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Oxidative stress indicators in populations of the gastropod *Buccinanops globulosus* affected by imposex

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The gastropod Buccinanops globulosus *is commonly used as a bioindicator of tributyltin (TBT) contamination due to its high imposex incidence in maritime traffic areas. The aim of this study was to evaluate both oxidative stress in* B. globulosus *at three sites with different maritime activity, and imposex incidence in Nuevo Gulf, Argentina. Oxidative stress parameters in digestive glands, like superoxide dismutase (SOD) and glutathione-S-transferase (GST) activities, reduced glutathione levels (GSH), and oxidative damage to lipids, estimated as thiobarbituric acid reactive substances (TBARs) as well as imposex parameters (% imposex and female penis length (FPL)) were measured in females. Gastropods from the harbour area showed 100% imposex, the highest FPL and TBARs content, as well as GSH levels and SOD activity.*

The different oxidative stress responses and high imposex incidence at the harbour site may indicate a negative effect on the organism's physiological state due to environmental pollution.

Keywords: Oxidative stress, imposex, marine pollution, *Buccinanops globulosus*, Nassariidae

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INTRODUCTION

Pollutants such as tributyltin (TBT), polyaromatic hydrocarbons (PAHs), organochlorinated compounds and trace metals are present in areas with intense maritime activity in Patagonian coasts (Gil *et al.*, 1999, 2006; Commendatore *et al.*, 2000; Esteves *et al.*, 2006; Commendatore & Esteves, 2007; Massara Paletto *et al.*, 2008; Bigatti *et al.*, 2009). Aquatic invertebrates, and molluscs in particular, are widely used as bioindicators of polluted environments (Meador *et al.*, 1995; Kim *et al.*, 2002; Antizar-Ladislao, 2008), while biomarkers are powerful tools to detect environmental damage and risk status (Dahlhoff, 2004). Pollutants could affect living organisms by inducing reactive oxygen species (ROS) formation (Winston & Di Giulio, 1991; Cheung *et al.*, 2001; Leonard *et al.*, 2004; Nicholson & Lam, 2005). Oxidative stress is the result of the imbalance between the generation and neutralization of ROS by antioxidant mechanisms (Davies, 1995). Oxidative stress responses (e.g. antioxidant enzyme activities and/or oxidative damage to lipids) have been used as biomarkers in molluscs to test and quantify the toxic effects of pollutants in the aquatic environment (de Almeida *et al.*, 2004; Belcheva *et al.*, 2011; Sabatini *et al.*, 2011a). The increased activity or *de novo* synthesis of antioxidant enzymes to mitigate oxidative damage has been considered as an adaptation of organisms to stress conditions (Young & Woodside, 2001). Among these enzymes are superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) which protect ROS scavenging cells (Karakoc *et al.*, 1997; Borković *et al.*, 2005) and glutathione S-transferase (GST) as well, a phase II detoxifying enzyme, exhibiting a protective mechanism against oxidative stress (Prohaska, 1980; Sheehan & Power, 1999; Doyen *et al.*, 2005). Moreover, aquatic organisms also present non-enzymatic antioxidant defences (e.g. vitamin E, reduced glutathione-GSH-, between others) contributing to minimize oxidative damage (Sayeed *et al.*, 2003; Wang *et al.*, 2008).

In molluscs, the digestive gland is the principal site for bioaccumulation and detoxification of pollutants and the main target of oxidative disruption (Malanga *et al.*, 2004). In several bivalve species exposed to pollutants, oxidative damage and an increased/decreased activity of antioxidant enzymes have been registered (Bainy *et al.*, 2000; Sabatini *et al.*, 2009, 2011a, b; Giarratano *et al.*, 2010, 2013; Di Salvatore *et al.*, 2013).

In marine gastropods from the Argentinean coast, the imposex phenomenon (penis or *vas deferens* neoformation) in females exposed to tributyltin (TBT) (Gibbs & Bryan, 1986) has been detected in all the harbour areas (Bigatti *et al.*, 2009). Many gastropod species have been affected by

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imposex in Argentina, while the nassarid *Buccinanops globulosus* showed high sensibility to TBT (Bigatti *et al.*, 2009). It has been demonstrated that TBT could induce imposex, shell malformation (Chagot *et al.*, 1990; Alzieu, 2000; Bigatti & Carranza, 2007; Márquez *et al.*, 2011) and also causes oxidative stress (Huang *et al.*, 2005; Wang *et al.*, 2005; Jia *et al.*, 2009; Zhou *et al.*, 2010).

The gastropod *Buccinanops globulosus* inhabits sandy or muddy bottoms of shallow waters (Pastorino, 1993) in Patagonian coasts, and most of the time lives buried in the sediment (Scarabino, 1977). It is distributed along the South-western Atlantic Ocean (Pastorino, 1993). *Buccinanops globulosus* is dioecious, with internal fertilization. Females attach the egg capsules to their own shells (Penchaszadeh, 1971), and are larger than the males. In general, the populations from Patagonia have shown variability in biological parameters such as growth, shell shape and ageing (Narvarte *et al.*, 2008; Avaca *et al.*, 2013; Bökenhans, 2014; Primost *et al.*, in press). This species is edible and is part of an expanding artisanal fishery (Narvarte *et al.*, 2008; Averbuj *et al.*, 2014). Sublethal effects and bioaccumulation of TBT and other pollutants (trace metals, hydrocarbons) have been detected in harbour areas (Bigatti *et al.*, 2009; Torres *et al.*, 2013; Primost, 2014). While signalling by retinoid X receptors (RXR) (Nishikawa *et al.*, 2004) could be involved on the imposex development in gastropods, although the induction mechanisms are under study, the determination of oxidative stress responses in imposex-affected gastropods still remains inconclusive in Argentina.

The aim of this study was to evaluate oxidative stress responses associated to maritime traffic contamination in imposex-affected *B. globulosus* from Nuevo Gulf, Argentina.

MATERIALS AND METHODS

Study area and imposex incidence

The study was performed in three sites of Nuevo Gulf, with decreasing maritime activity: harbour area at Luis Piedra Buena harbour (LPB) (42°43′57″S 65°1′53.9″W), Punta Cuevas beach (PC) (42°46′45″S 64°59′34″W) and Cerro Avanzado beach (CA) (42°49′37.66″S 64°51′29.19″W) (Figure 1). In the LPB site activity of large vessels is frequently present (~720 vessels per year) (APPM, 2013); in this area 100% imposex was reported in gastropods since 2000 (Bigatti & Penchaszadeh, 2005; Bigatti *et al.*, 2009; del Brío, 2011; Primost, 2014), while moderate pollution by PAHS, trace metals and TBT were previously recorded in sediments and molluscs (Gil *et al.*, 1999; Massara Paletto *et al.*, 2008; Bigatti *et al.*, 2009). The PC site is a recreational public area frequently presenting diving vessels, where low pollution by TBT and trace metals was measured (Primost, 2014) and lesser imposex parameters were reported (Bigatti *et al.*, 2009; Primost, 2014). The CA beach is a recreational area where very low or null imposex incidence was reported as well as no detectable TBT pollution (Bigatti *et al.*, 2009; del Brío, 2011; Primost, 2014); in this area there is low maritime traffic and sport vessels are present only occasionally. Table 1 summarizes the pollution levels previously detected in the sampling sites.

Adult female gastropods *Buccinanops globulosus* (25 approximately at each site) were collected using baited traps.

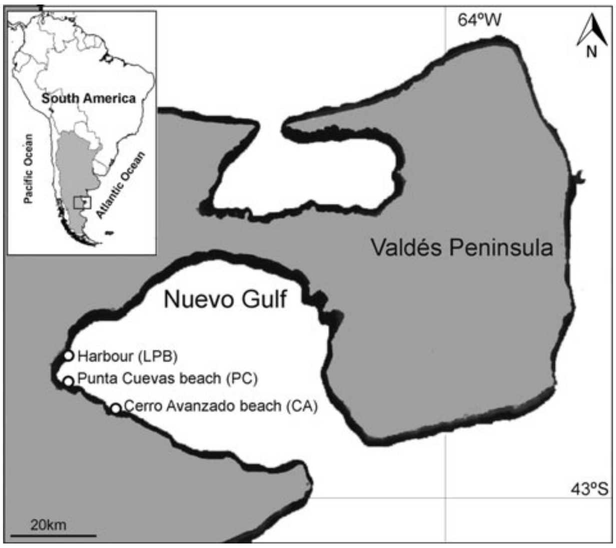

Fig. 1. Location of sampling sites in Nuevo Gulf, Patagonia, Argentina.

The sex was determined *in situ* by presence or absence of the ventral pedal gland (only present in females) used to fix egg capsules on its own shell. Total shell length (TSL) and body weight relative to size (BW) was recorded in the laboratory. Incidence of imposex (% I) was considered as the percentage of females with a penis or *vas deferens* development; correspondingly mean female penis length (FPL) was estimated only in females with penis development and using 0.1 mm precision digital caliper.

Oxidative stress parameters

In a subsample of nine females per site, oxidative stress parameters were determined. The digestive gland was carefully dissected, weighed (with a digital scale 0.01 g) and frozen at −80°C for later oxidative stress determinations.

Digestive glands were homogenized with 0.154 M KCl (1:5 w v^{-1}) containing 0.5 mM phenylmethylsulphonyl fluoride (PMSF) and 0.2 mM benzamidine (protease inhibitors) to study oxidative stress parameters. The homogenates were centrifuged at 12,000 × ***g*** during 30 min (4°C) and the supernatants were stored for later determinations.

Total soluble protein content was measured by the method of Bradford (1976), using bovine serum albumin as standard. The results were expressed as μg of total protein per mL.

Superoxide dismutase (SOD, EC 1.15.1.1) activity was assessed by inhibition of photoreduction of NBT (nitro blue tetrazolium) and monitoring absorbance at 560 nm according to Beauchamp & Fridovich (1971). The standard assay mixture contained 5, 10 and 15 μL enzymatic sample, 0.1 mM EDTA, 13 mM DL-methionine, 75 μM NBT and 20 μM riboflavin, in 50 mM phosphate buffer (pH 7.5), to a final volume of 3 mL. Samples were exposed for 15 min to intense cool-white light, and then kept in the dark until absorbance was measured at 560 nm. Results were expressed as U per mg protein. A SOD unit was defined as the enzyme amount necessary to inhibit the reaction rate by 50%.

Glutathione S-transferase (GST, EC1.11.1.9) activity was measured by monitoring the absorbance at 340 nm using 1-chloro-2 4-dinitrobenzene (CDNB) (100 mM) as substrate according to Habig *et al.* (1974). Briefly, we mixed 10 μL of

Table 1. Maximum values of different pollutants detected in gastropods (whole tissues) and sediments from sampling sites in Nuevo Gulf.

Pollutant	LPB harbour (LPBH)		Punta Cuevas beach (PC)		Cerro Avanzado beach (CA)		Reference
	Gastropods	Sediments	Gastropods	Sediments	Gastropods	Sediments	
TBTs (ng (Sn)g^{-1} dw)							
Tributyltin	171	175		1.9*	Nd	Nd	*Bigatti *et al.* (2009)
Dibutyltin	74	19			Nd	Nd	
Monobutyltin	345	72			Nd	Nd	del Brío (2011);
Booster biocides (ng g^{-1} dw)							
Diuron	Nd	Nd			Nd	Nd	
Irgarol	Nd	Nd			Nd	Nd	
Trace metals (µg g^{-1} dw)							
Al	5.5	12,958	16	8664	6	10,541	
Fe	126	13,581	89	12,175	89	10,492	Primost (2014)
Zn	182	33	119	19	108.5	16.84	
Cu	13	6.1	7.5	3	9	2.99	
Cd	8	Nd	7	Nd	24	Nd	
Pb	1.2	7.5	0.4	Nd	0.4	Nd	
PAHs (ng g^{-1} dw)							
Anthracene	174	30			Nd	Nd	
Fluoranthene	141	30			Nd	Nd	
Pyrene	28	20			Nd	Nd	
Benzo(b)fluoranthene	151	30			Nd	Nd	Torres *et al.* (2013)
Benzo(k)fluoranthene	44	40			Nd	Nd	
Benzo(a)anthracene	22	50			Nd	Nd	
Chrysene	0	30			Nd	Nd	
Dibenzoanthracene	0	20			Nd	Nd	
Total PAHs		2500		180			Massara Paletto *et al.* (2008)

Nd: Non detectable.

glutathione (GSH) (100 mM in phosphate buffer) and 20 µL of sample in 960 µL of 100 mM phosphate buffer (pH 6.5) and 10 µL CDNB. One GST Unit was defined as the amount of enzyme needed to catalyse the formation of 1 µmoL of GS-DNB per minute at 25°C.

Reduced GSH levels were determined monitoring the absorbance at 412 nm after 30 min incubation at room temperature following the Anderson (1985) procedure. Briefly, 100 µL supernatant from the 11,000 × g sample was acidified with 50 µL of 10% sulphosalicylic acid. After centrifugation at 8000 × for 10 min, supernatant (acid-soluble GSH) aliquots were mixed with 6 mM 5,5-dithiobis-(2-nitrobenzoic) acid (DTNB) in 0.143 M buffer sodium sulphate (pH 7.5) (containing 6.3 mM EDTA). Results were expressed as nmol GSH per mg of protein.

Lipid peroxidation was determined measuring thiobarbituric acid reactive substances (TBARs) according to Vavilin *et al.* (1998). Briefly, the 11,000 × g supernatant (175 µL) from total homogenate was mixed with thiobarbituric acid (TBA) (26 mM) solution and incubated at 95–100°C for 45 min. After cooling, the reaction mixture was centrifuged and the supernatant absorbance was determined at 535 nm. TBARs concentration was estimated using an extinction coefficient of 156 mM^{-1} cm^{-1} and absorbance determination at 535 nm. Results were expressed as µmol TBARs per mg of protein.

Statistical analysis

Normality and homogeneity of variances were tested by Lilliefors' and Bartlett's tests, respectively (Sokal & Rohlf, 1979). Results from size, weight and oxidative stress parameters were analysed by one way ANOVA followed by a Tukey's *post hoc* test. Results for imposex analysis were compared between sites by Kruskal–Wallis followed by a Dunn *post hoc* test. Differences were considered significant with $P < 0.05$. Statistica7 software was used for statistical analysis. A DistLM multiple correlations was performed using PRIMER software (Clarke & Gorley, 2006) to compare the effect of stress parameters (as co-variable) on penis length (as response variable). Prior to analysis, variables were

Table 2. Total shell length, body weight (means ± SD) and imposex parameters in *Buccinanops globulosus*.

SITE	Females (n)	Body weight/shell length	Total shell length (mm)	% Imposex	FPL (mm)
LPB harbour (LPBH)	25	0.34 ± 0.01*	40.72 ± 0.69*	100	4.51 ± 0.23*
Punta Cuevas beach (PC)	18	0.20 ± 0.01	32.85 ± 0.78	94.44	0.83 ± 0.11
Cerro Avanzado beach (CA)	23	0.23 ± 0.01	34.63 ± 0.95	4.34	–

FPL: female penis length.
*Significant differences between sites (LPB, PC and CA).

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transformed by Z-score using R software (https://www.r-project.org/).

RESULTS

Imposex incidence

A total of 66 females of *Buccinanops globulosus* were analysed for imposex incidence and a subsample of 27 females (nine per site) was used for the determination of oxidative stress parameters. Total shell length (TSL) and body weight (BW) were significantly different between sites (TSL: $F = 27.306$, $P < 0.0001$, df = 2, N = 66; BW: $F = 46.006$, $P < 0.0001$, df = 2, N = 66). In both cases, the highest values were obtained in the LPB site (Table 2).

The imposex incidence was 100% in LPB (Table 2) and significant differences in female penis length (FPL) between LPB vs. PC sites were observed ($U = 450.000$, $P < 0.0001$, N = 43). In CA site, the FPL was not calculated because only one female showed imposex development (with a small incipient penis).

Antioxidant defences

In order to analyse the antioxidant defences, results firstly showed that total protein content in the digestive gland did not differ between sampling sites ($F = 0.220$, $P = 0.804$, df = 2, $N = 27$) (data not shown). Therefore, all measured variables were standardized as a function of protein content.

Gastropods collected from the harbour area (LPB site) showed higher superoxide dismutase (SOD) activity than those from the other two sites (PC and CA) ($F = 13.277$, $P = 0.0001$, df = 2, N = 27) (Figure 2A). Also the reduced glutathione content (GSH) revealed a similar pattern, showing the highest values in the LPB site ($F = 8.148$, $P = 0.002$, df = 2, N = 27) (Figure 2C).

On the other hand, glutathione-S-transferase (GST) activity in digestive gland did not show significant differences between sampling sites ($F = 2.342$, $P = 0.118$, df = 2, N = 27) (Figure 2B).

Oxidative damage

In relation to oxidative damage, significant differences in lipid peroxidation among sites were obtained (TBARs: $F = 6.357$, $P = 0.006$, df = 2, N = 27); individuals collected in the LPB site showed the highest values (Figure 3).

Stress parameters and imposex response

Significant differences were obtained in DistLM for SOD, GSH and TBAR variables on penis length (as response variable). These results showed that 61.5% of variability in penis length was explained by stress parameters (Table 3).

DISCUSSION

The imposex incidence and female penis length (FPL) recorded in this work for *Buccinanops globulosus* could be related to maritime traffic and levels of TBT reported previously in Nuevo Gulf (Bigatti *et al.*, 2009; del Brío, 2011). Pollutants could be bioaccumulating in aquatic organisms

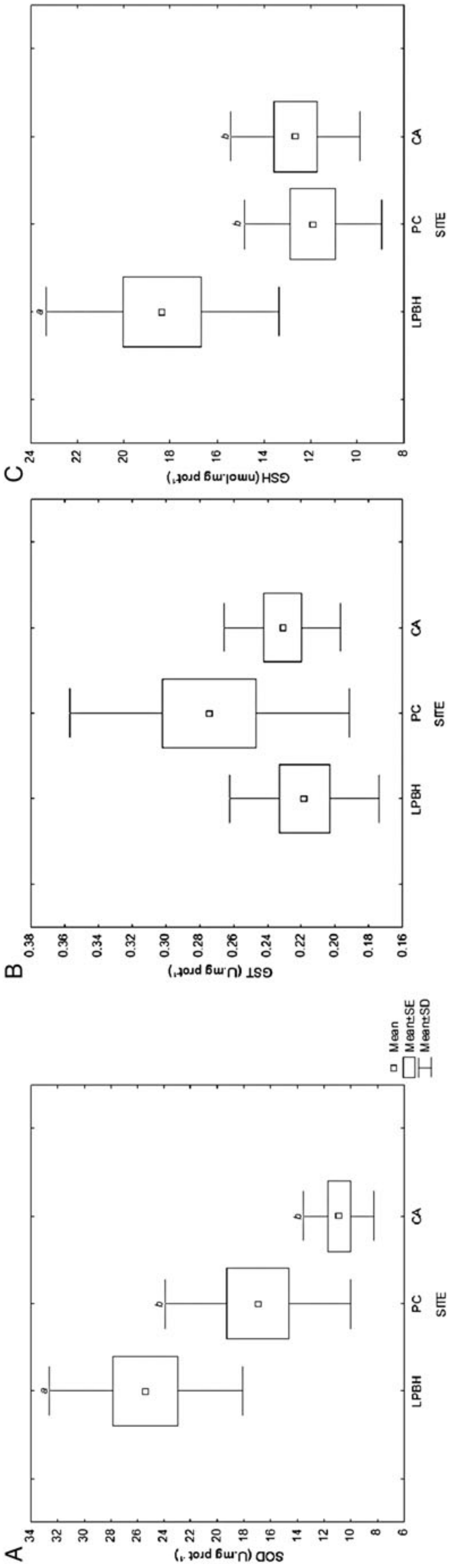

Fig. 2. Superoxide dismutase (SOD) (A), glutathione-S-transferase (GST) (B) activities expressed as U mg^{-1} prot and reduced glutathione (GSH) (C) levels expressed as nmol mg^{-1} prot, in digestive gland of *Buccinanops globulosus*. Results are expressed as mean ± SD (N = 9). Letters *a* and *b* indicate significant differences between sampling sites (LPB, PC and CA).

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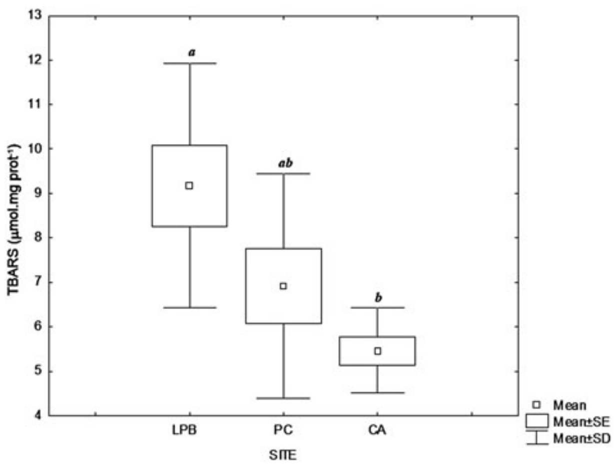

Fig. 3. Lipid peroxidation, expressed as µmol TBARS mg^{-1} prot, in digestive gland of *Buccinanops globulosus*. Results are expressed as mean ± SD (N = 9). Letters *a* and *b* indicate significant differences between sampling sites (LPB, PC and CA).

and affecting their defence mechanisms (Regoli & Principato, 1995; Chandran *et al.*, 2005; Chen *et al.*, 2011). In the LPB area, del Brío and colleagues detected butyltin levels (TBT + dibutyltin-DBT- + monobutyltin-MBT-) up to 265.8 ng (Sn) g^{-1} dry weight (dw) in sediments and up to 567.8 ng (Sn) g^{-1} (dw) in the tissues of the marine gastropod *Odontocymbiola magellanica*, the gonads and digestive gland being the organs with the highest TBTs concentration (del Brio *et al.*, in press). Also polyaromatic hydrocarbons such anthracene, benzo(b)fluoranthene (Torres *et al.*, 2013) and trace metals such as copper (Cu), iron (Fe), lead (Pb) and zinc (Zn) were detected in *B. globulosus* (Primost, 2014) in the LPB area confirming the capacity of these gastropod species to bioaccumulate different pollutants and potentially form reactive oxygen species (ROS).

ROS formation and changes in the oxidative balance have been observed as a result of exposure to environmental levels of TBT in bivalves (Huang *et al.*, 2005; An *et al.*, 2009) and gastropods (Jia *et al.*, 2009; Gopalakrishnan *et al.*, 2011). Imposex in *B. globulosus* was associated with TBT presence (Bigatti *et al.*, 2009) in the LPB area. In this work, the oxidative stress responses registered in imposexed gastropods could be attributed to TBT and other contaminants detected at the LPB harbour area such as trace metals and PAHs (Gil *et al.*, 1988, 1999, 2006; Commendatore *et al.*, 2000; Di Salvatore *et al.*, 2013; Torres *et al.*, 2013; Primost, 2014).

It is well known that a wide range of pollutants enhance enzymatic and non-enzymatic antioxidants in marine invertebrates to protect cells against oxidative damage (Livingstone, 2001; Brown *et al.*, 2004; Valavanidis *et al.*, 2006). Our results show highest SOD activities and highest concentrations of reduced glutathione in the digestive gland of gastropods from the LPB site. However, the glutathione-S-transferase (GST) activity showed a different pattern compared with these former antioxidant responses, where no significant differences in its activity were observed among the three sampling sites. Glutathione-S-transferase is a biotransformation enzyme which catalyses the conjugation of electrophilic pollutants with reduced glutathione (GSH). The resulting conjugates increase their water solubility favouring the excretion processes (Armstrong, 1997; Hayes *et al.*, 2005). In molluscs, the activity of GST usually increases in relation to detoxification processes (Almeida *et al.*, 2005; Huang *et al.*, 2005). However in 2005, Huang and colleagues determined that GST activity in the fish *Meretrix meretrix* may be increased or inhibited depending on high or low TBT concentrations in water, respectively (Huang *et al.*, 2005). Our results shows that pollution present in the PC site would induce a low increase in GST activity in the digestive gland of *B. globulosus*, while the pollutant presence in CA environment was not enough to produce changes in GST activity. In the present work, antioxidants (SOD and GSH) increased in the proximity of the harbour area, which was in concordance with higher imposex levels and penis length. Former studies, in the same sampling area, related to oxidative stress responses in the bivalve *Aulacomya atra* have detected seasonal changes in the antioxidant defences in relation to trace metal exposure and environmental pollution (Di Salvatore *et al.*, 2013; Giarratano *et al.*, 2013). In both studies, animals from the harbour area were the most affected, showing an increase in the antioxidant defences and also suffering higher oxidative damage to lipids. Meanwhile, a study in the fish *Sebastiscus marmoratus* exposed to TBT also revealed an increase in SOD activity in the liver (Wang *et al.*, 2005). In this sense, SOD increasing in *B. globulosus* probably could be related to TBT contamination detected recently in the area (Bigatti *et al.*, 2009; del Brío, 2011).

Lipid peroxidation has also been reported as a principal cause of cellular damage induced by oxidative stress conditions (Valavanidis *et al.*, 2006). Membrane alterations in molluscs are the major target of cellular damage in organisms exposed to trace metals and other toxic substances (Viarengo *et al.*, 1990, 1991). In the present work, *B. globulosus* showed a marked increment in lipid peroxidation in the digestive gland of gastropods collected from the harbour area compared with animals from CA and PC sites. In addition, our results are in accordance with those reported by Zhou *et al.* (2010), where TBT exposures increase lipid peroxidation (measured as malondialdehyde (MDA) levels) in the abalone *Haliotis diversicolor supertexta*. Similar results were also observed in laboratory studies where rats exposed to repeated TBT doses showed incremental MDA levels (Liu *et al.*, 2006); Bernat and colleagues also reported the same effect in the filamentous fungus *Cunninghamella elegans* exposed to TBT (Bernat *et al.*, 2014).

Our results suggest that the differences in terms of oxidative stress responses and high imposex incidence observed in *B. globulosus* at the harbour site indicate a negative effect on its physiological state due to the presence of pollutants in the aquatic environment. The possible relationship between the induction mechanism of imposex and oxidative stress

Table 3. Results from DistLM multiple correlations between stress parameters (co-variable) and penis length (response variable).

	R^2	*F*	*P*	df
U SOD mg^{-1} prot	0.404	16.921	0.0004*	25
Mmol TBARS mg^{-1} prot	0.586	10.539	0.0042*	24
U GST mg^{-1} prot	0.615	0.176	0.200	23
Nmol GSH mg^{-1} prot	0.615	0.015	0.907	22

Best solution: $R^2 = 0.6152$; N° Vars = 4; Selections = All. * indicates significant differences at $P < 0.05$ between sites (LPB, PC, CA) for these variables.

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should be tested in controlled experiments exposing normal and imposexed individuals to TBT, followed by comparative measurement of oxidative stress parameters in experimental groups.

CONCLUSIONS

In conclusion, both oxidative stress responses and imposex incidence were increased in gastropods inhabiting the harbour area. While *B. globulosus* suffers an increase of the antioxidant defences (SOD activity and GSH content), an oxidative damage to lipids (TBARs levels) was still observed.

This is the first study on oxidative stress responses associated with marine pollution in an edible gastropod affected by imposex in Argentina. Although TBT is not the only pollutant present in the harbour area, further integrated studies are necessary to evaluate the role of oxidative stress responses in *Buccinanops globulosus* as biomarkers of TBT presence.

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