

Bacciger bacciger (Trematoda: Fellodistomidae) infection effects on wedge clam *Donax trunculus* condition

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ABSTRACT: Wedge clams *Donax trunculus* inhabit high-energy environments along sandy coasts of the northeastern Atlantic Ocean and the Mediterranean Sea. Two sites were sampled monthly, one in Morocco (Mehdia), where the density was normal, and one in France (Biscarosse), where the density was very low. We tested the hypothesis that the difference in density between the sites was related to infection by the trematode parasite *Bacciger bacciger*. Identity of both the parasite and the host were verified using anatomical and molecular criteria. Parasite prevalence (i.e. the percentage of parasitized clams) was almost 3 times higher at Biscarosse. At this site, overall prevalence reached 32% in July and was correlated with the migration of several individuals (with a prevalence of 88%) to the sediment surface. After this peak, prevalence decreased rapidly, suggesting death of parasitized clams. The deleterious effect of *B. bacciger* on wedge clams was also supported by our calculations indicating that the weight of the parasite made up to 56% of the total weight of the parasitized clams. However, condition indices of trematode-free clams were also lower in Biscarosse than in Mehdia or other sites, suggesting that other factors such as pollutants or microparasites (*Microcytos* sp.) may alter wedge clam population fitness in Biscarosse.

KEY WORDS: *Donax trunculus* · *Bacciger bacciger* · Wedge clam · Parasitism · Trematodes · Condition index · Mortality · Molecular identification

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INTRODUCTION

Sandy beaches with highly energetic environments are usually inhabited by infaunal communities characterized by rather low species richness (Guillou & Bayed 1991). Abundance and biomass display contrasting values among sites, but biomass is often dominated by *Donax* spp. (L., 1758) (*Bivalvia*), especially in tropical waters, but also in warm temperate waters (Ansell 1983). Among the northeastern Atlantic and Mediterranean coasts, *Donax* spp. are the dominant biomass on exposed sandy beaches and sustain a fishery with an annual catch of ca. 1000 t (ranks: 1, Portugal; 2, Spain; 3, Italy; 4, France) (FAO, www.fao.org/fishery/statistics/software/fishstatj/en).

Identification among different species of the genus *Donax* can be tricky (Pereira et al. 2012), but the dominant species for this global area appears to be the wedge clam *D. trunculus* L., 1758 (Ansell & Lagardère 1980, Ramón et al. 1999). This suspension-feeding infaunal bivalve lives on shallow bottoms of the Mediterranean Sea and colonizes low tidal levels of Atlantic shores, from the wash zone to –2 m. Reported abundances vary considerably amongst sites, from fewer than 10 to several hundred per m² (Mouëza & Chessel 1976, Fishelson et al. 1999, Deval 2009, La Valle et al. 2011). Along with fishing exploitation (Charef et al. 2012) and pollution (Fishelson et al. 1999, Neuberger-Cywiak et al. 2003), numerous ecological factors can control these popu-

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lations, such as predation, parasitism, temperature, sediment grain size, and salinity (Ansell 1983, La Valle et al. 2011). Although a number of parasites such as *Rickettsia*, gregarines, nematodes, and trematodes have been listed as parasites of *Donax* (Sindermann & Rosenfield 1967, Comps & Raimbault 1978, Ansell 1983), few studies have reported specifically on *D. trunculus*, and little is known of the pathogens which may adversely affect the health of these populations. Trematodes are among the most important parasites of the family Donacidae (Lauckner 1983). Larvae of the families Monorchiiidae, Bucephalidae, and Gymnophallidae have been cited (Pelseneer 1906, Sindermann & Rosenfield 1967, Johnson 1968), but *Bacciger bacciger* (Rudolphi, 1819) of the Fellodistomidae seems to be the most prevalent in European waters. In fact, the cercariae of this trematode were first described in the clam *Venerupis decussata* (L., 1758) in Arcachon, France, by Lespès (1857), who named it *Cercaria lata*. Different authors reported *B. bacciger* in different bivalves including *D. trunculus* (Veneridae, Donacidae, Pholadidae) (Ramón et al. 1999), and the life cycle was proposed by Palombi (1934). After escaping from sporocysts parasitizing the bivalves' first intermediate host (presumably *D. trunculus* in the present study), cercariae penetrate and encyst as metacercariae in the amphipod *Erichthonius difformis* Milne-Edwards, 1830 that may be eaten by fish (*Atherina* L. 1758) where they develop into adults in the digestive tract. As a trematode using *D. trunculus* as the first intermediate host, with sporocysts intruding into the gonads, visceral mass, and eventually the whole body, it can be assumed by comparison with other bivalves being similarly infected by other trematode species that *B. bacciger* (Jonsson & André 1992, Thieltges 2006, de Montaudouin et al. 2012) will have a detrimental effect on its host. Ramón et al. (1999) noted castration in 2.4% of a *D. trunculus* population in the Gulf of Valencia (Spain). Sudden periodic and complete extinction was also observed in California (USA) for populations of the bean clam *D. gouldii* Dall, 1921, with the suspicion of an interaction between trematode occurrence and other environmental pressures (Johnson 1968).

In August 2010, a considerable number of *D. trunculus* were found moribund at the surface of the sediment of Biscarosse, a beach on the southwestern coast of France (Fig. 1). At that time, *D. trunculus* abundance was <1 ind. m^{-2} even though local fishermen claimed that historically the population had been able to sustain a fishery. Concomitantly, a small artisanal fishery is surviving in the Atlantic beach of



Fig. 1. Location of the 2 sampling sites, Biscarosse (France) and Mehdia (Morocco)

Mehdia (Morocco; Fig. 1), with a density around 12 ind. m^{-2} (H. Bazairi unpubl. data). The aim of this study was to monitor both populations over 1 year, focusing on trematode infection as a parameter to interpret and understand part of the difference in success observed in *D. trunculus* population stability between the 2 sites. In addition, following a recent molecular analysis of *D. trunculus* (Pereira et al. 2012), we had previously verified the identity of this bivalve at both sites. We also checked that *B. bacciger* was also the same species in Biscarosse (France) and Mehdia (Morocco) beaches using a molecular approach.

MATERIALS AND METHODS

Sites

Biscarosse beach is located on the Atlantic coast of southwestern France (44° 28' 34" N, 1° 15' 13" W; Fig. 1). The sediment consists of medium quartz sands (grain-size median = 390–400 μ m). The tide is of a macro type, with an average range of 3.2 m, extending up to 5 m during spring tides. Salinity is stable throughout the year (34.0–36.0), and sea surface temperature fluctuates seasonally between 10 and 20°C (Auby et al. 2011). Mehdia beach is also an exposed beach, on the Atlantic coast of Morocco

(34° 15' 22" N, 6° 40' 49" W; Fig. 1). Salinity ranges between 33 and 36, while temperature fluctuates from 12 to 25°C. Sediments are medium sands (median = 250–290 µm). Tidal elevation is 4.5 m at a tidal coefficient of 95 (Guillou & Bayed 1991).

Sampling and bivalve analysis

Wedge clams were collected by hand from August 2010 to November 2011, at a low tide of high coefficient (>90). A minimum of 20 individuals was sampled each month, the value of 20 being reached with difficulty at Biscarosse (range: 20–52 individuals), and more easily at Mehdia (61–241 individuals). All clams were measured (shell length to the nearest mm) and then opened, and the flesh was squeezed between 2 glass slides and screened for trematode identification using a stereomicroscope. Parasite prevalence was defined as the percentage of infected clams and was calculated for individuals with shell length >11 mm, corresponding to the smallest infected individuals identified to date. From December 2010 to November 2011, condition index (CI) was calculated for all parasitized clams and non-parasitized individuals of the same shell length as $CI = [\text{flesh dry weight (mg)}/\text{shell weight (g)}]$ and was expressed in %.

Finally, observations during this monitoring led us to complete this study by considering 2 more aspects: (1) due to the macroscopic appearance of parasitized clams, we attempted to assess how much of the flesh mass was in fact parasite mass. Sporocysts were separated from the clam flesh with forceps under a stereomicroscope, and weighed after 48 h at 60°C (dry weight). This dissection was performed from June to November 2011, for clams from Mehdia; (2) at Biscarosse, a mass mortality was observed in July 2011, with clams appearing at the surface of the sediment. A total of 34 surfaced clams were collected and dissected as previously described.

Table 1. Sequences of the primer pairs used to amplify the 18S and ITS regions from parasites of the genus *Bacciger* in Mehdia (Morocco) and Biscarosse (France). F: forward; R: reverse

Gene	Primer	Sequence 5'→3'
18S	F: Bb18S5	ACTGGAGGGCAAGTCTGGTG
	R: Bb18S3	CAGCTTTGCAACCATACTTCC
ITS	F: BbITS5	CTCCCATATGGTGCACCTGCA
	R: BbITS3	TTGACCGAAGCTTGATCATT

Identity of Biscarosse and Mehdia wedge clams

Identification of *Donax* species from Biscarosse and Mehdia was determined using the primer pairs described by Pereira et al. (2012) for the amplification of the 5S region. Length of the PCR products was observed via 1% p/v agarose gel electrophoresis.

Parasite comparison between Biscarosse and Mehdia wedge clams

Cercariae were observed under the microscope, and their anatomy was compared to a previous description of this species (Ramón et al. 1999).

Sporocysts from Biscarosse and Mehdia were also compared through molecular analysis. They were separated from bivalve flesh under the stereomicroscope, and total DNA was extracted from these samples as previously described by Cambier et al. (2010). Two regions, classically used in molecular taxonomy, were analyzed in this study: the rRNA 18S gene and the internal transcribed spacer (ITS) located between the 18S and the 28S genes. As no sequences of these genes were available for the genus *Bacciger* in any of the databases, the corresponding sequences from species belonging to the families Lepocreadidae and Fellodistomidae were aligned using ClustalW software. From these alignments, PCR primer pairs were determined in conserved regions. PCR reactions were then performed to amplify the 18S and the ITS regions using the primer pairs Bb18S5+Bb18S3 and BbITS5+BbITS3 (Table 1). Each PCR reaction included 10 µl of 5× buffer (Promega), 1 µl dNTPs (10 mM), 3 µl MgCl₂ (25 mM), 0.5 µl of each primer (100 µM), 1 µl of Go-Taq (1 U µl⁻¹, Promega), 1 µl of purified DNA, and water to a final volume of 50 µl. The program consisted of 40 cycles: 95°C for 60 s, 55°C for 60 s and 72°C for 90 s. PCR products were analyzed on a 1% p/v agarose gel and purified using the PCR purification kit (Qiagen), before being cloned in pGEM T (Promega) and sequenced (Millegen). The resulting sequences were assigned GenBank accession numbers KJ633827 (18S) and KJ633828 (ITS region).

Phylogenetic analysis

The nucleotide sequences of the 18S rRNA gene from *Proctoeces maculatus* (AY222161), *P. lintoni* (EU423073), *Olssonium turneri* (AJ287548), *Terghostia laticollis* (AJ287580), *Complexobursa* sp.

(AJ224462), *Coomera brayi* (AJ224469), *Fellodistomum fellis* (Z12601), *Lepidapedon rachion* (Z12607), *Steringophorus margolisi* (AJ287578), *S. furciger* (Z25818), and *B. bacciger* were aligned using ClustalW software. Phylogenetic analyses were performed using PHYLIP version 3.66 software. Phylogenetic trees were constructed from DNA sequences using the neighbor-joining and maximum likelihood methods, and genetic distances were calculated using the Kimura 2-parameter distance algorithm. Phylogenetic relationships among the sequences were evaluated to estimate the node reliability of the phylogenetic trees constructed using the 2 methods, with 1000 bootstrap replicates.

Statistical analysis

The CI of *D. trunculus* was compared in parasitized and non-parasitized (by *B. bacciger*) individuals and between months with a 2-way ANOVA. Prior to analysis, homogeneity of variance was verified by a Cochran test and data were log-transformed when necessary (normality was assumed). ANOVA was also performed with the CI calculated after subtracting the average parasite weight.

RESULTS

Identity of wedge clams and trematodes

Molecular identification using the approach previously described by Pereira et al. (2012) and targeting the 5S gene verified that the *Donax* species from both sites shared the same 300 bp PCR product and consequently were *D. trunculus*. PCR using the primer pairs Bb18S5-Bb18S3 and BbITS5+BbITS3 revealed the same results from samples collected at the 2 different sites (Fig. 2). A 673 bp fragment was obtained for the 18S region, while a 1335 bp fragment was determined for the ITS region. The alignments indicated that the 18S and ITS regions from *Bacciger* parasites in Mehdiya and Biscarosse were strictly identical, and no nucleotide or length variations were observed. A phylogenetic analysis based upon the V4 region of the small subunit rDNA sequences available in databases for Fellodistomidae, evidenced 2 different groups (Fig. 3). *Fellodistomum fellis*, *Tergestia laticollis*, *Olssonium turneri*, *Coomera brayi*, *Complexobursa* sp., and the 2 *Proctoeces* species were associated in the same group (59–100% bootstrap support),

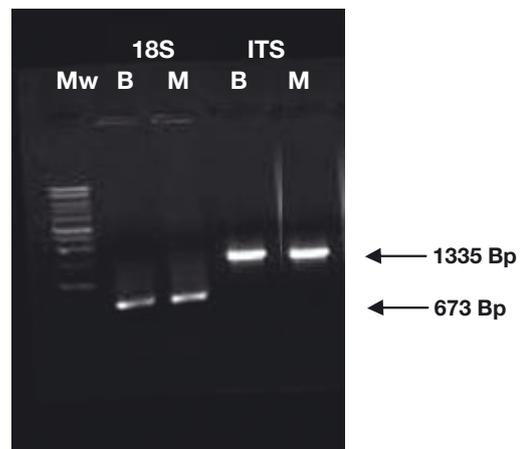


Fig. 2. Electrophoretic migration of PCR product obtained after amplification of the ITS and 18S region of the parasites isolated from wedge clams *Donax trunculus* from Mehdiya (M) and Biscarosse (B). Mw: Molecular weight marker (Promega)

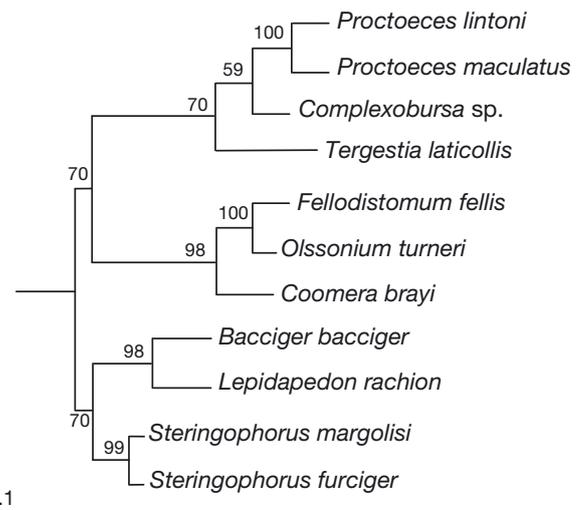


Fig. 3. Tree of phylogenetic relationships of the analyzed members of the Fellodistomidae based on small subunit rRNA gene sequences. Neighbor-joining and maximum likelihood show the same results. Values above branches indicate bootstrap percentages

while the 4 remaining species used in this analysis were closely related in a second group (70–99% bootstrap support).

Wedge clam/trematode dynamics

Shell length of wedge clams collected at Biscarosse varied between 10 and 44 mm with a peak at around 34 mm (Fig. 4). Among macroparasites, only 1 species of trematode was observed. It was present in the

digestive gland, the gonads, and eventually invaded surrounding tissues such as the foot and the gills. The color was bright orange. Cercariae were trichocercous (setiferous tail). The tail was composed of 27 pairs of finlets, with 8 longitudinal protruding rib-like supports. This description is consistent with those of the fellodistomatid trematode *Bacciger bacciger* (Ramón et al. 1999). The smallest infected clam was 22 mm long. Prevalence in clams ≥ 11 mm fluctuated between 0 and 32% with an 18% average (Fig. 5). There was no evidence of seasonal peaks in infection. CI of healthy clams was low between May and August, i.e. 47 to 72‰ (spawning season) and progressively increased to reach 81‰ in the pre-spawning period (spring; Fig. 6). On average, parasitized clams exhibited lower CI (-12%). There was a significant effect of parasitism on CI, with a significant interaction with months (Table 2). Overall CI was higher or similar in infected clams when the population had spawned and lower during periods of gametogenesis. For example in June, infected clams had 10% higher CI, while it was 37% lower in November (Fig. 6).

At Mehdiya, collected clams were smaller, with shell length ranging from 5 to 38 mm, with a peak at 23 mm (Fig. 7). Like at Biscarosse, the only parasite observed was similar in appearance to *B. bacciger* but infected clams at a much smaller size. Pooling all infected clams from each site (102 at Biscarosse and 162 at Mehdiya), 39% were ≤ 22 mm at Mehdiya (Fig. 7) while infection had just started at Biscarosse at this size (Fig. 4). Prevalence of clams ≥ 11 mm fluctuated between 1 and 14% with a 7% average, with the lowest prevalence recorded in autumn (Fig. 5). The CI of healthy clams was low in summer, similar to Biscarosse (62–73‰) and increased during gametogenesis to reach more than 100‰ in February (except a single low value in December; Fig. 6). In contrast to Biscarosse, there was no significant difference in the CI between parasitized and unparasitized clams (Table 2, Fig. 6).

The percentage of the flesh weight that was attributable to parasites was variable among individuals. Due to the unbalanced design (1 to 15 dissected clams per month) of the study, it was not possible to compare results among months. Out of 45 dissected clams, the mean \pm SD parasite percentage of total flesh weight was $19 \pm 10\%$ (range 6–56%). When subtracting 19% of the total flesh mass from

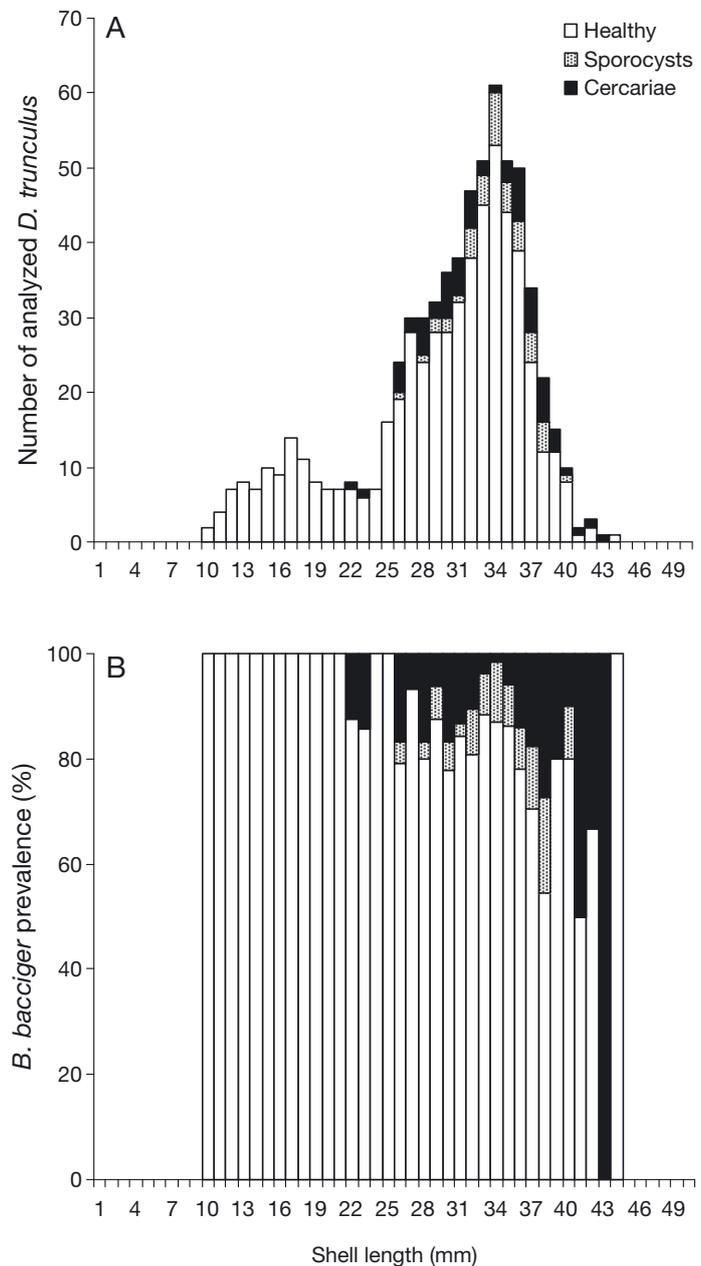


Fig. 4. (A) Shell length (mm) of analyzed *Donax trunculus* at Biscarosse (France) and (B) prevalence (%) of infection by the trematode *Bacciger bacciger*

parasitized clams, a significant effect of parasitism was observed on the CI of clams from both sites (Table 2).

In July 2011, a large number of clams were retrieved at the surface of the sediment. A total of 88% of these clams were infected by *B. bacciger*, most of them with developed cercariae. In comparison, prevalence in buried clams was 32% (the highest during the monitoring).

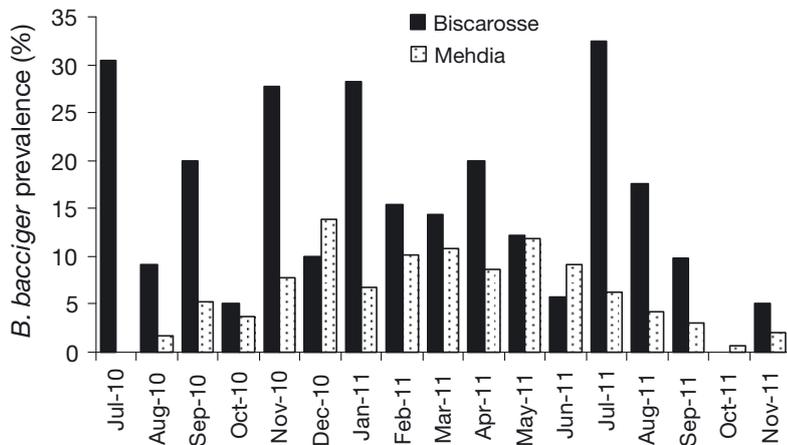


Fig. 5. Monthly prevalence (%) of *Bacciger bacciger* in *Donax trunculus* at Biscarosse and Mehdia in 2010–2011

DISCUSSION

Morphological criteria used to identify wedge clams (Tebble 1966) were corroborated by molecular identification proposed by Pereira et al. (2012) to confirm that the individuals collected at Biscarosse (France) and Mehdia (Morocco) were *Donax trunculus*, which was also in agreement with the geographical area of distribution for this clam.

Morphological criteria employed to identify cercariae found in *D. trunculus* matched the description of this trematode given by Ramón et al. (1999). Our molecular analysis, based on amplification and sequencing of the ITS and 18S regions, demonstrated that cercariae and sporocysts found in Biscarosse and Mehdia belonged to the same species, i.e. *Bacciger*

bacciger. No length or sequence differences were observed in the sequences of the ITS or the 18S. Knowledge of these genomic sequences would be of great interest in future studies to determine new detection and quantification methods for this trematode in bivalves, using techniques such as real-time quantitative PCR. No such tools currently exist for the genus *Bacciger*, although PCR detection is available for various other parasites such as *Perkinsus* species (Park et al. 2005, Abollo et al. 2006) or other trematodes (Baba et al. 2012, Le et al. 2012). Phylogenetic analysis based upon the V4 regions of the 18S gene revealed that species belonging to the Fellodistomidae could be separated into 2 distinct

groups. Relationships evidenced between *Fellodistomum fellis*, *Tergestia laticollis*, *Olssonium turneri*, *Coomera brayi*, *Compexobursa* sp., and the 2 *Proctoeces* species were fully in agreement with previous reports (Hall et al. 1999, Oliva et al. 2010). The authors of those studies proposed a possible monophyly of the Fellodistomidae (sensu stricto) and a polyphyly of the Fellodistomidae (sensu lato). However, the availability of new sequences, included in our phylogenetic analysis, for species belonging to this large family of digeneans suggested that the Fellodistomidae are more probably polyphyletic. Indeed, *Lepidapedon rachion*, *B. bacciger*, *Steringophorus margolisi*, and *S. fusciger* were clearly separated from the other species, and their relationships were supported by strong bootstrap values.

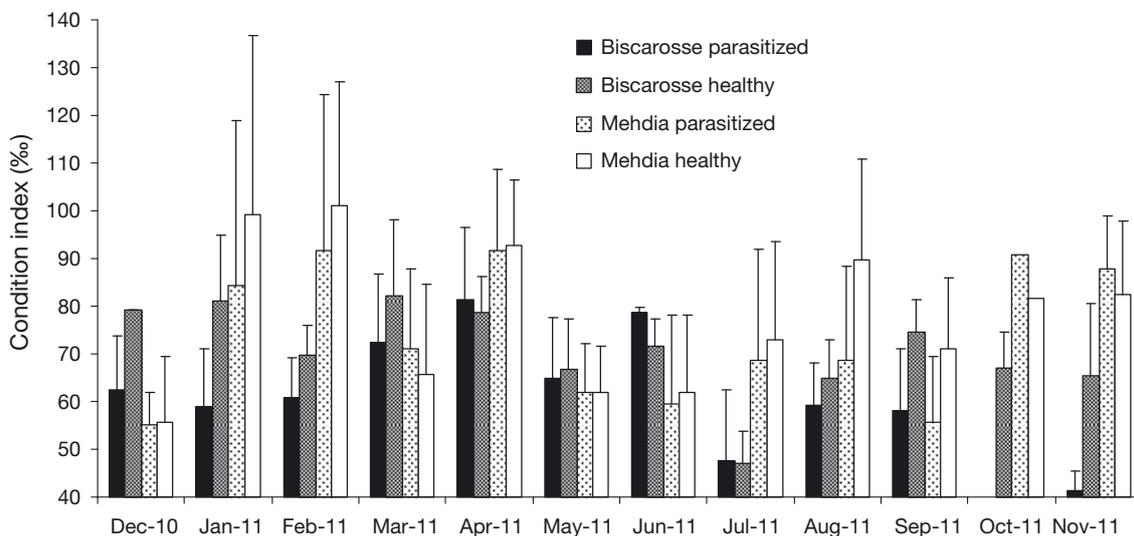


Fig. 6. Monthly condition index (‰) of *Donax trunculus* at Biscarosse and Mehdia in 2010–2011, in healthy and parasitized individuals

Sampling at Biscarosse confirmed the very low density of the *D. trunculus* population (<1 ind. m^{-2}) compared to that in Mehdia (several ind. m^{-2}), even though both sites display similar characteristics (sediments, temperature, salinity) that could promote dense populations. Among several biotic factors, at these low densities, competition alone cannot explain differences observed among sites. No data are avail-

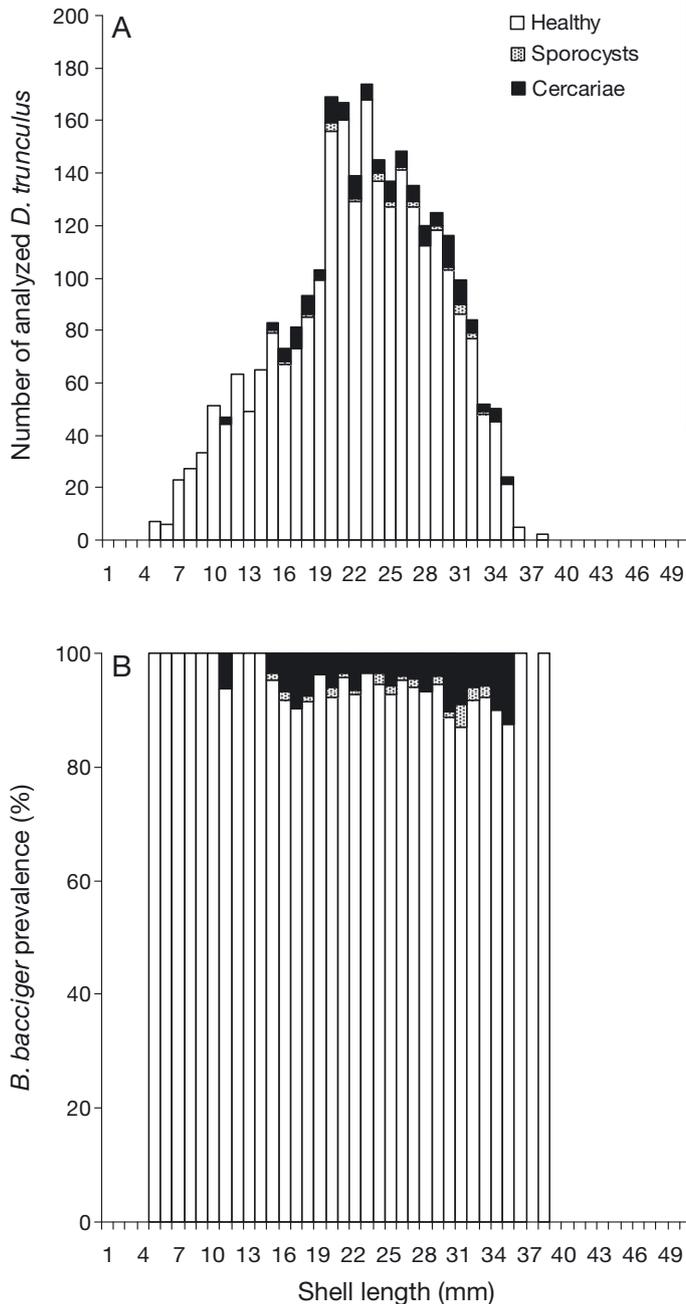


Fig. 7. (A) Shell length (mm) of analyzed *Donax trunculus* at Mehdia (Morocco) and (B) prevalence (%) of infection by the trematode *Bacciger bacciger*

Table 2. Statistical results of ANOVA comparing the condition index (CI) of wedge clams *Donax trunculus* and the CI of clam flesh with the parasite (*Bacciger bacciger*) weight removed according to date and parasite presence

Site	Treatment	df	F	p
Total CI				
Biscarosse	Date	10	11.36	<0.001
	Parasite	1	13.26	<0.001
	Date × Parasite	10	2.31	0.017
	Error	112		
Mehdia	Date	10	11.75	<0.001
	Parasite	1	3.74	0.054
	Date × Parasite	10	0.76	0.662
	Error	168		
CI (-19% flesh)				
Biscarosse	Date	10	12.08	<0.001
	Parasite	1	177.44	<0.001
	Date × Parasite	10	2.90	0.002
	Error	112		
Mehdia	Date	10	11.75	<0.001
	Parasite	1	122.32	<0.001
	Date × Parasite	10	0.76	0.662
	Error	168		

able about predation, but human fishing is sporadic in Mehdia and absent in Biscarosse. This could explain why Mehdia displayed smaller individuals than Biscarosse (i.e. removal of large specimens as a result of fishing). In this context, parasitism appears as a possible factor discriminating between the sites. Accordingly, the average prevalence of parasite infection within a year was almost 3-fold higher at Biscarosse (17.7% vs. 6.6% for individuals with shell length >11 mm). Very few data are available to which we can compare the results of our study. Ramón et al. (1999) found a prevalence $>4.5\%$ for clams over 22 mm, increasing to 15% in 34 mm clams. Conversely, within the same range of length, clams from Biscarosse displayed a prevalence varying between 13 and 15%, but reached values over 20% in clams with larger shell lengths.

Another argument in favor of parasite-dependent mortality was the occurrence of 2 summer (2010 and 2011) events when a large quantity of surfaced moribund *D. trunculus* was found. No survey was conducted in 2011, but in July 2011, surfaced *D. trunculus* were autopsied and ca. 90% of them were parasitized by *B. bacciger* (vs. 32% of buried *D. trunculus*, which is already a high value). After this peak in infection, prevalence dropped from 17% in August to 5% in November. Apart from recruitment or immigra-

tion, the decrease in parasite prevalence and/or abundance in a population can be interpreted as the death of the most severely parasitized hosts (Anderson & Gordon 1982, Kennedy 1984, Lester 1984, Desclaux et al. 2004). The detrimental effect of trematode parasites as the first intermediate host has already been observed in other bivalve/trematode systems, including *Cerastoderma edule* (edible cockle)/*Gymnophallus choledochus* (Thieltges 2006), *C. edule/Bucephalus minumus* (de Montaudouin et al. 2012), *C. edule/Monorchis parvus* (Jonsson & André 1992), *Perna perna* (brown mussel)/*Bucephalus* sp. (da Silva et al. 2002), *Dreissena polymorpha* (zebra mussel)/*Bucephalus polymorphus* (Lajtner et al. 2008), and *Ruditapes decussatus* (grooved carpet shell)/*Cercaria lata* (Gargouri Ben Abdallah et al. 2009). In all cases, the sporocysts or rediae progressively invade the whole host and it becomes difficult to distinguish parasite tissues from host tissues. In the present case, we showed that the mass of the parasite *B. bacciger* could represent up to 23% of the total flesh weight of the clam. This is in the same range of values that was calculated in cockles *C. edule* infected with *Bucephalus minumus* (up to 34%) and *M. parvus* (up to 22%; Baudrimont & de Montaudouin 2007, Dubois et al. 2009). These results highlight the necessity to be cautious when using CI as a proxy of bivalve health, because this index can overstate the real ratio of host body mass to host shell mass.

CONCLUSION

The difficulty experienced by the Biscarosse *Donax trunculus* population in sustaining itself may be related to trematode infection. However, parasitism could affect *D. trunculus* in its interaction with other factors (Johnson 1968). The CI, which provides an indication of the bivalves' fitness, remained relatively low in Biscarosse (47–82% in *B. bacciger*-free clams) compared to Mehdiya (56–101%) and other sites, e.g. in Tunisia (65–120%; Boussofoua et al. 2011). The next investigation should focus on analyzing which other factors could intervene to weaken the *D. trunculus* population at Biscarosse. The presence of treated wastewater output near the area could be considered as a source of pollution and stress for these sensitive bivalves (Fishelson et al. 1999, Bresler et al. 2003, Neuberger-Cywiak et al. 2003, 2007). In addition, other parasites have recently been pointed out as possible sources of mass mortality in this clam along the French Atlantic coasts, in particular the protozoan *Mikrocytos* sp. (Ifremer 2011).

Hence, further morphological studies are needed, e.g. through histopathology, in order to reveal the specific lesions linked to trematode parasite presence and in order to evaluate the presence of other pathogens in the examined population.

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