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Quantification of heat shock protein 70 and acetylcholinesterase over a time course suggests environmental adaptation in a foundational molluscan species

Andrew Ravaschiere, Caroline Cutler, Kristi Edleson, Zachery Halem, Henry Magun, Fred Meckler, Rachel Cox*

Lisman Laboratories, Riverdale Country School, 5250 Fieldston Rd., Bronx, NY 10471, USA

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ABSTRACT

Waterways in urban areas often act as repositories for sewage, industrial waste, and environmental contaminants. In response, inhabitants of these watersheds undergo physiological adaptations specific to their respective environments. Effects of these stressors can be assayed by quantification of various well-documented biomarkers in sentinel species such as the Atlantic Ribbed mussel, *Geukensia demissa*, a native to the Bronx River Estuary, Bronx, NY, USA. Heat shock protein 70 (Hsp70) is a universally expressed biomarker for an array of environmental stressors including toxins and low dissolved oxygen. To better understand the mechanisms by which organisms tolerate their contaminated environments, we monitored the constitutive and heat shock-induced levels of two proteins: Hsp70 and acetylcholinesterase (AChE) in natural populations of *G. demissa* from differentially impacted sites: the Bronx River and Greenwich Cove estuaries. We show that *G. demissa* from the Bronx River exhibits a higher level of constitutive Hsp70, and launches a more rapid and robust heat shock response than does its Greenwich Cove counterpart. In addition, AChE levels are recovered more quickly in Bronx River mussels. Based on response pattern investigations from heat stress as well as constitutive expression, we suggest that the Hsp70/AChE chaperone/client relationship exemplifies the unique adaptive mechanisms utilized by organisms in order to tolerate environmentally impacted habitats. Results from this study offer important insights from an ecological perspective into the molecular and cellular basis of stress response and provide valuable information regarding adaptation to the increased demands of challenging environments.

1. Introduction

Urbanization impacts the well-being of estuarine ecosystems more severely than any other form of human activity (Limburg et al., 2005; Chin et al., 2013). Changes in patterns of land use and water consumption stemming from human industrialization continually introduce harmful contaminants into nearby watersheds (Van Dolah et al., 2008; Astaraie-Imani et al., 2012). As urban communities become the most predominant form of human dwellings, it is important to understand the consequences of our actions on urban ecosystems and watersheds (Sanderson and Labruna, 2005). The two problems addressed in this article concern the extent to which animals tolerate rapidly urbanizing ecosystems and the means by which stress-tolerant organisms adapt to their changing environments.

The Bronx River originates at small tributaries near the Kensico dam in Westchester NY and enters the city of New York after 12 miles, where it flows through densely populated and industrialized neighborhoods. The Bronx River drains into the East River on the west end of the Long Island Sound where it becomes tidally influenced and more estuarine. Throughout the nineteenth and twentieth centuries, combined sewage outflow and industrial waste continued to contaminate this urban watershed with pathogens (Crimmens, 2002), chemical toxins (Litten et al., 2007) and sewage (Crimmens, 2002; Kriesberg and Larson, 2010; Enecio and Krakauer, 2014). The Bronx River Estuary experiences relatively low dissolved oxygen (DO) levels (as shown in Table 1 relative to our “clean test site”, Greenwich Cove, in Connecticut). These environmental factors may impact organisms living in the river. The Bronx River Estruary's native molluscan species *Geukensia demissa* (*G. demissa*) exhibits endocrine disruption and stunted growth (Halem et al., 2014). In addition, the foundational species *Spartina alterniflora* demonstrates elevated levels of the stress protein heat shock protein 70 (Hsp70), which is indicative of an adaptation to environmental stress

* Corresponding author.
E-mail address: rcox@riverdale.edu (R. Cox).

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Table 1
Average levels of dissolved oxygen and water temperature over a 7-year survey, and pH over a 5-year survey of the Bronx River and Greenwich Cove.

Estuary Site	Dissolved Oxygen (mg/L)	Temperature (°C)	pH
Bronx River	4.64 ± 1.22	22.8 ± 1.08	7.28 ± 0.05
Greenwich Cove	9.88 ± 1.83	23.9 ± 0.24	7.89 ± 0.24

All water collections (1–3 each year) occurred in June and July, around low tide. Data are presented as mean ± SD. $p < 0.0001$ for dissolved oxygen concentrations. $p = 0.0063$ for pH values. Revised from Halem et al. (2014).

(Decarlo et al., 2017).

With reference to comparisons made between the Bronx River Estuary, Bronx, NY and Greenwich Cove in Greenwich, CT, both of these watersheds are estuaries in the northeast region of the United States, roughly on the same latitude. The distance between these two collection points is 45.5 km (28.3 mi). A clear and important distinction is that the Bronx River runs through the city of New York, with concomitant exposure to urbanization, pollution, and contamination since the early 1900s. In contrast, Greenwich Cove is in a suburban area that has not experienced the same high level exposure to industrial contamination and sewage dumping. Low dissolved oxygen levels as those recorded in the Bronx River, (Table 1) approach hypoxic conditions that make life sustaining metabolic requirements very difficult if not impossible to meet (Diaz, 2001).

Despite extensive anthropogenic pollution, the Bronx River has remained a relatively resilient aquatic community (Rachlin, 2007). To better understand the cellular mechanisms utilized by stress-tolerant species, we compared the Hsp70 protein levels of two natural populations of ribbed mussels (*G. demissa*) under field conditions. One population originates from the Bronx River Estuary and one from Greenwich Cove, Connecticut, a relatively un-impacted site that has not undergone environmental disruption to the same degree.

As sessile estuarian filter feeders, mussels are well-established as sentinel organisms for biomonitoring environmental contamination (Goldberg, 1986). Specifically, *G. demissa* is well documented as a keystone bio-indicator species for the assessment of water quality (Bergen et al., 2001; Galimany et al., 2014; Giarratano et al., 2014; Halem et al., 2014). *G. demissa* reside in a wide range of geographical environments, from the Gulf of Lawrence, Canada to Florida, in mostly intertidal habitats, and experience a broad spectrum of salinities. *G. demissa* play an important role in the environmental flow of nitrogen (Abbott, 1974; Castagna and Chanley, 1973; Galimany et al., 2014; Jordan and Valiela, 1982).

Heat shock protein 70 (Hsp70) is a chaperone protein (Nover et al., 1996) that is biosynthesized both constitutively and in response to multiple stress factors in order to prevent or reverse protein denaturation, release stores of destroyed proteins, and guide the degradation of misfolded proteins (Bozaykut et al., 2014). The Hsp70 family is universally conserved, suggesting its relevance across evolutionarily diverse species (Gupta et al., 2010). Constitutive expression of Hsp70 has been maintained over at least 2.5 million years of evolution (Carpenter and Hofmann, 2000), perhaps in part because induction of Hsp70 synthesis facilitates the survival of organisms living under chronic environmental stress (Sanders et al., 1991). Hsp70 quantification is thus utilized as a consistent biomarker for environmental stress (Köhler et al., 1992; De Pomerai, 1996; Nadeau et al., 2001).

The enzyme acetylcholinesterase (AChE) breaks down the neurotransmitter acetylcholine, and plays a central role in neurotransmission. AChE responds to a variety of environmental triggers such as algal toxins (Cadavid, 2003; Lehtonen et al., 2003) and metal and pesticide exposure (Kopecka-Pilarczyk, 2010). In organisms exposed to carbamate and organophosphorus pesticides, AChE activity is inhibited (Singh and Agarwal, 1982) and such decreases are associated with several types of neurological disorders (Shinotoh et al., 2000). Due to its relevance with regard to environmental stress, AChE is an appropriate protein to study under the protection of Hsp70 acting as a potential chaperone.

The main aim of this research is to better understand the tolerance mechanisms utilized by these estuarine species. We measured both Hsp70 and AChE levels constitutively as well as after acute heat stress in two *G. demissa* field populations. Using AChE as a potential cognate protein under protection of Hsp70 chaperone, we propose that constitutive Hsp70 synthesis may be considered as a biochemical exaptation (Gould and Vrba, 1982) that allows mussels to survive under the pressure of chronic and multiple environmental stress factors (Koban et al., 1991; Sanders et al., 1991; Bednarek et al., 2016). This work provides empirical evidence for what appears to be a rapidly growing understanding that changing ecological conditions drive swift evolutionary change (Alberti, 2015). In addition, from the perspective of ecosystem services, which focus on the societal benefits offered by threatened species, we describe adaptive mechanisms in a highly tolerant foundational species (Gascon et al., 2015). We hope that these findings will better inform precautionary principles driving decisions that affect our urban waterways.

2. Materials and methods

2.1. Sample collection

Over the course of a three year study, *Geukensia demissa* specimens were collected between June 20 and July 10, at low tide, from Harding Lagoon, Bronx River Estuary, Bronx, NY, USA (40° 48′ 35.563″ N, 73° 51′ 40.893″ W), and Todd's Point, Greenwich Cove, Connecticut, USA (41° 0′ 31.296″ N, 73° 34′ 18.042″ W), see Fig. 1. Each collection resulted in twenty-four mussels overall. Organisms were transported to the laboratory in native water. Transport times varied from 30 to 90 min, field to laboratory. All samples, kept in native waters, were allowed to equilibrate at 24 °C for 24 h.

2.2. Heat shock treatment, time course, and dissection

Half of the mussels from each site were incubated at 24 °C for three hours. The remaining mussels were incubated in their respective site's waters at 37 °C for 2.5 h. After treatment, half of the mussels from the control group (24 °C) and heat shock group (37 °C) from each site (Bronx River Estuary and Greenwich Cove) equilibrated at 24 °C for 2.5 h before dissection. The other mussels equilibrated after heat shock at 24 °C for 27.0 h before dissection.

2.3. Protein extraction and concentration determination

Gills were pooled based on their site, treatment (heat shock vs. control), and equilibration time. Tissues were homogenized and lysed (Tissue PE, G-Biosciences #786181 and Halt PI #78425) in a 1:101 ratio (parts-to-whole). Gills were ground with a pestle and mortar before being further homogenized electronically. Samples were centrifuged for 20 min at 4 °C at 13,000 rpm. Protein containing supernatants were collected and concentrations were determined according to the Pierce BCA Protein Assay Kit (Thermo Scientific, #189966, Rockford, IL).

2.4. Determination of Hsp70 and AChE levels

Hsp 70 and AChE protein levels in *G. demissa* gills were determined by Western blot analysis. Gill homogenates were run on a 10% gel (30 µg) and transferred to PVDF membranes, which were Ponceau stained to visualize protein transfer. Membranes were blocked (Superblock, Thermo-scientific, # 37535, Rockford, IL) overnight at 4 °C. Blots were incubated in rabbit Hsp70 polyclonal antibody (1:2,000 Enzo SPA-812, Plymouth Meeting, PA) and in rabbit AChE polyclonal

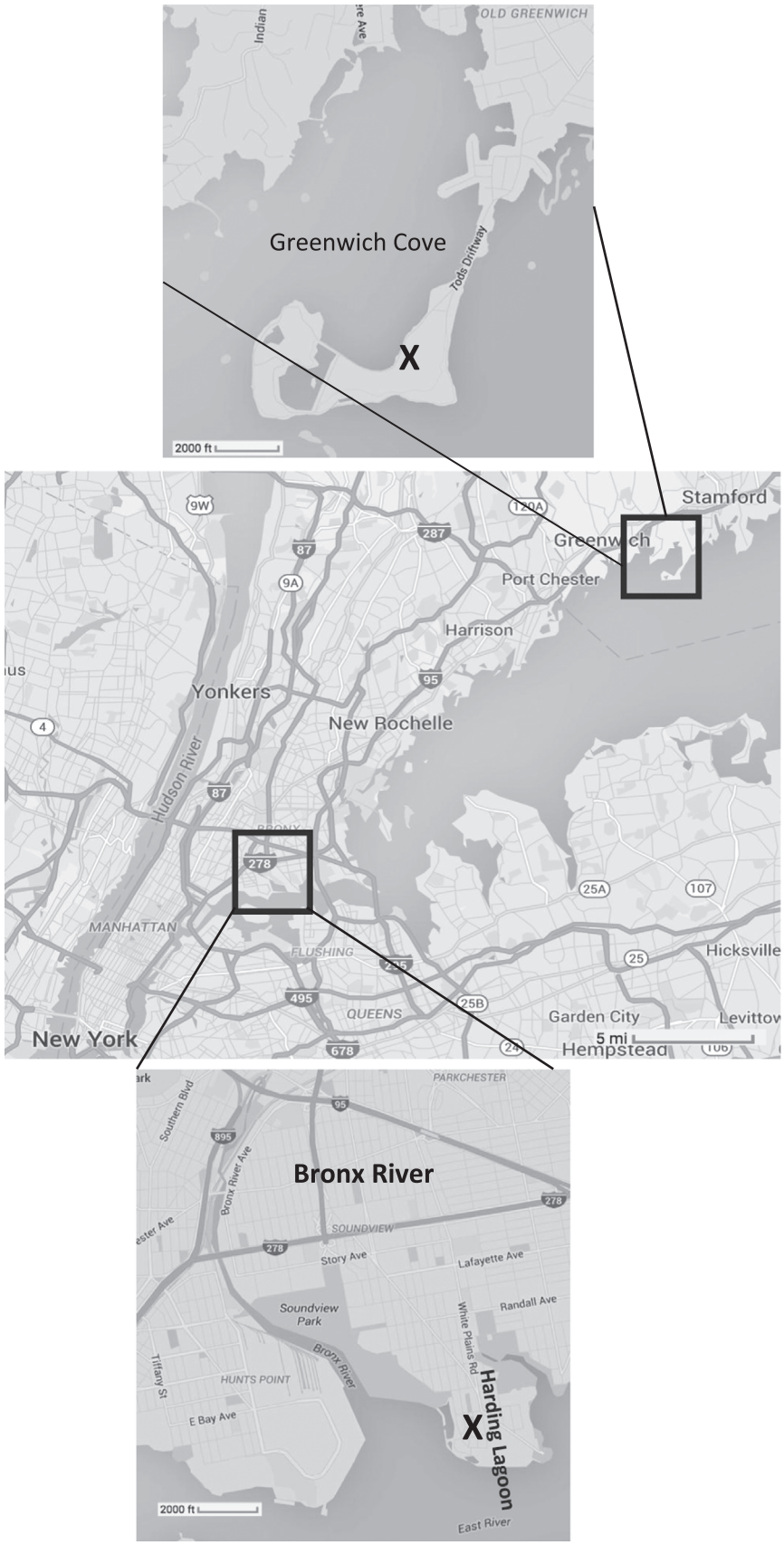

Fig. 1. Map of field areas. X represents collection sites.

antibody (1:1000 Protein Tech Anti AChE 17975-1-AP, Chicago, IL). After washing (1x Tris-Buffered Saline + Tween), the Hsp70 membranes were incubated in goat anti-rabbit secondary antibody (1:5,000, Promega W401B (GAR) HRP conjugated, Madison, WI) for 1 h at room temperature. The AChE membranes were incubated in goat anti-rabbit secondary antibody (1:10,000, Promega W401B (GAR) HRP conjugated, Madison, WI) for 1 h at room temperature as well. After washing the membranes (1x TBS + Tween), proteins were detected by Immun-Star luminol-peroxide (170–5060 Bio-Rad, Hercules, CA). Hsp 70 and AChE levels were quantified by protein densitometry using Quantity One program on a Bio-Rad Chemi-doc. As an internal protein loading control, glyceraldehyde 3-posphate dehydrogenase (GAPDH) was quantified using rabbit polyclonal antibody (1:2000, Enzo GAPDH, pAb ADI-905-734 Farmingdale, New York) and goat anti-rabbit polyclonal secondary antibody (1:10,000, Enzo Goat anti-rabbit (GAR) IgG, HRP conjugate ADI-SAB-300 Farmingdale, NY). Using the highest GAPDH level as the internal control, Hsp70 and AChE levels were calculated by multiplying Hsp70 and AChE intensities by the highest GAPDH expression number, and then dividing by each Hsp70 and AChE's corresponding GAPDH intensity, based on site, heat shock or control treatment, and equilibration time.

2.5. Statistical analyses

P-values were determined through unpaired t-tests for statistical comparisons of two discrete population and/or experimental groups, and through ANOVA tests for statistical comparisons of more than two experimental groups. P-values less than or equal to 0.05 are assumed to display statistical significance. MATLAB scripts were used for statistical analysis.

3. Results

3.1. Baseline and heat shock exposed Hsp70 protein levels

Through a purely comparative normalized metric, the Bronx River mussels showed consistently higher baseline levels of Hsp70 than their Greenwich Cove counterparts. Over the three-year study, levels of constitutive Hsp70 in the Bronx River mussels were found to be on average 10.42% greater with a 0.024% variance for an n = 12 sample size (Fig. 2A). Intra-site variation for both populations was found to be minimal and statistically inconsequential. Mussels from Bronx River exhibited an elevated heat shock response in comparison to Greenwich Cove. Bronx River mussels averaged a 9.0% higher percent increase from constitutive levels, and the resulting post-heat shock levels were found to be 2781.62 INT ± 100.46 INT greater (Fig. 2B). Heat-shock induced Hsp70 percent increase remained rather static on a year-to-year basis for Bronx River mussels, while increasing at a constant rate of 4–5% per year for Greenwich Cove mussels.

3.2. Time course for Hsp70 protein

Both Bronx River and Greenwich Cove mussels demonstrated increased Hsp70 levels as a result of heat shock. As shown in Fig. 3, after 2.5 h of equilibration post heat shock, Bronx River mussels displayed an 8.03 ± 0.49 greater percent increase than their Greenwich Cove counterparts. However, the two populations exhibited differing trends as heat shock recovery proceeded over a prolonged period. At 27 h post heat shock, Bronx River mussels showed Hsp70 levels at an average of 5.33% above the baseline concentration, demonstrating a 16.31% decrease from 2.5 h post heat shock. However, for Greenwich Cove mussels 27 h after heat shock, Hsp70 levels were 18.88% above their baseline, demonstrating a 5.27% increase from 2.5 h after heat shock.

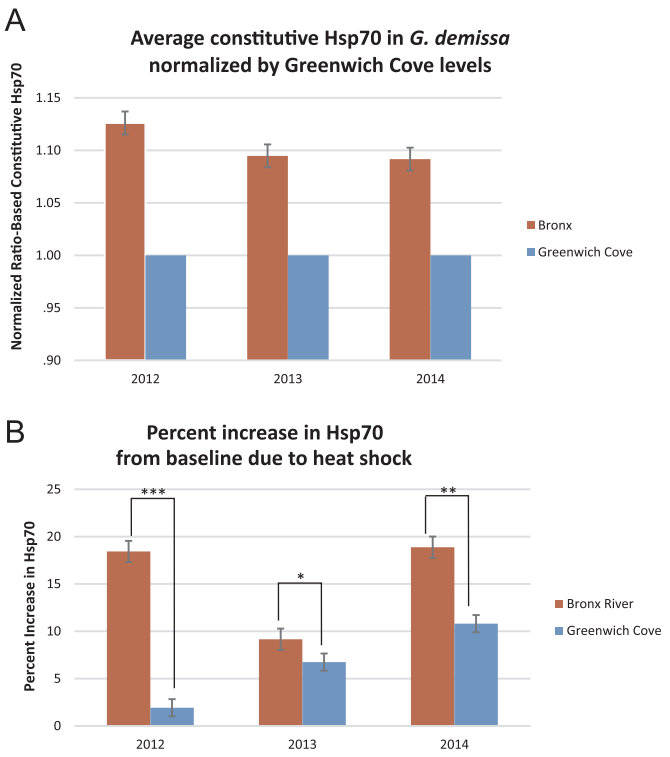

Fig. 2. A. Hsp70 from the Bronx River and Greenwich Cove, normalized by Greenwich Cove Western Blot intensity levels. Data prepared in duplicate (n = 12) are shown as mean ± S.E.M, while the basis of normalization has no uncertainty. 2B. Cumulative percentage change in Hsp70 protein levels (Western Blot intensity) of *Geukensia demissa* from baseline to post-heat shock equilibrium. Data prepared in duplicate (n = 12) are shown as mean ± S.E.M. *** = $p < 0.01$, ** = $p < 0.05$, * = $p < 0.12$, where p-values were determined through a t-test.

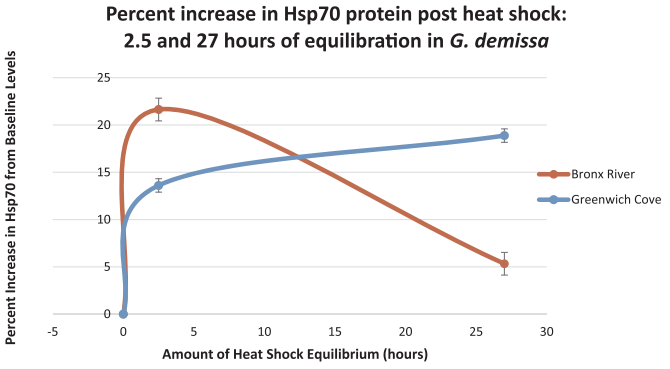

Fig. 3. Relative percentage change of Hsp70 protein levels in *Geukensia demissa* from both sites after 2.5 and 27.0 h of equilibration. Data are shown as mean ± S.E.M. For both 2.5 and 27 h, $p < 0.05$ based on the results of an ANOVA test.

3.3. Time course AChE protein

As shown in Fig. 3, after initial heat shock (2.5 h recovery), Bronx River and Greenwich Cove mussels both experienced diminishing AChE protein levels, with a 4.20% and 14.25% decrease respectively. After longer post heat shock equilibration (27.0 h), both populations displayed higher levels of AChE when compared to 2.5 h post heat shock and baseline. The Bronx River mussels exhibited a slightly larger increase in AChE levels between 2.5 h and 27.0 h of equilibrium, with a 27.05% percent increase as compared to 17.19% increase for Greenwich Cove. Over all, Bronx River mussels experienced a 22.85% increase from baseline to 27 h while for Greenwich Cove counterparts this change constituted a 2.94% increase.

4. Discussion

With accelerated rise in sea level and increased storm surges, urban salt marsh estuaries have assumed a vital role as providers of broad-ranging ecological services for their neighboring cities (Van Loon-Steensma and Vellinga, 2013). Emerging evidence from the field of ecotoxicology suggests that as estuarian populations adapt to urbanization and climate change, ecosystem stability will be impacted (Alberti, 2015). Here we describe important cellular mechanisms and adaptive responses in aquatic populations that face the challenges of survival in urban estuaries.

Filter feeding mussels, well documented as bio-indicator organisms, are uniquely suited for the monitoring of diverse local environmental changes, especially in urban settings (Sanders et al., 1991; Dietz and Somero, 1993; Hightower et al., 1999). These bivalves lend themselves well as model organisms for the study of urban adaptation as they are widely sensitive and resilient to abiotic fluctuation. In addition, their feeding habits favor bio-accumulation of xenobiotics. The gills, site of initial exposure to environmental challenge, are directly involved in physiological processes including gas exchange and nutrient uptake, thus making this organism and organ appropriate subjects of our study.

In order to better understand the impact of urbanization on organisms native to urban watersheds, we studied local populations of *Geukensia demissa* to monitor constitutive levels of the heat shock protein Hsp70. In addition, we measured Hsp70 and an esterase (AChE), before and after acute heat stress over a time course in *G. demissa*, from differentially impacted estuaries, the Bronx River and Greenwich Cove.

Heat shock proteins are the cellular products of rapid transcription and translation in response to various types of stress. They assist in the correct folding of both freshly-transcribed and stressfully-impacted proteins by inhibiting protein build-up and stimulating the selective breakdown of misfolded or denatured proteins (Schlesinger, 1990; Morimoto, 1993; Sikora and Grzesiuk, 2007; Saluja and Dudeja, 2008; Gupta et al., 2010; Bozaykut et al., 2014; Shiber and Ravid, 2014). Recent studies suggest that heat shock proteins may regulate transcriptional events that promote homeostasis and survival (Gao et al., 2015). Heat shock proteins respond to multiple types of stressors in addition to heat, including glucose deficit, low temperature, lack of oxygen, exposure to heavy metals and organic contaminants (Feder and Hofmann, 1999). Induction of Hsp70 synthesis facilitates the survival of organisms living under chronic environmental stress (Sanders et al., 1991).

Our three-year study demonstrates that mussels from the Bronx River consistently yield nine to thirteen percent higher constitutive Hsp70 levels than do their Greenwich Cove conspecifics. Elevated constitutive Hsp70 in Bronx River population may be indicative of an acclimation process that potentially facilitates fitness and survival under adverse conditions, a phenomenon that may be achieved through the constitutive production of heat shock proteins (Koban et al., 1991; Sanders et al., 1991; Todgham et al., 2005; Bednarek et al., 2016; Cheng et al., 2016).

The disparity in constitutive Hsp70 levels between mussels from the two sites could stem from the presence of toxins found in the Bronx River that are not as abundant in Greenwich Cove. At times, the Bronx River Estuary has contained the highest benzo (a) pyrene concentration out of all watersheds in the state of New York at 0.02 μg/L (Litten et al., 2007), which is significantly higher than the New York State Water quality standard. Benzo (a) pyrene increases Hsp70 expression (Vayssier-Taussat et al., 2001). Polychlorinated biphenyls (PCBs), long understood as environmental toxins (Safe, 1990) also contaminate the Bronx River watershed sediments, the result of past industrial discharges (Wiatllisbrer, 2011). PCB concentrations in the Bronx River Estuary are below aquatic life criteria limits, and thus not considered to be a major concern within the water column (Kriesberg and Larson, 2010). However, for sediment dwelling *G. demissa*, the impact of exposure to these chemicals could be more severe. Because these toxins have been shown to increase Hsp70 levels, as well (Cruz-Rodríguez et al., 2000), PCB exposure might contribute to the Bronx River mussels' higher constitutive Hsp70 levels.

Table 1 shows that over seven years, the Bronx River dissolved oxygen concentration (DOC) is significantly reduced compared to Greenwich Cove. Hypoxia is an environmental factor that renders biological adaptation across species. Drosophila (Zhou et al., 2008), hummingbirds and deer mice (Natarajan et al., 2013) demonstrate adaptations to high altitudes and hypoxic conditions. Aquatic organisms also adapt to low oxygen environments (Roesner et al., 2008; Richards, 2011).

Among cellular responses to hypoxia is a pre-induction of Hsp70, which has been shown to prevent acute hypoxic brain damage in mice (Zhang et al., 2009) and serve as a protective agent during ischemia/reperfusion injury in rats (Iwaki et al., 1993). Due to the Bronx River's hypoxic waters and the benefits to organisms who have elevated Hsp70 levels in these environments, Bronx River mussels may be better prepared to activate and complete an Hsp70 stress response to applied pressures. Greenwich Cove mussels, living in a relatively clean and oxygen-rich locale, may take longer to launch an Hsp70 response because they are not acclimated to environmental stress.

We tested these ideas by introducing heat stress to organisms from both populations in the laboratory under controlled conditions. Post heat shock, Bronx River mussels generated a more robust and quicker Hsp70 response after 2.5 h of equilibration, while Greenwich Cove organisms took longer (up to 27.0 h of equilibration, shown in Fig. 3) to launch a response that came close to the Bronx's 2.5-h level. These variable Hsp70 responses suggest that environmental stress differentially impacts patterns of acute stress response in these two conspecific natural mussel populations.

Bronx River mussels, potentially living under greater daily stress, may elicit a more rapid and robust response when placed under acute heat stress. Here, we see a perceived biological advantage to maintaining constantly engaged chaperone proteins: Bronx River mussels have developed a more efficient stress response. By 27.0 h after equilibration, Greenwich Cove mussels have yet to reach the Hsp70 levels that the Bronx River mussels reached after just 2.5 h of equilibration. This tendency is documented in the scientific literature, where in constitutive heat shock proteins are shown to be crucial to cellular functions such as protein folding and storage release (Hendrick and Hartl, 1995; Fink, 1999; Piano et al., 2002). Additionally, we analyzed the enzyme, AChE as a potential cognate, client protein to Hsp70. Responsible for the recycling of the neurotransmitter acetylcholine, AChE is involved in neural transmission across animal species. In molluscs, the enzyme controls filtration and feeding as well as valve opening and embryonic development (Corsi et al., 2007). AChE activity is considered a reliable marker for exposure to organo-phospahates from fertilizer and carbamates from pesticides (Lionetto et al., 2003). AChE activity and expression are reliably decreased in aquatic organisms such as bivalve molluscs and teleost fish living in industrial, anthropogenically impacted habitats (Fasulo et al., 2010; Benali et al., 2015; Natalotto et al., 2015). Inhibition of AChE activity causes acetylcholine to accumulate in the synapse, leading to significant physiological impairments related to feeding and filtration.

AChE levels decreased in mussels from both sites after 2.5 h of equilibration following heat shock, indicating possible denaturing as a result of heat shock treatment. Greenwich Cove mussels experienced a $-14.25\% \pm 1.19\%$ AChE drop-off, almost three times greater than the $-4.20\% \pm 1.94\%$ AChE decline found in their Bronx River counterparts. At 27.0 h of equilibration, both groups of mussels demonstrated an increase in AChE levels compared to their pre-heat shock baselines. Relative to baseline levels, Bronx River mussels showed a $22.85\% \pm 1.94\%$ rise in AChE. This is compared to the $2.94 \pm 1.19\%$ found in Greenwich Cove specimen, an almost eight times difference. The Bronx River samples' initial smaller percentage of AChE loss and

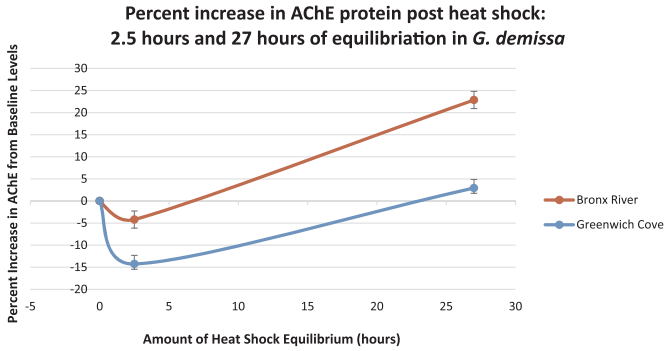

Fig. 4. Relative percentage change of AChE protein levels in *Geukensia demissa* from both sites after 2.5 and 27.0 h of equilibration. Data are shown as mean ± S.E.M. For both 2.5 and 27 h, $p < 0.05$ based on the results of an ANOVA test.

subsequent greater percentage of AChE increase suggest that Bronx organisms are comparatively better able to conserve their AChE when confronted with stress.

Differing degrees of AChE responses found in the Bronx River and Greenwich Cove organisms reflect patterns we observed regarding Hsp70. Bronx River mussels launched a stronger and quicker Hsp70 response, likely because of their higher constitutive Hsp70 levels. As a result, the Bronx River organism has a more engaged and powerful Hsp70 pathway, thus potentially preventing greater amounts of AChE loss or denaturation. This enhanced stress response and protein recovery system enables Bronx River mussels to better recover and maintain AChE when faced with stress, which is critical to physiological functions such as feeding and gas exchange. Greenwich Cove mussels, not exposed to chronic environmental stress in their native environment, take longer to recover from heat stress. Greenwich Cove mussels lose more AChE by 2.5 h post heat shock and by 27 h post heat shock, have not recovered their AChE as well as their Bronx River conspecifics (shown in Fig. 4). Greenwich Cove's mussels' slower reaction to heat stress could be indicative of their lack of "practice" or "experience" because their native environments rarely place them under stress. Mussels from the cleaner site do not have an Hsp70 pathway that is readily stimulated and thus "ready for battle" in the face of environmental pressure. This type of heightened stress experienced by organisms from impacted sites is well documented by other authors who suggest a "pre-adaptive," state of readiness in these urbanized organisms (Bednarek et al., 2016). This pre-adaptation gives organisms from environmentally impacted areas the capacity to acclimate more quickly to acute stress. Hypoxia in the Bronx River may serve as a contributing factor to the observed relationship between Hsp70 and AChE. Hypoxic conditions cause early induction of Hsp70, which is essential for maintaining expression levels of pre-synaptic proteins (Fei et al., 2007) such as AChE. Therefore, our work additionally demonstrates the inter-relationship between hypoxia, Hsp70, and pre-synaptic AChE, in a natural population.

Homeostatic plasticity may also edify the potential chaperone-client protein relationships we observed. In Drosophila, heat shock proteins trigger mechanisms that maintain neuronal activity during stress (Karunanithi and Brown, 2015). Because AChE plays a role in managing neuronal activity, it is reasonable to believe that organisms with higher constitutive and inducible Hsp70 levels would be better equipped to shepherd their AChE compared to organisms that do not have highly engaged Hsp70 pathways. Additionally, previous studies show that AChE is protected from denaturation by Hsp70, acting as a molecular chaperone (Eichler et al., 1991). Further examination of the heat shock response timeline with proteins taken at additional time intervals could lend further credence to the heat shock response model described here. A study that utilizes proteins in addition to AChE would help to confirm this type of chaperone-client relationship and its role in pre-adaptive mechanisms.

Halem et al. (2014) confirmed that the mussels found in the Bronx River and Greenwich Cove are the same species through DNA barcoding techniques. Therefore, the disparate constitutive and induced responses we describe here between the two populations is an example of phenotypic plasticity, in which phenotypes shift based on environmental fluctuation. Data presented here adds to the list of phenotypic differences – such as estradiol, testosterone, and progesterone concentrations, as well as shell and tissue size – we have documented between these two populations of *G. demissa* living in differently-impacted environments. By investigating the links between environmental factors and cellular protein responses over a time course at both a chaperone and client protein level, we have documented one potential way in which human-driven environmental factors enforce varying patterns of homeostasis, fitness and survival. Seawater temperatures are rising at a rate faster than in any period over the past million years (Zachos et al., 2001). Heat shock proteins such as Hsp70 are considered good candidates for use as universal biomarkers for climate change driven stress response across diverse species (Clark and Peck, 2009). Global climate change will increase biological stressors due not only to warmer water temperatures, but also to accelerated sea level rise and increased storm surge impact (Alberti, 2015). These environmental changes may contribute to stresses experienced by ecological providers, especially in urban estuaries. Results of this study will help to lay our foundational understanding of the complex and synergistic cellular mechanisms at play during biological adaptation in these vital ecological provider species.

5. Conclusion

Overall, this study provides further evidence for the "pre-adaptive" phenomenon observed with increasing frequency by ecologists and toxicologists. Pre-adaptation allows organisms that are exposed to prolonged stress to launch a more robust and efficient stress response when acutely challenged. We suggest that for highly stress-tolerant populations living over multiple generations in challenging environments, such as the Bronx River Estuary, the Hsp70/AChE client/chaperone relationship we describe here may play a role in tolerance, adaptation, and survival. With a better understanding of the cellular mechanisms that underlie maintenance of homeostasis in the face of environmental challenge, we hope that these findings will further contribute to critical conversations regarding land use management and species conservation of Earth's urban estuaries.

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