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

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Abstract

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

Keywords

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

1. INTRODUCTION

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

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

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

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

2. MATERIAL AND METHODS

2.1 Study area

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

2.2 Sampling procedure

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

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

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

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

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

2.3 Transcriptomic analysis

2.3.1 Total RNA extraction and reverse transcription

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

2.3.2 PCR and molecular cloning

The cDNAs were amplified using the specific primers. The amplification program consisted of 40 cycles of 95 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min and a final elongation step of 72 °C for 10 min. Amplified products were analysed on one percent agarose gels and fragments of the expected sizes were excised and purified 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 ΔCT 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 $MgSO_4$ 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 ε = 15.9×10^3 M^{-1} 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^{-1}). 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.

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

CAT activity was measured by the reaction of the enzyme with methanol in the presence of H_2O_2 (Johansson and Borg 1988). The standard curve was determined using formaldehyde standards (0 – 150 mM). After 20 min in an orbital incubator at room temperature, the formaldehyde formation in the presence of Purpald was spectrophotometrically measured at 540 nm. The enzymatic activity was expressed in U per mg of protein. One U is defined as the amount of enzyme that generated the formation of 1.0 nmol formaldehyde, per min.

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

2.5 Data analysis

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

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

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

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

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

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

3. RESULTS

3.1 Infection of *Donax trunculus*

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

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

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

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

3.2 Transcriptomic data

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

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

3.3 Biochemical data

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

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

GLY content of the NP clams was significantly higher in March and July compared to the other months, and a similar trend (higher GLY content in July because 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

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

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

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

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

When *B. bacciger* infection was observed (from July to January), this parasite showed to modulate the clams biomarkers response changing the seasonal pattern 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 detruncatum*
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

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

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

Gene	Accession number	Function	Sequence 5'-3'
Ef1α	MK388166	Protein synthesis and degradation (reference gene)	TCCCACTCCAGGACGTTTAC[a] TCCTGGGAGAGCTTCTGGTA[b]
β actin	MK388165	Cell structure (reference gene)	CCCACACCGTACCCATCTAC[a] GGGCAACATAGCAGAGCTTC[b]
Sod (Mn)	MK388163	Oxidative stress (manganese)	GCATCTTCTGGCAAGTCCTC[a] GAGAGCGTCCTGATTTGCTC[b]
Cat	MK388164	Oxidative stress	TGACCAGGGCATTAAGAACC[a] AGCACCATCTTACCCACAGG[b]

737 Abbreviations: [a]foward primer; [b]reverse primer

738

Table 2 Comparative basal expression expressed in terms of induction factor for the selected genes from parasitized and control clams (non-parasitized clams).

	Mar	May	Jul	Sep	Nov	Jan
sod (Mn)	4.9	—	0.3	0.3	—	—
cat	—	—	0.5	—	2.5	—

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