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I_h Block Reveals Separation of Timescales in Pyloric Rhythm Response to Temperature Changes in *Cancer borealis*

Kyra A Schapiro, **JD Rittenberg**, **Max Kenngott**, **Eve Marder**

Volen Center and Biology Department, Brandeis University, Waltham, MA 02454 USA

Abstract

Motor systems operate over a range of frequencies and relative timing (phase). We studied the contribution of the hyperpolarization-activated inward current (I_h) to frequency and phase in the pyloric rhythm of the stomatogastric ganglion (STG) of the crab, *Cancer borealis* as temperature was altered from 11°C to 21°C. Under control conditions, the frequency of the rhythm increased monotonically with temperature, while the phases of the pyloric dilator (PD), lateral pyloric (LP), and pyloric (PY) neurons remained constant. When we blocked I_h with cesium (Cs^+) PD offset, LP onset, and LP offset were all phase advanced in Cs^+ at 11°C, and the latter two further advanced as temperature increased. In Cs^+ the steady state increase in pyloric frequency with temperature diminished and the Q_{10} of the pyloric frequency dropped from ~1.75 to ~1.35. Unexpectedly in Cs^+, the frequency displayed non-monotonic dynamics during temperature transitions; the frequency initially dropped as temperature increased, then rose once temperature stabilized, creating a characteristic “jag”. Interestingly, these jags were still present during temperature transitions in Cs^+ when the pacemaker was isolated by picrotoxin, although the temperature-induced change in frequency recovered to control levels. Overall, these data suggest that I_h plays an important role in the ability of this circuit to produce smooth transitory responses and persistent frequency increases by different mechanisms during temperature fluctuations.

eLife assessment

This **important** study investigates neurobiological mechanisms underlying the maintenance of stable, functionally appropriate rhythmic motor patterns during changing environmental conditions - temperature in this study in the crab Cancer borealis stomatogastric central neural pattern generating circuits producing the rhythmic pyloric motor pattern, which is naturally subjected to temperature perturbations over a substantial range. The authors present **compelling** evidence that the neuronal hyperpolarization-activated inward current (Ih), known to contribute to rhythm control, plays a vital role in the ability of these circuits to appropriately adjust the frequency of rhythmic neural activity in a smooth monotonic fashion while maintaining the relative timing of different phases of the activity pattern that determines proper functional motor coordination transiently and persistently to temperature perturbations. This study will be of interest to neurobiologists studying rhythmic motor circuits and systems and their physiological adaptations.

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Introduction

Many repetitive motor patterns, such as breathing, walking, and swimming, operate over a wide range of frequency and relative timing of component movements (phase). The frequency and phase of these components dictates the function and efficiency of the overall motion. Many locomotor systems maintain effectiveness by changing phase concomitantly with frequency[1].

Some systems, such as lamprey[2] and leech[3] swimming, require constant phase across frequencies. In either case, when such systems transition to new steady frequencies, they normally do so in a smooth, monotonic fashion. However, control theory recognizes that while smooth transitions may be optimal, they are not inevitable. The constraints of a system can cause a non-monotonic response to a state change, in some cases even having an initial change opposite to the final appropriate response. While the dynamics of many motor patterns under stable conditions have been extensively studied, how such systems appropriately adjust their phase and frequency as conditions change and avoid turbulent dynamics has been less often addressed.

Many rhythmic neurons and muscles utilize the hyperpolarization-activated inward current, I_h, in generating their rhythmic activity[4–6]. I_h is mediated by the HCN-family of channels and is activated by hyperpolarization. This property allows excitable cells to depolarize after periods of inhibition, contributing to post-inhibitory rebound bursting behavior[4,5,7–9]. While some systems produce patterns myogenically, e.g. the vertebrate heartbeat[6,10–13], other systems rely on networks of cells to produce their activity, such as the leech heartbeat generator[14–19] or the mammalian respiratory system[20,21].

While I_h is utilized for rhythmogenesis in these systems, even a basic pattern of activity of a hard-wired neural circuit can be achieved through multiple combinations of intrinsic and synaptic properties[22–28]. The set of properties that allow functional activity during one set of conditions is likely inadequate for all conditions. Theoretical studies suggest that parameter sets that can produce similar activity in one environment display different robustness when the

environment changes[22,23]. As I_h is a key component of ubiquitous rhythmic activity, we explored its potentially changing role during transitions in conditions a rhythmic system might encounter.

To this end, we utilized the response of the pyloric rhythm of the stomatogastric ganglion (STG) of the crab, *Cancer borealis,* to temperature changes[22,29–31]. The pyloric rhythm controls food filtering by the crustacean pylorus[32]. Frequency is dictated by a three neuron electrically coupled pacemaker kernel, made up of the intrinsically oscillating anterior burster (AB) neuron and the two pyloric dilator (PD) neurons. The pacemaker kernel neurons make inhibitory synapses onto the lateral pyloric (LP) neuron and several pyloric (PY) neurons, whose properties are such that LP recovers from pacemaker inhibition before PY [29,33–35]. At ~11°C the frequency of the pyloric rhythm is typically about 1 Hz with a characteristic triphasic rhythm of activity in the PD, LP, and PY neurons[36].

As a poikilotherm, temperature changes are relevant to a crab in its natural environment. The pyloric rhythm frequency in *C. borealis* increases as temperature increases[22,30,31,37,38], but the phasing of PD, LP, and PY neurons remains constant[30,38]. Previous work has demonstrated that I_h increases in pyloric cells as temperature increases, but not whether this increase is necessary for normal responses of pyloric activity to temperature[30,39]. To probe the role I_h plays across changing conditions, we pharmacologically blocked I_h using cesium ions during temperature changes and observed the effect of this block on the frequency increase and phase constancy of the pyloric rhythm. This revealed unanticipated changes in frequency during acute transitions between temperatures.

Results

The steady-state frequency increase in response to elevated temperature was decreased by Cs^+

We used a combination of extracellular and intracellular recordings to monitor pyloric activity while the STG was superfused sequentially with saline or saline with 5mM CsCl to block I_h [39–45]. We began recording at the standard temperature of 11°C and changed the temperature of the superfused solution in steps of 2°C up to 21°C using a Peltier device. The dish temperature and pyloric activity were allowed to stabilize for 4 minutes between steps (**Figure 1A**). To confirm the effectiveness of Cs^+ in reducing I_h across this temperature range, we voltage clamped pyloric neurons (PD and LP) at the temperature extremes used in the experiment, 11°C and 21°C (**Figure 1**—figure supplement 1). We held cells at -50mV and hyperpolarized for 12s in -10mV steps from -100mV to -120mV. Because I_h is slow to activate, we quantified I_h as the difference between the initial current needed to hold the hyperpolarization and the current required to hold the hyperpolarization at steady state. At 11°C and -110mV, I_h was 1.5±0.83nA in saline and 0.23±.5nA in Cs^+, a reduction of 93±30% (4 PD cells, 3 LP cells, paired Student's t- test; p=0.0005). At 21°C and -110mV, I_h was 3.2±2.3nA in saline and 0.6±.8nA in Cs^+, a reduction of 78±20% (5 LP cells, 3 PD cells, paired Student's t-test; p=0.02).

An example of simultaneous intracellular PD and LP activity during both control saline and in Cs^+ at each temperature is shown in **Figure 1B**. As previously reported[30,31,38], the pyloric frequency increased as temperature increased in control (**Figure 1B**, top traces). In Cs^+ at 11°C, the pyloric frequency was significantly decreased compared to control conditions (Saline: 1.2±0.2 Hz; Cs^+ 0.9±0.2 Hz, paired Student's t-test; p<0.001) as has also been previously reported[39,44–46].

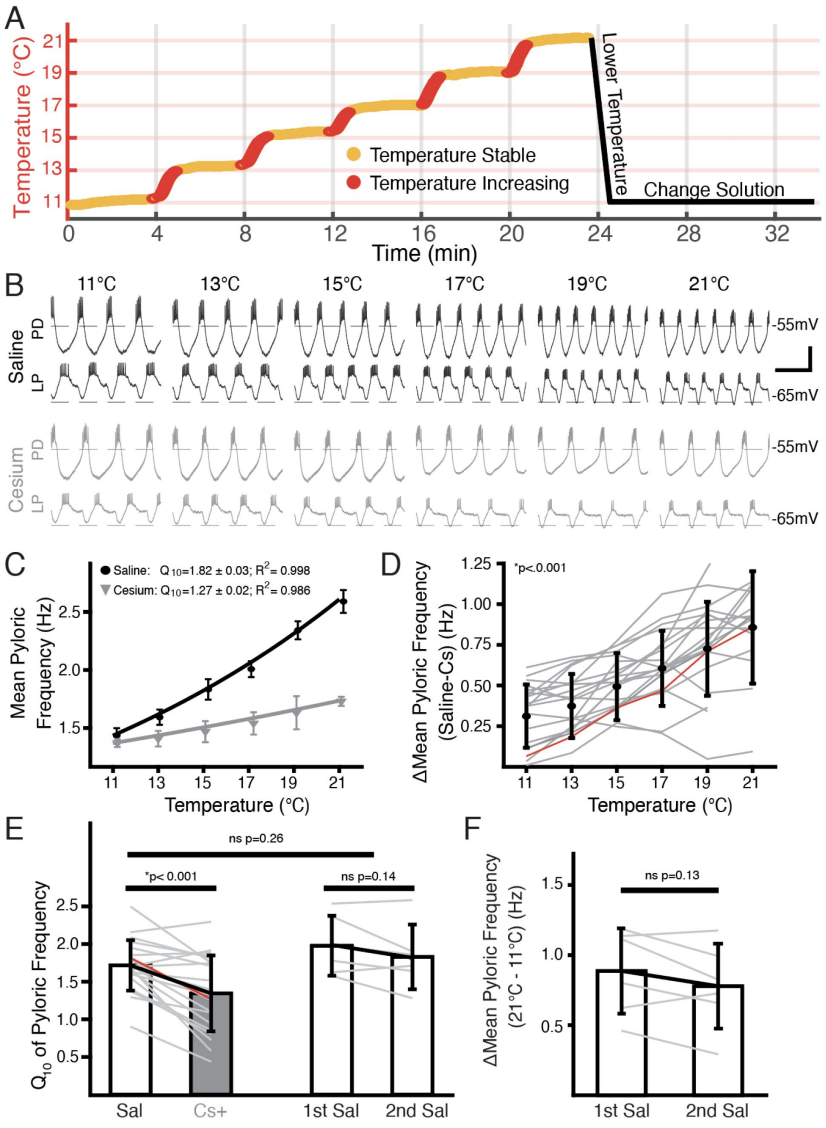


Figure 1

Temperature-induced frequency increase was diminished by Cs^+ application. **(A)** Basic experimental protocol. Experiment began in saline or Cs^+ solution (order randomized) at 11°C. The superfusion solution temperature was changed in steps of 2°C and allowed to stabilize over 4 minutes. Red represents times when the temperature was increasing, yellow when the temperature was considered stable. After the holding period at 21°C the preparation was returned to 11°C. The solution was then changed to the remaining condition over 10 minutes and the step protocol was repeated. **(B)** Intracellular recordings from a single preparation while in saline (**top**) and Cs^+ (**bottom**) at each holding temperature (yellow in **A**) from 11°C to 21°C. Within a condition the top trace is PD and the bottom trace is LP. Burst frequency increased as temperature increased in saline, but less so in Cs^+. Scalebar: Horizontal=1s, Vertical=20mV. **(C)** Mean frequency ± SD at each holding temperature across conditions from a different preparation than in **B**. Line represents the best fit Q_{10} equations for each condition; preparation in saline had a larger Q_{10} than in Cs^+ and as such a larger frequency increase with temperature. **(D)** Mean ± SD difference in frequency between saline and Cs^+ across temperatures and preparations. As temperature increased, the difference between a preparation's frequency in saline and Cs^+ significantly increased (1-way repeated measures ANOVA ($F(5,85) = 43.259$, $p < 0.001$, N = 21). Grey lines represent individual experiments, red line represents experiment depicted in **C**. **(E)** Q_{10} was significantly lower in Cs^+ than in saline (paired Student's t-test, $p < 0.001$, N=21). Q_{10} was not significantly different in two consecutive saline temperature changes (paired Student's t- test, $p = 0.14$, N=6). Q_{10} was also not significantly different between the mean Q_{10} of the experiments with two saline temperature changes those that experienced one saline and one Cs^+ temperature change (two-sample t-test, $p=0.26$). Bars are mean ± SD, individual lines represent individual experiments, red line is experiment depicted in **C**. **(F)** Overall change in mean pyloric frequency from 11°C to 21°C was not significantly different between first and second temperature change in saline (paired Student's t-test, $p=0.14$, N=6).

Interestingly, the degree of frequency increase with temperature was diminished in Cs^+ (**Figure 1B**, bottom traces). To quantify this effect, we took the mean pyloric frequency during times when the preparation was within 0.3°C of the target temperature and plotted the mean frequency as a function of temperature (**Figure 1C**). Within a preparation, the difference in frequency between control and Cs^+ at a given temperature significantly increased as temperature increased (**Figure 1D**). Additionally, we characterized the temperature sensitivity of each preparation in control and Cs^+ using Q_{10} as a measure of frequency change with temperature as described previously[30]. The mean Q_{10} of the pyloric frequency was significantly lower in Cs^+ (1.3±0.5) than in control (1.7±0.3) (**Figure 1E**). Together, these analyses show that the temperature-induced increase in pyloric frequency in control saline was greater than in Cs^+.

In addition to counterbalancing the starting condition, to further support the conclusion that the decline in pyloric frequency Q_{10} in Cs^+ was due to Cs^+ and not consecutive temperature changes, we performed two consecutive temperature changes in saline. The mean difference in frequency between 21°C and 11°C for the first (0.9±0.3 Hz) and second temperature changes (0.8±0.3 Hz) were not significantly different (**Figure 1F**). Moreover, the mean Q_{10} of the first temperature increase (2.0±0.4) was not significantly different than the mean Q_{10} of the second temperature increase (1.8±0.4) (**Figure 1E**). A two-sample t-test did not find a significant difference between the mean frequency Q_{10} in saline in experiments that underwent two saline temperature changes compared to those that experienced one in saline and one in Cs^+ (**Figure 1E**). Therefore, the decrease in frequency temperature sensitivity in Cs^+ was likely not a product of sequential temperature changes.

Cs^+ revealed dynamic transitory frequency response: frequency decreased while temperature increased, then increased when temperature stabilized

While the increase in frequency over the temperature steps was primarily monotonic in control, it was often non-monotonic in Cs^+. In Cs^+, during the increases between temperatures there was often either no increase or a decrease in frequency. **Figure 2** illustrates simultaneous intracellular recordings of a PD cell and an LP cell during the beginning of the step from 13°C to 15°C in both control (**Figure 2A**) and Cs^+ (**Figure 2B**). In control, when the temperature was increased (maroon vertical dashed line), there was a net increase in frequency. In contrast in Cs^+, there was an immediate decrease in frequency. In this particular example, the number of spikes per burst in LP decreased, though this effect was not seen in all preparations. Alignment of each PD cycle during this window to the first PD spike reveals that the decrease in frequency was due to elongation of the depolarizing phase and not the bursting, repolarization, or inhibitory phases, rather than a uniform expansion of all waveform components (**Figure 2C**).

Figure 3A shows an example of the entire frequency response during a temperature step between 17°C and 19°C. In control (**Figure 3A**, left), the frequency rose when the temperature increased, and stabilized when the temperature stabilized. Conversely in Cs^+, the frequency decreased when the temperature increased as in **Figure 2B**, and then rose when the preparation reached the holding temperature (**Figure 3A**, right). This activity produced a "jag" in the frequency-temperature curve (**Figure 3B**). We compared the distributions of change in mean frequency at times when the temperature was increasing or stable across conditions. "Increasing temperature" was defined as times when the change in mean temperature was greater than 0.01°C (red on **Figure 1A**, 3A,B). For these increasing-temperature pyloric cycles we calculated the proportion in which the mean frequency decreased by more than 0.002 Hz (blue points in **Figure 3A**; left of dashed line in overall distribution for one experiment in **Figure 3C** top) in both control and Cs^+. The proportion of cycles where frequency decreased while temperature increased was greater in Cs^+ than in control for 20 of 21 experiments. In Cs^+, the mean percent of temperature-increasing pyloric cycles in which the frequency decreased

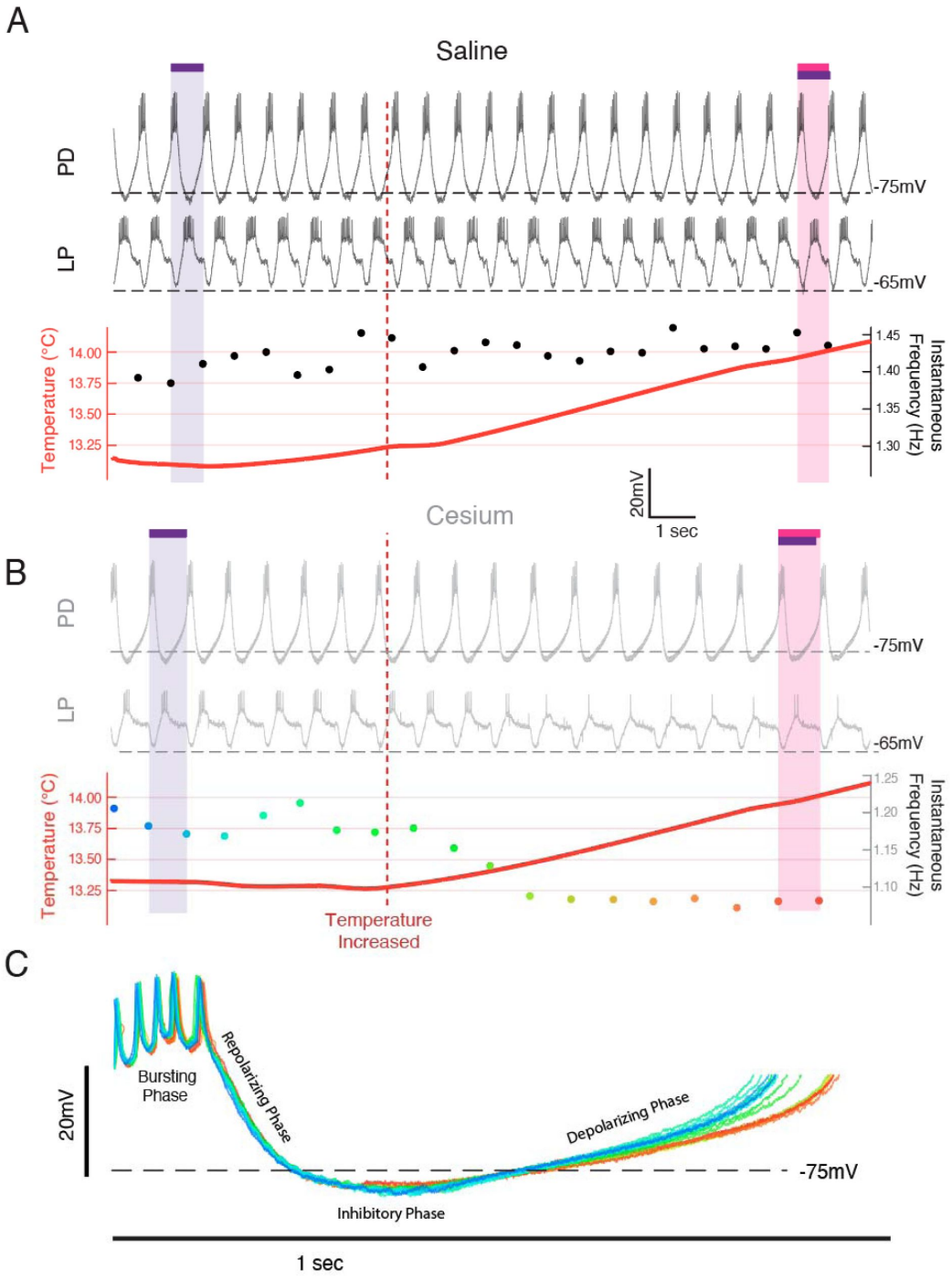


Figure 2

Frequency decreased when temperature was increased in Cs+ through an elongation of the depolarizing phase. Example from a single preparation as temperature was changed from 13°C towards 15°C in Saline**(A)** and Cs^{+}**(B,C). (A,B) Top:** the intracellular recording from PD; Middle: simultaneous intracellular recording from LP; Bottom: simultaneous temperature (red; left axis) and instantaneous frequency (right axis). Purple bar highlights one pyloric period before the temperature was increased, while the magenta bar highlights one pyloric period after. **(A)** In saline when the temperature increased (maroon line), the period decreased as highlighted by the magenta bar being shorter than the purple bar. **(B)** In Cs+, the period increased after the temperature shift, as highlighted by the magenta bar being longer than the purple bar. The same scale is used in both **A** and **B**. **(C)** Individual PD cycles from **B** aligned to the first spike. Coloration indicates when an individual cycle occurred as keyed by the instantaneous frequency trace in B. Increased period corresponds to an elongation of the depolarizing phase and a maintenance of the bursting and repolarization phases.

(31.4±15%) was significantly higher than in control (7.5±5%) (**Figure 3D**, paired Student's t-test, N=21, p <0.001). During pyloric cycles with temperature stability (yellow on **Figure 1A**, 3A,B), we calculated the proportion in which the mean frequency increased by more than 0.002 Hz (green points in **Figure 3A**; right of dashed line in overall distribution for one experiment in **Figure 3C** bottom). Again, this proportion was larger in Cs^+ than in control in 20 of 21 experiments. In Cs^+, the mean percent of temperature-stable cycles in which frequency increased in Cs^+ (27.6±6%) was significantly higher than in control (14.1±5%) (**Figure 3E**, paired Student's t-test, N=21, p <0.001). To summarize, in Cs^+ there was a distinct tendency for the frequency to decrease when temperature increased and then increase after the temperature stabilized.

In Cs^+, follower neurons determined frequency temperature sensitivity: pacemaker kernel determined jag response

We investigated to what extent the two effects of Cs^+, change in temperature sensitivity and non-monotonic frequency response, were caused by effects in the pacemaker kernel (AB and PD neurons) versus the follower neurons (LP and PY). To do so, we repeated the temperature step protocols in control saline, 5mM Cs^+ saline, and 5mM Cs^+ + 10^{-5} picrotoxin (PTX) saline.

PTX blocks the glutamatergic synapse that is the only feedback from the follower neurons to the pacemaker[31,47–49] (**Figure 4A**). **Figure 4B** illustrates that in Cs^++PTX, the pyloric rhythm did not show the diminished frequency-increase seen in Cs^+ alone but did demonstrate jags. We compared the change in frequency between 11°C and 21°C in control, Cs^+, and Cs^++PTX and found that there was a significant effect of condition; change in frequency in Cs^+ was significantly lower than either control or Cs^++PTX (**Figure 4C**) with no significant difference between control and Cs^++PTX. Therefore, the effect of Cs^+ on the temperature-induced frequency increase appears to require the activity of the follower neurons.

We also compared the probability of frequency decrease during temperature increase and probability of frequency increase during temperature stability. Both measures showed significant effects of condition. The probability of frequency decrease while temperature increased was lower in control than in either Cs^+ or Cs^++PTX (**Figure 4D**), with no difference between Cs^+ and Cs^++PTX. Interestingly, the probability of frequency increase while temperature was stable was significantly different between all three conditions: control was lowest, Cs^+ was in the middle, and Cs^++PTX was greatest. Therefore, the frequency jags appear to result from Cs^+ effects on the pacemaker kernel neurons, not the follower neurons, as they were still present when the isolated pacemaker underwent temperature steps in Cs^+.

One Cs^++PTX experiment in which elevating the temperature produced a particularly pronounced transient decrease in frequency is shown in **Figure 4F**. Overlay of the PD cycles during this window aligned to the first spike again show that the decrease in frequency is due to an elongation of the depolarizing phase rather than a uniform expansion of the waveform (**Figure 4G**). Additionally, without the inhibition from the LP to PD synapse, the hyperpolarization associated with increased temperature[50] becomes more apparent. When in this experiment the frequency increased after temperature had stabilized, the repolarizing phase shortened relative to the pre-temperature change, consistent with an overall maintenance of phase as temperature changed.

Phase of PD and LP advanced in Cs^+

We also examined the effect of Cs^+ on the firing time and phase relationships of PD, LP, and PY. **Figure 5A** illustrates the pyloric activity in both control (left) and Cs^+ (right) at 11°C recorded on the *pdn* (top), *lvn* (middle) and *pyn* (bottom). The time of PD bursting was significantly but modestly shorter in Cs^+ (**Figure 5B**, Saline: 164±34msec, Cs^+: 144±18msec).

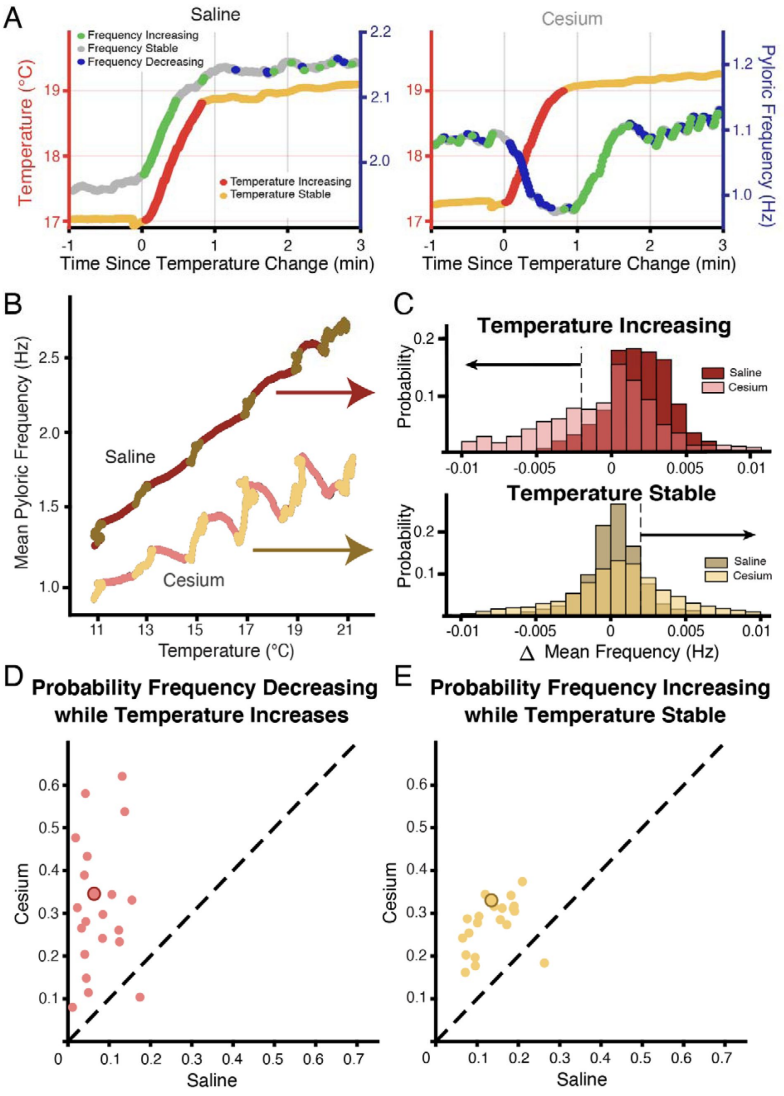


Figure 3

Across preparations frequency was more likely to decrease when temperature increased and increase when temperature was stable in Cs^+ compared to saline. **(A)** Example of a temperature step from 17°C to 19°C in saline **(left)** and Cs^+ **(right)**. Left axis and warm colors denote temperature, with reds denoting temperature increasing by more than 0.01°C/pyloric cycle, and yellows denoting stable temperature (change less than 0.01°C/pyloric cycle). Right axis and cool colors denote frequency, with green indicating mean frequency increase >0.002 Hz, blue indicating mean frequency decrease >0.002 Hz, and grey stable frequency between these bounds. In saline, frequency change closely mirrored temperature change. In Cs^+, frequency decreased (blue) when temperature increased (red) and increased (green) when temperature stabilized (yellow). **(B)** Mean pyloric frequency as a function of temperature for a single experiment in both saline and Cs^+. Note how in saline the frequency increased largely monotonically along the entire temperature range, increasing most when temperature increased (maroon). Conversely in Cs^+ the frequency displayed a non-monotonic, sawtooth-like pattern, decreasing when temperature increased (pink) and increasing when temperature was stable (light yellow). **(C)** For the same experiment as in **B**, distribution of mean change in frequency for cycles when the mean temperature was increasing (**top**) or when the temperature was stable (**bottom**), definition of temperature same as in **A**,**B**, **Figure 1A**. When the temperature increased, the change in mean frequency was more likely to decrease in Cs^+ than in saline. When temperature was stable, the change in mean frequency was more likely to increase in Cs^+ than in saline. Dashed line and black arrows highlight the criteria for decreasing and increasing in frequency used for quantification in **D** and **E**. **(D)** Across experiments, the probability of decreasing mean frequency while temperature increased in Cs^+ or in saline. For 20/21 of experiments, the probability was higher in Cs^+ than saline. Experiment from **B,C** is highlighted by the larger, outlined point. **(E)** Across experiments, the probability of having an increasing frequency while temperature was stable. For 20/21 of experiments, the probability was higher in Cs^+ than saline. Again, experiment from **B,C** is highlighted by the larger, outlined point.

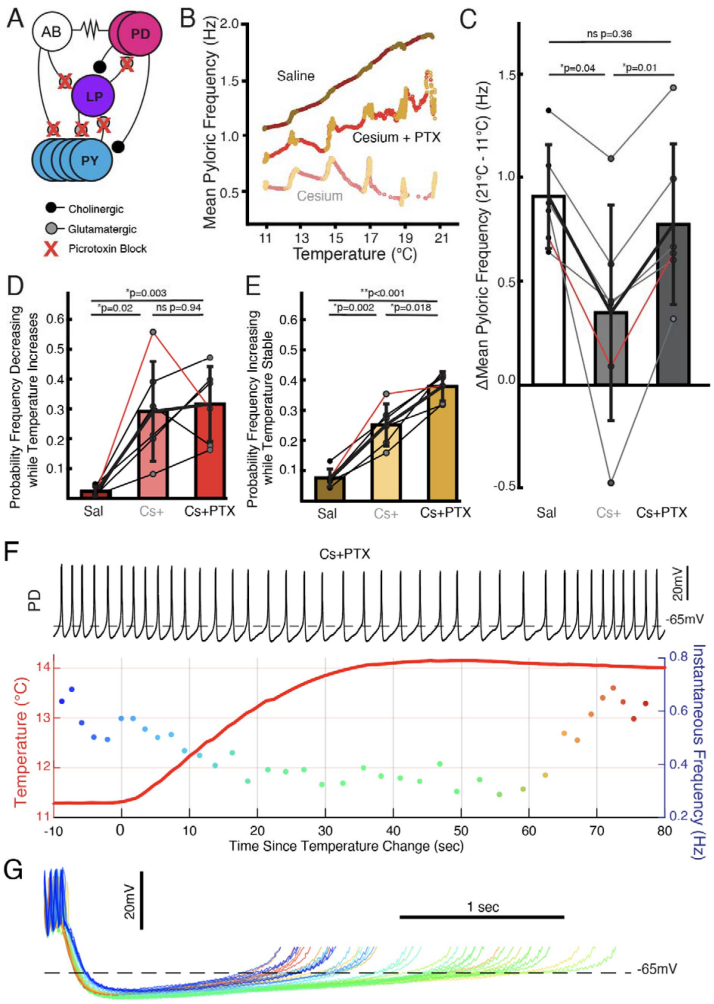


Figure 4

Follower neurons mediated pyloric frequency temperature sensitivity change by Cs^+, while non-monotonic temperature response was mediated by the pacemaker kernel neurons. **(A)** Wiring diagram of the pyloric network, illustrating the pacemaker kernel neurons (AB and PD) and the follower neurons (LP and PY). The only feedback synapse to the pacemaker kernel neurons, LP to PD, is blocked by picrotoxin (PTX). **(B)** Mean pyloric frequency as a function of temperature for a single experiment in saline, Cs^+, and Cs^+ + PTX. Red dots denote temperature increasing by more than 0.01°C/cycle, yellow dots denote stable temperature (change less than 0.01°C/cycle). In Cs^+ + PTX, the steady-state increase in frequency (yellows) as a function of temperature was similar to saline (trajectory roughly parallel). However, in Cs^+ + PTX pyloric frequency displayed non-monotonic responses to periods of transitory temperature increase as in Cs^+ alone. **(C)** There was a significant effect of condition on the change in frequency between 11°C and 21°C for N=6 experiments as a measure of temperature sensitivity (one-way repeated measures ANOVA; $F(2,10)=12.272$, $p=0.002$). Post hoc Tukey's Honestly Significant Difference (HSD) indicated the frequency changed significantly less in Cs^+ alone compared to either control or Cs^+ + PTX. Experiment from **B** is highlighted in red. **(D)** There was a significant effect of condition on the probability of frequency decrease during periods of temperature increase for N=6 experiments (one-way repeated measures ANOVA; $F(2,10)=13.077$, $p=0.002$). HSD indicated the probability was significantly higher in both Cs^+ and Cs^+ + PTX compared to control. Experiment from **B** is highlighted in red. **(E)** There was a significant effect of condition on the probability of frequency increase during periods of temperature stability for N=6 experiments (one-way repeated measures ANOVA; $F(2,10)=69.572$, $p < 0.001$). HSD indicated the probability was significantly greater Cs^+ than control and significantly greater in Cs^+ + PTX than in Cs^+. Experiment from **B** is highlighted in red. This elevated probability of increase may partially account for the lack of overall temperature-sensitivity decrease in Cs^++ PTX compared to Cs^+ alone in **C**. **(F)** Same scheme as **Figure 2B** and **C**. In Cs^+ + PTX frequency decreased when temperature rose and eventually increased once temperature stabilized. **(G)** Individual cycles from **F** aligned to the first spike, colored as in the instantaneous frequency trace in F. When the temperature increased (greenish traces), the depolarizing phase dramatically elongated and there was some increased hyperpolarization. After approximately 30 seconds of stable temperature, frequency increased and the depolarization accelerated again (reddish traces).

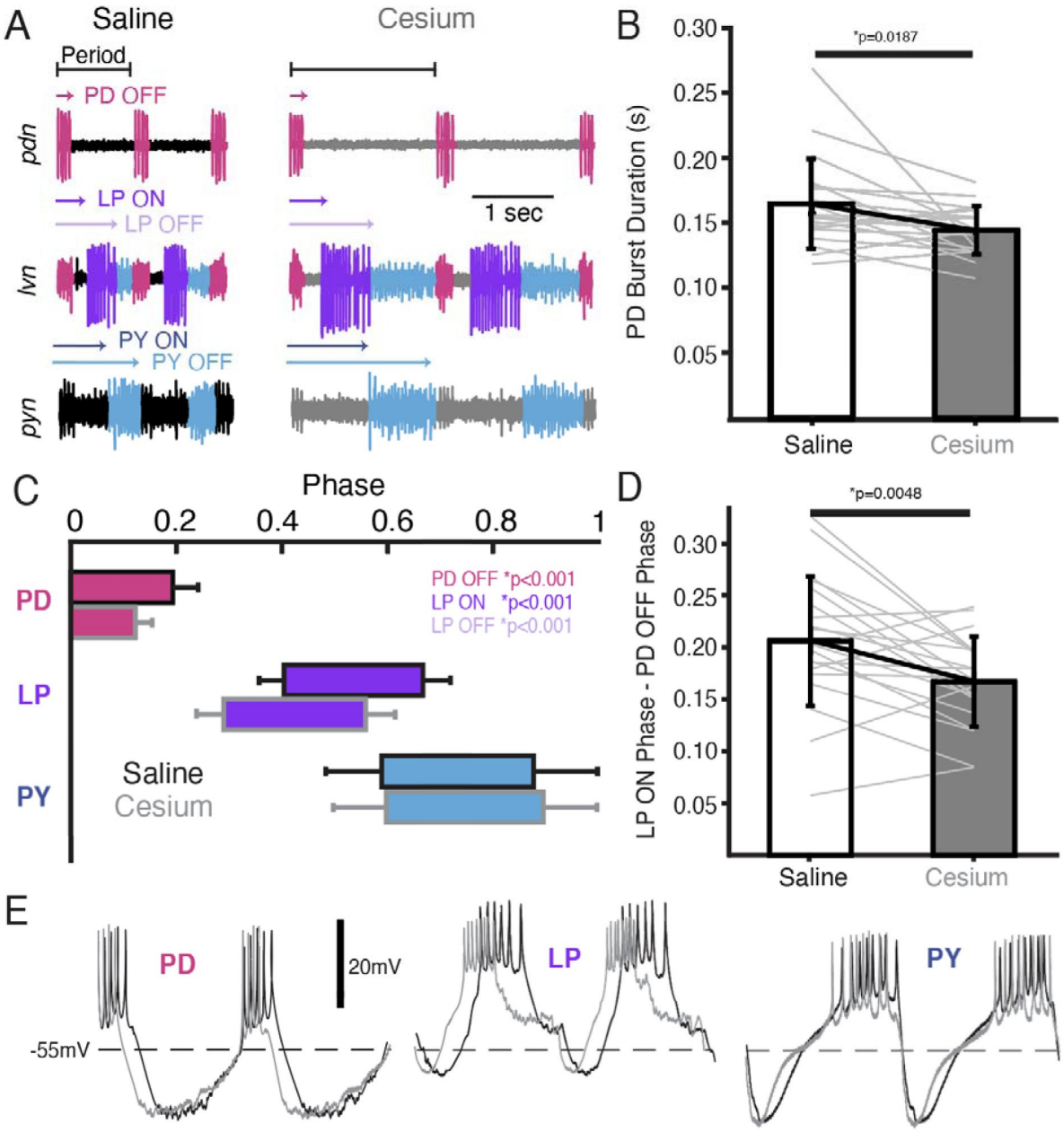


Figure 5

In Cs^+, PD OFF, LP ON, and LP OFF were all advanced compared to saline. **(A)** extracellular example traces of two pyloric cycles at 11°C recorded simultaneously on the *pdn* (**top**), *lvn* (**middle**), and *pyn* (**bottom**) in saline **(left)** and from the same experiment in Cs^+ (**right**). PD spikes are colored magenta, LP purple, and PY blue. Onset and offset delay of each neuron relative to the beginning of the period (PD burst) are indicated by arrows. **(B)** Duration of PD burst was marginally but significantly shorter in Cs^+ than saline (Wilcoxon Signed Rank Test, N=21, p=0.019). **(C)** Phase plot of saline and Cs^+ colored as in **A**. PD OFF, LP ON, and LP OFF are all advanced in Cs^+ compared to saline. (**D)** The phase delay between LP ON and PD OFF is smaller in Cs^+ than in saline (paired Student's t-test, N=21, p=0.0048). As such, LP ON is more advanced than one would expect if the system was maintaining a constant phase delay between PD OFF and LP ON. **(E)** Two periods of example intracellular recordings from PD (**left**), LP (**middle**), and PY (**right**) in saline (black) and Cs^+ (grey) scaled to the same period. Each column is from a different experiment. Note the advance of PD OFF, LP ON, and LP OFF with relatively little other change in the overall trajectory of the voltage trace.

Phase was quantified in **Figure 5C** and **Table 1**. Given the increase in period, it follows that the phase of PD OFF (percentage of period at which PD stops firing) was significantly advanced in Cs^+ compared to control. LP onset, which follows PD offset, was also phase advanced in Cs^+ conditions compared to control. However, this phase advance was more than would be expected given respective periods and the advanced PD OFF phase, as the delay between PD OFF and LP ON phase was significantly smaller in Cs^+ compared to control (**Figure 5D**, Saline: 0.21±0.06, Cs^+: 0.17±0.04).

LP OFF was also significantly advanced in Cs^+, and subsequently duty cycle (percent of the period a neuron is firing) was preserved (paired Student's t-test, N=21, p=0.448). PY ON and PY OFF were not significantly different between Cs^+ and control. The intracellular waveforms were similar between Cs^+ and control conditions apart from the noted phase-shifts. This similarity is illustrated in **Figure 5E** where two cycles of each pyloric neuron's intracellular activity have been scaled to the same period.

We identified cycles where period was matched between control at 11°C and Cs^+ (typically at a higher temperature for Cs^+). Although PD burst duration was only 51±30 msec shorter in Cs^+, LP fired 91±68 msec earlier. Conversely, PY latency did not change in these matched frequency samples (1.9± 100 msec earlier). There was no correlation between how advanced PD's offset was between control and Cs^+ conditions and how much LP's onset advanced in these matched period samples (Pearson correlation; $r(21)$=0.26, p=0.2928).

Phase constancy with increasing temperature was reduced by Cs^+; further advance with increasing temperatures in Cs^+

Having established that Cs^+ alters the phase relationships of PD and LP, we asked whether the phase constancy observed in control conditions in response to temperature increase was preserved in Cs^+. **Figure 6A** illustrates the phase of each pyloric component across temperature for both control and Cs^+ conditions. We fit a line to these points for each preparation and condition to determine as a first approximation whether there was a change in phase with temperature, though change in phase with temperature may not have been strictly linear (**Figure 6A**). In control conditions, LP OFF modestly advanced with temperature, with a mean advance of 0.07±0.07 per 10°C (Wilcoxon Signed Rank test, N=21, p<.01). This advance is demonstrated in the two *lvn* traces in **Figure 6B**, which depict one cycle of control activity at 11°C (top) and 21°C (bottom) scaled to the same period. The lilac bar highlights the advance of LP OFF at 21°C. All other pyloric components align, demonstrating phase constancy.

In Cs^+, LP ON and LP OFF advanced with temperature, with a mean advance of 0.05±0.05 per 10 °C and 0.14±0.09 per 10°C respectively (Wilcoxon Signed Rank test, N=21, p<.01). The advances of LP phase are shown in the two *lvn* traces in **Figure 6C**, which depict one cycle of Cs^+ activity at 11°C (top) and 21°C (bottom) scaled to the same period. The purple bar highlights the LP ON advance and the lilac bar highlights the advance of LP OFF at 21°C. PD and PY phase constancy was not significantly altered in either condition. Therefore, LP phase is most sensitive to temperature and is further phase advanced by increased temperatures in Cs^+.

Discussion

Motor patterns operate over a variety of conditions with varying phase and/or frequency to meet environmental needs. While the physiological mechanisms underlying many motor patterns have been investigated under stable conditions, studies of how these mechanisms change across conditions are rarer. We investigated how I_h contributes to frequency and phase of the pyloric rhythm in *C. borealis* during temperature changes using Cs^+ to decrease I_h. We conclude I_h plays

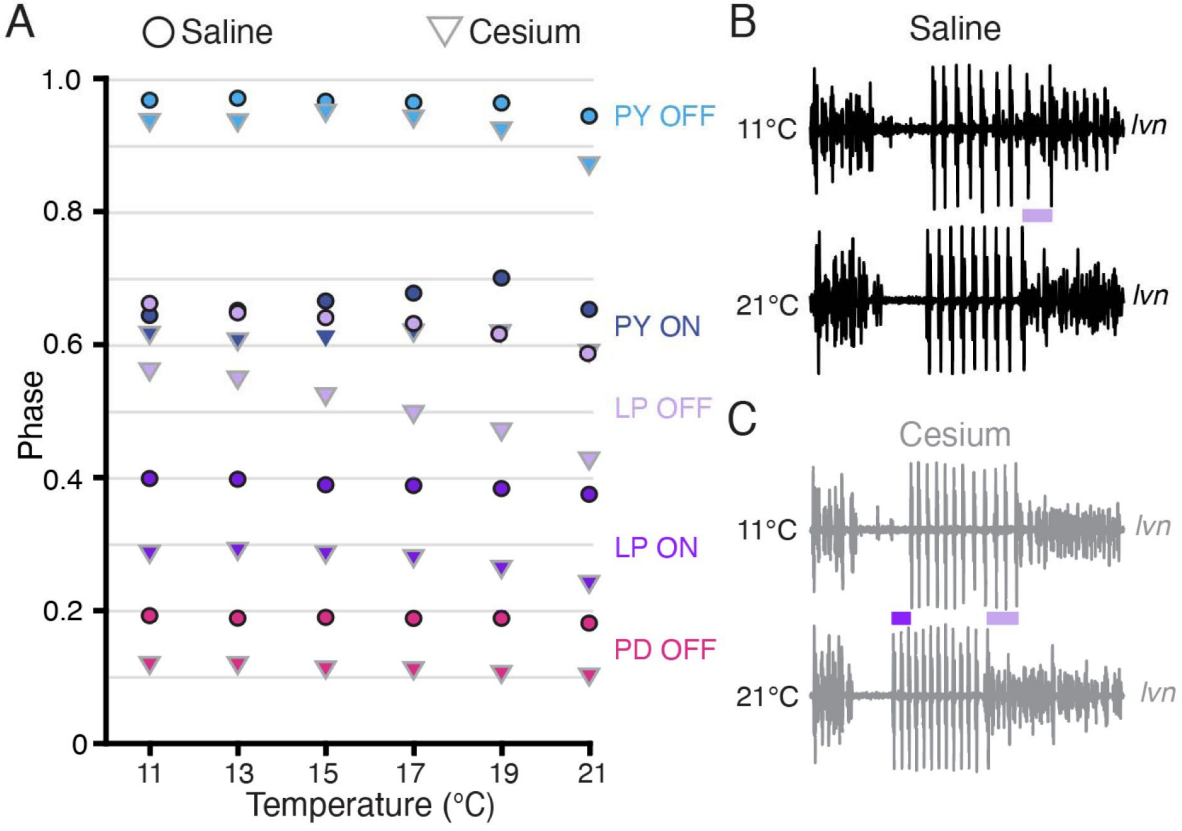
Table 1

Mean phase of pyloric neurons in saline and Cs^+ at 11°C. Phases that were significantly different in the two conditions are bolded. T=paired Student's t-test, W=Wilcoxon Signed Rank Test.

Phase	Saline	Cs^+	Significance Test	N	p-value
PD OFF	**0.19±0.04**	**0.12±0.03**	**T**	**21**	**<0.001***
LP ON	**0.40±0.05**	**0.29±0.05**	**T**	**21**	**<0.001***
LP OFF	**0.66±0.05**	**0.56±0.06**	**T**	**21**	**<0.001***
PY ON	0.58±0.11	0.60±0.11	T	12	0.86
PY OFF	0.87±0.20	0.90±0.19	W	12	0.91

Figure 6

Phase advanced across temperatures for LP OFF in both condition; LP ON phase also advanced with increased temperature in Cs^+. **(A)** Mean phase as a function of temperature in both saline (circles) and Cs^+ (triangles) (N=21 for PD and LP, N=12 for PY). Error bars have been omitted for clarity. LP OFF advanced as temperature increased in both saline and Cs^+, though the advance in Cs^+ was more dramatic. LP ON also significantly advanced with temperature in Cs^+. Criteria for maintaining constancy was a mean slope of phase vs temperature across experiments that was significantly different from zero according to a Wilcoxon Signed Rank Test (if not-normally distributed) or a paired Student's t-test (if normally distributed), Bonferoni-adjusted. **(B)** Single period of pyloric rhythm recorded extracellularly on the *lvn* at 11°C (**top**) and 21°C (**bottom**) scaled to the same period. The advance in LP OFF is highlighted by the lilac bar. (**C**) Same preparation as in **B** but while in Cs^+. Lilac bar highlights the LP OFF phase advance, purple bar highlights LP ON advance.



an important role in the ability of the system to appropriately adjust to temperature perturbations both transiently and persistently. Transiently, I_h removal revealed non-monotonic jags in the frequency response mediated by intrinsic properties of individual neurons. Persistently, I_h removal determined the overall frequency change (temperature sensitivity) through network effects.

The jags in frequency during transitory temperature changes upon I_h removal may be a consequence of imbalances in temperature sensitivities of individual neuron intrinsic properties

We unexpectedly found that in the presence of Cs^+, pyloric frequency decreased while temperature increased and then increased after the temperature stabilized (**Figs. 2**,**3**), producing jags in the frequency-temperature plot. This dynamic was mediated by the pacemaker alone (**Figure 4**). Previous observations of the effect of temperature on pyloric frequency showed the smooth increase seen in **Figure 3A** (left) and as such focused on the monotonic increase of steady state response as in **Figure 1C**[30,31,51]. We highlight for the first time the existence of a more dynamic response to increasing temperature. This dynamic occurred over the ~30 seconds of temperature increase and the first ~30 seconds of temperature stability (**Figure 2A** (right) and **Figure 4F**), suggesting the existence of a longer timescale process than previously recognized.

This longer timescale may be the result of interactions between two or more channels and/or channel-properties with fast kinetics[9,52–54]. Based on observations that the frequency decrease is associated with an elongation of the depolarizing phase of a burst (**Figure 2C**, 4G), we hypothesize that the decrease in frequency may be primarily caused by effects of temperature on the parameters of the transient potassium current, I_A. I_A is an inactivating, hyperpolarizing current activated by depolarization; its activation slows depolarization[30,40,55–57]. Each of its state transitions (activation, inactivation, deinactivation) may be differentially affected by temperature[30]. If deinactivation is more accelerated or altered by temperature than inactivation, then as temperature increases a larger pool of I_A channels would be available to be activated, resulting in transiently more I_A during the depolarizing phase of a PD cycle. This proposed transient increase in I_A would account for the decrease in frequency and elongated depolarization we observed. While temperature continued to change, the difference in parameters would continue to grow. Once the temperature stabilized a new equilibrium between activation, inactivation, and deinactivation could be achieved, potentially resulting in decreased total I_A during a pyloric cycle, an increase in the rate of depolarization, and an increase in frequency. I_h opposes and is correlated with I [25,44,55] and prevents a neuron from spending as much time in the I_A-deinactivating hyperpolarized state. Thus, when intact, we expect I_h obscures the dynamic frequency effects we propose to be caused by differential temperature sensitivities in I_A parameters.

Mathematically, activity patterns like those seen in Cs^+ where the output resulting from a step input first goes “in the wrong direction” have been described in control theory as having a “non-minimum phase dynamic”[58,59]. Typically, non-minimum phase systems are fragile and difficult to control, as they are prone to positive feedback loops unless the rate of sensor-induced adjustment is slow. While not uncommon in physics, such dynamics are generally avoided by biological systems to ensure robust responses[59]. Our data suggest I_h plays an important role in preventing the system from producing such turbulent behavior, resulting in the typical near-instantaneous and smooth increase in frequency upon changes in temperature. Future modeling may help demonstrate this principle in more general settings.

I_h dictates the steady state increase in pyloric frequency to temperature through network effects

Given that previous studies showed I_h increases in PD and LP cells at elevated temperatures[30,39], it is unsurprising that blocking I_h with Cs^+ had a larger effect at higher temperatures; the difference in I_h between control and Cs^+ is expected to be larger at elevated temperatures. As I_h is a depolarizing current, its removal would be consistent with the larger difference in frequency between saline and Cs^+ seen at higher temperatures (**Figure 1D**).

Unexpectedly, we found control levels of temperature-induced frequency increase in the presence of Cs^+ when the pacemaker kernel was isolated by PTX (**Figure 4** B,C). This result suggests that when temperature increases, the pacemaker kernel can increase the frequency of the pyloric rhythm without the contribution of depolarizing I_h. However, if the follower neurons can feed back to the pacemaker and I_h is blocked, the feedback limits the increase in frequency with increased temperature.

Potentially the temperature-induced increase in I_h seen in LP[30] is partially responsible for permitting the temperature-induced frequency increase seen in control conditions, and its removal by Cs^+ decreased the temperature-induced frequency increase. We noted that in Cs^+, LP phase advanced with a preserved duty cycle (**Figure 5C**) and advanced further with increased temperature compared to control conditions (**Figure 6A,C**). According to experiments on the effects of differently timed inhibition on pyloric frequency (phase response curve, PRC), phase advance of LP would be expected to either increase frequency or have no effect[60–65], contrary to what we observed. However, temperature-induced I_h increase in the pacemaker kernel[39] is expected to advance the PRC[46], which could permit the increase in frequency with temperature observed in control conditions despite LP inhibition. In Cs^+, this advance of the PRC would not occur[46], potentially causing the observed reduction in temperature-induced frequency increase in Cs^+ (**Figure 1B-E**). In Cs^++PTX, there is no inhibition from LP to potentially slow down frequency, so compensation by increased I_h in PD would be unnecessary. Overall, while I_h is often associated with rhythmogenesis in intrinsic oscillators[4,30,66], our results demonstrate its increasing role in appropriate regulation of persistent oscillatory frequency though network feedback at elevated temperature.

LP phase in Cs^+

Surprisingly, application of Cs^+ phase advanced LP, even more than might be expected when taking into account the advance in PD offset (**Figure 5C,D**). Previous studies reported no significant advance in LP, though these studies were in *Panulirus interruptus*[39,67]. Latency to LP firing increased after Cs^+ application but was insufficient to maintain or delay LP's phase given the increased period at 11°C. Increased period is known to advance LP onset[60,68,69], but our observed LP onset advance was maintained even in period matched samples between Cs^+ and control conditions. While in these matched samples PD bursting was shorter in Cs^+, the time by which PD offset advanced was almost half of what LP advanced and did not significantly correlate with how much LP advanced. The PD to LP synapse is depressing[70–72], such that longer recovery times between PD bursts, such as seen in period-matched Cs^+ samples, would be expected to strengthen the synapse, resulting in longer latencies to LP bursting, not shorter.

Additionally, PY onset did not advance in period-matched samples. While the synapse from the pacemaker cells to PY and LP are not the same, they have been reported to have similar properties[34] and there is no reason *a priori* to believe that Cs^+ differentially affected these synapses.

It was also surprising that when I_h was blocked, LP firing *further* advanced with temperature-mediated frequency increases. Phase maintenance is sometimes explained through a balance of synaptic depression and I [56,71–73]. The models of exploring phase constancy in oscillatory networks reminiscent of the STG [56,71] suggest that given I_h acts in opposition to I_A, normally I_h should impair phase constancy by *shortening* LP latencies as the period increases and advancing LP, and that this effect should be minimized when period is decreasing, such as was seen when temperature increased. By this logic, I_h removal would be expected to improve phase constancy and to have less effect on phase as period decreased, which is the opposite of what we observed. It therefore remains unclear why in Cs^+ LP was advanced and then further advanced as frequency increased with elevated temperatures. Again, more detailed modeling in the future may shed light on the precise property interactions that produce this unexpected further phase advancement.

While Cs^+ has been reported to block channels other than those responsible for I [20,74–78], there is no evidence that these other channels are playing significant roles in the current preparation[45]. Moreover, Cs^+ primarily blocks potassium-passing channels in a voltage dependent manner[79] such that only inward currents are blocked when Cs^+ is extracellular.

Additionally, we did not observe an increase in pyloric frequency or a consistent intracellular depolarization in Cs^+, both of which would be expected if Cs^+ were indirectly changing the extracellular potassium concentration through effects on other potassium channels. Therefore, as a first approximation most of the effects seen here can likely be attributed to Cs^+ block of I_h.

Concluding remarks and general implications

There was substantial animal to animal variability in the effect of Cs^+ block of I_h. Likely this variability stems from the degeneracy in the combinations of currents that can result in a particular motor pattern[23,24,26,80–83]. Some combinations likely rely more on I_h to produce the prototypical response to temperature. Despite this variability, without I_h, the pyloric network was unable to reliably produce either its typical rapid and smooth increase in frequency in response to temperature change, or its typical steady state increase in frequency. As such I_h may act a buffer to transient response hiccups that may be caused by intrinsic properties and as a buffer to the limiting effects of network inhibition.

Methods

Animals and Experimental Preparation

Cancer borealis were purchased from Commercial Lobster (Boston, MA) and maintained in tanks containing artificial seawater (Instant Ocean) at 11°C-13°C on a 12 hr light/dark cycle for at least 1 week before use. Crabs were acquired between November 2022 and December 2023. After placing the animals on ice for 30 minutes, the stomatogastric nervous system was dissected from the crab and pinned in a petri dish coated with Sylgard (Dow Corning) as described previously[84]. All preparations included the STG, the eosophageal ganglia, and two commissural ganglia. The STGs were desheathed to allow a combination of intracellular recordings and direct solution contact with the STG. The nervous system was kept in physiological saline composed of 440 mM NaCl, 11 mM KCl, 13 mM $CaCl_2$, 26 mM $MgCl_2$, 11 mM Trizma base, and 5 mM Maleic acid, pH 7.4–7.5 at 23°C (~7.7-7.8 pH at 11°C). All reagents were purchased from Sigma.

Temperature Protocol

Temperature of superfused saline solution was controlled using a SC-20 Peltier device controlled by model CL-100 temperature controller (Warner Instruments). Temperature was monitored with a Warner Instruments TA-29 thermocouple placed within 1cm of the STG. During ‘Step’ protocols,

temperature was set manually to within 0.2°C of 11°C. After 2 minutes of recording activity, the temperature was manually increased by 2°C and allowed to stabilize over 4 minutes. This process was repeated until the dish reached 21°C, after which the temperature was returned to 11°C (**Figure 1A**). The preparation was kept at 11°C for 10 minutes during which the solution was changed. Experiments started with either standard physiological saline or saline with 5mM CsCl obtained from Sigma to block I_h[39–43]. Experiments to test the importance of follower neurons in the various effects of Cs^+ were performed with first randomized Cs^+ or saline temperature steps protocol, followed by a temperature step protocol in Cs^+ + 10^{-5} M picrotoxin[49] from Sigma as picrotoxin does not easily washout.

Electrophysiology

Extracellular recordings were made by placing wells around the lower lateral ventricular nerve (*lvn*), pyloric dilator nerve (*pdn*) and pyloric constrictor nerve (*pyn*). Wells were made from 90% Vaseline 10% mineral oil solution. Stainless-steel pin electrodes were placed within the wells to monitor spiking activity and amplified using Model 1700 Differential AC Amplifiers (A-M Systems). Intracellular recordings from the soma of the PD, LP, and PY neurons were made using either discontinuous current clamp (one electrode) or two-electrode current and/or voltage clamp with 5-20MΩ sharp glass microelectrodes filled with 0.6 M K_2SO_4 and 20 mM KCl solution. For voltage clamp experiments, 3M KCl solutions were used to facilitate sustained current injections. Intracellular signals were amplified with an Axoclamp 900 A amplifier (Molecular Devices, San Jose). Cells were identified by matching intracellular activity with activity on the *pyn* (PY cells), *pdn* (PD cells), or *lvn* (LP cells). All amplified signals were digitized using a Digidata 1440 digitizer (Molecular Devices, San Jose) and recorded using pClamp data acquisition software (Molecular Devices, San Jose, version 10.5), sampling frequency of 10 kHz.

Analysis

Spikes were detected and assigned to a particular neuron using a custom software implemented in MATLAB ('Crabsort', *https://github.com/sg-s/crabsort*)[85,86] which used a machine-learning algorithm. Results of this automated process were double checked and edited by hand. Bursts were identified as two or more consecutive PD spikes with an inter-burst interval of at least 200 msec. Phase was defined as the difference between the first spike of a PD burst and the first (onset) or last (offset) spike of a neuron (LP, PY, or PD), normalized by the period (time between first PD spikes of two consecutive bursts). Data were tested for normality using the Shapiro-Wilks Test and appropriate parametric or non-parametric tests were used.

To quantify the non-monotonic responses to temperature change, we first smoothed frequency and temperature using a moving average over 30 and 10 pyloric cycles respectively to get a less noisy trajectory for each measure. We then divided pyloric cycles based on whether the smoothed temperature increased more than 0.01°C. For cycles that increased more than this criterion, we quantified the percent in which the smoothed frequency decreased more than 0.002Hz per cycle. For cycles where temperature did not increase above the criterion, we quantified the percent in which the smoothed frequency increased more than 0.002Hz per cycle.

To examine phase constancy, we found the best fit line to describe the relationship between mean phase and temperature for each experiment. We then used either Wilcoxon Signed Rank Test (non-normal data) or one-sample t-test (normal data) to determine whether the slope across experiments was significantly different from zero. Due to the 10 different tests (5 phases x 2 conditions), we Bonferroni-adjusted the p-values to reduce the false-positive rate.

Where not specifically noted otherwise, all summary statistic reports are (mean±SD). All analysis was performed using MATLAB.

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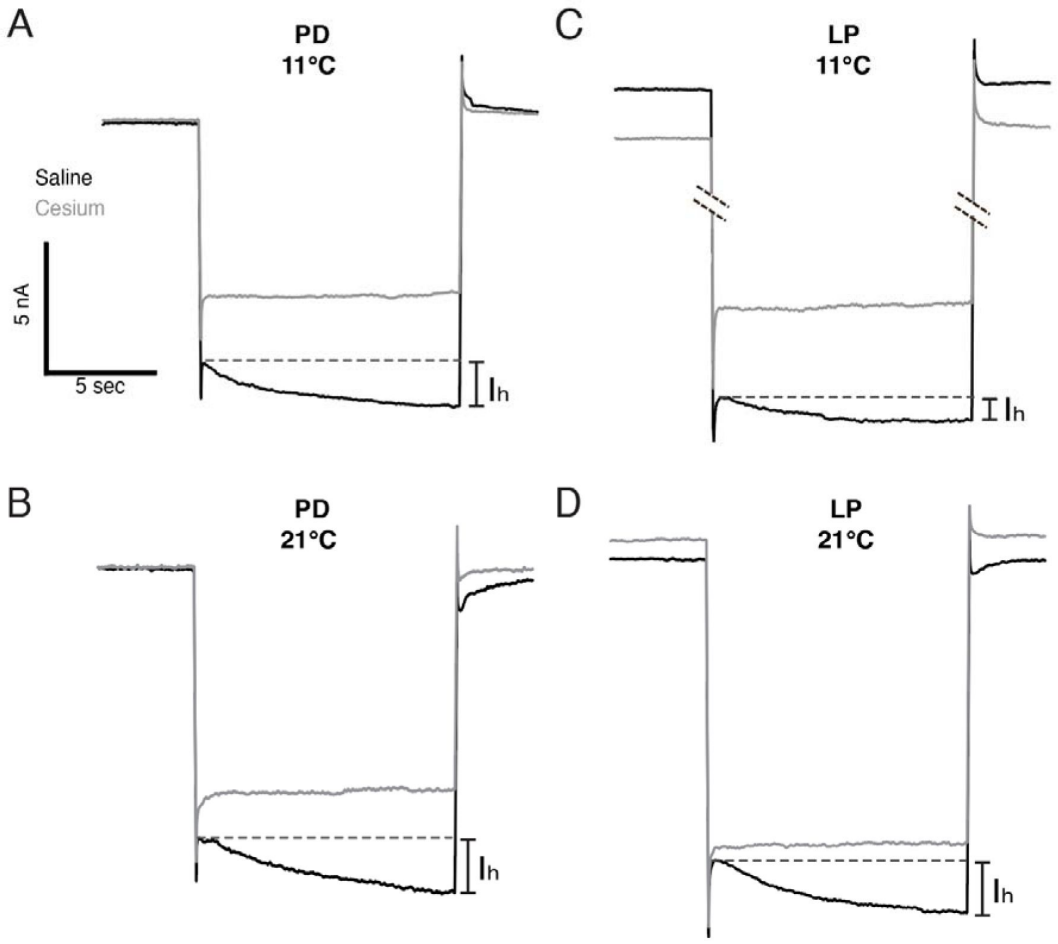

Figure 1—figure supplement 1

Voltage Clamp traces of PD and LP cells at the temperature extrema of these experiments in both saline and Cs^+. Cells were held at -50mV for 9 seconds, then -110mV for 12 seconds before being returned to -50mV for 4 seconds. In saline, there is a notable sag current (I_h) when the cell is held at -110mV (black traces, difference in initial and steady state current highlighted by dashed line). This sag current is absent when the cell is in Cs^+ (grey traces). **(A)** PD cell at 11°C. **(B)** PD cell at 21°C. **(C)** LP cell at 11°C. **(D)** LP cell at 21°C. Each cell is from a separate preparation.

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Editors

Reviewing Editor
Jeffrey Smith
National Institute of Neurological Disorders and Stroke, Bethesda, United States of America

Senior Editor
John Huguenard
Stanford University School of Medicine, Stanford, United States of America

Reviewer #1 (Public review):

Summary:

This important study investigates the neurobiological mechanisms underlying the stable operation and maintenance of functionally appropriate rhythmic motor patterns during changing environmental conditions - temperature in this study in the crab Cancer borealis stomatogastric neural pattern generating network producing the pyloric motor rhythm, which is naturally subjected to temperature perturbations over a substantial range. This study is relevant to the general problem that some rhythmic motor systems adjust to changing environmental conditions and state changes by increasing the cycle frequency in a smooth monotonic fashion while maintaining the relative timing of different network activity pattern phases that determine proper motor coordination. How this is achieved mechanistically in complex dynamic motor networks is not understood, particularly how the frequency and phase adjustments are achieved as conditions change while avoiding operational instabilities on different time scales. The authors specifically studied the contributions of the hyperpolarization-activated inward current (Ih), which is involved in rhythm control, to the adjustments of frequency and phases in the pyloric rhythmic pattern as the temperature was altered from 11 degrees C to 21 degrees C. They present compelling evidence that this current is a critical biophysical feature in the ability of this system to adjust transiently and persistently to temperature perturbations appropriately. After blocking Ih in the pyloric network with cesium, the network was unable to reliably produce its characteristic rapid and smooth increase in the frequency of the triphasic rhythmic motor pattern in response to increasing temperature or its typical steady-state increase in frequency over this Q10 temperature range.

Strengths:

(1) The authors addressed this problem by technically rigorous experiments in the crab Cancer borealis stomatogastric ganglion (STG) in vitro, which readily allows for neuronal activity recording in a behaviorally and architecturally defined rhythmic neural circuit in conjunction with the application of blockers of Ih and synaptic receptors to disrupt circuit interactions. This approach is an effective way to experimentally investigate how complex rhythmic networks, at least in poikilotherms, mechanistically adjust to environmental perturbations such as temperature.

(2) While previous work demonstrated that Ih increases in pyloric neurons as temperature increases, the authors here establish that this increase is necessary for normal responses of STG neural activity to temperature, which consist of a smooth monotonic increase in the frequency of rhythmic activity with increasing temperature.

(3) The data shows that blocking Ih with cesium causes the frequency to transiently decrease ("jags") when the temperature increases and then increases after the temperature stabilizes at a steady state, revealing a non-monotonic frequency response to temperature perturbations.

(4) The authors dissect some of the underlying neuronal and circuit dynamics, presenting evidence that after blocking Ih, the non-monotonic jags in the frequency response are mediated by intrinsic properties of pacemaker neurons, while in the steady state, Ih determined the overall frequency change (i.e., temperature sensitivity) through network interactions.

(5) The authors' results highlight more complex dynamic responses to increasing temperature for the first time, suggesting a longer timescale process than previously recognized that may result from interactions between multiple channels and/or ion channel kinetics.

Weaknesses:

(1) The involvement of Ih in achieving the frequency and phase adjustments as conditions change and allowing smooth transitions to avoid operational instabilities in other complex rhythmic motor networks, for example, in homeotherms, is not established, so the present results may have limited general extrapolations.

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Reviewer #2 (Public review):

Summary:

Using the crustacean stomatogastric nervous system (STNS), the authors present an interesting study wherein the contribution of the Ih current to temperature-induced changes in the frequency of a rhythmically active neural circuit is evaluated. Ih is a hyperpolarization-activated cation current that depolarizes neurons. Under normal conditions, increasing the temperature of the STNS increases the frequency of the spontaneously active pyloric rhythm. Notably, under normal conditions, as temperature systematically increases, the concomitant increase in pyloric frequency is smooth (i.e., monotonic). By contrast, blocking Ih with extracellular cesium produces temperature-induced pyloric frequency changes that follow a characteristic sawtooth response (i.e., non-monotonic). That is, in cesium, increasing temperature initially results in a transient drop in pyloric frequency that then stabilizes at a higher frequency. Thus, the authors conclude that Ih establishes a mechanism that ensures smooth changes in neural network frequency during environmental disturbances, a feature that likely bestows advantages to the animal's function.

The study describes several surprising and interesting findings. In general, the study's primary observation of the cesium-induced sawtooth response is remarkable. To my knowledge, this type of response has not yet been described in neurobiological systems, and I suspect that the unexpected response will be of interest to many readers.

At first glance, I had some concerns regarding the use of extracellular cesium to understand network phenomena. Yes, extracellular cesium blocks Ih. But extracellular cesium has also been shown to block astrocytic potassium channels, at least in mammalian systems (i.e., K-IR, PMID: 10601465), and such a blockade can elevate extracellular potassium. I was heartened to see that the authors acknowledge the non-specificity of cesium (lines 320-325) and I agree with the authors' contention that "a first approximation most of the effects seen here can likely be attributed to Cs+ block of Ih". Upon reflecting on the potential confound, I was also reassured to see that extracellular cesium alone does not in fact increase pyloric frequency, an effect that might be expected if cesium indirectly raises [K+]outside. If the authors agree, then I suggest including that point in their discussion.

In summary, the authors present a solid investigation of a surprising biological phenomenon. In general, my comments are fairly minor. Thanks for contributing an interesting study.

Strengths:

A major strength of the study is the identification of an ionic conductance that mediates stable, monotonic changes in oscillatory frequency that accompany changes in the environment (i.e., temperature).

Weaknesses:

A potential experimental concern stems from the use of extracellular cesium to attribute network effects specifically to Ih. Previous work has shown that extracellular cesium also blocks inward-rectifier potassium channels expressed by astrocytes, and that such blockade may also elevate extracellular potassium, an action that generally depolarizes neurons. Notably, the authors address this potential concern in the discussion.

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Reviewer #3 (Public review):

Summary:

This paper presents a systematic analylsis of the role of the hyperpolarization-activated inward current (the h current) in the response of the pyloric rhythm of the stomatogastric ganglion (STG) of the crab. In a detailed set of experiments, they analyze the effect of blocking h current with bath infusion of the h current blocker cesium (perfused as CsCl). They show interesting and reproducible effects that blockade of h current results in a period of frequency decrease after an upward step in temperature, followed by a slow increase in frequency. This contrasts with the normal temperature response that shows an increase in frequency with an increase in temperature without a downward "jag" in the frequency response. This is an important paper for showing the role of h current in stabilizing network dynamics in response to perturbations such as a temperature change.

Strengths of the paper:

The major effects are shown very clearly and convincingly in a range of experiments with combined intracellular recording from neurons during changes in temperature.

Weaknesses

The Marder lab has detailed models of the pyloric rhythm. These temperature effects have not yet been modeled and could be the focus of future modeling studies.

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Author response:

The following is the authors' response to the original reviews.

Response to Public Reviews:

We thank the reviewers for their kind comments have implemented many of the suggestion their suggestions. Our paper has greatly benefited from their advice. Like Reviewer 1, we acknowledge that while the exact involvement of Ih in allowing smooth transitions is likely not universal across all systems, our demonstration of the ways in which such currents can affect the dynamics of the response of complex rhythmic motor networks provides valuable insight. To address the concerns of Reviewer 2, we included a sentence in the discussion to highlight the fact that cesium neither increased the pyloric frequency nor caused consistent depolarization in intracellular recordings. We also highlighted that these observations suggest both that cesium is not indirectly raising [K+]outside and support the conclusion that the effects of cesium are primarily through blockade of Ih rather than other potassium channels.

Reviewer 3 raised some important points about modeling. While the lab has models that explore the effects of temperature on artificial triphasic rhythms, these models do not account for all the biophysical nuances of the full biological system. We have limited data about the exact nature of temperature-induced parameter changes and the extent to which these changes are mediated by intrinsic effects of temperature on protein structure versus protein interactions/modification by processes such as phosphorylation. With respects to the A current, Tang et al., 2010 reported that the activation and inactivation rates are differentially temperature sensitive but we do not have the data to suggest whether or not the time courses of such sensitivities are different. As such, we focus our discussion on the properties we know are modulated by temperature, i.e. activation rates. Within the discussion we now include the suggestion that future, more comprehensive modeling may be appropriate to further elucidate the ways in which reducing Ih may produce the here reported experimentally observed effects.

> ***Reviewer #1 (Recommendations For The Authors):***
>
> *Suggested revisions:*
>
> *A figure showing examples of the voltage-clamp traces for the critical measurements of the extent of Ih block by 5 mM CsCl in PD and LP neurons at the temperature extremes in these preparations is not shown, and the authors should consider including such a figure, perhaps as a supplemental figure.*

We have added Supplemental Figure 1 containing voltage-clamp traces demonstrating the extent of Ih block by 5mM CsCl in PD and LP neurons at 11 and 21°C. Due to technical concerns, different preparations were used in the measurements at 11°C and 21°C, but the point that the H-current is reduced is demonstrated in all cases.

> ***Reviewer #2 (Recommendations for The Authors):***
>
> *Specific (Minor) Comments:*

> *(1) Line 83: In Cs+ "at 11°C, the pyloric frequency was significantly decreased compared to control conditions (Saline: 1.2± 0.2 Hz; Cs+ 0.9± 0.2 Hz)".*
>
> *As above, the authors often report that cesium generally reduces pyloric frequency. Figure 5A demonstrates this action quite nicely. However, cesium's effect on pyloric frequency at 11°C seems less robust in Figure 1C. Why the discrepancy?*

There is variability in the effects of Cs+ on the pyloric frequency. As noted, the standard deviation in frequency in both conditions is 0.2Hz. As such, there are some cases in which the initial frequency drop in Cs+ compared to control was relatively small. 1C is one such case, but was selected as an example because of its clear reduction in temperature sensitivity.

> *(2) I don't understand what the arrows/dashed lines are trying to convey in Figure 3C.*

The arrows/dashed lines represent the criteria used to define a cycle as “decreasing in frequency” (Temperature Increasing) or “increasing in frequency” (Temperature Stable). We have amended lines 130 and 137 in the text to hopefully clarify this point, as well as the figure legend.

> *(3) Lines 118/168. The description of cesium's specific action on the depolarizing portion of PD activity is a bit confusing. In my mind, "depolarization phase" refers to the point at which PD is most depolarized. Perhaps restating the phrase to "elongation of the depolarizing trajectory" is less confusing. The authors may also want to consider labeling this trajectory in Figure 2C.*

We have changed “depolarization phase” to “depolarizing phase” to highlight that this is the period during which the cell is depolarizing, rather than at its most depolarized. We consider the plateau of the slow wave and spiking (the point at which PD is most depolarized) to be the “bursting phase”. We have labeled these phases in Figure 2C as suggested.

> *(4) Figure 3C legend: a few words seem to be missing. I suggest "the change in mean frequency was more likely TO decrease IN Cs+ than in saline".*

Thank you for catching this typo, it has been corrected.

> *(5) Line 165: Awkward phrasing. “In one experiment, the decrease in frequency while temperature increased and subsequent increase in frequency after temperature stabilized was particularly apparent in Cs+ PTX”.*
>
> *How about: “One Cs+ PTX experiment wherein elevating the temperature transiently decreased pyloric frequency is shown in Figure 4F.”*

We have amended this sentence to read, “One Cs++PTX experiment in which elevating the temperature produced a particularly pronounced transient decrease in frequency is shown in Figure 4F.”

> *(6) Line 186: Awkward phrasing. "LP OFF was also significantly advanced in Cs+, although duty cycle (percent of the period a neuron is firing) was preserved".*
>
> *The use of the word "although" seems a bit strange. If both LP onset and LP offset phase advance by the same amount, then isn't an unchanged duty cycle expected?*

“Although” has been changed to “and subsequently”.

> ***Reviewer #3 (Recommendations For The Authors):***
>
> *Major comments:*
>
> *(1) I know the Marder lab has detailed models of the pyloric rhythm. I am not saying they have to add modeling to this already extensive and detailed paper, but it would be useful to know how much of these temperature effects have been modeled, for example in the following locations.*
>
> *(2) Line 259 - "Mathematically..." - Is there a computational model of H current that has shown this decrease in frequency in pyloric neurons? If you are working on one for the future, you could mention this.*

There is not currently a model in which the reduction of the H-current results in the non-minimum phase dynamics in the frequency response to temperature seen experimentally. It should be noted that our existing models of pyloric activity responses to temperature are not well suited to investigate such dynamics in their current iterations. Further work is necessary to demonstrate the principles observed experimentally in computational modeling, and we have added a sentence to the paper to reflect this point (Line 268).

> *(3) Line 318 - "therefore it remains unclear" - I thought they had models of the circuit rhythmicity. Do these models include temperature effects? Can they comment on whether their models of the circuit show an opposite effect to what they see in the experiment? I'm not saying they have to model these new effects as that is probably an entirely different paper, but it would be interesting to know whether current models show a different effect.*

We have some models of the pyloric response to temperature, but these models were specifically selected to maintain phase across the range of temperature. When Ih was reduced in these models, a variety of effects on phase and duty cycle were seen. These models were selected to have the same key features of behavior as the pyloric rhythm, but do not capture all the biophysical nuances of the complete system, and therefore should not necessarily be expected to reflect the experimental findings in their current iterations. Furthermore, these models are meant to have temperature as a static, rather than dynamic input, and thus are ill-suited to examine the conditions of our experiments. The models in their current state are not sufficiently relevant to these experimental findings that we they can illuminate the present paper 2.

> *(4) "If deinactivation is more accelerated or altered by temperature than inactivation...While temperature continued to change, the difference in parameters would continue to grow" - This is described as a difference in temperature sensitivity, but it seems like it is also a function of the time course of the response to change in temperature (i.e. the different components could have the same final effect of temperature but show a different time course of the change).*

We know from Tang et al, 2010, that activation and inactivation rates of the A current are differentially temperature sensitive. We have no evidence to suggest that the time course of the response to temperature of various parameters differ. The physical actions of temperature on proteins are likely to be extremely rapid, making a time course difference on the order of tens of seconds less unlikely, though not impossible. Modeling of the biophysics might illuminate the relative plausibility of these different mechanisms of action, but we feel that our current suggested explanation is reasonable based on existing information.

> *(5) Is it known how temperature is altering these channel kinetics? Is it via an intrinsic rearrangement of the protein structure, or is it a process that involves phosphorylation (that could explain differences in time course?). Some mention of the mechanism of temperature changes would be useful to readers outside this field.*

It is not known exactly how temperature alters channel parameters. Invariably some, if not all, of it is due to an intrinsic rearrangement of protein structure, and our current models treat all parameter changes as an instantaneous consequence. However, it is possible that some effects of temperature are due to longer timescale processes such as phosphorylation or cAMP interactions. Current work in the lab is actively exploring these questions, but there is no definitive answer. Given that this paper focuses on the phenomenon and plausible biomolecular explanations based on existing data, we have not altered the paper to include more exhaustive coverage of all the possible avenues by which temperature may alter channel properties.

> *Specific comments:*
>
> *Title: misspelling of "Cancer" ?*

We are unsure how that extra “w” got into the earliest version of the manuscript and have removed it.

> *Line 66 "We used 5mM CsCl" - might mention right up front that this was a bath application of the substance.*

We have altered this line to read “used bath application of 5mM CsCl”.

> *Figure 4 - "The only feedback synapse to the pacemaker kernel neurons, LP to PD, and is blocked by picrotoxin" - I think the word "and" should be removed from this phrase in the figure legend.*

Fixed

> *Figure 4 legend - "Reds denote temperature...yellows denote..." - I think it should be "Red dots denote temperature...yellow dots denote...".*

Done

> *Figure 4B - Why does the change in frequency in cesium look so different in Figure 4B compared to Figure 1C or Figure 3B? In the earlier figures, the increase of frequency is smaller but still present in cesium, whereas, in Figure 4B, cesium seems to completely block the increase in frequency. I'm not sure why this is different, but I guess it's because 3B and 4B are just mean traces from single experiments. Presumably, 4B is showing an experiment in which the cesium was subsequently combined with picrotoxin?*

Figures 1C, 3B, and 4B are indeed all from different single experiments. As acknowledged in our concluding paragraph, there was substantial variability in the exact response of the pyloric rhythm to temperature while in cesium. The most consistent effect was that the difference in frequency between cesium and saline at a particular temperature increased, as demonstrated across 21 preparations in Figure 1D. It may be noted in Figure 1E that the Q10 was not infrequently <1, meaning that there was a net decrease in frequency as temperature increased in some experiments such as seen in the example of Figure 4B. The “fold over” (initial increase in steady-state frequency with temperature, then decrease at higher

temperatures) has been observed at higher temperatures (typically around 23-30 degrees C) even under control conditions but has not been highlighted in previous publications. The example in 4B was chosen because it demonstrated both the similarity in jags between Cs+ and Cs++PTX and an overall decrease in temperature sensitivity, even though in this instance the steady-state change in frequency with temperature was not monotonic.

> *Figure 6A - "Phase 0 to 1.0" - The y-axis should provide units of phase. Presumably, these are units of radians so 1.0=2*pi radians (or 360 degrees, but probably best to avoid using degrees of phase due to confusion with degrees of temperature).*

Phase, with respect to pyloric rhythm cycles, does not traditionally have units as it is a proportion rather than an angle. As such, we have not changed the figure.

> *Line 275 - "the pacemaker neuron can increase" - Does this indicate that the main effects of H current are in the follower neurons (i.e. LP and PY versus the driver neuron PD)?*

Not necessarily. We posit in the next paragraph that the effect of the H current on the temperature sensitivity could be due to its phase advance of LP, but that phase advance of LP is not particularly expected to increase frequency. We favor the possibility that temperature increases Ih in the pacemaker, which in turn advances the PRC of the rhythm, allowing the frequency increase seen under normal conditions. In Cs+, this advance does not occur, resulting in the lower temperature sensitivity. In Cs++PTX, the lack of inhibition from LP means compensatory advance of the pacemaker PRC by Ih is unnecessary to allow increased frequency.

> *Line 285 - "either increase frequency have no effect" - Is there a missing "or" in this phrase?*

Thank you, we have added the “or”.

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