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Comportements cellulaires et régulation génétique au cours des réactions d'immunité innée chez la moule *Mytilus galloprovincialis*

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A ma famille. A Yan.

A tous ceux qui m’ont soutenu et accompagné.

献给我的家人与我唯一的爱人

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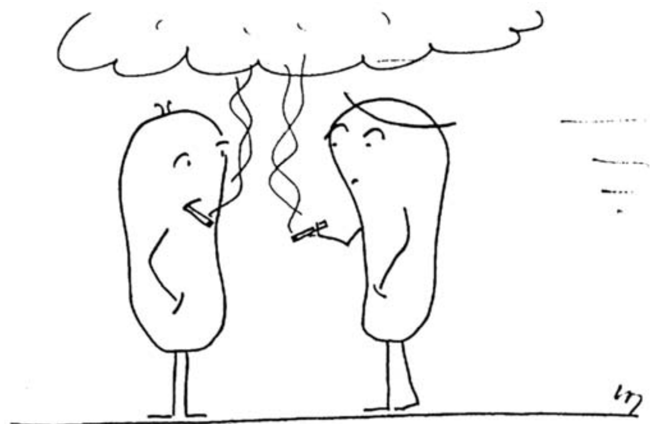
Introduction



(D’après « Funny Microbes », Leos Mandel)

Introduction

A. Historique de l'aquaculture

L'aquaculture est le terme générique qui désigne les activités de production animale ou végétale réalisées en milieu aquatique. L'aquaculture se pratique en bord de mer, dans les rivières, les lacs ou les étangs. Elle concerne la production de poisson (on parle alors de pisciculture), de coquillages ou conchyliculture avec les huîtres (ostréiculture) et les moules (mytiliculture), de crustacés (crabes, crevettes ou crevetticulture) ou encore d'algues (algoculture). L'aquaculture fournit actuellement 92 % de la consommation d'algues marines, 60 % des poissons d'eau douce, 40 % des mollusques, 30 % des crevettes et 5 % des poissons de mer. Sa production est en forte augmentation.

Le plus ancien ouvrage concernant l'aquaculture aurait été rédigé en Chine il y a 2500 ans par Fan Li et s'intitule « L'élevage des poissons de Tao Zhu Gong ». Ce traité est un ouvrage exceptionnel qui montre le niveau de technicité atteint par l'élevage des poissons en Chine au 5ième siècle av-JC. Il constitue un résumé des expériences accumulées sur l'élevage des carpes dans les étangs (Billard, 2000). En fait, on a découvert que des étangs étaient utilisés dès l'époque préhistoriques pour la stabulation et le maintien des animaux aquatiques à Hawaï (Iversen, 1976). En 1400 av-JC, il existait déjà des lois pour protéger les pisciculteurs des voleurs dans la région Indo-Pacifique. En Europe, Aristote mentionne la culture des huîtres en Grèce dès le 4ième siècle av-JC et, à la même époque, Pline donne une description de l'ostréiculture à Rome (Milne, 1972).

Jusqu'au 19ème siècle, la seule forme de mytiliculture connue en Europe était l'élevage sur « bouchots », réalisé en France depuis le 13ème siècle, mais dans une zone limitée. Partout ailleurs on se contentait d'exploiter les gisements naturels. Dans le courant du 19ième siècle, en même temps que les bouchots prenaient de l'extension sur le littoral français, deux nouveaux modes de culture ont apparu : la culture à plat, qui a commencé à être pratiquée aux Pays-Bas vers 1860, et la culture en suspension, qui a débuté en Espagne en 1846, mais ne devint vraiment importante qu'un siècle plus tard (Marteil, 1979).

A l'heure actuelle, on retrouve ces trois modes de culture en France. La culture sur bouchots est la plus répandue : on la trouve sur les côtes de l'Atlantique et de la Manche. L'élevage en suspension, important lui aussi, existe principalement en Méditerranée. La

culture à plat est moins pratiquée par rapport aux deux premières, à cause de son exigence en surface de terrain (Bompais, 1991; Marteil, 1979). Rappelons que les moules cultivées sur les côtes nord de l'Atlantique et de la Manche appartiennent à l'espèce *Mytilus edulis*, tandis qu'en Méditerranée, sur les côtes du Portugal et du sud de l''Espagne, c'est *Mytilus galloprovincialis* qui est exploitée (Fig. 1). Il existe aussi une importante zone hybride entre ces deux espèces de moules (Gosling, 1992).

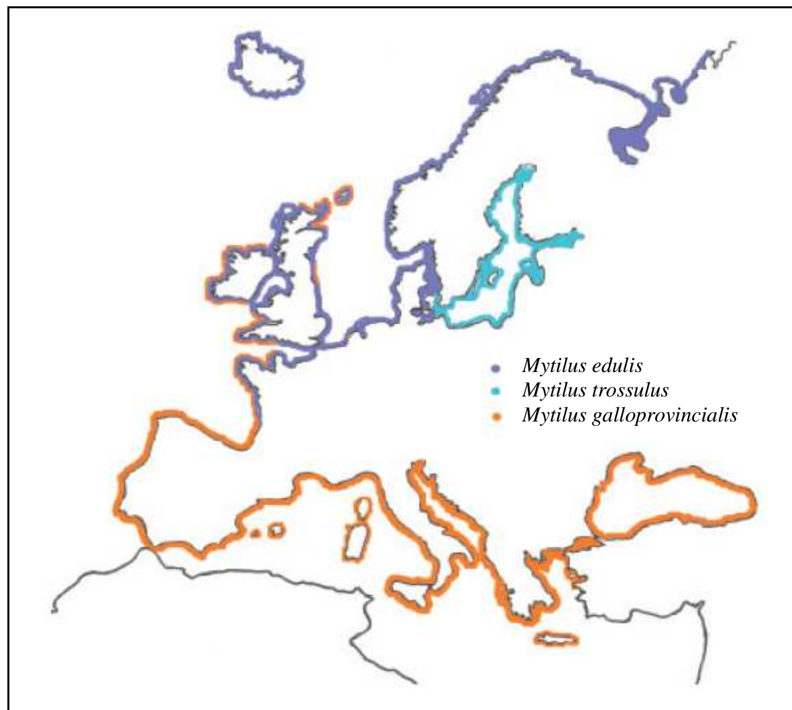


Figure 1 - Distribution géographique des espèces de *Mytilus* en Europe.

(i) La culture sur bouchots (Fig. 2) : mot d'origine celtique venant de *bout*, clôture, et *choat* ou *chot*, en bois. En 1855, seules les vases situées au sud de la rivière Sèvre Niortaise, au nord de La Rochelle, étaient exploitées. Peu à peu, les bouchots ont envahi la côte vers le nord et ont commencé à apparaître après 1860 dans d'autres régions: d'abord au sud de La Rochelle, puis en Bretagne et en Normandie (Marteil, 1979). A l'heure actuelle, la longueur totale de bouchots concédée en France excède 1.500 km, dont plus du tiers dans la seule région de La Rochelle (source Ageste 2001). Ils sont généralement établis sur des terrains dont l'élévation est comprise entre 0,70 m et 1 m par rapport au zéro des cartes.

L'histoire attribue l'origine des bouchots à un irlandais qui aurait fait naufrage en 1235 dans l'Anse de l'Aiguillon. Seul rescapé de cette aventure, Patrick Walton s'est installé à Esnandes où il a entrepris de capturer des oiseaux à l'aide d'un filet particulier, le filet « d'allouret ». Ce filet était tendu au-dessus du niveau de la pleine mer entre de grands piquets

enfoncés dans la vase. Walton s'est vite aperçu que les piquets se recouvraient de moules dont la croissance et la qualité étaient supérieures à celles des moules sauvages. Il a alors décidé d'exploiter cette propriété et il a organisé les premiers bouchots. Aujourd'hui, la culture sur bouchot se pratique sur une ligne de pieux plantés dans le sol. Les moules se fixent sur ceux qui sont situés le plus au large, dit « bouchots à naissain ». Au fur et à mesure de leur croissance, les petites moules sont transportées sur les pieux plantés plus près de la côte.

Figure 2 - La culture sur bouchots (Bretagne-France).

(ii) L'élevage en suspension : le naissain est récolté sur des cordes, dites « cordes de captage », ou péché directement sur des gisements naturels. Il est installé sur des « cordes d'élevage » qui pendent dans l'eau et sont donc constamment immergées. Il existe deux types d'installations (Bompais, 1991; Marteil, 1979) : **(a) les installations fixes** (Fig. 3), qui exigent des eaux peu profondes ne dépassant pas 10 m et une amplitude de marée faible. Cette sorte d'installation n'est donc retrouvée en France qu'en Méditerranée ; **(b) les installations flottantes** (Fig. 4), en revanche, ont l'avantage de pouvoir être employées indifféremment dans des zones où l'amplitude des marées est faible ou importante. Il faut noter que l'Etang de

Thau, qui est le principal centre français utilisant l'élevage en suspension, produit environ le quart de la production mytilicole française (source Ageste 2001).

Figure 3 - La culture en suspension sur une installation fixe (Etang du Prévost-France).

Figure 4 - La culture en suspension sur une installation flottante ou « batea » (Rias de Galicia-Espagne).

(iii) la culture à plat (Fig. 5): le naissain se fixe souvent en abondance dans des zones où la survie, la croissance et le grossissement sont faibles. Les jeunes moules sont pêchées sur ces gisements naturels et transférées dans des endroits où les conditions du milieu sont plus favorables. Pour améliorer encore le rendement, on veille à ce que la densité des mollusques sur le terrain ne soit pas excessive, et les prédateurs contrôlés. Le grand avantage de cette culture est qu'elle ne nécessite pas d'installation particulière, néanmoins, cette technique n'a

pas connu un grand succès en France du fait de son emprise territoriale (Bompais, 1991; Marteil, 1979).

Figure 5 - La culture à plat (Bretagne-France).

En 2000, la France se plaçait au 3ème rang en Europe pour la production de moules avec 73.000 t, loin derrière l'Espagne (250.000 t) et les Pays-Bas (100.000 t) (source IFREMER 2001). Comme on peut le constater sur la Figure 6, 85 % des moules consommées aujourd'hui proviennent de la mytiliculture.

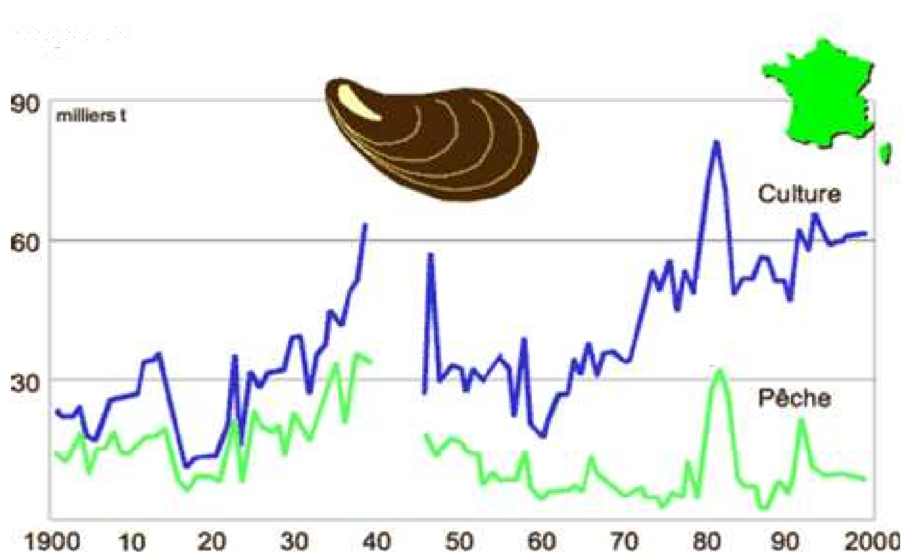


Figure 6 – L'évolution de la production des moules en France.

B. Production des bivalves et maladies

La mytiliculture française a connu une expansion importante depuis les années 80. La conséquence directe de l'intensification des cultures a été le développement rapide de diverses maladies infectieuses : bactérioses, viroses et surtout maladies liées à des protozoaires, qui ont probablement été à l'origine de pertes économiques importantes. Afin de mieux comprendre les raisons de ces pertes, il est nécessaire d'approfondir nos connaissances en pathologie, génétique et surtout sur le fonctionnement du système immunitaire des moules.

Du simple fait de leur maintien depuis des milions d'années, on peut supposer que les bivalves sont capables d'atteindre un équilibre avec leur environnement. Leur système immunitaire maitrise naturellement bien les infections ou les invasions « naturelles ». Cependant, les animaux en élevage sont soumis à des conditions environnementales souvent perturbées, telles une forte densité des animaux, une dégradation du milieu, un déséquilibre nutritionnel ou une forte pollution. Dans de telles conditions, les animaux sont affaiblis et en particulier, leur système immunitaire est moins performant. Toutes ces conditions favorisent le développement de maladies infectieuses qui vont ensuite se répandre en créant une épidémie d'autant plus dévastatrice que la culture sera intensive. Parmi toutes les maladies rencontrées chez les bivalves cultivés, les protozoaires sont décrits comme étant les agents les plus agressifs (Tableau 1).

(i) Protozoaires : les protozoaires les plus connus qui infectent les bivalves sont *Bonamia*, *Marteilia* et *Haplosporidium*. Chez l'huître plate *Ostrea edulis*, les protozoaires *Marteilia refringens* et *Bonamia ostreae* ont causé une baisse importante de la production française dans les années 70 (Comps, 1970; Pichot et al., 1979). En Espagne, la présence de *M. refringens* a été signalée chez des moules *M. galloprovincialis* de la région de Galice sans faire état de mortalité (Villalba et al., 1993). Un autre protozoaire *Perkinsus marinus* est considéré comme le pathogène le plus important pour l'huître *Crassostrea virginica* et ce depuis les années 50 sur la côte Est des USA (Oliver et al., 1998). Un autre *Perkinsus*, *P. atlanticus* est capable d'infecter les palourdes *Tapes decussatus* (Casas et al., 2002). En ce qui concernant les espèces d'*Haplosporidium*, *C. virginica* est sensible aux infections dues à *H. nelsoni* et à *H. costale* (Ford and Haskin, 1987).

(ii) Virus : la mortalité massive de l'huître portugaise *Crassostrea angulata,* entre 1967 et 1973 en France, est associée à l'infection par un Iridovirus (Comps and Bonami, 1977;

Comps et al., 1976). Des virus appartenant à la famille des Herpes ont été identifiés chez les bivalves dans différents pays producteurs tels les USA (Friedman et al., 2005), la Nouvelle Zélande (Hine, 1996), la France (Arzul et al., 2001; Renault et al., 2001), l'Australie (Hine and Thorne, 1997) et le Mexique (Vasquez-Yeomans et al., 2004).

(iii) Bactéries : les infections bactériennes sont surtout détectées dans les productions de larves de bivalves. Différentes bactéries du genre *Pseudomonas* et *Aeromonas*, ont été identifiées lors de la mortalité de larves d'huîtres en écloserie. Les bactéries les plus présentes dans le milieu marin, les *Vibrios*, sont considérées comme des opportunistes associées souvent à la mortalité des larves et des juvéniles (Lambert et al., 2001). Cependant, une souche particulière de *Vibrio splendidus*, la souche LGP32, a été décrite comme associée à la mortalité estivale de *C. gigas* en France (Lacoste et al., 2001).

Etiology	Pathogenic agent	Disease	Host species	Economical incidence	Reference
Viral	*Iridoviridae*	Gill disease	*Crassostrea angulata*	Disappearance of oysters along the European coasts	Comps et al. (1976)
	Iridoviridae	Oyster velar virus disease	*Crassostrea gigas*	Severe mortalities in larvae batches in USA	Kinne (1983)
	Virus-like particles	Infectious pancreatic necrosis virus (IPNV)	*Pecten maximus*	High mortalities in spat and adults scallops in Norway	Mortensen et al. (1990, 1992)
	Herpesvirus		*Crassostrea gigas*	Sporadic mortalities in hatchery-reared larval oysters in France	Renault et al. (1994)
	Papovavirus		*Crassostrea gigas*	Punctual mortalities in Australian oysters	Munday and Owens (1998)
Procaryote	*Rickettsiae*		*Crassostrea virginica*		Lauckner (1983)
			Ostrea edulis		Lauckner (1983)
			Ruditapes decussatus		Mialhe et al. (1986)
			Ruditapes philippinarum	Responsible for mass mortalities of scallops in France	Paillard et al. (1989)
			Pecten maximus		Le Gall et al. (1991)
	Bacteria	Vibriosis	*Bivalve molluscan larvae*		
			Crassostrea gigas		
			Crassostrea virginica	Higher larvae mortalities in hatchery	Tubiash et al. (1970)
			Mercenaria mercenaria	Higher summer mortality	Lodeiros et al. (1987)
		Brown ring disease	*Ostrea edulis*	Mortalities in cultured bivalves	Elston et al. (1982)
				Higher mortality in natural and reared clams in Europe (France, Spain, Italy)	Friedman et al. (1991)
					Paillard et al. (1994)
	Nocadia sp.	Nocardiosis	*Ruditapes philippinarum*		
			Ruditapes decussatus	Mortalities in adult oysters in USA	Friedman et al. (1988)
	Chlamidia sp.				
			Crassostrea gigas	Suspected to cause mortalities in bivalves	Comps et al. (1980)
					Cajaraville and Angulo (1991)
	Fungus	Fotsyke or shell disease	*Ruditapes decussates*	Unknown punctual mortality	
			Mytilus galloprovincialis		Davis et al. (1954)
			Ruditapes decussatus		Alderman and Gareth Jones (1971)
			Ostrea edulis		
Protozoan	*Bonamia ostreae*	Bonamiasis	*Ostrea edulis*	Higher mortality in natural and reared flat oysters in Europe and USA	Grizel (1985)
	Bonamia sp.	Dermo-disease	*Crassostrea gigas*	Associated with oysters mortality in USA	Perkins (1976)
	Perkinsus marinus	Digestive gland disease	*Crassostrea virginica*	Associated with mortalities in Portugal and Spain	Volety and Chu (1995)
	Perkinsus atlanticus	MSX-disease	*Ruditapes decussatus*	Associated with some mortalities in the Atlantic European coast	Comps and Chagot (1987)
	Marteilia refringens		*Ostrea edulis*	Dramatic mortalities in USA	Laukner (1983)
	Marteilia sp.		*Mytilus galloprovincialis*	Severe mortalities in USA	Villalba et al. (1993)
	Halosporidium nelsoni		*Crassostrea virginica*	Severe mortalities in Australia	Ford (1986)
	Mikrocytos mackini		*Crassostrea gigas*		Farley et al. (1988)
	Mikrocytos roughleyi		*Saccostrea commercialis*		Farley et al. (1988)
Metazoan	*Myticola intestinalis*	Digestive tract disease	*Mytilus edulis*	Associated with some mortalities	Lauckner (1983)
	Myticola orientalis		*Mytilus gallorprovincialis*	Unknown	Lauckner (1983)
			Crassostrea gigas		
			Mytilus edulis		

Tableau 1- Principaux agents pathogènes et maladies des bivalves en Europe et aux USA.
(Gestal et al., 2008)

C. Plan de la thèse

Au cours de ce travail, nous nous sommes concentrés sur la moule méditerranéenne *M. galloprovincialis*, une espèce à la fois économiquement et écologiquement importante. Outre l'étude détaillée de quelques manifestations de l'immunité innée, l'objectif principal de ce travail a consisté à rechercher si le milieu de vie des animaux a un effet sur leurs capacités immunitaires. Pour cela, nous considérerons les moules provenant de trois zones géographiques en Europe, l'océan Atlantique avec la Ria de Vigo en Espagne, le Golfe du Lion avec l'Etang du Prévost en France et le Lagune de Venise en Italie (Fig. 7), confrontées expérimentalement aux mêmes stimuli.

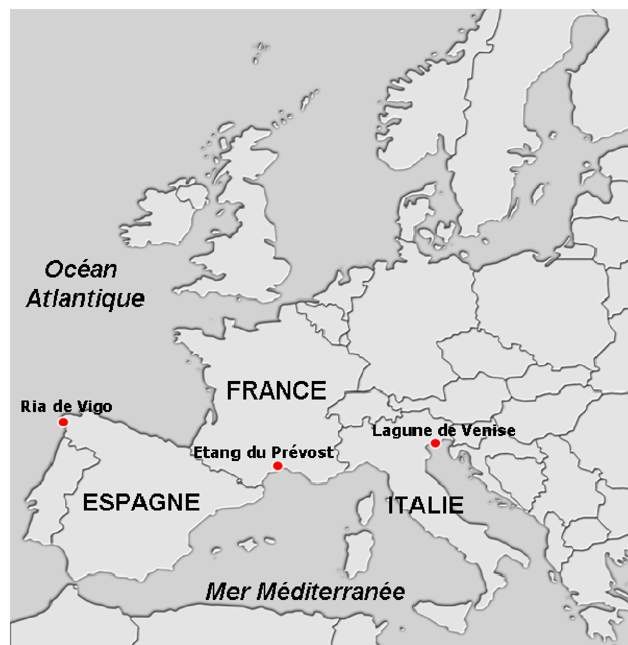


Figure 7 - Les trois localisations européennes des *M. galloprovincialis* étudiées

Le **chapitre 1** sera consacré à l'état de nos connaissances en matière d'immunité innée, principalement celle les bivalves. Nous y mettrons en lumière les bases moléculaires et cellulaires communes avec les systèmes immunitaires des invertébrés.

Dans les chapitre suivants, nous présenterons les publications, parues ou en cours de parution, qui regroupent les résultats obtenus au cours de ce travail. Dans le **chapitre 2**, nous verrons comment les différentes sous populations d'hémocytes réagissent aux divers stimuli. Le **chapitre 3** concerne le suivi de l'expression du gène de lysozyme à la suite de divers stimuli. Au cours de **chapitre 4**, nous comparerons l'expression des gènes qui codent trois

peptides antimicrobiens (AMP), le lysozyme et la protéine de choc thermique 70 (HSP70) entre les trois populations géographiques de *M. galloprovincialis* en réponse aux mêmes stimuli. Le **chapitre 5** consiste en un suivi de l'expression des mêmes gènes au sein de la population des moules de l'Etang du Prévost-France au cours de trois années consécutives. Dans le **chapitre 6**, nous traiterons les résultats obtenus récemment sur le polymorphisme d'ARN messager d'un peptide antimicrobien, la mytiline B. Enfin, ce manuscrit terminera par un chapitre **Discussion et Perspectives**.

D. Références

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Chapitre 1

(D'après « Funny Microbes », Leos Mandel)

Chapitre 1

Les réactions immunitaires chez les Invertébrés

Il existe deux types de réactions immunitaires, l'immunité adaptative et l'immunité innée. L'immunité adaptative est un système qui serait apparu il y a 400 millions d'années lors de l'émergence des vertébrés. L'élément central en est le lymphocyte dont chacun porte un récepteur antigénique unique, généré par clonage d'un répertoire diversifié. La capacité à générer un très grand nombre de molécules de reconnaissance ($>10^{14}$) par les lymphocytes B et T assure une détection spécifique des micro-organismes infectieux (McBlane et al., 1995; Pallavicini et al., 2008a), tandis que la sélection clonale des lymphocytes permet à un vertébré de mémoriser une infection et donc d'élaborer une réponse immunitaire plus rapide lors d'une deuxième infection (McHeyzer-Williams and Davis, 1995). Cependant, l'immunité adaptative a ses limites. Par exemples, les récepteurs ne sont pas capables de déterminer la source et le contexte de l'antigène pour lesquels ils sont spécifiques. De plus, le temps de mise en route du système est relativement long (McGuinness et al., 2003). Pour palier à ces imperfections, il y a l'immunité innée (Tableau 2), seul système immunitaire présent chez les invertébrés.

	Inate immune system		Adaptive immune system
Evolutionary history	Ancient (plants, insects, mammals) Billions of years old	Co-stimulation Education Cooperation ←→	Modern (jawed vertebrates) 400 million years old
Recognition	PAMPs (commonly carbohydrate and lipids)		Specific detail of molecular structure
Receptors	Fixed in genome (invariant) Rearrangement not necessary Non-clonal Diverse cellular distribution		Encoded in gene segments (variability) Rearrangement neccesary Clonal Lymphocytes
Self–nonself discrimination	Perfect		Imperfect; hence, autoimmune disease, allergy and allograft rejection
Time to onset	Immediate		Delayed
Memory	No		Yes

TRENDS in Parasitology

Tableau 2 - Le comparaison entre l'immunité innée et l'immunité adaptative.
(McGuinness et al., 2003)

I - Immunité innée

L'immunité innée, présente chez les invertébrés et les vertébrés, concerne les réactions non spécifiques d'un antigène, non adaptatives et ne générant pas de mémoire immunitaire. D'origine évolutive plus ancienne que l'immunité adaptative, l'immunité innée constitue une première ligne de défense présente chez tous les métazoaires et les plantes. Elle intervient également dans la mise en place de l'immunité adaptative. Au contraire de l'immunité adaptative, l'immunité innée reconnaît les micro-organismes par un nombre limité des récepteurs connus sous le nom de « Pattern-Recognition Receptors » (PRRs). Ces récepteurs possèdent des caractéristiques communes (Akira et al., 2006):

(i) la reconnaissance des composants microbiens ou « Pathogen-Associated Molecular Patterns » (PAMPs), qui sont essentiels pour la survie des micro-organismes mais absents des cellules eucaryotes. Ces motifs invariants sont, par exemple, les lipopolysaccharides (LPS) des bactéries à Gram-négatif, les peptidoglycanes (PGN) des bactéries à Gram-positif, les glycolipides des mycobactéries, les sucres de type mannans des levures et les ARN double-brins des virus,

(ii) une expression constitutive et la détection des agents pathogènes tout au long du cycle de vie,

(iii) leur codification par la lignée germinale de manière non-clonale, et par conséquence, dépourvus de mémoire immunologique.

I. 1 - Reconnaissance

Une grande partie des PRRs fait partie de la famille des « Toll-Like Receptors » (TLRs). Ces récepteurs font référence à « Toll » à la suite de la découverte chez la drosophile d'une protéine dont la première fonction connue a été un rôle dans la formation de l'axe dorso-ventral (Belvin and Anderson, 1996) (Hashimoto et al., 1988). Peu après, en la comparant à l'Interleukine-1 Receptor (IL-1R) humaine, qui joue un rôle très important dans la réponse immunitaire, on a découvert que le Toll de la drosophile est également impliqué dans la défense immunitaire de la mouche (Anderson, 2000; Engstrom et al., 1993; Lemaitre et al., 1996). C'est un an après sa découverte chez la drosophile que le premier TLR humain a été identifié : le TLR4 qui est capable de reconnaître spécifiquement les LPS des bactéries à Gram-négatif (Medzhitov et al., 1997). A l'heure actuelle, une dizaine de TLRs ont été décrits chez l'homme et chez la souris. Ils se distinguent les uns des autres par leur spécificité vis-à-

vis de différents ligands, leur expression, et probablement les gènes qu'ils induisent. Cependant, ils partagent une structure très similaire : **(i)** une partie extracellulaire contenant un certain nombre de motifs répétés riches en leucine (LRR), **(ii)** une partie transmembranaire, et **(iii)** une partie intracellulaire qui est homologue à l'IL-1R, d'où le nom de ce domaine, appelé TIR (Toll/IL-1R). Une fois activés, les TLRs utilisent les mêmes molécules qu'IL-1R pour aboutir à la production des cytokines ou à l'expression d'autres gènes immunitaires (Akira and Takeda, 2004) (Fig. 8).

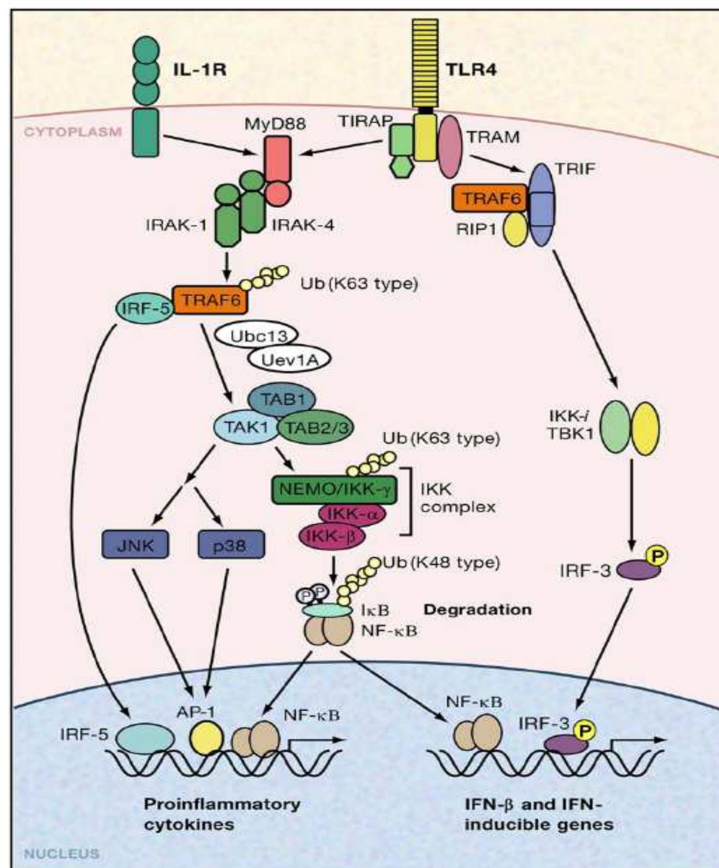

Figure 8 - Les TLRs partagent la voie de signalisation commune avec l'IL-1R.
(Akira and Takeda, 2004; Pallavicini et al., 2008a)

Toutefois, le Toll de la drosophile n'agit pas directement comme un PRR classique, car au lieu de reconnaître l'un des PAMPs, le ligand de ce Toll est une protéine endogène, Spatzle, qui est produite par protéolyse après l'infection (Weber et al., 2003). Chez la drosophile, le rôle des PRRs est assuré par une autre famille de protéines, appelées « Peptidoglycan Recognition Proteins » (PGRPs) (Kurata et al., 2006). Les PGRPs reconnaissent les peptidoglyganes de diverses bactéries et initient ensuite les réactions immunitaires appropriées, comme la libération des AMPs et l'activation de la cascade de la

pro-phénoloxidase (ProPO), qui permet d'effectuer la mélanisation et favorise la phagocytose. Ces protéines ont également été caractérisées chez le vers à soie *Bombyx mori* (Yoshida et al., 1996) et chez le papillon de nuit *Trichoplusia ni* (Kang et al., 1998), chez lesquels l'expression est induite par une infection bactérienne.

Un crustacé marin, la limule *Lymulus polyphemus*, utilise une stratégie différente de celle de la drosophile. Ariki *et al.* ont localisé sur les hémocytes une protéine reconnaissant les LPS et appelée « Factor C ». Il faut noter que les hémocytes de la limule ne répondent pas aux PAMPs autres que le LPS (Ariki et al., 2004). Après la stimulation par le LPS, les molécules dirigées contre les bactéries à Gram-positif et les champignons, comme le « β-1,3-glucan-sensitive protease zymogen factor G », les AMPs et les lectines, sont simultanément sécrétés par les hémocytes. Prenant en compte le fait que les microorganismes présents dans l'environnement de la limule sont principalement des bactéries à Gram-négatif, et que vraisemblablement chaque infection par des bactéries à Gram-positif ou des champignons s'accompagne d'une co-infection par des bactéries à Gram-négatif, la limule a développé un système de reconnaissance, certes très sensible aux bactéries à Gram-négatif, mais également efficace contre les autres micro-organismes (Kurata et al., 2006).

I. 2 - Elimination

L'étape suivante de la reconnaissance des micro-organismes infectieux sera leur élimination, faisant intervenir des composants cellulaires et des composants humoraux du système immunitaire (Fig. 9). Chez la drosophile, comme chez tous les invertébrés, les hémocytes sont les éléments clés de la défense cellulaire. Ces cellules dites immuno compétente protègent la drosophile des diverses invasions par trois actions cellulaires : **(i)** la phagocytose, très conservée tout au long de l'évolution, qui élimine les micro-organismes de petite taille comme les bactéries, les virus, les champignons, voire certains protozoaires. Elle comporte plusieurs étapes successives incluant la reconnaissance, l'attachement, la formation de pseudopodes, l'ingestion et la constitution de phagolysosomes (Bayne, 1990). **(ii)** l'encapsulation, qui prend en charge des organismes invasifs de taille supérieure à celle des hémocytes et qui ne peuvent donc pas être éliminés par phagocytose. Ce processus nécessite le dépôt de plusieurs couches d'hémocytes autour du pathogène entier afin de le détruire par action des enzymes lysosomiales. **(iii)** la formation d'un nodule par agrégation des hémocytes au site d'infection quand une grande quantité de particules étrangères a envahi l'hôte et que la phagocytose n'est plus suffisante pour les éliminer.

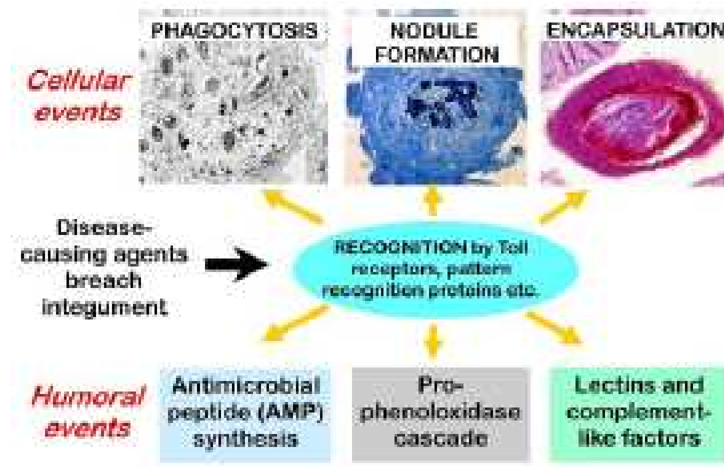

Figure 9 - La défense cellulaire et la défense humorale chez les invertévrés.
(Rowley and Powell, 2007)

En ce qui concerne la défense humorale, les AMPs sont sans doute les molécules les plus efficaces pour lutter contre les infections microbiennes. Ce sont des molécules de petite taille, au spectre d'activité très large. Chez la drosophile, 7 différentes familles d'AMPs ont été identifiées. Trois d'entre elles, diptéricine, drosocine, et attacine, sont très actives contre les bactéries à Gram-négatif, et tandis que la défensine est active contre les bactéries à Gram-positif. Drosomycine et metchnikowine sont des agents antifongiques. Enfin, la cécropine est un peptide à la fois antibactérien et antifongique. Les gènes qui codent ces AMPs sont silencieux ou faiblement exprimés chez les mouches non stimulées. Par contre, la mise en route de leur transcription est très rapide, quelques heures après l'infection, et ce phénomène peut durer jusqu'à 48 h.

Un autre mécanisme qui fait partie de la défense humorale est constitué par le système de l'activation des phénoloxidases (POs). Les phénoloxidases sont synthétisées sous la forme de précurseurs inactifs appelés pro-phénoloxidases (ProPOs), qui deviennent actifs après plusieurs étapes de protéolyses par des protéases à sérine appelées « ProPO activating enzymes » (ProPO-AEs) (Fig. 10) (Söderhäll and Cerenius, 1998). A la suite de la reconnaissance de divers PAMPs des envahisseurs, le précurseur inactif est transformé en PO, qui catalyse à son tour l'oxydation des phénols en quinones, produits intermédiaires instables qui vont polymériser en mélanines de façon non-enzymatique. Du fait de sa dangérosité pour l'hôte, l''activation de la cascade ProPO est strictement contrôlée par des inhibiteurs de protéase (Kromer et al., 1994; Söderhäll and Cerenius, 1998). Chez les invertébrés, le dépôt

de mélanine autour des tissus blessés ou des envahisseurs est un phénomène souvent observé. L'élimination des envahisseurs par les mélanines représente donc un mécanisme important de l'immunité innée.

Chez la plupart des invertébrés, les ProPOs sont synthétisées par les hémocytes, par exemple, dans les cellules à cristaux de la drosophile (Waltzer et al., 2002) ou dans les granulocytes des crustacés (Cerenius et al., 2003). Après libération par les hémocytes, les ProPOs peuvent être transportées au niveau de la cuticule et dans différents tissus par un mécanisme observé chez le vers à soie mais encore largement inconnu (Asano and Ashida, 2001a; Asano and Ashida, 2001b). Une activité de type PO a été également détectée dans les hémocytes et le plasma des bivalves comme *M. edulis* (Coles and Pipe, 1994), *Perna viridis* (Asokan et al., 1997) et *Crassostrea gigas* (Hellio et al., 2007).

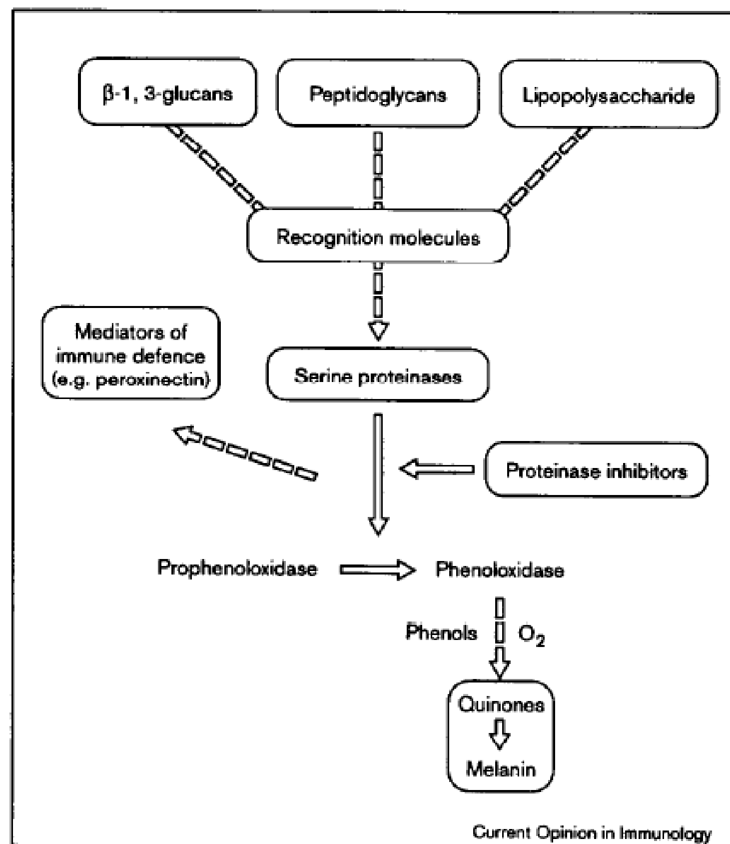

Figure 10 - Le mécanisme d'activation des phénoloxidases chez les arthropodes.
(Söderhäll and Cerenius, 1998)

II - Etat de nos connaissances sur le système immunitaire des mollusques bivalves

Les mollusques regroupent 8 % des espèces métazoaires répertoriées parmi les invertébrés, juste derrière les arthropodes qui en représentent plus de 80 %. Depuis une vingtaine année, les études pour comprendre leur système immunitaire attirent de plus en plus d'attention, parce que **(i)** les travaux comparant les systèmes immunitaires des différents invertébrés pourraient conduire à une meilleure compréhension des phénomènes beaucoup plus compliqués observés chez les vertébrés, et **(ii)** les enjeux socio-économiques demandent aussi des avancées sur les connaissances de la physiologie des réactions de défense des bivalves, qui sont souvent porteurs de maladies infectieuses pour les humains.

Même si les bivalves sont des organismes relativement simples, ils ont un long cycle de vie pendant lequel ils sont constamment exposés à différents microbes potentiellement pathogènes ou invasifs. Pour maintenir leur intégrité, les bivalves se sont adaptés à leur environnement et ont développé un système immunitaire très efficace. Comme c'est le cas de tous les autres invertébrés, cette immunité innée est leur seul système immunitaire et comprend deux actions complémentaires : une action impliquant directement les hémocytes (la défense cellulaire) et une action impliquant des molécules libres (la défense humorale) (Canesi et al., 2002) (Tableau 3).

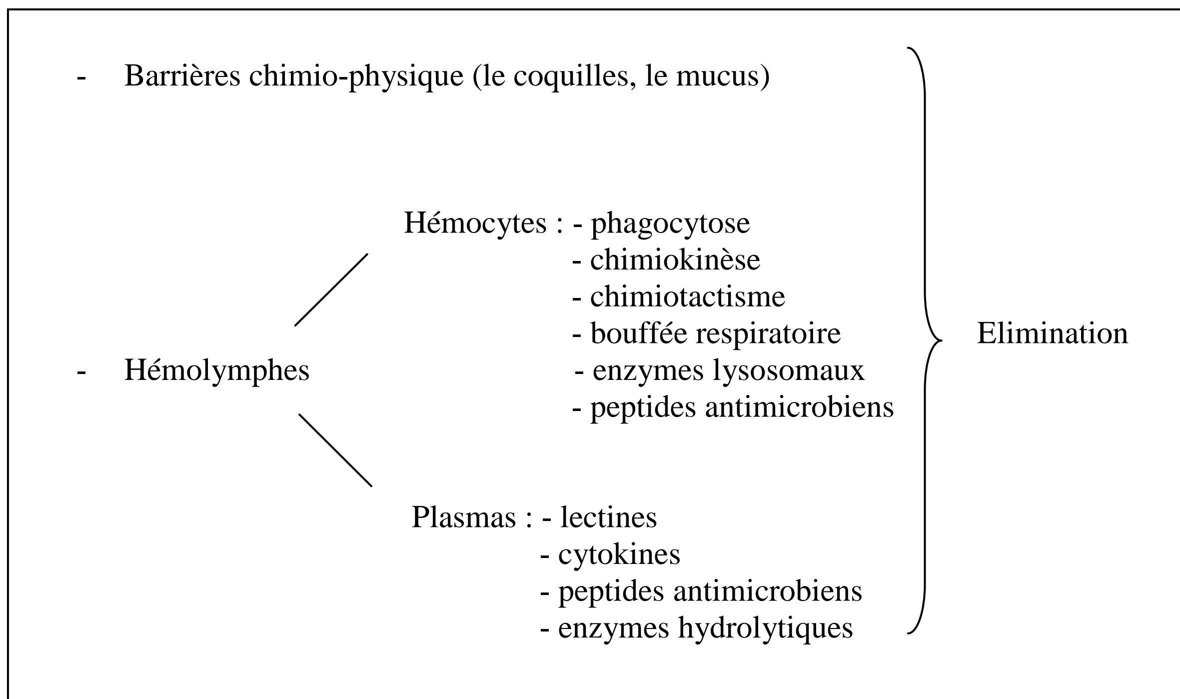

Tableau 3 - Les systèmes de défense chez les bivalves.
(Canesi et al., 2002)

II. 1 - Défense cellulaire

II. 1.1 - Les hémocytes

Cellules se déplaçant librement dans le système circulatoire, les hémocytes sont les effecteurs cellulaires essentiels de la réponse immunitaire des bivalves. A ce jour, aucune classification des hémocytes de bivalves ne fait consensus, à cause de notre méconnaissance d'un organe hémopoïétique et de l'absence de marqueur cellulaire. Cependant, on considère généralement qu'il existe deux types d'hémocytes : **(i)** les hyalinocytes, caractérisés par l'absence de granules cytoplasmiques et un rapport nucléo-cytoplamique élevé, et **(ii)** les granulocytes, qui possèdent des granules cytoplasmiques, un rapport nucléo-cytoplasmique faible et une activité phagocytaire (Carballal et al., 1997c; Cheng, 1981; Pipe, 1990a). Les granulocytes peuvent être subdivisés en fonction de la taille des granules. Des critères de coloration peuvent également servir à classer les hémocytes en **(i)** basophiles (environ 40% des hémocytes) comprenant la majorité des hyalinocytes et **(ii)** éosinophiles (environ 60%) comprenant les petits et les grands granulocytes (Dyrynda et al., 1997a; Pipe et al., 1997). Chez *M. galloprovincialis*, deux types d'hémocytes ont été décrits: **(i)** les hyalinocytes ayant des caractéristiques de cellules indifférenciées, et **(ii)** les granulocytes distingués d'après leur coloration en acidophiles et basophiles (Carballal et al., 1997a). Enfin, l'utilisation d'anticorps monoclonaux a révélé trois groupes d'hémocytes chez *M. edulis*: **(i)** les granulocytes basophiles, **(ii)** un sous-groupe comprend une partie des granulocytes basophiles et des hyalinocytes et **(iii)** des granulocytes éosinophiles (Dyrynda et al., 1997b).

II. 1.2 - La phagocytose

Une des armes les plus efficaces pour éliminer des éléments étrangers est constituée par la phagocytose (Fig. 11). Quand un pathogène envahit un hôte, de multiples réactions sont déclenchées. Le pathogène essaie d'échapper au système immunitaire, tandis que l'hôte essaie d'éliminer au plus vite cette particule étrangère pour maintenir son intégrité. Depuis plus d'un siècle, la phagocytose a été reconnue comme un phénomène essentiel, qui joue un rôle non seulement en nutrition, mais aussi en défense (Silverstein, 1995). Son but consiste à éliminer tous les matériaux étrangers, y compris des particules inorganiques, des organismes vivants étrangers (pathogène ou non-pathogène) et des cellules de l'hôte modifiées. Pour atteindre ce but, des cascades de réactions complexes sont mises en jeu, qui emploient de nombreuses molécules pour tout d'abord l'opsonisation, ensuite la reconnaissance et enfin l'élimination des particules étrangères (Stuart and Ezekowitz, 2005). Chez les bivalves, la phagocytose est

augmentée par une exposition de courte durée à des xénobiotiques en faible concentration (Coles et al., 1995), mais elle est affaiblie par une exposition plus longue ou pour des concentrations plus élevées (Robert S et al., 1981). Chez *M. edulis*, non seulement les hémocytes des moules adultes, mais aussi ceux des larves, sont capables de phagocytose (Dyrynda et al., 1995). En fait, l'effet bactéricide est une action combinée des phagocytes et de plusieurs facteurs humoraux, et cet effet varie en fonction de la saison (Canesi et al., 2002).

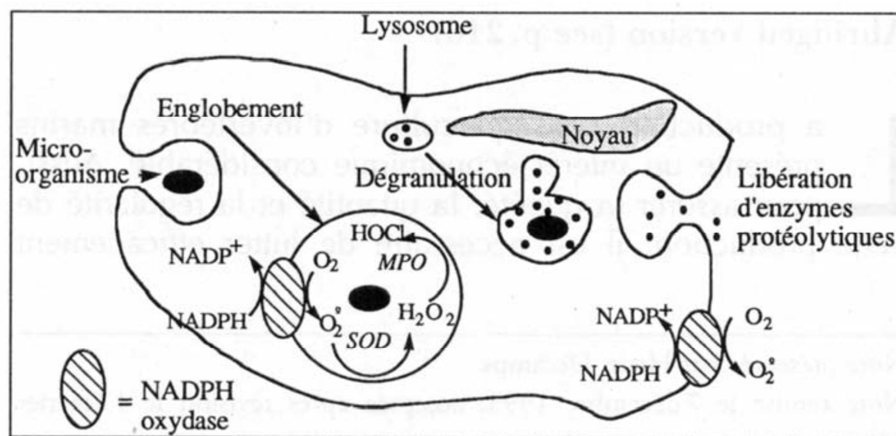

Figure 11 - Les voies de destruction des micro-organismes au cours de la phagocytose.
(Torreilles et al., 1996)

II. 1.3 - Les métabolites toxiques

Les hémocytes des bivalves sont capables de produire des métabolites toxiques, comme les radicaux oxygénés (EORs) et l'oxyde nitrique (ON). Ces deux produits sont souvent impliqués dans le processus de phagocytose.

(i) EORs : Chez les huîtres *C. gigas* et *Ostrea edulis* (Bachère et al., 1991) et chez les moules *M. edulis* et *M. galloprovincialis* (Pipe, 1992; Winston et al., 1996), les EORs participent activement à l'élimination intracellulaire des particules phagocytées. Curieusement, quelques espèces de bivalves, comme *Ruditapes decussatus*, *Mya arenaria* et *Mercenaria mercenaria*, ne possèdent pas de stimulation détectable de la production des EORs associée à la phagocytose (Anderson, 1994; Lopez et al., 1994). En fait, la production des EORs commence dès le contact des hémocytes avec un corps étranger, ce qui induit une augmentation de la consommation d'oxygène, due à une activité enzymatique de type NADH-oxydase liée à la membrane cellulaire et catalysant la réduction univalente de l'oxygène

moléculaire en O_2^-. Cet ion se transforme spontanément, ou sous l'action catalytique d'une superoxyde dismutase (SOD), en peroxyde d'hydrogène (H_2O_2), l'excès de H_2O_2 étant ensuite réduit en H_2O et O_2 par une catalase, ou transformé en acide hypochloreux (HOCl) par une myéloperoxydase (MPO) (Torreilles et al., 1996). Afin de se protéger des effets toxiques des EORs, les cellules des bivalves possèdent de nombreux systèmes antioxydants, comme c'est le cas chez les mammifères (Lemaire and Livingstone, 1993; Torreilles and Guérin, 1989). Il s'agit de la SOD qui transforme O_2^- en H_2O_2 , de la catalase et de la glutathion peroxydase qui réduisent H_2O_2 en eau, de la glutathion réductase qui régénère le glutathion réduit, et des composés piégeurs de radicaux, comme les vitamines A, C et E, etc.

(ii) NO : il est synthétisé à partir de l'acide aminé précurseur L-arginine dans une réaction d'oxydation, laquelle s'effectue en cinq étapes et est catalysée par l'enzyme NO synthase (NOS) (Fig. 12). Cette réaction complexe exige la présence des co-substrats O_2 et NADPH (nicotinamide adénine dinucléotide phosphate réduit). Le NO lui-même n'est pas toxique, par contre, en se combinant avec les anions superoxides O_2^-, il générera des péroxynitrites qui sont extrêmement toxiques. La génération de peroxynitrites associée à la phagocytose a été observée chez les bivalves via la chimioluminescence (Torreilles and Guérin, 1999). Par exemple, l'addition de zymosan, une molécule extraite de la membrane de levure, augmente rapidement la luminescence chez *M. galloprovincialis* (Torreilles and Guérin, 1999). Toujours chez la moule *M. galloprovincialis*, une augmentation importante de la production de NO est provoquée par l'IL-2 humaine (Novas et al., 2004b). En plus, l'incubation avec la bactérie *Vibrio tapetis* induit une augmentation significative de la production de NO par les hémocytes de *R. decussatus*. Néanmoins, le fait que la phagocytose des *E. coli* ne soit pas renforcée par l'addition de NO exogène, suggère que celui-ci n'intervient pas dans la phagocytose chez cette espèce (Tafalla et al., 2003).

II.1.4 - Le chimiotactisme

Grâce à leur mobilité, les hémocytes sont capables d'être attirés des pathogènes. Ce type de réaction est dépendant de la nature de la molécule attractive (Schneeweiss and Renwrantz, 1993). Chez l'huître *Crassostrea virginica*, les hémocytes ne sont attirés par des bactéries qui si celles-ci sont vivantes. De plus, ils migrent vers les bactéries *E. coli* mais pas vers *Vibrio parahaemolyticus* (Howland and Cheng, 1982). Au contraire, les hémocytes de la palourde *Corbicula japonica* migrent vers ces deux bactéries (Kumazawa and Morimoto, 1992). Chez la moule *M. edulis*, le chimiotactisme est stimulé par les LPS. Cependant, le N-formyl-methionyl-leucyl-phenylalanine (N-FMLP), un produit libéré par les bactéries à

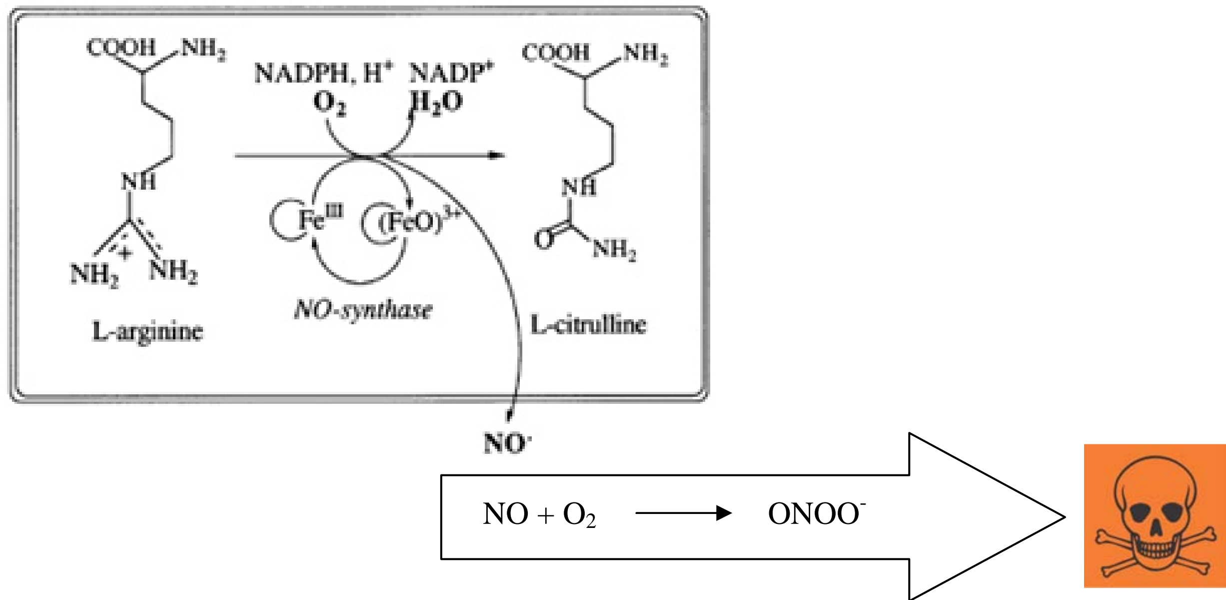

Figure 12 - La synthèse de l'oxyde nitrique par NO synthase.

Gram-positif et à Gram-négatif, stimule la migration cellulaire aléatoire, appelée chimiokinèse (Schneeweiss and Renwrantz, 1993). Chez l'huître *C. virginia*, la chimiokinèse est aussi induite par les bactéries vivantes *E. coli* ou par leur milieu de culture (Alvarez et al., 1995). Parce que le chimiotactisme et la chimiokinèse augmentent la probabilité du contact physique entre hémocytes et envahisseurs, Schneeweiss et Renwrantz suggèrent que les peptides formyles fonctionnent comme un signal universel pour activer les hémocytes en cas d'infections bactériennes (Schneeweiss and Renwrantz, 1993).

II. 2 - Défense humorale

II. 2. 1 - Les lectines

Les lectines appartiennent à la famille des glycoprotéines, qui réagissent spécifiquement avec les polysaccharides. Chez les bivalves, les études réalisées avec différentes espèces de bactéries ont permis de découvrir les diverses fonctions des lectines (Leclerc, 1996; Tunkijjanukij et al., 1998). Certaines d'entre elles pourraient agir simplement en se fixant directement à la surface des bactéries, causant leur agglutination. Par conséquence, l'immobilisation des envahisseurs pourrait conduire au renforcement de la phagocytose. D'autres lectines pourraient agir comme des molécules de liaison entre la surface d'une

bactérie et l'hémocyte. Ce processus favorise également la phagocytose, on parle alors d'opsonisation.

Chez *C. gigas*, les lectines sont capables d'agglutiner les érythrocytes humains ainsi que la bactérie *Vibrio anguillarum* (Olafsen et al., 1992). L'augmentation de l'activité des lectines a été observée après avoir exposé *C. gigas* aux *V. anguillarum* pendant 6 h (Olafsen et al., 1992). Les lectines ont été aussi identifiés chez *C. virginica* (Tripp, 1966) et chez *M. edulis* (Renwrantz and Stahmer, 1983), chez *Ostrea chilensis* (Minamikawa et al., 2004), chez *Saxidomus purpuratus* (Tatsumi et al., 1982) et chez le clam *Anadara granosa (Dam et al., 1993).* Chez le pétoncle *Chlamys farreri*, 2 séquences complètes d'ADNc de lectines ont été caractérisées (Wang et al., 2007; Zheng et al., 2008). L'augmentation de la quantité d'ARNm de la *Cf*Lec-1 a été observée au niveau des hémocytes après les injections de bactéries *V. anguillarum* et *Micrococcus luteus*. La protéine recombinante de *Cf*Lec-1 est capable d'agglutiner les bactéries *E. coli*, et cet effet est dépendant de l'ion Ca^{2+}. Cependant, cette protéine recombinante n'agglutine pas les bactéries *M. luteus* et *Candida lipolytica* ni les érythrocytes de lapin, de rat, de poulet et humains. Curieusement, la protéine recombinante d'une autre lectine, la *Cf*Lec-2, possède une activité agrégative envers la bactérie *Staphylococcus haemolyticus* indépendamment de l'ion Ca^{2+}. Récemment, une nouvelle isoforme de lectine, appelée MCL-4, a été purifiée et caractérisée chez la palourde *Ruditapes philippinarum* (Takahashi et al., 2008). Cette lectine agglutine les érythrocytes de mouton et de lapin, mais aucune des trois bactéries marines testées. La capacité de phagocytose des hémocytes de *R. philippinarum* pour la bactérie *Vibrio tubiashii* opsonizée préalablement par MCL-4 est significativement plus élevée que celle pour la bactérie non-traitée montrant l'importance de cette lectine dans l'élimination des bactéries (Takahashi et al., 2008).

II. 2.2 - Les cytokines

Ce sont des molécules de communication qui agissent sur d'autres cellules pour en réguler l'activité et la fonction. Les cytokines sont essentielles à la communication entre les cellules immunitaires permettant une réponse rapide lorsque survient une invasion ou une infection microbienne. Chez les vertébrés, ces molécules régulatrices comprennent les interleukines (IL), les interférons (IFN) et les chemokines. Par rapport aux vertébrés, les connaissances sur les cytokines des bivalves sont très limitées (DeVries et al., 2006).

Chez l'huître perlière *Pinctada fucata*, l'administration d'interféron-Ω recombinant de félin (rFeIFN-Ω) protège les animaux contre le virus akoya (Miyazaki et al., 2000) et les récepteurs pour rFeIFN- Ω sont présents sur les hyalinocytes de l'huître (Miyazaki et al.,

2002). Chez *C. farreri*, une sous famille de « Tumor Growth Factor-α » (TNF-α) appelée « LPS-induced TNF-α factor » (LITAF), a été découverte (Yu et al., 2007). Une injection de LPS entraîne une augmentation de l'expression de LITAF au niveau des hémocytes. Néanmoins, aucun changement significatif n'a été observé après la stimulation de PGN. Les hémocytes de *M. edulis* et *M. galloprovincialis* peuvent également répondre à l'administration de différentes cytokines hétérologues (Hughes et al., 1991; Hughes et al., 1992; Novas et al., 2004a; Ottaviani et al., 2000), comme le TNF, les IL-1, IL-2, IL-6 et IL-8. Récemment, Roberts *et al.* ont isolé par l'analyse d'une banque d' « Expressed Sequence Tags » (EST) chez l'huître *C. gigas,* deux clones codant une protéine similaire aux IL-17 des vertébrés (Roberts et al., 2008). Cette IL-17, appelée *Cg*IL-17, présente 27 % d'homologie avec l'IL-17D de la truite arc-en-ciel et 21 % d'homologie avec l'IL-17D humaine au niveau des acides animés. L'analyse des séquences protéiques montre que la position des cystéines chez *Cg*IL-17 est identique à celle de toutes les formes d'IL-17 des mammifères. Le gène de *Cg*IL-17 est exprimé de façon constitutive dans plusieurs tissus, mais l'expression la plus importante a lieu au niveau des branchies. L'injection d'un mélange de bactéries mortes entraîne une forte augmentation de la quantité d'ARNm de *Cg*IL-17 dans les hémocytes 6 h après cette injection, ce qui suggère que ce gène répond très vite aux pathogènes et qu'il pourrait participer à la stimulation des autres gènes immunitaires chez l'huître.

II. 2.3 - Les peptides antimicrobiens (AMP)

Les connaissances sur les peptides antimicrobiens chez les bivalves sont particulièrement importantes par les moules *M. edulis* et *M. galloprovincialis* (Mitta et al., 2000b). Par purification biochimique et clonage moléculaire, quatre familles d'AMP, appartenant à la classe des peptides cationiques riches en cystéines, ont été isolées à partir de l'hémolymphe (Charlet et al., 1996; Hubert et al., 1996; Mitta et al., 1999a): **(i)** defensines, **(ii)** mytilines, **(iii)** myticines et **(iiii)** mytimycine.

II. 2. 3. 1 - Caractéristiques : deux différents isoformes de **défensines** ont été caractérisées chez *M. galloprovincialis*: MGD1 et MGD2 (Charlet et al., 1996; Hubert et al., 1996; Mitta et al., 1999b). Ces deux défensines, de poids moléculaire 4,3 kDa, partagent un consensus de six cystéines avec leur homologue d'arthropodes. En plus de ces six cystéines, deux autres cystéines caractéristiques des défensines de moules révèlent l'originalité de ces peptides (Fig. 13). La structure tridimensionnelle de MGD1 comporte le motif typique en CSαβ (Cysteine-Stabilized α-helix β-sheet), avec quatre ponts disulfures entre les 8 cystéines (Yang et al.,

2000). La seconde famille d'AMP, les **mytilines**, comprennent cinq isoformes, A, B, C, D et G1, d'un poids moléculaire 4,3 kDa. Les isoformes A et B ont été isolées à partir du plasma de *M. edulis* (Charlet et al., 1996), tandis que les isoformes B, C, D et G1 ont été identifiées à partir des hémocytes de *M. galloprovincialis* (Mitta et al., 2000b). Ces cinq isoformes partagent un consensus de 8 cystéines. Récemment, la structure tridimensionnelle de la mytiline a été établie par NMR (Nuclear Magnetic Resonance) (Roch et al., 2008). Les 8 cystéines de la mytiline forment quatre ponts disulfures intra chaîne. Curieusement, les motifs structuraux observés pour la mytiline sont proches de ceux de la défensine, bien que les séquences primaires soient très différentes. La troisième famille, les **myticines**, comporte trois isoformes, A, B et C, de poids moléculaire 4,5 kDa (Mitta et al., 1999a). Les deux isoformes ont été isolées à partir des hémocytes de *M. galloprovincialis*, mais seule l'isoforme A a été caractérisée à partir du plasma de cette même espèce. A l'heure actuelle, la structure tridimensionnelle de la myticine n'est pas connue. Sa structure primaire montre qu'elle contient aussi 8 cystéines. Même si possédant une masse moléculaire similaire et un consensus de 8 cystéines, les mytilines et les myticines n'appartiennent à aucune famille de molécule connue. La quatrième famille, la **mytimycine**, de poids moléculaire 6,5 kDa, n'a été isolée qu'une seule fois à partir du plasma de *M. edulis* (Charlet et al., 1996). C'est un AMP partiellement caractérisé, qui comporterait 12 cystéines et est strictement antifongique.

Les études précédentes ont montré que les AMPs de moule sont produits dans les hémocytes puis sont stockés dans les granules des hémocytes (Mitta et al., 1999a; Mitta et al., 1999b; Mitta et al., 2000b). Les informations obtenues à partir des ARNm des AMPs ont révélé que ceux-ci sont tout d'abord synthétisés sous forme de précurseurs dans les hémocytes : 81 acides aminés pour les défensines, 103 pour les mytilines et 96 pour les myticines. Les précurseurs subissent ensuite une maturation intra-hémocytaire les transformant en peptides actifs, un phénomène similaire à celui observé chez la limule, *Tachypleus tridentatus* (Shigenaga et al., 1990) et chez la crevette, *Penaeus vannamei* (Destoumieux et al., 1997).

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                    A. Défensines

MGD-1      GFGCPNNYQCHRHCKSIPGRCGGY-CGGWHRLR-CTCYRCG
MGD-2      GFGCPNNYACHQHCKSIRGYCGGY-CAGWFRLR-CTCYRCG

                    B. Myticines

MC-A      HSHACTSYW-CGKFCGTAS--CTHYLCRVLHPGKMCACVHCSR
MC-B      HPHVCTSYY-CSKFCGTAG--CTRYGCRNLHRGKLCFCLHCSR
MC-C      QSVACTSYY-CSKFCGSAG--CSLYGCYLLHPGKICYCLHCSR

                    C. Mytilines

ML-A      GCASRCKAKCAGRRCKGWASASFRGRCYCKCFRC
ML-B      SCASRCKGHCRARRCGYYVSVLYRGRCYCKCLRC
ML-C      SCASRCKSRCRARRCRYYVSVRYGGFCYCRC..
ML-D      GCASRCKAKCAGRRCKGWASASFRRRCYCKCFRC
ML-E    VVTCGSLCKAHCTFRKCGYFMSVLYHGRCYCRCLLC

                    D. Mytimycine

MM-A    DCCRKPFRKHCWDCTAGTPYYGYSTRNIFGCTC.....
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Figure 13 - La diversité des peptides antimicrobiens chez *M. galloprovincialis*.

Les ADNc des divers AMPs ont montré que les précurseurs possèdent des caractères structuraux communs avec : **(i)** un peptide signal en N-terminal, suivi par **(ii)** une séquence correspondant au peptide mature, et **(iii)** une extension riche en résidus anioniques en C-terminal. Alors que le peptide signal est considéré comme une séquence nécessaire à la translocation des précurseurs, la fonction des extensions en C-terminal reste inconnue. Il a été suggéré que cette région pourrait interagit avec le peptide actif afin de : **(i)** neutraliser sa charge positive nette, ce qui permet un processus protéolytique convenable et/ou un envoi précis à un compartiment particulier des hémocytes, ou **(ii)** protéger les cellules des effets cytotoxiques des AMPs. Curieusement, une extension similaire en C-terminal a été également observée chez *T. tridentatus* (Shigenaga et al., 1990) et chez *Drosophila melanogaster* (Imler and Bulet, 2005).

II. 2. 3. 2 - Activités biologiques : les défensines et les myticines sont essentiellement actives contre les bactéries à Gram-positif, y compris certaines qui sont pathogènes pour les bivalves. Elles sont beaucoup moins efficaces contre les bactéries à Gram-négatif ou les champignons (Charlet et al., 1996; Hubert et al., 1996; Mitta et al., 1999b). Par contre, les mytilines ont un spectre d'activité plus large que les deux AMPs précédents, et ce en fonction des différentes isoformes : les isoformes B, C et D sont toxiques pour les bactéries à Gram-positif et à Gram-négatif, tandis que la mytiline E n'est active que contre les bactéries à Gram-positif (Mitta et al., 2000b). En fait, les différentes familles de peptides, voire les différents isoformes d'un même peptide, possèdent des propriétés complémentaires, ce qui permettrait une couverture antimicrobienne beaucoup plus large.

Tous les AMPs de moule exercent une action bactéricide, mais avec des cinétiques différentes. Par exemple, quand les divers AMPs sont incubés avec la bactérie Gram-positif, *Micrococcus lysodeikticus*, plus de 2 h et 6 h sont nécessaires à la mytiline D et à la myticine A, pour tuer toutes les bactéries. Cependant, seulement quelques minutes sont suffisantes en ce qui concerne les mytilines A, B, C ou la défensine MGD1 pour atteindre le même résultat. Une telle rapidité d'activité bactéricide ressemble à celle des défensines de la mouche, *Phormia terranovae* (Cociancich et al., 1993). Chez cet insecte, les défensines induisent une perméabilisation de la membrane cytoplasmique des bactéries à Gram-positif, ce qui conduit à la dépolarisation partielle, à la diminution de l'ATP (Adenosine Tri Phosphate) cytoplasmique et la mort cellulaire, probablement par apoptose.

II. 2. 3. 3 - Production et localisation : les hémocytes sont le site de production des précurseurs des divers AMPs chez les moules. Les hémocytes infiltrant tous les tissus, les AMPs sont partout présents dans le corps de moule, comme le manteau, les palpes, les branchies ou la glande digestive (Mitta et al., 1999a; Mitta et al., 2000b; Mitta et al., 2000c). En fait, l'hybridation *in situ* montre une distribution différentielle des hémocytes exprimant les gènes des divers AMPs. De nombreux hémocytes exprimant les défensines sont localisés au niveau de l'épithélium du tube digestif, alors que très peu de cellules exprimant les mytilines ou les myticines ont été trouvées à ce niveau. Les hémocytes exprimant les mytilines ou les myticines sont très présents au niveau des branchies alors qu'il n'y a pas d'hémocytes exprimant les défensines. Tous ces résultats suggèrent que les différents gènes sont exprimés dans différentes populations d'hémocytes. En conséquence, en plus des diverses activités des différents AMPs, la capacité antimicrobienne est renforcée par la distribution très large, mais spécifique, des AMPs dans les différents tissus de la moule.

Une fois produit dans les hémocytes, les AMPs sont stockés dans les granules des hémocytes (Fig. 14). Par la technique de l'immno localisation, il a été montré que défensines et mytilines sont stockées dans des différents organites sub-cellulaires (Mitta et al., 2000b; Mitta et al., 2000c). Les défensines sont localisées : **(i)** dans les vésicules des granulocytes à petits granules, et **(ii)** dans les gros granules des granulocytes à gros granules. En utilisant la même technique, les mytilines ont été observées exclusivement dans les granules des granulocytes à gros granules. En résumé, 37 % des hémocytes contiennent uniquement les mytilines, et 16 % des hémocytes contiennent uniquement les défensines. Le pourcentage des cellules contenant simultanément les deux AMPs est de 32 %, parmi lesquels 21 % ont montré une co-localisation des défensines et des mytilines dans les mêmes structures. Finalement, 15 % des hémocytes ne contiennent ni défensines ni mytilines.

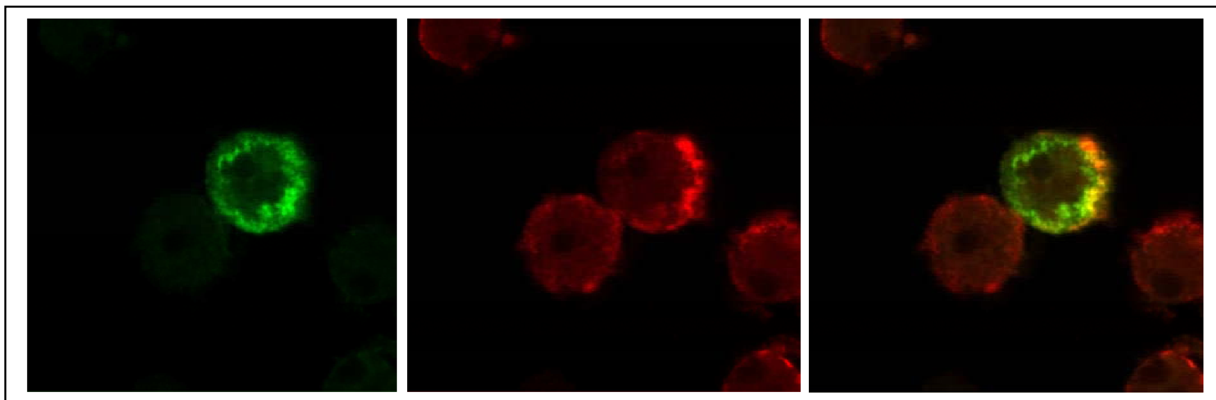

Figure 14 - La localisation des défensines (verte) et des mytilines (rouge) sur les hémocytes de *M. galloprovincialis*. (Mitta et al., 2000d)

II. 2. 4 - Les lysozymes

Les lysozymes constituent un composant important de la défense immunitaire contre diverses infections microbiennes. Ces petites enzymes sont capables d'hydrolyser la liaison glycosidique des peptidoglycanes, qui sont les composants majeurs de la paroi des bactéries à Gram-positif. Chez les bivalves, plusieurs études ont montré que les lysozymes sont aussi bactéricides contre les bactéries à Gram-négatif (Nilsen et al., 1999; Xue et al., 2004). Les activités anti protozoaires et anti fongiques des lysozymes par hydrolyse de la N-acétylglucosamine de la chitine, ont été également observées chez les vertébrés (Bierman et al., 1979; Samaranayake et al., 1997).

D'une manière générale, il existe plusieurs types de lysozymes, dont le type-poulet (type-c), le type-oie (type-g) (Prager and Jolles, 1996) et le type-invertébré (type-i) (Ito et al., 1999; Nilsen et al., 1999) sont les plus étudiés. La première séquence de la protéine et du gène d'un lysozyme de type-i viennent des travaux réalisés chez la palourde *Tapes japonica* (Ito et al., 1999) et le pecten *Chlamys islandica* (Nilsen et al., 1999). Ce lysozyme de type-i a été également identifié chez les moules, *M. edulis* et *M. galloprovincialis* (Bachali et al., 2002; Olsen et al., 2003). L'organisation du gène qui code le lysozyme chez *M. edulis* est différente de celle de *C. islandica* (Bachali et al., 2002; Nilsen and Myrnes, 2001). Chez ce dernier, il existe 4 exons qui sont typiques du lysozyme de type-c, tandis que 5 exons existent chez *M. edulis*.

Le lysozyme a été localisé dans les granules des hémocytes de *M. edulis* (Pipe, 1990b). L'activité est plus forte dans les extraits de granulocytes que dans le plasma à la fois chez *M. galloprovincialis* (Carballal et al., 1997b) et chez *R. decussatus* (Lopez et al., 1997). Chez l'huître *C. gigas*, l'ARNm du lysozyme est exprimé dans tous les tissus, sauf dans le muscle adducteur (Matsumoto et al., 2006). Par hybridation *in situ*, il a été montré que la quantité d'ARNm du lysozyme dans les palpes et le manteau est significativement plus élevée que dans les branchies, le tube digestif et les hémocytes chez *C. virginica* (Itoh et al., 2007). L'augmentation de l'expression du lysozyme au niveau des hémocytes a été observée après injection d'ADN bactérien chez la moule *Hyriopsis cumingii* (Hong et al., 2006). Un phénomène similaire a été observé après injection de *V. tapetis* chez *R. philippinarum*, mais il est moins important chez *R. decussatus* et inexistant chez *M. mercenaria* et *C. virginica,* suggérant une expression constitutive non inductible (Allam et al., 2006).

II. 3 - Cascade de régulation des gènes immunitaires

Les dernières avancées sur les connaissances moléculaires des bivalves ont permis de mettre en évidence la plupart des homologues de la voie de signalisation de Rel/NF-κB, qui joue un rôle très important dans l'induction des gènes immunitaires. En combinant les molécules identifiées chez les huîtres et les moules par divers auteurs employant diverses techniques, nous obtenons une voie de signalisation de type Rel/NF-κB qui ressemble à celle de la drosophile ou des mammifères (Montagnani et al., 2008)(Fig. 15). Parmi toutes les molécules de la cascade, TLR a été identifié chez *C. virginica* (Tanguy et al., 2004), tandis que la protéine adaptateur MyD88 (Tanguy et al., 2004), l'inhibiteur κB kinase (IKK) (Escoubas et al., 1999), l'inhibiteur κB (IκB) (Montagnani et al., 2008), l'homologue de

Facteur Nucleique κB (NF-κB) connu sous le nom de « Rel » (Montagnani et al., 2004) ont été découverts chez *C. gigas*. La protéine Rel a été également identifiée chez la moule *Bathymodiolus azoricus* (Bettencourt et al., 2007).

Les ligands qui se fixent aux TLR conduisent à l'activation des protéines adaptateurs (MyD88, Tube). Les diverses kinases sont ensuite activées, ce qui permet la phosphorylation et la dégradation des protéines inhibiteurs de Rel (cactus, IκB). Les inhibiteurs phosphorylés sont à leur tour dégradés afin de libérer les facteurs de transcription (Rel, NF-κB), qui vont être transportés dans les noyaux pour y activer les divers gènes immunitaires.

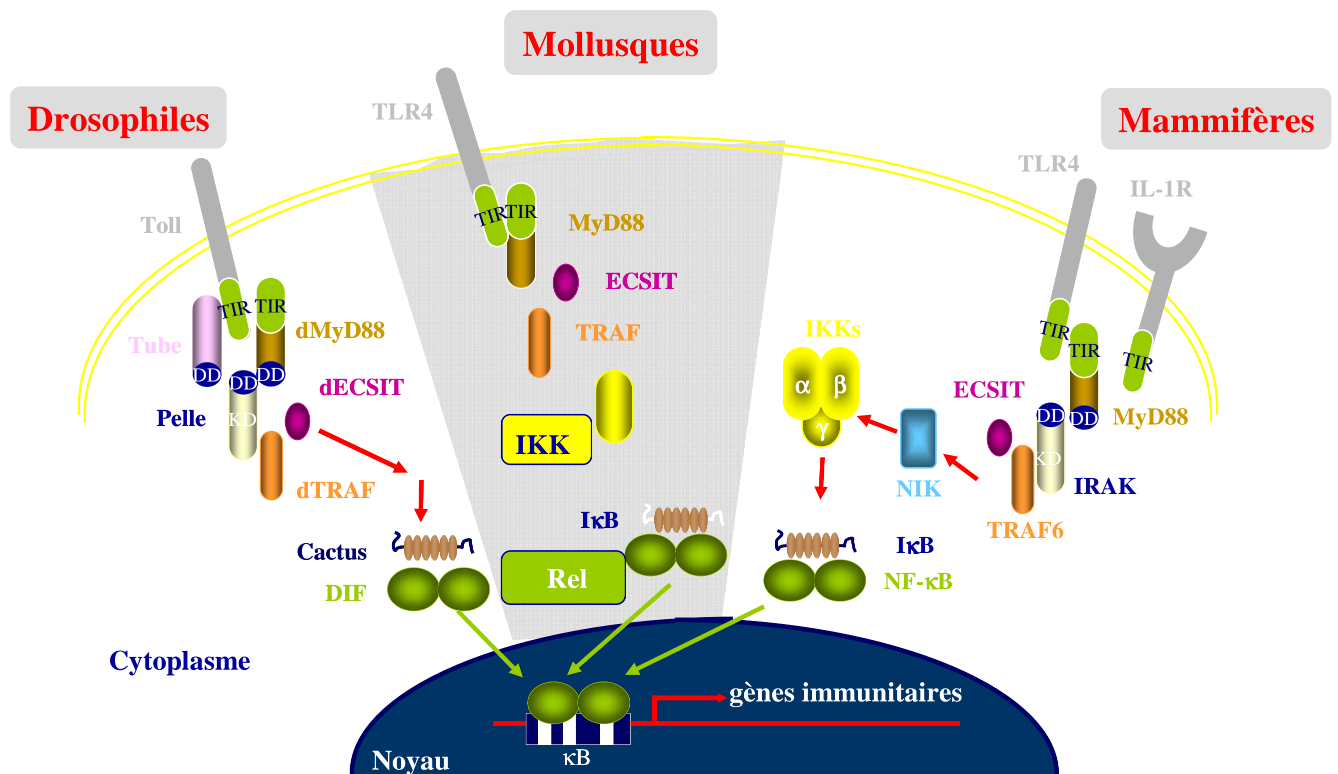

Figure 15 - La conservation de la voie de Rel/NF-κB chez les mammifères, les drosophiles et les bivalves.

(Montagnani et al., 2008)

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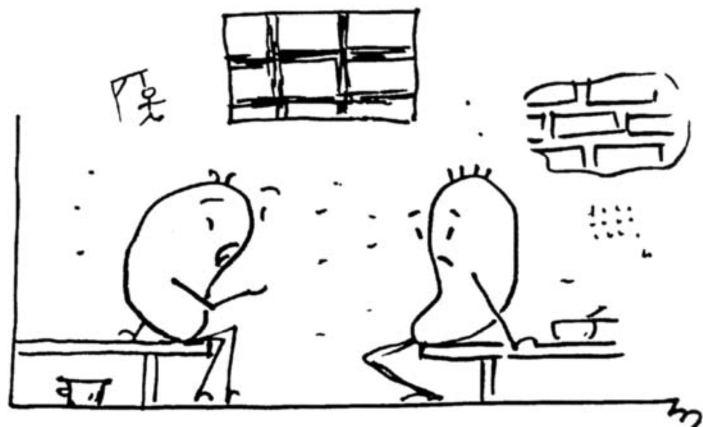
Chapitre 2



I never thought intracellular life could be so boring.

(D'après « Funny Microbes », Leos Mandel)

Chapitre 2

Elimination des bactéries par les hémocytes de *M. galloprovincialis*

Les hémocytes circulants sont les principales cellules qui interviennent lors de la réponse immunitaire à des invasions bactériennes chez la moule. Malgré les incertitudes sur leur classification, la population des hémocytes peut être subdivisée en 2 groupes : (i) les granulocytes et (ii) les hyalinocytes. Dans ce chapitre, nous allons étudier le comportement des différentes sous-populations hémocytaires identifiées par la technique de la cytométrie de flux, en réponse à l'injection de trois bactéries ayant des caractéristiques différentes. Ces trois bactéries, utilisées comme stimuli tout au long de la thèse, comprennent deux espèces à Gram-négatif de la famille des *Vibrionacea*, (i) *Vibrio splendidus* LGB32 qui a été isolée à partir de juvéniles de l'huître *Crassostrea gigas* lors de la moralité estivale de 2001, et (ii) *Vibrio anguillarum* qui n'est pas connue pour être pathogène des bivalves, et (iii) une bactérie à Gram-positif terrestre, *Micrococcus lysodeikticus*, qui possède une structure de paroi différente des deux précédentes. Ce travail a été rendu possible grâce à la coopération avec le laboratoire d'Elisabeth A. Dyrynda à Heriot Watt University, Edinburgh (UK), et au séjour de M-G. Parisi, doctorante de l'Université de Palerme (Italie).

Nos principaux résultats sont les suivants : **(i)** trois fenêtres en cytométrie de flux ont été déterminées pour quantifier trois sous-populations hémocytaires : les hyalinocytes, les petits granulocytes à petits granules et les grands granulocytes à gros granules; **(ii)** de nombreuse espèces bactériennes sont naturellement présentes dans le plasma, y compris plusieurs *Vibrios*, tandis que le contenu intracellulaire des hémocytes est stérile ; **(iii)** les trois bactéries injectées sont rapidement retrouvées dans les hémocytes, puis sont éliminées, rapidement pour *M. lysodeikticus* et plus lentement pour *V. anguillarum* ; **(iv)** il apparaît que les hyalinocytes participent également à l'élimination des bactéries, malgré leur faible capacité de phagocytose et le fait qu'ils ne contiennent pas de peptide antimicrobien.

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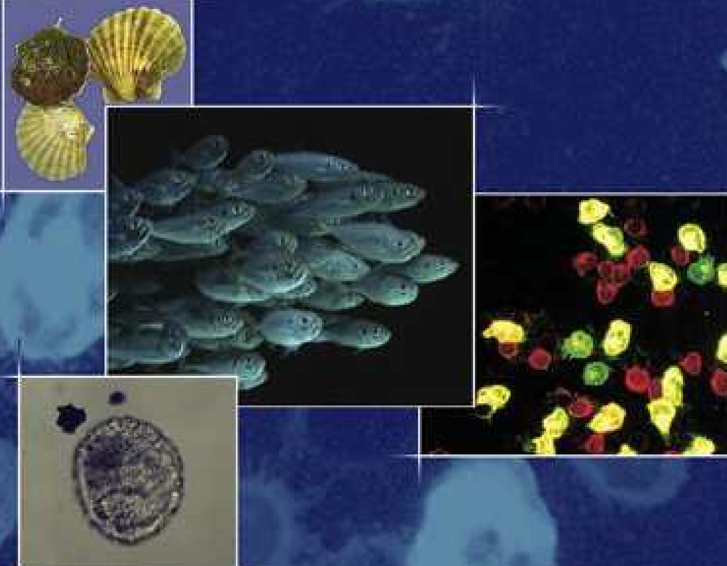

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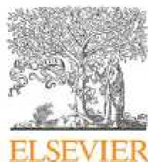

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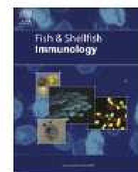

Differential involvement of mussel hemocyte sub-populations in the clearance of bacteria

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A B S T R A C T

Mussels are filter-feeders living in a bacteria-rich environment. We have previously found that numerous bacterial species are naturally present within the cell-free hemolymph, including several of the *Vibrio* genus, whereas the intra-cellular content of hemocytes was sterile. When bacteria were injected into the circulation of the mussel, the number of living intra-hemocyte bacteria dramatically increased in less than an hour, suggesting intense phagocytosis, then gradually decreased, with no viable bacteria remaining 12 h post-injection for *Micrococcus lysodeikticus*, 24 h for *Vibrio splendidus* and more than 48 h for *V ibrio anguillarum*. The total hemocyte count (THC) was dramatically lowered by the bacterial injections, as quantified by flow cytometry. *V. splendidus* induced the strongest decreases with −66% 9 h post-injection of living bacteria and −56% 3 h post-injection of heat-killed bacteria. Flow cytometry was used to identify three main sub-populations of hemocytes, namely hyalinocytes, small granulocytes and large granulocytes. When THC was minimal, i.e. within the first 9 h post-injection, proportions of the three cell categories varied dramatically, suggesting differential involvement according to the targets, but small granulocytes remained the majority. According to a decrease in their number followed by an increase (+90% at 12 h with living *V. splendidus*), hyalinocytes also appeared to be involved as cellular effectors of antibacterial immunity, despite possessing little capacity for phagocytosis and not containing antimicrobial peptides.

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1. Introduction

Bivalve hemocytes are responsible for cell-mediated immunity through a panel of activities such as phagocytosis [1], the release of cytotoxic and agglutinating molecules [2], and the production of several reactive oxygen intermediates ROIs) [3,4] and nitric oxide (NO) [5,6]. In addition, lysosomal enzymes [7], phenoloxidase/peroxidase activities [8–10] and lysozyme [11] were found within the granules of eosinophils [7,8]. Also associated with different granules are the antimicrobial peptides (AMPs) from *Mytilus edulis* and *Mytilus galloprovincialis*, acting against bacteria inside phagolysosomes, then released into the circulation [12,13]. Complex cell-signaling pathways occurring during the immune response emerged in different invertebrates, including the mussels [14], indicating close similarities with the mammalian kinase-mediated cascades.

The different cell types found in mussel hemolymph were described in detail in the early 1990s. The general assertion was that two cell categories existed in *M. edulis*: (i) hyalinocytes and (ii) granulocytes which might be further subdivided according to granule size [15]. Staining capacities also resulted in two cell types: (i) basophils (about 40% of the total hemocytes) including a large majority of hyalinocytes and (ii) eosinophils (about 60%) including small and large granules [16,17]. Also in the mussel, *M. galloprovincialis*, two cell types have been described on the basis of staining properties: (i) hyalinocytes with characteristics of undifferentiated cells, and (ii) granulocytes being acidophils, basophils or both [18]. Finally, monoclonal antibodies revealed three cell sub-groups in *M. edulis*: (i) basophilic granulocytes, (ii) a sub-group including basophilic granular and hyaline cells and (iii) eosinophilic granular cells [19].

Although granulocytes were largely suspected to play a prominent role in defense, few reports aimed to establish functional relationships between mussel hemocyte sub-populations and immune capabilities [20–22]. In previous studies on immune gene expressions in *M. galloprovincialis*, we showed that *HSP70* and *AMP*

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genes responded specifically to the challenges, confirming that at least some of the innate immune mechanisms are specifically orientated [23,24]. In addition, hemocyte sub-populations are capable of discriminating between two *Vibrio* species [25]. To compliment this, the present report addressed functional aspects bridging (i) differential clearance of Gram positive *versus* Gram negative bacteria, with (ii) variations in the total number of hemocytes, and (iii) the behavior of hemocyte sub-populations in response to various bacteria. To achieve such goals, mussels have been challenged with one injection of either *Vibrio splendidus* LGP32, *Vibrio anguillarum* or *Micrococcus lysodeikticus*. The remaining living bacteria have been quantified in hemocytes using the colony-forming unit (CFU) technique. Total circulating hemocytes and three sub-populations have been quantified by flow cytometry, considering both cell size and granularity.

2. Material and methods

2.1. Mussels and bacterial growth

Adult mussels, *Mytilus galloprovincialis* (6–7 cm shell length), were purchased from the marine farm Les Compagnons de Maguelone (Palavas-France). They were maintained in the laboratory in oxygenated sea water at 20 °C for 1–3 days prior to experimentation.

Vibrio splendidus LGP32 is a Gram negative marine bacterium isolated from juvenile oysters, *Crassostrea gigas*, during 2001 summer mortalities [26]. *V. anguillarum* was from the Institut Pasteur-France (ATCC 19264). Both *Vibrio* species (50 µl of overnight-cultured inoculum) were grown at 20 °C in 10 ml of trypsin–casein–soya (TCS, AES Laboratoire, Bruz-France) for 4–6 h to ensure bacteria were in exponential growth phase, centrifuged for 10 min at 500 × g, then adjusted to 10^8 bacteria/ml with phosphate buffered solution isotonic to sea water (PBS–NaCl: 2 mM KH_2HPO_4, 10 mM Na_2HPO_4, 3 mM KCl, 600 mM NaCl in distilled water, pH 7.4) according to 1 $OD_{600\ nm} = 5 \times 10^8$ bacteria/ml as established by counting the number of CFU. Gram positive *Micrococcus lysodeikticus* from Institut Pasteur-France (ATCC 4698) was grown at 37 °C in Luria Broth (LB, Sigma Chemical Co, St Louis, MO, USA) until the bacteria were in exponential growth phase, centrifuged for 10 min at 500 × g, then adjusted to 10^8 bacteria/ml with PBS–NaCl according to 1 $OD_{600\ nm} = 0.36 \times 10^8$ bacteria/ml as established by counting the number of CFU.

2.2. Bacteria injection and hemocyte sampling

Four batches of 10 mussels each per each sampling time point were injected with 100 µl (10^7 bacteria) into the posterior adductor muscle, through a hole created by light filing on their shells. After injection, mussels were returned to 20 °C sea water. Control injections consisted of 100 µl of PBS–NaCl. Four batches of 10 unchallenged mussels each (referred to as controls) were sampled at the time of the corresponding injection to minimize batch variations. The full experiment involved a grand total of 2520 mussels.

Hemolymph was collected from the posterior adductor muscle with a 1 ml syringe containing 120 µl of anti aggregate Alsever's solution, 0.5, 1, 3, 6, 9, 12, 24 and 48 h post-injection. To ensure the same quantity of hemolymph was collected from each mussel, we limited the sampling to 580 µl per mussel. Samples from 10 mussels were pooled and 3 ml were added to 3 ml of 3.7% formaldehyde in PBS–NaCl for later flow cytometry analysis. The remaining 4 ml were centrifuged (500 × g, 6 min, 4 °C), and the pellet of hemocytes was resuspended in 4 ml of PBS–NaCl for clearance measurement.

2.3. Clearance measurement

Aliquots of 50 µl of undiluted hemolymph or of hemolymph diluted 10- and 100-fold in PBS–NaCl, were plated in duplicate Petri dishes containing either TCS or LB agarose medium. CFU were counted after 24 h incubation at 20 °C for both *Vibrio* and 37 °C for *M. lysodeikticus*. Data from at least 2 consecutive dilutions (4 Petri dishes) were combined and presented as the arithmetic mean ± SEM. Statistically significant differences between some time point values were inferred from Student's *t*-test with $p < 0.05$.

2.4. Flow cytometry analysis

Aliquots (200 µl) from the formaldehyde-fixed hemocyte suspensions were added to 800 µl of PBS–NaCl. The samples were analyzed by flow cytometry (Cyflow® SL, data acquisition with Partec FloMax® software) using the parameters of relative size (FSC) and granularity (SSC). Each of the four different pools per time point were analyzed at least in duplicate. Data analysis and 3D graphs were generated with the software WinMDI 2.9 (Windows Multiple Document Interface for flow cytometry: http://facs.scripps.edu). A lower limit threshold was set up to exclude small events (<3 µm). Data are presented as the arithmetic mean ± SEM, with statistically significant differences between controls and selected time points values inferred from Student's *t*-test with $p < 0.05$.

3. Results

3.1. Clearance of bacteria

Previously, we have observed that no living bacteria could be recovered from circulating hemocytes collected from unchallenged mussels. In contrast, living bacteria from different species, including *Vibrio*, are normally present in the cell-free hemolymph as revealed by their capacity to form CFU of various morphologies (not shown). Consequently, CFU counts from cell-free hemolymph appeared unreliable. In our assays, the use of *Vibrio* selective thiosulfate citrate bile salts sucrose (TCBS) medium resulted in highly variable and largely under-estimated numbers of CFUs compared with the number of CFU with *Vibrio*-like morphology obtained with non-selective TCS medium (unpublished data). As a consequence, the present report considers only intra-hemocyte bacteria.

Following injection, both the quantities of living intra-hemocyte bacteria and the kinetics of clearance were dependent on the nature of the injected bacteria (Fig. 1). The three bacteria species were found inside hemocytes within the first hour post-injection. However, the number of recovered, living *V. anguillarum* 1 h post-injection (463 CFU) appeared about four times higher than the number of living *V. splendidus* (121 CFU, $p = 0.00011$) or *M. lysodeikticus* (133 CFU, $p = 0.00014$). Then, the number of CFU from the three bacteria species gradually decreased. *M. lysodeikticus* was the quickest to be eliminated as no living bacteria could be detected from 12 h post-injection onwards. Numerous CFU obtained from 6 h (239 CFU) to 24 h (261 CFU) post-injection samples revealed that injected *V. anguillarum* remained alive inside hemocytes but were reduced to 79 CFU at 48 h post-injection. Behavior of *V. splendidus* appeared different as, after a first minimum observed 3 h post-injection (60 CFU), a peak of living bacteria was observed 6 h post-injection (359 CFU, $p = 0.0008$) followed by a rapid decrease. Few living *V. splendidus* could be detected at 24 h post-injection (26 CFU).

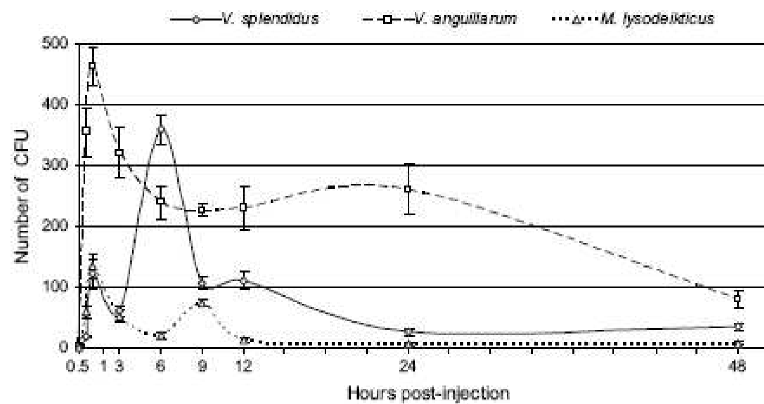

Fig. 1. Comparative numbers of living bacteria present in the circulating hemocytes from the mussel *Mytilus galloprovincialis* following one injection of Gram negative *Vibrio splendidus* and *V. anguillarum*, and of Gram positive *Micrococcus lysodeikticus*. Results are expressed as the mean ± SEM (bars) of CFUs counted in four different assays, each of them measured in duplicate.

3.2. Definition of hemocyte sub-populations

Flow cytometry was used to quantify the total hemocyte counts (THC) and the various cell types present in mussel hemolymph. Based on particle size and granularity (Fig. 2), five gates were set up distinguishing the three cell sub-populations as well as spermatozoa and debris, and any large aggregates, which were not considered in further analysis. The three cell groups included hyalinocytes, small and large granulocytes, as confirmed by optical microscope observations (not shown). Data inferred from the seven unchallenged mussel batches (referred to as controls), each of them including four pools of 10 mussels, revealed that among the $2.5 \pm 0.15 \times 10^6$ hemocytes per mussel, hyalinocytes represented 20% ($0.51 \pm 0.02 \times 10^6$), small granulocytes represented 51% ($1.27 \pm 0.02 \times 10^6$) and large granulocytes represented 29% ($0.71 \pm 0.02 \times 10^6$).

3.3. THC variations

Compared with the controls, one injection of living *V. splendidus* resulted in a statistically significant decrease in THC which reached a minimum of −66% ($p = 0.0001$) 9 h post-injection (Fig. 3). Normal THC was recovered 12 h ($p = 0.7$) post-injection and was above

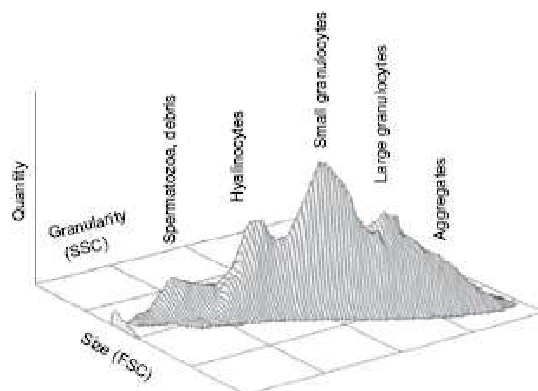

Fig. 2. Typical flow cytometry 3D graph of the hemolymph content from one pool of 10 unchallenged mussels. Five gates were set up to distinguish spermatozoa, hyalinocytes, small granulocytes, large granulocytes and aggregates.

normal 24 h ($p = 0.06$, not significant) to 48 h ($p = 0.02$, significant) post-injection. Injection of heat-killed *V. splendidus* also resulted in a statistically significant decrease in THC, but the minimum of −56% ($p < 0.0001$) was obtained 3 h post-injection, returning to normal 6 –9 h ($p = 0.1$) post-injection. Injection of living or heat-killed *V. anguillarum* resulted in a general decrease in THC 3 –12 h post-injection. Statistically significant minima were obtained with living *V. anguillarum*, 3 h (−33%, $p = 0.003$) and 12 h (−33%, $p = 0.003$) post-injection. Injection of *M. lysodeikticus* resulted in dramatic changes of THC with increase 1 h (+28%, $p = 0.04$) followed by a decrease 3 h (−31%, $p = 0.006$) post-injection with living bacteria, and a decrease 1 h (−47%, $p = 0.001$) followed by a prompt return to normal 3 h (+6%, $p = 0.3$) post-injection with heat-killed bacteria. THC of mussel injected with heat-killed *M. lysodeikticus* remained below normal even 48 h post-injection (−43%, $p < 0.0001$). Injection of PBS–NaCl did not statistically significantly modify the THC (data not shown).

3.4. Differential involvement of hemocyte sub-populations

According to the three gates which were set up for the flow cytometer, hyalinocytes, small and large granulocytes were quantified following the various injections (Fig. 4). Injecting living *V. splendidus* resulted in the decrease of the three sub-populations during the first hours post-injection. Minimum numbers of hemocytes were reached 9 h post-injection with small and large granulocytes being the most affected (−67%, $p = 0.005$, and −78%, $p < 0.0001$, respectively). The number of hyalinocytes was above normal 12 h (+90%, $p < 0.0001$) to 48 h (+59%, $p = 0.005$) post-injection, as well as the number of small granulocytes at 48 h (+14%, $p = 0.05$) post-injection, both cell categories being responsible for the elevated THC observed at these time points. The three sub-populations of hemocytes decreased in parallel following injection of heat-killed *V. splendidus* with minima reached 3 h post-injection: −55% of hyalinocytes ($p = 0.004$), −55% of small granulocytes ($p < 0.0001$) and −61% of large granulocytes ($p = 0.003$). In addition, the number of hyalinocytes and small granulocytes remained below normal even 48 h post-injection (−38%, $p = 0.005$, and −11%, $p = 0.02$, respectively).

Injection of living *V. anguillarum* resulted in several subsequent increases and decreases in the numbers of hyalinocytes and small granulocytes. The number of large granulocytes decreased 3 –6 h post-injection by about 60% ($p = 0.0007$). If small granulocyte numbers returned to normal 48 h post-injection, both hyalinocytes

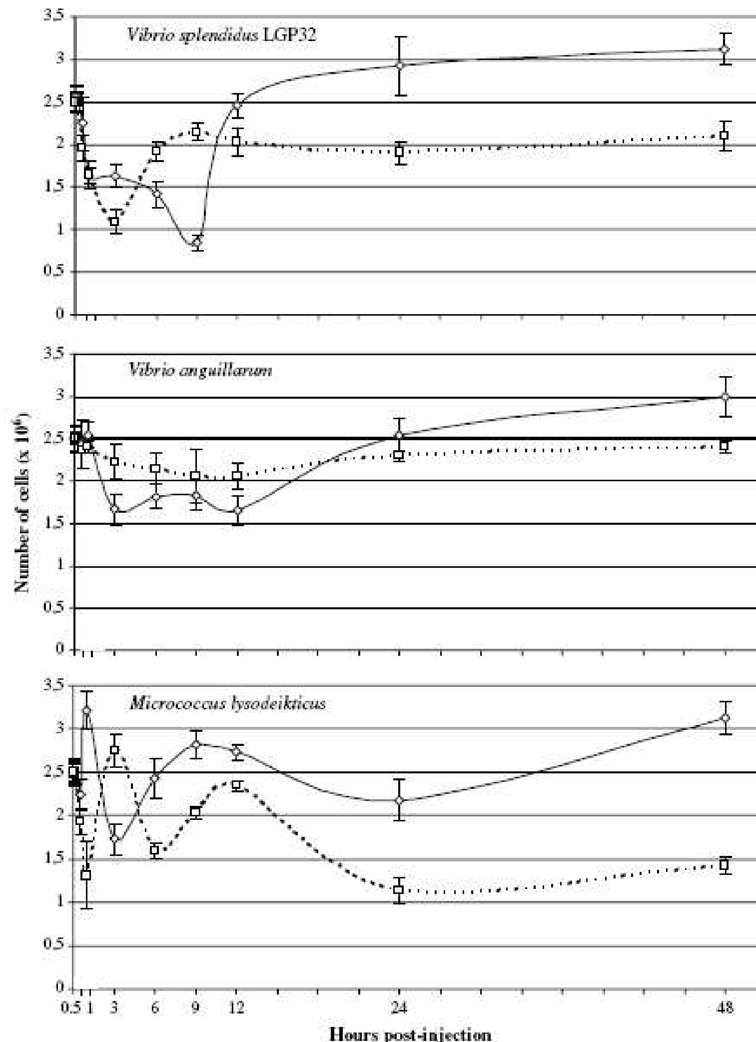

Fig. 3. Kinetics of THC collected from hemolymph of mussels injected with living (◇) or heat-killed (□) bacteria. Hemocytes were counted by flow cytometry on 2 aliquots of each of the 4 replicates per end point. Data are presented as the arithmetic mean ± SEM (bar).

(+47%, $p = 0.0003$) and large granulocytes (+31%, $p = 0.008$) remained elevated. Injection of heat-killed *V. anguillarum* resulted in a rapid decrease in the number of hyalinocytes (−29% after 30 min, $p = 0.003$) then returning gradually to normal. Small granulocytes were not statistically significantly affected, whereas large granulocyte number was reduced by 30%, 12 h post-injection ($p = 0.005$).

M. lysodeikticus injection resulted in a remarkable parallel evolution of the numbers of the three hemocyte sub-populations. Living *M. lysodeikticus* induced a general decrease 3 h post-injection (−32% of hyalinocytes, $p = 0.0009$, −30% of small granulocytes, $p = 0.0006$, and −35% of large granulocytes, $p = 0.0007$) followed by a general increase, ending 48 h post-injection with +34% of hyalinocytes ($p < 0.0001$), +25% of small granulocytes ($p = 0.001$) and +15% of large granulocytes ($p = 0.01$). Injecting heat-killed *M. lysodeikticus* induced only a light decrease in hyalinocyte number, with a minimum of −26% measured 9 h post-injection ($p = 0.005$). In contrast, both small and large granulocyte numbers decreased as quickly as 1 h post-injection (−49%, $p = 0.0003$, and −63%, $p < 0.0001$, respectively) and, after a brief increase 9 –12 h post-injection, they were still below normal values 48 h post-injection: −49% of small granulocytes ($p = 0.0004$) and −58% of large granulocytes ($p < 0.0001$).

4. Discussion

Numerous reports exist on the presence of bacteria in mussels, principally from the *Vibrio* genus [27–31]. As the aim of these studies was principally to trace human pathogens from seafood, bacterial growth assays were on diluted meat-and-liquor homogenates. Bacteria were suspected to be located at least in the gut, digestive gland and gills. In the Pacific oyster, *C. gigas* and the horse mussel, *Modiolus modiolus*, *Pseudomonas*, *Alteromonas*, *Aeromonas* and *Vibrio* were found in crude hemolymph [32]. In the present report, we found that cell-free hemolymph from unchallenged mussels contains numerous bacterial species, including several

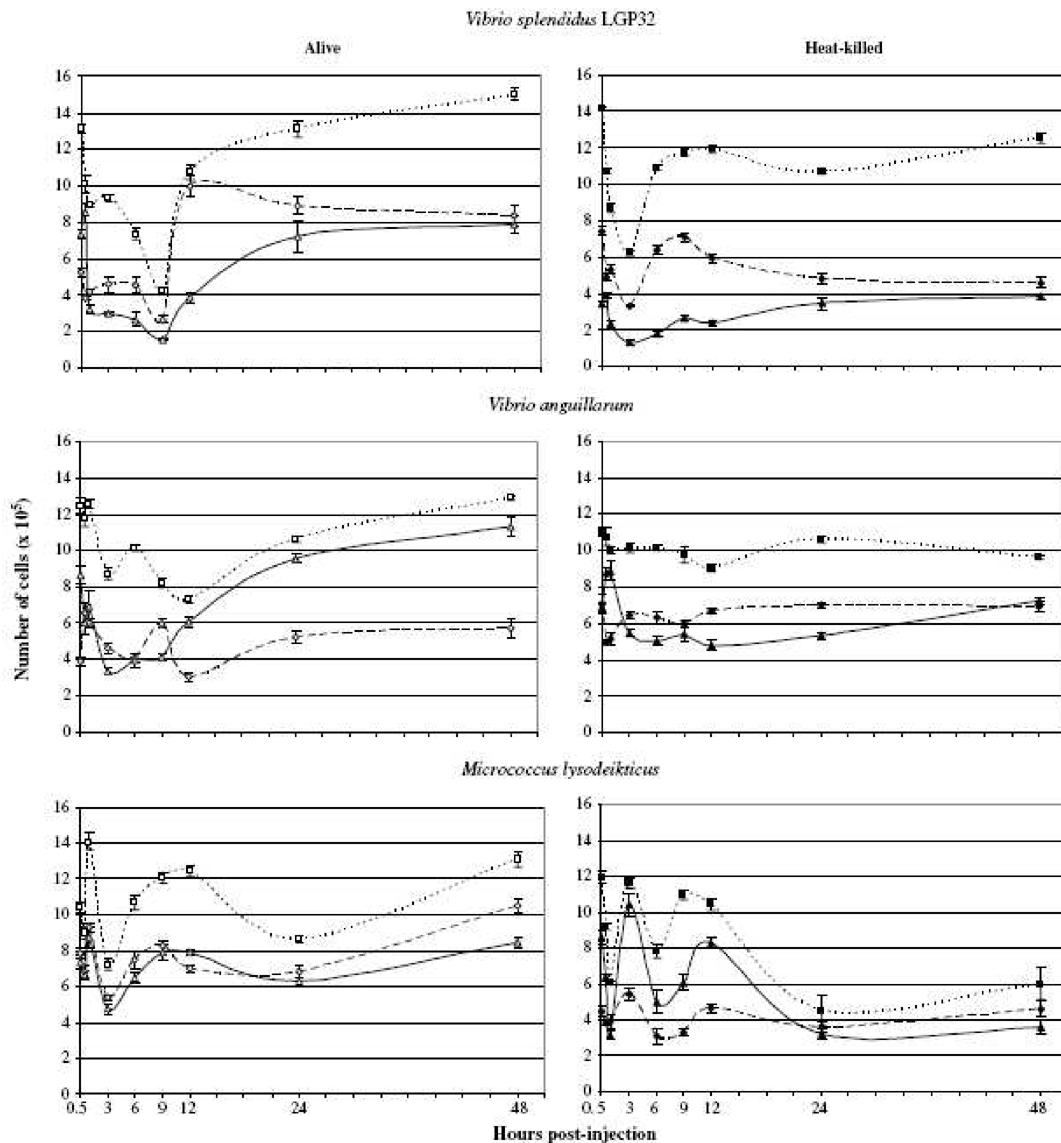

Fig. 4. Kinetics of hyalinocytes (◇), small granulocytes (□) and large granulocytes (△) collected from hemolymph of mussels injected with living (open symbols) or heat-killed (closed symbols) bacteria. Hemocyte sub-populations were determined and counted by flow cytometry on 2 aliquots of each of the 4 replicates per time point. Data are presented as the arithmetic mean ± SEM (bar).

Vibrio species, whereas the intra-cellular content of hemocytes was sterile. This means that bacteria from the hemolymph were not eliminated by phagocytosis due to circulating hemocytes, but tolerated as free micro-organisms. When injected into the circulation, numerous bacteria were rapidly found inside hemocytes, suggesting an intense process of phagocytosis during the first hour post-injection, as mentioned by previous reports on *in vitro* phagocytosis-associated activities [3,10].

Both Gram positive and Gram negative intra-hemocyte bacteria were gradually killed. However, living Gram positive *M. lysodeikticus* almost disappeared after 12 h post-injection, whereas *Vibrios* remained for longer times: 24 h for *V. splendidus* and up to 48 h for *V. anguillarum*. Interestingly, the majority of living phagocytised *V. splendidus* was at 6 h post-injection, compared to 1 h for *V. anguillarum*, suggesting hemocytes were reacting differently against the two *Vibrio* species. We previously reported similar different recognition/response concerning lysozyme gene expression [25]. The kinetics of clearance appeared very variable according to the particular bacteria/bivalve partners: elimination ranged from 2 h for *Escherichia coli* MG1655 in *M. galloprovincialis* [33], to 8 h for *E. coli* K-12 in the hard clam *Mercenaria mercenaria* [34], to 3 days for *Vibrio tapetis* (former *Vibrio* P1) in the carpet shell clam *Ruditapes decussatus* and 14 days in the Manila clam *R. philippinarum* [35], and to 13 days for *Vibrio vulnificus* in the American oyster, *Crassostrea virginica* [36]. This is not surprising considering that phagocytosis requires surface interactions prior to complex biochemical processes, as suspected long ago [37–39], reviewed by Canesi et al. (2002) [40] and still under investigation [41].

THC was dramatically affected by the bacterial injections. The effect of *M. lysodeikticus* was erratic during the first 3 h post-injection. *V. anguillarum* induced a minor decrease of about 36% of the normal THC during the first 12 h post-injection, whereas *V. splendidus* induced considerable THC decreases: −66% with living bacteria and −56% with heat-killed bacteria. Moreover, the decrease induced by living *V. splendidus* was maximum 9 h post-injection compared to 3 h post-injection of heat-killed *V. splendidus*. Comparable differing patterns observed with live versus killed bacteria have been reported for *V. tapetis* in *Ruditapes philippinarum*, i.e. faster elimination of killed bacteria [35]. One can hypothesize that such differences in kinetics resulted from the release of chemo-attractant factors by the living bacteria, whereas hemocytes briefly accumulated at the injection site of heat-killed bacteria, as reported for *E. coli* in the Eastern oyster, *C. virginica* [42]. The decrease we noticed after *Vibrio* injection is in agreement with our previous observations on mussels [12] but in opposition with the increase reported in other host-pathogen models: *V. tapetis* in the Manila clam, *Ruditapes philippinarum* [43,44], the protozoan parasite, *Haplosporidium nelsoni* in resistant Eastern oyster, *C. virginica* [45] or the protozoan parasite *Perkinsus marinus* and the Pacific oyster, *C. gigas* [46]. Not only pathogens/parasites significantly enhanced THC in mussels, but also experimental pollutants, such as copper or cadmium [47]. In our results, injection of living bacteria resulted in THC higher than normal 24 −48 h post-injection, suggesting multiplication and/or recruitment of hemocytes, a phenomenon never observed with the injection of heat-killed bacteria.

As in many invertebrates, mussel hemocytes appeared extremely heterogeneous when observed under the light microscope. The problem of their lineage is still under debate, principally due to the lack of specific markers. In addition, no clear stem cells, or hematopoïetic organ has been described. The classification of hemocytes on the basis of morphological, staining or labeling criteria does not give insight into the functionality of distinct sub-populations, and to focus on THC variations is not enough to explain physiological reactions. In the literature, the number of morphologically distinct mussel hemocyte sub-populations varied from two (hyalinocytes and granulocytes) [18,48] to four (large granulocytes, large semi-granulocytes, small semi-granulocytes and hyalinocytes) [22]. In agreement with Pipe et al. (1997) [16], the flow cytometer gates set in the present study identified three cell categories: large granulocytes (also referred to as eosinophils), small granulocytes (including both basophils and eosinophils) and hyalinocytes (exclusively basophils).

Confronted with bacteria *in vivo*, the general response of the mussels showed a rapid decrease of the three cell categories, confirming the decrease in THC. When THC were at their minima, i.e. within the first 9 h post-injection, proportions of the three cell categories varied dramatically, probably reflecting differential involvement according to the targets, but small granulocytes remained the majority. Although difficult to allocate one function to one cell category, it is clear that hyalinocytes also participate in the antibacterial response, despite being reported as showing characteristics of undifferentiated cells [18], not phagocytic, nor producing ROIs, but generating NO [22]. Meanwhile, other authors have reported that hyalinocytes were capable of phagocytosis, although they were less active than granulocytes [16,48]. The involvement of hyalinocytes was not through antimicrobial peptides as such immune effectors are stored into the granules of granulocytes [49] which accumulate at the bacteria injection site within the first 5 h post-injection [12]. If only granulocytes (small and large) were the immune active cells, their removal from the circulation by accumulation at the injection site would modify the percentage of circulating hyalinocytes, but not their number, which is not the case.

The present report confirms the existence of heterogeneous functionalities among circulating hemocytes. The presence of differentially engaged cell populations necessitates the existence of signaling molecules, reported in all vertebrates, but only putative lipopolysaccharide-induced TNF-α factor has been identified so far from the Zhikong scallop, *Chlamys farreri* [50] and the Pacific oyster, *Crassostrea gigas* [51], and interleukin-17 complete cds also from *C. gigas* [52]. There is no doubt that exploration of multiple mollusc EST libraries will give rise to major breakthroughs in the domain of cell-to-cell communication in invertebrates.

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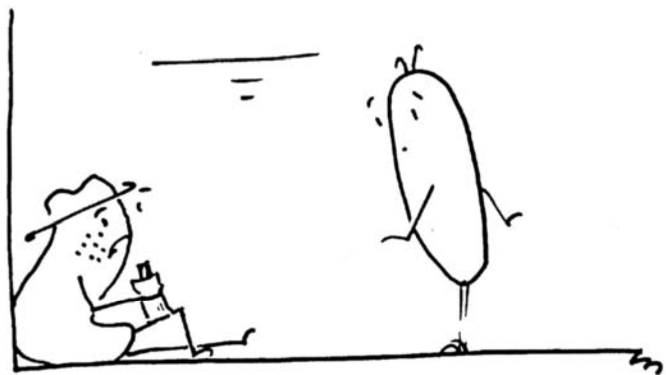
Chapitre 3



If you're right harsh, my lad, you can survive even in lysozyme.

(D'après « Funny Microbes », Leos Mandel)

Chapitre 3

Régulation de l'expression du gène du lysozyme

Dans le chapitre 2, nous avons vu l'évolution des trois populations d'hémocytes en réponse à des injections bactériennes. Dès ce chapitre, nous présenterons les résultats obtenus au niveau moléculaire sur l'expression du gène du lysozyme, après injection de bactéries vivantes et lors de deux stress de température : 30 et 5 °C. Cette expression a été quantifiée en Q-PCR durant 3 jours dans le cas des injections bactériennes et durant 24 h dans le cas des stress de température. Des précisions sur la nature et la localisation des cellules exprimant le lysozyme ont été apportées par la technique de l'ISH. Ce travail a été réalisé en coopération avec M-G. Parisi, doctorante au laboratoire d'Immunobiologie Marine de l'Université de Palerme (Italie).

Nos principaux résultats sont les suivants : **(i)** il y a une diminution générale de la quantité d'ARNm du lysozyme à la suite de l'injection de *V. splendidus*, tandis qu'une augmentation est enregistrée deux à trois jours après injection de *V. anguillarum* ou de *M. lysodeikticus* ; **(ii)** seule l'injection de *V. splendidus* produit une accumulation des hémocytes exprimant le lysozyme au niveau du site d'injection, soit dans le muscle d'adducteur postérieur ; **(iii)** le nombre totale d'hémocytes reste constant après l'injection de *V.anguillarum* ou de *M. lysodeikticus,* mais le pourcentage des hémocytes qui expriment le lysozyme augmente ; **(iv)** aucun de deux stress de température, ni l'injection d'une solution stérile de PBS-NaCl, ne change le profile d'expression du lysozyme. En conclusion, il semble que les hémocytes soient capables de faire la distinction entre la nature des différents stimuli.

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Lysozyme gene expression and hemocyte behaviour in the Mediterranean mussel, *Mytilus galloprovincialis*, after injection of various bacteria or temperature stresses

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Q-PCR;
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Mytilus;
Molluscs

Abstract The aim of the present study was to evaluate the expression of the *Mytilus galloprovincialis* lysozyme gene in different *in vivo* stress situations, including injection of bacteria *Vibrio splendidus* LGP32, *Vibrio anguillarum* or *Micrococcus lysodeikticus*, as well as heat shock at 30 °C and cold stress at 5 °C. Injection of *V. splendidus* LGP32 resulted in: (i) a general down-regulation of lysozyme gene expression, as quantified by Q-PCR; (ii) reduction in the number of circulating hemocytes; (iii) decrease in the percentage of circulating hemocytes expressing lysozyme mRNA which was now restricted to only small cells, as observed by ISH; and (iv) accumulation of hemocytes expressing lysozyme in the muscle sinus where injection took place. Injection of *V. anguillarum* or *M. lysodeikticus* induced significant up-regulation of lysozyme gene expression, but only 2—3 days post-injection, with no change in the total hemocyte counts but an increased percentage of hemocytes expressing lysozyme mRNA. Neither the control injection of PBS-NaCl nor temperature stress modified the lysozyme expression pattern. Consequently, the hemocyte population appears to be capable of discriminating between stress factors, and even between 2 *Vibrio* species.
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Introduction

Lysozymes are small ubiquitous antibacterial enzymes that hydrolyze β-1, 4-linked glycoside bonds of peptidoglycan, a major cell wall component of Gram-positive bacteria.

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Several studies have shown that lysozymes are also able to kill Gram-negative bacteria, demonstrated in bivalves [1,2] and in shrimp [3,4]. Anti-protozoan and anti-fungal activities of lysozymes, mediated by cleaving *N*-acetylglucosamine in chitin, have been also documented [5,6]. In addition, lysozyme can kill bacteria by non-enzymatic activity [7]. Thus, lysozymes appear to constitute an important component of immune defence against diverse microbial infections.

Several types of lysozymes have been purified, from the best-known chicken-type (c-type) and goose-type (g-type) [8], to the more recently identified invertebrate-type (i-type) [1,9]. Such i-type lysozyme has been identified in several bivalve molluscs, including the Icelandic scallop, *Chlamys islandica* [1], the blue mussel, *Mytilus edulis* [10], several mytilids and vesicomyids [11], and the Mediterranean mussel, *Mytilus galloprovincialis*, the hydrothermal-vent mussel, *Bathymodiolus azoricus* and the cold-seep clam, *Calyptogena* sp. [12]. Although numerous reports concern the different types of lysozymes and their activities [10,12–15], only the genes from the blue mussel, *M. edulis* [12], and from the Icelandic scallop, *Chlamys islandica* [16], have been sequenced. Curiously, the *M. edulis* lysozyme gene comprises five exons instead of the classical four exons of the c-type lysozyme gene, such as the *C. islandica* ones.

The Mediterranean mussel, *M. galloprovincialis*, is a filter-feeding bivalve and lysozymes are believed to be involved in digestive processes [17] as well as in host defence [18]. In *M. edulis*, lysozyme has been found localised within granular hemocytes [19] and higher levels of activity have been detected in hemocytes compared with plasma [20] in both *M. edulis* and the carpet shell clam, *Ruditapes decussatus* [21]. However, mRNA transcripts of g-type lysozyme were most abundantly expressed in gills, hepatopancreas and gonad, but only weak expression was evident in hemocytes and mantle from the Zhikong scallop, *Chlamys farreri* [22]. In the Pacific oyster, *Crassostrea gigas*, lysozyme mRNA was expressed in all tissues except the adductor muscle and ISH (*in situ* hybridization) analyses revealed strong expression in basophilic cells from the digestive tubules [23]. Similarly, ISH located lysozyme gene expression in the mantle and gill cells of the eastern oyster, *C. virginica*, with significantly higher mRNA content in labial palps and mantles than in gills, digestive glands and hemocytes [14]. For a long time, hemocytes have been considered as primary mediators of anti infectious defence, yet no work has been done on the kinetics of lysozyme gene expression in response to various challenges.

The aim of the present report was to study the kinetics of expression of the *M. galloprovincialis* lysozyme gene in response to various stress factors: high temperature, low temperature, and bacterial injection. Quantification of lysozyme transcripts was done using Q-PCR (quantitative polymerase chain reaction) with 28S ribosomal RNA as the house keeping gene. Visualisation of circulating hemocytes containing lysozyme mRNA was done by ISH and completed by histological observations of the posterior adductor muscles where injection took place.

Materials and methods

Bacterial growth and mussel challenges

Vibrio splendidus LGP32 is a Gram-negative marine bacterium isolated from juvenile oysters, *C. gigas*, during summer mortalities in 2001 [24], while *V. anguillarum* was from the Institut Pasteur-France (ATCC 19264). Both *Vibrio* species (50 μl of overnight-cultured inoculum) were grown at 20 °C in 10 ml trypsin-casein-soya (TCS, AES Laboratoire, Bruz, France) for 4–6 h to ensure bacteria were in the exponential growth phase, then centrifuged for 10 min at $500 \times g$, and adjusted to 10^8 CFU/ml with phosphate buffered solution isotonic to sea water (PBS-NaCl: 2 mM KH_2HPO_4, 10 mM Na_2HPO_4, 3 mM KCl, 600 mM NaCl in distilled water, pH 7.4) according to 1 $OD_{600\ nm} = 5 \times 10^8$ CFU/ml. *Micrococcus lysodeikticus* from the Institut Pasteur-France (ATCC 4698) was grown at 37 °C in Luria Broth (Sigma Chemical Co, St Louis, MO, USA) until bacteria were in the exponential growth phase, then centrifuged 10 min at $500 \times g$, and adjusted at 10^8 CFU/ml with PBS-NaCl according to 1 $OD_{600\ nm} = 0.36 \times 10^8$ CFU/ml.

Adult *M. galloprovincialis* were purchased from the marine farm Les Compagnons de Maguelone (Palavas, France) in May–June 2006 and April 2007. They were maintained in the laboratory in oxygenated sea water at 20 °C for 1–3 days prior to experimentation. Four batches of 10 mussels each per sampling end-point were injected with 100 μl (10^7 bacteria) into the posterior adductor muscle, through a hole created by light filing on the shells. After injection, mussels were returned to 20 °C sea water. Control injections consisted of 100 μl of PBS-NaCl. To test temperature stress, four batches of 10 mussels each per sampling end-point were subjected to 90 min immersion in sea water at either 30 °C or 5 °C, with their subsequent return to 20 °C sea water. Four batches of 10 untreated mussels each (referred to as untreated) were sampled simultaneously with each corresponding stress to minimize seasonal variations. Thus, the experiments involved a grand total of 2160 mussels.

Primers for lysozyme and 28S ribosomal RNA

Primers for lysozyme were designed from the *M. galloprovincialis* lysozyme mRNA (AF334665) [12]: forward 5′-ATGTGGAATCTGAAGGACTTGT-3′ (position 140–161) and reverse 5′- CCAGTATCCAATGGTGTTAGGG-3′ (position 486–507), giving an expected amplicon of 368 bp. Presence of a unique amplicon was checked by melting curve analysis (see Section 2.4) and gel electrophoresis on 2% agarose in Tris-borate-EDTA buffer stained with ethidium bromide. Specificity has been confirmed by several complete sequencings performed by Millegen (Labège, France).

28S rRNA was used as the house-keeping gene according to Cellura et al. [25] using the previously reported primer sequences: forward, 5′-AAGCGGAGGAAAAGAAACTAAC-3′ and reverse, 5′-TTTACCTCTAAGCGGTTTCAC-3′, giving an amplicon of 378 bp with a melting temperature of 90.17 ± 0.04 °C, also sequenced for confirmation of identity.

Hemolymph collection and cDNA synthesis

Hemolymph (0.8 ml per mussel) was collected from the posterior adductor muscle at 1, 3, 6, 9, 12, 24, 48 and 72 h post-injection, or immediately after the temperature shock (time 0) and after 3, 6, 9, 12, 15, 18 and 24 h recovery at 20 °C. Hemolymph was extracted into a 1 ml disposable syringe containing 0.2 ml of the anti-coagulant modified Alsever's solution (MAS) [26]. Hemolymph from 10 mussels was pooled and the hemocytes pelleted by 15 min centrifugation at 800 × g at 4 °C. Four pools of 10 mussels each, as replicates, were used for each sampling end-point. Total RNA was extracted with Trizol Reagent, according to the manufacturer's protocol (Invitrogen, Cergy Pontoise, France) and resuspended in 40 μl Tris-EDTA buffer. First strand cDNAs were synthesized from RNA adjusted to 5 μg using hexaprimers (Invitrogen, Cergy Pontoise, France) and murine leukemia virus reverse transcriptase (Promega, Charbonnières, France), and purified with Wizard SV gel and PCR clean-up system (Promega, Charbonnières, France), then kept in nuclease-free water at −20 °C until use.

Quantification of lysozyme transcripts by Q-PCR

Q-PCR was performed on the 4 pools for each sampling end-point of similarly treated mussels using SYBR Green chemistry on a LightCycler 480 384 well-plate (Roche Diagnostics, Meylan, France). The Q-PCR mixture contained the following: 1 μl first strand cDNA (10 ng), 0.75 μl of each specific primer at a concentration of 25 μM, and 2.5 μl of reaction mix (Roche Diagnostics, Meylan, France) containing FastStart Taq DNA polymerase, reaction buffer 2×, dNTP mix, SYBR Green 1 dye and $MgCl_2$. The PCR amplification programme started with initial Taq polymerase activation at 95 °C for 10 min, followed by 40 cycles at 95 °C for 10 s, 65 °C for 10 s and 72 °C for 15 s. Melting temperatures were measured by returning to 65 °C for 30 s and gradual heating to 95 °C. Negative control reactions contained sterile water in place of the cDNA template and were included in each run to ensure the absence of contamination. Calibration curves were obtained using 10-fold serial dilutions of lysozyme amplicon in 10 μg/ml sonicated salmon sperm DNA (Sigma-Aldrich Chimie, St Quentin, France).

Data analysis

Crossing point values expressed in cycle numbers were measured according to a threshold position of 4.2 and converted into equivalent target amounts (ETA) by the LightCycler 480 software, using calibration curves. Lysozyme expression level was calculated from the ratio of ETA for lysozyme on ETA for 28S rRNA. Normalization of the ratios was calculated considering each ratio equal to 1 in untreated mussels and expressed as x-fold the ratio for untreated mussels. Data were presented as the arithmetical mean of the four replicates ± SEM. Normality of data distribution was assayed using the Shapiro—Wilk test available at http://cran.fr.r-project.org. To compare the data of individual end-points with expression in untreated mussels, Student's *t*-test using t-Ease 2.8 ISI software was employed. Differences were considered significant when $p < 0.05$.

Tissue sampling

Hemocytes were collected in MAS and immediately fixed by dilution with 5 vol of 10% neutral buffered formalin (NBF: 46 mM Na_2HPO_4, 30 mM NaH_2PO_4, 3.7% formaldehyde in distilled water, pH 7). After centrifugation (800 × g, 15 min, 4 °C), hemocytes were resuspended in 10 ml 10% NBF and incubated overnight at 4 °C. After washing in 70% ethanol by centrifugation (800 × g, 15 min, 4 °C), hemocyte concentration was adjusted to 10^7 cells/ml and one drop of 5 μl was deposited on poly-lysine coated glass slides, air dried and kept at 4 °C. Total hemocyte numbers were determined in freshly collected hemolymph using Malassez' hemocytometer. Counts were done in duplicate for each of the 4 batches of untreated or experimentally treated mussels. Results are expressed as the arithmetic means ± SEM.

The adductor muscle from each sampled mussel was carefully detached from surrounding tissues and shell, and immediately immersed in Davidson fixative (33% of 95% ethanol, 22% of 37% formaldehyde, 11% glacial acetic acid and 34% distilled water). After 2 days of fixation, muscles were kept in 70% ethanol. They were embedded in paraffin after dehydration in gradual, increased ethanol concentrations, sectioned at 2 μm and laid on histological poly-lysine coated slides kept at room temperature.

Probe synthesis and ISH (in situ hybridization) assay

The lysozyme amplicon was synthesized by PCR using the same primers as for Q-PCR but using GoTaq DNA polymerase (Promega, Charbonnières, France). PCR conditions were: 2 min denaturation at 95 °C, followed by 30 cycles of 95 °C for 40 s, 60 °C for 30 s and 72 °C for 40 s, ended by final extension at 72 °C for 10 min. After analysis on 2% agarose gel, the PCR amplicon was cloned using TOPO TA Cloning kit (Invitrogen, Cergy Pontoise, France) with pCR II-TOPO plasmid and the inserted sequence controlled by sequencing (Millegen, Labège, France). Antisense and sense digoxigenin (DIG) labeled riboprobes were synthesized from the plasmid using the DIG RNA Labeling Kit (SP6/T7) (Roche Diagnostics, Meylan, France).

Cells on slides were permeabilised with 3 μg/ml proteinase-K (Sigma-Aldrich Chimie, St Quentin, France) for 10 min at 37 °C then fixed with 0.4% cold formaldehyde and rinsed 5 min with 2× sodium chloride/sodium citrate buffer (2× SSC: 150 mM NaCl, 15 mM sodium citrate in distilled water, pH 7). Histological sections of muscles were heated at 65 °C during 45 min, gradually hydrated in gradual, decreased ethanol concentrations, then permeabilised with 6 μg/ml proteinase-K for 10 min at 37 °C.

Lysozyme riboprobes (50 ng/ml) were denatured for 15 min at 95 °C, and hybridisation was performed overnight at 37 °C in a humid chamber with 50% formamide, 1× Denhard's, 5% dextran sulphate and 0.5 mg/ml salmon sperm DNA in 4× SSC. Unbound riboprobes were removed by washing twice (30 min, room temperature) with 2× SSC, then twice (5 min, 37 °C) with 1× SSC and twice (5 min, 37 °C) with 0.5× SSC. Cells and muscle sections were equilibrated for 5 min in Buffer 1 (1 M Tris—HCl, pH 7.5, 1.5 M NaCl) then incubated (15 min, 37 °C) with 500 μl of 0.5% blocking

reagent (Roche Diagnostics, Meylan, France) in Buffer 1. Cells and muscle sections were then incubated with sheep anti-DIG Fab fragments (Roche Diagnostics, Meylan, France) conjugated to alkaline phosphatase (30 min, 37 °C), then washed twice for 5 min in Buffer 1. The colorimetric reaction occurred during 3 h incubation in a freshly prepared solution of nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate (NBT/BCIP) (Promega, Charbonnières, France) diluted in Buffer 3 (100 mM Tris—HCl, pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$, 1% PolyVinyle Alcohol). The slides were subsequently rinsed with Buffer 4 (100 mM Tris—HCl, pH 8, 10 mM EDTA).

To enhance the contrast, counter-staining was done with 0.5% Bismarck Brown Y solution (Sigma-Aldrich Chimie, St Quentin, France). After being dehydrated in 95 and 100% ethanol and treated three times with non-toxic LMR-SOL solvent (Labo-Moderne, Paris, France), hemocytes and muscle sections were mounted with a cover-slip using the permanent mounting medium Histalaque (Labo-Moderne, Paris, France). Observations were made with a photonic microscope Leica DMR (Leica Microsystems, Wetzlar, Germany) equipped with a Leica DC200 camera.

Results

Specificity of lysozyme amplicon

The sequences of both mRNAs from *M. galloprovincialis* (AF334665) and the one deduced from *M. edulis* gene (AF334662) present a high degree of identity with only 43 nucleotides different out of 531, representing 8%. In addition, such differences were homogeneously spread all along the cDNA sequences with only a single substitution at each difference. The exception was 3 consecutive bases located in position 417—419. The only cDNA amplicon obtained in Q-PCR was of 368 bp (Fig. 1) and corresponded to the expected size deduced from the reported cDNA sequence. Amplicons from several Q-PCR were analyzed for DNA melting temperature revealing only one symmetrical peak at 82.88 ± 0.08 °C (Fig. 1). Definite confirmation of the primer specificity was obtained from several sequencings, showing total nucleotide sequence alignment with the expected *M. galloprovincialis* cDNA sequence reported in databank (AF334665) as belonging to i (invertebrate) type of lysozyme [12]. In addition, the nucleotide sequence of the *M. galloprovincialis* amplicon possessed only 45% and 51% identity with type-c lysozyme (*Marsupenaeus japonicus*, AB080238) and type-g lysozyme (*Chlamys farreri*, DF718947) including 9— 15 gaps to enforce alignment, respectively.

Relative concentrations of 28S rRNA transcript

The expression of 28S rRNA gene was measured by Q-PCR in all the cDNA samples collected from each time point. Samples collected from untreated mussels on the day of the corresponding challenge did not possess identical 28S rRNA content. ETA ranged from $3.69 \times 10^4 \pm 0.39 \times 10^4$ to $7.15 \times 10^4 \pm 0.56 \times 10^4$ according to the various batches of mussels, with no statistically significant differences (not shown). ETA was not stable throughout the duration of the various challenges but corresponded closely to the concentration in untreated animals (fold change from 0.77 to 1.19). None of the stress factors had a significant effect on 28S rRNA expression, confirming that the use of the 28S rRNA gene to normalize levels of gene expression in mussels is appropriate.

Effect of bacterial and PBS-NaCl injection on hemocyte lysozyme mRNA expression

Injection of *V. splendidus* LGP32 resulted in a general decrease in lysozyme mRNA levels as shown in Fig. 2. The maximum decrease of -3.47 ± 0.11- fold the *ratio* in untreated mussels ($p = 0.00001$) was observed 6 h after injection. Injections of both *V. anguillarum* and *M. lysodeikticus* resulted in an increase in lysozyme mRNA with a maximum observed after 48 h for *V. anguillarum* (4.15 ± 0.71; $p = 0.004$) and

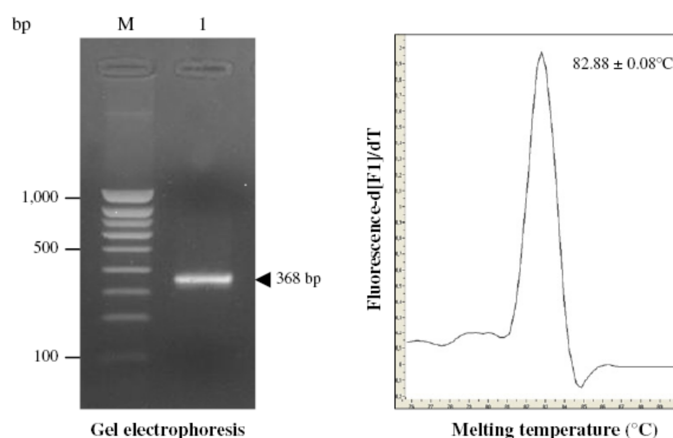

Figure 1 Gel electrophoresis migration and melting curve of Q-PCR lysozyme amplicon revealing the presence of a unique product of amplification at 368 bp and 82.88 ± 0.08 °C. M: DNA molecular weight marker. 1: Q-PCR amplicon.

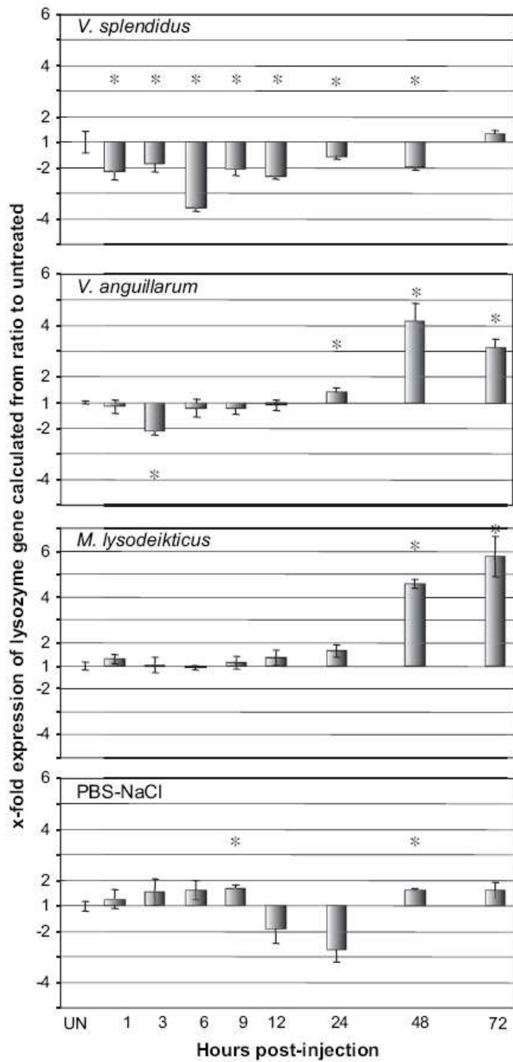

Figure 2 Kinetics of expression level of lysozyme gene following injection of *Vibrio splendidus* LGP32, *V. anguillarum*, *M. lysodeikticus* or PBS-NaCl. Values were inferred from 4 experiments performed in duplicate and plotted as mean ± SEM (bar). *: values statistically significantly different from untreated (UN) with $p < 0.05$.

72 h for *M. lysodeikticus* (5.78 ± 0.87; $p = 0.005$). Only a transient, statistically significant decrease was observed after 3 h for *V. anguillarum* (-2.10 ± 0.17; $p = 0.002$), whereas small, but statistically significant increases, were recorded with PBS-NaCl injection at 9 h (1.67 ± 0.14; $p = 0.033$) and 48 h (1.61 ± 0.07; $p = 0.19$).

Effect of heat shock and cold stress in hemocyte lysozyme mRNA expression

Limited increases in lysozyme mRNA levels were obtained after heat shock, with a maximum (but not significant value) of 2.60- fold the *ratio* in untreated mussels after 12 h of recovery (Fig. 3). The only statistically significant value was recorded after 3 h of recovery (2.16 ± 0.08; $p = 0.002$). In contrast, cold stress resulted in a general decrease in the quantity of lysozyme mRNA, being minimal after 15 h of recovery (-3.84 ± 0.08; $p = 0.022$), the only significant difference from untreated mussels.

Lysozyme expression in circulating hemocytes

ISH with antisense lysozyme riboprobe has been performed on hemocytes collected from all the sampling end-points of all the injected mussels, plus the untreated. In untreated mussels, labelling of lysozyme mRNA was observed only in some granulocytes (Fig. 4). The small hemocytes referred to as hyalinocytes appeared unstained. Six hours after injection of *V. splendidus* LGP32, the total number of circulating hemocytes was significantly lower than in untreated ($1.42 \times 10^6 \pm 0.14 \times 10^6$ versus $2.37 \times 10^6 \pm 0.22 \times 10^6$, $p = 0.005$; not shown) and very few hemocytes were labelled (Fig. 4). In addition, the labelling was restricted to small cells, the large cells, mainly granulocytes were no longer stained. Forty -eight hours after injection of *V. anguillarum* or *M. lysodeikticus*, numerous, but not all, small and large hemocytes were stained by the lysozyme riboprobe. At that time, total numbers of circulating hemocytes did not vary significantly from untreated, ranging from $2.50 \times 10^6 \pm 0.15 \times 10^6$ in the corresponding untreated to $3.00 \times 10^6 \pm 0.23 \times 10^6$ ($p = 0.094$) after injection of *V. anguillarum* and from $2.27 \times 10^6 \pm 0.21 \times 10^6$ in the corresponding untreated to $2.90 \times 10^6 \pm 0.19 \times 10^6$ ($p = 0.0502$) after injection of *M. lysodeikticus*. The specificity of hybridization was controlled by incubation with sense riboprobe, resulting in the absence of blue coloration.

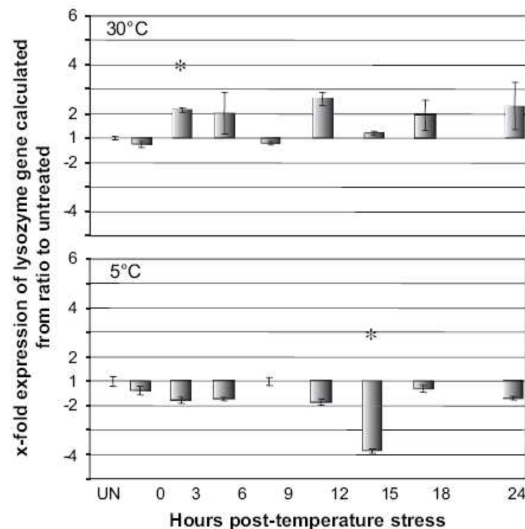

Figure 3 Kinetics of expression level of lysozyme gene after temperature stress at 30 °C and 5 °C. Values were inferred from 4 experiments performed in duplicate and plotted as mean ± SEM (bar). *: values statistically significantly different from untreated (UN) with $p < 0.05$.

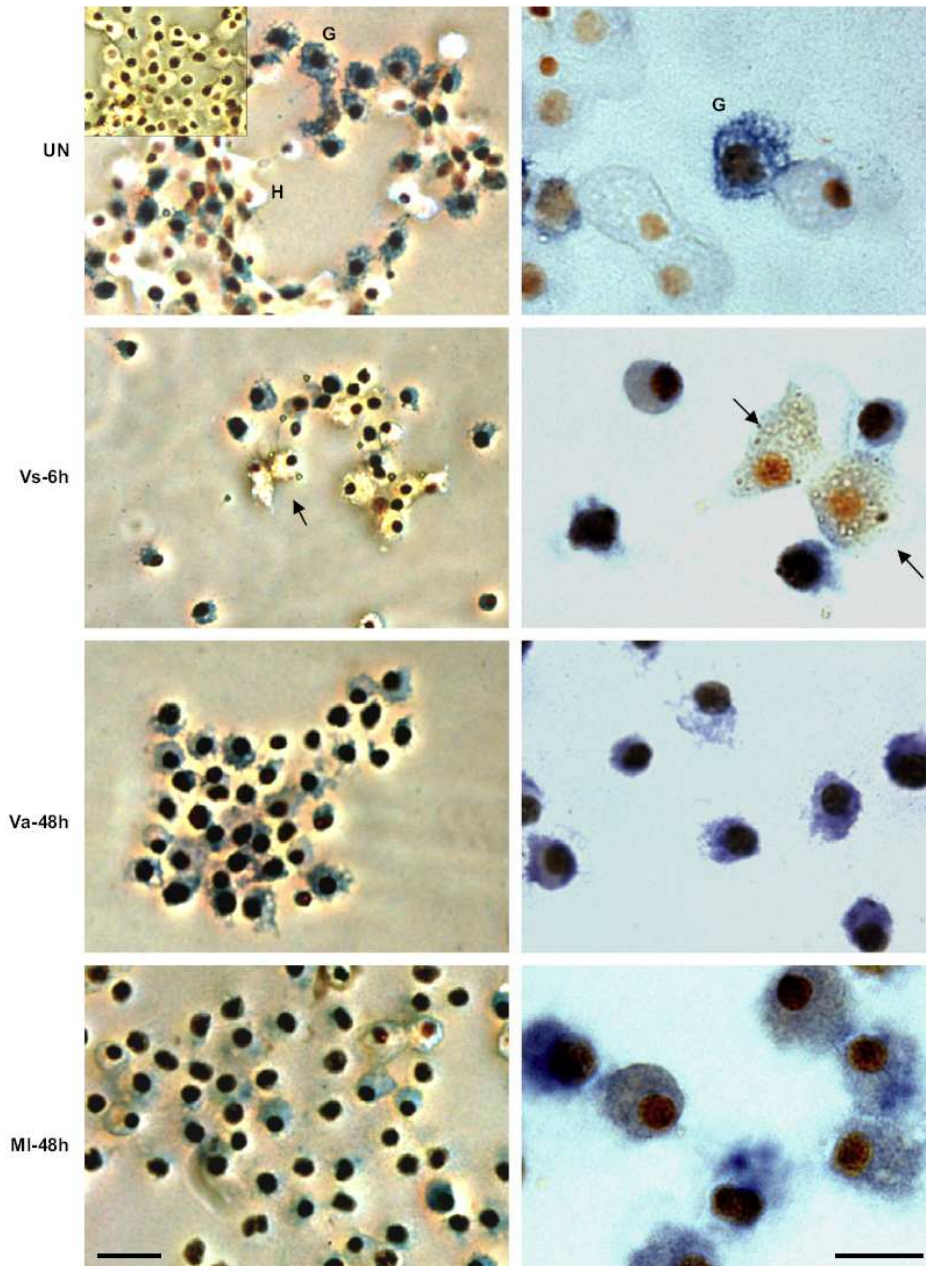

Figure 4 Optical microscopy observations of ISH on circulating hemocytes from untreated (UN) and bacteria-injected mussels revealing lysozyme gene expression in blue. Counter staining of nuclei was with Bismarck brown. Vs-6 h: *Vibrio splendidus* LGP32 6 h post-injection; Va-48 h: *V. anguillarum* 48 h post-injection; Ml-48: *Micrococcus lysodeikticus* 48 h post-injection. G: granulocytes expressing lysozyme gene; H: hyalinocytes not expressing lysozyme gene. Upper left window in UN corresponded to hybridization with sense lysozyme probe. Note the absence of blue coloration in granulocytes from Vs-6 h (arrows), the disappearance of granulocytes and the blue coloration of all the small hemocytes from Va-48 h, and the mixed population of blue granulocytes and small hemocytes from Ml-48 h. Magnification bars: 50 μm (left column) and 20 μm (right column).

Accumulation of hemocytes within muscle

Histological sections of adductor muscle from untreated mussels revealed the presence of narrow sinuses between the muscular fibres. The lumen of the sinuses contained few hemocytes including some labelled granulocytes, and liquid hemolymph was observed as open spaces after histological treatments (Fig. 5). Six hours after injection of *V. splendidus* LGP32, the sinuses were enlarged and the lumen was almost completely filled with numerous aggregated hemocytes, the cytoplasm of which being labelled. In muscles injected 48 h before with *V. anguillarum*, the size of the sinuses were similar to those of untreated mussels, but the lumen was completely filled with unstained aggregated hemocytes. In contrast, injection of *M. lysodeikticus* resulted in sinuses resembling the ones of untreated mussels, with open spaces, few hemocytes and rare labelling.

Discussion

For a long time, mollusc hemocytes have been reported to be responsible for bactericidal activity mediated by numerous toxic compounds, such as lysozyme [17], superoxide [27], lysosomal enzymes [21], nitric oxide [28], phenoloxidase [29] and antimicrobial peptides [30,31]. In addition, signal transduction pathways in *M. galloprovincialis* [32] and *Crassostrea gigas* [33,34] have been identified. Few studies, however, concentrated on the *in vivo* regulation of the immune genes, with those to date reporting that expression of immune-related genes have been modulated by physical stress, bacterial challenges or exposure to poly aromatic hydrocarbons [35–39]. In the present study, the kinetics of expression of the *M. galloprovincialis* lysozyme gene in response to different *in vivo* challenges was established. According to single melting curve of PCR amplicon, to total nucleotide sequence identity with type-i lysozyme, and to low nucleotide sequence identities with type-c and type-g lysozymes, we considered both mRNA quantification and ISH referred to the unique type-i lysozyme of *M. galloprovincialis*.

According to previous reports [25,39], we decided to maintain the quantification of 28S rRNA mRNA as representative of house keeping gene expression to be used in Q-PCR. However, the expression of 28S rRNA was not constant in untreated mussels collected during different weeks, at the time of the corresponding challenge. This observation confirmed the effect of the season on mussel physiology [29,40] and underlines the absolute necessity to perform the challenges during the same season when comparisons are required. In addition, no one gene expression is strictly unaffected by experimental treatments. In our challenges, the quantities of 28S rRNA transcripts varied. No significant down regulation was observed, and only the injection of *V. splendidus* LGP32 was able to significantly up-regulate this gene. Although mathematically significantly different ($p < 0.05$), the up-regulations were lower than 1.21- fold the expression in untreated mussels. Such slight variations may represent normal modulations of expression in a living organism. Consequently, and in agreement with the 4– 10-fold up-regulation previously observed for antimicrobial peptides (AMPs) and HSP70 [25,39], we decided that variations lower than 4- fold the one observed in untreated mussels will not be considered to reflect a biological response to the challenge.

Regarding lysozyme gene expression with this limitation, only up-regulations were observed, and only following *V. anguillarum* or *M. lysodeikticus* injection. Both were at the later end-points of 2– 3 days post-injection, revealing the delay in the triggering of the lysozyme gene expression. Curiously, *M. lysodeikticus* was the only bacterium not capable of up-regulating the gene expression of the 3 AMPs and of HSP70 [39]. Only *V. splendidus* LGP32 has been suspected to induce mortality in bivalves [24], but neither lysozyme (this report) nor mytilin, myticin and HSP70 [39] gene expressions were up-regulated after injection of *V. splendidus* LGP32. One can hypothesize relationships between the nature/structure of the injected bacteria and the immune-related gene response. Neither heat shock nor cold stress resulted in significant modulation of lysozyme gene expression. A much earlier study reported both individual and seasonal variability of hemolymph lysozyme activity in oysters [41]. More recently, heat shock was demonstrated to cause rapid induction of HSP70 and AMP myticin gene expression in *M. galloprovincialis* [39]. Subsequently, in the present study, lysozyme gene expression in hemocytes appeared variable, but not influenced by temperature shock.

The different cell types found in the hemolymph of mussels were reported in the 1990s. The general assertion was that two cell categories existed in *M. edulis*: (i) hyalinocytes; and (ii) granulocytes which might be subdivided according to granule size [42]. Staining capacities resulted also in two cell types: (i) basophils (about 40% of the total hemocytes) with a large majority of hyalinocytes; and (ii) eosinophils (about 60%) with small and large granules [43,44]. Also two cell types were described in *M. galloprovincialis*: (i) hyalinocytes with characteristics of undifferentiated cells; and (ii) granulocytes being acidophils, basophils or both [45]. Both phenoloxidase/peroxidase activities and lysozyme were found associated with the granules of eosinophils [19,29]. Despite the fact that monoclonal antibodies revealed 3 cell types [46], we decided to consider the two easily distinguishable categories, hyalinocytes and granulocytes. In the present report, lysozyme mRNA has been detected by ISH within the granulocytes in untreated mussels, however, injection of bacteria dramatically modified that profile. A few hours after injection of *V. splendidus* LGP32, the number of circulating hemocytes was dramatically reduced. The decrease in lysozyme mRNA transcript number revealed by Q-PCR was not due to the collection of less hemocytes, as cDNA concentrations had been adjusted to 10 ng in all the samples. ISH confirmed that few of the circulating hemocytes contained lysozyme mRNA, whereas histological observation revealed numerous hemocytes aggregated within the enlarged muscle sinuses and expressing the lysozyme gene. The accumulation of hemocytes containing mytilin has been also observed after bacterial challenge [47]. Presumably, this neutralizes localized infection, yet accumulation of hemocytes at the injection site resulted at least temporarily in lowering the number of circulating immune cells, consequently reducing the capacity to counter infection in another location.

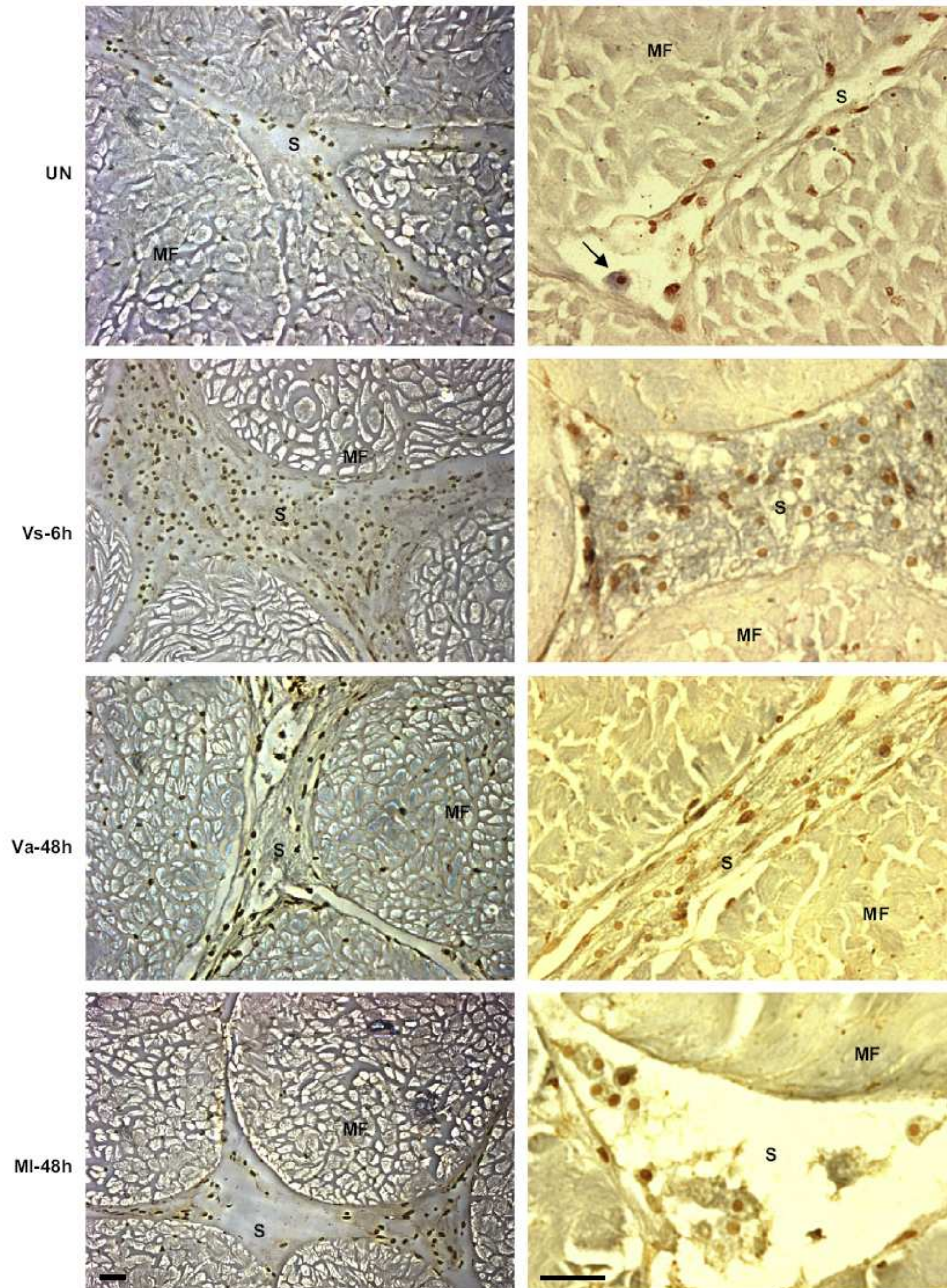

Figure 5 Optical microscopy observations of ISH on posterior adductor muscle from untreated (UN) and bacteria-injected mussels revealing lysozyme gene expression. Counter staining of nuclei was with Bismarck brown. Vs-6 h: *Vibrio splendidus* LGP32 6 h post-injection; Va-48 h: *V. anguillarum* 48 h post-injection; Ml-48: *Micrococcus lysodeikticus* 48 h post-injection. MF: muscle fibers; S: sinus. Note the open clear sinus in UN containing one blue stained granulocyte (arrow), the enlarged sinus containing aggregated blue stained hemocytes from Vs-6 h, the accumulation of unstained aggregated hemocytes within narrow sinus from Va-48 h, and the open sinus containing few free hemocytes from Ml-48 h. Magnification bars: 50 μm.

The response to *V. anguillarum* was totally different, with no difference in the number of circulating hemocytes but some accumulation in the muscle sinuses. The numerous hemocytes containing lysozyme observed in circulation, from both large and small cells, may explain the up-regulation detected by Q-PCR. Similarly, *M. lysodeikticus* challenge resulted both in up-regulation of the lysozyme gene expression, and observations of numerous hemocytes containing lysozyme in circulation. No obvious accumulation, however, was observed in the muscle sinuses and the number of circulating hemocytes was not significantly different to those from untreated animals, revealing different responses of the hemocytes according to the nature of the injected bacteria.

Other studies using clams have shown that defence-related factors were modulated in hemolymph in response to *V. tapetis* injection. For instance, lysozyme activity of cell-free hemolymph significantly increased 24—72 h post-challenge in *Ruditapes philippinarum* [48], as we observed in *M. galloprovincialis* challenged with both *V. anguillarum* and *M. lysodeikticus*. In contrast, no correlation was found between lysozyme activity from cell-free hemolymph and infection by the protozoan, *Bonamia ostreae* in the flat oyster, *Ostrea edulis* [49]. On the contrary to observations in mussels, lysozyme of the shrimp, *Litopenaeus vannamei*, is expressed in nearly all the circulating hemocytes [50]. Meanwhile, the shrimp response to an injection of *V. campbellii* resembled the one of *M. galloprovincialis* after injection of *V. splendidus* LGP32: i.e. decrease in lysozyme mRNA within the first hours following injection, with simultaneous recruitment and accumulation of hemocytes at the injection site.

In summary, based on quantification of lysozyme gene expression, total hemocyte counts, observation of hemocytes containing lysozyme mRNA and hemocyte accumulation at the site of injection, it appeared that hemocyte populations reacted differently according to the nature of the stress. Hemocytes were able to differentiate amongst bacterial species, even between two *Vibrio* species, whereas physical stress or PBS-NaCl injection did not strongly modulate the expression of the lysozyme gene. Further studies must address the behaviour of the other known immune molecules in order to obtain a complete overview of an innate immune response.

Acknowledgments

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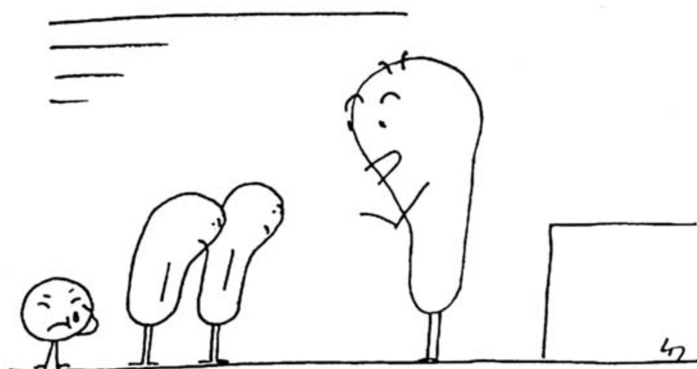
Chapitre 4



(D'après « Funny Microbes », Leos Mandel)

Chapitre 4

Effet de l'origine géographique des moules sur la capacité d'expression des gènes immunitaires

Comme mentionnés dans le chapitre 3, les mêmes stimulations (trois bactéries et deux températures) ont été employées sur les moules *Mytilus galloprovincialis* provenant de différentes localisations européennes : (i) l'océan Atlantique avec la Ria de Vigo en Espagne, (ii) le Golfe du Lion avec l'Etang du Prévost en France, et (iii) la Lagune de Venise en Italie, afin de tester l'effet de leur origine géographique sur leur capacité d'immunité innée. L'expression de divers gènes immunitaires (*défensine*, mytiline *B*, myticine *B* et *lysozyme*) et d'un gène de la famille des protéines de stress (*HSP70*), considéré comme répondant à un stress non-spécifique, a été quantifiée par la même technique de Q-PCR, et cette expression a été suivi sur la même durée que pour le *lysozyme* dans le chapitre 3.

Ce travail a été rendu possible grâce au contrat européen Imaquanim et a été réalisé en collaboration avec l'Instituto de Investigacions Marinas du CSIC à Vigo-Espagne, le Département de Biologie de l'Université de Padova-Italie et l'Istituto Zooprofilattico Sperimentale delle Venezie, Département de Physiologie des Poissons à Legnaro-Italie, au cours de plusieurs de mes visites dans ces laboratoires.

Les principaux résultats obtenus sont les suivants : **(i)** confirmation que les cinq gènes étudiés sont constitutivement exprimés dans les hémocytes de moule, le gène de la *défensine* étant le moins exprimé et celui de la *myticine* B le plus exprimé ; **(ii)** les moules provenant de la Ria de Vigo ont un niveau d'expression le plus bas pour les cinq gènes ; **(iii)** en général, les injections bactériennes ont provoqué une diminution de l'expression des gènes, principalement de ceux de la *mytiline* B et de la *myticine* B ; **(iv)** une élévation de la température a globalement augmenté l'expression des cinq gènes, sauf pour celui de la *mytiline* B ; **(v)** une baisse de la température n'a pas d'effet sur les gènes étudiés, sauf une augmentation significative sur le *défensine* observée chez la moule du Lagune de Venise. En conclusion, nos résultats renforcent l'idée de la spécificité de la réponse d'immunité innée. En outre, l'origine géographique des moules a fortement influencé la nature et l'intensité de l'expression des gènes immunitaires, suggérant un effet environnemental important sur l'immunité innée des animaux.

Ce chapitre correspond à une publication qui sera soumise au journal *Developmental and Comparative Immunology.*

Influence of the geographic origin of the mussels, *Mytilus galloprovincialis*, on the expression of some immune genes in response to experimental challenges

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Key words: antimicrobial peptide, defensin, mytilin, myticin, lysozyme, HSP70, real-time PCR, ISH, Vibrio, heat chock, innate immunity, *Mytilus*, Molluscs

Abstract

Mussels live in diverse environments in which they experience various physical, chemical and biological conditions. Here, we considered the expression levels of four immune genes (*defensin*, *mytilin B*, *myticin B* and *lysozyme*) and *HSP70* in *Mytilus galloprovincialis* collected during June-July from three different geographical locations (Atlantic Ocean-Ria de Vigo (RV-Spain), French Mediterranean Gulf of Lion-Prévost laguna (PP) and Northern Adriatic Sea-Venice (VI-Italy) in response to injection of three bacterial species or PBS-NaCl, and to heat shock or cold stress. We confirmed that the five genes are constitutively expressed in hemocytes, and that expression levels were different according to the considered genes, *defensin* being the less expressed, *myticin* B the highest. New was the fact that the same gene was differentially expressed according to the origin of the mussels, biggest difference being for *HSP70* and *lysozyme*. In addition, the five considered genes were statistically significantly less expressed in mussels from RV, the mussels living in the coldest water. The five

considered genes were differentially regulated according to the nature of the challenge. Most frequent effect of bacterial injections was down-regulation, particularly important for *mytilin B* and *myticin B*. Heat shock resulted in general up-regulation, except for *mytilin B*, whereas cold stress had no effect, except up-regulation of *defensin*, re-enforcing the idea of specificity of the innate immune response. In addition, the geographical origin of the mussels strongly influenced the nature and the intensity of the gene expression responses, suggesting important environmental influence.

1. Introduction

Mussels are common bivalve species capable to develop throughout the world seashores, including estuaries. Consequently, mussels experience a wide range of environmental conditions at these locations, and their ability to accumulate toxins (Dyrynda et al., 1997a; Marigomez et al., 2006) and human pathogens (Canesi et al., 2002; Croci et al., 2002; Hernroth et al., 2002; Le Guyader et al., 2000) has made them popular organisms extensively used in various biomonitoring programmes (Hamer et al., 2004; Viarengo et al., 2007). Most of these programmes aim to compare different biological capabilities between mussels from clean to contaminated areas, the oldest being the Mussel Watch (Goldberg, 1975). By means of chemical analyses and biomarker measurements mussels are used as bioindicator organisms of environment quality, therefore useful for evaluating/modeling ecological risk and for taking management decisions in sites defined as polluted or under recovery, e.g. detection of new environmental contaminants as for instance compounds with endocrine disruption potential, preventing fishing, prioritization of recovery actions, assessment of the effectiveness of actions undertaken. For instance, the level of total cytochrome P-450 and lipid peroxidation increased in the digestive gland tissue along a gradient towards the “Aegean Sea” oil spill in Galicia-1992 (Solé et al., 1996), elevations in hemocyte numbers and decreases in superoxyde generation and phagocytosis were observed following the “Sea Empress” oil spill in South West Walles-1996 (Dyrynda et al., 1997a), the level of stress-70 proteins was higher in urban and industrial polluted areas than near a mariculture area in Croatia (Hamer et al., 2004), and acetyl-CoA oxidase activity, lysosome membrane stability and hemocyte type replacement were modulated by the “Prestige” oil spill in the Bay of Biscay-2002 (Marigomez et al., 2006).

Numerous reports were on the *in vivo* effect of xenobiotics measured on mussels which have been displaced from a clean area to a contaminated one (Bodin et al., 2004; Honkoop et al., 2003). Other studies were on the effect of laboratory based experimental stressful situations on immune parameters in Bivalves, considering hemocyte counts and mortality, phagocyte-, cytotoxic-, metallothionein-, lysosomal enzyme and phenoloxidase-activities, lysosome membrane stability, production of intracellular superoxide, effect of cytokines, serum lysozyme concentration, and killing of *E. coli*. More recently, the development of suppression substractive hybridization method (Boutet et al., 2004; Escoubas et al., 1999; Gestal et al., 2007) and of EST technology (Jenny et al., 2002; Peatman et al., 2004; Song et al., 2006) revealed large panels of genes involved in immunity/stress response. Very prolific were the EST libraries constructed from pathogen-challenged oysters (Roberts et al., 2008; Tanguy et al., 2004) and mussels (Pallavicini et al., 2008b) with the discovery of new immune-related genes, including antimicrobial peptides (Roberts et al., 2008).

Here, we considered another aspect, i.e. the immune gene expression levels of *Mytilus galloprovincialis* collected in three different geographical locations in response to the same bacterial species. The three locations correspond to intensive mussel cultivation areas but with different environmental conditions: Atlantic Ocean-Ria de Vigo (Spain), French Mediterranean Gulf of Lion-Prévost laguna and Northern Adriatic Sea-Venice (Italy). Mussels were injected with the same dose of same bacterial species or submitted to high or low temperature stress. Some immune-related gene expressions were quantified: *defensin*, *mytilin B*, *myticin B* and *lysozyme*. *HSP70* was included as representing the most universal response to a variety of insults, not specific to immunity. As mussels rely only on innate immunity, considered as non specific and non adaptable, any differential gene expression response between the three populations will suggest differences in their innate immune capabilities, supposedly resulting from adaptation to the environment.

2. Material and methods

2.1. Geographic origin of mussels

Adult mussels, *Mytilus galloprovincialis* (6-7 cm shell length), were collected in three different locations during June-July 2005-2007 (Fig. 1). Mussels from Ria de Vigo (Vigo, Galicia-Spain) and referred as RV, were purchased from the shellfish farm Mariscos Ria de

Vigo S.L. Mussels from the French Mediterranean coast were purchased from the marine farm Les Compagnons de Maguelone (Prévost laguna, Palavas-France) and referred as PP. Mussels from the Adriatic Sea were purchased from the marine farm Mitilpesca (Alberoni, Venice-Italy) and referred as VI. They were acclimated for 24 h in the laboratory of the corresponding co-authors (Vigo, Montpellier and Padova) in a flow-through system of oxygenated sea water before any treatment.

2.2. Bacterial growth

Vibrio splendidus LGP32 is a Gram-negative marine bacterium isolated from juvenile oysters, *Crassostrea gigas*, during 2001 summer mortalities (Weber et al., 2003). *V. anguillarum* was from the Institut Pasteur-France (ATCC 19264). Both *Vibrio* species (50 µl of overnight-cultured inoculum) were grown at 20°C in 10 ml trypsin-casein-soya (TCS, AES Laboratoire) for 4-6 h to ensure bacteria were in exponential growth phase, centrifuged for 10 min at 500 x g, then adjusted to 10^8 bacteria/ml with phosphate buffered solution isotonic to sea water (PBS-NaCl: 2 mM KH_2HPO_4, 10 mM Na_2HPO_4, 3 mM KCl, 600 mM NaCl in distilled water, pH 7.4) according to 1 $OD_{600\ nm}$ = 5 x 10^8 bacteria/ml as established by counting the number of colony forming unit (CFU). Gram positive *Micrococcus lysodeikticus* from Institut Pasteur-France (ATCC 4698) was grown at 37°C in Luria Broth (LB, Sigma) until the bacteria were in exponential growth phase, centrifuged for 10 min at 500 x g, then adjusted to 10^8 bacteria/ml with PBS-NaCl according to 1 $OD_{600\ nm}$ = 0.36 x 10^8 bacteria/ml as established by counting the number of CFU.

2.3. Mussel treatments and hemolymph collection

Four pools of 10 mussels each per each sampling time point were injected into the posterior adductor muscle with 100 µl (10^7 bacteria) through a hole created by light filing on shells. After injection, mussels were returned to sea water at the corresponding temperature (Fig. 1). Control injections consisted of 100 µl of PBS-NaCl. Temperature stresses consisted in immersion of the mussels for 90 min in sea water at either 30°C or 6°C. After the stress, mussels were returned to sea water at the corresponding temperature for recovery. Four pools of 10 unchallenged mussels each (referred as control) were sampled at the time of the corresponding injection to minimize batch variations. The full experiment involved a

minimum of 6,480 mussels (2,160 per location), some challenges being duplicated due to abnormal mortalities or technical failures.

Hemolymph (0.8 ml per mussel) was collected from the posterior adductor muscle at 1, 3, 6, 9, 12, 24, 48 and 72 h post-injection, or immediately after the temperature shock (time 0) and after 3, 6, 9, 12, 15, 18 and 24 h recovery, with a 1 ml disposable syringe containing 0.2 ml of the anti-coagulant modified Alsever's solution (Torreilles et al., 1999). Hemolymph from 10 mussels was pooled and hemocytes pelleted by 15 min centrifugation at 800xg, 4°C, then resuspended in 1 ml Trizol Reagent (Invitrogen) and stored at -20°C until used. Four pools of 10 mussels each, as replicates, were used for each sampling time point.

2.4. cDNA synthesis and quantitative PCR (Q-PCR)

All RNAs were extracted in Montpellier according to manufacturer's Trizol instructions and resuspended in 45 µl of Tris-EDTA buffer (TE: 10 mM Tris-HCl, pH 8.0, 1 mM EDTA). RNA concentrations were measured on spectrophotometer ND-1000 (NanoDrop Technologies). First strand cDNAs were synthesized on 5 µg of total RNA using hexaprimers (Invitrogen) and murine leukemia virus reverse transcriptase (Promega), purified through QIAquick Column (Qiagen) and then kept in nuclease-free water at -20°C until use.

All Q-PCRs were performed in Montpellier by the same scientist, Hui LI, using the SYBR Green chemistry on the same LightCycler 480 384 well-plate (Roche) to maximize reproducibility. Primer sequences and specificity controls were previously reported (Cellura et al., 2007; Li et al., 2008b). Q-PCR mixture contained the following: 1 µl first strand cDNA (10 ng), 0.75 µl of each specific primers at a concentration of 25 µM, 2.5 µl of mix (Roche) containing FastStart Taq DNA polymerase, reaction buffer 2x, dNTP mix, SYBR Green 1 dye and $MgCl_2$. The PCR programme started with initial Taq polymerase activation at 95°C for 10 min, followed by 40 cycles at 95°C for 10 sec, 65°C for 10 sec and 72°C for 15 sec. Melting temperatures were measured by returning to 65°C for 30 sec and gradual heating to 95°C. Negative control reactions contained water in place of cDNA template and were included in each run to ensure the absence of contamination. Calibration curves were obtained using 10-fold serial dilutions of the corresponding amplicon in 10 µg/ml sonicated salmon sperm DNA (Sigma). House keeping gene was represented by 28S ribosomal RNA, as previously validated (Cellura et al., 2006).

2.5. Q-PCR data analysis

Crossing point values expressed in cycle numbers were measured according to the threshold position of 4.2 and converted into equivalent target amount (ETA) by the LightCycler 480 built-in software (Roche) using statistical calibration curves. Expression level of the gene of interest was calculated from the *ratio* of ETA for the considered gene on ETA for *28S rRNA*. Normalization of the *ratios* was calculated considering each *ratio* equal to 1 in untreated mussels and expressed as x-fold the *ratio* for untreated mussels. Data were presented as arithmetical mean of the four replicates ± SEM. Normality of data distribution was assayed using the Shapiro-Wilk test available at http://cran.fr.r-project.org. To compare the data of individual end-point with expression in untreated, Student's *t*-test using t-Ease 2.8 ISI software was employed. Differences were considered as significant for $p<0.05$. Meanwhile, and according to previous report (Li et al., 2008b), we decided the variations lower than 4-fold the one observed in untreated mussels will not be considered to reflect a biological response to the challenge or temperature stress.

2.6. Riboprobe synthesis and in situ *hybridization (ISH) assay*

The different amplicons were synthesized by PCR using the same primers as for Q-PCR but using GoTaq DNA polymerase (Promega). PCR conditions were: 2 min denaturizing at 95°C, followed by 30 cycles of 95°C for 40 sec, 60°C for 30 sec and 72°C for 40 sec, ended by final extension at 72 °C for 10 min. After analysis on 2 % agarose gel, PCR amplicons were cloned using TOPO TA Cloning kit (Invitrogen) with pCR II-TOPO plasmid and the inserted sequence controlled by sequencing (Millegen). Antisense and sense digoxigenin (DIG) labeled riboprobes were synthesized from the plasmid using the DIG RNA Labeling Kit (SP6/T7) (Roche).

Collected hemocytes were immediately fixed by dilution with 5 vol of 10 % neutral buffered formalin (NBF: 46 mM Na_2HPO_4, 30 mM NaH_2PO_4, 3.7 % formaldehyde in distilled water, pH 7). After centrifugation (800xg, 15 min, 4°C), hemocytes were resuspended with 10 ml 10 % NBF and incubated overnight at 4°C. After washing in 70 % ethanol by centrifugation (800xg, 15 min, 4°C), 5 µl of hemocyte suspension was deposited on poly-lysine coated glass slides, air dried and kept at 4°C.

Hemocytes were permeabilised with 4 µg/ml proteinase-K (Sigma) for 10 min at 37°C then fixed with 0.4% cold formaldehyde and rinsed 5 min with 2X sodium chloride/sodium citrate buffer (2X SSC: 150 mM NaCl, 15 mM sodium citrate in distilled water, pH 7). Riboprobes (50 ng/ml) were denatured during 15 min at 95°C, and hybridisation was performed overnight at 37°C in humid chamber. Unbound riboprobes were removed by washing twice (30 min, room temperature) with 2X SSC, then twice (5 min, 37°C) with 1X SSC and twice (5 min, 37°C) with 0.5X SSC. Cells were equilibrated during 5 min in Buffer 1 (1M Tris-HCl, pH 7.5, 1.5 M NaCl) then incubated (15 min, 37°C) with 500 µl of 0.5 % blocking reagent (Roche) in Buffer 1. Cells were then incubated with sheep anti-DIG Fab fragments (Roche) conjugated to alkaline phosphatase (30 min, 37°C), then washed twice 5 min in Buffer 1. The colorimetric reaction occurred during 3 h incubation in a freshly prepared solution of nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate (NBT/BCIP) (Promega) diluted in Buffer 2 (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$, 1% PolyVinyle Alcohol). Slides were rinsed with Buffer 3 (100 mM Tris-HCl, pH 8, 10 mM EDTA).

To enhance the contrast, counter-staining was with 0.5% Bismarck Brown Y solution (Sigma). After being dehydrated in 95 and 100% ethanol and treated three times with non-toxic LMR-SOL solvent (Labo-Moderne), hemocytes were mounted with a cover-slip by using the permanent mounting medium Histalaque (Labo-Moderne). Observations and pictures were done with a photonic microscope Leica DMR (Leica Microsystems, Wetzlar-Germany).

3. Results

3.1. Comparative expression in untreated mussels

The five tested genes were constitutively expressed in hemocytes from mussels collected in the three geographical origins (Fig. 2). However, important differences were noticed. *Defensin* was the lowest expressed, with marginal statistically significant difference (p=0.050) only between RV and VI. Expressions of *mytilin B, myticin B, lysozyme* and *HSP70* in RV were the lowest with always statistically significant differences with expressions in PP and VI. Regarding these four genes, highest expressions were always in PP but without statistically significant difference. Maximum differences of expression were for *HSP70* being

120-fold less expressed in RV than in PP (p=0.017) and for *lysozyme* being 70-fold less expressed in RV than in PP (p=0.004). In contrast, no statistically significant differences were found in gene expressions between PP and VI. In each of the three populations, highest expression was for *myticin B,* with remarkable similar change in fold-of-expression compared to *lysozyme* and to *myticin B*.

3.2. Comparative expression following injection

Mussels from the three geographical locations received one injection of 100 µl containing 10^7 bacteria. The three bacterial species were from the same stock maintained in the Montpellier's lab and freshly prepared in location immediately before injection to ensure treatments will be with identical batch of bacteria. To be consistent with previous report (Li et al., 2008b), Figures 3 to 8 included a scale line at -4 and +4-fold changes in expression between which modulation of expression was considered as non-biologically significant.

3.2.1. Injection of *Vibrio splendidus* LGP32 (Fig. 3): Major effect was a down-regulation of *mytilin B* in PP (minus 120-fold) as soon as 1 h post-injection and lasting at least 12 h (minus 12-fold). In contrast, no effect on *mytilin B* was observed in RV or RI. *Myticin B* expression appeared down-regulated in the three locations, but not before 2 days post-injection. *Defensin* expression was moderately down-regulated in RV from 6 h to 72 h with a minimum of minus 7-fold at 24 h, and in PP (minus 8-fold at 48 h), but up-regulated in VI, from 1 h (20-fold) to 9 h (10-fold) post-injection. *Lysozyme* expression was not modulated by *V. splendidus* injection, except a down-regulation in RV from 1 h (minus 5-fold) to 3 h (minus 11-fold) post-injection. No effect was recorder concerning *HSP70* expression.

3.2.2. Injection of *Vibrio anguillarum* (Fig. 4): Only moderated down-regulations were recorded for *defensin* (minus 7-fold at 72 h) and for *mytilin B* (minus 5-fold at 9 h) expressions in PP. In contrast, *myticin B* expression was down-regulated in RV (minus 10-fold at 48 h) and particularly in VI (from minus 10-fold at 6 h to minus 27-fold at 72 h), but not in PP. No effect was observed regarding *lysozyme* and *HSP70* expressions.

3.2.3. Injection of *Micrococcus lysodeikticus* (Fig. 5): *Defensin* expression was moderately down-regulated in RV (minus 6-fold at 3 h), but up-regulated in PP (5-fold at 3 h) and in VI (13-fold at 1 h and 10-fold at 3 h). Meanwhile, down-regulation was also observed in VI, but

only 72 h post-injection (minus 17-fold). *Mytilin B* expression was not affected by *M. lysodeikticus* injection, except in PP in witch extreme down-regulation was recorded as soon as 1 h (minus 400-fold) and still at 24 h (minus 10-fold) post-injection. *Myticin B* expression was down-regulated only in VI, from 24 h (minus 7-fold) to 72 h (minus 58-fold) post-injection. *Lysozyme* expression was slightly up-regulated (5-fold at 72 h) in both RV and PP, whereas *HSP70* expression was up-regulated in RV (5-fold at 6 h, 8-fold at 12 h and 11-fold at 72 h) and down-regulated in PP (minus 5-fold at 1 and 3 h).

3.2.4. Injection of PBS-NaCl (Fig. 6): The only up-regulation observed was in PP regarding *defensin* (5-fold at 9 h, 17-fold at 48 h and 11-fold at 72 h). Down-regulations were recorded for *defensin* in RV (from minus 12-fold at 3 h to minus 25-fold at 48 h) and to a less extend for *mytilin B* in PP (minus 11-fold at 3 h). *Myticin B, lysozyme* and *HSP70* expressions were not modified.

3.3. Comparative expression following temperature stress

3.3.1. Heat shock (Fig. 7): *Defensin* expression was up-regulated in both RV and VI with a maximum at 24 h with 7-fold and 20-fold, respectively. In contrast, *mytilin B* expression was only slightly down-regulated in PP (minus 4.5-fold at 3 h and 18 h). *Myticin B* expression was up-regulated in RV as soon as the end of the shock (7-fold) with a maximum of 23-fold at 18 and 24 h, but only marginally in PP (6.3-fold at 15 h) and no in VI. *Lysozyme* expression started to be up-regulated in RV since the end of the shock (11-fold) with a maximum at 9 h (33-fold) and still 8-fold at 24 h. *HSP70* expression undergone general up-regulation in RV with 14-fold at the end of the shock, a maximum at 9 h (102-fold) and still 80-fold at 24 h, and in PP with 4.2-fold at 6 h and 5-fold at 15 h.

3.3.2. Cold stress (Fig. 8): The only biologically significant modulation was the strong up-regulation of *defensin* expression in VI, with 27-fold at the end of the cold stress, and 8-fold after 12 h recovery. Whatever the geographical origin, no other gene was significantly modulated.

3.4. Changes in expression pattern in hemocytes from PP

Hemocytes from PP untreated mussels were incubated with DIG-labelled antisense AMP riboprobes. *Defensin* mRNA was observed in 68 % of the circulating hemocytes from

both granulocytes and hyalinocytes (Fig. 9), not all the granulocytes being labelled. In contrast, *mytilin* B and *myticin* B expressions were observed in about 7 % and 16 % of hemocytes, respectively, all belonging to small granulocytes. Expressions were also checked in hemocytes collected from some of the sampling point's post-bacterial injection. Both *defensin* and *myticin* B labelling patterns appeared as not modified by the injections. In contrast, *mytilin* B labelling was almost disappearing 3 h post-injection with *V. splendidus* or *M. lysodeikticus* (not illustrated).

4. Discussion

Mytilus galloprovincialis is the mussel species intensively farmed in the Mediterranean Sea and along the shores of Iberian Peninsula, although few hybrids with *M. edulis* were detected (Daguin et al., 2001). However, conditions of mussel farms from Ria de Vigo (Atlantic Ocean-NW Spain), Gulf of Lion (Palavas-France) and North Adriatic sea (Venice laguna-Italy), are different in many respects: temperature, UV, salinity, microbiological communities, nutrient content, density and cultural practices, for example. In contrast, due to the mode of cultivation on ropes always immersed, mussels used in this study were not submitted to rapid fluctuating habitats occurring during tidal episodes, such are changes in temperature, nutrients availability, salinity and oxygenation of the intrapallial water, possibly inducing stress responses.

Mussels were collected and treated during the months of June-July in the three locations to minimize fluctuations due to seasonal effect as well known for enzyme activities (Sheehan and Power, 1999), circulating hemocyte number (Carballal et al., 1998), protein content of hemolymph, lysozyme and agglutinin activities (Santarém et al., 1994), antibacterial response induced by LPS (Hernroth, 2003b), cytolytic activity (Ottaviani et al., 2000) and nitric oxide production (Novas et al., 2007). Concerning the expression of AMP genes, we demonstrated previously in Northern blot that *mytilin* and *myticin* were constitutively expressed in summer as well as in winter, whereas expression of *defensin* was not detectable in winter (Roch, 2001). In addition, expression of both *defensin* and *mytilin* were decreased following bacterial injection in winter, heat-shock resulting in no change in *mytilin* expression but suppression of *defensin* in winter and detectable expression in summer.

In the present report, we confirmed that *AMP* genes from mussels were constitutively expressed in hemocytes, as well as *lysozyme* and *HSP70* (Cellura et al., 2007; Li et al., 2008b),

and that expression levels were different according to the considered genes, *defensin* being the less expressed, *myticin B* the highest. New was the fact that the same gene was differentially expressed according to the origin of the mussels: *myticin B* was expressed 51-fold more in PP than in RV and *HSP70* was expressed 120-fold more in PP than in RV, for instance. In fact, the five considered genes were statistically significantly less expressed in mussels from RV. These mussels were also the ones living in the coldest water. No difference of gene expression was found between PP and VI, two mussel populations living at higher temperature. It is admitted that elevated temperature induced high metabolic state of the cells and high gene expression levels. We previously reported that heat shock did not provoke higher expression of *mytilin B* (Mitta et al., 2000a), *mytilin* (Cellura et al., 2007) or *lysozyme* (Li et al., 2008b) in mussels from PP, and the present data confirmed such results. Meanwhile, RV mussels behave differently with rapid and long-lasting up-regulations of *defensin*, *myticin B*, *lysozyme* and *HSP70*. It is not surprising that heat shock enhanced *HSP70* expression in animals living at low temperature. Cold stress did not enhanced *HSP70* expression in any of the three mussel populations, suggesting that such common gene is not triggered whatever the insult is. Similarly, *lysozyme* and *AMP* expressions were not affected by the cold stress, except a rapid and brief increase of *defensin* in VI, suggesting that these genes were not down-regulated by low temperatures. Adaptation to cold temperatures in the Atlantic coast and to warm temperatures in the Mediterranean coasts may explain the stronger response of RV mussels to heat shock compared to the Mediterranean mussels.

The main effects resulting from the injection of bacteria were observed at the *AMP* levels, with also the down-regulation of *lysozyme* expression after *V. splendidus* and the up-regulation of *HSP70* expression after *M. lysodeikticus* injection in RV mussels. Both *mytilin B* and *myticin B* expressions underwent remarkable parallel responses: (i) immediate down-regulation of *mytilin B* only in PP mussels, confirming previous data (Mitta et al., 2000a), (ii) late down-regulation of *myticin B* expression in the three populations after injection of *V. splendidus*, or in RV and VI after injection of *V. anguillarum*, or only in VI after injection of *M. lysodeikticus*. *Defensin* expression was also down-regulated in RV and PP mussels, as previously reported (Mitta et al., 2000a), except after injection of *M. lysodeikticus* in PP. In VI mussels, the expression of *defensin* gene appeared more complex with early up-regulation followed by late down-regulation after *V. splendidus* and *M. lysodeikticus* injection. In addition, relative quantities of AMP mRNAs were different within the same mussel population, *myticin B* being the most expressed, and between geographic areas, PP being the highest. Consequently, the biological meaning of variations appeared more complex than

suspected and the response to one bacteria species is not monogenic. Meanwhile, different bacteria provoked different gene expression responses, adding new data towards selectivity of innate immune response in molluscs (Cellura et al., 2007; Hernroth, 2003a) as demonstrated for insects (Imler and Hoffmann, 2000; Lemaitre et al., 1996).

Unexpectedly, control injections of PBS-NaCl also modulated the expression of *AMP* genes, with strong down-regulation of *defensin* in RV and to a less extend of *mytilin B* in PP, and up-regulation of *defensin* in PP. Only VI mussels were not affected by PBS-NaCl injection. *Myticin B*, *lysozyme* and *HSP70* genes did not respond to PBS-NaCl injection, validating the responses observed after bacterial injection, particularly concerning *mytilin B*, the most down-regulated gene.

Previous observations of AMPs in mussels revealed that 50 % of hemocytes contained defensin (Mitta et al., 2000c), which is in agreement with our observations performed by ISH at the mRNA level. Here, we evaluated in Q-PCR that *mytilin B* was 400-fold more expressed than *defensin*, which did not correspond to the low proportion of hemocytes (about 7 %) labelled with *mytilin B* riboprobe and to the 69 % of hemocytes labelled with anti-mytilin antibody (Mitta et al., 2000c). The discrepancy is even more intriguing concerning *myticin B*. We have no information on the percentage of hemocytes containing myticin, but *myticin B* was 27-fold more expressed than *mytilin B* with only the double of hemocytes containing *myticin B* mRNA. One can wonder that the delay to withdraw hemolymph and to prepare slides might explain the discrepancies of expression trends, which is probably not the case as hemocytes were fixed immediately after collection and appeared intact with numerous intracellular granules.

Defensin was reported as located in vesicles and in large clear granules of granulocytes (Mitta et al., 1999b). Using ISH, we observed the presence of defensin mRNA in numerous granulocytes, but also in hyalinocytes, even after bacterial injection, confirming our hypothesis on the active role of hyalinocytes in antibacterial immunity (Li et al., 2008a). Although observed in non-quantitative Northern, saline injection was reported as resulting in higher expression of *defensin* at 24 and 48 h (Mitta et al., 1999b), an unexpected effect we also observed in PP population by Q-PCR. Similarly, *defensin* was reported as over-expressed by heat shock, an effect we also observed. Mytilins are stored in granulocytes containing large and multivesicular granules (Mitta et al., 2000b) and we confirmed no hyalinocyte was labelled. The transient decrease of *mytilin B* expression following bacterial challenge was also confirmed herein.

Decrease of total hemocyte count (THC) was observed in mussels following injection of heat-killed bacteria (Mitta et al., 2000b), as well as of living bacteria (Li et al., 2008a; Li et al., 2008b). Such decrease in THC appeared specific to mussels, as the generally reported effect was an increase, as in the Manila clam, *Ruditapes philippinarum*, following *Vibrio tapetis* injection (Allam et al., 2006) or in the Pacific oyster, *Crassostrea virginica*, following exposure to the protozoa, *Perkinsus marinus* (La Peyre et al., 1995) or *Haplosporidium nelsoni* (Ford et al., 1993). However, resistance to the development of Brown Ring Disease symptom in the Manila clam was not related to the extend of hemocyte reaction measured following *in vivo* challenge, suggesting more complicated interactions (Allam et al., 2006).

In conclusion, we confirmed that hemocyte sub-populations and several immune-related genes are differentially regulated according to the challenge. In addition, the geographical origin of the mussels strongly influenced the constitutive level, the nature and the intensity of gene expression responses. Hopefully, the present picture of immune gene expressions given by the levels of a single isoform of each of the five genes should be confirmed and enriched by analyses on the ongoing first immunochip we constructed in mussels.

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	Geographical co-ordinates	Sea water temperature	Sea water salinity
RV	42°14'32 N – 08°48'26 O	17°C	33
PP	43°31'15 N - 03°54'33 E	20°C	37
VI	45°13'47 N - 12°15'59 E	27°C	32

Figure 1 - Geographic origin of the mussel populations used in these comparative studies: Ria de Vigo (RV), Prévost laguna-Palavas (PP) and Venice laguna-Italy (VI), with geographical co-ordinates and sea water temperature and salinity at the time of collection.

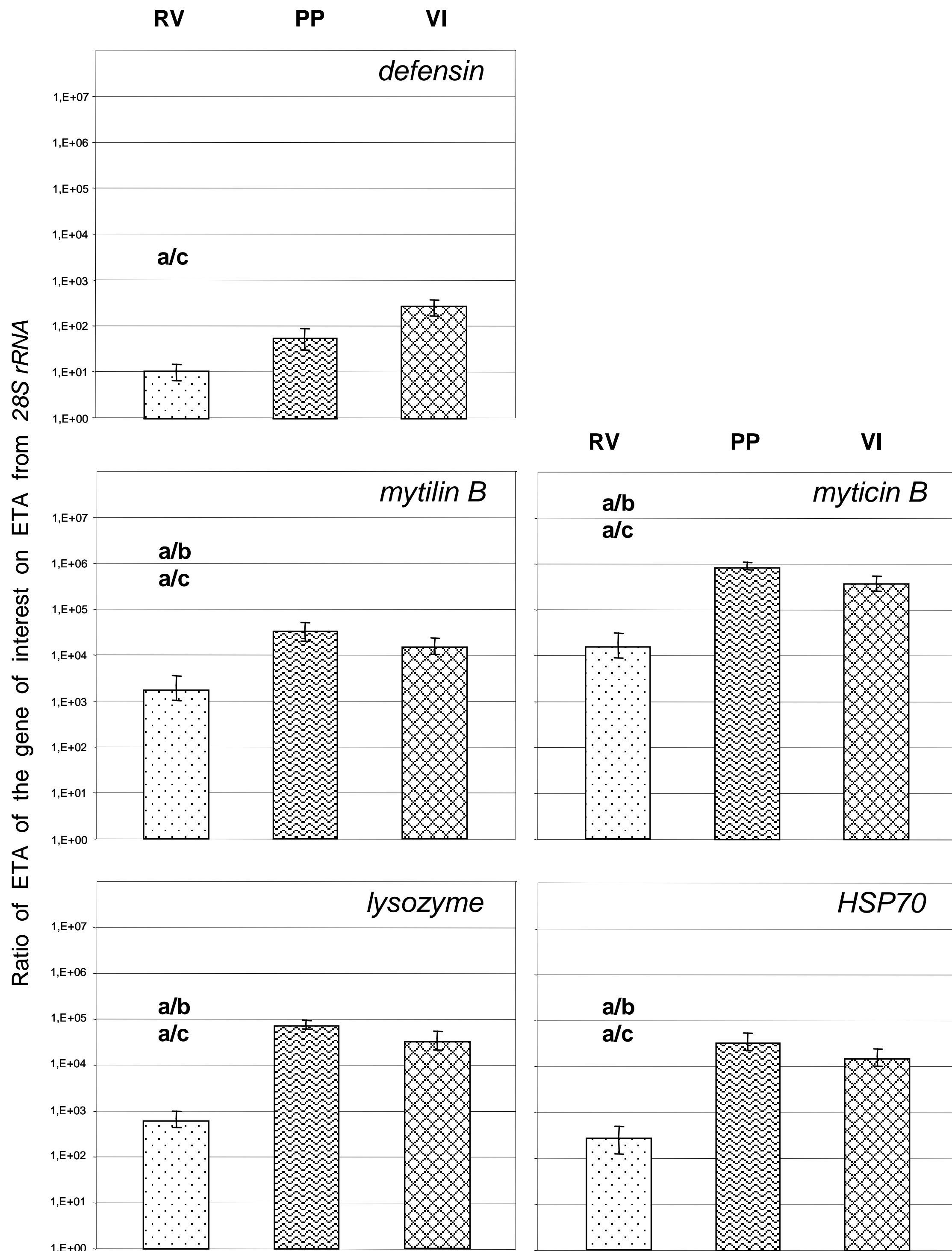


Figure 2 - Comparative expression of *defensin, mytilin B, myticin B, lysozyme* and *HSP70* genes in hemocytes from untreated mussels collected in the three geographic origins: Ria de Vigo (RV), Prévost laguna-Palavas (PP) and Venice laguna-Italy (VI). Expression data were presented as arithmetical mean of four replicates ± SEM. Statistically significantly difference ($p<0.05$) between the geographic origins were indicated by the letters a, b and c, referring to RV, PP and VI, respectively. Note the lowest expression of *defensin*, not significantly different between the three locations (a/c border line value of 0.050), the general lowest expressions in RV, the significant difference between RV and the two others and the non significant difference between PP and VI.

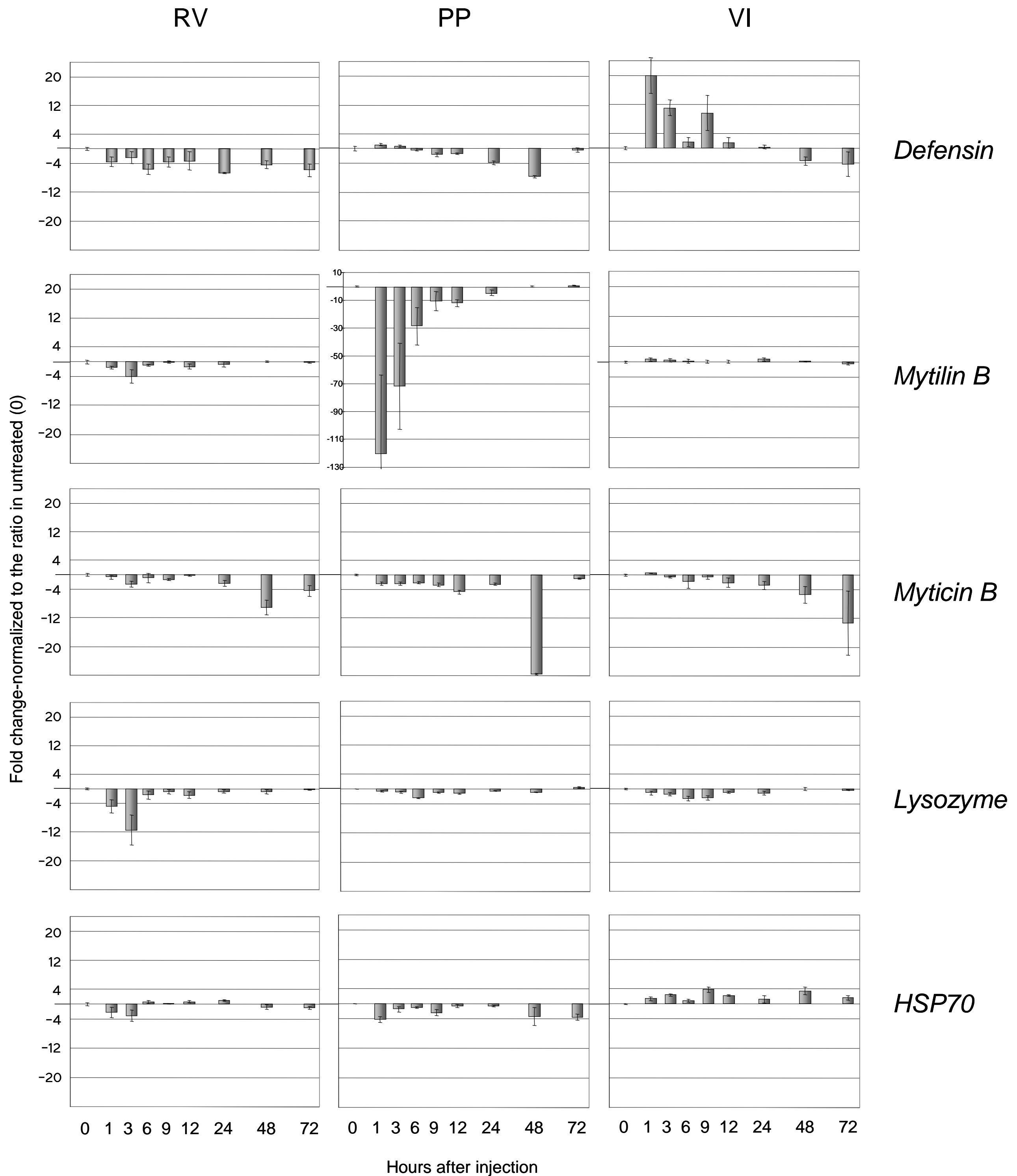


Figure 3 - Comparative effect of *Vibrio splendidus* LGP32 injection on the expression of some immune-related genes in mussels collected from 3 different geographical locations. RV: Ria de Vigo-Spain. PP: Prévost laguna Palavas-France. VI: Venice laguna-Italy. Scales are identical in each panel, except for PP-*mytilin B*. Note the up-regulation of *defensin* in VI, and the down-regulation of *mytilin B* and *myticin B* in PP and of *myticin B* and *lysozyme* in RV.

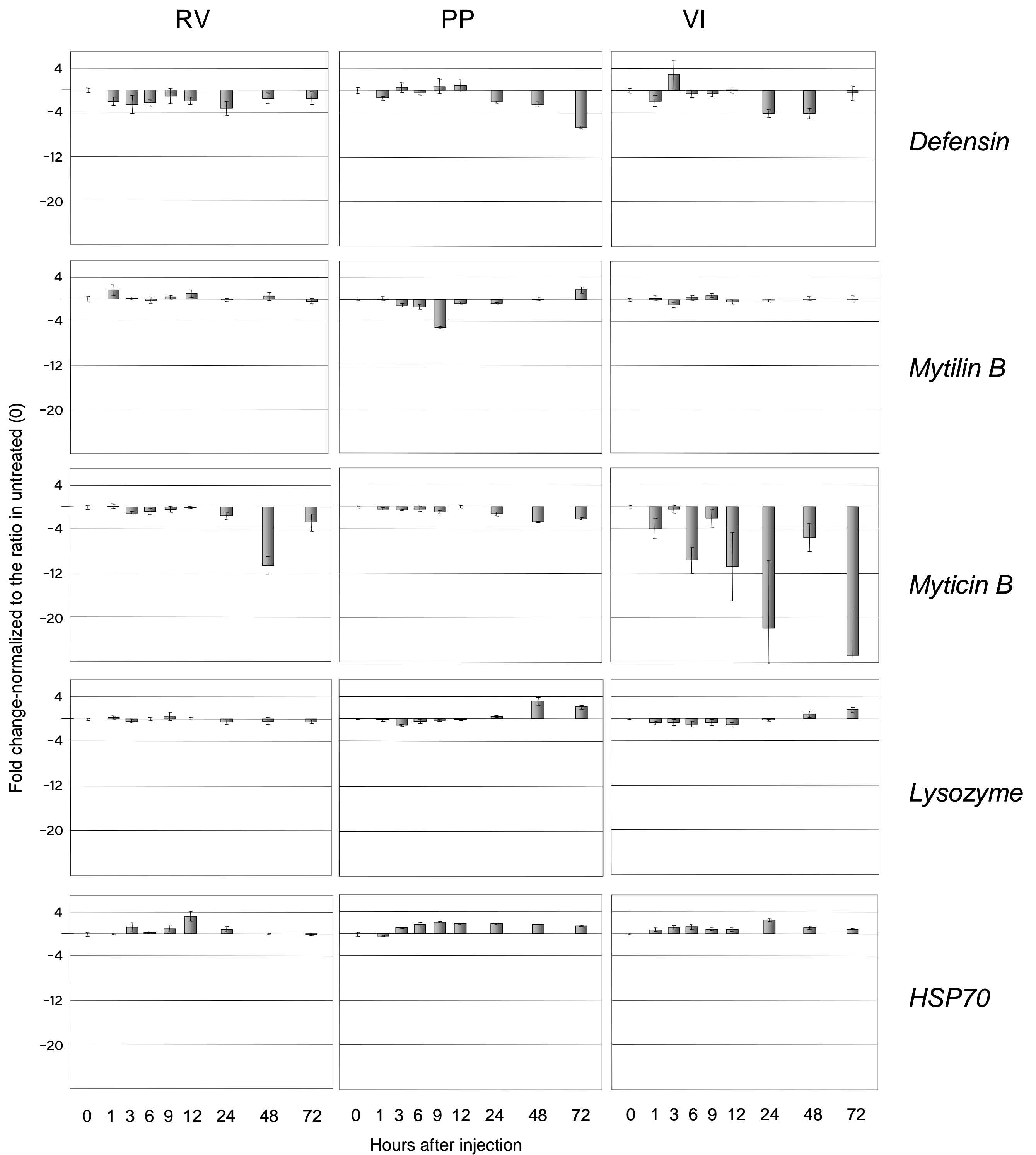


Figure 4 - Comparative effect of *Vibrio anguillarum* injection on the expression of some immune-related genes in mussels collected from 3 different geographical locations. RV: Ria de Vigo-Spain. PP: Prévost laguna Palavas-France. VI: Venice laguna-Italy. Scales are identical in each panel. Note the strong down-regulation of *myticin B* in VI.

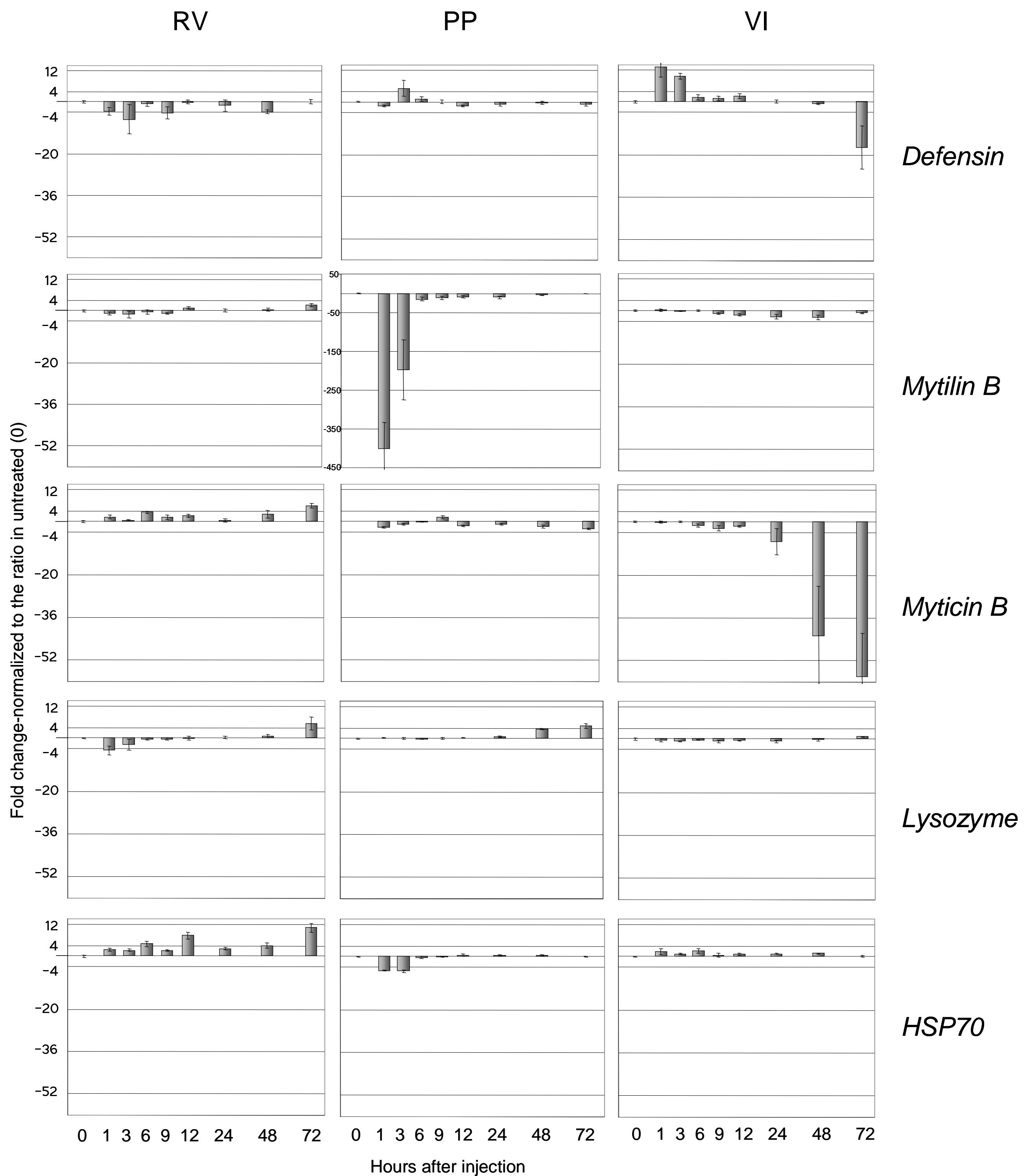


Figure 5 - Comparative effect of *Micrococcus lysodeikticus* injection on the expression of some immune-related genes in mussels collected from 3 different geographical locations. RV: Ria de Vigo-Spain. PP: Prévost laguna Palavas-France. VI: Venice laguna-Italy. Scales are identical in each panel, except for PP-*mytilin B*. Note the up-regulation of *defensin* in VI and of *HSP70* in RV, and the almost suppression of *mytilin B* expression in PP and of *myticin B* expression in VI.

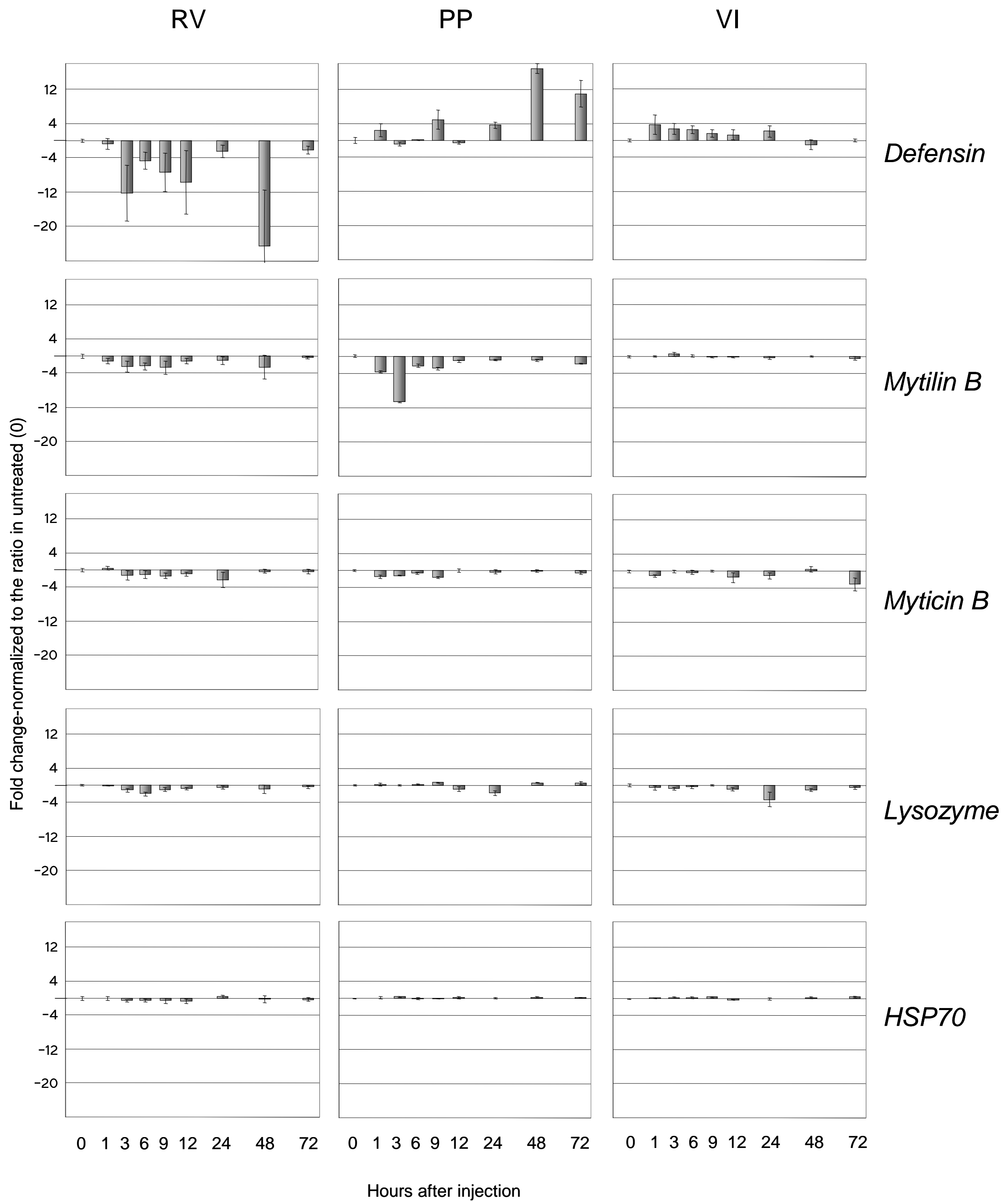


Figure 6 - Comparative effect of PBS-NaCl injection on the expression of some immune-related genes in mussels collected from 3 different geographical locations. RV: Ria de Vigo-Spain. PP: Prévost laguna Palavas-France. VI: Venice laguna-Italy. Scales are identical in each panel. Note the various effects on *defensin* expression, the down-regulation of *mytilin B* in PP and the non modulation of *myticin B*, *lysozyme* and *HSP70*.

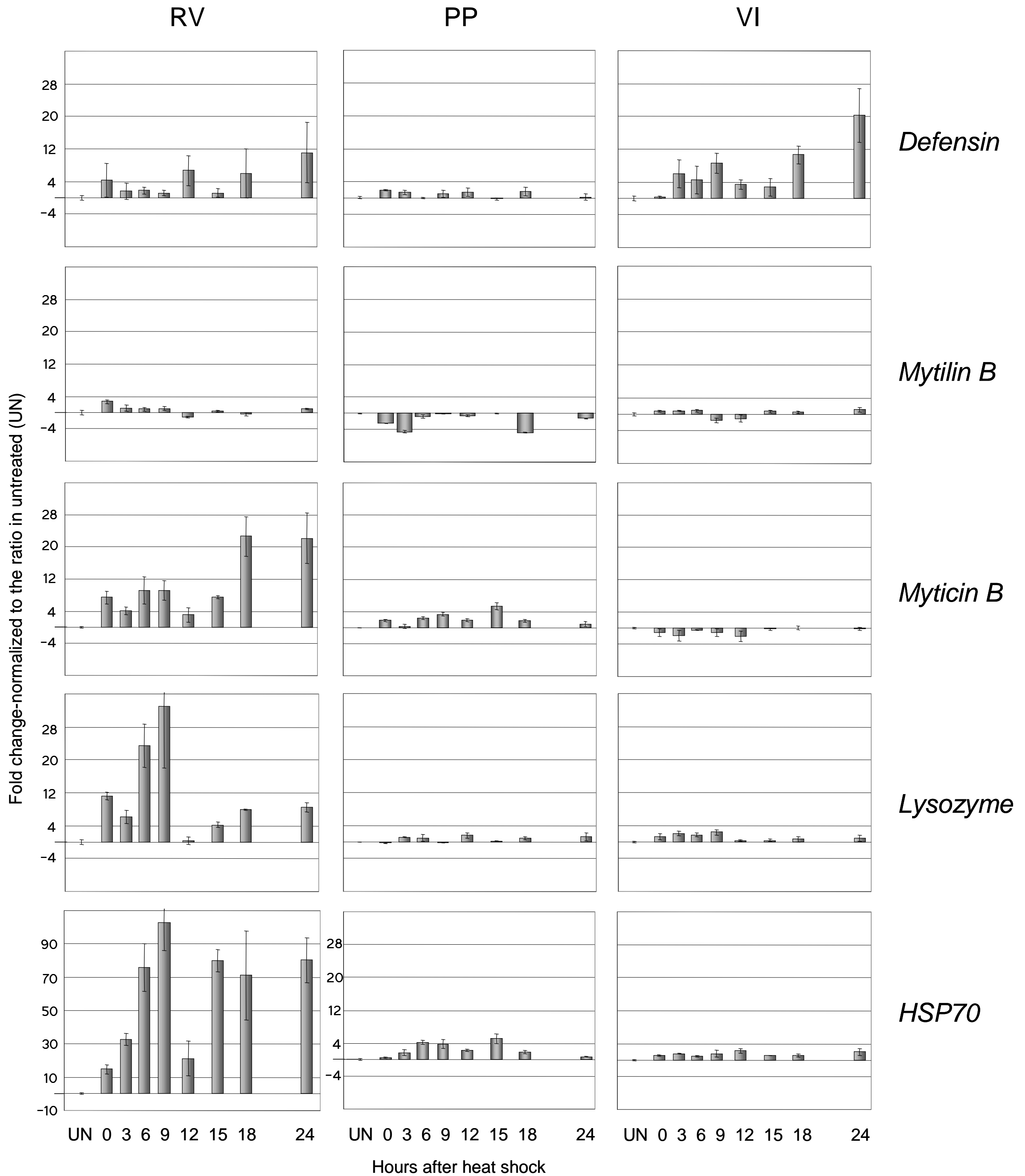


Figure 7 - Comparative effect of heat shock on the expression of some immune-related genes in mussels collected from 3 different geographical locations. UN: untreated mussels RV: Ria de Vigo-Spain. PP: Prévost laguna Palavas-France. VI: Venice laguna-Italy. Scales are identical in each panel except for *HSP70* in RV. Note the general up-regulation in mussels from RV (except for *mytilin B*) and for *defensin* in VI.

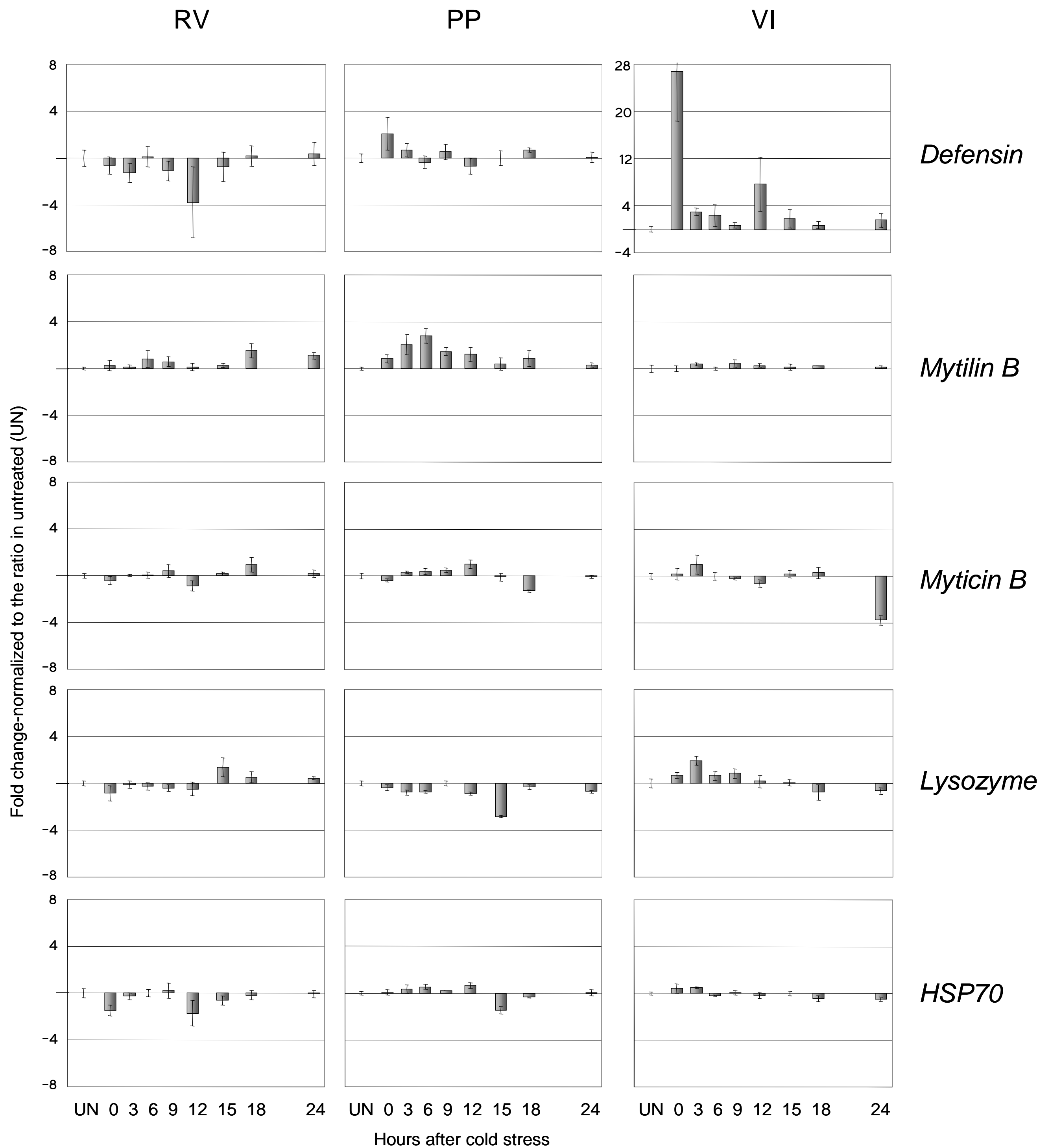
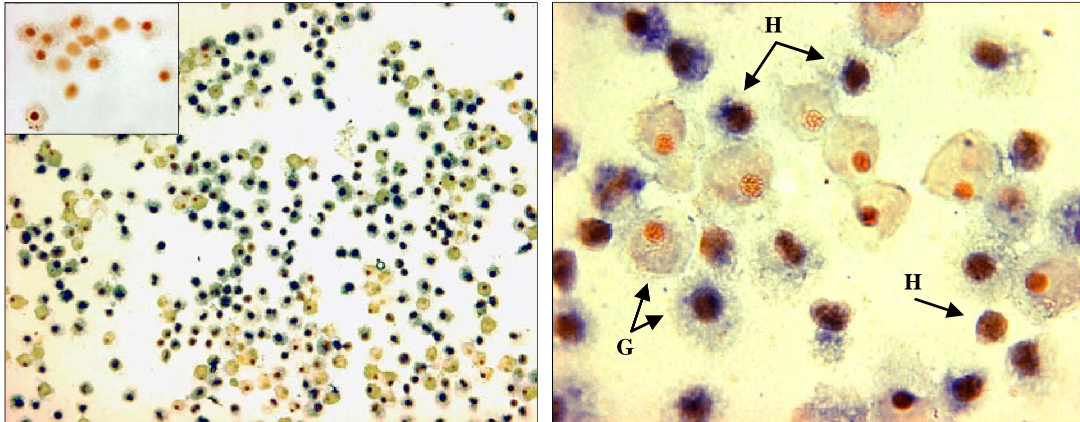
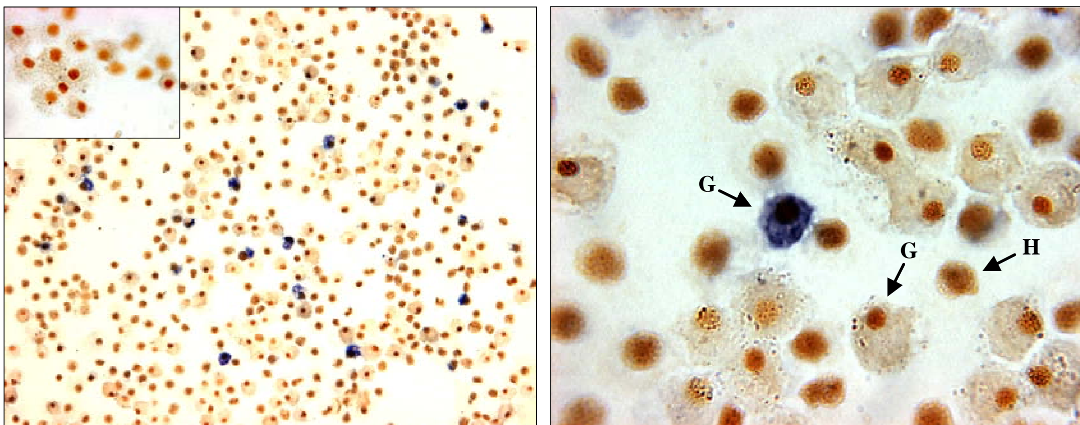


Figure 8 - Comparative effect of cold stress on the expression of some immune-related genes in mussels collected from 3 different geographical locations. UN: untreated mussels. RV: Ria de Vigo-Spain. PP: Prévost laguna Palavas-France. VI: Venice laguna-Italy. Scales are identical in each panel, except for *defensin* in VI. Note the general absence of effect, except for *defensin* in VI.

Defensin



Mytilin B



Myticin B

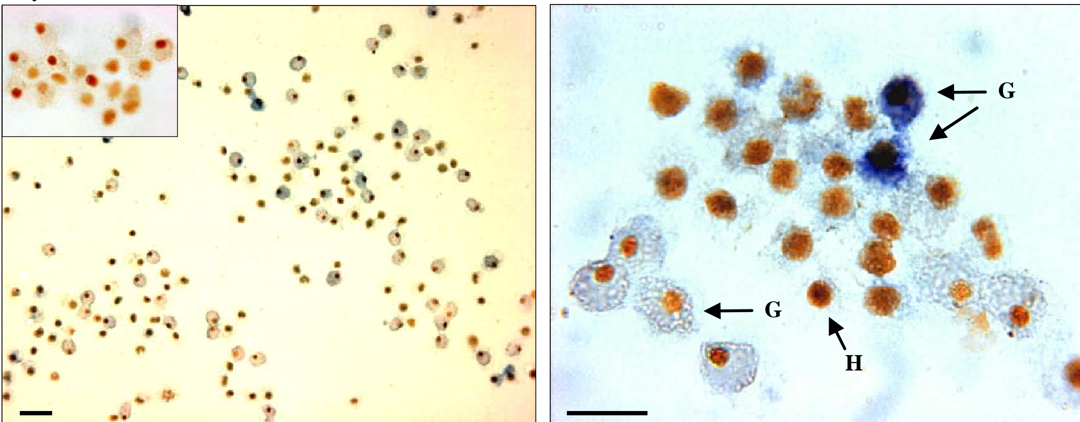
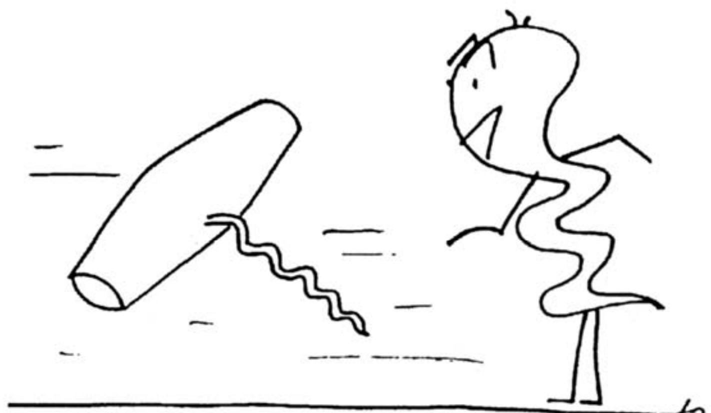


Figure 9 - Optical microscopy observations of ISH on circulating hemocytes from untreated mussels revealing *defensin*, *mytilin* B and *myticin* B gene expression in blue. Counter staining of nuclei was with Bismark brown. Upper left windows corresponded to hybridization with sense probe. Note the large proportion of granulocytes (G) and hyalinocytes (H) expressing *defensin* compared to few granulocytes expressing *mytilin* B or *myticin B*. Magnification bars: 20 µm.

Chapitre 5



Hey, don't you happen to be a vibrio too?

(D'après « Funny Microbes », Leos Mandel)

Chapitre 5

Suivi mensuel de l'expression de divers gènes immunitaires au sein de la même population de moules entre 2005 et 2008

Des études précédentes ont montré que de nombreuses activités d'immunité innée chez la moule sont en étroite relation avec la saison, tels le nombre d'hémocytes circulants, les activités du lysozyme et d'agglutination, la production d'oxyde nitrique, etc. En outre, le phénomène de mortalité massive observé chez les bivalves est supposé dépendre de la saison. Afin de voir si les capacités immunitaires des moules subissaient des variations d'intensité suivant les saisons, nous avons quantifié l'expression de divers gènes durant trois années consécutives, à raison d'une mesure par mois. Ces quantifications doivent être analysées en regard de diverses caractéristiques environnementales (salinité, température et concentration en coliformes).

Les analyses rapportées dans ce chapitre font partie de l'un des objectifs du contrat européen Imaquanim. Le dernier prélèvement a eu lieu en juillet 2008 et bien que les analyses en Q-PCR soient terminées, ce chapitre n'est pas suffisamment avancé dans sa réflexion pour être présenté sous la forme d'une publication. De plus, toutes les données environnementales ne sont pas encore disponibles. Néanmoins, plusieurs résultats marquants peuvent être dégagés : **(i)** l'expression du gène de *28S rRNA*, considéré comme un bon gène de ménage pour une période précise, n'est pas exprimé de façon constante tout long de l'année ; **(ii)** les expressions des quatre gènes immunitaires et celle de *HSP70* varient également au fil des années ; **(iii)** exception faite de *mytiline B*, les variations d'expression suivent un rythme saisonnier ; **(iv)** tous les gènes montrent une faible expression durant la période septembre 2006 à avril 2007.

Une publication est en cours de rédaction.

Constitutive expression of *HSP70* and four immune genes in the Mediterranean mussel, *Mytilus galloprovincialis* during the years 2005-2008.

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Key words: antimicrobial peptide, defensin, mytilin, myticin, lysozyme, HSP70, Q-PCR, immune survey, seasonal variations, innate immunity, *Mytilus*, Molluscs

Abstract

Several bivalves, including mussels, suffered from mortalities particularly important in summer. To look for possible seasonal effect on immune capacities, mussels were monthly collected from the same location in 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*, the expression of which was supposed to be constant, but observed to undergo seasonal variations. We applied a calculation mode based on *ratio* to compensate such variations. *Defensin*, *myticin B* and *lysozyme* appeared more expressed in spring-summer than in winter. In contrast, *HSP70* expression was higher in winter. No correlation to any particular season was observed for *mytilin B* expression.

1. Introduction

Fluctuations of numerous criteria were reported in mussels as related to seasons : enzyme activities (Sheehan and Power 1999), circulating hemocyte number (Carballal, Villalba et al. 1998), protein content of hemolymph, lysozyme and agglutinin activities (Santarém, Robledo et al. 1994), antibacterial response induced by LPS (Hernroth 2003),

cytolytic activity (Malagoli, Casarini et al. 2007) and nitric oxide production (Novas, Barcia et al. 2007), for instance. Also mortalities were frequently reported in relationships with the season, to such extend that they were called summer-mortalities, both in oysters (Lacoste, Jalabert et al. 2001; Friedman, Estes et al. 2005; Garnier, Labreuche et al. 2007) and in mussels (Myrand and Gaudreault 1995; Tremblay, Myrand et al. 1998). Concerning immunity, we previously demonstrated in Northern blot that the expression of AMP genes, *defensin*, *mytilin B* and *myticin B* were constitutively expressed all the year round, whereas *defensin* mRNA was not detectable in summer (Roch 2001). 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 at the same location, once a month during three consecutive years.

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 in a flow-through system of oxygenated sea water 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 the anti-coagulant modified Alsever's solution (27 mM sodium citrate, 115 mM glucose, 336 mM NaCl, 18 mM EDTA, pH 7.0). Hemolymph from 10 mussels was pooled and hemocytes pelleted by 15 min centrifugation at 800 g, 4°C, then resuspended in 1 ml Trizol Reagent (Invitrogen) and stored at -20°C until used. Four pools of 10 mussels each, as replicates, were used for each sampling day. The full survey involved 1,440 mussels.

2.2. cDNA synthesis and quantitative PCR (Q-PCR)

Total RNA was extracted according to manufacturer's instructions and resuspended in 45 µl of Tris-EDTA buffer (TE: 10 mM Tris-HCl, pH 8.0, 1 mM EDTA). RNA concentrations were measured on spectrophotometer ND-1000 (NanoDrop Technologies). First strand cDNAs were synthesized on 5 µg of total RNA using hexaprimers (Invitrogen) and murine leukemia virus reverse transcriptase (Promega), purified through QIAquick Column (Qiagen) and then kept in nuclease-free water at -20°C until use.

Q-PCR was performed using the SYBR Green chemistry on a LightCycler 480 384 well-plate (Roche). Primer sequences and specificity controls were previously reported (Cellura, Toubiana et al. 2007; Li, Parisi et al. 2008). Q-PCR mixture contained the following: 1 µl first strand cDNA (10 ng), 0.75 µl of each specific primers at a concentration of 25 µM, 2.5 µl of mix (Roche) containing FastStart Taq DNA polymerase, reaction buffer 2x, dNTP mix, SYBR Green 1 dye and $MgCl_2$. The PCR programme started with initial Taq polymerase activation at 95°C for 10 min, followed by 40 cycles at 95°C for 10 sec, 65°C for 10 sec and 72°C for 15 sec. Melting temperatures were measured by returning to 65°C for 30 sec and gradual heating to 95°C. Negative control reactions contained water in place of cDNA template and were included in each run to ensure the absence of contamination. Calibration curves were obtained using 10-fold serial dilutions of the corresponding amplicon in 10 µg/ml sonicated salmon sperm DNA (Sigma). House keeping gene was represented by *28S ribosomal RNA*, as previously validated (Cellura, Toubiana et al. 2006).

2.3. Q-PCR data analysis

Crossing point values expressed in cycle numbers were measured according to the threshold position of 4.2 and converted into equivalent target amount (ETA) by the LightCycler 480 built-in software (Roche) using statistical calibration curves. Expression level of gene of interest was calculated from the *ratio* of ETA for the considered gene on ETA for *28S rRNA*. Normalization of the *ratios* was calculated considering each *ratio* equal to 1 in untreated mussels and expressed as x-fold the *ratio* for untreated mussels. Data were presented as arithmetical mean of the four replicates measured in duplicate ± SEM. Statistical significant differences between data were established by Student's *t*-test using t-Ease 2.8 ISI software. Differences were considered as significant for $p<0.05$.

3. Results

3.1. Expression of 28S rRNA

The intensity of expression of *28S rRNA* was subjected to variations according to the month of collection. Lowest expressions were recorded from Aug 05 to Jan 06 (Fig 1) with statistically significant difference between Oct 05 and Oct 06 (p=0.0002) for instance, but not between Oct 06 and Oct 07 (p=0.51). Similarly, statistically significant difference was observed between Feb 06 and Feb 07 (p=0.035), but not between Feb 07 and Feb 08 (p=0.087). Meanwhile, some rhythm can be observed, with three period of higher expressions: Aug 05-Jan 06, Jun 06-Mar 07 and Sep 07-Mar 08. Lower expressions were consistent during Apr-Aug 07, i.e. during spring-summer times, with expression statistically significantly lower in Jun 07 than in Jun 06 (p= 0.00016), but not than in Jun 08 (p=0.96). In addition, no difference in expression was observed between Sep 07 and Aug 08.

3.2. Expression of AMPs and lysozyme

General observation of Figure 2 was on the existence of variations of expressions. Evident is that *defensin* expression was higher in summer, with a maximum in Aug-Sep, statistically significantly different between May and Aug 06 (p=0.0028) for instance. Particularly low expression of *defensin* was in winter, close to the detection sensitivity of the Q-PCR technology used, with no difference between Dec 05 and Dec 06 (p=0.23) and between Dec 06 and Dec 07 (p=0.96).

Except from Aug 05 to Mar 06 (significant difference between Dec 05 and Dec 06, p=0.0040, for instance), *mytilin B* did not seem to be up-regulated by the season. Minimum expression was from Apr 06 to Apr 07. No difference in *mytilin B* expression was recorded from May 07 to Jul 08, i.e. during 15 consecutive months (p=0.41 between Dec 07 and May 08, for instance).

Lower expression of *myticin B* was recorded during the winter times, with no significant difference between Jan 06 and Feb 07 (p=0.23) or between Feb 07 and Jan 08 (p=0.09). Higher expression was observed in spring, Apr 06 (p=0.015 with Jan 06), May 07 and Apr 08).

Observation of *lysozyme* expression revealed higher expression in summer, particularly in May, with no significant differences between the years: p=0.41 between May

06 and May 07, and p=0.54 between May 07 and May 08. Such higher expression of *lysozyme* was statistically different from the expression in winter: p=0.0048 between May 06 and Feb 06, p=0.029 between May 07 and Feb 07, and p=0.021 between May 08 and Mar 08, for instance.

3.3. Expression of HSP70

Although with different intensities, general higher values for HSP70 expression were recorded in Dec 05, Dec 06 and Dec 07, with significant difference with low expression measured in summer (p=0.0006 between Dec 06 and Jun 06, for instance) (Fig. 3). Exceptions were for Aug 07 and Jul 08 with high values not significantly different from Dec 07. Low expressions were recorded constantly from Feb 06 to Apr 07.

4. Discussion

The main criterium to decide for a housekeeping gene is its constant expression all along the experiment. In previous reports, we found *28S rRNA* as suitable housekeeping gene to study the variations of several immune-related genes following various treatments (Cellura, Toubiana et al. 2007; Li, Parisi et al. 2008). Such treatments were not statistically significantly affecting the expression of *28S rRNA*. During the course of the present multi annual survey, we also focussed on *28S rRNA* for Q-PCR calculations. Meanwhile, it was obvious that expression of such gene was not constant all along the year, but followed some rhythms, with higher expression from Jun 06-Mar 07 and from Sep 07-Mar 08. Even if some rhythm can be defined, with lower expression from Dec 05-May 06, Apr 07-Aug 07 and Apr 08-Jul 08, there was no clear relationships with particular season. One can speculate that such differences in expression were related to the level of mussel metabolism which is known to fluctuate according to season (Bodin, Burgeot et al. 2004).

The calculation mode based on the *ratio* of ETA for the considered gene on ETA for *28S rRNA*, compensated the variations in the house keeping gene. *Defensin* expressed in summer, from Apr to Oct, and quite undetectable in winter, from Dec to Mar, which is contradictory to previous observation done in Northern. *Mytilin B* constitutive expression appeared constant with a low level from Apr 06 to Apr 07 and with a little bit higher values from May 07 to Jul 08. Exception was during the winter 05-06 during which *mytilin B* expression was significantly up-regulated. Higher expression of *lysozyme* occurred in summer,

as well as of *HSP70*. All the five genes underwent dramatic change in May 07 with a breakdown of a long period of low expression, starting about a year before, particularly obvious for *mytilin B* and *HSP70*.

Global assertion was on the existence of seasonal variations of expression for the six considered genes. In addition, *defensin*, *lysozyme* and *HSP70* appeared more expressed in summer than in winter. In contrast, variations in the expressions of *mytilin B* and *myticin B* seemed not strictly correlated to any particular season. Consequently, the three *AMP* genes behave differently according to the season. We must notice the deviant comportments during the winter 05-06 with the highest expression of *mytilin B, lysozyme* and *HSP70*.

Global observations in *M. galloprovincialis* were on the highest concentrations/activities during summer times, with correlations to highest water temperature: hemolymph protein concentration, lysozyme and agglutinin activities (Santarém, Robledo et al. 1994), total hemocyte count (THC) (Carballal, Villalba et al. 1998), NO production (Novas, Barcia et al. 2007), and HSP70 and multixenobiotic resistance (MXR) protein accumulations (Minier, Borghi et al. 2000), for instance. However, intensity of cytolytic activity correlated to water temperature, but experiments in aquaria demonstrated this parameter is not the main cause of the fluctuation (Malagoli, Casarini et al. 2007). Also in *M. edulis*, activities such are glutathione (GSH), GHS-peroxidase and catalase were the highest in summer (Sheehan and Power 1999). Other observations in *M. galloprovincialis* were on higher prevalence of gonadal neoplasm between Apr and Jun (Alonso, Suarez et al. 2001) and on higher percentage of inflammatory lesions caused by parasites in summer than in winter (Bodin, Burgeot et al. 2004), exactly as reported for the Japanese pearl oyster, *Pinctada fucata*, infected by the marine birnavirus (MABV) (Kitamura, Jung et al. 2000). In contrast, greater concentrations of *HSP70* in winter in the horse mussel, *Modiolus modiolus*, suggested adjustment of such chaperone functions to cold temperature (5° versus 15°C) (Lesser and Kruse 2004).

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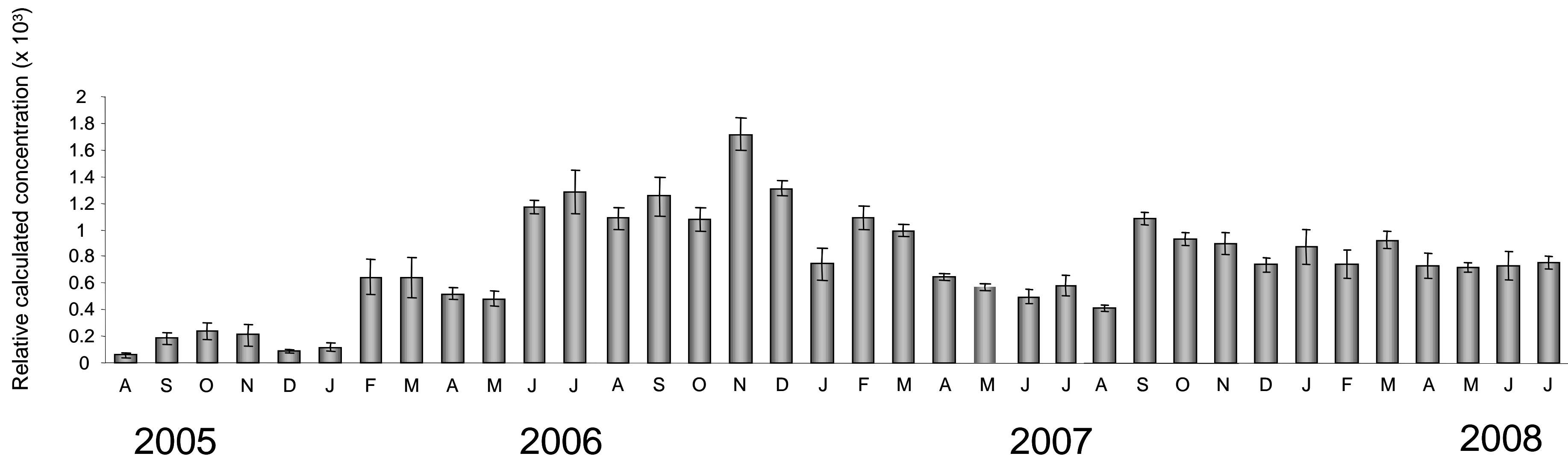


Figure 1 - Expression of *28S rRNA* as measured in Q-PCR from samples monthly collected from August 05 to July 08. Values were inferred from 4 replicates measured in duplicate ± SEM (bar). See the text for some statistical analysis. Note the lowest expression Aug 05-Jan 06, the highest expressions Jun 06-Mar 07 and Sep 07-Mar 08, separated by the low expression in Apr-Aug 07.

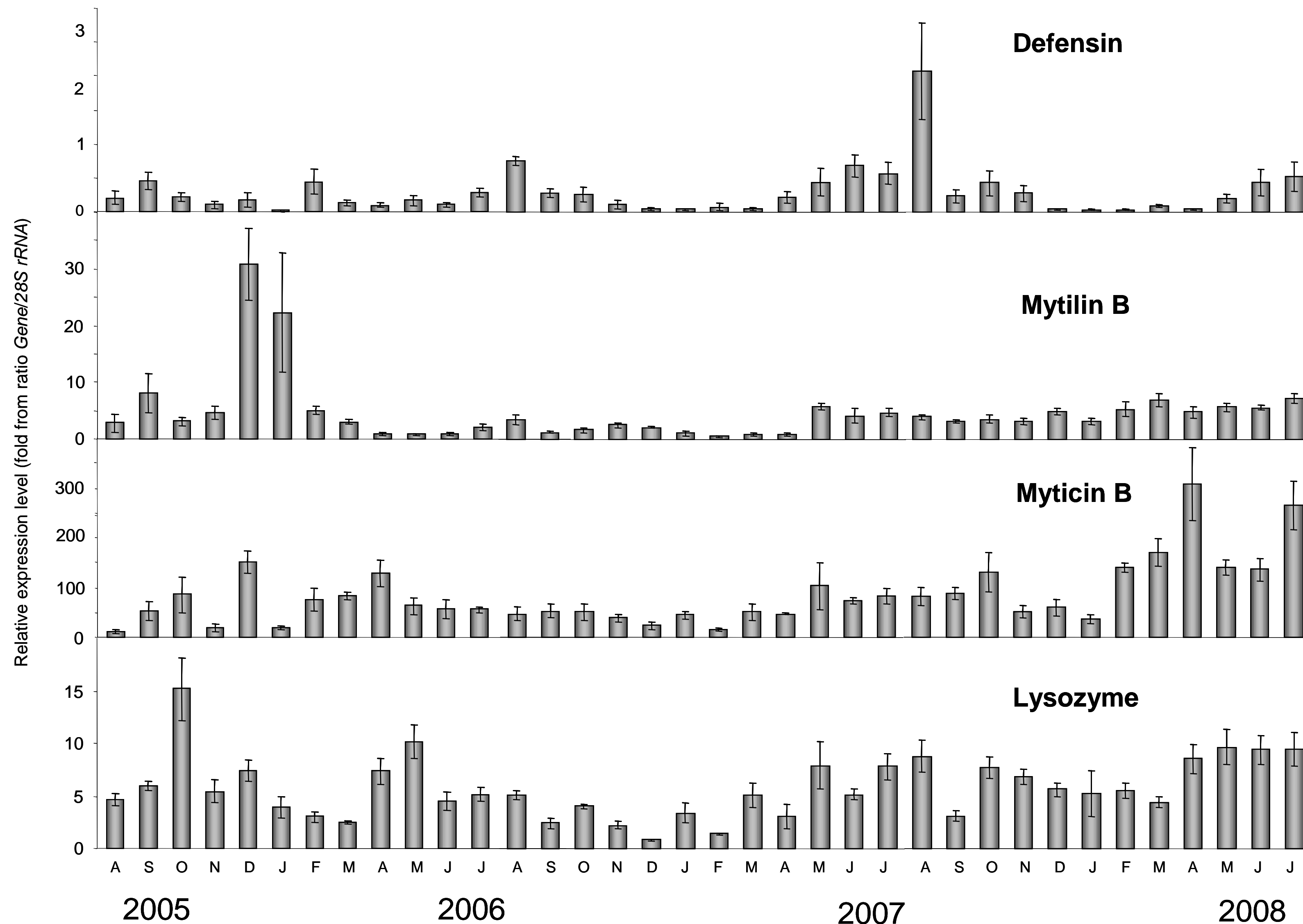


Figure 2 - Expression of *defensin, mytilinB, myticin B* and *lysozyme* as measured in Q-PCR from samples monthly collected from August 05 to July 08. Values were inferred from 4 replicates measured in duplicate ± SEM (bar). See the text for some statistical analysis. Note the existence of modulations of expressions, not identical between the four genes. General lower expressions were in winter 07.

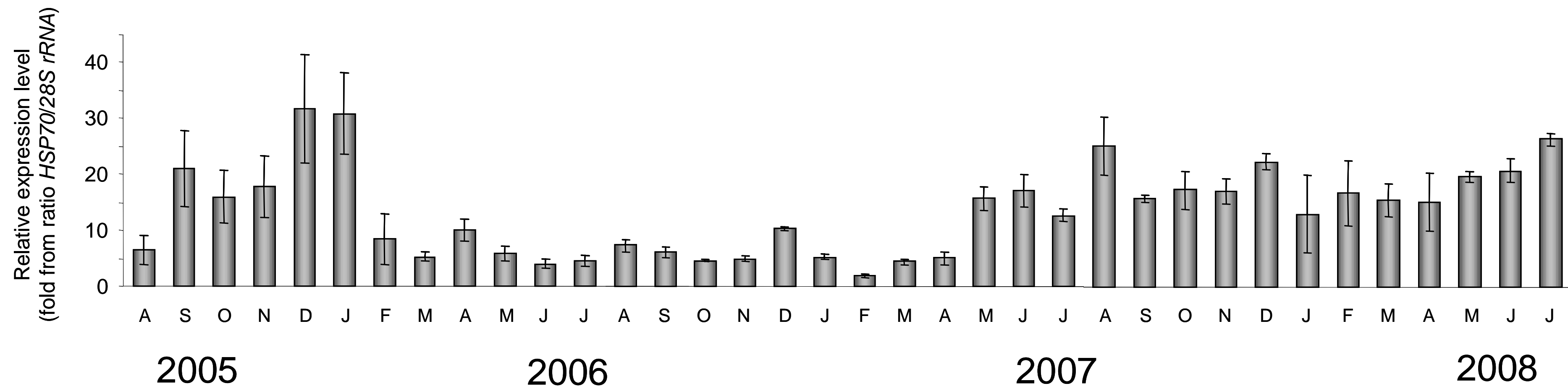
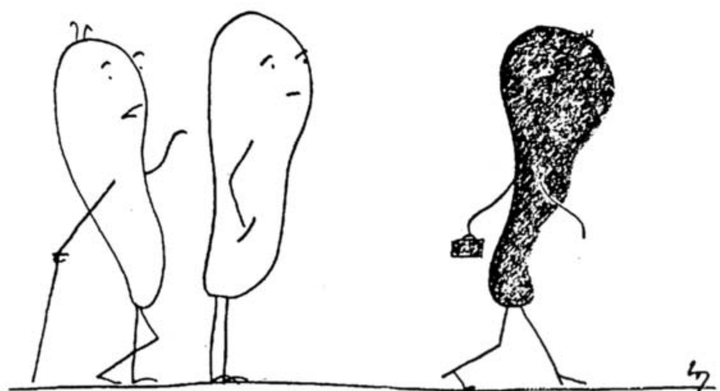


Figure 3 - Expression of *HSP70* as measured in Q-PCR from samples monthly collected from August 05 to July 08. Values were inferred from 4 replicates measured in duplicate ± SEM (bar). See the text for some statistical analysis. Note the higher expressions in Dec, with exceptions for Aug 07 and Jul 08. Low expressions were recorded from Feb 06 to Apr 07.

Chapitre 6



What a nice girl! And Gram-positive too!

(D'après « Funny Microbes », Leos Mandel)

Chapitre 6

Polymorphisme des ARNm de la mytiline B

Une nouvelle isoforme de la myticine, la myticine C, a été identifiée au cours de l'analyse d'une banque EST (Expressed Sequence Tags) générée par la technique SSH (Suppression Substractive Hybridization) par le laboratoire de P. Venier à l'Université de Padova (Italie) dans le cadre du partenariat financé par le contrat européen Imaquanim 2005-2010. A notre grande surprise, la myticine C est apparue comme extrêmement polymorphe puisque la traduction des ARNm a donné 74 pro-peptides et 25 peptides matures. Afin de vérifier que ce polymorphisme constituait un phénomène général, nous avons analysé la diversité des ARNm d'un autre peptide antimicrobien, la mytiline B, en utilisant une autre technique, la DGGE (Denaturing Gradient Gel Electrophoresis).

Le principe de la DGGE consiste en une séparation des 2 brins d'un petit fragment d'ADN (200-700 pb) en fonction de la température et de conditions dénaturantes. La plupart des fragments d'ADN ne se séparent pas d'une manière linéaire en suivant la hausse de la température, mais de manière discontinue. En outre, des portions de la double hélice d'ADN se séparent brutalement en simple-brin suivant une valeur précise des conditions dénaturantes crées par un mélange d'urée et de formamide. Les fragments d'ADN qui sont partiellement en simple-brin migrent beaucoup plus lentement qu'une double hélice complète lors d'une électrophorèse en gel de polyacrylamide. La sensibilité de la séparation en DGGE est accrue par l'addition d'une séquence de 40 nucléotides ne contenant que des C et G, en 5' de la sonde sens utilisée lors de la PCR précédant la migration.

Ce travail sur le polymorphisme des ARNm de la mytilin B a été réalisé en coopération avec M-G. Parisi, doctorante au laboratoire d'Immunobiologie Marine de l'Université de Palerme (Italie). Nos principaux résultats sont les suivants : **(i)** une moule exprime simultanément de 2 à 10 différents ARNm de la mytiline B ; **(ii)** 21 différents cds (Coding Sequence) ont été identifiés parmi les 57 moules analysées ; **(iii)** les nucléotides mutés sont principalement dans la partie carboxy-terminale et dans l'UTR (Untranslated Region) en 3' de la séquence d'ADNc ; **(iv)** la plupart des mutations sont silencieuses, ce qui fait que le nombre de pro-peptides n'est que de quatre et que le peptide mature soit toujours le même ; **(v)** contrairement à la myticine C, les tests de neutralité montrent une pression de

sélection négative sur le peptide signal et le peptide mature, mais suggèrent une pression de sélection positive sur la partie carboxy-terminale.

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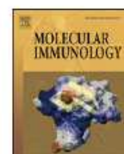

Polymorphism of mytilin B mRNA is not translated into mature peptide

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ABSTRACT

Diversity of mRNAs from mytilin B, one of the five mytilins identified in the Mediterranean mussel, *Mytilus galloprovincialis*, has been investigated from circulating hemocytes. One mussel expressed simultaneously two to ten different mytilin B mRNAs as observed in denaturing gradient gel electrophoresis (DGGE), defining 10 individual DGGE patterns (named A to J) within the mussels from Messina, Sicily (Italy). Three patterns accounted for 79% of the individuals whereas other patterns were found in only 2–7% of the 57 analyzed mussels. Base mutations were observed at specific locations, mainly within COOH-terminus and 3′UTR, leading to 36 nucleotide sequence variants and 21 different coding sequences (cds) segregating in two different clusters. Most of the base mutations were silent, and the number of pro-peptide variants was restricted to four. Finally, as the two amino acid replacements occurred within COOH-terminus, mature peptide from mytilin B appeared unique. Multiple sequencing of partial mytilin B gene from one mussel revealed that one to four randomly distributed mutation points occurred within intron-3. Only one sequence out of the 91 analyzed contained 16 mutation points. In addition, this sequence was the only one containing four out of the six mutation points occurring within exon-4, that code for most of the COOH-terminus domain, including the unique amino acid replacement. Statistical tests for neutrality indicated negative selection pressure on signal and mature peptide domains, but possible positive selection pressure for COOH-terminus domain.

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UNCORRECTED PROOF

1. Introduction

In invertebrates, hemocytes are the primary immune cells engaged in several innate immune reactions including phagocytosis, clotting, encapsulation, and the synthesis of several immune-related molecules. Among these molecules are the antimicrobial peptides (AMP) characterized by their extremely heterogenic structures. More than 2300 different AMPs were reported and are mainly available through three databases: Antimicrobial Sequence Database (AMSDb, http://www.bbcm.univ.trieste.it/~tossi/amsdb.html) with 1000 entries, Antimicrobial Peptide database (APD, http://aps.unmc.edu/AP/main.php) with 1008 records, and Antimic (http://www.ambis.org.sg/ppt/Manisha.ppt) with 1788 entries. Based on primary structure and consensus cysteine array, four AMP families have been identified in the mussels, *Mytilus edulis* (Charlet et al., 1996) and *Mytilus galloprovincialis* (Hubert et al., 1996; Mitta et al., 1999): defensin, mytilin, mytician and mytimycin. More recently, similar AMPs have been discovered in the bay mussel, *Mytilus trossulus* (GenBank AY730626), the Eastern oyster, *Crassostrea virginica* (Cunningham et al., 2006; Seo et al., 2005), the Manila clam, *Ruditapes philippinarum* (Kang et al., 2006), the Pacific oyster, *Crassostrea gigas* (Gonzalez et al., 2007; Gueguen et al., 2006b), the bay scallop *Argopecten irradians irradians* (Song et al., 2006; Zhao et al., 2007) and finally the carpet-shell clam, *Ruditapes decussatus* (Gestal et al., 2007).

Mytilin is composed of five different sequences, referred to as isoforms (Charlet et al., 1996; Mitta et al., 2000b). From one gene copy per genome, mytilin B mRNA is translated into pro-peptide and is matured inside hemocyte granules (Mitta et al., 2000a). The 3D structure of mytilin B has been established (Roch et al., 2008) revealing unexpected similarity to *M. galloprovincialis* defensin MGD-1 (Yang et al., 2000) considering primary amino acid sequences. In unchallenged *M. galloprovincialis* circulating hemocytes, mytilin B mRNA was evaluated as ten times less abundant than myticin B mRNA (Cellura et al., 2007). Myticin C appeared extremely polymorphic as 74 variants were identified with nucleotide mutations inducing amino acid replacements located on the entire pro-peptide sequence, i.e. the signal peptide, the mature myticin C and the COOH-terminus (Pallavicini et al., 2008). Individual sequences of myticin C are unique for each

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mussel and only animal belonging to the same family shared myticin C sequences, suggesting that its expression is probably a familial character (Costa et al., in press). Even more extended diversity was reported for penaeidins from different shrimp species, not only within individuals from the same species but also within a single shrimp (Gueguen et al., 2006a).

Denaturing gradient gel electrophoresis (DGGE) is an electrophoresis separation method based on differences in melting behaviour of double stranded DNA fragments (Fisher and Lerman, 1979). It is one of the most efficient and widely applied methods for detection of nucleotide differences through an increasing gradient of the denaturant formamide and urea (Abrams and Stanton, 1992; Lerman and Beldjord, 1998). DGGE has been applied to different biological purposes, from genetic mutations (Sheffield et al., 1989) to bacterial diversity (Muyzer et al., 1993; Van der Gucht et al., 2005) and genetic polymorphism (Ge et al., 1999). Sensitivity of DGGE allows to detect one single base substitution by adding a 40-base pair GC rich sequence (called GC clam) to the forward specific PCR primer used in amplification (Sheffield et al., 1989).

Here, we applied DGGE technology to demonstrate the diversity of *M. galloprovincialis* mytilin B mRNAs segregating in ten individual patterns in untreated mussels. Random cloning and sequencing of multiple PCR-generated mytilin B fragments established the relationships between patterns and nucleotide sequences, point mutations and deduced pro-peptide sequences. In addition, multiple random cloning and sequencing of partial intron-3-exon-4 fragment, analyzed with several tests for neutrality, suggested negative selection pressure for signal and mature peptide domains, but possible positive selection pressure for COOH-terminus domain.

2. Material and methods

2.1. Mussels and RNA extraction

Adult mussels, *M. galloprovincialis*, were collected from "Pantano piccolo" lagoon of Messina, Sicily (Italy) by the "Associazione ZOE". They were acclimated for two days in the laboratory of the Palermo University in a flow-through system of oxygenated sea water at 20 °C. Hemolymph from individual mussels was collected from the posterior adductor muscle using a 1 ml syringe containing 0.2 ml of the anti-coagulant modified Alsever's solution buffer (27 mM sodium citrate, 115 mM glucose, 18 mM EDTA and 336 mM NaCl in distilled water, pH 7.0). Hemocytes were pelleted by 10 min centrifugation at 800 × *g* and 4 °C, carefully resuspended in 500 μl RNALater (Sigma) and stored at 4 °C until used. After removal of RNALater by 10 min centrifugation at 12,000 × *g* and 4 °C, total RNA was extracted with 1 ml Trizol Reagent (Invitrogen Life Technologies) and resuspended in 20 μl of Tris-EDTA buffer (TE: 10 mM Tris–HCl, pH 8.0, 1 mM EDTA). RNA concentration was measured with spectrophotometer ND-1000 (NanoDrop Technologies, USA) and stored at −80 °C until use.

2.2. RT-PCR for DGGE and electrophoresis

Reverse transcription was performed at 45 °C for 30 min, followed by denaturizing at 94 °C for 10 min with the One Step RT-PCR kit (Invitrogen Life Technologies) according to the manufacturer's instructions using GC clamp forward primer Ml-F2 (GC-Ml-F2): 5′CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCGTGCTGA-CTATCTGAATCATACACATA3′, and reverse Ml-R2: 5′GTATAATG-TCAAACAGAACGGGTC3′.

Thereafter, the PCR program included: 30 cycles of denaturing at 94 °C for 45 s, primers annealing at 60 °C for 1 min and elongation at 68 °C for 2 min, and a final extension at 68 °C for 7 min. Uniqueness and expected size of amplicons were checked by 1% agarose gel electrophoresis. Polymorphism of amplicons was then analyzed by DGGE on a Vertical Electrophoresis DCODE system (Bio-Rad) with 6% (w/v) polyacrylamide gels and denaturizing gradient from 20% to 80%. The 100% denaturizing solution was composed of 7 M urea and 40% formamide (v/v). Samples were prepared by adding 7 μl of 6× loading dye solution (Fermentas; 0.03% bromophenol blue, 0.03% xylen cyanol, 60% glycerol, 60 mM EDTA in 10 mM Tris–HCl pH 7.6) to 7 μl of PCR amplicon. DGGE was performed in Tris-acetate-EDTA buffer (TAE: 40 mM Tris-base, pH 7.4 with HCl, 20 mM glacial acetic acid, 1 mM EDTA) at 60 °C during 16 h at 80 V constant voltage. Gels were stained for about 30 min in the dark with 3 μl concentrated 10,000× SybrGold (Molecular Probes) diluted in 30 ml TAE, and washed 15 min in TAE. They were photographed using the UV Imager Gel Doc XR (BioRad) using SYBR filter. Band profiles were analyzed on the basis of densitometry calculated for each lane through AlphaView gel acquisition software (Alpha Innotech).

2.3. RT-PCR and cDNA cloning

Reverse primer Ml-R2 was as above. Forward primer Ml-F2 was the specific part of the above GC-Ml-F2: 5′TGCTGACTATCTGAATCATACACATA3′. First strain cDNA was synthesized using the one-step Access RT-PCR kit (Promega) according to the manufacturer's instructions performed on 50–100 ng of total RNA from 10 selected mussels corresponding to the 10 patterns observed in DGGE. Briefly, RT was performed at 45 °C for 45 min, followed by denaturizing at 94 °C for 2 min. Thereafter, the PCR program included: 40 cycles of denaturing at 94 °C for 30 s, annealing at 60 °C for 1 min and elongation at 68 °C for 1 min, and a final extension at 68 °C for 7 min. Existence of expected size amplicons at 421 bp was monitored by 1% agarose gel electrophoresis. For each profile, amplicons were cloned according to the TOPO TA Cloning kit (Invitrogen Life Technologies) in the plasmid pCR 2.1 TOPO. White *E. coli* colonies were individually transferred to deep agar containing Luria-Bertoni medium and 50 μg/ml kanamycin, in 96 well microtiter plates and sent to Agowa GmbH (Berlin, Germany) for sequencing using M13 universal primers. Each clone was double strand sequenced and the released sequences corrected accordingly.

2.4. Analysis of cDNA and deduced amino acid sequences

Several adjustments and comparisons have been made: (i) the nucleotides from up-stream and down-stream the primers, including the primer sequences, were removed, giving identical lengths of 371 bp, (ii) for each of the 10 DGGE patterns, all the sequences were aligned using Multalin (http://bioinfo.genopole-toulouse.prd.fr/multalin/multalin.html) and clustered according to nucleotide sequences, (iii) UTRs were removed and cds compared using Multalin, (iv) the different cds were translated into pro-peptides (http://www.expasy.ch/tools/dna.html) and (v) the resulting amino acid sequences compared using Multalin. The evolutionary relationships were inferred using the Neighbor-Joining algorithm MEGA-4 (http://www.megasoftware.net) (Tamura et al., 2007) considering the different complete cds (312 nucleotides) and adding the mytilin B sequence from GenBank (AF162336). The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004). Predictable structures of pro-peptides were established using Phyre software available at http://www.sbg.bio.ic.ac.uk/phyre/. Isoelectric points were calculated by http://www.iut-arles.up.univ-mrs.fr/w3bb/d_abim/compo-p.html.

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Site-by-site frequency spectrum-based statistical assays were used to test the hypothesis of polymorphisms within loci being neutral: Tajima's D (Tajima, 1989), Fu and Li's D and Fu and Li's F (Fu and Li, 1993), available at http://www.ub.es/dnasp/.

2.5. Genomic cloning and sequence analysis

Genomic DNA from one mussel belonging to pattern A were extracted from hemocytes with DNAzol (Invitrogen Life Technologies). PCR amplification of the fragment coding for the COOH-terminus, where most of the mRNA mutation points were observed, was obtained using forward primer MI-F3 hand-designed within intron-3 based on AF177540 (5′AGCACCCGTAATTAGTCCAAAC3′), and the previous reverse primer MI-R2. PCR program included: denaturing 3 min at 94 °C followed by 35 cycles of denaturing at 94 °C for 30 s, annealing at 60 °C for 30 sec and elongation at 72 °C for 40 sec, and a final extension at 72 °C for 5 min. Amplicons were cloned, selected and sequenced as reported in 2.3. Sequence analysis included the removal of nucleotides from up-stream and down-stream the primers, including the primer sequences, giving identical lengths of 429 bp. The 91 double strand corrected sequences were aligned using Multalin to locate putative mutation points and to compare with mRNA sequences.

3. Results

3.1. mRNA polymorphism revealed by DGGE

Amplicons obtained with GC-MI-F2 and MI-R2 migrated in gel electrophoresis as a single intense band of about 461 bp whatever the mussel being analyzed (not shown). Meanwhile, DGGE migration revealed important differences between the individuals which clustered into ten different patterns according to number and position of bands (Fig. 1). The different patterns were not homogeneously represented within the 57 mussels, as three patterns accounted for 78.8% of the individuals (A 35%, B 28% and C 15.8%). The remaining ~~eight~~ patterns were present from no greater than 7%, i.e. one to four mussels (Fig. 1). Bands appeared with different staining intensities and their numbers per pattern might be underestimated due to limited staining sensitivity.

3.2. Differences in mRNA sequences

Mytilin B mRNA from one mussel representing each of the ten patterns was amplified using the primers MI-F2 and MI-R2. Amplicons were cloned and 21–34 clones for each of the patterns were randomly selected to be double strand sequenced. A grand total of 586 nucleotide sequences were analyzed. Patterns A and I resulted in nine different sequences, the frequency of which was not homogeneous, three sequences accounting for 70–71.6% of the clones analyzed (Table 1). Patterns F and J resulted in eight different sequences with three sequences representing 77% (pattern F) and 85% (pattern J) of the clones analyzed. Other patterns were composed of three to six different sequences also including one to four major sequences. Only pattern G included two sequences nearly equally represented.

When restricted to cds (312 nucleotides), i.e. removing partial UTRs, the diversity of sequences per pattern was reduced from nine to five (pattern A) or to six (pattern I), and from three to one (pattern B) (Table 1). Meanwhile, the diversity of patterns E, G and H did not change when restricted to cds. Of the 312 nucleotides, 299 were invariant, and as less as thirteen (4.1%) were heterogenic (Table 2). Mutations were more numerous within the COOH-terminus (eight sites within 141 nucleotides, 5.6%) and particularly the partial 3′UTR (five sites within 36 nucleotides, 13.8%). Both the signal peptide and the mature mytilin B contained few mutations: two sites within 66 nucleotides (3.0%) and three sites within 102 nucleotides (2.9%), respectively. At all the sites, only two alternate nucleotides were possible. Whatever their location, mutations occurred with extremely diverse frequencies, from rare (two sequences out of the 293 being analyzed, i.e. 0.7% for nucleotides −16, 9, 39, 105, 147, 258 and 319) to frequent (about 43% for nucleotides −13, 264 and 318). In addition, the signal peptide appeared stable (0.7% of maximum frequency of mutations) and to a less extent the mature mytilin B (0.7–5.1%). The highest frequencies of mutations (about 43%) were remarkable: one was recorded within the partial 5′UTR, another within the COOH-terminus, and one within the partial 3′UTR.

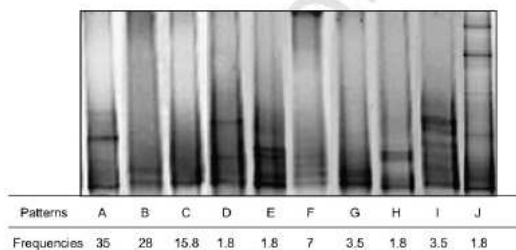

Fig. 1. Different patterns of mytilin B mRNA as observed with DGGE, and frequencies of occurrence (%) within the population from Messina, Sicily (Italy) ($n = 57$). Note the different numbers and positions of bands according to the patterns. Differences in band staining intensity might result in underestimation of the real number of bands per pattern.

3.3. Phylogenetic relationships of cds

Comparison of the 36 cds originating from the 10 different patterns revealed that some patterns shared identical cds. Consequently, the number of different cds was restricted to 21 (not illustrated). The bootstrap consensus tree inferred from 225 replicates was taken to represent the evolutionary changes of the mytilin B cds (Fig. 2). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed. As shown in Fig. 2, A1 sequence was identical to mytilin B deposited in GenBank (AF162336) and such sequence belong to one of the two clusters together with A3, C4, G2, H3, I1 and J2, accounting for 56.7% of the 293 cds analyzed during this study. In addition, the 3 cds A1, A4 and D2, segregating in different clusters, represented 80.9% of the analyzed sequences.

3.4. Evidence for neutral or positive selection pressure

To determine whether neutral selection was operating within the codon sites of the mytilin B cds variants, several parameters were defined using three different statistical tests (Table 3). All three tests were in agreement with the hypothesis of negative selection pressure regarding signal and mature domains. Concerning COOH-terminus domain, only Tajima's D value of 1.0093 (slightly above 1) suggested possible positive selection pressure, not confirmed by the two other tests. Similarly, only the Fu and Li's D test indicated possible positive selection pressure when applied to full cds. Fu and Li's F test was always in favour of negative selection pressure.

3.5. Consequences for peptide sequences

Nucleotide sequences from cds were translated into amino acids by computer. The number of different pro-peptide sequences

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Table 1
Number of DGGE patterns and corresponding different nucleotide sequences found in the mussel population from Messina, Sicily (Italy), with number of deduced amino acid sequences of pro-peptides, leading to a unique mature mytilin B.

DGGE patterns	Number of clone analyzed	partial mRNA sequences					Amino acid sequences		
		Different sequences			Different cds		Pro-peptides		Different mytilin B
		Number	Names	Fequency %	Names	Frequency %	Names	Frequency %	
A	34	9	A1	6	A1	26	P1-A	25	1
			A2	20			P2-A	75	
			A3	3	A3	3			
			A4	38					
			A6	6	A4	56			
			A7	12					
			A5	6	A5	6			
			A8	6	A8	9			
			A9	3					
B	34	3	B1	12	B1	100	P1-B	100	1
			B2	3					
			B3	85					
C	30	4	C1	57			P1-C	100	1
			C2	30	C1	93.5			
			C3	6.5					
			C4	6.5	C4	6.5			
D	30	6	D1	53	D1	60	P1-D	90	1
			D5	7			P2-D	10	
			D2	13	D2	23			
			D4	10					
			D3	10	D3	10			
			D6	7	D6	7			
E	28	3	E1	82	E1	82	P1-E	100	1
			E2	7	E2	7			
			E3	11	E3	11			
F	27	8	F1	44	F1	55	P1-F	70	1
			F2	7			P2-F	30	
			F3	4					
			F4	4	F4	15			
			F5	11					
			F6	22	F6	26			
			F7	4					
			F8	4	F8	4			
G	25	2	G1	52	G1	52	P1-G	52	1
			G2	48	G2	48	P2-G	48	
H	21	3	H1	81	H1	81	P1-H	100	1
			H2	4.5	H2	4.5			
			H3	4.5	H3	4.5			
I	31	9	I1	26	I1	35.6	P1-I	68	1
			I4	6.5			P2-I	32	
			I7	3.1					
			I2	22.6	I2	22.6			
			I3	22.6	I3	29.1			
			I6	6.5					
			I5	6.5	I5	6.5			
			I8	3.1	I8	3.1			
			I9	3.1	I9	3.1			
J	33	8	J1	64	J1	73	P1-J	76	1
			J3	9			P2-J	15	
			J2	12	J2	15	P3-J	6	
			J8	3			P4-J	3	
			J4	3	J4	3			
			J5	3	J5	3			
			J6	3	J6	3			
			J7	3	J7	3			

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Table 2
Location, occurrence and nature of nucleotide and amino acid replacements within mytilin B mRNAs. n = 293 sequences analyzed in double strand. The 2 locations with nucleotide replacements inducing different amino acids are shaded.

	mRNA	Replacement location	Nucleotide from sequence AF162336 and position in codon	Alternate nucleotide with frequency of occurrence	Corresponding amino acids
-26					
	Partial 5'UTR	-22	C	T - 13.6 %	
		-16	C	T - 0.7 %	
		-13	T	C - 43.4 %	
-1					
1					
	Signal peptide	9	A - 3rd	G - 0.7 %	Ala
		39	A - 3rd	G - 0.7 %	Ala
66					
67					
	Mature mytilin B	105	A - 3rd	G - 0.7 %	Arg
		135	C - 3rd	T - 5.1 %	Tyr
		147	C - 3rd	T - 0.7 %	Cys
168					
169					
	COOH-terminus	192	C - 3rd	T - 24.6 %	Phe
		232	C - 1st	G - 2.4 %	Gln or Glu
		258	A - 3rd	G - 0.7 %	Glu
		261	C - 3rd	T - 1.7 %	Phe
		264	T - 3rd	C - 43.3 %	Gly
		276	C - 3rd	A - 1.7 %	Pro
		305	G - 2d	A - 28.7 %	Gly or Asp
309		309	T - 3rd	A - 28.7 %	Ile
310					
	Partial 3'UTR	315	A	G - 32.8 %	
		318	T	A - 43 %	
		319	G	A - 0.7 %	
		322	C	T - 18.1 %	
		331	C	T - 2.1 %	
345					

ranged from four (pattern J) to a unique sequence (patterns B, C, E and H) revealing that most of the nucleotide substitutions were silent (Table 1). When 2 or 4 pro-peptide sequences were simultaneously present, one was always major (68–90%), exception being pattern G where the two pro-peptide sequences were nearly equally represented. When comparing the 18 pro-peptide sequences, only four different sequences were found (P1-A, P2-A, P2-J and P3-J), differing by two amino-acids in position 78 (Gln or Glu) and 102 (Gly or Asp) (Table 2, Fig. 3). The pro-peptide sequence including the combination Gln78-Gly102 was found in 70% of the 293 analyzed sequences, and corresponded to the original mytilin B mRNA sequence AF162336. Other combinations were less frequent: 27% for Gln78-Asp102, 2% for Glu78-Gly102 and less than 1% for Glu78-Asp102, i.e. found only twice. Comparison of predictable structures of the four pro-peptides combining the two amino acid replacements, shown identical secondary coil structure prediction of the COOH-terminus (not shown). We found remarkable that, whatever the number of variants observed in the different patterns, the mature mytilin B sequence was always unique, and identical to the one from GenBank AAD52661.

Table 3
Polymorphism and assays for neutrality in mytilin B cds variants of *M. galloprovincialis* from Messina, Sicily (Italy). n = 294. Note that no one test was statistically significant at 0.05% except the Tajima's D for COOH-terminus and the Fu and Li's D for total cds (*).

	Total cds	Signal peptide	Mature peptide	COOH-terminus
Domain positions	1–309	1–66	67–168	169–309
Number of nucleotides per domain	309	66	102	141
Number of variable sites (S)	13	2	3	8
Nucleotide diversity per site (π)	0.00661	0.00041	0.00122	0.01341
Nucleotide diversity based on the proportion of segregating sites (θw)	0.00672	0.00484	0.0047	0.00906
Neutrality tests				
Tajima's D	−0.0397	−1.1808	−1.1244	1.0093*
Fu and Li's D	1.4577*	0.6136	0.7464	1.1794
Fu and Li's F	1.0694	0.0419	0.1543	1.3412

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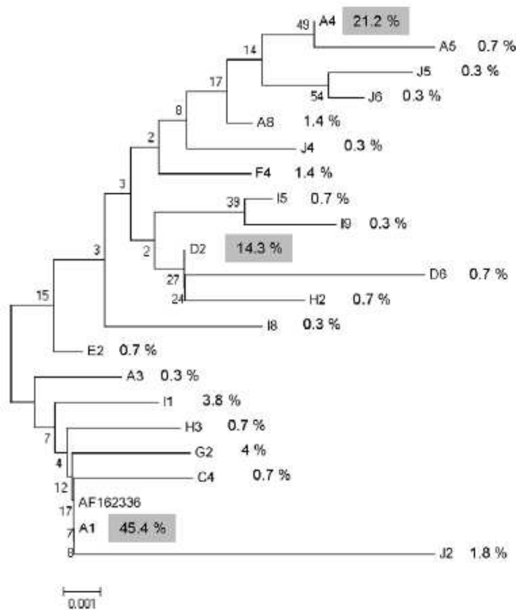

Fig. 2. Evolutionary relationships of the 21 different nucleotide sequences of mytilin B complete cds obtained in this study, joined to the mytilin B cds found in GenBank (AF162336). The evolutionary history was inferred using the Neighbor-Joining method. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated mytilin B cds clustered together in the bootstrap test (225 replicates) are shown next to the branches. The letter-digit referred to patterns and cds classification (see Table 1). Percentages referred to frequency of the related sequence among the 293 analyzed sequences. Scale bar: evolutionary genetic distance. Note the segregation in two clusters and the 3 cds A1, A4 and D2 (shaded area) accounting for 80.9% of the analyzed sequences.

3.6. Genomic analysis

3.6.1. Partial intron-3

Alignment of the 91 sequences obtained from PCR amplification of genomic mytilin B DNA from pattern A hemocytes revealed that 38 sequences were identical (Fig. 4). Among the 53 sequences showing differences, 52 sequences included one to four mutation points dispatched at random. Only one sequence contained 16 randomly distributed mutation points.

3.6.2. Partial exon-4

Among the 91 sequences, 90 were identical between them and identical to the mytilin B sequence deposited in GenBank (AF177540). Only one sequence was distinct and this particular sequence included four out of the six mutation points observed within the pattern A mRNAs: nucleotides 264, 305, 309 and 318 (Fig. 4). All four replacements were with the expected nucleotide according to mRNA sequences. Meanwhile, two mRNA mutation points (nucleotides 315 and 319), both located within the 3′UTR, were not observed within the genomic sequences. The presence of the mutation point 305, as observed within both DNA and mRNA with the same nucleotide replacement, was of particular interest. This resulted in the unique amino acid replacement. In addition, this was the same sequence possessing the highest intron-3 and exon-4 mutation points of 16 and 4, respectively.

4. Discussion

Previous biochemical purification of mussel mytilin revealed the presence of five different sequences of the mature peptide, referred to as isoforms (Mitta et al., 2000b). Complete gene sequence of mytilin B is the only one available, allowing knowledge of the complete sequence of the corresponding mRNA (Mitta et al., 2000a). Based on this sequence, specific primers for mytilin B were designed within the UTRs to ensure amplification of the full cds. The multiple sequences analyzed herein, identifying exclusively mytilin B, validated the specificity of the designed primers. Meanwhile, such high specificity is surprising when compared to the close relationships between the five mytilin amino acid sequences. As only the mytilin B mRNA sequence is known, we hypothesized that at least the respective UTRs are so different that cross-hybridization with the primers designed for mytilin B did not occur. Similar specificity of primers designed within the UTRs of myticin C were reported (Pallavicini et al., 2008), not amplifying myticin A nor B for which complete 5′UTR sequences are not known.

All the mussels constitutively expressed mytilin B in hemocytes, confirming our previous observations obtained by RT-PCR (Cellura et al., 2007). Using DGGE technology, we revealed that two to ten different sequences of mytilin B mRNAs were simultaneously present per mussel. Among the 57 mussels from the Messina, Sicily population, 10 original patterns were identified with three majors and four minors being encountered in only one mussel. By contrast, DGGE profile of myticin C was reported as specific of each individual mussel, the same pattern of bands being never observed, except within offspring from the same family (Costa et al., in press). All the patterns shared identical nucleotide sequences, reducing the number from 55 to 36 different mRNAs and to 21 different cds, a phenomenon also observed for myticin C (Costa et al., in press). The 21 mytilin B cds segregated in two clusters accounting for nearly half the sequences each. In addition, the most frequent cds (45%) was found in one cluster, the two others most frequent cds ~~(21 + 14 = 36%)~~ were found in the other cluster. Consequently, we hypothesized that the present diversity of mytilin B resulted from duplication of an ancestral gene.

The fact that nucleotide differences were precisely located in 21 mutation points, only nine being found in more than 10% of the

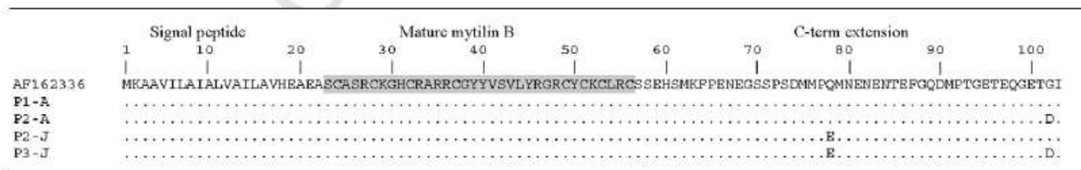

Fig. 3. The four pro-peptide sequences obtained by computer translation of the different cds clustered from the 10 DGGE patterns compared to mytilin B pro-peptide found in GenBank AF162336. Mature mytilin B is shaded. Note the constant composition of signal and mature peptides and the two replacements of amino acids occurring within COOH-terminus.

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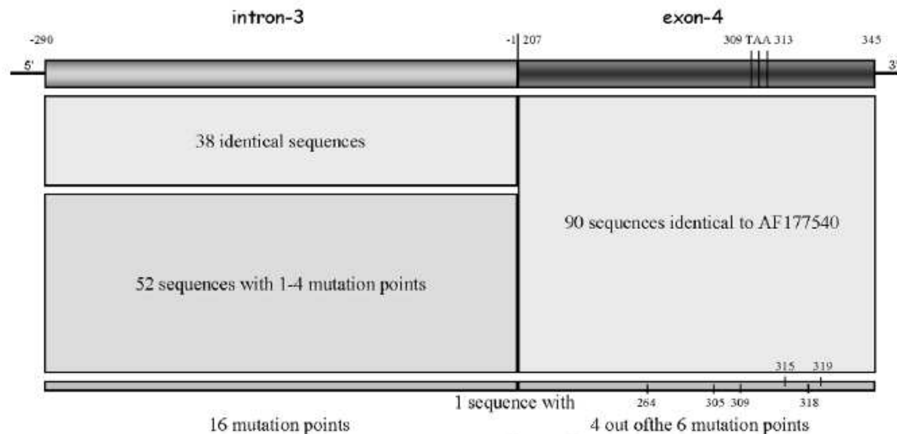

Fig. 4. Genomic analyses of the 91 sequences corresponding to part of the mytilin B gene obtained from hemocytes of one mussel from pattern A. Note the polymorphism within intron-3. The fact that only one sequence contained the most polymorphic intron and the only polymorphic exon was remarkable. However, mutation points 315 and 319 were missing from this sequence.

sequences, was remarkable. Both signal peptide and mature mytilin B included few mutation points, rarely found and not changing the amino acids. By contrast, COOH-terminus contained eight mutation points, four found in more than 24% of the sequences. It was also within this COOH-terminus that the only two mutation points (232 and 305) inducing different amino acids were located. The mutation 232, leading to Glu78, was found in only 2.3% of the sequences, and only in pattern J, which was encountered in only one of the 57 tested mussels. In contrast, the mutation 305, leading to Asp102, was found in six out of ten of the patterns, representing 29% of the analyzed sequences. The replacement of an uncharged polar side chain (Gln) or of a non-polar side chain amino acids (Gly) by acidic side chain amino acids (Asp) might induce different properties of the COOH-terminus. This COOH-terminus, found in many AMPs (reviewed by (Zasloff, 2002), was often considered to play a role in neutralizing the cationic bioactive mature AMPs (Mitta et al., 2000c). In the case of mytilin B, the different amino acid combinations resulted in predicted COOH-terminus iso-electric points that ranged from 3.49 to 3.60, compared to 12.05 for the mature peptide. Consequently, the replacements of the two amino acids resulting in only 0.11 *pI* value of difference, did not modify the COOH-terminus properties or the net charge of the pro-peptide.

Proteins with highly variable sequences are not rare in vertebrates (immunoglobulins, MHC and TLR for instance) and have been also reported in invertebrates: ascidians with the *fester* gene (Nyholm et al., 2006), sea urchin with TLR (Hibino et al., 2006), *Drosophila* with TLR (Tauszig et al., 2000), Dscam (Watson et al., 2005), PGRP (Royet et al., 2005) and AMPs (Imler and Bulet, 2005), shrimp with penaeidins (Padhi et al., 2007), gastropods with FREP (Zhang et al., 2004), bivalves with myticin C (Gestal et al., 2007; Pallavicini et al., 2008). All these polymorphic proteins belong to the immune system and our work extended the diversity phenomenon to another immune molecule. Meanwhile, only four different propeptides were identified and the mature mytilin B was unique, rejecting the existence of any genomic rearrangement or alternative splicing, as for instance in *Drosophila* Dscam (Schmucker et al., 2000) or ascidian *fester* (Nyholm et al., 2006). A recent bioinformatic analysis performed on data released by Pallavicini et al. (2008) concluded that polymorphism within myticin C loci was driven by positive selection, indicating adaptive evolution of certain amino acids (Padhi and Verghese, 2008). Similar analysis performed by the same authors, with identical conclusion concerned shrimp penaeidins (Padhi et al., 2007). In both cases, hypothesis suggested that interaction with surrounding pathogens in the changing environment caused molecular adaptation. Also mosquito's defensin (Dassanayake et al., 2007), termite's antifungal termicin (Bulmer and Crozier, 2004), frog's AMPs (Duda et al., 2002) and even mammal's defensins (Lynn et al., 2004), were reported as evolving rapidly under positive selection. In contrast, several studies failed to demonstrate rapid adaptive evolution of six different families of *Drosophila*'s AMPs (Jiggins and Kim, 2005; Lazzaro and Clark, 2003), although some evidence of positive selection were reported. Our data on mytilin B variants, not translated into different mature AMP, suggested that, on the opposite to myticin C, mytilin B did not co-evolve with pathogens (Fig. 5).

At least for pattern A, the diversity of the mytilin B genomic sequence corresponding to exon-4 did not match the diversity of corresponding mRNAs. Consequently, the diversity observed at the mRNA level most likely resulted from post-transduction modifications, which would not have been translated into amino acids. However, we found mutations also within partial intron-3: 57% of the analyzed sequences with one to four mutations, and one out of 91 with 16 mutations suggesting comparison restricted to the coding regions will result in an underestimated diversity. As demonstrated for mammal defensin, introns are more appropriated than exons for reconstruction of phylogenetic history data (Luenser et al., 2005). Coincidently, it was the same partial mytilin B gene sequence that showed the highest mutated intron and the unique mutated exon.

Among the three tests for neutrality applied for mytilin B cds, both Tajima's D and Fu &Li's suggested possible positive selection, the first for COOH-terminus, the second for full cds. They did not confirm each other and Fu and Li's S was in favour of neutrality. Consequently, the situation for mytilin B is definitively different from the one for myticin C (Padhi and Verghese, 2008). However, the two analyzed mussel populations belong to different geographical origins: Sicily (Italy) for mytilin B and Rias de Galicia (Spain) for myticin C. Reciprocal analysis of are in progress to explain the role of the different living environment on the polymorphism of mussel AMPs.

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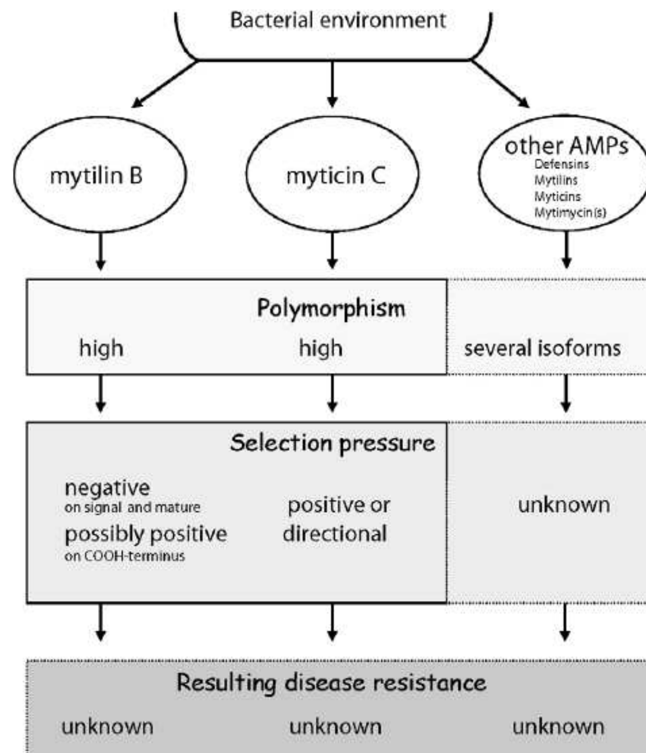

Fig. 5. Interpretive diagram of the putative influence of bacterial environment onto mussel AMP polymorphism, resulting from data on mytilin B and myticin C. Myticin C data are from Pallavicini et al. (2008) and Padhi and Verghese (2008).

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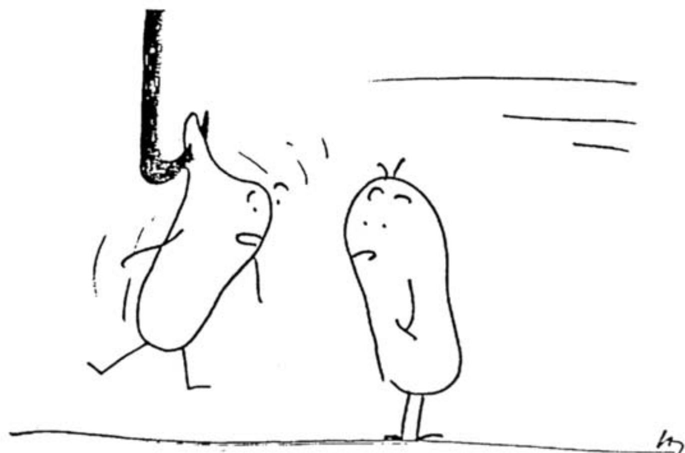
Conclusion et Perspectives



Don't stare at me in that silly way! Help me off this receptor!

(D'après « Funny Microbes », Leos Mandel)

Conclusion et Perspectives

L'ensemble des résultats obtenus au cours de cette thèse permet de mieux comprendre les comportements des hémocytes et la régulation des gènes immunitaires au cours des réactions d'immunité innée chez la moule méditerranéenne *Mytilus galloprovincialis*. Ces travaux ont confirmé la complexité et l'efficacité de l'immunité innée des invertébrés. La technique de la cytométrie de flux a permis de suivre l'évolution des différentes catégories d'hémocytes en réponse à l'introduction de diverses bactéries. Par ailleurs, la quantification de l'expression de quelques gènes immunitaires dans les hémocytes, tels le *lysozyme* et trois *AMPs*, jointe à celle de *HSP70* et de *28S rDNA*, a mis en évidence l'importance de l'origine géographique des moules, et l'influence de la saison, sur les caractéristiques de la réponse immunitaire. De plus, l'étude du polymorphisme des ARNm de l'un des AMPs de la moule, la mytiline B, a montré que la diversité des gènes/protéines immunitaires connue chez divers invertébrés existait également chez la moule de Méditerranée.

L'idée de la complexité du système immunitaire de la moule est renforcée par la découverte de la diversité des AMPs. Les moules vivent dans un environnement complexe et, de par leur activité de filtration de l'eau de mer, elles accumulent divers micro-organismes dans leur tissus. Le phénomène du polymorphisme de gènes immunitaires peut constituer la solution pour combattre un nombre élevé d'espèces de micro-organismes avec un nombre réduit de molécules antimicrobiennes. De fait, ce polymorphisme (ou cette diversité) n'est pas rare chez les invertébrés. Chez *M. galloprovincialis*, Pallavicini *et al.* (Dev Comp Immunol 2008;32:213-226) et Costa et al (Dev Comp Immunol 2008;doi:10.1016) ont montré récemment l'existence d'une troisième isoforme de la myticine, appelée myticine C, qui présente une forte diversité au niveau des ARNm ainsi qu'au niveau des acides animés. Contrairement à cette diversité de la myticine C observée chez la moule provenant de la côte Atlantique (Ria de Vigo-Espagne), nos résultats sur le polymorphisme de la mytiline B des moules de Sicile (Italie) montrent que le peptide mature est unique, malgré les mutations observées au niveau des ARNm. Les tests de neutralité suggèrent une pression de sélection positive sur le peptide mature de la myticine C, tandis qu'elle serait négative sur le peptide mature de la mytiline B. Ces différences de comportements entre deux AMPs pourraient avoir deux origines : **(i)** les populations de moules proviennent de deux localités géographiques ayant des caractéristiques environnementales très différentes, telles la température, la salinité,

les communautés microbiennes, le contenu en éléments nutritifs, etc ; **(ii)** les deux AMPs ne jouent pas le même rôle dans l'immunité innée de la moule et n'ont pas, par conséquence, suivi la même évolution. Dans un futur proche, il reste à comparer le polymorphisme de ces mêmes AMPs chez des moules provenant des localisations réciproques, ainsi que chez les moules de l'Etang du Prévost. Il serait également intéressant d'établir le degré de polymorphisme des autres isoformes des défensines, mytilines, myticines et mytimycines afin d'avoir une vue plus large sur l'étendue des molécules que la moule peut utiliser pour combattre les infections. Enfin, le fait que différents tissus d'une même moule présentent différentes myticine C, suggère que tous les hémocytes pourraient ne pas exprimer le même peptide suivant leur localisation tissulaire.

Pour la première fois, nous avons montré que l'expression d'un même gène immunitaire varie en fonction de l'origine géographique des moules : la *myticine B* est 51 fois plus exprimée chez les moules de l'Etang du Prévost-Palavas que chez celles de la Ria de Vigo. De même, l'expression de *HSP70*, représentant une réponse à un stress non-spécifique, est 120 fois plus élevée chez les moules de l'Etang du Prévost. En fait, c'est chez les moules de la Ria de Vigo que les cinq gènes étudiés (*défensine*, *mytiline B*, *myticine B*, *lysozyme* et *HSP70*) sont le moins exprimés. Ces faibles expressions observées chez les moules de la Ria de Vigo ne sont pas étonnantes si l'on considère l'existence d'une relation directe entre température et niveau métabolique.

En fonction de la nature du stress, nous avons montré que les cinq gènes sont régulés différemment dans les trois populations de moules : Vigo, Palavas et Venise. En général, les injections bactériennes provoquent une diminution de l'expression, principalement celles de la *mytiline B* et de la *myticine B*. Chez les moules de l'Etang du Prévost, un même profil d'expression de la *mytiline B* a été observé après l'injection de *V. splendidus* ou de *M. lysodeikticus* : l'expression du gène chute avec un minimum à 1 h, suivi par une récupération dès le stade 3 h avec un retour au niveau normal à 48 h. Cette baisse d'expression a été confirmée en hybridation *in situ* (HIS) : disparition du marquage de la *mytiline B* au stade 3 h. La même corrélation entre les résultats de Q-PCR et ceux d'HIS a également été trouvée pour le gène du *lysozyme*. Par exemple, 48 h après l'injection de *V. anguillarum*, l'augmentation du pourcentage des hémocytes exprimant le *lysozyme* observé en HIS correspond à un niveau d'expression de ce gène plus élevée mesuré en Q-PCR. La diminution de l'expression des gènes immunitaires telle que observée en Q-PCR résulte donc d'une accumulation au site

d'injection des hémocytes exprimant ces gènes et non pas d'une diminution de l'expression par les hémocytes compétents.

En ce qui concerne les stress de température, une élévation de la température a globalement augmenté l'expression des gènes, sauf celui de la *mytiline B*. En revanche, une baisse de la température n'a pas eu d'effet, sauf une augmentation de la *défensine* observée chez les moules de Venise. Nos résultats renforcent l'idée de la spécificité de la réponse d'immunité innée puisqu'une même bactérie n'induit pas une réponse identique des gènes immunitaires. En outre, l'origine géographique des moules a une forte influence sur la nature et l'intensité de l'expression de ces mêmes gènes, suggérant un effet environnemental important.

Comme nous l'avons mentionné plusieurs fois dans les chapitres précédents, les hémocytes circulants sont les principaux effecteurs de l'immunité innée de la moule. Après injections, les bactéries sont rapidement retrouvées dans les hémocytes, probablement par phagocytose, puis sont éliminées. Nous avons montré qu'à l'intérieur des hémocytes, la cinétique d'élimination dépend de la nature de la bactérie : 12 h pour la bactérie à Gram-positif *Micrococcus lysodeikticus*, 24 h pour la bactérie à Gram-négatif *Vibrio splendidus* et 48 h pour *V. anguillarum*. En outre, la majorité des *V. splendidus* vivants a été retrouvée à l'intérieur des hémocytes 6 h après l'injection, comparée à 1 h pour *V. anguillarum*. Ceci pourrait s'expliquer par l'existence de différents mécanismes de reconnaissance / réponse employés par les hémocytes vis-à-vis des bactéries. Cette explication a été partiellement confirmée par les résultats de la PCR quantitative obtenus avec les moules de l'Etang du Prévost : par exemple, une diminution générale de l'expression du *lysozyme* a été observée après injection de *V. splendidus*, tandis que l'injections de *V. anguillarum* a un effet d'augmentation sur les stades tardifs (deux jours après les injections). De même, l'injection de *V. splendidus* diminue fortement l'expression du gène de la *mytiline B* aussitôt après l'injection, tandis que l'injection de *V. anguillarum* n'a pas d'effet. Pour instant, nous n'avons que des résultats portant sur l'expression des gènes, soit bien en aval des voies de transduction intracellulaire des signaux. Pour compléter notre schéma, il est nécessaire d'étudier le comportement des autres gènes impliqués dans ces voies de transduction, et en premier lieu, ceux qui codent les récepteurs de type TLR.

D'après nos résultats, le nombre total d'hémocytes (THC) est modifié de façon différente en fonction de la bactérie injectée. *M. lysodeikticus* induit une augmentation 1 h après l'injection de bactéries vivantes puis une diminution au stade 3 h, et une diminution 1 h après l'injection de bactéries mortes puis un retour au niveau normal au stade 3 h. Quant aux deux *Vibrio*, l'injection de *V. anguillarum*, vivante ou morte, induit une faible diminution au stade 12 h, tandis que l'injection de *V. splendidus* fait considérablement chuter le THC : moins 66 % d'hémocytes 9 h après l'injection de bactéries vivantes et moins 56 % 3 h après l'injection de bactéries mortes. Une hypothèse pour expliquer ces différences de comportement vis-à-vis des bactéries mortes et vivantes, pourrait être que les hémocytes s'accumulent passivement au site d'injection des bactéries mortes, tandis qu'ils sont attirés par des molécules libérées par les bactéries vivantes, soit un processus d'élimination plus complexe. Un phénomène qui n'a jamais été observé avec les bactéries mortes, est que le THC est toujours plus élevé que le niveau normal 24 h ou 48 h après injections de bactéries vivantes, ce qui suggère une multiplication et / ou une mise en circulation intense des hémocytes.

La classification des hémocytes des bivalves fait toujours débat. Grâce à la technique de la cytométrie de flux, nous avons classé les hémocytes de moule en trois sous-populations : **(i)** les grands granulocytes qui représentent environ 30 % du THC; **(ii)** les petits granulocytes, 50 % ; et **(iii)** les hyalinocytes, 20 %. La réponse générale des moules consiste en une diminution rapide des trois sous-populations dans les 60 min suivant les injections de bactéries, ce qui confirme les diminutions de THC précédemment observées. Actuellement, même s'il est difficile d'attribuer des fonctions définies aux trois sous-populations d'hémocytes, la proportion de ces cellules varie énormément quand le THC est au minimum, suggérant une participation différentielle en fonction de la bactérie injectée. En outre, après une très forte augmentation du nombre de cellules des trois sous-populations, les hyalinocytes sont toujours en diminution tandis que le nombre des petits et des grands granulocytes continue d'augmenter. Ces variations laissent supposer que les hyalinocytes sont les cellules précurseurs des deux sous-populations de granulocytes. L'observation des diagrammes de cytométrie de flux montre une continuité morphologique entre les trois sous-populations d'hémocytes, suggérant l'existence d'une seule lignée cellulaire. Contrairement aux études précédentes, les hyalinocytes sont apparus comme agissant au même titre que les granulocytes dans l'immunité antibactérienne, parce que leur nombre varie dans les deux jours suivant les

injections, et ce en fonction des bactéries, i.e. plus 90 % 12 h après l'injection de *V. splendidus* vivante, et moins 37 % 3 h après l'injection de *M. lysodeikticus*.

La première étape de la réponse antibactérienne serait non-spécifique, car les trois sous-populations hémocytaires ont leur nombre qui diminue dans la circulation au stade 1 h, dû à leur accumulation au site d'injection (Fig. 16). Dès le stade 3 h, la seconde étape de la réponse montre une spécificité en fonction de la nature de la bactérie :
(i) *V. splendidus* continue à attirer les trois catégories d'hémocytes au site d'injection, ce qui rend leur nombre minimal au stade 9 h, malgré un faible recrutement entre 1 h à 3 h. Après cette baisse, une forte augmentation des trois sous-populations, particulièrement celle des hyalinocytes, résulte probablement d'une prolifération intense de ces derniers et de leur maturation en granulocytes. Au stade 12 h, le nombre des hyalinocytes diminue tandis que celui des deux sous-populations des granulocytes continue à augmenter, ce qui confirme que les hyalinocytes seraient bien les cellules précurseurs des granulocytes, et ce phénomène dure au moins jusqu'à 48 h ;
(ii) *V. anguillarum* a un effet plus complexe sur le comportement des trois sous-populations. Au stade 3 h, les hyalinocytes continuent à s'accumuler au site d'injection tandis que les deux sous-populations des granulocytes ont été recrutées dans la circulation avant de s'accumuler elles aussi au site d'injection. Au stade 6 h, le nombre des hyalinocytes commence à augmenter avant celui des granulocytes (9 h pour les grands et 12 h pour les petits). Au stade 12 h, le nombre des hémocytes circulants augmente jusqu'à au moins 48 h ;
(iii) *M. lysodeikticus* a un effet similaire sur le comportement des trois sous-populations, sauf pour la période entre 9 h et 12 h. Un recrutement intense dans la circulation suivi par une forte accumulation des hémocytes néo-recrutés au site d'injection pour les trois sous-populations pourrait expliquer le faible nombre d'hémocytes circulants observé au stade 3 h. Le nombre des trois catégories d'hémocytes circulants augmente ensuite grâce au recrutement de cellules. Au stade 9 h, la maturation des hyalinocytes en granulocytes commence à faire augmenter le nombre de ces derniers tout en diminuant celui des hyalinocytes. Cependant, les deux sous-populations des granulocytes continuent à s'accumuler au site d'injection entre 12 h et 24 h post-injection, probablement à cause de la persistance de cette bactérie. Au stade 24 h, la multiplication des hyalinocytes, puis leur maturation en granulocytes persiste jusqu'à au moins 48 h.

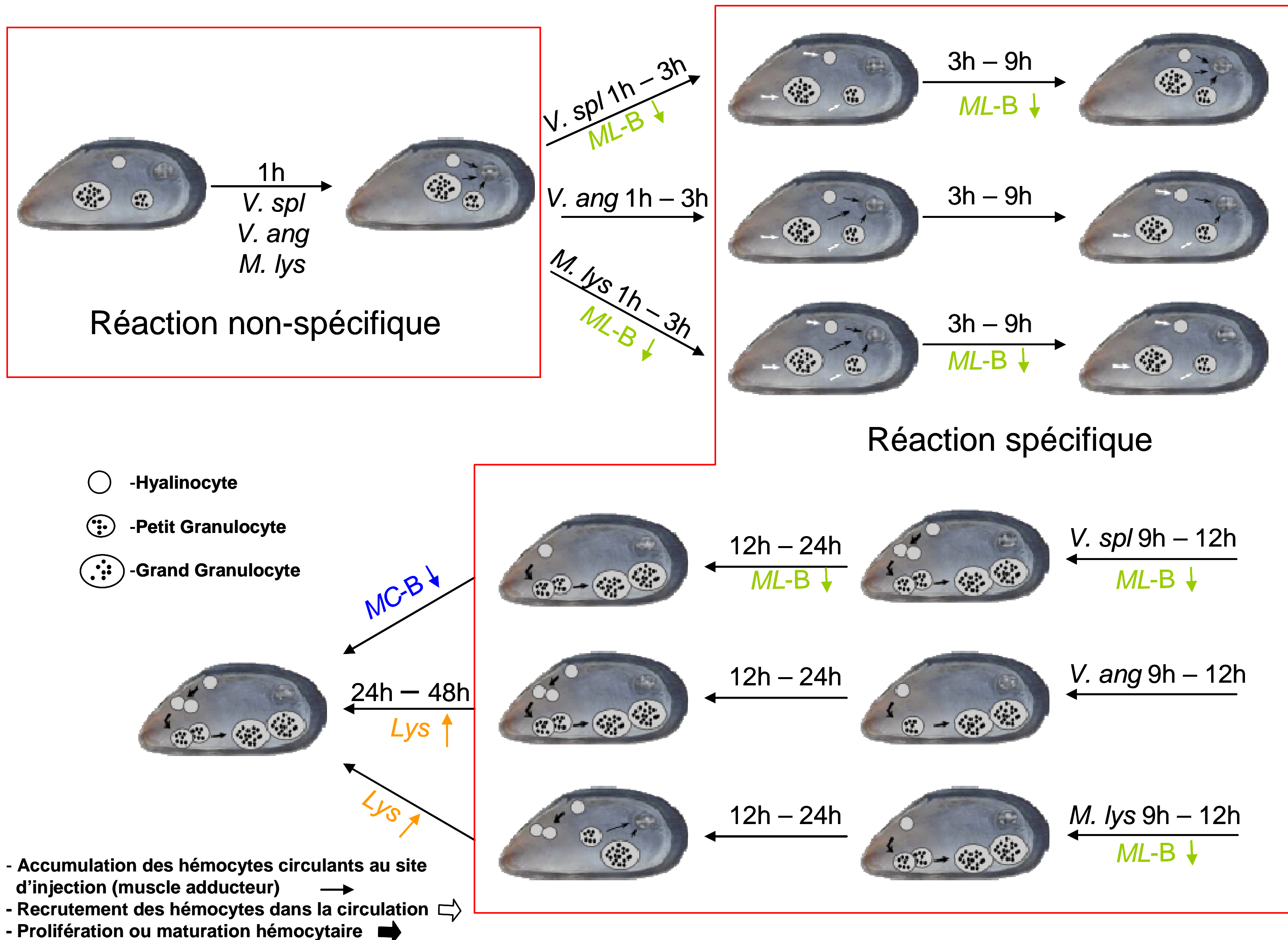


Figure 16 - Comportement des trois populations hémocytaires et régulation de divers gènes immunitaires en réponse aux injections bactériennes. *V.spl* : *V.splendidus* ; *V. ang* : *V. anguillarum* ; *M. lys* : *M. lysodeikticus*; *ML-B* : *mytiline B* ; *MC-B* :*myticine B* ; *Lys* : *lysozyme*.

En conclusion, basé sur les résultats obtenus par la cytométrie de flux, les hyalinocytes représenteraient une forme cellulaire précoce des petits et des grands granulocytes. Suite à la stimulation provoquée par l'injection de bactérie à Gram-positif ou à Gram-négatif, le système immunitaire de la moule réagit de la même façon durant la première heure: les deux sous-populations des granulocytes ainsi que les hyalinocytes s'accumulent au site d'injection, ce qui suggère une étape de réaction non-spécifique. Cependant, l'élimination des bactéries résulte de l'action combinée de composants cellulaires et de composants humoraux, qui sont contrôlés différemment par le système d'immunité innée de la moule en fonction de la bactérie rencontrée : les sous-populations d'hémocytes ont un comportement différent suivant la nature de la bactérie injectée. Ces différences confirment l'idée de la spécificité de l'immunité innée des invertébrés.

Comportements cellulaires et régulation génétique au cours des réactions d'immunité innée chez la moule *Mytilus galloprovincialis*

La mytiliculture occupe une place très importante dans l'aquaculture, cependant, le système immunitaire de la moule, qui joue un rôle décisif face aux diverses maladies infectieuses, reste mal connu. Comme tous les invertébrés, notre modèle d'étude, la moule méditerranéenne *Mytilus galloprovincialis*, ne possède qu'un système d'immunité innée souvent décrit comme peu complexe et non spécifique. Les résultats de cette thèse ont montré que le système immunitaire des moules **(i)** est capable de répondre différemment à diverses stimulations, ce qui suggère une reconnaissance spécifique ; **(ii)** répond différemment suivant l'origine géographique des animaux, ce qui montre une adaptation à l'environnement ; **(iii)** est influencé par la saison, ce qui pourrait être mis en relation avec les mortalités saisonnières ; **(iv)** présente un polymorphisme des peptides antimicrobiens, ce qui augmente d'autant le nombre d'effecteurs moléculaires. En conclusion, le système d'immunité innée de la moule apparaît comme complexe, impliquant des effecteurs polymorphes, capable de répondre spécifiquement à divers stimuli, adapté à l'environnement et influencé par la saison.
Mots clés : immunité innée, régulation génétique, polymorphisme, peptides antimicrobiens, hémocyte, *Mytilus*

Cellular behaviours and genetic regulation during innate immune reactions in the mussel *Mytilus galloprovincialis*

The mytiliculture is one of the most important aquaculture in the world, however, little is known on the mussel immune system, which plays a critical role in case of infectious diseases. Like all the other invertebrates, our model, the Mediterranean mussel *Mytilus galloprovincialis*, possesses only an innate immune system considered to be primitive and non-specific. In the present study, our results showed that the mussel immune system **(i)** is capable of responding differently to a variety of stimulations, suggesting a specific recognition process; **(ii)** reacts differently according to the geographic origin of the animals, showing an adaptation to their own environment; **(iii)** is influenced by the season, which might explain the seasonal mortalities; **(iv)** presents an extended polymorphism of antimicrobial peptides, which increases in turn the number of molecular effectors. In conclusion, the innate immunity system of the mussel appears more complex than suspected and involving polymorphic immune effectors. Moreover, this system is capable of responding specifically to different stimuli, of adaptation to the environment, and is influenced by the season.
Key words: innate immunity, genetic regulation, polymorphism, antimicrobial peptides, haemocyte, *Mytilus*

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