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Pen shell *Pinna nobilis* L. (Mollusca: Bivalvia) from different peculiar environments: adaptive mechanisms of osmoregulation and neurotransmission

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Abstract
The pen shell *Pinna nobilis* (Linnaeus, 1758) is the largest endemic bivalve mollusc of the Mediterranean Sea, listed as an endangered species in the European Union. Because no information is available about the adaptation of pen shells to different habitats, herein the fundamental conditions of adaptation of *P. nobilis* to peculiar natural environments, such as the Strait of Messina (SM) and the meromictic Faro Lake (FL; Sicily, Italy), were explored by assessing the morphology, mucous production, osmoregulation and neurotransmission of their gills. Although gills of the pen shells from both sites exhibited a regular morphology, a higher presence of acid mucous cells was detected in *P. nobilis* from FL than SM, as well as higher levels of osmolytes but without interfering the osmoregulatory processes. About the functioning of gills, the cholinergic (i.e. acetylcholine and AChE) neuronal system was unaltered between individuals from the two sites, whereas the GABAergic neurotransmission (i.e. 4-aminobutyrate or GABA) was significantly augmented in gills of *P. nobilis* from FL than SM. This may be an adaptive response to hypoxic conditions in FL, as supported by the increased hypoxia-inducible factor (HIF-1α) in gills of pen shells from FL than SM. Noteworthy, this study reports for the first time the presence of the GABA neurotransmitter within the metabolite profile, obtained by application of a protonic Nuclear Magnetic Resonance (^{1}H NMR)-based metabolomics approach, of a marine bivalve. Therefore, GABA may be suggested as a metabolite biomarker in pen shells. Overall, findings from this study provide new insights on the behavioural and adaptive responses of the pen shell *Pinna nobilis* settled in different peculiar environments.

Keywords: *Coastal brackish system, pen shells, NMR-based metabolomics, neurotransmitters, osmolytes*

Introduction

The pen shell *Pinna nobilis* (Linnaeus, 1758) is the largest endemic bivalve mollusc of the Mediterranean Sea (Butler et al. 1993). It is a filter-feeding mollusc with a long triangular shape, which can reach a size of up to 120 cm (Zavodnik et al. 1991). However, the average shell size of an adult is usually 65 cm. It was reported that at young life stages (1–3 years), the pen shells rapidly develop at growth rate of 10 cm per year, followed by slower growth of 10 cm every 3 years after sexual maturity is reached (Richardson et al. 1999). The pen shell is a sessile organism commonly inhabiting coastal and estuarine environments at depth ranging from 0.5 to 60 m, with the apex of the shell anchored by byssus threads in the substrate, mostly sand and muddy-sand bottoms preferentially overgrown by seagrass meadows of *Posidonia oceanica* or *Cymodocea nodosa* (Zavodnik et al. 1991; Richardson et al. 1999; Vazquez-Luis et al. 2014). The presence of pen shell populations in the Mediterranean Sea was documented in various areas with discrepancies in physico-chemical features, including the western Mediterranean along

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Mallorca Island and in the Cabrera Archipelago National Park (Natalotto et al. 2015), along the eastern Spanish coastlines in the Moraira Bay (García-March et al. 2006), in the southeastern France in the Embiez archipelago (Trigos et al. 2015), in the northeast Tunisia in the hyper-eutrophic Ghar El Melh Lagoon (Zakhama-Sraieb et al. 2011), along the northern and eastern Tunisian coastlines in Bizerta Lagoon, Gulf of Tunis and in the Monastir Bay (Rabaoui et al. 2008, 2009), in the eastern Mediterranean in Greece in the marine Lake Vouliagmeni (Katsanevakis 2007) and in the North Aegean Sea in the Thermaikos Gulf (Galinou-Mitsoudi et al. 2006), and in the central Mediterranean in the Croatian Adriatic Sea (Richardson et al. 2004), in Italy in the Gulf of Oristano (Addis et al. 2009), in the Ionian Sea in the Gulf of Taranto (Centoducati et al. 2007), in the Strait of Messina (Matozzo et al. 2016), and in the meromictic Faro Lake in Sicily (Bottari et al. 2017). However, over the last few decades, populations of *P. nobilis* have greatly declined due to habitat degradation resulting in reduction and loss of seagrass meadows, and increased anthropogenic activities such as reckless fishing, illegal trawling, and boat anchoring (Basso et al. 2015; Natalotto et al. 2015; Vazquez-Luis et al. 2017). As a consequence, in the European Union the pen shell *P. nobilis* has been listed as an endangered species, and nowadays it is under strict legal protection according to the European Council Directive 92/43/EEC (Habitats Directive; EEC 1992), and to the subsequent Council Directive 2006/105/EC, Annex IV (EC 2006).

Within the Central Mediterranean Sea, the Strait of Messina (Italy), with a length of 31 km and a width ranging from 3.2 to 14 km, represents the geographical point of connection between the Ionian Sea in the south and the Tyrrhenian Sea in the north. The hydrodynamic characteristics and ecological conditions of the Strait of Messina are peculiar, and strictly linked to its geomorphological structure (Spanò & De Domenico 2017). The particular geomorphology of the whole area, together with the different physical, chemical and oscillatory water characteristics between the Ionian and Tyrrhenian basins, determine peculiar hydrodynamic phenomena in the Strait of Messina (Mosetti 1988; Spanò & De Domenico 2017). These events contribute to the high biodiversity and distribution of benthic populations, including exclusive communities of Atlantic origin, in this complex and unique ecosystem with peculiar topography and hydrodynamics (Giacobbe & Spanò 2001; Maisano et al. 2013; Porporato et al. 2013; Smriglio et al. 2016; Spanò & De Domenico 2017).

Capo Peloro Lagoon is a coastal brackish system located in the north-easternmost part of Sicily (Italy), between the Ionian Sea and the Tyrrhenian Sea. It was declared a Nature Reserve in 2001 (Regional Administrative Decree 437/44 of 21.06.2001), and consists of two brackish basins, namely Ganzirri Lake and Faro Lake, connected to each other and to the sea through channels (Bottari et al. 2005; Leonardi et al. 2009; Maisano et al. 2016b). Faro Lake is a small and relatively deep meromictic coastal pond, covering a surface area of 26-ha and with a maximum depth of 28 m at its central part. It represents a rare example of a meromictic basin, characterized by the presence of anoxic waters generally confined below 15 m depth (Truper & Genovese 1968). For its particular characteristics, to date Faro Lake has been widely investigated, also with reference to its benthic communities (Leonardi et al. 2009; Giacobbe 2012; D'Agata et al. 2014; Capillo et al. 2018; Parrino et al. 2018; Spinelli et al. 2018).

Currently, no information is available in the literature about the adaptation of pen shells to different habitats. Therefore, the purpose of the present study was to explore the fundamental conditions of adaptation of the pen shell *P. nobilis* to natural environments with peculiar hydrodynamic, geomorphological and physico-chemical characteristics, such as the Strait of Messina and the meromictic Faro Lake. In order to achieve this aim, the gills of the pen shells were selected as target organs. Indeed, the gills of bivalve molluscs are a particularly sensitive organ responsible for numerous vital functions, including nutrient uptake being filter feeders, gas exchange, maintenance of the optimal osmotic pressure, acid-base balance, and neurotransmission (Bayne et al. 1976). The gills of bivalves are constantly in contact with the surroundings and are very sensitive to even minor chemical or physical changes in the external conditions. Indeed, mollusc gills are the first organ to be affected by environmental changes, and this may result in adaptive and reversible alterations in their structural pattern and biochemical profiles, as documented in marine mussel *Mytilus galloprovincialis* in response to low oxygen conditions (Giannetto et al. 2015, 2017), after exposure to meromictic environments with low hydrodynamics (D'Agata et al. 2014), and in response to petrochemical compounds (Cappello et al. 2013, 2015; Maisano et al. 2017) or heavy metals (Ciacci et al. 2012), as well as in blue mussel *Mytilus edulis* from sites with different anthropogenic influences (Manduzio et al. 2004), in clams *Tapes* spp. settled in environmentally different subbasins of a Mediterranean coastal lagoon (Maisano et al. 2016b), and in pen shell *P. nobilis* collected from anthropogenic impacted environments (Natalotto et al. 2015).

Materials and methods

Study area and sample collection

Sampling of the pen shell *Pinna nobilis* was carried out under municipal authorization (dated January 2016, reference number Prot. 454) in February 2016 from the Strait of Messina (SM; central Mediterranean Sea) and the Faro Lake (FL; Sicily, Italy) (Figure 1). Six individuals of *P. nobilis* (mean shell length ± standard deviation (S.D.): 45.7 ± 9.8 cm and mean shell width ± S.D.: 16.3 ± 1.7 cm for pen shells from SM; mean shell length ± S.D.: 32.3 ± 8.5 cm and mean shell width ± S.D.: 12.5 ± 3.5 cm for pen shells from FL) were collected at 15 m depth from SM and at 4–6 m depth from FL, and then carefully dissected in accordance to the protocol proposed by Yonge (1953), following the recommendations provided within the EU Directive 2010/63/EU for welfare of animals used for scientific purposes. Gills were then excised from each individual, flash-frozen in liquid nitrogen and then stored at −80°C until processing for metabolomic and enzymatic analysis. A small portion of each dissected gill tissue was also taken for morphological, histochemical and immunohistochemical investigations. Water physical and chemical parameters, such as temperature, salinity, pH, redox potential (Eh) and dissolved oxygen (DO) were measured in triplicate at both sampling sites by using a portable instrument (Multi 340i/SET, WTW Wissenschaftlich, Weilheim, Germany). In order to calibrate the oxygen sensor, the Winkler's method was used for an accurate determination of DO (Sanfilippo et al. 2016).

Histological and histochemical analysis

For histological evaluation, gills of the pen shells were fixed in 4% paraformaldehyde in 0.1 M phosphate buffered solution (PBS; pH 7.4) at 4°C, dehydrated in a graded series of ethanol, and finally embedded in Paraplast (Bio-Optica, Italy). Histological sections of 5 μm thickness were cut using a rotary automatic microtome (Leica Microsystems, Wetzlar, Germany), glass-slide mounted and stained with Hematoxylin/Eosin (Bio-Optica, Italy) to assess morphological features. Additionally, a combined method for polysaccharides based on Periodic Acid Schiff (PAS) staining and acid mucopolysaccharides based on Alcian Blue (AB; pH 2.5) staining was applied to detect the presence of neutral and acid mucous cells, respectively, in the gill epithelium of *P. nobilis* from the SM and FL sites.

Five fields (size of 250 μm each one, selected as field of view using the objective 10x) of one section per sample were therefore examined under a 40x oil-immersion objective using a motorized Zeiss Axio Imager Z1 microscope (Carl Zeiss AG, Werk Göttingen, Germany) equipped with an AxioCam digital camera (Zeiss, Jena, Germany).

Extraction of polar metabolites

Polar metabolites were extracted from gill tissues of pen shells by applying a "two-step" methanol/chloroform/water procedure, as described previously (Fasulo et al. 2012; Cappello et al. 2013, 2018b). In brief, gill tissues (c.a. 150 mg) were homogenized in 4 mL/g of

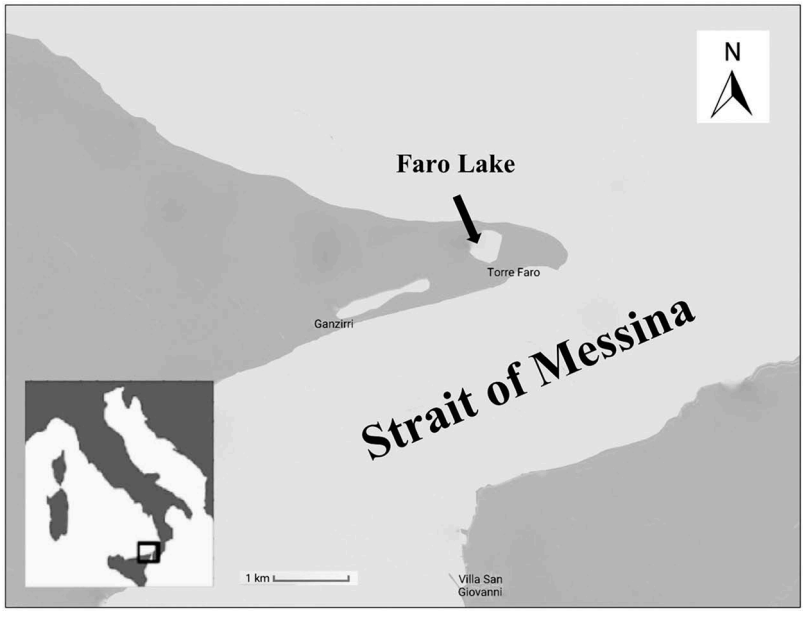

Figure 1. Map of the study area showing the Strait of Messina (SM) and Faro Lake (FL; Sicily, Italy).

cold methanol and 0.85 mL/g of cold water by a TissueLyser LT bead mill (Qiagen) with 3.2 mm stainless steel beads, for 8 min at 50 vibrations/s. The homogenates, after addition of 4 mL/g chloroform and 2 mL/g water, were vortexed for 60 s, left on ice for 10 min for phase separation, and centrifuged for 5 min at 2000*g* at 4°C. The upper methanol layer (600 µL) with polar metabolites was transferred into glass vials, dried in a centrifugal vacuum concentrator (Eppendorf 5301), and stored at −80°C. Prior to Nuclear Magnetic Resonance (NMR) analysis, the dried polar extracts were re-solvated in 600 µL of a 0.1 M sodium phosphate buffer (pH 7.0, 10% D_2O; Armar AG, Döttingen, Switzerland) with 1 mM 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) (Sigma-Aldrich Co), used as internal reference, and transferred to a 5 mm diameter NMR tube.

Extracts of gill tissues were analyzed on a Varian-500 NMR spectrometer working at a spectral frequency of 499.74 MHz at 298 K. One-dimensional (1-D) ^{1}H NMR spectra were obtained using a PRESAT pulse sequence to suppress the residual water resonance and 6 kHz spectral width with a 2.0 s relaxation delay. A total of 256 transients were collected into 16,384 data points requiring a 20 min acquisition time. All data sets were zero filled to 32,768 data points and exponential line-broadenings of 0.5 Hz were applied before Fourier transformation. All spectra were manually phased, baseline-corrected, and calibrated (DSS at 0.0 ppm) using Chenomx Processor, a module of Chenomx NMR Suite (version 5.1; Chenomx Inc., Edmonton, Canada) software. The peaks of interest, namely the metabolites related to osmoregulation (i.e. taurine, betaine, hypotaurine and homarine), and those related to the cholinergic (i.e. acetylcholine) and GABAergic (i.e. 4-aminobutyrate or GABA) neuronal systems, were identified within the ^{1}H NMR spectra and quantified using Chenomx Profiler, another module of Chenomx NMR Suite software, which uses the concentration of a known DSS signal to determine the levels of individual metabolites (Brandão et al. 2015; Cappello et al. 2015, 2017a, 2017b, 2017c, 2018a; Maisano et al. 2016a).

Enzymatic analysis

Gill tissues of the pen shells were homogenised in ten volumes (w/v) of 100 mM Tris-HCl buffer (pH 7.5). Homogenates were then briefly sonicated (2–3 s) by ultrasonic processor and centrifuged for 15 min at 9000*g* at 4°C (Manduzio et al. 2004). After centrifugation, supernatants were collected and immediately used for the determination of the activity of acetylcholine esterase (AChE), by applying the colorimetric method of Ellman et al. (1961), slightly modified. In brief, thiocholine derivatives are hydrolysed by AChE to produce thiocholine. Subsequent combination with 5,5-dithiobis-2-dinitrobenzoic acid (DTNB) forms the yellow anion 5-thio-2-nitrobenzoic acid, which absorbs strongly at 412 nm. The enzymatic activity of AChE was measured in duplicate by using a Shimadzu UV-2100 spectrophotometer operating at 25°C. Results were referred to the total protein content of the samples determined by the Bradford method (Biorad Protein Assay), using bovine serum albumin as standard. AChE activity was expressed as µmol/min/mg.

Immunohistochemical analysis

Histological sections of gills of the pen shells from SM and FL were also processed for immunolocalization of the hypoxia-inducible factor (HIF-1α) by application of the indirect immunofluorescence method (Maisano et al. 2017). In brief, gill sections were incubated with normal goat serum (NGS) in PBS (1:5) for 1 h in order to block the non-specific binding sites for immunoglobulins. The sections were then incubated overnight with the primary antibody anti-HIF-1α developed in mouse (Abcam, Cambridge, UK) and diluted 1:100, in a humid chamber at 4°C. After incubation, the slides were rinsed in PBS for 10 min and then incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Sigma) diluted 1:50 for 2 h at room temperature. The pre-absorption procedures, performed by incubating sections with the antiserum pre-absorbed with the respective antigen (10–100 g/mL), were carried out overnight at 4°C and used as positive control, while negative controls were performed by substitution of the primary antiserum with PBS.

The microscopical observations were made for each slide on five fields (size of 250 µm each one, selected as field of view using the objective 10x) of one section per sample, using a motorized Zeiss Axio Imager Z1 epi-fluorescence microscope (Carl Zeiss AG, Werk Göttingen, Germany), integrated with a digital camera (AxioCam, Zeiss, Jena, Germany). Appropriate filters for the excitation of FITC (480/525 nm) were used, and then the images were processed by AxioVision Release 4.5 software (Zeiss) for the count of HIF-1α immunopositive cells.

Statistical analysis

Results from all investigations were expressed as mean ± S.D. All data obtained were statistically analysed with the GraphPad software (Prism 5.0,

San Diego CA, USA) by one-way analysis of variance (ANOVA). The Tukey's multiple comparison test was applied for histochemical and immunohistochemical data and the Student's one-tailed *t*-test for metabolomic and enzymatic data, in order to assessing significant differences between pen shells from the SM and FL sites. Data were considered statistically significant at $p < 0.05$.

Results

Water physico-chemical parameters

The data of physico-chemical parameters of the pen shell sampling sites are herein reported. At SM, water temperature of 13.7 ± 0.4°C, salinity of 38.5 ± 0.2, pH 8.16 ± 0.06, Eh of 132 ± 2 mV, and DO of 8.27 ± 0.13 mL/L were recorded. Conversely, at FL water temperature was 14.7 ± 0.3°C, salinity 35.1 ± 0.4, pH 8.27 ± 0.09, Eh 115 ± 1 mV, and DO 6.65 ± 0.18 mL/L. Data are reported as mean ± S.D.

Histological and histochemical results

The histological observations of gills of the pen shell *P. nobilis* (n = 6 per sampling site) from both SM (Figure 2(a)) and FL (Figure 2(b)) revealed a regular morphology of the branchial tissue, displaying distinctive gill filaments arranged in parallel and ciliated cells with a regular distribution along the outer epithelial layer.

Additionally, the AB/PAS staining was also performed on gill tissue sections to visualize mucous cells. Neutral mucopolysaccharides were present in the branchial epithelium of specimens from both sites, whereas a higher number of acid mucous cells was detected in gills of the pen shells from FL in respect to those from SM, as clearly reported in Figure 2(c).

Osmoregulation

Proton chemical shift assignments and quantification of metabolites related to osmoregulation are reported in Table I. Overall, the NMR-based metabolomics analysis revealed that osmolytes are present at higher concentrations in gills of the pen shells (n = 6 per sampling site) from FL than SM. Specifically, a quantitative comparison of metabolomics data revealed a statistically significant ($p < 0.05$) increase of 64% in taurine, 32% in betaine, 683% in hypotaurine and 146% in homarine levels in gills of *P. nobilis* from FL compared to those from SM.

Neurotransmission

Proton chemical shift assignments and quantification of metabolites related to neurotransmission are reported in Table I. Data from the ^{1}H NMR-based metabolomics analysis highlighted that acetylcholine exhibited concentrations not significantly different in the gills of the pen shells (n = 6 per sampling site) collected from SM in comparison with those from FL. Conversely, the levels of GABA were found to be increased by 96% in the gills of *P. nobilis* from FL in respect to those from SM.

Also, by application of the enzymatic approach, it was revealed that the measurements of AChE activity showed no significant difference in gills of the pen shells collected from both sites, as showed in Figure 3.

Hypoxia-inducible factor

By using the antibody directed against HIF-1α, few immunopositive cells were found in the gills of *P. nobilis* (n = 6 per sampling site) collected from SM (Figure 4(a)). On the contrary, a high presence of both HIF-1α immunopositive cells and fibers was evidenced along the branchial epithelium of the pen shells from FL (Figure 4(b)). The statistical analysis on the average of cells immunopositive to HIF-1α ($p < 0.05$) is also shown in Figure 4(c).

Discussion

The pen shell *Pinna nobilis* (Linnaues 1758), the largest endemic bivalve mollusc of the Mediterranean Sea, commonly inhabits coastal and estuarine environments (Zavodnik et al. 1991; Richardson et al. 1999). The presence of pen shell populations in the Mediterranean Sea was documented in various areas with discrepancies in physico-chemical features. In spite of the scientific community interest for the conservation of *P. nobilis*, which is recognized as an endangered species in the European Union, knowledge about the adaptation of pen shells to contrasting habitats is still scarce. Therefore, in order to fill in this knowledge gap, the fundamental conditions of adaptation of the pen shell *P. nobilis* settled in the Strait of Messina (SM) and in the meromictic Faro Lake (FL), which are environments with peculiar hydrodynamic, geomorphological and physico-chemical characteristics, were herein explored.

The gills of bivalve molluscs are a particularly sensitive organ responsible for numerous vital functions (Bayne et al. 1976), and are very sensitive to even minor chemical or physical changes in the external conditions. Notwithstanding the substantial differences in the environmental conditions between the

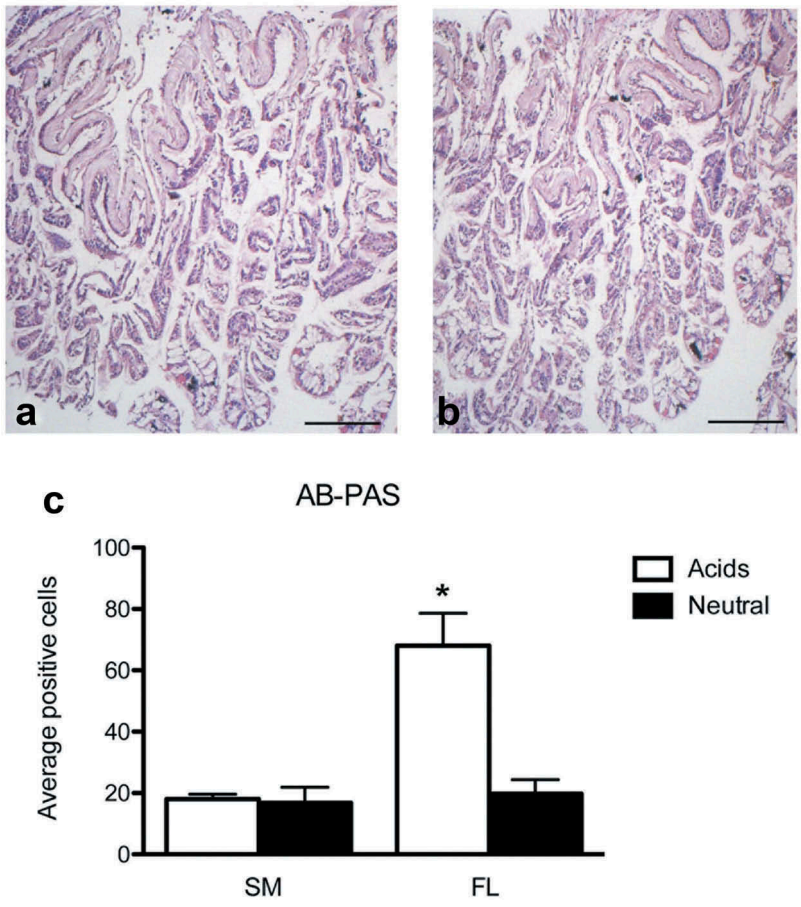

Figure 2. Histological sections of gills of the pen shell *Pinna nobilis* collected from SM (a) and FL (b), showing a regular morphology with parallel gill filaments and ciliated cells at the outer epithelial layer. Mean and S.D. of neutral and acid mucous cells detected in gills of the pen shells from both sites (c). Significance at $p < 0.05$. Scale bar = 20 μm.

Table I. ^{1}H NMR measurements (mM) expressed as means ± S.D. of metabolites detected in gills of pen shell *Pinna nobilis* collected from the Strait of Messina (SM) and Faro Lake (FL) (s: singlet; d: doublet; t: triplet; dd: doublet of doublets; m: multiplet) (* $p < 0.05$; Student's one-tailed *t*-test).

Metabolites	Chemical shift and peak shape, ppm	SM	FL
Osmolytes			
Hypotaurine	2.64 (t), 3.36 (t)	5.28 ± 1.1	39.15 ± 6.2*
Betaine	3.25 (s), 3.89 (s)	156.63 ± 13.7	207.07 ± 15.6*
Taurine	3.25 (s), 3.41 (t)	256.15 ± 14.2	420.92 ± 18.4*
Homarine	4.35 (s), 7.95 (dd), 8.02 (d), 8.53 (dd), 8.71 (d)	26.32 ± 3.6	64.97 ± 4.6*
Neurotransmitters			
4-Aminobutyrate	1.88 (m), 2.28 (t), 3.05 (t)	1.74 ± 0.4	3.42 ± 0.7*
Acetylcholine	2.15 (s), 3.20 (s)	3.39 ± 0.7	3.40 ± 0.8

Strait of Messina and the meromictic Faro Lake, surprisingly the pen shell naturally settled in both sites exhibited a regular morphology of their gills, although a higher presence of acid mucocytes was detected in the branchial tissues of *P. nobilis* from FL in respect to SM. The overproduction of acid mucosubstances, which form around the gill epithelium a protective coat that acts as a lubricant and possesses also antibacterial properties (Sanchez et al. 1997), may enhance the protective function of mucous against pathogens and natural toxins, and therefore it is indicative of an environmental adaptation of pen shell to the microbial communities present in FL (Truper & Genovese 1968; Leonardi et al. 2009).

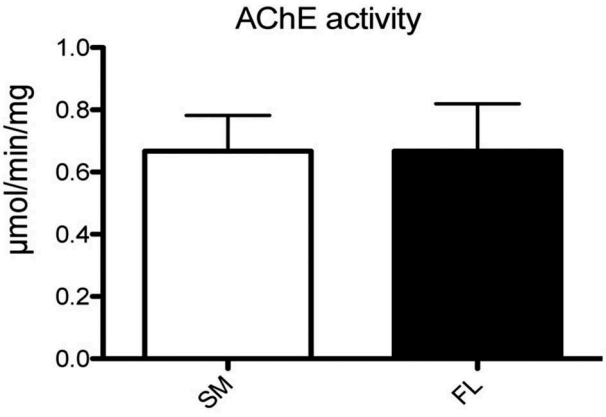

Figure 3. Data of AChE activity measured in gills of the pen shell *Pinna nobilis* collected from SM and FL.

Furthermore, in order to evaluate if the functioning of pen shell gills is influenced by the contrasting environmental conditions of SM and FL, a metabolomics approach was also herein applied. It is an innovative, sensitive and effective tool widely employed in the last decades to detecting differences in the metabolite profile of organisms in response to perturbations in environmental conditions or following exposure to contaminants (Brandão et al. 2015; Maisano et al. 2016a, 2017; Cappello et al. 2017a, 2017b, 2017c), having thus the potential to elucidate the interactions between individuals and their environment. In the present study, the NMR-based metabolomics analysis revealed no environmental interferences in the osmoregulatory processes in the gills of *P. nobilis* from SM and FL, though osmolytes, namely taurine, betaine, hypotaurine and homarine, were all found at higher concentrations in individuals from FL than those from SM. It is known that organic osmolytes are small molecules playing a crucial role in the osmotic balance of marine invertebrates, since their active accumulation or release at intracellular level enable cells to adapt very rapidly to fluctuations in the external saline environment (Somero & Bowlus 1983). Therefore, it is highly reasonable to hypothesize that the elevated levels of osmolytes detected in the pen shell from FL represent an adaptive response of *P. nobilis* to the rapid fluctuations in the value of salinity that occur in the meromictic lagoon in respect to the sea.

The environmental metabolomics applied in this study was also able to shed light on the neurotransmission systems, namely the cholinergic and GABAergic systems, in gills of the pen shells from SM and FL. In bivalve molluscs, the cholinergic system is responsible for the physiological functioning of the efferent nervous system and regulation of ciliary beating, and acetylcholine is in fact recognized as an excitatory neurotransmitter (Catapane et al. 1974). Acetylcholine is regulated by the enzyme acetylcholinesterase (AChE), which is responsible for its inactivity and breakdown by hydrolysis into choline and acetate. AChE is therefore essential for the normal functioning of the branchial epithelium, and measurements of AChE activity have been widely used as a validated biomarker of neurotoxicity in aquatic bivalves (Ciacci et al. 2012; D'Agata et al. 2014; Cappello et al. 2015; Maisano et al. 2015; Natalotto et al. 2015). In this study, the cholinergic neuronal system of pen shells was found not influenced by the different environmental conditions of the two sampling sites, as demonstrated by the comparable level of acetylcholine evaluated by metabolomics and the comparable enzymatic AChE activity measured in gill tissues of the pen shells from SM and FL.

Nevertheless, discrepancies in the GABAergic neurotransmission were also revealed by the metabolomics approach with the increased level of 4-aminobutyrate, or GABA, in gills of *P. nobilis* from FL respectively to those from SM. Although the presence of the relaxant neurotransmitter GABA has been previously documented in bivalve molluscs, such as in the freshwater mussel *Elliptio complanata* (Gagnè et al. 2010), its appearance within the ^{1}H NMR-based metabolite profile of marine bivalves, to the best of our knowledge, was never reported before. Therefore, it may be suggested as a metabolite biomarker in pen shells. However, it is

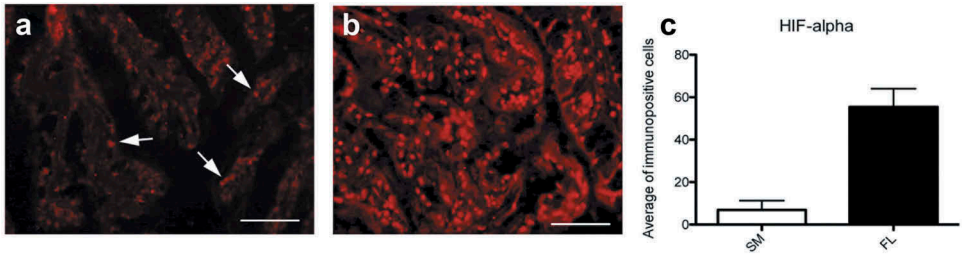

Figure 4. Immunolocalization of HIF-1α in gills of the pen shell *Pinna nobilis* collected from SM, showing few immunopositive cells (a; arrows), and from FL displaying numerous immunopositive cells and fibers (b). Mean and SD of HIF-1 α positive cells (c). Significance at $p < 0.05$. Scale bar = 20 µm.

worthy to note that modulation of GABAergic synaptic transmission has been previously observed in a variety of hypoxia-tolerant species in response to decreased oxygen levels, such as in the shore crab *Carcinus maenas* (Nillsson & Winberg 1993) and in the crucian carp (Hylland & Nilsson 1999). In fact, the increase in GABA concentration during anoxic conditions occurs because the conversion of glutamate to GABA by the glutamate acid decarboxylase is an anaerobic process (Milton & Prentice 2007). Taking into account the lower level of oxygen recorded in FL in respect to SM, the augmented concentrations of GABA detected in gills may be explained as an adaptive response of pen shell to the hypoxic conditions of the meromictic lagoon. This explanation gains further plausibility with the increased immunopositivity to the hypoxia-inducible factor (HIF-1α) observed in gills of the pen shells from FL than SM. Indeed, it is widely recognized that HIF proteins play a key role in hypoxia signal transduction and coordinate the compensatory responses to hypoxia in aquatic bivalves (Kawabe & Yokoyama 2012; Giannetto et al. 2015, 2017; Maisano et al. 2017).

Overall, findings from this study provide new insights on the behavioural and adaptive responses of the pen shell *Pinna nobilis* settled in contrasting habitats, characterized by peculiar hydrodynamics, geomorphology and physico-chemical features, therefore delineating the fundamental conditions of adaptation of the pen shell in different natural environments.

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