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

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ABSTRACT

Bivalve populations are controlled by several biotic and abiotic factors. Parasitism is among the biotic factors but is often neglected. In the present study, we focused on the transcriptomic and biochemical responses of *Cerastoderma edule* when parasitized as first intermediate host by the trematode *Bucephalus minimus* (sporocyst, the most damaging stage), and taking into account seasonal patterns. In order to test the hypothesis that the presence of *B. minimus* compromises cockle regular gene expression and biochemical performance and increases their vulnerability to other parasite species infection, cockles were sampled every other month during one year in Arcachon Bay (French Atlantic coast). Overall, results showed that *B. minimus* induced its first intermediate host defence mechanism against oxidative stress (mainly at gene level), increased host metabolism and energy demand especially in summer (revealed at both gene and biochemical level, although without significant differences) and was accompanied by a higher metacercariae abundance. Results allowed to accept the posted hypothesis and to conclude that transcriptomic and biochemical markers can provide additional and ecologically relevant information about parasite effects on their hosts, reflecting the invasion effects of pathogens but also the environmental conditions that animals experience.

1. Introduction

The edible cockle, *Cerastoderma edule* (Linnaeus, 1758) is a native, infaunal suspension-feeder bivalve, often dominant in semi-sheltered coastal waters along the north-eastern coast of Atlantic Ocean, from the Barents Sea to Mauritania (Honkoop et al., 2008; Tebble, 1966). This species plays a crucial role in the ecosystem, as cockles are an important food source and a link between food webs (Rakotomalala et al., 2015). They are also responsible for different ecosystem services (e.g. carbon storage and energy cycling (Morgan et al., 2013)). From the biological point of view, this species has been well-studied (Malham et al., 2012) and worldwide identified as a good sentinel (e.g. Freitas et al., 2012) and bioindicator species (e.g. Cheggour et al., 2001). Finally, cockle is also an extensively exploited species with high economic value (e.g. Rowley et al., 2014).

In the environment, bivalves including cockles are commonly affected by several diseases that can put at risk entire host populations. Ecological and economic consequences (Lafferty and Hofmann, 2016) make imperative to understand the impact of these diseases on the sustainability of this natural resource. Infectious diseases are frequent in cockles, especially by trematode parasites which can seriously interfere with host population performance (de Montaudouin et al., 2014). Trematodes are the most abundant and common macroparasites in coastal waters (Lauckner, 1983). They display a complex life cycle with alternation of parasitic and free-living stages, generally including three host species. As metacercariae (parasitic stage occurring in the second intermediate host), parasite usually displays high prevalence but their pathogenicity is reported as low, with little interaction between parasite and cockle (e.g. de Montaudouin et al., 2012a; Wegeberg and Jensen, 1999). As sporocyst (parasitic stage occurring in the first intermediate host), effects on host individual are often severe due to strong interaction between parasite and vital host organs like gonads, digestive tract and gills (Dubois et al., 2009). Some studies described sporocysts impact on hosts reproduction (Carballal et al., 2001), growth (Bowers, 1969) and behaviour (Babirat et al., 2004). Trematode parasite free-living propagules can easily infect cockles through their suspension-feeding activity, using cockles as first and/or second intermediate hosts (de Montaudouin et al., 2009).

In the edible cockle distribution area, *Bucephalus minimus* is the most prevalent trematode species using cockles as first intermediate

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host (de Montaudouin et al., 2009; Magalhães et al., 2015). 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). 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 and close-related trematode species using cockles as second intermediate host were able to modulate metallothioneins activity and related gene expression (Paul-Pont et al,. 2010, 2012), decreasing their synthesis (Baudrimont et al., 2003; Baudrimont et al., 2006; Desclaux-Marchand et al., 2007). In the Ria de Aveiro (Portugal), *B. minimus* displayed higher prevalence in the most contaminated areas (Freitas et al., 2014). However, it is difficult to collect infected hosts mainly due to the following reasons: on one hand, parasite prevalence is usually low (reviewed in Thieltges et al., 2008); and on the other hand, prevalence can reach high values but this outbreak is usually rapidly followed by mass host mortality (e.g. Thieltges, 2006).

Therefore, the present study focused on the *C. edule* (first intermediate host) – *B. minimus* (parasite) system, aiming to identify the consequences of heavy parasite infection in terms of cockle individual response as well as to recognize the influence in other associated trematode species abundance and diversity. Hence, this work combines, for the first time, host dissection for parasite identification, transcriptomic and biochemical analyses with bimonthly sampling in order to contribute for a better understanding of the sub-organism effect of *B. minimus* on its first intermediate host, when sporocyst is well developed (i.e. easily observed). The tested hypothesis was that the spread of *B. minimus* among tissues will compromise cockle regular gene expression, biochemical performance, increasing their vulnerability to other parasite species.

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 (Karray et al., 2015a). Nowadays, this technique has been used in cockles to assess the effects of inorganic contamination (Karray et al., 2015a). Previous studies also showed that 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., 2015a; Marques et al., 2016; Velez et al., 2016). In the present study, we intended to evaluate the capacity of these biomarkers to evaluate the impacts associated with the effects of *B. minimus* on *C. edule*.

2. Material and methods

2.1. Study area

C. edule individuals were collected in Ile aux Oiseaux in the inner part of Arcachon Bay, France (44°42′°N, 1°11′W). Arcachon Bay is a 156-km^2 macrotidal lagoon situated on the Atlantic south-west coast of France. It connects with the Atlantic Ocean by a wide 24-km^2 channel, characterized by the presence of a series of sand banks. At the sampling site sediments are classified as medium sand (grain-size median = 370 μm) (de Montaudouin et al., 2012b). The sampling site 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).

2.2. Sampling procedure

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 *Bm+*). 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 *Bm-*), 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 (*Bm-*). 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 *Bm+* and *Bm-* 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 *Bm-* cockles used in transcriptomic analysis) were also measured (shell length) and preserved, entire at - 80 °C. In order to identify *Bm+* and *Bm-*, 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 (*Bm+* and *Bm-*) 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).

2.3. Transcriptomic analysis

Samples (60 cockles, corresponding to 10 per sampling month, 5 *Bm+* and 5 *Bm-*), obtained and preserved as mentioned before, were homogenized with Lysing Matrix D Bulk® in a high-speed homogeneizer 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. 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, 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 1).