Neuromodulator-induced temperature robustness in a motor pattern: a comparative study between three decapod crustaceans

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Abstract.

Temperature fluctuations pose significant challenges to the nervous system, in particular in poikilothermic animals that lack active temperature regulation. Nevertheless, many vital neuronal systems in these animals function over a broad temperature range, making them ideal to study the mechanisms of temperature robustness. Using the gastric mill pattern generator in the Jonah crab, *Cancer borealis*, we previously demonstrated that temperature-induced increases in membrane leak conductance disrupt neuronal function, but also that neuropeptide modulation provided thermal protection by countering the temperature-induced leak currents. In this study, we test the hypothesis that temperature compensation via neuropeptide modulation is evolutionarily conserved, and not idiosyncratic to Jonah crabs.

Our results show that increasing temperature halted rhythmic gastric mill activity in three different crab species (*Cancer borealis*, *Cancer magister*, *Carcinus maenas*). Intracellular recordings revealed temperature-dependent changes in resting membrane potential, action potential amplitude, and postsynaptic potential amplitude. As predicted, higher temperatures diminished LG input resistance, and increasing descending projection neuron activity or applying neuropeptide modulators re-established and rescued rhythms at elevated temperatures.

In *C. borealis* and *C. magister*, we tested whether the observed changes in input resistance were sufficient to explain the termination of the rhythm at elevated temperature by altering input resistance with dynamic clamp. In *C. borealis*, decreasing input resistance at cold temperatures terminated rhythms, while increasing it at higher temperatures restored them, demonstrating the critical role of input resistance in temperature-induced rhythm crashes. In *C. magister*, this approach was successful in about half of the animals, suggesting that additional temperature-induced properties contributed to the termination of the rhythm at elevated temperature.

In summary, neuropeptide modulation rescued the gastric mill rhythm in all species, although the underlying cellular properties that caused the temperature crash may be distinct. Our study highlights the potential universality of neuropeptide-mediated temperature compensation.

Introduction.

Temperature changes are a particular challenge for the nervous system, as they can disrupt the well-balanced weighting of ionic intrinsic and synaptic conductances that is required to maintain neuronal function and behavioral performance[1-4]. Poikilothermic aquatic animals are especially prone to temperature changes, because they do not actively maintain their body temperature, live in an environment with high heat transfer, and are especially susceptible to climate change-induced global warming and associated heatwaves[5-9]. Yet, many vital behaviors in these animals function across a wide temperature range, making them ideal to study the mechanisms of temperature resilience. Recent studies have revealed that some neural circuits display automatic robustness to acute temperature perturbations that is either intrinsic to the circuit and arises from the emergent properties of cellular and synaptic ionic conductances[10]. Other neuronal circuits appear to rely less on intrinsic properties but gain robustness through external neuromodulatory influences. For example, the gastric mill central pattern generator in the stomatogastric nervous system of the Jonah crab *Cancer borealis*[11], shows very little intrinsic robustness against heating. A change of only a few degrees Celsius terminates the gastric mill motor pattern. We have previously shown[12, 13] that this response is due to an increase of membrane leak conductance with temperature in the lateral gastric (LG) neuron, which eliminates spike activity and shunts LG bursting. Since LG is a member of the gastric mill half-center circuit that drives the gastric mill rhythm, the rhythm terminates. However, we have also shown that neuropeptide modulation enables thermal robustness and allows normal neuronal functioning at much more elevated temperatures. Specifically, the substance P-related peptide CabTRP Ia (*Cancer borealis* tachykinin-related peptide Ia) enables neurons in the gastric mill central pattern generator to continue to produce bursts of action potentials at elevated temperature. CabTRP Ia is released from a pair of descending projection neurons (MCN1), and there are indications that MCN1 activity increases with temperature[12, 14]. CabTRP Ia rescues neuronal activity by activating a voltage-gated inward current (I_{MI}) that counterbalances temperature-induced increases in leak currents. Since neuromodulator release from descending projection neurons is a universal mechanism to adjust neuronal activity in many taxa, we hypothesized increasing temperature robustness via neuropeptide modulation is evolutionarily conserved. This study tests this hypothesis by comparing the effects of temperature and peptide neuromodulation on the LG neurons of several crab species that live in different habitats and show various temperature tolerances.

Materials and Methods.

Animals. Adult *Cancer borealis* were purchased from Ocean Resources Inc. (Sedgwick, ME) or Fresh Lobster Company (Boston, MA). Adult Cancer magister were purchased at a local store. Animals of both species were kept in filtered, aerated artificial seawater at 11°C until use. Adult *Carcinus maenas* crabs were provided by Dr. Markus Frederich (University of New England, Maine, USA) and Adam Smith (Perfect Limit Green Crabs, Massachusetts, USA). *C. maenas* crabs were kept in filtered, aerated artificial seawater at 20°C until use. Figure 1 gives am overview of which species were used, were they originated from, and what temperatures they encounter in their habitat[15, 16].

Cancer borealis - Jonah crab

Distribution: Atlantic ocean, U.S. East Coast

Trapped: Massachusetts, USA

Habitat: 3 - 18°C, 5 - 100 m water depth

Cancer magister - Dungeness crab

Distribution: Pacific ocean, U.S. West Coast

Trapped: Washington, USA

Habitat: 3 - 18°C, 5 - 100 m water depth

Carcinus maenas - Green crab

Distribution: Atlantic ocean, U.S. East Coast

Trapped: Massachusetts & Maine

Habitat: 0 - 34°C, intertidal, 0.5-15m water depth

Figure 1. The three crab species used in this study, their habitat, and their distribution.

Dissection: Established protocols were used. After ice anesthesia for 20 - 40 minutes, the stomatogastric nervous system was isolated according to[17], pinned out in a silicone lined (Wacker) petri dish and continuously superfused with physiological saline (10°C). We worked with fully intact and decentralized STNS preparations. In the latter, the STG was separated from the commissural ganglia (CoGs) by transecting the paired *ion*s and *son*s (Fig. 2A).

The animal species used in this study are not subject to ethics approval at Illinois State University. We still adhered to general animal welfare considerations regarding humane care and use of animals. Water quality, salinity and temperature were monitored daily. Crabs were sacrificed using ice which is a method recognized as acceptable under the AVMA guidelines for euthanasia of aquatic invertebrates. All animals were confirmed dead before use.

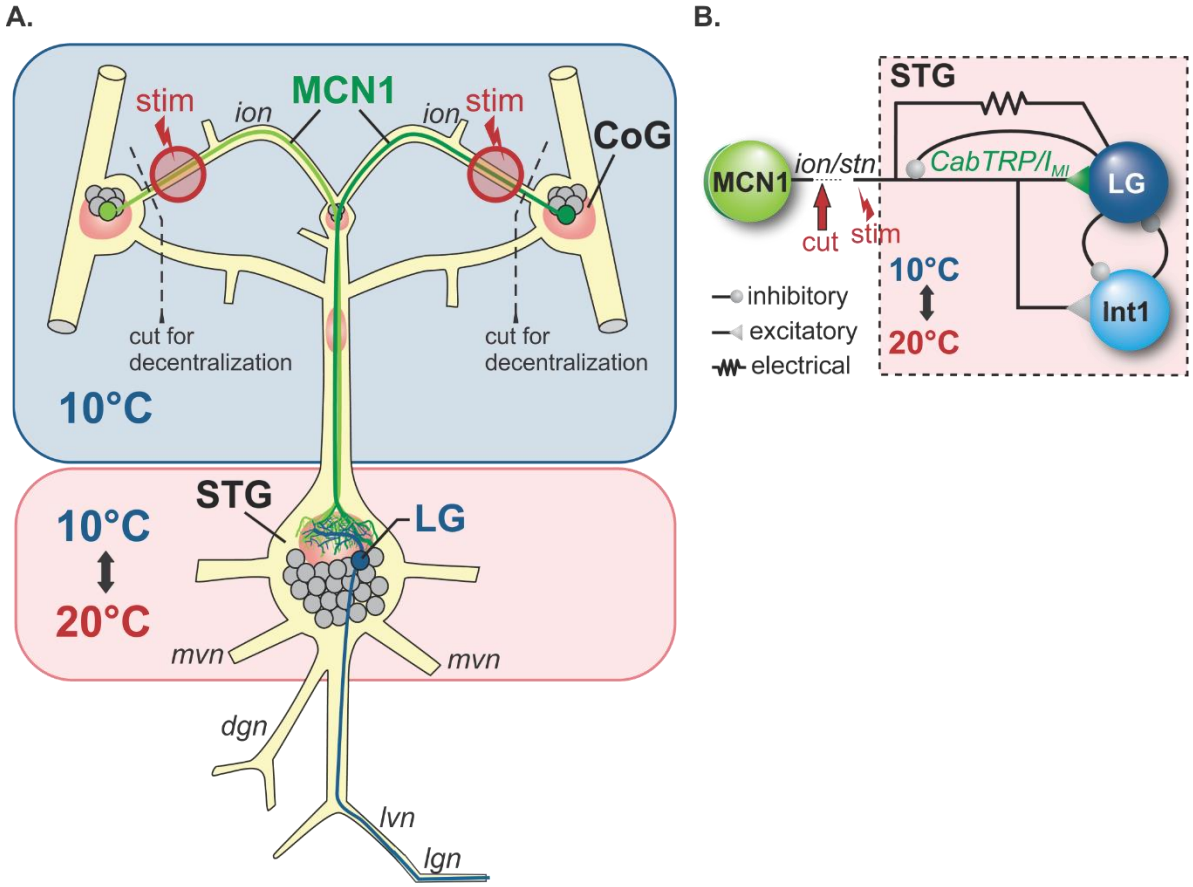

Figure 2. A. To study the effects of temperature on LG, we changed the temperature of the central pattern generating circuits in the STG (10 to 20°C), which contains the core gastric mill pattern generator. CabTRP Ia (0.1 -1 µM) was superfused exclusively to the STG. Upstream areas, including MCN1 in the paired commissural ganglia were kept at constant temperature (~10°C) in regular saline. Dashed lines indicate nerves cut for decentralization. B. We recorded intracellularly from LG, which builds the gastric mill core central pattern generator with Interneuron 1. LG was activated by selective stimulation of the descending modulatory projection neuron MCN1, which excites and modulates LG activity via an electrical synapse and chemically via paracrine release of the peptide CabTRP Ia. STG, stomatogastric ganglion; CoG, commissural ganglion; LG, lateral gastric neuron; Int1, Interneuron 1; ion, inferior oesophageal nerve; son, superior oesophageal nerve; mvn, medial ventricular nerve; dgn, dorsal gastric nerve; lvn, lateral ventricular nerve; lgn, lateral gastric nerve; stn. stomatogastric nerve.

Solutions. C. borealis and *C. magister* saline was composed of (in mM) 440 NaCl, 26 $MgCl_2$, 13 $CaCl_2$ x $2H_2O$, 11 KCl, 10 Trisma base, 5 maleic acid, pH 7.4-7.6 (Sigma Aldrich). *C. maenas* saline was composed of 410 NaCl, 11 KCl, 11 $CaCl_2$ x $2H_2O$, 21 $MgCl_2$ x $6H_2O$, 30 $NaHCO_3$, 11 Trizma base, 5.1 Maleic acid, pH 7.4-7.6 (Sigma Aldrich). In some experiments 100 nM CabTRP Ia (GenScript) was added to the saline. Solutions were prepared from concentrated stock solutions immediately before the experiment. Stock solutions were stored at -20°C in small quantities. Measurements were taken after 15 minutes wash in.

Electrophysiology. Extracellular action potentials were recorded, filtered and amplified with an AM Systems amplifier (Model 1700). Intracellular recordings were obtained from STG cell bodies using 20-30 MΩ glass microelectrodes (Sutter 1000 puller, 0.6 M K_2SO_4 + 20 mM KCl solution, or cytoplasm matched solution: 20 mM NaCl, 15 mM Na_2SO_4, 10 mM Hepes, 400 mM potassium gluconate, 10 mM $MgCl_2$[18]). Signals were filtered and amplified through an Axoclamp 900A amplifier (Molecular Devices) in bridge or two electrode current clamp mode. Files were recorded, saved and analyzed using Spike2 Software at 10 kHz (version 7.18; CED). Input resistance was measured using hyperpolarizing current pulses (-2nA, 2s duration). Membrane potential voltage deflections were measured in steady state (after 2s).

To elicit gastric mill rhythms in decentralized nervous system preparations, we extracellularly stimulated the axon of MCN1 in the part of the transected *ion* that remained connected to the STG (Fig. 2A). This elicits a specific version of the gastric mill rhythm at 10°C that has been characterized in detail before[19-21]. Both *ions* were tonically stimulated for 200s with the same frequency (Master-8 stimulator (AMPI), 1 ms pulse duration). The *ion* contains the axons of the MCN1 projection neurons, and MCN1 possesses the lowest activation threshold[19, 22]. The presence of LP PSPs during *ion* stimulation was used to confirm that MCN1 activation threshold was reached and maintained at all temperatures. Stimulation frequency was increased in 1 Hz steps until a gastric mill rhythm could be observed at 10°C (=*threshold frequency*).

Preparations were continuously superfused with physiological saline. Temperature was manipulated inside of a petroleum jelly well around the STG. The well thermally isolated the STG from the rest of the nervous system. Temperature inside and outside of the well was controlled independently with two saline superfusion lines, cooled by separate Peltier devices. Temperature was continuously measured within a few millimeters of the STG and the CoGs with separate temperature probes (Voltcraft 300K). We selectively altered STG temperature while the surrounding nervous system was kept constantly at 10°C. Temperature was changed by ~1°C/min unless otherwise mentioned. With intracellular recordings temperature was changed by ~1°C/6-7 min to prevent swelling of the neurons. Measurements were taken after 2 minutes at the target temperature.

Dynamic clamp[23] was used to reduce or increase LG input resistance. This was achieved using Spike2 software and two electrode current clamp mode. In each preparation input resistance and resting potential were measured at cold temperature and at the elevated temperature at which the rhythm had crashed to determine temperature-induced changes. Changes in input resistance were implemented through an additional leak conductance computed in dynamic clamp:

$$I_{dyn} = \Delta leak * (V - E)$$

where I_{dyn} is the injected current and $\Delta leak$ represents the difference in leak conductance between cold and elevated temperature. E was taken as the resting potential at elevated temperature. E and $\Delta leak$ were calculated separately for each preparation.

Data analysis. Figures were prepared with CorelDraw X7 (Corel Cooperation), Excel 365 (Microsoft), SigmaPlot (version 15, Jandel Scientific). Analyses were carried out using Spike2 (CED, UK). For some of the shown data (*C. borealis*), data and recordings files from publicly available repositories[12] were re-analyzed and pooled with newly collected data. Unless stated otherwise, data are presented as mean±SD. Alternatively, individual data points for each animal are given. Significant differences are stated as *p<0.05, **p<0.01, ***p<0.001.

Results.

We tested the influence of temperature on the gastric mill rhythm by temperature-controlling the saline superfusion to the STG. Discrete temperature steps of 1°C were used to assess the various properties of the gastric mill rhythm and its main pattern generating neuron, LG. Measurements were taken in steady-state, i.e., after keeping the temperature constant for at least 2 minutes. Temperature ramps of 1°C/min were used to change the temperature to new steady states. The gastric mill rhythm is two-phasic, episodic, and driven by half-center oscillations of Interneuron 1 (Int1) and the LG neuron (Fig. 2B). Rhythmic gastric mill activity requires modulatory input from descending projection neurons in the commissural ganglia (CoGs, Fig. 2A) that are activated by a diverse set of sensory pathways[11, 24-26].

Influence of temperature on the gastric mill rhythm.

Our previous data from *C. borealis* demonstrated that LG, one of the two half-center neurons without which the gastric mill rhythm cannot function, stops producing rhythmic bursts when the STG temperature is raised by only a few degrees Celsius[12-14]. We reaffirmed these findings by extracellularly recording the gastric mill motor nerves (Fig. 2A), including the *lgn* (lateral gastric nerve), which contains the LG axon, while we exposed the STG to increasing temperatures. The temperature of all non-STG nervous tissues, including the commissural ganglia that house the descending projection neurons that provide the excitation to start gastric mill rhythms[27, 28], remained unaltered (10°C, Fig. 2A). In a first set of experiments, we observed spontaneously occurring gastric mill rhythms. Selectively increasing the STG temperature from 10°C to 13°C stopped the spontaneous gastric mill rhythm, as previously reported (Fig. 3A).

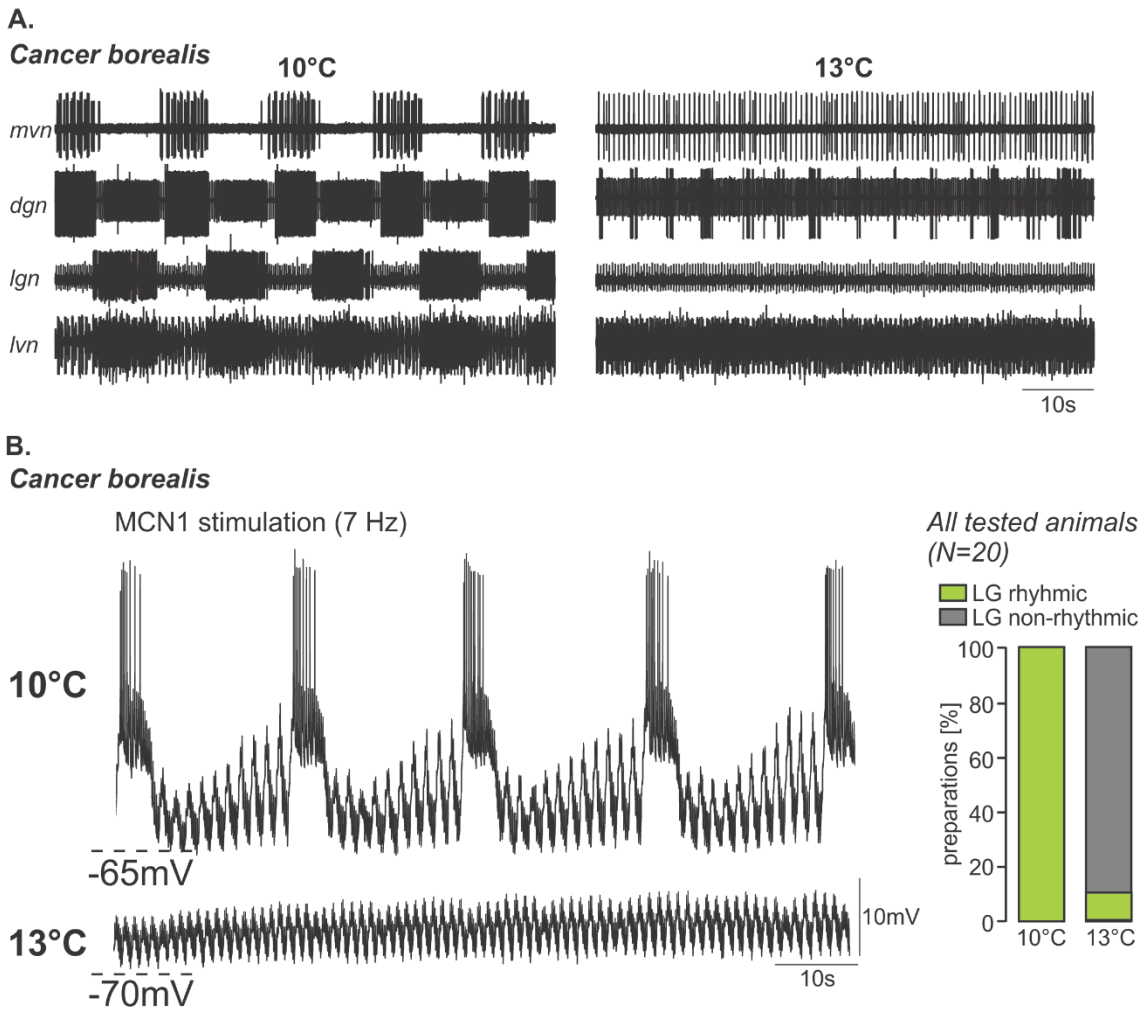

Figure 3. C. borealis gastric mill rhythms terminate at elevated temperature. A. Extracellular nerve recordings showing the spontaneous gastric mill rhythms at low (10°C) and elevated temperature (13°C). Four extracellular recordings are shown. Top: medial ventricular nerve showing the activities of the inferior cardiac and ventricular dilator neurons. Second from top: dorsal gastric nerve dgn showing the activity of the dorsal gastric (DG; large unit) and gastric mill (GM, small units) neurons. DG is a functional antagonist of LG and the GM neurons. Third from top: lateral gastric nerve lgn, showing the activity of LG. Bottom: lateral ventricular nerve lvn, showing the pyloric rhythm. The pyloric rhythm is a fast triphasic rhythm that is continuously active and shows a much larger intrinsic temperature robustness than the gastric mill rhythm. At 13°C, LG activity ceases, and the gastric mill rhythm terminates. Recordings are from the same preparation. B. Intracellular recording of LG during continuous extracellular MCN1 stimulation with 7 Hz at 10°C (top) and 13°C (bottom). LG was rhythmically active at 10°C, but activity ceased at 13°C. Right: percentage or preparations with rhythmically active LG during MCN1 stimulation.

Spontaneous gastric mill rhythms can vary in activity patterns because multiple descending projection neurons may contribute to their initiation. To better investigate the temperature effects on the rhythm, we thus selectively stimulated the modulatory commissural neuron 1 (MCN1), which is sufficient to activate the gastric mill rhythm, and elicits a distinct and well-described version of the rhythm[21, 29]. To recruit MCN1, we first transected the commissural ganglia to disable any spontaneous projection neuron activity[29] (Fig. 2A) and then we stimulated the MCN1 axon in the inferior oesophageal nerve (*ion*) at constant frequency (see Materials and Methods). In short, at threshold amplitude, the stimulation frequency was increased slowly until a stable gastric mill rhythm was observed[29]. This frequency was then maintained for the remainder of the experiment. We recorded intracellularly from LG in these experiments. Like previously reported, an increase to 13°C consistently prevented rhythmic LG bursting and stopped the MCN1-type gastric mill rhythm (Fig. 3B).

We used a similar approach in *C. magister*. Like in *C. borealis*, we observed spontaneous gastric mill rhythms, and when the STG was selectively heated, these rhythms stopped (Fig. 4A). Transecting the commissural ganglia and stimulating the *ion* consistently elicited an MCN1-type gastric mill rhythm when the STG was kept at 10°C. Like in *C. borealis*, the rhythm was identifiable by the rhythmic bursting of the LG neuron. Increasing the temperature terminated rhythmic bursting in LG (Fig. 4B), however the temperature had to be increased to 14°C for this to happen.

In *C. maenas*, no spontaneous gastric mill rhythms were observed. However, stimulation of the *ion* at 10°C elicited a gastric mill rhythm (Fig. 5), albeit less reliable than in the other crab species. In over 70% of our experiments, no gastric mill rhythm could be elicited. While failures to elicit gastric mill rhythms occasionally also occurred in *C. borealis* and *C. magister*, this was only the case in less than one in 10 preparations. The inability to elicit a rhythm in *C. maenas* was not due to a failure of the stimulation, as clear postsynaptic potentials from each stimulus pulse could be seen on the intracellular recording trace, even when no rhythm could be elicited. In addition, there was a clear excitatory effect of our stimulation on the pyloric rhythm, suggesting that the stimulation worked. We suspected that one reason could be that we were stimulating at the wrong temperature. We thus changed the STG baseline temperature to various temperatures between 10°C and 20°C in preparations that did not show a rhythm at 10°C. However, independently of temperature, no rhythm could be started in these preparations. Finally, increasing the stimulation frequency could not establish a rhythm either.

In those preparations where a gastric mill rhythm could be elicited, the rhythm was stable and showed similar activity patterns as in *C. borealis* and *C. magister*. Increasing the STG temperature by 4°C ended burst generation in LG and stopped the rhythm (Fig. 5).

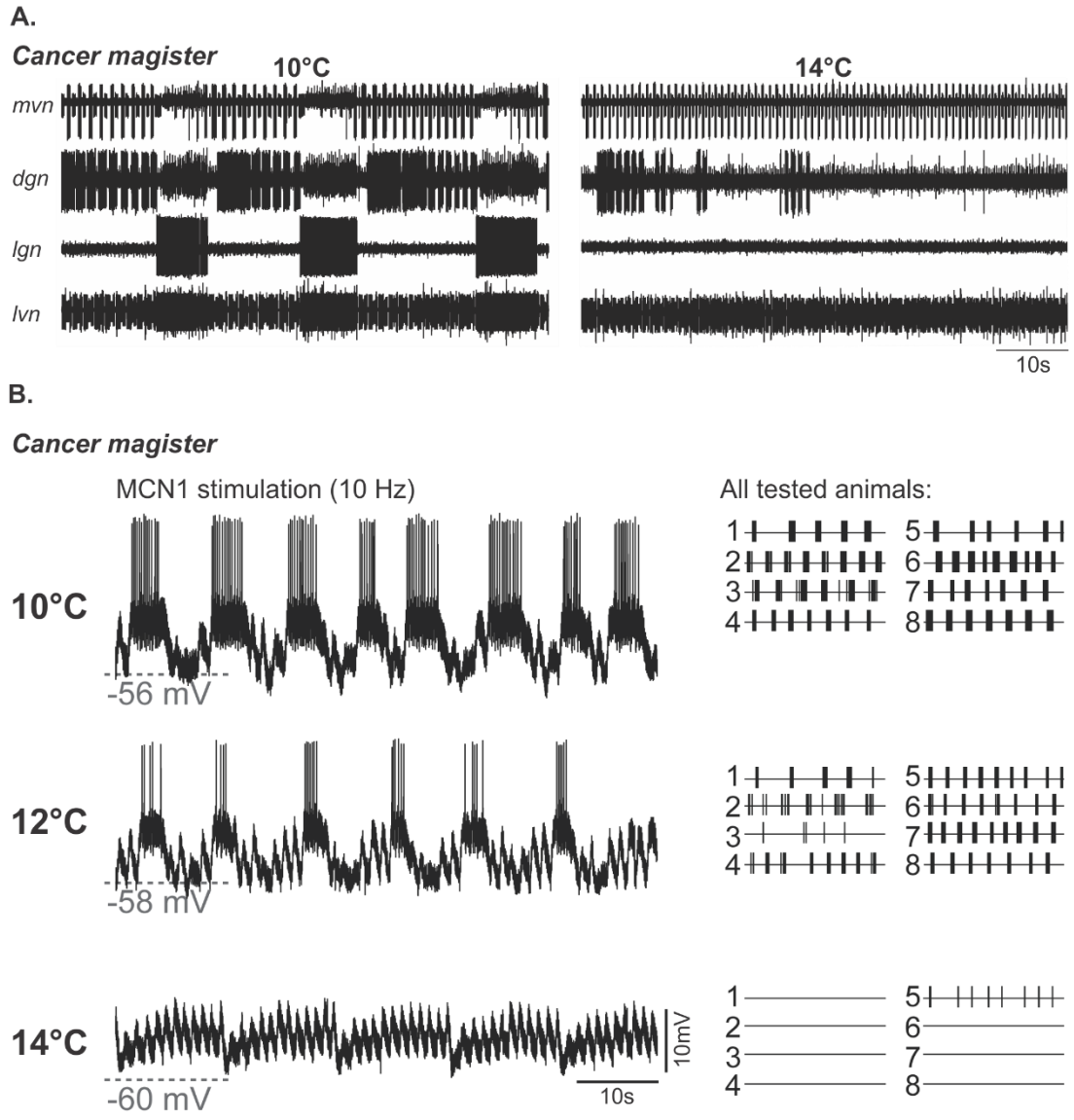

Figure 4. C. magister gastric mill rhythms terminate at elevated temperature. A. Extracellular nerve recordings showing the spontaneous gastric mill rhythms at low (10°C) and elevated temperature (14°C). Same order as in Figure 3. B. Intracellular recording of LG during continuous extracellular MCN1 stimulation with 10 Hz at 10°C, 12°C and 14°C. LG was rhythmically active at 10°C and 12°C, but activity ceased at 14°C. Right: Representation of LG spike activity for all preparations tested at 10°C, 12°C and 14°C. Each trace shows 100 s during continuous MCN1 stimulation, with each vertical line representing an action potential in LG.

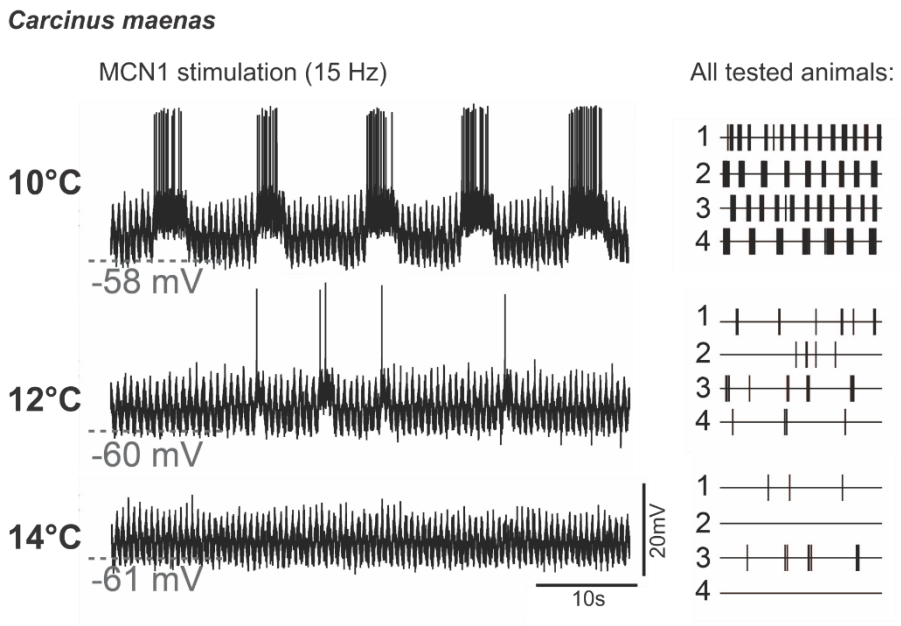

Figure 5. C. maenas gastric mill rhythms terminate at elevated temperature. Intracellular recording of LG during continuous extracellular MCN1 stimulation with 15 Hz at 10°C, 12°C and 14°C. LG was rhythmically active at 10°C, but activity ceased at 12°C and 14°C. Right: Representation of LG spike activity for all preparations tested at 10°C, 12°C and 14°C. Each trace shows 100 s during continuous MCN1 stimulation, with each vertical line representing an action potential in LG.

Temperature effects on resting membrane potential, action potential amplitude and postsynaptic potential amplitude.

To investigate the cellular effects of heating of the STG, we recorded intracellularly from LG and measured the changes of resting membrane potential, synaptic input, and action potential amplitude. For some aspects of this analysis, we re-analyzed existing *C. borealis* recordings[12] and pooled the resulting data with those from new experiments in order to compare them to new data from *C. magister* and *C. maenas*.

In *C. borealis*, the resting membrane potential hyperpolarized when STG temperature was increased (Fig. 6A). Concurrently, action potential amplitudes diminished with higher temperature. Similarly, the amplitude of the postsynaptic potential (PSP) elicited by each stimulus pulse, i.e., by each MCN1 action potential, diminished. MCN1-PSPs are of electrical nature and mediated by a gap junction between the MCN1 axon terminal and LG[29, 30] (Fig. 2B).

In *C. magister*, we found similar results. As temperature was increased, the resting membrane potential hyperpolarized (Fig. 6B). Concurrently, the amplitudes of the action potentials and of the PSPs diminished.

In *C. maenas*, the resting membrane potential also hyperpolarized when temperature was increased (Fig. 6C). Action potential and PSP amplitudes diminished as well.

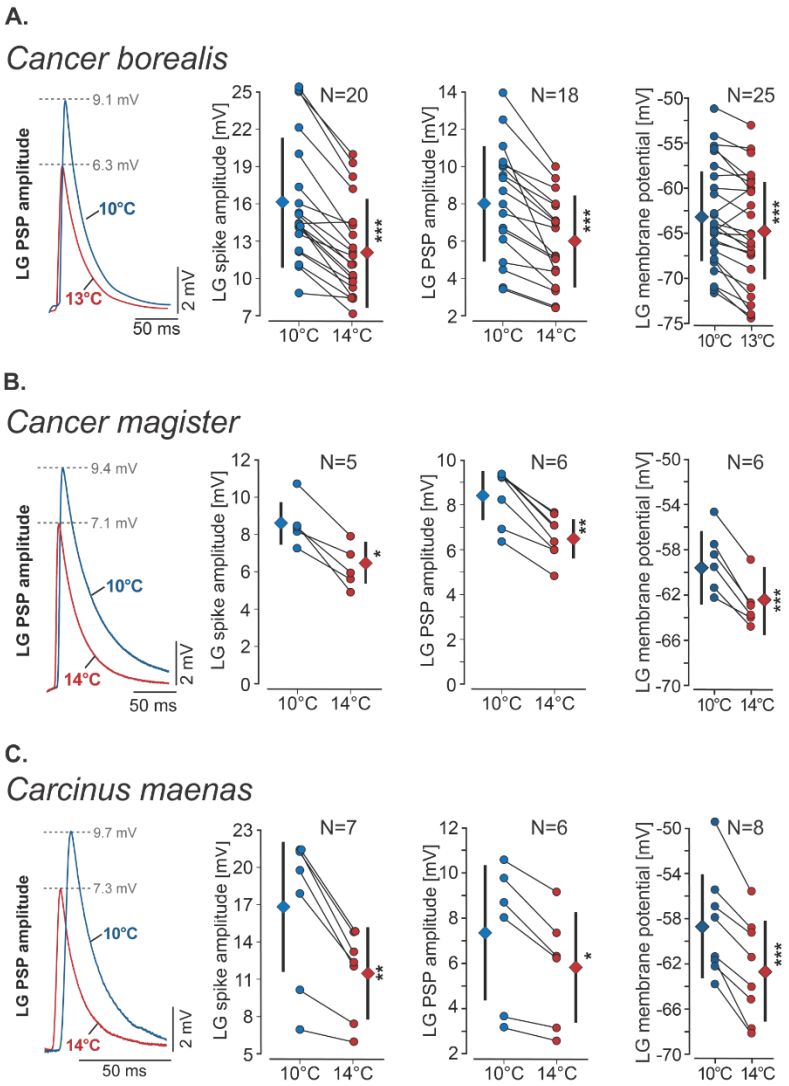

Figure 6. Comparison of temperature effects on LG's properties in the three different species. Left: Comparison of original recordings of the MCN1-elicited electrical PSPs in LG at two temperatures.

Increasing descending projection activity or supplementing released neuropeptide rescues the rhythm at high temperature.

We have previously shown that in *C. borealis*, MCN1 activity increases with higher temperature and that increasing MCN1 firing frequency is able to rescue the gastric mill rhythm at high temperature[12-14]. We also know that when MCN1 activity is maintained at its cold activity (with accordingly low spike activity), supplementing the released neuropeptide (CabTRP Ia, *Cancer borealis* tachykinin-related peptide Ia) with bath-applied CabTRP Ia can rescue the rhythm at high temperature as well. Figure 7 shows an original recording of the *C. borealis* LG, with rhythmic bursting at 10°C, and at 13°C when the rhythm had crashed. Increasing the MCN1 firing frequency by means of its stimulation frequency restored LG bursting and rescued the rhythm (Fig. 7, bottom). Similarly, when CabTRP Ia (10^{-7}M) was bath-applied at 13°C instead of increasing MCN1 stimulation frequency, the rhythm was also restored (Fig. 8).

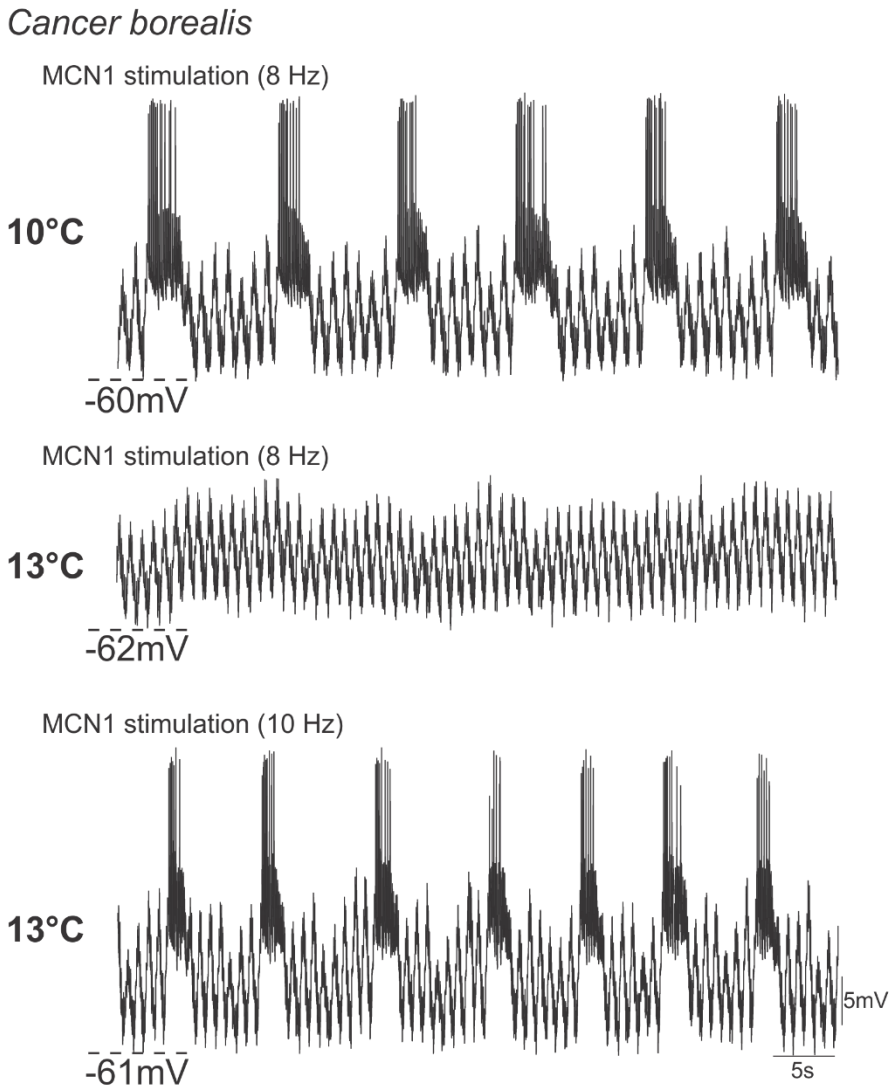

Figure 7. Increasing MCN1 firing frequency can rescue a crashed C. borealis gastric mill rhythm at elevated temperature. Intracellular recording of LG during continuous extracellular MCN1 stimulation with 8 Hz at 10°C and 13°C. LG was rhythmically active at 10°C, but activity ceased at 13°C. Increasing MCN1 stimulation frequency to 10 Hz re-established the rhythm at 13°C.

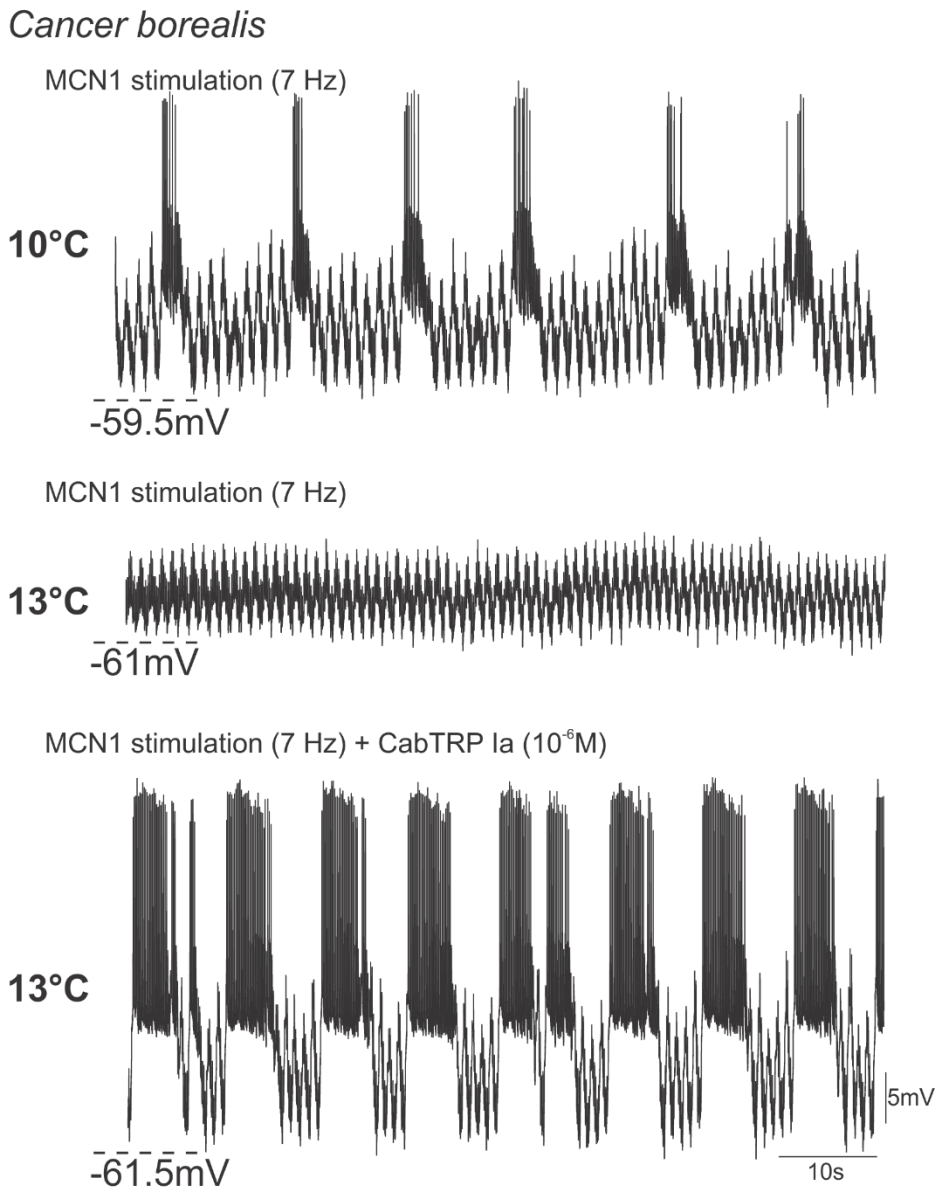

Figure 8. Supplementing the neuronal release of CabTRP Ia with bath-applied CabTRP Ia (10^{-6}M) can rescue a crashed C. borealis gastric mill rhythm at elevated temperature. Intracellular recording of LG during continuous extracellular MCN1 stimulation with 7 Hz at 10°C and 13°C. LG was rhythmically active at 10°C, but activity ceased at 13°C. Bath-applying CabTRP Ia re-established the rhythm at 13°C with unchanged MCN1 stimulation.

We found similar results in *C. magister*, in that higher MCN1 stimulation frequencies (Fig. 9) or CabTRP Ia (Fig. 10) application restored the rhythm at high temperature, suggesting that the activity of descending projection neurons (and the release of neuropeptide from them) is critical for temperature resilience of the gastric mill rhythm in this species as well.

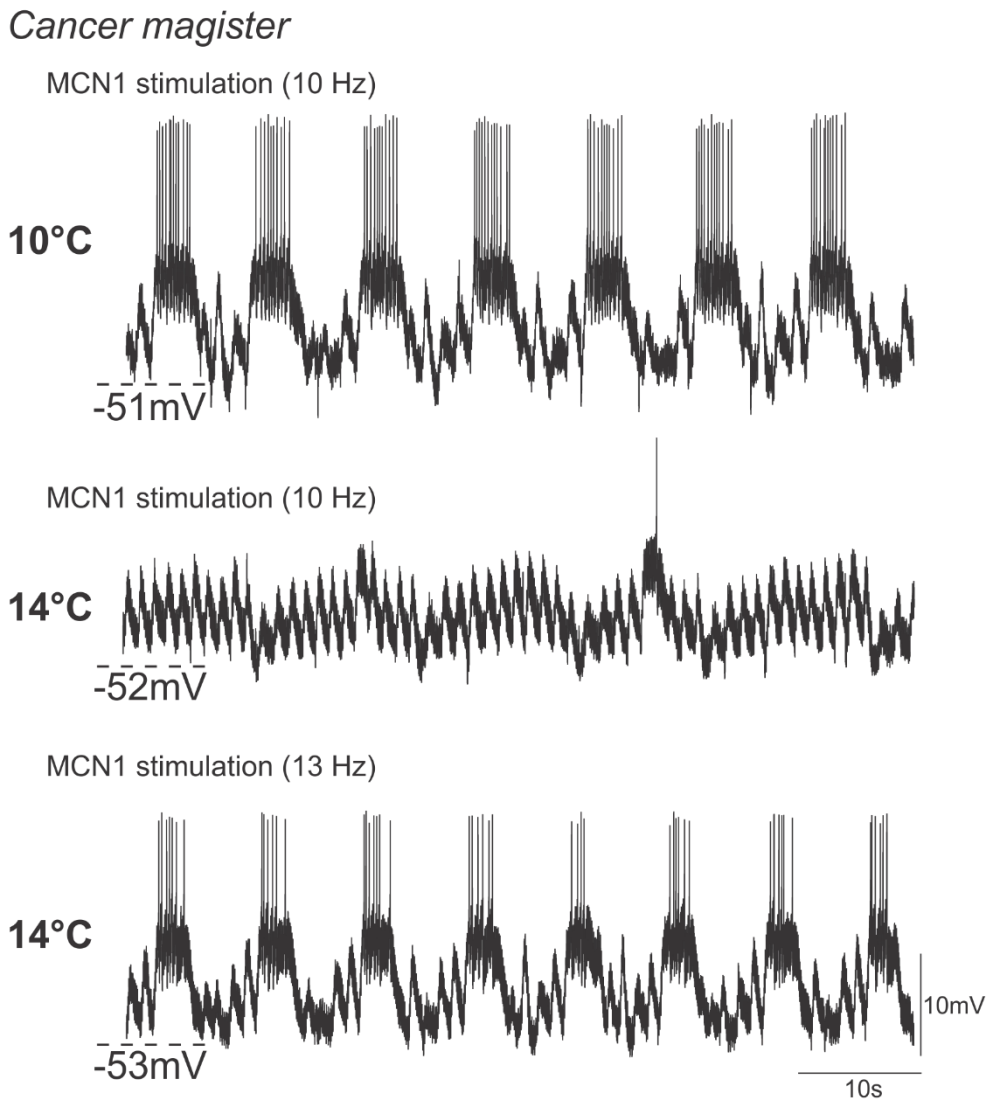

Figure 9. Increasing MCN1 firing frequency can rescue a crashed C. magister gastric mill rhythm at elevated temperature. Intracellular recording of LG during continuous extracellular MCN1 stimulation with 10 Hz at 10°C and 14°C. LG was rhythmically active at 10°C, but activity ceased at 14°C. Increasing MCN1 stimulation frequency to 13 Hz re-established the rhythm at 14°C.

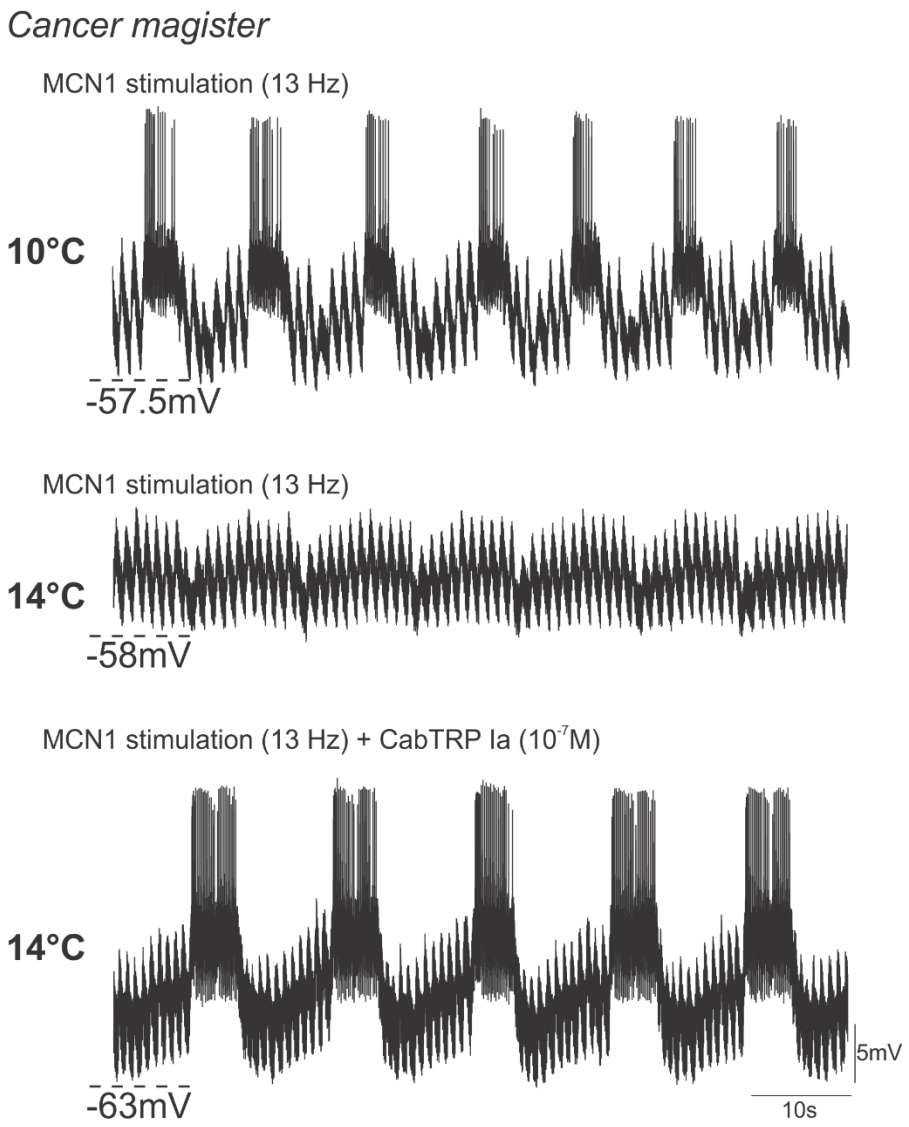

Figure 10. Supplementing the neuronal release of CabTRP Ia with bath-applied CabTRP Ia (10^{76}M) can rescue a crashed C. magister gastric mill rhythm at elevated temperature. Intracellular recording of LG during continuous extracellular MCN1 stimulation with 13 Hz at 10°C and 14°C. LG was rhythmically active at 10°C, but activity ceased at 14°C. Bath-applying CabTRP Ia re-established the rhythm at 14°C with unchanged MCN1 stimulation.

In *C. maenas*, in preparations where gastric mill rhythms could be elicited, higher MCN1 stimulation frequencies also restored a crashed rhythm at high temperature (Fig. 11). Unfortunately, because of the small number of successful preparations, we could not test the effects of CabTRP Ia on the gastric mill rhythm in this species. However, we tested whether higher MCN1 stimulation frequencies or CabTRP Ia application could elicit gastric mill rhythms in preparations where MCN1 stimulation was unable to elicit a rhythm in the first place. This was not the case.

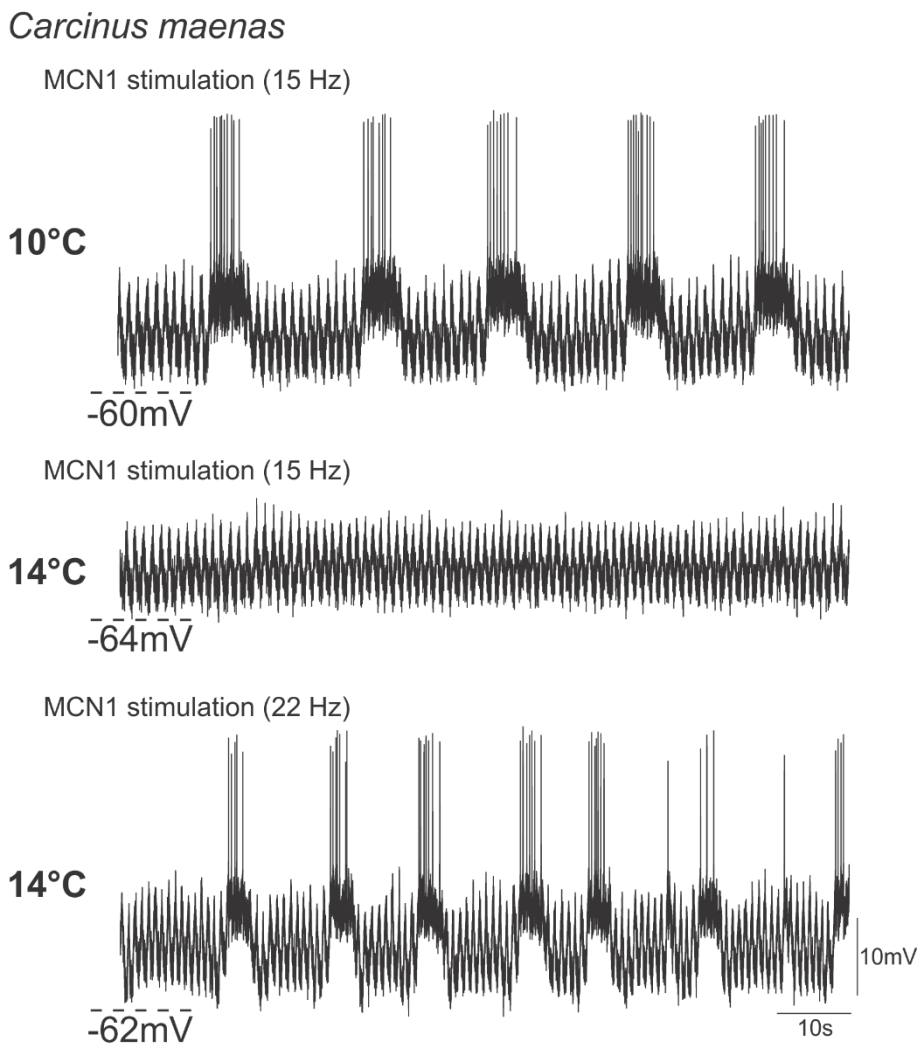

Figure 11. Increasing MCN1 firing frequency can rescue a crashed C. maenas gastric mill rhythm at elevated temperature. Intracellular recording of LG during continuous extracellular MCN1 stimulation with 15 Hz at 10°C and 14°C. LG was rhythmically active at 10°C, but activity ceased at 14°C. Increasing MCN1 stimulation frequency to 22 Hz re-established the rhythm at 14°C.

LG cell input resistance diminishes with increasing temperature.

To understand the cellular cause of the gastric mill rhythm crash at high temperature, we turned back to the cellular properties of LG. The diminishment of LG action potential and PSP amplitudes in all species indicated that LG's input resistance diminished with higher temperature. To test this, we injected negative holding currents for several seconds, measured the resulting voltage deviation, and calculated the input resistance. To accurately measure input resistance, LG was recorded in two-electrode current clamp in these experiments, with one electrode being used for current injection and the other for voltage recording. In all three species, input resistance was lower at higher temperatures (Fig. 12).

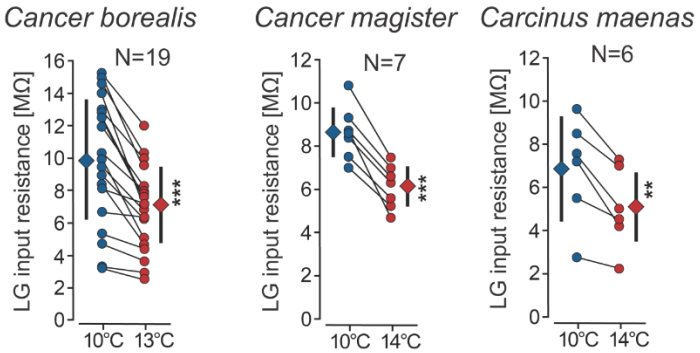

Figure 12. Comparison of temperature effects on LG's input resistance in all three species.

Restoring input resistance rescues the gastric mill rhythm in some species.

We tested whether the observed changes in input resistance are sufficient to explain the crash of the rhythm at high temperature. For these experiments, we reduced input resistance at cold temperature using dynamic clamp and measured whether this reduction stopped the rhythm. Conversely, we increased input resistance at high temperature to test whether this increase was sufficient to maintain the rhythm at that temperature. These recordings were carried out in two-electrode dynamic clamp, with one electrode measuring voltage, and the other injecting current. Changes in input resistance were matched to those measured during temperature changes in the same experiment (see Materials and Methods).

Figure 13A shows an original recording of the gastric mill rhythm in *C. borealis* in 4 conditions: at 10°C, at 10°C with dynamic clamp-reduced input resistance, at 13°C with no dynamic clamp manipulation, and at 13°C with dynamic clamp-increased input resistance. At 10°C, there was a regular gastric mill rhythm. Increasing the temperature to 13°C terminated the rhythm. The decrease in input resistance observed during this temperature manipulation was sufficient to terminate the gastric mill rhythm when applied through dynamic clamp at 10°C. Conversely, when input resistance was increased by the same amount at 13°C, the rhythm was restored. Figure 13B shows the quantitative data for all experiments.

In *C. magister*, the same experiment was only successful in half of the preparations. Figure 14A shows an original recording at 10°C, at 10°C with dynamic clamp-reduced input resistance, at 14°C, and at 14°C with dynamic clamp-increased input resistance. In this example, the rhythm was terminated by reducing the input resistance at 10°C by the amount that was caused by the temperature increase. Vice versa, increasing the input resistance at 14°C by the same amount rescued the rhythm. Figure 14B shows the quantitative data for 4 successful experiments.

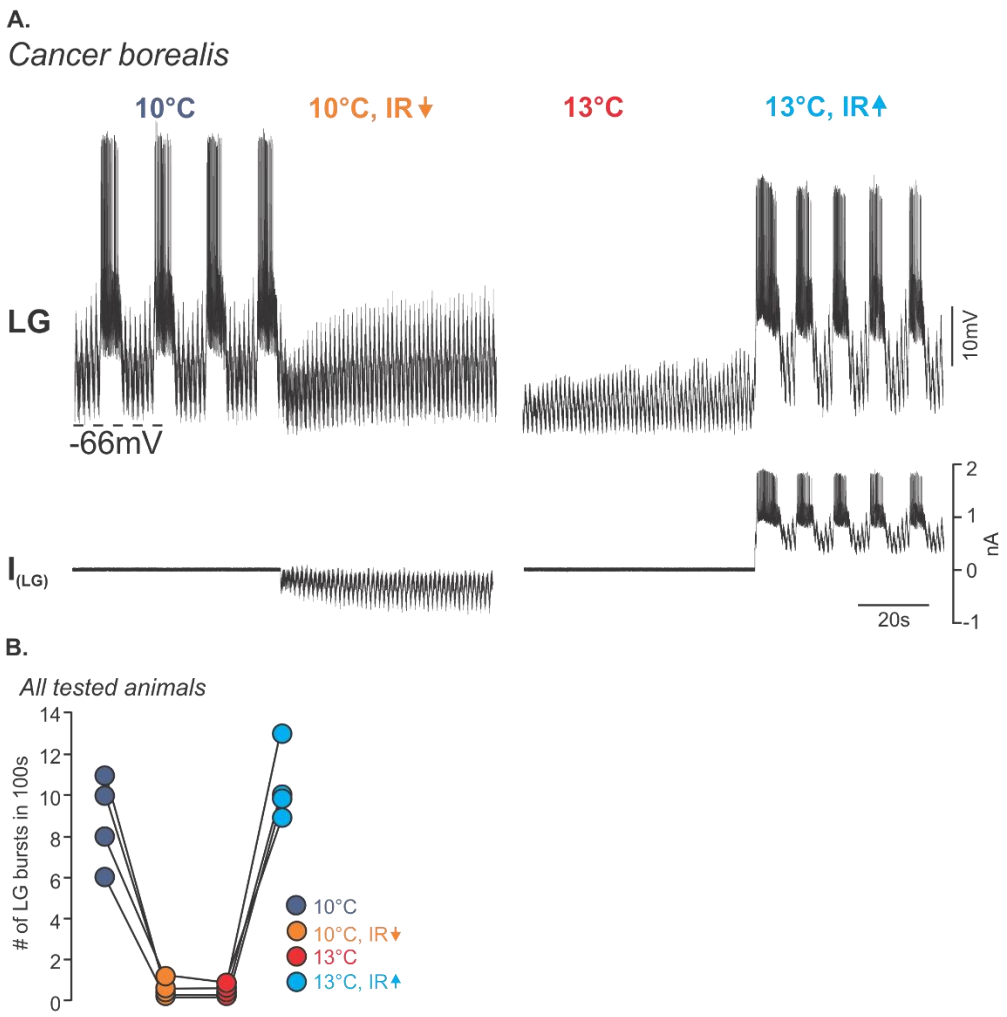

Figure 13. Changes in leak conductance are sufficient to terminate and rescue the rhythm in C. borealis. A. Top: Intracellular recording of LG during continuous MCN1 stimulation at 10°C. Rhythmic activity ceased when LG's input resistance was artificially reduced by dynamic clamp (10°C +IR$^{\vee}$). At 13°C, rhythmic LG activity had terminated even without artificial reduction of the input resistance. However, rhythmic activity was recovered when the input resistance was artificially increased (13°C+IR^), rescuing the rhythm. Bottom: Corresponding dynamic clamp current that was injected into LG. B. Quantification of LG bursts in the different dynamic clamp conditions.

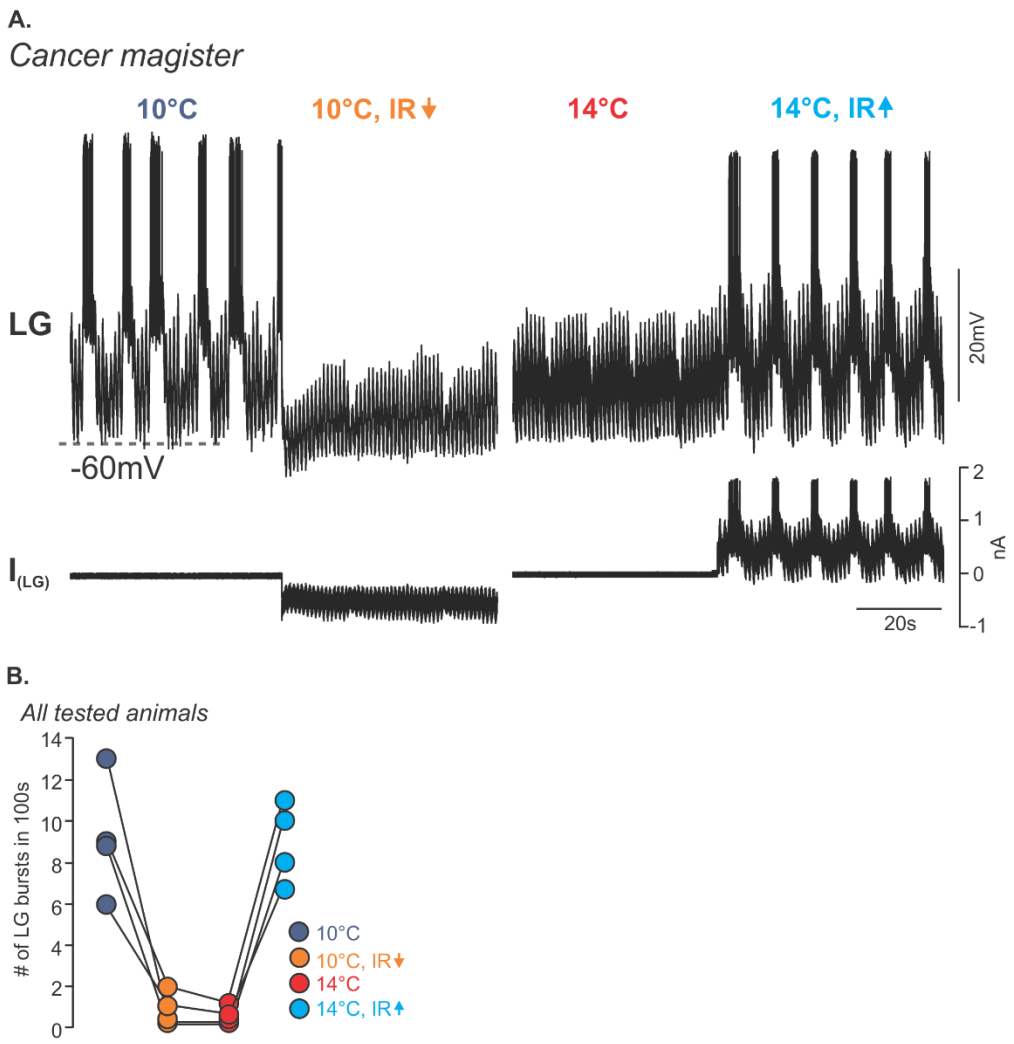

Figure 14. Changes in leak conductance are sufficient to terminate and rescue the rhythm in C. magister, in 50% of the preparations. A. Top: Intracellular recording of LG during continuous MCN1 stimulation at 10°C. Rhythmic activity ceased when LG's input resistance was artificially reduced by dynamic clamp (10°C +IR^{V}). At 14°C, rhythmic LG activity had terminated even without artificial reduction of the input resistance. However, rhythmic activity was recovered when the input resistance was artificially increased (14°C+IR^), rescuing the rhythm. Bottom: Corresponding dynamic clamp current that was injected into LG. B. Quantification of LG bursts in the different dynamic clamp conditions.

However, changing input resistance by the amount suggested by the temperature increase was not always successful. In another 4 experiments (not shown) the rhythm at 10°C was not terminated by the change in input resistance. Conversely, the rhythm could also not be restored at 14°C. Instead, LG continued to produced bursts, albeit often with fewer action potentials and at lower frequency. However, we noted that when we introduced larger changes in input resistance the rhythm at 10°C could be terminated in all preparations, and it could be restored in all preparation at 14°C as well.

Consequently, in these preparations, increasing the temperature was able to terminate the rhythm, but increasing the input resistance by the temperature-induced amount did not rescue the rhythm. This suggests that additional cellular or synaptic mechanisms must have contributed to the heat-induced termination of the rhythm.

Discussion.

The main finding of our study is that the intrinsic permissive ('stable') temperature range of the gastric mill rhythm was small in all tested species (3-4°C). This was despite the fact that all species experience larger temperature fluctuations in their habitat (Fig. 1). For *C. maenas*, for example, the gastric mill rhythm crashed more than 20°C below the temperature these animals can tolerate[31]. However, gastric mill activity could be extended to more elevated temperatures when extrinsic modulatory activity was increased, or when additional neuropeptide was bath applied. Consequently, temperature robustness in the gastric mill circuit is highly dependent on extrinsic modulatory input in these species. A consistent effect of elevated temperature in all three species was the hyperpolarization of the resting membrane potential, and a decrease in input resistance. The latter led to a shunt of postsynaptic potentials and action potentials, as evidenced by their diminished amplitude at elevated temperature. Temperature effects on input resistance are well described in other systems as well. For example, the input resistance in goldfish Mauthner cells is consistently smaller after acclimation to elevated temperatures[32]. Changes in input resistance are known to play an important role for network oscillations[33-36], regulation of excitability[37, 38] and switches in activity states of neurons[39]. Our results suggest that in all tested species, there is a contribution of input resistance to the termination of the rhythm at elevated temperature. However, our dynamic clamp experiments also demonstrate that additional factors may contribute to the termination of the rhythm in *C. magister*, because not in all experiments changing the input resistance was sufficient to recreate the effects of temperature. Additional factors may include effects on synaptic properties and voltage-gated ionic currents. Surprisingly, however, increasing MCN1's activity and application of MCN1's peptide co-transmitter (CabTRP Ia) was able to restore the rhythm in all preparations, indicating that whatever additional properties contribute to LG's temperature crash, they can be counterbalanced by neuropeptide modulation. Temperature effects on the ionic current elicited by CabTRP Ia release (I_{MI}) could also contribute to the temperature robustness. Its conductance may increase with temperature as well, which could boost temperature robustness, since I_{MI} has been shown to act as a negative leak conductance[36] and restore oscillations. However, if, and if so, how much I_{MI} changes with temperature is not known. It is surprising to us that our dynamic clamp experiments were successful in half of the experiments, while in the other half the injected conductance was not sufficient to mimic the temperature effects. It is possible that this reflects space-clamp problems in our approach. LG in *C. magister* is a very large cell. We estimate its cell body to >100µm, with a primary neurite of at least the same length. This could interfere with the ability of our (somatic) clamp to reach the distant dendritic processes of the cell,

where synaptic input arrive, and the spike initiation site where action potentials are generated. This may be particularly the case at elevated temperatures, because input resistance drops (Fig. 12), suggesting that the length constant of the neuron shortens. In fact, this effect was shown to be the present in the LG of *C. borealis*[13].

Counterbalancing detrimental temperature effects on the nervous system is critical for maintaining neuronal function and often essential for survival. This is particularly the case when circuits that drive vital body functions are affected. Hyperthermia during fever or heat stroke can, for example, cause failures of the respiratory pattern generator and induce apnea[40]. There is some evidence that synaptic influences in the respiratory network may help to counterbalance temperature effects, because the respiratory pacemakers are more temperature sensitive when they are synaptically isolated[40]. Generally, it is assumed that the permissible temperature range a neuronal circuit displays is related to the temperature fluctuation it experiences. For homeotherms, the permissive range is predicted to be small, given the rather small variations in body temperature that occur in a daily and monthly fashion[41]. In contrast, for poikilotherms, and particularly those that live in habitats with large temperature fluctuations, the permissive range must be much larger. In fact, recent studies on the crustacean pyloric rhythm have shown that neuronal circuits in species that live in the intertidal possess a larger permissive range than those in deeper-ocean species[31]. This suggests that neuronal mechanisms to counterbalance temperature effects are more sophisticated in animals that experience large temperature fluctuations in their environment.

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References.

1. Robertson, R.M., H.A. MacMillan, and M.K. Andersen, *A cold and quiet brain: mechanisms of insect CNS arrest at low temperatures.* Curr Opin Insect Sci, 2023. **58**: p. 101055.
2. Marder, E. and M.C.P. Rue, *From the Neuroscience of Individual Variability to Climate Change.* J. Neurosci., 2021. **41**(50): p. 10213-10221.
3. Robertson, R.M. and T.G. Money, *Temperature and neuronal circuit function: compensation, tuning and tolerance.* Curr. Opin. Neurobiol., 2012. **22**(4): p. 724-34.
4. Harding, E.C., N.P. Franks, and W. Wisden, *The Temperature Dependence of Sleep.* Front. Neurosci., 2019. **13**: p. 336.
5. Somero, G.N., *The physiology of global change: linking patterns to mechanisms.* Ann Rev Mar Sci, 2012. **4**: p. 39-61.
6. Somero, G.N., *The physiology of climate change: how potentials for acclimatization and genetic adaptation will determine 'winners' and 'losers'.* J. Exp. Biol., 2010. **213**(6): p. 912-20.
7. Reid, P.C., et al., *Chapter 1. Impacts of the oceans on climate change.* Adv Mar Biol, 2009. **56**: p. 1-150.

8. Oliver, E.C.J., et al., *Longer and more frequent marine heatwaves over the past century.* Nat. Commun., 2018. **9**(1): p. 1324.
9. Mann, M.E., et al., *Projected changes in persistent extreme summer weather events: The role of quasi-resonant amplification.* Sci Adv, 2018. **4**(10): p. eaat3272.
10. Alonso, L.M. and E. Marder, *Temperature compensation in a small rhythmic circuit.* Elife, 2020. **9**: p. e55470.
11. Stein, W., *Stomatogastric Nervous System*, in *Oxford Research Encyclopedia, http://dx.doi.org/10.1093/acrefore/9780190264086.013.153*. 2017, Oxford University Press.
12. Städele, C., S. Heigele, and W. Stein, *Neuromodulation to the rescue: Compensation of temperature-induced breakdown of rhythmic motor patterns via extrinsic neuromodulatory input.* PLoS Biol., 2015. **13**(9): p. e1002265.
13. DeMaegd, M.L. and W. Stein, *Neuropeptide Modulation Increases Dendritic Electrical Spread to Restore Neuronal Activity Disrupted by Temperature.* J. Neurosci., 2021. **41**(36): p. 7607-7622.
14. Stadele, C. and W. Stein, *Neuromodulation Enables Temperature Robustness and Coupling Between Fast and Slow Oscillator Circuits.* Front Cell Neurosci, 2022. **16**: p. 849160.
15. Prentice, E.F. and D.E. Schneider, *Respiration and thermal tolerance of the dungeness crab, Cancer magisterdana.* Comparative Biochemistry and Physiology--Part A: Physiology, 1979. **63**(4): p. 591-597.
16. Logan, L. and M. Frederich, *Differential behavior, habitat destruction, and stress tolerance in three populations of Carcinus maenas.* Integr. Comp. Biol., 2017. **57**: p. E100-E100.
17. Gutierrez, G.J. and R.G. Grashow, *Cancer borealis stomatogastric nervous system dissection.* J. Vis. Exp., 2009. **25**: p. e1207.
18. Hooper, S.L., et al., *Cell dialysis by sharp electrodes can cause nonphysiological changes in neuron properties.* J. Neurophysiol., 2015. **114**(2): p. 1255-71.
19. Coleman, M.J., P. Meyrand, and M.P. Nusbaum, *A switch between two modes of synaptic transmission mediated by presynaptic inhibition.* Nature, 1995. **378**(6556): p. 502-5.
20. Stein, W., et al., *Divergent co-transmitter actions underlie motor pattern activation by a modulatory projection neuron.* Eur. J. Neurosci., 2007. **26**(5): p. 1148-65.
21. Hedrich, U.B., F. Diehl, and W. Stein, *Gastric and pyloric motor pattern control by a modulatory projection neuron in the intact crab Cancer pagurus.* J. Neurophysiol., 2011. **105**(4): p. 1671-80.
22. Bartos, M. and M.P. Nusbaum, *Intercircuit control of motor pattern modulation by presynaptic inhibition.* J. Neurosci., 1997. **17**(7): p. 2247-56.
23. Sharp, A.A., et al., *Dynamic clamp: computer-generated conductances in real neurons.* J. Neurophysiol., 1993. **69**(3): p. 992-5.
24. Hedrich, U.B., C.R. Smarandache, and W. Stein, *Differential activation of projection neurons by two sensory pathways contributes to motor pattern selection.* J. Neurophysiol., 2009. **102**(5): p. 2866-79.
25. Beenhakker, M.P. and M.P. Nusbaum, *Mechanosensory activation of a motor circuit by coactivation of two projection neurons.* J. Neurosci., 2004. **24**(30): p. 6741-50.
26. Blitz, D.M., et al., *A newly identified extrinsic input triggers a distinct gastric mill rhythm via activation of modulatory projection neurons.* J. Exp. Biol., 2008. **211**(Pt 6): p. 1000-11.
27. Nusbaum, M.P., *Modulatory Projection Neurons*, in *Encyclopedia of Neuroscience* M.D. Binder, Hirokawa, N., Windhorst, U., Editor. 2013, Springer, Berlin, Heidelberg.
28. Nusbaum, M.P. and M.P. Beenhakker, *A small-systems approach to motor pattern generation.* Nature, 2002. **417**(6886): p. 343-50.
29. Bartos, M., et al., *Coordination of fast and slow rhythmic neuronal circuits.* J. Neurosci., 1999. **19**(15): p. 6650-60.

30. Stein, W., et al., *The dynamic range of voltage-dependent gap junction signaling is maintained by Ih-induced membrane potential depolarization.* J. Neurophysiol., 2022. **127**(3): p. 776-790.
31. Stein, W., et al., *Thermal acclimation and habitat-dependent differences in temperature robustness of a crustacean motor circuit.* Front Cell Neurosci, 2023. **17**: p. 1263591.
32. Szabo, T.M., et al., *Effects of temperature acclimation on a central neural circuit and its behavioral output.* J. Neurophysiol., 2008. **100**(6): p. 2997-3008.
33. Cymbalyuk, G.S., et al., *Bursting in leech heart interneurons: cell autonomous and network based mechanisms.* J. Neurosci., 2002. **22**(24): p. 10580–92.
34. Blethyn, K.L., et al., *Neuronal basis of the slow (<1 Hz) oscillation in neurons of the nucleus reticularis thalami in vitro.* J. Neurosci., 2006. **26**(9): p. 2474-86.
35. Koizumi, H., et al., *Functional imaging, spatial reconstruction, and biophysical analysis of a respiratory motor circuit isolated in vitro.* J. Neurosci., 2008. **28**(10): p. 2353-65.
36. Zhao, S., J. Golowasch, and F. Nadim, *Pacemaker neuron and network oscillations depend on a neuromodulator-regulated linear current.* Front. Behav. Neurosci., 2010. **4**: p. 21.
37. Rekling, J.C., et al., *Synaptic control of motoneuronal excitability.* Physiol. Rev., 2000. **80**(2): p. 767-852.
38. Brickley, S.G., et al., *TASK-3 two-pore domain potassium channels enable sustained high-frequency firing in cerebellar granule neurons.* J. Neurosci., 2007. **27**(35): p. 9329-40.
39. Gramoll, S., J. Schmidt, and R.L. Calabrese, *Switching in the activity state of an interneuron that controls coordination of the hearts in the medicinal leech (Hirudo medicinalis).* J. Exp. Biol., 1994. **186**: p. 157-71.
40. Tryba, A.K. and J.-M. Ramirez, *Hyperthermia modulates respiratory pacemaker bursting properties.* J. Neurophysiol., 2004. **92**(5): p. 2844-2852.
41. Stein, W. and S. Harzsch, *The Neurobiology of Ocean Change - insights from decapod crustaceans.* Zoology (Jena), 2021. **144**: p. 125887.