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Calcium response of KCl-excited populations of ventricular myocytes from the European sea bass (*Dicentrarchus labrax*): a promising approach to integrate cell-to-cell heterogeneity in studying the cellular basis of fish cardiac performance

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Abstract :

Climate change challenges the capacity of fishes to thrive in their habitat. However, through phenotypic diversity, they demonstrate remarkable resilience to deteriorating conditions. In fish populations, inter-individual variation in a number of fitness-determining physiological traits, including cardiac performance, is classically observed. Information about the cellular bases of inter-individual variability in cardiac performance is scarce including the possible contribution of excitation-contraction (EC) coupling. This study aimed at providing insight into EC coupling-related Ca2+ response and thermal plasticity in the European sea bass (Dicentrarchus labrax). A cell population approach was used to lay the methodological basis for identifying the cellular determinants of cardiac performance. Fish were acclimated at 12 and 22 A degrees C and changes in intracellular calcium concentration ([Ca2+](i)) following KCl stimulation were measured using Fura-2, at 12 or 22 A degrees C-test. The increase in [Ca2+](i) resulted primarily from extracellular Ca2+ entry but sarcoplasmic reticulum stores were also shown to be involved. As previously reported in sea bass, a modest effect of adrenaline was observed. Moreover, although the response appeared relatively insensitive to an acute temperature change, a difference in Ca2+ response was observed between 12- and 22 A degrees C-acclimated fish. In particular, a greater increase in [Ca2+](i) at a high level of adrenaline was observed in 22 A degrees C-acclimated fish that may be related to an improved efficiency of adrenaline under these conditions. In conclusion, this method allows a rapid screening of cellular characteristics. It represents a promising tool to identify the cellular determinants of inter-individual variability in fishes' capacity for environmental adaptation.

Keywords : Sea bass, Myocyte populations, Calcium signaling, Cardiac plasticity, Inter-individual variability

1. Introduction

Coastal environments are particularly affected by contemporary climate change. Moreover, with the continuous growth of human population, agricultural practices and industrial activities are intensifying, affecting the functioning of numerous marine ecosystems (Doney 2010). These human activities-related changes in environmental conditions combine with natural fluctuations to challenge the capacity of coastal fish populations to thrive in their native habitat (Roessig et al. 2004; Brierley and Kingsford 2009). Through phenotypic diversity, however, fish populations demonstrate remarkable resilience to these deteriorating circumstances (Hofmann and Todgham 2010; Crozier and Hutchings 2014). In fish populations, broad inter-individual variation in a number of performance traits is classically observed. These include growth (Mangel and Stamps 2001), swimming (Marras et al. 2010), hypoxia and temperature tolerance (Roze et al. 2013) or food deprivation tolerance (Dupont-Prinet et al. 2010). It is now well established that phenotypic diversity has an ecological and evolutionary significance (Bennett 1987; Vandamm et al. 2012; Claireaux et al. 2013) and that it is based on underlying functional trade-offs which allow various phenotypes to co-exist with equivalent fitness (Mangel and Stamps 2001).

The cardiovascular system plays a major role in fish environmental adaptation as it allows aligning energy production and allocation with environmental demand (Farrell et al. 2009). As for other major fitness-determining physiological traits, fish also display extensive within-population diversity in heart performance (Gamperl and Farrell 2004). In rainbow trout (*Oncorhynchus mykiss*) for instance, individual variation in heart morphology and capacity to generate force has been observed and was linked to variation in swimming performance (Claireaux et al. 2005).

Whereas the mechanisms involved in the acclimation of the cardiac muscle to different working conditions have been relatively well studied in teleosts (*e.g.*, McClelland et al. 2005; Castro et al. 2013; Anttila et al. 2014), information about the cellular and sub-cellular bases of inter-individual variation in heart performance is scarce. Chatelier et al. (2006) have shown in the European sea bass (*Dicentrarchus labrax*) that dietary fatty acids influenced swimming performance and that this effect correlated with changes in cardiac performance. Unfortunately, lack of information at myocyte level only allowed authors to speculate about the cellular mechanisms involved. They mentioned cellular and mitochondrial membrane lipid composition, as well as membrane-bound proteins. In this regard, they pointed out that unsaturated fatty acids protected the heart from arrhythmia at high workloads by preventing Ca^{2+} overload through an inhibitory effect on L-type Ca^{2+} current.

The excitation-contraction (EC) coupling is a key component of myocardium contraction (Vornanen et al. 2002a). Excitation-contraction coupling is a sequence of events that links the triggering of an action potential (AP; excitation) to the Ca^{2+}-mediated activation of cardiomyocytes' myofilaments (contraction). In fish, EC coupling has been studied mainly in rainbow trout (Hove-Madsen and Tort 1998; Shiels et al. 2002), tuna (*Thunnus albacares*; Galli et al. 2009), crucian carp (*Carassius carassus*) and burbot (*Lota lota*; Haverinen and Vornanen 2009), with particular attention to the effect of temperature. These studies particularly demonstrated the contribution of ion currents to the thermal acclimation of fish heart (Hove-Madsen and Tort 1998; Vornanen et al. 2002b; Haverinen and Vornanen 2004). However, the possible contribution of EC coupling to inter-individual variation in myocardium performance is unknown.

Available information about EC coupling has been essentially gathered on single-cells, using methodologies such as patch clamping and confocal imaging. Whereas these studies contributed to the fine scale description of the shape and time-course of the mechanisms involved, they did not allow the integration of this comprehension at organ level. For that,

information about cellular heterogeneity is required since, to some degree, cell-to-cell difference is always present in populations of "seemingly identical" cells (Altschuler and Wu 2010). It is believed that inter-cell phenotypic variability results from intrinsic factors (cellular components and chemistry) as well as extrinsic factors (cell microenvironment; Loewer and Lahav 2011). As shown in mammals (*e.g.*, Rathi et al. 2004), a cell population approach allows the integration of this diversity in cellular level responses and can provide a more accurate model of myocardium functioning. It should therefore be particularly relevant in identifying the cellular determinants of cardiac performance and their contribution to inter-individual variability in fishes' capacity for environmental adaptation.

The aim of the present work was to provide insight into EC coupling related-Ca^{2+} response and thermal phenotypic plasticity in sea bass cardiomyocytes. The study was conducted using cell populations to test an experimental approach allowing the investigation of the cellular basis of cardiac performance in fish.

2. Materials and methods

2.1. Fish

European sea bass (*Dicentrarchus labrax*; mean mass 205 ± 15 g, mean length 26 ± 1 cm) were obtained from a local fish farm (Aquastream, Lorient, France) and held in 400 L tanks supplied with recirculating, thermoregulated and biofiltered sea water (salinity 20-30 ‰). To ensure proper housing conditions, 30 % of the water was renewed every week. Fish were exposed to natural photoperiod and were fed twice a week with commercial dry pellets (Le Gouessant, Lamballe, France). Following acclimation to the laboratory conditions, fish were randomly divided among two experimental groups and maintained at either 12 °C (n= 11; 12.0 ± 0.1 °C) or 22 °C (n= 14; 21.9 ± 0.1 °C) for at least 3 weeks prior to experimentation. This experiment complied with all applicable international, national and institutional guidelines and ethical standards for the care and use of animals.

2.2. Ventricular myocyte isolation

Ventricular myocytes were isolated using a modified version of the protocol reported in Vornanen (1997). Fish were killed by a sharp blow on the head and the heart was quickly excised and the atrium removed. A 20-gauge cannula was then inserted through the bulbus arteriosus into the ventricle. The cannula was connected to a Langendorff apparatus and the heart placed in a petri dish on ice. The heart was first perfused during 12 min (1.7 mL/min) with a cooled and aerated isolation solution (in mM: NaCl 120, KCl 3.4, $MgSO_4$ 0.8, Na_2HPO_4 0.33, KH_2PO_4 0.44, $NaHCO_3$ 5, Hepes 10, Taurine 30, Glucose 10, BDM (2,3-Butanedione monoxime) 10, Creatine 5, L-Carnitine 2; pH7.63) containing Heparin (60 U/mL) and Tris-EGTA (0.4 mM). At the end of this 12-min period, the heart was perfused during 50 min (0.4 mL/min) with the same isolation solution added with bovine serum albumin BSA (0.3 mg/mL; Sigma Ref. A6003), Collagenase type IV (0.3 mg/mL; Sigma Ref. C5138), Collagenase type IA (0.3 mg/mL; Sigma Ref. C9891), Trypsine type XI-S (0.3 mg/mL; Sigma Ref. T0303) and $CaCl_2$ (0.3 mM). At the end of this second perfusion period, the cannula was removed and the bulbus arteriosus was excised. The ventricle was then gently triturated in cooled isolation solution containing $CaCl_2$ (0.3 mM) using a plastic pipette. After gravity sedimentation, the pellet, which mainly contained the rod-shaped, viable myocytes was retained while the supernatant, including most of the round-shaped, non-viable myocytes was discarded. The pellet was resuspended in isolation solution with $CaCl_2$ (0.3 mM) and cells were allowed to rest for at least 40 min at 4 °C. At the end of the resting period, ventricular myocytes were placed in Hank's balanced salt solution HBSS (in mM: NaCl 152,

KCl 3.4, $MgSO_4$ 0.8, Na_2HPO_4 0.33, KH_2PO_4 0.44, $NaHCO_3$ 5, Hepes 10, Glucose 10, $CaCl_2$ 1.2; pH7.63) containing BDM (10 mM) to protect cells from hypercontracture caused by the reintroduction of Ca^{2+} (calcium paradox). The cells were allowed to rest for an additional 40 min at 4 °C. At the end of the resting period, the cells were resuspended in HBSS and held on ice.

Visual observation of cell suspension showed the predominant presence of quiescent rod-shaped (viable) myocytes (85 - 90 %). Viability tests were performed using lactate dehydrogenase (LDH) cytotoxicity assay (Sigma Ref. TOX7).

2.3. Measurement of intracellular calcium concentration $[Ca^{2+}]_i$ using Fura-2

Isolated ventricle myocytes (~0.5 10^6 cells mL^{-1}) were incubated in the dark with 5 µM Fura-2 AM (final concentration) for 30 min. During the first 20 min of the loading period, the cells were gently agitated (50 rpm) whereas during the last 10 min they were allowed to settle. At the end of the loading period, cells were washed to remove any extracellular dye and an additional resting period of 10 min was allowed for deesterification. At the end of the deesterification period, samples of cell suspension were placed in semi-micro UV grade polymethylmethacrylate cuvettes for fluorescence measurements.

Changes in fluorescence intensity were monitored using a dual wavelength spectrofluorometer (FP-6200 Jasco) set at 340 and 380 nm excitation wavelengths and 510 nm emission wavelength. The spectrofluorometer was equipped with a cuvette chamber connected to a thermostatic bath (HUBER Polystat CC1) to control the test temperature. The spectrofluorometer was connected to a computer *via* data acquisition software Spectra manager. For each sample, two spectra were recorded:

- A first spectrum was recorded (100 s) to measure basal fluorescence intensity of resting myocytes.

- A second spectrum was recorded (400 s) immediately after addition of high KCl solution (in mM: NaCl 3.4, KCl 152, $MgSO_4$ 0.8, Na_2HPO_4 0.33, KH_2PO_4 0.44, $NaHCO_3$ 5, Hepes 10, Glucose 10, $CaCl_2$ 1.2; pH7.63) into the cell medium (17 mM final KCl concentration). To minimize mechanical shocks and resulting cell contractures and death, cell suspensions were not stirred during the measurements. However, cuvettes were gently turned upside down two to three times just after the addition of KCl solution to partially address the precipitation of cells.

For each cell suspension, autofluorescence estimated from non-loaded cells at both 340 and 380 nm was subtracted from fluorescence values measured on loaded cells at 340 nm and 380 nm respectively.

Intracellular calcium concentration ($[Ca^{2+}]i$) was estimated from the Grynkiewicz equation

(Grynkiewicz et al. 1985):

$$[Ca^{2+}]i\ (nM) = Kd \times \left(\frac{R-R_{min}}{R_{max}-R}\right) \times \left(\frac{Sf_2}{Sb_2}\right),$$

where Kd is the effective dissociation constant, R is the ratio between the fluorescence intensity at 340 nm and the fluorescence intensity at 380 nm (F340/F380), R_{max} is the F340/F380 ratio measured under saturating Ca^{2+} conditions following the addition of 0.1 % Triton X (final concentration), R_{min} is the F340/F380 ratio measured under zero Ca^{2+} conditions following the addition of 10 mM Tris-EGTA (final concentration) and Sf_2/Sb_2 is the

ratio between the fluorescence intensity at 380 nm under zero Ca^{2+} conditions (free) and the fluorescence intensity at 380 nm under saturating Ca^{2+} conditions (bound).

The values of Kd for Fura-2 have been measured *in vivo* over a range of temperatures (5 - 37 °C) in enterocytes of Atlantic cod (*Gadus morhua*) by Larsson et al. (1999). To take into account our experimental conditions (pH, temperature, ionic strength), Kd values from Larsson et al. (1999) were adjusted (Shiels et al. 2002) using the Maxchelator software (C. Patton, Stanford University, Pacific Grove, CA, USA). Accordingly, our Kd values were 389 nM and 346 nM at 12 °C and 22 °C respectively. R_{max} and R_{min} were calculated using non-linear regression (Microsoft Excel Add-in Solver).

2.4. KCl-induced Ca^{2+} response of myocyte populations and calcium handling experiments

This set of experiments was conducted at the test temperature of 12 °C on myocyte populations from 12 °C-acclimated sea bass.

The effects of increasing KCl concentration in the stimulation solution ($[KCl]_{stim}$: 12 mM, 17 mM, 22 mM and 27 mM final concentrations) on the Ca^{2+} response were tested by comparing times to 50 % maximal response (T_{50}). Times to 50 % maximal response (T_{50}) were calculated using non-linear regression (Microsoft Excel Add-in Solver). Equilibrium potentials for potassium ions calculated from Nernst equation (at 12 °C test) are -63 mV (12 mM KCl), -54 mV (17 mM KCl), -47 mV (22 mM KCl) and -43 mV (27 mM KCl) *versus* -93 mV in resting conditions (3.4 mM KCl).

The effects of antagonists of extra- and intracellular Ca^{2+} were also examined. Loaded cells were incubated during 30 min with L-type Ca^{2+} channel blocker nifedipine (10 µM final concentration) or with nifedipine plus sarcoplasmic reticulum (SR) Ca^{2+} store blockers ryanodine (10 µM final concentration) and thapsigargin (2 µM final concentration).

Finally, the effect of increasing adrenaline concentrations on the Ca^{2+} response was measured. Adrenaline was added to the cell medium a few seconds prior to spectrofluorometric measurements. Adrenaline concentrations 0 M, 10^{-12} M, 10^{-9} M and 10^{-6} M were tested.

2.5. Thermal acclimation experiments

The effect of thermal acclimation on Ca^{2+} response was also examined using myocyte populations issued from 12 °C-acclimated (n= 11) and 22 °C-acclimated (n= 14) sea bass.

Some myocyte populations (n= 4 at 12 °C acclimation and n= 7 at 22 °C acclimation) were tested in the presence of AD 10^{-9} M corresponding to the tonic adrenaline concentration in the circulation of resting fish. Others (n= 7 at 12 °C acclimation and n= 7 at 22 °C acclimation) were tested in the presence of AD 10^{-6} M corresponding to the adrenaline concentration under condition of extreme stress (Shiels and Farrell 1997).

All myocyte populations were tested at both test temperatures 12 °C and 22 °C. At each test temperature, control (average of n= 4 measurements at a given temperature) and ryanodine/thapsigargin experiments (average of n= 4 measurements at a given temperature) were performed. When the effects of ryanodine (10 µM) and thapsigargin (2 µM) were tested, cells were incubated in the presence of the agents for 30 min prior to measurements.

2.6. Chemicals

All chemicals were purchased from Sigma-Aldrich except Fura-2 AM and ryanodine which were purchased from Invitrogen (Ref. F1221) and Merck (Ref. 559276) respectively. The stock solution of ryanodine was prepared in HBSS whereas stock solutions of nifedipine (Sigma Ref. N7634) and thapsigargin (Sigma Ref. T9033) were prepared in Dimethyl sulfoxide (DMSO). When DMSO was the agent vehicle, control experiments were performed by adding the same volume of DMSO. Stock solutions of adrenaline (Sigma Ref. E4250) were prepared extemporaneously in 1N HCl to ensure the complete solubility and then diluted in HBSS.

2.7. Statistical analysis

Results are given as means ± SEM. Statistically significant differences ($p < 0.05$) were assessed using analysis of variance (ANOVA) followed by Tukey's *post hoc* test after checking normality of distribution and homeoscedacity. When necessary, $[Ca^{2+}]_i$ increases were log-transformed. All statistical analyses were performed using Statistica (Statsoft).

3. Results

3.1. Effect of $[KCl]_{stim}$ on the Ca^{2+} response of myocyte populations

The depolarization of ventricle myocyte populations (12 °C acclimation; 12 °C-test) using high KCl solution (17 mM) induced a rapid increase in F340/F380 ratio (Fig. 1), indicating an increase of intracellular calcium concentration ($[Ca^{2+}]_i$). This was followed by a plateau which was maintained for at least 400 s (end of our measuring period). The amplitude of the increase in $[Ca^{2+}]_i$ was not affected by $[KCl]_{stim}$ ($p > 0.05$). At 12 °C-test, increases in F340/F380 ratio were 1.93 ± 0.44, 1.96 ± 0.37, 1.86 ± 0.25, 2.01 ± 0.43 at 12, 17, 22 and 27 mM KCl, respectively. At 22 °C-test, increases in F340/F380 ratio were 1.64 ± 0.20, 1.96 ± 0.16, 2.20 ± 0.18, 2.18 ± 0.22 at 12, 17, 22 and 27 mM KCl, respectively. Test temperature had a significant effect on time to 50 % maximal response (T_{50}; $p = 0.017$; Fig. 2). The rise in $[Ca^{2+}]_i$ was slower when experiments were conducted at a test temperature of 12 °C compared to 22 °C. The rate of the $[Ca^{2+}]_i$ increase was also affected by KCl concentration in the stimulation solution. At $[KCl]_{stim}$ of 12 mM, Ca^{2+} response was found to be slower than at the other $[KCl]_{stim}$. However, further analysis revealed that this $[KCl]_{stim}$-related effect was statistically significant at 12 °C-test temperature but not at 22 °C. On the basis of the above, and to minimize risk of cell damage, a 17 mM KCl solution was used as a stimulation medium in all following experiments.

3.2. Calcium handling

As for the study of the effect of $[KCl]_{stim}$ on the Ca^{2+} response of myocyte populations, the study of calcium handling was conducted on 12 °C-acclimated fish at 12 °C-test. Following a 30-minute incubation with sarcolemmal L-type Ca^{2+} channel blocker nifedipine (10 µM), the increase in $[Ca^{2+}]_i$ was reduced by more than 60 % compared to the corresponding control (Fig. 3). The decrease in Ca^{2+} signal was not related to cell mortality as attested by LDH test ($p= 0.390$). When depolarization was performed in the presence of nifedipine (10 µM) combined with SR Ca^{2+} store blockers ryanodine (10 µM) and thapsigargin (2 µM), the Ca^{2+} response (increase in $[Ca^{2+}]_i$) was reduced by more than 75 % compared to the corresponding control. Note that ryanodine and thapsigargin had a significant impact on cell viability ($p= 0.036$). Since we used a ratiometric method, this significant cell mortality had, however, a limited impact upon our assessment of $[Ca^{2+}]_i$. Ratiometric indicators (including

Fura-2 dye) have the particular advantage of allowing the distinguishing between the Ca^{2+}-bound and Ca^{2+}-free forms of the dye through a shift in their fluorescence spectra as they bind with Ca^{2+}. The use of the ratio between the Ca^{2+}-bound and Ca^{2+}-free fluorescence intensities allows correction of artefacts resulting from changes in indicator concentration, cell number, cell volume, variations in laser intensity and bleaching. As these factors affect the signal equally at both wavelengths, they indeed cancel out when calculating the fluorescence ratio (Bruton et al. 2012).

Intracellular Ca^{2+} response was not significantly different when adrenaline concentration in the cellular medium was increased from 0 to 10^{-12} M and then to 10^{-9} M (Fig. 4). In the presence of the highest adrenaline concentration (10^{-6} M), the increase in $[Ca^{2+}]_i$ was significantly larger than that observed for the two lowest adrenaline concentrations (0 and 10^{-12} M).

3.3. Thermal acclimation

A significant difference between the two acclimation groups was observed (p= 0.019; Fig. 5). Overall, the increase in $[Ca^{2+}]_i$ measured in myocyte populations from 22 °C-acclimated fish was higher than that measured in myocyte populations from 12 °C-acclimated fish. The difference between the acclimation groups was mainly due to a greater effect of the highest level of adrenaline on the Ca^{2+} response of the 22 °C-acclimated fish. When comparing the controls AD 10^{-9} M *vs* AD 10^{-6} M within each acclimation condition, a significant difference was indeed observed in 22 °C-acclimated fish (p = 0.036) but not in 12 °C-acclimated fish (p = 0.357). The KCl-induced increase in $[Ca^{2+}]_i$ was generally less pronounced in the presence of SR Ca^{2+} store blockers ryanodine (10 µM) and thapsigargin (2 µM) compared to controls. Note that the interaction AD concentration X conditions (control or Ryano/Thaps) was found significant (p = 0.020), with a larger difference between control and Ryano/Thaps conditions observed under a high level of adrenaline (AD 10^{-6} M). Finally, regardless of experimental conditions, the test temperature (12 °C *vs* 22 °C) did not significantly modulate the Ca^{2+} response.

4. Discussion

The aim of the present work was to provide insight into EC coupling related-Ca^{2+} response and thermal phenotypic plasticity in the European sea bass. A cell population approach was used to lay the methodological basis for identifying the cellular determinants of individual variation in cardiac performance. We showed that the increase in $[Ca^{2+}]_i$ that rapidly occurs following KCl stimulation is mainly due to extracellular Ca^{2+} entry and, to a lesser extent, to Ca^{2+} release from intracellular SR stores. Moreover, we observed that compared to some other fish species, the response was relatively insensitive to adrenaline. Moreover, even though the Ca^{2+} response of sea bass ventricular myocytes appeared relatively insensitive to acute temperature change, a difference in Ca^{2+} response was observed between 12 °C- and 22 °C-acclimated fish. In particular, a greater increase in $[Ca^{2+}]_i$ at a high level of adrenaline was observed in 22 °C-acclimated fish that may be related to an improved stimulatory efficiency of adrenaline under these acclimation conditions.

The sustained depolarization of cell populations using high KCl solution induced a typical biphasic Ca^{2+} signal. First, the stimulation triggered a rapid increase in $[Ca^{2+}]_i$ (sharp rise) that was then maintained during at least 400 s (plateau phase). This response pattern has frequently been reported in various cell types *e.g.,* PC12 cells (Di Virgilio et al. 1987), rat vascular smooth muscle cells (Stepien and Marche 2000), rat cardiomyocytes (Rathi et al. 2004) or GH3 cells (Vela et al. 2007). This pattern has also been observed at higher

organization levels as reflected in the "phasic and tonic" components of the KCl-induced contracture response in various tissues *e.g.*, rat *vas deferens* smooth muscle (Langton and Huddart 1988), mouse aortic segments (Fransen et al. 2012) or guinea-pig ureter (Maggi and Giuliani 1995). The steady-state of the Ca^{2+} signal observed from about 200 s post stimulation onward could be explained by the existence of two populations of L-type Ca^{2+} channels (Fransen et al. 2012). Whereas a first population is rapidly inactivated during depolarization, a second population is only partly inactivated due to the KCl-induced sustained depolarization, resulting in a sustained non-inactivating Ca^{2+} influx (Fransen et al. 2012). Although the shape and dynamics of the Ca^{2+} signal obtained in the present work are in accordance with previous reports, KCl stimulus *i.e.*, sustained membrane depolarization, does not allow the investigation of Ca^{2+} removal pathways which return $[Ca^{2+}]_i$ to resting levels. Future experiments using electrical field stimulation are envisaged to examine this point.

We tested the effects of increasing $[KCl]_{stim}$ upon the intracellular Ca^{2+} response. The amplitude of KCl-induced increase in fluorescence ratios (indicative of $[Ca^{2+}]_i$) was not different between stimulation conditions. The velocity of the response, however, was influenced by $[KCl]_{stim}$. At 12 mM the rate of the response was half that observed at the other concentrations (At 12 °C-test: 99 ± 12 s *vs* 40 ± 4 s, 42 ± 11 s and 35 ± 8 s; At 22 °C-test: 30 ± 6 s *vs* 11 ± 1 s, 6 ± 1 s and 7 ± 1 s). This suggests that at 12 mM, membrane depolarization is possibly insufficient to activate all L-type Ca^{2+} channels as these are high voltage-activated channels. At concentration ≥ 17mM, on the other hand, full activation of these channels was observed. It is important to note, however, that this $[KCl]_{stim}$-related effect was found significant at 12 °C-test but not at 22 °C-test. The fact that the response to stimulation with 12 mM KCl solution was faster at 22 than at 12 °C-test may be explained by the fact that the reduced number of channels in open state is compensated for, at 22 °C, by faster Ca^{2+} influx.

After incubation of myocyte populations with L-type channel blocker nifedipine (10 µM), the increase in $[Ca^{2+}]_i$ was reduced by more than 60 % compared to the control. This is in line with the view that in fish, Ca^{2+} transient results primarily from extracellular Ca^{2+} entry (Tibbits et al. 1992; Keen et al. 1994; Shiels and Farrell 1997; Hove-Madsen and Tort 1998). However, incubation of cellular populations with nifedipine (10 µM), ryanodine (10 µM) and thapsigargin (2 µM) indicated that intracellular Ca^{2+} store from the SR is also involved. It is however important to keep in mind that since cells were kept quiescent before the stimulation by KCl, it is likely that the observed contribution of SR Ca^{2+} stores is overestimated in comparison to what would have been observed if myocytes had been paced, using electrical field stimulation for instance. The post-rest potentiation of myocyte responses has indeed been previously reported in fish (Tiitu and Vornanen 2002) and it has been attributed to a larger release of Ca^{2+} from the SR occurring at the first post-rest stimulation. Additionally, in the experimental condition where L-type channels, ryanodine receptors and SERCA pumps were all inhibited, we noted that the Ca^{2+} signal was not totally blocked. This finding indicates that an additional process may be involved in Ca^{2+} mobilization, such as sarcolemmal Na^+/Ca^{2+} exchanger (NCX; Vornanen 1999; Hove-Madsen et al. 2000).

Adrenergic stimulation of the heart is mainly mediated by β-receptors located on the cell surface. The β-binding of adrenaline induces the activation of the α-subunit of a stimulatory G-protein which in turn causes the activation of adenylyl cyclase. Resulting increase in cyclic adenosine monophosphate (cAMP) concentration causes the activation of protein kinase A (PKA) which phosphorylates L-type channels. This leads to the channel potentiation with the amplification of Ca^{2+} influx and subsequent increase in the contraction force. The positive inotropic effect of adrenaline has been reported in various fish species including rainbow trout (Graham and Farrell 1989; Keen et al. 1993; Gamperl et al. 1994), eel (*Anguilla dieffenbachii*; Franklin and Davie 1992) or tuna (Galli et al. 2009). In the present study, we did not observe a substantial positive effect of adrenaline on Ca^{2+} response, only the highest

adrenaline level (10^{-6} M) significantly increasing $[Ca^{2+}]_i$ above control level (0 M). The modest inotropic effect of adrenaline in sea bass has previously been reported by Farrell et al. (2007) as well as in a few other fish species such as winter flounder (*Pleuronectes americanus*; Mendonça and Gamperl 2009), Atlantic cod (*Gadus morhua*; Lurman et al. 2012) or tilapia (*Orechromis* hybrid; Lague et al. 2012). According to Farrell et al. (1996), explanation could be that sensitivity to adrenaline decreases at species optimum temperature (22 °C for sea bass; Claireaux and Largardère 1999). Our own results are, however, not in line with this hypothesis as calcium response appeared more AD-sensitive in 22 °C-acclimated than in 12 °C-acclimated fish. These seemingly inconsistent observations must, however, be considered carefully as Farrell et al. (1996) investigated the integrated response of *in situ* perfused heart preparations while our own experiment targeted a single cellular mechanism *i.e.*, intracellular Ca^{2+} mobilization. The possibility cannot be excluded that acclimation conditions influence other components of the contractile machinery such as the Ca^{2+} sensitivity of force generation, the rate of cardiac actomyosin ATPase or the phosphorylation of cardiac troponins (Klaiman et al. 2014), resulting in a different integrated response.

Quite clearly, additional experiments will be needed to clarify the role of adrenaline in setting the cardiac performance of sea bass. Among the issues that need to be explored is the effect of thermal acclimation on cardiac adrenoceptor density. In rainbow trout, a higher β-adrenoceptor density was observed in isolated sarcolemmal fractions of ventricles from fish acclimated to 8 °C compared to fish acclimated to 18 °C (Keen et al. 1993). It would also be very useful to assess whether β3-receptors are present in sea bass myocytes and how they may mediate the action of catecholamines. β3-receptors are activated at higher catecholamine concentrations than β1 and β2-receptors (Strosberg 1997; Gauthier et al. 2007) and they are believed to be involved in protecting the heart from overstimulation by catecholamine through a negative inotropic effect mediated by nitric oxide synthesis (Gauthier et al. 1998; Imbrogno et al. 2006; Angelone et al. 2008; Niu et al. 2012). Accordingly, the presence of these receptors, in addition to β1- and β2-receptors, in myocyte membrane has been proposed to explain the modest inotropic effect of adrenaline in some fish species. However, this remains to be verified. In addition, the density of the L-type Ca^{2+} current (I_{Ca}) should be examined. The L-type Ca^{2+} current has been shown to be insensitive to thermal acclimation in rainbow trout cardiac myocytes (Vornanen 1998; Tiitu and Vornanen 2003). At first glance, our experiments with SR blockers suggest that similar patterns may be found in sea bass as the signal strictly resulting from extracellular Ca^{2+} entry was unchanged between acclimation conditions. However, even though the Ca^{2+} influx is mainly due to the activation of L-type channels, it can also occur through the NCX, especially in cold-acclimated fish. In cold-acclimated fish, the rate of the inactivation of voltage-gated Na^+ channels is reduced and the density of sarcolemmal Na^+ current (I_{Na}) is increased, contributing to increased Na^+ influx (Haverinen and Vornanen 2004). It could be hypothesized that this increased Na^+ influx could stimulate the reverse mode of NCX, augmenting Ca^{2+} influx through this way. Obviously, the relative contribution of L-type channels and NCX needs to be fully assessed before determining the actual contribution of adrenaline to the full Ca^{2+} response.

Additional information on the impact of both adrenaline and thermal acclimation on the functioning of the SR is also required. Reticulum sarcoplasmic activity mainly consists in Ca^{2+} releasing in response to Ca^{2+} influx (Ca^{2+}-induced Ca^{2+} release CICR) and cytosolic Ca^{2+} reloading. Our results showed that a high level of adrenaline (AD 10^{-6} M) significantly increased the difference between the Ca^{2+} response measured in control condition and that measured in the presence of SR blockers. This may indicate a greater involvement of the SR in the Ca^{2+} response under demanding conditions, as it has been recently suggested in rainbow trout heart (Cros et al. 2014). Moreover, our results obtained under different acclimation conditions may reflect the thermal plasticity of the SR. It has been demonstrated in cold-active teleost species such as rainbow trout (Keen et al. 1994; Aho and Vornanen 1999) or perch (*Perca fluviatilis*; Bowler and Tirri 1990) that activity of the SR was enhanced

with cold acclimation. In particular, the expression and activity of SR Ca^{2+}-pumps were shown to increase in rainbow trout, resulting in enhanced Ca^{2+} sequestration by the SR (Keen et al. 1994; Aho and Vornanen 1998; Korajoki and Vornanen 2013). As we mixed ryanodine and thapsigargin to block CICR and SR loading respectively, our results did not allow distinguishing the two processes. However, it cannot be excluded that the lowest Ca^{2+} response observed in 12 °C-acclimated fish reflects an increased Ca^{2+} loading rate of the SR.

The less marked increase in $[Ca^{2+}]_i$ observed in myocyte populations from the 12 °C-acclimated fish compared to the 22 °C-acclimated fish ($p = 0.019$) is consistent with Imbert-Auvray et al. (2013) who reported, using single cells, lower amplitude of Ca^{2+} responses in 10 °C-acclimated sea bass compared to 20 °C-acclimated individuals. On the other hand, and consistent with our own results, these authors did not observe any significant effect of the test temperature. Sea bass are known to be highly eurythermic as they naturally occur at temperatures ranging from ~ 6 to 30 °C (Claireaux and Lagardère 1999). The present result suggests that within that thermal range, acute temperature changes do not affect significantly the capacity of myocytes to mobilize Ca^{2+}.

In conclusion, this work provides insight into EC coupling related-Ca^{2+} response and thermal phenotypic plasticity in the European sea bass. This investigation was conducted using a novel approach in fish as it involved populations of cardiac myocytes. This method offers the advantage of avoiding uncertainties associated with cell-to-cell heterogeneity while maintaining the ability to collect information on cellular events. It allows a rapid and global screening of cellular characteristics of individuals and therefore provides a promising tool to investigate the cellular basis of inter-individual variability in fish cardiac performance.

References

Aho E, Vornanen M (1998) Ca^{2+}-ATPase activity and Ca^{2+} uptake by sarcoplasmic reticulum in fish heart: effects of thermal acclimation. J Exp Biol 201:525–532.

Aho E, Vornanen M (1999) Contractile properties of atrial and ventricular myocardium of the heart of rainbow trout *Oncorhynchus mykiss*: effects of thermal acclimation. J Exp Biol 202:2663–2677.

Altschuler SJ, Wu LF (2010) Cellular heterogeneity: when do differences make a difference? Cell 141:559-563.

Angelone T, Filice E, Quintieri AM, Imbrogno S, Recchia A, Pulerà E, Mannarino C, Pellegrino D, Cerra MC (2008) Beta3-adrenoceptors modulate left ventricular relaxation in the rat heart via the NO-cGMP-PKG pathway. Acta Physiol Oxf Engl 193:229–239.

Anttila K, Jorgensen SM, Casselman MT, Timmerhaus G, Farrell AP, Takle H (2014) Association between swimming performance, cardiorespiratory morphometry, and thermal tolerance in Atlantic salmon (*Salmo sala L.*). Front Mar Sci 1:76.

Bennett AF (1987) Interindividual variability: an underutilized resource. In: Feder ME, Bennett AF, Burggren WW, Huey RB (eds) New Directions in Ecological Physiology. Cambridge University Press, pp 147-169.

Bowler K, Tirri R (1990) Temperature dependence of the heart isolated from the cold or warm acclimated perch (*Perca fluviatilis*). Comp Biochem Physiol A Physiol 96:177–180.

Brierley AS, Kingsford MJ (2009) Impacts of climate change on marine organisms and ecosystems. Curr Biol 19:R602–R614.

Bruton JD, Cheng AJ, Westerblad H (2012) Methods to detect Ca^{2+} in living cells. In: Islam MS (ed) Calcium Signaling. Adv Exp Med Biol 740:27-43.

Castro V, Grisdale-Helland B, Helland SJ, Torgersen J, Kristensen T, Claireaux G, Farrell AP, Takle H (2013) Cardiac molecular-acclimation mechanisms in response to swimming-induced exercice in Atlantic salmon. PLoS One 8:e55056.

Chatelier A, McKenzie DJ, Prinet A, Galois R, Robin J, Zambonino J, Claireaux G (2006) Associations between tissue fatty acid composition and physiological traits of performance and metabolism in the seabass (*Dicentrarchus labrax*). J Exp Biol 209:3429-3439.

Claireaux G, Lagardère J-P (1999) Influence of temperature, oxygen and salinity on the metabolism of the European sea bass. J Sea Res 42:157-168.

Claireaux G, McKenzie DJ, Genge AG, Chatelier A, Aubin J, Farrell AP (2005) Linking swimming performance, cardiac pumping ability and cardiac anatomy in rainbow trout. J Exp Biol 208:1775-1784.

Claireaux G, Theron M, Prineau M, Dussauze M, Merlin FX, Le Floch S (2013) Effects of oil exposure and dispersant use upon environmental adaptation performance and fitness in the European sea bass, *Dicentrarchus labrax*. Aquat Toxicol 130-131:160-170.

Cros C, Sallé L, Warren DE, Shiels HA, Brette F (2014) The calcium stored in the sarcoplasmic reticulum acts as a safety mechanism in rainbow trout heart. Am J Physiol Integr Comp Physiol 307: R1493-R1501.

Crozier LG, Hutchings JA (2014) Plastic and evolutionary responses to climate change in fish. Evol Appl 7:68–87.

Di Virgilio F, Milani D, Leon A, Meldolesi J, Pozzan T (1987) Voltage-dependent activation and inactivation of calcium channels in PC12 cells. Correlation with neurotransmitter release. J Biol Chem 262:9189–9195.

Doney SC (2010) The growing human footprint on coastal and open-ocean biogeochemistry. Science 328:1512–1516.

Dupont-Prinet A, Chatain B, Grima L, Vandeputte M, Claireaux G, McKenzie DJ (2010) Physiological mechanisms underlying a trade-off between growth rate and tolerance of feed deprivation in the European sea bass (*Dicentrarchus labrax*). J Exp Biol 213:1143-1152.

Farrell AP, Axelsson M, Altimiras J, Sandblom E, Claireaux G (2007) Maximum cardiac performance and adrenergic sensitivity of the sea bass *Dicentrarchus labrax* at high temperatures. J Exp Biol 210: 1216–1224.

Farrell AP, Eliason EJ, Sandblom E, Clark TD (2009) Fish cardiorespiratory physiology in an era of climate change. Can J Zool 87:835–851.

Farrell AP, Gamperl AK, Hicks JMT, Shiels HA, Jain KE (1996) Maximum cardiac performance of rainbow trout (*Oncorhynchus mykiss*) at temperatures approaching their upper lethal limit. J Exp Biol 199:663–672.

Franklin CE, Davie PS (1992) Myocardial power output of an isolated eel (*Anguilla dieffenbachii*) heart preparation in response to adrenaline. Comp Biochem Physiol Part C Comp Pharmacol 101:293–298.

Fransen P, Van Hove CE, van Langen J, Schrijvers DM, Martinet W, De Meyer GRY, Bult H (2012) Contribution of transient and sustained calcium influx, and sensitization to depolarization-induced contractions of the intact mouse aorta. BMC Physiol. 12, 9.

Galli GLJ, Shiels HA, Brill RW (2009) Temperature sensitivity of cardiac function in pelagic fishes with different vertical mobilities: yellowfin tuna (*Thunnus albacares*), bigeye tuna (*Thunnus obesus*), mahimahi (*Coryphaena hippurus*), and swordfish (*Xiphias gladius*). Physiol Biochem Zool PBZ 82:280–290.

Gamperl AK, Farrell AP (2004) Cardiac plasticity in fishes: environmental influences and intraspecific differences. J Exp Biol 207:2539–2550.

Gamperl AK, Pinder AW, Boutilier RG (1994) Effect of coronary ablation and adrenergic stimulation on in vivo cardiac performance in trout (*Oncorhynchus mykiss*). J Exp Biol 186:127–143.

Gauthier C, Leblais V, Kobzik L, Trochu JN, Khandoudi N, Bril A, Balligand JL, Le Marec H (1998) The negative inotropic effect of beta3-adrenoceptor stimulation is mediated by activation of a nitric oxide synthase pathway in human ventricle. J Clin Invest 102:1377–1384.

Gauthier C, Sèze-Goismier C, Rozec B (2007) Beta 3-adrenoceptors in the cardiovascular system. Clin. Hemorheol. Microcirc 37:193–204.

Graham MS, Farrell AP (1989) The effect of temperature acclimation and adrenaline on the performance of a perfused trout heart. Physiol Zool 62:38-61.

Grynkiewicz G, Poenie M, Tsien RY (1985) A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J Biol Chem 260:3440–3450.

Haverinen J, Vornanen M (2004) Temperature acclimation modifies Na^{+} current in fish cardiac myocytes. J Exp Biol 207:2823–2833.

Haverinen J, Vornanen M (2009) Responses of action potential and K^{+} currents to temperature acclimation in fish hearts: phylogeny or thermal preferences? Physiol Biochem Zool PBZ 82:468–482.

Hofmann GE, Todgham AE (2010) Living in the now: physiological mechanisms to tolerate a rapidly changing environment. Annu Rev Physiol 72:127–145.

Hove-Madsen L, Tort L (1998) L-type Ca^{2+} current and excitation-contraction coupling in single atrial myocytes from rainbow trout. Am J Physiol Regul Integr Comp Physiol 275:R2061–R2069.

Hove-Madsen L, Llach A, Tort L (2000) Na^{+}/Ca^{2+}-exchange activity regulates contraction and SR Ca^{2+} content in rainbow trout atrial myocytes. Am J Physiol Regul Integr Comp Physiol 279:R1856–R1864.

Imbert-Auvray N, Mercier C, Huet V, Bois P (2013) Sarcoplasmic reticulum: a key factor in cardiac contractility of sea bass *Dicentrarchus labrax* and common sole *Solea solea* during thermal acclimations. J Comp Physiol B 183:477–489.

Imbrogno S, Angelone T, Adamo C, Pulerà E, Tota B, Cerra MC (2006) Beta3-adrenoceptor in the eel (*Anguilla anguilla*) heart: negative inotropy and NO-cGMP-dependent mechanism. J Exp Biol 209:4966–4973.

Keen JE, Vianzon DM, Farrell AP, Tibbits GF (1993) Thermal acclimation alters both adrenergic sensitivity and adrenoceptor density in cardiac tissue of rainbow trout. J Exp Biol 181:27–48.

Keen JE, Vianzon D-M, Farrell AP, Tibbits GF (1994) Effect of temperature and temperature acclimation on the ryanodine sensitivity of the trout myocardium. J Comp Physiol B 164:438–443.

Klaiman JM, Pyle WG, Gillis TE (2014) Cold acclimation increases cardiac myofilament function and ventricular pressure generation in trout. J Exp Biol 217:4132–4140.

Korajoki H, Vornanen M (2013) Temperature dependence of sarco(endo)plasmic reticulum Ca^{2+} ATPase expression in fish hearts. J Comp Physiol B 183:467–476.

Lague SL, Speers-Roesch B, Richards JG, Farrell AP (2012) Exceptional cardiac anoxia tolerance in tilapia (*Oreochromis hybrid*). J Exp Biol 215:1354–1365.

Langton PD, Huddart H (1988) Voltage and time dependency of calcium mediated phasic and tonic responses in rat vas deferens smooth muscle--the effect of some calcium agonist and antagonist agents. Gen Pharmacol 19:775–787.

Larsson D, Larsson B, Lundgren T, Sundell K (1999) The effect of pH and temperature on the dissociation constant for fura-2 and their effects on $[Ca^{2+}]_i$ in enterocytes from a poikilothermic animal, Atlantic cod (*Gadus morhua*). Anal Biochem 273:60–65.

Loewer A, Lahav G (2011) We're all individuals: causes and consequences of non-genetic heterogeneity in mammalian cells. Curr Opin Genet Dev 21:753-758.

Lurman GJ, Petersen LH, Gamperl AK (2012) *In situ* cardiac performance of Atlantic cod (*Gadus morhua*) at cold temperatures: long-term acclimation, acute thermal challenge and the role of adrenaline. J Exp Biol 215:4006–4014.

Maggi CA, Giuliani S (1995) A pharmacological analysis of calcium channels involved in phasic and tonic responses of the guinea-pig ureter to high potassium. J Auton Pharmacol 15:55–64.

Mangel M, Stamps J (2001) Trade-offs between growth and mortality and the maintenance of individual variation in growth. Evol Ecol Res 3:583-593.

Marras S, Claireaux G, McKenzie DJ, Nelson JA (2010) Individual variation and repeatability in aerobic and anaerobic swimming performance of European sea bass, *Dicentrarchus labrax*. J Exp Biol 213:26-32.

McClelland GB, Dalziel AG, Fragoso NM, Moyes CD (2005) Muscle remodeling in relation to blood supply: implications for seasonal changes in mitochondrial enzymes. J Exp Biol 208:515-522.

Mendonça PC, Gamperl AK (2009) Nervous and humoral control of cardiac performance in the winter flounder (*Pleuronectes americanus*). J Exp Biol 212:934–944.

Niu X, Watts VL, Cingolani OH, Sivakumaran V, Leyton-Mange JS, Ellis CL, Miller KL, Vandegaer K, Bedja D, Gabrielson KL, et al (2012) Cardioprotective effect of beta-3

adrenergic receptor agonism: role of neuronal nitric oxide synthase. J Am Coll Cardiol 59:1979–1987.

Rathi SS, Saini HK, Xu Y-J, Dhalla NS (2004) Mechanisms of low Na^+-induced increase in intracellular calcium in KCl-depolarized rat cardiomyocytes. Mol Cell Biochem 263:151–162.

Roessig JM, Woodley CM, Cech Jr JJ, Hansen LJ (2004) Effects of global climate change on marine and estuarine fishes and fisheries. Rev Fish Biol Fish 14:251–275.

Roze T, Christen F, Amérand A, Claireaux G (2013) Trade-off between thermal sensitivity, hypoxia tolerance and growth in fish. J Therm Biol 38:98-106.

Shiels HA, Farrell AP (1997) The effect of temperature and adrenaline on the relative importance of the sarcoplasmic reticulum in contributing Ca^{2+} to force development in isolated ventricular trabeculae from rainbow trout. J Exp Biol 200:1607–1621.

Shiels HA, Vornanen M, Farrell AP (2002) Effects of temperature on intracellular Ca^{2+} in trout atrial myocytes. J Exp Biol 205:3641–3650.

Stepien O, Marche P (2000) Amlodipine inhibits thapsigargin-sensitive Ca^{2+} stores in thrombin-stimulated vascular smooth muscle cells. Am J Physiol Heart Circ Physiol 279:H1220–H1227.

Strosberg AD (1997) Structure and function of the beta 3-adrenergic receptor. Annu Rev Pharmacol Toxicol 37:421–450.

Tibbits GF, Moyes CD, Hove-Madsen L (1992) Excitation-contraction coupling in the teleost heart. In: Hoar WS, Randall DJ, Farrell AP (eds) Fish Physiology. Academic Press, pp 267-296.

Tiitu V, Vornanen M (2002) Regulation of cardiac contractility in a cold stenothermal fish, the burbot *Lota lota* L. J Exp Biol 205:1597-1606.

Tiitu V, Vornanen M (2003) Ryanodine and dihydropyridine receptor binding in ventricular cardiac muscle of fish with different temperature preferences. J Comp Physiol B 173:285–291.

Vandamm JP, Marras S, Claireaux G, Handelsman CA, Nelson JA (2012) Acceleration performance of individual European sea bass *Dicentrarchus labrax* measured with a sprint performance chamber: comparison with high-speed cinematography and correlates with ecological performance. Physiol Biochem Zool 85:704-717.

Vela J, Pérez-Millán MI, Becu-Villalobos D, Díaz-Torga G (2007) Different kinases regulate activation of voltage-dependent calcium channels by depolarization in GH3 cells. Am J Physiol Cell Physiol 293:C951–C959.

Vornanen M (1997) Sarcolemmal Ca influx through L-type Ca channels in ventricular myocytes of a teleost fish. Am J Physiol 272:R1432–R1440.

Vornanen M (1998) L-type Ca^{2+} current in fish cardiac myocytes: effects of thermal acclimation and beta-adrenergic stimulation. J Exp Biol 201:533–547.

Vornanen M (1999) Na^+/Ca^{2+} exchange current in ventricular myocytes of fish heart: contribution to sarcolemmal Ca^{2+} influx. J Exp Biol 202:1763–1775.

Vornanen M, Shiels HA, Farrell AP (2002a) Plasticity of excitation-contraction coupling in fish cardiac myocytes. Comp Biochem Physiol A Mol Integr Physiol 132:827–846.

Vornanen M, Ryökkynen A, Nurmi A (2002b) Temperature-dependent expression of sarcolemmal K^+ currents in rainbow trout atrial and ventricular myocytes. Am J Physiol Regul Integr Comp Physiol 282:R1191–R1199.

Figure captions

Fig.1 Representative time course of F340/F380 ratio after depolarization of cardiac myocyte populations using a high KCl solution (time 0; 17 mM final concentration), and following addition of Triton X and Tris-EGTA for estimation of maximum ratio (R_{max}) and minimum ratio (R_{min}) respectively

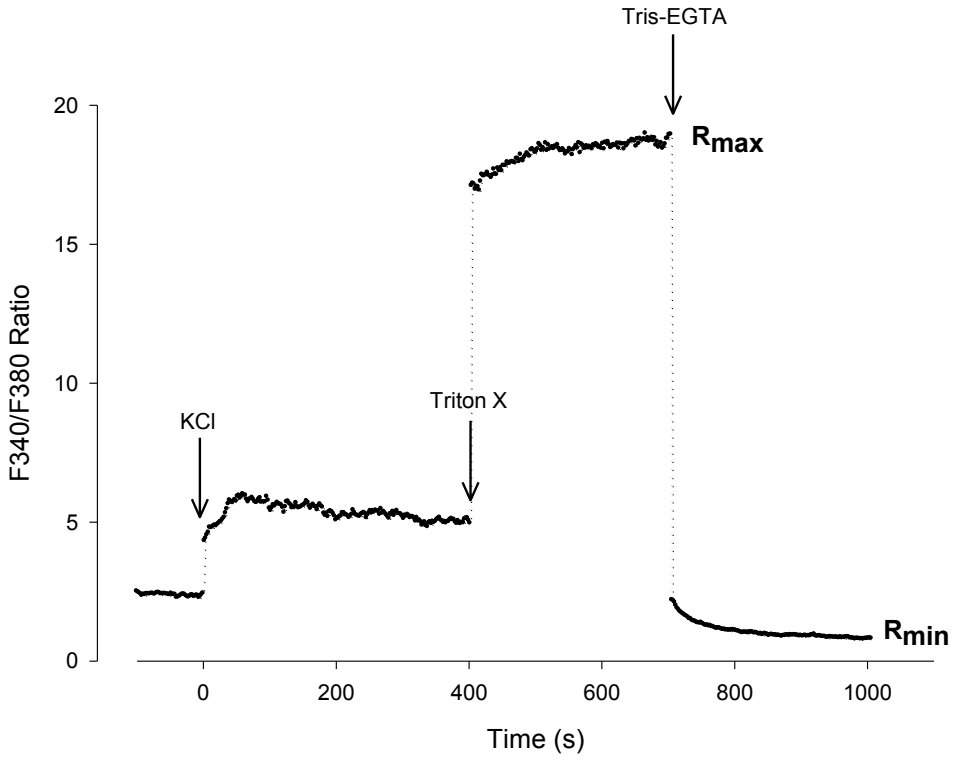

Fig.2 Effects of KCl concentration in the stimulation solution on time to 50 % maximal Ca^{2+} response (T_{50}) at 12 °C- and 22 °C-test. Values are means + S.E.M. (n= 5 measurements in each condition). Different letters above the bars indicate a significant difference ($p<0.05$). * indicates a significant difference between test temperature conditions ($p<0.05$)

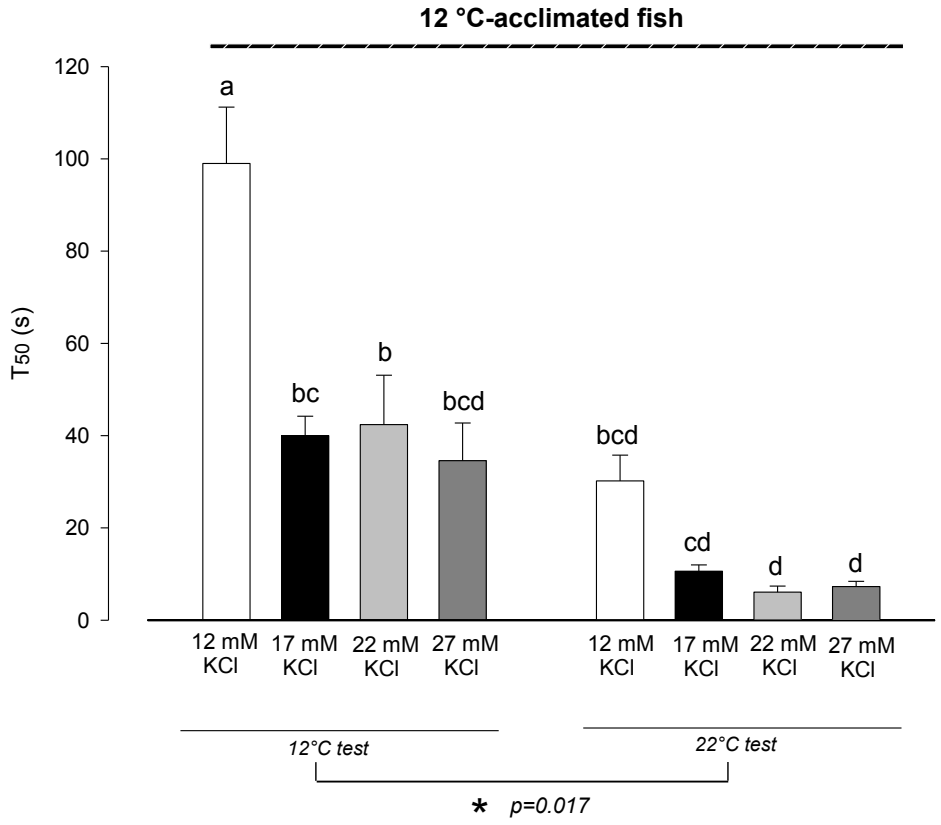

Fig.3 Effects of Ca^{2+} antagonists on KCl-induced increase in intracellular calcium concentration $[Ca^{2+}]_i$. Nif: nifedipine (10 µM); Ryano: ryanodine (10 µM); Thaps: thapsigargin (2 µM). Values are means + S.E.M. (n= 8 measurements in each condition). * indicates a significant difference compared to the corresponding control ($p<0.05$). # indicates a significant difference between control conditions ($p<0.05$). & indicates a significant difference between antagonist (Nif *vs* Nif+Ryano/Thaps) conditions ($p<0.05$)

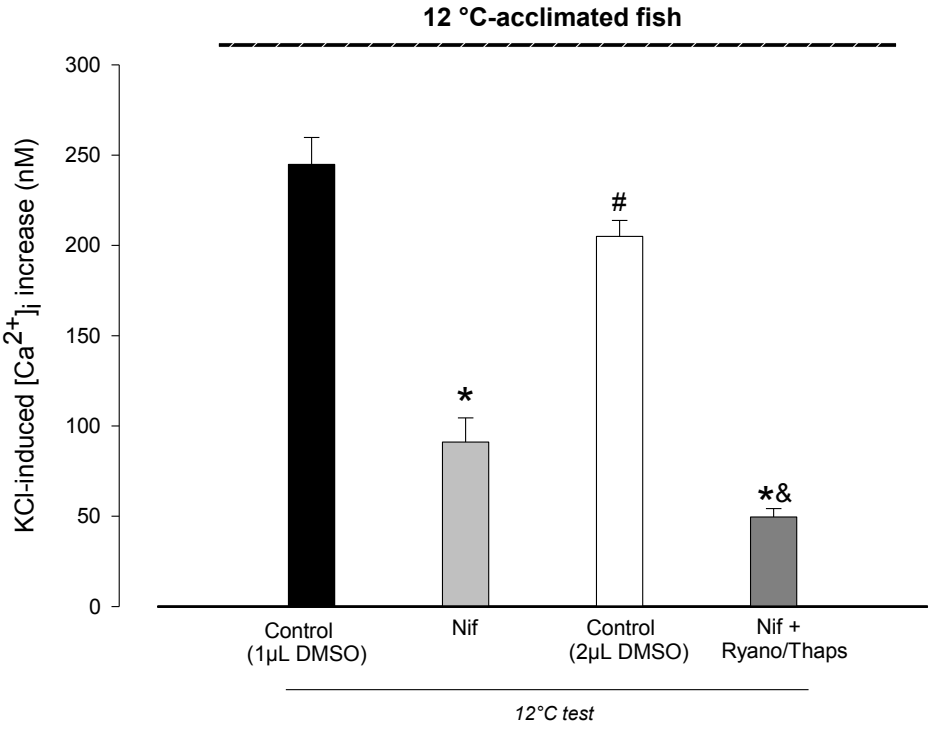

Fig.4 Effects of adrenaline concentration on KCl-induced increase in intracellular calcium concentration $[Ca^{2+}]_i$. Values are means + S.E.M. (n= 10, 11, 18 and 24 measurements for AD 0 M, AD 10^{-12} M, AD 10^{-9} M and AD 10^{-6} M respectively). Different letters above the bars indicate a significant difference ($p<0.05$)

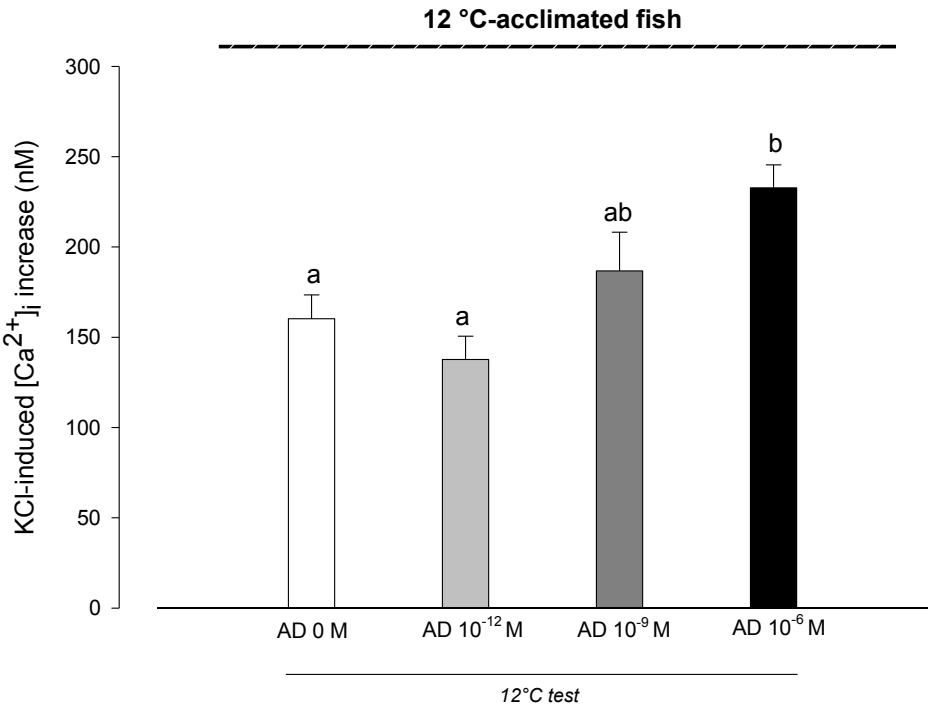

Fig.5 Effects of thermal acclimation on KCl-induced increase in intracellular calcium concentration $[Ca^{2+}]_i$. Myocytes were isolated from sea bass acclimated at 12 °C (n= 11 fish) or 22 °C (n= 14 fish). The increase in $[Ca^{2+}]_i$ was analyzed after KCl stimulation in the presence of adrenaline (AD) at 10^{-9} M (n= 4 and 7 fish for 12 °C- and 22 °C-acclimation respectively) or 10^{-6} M (n= 7 and 7 fish for 12 °C- and 22 °C-acclimation respectively), under control condition or following incubation with SR Ca^{2+} store blockers ryanodine (Ryano; 10 µM) and thapsigargin (Thaps; 2 µM). Measurements were made at 12 °C- and 22 °C-test. Values are means + S.E.M. (n= 4 measurements per fish for each condition). # indicates a significant difference ($p<0.05$) between conditions. * indicates a significant difference ($p<0.05$) with the corresponding control (*i.e.,* measured in identical conditions of acclimation temperature/AD concentration/ test temperature).