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Effect of combined stress (salinity and temperature) in European sea bass *Dicentrarchus labrax* osmoregulatory processes

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Abstract

European sea bass *Dicentrarchus labrax* undertake seasonal migrations to estuaries and lagoons that are characterized by fluctuations in environmental conditions. Their ability to cope with these unstable habitats is undeniable, but it is still not clear how and to what extent salinity acclimation mechanisms are affected at temperatures higher than in the sea. In this study, juvenile sea bass were pre-acclimated to seawater (SW) at 18 °C (temperate) or 24 °C (warm) for 2 weeks and then transferred to fresh water (FW) or SW at the respective temperature. Transfer to FW for two weeks resulted in decreased blood osmolalities and plasma Cl^- at both temperatures. In FW warm conditions, plasma Na^+ was ~15% lower and Cl^- was ~32 % higher than in the temperate-water group. Branchial Na^+/K^+ -ATPase (NKA) activity measured at the acclimation temperature (V_{apparent}) did not change according to the conditions. Branchial Na^+/K^+ -ATPase activity measured at 37 °C (V_{max}) was lower in warm conditions and increased in FW compared to SW conditions whatever the considered temperature. Mitochondrion-rich cell (MRC) density increased in FW, notably due to the appearance of lamellar MRCs, but this increase was less pronounced in warm conditions where MRC's size was lower. In SW warm conditions, pavement cell apical microridges are less developed than in other conditions. Overall gill morphometrical parameters (filament thickness, lamellar length and width) differ between fish that have been pre-acclimated to different temperatures. This study shows that a thermal change affects gill plasticity affecting whole-organism ion balance two weeks after salinity transfer.

Key words: gill plasticity, hydromineral balance, ionocytes, NKA activity, osmoregulation, teleost, thermal acclimation

1. Introduction

The European sea bass *Dicentrarchus labrax* (Linnaeus 1758) is an important aquaculture species. It is considered as highly euryhaline (tolerating salinities from fresh water (FW) up to 90‰) and eurythermic (tolerates temperatures from 5 to 28 °C) (Barnabé, 1989; Kousoulaki et al., 2015). *D. labrax* is commonly found on the coasts of the north-east Atlantic Ocean, the Mediterranean Sea and the Black Sea. In the Mediterranean region, *D. labrax* juveniles and adults undergo seasonal migrations (in spring) from the sea to lagoons occasionally travelling upstream into rivers (Dufour et al., 2009). Salinities of Mediterranean lagoon habitats range

from close to fresh water up to hypersaline waters (>45 ‰) (Newton and Mudge, 2003). In these habitats, it is not rare that temperatures reach mean temperatures close to 25 °C from late spring to end of August. In autumn, as temperatures of lagoons decrease to 5-6 °C, sea bass migrate back to the sea, where spawning occurs in winter (Dufour et al., 2009). In order to cope with the salinity drop caused by high precipitations that are common in autumn and spring season in Mediterranean lagoon habitats, sea bass have to be able to efficiently osmoregulate at different temperatures encountered in the wild. Gills are considered as the major organ involved in active ion absorption (review in Evans et al. 2005). In FW, mitochondrion-rich cells (MRCs) also called ionocytes are involved in transepithelial ion uptake, acid-base regulation and ammonia excretion (review in Hwang et al. 2011). The basolateral Na^+/K^+ -ATPase (NKA) is considered as a key pump enabling active transport of ions within these cells (review in Marshall, 2002).

Previous studies on juvenile sea bass transferred to FW at temperate conditions (18-20 °C) showed low and variable blood osmolalities (ranging from 214 to 316 mOsm.kg⁻¹) several weeks after salinity challenge (Nebel et al., 2005). In seawater, blood osmolality levels are more stable and ranged from 360 to 380 mOsm.kg⁻¹. FW transfer induced in this species the appearance of lamellar ionocytes, and an increase in branchial NKA activity (Jensen et al., 1998; Nebel et al., 2005). No data are available on osmoregulatory mechanisms in sea bass acclimated to increased temperatures.

In other teleost species, several studies are available on the effect of temperature on osmoregulatory mechanisms (Stuenkel and Hillyard, 1980; Kültz and Somero, 1995; Metz et al., 2003; Fiess et al., 2007; Sardella et al., 2008; Vargas-Chacoff et al., 2009; Radaelli et al., 2010; Uliano et al., 2010). Na^+/K^+ -ATPase activity can depend on acclimation temperature, but the effect of temperature depends on the range of temperatures that have been tested and the species considered (McCarty and Houston, 1977; Stuenkel and Hillyard, 1980; Imsland et al., 2003; Metz et al., 2003; Morrison et al., 2006; Fiess et al., 2007; Sardella et al., 2008; Mitrovic and Perry, 2009; Zydlewski and Zydlewski, 2012).

Several investigations reported differences in gill NKA activity according to the temperature used in the assay (acclimation (V_{apparent}) vs maximal temperature (V_{max})) (McCarty and Houston, 1977; Metz et al., 2003; Sardella et al., 2004a; Sardella et al., 2008; Michael et al., 2016). In FW carp, where three temperatures were tested (15, 22 and 29 °C), higher branchial NKA expression and activity (V_{max}) were reported at 15 °C (Metz et al., 2003). NKA activity assayed at acclimation temperature (V_{apparent}) however was not increased at this temperature and maximal NKA (V_{apparent}) levels were recorded at 29 °C. The authors state that cold

temperatures induce MRC number and NKA expression in order to compensate for the lower NKA enzyme activity. In Mozambique tilapia acclimated to FW or SW and then transferred from 26 °C to 15, 25 and 35 °C for 2 weeks, osmotic disturbances have been recorded in the low-temperature group in FW with decreased plasma osmolalities and NKA activities (assayed at the acclimation temperature, V_{apparent}). Osmotic alterations were also recorded in SW in this low-temperature group with increased plasma osmolalities and decreased NKA activities (V_{apparent}) (Sardella et al., 2008). Interestingly, maximal NKA activity (V_{max}) peaked at 15 °C in SW as well as the size of MRCs, which could be interpreted as a compensatory response following cold stress.

Temperature-dependent change in NKA activity depends on the considered salinity, as shown in several species such as pupfish and tilapia (Stuenkel and Hillyard, 1980; Fiess et al., 2007). In tilapia, a significant increase in NKA activity was recorded only in 200 ‰ SW but not in FW and SW at temperatures ranging from 20 to 35 °C (Fiess et al., 2007). In juvenile turbot exposed to different salinities (15, 25 and 33‰) and temperatures (10, 14, 18, and 22 °C) for 3 months, lowest gill NKA activities, plasma chloride and osmolality were recorded at 15‰, that corresponds to a salinity close to the iso-osmotic point (Imsland et al., 2003). Low gill NKA activity is an interesting parameter to consider for aquaculture studies as it leads to reduced energy expenditure for osmoregulation and possibly increased growth rate (Imsland et al., 2003; Árnason et al., 2014; Michael et al., 2016). In turbot, the optimal temperature-salinity combination for aquaculture was estimated at around 15.8 +/- 4.1‰ at 17.6 +/- 2.2 °C (Imsland et al., 2003).

In salmonids, it is well known that temperature is a key factor affecting the smoltification-related increase in branchial NKA activity (Handeland et al., 2013). In Atlantic salmon *Salmo salar*, where branchial NKA activity is supposed to increase during the smoltification process as a pre-acclimation to the seawater salinity, a different increase in NKA activity has been reported according to the temperature, with higher enzyme activities at high temperatures (19 °C vs 5 °C group), which can be linked to better hypo-osmoregulatory capacities in the 19 °C group (Handeland et al., 2000). In the same species, gill NKA activity, plasma Cl^- and growth-hormone have been shown to be affected by temperature (8.3 vs 12.7 °C) and photoperiod (Handeland et al., 2013).

In several species, gill morphology has been altered under temperature stress alone (Sollid et al., 2005) or combined with other stress (Metz et al., 2003; Tzaneva et al., 2011). Gill remodeling in response to environmental parameters (like salinity, temperature and oxygen) affects functional surface area that will either favor osmoregulation or respiration, a

phenomenon termed osmorepiratory compromise (Sollid et al., 2005; Sollid and Nilsson, 2006; Perry et al., 2010; Tzaneva et al., 2011). In goldfish *C. auratus* and carp *C. carpio*, important gill remodeling has been shown following temperature acclimation with the formation of an interlamellar cell mass (ILCM) in cold water (7 °C) to reduce respiratory surface area (Sollid et al., 2005; Mitrovic and Perry, 2009). Mitochondrion-rich cells (MRCs) of *C. auratus* have been shown to be larger in size, more abundant and differently located (confined in the outer part of the ILCM, that is devoid of a blood supply) after acclimation to cold temperatures (7 °C compared to 25 °C). Despite the higher number of MRCs, NKA activity (V_{\max}) was lower in cold conditions, probably linked to the decreased blood and oxygen supply at the basal part of MRCs (Mitrovic and Perry, 2009).

The aim of this study is to investigate the effects of temperature acclimation (18 °C or 24 °C) on the establishment of hyperosmoregulation in sea bass following SW to FW transfer. This study will analyze branchial NKA activity (V_{\max} , V_{apparent}), MRC density and size, as well as the morphometry of gills. Furthermore, blood osmolalities and plasma ion levels will be measured to assess osmoregulatory status of fish in order to get a comprehensive view on the effects of combined temperature and salinity stress in this species.

2. Materials and methods

2.1 Experimental conditions

European sea bass *Dicentrarchus labrax* were obtained from the Ifremer Station at Palavas-les-Flots (Hérault, France). Fish were brought to the Montpellier University and maintained for one week in 3,500 L tanks containing natural seawater (SW) from the Mediterranean Sea at 38‰ and 18 °C, under a 12 h light/12 h dark photoperiod. Fish were then transferred to 200 L tanks (14 fish/tank, density of 6-7 kg/m³, two replicates) to be acclimated either at 18 °C or 24 °C (with a temperature increase of 0.2 °C/h). After two weeks of temperature acclimation, fish were transferred directly either to dechlorinated tap water (fresh water, FW) or to SW (7 fish/tank, two replicates for each condition) and maintained in this salinity two weeks before sampling. Ionic composition (in mEq.L⁻¹) of the FW was Na⁺ (0.12), K⁺ (0.04), Ca²⁺ (5.70), Mg²⁺ (0.29), Cl⁻ (0.98), NO₃⁻ (0.06) and SO₄²⁻ (0.61). Water was aerated and mechanically/biologically filtered (Eheim System, Lens, Pas-de-Calais, France). Temperature, salinity, oxygen and nitrogen levels were checked daily. Fish were fed twice a week with fish granules (Aphymar feed, Mèze, Hérault, France) until 2 days before sampling. At the end of

the experiment, fish were anesthetized in a solution of phenoxy-2-ethanol ($0.24 \text{ mL}\cdot\text{L}^{-1}$) prior to any manipulation. The sampled fish average fork length was $20.77\pm 1.32 \text{ cm}$ (mean \pm SD) and average weight was $86.87\pm 20.23 \text{ g}$ (mean \pm SD). Four groups were compared: SW at $18 \text{ }^\circ\text{C}$ (temperate SW), SW at $24 \text{ }^\circ\text{C}$ (warm SW), FW at $18 \text{ }^\circ\text{C}$ (temperate FW) and FW at $24 \text{ }^\circ\text{C}$ (warm FW). These experiments were conducted according to the guidelines of the European Union (directive 86/609) and of the French law (decree 87/848) regulating animal experimentation.

2.2 Blood osmolality and plasma ion levels

Blood osmolality and plasma ions were measured in 8-12 fish per condition. Blood was sampled from the caudal vessel using a 1-mL syringe coated with heparin (Li-heparin, Sigma-Aldrich, France). The osmolality of $20 \mu\text{L}$ of blood was measured on an Advanced 3300 micro-osmometer using internal standards of 100, 300 and $1000 \text{ mOsm}\cdot\text{kg}^{-1}$. Plasma was obtained following centrifugation of 8 min at 10000 g at $4 \text{ }^\circ\text{C}$. $15 \mu\text{l}$ of plasma was used to determine chloride concentration using a chloride titrator (AMINCO, Maryland, USA). Plasma sodium levels were determined by flame photometry (Sherwood, Cambridge, UK) using a standard curve of Na^+ , K^+ from 0 to $400 \text{ mEq}\cdot\text{L}^{-1}$ and plasma at a 1/1000 dilution in milliQ water. Mean Na^+/Cl^- ratio was determined for each condition.

2.3 Branchial histology, morphometric analyses and immunolocalization of Na^+/K^+ -ATPase

The first left gill arch of three animals per condition was fixed for 48 h by immersion in Bouin's fixative. After rinsing in 70 % ethanol, samples were dehydrated in a graded ethanol series and embedded in Paraplast (Sigma). Transverse sections ($4 \mu\text{m}$) were cut on a Leitz Wetzlar microtome, collected on poly-L-lysine-coated glass slides and were stained using the Masson's Trichrome Staining Protocol. Slides were observed under Leica Diaplan microscope, and gill lamellae sections were photographed at a magnification of x400. For morphometric analyses, several parameters were measured using the software Image J (ImageJ 1.51f) (<http://rsbweb.nih.gov/ij/>): filament thickness ($n=18$ measurements per animal, $N=3$ animals per condition), lamellar length, lamellar width at top and base ($n=27$ measurements per animal, $N=3$ animals per condition) (Fig. 6).

For immunolabelling of the Na^+/K^+ -ATPase, sections were dewaxed (LMR), hydrated through a descending series of ethanol baths (from 100 % to 50 %) and rinsed in phosphate-buffered saline (PBS; 137 mM NaCl , 2.7 mM KCl , $10 \text{ mM phosphate buffer}$, pH 7.4, Sigma).

Slides were then immersed for 10 min into 0.02 % Tween 20, 150 mM NaCl in PBS, pH 7.3. After blocking in 5 % skimmed milk (SM) in PBS at 37 °C for 20 min, the slides were rinsed twice with PBS. Primary labelling was performed for 2 h at room temperature in a humidity chamber with the primary monoclonal antibody ($\alpha 5$) anti- Na^+/K^+ -ATPase (Hybridoma Bank, University of Iowa) diluted in PBS at $10 \mu\text{g}\cdot\text{mL}^{-1}$ in 0.5 % SM-PBS. After three washes in PBS to remove unbound antibody, the sections were incubated for 1 h with a secondary antibody (donkey anti-mouse Alexa Fluor[®] 488 (Invitrogen, Life Technologies) at $10 \mu\text{g}\cdot\text{mL}^{-1}$). Following washes, sections were mounted in an anti-bleaching mounting medium (Immunohistomount, Santa Cruz Biotechnology) and observed with a Leica Diaplan microscope equipped with a special filter for fluorescence set (450-490 nm) and coupled to a Leica DC 300F digital camera and FW4000 software. Gill sections were photographed using objectives with a magnification of x25 (MRC density) and x40 (MRC area) and images were analyzed with Image J software (ImageJ 1.51f). The number of MRCs located on the filaments and lamellae were counted on 300 μm of filament length in each picture on one side of the filament (n= 10 measurements per animal, N= 3 animals per condition). MRC area was measured using cells where the nucleus was visible (n=25 measurements per animal, N=3 animals per condition).

2.4 Scanning electron microscopy

A small section of the 1st left gill arch of four fish from each condition was fixed for 24 h at 4 °C by immersion in 2 % glutaraldehyde solution adjusted to 380 mOsm.kg⁻¹, buffered at pH 7.4 with 0.1 mol.L⁻¹ cacodylate buffer. Samples were then kept in 0.5 % glutaraldehyde with diluted SW adjusted at 380 mOsm.kg⁻¹ until proceeding. Then, samples were rinsed 3 times for 30 min in cacodylate buffer and post-fixed for 4 h at room temperature in buffered 1 % OsO₄. After extensive washes in buffer, the samples were thoroughly rinsed with water, dehydrated through an ethanol series (50 %-96 %), and 1,1,1, 3, 3, 3-hexamethyldisilazane (EMS, Hatfield, USA) (2 baths of 1 min), and were subsequently air-dried and attached to specimen stubs with adhesive carbon tabs. Samples were coated with gold for 160 seconds (560 mÅ), using the BAL-TEC SCD 050 sputter coater and examined with a FEI Quanta 200 ESEM using the conventional mode (low vacuum) and the Thornley-Everhart secondary electron detector.

2.5 Na^+/K^+ -ATPase activity measurements

NKA activity was measured on the first and second right gill arch from 8-12 fish per condition. After sampling, gills of each fish were stored at -80 °C in SEI buffer (300 mM sucrose, 20 mM Na₂EDTA, 100 mM imidazole, pH 7.4). Homogenization was performed in 300µl of buffer containing 250 mM sucrose, 5 mM MgCl₂, pH 7.4 and using a Retsch Mixer mill MM400 (Haan, Germany) (frequency : 30 Hz, 2 times for 30s). After centrifugation at 1800 g (5 min at 4 °C), the supernatant was used for protein quantification (Bradford ; Bio-Rad, France) and NKA activity determination. NKA activity, expressed in µmol Pi mg protein⁻¹ h⁻¹, was determined using a microplate method adapted from Flik et al. (1983) and previously validated in sea bass by Nebel et al. (2005). NKA specific activity was calculated from the difference in ATP hydrolysis at 37 °C (V_{max}), 24 °C (V_{app} for 24 °C acclimated fish) or 18 °C (V_{app} for 18 °C acclimated fish) for 20 min after the addition of 100 µl assay buffer (3 mM Na-ATP, 100 mM NaCl, 30 mM imidazole/Hepes, 0.1 mM EDTA, 5 mM MgCl₂, pH 7.4) amended with either 13.4 mM KCl or 1.4 mM ouabain. The enzyme reaction was stopped by the simultaneous addition of ice-cold trichloroacetic acid at 10 % and color reagent (660 mM H₂SO₄, 9.2 mM (NH₄)₆ Mo₇O₂₄.4H₂O, 330 µmol.L⁻¹ FeSO₄. 7H₂O), followed by a 10-min reaction at room temperature to quantify liberated P_i (630 nm absorbance, Infinite M200 plate reader, TECAN trading AG, Switzerland).

2.6 Statistics

Statistical analyses were performed using Graphpad Prism (version 6, GraphPad Software Incorporated, La Jolla, CA, 268 USA). Normality and homogeneity of variance were respectively checked using D'Agostino-Pearson test and Bartlett test. For data fitting homogeneity of variance requirement, a two-way factorial analysis of variance with temperature and salinity as the main factors was performed; critical differences between groups were appraised using the Fisher least-square difference test. For data not fitting homogeneity of variance and data issued from histology sections, a non-parametric Kruskal-Wallis test followed by a multiple comparison Dunn's test was used. Linear correlations were determined using the Spearman correlation. Data are presented as means ± SEM, and the level of statistical significance was set at p<0.05.

3. Results

3.1 Blood osmolality and plasma ion levels

For sea bass maintained in SW, blood osmolality was not significantly different between temperatures (Fig. 1A). After transfer to FW, blood osmolalities were significantly decreased by ~23 % and ~12 % in temperate and warm conditions, respectively. Plasma chloride levels were significantly decreased by ~35 % and ~17 % in temperate and warm FW conditions (Fig. 1C). Salinity, temperature and interaction between both exerted a significant effect on blood osmolality and plasma chloride (Fig. 1A, C, two-way ANOVA, $p < 0.0001$). Overall, blood osmolality was highly correlated to plasma chloride levels ($r = 0.9738$, $p < 0.0001$), but not to plasma sodium levels ($r = 0.09715$, $p > 0.05$). Plasma sodium levels were not significantly modified following FW transfer at temperate and warm temperature, but Na^+ levels were significantly lower in warm FW compared to temperate FW conditions (Fig. 1B). The temperature is the only factor exerting an effect on plasma Na^+ levels (Fig. 1B, two-way ANOVA, $p < 0.0001$). Na^+/Cl^- ratio in SW at 24 °C was significantly lower than in temperate conditions in SW and FW but was not significantly different from Na^+/Cl^- ratio in FW at 24 °C. Na^+/Cl^- ratio was slightly but not significantly increased in FW 18 °C compared to SW 18 °C (Table 1).

3.2 Branchial Na^+/K^+ -ATPase activity

Branchial NKA activity at acclimation temperatures (18 °C or 24 °C, V_{apparent}) did not change between different salinity and temperature conditions and was significantly lower than NKA activities assayed at 37 °C (V_{max}) (Fig. 2). When NKA activity was assayed at 37 °C, NKA activity was higher in FW compared to SW conditions by 67 % and 80 % at the temperate and warm temperature, respectively. Overall, lower NKA activities (V_{max}) were measured in warm conditions. There was a significant salinity effect ($p < 0.001$) and temperature effect ($p < 0.01$) but the interaction between temperature and salinity did not exert an effect on NKA activities (V_{max}) (Fig. 2, two-way ANOVA).

3.3 Branchial histology, morphometric analyses and immunolocalization of Na^+/K^+ -ATPase

MRCs were detected by NKA immunolabeling in gill filaments (in SW and FW conditions) and in lamellae (almost exclusively in FW conditions) (Figs 3, 4). Contrary to SW conditions, where temperature does not affect MRC density, fish acclimated to FW temperate conditions had significantly more MRCs on lamellae and filaments than fish acclimated to FW warm conditions (Figs 3, 4A). Moreover, the MRC area was significantly decreased in FW warm condition (Fig. 4B).

Lamellar width at the base (L.wd.b., ranging from 17-25 μm) and at the top (L.wd.t., ranging from 9-12 μm) was significantly higher in SW warm compared to the SW temperate condition whereas no effect was recorded in FW conditions (Fig. 5, 6A, 6B). Lamellar length (L.l.) however was significantly higher in FW warm compared to FW temperate conditions and no temperature effect was recorded in SW conditions (Fig. 5, 6C). Decreased filament thickness (F.th.) was measured in FW warm compared to FW temperate conditions whereas no effect was recorded between temperature regimes in SW (Fig. 5, 6D).

3.4 Scanning electron microscopy

The apical features of MRCs revealed different types of apical surfaces in sea bass exposed to different salinity regimes. Sea bass acclimated to FW showed some MRCs with wide and large apical surfaces with a few but well apparent microvilli (Fig. 7C, D). MRCs of sea bass acclimated to SW showed narrow and small, apical surfaces forming an apical pit (Fig. 7A, B). As a lot of mucous was detected on the gill surface (not shown), MRC apical surfaces could not be quantified among salinities and temperature regimes. Pavement cells (PC) showed well-developed microridges on apical surfaces at both salinities (Fig. 7A, C, D). In SW warm conditions, microridges were less developed, and nearly absent in the centre of the pavement cells (Fig. 7B).

4. Discussion

This study provides information on osmoregulatory mechanisms at two temperatures (18 and 24 °C, temperate and warm, respectively) in response to a salinity challenge from SW to FW.

4.1 Warm temperatures affect hydromineral status in fresh water

Direct transfer to fresh water resulted in decreased blood osmolalities as previously reported in sea bass (Bossus et al., 2011; Jensen et al., 1998). Blood osmolality is notably correlated to Cl^- levels ($r= 0.9738$, $p<0,0001$) and not to Na^+ levels ($r= 0.09715$, $p>0,05$). Changes in plasma ion levels as a result of temperature acclimation have been reported in many fish species (Metz et al., 2003; Fiess et al., 2007; Sardella et al., 2008; Mitrovic and Perry, 2009; Vargas-Chacoff et al., 2009) and could thus be expected in sea bass. This study has shown maintenance of overall hydromineral balance (blood osmolality and main plasma ion levels) in SW, 2 weeks after salinity transfer. In FW warm conditions however, there was an effect of temperature that seems to differ among ions (Na^+ levels were lower whereas Cl^- levels were

higher in FW warm compared to FW temperate conditions). Na^+/Cl^- ratio is slightly higher in FW (1.65 \pm 0.12) compared to SW (1.12 \pm 0.04) in temperate conditions unlike warm conditions, where Na^+/Cl^- ratio is similar in SW (0.93 \pm 0.02) and FW (1.07 \pm 0.03). A Na^+/Cl^- ratio of 1.50 \pm 0.01 was measured in FW-acclimated sea bass by Jensen et al. (1998), that is similar to our results in temperate FW. The authors concluded in this study that a high Na^+/Cl^- ratio in FW compared to SW could indicate a metabolic alkalosis. In sea bass maintained in warm conditions, the absence of a high Na^+/Cl^- ratio following FW transfer might indicate a different handling of acid-base status. This could be linked to an imbalance in branchial ion transporters/channels and uncoupled Na^+ vs Cl^- uptake. If branchial Na^+ uptake is more affected by warm temperatures, it is likely that the gill MRCs mainly involved in Na^+ uptake (comparable to HR cells in zebrafish (Lin et al., 2006) or PNA- MRCs in rainbow trout (Reid et al., 2003)) are more affected by warm temperatures than other MRC types involved in Cl^- uptake. Na^+ uptake is generally linked to H^+ excretion but has not been elucidated in sea bass. Transporters/channels like the Na^+/H^+ exchangers (NHE2 or 3), the acid sensing ion channel (ASIC) as well as the $\text{Na}^+/\text{HCO}_3^-$ cotransporter 1 (NBC1) and the V-H^+ -ATPase will be investigated in future studies (Hwang and Lee, 2007; Evans, 2011; Hwang, 2011; Hwang et al., 2011, Dymowska et al., 2014). Despite lower Na^+ levels, blood osmolality was higher in FW warm conditions than FW temperate conditions which indicate a strong capacity to osmoregulate and to compensate low Na^+ levels through increased Cl^- uptake at low salinity and high temperatures. This allows fish to stay in lagoon-like habitats characterized by instable (Newton and Mudge, 2003) and sometimes contrasted environmental conditions such as those tested in this study.

4.2 Branchial Na^+/K^+ -ATPase activity and mitochondrion-rich cell area and density

Gill parameters were affected by different temperature regimes. Maximal NKA activity (V_{\max}) (an estimate of the maximized NKA activity reflecting the total amount of enzyme, as stated by Metz et al. (2003)) was higher in FW-acclimated sea bass compared to SW-acclimated fish as was expected from previous investigations (Jensen et al., 1998; Nebel et al., 2005). Increased NKA activity (V_{\max}) in FW is certainly linked to the higher number of MRCs recorded in filaments and lamellae in FW-acclimated fish suggesting increased active transport for ion uptake. Overall, maximal NKA activity (V_{\max}) was lower in warm compared to temperate conditions which suggests decreased net branchial active ion transport in sea bass acclimated to warm temperatures. NKA activities (V_{apparent}) measured at the acclimation temperatures (24 °C and 18 °C) were significantly lower (by around 5-fold) than NKA

activities assayed at 37 °C (V_{\max}) and there were no significant differences between salinity and temperature conditions. In temperate conditions, however, there is a tendency of increased apparent NKA activity (V_{apparent}) in FW but without significant difference, due to the high variability of data. This inconsistency between NKA activities assayed at different temperatures (V_{\max} and V_{apparent}) has already been reported in previous studies (McCarty and Houston, 1977; Doneen, 1981; Metz et al., 2003; Sardella et al., 2004a; Sardella et al., 2008). Assaying apparent NKA activity seems essential for proper physiological interpretation and has, to our knowledge, been done for the first time in sea bass gills. Our results clearly show that using physiological assay conditions, FW transfer results in a slight though not significant increased NKA activity (V_{apparent}) in temperate conditions, contrary to warm conditions where NKA activity (V_{apparent}) does not change following salinity transfer. The difference between assay temperatures could be linked to the differential activation of different isoenzymes (like NKA alpha isoforms, see Blondeau-Bidet et al., 2016) in warm compared to temperate waters, which remains to be investigated in future studies.

As stated above, FW-acclimated sea bass have a higher density of MRCs on lamella and gill filaments, which is consistent with other studies dealing with FW transfer in euryhaline teleosts (Lin et al., 2003; Seo et al., 2009; Riou et al., 2012) and has been previously shown in sea bass (Varsamos et al., 2002; Nebel et al., 2005; Bossus et al., 2013). What is new in this investigation is that high temperatures negatively affect MRC number and area in FW. We hypothesize that this decrease could lower branchial ion uptake capacities and might also affect hydromineral balance after long term FW acclimation (>2 weeks). The lower size and number of MRCs may also partly explain that NKA activity (V_{apparent}) does not increase in FW warm compared to FW temperate conditions. In common carp, temperature seems also to affect MRC size with larger MRCs in 15 vs 29 °C acclimated fish (Metz et al., 2003). In sea bass, contrary to FW conditions, MRC size was not affected in SW conditions, which partially explains maintenance of hydromineral balance in warm SW-acclimated fish.

4.3 Gill remodeling, an essential response to environmental changes

Gill remodeling occurred in sea bass exposed to different salinity and temperature regimes. Several studies have addressed gill remodeling in fish related to temperature and/or salinity changes that are likely to affect the osmorepiratory compromise (Sollid et al., 2005; Sardella and Brauner, 2007; Mitrovic and Perry, 2009; Perry et al., 2010; Barnes et al., 2014). Negative interaction between gas exchange and ion regulation has been postulated in several studies (Randll et al., 1972; Mitrovic and Perry, 2009; Turko et al., 2012). In SW at 24 °C,

lamellar width was increased compared to SW at 18 °C. This is not linked to the MRCs, as these cells are located on the filament only. Increased lamellar width is probably linked to a thickening of respiratory cells and might increase blood to water distance and affect gas exchange in warm SW. Sea bass transferred to FW in control temperate conditions show higher lamella width and filament thickness, and lower lamellar length, all parameters probably linked to the recruitment of numerous MRCs required for effective ion uptake. At the temperature ranges that we have used in our study, low-salinity waters have generally higher dissolved O₂ levels than SW (5.2 ml/L in distilled water compared to 6.4 ml/L at 35 ‰ at a temperature of 20 °C, according to Ivanoff (1972)). We can thus suggest that sea bass maintained in temperate FW have higher dissolved O₂ levels than those maintained in temperate SW and seem to have a sufficient lamellar surface area to maximize diffusive gas transfer. The observed gill remodeling (following FW transfer in temperate conditions) seems to be in favor to maximize ion uptake. Chatelier et al. (2005) addressed oxygen uptake rate in sea bass maintained at 14 °C across salinities ranging from FW to SW and did not show any difference between salinities, however further studies should address oxygen uptake in this species, as only three fish were analyzed in FW. SW to FW transfer in warm conditions affected all measured parameters in an opposite way than what was observed in temperate conditions (decreased lamella width, increased lamella length and slightly decreased filament thickness in FW warm). This can be explained partially by a lower increase of MRC density in FW warm (compared to FW temperate) and the recruitment of smaller MRCs in FW warm conditions. The high lamella length in warm FW suggests a stronger need for O₂ uptake in this media, as O₂ is less available in warm waters. These data suppose that gill remodeling following FW transfer occurs to maximize gas exchange rather than osmoregulation in warm waters. Our results are inconsistent with the study on five coral reef fishes investigated at temperatures ranging from 29 °C to 34 °C, where the base of lamellae and length of the lamellae did not change with increasing temperatures (Bowden et al., 2014). It is likely that high temperatures are experienced differently between temperate and tropical reef fishes. Absence of gill remodeling could be disadvantageous in tropical fishes that have to maintain O₂ uptake mechanisms at high temperatures, except if other mechanisms compensate for low environmental O₂ conditions. In response to air exposure or hypoxia, gill morphology of *Kryptolebias marmoratus* and *Gymnocypris przewalskii* also showed high plasticity and remodeling capability (Ong et al., 2007; Matey et al., 2008).

Apical structure of the branchial epithelium of sea bass has already been shown by Varsamos et al. (2002) at different salinities. As shown in numerous species, salinity clearly affects

apical opening with small apical pits in SW to favor ion secretion and large MRC openings in FW to optimize ion uptake. No change in apical openings of MRCs could be observed in our study among temperatures, but measurements of apical areas could not be done due to numerous mucous cells masking apical cell surfaces. There was a clear difference regarding apical microridges of pavement cells (PVCs) between sea bass acclimated to different temperatures in SW. As more than 90 % of the gill surface correspond to PVCs, the convoluted microridges present on the PVCs have been defined as important interaction areas between the branchial epithelium and ambient water (Wilson and Laurent, 2002). Changes in apical microridges of pavement cells have been reported in other species during transfer to SW and/or FW (Daborn et al., 2001; Carmona et al., 2004; Sardella et al., 2004b; Seo et al., 2009; Choi et al., 2011; Chasiotis et al., 2012). In our study, changes in microridges surfaces were observed in warm temperature-challenged fish in SW only, probably as a response to increased temperatures and possibly linked to a change in membrane fluidity, but further investigations are required to prove that.

5. Conclusions

In summary, we have focused on the effect of salinity (SW vs FW) and temperature (18 temperate vs 24 °C warm) that are representative of environmental conditions encountered by migrating sea bass in the wild, with a focus on the onset of osmoregulatory mechanisms following FW transfer. Sea bass are euryhaline and eurythermic, as they are able to maintain relative high osmolalities in fresh water at both temperatures tested. At the gill level however, a high plasticity has been observed and gill remodeling seems to occur notably following FW transfer at both temperatures. It seems that gill remodeling occurs to maximize FW ion uptake in temperate water which is not the case in warm FW, where diffusive gas exchange seems to be maximized. NKA activity, MRC size and density seem to be lower in warm FW which also points to an altered branchial ion uptake capacity in warm FW, probably through an imbalance between Na^+ and Cl^- uptake mechanisms. We conclude that salinity and temperature stress seems to affect sea bass gill epithelium and it could be expected to encounter a more severe alteration of hydromineral balance following long-term FW transfer (> 2 weeks) at this warm temperature due to osmorepiratory compromise.

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Figure legends

Fig. 1. Blood osmolality (A), plasma sodium (B) and chloride concentration (C) of sea bass exposed to SW and FW at 18 °C (temperate) and 24 °C (warm). Different letters indicate significant differences between conditions (two-way ANOVA followed by a Fisher LSD post-hoc test, $p < 0.05$, $N = 8-12$). The table above each graph gives the results of two-way ANOVA with temperature and salinity as the main factors. Data are expressed as means \pm SEM. FW: fresh water; SW: seawater.

Fig. 2. Gill Na^+/K^+ -ATPase (NKA) activity of sea bass exposed to SW and FW at 18 °C (temperate) and 24 °C (warm). NKA activity was assayed at optimal temperature (V_{max}) and at the acclimation temperature of the fish (V_{apparent}). Different letters (V_{max}) or numbers (V_{apparent}) indicate significant differences between conditions (two-way ANOVA followed by a Fisher LSD post-hoc test, $p < 0.05$, $N = 8-12$). The table gives the results of two-way ANOVA with temperature and salinity as the main factors. Data are expressed as means \pm SEM. FW: fresh water; SW: seawater.

Fig. 3. Immunolocalization of gill Na^+/K^+ -ATPase of sea bass exposed to SW (A, B) and FW (C, D) at 18 °C (temperate, A, C) and 24 °C (warm, B, D). F: filament, L: lamellae, FW: fresh water, SW: seawater, Scale bar: 20 μm .

Fig. 4. Mitochondrion-rich cell number on filaments and lamellae per 300 μm of gill filament and MRC area (B) in sea bass exposed to SW and FW at 18 °C (temperate) and 24 °C (warm). Different letters (filaments) and numbers (lamellae) indicate significant differences between conditions (Kruskal-Wallis test followed by a Dunn's multiple comparison test, $p < 0.05$, $N=3$, 10-25 measurements per individual). Data are expressed as means \pm SEM. FW: fresh water; SW: seawater.

Fig. 5. Masson's Trichrome staining of gills in sea bass exposed to SW (A, B) and FW (C, D) at 18 °C (temperate, A, C) and 24 °C (warm, B, D) and schematic diagram (E). F: filament, L: lamellae, F.th.: filament thickness, L.l.: lamellar length, L.wd.b.: lamellar width at base, L.wd.t.: lamellar width at top, FW: fresh water, SW: seawater, Scale bar: 20 μm .

Fig. 6. Lamella width at the top (A), lamella width at the base (B), lamella length (C) and filament thickness (D) of sea bass exposed SW and FW at 18 °C (temperate) and 24 °C (warm). Different letters indicate significant difference between conditions (Kruskal-Wallis test followed by a Dunn's multiple comparison test, $p < 0.05$, $N=3$, 18-27 measurements per individual). Data are expressed as means \pm SEM. FW: fresh water; SW: seawater.

Fig. 7. Scanning electron micrographs of sea bass gills exposed to SW (A, B) and FW (C, D) at 18 °C (temperate, A, C) and 24 °C (warm, B, D). Arrows indicate apical pits of mitochondrion-rich cells (MRCs) in SW (A, B) and large apical MRC openings in FW (C, D). Note that apical microridges of pavement cells (PC) are less developed in warm SW (B). F: filament, L: lamellae, FW: fresh water, SW: seawater. Scale bar: 2 μ m.

Table 1. Plasma Na^+/Cl^- ratio of sea bass exposed to SW and FW at 18 °C (temperate) and 24 °C (warm). Data are expressed as means \pm SEM (N). Different letters indicate significant differences between conditions (Dunn's test, $p < 0.05$). FW: fresh water; SW: seawater.

SW 18 °C	FW 18 °C	SW 24 °C	FW 24 °C
1.12 ± 0.04^a (9)	1.65 ± 0.12^a (8)	0.93 ± 0.02^b (13)	$1.07 \pm 0.03^{a,b}$ (13)

Figure 1

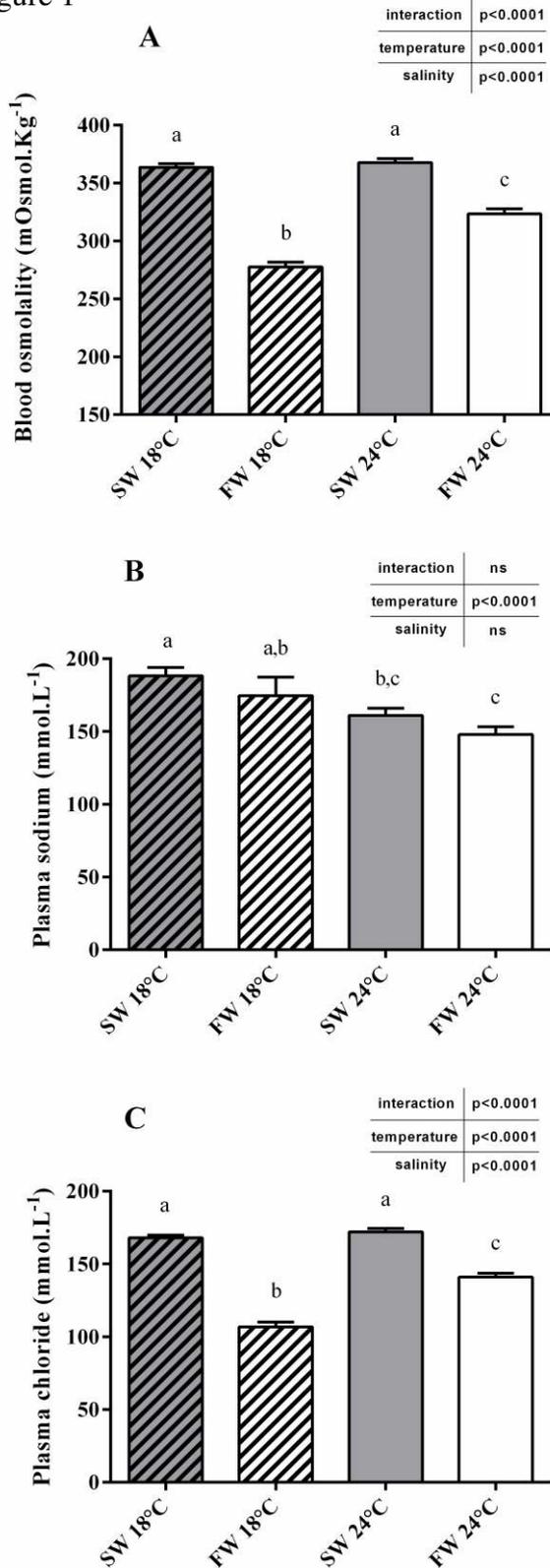


Figure 2

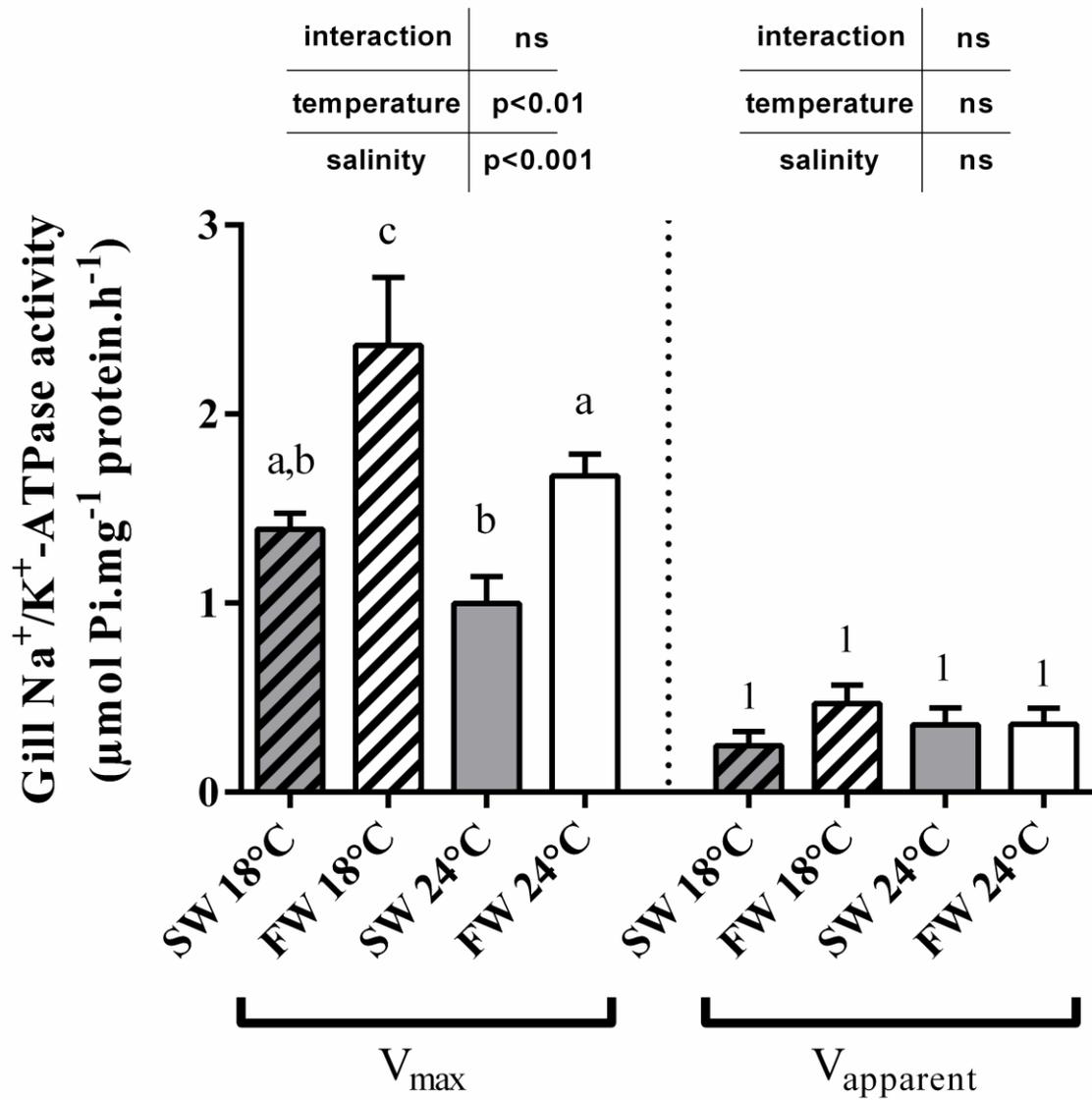


Figure 3

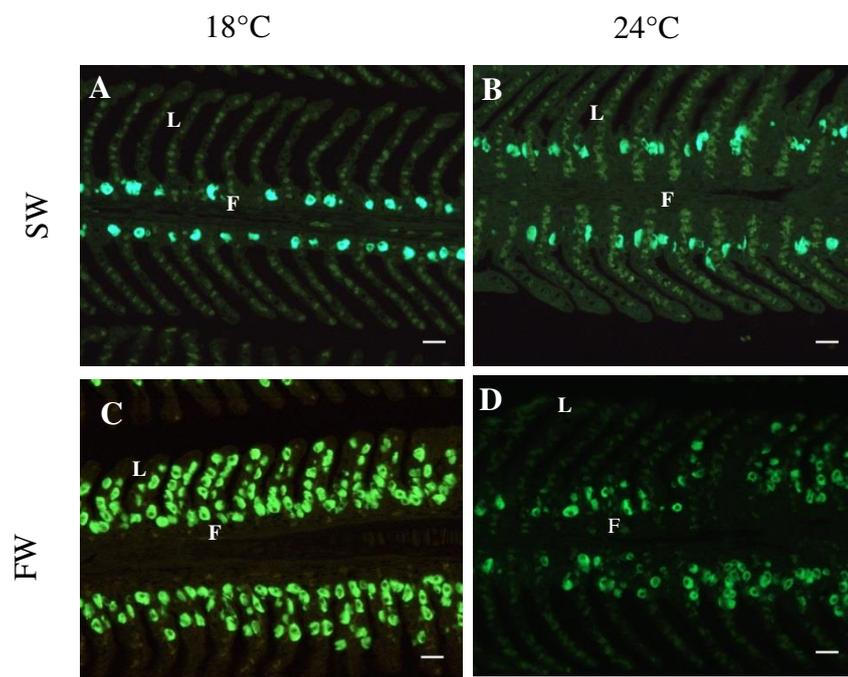


Figure 4

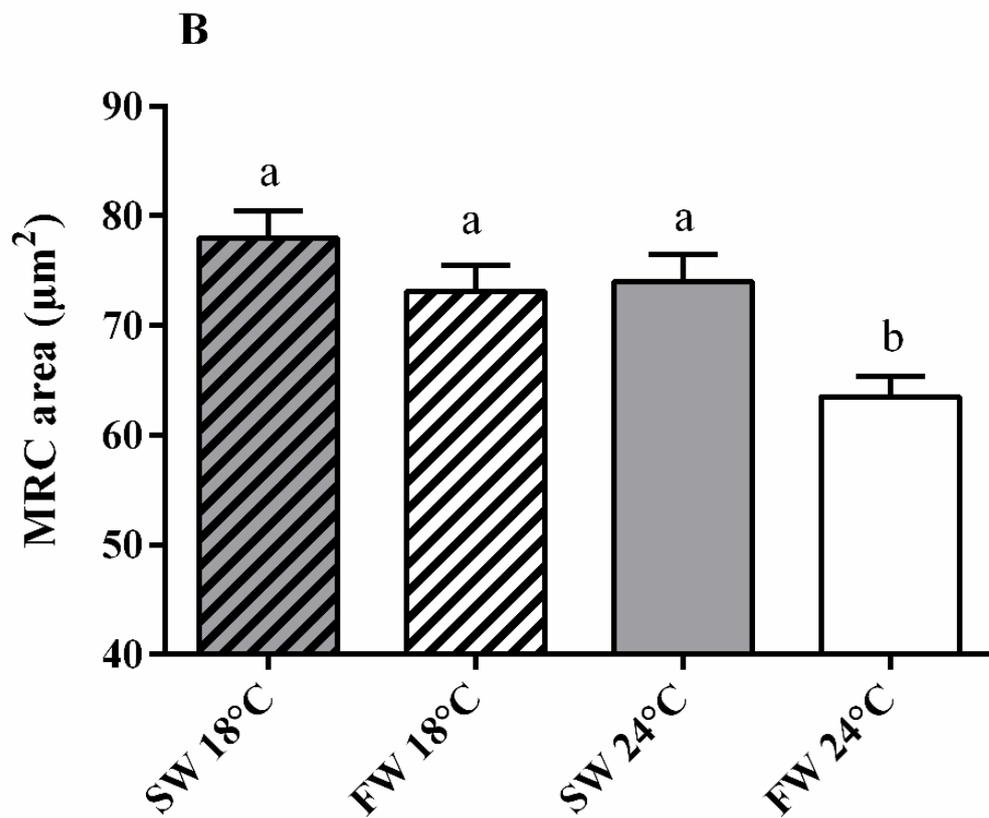
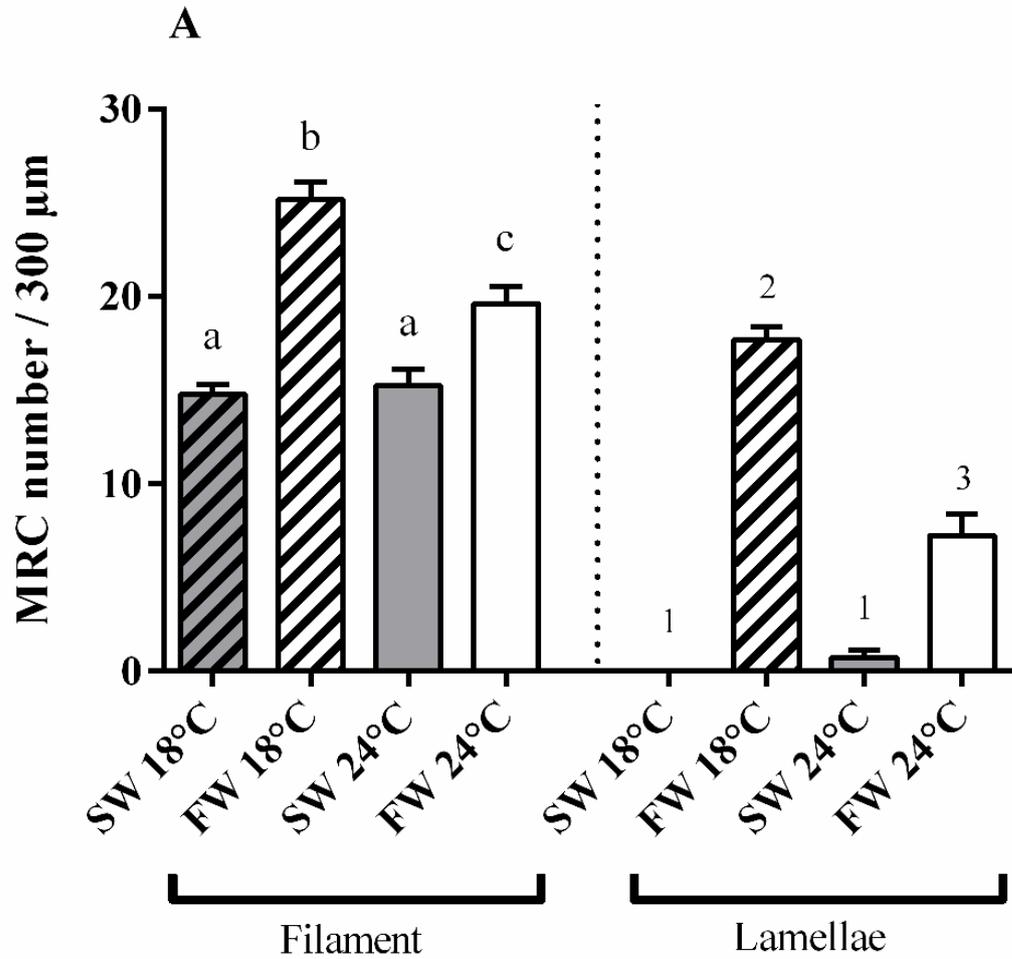


Figure 5

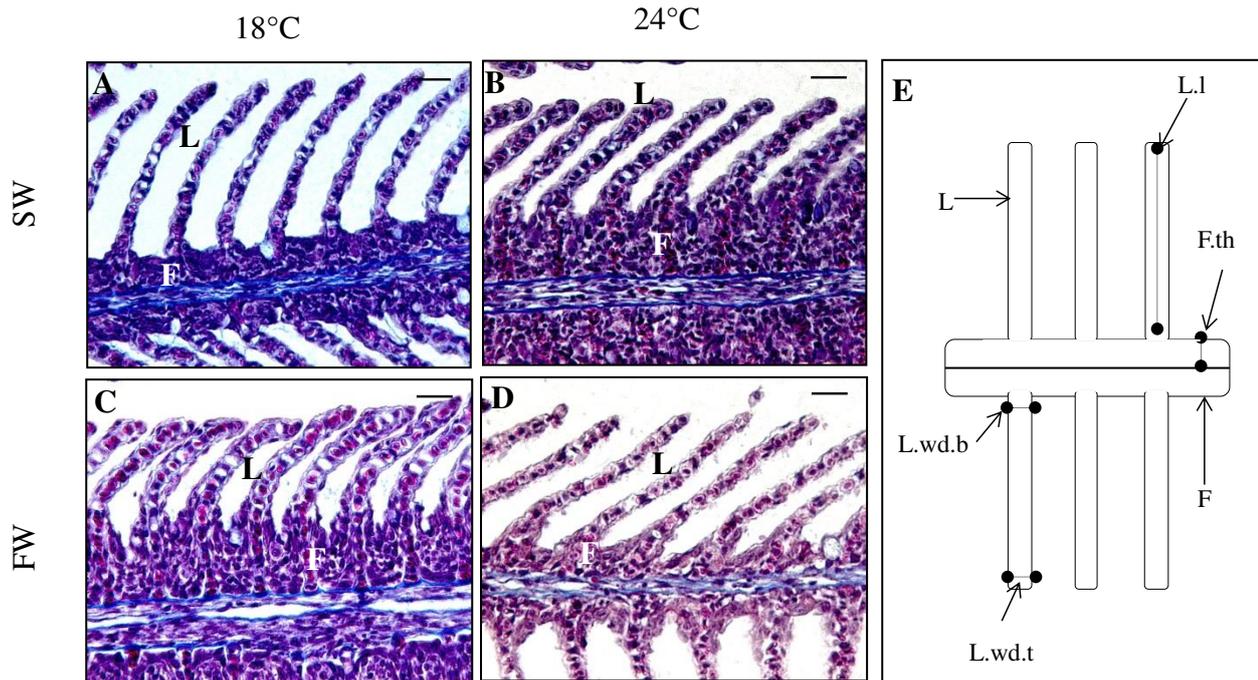


Figure 6

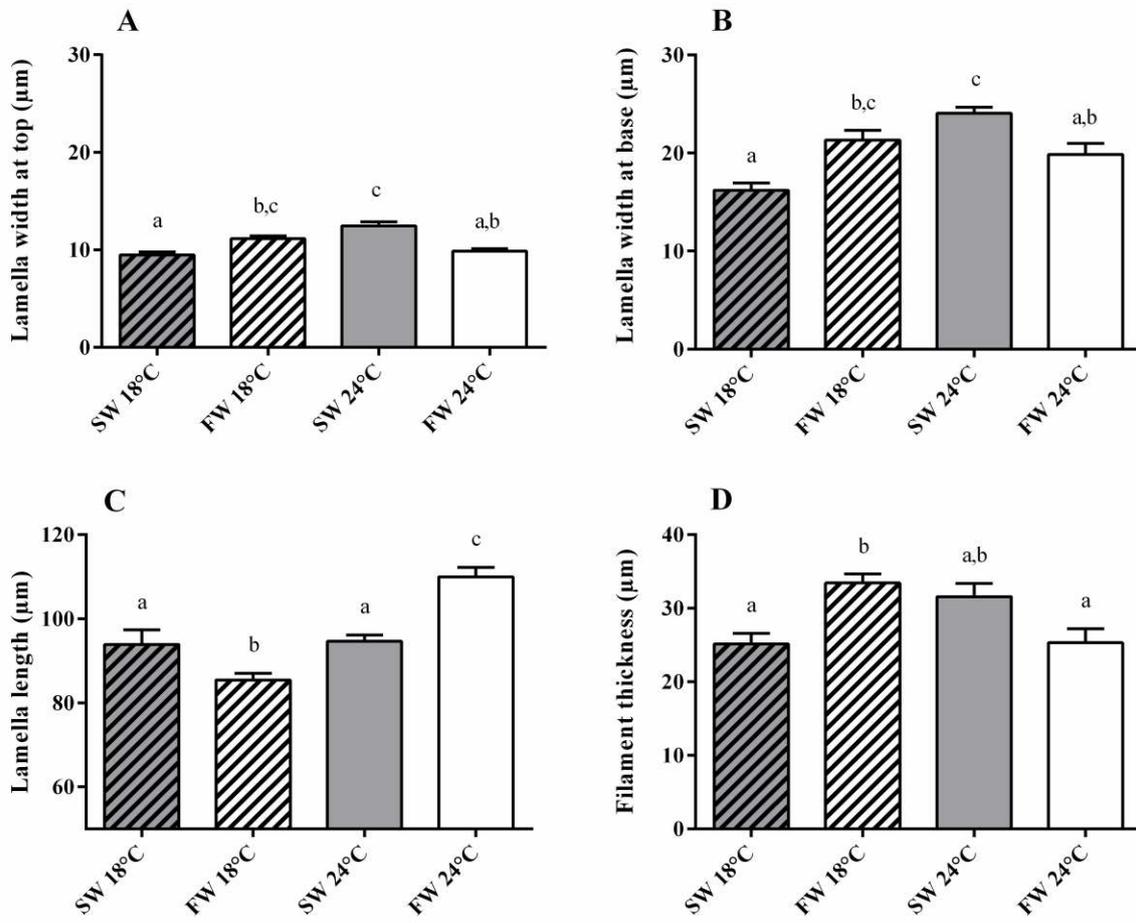


Figure 7

