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The synergistic effects of elevated temperature and CO_2-induced ocean acidification reduce cardiac performance and increase disease susceptibility in subadult, female American lobsters *Homarus americanus* H. Milne Edwards, 1837 (Decapoda: Astacidea: Nephropidae) from the Gulf of Maine

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ABSTRACT

Increased greenhouse gas emissions have caused rapid ocean warming (OW) and reduced ocean pH via acidification (OA). Both OW and OA will likely impact marine crustaceans, but they are often examined in isolation. We conducted an environmental-stressor experiment to understand how exposure to current summer conditions (16 °C, pH 8.0), OW only (20 °C, pH 8.0), OA only (16 °C, pH 7.6), or both acidification and warming, OAW (20 °C, pH 7.6), differentially influence thermal physiology and immune response of female subadults of the American lobster, *Homarus americanus* H. Milne Edwards, 1837. Following a 42 d exposure, cardiac performance was assessed during an acute thermal stress, and lobsters were subjected to a subsequent 21 d pathogen challenge with the bacterium *Aerococcus viridans* var. *homari*, the causative agent of gaffkemia. Lobsters under OAW had significantly lower ($P \leq 0.02$) Arrhenius break temperatures (ABT), an indicator of thermal limits of capacity, compared to lobsters exposed to all other treatments, suggesting these stressors act synergistically to reduce physiological performance. Individuals from the OW and OAW treatments also had significantly lower ($P \leq 0.035$) total hemocyte counts (THCs), an indicator of immune response, and showed a reduced median time to death (by up to 5 d sooner) post *A. viridans* injection compared to lobsters exposed to current summer conditions. Moreover, nearly twice as many lobsters exposed to OAW lost at least one claw during the pathogen challenge compared to all other treatment groups, potentially increasing the risk of mortality due to secondary infection. Together, these results suggest that OAW will impact the physiology and immune response of subadult *H. americanus*, potentially influencing successful recruitment to the fishery.

Key Words: Arrhenius break temperature, global climate change, immunity, ocean acidification, ocean warming, thermal physiology

INTRODUCTION

Human-induced climate change due to increased input of greenhouse gasses into the atmosphere has impacted a diversity of marine and terrestrial systems and species (IPCC (Intergovernmental Panel on Climate Change), 2014). Mean global surface temperatures have increased sharply compared to pre-industrial times and are now warming at a rate of 0.2 °C per decade (IPCC, 2019). The world's oceans have taken up the majority of Earth's excess heat, resulting in a doubling in the ocean's rate of warming since 1993 and leading to an increase in both the frequency and intensity of marine heatwaves across the globe (IPCC, 2019; Smale *et al.*, 2019). The oceans have also

likely absorbed 20–30% of total anthropogenic carbon dioxide (CO_2) emissions since the 1980s, resulting in a decline in surface seawater pH of 0.017–0.027 pH units per decade and more widespread ocean acidification events (IPCC, 2019). If greenhouse gas emissions continue to increase, it is likely that global water temperatures at the surface of the ocean will increase by 3.2–5 °C and pH levels will fall from ~8.0 to ~7.7 pH units by the year 2100 (following the RCP (representative concentration pathway) 8.5 emission scenario; IPCC, 2019).

Both ocean acidification (OA) and ocean warming (OW) have been linked to widespread impacts on marine organisms. Although some crustaceans may benefit from reduced larval development times and/or accelerated growth under warming conditions (MacKenzie, 1988; Arnberg *et al.*, 2013; Harrington *et al.*, 2019), OW has been linked to changes in phenology and geographic distribution in a number of species across taxa (Maynard *et al.*, 2016; IPCC, 2019; Schuetz *et al.*, 2019; Staudinger *et al.*, 2019), as well as increased levels of cellular stress (Somero *et al.*, 2017), disease susceptibility (Groner *et al.*, 2018; Shields, 2019), and elevated metabolic rates to meet the energetic demands of warming (Sokolova *et al.*, 2012; Pörtner *et al.*, 2017). Marine calcifiers may be particularly at risk to negative impacts of OA due to reduced growth, development, and calcification rates under a low pH environment (Kroeker *et al.*, 2013; Browman, 2016). OA, however, may also negatively impact a variety of other processes, including host-pathogen dynamics (Hernroth & Baden, 2018; Shields, 2019), internal chemistry (Klymasz-Swartz *et al.*, 2019), and energy allocation (Pan *et al.*, 2015). The impacts of OA may also be made worse under a warming ocean (ocean acidification and warming (OAW)), resulting in potential tradeoffs between various biological processes (Pörtner *et al.*, 2017; Hernroth & Baden, 2018). It is therefore critical to shift from single- to multi-stressor studies when making predictions about how organisms will respond to a changing environment (Przeslawski *et al.*, 2015; Hurd *et al.*, 2019).

We explored the potential individual and interactive effects of OAW on subadult American lobsters, *Homarus americanus* H. Milne Edwards, 1837. The American lobster sustains the most economically valuable fishery in the northeastern United States and Atlantic Canada (Steneck *et al.*, 2011). Distributed from North Carolina, USA to Newfoundland, Canada, a large portion of the *H. americanus* population is located within the Gulf of Maine, a region of the northwestern Atlantic that is experiencing some of the most accelerated rates of warming (Pershing *et al.*, 2015; Thomas *et al.*, 2017). The Gulf of Maine may also be vulnerable to both global and coastal acidification events due to high freshwater input and nutrient loading into the region (Gledhill *et al.*, 2015). A number of previous studies have explored the individual impacts of OA or OW on early life history stages (Keppel *et al.*, 2012; Barret *et al.*, 2017; McLean *et al.*, 2018; Harrington *et al.*, 2019; Menu-Courey *et al.*, 2019) and adults (Dove *et al.*, 2005; Camacho *et al.*, 2006; Ries *et al.*, 2009; Ries 2011), but few have explored the combined effects of OAW (Waller *et al.*, 2017; Klymasz-Swartz *et al.*, 2019). Further, the impacts of OAW on larger, adult *H. americanus* followed a short-term exposure (i.e., 14–16 d; Klymasz-Swartz *et al.*, 2019), potentially limiting the ability to determine longer-term acclimatory responses.

We previously demonstrated that pre-reproductive (subadult) *H. americanus* exposed to a low pH environment exhibited reduced physiological performance during acute thermal stress and reduced total hemocyte counts (THCs) as an indicator of immune response (Dove *et al.*, 2005; Hernroth *et al.*, 2012; Day *et al.*, 2019), suggesting the potential for increased disease susceptibility (Harrington & Hamlin, 2019). We describe herein a fully factorial experiment designed to further understand how a moderate duration exposure (i.e., 42 d) to predicted end-century warming and/or acidification influences cardiac performance during thermal stress, THCs, and survival during a subsequent pathogen challenge with *Aerococcus viridans* var. *homari*, a gram positive, tetrad-forming coccus bacterium that is the causative agent of gaffkemia (Stewart *et al.*, 2004; Cawthorn, 2011). Generally described as an impoundment disease (Bayer & Daniel, 1987), *A. viridans* has not been detected in the post-capture Maine lobster population or in holding facilities of Atlantic Canada since the early 2000s (Basti *et al.*, 2010; Bouchard *et al.*, 2010) and estimates of infection in the natural population are generally low (Stewart *et al.*, 1966; Lavallée *et al.*, 2001). Prevalence estimates in nature may be difficult to obtain, however, because infected lobsters exhibit lethargic behavior and reduced appetite, decreasing the likelihood of entry into experimental collection traps (Lavallée *et al.*, 2001; Cawthorn, 2011; Davies & Wootton, 2018). We chose *A. viridans* as a model infectious agent to more fully understand the potential impacts of OAW on the host-pathogen dynamic in *H. americanus* because the progression of gaffkemia is accelerated under warmer temperatures and results in observable behavioral impacts on infected lobsters (Stewart, 1975; Battison *et al.*, 2004; Robohm *et al.*, 2005). Our research aims to address the impacts of OAW on the physiological performance and immune response of the relatively understudied pre-reproductive stage of *H. americanus* to begin to more fully understand how a changing environment might influence this iconic fishery.

MATERIALS AND METHODS

Animal collection and husbandry

Female, subadult (50–60 mm carapace length (CL)) lobsters were obtained from the Maine Department of Marine Resources (ME DMR) Ventless Trap Survey in two collection rounds across sites in mid-coast Maine. Round 1 occurred on 24 April 2019, with lobstermen based out of Tenants Harbor, ME; round 2 occurred on 20 June 2019, with lobstermen based out of South Bristol, ME (Fig. 1). Following previous work (Harrington & Hamlin, 2019), we targeted the subadult size class as these individuals are in the process of sexually maturing, and it is possible that any negative effects of environmental stressors on the fitness of this stage could hinder reproductive success. We also chose only females to control for any potential sex-specific responses to environmental stressors (e.g., Ellis *et al.*, 2014).

Lobsters were held at the Aquaculture Research Center (ARC), Orono, ME, in recirculating holding tank systems filled with artificial seawater (Crystal Sea® Marine Mix; Marine Enterprises International, Baltimore, MD, USA). The tank systems were initially set to temperatures matching the natural environment from which individuals were collected, and lobsters were slowly acclimated to laboratory conditions (temperature 16 °C, pH ~8.0, salinity 35 ppt) over 17 weeks prior to use in experiments. Holding tanks were monitored twice daily for temperature, dissolved oxygen content (DO mg l^{-1} and % saturation), and salinity, and weekly for water quality. Lobsters were monitored twice daily to assess condition, and all molting events were recorded. Lobsters were fed a mixed diet of herring, shrimp, and invertivore gel (Repashy Bottom Scratcher, Repashy Ventures, Oceanside, CA, USA) every 2 d, but were not fed for at least 48 h prior to entering the environmental stress exposure experiment.

System design

We used four 708 l recirculating systems with four replicate and sealed 75 l tanks at the ARC to explore the independent and interactive effects of acidification and warming on subadult lobsters. Each system was randomly assigned one of four environmental-stressor treatments (Fig. 1): current summer conditions (16 °C, pH ~8.0; "current"); current summer temperatures but projected end-century acidification (16 °C, pH ~7.6; ocean acidification only, "OA"); projected warming but current pH (20 °C, pH ~8.0; ocean warming only, "OW"); or projected warming and acidification

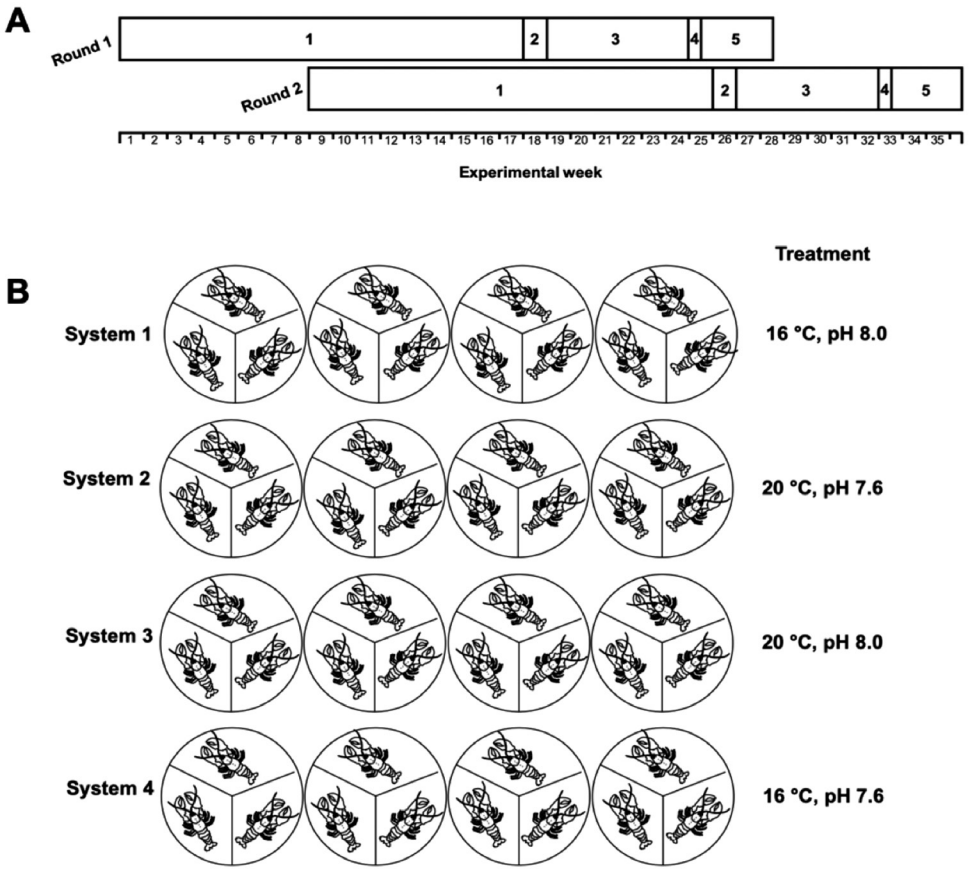

Figure 1. Experimental design assessing impacts of ocean acidification and warming on subadult *Homarus americanus*. Timeline for the components of the experiment (**A**): subadult lobsters were collected on April 24 and June 20, 2020 for rounds 1 and 2, respectively, and acclimated to laboratory conditions for 17 weeks prior to experimentation (1); lobster hemolymph was then sampled and lobsters measured for size pre-exposure, and then allowed to acclimate to environmental-stressor conditions over seven days (2); lobsters were fully exposed to one of four environmental-stressor combinations for a total of 42 d, after which post-exposure hemolymph sampling occurred (3); all lobsters were reacclimated to 16 °C over four days, and cardiac performance was assessed over the course of an additional four days during the temperature ramping experiment (4); and lobsters entered the 21 day pathogen challenge (5). Schematic of the four systems used in the environmental-stressor exposure (**B**). Each system was randomly assigned one of four temperature and pH treatments, and replicate treatment tanks ($N = 4$) were subdivided to house three lobsters each. Systems were reassigned a different temperature and pH treatment for the second experimental round.

(20 °C, pH ~7.6; ocean acidification and warming, "OAW"). We chose 16 °C and a pH of ~8.0 for our current conditions based on Gulf of Maine-wide measurements and estimates (Northeastern Regional Association of Coastal Ocean Observing Systems (www.neracoos.org); Salisbury & Jönsson, 2018). We chose a pH of 7.6 and a temperature of 20 °C for our end-century conditions following the RCP8.5 emission scenario (IPCC, 2019), and to target an aragonite saturation state (Ω_{arag}) that would be considered corrosive (Gledhill *et al.*, 2015). Systems and treatments were maintained by modifying the design of Harrington & Hamlin (2019). Replicate-treatment tanks were subdivided to house three lobsters each for a total of 12 individuals per system (Fig. 1). The structured barriers within each tank prevented lobsters from physically interacting with or visibly observing each other. Artificial seawater (Tropic Marin® Pro Reef Salt; Tropic Marin USA, Montague, MA, USA) was premixed (salinity 35 ppt, pH 8.1) and used in each system. System temperatures were maintained using Delta Star® in-line chillers (Aqua Logic, San Diego, CA, USA). Honeywell Durafet pH electrodes (Honeywell, Morris Plains, NJ, USA) were used in combination with a PENTAIR Point Four™ remote interface unit (RIU; Pentair Aquatic Eco-Systems, Cary, NC, USA) to monitor and maintain desired pH values. Carbon dioxide was removed from all incoming air prior to use in these systems with a Puregas™ CAS1-11 CO_2 Adsorber/Dryer (PUREGAS, Broomfield, CO, USA). The pCO_2 was monitored in the sealed head space of each tank using a LI-COR® LI-840A CO_2/H_2O gas analyzer (LI-COR Biosciences, Lincoln, NE, USA).

Environmental stress exposure

In each round, lobsters were randomly assigned one of the four environmental-stressor treatments ($N = 12$ lobsters per treatment, per round). Prior to beginning the experiment, all lobsters were measured for size (CL), assessed for any damage to the exoskeleton, and hemolymph was sampled (Fig. 1). Only lobsters without visible signs of damage or lesions were used in experiments, but any missing appendages (e.g., walking legs or shortened antennae) were noted. Following Harrington & Hamlin (2019), the dorsal side of the abdomen (between tergites) was disinfected with a sterile cotton ball soaked in 70% ethanol prior to collecting 200 µl of hemolymph using a 26 gauge needle and sterile 2.0 ml syringe. Hemolymph was placed in a glass vial containing 800 µl of fixative (10% buffered formalin in filtered, sterilized seawater) and used to perform total hemocyte counts (THCs) as an estimate of stress level and immune function. Briefly, three replicate 10 µl subsamples per lobster hemolymph sample were placed on KOVA Glasstic® Slide 10 with Grids hemocytometers (Kova International, Garden Grove, CA, USA) and the total number of hemocytes was counted three times and averaged. Final counts were averaged across subsamples and standardized to account

for the dilution of hemolymph in fixative (Harrington *et al.*, 2019; Harrington & Hamlin, 2019).

Following the pre-exposure sampling, lobsters were placed into treatment tanks and acclimated to experimental conditions (i.e., increasing temperature and/or decreasing pH from holding conditions) over the course of one week. Lobsters were then fully exposed to the environmental-stressor treatments for a total of 42 d. We recorded tank temperature, pH, dissolved oxygen content, pCO_2, and salinity daily; assessed lobster activity and overall health twice daily and fed lobsters every 2 d; monitored total ammonia nitrogen (TAN), nitrite, and nitrate using a LaMotte SMART 3 Colorimeter (LaMotte Co., Chestertown, MD, USA) and performed water changes twice weekly to maintain low nitrate concentrations in systems; and collected water samples to determine total alkalinity via titration twice weekly (Riebesell *et al.*, 2011). At no time during experiments did concentrations of TAN, nitrite, and nitrate reach toxic concentrations with respect to temperature and pH. We used CO2SYS to calculate carbonate chemistry in our tanks (Pierrot *et al.*, 2006), with constants from Mehrbach *et al.* (1973) that were refit by Dickson and Millero (1987), $KHSO_4$ from Dickson (1990), and [B]T from Uppström (1974) (Table 1).

At the conclusion of the 42 d exposure period, lobsters were again measured for size, and any damage was noted and compared to pre-exposure observations. Hemolymph was sampled as described above to provide "post-exposure" THCs. An additional 10 lobsters per round were maintained in holding trays under laboratory-acclimated conditions throughout the environmental stress exposure and subsequent pathogen challenge, and only handled during the pre- and post-exposure sampling periods. Lobsters used as handling controls were sampled for THCs and used to account for potential handling stress during the environmental stress exposure, and to provide an estimate of laboratory-based levels of mortality and/or molting throughout the entirety of the experiment.

Temperature ramping experiment

We conducted a thermal-challenge experiment following the environmental-stressor exposure to more fully understand the sublethal impacts of ocean acidification and warming on subadult lobsters. Following the post-exposure sampling period of each round, lobsters were returned to treatment tanks where pH values were maintained but temperatures of all systems were brought to 16 °C over the course of 4 d (Fig. 1). This was accomplished by lowering the temperature of the chiller units in the 20 °C treatment systems by 1 °C per day, resulting in a gradual decrease in temperature that was realized throughout the entire system over the course of several hours. We then used impedance pneumography to assess cardiac performance during a temperature ramping experiment for each lobster individually (Braby & Somero, 2006; Camacho *et al.*, 2006; Harrington & Hamlin, 2019; Harrington *et al.*, 2020). Following the detailed protocol of Harrington *et al.* (2020), a 1.6 mm drill bit was used to carefully hand-drill two small holes nearly through the carapace on either side of the pericardial space. A sterile dissecting needle was used to finish the holes, into which electrodes (36–38 gauge magnetic wire) were carefully placed. Electrodes were secured using a small drop of cyanoacrylate glue, and lobsters were then placed into an acclimation bath (16 °C) for ~15 min. Lobsters were then moved into the experimental arena where temperature was increased from 16 °C to ~30 °C over a 2 h period using a Fisher Scientific™ Isotemp™ refrigerated/heated bath circulator (Fisher Scientific, Hampton, NH, USA). Since *H. americanus* is capable of acclimating to and surviving at temperatures up to 30 °C (McLeese, 1956), we chose ~30 °C as our upper thermal limit to ensure lobsters experienced a thermally stressful scenario

Table 1. Water chemistry in systems over the course of the environmental-stressor experiment (mean ± SE) for round 1 and round 2. Current summer conditions; OA, ocean acidification only; OW, ocean warming only; OAW, combined effects of both ocean acidification and warming. Parameters calculated using CO2SYS are indicated by an asterisk (*).

Parameter	Current	OA	OW	OAW
Round 1				
Salinity (ppt)	35 ± 0.03	35 ± 0.01	35 ± 0.01	35 ±0.02
Dissolved oxygen (mg l^{-1})	8.67 ± 0.02	8.63 ± 0.02	7.92 ± 0.02	7.88 ± 0.01
Dissolved oxygen (%saturation)	109 ± 0.21	109 ± 0.21	108 ± 0.24	107 ± 0.19
Temperature (°C)	16.1 ± 0.006	16.1 ± 0.005	20.3 ± 0.010	20.4 ± 0.007
pH_{NBS}	8.08 ± 0.010	7.60 ± 0.005	7.91 ± 0.005	7.60 ± 0.004
A_T (µmol kg^{-1})	2,417.5 ± 54	2,326.0 ± 43	2,407.3 ± 46	2,390.1 ± 51
$*HCO_3^-$ (µmol kg^{-1})	1,962.4 ± 36	2,174.1 ± 40	2,085.7 ± 41	2,231.9 ± 49
$*CO_3^{2-}$ (µmol kg^{-1})	1,86.1 ± 16	61.6 ± 2	131.7 ± 6	64.7 ± 2
$*\Omega_{calcite}$	4.44 ± 0.39	1.47 ± 0.05	3.15 ± 0.15	1.55 ± 0.04
$*\Omega_{aragonite}$	2.86 ± 0.25	0.94 ± 0.031	2.05 ± 0.10	1.00 ± 0.03
$*pCO_2$ (ppm)	415.4 ± 45	1,414.5 ± 41	750.5 ± 47	1,709.2 ± 50
Round 2				
Salinity (ppt)	35 ± 0.03	35 ± 0.03	35 ± 0.03	35 ±0.03
Dissolved oxygen (mg l^{-1})	8.98 ± 0.01	9.04 ± 0.01	8.40 ± 0.01	8.35 ± 0.01
Dissolved oxygen (% saturation)	112 ± 0.10	114 ± 0.11	113 ± 0.12	113 ± 0.11
Temperature (°C)	16.1 ± 0.006	16.3 ± 0.003	20.0 ± 0.018	20.2 ± 0.023
pH_{NBS}	8.07 ± 0.003	7.60 ± 0.003	8.00 ± 0.003	7.60 ± 0.002
A_T (µmol kg^{-1})	2,421.4 ± 68	2,395.6 ± 81	2,423.5 ± 60	2,479.7 ± 67
$*HCO_3^-$ (µmol kg^{-1})	1,983.9 ± 61	2,234.7 ± 77	2,029.6 ± 55	2,311.4 ± 62
$*CO_3^{2-}$(µmol kg^{-1})	178.5 ± 6	65.5 ± 2	161.4 ± 6	69.4 ± 3
$*\Omega_{calcite}$	4.26 ± 0.14	1.56 ± 0.05	3.86 ± 0.14	1.66 ± 0.07
$*\Omega_{aragonite}$	2.74 ± 0.09	1.00 ± 0.04	2.51 ± 0.09	1.07 ± 0.06
$*pCO_2$ (ppm)	403.9 ± 19	1,409 ± 57	559.4 ± 33	1.695.3 ± 44

without exceeding the critical thermal maximum (Camacho *et al.*, 2006), potentially leading to death. Heart rate and arena temperature were recorded using a PowerLab® Data Acquisition System (ADInstruments, Colorado Springs, CO, USA; Harrington & Hamlin, 2019; Harrington *et al.*, 2020) and lobsters were returned to experimental tanks upon completion of the ramp. We were able to assess cardiac performance during a temperature ramp in a total of 12 lobsters per day using two experimental set-ups (see Harrington *et al.*, 2020), resulting in a total of 4 d needed to complete this experiment during each round (Fig. 1). Lobsters from each environmental-stressor experiment were distributed evenly across the 4 d period as well as time of day to account for potential biases. All but one lobster in experimental round 1 (from the current-conditions treatment) survived the ramping experiment and exhibited normal behavior within 24 h post-ramp.

Pathogen challenge

We conducted a discrete pathogen challenge following exposure to environmental stressors in addition to measuring THCs as an indicator of general immune function to understand the direct effects of acidification and warming on subsequent disease susceptibility (Fig. 1). *Aerococcus viridans* var. *homari* Rabin's strain was obtained from Dr. Spencer Greenwood (Department of Biomedical Sciences, Atlantic Veterinary College, University of Prince Edward Island, Charlottetown, PE, Canada) and prepared for use at the Diagnostic Research Laboratory (DRL), Orono, ME. The Rabin strain was cultured on trypticase soy agar (TSA) plates with 5% SBRC and 1.5% NaCl, checked for purity and then cryopreserved using CryoBeads™ (Hardy Diagnostics, Santa Maria, CA, USA) and stored at –80 °C. Beads were aseptically removed from cryopreservation and streaked onto TSA plates with 5% SBRC and 1.5% NaCl. Plates were incubated at 25 °C for 24–48 h, at which point growth was transferred to trypticase soy broth (TSB) with 1.5% NaCl using a sterile swab. TSB was incubated at 25 °C for an additional 18–24 h before it was aseptically transferred to a 50 ml conical tube and spun at 2,000 g for 10 min. The pelleted bacteria were then washed twice with a 3% NaCl solution, and bacteria were finally resuspended in 10 ml 3% NaCl. The titer of the bacterial suspension was calculated by performing a serial dilution in the 3% NaCl solution, plating 100 µl onto TSA plates with 5% SBRC and 1.5% NaCl, and incubating at 25 °C for 48 h. Colonies were counted to determine the colony forming units (CFUs). Due to a paucity of data working with subadult lobsters, we selected 1×10^5 CFUs per 100 µl as the target dose for this study as it represented an intermediate concentration compared to doses used in previous *A. viridans* infectivity studies on adults (Robohm *et al.*, 2005; Clark *et al.*, 2016). Given the target temperature for this study (see below; Table 2), we expected this dose would also result in a median survival time of at least 10 d (Robohm *et al.*, 2005), potentially allowing us to observe differential survival based on previous exposure to environmental stressors.

With the exception of the handling-control lobsters, all individuals used in each round were moved to the DRL within 24 h of completing the temperature ramping experiment at the ARC. Lobsters were carefully hand-netted and removed from tanks, tagged with a unique ID number using small cable ties, and placed in coolers. Although the drive to the DRL was approximately 5 min, lobsters were kept cool and moist using ice packs and paper towels that were dampened with system water. Lobsters were randomly assigned a tank position in one of two recirculating systems. Each system consisted of twelve 38 l holding aquaria that were subdivided to house pairs of lobsters such that agonistic interactions were minimized. In each round, lobsters from each environmental-stressor treatment were randomly assigned injection of either 100 µl *A. viridans* (target of 1×10^5 CFUs per ml in sterile 3% NaCl; Table 2) or a 100 µl sham injection (sterile 3% NaCl). We initially planned on randomly designating three lobsters per environmental-stressor treatment as shams (25% of total lobsters), but had to reduce this number to two in the current-conditions treatment of round 1 due to the mortality of one lobster during the temperature ramping study and an additional mortality during the first few days of the pathogen challenge. We therefore ensured that at least 20% of the lobsters in each treatment were assigned a sham injection (Table 3). Lobsters were injected using a 26 gauge needle and sterile 2.0 ml syringe at the base of the right leg in the fifth pair of walking legs. Systems were maintained at 15–16 °C and tank chemistry was checked daily (Table 2). The activity level of lobsters was recorded daily, and all dead lobsters were removed and sampled as close to time of death as possible.

Post mortality, the tissue between the cephalothorax and abdomen was disinfected using sterile cotton balls soaked in 70% ethanol and 100 µl hemolymph was removed using a 26 gauge needle and sterile 2.0 ml syringe. Several drops were plated and streaked onto a TSA plate with 5% SBRC and 1.5% NaCl and incubated at 20 °C for 24–48 h (Robohm *et al.*, 2005). Growth on the plates was categorized as positive (growth) or negative (no growth) within 48 h, and a Gram stain was performed on observed colonies to verify growth as *A. viridans*. Systems were maintained for 21 d post-injection, at which point all surviving lobsters were sacrificed and sampled as described.

Table 2. Water chemistry and actual dose levels of *Aerococcus viridans* var. *homari* for the two rounds of the pathogen challenge experiment (mean ± SE).

Parameter	Round 1	Round 2
Salinity (ppt)	35 ± 0.04	35 ± 0.01
Dissolved oxygen (mg l^{-1})	7.60 ± 0.02	7.40 ± 0.01
Temperature (°C)	15.4 ± 0.04	16.0 ± 0.02
Pathogen dose (CFUs 100 μl^{-1})	3.5×10^5	7.6×10^5

Data analysis

We used General Linear Models (GLMs) to determine the effects of tank, treatment, and round on the initial and final sizes (CL) of lobsters. We also performed bivariate correlation analyses between initial size, final size, ABT, and THC to determine the relationship between size and these biological endpoints. We used a GLM followed by post hoc LSD tests to determine the effects of tank, round, treatment, and the interactive effects of these parameters on THCs. Following Harrington & Hamlin (2019), we used a three-way mixed ANOVA to determine if lobster heart rate over the course of the temperature ramp was significantly impacted by round, treatment, or any interactions between terms. The three-way mixed ANOVA failed to meet the assumption of sphericity (Mauchly's test of sphericity: $\chi^2 = 346.09$, $df = 90$, $P < 0.001$), and we used the Greenhouse-Geisser correction when interpreting our test results (Maxwell & Delaney, 2004; Harrington & Hamlin, 2019). Heart rate data for each individual were also transformed and fit using a piecewise regression to determine the Arrhenius break temperature (ABT), the temperature at which heart rate begins to decrease with increasing temperature (Stenseng *et al.*, 2005; Camacho *et al.*, 2006; Harrington & Hamlin, 2019). We used a GLM followed by post hoc LSD tests to determine the effects of tank, round, treatment, and the interactive effects of these terms on ABTs. The assumptions of equal variance across groups and normality were assessed using Levene's test and Shapiro-Wilk's test, respectively ($P > 0.05$). Both THC and ABT data were $\log_{10}$-transformed to meet test assumptions. We observed no effect of tank (and no interactions with other terms) on any of the biological endpoints measured and as such omit this source of variability from our results.

Table 3. Results of the *Aerococcus viridans* var. *homari* pathogen challenge when replicate rounds were combined: number of subadult *Homarus americanus* that were sham injected (3% NaCl) or injected with *A. viridans* (Av); the number of *H. americanus* in each injection group that exhibited growth of *A. viridans* on blood agar plates; and cumulative mortality of those injected with *A. viridans* by environmental stressor treatment. Median days to death (DTD; 95% CI) and days to sustained lethargy (DTL; 95% CI) are reported from the Kaplan-Meier analyses (i.e., day at which 50% of population exhibited mortality or sustained lethargy) for only those lobsters injected with *A. viridans*. Current summer conditions; OA, ocean acidification only; OW, ocean warming only; OAW, combined effects of both ocean acidification and warming.

	N injected		*N* with growth		Av injected only		
Treatment	Sham	Av	Sham	Av	Mortality (%)	DTD	DTL
Current conditions	4	16	2	16	93.8	15 (9.8–20.2)	8 (4.5–11.5)
OA	6	16	2	16	75.0	16 (12.1–19.9)	8 (6.1–9.9)
OW	6	18	1	18	94.4	11 (8.5–13.5)	8 (5.3–10.7)
OAW	6	17	1	17	82.4	13 (8.9–17.0)	13 (11.4–14.6)
Total, round 1	11	32	4	32	81.2	19 (14.4–23.6)	9 (7.8–10.1)
Total, round 2	11	35	2	35	91.4	13 (8.0–18.0)	13 (8.7–13.4)

We used a Fisher's exact test to explore differences in overall survival across pathogen challenge rounds for lobsters injected with *A. viridans*. Since there was no significant effect of round on overall survival (see Results), data from the replicate rounds for each treatment were combined despite slight differences in tank chemistry (Table 2). We used a Pearson χ^2 test to further explore if pre-exposure to the various environmental-stressor treatments impacted overall survival in lobsters injected with *A. viridans*, although we note that ~50% of cells in our analysis exhibited expected cell counts < 5, suggesting caution when interpreting test results. Kaplan-Meier survival analysis (Kaplan & Meier, 1958) was used to compare time to death and time to sustained lethargy in lobsters infected with *A. viridans* (McLean *et al.*, 2018). Specifically, a log rank test was performed to determine if there were differences in the survival distributions of lobsters across rounds (i.e., treatments combined), and/or of lobsters pre-exposed to the different environmental stressors (i.e., rounds combined). Since lobsters suffering from gaffkemia may experience limb loss as a result of infection (Davies & Wootton, 2018), we used a Fisher's exact test to explore claw loss across rounds, and a Pearson χ^2 test to explore differences in claw loss across environmental-stressor treatments.

All statistical tests were performed using IBM® SPSS® Statistics Version 25 (IBM, 2017), with the exception of the piecewise regressions, which were performed using SigmaPlot Version 10.0 (Systat Software, San Jose, CA, USA).

RESULTS

Lobster size and condition

We observed no significant effect of round, treatment, or the interaction of these terms on either the initial or final sizes of lobsters used in this experiment (Supplementary material Table S1). Mean initial size (± SE) of lobsters in rounds 1 and 2 was 60.29 ± 0.65 and 61.22 ± 0.80 mm CL, respectively, and mean final size (± SE) for lobsters used in rounds 1 and 2 was 61.76 ± 0.83 and 61.22 ± 0.80 mm CL, respectively. There was no significant correlation between size and ABT (initial size: $r = 0.14$, $P = 0.18$; final size: $r = 0.12$, $P = 0.25$), but both initial and final size were significantly, negatively correlated with THC (initial size: $r = –0.37$, $P < 0.001$; final size: $r = –0.37$, $P < 0.001$). A total of 22 and 17 lobsters from rounds 1 and 2, respectively, molted during our experiment. Although the timing of these events was consistent across experimental rounds (i.e., all occurred late August through mid-October), all observed molts from round 1 occurred while lobsters were exposed to environmental stressors and those from round 2 occurred during the laboratory acclimation period prior to experimentation. We documented a total of five molting events in both the current and OW treatments during the exposure to environmental stressors of round 1, four in the OA treatment, and eight molting events in the OAW treatment. Importantly, we observed no significant damage to or formation of lesions on the exoskeletons of any lobsters over the course of both rounds of the environmental-stressor experiment.

Hemocyte counts

We were unable to process the pre-exposure THCs for the lobsters due to a sampling error in which samples were too clotted to count. With the post-exposure THCs, we observed no significant interactive effect of round and treatment (GLM: $F_{4,42} = 0.24$, $P = 0.91$) or individual effect of round (GLM: $F_{1,42} = 4.28$, $P = 0.06$) on mean values. Mean post-exposure THC was significantly different across treatments (GLM: $F_{4,42} = 6.92$, $P < 0.001$). The handling control lobsters exhibited a significantly higher mean post-exposure THC compared to all environmental stress treatments (post hoc LSD: $P < 0.05$; Fig. 2, Supplementary material Table S2), with the exception of the current-conditions treatment group (post hoc LSD: $P = 0.22$; Fig. 2, Supplementary material Table S2). Lobsters from the OA treatment were statistically similar to the current-conditions group (post hoc LSD: $P = 0.38$; Fig. 2, Supplementary material Table S2), although this treatment group had a mean post-exposure THC that was significantly less than the handling control group (post hoc LSD: $P = 0.04$; Fig. 2, Supplementary material Table S2). Both the OW and OAW treatment groups had significantly lower mean post-exposure THCs compared to both current and OA treatment groups and the handling control (post hoc LSD: $P \leq 0.035$; Fig. 2, Supplementary material Table S2), but they were not significantly different from one another (post hoc LSD: $P = 0.17$; Fig. 2, Supplementary material Table S2).

Cardiac performance

There was no significant three-way interaction between temperature, round, and environmental- stressor treatment on lobster heart rate during the temperature ramping experiment ($F_{7.96,63.68} = 0.89$, $P = 0.53$, $\eta^2 = 0.1$). There was a significant two-way interaction between round and treatment on lobster heart rate during the ramp ($F_{3,24} = 5.22$, $P = 0.006$, $\eta^2 = 0.40$). This was likely driven by the significant simple main effect of round only within the OA treatment ($F_{1,6} = 20.5$, $P = 0.004$, $\eta^2 = 0.77$; Bonferroni-adjusted alpha level of 0.025) as heart rate over the course of the ramp was not significantly different across rounds for any other treatment ($P > 0.20$; Fig. 3). There were no other interactive effects, but temperature significantly influenced lobster heart rate across all treatments and rounds ($F_{2,65,63.68} = 134.76$, $P < 0.001$, $\eta^2 = 0.85$) as expected (Fig. 3; Harrington & Hamlin, 2019).

We observed a significant effect of both treatment (GLM: $F_{3,24} = 13.10$, $P < 0.001$) and round (GLM: $F_{1,24} = 5.57$, $P = 0.027$) on mean ABT, but no interactive effect (GLM: $F_{3,24} = 0.94$, $P = 0.44$; Fig. 4). Lobsters from round 2 generally exhibited higher ABTs than those of round 1; however, differences in mean ABT based on environmental-stressor treatment were consistent across the two experimental rounds. Lobsters exposed to the OAW treatment had significantly lower ABTs than all other treatment groups (post hoc LSD: $P \leq 0.02$) with mean values (± SE) of 23.7 ± 0.85 and 25.7 ± 0.50 °C in rounds 1 and 2, respectively (Fig. 4). In contrast, lobsters exposed to the OW treatment exhibited significantly higher mean ABTs than the OA (post hoc LSD: $P = 0.003$) and the OAW (post hoc LSD: $P < 0.001$) treatment groups, whereby mean values were 27.5 ± 0.58 and 28.3 ± 0.50 °C in rounds 1 and 2, respectively (Fig. 4). The mean ABT of the OW group was not statistically significantly different from lobsters in the current-conditions treatment (post hoc LSD: $P = 0.54$) in which mean values were 27.3 ± 0.39 and 27.6 ± 0.36 °C in rounds 1 and 2, respectively (Fig. 4). Lobsters exposed to the OA treatment had significantly different ABTs compared to all other groups, (post hoc LSD: $P \leq 0.02$) whereby mean values were 25.7 ± 0.81 and 26.3 ± 0.52 °C in rounds 1 and 2, respectively (Fig. 4).

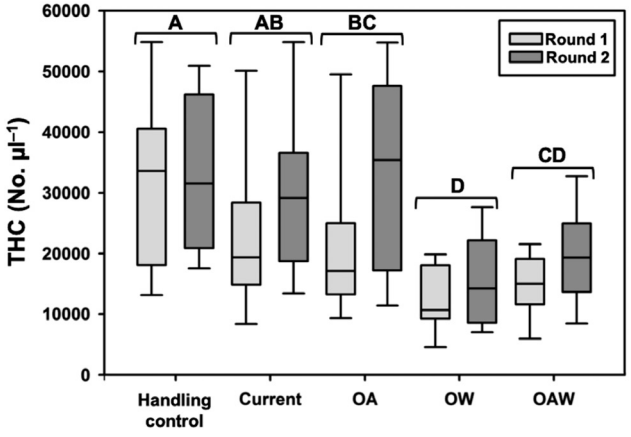

Figure 2. Post-exposure total hemocyte counts (THCs) for subadult *Homarus americanus*, rounds combined ($N \geq 10$ per treatment group). Letters indicated significant differences in mean THCs based on a GLM followed by post hoc LSD tests ($P < 0.05$). The upper and lower quartiles are represented by the top and bottom ends of each box, respectively, whereas the median is indicated by the solid, horizontal line within each box. Lines extending vertically from the box encompass variability outside of the upper and lower quartiles. THCs calculated from lobsters during the first experimental round are indicated by light bars, whereas those part of the second experimental round are indicated by dark bars.

Pathogen challenge

With the exception of one lobster in round 2, all lobsters given the sham injection survived and remained fully active during the 21 d pathogen challenge. When sampled upon the completion of the experiment, however, four of the shams in round 1 (36% of total) and two in round 2 (18% of total) exhibited positive growth on TSA plates with 5% SBRC and 1.5% NaCl that was confirmed via visual colony morphology, Gram stain, and microscopy to be *A. viridans* (Table 3). Of the lobsters injected with *A. viridans*, all mortalities and those that were sacrificed at the end of the 21 d challenge exhibited positive growth on blood agar plates that was confirmed to be *A. viridans* via visual colony morphology, Gram stain, and microscopy. We observed total mortality levels of 81.3% and 91.4% in rounds 1 and 2, respectively, but overall fate was not significantly different across rounds (Fisher's exact test: $P = 0.29$; Table 3). There were also no significant differences in overall mortality across environmental-stressor treatments (Pearson $\chi^2_3 = 3.77$, $P = 0.28$; Table 3).

The survival distributions of lobsters in each round of the pathogen challenge were not significantly different from one another (Kaplan-Meier log rank: $\chi^2_1 = 2.39$, $P = 0.12$). Survival distributions of *H. americanus* pre-exposed to the different environmental stressors were also not significantly different from one another (Kaplan-Meier log rank: $\chi^2_3 = 3.62$, $P = 0.31$; Fig. 5). Median time to death, however, was shorter in the warming treatments (OW and OAW) relative to current summer temperature treatments (current and OA) such that 50% of the warm treatment populations died 2–5 d sooner than the cooler temperature populations (Table 3). Although there was no significant difference in time to sustained lethargy across environmental-stressor treatments (Kaplan-Meier log rank: $\chi^2_3 = 6.10$, $P = 0.11$), the median amount of time it took lobsters to exhibit this behavior was 5 d longer in the OAW treatment compared to all other environmental-stressor treatments (Table 3).

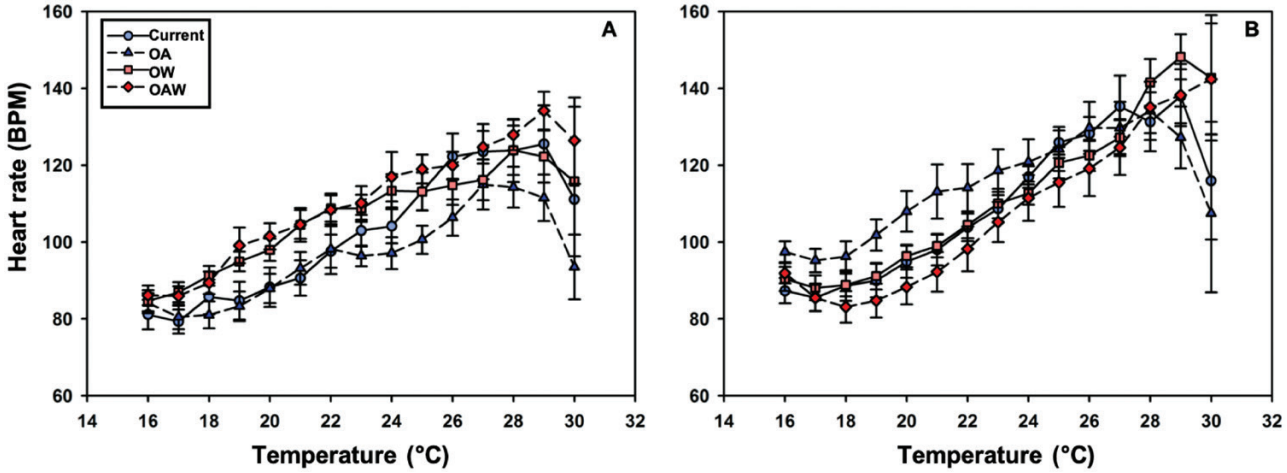

Figure 3. Mean heart rate (± SE) as beats per minute (BPM) over the course of the temperature ramp for subadult *Homarus americanus* exposed to various environmental-stressor treatments during round 1 (**A**) and round 2 (**B**). Light blue circles connected with a solid line represent lobsters exposed to current conditions, dark blue triangles connected by dashes represent lobsters exposed to ocean acidification (OA) only, pale red squares connected by a solid line represent lobsters exposed to ocean warming (OW) only, and bright red diamonds connected by dashes represent lobsters exposed to the combined effects of ocean acidification and warming (OAW) ($N = 12$ lobsters per treatment, per round). This figure is available in color at *Journal of Crustacean Biology* online.

There was no statistically significant difference in the number of lobsters infected with *A. viridans* that lost at least one claw across experimental rounds (Fisher's exact test: $P = 0.13$) and environmental-stressor treatments (Pearson $\chi^2_3 = 3.66$, $P = 0.30$). Nearly 53% of lobsters pre-exposed to OAW lost at least one claw during the pathogen challenge, a value that was nearly twice as large as those values calculated for all other treatment groups (Fig. 6). We observed no claw loss in any of the sham-injected lobsters. We also observed no mortalities and no loss of claws in any of the handling control lobsters monitored throughout the entirety of both rounds of this experiment.

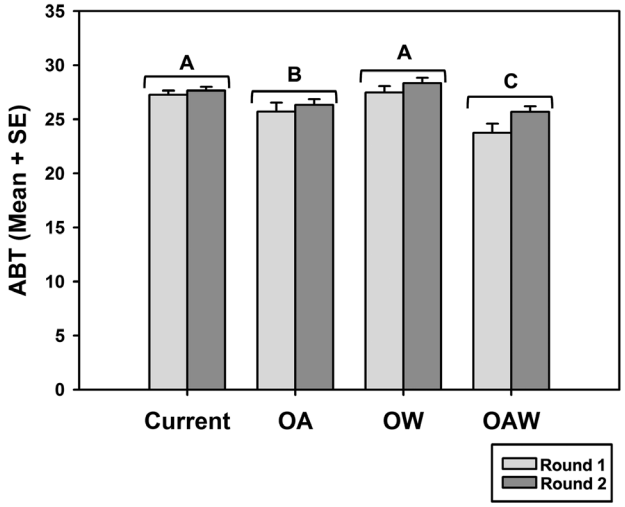

Figure 4. Mean Arrhenius break temperature (ABT +SE) in subadult *Homarus americanus* pre-exposed to different environmental-stressor conditions. Current, current summer conditions; OA, ocean acidification only; OW, ocean warming only; OAW, combined effects of both ocean acidification and warming. ABTs calculated from lobsters during the first experimental round are indicated by light bars, whereas those part of the second experimental round are indicated by dark bars ($N = 12$ lobsters per treatment, per round). Letters indicate significant differences in mean ABTs based on a GLM followed by post hoc LSD tests ($P < 0.05$).

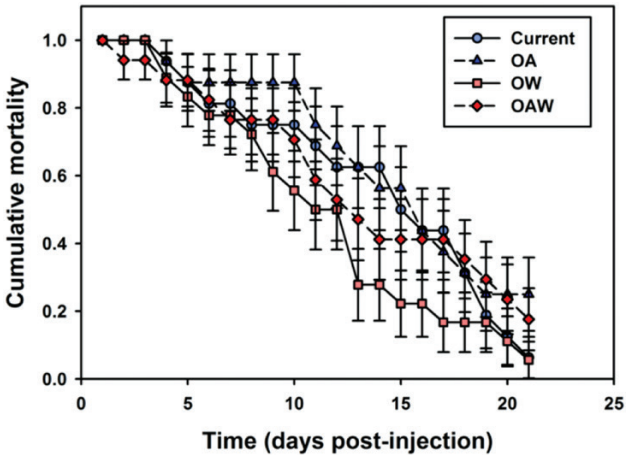

Figure 5. Kaplan-Meier estimates for cumulative mortality of both sham and subadult *Homarus americanus* infected by *Aerococcus viridans* var. *homari* infected over the course of the pathogen challenge (mean ± SE). Lobsters pre-exposed to current summer conditions (current) are represented by light blue circles connected with a solid line; those pre-exposed to ocean acidification only (OA) are represented by dark blue triangles connected by dashes; those pre-exposed to ocean warming only (OW) are represented by pale red squares connected by a solid line; and lobsters pre-exposed to both ocean acidification and warming (OAW) are represented by bright red diamonds connected by dashes. Replicate experimental rounds for each pre-exposure environmental-stressor treatment are combined ($N \geq 16$ lobsters per treatment). This figure is available in color at Journal of Crustacean Biology online.

DISCUSSION

This is the first study to explore the potential impacts of individual and/or interactive effects of ocean acidification and warming on the physiological scope and immune response in female, subadult *H. americanus*, a relatively understudied but important life-history stage. As expected, all treatment groups exhibited a significant increase in heart rate as temperature increased throughout the temperature ramp (Fig. 3), indicating an increase in metabolic rate to compensate for the increased energy demands of moderate thermal stress (Sokolova *et al.*, 2012; Pörtner *et al.*, 2017). Although we observed no significant interactive effect of temperature and environmental stress treatment on heart rate over the ramp, we detected significant differences in ABTs across treatments. Within both of the distinct temperature treatments (i.e., either 16 °C or 20 °C), mean ABT was significantly reduced in *H. americanus* exposed to acidified *versus* current pH conditions (Fig. 4), suggesting that exposure to OA alone significantly reduces thermal performance windows regardless of acclimation temperature. Further, in looking across all treatment groups, lobsters pre-exposed to OAW exhibited the lowest mean ABT, which was nearly 3 °C lower than the mean ABT of the current-conditions group (Fig. 4). This indicates that lobsters exposed to predicted end-century warming and acidification may be at risk of incurring greater energetic demands at lower temperatures relative to current conditions in order to deal will cellular stress and/or damage associated with acute warming events (Pörtner *et al.*, 2017; Somero *et al.*, 2017), which are also predicted to increase as a result of climate change (IPCC, 2019; Smale *et al.*, 2019). Together, these data support findings from Harrington & Hamlin (2019) that reduced pH alone narrows the thermal performance window, and also support the hypothesis that thermal performance windows are further compressed in the presence of multiple stressors (Sokolova *et al.*, 2012; Pörtner *et al.*, 2017). Most importantly, these results demonstrate that the

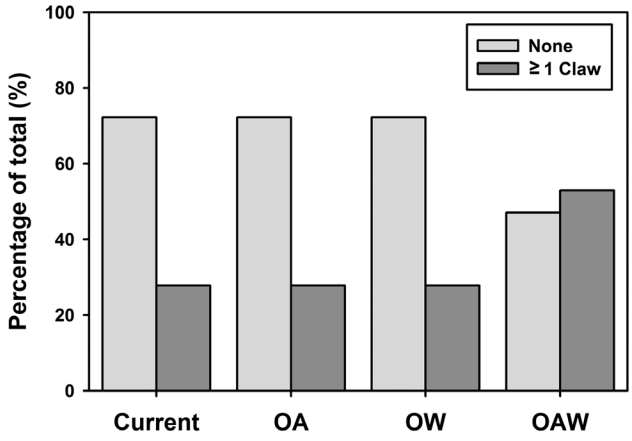

Figure 6. Claw loss in subadult *Homarus americanus* injected with *Aerococcus viridans* var. *homari* over the 21 d pathogen challenge grouped by pre-exposure to the environmental stressor treatments: current summer conditions (current), acidification only (OA), ocean warming only (OW), and both ocean acidification and warming (OAW). The percentage of total lobsters in each treatment that lost no claws is indicated by light bars, the percentage of lobsters that lost at least one claw by the dark bars ($N \geq 16$ lobsters per treatment).

combined effects of OAW on metabolic rates may be worse than the singular effects of either stressor as has also been demonstrated across a variety of other taxa, including the brachyuran crab *Hyas araneus* Linnaeus, 1758 (Walther *et al.*, 2009), the intertidal limpet *Cellana toreuma* (Reeve, 1854) (Wang *et al.*, 2018), and the fishes *Trematomus bernacchii* Boulenger 1902 (Davis et al., 2016, 2018) and *Boreogadus saida* (Lepechin, 1774) (Kunz *et al.*, 2018).

Adult *H. americanus* acclimated to temperatures similar to our OW treatment exhibited higher upper limits of cardiac function compared to those acclimated to cooler temperatures (Camacho *et al.*, 2006). We similarly expected lobsters from the OW treatment to exhibit significantly higher ABTs compared to the current-conditions treatment, but these groups were not statistically different (Fig. 4). Importantly, lobsters in the cooler temperature treatment of Camacho *et al.* (2006) were held at 4 °C, which greatly exceeded the temperature range assessed here. While reflective of the natural temperature gradient experienced across the species' distribution (Factor, 1995), Camacho *et al.* (2006) focused more on comparing acclimatory extremes and less on the physiological impacts of rising temperatures, which could explain the variation in results. It is also possible that the rapid rates of warming occurring in the Gulf of Maine, particularly during the summer and early fall (Thomas *et al.*, 2017; Fernandez *et al.*, 2020), as well as the two recent ocean heatwaves in 2012 and 2016 (i.e., temperatures above the 90th percentile of day-specific climatology for ≥ 5 d; Pershing *et al.*, 2018), have allowed lobsters to acclimatize to moderate periods of heat stress. We used the ABT as a non-lethal indicator of the thermal limit of capacity in order to ensure survival to the pathogen challenge, but it is possible that lobsters in the OW-only treatment may have exhibited a greater critical thermal maximum (CT_{max}: the upper limit of cardiac function, Pörtner *et al.*, 2017; Somero *et al.*, 2017) compared to the other groups. While including this metric may inform future efforts focusing on how rising temperatures will influence *H. americanus*, these lethal limits are often extreme and rarely encountered in the natural environment (Pörtner *et al.*, 2017) and would preclude the use of test subjects in additional experiments. It is also possible that lobsters in the 20 °C treatments were additionally stressed during the four-day adjustment to 16 °C in between the end sampling of the exposure to environmental-stress and the temperature ramping experiment (Fig. 1), which may have impacted their responses to the acute thermal stress.

Despite maintaining similar carbonate chemistry and temperature regimes for the environmental-stressor treatments across rounds, we did observe a significant effect of experimental round on mean ABT. Lobsters from round 1 generally exhibited lower ABTs than those from round 2 (Fig. 4), which we suggest may have been related to molt status. Although the total number of individuals that molted as well as the timing of molts were consistent across experimental rounds, all of the molting events from round 1 occurred while lobsters were exposed to environmental stressors and those from round 2 occurred during the laboratory acclimation period prior to experimentation. Molting is an energetically expensive process known to alter hemolymph chemistry and lobster behavior (Karnofsky *et al.*, 1989; Factor, 1995; Chang & Mykles, 2011), and the addition of environmental stressors may have resulted in metabolic suppression (Kelley & Lunden, 2017). We intended to run both experimental rounds over the spring and early summer months to avoid the major molting period, but system maintenance precluded us from achieving this timeline. Importantly, we did not observe any deaths related to molting, which may occur when lobsters are exposed to a low pH environment (Small *et al.*, 2016), and the pattern of differences in ABTs across environmental-stressor treatments were consistent across rounds (Fig. 4). Future efforts should benefit from direct assessment of molt condition, as well as greater control of the timing of exposure to environmental stressors to avoid or account for the peak time of molting in *H. americanus*; however, it is noteworthy that patterns of significance between treatments were consistent regardless.

Although we observed no significant differences in the estimates of the Kaplan-Meier survival curve based on previous environmental-stressor exposure, mean time to death in the pathogen challenge was consistent with previous investigations examining the Rabin's strain at a similar temperature (i.e., 12 d at 15 ± 0.5 °C: Stewart *et al.*, 2004). Previous research indicates that the rate of progression of gaffkemia is temperature-dependent (Battison *et al.*, 2004; Stewart *et al.*, 2004; Robohm *et al.*, 2005), but our results suggest that previous exposure to temperature stress may also increase susceptibility to *A. viridans*. Even though all lobsters were maintained at the same temperature during the pathogen challenge and all experienced a similar acute thermal stress during the temperature-ramping experiment, *H. americanus* pre-exposed to warmer temperatures (OW and OAW treatments) had a median time to death that was up to 5 d sooner than those exposed to current summer temperatures (current and OA treatments; Table 3). Lobsters from the warmer temperature groups also had mean THCs that were 1.5–2.0 times lower than those exposed to current summer temperatures (Fig. 2, Supplementary material Table S2). Although THCs would likely be impacted by the acute thermal stress, and conditions in lobster size and molt influence susceptibility to disease, these lower cell counts in the warmer-temperature treatments may have contributed to the progression of the disease. Phagocytosis of *A. viridans* by circulating hemocytes during infection proves ineffective and results in a decline of THCs, ultimately impairing the clotting mechanism of the hemolymph and increasing the risk of fatally hemorrhaging if wounded (Stewart, 1975; Stewart *et al.*, 2004; Cawthorn, 2011). It is therefore possible that *H. americanus* pre-exposed to predicted end-century warming were immunocompromised prior to the pathogen challenge due to temperature-related declines in the primary immune cells, which may have accelerated the invasion by *A. viridans*. Compounding low THCs, more than half of the lobsters pre-exposed to the OAW treatment lost at least one claw during the pathogen challenge (Fig. 6). Although not statistically significant, these data suggest a strong link between OAW exposure and claw loss during infection with *A. viridans*. American lobsters use their claws in a variety of ways, including grooming, mating, foraging and prey manipulation, protecting against predators, and during conspecific interactions (Factor, 1995). The loss of a claw therefore results in a number of potentially sublethal impacts on healthy *H. americanus* but may prove fatal during infection with *A. viridans* due to an increased risk of secondary infection and/or exsanguination (Stewart *et al.*, 2004; Cawthorn, 2011). Our work provides initial support for a connection between increased disease susceptibility under predicted climate change scenarios for subadult *H. americanus*, but future efforts should explicitly address how lobster size and condition, as well as acclimation time, influence this relationship. We also acknowledge that the used dose may have been too high to detect potential impacts of environmental stressors on susceptibility to *A. viridans* in subadult lobsters, and suggest investigating a range of doses in future efforts.

THCs have been used as indicators of immune function in a variety of lobsters (*H. americanus* (Battison et al., 2003, 2004), *H. gammarus* (Linnaeus, 1758) (Hernroth et al., 2012, 2015), and *Jasus edwardsii* (Hutton, 1875) (Day *et al.*, 2019)), but they are notoriously variable within an individual over time and across sampling locations. We were unable to process our pre-exposure THC samples to account for this individual variability, but the handling control group acted as sentinels for comparison throughout the entirety of the experiment. Despite the high variability in THCs, all lobsters exposed to any combination of predicted end-century conditions exhibited significantly lower THCs relative to the handling controls (Fig. 2), suggesting that both the individual and interactive effects of OAW may result in stress such that THCs begin agglutinating in response to cellular damage (Battison *et al.*,

2003; Zhou *et al.*, 2018). Similar to the current-conditions treatment, we found that mean THC in the handling control group was 2.1–2.5 times larger than OW treatment, and 1.7–2.1 times larger than OAW (Supplementary material Table S2). We expected THCs in lobsters exposed to predicted end-century warming to be higher relative to those lobsters exposed to current summer temperatures (as seen by Dove *et al.*, 2005). Individuals of *H. americanus* from the Dove *et al.* (2005) study, however, were collected from the southern portion of the species' distribution in the Long Island Sound, potentially resulting in regional variability in comparing THCs across studies. Although we observed much higher THCs in lobsters experiencing the same level of temperature stress as Dove *et al.* (2005), THCs measured here are in line with other previous reports on *H. americanus* (Battison *et al.*, 2003; Clark *et al.*, 2016). In the context of understanding how THCs relate to immune function, however, future efforts would benefit from conducting additional counts following the pathogen challenge (Battison, 2006), as well as analyses of phagocytotic activity, ROS production, and levels of apoptosis in hemocytes in addition to exploring overall mortality (Bibby *et al.*, 2008; Hernroth *et al.*, 2012; Meseck *et al.*, 2016; Wang *et al.*, 2016).

Of the 22 sham-injected individuals of *H. americanus* followed during the pathogen challenge, only one died while all others remained active throughout the experiment. We did not detect any growth of *A. viridans* in the hemolymph sample of the sham-injected lobster that died, but several of the survivors exhibited positive growth of *A. viridans* when sacrificed at the conclusion of the pathogen challenge (Table 3). We had intended to house each lobster individually to prevent agonistic interactions, but space limitations at DRL required lobsters to be housed as pairs in tanks. Since lobsters were randomized in tank placement, some of the shams were housed with lobsters injected with *A. viridans*. Although we constructed dividing barriers and did not directly observe any physical confrontations, it is possible that the sham lobsters interacted with their infected tank mates and acquired the bacteria through a small contact injury and subsequent opportunistic entry into the integument, which is the only route of infection for *A. viridans* (Stewart *et al.*, 2004; Cawthorn, 2011). *Aerococcus viridans* is able to survive outside *H. americanus* (Cawthorn, 2011), so it is also possible that cross-contamination via sampling nets inadvertently introduced the pathogen from infected to sham treatment tanks. Infection rates by *A. viridans* in natural populations are thought to be low (Stewart *et al.*, 1966; Lavallée *et al.*, 2001), but prevalence estimates are difficult to calculate due to alterations in behavior of infected lobsters that reduce entry into sampling traps (Lavallée *et al.*, 2001; Cawthorn, 2011; Davies & Wootton, 2018). Since lobsters were not tested for the presence of *A. viridans* prior to participating in these experiments, it is possible that shams that exhibited bacterial growth at the end of the experiment came into the laboratory as carriers with an avirulent strain of the bacterium (see Stewart *et al.*, 2004). Confirmation of virulence could be conducted through proteomic analysis of the bacterium (Clark & Greenwood, 2011), examination for the presence of a capsule (Clark *et al.*, 2016), or through histological examination of the hepatopancreas and cardiac tissues (Battison *et al.*, 2003). Future efforts should benefit from adoption of practices that reduce the potential for cross-contamination among individuals. It would also be prudent to reevaluate current estimates of *A. viridans* prevalence in the populations surveyed by Stewart *et al.* (1966) and Lavallée *et al.* (2001), as well as expand the geographic coverage of surveys, because environmental conditions have changed drastically since those studies were conducted (i.e., waters have warmed, potentially increasing the risk of mortality from infection).

Building on previous research exploring the impacts of climate change on subadult *H. americanus*, our study demonstrates that ocean acidification and warming clearly negatively affect this important life history stage by reducing physiological performance during an acute thermal challenge and lowering THCs, potentially increasing the risk of mortality due to disease. These findings could provide additional biological data to inform existing forecasting models (Mills *et al.*, 2017; Goode *et al.*, 2019; Oppenheim *et al.*, 2019) to more accurately predict how environmental change could influence the subadult population and their potential recruitment to the fishery. These results also demonstrate the importance of considering multiple drivers when examining environmental change, which may prove crucial in the context of assessing vulnerability and resiliency of this important fishery (Greenan *et al.*, 2019). While we explored the potential independent and interactive effects of acidification and warming on *H. americanus*, we acknowledge that lobsters will face a plethora of additional environmental stressors in the near future that could compound the negative impacts identified here such as hypoxia and osmotic stress (Robohm *et al.*, 2005; Hernroth & Baden, 2018). Future efforts would also greatly benefit by conducting additional immune challenges as novel pathogens arise. The potential for local adaptation to environmental change across the distribution of the species should also be addressed in future efforts through a comparative analysis of lobsters collected across northeastern North America.

SUPPLEMENTARY MATERIAL

Supplementary material is available at *Journal of Crustacean Biology* online.

S1 Table. Results of GLMs for the effects of round and treatment on the initial and final size of subadult *Homarus americanus* used in the experiments.

S2 Table. Post-exposure total hemocyte counts in subadult *Homarus americanus* across environmental stressor treatment conditions.

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