Please note that this is an author-produced PDF of an article accepted for publication following peer review. The definitive publisher-authenticated version is available on the publisher Web site

Developmental & Comparative Immunology
September 2009, Volume 33, Issue 9, Pages 974-979
http://dx.doi.org/10.1016/j.dci.2009.04.002
© 2009 Elsevier Ltd All rights reserved.

Archimer
Archive Institutionnelle de l'Ifremer
http://www.ifremer.fr/docelec/

Influence of temperature, salinity and *E. coli* tissue content on immune gene expression in mussel: Results from a 2005–2008 survey

Hui Li[a, 1], Mylène Toubiana[a], Patrick Monfort[a] and Philippe Roch[a, *]

[a] Ecosystèmes Lagunaires, JRU CNRS-IFREMER-Université Montpellier 2, cc093, place E. Bataillon, F-34095 Montpellier cedex 05, France
[1] Present address: College of Life Sciences, Shenzhen University, Shenzhen 518060, People's Republic of China.

*: Corresponding author : P. Roch, Tel.: +33 467 14 47 12; fax: +33 467 14 46 73, email address : proch@univ-montp2.fr

Abstract:

Several bivalves, including mussels, suffered from mortalities particularly in summer. To look for the possible effect of environmental parameters on immune capacities, *Mytilus galloprovincialis* were collected monthly from August 2005 to July 2008 from the Palavas Laguna, French Mediterranean coast. Q-PCR was used to quantify the expression of three antimicrobial peptide genes (*defensin*, *mytilin B* and *myticin B*), in addition to *lysozyme* and *HSP70*. House keeping gene was *28S rRNA*. *Defensin*, *myticin B* and *lysozyme* appeared more expressed in spring–summer than in winter. In contrast, *HSP70* expression was higher in winter. Statistical studies using principal component analysis (PCA) and multiple regression models revealed positive influence of temperature on *28S rRNA*, *defensin*, *myticin B* and *lysozyme* expressions, but not on *mytilin B* and *HSP70*. The positive influence was significant for *defensin* and *lysozyme* expression, but relationships cannot be quantified. Similarly, salinity appeared to influence *defensin* expression, but this relationship cannot be quantified neither. *E. coli* tissue content appeared without influence. Consequently, there was no clear relationship between environmental parameters and immune-related gene expressions, demonstrating anti-infectious capabilities cannot be evaluated using only the expression of such genes as markers.

Keywords: Antimicrobial peptide; *Defensin*; *Mytilin*; *Myticin*; *Lysozyme*; *HSP70*; Q-PCR; Immune survey; Seasonal variations; Innate immunity; *Mytilus*; Mollusks

1. Introduction

Effects of environmental parameters on immune functions of mussels have been extensively studied due to the sentinel role of such mollusk and numerous reports considered the effects of xenobiotics [1-4], but few considered bacteria [5-7] and none the *in situ* seawater micro fauna. Physical and biological parameters of the environment where the mussels live are largely dependent on the season and fluctuations of numerous criteria were reported in mussels as related to seasons : enzyme activities [8], circulating hemocyte number [9], protein content of hemolymph, lysozyme and agglutinin activities [10], antibacterial response induced by LPS [11], lipid composition [12], cytolytic activity [13] and nitric oxide production [14], for instance. Also mortalities were frequently reported in relationships with the season, to such extend that they were called summer mortalities, not only in mussels [15, 16] but also in oysters [17-19]. Concerning immunity, we previously demonstrated in Northern blot that the expression of AMP genes, *defensin*, *mytilin B* and *myticin B* were constitutively expressed in winter, whereas expression only *mytilin* and *myticin B* were detectable also in summer [20]. In addition, expression of both *defensin* and *mytilin B* were decreased following bacterial injection in winter, heat-shock resulting in no change in *mytilin B* expression but in suppression of *defensin* expression in winter and its induction in summer.

Here, we evaluated by Q-PCR the expression of several immune-related genes (*defensin*, *mytilin B, myticin B* and *lysozyme*) and of *HSP70* in mussels collected once a month at the same location during three consecutive years. Some environmental parameters (seawater temperature and salinity) and *E. coli* content of mussel tissues were joined to look for possible correlation with expression of such genes.

2. Material and methods

2.1. Mussels, hemolymph and hemocyte sampling

Adult mussels, *Mytilus galloprovincialis* (6-7 cm shell length), were purchased every 4 weeks from August 2005 to July 2008, from the marine farm Les Compagnons de Maguelone located in the Prévost Laguna of Palavas (French Mediterranean coast). They were acclimated for 24 h in the laboratory (15 mussels in 25 l of sea water per aquarium) in a flow-through air system before hemolymph collection.

Hemolymph (0.8 ml per mussel) was collected from the posterior adductor muscle with a 1 ml disposable syringe containing 0.2 ml of anti-coagulant modified Alsever's solution (27 mM sodium citrate, 115 mM glucose, 336 mM NaCl, 18 mM EDTA, pH 7.0). Hemolymphs from 10 mussels were pooled and hemocytes pelleted by 15 min centrifugation at 800 g, 4°C, then

71 resuspended in 1 ml Trizol Reagent (Invitrogen) and stored at -20°C until used. Four pools of 10
72 mussels each, as replicates, were used for each sampling month. The full survey involved 1,440
73 mussels.
74
75 *2.2. cDNA synthesis and quantitative PCR (Q-PCR)*
76 Total RNA was extracted according to manufacturer's instructions and resuspended in 45
77 µl of Tris-EDTA buffer (TE: 10 mM Tris-HCl, pH 8.0, 1 mM EDTA). RNA concentrations were
78 measured and quality controlled on spectrophotometer ND-1000 (NanoDrop Technologies). First
79 strand cDNAs were synthesized on 5 µg of total RNA using hexaprimers (Invitrogen) and
80 murine leukemia virus reverse transcriptase (Promega), purified through QIAquick Column
81 (Qiagen) then kept in nuclease-free water at -20°C until use.
82 Q-PCR was performed using the SYBR Green chemistry on a LightCycler 480 384 well-
83 plate (Roche). Primer sequences and specificity controls were previously reported [21, 22]. Q-
84 PCR mixture contained the following: 1 µl first strand cDNA (10 ng), 0.75 µl of each specific
85 primers at a concentration of 25 µM, 2.5 µl of mix (Roche) containing FastStart Taq DNA
86 polymerase, reaction buffer 2x, dNTP mix, SYBR Green 1 dye and $MgCl_2$. The PCR programme
87 started with initial Taq polymerase activation at 95°C for 10 min, followed by 40 cycles at 95°C
88 for 10 sec, 65°C for 10 sec and 72°C for 15 sec. Melting temperatures were measured by
89 returning to 65°C for 30 sec and gradual heating to 95°C. Negative control reactions contained
90 water in place of cDNA template and were included in each run to ensure the absence of
91 contamination. Calibration curves were obtained using 10-fold serial dilutions of the
92 corresponding amplicon in 10 µg/ml sonicated salmon sperm DNA (Sigma). House keeping
93 gene was *28S ribosomal DNA*, as previously validated [23].
94
95 *2.3. Q-PCR data analysis*
96 Crossing point values expressed in cycle numbers were measured according to the
97 threshold position of 4.2 and converted into equivalent target amount (ETA) by the LightCycler
98 480 built-in software (Roche) using statistical calibration curves. Expression level of gene of
99 interest was calculated from the *ratio* of ETA for the considered gene on ETA for *28S rDNA*
100 within the same sample. Data were presented as arithmetical mean of the four replicates
101 measured in duplicate ± SEM. Statistical significant differences between data were established
102 by Student's *t*-test using t-Ease 2.8 ISI software. Differences were considered as significant for
103 $p<0.05$.
104
105 *2.4. Environmental parameters*

106 Seawater temperature (measured by electronic probe) and salinity (measured by *in situ*
107 conductivity) values at the dates and unique location (43°31'15 N - 03°54'33 E) where mussels
108 have been collected for gene expression measurements, came from the Ifremer's network *Réseau*
109 *de surveillance du phytoplancton et des phytotoxines* (REPHY). *E. coli* tissue contents of
110 mussels at the same location, quantified by colorimetric enumeration based on β-glucuronidase
111 activity [24] were from Ifremer's network *Réseau de contrôle microbiologique des zones de*
112 *production de coquillages* (REMI). Data were uploaded from
113 http://www.ifremer.fr/envlit/surveillance, a website devoted to coastal environments.
114
115 *2.5. Statistics*
116 Statistics analysis were done with R software available at http://www.R-project.org, using
117 ade4 package [25] and ade4TkGUI package [26].
118 *2.5.1. Descriptive statistics:* Principal component analysis (PCA) was used to describe
119 data and to study the structures and correlations. Analysis was done on centred and scaled data.
120 *2.5.2. Multiple regression models:* To learn more about relationships between each gene
121 expression and environmental data, we tried to define 6 multiple linear models on centred and
122 scaled data. For each model, Fisher's method was used to look for global influence of
123 environmental parameters (Ho: regression coefficients = 0). Student's method was used to reveal
124 significant influence of each environmental parameter on expression (Ho: regression coefficient
125 of each parameter = 0). In the situation of non nullity of one or several coefficients, normality of
126 residuals of the model has been tested for validation using the Shapiro-Wilk's method.
127
128 **Results**
129
130 *3.1. Expression of* 28S rDNA
131 The intensity of expression of *28S rDNA* was subjected to variations according to the
132 month of collection. Lowest expressions were recorded from Aug 05 to Jan 06 (Fig. 1) with
133 statistically significant difference between Oct 05 and Oct 06 (p=0.0002) for instance, but not
134 between Oct 06 and Oct 07 (p=0.51). Similarly, statistically significant difference was observed
135 between Feb 06 and Feb 07 (p=0.035), but not between Feb 07 and Feb 08 (p=0.087). Lower
136 expressions were consistent during Apr-Aug 07, i.e. during spring-summer times, with
137 expression statistically significantly lower in Jun 07 than in Jun 06 (p= 0.00016), but not than in
138 Jun 08 (p=0.96). In addition, no difference in expression was observed between Sep 07 and Jul
139 08.
140

141 *3.2. Expression of* AMPs *and* lysozyme

142 Global observation on Figure 2 was on variations of expressions. Evident is that
143 *defensin* expression was higher in summer, with a maximum in Sep 05, Aug 06 and Aug 07,
144 statistically significantly different between May and Aug 06 (p=0.0028), for instance. Such
145 highest expressions ranged from 0.45 ETA (Sep 05) to 0.75 ETA (Aug 06) and 2.07 ETA (Aug
146 07), i.e. 4.6-folds the expression measured in Sep 05. Particularly low expression of *defensin* was
147 in winter, close to the detection sensitivity of the Q-PCR technology used, with no statistically
148 significant difference between the minimum values recorded from Jan 06 (0.01 ETA), Jan 07
149 (0.03 ETA) and Jan 08 (0.02 ETA).

150 *Mytilin B* expression did not seem to be regulated by the season, except during the winter
151 05, with the highest recorded values of 30.68 ETA (Dec 05) and 22.27 ETA (Jan 06). Such
152 increased expression was not observed during the winters 06 and 07 (p=0.0040 between Dec 05
153 and Dec 06). Minimum expressions were from Apr 06 (0.85 ETA) to Apr 07 (0.86 ETA) with no
154 statistically significantly difference. Similarly, no statistically significantly difference in *mytilin*
155 *B* expression were recorded from May 07 (0.57 ETA) to Jul 08 (0.71 ETA), i.e. during 15
156 consecutive months, with p=0.41 between Dec 07 and May 08, for instance. Meanwhile,
157 expression in Dec 07 was 2.2-fold (p=0.0019) higher from expression in Dec 06.

158 Lower expression of *myticin B* was recorded during the winter times (Nov 05 and Jan 06,
159 Dec 06 and Feb 07, Nov 07 and Jan 08), with no significant difference between Jan 06 (19.42
160 ETA) and Feb 07 (14.28 ETA, p=0.23) or between Feb 07 and Jan 08 (34.26 ETA, p=0.09).
161 Higher expressions were recorded during Dec 05 (151.67 ETA), Apr 06 (127.49 ETA), Oct 07
162 (124.24 ETA) and more consistent between Feb 08 (133.35 ETA) and Jul 08 (250.42 ETA).

163 *Lysozyme* underwent clear seasonal variations of expression with maximum in Oct 05,
164 Apr-Aug 06, May 07, Jul-Aug 07, Oct 07 and Apr-Jul 08, with no significant differences
165 between the years, with p=0.41 between May 06 (10.17 ETA) and May 07 (7.78 ETA), and
166 p=0.54 between May 07 and May 08 (9.56 ETA). Such higher expressions were statistically
167 different from expressions in winter 06: p=0.004 between May 06 and Feb 06 (3.00 ETA),
168 winter 07: p=0.029 between May 07 and Feb 07 (1.33 ETA), and winter 08: p=0.021 between
169 May 08 and Mar 08 (4.38 ETA), for instance. Exception was for Oct 05 with the highest
170 *lysozyme* expression recorded: 15.18 ETA.

171

172 *3.3. Expression of* HSP70

173 Higher values for *HSP70* expression were measured in Dec 05 (31.80 ETA), Jan 06
174 (30.88 ETA), Aug 07 (25.24 ETA), Dec 07 (22.34 ETA) and Jul 08 (26.38 ETA), with
175 statistically significantly difference with low expression generally recorded in summer (Fig. 2).

176 In addition to Aug 05 (6.51 ETA), low expressions were recorded constantly from Feb 06 (8.39
177 ETA) to Apr 07 (5.04 ETA).
178
179 *3.4. Environmental parameters*
180 Seawater temperature measured the day of mussel collection underwent expected
181 variations ranged from 9.2°C (Jan 06) to 23°C (Jul 07) with normal Mediterranean seasonal
182 rhythm (Fig. 2, upper panel). Salinity was simultaneously measured, revealing 4 low salinity
183 periods corresponding to heavy rainfalls in Sep 05 (24.2 g/l), Sep-Dec 06 (about 30 g/l), May 07
184 (27.5 g/l) and Jun 08 (21.3 g/l). The number of *E. coli* measured in 100 g of mussel tissues was
185 remarkably low (130-250 bacteria) and constant during summer 06 and the full year 07 until Jun
186 08, i.e. during 17 consecutive months. Four abnormally elevated contents were recorded in Sep-
187 Nov 05 (7,100-3,200 bacteria), Feb 06 (4,100 bacteria), Nov 06 (4,500 bacteria) and Jun 08
188 (8,600 bacteria).
189
190 *3.5. Correlations between gene expressions and environmental parameters*
191 *3.5.1. Descriptive statistics*. Six PCA have been performed to represent each gene
192 expression according to salinity, temperature and *E. coli* tissue content. In each case, only the
193 representation of variables on the main plan constructed by factorial axis 1 and 2, has been
194 analyzed because representing 70-80 % of total inertia. On the first axe and for all analysis,
195 negative correlation has been observed between salinity and *E. coli* tissue content. On the second
196 axe, correlation existed between the expression of each gene and the temperature, with the
197 exception of *HSP70*. The correlation was positive for *28S rDNA*, *defensin, myticin* and *lysozyme*
198 expression and negative for *mytilin* expression (Fig. 3).
199 *3.5.2. Multiple regression models.* Fisher's test for each model concerning global nullity
200 of coefficients was significant ($p<0.05$, Ho rejected) only for *defensin* and *lysozyme* expression,
201 revealing significant influence of environmental parameters on the expression of these 2 genes.
202 - Defensin model: Student's test was significant ($p<0.05$, Ho rejected) revealing significant
203 influence of temperature and salinity on *defensin* expression. Meanwhile, these relationships
204 cannot be quantified because normality of residuals was rejected ($p<0.05$) and the model could
205 not be validated.
206 - Lysozyme model: Student's test was significant ($p<0.05$, Ho rejected) revealing significant
207 influence of temperature on *lysozyme* expression. Meanwhile, as for defensin model, this
208 relationship cannot be quantified because normality of residuals was rejected ($p<0.05$) and the
209 model could not be validated.
210

211 **4. Discussion**

212

213 The main criterion to decide for a house keeping gene is its constant expression all along
214 the experiment. In previous reports, we found *28S rDNA* a suitable house keeping gene to study
215 the variations of several immune-related genes following various treatments [21, 22]. Such
216 treatments were not statistically significantly affecting the expression of *28S rDNA*. During the
217 course of the present multi annual survey, we also focused on *28S rDNA* for Q-PCR calculations.
218 It was obvious that *28S rDNA* expression was not constant all along the year, but followed some
219 rhythms, with lower expression Aug 05-Jan 06 and Apr 07-Aug 07, and higher expression Jun
220 06-Mar 07. One can speculate that such differences in expression must be related to the level of
221 mussel metabolism which is known to fluctuate according to season [27] and reproduction [28].

222 The calculation mode based on the *ratio* of ETA for the considered gene on ETA for *28S*
223 *rDNA*, compensated the variations in the house keeping gene expression. *Defensin* expressed in
224 summer, from Apr to Oct, and was nearly undetectable in winter, from Dec to Mar, which is
225 contradictory to our previous observations done in Northern [7]. *Mytilin B* expression appeared
226 constant with a low level from Apr 06 to Apr 07 and slightly higher values from May 07 to Jul
227 08. Exception was during winter 05 with significantly up-regulation of *mytilin B* expression.
228 Highest expression of *myticin B* and *lysozyme* occurred in summer whereas *HSP70* expression
229 appeared more erratic with maxima in Dec 05, Aug 07, Dec 07 and Jul 08. Consequently, the
230 five genes behave differently according to the season, but they all underwent almost a year of
231 low expression, starting Feb-May 06, particularly obvious for *mytilin B* and *HSP70*. We must
232 notice the deviant comportments during the winter 05-06 with the highest expression of *mytilin*
233 *B, lysozyme* and *HSP70*, the expression of the later being reported as showing significant
234 correlation with the seawater temperature [29].

235 Highest *E. coli* tissue content of mussel tissues, reflecting human faecal contamination
236 not correctly eliminated by public sewage systems, were observed at the time of lowest salinity
237 corresponding to heavy rainfalls during Sep-Nov 05, Nov 06 and Jun 08. Meanwhile, other low
238 salinity values (Apr 06, Sep 06 and May 07) were not associated with high *E. coli* tissue content.
239 Reciprocally, high *E. coli* tissue content measured in Feb 06 was not related to low salinity,
240 suggesting either not a strict relationship between the 2 phenomena or, most probably, a rapid
241 clearance of *E. coli* from the mussel tissues, as we observed for *Vibrio splendidus, V.*
242 *anguillarum* and *Micrococcus lysodeikticus* [30]. No particular relationship has been observed
243 between *E. coli* tissue content and seawater temperature, obviously more elevated in summer,
244 with a maximum of 23°C, than in winter, with a minimum of 8.5°C. When compared to the
245 constant *E. coli* tissue content during 07 and early 08, the up-regulations of gene expressions

246 observed in summer cannot be correlated to the reaction against more numerous bacteria.
247 Moreover, the two highest *E. coli* tissue content observed in Feb 06 and in Nov 06 corresponded
248 to the lowest expressions of *defensin*, *myticin B* and *lysozyme*, rejecting the hypothesis of
249 correlation between expression of these immune gene and the presence of bacteria. Highest
250 concentration of *E. coli* tissue content in Jun 08, not correlated to particularly high immune gene
251 expression, confirmed the above assertion. Meanwhile, both *lysozyme* and *mytilin B* were up-
252 regulated at the end of 2005 at the time salinity was increasing and *E. coli* tissue content
253 elevated, but decreasing. But such relationships were less obvious in Jun 08, and not confirmed
254 at the end of 2006. Statistical studies with PCA and multiple regression models revealed positive
255 influence of temperature on *28S rDNA*, *defensin*, *myticin B* and *lysozyme* expressions, but
256 negative influence on *mytilin B* expression. The positive influence was significant for *defensin*
257 and *lysozyme* expression, but relationships cannot be quantified. Similarly, salinity appeared to
258 influence *defensin* expression, but this relationship cannot be quantified neither.
259 General observations in *M. galloprovincialis* were on the highest concentrations/activities
260 during summer times, with correlations to highest water temperature: hemolymph protein
261 concentration, lysozyme and agglutinin activities [10], total hemocyte count (THC) [9], NO
262 production [14], and HSP70 and multi xenobiotic resistance (MXR) protein accumulations [31],
263 for instance. However, intensity of cytolytic activity correlated to water temperature, but
264 experiments in aquaria demonstrated this parameter is not the main cause of the fluctuation [13].
265 Also in *M. edulis*, activities such are glutathione (GSH), GHS-peroxidase and catalase were the
266 highest in summer [8]. Other observations in *M. galloprovincialis* were on higher prevalence of
267 gonad neoplasm between Apr and Jun [32] and on higher percentage of inflammatory lesions
268 caused by parasites in summer than in winter [27], exactly as reported for the Japanese pearl
269 oyster, *Pinctada fucata*, infected by the marine birnavirus (MABV) [33]. In contrast, greater
270 concentrations of *HSP70* in winter in the horse mussel, *Modiolus modiolus*, suggested
271 adjustment of such chaperone functions to cold temperature (5° versus 15°C) [34]. Correlation
272 between maxima seawater temperatures and occurrence of mortalities was proposed concerning
273 the European abalone, *Haliotis tuberculata* (Mollusk, Gastropod) [35] due to emerging vibriosis
274 [36]. In addition, diseases are known to be linked to reproductive cycle because reproduction is a
275 stressful event using energy that would otherwise be available for other functions. Such
276 relationships between spawning and mortality have been reported in oyster [37], mussels [28],
277 abalone [38] and Manila clam *Ruditapes philippinarum* [39]. One can expect the immune gene
278 expressions to be down-regulated during gonad maturation, lasting until spawning. In the present
279 report, up-regulation of *mytilin B* and *HSP70* was observed during the first reproductive period
280 of Dec 05-Jan 06, but not confirmed during the second reproductive period Sep-Oct 06, and

281 during the following years. Consequently, relationships between environmental parameters and
282 immune-related gene expression appeared not simple, demonstrating anti-infectious capabilities
283 cannot be evaluated using only such genes as markers.

284 In conclusion, different levels of expression have been observed for several immune
285 genes along the 3-years survey, with some seasonal variability. Statistical studies revealed
286 positive influence of temperature on *28S rDNA*, *defensin*, *myticin B* and *lysozyme* expressions,
287 but not on *mytilin B* and *HSP70*. Salinity positively influenced *defensin* expression only, whereas
288 *E. coli* tissue content appeared without influence.

289

290 **Acknowledgements**

291

292 HL and MT were fully supported by the EU programme Imaquanim (FOOD-CT-2005-
293 007103). Data from REMI and REPHY networks were collected with the help of Isabelle
294 Amouroux and Catherine Belin from Ifremer-Nantes (France).

295

296 **References**

297

298 [1] Pipe RK, Coles JA. Environmental contaminants influencing immune function in marine
299 bivalve molluscs. Fish Shellfish Immunol 1995;5:581-95.
300 [2] Dyrynda EA, Pipe RK, Burt G, Ratcliffe N. Modulations in the immune defences of
301 mussels (Mytilus edulis) from contaminated sites in the UK. Aquat Toxicol 1998;42:169-
302 85.
303 [3] Canesi L, Ciacci C, Betti M, Scarpato A, Citterio B, Pruzzo C, et al. Effects of PCB
304 congeners on the immune function of Mytilus hemocytes: alterations of tyrosine kinase-
305 mediated cell signaling. Aquat Toxicol 2003;63:293-306.
306 [4] Auffret M, Rousseau S, Boutet I, Tanguy A, Baron J, Moraga D, et al. A multiparametric
307 approach for monitoring immunotoxic responses in mussels from contaminated sites in
308 Western Mediterranea. Ecotoxicol Environ Saf 2006;63:393-405.
309 [5] Pruzzo C, Gallo G, Canesi L. Persistence of vibrios in marine bivalves: the role of
310 interactions with hemolymph components. Environ Microbiol 2005;7:761-72.
311 [6] Ordas M, Novoa B, Figueras A. Modulation of chemiluminescence response of
312 Mediterranean mussel (Mytilus galloprovincialis) hemocytes. Fish Shellfish Immunol
313 2000;10:611-22.
314 [7] Mitta G, Hubert F, Dyrynda EA, Boudry P, Roch P. Mytilin B and MGD2, two
315 antimicrobial peptides of marine mussels: gene structure and expression analysis. Dev
316 Comp Immunol 2000;24:381-93.
317 [8] Sheehan D, Power A. Effects of seasonality on xenobiotic and antioxidant defence
318 mechanisms of bivalve molluscs. Comp Biochem Physiol C Pharmacol Toxicol
319 Endocrinol 1999;123:193-9.
320 [9] Carballal MJ, Villalba A, Lopez C. Seasonal variation and effects of age, food
321 availability, size, gonadal development, and parasitism on the hemogram of Mytilus
322 galloprovincialis. J Invertebr Pathol 1998;72:304-12.

323 [10] Santarém MM, Robledo JAF, Figueras A. Seasonal changes in hemocytes and serum
324 defense factors in the blue mussel Mytilus galloprovincialis. Dis Aquat Organ
325 1994;18:217-22.
326 [11] Hernroth B. The influence of temperature and dose on antibacterial peptide response
327 against lipopolysaccharide in the blue mussel, Mytilus edulis. Fish Shellfish Immunol
328 2003;14:25-37.
329 [12] Saito H. Lipid and FA composition of the pearl oyster Pinctada fucata martensii:
330 influence of season and maturation. Lipids 2004;39:997-1005.
331 [13] Malagoli D, Casarini L, Ottaviani E. Monitoring of the immune efficiency of Mytilus
332 galloprovincialis in Adriatic Sea mussel farms in 2006: regular changes in cytotoxicity
333 during the year. Invertebr Survey J 2007;4:10-2.
334 [14] Novas A, Barcia R, Ramos-Martinez JI. Nitric oxide production by haemocytes from
335 Mytilus galloprovincialis shows seasonal variations. Fish Shellfish Immunol
336 2007;23:886-91.
337 [15] Tremblay R, Myrand B, Sevigny J-M, Blier P. Bioenergetic and genetic parameters in
338 relation to susceptibility of blue mussel, Mytilus edulis (L.) to summer mortality. J Exp
339 Mar Biol Ecol 1998;221:27-58.
340 [16] Myrand B, Gaudreault J. Summer mortality of blue mussels (Mytilus edulis L.) in the
341 Magdalen Islands (southern Gulf of St. Lawrence, Canada). J Shellfish Res 1995;14:395-
342 404.
343 [17] Lacoste A, Jalabert F, Malham S, Cueff A, Gelebart F, Cordevant C, et al. A Vibrio
344 splendidus strain is associated with summer mortality of juvenile oysters Crassostrea
345 gigas in the Bay of Morlaix (North Brittany, France). Dis Aquat Organ 2001;46:139-45.
346 [18] Friedman CS, Estes RM, Stokes NA, Burge CA, Hargove JS, Barber BJ, et al. Herpes
347 virus in juvenile Pacific oysters Crassostrea gigas from Tomales Bay, California,
348 coincides with summer mortality episodes. Dis Aquat Organ 2005;63:33-41.
349 [19] Garnier M, Labreuche Y, Garcia C, Robert M, Nicolas JL. Evidence for the involvement
350 of pathogenic bacteria in summer mortalities of the Pacific oyster Crassostrea gigas.
351 Microb Ecol 2007;53:187-96.
352 [20] Roch P. Behaviour of defense peptides in environmentally stressed mussels. Revue
353 Médicale Vétérinaire 2001;153:517-20.
354 [21] Cellura C, Toubiana M, Parrinello N, Roch P. Specific expression of antimicrobial
355 peptide and HSP70 genes in response to heat-shock and several bacterial challenges in
356 mussels. Fish Shellfish Immunol 2007;22:340-50.
357 [22] Li H, Parisi MG, Toubiana M, Cammarata M, Roch P. Lysozyme gene expression and
358 hemocyte behaviour in the Mediterranean mussel, Mytilus galloprovincialis, after
359 injection of various bacteria or temperature stresses. Fish Shellfish Immunol
360 2008;25:143-52.
361 [23] Cellura C, Toubiana M, Parrinello N, Roch P. HSP70 gene expression in Mytilus
362 galloprovincialis hemocytes is triggered by moderate heat shock and Vibrio anguillarum,
363 but not by V. splendidus or Micrococcus lysodeikticus. Dev Comp Immunol
364 2006;30:984-97.
365 [24] Adams MR, Grubb SM, Hamer A, Clifford MN. Colorimetric enumeration of
366 Escherichia coli based on beta-glucuronidase activity. Appl Environ Microbiol
367 1990;56:2021-4.
368 [25] Dray S, Dufour AB. The ade4 package: implementing the duality diagram for ecologists.
369 Journal of Statistical Software 2007;22:1-20.
370 [26] Thioulouse J, Dray S. ade4TkGUI: ade4 Tcl/Tk graphical user interface. R package
371 version 0.2-3. http://pbiluniv-lyon1fr/ade4TkGUI 2008:Mailing list http://listes.univ-
372 lyon1.fr/wws/info/adelist.
373 [27] Bodin N, Burgeot T, Stanisiere JY, Bocquene G, Menard D, Minier C, et al. Seasonal
374 variations of a battery of biomarkers and physiological indices for the mussel Mytilus

375 galloprovincialis transplanted into the northwest Mediterranean Sea. Comp Biochem
376 Physiol C Toxicol Pharmacol 2004;138:411-27.
377 [28] Myrand B, Guderley H, Himmelman J. Reproduction and summer mortality of blue
378 mussels Mytilus edulis in the Magdalen Islands, southern Gulf of St. Lawrence. Marine
379 Ecology Progress Series 2000;197:193-207.
380 [29] Hamer B, Hamer DP, Muller WE, Batel R. Stress-70 proteins in marine mussel Mytilus
381 galloprovincialis as biomarkers of environmental pollution: a field study. Environ Int
382 2004;30:873-82.
383 [30] Parisi MG, Li H, Jouvet LBP, Dyrynda EA, Parrinello N, Cammarata M, et al.
384 Differential involvement of mussel hemocyte sub-populations in the clearance of
385 bacteria. Fish Shellfish Immunol 2008.
386 [31] Minier C, Borghi VV, Moore MN, Porte C. Seasonal variation of MXR and stress
387 proteins in the common mussel, Mytilus galloprovincialis. Aquat Toxicol 2000;50:167-
388 76.
389 [32] Alonso A, Suarez P, Alvarez C, San Juan F, Molist P. Structural study of a possible
390 neoplasia detected in Mytilus galloprovincialis collected from the Ria of Vigo (NW
391 Spain). Dis Aquat Organ 2001;47:73-9.
392 [33] Kitamura S, Jung S, Suzuki S. Seasonal change of infective state of marine birnavirus in
393 Japanese pearl oyster Pinctada fucata. Arch Virol 2000;145:2003-14.
394 [34] Lesser MP, Kruse VA. Seasonal temperature compensation in the horse mussel,
395 Modiolus modiolus: metabolic enzymes, oxidative stress and heat shock proteins. Comp
396 Biochem Physiol A Mol Integr Physiol 2004;137:495-504.
397 [35] Huchette S, Clavier J. Status of the ormer (Haliotis tuberculata) industry in Europe.
398 Journal of Shellfish Research 2004;23:951-5.
399 [36] Travers MA, Basuyaux O, Le Goic N, Huchette S, Nicolas JL, Koken M, et al. Influence
400 of temperature and spawning effort on Haliotis tuberculata mortalities caused by Vibrio
401 Harveyi: an example of emerging vibriosis linked to global warming. Global Change
402 Biology 2008;doi:10/1111/j.1365-2486.2008.01764.x.
403 [37] Perdue J, Beattie J, Chew K. Some relationships between gametogenetic cycle and
404 summer mortality phenomenon in the Pacific oyster, C. gigas in Washington State.
405 Journal of Shellfish Research 1981;1:9-16.
406 [38] Nicolas JL, Basuyaux O, Mazurie J, Thebault A. Vibrio carchariae, a pathogen of the
407 abalone Haliotis tuberculata. Dis Aquat Organ 2002;50:35-43.
408 [39] Ngo TTT, Choi K. Seasonal changes of Perkinsus and Cercaria infections in the Manila
409 clam Ruditapes philippinarum from Jeju, Korea. Aquaculture 2004;239:57-68.
410

411 **Figure captions**

412

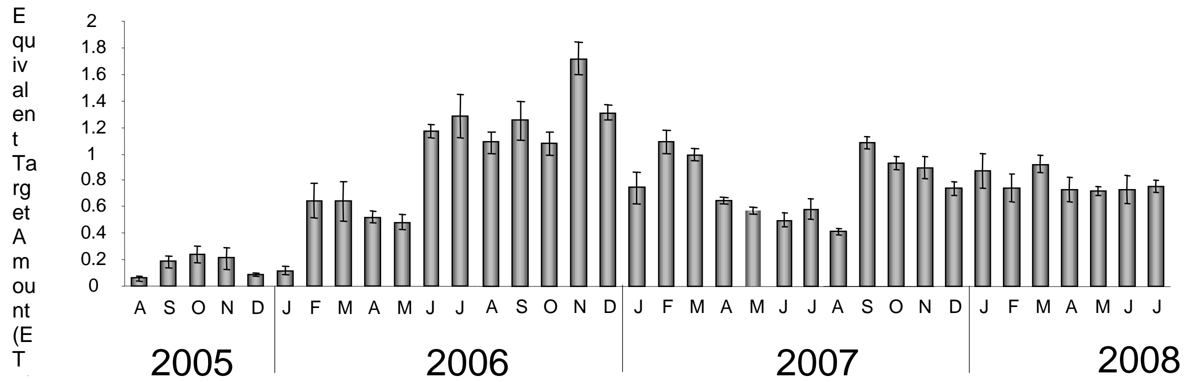
413 Figure 1 – Expression of *28S rDNA* as measured in Q-PCR from samples monthly collected from
414 August 05 to July 08. Values were inferred from 4 replicates measured in duplicate ± SEM (bar).
415 See the text for statistical analysis. Note the lowest expression Aug 05-Jan 06 and the highest
416 expressions Jun 06-Mar 07, then Sep 07-Jul 08.

417

418 Figure 2 - Salinity and temperature of seawater, and *E. coli* tissue content of mussels. Expression
419 of *HSP70*, *defensin, mytilin B, myticin B* and *lysozyme* as measured in Q-PCR from samples
420 monthly collected from August 05 to July 08. Values were inferred from 4 replicates measured
421 in duplicate ± SEM (bar). See the text for some statistical analysis. Note the 4 lowest salinities
422 corresponding to heavy rainfalls in Sep 05, Sep-Dec 06, May 07 and Jun 08, and the 4 peaks of
423 *E. coli* tissue content in Sep 05, Feb 06, Nov 06 and Jun 08. Note the existence of some
424 modulations of gene expressions, not identical between the five genes, and the global lower
425 expressions during winters 06 and 07.

426

427 Figure 3 – Representation of variables on the main plan by correlation circles related to *defensin*
428 (A) and to *lysozyme* (B). Note the negative correlations on axe 1 between salinity (SAL) and *E.*
429 *coli* tissue content (ECOLI) in both cases. Note also the positive correlations on axe 2 between
430 *defensin* (DEF) and temperature (TEMP)(A) and between *lysozyme* (LYS) and temperature (B).
431
432
433
434
435
436
437 Figure 1



438
439
440
441
442
443

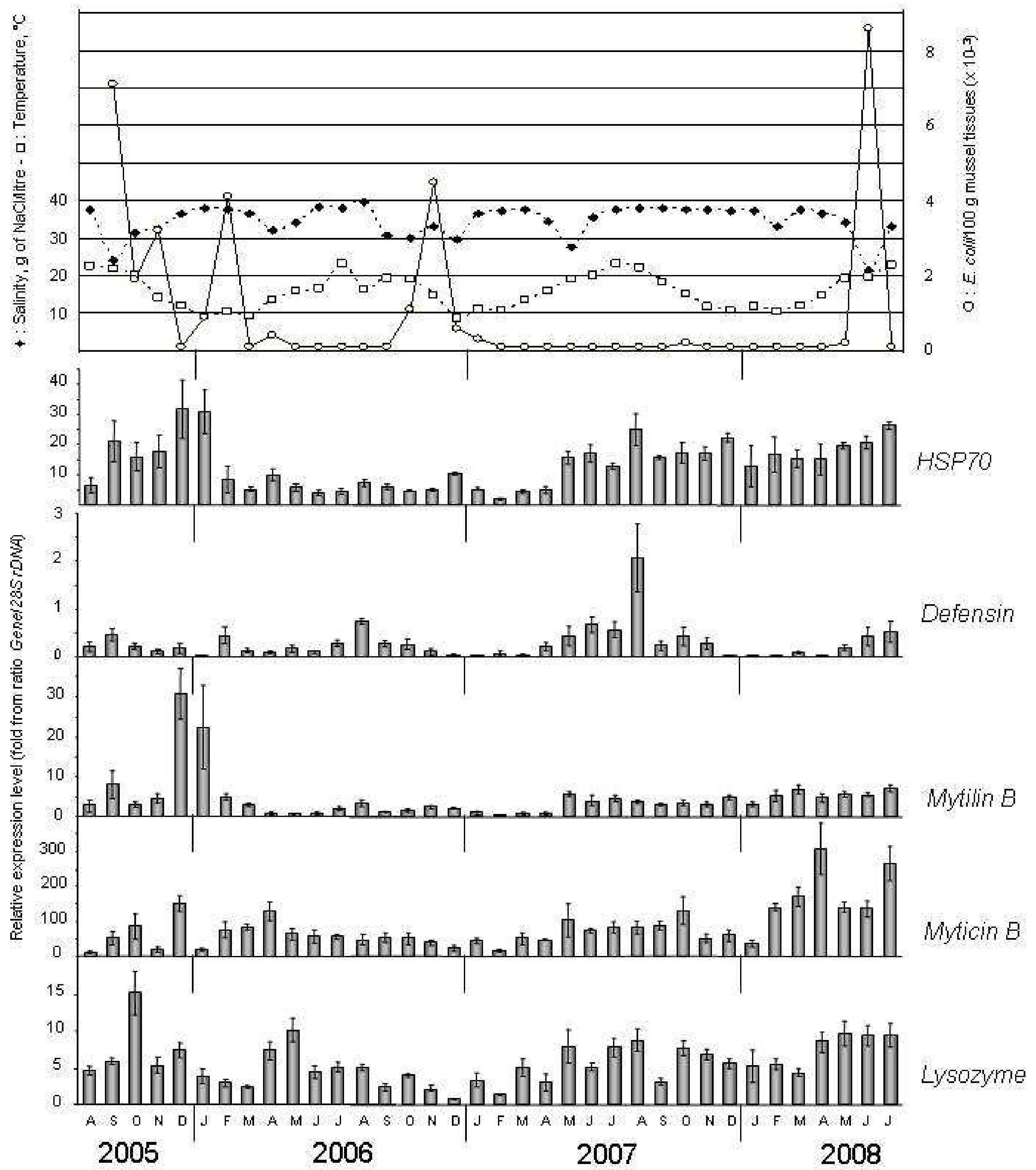
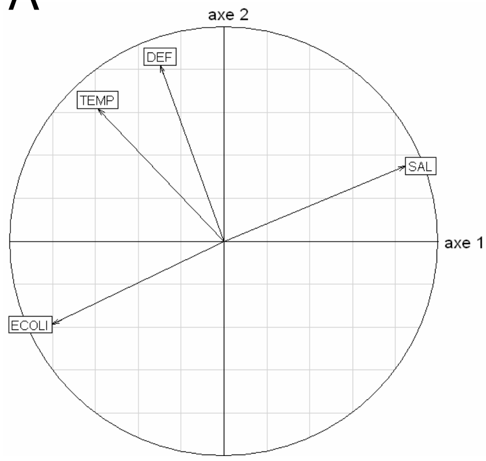
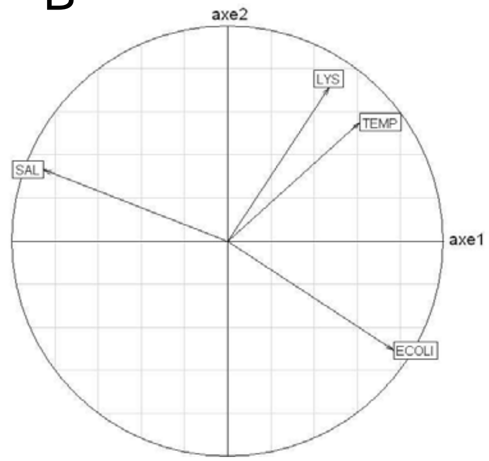


Figure 2

444
445
446
447
448
449
450
451

452 Figure 3





453
454
455
456