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EFFECTS OF THERMAL STRESS AND OCEAN ACIDIFICATION ON THE EXPRESSION OF THE RETROTRANSPOSON *STEAMER* IN THE SOFTSHELL *MYA ARENARIA*

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ABSTRACT The softshell *Mya arenaria* is an ecologically and commercially important bivalve mollusc that develops a fatal cancer known as disseminated neoplasia or hemocyte leukemia. This cancer, like many human cancers, involves the master control gene of apoptosis, p53. This gene is also known to interact with retrotransposons, and a growing body of evidence suggests that p53 actually controls retrotransposon expression and subsequently oncogenesis. The recent description of a retrotransposon (*i.e.*, *Steamer*) in adult *M. arenaria* and its potential involvement in the oncogenesis of hemocyte leukemia has been suggested. Juvenile clams, previously shown not to develop hemocyte leukemia, were exposed to climate-related changes in seawater temperature and pH predicted for the year 2100. All juvenile softshells do express the retrotransposon *Steamer*. When juvenile clams are exposed to predicted climate-related changes in temperature and pH in the Gulf of Maine, it significantly induces *Steamer* expression, specifically in treatments where clams were exposed to low pH. For juvenile individuals of *M. arenaria* and potentially other bivalves, *Steamer* may be a candidate biomarker for exposure to ocean acidification conditions.

KEY WORDS: *Steamer*, climate change, ocean acidification, *Mya arenaria*, softshell, clam, cancer, apoptosis, retrotransposon

INTRODUCTION

The softshell *Mya arenaria* (Linnaeus, 1758) is found worldwide in marine soft-bottom ecosystems where it is ecologically important in the coupling of water column productivity to benthic consumers and as food for numerous predators. Another critical function of softshells is the bioturbation of sediments and associated pore water constituents (*e.g.*, dissolved inorganic nutrients) to and from the sediment–water interface that mediates the biogeochemistry and ecotoxicology of sediment habitats (Norkko & Shumway 2011). In addition, although the softshell fishery in New England has increased in value, landings in recent years have declined significantly. Significant population declines of *M. arenaria* have been observed throughout New England, but specifically in the Gulf of Maine (GOM). Although some of these declines have been attributed to increasing seawater temperatures and ocean acidification (Salisbury et al. 2008, Balch et al. 2012), the most significant causes of population declines have been predation by the introduced European green crab (*Carcinus maenas*), overfishing, and a fatal cancer of softshells known as disseminated neoplasia or clam hemocyte leukemia (Beal & Kraus 2002, Walker et al. 2011). Interestingly, mortality by crab predation is focused on juvenile clams having less than 30 mm shell length (SL), whereas mortality from leukemia occurs in adult clams having 40–80 mm SL. Mortality by hemocyte leukemia can be as high as 10% of the adult population that survives green crab predation.

Despite the considerable amount of information available on the molecular mechanisms that characterize this disease (*e.g.*, Walker et al. 2011), the current data available on the etiology of clam hemocyte leukemia support environmental/toxicological and/or a viral/retrotransposon origin (Böttger et al. 2008, Arriagada et al. 2014). The strongest evidence, however, has suggested a viral origin with possible environmental induction of the disease (Böttger et al. 2008). Studies also show that the disease can be transferred to noncancerous clams by injection of hemocytes, lysed hemocytes, or hemolymph alone from leukemic clams (Taraska & Böttger 2013). There is also evidence supporting the presence of a retrovirus in diseased clams (House et al. 1998, AboElkhair et al. 2009, Walker et al. 2011, Taraska & Böttger 2013), as well as evidence against a viral origin casting doubt that the pathology of clam leukemia has a viral etiology (AboElkhair et al. 2012). Recently, a long terminal repeat retrotransposon, a transposable element named *Steamer*, has been described from the genomes of normal and leukemic clam hemocytes (Arriagada et al. 2014). The expression of this retrotransposon has been reported to be 50-fold to 200-fold greater in leukemic hemocytes than in normal hemocytes, and it has been suggested that transmission of the cancer between clam populations occurs by the release of cancerous hemocytes and subsequent uptake via filter feeding by naïve populations or the release of retrotransposons from cancerous clams and their uptake by naïve clams (Arriagada et al. 2014). A subsequent publication using microsatellite markers (Metzger et al. 2015) reported that populations of diseased clams along the eastern seaboard of the United States were genetically differentiated from each other but contained leukemic hemocytes of a single genetic (*i.e.*, clonal) lineage supporting the hypothesis that the disease is transmitted horizontally.

It is well known that malignant tumors starting from a single cell generally show a high degree of intragenomic heterogeneity. This heterogeneity results from the accumulation of genetic mutations and epigenetic changes as cells divide many times, resulting in cancerous cells that are genetically different from each other even at the level of individual cells within a single tumor (Crespi & Summers 2005), or in leukemia (Li et al. 2016). There is no *a priori* reason to expect rapidly dividing cancerous clam hemocytes to not exhibit some level of intragenomic heterogeneity as well. In addition, there is no direct evidence that transmission of this lineage occurred via filter feeding of

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released cancerous hemocytes over the last 40 y, from lower to higher latitudes, as suggested by the investigators. There is also no mechanism where a clonal cancerous cell lineage could remain genetically homogeneous over a 40-y-period of time as Metzger et al. (2015) suggest unless there is a selective sweep for every single mutation that arises.

Assessing whether these cells are similar to the respective host or whether they are clonal in nature using polymorphic markers such as microsatellites or single-nucleotide polymorphisms (SNP) is appropriate (Crawford & Oleksiak 2016). Both the microsatellite and SNP data from Metzger et al. (2015), however, do not appear to include a rigorous evaluation of the appropriate sample sizes required from each population, the use of an adequate number of microsatellites or SNP in the analysis of genetic differentiation, the neutrality of the markers to make sure they are not under selection, especially in the case of cancerous cells (Marusyk & Polyak 2010), and the linkage disequilibrium properties of the markers (Selkoe & Toonen 2006). In addition, retrotransposons are known to be expressed in response to environmental stress and are important regulators of gene expression as it relates to adaptation/acclimatization to stress (Casacuberta & Gonzáles 2013). Retrotransposons are also known to be an important source of genetic novelty that selection can act on and drive evolutionary changes (Tiwari et al. 2018). Patterns of expression for retrotransposons must, therefore, be interpreted cautiously when natural samples are taken across wide environmental gradients (*i.e.*, latitude).

From an ecological, or oceanographic, context, there is also no evidence for how the transmission of this disease would be accomplished from a southwest to northeast direction in the GOM, considering the prevailing physical oceanography where currents flow primarily from a northeast to southwest direction. Specifically, although the genetic evidence does provide support for the clonal nature of this retrotransposon in populations of leukemic hemocytes, the authors should have undertaken a seascape genetics approach, which would have incorporated many ecological aspects on the horizontal transmission of this disease as well as a more robust genetic analysis (*sensu* Selkoe et al. 2016). In addition, there are no data on whether cancerous hemocytes are actually released by clams, on the dose dependence required for cancerous hemocytes to establish disease, on the long-term survivability of hemocytes in seawater, on whether clams can actually filter and retain hemocytes, and if they can, how do these hemocytes then gain access into the hemolymph to proliferate?

The GOM is experiencing one of the fastest rates of increase in seawater temperature in the world, in addition to ocean acidification, and lower primary productivity in near coastal environments (Salisbury et al. 2008, Balch et al. 2012, Mills et al. 2013, Pershing et al. 2015). Given the correlation of *Steamer* expression with a cancerous state in *Mya arenaria* (Arriagada et al. 2014), and that retrotransposons can also differentially express themselves in response to environmental stress (Casacuberta & Gonzáles 2013), an experiment on the expression of *Steamer* in juvenile individuals of the softshell *M. arenaria* exposed to current, and predicted, changes in seawater temperature and pH (*i.e.*, ocean acidification) for the GOM was conducted. The specific aim was to test the role of abiotic factors in the expression of *Steamer* in clams that are not known to develop hemocyte leukemia.

MATERIALS AND METHODS

Animal Husbandry

One-year-old sibling clams having 1.5–2.4 cm SL, measured as the anterior/posterior axis of shell, from the Downeast Institute (Beals, ME) aquaculture hatchery facility were used for the experiment in spring 2014. Approximately 20 individual clams were placed in each of the aquaria described in the following paragraphs and fed every 4–5 days with Shellfish Diet 1800 (Red Mariculture Inc.) containing *Isochrysis* sp., *Pavlova* sp., *Thalassiosira weissflogii*, and *Tetraselmis* sp. Adult clams were simultaneously collected from mudflats on the coast of New Hampshire to ground truth the assay described in the following paragraphs. Bleeding of hemolymph from adult and juvenile clams was accomplished by withdrawing 50 µL of hemolymph from the pericardial sinus of each animal using a sterile 1-mL syringe and either a 21 or 26-gauge hypodermic needle. The samples of hemolymph were placed in 96-well flat-bottomed plates, incubated at 4°C for 2 h, and scored for their cancerous state using a Zeiss IM inverted microscope as described by Taraska and Böttger (2013). Clams were maintained at 12°C and 33 salinity, as measured with a calibrated YSI Model 63 pH and conductivity meter (YSI, Yellow Springs, OH) at the University of New Hampshire, and split into two experimental arrangements as described in the following paragraphs.

Experiments on the Effects of Temperature and pH Stress on Steamer *Expression*

Clams were subjected to bubbled CO_2 at 380 and 560 ppm and temperature treatments of 10–12°C and 16–18°C typical of current and predicted temperatures in coastal GOM waters on a 14:10 L/D cycle at 100 µmol quanta m^{-2} sec^{-1}. The experimental system (Aquatic Habitats benchtop) included two closed circulating systems, each using natural seawater run through a biofilter, particulate filter, and carbon filter, and constantly aerated. Each experimental unit has two shelves of individual aquaria ($n = 12$ each shelf) with a volume of 3 L and a flow rate 9 L h^{-1}. Two tanks of 60 L for each experimental unit contained natural seawater that was adjusted to the experimental pH determined using a feedback system of pH control (*i.e.*, pH stat control method) with computer-controlled solenoids for bubbling CO_2 gas using a Qubit Q-pH Control System-4 (Qubit Systems Inc., Kingston, ON, Canada). All aquaria were continuously monitored for temperature, salinity, and pH, although samples were taken at the end of the experiment to quantify carbonate chemistry by measuring pH, total alkalinity (TA), dissolved inorganic carbon (DIC), and CO_2 to be used with the salinity and temperature data to calculate the aragonite saturation state of the seawater. For these experiments, the seawater pH values, predicted from the CO_2 concentrations, local salinity, and temperature, were ~8.1 (natural seawater) and 7.9, respectively, based on the IPCC 2001, 2007 B2 climate model for CO_2 concentrations of 380 and 560 ppm (Caldeira & Wickett 2003). After 14 days of acclimating to ambient conditions, one experimental unit was maintained at 10–12°C, and the other unit had its temperature increased to 16–18°C. After 8 days of acclimation, a random collection of clams was made from each experimental unit ($n = 20$–30). After this collection, each experimental system was split and exposed to an ambient

CO_2 concentration of 380 ppm ($n = 6$ aquaria) and 560 ppm ($n = 6$ aquaria), based on the B2 climate model, for 13 days after which an additional set of clams ($n = 20$–30) was randomly collected from each experimental unit. Before freezing, experimental clam tissues were removed from their shells and placed in RNAlater (Qiagen) and stored at –20°C until analysis. As a result of this protocol, four treatments were tested: ambient T°C and pH (AmbNorm, $n = 31$), ambient T°C and low pH (AmbLow, $n = 11$), elevated T°C and normal pH (HiNorm, $n = 23$), and elevated T°C and low pH (HiLow, $n = 16$).

Treatment effects, temperature, pH, and their interaction in the experiment were analyzed using analysis of variance (ANOVA) after transformation as ratios are *a priori* not normally distributed. Significant effects were then subject to multiple-comparison testing and transformed values were back-transformed for presentation.

pH, DIC, TA, and CO_2 Measurements of Seawater

Independent samples of seawater ($n = 3$) were collected from the reservoir tanks of each experimental unit at the beginning, after 8d, and at the end of the experiment and analyzed for TA, pH, and DIC. Total alkalinity was analyzed using an Apollo Sci-Tech AS-A2 automated analyzer, which uses the Gran titration procedure. These analyses have a precision of 0.1%. The pH was measured from the same sample using a Thermo Orion combination electrode (precision ~ ±0.027 pH units). Dissolved inorganic carbon was analyzed from acidified 1–3 mL samples using an Apollo Sci-Tech DIC analyzer that typically achieves a precision of 0.1%. Certified reference materials were used to ensure the precision of the TA and pH determinations (Dickson et al. 2007). pCO_2 and the aragonite saturation state (Ω) were analyzed using CO2SYS software modified to reflect calcium ion (Ca^{++}) variability. These analyses were run at the directly measured experimental temperature and salinity using the inorganic carbon dissociation constants of Millero et al. (2006). The estimated accuracy of the pCO_2 determination using this method is ±9.9 µatm.

RNA Extraction, Sequencing, and Assembly

Clam samples equaling approximately 0.5 cm^2 were blotted to remove any excess RNAlater, and 1 mL Qiazol (Qiagen) containing a 5-mm steel bead (Qiagen) was added and homogenized for 2 min at 50 Hz. All centrifugations for RNA extraction, precipitation, and purification were performed at 4°C. The supernatant for all samples were transferred to a new tube, and 200 µL chloroform was added, followed by vigorous shaking for 15 sec. After incubating on ice for 15 min, the samples were centrifuged for 15 min at 12,000 × g. The upper aqueous phase was transferred to a new tube, and 500 µL ice-cold isopropanol was added. The samples were inverted five times before incubating overnight at –20°C. The samples were then centrifuged at 14,000 × g for 10 min, washed with 75% ethanol, and centrifuged at 14,000 × g for 5 min. The pellet was resuspended in 100 µL RNase-free water, followed by the addition of 10 µL 3M sodium acetate (pH 5.2) and 250 µL 100% ethanol. The samples were vortexed for 10 sec, incubated for 30 min at –20°C, and centrifuged at 12,000 × g for 15 min. A second wash was performed by aliquoting 1 mL of 75% cold ethanol after centrifugation at 14,000 × g for 5 min. The pellet was resuspended in RNase-free water, quantitated, and stored at –80°C.

RNA extracted from the posterior adductor muscle of adult clams ($n = 5$ noncancerous and $n = 5$ cancerous) included an additional PVP step in which 100 µL PVP/NaCl solution (18 M polyvinylpyrrolidone and 1.4 M NaCl) was added to 100 µL of sample and incubated for 10 min at room temperature. After centrifugation at 15,000 × g for 15 min at room temperature, 7.5 M LiCl was added to the supernate for a final concentration of at least 2.5 M LiCl. The samples were incubated for 45 min at –20°C and centrifuged for 20 min at 14,000 in 4°C. The resulting pellet was resuspended in RNase-free water, and its concentration quantified.

cDNA Synthesis and Real Time Quantitative PCR

Extracted RNA was treated with TURBO DNase (ThermoFisher) as per the manufacturer's directions followed by cDNA generation with ProtoScript II First Strand cDNA Synthesis Kit (New England BioLabs) as per the manufacturer's directions using random primers and the easy protocol. Reactions for real-time PCR included Luna Universal qPCR Master Mix (New England BioLabs), used as per the manufacturer's instructions, primers to amplify the *EF1*, and *Steamer* genes (Arriagada et al. 2014). The primers for *Steamer* were clamRT-F, 5′ tgc gtc gga aac cgg tct tgg; clamRT-R, 5′ caa cca ctc ggc gcc cgt at, and for the housekeeping gene *EF1* clamEF1F, 5′ gaa gga tga ggg aaa aga ggg; clamEF1R, 5′ cac att ttc ctg cta tgg tgc at working stock solutions of 100 nM for *Steamer* (clamRT-F/clamRT-R) and 200 nM for *EF1* (clamEF1F/clamEF1R).

Quantitative real-time PCR (qPCR) was performed in an Applied Biosystems 7300 fast real-time PCR system (ThermoFisher) using a thermoprofile of 95°C for 10 min and 40 cycles of 95°C for 10 sec and 60°C for 20 sec. Relative expression was calculated from the PCR efficiencies of *Steamer* and the housekeeping gene *EF1*, and the crossing point deviation of an unknown versus control sample as described by Pfaffl (2001).

RESULTS

Consistent with the pCO_2 measurements across treatments, the calculated pH_T for experimental seawater in those treatments representing normal pH_T varied from 8.06 to 8.08, whereas the low pH treatment group varied from 7.93 to 7.95 (Table 1). These values are consistent with current and predicted seawater pH_T for the B2 climate model (Caldeira & Wickett 2003).

The treatment effects on the expression levels of *Steamer,* relative to the housekeeping gene *EF1*, in adult clams with hemocyte leukemia were higher than those in normal clams but were not significantly different (two-tailed *t*-test, df 8, *t*-ratio = –1.294, $P = 0.23$) from each other (Fig. 1). All juvenile clams used in the experiment were noncancerous based on the cytological examination as described earlier by Taraska and Böttger (2013), and the treatment effects on the expression of *Steamer* were found to be significant (ANOVA: $F_{3,77} = 3.112$, $P = 0.031$). Multiple comparison testing showed that there was a significant interaction between temperature and pH (Tukey's HSD, $P < 0.05$) such that the independent effects of temperature and pH can no longer be considered as they are confounded. The AmbLow treatment exhibited the highest level of expression for *Steamer* (Tukey's HSD, $P < 0.05$), whereas the HiLow treatment had significantly lower expression (Tukey's HSD, $P < 0.05$). The Control and HiNorm showed intermediate

TABLE 1.

Values for directly measured and calculated carbonate chemistry parameters for the experimental treatments ($n = 3$ each treatment). Parameters of carbonate seawater chemistry were calculated from total alkalinity, pCO_2, temperature and salinity using the free-access CO2Calc package.

Treatment	TA (±SD)* (μmol kg^{-1} SW)	DIC (±SD)† (μmol kg^{-1} SW)	pCO_2 (±SD)* (μatm)	pH_T†	Ω_{Ca}†	Ω_{Ar}†
Ambient T°C and pH (AmbNorm)	2,272 ± 16	2,067 ± 23	369 ± 15	8.076	3.607	2.298
Ambient T°C and low pH (AmbLow)	2,401 ± 12	2,252 ± 23	573 ± 17	7.930	2.860	1.822
High T°C and Ambient pH (HiNorm)	2,198 ± 19	1,981 ± 27	377 ± 21	8.055	3.820	2.455
High T°C and low pH (HiLow)	2,418 ± 21	2,235 ± 12	555 ± 24	7.948	3.426	2.202

SW, seawater; DIC, dissolved inorganic carbon; pH_T, total scale; Ω_{Ca}, saturation state of calcite; Ω_{Ar}, saturation state of aragonite.
* Directly measured.
† Calculated.

levels of expression (Fig. 2). Taken together, the expression of *Steamer* in juvenile clams changes when exposed to pH and temperature for the year 2100, but only the low pH treatment is distinguishable from the other treatment groups.

DISCUSSION

Adult clams known to be cancerous express higher levels of the retrotransposon *Steamer* than noncancerous adult clams, but not significantly so in this study (Fig. 1). Adult clams in this study encompassed individuals in a moderate to fully leukemic state based on the morphology of the hemocytes. This variability in expression of *Steamer* has been previously shown (*e.g.*, Arriagada et al. 2014) and illustrates the lack of a consistent relationship between *Steamer* expression and cancerous state of the individual clams (see Fig. 2, Arriagada et al. 2014), where even moderately cancerous adult clams are not significantly different from noncancerous clams in their levels of *Steamer* expression. Juvenile clams less than 20 mm SL used here were free of hemocyte leukemia and have been shown in other studies not to develop leukemia after injection of leukemic hemolymph filtered at 0.2 μm (Taraska & Böttger 2013). Despite this, all clams used in this study expressed the retrotransposon *Steamer*, as has been shown in other studies on the hemocytes of noncancerous *Mya arenaria* (Arriagada et al. 2014, Metzger et al. 2015) and other species of bivalves (Metzger et al. 2016, 2018, Paynter et al. 2017). Exposure to predicted pCO_2 concentrations and temperatures for the year 2100, based on the B2 climate model, had a significant effect on *Steamer* expression (Fig. 2). Expression levels generally observed in this study were in the same range as observed in Arriagada et al. (2014) for normal clam hemocytes and for a large number of clams with hemocyte leukemia in that study. In this experiment, exposure to both higher temperatures and lower pH resulted in the lowest expression of *Steamer*, even lower than the control conditions,

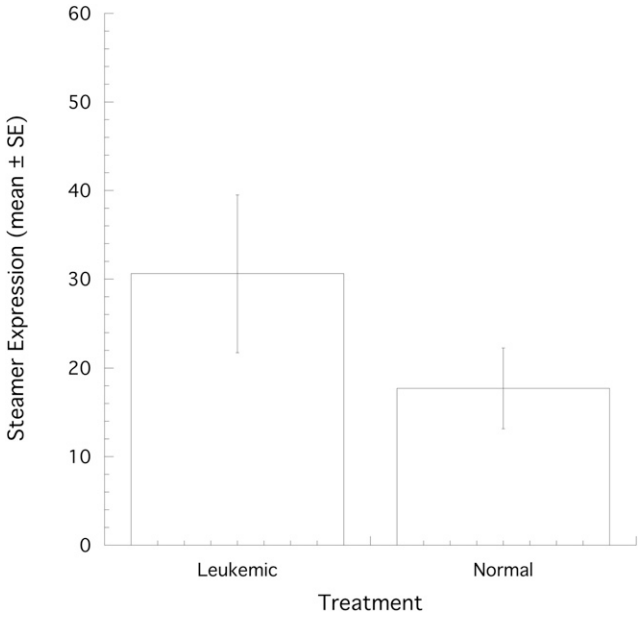

Figure 1. *Steamer* expression in adult softshells *Mya arenaria*. There were no significant differences (two-tailed *t*-test, df 8, *t*-ratio = –1.294, $P = 0.23$) between leukemic and normal clams.

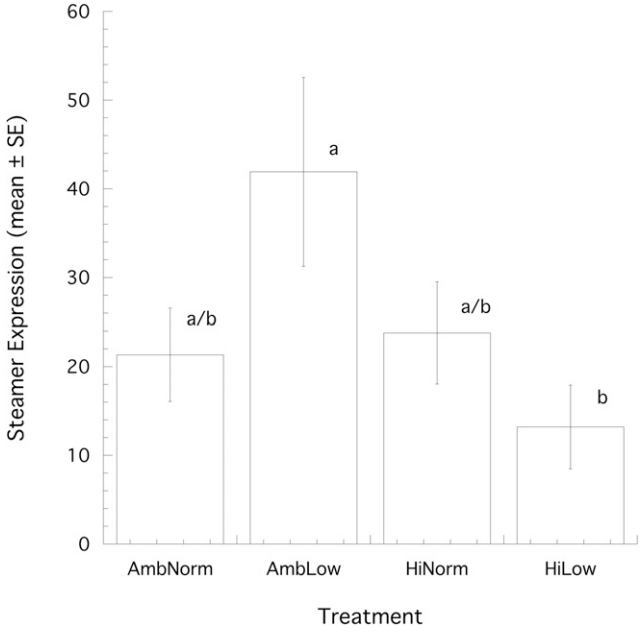

Figure 2. *Steamer* expression in juvenile clams exposed to varying seawater temperatures and pH. Significant treatment effects (ANOVA: $F_{3,77} = 3.112$, $P = 0.031$) were detected, and superscripts indicate the results of multiple comparison testing (Tukey's HSD). Similar superscripts indicate no significant differences between treatments. AmbNorm, ambient temperatures and pH; AmbLow, ambient temperatures and low pH; HiNorm, high temperatures and normal pH; and HiLow, high temperatures and low pH.

whereas the highest levels of *Steamer* expression occurred in the low pH–alone treatment. Ries et al. (2009) observed that the calcification rate decreased significantly when exposed to a range of elevated pCO_2 conditions in several species of molluscs, including *M. arenaria,* which indicates a physiologically stressful exposure to low pH. Although the range of pCO_2 conditions used by Ries et al. (2009) and the experiment described here include the predictions of the B1 climate model, they do not reflect the most physiologically extreme pH conditions that softshells might be exposed to (Waldbusser & Salisbury 2014). In the coastal habitats of the GOM, the sediments and associated porewaters exhibit low pH values and saturation states as a result of riverine input of low pH water (Salisbury et al. 2008). This causes low rates of calcification and a significant source of seasonal juvenile mortality of *M. arenaria* (Salisbury et al. 2008, Green et al. 2009, Ries et al. 2009). Neither of the temperature regimes in these experiments are representative of the maximum seasonal temperature exposures that clams would experience in late summer during immersion at low tide (Newell & Hidu 1986).

It is unknown whether clam leukemia occurs as a result of the high expression of *Steamer*, which then leads to genomic instability, mutations, and oncogenesis (Burns 2017), or whether the development of hemocyte leukemia results in higher *Steamer* expression. Clam hemocyte leukemia has highly conserved mechanistic underpinnings with several human cancers (*e.g.*, acute myelogenous leukemia and colon carcinoma), and previous studies have identified many of the genes in the p53 apoptogenic pathways that are conserved between this protostome and deuterostome humans after 400M+ years of separation from a common ancestor (Walker et al. 2011). In clams without leukemia, the expression of the control gene of apoptosis, p53 (Kruiswijk et al. 2015, Kastenhuber & Lowe 2017), can induce apoptosis in the hemocytes of environmentally stressed animals before they become leukemic (Walker et al. 2011). This occurs as the p53 protein enters the nucleus of stressed normal cells and induces gene pathways related to the initiation of apoptosis, thus eliminating unhealthy cells in clams from *in vivo* natural populations before they become cancerous; however, in fully leukemic hemocytes, the p53 protein is blocked from entering the nucleus through its binding to an overexpressed cytoplasmic Hsp70 protein called mortalin (Kelley et al. 2011, Walker et al. 2011). This results in the retention and inactivation of the p53 protein in the cytoplasm. As a result, clam hemocytes become immortalized, their cell division proceeds without normal cell cycle controls, and a fatal leukemia results (Walker et al. 2006, Walker & Böttger 2008, 2011, 2012). So, the identified common denominators in the development of clam hemocyte leukemia are the overexpression of mortalin protein, p53 and associated proteins, and the viral retrotransposon, *Steamer*. Although the mechanistic understanding of the relationship between mortalin and p53 in the oncogenesis of normal to leukemic hemocytes is very good, the role of *Steamer* is not.

Steamer is a member of the widespread Gypsy long terminal repeat family of retrotransposons which make up less than 1% genomes that have been examined (Thomas-Bulle et al. 2018) and are found in many bivalve molluscs. An emerging theme in cancer biology, especially those cancers involving mutations to p53 that represent pathologies directly related to the presence and genomic instability created by retrotransposons, is that a major role of p53 is to constrain the expression of transposable elements which have p53 DNA-binding sites (Levine et al. 2016, Wylie et al. 2016, Tiwari et al. 2018). It follows that if retrotransposons can cause DNA damage or DNA damage occurs as a result of environmental stress such as exposure to thermal stress or ocean acidification, with increased levels of oxidative stress and subsequent damage to DNA (Lesser 2006), then p53 will be involved because of its central role in sensing and repairing DNA damage. In addition, retrotransposon integration may be interpreted by the cell as DNA damage such that p53 would also be involved (Tiwari et al. 2018). Wylie et al. (2019) showed experimentally, using gene complementation studies in *Drosophila* and zebrafish models, that normal p53 alleles could suppress transposons, but that mutant p53 alleles could not. The conclusions drawn from this study, and others, is that from an evolutionary perspective, p53 evolved to constrain retrotransposon expression and that when retrotransposons are activated in the presence of mutated p53, genomic instability occurs and oncogenesis can proceed (Wylie et al. 2016, Tiwari et al. 2018). For hemocyte leukemia in *Mya arenaria,* this appears to be a reasonable path of study on the etiology of this regularly fatal disease of adult clams.

For juvenile clams that do not develop hemocyte leukemia, the combined effects of temperature stress and pH do not elicit expression levels of *Steamer* that have been associated with the presence of clam hemocyte leukemia, and where higher levels of *Steamer* expression were observed (*i.e.*, low pH treatment), they compare well with the mean value reported previously in leukemic adult clams (Arriagada et al. 2014). Given previous results on the inability to induce hemocyte leukemia in juvenile clams (Taraska & Böttger 2013), there is no reason to assume that leukemia was induced in these juvenile clams under the experimental conditions described as there was less than 3% mortality in clams across all treatments during the experiment.

Whether the high level of expression reported here is evidence of genomic instability and oncogenesis in clams exposed to low pH, or part of a more general stress response to changes in the environment, is unknown. Retrotransposons, however, have been reported to be a component of the acclimation response (Casacuberta & Gonzáles 2013), making *Steamer* a potential candidate marker for the response to ocean acidification that deserves additional study.

CONCLUSIONS

1. Juvenile *Mya arenaria* have not been observed to have, or develop, hemocyte leukemia, but do express the retrotransposon *Steamer*.
2. Experimental exposure to predicted climate-related changes in temperature and pH for the GOM significantly induced *Steamer* expression, specifically in treatments where clams were exposed to low pH.
3. In juvenile individuals of *Mya arenaria,* and potentially other bivalves, *Steamer* may be a candidate biomarker for exposure to ocean acidification conditions, and additional studies should be carried out to develop this potential molecular marker.

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LITERATURE CITED

AboElkhair, M., S. Synard, A. Siah, J. Pariseau, J. Davidson, G. Johnson, S. J. Greenwood, J. W. Casey, F. C. Berthe & A. Cepica. 2009. Reverse transcriptase activity in tissues of the soft shell clam *Mya arenaria* affected with haemic neoplasia. *J. Invertebr. Pathol.* 102:133–140.

AboElkhair, M., T. Iwamoto, K. F. Clark, P. McKenna, A. Siah, S. J. Greenwood, F. C. Berthe, J. W. Casey & A. Cepica. 2012. Lack of detection of a putative retrovirus associated with haemic neoplasia in the soft shell clam *Mya arenaria*. *J. Invertebr. Pathol.* 109:97–104.

Arriagada, G., M. J. Metzger, A. F. Muttray, J. Sherry, C. Reinisch, C. Street, W. I. Lipkin & S. P. Goff. 2014. Activation of transcription and retroposition of a novel retroelement, *Steamer*, in neoplastic hemocytes of the mollusk *Mya arenaria*. *Proc. Natl. Acad. Sci. USA* 111:14175–14180.

Balch, W. M., D. T. Drapeau, B. C. Bowler & T. G. Huntington. 2012. Step-changes in the physical, chemical and biological characteristics of the Gulf of Maine, as documented by the GNATS time series. *Mar. Ecol. Prog. Ser.* 450:11–35.

Beal, B. F. & M. G. Kraus. 2002. Interactive effects of initial size, stocking density, and type of predator deterrent netting on survival and growth of cultured juveniles of the soft-shell clam, *Mya arenaria* L., in eastern Maine. *Aquaculture* 208:81–111.

Böttger, S. A., E. C. Jerszyk & C. W. Walker. 2008. Genotoxic stress-induced expression of p53 and apoptosis in leukemic clam hemocytes with cytoplasmically sequestered p53. *Cancer Res.* 68:777–782.

Burns, K. H. 2017. Transposable elements in cancer. *Nat. Rev. Cancer* 17:415–424.

Caldeira, K. & M. E. Wickett. 2003. Anthropogenic carbon and ocean pH. *Nature* 425:365.

Casacuberta, E. & J. González. 2013. The impact of transposable elements in environmental adaptation. *Mol. Ecol.* 22:1503–1517.

Crawford, D. L. & M. F. Oleksiak. 2016. Ecological population genomics in the marine environment. *Brief. Func. Genomics* 15:342–351.

Crespi, B. & K. Summers. 2005. Evolutionary biology of cancer. *Trends Ecol. Evol.* 20:545–552.

Dickson, A. G., C. L. Sabine & J. R. Christian. 2007. Guide to best practices for ocean CO_2 measurements. PICES Special Publication 3, 191 pp.

Green, M. A., G. G. Waldbusser, S. L. Reilly, K. Emerson & S. O'Donnell. 2009. Death by dissolution: sediment saturation state as a mortality factor for juvenile bivalves. *Limnol. Oceanogr.* 54:1037–1047.

House, M. L., C. H. Kim & P. W. Reno. 1998. Soft shell clams *Mya arenaria* with disseminated neoplasia demonstrate reverse transcriptase activity. *Dis. Aquat. Organ.* 34:187–192.

Kastenhuber, E. R. & S. W. Lowe. 2017. Putting p53 in context. *Cell* 170:1062–1078.

Kelley, M. L., P. Winge, J. D. Heaney, R. E. Stephens, J. H. Farrell, R. J. Van Beneden, C. L. Reinisch, M. P. Lesser & C. W. Walker. 2001. Expression of homologues for p53 and p73 in the softshell clam (*Mya arenaria*), a naturally-occurring model for human cancer. *Oncogene* 20:748–758.

Kruiswijk, F., C. F. Labuschagne & K. H. Vousden. 2015. p53 in survival, deth and metabolic health: a lifeguard with a license to kill. *Nat. Rev. Mol. Cell Biol.* 16:393–405.

Lesser, M. P. 2006. Oxidative stress in marine environments: biochemistry and physiological ecology. *Annu. Rev. Physiol.* 68:253–278.

Levine, A. J., D. T. Ting & B. D. Greenbaum. 2016. p53 and the defense against genome instability caused by transposons and repetitive elements. *BioEssays* 38:508–513.

Li, S., C. Mason & A. Melnick. 2016. Genetic and epigenetic heterogeneity in acute myeloid leukemia. *Curr. Opin. Genet. Dev.* 36:100–106.

Marusyk, A. & K. Polyak. 2010. Tumor heterogeneity: causes and consequences. *Biochim. Biophys. Acta* 1805:105–117.

Metzger, M. J., C. Reinisch, J. Sherry & S. P. Goff. 2015. Horizontal transmission of clonal cancer cells causes leukemia in soft-shell clams. *Curr. Biol.* 161:255–263.

Metzger, M. J., A. Villalba, M. J. Carballal, D. Iglesias, J. Sherry, C. Reinisch, A. F. Muttray, S. A. Baldwin & S. P. Goff. 2016. Widespread transmission of independent cancer lineages within multiple bivalve species. *Nature* 534:705–709.

Metzger, M. J., A. N. Paynter, M. E. Siddall & S. P. Goff. 2018. Horizontal transfer of retrotransposons between bivalves and other aquatic species of multiple phyla. *Proc. Natl. Acad. Sci.* 115:4227–4235.

Millero, F. J., T. B. Graham, F. Huang, H. Bustos-Serrano & D. Pierrot. 2006. Dissociation constants of carbonic acid in seawater as a function of salinity and temperature. *Mar. Chem.* 100:80–94.

Mills, K. E., A. J. Pershing, C. J. Brown, Y. Chen, F.-S. Chiang, D. S. Holland, S. Lehuta, J. A. Nye, J. C. Sun, A. C. Thomas & R. A. Wahle. 2013. Fisheries management in a changing climate: lessons from the 2012 ocean heat wave in the northwest Atlantic. *Oceanography* 26:191–195.

Newell, C. R. & H. Hidu. 1986. Species profiles: life histories and environmental requirements of coastal fishes and invertebrates (North Atlantic)—softshell clam. U.S. Fish Wildl. Serv. Biol. Rep. 82(11.53). U.S. Army Corps of Engineers, TR EL-82-4. 17 pp.

Norkko, J. & S. E. Shumway. 2011. Bivalves as bioturbators and bioirrigators. In: Shumway, S. E., editor. Shellfish Aquaculture and the Environment. Hoboken, NJ: Wiley Science Publishers. pp. 297–317.

Paynter, A. N., M. J. Metzger, J. A. Sessa & M. E. Siddall. 2017. Evidence of horizontal transmission of the cancer-associated *Steamer* retrotransposon among ecological cohort bivalve species. *Dis. Aquat. Organ.* 124:165–168.

Pershing, A. J., M. A. Alexander, C. M. Hernandez, L. A. Kerr, A. Le Bris, K. E. Mills, J. A. Nye, N. R. Record, H. A. Scannell, J. D. Scott, G. D. Sherwood & A. C. Thomas. 2015. Slow adaptation in the face of rapid warming leads to collapse of the Gulf of Maine cod fishery. *Science* 352:809–812.

Pfaffl, M. W. 2001. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* 29:e45.

Ries, J. B., A. L. Cohen & D. C. McCorkle. 2009. Marine calcifiers exhibit mixed responses to CO_2-induced ocean acidification. *Geology* 37:1131–1134.

Salisbury, J., M. Green, C. Hunt & J. Campbell. 2008. Coastal acidification by rivers: a threat to shellfish? *EOS Trans. Am. Geophys. Union* 89:513–515.

Selkoe, K. A. & R. J. Toonen. 2006. Microsatellites for ecologists: a practical guide to using and evaluating microsatellite markers. *Ecol. Lett.* 9:615–629.

Selkoe, K. A., C. D. D'Aloia, E. D. Crandall, M. Iacchei, L. Liggins, J. B. Puritz, S. von der Heyden & R. J. Toonen. 2016. A decade of seascape genetics: contributions to basic and applied marine connectivity. *Mar. Ecol. Prog. Ser.* 554:1–19.

Taraska, N. G. & S. A. Böttger. 2013. Selective initiation and transmission of disseminated neoplasia in the sift shell clam *Mya arenaria* dependent on natural disease prevalence and animal size. *J. Invertebr. Pathol.* 112:94–101.

Thomas-Bulle, C., M. Piednoël, T. Donnart, J. Filée, D. Jollivet & E. Bonnivard. 2018. Mollusc genomes reveal variability in patterns of LTR-retrotransposons dynamics. *BMC Genomics* 19:821.

Tiwari, B., A. E. Jones & J. M. Abrams. 2018. Transposons, p53 and genomic security. *Trends Genet.* 34:846–855.

Waldbusser, G. G. & J. E. Salisbury. 2014. Ocean acidification in the coastal zone from and organisms perspective: multiple system

parameters, frequency domains, and habitats. *Annu. Rev. Mar. Sci.* 6:221–247.

Walker, C. W. & S. A. Böttger. 2008. A naturally occurring cancer with molecular connectivity to human diseases. *Cell Cycle* 7:2286–2289.

Walker, C. W., S. A. Böttger & B. Low. 2006. Mortalin-based cytoplasmic sequestration of p53 in a non-mammalian cancer model. *Am. J. Pathol.* 168:1526–1530.

Walker, C. W., R. J. van Beneden, A. F. Muttray, S. A. Böttger, M. Kelley, A. E. Tucker & W. K. Thomas. 2011. p53 Superfamily proteins in marine bivalve cancer and stress biology. *Adv. Mar. Biol.* 59:1–36.

Walker, C. W., B. Low & S. A. Böttger. 2012. Mortalin in invertebrates and the induction of apoptosis by wild-type p53 following defeat of mortalin-based cytoplasmic sequestration in cancerous clam hemocytes. In: Kaul, S. C. & R. Wadhwa, editors. Mortalin biology: stress, life and death. Dordrecht, The Netherlands: Springer. pp. 97–113.

Wylie, A., A. E. Jones & J. M. Abrams. 2016. p53 in the game of transposons. *BioEssays* 38:1111–1116.

Wylie, A., A. E. Jones, A. D'Brot, W.-J. Lu, P. Kurtz, J. V. Moran, D. Rakheja, K. S. Chen, R. E. Hammer, S. A. Comerford, J. F. Amatruda & J. M. Abrams. 2019. p53 genes function to retrain mobile elements. *Genes Dev.* 30:64–77.