



**HAL**  
open science

## **Seasonal variation of transcriptomic and biochemical parameters of *Donax trunculus* related to its infection by *Bacciger bacciger* (trematode parasite)**

Luisa Magalhaes, Xavier de Montaudouin, Simão Correia, Guillemine Daffe, Patrice Gonzalez, Etelvina Figueira, Jorge M.S. Gonçalves, Rosa Freitas

### ► **To cite this version:**

Luisa Magalhaes, Xavier de Montaudouin, Simão Correia, Guillemine Daffe, Patrice Gonzalez, et al.. Seasonal variation of transcriptomic and biochemical parameters of *Donax trunculus* related to its infection by *Bacciger bacciger* (trematode parasite). *Estuarine, Coastal and Shelf Science*, 2019, 219, pp.291-299. <10.1016/j.ecss.2019.02.012>. <hal-02324166>

**HAL Id: hal-02324166**

**<https://hal.science/hal-02324166v1>**

Submitted on 5 Jan 2021

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire HAL, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



HAL Authorization

1 **Seasonal variation of transcriptomic and biochemical parameters of**  
2 ***Donax trunculus* related to its infection by *Bacciger bacciger* (trematode**  
3 **parasite)**

4

5 **Magalhães, Luísa<sup>a,b</sup>, de Montaudouin, Xavier<sup>b</sup>, Correia, Simão<sup>a</sup>, Daffe,**  
6 **Guillemine<sup>b</sup>, Gonzalez, Patrice<sup>b</sup>, Figueira, Etelvina<sup>a</sup>, Gonçalves, Jorge M. S.<sup>c</sup>,**  
7 **Freitas, Rosa<sup>a\*</sup>**

8 <sup>a</sup> Departamento de Biologia & CESAM, Universidade de Aveiro, 3810-193 Aveiro,  
9 Portugal

10 <sup>b</sup> Univ. Bordeaux, CNRS, UMR EPOC, Station Marine, 2, rue du Pr Jolyet, F-33120  
11 Arcachon, France

12 <sup>c</sup> Centro de Ciências do Mar - CCMAR, Universidade do Algarve, FCT, Campus de  
13 Gambelas, 8005-139 Faro, Portugal

14

15 \* Corresponding author: Tel.: +351 234 370 350; fax. +351 234 372 587

16 E-mail address: [rosafreitas@ua.pt](mailto:rosafreitas@ua.pt)

17

18 **Abstract**

19           The wedge clam (*Donax trunculus*) is widely distributed along moderately  
20 exposed environments in the Atlantic coast, from France to Senegal. This species has  
21 high commercial importance, with the mean capture production on the last ten years of  
22 approx. 850 tonnes (50% represented by Portugal captures). *D. trunculus* populations  
23 are modulated by several drivers such as tidal range, temperature, sediment grain size,  
24 fishing pressure, predation and parasitism. Regarding parasitism, *D. trunculus* is the  
25 first intermediate host of *Bacciger bacciger* (trematode parasite) where the sporocysts  
26 develop. The sporocyst is the most damaging stage, reported as responsible for  
27 bivalve castration and flesh mass depletion. In order to test the hypothesis that *B.*  
28 *bacciger* infection modified wedge clam health status, including its biochemical  
29 performance and gene expression, clams were sampled every other month during one  
30 year in the Faro beach (south coast of Portugal). The results obtained revealed that *B.*  
31 *bacciger* total prevalence ranged between 0 and 33% in May and July, respectively.  
32 Overall, transcriptomic and biochemical results showed that *B. bacciger* induced in *D.*  
33 *trunculus* defense mechanisms against oxidative stress and increased the host  
34 metabolism and energy demand, especially in spawning and spent periods. In  
35 conclusion, the present work showed that the markers used can provide additional and  
36 ecologically relevant information about, not only the environmental conditions that  
37 animals experience but also, the invasion effects of pathogens. These findings can  
38 contribute to predict organisms chances of reproduction and survival in their natural  
39 context, which can be applied in bivalve conservation and disease episodes  
40 management.

41

42 **Keywords**

43           Bivalves; Host-parasite system, Gene expression; Biochemical markers;  
44 oxidative stress; metabolism.

## 45 1. INTRODUCTION

46 The wedge clam, *Donax trunculus* Linnaeus, 1758 (Bivalvia: Donacidae), is a  
47 warm-temperate species distributed from the Atlantic coast of France to Senegal  
48 (Tebble 1966) and the shallow bottoms of the Black Sea, Mediterranean Sea (Bayed  
49 and Guillou 1985) and Marmara Sea (Deval 2009). This species natural beds occur  
50 primarily in the intertidal zone, from 0 to 6 m (Gaspar et al. 2002), in highly energetic  
51 environments where it is exposed to the tidal rhythm, intense wave action and  
52 sediment instability (de la Huz et al. 2002). In many European regions (Nantón et al.  
53 2017) including Portugal (Gaspar et al. 1999, Pereira et al. 2016), *D. trunculus*  
54 constitutes one of the most important artisanal fisheries mainly due to its high  
55 economic value. Recorded European landings over the last 10 years were 9,408 tons,  
56 with a maximum yield of 1,353 tons in 2005 (FAO 2006-2018) and although fishing has  
57 slightly declined in recent years, as it has been occurring with other clams, namely  
58 carpet shells (*Ruditapes decussatus* (Linnaeus, 1758) and *Venerupis corrugata*  
59 (Gmelin, 1791) (FAO 2006-2018)), the wedge clam remains a significant commercial  
60 species.

61 In its habitat, *D. trunculus* population densities are highly variable (Fishelson et  
62 al. 1999, Gaspar et al. 1999, Delgado et al. 2017). Besides fishing exploitation, there  
63 are many other factors that can control these population densities, including  
64 environmental pollution (Fishelson et al. 1999, Neuberger-Cywiak et al. 2003, 2007),  
65 seawater acidification (Pereira et al. 2016), temperature (Botelho et al. 2018), sediment  
66 grain-size (de la Huz et al. 2002, La Valle et al. 2011) and parasitism. Concerning  
67 parasitism, among trematode species infecting the wedge clams, *Bacciger bacciger*  
68 (Rudolphi, 1819) Nicoll, 1914 is the most prevalent in European waters (Ramón et al.  
69 1999, de Montaudouin et al. 2014). This parasite has a complex life cycle using three  
70 different host species (Palombi 1934). *D. trunculus* is infected by miracidium free-living  
71 stage as first intermediate host, where the sporocyst parasitic stage develops. From  
72 mature sporocysts, cercariae larvae are released in the environment, another free-

73 living form, which rapidly penetrate an amphipod (*Erichthonius brasiliensis* (Dana,  
74 1853)) as second intermediate host where settle as metacercariae. When amphipods  
75 are predated by the fish *Atherina* sp., the definitive host, the parasite develops into its  
76 adult parasitic stage, sexually reproduces and releases its eggs in the environment.  
77 Despite its already studied detrimental effects on wedge clams condition index (de  
78 Montaudouin et al. 2014) and reproduction (Ramón et al. 1999), little is known about  
79 how this pathogen can affect the health of *D. trunculus* populations and its contribution  
80 for the population decline.

81         The present study aimed to identify the consequences of heavy parasite  
82 infection (*B. bacciger* visible sporocysts) in terms of individual response, evaluating the  
83 impacts induced in the host health status both at transcriptomic (antioxidant response  
84 related genes) and biochemical (metabolic activity, energy reserves, antioxidant activity  
85 and cellular damage) levels. The tested hypothesis was that the spread of *B. bacciger*  
86 among tissues will compromise *D. trunculus* regular gene expression and biochemical  
87 performance.

88

## 89 2. MATERIAL AND METHODS

### 90 2.1 Study area

91         *Donax trunculus* were collected in Praia de Faro (Faro's beach) in a single area  
92 (37° 00' 16"N, 7° 59'27"W). Praia de Faro is a narrow reflective beach (Balouin et al.  
93 2005) located in the Ancão Peninsula of the Ria Formosa coastal lagoon, south of  
94 Portugal. This Peninsula is approximately 10 km length and is the most western system  
95 of barrier islands of the Ria Formosa. Typical tidal range is between 2 and 3.8 m  
96 (Granja et al. 1984). The sampling area is composed mainly by fine (~ 125 µm) sands.  
97 The most recent data on Portuguese annual official landings of *D. trunculus* in 2016  
98 were of 252 ton with the sales value of 750 k€ (INE 2018).

99

### 100 2.2 Sampling procedure

101           Every other month from March 2016 to January 2017 (6 sampling months), *D.*  
102 *trunculus* were collected using a hand-dredger (Figure 1A) which is composed by a  
103 rectangular shape metal grid (Figure1B), a 2.8 cm mesh size bag (where the catch is  
104 retained) and a fixed wood beam (Figure 1C). Water temperature and salinity were  
105 recorded with a multiparametric probe.

106           In the laboratory, all wedge clams collected in a single haul were measured at  
107 least millimetre with a calliper and *Bacciger bacciger* prevalence, i.e. the percentage of  
108 infected hosts, calculated with two different methods. The two hundred largest  
109 organisms were individually placed in plastic containers filled with seawater extracted  
110 directly from the sampling area. After 12 hours, these containers were analysed under  
111 a stereomicroscope in order to check the presence of free *B. bacciger* cercariae  
112 emitted by the wedge clams in the water (this methodology was adapted from Jensen  
113 et al. 1999, de Montaudouin et al. 2016 and Magalhães et al. 2017). *B. bacciger*  
114 prevalence was calculated considering parasitized clams (P), those surrounded by free  
115 cercariae, and non-emitting clams, those with no cercariae in the surrounding water,  
116 here identified as emergence prevalence. Then, fifty wedge clams (from those found  
117 negative for the presence of *B. bacciger* cercariae) were opened and squeezed  
118 between two glass slides to check parasite presence (this methodology was adapted  
119 from de Montaudouin et al. 2000). *B. bacciger* prevalence was further calculated  
120 considering P clams, those that under stereomicroscope identification were positive for  
121 *B. bacciger* sporocysts presence, and non-parasitized (NP) clams, those that under a  
122 stereomicroscope were negative for *B. bacciger* sporocysts presence, identified as  
123 dissection prevalence.

124           Two *D. trunculus* specimens were randomly chosen and dissected to extract  
125 gills and digestive gland. These tissue samples were conserved in Ambion™ RNA  
126 later® (500 µL) at -80 °C prior to molecular cloning, consequent interest genes isolation  
127 and sequences determination.

128 For transcriptomic analysis, all wedge clams collected (excluding those found  
129 positive for *B. bacciger* cercariae emergence and the fifty clams opened) were opened  
130 and observed to check *B. bacciger* presence by the naked eye (orange sporocyst  
131 mass). Five first wedge clams found negative and positive for *B. bacciger* (a total of 10  
132 samples whenever possible) were immediately transferred into 500 µL of Ambion™  
133 RNA later® and conserved at - 80 °C.

134 For biochemical analyses, all the wedge clams left, as well as those found  
135 positive for *B. bacciger* cercariae emergence were preserved at - 80 °C. Wedge clams,  
136 were then quickly opened to check *B. bacciger* presence (orange mass) and pooled in  
137 groups of 2 accounting for 10 replicates, whenever possible, by condition (parasitized  
138 with *B. Bacciger*, P and non-parasitized with *B. bacciger*, NP) and by sampling month.  
139

## 140 2.3 Transcriptomic analysis

### 141 2.3.1 Total RNA extraction and reverse transcription

142 All *D. trunculus* samples (obtained and preserved as mentioned in the previous  
143 section) were homogenised at room temperature and 40 mg per individual were used.  
144 Total RNAs were extracted using SV Total RNA Isolation System kit (Promega) and  
145 reverse transcribed using oligo dT and random primers with the GoScript Reverse  
146 Transcription System kit (Promega), according to manufacturer instructions. The  
147 concentration of total RNAs was determined spectrophotometrically at 260 nm and  
148 purity checked by the 260/280 nm ratio.

149

### 150 2.3.2 PCR and molecular cloning

151 The cDNAs were amplified using the specific primers. The amplification  
152 program consisted of 40 cycles of 95 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min and  
153 a final elongation step of 72 °C for 10 min. Amplified products were analysed on one  
154 percent agarose gels and fragments of the expected sizes were excised and purified  
155 using Wizard SV Gel and PCR Clean-Up System (Promega), according to

156 manufacturer instructions. The resulting products were commercially sequenced  
157 (Sigma-Aldrich) and submitted to the GenBank. Partial cDNA sequence of *ef1α*, *β*  
158 *actin*, *Sod (Mn)* and *Cat* genes were successfully isolated using primers derived from  
159 conserved regions of the respective sequences.

160

### 161 2.3.3 Primer design

162 With the aim of recognizing *B. bacciger* effect on the wedge clam cell basic  
163 functions, two target genes were chosen: *Cat* and *Sod (Mn)* involved in the oxidative  
164 stress response. *Ef1α* and *β actin* were chosen as reference genes. Gene sequences  
165 from close related species, available in databases, were aligned using the Clustal  
166 Omega free software. From this alignment and for each gene, one forward and one  
167 reverse primer were deduced in conserved regions. All primer pairs used in this study  
168 are listed in Table 1.

169

### 170 2.3.4 Real-time quantitative PCR

171 Real-time PCR reactions were performed in a Lightcycler (Bio-rad CFX  
172 connect). The amplification program consisted of one cycle at 95 °C for 10 min and 50  
173 amplification cycles at 95 °C – 5 s, 60 °C – 5 s, 72 °C – 20 s. Each reaction contained  
174 17 µL of master mix including the SYBRgreen I fluorescent dye (Promega), 2 µL of the  
175 gene specific primer pair (final concentration 300 nM for each primer) and 1 µL of  
176 cDNA. Primers pairs were designed using the Primer 3 plus free software.

177 Relative quantification of each gene expression level was normalised according  
178 to the reference genes and generated using the  $2^{-\Delta CT}$  method described by Livak and  
179 Schmittgen (2001) where  $\Delta CT$  represents the difference between the cycle threshold of  
180 a specific gene and the cycle threshold of the reference genes. Inductor factor (IF) of  
181 each gene was determined in comparison with control (= without *B. bacciger*)  
182 corresponding to the following equation (Paul-Pont et al. 2010):

183

184 
$$IF = \frac{2^{-\Delta CT (with B. bacciger)}}{2^{-\Delta CT (without B. bacciger)}}$$

185

## 186 2.4 Biochemical analyses

187 Each replicate was homogenised with liquid nitrogen and separated into 0.3 g of  
188 soft tissue subsamples in order to perform the extraction with three different buffers.  
189 Supernatant of the subsample extracted with phosphate buffer (1:2 w/v) and  
190 centrifuged at 4 °C, 10000 g during 20 min was used to determine glycogen (GLY) and  
191 protein (PROT) concentrations, superoxide dismutase (SOD) and catalase (CAT)  
192 activities. Supernatant of the subsample extracted with 0.1 M Tris-HCl (pH 8.5), 15%  
193 (w/v) Poly Vinyl Pyrrolidone, 153 µM MgSO<sub>4</sub> and 0.2% (w/v) Triton X-100 buffer and  
194 centrifuged at 4 °C, 3000 g during 20 min was used to determine electron transport  
195 system (ETS) activity. Supernatant of the subsample extracted with 20% (w/v)  
196 trichloroacetic acid (TCA) and centrifuged at 4 °C, 10000 g during 20 min was used to  
197 determine lipid peroxidation (LPO) levels. All supernatants were then preserved at - 20  
198 °C or used immediately.

199 ETS activity is used as a metabolic capacity measure. Its activity was determined  
200 by the amount of formazan formed after adding p-IodoNitroTetrazolium (De Coen and  
201 Janssen 1997), calculated using  $\epsilon = 15.9 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$  and expressed in nmol of  
202 formazan formed per min per g of fresh weight (FW).

203 GLY was quantified by the phenol–sulphuric acid method described by Dubois  
204 et al. (1956). Absorbance was measured at 492 nm and results were expressed in mg  
205 per g FW.

206 PROT content was determined according to Robinson and Hogden (1940),  
207 following the Biuret method that uses Bovine serum albumin (BSA) as standard (0 – 40  
208 mg mL<sup>-1</sup>). After 10 min incubation at 30 °C the absorbance was read at 540 nm. The  
209 results were expressed in mg per g of FW and used to calculate enzymes activity.

210 SOD activity was measured using the method described by Beauchamp and  
211 Fridovich (1971). The standard curve was determined with SOD standards (0.25 – 60  
212 U mL<sup>-1</sup>). After 20 min in an orbital incubator set at room temperature, the enzyme  
213 activity was measured spectrophotometrically at 560 nm and expressed in unit of  
214 enzyme (U) per mg of protein. One U corresponds to a reduction of 50% of Nitro blue  
215 tetrazolium (NBT).

216 CAT activity was measured by the reaction of the enzyme with methanol in the  
217 presence of H<sub>2</sub>O<sub>2</sub> (Johansson and Borg 1988). The standard curve was determined  
218 using formaldehyde standards (0 – 150 mM). After 20 min in an orbital incubator at  
219 room temperature, the formaldehyde formation in the presence of Purpald was  
220 spectrophotometrically measured at 540 nm. The enzymatic activity was expressed in  
221 U per mg of protein. One U is defined as the amount of enzyme that generated the  
222 formation of 1.0 nmol formaldehyde, per min.

223 LPO was measured by the quantification of thiobarbituric acid reactive  
224 substances (TBARS), according to Buege and Aust (1978) protocol. This methodology  
225 is based on the reaction of LPO by-products, namely malondialdehyde (MDA), with 2-  
226 thiobarbituric acid (TBA) forming TBARS. The amount of MDA was quantified  
227 spectrophotometrically and measured at a wavelength of 532 nm ( $\epsilon = 156\text{mM}^{-1}\text{cm}^{-1}$ ).  
228 Results were expressed as nmol of MDA equivalents per g FW.

229

## 230 2.5 Data analysis

231 One-way ANOVA, followed by a Tukey post hoc analysis, was used to test  
232 differences between mean shell length of the dissected wedge clams used to calculate  
233 *B. bacciger* prevalence per sampling month.

234 Correlation between water temperature and *B. bacciger* prevalence was tested  
235 using nonparametric Spearman analysis.

236 One-way ANOVAs were performed and, whenever significant, followed by a  
237 post hoc analysis (Tukey test) for each gene expressed in order to test differences  
238 among sampling months in terms of induction factor.

239 One-way ANOVAs were performed and, whenever significant, followed by a  
240 post hoc analysis (Tukey test) for each biochemical marker in order to test: 1)  
241 differences among sampling months separately for NP and P clams; 2) differences  
242 between NP *and* P clams for each sampling month.

243 For all parametric tests, homogeneity of variances was checked with Cochran  
244 test while normality was assumed.

245 Three Principal Coordinates Ordination analysis (PCO) were separately  
246 performed for non-parasitized clams, parasitized clams and parasitized vs. non-  
247 parasitized clams. PCOs were based on biomarkers data matrices, containing all  
248 sampling months. Prior to visualization of the distance among centroids (i.e. the mean  
249 position of all the points representing a given sample) on PCO, data were normalised  
250 and the Euclidean distance calculated. In the PCO graphs, the variables (biomarkers)  
251 that better explained ( $r > |0.7|$ ) the samples spatial distribution were represented as  
252 superimposed vectors.

253

### 254 3. RESULTS

#### 255 3.1 Infection of *Donax trunculus*

256 Shell length of sampled wedge clams varied between 15 and 40 mm with a  
257 frequency peak at 25 mm (Figure 2). Regarding dissected clams, mean shell length  
258 was significantly higher in July, September and January (mean  $\pm$  standard deviation =  
259  $32.6 \pm 1.9$ ,  $33.2 \pm 2.0$  and  $32.6 \pm 1.6$  mm, respectively) comparing to March, May and  
260 November ( $29.0 \pm 2.9$ ,  $24.3 \pm 2.9$  and  $28.9 \pm 3.3$  mm, respectively) (One-way ANOVA:  
261  $F_{(5)} = 91.2$ ,  $p < 0.01$ ).

262 Only one macroparasite species was found, the trematode *Bacciger bacciger*  
263 identified at the naked eye as an orange mass invading all wedge clam tissues and

264 morphologically verified at the stereomicroscope following Ramón et al. (1999)  
265 description.

266 The dissection prevalence mean value ( $11.9 \pm 10.7$ ) was significantly higher  
267 than the mean emergence prevalence ( $1.3 \pm 1.9$ ). Total prevalence (considering  
268 infections detected by dissection and by emergence) showed a seasonal trend with the  
269 highest value in July (33%) and the lowest in May (0%) (Figure 3). Water temperature  
270 showed to be positively correlated to *B. bacciger* total prevalence ( $r = 0.83$ ,  $p < 0.05$ ,  
271 Figure 3).

272

### 273 3.2 Transcriptomic data

274 In July and September, the expression of *Sod (Mn)* gene was inhibited in  
275 parasitized compared to non-parasitized clams. In March, this gene was over-  
276 expressed in parasitized compared to non-parasitized clams (Table 2).

277 *Cat* gene was inhibited in July and induced in November for parasitized  
278 compared to non-parasitized clams (Table 2).

279

### 280 3.3 Biochemical data

281 For all biochemical parameters analysed there are no data concerning  
282 parasitized clams in March and May due to very low *B. bacciger* total prevalence found  
283 in these months.

284 Regarding ETS, in NP clams, the activity was significantly higher from March to  
285 July and significantly lower from September to January, while for P clams ETS activity  
286 was higher in July and January compared to September and November (Figure 4A). In  
287 July and January, ETS activity was significantly higher in P compared to NP clams  
288 (Figure 4A).

289 GLY content of the NP clams was significantly higher in March and July  
290 compared to the other months, and a similar trend (higher GLY content in July because  
291 there were no P clams in March) was observed for the P clams (Figure 4B). Overall,

292 there was lower GLY content in P compared NP clams (significant in July and  
293 November) (Figure 4 B). PROT content was not significantly different among all  
294 conditions (data not shown).

295 Taking into account NP wedge clams, results showed significantly higher SOD  
296 activity in September and significantly lower activity in January, with the remaining  
297 months displaying intermediate values (Figure 4C). Regarding P clams, significantly  
298 higher SOD activity was presented in November and lower in January (Figure 4C). The  
299 global trend showed lower SOD in P compared to NP but only significant in September  
300 (Figure 4C).

301 CAT activity for NP clams was significantly higher in September compared to  
302 the other sampling months (Figure 4D). Parasitized clams showed significantly lower  
303 CAT activity in November compared to the other sampling months (Figure 4D). The  
304 global trend showed lower CAT values in P clams compared to NP, with statistical  
305 significant in November (Figure 4D).

306 For NP clams, LPO values were significantly higher in March and May and  
307 significantly lower in November and January compared to the other months, while P  
308 clams showed slightly higher LPO in January (Figure 4E). Significant differences were  
309 observed between P and NP clams in July and January with lower and higher LPO  
310 levels for P clams, respectively (Figure 4E).

311 The horizontal dimension (Axis 1) of the PCO with NP clams explained 57% of  
312 the total variation separating March, May and July, in the negative side of the axis, from  
313 September, November and January, in the positive side of the axis. ETS, GLY and LPO  
314 biomarkers were the variables that better explained this variation presenting high  
315 negative correlation with axis 1 ( $r > |0.8|$ ) as well as CAT activity that presented high  
316 positive correlation with axis 1 ( $r > |0.7|$ ). Axis 2 explained 28% of the total variation which  
317 separated mainly January, in the positive side of the axis, from September with a strong  
318 positive correlation with SOD activity ( $r > |0.8|$ ) (Figure 5A).

319 The axis 1 of the PCO representing the P samples explained 70% of the total  
320 variation separating November, in the negative side of the axis, from July and January,  
321 in the positive side of the axis. SOD presented high negative correlation ( $r > |0.8|$ ) and  
322 ETS, CAT and LPO biomarkers high positive correlation ( $r > |0.8|$ ) with axis 1 and  
323 together were the variables that better explained the samples separation. Axis 2  
324 explained 26% of the total variation separating January, in the negative side of the axis,  
325 from July with a strong positive correlation with GLY content ( $r > |0.8|$ ) (Figure 5B).

326 The axis 1 of the PCO concerning P vs. NP samples explained 48% of the total  
327 variation separating mainly P clams of November but also NP clams of November and  
328 January and P clams of September in the negative side of the axis, from the other  
329 conditions in the positive side of the axis. ETS, CAT and LPO biomarkers were the  
330 variables that better explained the variation, presenting high positive correlation ( $r > |0.8|$ )  
331 with axis 1. The PCO vertical dimension (Axis 2) explained 25% of the total variation  
332 separating mainly NP clams of September but also NP clams of November, in the  
333 positive side of the axis, from the other conditions with a strong positive correlation with  
334 SOD activity ( $r > |0.8|$ ) (Figure 5C).

335

#### 336 4. DISCUSSION

337 Studies of first host – parasite systems and consequent predictions of disease  
338 events are very difficult to accomplish mostly due to generally low prevalence of these  
339 parasites and/or sudden high prevalence outbreaks that are followed by mass  
340 mortalities (Thieltges et al. 2008, Magalhães et al. 2015). The effects of sporocyst, the  
341 parasitic stage occurring in the first intermediate host, are often severe and negative  
342 impacts are described at the reproduction (Carballal et al. 2001), growth (Bowers 1969)  
343 and behaviour (Babirat et al. 2004) levels. Recently, it has been demonstrated that  
344 sporocyst can even interfere with host metabolism, energy reserves and antioxidant  
345 response (Magalhães et al. 2017). Particularly for the *Donax trunculus* – *Bacciger*  
346 *bacciger* host-parasite relationship, it was already demonstrated that this parasite is

347 able to damage the connective and foot tissue of the wedge clam (Ramadan and  
348 Ahmad 2010), leading to castration (Ramón et al. 1999) and decreasing its overall  
349 condition index (de Montaudouin et al. 2014). However, the present work increased  
350 considerably the knowledge in this field by: (1) primarily reporting the first record of *B.*  
351 *bacciger* infecting wedge clams in Portugal, expanding further west the known  
352 distribution boundary of this species (Margolis and Ching 1965) previously located in  
353 Mehdia, Morocco, and also (2) by representing the first effort to recognise the *B.*  
354 *bacciger* effects on *D. trunculus* gene expression and biochemical markers, over a year  
355 monitoring considering seasonal changes.

356         As previously demonstrated by other authors studying other 1<sup>st</sup> host-parasite  
357 systems (Curtis and Hubbard 1990, Magalhães et al. 2017), our findings demonstrated  
358 that prevalence calculated after host dissection was higher than prevalence calculated  
359 by cercariae emergence. This difference is due to parasite development, which can be  
360 already in the sporocyst form, i.e. visible at the stereomicroscope, but still not mature  
361 and so with no cercariae emergence. Therefore, we recommend dissection of the hosts  
362 as an important requirement for the correct prevalence calculation.

363         Overall, *B. bacciger* total (i.e. considering both methods) prevalence (mean =  
364 13%) was similar to values found in Mehdia, Morocco (6.6%) (de Montaudouin et al.  
365 2014), Cullera, Spain (8.4%) (Ramón et al. 1999) and Biscarosse, France (17.7%) (de  
366 Montaudouin et al. 2014), in Port Said, Egypt prevalence values were higher (73.7%)  
367 (Ramadan and Ahmad 2010). Total prevalence of infection showed a seasonal pattern  
368 being highest in July (33%) and lowest in May (0%) as opposite to what was observed  
369 in Spain (Ramón et al. 1999, Delgado and Silva 2018). Knowing that generally, the  
370 prevalence of trematodes infecting bivalves as first intermediate host is positively  
371 correlated to host size (Magalhães et al. 2015), the observed seasonal pattern could be  
372 related to the shell length confounding factor. Despite the clams sampled in May (*B.*  
373 *bacciger* total prevalence = 0%) have been smaller than clams sampled in July (*B.*  
374 *bacciger* total prevalence = 33%) conversely, there were other months displaying low

375 prevalence values in large organisms (e.g. January) excluding this feature as a  
376 prevalence explanatory variable.

377 In the present study, the most likely driver of the infection was the water  
378 temperature (as proxy of seasonality), a known trigger of trematode infection (de  
379 Montaudouin et al. 2016) which showed to be positively correlated with total  
380 prevalence. A similar trend was described by Ramadan and Ahmad (2010) studying  
381 the same host-parasite system in the north-east coast of Egypt. Seasonality in  
382 trematodes infecting their first intermediate host is not frequently observed, mainly due  
383 to the natural low prevalence of these parasites and consequent difficulty to find  
384 infected hosts (Magalhães et al. 2015). Nevertheless, most of the authors tend to  
385 converge by identifying summer and the positively correlated high temperatures as the  
386 season presenting the highest prevalence (demonstrated by Bowers (1969) in South  
387 Wales and by Desclaux et al. (2002) in France both studying *Cerastoderma edule*  
388 (Linnaeus, 1758) infected by *Bucephalus minimus* (Stossich, 1887) Nicoll, 1914).

389 As mentioned above, negative effects of *B. bacciger* on host health has been  
390 described by some authors, including impacts on reproduction (Ramón et al. 1999,  
391 Delgado and Silva 2018), soft tissues integrity (Ramadan and Ahmad 2010) and  
392 condition index (de Montaudouin et al. 2014). However, the present work represents  
393 the first assessment of *B. bacciger* effects at the level of gene expression and  
394 biochemical alterations. During an infection, host cells enhance reactive oxygen  
395 species (ROS) (Soudant et al. 2013). However, ROS have also important functions in  
396 some intracellular signalling cascades activation such as immunity response (Limón-  
397 Pacheco and Gonsebatt 2009). Nevertheless, when ROS production exceeds ROS  
398 elimination capacity, cellular damage may occur. Therefore, organisms need to  
399 balance ROS quantity and for that they mainly use antioxidant enzymes, such as  
400 superoxide dismutase (SOD) and catalase (CAT) (Regoli and Giuliani 2014). In  
401 accordance with such considerations, previous studies with a dinoflagellate parasitized  
402 by *Amoebophrya* (Lu et al. 2016) showed upregulation of genes involved in the calcium

403 signalling which in turn is related to the stress response. Furthermore, studying the disk  
404 abalone (*Haliotis discus discus*) challenged by bacteria and virus, De Zoysa et al.  
405 (2011) showed upregulation of the glutathione peroxidase gene. Similarly, glutathione  
406 S-transferases genes were upregulated in *Ruditapes decussatus* clams infected by  
407 *Perkinsus* sp. (Leite et al. 2013). All these genes are related to the antioxidant system,  
408 which is responsible for ROS elimination (Regoli and Giuliani 2014). Our results  
409 showed that *Sod (Mn)* and *Cat* oxidative stress related genes were upregulated in  
410 clams infected by *B. bacciger* in March (*Sod (Mn)*) and in November (*Cat*), while in July  
411 and September the same genes were downregulated in parasitized clams. These  
412 results may be explained by the fact that upregulation could represent a response to  
413 more than one stressful condition occurring at the same time. Particularly for the  
414 present case study, the combination of parasite infection and the peak of the spawning  
415 period occurring in March (Gaspar et al. 1999) and the combination of parasite  
416 infection and gametogenesis starting in November (Gaspar et al. op. cit). On the other  
417 hand, the expression of genes related to the antioxidant defense may have been  
418 modulated by the parasite patency. An infection is considered 'patent' when direct  
419 evidence of the parasite can be detected and so it is related to the development of the  
420 sporocyst in the host (Graczyk and Fried 2007). In March, the visible *B. bacciger* total  
421 prevalence was still low (2%), although it was already possible to observe a sporocyst  
422 (i.e. prepatent infection). In July and September, the highest *B. bacciger* total  
423 prevalence values were registered (33 and 21%, respectively) with mature sporocysts,  
424 and cercariae (patent infection). In November, the parasite returns to a prepatent  
425 infection. Following these arguments, downregulation of *Sod (Mn)* and *Cat* occurring  
426 when sporocysts were mature, can indicate the initiation of an alternative immune or  
427 stress response induced by higher parasite spread and consequent pathogenicity or an  
428 overall loss of ROS arrest signalling.

429           Taking only into account non-parasitized clams (sampled in all sampling  
430 months) and regarding biochemical parameters, these showed to be responsive to the

431 seasonal changes occurring at both biotic and abiotic levels. The metabolic rate  
432 (measured through ETS activity) and energy reserves (GLY content) registered a  
433 similar pattern with the highest values in March, May and July, overlapping the entire  
434 spawning period and the warmer seasons (spring and summer), and the lowest values  
435 in September, November and January, the post-spawning period followed by the colder  
436 season. Gametogenesis and spawning periods (frequently occurring during the warmer  
437 seasons) are usually characterized by higher metabolism demand. During these  
438 periods, GLY is an important energy source, which usually results in a higher condition  
439 index (Singh 2017). The same seasonal trend in terms of energy reserves was  
440 identified in a *D. trunculus* population from Tunisia (Tlili et al. 2013). After spawning  
441 period and during winter, a decrease in metabolic activities is probably a consequence  
442 of energy levels recovery after this reproduction process combine with adverse  
443 environmental conditions such as low temperature, low salinity and less quality and/or  
444 quantity of food. Previous studies already demonstrated that winter conditions tend to  
445 reduce the wedge clams fat content (Özden et al. 2009) and condition index (Tlili et al.  
446 2011). As it was reported for *R. philippinarum* (Anacleto et al. 2014) this decrease of  
447 metabolic rate and food intake could be explained by the bivalves strategy of closing  
448 their valves when under stressful conditions in order to enhance survival. Overall,  
449 higher antioxidant activity was registered in September (SOD and CAT activities)  
450 compared to the other sampling months corresponding to a post-spawning period, a  
451 stressful period, when *D. trunculus* condition index used to reach its lowest values  
452 (Gaspar et al. 1999). The described seasonal trend of metabolic rate and energy  
453 reserves (higher in March, May and July comparatively to September, November and  
454 January) as well as the relatively lower antioxidant activity resulted in a similar cellular  
455 damage trend.

456           When *B. bacciger* infection was observed (from July to January), this parasite  
457 showed to modulate the clams biomarkers response changing the seasonal pattern  
458 described above. Assessed biomarkers showed to be responsive not only to the

459 individual and environmental seasonal changes but also to the parasite infection.  
460 Parasitized clams showed higher metabolic rate (measured here by ETS activity) and  
461 lower GLY content. Correspondingly, the gastropod *Lymnaea stagnalis* infected with  
462 trematodes, showed an exhaustion of energy-cell resources experienced by the host  
463 which led to a decrease in CAT activity (Khomich et al. 2017). An infected clam has an  
464 additional metabolic requirement having to supply its own survival but also parasites  
465 with sufficient energy to grow (MacLeod 2017). Overall, *B. bacciger* infection inhibited  
466 the SOD and CAT activities, which is in agreement with the reduced *Sod (Mn)* and *Cat*  
467 expression registered in July and September. Similarly, *Clinostomum detrunctum*  
468 (trematode) infection proved to reduce the non-enzymatic antioxidant defenses with  
469 respect to pro-oxidant status in the muscle of the freshwater teleost *Rhamdia quelen*  
470 (Belló et al. 2000). Moreover, some vibrio species, such as *Vibrio tapetis*, shown to be  
471 able to inhibit reactive oxygen intermediates production (such as hydrogen peroxide) in  
472 hemocytes of *Crassostrea gigas* (Lambert et al. 2003). Transcriptional and biochemical  
473 results were not convergent in January, since *Sod (Mn)* and *Cat* genes were  
474 upregulated in parasitized clams but measured SOD and CAT activities were lower.  
475 Straight correlations between mRNA expression and the respective enzyme activity are  
476 still poorly studied and with controversial results but, certainly not always positively  
477 correlated and dependent on post-transcriptional, translational, regulation and protein  
478 degradation related processes (Vogel and Marcotte 2012). Finally, especially noticed in  
479 January, the increased metabolism and the reduction in the antioxidant enzymes  
480 activity led to oxidative stress (higher LPO level) and consequently to cellular damage  
481 in parasitized clams.

482

## 483 5. CONCLUSION

484 Overall, the present study showed that *B. bacciger* has a negative effect on its  
485 first intermediate host *Donax trunculus*, by increasing its metabolic rate, decreasing the  
486 energy reserves and inhibiting the antioxidant enzymes activity, which in some months

487 led to cellular damage (measured by LPO levels). Our findings showed that  
488 transcriptomic and biochemical markers can provide additional and ecologically relevant  
489 information regarding parasite effects on their hosts. Hence, these markers can not only  
490 reflect the environmental conditions that animals experience but also the invasion effects  
491 of pathogens, helping to predict organisms chance of reproduction and survival in their  
492 natural context. This approach can therefore help conservation practitioners to identify  
493 conservation threats to bivalve populations and to maximize the success of stock and  
494 disease episodes management. Moreover, the present study showed the importance of  
495 parasitology integration into physiological assessment of marine organisms exposed to  
496 stressful conditions to avoid incorrect identification of marine species as tolerant or  
497 susceptible to a given stress, when in fact the physiological response of the organism is  
498 modified by parasitic infection.

499

## 500 **Acknowledgements**

501       Luísa Magalhães benefited from a PhD grant (reference: PD/BD/52570/2014)  
502 given by the National Funds through the Portuguese Science Foundation (FCT),  
503 supported by FSE and Programa Operacional Capital Humano (POCH) and European  
504 Union. Simão Correia benefited from a research fellowship funded by INTERREG-  
505 ATLANTIC program through the research project COCKLES (EAPA\_458/2016  
506 COCKLES Co-Operation for Restoring Cockle Shellfisheries & its Ecosystem-Services  
507 in the Atlantic Area). Rosa Freitas benefited from a research position funded by the  
508 Integrated Programme of SR&TD “Smart Valorization of Endogenous Marine Biological  
509 Resources Under a Changing Climate” (reference: Centro-01-0145-FEDER-000018),  
510 co-funded by Centro 2020 program, Portugal 2020, European Union, through the  
511 European Regional Development Fund. Thanks are also due to CESAM  
512 (UID/AMB/50017) and to FCT/MEC, through national funds, for the financial support and  
513 the co-funding by the FEDER, within the PT2020 Partnership Agreement and Compete  
514 2020.

515            Authors are grateful to Isidoro Costa for collecting *Donax trunculus* specimens  
516    and to Carlos Afonso for helping with CCMAR lab facilities and work logistic.

517 References

518 Anacleto, P., A. L. Maulvault, V. M. Lopes, T. Repolho, M. Diniz, M. L. Nunes, A.  
519 Marques, and R. Rosa. 2014. Ecophysiology of native and alien-invasive clams in an  
520 ocean warming context. *Comparative Biochemistry and Physiology a-Molecular &*  
521 *Integrative Physiology* 175:28-37.

522 Babirat, C., K. N. Mouritsen, and R. Poulin. 2004. Equal partnership: two  
523 trematode species, not one, manipulate the burrowing behaviour of the New Zealand  
524 cockle, *Austrovenus stutchburyi*. *Journal of Helminthology* 78:195-199.

525 Balouin, Y., H. Howa, R. Pedreros, and D. Michel. 2005. Longshore sediment  
526 movements from tracers and models, Praia de Faro, South Portugal. *Journal of Coastal*  
527 *Research* 21:146-156.

528 Bayed, A., and J. Guillou. 1985. Contribution à l'étude des populations du genre  
529 *Donax*: La population de *D. trunculus* L. (Mollusca, Bivalvia) de Mehdiá (Maroc). *Annales*  
530 *de l'Institut océanographique* 61:139-147.

531 Beauchamp, C., and I. Fridovich. 1971. Superoxide dismutase: improved assays  
532 and an assay applicable to acrylamide gels. *Analytical Biochemistry* 44:276-287.

533 Belló, A. R. R., E. Fortes, A. Bello-Klein, A. A. Bello, S. F. Llesuy, R. B. Robaldo,  
534 and A. Bianchini. 2000. Lipid peroxidation induced by *Clinostomum detrunctum* in  
535 muscle of the freshwater fish *Rhamdia quelen*. *Diseases of Aquatic Organisms* 42:233-  
536 236.

537 Botelho, M. J., C. Vale, S. Joaquim, S. T. Costa, F. Soares, C. Roque, and D.  
538 Matias. 2018. Combined effect of temperature and nutritional regime on the elimination  
539 of the lipophilic toxin okadaic acid in the naturally contaminated wedge shell *Donax*  
540 *trunculus*. *Chemosphere* 190:166-173.

541 Bowers, E. A. 1969. *Cercaria Bucephalopsis haimeana* (Lacaze-Duthiers, 1854)  
542 (Digenea: Bucephalidae) in cockle, *Cardium edule* L. in south Wales. *Journal of Natural*  
543 *History* 3:409-422.

544 Buege, J. A., and S. D. Aust. 1978. Microsomal lipid peroxidation. Methods in  
545 enzymology 52:302-310.

546 Carballal, M. J., D. Iglesias, J. Santamarina, B. Ferro-Soto, and A. Villalba. 2001.  
547 Parasites and pathologic conditions of the cockle *Cerastoderma edule* populations of the  
548 coast of Galicia (NW Spain). Journal of Invertebrate Pathology 78:87-97.

549 Curtis, L. A., and K. M. Hubbard. 1990. Trematode infections in a gastropod host  
550 misrepresented by observing shed cercariae. Journal of Experimental Marine Biology  
551 and Ecology 143:131-137.

552 De Coen, W. M., and C. R. Janssen. 1997. The use of biomarkers in *Daphnia*  
553 *magna* toxicity testing. IV. Cellular Energy Allocation: a new methodology to assess the  
554 energy budget of toxicant-stressed *Daphnia* populations. Journal of Aquatic Ecosystem  
555 Stress and Recovery 6:43-55.

556 de la Huz, R., M. Lastra, and J. Lopez. 2002. The influence of sediment grain size  
557 on burrowing, growth and metabolism of *Donax trunculus* L. (Bivalvia: Donacidae).  
558 Journal of Sea Research 47:85-95.

559 de Montaudouin, X., H. Bazairi, K. A. Mlik, and P. Gonzalez. 2014. *Bacciger*  
560 *bacciger* (Trematoda: Fellodistomidae) infection effects on wedge clam *Donax trunculus*  
561 condition. Diseases of Aquatic Organisms 111:259-267.

562 de Montaudouin, X., H. Blanchet, C. Desclaux-Marchand, N. Lavesque, and G.  
563 Bachelet. 2016. Cockle infection by *Himasthla quissetensis* - I. From cercariae  
564 emergence to metacercariae infection. Journal of Sea Research 113:99-107.

565 de Montaudouin, X., I. Kisielewski, G. Bachelet, C. Desclaux. 2000. A census of  
566 macroparasites in an intertidal bivalve community, Arcachon Bay, France. Oceanologica  
567 Acta 23:453-468.

568 De Zoysa, M., I. Whang, C. Nikapitiya, C. Oh, C. Y. Choi, and J. Lee. 2011.  
569 Transcriptional analysis of disk abalone (*Haliotis discus discus*) antioxidant enzymes  
570 against marine bacteria and virus challenge. Fish and Shellfish Immunology 31:155-160.

571 Delgado, M., and L. Silva. 2018. Timing variations and effects of size on the  
572 reproductive output of the wedge clam *Donax trunculus* (L. 1758) in the littoral of Huelva  
573 (SW Spain). *Journal of the Marine Biological Association of the United Kingdom* 98:341-  
574 350.

575 Delgado, M., L. Silva, S. Gomez, E. Masferrer, M. Cojan, and M. B. Gaspar. 2017.  
576 Population and production parameters of the wedge clam *Donax trunculus* (Linnaeus,  
577 1758) in intertidal areas on the southwest Spanish coast: Considerations in relation to  
578 protected areas. *Fisheries Research* 193:232-241.

579 Desclaux, C., X. de Montaudouin, and G. Bachelet. 2002. Cockle emergence at  
580 the sediment surface: 'favourization' mechanism by digenean parasites? *Diseases of*  
581 *Aquatic Organisms* 52:137-149.

582 Deval, M. C. 2009. Growth and reproduction of the wedge clam (*Donax trunculus*)  
583 in the Sea of Marmara, Turkey. *Journal of Applied Ichthyology* 25:551-558.

584 Dubois, M., K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith. 1956.  
585 Colorimetric method for determination of sugars and related substances. *Analytical*  
586 *Chemistry* 28:350-356.

587 FAO 2006-2018. Fisheries and aquaculture software. FishStat Plus - Universal  
588 software for fishery statistical time series. FAO Fisheries and Aquaculture Department  
589 [online], Rome.

590 Fishelson, L., V. Bresler, R. Manelis, Z. Zuk-Rimon, A. Dotan, H. Hornung, and  
591 A. Yawetz. 1999. Toxicological aspects associated with the ecology of *Donax trunculus*  
592 (Bivalvia, Mollusca) in a polluted environment. *Science of the Total Environment*  
593 226:121-131.

594 Gaspar, M. B., L. M. Chicharo, P. Vasconcelos, A. Garcia, A. R. Santos, and C.  
595 C. Monteiro. 2002. Depth segregation phenomenon in *Donax trunculus* (Bivalvia:  
596 Donacidae) populations of the Algarve coast (southern Portugal). *Scientia Marina*  
597 66:111-121.

598 Gaspar, M. B., R. Ferreira, and C. C. Monteiro. 1999. Growth and reproductive  
599 cycle of *Donax trunculus* L., (Mollusca: Bivalvia) off Faro, southern Portugal. Fisheries  
600 Research 41:309-316.

601 Graczyk, T. K., and B. Fried. 2007. Human waterborne trematode and protozoan  
602 infections. Advances in Parasitology 64:111-160.

603 Granja, H., J. M. Froidefond, and T. Pera. 1984. Processus d'evolution morpho-  
604 sedimentaire de la Ria Formosa (Portugal). Bulletin de l'Institut de géologie du Bassin  
605 d'Aquitaine 36:37-50.

606 INE 2018. Fisheries statistics reports. INE Instituto nacional de estatística,  
607 Lisboa, <https://www.ine.pt>. Accessed on 15<sup>th</sup> March 2018.

608 Jensen, K. T., N. F. Castro, G. Bachelet. 1999. Infectivity of *Himasthla* spp.  
609 (Trematoda) in cockle (*Cerastoderma edule*) spat. Journal of the Marine Biological  
610 Association of the United Kingdom 79:265-271.

611 Johansson, L. H., and L. A. H. Borg. 1988. A spectrophotometric method for  
612 determination of Catalase activity in small tissue samples. Analytical Biochemistry  
613 174:331-336.

614 Khomich, A. S., D. V. Axenov-Gribanov, O. A. Bodilovskaya, Y. A. Shirokova, E.  
615 P. Shchapova, Y. A. Lubyaga, Z. M. Shatilina, V. A. Emshanova, and A. P. Golubev.  
616 2017. Assessment of the joint effect of thermal stress, pollution, and parasitic infestation  
617 on the activity of antioxidative enzymes in pulmonate mollusk *Lymnaea stagnalis*.  
618 Contemporary Problems of Ecology 10:157-163.

619 La Valle, P., L. Nicoletti, M. G. Finoia, and G. D. Ardizzone. 2011. *Donax*  
620 *trunculus* (Bivalvia: Donacidae) as a potential biological indicator of grain-size variations  
621 in beach sediment. Ecological Indicators 11:1426-1436.

622 Lambert, C., P. Soudant, G. Choquet, and C. Paillard. 2003. Measurement of  
623 *Crassostrea gigas* hemocyte oxidative metabolism by flow cytometry and the inhibiting  
624 capacity of pathogenic vibrios. Fish and Shellfish Immunology 15:225-240.

625           Leite, R. B., M. Milan, A. Coppe, S. Bortoluzzi, A. dos Anjos, R. Reinhardt, C.  
626 Saavedra, T. Patarnello, M. L. Cancela, and L. Bargelloni. 2013. mRNA-Seq and  
627 microarray development for the Grooved carpet shell clam, *Ruditapes decussatus*: a  
628 functional approach to unravel host -parasite interaction. *Bmc Genomics* 14:741.

629           Limón-Pacheco, J., and M. E. Gonsebatt. 2009. The role of antioxidants and  
630 antioxidant-related enzymes in protective responses to environmentally induced  
631 oxidative stress. *Mutation Research-Genetic Toxicology and Environmental*  
632 *Mutagenesis* 674:137-147.

633           Livak, K. J., and T. D. Schmittgen. 2001. Analysis of relative gene expression  
634 data using real-time quantitative PCR and the 2(T)(-Delta Delta C) method. *Methods*  
635 25:402-408.

636           Lu, Y. M., S. Wohlrab, M. Groth, G. Glockner, L. Guillou, and U. John. 2016.  
637 Transcriptomic profiling of *Alexandrium fundyense* during physical interaction with or  
638 exposure to chemical signals from the parasite *Amoebophrya*. *Molecular Ecology*  
639 25:1294-1307.

640           MacLeod, C. D. 2017. Parasitic infection: a missing piece of the ocean  
641 acidification puzzle. *Ices Journal of Marine Science* 74:929-933.

642           Magalhães, L., X. de Montaudouin, R. Freitas, G. Daffe, E. Figueira, and P.  
643 Gonzalez. 2017. Seasonal variation of transcriptomic and biochemical parameters of  
644 cockles (*Cerastoderma edule*) related to their infection by trematode parasites. *Journal*  
645 *of Invertebrate Pathology* 148:73-80.

646           Magalhães, L., R. Freitas, and X. de Montaudouin. 2015. Review: *Bucephalus*  
647 *minimus*, a deleterious trematode parasite of cockles *Cerastoderma* spp. *Parasitology*  
648 *Research* 114:1263-1278.

649           Margolis, L., and H. L. Ching. 1965. Review of the trematode genera *Bacciger*  
650 and *Pentagramma* (Fellodistomatidae) and description of *P. petrowi* (Layman, 1930) n.  
651 comb. from marine fishes from the pacific coast of Canada. *Canadian Journal of Zoology*  
652 43:381-405.

653 Nantón, A., A. Arias-Perez, R. Freire, J. Fernandez-Perez, S. Novoa, and J.  
654 Mendez. 2017. Microsatellite variation in *Donax trunculus* from the Iberian Peninsula,  
655 with particular attention to Galician estuaries (NW Spain). *Estuarine Coastal and Shelf*  
656 *Science* 197:27-34.

657 Neuberger-Cywiak, L., Y. Achituv, and E. M. Garcia. 2003. Effects of zinc and  
658 cadmium on the burrowing behavior, LC50, and LT50 on *Donax trunculus* Linnaeus  
659 (Bivalvia-Donacidae). *Bulletin of Environmental Contamination and Toxicology* 70:713-  
660 722.

661 Neuberger-Cywiak, L., Y. Achituv, and E. M. Garcia. 2007. Effects of sublethal  
662 Zn<sup>++</sup> and Cd<sup>++</sup> concentrations on filtration rate, absorption efficiency and scope for  
663 growth in *Donax trunculus* (Bivalvia; Donacidae). *Bulletin of Environmental*  
664 *Contamination and Toxicology* 79:622-627.

665 Özden, O., N. Erkan, and S. Ulusoy. 2009. Seasonal variations in the  
666 macronutrient mineral and proximate composition of two clams (*Chamelea gallina* and  
667 *Donax trunculus*). *International Journal of Food Sciences and Nutrition* 60:402-412.

668 Palombi, A. (1934) *Bacciger bacciger* (Rud.) trematode digenetic: fam.  
669 Steringophoridae Odhner. *Anatomia, sistematica e biologia. Pubblicazioni della Stazioni*  
670 *Zoologica di Napoli* 13:438–478

671 Paul-Pont, I., P. Gonzalez, M. Baudrimont, F. Jude, N. Raymond, L. Bourrasseau,  
672 N. Le Goic, F. Haynes, A. Legeay, C. Paillard, and X. de Montaudouin. 2010. Interactive  
673 effects of metal contamination and pathogenic organisms on the marine bivalve  
674 *Cerastoderma edule*. *Marine Pollution Bulletin* 60:515-525.

675 Pereira, A. M., P. Range, A. Campoy, A. P. Oliveira, S. Joaquim, D. Matias, L.  
676 Chicharo, and M. B. Gaspar. 2016. Larval hatching and development of the wedge shell  
677 (*Donax trunculus* L.) under increased CO<sub>2</sub> in southern Portugal. *Regional Environmental*  
678 *Change* 16:855-864.

679 Ramadan A. M. R and A. M. Ahmad. 2010. Infestation of *Donax trunculus*  
680 (Bivalvia, Donacidae) from Mediterranean Sea at Port Said coastal zone with *Bacciger*

681 *bacciger* (Trematoda, Fellodistomidae) and the role of the parasite in castration of the  
682 host. African Journal of Marine Sciences 6:83-94.

683 Ramón, M., M. Gracenea, and O. Gonzalez-Moreno. 1999. *Bacciger bacciger*  
684 (Trematoda, Fellodistomidae) infection in commercial clams *Donax trunculus* (Bivalvia,  
685 Donacidae) from the sandy beaches of the Western Mediterranean. Diseases of Aquatic  
686 Organisms 35:37-46.

687 Regoli, F., and M. E. Giuliani. 2014. Oxidative pathways of chemical toxicity and  
688 oxidative stress biomarkers in marine organisms. Marine Environmental Research  
689 93:106-117.

690 Robinson, H. W., and C. G. Hogden. 1940. The biuret reaction in the  
691 determination of serum proteins I. A study of the conditions necessary for the production  
692 of a stable color which bears a quantitative relationship to the protein concentration.  
693 Journal of Biological Chemistry 135:707-725.

694 Singh, Y. T. 2017. Relationships between environmental factors and biological  
695 parameters of Asian wedge clam, *Donax scortum*, morphometric analysis, length-weight  
696 relationship and condition index: a first report in Asia. Journal of the Marine Biological  
697 Association of the United Kingdom 97:1617-1633.

698 Soudant, P., F. L. E. Chu, and A. Volety. 2013. Host-parasite interactions: Marine  
699 bivalve molluscs and protozoan parasites, *Perkinsus* species. Journal of Invertebrate  
700 Pathology 114:196-216.

701 Tebble, N. 1966. British Bivalve Seashells. A Handbook for Identification.  
702 Trustees of the British Museum (Natural History), London.

703 Thieltges, D. W., K. T. Jensen, and R. Poulin. 2008. The role of biotic factors in  
704 the transmission of free-living endohelminth stages. Parasitology 135:407-426.

705 Tlili, S., I. Metais, N. Ayache, H. Boussetta, and C. Mouneyrac. 2011. Is the  
706 reproduction of *Donax trunculus* affected by their sites of origin contrasted by their level  
707 of contamination? Chemosphere 84:1362-1370.

708 Tlili, S., L. Minguéz, L. Giamberini, A. Geffard, H. Boussetta, and C. Mouneyrac.  
709 2013. Assessment of the health status of *Donax trunculus* from the Gulf of Tunis using  
710 integrative biomarker indices. *Ecological Indicators* 32:285-293.

711 Vogel, C., and E. M. Marcotte. 2012. Insights into the regulation of protein  
712 abundance from proteomic and transcriptomic analyses. *Nature Reviews Genetics*  
713 13:227-232.

714

715

716

717

718

719

720

721

722

723

724

725

726

727

728

729

730

731

732

733

734

735

736 **Table 1** Nucleotide sequences of specific primer pairs used in this study.

<b>Gene</b>	<b>Accession number</b>	<b>Function</b>	<b>Sequence 5'-3'</b>
<i>Ef1<math>\alpha</math></i>	MK388166	Protein synthesis and degradation (reference gene)	TCCCACTCCAGGACGTTTAC <sup>a</sup> TCCTGGGAGAGCTTCTGGTA <sup>b</sup>
<i><math>\beta</math> actin</i>	MK388165	Cell structure (reference gene)	CCCACACCGTACCCATCTAC <sup>a</sup> GGCAACATAGCAGAGCTTC <sup>b</sup>
<i>Sod (Mn)</i>	MK388163	Oxidative stress (manganese)	GCATCTTCTGGCAAGTCCTC <sup>a</sup> GAGAGCGTCCTGATTTGCTC <sup>b</sup>
<i>Cat</i>	MK388164	Oxidative stress	TGACCAGGGCATTAAAGAACC <sup>a</sup> AGCACCATCTTACCCACAGG <sup>b</sup>

737 Abbreviations: <sup>a</sup>forward primer; <sup>b</sup>reverse primer

738

739

740

741 **Table 2** Comparative basal expression expressed in terms of induction factor for the

742 selected genes from parasitized and control clams (non-parasitized clams).

	<b>Mar</b>	<b>May</b>	<b>Jul</b>	<b>Sep</b>	<b>Nov</b>	<b>Jan</b>
<b><i>sod (Mn)</i></b>	4.9	—	0.3	0.3	—	—
<b><i>cat</i></b>	—	—	0.5	—	2.5	—

743 expression not different (—); inhibition (expression ratio < 0.5); induction (expression ratio >2)

744

745

746