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Involvement of apoptosis in the dialogue between the parasite *Bonamia ostreae* and the flat oyster *Ostrea edulis*

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ABSTRACT

The protozoan parasite *Bonamia ostreae* has been associated with the decline of flat oyster *Ostrea edulis* populations in some European countries. Control of shellfish diseases mostly relies on prevention measures including transfer restrictions and stock management measures such as breeding programmes. These prevention and mitigation measures require a better understanding of interactions between host and pathogens. Previous *in vitro* studies allowed identifying apoptosis as a mechanism activated by the flat oyster in response to *B. ostreae*. However, these experiments also suggested that the parasite is able to regulate apoptosis in order to survive and multiply within hemocytes. By simplifying the conditions of infection, *in vitro* studies allow identifying most distinct features of the response of the host. In order to appreciate the relative importance of apoptosis in this response at the oyster scale, *in vivo* trials were carried out by injecting with parasites oysters from two French locations, Quiberon Bay (Brittany) and Diana Lagoon (Corsica). Apoptosis was investigated on pools of hemolymph from oysters collected at early and later times after injection using previously developed tools. Apoptotic cellular activities including intracytoplasmic calcium concentration, mitochondrial membrane potential and phosphatidyl serine externalization were analysed using flow cytometry. Moreover, the expression of flat oyster genes involved in both extrinsic and intrinsic pathways was measured using real time quantitative PCR.

1. Introduction

Populations of the European flat oysters, *Ostrea edulis,* have suffered from overfishing and several diseases including bonamiosis due to the parasite *Bonamia ostreae*. This protozoan was first detected in association with mortality of flat oysters in 1979 in Brittany [1] and was subsequently identified in other production sites in Europe. It is currently reported in several other European countries, in North America and New Zealand where it has recently been detected in another oyster species *Ostrea chilensis* [2].

Mortality due to *B. ostreae* mostly affects oysters older than two years although the parasite infects all developmental stages including larvae and spat [3–6]. Transmission of the parasite is direct from infected to non-infected oysters and does not seem to require intermediate host [7]. The parasite infects and multiplies within hemocytes and can be observed extracellularly in epithelium [1,8]. Routes of entrance and release of the parasite in the oysters remain undetermined. However, several studies have suggested the role of the gills in the infection and excretion of *B. ostreae* [9]. Although the parasite is not cultivable, the establishment of a protocol to purify *B. ostreae* from infected oysters [10] has allowed the development of *in vitro* and *in vivo* experimental infection protocols [11,12]. *In vitro* infection experiments consisting in contact between hemocytes and parasites allow investigating host parasite interactions at the cellular level and have been used to better understand mechanisms involved in the resistance of the flat oyster to *B. ostreae* infection. Previous studies have demonstrated that both hemocytes and *B. ostreae* are actively involved in the internalization of the parasite [13,14] and have notably showed the involvement of apoptosis in the defence against bonamiosis [15,16].

Apoptosis is a universal mechanism involved in different biological processes including defense against stressors like pathogens [17,18]. By degrading infected cells, apoptosis contributes indeed to limit pathogen multiplication and spread [18]. However, parasites have developed in turn strategies to modulate host cell apoptosis to successfully install

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into their hosts. Modulation of apoptosis appears as an important weapon in the pathogenic arsenal of multiple intracellular protozoan parasites including *Leishmania major*, *L. Mexicana* and *L. amazonensis* which inhibits macrophage apoptosis by inducing PI3-kinase/Akt a signalling pathway known to promote cell survival [19].

In vitro experiments have also previously suggested that *B. ostreae* inhibits hemocyte apoptosis after 4 h of contact notably by increasing overexpression of an *inhibitor of apoptosis* (*IAP*) [20]. By simplifying the conditions of infection, *in vitro* experiments allow identifying most distinct features of the response of the oyster. However, these responses might be mitigated by other processes at the oyster scale and relative importance of these features needs to be appreciated at the animal level through *in vivo* trials. Infection experiment by injecting parasites into the adductor muscle of the oyster allows subjecting oysters to a similar dose of pathogens and following the response of the oysters in time. Such approach has previously showed that infection with *B. ostreae* is associated with a decrease of phagocytosis and ROS production in «resistant » oysters contrary to wild oysters [21]. Furthermore, the response against bonamiosis is known to differ according to oyster populations [22].

In this context, the relative involvement of apoptosis in the dialogue between flat oyster and *B. ostreae* has been investigated *in vivo* on two genetically distinct oyster populations, from Quiberon bay in Brittany and from Corsica in South of France [22]. Apoptosis was evaluated during an experimental infection using flow cytometry and molecular tools previously developed [23].

2. Material and method

2.1. Flat oysters

Two year flat oysters, *O. edulis*, were collected from Quiberon bay (Brittany, France) and Etang de Diana (Corsica, France) and acclimatized over 30 days in Ifremer's Facilities. Oysters were maintained in 150 L raceway supplied with a constant flow of seawater enriched in phytoplankton (*Skeletonema costatum*, *Isochrysis galbana* and *Tetraselmis suecica*).

2.2. Parasite isolation

Parasites were purified according to the protocol developed by Ref. [10]. Examination of gill tissue imprints in light microscopy allowed selecting oysters highly infected with *B. ostreae*. All organs except the adductor muscle were homogenized and purified by differential centrifugation on sucrose gradients. *Bonamia ostreae* cells were washed and resuspended in filtered sea water (FSW) and counted with hemocytometer.

2.3. Experimental infection

The experimental design consisted of 10 tanks (5 for oysters injected with parasites and 5 for oysters injected with FSW). Each tank contained 30 oysters from Quiberon Bay and 30 oysters from Corsica (Fig. 1). Purified parasites ($7.5.10^5$ per individual) were injected into 150 oysters from Quiberon and 150 oysters from Corsica. The same amount of oysters was injected with 100 µl of FSW. Injection was performed into the adductor muscle after anesthesia using $MgCl_2$ (250 g) in 4 L of tap water and 1 L of seawater supplemented in phytoplankton. Flat oysters were maintained for one month in 150 L tank with constant flow of seawater enriched in phytoplankton. Potential mortality was checked daily.

2.4. Hemolymph collection

Ten oysters were collected from each tank at 1, 4, 7 30 and 34 days after injection. Hemolymph was withdrawn from the adductor muscle of the oysters using 1 mL syringe. Hemolymphs from 10 oysters subject to similar experimental conditions were pooled and kept on ice to avoid cellular aggregation. After filtration at 70 µm to remove debris, hemocyte concentration was estimated using a hemocytometer and adjusted at 5.10^5 cell.ml^{-1} with FSW for flow cytometry, estimation of DNA fragmentation and real time PCR.

2.5. Flow cytometry

Modulation of intracytoplasmic calcium concentration was monitored using Fluo-4/AM (Molecular Probes). Modulation of mitochondrial membrane potential ($\Delta\Psi m$) was measured using JC-10 dye (FluoProbes®) and externalization of phosphatidyl serine on plasma membrane and cell viability were tested using Annexin V and PI respectively (Eurobio). These activities were monitored by flow cytometry using an EPICS XL 4 (Beckman coulter) according to Ref. [23]. Six replicates were tested for each condition and at each sampling time.

2.6. Real time PCR for Bonamia ostreae detection

A piece of gill was sampled from 5 individuals per tank and time of sampling and fixed in 90% ethanol. Gill tissue (25 mg) was collected for DNA extraction using the QiaAmp DNA Mini-kit (Qiagen) according to the manufacturer's instructions. DNA was eluted and resuspended in 50 µl of sterile, deionized water and then diluted to a final concentration of 5 ng µl^{-1} for real time PCR analysis.

Real time PCR was performed using a MX3000 Thermocycler sequence detector (Stratagene) according to a previously published protocol Robert et al. (2009). All reactions were carried out in duplicate. Each PCR run included negative controls consisting in reaction mixtures without DNA and positive control consisting of plasmidic DNA including the target region.

2.7. Gene expression analysis

2.7.1. RNA isolation and cDNA synthesis

Samples of hemolymph were centrifuged at 4 °C during 10 min at 1500 × *g* and the supernatant was discarded and kept at −80 °C in TRIzol® Reagent (Ambion) until being processed. Total RNA was extracted using TRIzol® Reagent according to manufacturer's recommendations. Total RNA was treated with TurboTM DNase (Ambion) to remove genomic DNA. RNA concentration and quality was estimated using the NanoDrop 2000 (Thermo Scientific) and Bioanalyser 2100 (Agilent). Direct real time PCR with no retrotranscription was performed using EF1-α primers (Table 1) after each DNase treatment to control absence of genomic DNA. First strand cDNA synthesis was carried out from 500 ng of RNA treated using the SuperScript®III First-Strand Synthesis System (Invitrogen).

2.7.2. Real time quantitative PCR

Expression level of *O. edulis* apoptotic-related genes previously described in Ref. [15] was measured in each pool for both oyster groups after *B. ostreae* or FSW injection at each time of the experiment. Real time quantitative PCR was carried out in duplicate in 96-microwell plates using Mx3000 Thermocycler sequence detector (Stratagene). Amplification reactions contained 2 µL of each primer, 10 µL of Brillant III Ultra-Fast SYBR® Green QPCR Master Mix (Agilent Technologies) and 5 µL of diluted cDNA (1/30) in a final volume of 20 µL. Amplification was carried our using standard cycling conditions: 95 °C for 3 min, followed by 40 cycles of 95 °C for 5 s and 60 °C for 10 s. Efficacies were previously calculated for each primer pair in Article 3 (Table 1). Expression level of selected genes was analysed using the method described by Ref. [24]. Host gene expression was normalized using elongation factor-1α (EF-1α) according to Ref. [25]. Calibrators consisted of samples injected with FSW at each time of the experiment.

In addition, expression level of *B. ostreae* 18s was followed in oysters

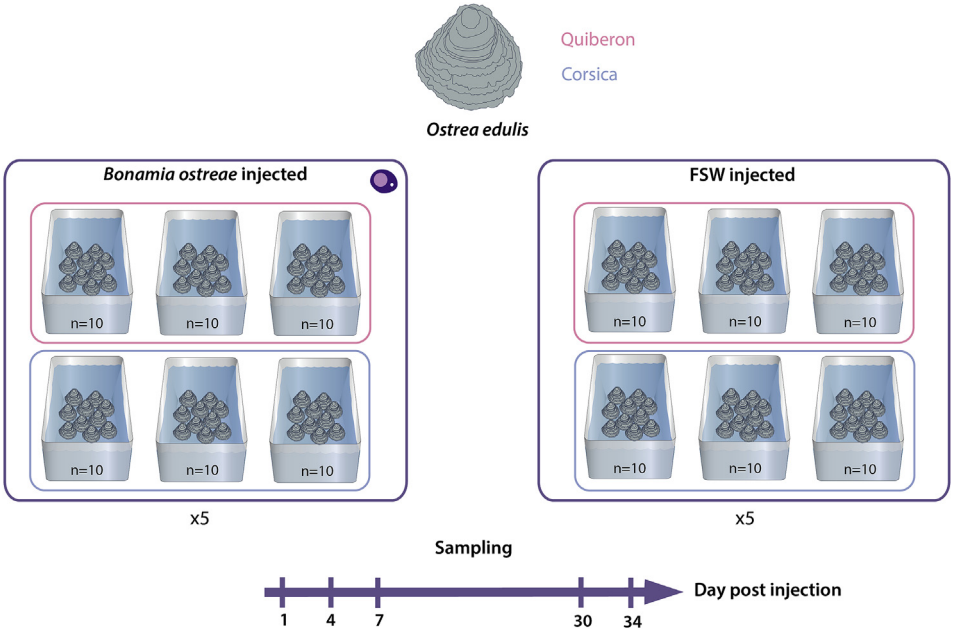

Fig. 1. Experimental design of infection by injection.

from Quiberon after parasite injection and in control condition. Real time quantitative PCR was carried out as previously described by Ref. [24].

2.8. Statistical analyses

Normality of all data sets was tested using Shapiro Wilk test and homogeneity of variances were assumed using Bartlett's and Levene test. Two-way ANOVA were used for normal data sets to determine whether significant differences existed between time and experimental treatments. Tukey's post hoc test was used to establish where significant differences occurred within the data set. Non normal distribution data sets were analysed using Wilcoxon test. Analyses were conducted using RStudio.

3. Results

3.1. Parasite detection

Parasite detection was investigated at the individual level in gills and in pool of hemocytes using real time PCR.

3.1.1. Parasite DNA in gills

No mortality was observed in challenged oysters during the experiment.

Quiberon bay is an endemic zone regarding infection with *B. ostreae*. The parasite was detected in up to 30% of tested oysters injected with FSW. *B. ostreae* was detected in 80% and then less than 50% of tested Quiberon oysters injected with parasites at 7 days and after day 30 respectively (Fig. 2A).

In contrast to Quiberon, prevalence of bonamiosis in Corsica is known to be very low and Corsican oysters injected with FSW appeared negative by PCR (Fig. 2B). Up to 60% of tested Corsican oysters injected with parasites were detected positive 1 and 30 days after injection.

3.1.2. Parasite RNA in hemolymph

To facilitate the reading of Fig. 3, results were represented by 40 (maximum ct value) - Ct value of sample.

B. ostreae 18s RNA was followed in order to quantify parasite infection in hemolymph collected from Quiberon oysters injected with

Table 1
Primer sequences and characteristics for quantitative real time PCR.

Species	Gene	Sequences 5′-3′		Primer concentration (μM)	Amplicon length (bp)	Tm (°C)	Efficacies (%)	Reference
O. edulis	*Bcl2*	Forward	TCCAGACTCGGAAGAAAGGA	4	263	84,3	96,3	Cahais et al., 2012
		Reverse	GGGGAAGGATGAATGGAGAT	4				
	casp 2	Forward	ATGACGGTGAGGAACAGGTC	3	242	81,2	98,2	Cahais et al., 2012
		Reverse	GGATTCCGAATGGCCTTAAT	3				
	casp3	Forward	GCAGTTTTTCCGGAATGAGA	3	188	81,7	97,3	Cahais et al., 2012
		Reverse	CCGATTCACTTGAGTGAGCA	3				
	TNFL 11	Forward	TTCCTAACGTCCCGAACAAC	4,5	224	84,3	97,6	Cahais et al., 2012
		Reverse	GCCACTCAGTCATCCTCCAT	4,5				
	TNFR	Forward	ATGGCGTCCCAATTAGTCTG	4	232	84,3	101,9	Cahais et al., 2012
		Reverse	TTCGGTTCCAAATCTTCGTC	4				
	endoG	Forward	TCCATCATCAATCGAACGAA	5	186	81,5	95	Cahais et al., 2012
		Reverse	GCCGATACCCTTCAAAAACA	5				
	TTRAP	Forward	AGTTAAAGCTGCTGCACTACACC	4	234	83,8	96,8	Cahais et al., 2012
		Reverse	CTGGAATGCCTTAGATACAATCG	4				
	EF1-α	Forward	GTCGCTCACAGAAGCTGTACC	4	162	82,9	99	Cahais et al., 2012
		Reverse	CCAGGGTGGTTCAAGATGAT	4				
B. ostreae	*18s*	Forward	TCAGCACTTTTCGAGAAATCAA	4,5	200	83	94,6	Cochennec et al., 2000
		Reverse	CCACCATGCATAGAATCAAGAA	4,5				

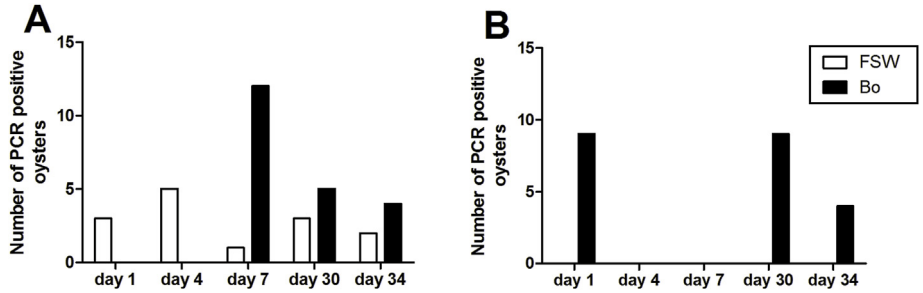

Fig. 2. Number of oysters detected positive by PCR in gills after injection with filtered sea water (FSW) or parasite (Bo) from Quiberon (A) and Corsica (B) at different times. n = 15.

FSW (Fig. 3B) and from all the oysters injected with parasite (Fig. 3A–C). Whatever the tested condition was, *B. ostreae* RNA was detected as soon as one day after injection. In Quiberon oysters, same amount of parasite RNA was detected when injected with parasites and with FSW. Less parasite RNA was detected in Corsican oysters injected with the parasite than in Quiberon oysters whether they were injected with parasites or FSW ($p < 0.05$) (Fig. 3C). Dynamics of *B. ostreae* 18s RNA was similar in oysters injected with parasites whatever their origin was, decreasing from day 1 to day 30.

3.2. Modulation of hemocyte cellular activities

Involvement of apoptosis response of flat oysters during an infection with *B. ostreae* was investigated at the cellular level by measuring by flow cytometry the intracytoplasmic calcium concentration, the mitochondrial membrane potential and the externalization of phosphatidyl serine on plasma membrane.

Whatever the origin was, injection of *B. ostreae* induced a significant decrease of cells with high intracytoplasmic calcium concentration compared to the control 7 and 34 days after injection (Fig. 4A). Modulation of this parameter was not significant at days 1, 4 and 30.

In oysters from Quiberon, percentages of hemocytes with low ΔΨm appeared slightly modulated compared to the control (Fig. 4B). In contrast, this parameter was significantly increased in Corsican oysters 1, 7 and 34 days after injection and decrease at day 30.

In oysters from Quiberon percentage of cells with externalization of phosphatidyl serine in oyster from Quiberon was higher only after 4 days of infection (Fig. 4C). In contrast Corsican oysters showed a decrease of cells with externalization of phosphatidyl serine compared to the control at 4 and 7 days post infection.

3.3. Ostrea edulis gene expression

Involvement of apoptosis in the response of flat oysters during an infection with *B. ostreae* was investigated at the molecular level by measuring the expression level of seven genes involved in both the extrinsic and intrinsic apoptotic pathways. These genes were chosen to provide an overview of the apoptotic response of the flat oyster.

3.3.1. Extrinsic pathway

Five genes involved in the extrinsic pathway were followed during the experiment: *TNFL*, *TNFR*, *TTRAP*, *caspase 2* and *caspase 3*.

In oysters from Quiberon injected with parasites, first significant gene expression modulation was observed at day 4 with an activation of caspase 3 which was followed at day 7 by an overexpression of *TNFL* and *TTRAP* (Fig. 5A,C,H). 30 days post injection, *TNFR* and *caspase 3* were down regulated. Finally at day 34, *caspase 2* appeared overexpressed.

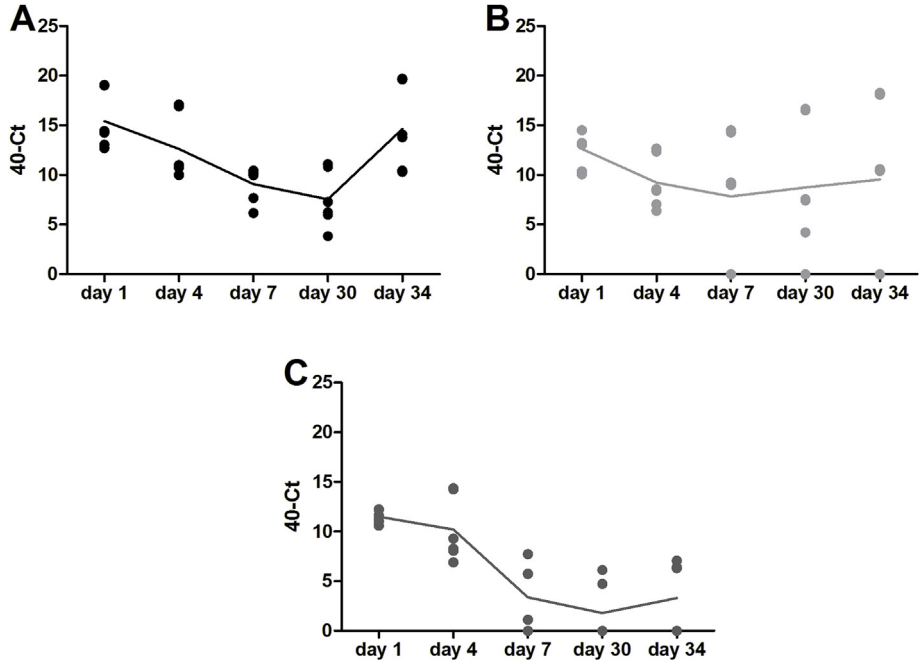

Fig. 3. Detection of *Bonamia ostreae* 18s rRNA in pools of hemolymph from Quiberon oysters injected with *B. ostreae* (A), injected with FSW (B) and Corsican oysters injected with *B. ostreae* (C) at different times. n = 6 (3 pools per time, 2 replicates per pool).

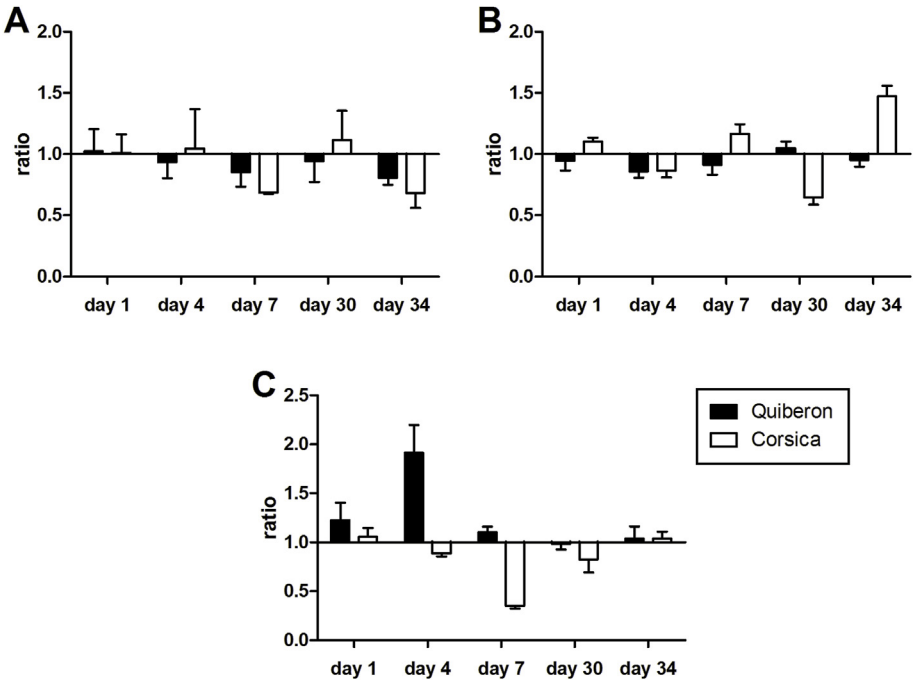

Fig. 4. Intracytoplasmic calcium concentration in hemocytes of flat oyster from Quiberon and Corsica (A). Ratios of labeled hemocytes with high calcium concentration between oysters injected with *B. ostreae* and with FSW. Mitochondrial membrane potential (ΔΨm) in hemocytes of flat oyster from Quiberon and Corsica (B). Ratios of labeled hemocytes with low ΔΨm between oysters injected with *B. ostreae* and with FSW. Plasma membrane integrity in hemocytes of flat oysters from Quiberon and Corsica (C). Ratio of AnnexinV positive and PI negative hemocytes between oysters injected with *B. ostreae* and with FSW. Results represent the mean ± SD. n = 6.

In Corsican oysters injected with *B. ostreae*, as soon as 1 day post injection TNFL and TNFR appeared under expressed and *caspase 3* over expressed compared to the control (Fig. 5A,B,E,H). *TNFL* expression increased at day 4 and was under expressed 30 days post injection like *TTRAP* and *caspase 2*. Finally, *caspase 3* appeared overexpressed at day 34 compared to the control.

3.3.2. Intrinsic pathway

Two genes involved in the intrinsic pathway were followed during the experiment: *Bcl2* and *endoG*. *Bcl2* is involved in the inhibition of apoptosis by the mitochondria and *endoG* is an enzyme that can induce DNA fragmentation.

In oysters from Quiberon, these two genes were upregulated 7 post injection with parasites and *endoG* appeared then under regulated at 30 days (Fig. 5F–G,H). In contrast, in Corsican oysters these two genes were under expressed after 30 days and endoG was overexpressed after 34 days.

4. Discussion

Successful establishment of infections in susceptible hosts requires means of avoiding host responses soon after initiating infection [26]. Installation of the parasite *Bonamia ostreae* into its host, the flat oyster *Ostrea edulis* seems to be a slow process. Experimental infections by cohabitation with naturally infected oysters showed that parasite detection was observed after 3 months in non-infected ones and first mortality was reported after 7 months [27]. In contrast, when the parasite source consisted of oysters previously injected with high doses of *B. ostreae,* first mortality was observed more quickly, after 2 months [21]. Injection of 50% ID (infectious dose) induced first mortality after 4 months [11]. Moreover, with higher parasite doses, *B. ostreae* can be detected as soon as first day after injection [21]. Moreover, previous studies have confirmed involvement of apoptosis in the response of the flat oyster, *O. edulis*, to *in vitro* infection by the parasite *B. ostreae* [15,16]. Considering these results, we have investigated response mechanisms and more particularly the apoptotic response of the oyster in the early and later phases of the infection after injection.

Experimental infection was carried out by injecting a fixed amount of parasites in the sinus of the adductor muscle of the oysters. Although injection might induce some stress and tissue lesions, no mortality was observed during the course of the experiment. Flat oysters from two natural locations were challenged concurrently in order to investigate the specificity of the response against *B. ostreae*. These populations were selected for their genetic differences demonstrated using microsatellite and SNP markers [22] and their prevalence regarding bonamiosis. Quiberon bay is an endemic location regarding bonamiosis with prevalence generally between 10 and 15% [28]. In contrast, prevalence in Corsica is low and associated with a congener species, *Bonamia exitiosa* [29,30].

Parasite DNA was detected in gills of oysters from both populations after parasite injection but also in control oysters from Quiberon. These results are concordant with prevalence of bonamiosis in both populations. Oysters from Quiberon showed a peak of detection 7 days after injection whereas Corsican individuals presented a peak of parasite DNA detection 1 day and then 30 days after injection. However, PCR allows detecting parasite DNA whether the parasite is alive or dead. In contrast, presence of RNA is indicative of live stages and live *B. ostreae* could be detected all sampling days in some pools of oysters injected with the parasite. Early detection of parasite DNA and RNA might correspond to injected parasites and, in Quiberon oysters, also to previously established infection. Later detection at days 30 and 34 showed that *B. ostreae* successfully installed in oysters from both populations. Previous studies also reported that the installation of the parasite into its host was effective around 30 days after injection [21].

Apoptosis response to *B. ostreae* was followed at the cellular and molecular levels using tools previously developed. Intracytoplasmic calcium concentration was reduced in both populations at 7 and 30 days post infection. It was previously showed that *in vitro* infection with the parasite also induced a decrease of intracytoplasmic calcium [16]. Calcium is important in cytoskeleton reorganization during parasite internalization [31]. This reduction could be due to an increase of internalization of *B. ostreae* by the hemocytes. The other tested cellular parameters showed differences between oyster populations. Oysters from Quiberon, showed indeed an increase of annexin V labelled cells at day 4 whereas this parameter was downregulated in Corsican oysters compared to the control at day 7. Finally, mitochondrial membrane potential appeared more modulated in Corsican than in Britain oysters. Presence of parasites into hemocytes was associated with an increase of

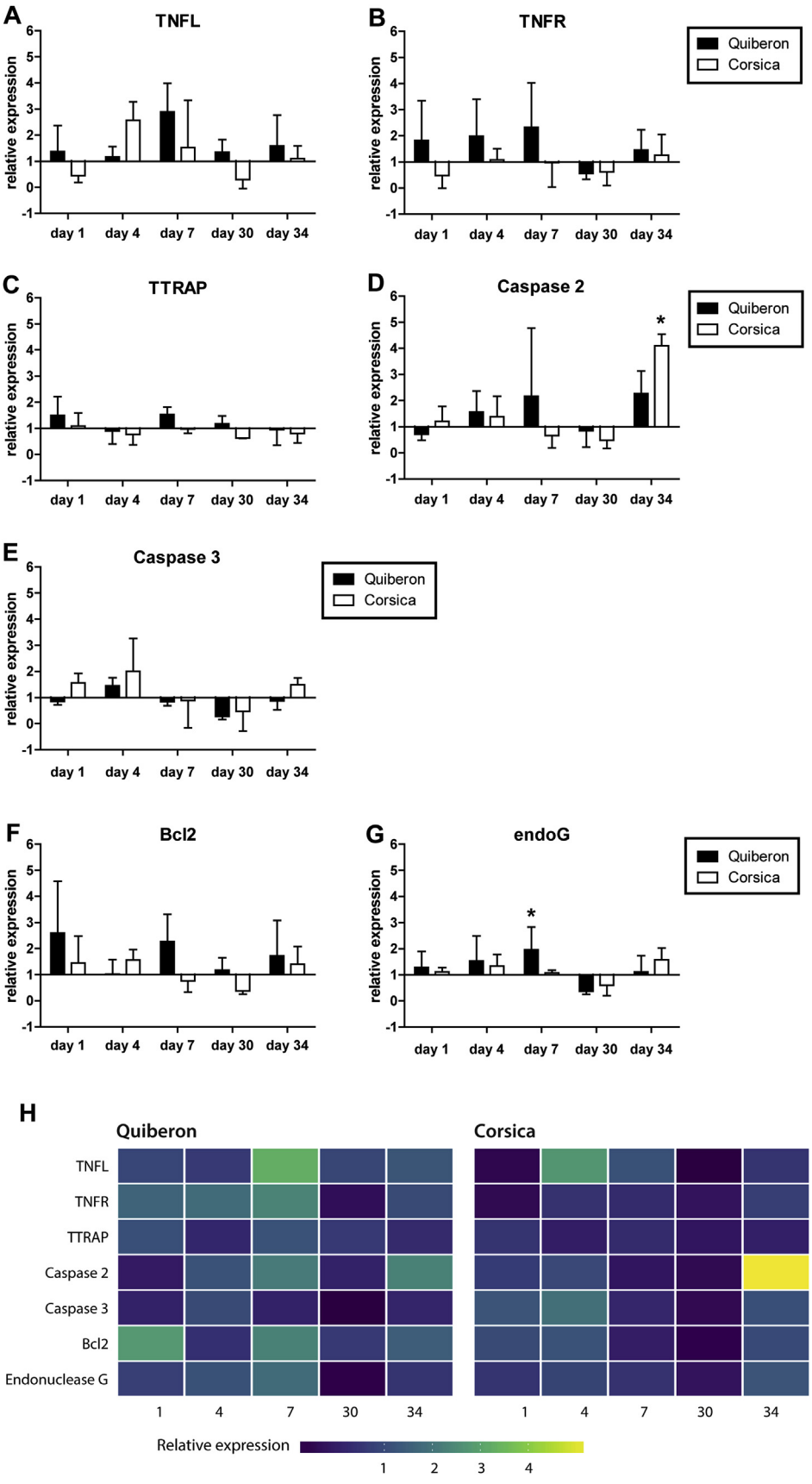

Fig. 5. Gene expression in Quiberon and Corsican oysters after parasite injection at different times. Relative expression by quantitative PCR of genes involved in the extrinsic pathway, TNFL (A), TNFR (B), TTRAP (C), Caspase 2 (D), Caspase 3 (E) and in the intrinsic pathway, Bcl2 (F), endoG (G). Expression levels were normalized to EF1-α and presented as relative expression to controls injected with FSW. *$p < 0.05$. Results represent the mean ± SD. $n = 3$. Heatmap of relative expression of apoptotic genes in Quiberon and Corsican oysters (H). The colour legend represents genes expression from violet (under expression) to yellow (over expression). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

some cellular tested activities but *B. ostreae* installation appeared to inhibit apoptosis induction. It was previously showed that *B. ostreae* can inhibit apoptosis in flat oyster hemocytes *in vitro* after 44 h [15].

Expression level of apoptotic genes as modulated differently in both populations after parasite injection. For oysters from Quiberon, the apoptotic response seemed to be activated at 7 days post-injection whereas after 30 days it appeared inhibited. In Corsican oysters, the apoptotic response was inhibited 30 days after injection, and, in contrast, activated after 34 days. For oysters from Quiberon, apoptosis activation observed at day 7 can be related to the high detection of parasites in gills. In contrast, inhibition of apoptosis in both groups may be associated with parasite installation in the flat oysters as observed at day 30 or decrease of parasite RNA detected on oyster hemolymph.

Apoptotic parameters monitored in the context of this study were modulated by the injection of parasites. However, they did not fluctuate all in the same way in the same time and, interestingly, were modulated differently in oysters originating from both oyster groups. Indeed, infection with *B. ostreae* develops differently between each individual and does not occur in the same way for both groups and can influence the apoptotic response. Our results confirm that interactions between parasite and their host are complex. Moreover, apoptosis is a process that can take hours or days to conduct to the cell death from the activation of initial trigger but once the mitochondria is compromised the cell death is very quick [32]. This pathway is the result of multiple activation and inhibition mechanisms that do not occur in the same time. Our experiments suggest that apoptosis is deeply involved in the response of the oyster against *B. ostreae* and differently depending on the stage of the infection. This balance between activation and inhibition of apoptosis might reflect the battle between the oyster and its parasite.

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