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The combinatorial effects of temperature and salinity on the nervous system of the American lobster, *Homarus americanus*

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An honors project for the Program of Neuroscience

By Katrina Carrier

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Table of Contents

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Abstract

The ability of nervous systems to continue to function when exposed to global perturbations such as elevated temperature and altered saline concentration is a non-trivial task, and the effects of such perturbations on circuit output are impossible to predict. The nervous systems of the American lobster (*H. americanus*), a marine osmoconformer and poikilotherm, must be robust to these stressors as they are exposed to fluctuations in temperature on a seasonal and a daily basis, and fluctuations in salinity as rainfall patterns change and as the lobsters move between areas of the ocean that have varied depths. Using the stomatogastric nervous system (STNS) of the American lobster, I characterized the effects of temperature on the output of the pyloric circuit, a central pattern generator contained within the STNS that controls the filtration of food that the lobster consumes, and established the maximum temperature that neurons within the pyloric circuit can withstand without "crashing" (ceasing function but recovering when returned to normal conditions). I then established a range of saline concentrations that did not cause the system to crash. Lastly, I determined whether altering the saline concentration within the permissible range affected the maximum temperature that the system was able to withstand. Although burst frequency increased as temperature was increased, phase constancy was observed. The PY neurons had a greater temperature tolerance than LP in each saline concentration tested, and, interestingly, the pyloric circuit could withstand higher temperatures upon exposure to lower than normal saline concentrations. Conversely, higher saline concentrations decreased the maximum temperature tolerated by the pyloric circuit. I also established the range of saline concentrations that the lobster's whole heart and cardiac ganglion (CG), the nervous system that controls the lobster's heartbeat contractions, could withstand. Then, I examined whether exposure to altered saline concentrations and in conjunction with elevated temperature alters the maximum temperature that the whole heart and CG can withstand. The CG was able to withstand a wider range of saline concentrations than the whole heart without crashing and crashed at higher temperatures than the whole heart in each saline concentration. Interestingly, as observed in the STNS, the whole heart and cardiac ganglion both crashed at higher temperatures in lower saline concentrations and higher temperatures in lower saline concentrations.

Introduction

Global perturbations: temperature and saline

Nervous systems must be able function despite environmental perturbations, a task that is particularly impressive when the systems are faced with perturbations that occur simultaneously. Such dual perturbations may limit the ability of a nervous system to continue to function to a greater extent compared to when each perturbation is presented individually (Hampton et al., 2024). This is likely to be true if cellular processes are differently affected by each perturbation, especially if the affected processes are maintained by overlapping compensatory mechanisms (O'Leary & Marder, 2016). On an evolutionary level, there may be trade-offs between susceptibility and resiliency to various environmental stressors. Marine organisms frequently encounter fluctuations in ambient seawater temperatures and salinity concentrations in their natural environments, so their nervous systems must be robust to these challenges. However, how these perturbations impact circuit output when presented individually and how they will alter circuit output when presented simultaneously is unpredictable.

It is impressive that marine poikilotherms can withstand fluctuations in ambient temperatures within a permissible range given that temperature differentially affects all the cellular process that determine the output of neural circuits, including neuronal excitability and rates of synaptic transmission (Soofi et al., 2014; Städele et al., 2015). Neuronal activity depends upon the balance of activity of ionic currents controlled by ion channel proteins, which temperature does not uniformly alter (Tang et al., 2010). The extent to which temperature fluctuations affect features like activation and inactivation rates and maximal conductance varies among individual ion channel types (Klöckner et al., 1990; Tang et al., 2010). Thus, the extent to which an individual neuron, nonetheless the output of an entire circuit, can withstand changes in

temperature is impossible to predict (Tang et al., 2012; Robertson and Money, 2012; Alonso et al., 2020).

Like temperature, changes in extracellular saline concentration affect the function of all neurons in a circuit (He et al., 2020; Marder and Rue, 2021; this thesis). Changing extracellular ionic concentrations can impact neuronal excitability because membrane potential is determined by the difference in extracellular and intracellular ionic concentrations as well as the ionic pumps and ion channels expressed by the neuron, and ionic pump stoichiometry can be affected by altering ionic concentrations (Rakowski et al., 1989). Furthermore, altering extracellular saline concentrations affects intracellular voltage, and because many ion channels are voltage sensitive, the extent to which a circuit can maintain output in the face of altered extracellular saline concentrations is also hard to predict (He et al., 2020).

For a species to be able to withstand global perturbations like temperature and salinity, the mechanisms that allow their neural circuits to continue to function must be able to do so despite inter-animal variability in underlying conductances and synaptic strengths within those circuits. Circuits that produce the same output can have variable synaptic strengths, and channel expression within cell types can vary drastically (Prinz et al., 2004; Schulz et al., 2006). Therefore, the solutions that confer the robustness of a circuit to perturbation must be able to do so for networks with a range of parameters (Ratliff et al., 2021).

Temperature and saline in the lobster's environment

The American lobster is one such marine organism that must be able to withstand changes in temperature and salinity as they are exposed to frequent fluctuations in both water temperature and saline concentrations (Cumberlidge et al., 2015; Tanaka et al., 2019). Understanding the extent to which they can withstand a shift in these parameters can elucidate

the potential implications of climate change for these animals. The internal environment of the lobster is directly impacted by ambient seawater because the lobster is a poikilotherm and an osmoconformer (maintains an internal environment that is isotonic to the salinity of its surrounding seawater) at salinities that exceed 20ppt (Dall, 1970; Jury et al., 1994).

Benthic saline concentrations off the Gulf of Maine have been measured to range from 25.8 ppt to 34.6 ppt, and saline concentrations fluctuate dramatically following weather events such as such as storms that incur increased rainfall (Tanaka et al., 2019). Climate-change is driving amplification of the global water cycle, where rates of both precipitation and evaporation are increasing, and this is expected to cause variability in saline concentrations across the ocean to become more pronounced (Durack et al., 2012). Simultaneously, fluctuations in water temperature as large as 12℃ that arise from changing tides and from wind patterns have been recorded over the course of a single day, and surface water temperatures have been rising at a rate of 0.27ºF (0.15ºC) per decade since the 1980s and are expected to continue to do so. This rise is especially dramatic off the Gulf of Maine where temperatures are rising at a rate of 0.86 ºF (0.48ºC) per decade (Gulf of Maine Research Institute, 2023).

Lobster stomatogastric nervous system and cardiac neuromuscular system

The perturbations I use here are found in the lobster's natural environment, so their nervous systems must be able to compensate for these stressors so as to continue to function when challenged with elevated temperature and altered saline concentrations. These circuits are composed of neurons whose role and function are known, and these circuits are well defined, with known connections between cells. Therefore, the lobster stomatogastric nervous system and cardiac neuromuscular systems are both ideal models for characterizing the ability of the nervous system to withstand these global perturbations.

The stomatogastric nervous system (STNS) in *Homarus americanus* controls the movement of the lobster foregut. The layout of the STNS is depicted in Figure 1A. When dissected, the STNS continues to generate fictive rhythmic activity *in-vitro* that resembles the *in-vivo* physiology. The pyloric network is contained within the STNS and innervates the muscles that control the pylorus, which are responsible for filtration of the food that the lobster consumes (Marder and Bucher, 2007). The pyloric rhythm is a triphasic motor pattern that consists of bursts of action potentials produced by four different groups of neurons (Fig. 1B and C). The pyloric dilator (PD) motor neurons, which are electrically coupled to the intrinsically oscillating anterior burster (AB) interneuron, serves as the pacemaker kernel for the circuit. Each AB/PD off-phase is followed by bursts of the lateral pyloric (LP) motor neuron, and then the pyloric (PY) motor neurons (Fig. 1C). PD innervates a set of muscles that dilate the pylorus, whereas LP and PY each activate distinct sets of muscles that subsequently constrict it, so the activation of each set of neurons in this particular order generates the peristaltic contraction driving food filtration (Harris-Warrick, 1992).

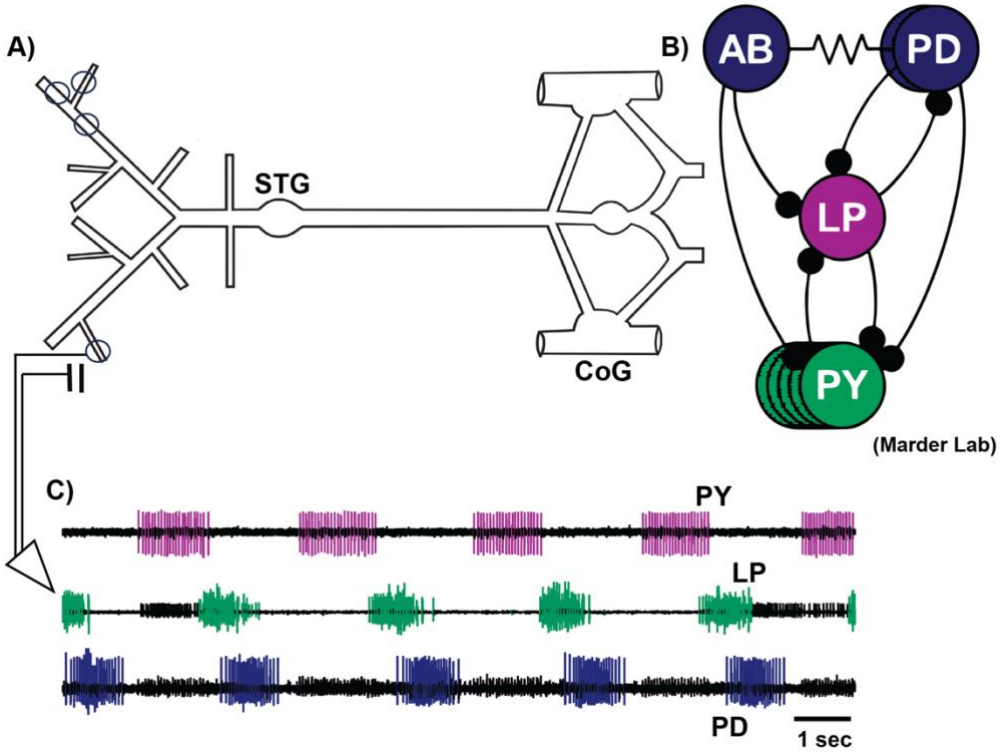

Figure 1. Stomatogastric nervous system, pyloric rhythm, and pyloric circuit wiring diagram. A) Schematic of the stomatogastric nervous system, with Vaseline wells (depicted as black circles [methods]) and extracellular recording setup also depicted. **B)** Connectivity diagram of the pyloric circuit. Electrical coupling is depicted with a resistor symbol, and chemical inhibitory connections are depicted as lines terminating in filled black circles. The image in panel B is available on the Marder Lab website (Brandies Univ.). **C)** Example simultaneous extracellular recordings of the LP, PY, and PD neurons, whose activity compose the pyloric rhythm.

The lobster cardiac ganglion (CG) controls the heartbeat, and, like the STNS, once dissected from the animal, produces fictive, rhythmic output *in-vitro* that closely resembles that which occurs *in-vivo*. The circuit is composed of four small pacemaker neurons that are electrochemically coupled to five large motor neurons, which are responsible for driving contractions of the neurogenic lobster heart. The layout of the isolated CG is depicted in Figure 2A, and a wiring diagram of the circuit is depicted in Figure 2B. The circuit produces rhythmic, monophasic, and synchronous bursts of action potentials (Cooke, 2002). Calcium mediated driver potentials that originate from the small cells elicit bursts of action potentials, which,

because of their synaptic and electrical coupling to the large cells, induce simultaneous activity in the large cells (Cooke, 2002; Hartline, 1979). The large cells innervate cardiac muscle, inducing muscle contractions at the neuromuscular junction (Cooke, 2002). Those cardiac muscle contractions constitute a heartbeat. The heartbeat continues *in-vitro* to resemble those produced *in-vivo* when the whole heart, including the cardiac ganglion, effector organs, and intrinsic feedback systems, is dissected from the lobster (Cooke, 2002).

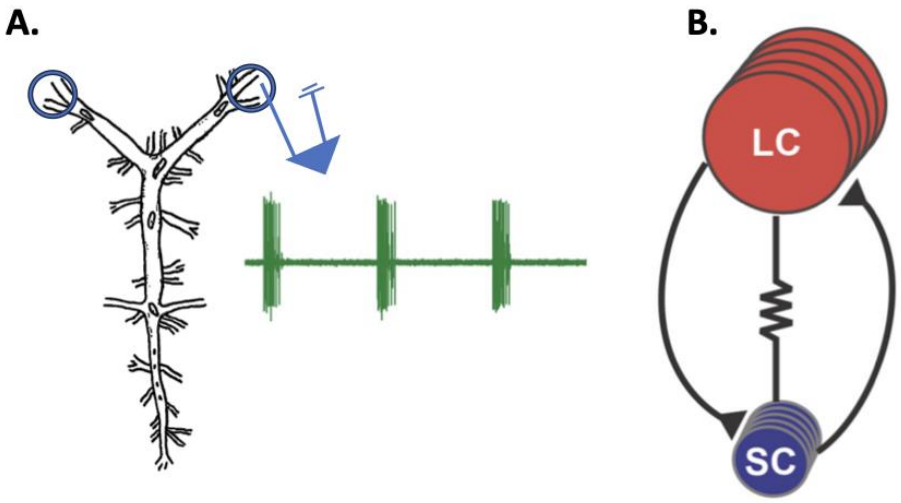

Figure 2. Cardiac ganglion experimental setup and wiring diagram. A) Schematic of the isolated cardiac ganglion with Vaseline wells (blue circles) and extracellular recording setup (methods). The physiology trace (green) to the right is an example extracellular recording from the CG. **B)** Wiring diagram of the cardiac ganglion. Resistor symbol denotes all-to-all electrical coupling and black triangles denote excitatory chemical synapses that exist between each group of neurons.

Effects of temperature on the nervous system

The ability of the STNS of another decapod crustacean, the Jonah crab, *Cancer borealis*, to withstand elevated temperature has been characterized. Within a permissible range of temperatures, exposure to elevated temperature causes the pyloric rhythm to increase in frequency. However, the phase relationships, or relative time for which each individual neuron is active within a single cycle of the pyloric rhythm, is maintained as temperature and burst

frequency increases (Tang et al., 2010). Given that the behavioral output of this circuit, food filtration by the pylorus, relies on activation of muscle groups in a particular order, phase constancy allows the animal to continue to filter its food even at elevated temperatures (Soofi et al., 2014). In other words, because successful filtration is dependent upon the activation of the three groups of pyloric muscles innervated by PD, LP, and PY neurons in that particular order and with a particular timing, even as these muscles are activated at a higher frequency, successful filtration can still occur (Harris-Warrick, 1992).

Within this permissible range, temperature compensation in this system arises, in part, from the similar temperature dependencies of processes that have opposing functional roles. Modeling work demonstrates that these 'antagonistic balances' can be achieved through maintaining linear correlations in conductance densities that have opposing functional roles, suggesting that evolutionary pressure has driven biological constraints on the regulatory balance of these and other processes with opposing functional roles (O'Leary & Marder, 2016). Temperature compensation that results from correlated temperature dependencies of opposing processes has been shown to play an important role in the temperature compensation of the STNS of *borealis*. For instance, simultaneous increases in membrane leak conductance and IPSC amplitude have been observed. Additionally, I_h and I_A currents both play important but opposing roles in latency to firing by follower neurons, where activation of I_h enhances rebound from inhibition whereas de-inactivation of I_A delays rebound from firing, and both of these processes have similar temperature dependencies (Tang et al., 2010).

Upon reaching a critical temperature, however, the system "crashes", or ceases to produce rhythmic bursts of action potentials until returned to a permissible range. At these temperatures, the underlying parameters that allow circuit output have likely become

incompatible. In the Jonah crab, *Cancer borealis*, the dynamics of crashes observed at elevated temperatures are variable between individual preparations, where, for example, the order of neurons that crashed and the ways in which their activity was disrupted differed (Gorur-Shandilya 2022; Haddad & Marder, 2018; Tang et al., 2012). These differences in crash dynamics likely arise, in part, because of individual differences in underlying circuit parameters (Schulz et al., 2006; Schulz et al., 2007; Tang et al., 2012).

The effects of elevated temperature on the lobster whole heart and cardiac ganglion have been previously characterized. Both the whole heart and cardiac ganglion crash upon reaching a critical temperature (Worden et al., 2006; Powell et al., 2023). Data from Powell et al. (2023) showed that as temperature increases, contraction frequency of the whole heart also increases up until around 18ºC. After reaching ~18ºC, however, the burst frequency begins to decrease. For the CG, burst frequency increases as temperature rises, until a few degrees below the crash temperature when the burst frequency rapidly decreases. As temperature increases, so too does variability in CG burst frequency. Interestingly, the temperatures at which the CG crash are significantly higher than those at which the whole heart crashes (Powell et al., 2023).

Effects of saline concentration on the nervous system

The effects of altering the ionic concentrations of all ion species that compose the extracellular physiological saline typically used for the lobster stomatogastric nervous system and cardiac neuromuscular system is unknown. Previous research has examined the effects of elevated potassium concentrations on the stomatogastric nervous system of *C. borealis* and found that the pyloric rhythm was negligibly affected by exposure to saline with 1.5 times the normal potassium concentration (1.5x), and that only upon exposure to 2.5x potassium did the

system crash and remain crashed until washed out. As expected, neuron membrane potentials depolarized in the presence of elevated potassium concentrations (He et al., 2020).

Previous work has examined the effects of altering extracellular concentrations of individual ions on the giant axon of the lobster. These experiments found that reducing extracellular sodium concentration does not change resting membrane potential but does decrease action potential spike amplitudes, increasing extracellular calcium concentrations causes the resting membrane potential to become more hyperpolarized, and no effects of altering extracellular magnesium concentration were observed (Dalton, 1958). As indicated by these data, changes in the concentration of all extracellular ions present in the seawater in which the lobster typically inhabits likely elicits changes in neuronal excitability and action potential dynamics. This can present challenges to continued neuronal and circuit function in ways that have yet to be explore in the lobster STNS and cardiac neuromuscular system.

Combinatorial effects of temperature and salinity

Given that changes in extracellular saline concentrations and exposure to elevated temperature each individually present challenges to continued function of the nervous system, I hypothesized that when presented in combination, the extent to which these systems could withstand elevated temperature would be lower than the maximum temperature that the system could withstand upon exposure to elevated temperature alone. The responses of the lobster STNS to elevated temperature were unknown, so I first sought to determine if the lobster STNS exhibits a similar response to elevated temperature as the Jonah crab. Furthermore, the permissible range of altered ionic concentrations was unknown, so I established a range of altered saline concentrations that did not cause the pyloric circuit to crash. Finally, using this range of

permissible saline concentrations, I determined how exposure to altered saline concentrations affected the maximum temperatures that the system could withstand.

I performed a similar set of experiments on the lobster whole heart and isolated cardiac ganglion, where I first established the range of saline concentrations each system could withstand, and then how exposure to altered saline concentrations affected the maximum temperature that the systems could withstand. These experiments allowed me to compare the effects of altered saline and temperature between circuits with cells whose underlying conductance sets vary drastically, and whose mode of pace-making generation differs. By comparing the responses to the same perturbations between these two different systems, I sought to elucidate differences in the response to them between a system whose activity relies, in part, upon rebound from inhibition (pyloric circuit) as compared to one whose activity relies on mutual excitation (CG). Although both nervous systems are present in each lobster, because these two circuits are differently configured, it was not clear if they would respond similarly to these combined perturbations.

Methods

Animals

Adult male and female American lobsters, *Homarus americanus*, were purchased from a local seafood wholesaler (Fisherman's Net, Brunswick, ME). Animals were housed in tanks with recirculating seawater and maintained at 10 to 12℃ in a vivarium kept on a 12-hour light/dark cycle at Bowdoin College, Brunswick, ME. They were fed with chopped squid or shrimp.

Stomatogastric nervous system preparation

Lobsters were anesthetized in a bucket of ice for 30 to 50 minutes prior to dissection. The stomach was dissected from the lobster, and the stomatogastric nervous system was further

dissected from the stomach, with as many nerves as possible left intact. The nervous system was pinned in a Sylgard-184 coated dish (Dow Corning, Midland, MI, USA) that was filled with chilled physiological saline. Once set up on the physiology rig, the nervous system was continuously superfused with cold saline. One saline inflow tube was placed next to the STG and a second next to a commissural ganglion. Outflow tubes were positioned such that saline would be drawn across both the STG and anterior ganglia. Saline temperature was controlled using a Peltier temperature device (CL-100 bipolar temperature controller and SC-20 in-line heater/cooler; Warner Instruments) and monitored using a temperature probe (Warner Instruments), which was placed next to the STG. Temperature was maintained at 10 to 12℃ in control conditions.

Whole heart and cardiac ganglion preparation

After anesthetization for 30 to 50 minutes in ice, the whole heart was dissected from the lobster and left attached to the overlaying portion of the dorsal thoracic carapace. It was then pinned with the ventral side up to a Sylgard-170 (Dow Corning, Midland, MI, USA) lined dish that was filled with chilled physiological saline. A short piece of plastic tubing was used to canulate the heart through the ventral, posterior artery, and physiological saline was continuously perfused through the heart. A second inflow tube was placed in the dish to superfuse saline over the external portions of the heart as to minimize any temperature gradient between the heart chamber and external environment. Temperature of saline was regulated and measured as previously described for the STNS. The temperature probe was placed in the heart through an ostium, so that the temperature of the saline internally perfused through the heart was measured. Temperature was maintained at 8 to 12℃ for control conditions.

Each CG was obtained by first removing the whole heart as previously described, then separating the heart from the carapace, pinning it ventral side up in a Sylgard dish containing chilled saline, and cutting the musculature of the heart along the ventral axis to open it. The CG, including the anterolateral branches and main trunk, was then dissected from the heart, and pinned in a clear Sylgard lined dish containing cold saline. Temperature inflow tubes were placed next to the trunk of the heart and next to an anterolateral branch, and the temperature probe was placed next to the trunk. Temperature was controlled and measured as previously described. Temperature was maintained at 10 to 12℃ for control conditions.

Solutions

Physiological saline was composed of 479.12 mM NaCl, 12.74 mM KCl, 13.67 mM CaCl2, 19.92 mM MgSO4, 3.91 mM Na2SO4, 11.45 mM Trizma base, 4.82 mM Maleic acid dissolved into ddiH_20 and pH was adjusted to be between 7.43 to 7.47 using either HCl or NaOH. Saline concentrations were made more dilute or more concentrated for saline experiments by adding or reducing the amount of deionized water used. All saline solutions had a pH between 7.43 to 7.47.

Extracellular recordings and data acquisition

For extracellular nerve recordings, Vaseline wells were created to electrically isolate sections of nerve for both the CG and STNS preparations (Fig. 1 and 2). The STNS recordings were usually sampled from the *lvn, pyn,* and *pdn* in order to fully capture each pyloric cycle. For the cardiac ganglion, a Vaseline well was created around an anterolateral nerve or the trunk. All nerve activity was measured using stainless-steel pin recording electrodes and was amplified using a differential amplifier (A-M Systems model 1700, Sequoia, WA). The signals were

digitized at 10 kHz using a CED Micro3 1401 Data Acquisition unit and visualized with Spike2 software (Cambridge Electronic Design, Cambridge, UK).

Whole heart recordings

To measure heartbeat contractions, a thread of suture silk was attached to a force-displacement transducer (Grass FT03, Grass Instruments, Quincy, MA [Discontinued]) and tied around the anterior arteries of the heart (Fig. 3). The force transducer was calibrated such that arteries were pulled by the suture silk to a baseline of two grams of force, which approximates the baseline stretch of cardiac muscles in the intact animals. Deflections from that baseline were recorded. The recordings were amplified using an ETH-250 Bridge amplifier (CB Sciences, Dover, NH, USA) and further amplified using a Brownlee Precision Model 410 Amplifier (Brownlee Precision, San Jose, CA, USA).

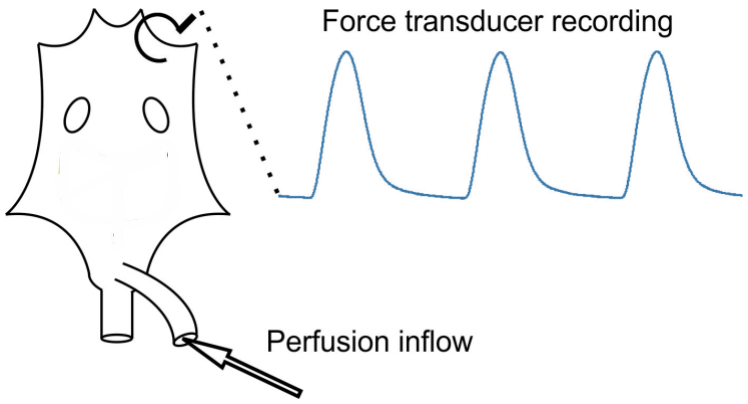

Figure 3. Whole heart experimental setup and recording. Whole heart experimental setup, including location of the perfusion inflow tube and the force transducer (left). Example force transducer recording of a heartbeat (blue trace).

Crashes of rhythmic activity

To measure the ability of these systems to withstand a range of elevated temperatures, altered saline concentrations, and the combination of these two perturbations, I needed to

determine the point at which these systems failed to produce meaningful activity (crash point). This definition of a crash contrasts with previous studies that used *C. borealis*, where a crash was defined as a reversable loss of *all* activity. Both definitions contrast with the death of a system, because activity was restored once the temperature was returned to within the permissible range. For the lobster STNS, a crash was defined as the point where both PY and LP neurons ceased to produce rhythmic bursts of action potentials for a period of 15 seconds. When both LP and PY cease to produce rhythmic bursts of action potentials, the behavioral output of this system, filtration, would no longer be functional. For the cardiac ganglion and whole heart, a crash was defined as the point at which fewer than 2 bursts of action potentials or heart beats, respectively, were observed over the course of 30 seconds. To be considered a crash, upon return to baseline conditions these systems must recover physiological output.

Saline experiments

To determine the ability of the STNS, CG, and whole heart to withstand altered saline concentrations, the system was exposed to a series of sequential 15-minute saline applications of different concentrations. The experiments began with 15-minute applications of physiological saline (1x). Then, for experiments measuring the effects of elevated saline concentrations, the saline was changed to increasingly concentrated solutions until a crash was identified, at which point the solution was returned to the 1x solution, and the saline concentration was not elevated further. The solutions applied were 1.25x, then 1.50x, 1.75x, and finally 2x solution, and the activity in each solution was measured for 15 minutes. Similarly, for the experiments that measured tolerance to lower saline concentrations, saline solutions were changed to 0.75x, 0.5x, and finally 0.25x, stopping at whichever solution causes a crash.

Temperature ramps

To determine the maximum temperature that the STNS and cardiac neuromuscular system were able to withstand, the temperature of saline inflow was increased from control temperatures by 2°C every 2 minutes until a given preparation crashed, at which point, the saline was set to cool by 5°C every minute. A temperature ramp sequencer script written in Spike2 by Maren Cooper and Patsy Dickinson was used to control the temperature of the saline to which the system was exposed. The system was manually set to cool when a crash was identified by the experimenter. For the experiments that examine the combined effects of temperature and saline concentration, the preparation was exposed to the altered saline solution for 5 to 15 minutes prior to beginning a temperature ramp.

Data analysis

For experiments that examine the effects of exposure to saline alone, the percent of STNS, CG, and whole heart preparations that crash in each saline solution was calculated. For the STNS, comparisons of PD burst frequency were made between 1x and 0.75x saline solutions and between 1x and 1.25x saline solutions. Thirty seconds of activity was extracted from the 15-minute saline application of these saline concentrations, and burst parameters, including PD frequency, were obtained using Spike2 scripts. GraphPad Prism (Dotmatics, Boston, MA, USA) was used to perform paired *t*-tests comparing burst frequency between saline concentrations (α=0.05).

For temperature experiments, thirty seconds of activity at each temperature step was extracted and burst parameters within that 30 second period were obtained using Spike2 scripts. Q_{10} values of PD frequency were calculated from these data with the equation $Q_{10} = (k2/k1)^{(10/T2-T1)}$ where k2 is the rate of a given parameter at the T2, and k1 is that parameter's rate at T1; T is

temperature. Paired *t*-tests of PD burst frequency were made between frequencies at 10ºC and 20ºC. Crash temperatures in each saline concentration for the STNS, CG, and whole heart were compared using Mixed-effects model and Tukey's multiple comparisons test. Comparison of crash temperatures between the LP and PY neuron and between the whole-heart and cardiac ganglion were made using unpaired *t*-tests (α=0.05). Statistical tests were performed using GraphPad Prism. For the STNS, phase data across temperature was calculated by taking 10 cycles of the pyloric rhythm at each temperature step, and calculating the percent of the total cycle, defined as the delay from either a neuron's burst start or burst end compared to the onset of the previous PD burst, divided by the total duration of that cycle. These calculations were only performed when all three neuronal units were active.

Results

Effects of elevated temperature on the pyloric rhythm

The ability of the STNS in the Jonah crab, *C. borealis*, to withstand a wide range of temperature has been previously established, but this was the first study to show that, like the Jonah crab, the American lobster *H. americanus* can withstand a wide range of elevated temperatures. In the lobster, the frequency of the pyloric rhythm increased with increased temperature (Q_{10}=1.87±0.31 between 10ºC and 20ºC; paired *t*-test of PD frequency between 10ºC and 20ºC $p<0.001$), and upon reaching an impermissible temperature, the rhythm crashed: defined by the LP and PY neurons both no longer producing rhythmic bursts of action potentials (Fig. 4A). Variation in burst frequency appeared to increase as temperature was increased (Fig. 4B). However, phase constancy was observed across a range of temperatures prior to the rhythm crashing. Over the temperatures in which all three of the neuronal units whose activity composes the triphasic rhythm had not crashed, the percent of time for which LP and PD neurons were

active over the course of a single cycle remained constant across temperature (Fig. 5, Table 1). PY on-phase increased slightly as temperature rose while PY off-phase did not change, which likely resulted from a decrease in strength of the inhibitory input from LP onto PY as the LP neuron approached its crash temperatures (Fig. 5, Table 1). Previous data from *C. borealis* found no change in phase across a range of temperature wider than those measured here, which could be explained by slight differences in pyloric phasing relationships between the crab and lobster at control temperatures.

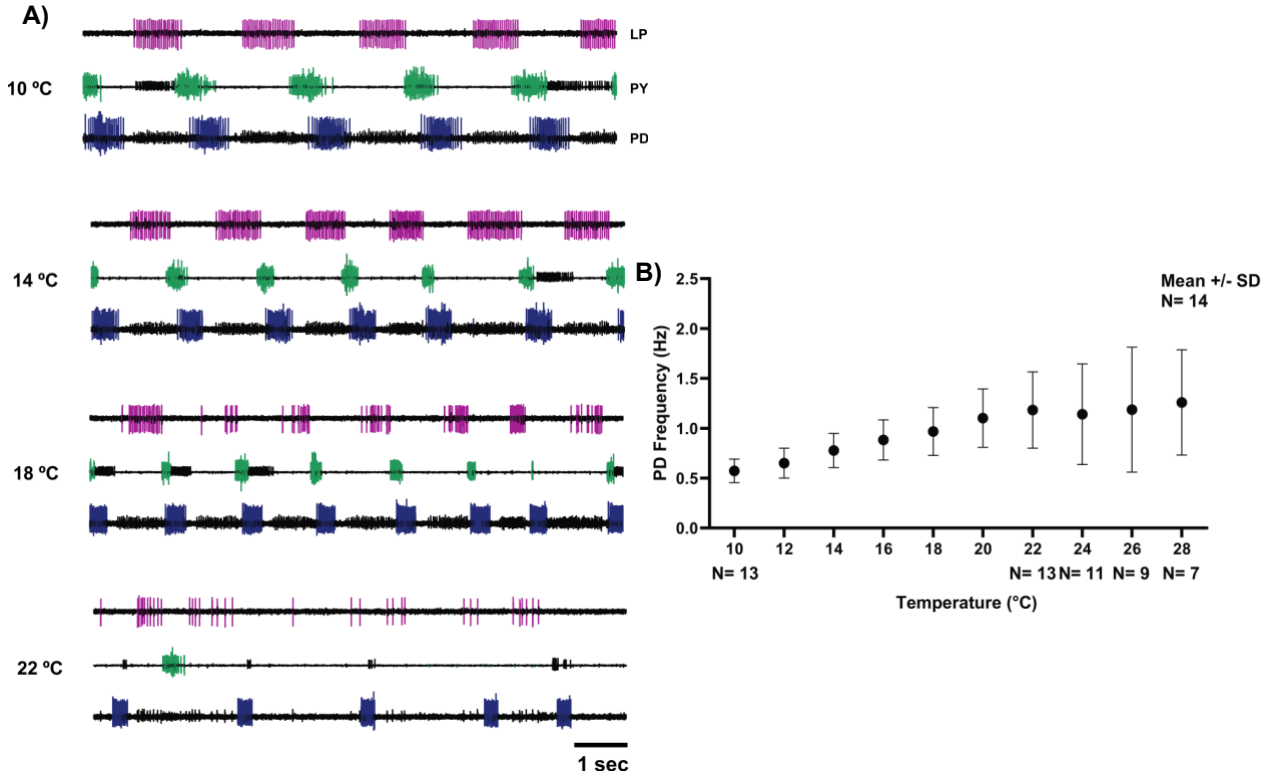

Figure 4. Pyloric rhythm frequency increases as temperature increases, until reaching an impermissible temperature that causes a crash. A) Example simultaneous extracellular recordings of the pyloric rhythm at discrete temperature steps from a single preparation. As temperature increases, the frequency of the bursts of action potentials increases. Upon approaching an impermissible temperature, 22ºC, the PY and LP neurons fail to burst. **B)** PD neuron burst frequency plotted as a function of temperature. Dots represent the mean of a 30 second bin and error bars represent the standard deviation. (*N*=14 until preparations begin to crash at 22ºC and for one preparation whose baseline temperature began at 12ºC).

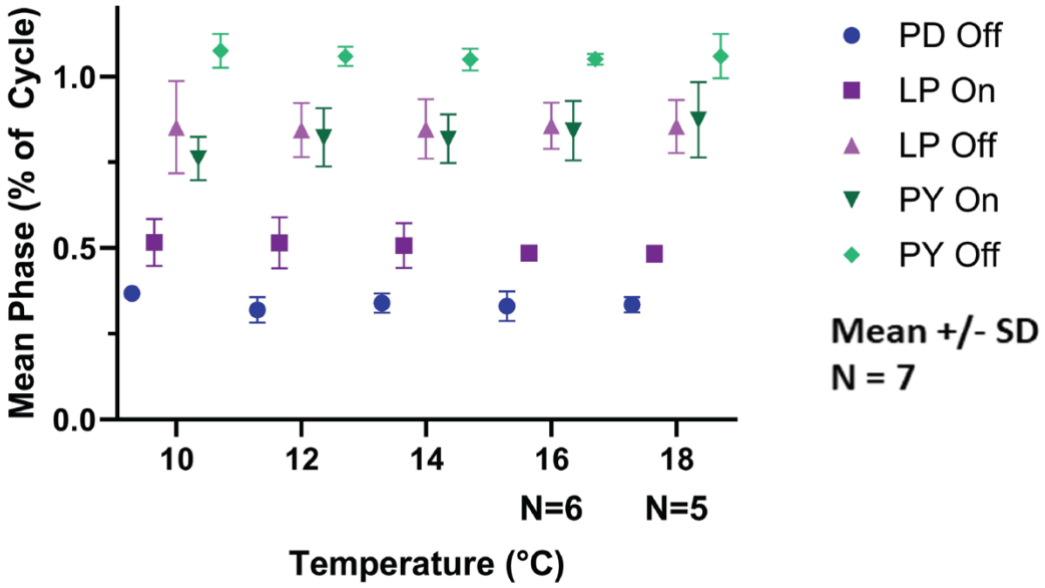

Figure 5. Phase relationships of the pyloric neurons are maintained across temperatures (10ºC to 18ºC). Average phase (methods) plotted as a function of temperature (mean ± SD**,** *N*=7 until neurons begin to crash at 16ºC). Note that phase is relatively constant across this temperature range.

Process	Q_{10}	S.D.	slope ≠ 0
PD off	0.88781599	0.08117614	no
LP on	0.97088405	0.12104351	no
LP off	1.02978472	0.14936874	no
PY on	1.23322161	0.188198	yes
PY off	1.00372525	0.07900395	no

Table 1. Q_{10} values of pyloric rhythm phase relationships. Q_{10} values for each phase relationship, calculated from 10 to 18ºC (mean±SD, *N*=5). Q_{10} slopes are compared to 0. PY is the only phase relationship with a slope that significantly differs from 0 (extra sum of squares F test, *p*=0.02).

Effects of altered salinity on the pyloric rhythm

After characterizing the response of the system to temperature alone, I then determined a range of saline concentrations that don't cause the system to crash. The pyloric rhythm did not crash in 1x, 0.75x, nor 1.25x saline concentrations, but upon exposure to 0.5x saline the system tended to crash (66.6% of preps crashed; *N*=9) (Fig. 6C). 100% of those exposed to 0.25x

crashed (*N* = 3) (Fig. 6C). Upon exposure to the higher saline concentrations, the triphasic rhythm of some preparations began to lose rhythmicity at 1.5x saline (12.5% of preps crashed; *N=8*), while 1.75x saline tended to cause most preparations to crash (71.42%; *N*=7) (Fig. 6A and C). PD frequency did not differ between either 1x and 0.75x or 1.25x, the altered saline concentrations that didn't cause the system to crash (Paired *t*-tests, 1x vs 0.75x: *p*=0.93; *N*=8; 1x vs 1.25x: *p*=0.89; *N*=8) (Fig. 6B).

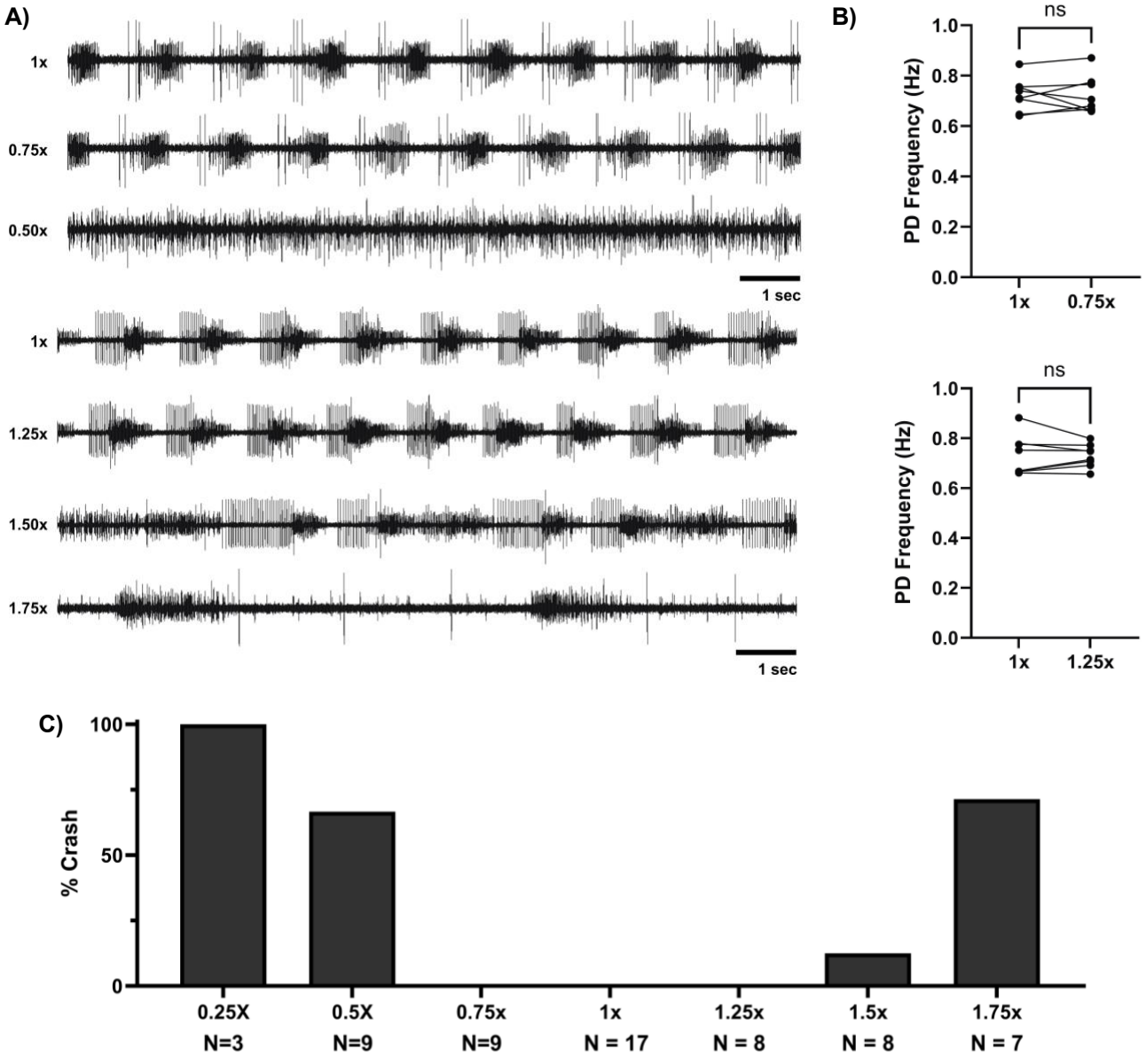

Figure 6. The STNS does not crash in saline concentrations ranging from 0.75x to 1.25x. A) Example extracellular recordings of the pyloric rhythm upon exposure to 1x, 0.75x, and 0.50x saline concentrations, and of a separate preparation upon exposure to 1x, 1.25x, 1.50x, and 1.75x saline concentrations. The triphasic rhythm is maintained across in 1x and 0.75x saline concentrations, but crashes in 0.50x. In 1x and 1.25x the triphasic rhythm is maintained, but in 1.50x it begins to deteriorate and in 1.75x the rhythm has crashed. **B)** PD burst frequency does not differ from 1x in 0.75x nor 1.25x saline concentrations. Each dot represents an individual preparation, and connected dots show that data is from the same preparation. (Paired *t*-tests, 1x vs 0.75x: p=0.93; N=8; 1x vs 1.25x: p=0.89; N=8). **C)** Percent of preparations that crash in a given saline concentration.

Effects of elevated temperature and altered salinity on the pyloric rhythm

I then examined whether the altered saline concentrations that did not independently cause the system to crash (0.75x and 1.25x) would alter the capacity of the system to withstand elevated temperature. As was observed in 1x saline, PD burst frequency and variation in burst frequency both increased as temperature increased in 1.25x and 0.75x saline concentrations (Fig. 7A, B). In 1x, 1.25x, and 0.75x saline concentrations, the LP neuron crashed at lower temperatures than the PY neuron (0.75x: p=0.0138; N=13; paired-test; 1x: paired *t*-test, p=0.006; N=18; 1.25x: paired *t*-test, p=0.0018; N=12) (Fig. 8).

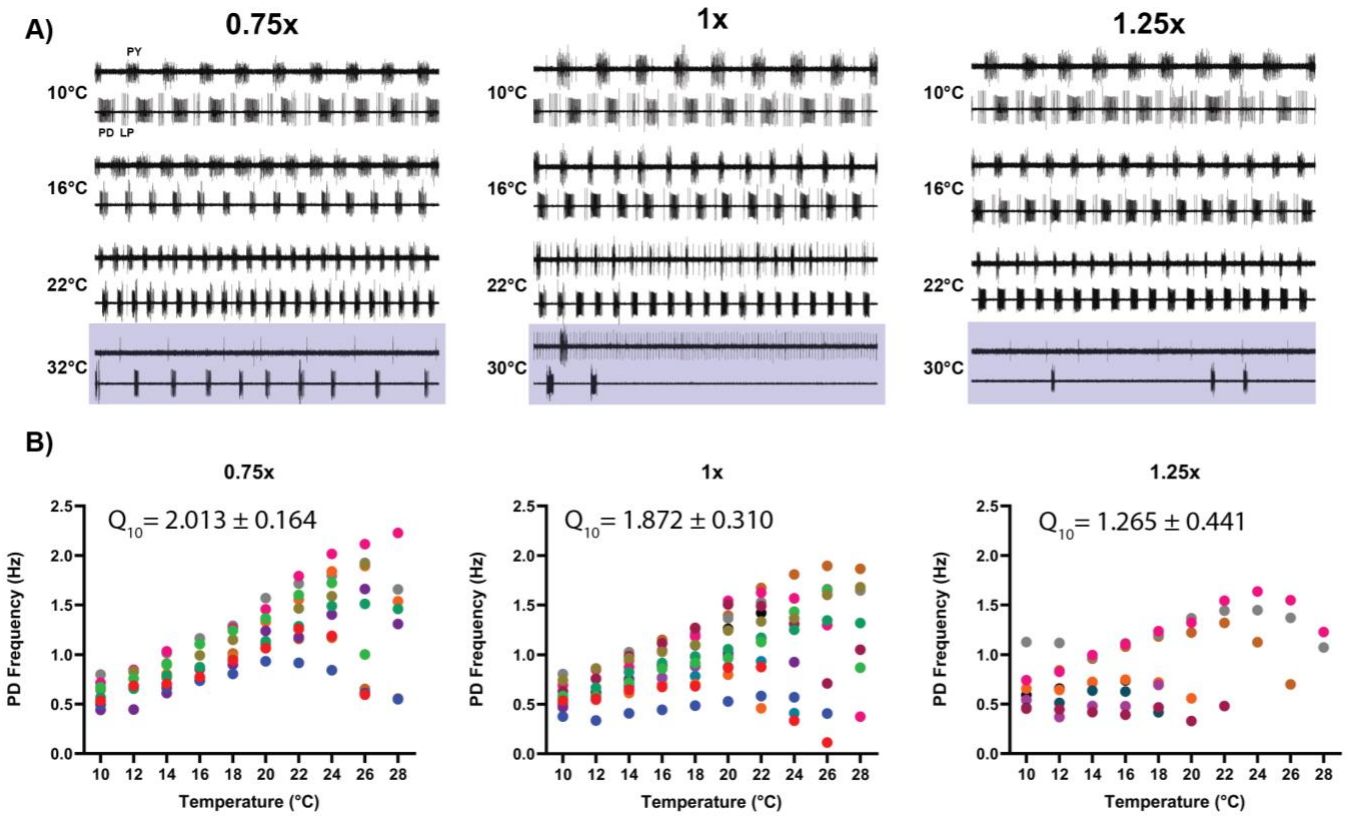

Figure 7. Burst frequency increases as a function of temperature A) Example extracellular recordings of the pyloric rhythm at discrete temperature steps in each saline concentration tested. As temperature increased so too did cycle frequency until reaching crash temperature, shown in trace shaded blue. **B)** PD burst frequencies plotted as a function of temperature in each saline concentration. Each dot represents the average of 30 seconds of PD burst frequency at each temperature step. Colors correspond to individual preparations. Q_{10} values for 0.75x (10 to 22ºC; *N*=9), 1x (10 to 20ºC; *N*=13), and 1.25x (10 to 16ºC, *N*=7) noted on each plot.

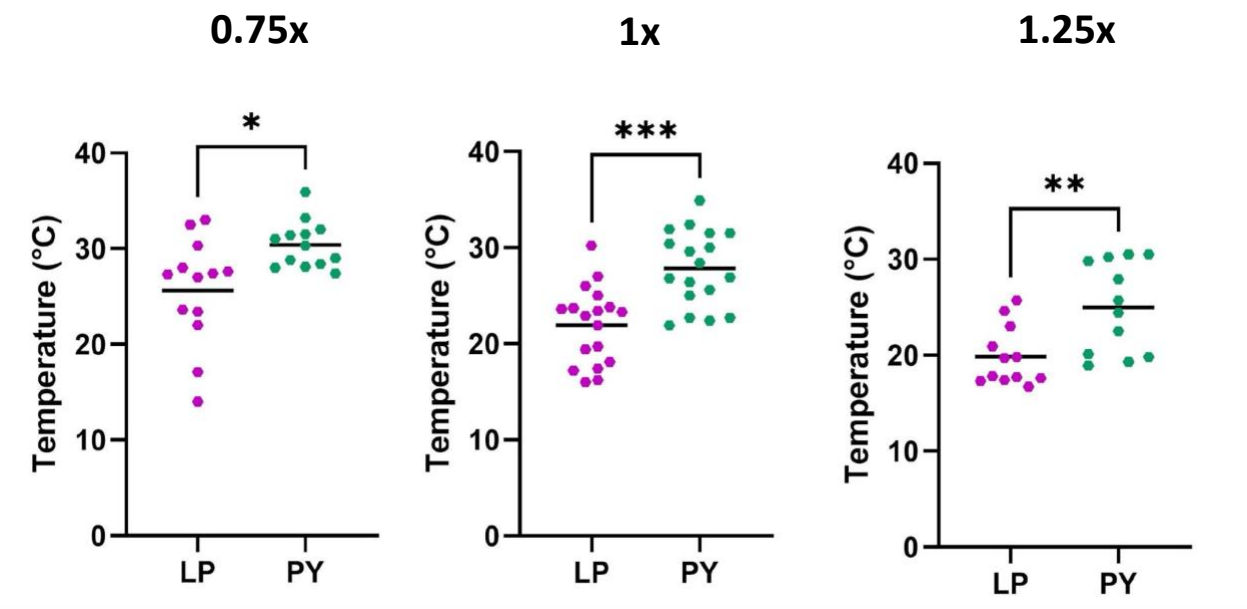

Figure 8. The PY neurons crash at higher temperatures compared to the LP in 0.75x, 1x, and 1.25x saline concentrations. The crash temperature of PY is significantly higher than LP in 0.75x saline (Paired *t*-test, p=0.0138, N=13), 1x saline (Paired *t*-test, p=0.006, N=18) and 1.25x saline (Paired *t*-test, p=0.0018, N=12).

The crash temperatures of both LP and PY neurons differed between 0.75x, 1x, and 1.25x saline concentrations. The crash temperature of PY was higher upon exposure to 0.75x saline as compared to 1x saline (Tukey's multiple comparisons test*;* p=0.0048; N=14) and was lower upon exposure to 1.25x saline as compared to 1x saline (Tukey's multiple comparisons test*;* p=0.0089; N=18) (Fig. 9). The same trend was observed in the LP neuron, where crash temperatures were significantly higher upon exposure to 0.75x as compared to 1x saline (Tukey's multiple comparisons test*;* p=0.0272; N=18), and significantly lower upon exposure to 1.25x as compared to 1x saline (Tukey's multiple comparisons test*;* p=0.0019; N=25) (Fig. 9).

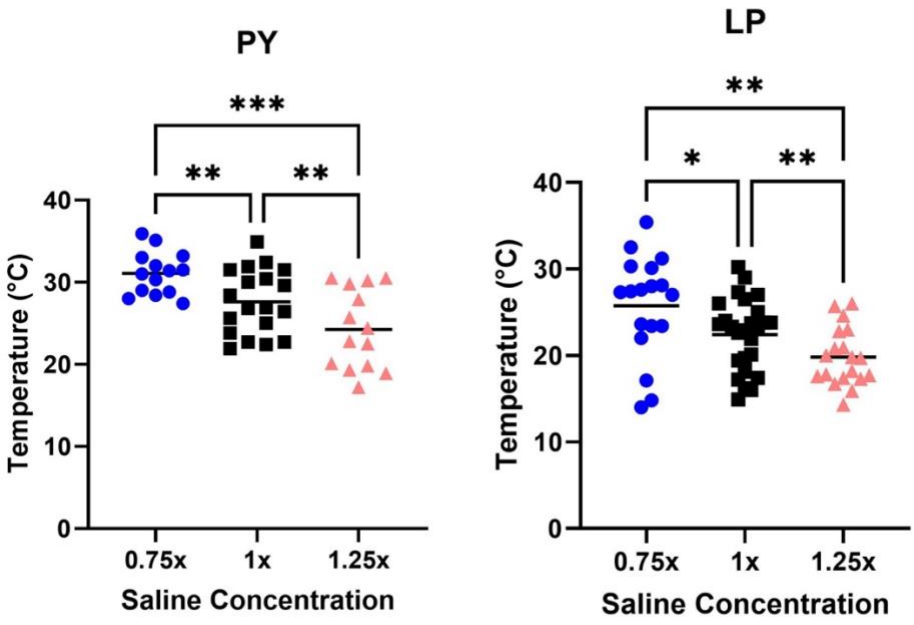

Figure 9. Both the LP and PY neurons crash at higher temperatures in 0.75x saline concentration and lower temperatures in 1.25x saline concentration. The crash temperature of PY was higher upon exposure to 0.75x saline as compared to 1x saline (Tukey's multiple comparisons test*; p*=0.0048; *N*=14) and was lower upon exposure to 1.25x saline as compared to 1x saline (Tukey's multiple comparisons test*; p*=0.0089; *N*=18). Similarly, for the LP neuron, crash temperatures were significantly higher upon exposure to 0.75x as compared to 1x saline (Tukey's multiple comparisons test*; p*=0.0272; *N*=18), and significantly lower upon exposure to 1.25x as compared to 1x saline (Tukey's multiple comparisons test*; p*=0.0019; *N*=25). Each dot represents an individual preparation and line represents the mean.

Effects of saline on the cardiac neuromuscular system

To examine whether the trend observed in the STNS would be observed in a circuit that drives a behavior necessary for the lobster's survival but whose properties differ drastically from the STNS, I then performed a similar set of experiments on the lobster's cardiac neuromuscular system. Like the STNS, the whole heart did not crash in 0.75x nor 1.25x saline concentrations, but exposure to saline concentrations more concentrated than 1.5x or less concentrated than 0.75x did cause the system to crash (Fig. 10A, C). The CG also could maintain function in 0.75x and 1.25x and did not crash in a wide range of saline concentrations (1x, 0.75x, 0.50x, 1.25x,

1.50x, 1.75x, and 2x saline) compared to the whole heart (Fig. 10A, C). All the whole heart preparations crash in 2x saline, and 50% crashed in 1.75x saline, while 20% crashed in 0.50x, and 100% crashed in 0.25x saline (Fig. 10C). Interestingly, in contrast, 100% of the CG preparations crashed in 0.25x but did not crashed in any other of the concentrations mentioned (Fig. 10C).

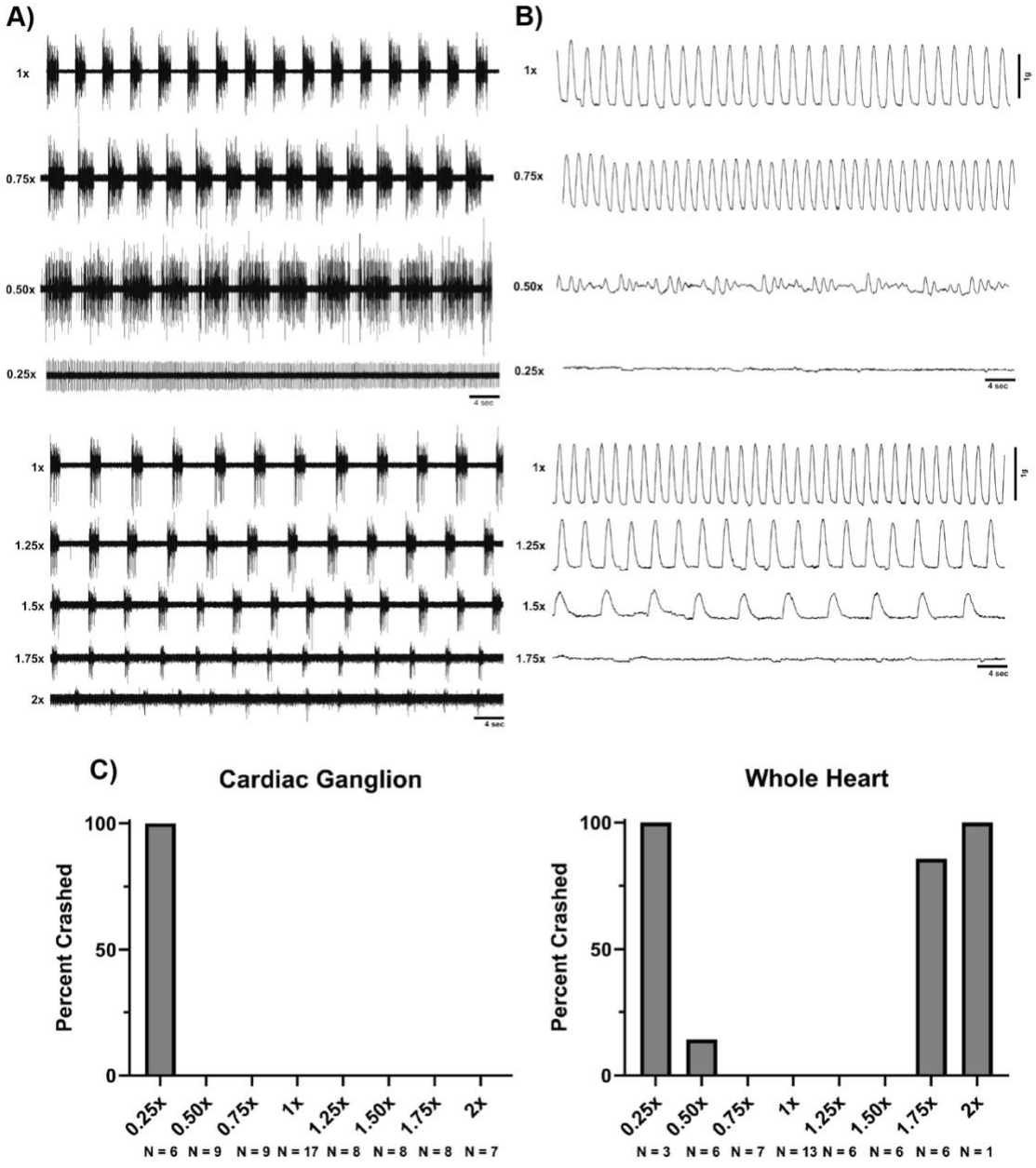

Figure 10. The CG can withstand a larger range of saline concentrations compared to the whole heart. A) Example extracellular CG recordings upon exposure to 1x, 0.75x, 0.50x, and 0.25x saline (upper traces), and example traces from recordings of a separate preparation upon exposure to 1x, 1.25x, 1.50x, 1.75x, and 2.0x (lower traces). **B)** Example force transducer recordings from a whole heart upon exposure to 1x, 0.75x, 0.50x, and 0.25x saline (upper traces), and from of a separate preparation upon exposure to 1x, 1.25x, 1.50x, 1.75x, and 2.0x (lower traces). **C)** The percent of CG (left) and whole heart (right) that crash upon exposure to a given saline concentration.

Effects of temperature and saline on the cardiac neuromuscular system

The crash temperatures of the CG were significantly higher than the whole heart in 1x saline (unpaired *t*-test; p=0.0253; WH N=15, CG N=16), 0.75x saline (unpaired *t*-test; p=0.0012; CG N=14, WH N=17) and 1.25x (unpaired *t*-test; p = 0.0210; CG&WH N=12) (Fig. 11). Similar to findings in the STNS, both the cardiac ganglion and the whole heart crashed at higher temperatures in 0.75x saline concentrations than 1x saline (Tukey's multiple comparisons test; CG: p <0.0001, 0.75x N=16, 1x N=14; WH: p <0.0001, 0.75x N=17, 1x N=21), and significantly lower temperatures in 1.25x compared to 1x for both the whole heart and CG (Tukey's multiple comparisons test; CG: p=0.003, 1x N=16, 1.25x N=13; WH: p<0.0001; 1x N=21, 1.25x N=18).

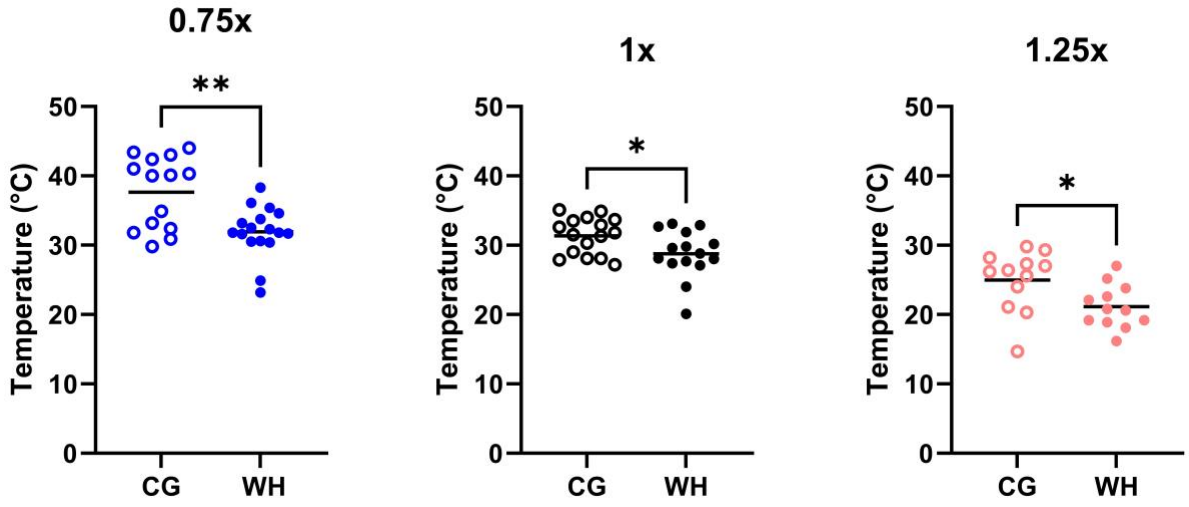

Figure 11. The CG crashes at higher temperatures than the whole heart in 0.75x, 1x, and 1.25x saline. The crash temperatures of the CG are significantly higher than the whole heart in 1x saline (unpaired *t*-test; p=0.0253; WH N=15, CG N=16), 0.75x saline (unpaired *t*-test;

p=0.0012; CG *N*=14, WH *N*=17) and 1.25x (unpaired *t*-test; *p*=0.0210; CG&WH *N*=12). This was rather unexpected given the anatomical and physiological differences between these two circuits.

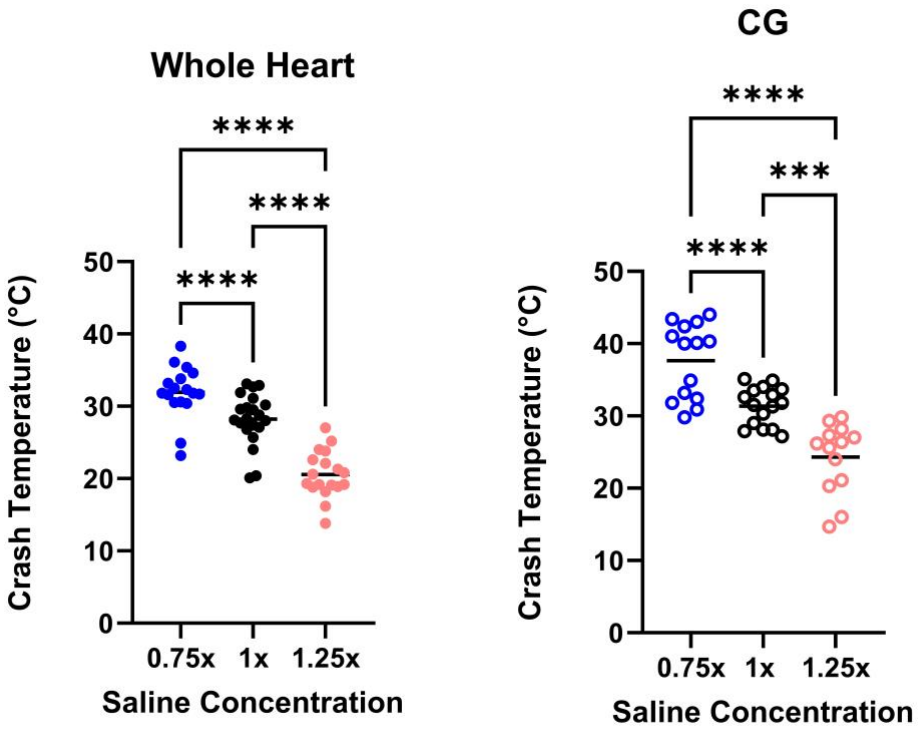

Figure 12. The whole heart and cardiac ganglion crash at higher temperatures in 0.75x saline and lower temperatures in 1.25x saline. The crash temperature in 0.75x is significantly higher in 0.75x as compared to 1x (Tukey's multiple comparisons test; CG: *p*<0.0001, 0.75x *N=16, 1x N*=14; WH: *p*<0.0001, 0.75x *N*=17, 1x *N*=21), and significantly lower in 1.25x compared to 1x for both the whole heart and CG (Tukey's multiple comparisons test; CG: *p*=0.003, 1x *N*=16, 1.25x *N*=13; WH: *p*<0.0001; 1x *N*=21, 1.25x *N*=18).

Discussion

The extent to which the stomatogastric nervous system and the cardiac neuromuscular system to withstand temperature and salinity perturbations described here, when those perturbations are presented both individually and in combination, are examples of the impressive capacity of neurons and neural circuits to maintain output in the face of perturbation. Temperature and saline concentration each globally affect the function of a neural network because biological processes, including rates of synaptic transmission and neuronal excitability,

are temperature dependent and altering extracellular saline concentrations also affects neuronal excitability. It was unexpected that rather than interfering with the capacity of the pyloric circuit to withstand elevated temperatures, the additional perturbation of lower saline concentration instead allowed the circuit to withstand higher temperatures. Given that this result in of itself was unexpected, it was interesting that the same trend was also observed in the CG, a circuit that differs drastically from the pyloric circuit in terms of mechanism of pattern generation and in its underlying conductances. While it is not clear if the mechanisms that allow each circuit to continue to function at relatively higher temperatures in lower saline concentrations are similar for the STNS and the CG, it is clear that both circuits have evolved a set of biological solutions that confer an ability to continue function in the face of simultaneous exposure to both perturbations, both within and across individuals.

Altered extracellular saline concentrations altered the pyloric rhythm's crash temperature

The system was able to withstand exposure to 0.75x and 1.25x saline concentrations without crashing and without altering the frequency of the pyloric rhythm at control temperatures, but it was unpredictable whether compensatory mechanisms (which, due to the timescale of these experiments are most likely to include second messenger pathway induced changes in channel densities or channel phosphorylation and de-phosphorylation), that permit the system to withstand these altered saline concentrations would interact with the effects of elevated temperature to lower the maximum temperatures that the system could withstand (He et al., 2020; Ransdell et al., 2012). While the system crashed at lower temperatures when exposed to higher saline concentrations, an interaction between the lower saline concentrations and elevated temperature caused the system to crash at higher temperatures as compared to those in 1x saline (Fig. 9).

It has been shown previously that in *C. borealis* elevated temperatures do not drastically alter the membrane potential trajectories of the LP neuron, illustrated by the Q_{10} of 0.92 of the total combined IPSPs onto the LP neuron. As previously described, this relative temperature insensitivity arises, in part, because even as LP's input conductance increases as a function of temperature, with Q_{10} of 1.56, while IPSCs onto the LP neuron have Q_{10} of 2.29 (Tang et al., 2010). Therefore, the increase in IPSC amplitude can offset the simultaneous increase in input conductance. However, in lower extracellular saline concentrations it would be expected that the cell's membrane potential would become hyperpolarized as the equilibrium potential of K^+ becomes hyperpolarized and K^+ exits the cell. Therefore, in lower saline concentrations when temperature is elevated, the cell may be more hyperpolarized as compared to when the cell is in more concentrated saline solutions.

This hyperpolarized membrane potential may help explain the capacity of the system to withstand higher temperatures without crashing when the nervous system is exposed to lower saline concentrations, because it may contribute to the continued activity of the I_h and I_A currents at elevated temperatures. It has been shown previously that in *C. borealis* activation of the $I_{h,}$ current, a hyperpolarization activated inward current that contributes to the ability of fire upon rebound from inhibition and that reduces delay to firing, and activation and inactivation of I_A, a transient outward current that induces a delay to firing, have similar Q_{10}s in LP. This likely contributes to the capacity of LP to continue bursting as temperature rises (Tang et al., 2010). Given that membrane potentials that are more hyperpolarized make both I_h current activation and de-inactivation of I_A more probable and that both currents are important in maintaining bursting, exposure to lower saline concentrations likely contributes to the ability of the cells in the pyloric circuit to continue to burst at higher temperatures in lower saline concentrations.

In extracellular saline concentrations that are higher, however, membrane potential may become more depolarized as the equilibrium potential of potassium becomes more depolarized. The pyloric network's burst frequency rises as temperature increases (with a Q_{10} of 1.872) and this increase in burst frequency, in conjunction with membrane depolarization, may contribute to the lower crash temperatures observed when preps were exposed to higher saline concentrations. This may be because burst frequency approaches or begins to exceed the rate of channel inactivation for one or multiple ion channels, and simultaneously, depolarized membrane potentials may cause channels to be less likely to be de-inactivated because of a shift in activation and inactivation probabilities. Therefore, it is possible that these changes compound to increase the likelihood of a depolarization block. If currents that mediate pace-making activity are no longer able to de-inactivate, this could lead to a decreased temperature tolerance for the pyloric rhythm in higher saline concentrations. To test the possibility that an interaction of increased burst frequency with a more depolarized membrane potential underlies the lower crash temperatures observed in higher saline concentrations, future experiments could isolate the PD neurons from AB to prevent intrinsic peacemaking, and instead artificially control the network's oscillatory activity to investigate how burst frequency alters the crash temperature of the pyloric rhythm when in more concentrated saline solutions.

The depolarization of potassium's equilibrium potential in higher extracellular saline concentrations also reduces potassium's driving force, which may contribute to lower crash temperatures observed in higher extracellular saline. The inhibitory synapses of the pyloric circuit are mediated, in part, by potassium, and so it is possible that this decrease in potassium's driving force reduces the IPSC amplitude at these synapses, thereby interfering with an important component of pyloric rhythm generation (Marder & Paupardin-Tritsch, 1978). Additionally,

LP's IPSP temperature compensation in *C. borealis* arises, in part, from an increase in IPSC amplitude that balances an increase in membrane conductance, so it is possible that a reduction of IPSC amplitude as saline concentration changes interferes with this mechanism of compensation (Tang et al., 2010). If because of a reduction of IPSC amplitude, the cells no longer hyperpolarize to voltages sufficient to initiate post-inhibitory rebound, this may contribute to system crashing at lower temperatures. Additionally, this reduction in potassium's driving force may also reduce the amplitude of I_h current, thereby inhibiting the ability of the cells to rebound from inhibition and causing the system to crash. In contrast with these hypotheses, Städele et al. (2015) shows that the LG neuron of *C. borealis*, which is contained within the STNS, becomes hyperpolarized at elevated temperatures. However, this neuron relies on different mechanisms of burst generation and is only indirectly influenced by the pyloric circuit, so it is unclear if this finding has implications for the activity of LP and PY neurons..

The CG and whole heart crash at higher temperatures in lower saline concentrations

The mode of pace-making and connectivity of the CG differs from the STNS, where the CG's pattern generation is driven by calcium mediated driver potentials whereas that of the STNS relies on the intrinsic oscillatory activity of the AB interneuron and subsequent rebound from inhibition of the follower neurons, and yet like the STNS, the CG and whole heart also crashed at higher temperatures in lower saline concentrations. CG burst frequency has been shown to increase as temperature rises until around 18-19ºC (Powell et al., 2023), and preliminary data from the Powell lab not shown here indicates that membrane potential becomes more depolarized in more concentrated saline solutions and more hyperpolarized in less concentrated saline solutions. Therefore, as in the STNS, it could be the case that exposure to less concentrated saline concentrations allows the system to continue to function at higher

temperatures because the more hyperpolarized membrane potential helps reduce the likelihood of a depolarization block by allowing driver potential Ca^{2+} channels to de-inactivate, whereas in higher temperatures the increase in frequency compounded with a more depolarized membrane potential could increase the likelihood of a depolarization block.

The PY neurons crash at higher temperatures than LP

The observation that the LP neuron crashes at lower temperatures than the PY neurons in each saline concentration might be explained by differences in channel expression between cell types, and by differences in the number of each cell type (Fig. 8). In *C. borealis*, there is considerable variability in channel expression between cell types in the stomatogastric ganglion and this is also likely true in the lobster given the difference in waveform between the cell types (Schulz et al., 2007). Neuronal activity relies on precise current activation and inactivation that are balanced with the activity of other currents, which have variable temperature dependencies (Klöckner et al., 1990). Therefore, it is possible that because of their different temperature dependencies, the currents that drive LP activity become desynchronized at lower temperatures as compared to PY, causing the neuron to stop bursting at comparatively lower temperatures. In future experiments, it would be interesting to compare the effects of altered extracellular saline concentrations on the membrane potentials of these neurons to discern if and how the effects of altering extracellular saline concentrations differ between them. Another possible explanation could be that because there is a single LP neuron in the pyloric circuit but multiple PY neurons. So, even if a subset of the PY neurons crash at temperatures more comparable to those at which LP crashes, PY activity of the remaining PY neurons was still observed on the extracellular recordings.

The CG crashes at higher temperatures than the whole heart and can withstand a wider range of saline concentrations without crashing

The CG crashes at higher temperatures compared to the whole heart, a trend observed here when the systems are exposed to 1x, 0.75x, and 1.25x saline concentrations, and which has been observed previously in 1x saline (Powell et al., 2023). It has been suggested that this trend may result from a lack of inhibition from nitric oxide in the CG that is otherwise present in the whole heart that contributes to the cessation of beating before bursting. In addition, a failure of muscles to contract even as the CG continues to produce rhythmic bursts of action potentials could underlie this trend (Powell et al., 2023). Similarly, the whole heart may crash in saline concentrations that do not cause the CG to crash because the altered saline concentrations have an impact on the muscle that prevent it from contracting that does not impact the isolated nervous system, or there may be an effect of altered saline concentration at the neuromuscular junction that causes the heartbeat to be less likely to contract. Further experiments are needed to fully understand the processes that limit cardiac physiology under these conditions.

Conclusions

This research characterizes the effects of global perturbations, presented individually and simultaneously, on the function of the lobster's pyloric circuit and cardiac neuromuscular system. Like the temperature responses observed in *C. borealis*, the pyloric rhythm of the lobster maintained constant phase across a range of elevated temperatures even as burst frequency increased. When exposed to altered extracellular saline concentration at the same time as elevated temperature, the maximum temperature that the system was able to withstand was affected by the presentation of the second global perturbation. Because the STG and CG circuits are differently configured, it was unexpected that they would each exhibit similar responses

when challenged with these two global perturbations: each circuit crashed at higher temperatures in lower saline concentrations and lower temperatures in higher saline concentrations. Interestingly, in the STG, I observed that the pyloric neurons LP and PY crashed at different temperatures, where the LP neuron consistently crashed at a lower temperature than PY. Furthermore, for the CG and whole heart, the CG crashed at higher temperatures than the whole heart in all saline concentrations tested.

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