Universidade de Aveiro Departamento de Biologia
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LUÍSA VIRGÍNIA DE SOUSA MAGALHÃES

INVENTÁRIO, DINÂMICA E IMPACTO DOS PARASITAS TREMATODES EM BIVALVES DE ELEVADA IMPORTÂNCIA ECONÓMICA

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Tese apresentada à Universidade de Aveiro e à Universidade de Bordéus para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia e Ecologia das Alterações Globais, realizada sob a orientação científica da Doutora Rosa Freitas, Investigadora do Departamento de Biologia da Universidade de Aveiro e do Doutor Xavier de Montaudouin, Professor catedrático da Universidade de Bordéus.

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Dedico este trabalho à Alice, minha filha, e ao Joel, meu marido.

o júri/the jury

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Palavras-chave

Zona intertidal, *Cerastoderma edule*, *Donax trunculus*, dinâmica populacional, *Bucephalus minimus*, *Bacciger bacciger*, metacercariae, expressão de genes, biomarcadores de stress, infeção experimental

Resumo

Entre os agentes que modulam a dinâmica populacional, o parasitismo é significativo mas muitas vezes negligenciado. É urgente não só inventariar as várias espécies de parasitas, bem como compreender a suscetibilidade dos hospedeiros à infeção (nomeadamente os bivalves) e investigar a interação entre os parasitas e outras condições ambientais. Pelo que, esta tese teve como objetivo principal caracterizar e quantificar os macroparasitas trematodes (os mais abundantes e prevalentes em águas costeiras) que infetam *Cerastoderma edule* (berbigão) e *Donax trunculus* (conquilha), dois dos bivalves mais importantes em Portugal e França tanto do ponto de vista ecológico como económico.

Primeiramente, a dinâmica populacional dos bivalves foi estudada, tendo em conta a relação entre a temperatura e o período de recrutamento e os efeitos recíprocos do recrutamento na biomassa de adultos. Para isso, foi analisada uma base de dados abrangendo 17 anos de observações mensais de uma população de berbigões que habitam uma área nacional protegida (Banc d'Arguin, Arcachon, França). Estas observações de longa duração mostraram que a sustentabilidade de uma população de berbigão é dependente do sucesso do recrutamento. Em berbigões, o sucesso do recrutamento mostrou ser em parte, mas não totalmente, dependente da temperatura. Por esta razão, a sustentabilidade de uma coorte pode estar a ser estabelecida mais cedo, isto é, por processos que acontecem antes do recrutamento. Seguindo esta pista, o verdadeiro papel do parasitismo na dinâmica populacional dos bivalves foi mais explorado.

De seguida e devido à elevada patogenicidade para os bivalves, foi dada especial atenção aos parasitas *Bucephalus minimus* e *Bacciger bacciger* que usam *C. edule* e *D. trunculus*, respetivamente, como primeiros hospedeiros intermediários (onde o estádio parasítico esporocisto se desenvolve). Métodos clássicos de dissecação dos hospedeiros para identificação dos parasitas foram combinados com abordagens de transcriptómica e bioquímica. Os resultados revelaram alguns aspetos essenciais da dinâmica hospedeiro-parasita: a relação positiva entre o tamanho do primeiro hospedeiro e a prevalência do parasita; a confirmação da temperatura como um agente importante da prevalência do parasita no seu primeiro hospedeiro e a descoberta de que uma maior prevalência do parasita (esporocistos) leva a uma maior abundância de metacercariae de outras espécies. Para ambos os casos de estudo (*C. edule* amostrado em Arcachon e *D. trunculus* amostrado em Faro, Portugal), foi demonstrado que o parasita tem um efeito negativo no seu primeiro hospedeiro aumentando a taxa metabólica, diminuindo as reservas de energia e inibindo a atividade de enzimas antioxidantes o que, em alguns meses, provocou danos celulares.

Depois, este estudo focou-se na infeção dos bivalves por metacercariae, ou seja, quando servem de segundos hospedeiros intermediários no ciclo de vida do parasita. Normalmente, esta relação é reportada como sendo menos perniciosa contudo, quando determinados limites de abundância são excedidos, as metacercariae são capazes de perturbar algumas das funções básicas dos bivalves. A variabilidade espácio-temporal da estrutura da comunidade de parasitas que infetam *C. edule* à escala da Ria de Aveiro (Portugal) foi então caracterizada e mostrou ser influenciada por múltiplos fatores abióticos e também pela densidade da população do hospedeiro alvo. O papel da densidade da população de bivalves na taxa de infeção individual foi então mais aprofundado numa monitorização de campo de longa duração e numa experiência de campo. Demonstrou-se que o efeito encontro-diluição pode também ser aplicado a populações naturais de bivalves.

Por fim, foi experimentalmente avaliada a suscetibilidade dos bivalves à infeção por parasitas quando desafiados por fatores relacionados com as alterações climáticas (salinidade, temperatura e pH) e contaminação (Arsénio). Os resultados mostraram que a exposição dos hospedeiros a condições de stress relacionadas com cenários de alterações globais podem modificar o sucesso da infeção parasitária e induzir alterações na resposta bioquímica do hospedeiro.

As descobertas apresentadas nesta tese melhoraram o conhecimento dos efeitos de diferentes variáveis nos bivalves, salientando o papel crucial do parasitismo. Se aplicados, estes novos pontos de vista podem promover a gestão sustentável dos bivalves, um recurso marinho tão importante, aumentando o seu potencial de produção e económico.

Mots clés

Zone intertidale, *Cerastoderma edule*, *Donax trunculus*, dynamique des populations, *Bucephalus minimus*, *Bacciger bacciger*, métacercaires, expression génique, biomarqueurs de stress, infection expérimentale

Resumé

Parmi les agents qui modulent la dynamique des populations, le parasitisme est important mais souvent négligé. Il est urgent non seulement d'inventorier les différentes espèces de parasites, mais aussi de comprendre la sensibilité des hôtes à l'infection (notamment des bivalves) et étudier les interactions entre les parasites et les autres facteurs environnementaux. Par conséquent, cette thèse avait comme objectif principal de caractériser et de quantifier les communautés de trématodes (les plus abondants et répandus des macroparasites de bivalves dans les eaux côtières) qui infectent *Cerastoderma edule* (coque) et *Donax trunculus* (telline), deux des bivalves les plus importants au Portugal et en France d'un point de vue écologique et économique.

Dans un premier temps, la dynamique des populations de bivalves a été étudiée en tenant compte de la relation entre la température et la période de recrutement et des effets en retour du recrutement sur la biomasse adulte. Pour cela, une base de données a été analysée couvrant 17 ans d'observations mensuelles d'une population de coques dans une réserve nationale (Banc d'Arguin, Arcachon, France). Ces observations à long terme ont montré que la durabilité d'une population de coques dépend du succès du recrutement. Pour les coques, le succès du recrutement a été montré comme étant en partie, mais pas totalement, dépendant de la température. Ainsi, la durée de vie d'une cohorte pourrait être estimée plus tôt, grâce à des indices se produisant en amont du recrutement. Suite à ces résultats, le rôle du parasitisme dans la dynamique des populations de bivalves a été étudié.

Premièrement, en raison de leur forte pathogénicité pour les bivalves, une attention particulière a été accordée aux parasites *Bucephalus minimus* et *Bacciger bacciger* qui utilisent *C. edule* et *D. trunculus*, respectivement, comme premier hôte intermédiaire (où se développe le stade parasitaire sporocyste). Les méthodes classiques de dissection de l'hôte pour la détection des parasites ont été combinées avec des approches transcriptomiques et biochimiques. Les résultats ont révélé certains aspects essentiels de la dynamique hôte-parasite: la relation positive entre la taille du premier hôte et la prévalence du parasite; l'importance de la température dans l'interprétation des fluctuations de prévalences parasitaires dans son premier hôte et la démonstration d'une relation positive entre l'infestation par les sporocystes et celle par les métacercaires d'autres espèces parasites. Pour les deux études de cas (*C. edule* échantillonné dans le Bassin d'Arcachon et *D. trunculus* échantillonné dans la Ria da Faro, Portugal), il a été démontré que le parasite a un effet négatif sur son premier hôte, augmentant le taux métabolique, diminuant les réserves d'énergie et inhibant l'activité des enzymes antioxydants qui, en quelques mois, causent des dommages cellulaires.

Deuxièmement, cette étude s'est concentrée sur l'infection des bivalves par les métacercaires, c'est-à-dire lorsqu'ils servent de second hôte intermédiaire dans le cycle de vie du parasite. Normalement, cette relation serait moins délétère, mais lorsque certains seuils d'abondance sont dépassés, les métacercaires sont capables de perturber les fonctions de base des bivalves. La variabilité spatio-temporelle de la structure communautaire des parasites qui infestent *C. edule* à l'échelle de Ria de Aveiro (Portugal) a ensuite été décrit et s'est montrée influencée par de multiples facteurs abiotiques mais aussi par la densité de population de l'hôte cible. Le rôle de la densité de population des bivalves dans le succès d'infection individuel a ensuite été étudié dans le cadre croisé du suivi pluriannuel d'une population de coques et d'une expérience sur le terrain. Il a été démontré que l'effet de rencontre-dilution peut également être appliqué aux populations naturelles de bivalves.

Enfin, la sensibilité des bivalves à l'infection parasitaire a été évaluée expérimentalement lorsqu'ils sont confrontés à des facteurs liés au changement climatique (salinité, température et pH) et à la contamination (arsenic). Les résultats ont montré que l'exposition de l'hôte à des conditions de stress liées à des scénarios de changement global peut modifier le succès de l'infection parasitaire et altérer les réponse biochimique de l'hôte.

Les résultats présentés dans cette thèse ont amélioré la connaissance des effets de différentes variables sur les bivalves, soulignant le rôle crucial du parasitisme. S'ils sont appliqués, ces nouveaux concepts peuvent promouvoir la gestion durable des bivalves, une ressource marine importante, en augmentant son potentiel de production et donc son potentiel économique.

Keywords

Intertidal, *Cerastoderma edule*, *Donax trunculus*, population dynamics, *Bucephalus minimus*, *Bacciger bacciger*, metacercariae, gene expression, stress biomarkers, experimental infection

Abstract

Among population dynamics drivers, parasitism is significant but often neglected. Beyond inventory of the various parasites, it is urgent to understand the susceptibility of hosts, namely bivalves, to infection, and to investigate the interaction among parasites and other environmental conditions.

In this way, the present study aimed to characterize and quantify the trematode macroparasites, the most abundant and prevalent in coastal waters, infecting *Cerastoderma edule* and *Donax trunculus*, which are among the most ecologically important and economically explored bivalve species in Portugal and France.

The first step was to study bivalve population dynamics, evaluating the relationship between temperature and recruitment timing and the reciprocal effects of recruitment on adult biomass. For this, a large database spanning 17 years of monthly observations of a cockle population inhabiting a national protected area (Banc d'Arguin, Arcachon, France) was analysed. Long-term observations showed that the sustainability of a cockle population is recruitment-success dependent. In cockles, recruitment success showed to be partly, but not only, dependent on temperature. Hence, the sustainability of a cohort could be set earlier, i.e. by processes happening before recruitment. Following this clue, the role of parasitism on the bivalve host population dynamics was explored.

Firstly, due to high pathogenicity for bivalves, special attention was given to the parasites *Bucephalus minimus* and *Bacciger bacciger* which use *C. edule* and *D. trunculus*, respectively, as first intermediate hosts (where their sporocysts parasitic stage develops). Classic methods of host dissection for parasites identification were combined with transcriptomic and biochemistry approaches. Results revealed some essential aspects of a host-parasite dynamics, such as the positive relationship between first host size and parasite prevalence, the statement of temperature as an important driver of parasite prevalence in its first intermediate host, and the higher parasite prevalence (as sporocysts) leading to higher metacercariae abundance of other species. For both case studies (*C. edule* collected in Arcachon, France and *D. trunculus* collected in Faro, Portugal), it was demonstrated that the parasite has a negative effect on its first intermediate host by increasing the metabolic rate, decreasing the energy reserves and inhibiting the antioxidant enzymes activity, which in some months led to cellular damages.

Then, the study focused on metacercariae infection in its bivalve second intermediate host, a relationship that is usually reported as less deleterious. However, when certain abundance thresholds are exceeded, metacercariae are able to disrupt some of the bivalve basic functions. The spatio-temporal variability of the structure of trematode community infecting *C. edule* at the scale of the Ria de Aveiro (Portugal) was characterized and showed to be influenced by multiple abiotic factors and target host population density. Thus, the role of bivalve population density on individual infection rate was further explored in a field long-term monitoring and field experiment study. It was demonstrated that the encounter-dilution effect can be applied also to natural bivalve populations.

Lastly, the susceptibility of bivalves to parasites infection when challenged by climate change related factors (salinity, temperature and pH) and contamination (Arsenic) was experimentally assessed. Main results showed that hosts exposure to stressful conditions related to global change scenarios can modify the parasite infection success and induced host biochemical response alterations.

The findings presented in this thesis improved the knowledge on the effects of different constraints on bivalves, highlighting the crucial role of parasitism. If applied, these new insights can promote the sustainable management of bivalves, such an important marine resource, with greater production and economic potential.

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CHAPTER 1. INTRODUCTION

1.1. State of the art

Bivalves constitute an essential component of estuarine and coastal ecosystems, representing a major proportion of the benthic fauna biomass, occurring also at high densities (Sousa et al., 2009). Bivalves include many keystone species (Taylor et al., 2018) that play an important role in ecological communities and on the ecosystem functioning (Morgan et al., 2013). Acting also as ecosystem engineers through their filter-feeding habit and, for the infaunal species, their burrowing activity, bivalves create, modify, and maintain habitat for other species (Philippart et al., 2007). They provide structural conditions for other invertebrates to settle, and occupy a crucial position within food webs (Rakotomalala et al., 2015). Bivalves are connected to primary producers by their suspension-feeding activity and related to higher trophic levels as prey for many bird, fish, crustacean and echinoderm species. Moreover, some bivalve species are the basis of important commercial activities (Beukema & Dekker, 2006; Oliveira et al., 2013; Rowley et al., 2014). Thus, bivalves contribute to biodiversity and ecosystem resilience, and therefore the identification of factors that modulate their population dynamics is of upmost importance.

Three factors determine bivalves population biomass, i.e. recruitment, individual growth and mortality. A fourth factor, migration, is rarely relevant for adult bivalves but may be associated to larval dispersion. Many authors have shown that long-term bivalve biomass presents high variability (Figure 1) and is mainly dependent on recruitment success (Beukema et al., 2010; Beukema & Dekker, 2006). Recruitment is, in its turn, characterized by high spatial and temporal variability (Bachelet et al., 1992b; Beukema et al., 2001; Dörjes et al., 1986; Ducrotoy et al., 1991) to which contribute hydrodynamic processes (André & Rosenberg, 1991), substratum type (de Montaudouin et al., 2003), predation (Andresen et al., 2013; Brock, 1980; Dekker & Beukema, 2014; Masski & Guillou, 1999), intraspecific competition (André & Rosenberg, 1991; Bachelet et al., 1992a, 1992b; Brock, 1980; de Montaudouin & Bachelet, 1996; Genelt-Yanovskiy et al., 2010; Jensen, 1992) but mainly temperature (Beukema & Dekker, 2005, 2014; Beukema et al., 2001). For example, temperature or more precisely, mean water temperature in the previous winter (January–March), is considered the major factor responsible for recruitment success in the Wadden Sea (Beukema et al., 2001). However, these authors also observed differences between the northern and southern Wadden Sea which could not be explained, at that scale, by differences in winter severity. Therefore, there must be other important drivers explaining bivalve stock biomass variability and consequent population sustainability.

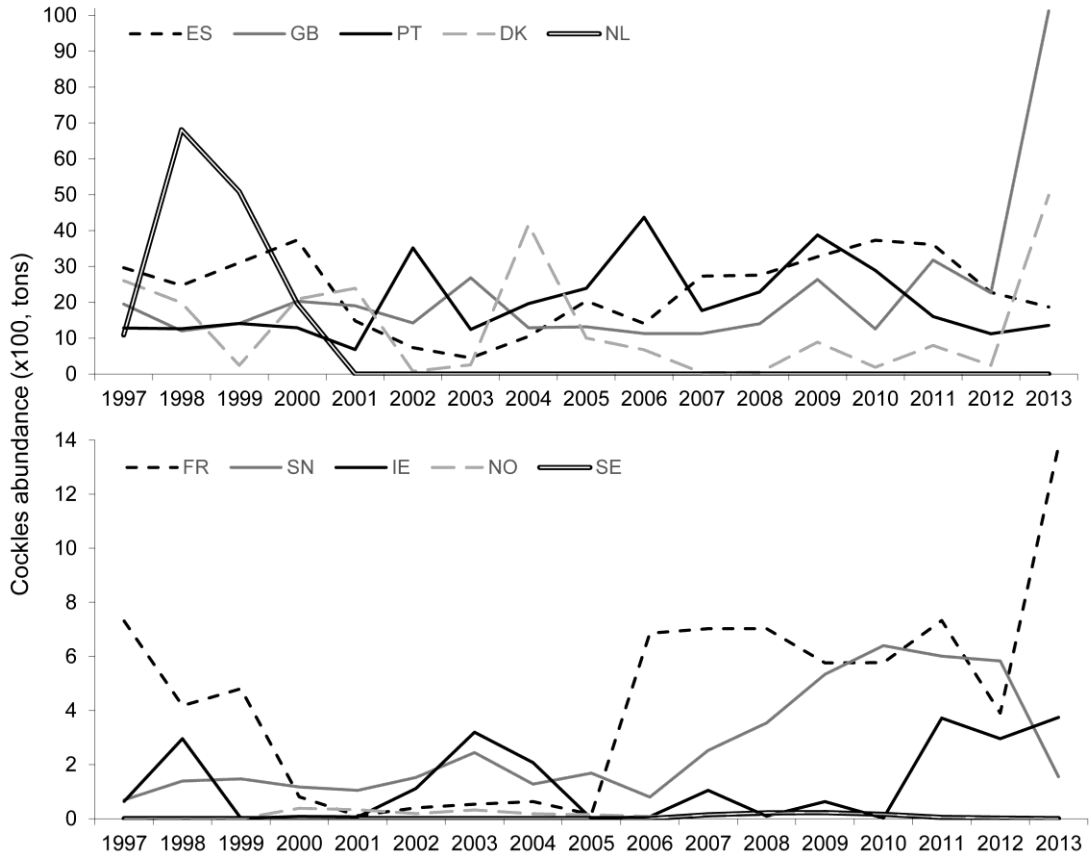

Figure 1 Total cockle, *Cerastoderma edule*, abundance (x100, tons) per year, for ten different countries (FAO, 2006-2018). Note different Y-axis scales.

Within these drivers is the parasitism. Parasitic fauna represents 40% of total known eukaryotic species (Dobson et al., 2008) being important elements of worldwide ecosystems. They can seriously interfere with host population performance (Curtis, 1995; de Montaudouin et al., 2014; Friesen et al., 2017; Gam et al., 2009; Jonsson & André, 1992) and change the composition and structure of natural animal communities (Poulin, 1999), although their precise contribution often remains neglected.

Trematodes are the most abundant and common macroparasites in coastal waters (Lauckner, 1983; Schmidt & Roberts, 2000). They display a complex life cycle with alternation of sexual and asexual generations and of parasitic and free-living stages. The typical life cycle of a trematode (Esch et al., 2002) includes (Figure 2): a) a ciliated and swimming larva, the **miracidium** (free-living stage) which penetrates the first intermediate host (a mollusk); b) this larva metamorphoses into a rather simple sac-like form, the **sporocyst** (parasitic stage). The sporocyst evolves in a more mature form of sporocyst (daughter-sporocyst) or in a developed form, with a mouth, the redia, depending on trematode species. In these sporocysts/rediae, new forms of larvae are

formed by asexual multiplication, the cercariae (free-living stage); c) the **cercariae** emerge from the first intermediate host and swim towards the second intermediate host that must be infected within the next 24 h after emission (Fried & Ponder, 2003; Pechenik & Fried, 1995); d) the cercariae infect the second intermediate host (invertebrate or vertebrate species) and transform into **metacercariae** (parasitic stage). The cycle will be achieved when the second intermediate host is predated by the final host, a vertebrate (parasitic stage); e) in the final host, the metacercariae transform into **adults** that will reproduce sexually, with emission of eggs, emitted in the environment with host faeces (Longshaw & Malham, 2013; Schmidt & Roberts, 2000); f) these **eggs** will hatch and liberate miracidium.

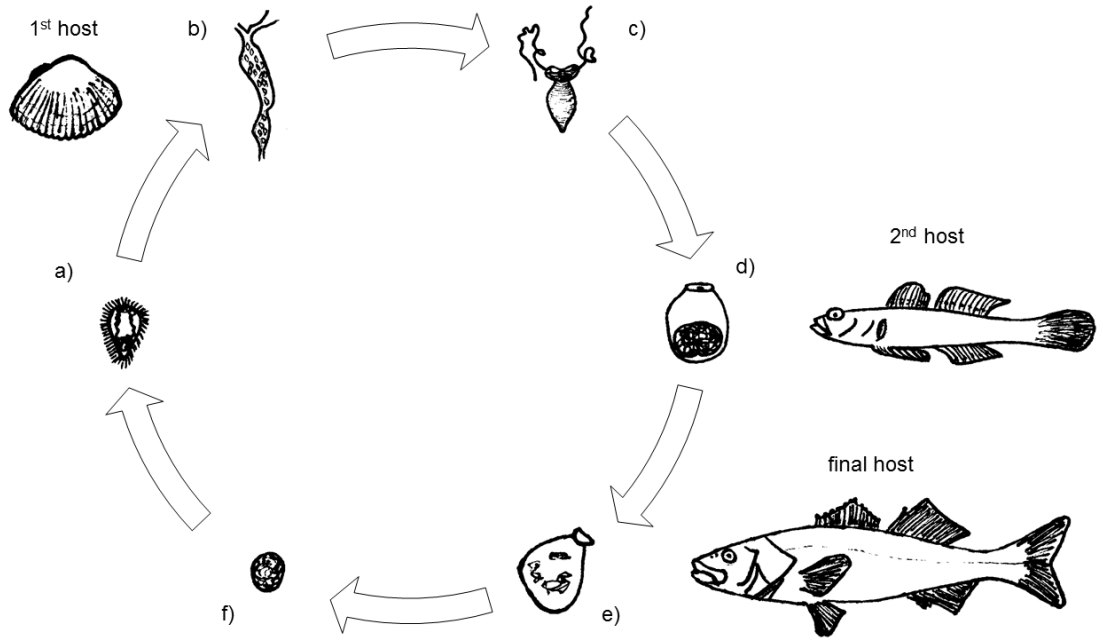

Figure 2 Schematic representation of a trematode life cycle taking *Bucephalus minimus* (trematode parasite) as an example: a) miracidium; b) sporocysts (developed in the first intermediate host – *Cerastoderma edule*); c) free-swimming cercaria; d) metacercaria (developed in the second intermediate host – *Pomastoschistus* spp.); e) adult stage (developed in the final host – *Dicentrarchus labrax*); f) egg.

Thus, during this complex life cycle, the parasite experiences totally different habitats, with different abiotic and biotic factors and consequent physiological adjustments that must be often made extremely rapidly (Schmidt & Roberts, 2000) in order to ensure the survival. Among these factors, that will affect parasite success and consequently its distribution pattern, temperature (de Montaudouin et al., 2016),

light:dark cycle (de Montaudouin et al., 2016), hydrodynamics (de Montaudouin et al., 1998), diversity of host species (Thieltges & Reise, 2007) and target host density (Mouritsen et al., 2003; Thieltges & Reise, 2007) are considered important drivers. The annual fluctuation of these parameters usually leads to a seasonal pattern with an optimal infection window occurring in the warmer season, at least for second intermediate host (de Montaudouin et al., 2016; Desclaux et al., 2004; Thieltges & Rick, 2006).

Molluscs are favourite hosts for trematodes both as first and second intermediate hosts (Lauckner, 1983). They get infected by free-living propagules through their feeding activity (grazing, suspension-feeding) and provide different tissular niches for parasites to settle (de Montaudouin et al., 2009). The complexity of the trematode life cycle described above, namely its multi-host nature, makes trematodes relevant ecosystem diversity and health indicators (Hechinger et al., 2006; Hudson et al., 2006). They have already been used to assess habitat stability over time, at the scale of several years (de Montaudouin et al., 2012b) and to detect global change effects through fish long-term monitoring (Dzikowski et al., 2003; Zander, 2005). On the other hand, at the individual level and by definition, the parasite exerts a negative impact on the host and can alter its basic biological functions (Babirat et al., 2004; Carballal et al., 2001). However, the impact of a parasite on a particular organism, or its pathogenicity, is species specific, depends on parasite prevalence (i.e. percentage of infected hosts) and/or abundance (i.e. number of parasite per potential host individual) and is also related to the parasitic stage.

As sporocyst (or redia), the parasitic stage (occurring in the first intermediate host) effects on host individual health are often severe due to strong interaction between parasite and vital host organs like gonads, digestive tract or gills (Dubois et al., 2009). Numerous studies described sporocyst impact on host reproduction (Carballal et al., 2001), growth (Bowers, 1969) and behaviour (Babirat et al., 2004). Nevertheless, measuring effects at the host population level remains difficult due to intrinsic low prevalence (< 5% of adults (Thieltges et al., 2008b), with some exceptions (Fredensborg et al., 2006)) or parasite prevalence gets high but this outbreak is generally correlated with a mass host population extinction (Jonsson & André, 1992; Thieltges, 2006) which makes difficult to collect infected hosts. Generally, as metacercariae (parasitic stage occurring in the second intermediate host), parasite usually displays high prevalence but, under standard environmental conditions, their pathogenicity is reported as low, with little interaction between parasite and host (e.g. de Montaudouin et al., 2012a; Wegeberg & Jensen, 1999). Occasionally, metacercariae abundance can reach relatively high values and disrupt bivalve basic

functions such as growth (de Montaudouin et al., 2014), burrowing capacity (Babirat et al., 2004), or even causing mortality (Desclaux et al., 2002, 2004; Gam et al., 2009; Marcogliese, 2004). For these reasons, it is important to recognize and predict processes that can enhance metacercariae abundance and lead to higher disease risk.

In the aquatic environment, abiotic and biotic drivers rarely occur isolated, with inhabiting organisms being exposed to a combination of factors, with a complex range of impacts (Sures, 2008). For example, predicted climate change (IPCC, 2014) is likely to impact many biotic interactions including host–parasite relationships which are particularly dependent on abiotic conditions (de Montaudouin et al., 2016). In fact, the negative effects of infectious diseases may become more severe in a global climate change scenario (Harvell et al., 2002). However, studies converging parasitology and climate change are still scarce and focused mainly on cercariae emergence and survival (MacLeod, 2017).

For Europe, by the end of the century, projections describe a higher frequency of heavy precipitation (Feyen & Dankers, 2009; Robins et al., 2016) with consequent impact on the salinity of the aquatic systems (Schmitt, 2008). Salinity plays an important role in defining structural and functional characteristics of aquatic biota (Little et al., 2017; Telesh & Khlebovich, 2010) and has been recognised as an important driver for parasitism and disease dynamics (Coffey et al., 2012; Messick et al., 1999). Some authors showed that cercariae emergence (from the first intermediate host) increase with increasing salinity (Lei & Poulin, 2011; Studer & Poulin, 2012), while other authors demonstrated an opposite trend (Koprivnikar & Poulin, 2009; Koprivnikar et al., 2014). By the end of this century, it is also expected an increase in the water temperature up to 4 °C (IPCC, 2014). Temperature is among the most pervasive and important physical factors in the environment of an organism and can have implications from molecular to biogeographical levels (Somero, 2011). Temperature effects on cercariae are well described, with authors demonstrating that an increase in temperature induces higher cercariae emergence until an optimum (Koprivnikar et al., 2014; Mouritsen, 2002; Studer et al., 2010; Thieltges & Rick, 2006) and higher cercariae infectivity (Studer et al., 2010; Thieltges & Rick, 2006) but lower cercariae survival (Mouritsen, 2002; Studer et al., 2010; Thieltges & Rick, 2006). Finally, CO_2 concentration in seawater is predicted to increase up to 1120 ppm (corresponding to 0.5 pH unit decrease) (Caldeira & Wickett, 2003). The specific effects of ocean acidification on host-parasite interactions are almost unknown although some evidences suggested significantly higher infection success in the environment subjected to reduced pH levels (Harland et al., 2016; Koprivnikar et al., 2010). Despite increasing efforts on parasitological studies over the last decades (Poulin et al., 2016),

parasites still play a discrete role in marine ecosystems and little is known about how parasites are influenced by environmental changes (Koprivnikar et al., 2010; Studer & Poulin, 2013).

In addition to climate change, pollution is present in aquatic ecosystems worldwide, particularly in areas of agricultural intensification and high human population density (de Sherbinin et al., 2007). The relationship between the bivalve contaminant body burden and the parasite infection is a complex question to address (Baudrimont & de Montaudouin, 2007, Baudrimont et al., 2006, Paul-Pont et al., 2010a, 2010c). Pollution can either favour or impair parasite infection success depending on a high number of interacting variables (Sures, 2008). Certain contaminants may increase parasite infection by excluding their natural predators, by reducing the resistance of their hosts, or by providing improved conditions for their intermediate hosts to live. For example, *Lymnaea truncatula* (gastropod) susceptibility to *Fasciola hepatica* miracidia (trematode) increases when exposed to detergent contamination (Abrous et al., 2001) and the prevalence of *Levinseniella byrdi* (trematode) in the amphipod *Orchestia grillus* increases when chronically exposed to nutrient enrichment (Johnson & Heard, 2017). On the other hand, contaminants can interfere with parasite transmission within their hosts or exert deleterious effects on intermediate hosts of the parasites with complex life cycles and thus, can reduce the parasite abundance. As an example, *L. truncatula* exposed to copper reduced *F. hepatica* infectivity (Rondelaud, 1995). Nevertheless, the available information concerning the effect of contaminants on host susceptibility to infection is still scarce, and especially regarding the combined impact of pathogen and contamination on host basic functions.

1.1.1. *Cerastoderma edule* as a host model

The edible cockle *Cerastoderma edule* (Bivalvia: Cardiidae) (Figure 3A) is an indigenous, infaunal suspension-feeder bivalve living in semi-sheltered marine systems along the north-eastern coast of the Atlantic Ocean, from the Barents Sea to Mauritania (Honkoop et al., 2008; Tebble, 1966) where it is often dominant. Cockles play a crucial role in the ecosystem being an important food source and a link within food webs (Rakotomalala et al., 2015). They are also responsible for several ecosystem services such as carbon storage and energy cycling (Morgan et al., 2013). In several regions, *C. edule* is an extensively exploited species with high economic value (Beukema & Dekker, 2006; Rowley et al., 2014). Cockles harvest has particular socio-economic relevance in Portugal (Pereira et al., 2014), representing 20% of total European captures in 2015 (FAO, 2006-2018) that corresponded to 5 tons captured and 4.5 million euros of revenue (INE, 2015).

From the biological point of view, it is a well-studied species (Malham et al., 2012). It lives buried in the sediment, its sexual maturity is usually reached when cockle shell length reaches 15–20 mm, although it may be as little as 12 mm in colder waters (Dutch Wadden Sea) (Cardoso et al., 2009; Morgan et al., 2013). Spawning can occur at temperatures around 13 °C (Boyden, 1971). The planktonic larval stage lasts 2–5 weeks (Creek, 1960), although it is much shorter in the hatchery, where food is abundant (Pronker et al., 2015). *C. edule* is considered as an euryhaline and an eurytherm species (Malham et al., 2012), i.e. an organism able to adapt to a wide range of salinities and temperatures, respectively. However, its optimal salinity is between 30 and 35 (Malham et al., 2012) and its thermal optimal levels of survival and overall functioning is between 20 and 23 °C (Verdelhos et al., 2015). Cockles are also worldwide identified as a good sentinel (Cheung et al., 2006; Karray et al., 2015a) and bioindicator (Cheggour et al., 2001; Freitas et al., 2012; Velez et al., 2016b) species. Furthermore, cockles are infected by several trematode species both as first and second intermediate host (de Montaudouin et al., 2009) and therefore, constitute a suitable model to study host-parasite interactions.

In the edible cockle distribution area, *Bucephalus minimus* is the most prevalent trematode species using cockles as first intermediate hosts, out of the four known species (de Montaudouin et al., 2009). Cockle infection by *B. minimus* starts on the gonad and digestive gland (Pina et al., 2009) ending up invading the entire organism (de Montaudouin et al., 2009). In the gonad, this parasite causes castration (Carballal et al., 2001). In the digestive gland, *B. minimus* causes starvation and autolysis of the digestive tract by consuming energy (Dubois et al., 2009). Regarding metacercariae

infection (Figure 3B, 3C), there are fourteen trematode species infecting cockles as second intermediate host along its north-eastern Atlantic distributional range (de Montaudouin et al., 2009).

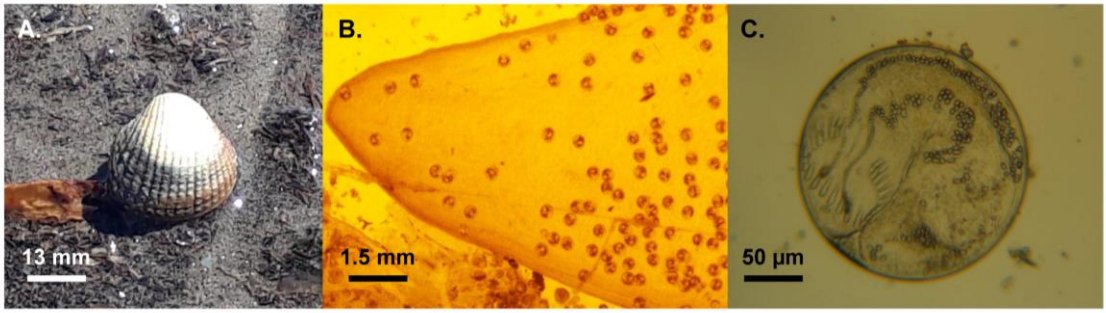

Figure 3 *Cerastoderma edule* – the edible cockle: **A.** Specimen in its natural habitat, but extracted from the sediment; **B.** Cockle foot infected by metacercariae of *Himasthla elongata*; **C.** *H. quissetensis* metacercaria.

1.1.2. *Donax trunculus* as a host model

The wedge clam, *Donax trunculus* (Bivalvia: Donacidae) (Figure 4A), is a warm-temperate species distributed from the Atlantic coast of France to Senegal (Tebble, 1966) and the shallow bottoms of the Black, Mediterranean (Bayed & Guillou, 1985) and Marmara (Deval, 2009) seas. 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. In 2015, wedge clams national fisheries represented 44% of total European captures (FAO, 2006-2018) with 308 tons and approx. 875 thousand euros of return (INE, 2015).

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). Its sexual maturity is usually reached in the first year of life, when clams shell length reaches 13–21 mm (Gaspar et al., 1999) although it may be as little as 9 mm in the coast of Huelva, Spain (Delgado & Silva, 2018). Spawning peaks occur in March and May-August periods (Gaspar et al., 1999) but this species presents a continuous spawning activity and oocytes emission (Delgado & Silva, 2018). In its habitat, *D. trunculus* population densities are highly variable (Delgado et al., 2017; Fishelson et al., 1999; Gaspar et al., 1999). 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 alteration (de la Huz et al., 2002; La Valle et al., 2011) and parasitism (Delgado & Silva, 2018).

Concerning parasitism, among trematode species infecting the wedge clams, *Bacciger bacciger* is the most prevalent in European waters (de Montaudouin et al., 2014; Ramón et al., 1999) using *D. trunculus* as first intermediate host (Palombi, 1934a) (Figure 4B – 4D). Despite some 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 to the population decline. Regarding metacercariae infection, records of trematode species infecting wedge clams as second intermediate host are rare (e.g. Carella et al., 2013).

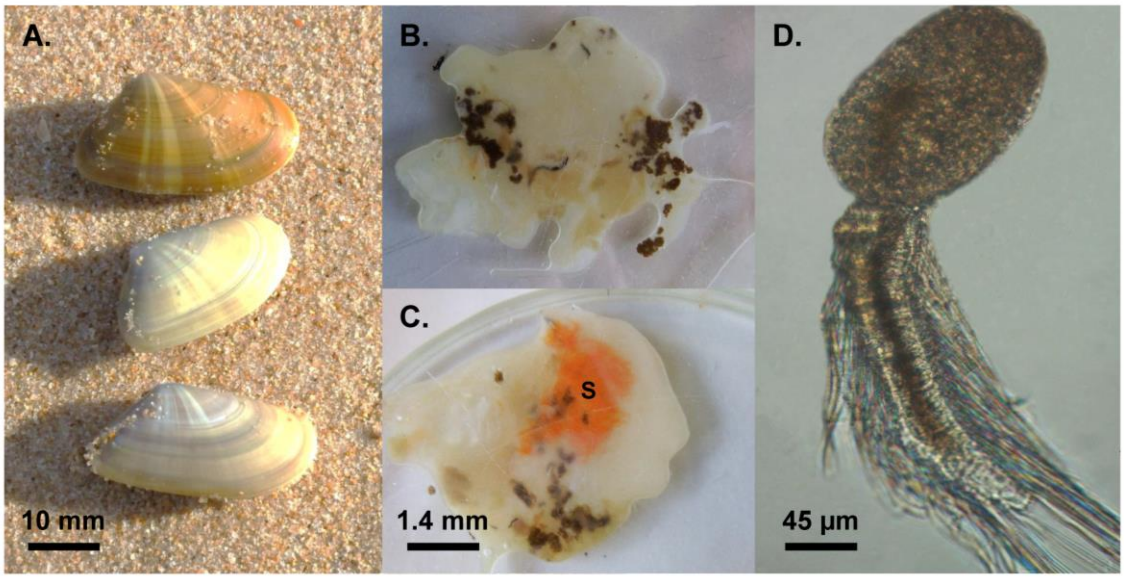

Figure 4 *Donax trunculus* – the wedge clam: **A.** Specimens in their natural habitat; **B.** non-parasitized clam tissue; **C.** clam flesh infected by *Bacciger bacciger* sporocysts (S); **D.** *B. bacciger* cercaria.

1.2. Thesis objectives

The cockle *Cerastoderma edule* and the wedge clam *Donax trunculus* are ecologically and economically important species in coastal environments such as the Arcachon Bay in France (*C. edule)*, the Ria de Aveiro (*C. edule*) and the Ria Formosa (*D. trunculus*) in Portugal, the study areas of this work. Among factors that can control these species population dynamics, trematode parasites may be dominant with possible deleterious effects but, however, remain often neglected. The main goal of the present work was to identify, quantify and characterize trematode communities infecting bivalves from the French and Portuguese coastal systems, recognizing some variables that can regulate these parasites population dynamics and assessing the effects of trematodes infection on their bivalve hosts in an environment that is also characterized by other sources of stress.

The thesis was divided into three core chapters with the following specific objectives:

- Chapter 2 – COCKLE POPULATION DYNAMICS: to evaluate the relationship between temperature and recruitment timing and the reciprocal effects of recruitment on adult biomass;
- Chapter 3 - BIVALVES AS FIRST INTERMEDIATE HOST: to review the literature to date and to analyse a long-term dataset of a host-parasite system phenology (3.1) and identify the consequences of heavy parasite infection in terms of bivalves individual response (3.2, 3.3);
- Chapter 4 - BIVALVES AS SECOND INTERMEDIATE HOST: to describe the spatio-temporal variability of the trematode community structure at the scale of the Ria de Aveiro coastal lagoon (Portugal) hierarchizing some environmental drivers of infection (4.1); to evaluate the effect of bivalve host density on individual parasite infection (4.2); to evaluate the susceptibility of bivalves to parasites infection when challenged by climate change related factors (4.3) and contamination (4.4).

CHAPTER 2. COCKLE POPULATION DYNAMICS

This chapter is published as:

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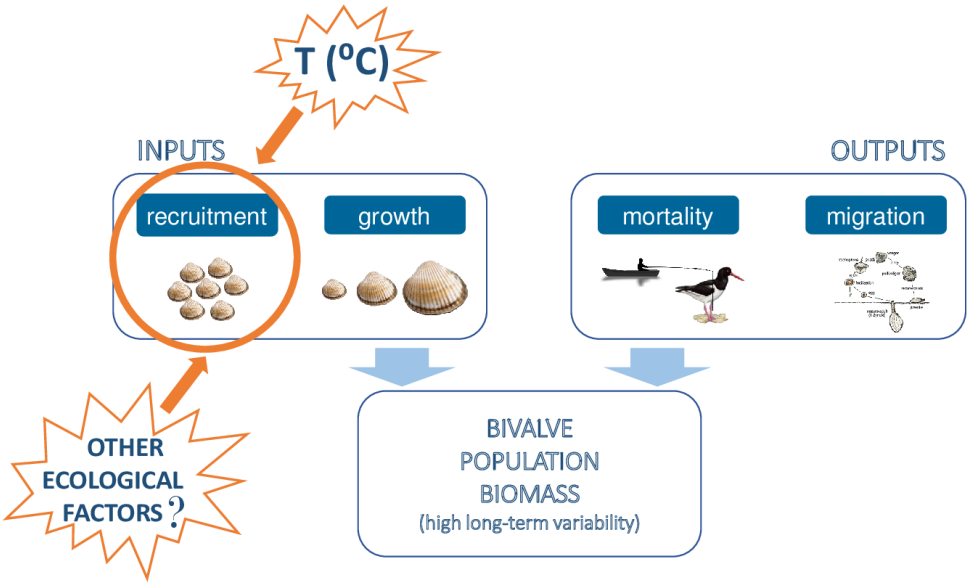
2. Cockle population dynamics: recruitment predicts adult biomass, not the inverse

GRAPHICAL ABSTRACT



2.1. Introduction

Bivalves are an essential component of marine ecosystems, playing an important role in community maintenance. The abundance of an exploited bivalve population depends on the balance between inputs (reproduction/recruitment and growth) and outputs (mortality and fishery removals). Excluding mass mortality events (e.g. diseases, overfishing, pollution (Burdon et al., 2014)), reproductive processes (including larval settlement and recruitment) are crucial for population maintenance (Dang et al., 2010).

One hypothesis that relates bivalve stock size to recruitment suggests that there is a reciprocal relationship between them: 1) **recruitment is needed for stock**. The early stages of recruitment and post-settlement mortality can determine relative abundances of adults. This postulate is true if mortality rate is constant among years, which is rarely the case (Roegner, 1991). However, while studying three bivalves in the Wadden Sea, Beukema et al. (2010) showed that variability in later recruitment explained a significant part of the variability observed in biomass values, particularly after years with non-exceptional mortality rates. Stock prediction from recruitment success is mandatory, for example to evaluate the trophic resource within the global food web, including migrating birds (Beukema et al., 2010), but also to manage fishing activity. The alternative hypothesis to the first is 2) **stock is needed for recruitment**,

through reproduction (Beukema et al., 2010; Honkoop & van der Meer, 1998). Notwithstanding, regarding reproduction, bivalves are r-strategists. They tend to overproduce offspring, and so a small number of adults could be sufficient for stock sustainability. In addition, reproduction is influenced by many biotic factors as well as temperature, which is often related to several reproductive characteristics such as gonad maturity or spawning season (Boyden, 1971; Honkoop & van der Meer, 1998; Morgan et al., 2013). Previous studies observed that recruitment was generally higher after cold rather than mild preceding winters. After a cold winter cockles settle in an environment with less predation because crustaceans are less abundant on the tidal flats. Strasser et al. (2003) suggested that the higher recruitment in the 1970s and 1980s was related to higher frequency and number of severe winters compared to the 1990s. However, there is still no conclusive explanation for this bivalve recruitment variability and its relationship to environmental parameters, mainly because these recruitment analyses have a high intrinsic variability that demands long-term studies, with a suggested minimum of 10 years of monitoring to stabilize the estimation of annual means (Beukema & Dekker, 2006).

In the present study, the relationship between temperature and recruitment timing and the reciprocal relationship between recruitment and adult stock biomass was investigated using a 17-year data base (1997–2014) on an unexploited *Cerastoderma edule* population from a national nature reserve in Arcachon bay, France. In the life cycle of many bentho-planktonic organisms, settlement is a crucial event. It is defined as the passage from a pelagic to a benthic way of life (Roegner, 1991). In the present chapter, the term recruitment will be used, which implies a certain lapse of time following settlement (Rodriguez et al., 1993). Following most other studies, this lapse of time was operationally defined as the one necessary for settlers to be retained on a 1-mm mesh sieve.

2.2. Material and methods

2.2.1. Study area

The sampling station was located in Banc d'Arguin (44.60°N, 1.25°W), Arcachon Bay, France (Figure 5). Arcachon bay is a 156-km^2 macrotidal lagoon situated on the Atlantic southwest coast of France. This lagoon opens to the Atlantic Ocean through a wide (24 km^2) channel. The junction with the Atlantic is characterized by the presence of several sand banks, one of them (Arguin) exposed also at high tide. Cockles (*Cerastoderma edule*) are distributed along the semi-sheltered, intertidal part

of this bank. The sediment is largely dominated by medium sands (median grain size = 350 µm, de Montaudouin et al. (2000)) with organic matter content not exceeding 2% (Baudrimont et al., 2003). Salinity is high (32–35) year round, while mean surface water temperature fluctuates between 9.5 °C in winter and 21.1 °C in summer (de Montaudouin et al., 2000). Since 1972, Arguin (25 km^2) has been a national reserve which is largely protected from anthropogenic activity. Sampling was performed under strict authorization.

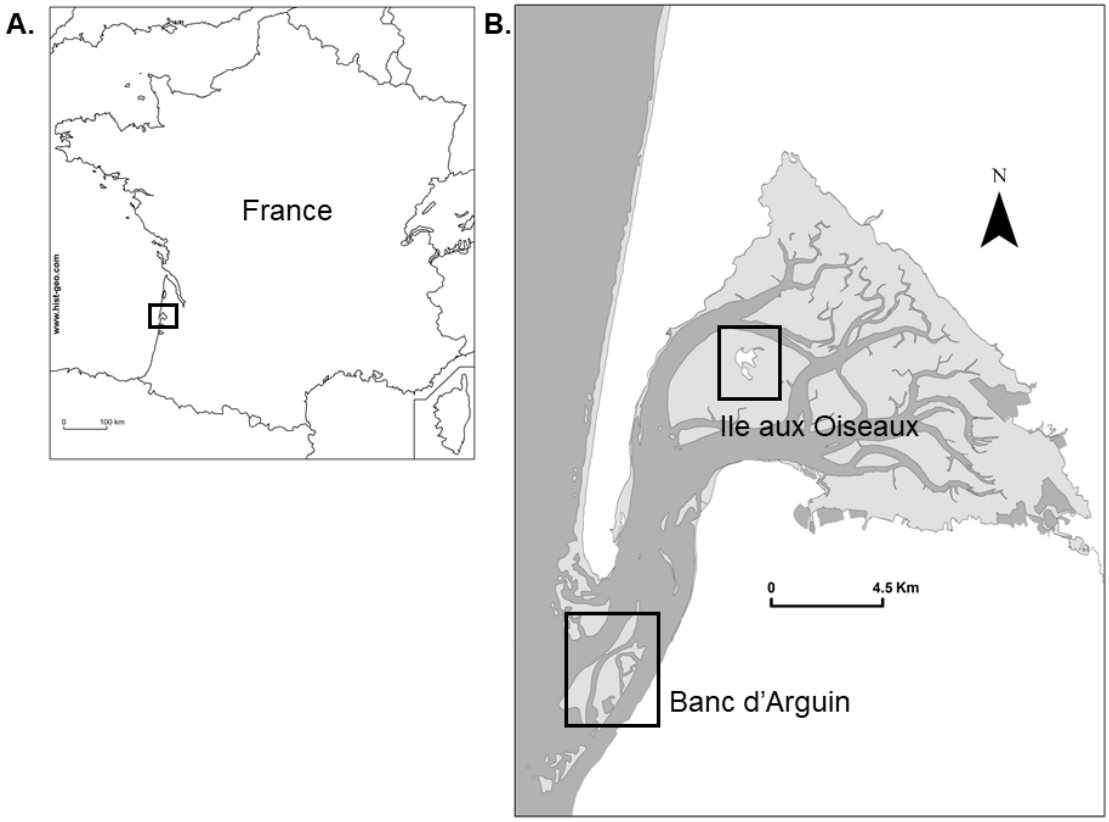


Figure 5 Study area. The Arcachon bay (Southwest France (A)) indicating the Banc d'Arguin and Ile aux Oiseaux location (B).

2.2.2. Sampling strategy

From October 1997–December 2014 (17 years and 3 months), cockles were collected monthly by sampling 6 quadrats (0.25 m^2 each), aligned along a 100 m parallel to the water transect, and by sieving the sediment over a 1-mm mesh. Cockle shell length (SL) was measured to the nearest mm with callipers.

Throughout the study, a temperature probe installed on the sediment surface where the cockles were sampled measured temperature every hour (HOBO® Water

Temp Pro v2 – U22-001). Mean temperature was calculated between consecutive sampling occasions.

2.2.3. Cohort analysis

The sampling date (monthly) length-frequency matrix was transformed into ind m^{-2} and analysed using the computer software package FISAT II (FAO – ICLARM stock assessment tool) (Gayanilo et al., 2005).

Modal class Progression Analysis (MPA) – Bhattacharya method (1967) was used to discriminate among the cohorts present in the dataset. This subroutine identifies cohorts by decomposing the polymodal size distribution into their normal distribution components. Cohorts were assumed to be single when the separation index was > 2.

Recruitment was defined as the peak in density of juveniles retained on a 1-mm sieve (acknowledging that some of these organisms had already spent several weeks on the bottom). The lifespan of a cohort was considered to begin at this recruitment peak and end when density declined to < 6 cockles $transect^{-1}$ (i.e. to a mean of one cockle $quadrat^{-1}$).

2.2.4. Growth analysis

The time series of the mean SL (± standard deviation (SD)) for each modal group (cohort) previously calculated with FISAT II was then linked and transformed into growth increment data using the same software. The Appeldoorn method (Gayanilo et al., 2005) was applied to estimate specific growth parameters L_∞ and K of the von Bertalanffy Growth Function (VBGF), to characterize each cohort:

$$VBGF: Lt = L_\infty \left[1 - e^{-K(t)}\right]$$

Where L_∞ is the asymptotic SL (mm) and K is the growth coefficient (y^{-1}).

The negative correlation between L_∞ and K invalidates comparisons based on individual parameters and, as a result, the comparison of bivalve population growth is better fitted by the growth performance index (ϕ') (Pauly & Munro, 1984) calculated for each cohort:

$$\Phi' = 2 \times log_{10}(L_\infty) + log_{10}(K)$$

Using all cockle cohorts, VBGF parameters were also calculated using a time-integrated average global cohort.

2.2.5. Biomass and production data treatment

Weight of the cockles present on each sampling date and within each discriminated cohort was calculated using the SL/weight relationship previously determined for the same study area (Gam et al., 2010) and described as:

$$Log_{10}\, W = 3.37 \times Log_{10}\, L - 5.54 \qquad (R^2 = 0.89)$$

where W is the cockle flesh dry weight (g DW), L is the SL (mm) and R^2 is the coefficient of determination.

Cockle weight data were then used to calculate biomass and production for each cohort during its lifespan, and also per population and per year. Biomass was determined by multiplying the individual weight by the density, and production (P) was calculated using an incremental summation method (Crisp, 1984), used for populations with non-continuous recruitment and distinguishable year-classes and described as:

$$P = \sum_{t=0}^{t=n} \left[\frac{N_t + N_{t+1}}{2} \times (w_{t+1} - w_t) \right]$$

where, N_t is the cockle density at time t and w_t is the mean individual dry flesh weight at time t. Annual productivity ratios (P/B) were also calculated.

2.2.6. Statistical analysis

Non-parametric Spearman correlations were performed to check the stock-recruit relationships: 1) individual growth performance vs. recruit density (peak) and individual growth performance vs. cohort lifespan, in order to understand the relationship between the individual growth performance and recruitment success; and 2) recruit density (peak) vs. cohort lifespan and recruit density (peak) vs. cohort production, to test if the density of recruits (peak) can predict the cohort 'strength' (lifespan, production). The same correlation test was used to analyse circumstances needed for a successful cohort: spawning adult biomass (mean biomass of adults present 3 months before the recruitment period) vs. recruit density (peak) and biomass of the adults present at the time of recruitment (mean biomass of adults present a month before, same month and a month after the recruitment peak, considered as the

recruitment period) vs. recruit density (peak), to evaluate whether stock influences the intensity of recruitment and to detect intraspecific competition, respectively. One-way ANOVAs (after log transformation and a Cochran homogeneity test) were used to evaluate the effect of recruitment month (May or June) on 1) recruit density (peak) and 2) cohort lifespan. One way ANOVAs (after log transformation and a Cochran homogeneity test) were also used to compare mean monthly temperature (from October to May, before recruitment) between years with early recruitment (May) and years with later recruitment (June). Analyses were performed with STATISTICA (StatSoft) software.

2.3. Results

2.3.1. Cohort characterization

During the 17-y study, cockle density on each sampling date ranged 1–2,390 ind m^{-2} (global average = 167 ind m^{-2}) and cockle total biomass was 0.0–53.0 g DW m^{-2} (global average = 12.4 g DW m^{-2}) (Figure 6). From these density data, as described in the 2.2.3 section, 19 cohorts were recognized and the mean SL (±SD) of cockles was depicted for each month, within each cohort. Cohort recruitment peaks ranged between 95 (in 2008) and 1,869 (in 2000) ind m^{-2} (Figure 7A). For all 19 cohorts, 10 months was the mean lifespan, which ranged 1–40 months (Figure 7A, 7B).

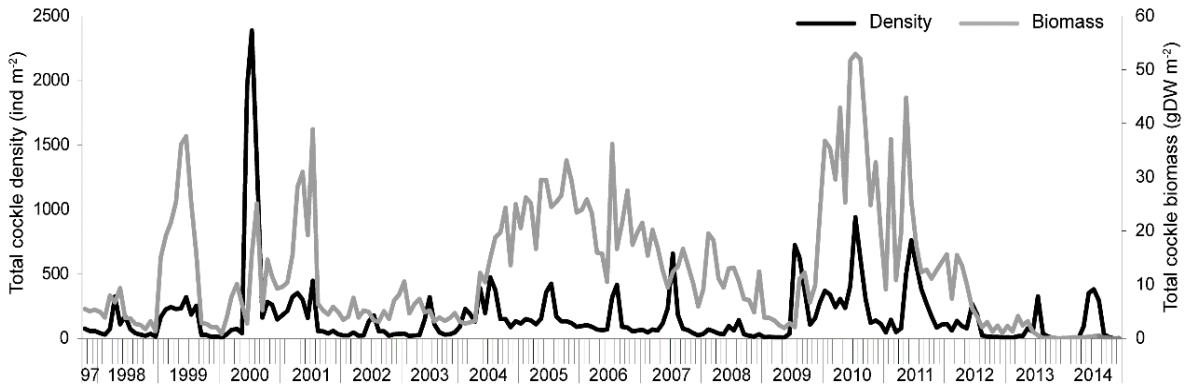


Figure 6 Total cockle density (ind m^{-2}) and total cockle biomass (g DW m^{-2}), October 1997 – December 2014.

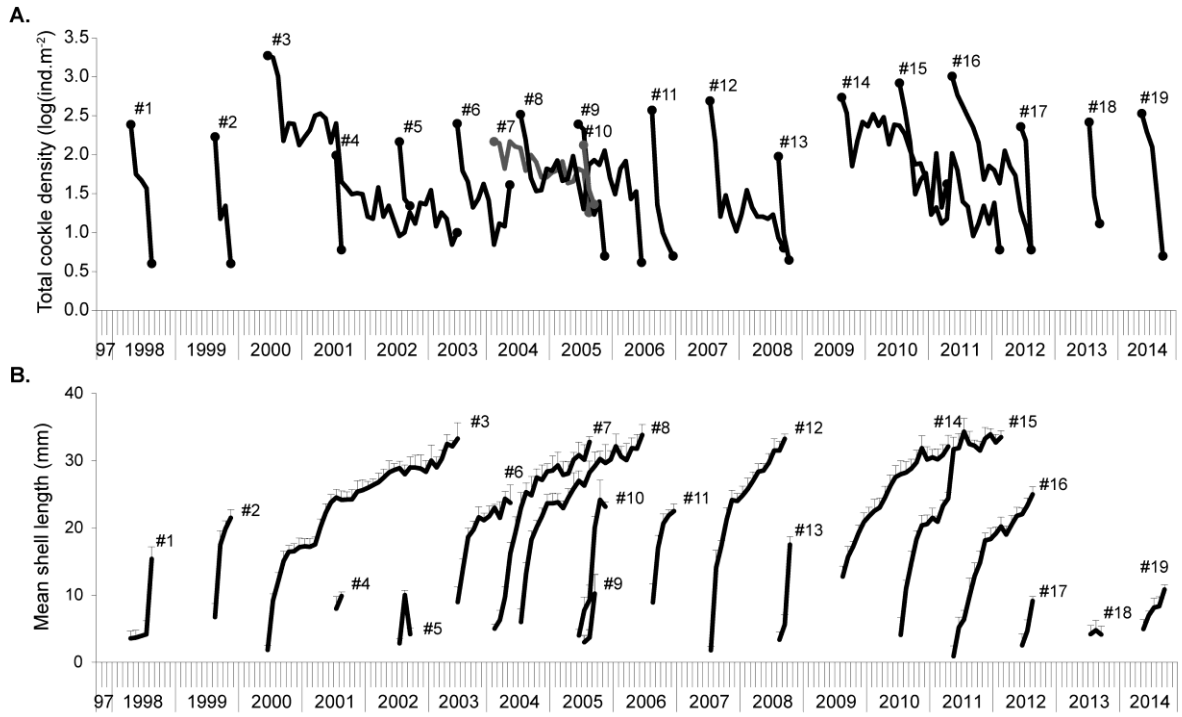


Figure 7 *Cerastoderma edule* cohorts represented as: **A.** total cockle density (log(ind m^{-2})) per month within each cohort; **B.** mean cockle shell length (mm) per month within each cohort.

2.3.2. Recruitment and sediment temperature

Most recruitment peaks occurred in May or June (37% and 47%, respectively, 84% of the 19 peaks) (Figure 8) with similar mean SL on the sampling dates, i.e. 4.5 mm (recruits retained on a 1-mm mesh ~ 2 w after settling).

In 2000, high recruitment occurred in June (1,869 ind m^{-2}) in the context of an exceptionally high temperature increase (7.9 °C) between March (11.8 °C) and June (19.7 °C), while the mean increase for that period was 4.4 °C (from 12.3 to 16.7 °C). Thus this year was not included in the following analyses but will be discussed.

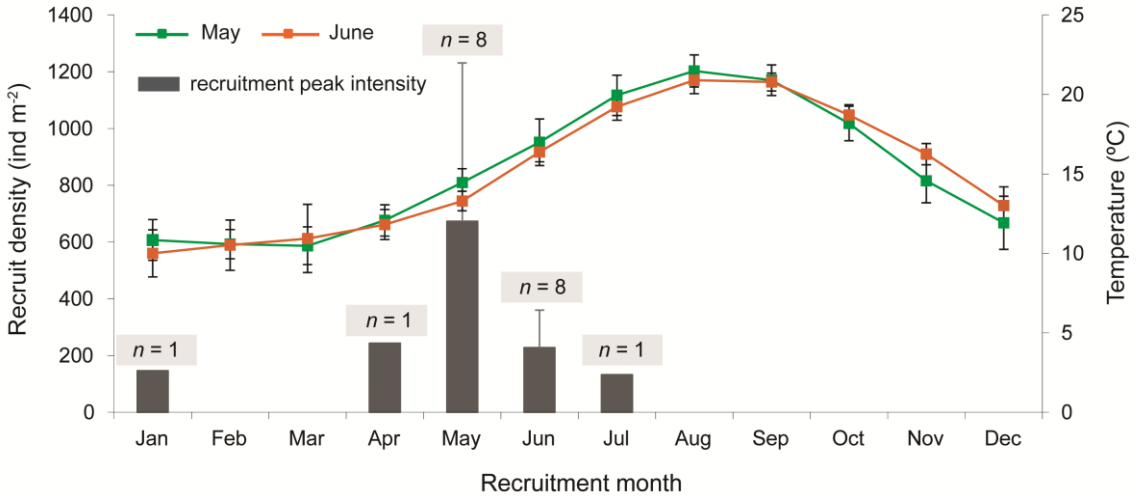


Figure 8 Mean monthly temperature (± standard deviation (SD)) for years in which cockle recruitment occurred in May or June. Mean recruitment density (ind m^{-2}) and respective SD bars (when $n > 1$) according to the month of recruitment.

When comparing mean monthly temperatures (from October to May before recruitment) between years with early recruitment (May) and years with later recruitment (June), there were no significant differences except in May (one-way ANOVA: $F = 11.4$, $p = 0.008$). Indeed, May temperature was higher in years with early recruitment (14.6 °C) than years with later recruitment (13.4 °C) (Figure 8). In order to compare our data with studies in the Wadden Sea (Beukema et al., 2001), we compared average January-March temperatures between years with high recruitment and years with low recruitment and no relationship was found ($p = 0.49$). Recruitment in June resulted in 2.2 times lower recruit density (228 vs. 502 ind m^{-2}) than an earlier recruitment in May (one-way ANOVA: $F = 5.4$, $p = 0.04$). Cohorts recruited in May had a longer lifespan (x 2.6) than cohorts recruited in June (one-way ANOVA: $F = 4.5$, $p = 0.05$). As an example, the 2011 cohort recruited in May (1,013 ind m^{-2}) and lasted 16 months while the 2012 cohort recruited in June (228 ind m^{-2}) and survived for 2 months (Figure 9). Recruitment did not occur before sediment temperature reached 14 °C.

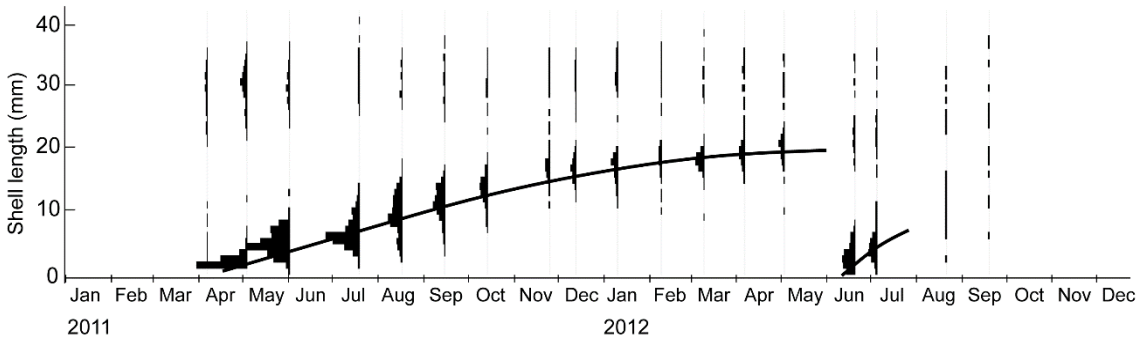


Figure 9 Cockles shell length histograms showing a successful cohort (2011) and an unsuccessful cohort (2012).

2.3.3. Growth

Growth parameters L_∞ and K, calculated for each cohort, varied from 25.7–42.1 mm and 0.8–3.9 y^{-1}, respectively (Table 1). Although, cohort growth performance indices (ϕ') were very stable (3.0–3.5) (Table 1), they were negatively correlated with the timing of the recruitment peak (r = - 0.68, $p \leq 0.05$) and cohort lifespan (r = - 0.83, $p \leq 0.05$). Data are not available for the short-lived cohorts because the Appeldoorn method could not be applied accurately.

VBGF parameters from a time-integrated average calculation based on all cohorts were L_∞ = 36 mm and K = 0.64 y^{-1} (ϕ' = 2.919).

Table 1 Appeldoorn method results for cockle cohort (time-integrated average): von Bertalanffy growth function parameters, asymptotic shell length (L_∞, mm), growth coefficient (K, y^{-1}) and respective growth performance index (ϕ').

Cohort	Year	L_∞	K	ϕ'
1	1998	28.3	1.45	3.064
2	1999	28.6	2.38	3.289
3	2000	36.0	0.84	3.037
4	2001	—	—	—
5	2002	—	—	—
6	2003	25.7	3.12	3.313
7	2004	34.3	1.66	3.291
8	2004	37.6	0.93	3.119
9	2005	35.2	2.24	3.442
10	2005	—	—	—
11	2006	37.8	1.83	3.418
12	2007	40.2	1.47	3.376
13	2008	38.9	1.36	3.314
14	2009	32.3	1.66	3.239
15	2010	35.1	1.60	3.294
16	2011	42.1	0.77	3.136
17	2012	27.7	3.88	3.473
18	2013	—	—	—
19	2014	—	—	—
Global		36.0	0.64	2.919

2.3.4. Recruitment, production and stock

As previously specified, the atypical year 2000 was not considered. Recruit peak density predicted 34% of cohort lifespan variation (p = 0.01) and 18% of cohort production during its lifespan (but p = 0.07) (Figure 10)**.** When recruit density was < 147 ind m^{-2}, cohort lifespan did not exceed 3–4 months (i.e. did not last beyond the first summer) and cohort production was < 7.9 g DW m^{-2}. On the other hand, when recruitment density was > 500 ind m^{-2}, the cohort lifespan was always > 1 y with a mean cohort production of 21.4 g DW m^{-2} (knowing that the maximum recorded value was 58.3 g DW m^{-2} for the successful cohort settling in 2009 and ending in 2011). There was a maximum of 3 years in a row of recruitment success (2009, 2010 and 2011 period), and 3 years in a row of recruitment failure (2012, 2013 and 2014 period). Annual cockle population production fluctuated between 0.0 (in 2013) and 46.9 (in 2010) g DW m^{-2} and annual productivity between 0.0 (in 2013) and 3.4 (in 2014) y^{-1} (Table 2).

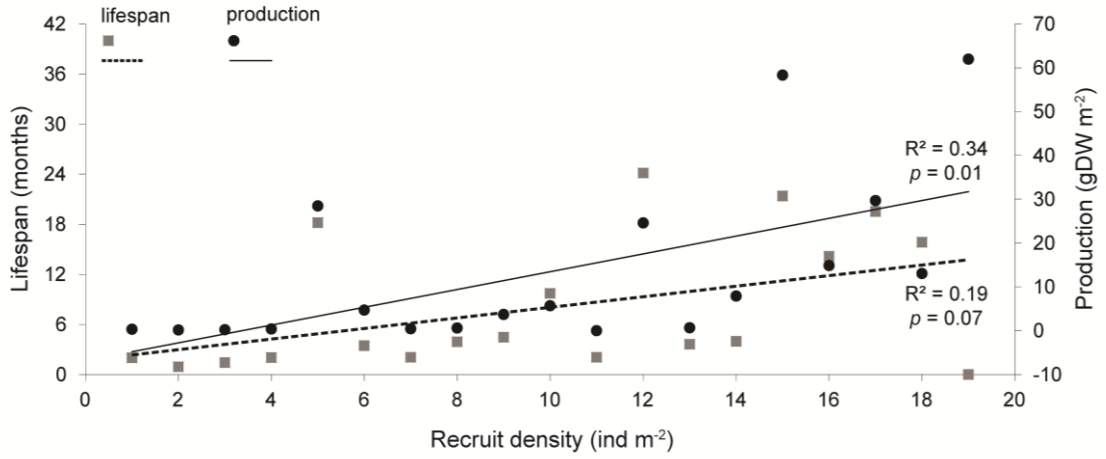


Figure 10 Lifespan (months) and production (g DW m^{-2}) of each cockle cohort with respect to recruit density (ind m^{-2}), with indication of coeficiente of determination.

Table 2 Annual cockle production (*P*, g DW m^{-2}), mean monthly biomass (*B*, g DW m^{-2}) and productivity (*P/B*, y^{-1}) for each cohort.

Year	*P*	*B*	*P/B*
1998	0.64	6.46	0.10
1999	4.85	14.68	0.33
2000	32.13	10.73	3.00
2001	26.85	13.96	1.92
2002	1.98	5.69	0.35
2003	8.16	4.43	1.84
2004	32.44	16.01	2.03
2005	20.50	26.08	0.79
2006	13.82	20.13	0.69
2007	12.19	13.34	0.91
2008	3.03	9.61	0.32
2009	31.89	12.34	2.58
2010	46.93	34.77	1.35
2011	26.76	17.06	1.57
2012	3.85	5.19	0.74
2013	0.02	1.03	0.02
2014	0.70	0.21	3.39

No correlation was found between spawning adult biomass (0.0–31.7 g DW m^{-2}) and recruit density (95–1,013 ind m^{-2}) ($R^2 = 2^{-5}$, $p > 0.05$, not illustrated), nor between adult biomass at the time of recruitment (0.0–48.0 g DW m^{-2}) and recruit density (R^2 = 0.03, $p > 0.05$, not illustrated).

2.4. Discussion

The present study showed that cockle density and biomass were highly variable from year to year, as already demonstrated by several other authors (e.g. Dörjes et al. (1986) in Germany and Möller (1986) in Sweden). Similarly, recruitment success among the cohorts over the 17-y study period was also variable. Cohort lifespans fluctuated from complete failure (1 month) to relatively successful (40 months). The few *C. edule* studies that provide cohort lifespan information give a wide range of values, from relatively short (3–11 months in Sweden (Möller & Rosenberg, 1983)) to relatively long (23 and 26 months in France and Morocco, respectively (Gam et al., 2009)), or even to extremely long (13 y recorded in the Barents Sea (Genelt-Yanovskiy et al., 2010)).

Our study was based on recruits and not settlers, meaning that cockles observed in the recruitment peak were already about two weeks post-settlement and had been affected by processes shaping cohort structure. In our long-term study, recruitment was generally unimodal, occurring once a year, in May or June. For comparison, some short-term studies recorded different cockle juvenile peaks from south to north along the eastern Atlantic coast: April–May in Merja Zerga, Morocco (Gam et al., 2009, 2010); April–July in Palmones, Spain (Guevara & Niell, 1989); June–July at Arcachon Bay, France (Gam et al., 2009, 2010). In Sweden, a maximum mean spat abundance (using a 200-µm mesh) was recorded from mid-June until mid-July (Möller & Rosenberg, 1983). These results show evidence of a progressively later recruitment going from south to north, suggesting a relationship between temperature (and perhaps food availability) to recruitment timing.

In fact, several previous studies have pointed out the importance of temperature to the cockle spawning season and consequent recruitment success, namely to temperature in the preceding winter (Beukema & Dekker, 2005, 2014; Beukema et al., 2001; Morgan et al., 2013). These researchers hypothesised that extremely cold winters promote a mismatch between larval settlement and predator arrival. However, this hypothesis was formulated mainly for Wadden Sea studies, an intertidal habitat that is part of the North Sea, which has frequent severe winters (mean water temperature < 2 °C (Beukema et al., 2010)). The present study brings new insights to cockle recruitment in the more southern part of their distribution, corresponding to conditions with relatively milder winters within the temperate zone.

Recruitment is key in determining cohort strength (lifespan) and consequently the level of annual production. Upstream, temperature is an important trigger of recruitment efficiency (Beukema et al., 2001, 2010). On the other hand, Strasser et al.

(2003) showed that temperature alone does not explain recruitment variability since differences have been observed at the ecosystem scale where the climate does not vary over the geographic extent. The present study showed that winter temperature had no effect on cockle recruitment and set 14 °C as the sediment temperature threshold for recruitment, consistent with a threshold for gamete discharge of 13 °C (Boyden, 1971). For all investigated years, there was no difference in temperature prior to recruitment until April. Then, checking all years, the present findings showed that this 14 °C temperature can be reached either in May or in June. But, the highest recruitment was observed when recruitment occurred earlier, in May rather than in June. Recruitment in May could coincide with the best conditions for newly-settled cockles (e.g. more and better quality food, fewer predators). Although it is not possible to extrapolate from a single year, it is relevant that the atypical year of 2000 (with an exceptional temperature increase March–June) was the best in terms of recruit density and cohort lifespan, in a year when the temperature increase between winter and spring was twice as high as in other years, despite late recruitment (June).

Von Bertalanffy Growth Function (VBGF) parameters varied among cohorts, but most of the variation was due to truncated cohorts (i.e. short-lived cohorts). Average values of L_∞ and K, calculated with all cohorts, were similar to the values from the two single cohorts of 2000 and 2004, which are considered complete in this Banc d'Arguin ecosystem (i.e. lifespan ≥ 2 years, with observable asymptotic length). The relatively short lifespan of cockle cohorts in Banc d'Arguin, even when recruitment was high, is related to large-scale movements of sand in this very dynamic oceanic system (Gam et al., 2010). Moving sand banks either cover the cockle population, or push (and gather) cockles in certain areas where consequently adult density (ind m^{-2}) increases. Sudden increases in cockle density, which usually occur in winter, prevent reliable mortality assessment. Cohort shell growth performance was stable over the whole study period but was negatively correlated with recruitment intensity and cohort lifespan. First, the possibility that cockle shell growth performance is a result of intraspecific competition for space and food can be excluded because the biomass found in the present study was low (maximum = 53.0 g DW m^{-2}). Therefore, this finding may be explained by previous evidence that cockle inter-annual fluctuations in recruitment in Brittany were also related to parental condition index (Guillou & Tartu, 1992). In other words, cockles under adverse environmental conditions were strongly limited in growth potential and to adapt to this, they tended to reduce reproductive effort in order to increase individual growth (Iglesias & Navarro, 1991). Therefore, in years characterized by less favourable recruitment conditions, cockles had higher individual growth which would assist population recovery (Ducrotoy et al., 1991).

For the present long-term data, variability in recruitment had a “one-way” relationship with stock size. Cohorts were short-lived (on average 1.7 months) if recruit density was < 150 ind m^{-2}, and its production was < 10 g DW m^{-2}. With 150–500 recruits m^{-2}, predicting cohort lifespan was difficult. For example, 328 recruits m^{-2} in 2004 corresponded to a 24-month lifespan while in 2014, a peak of 337 recruits m^{-2} corresponded to 4-month lifespan. Conversely, a recruit peak density > 500 ind m^{-2} allowed cockles to live more than a year (and to reproduce), and to significantly contribute to production (20–40 g DW m^{-2}). As an example, the year 2010 had the highest production (46.9 g DW m^{-2}) due to high recruitment in the same year (recruit peak = 832 ind m^{-2}). These results are certainly related to mortality rates which are relatively independent of recruitment rates (Beukema et al., 2010), allowing cohorts with higher recruitment to survive longer. Conversely, present results did not provide evidence of a reciprocal relationship, i.e. from stock to recruitment.

Intraspecific competition in *C. edule* was highlighted by previous authors (de Montaudouin & Bachelet, 1996; Genelt-Yanovskiy et al., 2010) who showed that the abundance of newly–settled juveniles was attenuated in the presence of a high density of conspecific adults. Adults compete for space and food but there is also a risk of adult cockles inhaling their own larvae. Signs of this competition were found in the presence of 400 adults m^{-2} (e.g. André & Rosenberg, 1991), or at high adult biomass, e.g. 70 gDW m^{-2} (de Montaudouin & Bachelet, 1996). In our study, these density and biomass thresholds were never attained, indicating there was no negative (competition) or positive (reproduction) feed-back from the adult presence.

Conclusion

These long-term observations showed that the sustainability of a cockle population is recruitment-success dependent. The direct relationship of cockle recruitment to temperature is less obvious than expected. In some bivalve species like *Macoma balthica*, global warming upsets the evolved reproduction strategy to tune its reproduction to the most optimal environmental conditions for the first vulnerable life stages (Philippart et al., 2003). In cockles, recruitment success is also partly (but not only) dependent on temperature and poses the question of stock sustainability in a global change scenario. Beukema & Dekker (2005) observed a substantial and significant decline of overall recruitment success in the Wadden Sea, and Beukema et al. (2010) suggested that this could be related to the infrequency of severe winters during the last 1–2 decades. On the other hand, Strasser et al. (2003) pointed out that temperature did not explain cockle recruitment heterogeneity at scales of several km.

In Arcachon Bay, i.e. further south in cockle distribution range where winters are milder than in the northern distribution, winter temperatures were not identified as an important driver of recruitment success. Whether temperature reached 14 °C in May rather than in June, both being possible depending on the year, was important but there were exceptions. These exceptions, and the different scenario between the Wadden Sea and Arcachon Bay, suggest that temperature alone is not the only driver of recruitment, that ecological factors involved in recruitment are certainly site-dependent (Strasser et al., 2003), and that the sustainability of a cohort could be set earlier, i.e. by processes happening before recruitment.

Final considerations

The present study showed that factors driving cockle recruitment success are highly site-dependent, temperatures at the site being only one component but not sufficient to explain all variability. Among other ecological factors that can be driving population performance, parasitism could be significant but is often underestimated. Bivalve species are used by trematodes, the most abundant and common macroparasites in coastal waters, as first and second intermediate hosts. These infections may exert deleterious effects on the host with consequent impacts at the population level. However, these host-parasite relationships are still poorly understood. Accordingly, using *Cerastoderma edule* and *Donax trunculus* as host models, the following thesis chapter proposes to characterize the relationship between trematode parasites and its bivalve first intermediate host, highlighting some of the parasite harmful effects.

CHAPTER 3. BIVALVES AS FIRST INTERMEDIATE HOST

This chapter is published as:

> Luísa Magalhães, Rosa Freitas & Xavier de Montaudouin (2015). Review: *Bucephalus minimus*, a deleterious trematode parasite of cockles *Cerastoderma* spp.. *Parasitology Research*, *114*, 1263-1278.
> Link: https://doi.org/10.1007/s00436-015-4374-6.

> Luísa Magalhães, Xavier de Montaudouin, Rosa Freitas, Guillemine Daffe, Etelvina Figueira & Patrice Gonzalez (2017). Seasonal variation of transcriptomic and biochemical parameters of cockles (*Cerastoderma edule*) related to their infection by trematode parasites. *Journal of Invertebrate Pathology*, *148*, 73-80.
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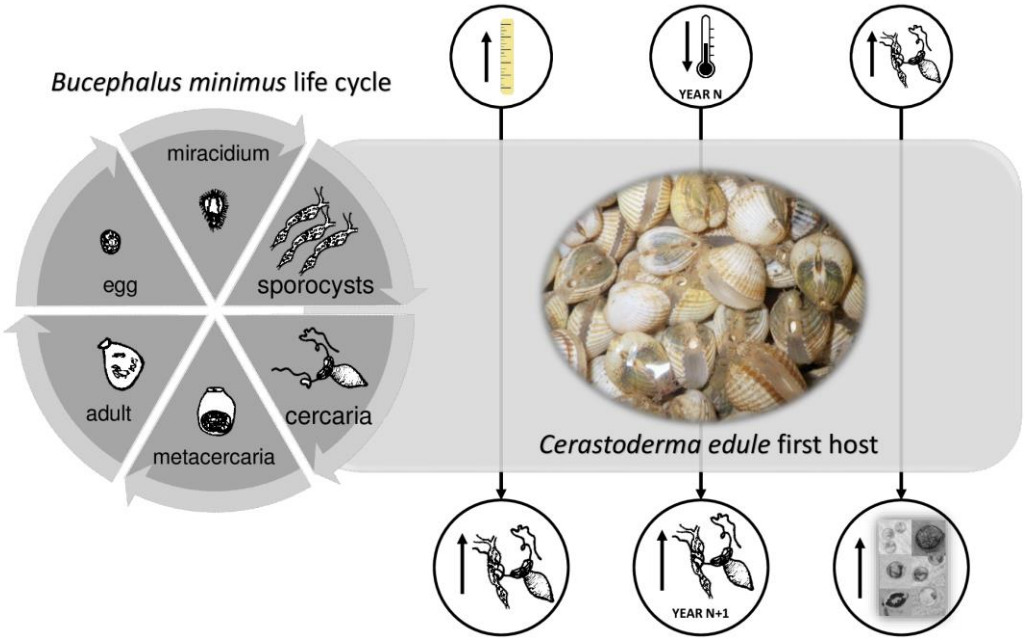
> Luísa Magalhães, Xavier de Montaudouin, Simão Correia, Guillemine Daffe, Patrice Gonzalez, Etelvina Figueira, Jorge Gonçalves & Rosa Freitas (submission number ECSS_2018_300). Seasonal variation of transcriptomic and biochemical parameters of *Donax trunculus* related to its infection by *Bacciger bacciger* (trematode parasite). *Estuarine, Coastal and Shelf Science*.

Cerastoderma edule (the edible cockle)

3.1. Review: *Bucephalus minimus*, a deleterious trematode parasite of cockles *Cerastoderma* spp..

GRAPHICAL ABSTRACT



3.1.1. Introduction

Trematodes are the most prevalent and abundant macroparasites in coastal waters (Lauckner, 1983; Schmidt & Roberts, 2000). They display a complex life cycle with alternation of free-living and parasitic stages generally involving three host species (Esch et al., 2002). In the first intermediate host, often a mollusc, the parasite penetrates as miracidium larva and asexually multiplicates in sporocysts or rediae to provide cercariae larvae. This is the most deleterious stage with described negative impacts at the reproduction, growth and behaviour level (Babirat et al., 2004; Bowers, 1969; Carballal et al., 2001) that may lead to population or community relevant changes. The actual impacts of sporocyst infection in its first intermediate host population dynamics are still unknown especially due to intrinsic low prevalence in ecosystems (Thieltges et al., 2008b), what makes difficult to study these systems.

The present work aimed to review all literature available concerning a parasite/first intermediate host system taking the example of the cockle (*Cerastoderma edule*), an exploited bivalve along North-Eastern Atlantic coasts, and *Bucephalus minimus*, its most prevalent parasite as first intermediate host (de Montaudouin et al., 2009). Furthermore, this study aimed to provide new information relating the host

individual features (such as size) and the environmental factors (such as temperature and other parasite species presence) to higher parasite prevalence occurrence. In this sense, data from a 16-years monthly monitoring study, performed at Banc d'Arguin (Atlantic coast of France), was used.

3.1.2. Material and methods

3.1.2.1. Literature review

Web of science was searched for publications containing several combinations and versions of the terms 'trematode', '*Cerastoderma* (or *Cardium*) *edule*' and '*Bucephalus* (or *Labratrema*) *minimus*' published before January 2015. The reference list of the papers found and theses were also consulted. The search was constrained to those studies that clearly identified the occurrence of *Bucephalus minimus*. A total of 51 publications (47 papers, 3 theses and 1 book section) were gathered and used in this study.

3.1.2.2. *Bucephalus minimus* 16-years monitoring at Banc d'Arguin

3.1.2.2.1. Study area

The sampling station was in Banc d'Arguin (44.60°N, 1.25°W), Arcachon bay, France (Figure 5) which is described in more detail in the section 2.2.1 of the previous chapter. Since 1972, Arguin (25 km^2) has been a national reserve with several marine bird species wintering, nesting or migrating, although Banc d'Arguin also undergoes an intense anthropogenic pressure (tourism and oyster farming).The surrounding waters are also inhabited by many fish. These birds and fish are potential final hosts of trematode parasites.

3.1.2.2.2. Sampling strategy and parasite identification

Between October 1997 and September 2013 (16 years), cockles were monthly collected by sampling 6 quadrats (0.25 m^2 each) and sieving them through a 1-mm mesh sieve. Cockle shell length (SL) was measured at the least mm with a caliper. Ten cockles per cohort were dissected and squeezed between two glass slides for trematode observation under a stereomicroscope (Figure 11). All digenean trematodes were identified to the species level following several authors (Bartoli et al., 2000;

Bowers, 1969; Bowers et al., 1996; de Montaudouin et al., 2009; Desclaux et al., 2006).

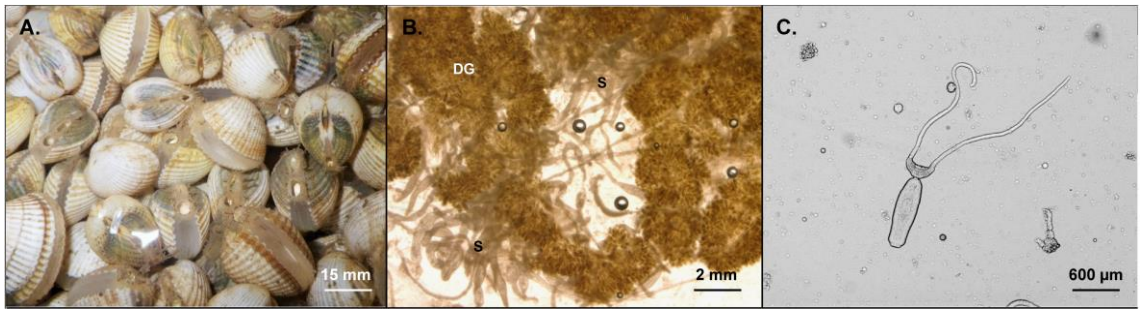


Figure 11 Photos of the host – parasite system under study: **A.** *Cerastoderma edule*, the edible cockle; **B.** *Bucephalus minimus* sporocysts (S) invading the cockle digestive gland (DG); **C.** *B. minimus* cercariae.

Metacercariae in second intermediate host were counted to assess parasite abundance, i.e. the number of metacercariae per cockle. For trematode using cockles as first intermediate host, it is not possible to count sporocysts and/or cercariae, due to dense mass created by those, so prevalence alone was calculated, i.e. percentage of infected cockles (Bush et al., 1997).

During the parasite monitoring, a temperature probe was settled in the sediment where cockles were sampled, measuring temperature every hour (HOBO® Water Temp Pro v2 – U22-001) during the whole study period.

3.1.2.2.3. Data analysis

Correlation between cockle SL classes and associated *B. minimus* prevalence was tested using nonparametric Spearman analysis.

In order to test the effect of seasons and years on *B. minimus* prevalence, two one-way ANOVAs were performed: 1) one to compare the different months, using the 16 years or replicates; and 2) one to compare the different years using the 12 months or replicates. One year was defined as the 12 months between October N and September N+1.

Prevalences of *B. minimus* were Arcsin $\sqrt{p}$ transformed prior to analysis to homogenize variance (with p = prevalence/100). Homogeneity of variance was then checked with Cochran test. Average prevalence per year was correlated (Spearman test) with mean temperature of each month, each year and each previous year, and also with mean yearly cockle abundance.

In order to compare abundance of each associated trematode species using cockle as second intermediate host between cockles infected and cockles not infected with *B. minimus*, a paired Wilcoxon test was performed. For each *B. minimus* infected cockle, a pair was constituted by randomly selecting a non-infected *B. minimus* cockle of the same SL and of the same sampling date. Bonferroni correction was applied to determine the threshold of significance (*i.e.*: 0.05/7 = 0.007). All statistical analyses were performed with STATISTICA (StatSoft) software.

3.1.3. Results

3.1.3.1. Literature review

All references used along this section were gathered in the Table 3.

3.1.3.1.1. *Bucephalus minimus* description and life cycle

Bucephalus minimus (Stossich, 1887), presented over time various synonyms: *Dolichoenterum lamirandi* Carrére, 1937, *Gasterostomum minimum* Stossich, 1887, *Prosorhynchoides minima* (Stossich, 1887), *Labratrema* (or *Bucephalus*) *lamirandi* (Carrére, 1937) Overstreet & Curran, 2002 and *Labratrema minimum* (Stossich, 1887) and sometimes was erroneously called as *Cercaria bucephalopsis haimeana* (Lacaze-Duthiers, 1854) or *Bucephalus haimeanus* (Lacaze-Duthiers, 1854). *B. minimus* is a parasite from the Platyhelminthes phylum, Trematoda class, Digenea subclass and Bucephalidae family.

Species of the bucephalidae family invariably occur in bivalves as sporocysts and cercariae (reported in 18 families of bivalves, the highest number for any other digenean family (Cribb et al., 2001)). The metacercariae usually live in cysts in various parts of the nervous system or in internal organs and the musculature of teleost fishes. Bucephalids adults inhabit the alimentary tract of fish predators (Lauckner, 1983). They differ from all other digeneans by the configuration of their digestive system and terminal genitalia and by the presence of an anterior rhynchus, for attachment, that is dissociated from the digestive system (Overstreet & Curran, 2002). Larval bucephalids are probably the most deleterious metazoan parasites of marine bivalves (Lauckner, 1983). There is a gradual destruction and replacement of the bivalve tissue by the sporocyst mass. Eventually, all of the tissues become significantly depleted reducing the host overall resistance to environmental stress and consequently the host survival probability (Lauckner, 1983; Longshaw & Malham, 2013).

To complete its life cycle, *B. minimus* infects three different hosts, one invertebrate and two vertebrate species (Maillard, 1975). This parasite infects a bivalve as first intermediate host in the form of sporocysts (series of elongated bags with diameter ranging between 80 and 500 µm (Deltreil & His, 1970)) and cercaria (length: 300-350 µm (de Montaudouin et al., 2009)). This bivalve is reported to be *Cerastoderma edule*, *C. glaucum* and some authors refer its occurrence also in clams *Ruditapes* spp.. The mature cercariae emerge from the first intermediate host, swim and invade several teleost fish species like gobbies *Pomatoschistus* spp., *Atherina* spp. and other small fish species which act as a second intermediate host for the metacercariae life stage of the parasite (400 µm length and 250 µm width (Maillard, 1975)). The adult life stage (size: 366-1088 µm (Pina et al., 2009)) of *B. minimus* develops in the final host, the seabass *Dicentrarchus labrax* which is infected by consuming the second intermediate host. The cycle resumes when parasite eggs are dropped in the environment with seabass faeces.

Besides occurrence records, many authors described this species in its first intermediate host: firstly with basic descriptive works regarding the sporocysts form (Deltreil & His, 1970; Pelseneer, 1906; Russell-Pinto, 1993) and the cercariae larvae (Deltreil & His, 1970; Matthews, 1973; Pelseneer, 1906; Russell-Pinto, 1993), and secondly with ultrastructure descriptions by electron microscopy (James et al., 1966; Pina et al., 2009; Russell-Pinto et al., 2006). Authors are unanimous referring that *B. minimus* infects preferentially adult cockles as first intermediate hosts (Baudrimont et al., 2003; Bowers, 1969; de Montaudouin et al., 2000; Derbali et al., 2009; Thieltges & Reise, 2006).

Table 3 Review of the literature regarding *Bucephalus minimus* infection. Data are chronologically exhibited according to published year. Reference; study location; intermediate host species (with respective mention to host size (mean and/or range of the shell length or age) and infected tissues when available; prevalence of infection in first intermediate host (mean, range or both) and main findings are specified.

Reference	Study location	Intermediate hosts (size: infected tissue)			Prevalence (%)	Main findings
		First	Second	Final		
Stossich, 1887	Mediterranean Sea	—	—	*Dicentrarchus labrax*	—	First description.
Vaullegeard, 1894	Luc-sur-Mer, France	*Ruditapes* spp.	—	—	—	Occurrence.
Johnstone, 1904	Lancashire, England	*Cerastoderma edule*	—	—	—	First reference in first intermediate host.
Pelseneer, 1906	Boulogne-sur-Mer, France	*C. edule*	—	—	—	Occurrence.
Lebour, 1911	Northumberland and Hampshire, England	*C. edule*	—	—	2.0-10.0	Occurrence.
Nicoll, 1914	English Channel, England	—	—	*D. labrax*	—	Occurrence.
Palombi, 1934b	Gulf of Naples, Italy	*Ruditapes* spp.	—	—	—	Occurrence.
Carrère, 1937	Camargue, France	—	*Atherina* spp. (liver)	*D. labrax*	—	First reference as metacercariae and experimental infestation in final host.
Hutton, 1952	Plymouth, England	*C. edule*	—	—	26.4	Occurrence.
Cole, 1956	Wales	*C. edule*	—	—	~2	Occurrence.
Rebecq, 1964	Camargue, France	—	*Atherina* spp.	*D. labrax*	—	Redescription of metacercaria and adult life stages.

Table 3 (continued)

James et al., 1966	Wales	*C. edule*	—	—	—	Sporocysts ultrastructure by electron microscopy.
James & Bowers, 1967	Burry Estuary, Wales	*C. edule*	—	—	9.5	Germ-cell cycle and reproduction.
Bowers, 1969	Burry Estuary, Wales	*C. edule* (>18 mm)	—	—	—	Causes a decrease in the shell growth rate and an improvement in the flesh yield.
Deltreil & His, 1970	Arcachon bay, France	*C. edule* (12.8 mm)	—	—	40.7	Sporocysts and cercariae description.
Matthews, 1973	Wales	*C. edule*	*Pomatoschistus microps Pleuronectes platessa*	—	—	Cercariae redescription and description of metacercaria with experimental infection.
Maillard, 1975	Languedoc, France	*C. glaucum*	*P.* microps Atherina spp (liver)	*D. labrax* (intestine)	—	1st, 2nd and final hosts stablish (with experimental infection).
Combes, 1980	Review work	—	—	—	—	Starts to being referred as *Labratrema minimum*.
Bartoli, 1984	Mediterranean coast, France	*C. glaucum*	—	—	—	Occurrence.
Bartoli & Combes, 1986	Brusc lagoon, France	*C. glaucum*	*P. microps*	*D. labrax*	—	Presents vertical dissemination, the 1st, 2nd and final host were established.
Faliex & Biagianti, 1987	Leucate lagoon, France	—	*Atherina boyeri*	—	—	Histo-cytological analysis as metacercariae.
Faliex, 1991	Leucate lagoon, France	—	*A. boyeri Liza ramada* (liver)	—	—	Effects in the second intermediate host.
Sauriau, 1992	Marennes-Oléron bay, France	*C. edule*	—	—	5-13	Occurrence.
Russell-Pinto, 1993	Ria de Aveiro, Portugal	*C.edule (digestive gland and gonad)*	—	—	2.65	Sporocysts and cercariae description.

Table 3 (continued)

Faliex & Morand, 1994	Salses-Leucate lagoon, France	—	*A. boyeri*	—	—	Population dynamics as metacercaria.
Paillard et al., 1994	Review paper	*Polititapes aureus*	—	—	—	Occurrence.
de Montaudouin et al., 2000	Arcachon bay, France	*C. edule* (21–29 mm: gonad and digestive gland)	—	—	2.6	Comparison: multi-host and multi-parasite infection.
Sasal et al., 2000	Salses-Leucate lagoon, France	*C. glaucum*	*P. microps*	—	—	Occurrence in the 1st host and experimental infection of the 2nd host.
Malek, 2001	Southwest Wales	—	*Pomatoschistus* spp.	—	—	Occurrence.
Desclaux et al., 2002	Arcachon bay, France	*C. edule* (gonad, digestive gland and then all tissues)	—	—	7.6	Occurs all year long but especially during warm season; surface cockles 2x more infected.
Baudrimont et al., 2003	Arcachon bay, France	*C. edule* (2-years old)	—	—	<5	Interfers with MTs synthesis by affecting its reproductive cycle.
Baudrimont et al., 2006	Arcachon bay, France	*C. edule*	—	—	—	Decreases MT synthesis.
Culurgioni et al., 2006	St. Gilla lagoon, Italy	*C. glaucum* (visceral mass and mantle)	—	—	0.7	Occurrence.
Russell-Pinto et al., 2006	Ria de Aveiro, Portugal	*C. edule* (digestive gland and gonad)	—	—	2.33	Cercaria description with electron microscopy.
Thieltges et al., 2006	Sylt Island, Germany	*Mytilus edulis*	—	—	0.3	Occurrence further north.
Thieltges & Reise, 2006	Sylt Island, Germany	*C. edule* (only adults)	—	—	0.1-1.0	Occurrence.

Table 3 (continued)

Bartoli and Gibson 2007	Mediterranean lagoons	*C. glaucum*	*A. boyeri* *P. microps* *Chelon labrosus* *L. ramada* *Sparus aurata*	*D. labrax*	—	Life cycle in the Mediterranean environment.
Desclaux-Marchand et al. 2007	Arcachon bay, France	*C. edule*	—	—	—	Infection and cadmium exposure increased the MT gene expression.
Gam et al. 2008	Merja Zerga, Morocco	*C. edule*	—	—	3.2	Occurrence.
de Montaudouin et al. 2009	Review paper	*C. edule* (start in gonads)	—	—	—	Occurrence.
Derbali et al. 2009	Gulf of Gabes, Tunisia	*C. glaucum* (only adults: digestive gland and gonads)	—	—	—	Prevalence tended to increase with cockle size.
Dubois et al. 2009	Arcachon bay, France	*C. edule*	—	—	1-17	Induced low or no shifts in isotopic signatures and feeds on digestive gland.
Pina et al. 2009	Ria de Aveiro, Portugal	*C. edule* (digestive gland and gonad)	*Mugil cephalus*	*D. labrax*	1.5-2.0	All life stages description.
Fermer et al. 2011	South coast, Ireland	*C. edule*	—	—	3.8 (2-6)	Occurrence.
Gargouri Ben Abdallah et al. 2011	Gulf of Gabes, Tunisia	*C. glaucum*	*A. boyeri*	—	0.5-20	Sporocyst and cercaria occurrence, description and behaviour, experimental infection of the 2nd host.
Meisterhans et al. 2011	Arcachon bay, France	*C. edule*	—	—	—	Infection increases bacterial community abundance (1.8x).
de Montaudouin et al. 2012	Arcachon bay, France	*C. edule*	—	—	17	Had a negative effect on host growth and condition index.
Morgan et al. 2012	Flaxfort Strand, Ireland	*C. edule*	—	—	3.8	Ocurrence.

Table 3 (continued)

Binias et al. 2014	Arcachon bay, France	*C. edule*	—	—	13	Occurrence.
Freitas et al. 2014	Ria de Aveiro, Portugal	*C. edule*	—	—	5	Prevalence higher in the most contaminated area.
Feis et al. in press	North Eastern Atlantic	*C. edule*	—	—	0.0–5.3	Strong population structure related with less vagile final host.

3.1.3.1.2. *Bucephalus minimus* distribution and prevalence

From the literature analysed, it was possible to show that *B. minimus* was observed for the first time in the Mediterranean Sea in the final host (Stossich, 1887). Since, it was described in the lagoon cockle *C. glaucum* with prevalence ranging from 0.5 to 20% (Culurgioni et al., 2006; Gargouri Ben Abdallah et al., 2011).

Concerning North-Eastern Atlantic, Johnstone (1904) referred for the first time the occurrence of *B. minimus* in *C. edule* in England but today this parasite is referenced as parasite of cockles from the United Kingdom and Wadden Sea (Germany) until Merja Zerga bay in Morocco (de Montaudouin et al., 2009).

The prevalence in *B. minimus* infecting *C. edule* as first intermediate host ranged from very low values (0.1% registered in Wadden Sea, Germany by Thieltges & Reise (2006)) to relatively high values (40.7% registered in Arcachon bay, France by Deltreil & His (1970)). Authors rarely referred seasonality of *B. minimus* infection in *C. edule*. Although, Bowers (1969) and Desclaux et al. (2002) showed that this parasite occurred all year long in Burry Estuary (Wales) and Arcachon bay (France), respectively, with higher prevalence in summer. These prevalences are calculated for adult cockles (*Cerastoderma* spp.) with a SL that is not always specified, which makes difficult to compare among studies.

3.1.3.1.3. Effects of *Bucephalus minimus* in the first host

B. minimus infection in *C. edule* starts on the gonad and in the digestive gland (de Montaudouin et al., 2000; Desclaux et al., 2002; Pina et al., 2009; Russell-Pinto, 1993; Russell-Pinto et al., 2006). On one hand, in the gonad, the parasite causes castration of the organism (Carballal et al., 2001). On the other hand, *B. minimus* consumes energy in the digestive gland (Dubois et al., 2009) and by infecting this organ causes starvation and consequently autolysis of the digestive tract. Then, the parasite proliferates to other microhabitats inside its host like gill and foot and ultimately invading all other host tissues (de Montaudouin et al., 2009; Desclaux et al., 2002). Bowers (1969) referred that this parasite species increase the flesh weight of cockles. In heavily infected cockles, *B. minimus* can represent 20 (Dubois et al., 2009) to 25% (Meisterhans et al., 2011) of the total host flesh dry weight. Bowers (1969) also concluded that *B. minimus* can cause a decrease in the host shell growth rate. However, transplant experiments suggested that effects of *B. minimus* on cockle growth and condition index depended on sites, with possible interaction with other

factors, including the occurrence of other trematode species (de Montaudouin et al., 2012a).

3.1.3.1.4. *Bucephalus minimus* in a multistress context

More recently, studies on *C. edule* infection by *B. minimus* focused on the relationship between this infection and cockle metallothioneins (MTs) synthesis. MTs are proteins with the capacity to bind physiological or xenobiotic elements providing defence against stressors like trace metals or parasites. The synthesis of MTs can also be stimulated by gametogenesis with MTs concentrations dropping after spawning period. However, the presence of *B. minimus* infection can modulate this synthesis. Indeed, *B. minimus* by causing castration of the host will consequently decrease MTs synthesis of mature cockles and will increase cockle vulnerability (Baudrimont et al., 2003, 2006). The same pattern was found regarding MTs gene expression (Desclaux-Marchand et al., 2007). These studies highlighted that the use of metallothioneins as a biomarker must integrate parasitism and host reproduction status.

Freitas et al. (2014) found higher prevalence of *B. minimus* in cockles living in the most contaminated areas at the Ria de Aveiro (Portugal). This work introduces a new topic of concern, suggesting that cockles that already experienced other sources of stress are more sensitive to *B. minimus* infection, and thus, emphasizing the advantage of using this parasite species as environment quality marker.

B. minimus infection has been reported inducing lower infection by other trematode species infecting cockles as second intermediate host (de Montaudouin et al., 2000).

B. minimus infection in cockles is correlated with an increase of the bacterial abundance (Meisterhans et al., 2011). Morgan and co-authors (2012) concluded that *B. minimus* infection along with other stress sources like spawning season, neoplasia or even metal contamination may immunocompromise the cockles.

3.1.3.2. *Bucephalus minimus* 16-years monitoring at Banc d'Arguin

During the total study period (October 1997 – September 2013), 5420 cockles were dissected and 13 different trematode species were identified. Cockle SL ranged from 1 to 39 mm, while those infected by *B. minimus* ranged from 16 to 39 mm. *B. minimus* prevalence fluctuated between 2.2% for 18-mm SL class and 15.6% for 34-mm SL class (Figure 12). Cockle SL and *B. minimus* prevalence showed a strong positive correlation ($r = 0.93$; $p < 0.001$).

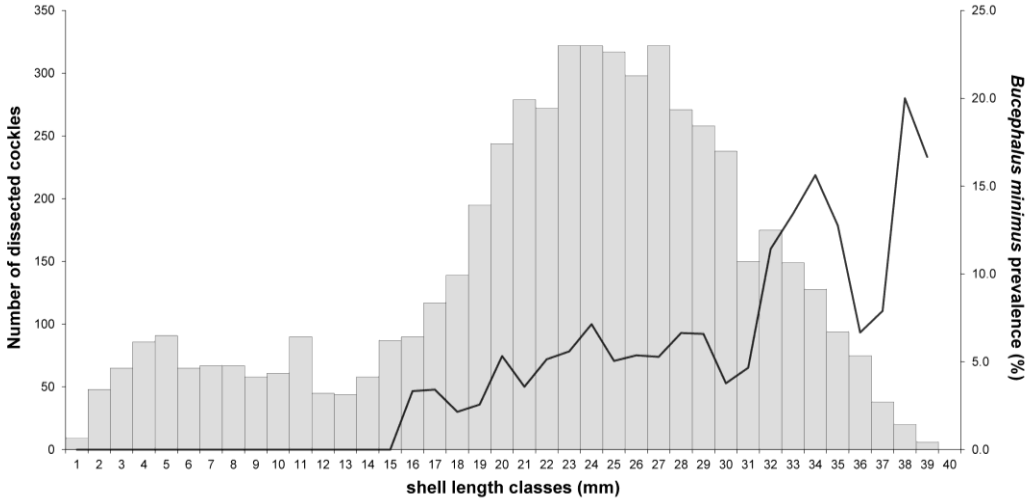


Figure 12 *Bucephalus minimus* prevalence (line: %) and corresponding number of monthly dissected *Cerastoderma edule* between October 1997 and September 2013 by shell length class (bars: mm).

Out of the total number of sampled cockles, those with SL over 15 mm (4520 individuals), i.e. cockles susceptible of being parasitized by *B. minimus*, were considered in the following analysis. With this selection, we avoid the “dilution” of prevalence values related to seasonal juvenile dominance. Out of these cockles, 276 were registered as infected with *B. minimus* with an overall prevalence of 6.2%.

The prevalence of *B. minimus* was analyzed per month (gathering all years) (Figure 13). *B. minimus* prevalence ranged between 3.8% in September and 8.7% in January without significant differences ($p > 0.05$).

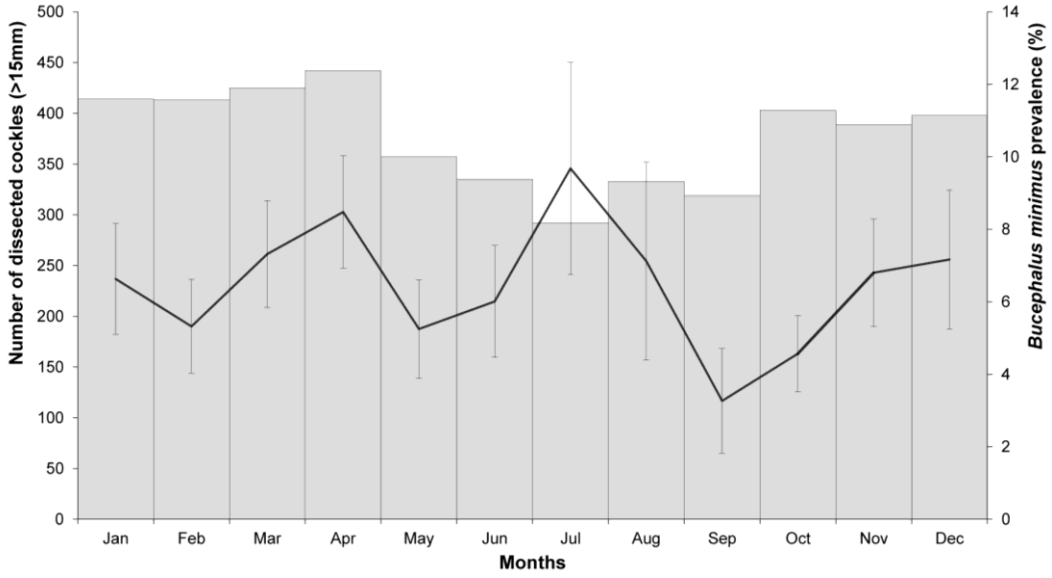


Figure 13 *Bucephalus minimus* prevalence (line: %) with respective standard error bars and corresponding number of dissected *Cerastoderma edule* susceptible to be infected (bars: >15 mm) per month gathering all sampled years.

The prevalence of *B. minimus* was different among years ($p < 0.05$). Post hoc analysis (Tukey test) discriminated three years different from all others ($p < 0.05$): 2002-03, 2007-08 and 2012-13, with 15.7%, 2.9% and 2.5% prevalence, respectively (Figure 14).

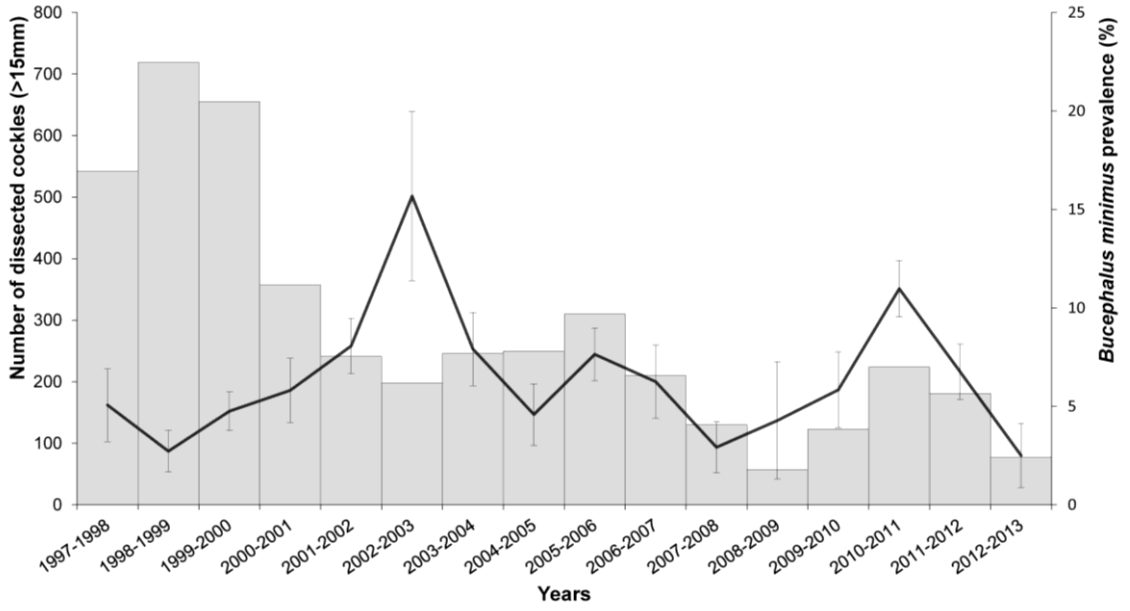


Figure 14 *Bucephalus minimus* prevalence (line: %) with respective standard error bars and corresponding number of the dissected *Cerastoderma edule* susceptible to be infected (bars: > 15 mm) per year.

From all tested correlations (see 3.1.2.2.4. section), the only significant negative correlation (r = - 0.64; p = 0.04) was found between *B. minimus* prevalence and mean temperature of the previous year. Therefore, when a year displayed a low mean temperature, higher *B. minimus* prevalence was observed the next year (Figure 15).

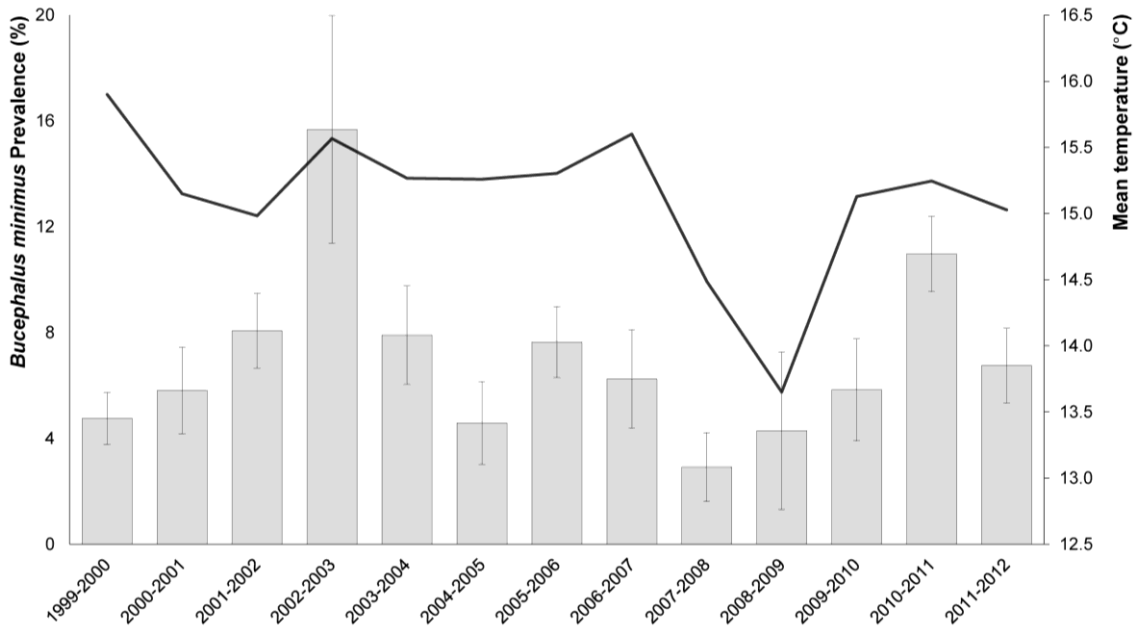


Figure 15 *Bucephalus minimus* prevalence (bars: %) with respective standard error bars and mean sediment temperature (line: ºC) per year.

A total of 276 pairs of cockles with and without *B. minimus* infection (for given SL and sampling date) were analyzed considering the abundance of the other trematode species using *C. edule* as second intermediate host (Table 4). Except *Renicola roscovitus*, parasite abundance of all other trematode species using cockles as second intermediate host was higher (between 2- in the case of *Diphterostomum brusinae* and 12-fold higher for *Parvatrema minutum* infection) when infected by *B. minimus* (p < 0.001) (Figure 16). Trematode species richness in *B. minimus* infected cockles (mean = 3.9) was also higher (p < 0.001) than in cockles free of *B. minimus* (mean = 2.8) (Figure 16).

Table 4 Trematode species mentioned along the manuscript with the respective description of the first, second and final host species and the respective literature references.

Trematode species	**1st intermediate host**	**2nd intermediate host**	**Final host**	**References**
Bucephalus minimus	***Cerastoderma edule***	Small teleost fishes (*e.g. Atherina* spp., *Pomatoschistus* spp.	*Dicentrarchus labrax*	Carrère, 1937; Maillard, 1975; Pina et al., 2009; Russell-Pinto et al., 2006
Curtuteria arguinae	Unknown	***C. edule***	Probably water birds	Desclaux et al., 2006; Russell-Pinto et al., 2006
Diphterostomum brusinae	*Tritia reticulata*	***C. edule***	Fishes of the genera *Blennius*, *Symphodus*, *Oblada* and *Diplodus*	Francisco et al., 2011; Prévot, 1966; Russell-Pinto et al., 2006
Himasthla interrupta	*Hydrobia* spp.	***C. edule***	Water birds	Desclaux, 2003; Lauckner, 1971, 1983; Russell-Pinto et al., 2006
Himasthla quissetensis	*T. reticulata*	***C. edule***	Water birds	Desclaux, 2003; Russell-Pinto, 1993; Russell-Pinto et al., 2006; Stunkard, 1938
Psilostomum brevicolle	*Hydrobia* spp.	***C. edule***	Water birds	Lauckner, 1971, 1983; Loos-Frank, 1968
Parvatrema minutum	*Scrobicularia plana*	***C. edule/C. glaucum***	Water bird *Haematopus ostralegus*	Bowers et al., 1996; Lauckner, 1971, 1983; Loos-Frank, 1968; Russell-Pinto & Bowers, 1998
Renicola roscovitus	*Littorina littorea*	***C. edule***	Water birds	Lauckner, 1971, 1983; Werding, 1969

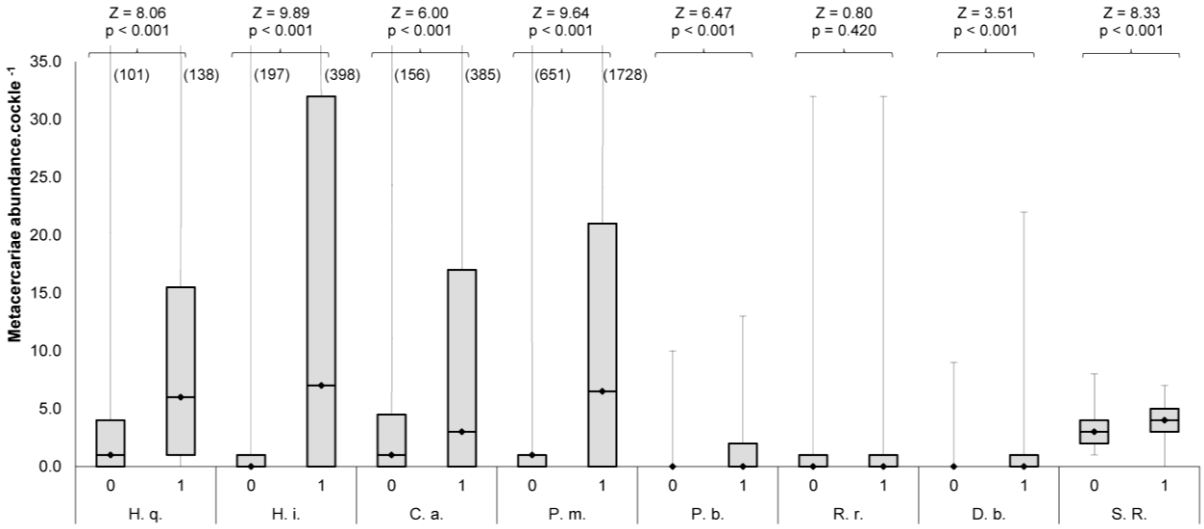


Figure 16 Compared trematode species abundance and species richness per cockle, not infected (0) or infected (1) by *Bucephalus minimus*. Box and whisker plots minimum, first quartile, median, third quartile and maximum values. When maximum value is over figure scale, it is written between brackets. H. q.: *Himasthla quissetensis*; H. i.: *Himasthla interrupta*; C. a.: *Curtuteria arguinae*; P. m.: *Parvatrema minutum*; P. b.: *Psilostomum brevicolle*; R. r.: *Renicola roscovitus*; D. b.: *Diphterostomum brusinae*; S.R.: Species richness. Results of Wilcoxon pairwise test with level of significance = 0.007 (after Bonferroni correction); N = 276.

3.1.4. Discussion

3.1.4.1. *Bucephalus minimus* 16-years monitoring at Banc d'Arguin

3.1.4.1.1. Size at first infection and size-dependent prevalence

Banc d'Arguin monitoring showed that *Cerastoderma edule* SL is positively correlated with *Bucephalus minimus* prevalence and that first size of infection by *B. minimus* is 16 mm SL. According to Gam et al. (2009), this SL corresponds at Banc d'Arguin to an age of approximately four months. Thus, it gives an order of magnitude of the necessary time for sporocysts to become visible (although the date of infection remains unknown). Since *C. edule* presents also a minimum size at first maturity of approximately 12 mm for females and 15 mm for males (Malham et al., 2012), it suggests that *B. minimus* infects only mature cockles. Several authors presented convergent ideas, for example referring that *B. minimus* infects only spent cockles (Bowers, 1969) or cockles with SL larger than 19 mm (de Montaudouin et al., 2000) or that trematode species using cockles as first intermediate host infects only adults (Thieltges & Reise, 2006). *Gymnophallus choledocus* and *Monorchis parvus*, both using *C. edule* as first intermediate host in Banc d'Arguin, also showed a preference for

larger SL classes, between 31 and 37 mm and between 19 and 40 mm, respectively (unpublished personal data). The same pattern was found for other bivalve first host – parasite systems: only larger *Scrobicularia plana* exhibit sporocysts of *Parvatrema minutum* (Fermer et al., 2009) and *Bacciger bacciger* prevalence in *Donax trunculus* (first intermediate host) is null in juveniles and increases with their SL (de Montaudouin et al., 2014; Ramón et al., 1999).

However, all of these studies, including the present monitoring work, refer to visible prevalence. In reality, the first size of infection corresponds to the moment when the miracidium infects its host, which is difficult to detect with optical methods. The relatively late observation of *B. minimus* infection in cockles could explain why no correlation was observed between prevalence and sediment temperature recorded for the same year, while a correlation was found with average temperature of the previous year. Colder temperature could favourize miracidium survival and infection (possible higher first host abundance and/or susceptibility to infestation) with visible effects due to sporocysts maturation few months later. Indeed, there is no reason for late “real” infestation by miracidium, *e.g.* considering that cercariae can infect 1-mm shell length cockles (Wegeberg et al., 1999).

3.1.4.1.2. Infection seasonality

Average prevalence of *B. minimus* with visible sporocyst fluctuated around 7% (of > 15-mm cockles) along the year without noticeable seasonal trend. In order to detect such seasonal trend, the best way would be to monitor host cohort infection along time as it has been performed with metacercariae in second intermediate host (Desclaux et al., 2004; Gam et al., 2009). However, *B. minimus* prevalence in cockle is too low to be monthly monitored and, as previously specified, infection time by miracidium remains unknown. This may partly explain why no infection seasonality was observed, even with a pooled 16-years monthly monitoring. It is also possible that there is really no seasonality because conditions are favourable all year long: 1) presence of hosts: *Pomastoschistus* spp. (second intermediate host) is a sedentary species which preferentially lives in semi-sheltered lagoon-like environment (Pampoulie et al., 2000) and *Dicentrarchus labrax* (final host) is an eurythermic and euryhaline species which is not particular sensitive to temperature drop, some of them over-wintering in coastal lagoons instead of returning to open sea (FAO, 2005-2018); 2) presence of miracidia all year long: miracidium is a non-feeding and free-living stage of trematodes life cycle and its survival/metabolism is not affected by temperature. It is, in general, well

adapted to the normal thermal ranges encountered by each miracidia species (Morley, 2012).

Finally, the lack of observable seasonality of sporocyst infection in cockles does not mean that there is no seasonality in cercariae emergence from these sporocysts, as it is deduced from observed infection seasonality in second intermediate host in other trematode – host systems (de Montaudouin et al., 2016; Desclaux et al., 2004; Meiβner, 2001; Thieltges & Rick, 2006).

3.1.4.1.3. Trematode metacercariae associated community

Cockles infected with *B. minimus* evidenced higher abundance and species richness of other trematode species (infecting as metacercariae). Sannia & James (1978) wrote that parasitism may increase the susceptibility of *C. edule* to other digenean species infection but de Montaudouin et al. (2000), with a much smaller data set, suggested that cockles with *B. minimus* infection displayed lower prevalence and abundance of other trematode species and, similar species richness than cockles without *B. minimus*. However, it is important to specify that the intensity of the associated parasite community in the presence of *B. minimus* is often under estimated due to the difficulty to observe and/or count metacercariae among sporocysts that invade the whole cockle flesh.

Trematode infection increases the total circulating hemocytes count and decreases their phagocytic capacity, thus modulating the cockle immune system (Dang et al., 2013). Consequently, cockles infected with *B. minimus* should be less resistant to infection by the other trematode species than cockles without *B. minimus*, or *vice-versa* cockles with rich and abundant metacercariae community could be more sensitive to *B. minimus* infection. Both scenarios lead to a higher abundance of metacercariae in *B. minimus* infected cockles.

From the host point of view, infection by *B. minimus* is then twice a handicap, firstly due to direct fitness alteration and secondly due to over infection by metacercariae. From the parasite point of view, multiple infection may become a benefit in a favourization framework (Combes, 1980; Dobson, 1988; Holmes & Bethel, 1972; Moore & Gotelli, 1990). Infection by *B. minimus*, for example, is often correlated with the migration of cockles at the surface of the sediment (Desclaux et al., 2002), certainly in interaction with other stressors like neoplasia (Le Grand et al., 2010) or prokaryotes (Meisterhans et al., 2011). This position makes cockles more susceptible to predators, including potential final hosts of many trematode species using cockles as second intermediate hosts (Babirat et al., 2004; Edelaar et al., 2003). The different

species (metacercariae) that were observed in cockles become “hitchhikers”, in the sense that they achieve a higher final host transmission rate without having to invest in modifying the phenotype of the cockle (Lafferty, 1999; Leung & Poulin, 2007; Mouritsen, 2001; Poulin et al., 2000; Thomas et al., 1998a, 1998b).

Conclusion

The edible cockle *C. edule*, the first host of *B. minimus*, is distributed from the Barents Sea to the Mauritanian coast, i.e. a concordant distribution with the final host *D. labrax*. However, seabass presents wider boundaries being more to north and more to south (Kaschner et al., 2013a) relative to cockles. *Pomastoschistus* spp. the most frequent second intermediate host of *B. minimus* is distributed from Norway to Morocco (Kaschner et al., 2013b) and *Atherina* spp. (other possible second intermediate host) is distributed from the French coast until Mauritania including Mediterranean Sea (Kaschner et al., 2013c). And, as previously referred, *B. minimus* is reported from Germany to Morocco coasts, a common area for the 3 hosts with *Pomastoschistus* spp. northern and *Atherina* spp. southern. This gives *B. minimus* the possibility to expand further north and further south from its actual distribution (Figure 17). However, the distribution of a parasite that has a fish as final host it is less efficient to disperse at long scale than a parasite with a more vagile final host like a bird (Feis et al., 2015).

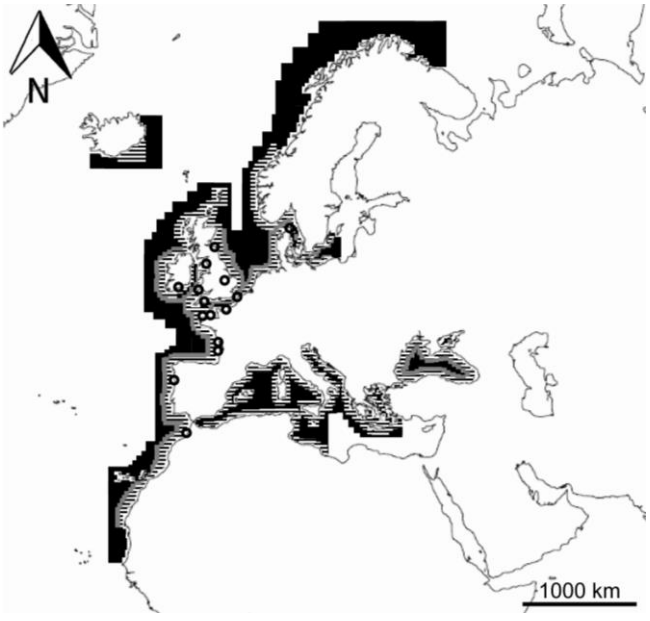


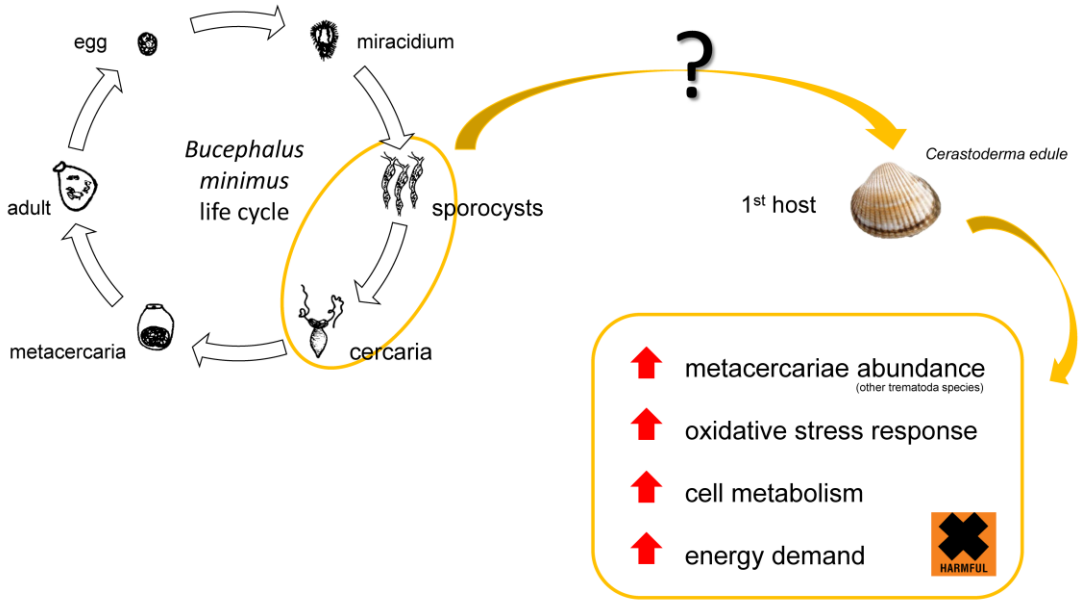
Figure 17 Schematic representation of the *Bucephalus minimus* hosts spatial distribution. Full black: *Dicentrarchus labrax* (final host); Black striped: *Pomastoschistus* spp. and *Atherina* spp. (second intermediate hosts); Full grey: *Cerastoderma edule* (first intermediate host); Black circles: reported sites for first host infection by *B. minimus*.

Final considerations

The review presented in the previous section allowed a better understanding of the relationship between trematode parasite and its bivalve first intermediate host, not only by compiling other authors studies on the subject but also bringing new important insights. The potential negative impact of the parasite on host was exacerbated by showing that for six out of seven trematode species using cockles as second intermediate host, metacercariae abundance per individual was 2 to 12 folds higher in *B. minimus* infected cockles. However, impact of trematodes on host populations is usually reported only when there is obvious disease symptoms or mass mortalities. Therefore, in the next section, cockles transcriptomic and biochemical responses were analysed together, an approach never used before, to assess the effects of sporocysts on host individual health, focusing at the sub-organism level. The potential of these techniques as predictive tools for disease outbreaks are discussed.

3.2. Seasonal variation of transcriptomic and biochemical parameters of cockles (*Cerastoderma edule*) related to their infection by trematode parasites

GRAPHICAL ABSTRACT



3.2.1. Introduction

Cerastoderma edule populations are distributed from the North Sea to West Africa and controlled by several abiotic factors, such as temperature (Beukema & Dekker, 2014) or hydrodynamic processes (de Montaudouin et al., 2003) but also by biotic interactions, such as predation (Masski & Guillou, 1999) and parasitism. Parasitism, although often understudied, is frequent and can seriously interfere with host population performance (de Montaudouin et al., 2014) leading to ecological and economic consequences (Lafferty & Hofmann, 2016).

C. edule is infected by several trematode species, four of them using this bivalve as first intermediate host (de Montaudouin et al., 2009) from which *Bucephalus minimus* is the most prevalent. The interaction between this parasite and *C. edule* has been proving to be highly deleterious with negative impacts at the growth (Bowers, 1969) and reproduction (Carballal et al., 2001) levels. When associated with other stressors, *B. minimus* increases cockle vulnerability, immunocompromising them and exacerbating parasite-dependent mortality (Morgan et al., 2012). Also, *B. minimus* infection in cockles was related with higher bacterial abundance (Meisterhans et al., 2011). Furthermore, in inorganic contamination experimental context, this parasite was

able to modulate metallothioneins activity and related gene expression (Paul-Pont et al., 2010c, Paul-Pont et al., 2012), decreasing their synthesis (Baudrimont et al., 2003; Baudrimont et al., 2006; Desclaux-Marchand et al., 2007).

Therefore, the present study focused on the *C. edule* (first intermediate host) – *B. minimus* (sporocyst) system, aiming to identify the consequences of heavy parasite infection in terms of cockle individual response as well as to recognize this infection influence in other associated trematode species abundance and diversity. The tested hypothesis was that the spread of *B. minimus* among tissues will compromise cockle regular gene expression, biochemical performance and increase their vulnerability to other parasite species. Thus, this work combined, for the first time, classic methods of host dissection for parasite identification to transcriptomic and biochemical analyses (taking into account seasonal patterns) in order to contribute for a better understanding of *B. minimus* effects on its first intermediate host, at the sub-organism level, when the sporocyst is well developed (i.e. easily observed). Quantitative real-time polymerase chain reaction (qRT-PCR) was the gene expression method used, a sensitive method requiring very small tissue samples (~40 mg). This analysis of gene transcription is one of the most robust tools of molecular biology and has been recently used in cockles to assess the effects of inorganic contamination (Karray et al., 2015a). In its turn, biochemical markers, namely indicators of oxidative stress, are powerful tools to determine the impact of several stressors on marine bivalves, including cockles (e.g. Freitas et al., 2012; Karray et al., 2015b; Marques et al., 2016; Velez et al., 2016b).

3.2.2. Material and Methods

3.2.2.1. Study area

C. edule individuals were collected in the inner part of Arcachon Bay, France (44°42'°N, 1°11'W) which is described in more detail in the section 2.2.1 of the previous chapter (cf. Figure 5). The sampling site is called Ile aux Oiseaux where sediments are classified as medium sand (grain-size median = 370 µm) (de Montaudouin et al., 2012b). Ile aux Oiseaux is situated at upper tidal level (+2.6 m above zero of chart datum, 50% immersion time). During the year, sediment temperature range is wide (min. = 0.2 °C, max. = 37.9 °C, mean = 16.1 °C) and salinity varies between 12.1 and 34.8 (mean = 29.6) (Dang et al., 2010).

3.2.2.2. Sampling strategy and parasite identification

Cockles were collected by hand every other month from January to November 2015 (6 sampling months). In the laboratory, 200 cockles from each sampling site were individually placed in plastic containers filled with seawater extracted directly from Arcachon Bay. After 12 h, these containers were analysed under a stereomicroscope in order to check the presence of free *B. minimus* cercariae emitted by the cockle in the water (here defined as P). However, cercariae can be absent but cockles still infected by immature *B. minimus* (sporocysts). Thus, for transcriptomic analyses and in order to find cockles not parasitized with *B. minimus* (here defined as NP), organisms not surrounded by swimming cercariae were individually measured and opened. The foot was extracted and squeezed between two glass slides to check sporocysts presence under a stereomicroscope. Specimens with no sporocyst observations were classified as negative or very moderately infected individuals (NP). The remaining tissues of the five first cockles found negative for *B. minimus* presence were immediately transferred into 500 µL of RNA later® and conserved at - 80 °C. Five out of the total cockles surrounded by *B. minimus* cercariae were also measured, opened and immediately transferred into 500 µL of RNA later® (the foot of these cockles was discarded to maintain the same conditions between P and NP samples) and preserved at - 80 °C.

For biochemical analyses, the remaining cockles (variable number = 200 – cockles surrounded by *B. minimus* cercariae – cockles dissected to find NP cockles used in transcriptomic analysis) were also measured (shell length (SL)) and preserved, entire at - 80 °C. In order to identify P and NP, these cockles were then cut in two equal parts using an electric hand saw without defreezing them. One half was used to check *B. minimus* sporocysts presence under a stereomicroscope and discarded, while the other half was kept at - 80 °C separated by sampling month and condition (P and NP) for further analysis.

To recognize all digenean trematodes present in each sampling month, more than the necessary number of cockles (over the 200) were collected. These cockles (a variable number between 50 and 179 depending on the cockle stock available) were measured, dissected, squeezed between two glass slides and observed under a stereomicroscope. All trematodes present (mainly metacercariae stage but also sporocyst and/or cercaria stage) were then identified to species level following the descriptions given by Bartoli et al. (2000), Bowers (1969), Bowers et al. (1996), de Montaudouin et al. (2009) and Desclaux et al. (2006).

3.2.2.3. Transcriptomic descriptors

3.2.2.3.1. Total RNA extraction and reverse transcription

Samples (60 cockles, corresponding to 10 per sampling month, 5 P and 5 NP), obtained and preserved as mentioned before, were homogenized 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.

3.2.2.3.2. Real-time quantitative PCR

Real-time PCR reactions were performed in a Lightcycler (Roche). The amplification program consisted of one cycle at 95 °C for 10 min and 50 amplification cycles at 95 °C – 5 s, 60 °C – 5 s, 72 °C – 20 s. Each reaction contained 17 µL of master mix including the SYBRgreen I fluorescent dye (Promega), 2 µL of the gene specific primer pair (final concentration 300 nM for each primer) and 1 µL of cDNA. Primers pairs were designed using the Lightcycler probe design software. With the aim of recognizing *B. minimus* effect on the cockle cell basic functions and then of comparing to biomarkers response, seven target genes were chosen: *cat, sod (Mn)* and *sod (Cu/Zn)* involved in the oxidative stress response, *coxI* and *12S* as part of the mitochondrial metabolism, *p53* responsible for the cell cycle regulation and *ras*, a cell growth cascade gene (Table 5). These are genes successfully used to stablish cause-effect responses in bivalve/disease interactions (Binias et al., 2014b) and to assess the effects of disseminated neoplasia (Ruiz et al., 2013).

Table 5 Nucleotide sequences of specific primer pairs used in this study. Abbreviations: a: upstream primer; b: downstream primer.

Gene	Accession number	Function	Sequence 5'-3'
β actin	EF520702	Cell structure (reference gene)	GCTACGTTGCCCTTGACT[a] CTGTTGTATGTGGTCTCATGGAT[b]
18S rRNA	EU660758	Ribosomal components (reference gene)	ATTGGAGGGCAAGTCTGGTG[a] GAAGGCCTGCTTTGAGGACT[b]
ef1α	JQ781170	Protein synthesis and degradation (reference gene)	GTCACATTCGCCCCTGC[a] GGTGCGTAGCCGTTTCG[b]
Cat	KY440113	Oxidative stress	GGCTGGTGAACGCTGAGGG[a] CTGAACTTCTCAGCTTCCTC[b]
coxI	JQ319608	Mitochondrial metabolism	GGTCGGCTTGGACGTAGA[a] CTCGCTAATACGACTCCAGTCA[b]
12S RNA	EF520704	Mitochondrial metabolism	AATACGGAAGTGTTGGGCG[a] AGAAGAATGGCGAAGCTCTTT[b]
sod (Mn)	HF947023	Oxidative stress (manganese)	CAGGGATCAGGATGGGG[a] GGCGTGCTCCCAGACATC[b]
sod (Cu/Zn)	HF947024	Oxidative stress (copper/zinc)	ATCTTGGCAATATAACTGCTGGT[a] GCGTGAACGACAAGTGTTCTAC[b]
p53	JN820317	Cell cycle arrest/apoptosis	TCTCGTCCGCTGCGAAC[a] TGACCCCAGGCACATGAA[b]
Ras	JN820318	Cell growth signalling cascade	ATGAGAACAGGAGAGGGGT[a] GGGAATCGTGTAACTATCGGC[b]

Relative quantification of each gene expression level was normalized according to the reference genes ***β actin***, ***18S rRNA*** and ***ef1α*** and generated using the $2^{-\Delta CT}$ method described by Livak & Schmittgen (2001) where ΔCT represents the difference between the cycle threshold of a specific gene and the cycle threshold of the reference genes. Inductor factor (IF) of each gene was determined in comparison with control (= without *B. minimus*) corresponding to the following equation (Paul-Pont et al., 2010c):

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

3.2.2.4. Biochemical descriptors

Samples, obtained and preserved as mentioned before, were pooled in groups of 4 accounting for 10 samples (corresponding to 40 halves of cockles), whenever possible, by condition (P and NP) and by sampling month. Each sample was homogenised with liquid nitrogen and separated into 0.3 g of soft tissue subsamples in

order to perform the extraction with specific buffers for each biochemical parameter (described in Carregosa et al., 2014). After buffer addition, all subsamples were centrifuged (10,000 g) for 20 min at 4 °C, supernatants were reserved and stored at -20 °C or used immediately.

With the aim of recognizing cockles biochemical alterations induced by *B. minimus* infection, five biomarkers were selected: protein (PROT) and glycogen (GLY) contents as energy reserves measure; superoxide dismutase (SOD) and catalase (CAT) activities, antioxidant enzymes involved in the oxidative stress response, and lipid peroxidation (LPO) levels as cellular damage indicator. These are biomarkers successfully used to assess the negative impact of several stressors in marine bivalves, including cockles (e.g. Freitas et al., 2012; Marques et al., 2016).

PROT content was determined according to Robinson & Hogden (1940), following the Biuret method that uses Bovine serum albumin (BSA) as standard (0 – 40 mg mL^{-1}). After 10 min incubation at 30 °C the absorbance was read at 540 nm. The results were expressed in mg and used to calculate enzymes activity.

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

The activity of SOD was measured using the method described by Beauchamp & 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 U per mg PROT. One U corresponds to a reduction of 50 % of Nitro blue tetrazolium (NBT).

The activity of CAT was measured by the reaction of the enzyme with methanol in the presence of H_2O_2 (Johansson & 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 unit of enzyme (U) per mg PROT. One U is defined as the amount of enzyme that generated the formation of 1.0 nmol formaldehyde, per min.

LPO levels were measured by the quantification of thiobarbituric acid reactive substances (TBARS), according to Buege & 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.

3.2.2.5. Data analysis

One-way ANOVA, followed by a Tukey post hoc analysis, was used to test differences between mean SL of the sampled cockles used to calculate *B. minimus* prevalence per sampling month.

B. minimus prevalence was calculated with two methods: 1) considering parasitized cockles (P), those surrounded by free cercariae, and non-parasitized cockles (NP), those with no cercariae in the surrounding water, here identified as emergence prevalence and 2) considering P cockles, those checked at the stereomicroscope and found positive for *B. minimus* sporocysts presence, and NP cockles, those checked at the stereomicroscope and found negative for *B. minimus* sporocysts presence, identified as dissection prevalence. Then, to assess differences between *B. minimus* prevalence, a Student *t*-test was performed for mean emergence prevalence vs. mean dissection prevalence.

The analysis of the associated trematode community (metacercariae) using cockle as second intermediate host and the presence/absence of *B. minimus* was performed taking only *Himasthla* spp. into account since this species complex alone accounted for 97.6 % of the total metacercariae abundance. Then, differences between P and NP cockles in terms of *Himasthla* spp. abundance were checked overall and per sampling month with two paired Wilcoxon test. For each P cockle, a pair was constituted by randomly selecting a NP cockle of the same SL and of the same sampling date. Differences among sampling months, both for P and NP cockles, were tested with a one-way ANOVA followed by a post hoc analysis (Tukey test).

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

These statistic analyses were performed with STATISTICA (StatSoft) software and for all parametric tests, homogeneity of variances was checked with Cochran test while normality was assumed.

Furthermore, three Principal Coordinates Ordination analysis (PCO) were separately performed using the software PRIMER-E: non-parasitized cockles, parasitized cockles and parasitized vs. non-parasitized cockles. 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 explained (r > |0.8|) the samples spatial distribution were represented as superimposed vectors.

3.2.3. Results

3.2.3.1. *Bucephalus minimus* infection and associated trematode metacercariae community

The mean SL of sampled cockles was higher (one-way ANOVA: $F = 18.4$, $p < 0.001$) in January (24.5 ± 2.2 mm) comparing with the remaining months (Figure 18A). For the other sampling months, SL fluctuated between 20.1 ± 1.2 and 21.9 ± 3.0 mm (Figure 18A). There was no difference, for each month, between P and NP cockle SL (one-way ANOVA: $F = 3.1$, $p = 0.08$).

B. minimus prevalence calculated by emergence technique was on average five fold lower than dissection prevalence (Student *t*-test: $t = -3.8$, $p = 0.004$) (Figure 18A). *B. minimus* dissection prevalence fluctuated between 12% in November and 61% in January (Figure 18A). Prevalence was considered high also in May (40%) and July (43%). High prevalence (over 40% in January and July) was followed by a prevalence decrease the consecutive month.

During the whole study period, cockles were parasitized by eight different trematode species: three infecting cockles as first intermediate host, *B. minimus*, *Gymnophalus choledochus* and *Monorchis parvus* (identified by the sporocyst stage); and five infecting cockles as second intermediate host (identified by the metacercariae stage), *Curtuteria arguinae* (total abundance = 5 metacercariae per cockle), *Diphterostomum brusinae* (total abundance = 8), *Himasthla interrupta* (total abundance = 12)*, Himasthla* spp. (a complex *H. quissetensis*/*H. continua*, total abundance = 1059) and *Psilostomum brevicolle* (total abundance = 1).

Overall, comparing P and NP cockles in terms of *Himasthla* spp. abundance (because represented 97.6% of the total metacercariae abundance), there was a higher metacercariae abundance in the presence of *B. minimus* (Wilcoxon test: $W = 2524, 2.8$, $p = 0.006$). Significant differences between mean *Himasthla* spp. abundance for P and NP cockles were found in January, July and November (Figure 18B). The seasonal trend was the same for both P and NP cockles with significantly higher *Himasthla* spp. abundance in July, September and November comparing to the 3 other months (Figure 18B). For all sampling months, trematode species richness was similar (Wilcoxon test: $W = 630, 1.8$, $p = 0.08$) between P (0.9 ± 0.05 species per cockle) and NP (0.8 ± 0.05 species per cockle) cockles.

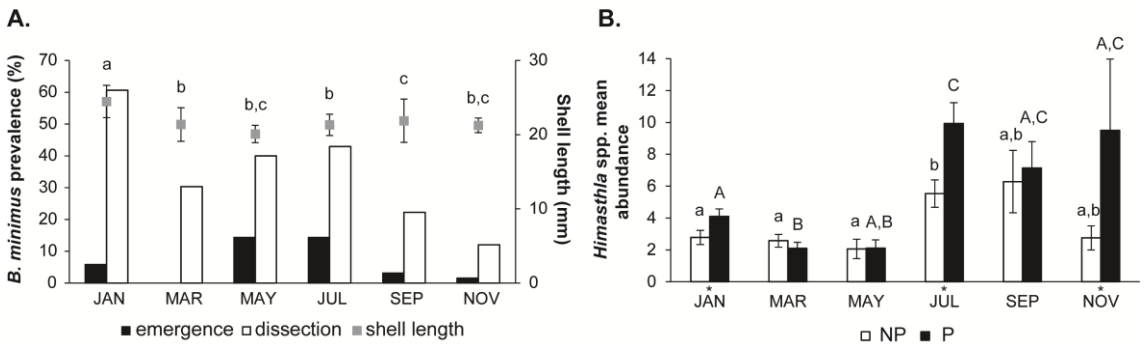


Figure 18 A. Seasonal trend of the *Bucephalus minimus* prevalence checked by two methods (cercariae emergence after 12 h and dissection) along with the mean shell length (± standard deviation) of the sampled cockles and respective significant differences represented with lower case letters; **B.** Seasonal trend of *Himasthla* spp. mean abundance (± standard error) for non-parasitized (NP) and parasitized (P) cockles with *B. minimus*. Significant differences among months are represented with lower (NP) and upper (P) case letters; significant differences between NP and P for each month are represented with an asterisk (*).

3.2.3.2. Transcriptomic data

Transcriptomic analysis showed a constant significant induction of *cat* expression in the presence of *B. minimus* (from March to September) (Table 6).

Results also demonstrated that *coxI* and *12S* were over-expressed only in July, when there was a significant induction of these genes for P cockles compared to NP individuals. On the contrary, *12S* in P cockles was inhibited in November compared to NP individuals.

Sod (*Mn*) and *sod* (*Cu/Zn*) genes did not show expression alterations influenced by the presence of *B. minimus* except in September when a significant under-expression of *sod* (*Cu/Zn*) was observed.

P53 gene showed inhibition of expression level in November and *ras* gene showed no significant differences between P and NP in all sampling months.

Table 6 Comparative basal expression expressed in terms of induction factor for the selected genes from parasitized and non-parasitized cockles (only significantly different expression is represented). Expression ratio not significantly different: —; inhibition: expression ratio < 0.5; induction: expression ratio >2.

	Jan	Mar	May	Jul	Sep	Nov
cat	—	3.2	8.6	3.3	3.0	—
coxI	—	—	—	2.9	—	—
12S	—	—	—	2.6	—	0.1
sod (Mn)	—	—	—	—	—	—
sod (Cu/Zn)	—	—	—	—	0.2	—
p53	—	—	—	—	—	0.1
ras	—	—	—	—	—	—

3.2.3.3. Biochemical data

Taking into account NP cockles, results showed a seasonal trend characterized by significantly lower GLY content from January to May compared to July, September and November months (Figure 19A). Similar results were obtained for P cockles except for September when a low value was displayed (Figure 19A). The global trend showed lower GLY values in P cockles, with significant difference to NP in March and July.

SOD activity for NP cockles was higher in March than in all other sampling months (Figure 19B). Parasitized cockles showed significantly lower SOD activity in January, July, September and November with higher values in March and May (Figure 19B). The global trend was higher SOD values in P cockles comparing with NP, significant in May and November.

Regarding CAT, the activity of this enzyme showed no significant difference among sampling months for P and NP cockles respectively (Figure 19C), and between P and NP cockles for each sampling month.

LPO values showed a similar trend for both P and NP cockles and were significantly higher in March and May (Figure 19D). In 4 out of 6 sampling months, there were significant differences between P and NP cockles, but without a defined pattern.

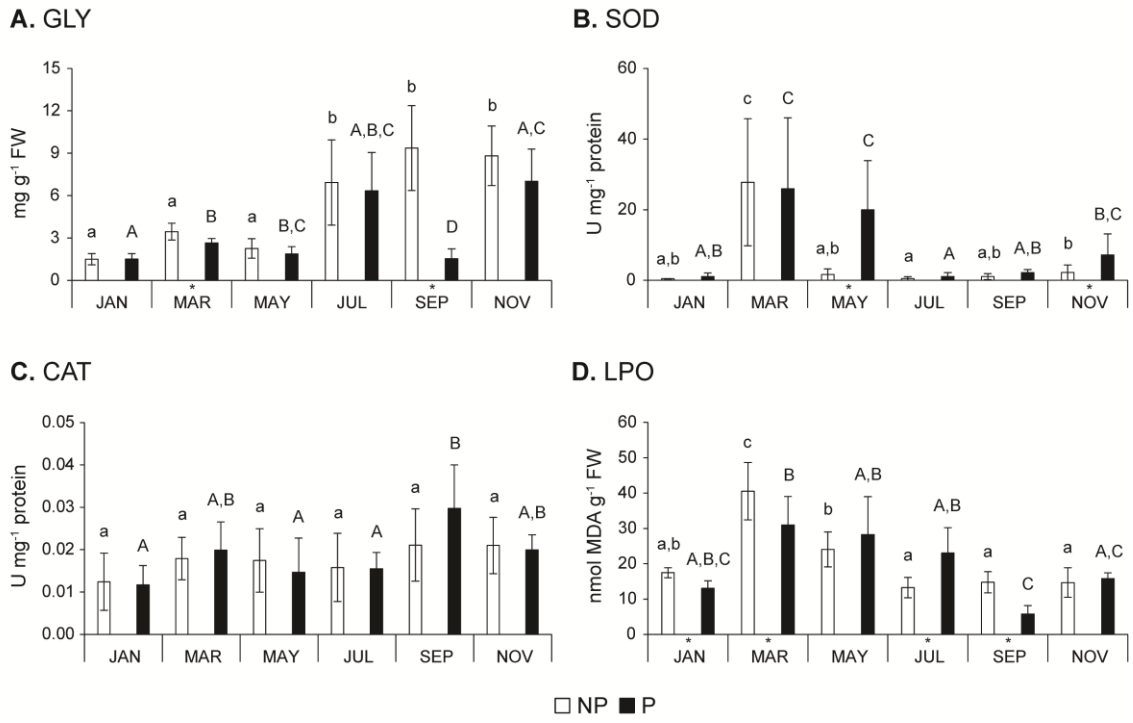


Figure 19 A. GLY, glycogen content; **B.** SOD, superoxide dismutase activity; **C.** CAT, catalase activity; **D.** LPO, lipid peroxidation levels mean values (± standard deviation) in cockles parasitized (P) and non-parasitized (NP) with *Bucephalus minimus* during one year of every other month sampling. Significant differences among months are represented with lower (NP) and upper (P) case letters; significant differences between NP and P for each month are represented with an asterisk (*).

The horizontal dimension (Axis 1) of the PCO with NP cockles explained 57% of the total variation separating July, September and November, in the negative side of the axis, from January, March and May, in the positive side of the axis. SOD activity and LPO levels were the variables that better explained this variation presenting high positive correlation with axis 1 ($r > 0.8$) as well as GLY content that presented high negative correlation with axis 1 ($r > - 0.8$). Axis 2 explained 38% of the total variation which separated March, September and November in the positive side of the axis, from January, May and July with a strong positive correlation to CAT activity ($r > 0.8$) (Figure 20A).

The axis 1 of the PCO representing the P samples explained 49% of the total variation separating March and May, in the negative side of the axis, from July, September, November and January, in the positive side of the axis. SOD and LPO presented high negative correlation ($r > - 0.8$) with axis 1. Axis 2 explained 30% of the total variation separating March, May and September, in the negative side of the axis,

from January, July and November with a strong positive correlation to GLY content (r > 0.8) (Figure 20B).

The axis 1 of the PCO concerning P vs. NP samples explained 51% of the total variation separating P and NP cockles of March and May in the negative side of the axis, from the other conditions in the positive side of the axis. SOD activity and LPO levels were the variables that better explained the variation, presenting high positive correlation (r > 0.8) with axis 1. The PCO vertical dimension (Axis 2) explained 26% of the total variation separating P and NP cockles of January, May and July, in the negative side of the axis, from the other conditions with a strong positive correlation to CAT activity (r > 0.8) (Figure 20C).

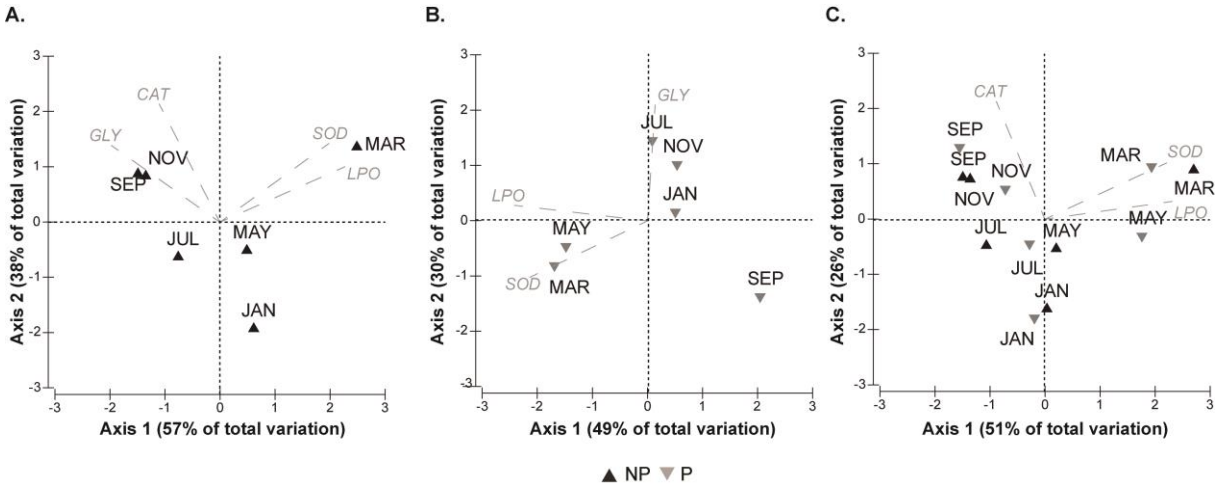


Figure 20 Principal coordinates ordination analysis (PCO) showing the variables that better explained samples distribution for **A.** Non-parasitized (NP) *Cerastoderma edule* cockles; **B.** Parasitized (P) cockles; **C.** NP vs. P cockles. GLY: glycogen content; SOD: superoxide dismutase; CAT: catalase; LPO: lipid peroxidation.

3.2.4. Discussion

In general, and bivalves are no exception, the study of 'trematode parasite - first intermediate host' relationship is a difficult issue to address due to the low prevalence of this parasite (except during some exceptional outbreaks that can cause massive mortalities). Therefore, the study of trematode effects on cockle fitness remains poorly explored, especially in what regards to the impacts on cockle gene expression and biochemical performance. Despite these limitations, efforts were already done to understand the influence of *B. minimus* infection on the classic metal contamination biomarkers response, revealing that metallothionein gene expression and protein concentrations can give false signals induced by this parasite presence (Desclaux-

Marchand et al., 2007). The present study represents the first attempt to investigate the concomitant effect of *B. minimus* on gene expression and biochemical markers on its first intermediate host (*C. edule*), over a year monitoring considering seasonal changes.

In terms of methodology, this work confirmed that host dissection is a much more reliable way to determine parasite prevalence (Curtis & Hubbard, 1990) than parasite shedding technique. Cockles can be infected with no cercariae emergence: after miracidium penetration, there is a gradual growth and maturation of the sporocyst mass that, after a still unknown period (prepatent infection), starts to release cercariae (patent infection). The parasite patency is correlated with the development of sporocyst in the host. Miracidium metamorphosis in its first intermediate host can be obtained within 12 h (Graczyk & Fried, 2001) but then, the delay between miracidium infestation and cercariae emission remains unknown due to difficulty (and failure) in experimental infestation (Maillard, 1976). However, this author succeeded in infesting first intermediate host with miracidium larvae of other trematode species, having obtained developed sporocyst (but still prepatent) within 2-3 months and cercariae (patent infection) after 2 to 5 months. With Echinostomatid trematodes, patent infection can even be reached in less than 8 weeks (Fried & Huffman, 1996; Muñoz-Antoli et al., 2007).

In the present study, the biomass of *B. minimus* was not measured, however it is known from the literature that it may vary between 2 and 25% of total flesh dry weight (Dubois et al., 2009). The highest *B. minimus* emergence prevalence registered was in May and July (14%) and the highest *B. minimus* dissection prevalence was in January (61%) followed by high values in May and July (40 and 43%, respectively). Knowing that *B. minimus* prevalence is positively correlated with first intermediate host SL (Magalhães et al., 2015), we can argue that the highest prevalence registered for January can be explained with the size (and age) of the collected cockles which were significantly larger at this month. The high prevalence values recorded in May and July, the spring/summer periods, allow us to predict a seasonal trend of *B. minimus* visible prevalence possibly moderated by the temperature raise experienced during this period of the year, an important factor stimulating the parasites life cycle (de Montaudouin et al., 2016). Seasonal trends in parasite infection were also found for other trematode species although using cockles as second intermediate host (e.g. de Montaudouin et al., 2016) but contested by Magalhães et al. (2015) in a *B. minimus* - *C. edule* long-term monitoring. The study by Magalhães et al. (2015) was based on the same parasite – host system but it was conducted in a different area of Arcachon Bay characterized by a much lower *B. minimus* visible (when sporocyst is already

developed) prevalence (between 2.2 and 15.6%). Furthermore, when *B. minimus* prevalence was higher than 40 % (January and July), the following sampling month (March and September respectively) displayed a lower prevalence. This fact suggests occurrence of cockle *B. minimus* dependent mortality, highlighting the deleterious effect of this parasite on its host as demonstrated elsewhere by Thieltges (2006) with a parasite species that also uses *C. edule* as first intermediate host. Finally, we must admit that in the present study, the term 'prevalence' rather referred to cockles with obvious sporocyst presence. Indeed, it was certainly impossible to detect *B. minimus* in very recently infected (by miracidium) cockles which were considered NP. In other words, the difference between P and NP was mainly related to expected disease severity.

The present study also revealed that metacercariae abundance, namely *Himasthla* spp., was significantly higher in the presence of *B. minimus* infection, as already referred by Magalhães et al. (2015) with long-term data. Sannia & James (1978) proposed that parasitism may increase susceptibility to other trematode species but, the difficulty remains to identify which parasite weakens first its host, *B. minimus* (sporocyst) or *Himasthla* spp. (metacercariae). Some arguments tend to privilege the hypothesis that metacercariae act first. Firstly, in terms of phenology, infection of cockles by *Himasthla* spp cercariae occurs very early in cockle life, i.e. in 2-4-mm SL individuals (de Montaudouin et al., 2005), while infection by *B. minimus* starts in 16-mm SL individuals (Magalhães et al., 2015). Secondly, infection by cercariae is dependent on cockle filtering activity and consequently on cockle fitness. Considering that *B. minimus* alters cockle fitness, the post-*B. minimus* infection of cockles should decrease the number of inhaled cercariae (de Montaudouin et al., 1998) and consequently the number of settled metacercariae. The process by which metacercariae affect cockles health is putative but, in other species, may involve molecular (Gorbushin et al., 2009; Loker & Bayne, 2001), immunological (Paul-Pont et al., 2010c) and physiological pathways (de Montaudouin et al., 2012a).

In order to understand transcriptomic and biochemical results it is important to note that in a bivalve immune system, when invaders are too large, the typical phagocytosis mechanism fails (Allam & Raftos, 2015). In this case, granulocytes (a type of hemocytes) are recruited in large number to surround and encapsulate the pathogen. Granulocytes, due to their higher lysosomal enzymes content than other kinds of hemocytes, are more efficient to eliminate pathogens producing consequently more reactive oxygen species (ROS) (Soudant et al., 2013). It has been shown in several studies that when ROS dramatically increase, they can cause cellular damage in organisms, including bivalves (e.g. Marques et al., 2016). This type of defence

mechanism that uses granulocytes although it failed in ark cockle (*Anadara trapezia*) hemocytes experimentally exposed to trematode sporocysts (Kawasaki et al., 2013), was found in mussels infected by trematodes (Huehner & Etges, 1981) and could be the same mechanism present in cockles infected by *B. minimus*. The present study used gene expression and biochemical markers in order to recognize *B. minimus* effects on cockles.

The obtained results showed gene expression changes induced by the presence of *B. minimus*, independently on the sampling month. In detail, *cat* (gene involved in the oxidative stress response) expression was induced due to the presence of the parasite during the entire year but especially during the warmer seasons (albeit without significant differences neither among sampling months nor between parasite condition in CAT activity). However, *sod* genes expression, another component involved in the oxidative stress response, was not significantly different among sampling months neither between parasite conditions. It has already been proved that the increase of *cat* expression represents an important defence mechanism against oxidative stress in bivalves (e.g. *Crassostrea gigas* exposed to metolachlor, a chloroacetanilide herbicide (Mai et al., 2014)). The present findings may indicate that cockles in the presence of *B. minimus* stressor tend to over-express *cat* genes in order to avoid oxidative stress damages (decreasing ROS levels). We can hypothesise that this response tended to be enhanced when cockles were facing more than one stressor at the same time (from March to September): i) first, during March and May cockles were under gametogenesis and gonad development (gametogenesis in February/March and gonad development in April/May (Malham et al., 2012)) which activates bivalve respiratory activity enhancing ROS production (Guerra et al., 2012; Soldatov et al., 2008); ii) then, in July and September, cockles were experiencing abiotic conditions shifts during the warm season, namely higher temperature. Studies conducted with mussel species and microarray techniques showed that high temperatures induced the expression of genes involved in the oxidative stress response (Lockwood & Somero, 2011; Lockwood et al., 2010).

CoxI gene encodes the enzyme cytochrome c oxidase or complex IV responsible for the transfer of electrons from reduced cytochrome c to molecular oxygen in the mitochondrial respiratory chain. *12S* is the small mitochondrial ribosomal RNA, with a sequence highly conserved, used in the present study to quantify mitochondria. In July, *coxI* and *12S* were clearly over-expressed in *B. minimus* parasitized cockles compared to non-parasitized individuals. This result suggests a stimulated mitochondrial activity and consequently an increase in the cell metabolism and energy demand when cockles were under more than one stressful condition:

abiotic factors (mainly higher temperatures) and parasitism. Infected organisms showing up-regulation of mitochondrial metabolism involved genes in summer was also found by Binias et al. (2014a) in *Ruditapes philippinarum* clams impacted by Brown Muscle Disease (etiological agent still unknown).

P53 is a gene involved in the cell cycle arrest and apoptosis (Levine, 1997), while *ras* is a gene responsible for the encoding of small membrane-bound proteins that play a central role in the cell growth signalling cascade (Buday & Downward, 2008). In the present study, these genes were up-regulated in March (and in July for *ras*), although without statistically significant differences, which may reveal some impact of ROS (caused by the parasite presence) although the antioxidant (*cat* gene) defence system was activated. Binias et al. (2014a) also demonstrated up-regulation of apoptosis related genes (*bax*), in *R. philippinarum* infected by Brown Muscle Disease, even in the presence of oxidative stress related genes induction (*cat* and *sod*). *P53* and *ras* also showed higher transcriptional levels in cockles with neoplasia compared to healthy ones (Ruiz et al., 2013). The inhibition of both genes observed in May (although with no significant differences among parasite conditions) and in November for *p53* in parasitized cockles compared to cockles without *B. minimus* can be related to the extreme harmful effects on cells caused by both parasite infection and probably gametogenesis and/or gonad development occurring during the first mentioned period. To support this hypothesis and as an example, human patients suffering acute lymphatic and myelogenous leukaemia also showed low transcriptional levels of *ras* gene (Mavilio et al., 1986).

In this study, biochemical markers response tended to be more sensitive to seasonality changes than to the presence of *B. minimus*. The seasonal trend showed lower GLY content, SOD activity and LPO levels in January suggesting a decrease in metabolic activities probably as consequence of adverse environmental conditions (such as low temperature, low salinity and less quality and/or quantity of food). As it was described by Newell & Bayne (1980), winter conditions tend to induce low metabolic rate in *C. edule* organisms. In *C. glaucum* from Tunisia, a low GLY content in winter was also observed (Karray et al., 2015b). We can argue that, as it was reported for *R. philippinarum* (Anacleto et al., 2014) this decrease of metabolic rate is related to bivalves strategy of closing their valves when under stressful conditions in order to enhance survival. During March and May, low values of GLY content were followed by an increase in SOD activity (in March for both conditions and in May for parasitized cockles) and LPO values suggesting a relationship with the coincident reproductive period. *C. edule* is a conservative species (termed by Bayne (1976)) therefore gametogenesis depends on the amount of GLY stored. As demonstrated by Karray et

al. (2015b), *C. glaucum* stores GLY to be later used during gametogenesis or/and as energy source which could be the case of *C. edule* in the present study. As already mentioned for gene expression, respiratory activity linked to gametogenesis in bivalves and the consequent ROS production may be the reason of having an increase of oxidative stress enzymes activity (SOD). Still, the activity of this enzyme was not sufficient to prevent cellular damage measured by LPO (higher cellular damage). Finally, in July – November period, GLY content increased (except for September parasitized cockles) and SOD activity and LPO values decreased suggesting the existence of less harmful abiotic conditions that allowed cockles to decrease antioxidant defences and to accumulate energy (higher GLY content at these months).

During the whole study period, CAT activity remained stable with two possible reasons: (1) there are other detoxification mechanisms degrading hydrogen peroxide produced by SOD activity, such as glutathione peroxidase (Regoli & Giuliani, 2014) which was not quantified in this study; alternatively or simultaneously (2) since *cat* gene is maintained highly expressed in cockles with *B. minimus* and especially during warmer seasons, CAT basal activity can be maintained stable with no significant differences between conditions (hypothesis never demonstrated for *C. edule* or any bivalve). However, straight correlations between mRNA expression and protein concentration or enzyme activity are still poorly studied, with controversial results but certainly dependent on post-transcriptional, translational and protein degradation related processes (Vogel & Marcotte, 2012).

Taking into account significant differences between P and NP cockles, and regarding biochemical markers there was a tendency for P cockles to present less energy reserves than non-parasitized individuals (significant in 2 particular months: March and September). As already demonstrated through *coxI* and *12S* gene expression results, parasitized cockles have a stimulated mitochondrial activity which increases cellular metabolism and energy demand. At the same time, because *B. minimus* consumes energy in the digestive gland (Dubois et al., 2009), this could be another factor causing lower GLY content. Infected cockles also presented higher SOD activity (significant for 2 months in particular: May and November) and an effect on LPO level (with different directions according to months). These results suggest that, as shown with *cat* gene (oxidative stress response) expression results, as well as with *p53* and *ras* (cell apoptosis and cell growth cascade, respectively), the parasite presence can cause oxidative stress or even severe cellular damage. As an example, Belló et al. (2000) obtained similar results showing that the freshwater fish *Rhamdia quelen* parasitized by *Clinostomum detruncatum* as second intermediate host (metacercariae) presented increased LPO levels.

Conclusion

This study showed that *B. minimus* has a negative effect on its first intermediate host *C. edule* demonstrated mainly through the influence on gene expression but also from the biochemical markers response (although without significant differences). The present findings showed that transcriptomic and biochemical markers can provide additional and ecologically relevant information about 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. Presently, more attention should be focused on the status of sporocyst in their first intermediate host, in terms of patency. Even though, pathological effects of parasites have been observed at both pre- and post-patent periods (Zakikhani & Rau, 1999), their intensity can vary according to the period (Muñoz-Antoli et al., 2007).

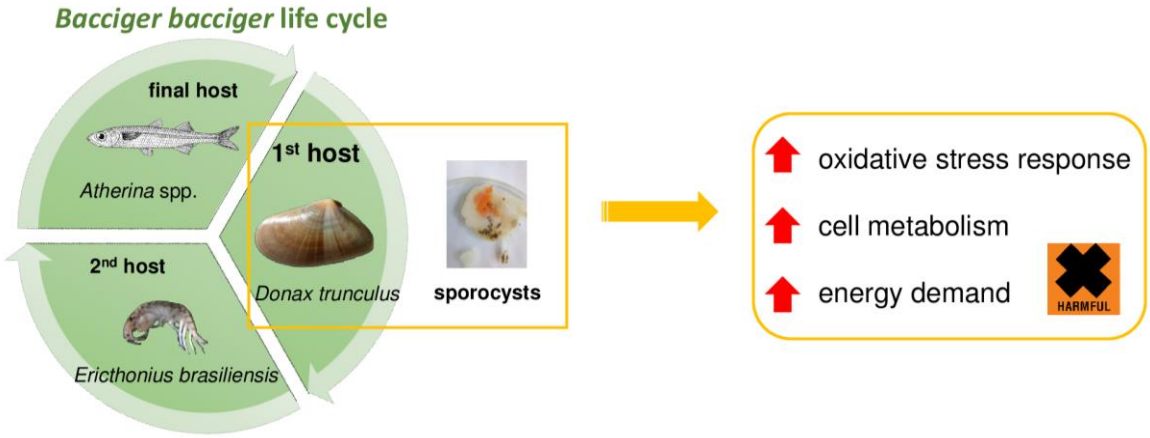
Final considerations

In the previous section, transcriptomic and biochemical tools were successfully used to assess *B. minimus* effects on cockle stress response and showed that this parasite compromises its first host regular gene expression and biochemical performance and increases its vulnerability to other parasite species infection. To test the specificity of the obtained results, another host-parasite system was selected, constituted by the first host *Donax trunculus* (the wedge clam) and the trematode parasite *Bacciger bacciger*. Thus, the next chapter section is devoted to assess parasite infection effect on the wedge clam health status, a much less studied model but still with high ecological and economic importance. The same approach of combining transcriptional and biochemical tools was applied. Results obtained for both bivalve species will be further compared.

Donax trunculus (the wedge clam)

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

GRAPHICAL ABSTRACT



3.3.1. Introduction

The wedge clam (*Donax trunculus*) is widely distributed along moderately exposed beaches in the Atlantic coast, from France to Senegal (Tebble, 1966). This species has high commercial importance, with the mean capture production on the last ten years of approx. 850 tons (50% represented by Portugal captures (FAO, 2006-2018)). *D. trunculus* populations are modulated by several drivers such as tidal range (Gaspar et al., 2002), temperature (Botelho et al., 2018), sediment grain size (de la Huz et al. 2002, La Valle et al. 2011), fishing pressure (Marie et al., 2016) and parasitism. Regarding parasitism, *D. trunculus* is the first intermediate host (Palombi, 1934a), where the sporocysts develop, of the trematode *Bacciger bacciger*. The sporocyst is the most damaging stage of a trematode life cycle, reported as responsible for bivalve castration and flesh mass depletion (de Montaudouin et al., 2014; Ramón et al., 1999).

Therefore, 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 oxidative stress both at transcriptomic (antioxidant response related genes) and biochemical (metabolic activity, energy reserves, antioxidant enzymes and cellular damage) levels, taking into account

seasonal patterns. The tested hypothesis was that the spread of *B. bacciger* among tissues will compromise clams regular gene expression and biochemical performance.

3.3.2. Material and methods

3.3.2.1. 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). This is a narrow reflective beach (Balouin et al., 2005) located in the Ancão Peninsula of the Ria Formosa coastal lagoon, south of Portugal (Figure 21). 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. Portuguese annual official landings in 2016 was of 252 tons with the sales value of 750 thousand euros (INE, 2016).

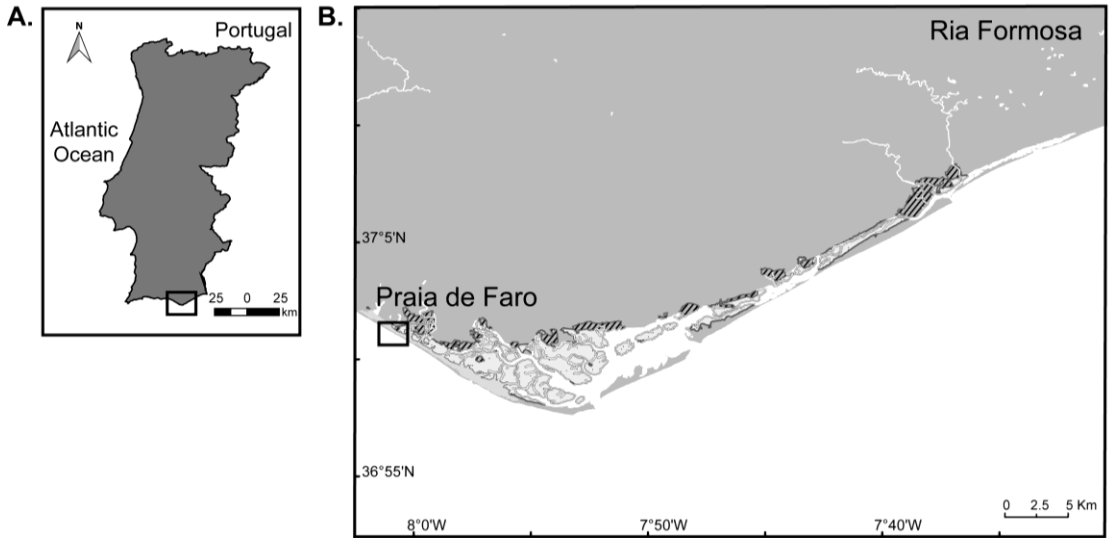


Figure 21 Study area. The Ria Formosa (South Portugal (A)) indicating the Faro beach location, where the wedge clams were collected (B).

3.3.2.2. Sampling strategy and parasite identification

Every other month, from March 2016 to January 2017 (6 sampling months), *D. trunculus* were collected using a hand-dredger (Figure 22A) which is composed by a rectangular shape metal grid (Figure 22B), a 2.8 cm mesh size bag (where the catch is retained) and a fixed wood beam (Figure 22C). 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 h, 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. *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. *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.

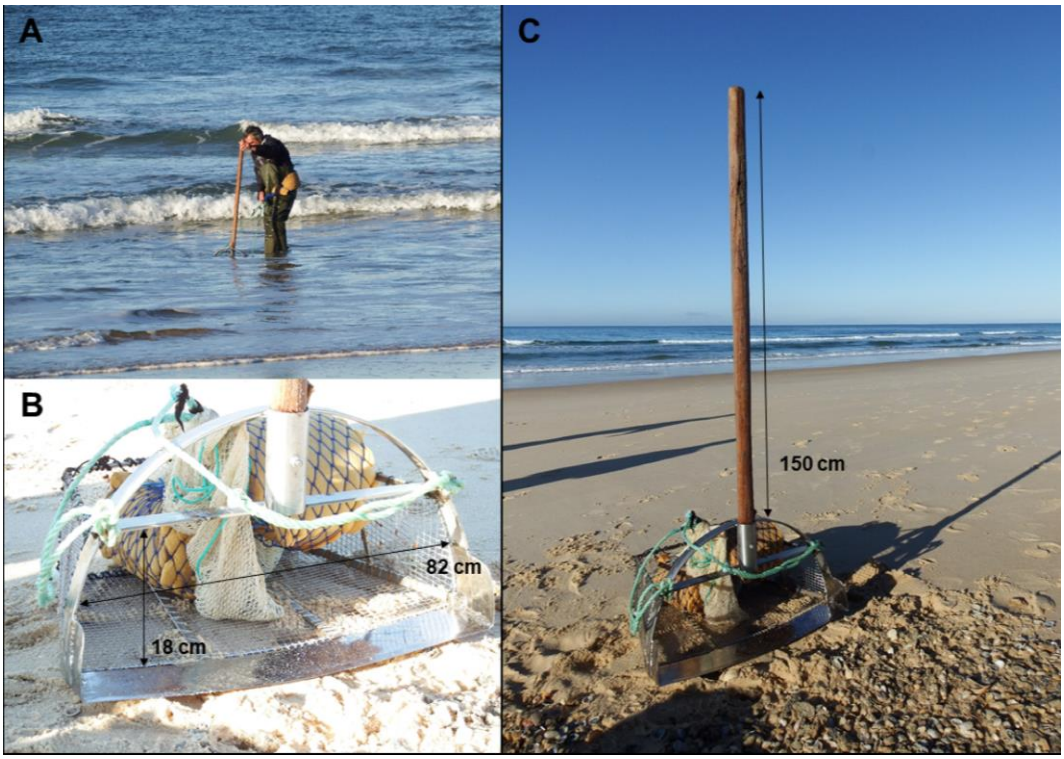


Figure 22 *Donax trunculus* sampling method: **A.** Fisherman handling the Portuguese hand-dredger; **B.** Details of the rectangular shape metal grid that composes the hand-dredger; **C.** General view of the Portuguese hand-dredger.

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.

3.3.2.3. Transcriptomic descriptors

3.3.2.3.1. Total RNA extraction and reverse transcription

The gill and digestive gland of two *D. trunculus* as well as the samples obtained and preserved as mentioned before, 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.

3.3.2.3.2. 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 1% agarose gels and fragments of the expected sizes were excised and purified using Wizard SV Gel and PCR Clean-Up System (Promega), according to manufacturer instructions. The resulting products were commercially sequenced (Sigma-Aldrich) and submitted to the GenBank. Partial cDNA sequence of *ef1α*, *β actin*, *sod (Mn)* and *cat* genes were successfully isolated using primers derived from conserved regions of the respective sequences.

3.3.2.3.3. Primer design

With the aim of recognizing *B. bacciger* effect on the wedge clam cell basic functions, two target genes were chosen: *cat* and *sod (Mn)* involved in the oxidative stress response. *Ef1α* and *β actin* were chosen as reference genes. This is the first study of gene expression using wedge clams and, therefore, gene sequences from close related species, available in databases, were aligned using the Clustal Omega free software. From this alignment and for each gene, one forward and one reverse primer were deduced in conserved regions. All primer pairs used in this study are listed in Table 7.

Table 7 Nucleotide sequences of specific primer pairs used in this study. a: upstream primer; b: downstream primer.

Gene	Function	Sequence 5'-3'
ef1α	Protein synthesis and degradation (reference gene)	TCCCACTCCAGGACGTTTAC[a] TCCTGGGAGAGCTTCTGGTA[b]
β actin	Cell structure (reference gene)	CCCACACCGTACCCATCTAC[a] GGGCAACATAGCAGAGCTTC[b]
sod (Mn)	Oxidative stress (manganese)	GCATCTTCTGGCAAGTCCTC[a] GAGAGCGTCCTGATTTGCTC[b]
Cat	Oxidative stress	TGACCAGGGCATTAAGAACC[a] AGCACCATCTTACCCACAGG[b]

3.3.2.3.4. Real-time quantitative PCR

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

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

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

3.3.2.4. Biochemical descriptors

Each replicate was homogenised with liquid nitrogen and separated into 0.3 g of soft tissue subsamples in order to perform the extraction with three different buffers. With the aim of recognizing clams biochemical alterations induced by *B. bacciger* infection, six biomarkers were chosen: electron transport system (ETS) activity, used as a metabolic capacity measure; protein (PROT) and glycogen (GLY) contents as energy reserves measure; superoxide dismutase (SOD) and catalase (CAT) activities, antioxidant enzymes involved in the oxidative stress response, and lipid peroxidation (LPO) levels as cellular damage indicator. Some of these biomarkers (namely CAT, GLY and LPO) were successfully used to assess the negative impact of metal contamination in wedge clams from Algeria and Tunisia (Amira et al., 2018; Tlili et al., 2010; 2013).

Supernatant of the subsample extracted with phosphate buffer (50 mM KH_2PO_4 (pH 7.0), 1 mM EDTA, 1% (v/v) Triton X-100, 1% (w/v) PVP, 1 mM DTT, 1:2 w/v) and centrifuged at 4 °C, 10,000 g during 20 min was used to determine GLY and PROT contents, as well as SOD and CAT activities. Supernatant of the subsample extracted with 0.1 M Tris-HCl (pH 8.5), 15% (w/v) Poly Vinyl Pyrrolidone, 153 µM $MgSO_4$ and 0.2% (w/v) Triton X-100 buffer and centrifuged at 4 °C, 3,000 g during 20 min was used to determine ETS activity. Supernatant of the subsample extracted with 20% (w/v) trichloroacetic acid (TCA) and centrifuged at 4 °C, 10,000 g during 20 min was used to determine LPO levels. All supernatants were then reserved and stored at - 20 °C or used immediately.

The activity of ETS was determined by the amount of formazan formed after adding p-IodoNitroTetrazolium (De Coen & Janssen, 1997), calculated using ε = 15.9×10^3 M^{-1} cm^{-1} and expressed in nmol of formazan formed per min per g of fresh weight (FW).

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

The PROT content was determined according to Robinson & Hogden (1940), following the Biuret method that uses Bovine serum albumin (BSA) as standard (0–40 mg mL^{-1}). After 10 min incubation at 30 °C the absorbance was read at 540 nm. The results were expressed in mg and used to calculate enzymes activity.

The activity of SOD was measured using the method described by Beauchamp & 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 PROT. One U corresponds to a reduction of 50 % of Nitro blue tetrazolium (NBT).

The activity of CAT was measured by the reaction of the enzyme with methanol in the presence of H_2O_2 (Johansson & 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 PROT. One U is defined as the amount of enzyme that generated the formation of 1.0 nmol formaldehyde, per min.

LPO levels were measured by the quantification of thiobarbituric acid reactive substances (TBARS), according to Buege & 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.

3.3.2.5. 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.

All parametric tests were performed using STATISTICA (StatSoft) software and homogeneity of variances was checked with Cochran test while normality was assumed.

Three Principal Coordinates Ordination analysis (PCO) were separately performed using the software PRIMER-E: 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 explained ($r > |0.7|$) the samples spatial distribution were represented as superimposed vectors.

3.3.3. Results

3.3.3.1. *Bacciger bacciger* infection

Shell length (SL) of sampled wedge clams varied between 15 and 40 mm with a frequency peak at 25 mm (Figure 23). Regarding dissected clams, mean SL was significantly higher in July, September and January (mean = 32.6 ± 1.9, 33.2 ± 2.0 and 32.6 ± 1.6 mm, respectively) comparing to March, May and November (mean = 29.0 ± 2.9, 24.3 ± 2.9 and 28.9 ± 3.3 mm, respectively) (one-way ANOVA: $F = 91.2$, $p < 0.01$).

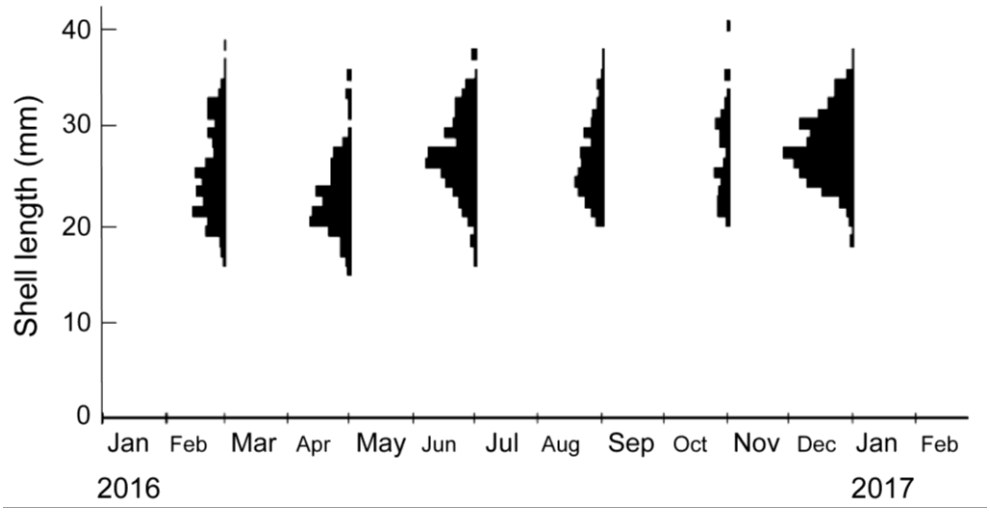


Figure 23 *Donax trunculus* shell length histograms per sampling month, showing the sampled cohort.

Only one macroparasite species was found, the trematode *B. 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 = 11.9 ± 10.7) was significantly higher than the emergence prevalence (mean = 1.3 ± 1.9). Prevalence showed a seasonal trend with the highest value in July (33%) and the lowest in May (0%) (Figure 24). Water temperature showed to be positively correlated to *B. bacciger* prevalence ($r = 0.83$, $p < 0.05$, Figure 24).

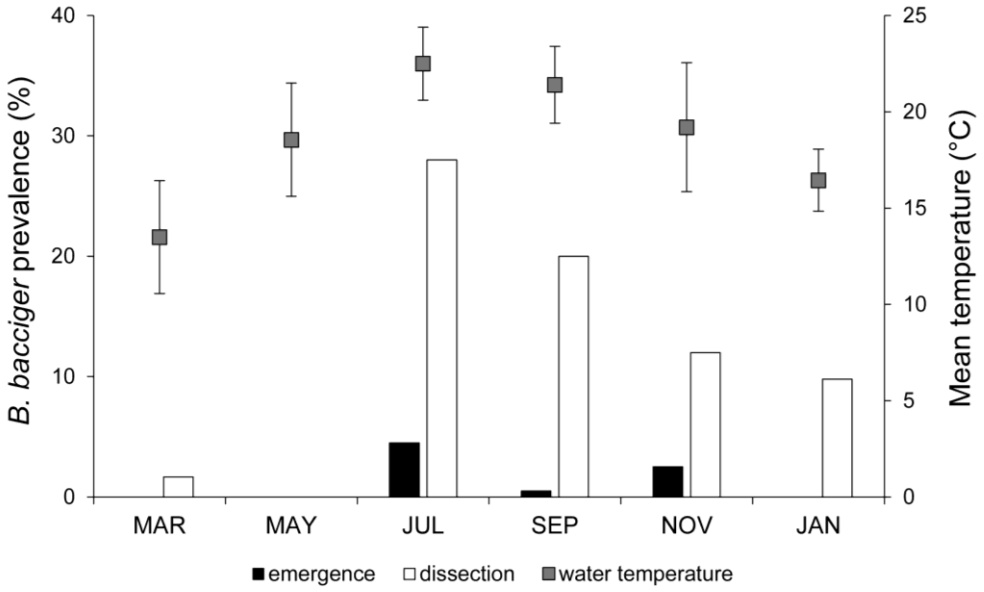


Figure 24 Seasonal trend of the *Bacciger bacciger* prevalence checked by two methods (cercariae emergence after 12 h and dissection), for each sampling month, along with the mean water temperature (± standard deviation) of the sampled area.

3.3.3.2. 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 8).

Cat gene was inhibited in July and induced in November for parasitized compared to non-parasitized clams (Table 8).

Table 8 Comparative basal expression expressed in terms of induction factor for the selected genes from parasitized and control clams (non-parasitized clams). Expression not significantly different: —; inhibition: expression ratio < 0.5; induction: expression ratio >2.

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

3.3.3.3. Biochemical data

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

Regarding ETS activity, in NP clams, the activity was significantly higher from March to July and significantly lower from September to January, while for P clams ETS was higher in July and January compared to September and November (Figure 25A). In July and January, ETS activity was significantly higher in P compared to NP clams (Figure 25A).

GLY content of the NP clams was significantly higher in March and July compared to the other months, and a similar trend was observed for the P clams (Figure 25B). Overall, there was lower GLY content in P compared NP clams (significant in July and November) (Figure 25B). PROT content was not significantly different among all conditions (data not shown).

Taking into account NP wedge clams, results showed significantly higher SOD activity in September and significantly lower activity in January, with remaining months displaying intermediate values (Figure 25C). Regarding P clams, significantly higher SOD activity was presented in November and lower in January (Figure 25A). The global trend showed lower SOD activity in P compared to NP but only significant in September (Figure 25C). CAT activity for NP clams was significantly higher in September compared to the other sampling months (Figure 25D). Parasitized clams showed significantly lower CAT activity in November compared to the other sampling months (Figure 25D). The global trend showed lower CAT values in P clams compared to NP, with statistical significance in November (Figure 25D).

For NP clams, LPO values were significantly higher in March and May and significantly lower in November and January compared to the other months, while P clams showed slightly higher LPO levels in January (Figure 25E). Significant differences were observed between P and NP clams in July and January with lower and higher LPO levels for P clams, respectively (Figure 25E).

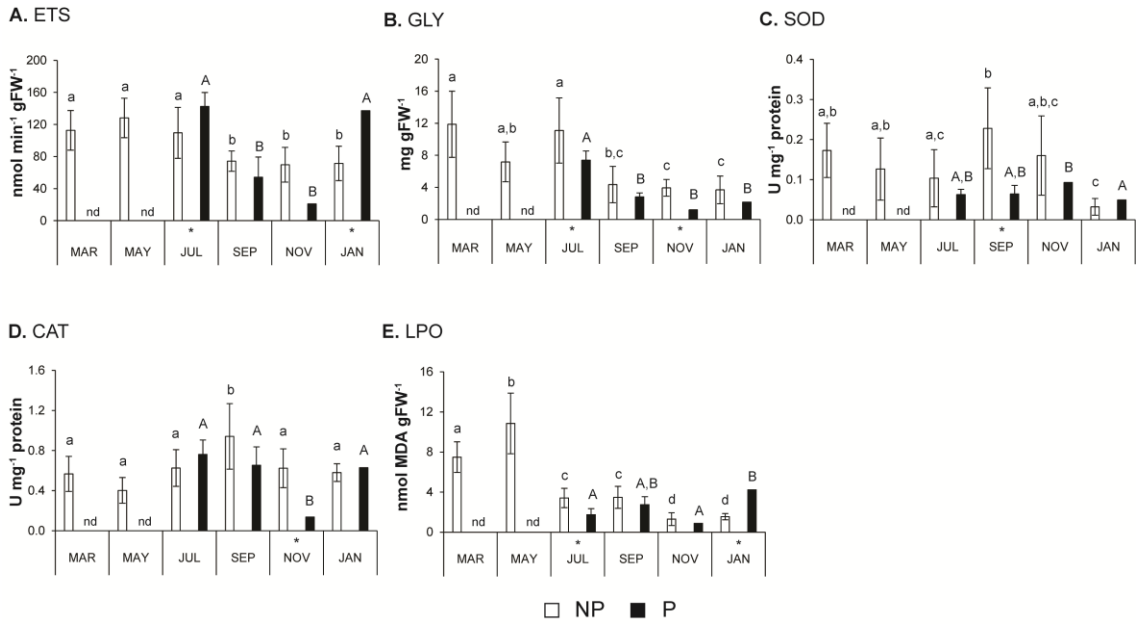


Figure 25 Mean values (± standard deviation) and significant differences represented with different lower (NP: non-parasitized), upper (P: parasitized) case letters and with asterisk (NP vs. P) of **A.** ETS, electron transport system activity; **B.** GLY, glycogen content; **C.** SOD, superoxide dismutase activity; **D.** CAT, catalase activity; **E.** LPO, lipid peroxidation levels in *Donax trunculus* wedge clams non-parasitized and parasitized with *Bacciger bacciger*. nd: no data.

Regarding PCO analysis with NP clams, the horizontal dimension (Axis 1) explained 57% of the total variation separating March, May and July, in the negative side of the axis, from September, November and January, in the positive side of the axis. ETS, GLY and LPO were the variables that better explained this variation presenting high negative correlation with axis 1 ($r > -0.8$) as well as CAT that presented high positive correlation with axis 1 ($r > 0.7$). Axis 2 explained 28% of the total variation which separated mainly January, in the positive side of the axis, from September with a strong positive correlation to SOD activity ($r > 0.8$) (Figure 26A).

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

The axis 1 of the PCO concerning P vs. NP samples explained 48% of the total variation separating mainly P clams of November but also NP clams of November and January and P clams of September in the negative side of the axis, from the other conditions in the positive side of the axis. ETS, CAT and LPO were the variables that better explained the variation, presenting high positive correlation ($r > 0.8$) with axis 1. The PCO vertical dimension (Axis 2) explained 25% of the total variation separating mainly NP clams of September but also NP clams of November, in the positive side of the axis, from the other conditions with a strong positive correlation to SOD activity ($r > 0.8$) (Figure 26C).

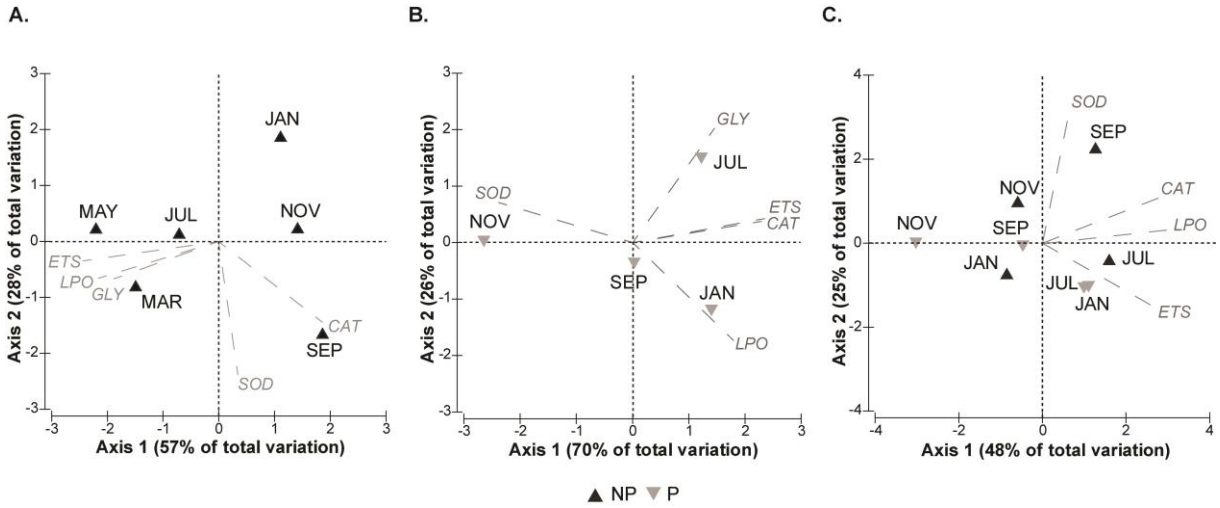


Figure 26 Principal coordinates ordination analysis (PCO) showing the variables that better explained samples distribution for **A.** Non-parasitized (NP) *Donax trunculus* wedge clams; **B.** Parasitized (P) wedge clams; **C.** NP vs. P wedge clams. ETS: eletron transport system; GLY: glycogen content; SOD: superoxide dismutase; CAT: catalase; LPO: lipid peroxidation.

3.3.4. Discussion

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

able to castrate the wedge clam (Ramón et al., 1999), decreasing its 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 & 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 & Hubbard, 1990), 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). 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 (Delgado & Silva, 2018; Ramón et al., 1999). 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 SL confounding factor. Despite the clams sampled in May (*B. bacciger* prevalence = 0%) have been smaller than clams sampled in July (*B. bacciger* prevalence = 33%) conversely, there were other months displaying low prevalence values in large organisms (e.g. January) excluding this feature as a prevalence explanatory variable.

In the present study, the most likely driver of the infection was the water temperature (as proxy of seasonality), a known trigger of trematode infection (de Montaudouin et al., 2016) which showed to be positively correlated with prevalence. Seasonality in trematodes infecting their first intermediate host is not frequently observed, mainly due to the natural low prevalence of these parasites and consequent difficulty to find infected hosts (Magalhães et al., 2015). Nevertheless, most of the authors converge by identifying summer and the associated high temperatures as the season presenting highest prevalence, as demonstrated by Bowers (1969) in South

Wales and by Desclaux et al. (2002) in France both studying *Cerastoderma edule* infected by *Bucephalus minimus*.

As mentioned above, negative effects of *B. bacciger* on host health has been described by some authors (de Montaudouin et al., 2014; Delgado et al., 2017; Ramón et al., 1999) but the present work represents the first assessment of *B. bacciger* effects at the level of gene expression and biochemical alterations. During an infection, host cells enhance reactive oxygen species (ROS) (Soudant et al., 2013). However, ROS have also important functions in some intracellular signalling cascades activation such as immunity response (Limón-Pacheco & Gonsebatt, 2009). Nevertheless, when ROS production exceeds ROS elimination capacity, cellular damages may occur. Therefore, organisms need to balance ROS quantity and for that they mainly use antioxidant enzymes, such as SOD and CAT (Regoli & Giuliani, 2014). In accordance with such considerations, previous studies with a dinoflagellate parasitized by *Amoebophrya* spp. (Lu et al., 2016) showed upregulation of genes involved in the calcium signalling which in turn is related to the stress response. Furthermore, studying the disk abalone (*Haliotis discus discus*) challenged by bacteria and virus, De Zoysa et al. (2011) showed upregulation of the glutathione peroxidase gene. Similarly, glutathione S-transferase genes were upregulated in *Ruditapes decussatus* clams infected by *Perkinsus* sp. (Leite et al., 2013). All these genes are related to the antioxidant system, which is responsible for ROS elimination (Regoli & Giuliani, 2014). Our results showed that *sod (Mn)* and *cat* oxidative stress related genes were upregulated in clams infected by *B. bacciger* in March (*sod (Mn)*) and in November (*cat*), while in July and September the same genes were downregulated in parasitized clams. These results may be explained by the fact that upregulation could represent a response to more than one stress at the same time. In the present case, parasite infection and the peak of the spawning period occur in March (Gaspar et al., 1999) and parasite infection and gametogenesis start in November (Gaspar et al., 1999). On the other hand, the expression of genes related to the antioxidant defence may have been modulated by the parasite patency. An infection is considered as patent when direct evidence of the parasite can be detected and so is related to the development of the sporocyst in the host (Graczyk & Fried, 2007). In March, the visible *B. bacciger* prevalence was still low (2%), although it was already possible to observe a sporocyst (i.e. prepatent infection). In July and September, the highest *B. bacciger* prevalence values were registered (33 and 21%, respectively) with mature sporocysts, and cercariae (patent infection). In November, the parasite returns to a prepatent infection. Following these arguments, downregulation of *sod (Mn)* and *cat* occurring when sporocysts were mature, can indicate the initiation of an alternative immune or stress response induced by the higher

parasite spread and consequent pathogenicity or an overall loss of ROS arrest signalling.

Taking only into account non-parasitized clams (sampled in all sampling 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 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, a higher antioxidant activity was registered in September (SOD and CAT activity) 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. Thus, assessed biomarkers showed to be responsive not only to the individual and environmental seasonal changes but also to the parasite infection.

Parasitized clams showed higher metabolic rate (measured here by ETS activity) and lower GLY content. Correspondingly, the gastropod *Lymnaea stagnalis* infected with trematodes, showed an exhaustion of energy-cell resources experienced by the host which led to a decrease in CAT activity (Khomich et al., 2017). An infected clam has an additional metabolic requirement having to supply its own survival but also

parasites with sufficient energy to grow (MacLeod, 2017). Overall, *B. bacciger* infection inhibited SOD and CAT activities, which is in agreement with the reduced *sod (Mn)* and *cat* expression in July and September. Similarly, *Clinostomum detruncatum* (trematode) infection proved to reduce the non-enzymatic antioxidant defences with respect to pro-oxidant status in the muscle of the freshwater teleost *Rhamdia quelen* (Belló et al., 2000). Also, some vibrios such as *Vibrio tapetis* were shown to inhibit ROS intermediates production (such as hydrogen peroxide) in hemocytes of *Crassostrea gigas* (Lambert et al., 2003). Transcriptional and biochemical results were not convergent in January, since *sod (Mn)* and *cat* genes were upregulated in parasitized clams but measured SOD and CAT activities were lower. Straight correlations between mRNA expression and enzyme activity are still poorly studied, with controversial results but certainly not always positively correlated and dependent on post-transcriptional, translational, regulation and protein degradation related processes (Vogel & Marcotte, 2012). Finally, especially noticed in January, the increased metabolism and the reduction in the antioxidant enzymes activity led to oxidative stress (higher LPO level) and consequently to cellular damage in parasitized clams.

Conclusion

Overall, the present study showed that *B. bacciger* has a negative effect on its first intermediate host *D. trunculus,* by increasing its metabolic rate, decreasing the energy reserves and inhibiting the antioxidant enzymes activity, which in some months led to cellular damages. These 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.

Final considerations

For both species studied, sporocysts infection showed to induce host defence mechanism against oxidative stress and to increase the host metabolism and energy demand, highlighting the harmful effects of these infections on their hosts, occurring even at the sub-individual level. These negative impacts were especially noticed during summer for cockles and during spawning and spent periods for wedge clams. By showing the same trend with two different models, present results revealed the use of transcriptomic and biochemical markers as valuable tools, capable of providing additional and ecologically relevant information about, not only the environmental conditions that animals experienced, but also the invasion effects of pathogens. These findings can help to predict organisms chances of reproduction and survival in their natural context, which can be applied in bivalve conservation and disease episodes management.

On the other hand, effects on host fitness are different or display different magnitudes according to the parasite stage. Less deleterious effects are usually described for trematodes infecting bivalves as second intermediate host. However, their prevalence is often high and occasionally, when high abundances are reached, some bivalve basic functions can be disrupted. For these reasons, it is important to recognize processes that can enhance metacercariae abundance leading to disease risk. The first step, described in the first section of the next chapter, is to characterize trematode distribution in the investigated areas, evaluating the spatial distribution and the temporal persistence of trematode communities in bivalves and identifying the triggers of variability.

CHAPTER 4. BIVALVES AS SECOND INTERMEDIATE HOST

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Link: https://doi.org/10.1017/S0025315416001107.

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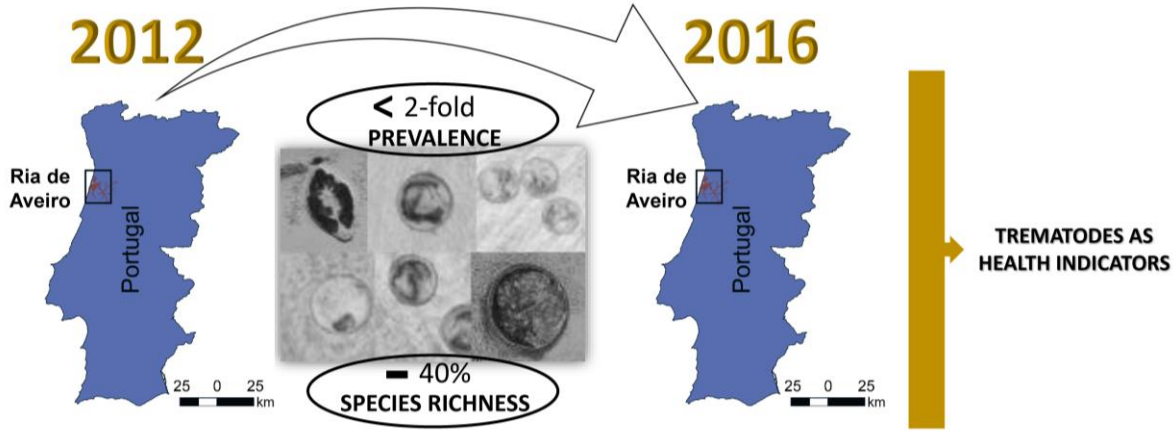
Link: https://doi.org/10.1016/j.envpol.2018.09.102

Cerastoderma edule field monitoring

4.1. Spatio-temporal variation of trematode parasites community in *Cerastoderma edule* cockles from Ria de Aveiro (Portugal)

GRAPHICAL ABSTRACT



4.1.1. Introduction

Cerastoderma edule is among the most exploited bivalves in Europe playing an important socio-economic role (Pereira et al., 2014). Cockles live in estuaries and lagoons where their population is controlled by several environmental factors including parasitism (Gam et al., 2009).

Parasites represent an important part of the world known biodiversity (Dobson et al., 2008) however, their ecological role still remains not fully understood. Trematodes are the most prevalent macroparasites of cockles being able to exert an impact both at the individual (e.g. Babirat et al., 2004) and population levels (e.g. Marcogliese, 2004). On the other hand, the complexity of the trematode life cycle, that includes, generally, three host species, make it suitable ecosystem diversity and health indicators (Hechinger et al., 2006; Hudson et al., 2006). Therefore, it is of prime relevance to recognize and understand the parasite/host system dynamics in order to better predict potential conservation threats to bivalve populations and to maximize the success of stock and the disease episodes management.

The present study focused on spatio-temporal variability of the structure of trematode community infecting *C. edule* at the scale of the Ria de Aveiro coastal lagoon (Portugal), with two main objectives. Firstly, to map trematode parasites in the

Ria de Aveiro and to hierarchize some environmental drivers of infection. Based on former studies and highlighting the importance of abiotic factors (e.g. temperature, salinity), the advanced hypothesis was that the structure of parasite communities in cockles should follow an oceanic-continental gradient and seasonal cycles. Secondly, to compare trematode communities in cockles from a previous field study with similar sampled stations and season in the Ria de Aveiro. The hypothesis was that the parasite community structure is relatively stable over time as long as environmental parameters remain stable too. Thus, a significant difference in trematode parasite community structure could alert for environmental changes and alterations in ecosystem functioning.

4.1.2. Material and methods

4.1.2.1. Study area

The Ria de Aveiro is a coastal lagoon (Northwest of Portugal) with four main channels which radiate from the ocean mouth with several branches, islands and mudflats (Figure 27). Tides are semi-diurnal and constitute the main forcing water circulation agent. The minimum and maximum tidal height ranges are about 0.6 m and 3.2 m at neap and spring tides, respectively (Dias et al., 2000). The most important freshwater input (the Vouga River) of the Ria de Aveiro flows through the Espinheiro channel, one of the present study areas (Figure 27) that is about 17 km long and is characterized by a strong horizontal gradient of salinity and water temperature which migrates back and forth with the spring/neap cycle (Lillebø et al., 2015; Vaz et al., 2005). The other freshwater sources are smaller, namely the Boco, Mira and Cáster rivers, the latter flowing through the 29 km long São Jacinto-Ovar channel, the other study area (Figure 27). According to the Water Framework Directive, the Ria de Aveiro is divided in five water bodies (WB), identified in Figure 27, with WB2 gathering both study areas (the two channels) and is classified with a “moderate” water ecological status (MAMAOT/ARHCentro, 2012).

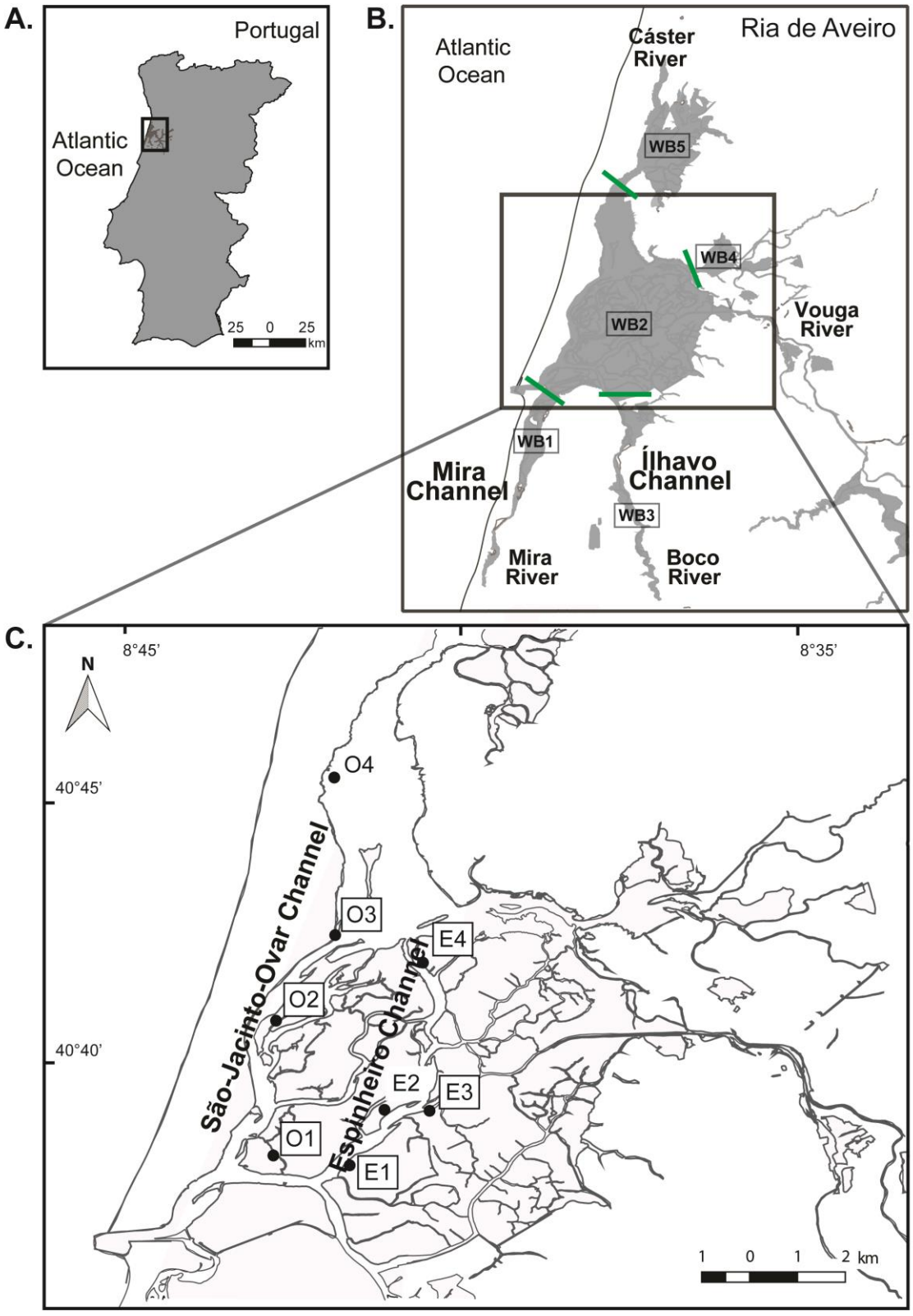


Figure 27 Study area. The Ria de Aveiro coastal lagoon (Northwest Portugal (A)) indicating the positions of the seasonal sampling stations (São-Jacinto-Ovar channel: O1, O2, O3, O4 and Espinheiro channel: E1, E2, E3, E4) with indication of the lagoon division into five transitional water bodies (WB) which borders are indicated by green lines (B). Compatible stations used for 2012-2016 comparisons are identified by a square (C).

4.1.2.2. Sampling strategy and parasite identification

Field monitoring occurred during one year (from December 2015 to November 2016) with seasonal sampling in 8 stations along the two channels, Espinheiro and São Jacinto-Ovar (Figure 26), identified with different codes: first two letters represent the season (WI: winter, SP: spring, SU: summer, FA: fall); third letter represents the channel (E: Espinheiro channel, O: São Jacinto-Ovar channel); and the number represents the sampling station (from 1, the most oceanic station, to 4, the most continental station). At each station, temperature and salinity were measured and two sediment samples (2 replicates each) were collected in order to estimate median grain size and total organic matter (TOM) content. At each station, cockles were collected by sampling six quadrats (0.25 m^2 each) and sieving them through a 1-mm mesh sieve to estimate density (cockles m^{-2}). Cockle shell length (SL) was measured at the least mm with a calliper. Size-histogram analysis allowed discriminating different cohorts. In order to compare infection in cockles with similar age, we decided to concentrate on the most represented cohort, i.e. 2014. Twenty cockles per station and season were dissected. All trematodes were identified to the species level following several authors descriptions (Bartoli et al., 2000; Bowers, 1969; Bowers et al., 1996; de Montaudouin et al., 2009). Metacercariae identified in each of the observed cockles were counted to assess parasite abundance (number of metacercariae per cockle) and prevalence (percentage of infected cockles) (Bush et al., 1997). For parasite using cockles as first intermediate host, only prevalence was calculated.

A survey of the trematode species infecting cockles in the Ria de Aveiro has already been conducted in 2012 (Freitas et al., 2014). In order to assess interannual variability of the structure of trematode communities, 2012 (October sampling) and 2016 (September sampling) databases were compared. Overlapping areas from both studies were identified and selected to perform the temporal comparison, corresponding to six comparable stations (Figure 26).

4.1.2.3. Sediment characterization

Sediment samples for grain-size analysis were dry and wet sieved through a column of sieves with decreasing mesh sizes following the procedure described by Quintino et al. (1989).

Sediment TOM content was measured as the percent weight loss in 1 g of dried sediment, after combustion at 450 °C, during 5 h (Kristensen & Andersen, 1987).

4.1.2.4. Data analysis

Prevalence, i.e. the percentage of hosts infected with one or more individuals of a particular parasite species (Bush et al., 1997), was calculated for all trematode species found: *Diphterostomum brusinae* (Stossich, 1889), *Himasthla elongata* (Mehlis, 1831), *H. interrupta* Loos-Frank, 1967, *H. quissetensis* (Miller and Northup, 1926) Stunkard, 1938, *Renicola roscovitus* (Stunkard, 1932), *Parvatrema minutum* (Cobbold, 1859), *Bucephalus minimus* (Stossich, 1887) and *Monorchis parvus* Looss, 1902. Parasite abundance, i.e. the total number of individuals of a particular parasite species in a sample of a particular host species (Bush et al., 1997), was calculated for 6 out of 8 species found, excluding those infecting cockles as first intermediate host (*B. minimus* and *M. parvus*).

Regarding 2016 data, due to high heterogeneity of metacercariae abundance in cockles and because data from fall season were missing, we performed two separated ANOVAs with mean metacercariae abundance per cockle in each station or season as dependent variable. Prior to analysis, data were log (x+1) transformed in order to achieve homogeneity of variance which was verified with Cochran test. The first was a two-way ANOVA in order to test the effect of the channel (E vs. O), the proximity to ocean (stations 1 to 4) and the interaction between factors on the total number of metacercariae in cockles. The second test was a one-way ANOVA assessing seasonal effect (with all stations as replicates) on the mean number of metacercariae per cockle. The same analysis (two-way ANOVA with channel and stations followed by a one-way ANOVA with seasons) was repeated for *P. minutum*, the most abundant species.

Comparison of trematode communities in cockles among stations in each season was performed by a cluster analysis, i.e. an exploratory technique that aims to join together objects into successively larger clusters, using some measure of community similarity or distance: a short distance between two stations means that the community of parasites in cockles is similar in terms of species composition and species abundance. Dataset consisted of a “season/station (categorical factor) × trematode species (dependent variables)” matrix. In order to include the two trematode species found using cockles as first intermediate host (*B. minimus* and *M. parvus*), each dependent variable corresponded to the mean infection prevalence (mean percentage of infected hosts) of a given trematode species in cockles from a given station at a given season. The cluster analysis was performed on the matrix of Euclidean distance between variables (Ward method of aggregation, ascendant hierarchical method). Due to heterogeneity of trematode infection in relation with the scale of analysis, we assigned a threshold at 30% of dissimilarity to separate the

different groups. Groups were then characterized in terms of parasite community, environmental and host population features. For each of these parameters (dependent variables), differences among affinity groups (categorical factor) given by the cluster 30% dissimilarity threshold were tested using one-way ANOVAs followed by post-hoc Tukey test for comparison of means, identifying homogenous groups which are represented in tables with superscript lower case letters. Prior to ANOVA, homogeneity of variance was verified with Cochran test.

To compare trematode communities in cockles between October 2012 and September 2016, a cluster analysis followed by one-way ANOVAs were performed as previously described for 2016 data.

All statistics were performed with STATISTICA (StatSoft) software.

4.1.3. Results

4.1.3.1. Trematode community: 2016 spatial and seasonal patterns

A total of 8 stations and 4 seasons were sampled but cockles (from the 2014-cohort) were absent at 9 occasions, especially during fall campaign (in 6 stations). During the present study 387 cockles were dissected, 166 of them were uninfected. Eight trematode species (belonging to 6 different families, Table 9) were observed in 221 cockles, six species using cockles as second intermediate host: *D. brusinae* (mean prevalence (P) = 0.5%), *H. elongata* (P = 23.9%), *H. interrupta* (P = 0.9%), *H. quissetensis* (P = 0.9%), *P. minutum* (P = 48.9%) and *Renicola roscovitus* (P = 0.2%) and two using cockles as first intermediate host: *B. minimus* (P = 1.1%) and *M. parvus* (P = 0.2%, registered in the present study only in sporocyst form) (Table 9). Among the sampling stations, species richness varied from 0 to 4 species per cockle. Overall, trematode mean abundance ranged between 0 and 132 metacercariae cockle^{-1}, with an average of 43 metacercariae cockle^{-1} (Figure 28). The most abundant species was *P. minutum* which represented 65 to 100% of total metacercariae in cockles from each station, occurring in 96% of the sampling occasions (station x season). *H. elongata* was present in cockles in 83% of sampling occasions but with a very low average abundance (< 1 metacercariae cockle^{-1}). *H. quissetensis*, *H. interrupta*, *D. brusinae* and *R. roscovitus* were rare, infecting 4, 3, 2 and 1 out of the 387 total number of cockles dissected, respectively, without spatial and/or seasonal significant differences.

Table 9 List of trematode species identified using *Cerastoderma edule* as their first and/or second intermediate host and the other hosts of the life cycle.

Trematoda species	**Family**	**1st intermediate host**	**2nd intermediate host**	**Final host**
Diphterostomum brusinae	Zoogonidae	*Tritia reticulata*	*Cerastoderma edule*	Fish
Himasthla elongata	Himasthlidae	*Littorina littorea*	*C. edule*	Water birds
Himasthla interrupta	Himasthlidae	*Hydrobia* spp.	*C. edule*	Water birds
Himasthla quissetensis	Himasthlidae	*T. reticulata, T. neritea*	*C. edule*	Water birds
Renicola roscovitus	Renicolidae	*L. littorea*	*C. edule*	Water birds
Parvatrema minutum	Gymnophallidae	*Scrobicularia plana*	*C. edule*	*Haematopus ostralegus*
Bucephalus minimus	Bucephalidae	*C. edule*	*Pomatoschistus* sp.	*Dicentrarchus labrax*
Monorchis parvus	Monorchiidae	*C. edule*	*C. edule*	*Diplodus* spp.

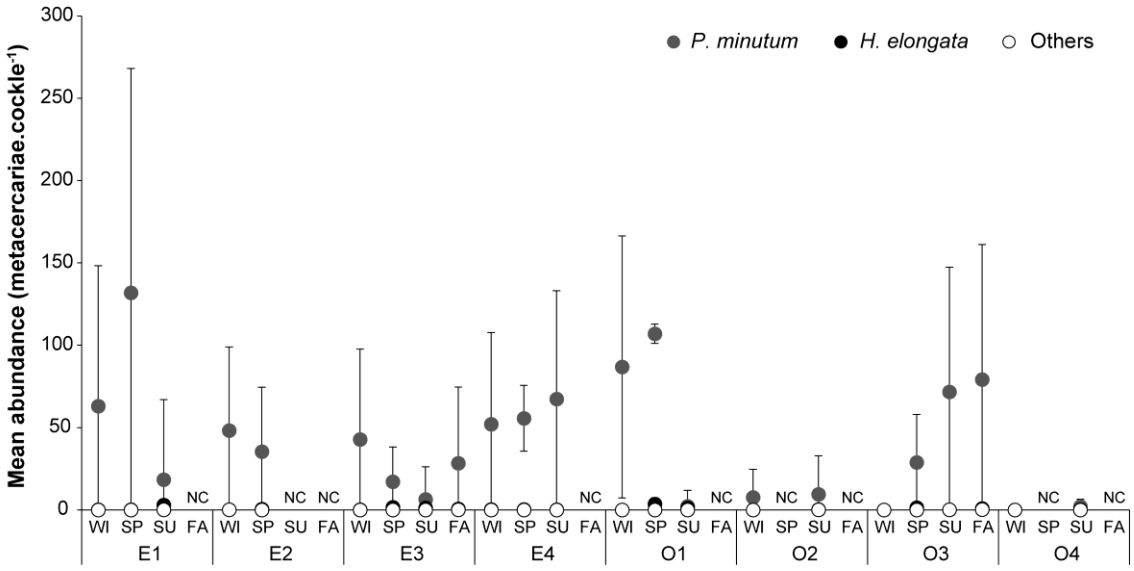


Figure 28 Mean metacercariae abundance (± standard deviation) per cockle and per season (WI: winter, SP: spring, SU: summer, FA: fall) in each sampling station (São-Jacinto-Ovar channel: O1, O2, O3, O4 and Espinheiro channel: E1, E2, E3, E4) for *Parvatrema minutum*, *Himasthla elongata* and others (gathering the other four less represented species). NC: absence of cockles.

Spatially, the mean number of metacercariae per cockle in 2016 was significantly higher in Espinheiro channel (47.9 ± 32.3 metacercariae cockle^{-1}) than in São-Jacinto-Ovar channel (36.6 ± 42.1 metacercariae.cockle^{-1}) (two-way ANOVA: F = 6.6, p = 0.02) with no significant differences related to ocean proximity (two-way ANOVA: F = 1.3, p = 0.32, Table 10) and no interaction between both independent factors (two-way ANOVA: F = 2.1, p = 0.14, Table 10). Seasonally, the mean number of metacercariae per cockle ranged between 26.2 (± 30.3) and 63.8 (± 46.5), with no significant difference among seasons (one-way ANOVA: F = 1.3, p = 0.31, Table 10). The most abundant species, *P. minutum*, presented the highest mean abundance in WIO1, SPO1 and SPE1 (on average, 87, 107 and 132 metacercariae cockle^{-1} respectively) following the same pattern as total metacercariae, with significantly higher abundance in Espinheiro channel than São Jacinto-Ovar channel, with no significant differences related to ocean distance nor interaction between factors (two-way ANOVAs: F = 6.2, p = 0.02, F = 1.1, p = 0.40 and F = 2.0, p = 0.16 respectively, Table 10) as well as no seasonal trend (one-way ANOVA: F = 1.3, p = 0.31, Table 10).

Table 10 Two two-way ANOVA results performed to test differences among Channels, Ocean proximity (stations 1 to 4) and interaction between factors in terms of total metacercariae abundance (total number of metacercariae in cockles) and *Parvatrema minutum* abundance. Two one-way ANOVAs results performed to test differences among seasons in terms of total metacercariae abundance and *P. minutum* abundance. df: degrees of freedom; MS: mean square; *F*: *F* value; *p*: *p* value. Bold letters indicate significant differences ($p < 0.05$).

Dependent variable	**Statistical test**	**Factors**	**df**	**MS**	***F***	***p***
Total metacercariae abundance	Two-way ANOVA	Channel	**1**	**1.9**	**6.6**	**0.02**
		Ocean proximity	3	0.4	1.3	0.32
		Channel*Ocean proximity	3	0.6	2.1	0.14
		Error	15	0.3		
	One-way ANOVA	Season	3	0.5	1.3	0.31
		Error	19	0.4		
Parvatrema minutum abundance	Two-way ANOVA	Channel	**1**	**1.9**	**6.2**	**0.03**
		Ocean proximity	3	0.3	1.1	0.40
		Channel*Ocean proximity	3	0.6	2.0	0.16
		Error	15	0.3		
	One-way ANOVA	Season	3	0.5	1.3	0.31
		Error	19	0.4		

In terms of parasite community in cockles, cluster analysis discriminated 3 groups at a threshold at 30% of dissimilarity (Figure 29). Total mean prevalence was not significantly different among groups (one-way ANOVA: $F = 1.7$, $p = 0.20$, Table 11). *D. brusinae* and *H. elongata* were the main species contributing for clusters dissimilarity, the first was exclusive of Group *1* (3 sampling stations, 2 of them in the most oceanic part, Figure 30) where *H. elongata* presented the highest mean prevalence. Group *2* (5 sampling stations) included mainly the stations located in the middle of the sampled channels (stations E3 and O3, Figure 30). This group displayed intermediate values of *H. elongata* mean prevalence compared to groups *1* and *3* (Table 11). Finally, group *3* gathered 13 sampling stations, including all the upstream positions, characterized by the lower values in terms of *H. elongata* prevalence (Figure 29, Table 11). Comparing mean temperature, mean salinity, mean TOM content in the sediment, sediment median grain-size, mean cockle SL and cockle density, only grain-size and cockles density showed significant differences among cluster affinity groups (one-way ANOVA: $F = 3.5$, $p = 0.05$ and $F = 4.1$, $p = 0.03$, respectively), with group *3* displaying the lowest (statistically significant) median grain-size and the highest (statistically significant) cockle density values (Table 11). In terms of seasonal patterns, spring, summer and fall were relatively dispersed in all groups but group *3* (lower trematode prevalence) included all winter samples.

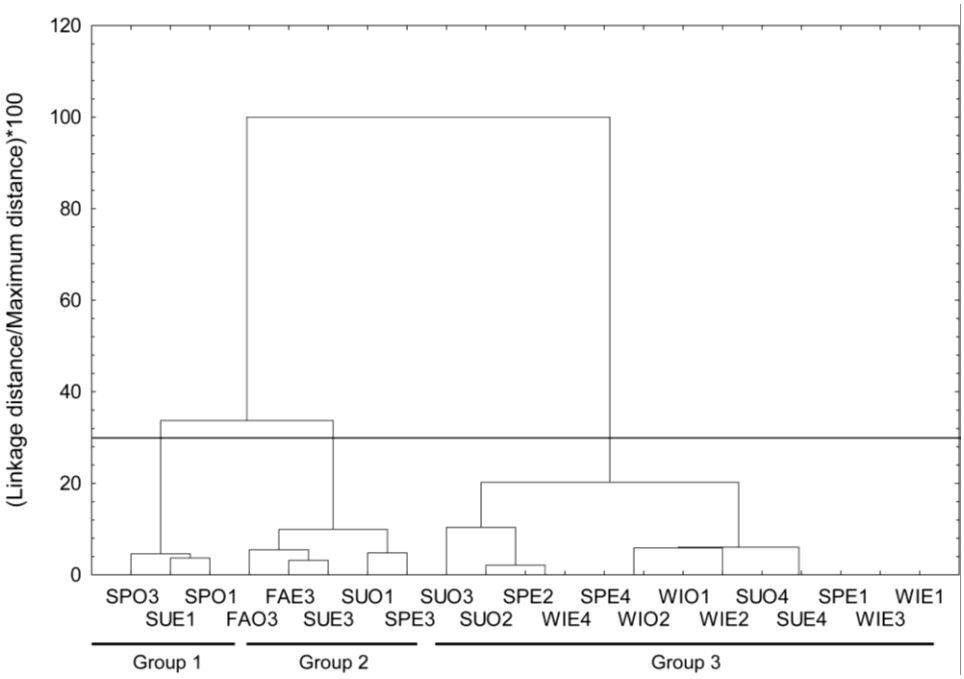


Figure 29 Cluster analysis on trematode parasites community in adult cockles sampled seasonally in 2016 (WI: winter, SP: spring, SU: summer, FA: fall) in eight different stations in the Ria de Aveiro coastal lagoon (São-Jacinto-Ovar channel: O1, O2, O3, O4 and Espinheiro channel: E1, E2, E3, E4). The 30% distance of dissimilarity threshold is drawn.

Table 11 Characterization of the affinity groups identified in the Ria de Aveiro spatial and seasonal analysis, in 2016. Each variable is represented by mean ± standard deviation (SD). Bold values indicate significant differences in the ANOVA main test while superscript lower case letters indicate the homogenous groups identified by the Tukey post-hoc comparison of means test.

		Affinity groups			**One-way ANOVAs results**	
		Group *1*	**Group *2***	**Group *3***	*F*	*p*
	Nr. of sampling stations	3	5	13		
Parasites	Trematoda abundance (mean ± SD)	54.03 ± 49.11	11.88 ± 11.74	51.99 ± 35.32	2.8	0.09
	Species richness (mean ± SD)	2.33 ± 0.58	2.80 ± 0.84	2.15 ± 0.99	0.9	0.43
	Prevalence (mean ± SD, %)	92.67 ± 12.70	66.00 ± 19.49	64.54 ± 26.40	1.7	0.20
	Diphterostomum brusinae	**3.70 ± 6.42^{a}**	**0.00 ± 0.00^{a,b}**	**0.00 ± 0.00^{b}**	**3.9**	**0.04**
	Himasthla elongata	**80.09 ± 6.56^{a}**	**41.69 ± 11.60^{b}**	**7.69 ± 7.53^{c}**	**99.1**	**< 0.001**
	Himasthla interrupta	0.00 ± 0.00	4.08 ± 6.68	0.00 ± 0.00	3.2	0.06
	Himasthla quissetensis	0.00 ± 0.00	0.00 ± 0.00	1.54 ± 3.15	0.9	0.43
	Parvatrema minutum	61.57 ± 38.90	35.31 ± 25.88	58.75 ± 28.07	1.3	0.29
	Renicola roscovitus	0.00 ± 0.00	0.00 ± 0.00	0.38 ± 1.39	0.3	0.75
	Bucephalus minimus	0.00 ± 0.00	1.00 ± 2.24	1.54 ± 5.55	0.1	0.87
	Monorchis parvus	0.00 ± 0.00	1.00 ± 2.24	0.00 ± 0.00	1.7	0.21
Environmental data	Temperature (mean ± SD; °C)	15.33 ± 0.83	17.48 ± 4.29	18.91 ± 2.82	1.8	0.20
	Salinity (mean ± SD)	31.00 ± 4.58	30.00 ± 3.16	31.38 ± 6.42	0.1	0.90
	TOM content (mean ± SD; %)	0.84 ± 0.32	1.68 ± 0.72	2.22 ± 1.54	1.4	0.26
	Median grain size (mean ± SD, mm)	**0.25 ± 0.0^{a}**	**0.14 ± 0.07^{a,b}**	**0.13 ± 0.08^{b}**	**3.5**	**0.05**
Host population	Shell length (mean ± SD; mm)	28.55 ± 1.91	30.26 ± 1.97	28.73 ± 2.59	0.8	0.46
	Density (mean ± SD; ind.m^{-2})	**10.89 ± 10.12^{a,b}**	**2.40 ± 2.77^{a}**	**55.79 ± 109.41^{b}**	**4.1**	**0.03**

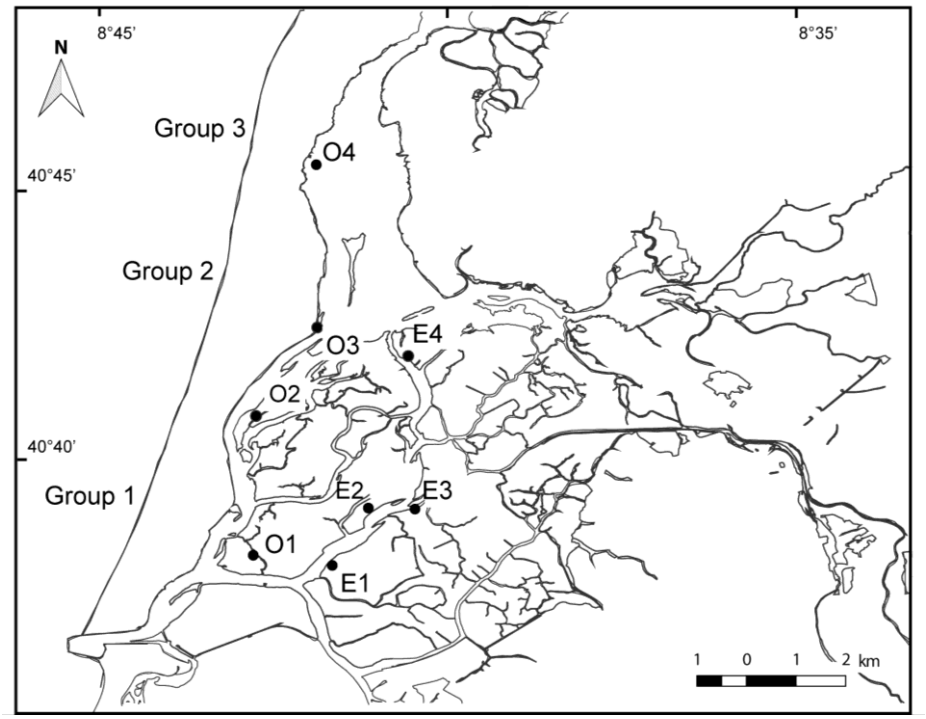


Figure 30 Spatial representation and distribution pattern of the 3 trematode assemblages (nominated as group *1*, *2* and *3*) resulting from the cluster analysis.

4.1.3.2. Trematode community: 2012-2016 comparison

In the 2012 survey (Freitas et al., 2014), in the six sampling stations in common with the present study, cockles were infected by 8 different trematode species: *D. brusinae*, *H. elongata*, *H. interrupta*, *H. quissetensis*, *P. minutum* and *R. roscovitus* (metacercaria) and *B. minimus* and *M. parvus* (sporocyst). Comparing to 2012 survey, in the 2016 samples three species were lacking (*H. interrupta*, *M. parvus* and *R. roscovitus*). Gathering both surveys data (6 stations per survey), cockles were distributed by 3 clusters that displayed 30% of dissimilarity (Figure 31). Groups *A* and *B* gathered three sampling stations each and included all 2012 sampling year, group *C* assembled 2016 sampling stations. *H. elongata* (significantly higher prevalence in group *A*), *H. quissetensis* (significantly higher prevalence in group *A*) and *D. brusinae* (significantly lower prevalence in group *C*) were the main species contributing for the separation of the three cluster groups (70% similarity). Group *C* (2016 samples) was characterized by lower mean species richness compared to group A (one-way ANOVA: $F = 12.3$, $p < 0.01$, Table 12) and lower trematode mean prevalence (one-way ANOVA: $F = 16.1$, $p < 0.01$) compared to groups *A* and *B* (2012). Regarding host population characteristics and environmental variables (Table 12), only TOM content displayed a

significant variation, showing to be significantly higher (one-way ANOVA: $F = 4.8$, $p = 0.03$) in group *A* (2012) than in group *C* (Table 12).

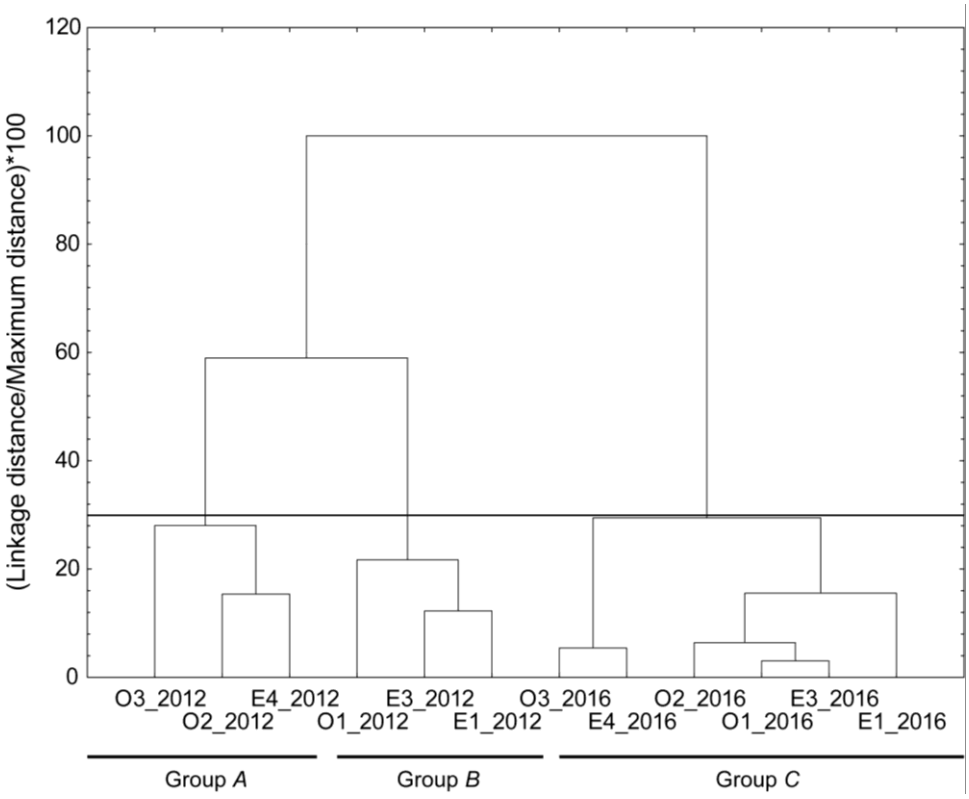


Figure 31 Cluster analysis on trematode parasites communities in adult cockles sampled in 2012 (Freitas et al., 2014) and in 2016 (present study) in six stations of the Ria de Aveiro coastal lagoon (São-Jacinto-Ovar channel: O1, O2, O3 and Espinheiro channel: E1, E3, E4). The 30% distance of dissimilarity threshold is drawn.

Table 12 Characterization of the affinity groups identified in the Ria de Aveiro interannual analysis. Each variable is represented by mean ± standard deviation (SD). Bold values indicate significant differences in the ANOVA main test while superscript lower case letters indicate the homogenous groups identified by the Tukey post-hoc comparison of means test. NA: not available.

		Affinity groups			**One-way ANOVAs results**	
		Group *A*	**Group *B***	**Group *C***	*F*	*p*
	Nr. of sampling sites	3	3	6		
Parasites	Trematoda abundance (mean± SD)	549.20 ± 849.46	17.09 ± 7.41	30.21 ± 31.16	3.4	0.08
	Species richness (mean ± SD)	**6.00 ± 1.00^{a}**	**4.33 ± 1.15^{a,b}**	**2.83 ± 0.75^{b}**	**12.3**	**< 0.01**
	Prevalence (mean ± SD, %)	**100.00 ± 0.00^{a}**	**97.67 ± 4.04^{a}**	**54.67 ± 17.91^{b}**	**16.1**	**< 0.01**
	Diphterostomum brusinae	**66.67 ± 24.04^{a}**	**97.78 ± 3.85^{a}**	**1.85 ± 4.54^{b}**	**72.6**	**< 0.001**
	Himasthla elongata	**91.11 ± 10.18^{a}**	**15.56 ± 16.78^{b}**	**32.13 ± 26.95^{b}**	**10.1**	**< 0.01**
	Himasthla interrupta	**55.56 ± 50.92^{a}**	**31.11 ± 30.06^{a,b}**	**0.00 ± 0.00^{b}**	**4.2**	**0.05**
	Himasthla quissetensis	**66.67 ± 11.55^{a}**	**2.22 ± 3.85^{b}**	**1.67 ± 2.58^{b}**	**18.2**	**< 0.001**
	Parvatrema minutum	**88.89 ± 10.18^{a}**	**48.89 ± 34.21^{a,b}**	**27.87 ± 23.70^{b}**	**6.3**	**0.02**
	Renicola roscovitus	8.89 ± 10.18	6.67 ± 6.67	0.00 ± 0.00	2.9	0.11
	Bucephalus minimus	11.11 ± 19.25	0.00 ± 0.00	4.17 ± 8.01	0.8	0.47
	Monorchis parvus	2.22 ± 3.85	0.00 ± 0.00	0.00 ± 0.00	1.7	0.24
Environmental data	Temperature (mean ± SD; °C)	NA	NA	19.80 ± 2.89	—	—
	Salinity (mean ± SD)	34.00 ± 8.72	35.67 ± 3.79	28.67 ± 5.20	1.7	0.24
	TOM content (mean ± SD; %)	**2.51 ± 0.84^{a}**	**1.97 ± 0.82^{a,b}**	**1.15 ± 0.43^{b}**	**4.8**	**0.04**
	Median grain size (mean ± SD mm)	0.17 ± 0.07	0.13 ± 0.00	0.16 ± 0.08	0.3	0.72
Host population	Shell length (mean ± SD; mm)	24.62 ± 2.22	26.44 ± 1.24	27.82 ± 1.89	3.0	0.10
	Density (mean ± SD; ind.m^{-2}))	NA	NA	4.33 ± 4.98	—	—

4.1.4. Discussion

In its distribution area, *C. edule* is known to be infected by sixteen different trematode parasite species (de Montaudouin et al., 2009). Eight species were identified in the present study, performed in the Ria de Aveiro. Similar values of species richness were registered in the south coast of Ireland (Fermer et al., 2010, eight species) and in the Merja Zerga, a Moroccan coastal lagoon (Gam et al., 2008, nine species). Ten trematode species were found infecting cockles from the Northern Wadden Sea, Germany (Thieltges & Reise, 2006). Eleven species were recorded in the Ria de Aveiro, Portugal (Freitas et al., 2014; Russell-Pinto et al., 2006) and a maximum of 13 species in Arcachon bay, France (de Montaudouin et al., 2009). Parasite community from the Ria de Aveiro seasonal campaign was dominated by *P. minutum* (Gymnophallidae) presenting the highest prevalence and mean metacercariae abundance per cockle. Former studies performed in the Ria de Aveiro revealed a similar trend (Freitas et al., 2014; Russell-Pinto, 1990) as well as in the Exe Estuary, England (Goater, 1993), Arcachon bay, France (de Montaudouin et al., 2000, 2010) and south coast of Ireland (Fermer et al., 2009, 2010). There are some species that display a restricted distributional range: *Asymphylodora demeli* Markowski, 1935, registered only for the German Baltic coast and *Gymnophallus somateriae* (Levinsen, 1881) only for the German North Sea coast showing thus a northern distribution (Kesting et al., 1996; Thieltges & Reise, 2006) and an undescribed species registered only in Morocco (de Montaudouin et al., 2009). However, overall species composition of the trematode communities is similar among the cockle populations sampled in different locations from south Ireland to Morocco (including the present study area). This suggests a high distributional range of these different trematode species, possibly related to final hosts migration (mainly birds) (Feis et al., 2015).

At lower scale, comparing different stations within the Ria de Aveiro, mean parasite species richness per cockle and mean metacercariae abundance per cockle displayed low values, especially in summer. The mean and maximal number of metacercariae per cockle reached 43 and 132, respectively, contrasting with other works where metacercariae number per cockle frequently surpasses thousands (e.g. Fermer et al. (2010) 5585 metacercariae cockle^{-1}, among others). Low mean number of metacercariae per cockle can be due to parasite-dependent mortality, i.e. mortality of heavily parasitized cockles before sampling. However, infection levels observed in the present work were always too low to induce mortality outbreak. Cockle mortality thresholds were reported at 10-50 for Himasthlidae (Desclaux et al., 2004, 2006; Gam et al., 2009) and 500 metacercariae cockle^{-1} for Gymnophallidae (Gam et al., 2009).

Low metacercariae abundance could also be the result of low diversity of the potential intermediate and final hosts. However, this hypothesis is doubtful because the Ria de Aveiro is considered as a hotspot of biodiversity, is part of the Natura 2000 network (EU habitats directive), designated Special Protected Area and protected by the EU Birds Directive (79/409/CEE). The scarcity of trematode parasites in the Ria de Aveiro could therefore be related to much more multifactorial habitat characteristics, comparing to the few other coastal systems where spatial distribution of these parasites were performed. The success of infection processes, i.e. the efficiency to accomplish trematode parasite life cycle, appears related to the more or less sheltered status of the habitat. In inner areas of coastal ecosystems with more continental influence, more pronounced seasonal variation of temperature and salinity (more extreme values), less hydrodynamics and lower water mass turnover, and sometimes seagrass occurrence and salt marsh proximity, trematode parasite abundance is often low. This is the case in the inner part of Arcachon Bay (France), Merja Zerga (Morocco) (de Montaudouin & Lanceleur, 2011; Gam et al., 2008) and the present study (all stations and seasons) where parasite abundance is < 30 metacercariae cockle^{-1} (excluding *P. minutum*). Conversely, more oceanic influenced habitats with more buffered temperature and salinity fluctuations, and higher hydrodynamics features like the outer part of Arcachon Bay, Merja Zerga or Sylt area (Germany) are generally characterized by higher metacercariae abundance (Gam et al., 2009; Thieltges & Reise, 2007). These observations are not true for the gymnophallid *P. minutum* which can be very abundant in cockles in highly contrasted environments as long as first intermediate host (*Scrobicularia plana*) is present (Fermer et al., 2010).

There are two immediate consequences of trematode scarcity in the Ria de Aveiro. Firstly, and conversely to what is generally expected, sampled trematode community showed no evident seasonality. Typically, trematode parasites using cockles as second intermediate host present a seasonal pattern of increasing infection in the warmer seasons and decreasing or no infection in the cold season (Desclaux et al., 2004; Goater, 1993). For example, in Arcachon bay, a synchrony was observed between parasite emergence from the first intermediate host and infection on the second intermediate host, in May-October period when water temperature was above 15 ºC (de Montaudouin et al., 2016). Absence of seasonality in the present study could result from narrow abiotic (e.g. temperature, salinity) changes occurring throughout the year as the example of the Cachoeira estuary, Brazil (Boehs et al., 2010). However, it is known that the Ria de Aveiro undergoes sharp seasonal salinity and temperature fluctuations (Vaz et al., 2005). In fact, the absence of seasonal fluctuation in parasite

infection is rather interpreted as the result of a general low infection level in cockles preventing any discrimination between high and low infection periods.

Secondly, another consequence of general low trematode infection in cockles is the lack of distinct trematode community between stations. In other words, our results showed a great homogeneity of trematode community in the Ria de Aveiro, characterized by poor cockle infection compared to less sheltered sites along the Atlantic coasts. A general homogeneity was observed, especially influenced by the wide and prevalent distribution of *P. minutum*, which in turn could be related to *S. plana* (first intermediate host) presence and density, one of the most abundant taxa in the Ria de Aveiro (Nunes et al., 2008). Nevertheless, it was evidenced a significant downstream-upstream gradient of prevalence, with the highest overall values registered on the ocean side sampling stations that was mainly shaped by the *H. elongata* prevalence. Taking into account that the first intermediate host of *H. elongata* (*Littorina littorea*) is also widely distributed in the Ria de Aveiro lagoon (Laranjeiro et al., 2015) and beyond arguments concerning the effect of abiotic factors in more or less oceanic areas (as developed above), the host population itself can regulate parasite infection by dilution effect (Magalhães et al., 2017b), also referred as interference effect (Goedknegt et al., 2016). In the present study, target host (cockle) density showed to be negatively linked with trematode prevalence and could have been an important driver of the trematode distribution. These results may be corroborated by those stating that the negative consequences of intraspecific competition at high density can be mitigated by lower parasite burden (Magalhães et al., 2017b).

Knowing that trematodes are useful ecological indicators and can be used even in poorly studied systems (Hechinger et al., 2006), the present study compared 2012 and 2016 (4 years gap) trematode communities in cockles from the same sampling stations and season. Cockles sampled in the present study (group *C*), together with cockles from 2012 affinity group *B* (O1, E1 and E3), presented lower trematode species richness compared to group *A* (O2, O3 and E4 from 2012), i.e. a maximum of five species instead of eight and a 2-fold decrease in species mean prevalence. On one hand this result could indicate a naturally strong interannual variability in terms of trematode communities. Dynamics of trematode larvae community was mostly described with snails as first intermediate hosts, i.e. in a situation where multi-infection is rather rare (Esch & Fernandez, 1994; Esch et al., 2001; Soldánová et al., 2012). In this case, it is expected that some trematode species, especially those of migratory birds, undergo some periodical extinctions (Esch et al., 2001), therefore temporal factors are likely to affect the trematode prevalence as well as overall species richness and composition until the ecosystem reaches a dynamic equilibrium (Esch &

Fernandez, 1994). However, in the second intermediate host, the interaction is different and multispecies infection is generally the rule. Few studies dealing with metacercariae temporal dynamics reported that, in a low impacted/stable environment, trematode community also tends to display significant stability (Campbell et al., 2007; de Montaudouin et al., 2012b; Thieltges & Reise, 2006). Actually, the ecological quality status (EcoQS) of the Ria de Aveiro water bodies, particularly the WB2 where the study area is inserted, registered an improvement from "Moderate EcoQS" (MAMAOT/ARHCentro, 2012) to "High EcoQS" (Marín et al., 2015) based only on benthic habitats density and species composition. Parasites presence is mainly dependent on the diversity and density of any other organisms that participate in the various parasites life cycles (Fredensborg et al., 2006; Hechinger et al., 2006; Hudson et al., 2006; Morley & Lewis, 2007) which is not the limiting factor in the Ria de Aveiro (Rodrigues et al., 2011). However, the parasite infection success is also negatively impacted by several anthropogenic activities (such as roads and consequent nitrogen and metals input (Altman & Byers, 2014)), i.e. low water quality, for example contamination with metals and acidification (Blanar et al., 2009). Indeed, human population in the watershed area of the Ria de Aveiro increased in the last decades, with 250,020 inhabitants registered in 2001 and 353,688 in 2011 (INE, 2001, 2011) which resulted into an increase of exposure to several anthropogenic-derived pressures such as pharmaceuticals (Calisto et al., 2011), metals and other elements contamination (Velez et al., 2015), endocrine disruptor compounds (Rocha et al., 2016) and non-point nitrogen sources (Lopes et al., 2017), among others, not included in the EcoQS evaluation. Besides, official data showed that fisheries effort has been increasing with 127 licensed shellfish fishermen in 2006 and 208 in 2016 (INE, 2006, 2016). Then, on the other hand, and considering trematodes as early warning indicators of deteriorating conditions (MacKenzie, 1999) there is a strong possibility of the Ria de Aveiro being a less healthy ecosystem in 2016 than it was 4 years before. This presumable ecosystem ecological quality loss, taking trematode community changes as indicator, could be already noticed when comparing 2012 results (Freitas et al., 2014) to a former work performed in the same coastal lagoon (Russell-Pinto et al., 2006). Species richness was the same (11 species) but prevalence decreased from 2006 to 2012. However, it is important to refer that while Freitas et al. (2014) analysed cockles from 28 stations, Russell-Pinto et al. (2006) study was performed in a single sampling station which could not represent the entire system. The 2016 lower trematode species richness and prevalence comparing to 2012 data can also represent an evidence of climate change phenomena occurring in the Ria de Aveiro coastal lagoon. Trematode infection is dependent on temperature (and closely related physico-

chemical parameters) thresholds (de Montaudouin et al., 2016) and the data from the Portuguese Institute of the Sea and Atmosphere (IPMA, 2012, 2016) indicated that 2016 fall was the seventh hottest since 1931 (0.8 °C higher comparing to average values) with mean temperature 1.3 °C higher than 2012 fall.

Conclusion

The present study showed a spatial and seasonal homogeneity in terms of trematode parasites prevalence in cockles living in the Ria de Aveiro. However, it was described an influence of abiotic factors and target host population density on the trematode spatial distribution. This work most important outcome was the effective use of trematode communities as possible early warning indicators of global changes and deteriorating conditions occurring in an ecosystem. This finding is comparable to what was demonstrated by Turner (1985) using trematodes infecting oysters as indicators of water quality and Schmidt et al. (2003) using several parasite species infecting flounders as a valuable tool for the assessment of chemical contamination in a habitat, as also reviewed by MacKenzie et al. (1995) and MacKenzie (1999).

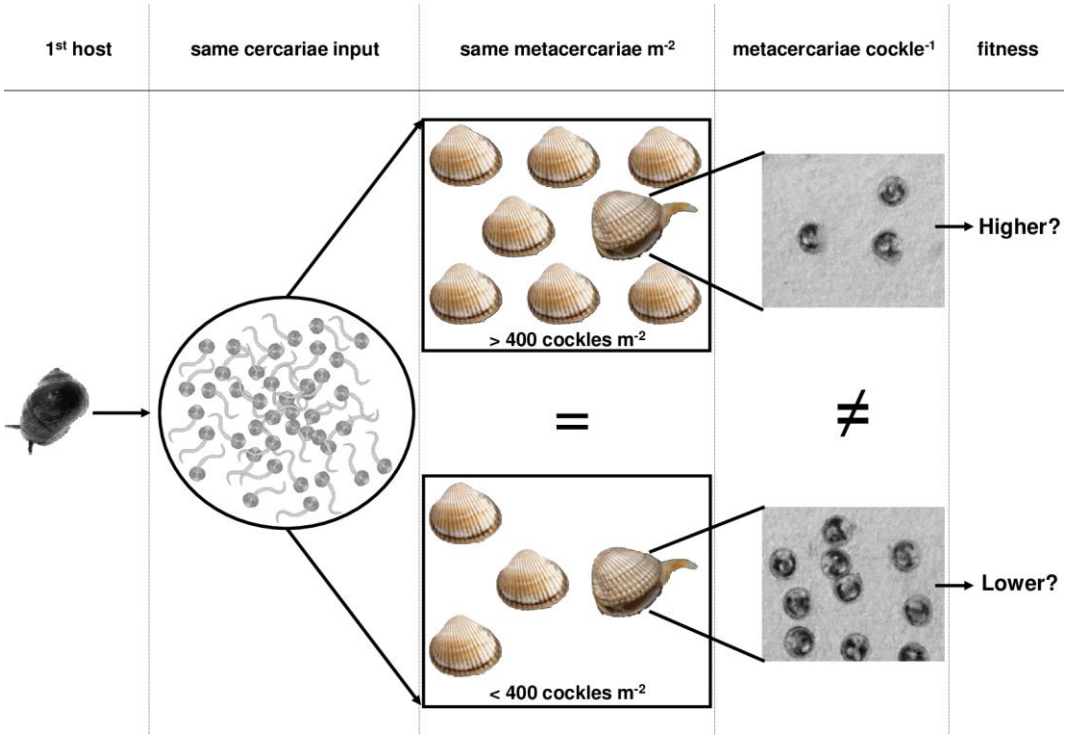
Final considerations

The main result of the previous section showed that despite a relative homogeneity of the parasite community structure in cockles, the among-years heterogeneity of trematode communities was higher than among-stations and among-seasons heterogeneity rejecting the postulated hypothesis. Results demonstrated that trematode communities from the Ria de Aveiro are characterized by low abundance, which resulted in a spatial and seasonal trematode homogeneity (despite an overall channel difference and a slight downstream-upstream gradient). The interannual analysis showed a worrisome loss of trematode diversity and prevalence which could consequently indicate an important loss of overall diversity and/or ecosystem quality status reflecting the negative effects of global change (mean temperature rise and overharvesting, among others). The present study highlighted the importance of trematodes in characterising their associated environment and respective biodiversity which might be helpful to assess ecosystem ecological status and to identify threatened areas. These findings, together with other authors studies, showed a relevant influence of many abiotic factors on cercariae infection in its second intermediate host, such as temperature, light:dark cycle and oxygen concentration. On the other hand, because the role of biotic factors remains less explored, the next

section of this chapter aimed to evaluate the effect of host population dynamics, namely host density, on individual parasite infection (metacercariae abundance).

4.2. Can host density attenuate parasitism?

GRAPHICAL ABSTRACT



4.2.1. Introduction

Among biotic interactions, competition and predation are by far the most studied and their role in determining population dynamics and community structure is clearly evidenced (e.g. Bachelot et al., 2015; Chesson, 2000). Particularly for *Cerastoderma edule*, population success can be negatively influenced by intra-specific competition, through density (de Montaudouin & Bachelet, 1996; Jensen, 1992; 1993) and by intra-specific competition, e.g. through *Mytilus edulis* presence (Ramón, 1996), with effects on the individual growth and consequent life-history traits modification. Similarly, predation showed to strongly regulate the structure of cockle populations being responsible for more than 86% of juveniles mortality (Masski & Guillou, 1999).

Recently, more complex processes have been identified such as facilitation (Bruno et al., 2003). Facilitation corresponds to positive interactions between organisms where at least one of the participants is benefited without any harm for the others. One interesting facilitation output is the positive density dependence at high population densities (e.g. at high densities, neighbors buffer each other from potential lethal thermal stress (Bruno et al., 2003)) that contradicts dogmas stating that there is a negative relationship between population density and individual fitness.

Among the different life histories taking place in marine systems (Bulleri, 2009) here, it was investigated a case of intraspecific facilitation where host density could negatively affect parasite abundance through a dilution effect. The dilution effect theory suggests that a community with high diversity presents a lower parasitism rate. Even though it has been formulated based on microparasite patterns, usually related with contagious diseases (reviewed in Service, 1991), the same assumptions are also valid for macroparasites with complex life cycles that actively search for their hosts (Johnson & Thieltges, 2010). This theory predicts that a community characterized by a greater fraction of unsuitable hosts or parasite predators, i.e. higher diversity, presents a lower individual disease risk (e.g. Keesing et al., 2006; Thieltges et al., 2008a, 2008c). However, concerning the target-host density effect on individual parasitism rate, it is hard to find unanimous assumptions. In the case of contagious diseases, the fact that crowding can increase the transmission rates is empirical and many authors have shown positive correlations between host density and parasite prevalence (reviewed in Patterson & Ruckstuhl, 2013). On the other hand, the host density effect is different for parasites with more complex life cycles, for example the case of mites parasitizing lizards (Sorci et al., 1997), flies parasitizing reindeers (Fauchald et al., 2007) or sea lice parasitizing Atlantic salmons (Samsing et al., 2014). It has been suggested that these highly vagile and social host species tend to aggregate in dense assemblages resulting in lower parasite loads so, applying in a different way, the dilution effect theory, termed as encounter-dilution effect by Mooring & Hart (1992) and Hart (1994). The similar effect of population density on individual infection level remains poorly studied for non-social host species.

For bivalves, some studies are already published on population density effect on individual parasite abundance, one performed in two sites with no replication (Mouritsen et al., 2003) and another with an in situ experimental approach (Thieltges & Reise, 2007). In the present study, using the edible cockle (*C. edule*) as a model and combining in situ experimental and long-term field monitoring approaches, the objective was to investigate the effect of population density on individual infection intensity by trematode parasites. Taking into account that these parasites infect cockles through filtration activity, the first hypothesis was that high host density will have a dilution effect so that infection intensity decreases with host density. Conversely, high cockle density could attract other hosts used by these trematode parasites to complete their life cycle.

4.2.2. Material and methods

4.2.2.1. Study area

Cockles were collected in Banc d'Arguin (44.60°N, 1.25°W), Arcachon bay, France (cf. Figure 5) which is described in more detail in the section 2.2.1 of the chapter 2. Since 1972, Arguin (25 km^2) has been a national reserve with no access allowed and without direct anthropogenic pressure including fishing, which supports several trematode final hosts preying on cockles, including shorebirds (particularly oystercatchers, *Haematopus ostralegus*) and various fish species, e.g. the European seabass (*Dicentrarchus labrax*). Therefore, sampling was performed in this area under strict authorization.

4.2.2.2. Field monitoring

Field monitoring was conducted between 1998 and 2014 (17 years), on a monthly basis. Data on five different variables were collected and analysed: cockle density, cockle shell length, metacercariae abundance (per cockle), metacercariae density (per m^2) and sediment temperature. Cockles were collected using six quadrats (0.25 m^2 each) by sieving sediment through a 1-mm mesh. Shell length (SL) was measured to the nearest mm, with a calliper. Number of cockles per m^2 (density) and per cohort were estimated. The cohorts were separated using Modal class Progression Analysis (MPA) – Bhattacharya method (1967). This subroutine (FISAT II (FAO – ICLARM stock assessment tool)) identifies cohorts by decomposing the polymodal size distribution into their normal distribution components. Cohorts were assumed to be single with a separation index greater than 2 (Gayanilo et al., 2005). For metacercariae abundance (per cockle) and density (per m^2) calculation, ten cockles per cohort were monthly dissected and squeezed, between two glass slides, under a stereomicroscope. All digenean trematodes were identified to species level following several authors descriptions (Bartoli et al., 2000; Bowers, 1969; Bowers et al., 1996; de Montaudouin et al., 2009; Desclaux et al., 2006). Because water temperature potentially is an important driver of trematode transmission, e.g. via its positive effect on cercarial emergence from the first intermediate host (de Montaudouin et al., 2016), sediment temperature was measured (Probe HOBO® Water Temp Pro v2 – U22-001) as proxy during the whole study (except for 1998).

4.2.2.3. Field experiment

In parallel and in the same study area as the monitoring work, a field experiment was conducted between March and November 2005 (9 months). Eight enclosures were settled in a single row parallel to the shoreline, 1 m vertically above mean low water level. The enclosures were regularly spaced with 2 m access paths between them, each consisting of a 50 x 50 x 25 cm metal frame meshed with 10-mm plastic net, buried 20 cm and projecting 5 cm above the sediment surface. After removing all natural large bivalves (> 10 mm) by hand, enclosures were assigned to two treatments: low cockle density (50 cockles per enclosure corresponding to 200 cockles m^{-2}) and high cockle density (200 cockles per enclosure corresponding to 800 cockles m^{-2}). Four enclosure replicates for each treatment were established following an alternate design. Cockles from the same area were sampled and belonged to the 2003 cohort. Their mean shell length at the experiment beginning was 24 ± 1 mm. In order to maintain constant density pressure, cockle mortality was verified every month by collecting empty cockle shells at the sediment surface and by checking for buried shells with fingers. Tagged cockles substitutes of the same shell size were added. Tag consisted in a spot of nail varnish on each valve, each month corresponding to a new colour or pattern. Considering also that no recruits were found inside the enclosures, the density of cockles in enclosures was considered constant during the experiment. Before the beginning of the experiment, thirty cockles were dissected for trematode count and identification, with the same methodology as for field monitoring. After 9 months, all enclosures were removed from the field and the entire surface sediment in the enclosures was sieved through a 1-mm mesh and cockles present from the beginning of the experiment were dissected for trematode count and identification.

4.2.2.4. Data analysis

4.2.2.4.1. Field monitoring

In order to correlate cockle density and metacercariae abundance per cockle from the 17 years database, the period between August and September (2 months) of each year was chosen. This is the period after recruitment season (Magalhães et al., 2016), when the new cohort is already established, with recruits having reached a size highly vulnerable to trematode infection (> 10 mm) and also corresponding to the optimal annual metacercariae infection period (de Montaudouin et al., 2016).

Multiple linear regression analysis was performed to find the significant predictors of cockle metacercariae mean abundance at August-September period. The

dependent variable was the mean number of metacercariae per cockle (mean abundance log-transformed), the independent variables were mean cockle density, mean cockle SL, mean sediment temperature and mean number of metacercariae settled during this period (metacercariae density, per m^2). Metacercariae density at August-September period of each year was calculated assuming random mortality across cohorts and infection levels. For the younger cohort, mean infection of the dissected cockles (n = 10 per cohort) was multiplied by the cockles cohort mean density (ind m^{-2}). Regarding adult cockles (the ≥ 1^+-cohorts), only new infection was considered, and was calculated by subtracting mean August-September metacercariae abundance of the dissected cockles (n = 10) to the mean metacercariae abundance of the two former months (June–July). Finally, prior to multilinear regression analysis, we checked if independent variables were correlated with each other.

In order to determine a threshold of cockle density below which there is a significant increase of mean metacercariae abundance per cockle, a classification analysis was performed using software PRIMER-E. Variables were normalised, the Euclidean distance between samples calculated and an agglomerative hierarchical clustering performed, using the unweighted pair-group mean average (UPGMA) algorithm. To verify the existence of confounding factors influencing the cockles density groups separation, Student *t*-tests were performed to compare mean cockle SL (cohort 0+), sediment temperature and metacercariae density (as a proxy of cercariae input) between both cockle density groups. This threshold was also checked for the four most abundant trematode species by performing a two-way ANOVA with repeated measures testing the influence of metacercariae $cockle^{-1}$ (abundance) and trematode species on cockle density groups.

4.2.2.4.2. Field experiment

A Student *t*-test was performed to assess the effect of cockle density on mortality. Mortality rates in enclosures were (arcsin$\sqrt{p}$) transformed.

Three two-way nested ANOVAs were performed with cockle density as fixed factor (two levels: 200 and 800 cockles per m^2), enclosure as random factor nested in density factor and metacercariae abundance as dependent variable. This test was used for mean cockle SL and both dominant parasite species, *Himasthla interrupta* and *Parvatrema minutum*.

All parametric statistics were performed using STATISTICA (StatSoft) software and homogeneity of variance was verified with Cochran test while normality was assumed.

4.2.3. Results

4.2.3.1. Field monitoring

Mean cockle density at August-September period fluctuated between 19 and 756 ind m^{-2} from 1998 to 2014. Four years were left out of our analysis (2001, 2008, 2013 and 2014) because of the absence of 0^+-cohort.

Sampled cockles were infected by two trematode species at sporocyst stage, *Bucephalus minimus* (Prevalence = 8.3%) and *Monorchis parvus* (Prevalence = 1.0%), and ten different trematode species at metacercariae stage: *Curtuteria arguinae*, *Diphterostomum brusinae*, *Himasthla continua*, *H. elongata*, *H. interrupta*, *H. quissetensis*, *Parvatrema fossarum*, *P. minutum*, *Psilostomum brevicolle* and *Renicola roscovitus*. Four of these species represented 97% of the total metacercariae abundance: *C. arguinae* (37%), *H. interrupta* (27%), *H. quissetensis* (21%) and *P. minutum* (12%).

Multiple regression analysis used to find the significant predictors of cockle metacercariae mean abundance at August-September period, revealed a significant negative relationship between metacercariae mean abundance (*M*) of the newly settled cockles and cockle mean density (*C*) (Figure 32A, Table 13). No relationship ($p > 0.05$) was found between metacercariae mean abundance and cockle *SL*, sediment temperature (*T*) and metacercariae density (*D*) (Figure 32B, 32C and 32D). The model:

$$\log M = -0.001\,C + 0.161\,SL - 0.001\,T + 0.000\,D - 0.840$$

explained 72% of the decrease of metacercariae in cockles among different investigated years (Table 13).

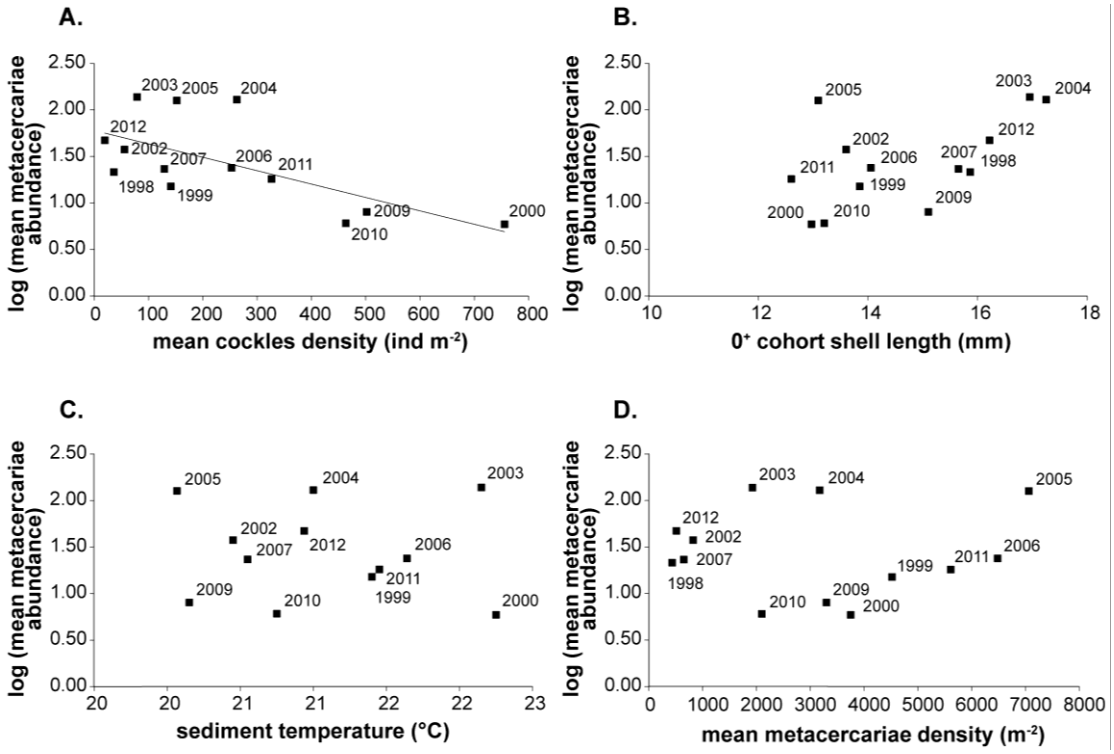


Figure 32 Field monitoring. Relationship between log transformed mean metacercariae (all species combined) abundance of the newly settled cockles at August-September period (y-axis) and **A.** *Cerastoderma edule* density (ind m^{-2}, x-axis) with negative linear regression line representation; **B.** 0^+-cohort shell length (mm, x-axis); **C.** sediment temperature (°C, x-axis); **D.** metacercariae density (metacercariae m^{-2}, x-axis).

Table 13 Multiple linear regression analysis used to find the significant predictors (*Cerastoderma edule* density (ind. m^{-2}), *C. edule* shell length (mm), sediment temperature (°C) and metacercariae density (m^{-2})) of cockle metacercariae mean abundance (dependent variable, log-transformed) during the reference period (August-September) between 1998 and 2014. SE: standard error; *p: p*-value related to partial correlation coefficient; R^2: coefficient of determination.

	Constant	Cockle density	Shell length	Sediment temperature	Metacercariae density	Model
Estimate	-0.840	-0.001	0.161	-0.001	0.000	
SE	3.025	0.001	0.077	0.145	0.000	0.330
P	0.789	0.034	0.074	0.994	0.108	0.041
R^2						0.721

The cluster analysis grouped the different years in two affinity groups according to cockle density and individual metacercariae abundance (Figure 33). These groups were separated by a 400-cockles m^{-2} density threshold (Figure 32A). When density of

cockles was higher than 400 ind m^{-2}, mean metacercariae abundance was 6.6 ± 0.7, and when cockles density was lower than 400 ind m^{-2}, mean metacercariae abundance was 57.9 ± 16.3 (Table 14). Conversely, these two groups of years, i.e. groups of cockle density, presented similar characteristics in terms of mean cockle SL, sediment temperature during the August-September period and total metacercariae density (Table 14).

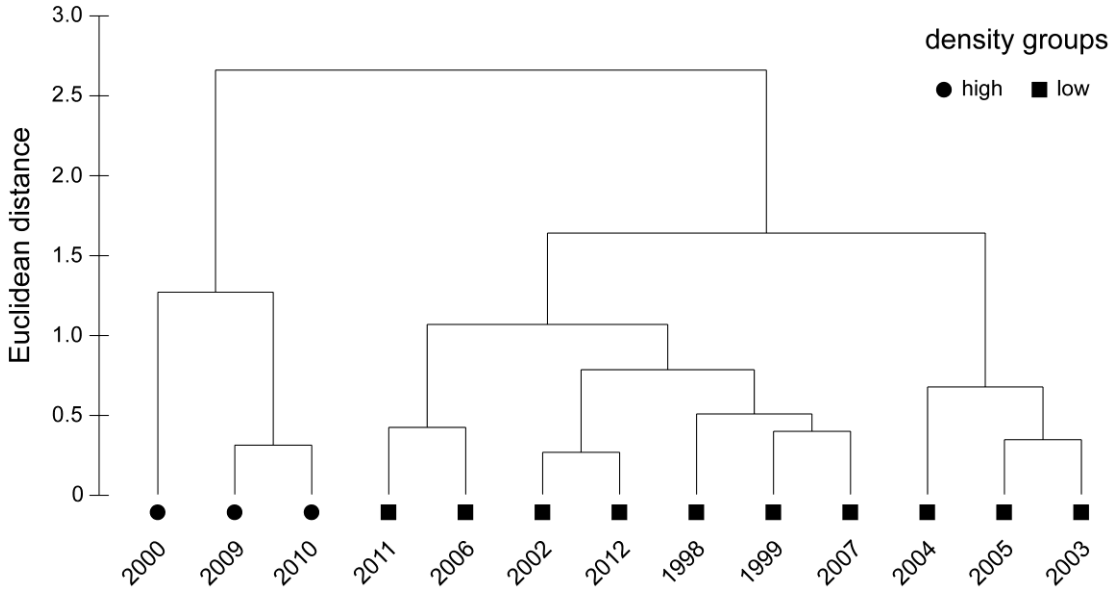


Figure 33 Field monitoring. Classification analysis identifying the cockle density affinity groups in terms of metacercariae individual abundance.

Table 14 Student *t*-test results performed to check differences in terms of cockle density, mean shell length, mean sediment temperature and mean metacercariae density between the two cockle density groups (separated by the 400 cockles m^{-2} threshold). Mean ± standard error (SE) are indicated by cockle density group and for each variable. df: degrees of freedom; *t*: *t* value; *p*: *p* value.

	mean ± SE		**df**	***t***	***p***
	< 400 ind m^{-2}	**> 400 ind m^{-2}**			
cockles density	145.4 ± 33.1	573.6 ± 91.7	11	5.6	< 0.001
shell length (mm)	14.9 ± 0.5	13.8 ± 0.7	11	-1.1	0.29
sediment temperature (°C)	21.1 ± 0.2	21.0 ± 0.6	10	-0.04	0.97
metacercariae density (meta m^{-2})	3119.6 ± 828.9	3050.9 ± 493.0	11	-0.04	0.97

Two-way ANOVA with repeated measures showed the same pattern for the four most abundant trematode species (Figure 34, Table 15). Metacercariae abundance per cockle was higher for lower cockle density. Besides, there was no significant interaction between both factors ("cockle density" and "parasite species"), i.e. the effect of cockle density on metacercariae abundance was similar for the four dominant trematode species.

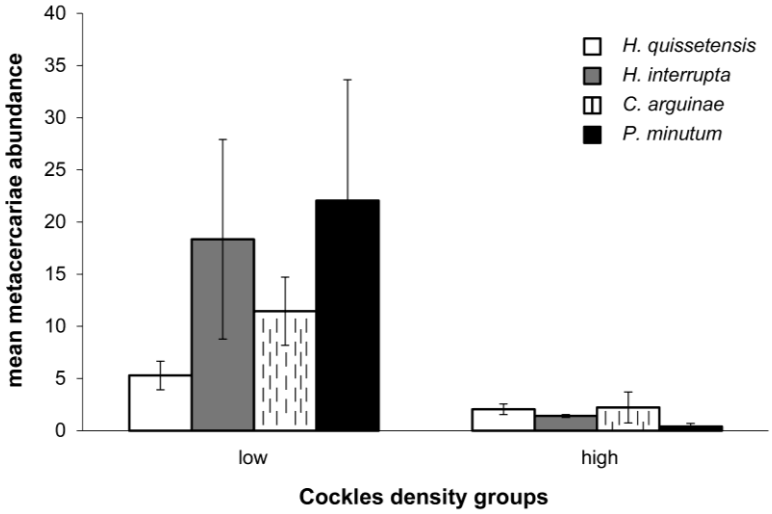


Figure 34 Field monitoring. Newly settled cockles mean metacercariae abundance (metacercaraie cockle^{-1}) for the most abundant trematode species (*Curtuteria arguinae*, *Himasthla interrupta*, *H. quissetensis* and *Parvatrema minutum*) according to *Cerastoderma edule* density groups (high density: > 400 cockles m^{-2} and low density: < 400 cockles m^{-2}).

Table 15 Two-way ANOVA with repeated measures testing the influence of trematode species and cockle density groups on metacercariae abundance (metacercariae cockle^{-1}). df: degrees of freedom; MS: Mean square; *F*: *F* value; *p*: *p* value.

	Df	**MS**	***F***	***p***
cockle density group	1	5.7	15.4	0.004
error	8	0.4		
Species	3	0.3	0.8	0.53
species x density group	3	0.8	2.3	0.10
error	24	0.3		

4.2.3.2. Field experiment

At experiment completion, cockle SL was slightly different (p = 0.05, Table 16) between treatments, 27.4 ± 0.5 and 27.6 ± 0.5 mm for 200 and 800 ind m^{-2}

respectively. Mortality rate of cockles initially introduced in enclosures (65% in 9 months) was not influenced by density treatment (Student *t*-test: $t = -0.7$, $p = 0.53$).

Table 16 Field experiment. Three two-way nested ANOVA results for shell length and both dominant parasite species, *Himasthla interrupta* and *Parvatrema minutum*. Cockle density used as fixed factor (two levels: low and high density (200 and 800 cockles m^{-2} respectively), enclosure as nested factor and metacercariae abundance as dependent variable. df: degrees of freedom; MS: Mean square; *F*: *F* value; *p*: *p* value.

	df	**MS**	***F***	***p***
Shell length				
cockle density	1	1.0	4.1	0.05
enclosure (cockle density)	6	0.2	0.9	0.53
error	72	0.3		
Himasthla interrupta				
cockle density	1	0.0	0.1	0.71
enclosure (cockle density)	6	0.1	1.5	0.20
error	72	0.1		
Parvatrema minutum				
cockle density	1	0.8	15.8	< 0.001
enclosure (cockle density)	6	0.1	2.1	0.07
error	72	0.1		

At the end of the experiment, cockles were infected by seven different trematode species at metacercariae stage: *Curtuteria arguinae*, *Diphterostomum brusinae*, *Himasthla interrupta*, *H. quissetensis*, *Parvatrema minutum*, *Psilostomum brevicolle* and *Renicola roscovitus*. Only two parasite species dominated: *H. interrupta* and *P. minutum*, representing 99.6% of the total number of metacercariae. For *H. interrupta* the mean metacercariae abundance per cockle decreased significantly between March (64 ± 14 metacercariae $cockle^{-1}$) and November, for high (18 ± 2 metacercariae $cockle^{-1}$, Student *t*-test: $t = 6.0$, $p < 0.001$) and low (20 ± 3 metacercariae $cockle^{-1}$, Student *t*-test: $t = 5.1$, $p < 0.001$) cockle density treatments (Figure 35). Furthermore, there were no significant differences between low and high cockle density treatments (Table 16). Conversely, there was a significant increase of *P. minutum* during the experiment period, starting with 134 ± 8 metacercariae per cockle in March and reaching 599 ± 42 metacercariae $cockle^{-1}$ (Student *t*-test: $t = -5.5$, $p < 0.001$) in high density enclosures and 923 ± 94 metacercariae $cockle^{-1}$ (Student *t*-test: $t = -4.2$, $p < 0.001$) in low density ones (Figure 35, Table 16). For both species there was no enclosure (nested factor) effect.

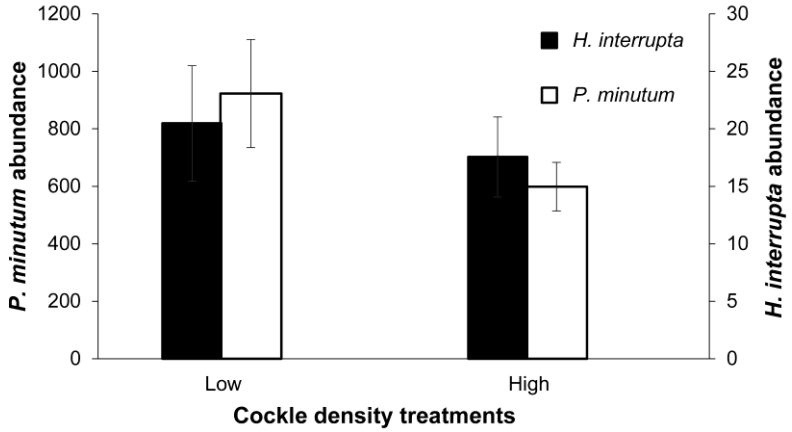


Figure 35 Field experiment. Metacercariae abundance in cockles *Cerastoderma edule* individuals (mean metacercariae ind^{-1} ± standard error) at low (50 cockles enclosure^{-1}, 200 cockles m^{-2}) and high (200 cockles enclosure^{-1}, 800 cockles m^{-2}) host density treatments for the two dominant trematode species, *Himasthla interrupta* and *Parvatrema minutum*.

4.2.4. Discussion

The effect of biodiversity on mollusc target-host individual infection has been explored by several authors. Mouritsen & Poulin (2003b), for example, demonstrated that the non-obligate mutualistic relationship between the intertidal cockle *Austrovenus stutchburyi* and a mud flat anemone significantly depress the rate by which cockles accumulate parasites. In mesocosm experiments, Johnson et al. (2012) found a strong negative effect of the non-host species richness on the transmission success of *Ribeiroia ondatrae* (trematode) to *Helisoma trivolvis* (snail). Welsh et al. (2014), with the *Himasthla elongata* (trematode) – *Littorina littorea* (snail) parasite-host system, proved the existence of several dilutors on a trematode life cycle, i.e. non-host species that interfere with cercariae transmission and consequently reduce the individual host parasite infection. Similarly, Thieltges et al. (2009) showed experimentally that both the presence of two invasive species (*Crassostrea gigas* and *Crepidula fornicata*) and also the density of *Mytilus edulis* reduces (in a lower rate than the invasive species) the native host risk to become infected. On the other hand, the effect of mollusc target-host density on individual infection has been poorly explored. Hence, the present study showed both with in situ experimental approach and, for the first time, with bivalve natural population long-term monitoring that, density (cockles m^{-2}) and mean infection per cockle (metacercariae abundance per cockle) are negatively correlated. Our study suggests a 400-cockle m^{-2} density threshold below which there is a significant increase

of individual infection. This finding supports our first hypothesis: high host density is correlated to lower individual infection, suggesting a dilution effect.

However, knowing that several factors can influence metacercariae infection success, we tested possible confounding factors which could also vary among both cockle density groups and influence cockle infection. Firstly, the mean SL of the sampled cockles (recently settled) was tested between both cockle density groups. It is known that older and consequently larger cockles may be more heavily infected because they have had a longer life period to encounter and accumulate parasites, and also because larger cockles present higher filtration rate which is the main way of infection (Mouritsen et al., 2003; Wegeberg et al., 1999). However, mean SL of sampled cockles was not significantly different between cockle density groups and thus did not explain observed differences on individual infection. Sediment temperature also drives cercariae emission and influences metacercariae infection success (de Montaudouin et al., 2016). Although, in the present case, sediment temperature was similar in both density groups (corresponding to groups of years and respective summer period) and cannot explain cockle mean metacercariae abundance differences. A third possible confounding factor was cercariae abundance in the ecosystem. It was not possible to directly measure cercariae density, as it is a free-living stage very difficult to sample and quantify (de Montaudouin et al., 2016). However, employing metacercariae density (metacercariae m^{-2}) as a proxy of cercariae input allowed to infer that metacercariae density was similar among both density groups. Therefore, cockle population density remains the only addressed factor explaining different individual infections (mean metacercariae $cockle^{-1}$).

The 400-cockles m^{-2} density threshold found, conceded a clear distinction between two main groups: high cockle density group, presenting less metacercariae in each organism, and low cockle density group characterized by higher individual metacercariae infection. According to the literature, this value is also consistent with the threshold beyond which intraspecific competition impairs individual growth, recruitment and survivorship (André & Rosenberg, 1991; Beukema & Dekker, 2015; de Montaudouin & Bachelet, 1996).

The present findings suggested that the negative aspect related to intraspecific competition (and high density) could be balanced by lower parasite infection and consequently better fitness. Although it is necessary to increase knowledge in terms of the real effect of metacercariae on hosts fitness, it has been now established that high metacercariae numbers induce reductions in growth, condition index and burrowing capacity (e.g. Babirat et al., 2004; de Montaudouin et al., 2012a; O'Connell-Milne et al., 2016) and is correlated with higher mortality rates, namely for trematode species that

were observed in this study, i.e. *C. arguinae* (Desclaux et al., 2006), *H. interrupta* (de Montaudouin et al., 2012a), *H. quissetensis* (Desclaux et al., 2004) and *P. minutum* (Gam et al., 2009).

The field experiment strengthens the 17-year survey result: individual infection was higher in cockles living at lower densities. This is in agreement with Mouritsen et al. (2003) for *A. stutchburyi*, the New Zealand cockle (although comparing different sites with no replicates) and Thieltges & Reise (2007) in the Wadden Sea, with field experiments, who concluded that low parasitism rate is a consequence of much higher *C. edule* densities. More in detail, *P. minutum*, the most abundant trematode species found in the field experiment, showed significantly higher infection in cockles in lower density treatments. In this treatment, *P. minutum* infection reached on average 923 metacercariae cockle^{-1}, which is over the threshold causing mortality for gymnophalids (> 500 metacercariae cockle^{-1}) described by Gam et al. (2009). The fact that metacercariae abundance is multiplied by two whereas cockle density is decreased by a four-fold factor suggests that the relationship between both variables is not linear. The reason of non-linearity could be that cercariae were not available *ad libitum*, i.e. that even if cockle density becomes low the number of infecting cercariae is not infinite. Conversely, the second most abundant trematode species found in the field experiment (*H. interrupta*) showed no significant differences between density treatments. In fact, this parasite abundance even declined during the experiment, suggesting that either there was parasite mortality, e.g. parasite lifespan can be shorter than its host (de Montaudouin et al., 2012a) or cockle mortality was parasite-dependent as already demonstrated with a congeneric species (Desclaux et al., 2004). The fact that mortality and growth rates were similar between both treatments could be in favor of the hypothesis that high host density would be compensated by low parasite abundance and vice versa.

The present study demonstrated the positive effect for cockles to be at high density (dilution for parasites), although we did not observe negative effects neither on cockle mortality (field experiment) nor on shell growth (field experiment and monitoring). These results contradict many previous studies describing the negative density-dependent interactions in terms of cockle population dynamics (Jensen, 1992; de Montaudouin & Bachelet, 1996). The absence of negative effects of intraspecific competition on growth could be due to the fact that, at the beginning of the experiment, cockles were already large (SL = 24 mm) which gave them less potential for growth. Another reason could also be that the trophic resource is non-limiting at this cockle density. Indeed, present monitoring and experiment site is characterized by a high concentration of chlorophyll-a (Gam et al., 2010). Previous experiments performed

close to this area and among different suspension feeder species (*Crepidula fornicata* and *Crassostrea gigas*) also showed no manifestation of competition (de Montaudouin et al., 1999), like in artificial systems where food is provided *ad libitum* (Kamermans et al., 1992).

Conclusion

The present field long-term monitoring and field experiment study showed that the encounter-dilution effect can be applied also to natural bivalve populations. This study emphasizes the role of parasitism on host population dynamics leading to implications in decision-making, related to cockle larvae settling within the intertidal zone. Settlement in areas supporting high densities of adult cockles is associated with a high mortality risk, but with this new insight, the fact that high densities will provide safety not only against predators but also parasites was demonstrated, at least those with complex life-cycle. This process may be selectively advantageous because, as mentioned before, high metacercariae abundance is known to affect cockle growth, reducing fitness, and can impair the mobility of the cockle foot. As a result, the re-bury capacity handicap dislodges cockles to the sediment surface (Babirat et al., 2004) making them 7-fold more susceptible to bird predation risk than their buried conspecifics (Thomas & Poulin, 1998) or easily predated by non-target hosts (Mouritsen & Poulin, 2003a). However, density can also stimulate some density dependent pathologies where parasites are horizontally transmitted (i.e. with no need for intermediate hosts). Thus, the “ideal” host density to reach the highest production remains a difficult issue and that the answer certainly depends on local environment.

Final considerations

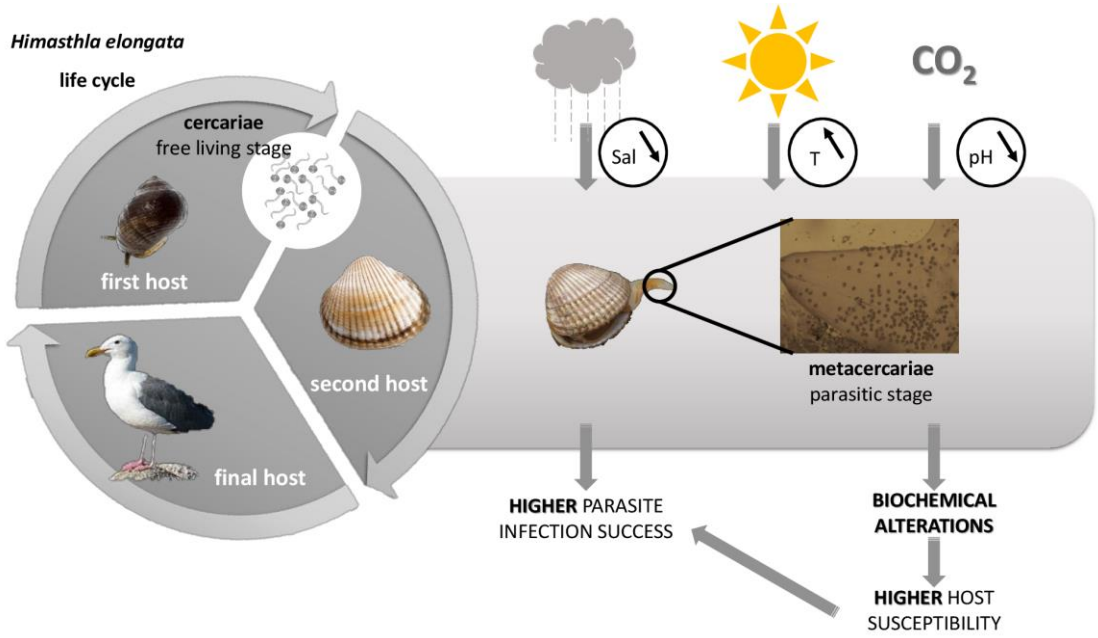
Results presented in the previous chapter section allowed to recognize host population dynamics, namely host density, as an important factor modulating trematode infection rate. This study emphasizes that for certain environments, negative consequences of bivalve intraspecific competition at high densities can be mitigated by lower parasite pressure, leading to implications in decision-making, related to cockle larvae settling within the intertidal zone.

So far, the work included in this thesis has been shown that parasites are everywhere, all the time, depending on different abiotic and biotic environmental conditions. The next section of this chapter will be focused on fill the gap in knowledge about the effects of parasites on cockles in interaction with environmental factors that may change in a climate change scenario.

Cerastoderma edule experimental infection

4.3. Trematode infection modulates cockles biochemical response to climate change

GRAPHICAL ABSTRACT



4.3.1. Introduction

Mainly due to rising anthropogenic activities, such as fuel combustion and deforestation, atmospheric carbon dioxide (CO_2) concentration have increased from 280 ppm, in the preindustrial time, to 430 ppm in 2011 (IPCC, 2014). A significant part of this CO_2 is uptaken by the oceans causing changes in seawater chemistry and consequent pH decrease (Caldeira & Wickett, 2005). Seawater pH is predicted to decrease up to 0.5 pH unit (corresponding to 1120 ppm CO_2 concentration increase (Caldeira & Wickett, 2003)) by the end of the century. Besides, and resulting also from CO_2 build-up, the temperature world regime is changing leading to seawater temperature rise which is among the most important environmental factors affecting costal marine ecosystems (Somero, 2011). Furthermore, global warming will have implications on the water cycle (Schmitt, 2008), increasing the risk of heavy rainfall (Robins et al., 2016) and consequent freshwater input into the oceans but also, increasing the frequency of extreme drought periods with consequent salinity increase (Feyen & Dankers, 2009). For Europe, projections describe an increase in the water temperature up to 4 °C and a higher frequency of heavy precipitation (IPCC, 2014).

These changes are expected to have negative consequences particularly for the biota of intertidal and shallow marine areas (Ringwood & Keppler, 2002). Especially for bivalves, some studies have demonstrated that seawater acidification, temperature rise and salinity shifts result in a negative impact, namely on the condition index (Ong et al., 2017), activity (Verdelhos et al., 2015) and physiological pathways (Gonçalves et al., 2017), respectively. Ultimately, the negative consequences can lead to biodiversity and habitat reduction, shifts of species distributional patterns, and/or even ecosystem loss (Brierley & Kingsford, 2009; Cheung et al., 2009; Doney et al., 2012; Sarà et al., 2014). Effects on one species can drive changes up to the population or community levels. For example, effects at the individual reproduction level can drive changes on the population recruitment which in turn can influence the interactions among populations at the community level, such as predation and competition (Harley et al., 2006). Therefore, predicted changes are likely to impact many biotic interactions, including host–parasite relationships which are particularly dependent on abiotic conditions (de Montaudouin et al., 2016).

The present study aimed to investigate the interactive effects of each abiotic, variable tested (salinity, temperature and pH) and trematode infection on the host biochemical performance. The tested hypothesis was that the edible cockle, *Cerastoderma edule*, exposed to different salinity, temperature and pH levels, as proxy for climate change, modify the infection success of the trematode parasite *Himasthla elongata,* with consequences to cockles biochemical performance. Accordingly, a series of laboratory experiments were conducted by exposing cockles as second intermediate host of *H. elongata* cercariae, to the three distinct experimental conditions.

4.3.2. Material and methods

4.3.2.1. Hosts and parasites

The parasite species used was *Himasthla elongata*, a marine trematode, i.e. the most dominant clade of macroparasites in coastal waters (Lauckner, 1983). This parasite has a complex life cycle with three host species: i) a water bird as definitive host, where the adult parasitic stage develops and sexually reproduces; ii) the gastropod *Littorina littorea* as first intermediate host, where the sporocyst parasitic stage matures and the cercariae are formed and released (asexual multiplication). Usually, cercariae display a short lifespan (< 48 h) and their functional ability to infect has a duration of less than 12 h (de Montaudouin et al., 2016); iii) next, *H. elongata* cercariae penetrate a cockle as second intermediate host and settle as metacercariae.

Potential first intermediate host *L. littorea* infected with *H. elongata* were collected from NIOZ harbour, Texel, the Netherlands (53°00'32.1''N, 4°47'36.5''E) in September 2017. Returning from the field, snails were screened for infections by incubating them in wells (6-well plate) with ~16 mL seawater (salinity = 35 ± 1) at 25 °C under constant illumination for 4 h. After, each well of each plate was observed at the stereomicroscope to check for the presence or absence of emitted cercariae in the water where the snails were immerged. Non-infected snails were returned to their natural habitat, while infected snails, a stock composed by six individuals (shell height ranged between 14 and 18 mm), were transported to Portugal at room temperature and dark conditions. When in the laboratory, snails were kept in the dark, in a 70 L aquarium filled with artificial seawater (salinity = 35 ± 1) constantly refreshed by a chiller to maintain 14 ± 1 °C and fed with fresh sea lettuce (*Ulva* sp.) *ad libitum*.

Potential second intermediate host *Cerastoderma edule* (edible cockle) was collected from the Mira channel, Ria de Aveiro coastal lagoon, Portugal (40°38'31.7''N, 8°44'10.9''W) in September 2017. After collection, cockles were transferred to the laboratory and acclimated for two weeks prior to exposure. The cockles were kept randomly distributed in two aquaria with constant filtration under controlled (CTL) conditions: salinity = 30 ± 1, temperature = 17 ± 0 °C, pH = 8.2 ± 0.0 and photoperiod = 12:12 h (light/dark). Cockles were fed with a heterotrophic and phototrophic species mixture (Algamac Protein Plus®) at a concentration of 720 cells μL^{-1} day^{-1} adapted from Pronker et al. (2015). Cockles shell length (SL) ranged between 13 and 17 mm, i.e. young individuals, in order to limit natural trematode former infection.

4.3.2.2. Experimental design

In order to stimulate cercariae emergence from the infected snails, these organisms were individually transferred to a plate well (6-well plate) with ~16 mL artificial seawater (salinity = 35 ± 1) and exposed to constant illumination and, consequently, higher temperature (~25 °C) during a period that ranged between 4 and 6 h. Emitted cercariae were then individually collected with a pipette and separated into groups of twenty five for immediate cockles infestation.

After cockles acclimation period (see 4.3.2.1 section), three different experiments were carried out with sixty cockles (thirty cockles per treatment from which fifteen were infected) and 750 cercariae (Figure 36). Experiments lasted for 144 h (96 h + 48 h) and were performed in three interspersed weeks to allow snails and parasites recovery and consequent maintenance of the cercariae production.

Each experiment consisted on two phases: (1) incubation period – during 96 h, cockles were exposed to the different treatments of salinity, temperature or pH (according to the experiment) with two levels per condition; (2) infection period – fifteen cockles per treatment (thirty cockles per experiment) were individually infested with *H. elongata* by dropping twenty-five cercariae from a microtube to the water of the cockles containers. During 48 h, cockles were simultaneously exposed to both factors: salinity and parasites, temperature and parasites or pH and parasites, according to the experiment (Figure 36). All experiments were conducted with artificial seawater prepared at required salinity with reverse osmosis water and artificial sea salt (RedSea Salt®).

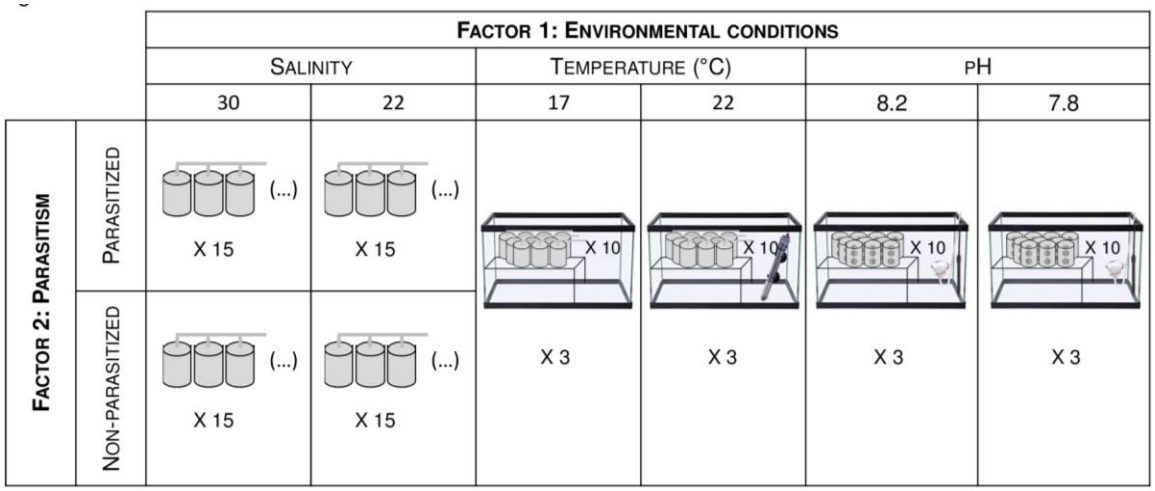


Figure 36 Schematic representation of the experimental designs. Each experiment contained sixty replicates, thirty per each treatment level (two salinity, temperature and pH levels) and fifteen per each treatment level x infection condition (parasitized and non-parasitized).

4.3.2.2.1. Salinity experiment

The levels tested were salinity 30 (salinity control, CTL), resembling conditions at the sampling area (Magalhães et al., 2018), and salinity 22, corresponding to a salinity decrease derived from an increase in the frequency and intensity of precipitation events predicted for Europe, especially in winter (IPCC, 2014). During the first week of the experimental period, to achieve testing salinity 22, the salinity of one group of cockles was lowered at a rate of 2 every other day. After this procedure, for each salinity level (2 levels), thirty cockles were individually placed in glass containers, filled with 50 mL seawater at the required salinity (30 or 22) and with constant aeration (Figure 36). Per salinity level (2 levels) and after the incubation period (96 h of exposure at each salinity), fifteen cockles were individually parasitized (for 48 h) with twenty five cercariae per cockle.

At the end of the experiment (144 h), nine cockles were dissected to measure infection success and six were properly tagged and conserved at - 80 °C for biochemical analysis. Temperature and pH were maintained stable at control conditions (17.3 ± 0.3 ° C and 8.2 ± 0.0, respectively) with 12:12 h (light/dark) photoperiod.

4.3.2.2.2. Temperature experiment

The levels tested were 17 °C (temperature control, resembling conditions at the sampling area (Magalhães et al., 2018), CTL) and 22 °C to test the effects of the predicted ocean warming (IPCC, 2014). For each temperature level, three aquaria were used, each one with ten cockles individually placed in glass containers, filled with 50 mL seawater and with constant aeration (Figure 36). Increased temperature level was obtained by heating with a thermostat the aquaria water where cockles were placed (Figure 36). When expected temperature was reached, cockles were maintained under experimental conditions (2 temperature levels) during 96 h and then five cockles per aquarium (a total of 15 cockles per treatment) were infected (for 48 h) with twenty five cercariae each.

At the end of the experiment (144 h), cockles were dissected to measure infection success (nine individuals) or properly tagged and conserved at - 80 °C for biochemical analysis (six individuals). Salinity and pH were maintained stable at control conditions (30 ± 1 and 8.2 ± 0.0, respectively) with 12:12 h (light/dark) photoperiod.

4.3.2.2.3. pH experiment

The levels tested were 8.2 (pH control, based on the sampling area registered values, CTL) and 7.8 obtaining 403 ± 19 µatm of pCO_2 and 1112 ± 149 µatm of pCO_2 respectively (Table 17), an increase considered within pCO_2 predictions by the end of the twenty first century (IPCC, 2014; Raven et al., 2005). For each pH level, three aquaria were used with ten cockles each. In order to separate cockles (for later individual infection) but still maintain water circulation and pH conditions, cockles were isolated by pierced plastic containers covered by a 250 µm mesh net and filled with 50 mL seawater. Since *H. elongata* cercariae mean size is 400 x 180 µm (Krupenko & Dobrovolskij, 2015), this net avoided larvae escape. Acidification was obtained by directly diffusing CO_2 into aquaria and continuously monitored and controlled using a pH Stat system (Aquamedic®) (Figure 36). When expected pH was reached, cockles were maintained under experimental conditions (2 pH levels) for 96 h, infected (for 48

h) with twenty five cercariae each and at the end of the experiment (144 h) dissected to measure infection success (nine individuals) or properly tagged and conserved at - 80 °C for biochemical analysis (six individuals). Salinity and temperature were maintained stable (30 ± 1 and 17.3 ± 0.3 ° C, respectively) with 12:12 h (light/dark) photoperiod.

Water samples were collected from each aquarium at the experiment start (T0 h), middle (T72 h) and end (T144 h) for further total alkalinity (TA) calculation using the Alkalinity calculator (USGS, 2012) after potentiometric titration (Gran, 1952). TA values and measured parameters (temperature, salinity and pH values, read at the time of water sampling) were plotted in CO_2Calc software (Robbins et al., 2010) and carbonate chemistry determined using dissociation constants K1, K2 (Dickson & Millero, 1987; Mehrbach et al., 1973) and KHSO4 (Dickson, 1990). Mean values of each parameter were calculated considering all samples and collection periods.

Table 17 Mean ± standard deviation of carbonate system physicochemical parameters for each condition. Measured parameters: salinity, temperature and pH; Determined parameters: Total alkalinity (TA), Partial CO_2 pressure (pCO_2), bicarbonate (HCO_3^-), carbonate ion concentration (CO_3^{2-}) and saturation states of calcite (Ω Cal) and aragonite (Ω Ag). CTL: control (field condition).

	CTL (8.2)	**CTL − 0.4 units (7.8)**
Salinity	30.0 ± 0.0	30.0 ± 0.0
Temperature	17.4 ± 0.1	17.2 ± 0.1
pH	8.18 ± 0.01	7.78 ± 0.04
TA	2291 ± 101	2241 ± 189
***p*CO_2 (µatm)**	403 ± 19	1112 ± 149
HCO_3^- (µmol kg^{-1})	1906 ± 85	2074 ± 181
CO_3^{2-} (µmol kg^{-1})	158 ± 8	68 ± 7
Ω Cal	3.89 ± 0.20	1.68 ± 0.18
Ω Ara	2.48 ± 0.13	1.07 ± 0.11

4.3.2.3. Biochemical descriptors

Several studies showed that biochemical markers are useful tools to determine the impact of several stressors on marine bivalves (Faggio et al., 2016), including cockles (Freitas et al., 2012; Marques et al., 2016). In the present study, biochemical alterations induced by abiotic factors, by parasitism or by the interactive effects, were assessed measuring the energy reserves content namely protein (PROT) and glycogen (GLY); the activity of antioxidant enzymes namely superoxide dismutase (SOD), that converts superoxide anion (O_2^-) to hydrogen peroxide (H_2O_2), catalase (CAT) and

glutathione peroxidase (GPx) both converting the SOD product (H_2O_2) to water; the activity of biotransformation enzymes namely glutathione S- transferases (GSTs) that catalyse the conjugation of the reduced form of glutathione to xenobiotics acting as cell detoxifier; the metabolic capacity through the electron transport system (ETS) activity, and the level of cellular damage through the lipid peroxidation (LPO) quantification in organisms after exposure.

Cockles were pooled in groups of two specimen accounting for three replicates (corresponding to six cockles) per condition (2 salinity, temperature or pH levels x 2 infection conditions: parasitized (P) and non-parasitized (NP)). Each replicate was homogenised with liquid nitrogen and separated into 0.3 g of soft tissue subsamples in order to perform the extraction with three different buffers (1:2 w/v). Supernatant of the subsample extracted with phosphate buffer (50 mM KH_2PO_4 (pH 7.0), 1 mM EDTA, 1% (v/v) Triton X-100, 1% (w/v) PVP, 1 mM DTT, 1:2 w/v) and centrifuged at 4 °C, 10,000 g during 20 min was used to determine PROT and GLY concentrations, SOD, CAT, GPx and GSTs activities. Supernatant of the subsample extracted with 0.1 M Tris-HCl (pH 8.5), 15% (w/v) Poly Vinyl Pyrrolidone, 153 µM $MgSO_4$ and 0.2% (w/v) Triton X-100 buffer and centrifuged at 4 °C, 3,000 g during 20 min was used to determine ETS activity. Supernatant of the subsample extracted with 20% (w/v) trichloroacetic acid (TCA) and centrifuged at 4 °C, 10,000 g during 20 min was used to determine LPO. All supernatants were used immediately after extraction or preserved at - 20 °C for a short period of time.

Total PROT content was determined according to Robinson & Hogden (1940), following the Biuret method that uses Bovine serum albumin (BSA) as standard (0–40 mg mL^{-1}). After 10 min incubation at 30 °C the absorbance was read at 540 nm. The results were expressed in mg and used to calculate enzymes activity.

The GLY content was determined by the phenol-sulphuric acid method (Dubois et al., 1956). Absorbance was measured at 492 nm and results were expressed in mg per grams (g) of fresh weight (FW).

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

The activity of CAT was measured by the reaction of the enzyme with methanol in the presence of H_2O_2 (Johansson & Borg, 1988). The standard curve was determined using formaldehyde standards (0–150 mM) and the reaction was

performed during 20 min in an orbital incubator set at room temperature. The formaldehyde formation in the presence of Purpald was spectrophotometrically measured at 540 nm and the enzymatic activity was expressed in U per mg of PROT. One U is defined as the amount of enzyme that generated the formation of 1.0 nmol formaldehyde per min.

The activity of GPx was determined by the reaction of reduced glutathione, cumene hydroperoxide, glutathione reductase and NADPH (Paglia & Valentine, 1967). The NADPH oxidation is accompanied by a decrease in absorbance spectrophotometrically measured at 340 nm. The activity was calculated using $\varepsilon = 6.22 \times 10^{-3}\ M^{-1}\ cm^{-1}$ and expressed in U per mg of PROT. One U is defined as nmol of NADPH oxidized per min.

The activity of GSTs was determined using CDNB as substrate according to Habig et al. (1974) method. The increase in absorbance was spectrophotometrically measured at 340 nm. The activity was calculated using $\varepsilon = 9.60 \times 10^{3}\ M^{-1}\ cm^{-1}$ and expressed in U per mg of PROT. One U is defined as nmol of CDNB conjugate formed per min.

The ETS activity was determined by the amount of formazan formed after adding p-IodoNitroTetrazolium (De Coen & Janssen, 1997), calculated using $\varepsilon = 15.9 \times 10^{3}\ M^{-1}\ cm^{-1}$ and expressed in nmol of formazan formed per min per g of FW.

Finally, LPO levels were measured by the quantification of thiobarbituric acid reactive substances (TBARS) (Buege & Aust, 1978). 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 ($\varepsilon = 156 \times 10^{3}\ M^{-1}\ cm^{-1}$). Results were expressed as nmol of MDA equivalents per g FW.

4.3.2.4. Data analysis

For each experiment, two two-way ANOVAs were performed. The first used to verify cockle SL similarity among treatments and the second used to test treatment (salinity, temperature and pH levels), infection condition (parasitized and non-parasitized) and the interaction between factors on the number of *H. elongata* metacercariae infecting cockles after exposure. Prior to analysis, homogeneity of variance was verified with Cochran test. Two-way ANOVAs were followed by post-hoc Dunnett test for comparison of means. These analysis were performed using STATISTICA (StatSoft) Software.

Due to a lack of homogeneity of variance, PROT, GLY, SOD, CAT, GPx, GSTs, ETS and LPO were separately submitted to a non-parametric permutational analysis of variance (PERMANOVA Add-on in PRIMER-E software) with a two factors design: abiotic treatment (salinity, temperature and pH) as factor 1 and infection condition (non-parasitized (NP) and parasitized (P)) as factor 2. PERMANOVA main test was performed to test the effect of treatment, infection condition and the interaction between these two factors on each biomarker. PERMANOVA main tests were considered significant when $p < 0.05$ and followed by PERMANOVA pair-wise tests. Pair-wise tests were used to test the effect of infection condition (NP and P) within each treatment level (two levels per experiment) and the effect of treatment level within each infection condition. PERMANOVA main tests results are detailed described in a table and pair-wise tests results are represented in figures with lower case letters and in the main text by *p* values.

The matrix of each experiment containing biomarkers results per treatment and infection condition was normalised and the Euclidean distance calculated. Then, distance among centroids (i.e. the mean position of all the points representing a given sample) was visualized in Principal Coordinates Ordination analysis (PCO). In the PCO graph, the variables (biomarkers) that best explained the samples spatial distribution were represented as superimposed vectors.

4.3.3. Results

4.3.3.1. Infection success

Cockles used on each experimental treatment (two levels of salinity, temperature and pH) and infection condition (non-parasitized and parasitized) of each experiment showed similar mean SL ($p > 0.05$), i.e. 15.7 mm, ranging between 13 and 17 mm.

For the three experiments, experimentally infected cockles, from thereafter named as “parasitized cockles (P)”, presented significantly higher number of *H. elongata* metacercariae compared to cockles naturally infected, from thereafter named as “non-parasitized (NP) cockles” (Tables 18 and 19). Cockles presented similar infection level when exposed to lower salinity (7.1 ± 2.1 metacercariae cockle^{-1}) compared to control salinity (5.6 ± 1.5 metacercariae cockle^{-1}) (Tables 18 and 19). Likewise, *H. elongata* abundance was similar in cockles infected at 22 °C (7.1 ± 2.9 metacercariae cockle^{-1}) compared to cockles infected at control temperature, 17 °C (6.5 ± 5.3 metacercariae cockle^{-1}) (Tables 18 and 19). In the pH experiment, *H.*

elongata infection was higher when cockles were infected at lower pH (7.8) (6.9 ± 2.2 metacercariae cockle^{-1}) compared to control pH (8.2) (4.1 ± 2.0 metacercariae cockle^{-1}) (Tables 18 and 19).

Table 18 Mean (± standard deviation) number of *Himasthla elongata* metacercariae found in dissected cockles for each experiment (salinity, temperature and pH) and each treatment (two levels per experiment). NP: non-parasitized cockles; P: parasitized cockles.

	Mean ± SD			
	30		22	
Salinity	NP	P	NP	P
	0.7 ± 0.9	5.6 ± 1.5	0.8 ± 1.0	7.1 ± 2.1
	17 °C		22 °C	
Temperature	NP	P	NP	P
	0.2 ± 0.9	6.5 ± 5.3	0.7 ± 0.8	7.1 ± 2.9
	8.2		7.8	
pH	NP	P	NP	P
	0.3 ± 0.5	4.1 ± 2.0	0.6 ± 0.5	6.9 ± 2.2

Table 19 Two-way ANOVA results performed to test the effects of experimental treatments, infection condition and interaction of factors on the number of *Himasthla elongata* metacercariae found in dissected cockles. MS: mean square; *F*: *F* value; *p*: *p* value. Bold letters indicate significant differences ($p < 0.05$).

Two-way ANOVAs				
		MS	*F*	*p*
Salinity	Treatment	0.2	0.8	0.37
	Infection condition	**69.0**	**377.6**	**< 0.001**
	Interaction	0.1	0.4	0.55
	Error	0.2		
Temperature	Treatment	0.5	2.1	0.16
	Infection condition	**18.9**	**79.5**	**< 0.001**
	Interaction	0.0	0.0	0.87
	Error	0.2		
pH	Treatment	**0.8**	**5.9**	**0.02**
	Infection condition	**18.6**	**132.9**	**< 0.001**
	Interaction	0.2	1.5	0.23
	Error	0.1		

4.3.3.2. Biochemical data

4.3.3.2.1. Salinity experiment

Salinity treatment and infection condition as well as the interaction between factors presented no significant effect on PROT and GLY content (Table 20).

Salinity treatment did not affect SOD activity in NP neither P cockles (Table 20). Trematode infection exerted an effect on SOD activity (Table 20) with significantly higher SOD values in NP compared to P cockles within both salinity treatments ($p < 0.05$, Figure 37A). The interaction between salinity and infection conditions presented no effect on SOD activity (Table 20).

Overall, salinity treatment presented no significant effect on CAT activity (Table 20). However, within P cockles, salinity proved do exert an effect on CAT activity with lower values when cockles were exposed to salinity 22 compared to salinity 30 ($p < 0.05$, Figure 37B). Trematode infection significantly affected CAT activity (Table 20), especially noticed at the lowest salinity treatment when NP cockles presented significantly higher CAT activity compared to P cockles ($p < 0.01$, Figure 37B). PERMANOVA revealed a significant effect of the interaction between salinity and infection condition on CAT activity (Table 20).

Salinity treatment showed to have no influence on GPx activity (Table 20). Infection condition significantly affected GPx activity (Table 20) within salinity treatment 22, with higher values registered for NP compared to P cockles (Figure 37C). There was also a significant effect of the interaction of factors (salinity and infection) on the GPx activity (Table 20).

Salinity treatment and infection condition as well as the interaction between factors presented no significant effect on GSTs activity (Table 20).

Salinity treatment presented an effect on ETS levels (Table 20) with a different trend according to infection condition. Within NP cockles, ETS activity was significantly lower at salinity 30 compared to the lowest salinity treatment ($p < 0.05$, Figure 37D), while within P cockles ETS activity was significantly higher at control salinity (30) compared to salinity 22 ($p < 0.01$, Figure 37D). Infection condition exerted a significant effect on ETS activity (Table 20) with significantly higher activity in P cockles compared to NP cockles within control salinity (30) ($p < 0.01$, Figure 37D) and significantly lower activity in P cockles compared to NP cockles exposed to low salinity treatment ($p < 0.01$, Figure 37D). PERMANOVA showed also a significant effect of the interaction between salinity and infection on the ETS activity (Table 20).

Table 20 PERMANOVA results performed to test the effects of experimental treatments, infection condition and interaction of factors on the biochemical descriptors. PROT: protein; GLY: glycogen; SOD: superoxide dismutase; CAT: catalase; GPx: glutathione peroxidase; GSTs: glutathione S-transferases; ETS: electron transport system; LPO: lipid peroxidation; MS: mean square; *F*: *F* value; *p*: *p* value. Bold letters indicate significant differences ($p < 0.05$).

		Treatment			**Infection condition**			**Interaction**			**Error**
		MS	***Pseudo-F***	***p***	***MS***	***Pseudo-F***	***p***	***MS***	***Pseudo-F***	***p***	***MS***
Salinity	PROT	0.0	0.1	0.82	7.1	23.2	0.06	1.5	4.9	0.06	0.3
	GLY	0.6	0.7	0.42	1.3	1.7	0.23	2.7	3.4	0.11	0.8
	SOD	0.0	0.0	0.95	**7.9**	**21.4**	**0.002**	0.1	0.4	0.56	0.4
	CAT	0.4	1.2	0.30	**3.8**	**10.8**	**0.01**	**4.0**	**11.5**	**0.01**	0.4
	GPx	0.0	0.0	0.86	**6.1**	**30.9**	**< 0.001**	**3.5**	**17.0**	**0.003**	0.2
	GSTs	1.0	1.0	0.35	1.1	1.1	0.33	1.1	1.1	0.33	1.0
	ETS	**1.8**	**13.3**	**0.008**	**1.0**	**7.7**	**0.02**	**7.1**	**52.5**	**< 0.001**	0.1
	LPO	**2.3**	**7.4**	**0.04**	**4.0**	**12.9**	**0.008**	**2.2**	**7.2**	**0.03**	0.3
Temperature	PROT	2.7	8.6	0.06	4.0	12.5	0.06	1.7	5.4	0.06	0.3
	GLY	0.3	0.3	0.60	1.10^{-4}	1.10^{-4}	0.99	2.0	1.8	0.21	1.1
	SOD	**4.5**	**11.6**	**0.008**	**3.2**	**8.1**	**0.02**	0.2	0.6	0.48	0.4
	CAT	0.0	0.0	0.93	**3.6**	**6.1**	**0.04**	2.6	4.5	0.06	0.6
	GPx	**6.7**	**24.4**	**0.001**	**2.2**	**7.9**	**0.03**	2.10^{-4}	6.10^{-4}	0.98	0.3
	GSTs	1.1	1.2	0.29	2.2	2.4	0.16	0.5	0.6	0.48	0.9
	ETS	**2.5**	**29.9**	**0.002**	**7.0**	**57.2**	**< 0.001**	0.5	4.3	0.07	0.1
	LPO	**6.8**	**35.4**	**< 0.001**	**2.1**	**11.1**	**0.01**	0.6	3.0	0.12	0.2
pH	PROT	2.0	2.4	0.16	1.6	1.9	0.20	0.8	1.0	0.34	0.8
	GLY	6.2	10.6	0.06	4.10^{-6}	6.10^{-6}	0.99	0.0	0.1	0.81	0.6
	SOD	1.9	5.7	0.05	**6.1**	**18.0**	**0.003**	0.2	0.7	0.43	0.3
	CAT	1.6	2.7	0.13	0.1	0.2	0.70	**4.5**	**7.5**	**0.02**	0.6
	GPx	2.3	2.4	0.16	1.2	1.2	0.30	0.1	0.1	0.77	1.0
	GSTs	0.3	0.3	0.58	2.5	2.8	0.14	1.3	1.5	0.27	0.9
	ETS	2.1	3.7	0.09	0.5	0.8	0.39	**4.0**	**7.3**	**0.03**	0.6
	LPO	1.2	1.2	0.30	0.4	0.4	0.56	1.5	1.5	0.26	1.0

Salinity treatment affected LPO levels (Table 20). Nevertheless, this effect was only significant within P cockles ($p < 0.05$, Figure 37E) with significantly higher LPO levels at salinity 30 compared to low salinity treatment (22). Infection condition presented a significant effect on LPO levels (Table 20) at low salinity treatment (22), with LPO levels significantly lower in P compared to NP cockles ($p < 0.01$, Figure 37E). Interaction between salinity and infection showed a significant effect on LPO levels (Table 20).

The PCO horizontal dimension (Axis 1) explained 69% of the total variation separating NP cockles, in the negative side of the axis, from P cockles, in the positive side of the axis. SOD, CAT, GPx and LPO were the variables that better explained this variation presenting high negative correlation with axis 1 (r > - 0.8). Axis 2 explained 25% of the total variation which separated P cockles exposed to control salinity, in the positive side of the axis, from the other conditions with a strong positive correlation with ETS activity (r > 0.8) (Figure 37F).

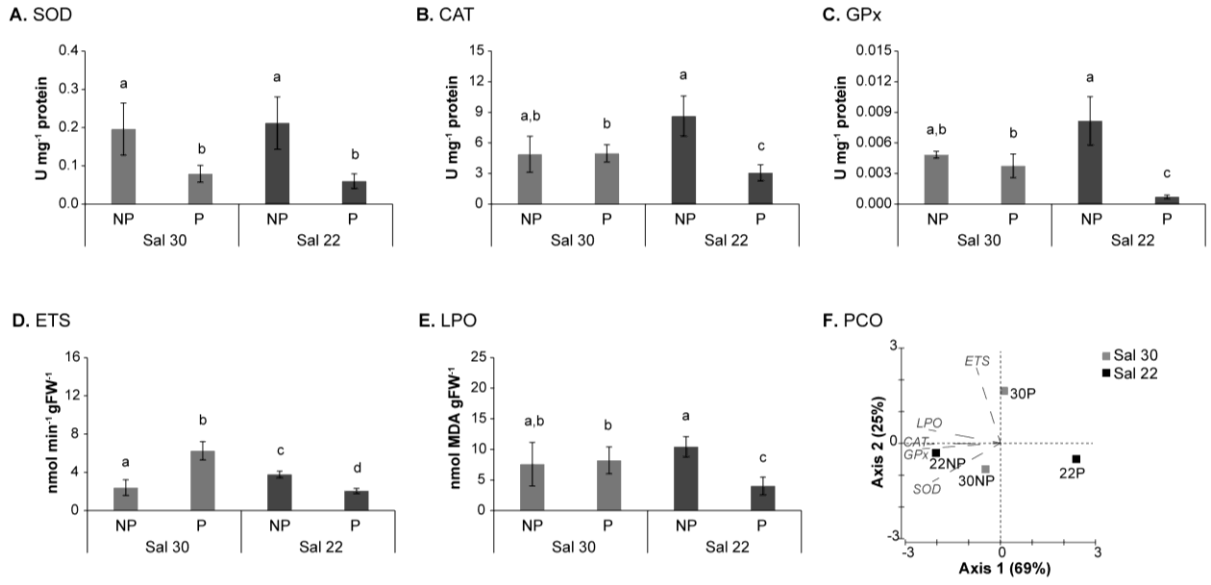


Figure 37 *Salinity experiment.* Mean values (± standard deviation) and significant differences represented with different lower case letters of **A.** SOD, superoxide dismutase activity; **B.** CAT, catalase activity; **C.** GPx, glutathione peroxidase activity; **D.** ETS, electron transport system activity; **E.** LPO, lipid peroxidation levels in non-parasitized (NP) and parasitized (P) cockles with *Himasthla elongata*; **F.** Principal coordinates ordination analysis (PCO) showing the variables that better explained samples distribution.

4.3.3.2.2. Temperature experiment

Temperature treatment, infection condition and interaction between factors presented no significant effect on PROT and GLY content (Table 20).

Temperature treatment showed an effect on SOD activity (Table 20), mainly within NP cockles, with significantly higher levels when cockles were at control temperature (17 °C) compared to cockles from the 22 °C treatment ($p < 0.05$, Figure 38A). Infection condition presented a significant effect on SOD activity (Table 20) especially noticed when cockles were exposed to the higher temperature treatment (22 °C) and with significantly higher SOD values in NP compared to P cockles ($p < 0.05$, Figure 38A). Despite lower SOD activity recorded in P cockles exposed to 22 °C, no significant effect of the interaction between temperature and infection on SOD activity was observed (Table 20).

Temperature treatment affected CAT activity within P cockles, with significantly lower CAT values when cockles were exposed to the higher temperature compared to control temperature ($p < 0.05$, Figure 38B). Infection condition presented a significant effect on CAT activity (Table 20) within 22 °C treatment with NP cockles presenting significantly higher CAT than P cockles ($p < 0.05$, Figure 38B). The interaction between temperature and infection did not affect CAT activity (Table 20).

Temperature presented a significant effect on GPx activity (Table 20) with significantly higher levels at 17 °C compared to 22 °C ($p < 0.05$, Figure 38C). Infection condition affected the GPx activity (Table 20) within the control temperature treatment with NP cockles presenting significantly higher GPx levels compared to P cockles ($p < 0.01$, Figure 38C). PERMANOVA demonstrated no significant effect of the interaction between factors on GPx activity (Table 20).

Temperature treatment, infection condition and interaction between factors presented no significant effect on GSTs activity (Table 20).

Temperature affected ETS activity (Table 20) noticed within NP cockles with significantly higher values in cockles exposed to temperature control in comparison to cockles exposed to 22 °C treatment ($p < 0.01$, Figure 38D). Infection condition significantly affected ETS levels (Table 20) with significantly higher activity in P compared to NP cockles ($p < 0.05$, Figure 38D). PERMANOVA results demonstrated that the interaction between temperature and infection did not affect ETS levels (Table 20).

Temperature presented a significant effect on LPO levels (Table 20) with significantly higher LPO values when cockles were exposed to 22 °C compared to cockles from the control temperature treatment ($p < 0.04$, Figure 38E). Infection

condition also affected LPO levels (Table 20) within temperature treatment 22 °C with P cockles presenting significantly higher LPO levels than NP cockles ($p < 0.05$, Figure 38E). There was no significant effect of the interaction between temperature and infection on LPO levels (Table 20).

The PCO axis 1 explained 63% of the total variation separating the control condition, i.e. NP cockles exposed to 17 °C, in the negative side of the axis, from P cockles exposed to higher temperature (22 °C), in the positive side of the axis. SOD and GPx presented high negative correlation (r > - 0.8) and LPO high positive correlation (r > 0.8) with axis 1 and together were the variables that better explained the samples separation. Axis 2 explained 32% of the total variation separating NP cockles exposed to higher temperature (22 °C), in the negative side of the axis, from the remaining conditions with a strong positive correlation with ETS activity (r > 0.8) and a strong negative correlation with CAT activity (r > ─ 0.7) (Figure 38F).

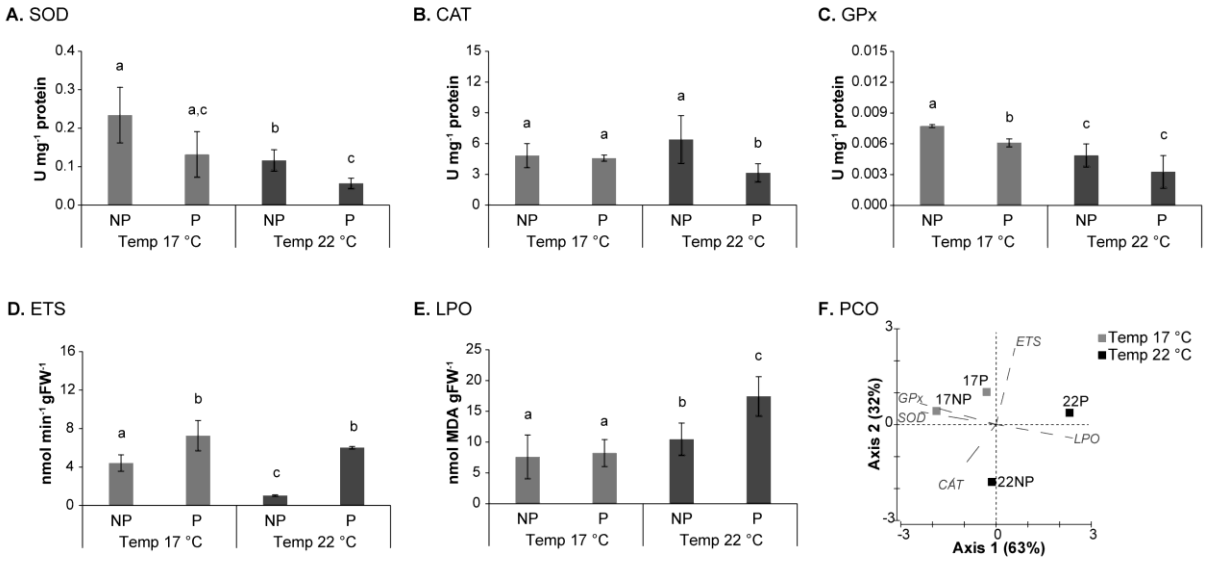


Figure 38 *Temperature experiment.* Mean values (± standard deviation) and significant differences represented with different lower case letters of **A.** SOD, superoxide dismutase activity; **B.** CAT, catalase activity; **C.** GPx, glutathione peroxidase activity; **D.** ETS, electron transport system activity; **E.** LPO, lipid peroxidation levels in non-parasitized (NP) and parasitized (P) cockles with *Himasthla elongata*; **F.** Principal coordinates ordination analysis (PCO) showing the variables that better explained samples distribution.

4.3.3.2.3. pH experiment

There was no effect of pH, infection neither interaction between those on the PROT and GLY content (Table 20).

Different levels of pH exerted no effect on SOD activity (Table 20). Infection condition presented a significant effect on SOD levels (Table 20) with significantly higher activity in NP compared to P cockles ($p < 0.05$, Figure 39A). PERMANOVA results demonstrated no significant effect of the interaction on SOD activity (Table 20).

The pH treatment and infection condition did not affect CAT activity (Table 20). However, there was a significant effect of the interaction between pH and infection on CAT activity (Table 20) with significantly lower CAT when cockles were exposed to both lower pH and infection (Figure 39B).

There was no effect of pH, infection neither interaction between those on the GPx (Table 20, Figure 30C) and GSTs activity (Table 20).

The pH treatment and infection condition exerted no effect on ETS activity (Table 20), except for NP cockles between both pH levels (Figure 39D). PERMANOVA revealed a significant effect of the interaction of factors (Table 20).

There was no effect of pH, infection neither interaction between those on the LPO levels (Table 20, Figure 39E).

The PCO axis 1 explained 60% of the total variation separating the control condition, i.e. NP cockles exposed to pH 8.2, in the negative side of the axis, from the other three conditions, in the positive side of the axis. SOD and ETS activities were the variables that better explained the variation, presenting high negative correlation ($r > -0.8$) with axis 1. The PCO vertical dimension (Axis 2) explained 32% of the total variation separating P cockles exposed to lower pH (7.8), in the positive side of the axis, from the other conditions with a strong negative correlation with CAT activity ($r > -0.8$) (Figure 39F).

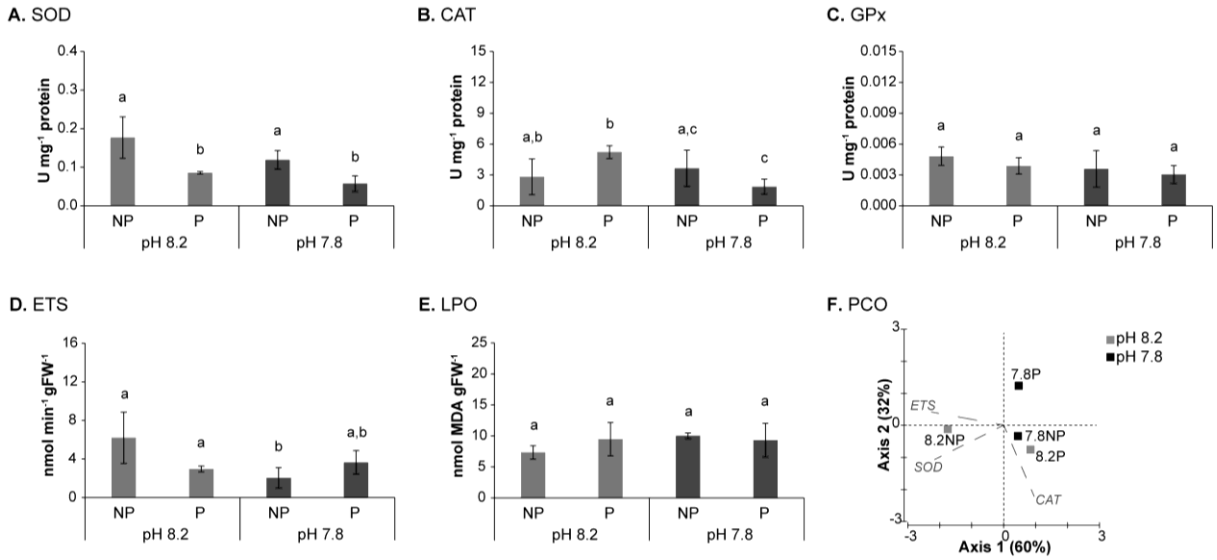


Figure 39 *pH experiment.* Mean values (± standard deviation) and significant differences represented with different lower case letters of **A.** SOD, superoxide dismutase activity; **B.** CAT, catalase activity; **C.** GPx, glutathione peroxidase activity; **D.** ETS, electron transport system activity; **E.** LPO, lipid peroxidation levels in non-parasitized (NP) and parasitized (P) cockles with *Himasthla elongata*; **F.** Principal coordinates ordination analysis (PCO) showing the variables that better explained samples distribution.

4.3.4. Discussion

Parasites are strongly influenced by environmental conditions and rely on their hosts for completion of the life cycle. A change in these factors, particularly salinity, temperature and pH, are very likely to differentially affect the parasite, the host and/or their interaction. The outcome of such changes on host–parasite relationships in marine systems has rarely been assessed, the present study being one of the first that experimentally investigated the impact of predicted climate change on trematode parasitism by testing different levels of salinity, temperature and pH on an intertidal trematode – bivalve system.

It is generally admitted that older and consequently larger cockles are more heavily infected due to a longer exposure period to parasites, and also because larger cockles present higher filtration rate which is the main way of infection (Mouritsen et al., 2003; Wegeberg et al., 1999). On the other hand, it was also demonstrated that cockles from a particular SL range could be more vulnerable to infection (de Montaudouin et al., 2012a). For these reasons, it is important to mention that the mean SL of the cockles used in the experiments were all similar (mean = 15.7 mm). Moreover, and by choosing small size cockles, we obtained cockles with very low

natural infection (mean infection ranged between 0.2 and 0.8 metacercariae per cockle).

4.3.4.1. Salinity effects in cockles trematode infection

The present study showed that, although without statistical significance, cockles from the lowest salinity treatment presented higher number of *H. elongata* metacercariae than cockles from the control salinity. There are few studies reporting effects of salinity on marine trematode parasites and the majority of them are related to cercariae emergence from its snail first intermediate host (Lei & Poulin, 2011; Mouritsen, 2002; Studer & Poulin, 2012). Lei & Poulin (2011) and Studer & Poulin (2012) showed that cercariae emergence from first intermediate host increased with increasing salinity, while Mouritsen (2002) demonstrated the same trend but only under elevated temperatures. In contrast, Koprivnikar & Poulin (2009) and Koprivnikar et al. (2014) showed cercariae emergence increase under decreasing salinities. Considering that Studer & Poulin (2012) studies, on a snail (1st host) - trematode - amphipod (2nd host) system, showed the highest cercariae survival at salinities ranging between 30 and 40 leading to greater successful transmission, our results demonstrate that cockles infection: i) was independent on the different cercariae performance according to salinity; ii) but was affected by the performance of cockles at lower salinities. Previous studies developed by Malham et al. (2012) demonstrated that *C. edule*, considered as an euryhaline species, i.e. an organism able to adapt to a wide range of salinities, shows its optimal salinity between 30 and 35 (Malham et al., 2012). Salinities outside this range could be stressful to cockles, in particular lower salinities because it might be expected that intertidal mollusks would more routinely experience elevated rather than decreased salinities, particularly during sunny low tides (Koprivnikar & Poulin, 2009). Therefore, higher number of cercariae in cockles exposed to lower salinity may result from cockles abnormal performance under this condition, being more susceptible to infection. In fact, non-parasitized cockles from the low salinity treatment showed higher antioxidant activity than non-parasitized cockles at control salinity, evidencing the negative impacts induced to cockles by low salinity. Similar harmful effects of low salinities were observed in cockles (Gonçalves et al., 2017), in *Mytilus galloprovincialis* mussels (Freitas et al., 2017) and in *Ruditapes philippinarum* clams (Velez et al., 2016a) from the same coastal lagoon. However, the observed response was different when cockles were parasitized. In general, when a trematode invades a bivalve, its immune system answers by recruiting granulocytes (a type of haemocytes). Granulocytes have dense cytoplasmic granules and are known to be the main

immunoreactive cells in the bivalve immune system, presenting the highest phagocytic capacity, destroying the invader with lysosomal enzymes and producing more reactive oxygen species (ROS) (Lin et al., 2013; Soudant et al., 2013). However, under both salinities tested, parasitized cockles seemed to be subjected to lower oxidative stress than non-parasitized cockles, since antioxidant defences and cellular damages were lower in infected cockles. This response was also accompanied by a reduction in the metabolic activity (lower ETS) when cockles were parasitized and exposed to low salinity. The apparent less stressful condition, in parasitized cockles, could instead indicate that cockles are experiencing an enzymatic inhibition by any mechanism triggered by parasites such as a reduction in some or all functions of the immune system described above. Moreover, cockles experiencing two stressful conditions at the same time, low salinity and trematode infection, seem to react with a metabolic reduction emphasizing the significant capacity of trematodes in modifying the hosts response to a given stress (e.g. Macleod & Poulin (2016)). Low metabolic capacity measured by the activity of the mitochondria respiratory chain (ETS) resulted into low ROS generation which may prevent cellular damages, explaining low LPO levels in parasitized cockles at low salinity. *R. philippinarum* showed a similar response when exposed to both low salinity and *Vibrio tapetis* by significantly reducing the lysozymes level, a possible sign of overall metabolism reduction, although in this case the strategy of defence adopted led to a higher disease prevalence and progression (Reid et al., 2003).

4.3.4.2. Temperature effects in cockles trematode infection

It is well stablished that under increasing temperatures there is an increase on cercariae activity leading to a faster depletion of their finite energy reserves which in turn reduces their survival rates (Studer & Poulin, 2013; Studer et al., 2010). However, due to this short-term increasing activity, with warmer temperatures, the number of interactions between cercariae and hosts per unit of time is higher (Evans, 1985) which may increase the effective infectivity (Studer et al., 2010; Thieltges & Rick, 2006). In one hand, these arguments can explain the slightly higher number of *H. elongata* metacercariae infecting cockles at 22 °C compared to cockles maintained at 17 °C, but on the other hand, the difference was not statistically different maybe because both temperatures are in the range of the optimal infection temperature (between 15 °C and 22 °C) described for other Himasthlidae parasites (de Montaudouin et al., 2016). Biomarkers showed that temperature affected cockles enzyme activity, with high temperature treatment inhibiting the antioxidant response in both infection conditions.

Under temperature stressful conditions (22 °C) and compared to control (17 °C), a strong reduction in non-parasitized cockles metabolic capacity was observed, which may explain that cockles were not able to activate their antioxidant defence system, leading to increased LPO levels as previously described for *R. decussatus* (Velez et al., 2017). Despite an overall increase in the metabolic activity in parasitized cockles compared to non-parasitized cockles regardless temperature treatment, when cockles were facing two stressful conditions at the same time (trematode infection and high temperature) an inhibition of their antioxidant capacity was observed. This increase of the metabolic rate (measured through ETS activity) in parasitized cockles exposed to temperature 22 °C generated higher ROS levels, which in turn resulted in the higher production of ROS that, associated to lower antioxidant capacity lead to higher LPO levels in parasitized cockles. Generally, infected cockles have to face an additional metabolic requirement in order to supply parasites with sufficient energy to grow (MacLeod, 2017). Overall, the present results demonstrated that higher temperature may induce an additional stress to parasitized cockles, inducing higher metabolic activity and cellular damages.

4.3.4.3. Water acidification effects in cockles trematode infection

Research on the ecological impacts of ocean acidification has expanded (Poulin et al., 2016) but, the interactive effects of parasitism and ocean acidification on marine organisms is still rudimentary. Nevertheless, there are already some evidences of cercariae longevity reduction (MacLeod & Poulin, 2015), although with higher trematode infection success, under low pH conditions (Harland et al., 2015, 2016). In comparison to the most studied bivalve species (e.g. *Mytilus edulis*), *C. edule* has a thinner periostracum (Richardson et al., 1981) and its shell is only composed by aragonite, the most soluble polymorph of carbonate (Cubillas et al., 2005). Therefore, cockles are particularly vulnerable to acidification which decreases their body condition (Schade et al., 2016) likely inducing a shift on their immune system and increasing the cockles susceptibility to diseases. Accordingly, in the present study, cockles from the lower pH treatment presented significantly higher number of *H. elongata* metacercariae, possibly related to higher host susceptibility mostly evidenced by the antioxidant system inhibition, lower metabolism and higher cellular damages experienced by cockles exposed to these conditions. Biomarkers showed that pH affected non-parasitized cockles enzyme activity, with low pH treatment inhibiting the antioxidant response. Under pH stressful conditions (7.8), a strong reduction in non-parasitized cockles metabolic capacity was observed as previously demonstrated for

M. galloprovincialis mussels (Freitas et al., 2017). Under both pH treatments, but especially noticed at 7.8, parasitized cockles tended to decrease their enzymatic activity in comparison to non-parasitized cockles. Previous studies already demonstrated that higher *p*CO2 levels impact bivalves immune system reducing the bactericidal activity (Ellis et al., 2015) and the phagocytic activity by decreasing the lysosomes health (Bibby et al., 2008). This mechanism, similarly to what was described for low salinity effects, reduces overall ROS production preventing the occurrence of LPO and limiting the activation of antioxidant enzymes. Notwithstanding, it is important to note that this was a short-term experiment and therefore, careful should be taken when extrapolating this ilations to the natural environment where cockles are exposed to mechanisms of transgenerational acclimation. These mechanisms can either result into decreased sensitivity to stressors of the next generations (Thomsen et al., 2017) or induce carryover effects and lead to a more sensitive offspring (Griffith & Gobler, 2017).

Considering the three experiments and taking into account non-parasitized cockles, salinity was the factor exerting the highest stress levels on cockles. This was demonstrated by higher antioxidant response resulting into greater injuries to cockles when exposed to lower salinity condition. Considering parasitized cockles, our findings may indicate that the interaction trematode infection x temperature and trematode infection x pH will result into major shifts in the cockles biochemical performance, with greater impacts at high temperature and low pH leading to possible higher disease susceptibility.

Conclusion

Present results suggest that changes forecasted by many models may promote the proliferation of the parasites infective stages in many ecosystems leading to enhanced transmission, especially on temperate regions, that will influence the geographical distribution of some diseases and, probably, the survival capacity of infected bivalves. As parasites have the ability to influence host population behaviour, reproduction and survival, small changes will have a multitude of potential subsequent effects on host populations and communities. An increase in overall parasite abundance could alter marine ecosystems in significant ways: populations of first intermediate host snail species, usually castrated by trematode infection (Lafferty & Kuris, 2009), would have lower reproductive potential; second intermediate hosts parasite-mediated dynamics, such as predator-prey relationships (Thomas et al.,

1998a, 1998b) or bottom-up control (Johnson & Heard, 2017) would be maximised; and any host species that experience increased mortality as a consequence of parasitic infection (Desclaux et al., 2004; Thieltges, 2006) may become rare.

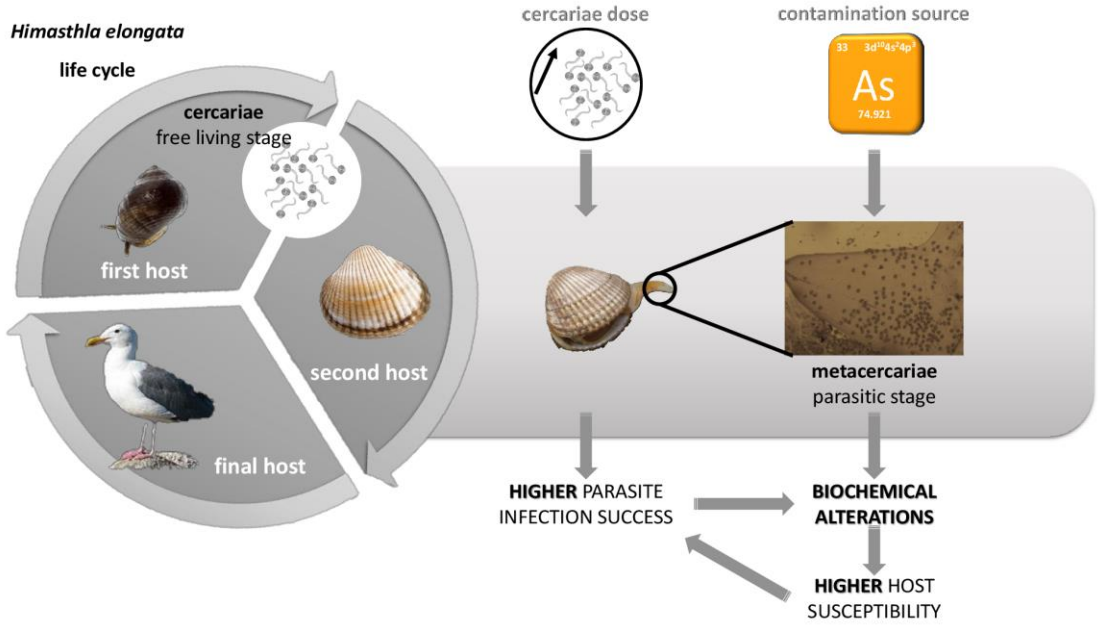
Final considerations

In this section it was demonstrated that the integration of parasitology into the physiological assessment of marine organisms exposed to simulated climate change conditions is urgently required. A failure to do so may lead to the incorrect identification of some marine species as tolerant or susceptible to a given stress, when in fact the physiological response of the organism is modified by parasitic infection.

Considering that, in a scenario of anthropogenic-derived contamination the interaction between host/parasite may also be modified, the next section will investigate the effect of a contaminant (taking asenic as an example) on the parasite infection success and in the cockles response to infection.

4.4. Interactive effects of contamination and trematode infection in cockles biochemical performance

GRAPHICAL ABSTRACT



4.4.1. Introduction

Anthropogenic activities, especially those involving chemical contaminants that pollute the environment can seriously interfere with inhabiting organisms (Ventura-Lima et al., 2011).

Among inorganic elements, arsenic (As) is one of the most widely distributed pollutants in the world (Mandal & Suzuki, 2002), with known negative effects in aquatic environments that can put at risk marine populations, including bivalves (Neff, 1997). Several activities have been contributing to the increasing As concentration in the environment, including wood preservation, insecticides and herbicides use as well as coal burning and mining (Corzo & Gamboa, 2018; Mandal & Suzuki, 2002; Ranft et al., 2003). In the marine environment, As occurs in both seawater and sediment fractions (Mandal & Suzuki, 2002). Average As concentration in seawater is typically around 1.5 µg L^{-1} reaching up to 8.8 µg L^{-1} (Smedley & Kinniburgh, 2002) in more estuarine and industrial influenced areas. In sediments, As concentrations range is wider, varying between 0.8 (Velez et al., 2015) and 33.4 mg kg^{-1} (Wang et al., 2010). In the environment, As is commonly present in its inorganic form (arsenite and arsenate) where it may be transformed into less toxic organic forms, such as arsenobetaine and

arsenocholine, and accumulated by marine organisms (Fattorini et al., 2006). Recent studies demonstrated that bivalves can bioaccumulate As (Fattorini et al., 2006; Figueira et al., 2011) which can interfere, for example, with their oxidative stress response (Freitas et al., 2016) and metabolism (Coppola et al., 2018).

Parasitism is a fundamental ecological interaction but we still understand relatively little about the ecological role of parasites compared to what is known about free-living organisms. In natural environments, marine organisms are concomitantly exposed to pollutants and parasites, a combination with synergistic, antagonistic or additive effects that can represent a serious threat to the health of aquatic communities. Hence, the main objective of the present study was to assess host biochemical response, through energy reserves, metabolism and oxidative stress biomarkers, 1) when challenged by different parasite burdens, and 2) when performing a combined exposure to parasite infection and As contamination. *Cerastoderma edule* (the edible cockle) and *Himasthla elongata* (a trematode parasite) were used as a host – parasite model to test the hypotheses “The greater the infection burden, the greater the stress response” and “Arsenic will change parasite infection levels and, consequently, cockles stress response to infection”.

4.4.2. Material and methods

4.4.2.1. Live material

Snails (*Littorina littorea*) infected only by *Himasthla elongata* (trematode parasite) as first intermediate host were collected from the NIOZ harbour, Texel, the Netherlands (53°00’32.1’’N, 4°47’36.5’’E). Potential second intermediate hosts (*Cerastoderma edule*) were collected from the Mira channel, Ria de Aveiro coastal lagoon, Portugal (40°38’31.7’’N, 8°44’10.9’’W). *H. elongata* was identified in its metacercariae stage following de Mountaudouin et al. (2009) description. Hosts and parasites were obtained and kept as described in the previous section of this chapter. Shell length of the collected cockles ranged between 14 and 17 mm, i.e. young individuals, in order to limit natural trematode former infection (de Montaudouin et al., 2012b; Mouritsen et al., 2003; Wegeberg et al., 1999).

4.4.2.2. Infestation levels experiment

After two weeks of acclimation to laboratory conditions, twenty-four cockles were individually placed in glass containers filled with 50 mL seawater, with constant

aeration (Figure 40A) and under controlled abiotic conditions: salinity = 30 ± 1, temperature = 17 °C, pH = 8.2 and photoperiod = 12:12 h (light/dark).

In order to obtain cercariae, infected snails (kept at ~14 °C) were individually transferred to a 6-well plate with ~16 mL artificial seawater (salinity = 35 ± 1) per well and exposed to constant illumination and consequent temperature boost (~25 °C) during 4 to 6 hours. Cercariae were collected with a pipette, pooled, counted and separated into groups of twelve, twenty-five and fifty cercariae and then used for cockle immediate infestation.

Experimental design included one factor (trematode infection) with four levels, i.e. control (CTL = 0 cercariae), twelve, twenty-five and fifty cercariae (inf 12, inf 25 and inf 50, respectively), with six replicates per level/condition (Figure 40A). At the end of the infestation experiment (48 h after cercariae addition, to allow encystment (de Montaudouin et al., 2016)) cockles were conserved at - 80 °C for further *H. elongata* infection success calculation (percentage of administered cercariae that infected cockles), metacercariae abundance determination and for biochemical responses evaluation.

4.4.2.3. Contamination experiment

After two weeks of acclimation period, twenty-four cockles were individually placed in glass containers filled with 50 mL seawater, with constant aeration and under the same controlled abiotic conditions described above. These cockles were exposed to two different As treatments, with 12 replicates per treatment (Figure 40B), during 96 h. This exposure period was selected based on standard guides for conducting toxicity tests with macroinvertebrates (ASTM E729-96, 2002). The tested treatments were 0.0 µg L^{-1} (control) and 5.2 µg L^{-1}, corresponding to maximum As levels found dissolved in the water of the Ria de Aveiro (Ereira et al., 2015). Arsenic stock solution (10.4 mg L^{-1}) was prepared using Sodium Arsenate (Sigma-Aldrich).

After 96 h of contamination, infected snails were exposed to a new temperature boost, cercariae were obtained as mentioned before and separated into groups of twenty-five. Then, cercariae were used to infest six cockles per As treatment (Figure 40B).

At the end of the experiment (96 h of As exposure + 48 h of combined As and cercariae exposure) cockles were conserved at - 80 °C for further *H. elongata* infection success calculation (percentage of administered cercariae that infected cockles), metacercariae abundance determination (cockles susceptibility to parasites) and responses assessment.

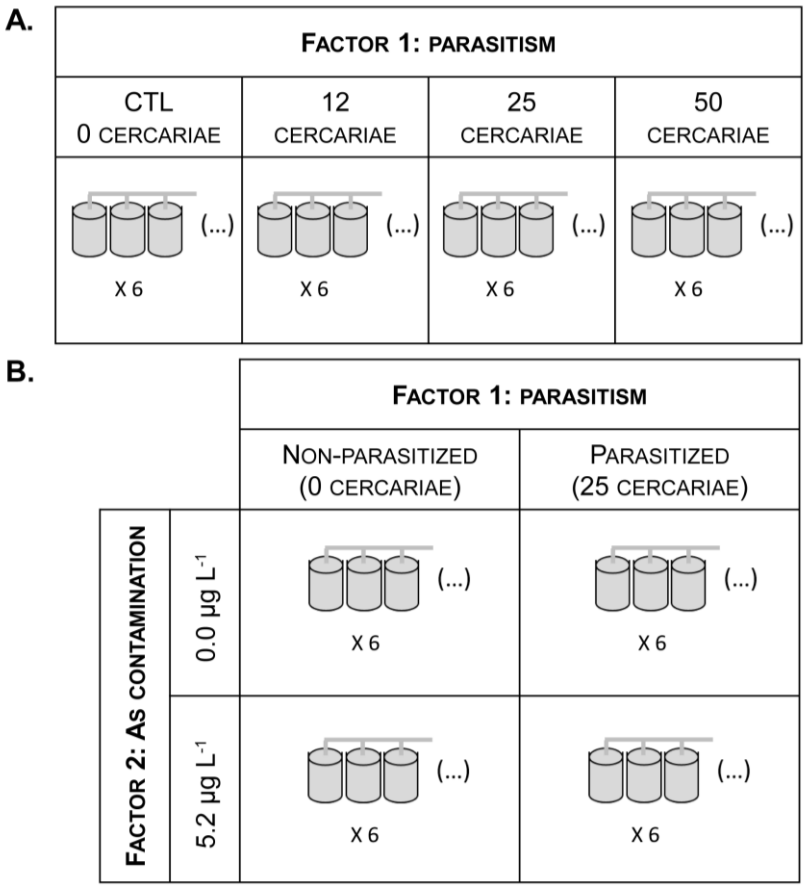


Figure 40 Schematic representation of the experimental designs. **A.** Infestation levels experiment; **B.** Contamination experiment. As: Arsenic.

4.4.2.4. Biochemical descriptors

After experimental period, cockles from the two experiments (infestation levels and contamination), making a total of eight treatments were dissected and observed using a stereomicroscope to assess *H. elongata* infection success and metacercariae number. Then, in order to obtain enough flesh for further analysis, cockles were pooled in groups of two entire organisms per replicate, three replicates (corresponding to six cockles) per treatment. After homogenization with liquid nitrogen, each replicate was separated into at least 3 aliquots containing 0.3 g of soft tissue. Eight different biochemical markers were determined after aliquots extraction using specific buffers (described in detail in the previous section of this chapter): protein (PROT) and glycogen (GLY) contents, for energy storage measure; electron transport system (ETS) activity, representing a proxy of cellular respiratory potential; superoxide dismutase (SOD) activity, the enzyme responsible for the removal of superoxide anion (O^{2-}) with hydrogen peroxide (H_2O_2) formation; catalase (CAT) activity, the enzyme that reduces the SOD product to water; glutathione peroxidase (GPx) activity, the enzyme that

catalyses the reduction of several hydroperoxides to water; glutathione S-transferases (GSTs) activity, a group of biotransformation enzymes that act as cell detoxifiers and lipid peroxidation (LPO) level, an indicator of cellular damage. Details on biomarkers methods are described in Table 21.

4.4.2.5. Data analysis

For the infestation levels experiment, one-way ANOVA was performed in order to compare initial cockle shell length between treatments and to test the effect of treatments (CTL, 12, 25 and 50 cercariae) on the infection success and number of *H. elongata* metacercariae infecting cockles after exposure. Prior to analysis, homogeneity of variance was verified with Cochran test. One-way ANOVA was followed by post-hoc Tukey test for comparison of means.

For the contamination experiment, two-way ANOVA was performed in order to compare initial cockle shell length between treatments and to test the effect of treatment (0.0 and 5.2 µg L^{-1}), infection condition (parasitized and non-parasitized) and the interaction between factors on the number of *H. elongata* metacercariae infecting cockles after exposure. Prior to analysis, homogeneity of variance was verified with Cochran test. Two-way ANOVA was followed by post-hoc Tukey test for comparison of means.

Due to a lack of homogeneity of variance, PROT, GLY, ETS, SOD, CAT, GPx, GSTs and LPO, were separately submitted to a non-parametric permutational analysis of variance (PERMANOVA Add-on in PRIMER-E software) with one fixed factor (infection level) and four levels (CTL, 12, 25 and 50 cercariae). Main test with p values lower than 0.05 were considered as significant and followed by pair-wise tests. Pair-wise tests were used to identify statistical differences and represented in figures with lower case letters. The effect of contamination (0.0 and 5.2 µg L^{-1}), infection condition (parasitized and non-parasitized) and the interaction between factors were tested on each biomarker using the same analysis described above but taking into account two fixed factors (arsenic treatment and infection condition) with two levels each.

The Euclidean distance of the matrix of each experiment containing biomarkers results per treatment was calculated after samples normalisation. Distances among centroids were then plotted in a Principal Coordinates Ordination analysis (PCO). Superimposed vectors were used to represent the variables (biomarkers) that better ($r > |0.8|$) explained samples spatial distribution.

Table 21 Method principle, function and respective reference of the eight biomarkers used in the present study.

Biomarker	Method Principle	Function	Reference
Protein	Under alkaline conditions, the peptide bonds form a purple complex (measured at 540 nm) with copper salts contained in the biuret reagent	Energy reserve	Robinson & Hogden, 1940
Glycogen	Under acidic conditions, carbohydrates are dehydrated forming a colored product with phenol spectrophotometrically measured at 492 nm	Energy reserve	Dubois et al., 1956
Electron Transport System (ETS)	ETS reduces tetrazolium with formation of formazan, a chromogenic product spectrophotometrically measured at 490 nm	Mitochondrial metabolism	De Coen & Janssen, 1997
Superoxide Dismutase (SOD)	Nitroblue tetrazolium (NBT) is converted to NBTdiformazan (formazan dye) via superoxide radical, which is the SOD substrate. SOD activity is measured in decreased absorbance at 560 nm	Antioxidant enzyme	Beauchamp & Fridovich, 1971
Catalase (CAT)	CAT reacts with methanol in the presence of H_2O_2 producing formaldehyde that is spectrophotometrically measured at 540 nm using Purpald as a chromogen	Antioxidant enzyme	Johansson & Borg, 1988
Glutathione Peroxidase (GPx)	GPx catalyzes the reduction of cumene hydroperoxide oxidizing reduced glutathione (GSH) to form disulfide glutathione. The oxidized glutathione is then reduced by glutathione reductase and NADPH forming NADP+, resulting in decreased absorbance at 340 nm and recycling the GSH	Antioxidant enzyme	Paglia & Valentine, 1967
Glutathione S-transferases (GSTs)	GSTs catalyse the conjugation reaction of CDNB (used as substrate) to GSH with formation of a spectrophotometrically measured (at 340 nm) thioether	Biotransformation enzyme/ cell detoxifier	Habig et al., 1974
Lipid peroxidation	Malonaldehyde, a product from membrane deterioration, is determined by thiobarbituric acid (TBA)-measurements forming TBA reactive substances, spectrophotometrically measured at 532 nm	Cellular damage	Buege & Aust, 1978

4.4.3. Results

Cockles used on each treatment level of each experiment showed similar mean shell length ($p > 0.05$), mean = 15.8 ± 0.8 (standard deviation) mm) (N = 2 experiments x 4 treatments x 6 replicates = 48).

4.4.3.1. Infestation levels experiment

Different infestation levels resulted in similar *H. elongata* infection success (one-way ANOVA, F = 0.5, $p = 0.61$, Figure 41A) and significantly different number of *H. elongata* metacercariae (One-way ANOVA, F = 14.7, $p < 0.001$). *H. elongata* abundance was the lowest in naturally infected cockles (0.8 ± 1.0 metacercariae cockle^{-1}), from hereafter named as "non-parasitized cockles (NP)" and the highest in experimentally infected cockles, from hereafter named as "parasitized cockles (P)", infested with fifty cercariae (11.7 ± 1.2 metacercariae cockle^{-1}) (Figure 41B). Cockles infested with twelve and twenty-five cercariae showed intermediate and similar *H. elongata* abundance (4.3 ± 3.6 and 6.5 ± 2.0 metacercariae cockle^{-1}, respectively) (Figure 41B).

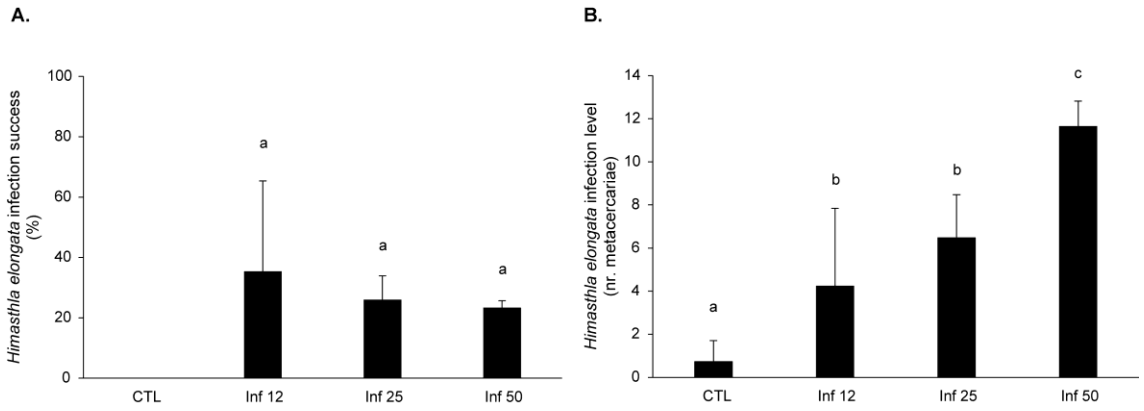


Figure 41 Mean (± standard deviation) of *Himasthla elongata* infection success (**A**) and metacercariae infection (**B**) found in control and three levels of experimentally parasitized cockles (infection levels: 12, 25 and 50). Post-hoc Tukey test homogenous groups ($p < 0.05$) are represented by lower case letters.

4.4.3.2. Contamination experiment

Different As treatments resulted in similar *H. elongata* infection success (One-way ANOVA, F = 1.1, $p = 0.33$, Figure 42A). Cockles presented similar infection when exposed to As compared to non-contaminated cockles (7.0 ± 4.1 metacercariae cockle-

[1]) (Figure 42B, Table 22). P cockles presented significantly higher number of *H. elongata* metacercariae compared to control, i.e. NP cockles (Figure 42B, Table 22), regardless As treatment.

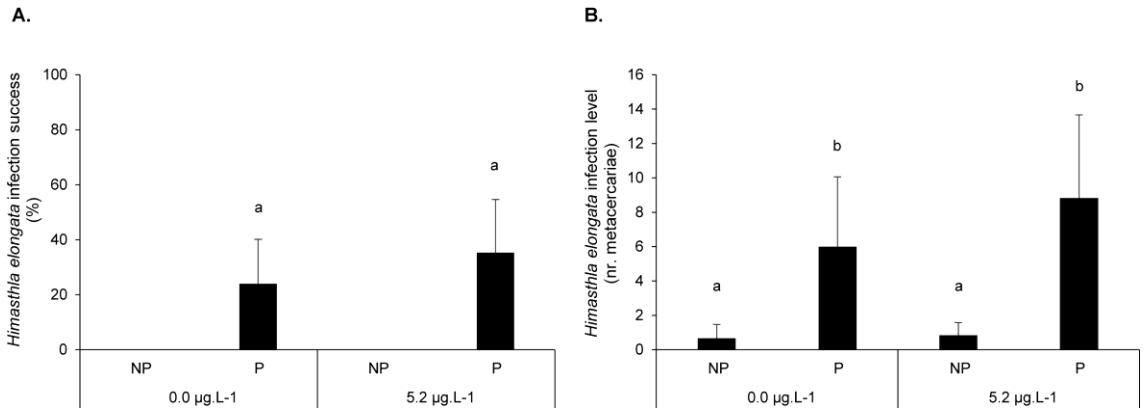


Figure 42 Mean (± standard deviation) of *Himasthla elongata* infection success (**A**) and metacercariae infection (**B**) found in not experimentally parasitized (NP) and experimentally parasitized (P) cockles of each Arsenic treatment (0.0 and 5.2 µg L^{-1}). Post-hoc Tukey test homogenous groups ($p < 0.05$) are represented by lower case letters.

Table 22 Two-way ANOVA results performed to test the effects of experimental treatment (0.0 and 5.2 µg L^{-1} of arsenic), infection condition and interaction of factors on the number of *Himasthla elongata* metacercariae found in dissected cockles. MS: mean square; *F*: *F* value; *p*: *p* value. Bold letters indicate significant differences ($p < 0.05$). N = 24.

Two-way ANOVA			
	MS	***F***	***p***
Treatment	0.5	1.3	0.27
Infection condition	**12.0**	**29.5**	**< 0.001**
Interaction	0.2	0.5	0.50
Error	0.4		

4.4.3.3. Biochemical descriptors

4.4.3.3.1. Infestation levels experiment

Infection levels did not affect PROT content (Table 23).

GLY content was significantly higher in P cockles compared to CTL cockles, with significantly higher values at the highest infection level, while infection levels 12

and 25 displayed intermediate and not significantly different GLY contents (Figure 43A, Table 23).

Cockles exposed to the highest infection level (50 cercariae) presented significantly higher ETS values compared to CTL and cockles exposed to the lowest infection level (Figure 43B, Table 23).

SOD activity showed no significant differences between cockles exposed to 50 cercariae and CTL that presented higher values compared to cockles from the infection levels 12 and 25 (Figure 43C, Table 23).

CAT activity was significantly higher in P cockles compared to CTL. The highest CAT activity was observed at the highest infection level, with significant differences to the remaining conditions (Figure 43D, Table 23).

GPx activity showed no significant difference between cockles exposed to 50 cercariae and CTL that presented higher values compared to cockles from the infection levels 12 and 25 (Figure 43E, Table 23).

GSTs activity showed no significant difference between cockles exposed to 50 cercariae and CTL. The lowest GSTs levels were registered for cockles exposed to 25 cercariae, with significant differences to the remaining conditions (Figure 43F, Table 23).

Regarding LPO levels, no significant difference were observed between cockles exposed to the highest level of infection and CTL. LPO was significantly higher in cockles exposed to 25 cercariae and significantly lower in cockles from the infection level 12 (Figure 43G, Table 23).

The PCO1 (horizontal axis) explained 49% of the total variation separating infection level 50, in the negative side of the axis, from CTL and infection levels 12 and 25, in the positive side of the axis. GLY, ETS and CAT presented high negative correlation ($r > - 0.8$) to PCO1 and were the variables that better explained samples separation. The vertical axis (PCO2) explained 37% of the total variation separating CTL and infection level 50, in the negative side of the axis, from infection level 25, with a strong negative correlation to GPx and GSTs activities ($r > - 0.8$) and a strong positive correlation to LPO ($r > 0.8$, Figure 43H). SOD did not present significant correlation with none of the axes ($r < |0.7|$).

Table 23 PERMANOVA results performed to test the effects of infection conditions (0, 12, 25 and 50) on the biochemical descriptors. PROT: protein; GLY: glycogen; ETS: electron transport system; SOD: superoxide dismutase; CAT: catalase; GPx: glutathione peroxidase; GSTs: glutathione S-transferases; LPO: lipid peroxidation; MS: mean square. Bold letters indicate significant differences ($p < 0.05$). N = 12.

	Infection condition			**Error**
	MS	***Pseudo-F***	***P***	***MS***
PROT	3.2	18.5	0.06	0.2
GLY	**3.5**	**74.0**	**< 0.001**	**$4.8.10^{-2}$**
ETS	**3.1**	**14.0**	**0.002**	**0.2**
SOD	**3.3**	**26.1**	**< 0.001**	**0.1**
CAT	**3.5**	**65.1**	**< 0.001**	**0.1**
GPx	**3.4**	**28.9**	**< 0.001**	**0.1**
GSTs	**2.9**	**10.6**	**0.004**	**0.3**
LPO	**3.5**	**53.1**	**< 0.001**	**$6.6.10^{-2}$**

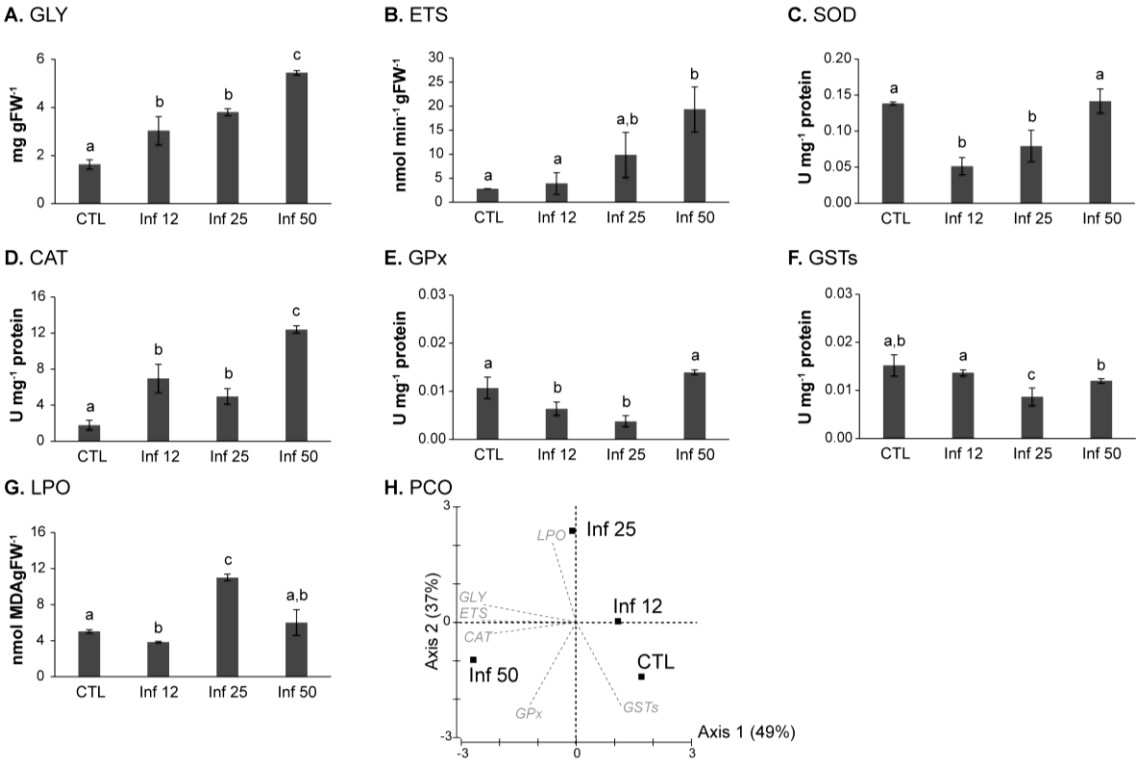


Figure 43 Mean values (± standard deviation) and significant differences represented with different lower case letters of **A.** GLY, glycogen content; **B.** ETS, electron transport system activity; **C.** SOD, superoxide dismutase activity; **D.** CAT, catalase activity; **E.** GPx, glutathione peroxidase activity; **F.** GSTs, glutathione S-transferases activity; **G.** LPO, lipid peroxidation levels in four levels of cercariae infection: 0 (CTL), 12, 25 and 50; **H.** Principal coordinates ordination analysis (PCO) showing the variables that better explained samples distribution.

4.4.3.3.2. Contamination experiment

As treatment and infection condition did not affect PROT content (Table 24).

Results revealed no significant effect of the interaction 'As x trematode infection' on cockles GLY content (Table 24). Arsenic treatments (0.0 and 5.2 µg L^{-1} of As) did not affect cockles GLY content (Table 24). Within both As treatments, significantly higher GLY levels were observed in P compared to NP cockles (Figure 44A).

Results showed a significant effect of the interaction between As contamination and infection condition on ETS levels (Table 24). Within P cockles, there was a significantly lower ETS activity when exposed to As compared to control (Figure 44B). Within As control treatment (0.0 µg L^{-1}), P cockles presented significantly higher ETS activity compared to NP cockles (Figure 44B).

Results showed no significant effect of As and infection interaction on SOD and CAT levels (Table 24). Arsenic contamination presented no effect on SOD activity (Figure 44C, Table 24) but resulted in significantly higher CAT values (Figure 44D). Infection condition showed to exert an effect on SOD and CAT activities with higher values in NP compared to P cockles (Figure 44C, 44D).

A significant interactive effect of As and infection on the GPx activity (Table 24). Cockles GPx activity, within NP condition, was significantly lower when exposed to As contamination (Figure 44E). Within As control treatment (0.0 µg L^{-1}), GPx levels were higher in NP compared to P cockles (Figure 44E, Table 24).

As treatment and infection condition did not affect GSTs activity (Figure 44F, Table 24).

Similarly to what was described to ETS and GPx activities, As and infection exerted a significant interactive effect on LPO levels (Table 24). Within P condition, cockles presented higher LPO in the As control treatment (0.0 µg L^{-1}) compared to contaminated treatment (Figure 44G). Within non-contaminated cockles, P presented higher LPO than NP (Figure 44G).

The PCO1 explained 74% of the total variation separating P cockles, in the negative side, from NP cockles, in the positive side of the axis. GLY, ETS and LPO presented high negative correlation ($r > - 0.8$) while SOD, CAT and GPx presented high positive correlation ($r > 0.8$) to PCO 1. These variables presented the most influence on samples space distribution. The PCO2 explained 21% of the total variation, separating cockles from control treatment (NP and P), in the positive side of the axis, from cockles exposed to As contamination (NP and P) in the negative side, with a strong positive correlation to GSTs activity ($r > 0.8$, Figure 44H).

Table 24 PERMANOVA results performed to test the effects of arsenic experimental treatments (0.0 and 5.2 µg L^{-1}), infection condition and interaction of factors on the biochemical descriptors. PROT: protein; GLY: glycogen; ETS: electron transport system; SOD: superoxide dismutase; CAT: catalase; GPx: glutathione peroxidase; GSTs: glutathione S-transferases; LPO: lipid peroxidation; MS: mean square. Bold letters indicate significant differences ($p < 0.05$).

	Treatment			**Infection condition**			**Interaction**			**Error**
	MS	*Pseudo-F*	*p*	*MS*	*Pseudo-F*	*p*	*MS*	*Pseudo-F*	*p*	*MS*
PROT	2.6	11.6	0.06	6.5	29.1	0.06	$6.0.10^{-2}$	0.3	0.62	0.2
GLY	0.3	1.0	0.36	**7.8**	**22.9**	**0.002**	0.2	0.6	0.47	0.3
ETS	**1.8**	**9.3**	**0.02**	**5.1**	**26.1**	**0.001**	**2.6**	**13.2**	**0.008**	**0.2**
SOD	1.0	3.3	0.12	**6.8**	**22.1**	**0.001**	0.7	2.3	0.17	0.3
CAT	**3.6**	**53.4**	**< 0.001**	**6.7**	**100.0**	**< 0.001**	0.2	3.0	0.13	$6.7.10^{-2}$
GPx	**1.4**	**6.7**	**0.03**	**6.0**	**27.5**	**< 0.001**	**1.9**	**8.7**	**0.02**	**0.2**
GSTs	2.3	2.3	0.17	0.4	0.4	0.56	0.2	0.2	0.67	1.0
LPO	**3.3**	**16.3**	**0.003**	**2.3**	**11.4**	**0.009**	**3.7**	**18.1**	**0.003**	**0.2**

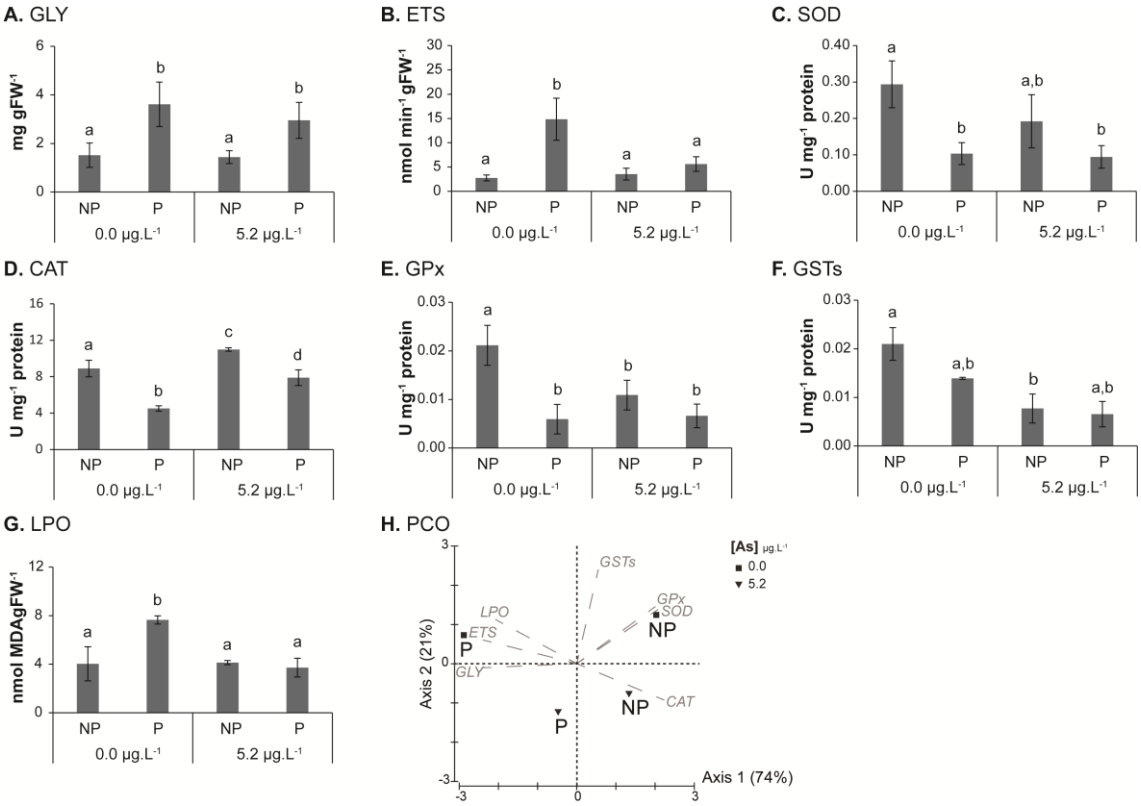


Figure 44 Mean values (± standard deviation) and significant differences represented with different lower case letters of **A.** GLY, glycogen content; **B.** ETS, electron transport system activity; **C.** SOD, superoxide dismutase activity; **D.** CAT, catalase activity; **E.** GPx, glutathione peroxidase activity; **F.** GSTs, glutathione S-transferases activity; **G.** LPO, lipid peroxidation in non-parasitized (NP) and parasitized (P) cockles exposed to two levels of Arsenic contamination (0.0 and 5.2 µg L^{-1}); **H.** Principal coordinates ordination analysis (PCO) showing the variables that better explained samples distribution.

4.4.4. Discussion

Experimental studies on parasite transmission events have proved to be extremely useful for the knowledge on parasite ecology (Poulin, 2010). In the present study, the experimental approach contributed to understand the effects of a classical contaminant on the infectious success of a trematode parasite and, for the first time, the repercussions on host biochemical performance and consequent susceptibility to infection.

In what regards to infection success, different authors agreed that, in a natural context, few hosts carry the majority of the parasites (Ebert et al., 2000), i.e. parasites distribute aggregatively within their population host, which is possibly explained by a snow ball effect, i.e. the more parasites infect a host, the more susceptible this particular host is for new infections. However, and experimentally speaking, results are

controversial with some studies showing that infection success is negatively dose-dependent (Poulin, 2010), but others demonstrated that infection intensity tends to proportionally increase with the number of cercariae exposure (Liddell et al., 2017). Our findings showed that the highest the cercariae exposure (at least within the tested range of 50 cercariae), the highest the trematode infection and consequent metacercariae encystment in the second intermediate host (*Cerastoderma edule* cockles). Moreover, the present study showed that the more parasites infect a single host, the stronger are the parasite-induced effects on the host biochemical response.

The bivalve immune system answers to trematode invasion by recruiting granulocytes. This type of haemocytes are the bivalve immunoreactive cells that present the highest phagocytic capacity, encapsulating and destroying the pathogen with lysosomal enzymes, which activity produces high amount of reactive oxygen species (ROS) (Lin et al., 2013; Soudant et al., 2013). The adverse effects of ROS in cells are usually prevented by the antioxidant system, which may result in energy cost (Regoli & Giuliani, 2014). However, the present results demonstrated that increased infection levels resulted into proportional increasing GLY content (energy storage). The increase in GLY content in infected individuals might be explained by the use of other carbohydrates, such as lipids, as an energy source to fuel up defence mechanisms (e.g. Xiao et al., 2014), by an increased uptake of nutrients due to extended energy requirements of the infected host or due to a quick mobilization of energy storage during the encystment phase of the parasite as it was observed in *Gammarus fossarum* infected by both *Polymorphus minutus* (acanthocephalan) and *Dictyocoela duebenum* (microsporidian) (Chen et al., 2015). Increasing parasite burden showed to be positively related to higher ETS activity (metabolic rate). Generally, infected hosts tend to increase their metabolism to face an additional metabolic requirement to supply parasites with sufficient energy to subsist (MacLeod, 2017) and, as shown in the present study, also to fuel defence mechanisms. Regarding the antioxidant and biotransformation enzymes, cockles that were moderately infected (infection levels 12 and 25) showed an overall (except for CAT) reduction of their activities compared to CTL and infection level 50. It is known that the Ria de Aveiro (the coastal lagoon where cockles were collected) is characterized by low trematode metacercariae abundance and prevalence (Magalhães et al., 2018), which lead us to hypothesise that these cockles immune system may be poorly adapted to rapidly react to trematode infection. Although bivalves lack the mechanisms conferring adaptive immunity in vertebrates, bivalves immune system is strongly influenced by endogenous and environmental related factors (Zannella et al., 2017) and there are already some evidences of a certain level of immune specificity and immune memory (Zhang et al., 2014), or at least

some “memory mechannisms” related to the life history of these bivalves (Paul-Pont et al., 2010b). Thus, a moderate infection (represented by 12 and 25 cercariae addition) may not be enough for cockles to efficiently activate their antioxidant system and resulting into higher cellular damages (higher LPO). On the other hand, when these young and small cockles were exposed to 50 cercariae and, consequently, metacercariae infection reached the threshold of more than 10 metacercariae per cockle, cockles increased the activity of all enzymes studied leading to an efficient reduction of the cellular damages.

Regarding contamination experiment results, the present study revealed that when cockles were exposed to As contamination and were then challenged with a moderate level of *H. elongata* cercariae (25), they presented similar number of metacercariae compared to not contaminated cockles (8.8 vs. 6.0 metacercariae cockle^{-1}, respectively). Conversely, several examples from the literature showed higher infection in contaminated conditions: snails (*Physa fontinalis* and *Lymnaea stagnalis*) previously exposed to toxicants (cadmium, zinc and a cadmium/zinc mixture used at concentrations of 100, 1,000 and 10,000 µg L^{-1}) have shown an increase in parasite Echinoparyphium recurvatum prevalence and intensity (Morley et al., 2002) and *M. edulis* mussels exposed to neurotoxins through ingestion of harmful algal species showed a significant increase in the occurrence of Gymnophallidae metacercariae (Galimany et al., 2008). These environmental pollutants may have weakened host defences and contributed for the higher infection success of the parasites. However, the cercariae pressure (25) and the contamination level (5.2 µg L^{-1} As) used in the present work, as well as the duration of the exposure (96 h) may not have been sufficient to exert similar effect.

During several cellular pathways, mainly related to aerobic metabolism, ROS are naturally produced (Murphy, 2009). Under normal conditions, bivalves can regulate ROS quantity and prevent oxidative stress using the antioxidant system. However, chemical toxicity can interfere with the cellular balance between prooxidants and antioxidants resulting into antioxidants capacity depression and increase of intracellular ROS formation (Regoli & Giuliani, 2014). Nevertheless, in the present study, results showed lower impact of As contamination compared to trematode infection effects. As mentioned before, this can be explained by the As contamination level used (5.2 µg L^{-1}, which is under reported *C. edule* bioaccumulated As (Figueira et al., 2011)) and by the short exposure period (96 h) that was not enough to induce cellular injuries. Low impact of As (1 mg L^{-1}), acting under regular temperature conditions (17 °C), was also demonstrated in mussels, *M. galloprovincialis*, with similar SOD, CAT and ETS activities as well as similar LPO levels compared to control (no As contamination)

(Coppola et al., 2018). In its turn, trematode infection alone caused a significant increase in cockles GLY content and on ETS activity that, as previously discussed, is related to higher energy storage and metabolic requirements induced by the parasite presence. Moreover, comparable to what was discussed above, regarding the infection levels experiment and particularly concerning the infestation level 25, the antioxidant response was not activated by the presence of trematode infection leading to higher LPO levels.

Acting in interaction, As contamination and trematode infection did not affect the GLY accumulation pattern described above, i.e. parasitized cockles presented higher GLY content than non-parasitized cockles regardless As exposure. A similar case was found in a freshwater clam (*Pisidium amnicum*) infected by trematodes and exposed to petachlorophenol (Heinonen et al., 2001). Conversely, in the present study, the stressors interaction (As contamination and parasite infection) induced an effect in the host metabolism reducing the ETS activity. This change in the metabolism of infected cockles under contaminated conditions can indicate a change in some physiological parameters such as feeding or respiration when cockles were facing more than one stressful condition at the same time. Similarly, it was registered a reduction in the cardiac rate of both bivalve and pulmonate molluscs, compared to controls, when under the combined effect of pollutants (0.2, 1.0, 1.8 mg L^{-1} of copper sulfate) and trematodes (Morley et al., 2006). Surprisingly, and taking also into account no significant activation of the antioxidant system, LPO levels of cockles simultaneously exposed to As and trematode infection were low compared to parasitized cockles not exposed to As, displaying values that were even closer to control (NP, 0.0 µg L^{-1} As). These low LPO levels are certainly related to lower metabolic activity and consequent lower ROS production (Murphy, 2009) induced also by As and trematode infection interaction. Lower oxidative burst could be simultaneously explained by a phagocytosis suppression induced by the stressors interaction (Sauvé et al., 2002). Therefore, to a certain extent and in a contamination scenario, trematode infection may be beneficial to cockles, working as a protection for the pollutant accumulation in the organisms, reducing overall ROS production, which consequently led to less toxic effects. Other authors with convergent findings showed a decrease in *Littorina littorea* iron, copper and nickel accumulation (field monitoring) related to digenean trematode infection (Evans et al., 2001) and a significant decrease in cadmium (used at 15 µg L^{-1}) bioaccumulation due to the presence of pathogens (trematode *H. elongata* and bacteria *Vibrio tapetis*) on *C. edule* (Paul-Pont et al. 2010c).

Conclusion

These results allowed acceptance of the first postulated hypothesis, i.e. when the infection burden was higher, the cockles stress response was also higher. However, concerning the second hypothesis, As contamination (at least at the single As concentration used = 5.2 µg L^{-1}) did not change parasite infection success but modified cockles stress response to infection mostly at the metabolism reduction level. The present findings highlighted the deleterious effects of trematode infection in their hosts biochemical performance. Biochemical markers proved to be useful tools in reflecting the invasion infects of pathogens however, may give false-positive or false-negative results modulated by several environmental variables such as contamination. In fact, caution must be taken when extrapolating simplified laboratory experiments into complex ecosystems which encounter a variety of stresses.

CHAPTER 5. CONCLUDING REMARKS AND PERSPECTIVES

Concluding remarks

Intertidal areas include complex ecosystems, which are under constant natural pronounced abiotic and biotic shifts and are also significantly influenced by human activities. As a result, organisms inhabiting these ecosystems must be able to rapidly respond and adapt to anthropogenic and natural stress (which are often difficult to distinguish (Elliott & Quintino, 2007)) otherwise, may be subjected to negative impacts on their health and survival rate.

Bivalves are among the most important organisms living in intertidal systems, not only by their crucial role in the ecosystems functioning, often considered as keystone species, but ultimately by their role as an important human food resource at least since the Roman times. However, in the environment, bivalves are commonly affected by several diseases that can put at risk entire populations with subsequent ecological and socio-economic consequences (Lafferty, 2017). The magnitude of the impact of these diseases on bivalve populations may differ accordingly to a variety of other stressors that rarely occur independently and, on the contrary, often interact (Sures et al., 2017a). It was in this context of urgency in recognizing the processes that enhance disease episodes and in understanding the impact of these diseases on the sustainability of natural resource that this thesis subject arised.

The sporocyst (or redia) stage of a trematode life cycle multiplies asexually. This is a strategy, evolutionarily adopted by the parasite in order to successfully invade the host tissues and have a short term rapid population growth and expansion due to cercariae larvae dispersal. Especially due to this feature, sporocysts are the most deleterious stage in a trematode life cycle, able to impair some of the basic functions of its host. Taking the unique opportunity of analyse a long-term (1997-2013) database of a cockle population living in a natural reserve (Banc d'Arguin, Arcachon, France) and respective infection by *Bucephalus minimus* sporocysts, this thesis started providing new valuable information concerning this host/parasite system and consequent basis for future research. Parasite prevalence increased with cockle shell length and the sporocysts were not visible before cockle reached 16-mm shell length. This finding will support further works by setting a minimum recommended size to sample cockles infected as first intermediate host. Besides, by gathering all these years of sampling, it was possible to conclude that cockles infected by *B. minimus* presented as well a significant higher metacercariae abundance of other trematode species, highlighting the possible harmful effects of a sporocyst invasion in the host bivalve fitness.

In this thesis, it was applied, for the first time, a combination of transcriptomic and biochemical techniques to evaluate the actual effects of the sporocysts invasion in its bivalve host. It was found that this trematode stage impacts are not limited to morphologic alterations but has implications also at sub-cellular level. Same methodology was applied to cockles (*Cerastoderma edule*) inhabiting an intertidal area in Arcachon bay (France) and to wedge clams (*Donax trunculus*) inhabiting a sand beach in the Ria Formosa coastal lagoon (Portugal). These species are similar, regarding their distribution, latitudinal range, taxonomy and trophic behaviour but different in terms of habitat. While cockles tend to occupy intertidal areas characterized by sandy-mud sediments, wedge clams are usually found in highly energetic sand beaches. Hence, cockles are more often exposed to abrupt abiotic environmental changes, in a daily and seasonal basis, compared to wedge clams. Nevertheless, these two bivalves showed a similar defence response when parasitized by a trematode in the sporocyst form. Namely by inducing their mechanism defence against oxidative stress (showed mainly by gene expression markers) and reducing the overall energy reserves. The applied biomarkers reflected the impacts induced by environmental conditions that animals experienced, sometimes acting as confounding factors, but essentially, they showed potential to reflect the invasion effects of parasites. If implemented, these techniques can help to predict the organisms chances of reproduction and survival in their natural context. Consequently, these methods will help management practitioners to identify conservation threats to bivalve populations before disease and mass mortality outbreaks.

Due to the complex nature of a trematode life cycle that uses generally, three host species, the presence of a trematode in a given population can be assumed as indicator of the presence of all species it needs to complete the life cycle, i.e. high species richness (Frainer et al., 2018; Hechinger & Lafferty, 2005; Hechinger et al., 2008). Besides, a trematode presence in a given population can also be indicator of favourable environmental conditions (biotic and abiotic) for parasites free-living stages to transfer from one host to the other (MacKenzie et al., 1995; Pietrock & Marcogliese, 2003). In this thesis content, it is included the first study of trematode communities in the Ria de Aveiro coastal lagoon being also the first performed in Portugal, with spatial and seasonal monitoring. Trematode infection found was surprisingly low and explained by multifactorial habitat characteristics (such as high continental influence, low hydrodynamics and low mass turnover) that reduce the success of trematode infection processes. Nevertheless, trematode infection was numerous enough to give a clear sign of ecosystem fitness decline. This work showed the reliable use of trematode

communities as early warning indicators of changes occurring in an ecosystem that can represent an extremely useful management and conservation tool.

The metacercariae stage of a trematode life cycle, often occurs in bivalves, using it as second intermediate host. Metacercaria uses the host as its habitat and food resource but, conversely to sporocyst stage, do not reproduce inside the host. Occupying much less space with consequent lower interaction between the parasite and the host, metacercariae are generally reported as having low pathogenicity. This stage has been identified as the occasion for parasite to “mix” their genotypes as cercariae are coming from different upstream hosts (Leung et al., 2009). However, there are already some evidences of significant negative effects on bivalve hosts when metacercariae abundance gets high (Addino et al., 2010; Desclaux et al., 2004; Thieltges & Rick, 2006). In this sense, it is important to recognize the most significant variables causing infection outbreaks and subsequent host population dynamics modulation. In this thesis, it was successfully applied the encounter-dilution effect to a natural bivalve population (Buck & Lutterschmidt, 2017). This theory describes that host species aggregation in dense assemblages result in lower parasite loads. Settlement in areas supporting high densities of adult cockles is generally associated with a high mortality risk, but with this new insight, it was demonstrated that high densities will provide safety against high parasite burden. On the other hand, this finding alert to fact that when in an overfished bank, the stock decrease will be twice a handicap: the decrease of the population density will consequently decrease the reproduction probability and the population sustainability but will simultaneously increase the parasite infection of the cockles that remain. The present findings may lead to implications in decision-making, especially related to bivalve larvae settling within the intertidal zone. Besides, this knowledge can help in fishing activities and stock management as well as aquaculture practices.

In the environment, bivalves are not only exposed to parasites but to a wide range of different abiotic variables that can change (de Montaudouin et al., 2010) or by anthropogenic intervention (Beketov & Liess, 2012; Mearns et al., 2017). This thesis included new content about the influence of climate change related factors, namely salinity, temperature and pH alterations, on the trematode success to invade the bivalve host. It was shown that these drivers may promote the parasite infection success and consequent proliferation, and that trematode infection as metacercariae parasitic stage, usually associated to lower risk, induced alterations on the regular bivalve biochemical responses. In many ecosystems, this will influence the

geographical distribution of some diseases and, probably, the survival capacity of infected bivalves. As parasites have the ability to influence host population behaviour, reproduction and survival, small changes in parasite dynamics will have several potential subsequent effects on host populations and communities with consequences even at the ecosystem level (Benesh & Kalbe, 2016; Larsen & Mouritsen, 2014; Mouritsen & Poulin, 2010). Moreover, in this thesis it was shown that in a contamination scenario (using ecologically relevant concentrations), the host may surprisingly benefit from the parasite presence that, in interaction with the contaminant shows an antagonistic effect, decreasing the bivalve stress response. The parasite presents an interesting altruistic behaviour, trying to keep the host alive (its food and shelter resource) under stressful conditions. Highlighting the fact that the response of an organism to a given anthropogenic derived stress can be modified by parasitic infection, the present study contributed to emphasize the importance of parasitology integration into physiological and ecotoxicological assessment of marine organisms exposed to stressful conditions.

This thesis clearly demonstrated that parasites do matter!

Perspectives

Host-parasite interactions are very rarely a focus of ecosystem ecology research. The understanding of marine parasite dynamics remains limited, even in what concerns to baseline knowledge such as species inventory or infection levels (Poulin et al., 2016). Counteracting this trend, this thesis content focused on parasites diversity, population dynamics and effects on their hosts fitness, providing a broad image of parasites role in the natural environment. It was shown that, on one hand, parasites are positive actors, being part of the biodiversity and useful as indicators of ecosystem quality. On the other hand, parasites can be considered as negative actors, exerting a harmful impact on the bivalves health. Taking into account these new insights and all the information produced in this thesis, future research should certainly study parasites, namely trematodes, as integral parts of the ecosystems and should finally recognize their actual contribution to ecosystems functions.

Some of the questions that I would like to see addressed in a near future are:

1. *In a given ecosystem, what is the importance of trematodes populations in terms of productivity and biomass?*

Productivity and biomass of organisms are traditionally used to estimate the energy flow of an ecosystem from which, parasites are frequently excluded. Available data of trematodes yearly productivity and biomass have been showing that it can be comparable to many groups of invertebrates and can even exceed that of predatory birds (Preston et al., 2013; Thieltges et al., 2008b).

The answer to this question can pass through the use of novel methods of direct biomass estimation especially created to small organisms, i.e. which mass is difficult to obtain. The estimation of these parameters for as many ecosystems as possible, will highlight the trematodes role in the ecosystem energetics and will represent a further step towards the recognition of the structuring role of parasites in the communities.

2. *Do trematodes follow same latitudinal gradients of diversity as their hosts?*

Latitudinal gradients of species diversity are among the best-studied biogeographical patterns. However, these well-known and accepted macro-ecology laws may not be valid for parasites, i.e. organisms which habitat is inside another organism and hence, influenced by different constrains. Available studies are often limited to one region and focused in a given host class and so, the results are not

consensual and showed that parasites may be following more complex diversity patterns than their hosts (Jorge & Poulin, 2018; Poulin & Leung, 2011).

The answer to this question can be found for instance, gathering large-scale geographic historical data and/or performing a large-scale trematode sampling for further analysis of population genetics and demographic connectivity. The increasing knowledge of parasite diversity patterns and consequent identification of the main modulators of parasites transmission will help to predict increasing infection levels and higher potential negative impacts to populations in a global climate change scenario.

3. *If the parasite can manipulate its bivalve host behaviour (e.g. Friesen et al., 2017), will it have consequences in the food web structure?*

Parasites can not only influence ecosystem energy flow due to possible strong representativeness in the community productivity and biomass, as mentioned before, but also due to manipulation of their host behaviour and/or phenology with probable consequences at the food chain level.

Experimental tests in mesocosm environment could be a way to answer to this question. By examining the natural environment but under controlled conditions and studying multiple trophic levels at the same time (at least the ones that compose a trematode life cycle), mesocosm experiments can help to find the answer to this question and many other issues related to the parasites influence in the community functioning.

4. C*an parasites be used to understand the biological availability of pollutants in the ecosystems?*

There is some evidences that trematodes are able to concentrate and withstand levels of contaminants far above the background levels and greater than levels sustained by their hosts (Sures et al., 2017b). This high capacity of trematodes bioaccumulation, may help to detect and quantify some contaminants (even those emergent contaminants called as technology-critical elements) that are present in the environment but in low concentrations, using the traditional analytical techniques. If true, trematodes can become a model sentinel species, i.e. a species used to measure the amount of a pollutant that is biological available in a given ecosystem which will help to overcome some difficulties found in biomonitoring programs.

All the information described to date in the literature (including this manuscript and many of the references that gathered), together with the answers to these questions (and others that will certainly arise) will provide valuable information on the

effective impact of parasites infection at the ecosystem level and will help to fully describe ecosystem services provided by parasites. An interesting way of understand how parasite communities can structure the ecosystem functioning and consequently be able to predict and manage changes, it would be the incorporation of all relevant information available in a traits-based approach. This type of approach is based on patterns and has been successfully applied to understand the responses of biodiversity to drivers of change and the effect of biodiversity on the ecosystem properties improving conservation practices.

CHAPTER 6. REFERENCES

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