Approche intégrative de la réponse d'un organisme marin face au changement climatique : la coquille Saint-Jacques Pecten maximus et les stress thermique et hypoxique

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Liste des principales abréviations utilisées

2-DE :	Two-Dimensional Electrophoresis
2-PG :	2-phosphoglycerate
ACN :	Acetonitrile
ACOT4 :	Acyl-Coenzyme A Thioesterase 4
AFMID :	N-Formylkynurenine Formamidase
AKT :	Proteine Kinase B
AMACR :	Alpha-methylacyl-CoA racemase
AOX :	Alternative Oxidase
APMAP :	Adipocyte Plasma Membrane Associated Protein
ARA :	Arachidonic acid
ATP :	Adénosine triphosphate
BATF :	Basic leucine zipper transcription factor, ATF-like
BER :	Base Excision Repair
BF3 :	Boron triFluoride
BICAM :	Ammonium bicarbonate
BIRC7 :	Baculoviral IAP Repeat Containing 7
Blast :	Basic Local Alignment Search Tool
CA2 :	Carbonic anhydrase II
CALR :	Calreticulin
CASP :	Caspase
CCAR1 :	Cell Cycle And Apoptosis Regulatory Protein 1
CCBL2 :	Kynurenine--Oxoglutarate Transaminase
CHAPS :	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate
CROP :	Cisplatin Resistance associated Overexpressed Proteins
CSDE1 :	Cold Shock Domain Containing Protein E1
CSTF3 :	Cleavage stimulation factor 77 kDa
DG :	Digestive Gland
DHA :	Docosahexaenoic acid
DTT :	Dithiothréitol
EDTA :	Ethylene Diamine tetraacetic acid
ENO3 :	Enolase 3
EPA :	Eicosapentaenoic acid
ERO :	Espèce réactive de l'oxygène
ESI :	ElectroSpray Ionization
EST :	expressed sequence tag
FA :	Fatty Acids
fdr :	false discovery rate
GAPDH :	Glyceraldehyde 3 Phosphate Dehydrogenase
GC :	Gas Chromatography
GNRH :	Gonadotropin Releasing Hormone
GSN :	Gelsolin
HCCA :	α-cyano-4-hydroxycinnamic acid
HIF-1 :	Hypoxia Inducible Factor
HNRPD :	Heterogeneous nuclear ribonucleoprotein D
HSF :	Heat Shock Factor
HSP :	Heat Shock Protein

HSR :	Heat Stress Response
IEF :	IsoElectric Focusing
IPG	Immobilized pH gradient
iTRAQ :	Isobaric tag for relative and absolute quantitation
JNK :	c-JUN N terminal Kinase
MALDI :	Matrix-Assisted Laser Desorption Ionization
MS :	Mass Spectrometry
MT :	MetalloThionein
mTOR :	Mammalian target of rapamycin
NAD^+ :	Nicotinamide Adenine Dinucleotide (oxidized)
NADH2 :	Nicotinamide Adenine Dinucleotide (reduced)
NF-kB :	Nuclear Factor-kB
NGS :	Next generation sequencing
NGS :	Next Generation Sequencing
NTF2 :	Nuclear transport factor 2
OCLTT :	Oxygen and Capacity Limitation of Thermal Tolerance
ODH :	Octopine Dehydrogenase
PAGE :	Polyacrylamide Gel Electrophoresis
PcO_2 :	Pression partielle critique en O_2
PDIA :	Protein Disulfide Isomerase Family A
PFF :	Peptide Fragment Fingerprint
PGM :	PhosphoGlycerate Mutase
Pi3K :	PhosphatidylInositol 3-Kinase
PLA2G16 :	Phospholipase A2, Group XVI
PMF :	Peptides Mass Fingerprinting
POL3RC :	DNA-Directed RNA Polymerase III Subunit C
PPIB :	Peptidylprolyl Isomerase B (Cyclophilin B)
RAPD :	Random Amplified Polymorphic DNA
RFLP :	Restriction Fragment Length Polymorphism
RNA pol II :	ARN polymerase type II
ROS :	Reactive Oxygen Species
SCD :	Stearoyl CoA desaturase
SDS :	Sodium Dodecyl Sulfate
SHM :	Somatic HyperMutation
SILAC :	Stable isotope labeling by amino acids in cell culture
SMC1A :	Structural maintenance of chromosomes protein 1A
SNAP :	Sophisticated Numerical Annotation Procedure
SYNCRIP :	Heterogeneous Nuclear Ribonucleoprotein Q
TBK1 :	TANK-binding kinase 1
TCA :	TrichloroAcetic acid
TFA :	trifluoroacetic acid
TLR4 :	Toll Like Receptor 4
TNF :	Tumor Necrosis Factor
TOF :	Time-Of-Flight
TRAF3 :	Tumor necrosis factor receptor (TNFR)-associated factor 3
UDG :	Uracile DNA Glycosylase
UFA :	Unsaturated Fatty Acid
UV :	Ultra Violet
WoRMS :	World Register of Marine Species

Sommaire

Introduction Générale

1 L'impact du changement climatique sur les écosystèmes côtiers

1.1 Le changement climatique en milieu côtier

Bien que n'occupant que 10% de la surface océanique, les écosystèmes côtiers (depuis le bord du plateau continentale jusqu'aux estuaires) sont parmi les systèmes les plus importants de la planète. Ce sont des zones de fortes productions biologiques, environ 20 fois supérieure à la productivité terrestre, qui contribuent pour environ un quart de la production primaire globale et 80% de la biomasse marine. Il a été estimé qu'en terme de biens (nourriture, matières premières…) et de services (protection des côtes, recyclage des nutriments…), les écosystèmes côtiers fournissent une valeur équivalente à 140 000 milliards de dollars par an (Costanza *et al.*, 1997). Ainsi, les côtes sont les zones les plus peuplées de la planète, et environ 3 milliards de personne vivraient dans une zone côtière (Creel, 2003). Le fort impact anthropique exercé sur ces zones menace directement les espèces qui y vivent. En plus de cet impact direct sur les écosystèmes côtiers, il existe désormais un consensus qui reconnaît le rôle de l'Homme dans le processus actuel d'évolution du climat vers une augmentation des températures moyennes (GIEC, 2013).

Le dernier rapport du groupe d'experts intergouvernemental sur l'évolution du climat (GIEC) décrit 4 scénarios possibles d'évolution du climat basés sur des modèles de circulation générale couplés atmosphère-océan. Chacun de ces scénarios est associé à une gamme probable d'augmentation de la température (GIEC, 2013). Ainsi, selon le scénario choisi, dépendant des prédictions d'émission de gaz à effet de serre et compte tenu des rétroactions climat-cycle du carbone, la température moyenne de l'atmosphère pourrait augmenter de 0,3°C à 4,8°C d'ici la fin du siècle. Dans un même temps, la température globale de la couche de surface des océans pourrait augmenter de 1,5°C à 2,6°C (GIEC, 2007).

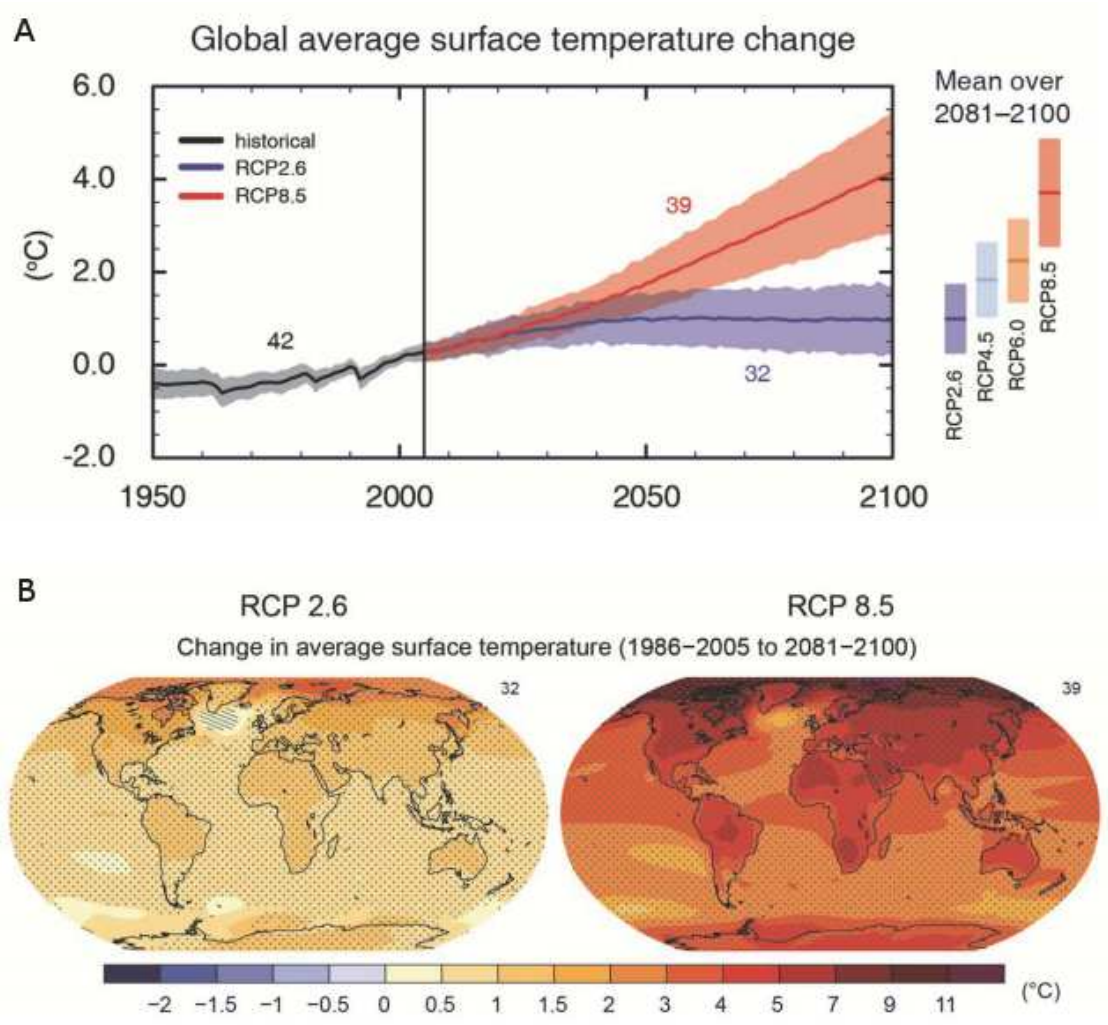

Figure 1: A) Projections d'évolution de la température moyenne de surface et B) évolution projetée de la température moyenne en surface pour la fin du XXI^{e} siècle (2081-2100) par rapport à la période 1986-2005. Les résultats montrés correspondent à deux scénarios d'émissions de gaz à effet de serre : élevées (RCP8.5, rouge) et faibles (RCP2.6, bleu) (GIEC, 2013).

Si ces prédictions s'accompagnent d'un haut niveau d'incertitude, il est néanmoins fortement probable, quelque soit le modèle ou le scénario envisagé, que la température moyenne de surface ainsi que la température de la couche supérieure des océans, vont avoir une tendance significative à la hausse durant le prochain siècle.

La planète Terre a connu de nombreuses évolutions de son climat depuis sa création, permettant à la vie de prospérer ou au contraire de péricliter, voire de subir des déclins massifs. Il existe désormais de nombreuses preuves que les changements actuellement à l'œuvre dans les systèmes côtiers sont du même ordre que certains des évènements climatiques passés ayant entrainé des conséquences dramatiques pour les écosystèmes (GIEC, 2007; Hoegh-Guldberg & Bruno, 2010).

Les modifications des paramètres physico-chimiques des systèmes côtiers entrainent des réponses biologiques très variables à des échelles spatio-temporelles diverses. La température a un effet fondamental sur tous les processus biologiques de par son influence sur l'énergie régissant des processus fondamentaux comme les réactions enzymatiques, la diffusion et le transport membranaire (Hochachka & Somero, 2002). Les variations de température ont ainsi un impact majeur sur les espèces biologiques modifiant la physiologie, la phénologie et la distribution de ces espèces (Peñuelas & Filella, 2001; Walther *et al.*, 2002).

Ces modifications au niveau spécifique rejaillissent sur la fonctionnement des écosystèmes (Hoegh-Guldberg & Bruno, 2010). La composition spécifique, la dynamique et la structure des écosystèmes s'en trouvent modifiées, des processus biologiques clés pour le fonctionnement des écosystèmes côtiers peuvent ainsi être altérés. Par exemple, la distribution et l'abondance des communautés phytoplanctoniques se sont modifiées en réponse au réchauffement, à l'acidification et à la stratification de l'océan (Polovina *et al.*, 2008; Doney *et al.*, 2009). La production primaire annuelle a ainsi diminué d'au moins 6% depuis le début des années 80. Les conséquences de telles modifications se répercutent à tous les niveaux des réseaux trophiques côtiers (Gregg *et al.*, 2003).

Les espèces tentent de s'adapter à ces conditions changeantes, cependant tous les organismes n'ont pas les mêmes capacités d'adaptation. Ainsi, l'impact biologique d'une augmentation de la température peut avoir des conséquences variées selon l'espèce considérée mais aussi selon la population au sein d'une même espèce (Harley

et al., 2006). Un des paramètres majeurs limitant les capacités adaptatives des espèces est l'aptitude de leurs systèmes d'approvisionnement à fournir l'oxygène nécessaire au maintien du métabolisme aérobie lorsque la demande en énergie augmente (Pörtner, 2010). Cette capacité dépend notamment du système cardio-circulatoire et de l'oxygène disponible dans l'environnement définissant une « fenêtre thermique » dans laquelle les performances aérobies sont optimales.

Au regard des changements actuels, les espèces marines, et plus particulièrement les espèces ectothermes sessiles, apparaissent particulièrement menacées. D'une part, beaucoup d'espèces marines vivent à des températures proches de leurs limites de tolérance thermique (Somero, 2002; Hughes *et al.*, 2003). D'autre part, avec le changement climatique global, les épisodes hypoxiques ont tendance à augmenter en intensité et en fréquence, limitant la disponibilité en oxygène et potentiellement la capacité d'adaptation des organismes marins sessiles.

1.2 L'impact du changement climatique sur les organismes marins

Les organismes vivants survivent en maintenant en leur sein un équilibre dynamique complexe, appelé homéostasie. La notion de stress peut être définie comme une menace ou une perturbation de l'homéostasie par des stimuli internes ou externes (Wendelaar Bonga, 1997). Les stimuli externes, ou éléments stressants, correspondent à un changement dans l'environnement de l'organisme considéré. Les organismes tentent de répondre à ces menaces en fournissant une réponse appropriée permettant de rétablir l'équilibre : c'est la réponse au stress.

Au delà du changement climatique, le milieu côtier est par nature un environnement extrêmement dynamique, aux conditions changeantes, potentiellement source de nombreux stress pour les organismes qui y vivent. Les organismes marins peuvent ainsi être soumis à des variations de température, de salinité, de disponibilité en oxygène, de nourriture (tant qualitatives que quantitatives), à la présence d'espèces compétitrices ou prédatrices, à la présence de toxines d'origines naturelles ou encore à des contaminants d'origine anthropique.

1.2.1 L'impact de la température sur les organismes marins

La température est reconnue comme un des facteurs abiotiques les plus importants pour les individus et les chercheurs se sont intéressés de longue date à l'étude des effets de la variation de température sur les organismes (Krogh, 1916). Le changement climatique en cours a entrainé un regain d'intérêt pour ces études liées à la température et ses effets sur de nombreux organismes marins à différents niveaux d'intégration (*e.g.*, Buckley *et al.*, 2006; Anestis *et al.*, 2007; Brun *et al.*, 2008; Lockwood *et al.*, 2010; Tomanek & Zuzow, 2010).

La plupart des phénomènes physico-chimiques qui se déroulent au sein de la cellule dépendent largement de la température (*e.g.*, la diffusion à travers les membranes, l'activité enzymatique …). Chez les ectothermes, la température du milieu extérieur influence directement le métabolisme (Hochachka & Somero, 2002). L'augmentation de la température dans le milieu extérieur entraine ainsi l'augmentation des besoins en énergie et en oxygène chez les organismes ectothermes, jusqu'à ce que les besoins en oxygène ne puissent plus être couverts par les systèmes cardio-circulatoire des animaux : c'est le concept de la limitation des capacités de tolérance thermique par l'oxygène (Pörtner, 2001, 2010; Pörtner & Knust, 2007; Oxygen and capacity limitation of thermal tolerance, OCLTT, Fig. 2).

Dans ce contexte, Pörtner (2001, 2010) définit pour chaque organisme une fenêtre thermique permettant le fonctionnement optimal de l'organisme. Cette fenêtre est délimitée par les températures "pejus" (Tp) en deçà- ou au delà- desquelles les performances de l'organisme diminuent, entraînant le développement d'une réponse au stress (Fig. 2). Cette réponse au choc thermique est bien caractérisée et induit des mécanismes qui protègent les cellules des changements abrupts pouvant se produire dans leur environnement, prévenant les risques de modifications des macromolécules (Kültz, 2003).

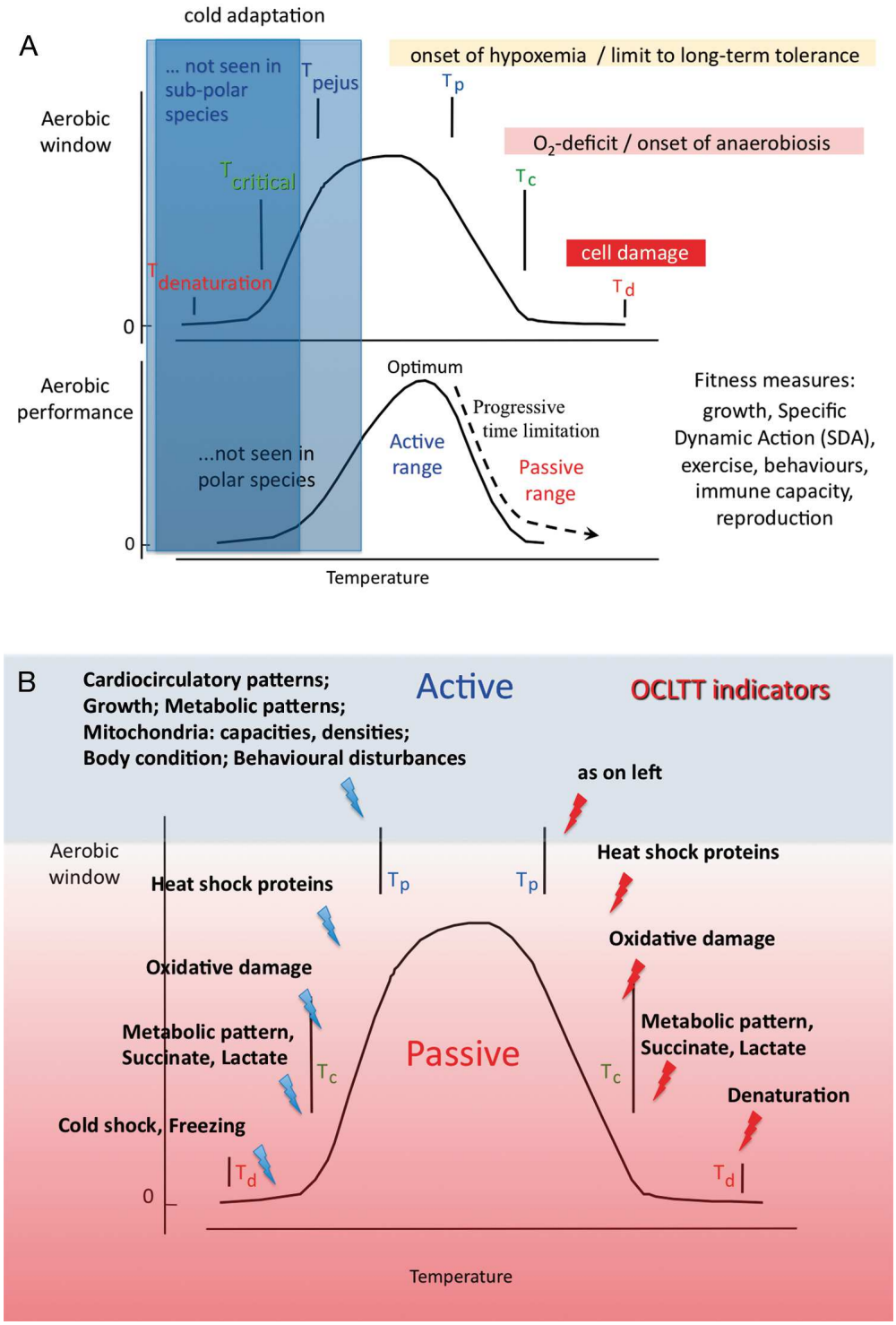

Figure 2: Concept de la limitation des capacités de tolérance thermique par l'oxygène « OCLTT » (Oxygen and capacity-limited thermal tolerance). Ce concept intègre de multiples facteurs de stress et les divers processus et leurs indicateurs (Pörtner, 2011).

De nombreuses protéines sont synthétisées en réponse au stress thermique. Or, pour être fonctionnellement actives, les protéines néo-synthétisées doivent se conformer en une structure tridimensionnelle unique qui dépend essentiellement de l'information contenue dans leur séquence en acides aminés. Les chaînes polypeptidiques ayant un haut degré de flexibilité, et les chaines latérales des acides aminés permettant de nombreuses interactions non-covalentes, il existe potentiellement un grand nombre de conformations possibles. Or, si certaines protéines prennent spontanément leur structure tridimensionnelle active *in vitro*, *in vivo* l'implication de protéines chaperonnes est requise. D'autre part, les interactions les moins stables sont facilement affectées par les facteurs environnementaux tels que la température (Feder, 1999). Les altérations de ces liaisons faibles peuvent résulter en une perte de la structure fonctionnelle des protéines et, *in fine*, avoir des conséquences dramatiques pour la survie de l'individu (Somero, 1995). De plus, les protéines dénaturées peuvent se lier avec d'autres protéines et ainsi former des agrégats qui réduisent la quantité de protéines disponible pour la cellule. Ces agrégats peuvent se révéler toxiques pour les cellules et être difficiles à éliminer (Feder, 1999). Les protéines dénaturées doivent donc être soit dégradées, soit remises dans leur structure tridimensionnelle fonctionnelle originelle, c'est le rôle des chaperonnes moléculaires (Hartl, 1996; Wickner *et al.*, 1999).

Les chaperonnes sont universelles et jouent un rôle important dans le fonctionnement normal des cellules. Leur rôle prédominant dans les mécanismes moléculaires de tolérance au stress a été particulièrement bien documenté (Feder, 1999).

Les cellules de tous les organismes vivants contiennent au moins deux systèmes principaux de chaperonnes : le système des chaperonines et les « Heat Shock Proteins » (HSPs). :

- Les chaperonines : Cette famille contient 2 sous-groupes. Les chaperonines de type I (appelé aussi la famille GroEL) présentent dans les eubactéries, les mitochondries et les chloroplastes. Les chaperonines de type II, membres de la famille des TRiC (TCP-1 ring complex) sont quant à elles présentes dans les archæbactéries et le cytosol des eucaryotes (Llorca *et al.*, 1999). Les membres de la famille des TRiC ne sont pas induites en cas de stress chez les eucaryotes et ont un nombre de substrats réduit (Hartl, 1996).

- Les Heat Shocks Proteins (HSPs, « protéines de choc thermique ») : Ce sont les protéines chaperonnes les plus étudiées. Les HSPs sont très conservées dans tous les domaines du vivant, présentes dans tous les compartiments de toutes les cellules, des procaryotes aux eucaryotes. Même si elles ont un rôle constitutif dans le fonctionnement cellulaire, elles ont été originalement décrites comme des protéines induites en réponse au choc thermique (Ritossa, 1962). Chaque HSP est désignée par son poids moléculaire apparent (p. ex., Hsp70, 72, 73, etc …), et elles sont regroupées en famille en fonction de leur poids (*e.g.* la famille des Hsp 70).

Une réponse au choc thermique impliquant des HSPs a été décrite chez toutes les espèces d'invertébrés marins étudiées à ce jour (Feder & Hofmann, 1999; Barral *et al.*, 2004; Fabbri *et al.*, 2008; Brun *et al.*, 2009), à l'exception de quelques espèces antarctiques (Clark & Peck, 2009). Des Hsp70, Hsp90, Hsp60 (mitochondriale), Hsp40 et des Hsp de plus petits poids moléculaires (sHSPs pour « small heat shock proteins ») ont ainsi été identifiées chez les moules (Buckley *et al.*, 2001; Lockwood *et al.*, 2010; Tomanek & Zuzow, 2010), les huîtres (Hamdoun *et al.*, 2003), les ormeaux (Farcy *et al.*, 2007) ou les pétoncles (Gao *et al.*, 2007; Brun *et al.*, 2009).

Les HSPs permettent aux protéines de retrouver leur conformation fonctionnelle. Cette activité est généralement régulée par une activité ATPase et des cofacteurs variés. En outre, les HSPs assument également de nombreuses fonctions essentielles dans la transduction du signal, l'apoptose, la réponse immunitaire et d'autres processus physiologiques (van Noort, 2008).

1.2.2 L'impact de l'hypoxie sur les organismes marins

Les valeurs de concentration en oxygène dissous dans les eaux côtières ont radicalement changé durant les dernières décennies, probablement plus que toutes les autres variables écologiques importantes (Diaz & Rosenberg, 1995; Diaz, 2000). Ces changements ont abouti au développement de zones d'hypoxie sur les côtes du monde entier (Diaz & Rosenberg, 2008) dont le nombre a ainsi augmenté de façon exponentielle depuis les années 70 (Vaquer-Sunyer & Duarte, 2008). Les événements hypoxiques se sont aussi aggravés en terme d'intensité, de durée et de fréquence (Conley *et al.*, 2007; Chan *et al.*, 2008; Stramma *et al.*, 2008; Turner *et al.*, 2008).

Selon les prévisions actuelles, la fréquence et l'intensité des événements hypoxiques en zone côtière vont continuer de se développer sous l'effet i) de l'augmentation des phénomènes d'eutrophisation, due à la présence excessive de matière organique (*e.g.* Service, 2004) et ii) de l'augmentation de la température due au changement climatique, qui augmente la demande en oxygène des organismes (Harris *et al.*, 2006), diminue la solubilité de l'oxygène (Carpenter, 1966) et augmente les phénomènes de stratification des eaux côtières (Stow *et al.*, 2004).

En situation d'hypoxie, les organismes marins mobiles vont se déplacer vers des zones où la concentration en oxygène dissous est moins critique dans des zones où l'eau est mieux aérée (*e.g.* l'amphipode *Corophium arenarium* ; Gamble, 1971; Grieshaber *et al.*, 1994), ou même tenter de sortir de l'eau pour respirer de l'air (*e.g.* le crabe vert, *Carcinus maenas* ; Taylor *et al.*, 1973, 1977). Cependant beaucoup d'espèces d'invertébrés marins sont sessiles et doivent s'adapter à ces événements hypoxiques.

En réponse à une hypoxie modérée, la plupart des invertébrés marins sessiles commencent par réguler leur activité ventilatoire (Grieshaber *et al.*, 1994). Les taux de filtration peuvent ainsi augmenter de 1,2 à 8 fois par rapport à la normoxie selon l'espèce et le degré d'hypoxie (van Dam, 1938, *dans* Grieshaber *et al.*, 1994 ; McMahon & Wilkens, 1975; Toulmond & Tchernigovtzeff, 1984). La quantité d'oxygène rendue disponible par l'augmentation de l'activité de filtration dépend aussi des capacités du système circulatoire. L'augmentation de l'activité cardiaque par des effets chronotropes (*i.e.* la fréquence) ou inotropes (*i.e.* le volume) peut ainsi permettre un afflux accru d'hémolymphe au niveau des branchies et des organes consommateurs d'oxygène. Chez beaucoup d'espèces d'invertébrés marins, néanmoins, le système circulatoire ne permet qu'une augmentation chronotrope de l'activité cardiaque (Grieshaber *et al.*, 1994).

Chez les invertébrés marins, l'évolution de la consommation d'oxygène en fonction du niveau d'hypoxie n'est pas identique selon les espèces (Pörtner & Grieshaber, 1993). Ainsi certains organismes sont capables de maintenir une consommation d'O_2 relativement constante sur une large gamme de concentrations en oxygène, et ont été appelés « oxyrégulateurs ». D'autres, en revanche, ont été appelés « oxyconformeurs », c'est à dire que la consommation d'oxygène de ces espèces dépend de la concentration en oxygène dans l'environnement. La distinction entre ces

deux modes de réponse à l'hypoxie n'est toutefois pas complétement cloisonnée, d'une part parce que des réponses intermédiaires existent (Mangum & von Winkle, 1973), et d'autre part parce que les oxyrégulateurs se comportent généralement comme des oxyconformeurs en deçà d'un seuil critique en pression partielle d'oxygène. Ce seuil, la pression partielle critique en O_2 (PcO_2) coïncide avec la mise en place du métabolisme anaérobie (Pörtner & Grieshaber, 1993).

En réponse à une hypoxie sévère , durant les 3 à 6 premières heures, l'utilisation de la glycolyse anaérobie entraine l'accumulation de lactate ou d'opines dans le cytosol (Fig. 3; Grieshaber *et al.*, 1994). Dans cette voie des opines, très répandue chez les invertébrés marins (Harcet *et al.*, 2013), la lactate déshydrogénase est remplacée par une autre pyruvate réductase (une opine déshydrogénase). Dans cette réaction, le pyruvate est condensé avec un acide aminé pour former un acide iminé (octopine, alanopine, strombine, tauropine, ou nopaline), tout en régénérant une mole de NADH,H+ (forme réduite) en NAD+ (forme oxydée). Si cette voie est sans doute plus ancienne que la fermentation impliquant l'accumulation de lactate comme produit final (Harcet *et al.*, 2013), elle présente néanmoins l'avantage de ne pas influencer le pH et d'être moins perturbant pour les enzymes que le lactate (Zammit & Newsholme, 1976; Bowlus & Somero, 1979). En revanche, alors que le lactate est excrété à l'extérieur des cellules, les opines, elles, s'accumulent.

Si l'hypoxie se prolonge, la production d'ATP devient essentiellement mitochondriale, par la voie « aspartate-succinate ». Dans ce cas, la transamination cytosolique de l'aspartate aboutit à la formation de malate, qui est utilisé par une partie du cycle de Krebs dans la mitochondrie pour être réduit en succinate. Le propionate produit à partir du succinate est excrété dans le milieu extérieur et n'est pas accumulé dans les cellules (Grieshaber *et al.*, 1994; Fig. 3).

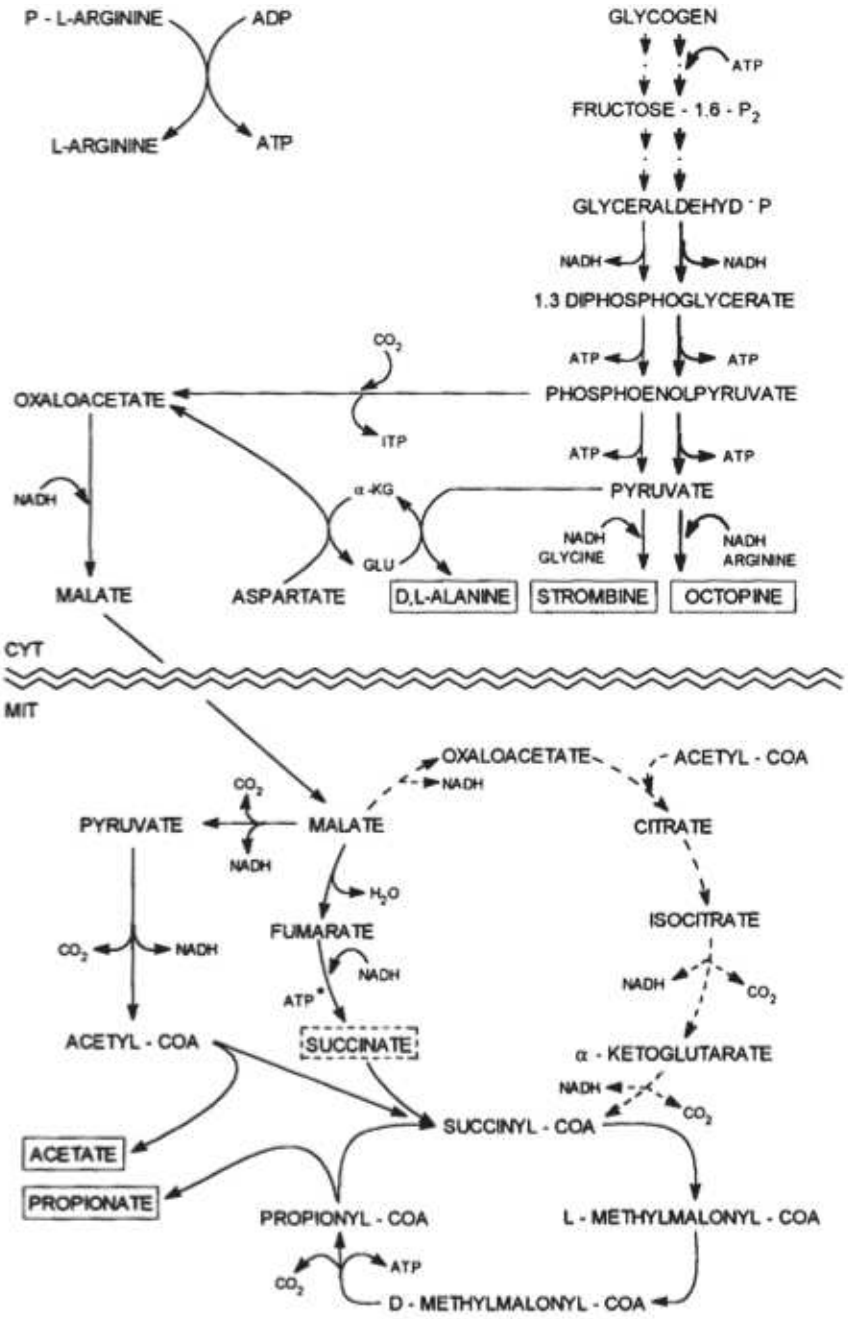

Figure 3: Schéma de fonctionnement du métabolisme anaérobie chez les invertébrés marins. (Grieshaber *et al*, 1994)

Le glycogène est la forme principale de stockage du glucose chez les animaux et la principale source d'énergie du métabolisme anaérobie. Les importantes réserves de glycogène trouvées chez les invertébrés marins, jusqu'à 35% de la masse sèche (de Zwaan & Zandee, 1972), contribuent à la grande tolérance à l'hypoxie de ces animaux.

Malgré l'efficacité du métabolisme anaérobie chez les animaux marins tolérants à l'hypoxie, les stratégies de survie de ces animaux ne reposent pas sur une compensation de la production en énergie par rapport à la normoxie (qui serait difficile à atteindre sur une période étendue) mais sur une conservation de l'énergie (Fig.4; Gorr *et al.*, 2006). La capacité d'entrer dans un état de dépression métabolique régulé, caractérisé par le maintien d'une balance réduite mais équilibrée entre production et consommation d'ATP, permet de prévenir une chute de la concentration cellulaire en ATP à des niveaux létaux. Cette capacité est la plus protectrice et la plus conservée parmi les mécanismes de tolérance à l'hypoxie, chez les animaux hypoxie-tolérants (Hochachka *et al.*, 1996; Boutilier & St-Pierre, 2000; Hochachka & Somero, 2002). L'ampleur de cette dépression métabolique (ou état "hypométabolique"), est inversement proportionnelle au temps passé en hypoxie (Guppy & Withers, 1999). Comme environ 80% de l'oxygène consommé par la mitochondrie est couplé à la synthèse d'ATP (Rolfe & Brown, 1997) le passage à l'état hypométabolique réduit considérablement la consommation d'oxygène. Au niveau moléculaire, l'état hypométabolique implique la réduction coordonnée de toutes les fonctions les plus consommatrices en ATP de la cellule telles que la synthèse de protéines (~30% de la consommation de l'ATP en état normoxique), la dégradation protéique (~20%), les transports ioniques liés à des ATPases (~25% ; notamment la Na^+/K^+ ATPase) (Land *et al.*, 1993; Land & Hochachka, 1994; Hochachka *et al.*, 1996; Rolfe & Brown, 1997; Hochachka & Somero, 2002).

Certains auteurs (Pörtner, 2010) ont attribué la diminution progressive du métabolisme respiratoire en hypoxie chez les oxyconformeurs à l'existence d'une oxydase alternative (AOX ; Abele *et al.*, 2007). Cet enzyme shunterait les complexes III et IV de la chaine respiratoire pour venir réduire l'O_2 en eau, permettant le maintien de la respiration et de la synthèse d'ATP à un niveau réduit (McDonald *et al.*, 2009). Toutefois, de récentes études chez l'huître du Pacifique, *Crassostrea gigas*, ont montré que l'expression et l'activité de l'AOX sont augmentées pendant la phase

de réoxygénation après une hypoxie (Sussarellu *et al.*, 2012, 2013) et que l'inhibition simultanée de l'AOX et du complexe III de la chaine respiratoire mitochondriale n'augmentent pas la production de ROS (Donaghy *et al.*, 2012). Ces auteurs suggèrent l'existence d'une réductase terminale alternative non-identifiée jouant un rôle équivalent à l 'AOX mais utilisant un accepteur d'électron final autre que l'oxygène (Donaghy *et al.*, 2013). Ainsi, la moule *Mytilus edulis* aurait la capacité de shunter les électrons vers la fumarate réductase en anaérobiose (Tielens *et al.*, 2002).

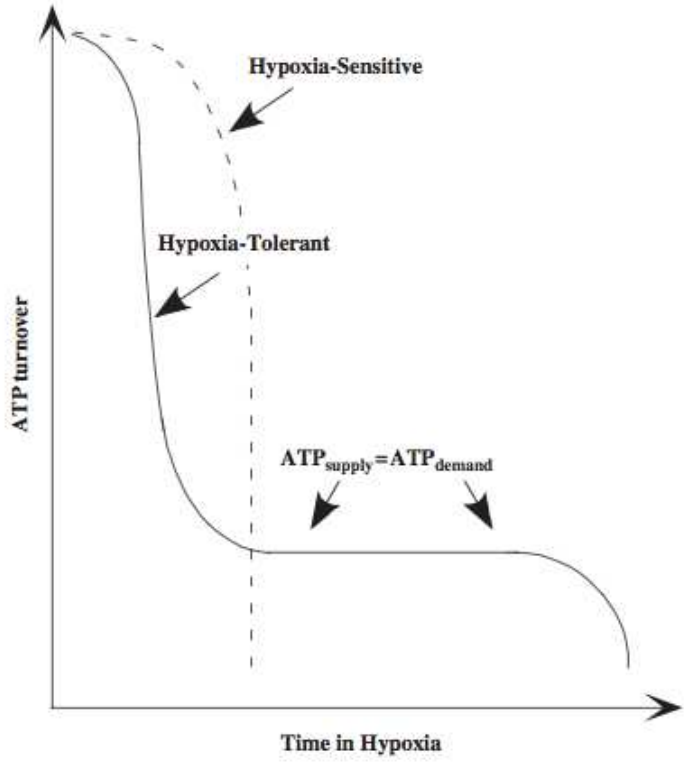

Figure 4: Comparaison simplifié du turnover d'ATP entre les espèces sensibles et tolérantes à l'hypoxie (Gorr *et al*, 2006).

Lorsque la concentration en oxygène dans le milieu devient limitante, c'est à dire en dessous de la PcO_2, pour les oxyrégulateurs comme pour les oxyconformeurs, le métabolisme anaérobie est nécessaire pour couvrir les besoins en énergie.

La mise en place des différents processus permettant aux organismes marins une tolérance accrue à l'hypoxie serait principalement contrôlée par un facteur de transcription très répandu dans le monde animal, HIF-1 (hypoxia inducing factor ; Gorr *et al.*, 2006). Structurellement, HIF-1 est un hétérodimère composé de 2 sous-unités (α et β) qui se lient spécifiquement à certaines séquences d'ADN, les HREs (« hypoxia response element »), induisant la synthèse d'un grand nombre de gènes (p. ex, 2 à 5% des gènes chez l'humain peuvent être contrôlés par HIF ; Manalo *et al.*,

2005). En normoxie, les deux sous-unités d'HIF-1 sont synthétisées, toutefois si HIF-1β est stable, HIF-1α est directement hydroxylé et dégradé par la cellule. En hypoxie, les faibles valeurs d'O_2 intracellulaires induiraient une production accrue d'espèces réactives de l'oxygène (ROS, « reactive oxygen species ») par la mitochondrie qui stabiliseraient HIF-1α, permettant sa dimérisation avec HIF-1β et donc sa mise en forme fonctionnelle (Schroedl *et al.*, 2002). La stabilisation d'HIF-1α serait due à l'inhibition de prolyl-hydroxylases par les ROS (responsable de sa dégradation en normoxie). Cette hypothèse a cependant récemment était remise en cause par Chua et ses collaborateurs (2010), qui décrivent une stabilisation d'HIF-1α indépendante de la production de ROS mais vraisemblablement régulée par la mitochondrie via la disponibilité en O_2 intracellulaire.

L'efficacité de ce mode de contrôle au niveau de la protéine est illustrée par la courte demi-vie d'HIF-1α lors de la ré-oxygénation après une phase hypoxique (< 5 min ; Huang *et al.*, 1996) et inversement par l'accumulation instantanée d'HIF-1 lorsque la concentration en O_2 décline (Jewell *et al.*, 2001).

1.2.3 Effets combinés de la température et de l'hypoxie sur les organismes marins

L'hypoxie et la température ont été traditionnellement considérées comme des facteurs environnementaux distincts, chacun ayant des implications spécifiques sur le fonctionnement des organismes. Néanmoins, l'activation du métabolisme anaérobie est généralement observée aux deux extrémités de la fenêtre thermique des organismes. Ces observations ont mené au développement du concept de limitation des capacités de tolérance thermique par l'oxygène (Pörtner, 2001; Oxygen and Capacity Limitation of Thermal Tolerance, OCLTT). Dans ce concept, la capacité d'adaptation à la thermie est limitée par les possibilités de l'organisme à fournir l'oxygène nécessaire à une demande en énergie croissante (Fig. 2). L'interdépendance de ces facteurs implique que les possibilités d'adaptation à l'hypoxie sont maximum dans la fenêtre thermique des espèces (où la PcO_2 est minimale). *A contrario*, l'hypoxie systémique qui se met en place aux abords des températures critiques de la fenêtre thermique entraine une augmentation de la PC_{O2} et donc une limitation des capacités de tolérance à l'hypoxie. Inversement, l'hypoxie (fig portner 2 B) entraine

un rétrécissement de la fenêtre thermique des organismes et une diminution des performances température-dépendantes.

Quelques travaux ont étudié les réponses simultanées de l'hypoxie et de la température de bivalves marins. L'huître *Crassostrea gigas*, a ainsi été définie comme un oxy-régulateur à 12°C,15°C et 20°C, mais pour une gamme limitée d'oxygène(Le Moullac *et al.*, 2007). Par l'analyse de l'activité des enzymes du métabolisme (PK, PEPCK) et de l'accumulation des métabolites (Alanine, Succinate), les capacités anaérobies des huîtres ont été décrites comme diminuées à 20°C par rapport à 12°C, confirmant le concept d'OCLTT (Le Moullac *et al.*, 2007). , Il a également été observé chez la moule *Mytilus galloprovincialis* qu'une augmentation de la température de 10°C à 27°C diminue les capacités d'oxyrégulation (Jansen *et al.*, 2009).

2 Evaluer la réponse au stress à différentes échelles

Les réponses biologiques à un changement dans l'environnement peuvent se mesurer à de nombreuses échelles, depuis l'échelle dite « moléculaire » (p. ex. Clark & Peck, 2009 ; Sussarellu *et al.*, 2010) jusqu'au niveau comportemental (p. ex. Long *et al.*, 2008), en passant par les niveaux physiologiques et cellulaires (p. ex. Strahl *et al.*, 2011; Donaghy *et al.*, 2013). Les résultats obtenus lors des études écophysiologiques renseignent sur la physiologie générale d'un individu et correspondent à l'intégration des réponses au stress à plus petites échelles. D'autre part, ces résultats permettent de faire des projections au niveau de la population ou de l'écosystème de façon plus directe que les réponses moléculaires. Cependant, les études se situant à un niveau moléculaire ont fourni un aperçu détaillé des capacités des espèces à s'adapter (Kültz *et al.*, 2007; Brun *et al.*, 2008; Tomanek *et al.*, 2011). De plus, il est généralement accepté que les signes de stress apparaissent initialement au niveau moléculaire. Ces changements précoces, tels que les niveaux d'expression des gènes et des protéines, peuvent ainsi être utilisés comme marqueurs biologiques annonçant des changements à un niveau d'organisation supérieur (Cajaraville *et al.*, 2000; Dondero *et al.*, 2006). L'utilisation de ces marqueurs biologiques permettent la

détection précoce des effets de changements environnementaux et ont des applications diverses dans des domaines tels que l'écologie, la biogéographie ou l'aquaculture.

2.1 Ecophysiologie

L'écophysiologie (ou encore « écologie physiologique » ou « physiologie environnementale ») peut se définir comme « l'étude des mécanismes physiologiques d'un organisme en interaction avec son environnement. » (Bradshaw, 2003). En pratique, l'écophysiologie cherche donc à mesurer l'impact de différents facteurs environnementaux sur les grands processus physiologiques. La respiration est l'un de ces processus les plus utilisés en écophysiologie. Deux systèmes sont classiquement utilisés pour mesurer la respiration chez les bivalves : les systèmes en circuit ouvert ou en circuit fermé.

Dans les systèmes en circuit ouvert, le taux de respiration est estimé à partir des différences en concentration en oxygène entre l'entrée et la sortie des enceintes contenant le ou les animaux. L'un des principaux intérêts de ce type de système est la possibilité d'effectuer des mesures sur une longue période. En revanche, deux mesures étant nécessaires pour estimer le taux de respiration, chacune comprenant une incertitude, la précision de la mesure est difficile à déterminer (Flye Sainte-Marie, 2007).

Les systèmes en circuit fermé consistent à incuber le ou les animaux dans une enceinte close. L'estimation du taux de respiration se fait en mesurant la décroissance en oxygène dans l'enceinte. Dans ce type de système, la diminution de la concentration en oxygène dans l'enceinte peut potentiellement avoir un impact pour l'organisme. Toutefois, des incubations courtes et répétées permettent de limiter ce biais technique et d'obtenir une mesure fiable du taux de respiration.

2.2 Protéomique

Le terme de « protéome » a été proposé par Wilkins et ses collaborateurs (1996) pour définir « toutes les protéines produites par le génome ». Le protéome n'est cependant pas un catalogue de toutes les protéines possibles produites par le génome mais correspond au résultat de processus dynamiques, dépendants de l'état

physiologique des organismes. Les protéines sont les effecteurs de la réponse cellulaire. Identifier les protéines impliquées dans une réponse à un stress, à un stade de développement, etc. est donc d'un intérêt majeur pour comprendre les mécanismes biologiques sous-jacents. Le protéome peut aussi être interprété comme un « phénotype moléculaire », plus proche du phénotype que le transcriptome (Biron *et al.*, 2006).

Quelle que soit la méthode choisie, une étude protéomique requiert (i) la préparation de l'échantillon (*e.g.* des étapes de prefractionation), (ii) la séparation et l'identification des protéines d'intérêt et (iii) l'analyse des données grâce à des outils bioinformatiques.

2.2.1 Les techniques de séparation et de quantification

Il existe plusieurs techniques de séparation des protéines dont les plus utilisées en écologie pour l'étude des protéines sans *a priori* sont l'électrophorèse bidimensionnelle et la chromatographie liquide (Slattery *et al.*, 2012).

2.2.1.1 L'électrophorèse bidimensionnelle

L'électrophorèse bidimensionnelle (2-DE) a été développée indépendamment par O'Farrell (1975) et Klose (1975), et permet la séparation de mélanges protéiques complexes en 2 dimensions. Son fonctionnement consiste tout d'abord en une focalisation isoélectrique (IEF, « isoelectric focusing ») séparant les protéines selon leurs propriétés acido-basiques. Les protéines migrent dans un gradient de pH jusqu'à atteindre une position stationnaire à la valeur de leur point isoélectrique (pI), où leur charge nette est nulle. Le résultat de la focalisation est ensuite séparé de façon orthogonale par électrophorèse en gel de polyacrylamide en présence de SDS (sodium dodecyl sulfate). La migration des protéines dans un tel gel dépend de leur masse relative. Les protéines sont ensuite colorées de façon non spécifique, le plus souvent avec du Bleu de Coomassie ou du nitrate d'argent, permettant de visualiser les protéines (quelques centaines à env. 1000) comme des points sur une carte.

Après l'acquisition des images des gels via un scanner, des logiciels d'analyses d'images permettent d'identifier les différents tâches correspondant à des

protéines, de faire correspondre ces tâches entre les différents gels et, enfin, d'évaluer le volume des tâches (protéines). Le but d'une étude de protéomique est d'identifier des protéines liés à une condition particulière, pour ce faire des conditions contrastées sont souvent comparées. Aussi, les volumes des protéines issus de ces différentes conditions doivent-ils être comparés de façon statistiquement fiable afin de mettre en évidence les protéines dérégulées (sur- ou sous-exprimés). Ce dernier point est l'objet du premier chapitre de ce travail, où une nouvelle méthode pour la comparaison des volumes des protéines est proposée.

2.2.1.2 La chromatographie liquide

Au delà des aspects purement techniques, la chromatographie liquide diffère de l'électrophorèse en premier lieu parce qu'elle n'est pas utilisée pour séparer directement les protéines mais les peptides issus de la digestion des protéines par des protéases comme la trypsine. Dans ce type d'approche, un mélange complexe de protéines issues de l'extraction de tissus ou de lysats cellulaires est digéré, puis injecté dans une colonne (souvent une colonne C18). Les peptides sont ensuite élués par l'augmentation de solvant organique (acétonitrile ou méthanol) dans le mélange d'élution. Le développement de pompes à nano débit (<1 µL/min) a permis l'augmentation de la résolution de ces approches. D'autre part, le couplage avec un spectromètre de masse « in line » (injection directe dans une source de type ESI, "electron spray ionisation") ou « off line » (avec dépôt sur des plaques de MALDI) rend cette technique extrêmement performante permettant l'identification d'un grand nombre de protéines (e.g. 2990 protéines identifiés en 1 run en triplicat chez la levure et 5376 dans les mêmes conditions pour des lignées cellulaires de mammifères ; Thakur et al., 2011).

Les comparaisons quantitatives d'échantillons pour les approches protéomiques utilisant la chromatographie liquide dépendent du couplage avec un spectromètre de masse. Il existe plusieurs façons de comparer ces échantillons (Neilson *et al.*, 2011) : le marquage différentiel des échantillons avec des isotopes (iTRAQ, SILAC…), la comparaison de l'intensité des pics détectés pour les peptides ou encore la comparaison du nombres de spectrogrammes pour un peptide donné (« spectral counting »).

Si ces méthodes sont prometteuses, elles sont néanmoins encore en développement et apparaissent aujourd'hui limitées, notamment pour la résolution d'échantillons complexes (même si les systèmes en 2 dimensions se développent) et du fait du peu d'outils bioinformatiques adaptés actuellement disponibles.

2.2.2 L'identification des protéines

L'identification de protéines à l'aide de la spectrométrie de masse (MS), quelque soit les étapes préliminaires, nécessite la digestion des protéines en peptides par une protéase coupant les protéines à des sites prédéterminés identifiables. Dans ce contexte, l'enzyme la plus utilisée est la trypsine (Tomanek, 2011).

La première étape de l'analyse en MS mesure la masse des peptides, en terme de rapport masse sur charge (m/z), donnant ainsi une empreinte peptidique de la protéine supposée unique (i.e. PMF, « peptide mass fingerprint »). Cette empreinte peut être comparée à une empreinte théorique issue de la digestion *in silico* des séquences protéines incluses dans les bases de données. Pour augmenter l'efficacité de l'identification, les peptides peuvent être fragmentés plus avant en ions fils, pour produire une empreinte du peptide (i.e. PFF, « peptide fragment fingerprint »). Ce processus s'appelle la spectrométrie de masse en tandem (tandem MS ou MS/MS) et permet, en plus d'une recherche plus efficace dans les bases de données, l'interprétation des spectres pour identifier les séquences en acide-aminés, ou séquençage *de novo*. Le séquençage *de novo* reste néanmoins très dépendant de la qualité des spectres et est surtout utilisé en complément des recherches s'appuyant sur des bases de données.

D'une manière générale, l'identification de protéines à l'aide de la spectrométrie de masse reste tributaire des informations contenues dans les bases de données. Le peu d'informations actuellement disponibles pour les organismes dits « non modèles » limite en pratique l'identification (Forné *et al.*, 2010; Dowd, 2012). Toutefois, ces limites sont sur le point de tomber, avec le rapide développement et la baisse des coûts du séquençage d'acides nucléiques à haut débit. Les informations disponibles pour toutes les espèces devraient massivement augmenter dans les prochaines années.

2.3 Transcriptomique

Les récents développements des techniques de séquençage dites de nouvelle génération (NGS, « next generation sequencing ») comme le 454, SOLiD ou l'Illumina HiSeq ont minimisé les coûts et le travail associés au séquençage haut débit (Tableau 1.1). Bien que ces plateformes diffèrent en termes de longueur et de nombres de « reads» produits, elles sont similaires en terme de capacité à produire un grand nombre de « reads » simultanées comparés aux méthodes traditionnelles. L'amélioration en nombres de séquences par « run » par rapport à la méthode de Sanger est d'environ 10^{12} kpb (Liu *et al.*, 2012).

Tableau 1 : Comparaison des différentes méthodes de séquençage (adapté de Liu et al, 2012)

Séquenceur	**454 GS FLX**	**HiSeq 2000**	**SOLiDv4**	**Sanger 3730xl**
Mécanismes de séquençage	Pyroséquençage	Séquençage par synhèse	Ligation et codage 2 bases	Terminaison de chaînes (Dideoxy)
Longueur de « reads »	700 pb	50SE, 50PE, 101PE	50 + 35 pb ou 50 + 50 pb	400-900 pb
Précision « reads »	99,9%	98%, (100 PE)	99,94%	99,999%
Données générés/ « run »	0,7 Gb	600 Gb	120 Gb	1,9-84 kB
Temps/ « run »	24 h	3-10 j (selon méthode)	7-14 j (simple ou paired ends)	20 min – 3h
Avantages	Taille des « reads », rapidité	Haut débit	Précision	Haute qualité, taille des « reads »
Inconvénients	Taux d'erreurs (si plus de 6 répétitions de base), cout élevé, débit plus faible	Assemblage (problème taille des « reads »)	Assemblage (problème taille des « reads »)	Coût très élevé, débit très faible
Coût/ million bases	$10 (~7,40€)	$ 0,07 (~0,05 €)	$ 0,13 (~0,10 €)	$ 2400 (~1775 €)

Ces nouvelles technologies reposent notamment sur des modifications de la méthode PCR, permettant des PCR par patch (emulsion PCR (454), bridge PCR (Illumina)). Si ces méthodes possèdent des limitations, notamment l'analyse des données qui demande d'une part des ressources informatiques importantes et d'autre part une expertise et un temps d'analyse non négligeables, elles apparaissent

néanmoins aujourd'hui suffisamment matures pour remplacer avantageusement d'autres techniques d'analyses transcriptomiques comme les microarray ou la création de banques d'EST substractives.

3 La coquille Saint Jacques, *Pecten maximus*

3.1 Position systématique

La coquille Saint-Jacques, *Pecten maximus* (Linné, 1758) fait partie des 17 espèces appartenant au genre *Pecten* (Muller, 1776) selon le WoRMS (World Register of Marine Species, www.marinespecies.org), dont la position systématique est détaillée ci-après.

Embranchement *Mollusca*
Classe *Bivalvia*
Ordre *Pectinoida*
Super Famille *Pectinoidea*
Famille *Pectinidae*
Genre *Pecten*

Figure 5: Pecten maximus dans son milieu naturel (photo Erwan Amice).

3.2 Répartition des populations

3.2.1 Aire de répartition géographique

La coquille Saint-Jacques est présente sur toute la côte est de l'Atlantique Nord, du nord de la Norvège à la côte ouest de l'Afrique, et également aux Açores, aux Maldives et à Madère (Brand, 2006). A l'est de sa répartition elle s'étend au sud-est de l'Espagne et dans la Méditerranée jusqu'à Malaga, puis est remplacée par une autre espèce de pétoncle, *P. jacobaeus* (Brand, 2006). Reconnue pour ses qualités gustatives comme un produit de choix, *P. maximus* a une longue histoire d'exploitation commerciale. Les principales pêcheries par drague se localisent sur les côtes ouest des îles britanniques et de la France, ainsi qu'au nord-est de l'Ecosse (Brand & Shumway, 1991).

3.2.2 Génétique

D'un point de vue génétique, *P. maximus* et *P. jacobaeus* sont très proches. Des études se basant sur l'électrophorèse d'allozymes (Huelvan, 1985), d'ADN mitochondrial (Wilding *et al.*, 1999) et des séquences partielless du gène codant pour l' ARN ribosomique 16S et 12S (Canapa *et al.*, 2000; Barucca *et al.*, 2004) ont suggérés que la distance génétique entre *P. maximus* et *P. jacobaeus* n'était pas suffisante pour les considérer comme des espèces formellement distinctes. Cependant d'autres études sur les fréquences d'allozymes (Ríos *et al.*, 2002) ont trouvé une différenciation significative correspondant aux différences de morphologie observées, ce qui confirmerait l'existence de deux espèces à part entière.

Concernant la structuration génétique des populations de *P.maximus*, les résultats sont variables selon les méthodes et les populations étudiées. Ainsi, deux études ont été réalisées sur les populations du Royaume-Uni et de France en utilisant des loci allozymiques polymorphes (8 loci sur 13 populations, Beaumont *et al.*, 1993; 7 loci sur 9 populations, Wilding *et al.*, 1998). Aucune de ces études n'a mis en évidence l'existence d'une sous-population. D'autres études ont révélé une séparation génétique de la population de *P. maximus* de la baie de Mulroy en Irlande, par utilisation de RFLP (Restriction Fragment Length Polymorphism) et de RAPDs (Random Amplified Polymorphic DNA) sur des marqueurs d'ADN mitochondriaux

(Wilding *et al.*, 1997; Heipel *et al.*, 1998). D'autre part l'étude de fréquences haplotypiques montrerait une différenciation des populations de Norvège du reste de l'Europe (Beaumont, 2006).

Figure 6: Aire de répartition de la Coquille Saint-Jacques Pecten maximus. (Carte Romain Lavaud, d'après données FAO, www.fao.org)

3.3 Biologie et écologie

P. maximus atteint dans de nombreux endroits une taille maximale de 150 mm (hauteur de coquille), même si certains individus de tailles plus importantes ont pu être signalées (210 mm, Minchin, 1978; 230 mm, Jolivet, comm. pers.). C'est une espèce aux valves asymétriques, avec une valve supérieure plate (valve gauche morphologique) et une valve inférieure (droite) incurvée, qui dépasse légérement la gauche en son bord. Les deux valves portent de larges nervures rayonnantes (env. 15 à 17) et de nombreuses ondulations concentriques ainsi que de fines stries. La valve gauche est habituellement d'un marron rougeâtre mais cette coloration peut varier du rose pâle à quasiment noir. La valve droite est généralement blanc cassé, jaunâtre ou marron claire. Les anneaux de croissance « dits hivernaux » sont normalement bien marqués sur les deux valves, particulièrement sur la valve gauche (Brand, 2006).

P. maximus peut vivre juste en dessous de la zone tidale jusqu'au bord du plateau continental, à une profondeur de plus de 210 m (Forbes & Hanley, 1853; Tebble, 1966; Nerot *et al.*, 2012). Cette espèce est cependant essentiellement côtière et est plus communément trouvée entre 20 et 45 m de profondeur, sur des fonds sableux, de fins graviers, de maërl, avec parfois un mélange de boue (Brand, 2006).

P. maximus s'enfouit généralement sur le fond de façon à ce que la valve supérieure soit au niveau, ou juste en deçà, de la surface du sédiment (Baird, 1958). Le comportement d'enfouissement est un processus relativement complexe qui implique l'expulsion d'eau via la cavité palléale et permet le recouvrement de la valve supérieur par le sédiment préalablement suspendus (Brand, 2006). Les coquilles enfouies sont plus difficilement détectées par les prédateurs visuels et ce comportement de protection est ainsi adopté dès qu'elles atteignent une taille de 6-10 mm (Minchin, 1992; Minchin *et al.*, 2000). Les jeunes coquilles (< 15 mm) sécrètent du byssus et sont attachés sur le fond. Toutefois, attachées ou non, les coquilles Saint-Jacques, comme la plupart des pectinidés, sont capables de nager en réponse à des stimuli variés (présence de prédateurs, vibrations, etc. ; Brand, 2006). Le mouvement de nage (aussi qualifié de réponse de fuite) est dû à l'expulsion de puissants jets d'eau de la cavité palléale, générés par l'adduction des valves. Ce mouvement repose principalement sur le métabolisme anaérobie de l'arginine phosphate du muscle adducteur (Ansell, 1977; Grieshaber & Gäde, 1977). En conséquence, il sert à se

déplacer sur des distances réduites et nécessite une phase de récupération longue (Grieshaber & Gäde, 1977).

La croissance de *P. maximus* en terme de taille de coquille et de poids est considérée comme conforme à la loi de von Bertalanffy (Broom, 1976; Murphy, 1986; Allison, 1993). Chauvaud *et al.* (1998) ont avancé que la croissance de *P. maximus* s'expliquerait essentiellement par la température de l'eau de fond, la salinité, et dans une moindre mesure des flux fluviaux. La nourriture aurait un rôle mineur, exception faite des efflorescences d'algues toxiques qui provoquent une diminution drastique du taux de croissance journalier (Lorrain *et al.*, 2000).

Il existe de larges différences de croissance entre les populations, à la fois entre des populations éloignées géographiquement (*e.g.*, Norvège et Rade de Brest) et entre des populations plus proche (*e.g.*, Rade de Brest et Baie de St Brieuc). D'un point de vue général, une différence de taille asymptotique est observé le long du gradient latitudinal, caractérisé par un gradient de température avec des coquilles plus petites pour les populations du Nord (Chauvaud *et al.*, 2012). Les faibles taux de croissance annuels observés pour les populations du Nord résulteraient non pas d'un taux de croissance journalier plus faible mais d'une période de croissance plus courte (328 jours de croissance/ an à Vigo contre 130 jours de croissance en moyenne en Norvège). Cependant les performances de croissance annuelle des populations nordiques persistent alors qu'elles diminuent dès la seconde année de croissance dans les populations du sud (Chauvaud *et al.*, 2012).

En dehors de la croissance, les cycles de stockage d'énergie et de reproduction varient aussi largement selon la position géographique (Brand, 2006). Le stockage de l'énergie paraît fortement lié aux paramètres environnementaux (Saout *et al.*, 1999; Brand, 2006), ainsi que certains facteurs du cycle de reproduction comme le déclenchement et la durée de la ponte (Mackie & Ansell, 1993). Des études ont cependant mis en évidence une composante génétique marquée dans le contrôle du cycle de reproduction (Paulet *et al.*, 1988; Mackie & Ansell, 1993). L'influence relative des facteurs génétiques et de l'environnement diffère dans les cycles de reproduction et de stockage de l'énergie, ce qui peut potentiellement être un problème pour les populations transplantées pour le repeuplement des pêcheries (Brand, 2006).

4 Contexte de l'étude

4.1 Intérêt des approches protéomique et transcriptomique en biologie marine

Un des intérêts majeurs de la transcriptomique et de la protéomique est que ces approches sont sans *a priori* et reposent sur la comparaison de conditions contrastées permettant d'identifier des différences à l'échelle de l'ensemble du transcriptome ou du protéome.

Dans l'étude de l'effet du changement global sur les organismes marins, ces outils ont été employés avec succès. En effet, l'électrophorèse bidimensionnelle a été utilisée pour comparer la réponse à un stress thermique aigu de deux espèces de moules, une espèce adaptée aux températures plus froides, *Mytilus trossulus* et une espèce adaptée aux températures plus chaudes, *M. galloprovincialis* (Tomanek & Zuzow, 2010). Si les résultats obtenus montrent une différence attendue pour des Heat Shock Proteins, ils ont également permis de montrer l'implication de protéines du cytosquelette, du cycle de Krebs et de protéines anti-oxydantes. Ces changements étant plus prononcés chez *M. trossulus*, les auteurs ont suggéré que la réduction de l'aire de répartition de cette espèce pourrait être en partie liée à une adaptation relativement moins efficace au stress oxydatif induit par la température. D'autre part, ces études ont permis l'identification de protéines nouvelles, indicatrices d'un stress thermique, comme la sirtuine, une enzyme impliquée dans la modification épigénétique de certains gènes et qui jouerait un rôle clé dans la production d'énergie et la mort cellulaire programmée (Finkel *et al.*, 2009; Tomanek & Zuzow, 2010; Tomanek *et al.*, 2011).

Le développement et la diminution des coûts du séquençage de nouvelle génération (*cf.* § 2.3), ont rendu ces techniques accessibles pour les sciences environnementales. En conséquence, ces approches commencent à être utilisées pour l'étude des réponses aux stress chez les organismes marins (*e.g.* Burns *et al.*, 2012; Clark *et al.*, 2013; Fu *et al.*, 2013). Par exemple, la réponse transcriptomique de l'huître *Crassostrea gigas* à une diminution chronique (3 mois) du pH (-0,4) et une augmentation de la température (19°C et 24°C) a été évaluée par séquençage haut-débit SOLiD (Clark *et al.*, 2013). Parmi les résultats obtenus, l'implication de la voie PI3K/AKT/mTOR dans la régulation chronique du stress a été suggérée. Cette voie

serait impliquée dans la régulation du taux de croissance cellulaire, notamment par son rôle dans l'apoptose (Clark *et al.*, 2013).

4.2 Complémentarité des approches transcriptomique et protéomique chez les espèces non modèles

Les espèces non-modèles sont celles pour lesquelles il n'y a ni lignée pure, ni stock d'élevage, un historique de recherche limité et sur lesquelles il n'y a pas d'études de manipulation génétique (Wilson *et al.*, 2005). Si l'historique de recherche sur *P. maximus* est loin d'être famélique, on peut néanmoins la considérer comme une espèce non modèle, vu le peu d'information génétique disponible sur cette espèce (tableau 2). Aussi, la possibilité d'identification de protéines en spectrométrie de masse s'en trouve fortement limitée (*cf.* § 2.2.2).

Des études chez des espèces marines non-modèles ont combiné le séquençage massif d'ARN messager à des approches de protéomique. Les séquences générées permettent de créer une base de données utilisable par la suite pour l'identification de protéines. Wang *et al.* (2010) ont ainsi étudié les mécanismes de la métamorphose et de la fixation chez le bryozoaire *Bugula neritina*. La combinaison des approches a permis la génération de 15192 séquences uniques par pyrosequençage de type 454 et l'utilisation de chromatographie liquide (2 dimension) couplé à un spectromètre de masse à permis d'identifier 882 protéines à l'aide cette base de donnée.

La corrélation entre la concentration mesurée d'une protéine et de son ARNm est relativement faible (estimée à env. 40%, Vogel & Marcotte, 2012), ce qui s'explique principalement par les nombreuses étapes possibles de régulation entre un transcrit nouvellement synthétisé et la protéine active correspondante. L'utilisation de méthodes à la fois transcriptomique et protéomique permet donc théoriquement non seulement d'identifier les protéines impliquées, mais également d'estimer les niveaux de régulation des protéines, donnant ainsi une information majeure sur la mise en place et la cinétique des processus biologiques impliqués (Dondero *et al.*, 2010; Vogel & Marcotte, 2012).

Tableau 2 : Nombre de séquences (Nucléotides, EST et protéines) disponibles sur le site du NCBI (www.ncbi.nlm.nih.gov) en Octobre 2013 pour des organismes modèles (*H. sapiens*, *D. melanogaster*, *C. elegans*) et non-modèles (*C. gigas*, *M. galloprovincialis*, *P. maximus*).

Espèces	Nucléotides	EST	Protéines
Homo sapiens	13 192 543	8 863 664	10 190 419
Drosophila melanogaster	305 559	848 559	194 538
Caenorhabditis elegans	80 313	536 988	75 839
Crassostrea gigas	11 421	206 647	28 682
Mytilus galloprovincialis	48 835	68 209	7937
Pecten maximus	241	1133	197

4.3 Réponses de *Pecten maximus* à des changements environnementaux

Les approches moléculaires (protéomique, transcriptomique) n'ont, à notre connaissance, jamais été utilisées pour mesurer la réponse au stress chez *P. maximus*. En revanche, beaucoup de travaux ont été menés sur la réponse à divers stimuli des cellules circulantes de cette espèce, les hémocytes. Avec le développement de l'aquaculture pour cette espèce, les interactions avec les pathogènes (Rickettsia like organismes, Vibrios) ont été un champ de recherche particulièrement actif (Le Gall *et al.*, 1991; Mortensen & Glette, 1996; Lambert *et al.*, 2001), mais aussi l'interaction de la réponse immunitaire avec les polluants (Hannam *et al.*, 2010 a,b). En dehors des hémocytes, toujours dans une perspective d'optimisation de l'aquaculture, les effets sur les larves de pathogènes (Nicolas *et al.*, 1996; Lambert *et al.*, 1998) ou des polluants ont aussi été investigués (Beaumont *et al.*, 1987). D'autres études ont révélé la vulnérabilité de *P. maximus* aux pratiques de l'aquaculture comme le transport et l'émersion (Duncan *et al.*, 1994; Maguire, 2007).

La variation de quelques paramètres abiotiques a aussi été investiguée, ainsi une baisse de la salinité réduirait la croissance aux stades précoces de développement de *P. maximus* (Laing, 2002). Une étude reporte aussi une diminution de la respiration et du rythme cardiaque lorsque la concentration en oxygène décline dans le milieu,

suggérant que *P. maximus* est un oxyrégulateur mais sur une gamme de concentration en oxygène très limitée (Brand & Roberts, 1973).

4.4 Objectifs de l'étude

Dans le contexte des changements globaux actuels, les écosystèmes côtiers apparaissent comme particulièrement vulnérables aux changements globaux actuels, notamment face à l'augmentation de la température de l'eau et à la fréquence des épisodes hypoxiques. La coquille Saint-Jacques, *Pecten maximus*, est un mollusque bivlave vivant juste au dessous de la zone tidale jusqu'au bord du plateau continental, à une profondeur de plus de 210 m. Sur un gradient latitudinal, son aire de répartition couvre toute la côte Atlantique Est depuis le Nord de la Norvège jusqu'au Maroc. Malgré un intérêt commercial important, un rôle écologique majeur et un historique de recherche conséquent, notamment au LEMAR, cette espèce n'a fait l'objet que de peu d'études au niveau moléculaire.

L'objectif de départ du travail présenté dans cette thèse était de caractériser les mécanismes moléculaires régissant l'acclimatation de cette espèce aux contraintes thermique et hypoxique. L'utilisation d'une approche intégrative plutôt que l'utilisation d'une discipline unique pour l'étude de ces stress est une des originalités du travail présenté ici. En effet, dans un premier temps, une approche protéomique pour l'étude de ces stress en conditions contrôlées a été privilégiée. Pour ce faire il nous a parus nécessaire de développer des méthodes statistiques appropriées. L'utilisation du logicel R (R Core Team, 2013) et de la suite de packages pour R spécialisés en bioinformatique Bionconductor (www.bioconductor.org) ont permis le développement de méthodes spécifiques pour la comparaison de données protéomiques, l'ensemble de ces méthodes ont été regroupés dans un package pour R « prot2D », ces méthodes ont été rendus librement disponible par son intégration au sein de la suite Bioconductor. Ces méthodes ont été évaluées et ont fait l'objet d'une publication dans Bioinformatics, présentée dans le chapitre 1 (article 1). D'autre part, la documentation pour le package prot2D est présentée en annexe.

L'intérêt de compléter les approches protéomiques avec des approches transcriptomiques « NGS » pour l'étude du stress thermique nous est très vite apparu, en premier lieu pour la génération de séquences en vue de l'identification de protéines

en spectrométrie de masse et ensuite parce que le couplage de ces deux approches renseigne sur les différents niveaux de régulations et permet ainsi d'identifier, dans une même voie, quels sont les facteurs régulés au niveau protéique et lesquels sont régulés au niveau de leur transcrit. Ce travail a pus être effectués grâce à une collaboration avec Melody S. Clark, Michael A. Thorne et Lloyd S. Peck du British Antartic Survey, à Cambridge, au Royaume Uni. Les résultats obtenus sont présentés et discutés sous la forme d'une publication scientifique (article 2) dans le chapitre 2.

Pour l'étude de la réponse à l'hypoxie et plus spécifiquement, de l'influence de la température sur la réponse à l'hypoxie, il nous paraissait primordial de caractériser la réponse au niveau physiologique et notamment l'évolution de la respiration en fonction du niveau d'oxygène dans l'eau de mer. La comparaison avec la moule bleue, une espèce intertidale, fréquemment exposée à des conditions plus extrêmes que *P. maximus* donne des points de repères pour situer la réponse de P. maximus face à l'hypoxie. Les résultats de cette étude sont présentés et discutés sous la forme d'un article scientifique dans le chapitre 3 (article 3). Logiquement, nous avons ensuite caractérisés les réponses protéiques à l'hypoxie de P. maximus à différentes températures, ces résultats sont présentés à la suite dans le chapitre 3 (article 4).

Enfin, une fois les outils protéomique matures pour la Coquille Saint-Jacques, il semblait pertinent de comparer les expressions protéiques des populations de *P. maximus* pour lesquels on observe d'importantes différences physiologiques. L'expression des Coquilles Saint-Jacques brestoises et norvégiennes ont ainsi été comparés grâce à l'accueil de Øivind Strand et Tore Strohmeier de l'Institute for Marine Research à la station d'Austevoll, en Norvège. Les résultats sont présentés dans le chapitre 4 et discutés, sous la forme d'une publication scientifique, au regard des différences de croissance observées entre ces deux populations. D'autre part les différences de croissance pourraient s'expliquer par une différence de régime alimentaire, la comparaison des deux régimes passent donc par la caractérisation des régimes dans les deux populations. Dans le travail mené par Romain Lavaud, avec lequel j'ai étroitement collaboré, présenté dans le chapitre 4 (article 6) l'utilisation de combinaisons de marqueurs trophiques permet d'émettre de nouvelles hypothèses sur le régime alimentaire de *P. maximus* en Rade de Brest qui pourraient avoir des implications importantes sur la compréhension de la croissance de *P. maximus*.

Chapitre I : Développement d'un outil bioinformatique pour les analyses protéomiques

I/ Introduction

Dans l'optique de caractériser les mécanismes moléculaires impliqués dans la réponse des organismes, des tissus ou des cellules, à des conditions inhabituelles, le développement d'approches permettant d'aborder la réponse dans sa globalité et sans a priori, est particulièrement séduisante. Ainsi, les approches transcriptomiques basées sur l'utilisation de puces à ADN ou plus récemment, sur le séquençage d'ADN à haut débit, rendu possible par les nouvelles générations de séquenceurs se sont particulièrement développées ces dernières années, y compris pour des problématiques d'écologie moléculaire. Dans un tel contexte, les approches de protéomique comparative, permettant d'identifier les acteurs protéiques dérégulés en réponse à une situation donnée, sont également particulièrement séduisantes.

L'un des objectifs majeurs de cette thèse a été de développer des approches de protéomique environnementale pour comprendre les mécanismes d'adaptation de la coquille Saint Jacques à la thermie et l'hypoxie. Il faut noter qu'au démarrage de cette thèse, ce type d'approches n'était absolument pas mené au laboratoire, et n'étaient, d'ailleurs, que très peu utilisées dans des problématiques d'écologie. Ainsi, outre les aspects techniques, de l'extraction des protéines à la réalisation des électrophorèses bidimensionnelles, qu'il a fallu mettre en place et développer, nous nous sommes rapidement rendus compte, au vu des premiers résultats que l'absence de données génomique pour *P. maximus* constitueraient un problème majeur pour la suite du travail, de même que l'absence d'outils statistiques fiables pour l'exploitation de données obtenues à partir de populations naturelles (pour lesquelles la variabilité est souvent très importante).

Pour pallier au premier problème, nous avons décidé de nous orienter, au moins en partie, vers une approche transcriptomique de type RNAseq qui, outre la richesse des informations classiquement apportées par ce type de démarche, présentait l'avantage de conduire à la génération de données génomiques pour notre organisme d'intérêt (voir chapitre 2). Concernant le second problème, nous avons décidé de développer des outils bioinformatiques adaptés au type d'études protéomiques que nous souhaitions mettre en place.

L'analyse statistique des données, en particulier les problèmes de normalisation et d'identification du caractère différentiel de ces données, ont été particulièrement bien

développés pour la transcriptomique. Malgré des jeux de données tout à fait différents, il apparaît évident que les problématiques statistiques en protéomique et en transcriptomique sont similaires. Nous avons donc évalué la valeur, pour l'analyse de jeux de données protéomiques, de différentes procédures de normalisation, tests statistiques, et méthodes de calcul des FDR (False Discovery Rates) précédemment développés pour la transcriptomique. Nous appuyant sur des jeux de données à la fois réels (acquis au laboratoire) et simulés *in silico*, nous avons construit et validé une séquence d'analyse ("workflow") de la normalisation à l'identification des points exprimés de manière différentielle. Nous avons assemblés ces différentes procédures dans un package librement accessible par la communauté scientifique (« prot2D », dont la documentation est présentée en annexe 1), fonctionnant sous R (R Core Team, 2013), dont nous avons également obtenu l'intégration dans la suite Bioconductor (www.bioconductor.org), largement utilisée par la communauté scientifique.

II/ Article n°1 :

Identifying differentially expressed proteins in two-dimensional electrophoresis experiments: inputs from transcriptomics statistical tools

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Gene expression

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Identifying differentially expressed proteins in two-dimensional electrophoresis experiments: inputs from transcriptomics statistical tools

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ABSTRACT

Background: Two-dimensional electrophoresis is a crucial method in proteomics that allows the characterization of proteins' function and expression. This usually implies the identification of proteins that are differentially expressed between two contrasting conditions, for example, healthy versus diseased in human proteomics biomarker discovery and stressful conditions versus control in animal experimentation. The statistical procedures that lead to such identifications are critical steps in the 2-DE analysis workflow. They include a normalization step and a test and probability correction for multiple testing. Statistical issues caused by the high dimensionality of the data and large-scale multiple testing have been a more active topic in transcriptomics than proteomics, especially in microarray analysis. We thus propose to adapt innovative statistical tools developed for microarray analysis and incorporate them in the 2-DE analysis pipeline.

Results: In this article, we evaluate the performance of different normalization procedures, different statistical tests and false discovery rate calculation methods with both real and simulated datasets. We demonstrate that the use of statistical procedures adapted from microarrays lead to notable increase in power as well as a minimization of false-positive discovery rate. More specifically, we obtained the best results in terms of reliability and sensibility when using the 'moderate t-test' from Smyth in association with classic false discovery rate from Benjamini and Hochberg.

Availability: The methods discussed are freely available in the 'prot2D' open source R-package from Bioconductor (http://www.bioconductor.org//) under the terms of the GNU General Public License (version 2 or later).

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1 INTRODUCTION

Comparative proteomics based on 2D-polyacrylamide gel electrophoresis aims at identifying significant biologically relevant proteins. In most cases, protein samples from two conditions are compared, e.g. healthy versus diseased in human proteomics biomarker discovery (Kim *et al*., 2004), or stressful conditions versus control in animal experimentation (Tomanek *et al*., 2011). Besides the laboratory bench, informatics takes a large place in such experiments with digitalization of gels, spots detection and matching, evaluation of spots' volumes and identification of differentially expressed proteins.

A large variety of software designed for the analysis of 2D digitized images exist, both commercial (e.g. Delta2D from Decodon, SameSpot Progenesis from Nonlinear dynamics, PDQuest from Bio-rad laboratories) and custom researcher-developed (e.g. Pinnacle from Morris *et al*., 2010; RegStatGel from Li and Seillier-Moiseiwitsch, 2011). These programs perform three main tasks: identification of spots, matching of spots from different gels and evaluation of spots' volumes. Each program has its own way to perform these steps; therefore, software choice has a great impact on the result of the analysis (Millioni *et al*., 2012; Morris *et al*., 2010; Stessl *et al*., 2009; Wheelock and Buckpitt, 2005). A normalization step is also needed to remove the systemic variation before data analysis. An issue with commercial software is the lack of information concerning this critical step as well as the inability to customize the normalization procedure. Ultimately, once spots' volumes have been estimated and normalized across gels, proteins that are differentially expressed between groups are identified with software-specific methods.

Normalization and identification are also essential steps in 2-DE-based proteomics analysis and, as compared with other critical steps of the 2-DE procedure (e.g. protein extraction, staining, image analysis), are less studied and under-reviewed in the literature. On the other hand, the same issues have been widely studied and reviewed for transcriptomic analysis, especially for microarray studies. As pointed out by some authors, the underlying statistical issues are similar between microarray- and 2-DE analyses. A number of the statistical tools developed for microarray analysis have begun to be incorporated in the 2-DE toolbox. More specifically, advances in normalization have been adapted to Difference Gel Electrophoresis (DIGE) analysis (Fodor *et al*., 2005; Miecznikowski *et al*., 2011). Statistical tests developed for microarrays have also been evaluated (Fodor *et al*., 2005; Meunier *et al*., 2005). Finally, numerous authors highlight the need of correction for multiple testing, such as false discovery rate (FDR), but few actually evaluate the performance of the different procedures (Chang *et al*., 2004; Karp *et al*., 2007; Morris, 2012).

The aim of the present work is to evaluate different methods, initially developed for microarray analysis, to identify differential

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spots in 2-DE experiments. To compare these methods, real datasets as well as simulated data were used. We also compare methods for normalization of volumes' data before statistical analysis. By evaluating the key steps of the analysis workflow, from normalization to identification of differentially expressed spots, our motivation is to provide a simple and adapted workflow to non-statistics expert biochemists. All the methods discussed are available as a free, open-source R package (R Core Team, 2012) with highly customizable options.

2 METHODS

2.1 Datasets description

2.1.1 Platichthys flesus *liver dataset* The *Platichthys flesus* liver dataset (Pfl DS)corresponded to a 2-DE-based comparison of liver proteomes of two populations of *Platichthys flesus* living in contrasted estuaries, i.e. the Seine estuary, known to be highly polluted, and the Canche, which is often considered as a pristine area. The whole study was previously published in Galland *et al.* (2013). It included analyzing the liver proteomes of four juvenile individuals from each estuary.

2.1.2 Pecten maximus *gills dataset* The *Pecten maximus* gills dataset (Pmg DS) is issued from a 2-DE experiment performed on proteins from *Pecten maximus* gills subjected to a temperature challenge. Briefly, scallops were kept in two separate tanks at 16.4°C (±0.3°C) (Huber *et al.*, 2002) and fed *ad libitum* for 2 weeks. One tank was then heated at 1°C per day and allowed to reach 27°C ('hot condition'), whereas the other tank was kept at 16.5°C ('control'). At the end of the experiment, six animals per condition were sampled. Gills were snap-frozen in liquid nitrogen and kept at −80°C until protein extraction. Gills were crushed (using Retsch® MM400) device kept frozen using liquid nitrogen. Hundred milligrams of the obtained powder was homogenized in 100 mM Tris–HCl (pH 6.8), centrifuged (4°C, 50 000 *g*, 5 min) and supernatants were pipetted in other tubes. Protease inhibitor mix (GE Healthcare) was then added and nucleic acids were removed (nuclease mix, GE Healthcare, following manufacturer's instructions). Samples were precipitated overnight at −20°C using TCA 20% (1/1:v/v, overnight). After centrifugation (4°C, 20 000 *g*, 30 min), pellets were washed with acetone 70% and 0.1% DTT and re-suspended in urea/thiourea buffer (2 M thiourea, 7 M urea, 4% CHAPS, 1% DTT) containing 1% IPG (pH 3–10, GE Healthcare). Protein concentration was determined using a modified Bradford assay12, and all samples were adjusted to 400 mg of proteins in 250 µl. Electrophoresis and staining procedures were performed as described in Galland *et al.* (2013).

2.2 Preprocessing of data

2.2.1 Image Analysis All gels were digitized using a transparency scanner (Epson Perfection V700) in gray scale with 16-bit depth and a resolution of 600 dpi. Images were aligned and spots were detected and quantified with the Progenesis SameSpots software (Nonlinear dynamics, v.3.3) using the automated algorithm. All detected spots were manually carefully checked and artifact spots were removed. Datasets were exported as raw values in the form of a matrix of volume data X_{ij} with spots i as rows and gels j as columns. In all, 611 spots were identified in the Pfl dataset from Galland *et al.* (2013) with four replicates in two conditions (611 × 8 matrix), and 766 spots were identified in the Pmg dataset with six replicates per condition (766 × 12 matrix).

2.3 Visualization and normalization of datasets

Dudoit *et al.* (2002) proposed a method for visualization of artifacts in microarray datasets, called the MA-plot, which was transposed for proteomics data as the ratio-intensity plot (Meunier *et al.*, 2005; R-I plot). It consists in plotting the intensity $\log_2$-ratio (R) against mean $\log_{10}$ intensity (I):

$$R = \log_2 \frac{mean(V_{Cond2})}{mean(V_{Cond1})}$$

$$I = \log_{10}(mean(V_{Cond2}) \times (mean(V_{Cond1}))$$

where V_{Cond1} and V_{Cond2} are spots' volumes for conditions 1 and 2, respectively.

R-I plots allow to directly visualize artifacts in the original dataset as well as the effects of normalization (Fig. 1). Artifacts were already described for Coomassie blue-stained 2-DE gels, but have also been reported in DIGE, SYPRO ruby and silver-stained experiments. In the original datasets (Fig. 1A and B), the cloud of points seems to be off-centered, especially for low-intensity spots, either down-shifted in Pmg DS or up-shifted in Pfl DS.

Among the variety of methods for normalization recently proposed in the literature, both for proteomics and microarray experiments (Podrabsky and Somero, 2004; Quackenbush, 2002; Yang *et al.*, 2002), two widely used methods are compared, the 'Variance Stabilizing Normalization' (VSN; Huber *et al.*, 2002) and the 'Quantile Normalization' (Qt; Bolstad *et al.*, 2003). The principle of the 'quantile normalization' is to set each quantile of each column (i.e. the spots' volume data of each gels) to the mean of that quantile across gels. The intention is to make all the normalized columns have the same empirical distribution. Whereas the VSN method relies on a transformation h of the intensities, of the parametric form $h(x) = arsinh(a + bx)$ .The parameters of h are estimated with a robust variant of maximum-likelihood estimation. The R-I plots of normalized data (Fig. 1) show that both methods center the data around a log ratio of 0. Nevertheless, for low values of intensities the VSN normalized data (Fig. 1E and F) seem to be less efficient to recenter the cloud of points.

2.4 False discovery rate and significance

Transcriptomics and 2-DE experiments analyses have in common that they require a variant of a t statistic that is suitable for high-dimensional data and large-scale multiple testing. For this purpose, in the past few years, various test procedures have been suggested. We decided to compare a simple method (the classical Student's t-test), two tests especially

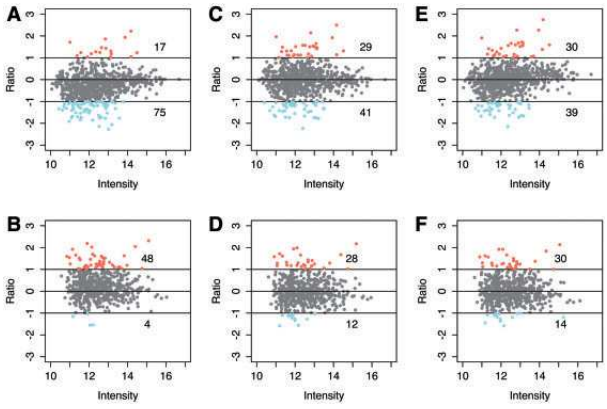

Fig. 1. Comparison of the effect of normalization procedures using R-I plots. Values on the plots are the number of spots with a ratio >1 (upper values) or <−1 (lower values). (**A**) Pmg DS without normalization. (**B**) Pfl DS without normalization. (**C**) Pmg DS with Qt. (**D**) Pfl DS with Qt. (**E**) Pmg DS with VSN. (**F**) Pfl DS with VSN

modified for microarray analysis (Efron's *t*-test; Efron *et al.*, 2001), the modified *t*-test used in significance analysis for microarray (SAM; Tusher *et al.*, 2001) and two methods that take advantage of hierarchical Bayes methods for estimation of the variance across genes: the 'moderate *t*-test' from Smyth (2004) and the 'Shrinkage *t*' statistic test from Opgen-Rhein and Strimmer (2007).

Statistical tests allowing the identification of differentially expressed proteins must take into account a correction for multiple tests to avoid false conclusions. Testing for expression changes across hundreds or thousands of proteins with univariate test, such as Student's *t*-test, causes false positives to accumulate. To address this issue in multiple comparison procedure, Benjamini and Hochberg (1995) proposed a method for estimating and controlling the FDR, allowing for 'FDR-driven' decision of significance.

In proteomics, the FDR is usually defined as the ratio of the proteins not differentially expressed in reality but declared differentially expressed by the test, over the total number of proteins declared differentially expressed by the test [Table 1; FDR = FP/(TP + FP)].

From a statistical point of view, there are two types of FDR, the 'classic' tail area-based FDR (*Fdr* in Efron, 2008) and the local FDR (*fdr* in Efron, 2008). As it is easier to interpret for non-specialists, we focused our study on tail area-based FDR methods.

We decided to compare four different FDR estimators: (i) the classical FDR estimator of Benjamini and Hochberg, (ii) Strimmer's FDR (based on local FDR calculation; Strimmer, 2008a), (iii) the 'robust Fdr' estimator of Pounds and Cheng (2006) and (iv) the widely used FDR method known as 'q-value' defined by Storey (2002) and improved in 2004 (Storey *et al.*, 2004).

To evaluate the impact of the normalization on the number of spots declared significant as well as the impact of the statistical test and mode of calculation for FDR, the following workflow was used for each dataset:

(1) Normalization of raw volume data with either VSN or quantile methods.
(2) Calculation of statistic value for normalized volume data using Student's *t*-test, Moderate *t*-test, SAM statistic, Efron's *t*-test and shrinkage *t*-test.
(3) Computation of *P*-values for each test based on a null distribution estimated using the fdrtool package (Strimmer, 2008b).
(4) FDR values were calculated using *P*-values as input with Benjamini and Hochberg ('BH'), Strimmer ('Stri'), Pounds and Cheng ('PC') or Storey ('Sto') procedures.
(5) Spots under a cut-off of 0.1 were declared significantly differentially expressed between the conditions.

2.5 Simulations study

To compare FDR and the responses of the different tests as well as the influence of the number of replicates, simulated data were used. Data were simulated based on parameter estimates of Pmg dataset, following these steps:

(1) Log_2 mean volumes from Pmg DS were computed for each spot.
(2) Means were used as input parameters to simulate a normal distribution (with no differential expression between conditions) for each spot with standard deviations computed as described by Smyth (2004).
(3) Ten percent of spots were randomly picked for introducing differential expression in both conditions (5% in each condition).

Briefly, in this hierarchical Bayesian model, the distribution is controlled by hyperparameters s_0^2 (estimator of the standard deviation) and d_0 (the degrees of freedom of the $\chi 2$ distribution used in the calculation of the distribution; see Smyth, 2004 for details). To simulate realistic data, we used $s_0^2 = 0.2$ and $d_0 = 3$.

Table 1. Output of statistical test versus reality of protein expression

	Declared non-significant	Declared significant
Protein *is not* differentially expressed	True negative	False positive
Protein *is* differentially expressed	False negative	True positive

3 RESULTS AND DISCUSSION

3.1 Results from Pmg and Pfl datasets

As seen in Table 2, both the moderate *t*-test and the classical Student's *t*-test seem to (i) be the most coherent among the compared tests and (ii) detect a greater number of differentially expressed spots. The effectiveness of the moderate *t*-test stems from its very definition: a modified *t*-test for which the standard errors have been moderated across spots, i.e. shrunk toward a common value, using a simple Bayesian model. As already demonstrated with microarray data, the smoothing of the standard errors increases the reliability of the test (Smyth, 2004).

Table 2. Spots declared significant (FDR < 0.1) for Pmg and Pfl DS with two methods for normalization: VSN and Qt. Four methods of FDR calculation are also compared: BH (Benjamini and Hochberg, 1995); Stri (Strimmer, 2008a); PC (Pounds and Cheng, 2006); Sto (Storey *et al*, 2004)

Normalization	FDR	Student's *t*-test	Moderate *t*-test	SAM's *t*-test	Efron's *t*-test	Shrinkage *t*-test
Pmg DS						
VSN	BH	4 (1)	7 (2)	4 (4)	3 (3)	1 (1)
	Stri	4 (1)	7 (2)	4 (4)	3 (3)	1 (1)
	PC	0	7 (2)	160 (15)	172 (15)	4 (4)
	Sto	4 (1)	7 (2)	4 (4)	3 (3)	1 (1)
Qt	BH	2 (1)	16 (3)	4 (4)	5 (5)	1 (1)
	Stri	3 (1)	20 (9)	4 (4)	5 (5)	1 (1)
	PC	2 (1)	16 (3)	0	85 (17)	0
	Sto	2 (1)	21 (9)	4 (4)	5 (5)	2 (2)
Pfl DS						
VSN	BH	9 (1)	14 (7)	1 (1)	1 (1)	1 (1)
	Stri	9 (1)	14 (7)	1 (1)	1 (1)	1 (1)
	PC	40 (9)	19 (8)	19 (16)	1 (1)	1 (1)
	Sto	10 (1)	14 (7)	1 (1)	1 (1)	1 (1)
Qt	BH	6 (0)	9 (5)	0	0	0
	Stri	6 (0)	9 (5)	0	0	0
	PC	6 (0)	9 (5)	0	0	0
	Sto	6 (0)	9 (5)	0	0	0

Note: Values in parentheses are the number of spots with an absolute log_2 ratio > 1.

Another striking fact is the inconstancy of Pounds and Cheng's 'robust Fdr' method throughout the results (e.g. from 0 spots detected for classic *t*-test to 172 for Efron's *t*-test for Pmg VSN data). This might reflect that 'robust Fdr' is not well adapted to 2D gel data, where the null proportion (the proportion of spots not differentially expressed) is high. Actually, the authors warned about the instability of the FDR estimates when this null proportion nears 1 (Pounds and Cheng, 2006). Putting aside 'robust Fdr' results, SAM's, Efron's and the shrinkage *t*-test are relatively consistent with both normalization methods but detect almost no significant proteins in the Pfl dataset. Finally, the standard Student *t*-test is consistent among all conditions but detected less significant proteins than the moderate *t*-test.

Concerning the normalization method, the results from Table 2 do not allow making a clear decision between VSN or quantile methods. Nevertheless, as shown in R-I plots (Fig. 1), Qt could be more appropriate for normalization of 2-DE volume data, especially with low intensities.

3.2 Validity of simulated data

To validate the simulated data, we compared the distribution of simulated data with real datasets (Fig. 2). Simulated data with four replicates per condition were compared with Pfl DS and simulated data with six replicates were compared with Pmg DS. The distribution of standard deviation and the R-I plot is illustrative of real proteomic datasets.

3.3 Comparisons of FDR modes of estimation

Simulations were used to evaluate the effect of FDR mode of calculation. For each mode, 100 simulations were run (with 10 replicates), and the number of significant differentially expressed proteins for each test and each Fdr was computed. To compare Fdr threshold with actual FDR values, 10 different threshold values were used (from 0.05 to 0.9). The real value of the FDR was then calculated and compared with the threshold used (Fig. 3). Results show a good correlation between the threshold and the different calculated FDR for high-FDR values. However, for lower values, which are more crucial in this kind of analysis (acceptable FDR is generally acknowledged to be 10% in proteomics), the robust FDR method tended to underestimate actual FDR, thus increasing the discovery of proteins falsely declared differentially expressed. Nevertheless, the other methods appeared appropriate in the whole range of values, and are thus potentially more useful for these kind of data.

Furthermore, we evaluated tests and FDR mode by running 100 simulations without differential expression (by skipping step 3 of the data simulation process). For 'robust Fdr', an average of 31.24–4.12 spots (depending on the test used) were declared significant, whereas for the other methods, the average proportion of spots declared differentially expressed was <1 for all tests (from 0.08 to 0.85). This clearly indicates that the robust FDR tends to select undifferentially expressed spots when the null proportion is high.

3.4 Influence of the number of replicates depends on the test and the FDR calculation mode

One of the main issues reported in proteomics studies is the low number of replicates per condition. We have simulated data with 20, 10, 8, 6, 4 and 3 replicates to determine how this affected the different statistical tests results in terms of false positives and false negatives (Fig. 4). For all these tests, the FDR cut-off was set to 0.1. To assess the power (or sensitivity) of the tests, the false non-discovery rate (FnDR) was also calculated as the number of false negatives over the total number of significant proteins [FnDR = FN/(FN + TN)].

As previously observed, the robust FDR method clearly underestimated the false-positive discovery, even for large numbers of replicates. The method from Storey also showed a relatively high number of false positives for small numbers of

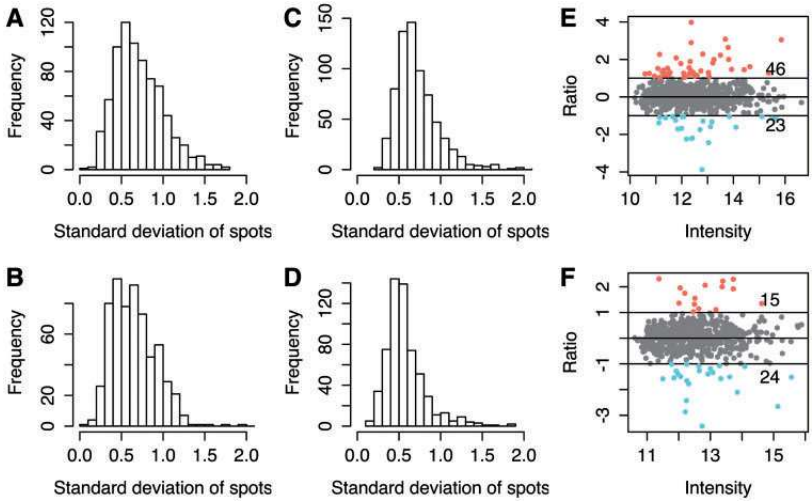

Fig. 2. Comparison between simulated data and real datasets. (**A**) Distribution of standard deviation for Pmg DS. (**B**) Distribution of standard deviation for Pfl DS. (**C**) Distribution of standard deviation for simulated data with 700 spots and six replicates. (**D**) Distribution of standard deviation for simulated data with 700 spots and four replicates. (**E**) R-I plot for simulated data with $n = 6$. (**F**) R-I plot for simulated data with $n = 4$

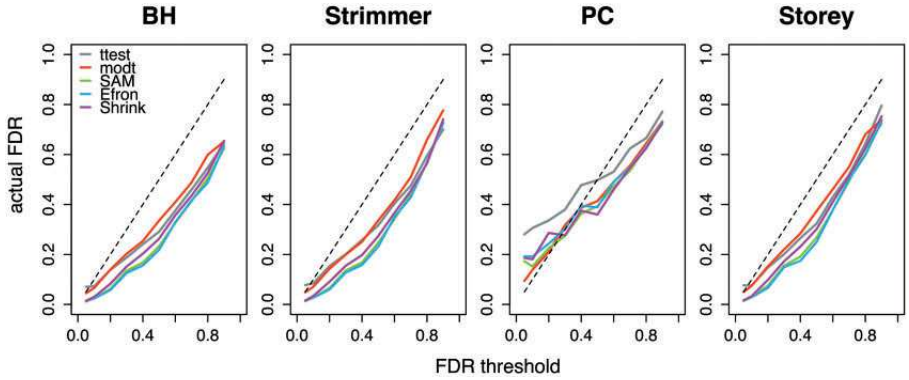

Fig. 3. Threshold versus actual FDR for five statistical tests and four modes of calculation of FDR. Actual FDRs are calculated as the average of 100 simulations with 10 replicates per condition. For comparison purposes, a perfect correlation between threshold and actual FDR is represented as a dashed line. BH (Benjamini and Hochberg, 1995); Strimmer (Strimmer, 2008a); PC (Pounds and Cheng, 2006); Storey (Storey *et al.*, 2004)

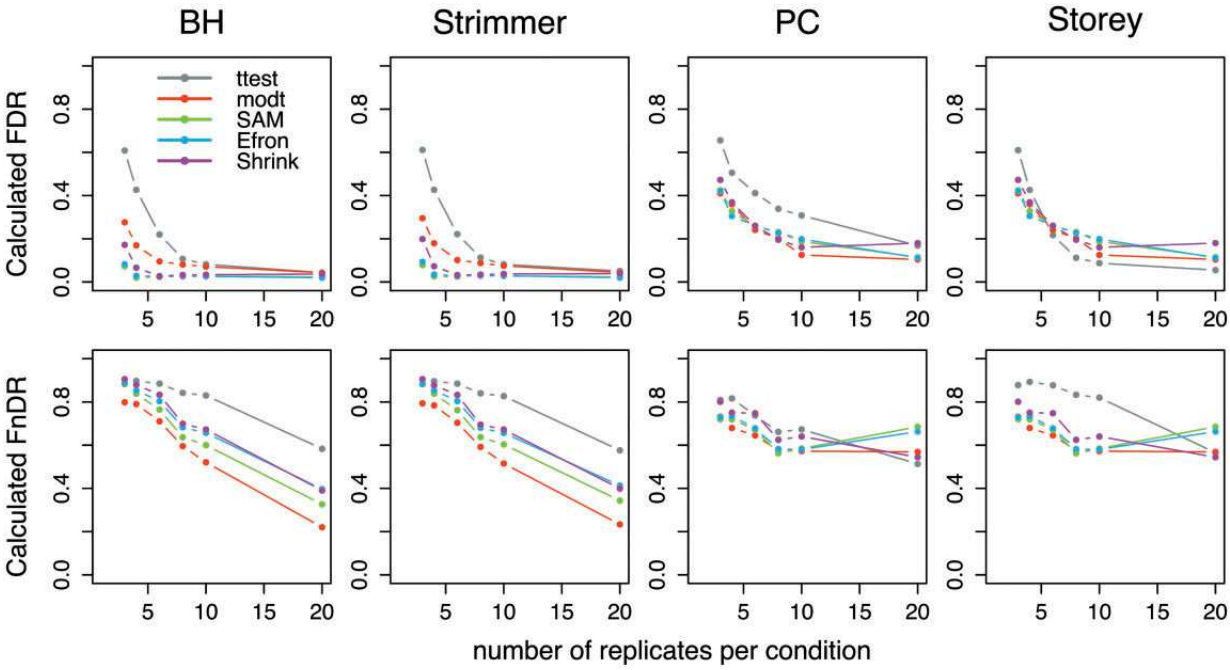

Fig. 4. FDR and False non-Discovery Rate as a function of number of replicates per condition. Calculated values are an average on 100 simulations. BH (Benjamini and Hochberg, 1995); Strimmer (Strimmer, 2008a); PC (Pounds and Cheng, 2006); Storey (Storey *et al.*, 2004)

replicates, but these quickly decrease as the number of replicates increases. We observed similar patterns for classic FDR ('BH') and Strimmer, i.e. the calculated FDR decreased as we increased the number of replicates. Considering the FnDR, we also observed a similar pattern between these two methods, i.e. FnDR decreased as a function of the number of replicates. All the tests displayed this pattern. However, we showed that the classical *t*-test strongly increased the FDR, especially in combination with low numbers of replicates. As for FDR calculation, FnDR did not decrease as rapidly with the classical *t*-test, as compared with the other tests. By contrast, the test that appeared the most sensitive (FnDR systematically lower, regardless of the number of replicates) was the moderate *t*-test. Based on the analysis of both real and simulated data, the moderate *t*-test thus appears to be the most reliable for finding differentially expressed proteins in 2-DE experiments.

4 CONCLUSION

The 2-DE gel-based experiments are often used in comparative proteomics to identify differentially expressed, or accumulated, proteins between two or more conditions. The 2D gels classically allow the visualization of 500–1000 proteins per gel, and replication of experiments is needed to minimize the effects of (i) biological variations in protein expression between individuals and (ii) known technical limits such as differential protein extraction

efficiencies, comigration of proteins, lack of penetration of some proteins in gels or limit of detection of the staining procedure.

Recently, different techniques based both on 2D gel (e.g. DIGE) and gel-free (e.g. iTRAQ) techniques have been developed to reduce these technical biases. However, it seems obvious that, whichever technique is used, statistical analysis remains a crucial step in the proteomics workflow. To date, only a few statistical studies have been dedicated to the specific needs of proteomics, and researchers often use the statistical tools offered in commercial software. The statistical possibilities are restricted, and researchers have to make decisions based only on a *P*- or q-value, without knowing the details of the statistical procedure.

By contrast with proteomics, statistical tools have been widely developed for transcriptomics applications. From a statistician point of view, and notwithstanding different forms of data, the problem is similar in both applications, i.e. extracting statistically significant expression from huge datasets. In this article, we took advantage of many freely available open source statistical tools developed for transcriptomics, evaluated their performance to analyze both real and simulated 2-DE proteomics datasets and developed an R-package adapted to the specific needs of proteomists.

This R-package, called 'prot2D', is freely available as part of Bioconductor (www.bioconductor.org) and includes functions implementing all the methods used in the present article. The Qt method, the FDR calculation method and the moderate *t*-test, that were shown in this article to be the best compromises to analyze proteomics data, are preset in the package with the optimal parameters we determined. For the FDR calculation mode, method from Benjamini and Hochberg and method from Strimmer were shown to be efficient and appropriate for 2-DE volume data.

In all, as statistics is one of the most determining step in the comparative proteomics workflow, and paradoxically, one of the less studied to date, we hope that this new package will help improving future 2-DE-based proteomics studies.

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Conflict of Interest: none declared.

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Chapitre II : Caractérisation de la réponse moléculaire à un stress thermique prolongé chez *Pecten maximus*

I/ Introduction

Les écosystèmes marins côtiers sont particulièrement sensibles aux changements globaux actuels. En particulier, l'augmentation des températures impacte fortement les organismes marins ectothermes, entrainant des modifications majeures dans la structure et le fonctionnement des communautés biologiques. Les mécanismes moléculaires impliqués dans l'adaptation des organismes au stress thermique ont été particulièrement bien étudiés, notamment dans les espèces modèles. Néanmoins, l'essentiel de ces études ont concerné des expositions à des contraintes thermiques sévères, mais de courtes durées, ne reflétant pas une situation pertinente d'un point de vue environnemental, notamment si l'on considère les organismes subtidaux.

Dans l'optique de se rapprocher au mieux des conditions environnementales rencontrées par *Pecten maximus* dans son environnement naturel, de jeunes coquille Saint-Jacques (1 an) ont été maintenues au laboratoire à 15°C (température de l'eau en Rade de Brest au moment du prélèvement) avant d'être soumises expérimentalement à une augmentation progressive de la température (1°C/jour), puis maintenues sur une longue durée (56 jours) à 21°C ou 25°C. Durant cette période, 5 prélèvements ont été effectués, lors desquels nous avons extrait les protéines et les ARN messagers à partir du manteau, en vue d'analyses protéomiques et transcriptomiques. Il est à noter que de nombreux autres prélèvements ont été effectués; ils sont toujours en cours d'analyse et devront renseigner sur l'impact de ce stress thermique prolongé sur la physiologie des animaux.

L'analyse transcriptomique a été effectuée par séquençage massif (Illumina HiSEQ 100PE) de 21 banques de cDNA préparées à partir des ARN messagers. L'assemblage de ces séquences a conduit à une base de données de 26064 contigs, d'une taille moyenne d'environ 1kpb. Dans le but d'identifier les transcrits dérégulés de manière stable en réponse au stress thermique prolongé, nous avons fait le choix de ne retenir que ceux présentant un facteur d'induction d'au moins 2 sur les trois derniers temps de prélèvements de l'expérience. Au total, nous avons pu identifier 531 contigs dérégulés, dont seulement 177 ont donné des résultats dans les recherches d'homologies par Blast. L'analyse protéomique a été effectuée sur les 2 derniers prélèvements, pour les températures de 15 et de 25°C. Nous avons observé en tout 24 spots dérégulés, et avons pu identifier 9 d'entre eux par spectrométrie de masse de type MALDI TOF-TOF.

L'analyse de la fonction des gènes-protéines identifiés, et des réseaux d'interactions a été effectuée en utilisant l'algorithme STRING 9.05. Au total, l'analyse a été effectuée sur

seulement 144 gènes, car l'analyse par STRING exclue les gènes (i) de fonction inconnue, et (ii) n'ayant pas d'homologue connu chez les mammifères. Nous avons ainsi pu mettre en évidence une place centrale de l'enzyme GAPDH (10 interactants), faisant le lien entre les voies AP-1 (10 interactants), CALR (7), HSP90 (5), CA2 (5), SYNCRIP (3) et UDG (2). L'analyse des résultats dans leur globalité suggère un rôle essentiel de (i) la voie de signalisation AP-1, (ii) l'inhibition des processus apoptotiques, (iii) la dégradation des réserves lipidiques, (iv) la désaturation des lipides membranaires, (v) une implication limitée des HSPs. En outre, la régulation négative de nombreux systèmes de réparation de l'ADN suggère que le stress thermique prolongé pourrait accroître la fréquence d'apparition de mutations dans les cellules.

II/ Article n°2 :

Coupling RNAseq to proteomic- analyses to decipher molecular adaptation of the great scallop (*Pecten maximus*) to heat stress

Article en préparation.

Coupling RNAseq to proteomic-analyses to decipher molecular adaptation of the great scallop (*Pecten maximus*) to heat stress

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Abstract

The adaptive capacity of marine populations to chronic heat stress is the cornerstone of their ability to survive the ongoing global warming. In this study, as an attempt to decipher the molecular adaptation of the subtidal bivalve *Pecten maximus*, individuals were eventually subjected to a progressive warming, and maintained at three different temperatures (15, 21 and 25°C) during 56 days. Changes in genes and proteins expressions in mantle tissue were monitored by using both a transcriptomic (RNAseq) and a proteomic (2-DE) approaches. Over the 26,064 assembled contigs, our RNAseq experiment allowed to show the thermal deregulation of 531 ones, of which 177 could be annotated. The proteomic approach evidenced 24 proteins, which 9 were identified by mass spectrometry. In all, the main results suggested a deep remodeling of the cell structure, as revealed by the deregulation of many genes implied in the formation of the cytoskeleton and of cell membranes properties (as evidenced by the heat-promoted downregulation of the Δ^9-desaturase gene). They also suggested the diversion of energetic metabolism towards the mobilization of lipid storage. Considering both genes functions and network analysis, we postulated a central role for the AP-1 signaling pathway which, together with many other deregulated genes known to be involved in apoptosis, should lead to an inhibition of apoptosis. Interestingly, several genes involved in DNA repair (eg, UDG) appeared downregulated, suggesting that cells could turn towards a mutator state in response to thermal stress. Finally, we could propose a pivotal role for GAPDH, a quite common glycolytic enzyme which was recently shown to carry crucial functions in most aspects of cells functioning. In all, this work gave very interesting insights into the response of *P. maximus* to long-term exposure to hyperthermal stress. Future studies will determine the actual role of the genes/proteins that were pointed out.

Introduction

Coastal marine environments are of particular ecological-, economical- and societal-importance. The particular physico-chemical structure of these ecosystems makes them highly fluctuating environments, particular sensitive to the ongoing global changes. In particular, many coastal environments were shown to experience recent changes in temperature and oxygen availability, thus triggering strong modifications in biological communities (Harley *et al.*, 2006). In this context, the ability of marine species and populations to cope with environmental changes appears as a cornerstone of marine ecosystems maintenance.

Temperature is a key factor affecting the physiology of ectotherms. The molecular mechanisms underlying adaptation to thermal stress have been well studied in the last decades, especially in model species. Adaptation of organisms to high temperatures has been relied on the expression of a series of evolutionary conserved stress-responsive genes that include genes controlling protein folding and repair (the so-called HSPs), cell cycle, chromatin stabilisation and repair and energy metabolism. Although marine ecosystems are among the most vulnerable to climate change, only few studies to date dealt with the adaptation of marine mollusks to temperature fluctuations. A few studies dealt with short acute thermal stresses. For example, Ivanina *et al.* (2009) showed the overexpression of selected HSPs and MT in *Crassostrea virginica* exposed for 1 h at 40°C, and Brun *et al.* (2008, 2009) characterized the heat shock response and the acquisition of thermotolerance in selected pectinidaeae (*Argopecten irradians*, *Placopecten magellanicus*) at the level of HSPs synthesis.

Only few global analyses were performed to date. Lang *et al.* (2009) used a microarray containing 1,675 ESTs from *Crassostrea gigas* and *C. virginica* to characterize the transcriptomic response of different families of the pacific oyster showing contrasting degrees of thermotolerance. Of note, they showed differential expression of genes encoding HSPs, and genes involved in lipids metabolism, protection against bacterial infections and structure (e.g. collagen) in response to an acute thermal stress (40°C, 1 h). However, if studies concerning short exposure to acute stress can provide interesting insights into stress pathways, they may not reflect the response to a permanent gradual shift such as the predicted increase in sea surface temperature (Tomanek, 2011). In a matter of fact, short- and long-term exposures can produce very different responses in genes expression profiles (Clark & Peck, 2009). In a very recent study, the effect of both acute- (within a day) and long-term- (up to 14 days) exposures to heat stress on the genes expression of *Chlamys farreri* was studied (Fu *et al.*, 2013). To our

knowledge, the only other recent study dealing with long-term adaptation to thermal challenge concerned *C. gigas* (3 months, 24°C; Clark *et al.*, 2013). This study showed the essential role of lipid breakdown and of the mTOR regulatory pathway, and ultimately the inhibition of apoptosis in heat stress adaptation of oyster. Both of these studies used next generation sequencing technologies (NGS).

Studying organisms' adaptation to changing environments is an extraordinary challenge in the field of ecological genomics. In particular, transcriptomic and proteomic characterizations of the responses of organisms to environmental change offer an opportunity to understand the underlying molecular basis for adaptation. However, only few genomic data are currently available for marine organisms, thus strongly limiting genome wide- and proteomic- studies. Nevertheless, next generation sequencing technologies (NGS) have changed the deal, allowing innovative transcriptomics and proteomics studies in the environmental sciences field.

The great scallop *Pecten maximus* is naturally distributed along a large latitudinal gradient (from 31°N to 69°N) and lives in the subtidal zone (depth from 0 to 250m , www.fao.org), making it a good model for studying the adaptation to chronic heat stress. In this paper, we describe the first wide genome transcriptome analysis in the great scallop, *P. maximus*, subjected to a 56-days thermal challenge at 3 different temperatures. The kinetic process is described using several time points coupling transcriptomics and proteomic approaches, thus giving a system biology view of heat adaptation/acclimation in this species.

Methods

Biological material

A total of 1270 *Pecten maximus* (1 year old; average length , standard deviation : 34.0 , 4.1 mm) were used for this experiment. By convention, the age class is the number of first January experienced by the individual. All the individuals used in this experiment came from a cohort of the Tinduff hatchery (Bay of Brest, France) born in spring 2010 and thus called scallops of age class I. The individuals were then put *in situ* in small cages at Ste-Anne du Portzic, Bay of Brest from June 2010 to June 2011. The 1270 individuals for experiment were transferred in the tanks of the French Research Institute for Exploitation of the Sea (IFREMER) Argenton Shellfish Laboratory (North Finistère, France) on the 6th of June 2011.

Experimental conditions

All the scallops were randomly divided into three batches of 440 individuals placed into 250 L tanks. In each tank, the lot of 440 individuals were divided in two and placed in plastic cages without sediment.

Each tank was supplied with 1µm filtered, UV sterilized seawater at a controlled temperature of 15 °C with a flow rate of 80 L.h^{-1} and complete water changes were made once a week. During all the experiment, the photoperiod (12/12) remained constant. Water flowing into the 250 L tanks was supplemented continuously with cultured microalgae (50% *Isochrysis affinis galbana* (Tahitian strain *T.iso*) and 50% *Chaetoceros calcitrans*). The food was distributed with a peristaltic pump regulated once a day by counting the microalgae at the inlet and outlet of the tank with a counter Coulter to have a similar quantity at the outlet of the tank of 1.5 µm^3.ml^{-1} independently of the temperature and of the number of individuals in the tank. This quantity of microalgae was chosen so the scallops are considered fed *ad libitum* and an aquarium pump maintained homogeneity in the tanks.

Initially, the scallops were left at ambient temperature (15.1 ± 0.2 °C) for 16 days to allow animals to acclimatise to the rearing conditions and to identify any damaged or unhealthy individuals prior to experimentation. Then, from the 22th of June, one tank was kept at 15°C, while temperature was increased at a gradual change of 1°C/day to reach 21°C and 25°C in the two other tanks. Temperature was recorded every 15 min in the three tanks using button temperature data logger (EBRO, Germany) during all the experiment. During the experiment the three temperatures were thus maintained at: 14.8 ± 0.6°C as control, 21.4 ± 0.2°C and 25.2 ± 0.9°C (Fig. 1).

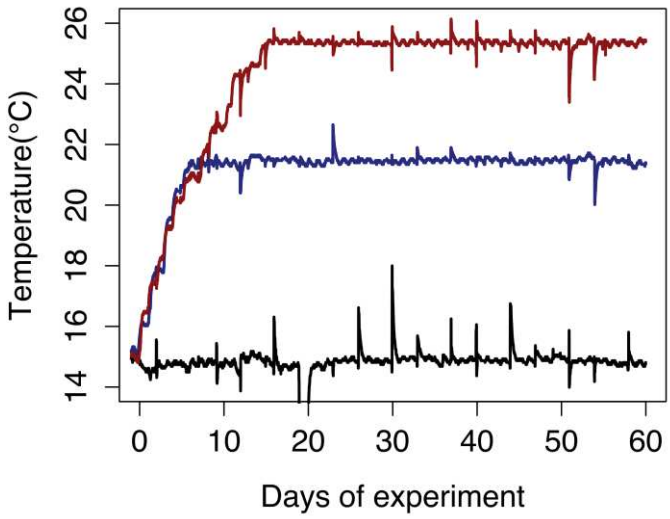

Figure 1: Evolution of the temperature in the three tanks during the experiment.

Molecular analyses

At the beginning of the experiment (T0), 6 individuals were sampled and then 6 individuals were sampled in each temperature treatment after 3 days (T1), 14 days (T2), 21 days (T3), 27 days (T4) and 56 days (T5). The scallops were quickly dissected and mantle tissue was flash frozen in liquid nitrogen and stored at -80°C until further analysis. All molecular analyses were carried out using the mantle tissue. This organ has multiple functions including shell formation, secretion of the ligament and sensorial activities. A number of transcriptome studies have been published for this organ, enhancing the annotation potential of transcripts (Clark *et al*., 2010; Jackson *et al*., 2010; Kinoshita *et al*., 2011). Additionally, gene chip studies have shown that bivalve tissues directly in contact with the external environment are more responsive to environmental perturbation than internal tissues (Clark *et al*., 2013). Hence, transcription profiles of the mantle can act as an effective proxy of whole-animal response. The scope of the molecular analyses was to study the transcriptional and proteins profiling patterns in mantle tissue.

RNA extraction and reverse transcription

Total RNA was extracted from mantle tissue of 4 individuals per conditions at each time point using TRI Reagent® Solution (Life Technologies) according to manufacturer's instructions. RNA from the 4 individuals was then pooled for each time point separately. RNA quality and concentration were determined by Agilent 2100 RNA Nanochip (Agilent, Santa Clara, CA, USA) and by NanoDrop ND-1000 Spectrophotometer (260/280 nm, NanoDrop Technologies, Wilmington, DE, USA), respectively. Samples were then treated with a DNA-free Kit (Ambion, Austin, TX) to remove genomic DNA, and reverse transcription was carried out using 500 ng of total RNA, random hexamers, and MMLV reverse transcriptase (Promega) according to manufacturer's protocols.

Library construction and sequencing (incl assembling)

The production of Illumina libraries for mRNA-seq and the transcriptome sequencing using the Illumina HiSeq^{TM}2500 (HiSeq 100 pair-ends) was conducted by the Genome Analysis Centre (Norwich, UK).

RNA-Seq data sets

The RNA libraries yielded 806 million paired end reads. Raw reads were filtered and trimmed using the FASTX-toolkit (Version 0.0.13 from Assaf Gordon Hannon lab) and rRNA contamination removed using ribopicker (Schmieder *et al*., 2012) and cutadapt (Version 1.1; Martin, 2011), with a final quality check performed using fastQC (Version 0.10.0 ; http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc/).

The contigs were assembled using Newbler (Version 2.6, Margulies et al., 2005) and a paired end assembly strategy. Redundancy was determined by self-Blasting and the use of CD-HIT (95% similarity, Li & Godzik, 2006). De novo assembly led to a total number of 26 064 contigs. The transcripts were then processed through the Blast2GO pipeline to produce putative annotations and functional classifications based on Blastx results against the GenBank NR database release 190.

Differential genes expression analyses

Normalization was carried out by dividing counts by library size. The metrics of TPM (Transcripts per million) was used. Ratios were calculated for each time point with 25°C

versus 15°C treatment and 21°C versus 15°C. Only the transcripts with a ratio higher than 2 successively at T3, T4 and T5 were kept for further analysis.

Proteins extraction and separation

Mantle tissues from 6 individuals of 25°C and 15°C treatment at the two last time points (T4 and T5) were crushed with a mixer mill (MM400, RETSCH, Haan, Germany). One hundred milligrams of the obtained powder was homogenized in 100 mM Tris-HCl (pH 6.8) with 1% of Protease inhibitor mix (GE Healthcare), centrifuged (4°C, 50 000 g, 5 min) and supernatants were pipetted in other tubes. Nucleic acids were then removed (nuclease mix, GE Healthcare, following manufacturer's instructions). Samples were precipitated at 4°C using TCA 20% (1/1:v/v, overnight). After centrifugation (4°C, 20 000 g, 30 min), pellets were washed with acetone 70% and re-suspended in urea/thiourea buffer (2 M thiourea, 7 M urea, 4% CHAPS, 1% DTT) containing 1% IPG (pH 4–7, GE Healthcare). Protein concentrations were determined using a modified Bradford assay (Ramagli, 1998), and all samples were adjusted to 400 µg of proteins in 250 µl.

Prior to isoelectric focusing, IPG strips (pH 4–7, 13 cm, GE Healthcare) were passively rehydrated with 250 µl of protein solution in wells for 14 h. Isoelectric focusing was conducted using the following protocol: 250 V for 15 min, 500 V for 2 h, gradient voltage increase to 1000 V for 1 h, gradient voltage increase to 8000 V for 2 h 30, 8000 V for 3 h, and reduced to 500 V (Ettan IPGphor3, GE Healthcare). To prepare for second dimension SDS-PAGE electrophoresis, strips were incubated in equilibration buffer (50 mM Tris-HCl pH 8.8, 6 M urea, 30% glycerol, 2% SDS and 0.002% Bromophenol Blue) for two 15 min intervals, first with 1 $g.l^{-1}$ dithiothreitol and then with 48 $g.l^{-1}$ iodoacetamide. IPG strips were then placed on top of 12% polyacrylamide gels, which were run in 10°C thermo-regulated device (SE 600 Ruby, Amersham Biosciences) at 10 mA per gel for 1 h and then 30 mA per gel until complete migration. Gels were subsequently stained with Coomassie Blue (PhastGel, GE Healthcare) and unspecific coloration was destained with a solution containing 30% methanol and 7% acetic acid. The resulting gels were scanned with a transparency scanner (Epson Perfection V700) in gray scale with 16-bit depth and a resolution of 400 dpi.

Differential proteomics analysis

Images were aligned and spots were detected and quantified using the Progenesis SameSpots software (version 3.3, Nonlinear Dynamics) applying the automated algorithm. All detected spots were manually carefully checked and artifact spots were removed. Data

were exported as volume raw values and statistical analyzes were conducted in R (R Core Team, 2013) using the packages prot2D (Artigaud *et al*., 2013) and Limma (Smyth, 2004). Data were normalized (quantile normalization) and the samples from the 25°C treatment were paired-compared using moderated *t*-test to the samples from 15°C treatment (control) for each sampling date (with 6 replicates per group). A global correction by false discovery rate (fdr ; Benjamini & Hochberg, 1995) was used, in order to take into account multiple comparisons issues and paired-comparison correction. Spots with an fdr threshold lower than 0.1 and an absolute fold change superior to 2 were considered as differentially expressed.

Mass spectrometry and protein identification

Proteins which abundance significantly changed between 25°C and 15°C conditions were excised from gels and prepared for analysis by mass spectrometry (MS). Gel pieces were first washed in 50 mM ammonium bicarbonate (BICAM), and then dehydrated in 100% acetonitrile (ACN). Gel pieces were vacuum-dried, and rehydrated with BICAM containing 0.5 µg sequencing grade porcine trypsin (Promega), and incubated overnight at 37°C. Peptides were extracted from the gels by alternative washing with 50 mM BICAM and ACN, and with 5% formic acid and ACN. Between each step, the supernatants were pooled, and finally concentrated by evaporation using a centrifugal evaporator (SpeedVac). Samples were then resuspended in trifluoroacetic acid (TFA; 0.1% in water). Peptide solutions were mixed with the α-cyano-4-hydroxycinnamic acid (HCCA, 10 $mg.ml^{-1}$ of a ACN/TFA/water (60/4/36:v/v/v) solution), and spotted on a polished steel target using the dried droplet method. Peptides were then analyzed by Matrix-Assisted Laser Desorption Ionization Time-Of-Flight tandem mass spectrometry (MALDI TOF-TOF) in positive ion reflector mode, using an Autoflex III (Bruker Daltonics) mass spectrometer. The FlexControl software (v3.0, Bruker Daltonics) was set up to acquire successively PMF spectra and MS/MS from the dominant peaks. Mass spectra were analyzed with FlexAnalysis (v 3.0; Bruker Daltonics) by applying the following conditions: TopHat algorithm for baseline subtraction, Savitzky-Golay analysis for smoothing (0.2 m/z; number of cycles: 1) and SNAP algorithm for peaks detection (signal-to-noise ratio: 6 for MS and 1.5 for MS/MS). The charge state of the peptides was assumed to be +1. Fragments of porcine trypsin were used for internal mass calibration.

Proteins were subsequently identified with PEAKS (v 5.2, Bioinformatics Solutions) using MS/MS-based identification and de novo sequencing. The search parameters against a custom-made database were set as follows: carbamidomethylation of cysteine was set as a

fixed modification, oxidation of methionine and phosphorylation of serine, threonine and tyrosine were set as variable modifications, one missing cleavage during trypsin digestion was allowed and the tolerance for precursor-ion mass tolerance was set to 1 Da. The database was constructed by combining *P*. *maximus* sequences from two sources: sequences acquired in this study and sequences from the REPROSEED project. Overall, the database included a total of 252 888 *P*. *maximus* expressed sequence tags (ESTs). Protein identification was considered as unambiguous when a minimum of two peptides matched with a minimum score of 20. False discovery rates were also estimated using a reverse database as decoy. EST database sequences were annotated by homology searches against a non-redundant database using the Blast algorithm from NCBI (http://www.ncbi.nlm.nih.gov/BLAST).

Network analysis

As an attempt to better understand the relationships between the proteins and genes evidenced in this study, we performed network analyses using the String 9.05 algorithm available at http://string-db.org. STRING is a database of known and predicted protein interactions, which includes both direct (physical) and indirect (functional) associations. This kind of network analysis is strongly dependent on the quality of the used database, and is thus much more efficient using databases from the most studied model organisms. As a consequence, we searched for the human homologs of the genes and proteins deregulated in our study, and asked STRING for interaction between them.

Results

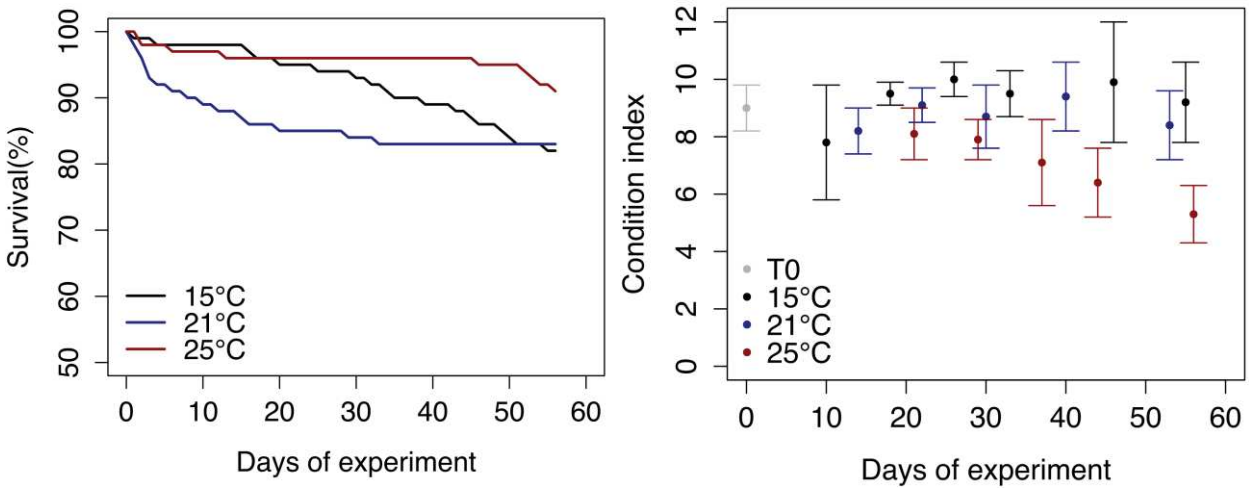

Figure 2: Survival (left) and evolution of condition index (right) of *Pecten maximus* during a long-term exposure (56 days) to three different temperatures (15°C, 21°C and 25°C).

Physiological response to heat stress

All the scallops used in this study came from the same 2010 production of the Tinduff hatchery, and were held under identical conditions prior the start of the experiment, with a year's cultivation in the bay of Brest, followed by a 16-days acclimation period to the laboratory environment during which they were maintained at 15°C (the seawater temperature in the bay of Brest at that time). Thus, they were similar in size, age and physiological conditions. At the beginning of the experiment, animals were splitted in three tanks and either maintained to 15°C, or subjected to a gradual temperature increase of 1°C/day, and then maintained at 21°C or 25°C for the rest of experiment. The whole experiment lasted 56 days. Amongst the environmental parameters monitored, we did not observe any significant modification for salinity and pH (that were maintained to 35.8‰ ± 0.13 and 8.1 ± 0.11, respectively, during the experiment), while slight modifications of O_2 (that dropped from 9.23 ± 0.63 mg/L to 7.9 ± 1.08 and 7.43 ± 1.28 mg/L at 21 and 25°C, respectively) and ammonia (that increased from 4.68 µM ± 1.6 to 5.96 µM ± 1.9 and 6.5 µM ± 2.3 at 21 and 25°C, respectively), were observed.

Mortalities occurred in each tank during the experiment (Fig. 2). However, this mortality was not correlated to the increased temperature (as we observed more mortality in the tanks maintained at 15°C and 21°C than in the 25°C treatment; Fig. 2). However, more

than 80% of the animals survived in all conditions (Fig. 2). By contrast, the condition index (CI), that reflects the physiological condition of the animals, appeared significantly reduced at the end of the experiment for the animals kept at 25°C (Fig. 2). For this study, focused on the molecular response to heat stress, we chose to use the mantle tissue, that carries multiple essential functions in bivalve mollusks (eg., shell formation, sensorial activities).

Illumina sequencing data and quality statistics

Total RNAs were extracted at 6 different time points all along the experiments, reverse transcribed, and the resulting cDNAs libraries were massively sequenced using NGS (Illumina HiSEQ 100 paired ends). In all, sequencing of the 22 created libraries resulted in a total of 806 millions pairs of raw reads.

After trimming and quality filtering, 17.2% of reads were discarded, leaving 667.5 millions reads for downstream analyses. The mean final number of reads for each time point was 30.3 millions. De novo assembly led to a total number of contigs of 26,064, that had an average size of 1,022 bp.

Differential genes expression analyses

This study aimed at better understanding the molecular mechanisms underlying adaptation to long-term exposure to heat stress in *Pecten maximus*. Therefore, we chose to adopt a very stringent strategy to identify the durably deregulated transcripts, by taking into account only those displaying a ratio (treatment/ control) of at least 2 in all the last 3 time points. In all, over the 26,064 transcripts analyzed, only weak changes were observed in pooled individuals subjected to 21°C with only 46 assembled contigs appeared up-regulated, and 49 down-regulated. By contrast, we could identify an amount of 164 down-regulated and 272 up-regulated transcripts at 25°C, which represented 1.67% per cent of the whole coverage.

Of the 531 deregulated contigs, only 177 (33%) showed a blast match (using Blast2Go) vs the NCBI non-redundant database, using an E-value cut off of 1E-15. Regarding the whole 26k contigs, 6 138 could be annotated in the first annotation (23.6%). It is noteworthy that this poor annotation level was similar to those obtained for other non-model organisms (e.g., Clark *et al*., 2010; Kinoshita *et al*., 2011). A summary of differentially expressed contigs with significant Blast hits is available as supplemental data.

Differential proteins expression / accumulation

Table 1: Values of Log_2 Fold Change for spots differentially expressed between animals maintained at 15°C and animals maintained at 25°C, after 27 days (T4) and 56 days (T5)

	Log_2 FC (25°C/15°C)	
Spot	T4	T7
HiT-1	2.22	0.97
HiT-2	2.07	2.85
HiT-3	1.10	0.33
HiT-4	0.95	1.20
HiT-5	0.62	1.42
HiT-6	0.43	1.22
HiT-7	0.36	1.80
HiT-8	0.27	1.50
HiT-9	0.11	1.16
HiT-10	-0.50	1.11
HiT-11	-0.41	1.23
HiT-12	-1.14	1.42
LoT-1	-1.91	1.04
LoT-2	-2.31	0.98
LoT-3	-1.62	0.87
LoT-4	-2.14	0.80
LoT-5	-1.43	0.74
LoT-6	-1.77	0.38
LoT-7	-1.75	0.14
LoT-8	-1.50	-0.23
LoT-9	-1.37	-0.61
LoT-10	-0.14	-1.00
LoT-11	-0.30	-1.01
LoT-12	-0.33	-1.77

Mantle proteins from 6 individuals per condition (ie 15°C and 25°C, and 2 time points, 27 and 56 days) were extracted and analyzed by 2-DE (Fig. 3). In all, we could observe a mean proteome of 956 spots observable on gels. Normalization volumes of protein spots and statistical analysis were performed using our newly developed Prot2D R-package. In all, this allowed to discover 24 protein spots significantly differentially accumulated at 25°C at one or the other time point, of which 12 revealed overproduced and 12 were downregulated. Of note, only few protein spots clearly displayed the same accumulation profile at both time points (see Table 1).

All of these 24 protein spots were subjected to trypsin digestion and MALDI-TOF-TOF mass spectrometry analysis of the released peptides. This led to the identification of 9 proteins (Table 2), which corresponds to a quite low rate of identification (37.5%), but comparable to those classically obtained for non-model organisms (Forné *et al*., 2010; Dowd, 2012). It should be recalled that the main limitation to proteins identification by mass spectrometry is the availability of proper databases. In this context, no convincing identification results were obtained using classical non-redundant protein databases from NCBI and SwissProt, which contained only few proteins from scallop. In fact, 5 out of the 9 proteins identified here came from the ESTs database constructed de novo from the RNAseq experiments described in this study. The 4 other identifications were allowed by an additional scallop EST database constructed in the context of the Reproseed FP7 European program.

Network Analysis

In all, network analysis using STRING was performed on 144 genes (including the redundant ones) over the 177 for which we obtained significant Blast hits. Network analysis highlighted relationships between 35 of them (Fig. 4). The main result showed a central role of GAPDH (10 interactants), which filled the gap between the c-JUN pathway (which displayed 8 interactants), CALR (7), HSP90 (5) CA2 (5), SYNCRIP (3) and UNG (2). Other small independent networks were also found, e.g. one centered on CCAR1 and comprising 4 genes, and two others including one grouping two enzymes implied in the biosynthesis of nicotinamide (AFMID and CCBL2).

Table 2: List of *Pecten maximus* mantle tissue proteins identified by MS/MS whose abundance change between animals maintained at 15°C and animals maintained at 25°C (moderate t-test paired-comparison, fdr <0.1, absolute fold change >2).

Spot	Blast Hit Name	Acc. Nr	EST Sequence Name	Sequenced peptides	Peaks score
HiT-1	calumenin-B-like (CALUB)	XP_003704568	Contig22840	TEFMYFVHPEEGK DVTVCEYTDR	83.6
HiT-2	calumenin-like isoform X3 (CALU)	XP_005110308	Contig_Reproseed325	DVLVLEYTDR DNAEEFEHATEQESK CSWPNYVR	98.0
HiT-9	No hit	-	Contig_Reproseed34795	TFVFPPADTQKPVITGIM(+15.99)R GESYIHVK TFVFPPADTQKPVITGIMR TFQM(+15.99)FDALQYIEQGNM(+15.99)IQGR	88.0
LoT-2	Putative phosphoglycerate mutase (PGAM)	EKC26210	Contig_Reproseed34491	VLISAHGNSLR SYDVPPPAREDGDER YAHKDASVVPR	94.3
LoT-3	glyceraldehyde 3-phosphate dehydrogenase	XP_002434347	scallop_rep_c46254	SSXFDANAGHAYNNNFVK VPVPDVSVVDHTC(+57.02)R VVSQDFC(+57.02)GDSR	98.3
LoT-6	gelsolin-like protein 2-like isoform X1 (GSN)	XP_005100380	Contig28884	AWDGAGQEPGTQFWR QSWQVGNR NSGNSGDVYXPDGGR	85.7
LoT-7	Kynurenine--oxoglutarate transaminase 3 (CCBL2)	EKC20610	Contig34767	NLGENFLR AVNSDNANWAQYAR GQVPDDGSDDPYDYK IASLPGM(+15.99)WDR	97.3
LoT-9	peptidyl-prolyl cis-trans isomerase B precursor (PPIB)	NP_990792	Contig2622332	DFMVQGGDFSEGDGTGSK VSEGMDVVR KLENTEVDIENR YFADENFK	98.4

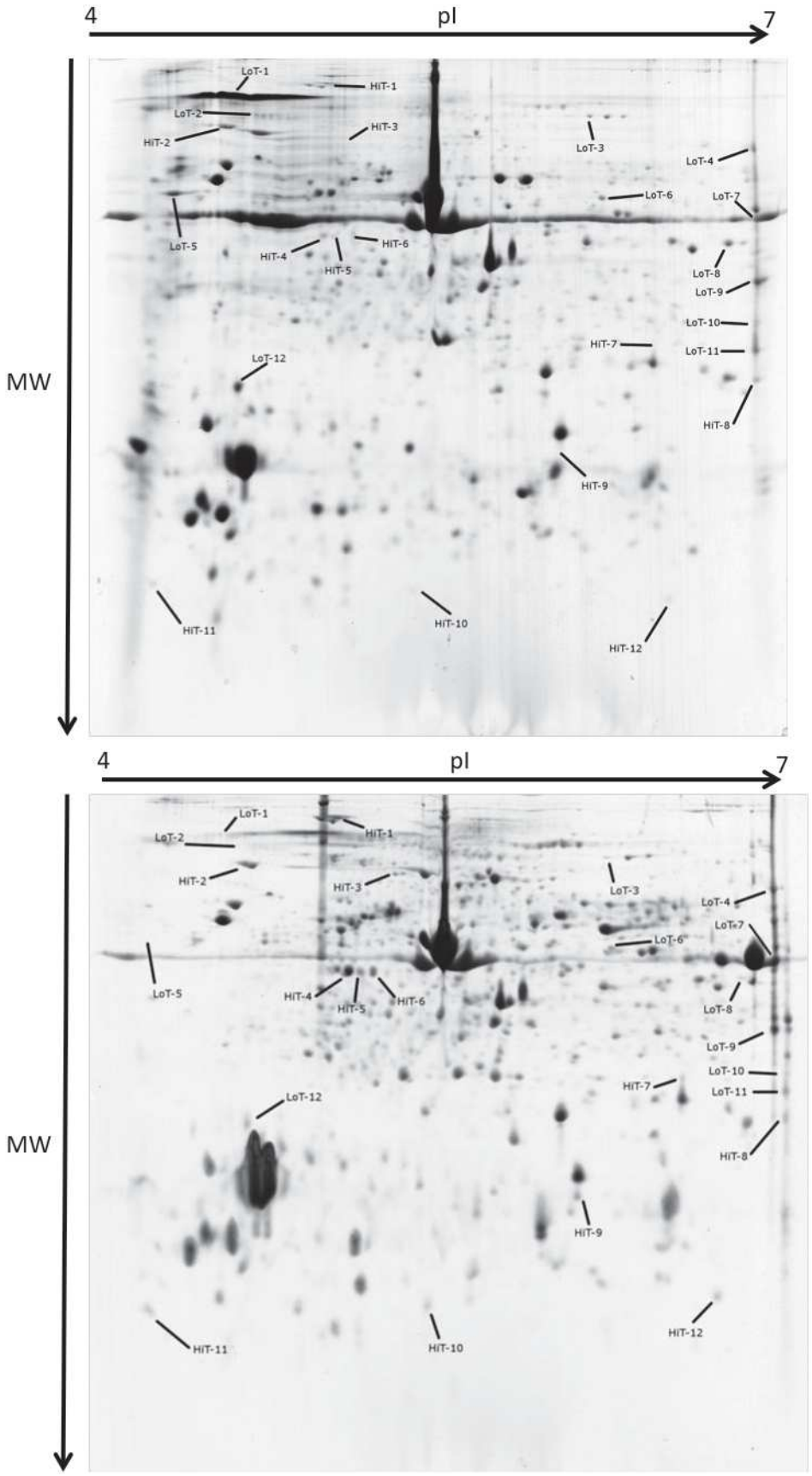

Figure 3: Representative bi-dimensional gels (pH 4-7, SDS-PAGE 12%) for *Pecten maximus mantle* proteins maintained at 15°C (upper gel) and 25°C (lower gel). Differentially expressed spots are arrowed.

Discussion

In the context of global warming, temperature is one of the most important environmental threat affecting marine Mollusks, and characterizing the molecular bases for thermal acclimation/adaptation is an essential challenge to understand their ecology. Numerous studies aimed at characterizing the effects of short term exposure to heat stress, emphasizing the role of some proteins such as HSPs, but only few numbers addressed the effects of long term (chronic) exposure which is quite more relevant in the context of global warming. In this paper, we used transcriptomic and proteomic tools to characterize the adaptation/acclimation of *Pecten maximus* subjected to a 56-days exposure to thermal stress.

Proteins exert almost all functions in cells. Proteins expression depends both on genes expression and a variety of post-transcriptional modifications including mRNA processing, translational efficiency and the so-called post-translational modifications such as phosphorylation. Proteomics is then closer to phenotypes than transcriptomics. However, 2D-based proteomics is naturally biased towards highly abundant proteins, and is often limited by the availability of genomic data, especially for non-model organisms. As a consequence, in spite of its physiological relevance, proteomic strategies classically give a lower amount of results than transcriptomics. Indeed, the modern transcriptomic tools can give a quite extensive view of genes expression, even for non-model organisms when using those based of NGS (ie, RNAseq), and in a same time allow for the generation of high amounts of genomic data. Therefore, transcriptomic and proteomic approaches are very complementary. In our study, we found that 1.67% out of the 26k assembled contigs analyzed appeared deregulated during thermal stress. This represented 531 deregulated contigs, which 177 could be assigned to a known gene through Blast comparisons. In the proteomic study, 24 proteins appeared differentially accumulated under thermal stress. This represented 2.5% of the protein spots identified in 2-DE gels.

Analyzing the presumed functions of these genes may give new insights on the mechanisms by which *P. maximus* does adapt to prolonged temperature stress. Regarding functional categories, we observed that these genes/proteins belong to different classes, and should be implied in functions highly diverse as the stress response, metabolism, alternative expression of genes including signaling, cytoskeleton, genome maintenance, etc. .. Below we discuss the main information gathered from our data.

Only few Heat Stress Proteins are deregulated upon long-term exposure to heat stress

Considering what is known in classical models, and classical heat stress adaptation, the first category of proteins that one could expect to see deregulated upon heat stress are the so-called Heat Shock Proteins (HSPs). These proteins are classically regulated at the transcriptional level by the heat shock factor (HSF). Accordingly, we found positively deregulated the genes encoding two major HSPs, i.e. HSP70, HSP90 and the small HSP P40. Several related genes, including an activator of HSP90, different folding proteins (i.e. chaperones, e.g. PPIB, PDIA, CALR...) and proteins acting in proteins recycling (linked to ubiquitin dependent protein recycling) were also found. However, as compared to the huge armada of HSP genes in mollusks (for example, 88 genes encoding HSP70 were identified in the oyster genome, Zhang *et al*., 2012), or to the genes known to be HSF dependent in other species (Yamamoto *et al*., 2005), only poor coverage of the whole classical Heat Stress Response (HSR) appeared induced in our experimental conditions. Nevertheless, HSF activation is fast, but transient, as it occurs only when cells are experiencing a proteotoxic stress (Voellmy, 2004). As a consequence, we ought that our experimental conditions (especially considering the temporal factor), and the design of our bioinformatic analysis of data, which aimed at identifying only the stable modifications of genes expression, were presumably not adapted to visualize these modifications.

Heat stress adaptation does not only imply HSPs. Indeed, heat stress is likely to affect all of the cellular constituents, and the membranes are known to be highly sensitive components within cells. Temperature highly impacts the fluidity of membranes, thus highly impacting its functionalities. As a consequence, maintaining stability and physical properties of membranes are crucial for cell survival. It is well known that one of the main difference in the composition of cell membranes of organisms living at contrasted temperatures is the relative amounts of unsaturated fatty acids (UFAs) in their lipids, i.e. organisms living in cold environments produce more UFAs than those living in temperate- or warm- ones. Similarly, studies on ectotherms living in thermally fluctuating environments showed that they can dynamically modulate their degree of unsaturation of fatty acids (FA), thus regulating the fluidity of their membranes to ensure an optimal functionality, a phenomenon commonly designated as “homeoviscous acclimation” (Sinensky, 1974; Los & Murata, 1998; Guschina & Harwood, 2006). In animals, the main enzyme implied in FA desaturation is the Δ^9 desaturase. In our study, we showed that the gene encoding this enzyme (i.e., SCD) appears strongly down-regulated in the mantle cells of scallops subjected to 25°C. This most probably

aims at decreasing the level of unsaturation in membrane FAs, thus reducing the fluidity of membranes.

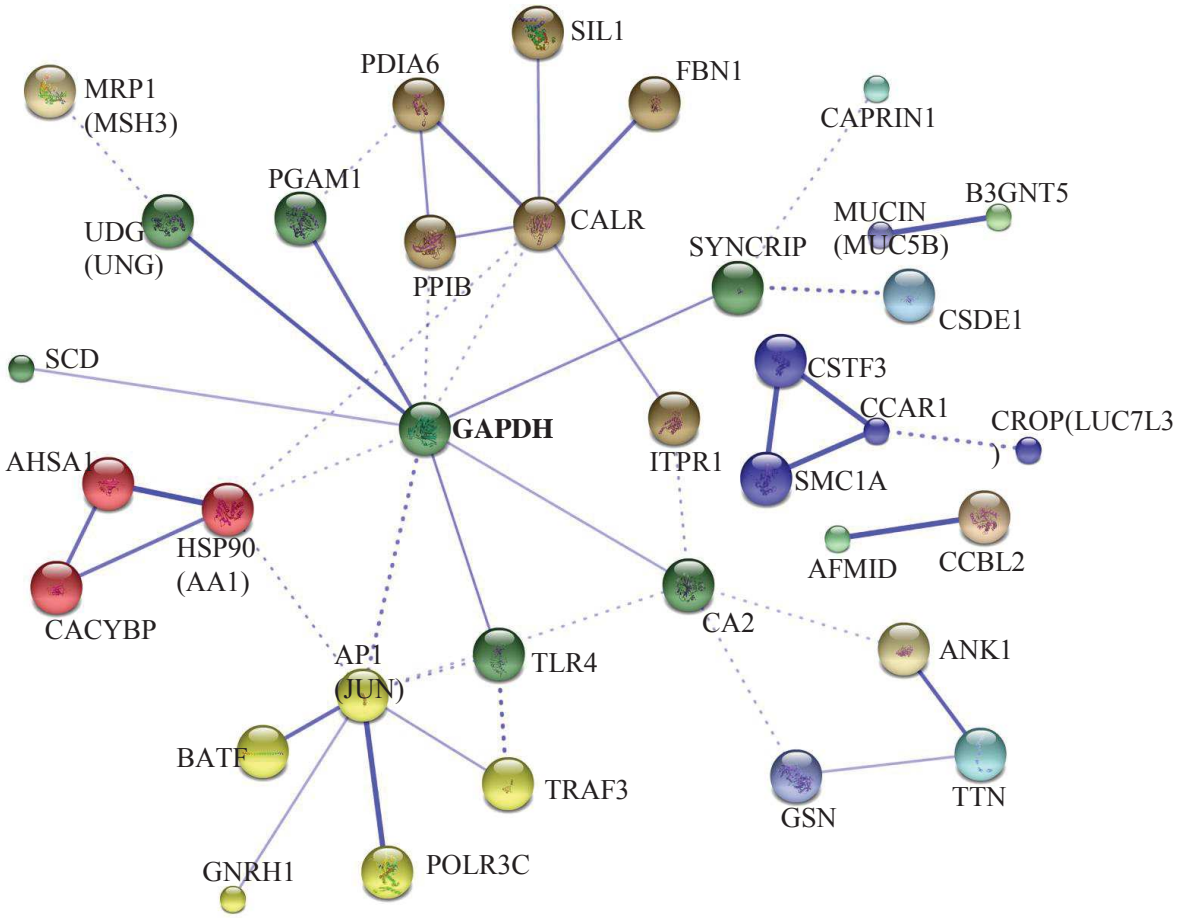

Figure 4: Network showing interactions of genes/proteins (n=134) found in this study. Network is constructed from the human homologs, using the String 9.05 algorithm.

Heat stress would impact the metabolism through upregulation of lipids breakdown.

Many processes involved in the cellular stress response require ATP (Kültz, 2005), so energy metabolism is crucial to mounting a robust stress response. Accordingly, numerous global studies revealed up-regulation of metabolism genes in hyperthermally stressed organisms such as the yeast *Saccharomyces cerevisiae* (Gasch *et al*., 2000), the goby *Gillichthys mirabilis* (Buckley *et al*., 2006), the Antarctic fish *Trematomus bernacchii*

(Buckley & Somero, 2009), the mussels *Mytilus californianus* (Gracey *et al*., 2008) and *M. trossulus* (Lockwood *et al*., 2010). In theses studies, most of genes implied in energy metabolism shown deregulated were related to glycolysis and the subsequent respiratory metabolism. In our transcriptomic study, it should be noted that no gene encoding proteins involved in these pathways were found deregulated. Rather, two glycolytic enzymes, i.e. GAPDH and PGM, appeared reduced in the proteomic counterpart of our study. Independently on the possible extra-glycolytic roles of such enzymes (discussed thereafter), it should be noted that Tomanek & Zuzow (2010) performed a proteomic analysis on the same animals as Lockwood *et al*. (2010), showing opposite results in proteomics compared to the transcriptomics approach. In addition, the fundamental role of nicotinamide intermediates (NAD+, NADH2) on metabolism should be recalled. Negative regulation of enzymes implied in the synthesis of these molecules (i.e. enzymes of the kynurenine metabolism KFase and CCBL2) was found in both our transcriptomics and in our proteomics approaches.

Interestingly, RNAseq experiment revealed the deregulation of several genes associated with lipids metabolism, i.e. APMAP, PLA2G16 (2 contigs), AMACR, and ACOT4. All these genes appeared up-regulated, most probably leading to an increase in lipid mobilization and degradation. This result thus suggest, that chronic heat stress induces the use of fats rather than sugars as energy source, in order to maintain energy homeostasis. It should be recalled that lipids are the long-term energy storage molecules. A similar observation was addressed in one of the rare study dealing with the adaptation to prolonged exposure to heat stress in a mollusk bivalves, the Pacific oyster (Clark *et al*., 2013).

GAPDH could be a pivotal protein in adaptation to prolonged heat exposure, through its extra-glycolytic roles

Our network analysis (Fig. 4) gave a central place of GAPDH, making the link between several other pathways. As previously evoked, GAPDH is a key enzyme in the glycolytic pathway, catalysing the conversion of glyceraldehyde-3-phosphate (G3P) to 1,3-biphosphoglycerate in the presence of NAD+ and inorganic phosphate. Until recently, this enzyme was only considered as a house-keeping gene. However, new extraglycolytic roles for GAPDH in a diverse range of cellular processes were recently discovered. Like other glycolytic enzymes such as enolase (Boël *et al*., 2004), GAPDH has frequently been found in the extracellular environment, in which its exact role still remain unclear (Sánchez *et al*., 2010). This protein was also found to bind nucleic acids, especially certain mRNAs and

telomeric DNA (Nicholls *et al*., 2012). Other evidence showed that this protein may function as a transcription factor interacting with RNA pol II (Mitsuzawa *et al*., 2005), and acts as a signaling protein, by regulating the activity of the mTOR signaling pathway through sequestring the GTPase Rheb (Lee *et al*., 2009). Several links between GAPDH and apoptosis were also established. These links, that were recently reviewed (Sirover, 2005; Nicholls *et al*., 2012), would occur through its interaction with the ubiquitin ligase Siah1, but also through its role in p53 and caspase-3 dependent apoptosis. Interestingly, the recent study concerning oyster adaptation to long-term thermal challenge showed, in addition to the essential role of lipid breakdown, a major role of the mTOR signaling pathway and apoptosis (Clark *et al*., 2013).

GAPDH was found only in our proteomic analysis, and did not appear to undergo clear (significant) transcriptional regulation in our experimental conditions. It should be noted that GAPDH acts in cells as a homotetramer, and is known to be mainly regulated through post-translational modifications (e.g. phosphorylation; Tisdale, 2002), that play a prominent role in both its glycolytic- and in its extra-glycolytic- activities. In all, our network analysis suggests a pivotal role for this enzyme in the response to long-term thermal stress; its relationships to the different signaling pathways (e.g., AP-1, CALR) should be further defined in future experiments.

A major role for signaling through the AP-1 pathway ?

Numerous genes encoding proteins exerting their roles within the nucleus, or involved in protein trafficking in the nucleus (e.g. NTF2), were found deregulated in our study. As revealed by the network analysis, and given its importance in other species adaptation to environmental fluctuation, the most relevant seems to be the deregulation of the AP-1 system.

The AP-1 family represents a paradigm for signal-responsive transcription factors. This family consists of several groups of bZIP-domain (bZIP=basic region leucine zipper) proteins: JUN, FOS, and ATF-2 subfamilies (Hess *et al*., 2004). In our study, one contig was annotated as AP-1 (JUN) and appeared highly up-regulated at 25°C, as well as two other related CREB/ATF bZIP transcription factors. Moreover, one contig identified as BATF and an hypothetical protein containing a bZIP domain, which belongs to same class of transcriptional regulators, were both found up-regulated at 25°C. Like all bZIP transcription factors, AP-1 proteins must dimerize before binding to theirs DNA target sites. Dozens of different homo- and heterodimeric combinations of AP-1 proteins can be formed, with

different transcriptional regulatory properties (Hess *et al*., 2004). Furthermore, the different forms of AP-1 can be regulated at different levels.

Network analysis using STRING 9.05 placed AP-1 (JUN) at the center of a network including 10 genes of our study, known to be regulated by this transcriptional factor or interacting with it, logically including BATF, but also TLR4, GNRH, POLR3C, CA2, HSP90, TRAF3 (which was found as three different contigs) and GAPDH. It should be recalled that this analysis was made relatively to what is known in human, and that three other deregulated uncharacterized bZIP regulators may also participate to this regulation. Moreover, JUN was found to regulate other genes in other species such as gelsolin, collagen, phospholipases, DNA polymerases, it is not excluded that other contigs found deregulated in our study are in fact members of the JUN regulon.

Interestingly, we also found several components of a multi subunit autoregulatory ribonucleoprotein complex, potentially involved in translationally coupled mRNA turnover (Grosset *et al*., 2000). This complex may associate with the mCRD domain of c-FOS (a component of AP-1), and includes, PAIP1, HNRPD, CSDE1 and SYNCRIP (for which 2 contigs were found deregulated in our study). The two genes SYNCRIP and CSDE1 were found overexpressed at 25°C in our study. Interestingly, SYNCRIP was also given to interact with CAPRIN1 and with GAPDH in our network analysis.

The interaction of JUN with TRAF3 is of particular interest. Tumor necrosis factor receptor (TNFR)-associated factor 3 (TRAF3) has been one of the first identified TRAFs, initially isolated by virtue of its binding to the cytoplasmic domain of TNFR-superfamily (TNFR-SF) member CD40 (Hu *et al*., 1994). TRAF3 is a pivotal protein in signalisation, that can interact with several receptors (TLR, RLR, CD40, ...), thus participating to sensing and transduction of many signals. Interestingly, its action may require ubiquitination processes (Hildebrand *et al*., 2011), and may lead to the activation of the JNK pathway and of the negative regulation of NFkB2 (thus leading to its designation as a "tumor suppressor"). It should be recalled that AP-1 is a major target of the JNK MAP kinase pathway, and is known to regulate a number of major physiological processes including cell proliferation, stress, apoptosis, etc. (Leppä & Bohmann, 1999). More particularly, JUN is known as anti-apoptotic (Wisdom *et al*., 1999). Overall, this suggest that long term-exposure to heat stress should inhibit apoptosis, in an AP-1 dependent manner.

Long term-exposure to heat stress should inhibit apoptosis through several mechanisms

As evoked above, the activation of the AP-1 pathway should lead to the inhibition of apoptosis. The deregulations observed for several other genes in our study argue in the same sense. The development of the heat shock response helps cells limiting the damage caused by stress, and thus facilitates cellular recovery. However, it is acknowledged that the inevitable presence of cellular damage under hyperthermia triggers apoptotic cell death, that may commonly be identified by typical ultrastructural changes and inter-nucleosomal DNA fragmentation in heat stressed cells. In this context, a number of studies were dedicated to understanding the interplays between the heat stress response and apoptosis. Interestingly, it was established that HSPs may regulate the expression of pro-inflammatory cytokine genes and downstream targets, resulting in apoptotic cell death. However, HSPs have a wider role in relation to apoptosis and various facets of the inflammatory process (Roberts *et al*., 2010). Experimental induction of HSPs has been shown, in a variety of mammalian tissues, to inhibit apoptosis and considerably enhance cell survival (Landry *et al*., 1989; Musch *et al*., 1999). As a consequence, in addition with the induction of the AP-1 pathway, the deregulation of HSPs shown in our study may negatively interfere with apoptosis.

Not unsurprisingly, a number of genes deregulated encoded apoptosis related proteins. In particular, 4 genes clearly annotated as inhibitors of apoptosis (3 were annotated as BIRC7, and 1 as PIAP), as well as several genes related to the ubiquitin related processes. In particular, the BIRC7 proteins repress apoptosis through the inhibition of CASP3, CASP7 and CASP9, and through their E3 ubiquitin-protein ligase activity. In addition, the major regulator of apoptosis CCAR1 was also found up-regulated during heat stress. Network analysis showed interactions with CROP, which encodes a cisplatin resistance associated overexpressed proteins (it may be recalled that cisplatin induces cell death through induction of apoptosis, Henkels & Turchi, 1999), CSTF3 (Cleavage stimulation factor 77 kDa), and SMC1A (Structural maintenance of chromosomes protein 1A). In all, our results strongly suggest that the response developed by *P. maximus* mantle tissue cells to long-term heat stressed negatively interferes with the development of apoptosis.

Does long-term exposure to heat stress increase genetic variability in mantle cells of scallop?

The discovery of stress-induced mutagenesis, some years ago, has challenged the claim of independence between the evolutionary forces of mutation and selection. In bacteria, exposure to a variety of stresses induces a global stress response, which includes functions

that can increase genetic variability (Bjedov *et al*., 2003; Foster, 2007). In particular, up-regulation and activation of error-prone DNA polymerases, down-regulation of error-correcting enzymes, and movement of mobile genetic elements are common features of several stress responses (Foster, 2007). The result is that under a variety of stressful conditions, bacteria are induced for genetic change, leading to an increased genetic polymorphism in populations that may be important for adaptive evolution. Numerous studies have indicated that environmental stress can also affect genome stability in eukarya (Galhardo *et al*., 2007). For instance, in mammalian cells, hypoxia and starvation can suppress error-free DNA repair pathways (e.g. mismatch repair and homologous recombination) and cause an increase in mutagenesis (Goncharova *et al*., 1996; Yuan *et al*., 2000; Mihaylova *et al*., 2003; Koshiji *et al*., 2005) and Shor *et al*. (2013) recently discovered that the yeast environmental stress response regulates mutagenesis.

Base excision repair (BER) is one of the most important mechanism protecting cells from the deleterious effects of endogenous DNA damage induced by hydrolysis, reactive oxygen species or other intracellular metabolites that modify the DNA base structure. The pivotal enzymes involved in BER are DNA glycosylases, which remove different types of modified or damaged bases by cleavage of the N-glycosidic bond between the base and the 2-deoxyribose moieties of the nucleotide residues. Different DNA glycosylases, including Uracil DNA glycosylase (UDG), remove different kinds of damage, and the specificity of the repair pathway is determined by the type of glycosylase involved. Uracil appears in DNA as a result of cytosine deamination, one of the most common DNA spontaneous modification (that naturally occurs in genomes at a rate of around 100 lesions per cell per day). Since this lesion is directly mutagenic, producing C→T transition, the production of UDG is essential for all living organisms. Consistently, the corresponding gene is extremely highly conserved from bacteria to man (56% identity, Sinha & Häder, 2002). Interestingly, the mammal UNG2 is also essential in the acquired immune response, including somatic hypermutation (SHM) required for antibody affinity maturation and class switch recombination (CSR) mediating new effector functions, e.g. from IgM to IgG in B-lymphocytes (Kavli *et al*., 2007).

Our transcriptomic study revealed the deregulation of numerous genes encoding proteins potentially implied in DNA repair and replication. In particular, we observed the down-regulation of not less than 5 different contigs annotated as UDG. Considering the particular role of the encoded proteins in DNA integrity maintenance, one could expect that such a down-regulation may lead to decreased UDG activity in cells, thus leading to increased mutation rates. In addition, we also showed the regulation of several DNA polymerases and of

genes encoding pol-like proteins. We have only poor information about the fidelity of molluskan DNA polymerases. The ecological significance of such a diversification occurring in somatic mantle cells is not so clear. Somatic hypermutations in animal cells is often associated with the acquired immune system, which is absent in invertebrates. Future studies should determine whether it could also be observed in germ cells, thus reflecting a population strategy to encompass chronic heat stress.

Concluding remarks

The aim of this study was to assess the molecular mechanisms underlying adaptation of the great scallop to prolonged heat stress, a situation which is closer to that encountered by marine organisms as a result of the ongoing global warming. Combining a RNAseq based transcriptomics approach and a proteomics one, we showed few relations to the highly conserved classical response to heat shock, as only few HSPs genes appeared deregulated under these conditions. Rather, our main results suggested a deep remodeling of the cell structure as revealed by the deregulation of many genes implied in the formation of the cytoskeleton and of cell membranes properties (through the modulation of fatty acids unsaturation), and a diversion of energetic metabolism towards the mobilization of lipid storage. The whole results also suggested a central role of the AP-1 signaling pathway which, together with many other deregulated genes known to be involved in apoptosis, should lead to an inhibition of apoptosis. Interestingly, several genes involved in DNA repair appeared downregulated, suggesting that cells turn towards a mutator state in response to stress. Finally, our network analyses led us to propose a pivotal role for GAPDH, a quite common glycolytic enzyme which was recently shown to carry crucial functions in most aspects of cells functioning. In all, this work gave very interesting insights into the response of *P. maximus* to long-term exposure to hyperthermal stress. Future studies will determine the actual role of the genes/proteins that were pointed out here.

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Supplemental data

Table S1: List of genes found deregulated at 25°C. E-values, gene names and accession numbers are those given by Blast2GO for the best hit. Short names are, when possible, those of the best human homolog of the best hit (these names were used for String 9.05 analyses). Induction factors are given as Log2.

Functional Classification	Gene Name	Contig ID	E-value	Id (%)	Acc	Short Name	3 d	14 d	21 d	27 d	56 d	25°C
Cytoskeleton/ Cell Binding	Protocadherin-11 X-linked	Contig14009	0	67	EKC36072	PCD11X	-0.95	0.48	1.48	1.38	1.38	up
	Putative tubulin polyglutamylase TTLL2	Contig24823	8.96E-58	78	EKC20821	TTLL2	-2.23	0.50	1.11	1.69	1.73	up
	hypothetical protein CGI_10013101	Contig31880	1.15E-80	72	EKC18364	PH-like	-1.91	0.69	1.67	1.21	1.47	up
	Ankyrin-1	Contig4777	9.70E-28	56	EKC31490	ANK1	-1.35	-0.30	1.09	1.46	1.65	up
	intraflagellar transport protein 20 homolog	Kmer2588320	4.89E-45	84	XP_798031	IFT20	-0.24	0.55	1.68	1.68	1.05	up
	Neurofilament heavy polypeptide. partial	Contig31123	1.19E-33	50	ELR55547	NEFH	-3.73	1.75	-1.08	-1.63	-2.56	down
	cornifelin-like protein	Kmer2590676	1.46E-20	56	XP_002730715		-1.77	-0.67	-2.56	-1.33	-3.26	down
	Fibrillin-1	Kmer2618224	1.66E-24	50	ELK31446	FBN1	-1.70	0.31	-1.27	-2.43	-1.51	down
	hypothetical protein CAPTEDRAFT_111329. partial	Contig34524	1.38E-33	57	ELT87078	FBN3	3.35	1.37	1.16	1.86	2.65	up
	neogenin-like	Contig35282	7.97E-57	57	ELU03642		4.14	-6.90	-3.15	-4.30	-4.78	down
	Tenascin-X	Contig24522	3.13E-42	54	EKC28247	TNX	-0.84	2.61	3.82	2.80	3.57	up
	Keratin-associated protein 10-8	Contig32243	2.67E-07	45	XP_002719196	KRTAP10-8	-0.54	1.26	2.01	1.31	1.13	up
	Collagen alpha-5(VI) chain	Contig34595	2.73E-83	50	EKC40283	COL6A5	-3.04	-1.00	-1.25	-2.10	-2.08	down
	hypothetical protein (mucin like)	Contig5092	6.40E-25	50	XP_001283171		-4.94	0.15	-1.28	-4.51	-1.96	down
	Tenascin-X	Contig26966	2.00E-18	42	EKC34579.1	TNX	-2.16	0.47	2.42	3.31	6.13	up
	predicted protein (protocadherin 16)	Kmer2596774	1.24E-13	50	XP_001632533	DCHS1	0.18	0.70	1.59	1.17	2.39	up
	hypothetical protein CAPTEDRAFT_224751 (Lachesin)	Contig22445	3.22E-36	52	ELT92279	LAC	-1.88	1.90	1.29	1.65	2.12	up
	hypothetical protein CGI_10012792	Contig29817	1.29E-142	80	EKC28948		-0.79	1.73	1.11	1.08	1.89	up
	Leucine-rich repeat and fibronectin type III domain-containing protein 1-like protein	Contig34753	1.17E-08	52	EKC34931	LRFN2	-0.64	1.56	1.84	1.79	1.06	up
	Neuroglian	Contig30563	9.94E-115	69	EKC24451	NRG	-1.10	1.15	1.10	1.70	1.18	up
	AP-1 protein	Contig14186	2.57E-61	66	ADZ48236	AP1	-0.93	0.91	1.20	1.82	1.52	up
	CREB/ATF bZIP transcription factor	Contig6686	4.32E-11	68	EKC41122	CREBZF	-2.22	1.72	1.99	1.56	1.77	up
	CREB/ATF bZIP transcription factor	Contig13363	2.10E-19	53	EKC41122	CREBZF	-1.04	1.81	1.97	2.51	1.98	up
	Basic leucine zipper transcriptional factor ATF-like 3	Contig24201	1.82E-05	59	EKC39151	BATF	-0.73	3.34	1.60	4.42	2.65	up
	Cold shock domain-containing protein E1	Contig21934	0	81	EKC28502	CSDE1	-1.31	1.02	1.56	1.51	1.56	up

WD repeat-containing protein 55. partial	Contig23964	6.40E-158	85	EKC18506	WDR55	-1.14	0.87	1.49	1.63	1.52	up
Conserved domain : NTF2	Contig2406	1.70E-06	63	ELT94896	NTF2	-1.72	-0.04	1.16	1.54	1.41	up
hypothetical protein CGI_10024367	Contig24111	1.62E-35	54	XP_002741750		-3.10	1.11	2.54	2.16	2.68	up
Pre-mRNA-splicing factor SYF1	Contig25944	0	92	EKC32877	SYF1	-1.70	0.70	1.56	1.47	1.55	up
hypothetical protein CGI_10010503	Contig27155	9.91E-102	64	EKC23497		-1.74	0.15	1.04	1.04	1.30	up
Helix-loop-helix protein delilah	Contig34688	3.63E-25	87	EKC41081	DEI	-0.33	1.13	1.64	1.55	1.23	up
Transposable element Tcb2 transposase	Contig34873	9.99E-15	69	EKC40022	TCB2	-1.02	0.80	1.61	1.56	1.30	up
Cisplatin resistance-associated overexpressed protein	Contig35733	4.22E-118	86	EKC39141	CROP	-1.28	0.34	1.22	1.49	1.16	up
Nucleolar GTP-binding protein 1	Contig4773	1.43E-60	80	EKC19642	GTPBP1	-0.68	1.06	2.41	2.50	1.84	up
Sin3 histone deacetylase corepressor complex component SDS3	Kmer2605584	1.37E-82	84	EKC32335	SDS3	-0.39	0.61	1.65	1.62	1.20	up
HIV Tat-specific factor 1 homolog isoform 2	Kmer2605766	2.45E-73	75	XP_003727819	HTATSF1	-2.35	0.86	2.49	2.70	2.12	up
Oxidoreductase HTATIP2	Contig17480	1.00E-73	49	EKC37161	HTATIP2	-0.08	1.19	1.64	1.99	1.51	up
Hairy/enhancer-of-split related with YRPW motif protein	Contig20067	6.60E-18	52	EKC31110	HEY1	1.55	2.55	1.55	1.37	1.66	up
Cleavage stimulation factor 77 kDa subunit	Contig17300	2.89E-139	85	EKC20054	CSTF3	1.04	0.09	2.21	1.81	1.14	up
Cleavage stimulation factor 77 kDa subunit	Kmer2612942	1.74E-129	87	EKC20054	CSTF3	0.78	-0.74	1.70	1.31	1.03	up
Histone H1-delta	Kmer2595300	1.33E-22	84	EKC17653	H1	-1.17	0.69	2.25	1.48	1.43	up
Dermal papilla-derived protein 6-like protein	Kmer2612148	2.19E-62	63	EKC36760	DERP6	-2.56	-0.79	3.17	1.26	1.52	up
Zinc finger protein 26	Contig27896	3.41E-48	71	EKC19913	ZNF26	-2.05	0.82	1.02	1.57	1.94	up
eukaryotic translation initiation factor 5A-1	Contig31784	1.75E-64	81	NP_998350	EIF5A	-2.62	0.59	2.55	3.36	4.53	up
NTF2-related export protein 2	Contig24619	1.18E-46	83	EKC36366	NXT2	-1.77	1.55	1.06	1.14	1.43	up
hypothetical protein CGI_10001573 (b_ZIP superfamily protein)	Contig26918	2.05E-38	56	EKC27007		-2.62	1.16	1.10	1.08	1.76	up
HSPC148	Contig27021	2.32E-66	80	AAF29112	HSPC148	-1.42	1.20	1.00	1.09	1.00	up
Transcription elongation regulator 1	Contig29941	3.23E-88	89	EKC23634	TCERG1	-2.94	1.58	1.06	1.36	1.04	up
Caprin-1	Contig30972	7.52E-54	76	EKC23296	CAPR1	-1.24	1.35	1.66	1.11	1.64	up
Heterogeneous nuclear ribonucleoprotein Q	Contig12409	4.01E-39	92	EKC37135	SYNCRIP	1.04	0.93	1.83	2.80	1.03	up
RNA-binding protein (2nd hit = hn-RNP Q)	Contig12883	2.55E-65	92	ABP04054	SYNCRIP	0.88	1.60	2.58	2.87	1.54	up
high mobility group protein D	Contig34400	5.94E-17	76	BAM17877	HMGD	-1.04	0.30	1.16	2.34	1.17	up
Zinc finger CCCH domain-containing protein 3	Contig35254	2.66E-58	59	EKC35995	ZC3H3	-0.68	1.34	1.48	2.27	1.82	up
hypothetical protein CGI_10006979 (N151 protein)	Contig11211	9.29E-34	93	BAI49993	N151	-1.46	0.93	1.41	1.37	1.48	up
N151	Contig35661	5.35E-09	69	BAI49993	N151	0.23	1.19	1.16	3.26	2.18	up
U11/U12 small nuclear ribonucleoprotein 25 kDa protein	Kmer2606506	2.45E-53	83	EKC41736	SNRNP25	-1.89	0.93	1.08	1.75	1.08	up
hypothetical protein CAPTEDRAFT_222987	Kmer2612936	5.84E-78	71	ELT89986		-3.01	0.50	1.27	2.26	1.33	up
Putative ATP-dependent RNA helicase DDX27	Kmer2614026	5.16E-96	78	EKC21239	DDX27	-1.55	-0.60	1.41	3.06	1.48	up
Poly [ADP-ribose] polymerase 14	Contig30195	2.60E-29	60	EKC32942	PARP14	-2.88	0.68	1.80	1.76	1.80	up

	MRG-binding protein	Contig31296	2.49E-38	68	EKC26944	MRGBP	-2.15	0.69	1.50	1.73	1.30	up
	DNA-directed DNA polymerase epsilon 3	Contig33716	7.65E-52	92	NP_001140051	POLE3	-1.27	0.16	1.12	1.22	1.15	up
	pol-like protein	Contig14232	3.16E-67	57	BAC82626		-4.77	1.38	-1.88	-1.75	-3.82	down
	endonuclease-reverse transcriptase	Contig29988	7.32E-29	63	EHJ67413	RTase	-1.72	0.31	-4.81	-2.86	-3.13	down
	uracil-DNA glycosylase	Contig33560	6.09E-14	66	YP_001219471	UDG	0.04	0.59	-1.58	-4.54	-2.96	down
	predicted protein	Contig29412	1.01E-102	74	XP_001632570		1.04	0.78	2.21	2.10	1.31	up
	uncharacterized protein LOC764672	Contig31538	4.83E-29	57	XP_003724962		-2.62	1.00	4.51	2.24	1.77	up
	unnamed protein product	Kmer2589196	6.27E-92	87	BAC34934		-1.81	-2.10	1.73	1.08	1.02	up
	CWF19-like protein 1	Kmer2601862	9.39E-75	73	EKC30101	CWF19L1	-1.94	1.49	2.32	1.05	1.33	up
Replication / DNA Repair	Uracil-DNA glycosylase	Contig21363	2.08E-60	70	EKC38003	UDG	-4.04	0.09	-1.93	-1.11	-1.70	down
	hypothetical protein	Contig34404	8.57E-47	44	AEO32622		-2.05	-1.95	1.06	1.06	3.70	up
	pol-like protein	Kmer2621110	4.10E-53	47	BAC82624		0.23	1.57	2.23	2.08	3.75	up
	Exonuclease 3'-5' domain-containing protein 2	Contig21482	6.73E-114	60	EKC41618	EXD2	-1.12	1.48	1.29	1.67	1.31	up
	Putative RNA-directed DNA polymerase from transposon BS	Contig22409	2.49E-18	56	EKC31648	RTase	-0.99	1.48	1.27	1.54	1.53	up
	uncharacterized protein LOC101238120	Contig29133	2.72E-55	66	XP_004208670		-4.60	2.70	2.09	2.47	3.20	up
	polymerase (RNA) III (DNA directed) polypeptide C (62kD)-like	Contig28898	7.25E-88	72	XP_002740482	POLR3C	-0.98	0.04	1.24	1.78	1.11	up
	Structural maintenance of chromosomes protein 1A	Kmer2615746	1.30E-158	88	EKC29388	SMC1A	-1.47	0.46	1.29	2.16	1.04	up
	hypothetical protein BRAFLDRAFT_128896	Contig20242	1.03E-43	49	XP_002611771		-0.40	1.05	2.09	2.05	1.47	up
	Peptide-N(4)-(N-acetyl-beta-glucosaminyl)asparagine amidase	Contig28726	4.65E-97	80	EKC20424	NGLY1	-1.13	1.07	1.34	1.51	1.87	up
	ariadne-1-like protein	Contig28961	1.30E-24	49	EKC29163	ARIH1	-3.56	0.82	2.24	1.59	2.51	up
	hypothetical protein CGI_10019403	Contig29154	1.40E-38	73	EKC37854		-1.70	0.31	2.04	2.19	2.39	up
	hypothetical protein CGI_10024659	Contig32670	4.16E-88	82	EKC42154		-1.89	0.40	1.16	1.20	1.17	up
	Carbohydrate sulfotransferase 15	Contig3509	5.48E-23	61	EKC27439	CHST15	-0.93	0.00	1.35	1.34	1.23	up
Protein modification/ maturation	dolichyl-phosphate beta-glucosyltransferase	Kmer2588360	4.19E-11	89	NP_001080634	ALG5	0.23	-0.07	1.24	1.66	1.55	up
	E3 ubiquitin-protein ligase MIB2	Kmer2591220	3.16E-36	70	EKC39101	MIB2	0.06	0.38	1.19	1.69	1.74	up
	UDP-GlcNAc:betaGal beta-1.3-N-acetylglucosaminyltransferase 5	Kmer2611298	2.74E-10	60	CAX69340	B3GNT5	-1.94	-1.59	-2.02	-2.21	-3.06	down
	Carbohydrate sulfotransferase 15	Contig31979	8.13E-41	67	EKC36000	CHST15	-0.15	-0.13	2.83	1.06	2.04	up
	hypothetical protein CGI_10007323	Kmer2588384	6.80E-56	77	EKC23848		-1.77	1.03	1.38	1.99	1.36	up
	SIL1 protein-like	Kmer2589504	6.44E-31	73	XP_002737127	SIL1	-4.26	1.47	1.35	2.25	1.49	up
	Serine/threonine-protein kinase SBK1	Kmer2616510	1.67E-176	86	EKC36229	SBK1	-0.20	0.70	1.32	2.15	1.74	up
	FAM50-like protein	Contig27547	9.41E-112	89	EKC29307	FAM50	-0.51	0.78	1.76	2.24	1.86	up
Signaling	Calcyclin-binding protein	Contig30000	7.08E-39	65	EKC28333	CACYBP	-2.23	1.39	1.64	2.18	1.70	up
	Ras-related protein Rab-9A	Kmer2616396	2.79E-68	90	EKC42702	RAB9A	-0.42	1.06	1.44	1.36	1.47	up

	Ectonucleotide pyrophosphatase/phosphodiesterase family member 5	Contig27767	5.22E-71	62	EKC40323	ENPP5	-1.87	0.95	-1.09	-2.01	-1.69	down
	L-rhamnose-binding lectin CSL3	Kmer2588308	5.04E-11	80	EKC38532	CSL3	-1.43	-2.96	-2.15	-1.71	-2.30	down
	tumor necrosis factor receptor-associated factor 3	Contig22790	4.97E-17	75	AFL03408	TRAF3	-0.90	2.64	1.93	1.64	1.99	up
	hypothetical protein CGI_10008481	Contig28040	2.45E-148	72	EKC19449		-0.83	2.54	1.86	1.24	2.36	up
	hypothetical protein BRAFLDRAFT_123623	Contig28118	6.62E-16	63	XP_002590580		-1.65	-0.78	-1.24	-1.56	-1.21	down
	Neurocalcin-like protein	Contig24768	2.46E-49	66	EKC27427	NCALD	-1.37	0.27	1.05	1.11	2.39	up
	similar to tumor necrosis factor receptor-associated factor 3	Contig30315	1.43E-53	72	EKC42493	TRAF3	-0.86	1.57	1.40	2.07	3.50	up
	canopy-like protein 2	Contig32516	1.15E-56	70	EKC42608	CNPY2	-2.94	1.07	1.49	1.25	2.38	up
	calumenin-B-like	Contig22840	2.00E-47	45 %	XP_005816427	CALUB	-1.66	3.87	3.59	3.10	3.40	up
	Teneurin-3	Contig33008	1.30E-11	47	EKC39942	TENM3	-0.62	1.67	1.85	1.68	1.23	up
	UPF0556 protein C19orf10 (interleukine 25)-like protein	Contig31721	1.98E-13	51	EKC42343	IL25	-2.02	1.14	1.44	1.09	1.77	up
	tumor necrosis factor receptor-associated factor 3	Contig20819	9.21E-128	61	AFL03408	TRAF3	-1.30	2.19	1.34	2.90	2.15	up
	toll-like receptor D	Contig33472	3.11E-37	51	AFU48616	TLR4	-0.43	1.28	2.13	2.60	1.86	up
	28 kDa heat- and acid-stable phosphoprotein	Contig35358	2.17E-27	70	EKC25746	PDAP1	-0.83	0.90	1.05	1.86	1.59	up
	calmodulin-like protein	Contig4442	6.28E-66	82	XP_003766688	CALM5	0.16	0.63	1.10	2.22	1.34	up
	Cubilin	Contig14685	4.47E-22	42	EKC18187	CUBN	-2.03	0.33	1.12	1.09	1.55	up
	Kynurenine formamidase	Contig26371	3.00E-45	62	EKC35917	KFASE	-1.50	2.16	-1.22	-1.47	-1.77	down
	DDI1-like protein 2	Contig25538	6.47E-31	74	EKC18588	DDI1	-5.05	0.77	1.27	1.75	1.49	up
	Adipocyte plasma membrane-associated protein	Contig25844	9.16E-80	80	EKC42337	APMAP	0.32	0.93	1.37	1.31	1.35	up
	Dipeptidyl-peptidase 1. partial	Contig34949	9.00E-120	64	EKC33922	DPP1	-3.13	-1.71	-1.14	-1.59	-1.00	down
	Carboxypeptidase B	Contig35864	1.00E-78	53	EKC34650	CPB1	-1.27	0.79	1.00	1.54	1.70	up
	Tetratricopeptide repeat protein 19	Kmer2601832	2.19E-10	48	EKC34342	TTC19	-1.35	-0.12	1.33	1.38	1.39	up
Metabolism/	GnRH-related peptide precursor	Kmer2609810	2.66E-44	95	BAH47639	GNRH	-0.84	0.63	2.22	2.02	1.48	up
Energy related	Group XVI phospholipase A2	Contig23068	2.30E-24	56	EKC23544	PLA2G16	0.50	1.51	-6.19	-1.27	-1.58	down
	serine protease CFSP3	Contig31517	2.65E-62	58	ABB89132	CFSP3	-1.19	-0.61	-1.48	-1.81	-4.32	down
	predicted protein	Kmer2586618	1.82E-14	50	XP_001623807		-0.64	1.70	-1.05	-1.60	-1.32	down
	carbonic anhydrase. putative	Kmer2613940	1.02E-10	46	XP_002428435	CA2	-1.16	-0.50	-1.04	-1.22	-3.62	down
	Serum paraoxonase/arylesterase 1	Kmer2590048	3.90E-19	61	EKC25343	PON1	1.59	-0.36	2.85	1.16	1.49	up
	Alpha-methylacyl-CoA racemase	Kmer2593398	5.99E-78	77	EKC18886	AMACR	0.25	-0.17	2.68	1.75	1.17	up
	Acyl-coenzyme A thioesterase 4	Kmer2613834	2.57E-30	53	EKC26987	ACOT4	-3.81	0.07	2.14	1.09	1.64	up
	group XVI phospholipase A1/A2-like. partial	Contig16720	5.00E-14	40	XP_004085715	PLA2G16	-2.37	1.67	1.81	1.71	1.72	up

	Protein C20orf11. glucose induced degradation protein 8	Contig9673	1.27E-107	92	EKC24371	GID8	-1.48	1.31	1.16	1.41	1.31	up
	Heat shock 70 kDa protein 12A	Contig25894	1.38E-128	72	EKC18507	HSPA12A	-0.73	0.45	1.12	1.06	1.12	up
	stearoyl-CoA desaturase-1	Contig28270	4.49E-19	72	AET74083	SCD	-0.80	-0.40	-1.17	-1.07	-1.54	down
	Laccase-1	Contig26352	3.40E-80	64	EKC25936	LAC1	-1.06	-1.30	-3.37	-2.26	-3.58	down
	hypothetical protein BRAFLDRAFT_67911	Contig31568	4.59E-138	75	XP_002595129		-0.21	0.86	1.75	1.40	1.04	up
	Neutral and basic amino acid transport protein rBAT	Contig32431	1.10E-110	63	EKC19380	RBAT	-0.51	0.32	1.83	1.38	1.02	up
	Laccase-1	Contig8350	6.31E-100	61	EKC25936	LAC1	-2.39	-1.86	-1.09	-1.50	-1.80	down
	hypothetical protein CAPTEDRAFT_220209 (immunophilin)	Contig9638	3.54E-47	85	ELT92262	FKBP45	-5.60	1.39	2.93	1.05	2.11	up
Stress Response	glutathione S-transferase sigma 3	Contig20068	5.75E-32	66	AFQ35985	GSTS3	-1.83	-2.21	-1.45	-2.74	-1.13	down
	calreticulin precursor	Kmer2615304	3.25E-177	91	NP_001191523	CALR	-2.08	1.50	1.13	1.67	2.31	up
	peptidoglycan recognition protein 4	Contig10551	1.76E-40	73	AAY27976	PGLYRP4	0.58	4.05	3.99	4.44	3.87	up
	Steroid 17-alpha-hydroxylase/17.20 lyase	Contig28362	9.75E-67	66	EKC22297	CYP17A1	-0.23	2.07	1.76	1.47	1.93	up
	heat shock protein 90	Contig33569	0	99	ABS50431	HSP90	-2.03	1.63	1.13	2.00	2.34	up
	hypothetical protein CAPTEDRAFT_183144	Contig35018	1.50E-125	83	ELT99118		-1.53	1.29	1.65	1.06	1.37	up
	Major egg antigen	Contig28432	3.68E-122	72	EKC31248	P40	-0.08	1.15	1.56	2.29	1.37	up
	Activator of 90 kDa heat shock protein ATPase-like protein 1	Contig9726	1.24E-163	84	EKC32134	AHSA1	-2.39	1.70	1.47	2.49	2.34	up
	Baculoviral IAP repeat-containing protein 7-A	Contig27457	1.84E-96	60	EKC32616	BIRC7	-0.39	1.34	1.22	1.89	2.01	up
	Inositol 1.4.5-trisphosphate receptor type 1	Kmer2589886	4.91E-59	77	EKC35464	ITPR1	-0.35	-0.47	-1.20	-1.82	-1.32	down
	Baculoviral IAP repeat-containing protein 7-A	Contig36037	6.11E-86	73	EKC32616	BIRC7	-1.29	1.84	2.39	1.81	1.53	up
	ariadne-1-like protein	Contig28903	1.02E-63	47	EKC29163	ARI1	-2.74	1.14	1.60	1.85	3.35	up
	Tripartite motif-containing protein 2	Contig34345	1.00E-65	51	EKC26008.	TRIM2	-1.41	-0.35	1.06	2.51	3.14	up
	hypothetical protein	Kmer2620970	7.32E-17	43	XP_002732174		-0.47	0.97	2.11	1.63	3.88	up
Apoptosis	Baculoviral IAP repeat-containing protein 7-A	Contig18058	2.83E-80	63	EKC38618	BIRC7	-1.30	1.68	2.04	2.00	1.30	up
related	Ubiquitin carboxyl-terminal hydrolase 27	Contig19723	1.42E-43	51	EKC20536	USP27X	-1.24	2.37	2.20	2.53	2.48	up
	Putative inhibitor of apoptosis	Contig26840	8.42E-56	58	EKC17690	PIAP	-0.77	2.40	2.15	1.88	1.60	up
	hypothetical protein CGI_10022970	Contig35119	5.53E-12	46	EKC38301		-1.12	1.57	1.34	1.44	1.55	up
	Tripartite motif-containing protein 13	Contig20284	5.75E-24	52	EKC30865	TRIM13	-3.16	2.98	1.76	4.90	2.40	up
	Cell division cycle and apoptosis regulator protein 1	Kmer2621852	1.40E-52	84	EKC29909	CCAR1	-1.66	0.84	1.62	2.14	1.34	up
	Solute carrier family 43 member 3	Contig33561	3.10E-18	48	EKC31271	SLC43A3	0.82	-0.10	1.40	1.52	1.23	up
	Sodium-dependent phosphate transport protein 2B	Contig21791	0	74	EKC36780	SLC34A2	0.69	1.22	1.13	3.08	4.05	up
Transport/	uncharacterized protein LOC764526 isoform 1	Kmer2596006	1.58E-39	62	XP_001200862		-0.23	0.85	1.03	1.37	3.75	up
Homeostasis	hypothetical protein CGI_10017460	Contig25397	1.83E-20	60	EKC30689		0.93	1.30	1.43	1.94	1.22	up
	annexin A7-like	Contig31351	6.37E-38	76	XP_003383438	ANXA7	-1.88	-1.53	-1.52	-1.35	-2.09	down
	Protein disulfide-isomerase A6	Contig32100	0	84	EKC19533	PDIA6	-0.73	0.80	1.15	2.21	1.61	up

	Multidrug resistance-associated protein 1	Contig35841	0	76	EKC32378	MRP1	1.50	6.07	3.49	7.99	2.36	up
	Sodium- and chloride-dependent glycine transporter 2	Kmer2603250	3.46E-26	56	EKC40069	SLC6A5	0.26	0.08	1.93	2.50	1.58	up
	alpha macroglobulin	Contig9823	0	90	AAR39412	A2M	-1.63	-0.18	-1.24	-1.57	-1.10	down
	hypothetical protein LOTGIDRAFT_237271	Kmer2613326	3.00E-22	35	ESP04739		-2.97	-0.41	-1.91	-1.60	-2.36	down
	hypothetical protein BRAFLDRAFT_171338	Contig26581	6.91E-15	50	XP_002600473		-0.67	2.01	1.28	1.37	2.31	up
	Protein TSSC4	Contig25750	1.17E-23	61	EKC20638	TSSC4	-1.38	1.73	1.93	1.49	1.72	up
other	NEFA-interacting nuclear protein NIP30	Kmer2597016	3.83E-36	66	EKC19734	NIP30	-2.09	1.22	1.60	1.16	1.91	up
	hypothetical protein CAPTEDRAFT_190177	Contig34305	1.07E-11	54	ELU07255		0.45	2.20	1.06	2.82	1.34	up
	Kelch-like protein 24	Contig35538	1.54E-25	54	EKC17247	KLHL24	-0.66	1.79	1.62	2.84	1.60	up
	UPF0420 protein C16orf58-like protein	Kmer2592130	1.55E-98	90	EKC39300	C16ORF58	0.32	1.63	1.12	2.77	1.24	up
	Kelch-like protein 24	Contig16700	3.26E-27	50	EKC17247	KLHL24	0.32	2.71	2.35	3.34	2.83	up

Table S2: List of genes found deregulated at 21°C. E-values, gene names and accession numbers are those given by Blast2GO for the best hit. Short names are, when possible, those of the best human homolog of the best hit (these names were used for String 9.05 analyses). Induction factors are given as Log2.

Functional Classification	Gene Name	Contig ID	e-value	Id (%)	Acc	Short Name	3 d	14 d	21 d	27 d	56 d	21°C
Cytoskeleton/ Cell Binding	Titin	Contig29570	1.00E-62	39	EKC32610.1	TTN	-2.50	0.48	7.08	1.69	2.31	up
	Mesenchyme-specific cell surface glycoprotein	Contig23465	7.00E-72	48	EKC20477.1	MSP130	2.92	-0.65	1.57	2.79	1.01	up
	Cartilage matrix protein	Contig30009	1.00E-45	30	EKC20505.1	MATN1	-2.52	-1.07	1.50	1.14	2.70	up
	Kelch-like protein 24	Contig16700	5.00E-27	30	EKC17247.1		2.21	1.02	1.07	2.87	1.33	up
Replication / DNA Repair	pol-like protein	Kmer2621110	6.00E-53	27	BAC82624.1		2.48	1.74	1.73	1.05	1.96	up
	Uracil-DNA glycosylase	Contig31738	4.00E-67	62	EKC38003.1	UDG	-2.75	-1.87	-2.58	-2.30	-1.25	down
	Uracil-DNA glycosylase	Contig22819	3.00E-81	58	EKC38003.1	UDG	-2.12	-1.96	-2.89	-1.41	-1.21	down
	Uracil-DNA glycosylase	Contig21363	3.00E-60	60	EKC38003.1	UDG	-5.74	-1.66	-4.08	-2.92	-1.16	down
	pol-like protein	Kmer2598858	1.00E-35	42	BAC82626.1		2.81	1.18	2.33	1.38	4.26	up
	hypothetical protein	Contig34404	1.00E-44	32	XP_003391328.1		1.86	0.97	2.77	1.67	3.04	up
	RNA-directed DNA polymerase from mobile element jockey-like	Contig1391	1.00E-34	52	XP_003725849.1		1.21	3.25	-1.36	-3.38	-1.09	down
	Uracil-DNA glycosylase	Contig32735	2.00E-70	53	EKC38003.1	UDG	-2.84	1.53	-3.01	-2.04	-1.02	down
	transposase	Kmer2621654	1.00E-26	29	AER39694.1		-5.13	1.00	-1.68	-1.34	-1.87	down
	Putative nuclease HARBI1	Kmer2607486	2.00E-23	35	EKC43123.1	HARBI1	-0.31	2.26	-1.65	-1.68	-1.10	down
	uncharacterized protein	Contig34877	5.00E-40	53	XP_004209790.1		-2.01	-1.85	1.75	1.39	1.17	up
	pol-like protein	Kmer2621110	6.00E-53	27	BAC82624.1		2.48	1.74	1.73	1.05	1.96	up
	Uracil-DNA glycosylase	Contig31738	4.00E-67	62	EKC38003.1	UDG	-2.75	-1.87	-2.58	-2.30	-1.25	down
	Uracil-DNA glycosylase	Contig22819	3.00E-81	58	EKC38003.1	UDG	-2.12	-1.96	-2.89	-1.41	-1.21	down
Protein modification/ maturation	hypothetical protein	Contig24181	2.00E-15	18	XP_001619756.1		1.89	2.01	1.76	1.77	1.41	up
	VPS4A protein	Kmer2605336	9.00E-17	61	AAH35121.1	VPS4A	0.92	0.73	2.61	1.08	1.04	up
Signaling	TNF receptor-associated factor 3	Contig30315	2.00E-53	54	EKC42493.1	TRAF3	-0.66	1.32	1.15	2.55	1.81	up
	Prostaglandin reductase 1	Contig25288	1.00E-61	57	EKC24838.1	PTGR1	0.27	-0.82	1.44	1.43	1.06	up
	hypothetical protein	Kmer2589494	1.00E-57	69	ESO84907.1		0.45	-0.25	1.37	1.04	1.57	up
	Putative inhibitor of apoptosis	Contig12878	4.00E-57	31	EKC26950.1	XIAP	0.34	-0.45	1.25	1.14	1.28	up
	ariadne-1-like protein	Contig28903	1.02E-63	47	EKC29163		-0.78	-0.21	1.58	1.92	1.30	up
	Sodium- and chloride-dependent glycine transporter 2	Contig31254	4.00E-58	49	EKC21522.1	GST	1.48	1.29	2.91	1.38	1.13	up
	peptidoglycan recognition protein 4	Contig10551	3.00E-40	57	AAY27976.1	PGLYRP4	1.68	1.79	1.52	3.49	2.84	up
other	hypothetical protein BRAFLDRAFT_122687	Contig29522	2E-56	1	XP_002591716.1		-0.42	0.68	1.52	1.46	1.51	up

Chapitre III : Réponses physiologiques et protéomiques de *Pecten maximus* à la combinaison d'un stress thermique et hypoxique

I/ Introduction

A l'instar de la température, la diminution de la disponibilité en oxygène dans l'eau de mer est un paramètre majeur de l'environnement impactant la physiologie des organismes marins. En fonction de leur capacité à adapter leurs taux de respiration en situation d'hypoxie, les organismes "oxyregulateurs" sont ceux capables de maintenir leur consommation d'oxygène indépendamment de la concentration en oxygène externe. Ceux dont la consommation d'oxygène diminue avec la concentration en oxygène dissous sont qualifiés d'"oxyconformeurs". La réponse des organismes à l'hypoxie peut en outre être caractérisée par le point critique en oxygène (PcO_2). Du point de vue métabolique, ce seuil reflète la bascule du métabolisme énergétique de la voie respiratoire aérobie vers la voie fermentaire anaérobie.

La coquille Saint-Jacques, précédemment qualifiée d'oxyrégulateur limité, se révèle comme une espèce relativement tolérante à l'hypoxie en l'absence de stress thermique, avec des taux de régulation élevés et des PcO_2 faibles. A 25°C néanmoins, le pourcentage de régulation diminue (86%) et la PcO_2 double par rapport à 10°C (36% d'O_2), suggérant que les animaux se trouvent, à cette température, en dehors de leur fenêtre thermique optimale. Il faut noter que cette hypothèse est largement appuyée par le fait qu'un passage de 24 h en hypoxie en dessous de la PcO_2 entraîne un fort taux de mortalité (50%) pour les coquilles Saint-Jacques maintenues à 25°C. En outre, les dosages de l'activité de l'Octopine Déshydrogénase (ODH) montrent que cette enzyme, qui est considérée comme un marqueur du métabolisme fermentaire chez les bivalves, est déjà activée en normoxie à 25°C (et ne l'est donc pas plus en situation hypoxique). Ces résultats sont confirmés par la diminution des teneurs en arginine, l'un des substrats de cette enzyme, à 25°C.

La comparaison des réponses observées dans différents modèles biologiques est souvent très informative. Les données disponibles dans littérature suggèrent que les moules *Mytilus edulis* et *M. galloprovincialis* présentent un comportement similaire à *P. maximus* en situation d'hypoxie. Néanmoins, nos résultats ont montré une réponse très différente dans les deux modèles. Ainsi, alors que *P. maximus* maintient un fort taux de respiration lorsque les concentrations en O_2 chutent, avant de basculer vers l'oxyconformité, les moules voient leurs taux de respiration décroitre lentement à mesure que les concentrations en oxygène diminuent, et finissent

également par basculer vers l'oxyconformité, mais pour des valeurs d'O_2 plus élevées. Le point de rupture, pour les 2 espèces, a été appelé PcO_2 (point critique en O_2). Nous proposons en outre dans cette publication une nouvelle méthode de détermination de ce paramètre essentiel dans la compréhension de la réponse des mollusques à l'hypoxie.

Dans l'optique de mieux comprendre les mécanismes moléculaires sous-tendant l'adaptation de la coquille Saint-Jacques à l'hypoxie, nous avons effectué une analyse protéomique sur l'organe probablement le plus exposé à ce stress, ie les branchies. Les signatures protéomiques obtenues aux trois températures testées se sont révélées extrêmement différentes. En effet, le nombre de protéines dérégulées significativement par le stress hypoxique (0 à 10°C, 15 à 18°C et 11 à 20°C), mais également leur nature (un seul spot commun entre 18 et 25°C), suggèrent un effet crucial de la température sur les capacités d'adaptation de cette espèce.

La nature des protéines identifiées donne des indications sur les fonctions cellulaires dérégulées dans ces conditions particulières d'hypoxie. Ainsi, nous avons montré l'accumulation de la caséine kinase 2 lors de l'exposition hypoxique à 18°C. Cette protéine, qui joue un rôle essentiel chez les animaux chez lesquels catalyse la phosphorylation de plus d'une centaine de protéines, pourrait influencer l'activité transcriptionnelle du facteur majeur de la réponse hypoxique HIF-1 (hypoxia inducible factor 1) via la dégradation de p53, un inhibiteur compétitif de HIF-1. Cette protéine a également été montrée comme inhibitrice de l'apoptose (par le biais de la phosphorylation de la protéine pro-apoptique Bid, entrainant son inactivation).

Le métabolisme des acides gras semble également être impliqué dans la réponse au stress hypoxique à 18°C, alors que le métabolisme glucidique (plus spécialement la synthèse de glycogène, étant donnée la fonction postulée d'ENO3) semble quant à lui être régulé à la baisse chez les animaux acclimatés à 25°C en situation d'hypoxie. Dans ces conditions, des protéines impliquées dans l'apoptose ont également été identifiées (Gelsolin, kinase TBK1). Néanmoins, l'impact de ces changements d'abondance de ces protéines semble difficile à prévoir puisqu'ils semblent être antagonistes.

II/ Article n°3 :

Respiratory response to combined heat and hypoxia in the marine bivalves *Pecten maximus* and *Mytilus* spp.: a comparative study

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Respiratory response to combined heat and hypoxia in the marine bivalves *Pecten maximus* and *Mytilus spp.*: a comparative study

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Abstract

In a context of current global change, coastal ecosystems are increasingly endangered by augmentation of mean sea surface temperature and expansion of hypoxic areas. Objectives of the present work were to describe and compare respiratory responses to combined heat and hypoxia in two bivalve species (*Pecten maximus and Mytilus spp*.) living in two different coastal habitats (respectively subtidal and intertidal). Results were consistent with vertical zonation of both species. *Mytilus spp*. seemed to cope better with a temperature increase whereas *P*. *maximus* was found outside its optimal thermal window at 25°C. Concerning respiratory responses to hypoxia at a given temperature, *P*. *maximus* exhibited a more important oxyregulation capacity that was maintained over a larger range of O_2 levels compared to *Mytilus spp*., indicating both species rely on different strategies to survive temporary hypoxia. When acclimation temperatures increased, both species showed a decrease in regulation capacity that went along with a reduction of aerobic performances, especially for *P*. *maximus*. Thus, results gave further proofs of the vulnerability of marine organisms to global change. Lastly, this study highlighted the usefulness of segmented linear models to estimate PcO2 and regulation percentages in marine organisms exposed to hypoxia.

Introduction

Among the consequences of current global change, the augmentation of mean sea surface temperatures and the frequency of hypoxic events, as well as the extension of hypoxic areas, are well-described phenomena (Levitus *et al*., 2000; Stramma *et al*., 2008; Vaquer-Sunyer & Duarte, 2008; Keeling *et al*., 2009). These two environmental factors have a major impact on marine organisms (Doney *et al*., 2011). Great scallops (*Pecten maximus*) and blue mussels (*Mytilus spp*.) are both environmentally and commercially important marine bivalves living in coastal areas potentially impacted by these global-change-related stressors (Grieshaber *et al*., 1994). As sedentary ectothermic filter-feeders, they are at the basis of the trophic food chain and may play an important role in coastal ecosystem maintenance (Newell, 2004). Both species are also particularly threatened by hypoxic and heat stress events (Wang & Widdows, 1991; Anestis *et al*., 2007).

Impacts of hypoxia and temperature on marine organisms are interdependent, as shown by a number of studies on temperature-dependent metabolisms and thermal adaptation mechanisms that suggested a role of oxygen supply in thermal limitation (Pörtner & Grieshaber, 1993; Pörtner, 2001). Consequently, hypoxia affects thermal tolerance by reducing the width of organism thermal window and by decreasing aerobic performance (Pörtner & Lannig, 2009). In previous works, organisms have been characterized by their capacity to adapt their respiration rates during hypoxia (Grieshaber *et al*., 1994). The ones that are able to maintain their oxygen consumption independently from external oxygen concentration are qualified as “oxyregulators”, whereas the ones whom oxygen consumption decreases as the oxygen concentration in water declines are named “oxyconformers”. Organisms responses to hypoxia can be further characterized by the concept of oxygen critical point (PcO_2), which was introduced to characterize the respiration rates threshold below which an oxyregulator cannot maintain its oxygen uptake rate (Grieshaber *et al*., 1988). From a metabolic perspective, this threshold reflects a change from an aerobic towards an anaerobic pathway of energy production (Pörtner & Grieshaber, 1993).

According to previous works, responses to hypoxia of subtidal *Pecten maximus* and intertidal *Mytilus spp*. were both classified as poor to moderate oxyregulators, depending on the studies (Bayne, 1971a, 1971b; Brand & Roberts, 1973; Bayne & Livingstone, 1977; Famme & Kofoed, 1980; Davenport, 1983; de

Zwaan *et al*., 1991). Combined effects of hypoxia and temperature were also assessed in different marine bivalves by several authors with the aim to assess the evolution of oxyregulation ability when temperature rises. In non-temperature acclimated *M. galloprovincialis*, results obtained by Jansen *et al*. (2009) indicated an increasing loss of oxyregulation capacity towards high temperatures whereas Hicks & McMahon (2002) observed a positive temperature-dependent ability to regulate O_2 uptake in the brown mussel (*Perna perna)*. In the zebra mussel *(Dreissena polymorpha)*, Alexander & McMahon (2004) observed an acclimation effect on oxygen regulation as the degree of oxygen regulation increased with increasing test temperature but decreased with increasing acclimation temperature.

In the present study, we compared temperature effects on O_2 consumption rate of *P. maximus* and *Mytilus spp*. during progressive hypoxia after acclimation over a range of temperatures spanning their natural ambient range (10°C and 18°C) and more stressful (25°C) conditions. In addition, we proposed a new method to estimate the PcO_2, a crucial characteristic of organism responses to hypoxia, allowing more accurate comparisons between species. To our knowledge, this is the first study of comparative physiology estimating the effect of temperature acclimation on hypoxia regulation on two bivalve species living in two different marine habitats (intertidal and subtidal zones) under identical experimental conditions.

Materials and methods

Biological material

In March 2013, wild intertidal adult mussels (*Mytilus spp*.) (length 57.3 +/- 5.3 mm) were sampled in the Bay of Brest (48° 19' 20.29"N, 4° 27' 13.51"W, Brittany, France) and six-month scallops (*Pecten maximus*) (length 32.5 +/- 3.4 mm) were provided by the Tinduff hatchery (Brittany, France). It should be noticed that, in the study area, mussel population is composed of *Mytilus galloprovincialis*, *M*. *edulis* and their hybrids (Bierne *et al*., 2003). Both species can hardly be morphologically distinguished (Lewis & Seed, 1969). Nevertheless, in a previous work, 165 mussels sampled in the Bay of Brest were genotyped by study of the adhesive protein gene length polymorphism as described by Inoue *et al*. (1995). Mussel population is mainly composed of *M*. *galloprovincialis* (64%) and hybrid genotypes (33%), and only 2% were genotyped as *M*. *edulis* (C. Lacroix, unpublished data). As species composition requires further investigation using additional markers, mussels are referred as *Mytilus spp*. in this study.

Once in the laboratory, both *P*. *maximus* and *Mytilus spp*. were divided into 3 homogenous groups ("10°C", "18°C" and "25°C") and were maintained in flow-through tanks containing sand-filtered air-saturated seawater at 10 °C (ambient field temperature) for one week. Then two tanks were heated at a rate of 1°C per day until reaching 18 and 25°C, respectively. Animals were acclimated at least one week at targeted temperatures (10, 18 and 25°C) before hypoxia/respirometry experiments started. Temperatures in the 3 tanks were monitored continuously during the whole experiment using an autonomous temperature logger (EBRO, Germany). Temperatures were maintained homogenous in each tank using pumps and O_2-saturation was kept at 100% until respiration rate measurements started. Seawater parameters (temperature, pH, salinity and O_2 level) and mortality were assessed daily in each tank during the whole experiment and a day-night (12/12) alternation was maintained.

Respiration rate and hypoxia experimental system

Respiration rate measurements were performed using 6 acrylic chambers (0.59 L). Each chamber was equipped with two pumps and an oxygen probe (FDO 925-3

dissolved oxygen probe, WTW, USA) connected to a Multi 3430 Multiparameter (WTW, USA) that recorded the oxygen concentration within the chamber every 30 seconds. One of the two pumps was continuously turned on and allowed both an homogenization of the water within the chamber and a circulation of water on the oxygen probe (Fig. 1). The second pump allowed renewing of the water within the chamber. This renewing pump was automatically activated for 5 minutes every 25 minutes, the system thus allowed a succession of cycles of 20-min incubation followed by 5-min renewing period during all the experiments. For all experiments, 5 out of the 6 chambers contained 4 animals (either mussels or scallops) and the 6^{th} one was used as a control and only contained empty shells.

Oxygen concentration within the experimental tank was controlled by injection of nitrogen into the seawater. For this purpose, nitrogen was injected in the inflowing water of a 1000 L/h pump, which pumped the water through an acrylic chamber allowing an efficient gaseous exchange between water and nitrogen. An oxygen probe (FDO 925-3 dissolved oxygen probe, WTW, USA) measured continuously the oxygen concentration within the experimental tank and was interfaced with a computer that controlled the injection of nitrogen by the mean of an electric-valve. This system allowed controlling the oxygen concentration within the tank at any desired value.

Hypoxia exposure and respiration measurement procedure

Before each assay, chambers and oxygen probes were carefully washed in order to limit bacterial metabolism within the chambers. Animals were then removed from their acclimation tank, gently cleaned from their fouling and transferred in respiration chambers. Once measurements were started, maximum O_2-saturation level was maintained for 2 hours before being decreased by stages (75, 50, 40, 30 and 20% dissolved O_2) of 2 hours to a final concentration of 10% dissolved oxygen. Whole measurements were repeated twice for each species and at each temperature (10, 18 and 25°C), leading to a total of 10 replicates for each condition (species/temperature). For each incubation (time period between two water renewals), the slope of the linear regression for oxygen concentration values (mg O_2 L^{-1}) over the time of incubation (expressed in h) was calculated. Individual respiration rate value (mg O_2 h^{-1}) was then

subsequently obtained by multiplying the absolute value of the slope (mg O_2 L^{-1} h^{-1}) by the volume of water in the measurement chamber (in L) and divided by the number of animals in the chamber (4). Volumes of water in the chambers were calculated by deducting the volume of animals in the chamber to the volume of the chamber.

Because average size of animals within the chambers were slightly variable, respiration rate were standardized to a standard shell weight of 5 grams using the Bayne *et al*. (1987) formula :

$$Ys = \left(\frac{Ws}{Wm}\right)^{b} \times Ym$$

where Ys is the respiration rate for an individual of standard shell dry weight Ws, Ym is the measured respiration rate for an individual of shell dry weight Wm and b the weight exponent for the respiration rate function. Reviews concerning weight exponents for respiration calculated for several bivalves (Savina & Pouvreau, 2004) showed that average weight exponents are generally around 3/4 for respiration, these value were thus chosen for standardization of respiration rates.

Estimation of regulation and P_CO_2

In order to estimate regulation percentage as well as the critical point in oxygen (P_CO_2), respiration rates were expressed as a percentage of the maximum value. Regulation was then estimated according to Hicks & McMahon (2002), i.e. area under the curve (respiration rates (%) ~ oxygen saturation) were integrated and divided by the value that would be obtained by a perfect regulator (displaying a respiration of 100% whatever the level of oxygen saturation). Furthermore, a segmented linear model was fitted to the value, allowing estimation of a "break-point", dividing the curve into two linear parts (Muggeo, 2003, 2008). All data treatment and subsequent statistical analyses were performed using R software (R Core Team, 2013). Data were tested for normality using Shapiro test, as the normality condition were not respected (p-value <0.05), a Kruskal-Wallis Rank Sum Test were performed and a paired comparison by Steel-Dwass test was used as a post-hoc when necessary.

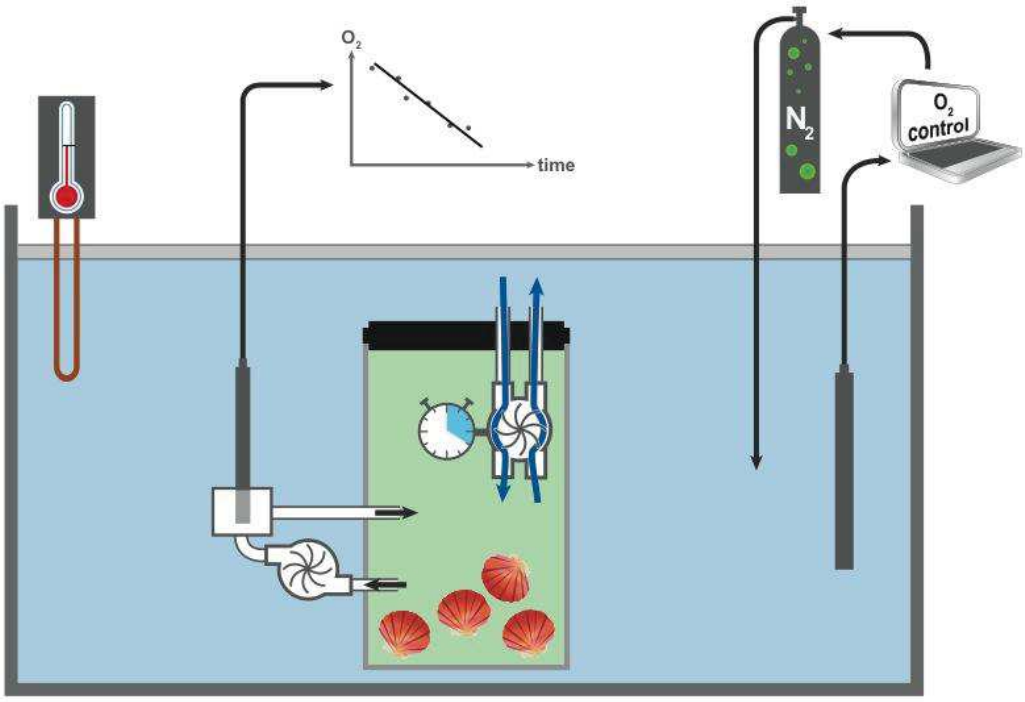

Figure 1: Scheme of the installation for measuring oxygen consumption under hypoxia. A chamber containing 4 animals is represented with its 2 pumps: one for renewal from the tank (turned on for 5 min every 25 min) and another for water circulation into the chamber, passing through an oxygen-sensing probe. The decrease of oxygen recorded from this probe allows calculation of oxygen consumption of animals. In the tank, oxygen concentration is computer-controlled via nitrogen injection into the water with oxygen concentration feedback given by oxygen sensing probe plugged to a computer.

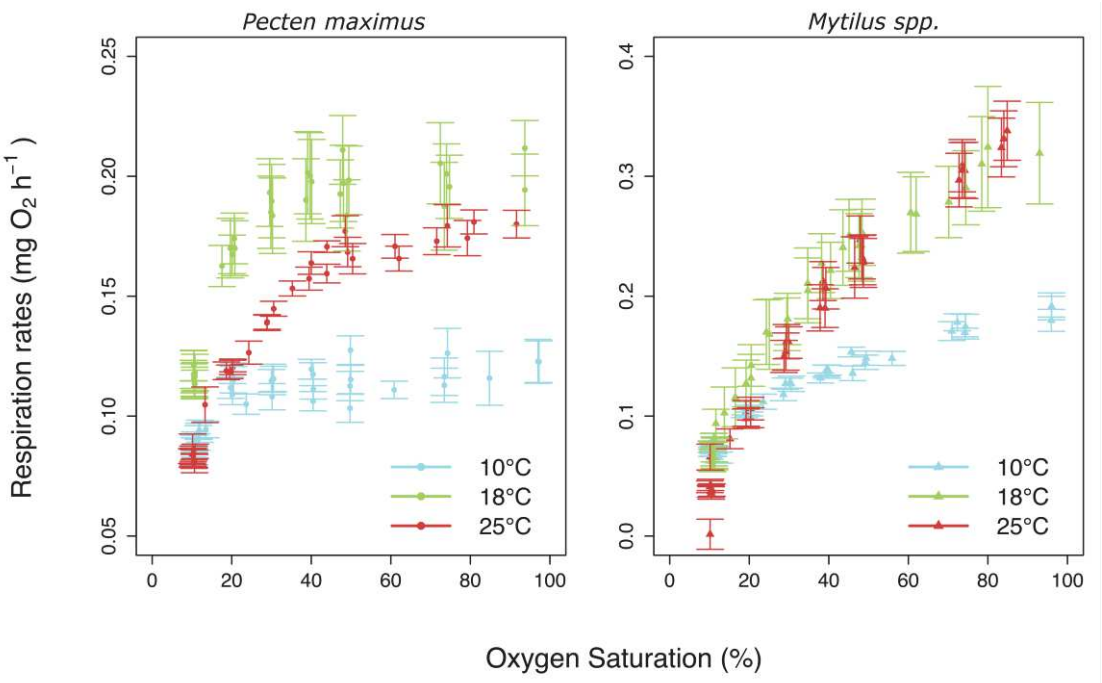

Figure 2: Respiration rates (in mg O_2 h^{-1}) of *Pecten maximus* and *Mytilus spp.* as a function of oxygen saturation at 3 temperatures (10°C, 18°C and 25°C). Bars represent standard error of the mean of 10 individuals (except *P. maximus* 10°C, 9 individuals).

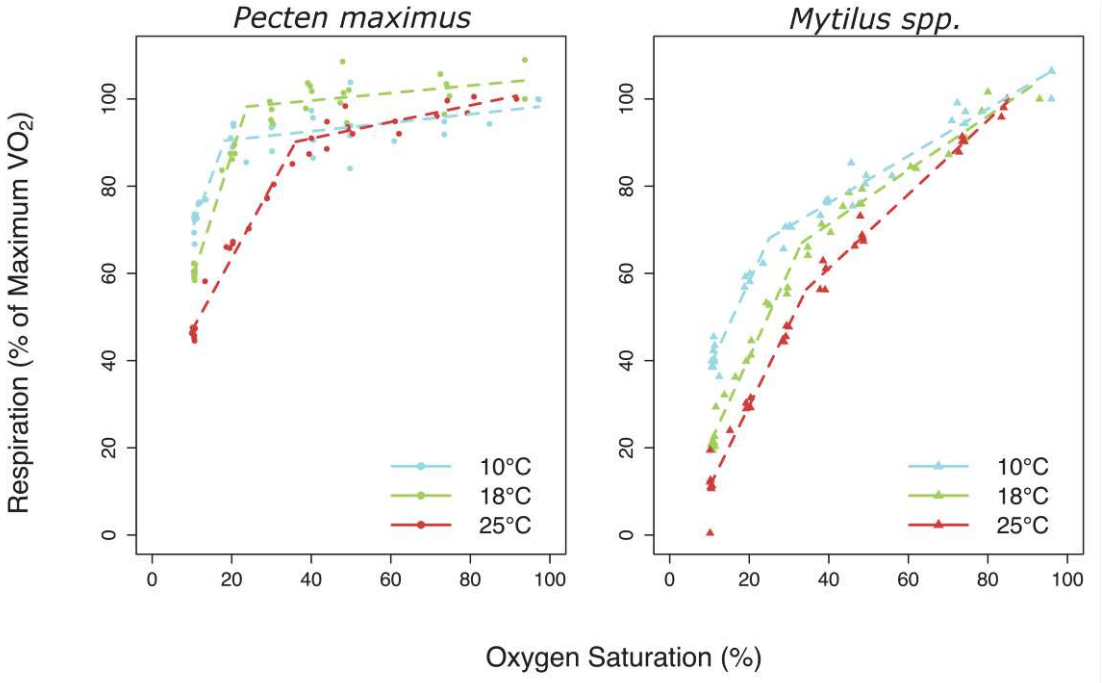

Figure 3: Respiration rate (expressed as a percentage of the maximum value) of *Pecten maximus* and *Mytilus spp.* as a function of oxygen saturation at 3 temperatures (10°C, 18°C and 25°C). Dashed lines represent a linear segmented model fitted to the values (see text for details).

Effects of temperature on respiration rates

Results showed that, at high O_2-saturation levels (80-100%), respiration rates were significantly higher at 18°C and 25°C than at 10°C for both *Mytilus spp*. and *P*. *maximus* (Steel-Dwass test, p-value <0.05, fig. 2). Additionally, at these same saturation levels, a significant decrease in respiration rates between 18°C and 25°C was observed for *P*. *maximus* (Steel-Dwass test, p-value = 0.03) but not for *Mytilus spp*. (Steel-Dwass test, p-value = 0.45). When oxygen level decreased, the reduction of respiration observed in *Mytilus spp*. between high (80-100% of saturation) and low (10-15% of saturation) oxygen level was more important for high temperatures (-77% at 18°C and -87% at 25°C) than at 10°C (-61%). Although less pronounced, similar results were observed for *P*. *maximus* (-25%, -42%, -52% at 10°C, 18°C and 25°C, respectively).

Segmented linear regression

As compared to a linear model, segmented linear regression fitted more accurately respiration rate data (expressed as percentage of maximum value) for both species at all temperatures, as indicated by the systematically lower BIC value and residual sum of squares for this model (fig. 3, table 1). In *P*. *maximus*, breakpoints estimated by the segmented linear model, corresponded to oxygen levels at which the organisms switched from respiration rates lowly dependent on ambient oxygen level, (slight decrease in respiration rates as oxygen saturation decrease; 2^{nd} segment slope, table 2), to respiration rates that are highly dependent on ambient oxygen concentrations (1^{st} segment slope, table 2). Such a segmented profile described the decrease in respiration rates as oxygen saturation decreased in *Mytilus spp*. However, for oxygen levels above the breakpoint value, the observed respiration rates decrease was more important than in *P*. *maximus* and the differences of slopes between the two parts of the segmented linear model appeared lower (Table 2). According to our results, breakpoint values were positively influenced by temperature for both species. However, this effect was more pronounced for *P*. *maximus*, as the breakpoint was twice more important at 10°C than at 25°C (from 18 to 36%, table 2).

Table 1: Model fitting comparison for linear and segmented linear regression for respirations rates expressed as a percentage of maximum value (%) as a function of oxygen saturation (% O_2) for each species (*Pecten maximus* and *Mytilus spp.*), at the three temperatures tested. See fig. 2 for a graphical representation. A lower value of Residual sum of squares or BIC (Bayesian Information Criterion) indicates a better fit.

	Segmented linear model		**Simple linear model**	
	residual sum of squares	BIC	residual sum of squares	BIC
Pecten maximus				
10°C	4.01	210	7.24	246
18°C	3.27	185	11.76	265
25°C	2.70	177	9.07	255
Mytilus spp				
10°C	2.97	189	6.86	243
18°C	2.92	183	8.19	248
25°C	3.16	183	6.71	228

Table 2: Estimation of the parameters of segmented linear regression between respiration rates (expressed as a percentage of the maximum value) and oxygen saturation (%O_2) for *Pecten maximus* and *Mytilus spp.* at three different temperatures (10°C, 18°C and 25°C). Break points are values of oxygen saturation separating the data into two linear models. Slope is the estimated value of the slope for linear model for part 1 (0% oxygen – break-point) and part 2 (break-point – 100%), respectively. Values after ± are confidence interval at 95%.

	Break-point (%0_2)	Part 1		Part 2	
		Slope	Regul. (%)	Slope	Regul. (%)
Pecten maximus					
10°C	18.3 ± 8.5	2.46 ± 2.81	55.7 ± 28.5	0.10 ± 0.07	77.0 ± 11.2
18°C	23.8 ± 1.8	2.87 ± 0.40	53.7 ± 6.1	0.09 ± 0.07	84.9 ± 6.1
25°C	36.1 ± 2.8	1.66 ± 0.15	56.1 ± 5.3	0.19 ± 0.09	89.2 ± 8.5
Mytilus spp.					
10°C	25.0 ± 3.3	1.97 ± 0.35	42.4 ± 5.5	0.54 ± 0.06	81.5 ± 5.1
18°C	33.2 ± 3.3	1.99 ± 0.21	35.2 ± 5.3	0.62 ± 0.08	85.0 ± 8.3
25°C	34.4 ± 4.7	1.86 ± 0.20	24.2 ± 1.2	0.85 ± 0.09	83.4 ± 10.6

Oxygen uptake regulation

For each segment of the segmented linear regression, an estimation of regulation was deduced from the area under the curve (fig. 3). For both *P. maximus* and *Mytilus spp.*, regulation percentages were very different between the two segments of each curve. Above the breakpoint, regulation values were very similar whatever the temperature or the species and a strong decrease occurred for both species for level of oxygen below the breakpoint. But, for low O_2 saturation levels (0% - breakpoint), differences between species were observed. Regulation values were similar for *P. maximus* but were slightly lower for *Mytilus spp.* at high temperature. Overall regulation values (fig. 4) were high at all temperatures in *P.*

maximus with a slight decrease at 25°C (fig. 4; 94% at 10°C, 96% at 18°C and 86% at 25°C) whereas a constant decrease was observed as temperature increased (81%, 76%, and 63% at 10°C, 18°C and 25°C, respectively) in *Mytilus spp*.

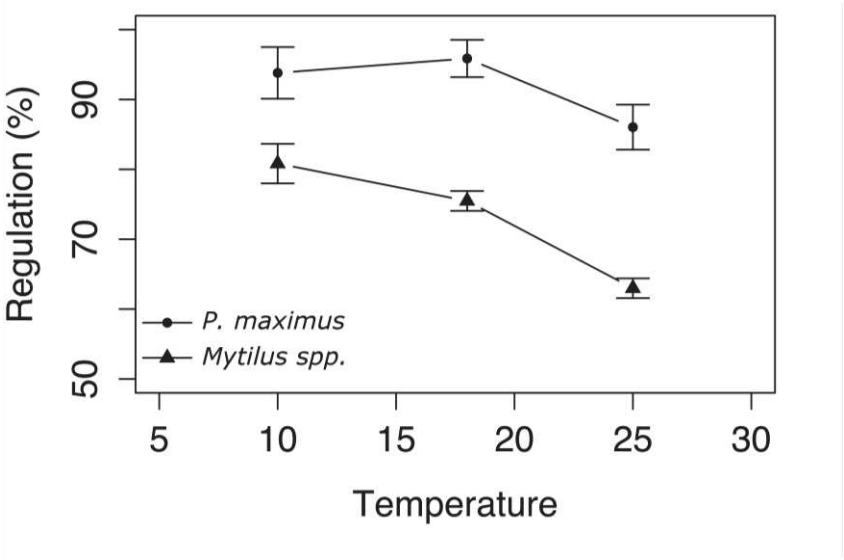

Figure 4: Estimation of the overall respiration rates regulation under declining oxygen level for *P.maximus* and *Mytilus spp.* at the 3 tested temperatures. Values are means and bar represent standard error (n=10).

Discussion

Range of optimal thermal window

The optimal thermal window of an organism is marked out by "Pejus temperatures", beyond which the aerobic performance is reduced (Pörtner, 2001). *P. maximus* respiration rates at maximum oxygen saturation and 25°C were significantly lower than at 18°C, indicating a decrease in aerobic performance and indicating that the "Pejus temperature" was outreached and consequently *P. maximus* was out of its optimal thermal window. In contrast, *Mytilus spp*. may not be out of its optimal thermal window at 25°C, as increase in respiration rates in oxygen-saturated water were almost equal at 18°C and at 25°C and superior to the values measured at 10°C. However, because no clear increase in oxygen uptake was observed between 18 and 25°C, we hypothesize that maximum respiration rates were reached and that 25°C might be close to the upper Pejus temperature. This hypothesis is supported by work of Jansen *et al*. (2009) who determined the upper thermal limit to be between 27°C and 30°C for *Mytilus galloprovincialis*. The three temperatures used in this study were chosen to reflect both temperatures experienced in the field (10°C in winter and 18°C in summer) and a more stressful thermal condition (25°C). Marine animals generally live close to their upper thermal limit (Hughes *et al*., 2003; Anestis *et al*., 2007) and the decrease in respiration rates observed between 18°C and 25°C for *P. maximus* are along those lines, highlighting the vulnerability of marine organisms to climate change. *Mytilus spp*. seems to cope better with high temperatures, reflecting adaptation to more elevated temperature which can be explained by its intertidal habitats as organisms thermal tolerance is correlated with their vertical position along the subtidal to intertidal gradient (Somero, 2002).

Responses to hypoxia and their alterations by thermal stress

The range of optimal thermal window is determined by the ability of organisms to sustain oxygen needs for aerobic metabolism (Pörtner, 2001). Ambient hypoxia reduces oxygen availability and thereby thermal tolerance (Pörtner, 2001; Pörtner & Lannig, 2009). As these two factors are closely tied, changes in temperature have a major impact on organisms responses to hypoxia (Hicks & McMahon, 2002; Jansen *et al*., 2009; Pörtner, 2012). In the absence of thermal stress, *Mytilus edulis* and *M. galloprinvincialis* were described as poor to moderate oxyconformers, faintly

regulating their oxygen uptakes under declining oxygen concentration (Bayne, 1971a, 1971b; Famme & Kofoed, 1980; Davenport, 1983; Bayne *et al*., 1987; de Zwaan *et al*., 1991), and so was *P*. *maximus* (Brand & Roberts, 1973). Nevertheless, our results indicated that *P*. *maximus* and *Mytilus spp*. displayed two very contrasting responses to hypoxia. When O_2 concentration decreased, *P*. *maximus* maintained a high respiration rate, before switching to oxyconformity. In *Mytilus spp*., the respiration rate moderately decreased as soon as the oxygen level started to fall, then eventually, like *P*. *maximus,* switched to oxyconformity but at a higher oxygen threshold. The point at which organism flip to oxyconformity has been defined as the critical point in oxygen (PcO_2), which is represented in the present study by a breakpoint in respiration rates when O_2 saturation decreases. This transition to oxygen-dependent respiration rates goes along with the setting up of the anaerobic metabolism (Grieshaber *et al*., 1988; Pörtner & Grieshaber, 1993). The two different patterns observed in this study illustrated the difference in adaptation strategies to hypoxia between species. Mussels live in the tidal area, where they experience frequent aerial exposures. Instead of trying to regulate their respiration rates at a high level, mussels rely on a regulated shutdown of high-energy costs mechanisms (Pörtner & Lannig, 2009) and on some level of aerial respiration (Tagliarolo *et al*., 2012). Unlike *Mytilus spp*., *P*. *maximus,* which is usually less exposed to hypoxia, maintains its respiration rates high in order to maintain a full aerobic metabolism. *Mytilus spp*. and *P*. *maximus* not only differed in their primary response to hypoxia, but also in their response to combined hypoxia and heat. Indeed, *P*. *maximus* exhibited high regulation rates at the three different temperatures tested with a slight decrease at 25°C associated with a concomitant strong increase in PcO_2. *P*. *maximus* ability to maintain a steady respiratory state over decreasing oxygen level, relies on both enhanced ventilation and heartbeat rates (Brand & Roberts, 1973; Pörtner & Grieshaber, 1993) until a failure of oxygen transport system occurs at the PcO_2 level, which triggers anaerobic metabolism. The rise of oxygen needs at more elevated temperatures causes failure to come at higher level of oxygen saturation, explaining the more elevated PcO_2 observed. The patterns observed for *Mytilus spp*. were much more different with a marked decrease in regulation rates and a more modest increase in PcO_2. *Mytilus spp*. regulation capacity was reduced, and this ability decreased as the temperature rose. Jansen *et al*. (2009) showed that at 27°C, *Mytilus galloprovincialis* did not regulate its respiration rates anymore. Overall, during hypoxia, *P*. *maximus* exhibited a more

important oxyregulation capacity that was maintained over a larger range of O_2 concentrations, as compared to *Mytilus spp*.. When acclimation temperatures increased, both species showed a decrease in regulation capacity. This decrease went along with a reduction of aerobic performances, especially for *P*. *maximus* as traduced by a strong increase of the PcO_2 at 25°C.

Interest of segmented linear model for comparison of species responses to hypoxia

Species are usually classified between the so-called "oxyregulator" and "oxyconformer", but these categories are rather a quality factor than a quantitative one. If they can be useful as a first approach, they cannot describe the hypoxia response in terms of quantity and, as a consequence, it can be difficult to compare species responses based solely on these two categories. To overcome this difficulty, the concept of PcO_2 was introduced by Grieshaber *et al*. (1988). It constitutes a useful parameter in order to compare species (Pörtner & Grieshaber, 1993), allowing a more accurate comparison based on a quantitative value. Nevertheless, some species could share the same PcO_2 but respond to hypoxia in a completely different way. In their works, Hicks & McMahon (2002) and Alexander & McMahon (2004) added a regulation percentage as a practical statistical feature to compare species response to hypoxia, reflecting the ability to regulate oxygen uptake under hypoxia. Hicks & McMahon (2002) classified oxygen regulator ability as follows: $\geq$ 90% indicating excellent O_2 regulation, 80 - 89% indicating good O_2 regulation, 70 - 79% indicating moderate O_2 regulation, 60 - 69% indicating poor O_2 regulation, and < 60% indicating no O_2 regulation. These features can be particularly useful to compare species as well as the effect of additional/other factors, such as temperature.

In our study, PcO_2 and regulation percentage were easily estimated by using segmented linear models. As a consequence, efficient comparisons were made between mussel and scallop responses and we believe that this approach could be useful for further studies in order to describe and compare the responses of different species to hypoxia.

Conclusion

In the present work, further proofs were given that marine bivalves live close to their upper thermal limit. At 25°C, results indicated that the subtidal *P. maximus* was outside its optimal thermal window, whereas the intertidal *Mytilus spp*. seemed to cope better with a temperature increase, which is consistent with their different vertical tidal zonation.

Regarding hypoxia response, *P. maximus* and *Mytilus spp*. displayed different patterns. *P. maximus* exhibited a more important oxyregulation capacity that was maintained over a larger range of O_2 levels compared to *Mytilus spp*., indicating that both species rely on different strategies to survive temporary hypoxia. When acclimation temperatures increased, both species showed a decrease in regulation capacity that went along with a reduction of aerobic performances, especially for *P. maximus*.

Taken together, our results confirmed the vulnerability of marine organisms to global change. Our work also highlighted the usefulness of segmented linear models to analyze hypoxia respirometry data. Indeed, this approach allows an easy estimation of PcO_2 and regulation percentage and may be very useful for further studies aiming at describing and comparing the responses of different species to hypoxia, which is of particular importance in the current context of increasing frequency of hypoxic events, and of expansion of hypoxic areas.

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III/ Article n°4 :

Proteomic responses to hypoxia at different temperatures in the Great Scallop (*Pecten maximus*)

Article à soumettre (dec 2013)

Proteomic responses to hypoxia at different temperatures in the Great Scallop (*Pecten maximus*)

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Abstract

Hypoxia and hyperthermia are two connected consequences of the ongoing global changes, that constitute major threats for coastal marine organisms. We recently characterized the physiological response to hypoxia of *Pecten maximus* (Artigaud *et al*., In prep). In this paper, We used a proteomic approach to characterize the changes induced by hypoxia in *P. maximus* individuals subjected to three different temperatures, ie, 10°C, 18°C and 25°C. We did not observe any significant change induced by hypoxia in 10°C-animals. Contrastingly, 15- and 11- protein spots revealed differentially accumulated in 18°C- and 25°C- scallops, respectively. We also present biochemical data, ie octopine dehydrogenase activity- and arginine-assays, that showed that 25°C animals switched their metabolism towards fermentation, thus suggesting that this temperature is out of the optimal thermal window of scallops. In all, 11 proteins could be unambiguously identified by mass spectrometry. These proteins are implied in proteins modifications (eg. CK2, TBK1), metabolism (eg. ENO3) or cytoskeleton (GSN). The potential roles of these proteins in the thermal-dependent response of scallops to hypoxia are discussed.

Introduction

Temperature and oxygen availability are two of the most prominent abiotic factors impacting marine organisms in natural environments. Due to Global Change, both are currently experiencing severe changes (Harley *et al*., 2006; Diaz & Rosenberg, 2008). In particular, air temperature dramatically rose in the last decades, and this will most probably continue during the next century (IPCC, 2013). Interactions between climate and ocean are complex. However, most models predict that the sea surface temperature will follow the lead. On the other hand, the extent, severity, and persistence of marine hypoxic zones, i.e. the areas where oxygen availability is reduced, have been observed to be increasing on an exponential basis (Diaz & Rosenberg, 2008; Vaquer-Sunyer & Duarte, 2008). Hypoxia and temperature are not independent factors, as the temperature has a major effect on the development of hypoxic areas through the eutrophication process.

These major changes in marine environments are a threat for marine organisms, and especially for non-mobile benthic animals, such as bivalves. Bivalves are a class in the Mollusca phylum which was qualified as "evolutionary successful" (Gould & Calloway, 1980; Miller, 1998). From their appearance on Earth, some 530 million years ago, they eventually came to dominate over brachiopods and are nowadays often the major macrofauna on rocky substrates of littoral and shallow sub-littoral (Bayne, 1976). Their ecological significance stems from their key roles in coastal ecosystems: they are major calcium and carbon accumulators, they link primary producers (bacteria and phytoplankton) to higher organisms in marine food-networks and are responsible for filtration of the water body in tidal zones (Newell, 2004). In addition to their ecological importance, some species, such as *Pecten maximus*, have acquired a non-trivial economic value through their utilization as food-sources for human populations. In a context of global change, *P. maximus* that lives under the tidal area, as a non-mobile species, has therefore to adapt to the currently changing environments.

Impacts of declining oxygen concentrations and variations in temperature have a synergistic effect on the physiology of marine organisms. Specifically, thermal windows are limited by the capacity of marine organism to sustain a rise in aerobic demand (Pörtner, 2001). The interaction between these factors goes both ways, and thermal stress also reduces the adaptation capacity of organisms to hypoxia (Pörtner,

2005). As molecular oxygen (O_2) is required by most organisms in order to achieve essential metabolic processes, a decrease in such a crucial component is a major concern for organisms. Nevertheless, all organisms do not display the same responses to an oxygen decline in their environment. Based on the evolution of oxygen consumption in a context of decreasing oxygen concentration, marine organisms were divided into two categories according to their physiological response. Animals whom oxygen consumption is relatively stable when oxygen concentration falls were called "oxyregulators"; conversely, animals unable to maintain their oxygen consumption were classified as "oxyconformers". However, oxyregulators eventually switch to oxyconformity below a certain level of oxygen. This level of oxygen, the PcO_2 (critical point in oxygen) also corresponds to the setting up of anaerobic metabolism. Hicks & McMahon (2002), added a regulation percentage estimated by integrating area under the curve (respiration rates (%) ~ oxygen saturation) divided by the value that would be obtained by a perfect regulator (displaying a respiration rate of 100% whatever the level of oxygen saturation), this parameter thus reflects the ability to regulate oxygen uptake under hypoxia.

Regulation percentages and PcO_2 were estimated for *P. maximus* in a previous study at 10°C, 18°C and 25°C (article 3). Results showed that PcO_2 increases with temperature along with a decline in regulation, indicating an altered response to hypoxia at higher temperatures.

In order to further investigate *P. maximus* response to hypoxia at different temperatures, proteomics is a method of choice as changes in proteins abundances represent modifications of the molecular phenotype of the cell and therefore functional changes (Feder & Walser, 2005). The molecular responses of *P. maximus* to long-term changes in water temperature will be addressed in another work (article 2). The present study focuses on the responses to short-term hypoxic stress (24 h) as a function of water temperature. To assess the differences of regulation at three different temperatures, proteins expression between normoxic and hypoxic conditions were compared using 2-DE and proteins differentially expressed were analyzed by MS/MS. Furthermore, anaerobic metabolism was estimated through enzymatic assays of octopine dehydrogenase (ODH) activity and arginine content.

Several proteomic studies already described the effects of different stressors on bivalves (recently reviewed in Sheehan & McDonagh, 2008; Tomanek, 2011; Campos *et al*., 2012), as well as studies of hypoxia at the transcriptomic level (David

et al., 2005; Le Moullac *et al*., 2007; Sussarellu *et al*., 2010) and targeted proteomic approach (Guévélou *et al*., 2013). However, to our knowledge, the work presented here is the first study exploring the proteomic response to hypoxia in bivalves, and its connection with water temperatures.

Materials and methods

Animals Experimentation

Scallops aged of six-month (height average, standard deviation 35.5 mm, 2.6) were provided by the Tinduff hatchery (Brittany, France). Animals were divided into 3 groups and acclimated in separate flow-through tanks containing sand-filtered air-saturated seawater maintained at 10°C. After one week of acclimation, two of the tanks were heated (1°C per day) until they reached 18°C and 25°C, respectively. Temperatures in the 3 tanks were monitored continuously during the whole experiment thanks to temperature logger (EBRO, Germany). Each tank was equipped with a pump to ensure that seawater temperature was homogenous and care was taken to maintain an O_2-saturation level of 100% until the beginning of experimentation.

Hypoxia challenge and Sampling

For the hypoxia challenge, scallops were placed in a thermo-regulated tank in which O_2 concentrations were controlled by a computer through nitrogen injection. A feedback was provided to the computer by an O_2 sensing probe (FDO 925-3, WTW, USA) placed in the tank and nitrogen flow was adjusted in order to maintain O_2 levels to a set-value. Once in the experimental tank, animals were allowed to acclimate for at least 2 hours before oxygen level was decreased (within 1 hour) to the given set-value. Animals were left for 24 h in hypoxic seawater before being sampled. To ensure that animals were in hypoxic environment, they were exposed to a level of oxygen below their PcO_2 at the given temperature. PcO_2 in these conditions were determined in a previous study (article 3). Depending on their temperature of acclimation, animals were exposed as follows: animals acclimated at 10°C were exposed to 7.6% ± 0.3 of O_2 saturation (PcO_2 at 10°C: 18.3%), animals at 18°C were exposed at 7.7% ± 0.2 (PcO_2 at 18°C: 23.8%), and animals at 25°C were exposed at 14% ± 0.3 (PcO_2 at 25°C: 36.1%).

After 24-h of hypoxia challenge, animals were quickly dissected, muscles and gills were snap-frozen in liquid nitrogen and kept at -80°C until further analysis. Care was taken to minimize air exposure during the sampling. Scallops acclimated at the same temperature but maintained in normoxic conditions were dissected in similar conditions.

Determination of ODH activity and Arginine content

Muscles were crushed with a mixer mill (MM400, RETSCH, Haan, Germany) and kept frozen using liquid nitrogen. For octopine dehydrogenase (ODH; EC1.5.1.11) activity, muscle powder was homogenized on ice with an Ultra-Turrax (Model Pro 200, PRO Scientific Inc., Oxford, USA) after adding a 6-fold volume (w/v) of homogenizing-buffer (20 mM Tris–HCL, 1 mM EDTA, 1 mM DTT, pH 7.5). The homogenate was centrifuged for 15 min at 12 000 g and 4°C. ODH activity was then determined in supernatants according to Livingstone *et al*. (1990). The absorbance decrease of $NADH_2$ at 340 nm was recorded over 10 min in 15 s intervals in a fluo-spectrometer microplate reader (POLARStar Omega, BMG Labtech) at 25°C. No activities were recorded in the absence of L-arginine (thus showing the lack of lactate dehydrogenase activity).

For determination of arginine contents, muscle powder was homogenized on ice with an Ultra-Turrax after adding a 6-fold volume (w/v) of 0.5 M perchloric acid. The homogenate was centrifuged for 15 min at 12 000 g and 4°C and the supernatant of each sample was neutralized with 2 M KOH and centrifuged for 5 min at 12 000 g and 4°C. Arginine contents were determined enzymatically according to Gaede & Grieshaber (1975), except that the incubation time in the presence of a purified ODH (purchased from Sigma-aldrich) was prolonged to 2 hours, to allow complete reaction of all the arginine in the samples. The fluorescence of $NADH_2$ was measured in a fluo-spectrometer microplate reader (355nm excitation / 460 nm emission) and arginine contents were deduced from standards of arginine (0 – 120 µM).

Proteins extraction

Gills were crushed as described for the determination of ODH activity and arginine content. One hundred milligrams of the obtained powder was homogenized in 100 mM Tris-HCl (pH 6.8) with 1% of Protease inhibitor mix (GE Healthcare), centrifuged (4°C, 50 000 g, 5 min) and supernatants were pipetted in other tubes. Nucleic acids were then removed (nuclease mix, GE Healthcare, following manufacturer's instructions). Samples were precipitated at 4°C using TCA 20% (1/1:v/v, overnight). After centrifugation (4°C, 20 000 g, 30 min), pellets were washed with acetone 70% and re-suspended in Destreak buffer (GE Healthcare)

containing 1% IPG (pH 4–7, GE Healthcare). Protein concentrations were determined using a modified Bradford assay (Ramagli, 1998), and all samples were adjusted to 200 µg of proteins in 250 µl.

Two-dimensional electrophoresis

Prior to isoelectric focusing, IPG strips (pH 4–7, 13 cm; GE Healthcare) were passively rehydrated with 250 µl of protein solution in wells for 14 h. Isoelectric focusing was conducted using the following protocol: 250 V for 15 min, 500 V for 2 h, gradient voltage increase to 1000 V for 1 h, gradient voltage increase to 8000 V for 2 h30, 8000 V for 3 h, and finally reduced to 500 V (Ettan IPGphor3, GE Healthcare). To prepare for the second-dimension SDS-PAGE electrophoresis, strips were incubated in equilibration buffer (50 mM Tris-HCl pH 8.8, 6 M urea, 30% glycerol, 2% SDS and 0.002% Bromophenol Blue) for two 15 min periods, first with 1 $g.l^{-1}$ dithiothreitol and then with 48 $g.l^{-1}$ iodoacetamide. IPG strips were then placed on top of 12% polyacrylamide gels, which were run in 10°C thermo-regulated device (SE 600 Ruby, Amersham Biosciences) at 10 mA per gel for 1 h and then 30 mA per gel until complete migration. Gels were subsequently stained with "Blue Silver" (Candiano *et al*., 2004) and destained with Milli-Q water for 48 h. The resulting gels were scanned with a transparency scanner (Epson Perfection V700) in gray scale with 16-bit depth and a resolution of 400 dpi.

Gel image analysis and statistical analysis of proteins abundances

Images were aligned and spots were detected and quantified using the Progenesis SameSpots software (version 3.3, Nonlinear Dynamics) using the automated algorithm. All detected spots were manually carefully checked and artifact spots were removed. Data were exported as raw values and statistical analyses were conducted in R (R Core Team, 2013) using the packages prot2D (Artigaud *et al*., 2013) and limma (Smyth, 2004). After normalization by quantiles, volume data were paired compared between hypoxia and normoxia conditions using moderated *t*-test at each temperature with 5 replicates per condition. A global correction by false discovery rate (fdr; Benjamini & Hochberg, 1995) was used, taking into account multiple comparisons issues and paired-comparisons correction. Spots with an fdr

threshold below 0.1 and an absolute fold change higher than 2 were considered as differentially expressed.

Mass Spectrometry

Proteins that changed in abundance in response to hypoxia at the different temperatures were excised from gels and prepared for analysis by mass spectrometry (MS). Gel pieces were first washed in 50 mM ammonium bicarbonate (BICAM), and then dehydrated in 100% acetonitrile (ACN). Gel pieces were vacuum dried, and rehydrated with BICAM containing 0.5 μg of Porcine recombinant trypsin (sequencing grade, Promega), and incubated overnight at 37 °C. Peptides were extracted from the gels by alternative washing with 50 mM BICAM and ACN, and with 5% formic acid and ACN. Between each step, the supernatants were pooled, and finally concentrated by evaporation using a speed vac. Samples were then resuspended in Trifluoroacetic acid (TFA; 0.1% in water). Peptides solutions were mixed with the α-Cyano-4- hydroxycinnamic acid (HCCA, 10 mg/ml of a ACN/TFA/water (60/4/ 36:v/v/v) solution), and spotted on a polished steel target using the dried droplet method. Peptides were then analyzed by Matrix-Assisted Laser Desorption Ionization Time-Of-Flight tandem mass spectrometry (MALDI TOF-TOF) in positive ion reflector mode, using an Autoflex III (Bruker Daltonics) mass spectrometer. The flexControl software (v3.0, Bruker Daltonics) was programmed to acquire successively PMF spectra and MS/MS from the dominant peaks. Mass spectra were analyzed with flexAnalysis (v 3.0; Bruker Daltonics) by applying the following conditions: TopHat algorithm for baseline subtraction, Savitzky-Golay analysis for smoothing (0.2 *m*/*z*; number of cycles: 1) and SNAP algorithm for peak detection (signal-to-noise ratio: 6 for MS and 1.5 for MS/MS). The charge state of the peptides was assumed to be +1. Porcine trypsin fragments were used for internal mass calibration.

Proteins were subsequently identified using PEAKS (v 5.3, Bioinformatics Solutions) using MS/MS-based identification and *de novo* sequencing. The search parameters against a custom-made database were set as follows: carbamidomethylation of cysteine was set as a fixed modification, oxidation of methionine and phosphorylation of serine, threonine and tyrosine were set as variable modifications, one missing cleavage during trypsin digestion was allowed and the

tolerance for precursor-ion mass tolerance was set to 1 Da. The database was constructed by combining *Pecten maximus* sequences from two sources: sequences from a RNAseq previous study and sequences from the REPROSEED project. Overall, the database comprised a total of 252 888 *Pecten maximus* ESTs. Proteins identifications were considered as unambiguous when a minimum of two peptides was matched with a minimum score of 20. False discovery rates were also estimated using a reverse database as decoy. EST database sequences were annotated by homology searches against a non-redundant database using Blast software from NCBI (http://www.ncbi.nlm.nih.gov/BLAST).

Results and Discussion

General patterns of proteomic response

In natural environments, marine mollusks are subjected to an array of stresses among which high temperature and hypoxia are of the most significant. The aim of this study was to decipher the proteomics signatures of hypoxia in animals previously exposed to three different temperatures: 10, 18 and 25°C. Hypoxia level was adjusted in function of the specific PcO_2 previously determined for each temperature. After a 24h-exposure to hypoxic seawater, all animals were dissected and proteins from the gills were subsequently extracted and analyzed by two-dimensional gel electrophoresis. No mortality was observed after hypoxia exposure except at 25°C, at which 50% of mortality occurred, traducing a severe hypoxic stress.

Table 1: Values of Log_2 Fold Change for spots differentially expressed between animals in hypoxic and normoxic conditions at the 3 temperatures tested (10°C, 18°C and 25°C)

	Log FC (Hypoxia/Normoxia)		
Spot	10°C	18°C	25°C
1	-0,13	-1,24	-0,84
2	-0,73	-1,74	-0,7
3	-0,8	-1,92	-0,49
4	-0,26	-1,23	-0,07
5	-0,48	-1,16	0,53
6	-0,31	-0,16	-1,02
7	0,22	-1,19	-1,15
8	0,03	-0,28	-1,16
9	-0,3	-0,74	-1,89
10	0,14	-0,31	-1,32
11	-0,01	-0,83	-1,98
12	-0,08	-0,39	-1,07
13	-0,21	-0,62	-1,1
14	-0,07	-0,15	-1,02
15	-0,79	0,35	-1,72
16	0,12	1,31	-0,12
17	-0,29	1,34	0,28
18	-0,38	1,81	-0,52
19	0,21	1,35	-0,21
20	0,34	1,3	-0,42
21	0,29	1,3	-0,32
22	0,15	1,18	0,07
23	0,15	1,61	0,02
24	0,5	1,42	-0,84
25	0,23	1,93	-0,95
26	0,52	-0,73	1,18

Overall 647 spots were identified in the 30 gels analyzed (Fig. 1). Proteins expression changes were examined between normoxia and hypoxia conditions, at the three different temperatures (10°C, 18°C and 25°C). Interestingly, at 10°C, we did not observe any significant changes in protein abundances between normoxia and hypoxia conditions (paired moderate t-test , fdr <0.1, absolute fold change >2), suggesting animals acclimated at 10°C may not suffer from the subsequent hypoxia. The transition to hypoxia should force animals to shift their metabolism towards anaerobic metabolism. The main fermentative metabolism in mollusk is the octopine dehydrogenase (ODH) pathway, that catalyses the condensation of the glycolytic pyruvate to arginine, leading to the final fermentative product octopine (Storey & Storey, 2005). This reaction allows restoring the pyridines reduced during glycolysis (ie $NADH_2$). As an attempt to evaluate the metabolic state of animals in our experimental conditions, the arginine contents and ODH activities were assayed in all conditions. The results, presented in figure 2, showed that arginine

contents significantly decreased during hypoxia at 18°C, thus showing the fermentative shift. By contrast, in animals maintained at 25°C prior to hypoxia, arginine amounts appeared already very low, and did not further decrease under hypoxia. Regarding the ODH activity, it was shown to increase significantly at 25°C, but no significant difference was shown between animals subjected or not to hypoxia.

At 18°C, 15 proteins were found to be significantly differentially accumulated between hypoxic and normoxic conditions, ten of which being accumulated during hypoxia whereas 5 appeared down-regulated (table 1). At 25°C only 1 protein was significantly up-regulated and 10 proteins were down-regulated. Surprisingly, only one protein (spot 7; Fig. 1, Table 1), displayed a similar changed accumulation profile under hypoxic conditions at both 18°C and 25°C.

In all, at the three temperatures tested, a total of 26 proteins were found to change in abundance between the hypoxic and normoxic conditions. All of them were subjected to MALDI TOF/TOF mass spectrometry, but only 11 matched to an EST sequence (Table 2). However, one EST could not be annotated using BLAST (sequence scallop_rep_c9282) and two other sequences were attributed to hypothetical proteins with no evidence for functions (Contig_RS_4016 and Contig_BAS15570).

Hypoxia response at 18°C

A Casein Kinase 2 alpha catalytic subunit (CK2α) was identified as accumulated in hypoxia at 18°C (spot 23, Fig. 1, Table 1 & 2). CK2 is an ubiquitous serine/threonine protein kinase found in eukaryotic cells, involved in a variety of cellular processes including metabolism, signal transduction and transcription (Mottet *et al*., 2005). Interestingly, CK2α have recently been observed to be overexpressed during hypoxia (Majmundar *et al*., 2010). Moreover, some authors proposed a key role of CK2α in hypoxia regulation, through the mediation of HIF-1 activity (Hypoxia Inducible Factor-1; Mottet *et al*., 2005; Hubert *et al*., 2006). HIF-1 is a major transcriptional regulator of cell responses under reduced oxygen level and it has been shown to be up-regulated under hypoxia in multiple species (Gorr *et al*., 2006; De Palma *et al*., 2007), including bivalves (Kawabe & Yokoyama, 2012). It seems that the activity of CK2α increases the transcriptional activity of HIF-1 without increasing

HIF-1 at the protein level (Mottet *et al*., 2005). Indeed, the CK2 protein could phosphorylate the p53 protein, a competitive inhibitor of HIF-1, thus targeting its degradation through the proteasome (Hubert *et al*., 2006). The increase of CK2 in hypoxia could thus lead to a down-regulation of p53 and thereby to an enhanced transcriptional activity of HIF-1. Furthermore, CK2 is also know to play a major role in inhibition of apoptosis through phosphorylation of the Bid protein, and subsequent inhibition of caspases pathway (Yamane & Kinsella, 2005; Ahmad *et al*., 2008).

Two proteins identified as down-accumulated under hypoxia at 18°C corresponded to a ZDHHC type palmitoyl transferase and a NADH cytochrome b5 reductase (spot 4 and 5, respectively). NADH-cytochrome b5 reductases are notably involved in the desaturation and elongation of fatty acids, and in cholesterol biosynthesis. These results suggested a decrease in unsaturated fatty acid content along with a diminution in sterol biosynthesis under hypoxic conditions. Of note, a similar decrease was observed in metabolomic studies in yeast (Gleason *et al*., 2011), rat (Bruder *et al*., 2004) and human aorta (Filipovic & Rutemöller, 1976). Fatty acids metabolism under hypoxia is poorly known. Nevertheless such a shift in fatty acid composition could have deep implications for membrane structure and/or energy metabolism. ZDHHC-type palmitoyl transferases catalyse the transfer of palmitate, a 16-carbon saturated fatty acid, on proteins. Their roles in hypoxia response are difficult to assess, as palmitate was reported to be linked to more than 100 proteins (Resh, 2006). Palmitoylation is a reversible modification of proteins mainly associated with their anchoring in biological membranes. In particular, this post-translational modification plays an important role in regulating ion channel localization and activity (El-Husseini & Bredt, 2002). This is accomplished by effects of palmitoylation on the ion channels, many of which are also receptors, as well as on the scaffolding proteins that bind to the channels (Smotrys & Linder, 2004). Therefore, the down-regulation of palmitoyl transferase could reflect a change in a signalling pathway under hypoxic conditions.

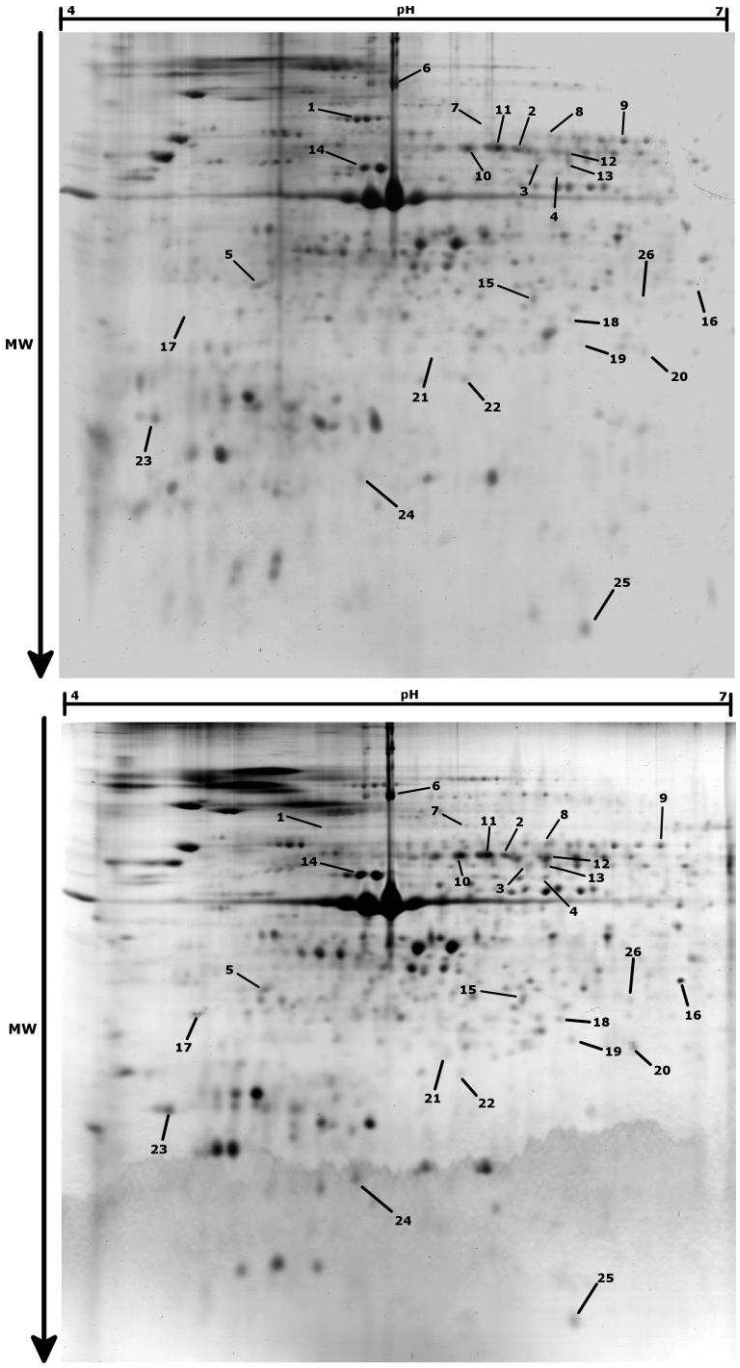

Figure 1: Representative bi-dimensional gels (pH 4-7, SDS-PAGE 12%) for *Pecten maximus* gills proteins in Normoxic (upper gel) and Hypoxic (lower gel) conditions at 18°C. Spots showing significant differential accumulation are arrowed.

Hypoxia responses at 25°C

Intriguingly, the proteomic responses of scallop gills to hypoxia at 25°C strikingly differed from that observed at 18°C. Indeed, only 11 spots appeared deregulated in this condition, which only 1 (non-identified) significantly differentially accumulated between normoxic and hypoxic conditions at both temperatures. It is noteworthy that at 25°C, *P. maximus* is out of its optimal thermal window (article 3) and the observed differences may be explained by the acute heat stress experienced at this temperature. Indeed, it could be hypothesized that the severe heat stress may prevent animals to develop the whole hypoxia response, which is consistent with results obtained at the enzymatic level (Fig. 2) and with the mortalities observed during this experiment.

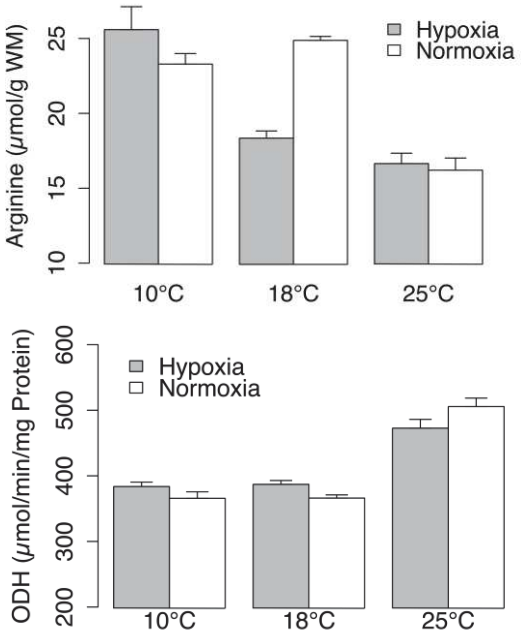

Figure 2 : Evolution of ODH activity (A) and arginine contents (B) at 3 different temperatures in normoxic condition (blank) and following 24 h hypoxia (grey) in *Pecten maximus*.

Among the proteins differentially expressed in hypoxia at 25°C, only 1 protein was up-regulated and it could not be identified by mass spectrometry. 5 of the 15 down-regulated proteins in hypoxia at 25°C were formally identified by mass spectrometry (spots 11 to 15).

Two identified proteins should be involved in signalling, ie the protein kinase TBK1 (spot 15) and a channel cotransporter of oligopeptides (Solute carrier family 15 member 4; spot 13). TBK1 promotes the TNF-induced NF-kB activation by phosphorylating I-kB, thus promoting its degradation and the subsequent activation of the NF-kB transcriptional regulator (Tojima *et al*., 2000). In addition, TBK1 was suggested to act as an inhibitor of apoptosis, as RNA interference analyses showed an increase in apoptosis induced by TNF (Fujita *et al*., 2003). As we showed that this protein is down accumulated, one could then expect that our experimental conditions should result in increased apoptosis of gill cells.

Gelsolin (spot 14) is an actin binding protein involved in the regulation of actin dynamics (Li *et al*., 2012). However, increasing evidence showed that gelsolin is a multifunctional regulator of cell metabolism involved in multiple mechanisms, independently of its actin regulatory functions (Sun *et al*., 1999; Silacci *et al*., 2004). One function worth noting, is the apoptotic activity of gelsolin through the gelsolin-HIF1α-DNase I pathway (Li *et al*., 2009). A decrease in gelsolin could thus be considered as anti-apoptotic, contrasting with the decrease of TBK1, which could be viewed as pro-apoptotic. Apoptosis is a complex phenomena and a matter of balance between "life" and "death" signals (Spyridopoulos *et al*., 1997). As an example, the TNF activation of the NF-κB pathway was shown to promote either pro- or anti-apoptotic effects, depending on the nature of the stimulus (Kaltschmidt *et al*., 2000). Therefore, further studies will be needed in order to elucidate whether an increase or decrease in apoptosis occurs under hypoxic conditions at 25°C in *Pecten maximus*.

Two down-regulated proteins identified are involved in energy metabolism, ie the enolase (spot 11) and the alpha-L-fucosidase (spot 12). Enolase is an enzyme involved in glycolysis, catalysing the conversion of 2-phosphoglycerate (2-PG) to phosphoenolpyruvate (PEP). Specifically the enolase 3 should be linked to glycogen utilization, as a mutation of the gene encoding enolase 3 in human (ENO3) has been reported to trigger glycogen storage disorder (Comi *et al*., 2001). Nevertheless, down-regulation of enolase 3 in hypoxic conditions has already been observed at the protein level in trouts (Wulff *et al*., 2012) and rats (De Palma *et al*., 2007). Considering the specific role of ENO3, this down-regulation could therefore mean a decrease in glycogen utilization, resulting from a shift in an hypometabolic state, as an attempt to limit energy consumption. Anaerobic metabolism is the major source of energy for marine organisms under hypoxia (Grieshaber *et al*., 1994). Therefore, as the transition to fermentative metabolism generally implies increased glycolytic fluxes to produce high amounts of pyruvate, the down-regulation of glycolytic enzymes is not expected in hypoxia. In mollusks, it should be recalled that anaerobic metabolism uses the ODH pathway, which allows for the condensation of pyruvate to arginine, while recycling the $NADH_2$ produced during glycolysis. Our arginine and ODH assays suggested that the animals subjected to 25°C prior to the hypoxia experiment had already shifted their metabolism towards fermentation. As 25°C is outside of the optimal thermal window for scallop, the animals most probably already encountered a

maximal oxygen demand, so as the animals could most probably not further modify their metabolism to adapt to hypoxia.

Fucosidases are enzymes associated with carbohydrates metabolism, as they removes terminal L-fucose residues present on the oligosaccharide chains of glycoconjugates (Johnson & Alhadeff, 1991). A wide variety of conjugates can be fucosylated, and fucosidases act on glycoproteins, glycolipids and glycans (Johnson & Alhadeff, 1991; Becker & Lowe, 2003). Fucosidases are located in lysosomes where their actions are required as the first step in degradation of glycoproteins containing complex N-linked chains (Varki *et al*., 1999). Therefore a decrease in hypoxia at 25°C of alpha-L-fucosidases could be part of an energy saving strategy by reducing the proteins turnover.

Table 2: List of *Pecten maximus* gills tissue proteins identified by MS/MS whose abundance change between Hypoxic and Normoxic conditions either at 18°C or 25°C (moderate t-test paired-comparison, fdr <0.1, absolute fold change >2).

#Spot	Score	%Cov.	Peptides Sequences	Assignation	Acc.	EST
1	21.34	4	IHS(+79.97)FC(+57.02)IS(+79.97)R.C SPSSMSWMR.C SFSAPPTPSR.G	hypothetical protein CGI_10020036 [Crassostrea gigas]	EKC39433	Contig_RS_4016
4	75.73	3	ALSSDRHSTVSR.T QPS(+79.97)IT(+79.97)PSR.C	Putative ZDHHC-type palmitoyltransferase 5 [Crassostrea gigas]	EKC28431	Contig_RS_3827
5	52.72	11	PVLPQS(+79.97)PR.C GSLSRGFS(+79.97)R.G	NADH-cytochrome b5 reductase-like protein [Crassostrea gigas]	EKC35862	Contig_RS_5999
11	117.50	7	AAVPSGASTGIYEALELR.G EANWGVM(+15.99)VSHR.A LGANAILGVSLAVC(+57.02)R.G M(+15.99)GSETYHHKK.G	enolase 3 [Salpingoeca rosetta]	XP_004994770	Contig_RS_391
12	91.71	2	VIPIFAER.C SSRS(+79.97)AS(+79.97)R.T	Plasma alpha-L-fucosidase [Crassostrea gigas]	EKC34412	Contig_RS_12960
13	49.83	2	VLYPLLAR.C VTMVMGC(+57.02)PRR.C QRC(+57.02)YAS(+79.97)R.T	Solute carrier family 15 member 4 [Crassostrea gigas]	EKC40000	Contig_RS_4977
14	160.47	4	NSINSGDVYILDLGR.G NIEVVEVPLSR.A AWDGAGQEPGIQIWR.A	gelsolin [Suberites ficus]	CAF21863	Contig_RS_292
15	40.72	1	LLYS(+79.97)RY(+79.97)IAR.T VWHSC(+57.02)NSR.C	Serine/threonine-protein kinase TBK1 [Crassostrea gigas]	EKC21054	Contig_RS_3221
21	300.00	9	DLYASLQSELK.C AIVLFVDGNADDANAAK.G	-	-	scallop_rep_c9282
22	85.73	2	YQGPFNAEDQGTVR.G VYDSVTWVGR.T NACCS(+79.97)SGAPCGAGGAGADLAD	hypothetical protein LOC100635635 [Amphimedon queenslandica]	XP_003383336	Contig_BAS_15570

			DCK.A			
23	43.80	3	GNPY(+79.97)IS(+79.97)LISRSQYH.T M(+15.99)PACLS(+79.97)VR.T	protein kinase CK2 alpha catalytic subunit [Mytilus galloprovincialis]	CBK38915	Contig_RS_4949

Concluding remarks

In conclusion, our results highlighted a strong temperature effect on the response of *P. maximus* to hypoxia. Very different proteomic signatures between normoxic and hypoxic conditions were observed at 10, 18 and 25°C. Indeed, although no proteomic phenotype was observed at 10°C, suggesting that the low energy demand due to hypoxia did not require extra proteins, we could identify 26 spots differentially accumulated at either 18°C or 25°C. We could sequence peptides from 11 proteins, and assign functions for 8 of them. The whole results suggested a down regulation of some parts of the energetic metabolism, and a role for apoptosis in the hypoxia response following thermal acclimation. Several proteins could be linked to HIF related metabolisms. Further studies will determine the exact role of these protein effectors of the response to hypoxia, with a special focus on their interaction with HIF and with apoptosis.

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Chapitre IV : Analyse écophysiologique et protéomique de *Pecten maximus* dans son environnement

I/ Introduction

Il est extrêmement important de replacer les approches physiologiques, et à plus forte raison moléculaires, dans un contexte environnemental. Dans ce cadre, nous avons dragué des coquilles en rade de Brest en novembre 2010, et les avons replacées sur le site de Lanvéoc, à 12 m de profondeur. Dans le cadre du projet CHIVAS, ce site a été instrumenté de manière à monitorer les principaux des paramètres physico chimiques susceptibles d'influencer la physiologie des mollusques. Les coquilles ont été récupérées à partir du printemps suivant. L'idée initiale était de comparer la physiologie et les signatures protéomiques de cette population avec une population évoluant en limite nord de l'ère de répartition de l'espèce, à Bjørnafjorden, en Norvège. Seule la partie protéomique a pu être réalisée dans ce dernier contexte.

La qualité et la quantité des ressources trophiques constitue l'un des facteurs environnementaux les plus importants influençant la physiologie des mollusques. Le premier travail présenté dans ce chapitre utilise une approche innovante pour tracer la quantité, la qualité et les variations temporelles des sources trophiques disponibles pour *P. maximus* en rade de Brest en combinant trois marqueurs trophiques : les pigments, les acides gras et les stérols.

Les principaux résultats obtenus, notamment les niveaux de DHA et de péridine dans la glande digestive et l'estomac, suggèrent que la coquille Saint-Jacques filtre activement certaines espèces de dinoflagellées, ouvrant de nouvelles perspectives dans la compréhension du régime alimentaire de cette espèce en situation naturelle. D'importantes différences de croissance ont été observées entre les populations de *P. maximus* évoluant à différentes latitudes, qui pourraient être liées avec ce régime alimentaire. Néanmoins, la comparaison avec la population norvégienne n'a malheureusement pas pu être effectuée.

Les différences de phénotypiques entre les populations évoluant en Norvège et à Brest soulèvent des questions quant aux caractéristiques génétiques de ces populations. Dans le cadre de cette thèse, nous avons entrepris de comparer les signatures protéomiques de ces deux populations. Cette étude a été permise par l'obtention d'un financement LabexMER, qui m'a permis d'effectuer un séjour à la station d'Austevoll, en Norvège, pendant ma thèse.

Afin de caractériser les signatures protéomiques des deux populations d'intérêt, des animaux de tailles similaires ont été échantillonnés, à une période de l'année correspondant à un état physiologique comparable pour les deux populations. D'autre part, afin de réduire le biais temporel et l'expression de protéines liées à des conditions environnementales particulières, les animaux ont été échantillonnés à deux semaines d'intervalle dans les deux sites.

Globalement, nos résultats ont montré l'expression différentielle de 23 spots protéiques. 11 ont pu être identifiées par spectrométrie de masse. La grande majorité de ces protéines font partie du cytosquelette (10 sur 11). Ainsi 8 protéines plus abondantes dans les animaux de la rade de Brest ont été identifiées comme étant de l'actine, tandis que 2 des protéines surabondantes dans la population nordique ont été identifiées comme étant de la filamine. La dernière protéine identifiée, accumulée dans les individus évoluant en Norvège, est l'élastase. Des différences aussi importantes, pour des protéines impliquées dans une fonction aussi essentielle pour la cellule que le cytosquelette, apparaissent étonnantes. Nous discutons ici d'un rôle éventuel de ces protéines dans d'autres fonctions que le seul soutien structural.

II/ Article n°5 :

Seasonal feeding behavior of the great scallop assessed by pigment, fatty acid and sterol analysis

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Seasonal feeding behavior of the great scallop assessed by pigment, fatty acid and sterol analysis.

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Abstract

In the present study we combined the use of pigments, fatty acids and sterols as food source biomarkers in a seasonal trophic study of a marine suspension-feeding invertebrate, the great scallop *P. maximus*. During eight months (March-October), a biweekly to twice-weekly sampling was conducted in the Bay of Brest (Brittany, France). Along with phytoplankton monitoring, seawater from the water column and the water-sediment interface was filtrated and three scallops were collected. Pigments concentration in the filters and in the stomach and rectum content of the scallops were analyzed in HPLC. Lipid extraction was also conducted on seawater filters and on the digestive gland (DG) tissue. Fatty acids form the polar and neutral fractions and sterols were isolated and analyzed in gas chromatography. Ingestion, assessed by the total amount of pigment in the stomach content was found to follow the phytoplankton dynamic in the water. Ingestion seemed to be more correlated to the dynamic of bottom water in the beginning of spring and to the water column for the rest of the study period. Fucoxanthin, a specific diatom marker, was the major pigment recorded in the gut of *P. maximus*. Peridinin, mainly found in dinoflagellates, occurred in high proportions compared to the low availability in the water. Proportions of these two pigments in the stomach and rectum content alternated in time. The same tendency was captured by the proportion of the 20:5n-3 and 22:6n-3 FA and the sterols 24 methylene and brassicasterol in the DG. Chlorophyceae and green macroalgae, traced by chlorophyll-*b*, 18:2n-6 and 18:3n-3 were found to be negatively ingested and accumulated. Markers of prymnesiophyceae (19'HF and 18:4n-3) were also registered in significant levels. Degradation pigments peaks measured in the gut of *P. maximus* were observed during low ingestion periods, just before or just after high feeding activity. Switches from one trophic source to another are discussed as well as the selectivity in feeding relative to the season.

Keywords: Food sources, Pigments, Fatty acids, Sterols, Trophic marker, *Pecten maximus*

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1. Introduction

Scallops are sessile suspension-feeding animals living half-buried on the sea floor. They rely on the availability of the trophic resource in their close surrounding water to obtain their food. As coastal environments are very changing and have strong seasonality, suspension-feeding bivalves have evolved to develop a plastic trophic niche (Rossi et al., 2004; Nerot et al., 2012). Moreover, bioenergetic studies require precise knowledge about the energy entry in an organism, which is necessary to complete physiological processes and metabolism work. This energy comes from one or several substrates (e.g. a mineral, an organic molecule or a prey) depending on the ecological position of the species and its feeding strategies. The determination of its trophic sources is essential to better comprehend the dynamics of energy intake and further the variability of any physiological functions.

The study of trophic sources makes use of various chemical, biological, biochemical or physiological indicators to identify the origin of the food for an organism. Among these markers, stable isotopes are often used (Rossi et al., 2004; Marín Leal et al., 2008). However, although very relevant to discriminate trophic position between superior taxa, they have not been reported to be good indicators of different classes of organisms in a same trophic level. To the contrary, pigments and lipids (fatty acids and sterols) have long been used to characterize classes, gender and even species of microalgae (Pastoureaud et al., 1995; Jeffrey, 1997; Loret et al., 2000; Louda et al., 2008 for pigments, Parrish et al., 1995; Soudant et al., 1996; Bachok et al., 2003, 2009 for fatty acids and Napolitano et al., 1993; Soudant et al., 1998a for sterols).

This study aims at better understanding the temporal qualitative and quantitative variations in trophic sources foraged by *P. maximus*, in an innovative approach combining three well established trophic markers: pigments, fatty acids and sterols composition, in the digestive gland and gut contents.

2. Material and methods

2.1. Study site and sampling

The study was conducted in the Bay of Brest (Brittany, France); where scallops dredged in the bay in November 2010 and then placed in the location of Lanvéoc (average depth of 12 m). From March 14th 2011 until October 24th 2011, three individuals of *P. maximus* were collected by scuba-diving every two weeks and twice a week (during April and May). Only animals over three years old and fully mature were selected for this study. The stomach content was collected in 2 mL Eppendorf tubes by squeezing the digestive gland (DG). In the same way, the gut content was obtained from pressing the rectum. Two aliquots of DG tissue were encapsulated into aluminium cups. DG content and tissue samples were fixed in liquid nitrogen and stored in -80 °C until sample treatment.

Seawater was also sampled using a five liter Niskin bottle at two meters under the surface all along the study period. An other set of samples was carried out from late April at the water-sediment interface using a 450 mL syringe. Species

composition of phytoplankton was determined both in the water column samples and for the water-sediment interface. A volume of 250 mL of each depth sample was fixed with acetic Lugol's. Quantitative and qualitative analyses were carried out from settled cells using an inverted phase microscope. Vacuum filtration of seawater was conducted on GF/F filters previously heated 6 h at 450 °C. Between 500 mL and 1.5 L of sub-surface seawater were filtrated on each sampling time ; the volume of filtrated bottom water ranged from 50 mL to 150 mL, depending on the filter clogging. Two filtrations were realized for both sampled levels and filters were stored in sealed aluminium folds at -80 °C until sample treatment. Simultaneous analyses of pigment composition of the seston from the water column and from the water-sediment interface were conducted on each sampling occasion, though they are not available for the two first months of the study. Nevertheless, the occurrence, timing and intensity of the spring phytoplankton at the bottom are documented in a previous study carried out at the same time scale and on the very same location (Chatterjee et al., 2013). Pigment and lipid composition of all samples (DG content, DG tissue of scallops and filters) were conducted as described hereafter.

2.2. Pigment analysis

The procedure used to identify and quantify pigments in the filters and in the DG contents is the LOV method, described by Claustre and Ras (2009). The extraction of pigments was realized from 200 µL of stomach content, 50 µg of rectum content and seawater filters from the two depths, in 100 % methanol enriched with vitamin E acetate (acting as internal standard, Sigma-Aldrich). Samples were disrupted by sonication using a S-4000 sonicator (Misonix Inc.) and stored at -20 °C. Sonication was repeated after two and four hours of extraction. Samples were centrifugated for 10 min at 3000 rpm (4 °C) and passed through 13-mm syringe filters (Puradisc, 0.2 µm, PTFE, Fischer). Pigment analyses were carried out on a complete Agilent Technologies 1200 series HPLC system, equipped with a ZORBAX Eclipse XDB-C_8 silica column (3x150 mm^2, 3.5 µm particle size) and a diode array detector, which permits automatic pigment identification based on absorption spectra. The elution was run at a flow rate of 1 OR 0.55 $mL.min^{-1}$ using solvent A (TetraButylAmmonium Acetate and methanol, 30:70 v/v) and solvent B (methanol) in the following elution procedure (min, solv. A, solv. B): (0, 90, 10), (22, 5, 95), (27, 5, 95), (28, 90, 10) and (33, 90, 10). Pigments optical densities were monitored at 450 nm (chloropigments and carotenoids) and at 667 nm (chlorophyll-*a* and derived pigments), automatically compared to the retention times of 15 pigment standards (DHI and Sigma-Aldrich) previously calibrated. The injection precision of the method is estimated at 0.4 %, and the effective limits of quantification for most pigments are low (0.0004 $mg.m^{-3}$ for chlorophyll-*a* and 0.0007 $mg.m^{-3}$ for carotenoids). The off-line version of the Chemstation software was used for verification and eventual correction of the peak integrations in each chromatogram.

2.3. Lipid analysis

Chemicals

High performance liquid chromatography (HPLC) grade solvents were purchased from VWR International. Boron trifluoride (BHT, 14 % by weight in methanol), tricosanoic acid (C23:0), cholestane and 37-component FAME mix were obtained from Sigma–Aldrich. Silica gel 60 (63-230 μm mesh) was purchased from Merck. Gas chromatography (GC) capillary column was a DBWAX (30 mm x 0.25 mm i.d.; 0.25 μm thickness) for FAME analysis and a Rtx65 (15 m x 0.25 mm i.d.; 0.25 μm thickness) for sterol analysis and were respectively obtained from Agilent and Restek.

Lipid extraction

Lipid extraction was conducted by resuspension of the filters or 200 mg aliquots of digestive gland (DG) previously grounded by ball milling under liquid nitrogen. Samples were put in glass tubes containing 6 mL of chloroform-methanol (2:1, v/v) and stored at -20 °C before analysis.

Separation

After centrifugation (2 min, 1000 rpm), lipid analyses were carried out on 1 mL of DG lipid extracts and on 5 mL of filter lipid extracts (due to the small amount of material in the seawater compared to the DG). Lipid extracts were then evaporated to dryness under nitrogen, recovered with three chloroform-methanol (98:2, v/v) washings of 500 μL each and deposited at the top of a silica gel microcolumn (Pasteur pipette of 5-mm inner diameter, plugged with glass wool and filled with silica gel 60 both previously heated at 450 °C and deactivated with 6 % water by weight). Neutral lipids (triglycerides, free fatty acid, free sterols and sterol esters) were eluted with 10 mL of chloroform-methanol (98:2 v/v) and polar lipids were eluted with 20 mL of methanol, and were both collected in 20 mL vials.

Transesterification

After evaporation to dryness under nitrogen, polar and neutral lipid fractions were recovered and transferred in 8 mL vials with three chloroform-methanol (98:2, v/v) washings of 1 mL each. Each neutral lipid fraction was then equally divided into two vials. The totality of the polar lipid fractions and half of the neutral lipid fractions were dedicated to fatty acid (FA) analysis whereas the other part of the neutral lipid fractions were dedicated to sterol analysis. As internal standards, 2.3 μg of C23:0 was added to the fractions dedicated to FA analysis and 2.5 μg of cholestane was added to the fractions dedicated to sterols analysis. After evaporation to dryness under nitrogen, 800 μL of boron-trifluoride (10-14 % BF_3 in methanol) was added to the fractions dedicated to FA analysis that were then vortexed and heated for 10 min at 100 °C (acid transesterification). For the sterol analysis, 2 mL of a sodium methoxyde solution (NaOH, 27 $\mu g.\mu L^{-1}$ in methanol) was added to the corresponding fractions, which were maintained under agitation for 90 min at room temperature (basic transesterification). Before GC analysis, lipid fractions were washed three times with 1 mL of distilled water and 800 μL of hexane and centrifugated (1 min at 1000 rpm) before

eliminating the aqueous phase each time. Finally organic phases were transfered into tapering vials and stored at -20 °C.

Gas chromatography analysis

Fatty acid were analyzed in a Agilent 6890 gas chromatograph equipped with an on-column injector and a flame-ionization detector, with hydrogen as carrier gas. They were identified by their retention times with reference to those of a standard 37-component FAME mix and other known standard mixtures from marine bivalves (Soudant et al., 1995) and designated following the formula C:Xn-Y where C is the number of carbon atoms, X is the number of double bonds and Y is the position of the first double bond counted from the CH, terminal. The sterols were separated by gas chromatography, in a Chrompack 9002 equipped with an on-column injector and a flame-ionization detector, with hydrogen as carrier gas. Sterols were identified by comparison of their retention time with standards as described in (Soudant et al., 1998a).

3. Results

3.1. Pigment concentration in the seston

First of all, the GC analysis of fatty acids (FA) and sterols in the seawater filters revealed extremely low quantities in the samples. Only the most prominent FA could be monitored, showing tiny peaks hardly observed on the chromatograms which could not be interpreted. Even after several tries to concentrate the samples we were not able to workout a descent set of data. This is probably due to a failure in the analytical rather than in the sampling or conservation process as the HPLC analysis of pigments produced exploitable results.

The general pattern of phytoplankton primary production during the season can be seen in Fig. 1, showing the dynamic of total pigment concentration in the seston from the water column as well as in the bottom water. Phytoplankton first bloomed at the water-sediment interface in April before seeing a development of microalgae in the water column the month after. In June, another important microalgae bloom occurred in the water column while pigment concentrations in the bottom water started to decrease. During the summer few events were recorded in the water column and dramatically low pigment amounts were found at the bottom. In the autumn period, a renewal of primary production was monitored both at the water-sediment interface and in the water column. The pelagic pigment concentration crashed down at the end of the studied period, while bottom production reached high levels, comparable to spring time. As chlorophyll-*a* is present in all marine algae species and because the goal of this study is to identify specific pattern of the dynamic of several algae classes, chlorophyll-*a* was removed from the total amount of pigments and each pigment quantity was therefore expressed as its contribution to the total amount of pigment without chlorophyll-*a*.

In more details, Fig. 2 shows that primary production started in 2011 at the water-sediment interface appeared at the same time as in the water column during the last week of March with an increase of the pigment concentration relying on variations of alloxanthin and peridinin concentration, both increasing up to 0.045 $\mu g.L^{-1}$. This increase was associated to the observation of cryptophyceae and dinophyceae (*Heterocapsa triquetra* and *Gyrodinium flagellare*). Then, the chlorophyll-*a* content of bottom water showed a critical peak in April, synchronous with blooms of *Navicula sp*. and *Fragillaria sp*., before starting to decrease at the end of the month. This primary production event is the most important of the season at this depth. The total pigment concentration in the pelagic water and at the water-sediment interface both increased in late April, mainly due to the elevation of fucoxanthin. In the same time, overlapping blooms of cryptophyceae and diatoms from the genders *Chaetoceros*, *Cerataulina* and *Pseudo-Nitzschia* were observed. Fucoxanthin was at its highest level in early May (0.8 $\mu g.L^{-1}$) in the water column, contributing to 33.6 % of the total of pigments, when blooms of diatom species (*Chaetoceros sp*., *Cerataulina pelagica* and *Dactiliosolen fragilissima*) were registered. Almost all pigments presented an increase during this bloom, except for the peridinin. The second high pigment concentration event was measured in early June and caused by an increase in fucoxanthin concentration from 0.1 to 0.8 $\mu g.L^{-1}$. This bloom was mainly constituted of the same diatom species than in May. From there, fucoxanthin concentrations remained at lower levels both in the water column and at the water-sediment interface, whereas chlorophyll-*b* started to increase from an average of 0.02 $\mu g.L^{-1}$ to a mean of 0.06 $\mu g.L^{-1}$ in June for the water column. The same occurred at the bottom from July, also accompanied by a similar increase of peridinin and zeaxanthin concentrations.

3.2. Pigment composition of the digestive contents

The pigment quantities measured in the stomach content were ten times as much as in the water. Furthermore, from the stomach to the rectum concentrations increased by a factor of five. The total quantity of pigment was well correlated with the availability in the water (Fig. 3). Chlorophyll-*a* was the main pigment in seawater, accounting for more than 50 % of the total pigment concentration in average in the water column and around 40 % at the sea bottom (Fig. 1). However in the gut of *P. maximus* it only represented 10 to 30 % (Table 1) and fucoxanthin was the major pigment found both in the stomach content and in the output of the digestive system (up to 55 and 50 % respectively). Thus the contribution of each pigment measured in the gut of the scallops was calculated as a proportion of the total quantity of pigment in the compartment considered. Peridinin was also highly present with a maximum occurrence at 43 % in the stomach and at 28 % in the rectum. The dynamic of these two prominent pigments were negatively correlated, as shown in Fig. 6. Fucoxanthin in the stomach showed highly fluctuating profile during spring, with sharp increases from 10-20 % up to 50-55 % in late April, mid-May, late May and early June. The minimum was observed on May 30th (11.4 %) and the maximum in April 28th at 53.6 %. As observed in the water, a significant increase in autumn

was registered. The proportion of fucoxanthin in the rectum was very similar to what was observed in the stomach, as shown by a linear regression coefficient of $r^2 = 0.78$ (Fig. 4). Peridinin showed a similar pattern, with a fluctuating spring period followed by low proportions in early summer and medium levels until autumn when it dropped to its minimum (2.4 %) on October 3rd. Maximum was reached on May 2nd at 42.3 %. Compared to fucoxanthin, the linear regression between the proportion of peridinin in the rectum and in the stomach was less significant ($r^2 = 0.41$). For instance, at the end of May as well as in July and August, the discrepancy between the two compartments was of 20 % between the entry and the exit of the digestive tract. The occurrence of chlorophyll-*b* was not observed in the gut of the scallops before an important peak of 13.5 % during June and July and a shorter one in mid-August (12 %). In the rectum, proportions during the first increase were found to be lower (10.5 %) and higher during the second peak (16 %). Nevertheless, the correlation between the two compartments was rather elevated ($r^2 = 0.76$). To a lesser extent, two peaks of 19'hexanoyloxyfucoxanthin (19'HF) were recorded, at 14 and 10 % respectively, in the stomach on April 11th and on May 2nd and another increase was reported during August, yet only reaching 5 %. During the first peak and the late summer increase, the proportion of this pigment in the rectum did not change whereas it increased up to 8 % just after the second spring peak. Finally, several events of increased proportion of 19'betanoyloxyfucoxanthine (19'BF) up to 5 % were observed in the stomach as well as in the rectum during April and the first week of May, whereas only one peak occurred in the seawater in the end of April.

Degradation pigment, namely phaeophorbid-*a* and phaeophytin-*a*, were also observed both in the stomach and in the rectum (Fig. 5). High proportions (17-24 %) of these two pigments were already present in the stomach at the beginning of the study and dropped down to zero on April 11th. A brief increase of phaeophytin-*a* of 24 % was noticed on April 18th. A peak of degradation pigment was reached between May 26th and June 6th and a second event occurred between July 27th and August 30th. In the rectum the level of degradation pigments followed very closely the pattern described for the stomach. However, phaeophytin-*a* was less fluctuating from June and varied between 6 and 16 %. Moreover, when compared to the ingestion dynamic (Fig. 5), the proportions of degradation pigments seems rather disconnected from the periods of increased ingestion. During the four periods of high pigment content in the gut proportion of degradation pigments do not show any strong dynamic. Conversely, the three periods of intense proportions identified in the gut of scallops occurred just before. Furthermore, it seems that high levels of degradation pigments are observed after low ingestion periods.

3.3. Fatty acids and sterols composition of the digestive gland

The total amount of FA and sterols in the DG of *P. maximus* showed particularly high variations during spring (Fig. 7). Total FA content of the digestive gland (DG) increased from 45 to 75 $mg.g^{-1}$ between the start and the end of the study, the highest value of 172 $mg.g^{-1}$ being reached in mid-June (Table 2). The main FA found in the polar

fraction were 20:5n-3 (EPA), 22:6n-3 (DHA), 16:0, each accounting for about 15 % in average, 18:4n-3, 20:4n-6 (ARA), around 5 % and to a lesser extent 18:2n-6, 18:3n-3, 18:1n-9, accounting for 1 to 2 %. In the neutral fraction, 20:5n-3 was the most present (25 %), 16:0 accounted for about 15 %, 14:0, 16:1n-7 and 22:6n-3 ranged between 6 and 12 %, ARA was found around 3.5 % and 18:1n-9, 18:3n-3, 18:4n-3 were each accounting for 2 % in average. Within the polar fraction, the ratio between EPA and DHA showed two periods of increase in late April-early May and in the beginning of June. Lower levels were observed in the beginning of April, in the end of May and in August. Finally, Fig. 7 shows the occurrence of a peak in 18:4n-3 at the end of April at 5 % and a regular increase from June to October in the proportion of this FA. A similar pattern was observed for 18:2n-6, 18:3n-3 which also exhibited an important increase of twice their initial amount in late May.

Sterol content in the DG increased from 35.9 to 105.3 $\mu g.g^{-1}$ with a peak at 183.0 $\mu g.mg^{-1}$ on the 19th May (Table 3). Cholesta-5-en-3β-ol(cholesterol) was, in average, the most present compound (24 %), followed by 5β-cholestan-3β-ol (coprostanol, 21.6 %), 24β-methylenecholesta-5,24(28)-dien-3β-ol (24 methylene, 15.8 %), 24β-methylcholesta-5,22-dien-3β-ol (brassicasterol, 11.8 %) and 24β-ethylenecholesta-5-en-3β-ol (β-sitosterol, 5.4 %). Cholesterol decreased slowly from 29.5 % to 20.5 % along the study period, without any strong variation (Fig. 8). The evolution of 24 methylene followed the same dynamic as fucoxanthin in the stomach content and the EPA in the stomach tissue. A first increase in May is observed, lasting for two weeks with a maximum at 22 %. It was followed by a second more intense peak in early June up to 24 % and a final increase in late September of about the same level as the first spring peak. Brassicasterol showed an inverse dynamic, it decreased from mid-April to mid-May down to 10 %, then increased at the end of the month before reaching a plateau in summer and it slightly decreased from mid-August until the end of the study.

4. Discussion

4.1. Validation of biomarkers

Recently, Hurtado et al. (2012) conducted a study focused on the seasonal dynamic of pigments, fatty acids (FA) and sterols in relation to the reproductive activity of female *Crassostrea corteziensis*. They concluded that these indicators were relevant when trying to infer the energy reserve dynamic, the essential membrane components provided by diet and food availability. The present study is the first to combine the use of pigments, FA and sterols as food source biomarkers in a seasonal trophic study of a marine suspension-feeding invertebrate.

First of all, there is a clear relationship between pigment concentration in the filters and the phytoplankton cell identification. The variations of fucoxanthin proportion match well the different diatom blooms observed in early May, early June and late September. Indeed, this pigment has been reported to be a specific pigment of diatoms (Jeffrey,

1997; Lampert, 2001). Likewise, the highly fluctuated dynamic of dinophyceae blooms is very well reproduced by the proportion of peridinin in the water. Then, alloxanthin amounts recorded in the filters was precisely in accordance with the identification of cryptophyceae at the two depths sampled, which is also in accordance with previous founding (Jeffrey, 1997; Lampert, 2001). The prymnesiophyceae algae class has also been found to contain a specific pigment, 19'HF (Jeffrey, 1997; Lampert, 2001), which in our results seems to be well correlated to the development of this microalgae. However, concerning the phylum of chlorophyceae we have to admit that the agreement between cell identification and measurements of chlorophyll-*b*, a pigment usually linked to this algae class (Jeffrey, 1997; Lampert, 2001), is not obvious. Indeed, while the proportion of this pigment in the filtrated water was substantial (up to 15 % in the water column and 25 % at the bottom, especially during summer), only one bloom of chlorophyceae was registered in late May. Nevertheless, this pigment is also a major component of green macroalgae, which are often observed in the area of the bay. The important level registered in our filters could be attributed to discarded pieces of macroalgae in the water (Jeffrey, 1997; Lampert, 2001).

The dynamics of lipids in the digestive gland are less fluctuating than the pigments in the gut content. However, the incorporation of lipids in the tissues of the organism is a regulated process, smoothed by dilution and selective incorporation mechanisms (Soudant et al., 1996). On the other side, pigments are not edible compounds to be assimilated by the scallop. For the majority, they are released in a more or less degraded form with feces. Considering the properties of these two biochemical compounds, the brutal changes observed in both FA from the polar lipids and sterols are important and show critical modifications of the cell membrane of the digestive gland tissue, although rather strong homeostasis would be expected from this class of compounds.

4.2. Feeding ecology

The present study shows the complexity of the diet of *P. maximus*, which seems to apply a selection on the various algae classes available in the water. Moreover, the great scallop seems to be able to switch from one to another depending on the season. Indeed, despite the predominant proportion of bacillariophyceae (diatoms) as food source for scallops, Fig. 6 shows that their is an alternation of two pigments in the stomach content between fucoxanthin, found mainly in diatoms (Jeffrey, 1997; Lampert, 2001) and peridinin, a specific pigment of the dinophyceae (Jeffrey, 1997; Lampert, 2001). These shifts can be observed in Fig. 7 which presents the proportion of EPA and DHA, respectively representative of diatoms and dinoflagellates (Parrish et al., 1995; Dalsgaard et al., 2003; Parrish, 2013). Finally, Fig. 8 brings an ultimate evidence of this alternation by showing the evolution of 24 methylene and brassicasterol in the digestive gland tissues, which have also been reported to be major sterols of these two algae classes respectively (Volkman, 1986). During the biggest bloom of the water column in early May, proportions of fucoxanthin rapidly increased before dropping on May 2nd and rise back to previous high level four days after. This temporary stop in

diatom ingestion while cell concentrations in the water were at their maximum has already been reported as a negative effect of too elevated food particles in the surrounding of the scallop (Lorrain et al., 2000).

The proportion of DHA in the total lipid of the digestive gland doubled during the monitoring. Moreover, the average level of peridinin, and thus of dinoflagellate, in the digestive tract of *P. maximus* reaches 18.1 % in the stomach, indicating a significant ingestion and assimilation of nutrients from dinophyceae. This is quite surprising while the peridinin amount in the water does not exceed 1.4. Dinoflagellates are not the dominating phylum among the phytoplankton taxa identified in the water. The fact that despite the relatively low occurrence of dinoflagellates in the water, biomarkers of this phylum are found in the scallop allow us to assume that *P. maximus* is actively feeding on dinophyceae. And yet, many of dinoflagellates species are known for their toxicity. Several studies have reported the negative consequences of bloom events on the physiology of *P. maximus* (Erard-Le Denn et al., 1990; Lorrain et al., 2000; Chauvaud et al., 2001). Not all the species of dinoflagellates produce toxins but the connection is often made between the occurrence of dinophyceae and a potential toxicity for suspension-feeding bivalves. And it has long been considered more as a threat than as a food source for the great scallop (Smolowitz and Shumway, 1997; Lorrain et al., 2000; Chauvaud et al., 2001; Bougrier et al., 2003). However, no harmful species were observed during year 2011. Moreover, DHA (as well as EPA) are essential FA for bivalves (Soudant et al., 1998b). Furthermore, DHA is a FA known to be largely present in dinophyceae (Parrish et al., 1995; Dalsgaard et al., 2003; Parrish, 2013). Thus, in the light of our results, the place of dinoflagellates in the diet of *P. maximus* should be reconsidered.

P. maximus, as other suspension feeding bivalves is known to feed mainly on microalgae, filtrated from the surrounding seawater (MacDonald et al., 2006). The "bottom-up" relationship between the great scallop and its food implies an adaptability, a feeding plasticity in response to the environmental availability. This study, conducted over eight months, during witch food is abundant for *P. maximus*, show that its diet is actually changing over time. Indeed, three contrasted periods can be observed. First, from the very beginning of the study until the first big bloom in the water column in early May, scallops seemed to feed on dinoflagellates, prymnesiophyceae and chrysophyceae, given the respective proportions of peridinin, 19'HF and 19'BF in their stomach. This is also supported by the evolution of 18:4n-3, a common FA in prymnesiophyceae and DHA, found in high proportion in the dinophyceae. Then, during spring and the renewal of primary production in the water, *P. maximus* seems to feed mainly on diatoms and dinoflagellates, which respective specific pigments account for more than 90 % of the total pigments of the stomach content. After the last spring bloom in early June, the level of chlorophyll-*b* that was remaining under 2 % in the gut of the scallop increased up to 15 % during the first part of summer. In the FA composition we see the increasing proportion of 18:3n-3 and 18:2n-6 in the polar lipids, two indicators of the consumption of green macroalgae or chlorophyceae (Claustre et al., 1988; Parrish et al., 1995; Dalsgaard et al., 2003; Parrish, 2013). The investigated biomarkers are then

rather stable toward the of the summer, the total quantities of pigment and FA decreasing slowly. Finally in the end of the monitoring (late September/early October), sharp increases of 24 methylene, fucoxanthin and EPA bring the evidence of a diatom based diet.

On the other hand, some pigments detected in the water did not appeared in the gut of the scallops. For instance, zeaxanthin was measured at levels varying between 5 and 10 percent in the water column as well as at the water-sediment interface during the whole duration of the study. However, except in the first sampling point, the contribution of zeaxanthin was close to zero for the rest of the time (Table 1). Zeaxanthin is often associated to cyanophyceae (Jeffrey, 1997; Lampert, 2001), which size is under 2 μm. It is well known that the gill retention of most bivalve is not fine enough to capture these tiny particles (Pouvreau et al., 1999). But some studies hypothesized the fact that aggregates or adduction on bigger particle could permit an indirect ingestion of cyanophyceae by bivalves (Ward and Kach, 2009). This could explain the occurrence of zeaxanthin in relatively high amounts (3 %) in the first sample. Moreover, several experimental studies have pointed out the capacity of *P. maximus* to select different algae species on the basis of their size, their nutritional value, their physiological state or their lipid content (Shumway et al., 1985; Soudant et al., 1998b; Ward and Shumway, 2004). In the end of May and during summer 2011, increases of chlorophyll-*b* were observed in the seawater, though not always in the scallops. In spring and in the middle of summer, chlorophyll-*b* proportion was close to zero. Meanwhile, the amount of peridinin and thus of dinophyceae showed an increase in the stomach content.

Furthermore some pigments found in the stomach, are found in the rectum in similar proportions, possibly meaning that they were not subject to digestion. It is the case for fucoxanthin during the month of May and in the end of July, which shows a positive linear correlation between the two compartments. A strong correlation is also observed for chlorophyll-*b*. Moreover, lipid composition of the digestive gland also show that 18:3n-3 and 18:2n-6 FA, usually representative of chlorophyceae (Parrish et al., 1995; Dalsgaard et al., 2003; Parrish, 2013), were not accumulated in the tissue of *P. maximus* during and after the ingestion peak of this microalgae. This could reflect a negative selectivity against this trophic source, which although sometimes ingested, is not actively assimilated.

4.3. Digestion and assimilation

Beyond the matter of ingestion stands the question of the digestion and assimilation of the ingested products. This study provides a large data set allowing to explore the fate of food after having undergo several steps of digestion. Phaeophorbid-*a* and phaeophytin-*a* are two pigments resulting from the degradation of chlorophyll-*a* (Louda et al., 2008). Significant amounts (up to 36 % of phaeophorbid-*a* and 25 % of phaeophytin-*a*) were found in the stomach, suggesting a first step of food processing (Fig. 5). Digestion in *P. maximus* is both extra- and intracellular and occurs in several sites of the digestive tract (Beninger and Le Pennec, 2006). The first step of degradation occurs in the

stomach with the both mechanical and chemical action of the crystalline style that break and attack the microalgae frustule. Pigments contained in microalgae undergo different levels of degradation by the enzymatic activity of digestion (Bayne et al., 1987; Pastoureaud et al., 1995). The fact that degradation peaks are observed after periods of low ingestion could result from the degradation of the food remains in the stomach. Indeed, scallops have to cope with periods of low food availability like during the winter or between two phytoplankton blooms. The degradation of the last food particles in the stomach could provide them with nutrients while awaiting for a renewal of food supply. Another possible explanation could be the passive deterioration (not due to active digestion) of remaining products of the digestion inside the stomach.

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Tables

TABLE 1: Pigments contribution (%) to total pigment concentration in the stomach and the rectum of *P. maximus* in the Bay of Brest during year 2011. PHB: Pheophorbid-*a*, PER: Peridinin, BFU: 19'BF, FUC: Fucoxanthin, HFU: 19'HF, ALO: Alloxanthin, ZEA: Zeaxanthin, LUT: Lutein, CHB: Chlorophyll-*b*, PHT: Phaeophytin-*a*. Standard deviation are in brackets.

	PHB	PER	BFU	FUC	HFU	ALO	ZEA	LUT	CHB	PHT
Stomach										
21/03	18.7 (4.6)	16.9 (1.6)	0.0 (0.0)	14.4 (1.4)	0.9 (0.8)	0.7 (1.2)	2.7 (1.6)	1.5 (0.7)	2.3 (0.3)	27.1 (3.3)
04/04	31.1 (7.2)	9.7 (3.1)	0.0 (0.0)	23.6 (0.8)	0.9 (1.5)	3.1 (1.0)	0.0 (0.0)	0.0 (0.1)	0.5 (0.9)	23.1 (6.3)
11/04	0.0 (0.0)	44.1 (1.2)	2.0 (1.0)	22.7 (8.4)	16.0 (4.5)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
18/04	7.9 (1.1)	18.8 (1.3)	0.0 (0.0)	34.0 (0.4)	3.8 (0.4)	0.3 (0.5)	0.0 (0.1)	0.1 (0.2)	0.3 (0.5)	28.9 (1.3)
26/04	5.2 (1.1)	28.1 (5.9)	3.2 (0.5)	50.1 (6.0)	1.7 (0.6)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	5.1 (1.6)
28/04	0.0 (0.0)	19.8 (5.4)	0.7 (1.3)	60.4 (5.0)	1.3 (0.3)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	9.1 (0.9)
02/05	0.0 (0.0)	48.0 (0.1)	5.1 (0.3)	19.8 (2.2)	11.5 (1.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	1.8 (1.8)	0.2 (0.3)
09/05	0.0 (0.5)	25.6 (4.1)	0.0 (0.0)	56.6 (4.5)	3.8 (0.9)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	4.2 (1.2)
12/05	0.0 (0.9)	13.3 (6.9)	0.0 (0.0)	66.0 (5.0)	2.0 (0.7)	0.0 (0.0)	0.1 (0.1)	0.0 (0.0)	0.8 (0.8)	1.8 (1.7)
16/05	0.0 (0.0)	35.0 (4.3)	0.0 (0.0)	45.1 (3.1)	2.3 (0.6)	0.6 (0.1)	0.0 (0.0)	0.0 (0.0)	0.4 (0.7)	3.9 (0.5)
19/05	0.0 (0.0)	40.7 (7.9)	0.0 (0.0)	37.9 (6.2)	4.2 (1.5)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	6.9 (2.2)
23/05	0.8 (1.2)	13.2 (1.2)	0.0 (0.0)	60.3 (3.2)	1.3 (0.4)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	4.7 (0.1)
26/05	22.7 (1.2)	23.4 (0.9)	0.0 (0.0)	30.9 (3.2)	0.9 (0.0)	5.4 (0.9)	0.2 (0.1)	0.4 (0.2)	0.3 (0.3)	3.9 (0.9)
30/05	41.1 (2.0)	17.5 (2.4)	0.0 (0.0)	13.2 (2.0)	1.0 (0.6)	4.6 (2.2)	0.0 (0.0)	0.0 (0.0)	1.2 (1.0)	15.5 (3.6)
06/06	0.0 (0.0)	4.6 (1.3)	0.7 (0.6)	50.9 (3.4)	0.8 (0.8)	0.0 (0.0)	0.0 (0.0)	0.4 (0.4)	2.4 (0.6)	26.0 (6.7)
14/06	2.4 (2.5)	8.5 (0.3)	0.0 (0.0)	59.2 (2.4)	1.2 (1.1)	2.1 (0.5)	0.0 (0.0)	0.1 (0.1)	4.9 (1.2)	5.7 (1.7)
27/06	5.7 (1.9)	15.6 (3.6)	0.0 (0.0)	23.2 (7.0)	4.1 (0.3)	2.0 (0.9)	0.0 (0.0)	2.1 (0.6)	20.6 (1.5)	14.0 (3.6)
11/07	5.9 (0.8)	14.3 (3.7)	0.0 (0.0)	21.6 (5.2)	4.1 (0.7)	2.1 (0.2)	0.0 (0.0)	2.5 (0.2)	19.9 (2.5)	16.0 (3.7)
27/07	22.7 (1.3)	23.4 (0.9)	0.0 (0.0)	30.9 (3.2)	0.9 (0.0)	5.4 (1.0)	0.2 (0.1)	0.4 (0.2)	0.5 (0.1)	3.9 (0.9)
16/08	29.1 (3.0)	19.1 (3.0)	0.0 (0.0)	27.8 (2.5)	5.0 (1.1)	4.6 (0.6)	0.0 (0.0)	3.8 (0.4)	0.0 (2.4)	5.4 (0.9)
30/08	8.2 (1.2)	26.2 (2.5)	0.0 (0.0)	31.0 (0.5)	6.3 (1.5)	5.9 (1.1)	0.0 (0.0)	3.7 (1.5)	0.0 (0.0)	28.3 (1.8)
15/09	10.2 (9.5)	24.4 (9.7)	0.0 (0.0)	30.3 (5.7)	2.3 (1.1)	9.0 (1.4)	0.0 (0.0)	4.3 (0.3)	0.0 (0.0)	5.8 (5.1)
03/10	4.3 (2.6)	3.4 (1.8)	0.0 (0.0)	62.0 (1.1)	1.1 (0.4)	1.8 (0.2)	0.1 (0.0)	0.0 (0.1)	0.3 (0.6)	9.5 (4.4)
24/10	18.7 (1.2)	27.2 (3.2)	0.0 (0.0)	15.1 (4.0)	4.6 (0.6)	8.3 (1.2)	0.0 (0.0)	0.3 (0.0)	0.0 (0.0)	6.0 (0.2)
Rectum										
21/03	31.4 (8.8)	13.9 (3.2)	0.0 (0.0)	15.0 (3.7)	1.1 (2.0)	6.0 (0.6)	1.1 (0.4)	1.2 (0.8)	0.0 (0.0)	14.2 (0.8)
04/04	31.1 (8.1)	0.0 (0.0)	0.0 (0.0)	28.7 (3.8)	1.9 (3.4)	8.6 (4.0)	0.4 (0.2)	0.6 (0.3)	0.0 (0.0)	11.0 (1.9)
11/04	6.5 (2.1)	18.4 (4.4)	3.6 (0.6)	33.0 (2.0)	4.0 (2.1)	4.2 (1.2)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	10.8 (0.8)
18/04	0.0 (0.0)	36.2 (6.9)	2.9 (2.8)	19.5 (17.5)	2.9 (0.6)	5.1 (2.4)	0.3 (0.3)	1.4 (0.8)	0.0 (0.0)	7.9 (7.7)
26/04	15.5 (1.4)	19.3 (3.8)	3.3 (1.0)	36.8 (3.5)	1.9 (1.3)	0.0 (0.0)	0.2 (0.2)	0.1 (0.2)	0.0 (0.0)	6.4 (0.8)
28/04	0.0 (0.0)	12.1 (1.0)	6.0 (1.2)	48.1 (5.3)	1.4 (0.7)	1.6 (0.8)	0.1 (0.1)	0.1 (0.1)	0.0 (0.0)	17.0 (5.2)
02/05	1.5 (0.4)	41.5 (1.8)	7.7 (1.1)	12.9 (0.9)	5.3 (0.7)	2.4 (0.2)	0.4 (0.1)	0.4 (0.1)	2.8 (0.4)	4.6 (0.7)
09/05	12.4 (5.2)	0.0 (0.0)	1.3 (0.4)	54.1 (0.9)	8.1 (1.6)	1.8 (1.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	11.5 (1.9)
12/05	7.8 (1.8)	8.1 (1.1)	0.0 (0.0)	57.3 (0.9)	1.8 (1.5)	0.0 (0.0)	0.2 (0.1)	0.1 (0.1)	0.0 (0.0)	6.6 (0.8)
16/05	4.2 (1.9)	18.7 (2.3)	1.5 (0.3)	46.7 (2.4)	4.6 (2.6)	1.1 (0.2)	0.2 (0.1)	0.1 (0.1)	1.4 (1.3)	7.8 (1.4)
19/05	6.5 (4.9)	34.7 (10.2)	3.0 (0.6)	26.9 (9.1)	3.2 (0.1)	1.6 (0.1)	0.2 (0.0)	0.1 (0.0)	1.0 (1.7)	6.6 (0.6)
23/05	3.3 (1.2)	5.1 (1.4)	1.7 (1.4)	52.6 (8.4)	1.0 (0.4)	1.8 (1.1)	0.1 (0.1)	0.1 (0.1)	0.0 (0.0)	20.1 (4.2)
26/05	35.5 (4.4)	0.0 (0.0)	0.0 (0.0)	26.1 (1.8)	0.6 (0.2)	4.4 (0.3)	0.2 (0.2)	0.3 (0.3)	0.0 (0.0)	15.5 (2.6)
30/05	50.5 (8.9)	2.3 (2.1)	0.0 (0.0)	14.9 (6.2)	0.0 (0.0)	3.7 (1.5)	0.1 (0.2)	0.2 (0.4)	0.0 (0.0)	14.8 (1.3)
06/06	0.0 (0.0)	5.2 (1.2)	0.8 (0.7)	52.6 (4.8)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	26.6 (5.8)
14/06	6.6 (1.6)	7.4 (1.9)	0.0 (0.0)	39.6 (4.2)	1.8 (1.7)	1.3 (0.5)	0.0 (0.0)	0.7 (0.1)	2.4 (0.8)	20.2 (3.4)
27/06	9.8 (0.9)	7.4 (0.6)	0.0 (0.0)	12.6 (0.8)	1.2 (0.1)	0.4 (0.1)	0.0 (0.0)	7.9 (1.3)	9.7 (0.5)	19.7 (2.8)
11/07	11.7 (0.6)	8.8 (0.8)	0.0 (0.0)	14.8 (1.4)	1.5 (0.3)	0.5 (0.1)	0.0 (0.0)	9.2 (1.6)	11.3 (0.5)	21.6 (3.8)
27/07	33.9 (3.8)	0.0 (0.0)	0.0 (0.0)	26.5 (2.1)	0.6 (0.2)	4.5 (0.2)	0.2 (0.2)	0.3 (0.3)	0.7 (0.6)	15.7 (2.2)
16/08	0.0 (0.0)	16.0 (2.1)	0.0 (0.0)	23.5 (3.2)	0.0 (0.0)	0.0 (0.0)	3.0 (1.4)	10.2 (1.7)	16.6 (2.7)	9.3 (0.5)
30/08	22.5 (7.3)	12.7 (5.0)	0.0 (0.0)	22.4 (2.1)	0.5 (0.5)	3.6 (3.2)	2.9 (2.2)	1.1 (0.2)	0.3 (0.4)	9.2 (2.8)
15/09	20.2 (8.5)	9.2 (0.3)	0.0 (0.0)	22.5 (4.0)	0.8 (0.1)	4.9 (0.6)	1.6 (0.1)	1.2 (0.4)	0.0 (0.0)	19.1 (1.5)
03/10	10.4 (4.0)	3.8 (0.6)	0.0 (0.0)	48.0 (3.1)	1.2 (0.0)	2.1 (0.8)	0.2 (0.3)	0.1 (0.2)	0.1 (0.1)	14.7 (3.2)
24/10	10.1 (4.8)	5.3 (3.1)	0.0 (0.0)	40.2 (18.8)	0.2 (0.1)	3.3 (1.5)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	5.9 (2.9)

TABLE 2: Fatty acids composition of the polar lipids in the digestive gland of *P. maximus* (expressed as fatty acid molar percentage of total) in the Bay of Brest, during year 2011. Standard deviation are in brackets.

	21/03	04/04	11/04	18/04	26/04	28/04	02/05	09/05	12/05	16/05	19/05	23/05
TMTD	8.28 (7.82)	5.18 (8.97)	1.92 (3.33)	7.09 (6.43)	8.04 (1.80)	6.02 (5.27)	6.62 (2.05)	4.85 (2.08)	5.92 (0.80)	10.09 (0.60)	6.96 (6.14)	8.83 (1.29)
14:0	2.99 (0.15)	3.23 (0.45)	3.20 (0.29)	3.93 (0.55)	3.94 (0.06)	3.99 (0.58)	4.35 (0.33)	3.97 (1.31)	3.97 (0.52)	4.54 (0.31)	4.35 (0.51)	4.58 (0.22)
16:0	11.51 (0.66)	11.27 (0.73)	13.26 (0.94)	12.23 (0.98)	14.01 (0.98)	11.90 (0.79)	12.97 (1.33)	14.87 (1.63)	14.33 (1.25)	14.69 (0.22)	15.19 (0.91)	15.84 (0.46)
18:0	8.65 (1.08)	8.65 (1.08)	8.95 (0.60)	7.82 (1.36)	7.91 (0.30)	8.16 (0.34)	5.26 (4.56)	9.02 (0.18)	5.43 (4.71)	10.63 (0.47)	9.43 (0.55)	9.79 (0.17)
16:1n-7	3.37 (0.69)	3.23 (0.71)	2.54 (0.11)	3.49 (0.52)	4.09 (0.29)	3.79 (0.42)	4.77 (0.19)	5.38 (0.94)	4.98 (0.52)	4.98 (0.60)	4.28 (0.54)	4.19 (0.45)
18:1n-9	1.29 (0.13)	1.23 (0.15)	1.04 (0.08)	1.29 (0.02)	1.71 (0.11)	1.20 (0.05)	1.23 (0.24)	1.50 (0.08)	1.35 (0.08)	1.44 (0.15)	1.27 (0.10)	1.77 (0.08)
18:1n-7	0.95 (1.02)	1.36 (1.18)	1.99 (0.21)	2.08 (0.24)	2.47 (0.19)	2.59 (0.16)	3.52 (0.21)	4.73 (0.26)	5.27 (0.43)	4.48 (0.28)	4.20 (0.08)	3.52 (0.21)
20:1n-9	0.63 (0.09)	0.64 (0.09)	0.86 (0.05)	0.68 (0.13)	0.71 (0.06)	0.74 (0.04)	0.83 (0.10)	0.81 (0.16)	0.75 (0.02)	0.74 (0.04)	0.71 (0.01)	0.79 (0.07)
20:1n-7	0.63 (0.11)	0.62 (0.12)	0.60 (0.02)	0.63 (0.15)	0.75 (0.01)	0.70 (0.05)	0.93 (0.12)	0.94 (0.16)	0.93 (0.05)	1.02 (0.15)	0.86 (0.11)	0.80 (0.02)
16:3n-3	0.89 (0.95)	1.32 (1.14)	1.75 (0.28)	1.26 (0.25)	0.73 (0.05)	1.14 (0.10)	0.97 (0.15)	0.68 (0.61)	0.84 (0.02)	0.77 (0.08)	0.92 (0.11)	0.91 (0.06)
18:2n-6	0.74 (0.10)	0.72 (0.11)	0.74 (0.00)	0.91 (0.14)	1.00 (0.08)	0.77 (0.02)	0.76 (0.12)	0.90 (0.11)	0.86 (0.05)	0.63 (0.06)	0.63 (0.04)	0.88 (0.01)
18:3n-3	1.02 (0.07)	1.03 (0.07)	1.08 (0.05)	1.27 (0.18)	1.38 (0.09)	1.14 (0.12)	1.10 (0.17)	1.12 (0.13)	1.05 (0.11)	0.93 (0.06)	0.76 (0.08)	1.14 (0.03)
18:4n-3	2.67 (0.09)	2.72 (0.12)	3.04 (0.36)	3.77 (0.68)	4.99 (0.32)	3.78 (0.79)	3.40 (0.19)	2.63 (0.27)	3.11 (0.41)	2.00 (0.09)	2.19 (0.11)	2.83 (0.19)
20:4n-6	3.94 (0.44)	3.69 (0.59)	3.59 (0.52)	3.29 (0.80)	3.00 (0.24)	3.82 (0.67)	5.00 (0.50)	4.46 (0.50)	4.13 (0.34)	3.59 (0.20)	3.60 (0.80)	3.31 (0.37)
20:5n-3	16.21 (0.15)	16.04 (0.36)	14.13 (1.04)	15.98 (1.37)	17.88 (0.71)	17.48 (1.06)	18.66 (1.53)	16.03 (1.95)	18.08 (1.29)	13.03 (0.31)	13.59 (1.44)	11.56 (0.55)
21:5n-3	1.17 (0.05)	1.26 (0.15)	1.01 (0.06)	1.10 (0.06)	1.09 (0.01)	1.17 (0.10)	1.19 (0.11)	0.95 (0.02)	1.00 (0.09)	0.70 (0.01)	0.69 (0.07)	0.66 (0.02)
22:4n-9t	1.91 (0.24)	1.97 (0.25)	2.52 (0.30)	1.55 (0.13)	1.14 (0.17)	1.42 (0.10)	1.32 (0.20)	1.65 (0.24)	1.35 (0.16)	1.71 (0.03)	1.73 (0.15)	1.65 (0.14)
22:5n-3	0.95 (0.06)	1.03 (0.16)	1.08 (0.12)	0.86 (0.12)	1.08 (0.15)	1.11 (0.11)	1.20 (0.18)	1.12 (0.12)	1.29 (0.04)	0.86 (0.06)	0.94 (0.10)	0.84 (0.04)
22:6n-3	14.88 (1.21)	15.74 (1.81)	16.94 (0.70)	13.21 (1.63)	11.42 (0.35)	12.38 (1.29)	10.82 (1.47)	10.21 (0.66)	11.52 (0.58)	9.02 (0.48)	11.94 (1.15)	11.09 (0.61)
18:0DMA	6.76 (1.17)	7.34 (1.45)	6.48 (0.73)	5.83 (0.93)	4.14 (0.19)	5.81 (0.38)	5.01 (0.39)	4.64 (0.69)	4.43 (0.27)	4.16 (0.70)	4.51 (0.55)	4.11 (0.25)
20:1n-7DMA	1.56 (1.16)	1.99 (1.30)	2.23 (0.18)	1.84 (0.37)	1.15 (0.10)	1.71 (0.03)	1.74 (0.20)	1.58 (0.48)	1.51 (0.05)	1.25 (0.10)	1.59 (0.33)	1.26 (0.18)
T.SAT.	24.95 (0.63)	25.04 (0.63)	27.57 (1.98)	25.71 (1.68)	26.67 (0.73)	25.58 (1.54)	23.71 (3.58)	29.11 (2.41)	25.26 (2.96)	31.72 (0.30)	31.19 (1.68)	32.27 (0.65)
T.MONO	8.58 (0.97)	8.77 (0.98)	8.80 (0.28)	9.66 (1.00)	11.12 (0.81)	10.74 (0.47)	12.73 (0.82)	14.85 (0.89)	14.48 (1.21)	14.63 (0.77)	13.28 (0.46)	13.28 (0.73)
T.POLY	47.34 (2.47)	48.10 (2.66)	49.49 (1.38)	47.27 (2.81)	47.46 (1.65)	47.67 (3.27)	48.21 (4.41)	42.86 (3.59)	46.64 (2.23)	36.20 (0.50)	40.78 (3.85)	38.36 (0.84)
T.DMA	10.35 (3.75)	12.08 (4.54)	11.73 (1.28)	9.90 (1.73)	6.44 (0.34)	9.64 (0.24)	8.40 (0.67)	7.91 (1.22)	7.40 (0.19)	7.06 (0.77)	7.48 (1.58)	6.96 (0.33)
T.n-6	2.42 (0.34)	2.37 (0.35)	2.90 (0.11)	2.58 (0.22)	2.45 (0.46)	2.31 (0.06)	2.63 (0.33)	2.66 (0.39)	2.60 (0.28)	2.11 (0.07)	2.19 (0.16)	2.33 (0.17)
T.n-3	38.47 (2.64)	39.68 (3.18)	40.22 (1.83)	38.80 (2.37)	39.87 (0.80)	39.06 (3.29)	38.35 (3.79)	33.48 (2.46)	37.70 (2.26)	27.93 (0.33)	31.77 (2.88)	29.95 (0.42)
T.nmi	0.18 (0.17)	0.11 (0.19)	0.00 (0.00)	0.11 (0.20)	0.12 (0.20)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.22 (0.38)	0.00 (0.00)
n-3/n-6	16.02 (1.12)	16.88 (1.89)	13.90 (0.93)	15.14 (1.64)	16.68 (3.34)	16.87 (1.29)	14.64 (0.38)	12.73 (1.62)	14.63 (2.25)	13.23 (0.47)	14.55 (1.13)	12.87 (0.76)
16PUFA/18PUFA	0.28 (0.25)	0.35 (0.26)	0.46 (0.05)	0.34 (0.06)	0.18 (0.03)	0.33 (0.01)	0.38 (0.03)	0.27 (0.17)	0.28 (0.10)	0.29 (0.09)	0.37 (0.06)	0.30 (0.05)
20:5n-3/22:6n-3	1.09 (0.08)	1.03 (0.13)	0.83 (0.06)	1.22 (0.16)	1.57 (0.11)	1.42 (0.07)	1.73 (0.12)	1.57 (0.10)	1.57 (0.04)	1.45 (0.11)	1.14 (0.04)	1.05 (0.10)

	26/05	30/05	06/06	14/06	27/06	11/07	27/07	16/08	30/08	15/09	03/10	24/10
TMTD	6.87 (1.33)	8.33 (1.42)	7.59 (0.03)	6.06 (1.49)	6.02 (1.44)	5.80 (1.69)	4.67 (1.27)	4.75 (0.31)	4.40 (1.22)	3.15 (0.31)	3.25 (0.44)	4.14 (0.52)
14:0	3.92 (0.82)	4.65 (0.79)	5.68 (0.40)	5.44 (0.35)	4.87 (0.63)	4.73 (0.42)	4.14 (0.07)	3.15 (0.32)	3.29 (0.48)	3.78 (0.13)	2.76 (1.89)	2.73 (1.88)
16:0	15.34 (1.27)	15.15 (0.91)	12.41 (0.87)	13.59 (1.42)	14.11 (1.37)	14.83 (0.72)	15.53 (0.76)	13.79 (0.19)	14.25 (1.16)	15.24 (1.15)	15.55 (0.06)	14.62 (1.24)
18:0	9.54 (0.62)	8.91 (0.65)	8.10 (0.56)	8.43 (0.34)	5.20 (4.52)	8.50 (0.57)	7.69 (0.51)	7.73 (0.61)	7.58 (0.41)	7.73 (0.29)	7.22 (1.43)	7.18 (1.48)
16:1n-7	3.79 (0.51)	4.06 (0.77)	4.18 (0.46)	5.09 (0.54)	4.77 (0.43)	4.58 (0.61)	4.39 (0.61)	2.79 (0.38)	3.01 (0.69)	3.78 (0.14)	4.43 (1.09)	4.25 (1.35)
18:1n-9	1.97 (0.20)	1.76 (0.29)	1.18 (0.06)	1.36 (0.14)	1.54 (0.14)	1.70 (0.31)	2.15 (0.36)	1.40 (0.12)	1.46 (0.17)	1.65 (0.20)	1.77 (0.21)	1.75 (0.25)
18:1n-7	3.10 (0.58)	2.89 (0.44)	2.49 (0.07)	2.79 (0.22)	2.92 (0.24)	2.73 (0.21)	2.72 (0.13)	2.37 (0.14)	2.53 (0.12)	2.73 (0.07)	2.71 (0.07)	2.58 (0.20)
20:1n-9	0.85 (0.09)	0.86 (0.04)	0.67 (0.02)	0.64 (0.01)	0.68 (0.06)	0.65 (0.08)	0.55 (0.09)	0.74 (0.07)	0.78 (0.00)	0.79 (0.02)	0.69 (0.16)	0.67 (0.14)
20:1n-7	0.72 (0.14)	0.65 (0.05)	0.59 (0.03)	0.64 (0.09)	0.68 (0.05)	0.65 (0.16)	0.68 (0.09)	0.50 (0.07)	0.57 (0.01)	0.56 (0.03)	0.65 (0.11)	0.56 (0.19)
16:3n-3	0.98 (0.08)	0.99 (0.09)	0.27 (0.48)	0.96 (0.21)	1.00 (0.02)	0.58 (0.50)	0.94 (0.18)	0.23 (0.41)	0.33 (0.57)	1.06 (0.12)	0.72 (0.45)	0.73 (0.46)
18:2n-6	0.96 (0.06)	0.92 (0.11)	0.60 (0.04)	0.70 (0.16)	0.86 (0.09)	0.92 (0.21)	1.14 (0.16)	0.98 (0.06)	1.01 (0.13)	1.10 (0.16)	1.12 (0.06)	0.96 (0.09)
18:3n-3	1.42 (0.08)	1.27 (0.12)	0.86 (0.03)	0.83 (0.16)	1.27 (0.11)	1.19 (0.19)	1.54 (0.23)	1.46 (0.04)	1.53 (0.14)	1.59 (0.19)	1.71 (0.02)	1.56 (0.15)
18:4n-3	3.61 (0.81)	3.19 (0.20)	1.88 (0.35)	2.13 (0.54)	3.62 (0.34)	2.90 (0.59)	4.02 (1.02)	3.85 (0.40)	3.94 (0.49)	4.24 (0.30)	4.74 (0.41)	4.44 (0.74)
20:4n-6	2.96 (0.30)	3.28 (0.33)	6.69 (0.56)	6.16 (0.46)	4.83 (0.10)	4.87 (0.65)	4.34 (0.33)	3.82 (0.13)	3.89 (0.36)	4.45 (0.45)	4.82 (0.90)	5.30 (0.48)
20:5n-3	13.52 (2.54)	13.13 (1.53)	15.32 (1.40)	16.70 (1.33)	16.56 (0.83)	17.01 (0.75)	17.50 (1.57)	15.43 (1.55)	15.43 (1.00)	16.54 (0.22)	17.52 (1.61)	17.15 (1.94)
21:5n-3	0.80 (0.13)	0.78 (0.06)	0.88 (0.08)	0.76 (0.02)	0.87 (0.07)	0.57 (0.41)	0.81 (0.03)	0.86 (0.05)	0.91 (0.06)	0.90 (0.07)	0.66 (0.56)	0.61 (0.51)
22:4n-9t	1.62 (0.12)	1.91 (0.31)	1.47 (0.18)	1.57 (0.26)	1.77 (0.19)	1.53 (0.31)	1.47 (0.18)	1.69 (0.14)	1.55 (0.01)	1.80 (0.21)	1.75 (0.33)	2.02 (0.20)
22:5n-3	0.81 (0.07)	0.76 (0.08)	1.08 (0.07)	0.98 (0.12)	1.06 (0.10)	1.08 (0.17)	0.94 (0.11)	1.04 (0.08)	0.97 (0.17)	0.88 (0.10)	0.81 (0.05)	0.96 (0.10)
22:6n-3	12.30 (1.99)	11.61 (1.92)	10.56 (0.83)	9.79 (1.12)	10.73 (0.68)	11.66 (0.18)	12.74 (0.48)	15.99 (0.55)	15.12 (0.88)	13.77 (0.39)	13.80 (0.53)	14.09 (0.88)
18:0DMA	4.76 (0.11)	4.69 (0.49)	3.18 (0.74)	4.79 (0.54)	4.89 (0.38)	4.02 (0.70)	3.77 (0.70)	4.28 (0.09)	4.36 (0.02)	4.54 (0.33)	4.24 (0.26)	4.61 (0.59)
20:1n-7DMA	1.24 (0.03)	1.20 (0.10)	1.05 (0.02)	1.31 (0.18)	1.14 (0.09)	0.97 (0.33)	0.82 (0.25)	0.97 (0.06)	0.91 (0.10)	0.94 (0.17)	0.54 (0.44)	0.65 (0.54)
T.SAT.	30.58 (2.80)	30.57 (1.34)	27.54 (0.88)	29.25 (1.28)	26.02 (2.35)	29.30 (0.72)	28.66 (0.33)	25.91 (1.00)	26.30 (2.53)	28.51 (1.27)	27.42 (3.12)	26.28 (2.26)
T.MONO	12.43 (1.91)	12.13 (1.82)	11.01 (0.61)	12.33 (0.87)	12.69 (1.07)	11.88 (0.91)	11.04 (0.79)	9.21 (0.89)	9.57 (0.86)	10.84 (0.33)	11.63 (1.83)	11.29 (2.18)
T.POLY	41.95 (5.91)	40.90 (4.45)	47.55 (1.73)	44.13 (0.48)	47.13 (2.57)	46.31 (1.06)	49.39 (2.17)	52.99 (1.44)	52.61 (1.85)	50.10 (0.51)	51.70 (2.12)	51.76 (2.14)
T.DMA	7.82 (0.20)	7.62 (0.62)	6.05 (0.60)	7.90 (1.14)	7.69 (0.53)	6.26 (0.93)	5.83 (1.25)	6.89 (0.16)	6.84 (0.40)	7.05 (0.90)	5.59 (1.36)	6.09 (1.82)
T.n-6	2.41 (0.15)	2.32 (0.10)	2.65 (0.19)	2.51 (0.12)	2.82 (0.20)	2.81 (0.65)	2.67 (0.23)	2.55 (0.20)	2.67 (0.04)	2.66 (0.05)	2.78 (0.11)	2.88 (0.16)
T.n-3	34.24 (5.66)	32.79 (3.58)	32.12 (2.55)	32.92 (0.77)	36.17 (1.69)	36.12 (1.67)	39.99 (2.94)	40.28 (2.04)	39.69 (1.11)	40.30 (0.58)	41.24 (0.47)	40.66 (1.04)
T.nmi	0.00 (0.00)	0.00 (0.00)	0.41 (0.36)	0.00 (0.00)	0.23 (0.39)	0.32 (0.33)	0.09 (0.16)	0.27 (0.24)	0.29 (0.25)	0.00 (0.00)	0.00 (0.00)	0.04 (0.07)
n-3/n-6	14.25 (2.41)	14.11 (1.12)	12.23 (1.92)	13.15 (0.41)	12.86 (0.33)	13.33 (3.01)	15.11 (2.30)	15.94 (2.18)	14.85 (0.18)	15.14 (0.07)	14.83 (0.39)	14.16 (0.94)
16PUFA/18PUFA	0.24 (0.03)	0.28 (0.02)	1.03 (0.51)	0.46 (0.12)	0.35 (0.02)	0.21 (0.13)	0.26 (0.04)	0.67 (0.41)	0.69 (0.37)	0.27 (0.03)	0.21 (0.09)	0.19 (0.07)
20:5n-3/22:6n-3	1.10 (0.09)	1.14 (0.08)	1.45 (0.03)	1.73 (0.32)	1.55 (0.14)	1.46 (0.08)	1.37 (0.13)	0.97 (0.12)	1.03 (0.13)	1.20 (0.05)	1.27 (0.17)	1.23 (0.21)

TABLE 3: Sterol composition of the digestive gland of *P. maximus* (expressed as sterol % of the total sterol) in the Bay of Brest during year 2011. NOR: Norcholesterol, COP: Coprostanol, CDH: Cdehydro, TDH: Tdehydro, DHY: Dihydro, CHO: Cholesterol, BRA: Brassicasterol, DES: Desmosterol, CAM: Campesterol, 24M: 24 Methylene, STI: Stigmasterol, BSI: β-Sitosterol, FUS: Fucosterol, TOT: total sterol concentration (in $\mu g.g^{-1}$). Standard deviation are in brackets.

	NOR	COP	CDH	TDH	DHY	CHO	BRA	DES	CAM	24M	STI	BSI	FUS	TOT
21/03	4.0 (0.4)	9.4 (2.4)	1.6 (0.2)	6.8 (0.5)	1.5 (0.6)	29.6 (2.1)	15.8 (1.1)	4.6 (0.3)	2.5 (0.2)	12.4 (1.0)	3.3 (0.6)	6.9 (0.4)	1.7 (0.2)	46.8 (7.0)
04/04	4.3 (0.2)	13.7 (9.4)	1.8 (0.2)	6.6 (0.1)	1.8 (0.6)	29.8 (1.9)	13.6 (3.3)	4.0 (1.3)	2.3 (0.5)	11.6 (0.4)	3.0 (1.0)	5.9 (1.4)	1.5 (0.4)	50.9 (15.0)
11/04	4.1 (0.5)	13.4 (9.1)	1.8 (0.1)	7.0 (1.1)	1.2 (0.0)	28.1 (3.8)	14.7 (2.3)	3.7 (0.3)	2.5 (0.3)	13.3 (1.5)	2.9 (0.3)	5.9 (0.8)	1.7 (0.3)	35.9 (7.4)
18/04	4.3 (0.2)	11.0 (5.3)	2.0 (0.1)	7.2 (0.3)	1.6 (0.1)	29.1 (2.2)	15.4 (1.0)	2.9 (0.2)	2.1 (0.1)	14.4 (0.8)	2.9 (0.4)	6.1 (0.6)	1.6 (0.2)	55.7 (18.8)
26/04	3.6 (0.5)	20.7 (1.4)	2.2 (0.2)	5.4 (0.2)	1.5 (0.1)	25.4 (0.4)	12.1 (0.7)	2.2 (0.8)	1.9 (0.1)	15.7 (0.7)	2.1 (0.3)	5.3 (0.8)	1.9 (0.2)	58.8 (15.0)
28/04	2.7 (0.1)	21.4 (5.4)	2.2 (0.2)	5.0 (0.4)	1.5 (0.1)	24.3 (1.8)	10.7 (0.4)	2.8 (0.6)	1.7 (0.1)	18.5 (2.2)	2.5 (0.3)	5.6 (1.1)	1.6 (0.2)	53.7 (5.4)
02/05	3.3 (0.4)	19.5 (8.6)	2.2 (0.1)	5.0 (0.1)	1.8 (0.2)	23.9 (2.8)	9.9 (0.9)	2.2 (0.2)	1.9 (0.1)	21.7 (3.8)	2.5 (0.4)	5.0 (0.9)	1.3 (0.0)	50.75 (6.6)
09/05	3.6 (0.3)	18.4 (2.4)	2.1 (0.1)	5.7 (0.3)	1.6 (0.2)	24.5 (0.4)	10.0 (0.9)	1.8 (0.2)	1.7 (0.2)	21.6 (3.7)	2.5 (0.5)	5.0 (0.2)	1.3 (0.1)	49.8 (9.8)
12/05	3.2 (0.1)	16.5 (3.4)	2.0 (0.1)	5.1 (0.1)	1.8 (0.1)	23.6 (1.4)	10.4 (0.6)	2.9 (0.3)	1.8 (0.3)	21.9 (1.1)	3.0 (0.5)	6.4 (0.3)	1.3 (0.5)	47.3 (3.2)
16/05	3.4 (0.3)	20.4 (3.1)	1.9 (0.1)	5.3 (0.0)	2.4 (0.3)	24.4 (1.4)	10.2 (0.1)	2.9 (0.7)	1.7 (0.5)	16.5 (1.2)	3.1 (0.3)	6.2 (0.6)	1.4 (0.1)	44.4 (3.5)
19/05	0.0 (0.0)	10.6 (5.5)	0.9 (0.5)	6.1 (0.3)	3.3 (0.3)	26.4 (0.6)	12.6 (1.1)	3.9 (0.5)	2.3 (0.1)	20.4 (1.0)	4.1 (0.7)	7.3 (0.8)	2.0 (0.4)	41.2 (8.4)
23/05	3.7 (0.2)	17.2 (5.7)	1.7 (0.3)	5.7 (0.6)	3.0 (0.7)	24.4 (1.9)	12.6 (1.1)	2.9 (0.4)	2.2 (0.1)	15.4 (0.9)	3.3 (0.2)	6.2 (0.6)	1.7 (0.3)	48.9 (4.4)
26/05	3.6 (0.1)	20.4 (8.0)	1.9 (0.2)	5.6 (0.3)	2.7 (0.2)	22.6 (1.7)	13.5 (2.3)	2.7 (0.5)	2.3 (0.2)	12.9 (2.0)	3.4 (0.7)	6.3 (1.0)	2.0 (0.3)	52.7 (15.8)
30/05	3.0 (0.4)	24.4 (8.1)	1.9 (0.4)	5.5 (0.1)	3.0 (0.6)	21.7 (2.4)	12.1 (1.7)	3.2 (0.6)	2.0 (0.2)	12.6 (0.9)	3.0 (1.0)	5.7 (1.4)	1.7 (0.4)	73.7 (40.5)
06/06	3.4 (0.2)	16.7 (4.1)	1.7 (0.1)	5.5 (0.1)	2.6 (0.3)	23.8 (1.9)	10.5 (0.7)	2.2 (0.1)	2.4 (0.4)	24.0 (2.9)	2.5 (0.3)	4.8 (0.6)	1.1 (0.0)	183.0 (35.7)
14/06	2.6 (0.1)	21.9 (4.6)	1.7 (0.2)	5.0 (0.1)	2.4 (0.1)	21.4 (0.6)	12.9 (1.2)	2.8 (0.4)	3.2 (0.4)	17.9 (1.4)	2.0 (0.4)	4.9 (0.8)	1.2 (0.2)	66.1 (21.9)
27/06	3.3 (0.1)	23.6 (4.1)	1.9 (0.2)	5.4 (0.5)	2.7 (0.1)	23.4 (1.3)	12.4 (0.9)	2.4 (0.1)	2.2 (0.2)	13.9 (0.9)	2.7 (0.2)	4.7 (0.1)	1.4 (0.1)	62.3 (2.9)
11/07	2.5 (0.3)	24.6 (1.6)	1.7 (0.1)	5.9 (0.3)	2.0 (0.8)	22.4 (1.3)	11.1 (0.3)	3.5 (0.7)	1.9 (0.2)	16.0 (0.6)	2.4 (0.3)	4.9 (0.3)	0.9 (0.5)	63.8 (2.9)
27/07	2.4 (0.1)	28.5 (7.5)	1.8 (0.2)	5.3 (0.5)	1.9 (1.0)	22.4 (2.6)	11.3 (0.8)	2.8 (1.2)	2.3 (0.2)	12.9 (1.3)	2.6 (0.4)	4.9 (0.5)	1.8 (0.5)	54.4 (23.3)
16/08	2.8 (0.6)	33.0 (2.9)	1.9 (0.2)	5.7 (1.0)	1.7 (0.3)	22.7 (4.7)	11.4 (2.4)	2.3 (1.1)	2.3 (0.1)	9.8 (0.5)	2.7 (0.2)	6.4 (2.2)	2.0 (0.1)	42.9 (21.8)
30/08	2.4 (0.3)	36.7 (0.3)	1.7 (0.1)	5.2 (0.2)	1.6 (0.2)	19.7 (0.6)	9.6 (0.0)	2.7 (0.3)	2.1 (0.1)	9.3 (0.1)	2.6 (0.3)	4.5 (0.3)	1.6 (0.4)	91.1 (5.7)
15/09	2.9 (0.2)	36.1 (5.6)	1.8 (0.2)	5.2 (0.4)	1.6 (0.2)	21.1 (2.7)	10.3 (1.7)	1.5 (0.9)	2.2 (0.4)	10.2 (1.4)	2.5 (0.7)	3.2 (1.5)	1.5 (0.4)	98.5 (12.2)
03/10	1.9 (0.3)	30.1 (2.1)	1.2 (0.2)	3.8 (0.8)	1.1 (0.3)	22.0 (0.4)	8.7 (2.1)	1.3 (0.9)	1.8 (0.3)	22.0 (5.6)	2.3 (0.5)	2.6 (1.7)	1.3 (0.2)	65.4 (7.2)
24/10	3.0 (0.4)	30.9 (7.5)	1.8 (0.1)	5.3 (0.2)	1.5 (0.2)	20.5 (0.8)	10.6 (1.8)	2.0 (0.6)	2.2 (0.3)	14.8 (5.2)	2.1 (0.1)	4.4 (0.6)	1.6 (0.2)	105.3 (23.7)

Figure Captions

FIGURE 1. Total pigment (dotted line, full dots) and chlorophyll-*a* (solid line, empty squares) concentration in the water column (A) and in the water-sediment interface (B) in the Bay of Brest during year 2011. Chlorophyll-*a* concentration (gray area) at the sea floor are from Chatterjee et al. (2013).

FIGURE 2. Proportions of fucoxanthin (A), peridinin (B), chlorophyll-*b* (C), 19'HF (D) and 19'BF (E) in the water from the column (light gray area) and from the water-sediment interface (dark gray area) and in the stomach (dotted dark line, full dots) and the rectum content (solid dark line, empty squares) of *P. maximus* in the Bay of Brest during year 2011.

FIGURE 3. Total quantity of pigment measured in the water column (light gray area, dotted white line), at the water-sediment interface (gray area) and in the stomach content of *P. maximus* (dark line, full dots) in the Bay of Brest during year 2011. Data from the water-sediment interface from the study of Chatterjee et al. (2013) are also plotted (dark gray area, dotted dark line) to complete the present data set.

FIGURE 4. Linear regression ($r^2 = 0.78$) between the proportion of fucoxanthin measured in the stomach and in the rectum of *P. maximus* (expressed as % of the total pigment) in the Bay of Brest, during the year 2011.

FIGURE 5. Total pigments (without degradation pigments) in the stomach content of *P. maximus* (gray area) and proportions of phaeophorbid-*a* (dotted lines, full dots) and phaeophytin-*a* (solid line, empty squares) in the stomach content (gray) and the rectum content (dark) of *P. maximus* in the Bay of Brest during year 2011.

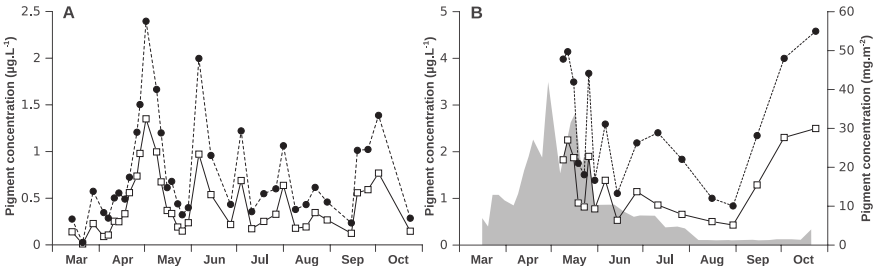
FIGURE 6. Proportions of fucoxanthin (solid gray line, full dots) and peridinin (dotted gray line, empty squares) in the stomach content of *P. maximus* in the Bay of Brest during year 2011.

FIGURE 7. Proportion of six major fatty acids of the polar lipids in the digestive gland of *P. maximus* (expressed as fatty acid molar percentage of total) in the Bay of Brest, during the year 2011. A: 20:5n-3 (dotted line, full dots) and 22:6n-3 (solid line, empty squares); B: 18:4n-3 (dotted line, full dots) and 20:4n-6 (solid line, empty squares); C:

18:2n-6 (dotted line, full dots), 18:3n-3 (dashed line, gray squares) and 20:4n-3 (solid line, empty triangles); D: total fatty acid concentration (polar and neutral fractions, expressed in $\mu mol.g^{-1}$) .

FIGURE 8. Proportion of cholesterol (dotted line, full dots), 24 methylene (dashed line, gray squares) and brassicasterol (solid line, empty triangles) in the digestive gland of *P. maximus* (expressed as sterol % of the total sterol) in the Bay of Brest, during the year 2011.

Figures



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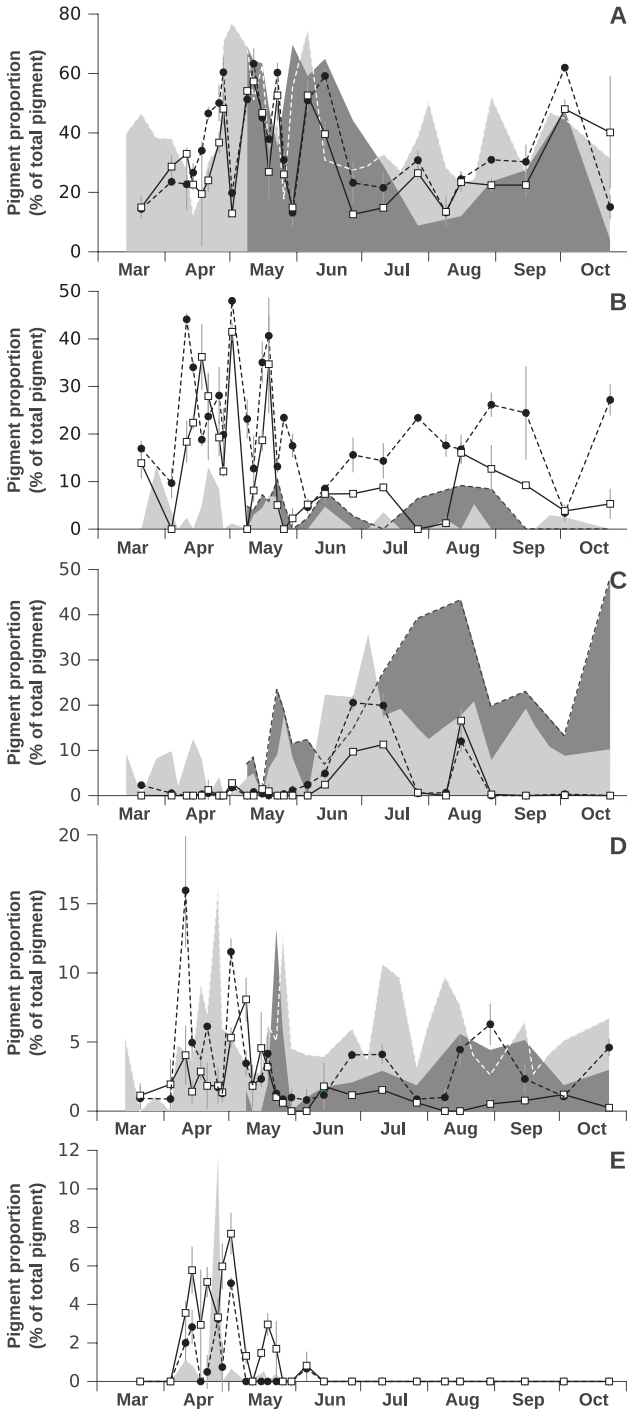

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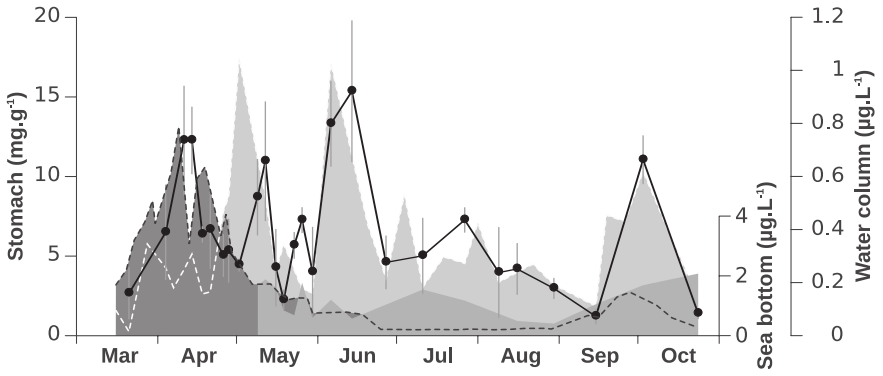

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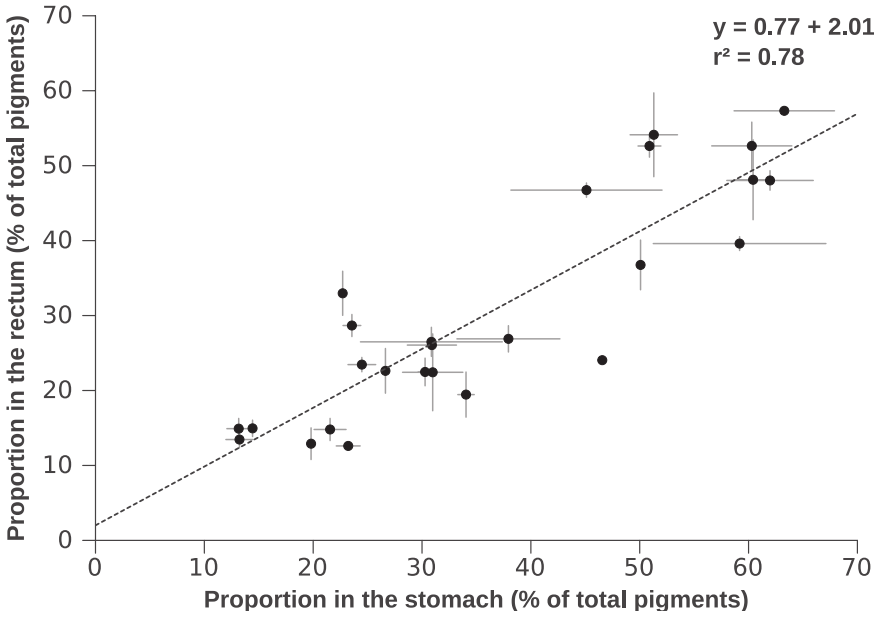

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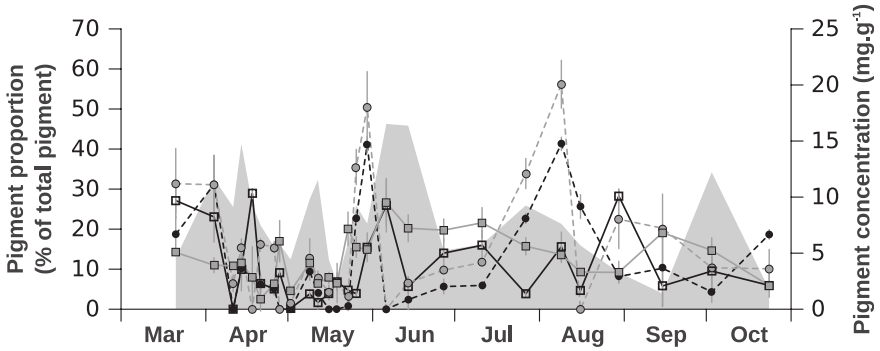

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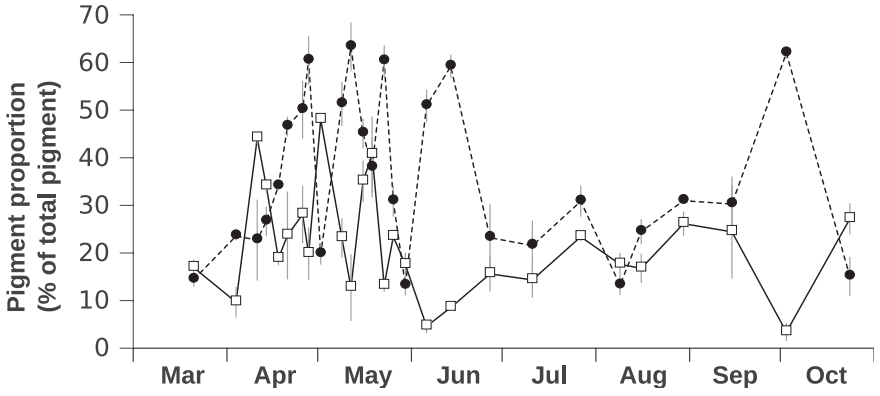

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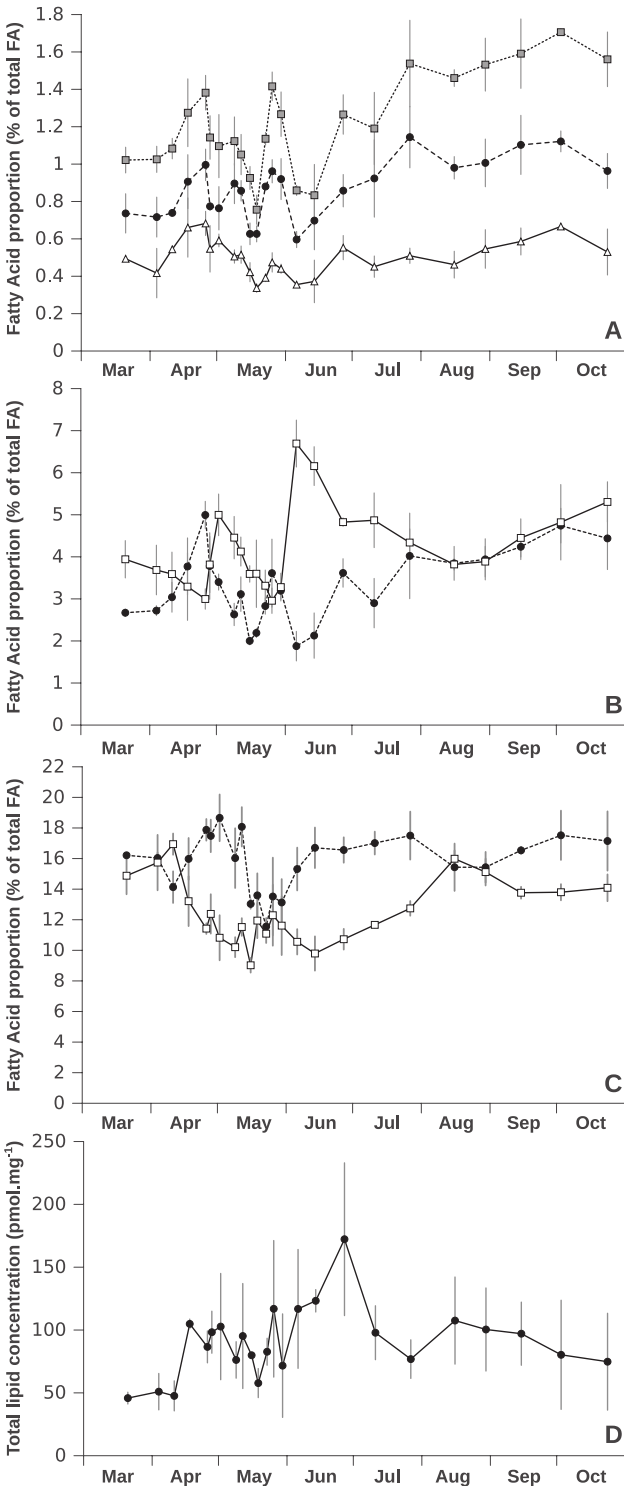

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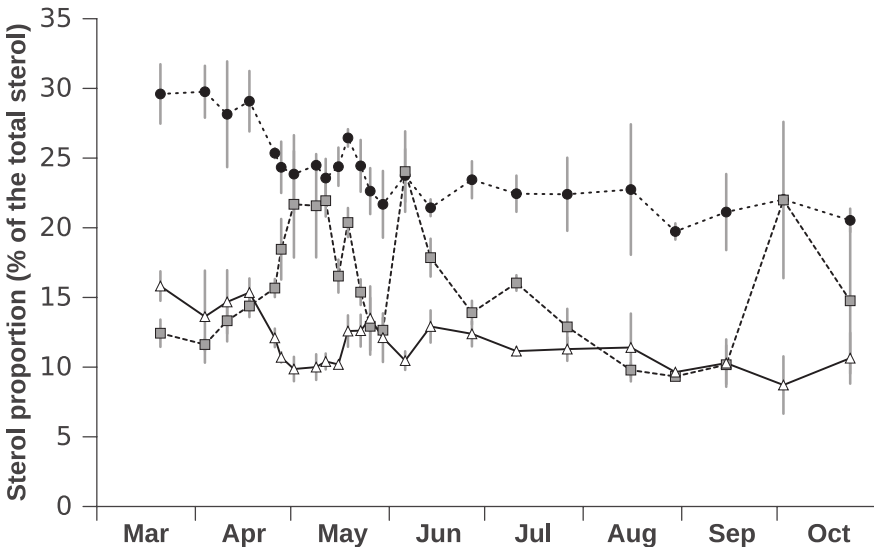

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III/ Article n°6 :

Proteomic-based comparison of *Pecten maximus* populations along a latitudinal gradient

Article à soumettre dans Journal of Sea Research (dec 2013)

Proteomic-based comparison of *Pecten maximus* populations along a latitudinal gradient

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Abstract

Comparing populations living in contrasted habitats is an efficient way to decipher how organisms do modulate their physiology as a function of environmental conditions. Here we determine the proteomic signatures of two populations of the great scallop *Pecten maximus*, living at the northern limit- (Hordaland, Norway) and in the center- (Brest, France) of this species' latitudinal distribution range. The results showed 38 protein spots significantly differentially accumulated in mantle tissues from individuals of the two populations. We could unambiguously identify 11 of them by Maldi TOF-TOF mass spectrometry. 8 of them corresponded to actin isoforms, and two were identified as Filamin, another protein related to the cytoskeleton structure. The last one corresponded to the protease elastase. In all, our results strongly suggest a crucial importance of the cytoskeleton structure in the differential ways of life of scallops poputations, as a function of their biogeographical characteristics.

Introduction

Pecten maximus is a filter-feeding bivalve mollusk, living along the north-east Atlantic coasts from Norway to Morocco and entering the Mediterranean in the western part of the Alboran Sea. Further east, it is replaced by a close species, *P. jacobeus*. Like many other marine bivalves (Gosling, 2003), *P. maximus* is characterized by populations of sessile adults living on the sea bottom and a long planktonic larval phase, lasting up to 6 weeks (Beaumont & Barnes, 1992). Giving these characteristics, *P. maximus* populations are expected to show low genetic differentiation among populations. Nevertheless, marine barriers such as eddies, fronts and gyres may restrict the gene flow between populations during larval transport and the highly variable recruitment implying demographic instability as well as the natural selection can lead to more marked differences than expected between populations (Saavedra & Peña, 2005).

Population genetics studies of *P. maximus* are controversial to some extent. Two studies were carried out on *P. maximus* populations in United Kingdom and France using allozyme polymorphic loci (Beaumont *et al*., 1993; Wilding *et al*., 1998), and none of them revealed well-defined structuration at the sub-population scale. However, other studies based on RFLP (Restriction Fragment Length Polymorphism) and RAPD (Random Amplified Polymorphic DNA) of mitochondrial DNA (Wilding *et al*., 1997; Heipel *et al*., 1998) showed a genetic separation in Britain populations but only for the population of Mulroy Bay in Ireland, an enclosed area. Studies on Norwegian populations based on mitochondrial haplotypes, showed negligible (Ridgway & Dahle, 2000) to significant differences (Beaumont, 2006) with samples from UK, France and Ireland, depending on the mode of analysis. The relative lack of genetic structuration is somewhat surprising, given the differences observed in life-history traits (e.g. reproduction, shell growth) in the different studied areas (Paulet *et al*., 1988; Mackie & Ansell, 1993; Chauvaud *et al*., 2012).

The use of proteomic tools in population comparison perspectives, called "population proteomics", is a new field, formerly developed to find cancer-specific biomarkers in human population studies (Nedelkov, 2005; Nedelkov *et al*., 2006). Since its introduction, this new research field has found its way to environmental sciences, leading to some interesting conclusions, e.g. in ecotypes comparisons of angiosperms *Arabidopsis* (Chevalier *et al*., 2004), snails (Martínez-Fernández *et al*.,

2008) or mussels (López *et al*., 2001). 2D gel based studies carried out at a wider scale have also provided useful considerations, such as the identification and distinction of hake populations (Gonzalez *et al*., 2010), marine mussels (López *et al*., 2002), fishes (Carrera *et al*., 2006, 2007) and shrimp species (Ortea *et al*., 2009).

In this study we compared the mantle tissue proteomes of *Pecten maximus* from two different populations, located in the north (i.e. Norwegian population) and in the center (i.e. Bay of Brest population) of its latitudinal distribution range. The expression of proteins is known to depend on both environmental factors and genetic factors. The aim of this study was not to assess genetic differences between populations, but to identify potential biomarkers at the protein level that could be used to discriminate populations. Moreover, since proteins are the effectors of cells, the identification of such biomarkers could provide valuable insights of the potential physiological and phenotypical differences between the populations of Norway and France. In order to identify valuable biomarkers, efforts were made to avoid environmental biases. Animals of similar sizes were collected and sampling periods were chosen to match a similar physiological state for the scallops. In order to reduce temporal variability, animals were sampled at two weeks intervals, before and during a major change in the scallop's environment, i.e. the first phytoplankton bloom after winter. Protein expression was then paired-compared at each sampling date and only proteins systematically differentially expressed between populations at each sampling date were considered as valuable candidates.

Materials and methods

Sampling

Individuals of *Pecten maximus* were collected from the Bay of Brest (Brittany, France) by SCUBA diving in Spring 2011, at a depth of 10 m (Fig.1). Five animals were collected before the first phytoplankton bloom (21/03/2011; average shell height, standard deviation: 93.2 mm, 5.0 mm) and five more during the first bloom (04/04/2011; 104.3 mm, 6.9 mm). Similarly, specimens were sampled from Bjørnafjorden (Hordaland, Norway; Fig. 1) in Spring 2012 before (04/04/2012; 103.0 mm, 6.0 mm) and during the first phytoplankton bloom (17/04/2012; 108.4 mm, 2.7 mm). At both locations, animals were quickly dissected once in the laboratory, the

mantle tissue was snap-frozen in liquid nitrogen and kept at -80°C until protein extraction.

In the Bay of Brest, environmental parameters were monitored using a CTD profiler (Sea-Bird SBE-911), which was immersed at the site of sampling, as described in Chatterjee *et al*. (2013). For Bjørnafjorden location, a CTD profiler (AS model 204, SAIV Bergen Norway) was immersed from December 2011 to April 2012 at the sampling site and data were collected weekly (Table 1).

Table 1: Details of Sampling Site and parameters at the time of sampling

Site	Coordinates	Date	Temperature	Chl-a (µg/l)	Salinity
Bay of Brest	48° 17' 43.32" N, 4° 27' 12.78" W	21/03/11	9.8°C	0.89	34.10
		04/04/11	11.3°C	1.69	34.10
Bjørnafjorden	60° 5' 1.14" N, 5° 16' 26.04" E	04/04/12	7.1°C	0.46	30.36
		17/04/12	7.3°C	1.53	31.89

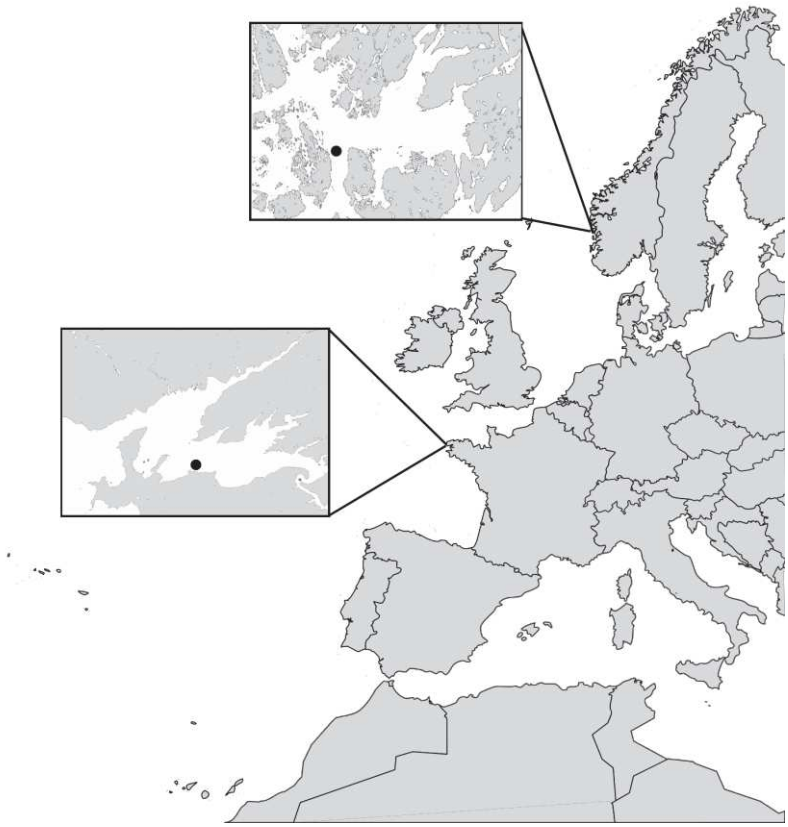

Figure 1: Sampling sites of Pecten maximus populations from Bjørnafjorden (Hordaland, Norway) and the Bay of Brest (Brittany, France). Black points indicate precise sampling sites.

Proteins extraction

Mantles were crushed with a mixer mill (MM400, RETSCH, Haan, Germany) and kept frozen during the crushing using liquid nitrogen. One hundred milligram of the obtained powder was homogenized in 100 mM Tris-HCl (pH 6.8) containing 1% of Protease inhibitor mix (GE Healthcare). After centrifugation (50 000 g, 5 min, 4°C) pellets were discarded and supernatants were pipetted in other tubes, nucleic acids were then enzymatically removed following manufacturer's instructions (nuclease mix, GE Healthcare). Samples were precipitated at 4°C using trichloroacetic acid 20% (1/1:v/v, overnight). After centrifugation (20 000 g, 30 min, 4°C), pellets were washed with acetone 70% and re-suspended in urea/thiourea buffer (2 M thiourea, 7 M urea, 4% CHAPS, 1% DTT) containing 1% IPG (pH 4–7, GE Healthcare). Protein concentrations were determined using a modified Bradford assay (Ramagli, 1998), and all samples were adjusted to 400 µg of proteins in 250 µl.

Two-dimensional electrophoresis

Prior to isoelectric focusing, IPG strips (pH 4–7, 13 cm, GE Healthcare) were passively rehydrated with 250 µl of protein solution in wells for 14 h. Isoelectric focusing was conducted using the following protocol: 250 V for 15 min, 500 V for 2 h, gradient voltage increase to 1000 V for 1 h, gradient voltage increase to 8000 V for 2 h 30, 8000 V for 3 h, and reduced to 500 V (Ettan IPGphor3, GE Healthcare). To prepare for second dimension SDS-PAGE electrophoresis, strips were incubated in equilibration buffer (50 mM Tris-HCl pH 8.8, 6 M urea, 30% glycerol, 2% SDS and 0.002% Bromophenol Blue) for two 15 min intervals, first with 1 $g.l^{-1}$ dithiothreitol and then with 48 $g.l^{-1}$ iodoacetamide. IPG strips were then placed on top of 12% polyacrylamide gels, which were run in 10°C thermo-regulated device (SE 600 Ruby, Amersham Biosciences) at 10 mA per gel for 1 h and then 30 mA per gel until complete migration. Gels were subsequently stained with Coomassie Blue (PhastGel, GE Healthcare) and unspecific coloration was destained with a solution containing 30% methanol and 7% acetic acid. The resulting gels were scanned with a transparency scanner (Epson Perfection V700) in gray scale with 16-bit depth and a resolution of 400 dpi.

Gel image analysis and statistical analysis of protein abundances

Images were aligned and spots were detected and quantified using the Progenesis SameSpots software (version 3.3, Nonlinear Dynamics) applying the

automated algorithm. All detected spots were manually carefully checked and artifact spots were removed. Data were exported as volume raw values and statistical analyzes were conducted in R (R Core Team, 2013) using the packages prot2D (Artigaud *et al*., 2013) and Limma (Smyth, 2004). Data were normalized (quantile normalization) and the samples from France were paired-compared using moderated *t*-test to the samples from Norway for each sampling date (with 5 replicates per group). A global correction by false discovery rate (fdr ; Benjamini & Hochberg, 1995) was used, in order to take into account multiple comparisons issues and paired-comparison correction. Spots with an fdr threshold lower than 0.1 and an absolute fold change superior to 2 were considered as differentially expressed.

Mass spectrometry

Proteins which abundance changed between animals from Norway and France were excised from gels and prepared for analysis by mass spectrometry (MS). Gel pieces were first washed in 50 mM ammonium bicarbonate (BICAM), and then dehydrated in 100% acetonitrile (ACN). Gel pieces were vacuum-dried, and rehydrated with BICAM containing 0.5 µg sequencing grade porcine trypsin (Promega), and incubated overnight at 37°C. Peptides were extracted from the gels by alternative washing with 50 mM BICAM and ACN, and with 5% formic acid and ACN. Between each step, the supernatants were pooled, and finally concentrated by evaporation using a centrifugal evaporator (SpeedVac). Samples were then resuspended in trifluoroacetic acid (TFA; 0.1% in water). Peptide solutions were mixed with the α-cyano-4-hydroxycinnamic acid (HCCA, 10 $mg.ml^{-1}$ of a ACN/TFA/water (60/4/36:v/v/v) solution), and spotted on a polished steel target using the dried droplet method. Peptides were then analyzed by Matrix-Assisted Laser Desorption Ionization Time-Of-Flight tandem mass spectrometry (MALDI TOF-TOF) in positive ion reflector mode, using an Autoflex III (Bruker Daltonics) mass spectrometer. The FlexControl software (v3.0, Bruker Daltonics) was set up to acquire successively PMF spectra and MS/MS from the dominant peaks. Mass spectra were analyzed with FlexAnalysis (v 3.0; Bruker Daltonics) by applying the following conditions: TopHat algorithm for baseline subtraction, Savitzky-Golay analysis for smoothing (0.2 m/z; number of cycles: 1) and SNAP algorithm for peaks detection (signal-to-noise ratio: 6 for MS and 1.5 for MS/MS). The charge state of the peptides

was assumed to be +1. Fragments of porcine trypsin were used for internal mass calibration.

Proteins were subsequently identified with PEAKS (v 5.3, Bioinformatics Solutions) using MS/MS-based identification and de novo sequencing. The search parameters against a custom-made database were set as follows: carbamidomethylation of cysteine was set as a fixed modification, oxidation of methionine and phosphorylation of serine, threonine and tyrosine were set as variable modifications, one missing cleavage during trypsin digestion was allowed and the tolerance for precursor-ion mass tolerance was set to 1 Da. The database was constructed by combining *P. maximus* sequences from two sources: sequences from a previous RNAseq study and sequences from the REPROSEED project. Overall, the database included a total of 252 888 *P. maximus* expressed sequence tags (ESTs). Protein identification was considered as unambiguous when a minimum of two peptides matched with a minimum score of 20. False discovery rates were also estimated using a reverse database as decoy. EST database sequences were annotated by homology searches against a non-redundant database using the Blast algorithm from NCBI (http://www.ncbi.nlm.nih.gov/BLAST).

Results

A total of 648 spots were detected in all the replicates from each location in 2-DE gels (fig. 2). Paired comparisons between scallops from France and Norway for each week of sampling showed that most of the differences between populations are shared whatever the sampling date, resulting in a total of 38 protein spots significantly differentially accumulated (fig. 3). Overall, 23 proteins were more abundant in mantle tissues from French scallops (spots Fr-1 to 23, fig. 2) and 15 were significantly more abundant in Norwegian samples (spots No-1 to 15, fig. 2). Contrastingly, direct comparisons between samples from the same location at different sampling dates showed no difference for the French scallops, and only 8 differentially accumulated proteins for the Norwegian samples. The 38 proteins differentially expressed allowed to establish a clear differentiation between French and Norwegian populations, as showed in the figure 4.

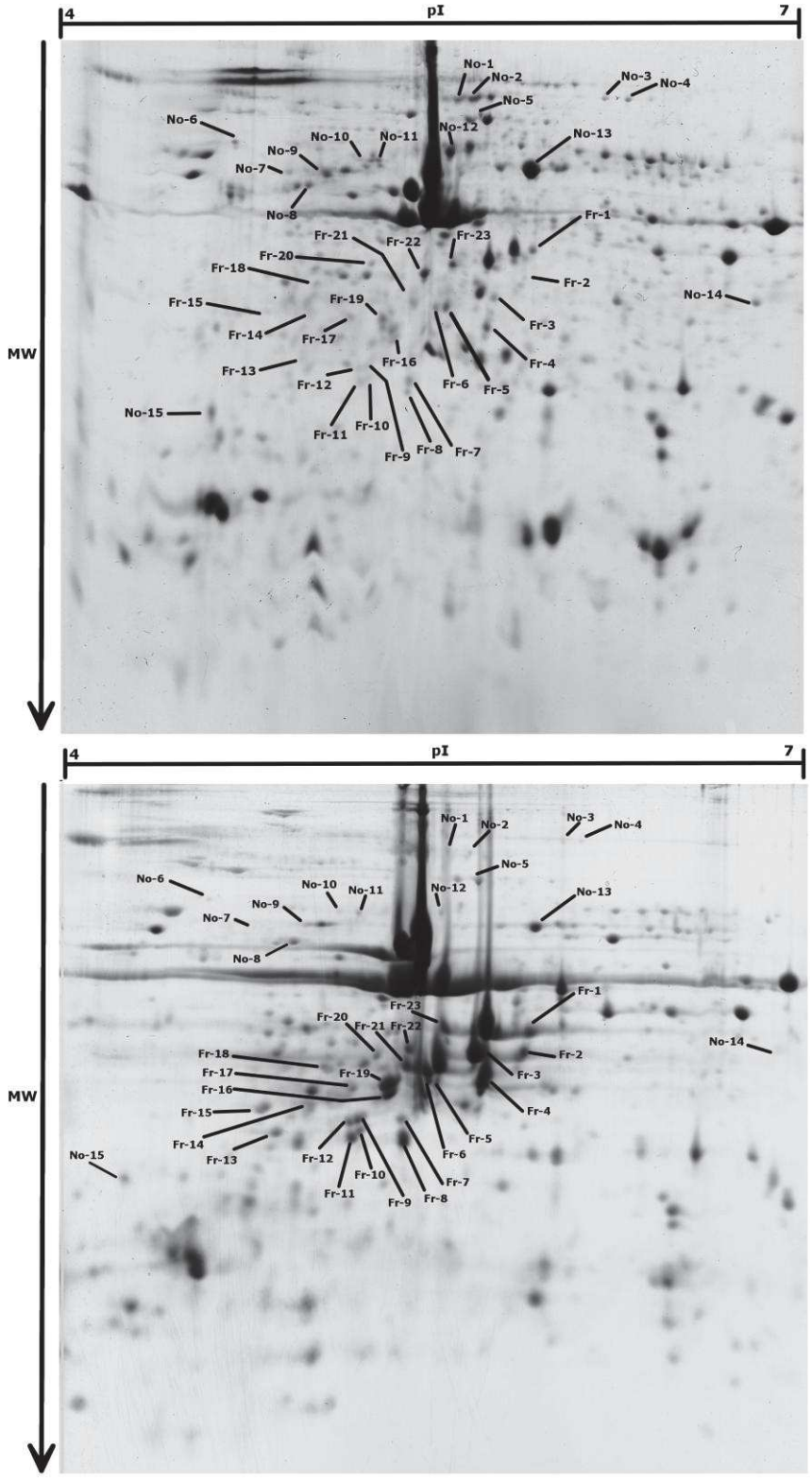

Figure 2: Representative bi-dimensional gels (pH 4-7, SDS-PAGE 12%) for *Pecten maximus mantle* proteins from Norway (upper gel) and France (lower gel). Differentially expressed spots are arrowed.

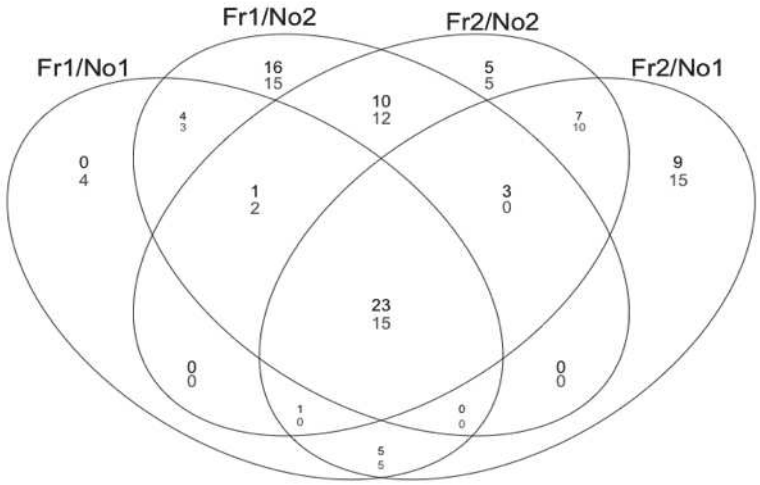

Figure 3: Venn diagram representing differentially expressed *Pecten maximus* mantle proteins (moderate t-test paired-comparison, fdr <0.1, absolute fold change >2) between samples from France and Norway at different sampling date (Fr1: Bay of Brest, 21/03/11; Fr2: Bay of Brest, 04/04/11; No1: Bjørnafjorden, 04/04/12; No2: Bjørnafjorden, 17/04/12). Each ellipse represents a comparison between the two populations at a given date, e.g. Fr1/No2 is the comparison between animals from France sampled in week 1 (21/10/11) and animals from Norway sampled in week 2 (17/04/12). Upper and lower numbers are the numbers of proteins more abundant for French or Norwegian samples, respectively.

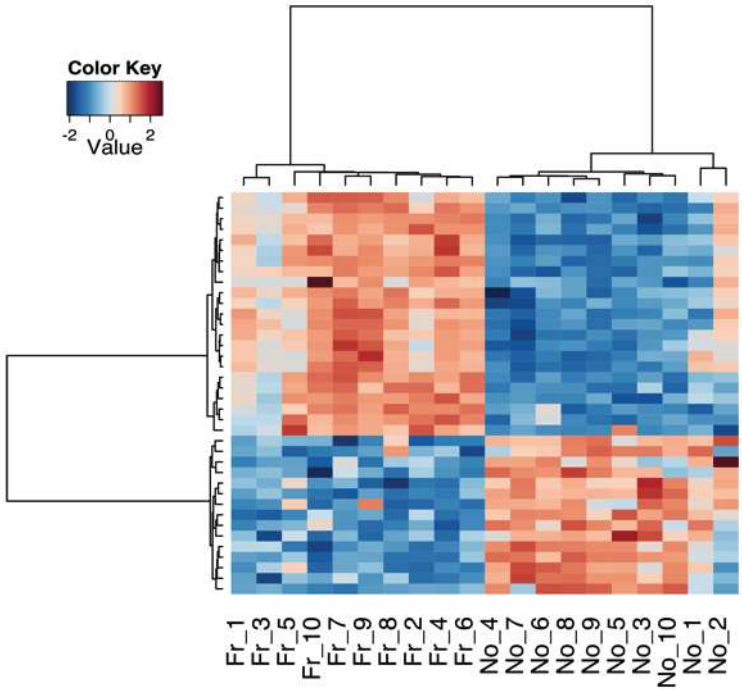

Figure 4: Two-way hierarchical clustering analysis with Euclidean distance and Ward's aggregation method of proteins from *Pecten maximus* mantle differentially expressed between French and Norwegian populations. Columns represent gels replicates where Fr-1 to 5 are the samples from first sampling date in France and Fr-6 to 10 for the second sampling date, similarly No-1 to 5 are the samples from first sampling date in Norway and No-6 to 10 for the second one. Rows represent the 38 differentially expressed proteins between the populations.

Table 2: List of *Pecten maximus* mantle tissue proteins identified by MS/MS whose abundance change between samples from France and Norway (moderate t-test paired-comparison, fdr <0.1, absolute fold change >2). Mass weight (MW) and number of amino acids (AA) are computed from NCBI sequences.

Spot	-10logP	% Coverage	Peptides sequences	Assignation	NCBI	EST	MW	AA
Fr-4	666.54	18	SYELPDGQVSTRGNER.C GYSFTTTAER.G QEYDESGPSIVHR.A	Actin [*Pecten sp*.]	1101351B	scallop_rep_c_68146	42kDa	374
Fr-23	521.94	13	SYELPDGQVSTRGNER.C QEYDESGPSIVHR.A					
Fr-2	1452.26	14	QEYDESGPSKVHR.C SYEEPDGQVATDGNER.A VAPEEHPVYATEAPTNPK.C GYSFTTTAER.T	Actin-2 [*Crassostrea gigas*]	EKC38058.1	Contig_35831	42kDa	376
Fr-6	1341.09	12	GYSFTTTAER.G SYEEPDGQVATDGNER.A VAPEEHPVLLTEAPLNPK.G					
Fr-9	594.56	12	SYEHPDGQVRTXGNER.K GYSFTTTAER.G	beta-Actin, partial [*Cipangopaludina cathayensis*]	ACT22633.1	scallop_rep_c100421	21kDa	184
Fr-10	492.53	12	SYEHPDGQVRTXGNER.K GYSFTTTAER.G					
Fr-21	583.29	17	SYEHPDGQVRTXGNER.K GYSFTTTAER.G					
Fr-5	1361.49	6	GYSFTTTAER.G QEYDESGPSIVHR.A SYEXPDGQVKTRGNER.C NHC(+57.02)PSSTNNENQDHRS(+79.97)TR.G	Actin [*Azumapecten farreri*]	AAP88387.1	Contig_6250	42kDa	376
No-3	799.91	4	GEINQPC(+57.02)EFNIYTR.G VTESDIGSLMATIR.A FNDEHIPQSPYR.G VYVTPSIGDAR.A	Filamin-C [*Crassostrea gigas*]	EKC28512.1	Contig_13763	324kDa	3016
No-12	981.70	3	FNDEHXPQSPYR.T GEENQPC(+57.02)EFNXYTR.A VYVTPSIGDAR.A					
No-10	214.01	3	SAYDQGQNPTPYYR.G IGTWENDEEGR.A	Elastase [*Lottia gigantea*]	ESO85431.1	Contig_1	75kDa	645

The 38 differentially expressed protein spots were excised from gels, trypsinolysed, and analyzed by mass spectrometry, leading to the formal identification of 11 proteins (Table 2). Within these 11 proteins, 8 were identified as actin. Given these rather surprising results, spots were re-analyzed by excising other gels and particular care was taken in order to avoid contamination, however mass spectrometry results were similar (data not shown). Although the 8 proteins were all identified as actin, they matched to 4 different EST sequences. Theses sequences were aligned along with actin sequences from *Pecten sp*. (Accession n°1101351B) and closely related species *Crassostrea gigas* (EKC38058.1) and *Azumapecten farreri* (AAP88387.1). Only one sequence could be considered as complete (Contig_6250). This sequence showed 91% similarity with actin from *Pecten sp*. and *C*. *gigas* and 94% with the sequence from *A*. *farreri*. Among the most expressed proteins in Norwegians samples, 2 were identified as Filamin C and 1 was identified as elastase, a metalloprotease.

Discussion

Among the 38 proteins analyzed by mass spectrometry, only 11 were identified, but a poor ratio of identification is common in non-model species (Forné *et al*., 2010; Dowd, 2012). Surprisingly, within the 11 proteins identified, 10 were related to the cytoskeleton. Moreover, 8 distinct spots were identified as actin. One reasonable hypothesis for this is the presence of several isoforms. Isoforms of actin are widespread in eukaryotes (Khaitlina, 2001). Mammals and birds are known to contain 6 different isoforms encoded by separate genes. Four of these isoforms are related to muscular functions ($\alpha_{skeletal}$-actin, $\alpha_{cardiac}$-actin, α_{smooth}-actin, γ_{smooth}- actin) and two are related to the cytoskeleton (β_{cyto}-actin and γ_{cyto}-actin) (Perrin & Ervasti, 2010). In marine invertebrates, isoforms are less known and no classification of isoforms has yet been reported. Nevertheless, some studies have described the existence of actin isoforms in some phyla, like cephalopods (Carlini *et al*., 2000), gastropods from the Planorbidae family (Adema, 2002) or abalone (Bryant *et al*., 2006). In bivalve mollusks, southern hybridization in *Placopecten magellanicus* revealed the existence of 12 to 15 genes coding for actin (Patwary *et al*., 1996) and the presence of different isoforms within the cytoplasm of *Chlamys farreri* has also

been inferred (Ma *et al*., 2007). Moreover, for *Crassostrea gigas*, which genome was recently sequenced (Zhang *et al*., 2012), 13 non-redundant actin sequences of similar sizes (354-379 AA) were found in GenBank (http://www.ncbi.nlm.nih.gov/).

The complete sequence found in our database (Contig_6250) could be one of the *P*. *maximus* actin isoforms as it differs from actin sequence found in GenBank (accession number 1101351B) only by 20 amino acids (Supplemental Data 1). Isoforms of actin are generally very close and differ only by a few numbers of amino-acid in their N-terminal part (Khaitlina, 2001; Perrin & Ervasti, 2010). Nevertheless all the spots identified as actin are not necessary isoforms and could also result from alternate splicing or post translational modifications (PTMs). It is noteworthy that actin is known to be a major target of PTMs such as acetylation, ADP-ribosylation, arginylation, methylation, and phosphorylation (Terman & Kashina, 2013).

Actin is one of the most essential and abundant intracellular proteins. Actin microfilaments are involved in numerous major cellular functions, e.g. maintaining cell shape, cell division, endocytosis, exocytosis, secretion, signal transduction, and regulation of enzyme activities (Pollard & Cooper, 1986; Welch *et al*., 1998). Interestingly, actin isoforms have both overlapping and unique cellular functions (Perrin & Ervasti, 2010). Thus, biochemical evidence suggests that properties, and therefore functions, of actin microfilaments may vary according to the mix of isoforms in the filament (Bergeron *et al*., 2010). In a way actin can actually be compared to "cellular steel" (Perrin & Ervasti, 2010). As for steel, actin is an alloy which characteristics depend on the ratio of each components and different kind of actin are designed to meet specific needs in tensile strength, flexibility, etc. and taking into accounts the costs of building.

Filamin is another protein related to the cytoskeleton that has been identified in our study. Two different spots were identified as filamin and they are likely to be isoforms of this protein. Isoforms of filamin are expected in *P*. *maximus* as they have been observed in numerous phyla (*e*.*g*., Filamin A, B and C in mammals; van der Flier & Sonnenberg, 2001), and three isoforms have already been characterized in bivalves (Méndez-López *et al*., 2012). Filamins are a family of protein initially considered as cytoskeleton organizers as they cross-link microfilaments of actin through their actin binding domain (van der Flier & Sonnenberg, 2001). Nevertheless, recent studies in mammalians have shown that filamins can interact with several cell proteins other

than actin, including membrane receptors, ion channels, enzymes, signaling pathway proteins and transcription factors (Popowicz *et al*., 2006; Nakamura *et al*., 2011).
Intriguingly, in our data, 8 isoforms of actin were overabundant in French populations of scallops, whereas the 2 isoforms of filamin were more abundant in Norwegian specimens. These differences in cytoskeleton organization and regulation might impact numerous functions associated with cytoskeleton and finally rain down upon the whole physiology of animals.

One of the major differences between French and Norwegian populations is the rate and scope of growth (Strand & Parsons, 2006; Chauvaud *et al*., 2012). Indeed, French scallops grow faster and have asymptotic sizes much lower than Norwegian scallops (Strand & Parsons, 2006; Chauvaud *et al*., 2012). Chauvaud *et al* (2012) explained the lower annual growth rate in Northern populations by a similar daily growth rate but a reduced number of days spent each year to achieve growth in the North as compared to the South. The same authors proposed that reduced costs in maintenance associated with higher growth efficiency might explain the higher asymptotic sizes in the Northern population (Chauvaud *et al*., 2012). Specifically, the annual growth rate of scallops decrease with age, i.e. as scallops are aging the annual number of growth days are reduced, but this decrease is much less pronounced in Northern populations, leading to higher asymptotic sizes (Chauvaud *et al*., 2012).
The differences observed in the cytoskeleton organization and regulation might play a key role in the growth efficiency. The cytoskeleton might have a major role in adjusting the biological rhythms through the sensing of environmental parameters and transforming it into biochemical signals (Shweiki, 1999). Therefore, a change in the cytoskeleton organization could reflects a difference in sensing and/or signaling pathways, leading to temporal variations of physiological processes according to the location. In addition, filamin might be of particular interest for further studies, as it regulates the transforming growth factor-β (TGF-β) signaling, and thus mediates cell proliferation (Zhou *et al*., 2010).

In conclusion, future genetic studies might focus on actin and filamin and their respective isoforms. An extensive analysis of these particular sequences could provide useful information on the genetic local adaptation versus phenotype plasticity debate.

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Supplemental Data 1: Multiple Sequence Alignment (Clustal O, version 1.2.0) of actin sequences from *Pecten sp* (NCBI accession number: 1101351B), *Crassostrea gigas* (EKC38058) and *Azumapecten farreri* (EKC28512) with EST sequences from *Pecten maximus* identified as actin in this study.

```
scallop_rep_c_68146  ------------------------------------------------------------
Contig_35831         ------------------------------------------------------------
scallop_rep_c_100421 ------------------------------------------------------------
Contig_6250          MCDDDVAALVVDNGSGMCKAGFAGDDAPRAVFPSIVGRPRHQGVMVGMGQKDSYVGDEAQ 60
Actin[Pecten_sp.]    --DDEVAALVVDNGSGMCKAGFAGDDAPRAVFPSIVGRPRHQGVMVGMGQKDSYVGDEAQ 58
Actin-2[C._gigas]    MGDEDVAALVIDNGSGMCKAGFAGDDAPRAVFPSIVGRPRHQGVMVGMGQKDSYVGDEAQ 60
Actin[A._farreri]    MCDDDVAALVVDNGSGMCKAGFAGDDAPRAVFPSIVGRPRHQGVMVGMGQKDSYVGDEAQ 60


scallop_rep_c_68146  ------------------------------------------------------------
Contig_35831         ------------------------------------------------------------
scallop_rep_c_100421 ------------------------------------------------------------
Contig_6250          SKRGILTLKYPIEHGIVTNWDDMEKIWHHTFYNELRVAPEEHPVLLTEAPLNPKANREKM 120
Actin[Pecten_sp.]    SKRGILTLKYPIEHGIVTNWDDMEKIWHHTFYNELRVAPEEHPVLLTEAPLNPKANREKM 118
Actin-2[C._gigas]    SKRGILTLKYPIEHGIVTNWDDMEKIWHHTFYNELRVAPEEHPVLLTEAPLNPKANREKM 120
Actin[A._farreri]    SKRGILTLKYPIEHGIVTNWDDMEKIWHHTFYNELRVAPEEHPVLLTEAPLNPKANREKM 120


scallop_rep_c_68146  ------------------------------------------------------------
Contig_35831         ------------------------------------------------------------
scallop_rep_c_100421 ------------------------------------------------------------
Contig_6250          TQIMFETFNAPAMYVAIQAVLSLXASGRTTGIVFDAGDGVSHTVPIYEGAVVSTQSTGGD 180
Actin[Pecten_sp.]    TQIMFETFNAPAMYVAIQAVLSLYASGRTTGIVLDSGDGVTHTVPIYEGYALPHAILRLD 178
Actin-2[C._gigas]    TQIMFETFNSPAMYVAIQAVLSLYASGRTTGIVLDSGDGVTHTVPIYEGYALPHAIMRLD 180
Actin[A._farreri]    TQIMFETFNSPAMYVAIQAVLSLYASGRTTGIVFDAGDGVSHTVPIYEGYALPHAILRLD 180


scallop_rep_c_68146  ----------MKILTERGYSFTTTAEREIVRDIKEKLCYVALDFENEMSTAASSSSLEKS 50
Contig_35831         ----------MKILTERGYSFTTTAEREIVRDIKEKLCYVALDFEQEMQTAASSSSLEKS 50
scallop_rep_c_100421 ---------------------------------------VALDFEQEMQTAASSSSLEKS 21
Contig_6250          LAGRDLTDYLMKILTERGYSFTTTAEREIVRDIKEKLAYVALDFEQEMQTAASSSSLEKS 240
Actin[Pecten_sp.]    LAGRDLTDYLMKILTERGYSFTTTAEREIVRDIKEKLCYVALDFENEMATAASSSSLEKS 238
Actin-2[C._gigas]    LAGRDLTDYLMKILTERGYSFTTTAEREIVRDIKEKLCYVALDFEQEMATAASSSSLEKS 240
Actin[A._farreri]    LAGRDLTDYLMKILTERGYSFTTTAEREIVRDIKEKLCYVALDFEQEMQTAASSSSLEKS 240
                                                       ******:** ***********


scallop_rep_c_68146  YELPDGQVITIGNERFRCPESLFQPSFLGMESAGIHETTYNSIMKCDVDIRKDLYANTVL 110
Contig_35831         YELPDGQVITIGNERFRCPEALFQPSFLGMESAGIHETTYNSIMKCDVDIRKDLYANTVL 110
scallop_rep_c_100421 YELPDGQVITIGNERFRCPEALFQPSFLGMESAGIHETTYNSIMKCDVDIRKDLYANCVL 81
Contig_6250          YELPDGQVITIGNERFRCPEALFQPSFLGMESAGIHETTYNSIMKCDVDIRKDLYANCVL 300
Actin[Pecten_sp.]    YELPDGQVITIGNERFRCPESLFQPSFLGMESAGIHETTYNSIMKCDVDIRKDLYANTVL 298
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Conclusions et Perspectives

1 Le stress thermique chez *Pecten maximus*

Les mollusques bivalves, comme la coquille Saint-Jacques *Pecten maximus*, sont des organismes ne régulant pas la température de leur milieu intérieur (poïkilothermes) et dont la principale source de chaleur provient du milieu extérieur (ectothermes). Une variation de la température dans le milieu extérieur se répercute donc de façon directe sur leur organisme. L'effet d'une augmentation de la température sur la respiration a notamment été étudié dans le chapitre 3. Une augmentation des taux de respiration mesurés à 10°C et 18°C a ainsi été observée mais des taux singulièrement plus faibles à 25°C par rapport à 18°C on été mesurés (Fig.1). L'essentiel de l'oxygène moléculaire est utilisé dans les cellules au niveau de la chaine respiratoire mitochondriale pour la production d'ATP (90% ; Rolfe 1997). Une augmentation de la respiration traduit donc une augmentation de la production d'ATP via la mitochondrie. Or, cette augmentation de la production est due à une augmentation de la demande en énergie qui augmente avec la température, par l'effet direct de la température sur les processus biochimiques.

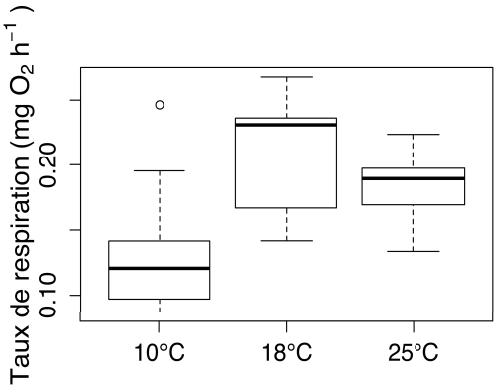

Figure 1: Taux de respiration (en mg O_2 h^{-1}) de *Pecten maximus* à différentes températures (10°C, 18°C et 25°C) (n=10, sauf pour 10°C , n=9)

La relative diminution de la consommation en oxygène des organismes observée entre 18°C et 25°C est, quant à elle, liée à un phénomène physiologique dépendant de l'adaptation des organismes à la température. En effet, avec l'augmentation de la température, les besoins en énergie continuent de croître, mais un plafond est atteint lorsque les capacités aérobies de production d'énergie de l'organisme sont limitées par sa capacité à apporter l'oxygène au niveau des tissus (Pörtner, 2001). C'est le concept de limitation thermique par la capacité en oxygène (OCLTT, Pörtner, 2001). Cette capacité détermine une fenêtre thermique optimale, bordée par les températures pejus. Or, à 25°C nos résultats portent à croire que *P.*

maximus a dépassé sa température pejus supérieure, on observe alors une diminution de ses capacités aérobies.

Les besoins en énergie sont donc supérieurs à 25°C et les capacités aérobies sont réduites. Une augmentation de l'activité de l'octopine déshydrogénase (ODH) a été observée à 25°C par rapport aux activités à 10°C et 18°C (Article 3, chapitre 3) coïncidant avec une diminution de la concentration en arginine (Article 3, chapitre 3), mettant ainsi en évidence la mise en place du métabolisme anaérobie pour les coquilles Saint-Jacques acclimatés à 25°C. L'ODH est en effet une enzyme clé du métabolisme fermentaire chez les bivalves, permettant le renouvellement des espèces nicotiniques réduites ($NADH_2$) en oxydées, en catalysant la réaction entre l'arginine et le pyruvate et aboutissant à l'accumulation de l'octopine (Grieshaber *et al.*, 1994). Les forts besoins en ATP des coquilles à 25°C sont donc couverts, au moins en partie, par l'apport en énergie imputable au métabolisme fermentaire.

Pour l'expérience du chapitre 3, les coquilles Saint-Jacques on été acclimatées pendant une semaine à une température de 18°C ou 25°C, après une montée d'un degré par jour. Les expériences réalisées dans le chapitre 1 nous renseignent sur les voies d'adaptation à plus long terme, puisque les animaux ont été maintenus à des températures élevées (21°C et 25°C) sur une période de 56 jours.

Les expériences réalisées par l'analyse haut débit de données transcriptomiques (RNAseq) n'ont pas permis d'identifier de transcrits codant des protéines impliquées dans le métabolisme glucidique surexprimés dans les animaux maintenus à 21°C ou 25°C pendant une période de 56 jours. Cette observation laisse penser que l'activité du métabolisme anaérobie n'est pas plus importantes dans ces animaux que dans les contrôles (animaux maintenus à 15°C). Toutefois, la plupart des régulations du métabolisme glucidique ont lieu au niveau protéique plutôt que de l'activité transcriptionelle. L'analyse des protéines par électrophorèse bidimensionnelle sur les mêmes animaux ne montre cependant pas plus d'augmentation de protéines du métabolisme glucidique. Au contraire, la glycéraldéhyde-3-phosphate déshydrogénase (GAPDH) et la phosphoglycérate mutase (PGM) on été identifiées comme significativement moins abondantes chez les animaux à 25°C après 27 jours d'exposition.

Cette diminution des protéines impliquées dans le métabolisme glucidique s'accompagne de l'augmentation d'ARNm impliqués dans le métabolisme des acides gras (comme la phospholipase A2, l'adipocyte plasma membrane-associated protein ou l'alpha-methylacyl-CoA racemase). Ces résultats suggèrent que l'adaptation au stress thermique chronique chez *P. maximus* dépend de l'utilisation des réserves lipidiques plus que du métabolisme purement fermentaire. Il est intéressant de noter qu'une étude du stress thermique sur une échelle de temps similaire (3 mois) chez un autre mollusque bivalve, l'huître *Crassostrea gigas*, a obtenu des résultats comparable (Clark *et al.*, 2013).

Un autre élément nouveau, révélé par la combinaison des études transcriptomique et protéomique du stress thermique dans le chapitre 1, est l'implication relativement faible des protéines de choc thermique (HSP) dans l'adaptation thermique chronique. En effet, peu de gènes et aucune protéine de la réponse « classique » au choc thermique n'ont été identifiés dans cette étude. Il faut cependant noter que le parti pris pour l'analyse bioinformatique ne permet pas de considérer les transcrits exprimés de façon transitoire dont les HSPs font peut être partis.

Enfin, l'identification de la diminution de l'expression de la GAPDH au niveau protéique à 25°C, pourrait être de première importance. En effet, de nombreuses études récentes attribuent à cette protéine des rôles essentiels dans le fonctionnement cellulaire, en dehors de son rôle dans la glycolyse. Notre analyse, à partir de nos résultats, des possible réseaux de régulation, suggère un rôle pivot de la GAPDH dans la réponse cellulaire à un stress thermique chronique (Chapitre 1). En particulier, les interactions de cette protéine avec la voie de signalisation anti-apoptique AP-1, mais également avec d'autres voies. En outre, nos résultats suggèrent l'importance de la régulation négative de l'apoptose dans la réponse, ainsi que de systèmes de réparation de l'ADN, ce qui conduirait à un état de mutagénèse somatique accrue dont le rôle dans le développement de la réponse est difficile à comprendre.

En conclusion, les résultats obtenus sur l'adaptation au stress thermique de *P. maximus* au cours des différentes études présentées dans ce travail soulignent l'importance et la complémentarité des différentes approches. En effet, les études physiologiques et métaboliques du stress thermique sur une courte période sont nécessaires pour permettre d'approcher de façon générale la réponse de *P. maximus* à

des températures élevés dans son milieu. Cependant, les approches transcriptomiques et protéomique d'un stress thermique plus long permettent de comprendre la mise en place de mécanismes plus fins, plus susceptibles de refléter la réalité du fonctionnement de *P. maximus* dans son milieu et dans le contexte actuel de changement global. D'autre part, ces études montrent aussi l'intérêt des approches « sans a priori » en protéomique et en transcriptomique, qui permettent de mettre en lumière des voies de régulation nouvelles, comme le rôle central de la GAPDH, et de relativiser l'importance, dans le contexte particulier de notre étude, de voies bien caractérisés comme la régulation des HSPs.

2 Combinaison des stress thermique et hypoxique chez *Pecten maximus*

Tout comme l'augmentation de température, la diminution de la disponibilité en oxygène dans l'eau de mer est une des conséquences du changement global qui peut avoir un impact majeur sur les animaux marins (Grieshaber *et al.*, 1994; Harley *et al.*, 2006). L'évolution des taux de respiration de *P. maximus* en fonction de la saturation en oxygène dans l'eau de mer étudiée dans le chapitre 3, a permis d'estimer deux paramètres caractérisant la réponse d'un organisme à l'hypoxie : le pourcentage de régulation et le point critique en oxygène (PcO_2).

Le pourcentage de régulation représente la capacité d'un organisme à maintenir sa consommation en oxygène lorsque le niveau d'oxygène décroit dans le milieu extérieur. Un organisme capable de maintenir une consommation en oxygène complètement indépendante de la concentration en oxygène dans le milieu extérieur aura ainsi un pourcentage de régulation de 100%. La PcO_2, quant à elle, correspond à la concentration en oxygène pour laquelle la respiration devient dépendante de la concentration dans le milieu extérieur (oxyconformité).

La coquille Saint-Jacques, précédemment qualifiée d'oxyrégulateur limité (Brand & Roberts, 1973), se révèle comme une espèce relativement tolérante à l'hypoxie en l'absence de stress thermique, avec des taux de régulation élevés (94% à 10°C, 96% à 18°C) et des PcO_2 faibles (18% et 24% d'O_2 à 10°C et 18°C, respectivement). Cependant les individus utilisés pour cette étude sont jeunes et de petite taille (environ

6 mois), alors que Brand & Roberts (1973) avaient travaillé sur des individus adultes, ce qui peut expliquer les différences de résultats obtenus.

A 25°C néanmoins, le pourcentage de régulation diminue (86%) et la PcO_2 double par rapport à 10°C (36% d'O_2). Ces évolutions laissent à penser qu'un stress hypoxique ajouté au stress thermique, *P. maximus* étant en dehors de sa fenêtre thermique optimale à 25°C, induit une situation critique pour ces organismes. Ces prédictions se confirment après un passage de 24 h en hypoxie en dessous de la PcO_2, où un fort taux de mortalité (50%) est observé pour les coquilles Saint-Jacques maintenues à 25°C.

Les dosages métaboliques réalisés sur les animaux après 24 h d'hypoxie aux trois différentes températures testées viennent fournir une piste d'explication. L'activité de l'ODH est relativement similaire entre les conditions hypoxiques et normoxiques, et les différences observés sont liées essentiellement à la température avec un augmentation notable à 25°C. Pour les concentrations en arginine, en revanche, une différence est observée ente les conditions hypoxique et normoxique mais seulement à 18°C. Tandis qu'à 10°C, des concentrations élevées sont observées dans les deux conditions, et qu'à 25°C des concentrations plus faibles sont observées dans les deux conditions. Ces résultats suggèrent que le métabolisme anaérobie est peu utilisé après 24h d'hypoxie à 10°C, alors qu'il est fortement utilisé à 18°C. A 25°C, il semble que les réserves d'arginine mobilisables pour le métabolisme anaérobie soient déjà consommées en normoxie. Ainsi à 25°C, la diminution de production d'énergie due à la diminution des capacités aérobies ne peux être palliée par un déclenchement du métabolisme anaérobie, tous les besoins métaboliques ne peuvent donc pas être couverts et les coquilles Saint-Jacques se trouvent en situation de vulnérabilité aigue.

Les analyses protéomiques effectuées sur ces même animaux viennent confirmer la singularité des réponses à l'hypoxie en fonction de la température. En premier lieu, le nombre de protéines dérégulés en hypoxie n'est pas le même selon la température.

A 10°C, aucune protéine n'a été identifiée comme différentiellement accumulée entre les conditions hypoxique et normoxique. Ce résultat suggère que, dans ces conditions expérimentales, la réduction d'oxygène pendant 24 h à 10°C n'est pas une condition stressante pour la coquille Saint-Jacques et ne nécessite pas d'ajustements importants des protéines. Il est cependant possible que des changements transitoires

ait eu lieu, notamment par le biais de modifications post-traductionnelles (e.g. , phosphorylation).

Aux températures plus élevées, des différences d'accumulation de protéines ont été identifiées entre les conditions hypoxiques et normoxiques. Ainsi, 15 et 11 protéines on été identifiées comme dérégulées à 18°C et 25°C, respectivement. Les réponses à l'hypoxie diffèrent largement selon la température car sur ces 26 protéines, une seule a été à la fois identifiée comme dérégulée à 18°C et 25°C.

Parmi les protéines dérégulées après 24 h d'hypoxie pour les coquilles maintenues à 18°C, une caseine kinase 2 a été identifiée comme surexprimée en hypoxie. Cette protéine pourrait jouer un rôle majeur dans la réponse de *P. maximus* à l'hypoxie en améliorant l'activité de transcription de HIF-1 (hypoxia inducible factor 1) via la dégradation de p53, un inhibiteur compétitif de HIF-1 (Mottet *et al.*, 2005; Hubert *et al.*, 2006).

D'autre part, le métabolisme des acides gras semble être impliqué dans la réponse au stress hypoxique à 18°C via la régulation de la NADH-cytochrome b5 reductase. Tandis que le métabolisme glucidique semble être régulé à la baisse chez les animaux en situation d'hypoxie à 25°C, comme le montre la diminution de l'enolase 3 et de l'alpha-L-fucosidase.

Chez les animaux acclimatés à 25°C en situation d'hypoxie, des protéines impliquées dans l'apoptose ont aussi été identifiées (Gelsolin, kinase TBK1). L'effet d'un changement d'abondance de ces protéines n'est cependant pas clairement identifiable puisqu'ils semblent être antagonistes. Toutefois l'importance des mortalités observées après 24 h d'hypoxie ainsi que la probable incapacité du métabolisme anaérobie à pallier au déficit de production d'énergie aérobie, pointent en direction d'une défaillance cellulaire énergétique. Dans ce cas, l'induction de l'apoptose serait la voie privilégiée (Wang *et al.*, 2003; Festjens *et al.*, 2007).

Pour conclure, la combinaison des approches écophysiologiques et protéomiques dans l'étude de l'hypoxie à différentes températures chez *P. maximus* montre la cohérence des réponses observées à différentes échelles, à savoir, des réponses à l'hypoxie profondément modifiées en fonction de la température. Enfin, l'implication du métabolisme des acides gras ainsi que la diminution de la glycolyse en hypoxie, même si ils ne sont pas observés pour la même température, ne sont pas sans rappeler

les mécanismes impliqués dans les réponses au stress thermiques vus précédemment. L'importance du métabolisme lipidique dans la réponse au stress est souvent sous-évaluée, ce qui souligne la pertinence de mener des études à plus long terme pour évaluer la réponse au stress chez les organismes marins.

3 *Pecten maximus* dans son environnement

Les populations de *Pecten maximus* sont notamment caractérisées par d'importantes différences de croissance suivant un gradient latitudinal (Chauvaud et *al.*, 2012). Les populations au Nord de l'aire de répartition ont en effet une croissance annuelle plus faible mais une taille asymptotique plus grande que les populations du Nord.

Parmi les facteurs de l'environnement conditionnant la croissance, la qualité et la quantité des sources trophiques utilisables par *Pecten maximus* sont de première importance. L'étude menée par Lavaud *et al.* (Article 5) présentée dans le chapitre 4, utilise une approche innovante pour tracer la quantité, la qualité et les variations temporelles des sources trophiques disponibles pour *P. maximus* en rade de Brest en combinant trois marqueurs trophiques : les pigments, les acides gras et les stérols.

Les algues phytoplanctoniques dinoflagellées ont beaucoup été étudiées sous le prisme de leur toxicité. Ainsi, des études ont montré l'impact négatif d'espèces de dinoflagellées toxiques sur la croissance de *P. maximus* (Lorrain et *al.*, 2000). Les résultats obtenus lors de l'étude présentée dans le chapitre 4, notamment les niveaux de DHA et de péridine dans la glande digestive et l'estomac, suggèrent néanmoins que la coquille Saint-Jacques filtre activement certaines espèces de dinoflagellées. Cette remise en cause des sources trophiques disponibles pour *P. maximus* pourrait avoir un impact majeur, notamment dans la comparaison des sources trophiques avec des populations nordiques utilisables pour la croissance.

Idéalement une étude utilisant les même marqueurs et avec le même effort d'échantillonnage, menée sur des populations nordiques de *P. maximus*, pourrait donner des moyens de comparaison des régimes alimentaires entre populations et leur influence sur la croissance. Dans ce contexte, il semble également que la caractérisation du régime alimentaire de *P. maximus*, par des approches post-

génomiques (métagénomiques et/ou métaprotéomiques) devraient se révéler extrêmement intéressante. Il est à noter que j'ai participé dans ce cadre au montage d'un petit projet, MetaMax (PI: V. Pichereau), visant à la caractérisation métaprotéomique (par couplage SDSPAGE 1D/nanoLC/Maldi TOF-TOF) du régime alimentaire de *P. maximus*, pour lequel j'ai réalisé des expériences préliminaires. D'importantes différences de croissance ont été observées entre les populations de *P. maximus* évoluant à différentes latitudes. Cela pose bien entendu des questions à propos de la structure génétique de ces populations (qui sera étudiée au laboratoire). Dans le cadre de cette thèse, nous avons entrepris de comparer les signatures protéomiques de deux populations évoluant an nord de l'aire de répartition de l'espèce, i.e. en Norvège (Bjørnafjorden, plus précisément), en en plein centre de cette aire, i.e. en rade de Brest. Cette étude a été permise par l'obtention d'un financement LabexMER, qui m'a permis d'effectuer un séjour à Bjørnafjorden, en Norvège, pendant ma thèse.

L'expression des protéines de coquilles Saint-Jacques issues des deux populations ont été comparées dans le chapitre 4 (Article 6). Afin d'identifier les protéines exprimées de façon différentielle entre les deux populations, des animaux de tailles similaires ont été échantillonnés, à une période de l'année correspondant à un état physiologique comparable dans les deux populations. D'autre part, afin de réduire le biais temporel et l'expression de protéines liées à des conditions environnementales particulières, les animaux ont été échantillonnés à deux semaines d'intervalle dans les deux sites.

La grande majorité des protéines qui on été identifiées dans cette étude font partie du cytosquelette (10 sur 11). Ainsi 8 protéines surexprimées dans les animaux de la rade de Brest on été identifiées comme étant de l'actine, tandis que 2 des protéines surexprimés dans la population nordique ont été identifiés comme étant de la filamine. Des différences aussi importantes, pour des protéines impliquées dans une fonction aussi essentielle pour la cellule que le cytosquelette, apparaissent étonnantes.

La filamine ne se cantonne pas à son rôle de soutien dans l'organisation du cytosquelette, mais joue bien d'autres rôles, notamment dans la signalisation cellulaire. Son implication dans la régulation de l'activité du facteur de croissance TGF-β semble plus particulièrement intéressante, au regard des différences de croissance observées entre les deux populations.

Étant données les importantes différences dans l'organisation du cytosquelette entre les deux populations, il paraît raisonnable de penser que le rôle premier du cytosquelette, son rôle de soutien, soit impacté. En conséquence, il est possible que les contraintes d'ordre physique selon la localisation impliquent une adaptation du cytosquelette pour conserver son rôle de soutien et ses propriétés (de viscosité, rigidité, etc.).

Une autre hypothèse impliquerait le cytosquelette comme récepteur des signaux de l'environnement permettant d'ajuster l'horloge biologique des animaux (Shweiki, 1999). Dans ce cas, les différences observées dans l'organisation du cytosquelette pourraient indiquer une différence de sensibilité aux signaux de l'environnement et, *in fine*, se traduire par un décalage chronologique dans les fonctions biologiques essentielles entre les deux populations, comme la croissance. Cette hypothèse est d'autant plus séduisante que les différences de croissance observées entre les populations de *Pecten maximus* tiennent avant tout à une différence dans le nombre de jours alloués à la croissance chaque année (Chauvaud *et al.*, 2012).

4 Perspectives

Rôle du métabolisme lipidique dans l'adaptation à long terme à des conditions stressantes chez Pecten maximus

Le métabolisme lipidique semble être impliqué dans l'adaptation au stress thermique, comme le montre dans notre étude la régulation de certains ARNm, et dans la réponse au stress hypoxique, comme le laisse penser la régulation de la cytochrome b reductase. La composition en acides gras de la coquille Saint-Jacques a été largement étudiée et est donc bien caractérisée (Soudant *et al.*, 1996). Des études permettant de déterminer une modification des différentes classes d'acides gras, des stérols etc... chez *P. maximus* en fonction de l'exposition au stress thermique et hypoxique pourrait permettre de mettre en évidence de façon directe l'implication du métabolisme lipidique et de préciser son rôle dans l'adaptation au stress à plus long terme.

Caractérisation fonctionnelle et identification des régulations des protéines clés du stress thermique et hypoxique chez Pecten maximus.

Parmi les protéines identifiées dans notre étude, certaines semblent jouer un rôle clé dans la réponse au stress. Notamment les rôles de la GADH et de AP-1, qui semblent au centre de la régulation face au stress thermique, tandis que CK2-α pourrait être de première importance dans la réponse au stress hypoxique.

Un des moyens pour préciser le rôle et la régulation de ces protéines consisterait à utiliser des primo-cultures de *P. maximus* et à investiguer les effets des protéines cibles via l'utilisation d'ARN interférence. Chez *P. maximus*, des primo-cultures de cellules cardiaques ont déjà été maintenues pendant 1 mois (Le Marrec-Croq *et al.*, 1999) et d'autres études sur des cellules isolées ont déjà été utilisés pour évaluer les effets de différents facteurs de croissance (Giard *et al.*, 1998). D'autre part l'utilisation d'ARN interférence (ARNi) dans des expériences *in vivo* chez les bivalves ont déjà prouvé la viabilité et l'intérêt de l'utilisation de telles démarches (Fabioux *et al.*, 2009; Béguel *et al.*, 2013).

L'inhibition des protéines d'intérêt suite à l'utilisation d'ARNi dans les primo-cultures de *P. maximus* pourrait permettre l'identification d'autres protéines dont la régulation est liée aux protéines cibles. Ainsi, en combinant des approches transcriptomiques et protéomiques, et en faisant varier les conditions de culture des cellules et les protéines ciblées par ARNi, ces études pourrait permettre une caractérisation complète de la réponse moléculaire aux stress hypoxique et thermique.

Recherche de polymorphisme pour les gènes identifiés dans la réponse aux stress entre les populations de Pecten maximus

Les gènes identifiés dans cette étude apparaissent d'un intérêt particulier dans la perspective d'identifier des différences génétiques, soit entre des populations de *P. maximus* éloignés géographiquement, soit entre des familles d'individus issus de géniteur identifiés, une démarche actuellement en cours au Lemar.

Le génotypage et l'analyse des séquences d'ADN d'actines et de filamines dans différentes populations de *P. maximus*, le long de son aire de répartition, pourraient ainsi permettre d'identifier des adaptations locales, en lien avec les différences de phénotypes observés.

D'autre part l'identification de variants pour les gènes identifiés dans cette étude comme liés à la réponse au stress, pourrait permettre la recherche d'un lien entre ces variants et des réponses plus ou moins performantes aux situations stressantes chez *P. maximus* selon les individus. A plus long terme, la recherche de ces marqueurs dans les populations naturelles pourrait permettre une identification des populations les plus vulnérables au changement global.

Développement des approches multiples pour l'étude de la réponse au stress chez les organismes marins

Le développement des outils de séquençage haut débit représentent de formidables perspectives pour l'étude des organismes marins. Ainsi le séquençage massif d'acides nucléiques, en plus de son aspect analytique, accroît l'information génétique disponible pour de plus en plus d'organismes marins et peux ainsi servir de support pour d'autres études, notamment protéomiques, sur ces espèces. En parallèle

de ces nouvelles approches, le développement des méthodes protéomiques haut débit « gel free» promet de nouvelles perspectives pour la caractérisation fonctionnelle des protéines. L'étude de l'expression de protéines par électrophorèse bidimensionnelle a cependant encore de beaux jours devant elle, notamment dans l'étude des modifications post-traductionnelle. Dans cette perspective, le développement de méthodes statistiques appropriées, telles que celle détaillée dans le chapitre 1, devrait permettre l'amélioration des comparaisons effectuées et la fiabilité des protéines découvertes.

D'autre part l'estimation de paramètres critiques pour l'évaluation de la réponse à l'hypoxie, par les méthodes détaillés dans le chapitre 3, devrait permettre une meilleure comparaison des réponses des espèces à l'hypoxie ainsi que de l'évaluation de l'impact d'autres stress sur la réponse à l'hypoxie dans une perspective d'approche multistress.

Enfin, cette étude a permis de mettre en évidence l'utilité de multiplier les approches dans l'étude de la réponse au stress. L'étude notamment à différentes échelles d'organisation (physiologique, protéines, ARN) ainsi qu'à différentes échelles de temps permet d'appréhender les différents aspects de la réponse et de l'adaptation des organismes marins à des conditions stressantes.

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Annexe

prot2D : Statistical Tools for volume data from 2D Gel Electrophoresis

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Contents

1 Introduction

This document briefly describes how to use the *prot2D* package. An R package designed to analyze (i.e. Normalize and select significant spots) data issued from 2D SDS PAGE experiments. *prot2D* provides a simple interface for analysing data from 2D gel experiments. Functions for normalization as well as selecting significant spots are provided. Furthermore, a function to simulates realistic 2D Gel volume data is also provided.

2 Input data for *prot2D*

prot2D uses raw 2D volume data intensities as input, datasets must be exported from specialized Image Software in the form of a dataframe of volume data $X_{j,i}$ with gels j as columns and spots i as rows. Note that the name of columns

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Table 1: Example of input data for prot2D with 3 replicates gels in each condition

	Replicates Condition 1			Replicates Condition 2		
	$Gel1$	$Gel2$	$Gel3$	$Gel1'$	$Gel2'$	$Gel3'$
$Spot_1$	$X_{1,1}$	$X_{2,1}$	$X_{3,1}$	$X_{1',1}$	$X_{2',1}$	$X_{3',1}$
$Spot_2$	$X_{1,2}$	$X_{2,2}$	$X_{3,2}$	$X_{1',2}$	$X_{2',2}$	$X_{3',2}$
$Spot_i$	$X_{1,i}$	$X_{2,i}$	$X_{3,i}$	$X_{1',i}$	$X_{2',i}$	$X_{3',i}$

should therefore corresponds to the names of the gels and the names of the rows to the name of the spots. The replicates for each condition should be ordered in the following columns (see Table 1). Furthermore, another dataframe is needed to describe the experiment with the names of gels as rownames and a single column giving the two level of condition for data.

3 Getting started

To load the *prot2D* package into your R envirnoment type:

```
> library(prot2D)
```

In this tutorial we will be using the `pecten` dataset obtained from a 2-DE experiment performed on proteins from the gills of *Pecten maximus* subjected to a temperature challenge. 766 spots were identified with 6 replicates per condition, therefore the dataset is a dataframe of 766 rows and 12 columns (for details, see Artigaud et al., 2013). `pecten.fac` describe the data by giving the names of the gels (as rownames) and the condition for the temperature challenge (15C = control vs 25C) in the "Condition" column, load by typing:

```
> data(pecten)
> data(pecten.fac)
```

4 *prot2D* workflow

4.1 Vizualize and Normalize volume data

Vizualization

Dudoit et al. (2002) proposed a method for the visualization of artifacts in microarray datasets, called the MA-plot, which was transposed for proteomics data as the Ratio-Intensity plot (Meunier et al., 2005; R-I plot). It consists in plotting the intensity log_2-ratio (R) against mean log_{10} intensity (I):

$$R = log_2 \frac{mean(V_{Cond2})}{mean(V_{Cond1})} \tag{1}$$

The intensity (I) is the log10 of the mean of volume data in condition 2 by the mean of volume data in condition 1 :

$$I = log_{10}(mean(V_{Cond2}) \times mean(V_{Cond1})) \tag{2}$$

Where V_{Cond1} and V_{Cond2} are spot volumes for conditions 1 and 2, respectively. In *prot2D*, R-I plot can be easily displayed with `RIplot`:

```
> RIplot(pecten, n1=6, n2=6)
```

`RIplot` requires data supplied as a dataframe or a matrix as well as the number of replicates for each conditions.

Normalization

2D Gel Volume data must be normalized in order to remove systemic variation prior to data analysis. Two widely used methods are provided, the "Variance Stabilizing Normalization" (vsn) and the "Quantiles, Normalization". The principle of the "quantiles normalization" is to set each quantile of each column (i.e. the spots volume data of each gels) to the mean of that quantile across gels. The intention is to make all the normalized columns have the same empirical distribution. Whereas the vsn methods relies on a transformation h, of the parametric form $h(x) = arsinh(a+bx)$ (Huber et al., 2002). The parameters of h together with those of the calibration between experiments are estimated with a robust variant of maximum-likelihood estimation. Both methods recentered the data around a zero log ratio, nevertheless for low values of intensities the vsn normalized data seems to be less efficient in order to recentered the cloud of points. Users are thus advised to use the quantiles normalization via a call to `Norm.qt`.

```
> pecten.norm <- Norm.qt(pecten, n1=6, n2=6, plot=TRUE)
```

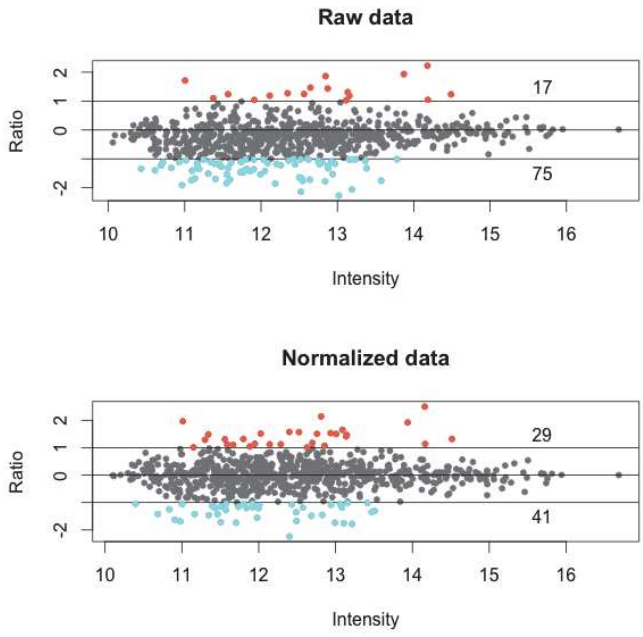

Figure 1: Ratio-Intensity plot showing Quantiles normalization

```
>
```

4.2 Coerce data into an ExpressionSet

Prior to analysis for finding differentially expressed proteins, data must be coerced into an `ExpressionSet`. This can be done easily with `ES.prot`, which requires a matrix of normalized volume data, the number of replicates in each condition and a dataframe giving the condition for the experiment.

```
> ES.p <- ES.prot(pecten.norm, n1=6, n2=6, f=pecten.fac)
```

The matrix of spots intensities (i.e. Volume) is log2 transformed and stored in the `assayData` slot of the `ExpressionSet`. Furthermore, the log2-ratio is computed and stored in the `featureData` slot.

4.3 Find differentially expressed proteins

As described in Artigaud et al (2013) *prot2D* provide functions adapted from microarray analysis (from the *st, samr, limma, fdrtool* package). 2-DE experiments analysis require a variant of the t-statistic that is suitable for high-dimensional data and large-scale multiple testing. For this purpose, in the last few years, various test procedures have been suggested. *prot2D* provides:

- the classical Student's t-test (in `ttest.Prot` function)
- two tests especially modified for micro-array analysis : Efron's t-test (2001; `efronT.Prot` function) and the modified t-test used in Significance Analysis for Microarray (Tusher et al, 2001; `samT.Prot` function).
- two methods that take advantage of hierarchical Bayes methods for estimation of the variance across genes: the "moderate t-test" from Smyth (2004; `modT.Prot` function) and the "Shrinkage t" statistic test from Opgen-Rhein and Strimmer (2007; `shrinkT.Prot` function).

As statistical tests allowing the identification of differentially expressed proteins must take into account a correction for multiple tests in order to avoid false conclusions. *prot2D* also provide different methods to estimate the False Discovery Rate :

- the classical FDR estimator of Benjamini and Hochberg (1995).
- the local FDR estimator of Strimmer (2008).
- the "robust FDR" estimator of Pounds and Cheng (2006).

`ttest.Prot` function, `modT.Prot` function, `samT.Prot` function, `efronT.Prot` function or `shrinkT.Prot` function can be used to find differentially expressed proteins and the different FDR mode of calculation are implemented with the `method.fdr` argument. However, the moderate t-test with the FDR correction of Benjamini and Hochberg was find to be the best combination in terms of sensitivity and specificity. Thus, users are advised to use this combination by typing :

```
> ES.diff <- modT.Prot(ES.p, plot=TRUE, fdr.thr=0.1,
+                      method.fdr="BH" )
```

```
Number of up-regulated spots in Condition 2
[1] 0
Number of down-regulated spots in Condition 2
[1] 1
```

The function returns an `ExpressionSet` containing only the spots declared as significant. A plot can also be generated to vizualize the FDR cut-off. Additionally, it can be usefull to select only the spots with an absolute ratio greater than 2, as they are often considered as the most biologically relevant proteins, this can be done by adding the command `Fold2=T`. The names of the selected spots can be retrieved with :

```
> featureNames(ES.diff)

[1] "2607"
```

Displaying fold change (as log2(ratio)) for selected spots

```
> head(fData(ES.diff))

         ratio
2607 -1.912657
```

Volume normalized data for selected spots

```
> head(exprs(ES.diff))

     Br_23865 Br_23883 Br_23884 Br_23728 Br_23729 Br_23730 Br_23731 Br_23732
2607 23.21036   23.454 20.22889 22.34141 23.07774  23.0274 22.63096 20.07119
     Br_23733 Br_23875 Br_23876 Br_23877
2607 21.07323 20.52232 20.02905 19.53711
```

5 Simulation of 2D Volume data

In order to compare FDR and the responses of the different tests as well as the influence of the number of replicates, simulated data ca be used. `Sim.Prot.2D` simulates realistic 2D Gel volume data, based on parameters estimate from real dataset. Volume data are computed following these steps (see Smyth, 2004 and Artigaud et al., 2013 for details) :

- Log2 mean volumes from data are computed for each spot.
- Means are used as input parameters in order to simulate a normal distribution (with no differential expression between conditions) for each spot with standard deviations computed as described by Smyth (2004).
- A define proportion `p0` of the spots are randomly picked for introducing differential expression in both conditions (`p0`/2 in each condition).

The `Sim.Prot.2D` returns an `ExpressionSet` of simulated volume data (log2 transformed) with 2 conditions (”Cond1” and ”Cond2” in `phenoData`) slot of the `ExpressionSet`.The spots differentially generated can be retrieve with `notes`. Simulate data based on ”pecten”

```
> Sim.data <- Sim.Prot.2D(data=pecten, nsp=700,
+                         nr=10, p0=0.1, s2_0=0.2, d0=3)
```

Compare different methods for finding differentially expressed proteins

```
> res.stud <- ttest.Prot(Sim.data, fdr.thr=0.1, plot=FALSE)

Number of up-regulated spots in Condition 2
[1] 9
Number of down-regulated spots in Condition 2
[1] 13

> res.mo <- modT.Prot(Sim.data, fdr.thr=0.1, plot=FALSE)

Number of up-regulated spots in Condition 2
[1] 23
Number of down-regulated spots in Condition 2
[1] 20
```

Names of the spots selected by student's t-test with an FDR of 0.1

```
> featureNames(res.stud)

 [1] "68"  "79"  "121" "147" "151" "153" "211" "213" "258" "268" "284" "300"
[13] "304" "343" "385" "387" "410" "499" "507" "518" "617" "660"
```

Names of the spots selected by modT-test with an FDR of 0.1

```
> featureNames(res.mo)

 [1] "23"  "43"  "68"  "69"  "79"  "80"  "111" "121" "143" "147" "153" "169"
[13] "173" "174" "211" "238" "242" "268" "283" "284" "300" "301" "342" "343"
[25] "356" "385" "387" "402" "408" "410" "441" "499" "518" "532" "553" "564"
[37] "580" "617" "629" "660" "664" "666" "670"
```

Names of the differentially generated spots

```
> notes(Sim.data)$SpotSig

 [1] "1"   "67"  "101" "111" "121" "133" "143" "151" "153" "211" "250" "345"
[13] "353" "356" "359" "385" "386" "405" "410" "460" "465" "483" "499" "505"
[25] "518" "528" "553" "564" "565" "599" "617" "629" "646" "23"  "51"  "69"
[37] "79"  "80"  "83"  "115" "147" "169" "173" "238" "242" "266" "268" "283"
[49] "284" "300" "301" "343" "359" "387" "400" "402" "405" "408" "432" "441"
[61] "460" "526" "528" "569" "577" "580" "582" "607" "674"
```

6 Session Info

```
> sessionInfo()
```

```
R version 3.0.2 (2013-09-25)
Platform: x86_64-unknown-linux-gnu (64-bit)

locale:
 [1] LC_CTYPE=en_US.UTF-8       LC_NUMERIC=C
 [3] LC_TIME=en_US.UTF-8        LC_COLLATE=C
 [5] LC_MONETARY=en_US.UTF-8    LC_MESSAGES=en_US.UTF-8
 [7] LC_PAPER=en_US.UTF-8       LC_NAME=C
 [9] LC_ADDRESS=C               LC_TELEPHONE=C
[11] LC_MEASUREMENT=en_US.UTF-8 LC_IDENTIFICATION=C

attached base packages:
[1] grid      parallel  stats     graphics  grDevices utils     datasets
[8] methods   base

other attached packages:
 [1] statmod_1.4.18     prot2D_1.0.0       qvalue_1.36.0      MASS_7.3-29
 [5] Mulcom_1.12.0      fields_6.8         maps_2.3-6         spam_0.40-0
 [9] limma_3.18.0       Biobase_2.22.0     BiocGenerics_0.8.0 samr_2.0
[13] matrixStats_0.8.12 impute_1.36.0      st_1.2.1           sda_1.3.1
[17] corpcor_1.6.6      entropy_1.2.0      fdrtool_1.2.11

loaded via a namespace (and not attached):
[1] R.methodsS3_1.5.2 tcltk_3.0.2       tools_3.0.2
```

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