Seasonal Changes of Oxidative Stress Biomarkers in White Muscle of Longfin Gurnard (*Chelidonychthys obscurus*) from the Adriatic Sea

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Abstract

The aim of this study was to investigate the activity of oxidative stress biomarkers (total superoxide dismutase-Tot SOD, manganese containing superoxide dismutase-Mn SOD, copper zinc containing superoxide dismutase-CuZn SOD, catalase-CAT, glutathione peroxidase-GSH-Px and glutathione reductase-GR), as well as biotransformation phase II enzyme glutathione-S-transferase (GST) in the white muscle of longfin gurnard (*Chelidonychthys obscurus*) at two localities: Platamuni (PL) and the Estuary of the River Bojana (EB) in the south-eastern Adriatic Sea (Montenegro) in winter and spring seasons. Our study represents the first investigation of this kind in the white muscle of longfin gurnard from the south-eastern Adriatic Sea and shows site specific differences between some investigated enzymes, with seasonal effects being the main influencing factor on investigated enzymes at both localities PL and EB.

Keywords: Longfin gurnard, *Chelidonychthys obscurus*, oxidative stress biomarkers, seasonal changes, white muscle.

Introduction

Oxidative stress is an important component of the stress response in marine organisms, which are exposed to wide variety of environmental stressors, such as anthropogenic contamination or seasonal influences (Vinagre *et al.*, 2012). Oxidative stress is the result of the over-production of reactive oxygen species (ROS). ROS, such as superoxide anion radicals, hydrogen peroxide and hydroxyl radicals are natural by-products of aerobic metabolism, but can additionally be produced intracellularly by different xenobiotics. They also can play a beneficial role in cells by contributing to pathways of intracellular signaling and redox regulation (Grim *et al.*, 2013). Their toxicity to the main biological components (proteins, lipids and DNA) is counteracted by the activities of many cell defence mechanisms (Stohs *et al.*, 2000). Cellular antioxidant defence system (AOS) is one of the important biochemical strategies that give protection to cells against deleterious effects of endogenous ROS by keeping their level relatively low (Paital and Chainy, 2010). AOS comprises of both non-enzymatic small antioxidant molecules (reduced glutathione, acsorbic acid, carotenoids) and a cascade of antioxidant defence enzymes: superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px) and glutathione reductase (GR), as well as phase II biotransformation enzyme glutathione-S-transferase (GST), (Halliwell and Gutteridge, 2007; Van der Oost *et al.*, 2003).

Field studies mainly considered the influence of pollution on antioxidant enzyme activities without examination of the influence of seasonal changes on enzyme activities. An important issue in biomonitoring studies of aquatic organisms is the complexity of biochemical and physiological responses to seasonal variables (Da Rocha *et al.*, 2009). Fishes are thermo and oxygen-conformer organisms and production of ROS levels and antioxidant defence depends of these physical variables (Wilhelm Filho, 2007). In fish, changes in antioxidant defense enzyme activities can be influenced both by intrinsic factors (age, feeding behavior, food consumption), and also by extrinsic factors, such as toxins present in the water, seasonal and daily changes in dissolved oxygen and water temperature (Bayir *et al.*, 2011). In their natural habitats, fish often have periods of poor food supply as a result of lower environmental temperature, spawning, migration and reproduction (Furné *et al.*, 2009) and changes of these variables

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are accompanied with seasonal fluctuations. For this reason, investigation of antioxidant enzymes in fish in biomonitoring studies, have also taken into account seasonal effects beside the effects of various pollutants.

Longfin gurnard *Chelidonichthys obscurus* (Walbaum, 1792) is a perciform fish which occurs in benthic zones and inhabits sand, muddy sand or gravel bottoms. It feeds mainly on fish, crustaceans and mollusks. Longfin gurnard has three isolated rays on the pectoral fin which function as legs on which the fish rests and also help in locating food on the soft bottom (Frimodt, 1995). Moreover, this species maintains a close association with sediments for food and protection and it is therefore more likely to be exposed to sediment-associated pollutants.

The aim of the present work was to compare the activity of oxidative stress biomarkers in the white muscle of longfin gurnard (*Chelidonichthys obscurus*) between two localities, Platamuni and the Estuary of the River Bojana (south-eastern Adriatic Sea) in two seasons: winter and spring. We examined the activities of total superoxide dismutase (Tot SOD), manganese containing superoxide dismutase (Mn SOD, EC 1.15.1.1), copper zinc containing superoxide dismutase (CuZn SOD), catalase (CAT, EC 1.11.1.6), glutathione peroxidase (GSH-Px, EC 1.11.1.9), glutathione reductase (GR, EC 1.6.4.2), as well as the activity of biotransformation phase II enzyme glutathione-S-transferase (GST, EC 2.5.1.18).

Materials and Methods

The specimens of longfin gurnards (*Chelidonychthys obscurus*) were caught by trawling in winter (February) and late spring (May) at two localities: Platamuni (PL) and the Estuary of the River Bojana (EB), (Fig. 1). These localities receive different amounts of industrial and agricultural discharges and were selected in order to compare the activity of oxidative stress biomarkers between sites, as well as between periods of lower metabolic activity (winter) and higher metabolic activity (spring). At Platamuni, 20 specimens (10 in the winter and 10 in the spring) and at the Estuary of the River Bojana, 20 specimens (10 in the winter and 10 in the spring) of longfin gurnard were collected. Specimens of fish were collected and immediately transferred to seawater tanks where they were identified. Fish were killed on board by severing the spinal cord and dissected within 3 minutes on ice. The white muscle was rapidly dissected from each sample, washed in ice-cold 0.65% NaCl and frozen in liquid nitrogen (-196°C) before storage at -80°C. White muscle was ground and homogenized in 5 volumes of 25 mmol/L sucrose containing 10 mmol/L Tris-HCl, pH 7.5 (Lionetto *et al.*, 2003) using Janke & Kunkel (Staufen, Germany) IKA-Werk Ultra-Turrax homogenizer at 4°C (Rossi *et al.*, 1983). The homogenates were sonicated for 30s at 10 kHz on ice to release enzymes (Takada *et al.*, 1982) and sonicates were then centrifuged at 4°C at 100000 g for 90 minutes. The resulting supernatants were used for biochemical analyses.

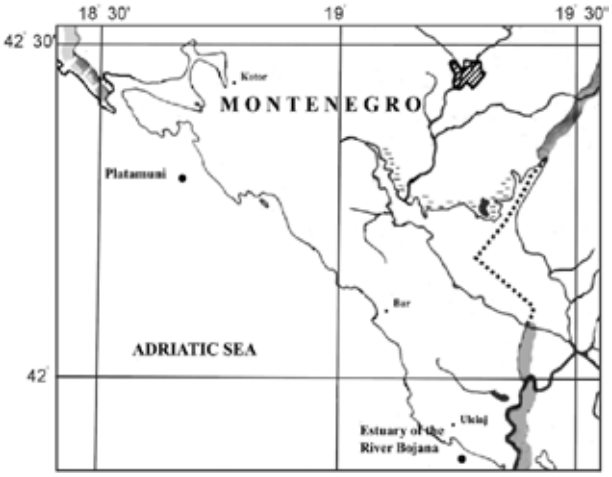

Figure 1: The geographical position of two investigated localities: Platamuni (PL) and the Estuary of the River Bojana (EB) in the south-eastern Adriatic Sea (Montenegro).

Total protein concentration in the supernatant was determined according to the method of Lowry *et al.* (1951) and expressed in mg/g wet mass. The activity of antioxidant defence enzymes was measured simultaneously in triplicate for each sample using a Shimadzu UV-160 spectrophotometer and a temperature controlled cuvette holder. The total activity of SOD was assayed using the epinephrine method (Misra and Fridovich, 1972) and expressed as specific activity (U/mg of protein). For the determination of Mn SOD activity the assay was performed after the preincubation with 8 mmol/L KCN. CuZn SOD activity was calculated as a difference between total SOD and Mn SOD activities. CAT activity was evaluated by the rate of hydrogen peroxide (H_2O_2) decomposition and expressed as μmol H_2O_2/min/mg protein (Claiborne, 1984). The activity of GSH-Px was determined following the oxidation of nicotine amide adenine dinucleotide phosphate (NADPH) as a substrate with t-butyl hydroperoxide (Tamura *et al.*, 1982) and expressed in nmol NADPH/min/mg protein. The activity of GR was assayed as described by Glatzle *et al.* (1974) and expressed as nmol NADPH/min/mg protein. The activity of biotransformation phase II enzyme GST towards 1-chloro-2,4-dinitrobenzene (CDNB) was measured using the method of Habig *et al.* (1974) and expressed as nmol GSH/min/mg protein. All chemicals were the products of Sigma (St. Louis, MO, USA).

The data are expressed as mean ± S.E. (standard error). The non-parametric Mann-Whitney U-test was used to seek significant differences between means. A minimum significance level of $p<0.05$ was accepted. In addition, Principal Component Analysis (PCA) was employed to detect variables that significantly contributed to differences in the activities of investigated enzymes between the examined sites and seasons. Analytical protocols described by Darlington *et al.* (1973) and Dinneen and Blakesley (1973) were followed.

Results and Discussion

Table 1 represents geographical coordinates of investigated locations (PL and EB) in the south-eastern Adriatic Sea in winter and spring season, while Table 2 represents physico-chemical parameters of sea water (depth, salinity, temperature, O_2 concentration and O_2 saturation) at the examined locations (spot measurements). As we expected, seawater temperature was markedly higher in spring season in respect to winter at both localities. The rate of biological activity and consequently metabolic activity in ectotherms are deeply influenced by environmental temperature (Crocket, 2008). At cold temperatures, intracellular lipids are elevated (Sidell, 1998), and in biological membranes the degree of unsaturation and ratio of phospholipids are increased (Hazel, 1995). Consequently, ectotherms are exposed to elevated risk of lipid peroxidation. They also maintain oxidative metabolism of skeletal muscle by increasing the density of mitochondria and thus maintain elevated production of ROS, even at very low temperatures (Mueller *et al.*, 2011).

Table 1: Geographical coordinates of two investigated locations (Platamuni-PL and the Estuary of the River Bojana-EB) in the south-eastern Adriatic Sea in winter and spring.

		Longitude	Latitude
Winter	**PL**	42°19'56''	18°35'05''
	EB	41°52'60''	19°14'87''
Spring	**PL**	42°16'56''	18°41'66''
	EB	41°52'60''	19°14'87''

Table 2: Physico-chemical parameters of the seawater (depth, salinity, temperature, O_2 concentration and O_2 saturation) at two examined locations (Platamuni-PL and the Estuary of the River Bojana-EB) in the south-eastern Adriatic Sea in winter and spring.

		Depth (m)	Salinity (‰)	Temperature (ºC)	O_2 conc. (mg/l)	O_2 sat. (%)
Winter	**PL**	80	33.07	12.8	8.1	91
	EB	30	37.70	11.6	8.4	95
Spring	**PL**	80	38.00	17.2	7.6	98
	EB	28	37.20	17.9	7.4	97

The major antioxidant defence enzymes in marine fish are SOD, CAT and GSH-Px (Winston and Di Giulio, 1991). Antioxidant status in species of marine fish is related to tissue oxygen consumption and to overal activity of the organism (Wilhelm Filho *et al.*, 2000). At the same time, many enzyme activities, including antioxidant enzymes show considerable seasonal fluctuations. Seasonality is known to exist in internal and natural exposure to oxidative stress as well (Pavlović *et al.*, 2010). The overall trend obtained in our study revealed decreased activities of the investigated enzymes in spring when compared to the winter. The SODs are a group of metalloenzymes that catalyse the conversion of superoxide anion radicals to hydrogen peroxide. The activities of Tot SOD, Mn SOD and CuZn SOD obtained in our experiment are presented in Fig. 2. Our results show decreased activities of all three enzymes at PL and only Mn SOD at EB in winter season ($p<0.05$). The effect of locality was detected for Tot SOD and CuZn SOD whose activities were increased at EB in spring season ($p<0.05$). Some laboratory studies have reported an increased SOD activity in fish exposed to various contaminants, but some studies reported a decreased activity, too. The main difficulty in understanding SOD behavior is the notable difference between responses observed in laboratory studies and in the field (Van der Oost *et al.*, 2003).

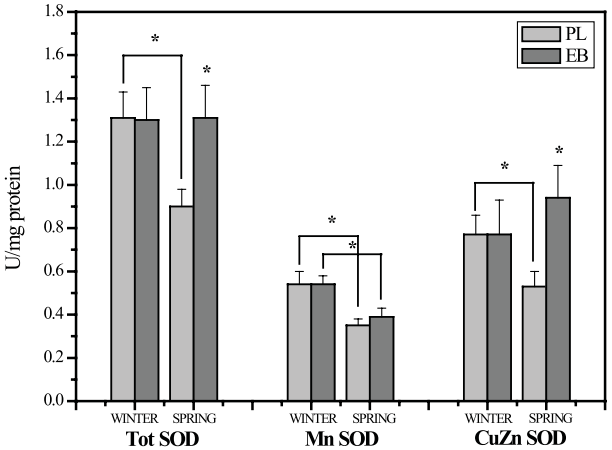

Figure 2: Tot SOD, CuZn SOD and Mn SOD activities (U/mg protein) in the white muscle of longfin gurnard (*Chelidonychthys obscurus*) from the Platamuni (PL) and the Estuary of the River Bojana (EB) in winter and spring. The data are expressed as mean ± S.E. The non-parametric Mann-Whitney U-test was used to seek significant differences between means. $p<0.05$ represents a minimal significant level.

The activities of CAT and GSH-Px are shown in Fig. 3. Our analysis show that seasonal effect was obtained only in CAT activity with decreased activity at both PL and EB in spring season ($p<0.05$). At the same time, the activity of CAT was increased at

EB in respect to PL in winter season ($p<0.05$) and activity of GSH-Px was decreased at EB compared to PL in spring season ($p<0.05$). CATs are hematin-containing enzymes localized in the peroxisomes of most cells that facilitate the removal of hydrogen peroxide. Similarly to SOD, in various studies, opposite results for CAT activity were obtained (Van der Oost *et al.*, 2003). At the same time, glutathione-dependent enzymes (GSH-Px and GST) showed decreased activities in spring in respect to the winter. At low temperatures, the increased polyunsaturation of mitochondrial membranes in fish should raise rates of mitochondrial respiration, which would in turn enhance the formation of ROS, increase proton leak and favour peroxidation of these membranes. The mitochondria show seasonal cycles of the maximum rates of protein-specific substrate oxidation at any given temperature. Increases in the maximal capacity of pyruvate oxidation were sufficient to compensate for seasonal changes in temperature, except during the winter months when rates at habitat temperature were approximately half the rates over other periods (Guderley, 2004). Also, higher levels of organic hydroperoxides are formed by enhanced lipid mobilization which leads to induction of higher GSH-Px activity. This induces enhanced utilization of reduced glutathione (GSH) forming their oxidized form (GSSG).

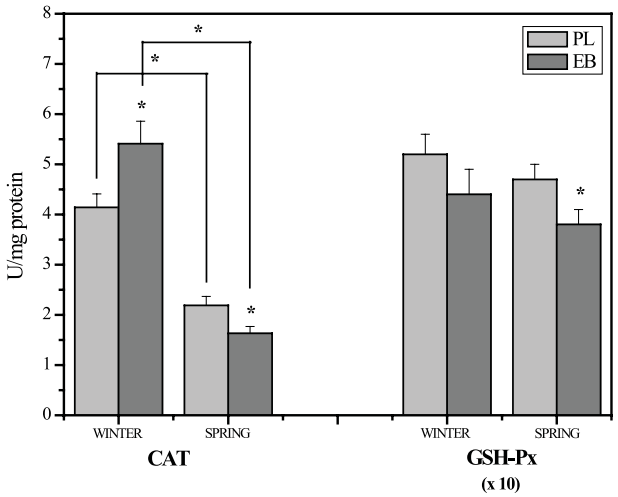

Figure 3: CAT and GSH-Px activities (U/mg protein) in the white muscle of longfin gurnard (*Chelidonychthys obscurus*) from the Platamuni (PL) and the Estuary of the River Bojana (EB) in winter and spring. The data are expressed as mean ± S.E. The non-parametric Mann-Whitney U-test was used to seek significant differences between means. $p<0.05$ represents a minimal significant level.

Changes in GR activity either for the season or for locality was not observed (Fig. 4). The activity of phase II biotransformation enzyme GST was significantly decreased at EB in spring season ($p<0.05$), (Fig. 4). In winter, the activity of this enzyme was increased, while in spring season was decreased at EB in respect to PL. These data suggest that GST enzyme react differently in winter and spring season (increase in winter and decrease in spring at EB in respect to PL) suggesting that this enzyme exhibit different behavior to contaminants in respect to season. This indicates that GST is a sensitive and suitable biomarker of environmental status. In winter, possible effects of organic pollutants on GST activity are more pronounced, according to the synergism between cold-stress and toxic effects and chemical pollutants (Pavlović *et al.*, 2004).

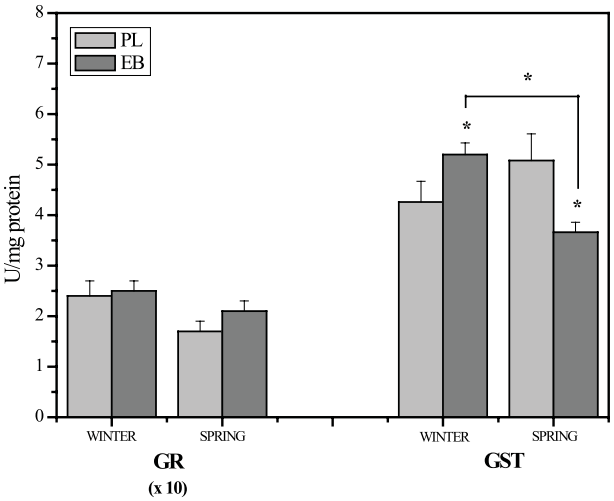

Figure 4: GR and biotransformation phase II enzyme GST activities (U/mg protein) in the white muscle of longfin gurnard (*Chelidonychthys obscurus*) from the Platamuni (PL) and the Estuary of the River Bojana (EB) in winter and spring. The data are expressed as mean ± S.E. The non-parametric Mann-Whitney U-test was used to seek significant differences between means. $p<0.05$ represents a minimal significant level.

In Fig. 5 the Principal component analysis (PCA) of all investigated antioxidant defence enzymes is presented. The PCA referred to relative contribution of every antioxidant enzyme in the white muscle and show that Factor 1 and Factor 2 explain over 60% of the total variance in the data matrix. Factor 1 explains 38.30% of the total variance and is mainly characterized by negative loading of the variables GSH-Px and GST. Factor 2 explains 22.53% of the total variance and is mainly characterized by positive loading of the Tot SOD and CuZn SOD and negative loading of Mn SOD, CAT and GR.

Fig. 6 represents summary results of PCA for both investigated sampling localities in winter and spring seasons and shows that Factor 1 and Factor 2 explain over 90% of total variance. Relating to the position of sites, Factor 1 (51.99%) discriminates between PL-winter/EB-winter and PL-spring/EB-spring. Factor 2 (38.48%) discriminates between EB-spring and PL-winter/EB-winter/PL-spring.

Several studies have shown that the physiological status of marine organisms changed when exposed to contaminants, but little work has been devoted to mutual effects of seasonal fluctuations of temperature and influence of pollutants. The annual variation of a particular biomarker response should be known and well understood prior its use in biomonitoring studies in order to separate successfully the contamination effects from the effects caused by normal physiological variations and thus for correct interpretation of results (Petrović *et al.*, 2004). In general, many enzymes react differently to contaminants in respect to the season and this fact must be incorporated in the biomonitoring studies (Pavlović *et al.*, 2004).

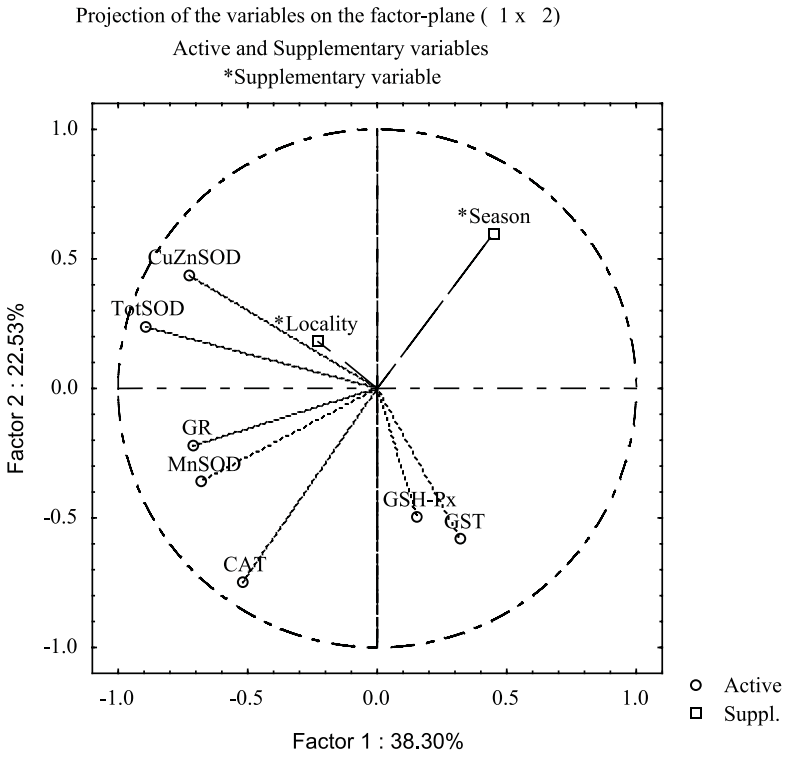

Figure 5: Principal component analysis (PCA) based on correlations, projection of all investigated antioxidant defense enzyme activities on the factor plane.

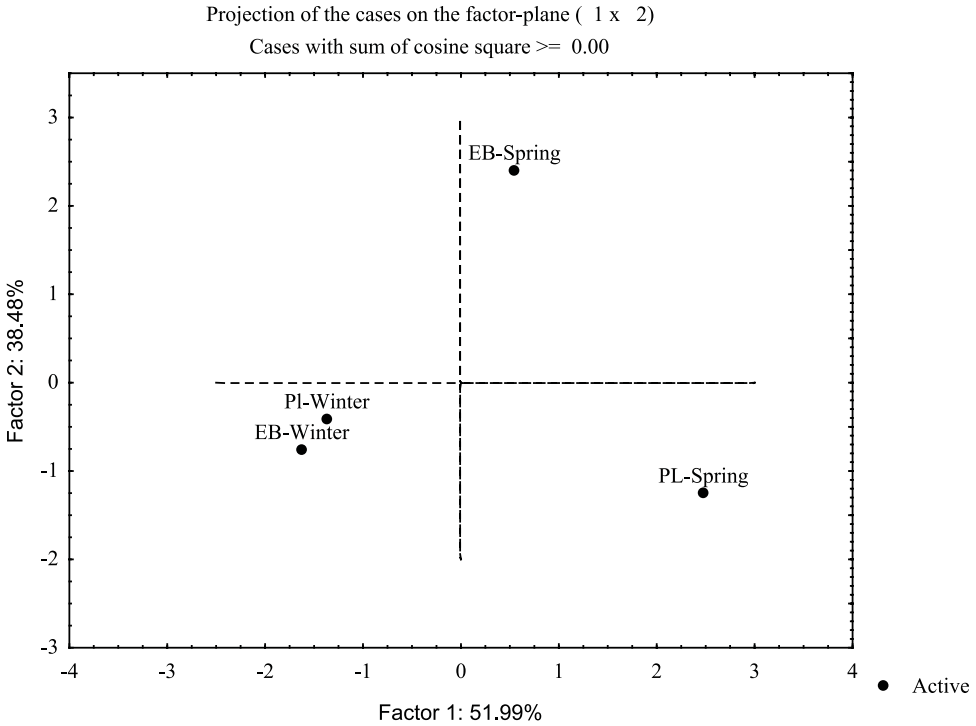

Figure 6: Principal component analysis (PCA) of each site (Platamuni-PL and the Estuary of the River Bojana-EB) and season on the factor plane.

Conclusions

In this study, both the effects of locality and season on oxidative stress biomarkers analyzed were observed, but statistical analysis reveal stronger effect of season on investigated parameters in the white muscle of longfin gurnard (*Chelidonychthys obscurus*) at investigated locations. Our study represents the first investigation of this kind in the white muscle of longfin gurnard (*Chelidonychthys obscurus*) from the south-eastern Adriatic Sea (Montenegro) and requires more detailed analysis in future.

Acknowledgements

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