Fish & Shellfish Immunology 36 (2014) 1–8

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

The impacts of handling and air exposure on immune parameters, gene expression, and susceptibility to vibriosis of European abalone *Haliotis tuberculata*

Marion Cardinaud [a], Clément Offret [a], Sylvain Huchette [a,b], Dario Moraga [a], Christine Paillard [a,*]

[a] UMR 6539 – LEMAR (Laboratoire des Sciences de l'Environnement Marin), IUEM (Institut Universitaire Européen de la Mer), Université de Bretagne Occidentale (UBO), CNRS, IRD, Ifremer, Technopôle Brest Iroise, 29280 Plouzané, France
[b] France Haliotis, Kerazan, Lilia, 29880 Plouguerneau, France

ARTICLE INFO

Article history:
Received 21 December 2012
Received in revised form
26 September 2013
Accepted 28 September 2013
Available online 9 November 2013

Keywords:
Abalone
Stress
Immunity
Gene expression
Vibriosis

ABSTRACT

Wild or farmed abalone are regularly exposed to stressors, such as air exposure and handling. Immune and transcriptional responses as well as susceptibility to vibriosis of sexually mature or immature European abalone acclimated at 16 or 19 °C were determined following handling or air exposure. Hemocyte density and H_2O_2 production increased while hemocyte viability and phagocytic index decreased following handling. Air exposure induces a decrease of hemocyte density and phagocytic index. Measurement of the expression of genes implicated in general metabolic, immunological and stress responses in gills, foot-muscle and hemocytes by real time q-PCR suggested that both stressors lead to a metabolic rate depression, characterized by a general inhibition of transcription. Finally, following handling a *Vibrio harveyi* challenge enhances almost 100% mortality of sexually immature animals at 19 °C while it has been previously demonstrated that only mature are susceptible to vibriosis.

1. Introduction

Wild and farmed populations of the European abalone, *Haliotis tuberculata*, have suffered massive mortalities along the French north Atlantic coasts beginning in the late 1990s. These mortalities occurred when seawater temperature exceeded 17 °C and have been attributed to the bacterial pathogen *Vibrio harveyi* [1]. This vibriosis has generally been reported to develop in sexually mature abalone but not in immature or post-spawning individuals [2]. Immune depression characterized by a decrease in hemocyte density, hydrogen peroxide (H_2O_2) production and phagocytosis index measured in spawning European abalone may explain their increasing susceptibility to vibriosis [3]. Mortalities have also been observed in farmed animals and in laboratory studies following handling and air exposure (Huchette, pers. comm.; personal observations). The effects of handling and air exposure on the susceptibility to *V. harveyi* of European abalone at different stages of sexual maturity and temperature however remain to be determined. Studies on the impacts of air exposure and mechanical disturbance such as handling or shaking in marine mollusks have mostly focused on immune responses, particularly hemocyte functions [4–7]. Hemocyte density, phagocytosis index and production of reactive oxygen species decreased during oxygen deprivation due to nitrogen gazing in the Taiwan abalone *Haliotis diversicolor supertexta* [8], and after shaking in the European abalone [9], while hemocyte density in the Australian abalone [10] increased following detachment and handling. Hemocyte responses therefore appear to vary depending on abalone species and stressor types. Furthermore, no study has been conducted on transcriptional effects of air exposure or handling in abalone. Coupling immune and transcriptional analyses should lead to a better understanding of the molecular and cellular responses of the European abalone to handling and air exposure.

* Corresponding author. LEMAR – UMR 6539, IUEM, Université de Bretagne Occidentale, Technopôle Brest Iroise, 29280 Plouzané, France. Tel.: +33 2 98 49 86 50; fax: +33 2 98 49 86 45.
E-mail addresses: marion.cardinaud@gmail.com (M. Cardinaud), dario.moraga@univ-brest.fr (D. Moraga), christine.paillard@univ-brest.fr, paillard@univ-brest.fr (C. Paillard).

1050-4648/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.fsi.2013.09.034

Mortalities observed in European abalone after handling and air exposure may be due to a low capacity of the abalone to tolerate these stressors resulting in alterations of immune parameters and transcription of candidate genes, and/or enhancement of invasion by opportunistic pathogen such as *Vibrio* bacteria. Our objectives were therefore to: (i) determine the immune and transcriptional responses of *H. tuberculata* to handling and air exposure, and (ii) assess the impact of these stressors on the development of vibriosis in *H. tuberculata*. Studies were conducted out of the boundary condition of *V. harveyi* infection, i.e. in sexually mature abalone at 16 °C and in sexually immature abalone at 19 °C.

2. Material and methods

2.1. Study 1: effects of handling and air exposure on immune and transcriptional responses

2.1.1. Abalone

Three years old abalone ($n = 36$, 42.3 mm ± 2.7) were transported in containers filled with macroalgae *Palmaria palmata* from the *France Haliotis* hatchery in Plouguerneau, France, (francehaliotis.com) to our laboratory at the European Institute for Marine Studies in Brest in September 2011. Sexually mature and immature animals were separated and placed into twelve 5 L aerated seawater tanks ($n = 4$ per tank). Sexually mature abalone had well-developed gonads while the immature abalone were induced to spawn 10 weeks prior to their transfer to our lab and had no visible gonads. The seawater from each tank was replaced daily with seawater pumped from Brest Bay (dissolved oxygen concentration > 7.5 mg L^{-1}, salinity 34.3–34.7), sand-filtered and UV-sterilized.

2.1.2. Experimental design

Sexually mature abalone were divided into two sets of animals. The first set was kept at 16 °C while the second set was acclimated to 19 °C by increasing the temperature by 1 °C every other day. After three weeks of acclimation, the sexually mature abalone at each temperature were either exposed to air for 3 h at room temperature (16.0 ± 0.5 °C), or detached, handled and placed down with the foot-muscle upwards, three times over a period of 24 h, or left alone (control). They were then sampled at the same time at the end of the stress period to measure their immune and transcriptional responses. Sexually immature animals were acclimated at 19 °C and subjected to the same treatments as the sexually mature abalone and sampled at the same time.

2.1.3. Hemolymph collection and hemocyte analysis

About 1 mL of hemolymph was collected from the pedal sinus of each abalone with a 2.5 mL sterile syringe containing one mL of ice-cold sterile seawater to avoid hemocyte aggregation. Hemolymph was sampled within 30 s after removal of individual abalone from each tank to prevent sampling stress and was kept on ice. Five hundred microliters of hemolymph were centrifuged at 2500 *g* for 15 min, the supernatant was discarded and the hemocyte pellets were frozen in liquid nitrogen and stored at −80 °C for gene expression analysis. Hemocyte density, viability, phagocytic index and H_2O_2 production in the remaining 1.5 mL were measured using a FACS Calibur flow cytometer (BD Biosciences, SanJose, CA) equipped with a 488 nm laser as described in Travers et al. [3].

2.1.4. Gene expression analysis

2.1.4.1. Sampling. Immediately after hemolymph collection, each abalone was labeled, frozen in liquid nitrogen and stored at −80 °C. After thawing, the gills and the central part of the foot-muscle were dissected in sterile condition, weighed and kept on ice just prior to RNA extraction.

2.1.4.2. RNA isolation and cDNA synthesis. Total RNAs were extracted in TRIzol® Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions and resuspended in RNase-free water. RNAs were quantified by spectrophotometry with a Nanodrop ND-1000 (Thermo Fisher scientific, USA) and RNAs quality was assessed using 1% agarose electrophoresis and analysis on an Agilent Bioanalyzer 2100 with RNA 6000 Nano Kit (Agilent Technologies). Reverse transcription was done with 1 μg of RNA from each sample using random hexamer primers and a RevertAid™ kit (Fermentas International Inc., Burlington, Canada). DNA contamination in RNA samples was checked by amplification of a non-reverse transcribed fraction (minus-RT). A minimal difference between minus-RT and cDNA samples was fixed at eight amplification cycles to accept a sample in gene expression results.

2.1.4.3. Selected genes. Twelve genes were measured by quantitative real time PCR (RT-qPCR): 4 putative reference genes, 4 genes involved in metabolic responses and 4 genes involved in immune and stress responses according to the few genomic sequences available in *H. tuberculata* (Table 1). *H. tuberculata* ribosomal protein 18S, actin, Elongation Factor 1 -EF1 and 2 -EF2 genes were selected as putative reference genes as their sequences were available at the time of the study in NCBI database. The algorithm NormFinder was used to identify the reference gene with the most stable expression as described by the developer [11].

2.1.4.4. Primer design and efficiency estimation. Primer sequences for 9 of the selected genes were available in the literature (Table 1). RT-qPCR primers of EF1 and ATP synthase FO subunit 6 were designed from cDNA sequences of *H. tuberculata* available in NCBI database. For Macrophage-expressed protein (MEP), a cDNA consensus sequence was created by the software Bioedit Sequence Alignment Editor 7.1.3.0 [12] from other *Haliotis* species.

Table 1
Primer sequences used in real time qPCR analysis.

Gene name	Sequence (sense and antisense)	Source	Metabolism
Ribosomal protein 18S	AGAAACGGCTACCACATCCAA TTCGTCACTACCTCCTCGTATCG	Travers et al., 2010[a]	Reference gene
EF1	GGCCACGGTCTGCTTCAT TTTCTCCTCCTACCCACCATTG	This study	Reference gene
EF2	ATGGAGTTTTGTGCGATGAGAA TCGGCGTGGAGAGTCACAT	Travers et al., 2010[a]	Reference gene
Actin	GCCTCTCTGTCCACCTTCCA GGGCCGGACTCATCGTACT	Farcy et al., 2007[b]	Reference gene/ muscular activity
ATP synthase FO	CTCTGCTCAGCCATCTTTATTTACC CAGGGACACACCGATTTCG	This study	Energetic metabolism
Cytochrome c oxidase III	TTGGAAACCTTCTTTTTTATCACCTT TTCCAAGTTCCCCTCCTCAA	Travers et al., 2010[a]	Cellular respiration
Glutamine synthase	GCGACACCTGCCGAGAAGT AGTTATTGAAGAAGCCATCGAGAGA	Travers et al., 2010[a]	General metabolism
Macrophage-expressed protein	AGGTWGCTTCGCTRTGGT TCAYTCCTGCTGGYTGTTCT	this study	Immunity
Ferritin	CGTGACGACTGGGCATTACTC GGACCACATCACCAACCTGAA	Travers et al., 2010[a]	Stress response/ Immunity
Heat-shock protein 71	5′ CGGTGAGCGCAATGTTC 3′ 5′ CCAAGTGGGTGTCTCCA 3′	Farcy et al., 2007[b]	Stress response
Heat-shock protein 84	GATTCGCCATTCGGTTTGTT TGTGGCGATGTTATTGTTGGA	Travers et al., 2010[a]	Stress response

[a] [14].
[b] [47].