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1 **Characterization of Glutathione Peroxidase Diversity in the Symbiotic Sea Anemone**
2 ***Anemonia viridis***

3
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15
16 † The authors dedicate this article to the memory of their friend and colleague, Pierre-Laurent
17 Merle, who died February 1st, 2015. The scientific world has lost a man fascinated by the sea with
18 great humanity. Without his involvement at all stages of the project this work would not have been
19 possible.

Abstract

21

22 Cnidarians living in symbiosis with photosynthetic dinoflagellates (commonly named
23 zooxanthellae) are exposed to high concentrations of reactive oxygen species (ROS) upon
24 illumination. To quench ROS production, both the cnidarian host and zooxanthellae express a full
25 suite of antioxidant enzymes. Studying antioxidative balance is therefore crucial to understanding
26 how symbiotic cnidarians cope with ROS production. We characterized glutathione peroxidases
27 (GPx) in the symbiotic cnidarian *Anemonia viridis* by analysis of their isoform diversity, their
28 activity distribution in the three cellular compartments (ectoderm, endoderm and zooxanthellae) and
29 their involvement in the response to thermal stress. We identified a GPx repertoire through a
30 phylogenetic analysis showing 7 GPx transcripts belonging to the *A. viridis* host and 4 GPx
31 transcripts strongly related to *Symbiodinium* sp. The biochemical approach, used for the first time
32 with a cnidarian species, allowed the identification of GPx activity in the three cellular
33 compartments and in the animal mitochondrial fraction, and revealed a high GPx electrophoretic
34 diversity. The symbiotic lifestyle of zooxanthellae requires more GPx activity and diversity than
35 that of free-living species. Heat stress induced no modification of GPx activities. We highlight a
36 high GPx diversity in *A. viridis* tissues by genomic and biochemical approaches. GPx activities
37 represent an overall constitutive enzymatic pattern inherent to symbiotic lifestyle adaptation. This
38 work allows the characterization of the GPx family in a symbiotic cnidarian and establishes a
39 foundation for future studies of GPx in symbiotic cnidarians.

40

Keywords

42

43 Oxidative stress; Cnidaria; Glutathione Peroxidase; Symbiosis; Zooxanthellae

44 1. Introduction

45

46 For a long time, symbiotic cnidarians have ensured their evolutionary success by a life in symbiosis
47 with dinoflagellates of the genus *Symbiodinium*, also commonly named zooxanthellae.
48 Zooxanthellae are endosymbionts, living in the animal host cells. This intimate association offers
49 advantages for both partners. On the one hand, zooxanthellae find a protected and stable
50 environment and the animal cells actively provide inorganic compounds such as nitrogen,
51 phosphorus and sulfate used for the algal photosynthetic activity [1]. On the other hand, some of the
52 organic compounds produced by algal photosynthesis are transferred from the zooxanthellae to the
53 animal host, which makes them less dependent on predation. Such metabolic relationships also
54 confer costs and disadvantages, which must be tolerated by both partners. In particular, when
55 photosynthesis occurs, a great amount of molecular oxygen is produced. Although oxygen is
56 unavoidable, it can be transformed and reduced into harmful reactive oxygen species (ROS; [2]). As
57 a result, the animal tissues face both an intense diurnal hyperoxic state and consequently a
58 concomitant ROS overproduction [3-6]. Consequently, both partners must have the pathways for
59 cross-regulating many metabolic processes, especially those involved in ROS resistance [7-9]. This
60 explains why symbiotic cnidarians are considered interesting biological models for investigating
61 ROS resistance.

62

63 The study of ROS synthesis and removal in these organisms is also of great environmental interest.
64 Environmental perturbations (especially variations in temperature and UV radiation) often induce
65 symbiosis breakdown, a process commonly called bleaching (for reviews see [10-12]). Under
66 stressful conditions, zooxanthellae can be eliminated from or exit the host through different cellular
67 processes involving oxidative stress. Usually, both the cnidarian host and zooxanthellae express a
68 full suite of antioxidant enzymes to avoid damage from ROS production. But under stressful
69 conditions, imbalance between ROS overproduction and antioxidant defenses leads to cellular
70 damage (lipid peroxidation, protein oxidation, DNA degradation) resulting in the disruption of the
71 symbiotic association [13-16].

72

73 Many reports corroborate the fact that these symbiotic cnidarians possess extensive and specific
74 enzymatic and non-enzymatic antioxidant defenses [17]. In previous work, we focused our interest
75 on superoxide dismutases (SOD, EC 1.15.1.1), which are at the forefront of the defenses against
76 ROS by the dismutation of superoxide anions to H₂O₂ and O₂ [4,14,18]. At low levels, hydrogen
77 peroxide may play important roles in different cellular signaling pathways and be catabolized by
78 peroxidase-driven reactions (for review see [2,19]). However, at higher doses, H₂O₂ is a very

79 cytotoxic molecule, diffusing through biological membranes and therefore causing damage far from
80 its original location. Therefore, subsequent antioxidant systems are needed to counteract potential
81 H₂O₂ accumulation and cytotoxicity [20]. Within most cellular defense systems, the enzymes
82 glutathione peroxidase (GPx, EC 1.11.1.9) and catalase (CAT, EC 1.11.1.6) are the major
83 degradation enzymes of peroxides and organic peroxides. GPx inhibits production of high levels of
84 oxidant free radicals, such as the hydroxyl radicals derived from H₂O₂ and alkoxy radicals derived
85 from organic peroxides. CAT has a direct effect on H₂O₂ through a dismutation reaction [2]. GPx
86 and CAT can be considered as the essential partners of SOD, directing the flow of superoxide
87 radicals towards the formation of water molecules. Thus, the cytotoxic action of SOD depends on
88 the equilibrium between SOD, GPx and CAT, confirming the importance of this balance in
89 maintaining cellular integrity and function.

90

91 The GPx family (EC.1.11.1.9) is known to present an ubiquitous distribution within the 'tree of life'
92 and to possess a high variety of isoforms [21]. For example, in mammals, there are up to 8 isoforms
93 of GPx with specific cellular and subcellular localizations and activities [21,22,23]. In cnidarian
94 species, Hawkrige et al. [24] localized GPx proteins in the sea anemone *Anemonia viridis* and in
95 their symbiotic algae with immunocytochemical techniques. Moreover, previous transcriptomic
96 studies on *A. viridis*, revealed that some isoforms of glutathione peroxidases were up-regulated in
97 symbiotic specimens vs. aposymbiotic ones [25], and down-regulated in response to thermal stress
98 [26]. These patterns suggested an important role for GPx enzymes in maintenance of the cnidarian-
99 dinoflagellate symbiosis. Thus, to gain further insights into the cnidarians-dinoflagellate symbiosis,
100 studies of the activity of GPx proteins, and their role in symbiosis maintenance and disruption are
101 necessary. In this study, we characterized the GPx isoforms in the symbiotic cnidarian *A. viridis*, by
102 analysis of their isoform diversity and their activity distribution in the three cellular compartments -
103 ectoderm, endoderm and freshly isolated zooxanthellae - and in mitochondria. In addition, we
104 compared the influence of the symbiotic lifestyle on the zooxanthellae by comparing the GPx
105 activities of freshly isolated zooxanthellae to those of cultured zooxanthellae. Furthermore, we
106 investigated the induction of GPx activity in response to thermal stress.

107 2. Materials and Methods

108

109 All chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

110

111 2.1. *Biological material and experimental design*

112 Specimens of the Mediterranean sea anemone *Anemonia viridis* (Forskål 1775) were collected from
113 'Baie des Croutons' (Antibes, France) and maintained in a closed-circuit natural seawater aquarium.
114 Half the volume of seawater was exchanged with fresh seawater once a week and the temperature
115 was kept at $20^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$. Artificial light was provided by metal halide lamp (HQI-TS 400W,
116 Philips), with a photosynthetic photo flux density of $200 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ and a 12h:12h
117 photoperiod. Specimens were fed once a week with extracts of frozen adults of *Artemia salina*. For
118 the thermal stress experiment, three aquaria, each containing one specimen of *A. viridis*, were
119 heated from 20°C (control temperature) to 29°C (stress temperature) within 2 hours and maintained
120 at this maximal temperature for 15 days. For glutathione peroxidase (GPx) activities, 3-4 tentacles
121 were sampled from each specimen at day 0 (control condition), and after 1, 2, 5, 7, 9 and 15 days of
122 consecutive thermal stress. Cultured zooxanthellae were originally extracted from *A. viridis*,
123 maintained in f/2 medium [27] at pH 8.2 and incubated at $26^{\circ}\text{C} \pm 0.1^{\circ}\text{C}$ under an irradiance of
124 $100 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ (Sylvania Gro-Lux, Loessnitz, Germany), on a 12h:12h photoperiod.
125 Stock cultures were transferred monthly.

126 All experiments were conducted in accordance with the NIH guidelines for the care and handling of
127 experimental animals (NIH publication no. 85-23, revised 1985).and the directive of the European
128 Communities Council (2010/63/EU).

129

130 2.2. *Tissue separation and protein extraction*

131 The protocol used (adapted from [28]) allowed the specific separation and extraction of soluble
132 proteins from the three tissue compartments of *A. viridis*: ectoderm (C), endoderm (D), and freshly
133 isolated zooxanthellae. Soluble proteins from 3 independent flasks of cultured zooxanthellae were
134 also processed. The extraction medium was 50 mM potassium phosphate pH 7, 1 mM EDTA, and
135 1/1000 dilution of a protease inhibitor cocktail (P-8340). The protein concentrations obtained in the
136 extracts were determined using the Bradford method [29], with the Bio-Rad Protein Assay reagent
137 and bovine serum albumin as standard.

138

139 2.3. *A. viridis mitochondrial enrichment*

140 The enrichment technique for the animal mitochondrial fraction was specifically developed with *A.*
141 *viridis*. Fifteen tentacles were soaked in cold enrichment buffer (sucrose 450 mM, KCl 100 mM,

142 NaCl 50 mM, EGTA 3mM, HEPES 30 mM, K_2HPO_4 2 mM, pH 7.6) with 0.5% fatty acid free and
143 $10 \mu\text{g ml}^{-1}$ protease inhibitor cocktail, and homogenized using a glass homogenizer. Homogenate
144 was centrifuged at 3,000xg for 10 min at 4°C to remove zooxanthellae and the supernatant was
145 recovered for a second centrifugation at 3000xg for 10 min. The resulting supernatant, containing
146 the animal mitochondrial fraction without zooxanthellae, was recovered and centrifuged at
147 15,000xg for 10 min. The pellet, containing mitochondria, was resuspended in the cold enrichment
148 buffer and mitochondrial membranes were broken by pipette stirring.

149

150 2.4. Spectrophotometric measurement of GPx activity

151 GPx activity (selenium-dependent and non-selenium-dependent) was measured according to the
152 method of Paglia and Valentine [30] and Weydert and Cullen [31]. This method is based on the
153 GPx catalytic oxidation of glutathione by cumene hydroperoxide in the presence of glutathione
154 reductase and NADPH. Oxidized glutathione formed by the GPx reaction ($H_2O_2 + 2GSH \rightarrow GSSG$
155 $+ 2H_2O$) is continuously reduced by glutathione reductase activity ($GSSG + NADPH + H^+ \rightarrow$
156 $2GSH + NADP^+$). GPx activity was therefore calculated from the decrease in NADPH absorbance
157 at 340 nm over 10 min. Samples containing 100 μg of protein were added into the assay mixture
158 and the reaction was initiated by the addition of the cumene hydroperoxide. The assay mixture
159 included 0.5 mM cumene hydroperoxide (247502), 10 mM GSH (G-4251), 30 mM sodium azide
160 (S-2002), 3.6 mM NADPH (N-7505) and 5 u/ml glutathione reductase (G-3664) in 50 mM
161 phosphate buffer pH 7.6. GPx units, defined as the degradation of 1 μmol of NADPH per minute,
162 were calculated using a molar extinction coefficient for NADPH at 340 nm of 6.22 mM^{-1} and
163 normalized to mg total protein.

164

165 2.5. Electrophoresis separation and activity staining of GPx.

166 GPx activities in each tissue compartment were monitored by 10% non-denaturing polyacrylamide
167 gel electrophoresis (PAGE) and GPx isoforms were highlighted by staining the gel with 3-(4,5-
168 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), as described by Lin et al. [32]. The
169 gel was loaded with 100-200 μg of soluble protein, depending of the animal compartment. GPx
170 from bovine erythrocytes (G-6137) was used as the internal standard (50 mU per lane). After
171 electrophoretic separation, the gel were equilibrated for 15 min in tris HCl 50 mM, pH 7.7 at 4°C,
172 and incubated in GSH with H_2O_2 solution (13 mM GSH, 0.004% H_2O_2 , tris HCl 50 mM, pH 7.7)
173 for 10 min at 4°C in the dark. GPx activities were revealed by soaking the gel in the dark for 5 min
174 in a solution of 24 mM MTT (M-2128), 160 mM phenazine methosulfate (P-9625) and tris HCl 50
175 mM, pH 7.7. When achromatic bands began to form, the stain was poured off and rinsed
176 extensively with double-distilled H_2O . Achromatic bands demonstrated the presence of GPx

177 activity.

178

179 2.6. *A. viridis* transcriptome production and cnidarian genes encoding GPx sequences

180 Total RNAs of 12 independent tentacle tissue samples were extracted from two specimens of *A.*
181 *viridis*. After extraction, cleaning and quality checks of mRNA, 3 to 5 independent lanes of paired-
182 end HiSeq Illumina sequencing were done. All R1 and R2 fastq files were pooled and Illumina
183 adapters removed with Trimmomatic software [33]. A complete transcriptome was generated by
184 Trinity [34]. This generated 554 092 alternative transcripts, corresponding to 331 933 “genes”.

185 *A. viridis* genes encoding GPx sequences were isolated from the transcriptome by Blastn using
186 *Homo sapiens* GPx family gene sequences. Data validation was then performed by tBLASTx and
187 tBLASTn searches in the databases at NCBI (National Center for Biotechnology Information). GPx
188 sequences from *Nematostella vectensis*, *Acropora digitifera* and its symbiont, *Symbiodinium* sp.
189 (clade A and type B1) were isolated from transcriptome or genome public databases (detailed in
190 table 1), using *H. sapiens* and *A. viridis* GPx family gene sequences, by the same procedure. Gene
191 analysis was completed *via* identification of the presence of the Sec codon, the oligomerization
192 interface and the features to determine thioredoxin specificity in the isolated *A. viridis* GPx
193 sequences [35,36]. *A. viridis* sequences were deposited in the NCBI GenBank database (NCBI
194 accession numbers in Table 1).

195

196 Table 1: Cnidarian GPx homologs identified by Blast searches in available transcriptome and genome databases

Taxons	Name used in the phylogenetic analysis	Database ID	Database Source	Database References	MW*	Presence of Seleno-cystein site
<i>Acropora digitifera</i>	A.digitiferaGPxa	adi_EST_assem_4930	Transcriptome	[37]	188	+/+
	A.digitiferaGPxb	adi_EST_assem_6302	Transcriptome	[37]	231	+/+
	A.digitiferaGPxc	adi_EST_assem_10238	Transcriptome	[37]	228	+/+
	A.digitiferaGPxd	adi_EST_assem_2566	Transcriptome	[37]	191	-/-
	A.digitiferaGPxe	adi_EST_assem_811	Transcriptome	[37]	204	+/-
<i>Nematostella vectensis</i>	N.vectensisGPxa	XP_001641220.1	Genome	[38]	203	+/+
	N.vectensisGPxb	XP_001641323.1	Genome	[38]	221	-/+
	N.vectensisGPxc	XP_001641219.1	Genome	[38]	230	+/+
	N.vectensisGPxd	XP_001625812.1	Genome	[38]	193	-/-
	N.vectensisGPxe	XP_001617695.1	Genome	[38]	159	-/-
<i>Anemonia viridis</i>	A.viridisGPxa	TR139497**	Transcriptome	This study	212	+/+
	A.viridisGPxb	TR88767**	Transcriptome	This study	206	+/+
	A.viridisGPxc	TR176747**	Transcriptome	This study	217	+/+
	A.viridisGPxd	TR800**	Transcriptome	This study	242	+/+
	A.viridisGPxe	TR150403**	Transcriptome	This study	233	+/+
	A.viridisGPxf	TR74656**	Transcriptome	This study	197	-/-
	A.viridisGPxg	TR50351**	Transcriptome	This study	306	+/+
	A.viridisGPxh	TR159974**	Transcriptome	This study	273	+/-
	A.viridisGPxi	TR92513**	Transcriptome	This study	192	-/-
	A.viridisGPxj	TR146728**	Transcriptome	This study	nd	+/-
A.viridisGPxk	TR122461**	Transcriptome	This study	217	+/-	
<i>Symbiodinium</i> sp. clade A	Symbiodinium_cladeA_GPxa	kb8_rep_c49	Transcriptome	[39]	244	+/-
<i>Symbiodinium</i> sp. clade B1	Symbiodinium_cladeB1_Gpxa	symbB.v1.2.004888	Genome	[40]	544	+/+
	Symbiodinium_cladeB1_GPxb	symbB.v1.2.008497	Genome	[40]	287	+/-
	Symbiodinium_cladeB1_GPxc	symbB.v1.2.010998	Genome	[40]	271	+/-
	Symbiodinium_cladeB1_GPxd	symbB.v1.2.010038	Genome	[40]	257	+/-

197 Nd: non determined because incomplete sequence ; +/-: presence or absence of selenocystein site; *:predicted molecular weight amino acid sequence,

198 **: temporary sequence assignment (genebank accession number in submission)

199 2.7. *Phylogenetic GPX analysis*

200 Sequence analysis was conducted by aligning protein sequences of cnidarian and *Symbiodinium* sp.
201 GPx proteins (isolated as described previously), and animal, fungal or plant sequences obtained
202 from the Peroxibase database [41] (<http://peroxibase.toulouse.inra.fr>) and lists in Supplementary
203 Table 1S. The multiple sequence alignment was performed using the MUSCLE algorithm and
204 manual adjustment. After identification of the best protein substitution model predicted by ProtTest
205 3.4, maximum likelihood tree construction was performed using Seaview software [42], with
206 bootstrap support calculated using 100 bootstrapping events. The complete alignment is available
207 upon request.

208

209 2.8. *Statistical analysis*

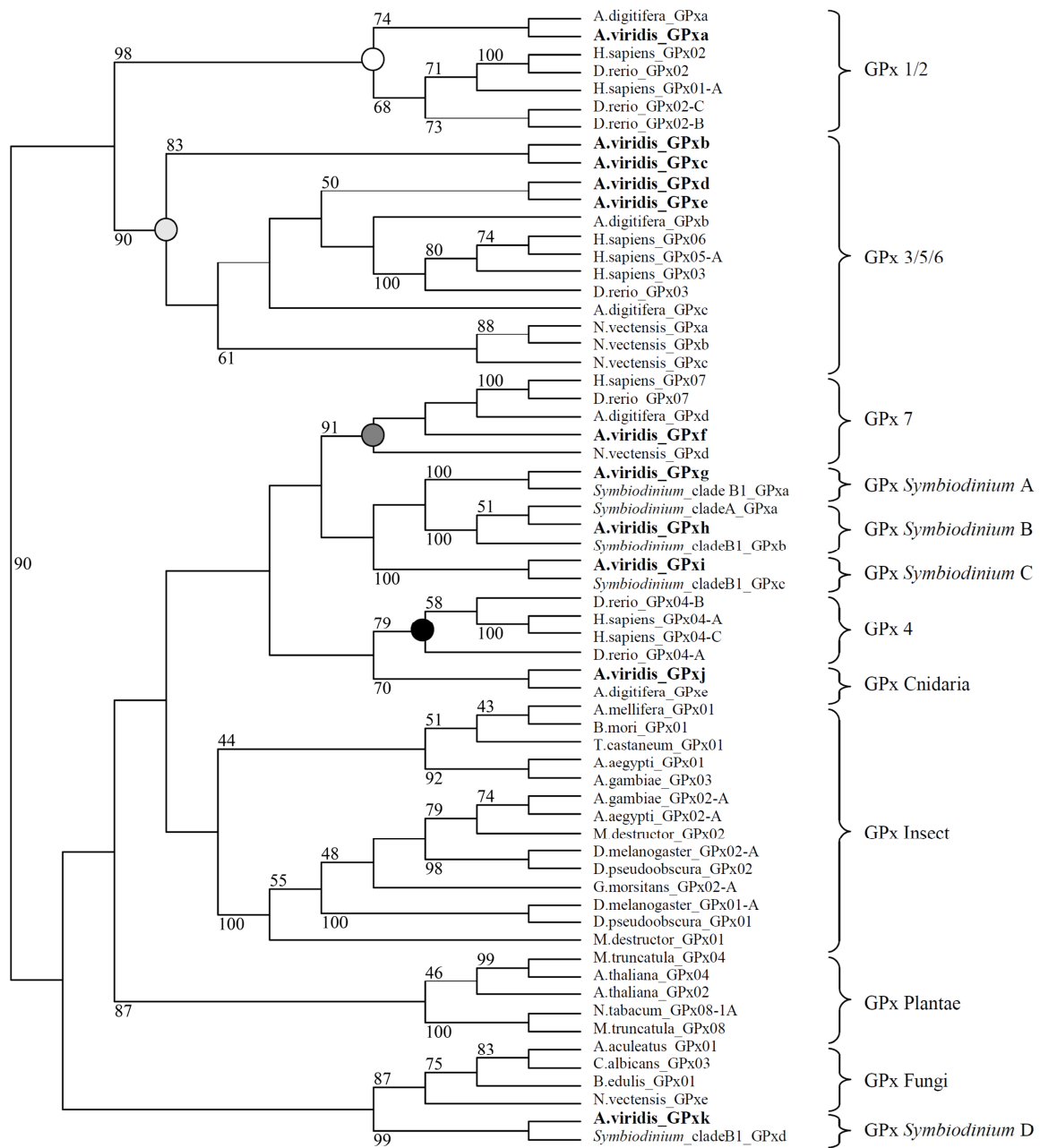
210 Results are given as mean \pm S.E.M. The GPx activities of each compartment were compared and
211 analyzed using Kruskal–Wallis analysis followed by a Nemenyi *post hoc* procedure [43]. The
212 change in GPx activities during thermal stress, in each compartment, was analyzed using
213 Friedman's nonparametric two-way ANOVA. Differences were considered statistically significant
214 when $p < 0.05$.

215 **3. Results**

216

217 *3.1. GPx phylogenetic analysis*

218 We queried the available transcriptome of *A. viridis* for the presence of GPx sequences. We
219 identified 11 *A. viridis* putative GPx-encoding transcripts belonging to different GPx families
220 (Table 1). Similarity searches and detailed analyses of the phylogenetic affiliation of GPx *A. viridis*
221 gene sequences revealed the presence of 7 GPx transcripts belonging to the 4 main metazoan groups
222 (5 having a tetrameric structure and 2 a monomeric structure). 4 GPx transcripts strongly related to
223 *Symbiodinium* sp. GPx were observed (1 having a dimeric structure and 3 a monomeric structure)
224 (Fig. 1 and Table 1). The comparison with two other cnidarians, *A. digitifera* and *N. vectensis*,
225 shows a similar GPx group distribution, but no *N. vectensis* GPx related to GPx1/2 and GPx4 were
226 identified in its genome. On the other hand, the absence of selenocysteine site in *A.viridis*_GPxf, *A.*
227 *digitifera*_GPxd and *N.vectensis*_GPxd protein sequences and their predicted monomeric structure
228 confirm their close affiliation to the GPx7 group (Table 1). None of the 11 *A. viridis* putative GPx
229 sequences presented the resolving cysteine in the cysteine-block, which is important for thioredoxin
230 specificity (data not shown). Finally, we noticed the presence of a GPx-like fungal sequence (Fig.1)
231 and an incomplete bacterial sequence (results not shown) in the *N. vectensis* genome, presumably
232 resulting from cultured medium contamination.



233

234 Fig. 1: Phylogenetic tree analysis for GPx protein. The phylogenetic tree of GPx amino acid
 235 sequences was constructed as described in Materials and Methods and was represented as an
 236 unrooted ML cladogram. Bootstrap confident values are expressed as percentages with a bootstrap
 237 threshold fixed to 40%. White, grey and black circles indicate the clusters related to animal GPx1/2,
 238 GPx3/5/6, GPx7 and GPx4. *A. viridis* squared representation of the phylogenetic tree is available in
 239 Supplementary Fig. 1S.

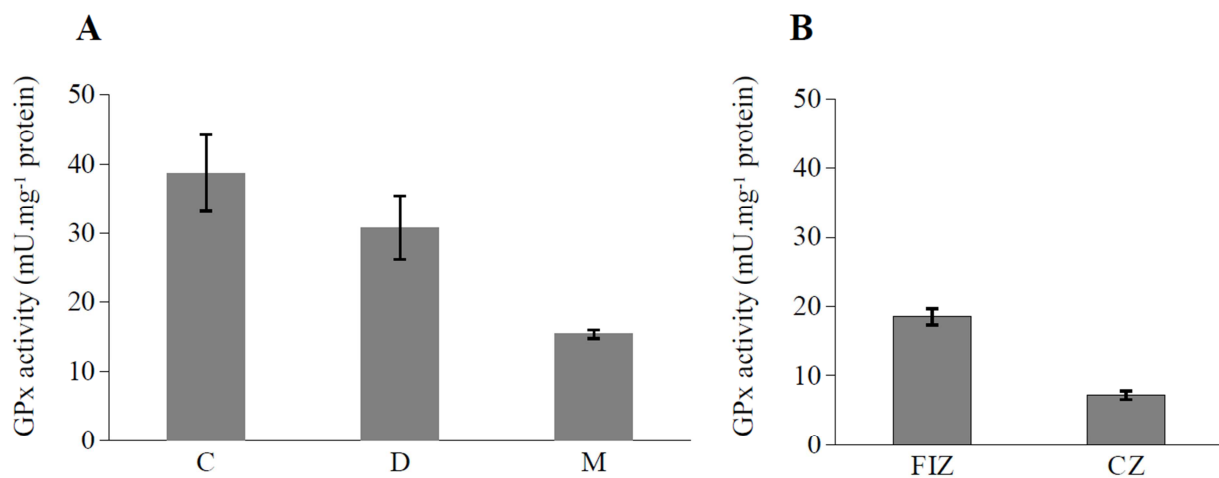
240

241

242 3.2. Spectrophotometric analysis of GPx activity

243 Active GPx isoforms and tissue-specific distributions of their activity were evaluated in the animal
 244 compartments of *A. viridis* (ectoderm and endoderm), the freshly isolated zooxanthellae, and the
 245 animal mitochondrial fraction (Fig. 2). The ectodermal and endodermal fractions displayed similar
 246 GPx activities of, respectively, 38.7 ± 5.5 and 30.9 ± 4.6 mU mg⁻¹ protein ($p=0.31$ Nemenyi test). In
 247 contrast, the animal mitochondrial fraction displayed much reduced GPx activity, with 15.5 ± 0.6
 248 mU mg⁻¹ protein (Nemenyi test, $p<0.001$). GPx analysis of freshly isolated zooxanthellae and
 249 cultured zooxanthellae showed significant differences, with minimal activity in cultured cells ($7.2 \pm$
 250 0.6 mU mg⁻¹ protein; Nemenyi test, $p<0.001$).

251



252

253 Fig. 2: Spectrophotometric analysis of GPx activity in *Anemonia viridis* and zooxanthellae. A)
 254 Ectoderm (E), endoderm (D) and mitochondrial fraction (M). B) Freshly isolated zooxanthellae
 255 (FIZ) and cultured zooxanthellae (CZ). Data are represented as means \pm S.E.M of four independent
 256 analyses. Bars marked with the same letters are not significantly different from one another
 257 ($p<0.05$, Nemenyi test).

258

259 3.3. GPx activity staining on native gels

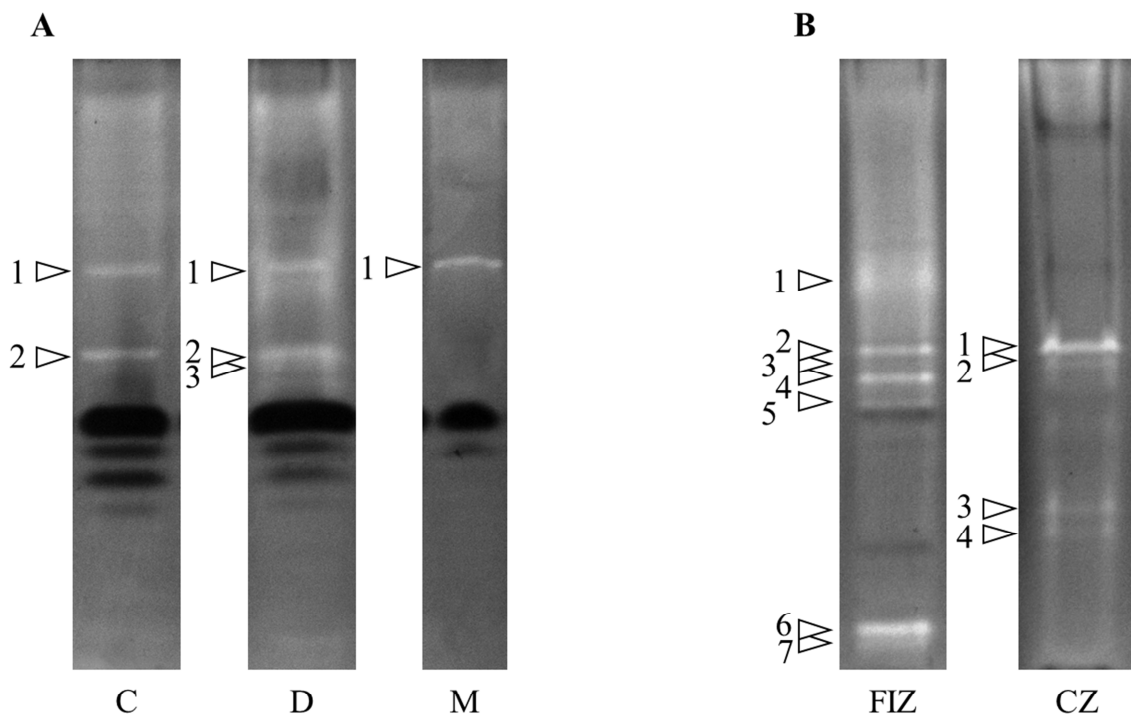
260 To determine GPx electrophoretype diversity in the various *A. viridis* compartments, different
 261 amounts of protein extract were resolved by native PAGE staining for GPx activities. Fig. 3 shows
 262 a representative tissue-specific electrophoresis pattern of GPx activity. The different isoforms,
 263 revealed as achromatic bands, were numbered in order of their migration distance. In the ectoderm,
 264 two bands were detected. These bands, named electrophoretotypes 1 and 2, were also detected in the
 265 endoderm extract with identical migration characteristics. In the endoderm, a third band, less
 266 intense than the two others, was observed below electrophoretype 2. For the animal mitochondrial
 267 fraction, only one band was detected, at the same migration distance as electrophoretype 1 of the

268 ectoderm and endoderm extracts. The GPx patterns of the freshly isolated and cultured
 269 zooxanthellae underlined the major differences between their two life-styles. In freshly isolated
 270 zooxanthellae, at least 7 distinct electrophoretotypes were seen with different intensities, with
 271 electrophoretotypes 2, 4 and 6 showing the highest band intensities. In cultured zooxanthellae, GPx
 272 activities were characterized by 4 electrophoretotypes, with intense electrophoretotype 1 activity.

273

274 Surprisingly, this staining method also revealed dark chromatic bands in the animal fraction, which
 275 could hide the presence of additional GPx activities (Fig. 3). In order to characterize the dark
 276 chromatic bands, we performed additional GPx native PAGE assays in the presence of new internal
 277 standards: *Escherichia coli* MnSOD (S-5639), yeast glutathione reductase (G-3664), horseradish
 278 peroxidase (HRP) (P-8250) and bovine catalase (C-9322). No correlation between the dark
 279 chromatic bands and the tested standards was seen (data not shown).

280



281

282 Fig. 3: GPx electrophoresis patterns in compartments of *Anemonia viridis* and zooxanthellae. A)
 283 ectoderm (C), endoderm (D) and mitochondrial fraction (M) were loaded onto 10% resolving native
 284 PAGE with 100 µg, 200 µg and 200 µg of protein, respectively. B) Cultured zooxanthellae (CZ) and
 285 freshly isolated zooxanthellae (FIZ) were loaded onto 10% resolving native PAGE with 150 µg of
 286 protein. White arrows indicated the GPx band activities.

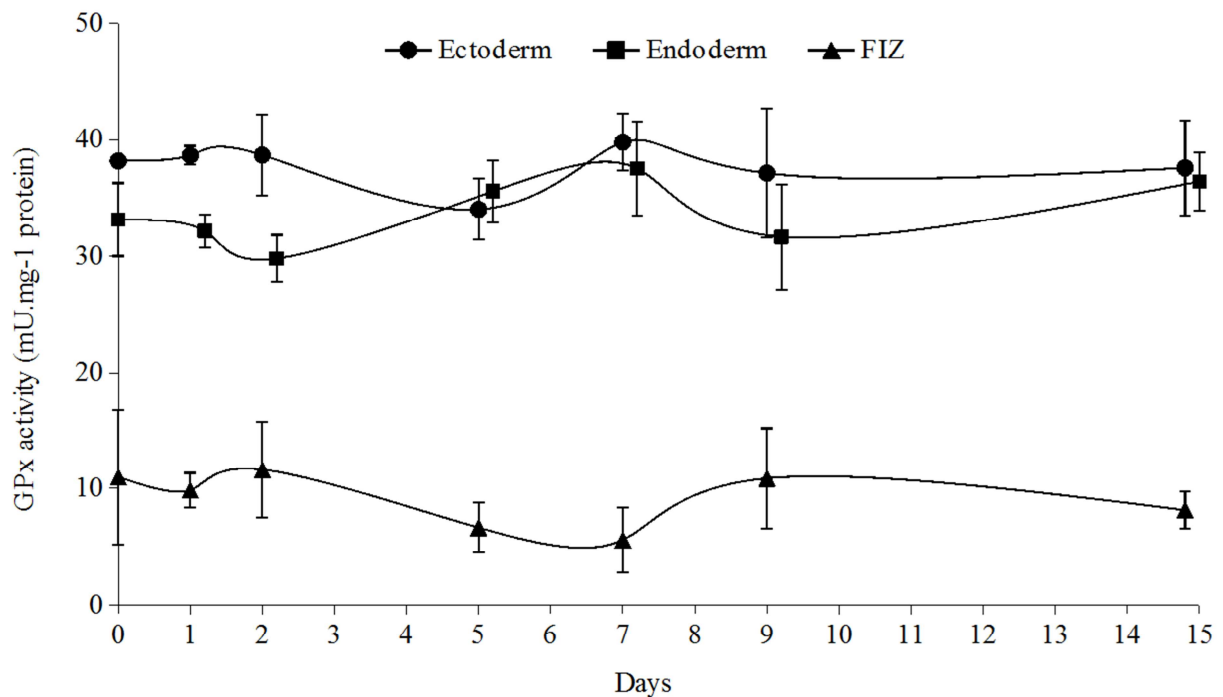
287

288

289 3.4. Impact of thermal stress on GPx activity

290 GPx activities were followed in the various *A. viridis* compartments during the + 9°C heat stress
 291 (Fig. 4). While heat stress induced the sea anemone to bleach [27], GPx activity did not reveal
 292 significant modification during the 15 days of stress, irrespective of symbiosis compartment, i.e.
 293 ectoderm, endoderm or freshly isolated zooxanthellae (Friedman ANOVA, $p>0.05$). In addition,
 294 GPX electrophoretype diversity in the different compartments did not show modification in response
 295 to stress (data not shown).

296



297

298 Fig. 4: Quantitative evaluation of the GPx activity during thermal stress in *Anemonia viridis*.
 299 Spectrophotometric analysis of GPx activity was followed during the +9 °C heat stress in the
 300 various *A. viridis* compartments: ectoderm, endoderm and freshly isolated zooxanthellae (FIZ).
 301 Data are represented as means \pm S.E.M of three independent analyses.

302

303 **4. Discussion**

304

305 To protect and fight against oxidative stress, symbiotic cnidarians have developed adaptive
306 mechanisms thanks to the presence of antioxidant defenses. The efficiency of the response depends
307 on the equilibrium of three detoxifying enzymes, which are the cornerstones of this protection:
308 superoxide dismutase (SOD), peroxidase including glutathione peroxidase (GPx), and catalase
309 (CAT). Many studies of SOD and CAT have highlighted their roles in the maintenance and
310 disruption of cnidarian-dinoflagellate symbioses, but few extensive studies of GPx in symbiotic
311 cnidarians have been reported [3,28,44].

312

313 In the present study, we report the first identification and characterization of the GPx isoform
314 repertoire in the transcriptome of *A. viridis* and the genome of two others cnidarians, *N. vectensis*
315 and *A. digitifera*. Phylogenetic analyses showed the presence in Cnidaria of the 7 main metazoan
316 GPx classes (Fig. 1). This analysis confirmed that the common ancestor diverged in two groups,
317 GPx7/4 and GPx1/2/3/5/6, followed by multiple duplication events in the early stage of GPx
318 evolution [21,45]. A comparison between the non-symbiotic cnidarian *N. vectensis* and the two
319 symbiotic cnidarians, *A. viridis* and *A. digitifera*, highlights a higher number of GPx transcripts in
320 these symbiotic species, with *N. vectensis* lacking GPx1/2 and GPx4. This trend could be correlated
321 to an adaptation to the highly oxidative state encountered by these symbiotic organisms, and which
322 is controlled by a diverse array of antioxidant defenses as already demonstrated with respect to
323 SOD diversity [13,28], or alternatively by the selection in *N. vectensis* of other H₂O₂-scavenging
324 enzymes, such as catalases or other peroxidases. In *Symbiodinium* sp., the GPx transcripts examined
325 showed less diversification, with two major evolutive groups within the GPx7/4 branch.

326

327 In metazoans, different GPx isoforms have specific cellular and subcellular localizations, and
328 specific reducing functions [19,21]. The phenotypic role of the *A. viridis* GPx repertoire was
329 determined with protein extracts specifically isolated from both animal epithelia and the symbiont,
330 enabled by a method that readily separates the tissue layers. The qualitative analysis of GPx by
331 electrophoretic method, conducted for the first time in a cnidarian by native PAGE, revealed the
332 presence of several active GPx isoforms. At least three active GPx isoforms were observed in the
333 animal tissues, with two isoforms identified in both ectodermal and endodermal tissues. However,
334 the presence of dark chromatic bands in animal epithelia, possibly hiding additional GPx proteins,
335 prevented the fully characterization of the GPx diversity pattern in the animal tissues.
336 Complementary studies have to be carried out to determine the nature of this sharp reduction of
337 MTT to formazan. One way would be to explore the presence of other thiol compounds that GSH

338 revealing the activity of enzymes such as cystathionine gamma-lyase, methionine gamma-lyase and
339 cysteine lyase (Ukai and Sekiya, 1997). Among the three active GPx isoforms, the identification of
340 the electrophoretype 1 in the mitochondrial extract, suggests its specificity to this subcellular
341 compartment. The mitochondrial respiratory chain is the second main producer of ROS, so it was
342 unsurprising to measure GPx activities in animal extracts enriched in mitochondria. The
343 electrophoretype 1 could correspond to AviridisGPxa or AviridisGPxj, for which phylogenetic
344 analysis suggested their close affiliation to the mitochondrial GPx1 or GPx4 isoforms [50]. With
345 respect to the symbionts, the absence of dark chromatic bands in both symbiont fractions allows to
346 identify the complete pattern of their GPx diversities. Cultured zooxanthellae possessed at least 4
347 distinct electrophoretotypes and freshly isolated zooxanthellae possessed at least 7 distinct
348 electrophoretotypes that could correspond to different isoforms of the four *Symbiodinium* GPx
349 sequences found in the transcriptome.

350

351 With regards to the similarity between host and the symbiont activities, one interesting result was
352 the presence of a common band, for electrophoretype 2, in the two symbiotic partners. The
353 molecular characterization of these proteins could increase our knowledge of the mechanisms that
354 maintain life in symbiosis and the possible exchanges between partners. The sequencing of these
355 GPx activity bands could elucidate whether the shared band between host and symbiont may result
356 from lateral transfer of genes or proteins; several such cases exist, and transfer of APx and MnSOD
357 has been hypothesised [55,56]. Another element supporting this hypothesis is the, recent genome
358 analyses that have highlighted similarities between the genomes of the coral *Acropora digitifera*
359 and the symbiotic dinoflagellate *Symbiodinium kawagutii* [57].

360

361 The quantitative analysis by spectrophotometry revealed that the ectodermal and endodermal
362 fractions displayed the highest GPx activities, with the zooxanthellae displaying two times less
363 activity (Fig. 2). These results corroborate the host and symbiont transcript diversity (Fig.1), and a
364 previous study of GPx immunolocalization in *A. viridis* tissue [24]. Moreover, contrary to the direct
365 ROS enzymatic defenses (i.e. CAT and ascorbate peroxidase (APx, EC 1.11.1.11)), GPx4 has an
366 important affinity for fats and lipids, and can interact with lipophilic substrates such as peroxidized
367 phospholipids and cholesterol [46,47]. Thus, the higher activities observed in the animal can be
368 correlated to the significant presence of highly polyunsaturated fatty acids in the tissues of the sea
369 anemone compared to the low concentration present in the zooxanthellae [48,49]. Despite the
370 localization of the symbiont, the main ROS producer, to the endodermal tissue, our results revealed
371 the same level of GPx activity in both the ectoderm and endoderm. This identical level can be
372 explained by high hydroperoxide tissue permeability and the simultaneous presence of GPx and

373 catalase owing a high detoxification capacity [44].

374

375 Concerning the symbiont, a low level of total GPx activity was measured in the freshly isolated
376 zooxanthellae when compared to activity in the animal. The production of H₂O₂ and its diffusion
377 through biological membranes to the external seawater [6] may then be partially offset by other
378 scavenging systems of H₂O₂, such as CAT or APx, as reported for the algal symbiont [44,51,52]. In
379 addition, the analysis of GPx in freshly isolated zooxanthellae and in cultured zooxanthellae
380 revealed that freshly isolated zooxanthellae require more GPx activity. Cultured zooxanthellae may
381 require less protection against H₂O₂, since they are affected only by their own production that is
382 released directly into the external seawater, and by other important peroxidase defenses (APx or
383 CAT [52]). Moreover, the presence of a higher proportion of fatty acids (including PUFA) in
384 freshly isolated zooxanthellae than in cultured ones could justify higher GPx protection when the
385 *Symbiodinium* cells are *in hospite* [53,54]. Finally, the low level of GPx activity in cultured
386 zooxanthellae could also be related to reduced selenium availability or absorption for GPx activity
387 in the free-living state or, conversely, an increase in selenium uptake in the symbiotic state. No data
388 are currently available but the analysis of the selenium concentration in the cnidarian-zooxanthella
389 symbiosis could provide further insight.

390

391 In the view of its strategic localization in both partners, GPx may play a key role in symbiotic
392 equilibrium and adaptation to global temperature increase, especially since GPx have demonstrated
393 to be heat-stress inducible, as in mammals [58] and invertebrates [59]. Moreover, in a recent study,
394 we investigated the transcriptomic response to thermal stress in *A. viridis* and found that the
395 transcription of some GPx was repressed under heat stress [26], but in the present study we showed
396 no modification of protein expression during heat stress. An identical result has also been observed
397 by Richier et al. [4,13] for SOD activity during the same heat stress in *A. viridis*. Thus, both
398 electrophoretic patterns and GPx activities, which are stable under heat stress, suggest an overall
399 constitutive and non-inducible activity, which may be an inherent adaptation to a symbiotic
400 lifestyle. Taken together, these results suggest that the absence of antioxidant enzyme activation
401 during stress is the result of a preconditioning of the animal by daily endogenous oxygen variations
402 that push the antioxidant system to the upper limit of its plasticity.

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404

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410 Conflict of interest

411

412 The authors declare no conflict of interest.

ACCEPTED MANUSCRIPT

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