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Combined use of environmental data and biomarkers in fish (*Liza aurata*) inhabiting a eutrophic and metal-contaminated coastal system - gills reflect environmental contamination

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ABSTRACT

An investigative biomonitoring study was carried out in a eutrophic coastal system with a moderate contamination by metals (Óbidos lagoon, Portugal), combining the evaluation of exposure concentrations with metals accumulation and oxidative stress responses in gills of the golden grey mullet (*Liza aurata*). Two contrasting seasons (winter and summer) were considered at three sites: Barrosa (BB) and Bom-Sucesso (BS) branches; Middle lagoon (ML). Data on the water column pointed to a higher metals and nutrients availability at BB that was reflected in the higher metal concentrations in gills, particularly in winter. Similarly, oxidative stress responses demonstrated a pro-oxidant challenge at BB (winter and summer), which was corroborated by an integrated biomarker response index (IBR). Metal concentrations in gills were higher in summer than winter, reflecting the increased environmental concentrations in combination with elevated metabolic rates. Catalase (CAT), glutathione-S-transferase (GST), total glutathione (GSH_t) and lipid peroxidation (LPO) increases observed in winter at BB were related with metal accumulation, while summer enhancement of glutathione peroxidase (GPx), glutathione reductase (GR), GST and GSH_t was associated with other stressors. Inter-site differences on the basis of IBR were more accentuated in winter. Gills can be considered as an important route of entry for contaminants and were demonstrated to reflect water contamination and are therefore useful in the context of environmental assessment.

KEYWORDS: Oxidative stress; fish; metals; coastal lagoon; temporal variability.

1. INTRODUCTION

The suitability of fish gills for environmental biomonitoring was recommended by several recent works (Fernandes et al., 2008; Dautremepuits et al., 2009; Oliveira et al., 2009). In fact, gills have a wide surface area that is continuously in contact with the external medium and are thus the main uptake route of contaminants from aqueous phase (Playle, 1998). On the other hand, fish gills are able to accumulate chemicals that were taken up by other exposure routes, due to their position between the venous and arterial circulation, thus receiving nearly all of the cardiac output (Levine and Oris, 1999). Moreover, gills play a key role in fish physiology, such as gaseous exchange, osmotic and ionic regulation, acid-base balance and nitrogenous waste (Ahmad et al., 2008). It is documented that fish gills respond earlier than kidney and liver under field contamination exposure (Ahmad et al., 2004; Santos et al., 2004). Its applicability is also enhanced by the fact that gills' epithelium suffers an increased cell division to counteract its exfoliation and erosion and by this feature gills usually reflect current exposures (Mieiro et al., 2009). This seems to be particularly relevant when fish, with their non-sessile behaviour, are used as bioindicators.

Monitoring the state of coastal systems has traditionally been largely based on measurements of concentrations of contaminants in sediment and water column. However, this chemical monitoring alone is no longer considered meaningful or cost-effective. Additionally, it has become evident that measuring concentrations in the tissues does not provide the information needed to assess biological effects that contaminants may cause in organisms (Lehtonen and Schiedek, 2006). In fact, contaminants usually appear in the environment as very complex mixtures that can cause interactive effects on the biota impossible to evaluate only by means of chemical analyses. Thus, recent investigative monitoring studies have involved the combined measurement of environmental concentrations and body burdens, together with the evaluation of biomarkers, which represent early signs of biological effects (Van der Oost et al., 2003). In this context, many pollutants are capable of inducing oxidative stress in aquatic animals by disturbing the antioxidants efficiency and enhancing the intracellular reactive oxygen species (ROS), which often prelude in DNA damage, lipid peroxidation (LPO) and enzyme inhibition (Van der Oost et al., 2003). Antioxidant defence systems include antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione

peroxidase (GPx) and glutathione reductase (GR), as well as non-enzymatic antioxidants (e.g. glutathione – GSH) which have been extensively used as biomarkers of oxidative stress (Ahmad et al., 2006; 2008; Guilherme et al., 2008). LPO estimation has also been found to have a high predictive value as a biomarker of effect (Guilherme et al., 2008). To our knowledge, only a few studies have combined the measurement of oxidative stress in fish gills with concentrations of accumulated contaminants (Hansen et al., 2006; Fernandes et al., 2008). Additionally, an investigative approach with the previous information and environmental contamination levels is more uncommon (Hansen et al., 2006).

Previous studies have employed the golden grey mullet (*Liza aurata*) as a model species on field studies (Oliveira et al., 2009; Guilherme et al., 2008) and under laboratory conditions (Cionna et al., 2006; Oliveira et al., 2007). In fact, *L. aurata* has favourable features as a sentinel of contaminated coastal systems. This is a pelagic species that is in frequent contact with sediments, filtering its superficial layer and suspended particulate matter (SPM) in the water column. Moreover, mullets play an important role in the estuarine trophic web (Almeida, 2003).

In order to access the environmental health of a coastal lagoon impacted by eutrophication and moderate metal contamination (Óbidos lagoon, Portugal), gills of *Liza aurata* were monitored for metal accumulation and oxidative stress in two contrasting seasons: winter and summer. Peroxidative damage and antioxidant (enzymatic and non-enzymatic) responses were measured in *L. aurata* gills and their relationship with metal accumulation as well as with environmental chemical data (metals and nutrients) was assessed. It was also intended to evaluate the suitability of the adopted combined strategy (external levels of exposure/bioaccumulation markers/oxidative stress responses) applied to fish gills on monitoring coastal ecosystems.

2. MATERIALS AND METHODS

2.1. Study area

The Óbidos lagoon is a shallow coastal ecosystem, located on the west coast of Portugal with a wet area of 7 km², permanently connected to the sea through a narrow inlet (Figure 1). It comprises areas of different morphological and sedimentary characteristics: sand banks and

narrow channels in the lower/middle lagoon; muddy bottom sediments in the two inner branches (Barrosa and Bom-Sucesso). The Barrosa branch is shallower (mean depth 0.5-1 m) compared to the Bom-Sucesso (mean depth 1-2 m) and water circulation is mostly driven by tides and by a small tributary (Cal River) that drains agricultural fields. Urban effluents from a nearby town (Caldas da Rainha, 50 000 inhabitants) also have been discharged to the Barrosa branch by the Cal River. Consequently, this area presents the highest nutrient availability of the lagoon, being classified as eutrophic (Pereira et al., 2009a). High nutrient concentrations were in line with abundant macroalgae (*Ulva* sp. and *Enteromorpha* sp.) and a broad daily variation of dissolved oxygen concentration during the summer months (Pereira et al., 2009b). Previous water quality surveys of the lagoon showed maximum concentrations of Mn, Ni, Cu and Cd in Barrosa branch. Major metal sources were identified to be the Cal River in periods of higher inflow and remobilization from sediments in summer months (Pereira et al., 2009b). The Bom-Sucesso branch is also a confined area but receives a smaller freshwater flow (Vala do Ameal) with better water quality than the Cal River, according to the Portuguese categorization of freshwater systems. In this area, metal remobilization from sediments is less likely due to the greater depth. The middle lagoon was previously characterised by coarser sediments with a low affinity for metals (Carvalho et al., 2006), as well as by a better water quality (Pereira et al., 2009a). Moreover, previous ecotoxicological studies with *Ulva* sp. and *Carcinus maenas* pointed out Barrosa branch as a critical area of the lagoon with potential impact on autochthonous populations, whereas the lower/middle lagoon was considered the reference area (Pereira et al., 2009c; 2009d).

2.2. Sampling

Two surveys were carried out at Óbidos lagoon in winter (December 2006) and summer (July 2007) during low-tide and juveniles of the golden grey mullet (*L. aurata*) were collected (n=10) using a traditional beach-seine net named “chinha”. Three sampling areas were selected taking into account previous ecotoxicological studies (Pereira et al., 2009c, 2009d) (Figure 1): Middle lagoon (ML); Bom-Sucesso (BS) and Barrosa (BB) located in the inner branches of the lagoon. Fish biometrical parameters, such as weight and length ranged from 20-40 g and 13-17 cm. Juveniles were selected in order to minimize any gender interference

and reduce the background arising from previous exposures to contaminants (Ahmad et al., 2008). Immediately after catching, fish were sacrificed and gills were removed and frozen in liquid nitrogen in two sets of samples: one for oxidative stress quantifications and other corresponding to the rest of the organ for metal determinations. In the laboratory, samples were preserved until further processing at -80 and -20 °C for oxidative stress and metal determinations, respectively.

Surface water (0.2 m depth) was sampled in the three areas for determinations of nutrients, chlorophyll a and metals.

2.3. Determination of physicochemical parameters and metals in water

Water temperature, salinity and dissolved oxygen were measured in situ using a YSI, 650 meter. Water samples for determinations of nitrate+nitrite ($\text{NO}_3^- + \text{NO}_2^-$), ammonium (NH_4^+), phosphate (PO_4^{3-}) and silicate $\text{Si}(\text{OH})_4$ were filtered through MSI Acetate Plus filters and analyses carried out using an autoanalyser TRAACS 2000 (Bran+Luebbe). For chlorophyll a determinations, water was filtered through a Whatman GF/F (0.7 μm) filter that was immediately frozen at -20 °C and later extracted in 90% acetone, for analysis in a Perkin Elmer Fluorometer using the protocol modified by Lorenzen (1966).

Manganese, Cu, Ni, Cr, Pb and Cd in the collected waters (triplicate samples) were measured using diffusive gradients of thin films (DGT). All DGT holders, Chelex-100 resins and diffusive gels (type APA, 0.8-mm thickness, open pore > 5 nm) (Zhang and Davison, 1999) were purchased from DGT Research (Lancaster, UK). The DGT devices were deployed in 2-L polypropylene bottles with unfiltered sampled water and stirred at constant temperature for 48 hours. After devices retrieval, resins were eluted by immersion in 5 mL of 1 M HNO_3 (prepared from suprapur nitric acid) at a minimum of 24 h. Eluates were analysed directly by a quadrupole inductively coupled plasma mass spectrometer (ICP-MS) (Thermo Elemental, X-Series). All eluates were analysed with reagents blanks and an international standard of river water (SLRS-4) used to control eventual contaminations during the analytical procedure and the procedure accuracy, respectively. The eluate concentration was converted into the mass of metal accumulated on the resin using an elution factor with a yield value of 0.8 (Zhang and Davison, 1995) and a resin gel volume of 0.15 mL. DGT-metal concentrations were calculated according

to the following equation: $[Metal] = (M \Delta g) / (tAD)$; where M is the mass of metal accumulated on the resin during the emersion time (t); Δg the diffusive gel thickness (0.08 cm); A the exposure area (3.14 cm²); and D the diffusion coefficient of the metal in the gels as provided by DGT Research.

Water was collected in triplicates for separation of suspended particulate matter (SPM), which was retained on 0.45 µm membranes that were mineralized completely with HF (40%) and Aqua Regia (HCl-36%:HNO₃-60%; 3:1) in closed Teflon bombs (100°C for 1 h), evaporated to near dryness (DigiPrep HotBlock – SCP Science), redissolved with 1 mL of doubled-distilled HNO₃ and 5 mL of ultra-pure water, heated for 20 min at 75 °C, and diluted to 50 mL with ultra-pure water. Reagents blanks and international certified standards of sediments from the National Research Council of Canada (1646a; BCSS-1; MESS-3) were prepared in a similar way of samples to control the accuracy of the procedure. The concentrations of Mn and Zn were determined by flame atomic absorption spectrometry (Perkin Elmer Analyst 100) whereas Cu, Ni, Cr, Pb and Cd were determined by ICP-MS. Levels of the analysed elements obtained in the reference materials were consistently within the ranges of certified values.

2.4. Metal analyses in gills

Approximately 50 mg of freeze dried tissue was digested with a mixture of HNO₃ (doubled distilled from 65%) and H₂O₂ (suprapure, 30%) at 60 °C for 12 h, at 100 °C for 1 h and at 80 °C for 1 h according to the method described in Pereira et al. (2009e). Procedural blanks were prepared using the same analytical procedure and reagents. Concentrations of Mn, Zn, Cu, Ni, Cr, Pb and Cd were determined by ICP-MS. International certified standards (TORT-2, DOLT-2 and DOLT-3) were used to control the accuracy of the analytical procedure.

2.5. Biochemical analyses in gills

Tissue handling and preparation of post-mitochondrial supernatant (PMS) fraction - Tissue samples were homogenized, using a Potter-Elvehjem homogenizer, in chilled phosphate buffer (0.1 M, pH 7.4) (1 g of tissue/10 ml buffer). This homogenate was then divided in two aliquots for LPO and PMS preparation. The PMS preparation was obtained by

centrifugation in a refrigerated centrifuge (Optima TL, Beckman) at 13,400 g for 20 min at 4 °C. Aliquots of PMS were divided in microtubes and stored at -80 °C until analyses.

Estimation of lipid peroxidation (LPO) - LPO was determined in the previously prepared homogenate as adapted by Filho et al. (2001). Briefly, to 150 µl of homogenate, 10 µl of 1-1 butylated hydroxytoluene (4% in methanol) was added and mixed well to prevent oxidation. The absorbance of each sample was measured at 535 nm. The rate of LPO was expressed in nmol of thiobarbituric acid reactive substances (TBARS) formed per milligram of fresh tissue using a molar extinction coefficient of $1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$.

Catalase (CAT) activity measurement - CAT activity was assayed in PMS by Claiborne (1985) method (at 25 °C). Briefly, the assay mixture consisted of 1.95 ml phosphate buffer (0.05 M, pH 7.0), 1 ml hydrogen peroxide (0.019 M) and 50 µl of sample in final volume of 3 ml. Change in absorbance was recorded at 240 nm and CAT activity was calculated in terms of µmol H₂O₂ consumed min⁻¹mg⁻¹ protein using a molar extinction coefficient of $43.5 \text{ M}^{-1} \text{ cm}^{-1}$.

Glutathione peroxidase (GPx) activity measurement - GPx activity was determined in PMS according to the method described by Mohandas et al. (1984) with some modifications (at 25 °C). The assay mixture consisted of 0.72 ml phosphate buffer (0.05 M, pH 7.0), 0.05 ml EDTA (1 mM), 0.05 ml sodium azide (1 mM), 0.025 ml GR (1 IU/ml), 0.05 ml GSH (4 mM), 0.05 ml NADPH (0.8 mM), 0.005 ml H₂O₂ (1.0 mM) and 0.05 ml of PMS in a total volume of 2 ml. Oxidation of NADPH was recorded at 340 nm, and GPx activity was calculated in terms of nmol NADPH oxidized min⁻¹mg⁻¹ protein using a molar extinction coefficient of $6.22 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$.

Glutathione reductase (GR) activity measurement - GR activity was assayed by the method of Cribb et al. (1989) with some modifications (at 25 °C). Briefly, the assay mixture contained 0.025 ml of PMS fraction and 0.925 ml of NADPH (0.2 mM), GSSG (1 mM) and DTPA (0.5 mM). The enzyme activity was quantified by measuring the disappearance of NADPH at 340 nm during 3 min. The enzyme activity was calculated as nmol NADPH oxidized min⁻¹mg⁻¹ protein using a molar extinction coefficient of $6.22 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$.

Glutathione-S-transferase (GST) activity measurement - GST activity was determined using CDNB (1-chloro-2,4-dinitrobenzene) as substrate according to the method of Habig et al. (1974) (at 25 °C). The assay was carried out in a quartz cuvette with a 2 ml mixture of phosphate buffer (0.2 M, pH 7.4), CDNB (0.2 mM) and 0.2 M GSH. The reaction was initiated by addition of 10 µl of PMS and the increase in absorbance was recorded at 340 nm during 3 min. The enzyme activity was calculated as nmol CDNB conjugate formed $\text{min}^{-1}\text{mg}^{-1}$ protein using a molar extinction coefficient of $9.6 \text{ mM}^{-1}\text{cm}^{-1}$.

Total glutathione (GSH_t) content measurement - Protein content in the tissue homogenate was precipitated with TCA (5%) for 1 h and then centrifuged at 13,400 g for 20 min at 4 °C. GSH_t content was determined in the resulting supernatant (deproteinated PMS) (at 25 °C) adopting the enzymatic recycling method using glutathione reductase (GR) excess, whereby the sulfhydryl group of GSH reacts with DTNB (Ellman's reagent) producing a yellow coloured 5-thio-2-nitrobenzoic acid (TNB). The rate of TNB production is directly proportional to this recycling reaction, which is in turn directly proportional to the concentration of GSH in the sample. Formation of TNB was measured by spectrophotometry at 412 nm. It should be noted that GSSG is converted to GSH by GR in this system, which consequently measures total GSH. The results were expressed as nmol TNB formed $\text{min}^{-1} \text{mg}^{-1}$ protein using a molar extinction coefficient of $14.1 \times 10^3 \text{ M}^{-1}\text{cm}^{-1}$.

Protein measurement - Total protein contents were determined according to the Biuret method (Gornall et al., 1949), using bovine serum albumin (E. Merck-Darmstadt, Germany) as a standard.

2.6. Statistical analyses

A method for combining all the measured oxidative stress endpoints into a general index - Integrated Biomarker Response (IBR) (Beliaeff and Burgeot, 2002) - was applied to data of both surveys. The basis of data processing is described here briefly. First, the mean value (X_i) for each biomarker at each sampling area and season was calculated. In addition, the general mean (m_i) and standard deviations (s_i) of each biomarker were estimated for all

sampling areas and seasons. The value of X_i was then standardized to obtain Y_i , where $Y_i = (X_i - m_i)/s_i$. Then Z_i was computed via the equation $Z_i = -Y_i$ or $Z_i = Y_i$ in the case of a biological effect corresponding, respectively, to inhibition or activation. The minimum value (Min_i) of Z_i for each biomarker was calculated for all sampling areas and seasons, and then the score S_i was computed as $S_i = Z_i + |\text{Min}_i|$, where $|\text{Min}_i|$ is the absolute value. Finally, IBR for each sampling site and season was calculated via the following formula: $\text{IBR} = (S_1 \times S_2)/2 + (S_2 \times S_3)/2 + \dots + (S_{n-1} \times S_n)/2 + (S_n \times S_1)/2$; in which the obtained score for each biomarker (S_i) is multiplied with the score of the next biomarker (S_{i+1}), arranged as a set, dividing each calculation by 2 and summing-up of all values.

Statistical software (Statistica 7.0) was used for statistical analyses. The assumptions of normality and homogeneity of data were verified. ANOVA analysis was used to compare sampling sites and Tukey test was applied for Post-hoc comparison (Zar, 1996). Differences between means were considered significant when $p < 0.05$. The correlation between metals in the water column (DGT-measured and SPM fractions) and accumulated in gills, as well as between accumulated metals and oxidative stress responses in gills was tested (considering the mean values of the two surveys at each site) by Pearson analysis ($p < 0.05$).

3. RESULTS

3.1. Water characteristics

Water temperature was slightly higher at BB and BS in summer and winter, while salinity was lower at BB than at the other sites, particularly in winter (Table 1). In general, dissolved oxygen was around 100% but undersaturation was recorded at BB in summer and BS in winter. In general, the maximum values of nitrogenous compounds, Si(OH)_4 , PO_4^{3-} and chlorophyll a were recorded at BB in both surveys. NH_4^+ , $\text{NO}_3^- + \text{NO}_2^-$ and Si(OH)_4 exhibited a typical temporal fluctuation in the lagoon with lower values in summer. These site and seasonal differences were consistent with previous measurements (Pereira et al., 2009a).

Table 2 presents the levels of metals in water (DGT-measured fraction) at the three sites in winter and summer. BB presented significantly higher levels of Cu in winter than ML. In summer, Cu was significantly higher at BB (than ML and BS), while the opposite pattern was observed for Mn. Moreover, in summer Cr was significantly higher at BB and BS than at ML.

Concerning temporal differences, significantly higher levels of Mn and Cd were found in winter, while Cu and Cr enhanced in summer.

Copper and Cd in SPM collected in winter were significantly higher at BB (than ML, ML and BS, respectively) (Table 3). In summer, Cu, Ni and Pb in SPM were higher at BB, while SPM from ML contained higher levels of Mn, Zn and Cd than other sites (though without statistical significance). Moreover, Ni increased significantly in summer in relation to winter (BB) whereas Pb (ML) and Cd (BB) were significantly higher in winter than in summer.

3.2. Metals in *L. aurata* gills

In winter, gills of fish from BB showed significantly higher accumulation of Zn, Cu, Cr and Cd than ML (Figure 2). Moreover, concentrations of Cr were significantly higher in gills of fish from BB than BS in winter, whereas at the BS site Zn and Pb were elevated in comparison with ML. In summer, gills at BB showed lower concentrations of Cu (than BS) and Cr (than BS and ML).

Differences between the two surveys were found for all metals, except Mn and Pb. Gills in winter presented higher contents of Cu and Ni (BB) than in summer. In contrast, in summer gills presented higher concentrations of Zn (all sites), Cu (BS), as well as Cr and Cd (ML, BS).

3.3. Oxidative stress in *L. aurata* gills

A spatial analysis of gills antioxidant defences revealed significant increases of CAT (winter), GPx and GR (summer) activities, as well as GSH_t content (winter and summer) at BB relatively to ML and BS (Figure 3). Moreover, GST activity in winter and summer was enhanced significantly at BB in comparison to ML. In terms of peroxidative damage, significantly higher levels were also found at BB (versus ML and BS) in winter. Concerning temporal differences, significantly higher levels were found in summer for: CAT, GPx and GR at all sites; GST at ML and BS; and GSH_t at BB. In addition, LPO was higher in winter than summer at BB.

3.4. Correlations between parameters and IBR calculation

The significant correlations obtained between metal levels in gills versus oxidative stress endpoints are shown in Figure 4 (non-significant correlations were not presented). Positive

correlations were found between activities of GPx, GR and GST and accumulated levels of Zn, Cd and Cu, respectively. Significant correlations were not found between metals in the water column (DGT-measured fraction and SPM) and accumulated in *L. aurata* gills.

The antioxidants and LPO values measured in gills were processed in order to calculate the IBR index (Figure 5). IBR values showed different variation ranges in winter and summer, being higher in summer. Even so, in both surveys the computed IBR values were always higher at BB than other sites. In particular, the estimated IBR value for BB was 100 times higher than ML in winter, whereas in summer the difference between the two sites was approximately 3 times.

4. DISCUSSION

4.1. Water quality and metal availability

In winter and summer BB exhibited a higher metal availability than BS and particularly ML. This was particular evident for Cu in water (DGT-measured fraction) and SPM, Cr in water, as well as Mn, Ni and Cd in SPM. The tributary that discharges into the BB branch probably contributed to metal inputs, mainly in periods of higher freshwater flows (Pereira et al., 2009b). In fact, levels of water Cd and Mn (all sites) as well as particulate Pb (all sites) and Cd (BB) were higher in winter than in summer. Conversely, values of Cu (all sites) and Cr (BS, BB) in water were higher in summer than winter, when freshwater flows into the lagoon are almost insignificant. Nickel associated with particles also increased in summer at BB. These summer enhancements are probably related with the pulse release of metals from sediments during periods of low oxygenation as indicated by the simultaneous undersaturation of oxygen recorded mainly at BB. This internal source was hypothesized to occur in BB branch (Pereira et al., 2009b). The importance of these sediment-related processes for the water quality is probably lower at BS and ML than at BB due to the higher trophic state of these two areas (Pereira et al., 2009b). In fact, BB was already proposed as a major impacted area in the lagoon being classified as eutrophic (Pereira et al., 2009a). Accordingly, BB exhibited the highest levels of nutrients and chlorophyll a in both seasons. In wet seasons, the tributary that discharges at BB branch introduce large amounts of nutrients into that area of the lagoon (Pereira et al., 2009a). Since PO_4^{3-} regeneration could occur in organically-rich sediments with upwards

diffusion during periods of low oxygenation (mainly in summer), this should be considered as an additional input to the water column at BB as previously recorded (Pereira et al, 2009b).

4.2. Metal concentrations in *L. aurata* gills and associations with environmental factors

Gills are the first organ to be in contact with water and resuspended sediment particles, so they can be relevant sites of interaction with metal ions (Fernandes et al., 2007). In winter, gills from BB presented higher concentrations of Cu, Cr and Cd in comparison with ML, which indicates a higher uptake from water column and is in line with the spatial patterns recorded for those metals in SPM and water (DGT-measured fraction). This gills feature was previously observed in other fish species (Reynders et al., 2008). The Pb enhancement at BS in winter (in comparison with ML) is also probably a response to a higher availability as suggested by levels in SPM.

Gills from BB and BS showed higher contents of Zn in winter but this was not accompanied by increases in concentrations in the water or SPM, which suggests other factors explaining its accumulation rather than simply Zn availability. This discrepancy was already observed by other authors for essential elements in fish under field exposure (Demirak et al., 2006; Reynders et al., 2008). An inconsistency between environmental data and accumulation was also found in summer for Cu and Cr. Despite the higher availability of those elements at BB their uptake and/or accumulation was lower than at other sites.

Manganese accumulation in gills did not reflect the spatial differences recorded in water or particles in summer. These results suggested that *L. aurata* was able to regulate tissue Mn concentrations as previously reported in the Óbidos lagoon for crabs (Pereira et al., submitted). Indeed, the concentrations of essential metals (as Mn) in organisms tend to be highly regulated compared to nonessential. Fish, in particular, can use distinct strategies of metal homeostasis to achieve a steady-state balance: reducing metal accumulation and toxicity including uptake inhibition, increased elimination and detoxification, and storage (Fernandes et al., 2007).

Pearson analysis did not provide any association between accumulated metals in gills and the corresponding levels in water or particles. Since data of the two seasons were considered jointly in the Pearson analysis and the associations above discussed did not occur in summer, non-significant correlations were obtained. The absence of significant correlations

between water data and concentrations in the gills may have been due to the discrete water sampling compared to the time-integrated measurements in organisms.

In general, gills exhibited higher metal concentrations in summer. For instance, Cr was higher in summer (ML and BS) in line with the increases recorded in water (DGT-measured fraction). Identically, a higher availability could also be on the basis of Cu enhancement in gills during the summer at BS. However, gills concentrations of Zn and Cd in the gills increased in summer in contrast to relatively constant concentrations in the environment or enhancements in Cd in the winter. Considering that Zn is an essential element (Fernandes et al., 2007), the enhancement of its uptake and subsequent accumulation in gills could represent an adaptation strategy in order to compensate for a higher requirement during the summer period. Nonetheless, it is well known that the rates of metal uptake and accumulation increase with increasing temperature in ectothermal organisms (Sokolova and Lannig, 2008). An increase in metabolic rates at elevated temperatures may contribute to metal accumulation in ectotherms due to a higher energy demand, which results in elevated ventilation (Pörtner, 2002). Under conditions of moderate contamination, as in the Óbidos lagoon, the seasonal difference of metabolism probably dominated and masked any differences in metal environmental availability, thus explaining the limited inter-site differences in gill metal concentrations observed in the summer.

4.3. Oxidative stress in *L. aurata* gills and causative factors

4.3.1. Spatial variations

Enzymatic antioxidant responses showed that fish are experiencing from pro-oxidant challenges at BB in winter and summer. This site presents compounds that stimulate the production of H_2O_2 as depicted by the increase of CAT (winter) and GPx activities (summer). Hydrogen peroxide is the main cellular precursor of the hydroxyl radical that is the most toxic ROS. Those enzymes are known to protect the cell by reducing H_2O_2 to H_2O . The induction of both enzymes has been described previously in gills of *L. aurata* exposed to contaminants under field conditions (Oliveira et al., 2009). In particular, metals were previously associated with CAT induction in fish gills (Fernandes et al., 2008). In the Óbidos lagoon, CAT enhancement at BB in winter may be associated with higher accumulation of metals (particularly

Cu, Cr and Cd). On the other hand, it is unlikely that GPx induction at BB in summer could be due to accumulated metals since substantial increases were not recorded in gills. In this direction, the significant correlation found between GPx and Zn accumulated in gills (provided by data of the two seasons jointly) has a limited value in establishing causal relationships strictly in summer. It is more plausible that other stressors rather than metals could be the basis of GPx induction in summer. The broad fluctuations of dissolved oxygen between day (180%) and night (30%) that were recorded in summer (Pereira et al., 2009a) could lead to an activation of oxidative stress defences. In fact, it is well known that organisms exposed to fluctuating levels of oxygen are subjected to marked oxyradical burst, which can be counteracted by elevated levels of antioxidants to prevent oxidative damage (Storey, 1996).

GR plays a major role in GPx and GST reactions as an adjunct in the control of peroxides and free radicals by maintaining the GSH redox status (Bompart et al., 1990). Thus, exposure to pro-oxidant stressors leads to elevated activities of GR in aquatic organisms (Regoli et al., 2002) as in *L. aurata* gills (Oliveira et al., 2009). This is also occurring at BB site of the Óbidos lagoon in summer but was not associated with concomitant elevation of metals in the gills. The high GR activity at BB reflects increased glutathione recycling (replenishing GSH in order to avoid its depletion), suggesting that the ratio GSSG/GSH has been increased as consequence of pro-oxidants. As stated for GPx induction at BB in summer, other stressors rather than metals are probably on the basis of GR response. Nevertheless, GR was correlated with the Cd accumulated in the gills. As previously mentioned for GPx, this correlation has limited value in explaining summer responses.

In response to environmental changes, GSTs (a multigenic superfamily of multifunctional enzymes) may play a dual protective role associated with their activity on conjugation of electrophilic compounds (or phase I metabolites) with GSH (Van der Oost et al., 2003) and to a direct antioxidant action carried out by GST α -class catalyzing the reduction of organic hydroperoxides by GSH (Wang and Ballatori, 1998). Given that the adopted methodology (using CDNB, which is conjugated by all GST isoforms with the exception of the q-class) determines total GST activity, the observed GST activity increase at BB in summer, compared with other sites, is not easily interpreted. When examining significant correlations (Pearson analysis), the GST induction was correlated with levels of Cu in the gills. In fact, several studies have shown

that exposure to metals can lead to an increase of GST in liver (Amado et al., 2006; Guilherme et al., 2008; Oliveira et al., 2008) and to a lesser extent in gills (Dautremepuits et al., 2009).

GSH act against ROS protecting the cells from the pernicious effects of metals and other electrophilic compounds (Wang and Ballatori, 1998). In this study, GSH_t was higher at BB in winter and summer, which indicates the presence of contaminants challenging cell defences depending on this thiol. Indeed, the variation of the cellular glutathione status in fish is considered an indicator of the degree and duration of exposure to oxidant pollutants (Dautremepuits et al., 2009). The protective and adaptive role of GSH was previously observed in *L. aurata* gills from a contaminated ecosystem (Oliveira et al., 2009). Although the Pearson analysis did not provide any significant relationships between accumulated metals and GSH_t, the interference of metals (mainly Cu, Cr and Cd) in winter should be considered. In fact, metal metabolism involves the formation of GSH-metal complexes (Atli and Canli, 2008). However, in summer other compounds are probably challenging cell defences depending on this thiol. Elevated GSH_t levels represent a great ability to destroy ROS and are a result of a transfer from other organs (Oliveira et al., 2008). Indeed, most of the GSH is synthesized in liver and then exported to other tissues (Deneke and Fanburg, 1989). In addition, the concomitant increase of GR and GSH_t (summer) indicates that the turnover of reduced glutathione pools was not enough to maintain the redox cycling and cell homeostasis, forcing a translocation from other organs to the gills. Moreover, this evidence of high GSH requirements is in agreement with the observed concomitant increases in GPx and GST activities.

Free radical reactions in biological membranes could form lipid hydroperoxides that decompose double bonds of unsaturated fatty acids, damaging lipid membranes (Van der Oost et al., 2003). This complex process is known by lipid peroxidation. Our study revealed higher LPO at BB in winter, which is an indication of oxidative stress. This suggests that the observed mobilization of antioxidant defences was not able to prevent the gill LPO, probably induced by metal exposure in winter. Nevertheless, no associations were established between LPO and accumulated metals.

The higher levels of NH_4^+ , $\text{NO}_3^- + \text{NH}_2^-$ and PO_4^{3-} registered at BB in winter could be associated with the observed oxidative stress responses. In fact, it has been suggested that ammonia and nitrite can be important pro-oxidants in fish, mussels and aquatic plants, leading

to nitric radicals' production (Lima et al., 2006; Nimptsch and Pflugmacher, 2007). At the Óbidos lagoon, the importance of those compounds on oxidative stress induction in crabs was previously invoked (Pereira et al., 2009d).

By combining the different biomarker signals, the IBR index provides a simple mean for a general description of populations' health status (Broeg and Lehtonen, 2006). In the present work, IBR clearly demonstrated and confirmed that BB is the most impacted site within the lagoon.

4.3.2. Seasonal variations

In general, oxidative stress responses were enhanced in summer, namely activities of CAT, GPx, GR and GST and GSH_t content. The sole exception to this temporal pattern occurred for LPO at BB. It should be also emphasised that despite the higher values in summer, GST and GSH_t were the only endpoints exhibiting and identical spatial patterns in both seasons (characterised by increases at BB).

Metal concentrations in tissues are likely to change with the season, reflecting variability in the environmental inputs, but also changes in the metabolism (Regoli, 1998). Herein, seasonal variations of metals in gills were mainly associated with the effect of temperature on metals uptake, which enhanced in summer. Maximum enzymatic activities and GSH_t content were recorded in summer when higher concentrations of Zn (at all sites) and Cd (at ML and BS) were found. These results point to a major influence of metals for the recorded temporal variations.

Nonetheless, since water temperature rose 11 to 13 °C in summer in relation to winter, its influence on oxidative stress responses is a possibility. Previous work carried out at the Óbidos lagoon, with crabs, also demonstrated activation of oxidative stress responses in summer (Pereira et al., submitted). Low temperature reduces the metabolic rates in ectothermal organisms, and hence lower enzymatic activities are in general observed in colder seasons. This was reported for CAT and GST in fish (Gorbi et al., 2005; Amado et al., 2006). Moreover, higher temperature lead to an increase in oxygen consumption and consequently to an enhancement of ROS generation (Amado et al., 2006). Most aquatic organisms are able to adjust antioxidant defences as a mechanism to temperature adaptation (Filho et al., 2001). The

current GSH_t increase in summer is in agreement with previous studies with ectothermal marine animals (Heise et al., 2006). This variation was related to spontaneous, non-enzymatic GSH oxidation by emerging ROS under hyperthermia. Elevated temperatures accelerate mitochondrial respiration and increase mitochondrial ROS formation (Heise et al., 2003). As the current summer GSH_t increase was only measured at BB, it appears as an indication that glutathione pool responds to stressful conditions particularly in that area of the lagoon.

Conversely to other oxidative stress endpoints, LPO was enhanced in winter reinforcing the hypothesis that metals are inducing responses in this season.

The computed IBR values were higher in summer than winter at all sites, which is in agreement with the conclusions provided by the analysis of individual oxidative stress endpoints. Nevertheless, an identical configuration of star plots representing IBR was found in winter and summer, highlighting BB as a major impacted area in the Óbidos lagoon. Despite this clear conclusion for both seasons, IBR seemed to have a higher capacity to discern sites in winter than in summer. In fact, the estimated IBR value for BB was 100 times higher than ML in winter, whereas in summer the difference between the two sites was approximately 3 times. Interestingly, it was in winter that spatial differences in individual oxidative stress endpoints were associated with a higher metal accumulation. In general, IBR represented a potential measure of *L. aurata* health at the Óbidos lagoon, providing stronger evidence of the existing impact, as previously reported in the Óbidos lagoon (Pereira et al., 2009c) and in other systems (Beliaeff and Burgeot, 2002).

5. Conclusions

Results of this work provided these main findings:

- Oxidative stress responses of *L. aurata* gills were able to detect inter-site differences pointing BB as a critical area in the Óbidos lagoon, which was confirmed by a general stress index (IBR). Higher concentrations of accumulated Cu, Cr and Cd in winter could be on the basis of the pro-oxidant challenge at BB.
- Winter-summer variations were prevalent in gills of *L. aurata*, which exhibited a higher accumulation of metals in summer as well as increases of oxidative stress responses. IBR values corroborated a higher stress syndrome in summer at all sites than in winter.

Nevertheless, peroxidative damage occurred at BB only in winter despite the activation of antioxidant defences. In terms of metals accumulation, gills were more responsive in winter than summer, while oxidative stress endpoints provided evidence of existing impact at BB in both seasons.

- The applied approach combining biomarkers in gills (metal concentrations and oxidative stress) and environmental data (mainly metals and nutrients) was shown to be a useful means of assessing environmental health, particularly under a moderate contamination scenario. Gills are not only an important route of entry for contaminants, but the current study has demonstrated the ability of gills to reflect environmental status (through metal bioaccumulation and oxidative stress responses).

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Figure captions

Figure 1 - Location of the sampling sites at the Óbidos lagoon: middle lagoon (ML) (39°24'50.48"N, 9°12'42.82"W); inner branches Bom-Sucesso (BS) (39°23'44.88"N, 9°13'01.75"W) and Barrosa (BB) (39°26'16.84"N, 9°11'33.18"W).

Figure 2 – Metal concentrations in gills of *L. aurata* captured in winter and summer in the middle lagoon (ML) and in the inner branches (Bom-Sucesso – BS, Barrosa - BB) at Óbidos lagoon. Letters denote statistically significant differences ($p < 0.05$) between sites (in the same survey) and between surveys (for each site): (a) vs. ML; (b) vs. BS; (w) vs. winter. (Means \pm standard errors).

Figure 3 – Oxidative stress responses in gills of *L. aurata* captured in winter and summer in the middle lagoon (ML) and in the inner branches (Bom-Sucesso – BS, Barrosa - BB) at Óbidos lagoon. Letters denote statistically significant differences ($p < 0.05$) between sites (in the same survey) and between surveys (for each site): (a) vs. ML; (b) vs. BS; (w) vs. winter. (Means \pm standard errors; mv – missing value).

Figure 4 – Significant correlations between metal concentrations and oxidative stress responses in gills of *L. aurata*. Statistical significance and correlation coefficient are represented by p and r, respectively. Non-significant correlations are not presented.

Figure 5 - Integrated biomarker response (IBR) index in gills of *L. aurata* captured in winter and summer in the middle lagoon (ML) and in the inner branches (Bom-Sucesso – BS, Barrosa - BB) at Óbidos lagoon.

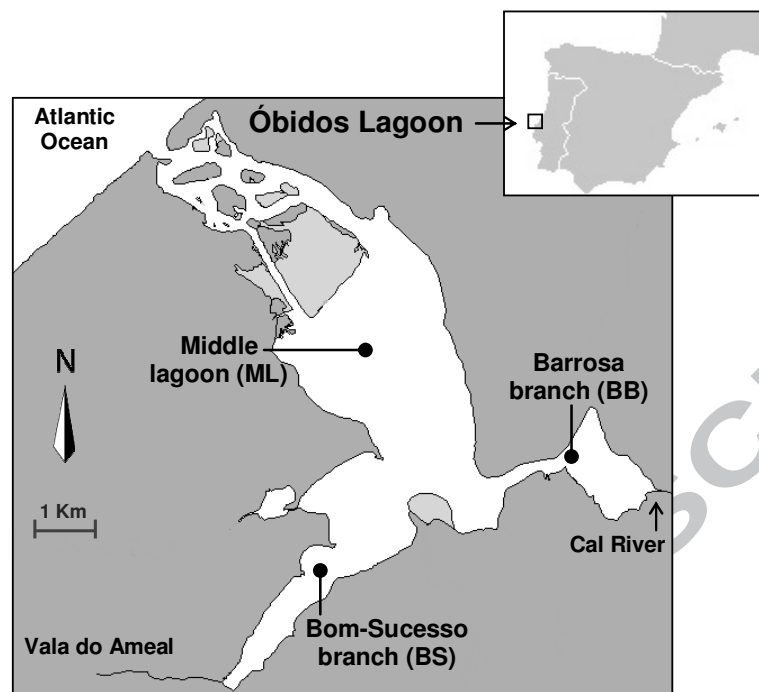


Figure 1

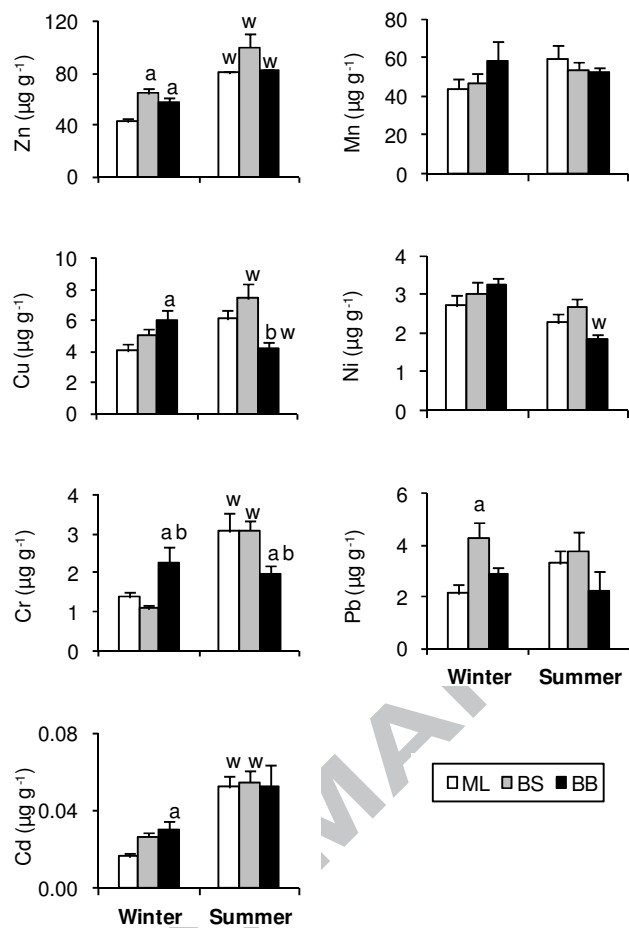


Figure 2

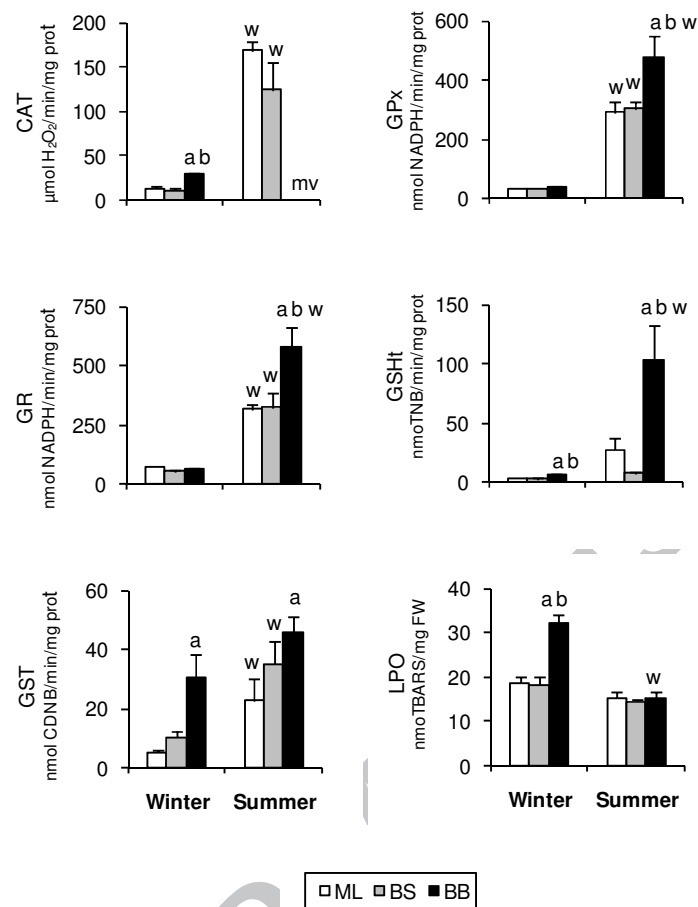


Figure 3

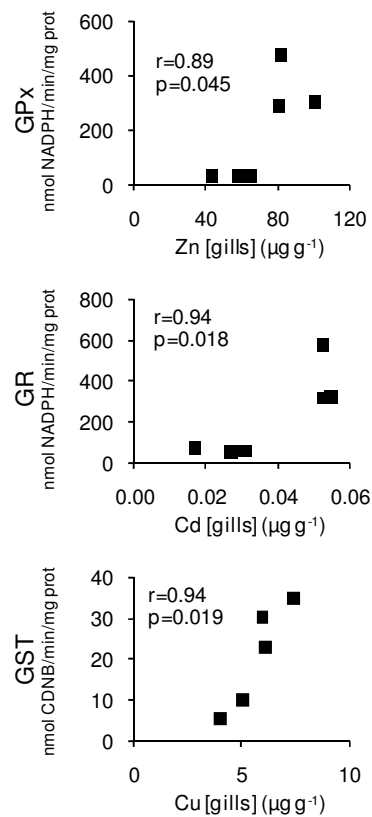


Figure 4

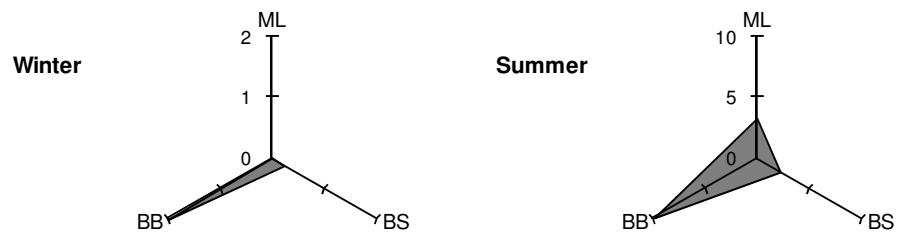
**Figure 5**

Table 1 – Water temperature (T), salinity, dissolved oxygen (DO), ammonium (NH_4^+), nitrate+nitrite ($\text{NO}_3^- + \text{NO}_2^-$), phosphate (PO_4^{3-}), silicate (Si(OH)_4) and chlorophyll a (Chl a), in winter and summer of low-tide, in middle lagoon (ML) and in inner branches (Bom-Sucesso - BS, Barrosa - BB) at Óbidos lagoon.

Season	Site	T (°C)	Salinity	DO (%)	NH_4^+ (μM)	$\text{NO}_3^- + \text{NO}_2^-$ (μM)	PO_4^{3-} (μM)	Si(OH)_4 (μM)	Chl a ($\mu\text{g L}^{-1}$)
Winter	ML	9	34.6	101	2.0	9.2	0.5	6.0	1.7
	BS	11	31.2	87	18	31	2.3	20	0.7
	BB	11	28.1	96	45	40	5.6	41	1.8
Summer	ML	20	34.6	95	1.0	1.0	2.9	5.1	4.9
	BS	23	35.2	108	1.0	1.2	2.1	4.6	3.7
	BB	24	33.3	83	0.9	1.3	6.5	12	10

Table 2 – Concentrations of metals in water at low-tide, in winter and summer, in the middle lagoon (ML) and in inner branches (Bom-Sucesso - BS, Barrosa - BB) at Óbidos lagoon. Means \pm standard errors with significant differences highlighted.

Season	Site	Mn (μ M)	Cu (nM)	Ni (nM)	Cr (nM)	Pb (nM)	Cd (nM)
Winter	ML	0.22 \pm 0.06	2.3 \pm 0.14	7.5 \pm 2.0	7.5 \pm 1.4	0.22 \pm 0.06	0.16 \pm 0.01
	BS	0.68 \pm 0.01	3.9 \pm 0.17	7.0 \pm 0.85	8.3 \pm 0.17	0.13 \pm 0.005	0.18 \pm 0.01
	BB	1.0 \pm 0.55	4.9 \pm 0.17 ^a	7.7 \pm 0.87	8.5 \pm 0.17	0.26 \pm 0.08	0.24 \pm 0.06
Summer	ML	0.13 \pm 0.01	3.3 \pm 0.21 ^w	8.6 \pm 2.3	6.4 \pm 1.2	0.47 \pm 0.12	0.07 \pm 0.006 ^w
	BS	0.12 \pm 0.01 ^w	4.9 \pm 0.18 ^w	6.3 \pm 0.81	14 \pm 1.9 ^{aw}	0.72 \pm 0.02	0.09 \pm 0.007 ^w
	BB	0.04 \pm 0.02 ^{ab}	7.9 \pm 1.0 ^{abw}	8.0 \pm 0.38	14 \pm 1.3 ^{aw}	0.99 \pm 0.21	0.14 \pm 0.03 ^w

a – significantly different from ML; b – significantly different from BS; w – significantly different from winter

Table 3 – Concentrations of metals ($\mu\text{g g}^{-1}$) in suspended particles of low-tide, in winter and summer, in the middle lagoon (ML) and in inner branches (Bom-Sucesso - BS, Barrosa - BB) at Óbidos lagoon. Means \pm standard errors with significant differences highlighted.

Season	Site	Mn	Zn	Cu	Ni	Cr	Pb	Cd
Winter	ML	505 \pm 30	161 \pm 32	29 \pm 4.2	22 \pm 3.1	55 \pm 6.7	40 \pm 0.31	0.19 \pm 0.08
	BS	458 \pm 48	113 \pm 9.1	35 \pm 12	23 \pm 6.7	56 \pm 14	50 \pm 10	0.18 \pm 0.08
	BB	749 \pm 18	142 \pm 4.1	52 \pm 0.35 ^a	28 \pm 2.0	68 \pm 2.6	44 \pm 3.4	0.36 \pm 0.11 ^{ab}
Summer	ML	1515 \pm 92	153 \pm 3.1	24 \pm 3.0	28 \pm 3.6	48 \pm 0.32	24 \pm 1.2 ^w	0.20 \pm 0.05
	BS	623 \pm 9.1 ^a	128 \pm 17	24 \pm 3.5	28 \pm 4.1	23 \pm 3.9	21 \pm 1.5	0.18 \pm 0.05
	BB	812 \pm 13 ^a	134 \pm 8.2	52 \pm 2.3 ^a	61 \pm 2.7 ^{aw}	51 \pm 3.3	31 \pm 1.7 ^b	0.18 \pm 0.01 ^w

a – significantly different from ML; b – significantly different from BS; w – significantly different from winter