In vivo Effects of Temperature on the Heart and Pyloric Rhythms in the Crab, *Cancer borealis*.

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Running title: *In vivo* heart and pyloric rhythms

Key words – photoplethysmography, crustaceans, stomatogastric nervous system, cardiac ganglion, central pattern generators, Q_{10}

Author Contributions: Experiments, data analysis and figure preparation, D.K. Study design and manuscript writing and editing, D.K and E.M.

Summary Statement

Elevation of ambient temperature increases correlations between the heart and pyloric rhythms in *Cancer borealis*.

Abstract

The heart and pyloric rhythms of crustaceans have been studied separately and extensively over many years. Local and hormonal neuromodulation and sensory inputs onto these central pattern generating circuits play a significant role in the animals' responses to perturbations, but are usually lost or removed during *in vitro* studies. To examine simultaneously the *in vivo* motor output of the heart and pyloric rhythms, we developed a technique using photoplethysmography (PPG). In the population tested (n = 53), heart rhythm frequencies were bimodal (peaks at ~ 0.7 Hz and ~ 1.1 Hz). The pyloric rhythms were normally distributed with a mean frequency of 0.8Hz. Over 24 hour recordings, animals held at baseline conditions cycled in frequency. We calculated the correlations between the two rhythms at baseline conditions and found that in some animals the rhythms were highly correlated while in others they were not. To investigate how these rhythms behave in response to perturbation, we applied temperature ramps to animals by heating or cooling the saline bath while recording both the heart and pyloric muscle movements. Q_{10}s, critical temperatures (temperatures at which function is compromised), and changes in frequency were calculated for each of the rhythms tested. Changes in frequency and Q_{10} values were not significantly different between the two rhythms while critical temperatures were significantly different, and the correlations between the two rhythms during these times were high.

Introduction

Central pattern generators (CPGs) are responsible for the generation of many rhythmic motor patterns that are vital for muscle movements of an animal's life. In crustaceans, mechanisms of central pattern generation have been studied using the cardiac ganglion (CG), which produces heart movements, and the stomatogastric ganglion (STG) which generates the rhythmic movements of the stomach (Cooke, 2002; Maynard, 1972). These two rhythms are subject to both local and global modulation. These rhythms have been studied separately, both *in vivo* and in dissected and isolated preparations, but now we ask whether these rhythms appear to be coordinated.

The heart of the crustacean is neurogenic, controlled by the motor neuron discharges produced by the CG. In normal conditions, the heart continuously pumps hemolymph through an open circulatory system to distribute oxygen and neuromodulators to the animal's tissues (McMahon, 1995; McMahon, 2001a; McMahon, 2001b). This circulatory system is formed by elaborate capillary beds in many of the animal's tissues, paired with valves that allow for selective hemolymph distribution (McMahon, 1995; McMahon, 2001a; McMahon, 2001b). The cardiac output, calculated as the stroke volume/heart rate, must be maintained to ensure adequate hemolymph distribution (McGaw and McMahon, 1996). Hemolymph flow rate and stroke volume are subject to wide variations both within and across animals (McGaw and McMahon, 1996). Changes in flow rate are likely due to modifications in stroke volume or valve activation, and may occur in response to local tissue demands (McGaw and McMahon, 1996; Wilkens and McMahon, 1994). Changes in cardiac output may

also be the result of spontaneous firing events in the CNS, CG, or cardioarterial valves of the heart (McGaw and McMahon, 1996; Wilkens and McMahon, 1994).

The CG, controlling the movement of the heart, contains an intrinsically oscillating pacemaker to form a central pattern generated rhythm. Additionally, the heart is innervated by extrinsic fibers, one inhibitory and two excitatory, that modify heart rate in relation to behavioral demands (Cooke, 2002). The CG of the crab, *Cancer borealis,* is comprised of nine neurons, 4 small and 5 large cells, that burst in time to produce muscle movements. The CG also produces patterned bursts of impulses in response to simple stimuli, such as excitation from stretch sensitive dendrites (Cooke, 2002). This allows the heart to adjust its frequency and strength of contractions when faced with different metabolic needs (Dickinson et al., 2016a; Dickinson et al., 2015a).

The stomatogastric nervous system (STNS) has long been used to study central pattern generation and mechanisms underlying rhythmic and continuous motor patterns (Maynard, 1972). The STNS controls the movement of the crustacean stomach (Maynard and Dando, 1974)(Morris and Hooper, 1997; Morris and Hooper, 1998; Morris and Hooper, 2001). Foregut movements allow for feeding behavior, including chewing, swallowing, and processing of waste (Clemens et al., 1998b; Johnson and Hooper, 1992). Oxygen tension alters the neuronal activity of the STG and therefore its motor output (Clemens et al., 2001). The stomach is a complex mechanical structure with ossicles that provide mechanical support and insertions for intrinsic stomach muscles (Maynard and Dando, 1974). The STNS generates the continuously active and rapid pyloric rhythm and the slower, episodic gastric mill movements (Clemens et al., 1998a; Clemens et al., 1999; Clemens et al., 1998b; Clemens et al., 2001; Clemens et al.,

1998c; Heinzel, 1988; Heinzel and Selverston, 1988; Rezer and Moulins, 1983; Soofi et al., 2014).

The pericardial organs (POs) are secretary organs that deliver neurohormones to the animal's hemolymph (Alexandrowicz and Carlisle, 1953; Chen et al., 2010; Christie et al., 1995; DeKeyser et al., 2007; Hui et al., 2012). These neuromodulators may increase the frequency and amplitude of the heartbeat (Christie et al., 2008; Cruz-Bermudez and Marder, 2007; Sullivan and Miller, 1984; Williams et al., 2013), or change the frequency of the pyloric rhythm (Hooper and Marder, 1987; Marder, 2012; Marder and Bucher, 2007). Hemolymph containing modulator, flowing through the ophthalmic artery, directly bathes the STG. The modulators released into the hemolymph act hormonally, and therefore may affect the animal's various organs in the same manner, or may have varied effects depending on the receptors present in the tissues. This provides a feedback system within the animal, allowing for the simultaneous adjustment of organ activity when needed in the animal.

Throughout its life, an organism must respond to perturbations that affect the nervous system and the biomechanical structures it controls. Crustaceans are poikilotherms and therefore may experience wide variations in body temperature due to temperature changes in the environment. Because of this, it is relevant to study the effect of temperature on the activity of the STNS and CG, as temperature changes may simultaneously affect cellular processes of both systems, and disrupt neuronal function. Throughout their lives, crustaceans experience both short term temperature fluctuations, due to changing tidal patterns during a single day, and long term temperature fluctuations due to seasonal temperature variations (Soofi et al., 2014;

112 Tang et al., 2010; Tang et al., 2012). Despite these varied temperature changes, animals
113 must maintain rhythmicity and performance of the foregut and heart. Therefore,
114 temperature is a useful perturbation to study network stability in the face of
115 perturbation.

116 Previous work on crustaceans that both the heart and pyloric rhythms are robust
117 to temperature changes. In the heart, studies have shown that strength of a heartbeat
118 decreases and heart rate increases with increases in temperature (Camacho et al., 2006;
119 Worden et al., 2006). Therefore, the increase in heart rate partially compensates for the
120 decrease in stroke volume as the CG is further pushed from its normal state at 11°C. The
121 pyloric triphasic rhythm is maintained across a wide range of temperatures. In both the
122 heart and the pyloric rhythm, the maximum frequency attained at highest temperatures
123 *in vivo* are lower than those of *in vitro* conditions, likely due to the presence of sensory
124 feedback and neurohormonal input (Soofi et al., 2014; Worden et al., 2006).

125 The Q_{10}, is a measure of the sensitivity of a biological process to a 10°C change in
126 temperature. Many biological processes have Q_{10}s between two and three, while some
127 temperature sensitive ion channels have Q_{10}s as high as 50 or 100 (Marder et al., 2015).
128 If all Q_{10}s of the components involved in a biological process are similar, this process is
129 likely to be temperature compensated (Caplan et al., 2014; Marder et al., 2015; O'Leary
130 and Marder, 2016; Robertson and Money, 2012). In both the heart and pyloric rhythms,
131 the Q_{10}s for the rhythm frequencies are similar *in vivo* and *in vitro* (Soofi et al., 2014;
132 Worden et al., 2006). Worden and colleagues (2006) noted that in the heart, the Q_{10}s of
133 various parameters were 1- 3.5. The pyloric rhythm increases in frequency with Q_{10}s

134 between 2 and 2.5 while conserving its phase relationships within the triphasic rhythm
135 (Soofi et al., 2014; Tang et al., 2010).

136 Temperature increases beyond 23°C lead to severely disrupted motor patterns
137 and subsequent loss of rhythms in the heart and pyloric rhythms (Rinberg et al., 2013;
138 Tang et al., 2012). Recordings of the pyloric rhythm show that preparations appear
139 similar at permissive temperatures, but extreme temperatures disrupt, or crash them, sh
140 in dissimilar ways revealing their variability (Marder et al., 2015; Rinberg et al., 2013;
141 Tang et al., 2012).

142 In this study we systematically explore the potential relationships between
143 stomach and heart rhythms, and ask whether they are coordinately sensitive to
144 perturbation by temperature.

145

146

147 **Materials and Methods**

148 Animals

149 Adult male Jonah crabs (*Cancer borealis)* between 400 and 700 grams were
150 obtained from Commercial Lobster (Boston, MA). Animals were housed in tanks with
151 artificial seawater (Instant Ocean) between 10°C and 13°C on a 12 hour light/dark cycle
152 without food for a maximum of 10 days. For the *in vivo* experiments, animals were
153 placed in a 25 liter tank filled with approximately 10 liters of artificial seawater inside an
154 incubator at 10°C to 12°C. Experiments were done between 9/1/16 and 7/12/17.

155 Prior to each experiment, crabs were weighed and anesthetized on ice for 10
156 minutes. Photoplesmogram (PPG) sensors (Vishay CNY70331) (Fig. 1), as described in
157 Depledge (1983), were placed on the carapace above the heart and pyloric muscles to
158 record the heart and pyloric rhythms, respectively. Sensors were secured to the carapace
159 using dental wax and cyanoacrylate glue (Starbond, EM-2000) and covered in Marine
160 Adhesive Sealant (3M, Fast Cure 5200) to waterproof and ensure the stability of the
161 sensors over time. After the sensors were attached to the animal, we waited a minimum
162 of 16 hours prior to experimental recordings, during which time the animals were not
163 handled and the door to the incubator was not opened.

164

165 Temperature Experiments

166 After a period of baseline (10°C to 12°C) recording, water temperature was
167 manipulated by flowing either cold or warm saline into the tank through a tube inserted
168 through the door of the incubator. A vacuum line was used to pump water out of the
169 tank to maintain a constant volume. Temperature was slowly ramped from 11°C to 32°C

over 1.5 to 2 hours. Heart rate was closely monitored to ensure health during the temperature changes and ramps were halted once the heart rate developed an arrhythmia or decreased to baseline frequencies, indicating that a 'critical temperature' had been reached.

Data Acquisition and Analysis

PPG data were acquired through the PPG amplifier (Newshift AMP03) and recorded digitally through a digitizer (Axon Digidata 1550B) into computer software (AxoScope 10.6) Data were analyzed through code written for MATLAB. PPG waveforms were analyzed for frequency data by marking points of peaks in waveform as a single muscle movement.

Inhibitory Bouting Analysis

Heart inhibitory bouts were defined as spontaneous (non-perturbation induced) events characterized by at least a 33% decrease in frequency and amplitude of heart muscle movement. Inhibitory bouts occurring during baseline conditions of experiments were analyzed for period, duration, and change in amplitude following bout. Change in amplitude was calculated by subtracting the mean amplitude 5 seconds after the end of a bout from the mean amplitude 5 seconds prior to the beginning of a bout.

Change in Frequency Analysis

Temperature experiments were evaluated for changes in frequency following perturbation. Change in frequency was the difference between mean frequency 30 minutes prior to ramp initiation and max frequency during temperature ramp.

Q_{10} Analysis

Q_{10} analysis was performed to evaluate the temperature dependence of the heart and pyloric rhythms *in vivo*. Frequency data (P) were plotted against temperature (T) in a semilog plot and the Q_{10} was extracted from the slope of the linear regression (m), following the equation:

$$Q10 = 10^{10M}$$

$$M = \frac{dlogP}{dT}$$

Critical Temperature Analysis

Critical temperatures were defined as temperatures (in °C) at which the heart or pyloric movement was lost for at least 15 seconds with a subsequent drop in contraction frequency to near-baseline values (Worden et al., 2005). Critical temperatures of the pyloric rhythm were often impossible to determine due to global irregularity in pyloric rhythm signal.

Results

Heart and pyloric muscle movements of *Cancer borealis* were recorded *in vivo* using a photoplesmography (PPG) system (Fig. 1). PPG sensors were glued onto the carapace of animals above the heart and dorsal dilator muscles to monitor the heart and pyloric rhythms, respectively. Animals were allowed to recover from handling for a minimum of 16 hours before recording to limit stress artifacts.

Heart rhythms. Figure 2A shows the raw plesmograph signal from the hearts of two crabs. The heart rate of Animal 1 was considerably slower (0.6 Hz) than that of Animal 2 (1.4 Hz), but both showed strong and regular rhythms. Figure 2B shows stable recordings from an animal over 30 minutes. In total, we measured heart activity from 53 animals (Fig. 2), held between 11 and 13°C in artificial seawater with a 12-hour light/dark cycle. Within a baseline recording period, PPG waveforms (indicating muscle movement) maintained similar frequency, amplitude, and shape with minimal drift. Some animals maintained faster heart rhythm movements over time than others.

For each of the 53 animals, we recorded baseline heart rate frequency over 30 minute periods. Figure 2C shows that these baseline recordings had a multimodal distribution (Hartigan's dip test, $p = 0.024$). Heart rhythm frequencies ranged between 0.4 Hz and 1.7 Hz (mean = 1.0, s.d. = 0.34) (Fig. 2C). No correlation was found between heart rhythm frequency and time of year of experiment ($p = 0.25$, Pearson's correlation coefficient). Additionally, no correlation was found between average heart rhythm frequency and weight of crab ($p = 0.37$, Pearson's correlation coefficient).

Long term experiments (Figure 3), beginning at 10 AM and ending at 10 AM the following day, indicate drifts in heart rate. These changes in frequency of individual

233 crabs occurred over the light/dark cycle, as seen in the example shown in Figure 3A.
234 Animals tested (n = 6) showed relatively rapid and large swings in frequency during
235 daytime hours and switched to a more constant pattern during nighttime hours (Fig.
236 3A). To determine the overall activity of the heart during these extensive recording
237 sessions, we plotted histograms of the frequencies, in 5 second bins (Fig. 3B).

238 Heart rhythms were often accompanied by periods of bradycardia, during which
239 the heart considerably slowed or halted for a significant period (Fig. 4). We defined
240 these periods as inhibitory bouts, marked by a decrease in both amplitude and
241 frequency of heart rhythm PPG recordings by at least 33% and lasting for at least 10
242 seconds. An example of two such bouts can be seen in Figure 4A. Termination of
243 inhibitory bouts was associated with a return of amplitude and frequency above the 33%
244 decrease line. Inhibitory bouts were seen in 13 animals of the population tested (25%).
245 Inhibitory bouts persisted across long periods of time, indicated in the long term 24-
246 hour recordings (Fig. 4B). Inhibitory bouts occurred in a regular pattern, with small
247 variation in frequency within animals (Fig. 4D). Bout durations (Fig. 4C) were variable
248 both across and between animals.

249 The occurrence of inhibitory bouts was not significantly correlated with the time
250 of year ($p = 0.79$, t-test) or weight of animal ($p = 0.41$, t-test).

251 Pyloric Rhythms. Recording of the pyloric rhythm with PPG yielded complex,
252 highly variable waveforms due to the angle of muscle movement with respect to the PPG
253 sensor placement on the carapace of the animal. Figures 5A and 5B show raw traces of
254 simultaneous recordings of heart and pyloric rhythms. In Figure 5A the pyloric rhythm
255 was substantially slower than the heart rhythm, while in Figure 5B, the two rhythms are

256 closer in frequency. Figure 5C shows stretches of pyloric rhythm activity during a longer
257 recording, illustrating the waveform and frequencies seen during a longer recording.
258 Pyloric rhythms were recorded from 29 animals (Fig. 5D). In this compilation, we
259 excluded experiments in which data were too noisy or unreliable. To exclude the
260 possibility of cross-talk between the sensors, pyloric rhythm data were also discarded
261 they too closely resembled that of the heart recording waveforms, determined by
262 plotting heart against pyloric waveform with comparison to the heart identity line. Mean
263 pyloric rhythm data (Fig. 5D) was unimodal (Hartigan's dip test, $p = 0.97$), ranging from
264 0.4 Hz to 1.4 Hz with a mean of 0.8 Hz (s.d. = 0.29).

265 Relationships between the heart and pyloric rhythms. We were interested to
266 determine if there was a relationship between the muscle movement of the heart and
267 pylorus in an individual animal. By plotting the time of muscle movement of the pylorus
268 within the phase of the heart rhythm, we determined that muscle movements of the two
269 rhythms were not phase locked. Nonetheless, in several cases, such as times of an
270 inhibitory bout of the heart, the pyloric rhythm slowed when the heart rhythm had
271 slowed or stopped. Figure 6A illustrates one such case where the pyloric rhythm was
272 reliably moving with a frequency of 1.3 Hz while the heart was beating at 1.5 Hz.
273 However, during a loss of heart beat, the pyloric rhythm slowed to 0.9 Hz. Throughout
274 the population with little exception, the frequency of the pyloric rhythm was lower than
275 that of the heart rhythm in the same animal (Fig. 6B).

276 To compare the frequencies of the heart and pyloric rhythm and determine if they
277 change in similar ways across time, we calculated the cross-correlation factors between
278 the frequencies, in 9 second bins (Fig. 6C). When comparing the rhythms at baseline

conditions over 30 minutes, we found that some animals had highly positive correlation coefficients, some had negative correlation coefficients, and still others had very small correlation coefficients. Overall, correlation coefficients ranged from -0.45 to 0.64, and had a mean of 0.28 (s.d. = 0.29).

Effects of temperature on heart and pyloric rhythms. We tested the effects of increasing temperature upon the muscle movements of nine animals. Temperature ramps caused increases and subsequent decreases in both the pyloric and heart muscle movement frequencies (Fig. 7). Figure 7A, B show raw data from a typical experiment showing heart and pyloric movements during a temperature ramp. In heart movement recordings, waveform amplitudes often decreased and frequency increased with increasing temperature until crashing of the heart occurred. In pyloric movement recordings, waveforms changed in overall shape, often exhibiting peaks followed by long plateaued waveforms (Fig. 7B). Similarly to heart activity, frequency of pyloric rhythm also increased with increasing environmental temperature.

Overall change in frequency (Fig. 7C) was calculated for both heart and pyloric rhythms. Changes in frequency were determined by subtracting the average frequency 30 minutes prior to temperature ramp initiation from the maximum frequency during temperature ramps. Change in frequency ranged from 0.7 Hz to 2.4 Hz for heart rhythms with a mean of 1.4 Hz (s.d. = 0.5), and 0.7 Hz to 1.4 Hz for pyloric rhythms with a mean of 1.1 (s.d. = 0.2). The change in frequency of the two rhythms was not significantly different ($p = 0.0589$, pairwise t-test). We also determined percent change in frequency (Fig. 7D), and found that these were not significantly different between the two groups ($p = 0.38$, pairwise t-test).

302 Q_{10}, a measurement of the rate of change of a biological process in response to a
303 change in temperature, was calculated for both the heart and pyloric rhythm for each
304 animal tested (Fig. 7E). Q_{10}s for heart rhythms, ranged from 1.2 to 3.9 with a mean of
305 2.2 (s.d. = 0.84). Pyloric rhythm Q_{10}s ranged from 0.9 to 5.9 with a mean of 2.2 (s.d. =
306 1.4). The Q_{10}s of the heart and pyloric rhythms were not significantly different ($p = 0.87$,
307 pairwise t-test).

308 Both rhythms crashed at distinct temperatures during experiments. Critical
309 temperatures were defined as the temperature at which cardiac or pyloric muscle
310 movement stability and regularity was lost for at least 15 seconds, with a subsequent
311 drop in contraction frequency to near baseline values. Critical temperatures of the heart
312 and pyloric muscle movements were collected for all nine animals (Fig. 7F). The pyloric
313 rhythm consistently crashed at lower temperatures than the heart rhythm in the same
314 animal. Mean heart critical temperature was 28.5°C (s.d. = 1.7) while mean pyloric
315 critical temperature was 21.6°C (s.d. = 3.4). The critical temperatures of the heart and
316 pyloric muscle movements were significantly different ($p = 0.00006$, pairwise t-test).

317 We calculated cross correlation coefficients between the two rhythms during
318 times of increasing and decreasing environmental temperature (Fig. 8A-B). Correlation
319 coefficients were high, indicating a high correlation between the frequency of the
320 rhythms when faced with changes in temperature. Temperature correlation coefficients
321 of the rhythms during temperature increases (Fig. 8A) were determined by calculating
322 the cross correlation from the initiation of the temperature ramp to the pyloric rhythm
323 critical temperature. Temperature correlation coefficients of the rhythms during
324 temperature decreases (Fig. 8B) were determined by calculating the cross correlation

from the stabilization of the pyloric rhythm following its critical temperature to the return to the original temperature. Correlation coefficients for the up ramp ranged from 0.41 to 0.95 with a mean of 0.69 (s.d. = .21) while correlation coefficients for the down ramp ranged from 0.27 to 0.99 with a mean of 0.86 (s.d. = 0.24).

330 **Discussion**

331 It is always interesting to determine the extent to which different body rhythms
332 are correlated in their activity. This is especially interesting with respect to the heart
333 and stomach rhythms in crustaceans because the stomatogastric ganglion is situated
334 just anterior to the heart in the dorsal artery, where it is directly perfused by
335 hemolymph containing hormones released into the circulatory system. To the best of
336 our knowledge, ours is the first study to record simultaneously the heart and stomach's
337 pyloric rhythms both under control conditions and in response to temperature changes.

338 <u>Baseline Functioning of the Heart Rhythm</u>

339 The crustacean heart is neurogenic, and therefore the activity of the cardiac
340 ganglion directly impacts the heart beat frequency. The CG must be able to produce
341 activity regularly and across a wide range of perturbations. It must be modifiable to
342 adjust to the animal's metabolic needs (Dickinson et al., 2016b; Dickinson et al., 2015b;
343 Robertson and Money, 2012) and reliable enough to ensure that the heart continues to
344 pump hemolymph at all times. Both frequency and contraction amplitude, two factors
345 which must be regulated to ensure the animals' success, are expressions of the activity of
346 the CG, specifically its interburst interval, rate, and firing patterns.

347 We captured heart activity using PPG recording techniques while limiting the
348 invasiveness and stress placed on the animal. PPG recordings of the heart musculature
349 were reliable over time, and show differences in frequency of heart beats within the
350 population (Fig. 2C). In the majority of experiments, an animal's baseline heart rate
351 over a 30-minute period was stable (Fig. 2B). During baseline recordings, animals were
352 not subjected to changes in temperature, light, salinity, stress or environmental factors

353 that could interact with the metabolic needs of the animal or the functions of the CG.
354 During this time, the firing of the CG, and therefore the activity of the heart, would not
355 be expected to appreciably change. Nonetheless, the heart rate of resting animals falls
356 into a multimodal distribution. These data suggest that heart activity, and therefore
357 activity of the CG, falls into states of higher or lower activity. These states may be a
358 consequence of variable metabolic needs of the animal during the time of recording,
359 such as digestion, movement, or excretion. The CG may therefore have mechanisms for
360 switching between high and low activity states through neuromodulation or extrinsic
361 neural input.

362 Alternatively, the high and low activity states may reflect circatidal (rhythms that
363 are governed by the tide) (Chabot and Watson, 2010a) or circadian rhythms the animal
364 experiences (Chabot and Watson, 2010b; De La Iglesia and Hsu Y., 2010). During 24-
365 hour long term experiments animals experienced significant shifts in heart rhythm
366 activity throughout the day (Fig. 3A). Shifts in heart rhythm may result from increased
367 metabolic processes occurring in the animal because of its natural circatidal rhythm, or
368 because of its circadian rhythm and patterned sleep periods during the day and night.
369 Circatidal and circadian rhythms are known to influence crustacean locomotion with
370 two independent daily bouts of activity (Chabot and Watson, 2010a; Chabot and
371 Watson, 2010b).

372 Several animals displayed heart inhibitory bouts, periods of bradycardia during
373 which the heart slowed or completely stopped for a minimum of 10 seconds. These
374 inhibitory bouts persisted throughout an entire experiment, and likely through long
375 periods in an animal's life. Within an animal, most inhibitory bouts had a constant

duration and inter-inhibitory bout frequency. Inhibitory bouts occur at low frequencies (Fig. 4D) and maintain these frequencies with little deviation across the recording session, indicating a regularity within the system controlling these events. Inhibitory bouts have previously been observed as linked to times of gill ventilation during reversal of pumping of the scaphognathite (McMahon, 1999). This is likely due to overflow of ocean water, and therefore oxygen, in the gills, causing the organism's heart to stop for a significant period. It is interesting, however, that this occurs in only about 25% of animals tested, and persists in animals at regular intervals. This implies a mechanism affecting both the heart and the gills that takes place at regular intervals and influences the overall levels of dissolved O_2 within the animal.

Baseline Function of the Pyloric Rhythm

The STG maintains its triphasic rhythm to control the movement of the muscles, and is robust to several global perturbations, including temperature (Soofi et al., 2014; Tang et al., 2010; Tang et al., 2012). Previous work has shown the importance of animal-to-animal variability in responses to such perturbations (Hamood et al., 2015; Hamood and Marder, 2014; Hamood and Marder, 2015). However, no one has previously compared the responses of both heart and pyloric rhythms to such perturbations.

In the experiments presented here, PPG sensors recording the muscle movement of the pylorus were placed on the carapace above the dorsal dilator muscle. While heart rhythm waveforms were relatively simple, with one maximum and minimum, pyloric rhythm waveforms could be grouped into categories of simple, dynamic, or complex (Fig. 5A-B). The complexity of the PPG waveform is likely due to the positioning of the muscles being recorded and their movements in relation to the PPG sensor. *In vivo,* the

399 pyloric rhythm frequency drifts more than that of the heart rhythm frequency over a 30-
400 minute period. Across the population of tested and analyzed animals, the pyloric rhythm
401 had a unimodal distribution, unlike that of the heart rhythm.

402 Comparison of the Heart and Pyloric Rhythms

403 While the generation and movement of the heart and pylorus have been
404 extensively studied separately in the past, here we examined the interactions between
405 these two central pattern generated rhythms. To determine how these two essential
406 rhythms may interact in a single animal, we compared the frequencies of the rhythms of
407 animals in controlled environments. The heart and pyloric rhythms (Fig. 5A-B) are not
408 phase coupled; instead the timing of the movements of the pylorus drift between the
409 phases of the heartbeat. From this, we can conclude that the two rhythms are not tightly
410 regulated by the same top down control, nor do they tightly control each other.

411 Across time, there are instances where changes in the heart rhythm are mirrored
412 by changes in the pyloric rhythm. In one instance of heart inhibitory bouting (Fig. 6A),
413 we see that prior to the inhibitory bout both the heart and pyloric rhythm are regular.
414 However, when a cardiac inhibitory bout occurs and the pyloric rhythm slows. This
415 change indicates that some mutual control exists between the cardiac and pyloric
416 rhythm to cause both to change at the same time.

417 To compare directly the heart and pyloric rhythm frequencies to find if they
418 change in frequency together over time, we calculated the cross-correlation coefficients
419 for the frequencies of 30-minute baseline recordings. We found a wide range of
420 correlation coefficients, indicating that the correlation between the rhythms was
421 different within each animal. Several of the animals tested, however, had a significantly

422 positive correlation coefficient, indicating that the two rhythms do change in similar
423 ways during baseline conditions.

424 The similarity in changes across time in these animals may be explained by many
425 factors. One may be that when the heart moves slower or halts completely (such as
426 during an inhibitory bout) less hemolymph and less oxygen is delivered to the pylorus
427 and therefore it moves more slowly due to its lack of oxygen. Oxygen has been shown to
428 affect the rhythm in similar ways as a neuromodulator would (Clemens et al., 2001).
429 Alternatively, the frequency of both rhythms may be affected by neuromodulators
430 released into the pericardial organ, acting in similar ways.

431 Temperature as a Perturbation to Expose the Mutual Changes in the Heart and Pyloric
432 Rhythms

433 Previous work on temperature has shown that both the heart and pyloric rhythms
434 increase in frequency with increases in temperature (Marder et al., 2015; Tang et al.,
435 2010; Worden et al., 2006). Both rhythms are also known to crash beyond critical
436 temperatures, at which point the muscles no longer move in comprehensive patterns
437 (Soofi et al., 2014; Tang et al., 2012). In intertidal worms, the heart critical temperature
438 likely corresponds with the onset of anaerobic metabolism, due to a mismatch between
439 the animal's oxygen needs and the supply being delivered to the heart (Zielinski and
440 Portner, 1996).

441 In the data presented here, it is clear that temperature affects both rhythms and
442 increases their overall frequency, with similar Q_{10}s (Fig. 7E). This indicates that
443 increases in temperature cause similar changes in the two frequencies, until a crash

point occurs. Interestingly, the heart is more robust to extreme temperature changes than the pyloric rhythm.

These measurements indicate that during times of global perturbation or stress upon the animal, the two rhythms respond in similar ways to maintain homeostasis within the animal (Fig. 7E, Fig. 8). Nonetheless, during times of low stress or perturbation, such as during controlled, baseline conditions, the rhythms are less likely to be coordinated and have lower correlation coefficients. It is likely that during times of extreme perturbation, such as during drastic increases in temperature, the animal's top down regulatory systems (possibly through release of neuromodulators into the hemolymph) begin to tightly control processes occurring within the animal, and the rhythms respond in a coordinated manner.

Acknowledgements

We thank Dr. Markus Frederich for an introduction into the use of PPG system, and Jessica Haley for assistance in early data analysis. Data in this paper were first presented for partial fulfilment of requirements for BS/MS program (DK) at Brandeis University.

Competing Interests

The authors declare no competing or financial interests.

Funding

This work was supported by National Institutes of Health R35 NS 097343 to E.M.

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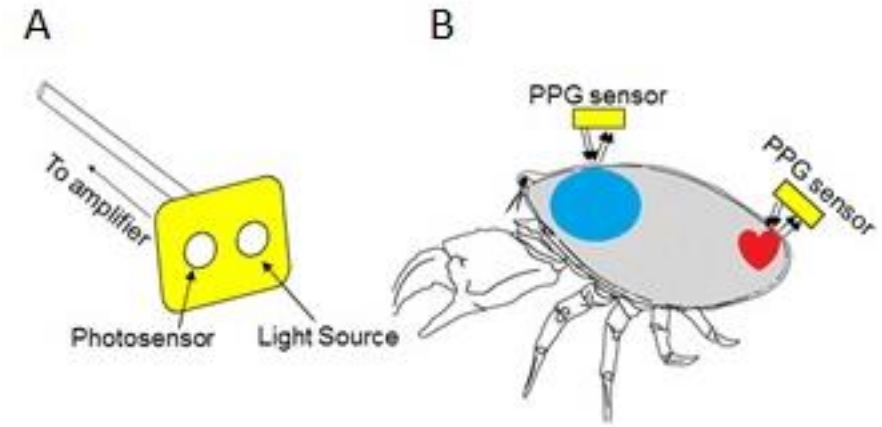

Figure 1. Use of photoplesmography (PPG) allows for continuous, noninvasive recordings of the heart and pyloric muscles. A. Drawing of PPG sensor system, indicating light source and photosensor on device. **B.** Drawing of the PPG sensor positioning for detection of heart (red) and pylorus (blue) movements when placed on the carapace of an animal.. Infrared light from the sensors are emitted and travel through an animal's carapace before being reflected from the muscle directly below the PPG sensor. Crab image modified from http://stg.rutgers.edu/Resources.html.

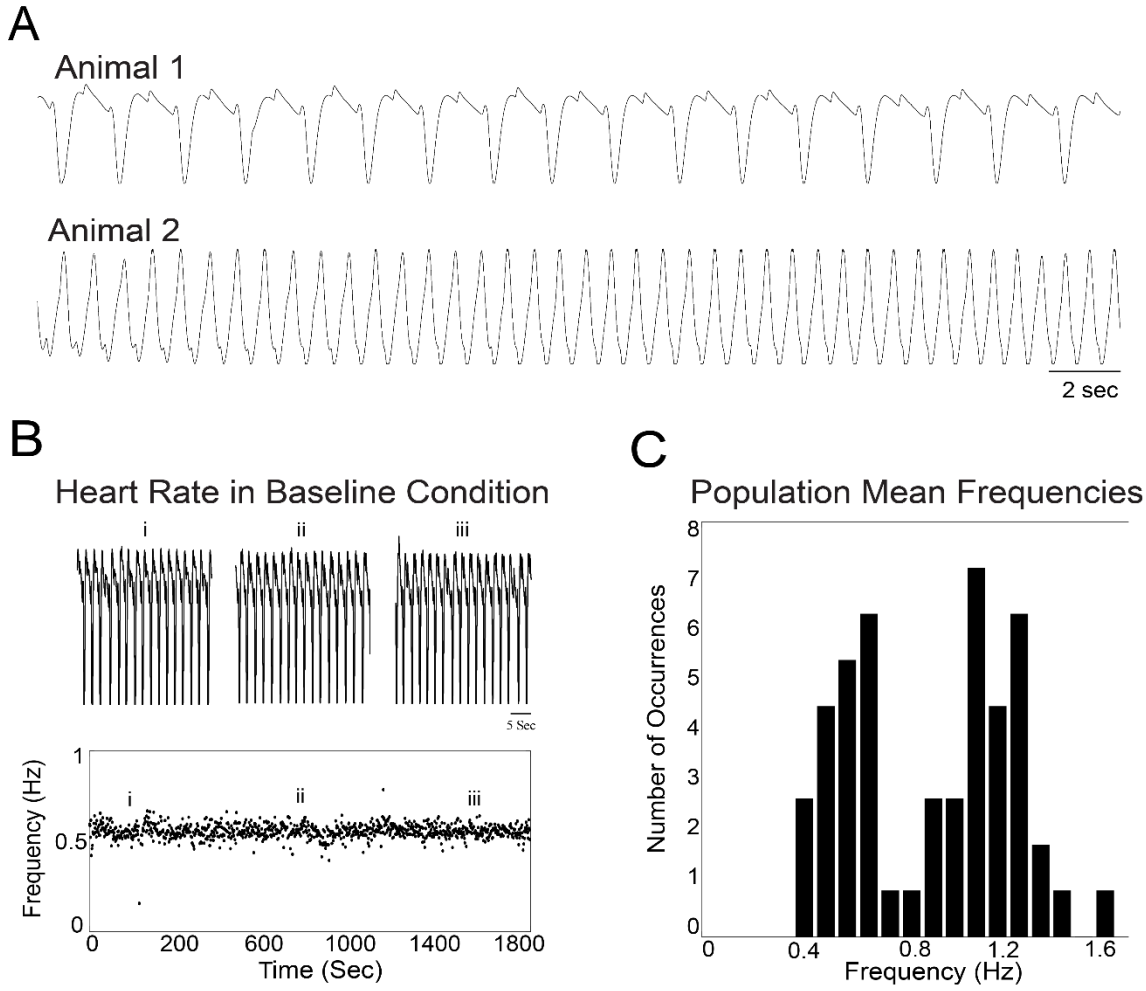

Figure 2. Heart activity is variable across population at baseline conditions. A. PPG recordings of heart movement of two animals in baseline conditions. 30-second long recordings of animal 1 and animal 2 show animals have different frequency rhythms at baseline conditions at 0.6 Hz and 1.4 Hz, respectively. **B.** Cardiac rhythm waveforms and instantaneous frequency of one animal. Three 30 second recordings from one animal over a 30-minute period demonstrates that PPG waveforms are relatively consistent in both frequency and amplitude across short periods of time. Below, instantaneous frequency across a 30-minute recording session indicates that the frequency of the cardiac rhythm is stable, with a mean of 0.51 Hz. Roman numerals mark where 30-second raw traces fall in 30-minute frequency data. **C.** 30-minute averaged frequencies of heart rhythm at baseline conditions of 52 animals are multimodal (Hartigan's dip test, $p = 0.024$). The distribution ranges from 0.4 Hz to 1.7 Hz, with a mean of 1 Hz.

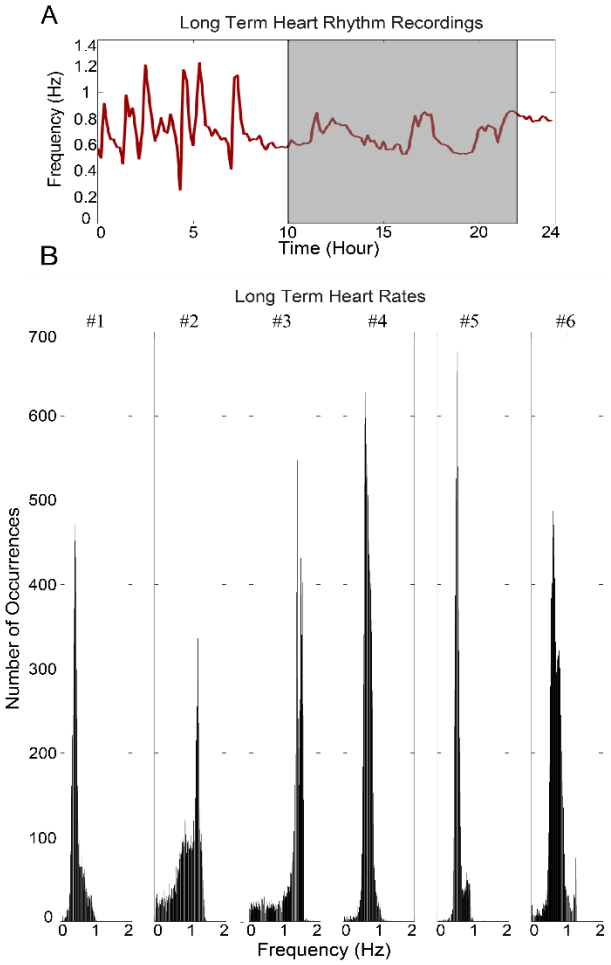

Figure 3. Long-term experiments display patterned changes in heart rate during a day. A. 24-hour recordings of animals held at baseline conditions indicate patterned increases and decreases in cardiac rhythm. Frequency was binned into 10-minute windows. A gray box indicates time that the animal's environment was dark. **B.** Histograms of 5-second binned frequencies of long term experiments. Each plot displays frequencies of an individual animal tested, for a total of 6 different animals.

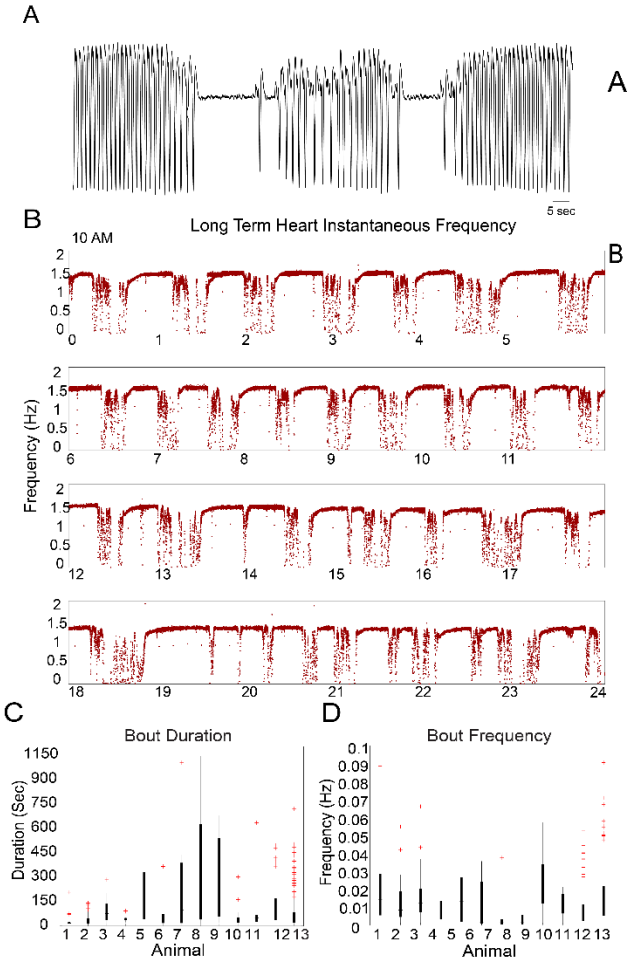

Figure 4. Inhibitory bouts persist through recording sessions. A. 150-second PPG recording of the heart rhythm shows inhibitory bouts, followed by recovery of both frequency and amplitude of heart rhythm. **B.** Instantaneous heart rhythm frequency over a 24-hour recording session shows inhibitory bouts occur at constant intervals. Each row includes 6 hours of recording time. Heart rhythm in between inhibitory bouts maintains a frequency at 1.5 Hz. **C.** Bout duration is highly variable both within and across animals. Bout duration was calculated from the beginning of slowing or loss of muscle movement to the time of regaining heart frequency and amplitude. Animals 12 and 13 were tested over 24-hour periods, while all others were over 30-minute to 1.5-hour recording periods. **D.** Inhibitory bout frequency is variable within and across animal. Frequency of inhibitory bouts is relatively low.

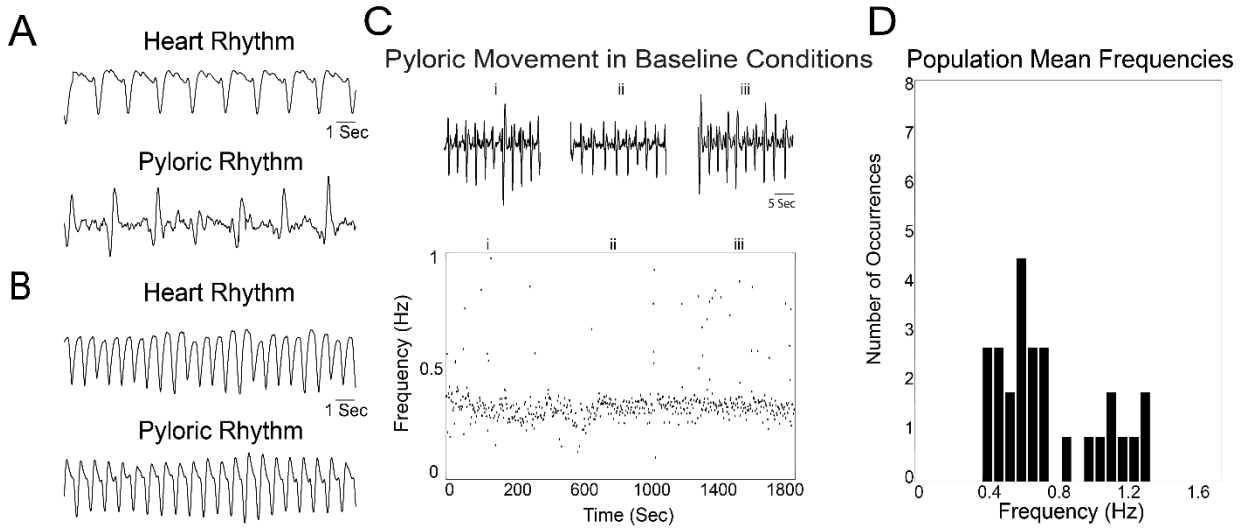

Figure 5. Pyloric rhythm recordings in animals are variable but not phase locked with their heart rhythms. A. Traces of the heart and pyloric rhythm in a single animal over 15-seconds at baseline conditions. The pyloric rhythm displays a complex waveform, with peaks followed by long plateaus. **B.** Traces of the heart and pyloric rhythm in a single animal over 30 seconds at baseline conditions. The peak times of the heart and pyloric rhythms drift past each other, indicating that they are not phase locked. **C.** Pyloric rhythm waveforms and instantaneous frequency of one animal. Three separate, 30-second long recordings from an individual animal over a 30-minute recording period demonstrates that PPG waveforms are relatively consistent in both frequency and amplitude across short periods of time. Roman numerals mark locations of 30-second raw traces in the experiment. Below, instantaneous frequency across a 30-minute recording session indicates that the frequency of the cardiac rhythm is stable in this animal, with a mean of 0.3 Hz. **D**. Histogram of the population of animals analyzed for pyloric rhythm activity. Pyloric rhythms of 29 animals were analyzed. Population distribution was unimodal (Hartigan's dip test, $p = 0.97$), with a range from 0.4 Hz to 1.4 Hz, and mean of 0.8 Hz.

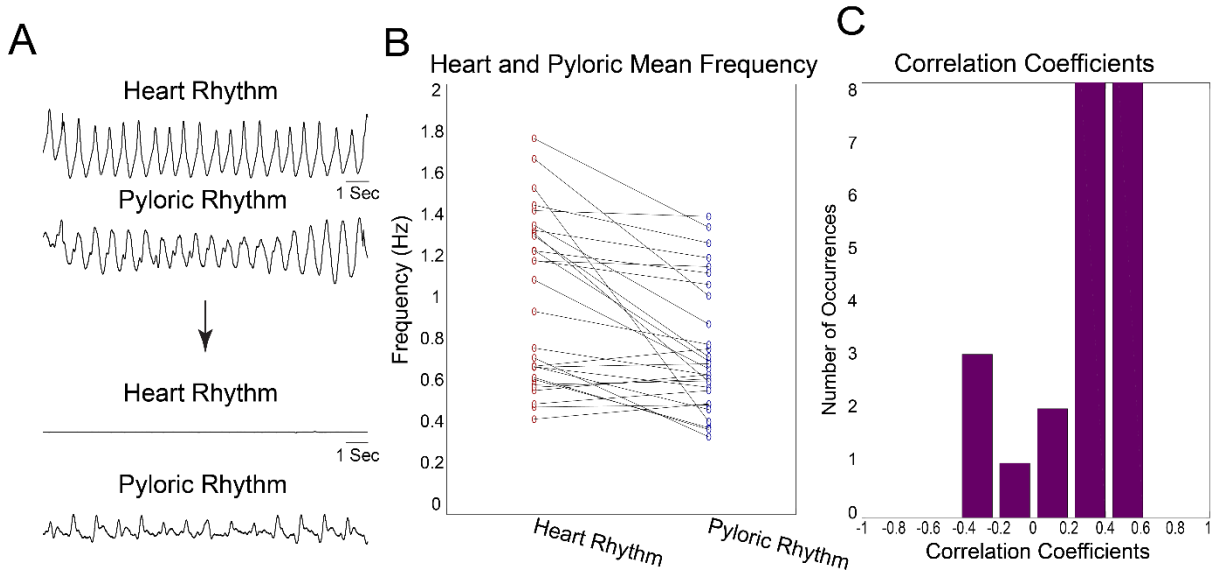

Figure 6. Heart rhythm activity has high correlation with pyloric rhythm activity in some animals, while low correlation in other animals. A. Raw PPG waveforms of heart and pyloric movements before and during a heart inhibitory bout. 15-second traces are shown of parallel heart and pyloric activity. Before inhibitory bout, both cardiac and pyloric rhythm have fast activity (frequencies of 1.4 Hz and 1.3 Hz, respectively). As the heart rhythm enters an inhibitory bout, pyloric rhythm frequency decreases to 0.9 Hz. **B.** Paired mean heart and pyloric rhythm frequencies. 29 animals were analyzed for both heart and pyloric activity over baseline conditions. Lines connect heart and pyloric mean frequencies of the same animal. **C.** Histogram of correlation coefficients using a cross-correlation between 30-minute baseline activity in 9 second bins of the cardiac and pyloric rhythms of a single animal. Correlation coefficients suggest that some animals have highly correlated heart and pyloric rhythms, while others do not. Still others have negative correlations between the heart and pyloric rhythm

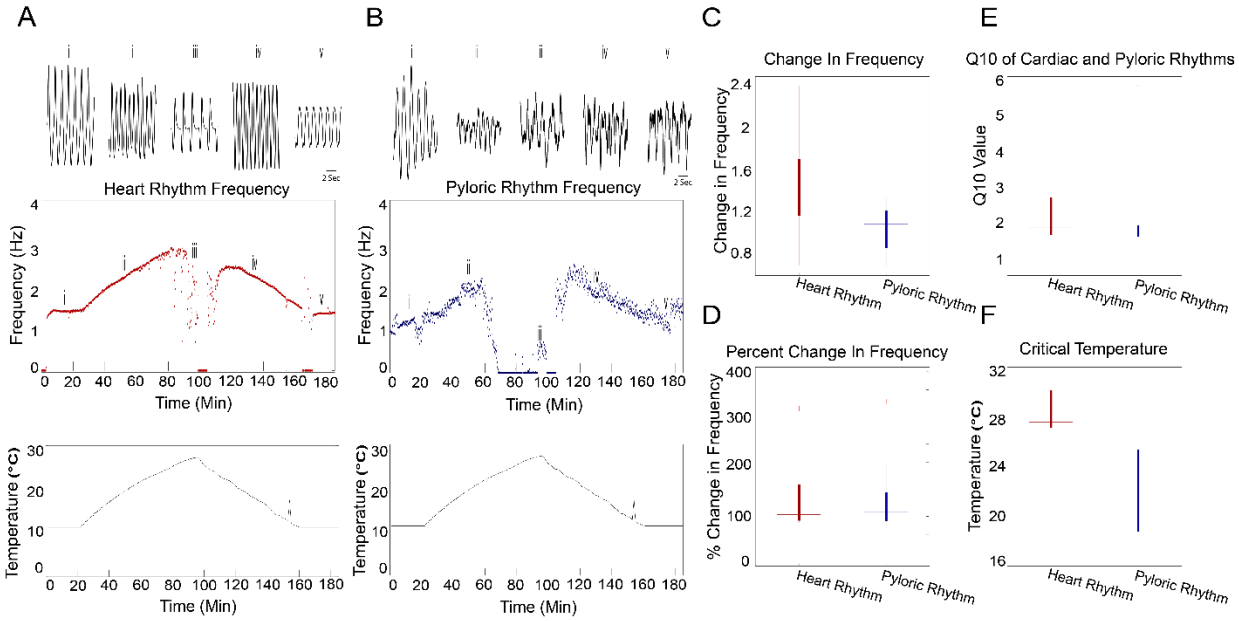

Figure 7. Temperature perturbations affect the heart and pyloric rhythms in similar ways. A. Increasing temperature affects the frequency of the heart rhythm. Raw traces, 10 seconds in length, indicate increases and decreases in heart rhythm as experiment takes place. Location of raw waveforms in experiments are indicated by Roman numeral markings in frequency graph. Instantaneous frequency of heart rhythm was binned into 10 second bins, starting at 25 minutes before initiation of temperature ramp and 25 minutes following end of temperature ramp. Temperature in tank during experiment in 1-minute bins is plotted below. Heart rhythm frequency increases with increase in environmental temperature until heart critical temperature and following recovery, decreases with decreasing temperature. **B.** Increasing temperature affects the frequency of the pyloric rhythm. Setup identical as A, displaying data for pyloric rhythm recordings. Pyloric rhythm increases with increasing temperature until its critical temperature. **C.** Change in frequency was significantly different between heart and pyloric rhythm. Change in frequency was calculated by subtracting the maximum frequency obtained from temperature ramp by average frequency 30 minutes before the initiation of a temperature ramp. Mean change in frequency for heart rhythm was 1.4 Hz while mean change in frequency for pyloric rhythm was 1.1 Hz. **D.** Percent change in frequency was not significantly different between heart and pyloric rhythm activity. **E.** Q_{10} was not significantly different between the heart and pyloric rhythms. Mean Q_{10} of the heart and pyloric rhythms were 2.2. **F.** Critical temperatures of the heart and pyloric rhythms were significantly different. Critical temperatures of the pyloric rhythm (mean = 21.6°C, s.d. = 3.4) and heart rhythm (mean = 28.5°, s.d. = 1.7).

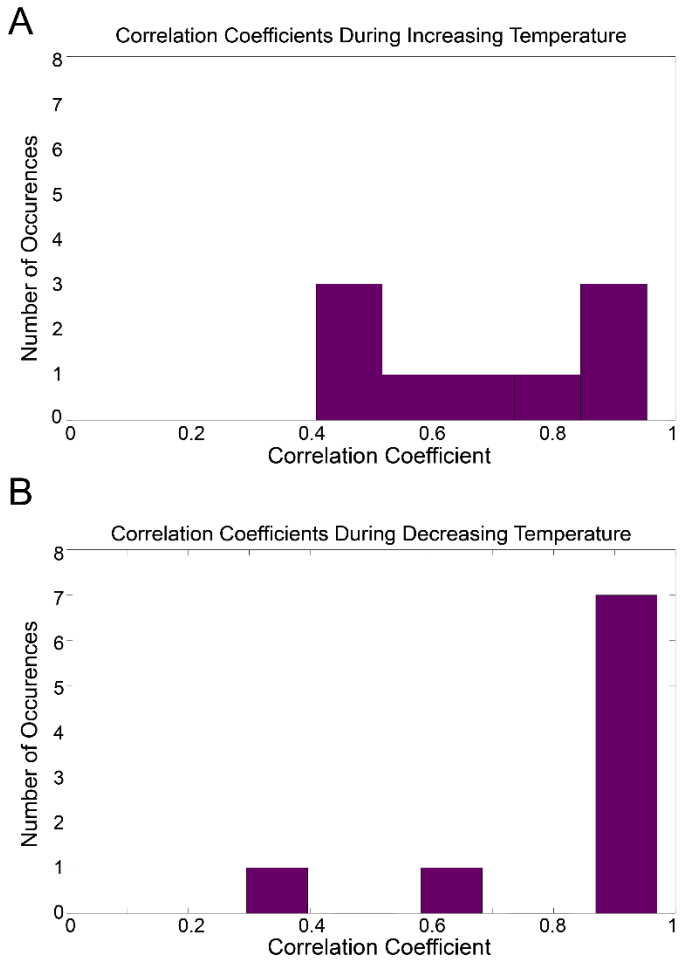

Figure 8. Correlation coefficients during temperature ramp experiments. A. Correlation coefficients of the heart and pyloric rhythms while increasing environmental temperature showed that the rhythms were highly correlated. Correlation coefficients were determined by using a cross-correlation between the two rhythms frequencies in 9 second bins, until the critical temperature point of the pyloric rhythm. Correlation coefficients are higher than those between heart and pyloric rhythms at baseline conditions, with a mean of 0.69. **B.** Correlation coefficients of the heart and pyloric rhythms while decreasing environmental temperature following recovery of the pyloric rhythm show that the two rhythms are highly correlated. Correlation coefficients were determined by using a cross-correlation between the two rhythms frequencies in 9 second bins. Correlation coefficients in this group have a mean of 0.86.