pumping of calcium back into sarcoplasmic reticulum ([Bers, 2002](#)). Furthermore, cardiac SERCA activity has recently been shown to link with the maximum cardiac performance, especially maximum stroke volume ([Anttila et al., 2019](#)). Therefore, it seems that also at the cellular level the fish with high thermal tolerance were having more efficient contraction machinery, at least when it comes to SERCA.

Following an acute heat shock, SERCA activities measured at 27 °C were, however, similar between the groups. This result was unexpected as it suggests that the heat shock clearly impacted the SERCA activity of thermally tolerant fish while hardly affecting that of the less tolerant fish. The mechanistic basis of this phenomenon remains to be clarified and in particular, whether the elements responsible for the thermal resilience of SERCA in the tolerant fish are impacted by a heat shock treatment. On the other hand, the lack of effects in thermally less tolerant animals may indicate that SERCA activity in those animals is minimal and cannot be further decreased.

Interestingly, there were also differences in the activities of enzymes involved in the cardiac energy metabolism between the groups especially after the heat shock. The fish with high thermal tolerance were able to increase the LDH activity of ventricles after the heat shock while the fish with poor tolerance were not (no differences between groups before the heat shock). Since this increased activity was still visible one week post-heat shock, de novo synthesis of enzymes with different functional characteristics is likely. [Gesser and Poupa \(1973\)](#) have suggested that increased cardiac LDH activity is associated with increased lactate production in fish muscle. In heart, lactate is converted into pyruvate with LDH, avoiding accumulation of lactate in the tissues and the build-up of an oxygen debt. Thus, it seems that fish with high temperature tolerance were, first, able to utilize anaerobic metabolism more efficiently after heat shock for producing ATP (to respond to increased need for ATP due to increased metabolic rate at high temperature) and, second, to pay oxygen debt better. The utilization of anaerobic metabolism in highly tolerant fish after heat shock is supported by the observation of liver mass decreasing significantly after the heat shock in fish with good temperature tolerance while that of fish with poor temperature tolerance did not. It seems that fish with high tolerance were utilizing energy reserves more than fish with low tolerance. There were not, however, any differences between groups in aerobic enzyme citrate

synthase activity and also ventricular myoglobin levels were similar in the groups. This contrasts with previous findings in Atlantic salmon in which CT_{MAX} correlated with cardiac myoglobin level ([Anttila et al., 2013](#)). The contradicting results could be due to species differences, the salmonids relying more on the aerobic cardiac capacities (e.g. the blocking of coronary flow reduced CT_{MAX} in salmonids, [Ekström et al., 2019](#)) while seabass might rely more on anaerobic capacities especially in stressful situations ([Joyce et al., 2016](#)).

In seabass the oxygen transport chain parameters did not show clear differences between the groups. The length of gill secondary lamellae in contact with water, for example, did not differ significantly between the experimental groups, suggesting that this parameter of oxygen extraction capacity is not critical in determining CT_{MAX} . Clearly, measurements of the diffusion distance between water and blood would be required to fully ascertain this point. These results must also be considered in the light of work showing that the manipulation of water oxygen level (hyperoxia/hypoxia) has no major influence on CT_{MAX} of some fish species ([Ern et al., 2016](#)). Interestingly, fish with poor thermal tolerance displayed higher hematocrit values than the ones with good tolerance. The high hematocrit could be either due to a higher number of red blood cells or to a bigger size of the cells. The reason why the poorly tolerant fish had high hematocrit is uncertain and can only be speculated. Previous studies have, however, found that the change in hematocrit did not have a marked effect on CT_{MAX} of the seabass ([Wang et al., 2014](#)). Here the high hematocrit in fish with poor thermal tolerance could be linked, e.g., to lower threshold to release red blood cells from spleen or easier swelling of red blood cells of poorly tolerant fish or even with their lower heart rate. The lower heart rate with combination of possibly lower stroke volume due to smaller hearts in thermally less tolerant fish could mean that their systolic blood pressure might be so low that it can, speculatively, reduce red blood cell entry to capillaries. Reduced capillary entry of erythrocytes would be seen as increased hematocrit value measured in major vessels ([Nikinmaa et al., 1981](#)). The possible low blood supply to tissues will lead to endogenous hypoxia especially at high temperatures when oxygen demand increases. In many fish species endogenous hypoxia increases hematocrit ([Gallaugh and Farrell, 1998](#)) by increasing the adrenalin level which increases the production of new red blood cells and causes increased volume of the cells ([Nikinmaa, 1982](#); [Salama and Nikinmaa, 1988](#)). Poorly tolerant fish might also be more sensitive to adrenalin which would increase their red blood cell size and/or red blood cell release from spleen. This could also explain why fish with poor thermal tolerance had higher hematocrit levels and why this level increased even further with heat shock. Furthermore, we observed that groups had dissimilar expression of hemoglobin mRNAs and the differences were especially pronounced after the heat shock. Currently it is not known if the functional properties of these Hb isoforms are different ([Feng et al., 2014](#)), but since the groups with different tolerances had different translational efficiency and subunit assembly of hemoglobin there probably are some

differences in biochemical/physiological properties of isoforms as well (Feng et al., 2014). However, the role of different hemoglobin isoforms in temperature responses has not been clarified.

Histological analyses bring support to the hypothesis that fish with poor tolerance have possibly lower capacity to ensure oxygen delivery to the tissues. Although there were no differences in capillary-to-cell ratio or in capillary density between the groups, the thermally less tolerant fish had bigger myocytes with lower myoglobin level in red muscle than the tolerant ones. This suggests that the rate of oxygen diffusion to the mitochondria is decreased in the individuals with poor thermal tolerance. Although the capillary density did not differ significantly between experimental groups, the capacity for vascular formation may be lower in fish with poor than in ones with good temperature tolerance. We observed that the former tended to have lower level vascular endothelial growth factor (VEGF) in liver than the latter especially after heat shock even though the differences were not statistically significant ($p = 0.06$). However, possibly reduced oxygen diffusion efficiency to tissues cannot be a major factor influencing CT_{MAX} since we did not find statistically significant correlation between CT_{MAX} and cell size or myoglobin level even though there was tendency towards that direction.

In order to assess if the cellular damage at high temperatures could play a role in thermal tolerance (Heise et al., 2006; Madeira et al., 2013; Cheng et al., 2015) we measured parameters related to oxidative stress and enzymes activated by oxidative stress before and after a heat shock. Measurements of ROS suggest that fish with poor thermal tolerance suffered from cellular hypoxia or impaired ATP production. The fish with poor temperature tolerance displayed much higher liver glutathione peroxidase activity than fish with good temperature tolerance. This observation suggests that the former required more ATP and NADH for maintaining their redox balance in relation with their higher CS activity. Furthermore, following heat shock, the activities of the glutathione reductase and glucose-6-phosphate-dehydrogenase were higher in fish with poor than in fish with good temperature tolerance. These results indicate the possibility of a trade-off in energy allocation between redox balance and other functions, like heart rate, in fish with poor thermal tolerance. ROS production, mitochondrial function and cardiac properties may be interrelated, as Gerber et al. (2021) have observed that during acclimation to high temperatures the mitochondrial production of ROS is reduced.

As a conclusion, we provide evidence that several parameters of cardiovascular performance, from molecular to functional level, plays a decisive role in determining the upper thermal tolerance of European seabass. This conclusion can be reached, since many parameters from morphology to function (anaerobic energy metabolism, SERCA, RVM, f_{Hmax}) were related to upper thermal tolerance of the fish as determined by CT_{MAX} . Especially the capacity to utilize anaerobic cardiac performance in stress situation seems to be the key in European seabass (Joyce et al., 2016) which could save the fish from endogenous hypoxia and oxidative stress. Besides showing that the upper thermal tolerance is dependent on cardiovascular function and oxygenation capacity, we showed that there is an extremely large inter-individual variance in CT_{MAX} . The variance needs to be incorporated into the models predicting the effect of climate change, since selection happens at individual level. In future, it is important to study to what degree the thermal tolerance and its variability are heritable, as this will have significant impact on the vulnerability of species to global warming (Anttila et al., 2013).

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Ethics approval

All the experiments were approved by Ministère Délégué à

l'Enseignement Supérieur et à la Recherche.

Declaration of Competing Interest

The authors declare no conflict of interest.

Data availability

Data will be made available on request.

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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.2022.111340>.

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