Phosphorylation Events Catalyzed by Major Cell Signaling Proteins Differ in Response to Thermal and Osmotic Stress among Native (*Mytilus californianus* and *Mytilus trossulus*) and Invasive (*Mytilus galloprovincialis*) Species of Mussels

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ABSTRACT

Sharp environmental gradients encountered within the intertidal zone have driven the evolution of physiological adaptations that allow its inhabitants to maintain cellular function in the presence of fluctuating abiotic factors. These adaptations are mediated by gene-regulatory networks that, despite their inherent complexity, must remain evolvable and capable of responding to different selection pressures associated with specific ecological niches. Phosphorylation events catalyzed by cell-signaling enzymes represent a parsimonious mechanism to integrate new functional or regulatory properties into these gene-regulatory networks. In this study, proteins phosphorylated on consensus sequences for protein kinases A, B, and C; cyclin-dependent kinases; and mitogen-activated protein kinases, as well as the abundance of phosphorylated stress-activated protein kinase (phospho-SAPK/JNK), were quantified in order to ascertain whether phosphorylation events are divergent among native (*Mytilus californianus* and *Mytilus trossulus*) and invasive (*Mytilus galloprovincialis*) species of mussels that differ in their tolerance toward environmental stress. Abundances of phosphorylated substrate proteins for each of the major signaling proteins that were investigated, as well as the abundance of phospho-SAPK/JNK, differed both within and between species during thermal and osmotic stress. These data suggest that modulating protein function via phosphorylation may be an important mechanism to integrate novel properties into stress-regulatory networks. In turn, differential phosphorylation during environmental stress may contribute to species-specific tolerances toward abiotic stress, interspecies dynamics, and biogeographic patterns in *Mytilus* congeners.

Introduction

Abiotic factors such as temperature and osmolality are critical in governing the structures and functions of marine intertidal communities (Tomanek and Helmuth 2002). As a result, physiological and/or biochemical adaptations that promote cellular function in the presence of changing abiotic conditions have become fundamental components of intertidal lifestyles (Hochachka and Somero 2002). Interplay between the abiotic environment and the functional limits of these adaptive responses appear to regulate the distribution of intertidal species, where differential tolerances toward the exigencies of specific habitats create the conspicuous biogeographic patterns within intertidal zones (Hochachka and Somero 2002; Somero 2002; Tomanek and Somero 2002; Denny and Harley 2006; Denny et al. 2006; Helmuth 2006).

Mussels of the genus *Mytilus* are important components of temperate marine intertidal communities, representing some of the most abundant invertebrate species in these assemblages and playing important roles that govern overall species composition (Ricketts et al. 1962). The Pacific coast of North America is occupied by three species of *Mytilus* mussels: the ribbed mussel *Mytilus californianus* Conrad and the blue mussels *Mytilus galloprovincialis* Lamarck and *Mytilus trossulus* Gould. Habitat characteristics of these closely related *Mytilus* congeners vary widely and include wave-exposed rocky shores and relatively protected estuarine bays and sloughs (Braby and Somero 2006*a*, 2006*b*). For example, native *M. californianus* mussels are competitively dominant on exposed rocky shores, where thermal regimes are variable and the mussels' more physically robust shells provide protection against wave action and predation (Bayne et al. 1976). Although *M. galloprovincialis* and *M. trossulus* have been collected at rocky intertidal sites (Sarver and Foltz 1993; Rawson et al. 1996, 1999; Suchanek et al. 1997), abundances at these sites are limited by tolerances toward extreme abiotic stresses such as temperature and mechanical

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forces, as well as by biotic factors that include preferential predation by the abundant *Nucella* spp. whelks (which exhibit a strong preference for blue mussels over ribbed mussels) and by low recruitment rates in California relative to Oregon (Connolly et al. 2001). Conversely, *M. californianus* is presumed to be competitively excluded from protected bays, estuaries, and marinas by its lower tolerance to osmotic gradients, vulnerabilities to siltation and suffocation, and early life-history factors that limit larval settlement (Heath et al. 1996).

Protected coastal sites along the North Pacific are instead dominated by the blue mussels *M. trossulus* and *M. galloprovincialis*, which inhabit estuarine environments characterized by naturally occurring salinity and temperature changes. Historical records suggest that the northeastern Pacific native *M. trossulus* was once abundant within these habitats along much of the coastline (Geller 1999). However, current distributions are mostly limited to central California and northward (Braby and Somero 2006*a*; Dutton and Hofmann 2008). This biogeographical shift has been attributed to the introduction of the nonnative European blue mussel *M. galloprovincialis*, a vigorous coastal invader native to the Mediterranean Sea, where habitats are characterized by warmer water temperatures, higher salinity, lower-magnitude tidal fluxes, and less seasonal variation than is found in the northeastern Pacific. As a reflection of this evolutionary history, *M. galloprovincialis* has evolved a warm-adapted physiology that allows it to competitively displace *M. trossulus* along its warmer southern range, from Baja California, Mexico, north to central California (Braby and Somero 2006*b*; Fields et al. 2006). Conversely, *M. trossulus* appears to be less tolerant of high temperatures and more tolerant of low temperatures and variable salinities, conferring a competitive advantage in higher latitudes and strongly estuarine environments (Sarver and Foltz 1993; Gardner 1994; Rawson et al. 1996, 1999; Suchanek et al. 1997). All physiological and biochemical comparisons of the native and invasive blue mussels published to date show the latter species to be more warm adapted (heart function: Braby and Somero 2006*b*; enzyme kinetics: Fields et al. 2006; and heat shock response: Hofmann and Somero 1995; Buckley et al. 2001; Halpin et al. 2002) but less tolerant of reduced salinity (Braby and Somero 2006*b*).

Habitat and physiological characteristics of *Mytilus* spp. strongly suggest that differential tolerance toward temperature and salinity are likely the dominant forces governing interspecies dynamics, and they imply that physiological adaptations limited to a discrete range of thermal and osmotic conditions may dictate their sympatric existence along the northeastern Pacific coast. In sessile organisms like *Mytilus* spp., modulating gene expression represents one of the most rapid and versatile responses available to mitigate the effects of environmental stress (Gracey and Cossins 2003; Gracey et al. 2008). Adaptive shifts in gene expression typically terminate at so-called "effector" proteins, which work synergistically to actively restore cellular homeostasis (Fiol and Kültz 2007; Evans and Somero 2008). However, the ability to actively regulate effector-protein expression during environmental stress is ultimately dependent on upstream environmental stress sensors and signal transducers, which relay molecular messages to specific effector molecules. As a result, adaptive responses are contingent on the systematic actions of sensors, signal transducers, and effectors (Fiol and Kültz 2007; Evans and Somero 2008, 2009), and they have evolved into complex, multicomponent regulatory networks (Hartwell et al. 1999; Feder 2007; Singh et al. 2008). However, the appearance of different ecological lifestyles, even among closely related species such as *Mytilus* congeners in the northeastern Pacific, suggest that despite this inherent complexity, the networks underlying stress adaptation have remained evolvable and capable of responding to different selection pressures associated with specific ecological niches (Singh et al. 2008).

Recent analyses of stress-regulatory network structure indicate that most of the variation in environmentally responsive gene networks (in both gene sequence and gene content) lies in the sensing and signal-transduction genes that operate at the onset of stress (Singh et al. 2008). Phosphorylation events that alter the functional properties of existing cellular proteins are integral to stress sensing and signal transduction. Consequently, altering the phosphorylation states of key proteins may be important in moderating evolutionary selection pressures and integrating novel adaptive mechanisms into stress-regulatory networks. Despite these conjectures, only modest attention has been directed toward characterizing such changes during stress sensing and signal transduction. To address this issue, we investigated the role of phosphorylation during thermal and osmotic stress responses in *M. californianus*, *M. trossulus*, and *M. galloprovincialis*, using a series of phosphospecific antibodies. Prominent are the novel application of substrate-specific antibodies, which react to phosphorylated amino acid consensus sequences optimized toward major cell-signaling proteins and allow the quantification of total phosphorylation levels (the net result of kinase-catalyzed phosphorylation and phosphatase-mediated dephosphorylation) on multiple target proteins. By comparing phosphorylation levels during both thermal and osmotic stress in *Mytilus* congeners, we gain insight into how environmental stress is differentially sensed and transduced within these organisms and how phosphorylation may affect larger-scale downstream cellular outcomes that likely underlie species-specific tolerances toward thermal and osmotic stress.

Material and Methods

Specimen Collection and Acclimation

Mytilus californianus (average length ± SE = 60 ± 3 mm) were collected from mussel beds in the exposed rocky intertidal zone at Hopkins Marine Station, Pacific Grove, California (36°37′N, 121°54′W). *Mytilus trossulus* (average length ± SE = 53 ± 3 mm) were collected from dock pilings in Yaquina Bay, Newport, Oregon (44°38′N, 124°03′W). *Mytilus galloprovincialis* (average length ± SE = 58 ± 3 mm) were collected from pilings in Mission Bay, San Diego, California (32°46′N, 117°14′W). *Mytilus trossulus* and *M. galloprovincialis* individuals

were shipped to Hopkins Marine Station in insulated boxes wrapped loosely in net bags containing kelp. All three species were maintained without emersion in 400-L aquaria held at 14°C and containing recirculating seawater pumped from Monterey Bay at a salinity of 31 parts per thousand (ppt) for at least 28 d before experimentation. During acclimation, mussels were fed diluted shellfish diet (Reed Mariculture, Campbell, CA) as per the manufacturer's instructions.

Thermal and Osmotic Stress Exposures

Following the acclimation period, 36 mussels from each species were transferred to an exposure aquarium. To eliminate the effects of handling stress, these mussels were once again allowed to acclimate to ambient Monterey Bay seawater (14°C, 31 ppt) for at least 4 d in the exposure aquarium before treatment. Six time-zero (control) mussels from each species were removed before the exposures were initiated and were immediately flash frozen in liquid nitrogen. Thermal stress exposures were initiated by cutting the flow of recirculating Monterey Bay seawater and subsequently pumping aquarium water through heaters programmed to increase water temperatures by 5.5°C h^{-1}, a natural heating rate for the intertidal zone at this latitude (Denny et al. 2006; Dong et al. 2008). Six individual mussels from each species were removed as the water reached the desired sampling temperature. Mussels were sampled at five ecologically relevant temperatures: 20°, 24°, 28°, 32°, and 36°C (Denny et al. 2006) and were then immediately flash frozen in liquid nitrogen. Mortality during exposures was scored if mussels failed to close their shells after external stimulation. Mussels that did not display this obvious sign of mortality were considered to be viable and were used in subsequent protein extractions. Mortality rates were 0%, 0%, and 6% of the total number of mussels killed in *M. californianus*, *M. galloprovincialis*, and *M. trossulus*, respectively. Mortality in *M. trossulus* was restricted to two individuals in the 28°C exposure group.

Hypoosmotic stress exposures were performed concurrently with thermal stress exposures in an identical but separate exposure aquarium containing 12 mussels from each species. As in the thermal stress exposures, mussels were allowed to acclimate to ambient Monterey Bay seawater (14°C, 31 ppt) for at least 4 d in the exposure aquarium before treatment. To initiate hypoosmotic stress, the flow of recirculating Monterey Bay seawater was halted and an equal volume of distilled water was dripped into the exposure aquarium at a rate of 1.3 L h^{-1}, resulting in a decrease in salinity of approximately 3 ppt h^{-1}. Mixing of distilled water and seawater was achieved using standard aquarium pumps. Salinity was monitored using refractometry and was decreased in a linear fashion over the course of the exposure. Temperature was maintained at 14°C throughout the hypoosmotic stress exposure by placing the exposure aquarium in a large water bath containing recirculating seawater from Monterey Bay. Six individual mussels from each species were removed as the water reached the desired salinity. Mussels were sampled at the ecologically relevant salinities of 23 ppt (75% seawater) and 15 ppt (50% seawater; Stickle and Denoux 1976; Shumway 1977) and were immediately flash frozen in liquid nitrogen. Mortality rates during hypoosmotic stress exposures were 6%, 0%, and 6% in *M. californianus*, *M. galloprovincialis*, and *M. trossulus*, respectively. Mortality was attributed to one individual in the 23-ppt exposure group for *M. californianus* and one individual in the 23-ppt exposure group for *M. trossulus*.

Tissue Dissection and Protein Extraction

Gill tissue was used exclusively in this study because it has been previously shown to be highly responsive to thermal and osmotic stress in mussels, denoting the important role of gills in maintaining organismal homeostasis (Hofmann and Somero 1995; Neufield and Wright 1996; Hofmann 2005; Gracey et al. 2008). Partially thawed gill tissue was dissected and placed into ice-cold homogenization buffer (32 mM Tris-Cl pH 6.8, 2% SDS, 1 mM EDTA with protease inhibitors [complete Mini, Roche Applied Science, Indianapolis, IN]). Dissected tissue was homogenized using a TissueLyzer (Qiagen, Valencia, CA) for 2 min at 25 strokes s^{-1}. Homogenates were then heated for 5 min at 100°C and centrifuged at 12,500 rpm for 10 min. Pellets were discarded, and the total protein content in the soluble fraction was determined by Pierce BCA protein assay (Pierce, Rockford, IL). Samples with total protein concentrations that were too low to allow detection of specific proteins were not subjected to further analysis. However, each sampling point from both the thermal and the osmotic stress exposures had at least four individuals from each species, with the exception of the control group for *M. trossulus* in the phospho-SAPK/JNK experiments, which contained three individuals (overall mean, $n = 5.5$; mode, $n = 6$).

Antibodies

A total of seven primary antibodies were employed in this study, including five substrate antibodies that react specifically to phosphorylated residues occurring within a consensus sequence optimized toward major signaling enzymes. The phosphoserine/threonine (Ser/Thr) protein kinase B (Akt)–substrate antibody (Cell Signaling Technologies 9611, Danvers, MA) preferentially recognizes peptides containing a phosphorylated Ser or Thr residue preceded by a lysine (Lys) at amino acid position −5 and an arginine (Arg) at amino acid position −3, relative to the phosphorylation site. The phospho-(Ser/Thr) protein kinase A (PKA)–substrate antibody (Cell Signaling Technologies 9621) detects peptides containing a phosphorylated Ser/Thr with an Arg residue at the −3 position. The phospho-(Ser) protein kinase C (PKC)–substrate antibody (Cell Signaling Technologies 2261) detects cellular proteins phosphorylated at Ser residues surrounded by Lys or Arg at the −2 and +2 positions and a hydrophobic residue at the +1 position. The phospho-(Ser) cyclin-dependent kinase (CDK)–substrate antibody (Cell Signaling Technologies 2324) recognizes phosphorylated Ser residues within the consensus sequence Lys/Arg-Ser-Proline (Pro)-X-Lys/Arg, whereby X denotes any of the 20

amino acids. Finally, the phospho-(Thr) mitogen-activated protein kinase (MAPK)–substrate antibody (Cell Signaling Technologies 4391) detects phosphorylated Thr residues within the consensus sequence Pro-X-Ser/Thr-Pro. None of the substrate antibodies described above cross-react with nonphosphorylated consensus sequences. All substrate antibodies were diluted 1 : 1,000 in the working solution. In addition to these substrate antibodies, a phosphorylated stress-activated protein kinase/c-Jun-amino terminal kinase (phospho-SAPK/JNK; Cell Signaling Technologies 9255) antibody was also employed. The SAPK/JNK antibody was diluted 1 : 500 in the working solution. A β-actin antibody (sc-47778; Santa Cruz Biotechnology, Santa Cruz, CA) was used to demonstrate that the observed expression patterns were not a reflection of differences in total protein.

Western Blot Analysis

Total protein (30 ng) was diluted in 1 × Laemmli sample buffer (Bio-Rad, Hercules, CA), heated for 5 min at 100°C, and loaded onto precast 10% Tris-HCl polyacrylamide gels (Bio-Rad). Electrophoretically separated proteins were wet transferred to nitrocellulose membranes for 2 h at 4°C. Resulting blots were blocked for 1 h in 5% blocking-grade nonfat dried milk (Bio-Rad) dissolved in Tris buffered saline (250 mM Tris-Cl pH 7.5, 1.5 M NaCl) containing 0.1% Tween-20 (TBST), washed for 2 × 5 min in TBST, and incubated in the primary antibody diluted in 5% bovine serum albumin (BSA) in TBST. Following 3 × 5-min washes in TBST, blots were incubated in the secondary antibody (either goat antirabbit [sc-2004] or goat antimouse [sc-2055]; Santa Cruz Biotechnology). All secondary antibodies were diluted 1 : 5,000 in 5% BSA in TBST and incubated for 60 min at room temperature with gentle agitation. Following 6 × 5-min washes in TBST, blots were treated with enhanced chemiluminescent reagent (Amersham, Piscataway, NJ) for 2 min. Finally, blots were exposed to film (Blue Lite Auto Rad F-9024, ISC BioExpress, Kaysville, UT) and developed. Representative Western blots are available as supplemental online materials (appendix).

Densitometric analyses were performed using ImageJ software (http://rsb.info.nih.gov/ij/). For substrate antibodies, density was calculated as the cumulative intensity of multiple bands (with each band representing a different phosphorylated target protein) in a single lane, multiplied by the total area of that lane. For the phospho-SAPK/JNK antibody, density was calculated as the intensity of the band at 46 kDa multiplied by the area of that band. Relative intensity values were calculated by normalizing samples against the average density of a protein standard loaded twice onto each gel and used repeatedly in all experiments. Statistical significance of densitometric data was determined by two-way ANOVA with temperature and species or salinity and species as factors. A least significant difference (LSD) post hoc test ($P < 0.05$) was used to resolve statistically significant differences between treatment groups or species. Expression data were either log or square-root transformed in order to meet the assumption of homoscedasticity (Levene's test $P < 0.05$). Despite transformation, expression data using the MAPK-substrate antibody remained significantly heteroscedastic. Five outlier individuals from this dataset were subsequently identified and removed in order to meet the assumption of homoscedasticity when performing the ANOVA.

Results

Net levels of phosphorylated targets for major cell-signaling proteins (PKA, Akt, PKC, MAPKs, and CDKs; Figs. 1–5), as well as the abundance of phospho-SAPK/JNK (Fig. 2*B*, 2*D*), were all responsive to environmental stress in the three species of *Mytilus*. During thermal stress, significant differential phosphorylation ($P < 0.05$) was observed for at least one temperature sampling point in all three species across all five substrate antibodies. Statistically significant differences in phosphorylation were manifested both within a single species, as determined by deviation from controls (time = 0) at various sampling points, and between species, as determined by species-specific differences in magnitude at particular stress levels. Phosphorylation levels at 36°C, which is a temperature that likely exceeds the lethal limits for all three *Mytilus* congeners following 4 h of total thermal stress exposure, were generally reduced.

Significant effects on levels of phosphorylated proteins were observed in five of the six proteins analyzed during hypoosmotic stress in *Mytilus* spp. The exception, phospho-SAPK/JNK, did not respond significantly to osmotic stress in any of the three species. Nonlinear regression analyses demonstrated that mussel length did not significantly affect the expression of any protein investigated in this study (data not shown; *F*-test $P < 0.05$), and they suggest that the gene-expression patterns observed here are a reflection of species-specific responses to the environment rather than biases in the size distribution of mussels.

Protein Kinase B (Akt)

The Akt signaling pathway is recognized as a critical regulator of cell survival, acting through multiple substrates as a potent inhibitor of apoptosis. Promoting cell survival during environmental stress is likely a key endpoint of adaptive networks in *Mytilus* spp. To gain insight into the role of Akt signaling in *Mytilus* congeners during environmental stress, we employed an Akt-substrate antibody, which detects proteins that are phosphorylated on Akt consensus sequences, and we quantified these events via immunoblotting (Fig. 1). Levels of Akt-substrate phosphorylation in controls did not differ significantly between species, indicating that steady state levels of phosphorylation during nonstress conditions are comparable. However, in mussels exposed to thermal stress, the abundance of Akt-phosphorylated substrates varied significantly both within and between species. Akt-substrate phosphorylation differed significantly from controls at all five temperatures inves-

tigated in all three species. Peak phosphorylation levels in the more heat-tolerant *Mytilus californianus* and *Mytilus galloprovincialis* occurred at 24°C, and there they exhibited a much greater magnitude than did the more cold-tolerant *Mytilus trossulus*, whose peak occurred at 28°C and was only about one-half to two-thirds as great as the responses observed in its two congeners at 24°C (Fig. 1*A*).

During hypoosmotic stress, the abundance of Akt-phosphorylated substrates differed within a single species but did not differ significantly between species. All three species exhibited similar and statistically significant increases at the more severe osmotic stress level of 15 ppt (Fig. 1*B*).

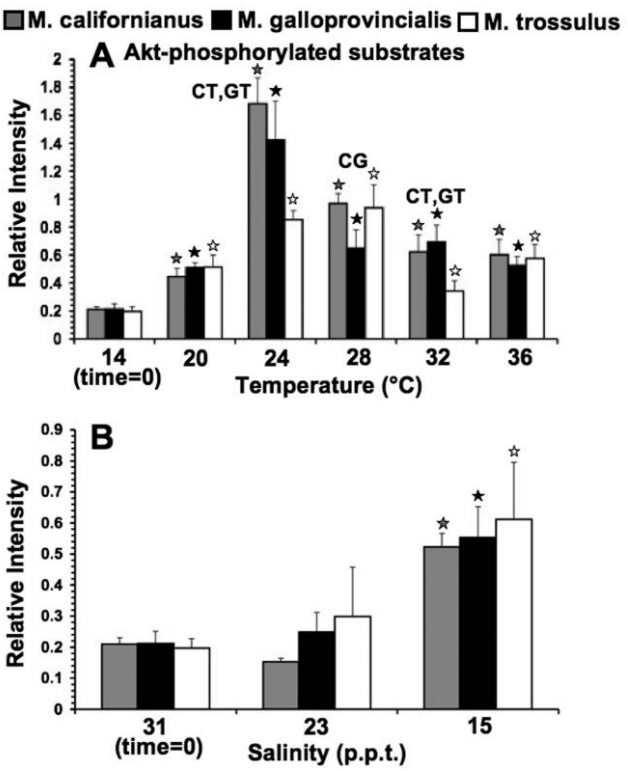

Figure 1. Levels of protein kinase B (Akt)–substrate phosphorylation in *Mytilus* spp. during environmental stress. Relative intensity of Akt-phosphorylated substrates in *Mytilus californianus* (*gray bars*), *Mytilus galloprovincialis* (*black bars*), and *Mytilus trossulus* (*white bars*) during thermal (*A*) and osmotic (*B*) stress as detected by an Akt substrate–specific antibody and immunoblotting. Statistical significance was determined by two-way ANOVA with a least significant difference post hoc test ($P < 0.05$). Stars denote statistical significance within a given species relative to controls (time = 0). Letters denote statistical significance between species, whereby CG indicates significance between *M. californianus* and *M. galloprovincialis*, CT indicates significance between *M. californianus* and *M. trossulus*, and GT indicates significance between *M. galloprovincialis* and *M. trossulus*. Relative intensity values are shown + SE.

Mitogen-Activated Protein Kinases (MAPKs)

MAPK cascades are essential for adaptive responses to environmental stress, and they regulate major cellular endpoints such as cell proliferation and apoptosis (Kültz and Burg 1998). Steady state levels of MAPK-phosphorylated substrates were elevated in the heat-tolerant *M. galloprovincialis* relative to the more thermally sensitive *M. trossulus* (Fig. 2*A*, 2*C*). During thermal stress, peak phosphorylation levels in all three species were ultimately of similar magnitude; however, these levels were achieved at the 20°C temperature point in *M. galloprovincialis* and *M. trossulus*, while *M. californianus* did not reach this level until 28°C. MAPK-substrate phosphorylation in the most thermally sensitive congener, *M. trossulus*, was significantly depressed relative to that of the more heat-tolerant species *M. californianus* at 28°C and *M. galloprovincialis* at 28° and 32°C (Fig. 2*A*). This pattern was also reflected in the expression of phospho-SAPK/JNK, a MAPK-family signaling protein (Fig. 2*B*, 2*D*). SAPK/JNK is activated by many types of cellular stress and functions to regulate a variety of cellular processes, including cell proliferation, differentiation, and apoptosis. Abundance of phospho-SAPK/JNK differed significantly in two of the three species, but as with MAPK-substrate proteins, expression of phospho-SAPK/JNK in *M. trossulus* was significantly reduced at the 32°C temperature point relative to that of the more heat-tolerant *M. galloprovincialis* (Fig. 2*B*).

Exposure to hypoosmotic stress significantly upregulated MAPK-dependent phosphorylation of substrate proteins in both *M. californianus* and *M. trossulus*, while expression in *M. galloprovincialis* remained unchanged relative to controls at both salinity points (Fig. 2*C*). The most osmotic stress–tolerant species, *M. trossulus*, was the only congener in which MAPK phosphorylation was significantly elevated at both 23 and 15 ppt. Phosphorylation levels in *M. trossulus* at 23 ppt were also significantly higher than they were in *M. galloprovincialis* and *M. californianus*, which are presumed to be more sensitive to osmotic stress than *M. trossulus*. Unlike during thermal stress, phospho-SAPK/JNK abundance did not correlate with levels of MAPK phosphorylation during osmotic stress, and phospho-SAPK/JNK abundance did not significantly differ within or between any species at the salinities investigated (Fig. 2*D*).

Cyclin-Dependent Protein Kinases (CDKs)

CDKs regulate cell-cycle transitions by interacting with and subsequently phosphorylating proteins that drive progression through various phases of the cell cycle (Malumbres and Barbacid 2009). Changes in proliferation and differentiation can be important to environmental stress adaptation, but they may also affect longer-term processes such as growth, which has been shown to impact interspecies dynamics in *Mytilus* spp. (Schneider 2008). Therefore, quantifying changes in the phosphorylation status of CDK substrates during environmental stress in *Mytilus* spp. may reveal not only trends that contribute to differential stress tolerances but also larger-scale interspecific differences in growth. During thermal stress, significant differences in the phosphorylation status of CDK substrates occurred in all three species (Fig. 3*A*). Levels of CDK-substrate phos-

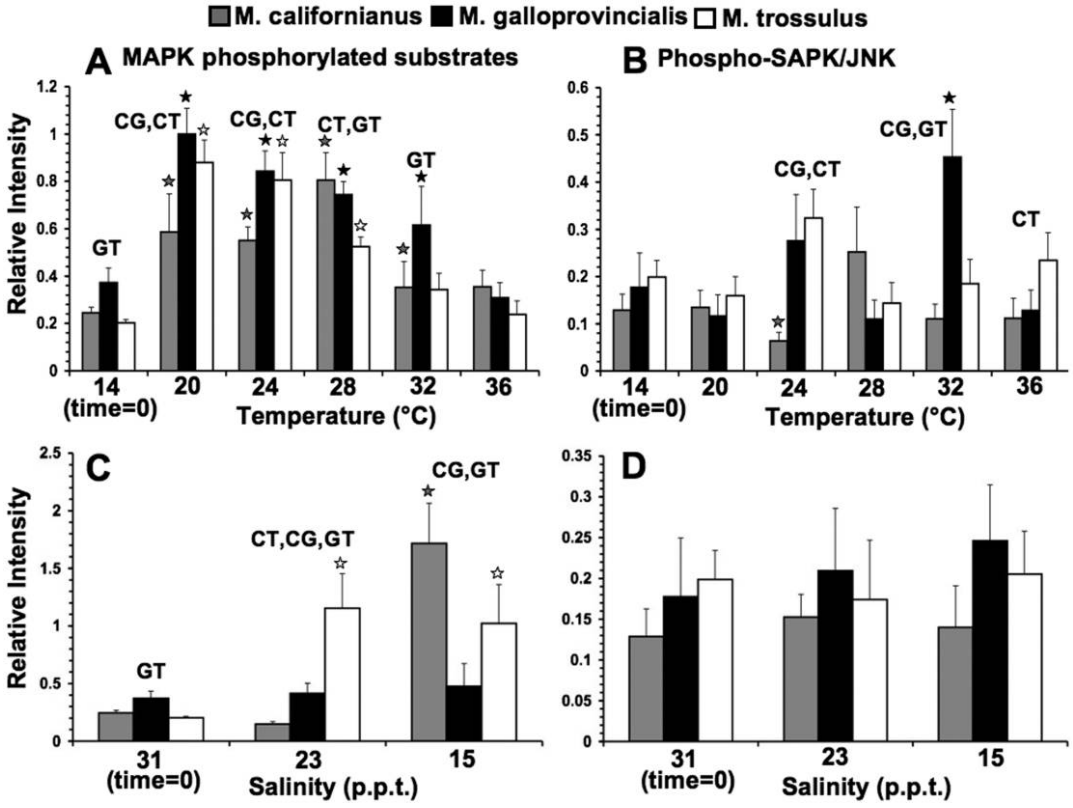

Figure 2. Levels of mitogen-activated protein kinase (MAPK)–substrate phosphorylation in *Mytilus* spp. during environmental stress. Relative intensity of MAPK-phosphorylated substrates (*A*, *C*) and phospho-SAPK/JNK (*B*, *D*) in *Mytilus californianus* (*gray columns*), *Mytilus galloprovincialis* (*black columns*), and *Mytilus trossulus* (*white columns*) during thermal (*A*, *B*) and osmotic (*C*, *D*) stress as detected by immunoblotting. Statistical significance was determined by two-way ANOVA with a least significant difference post hoc test ($P < 0.05$). Stars denote statistical significance within a given species relative to controls (time = 0). Letters denote statistical significance between species, whereby CG indicates significance between *M. californianus* and *M. galloprovincialis*, CT indicates significance between *M. californianus* and *M. trossulus*, and GT indicates significance between *M. galloprovincialis* and *M. trossulus*. Relative intensity values are shown + SE.

phorylation increased dramatically at the 20°C temperature point in *M. trossulus* and were significantly elevated relative to *M. galloprovincialis* at 20°, 24°, and 28°C and to *M. californianus* at 20° and 24°C. In contrast, peak levels of CDK-substrate phosphorylation were not observed until 32°C in the more heat-tolerant *M. californianus* (Fig. 3*A*).

During osmotic stress, peak levels of CDK phosphorylation once again occurred in *M. trossulus* and differed significantly from controls at both 23 and 15 ppt. A similar pattern was observed for *M. californianus*. *Mytilus galloprovincialis* did not significantly modulate CDK-dependent phosphorylation in response to osmotic stress, and phosphorylation levels were significantly reduced relative to the more osmotic stress–tolerant congener *M. trossulus* at 15 ppt (Fig. 3*B*).

Protein Kinase A (PKA)

PKA is a cAMP second messenger–dependent enzyme that plays a major role in the regulation of metabolism. Steady state levels of PKA-phosphorylated substrates were significantly elevated in *M. galloprovincialis* relative to in *M. californianus* and *M. trossulus* (Fig. 4). All three species modified PKA phosphorylation during thermal stress (Fig. 4*A*). *Mytilus californianus* increased phosphorylation starting at 24°C, and phosphorylation levels remained significantly elevated through to 36°C. *Mytilus galloprovincialis* and *M. trossulus* exhibited similar PKA phosphorylation profiles during thermal stress, with the exception of at the 32°C sampling point, where the more heat-tolerant *M. galloprovincialis* displayed significantly elevated levels of phosphorylation relative to the more heat-sensitive *M. trossulus* (Fig. 4*A*).

Levels of PKA-phosphorylated substrates were also significantly different during osmotic stress in all three *Mytilus* congeners. PKA phosphorylation was significantly upregulated relative to controls in *M. californianus* at both 23 and 15 ppt. Peak levels in *M. galloprovincialis* occurred at 23 ppt, and phosphorylation levels in both *M. californianus* and *M. galloprovincialis* were significantly higher than the most osmotic stress–tolerant species at this salinity, *M. trossulus*. In contrast, *M. trossulus* did not display significantly elevated levels of PKA-

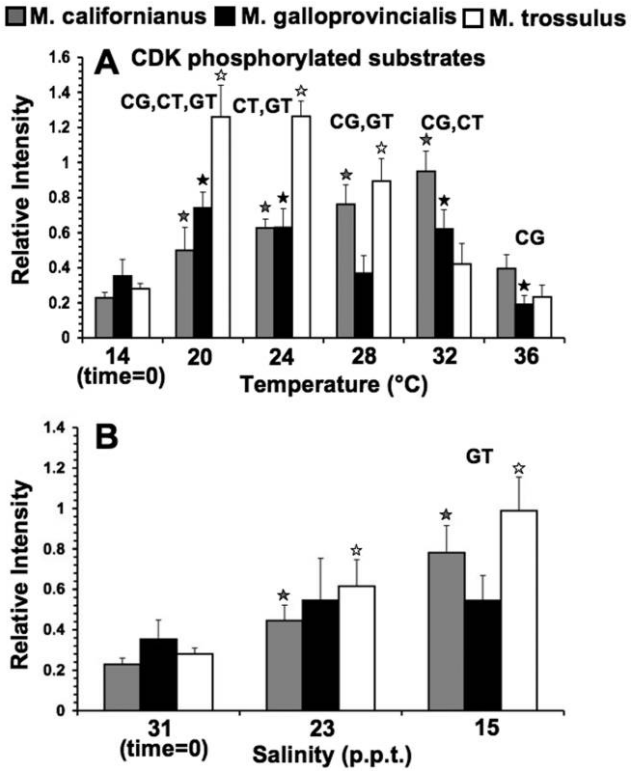

Figure 3. Levels of cyclin-dependent protein kinase (CDK)–substrate phosphorylation in *Mytilus* spp. during environmental stress. Relative intensity of CDK-phosphorylated substrates in *Mytilus californianus* (*gray bars*), *Mytilus galloprovincialis* (*black bars*), and *Mytilus trossulus* (*white bars*) during thermal (*A*) and osmotic (*B*) stress as detected by a CDK substrate–specific antibody and immunoblotting. Statistical significance was determined by two-way ANOVA with a least significant difference post hoc test ($P < 0.05$). Stars denote statistical significance within a given species relative to controls (time = 0). Letters denote statistical significance between species, whereby CG indicates significance between *M. californianus* and *M. galloprovincialis*, CT indicates significance between *M. californianus* and *M. trossulus*, and GT indicates significance between *M. galloprovincialis* and *M. trossulus*. Relative intensity values are shown + SE.

phosphorylated substrates until 15 ppt, and this response was of similar magnitude to the responses of *M. californianus* or *M. galloprovincialis* at this salinity (Fig. 4*B*).

Protein Kinase C (PKC)

PKC is a major cell-signaling intersection involved in a vast number of cellular processes, including modulating membrane structure, regulating transcription and cell growth, and governing immune responses (Mellor and Parker 1998). Some of these diverse functions may contribute to adaptive environmental stress networks in *Mytilus* spp. Steady state levels of PKC-phosphorylated substrates were markedly reduced in *M. galloprovincialis* relative to those of both *M. californianus* and *M. trossulus* (Fig. 5*A*). While *M. galloprovincialis* appeared to be able to modulate PKC activity in response to thermal stress at 24°, 28°, 32°, and 36°C, phosphorylation levels remained significantly depressed relative to those of *M. californianus* through to 24°C and those of *M. trossulus* at all but the 32°C temperature-sampling point. The phosphorylation levels of PKC substrates in *M. californianus* and *M. trossulus* were coordinate during thermal stress, with peak levels observed at 28°C and a subsequent reduction to near–steady state levels by 36°C (Fig. 5*A*). The coordinate expression of *M. californianus* and *M. trossulus* during thermal stress was also apparent during osmotic stress, where PKC-substrate phosphorylation dropped significantly by 23 ppt and remained reduced relative to controls at 15 ppt. In contrast to both *M. californianus* and *M. trossulus*, *M. galloprovincialis* increased PKC-substrate phosphorylation at 15 ppt (Fig. 5*B*).

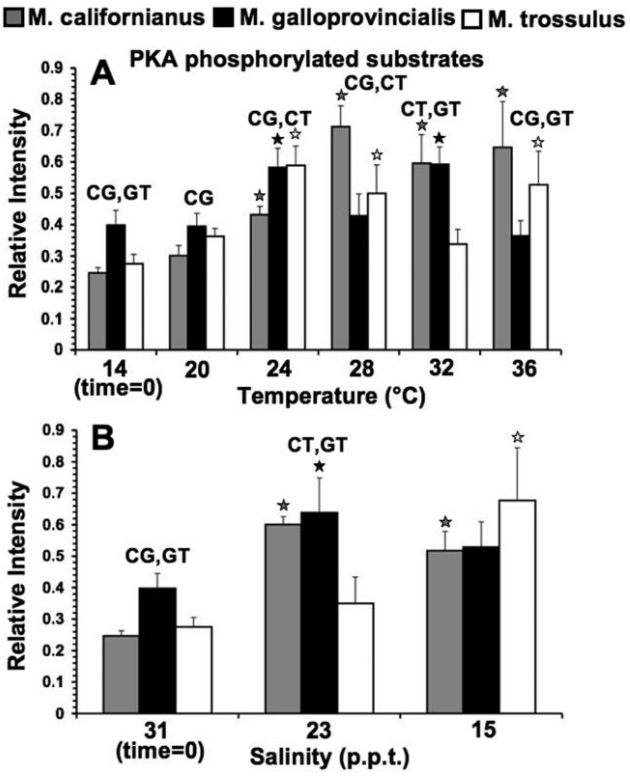

Figure 4. Levels of protein kinase A (PKA)–substrate phosphorylation in *Mytilus* spp. during environmental stress. Relative intensity of PKA-phosphorylated substrates in *Mytilus californianus* (*gray bars*), *Mytilus galloprovincialis* (*black bars*), and *Mytilus trossulus* (*white bars*) during thermal (*A*) and osmotic (*B*) stress as detected by a PKA substrate–specific antibody and immunoblotting. Statistical significance was determined by two-way ANOVA with a least significant difference post hoc test ($P < 0.05$). Stars denote statistical significance within a given species relative to controls (time = 0). Letters denote statistical significance between species, whereby CG indicates significance between *M. californianus* and *M. galloprovincialis*, CT indicates significance between *M. californianus* and *M. trossulus*, and GT indicates significance between *M. galloprovincialis* and *M. trossulus*. Relative intensity values are shown + SE.

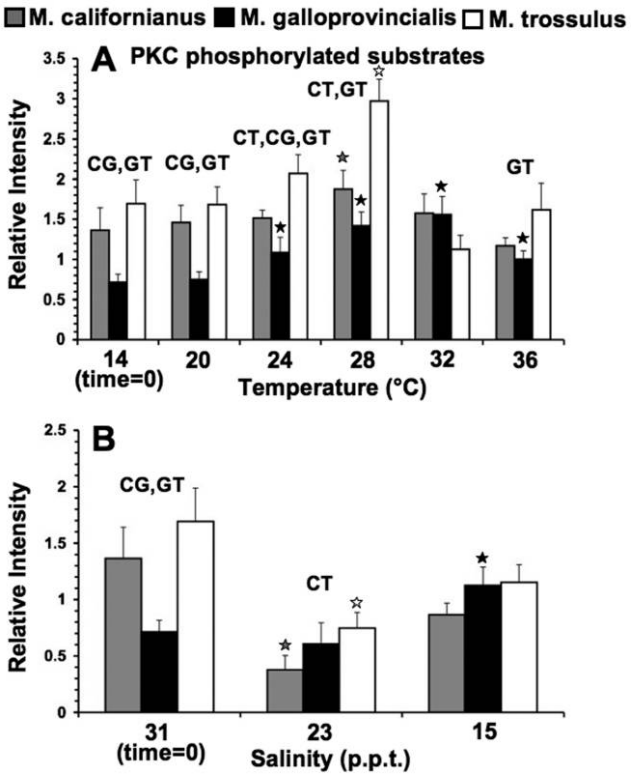

Figure 5. Levels of protein-kinase C (PKC)–substrate phosphorylation in *Mytilus* spp. during environmental stress. Relative intensity of PKC-phosphorylated substrates in *Mytilus californianus* (*gray bars*), *Mytilus galloprovincialis* (*black bars*), and *Mytilus trossulus* (*white bars*) during thermal (*A*) and osmotic (*B*) stress as detected by a PKC substrate–specific antibody and immunoblotting. Statistical significance was determined by two-way ANOVA with a least significant difference post hoc test ($P < 0.05$). Stars denote statistical significance within a given species relative to controls (time = 0). Letters denote statistical significance between species, whereby CG indicates significance between *M. californianus* and *M. galloprovincialis*, CT indicates significance between *M. californianus* and *M. trossulus*, and GT indicates significance between *M. galloprovincialis* and *M. trossulus*. Relative intensity values are shown + SE.

Discussion

Modifying existing cellular proteins through phosphorylation may represent a means to integrate novel functional properties into environmental stress–regulatory networks. To address this hypothesis, changes in phosphorylation levels (i.e., the net result of kinase-catalyzed phosphorylation and phosphatase-mediated dephosphorylation) were quantified during thermal and osmotic stress in three closely related species of *Mytilus* mussels. Net phosphorylation levels were determined by the application of substrate-specific antibodies, which provides a tractable approach to quantifying the effects of altered kinase or phosphatase activity during environmental stress. The validity of this approach has been demonstrated in a previous study showing that reductions in PKC mRNA in response to osmotic stress in a euryhaline fish are accompanied by a reduction in PKC-specific phosphorylation, as measured by the same PKC-substrate antibody used in this study (Evans and Somero 2008). Data presented in this study suggest that disparate phosphorylation may contribute to the sum of adaptive processes that constitute environmental stress responses in *Mytilus* spp. Environmentally regulated phosphorylation levels also differed significantly between species, suggesting that divergent phosphorylation of specific proteins may, at least in part, contribute to species-specific tolerances toward thermal and osmotic stress. Because biogeographic patterns within the intertidal zone are so tightly linked to the functional limits of these adaptive responses, the phosphorylation events reported in this study may in turn reinforce the biogeographic dynamics of *Mytilus* congeners inhabiting the northeastern Pacific.

Downstream Regulation of Larger-Scale Cellular Processes

While assigning specific cellular outcomes to the activities of the six multifunctional and interconnected proteins investigated in this study is difficult, the functional properties of most of the signaling proteins we investigated are skewed toward certain larger-scale cell processes. This information allows conjectures to be developed about the downstream effects of the changes in phosphorylation status that were observed in this study. Accordingly, we hypothesize that the differences in phosphorylation presented in this study serve to optimize the balance between maintaining cell survival, growth, and proliferation during environmental challenges and eliminating dysfunctional cells via apoptosis when stress thresholds have been breached (Gabai and Sherman 2002). Maintaining optimal growth rates across a variety of environmental conditions has clear selective advantages, and it likely plays a considerable role in the biogeographic patterning of intertidal species. For example, larval growth of *Mytilus galloprovincialis* was depressed relative to that of *Mytilus trossulus* when maintained at low salinities (Matson et al. 2003), while increased growth rates allow *M. galloprovincialis* to overgrow other mussel competitors during space limitation (Harger 1968; Hockey and Schurink 1992). As a second major outcome, PKA and PKC may be involved in the adjustment of cellular metabolism to cope with anoxic conditions following valve closure during moderate stress and to provide fuel to drive larger-scale adaptive responses that involve costly cellular processes, such as protein synthesis, at more severe stress levels. Finally, because the 36°C sampling point likely exceeds the lethal limit in all three species, the generally muted levels of phosphorylation at this extreme temperature may be indicative of "end of life" responses that are pathological rather than adaptive or restorative.

Balancing Cell Division, Growth, and Apoptosis during Environmental Stress

Mitogen-Activated Protein Kinases (MAPKs). MAPK cascades represent critical intermediaries in the transmission of cues from the environment to the transcriptional machinery in the nucleus. Major cellular outcomes of the MAPK information-processing system involve regulation of the cell cycle, cell

growth and differentiation, and cell death (Karin 1998; Cowan and Storey 2003). MAPK expression in *Mytilus* spp. is highly responsive to environmental perturbations, including temperature, osmolality, hypoxia, and heavy metals (Gaitanaki et al. 2004; Kefaloyianni et al. 2005; Anestis et al. 2007). In this study, thermal stress elicited rapid and sizable increases in MAPK-related phosphorylation. These data were corroborated by changes in the abundance of phospho- (i.e., activated) SAPK/JNK. MAPK-substrate phosphorylation was significantly upregulated in all three species at 20°C, suggesting that MAPK-mediated signaling events are tightly linked to the environment and regulate early adaptive responses to environmental stress. MAPKs have been previously implicated as stress-sensing proteins (Kültz and Avila 2001). The greatest magnitude and most prolonged response for both MAPK-substrate protein phosphorylation and phospho-SAPK/JNK abundance occurred in the warm-adapted *M. galloprovincialis*. These trends suggest that this species may be able to maintain MAPK-mediated adaptive processes at relatively higher temperatures than the similarly thermotolerant species *Mytilus californianus* and the less thermotolerant species *M. trossulus*, where MAPK-substrate phosphorylation decreased dramatically at 32°C and 28°C, respectively. A role for MAPK expression in promoting adaptive processes during osmotic stress is also evident, wherein the most euryhaline species, *M. trossulus*, exhibited significantly elevated levels of MAPK-substrate phosphorylation relative to *M. californianus* and *M. galloprovincialis* at 23 ppt and relative to controls at 15 ppt. These data corroborate an extensive literature demonstrating MAPK cascades as being central to osmotic stress responses (Kültz and Burg 1998).

Because MAPK activity is generally highest in those species that are most tolerant of thermal and osmotic stresses, these signaling events may promote cell survival, possibly through an interaction with the heat shock response and the proximate upregulation of heat shock proteins (Hsps; Dorion and Landry 2002). It has been suggested that the heat shock response is facilitated by the MAPK-catalyzed phosphorylation of heat shock factor 1 (HSF1), the transcription factor responsible for the stress-induced synthesis of Hsps (Sheikh-Hamad et al. 1998; Uehara et al. 1999; Rafiee et al. 2003). In support of this mechanism, Anestis and colleagues (2007) demonstrate that the abundances of phospho-SAPK/JNK and phospho-p38 MAPK increase markedly in *M. galloprovincialis* exposed to temperatures above 24°C; these changes in expression parallel the induction of Hsps (Anestis et al. 2007). Similarly, a separate study investigating phospho-p38 MAPK expression in *M. galloprovincialis* during thermal stress demonstrated that activation of this pathway induces antiapoptotic events and promotes cell survival in concert with the accumulation of Hsp70 in gill tissue (Kefaloyianni et al. 2005). These data suggest that increased levels of MAPK-substrate phosphorylation among the most environmentally tolerant *Mytilus* spp. may promote cell survival through an inhibition of apoptosis related to the induction of Hsps.

Protein Kinase B (Akt). The Akt pathway is widely recognized as a critical regulator of cell survival (Datta et al. 1999; Downward 2004; Song et al. 2005). The activation of Akt appears to provide cells with a survival signal that promotes resistance to apoptosis (Yao and Cooper 1995). The antiapoptotic functions of Akt are at least in part mediated by inhibitory phosphorylation of proteins involved in the execution of apoptosis (Zha et al. 1996; Datta et al. 1997, 1999; Cardone et al. 1998; Zhou et al. 2000). These mechanisms likely underlie previous reports of Akt activation during thermal and osmotic stress (Konishi et al. 1996), and they imply that phosphorylation events are central to the execution of Akt-mediated cellular processes. In this study, Akt-substrate phosphorylation significantly increased relative to controls in all three *Mytilus* congeners at each temperature point, suggesting that Akt activity is tightly linked to the environment and performs conserved functions in adaptive responses to environmental stress. The most plausible explanation is that increases in Akt-substrate phosphorylation are functioning to promote cell survival during thermal and osmotic stress. In support of this hypothesis, the greatest-magnitude changes during thermal stress occur in the more thermotolerant species *M. californianus* and *M. galloprovincialis*, in which expression levels are significantly elevated at 24° and 32°C relative to the more thermally sensitive species *M. trossulus*. These data imply that greater tolerances toward heat stress in *M. californianus* and *M. galloprovincialis* may in part be due to an increased ability to promote cell survival and inhibit apoptosis at elevated temperatures. Greater heat-induced damage in cells of *M. trossulus* might necessitate apoptosis in order to remove irreversibly damaged cells. Akt likely promotes cell survival during osmotic stress as well, as all three species exhibited a similar marked increase in Akt-substrate phosphorylation at the more severe 15 ppt osmotic stress sampling point.

Cyclin-Dependent Kinases (CDKs). CDKs are defined by their regulatory control of cell-cycle progression, where they coordinate events required for proper cell division (Cross 1995; Küntzel et al. 1996; Doonan and Kitsios 2009). These processes are driven in a highly regulated manner by CDK-dependent phosphorylation of various cell cycle–related target proteins that allow passage through specific stages of cell division (Loog and Morgan 2005). Therefore, modulation of the cell cycle is heavily influenced by the phosphorylation status of CDK substrates. Bioinformatic-based screens for CDK target proteins using the same amino acid consensus sequences recognized by the CDK-substrate antibody used here have identified a variety of phosphorylated target proteins associated with diverse aspects of the cell cycle (Ubersax et al. 2003; Chang et al. 2007). Emerging from these studies is the finding that increased CDK-dependent phosphorylation is not necessarily associated with increases in cell division, as a number of CDK-substrate proteins are involved in DNA repair, cell-cycle checkpoints, and cell-cycle arrest. Consequently, increases in the abundance of CDK-phosphorylated substrates could be a reflection of increased, arrested, or decreased proliferation.

Phosphorylation of CDK-specific consensus sequences during thermal and osmotic stress in *Mytilus* spp. suggests that

cell-cycle dynamics are indeed affected by environmental stress, and that these responses are divergent between *Mytilus* congeners. It seems dubious that CDK-related phosphorylation promotes cell division during environmental stress, given such uncertain cellular conditions (Gasch et al. 2000; Kültz 2003; Kassahn et al. 2009). More conceivably, increases in CDK phosphorylation may be indicative of cell-cycle arrest and/or decreased cell proliferation. This hypothesis is supported by the marked increase in CDK-substrate phosphorylation at 20°, 24°, and 28°C in the most thermally sensitive congener, *M. trossulus*. In contrast, the more thermally tolerant species *M. galloprovincialis* and *M. californianus* exhibited reduced phosphorylation levels relative to those of *M. trossulus* at 20°, 24°, and 28°C and at 24° and 28°C, respectively. Reduced cell division in *M. trossulus* at elevated temperatures may contribute to displacement by *M. galloprovincialis* in the warm southern portions of its former range.

Adjusting Metabolism: Managing Temporary Anoxia and Fueling Large-Scale Adaptive Responses

Protein Kinase A (PKA). The effects of environmental stress on energy expenditure appear to result in a biphasic metabolic response in *Mytilus* mussels. The first phase is characterized by metabolic depression as a means of limiting oxygen consumption during anoxic conditions resulting from valve closure, an initial behavioral response to environmental perturbations such as temperature or osmotic stress (Ortmann and Grieshaber 2003; Anestis et al. 2007). For example, *M. galloprovincialis* individuals acclimated to 24°C display extended periods of valve closure that are paralleled by a reduction in pyruvate kinase activity, which is indicative of low glycolytic rate and reduced energy turnover (Anestis et al. 2007). Although appropriate for moderate stress levels, this measure occurs at the expense of aerobic capacity and is therefore unfeasible as a long-term adaptive strategy. Prolonged or severe environmental stress responses demand augmented metabolic inputs to drive costly adaptive processes such as protein synthesis (Kültz 2003). For example, significant increases in the enzymatic activities of pyruvate kinase, hexokinase, and aldolase were observed in *M. galloprovincialis* individuals acclimated to 26° or 28°C, and they were coincident with gaping behavior (indicative of increased oxygen demand) and the synthesis of Hsps (Anestis et al. 2007). The prominent role of PKA as a regulator of cellular metabolism may allow it to act as an intermediary to facilitate transitions from a depressed to an elevated metabolic state. For example, PKA-dependent protein phosphorylation has been shown to stimulate metabolic-rate depression used to survive extended periods of anoxia during valve closure in the related congener *Mytilus edulis* (Michaelidis and Storey 1990, 1991). In contrast, the key metabolic enzyme phosphofructokinase was activated when it was phosphorylated by PKA in *M. galloprovincialis* (Fernández et al. 1997, 1998). PKA-substrate phosphorylation as quantified in this study did fluctuate at temperatures beyond 24°C in *M. galloprovincialis* and *M. trossulus*, possibly illustrating a shift from short-term suppressed metabolic activity in association with valve closure and anoxia to enhanced metabolic activity coordinate with gaping behavior and increased oxygen demand, to drive large-scale adaptive responses.

Protein Kinase C (PKC). The well-characterized roles of CDKs as critical regulators of the cell cycle, of Akt in mediating cell-survival pathways, and of PKA in regulating metabolism, permit more focused interpretations regarding their functions during thermal and osmotic stress in *Mytilus* spp. In contrast, PKC represents a signaling molecule characterized by a much broader spectrum of functions (Mellor and Parker 1998). Previous investigations of PKC function in *Mytilus* mussels during environmental stress are also limited, providing little background from which to draw conclusions. PKC has been shown to be involved in the signal-transduction pathway elicited by cadmium exposure in *M. galloprovincialis* (Dailianis and Kaloyianni 2004). In this case, PKC stimulated both the Na^+/H^+ exchanger and pyruvate kinase, which were ultimately attributed to the regulation of intracellular pH and the acceleration of glycolysis to fuel adaptive responses to stress, respectively. These functions during cadmium stress provide perhaps the best insight into possible PKC functions during thermal and osmotic stress in this study. For example, anoxic conditions stemming from increased valve closure at elevated temperatures increase hemolymph CO_2 concentrations and subsequently lower pH (Thompson et al. 1980). Increased Na^+/H^+ exchanger activity mediated by PKC may serve to restore homeostatic pH during thermal and osmotic stress. The coordinate activation of pyruvate kinase by PKC is also congruent with the previously mentioned importance of increasing glycolytic activity during severe or prolonged episodes of environmental stress in *Mytilus*. From a comparative perspective, the consistently higher levels of PKC-substrate phosphorylation in *M. trossulus* during thermal stress may be indicative of endogenous sensitivity toward pH changes or relatively increased metabolic demands during both stress and nonstress conditions. These factors may limit resources that can be directed toward thermal stress responses and contribute to the more heat-sensitive physiology of *M. trossulus*. However, PKC-substrate phosphorylation decreased during osmotic stress in *M. trossulus*. Therefore, it may be somewhat contradictory to predict that PKC is directly regulating the activity of the Na^+/H^+ exchanger, assuming that changes in Na^+/H^+ exchanger activity would also be beneficial in restoring ion homeostasis during osmotic stress.

Concluding Remarks

The nature of major cell-signaling proteins as focal points of diverse cellular responses makes it challenging to appropriate changes in their activity to specific biological processes. However, this task is aided by the relatively restricted functions of some signaling proteins during environmental stress, such as CDKs, Akt, MAPKs, or PKA. These signaling proteins likely regulate fundamental cell processes such as cell proliferation, growth, and apoptosis during environmental stress. Differential

ability to optimize these processes during environmental stress would have clear selective consequences that may influence species-specific tolerances toward abiotic stresses. Nonetheless, the difficulty in assigning functions to more indiscriminate proteins such as PKC is obvious. This trend is further complicated by the possibility that changes in cell signaling–related gene expression may be representative of either sensitivity toward environmental stress or the possession of enhanced adaptive response mechanisms. Despite these inherent complications, data presented in this study at minimum demonstrate that phosphorylation events catalyzed by major signaling proteins are divergent within and between *Mytilus* spp. during environmental stress. Studies of this nature represent an important first step in analyzing the role of posttranslational modifications during environmental stress responses, and they illustrate the need for more directed research. As transcriptomic and proteomic studies continue to gain prominence as means of characterizing gene expression during environmental stress, the need to investigate the adaptive significance of responses that occur in the absence of changes in the abundance of gene products will likely become reinforced.

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