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Molecular Physiology of Stress Tolerance in Marine Invertebrates: The Heat Shock Response and Multidrug Resistance.

by

AMRO M. HAMDOUN
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DEDICATION

This dissertation is dedicated to my mother and father who define what it means to be committed to education no matter the obstacles.
This work is also dedicated to my best friend, companion and lover, Julia Cardosa.

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First and foremost special thanks are owed to those individuals who recognized my talents and encouraged my efforts to complete this journey. Fred Frye, in particular, for being a role model, friend and colleague all at once. Doug Conklin is owed thanks for being a trusty compass as I navigated through my undergraduate education- without him there would have been no trip to BML.

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This work is a credit to my family in Egypt and especially the community of Ezbet el-Borg for being my models for inventiveness and perseverance and for always being sources of great inspiration.

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Physiology

Molecular Physiology of Stress Tolerance in Marine Invertebrates

Abstract

A number of molecular mechanisms have been implicated in biological tolerance of marine environmental stress. Among the best characterized of these is the heat shock response, which has been shown to play an important role in determining the distribution of marine invertebrates along natural gradients of stress (eg. thermal stress in the intertidal). The heat shock response also appears to play a critical role in determining the susceptibility of invertebrates to anthropogenic stress. In a parallel context, it has been proposed that multidrug resistance may play an important role in determining the survival of invertebrates along natural and anthropogenic gradients of toxic organic compounds.

This study characterized the role of both of these systems in the tolerance of natural stressors in model marine invertebrate systems. In the first section of the dissertation I demonstrated that, although Pacific oysters are capable of significant adaptive regulation of heat shock protein (HSP) expression, adaptive plasticity occurs at a physiologic cost to inducible stress tolerance. Moreover, although adaptive regulation of HSPs is via a transcriptional mechanism during conditions of acute thermal stress, the response to chronic stress may not require continual transcriptional activation of HSP genes. In the second section of the dissertation I focused on the role of multidrug resistance (MDR) transporters in oocytes, embryos and larvae in model (sea urchin, starfish and urechis) systems. In the first chapter of this section I described species-

specific differences in transporter expression and consequent differences in susceptibility to naturally degraded hydrocarbons. In the second chapter I hypothesized that the reason for organic susceptibility in echinoderms is not due to the absence of a transporter, but rather due to the specificity of the predominant transporter family expressed in each species. Consistent with this hypothesis I showed that the predominant type of transporter expressed in echinoderm oocytes and embryos bear homology to the multidrug resistance associated protein (MRP) family rather than the multidrug resistance (P-gp/MDR) family. I examined the potential consequences of this difference for transporter substrate specificity and we measured the level of MRP expression during early development. I show that echinoderms possess a characteristic MRP transport phenotype that is activated at fertilization.

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Chapter 3

INTRODUCTION

A central theme in the discipline of physiology is the concept of homeostasis. Homeostasis can be loosely defined as the directed regulation of the internal environment of organisms. Homeostatic mechanisms have been exhaustively studied at various levels of biological organization. In 'complex' animals, homeostasis is an integrated process that involves specialized sensory structures that monitor the external environment and transmit signals to the central nervous system where responses are initiated and coordinated. These responses may be measurable in changes of the systemic physiology of an organism. However, regardless of the complexity of these responses, at their basis is a group of specific cellular and molecular changes that collectively represent a homeostatic mechanism. Because different organisms face the same types of challenges to homeostasis, evolution has resulted in conservation of those cellular and molecular response mechanisms that have proven useful for dealing with homeostatic challenges.

What is remarkable about homeostasis is that it occurs in a constantly fluctuating external environment. Many organisms defy our preconceptions about the logical environmental limits for life. Bacteria, fungi, plants and animals have been found in environments that impose extreme physical challenges ranging from thermal springs with boiling hot temperatures and acidic pH, to icy-cold deep sea vents with crushing external pressures and sulfide rich waters. Fortunately for physiologists, most living things, exploit niches that are far from these extremes. Nonetheless, in the marine environment large changes in temperature, pH and the external osmotic environment can occur multiple times per day. In these environments the mechanisms required for adaptation to

external environment are sufficiently conserved between widely divergent taxa. For this reason, the study of physiology in marine invertebrates can illuminate the path to understanding the basic physiology of homeostasis in all organisms.

Although most changes in the external environment could be defined stresses, a more accurate definition of stress is one that integrates the concept of homeostasis. Many stresses are generally believed to be challenges to the homeostatic mechanisms of organisms. However, natural challenges to homeostasis occur with such intensity and frequency in the marine environment that organisms have necessarily evolved the capacity to adapt to them. Moreover, these mechanisms are often robust enough to ameliorate the effects of anthropogenic influences on the environment that would otherwise be clearly classified as stressors. Thus, I propose that it is only at the limit of homeostatic adaptation to external influences that the organism encounters stress. Consequently it is not only important to characterize the mechanisms by which organisms maintain homeostasis, but also the limits of those mechanisms.

The diversity of these adaptive responses is not only reflected in individual differences observed within a given life history stage, but also in differences observed between life history stages. Among various life history stages, early developmental stages are often thought to be the most susceptible to stress. In the earliest stages of development (prior to the mid-blastula transition) zygotic gene transcription is quiescent, limiting induction of stress responses to a fixed pool of maternal mRNA. Consistent with this observation early embryos often have relatively fixed physiologic limits. The timing of initiation of zygotic "stress gene" expression can vary significantly from gene to gene

and species to species. Consequently critical physiological limits can be set very early in the life cycle of various invertebrates.

In this dissertation I have chosen to study two, well characterized, homeostatic mechanisms that have also been described as "stress responsive" systems. These two mechanisms are the heat shock response and multi-drug resistance. Within species, I examine the roles of these systems under a variety of conditions ranging from chronic to acutely stressful, and consequently, I begin to test the limits of these systems. By characterizing the developmental and taxonomic variation in these two stress responsive mechanisms, I attempt to elucidate both the mechanisms of stress responsive gene regulation and some of the environmental factors that may be important in driving their evolution.

HSPs: DISCOVERY, FUNCTION and CURRENT ISSUES

As was the case with Fleming's discovery of the lysosome, the discovery of the heat shock response also follows an indirect and perhaps fortuitous route. Ferruccio Ritossa is often credited with the initial discovery that suggested the presence of specific cellular and molecular responses to thermal stress (Ritossa, 1996). Ritossa was conducting experiments to determine what type of nucleic acid macromolecule is synthesized in *Drosophila* chromosome "puffs". Ritossa made the observation that elevated incubation temperature of *Drosophila* larvae results in a dramatic change in the pattern of chromosome puffs and rapid transcription of mRNA at those new puffs. By the 1970's several groups had shown that heat shock causes specific changes in the profile of expressed proteins. However, the first evidence that this respose is a universal phenomenon emerged from the experiments of Phil Kelly, Milton Schlesinger and Lawrence Hightower (Schlesinger, 1996). Two key findings of their experiments were that the heat shock response is conserved and that the protein(s) produced in response to thermal stress are also conserved even among widely divergent taxa. Numerous authors would confirm the presence of several evolutionarily conserved families of proteins, heat shock proteins (HSP) that are involved in the cellular response to stress (Gething, 1997).

Subsequent experiments by Schlesinger and others, would confirm that at least one function of these stress inducible proteins is associated with protein folding and conformation. Simultaneously (see Ellis, 1996 for review) John Ellis and his colleagues would also show that these proteins have "non-stress" roles as chaperones of nascent polypetides, andr in stabilization of unfolded proteins during protein translocation. Many subsequent details of heat shock responses were elucidated by Susan Lindquist and her colleagues. Lindquist demonstrated that although HSPs are massively induced in response to protein denaturing stress, synthesis of all other proteins is reduced. Moreover, the induction of HSPs results in simultaneous induction of elevated thermal tolerance (Lindquist, 1986). Morimoto (1993) and colleagues would later describe a heat shock transcriptional factor (HSF) that would be implicated in regulating the induction of HSPs.

With this basic foundation established, the field of HSP biology has experienced explosive growth that is reflected in the overwhelming number of publications on this topic. Many recent studies have emphasized the use of non-model organisms to elucidate mechanisms of HSP regulation, and their physiological and evolutionary implications. Marine invertebrates, and particularly intertidal molluscs, have received considerable attention (see Chapter 1 for review). Because of their life history strategies many, intertidal molluscs experience wide daily and seasonal temperature fluctuations. These fluctuations are often reflected in corresponding changes of the levels and forms of HSPs expressed. The mechanism of this regulation is a major question that is not yet explained by existing models for HSP expression.

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MULTIDRUG RESISTANCE: PHENOPTYPE, GENOTYPE, FUNCTION.

Multidrug resistance is a term used to describe a number of complex cellular phenotypes. The most notorious examples of multidrug resistance have typically been those phenotypes that are associated with human disease pathologies (Litman, 2001, Gottesman and Pastan, 1993) One such is example is enhanced tolerance of cancer cells, relative to normal cells, to chemotherapeutic agents. Although several cellular mechanisms may be responsible for drug resistance of various cells, one mechanism in particular has emerged as the most important causative factor in multidrug resistance.

The discovery of multidrug resistance began with the simple observation that animal cells in culture could be selected for resistance to a given drug. More importantly, this resistance to one drug would often be associated with acquired resistance to a broad spectrum of other drugs. A perplexing aspect of this acquired tolerance was the remarkable structural diversity of compounds that appeared to be "detoxified". Early authors described a wide array of compounds that share the general physical property of moderate hydrophobicity. Thus, the mechanism of this resistance remained unclear until in 1976 Juliano and Ling described a glycoprotein, named Permeability glycoprotein (P-gp), in Chinese hamster ovary cell lines that appeared to be associated with changes in drug resistance. Because many of the drugs were moderately hydrophobic, it was proposed that this protein effected drug resistance via alterations in membrane permeability to drugs.

By the mid 1980's mounting molecular evidence suggested that P-gp and its homologues are members of the well known ATP binding cassette (ABC) superfamily (Dean et al, 2001) The ABC gene family is huge including at least 50 genes in humans,

and more than 100 genes in other eukaryotes (eg. *Arabidopsis;* Martinoia, et al 2002). The products of these genes are typically large (>150kD) membrane spanning proteins that are involved in the transport of a wide array of substrates including simple ions and ion conjugates, lipids and lipid derivatives, and a wide array of moderately hydrophobic exogenous compounds (Ambudkar et al 1999). In animals, the ABC transporter gene superfamily is subdivided into seven families that group these transporters based on their general transport characteristics. So far, only three of these families have been shown to encode proteins that are involved in drug resistance.

Of the three proteins involved in drug resistance the MDR/P-gp gene family has received the most widespread attention . P-gp and related proteins have been described in several marine vertebrate and invertbrate systems. Most of the evidence suggests that these transporters act as "xenobiotic" transporters, that are important in protecting animals in the marine environment from a wide array of natural and anthropogenic toxic compounds (Leslie et al 2001). Several studies, including one presented here, have suggested that byproducts of microbial metabolism are important substrates for these transporters in the marine environment. However it is not known if these transporters are sufficiently specific to exclude endogenous substrates from extrusion . If not, they may play important roles in normal physiologic processes, particularly during development when rapid changes in intracellular concentrations of various molecules can regulate differentiation.

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CHAPTER 1

Phenotypic plasticity of HSP70 and HSP70 gene expression in the Pacific oyster (*Crassostrea gigas*): Implications for Control Thermal Limits and Induction of Thermal Tolerance

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Abstract:

Pacific oysters, *Crassostrea gigas*, living at a range of tidal heights, routinely encounter large seasonal temperature fluctuations. We demonstrate that the thermal limits of oysters are relatively plastic, and that these limits are correlated with changes in HSP70 family expression.

Sibling oysters were cultured along a tidal height gradient and monitored for changes in cognate and inducible (HSC/HSP) heat shock protein expression during the progression from spring through winter. We found that the "control" levels (ie. prior to laboratory heat shock) of HSC77 and HSC72 protein are positively correlated with increases in ambient temperature and were significantly higher in August than in January. The elevated levels of HSC70 family members were also associated with moderate increases (2-3 degrees) in upper thermal limits. Similar changes were observed in the threshold temperatures ("T_{on}") for HSP70 family induction. Total *HSP70* mRNA expression was closely correlated with the observed changes in inducible HSP70 protein accumulation. A potential cost of these changes in T_{on} is that there was no induction of thermal tolerance after sub-lethal heat shock at temperatures that result in induced thermal tolerance during the winter months.

Keywords: HSP70, Crassostrea gigas, induced thermotolerance, thermal limits, gene expression, natural stress.

Introduction:

Marine invertebrates tolerate a remarkable array of natural and anthropogenic stresses. Although the near shore environment supports great invertebrate diversity, it is physiologically challenging. Littoral invertebrates routinely encounter anoxia, osmotic stress and wide thermal fluctuations. Withstanding these conditions usually requires the initiation of coordinated cellular stress responses. These responses are particularly important in the adaptive response of sessile intertidal invertebrates such as mussels and oysters.

Many of these natural stresses can directly alter protein conformation and stability. Because proteins must be sufficiently labile to interact with their substrates they are often highly susceptible to protein denaturing stresses such as reduced intracellular pH or elevated temperature (Hochachka and Somero, 1984; Somero 1995). Protein denaturing stressors such as heat can result in the exposure of hydrophobic domains of proteins and cause non-specific protein interactions. Under these conditions, several families of proteins known as heat shock proteins (HSP) or molecular chaperones perform critical protein-stabilizing functions (Lindquist, 1986; Gething and Sambrook, 1992; Morimoto et al 1994). Under "non-stress" conditions, molecular chaperones are required to stabilize hydrophobic protein domains exposed to aqueous cellular environments during protein synthesis and translocation (for review please see Hartl and Hayer-Hartl, 2002). Stress induced expression of HSPs contributes dramatically to the acquisition of tolerance to otherwise lethal conditions (Parsell and Lindquist, 1994).

Natural variation in the heat shock response appears to be correlated with distribution along environmental gradients of stress (Hofmann and Somero, 1996; Tomanek and Somero 1999). Threshold induction temperatures for HSPs are positively correlated with ambient environmental temperatures (Dietz and Somero, 1992; Buckley et al, 2001). In the marine mussel, *Mytilus*, threshold temperatures can be higher in animals living at higher tidal heights (Roberts et al, 1997). In congeneric species of the marine snail *Tegula*, threshold temperatures are positively correlated with the maximal tidal height at which each species is found. In *Tegula*, these differences are maintained even after laboratory acclimation, suggesting a role for gene regulatory factors that establish set points for hsp induction and/or interspecific variation in the stability of cellular proteins (Tomanek and Somero, 1999).

The basic features of the heat shock response in Pacific oysters, *Crassostrea gigas*, have been well characterized (Clegg et al, 1998). HSP70 has been shown to be the primary family of HSP that is responsive to thermal stress. Three isoforms of HSP70 family protein are resolved after one-dimensional electrophoresis and western blotting. Two proteins, HSC77 and HSC72 are constitutively expressed and can also be induced by thermal stress. In contrast to other bivalve species such as Mytilus, a third inducible protein HSP69 can also be detected and it is specifically expressed after acute thermal stress at levels that account for roughly one third of total HSP70. At least two genes encoding mRNAs that correspond to cognate and inducible forms of these three protein products have been sequenced and characterized. These results indicated that distinct gene products encode a cognate protein of approximately 72kd (Gourdon et. al., 2000) and an inducible protein of approximately 68-70kd (Boutet, et al., 2002). In Pacific

oysters a single sub-lethal heat shock, sufficient to cause significant HSP69 expression, also results in elevated thermal tolerance for a remarkably prolonged period of up to two weeks (Clegg et al 1998).

In this study we attempted to determine the extent and mechanism of adaptive plasticity of the heat shock response in a group of sibling Pacific oysters planted at two tidal heights (+0.3m and +1.2m) at Totten Inlet, Southern Puget Sound, Washington State, USA. Our findings suggest that phenotypic plasticity of the heat shock response is a significant component of adaptation to natural thermal stress. Moreover, we found that natural adaptation of the heat shock response is associated with concomitant changes in control (ie. prior to laboratory heat shock) and inducible thermal limits.

Materials and Methods

Animals and Study Site

A commercial oyster bed in Totten Inlet, south Puget Sound, Washington was selected as an experimental study site. The juvenile "seed" oysters for this experiment, 1999 age class, were obtained in late June, 1999 and planted at two tidal elevations: a low intertidal elevation plot at 0.3m mean lower low water (MLLW) and a high intertidal elevation plot at 1.2m MLLW. The seed were placed in small mesh grow-out bags set on top of metal racks at each tidal height. The oysters were later transplanted to larger mesh grow-out bags. Oysters were sampled monthly during low tide events starting in April 2000 and continuing until January 2001.

External site temperature data were recorded every 15 minutes using Hobo© temperature sensors. Internal temperature data were recorded using Hobo© 4 channel

external data loggers and TMC6-HA temperature sensors. Temperature data were recorded at each tidal height from 3 oysters with internally mounted sensors and 1 externally mounted sensor (TMC6-HA sensor), which was placed next to the oysters either in a grow-out bag or on the ground. The internal temperature sensors were placed inside the shell of live oysters through a 6mm opening. The sensors were secured and sealed by using marine epoxy.

Heat Shock

Immediately after collection oysters were shipped overnight in chilled coolers to Bodega Marine Laboratory, Bodega Bay, CA. for use in experiments. Animals were allowed to recover for 24 hours in flow through tanks, with raw Bodega Bay seawater (RSW; 13°C, +/- 2°C) prior to heat shocks. Animals were placed in mesh bags and heat shocked submerged in preheated, aerated filtered seawater baths. All heat shocks were for one hour following a 5 minute temperature equilibration period. Animals were returned to the flow through seawater tanks immediately after heat shock. 5-6 oysters were heat shocked per temperature for sampling of gills for northern and western blots. After heat shock oysters were returned to RSW tanks for 48 hours prior to sampling. Sampling of gill for protein and mRNA analysis was performed at 48hrs because this time point has been previously shown to be the point at which accumulation of HSP70 protein is maximal, and *de novo* synthesis of HSP70 is elevated relative to control *Crassostrea gigas* (Clegg et. al, 1998). Moreover this allowed us to compare the level of HSP70 protein and mRNA from identical gill sections. It is important to note that HSP70

levels remain elevated in oysters for at least one week following heat shock. Thermal limits were determined by heat shocking triplicate bags of 7 animals at each temperature. Survival was determined after a 10 day recovery in RSW tanks.

Tissue Sampling and Sample Preparation

Oysters were opened and gills were dissected and divided into two sections for protein and RNA isolation. Gill samples for western blots were prepared according to Clegg et al. 1998. Briefly, they were rinsed in ddH_20, blotted dry, weighed, and flash frozen in liquid nitrogen. These samples were stored and -70°C and later homogenized on ice in glass tissue grinders containing potassium gluconate buffer (5mM $MgSO_4$, 5mM NaH_2PO_4, 40mM Hepes, 70mM Gluconic acid, 150mM Sorbitol; pH 7.5) at a concentration of 100mg tissue/ml buffer. Homogenates were prepared for electrophoresis by mixing 1:1 in 2x sample buffer and boiling for 5 minutes.

Gill sections for RNA isolation were immediately preserved in 5 volumes of RNAlater (Ambion) solution, frozen in liquid nitrogen, and stored at -70°C. Gills were later homogenized in 3ml guanidine thiocyanate denaturing solution. 900µl of this homogenate were mixed with 100µl, 2M sodium acetate and incubated on ice for 5 minutes. Samples were next mixed 1:1 with ice cold phenol/chloroform (pH 4.3; Sigma) and incubated on ice for 20 minutes. After centrifugation for 25 minutes at 10,000g the aqueous layer was removed and RNA was precipitated overnight in an equal volume of isopropyl alcohol. The next day the RNA pelleted was washed, resuspended in DEPC H_2O and checked for purity and concentration using a spectrophotometer.

cDNA Cloning and Probe Synthesis

Total RNA was isolated from heat shocked oysters, and used to synthesize cDNA with Superscript Reverse Transcriptase (Gibco-BRL) according to manufacturer's instructions. A 660bp fragment of oyster HSC72 cDNA was amplified from this template using degenerate HSP70 primers (Cochrane et al, 1994). PCR products were checked for size and yield on 1% agarose gel prior to TA cloning using the dual promoter pCRII-TOPO vector (Invitrogen) and cloning kit. Aliquots of plasmid minipreps (Qiagen) of positive clones were sent to Davis Sequencing for sequencing. Results were analyzed by BLAST comparison of existing sequences at NCBI. A 660bp fragment was cloned that was identical to a portion of a previously submitted sequence of Pacific oyster HSC72 mRNA (Gourdon et al, 2000).

An antisense RNA probe for total HSP70 probe was synthesized from minipreps (confirmed to be positive for the HSC72 gene product by sequencing). Briefly, a digoxigenin-UTP (Roche) labeled probe was synthesized by in vitro transcription (Maxiscript; Ambion) of the anti-sense strand. Because of the high homology in this region of the known oyster HSP70 genes it was expected that this probe would hybridize to all of the known HSP70 family gene products. The yield of resulting probe was determined by using a dot blot of the probe versus a digoxigenin-RNA probe standard (Roche) according to manufacturer's instructions.

Western Blotting

Western blots of oyster HSP70 family were prepared according to Clegg et al, 1998. 10µl of each solubilized sample was resolved on a 10% SDS-Polyacrylamide gel.

In order to normalize between blots, multiple aliquots of a single sample of heat shocked oyster gill were made. Each time gels were run an aliquot of this sample was loaded on the center lane of each gel. The internal control sample was arbitrarily chosen from a previous experiment and was known to have all three isoforms of HSP70 family protein. Therefore, levels of each HSP70 family member were normalized relative to the level of the corresponding band from the internal standard (the maximal variation in standard level between gels was less than 10 fold). To ensure that the large differences between summer and winter control HSP70 protein levels were not attributable to blot variation the summer and winter samples were run on a single blot and their values relative to heat shocked oysters were adjusted according to the level of the corresponding standard sample run on that blot. Control levels of HSP70mRNA were compared from samples run on a single northern blot.

Proteins were transferred onto nitrocellulose and probed using a monoclonal rat anti-HSP 70 antibody (Affinity Bioreagents, MA3-001), an HRP conjugated goat secondary (Sigma). Signal was detected by chemiluminescence using enhanced detection reagents (Supersignal, Pierce) on a Bioimaging Systems digital gel scanner or by exposing blots to photographic film (Kodak, Biomax MR). Relative levels of band intensified were using gel plotting macros available on Scion Image software.

Northern Blotting

For each northern blot 4μg of denatured total RNA were loaded onto each lane of 1% agarose (in 1x MOPS), 6.66% formaldehyde gels and run for 3-5hours at 50V. RNAs were transferred overnight onto nylon membranes (Hybond) using standard capillary

methods in 10xSSC buffer. After transfer RNA was cross-linked to the blots (Stratalinker), and blots were stored at -20°C until probed. DIG-labeled RNA probes were prepared at a final concentration of 50ηg/mL in high SDS buffer (all buffers in RNA detection were prepared according to instructions from DIG applications manual, Roche). Blots were incubated overnight at 50°C in probe solution with gentle rotation. The next day nonspecific hybridization was removed using four, 15 minute stringency washes at 60°C in decreasing concentration of SSC solution (2X-0.5X). Blots were then washed twice in maleic acid buffer containing 0.3% tween 20, and blocked for 30minutes using 1x blocking buffer (Roche). Blocked blots were incubated for 1hr with gentle rotation at RT in a 1:10000 solution of anti-dig AP fab fragments (Roche) in blocking buffer. CSPD solution (Roche) was used 1:100 in detection buffer to detect hybridized probe. Chemiluminescence was enhanced by 15 minute incubation of the blot at 37°C followed by detection using a Bioimaging Systems digital gel scanner.

Statistics

Differences in protein and mRNA level were analyzed for statistical significance using one way ANOVA, and multiple pairwise comparisons were performed according to Dunn's method; all tested data conformed to equal variance and normality assumptions. All analyses were conducted using Sigmastat software v.2.03.

Results

Animals and Study Site

Internal measurements of oyster temperatures at high and low tidal height are shown in Figure 1. Temperatures recorded in living oysters generally matched those recorded by external data temperature loggers placed alongside the oysters in the culture bags. This suggests that despite the thickness of oyster shell it acts as relatively poor insulator against thermal stress. Oysters at 1.2m MLLW generally experienced higher temperatures and longer durations of exposure to elevated temperature. In order to estimate the duration of exposure to elevated (>20°C) temperatures, measurements from data loggers were sorted into 5°C intervals and measurements in each class were summed together. Each data point was assumed to represent approximately 15 minutes of exposure to the recorded temperature. Figure 2 summarizes the estimated hours of exposure to warm temperatures (>20°C) for the two weeks preceding our summer sampling. In nearly all cases the approximate duration of exposure to warm temperatures was higher at the high tidal height. During the summer 2000 period, the total exposure to temperatures in excess of 20°C was approximately 67 hours at high tidal height versus 44 hours at the lower tidal height.

For the duration of the experiment no significant difference in mortality was recorded in animals at either site. Total mortality at both sites was less than 20%. Totten high tidal height (1.2m) animals showed a cumulative mortality of 16.5% while Totten low tidal height (0.3 m) animals experienced a 18.4% mortality.

HSP70 and HSP70 mRNA Levels

The control levels of HSC70 in Pacific oysters are associated with the level of environmental thermal stress (Figure 3, 5A). At both study sites the control levels of HSC77 and HSC72 were as much as 100 fold greater in summer than in winter ($P<0.001$). These differences were not attributed to differences in total protein levels as control protein levels were 41.5 mg/g wet weight and 46.2 mg/g wet weight in winter and summer, respectively (Bradford protein assay, Bio Rad), and these differences were not significantly different ($P>0.07$). During the winter months there was no statistically significant difference in the control levels of HSC70 between the high and low tidal height sites ($P=0.917$). In contrast, during the warm summer months oysters at 1.2m had significantly higher levels of HSC77 and HSC72 than their counterparts living at 0.3m ($P<0.001$; Fig 3). The elevated summertime levels of HSC70 approximated those observed in oysters during the winter after acute heat shock at 37°C.

Figure 4 shows the changes in HSP70 family mRNA in high tidal height oysters in the summer. The level of HSP70 mRNA in summertime totten high oysters increased dramatically after heat shock at 40 and 43°C, but not after heat shock at 33 and 37°C. This result was in agreement with the observed increases in HSP69 protein after heat shock at these temperatures in the same animals. Although there are slight increases in mean levels of control HSP 70 mRNA level between summer and winter oysters, the differences are not significant ($P=0.13$; Fig 6).

The level of environmental thermal stress (Fig. 1 and Fig. 2) appeared to be associated with the threshold temperature for induction of HSP69 (Fig.3). In January, HSP69 accumulation was induced in oysters from both sites after heat shock at 37°C. In contrast limited induction of HSP69 was observed following heat shock at 37°C in

oysters living at high tidal height in August (Fig 3, Fig 5A). Full induction (ie. HSP69 expression at levels approximating those of HSC77 or HSC72) of HSP69 was not observed in August until after heat shock at 40-43°C. Thus, both the threshold temperature for HSP69 induction and the control levels of HSC70 exhibited significant seasonal and tidal height specific plasticity.

We observed the greatest change in threshold temperature for HSP69 induction and control level of HSC77/HSC 72 temperature to occur some time between our June and July sampling dates (Figure 5B). In June Totten 1.2m oysters completely induced HSP69 after a 37°C heat shock. In contrast by July induction of HSP69 was much more limited in these oysters. A marked increase in control levels of HSC70 was also observed during this period.

Thermal Limits and Induction of Thermal Tolerance

Laboratory heat shock of oysters at both tidal heights suggested both seasonal and tidal height influences on upper thermal limits (Figure 7). In January there was no significant difference in thermal limits among oysters at both tidal heights. However by August there were significant increases in baseline thermal limits of oysters at both study sites. Moreover, Totten 1.2m oysters appeared to exhibit elevated thermal limits relative to 0.3m counterparts during the summer. August survival of high tidal height oysters after 44°C heat shock was significantly higher than the low tidal height oysters ($P=0.03$). These differences occurred in the absence of HSP69 expression, suggesting that control thermal limits in Pacific oysters are likely to be more closely associated with cognate HSP70 expression than to inducible HSP70 expression.

In contrast, induced thermal tolerance appeared to be associated with inducible HSP70 expression (Figure 8). Totten 1.2m oysters heat shocked at 37°C during the winter were able to completely withstand a subsequent lethal treatment of 44°C. Heat shock at 37°C had no impact on tolerance of lethal treatment (46°C) during the summer. However, when August oysters were heat shocked at 40°C, they were able to withstand subsequent lethal treatment. These results suggest that induction of thermal tolerance is more closely associated with inducible HSP70 family expression.

Discussion

Our results demonstrate significant phenotypic plasticity in the heat shock response of Pacific oysters. Massive changes (10-100 fold increases) in the amounts of constitutively expressed HSP70 proteins were measured in summer as compared to winter. These changes are associated with simultaneous increases in thermal limits and set-point for HSP69 induction. In both high and low tidal height oysters the threshold for complete induction of HSP69 increased from approximately 33-37°C in winter to 37-40°C in summer. In high tidal height oysters the temperature required for induction of thermal tolerance also increased from 37°C in winter to 40°C in summer.

Although we did not determine the changes to the absolute limits for extension of thermal tolerance these changes suggest potential costs of plasticity of the heat shock response since it is clear that seasonal increases in thermal limits are associated with concomitant increases in the temperatures required for induction of tolerance to otherwise lethal temperatures. However it is important to note that induction of thermal tolerance is laboratory phenomenon and may not directly relate to changes in thermal limits in nature.

Clearly most of the temperatures encountered in nature are far below those required to measure changes in thermal limits. Thus the consequences of changes in 'inducible' thermal tolerance may be most significant during periods when elevated temperatures are extreme or when they occur in concert with other stresses.

Over-expression of HSPs is known to have deleterious consequences. Initiation of heat shock responses usually results in a concomitant reduction in synthesis of all other proteins (Lindquist et al, 1994). During stress, HSPs can account for a large fraction of cellular protein and thus it is hypothesized that their synthesis represents a significant energetic cost. In *Drosophila*, artificially high levels of HSP70 can reduce thermotolerance, and slow the development of *Drosophila* larvae (Krebs and Feder, 1998). Thus it is possible that the observed plasticity of the heat shock response in Pacific oysters represents a trade-off between the cost of maintaining elevated control thermal tolerance versus

Previous work has described differences in threshold temperatures either between congeners or in conspecifics naturally distributed along environmental gradients of stress such as tidal height gradients. Other bivalve species such as *Mytilus* exhibit significant plasticity in various features of the heat shock response, in the laboratory (Buckley et al, 2001). However two confounding factors complicate the interpretation of results from experiments with adult bivalves collected from the field. First, significant selection is known to occur at settlement (Launey and Hedgecock, 2001) and it is likely to correspond with distribution along gradients of stress (Michener and Kenny, 1991). Second, large-scale mortality may occur within adult bivalve beds during warm seasons (Cheney et al, 2000). Because there was no significant differential mortality during our

experiments we can reduce the possibility of a strong, site-specific effect due to selection. Moreover, because we planted sibling juvenile oysters at each site we limited any confounding effect of selection for thermal tolerance at settlement. Thus, we conclude that our results and perhaps those previously observed in *Mytilus* (Roberts, et al 1997; Buckley et al 2001) are likely the consequence of significant phenotypic plasticity in the heat shock response rather than the consequence of fixed genotypic differences between bivalves living along gradients of stress.

Further experiments using genetically characterized families of bivalves, and other model organisms, may be the most useful in elucidating what components of the heat response are genetically "fixed" versus those that exhibit adaptive plasticity or intra-specific variability. Oysters may be an ideal system for these studies. Because oysters are a commercially valuable species, recent studies have focused on characterizing the genetics (Launey and Hedgecock, 2001) of Pacific oysters from known lineages. The possibility for producing oysters with characterized genotypes and improved culture performance under a wide range of environmental conditions is being explored (Langdon et al, 2002). A major challenge facing oyster growers is reducing mortality during the warmest summer months (Cheney et al, 2000). It is not yet known if the improved performance of these characterized families of oysters is related, at least in part, to genetically fixed differences in their ability to mount functional heat shock responses and withstand thermal stress.

Our results suggest that both HSP accumulation and gene expression rates are responsive to environmental stress, but that changes in gene expression alone are not sufficient to account for the differences in protein accumulation. It is not clear how

oysters are able to dramatically increase total HSP70 protein levels in the summer without concomitant changes in total HSP70 mRNA level. These results suggest that in oysters cognate HSP70 protein expression is not directly controlled at the transcriptional level. It is possible that changes in the rate of HSP70 protein synthesis and degradation may be important regulatory components of the long-term modulation in control HSP70 level such as those observed during adaptation to seasonal stress. Other authors (Tomanek and Somero, 2002) have proposed that level of cognate HSP70 isoforms, may be an index of protein synthesis capacity whereas the levels of inducible isoforms may be more accurate indices adaptation to heat stress. In our study we found that it was the threshold for HSP69 expression that reflected adaptation to thermal stress associated with seasonal changes. In contrast the large seasonal changes in the levels of HSC77 and HSC72 suggest that these isoforms may also play important roles in mediating the effects of thermal stress.

In laboratory selection experiments using *Drosophila* (Lerman and Feder, 2001) there is evidence that the heat shock response may be regulated by genetic factors that are fixed during evolution in a particular environment. However in sessile intertidal species several key features of the heat shock response (eg. control levels of hsp and threshold temperatures of hsp induction) appear to be sensitive to acclimation to laboratory and natural seasonal conditions (Roberts et al 1997; Buckley et al 2001). In sessile species, such as oysters or mussels, behavioral adaptation plays a more limited role in regulation of environmental stress exposure. Thus, we hypothesize that the heat shock response, and particularly the control levels of HSC70 in Pacific oysters must exhibit sufficient

phenotypic plasticity to allow organisms to cope with a wide array of environmental conditions.

There are two key limitations to most studies of ecological physiology of stress proteins (Feder and Block, 1991; Feder and Hofmann, 1999). First, most relevant stressors initiate responses from a variety of cellular and systemic stress response systems. Similarly, single stresses are rarely encountered in the environment. More typically, organisms encounter multiple stresses that interact with a variety of stress responsive systems. In the case of sessile bivalves a critical stressor that has been overlooked is the simultaneous exposure to anoxia/hypoxia during periods of thermal stress. Since most periods of elevated ambient temperatures occur during emersion (Figure 9), it will be important to address the question of whether or not anoxic/hypoxic conditions interact with heat shock responses, in intertidal bivalves.

Plasticity of the heat shock response appears to be a common feature in sessile intertidal invertebrates but not other marine invertebrates (Tomanek and Somero, 1999; Spees et al., 2002). This suggests that the potential costs of adaptive plasticity of the heat shock response outweigh the benefits in species and life history stages where behavioral regulation of thermal environment is possible. Perhaps more significantly, these results could indicate that although heat shock proteins and the genes that encode them are highly conserved, the composition and expression of the regulatory components that collectively comprise the "cellular thermostat" may exhibit significant interspecific variability. Thus, oysters, mussels and other species that exhibit significant adaptive plasticity of the heat shock response may have evolved the most complex and robust regulatory mechanisms. An unresolved question is the mechanism by which control or

"resting" levels of HSP70 are regulated during chronic stress exposure. It is not yet clear if these mechanisms provide distinct regulatory pathways that might be adaptive in specific tolerance of acute versus chronic stress.

This study has shown that a change in threshold temperature for HSP70 induction, in response to chronic stress is associated with a concomitant change in the threshold temperature required for induction of thermotolerance. We have shown that the changes in threshold temperature for induction of thermal tolerance match the seasonal changes in threshold temperature for HSP70 induction/expression. Of particular ecological significance is the finding that an increase in threshold temperature narrows the range of temperatures at which HSP induction can contribute to the acquisition of thermotolerance in oysters. Although this would suggest a cost of adaptive plasticity of the heat shock response, the impact this has on survival in the field is not clear.

Acknowledgements

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Figure Legends

Figure 1. Internal measurements of temperatures of three living oysters at two tidal heights (1.2m and 0.3m at Totten Inlet, Washington) are plotted with the results from a HOBO temperature probe placed in the culture bags alongside the oysters.

Figure 2. The approximate hours of exposure to elevated (>20°C) air temperatures in high and low tidal height oysters for the two weeks prior to the summer sampling. Note that the vast majority of the time oysters experienced temperatures (15-20°C) below the range shown.

Figure 3. The relative levels of HSP 70 as determined from western blots are shown for animals sampled in August and later in January. Bars represent the means of total HSP70 for 5-6 animals (+/- s.e.m. for each isoform). The relative amount of each isoform is indicated within the total amount. Three isoforms were resolved by one dimensional electrophoresis, two cognate forms (HSC77 and HSC72) and one inducible isoform (also see Figure 5).

Figure 4. Northern blots of *HSP70* mRNA in Totten high tidal height (1.2m) oysters 48hr after heat shock (HS) in August. Lanes are loaded with equivalent amounts (4μg) total mRNA. HSP70 was probed with an antisense digoxygenein-UTP labeled probe corresponding to a 660bp portion of oyster HSC72. Each lane represents mRNA from one oyster. 4 oysters were subjected to each treatment (1-4 are Controls, 5-8 are 33°C HS, 9-12 are 37°C HS, 13-16 are 40°C HS, 17-20 are 43°C HS).

Figure 5. A. Western blots, captured on digital scanner, of HSP70 family expression high tidal height (1.2m) oysters (3 individuals per treatment) in August and in January. HSC77 and HSC72 are dramatically elevated in control oysters sampled in August.

*B.*Western blots, exposed to film, of HSP 70 protein family in oysters (5 individuals per treatment) living at high tidal height (1.2m). In June a one hour heat shock completely induces HSP69 expression. In July HSP69 expression is absent in most animals sampled, but the control levels of HSC77 and HSC 72 are elevated relative to June.

Figure 6. Control levels of total HSP70 mRNA (n=4) and total HSP70 (n=5) prior to heat shock in summer and winter from oysters living at a high tidal height site (1.2m). Summer and winter protein samples were run on the same blot, as were mRNA samples, and relative levels were measured in arbitrary units using Scion Image.

Figure 7. Survival of oysters after one hour submerged heat shock in summer and winter. Points are the means of three group of seven oysters (+/- s.e.m.).

Figure 8. Induced thermal tolerance in oysters from Totten 1.2m. Oysters were either heat shocked directly at lethal temperature or shocked for one hour at a sublethal temperature and then allowed two days to recover in before lethal temperature treatment. Numbers below bars indicate the initial treatment temperature, followed by the temperature of the subsequent treatment ("LT" = lethal temperature). Bars represent means of three groups of seven oysters (+/- s.e.m.).

Figure 9. Mean meters emersion is plotted for each 5°C temperature interval encountered during the duration of the experiments. Note that the vast majority of time was spent at 15-20°C where the low tidal height oysters were completely submerged, and the high tidal height oysters were near emersion. All elevated and reduced temperatures occurred while oysters were on average significantly higher than the tide.

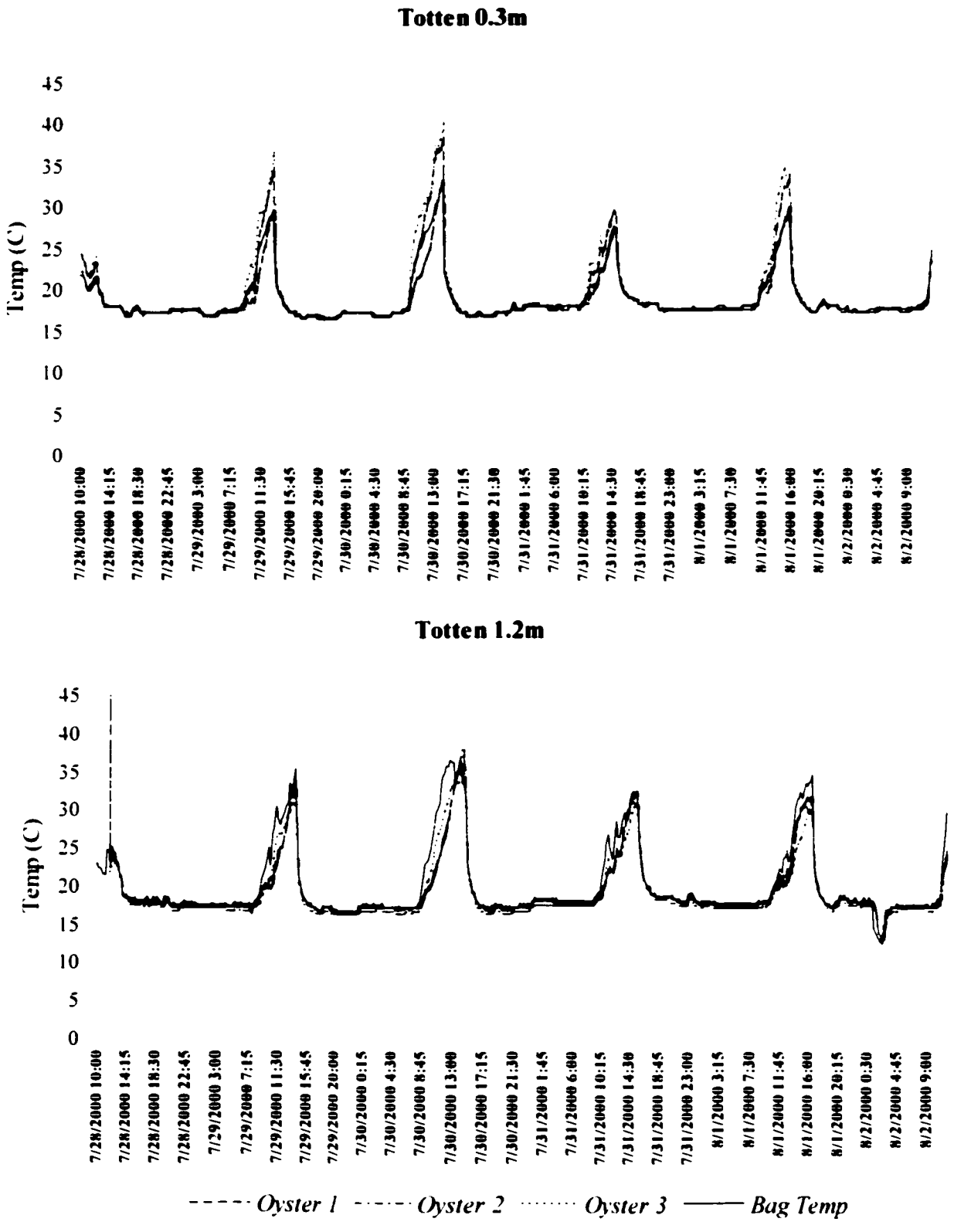
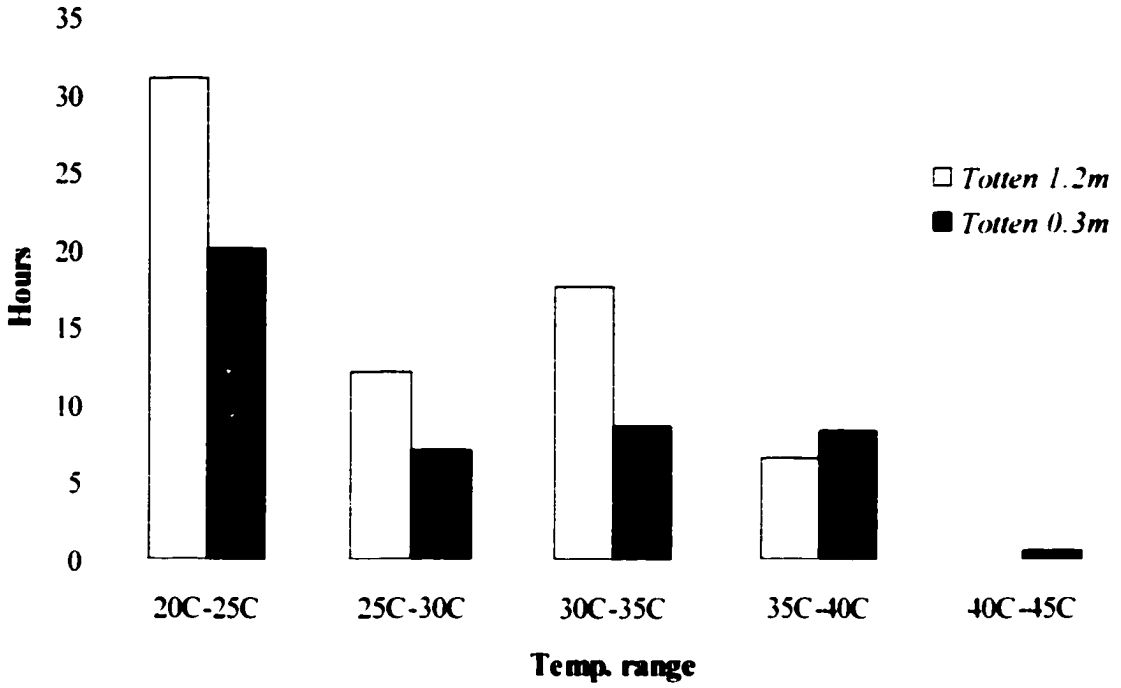


Figure 1.



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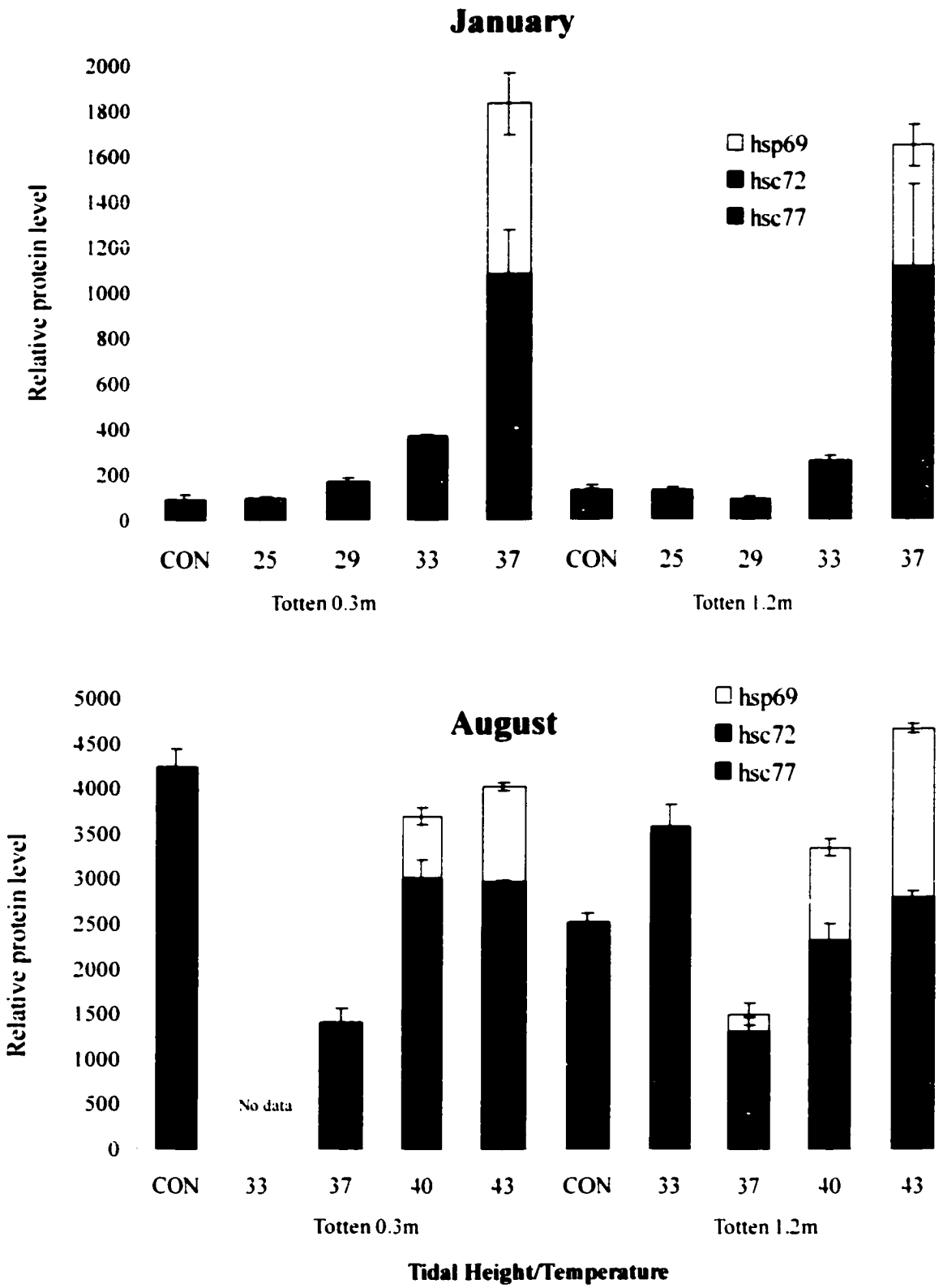

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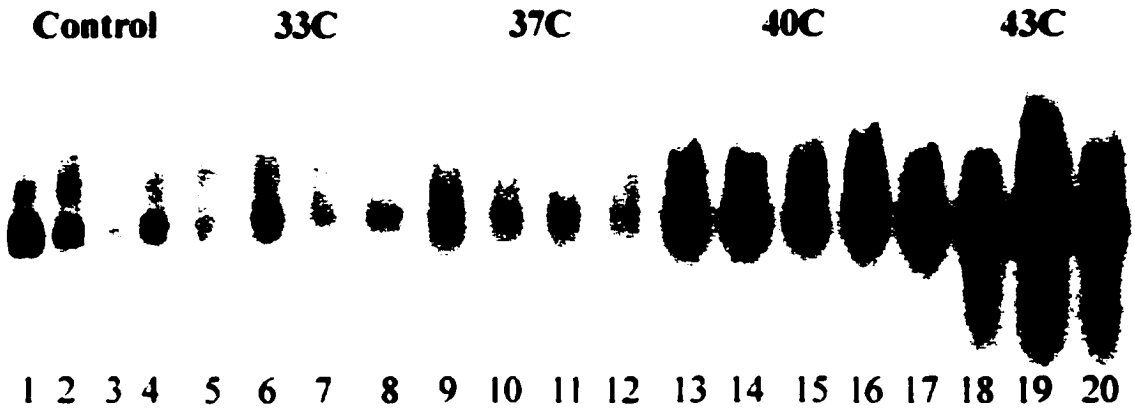
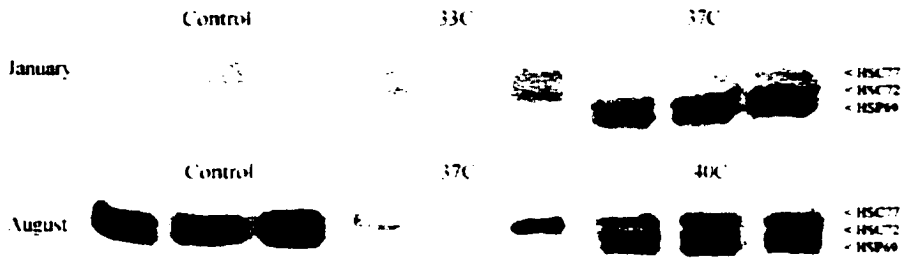


Figure 4.

A.



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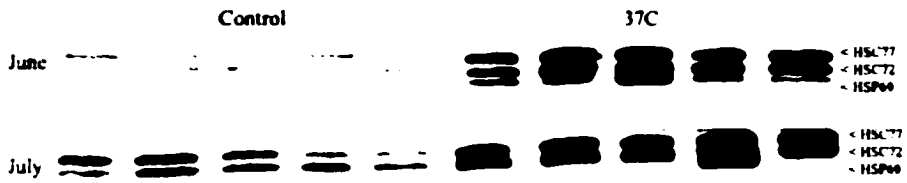

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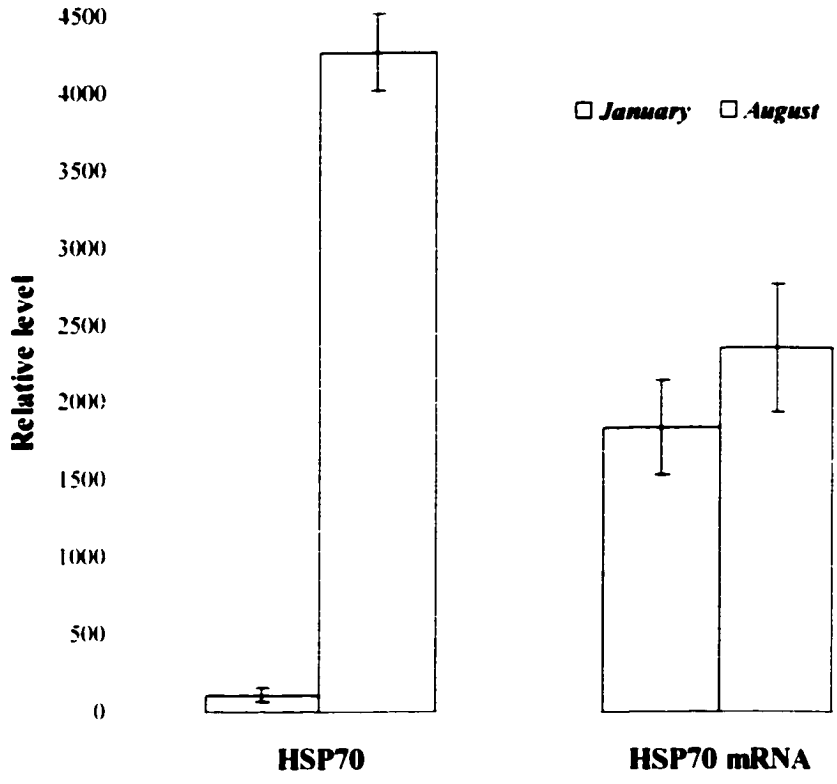

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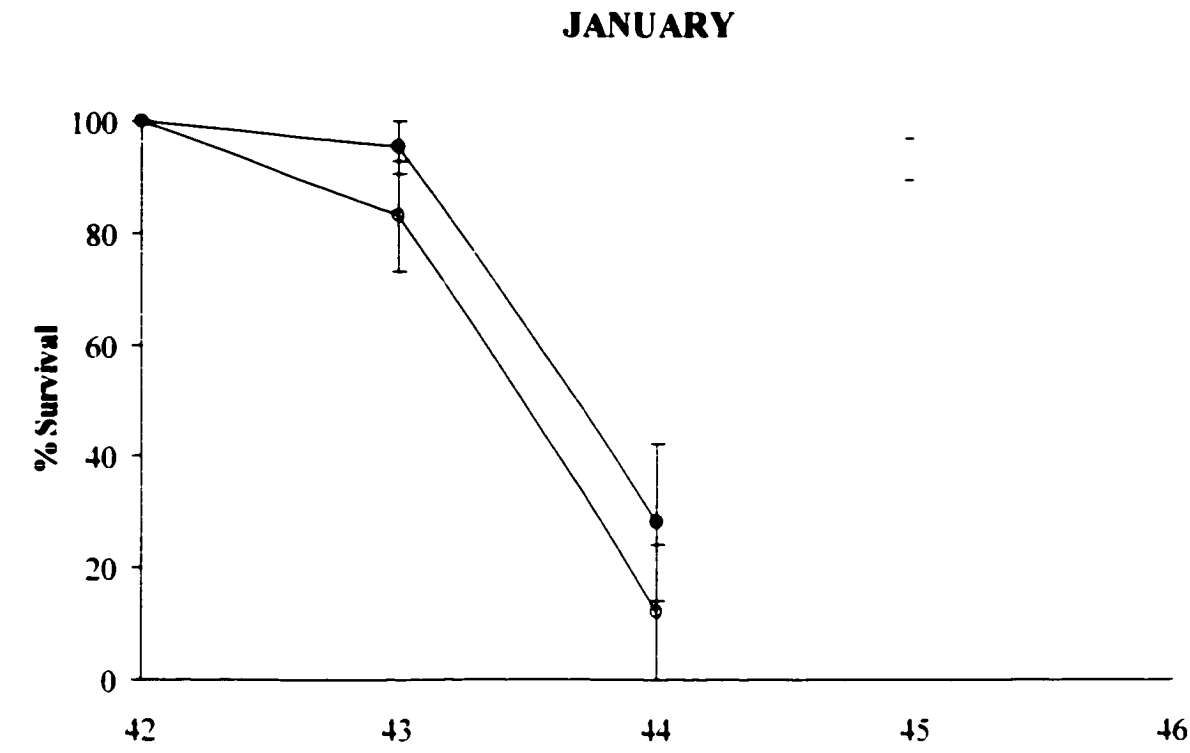

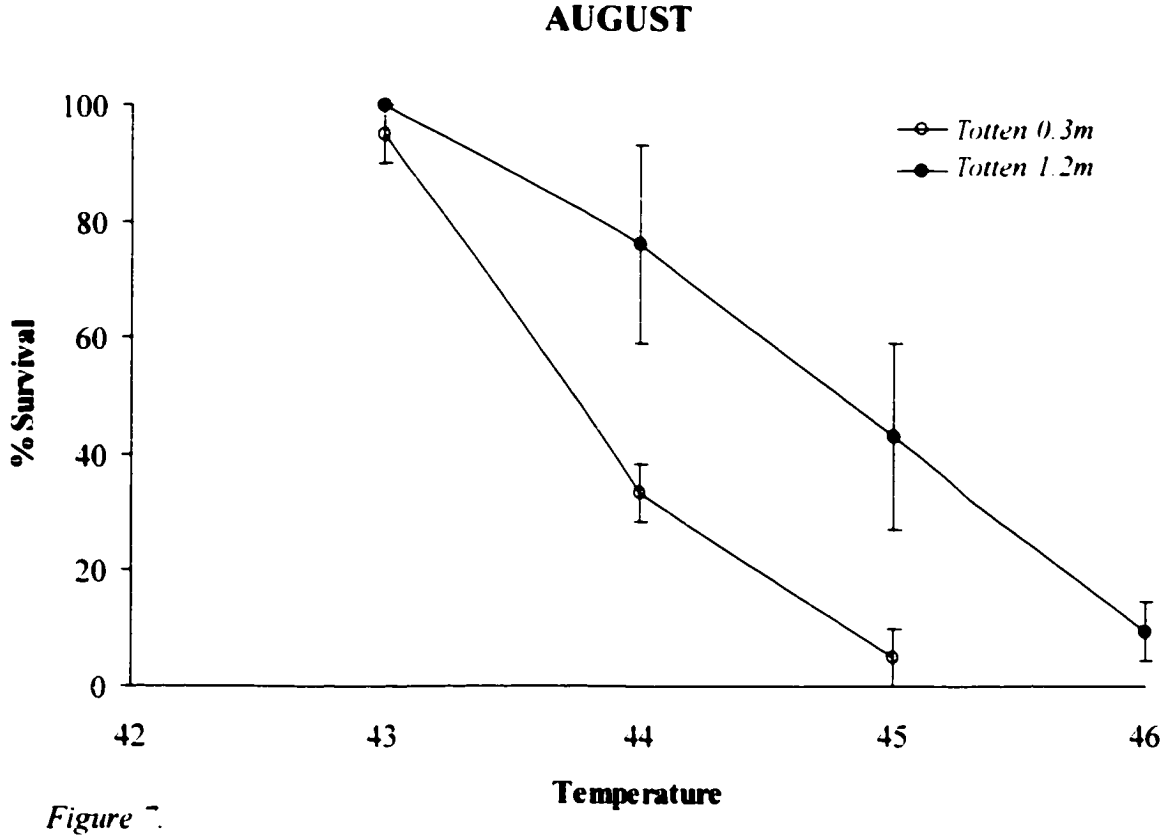

Figure 7.

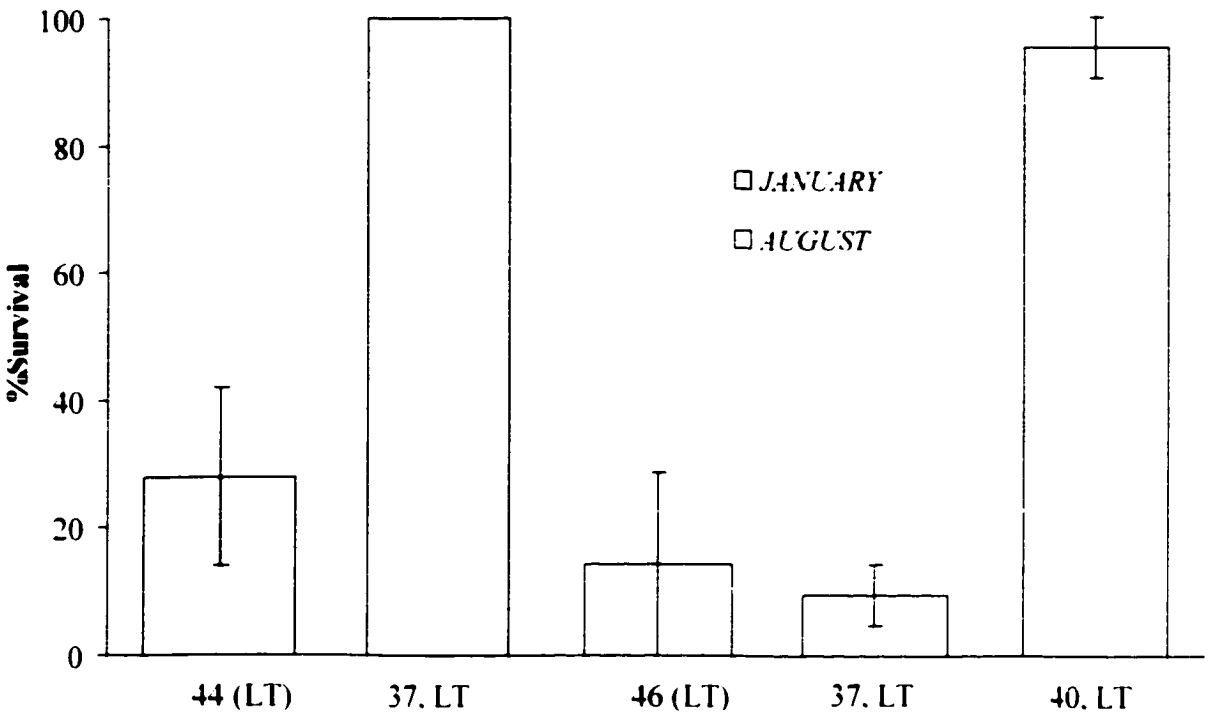

Figure 8.

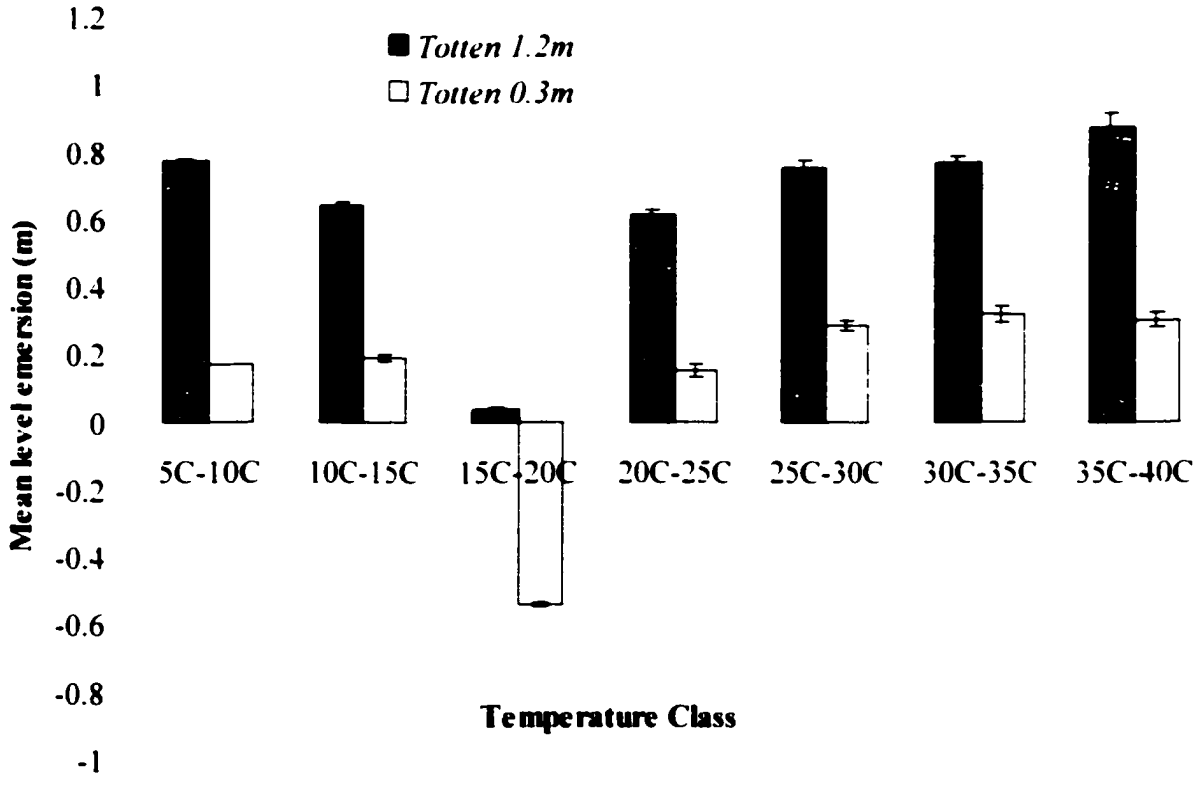

Figure 9.

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CHAPTER 2

Tolerance to Biodegraded Crude Oil in Marine Invertebrate Embryos and Larvae is Associated with Expression of A Multidrug Resistance (*P-gp*) Transporter

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Abstract

The toxicity of water soluble fractions of biodegraded crude oil (BWSF) to embryos and larvae of two marine invertebrates the white sea urchin (*Lytechinus anamesus*) and the fat innkeeper (*Urechis caupo*) was studied. Santa Barbara Channel crude oil was artificially weathered and subjected to biodegradation using a mixed microbe culture obtained from naturaloil seep sites. The degradation culture inoculated with seep sediment microbes accumulated 43.7μg/L water-soluble hydrocarbons. In contrast water-soluble fractions from the non-degraded cultures (NWSF) only accumulated 3.05μg/L. BWSF proved deleterious to *Lytechinus* embryo development at low concentrations (EC50 = 0.33 mg/L) but was essentially non-toxic to Urechis embryos/larvae up to 3.0 mg/L. An established mechanism for handling of a wide array of xenbiotics in *Urechis* embryos is the multixenobitoic resistance (MXR) transporter (also known as MDR). This mechanism is primarily mediated by ATP –dependent, efflux pumps that extrude a wide array of xenobiotic compounds. In this study we show that *Lytechinus* embryos/larvae do not appear to express MDR-1 efflux protein nor MDR-like dye efflux capacity. In contrast, BWSF acts as a competitive inhibitor of MDR transport-mediated dye efflux in *Urechis* embryos and larvae. These results suggest that MDR may be an important mechanism for handling the by-products of crude oil degradation by microbes, and that the level of its expression may determine the susceptibility of organisms to degraded oil hydrocarbons.

Keywords: Embryos, Larvae, Biodegraded Oil, *Lytechinus*, MDR, MXR, Oil Seep, Sea Urchin, *Urechis, P-gp*

1. Introduction

Crude oil enters the marine environment from a number of natural and anthorpogenic sources. Increasingly, it is introduced as an indirect result of crude oil production and transport rather than catastrophic spills. Crude oil is relatively unstable in the marine environment and it immediately undergoes a process termed 'weathering' during which time it can undergo adhesion to sediments, mixing with seawater and loss of volatile constituents, and photo-degradation (Boehm, 1987; Payne, et al 1991; Venkateswaran et al, 1995) The weathering process results in release of low boiling point aromatic and saturated hydrocarbons (Galt, et al 1991). Prolonged mixing with seawater results in release of water-soluble fractions that have been shown to be especially toxic to fish and invertebrate embryos from pristine environments (Davis et al 1981; Middaugh and Whiting, 1995). Furthermore, exposure to ultraviolet radiation from sunlight is known to increase the toxicity of oil hydrocarbons (Cleveland et al, 2000; Pelletier, et al 1997). Weathered crude oil is also susceptible to biodegradation by various naturally occurring, hydrocarbon degrading microbes (Galt, et al 1991).

At sites of natural seepage, such as Coal Oil Point in the Santa Barbara Channel, naturally occurring populations of sediment-associated, oil metabolising microbes have been described (Spies and Davis, 1979). Since several marine bacteria are suited to metabolizing hydrocarbons of weathered oil, they are often employed in cleansing oil-contaminated sites (Mueller et al 1992; Bragg et al 1994). Fertilizers have been used to enhance the growth of crude oil degrading microbes at several sites contaminated with oil from the *Exxon Valdez* in 1989. Fertilized plots demonstrated reductions in deposited oil and a loss of extractable organic matter from the remaining oil (Claxton et al 1991).

Similarly, microbial degradation at sites of natural oil seepage causes a decrease in sediment crude oil along with measurable organic enrichment (Spies, 1987).

Numerous studies have documented the adverse effects of catastrophic crude oil spills on marine environments. Recently the toxicity of biodegraded water-soluble fractions (BWSF) of crude oil has been demonstrated in embryos and larvae of several marine species. The increased water solubility of degraded crude oil is thought to increase oil toxicity to fish (*Clupea pallasi.)* embryos by as much as 10 fold. (Middaugh et al, 1998). Specific toxicity of water-soluble constituents of biologically degraded oil, rather than water soluble constituents of oil itself, has also been demonstrated in two estuarine crustacean species *Palaeomonetes pugio* and *Mysidopsis bahia* (Shelton et al, 1999). Paradoxically, sediments surrounding sites of natural seepage support elevated numbers of several invertebrate species (Steichen et al 1996). Several studies have suggested that organisms living near sites of natural seepage have evolved specific mechanisms for coping with constant exposure to potentially toxic by-products of oil exposure (Spies et al, 1982; Spies et al 1996).

Many marine invertebrates are known to live and reproduce in other environments rich in potentially toxic chemicals (Kurelec, 1997). Chemical concentrations in tissues of these organisms are often maintained at levels below those in the surrounding environment. A primary molecular detoxification mechanism, the multixenobiotic response (MXR), has been strongly implicated in this. This phenomenon is similar to multidrug resistance (MDR) in humans, which is associated with the expression of a membrane transport "P-glycoprotein" (P-gp) belonging to the ABC transporter protein family. P-gp is thought to confer drug resistance by acting as an energy dependent efflux

pump (Horio et al, 1988). Over-expression of the *MDR*1 gene product, and associated proteins, has been linked to the resistance of cancer cells to chemotherapeutic agents (Roninson, 1992). In normal tissues the MDR transport proteins have been implicated in transport of lipids, metabolites, ions and both endogenous and exogenous toxins (Shinkel, 1997).

Recent studies have emphasized the importance of MDR-like proteins in aquatic systems (Epel, 1998; Bard 2000). Kurelec and his colleagues (Kurelec, 1997 for review) have described verapamil sensitive, MXR proteins in several adult bivalve and sponge species, including *Anodonta sp.* (Kurelec and Pivceviac; 1989). Minier and his colleagues found that oysters from polluted sites have higher frequencies of expression of MXR proteins than those from sites with less pollutants (Minier, et al 1993). The MXR transporter protein also appears to be important for juvenile and adult forms of the echiuroid worm, *Urechis caupo*, which live in habitats naturally rich in environmental toxins (Toomey and Epel, 1993). In this study we investigate the toxicity of biodegraded, water-soluble fractions (BWSF) of crude oil on embryos from two marine invertebrate model species, *Urechis caupo* and *Lytechinus anameus*. *Urechis* and *Lytechinus* embryos have previously been shown to differ dramatically in their MXR mediated ability to actively extrude toxic organic compounds thus, we examined the role of Multixenobiotic response (MXR) in the differential response of each to BWSF .

2. Materials and Methods

2.1 Animals

Adult *Lytechinus anamesus* were maintained year round at the Bodega Marine Lab (BML) in continuous flow through, sand filtered Bodega Bay sea water (13°C +/- 2°) with weekly feedings of locally collected kelp. *Lytechinus* were spawned by injection of 0.25-0.5 mL of 0.5M KCl into the body cavity via the peristomial membrane. Gametes were collected into chilled 0.7μm filtered Bodega Bay seawater (FSW) and kept on ice until use. Adult *Urechis caupo* were collected from mudflats at Bodega Harbor and Pillar Point and maintained in plastic tanks with flow through seawater and several inches of mud and sand. Gametes were collected within several weeks of collection.

2.2 Crude Oil Weathering and Degradation

Santa Barbara Channel crude oil was collected directly into clean 4L amber bottles at the Gaviota platform in the Santa Barbara Channel and shipped overnight to the Bodega Marine Laboratory at 4°C. The crude oil was artificially weathered according to the method of Middaugh et al (1996). The oil was autoclaved at 374°C for 1hour, distilled at 1 atm, and then stored in aliquots at -20°C. This oil is subsequently referred to as artificially weathered oil.

Sediment samples were collected from oil seep sites 60 feet below the surface of the Santa Barbara channel at Coal Oil Point. 100-200mL of surface sediment was directly scraped into 250ml, Nalgene polypropylene bottles. Excess seawater was poured off and samples were put on ice and transported to the Bodega Marine Laboratory. Upon arrival,

a primary 'enrichment' culture was inoculated with these sediments to enrich for the presence of hydrocarbon degrading microbes.

A reduced scale adaptation of the method of Middaugh et al (1996) was used for microbial enrichment and subsequent biodegradation. Degradation vessels were 4 L amber bottles that had been acid-washed, and pre-rinsed several times with reagent grade methylene chloride, acetonitrile and finally distilled water. An enrichment culture, containing 3L of 0.7μm filtered, full strength (32-34ppt) Bodega Bay seawater (FSW) fertilized with Bushnell and Haas nutrients (DIFCO) was autoclaved, cooled to room temperature (18 °C) and then inoculated with 0.5% artificially weathered crude oil and 15mL of fresh seep sediment. The enrichment culture was vigorously aerated with 0.2μM filtered air in a fume hood at room temperature (17-20 °C). After 14 days the culture was passed over a 8.0μm paper (Whatman) filter to remove large particles and emulsified oil, and the resulting filtrate was stored in aliquots containing 10% DMSO at -70 °C.

Final degradation cultures were prepared identically to the enrichment cultures except they were inoculated with 50mL of the enrichment culture. A negative control (for the effect of the enrichment culture microbes) was prepared by inoculating an identically prepared biodegradation vessel with 50mL of FSW containing Bushnell and Haas nutrients and 10% DMSO. Incubation was conducted in the same way used for the enrichment culture.

After 14 days, biodegraded water-soluble fractions (BWSF) of crude oil were prepared by sequential filtration of the degradation culture inoculated with 'enrichment' microbes. Large particles and remaining oil particles were removed by sequential filtration through a 5.0μM followed by 2.0μm cellulose acetate filters (Millipore) and

stored at -70 °C in aliquots. 'Non-degraded' water-soluble fractions (NWSF) were prepared from the control culture in the same way. Aliquots of BWSF and NWSF for larval exposures were further filtered to 0.45μM prior to use with embryos. Smaller aliquots were sterile filtered to 0.22μm for use with cell cultures.

Approximate concentrations of solubilized crude oil hydrocarbons in BWSF and NWSF were determined by fluorescence excitation scanning (Photon Technologies Inc. Spectrofluorometer, South Brunswick, NJ) using a modification of the methods of De Alda-Villaizan et al. (1995). A standard curve (r^2=0.96) for crude oil hydrocarbons was prepared by sequentially dissolving a known quantity of artificially weathered crude oil in methylene chloride, DMSO and finally FSW and measuring fluorescence (353ηm) at peak excitation for oil hydrocarbons (290ηm). All standard values were adjusted for the fluorescence of a DMSO/Methylene chloride control.

2.3 Larval Exposures

Eggs of the various species were fertilized and fertilization success was assessed as elevation of the fertilization envelope and successful completion of the first cell division. Only batches with 90% or greater fertilization success were used for experiments. Gametes from different individuals were not mixed prior to fertilization, thus each replicate was the product of a unique male and female pairing. At least 3 replicates were used for all experiments. After fertilization groups of 150-250 embryos were transferred to 60mL shell vials (Fisher) containing 20mL of FSW and concentrations ranging from 0-3mg/L degraded hydrocarbons (corresponding to 0-7% of BWSF or NWSF). Vials were incubated at 15 °C through embryonic development.

Lytechinus and *Urechis* larvae were fixed in 0.5% paraformaldehyde and 50-100 larvae per vial were scored for successful development to pluteus or trochophore larval stages, respectively.

2.4 Dye Efflux Assays

To assess whether BWSF could be acting as a competitive inhibitor of the MDR transporter we conducted competitive dye transport assays according to the methods of (Toomey and Epel, 1993). Briefly, unexposed embryos of each of three species were incubated for 30 minutes at 15°C either with 1μM rhodamine alone, 5μM rhodamine and 20μM verapamil or 1μM rhodamine and 2.2ppm BWSF hydrocarbons. After incubation embryos were washed twice by hand centrifugation and resuspended in FSW.

Bulk fluorimetry was conducted by placing groups of 1000-2000 larvae into a microcuvette (at a total volume of 1.5ml) with constant stirring in a PTI microfluorimeter. Fluorescence was measured at 550ηm excitation and 580ηm emission.

2.5 Western Blotting

Two batches of embryos and larvae from each species were collected into conical tubes from cultures, then chilled on ice for 15 minutes and gently concentrated by hand centrifugation. Pellets of 100-200ul of embryos were transferred to microfuge tubes containing an equal volume of ice-cold hypotonic lysis buffer (Morris, et al 1991) modified with the addition of with general protease inhibitor cocktail (Sigma). Embryos were disrupted by brief sonication. Aliquots of embryo homogenates were mixed with equal volumes of 2x sample buffer and solubilized by boiling for 5 minutes. Protein

concentrations of homogenates were determined using a standard BCA protein assay (Pierce). Solubilized homogenates (15μg of total protein/sample) were loaded onto 10% SDS-polyacrylamide gels and run at 155V for 90 minutes at room temperature. Proteins were transferred onto nitrocellulose for 3h at 115V in a cooled chamber. Blots were blocked overnight in 4% non-fat milk in Tris-buffered saline (10mM Tris base, 150mM NaCl, pH 7.4) MXR protein was labelled by incubating blots with the mouse monoclonal antibody C219 (Fujirebio Diagnostics) at 1ug/mL, followed by the horseradish peroxidase (HRP) conjugated goat anti-mouse (1:5000) secondary (Sigma). HRP labelled MXR protein was visualized by reaction with chemiluminescent reagent (Pierce) according to manufacturers instructions and exposure to photographic film (Biomax MR, Kodak). Since *Urechis* embryos are known to express MDR 1 (Toomey and Epel, 1993), they served as a positive control.

2.6 GC-MS Analysis of BWSF and NWSF

Samples were prepared for analysis by gas chromatography – mass spectroscopy (GC-MS) using C-18 and silica columns (Alltech), using an adaptation of the methods of (Vines et al, 2000). Columns were first conditioned by rinsing with the appropriate solvent (methanol for C-18 columns and hexane for Silica columns) followed by FSW. BWSF and NWSF corresponding to approximately 300ppm of each sample was loaded onto each and withdrawn, by vacuum filtration into a clean flask. The filtrate was retained for toxicity testing in embryos. The columns were then washed with ddH_20 followed by methanol (C-18) or hexane (silica). Columns were eluted with 0.5-1mL of hexane (C-18) or methanol (silica) onto a clean flask in ice. Samples were sent to the

Facility for Advanced Instrumentation, UC Davis for analysis on a Varion Saturn 4 GC - ion trap - MS instrument. A 30m (0.25mm thickness) capillary column was used with the following temperature program: 60°C, 1min, increased by 10°C/min to 300°C and maintained at 300°C for 10min. Peaks were compared to those from the National Institute of Standards and Technology (NIST) library of known compounds to give approximations of their identity based on probability based matching algorithms. Only structures with purity fit values above 700 are listed.

2.7 Data analysis

EC 50 values were calculated by logit-log analysis of toxicity data. Differences between species in toxicity and dye extrusion were calculated by using t-test of means on normalized (log transformed) data. Analyses were done using Sigma Stat (version 2.03) software.

3.Results

3.1 Crude Oil Degradation

Cultures inoculated with Santa Barbara Channel microbes produced visibly greater levels of crude oil emulsification than controls. Little or no oil remained adherent to the sides of the glass culture vessels in the microbe inoculated cultures. After filtration the resultant BWSF and NWSF were acidic, pH 6.5. The addition of these fractions to seawater for toxicity assays had no effect on seawater pH at the concentrations used. Fluorometric determination of soluble hydrocarbon concentrations showed dramatically

higher levels of hydrocarbons in BWSF versus NWSF, at 43.7mg/L versus 3.05mg/L, respectively. These values were based on the standard curve developed for crude oil (see Materials and Methods).

3.2 Larval Exposures

Urechis embryos were insensitive even to relatively high (3 mg/L) concentrations of BWSF (Fig. 1). In contrast sea urchin development was significantly ($P<0.05$) delayed by exposure to BWSF at concentrations as low as 0.1 μg/L (Fig. 1). Delayed urchin development was observed in a dose dependent fashion both at hatching (about 12 hours after fertilization) and later at the pluteus larval stage (Fig. 2). At high concentrations of BWSF (2.2 mg/L) sea urchin embryos showed more severe developmental delay and abnormalities including abnormal cell division and poor ciliary movement. Urchin embryos that hatched in high concentrations of BWSF were often irregular in shape and observed on the bottom of vials rotating in circles (in contrast to normal circular gastrulae which swim in the water column).

To compare the relative toxicity of BWSF and NWSF, sea urchin embryos were exposed to NWSF ranging from 0.5-5% of the culture volume (2.5 - 25 mg/L). These proportions correspond to the range of proportions of BWSF used in the initial exposures (0.22-2.2 mg/L). At equivalent proportions in seawater, BWSF is more toxic than NWSF (Fig 3). Calculated EC50 values for BWSF and NWSF were 0.74% and 1.07% respectively. However, based on differences in hydrocarbon concentration in each of the two fractions these results suggest that the constituents of NWSF are significantly more toxic than those from BWSF, with EC50 values of 0.03 and 0.33 mg/L respectively. The

enhanced toxicity of BWSF may therefore be attributed to the dramatic increase in water-soluble hydrocarbons observed after microbial degradation of oil. Sea urchin embryos were only slightly less susceptible to the effects of BWSF if allowed to develop to hatching in clean FSW prior to exposure to BWSF (Fig. 4), thus toxicity of BWSF is not specific to early developmental stages.

At 15°C *Lytechinus* typically developed to the pluteus stage in approximately 93 hours while *Urechis* develop to trochophore was less than 48 hours. Thus to determine if the increased exposure time of sea urchin embryos to BWSF had any significant effect on their sensitivity, additional experiments were conducted where *Urechis* embryos were also exposed for 93 hours (Fig. 5). This extended exposure to BWSF still had no significant effect on *Urechis* embryo/larval development.

3.3 Dye Efflux

Lytechinus anamesus larvae do not appear to possess functional MDR-like rhodamine dye extrusion capacity. No significant differences were observed between verapamil or BWSF treated embryos and controls (Fig 6). In contrast, *Urechis* larvae were able to extrude significantly more dye in the absence of MDR inhibitors, including verapamil or BWSF. BWSF seemed to induce relatively large increases in dye accumulation in *Urechis* larvae that were not significantly different from those observed with verapamil ($P<0.001$).

3.4 Western Blots

P-gp appears to be expressed relatively homogenously throughout Urechis embryo development and does not appear to be induced by exposure to high (2.2 mg/L) concentrations of BWSF, nor does it appear to be expressed in a stage-specific manner (Fig.7). In agreement with earlier findings in purple sea urchins (Toomey and Epel, 1993), *Lytechinus* embryos and larvae do not appear to express an MDR 1-like protein that cross reacts with monoclonal antibody C219. Exposing urchins to 0.22 mg/L did not result in expression of a cross-reactive protein.

3.5 GC/MS Analysis of BWSF/NWSF

Column filtrates were tested for their toxicity to urchin embryos. Dramatic differences were observed at the 1.1ppm level of BWSF where abnormal development was reduced from 86% in controls to 42% and 0% in C-18 and silica extracted fractions respectively. These reductions in toxicity suggest that the columns are removing many of the water-soluble toxic constituents of degraded and non-degraded oil hydrocarbons. The complete removal of toxicity by silica columns suggests that most of the toxicity is mediated by smaller, polar constituents. Previous studies have shown significant enrichment of small hydrocarbons during degradation (Middaugh et al 1996). Table 1 summarizes the composition and structural homologies (from existing databases) of major peaks found by GC MS analysis of water-soluble oil fractions. Constituents are listed in descending order of their relative abundance. Silicon atoms associated with silica column extracts were likely contaminants associated with the elution process. *However,*

even after accounting for their contribution to molecular weight of silica extracts of BWSF and NWSF, there were reductions in mean molecular weight of the top seven constituents from 295.9 g/Mol in NWSF to 234.7 g/Mol in BWSF.

4. Discussion

In this study we have examined the toxicity of biodegraded crude oil similar to that naturally occurring in the Santa Barbara channel. Our results provide further evidence that biodegradation of crude oil may actually result in enhanced oil toxicity to some marine invertebrate embryos. As has been previously reported (Shelton et al, 1999) the mechanism of this enhanced toxicity after biodegradation appears to be as a result of increased accumulation of small, water-soluble hydrocarbon fractions. Consistent with this hypothesis silica extraction of BWSF caused greater reductions in toxicity than C-18 extraction. The low levels of water-soluble hydrocarbons measured in NWSF suggest that although water-soluble fractions of non-degraded oil are highly toxic, they are probably less likely to accumulate at high concentrations without enhanced microbial degradation. Levels of toxicity were observed at concentrations relevant to those previously measured in laboratory and environmental samples. Stuermer et al, 1981 have reported that water soluble fractions of Santa Barbara Channel seep oil prepared in the laboratory contained hydrocarbon concentrations as high as 9.7ppm. Environmental concentrations of oil hydrocarbons are more variable and depend on several environmental factors including mixing and temperature. Previous studies have suggested that these concentrations likely range from 10 μg/L in near-seep water to as high 0.5 mg/L directly at the sediment-water interface (Spies, 1987). Recent research has shown

that, sediment-oil mixtures can be transported long (50km) distances before completion of the degradation process (Philips, et al 1998). This would suggest that some of the impacts of crude oil degradation would occur at distances farther from the initial site of oil release than previously thought.

To our knowledge, this is the first report that demonstrates species-specific embryo tolerance to high levels of water-soluble fractions from degraded oil. Over the range of concentrations used, the effects of BWSF on *Urechis* embryos/larvae were negligible. Consequently, these species-specific differences in susceptibility to BWSF are important considerations when evaluating the risk of crude oil degradation on marine invertebrates. These results suggest that animals that live in close contact with microbe rich sediments, such as *Urechis caupo*, are likely to have evolved mechanisms for coping with toxic by-products of microbial metabolism of a range of organic constituents. Previous studies have shown that this is also true of oil seep-dwelling organisms. One mechanism implicated in tolerance to the effects of petroleum hydrocarbons (Klotz et al, 1983; Spies, et al 1982) has been the mixed function oxygenases (MFO). In surfperch living near seeps levels of cytochrome P450 1A are significantly elevated as compared to counterparts from clean sites (Spies et al, 1996).

MDR mediated efflux appears to be another mechanism that may be important in determining susceptibility to degraded oil hydrocarbons. Many of these compounds have structures that one would expect to interact well with MDR transporters (Ambudkar et al, 1999). Previous studies have demonstrated that oil hydrocarbons can result in enhanced MDR-like activity (Kurelec et al, 1995) in marine invertebrates. Our results show that biodegraded hydrocarbons can act as competitive inhibitors of MDR mediated efflux in

Urechis embryos/larvae. These results are not unexpected, given the promiscuous nature of MDR transporters (Gottesman and Pastan, 1993; Sharom, 1997). A significant advantage of MDR-like efflux of hydrocarbons is that it likely results in reduced accumulation of potentially hazardous compounds. Because drugs appear to bind to MDR transporters while in solution in the phospholipid bilayer (Ueda et al, 1997; Romiski and Sharom, 1999), MDR represents a first line of defense against environmental toxins (Epel, 1998). It is thus possible that elevated levels of MFO in response to petroleum hydrocarbons occurs only after primary mechanisms of drug extrusion have been overwhelmed. Under these conditions cytotoxicity may be mediated by constitutively occurring compounds that are otherwise maintained at non-toxic concentrations.

Several studies have also suggested that MDR transporter expression may be part of a more generalized cellular stress response and that its expression may be coordinated with the expression of other stress proteins. In fish and mammalian liver (Cooper et al, 1999; Silverman and Schrenk, 1997) expression of MDR is also associated with expression of MFO. In *Mytilus californianus* (Eufemia and Epel, 2000) and several mammalian systems (Chin 1990; Vilaboa, 2000) protein denaturing stresses such as metal exposure and heat shock also result in enhanced MDR expression. Collectively, these observations suggest that simultaneous induction of several cellular stress tolerance mechanisms may act to confer tolerance to chronic stresses such as exposure to BWSF and related compounds at natural seep sites.

This study reinforces the previous observation suggesting absence of MDR-like activity in sea urchin embryos (Toomey and Epel, 1993). This is the second study that has found an absence of verapamil-sensitive rhodamine dye efflux and P-gp expression

(as determined by labelling with C219) in sea urchin embryos. Nonetheless, these results are not conclusive. Given the high level of conservation among ABC transporter proteins and their nearly ubiquitous expression (van Veen and Konings, 1997) it seems likely that some form of these proteins should be expressed in the sea urchin embryo/larva. In mammalian systems several efflux proteins have been shown to be associated with the MDR phenotype. In many cases ABC transporter proteins other than P-gp (Cole et al, 1992) confer drug resistance via active extrusion of drugs. Moreover, increased expression of detoxification enzymes may also be associated with drug resistance. Thus, absence of P-gp expression alone may not be an adequate measure of susceptibility to xenobiotics.

An alternate hypothesis for the observed species-specific differences in toxicant resistance is the possibility that the protein(s) responsible for efflux of endogenous compounds and xenobiotics in sea urchin embryos differ from those expressed in *Urechis* embryos. The various families of ABC transporter proteins are known to differ significantly in substrate specificity (Cole and Deely, 1998; Ambudkar et al 1999). Because of these differences in substrate specificity among these proteins their activity may not be readily detected using standard rhodamine efflux assays (Twentyman et al, 1994). This has important implications for determining whether or not a given compound will act as a "chemosensitizing" agent (Kurelec, 1997) in the environment. MDR-like transporter proteins are known to play important roles during development via efflux of endogenously produced molecules such as differentiation factors (Good and Kuspa, 2000). It is not known if a subset of transporters expressed during development is primarily involved in the efflux of exogenous substrates. Characterizing species and stage

specific patterns of drug resistance protein expression is necessary in order to compose a complete picture of species-specific differences in susceptibility to environmental toxins. In order to address this question it is likely that multiple and/or degenerate probes targeted against various MDR proteins and gene sequences will be required. Such an approach has already been successfully employed for the cloning of transporters from model organisms (Allikmets and Dean, 1998) and for the specific identification of various MDR related proteins (Scheffer et al, 2000).

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Figure Legends

Figure 1. Effect of continuous exposure to BWSF on development to larval stage in urchins and urechis (n=3 batches embryos per species).

Figure 2. BWSF delays development to pluteus (A) in urchins at low concentrations (0.44ppm). At higher concentrations complete developmental arrest occurs at the early gastrula stage.

Figure 3. Relative toxicity of BWSF and NWSF. To account for the difference in relative hydrocarbon concentration between the two fractions concentrations are shown as percentage of culture volume. NWSF is more toxic to urchins than BWSF, but it contains significantly lower concentrations of soluble hydrocarbons.

Figure 4. Toxicity of BWSF is only moderately reduced if urchins are allowed to develop to hatching in clean FSW prior to BWSF exposure.

Figure 5. 96hr BWSF exposure. Prolonged exposure to BWSF is not deleterious to urechis.

Figure 6. Competitive dye efflux experiment. Both BWSF and Verapamil cause significant increases in rhodamine B dye accumulation in urechis but not urchin. Dotted line indicates control fluorescence level.

Figure 7. Western blot of P-gp using monoclonal Ab. C219 in urchin and urechis. Urchins do not appear to express P-gp at any point during development. In contrast urechis appear to express P-gp continuosly.

Table Legend

Table 1. Results of GC-MS analysis of C-18 and silica column extracts of BWSF and NWSF. Predicted structures with purity fit values (to known NIST standards) of greater than 700 are listed.

BWSF C-18	BWSF Silica	*NWSF* C-18	NWSF Silica
$C_{41}H_{-2}0_2$; MW 592	$C_{13}H_{21}N0_3$; MW 239	$C_{24}H_{38}O_4$; MW 390	C-H_4F_2O; MW 142 2,5 Difluorobenzaldehyde
$C_{19}H_{19}N$; MW 261 Butylated hydroxytoluene	$C_8H_{14}O$; MW 126 Ethanone, 1-cyclohexyl	$C_{10}H_{12}O_2$; MW 164	$C_8H_{14}O$; MW 126
$C_{41}H_{-2}0_2$; MW 592	$C_{10}H_{26}O_2Si_2$; MW 234	$C_{41}H_{-2}O_2$; MW 596	C_1-H_2-NO_2; MW 277 Phenol, 2,6-bis(1,1-dimethylethyl)-4-methyl, methylcar
$C_{14}H_{22}0$; MW 206	$C_{14}H_{20}O_2$; MW 220	$C_{15}H_{24}O$; MW 220	$C_{16}H_{48}O_6Si$-; MW 532
$C_{19}H_{19}N$; MW 261	$C_{15}H_{24}O$; MW 220 Butylated hydroxytoluene	$C_{22}H_{14}O_6$; MW 374	$C_{13}H_{40}O_5Si_6$; MW 444 1,1,1,5,7,7,7-Heptamethyl-3,3-bis(trimethylsiloxy)tetra
	C_1-$H_{34}O_2$; MW 270		$C_{13}H_{24}O_2Si_2$; MW 268
	$C_{22}H_{34}O_4$; MW 362		$C_{19}H_{54}O$-Si-; MW590
			$C_8H_{24}O_4Si_4$; MW 296 Cyclotetrasiloxane, octamethyl

Table 1.

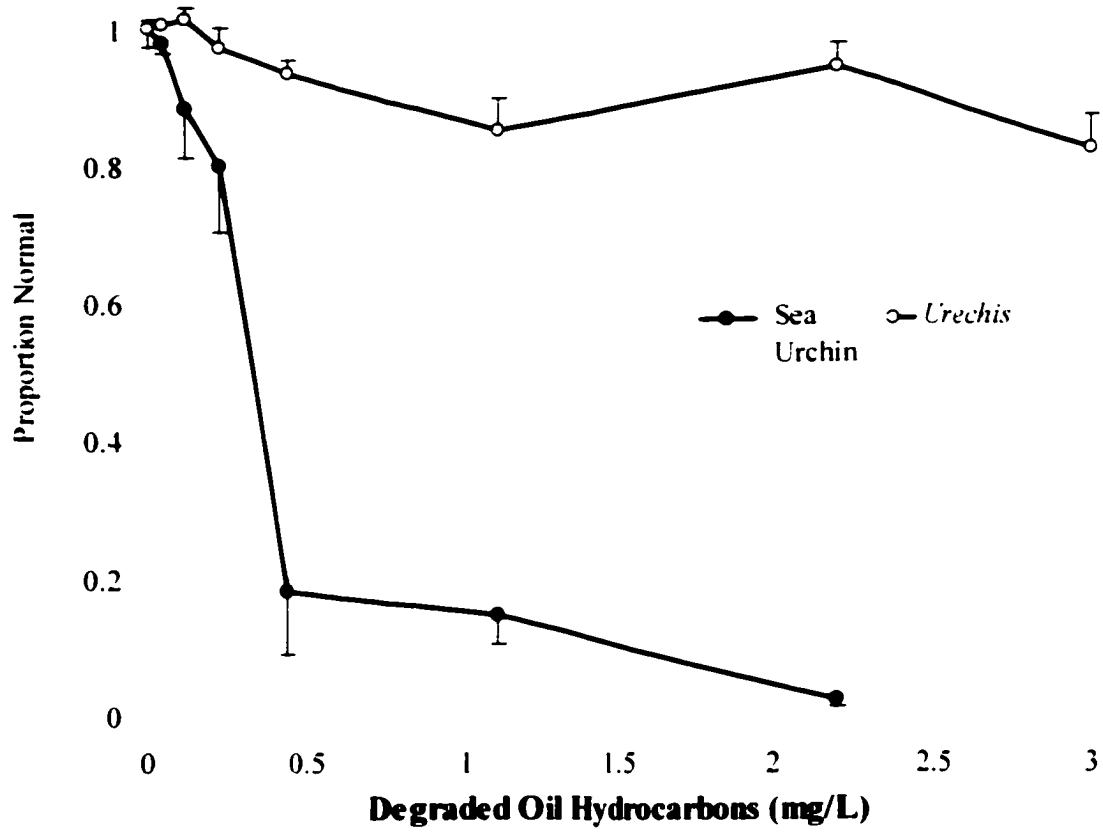
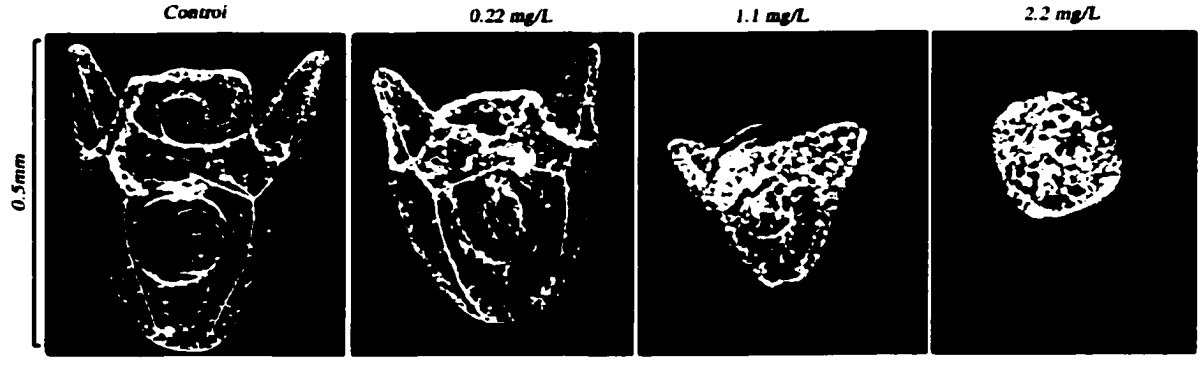


Figure 1 (above). Figure 2 (below)



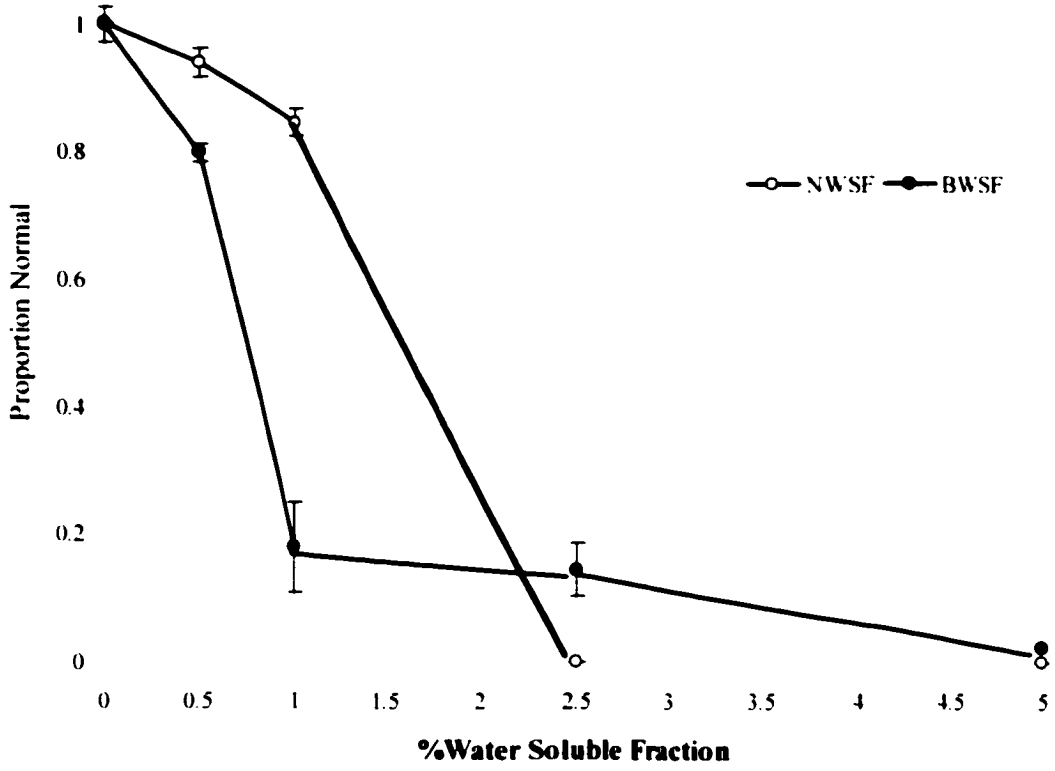
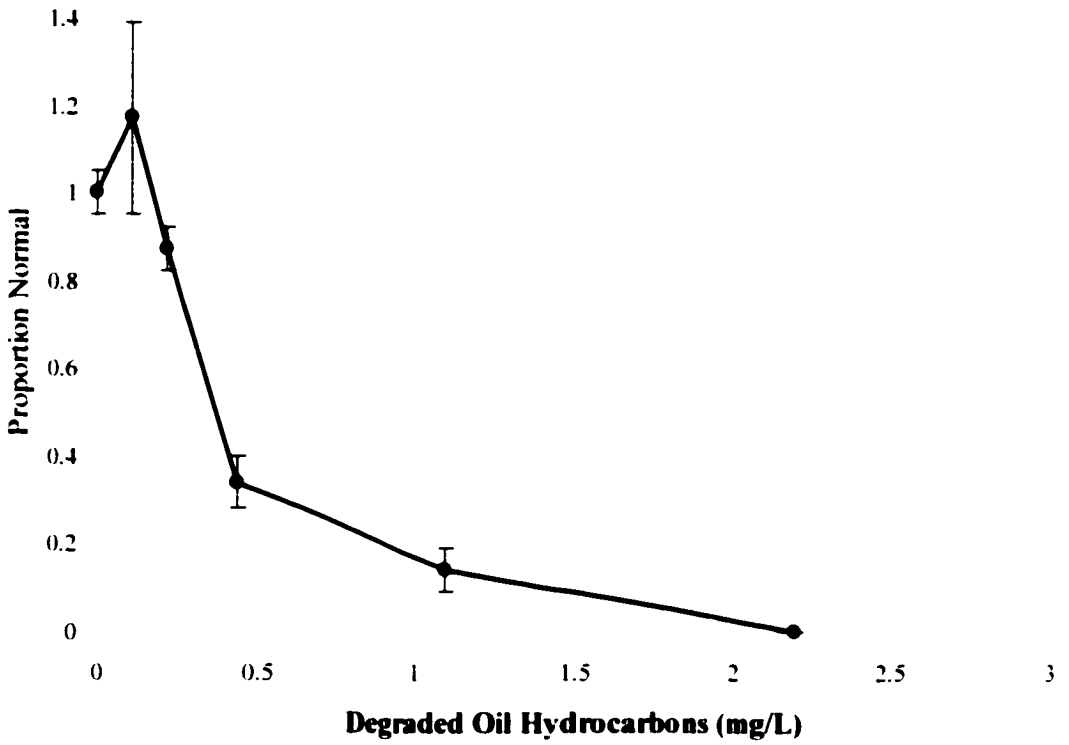


Figure 3.



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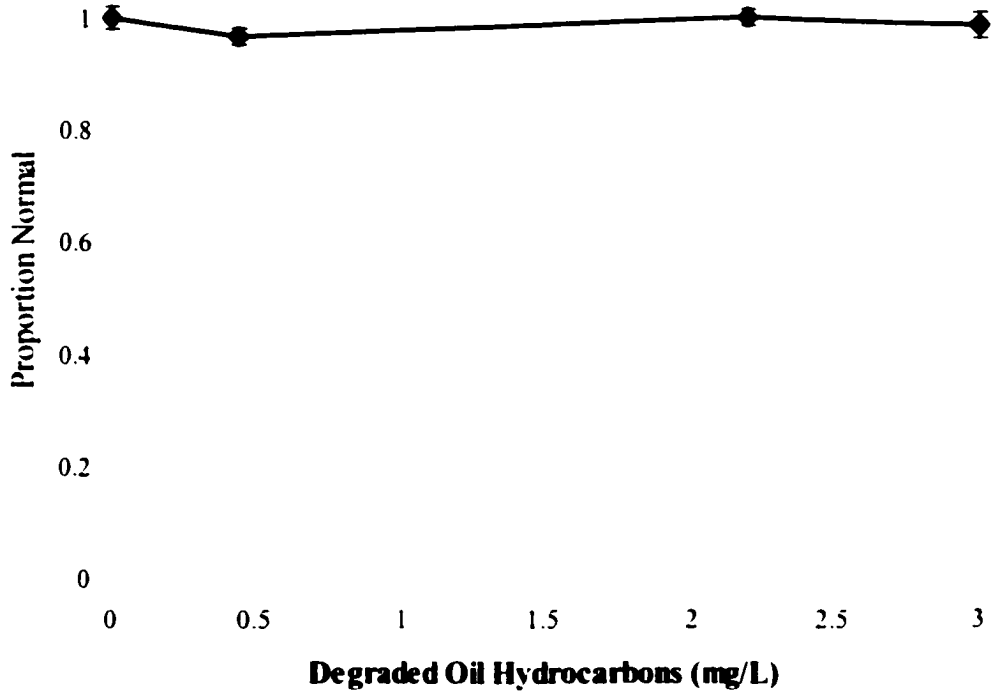

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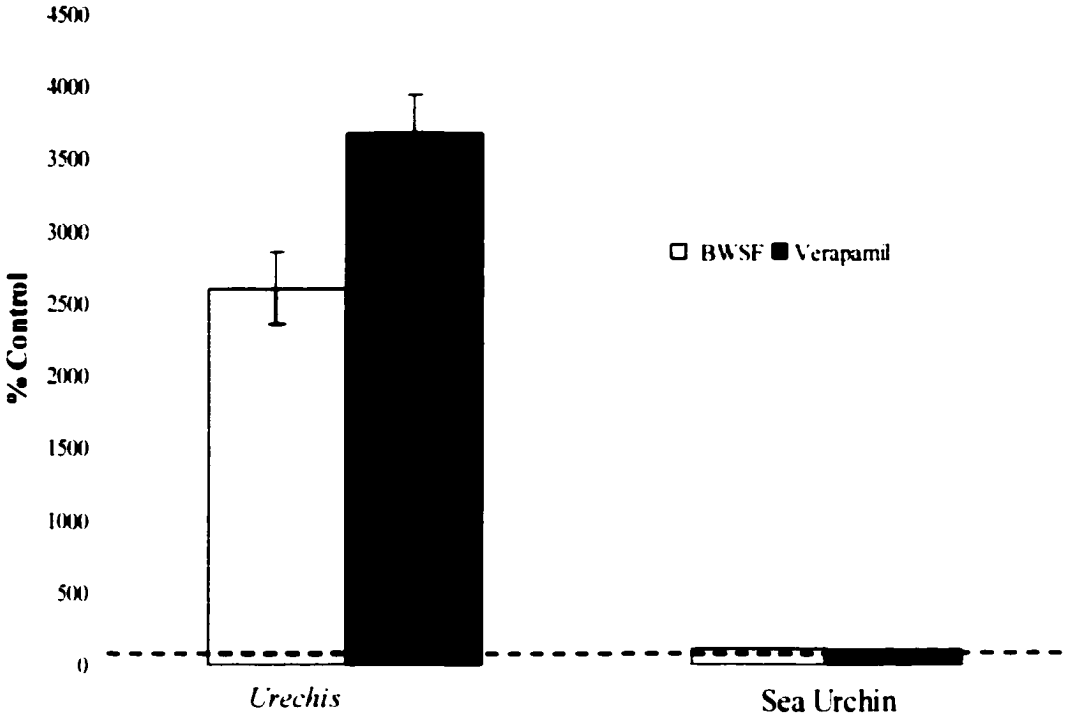
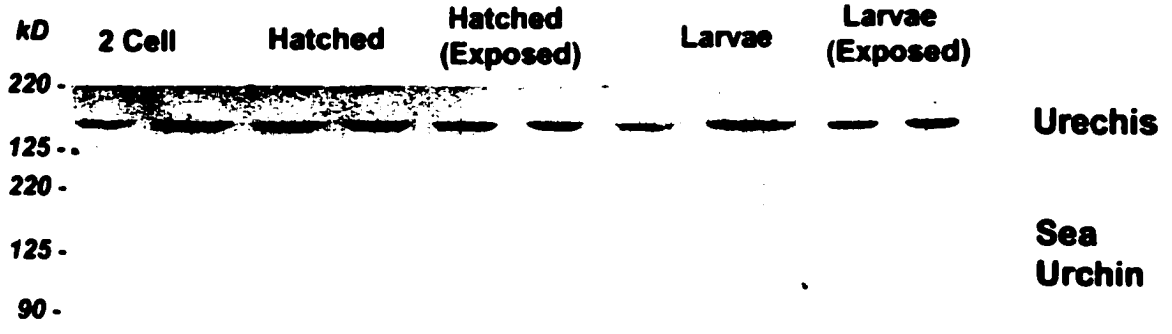


Figure 6 (above).

Figure 7 (below).



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CHAPTER 3

Evidence for Multidrug Resistance Associated Protein (MRP) Mediated Xenobiotic Extrusion in Echinoderm Embryos and Larvae: Activation at Fertilization

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Abstract

In this study we present functional and molecular evidence for a multidrug resistance associated protein (MRP) that mediated a drug efflux phenotype in eggs embryos and larvae of several echinoderm species, purple sea urchins *Strongylocentrotus purpuratus*, white sea urchins *Lytechinus anamesus* and sea stars *Asterina miniata*. Using a cDNA probe corresponding to the highly conserved nucleotide binding domains of ABC transporters we identified several transcripts encoding multidrug resistance transporters from purple sea urchin and sea star egg cDNA libraries. Comparison of these transcript sequences to known MRP transporter sequences suggests that echinoderm eggs possess at least two unique MRP-like transcripts. A third transcript with homology to the Permeability-glycoprotein (P-gp/MDR) family also appears to present in these eggs. Functional analyses of echinoderm egg, embryo and larval drug efflux supported the hypothesis that an MRP phenotype predominates in these species. Specifically we show that echinoderm embryos and larvae can extrude fluorescent substrates (Calcein-AM) of MRP, and that this transport is relatively insensitive to the presence of P-gp-specific substrates (Rhodamine). Comparison of drug efflux in eggs versus early embryos suggest that transport is activated at fertilization.

Introduction

A remarkable feature of most cells is their ability to actively extrude endogenous and exogenous substances that would otherwise accumulate to toxic concentrations. This phenomenon has been extensively studied because of its importance in determining the susceptibility of various cell types and organisms to toxic compounds. The best characterized mechanism for active toxicant extrusion is via the function of membrane transporters belonging to the ATP Binding Cassette (ABC) super-family (Dean et al 2001). This family of proteins includes proteins with a wide array of functions, and consequently, a large number of potential substrates (ligands). Of the 12 families of ABC transporters identified in humans so far only 3 have been shown to play major roles in the transport of xenobiotic compounds such as chemotherapeutic agents. These three gene families encode multidrug resistance (MDR) proteins, multidrug resistance associated proteins (MRP) and White family resistance proteins.

These three transporter families share structural and functional similarities. In humans each of these proteins are assembled from one or more "subunits" each of which typically possesses several membrane spanning domains which encoding the drug binding domain and one or two cytosolic domains. In the case of White family proteins a single subunit is sufficient to handle drug extrusion. In the case of MDR and MRP two or three subunits, respectively, are linked to form a large transporter with multiple drug binding domains (Ambudkar et al 1999, Cole and Deely, 1998). These variations are thought to be the consequence of multiple chromosome duplication and gene

recombination events through the long evolution of these proteins. Within each of these three families, genes encoding highly promiscuous "drug resistance" transporters have been identified. In addition to their role in toxicant transport, each family encodes members with more specified roles in normal cellular transport of a variety of compounds ranging from complex lipids to ions. MRP family transporters are distinguished by their additional ability to transport glutathione conjugates (Leslie et al 2001).

MDR like phenotypes have been described in a large number of vertebrate and invertebrate animal systems. In marine organisms a number of transporters have been shown to play important roles in the extrusion of potentially toxic, exogenous substrates (Eufemia 1996, Eufemia 2002). There is clear evidence for the presence and activity of homologues of MDR-like proteins in fish liver from several species (Albertus and Laine 2001, Cooper et al 1999). Similarly, in marine invertebrates, MDR-like phenotypes, and gene products, have been described in a number of adult and embryonic life history stages including those of echinoderms, echuroid worms and mollusks (see Epel, 1998 and Kurelec 1997 for review). In many cases these transport phenotypes are also associated with reduced susceptibility to a number of anthropogenic toxicants that might be encountered in the marine environment.

Although many of these transporters have been named multi-xenobiotic transporters (MXR), molecular evidence suggests that typically they bear homology to known MRP and MDR transporters. Previous observations in marine invertebrate oocytes, eggs and embryos suggest that there may be clear differences in the type of transporter expressed in different species. For example, Toomey and Epel (1993) found that sea urchin (*Strongylocentrotus purpuratus*) eggs lack Permeability glycoprotein (*P-*

gp) mediated MDR activity, as determined by verapamil sensitive rhodamine dye efflux capacity. In contrast oocytes and embryos of the echiuroid worm (*Urechis caupo*) to express a functional *P-gp* - like protein, and functional dye efflux capacity. Several authors have shown that these differences are also correlated with differences in susceptibility to, naturally occurring, algal and microbial metabolites and byproducts (Eufemia et al 2002, Hamdoun et al, 2002, Toomey et al 1996) in marine invertebrates.

Little is known about the endogenous function of these transporters during embryonic development. In *Dictyostelium*, an ABC transporter, *RhT*, is known to handle the endogenously produced differentiation factor (DIF-1) that regulates terminal stalk cell differentiation. Several MDR homologues are also expressed in embryos of the soil nematode *C. elegans* (Broeks, 1996). However, it is not yet known if MDR transporters could play a similar role in early development of animals. Ebling, (1993), and co-workers have described an MDR phenotype in mouse embryos that appears to be mediated by the *P-gp* family of MDR transporters. In this system *P-gp* plays a clear role in regulating chemosensitivity during development.

Since the expression of these transporters appears to be ubiquitous, we hypothesized that the previously described absence of MDR activity in sea urchin eggs is a consequence of a difference between echinoderms and other marine invertebrate embryos in the predominant type of multidrug resistance transporter expressed. In order to test the hypothesis that sea urchins and other echinoderms such as sea stars express different transporters than species such as *Urechis* we used a number of approaches in order to clone and characterize echinoderm transporters. Previous functional assays of MDR activity, using several fluorescent dyes as indices of transporter protein efflux

capacity, suggest that the efflux kinetics for a given fluorophore may vary significantly between cell types based on the predominant type of transporter expressed. For example although P-gp can transport both rhodamine and Calcein-Acetoxymethyl ester spell out "AM" (C-AM), MRP family proteins handle C-AM efficiently but not rhodamine. In this study we use calcein-AM (C-AM) to characterize the transport phenotypes of sea stars and sea urchins (Hollo 1996, Sarkadi 1994). Using a high-affinity peptide inhibitor Reversin 205 of drug resistance transporter proteins we test the hypothesis that echinoderms possess a drug efflux transport phenotype that is unique from P-gp mediated drug efflux described in other marine invertebrates. Our results demonstrate the presence of reversin sensitive C-AM efflux in echinoderms Moreover we demonstrate that the echinoderm drug efflux transport is relatively insensitive to the presence of rhodamine. Additionally, we present molecular evidence that sea urchins and sea stars possess a number of MRP-like gene products. In this study, we report on the cloning and preliminary characterization of an MRP-like transporter in sea star and sea urchin oocytes, eggs and embryos, and we track the expression of the transporter gene during early development. Because sea urchins and sea stars are model developmental systems we propose that these species may also be ideal systems for elucidating the developmental roles of MDR transporters.

Materials and Methods

Animal Collection and Maintenance

Sea stars (*Pisaster sp.* and *Asterina miniata*) and purple urchins (*Strongylocentrotus purpuratus*) were collected in Bodega Bay, California and transported immediately to the Bodega Marine Laboratory (BML) where they were kept in flow through sea water tanks (approximately 13°C). White sea urchins (*Lytechinus anamesus*) were originally collected in the Santa Barbara Channel and were maintained in flowing sea water tanks at BML. Sea stars were fed fish and squid, while sea urchins were fed kelp several times weekly. With this regime animals remained sexually mature most of the year. Gametes were collected from from sea urchins and sea stars using 0.5M KCl or 1mM 1-methyl adenine injection respectively. Eggs and oocytes were washed several times in 0.7μM filtered seawater (FSW) prior to use in experiments. In the case of sea stars, only batches of eggs with >95% germinal vesicle breakdown were used for experiments. Embryos were prepared by mixing eggs from 2-3 females, with diluted sperm from one male. Only batches of embryos with >95% successful development (assessed at the two-cell stage) were used. MDR protein expression in echinoderms was compared to the expression of P-gp in Urechis caupo embryos according to Hamdoun et al, 2002.

Degenerate PCR and cDNA Library Screening

Total RNA was isolated from eggs and embryos using a modification of standard methods. Brifely, packed eggs or embryos were flash frozen in liquid nitrogen and then homogenized in a liquid nitrogen cooled ceramic mortar and then thawed into 10

volumes of homogenization buffer (Kalinowski et al., 2002) containing proteinase K (200µg/ml). Homogenates were incubated for 45 minutes at 37°C before standard phenol-chloroform extraction and lithium chloride precipitation (Sambrook and Russell 2001). cDNA was synthesized with Superscript Reverse Transcriptase (Gibco-BRL) according to manufacturer's instructions. Degenerate MDR and MRP primers (Allikmets and Dean 1998, Lee et al 1998) were used to amplify fragments of the corresponding transporter cDNA. PCR products were checked for size and yield on 1% agarose gel prior to TA cloning using the dual promoter pCRII-TOPO vector (Invitrogen) and cloning kit. Aliquots of plasmid minipreps (Qiagen) of positive clones were sent to Davis Sequencing (Davis, CA) for sequencing. Results were analyzed by BLASTX comparison to existing sequences at NCBI.

Once a positive clone was identified (Figure 1a) encoding a 230bp conserved portion of MDR/MRP transporters, the fragment was random primed and used to screen cDNA macroarray filter libraries (Clark, 1999) of *S.purpuratus* (Cameron et al 2000) and *A.miniata* (Kalinowski et al 2002) using standard filter hybridization techniques (Sambrook and Russell 2001).

Western Blotting

Oocytes, eggs and embryos from each species were collected into conical tubes from cultures and gently concentrated by hand centrifugation. Pellets of 100-200ul of embryos were transferred to glass tissue grinders (Kontes) tubes containing 5 volumes of ice cold Tris-Hepes-EDTA (5mM EDTA, 50mM HEPES, 50mM Tris, 1.25M Sucrose; pH7.2) buffer containing a general use protease inhibitor cocktail (Sigma). Embryos

were homogenized by hand while being kept cold on ice. Aliquots of embryo homogenates were mixed with equal volumes of 2x sample buffer and solubilized by boiling for 5 minutes. Membrane fractions of sea urchin and *Urechis* embryo proteins were prepared by centrifugation of THE homogenates at 6000g for 15min at 4°C, followed by ultracentrifugation of the supernatants at 50,000g for 30min at 4°C. The resulting membrane fraction pellet was resuspended in small volume of THE buffer.

Protein concentrations of homogenates were determined using a standard BCA protein assay (Pierce). Solubilized homogenates (15μg of total protein/sample) were loaded onto 10% SDS-polyacrylamide gels and run at 155V for 90 minutes at room temperature. Proteins were transferred onto nitrocellulose for 3h at 115V in a cooled chamber. Blots were blocked overnight in 4% non-fat milk in TBS. MXR protein was labelled by incubating blots with the mouse monoclonal antibody C219 (Fujirebio Diagnostics) at 1ug/mL, followed by the horseradish peroxidase (HRP) conjugated goat anti-mouse (1:5000) secondary (Sigma). HRP labelled MXR protein was visualized by reaction with chemiluminescent reagent (Pierce) according to manufacturers instructions in a Bioimaging Systems digital gel scanner.

Fluorescence Imaging and Quantitation

To assess whether or not echinoderms possess a drug resistance transporter extrusion of calcein-acetoxy methylester (C-AM) a known substrate for mammalian MDR and MRP proteins. Accumulation of the fluorescent calcein occurs when intracellular esterases cleave the AM group, rendering the molecule relatively membrane

insoluble. Reversin (R205; Sigma) is a novel, high affinity, tripeptide substrate of mammalian MDR transporters (Sharom et al, 1999; initially obtained by kind gift of Dr. Balzas Sarkadi) was used.

To test the hypothesis that calcein efflux occurs via a transporter that is unique from the P-gp transporter that has been previously shown to handle rhodamine dye in other species (Toomey and Epel, 1993), we conducted competitive dye efflux experiments in which sea urchins and sea stars were simulateneously incubated (15°C, gentle rocking) in 0.25μM calcein-AM and a 4 fold (1.0μM) molar excess of rhodamine. Aliquots of embryos were removed from incubation tubes and the intracellular accumulation of calcein dye was measured.

Eggs and embryos were incubated at 15°C with gentle rocking, in 0.5μM Calcein-AM in the presence or absence R205. After 2 hours of incubation were transferred to ice and immediately analysed. Fluorescence microscopy was conducted using a fixed stage upright microscope (Olympus BX50-WI) using xenon illumination and a cooled (4°C) stage. Excitation was set to 488ηm with emission measured at 520ηm. Images were captured using a Photometrics Coolsnap FX cooled CCD camera. Companion Nomarski and fluorescence images from several fields of embryos within each treatment were captured into MetaMorph software (Universal Imaging). Mean pixel fluorescence intensity was measured from individual (n=10-60 per treatment) embryos using image analysis macros provided with MetaMorph software.

Results

Degenerate PCR and cDNA Library Screening

Degenerate PCR of *Asterina* cDNA yielded a 230bp fragment with high homology to the highly conserved ATP binding domains of known MRP transporters (Figure 1a). Using this fragment as a probe we were able to identify cDNA clones from the echinoderm egg macroarrays that encode several echinoderm MDR transporters. Based on BLASTX comparison (E<1e-10 for all comparisons) of at least 800bp of open reading frame from each clone to existing sequences at NCBI we identified several echinoderm cDNA sequences that correspond to *MRP* family gene products. Figure 1b shows a comparison of the partial translated sequence of a putative sea urchin and a sea star MRP sequence in comparison to several known MRP sequence. This comparison demonstrates the previously suggested conservation of key functional domains MRPs among diverse taxa.

In both *Asterina* and *S. purpuratus* egg libraries, we were able to detect a cDNA corresponding to an MRP with high homology to the mammalian sulfonylurea receptor (*MRP 8* and *MRP 9*). These transcripts were characterized by the presence of approximately 14 additional (relative to P-gp) amino acids between the highly conserved Walker A and Walker B motifs of ABC transport proteins (Walker et al, 1982, Cole and Deely, 1998). We also identified several *Asterina* cDNA clones that suggest the presence of a transcript with homology to mammalian *MRP 5*, a canalicular organic anion transporter, although additional sequence information will be required to fully characterize this transcript.

Fluorescence Imaging and Analyses

Figure 3 shows the effect of rhodamine and reversin on the inhibition of C-AM transport in sea urchin eggs and embryos. Calcein-AM dye accumulation appears to be relatively insensitive to the presence of rhodamine or reversin prior to fertilization (Figure 2 and 3a). Addition of reversin to sea urchin or sea star eggs results in small increases in dye accumulation relative to control (Figure 2). However, in both *S. purpuratus* and in *Asterina*, these differences are significantly less than those observed after fertilization (Figure 3b and 4). Within 90 minutes post fertilization (two cell stage) the rate of dye accumulation is dramatically reduced by the presence of reversin, but not rhodamine (Figure 3b) and the total dye accumulation after 120minutes is reduced (Figure 3 and 4). These results suggest the activation of a C-AM transport mechanism at fertilization. In both cases the control level of C-AM accumulation after 120min is elevated in eggs relative to two cell embryos.

Reversin sensitive calcein-AM efflux was also measured in larvae of both echinoderms aand other marine invertebrates (Figure 5a). The level of reversin sensitive C-AM efflux appears to be similar in both white sea urchins (*Lytechinus anamesus*) and *Urechis caupo* (Figure 5b). These results are consistent with the observation that cells with P-gp mediated MDR (eg. *Urechis*) can transport both C-AM and rhodamine (Hollo, 1998) while cells that possess a MRP transporter do not transport rhodamine efficiently (Litman et al 2001, Twentyman and Rhodes 1994). However the sensitivity of C-AM efflux in larval urchins (Figure 5c) to reversin is approximately one order of magnitude lower than has been previously reported in mammalian cells (Sharom et al 1999). This may be a consequence of both high levels of MDR/MRP activity in larval urchins and

reduced affinity of mammalian MDR reversing drugs for marine invertebrate transporter proteins.

Western Blot Analyses

Figure 6a shows a western blot of whole sea urchin and sea star egg lysates probed with the anti P-gp antibody C-219. In *Urechis* oocytes a high molecular weight band, consistent with P-gp, can readily be detected with this antibody, but neither sea urchins nor sea stars appear to express this protein. In sea stars a low molecular weight (~50kd) band is specifically detected with this antibody. Other authors (Kawai et al, 1994, Zaman et al, 1994) have reported the presence of a similar low molecular weight protein in both MDR and MRP expressing cells. In membrane protein preparations of sea urchin and *Urechis* several low (~ 50kd) molecular weight protein were detected with the anti MDR antibody C219 (Figure 6b). An interesting observation is that several proteins of similar molecular weight are also detected in monkey liver membrane fractions which are known to express high levels of transporters. In both sea urchins and *Urechis* the level of this protein is elevated in larvae relative to eggs/oocytes (Figure 6b), consistent with the observed changes in dye efflux function. Moreover this protein appears to increase after 1MA maturation in starfish eggs (Figure 6a). Similarly, we have also observed increases in C-AM dye efflux capacity during maturation of starfish eggs (not shown). However we interpret these results with caution because it is also possible that other conserved membrane ATPases are recognized by this antibody, or that the low molecular weight bands are artifacts caused by degradation of a higher molecular weight

band(s) that are unstable under the reducing conditions used to prepare our samples. The latter possibility seems unlikely since this is not the case with the echuroid *P-gp*.

Discussion

Our results clearly demonstrate the presence of an MRP-like phenotype in early echinoderm embryos. It is not yet clear if high levels of this transport activity are also present in mature echinoderm eggs. In our results the accumulation of Calcein-AM in eggs was less sensitive to the presence of reversin, a potent inhibitor of MDR, than in two cell embryos. In contrast, early embryos appear to have active Calcein-AM extrusion capacity, and although Calcein-AM extrusion appears to be relatively insensitive to the excess presence of Rhodamine B, it is highly sensitive to reversin. This would suggest a unique transporter for each of these fluorescent substrates. Previous studies (Hollo et al 1996) have demonstrated broad cross-inhibitory properties of *P-gp* and *MRP* reversing drugs. However important differences in the specificity of fluorophores used to detect the transport phenotypes in cells with the different families of transporter protein have also been noted. (Litman et al 2000, Litman et. al, 2001) have demonstrated that while Calcein-AM is extruded by both P-gp and MRP proteins, rhodamine is only extruded effectively by cells that overexpress P-gp. Consistent with this observation, it has been demonstrated that *Urechis* embryos and larvae, which are known to express *P-gp*, are capable of both rhodamine (Hamdoun et al 2002, Toomey and Epel 1993) and C-AM extrusion. In contrast sea urchin embryos, that are not thought to express high levels of *P-gp*, extrude C-AM but not rhodamine.

Activation of inwardly directed transporters at fertilization has been previously observed various marine invertebrates (Epel, 1972), and it is widely known that there are massive reorganizations of the plasma membrane of invertebrate eggs at fertilization. The activation of MRP-like activity in echinoderms at fertilization may share a common mechanism with the activation of other transport mechanisms. If so, it is possible that vesicles containing a both inwardly and outwardly directed, mature transporter proteins are translocated to the plasma membrane at fertilization. This may prepare the embryo for exposure to toxic MRP substrates in the external environment. Although, many stress inducible proteins, including MDR/MRP proteins are thought to be transcriptionally regulated in response to specific stress signals (Shin, 1986), it is not until mid-blastula transition that most echinoderm embryos begin transcription of the zygotic genome, even of stress responsive genes such as heat shock proteins. Thus we hypothesize that a number of post transcriptional processes are required for the dramatic changes in transport activity observed at fertilization The presence of maternal transcripts in the echinoderm egg may represent a reserve of transporter that can be mobilized in the event of stress exposure.

It is not yet clear how the selective expression of one or more of these transporters has been adaptive during evolution in a particular environment. Many studies have demonstrated the important roles of these transporters in handling xenobiotic compounds (for review please see Epel, 1998, Kurelec, 1997) in the aquatic environment. Thus one hypothesis is that expression of these transporters is driven by environmental exposure to naturally occurring exogenous substrates. If this is true we would expect MDR phenotypes to be associated with distribution along naturally occurring gradients of toxic

organic substrates. However, although differences in drug efflux transporter expression has been linked to exposure to natural and anthropogenic compounds in the laboratory, evidence of natural modulation of MDR transporter expression *in situ* is limited. Moreover, it will be important to correlate inter-specific variation in drug efflux transport physiology with differences in the environment encountered by different marine invertebrate embryos.

An alternate hypothesis regarding the evolution of these transporters in marine invertebrate embryos, is that both endogenous and exogenous substrates play an important role in driving the evolution of MDR transporters. In this scenario the toxic effects of exogenous MDR substrates may, in fact, be attributed to indirect effects caused by competitive inhibition of endogenous substrate extrusion. This may be particularly relevant during early development where gene expression is limited and a fixed pool of maternally derived transporters and transporter mRNA are required to handle a wide array of functions. However, transcription of *P-gp* is thought to be initiated at both TATA and TATA-less upstream promoters (Scotto and Egan, 1998), suggesting that in some systems transcriptional activation of MDR could occur prior to the onset of zygotic RNA polymerase II (Martianov et al, 2002) transcription via TATA binding proteins. Moreover, both transcriptional and post-transcriptional processes are involved in the regulation of *P-gp* expression even in adult tissues (Lee, 2001) suggesting that the early embryo could maintain a level of chemoprotection without activation of MDR transcription.

Recent observations in various populations of stem cells reinforce the notion that these transporters may also play important roles in the regulation of cellular

differentiation (Bunting, 2001). In this study we show an example of developmental regulation of MRP that is independent of exposure to environmental toxins. Studies in *Dictyostelium* (Good and Kuspa, 2000) have demonstrated endogenous roles for other ABC transporters in development. In *Arabidopsis*, the huge number of ABC transporters are thought be involved both in detoxification processes and more generalized metabolic processes (Martinoia, 2001). Less is known about the developmental role of these transporters in animals but in one example, *C.elegans*, the total level of MRP mRNA appears to be relatively constant through early development (Broeks, et al 1996).

Molecular analyses of MRP suggest that there is little evidence for biologically regulated splice regulation of transporter mRNA expression (Grant et al 1997). Combined with the observation that the level of MRP is constant during nematode development, it may be reasonable to postulate that selective regulation of multiple drug efflux transporter genes and their products is required for successful transport of both endogenous and exogenous substrates. Consistent with this hypothesis multiple transcripts, *P-gp* and MRP, appear to be present in the echinoderm egg although the transport phenotype in the early embryo appears to be predominated by a single transporter (MRP). It is not yet clear if this is simply a consequence of different levels of abundance of each transcript and its product(s), or if it is a result of specific regulation of each transcript. Previous studies in *Urechis* eggs and embryos have failed to find consensus regions (Rosenthal et al 1993, Rosenthal 1993) in maternal mRNAs that are associated with the polyadenylation and translation of these gene products at fertilization, even though there is clear evidence for selective mRNA translation through oogenesis and early development. The diversity of the ABC transporter family, coupled with the

variation in function and regulation of its products, may provide an ideal system for elucidating both some of the general aspects of gene regulation during development and the details of transporter specific regulation.

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Figure Legends

Figure 1. a. Sequence of the cDNA MRP probe used to screen echinoderm egg cDNA libraries. *b.* Partial translated sequence of cloned *Asterina* and *S. purpuartus* MRP transporter proteins compared with other MRP sequences. This sequence corresponds to the highly conserved nucleotide binding domains.

Figure 2. The mean increase in fluorescence intensity (after 120 minutes of incubation) of sea star and sea urchin eggs when treated with reversin (a high affinity MDR inhibitor) or rhodamine, a P-gp specific fluorophore. Calcein-AM accumulation was measured as the increase of free intracellular calcein fluorescence In all cases increases relative to control are modest (<150% control) but significant ($P<0.05$ by one way anova).

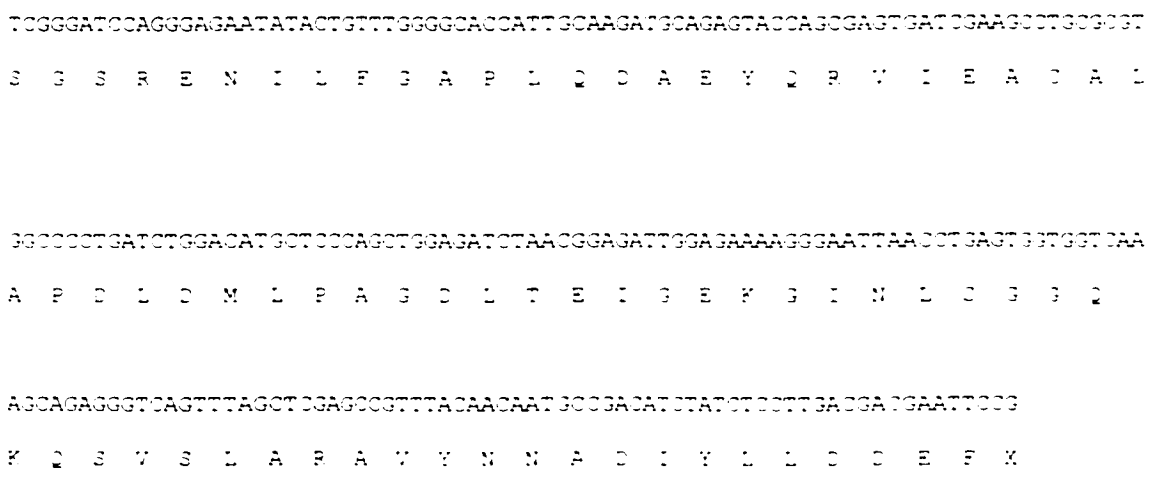
Figure 3. a. Calcein accumulation is rapid in sea urchin (*S. purpuratus*) eggs and is relatively insensitive to the presence of inhibitors (see also figure 2). *b.* Eggs were fertilized and allowed to begin cleavage (~90min) prior to fluorophore incubation. The total level of calcein accumulation is much lower in early embryos after 120 min, than in eggs. Moreover the level of calcein accumulation is sensitive to the presence of reversin but not rhodamine. (n=10-60 eggs or embryos for all measurements).

Figure 4. Calcein accumulation (0.25μM, C-AM) in sea star eggs and early embryos is shown at a single time point after 120 minutes of incubation. As in sea urchins (Figure 3) the total level of calcein accumulation is lower after fertilization. Similarly the relative level of sensitivity to inhibitor (reversin) is low prior to fertilization (n=15-35 for all measurements). Reversin treated embryos accumulate similar amounts of calcein as untreated eggs ($P>0.05$) suggesting that 1μM reversin is nearly completely reversing the induction of MRP at fertilization in these embryos.

Figure 5.a. Accumulation of calcein (0.5μM, C-AM) in sea urchin (*Lytechinus*) pluteus larvae and *Urechis* trocophore larvae after 120 minutes of incubation. Addition of inhibitors increases the level of accumulation dramatically see figure 5b; letters indicate diginifcant differences P<0.01). The level of calcein accumulation is only moderately sensitive to the presence of high concentrations of microbial hydrocarbon metabolites (2.2ppm PAH = 5.0%BWSF), that have been previously shown to increase rhodamine dye accumulation in *Urechis*. Thus, calcein may be extruded by a transporter that is unique from the echiuroid xenobiotic transporter.

Figure 6 a. Western blot of total protein from immature sea star oocytes(lane 2), sea star eggs (lane 3) sea urchin egg (lane 4) and *Urechis* oocytes (lane 5) with the anti MDR antibody C-219. Lane 1 is a molecular weight standard. A low molecular weight isoform is detected in sea stars, and *Urechis*. The level of this band increases after starfish oocyte maturation. A band of the expected high molecular weight is only detected in *Urechis*. *b.* Western blot of membrane fractions from sea urchin eggs (lane 1), sea urchin larvae (lane 2) *Urechis* oocytes (lane 3), *Urechis* larvae (lane 4) and monkey liver (lane 5) with mAb C219. A number of high and low molecular weight bands are detected although in monkey liver (a putative positive control) the low molecular weight bands are labeled most specifically. A band of approximately 50kD (arrow) is also labeled in sea urchin and *Urechis* and its level seems to increase in larvae relative to eggs. 15μg of total protein were loaded in all lanes.

a.



b.

Asterina	RENILFGAPLQDAEYQRVIEACALAPDLDMLPAGDLTEIGEKGINLSGGQKQRVSLARAV
S. Purpuratus	KDNVLFGEKLRDSKYRKVIGACALNPDIDMLPAGDQTEIGEKGINLSGGQKQRVSVARAL
Rat	RENILFGRPLQEHCYKAVMEACALLPDLEILPSGDLTEIGEKGVNLSGGQKQRVSLARAV
Anopheles	KDNILFGKPMDQRRYARVIEACALKPDIEMLPGGDMTEIGEKGINLSGGQKQRVSLARAV
Raja	QDNILFGMKMEDSRYQEVLEACALPQDLELLPAGDLTEIGERGINLSGGQKQRVSLARAV
Consensus	**N LFG Y V ACAL P D LP GD TEIGE G NLSGGQKQRVS ARA**

Figure 1.

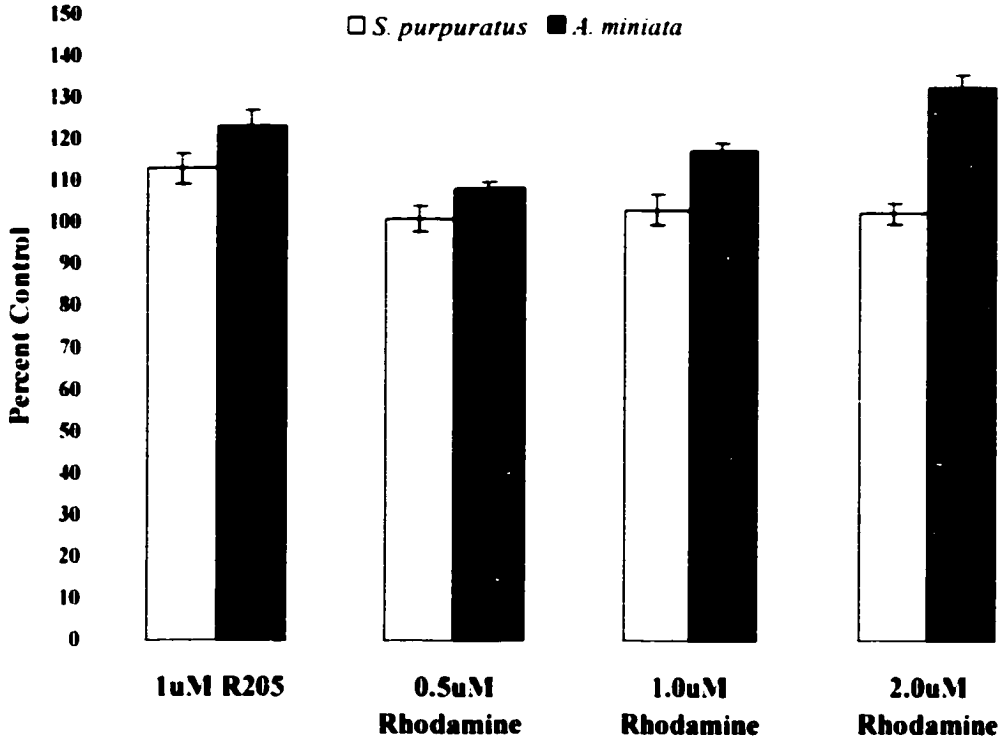


Figure 2.

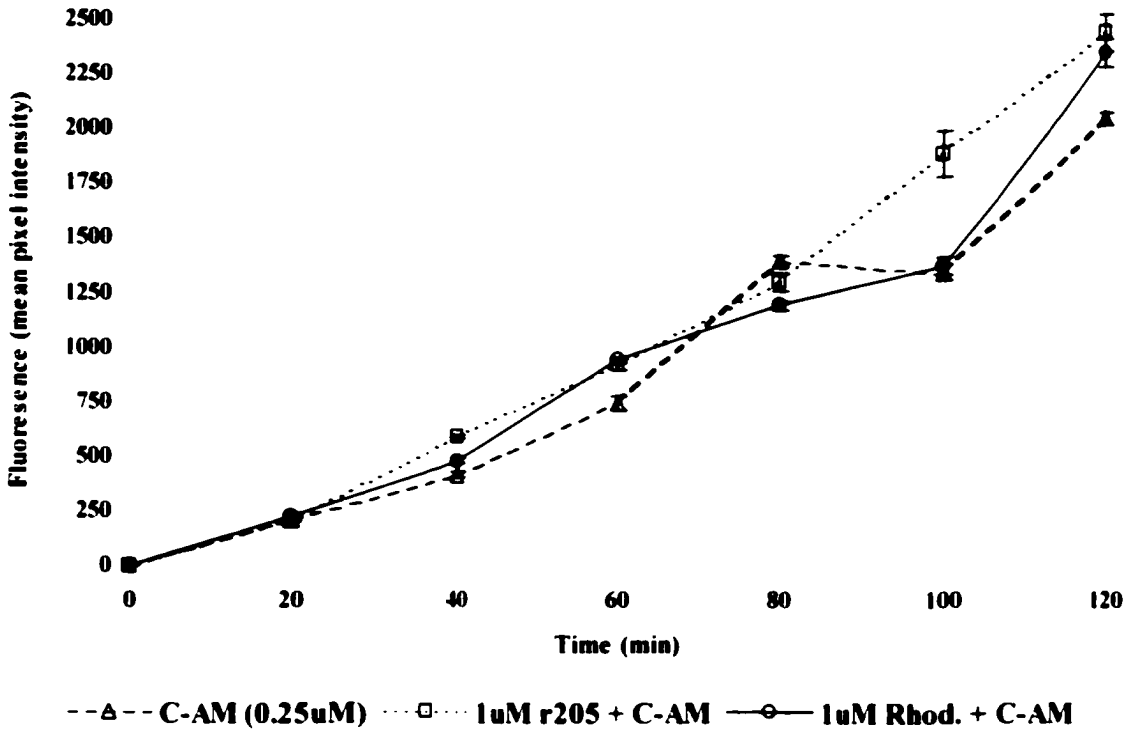
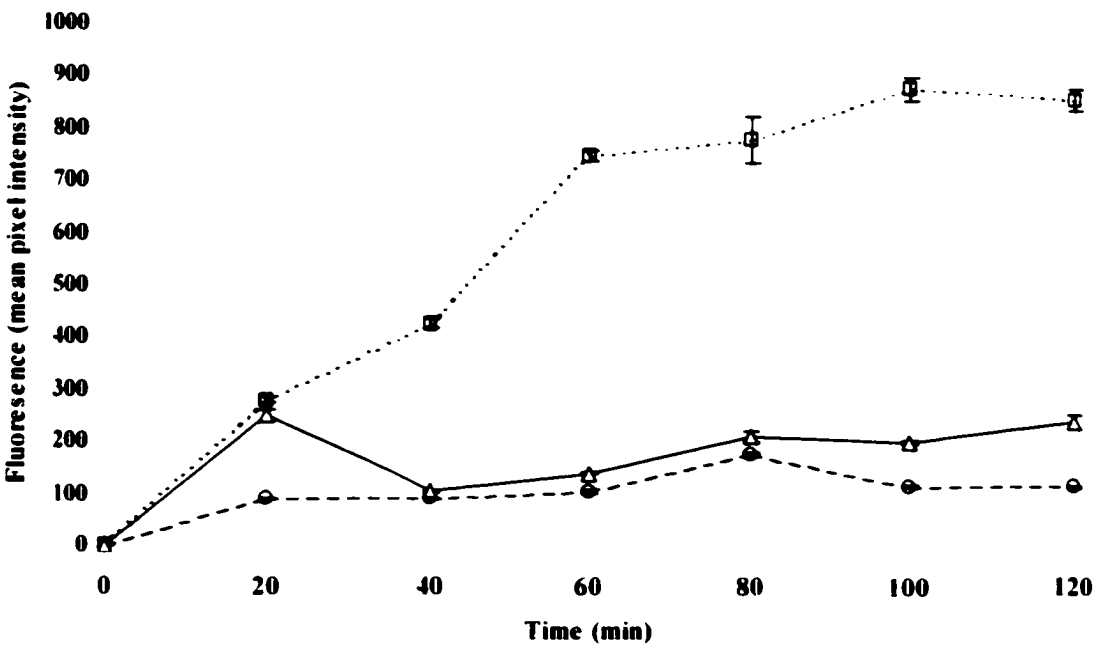


Figure 3 a. (above)
b. (below)



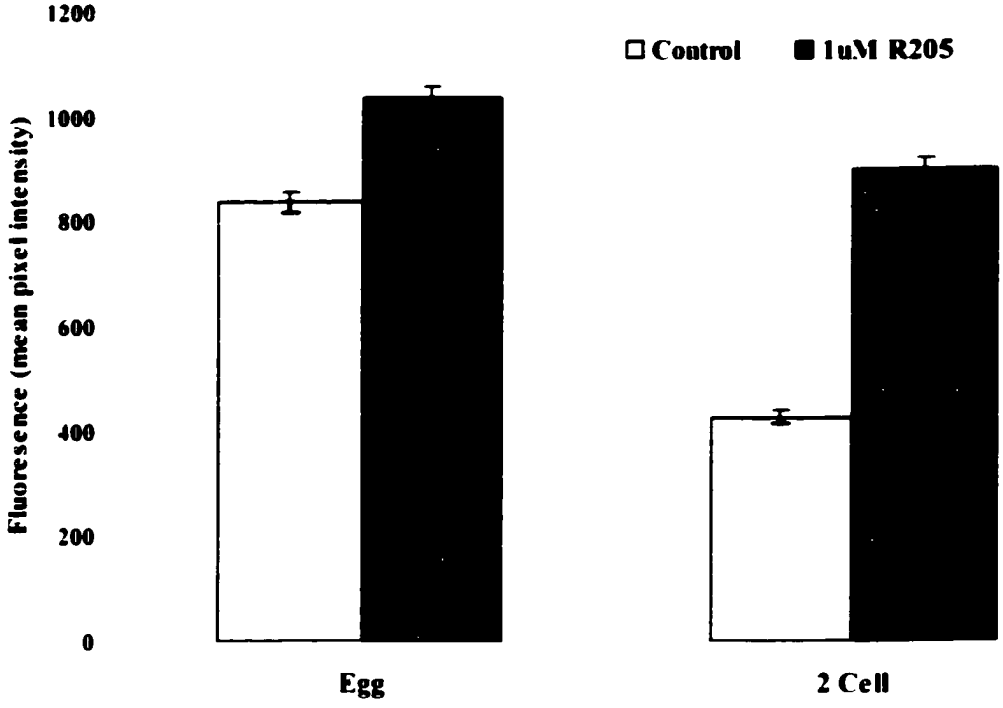


Figure 4.

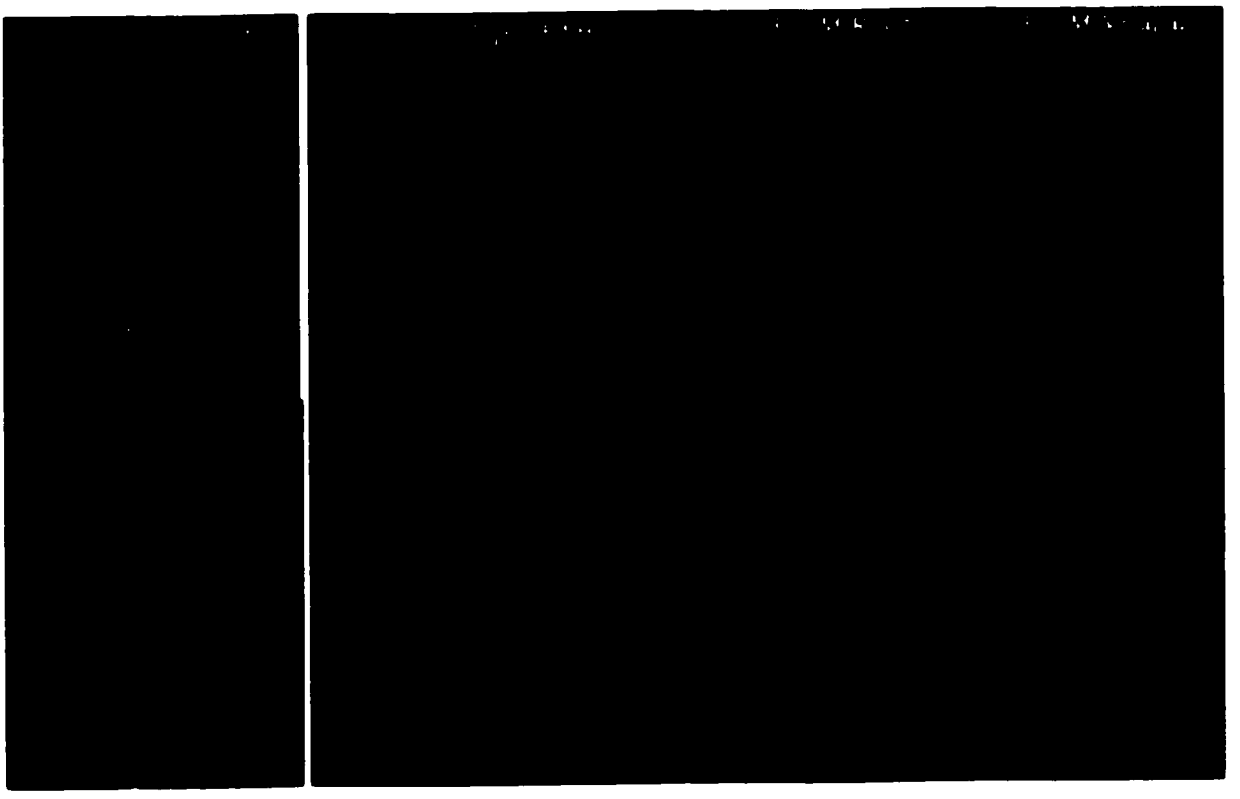
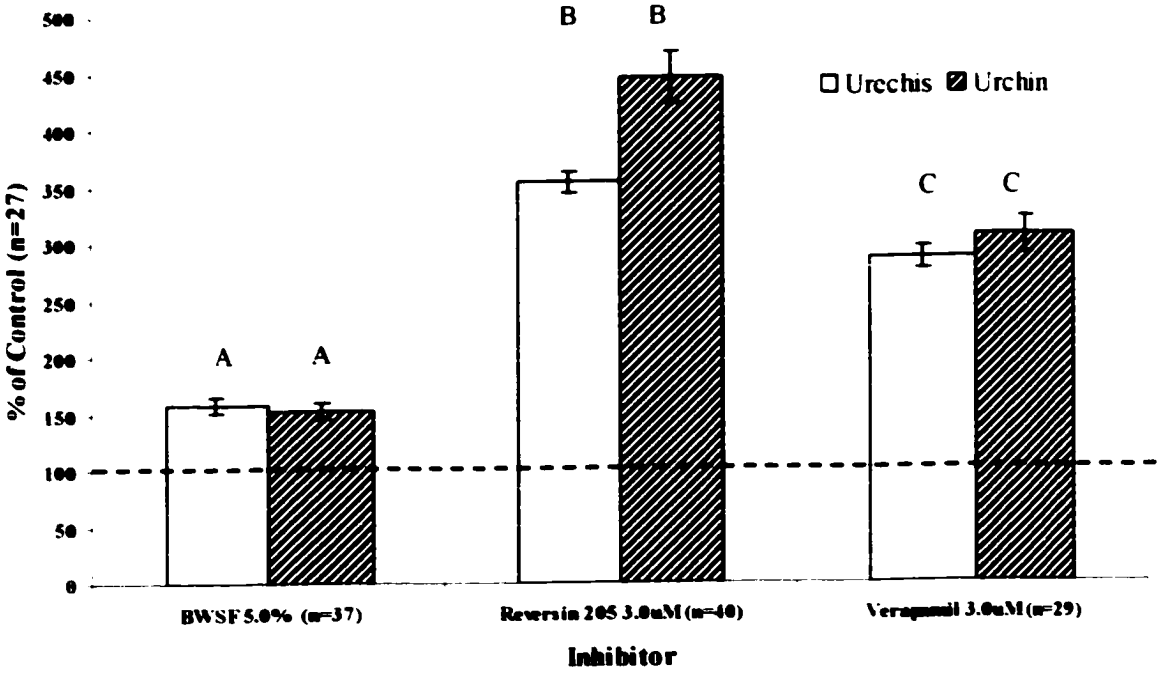


Figure 5a. (above) b. (below)



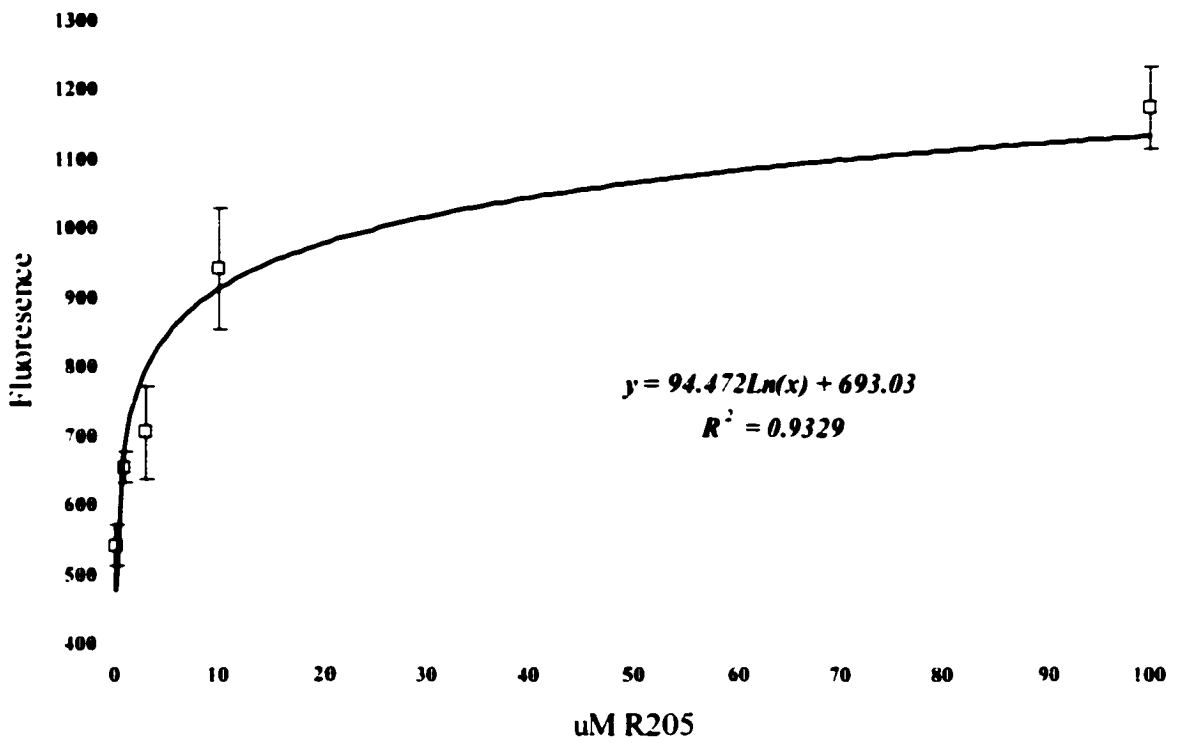


Figure 5c.

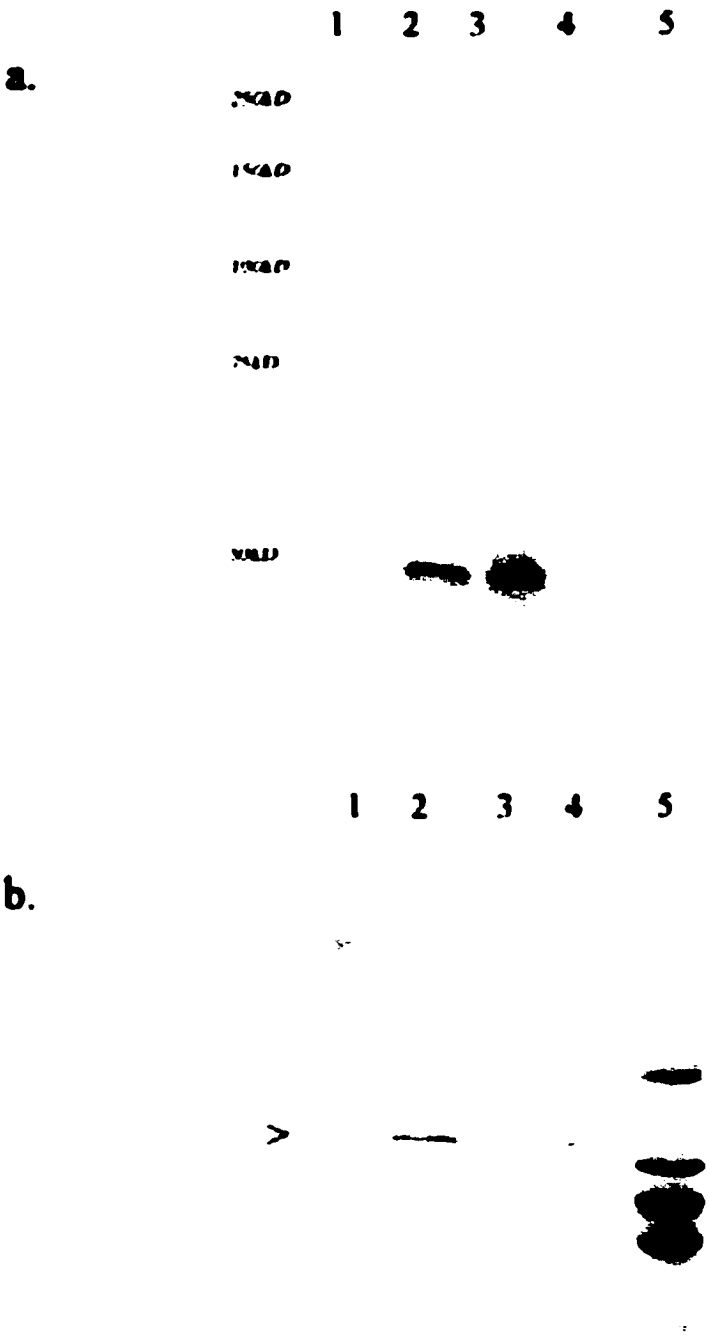


Figure 6

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