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Dietary diversity correlates with the neuromodulatory capacity of the stomatogastric nervous system in three species of *majoid* crabs

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An honors project for the Program of Neuroscience

By Elise Martin

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Table of Contents

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ABSTRACT

This project sought to answer the following question: what is the relationship between the extent of neuromodulation in a nervous system, and the behavioral demands on that system? A well-characterized CPG neuronal circuit in decapod crustaceans, the stomatogastric nervous system (STNS), was used as a model circuit to answer this question. The stomatogastric ganglion (STG) in the STNS is responsible for muscular contractions in the stomach that aid in digestion. It has been shown that the neural networks in the STG are subject to neuromodulation. One feature of neuromodulation is that it enables circuit flexibility, which confers upon a system the ability to produce variable outputs in response to specific physiological demands. It was hypothesized that opportunistic feeders require more extensively modulated digestive systems compared to exclusive feeders, because opportunistic feeders require a greater variety of digestive outputs to digest their varied diets. In this study, *Chionoecetes opilio* and *Libinia emarginata*, the opportunistic feeders, showed greater neuromodulatory capacity of the STNS than *Pugettia producta*, the exclusive feeder. The hypothesis that neuromodulatory capacity of the STNS correlates with dietary diversity was supported. The results detailed in this study lend credence to the idea that evolutionary basis for neuromodulatory capacity of a system is related to the behavioral demands on that system.

INTRODUCTION

Neuromodulation and n*euromodulatory capacity*

Neuromodulators enable flexibility of a neural circuit by altering the firing activity and therefore the patterning of the circuit. The most abundant class of neuromodulators in nervous systems are neuropeptides. Neuropeptides are short alpha amino acid chains linked by amide bonds. They are released both locally via synapse or distally via release from neurosecretory structures and subsequently travel to their neural target (Christie et al., 2010). Neuromodulatory capacity describes the extent to which a system can be modulated. Even closely related species may have different neuromodulatory capacities. Dickinson et al. (2008) have shown that that the STNS of the opportunistically feeding Jonah crab *Cancer borealis* showed greater neuromodulatory capacity, represented by the number of neuromodulators that the species responded to, than the exclusively feeding kelp crab *P. producta*. Based on these results, Dickinson et al. hypothesized that the neuromodulatory capacity of a system depends on the flexibility of output that is required of that system (2008). However, *C. borealis* and *P. producta* are distantly phylogenetically related, so any differences in neuromodulatory capacity between the two species could potentially be attributed to phylogenetic distance instead of functional correlation. This project examined the neuromodulatory capacity of three closely-related *Majoid* crabs (*P. producta, C. opilio,* and *L. emarginata*) in order to answer the question: is the extent to which a nervous system can be modulated related to the diversity of neural output required of that system?

Central pattern generators

To test our hypothesis, a neural circuit that is known to be modulated was needed. Suitable neural circuits for study are central pattern generators (CPGs), neural networks that

generate rhythmic motor outputs such as walking, breathing, and chewing. CPGs continue to fire rhythmically and generate muscle contractions if still innervating muscle tissue even in the absence of patterned input from the rest of the nervous system, making them good model systems. However, a particular CPG's neural activity is not fixed. CPG motor output can be flexibly modulated by molecules called neuromodulators, which alter neuronal activity and thus produce variable motor outputs (Katz & Warrick, 1990; Hultborn & Kiehn, 1992; Marder & Thirumalai, 2002). For example, neuromodulation may cause the heart to speed up or slow down, and running to progress to walking, or vice versa (Nusbaum & Beenhakker, 2002). Two well-characterized CPG model systems found in decapod crustaceans are the cardiac ganglion (CG) and the stomatogastric nervous system (STNS). The CG drives the heartbeat, while the stomatogastric ganglion (STG) in the stomatogastric nervous system (STNS) is responsible for muscular contractions in the stomach that move food through the foregut (Marder & Bucher, 2007). These particular CPGs continue to produce fictive motor patterns when isolated and removed from the organism, and these fictive motor patterns closely resemble those muscular movements they evoke *in vivo*, with the benefit of being more easily studied once removed from the animal (Marder & Bucher, 2001).

The CG as a model CPG

In crustaceans, the heart is neurogenic (Hartline, 1967), meaning that rhythmic heart contractions are driven by a neural circuit called the cardiac ganglion (CG), as opposed to the myogenic heartbeat observed in vertebrates (Cooke, 2002). The CG, which is located inside the heart of decapod crustaceans, is composed of a network of nine neurons that fire in unison to produce heart contractions. There are four small cells (premotor neurons) and five large cells

(motor neurons). The small cells are referred to as the pacemaker neurons because they generate the excitatory activity necessary to coordinate the large cells to fire synchronously. The synchronous firing of the premotor neurons and motor neurons causes the heart muscles to contract rhythmically (Cooke, 2002).

The activity of the CG is modulated via multiple pathways, including nitric oxide-mediated feedback from the cardiac muscle, stretch feedback from the cardiac muscle, and by signaling ligands such as intrinsic and extrinsic amines, amino acids, and peptides (Goy, 2005; Cooke, 1988; Sakurai & Wilkins, 2003; Christie et al., 2010, Cruz-Bermudez & Marder, 2007). These neuromodulators are released from neurons in the CG (intrinsic modulation), originate in the neuroendocrine organs outside of the heart and circulate through the heart (extrinsic neuromodulation) and are released by neurons innervating the CG (another form of extrinsic modulation) (Christie et al., 1995; Christie, 2011). Neuromodulators act on the cardiac muscle and neuromuscular junctions as well as the CG itself (Fort et al., 2007, Stevens et al., 2007). For this reason, whole heart recordings are favored over isolated nerve recordings to capture any neuromodulatory effects on the heart regardless of the site of action. The whole heart is easily extracted from the animal and will continue to beat for many hours following extraction, making it an ideal system for recording the effects of neuromodulator application over time.

The STNS as a model CPG

In *C. borealis*, the STNS is composed of four separate ganglia: the bilateral commissural ganglia (CoGs), the oesophageal ganglion (OG), and the stomatogastric ganglion (STG). The STG is composed of approximately 25-26 neurons, depending on the species. The STG contains motor neurons and interneurons that generate two motor rhythms of interest: the pyloric rhythm

and the gastric mill rhythm (Marder & Bucher, 2007). The pyloric rhythm is continuously active and consists of three phases: first there is bursting of the PD neuron driven by the intrinsically bursting AB interneuron. This is followed by bursting of the LP neuron (often accompanied by firing of the IC neuron), and finally bursting of the PY neurons (often accompanied by firing of the VD neuron). The AB neuron is electrically coupled with PD (Marder & Bucher, 2007). The electrical coupling of AB with PD allows AB to induce PD to fire in bursts. At the same time, AB and PD inhibit LP and PY. LP recovers from inhibition and fires after PD, inhibiting PY; then PY finally recovers and bursts while inhibiting LP and PD (Marder & Bucher, 2007). The sequence then repeats itself. Because there are only four types of neurons that play a fundamental role in the generation of the pyloric rhythm and they project axons through the same nerve, extracellular recordings can be placed such that they are able to record the activity of all four types of neurons, with the addition of IC and VD. Despite their evolutionary distance, the structure and function of the STG in *C. borealis* and the American lobster *H. americanus* are very similar. Therefore, it is expected that the crabs from the more closely-related *Majoid* family will have similar ganglia to *C. borealis*. Additionally, at the outset of this experiment, it was already known that the motor pattern exhibited by *P. producta* closely resembled that of *C. borealis*, supporting this assumption (Dickinson et al., 2008).

The activity of the STG is substantially modulated (Marder & Thirumalai, 2002; Marder & Bucher, 2007). It is a target for various amines, amino acids, and neuropeptides, many of which are synthesized in the pericardial organ, released into the hemolymph, and subsequently travel to the STG (Christie et al., 1995; Christie, 2011). The STG is also modulated by descending modulatory neurons that evoke a variety of motor patterns in the STG depending on the neuromodulators that are released and the target neurons that they act upon. Regardless of

where they originate, neuromodulators enable flexibility of motor output in the STNS (Marder & Bucher, 2007). Every neuron and every synapse within the STG is modulated, underscoring the importance of neuromodulation to the effective function of the STG. Changes in the firing activity of neurons within the STG can be observed by recording extracellularly nerves in the STNS and quantifying changes in the recording.

Neuromodulation in crustaceans

Crustaceans have two neuroendocrine organs where most neuropeptides are synthesized: the X-organ-sinus gland in the eyestalks, and the pericardial organs (PO) in the cephalothorax. Neuropeptides synthesized in the neuroendocrine organs are released into the hemolymph and then are circulated through the circulatory system (Christie et al., 1995; Christie, 2011). Both the STNS and the CG in the *Cancer borealis* crab are known to be highly modulated. Mass spectrometry identified 42 neuropeptides present in the STG of the Jonah crab, *Cancer borealis,* that were not state-dependent—in other words, even when the STG was removed from its descending neuromodulatory input neurons, 42 neuromodulators were still present in STG tissue (DeLaney et al., 2021). In the STNS, neuromodulators alter the triphasic firing pattern of the STG, changing phase relationships, bursting duration, frequency of spiking, and sometimes activating the gastric mill pattern, among other parameters. In the heart, neuromodulators act on the CG and the muscles/neuromuscular junction activated by the CG, changing heartbeat strength, heartbeat frequency, and heartbeat duty cycle, among other parameters (Cruz-Bermudez & Marder, 2007; Stevens et al., 2009).

An evolutionary hypothesis for neuromodulatory capacity

It was hypothesized that neuromodulatory capacity evolves in relation to the extent of behavioral diversity required of a system. This hypothesis was first posited by Dickinson et al. (2008). In this study, the STNS of an opportunistic feeder, *C. borealis,* was shown to respond to more neuromodulators than the STNS of *P. producta,* a limited feeder. In other words, the STNS of *C. borealis* had greater neuromodulatory capacity than *P. producta.* In the same study, Dickinson et al. showed that neuromodulators that do not show any functional effect on the STNS in *P. producta* are still present in the STNS and the amino acid sequence is highly conserved between decapod species (Dickinson et al., 2018). Therefore, it seems plausible that at one point in evolutionary history, an opportunistically-feeding ancestor of *P. producta* responded to the neuromodulators present in its system and had a higher neuromodulatory capacity due to greater dietary demands, but over time, as it limited its diet to kelp, it evolved to have a lower neuromodulatory capacity even though "vestigial" neuromodulators are still present in its current form. One possible explanation for their presence is that they are modulating systems other than the STNS, which presumably still require the same degree of neuromodulatory flexibility as this hypothetical opportunistically-feeding ancestor. *P. producta* is a member of the *majoid* family of crabs, but most of its *majoid* relatives are opportunistic feeders. Comparing the neuromodulatory capacity of *P. producta* to its opportunistic relatives sheds more light on the evolutionary basis for neuromodulatory capacity. Because neuromodulatory capacity appears to facilitate the functional flexibility of a system, it is theorized that neuromodulatory capacity evolves alongside the variety in neural output required of the system.

I assessed the neuromodulatory capacity of crabs from three species of the *majoid* family, all of which are closely related: *P. producta* (the kelp crab)*, L. emarginata* (the spider crab)*,* and *C. opilio* (the snow crab) (Figure 1)*. L. emarginata* and *C. opilio* are opportunistic feeders, while

P. producta, as previously mentioned, is a specialized feeder, eating only kelp. I hypothesized that dietary diversity may require different motor patterns for digestion and thus the STNS of varied feeders evolved to have higher neuromodulatory capacity, while the STNS of exclusive feeders does not require the same degree of flexibility and thus evolved to have lower neuromodulatory capacity. In terms of experimental data, I would expect *P. producta*, the exclusive feeder, to respond to fewer neuromodulators than *L. emarginata* or *C. opilio*, the opportunistic feeders. I applied neuromodulators that have been well-characterized in *C. borealis* and established to be highly conserved within the *majoid* family to the STNS of each species and recorded the responses extracellularly. To determine whether the neuromodulatory capacity of a species is system-specific or organism-wide, the responses of the whole heart to the same neuromodulators that were used in the STNS were assessed. Because dietary demands have very little, if any, effect on the activity of the heart, it was hypothesized that there would be no correlation between diet and the neuromodulatory capacity of the heart.

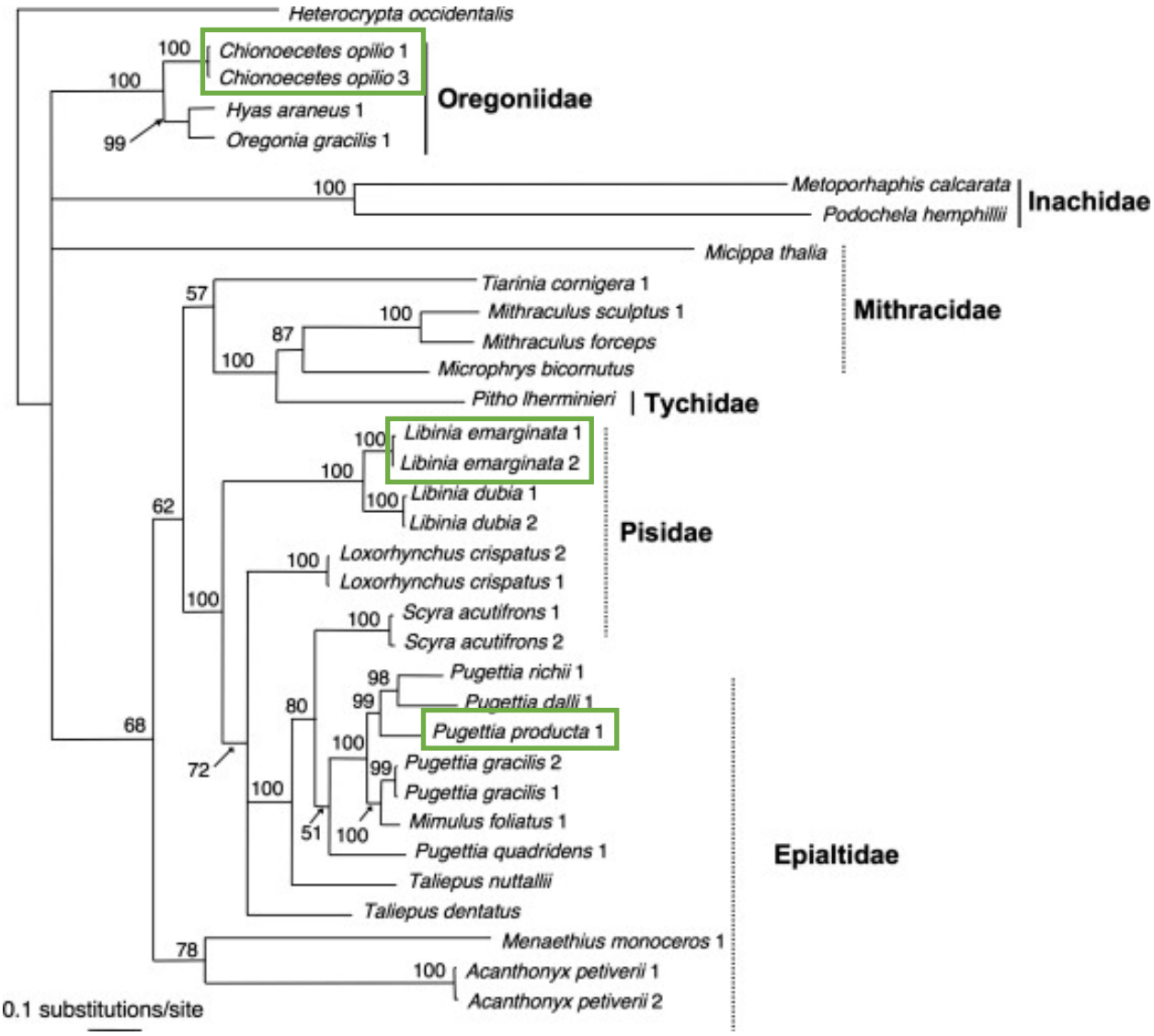


Figure 1. Phylogenetic tree of the Majoidea superfamily generated using genetic analysis of three gene loci. *P. producta* and *L. emarginata* are more closely related to each other than *C. opilio* is to either, but all three species are phylogenetically similar and share a recent common ancestor. Adapted from Hultgren & Stachowicz, 2008.

METHODS

Specimens

P. producta specimens were collected by hand from kelp beds in the San Juan archipelago in Washington State and shipped to Maine by the Friday Harbor Laboratory at the University of Washington. *C. opilio* specimens were collected from the Gulf of St. Lawrence by commercial crabbing operations in Canada and were purchased at the Fishermen's Market International Inc. in Halifax, Canada, shortly after they were caught. *L. emarginata* specimens were purchased from Gulf Specimen Marine Laboratory in Panacca, Florida. Specimens were housed in natural seawater aquaria and kept at 7-10°C, 16-21°C, and 10-15°C for *C. opilio*, *L. emarginata*, and *P. producta*, respectively.

Dissection

Specimens were removed from tanks and immediately anesthetized by packing into ice for approximately 30 minutes. After 30 minutes had elapsed, the rostral dorsal carapace was removed, and the stomach was extracted. A vertical cut down the ventral side of the esophagus and through the stomach to the pyloris was made, exposing the interior of the stomach. The stomach was placed dorsal-side-up in a 180 Sylgard-lined dish filled with *C. borealis* chilled 10°C saline (mmol/L l–1:NaCl, 440.0; KCl, 11.0; CaCl2, 13.0; MgCl2, 26.0; Trizma base, 12.0; maleic acid, 1.22; pH 7.4–7.5), pinned out, and chilled throughout the dissection.

Following the gross dissection of the stomach, the heart was extracted with the dorsal carapace still attached. The heart was placed ventral-side-up in a 180 Sylgard-lined dish filled with *C. borealis* physiological saline and pinned to the dish. For *L. emarginata*, a drill was necessary to create holes for the pins due to the rigidity of the shell.

The commissural ganglia (CoG), oesophageal ganglion (OG), and stomatogastric ganglion (STG), as well as the connecting and motor neurons of the STNS were dissected from the foregut of the stomach, taking care to ensure the STNS was fully intact and centralized. The STNS was then pinned out in a Sylgard-lined dish.

Drugs and dilutions

Crustacean cardioactive peptide (CCAP; PFCNAFTGCamide), crustacean tachykinin neuropeptide (CabTRP; APSGFLGMRamide), proctolin (RYLPT), red pigment concentrating hormone (RPCH; pELNFSPGWamide), myosuppressin (pQDLDHVFLRFamide), calcitonin-like diuretic hormone (CLDH, also known as DH31; GLDLGLGRGFSGSQAAKHLMGLAAANFAGGPamide), HIGSLYRamide, NRNFLRFamide, G-SIFamide (GYRKPPFNG-SIFamide), and AKH/corazonin-related peptide (ACP; pQVTFSRDWNAamide) were ordered from and synthesized by GenScript. Dopamine and oxotremorine were purchased from Sigma Aldrich. All neuromodulators except for RPCH, G-SIFamide, and dopamine were dissolved in deionized H_2O and diluted to 10^{-3} M. RPCH and G-SIFamide were dissolved in 15% DMSO and diluted to 10^{-3} M. Dopamine was stored in powdered form and diluted to 10^{-3} M in deionized H_2O immediately before use due to its light-sensitive properties. All drugs except dopamine were stored at -20°C. Dopamine was stored at room temperature. Modulator stock solutions were dissolved in room temperature saline and diluted to 10^{-7} M for application to the heart, and dissolved in cold saline and diluted to 10^{-6} M for application to the STNS.

Extracellular STNS recordings

The STG was desheathed (neuronal sheath removed) to allow the neuromodulator solution to reach the somata. Vaseline wells were built around the lateral ventricular nerves (*lvn*), median ventricular nerve (*mvn*), and pyloric dilator nerve (*pdn*) to isolate electrical currents caused by neural activity from the surrounding saline bath. Two-pronged stainless steel electrodes were used and standard extracellular recording protocol was followed. Activity was amplified with a Model 1700 Systems Differential AC Amplifier (Sequim, WA, USA). A CED Micro Board 1401 Converter (Cambridge Electrical Design, Cambridge, UK) was used to digitize data and Spike2 v7/v8 recording software was used to record the traces.
C. borealis physiological saline was temperature-regulated with a Peltier temperature regulator (Warner Instrument, Hamden, CT) at a temperature of 9-10°C for *C. opilio* and *P. producta*, and 10-12°C for *L. emarginata* and superfused into the dish for 40 minutes to allow the neurons to stabilize. After the stabilization period ended, neuromodulator trials began. Each neuromodulator had a two-minute control period during which saline was superfused into the STNS. Following the control period, neuromodulator solution was applied for ten minutes. Then a saline wash was applied for 30 minutes to wash out the neuromodulator solution and return the STNS to its baseline activity. Six neuromodulators were applied to each preparation.

Data analysis of the STNS

A custom Spike2 script modified by Dan Powell from scripts originally written by Dirk Bucher (NJIT/Rutgers) was used to generate a dataset quantifying a variety of burst metrics from extracellular nerve recordings. Duty cycle of the pyloric rhythm and cycle period, burst duration, and spike frequency were assessed for PD, PY, and LP neurons. Data from the first two minutes of recording during the control period was compared to data from the two minutes of greatest

effect from the neuromodulator application period. These parameters were assessed for the first two minutes of the control and the two minutes of greatest effect during the modulation period. The data points from each parameter were averaged. A paired two-tailed t-test t was used on the averaged value of each parameter ($p < 0.05$) to determine whether there was a significant difference between the control period and the neuromodulator application period. However, the percent of control was graphed in figures to facilitate comparison between species.

Whole-heart preparation recordings

For *C. opilio,* the sternal posterior artery of the whole heart was cannulated with polyethylene tubing. For *L. emarginata* and *P. producta*, a smaller bent metal tube was used to cannulate the heart owing to the species' small size and the positioning of the sternal posterior artery. Saline was continuously perfused into the heart. Saline temperature was regulated with a Peltier temperature regulator (Warner Instrument, Hamden, CT) at a temperature of 9-10°C for *C. opilio* and *P. producta*, and 10-12°C for *L. emarginata*. Continuous rate and flow of saline was maintained with a Rabbit peristaltic pump (Gilson, Middleton, WI). Occasionally cannulation of the sternal posterior artery was unfeasible due to damage to the sternal posterior artery; in these cases, an incision was made in the lumen of the heart and the cannula was inserted through the incision. The heart was monitored to make sure that no significant muscular injury had occurred.

The force of heart contraction was measured with a FTO3 force transducer (Grass Natus Technologies, CA) and ETH-250 Bridge/Bio amplifier, filtered with a model 410 Brownlee Precision Instrument Amplifier, digitized with CED Micro Board 1401 Converter (Cambridge Electrical Design, Cambridge, UK), and recorded with Spike2 v7/v8 software. 6-0 surgical silk was tied with a box knot around the five anterior arteries of the heart and attached on the other end to the force transducer at a 45-degree angle relative to the surface the heart was placed on.

The string was made to be sufficiently taut for the heart at rest to exert about 0.2 grams of force for *L. emarginata* and *P. producta*, and 2.5 force grams for *C. opilio* (which has a larger heart, and thus exerts more force during the heartbeat). After the heart was cannulated and attached to the force transducer, it was perfused with saline for one-hour minutes to allow the contractions to stabilize.

After the stabilization period, neuromodulators were sequentially applied to the heart. Neuromodulator solution was applied for ten minutes. Then a saline wash was applied for 50 minutes to wash out the neuromodulator solution and return the heart to its baseline activity.

Data analysis of the whole heart

A custom Spike2 script modified by Dan Powell and myself was used to generate a dataset quantifying the amplitude and frequency of the force of the heart. The dataset was then analyzed in MatLab to produce eight different parameters describing the activity of the heart: cycle period, width at ½ max (Figure 2A), duty cycle (beat duration/cycle period), beat duration (Figure 2B), peak force (force exerted by the heart at the apex of its contraction), minimum force (minimum force exerted by the heart between beats; a measure of baseline), area under the curve, and time to peak (from the beginning of the contraction to its apex). These parameters were assessed for the first two minutes of the control and the two minutes of greatest effect during the modulation period and the data points. The data points from each parameter were averaged. A paired two-tailed t-test t was used on the averaged value of each parameter ($p < 0.05$) to determine whether there was a significant difference between the control period and the neuromodulator application period. However, the percent of control was graphed in figures to facilitate comparison between species.

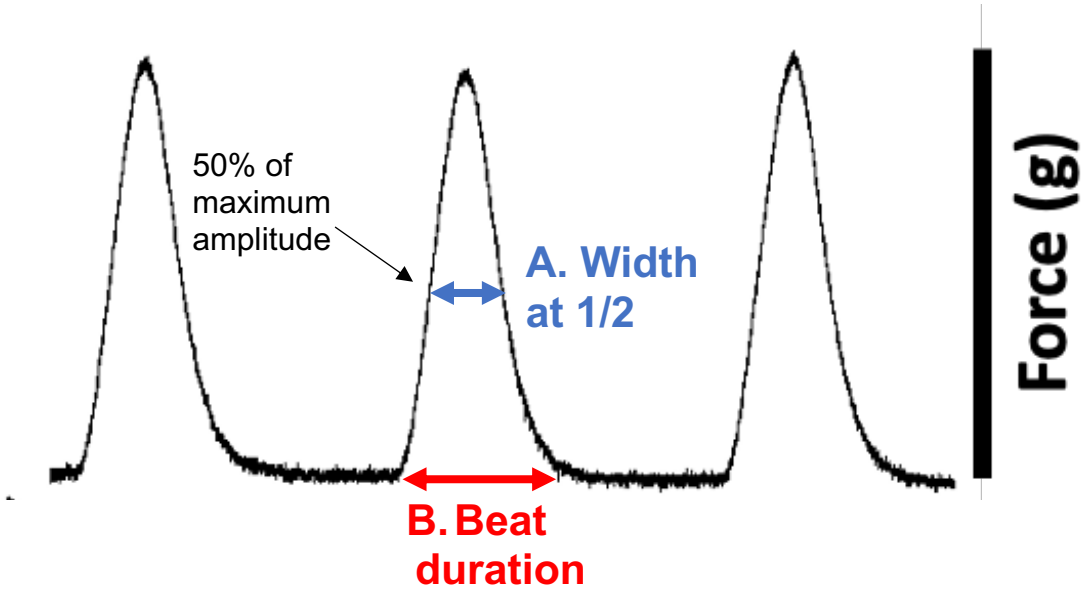


Figure 2. Beat duration and width at ½ max measure are two different measurements of the length of time it takes for the heart to contract and relax. Beat duration measures the time elapsed from the start of the contraction to the end of relaxation, whereas width at ½ max measures the time elapsed from 50% of maximum amplitude during the contraction to 50% of maximum amplitude during the relaxation.

RESULTS

STNS

Species sensitivity

My data were pooled with *C. opilio* and *L. emarginata* data collected during previous years by Jacob Kazmi and Alexandra Miller (Kazmi, 2020; Miller; 2018). *C. opilio* responded to 11/12 neuromodulators, *L. emarginata* responded to 11/12 neuromodulators, and *P. producta* responded to 8/12 neuromodulators (Table 1). The opportunistic feeders *C. opilio* and *L. emarginata* were sensitive to more neuromodulators than the exclusive feeder, *P. producta.* The neuromodulator that *C. opilio* did not respond to was proctolin (Table 1). The only neuromodulator that *L. emarginata* did not respond to was CabTRP (Table 1). The neuromodulators that *P. producta* did not respond to were CabTRP, CCAP, RPCH, and ACP (Table 1). Note that the *P. producta* STNS data for CabTRP, CCAP, dopamine, myosuppressin, oxtremorine, proctolin, and RPCH was previously reported on in Dickinson et al. (2008) so those data are not analyzed here. Additionally, the STNS data for ACP, CLDH, NRNFLRFamide, G-SIFamide, and HIGSLYRamide have not been analyzed for significant difference yet. For the present purpose, modulators that elicited changes in the rhythm that were visually apparent were considered to have had modulatory effects (Figure 10, Figure 11, Figure 12). Overall, the neuromodulatory capacity of the STNS correlated with a species' diversity of diet.

CabTRP

CabTRP caused a significant change in the pyloric pattern in *C. opilio* and *L. emarginata*. It increased the PD neuron duty cycle of *L. emarginata* (Figure 3B). It also decreased the PD neuron spike frequency in *C. opilio* (Figure 3C). However, it appeared not to affect the PY or LP neurons of either species.

CCAP

CCAP caused a significant change in the pyloric pattern in *C. opilio* and *L. emarginata.* In *C. opilio*, it increased the LP neuron burst duration (Figure 4G), but did not affect PY or PD. In *L. emarginata,* it decreased the PD neuron spike frequency (Figure 4E) but did not affect LP or PY.

Dopamine

Dopamine caused a significant change in the pyloric pattern in *C. opilio* and *L. emarginata.* In *C. opilio,* it tended to increase PY neuron spike frequency (Figure 5G) and increase LP neuron burst duration (Figure 5G), but it did not affect the PD neuron. In *L. emarginata*, it tended to increase PD neuron burst duration, PD duty cycle, and PY neuron spike frequency (Figure 5A, 5B, 5F), but did not affect the LP neuron.

Myosuppressin

Myosuppressin caused a significant change in the pyloric pattern of *C. opilio* and *L. emarginata*. It caused a significant increase in the cycle period of the *C. opilio* pyloric rhythm (Figure 6J). It also caused a significant decrease of PY neuron spike frequency (Figure 6F). However, it did not affect the PD or LP neurons. In *L. emarginata*, it caused a significant decrease in the PY neuron spike frequency (Figure 6F), but, like *C. opilio*, did not affect the PD or LP neurons.

Oxotremorine (muscarinic ACh agonist)

Oxotremorine caused a significant change in the pyloric pattern of both *C. opilio* and *L. emarginata*. In *C. opilio,* it caused a decrease in LP neuron burst duration (Figure 7H), but no change in the PD or PY neurons. In *L. emarginata,* it caused an increase in PD neuron burst duration and duty cycle (Figure 7A, 7B), PY spike frequency (Figure 7F), and LP duty cycle (Figure 7H). It caused a decrease in PY duty cycle (Figure 7E). Oxotremorine's extensive effect in *L. emarginata* was surprising because it elicited such a limited effect in *C. opilio.*

Proctolin

Proctolin caused a significant change in the pyloric pattern in *L. emarginata*, but not *C. opilio*. In *L. emarginata*, it caused a significant increase in PY neuron spike frequency (Figure 8F) and LP neuron duty cycle (Figure 8H), but did not affect the PD neurons.

RPCH

RPCH caused a significant change in the pyloric pattern in *C. opilio* and *L. emarginata*. In *C. opilio,* it increased overall cycle period of the pyloric rhythm (Figure 9J) and decreased the PD spike frequency (Figure 9B), but did not affect the LP or PY neurons. In *L. emarginata*, it decreased overall cycle period of the pyloric rhythm (9J) and decreased PY neuron duty cycle (9E). It increased PY neuron spike frequency (9F) and increased LP neuron burst duration and LP neuron duty cycle (9G, 9H). However, it did not affect the PD neurons. Surprisingly, RPCH caused opposite results in *C. opilio* (increased cycle period, change in PD neurons) as compared to *L. emarginata* (decreased cycle period, change in LP and PY neurons).

ACP, CLDH, NRNFLRFamide, G-SIFamide, & HIGSLYRamide (+ myosupressin for *P. producta)*

These neuromodulators were assessed observationally because data analysis has not yet been completed, so statistical significance of results could not be assessed. That said, in *C. opilio*, all neuromodulators except HIGSLYRamide caused a visually apparent change in at least one of the following: the total cycle period, the burst duration of any of the three neurons, or the spike frequency of any of the neurons in the pyloric rhythm (Figure 10B, 10C, 10D, 10E). In *L. emarginata*, all neuromodulators caused a visually apparent change in in at least one of the following: the total cycle period, the burst duration of any of the three neurons, or the spike frequency of any of the neurons in the pyloric rhythm (Figure 11A, 11B, 11C, 11D, 11E). And in *P. producta,* all neuromodulators except ACP caused a visually apparent change in in at least one of the following: the total cycle period, the burst duration of any of the three neurons, or the spike frequency of any of the neurons in the pyloric rhythm of *P. producta* (Figure 12A, 1210C, 12D, 12E, 12F, 12G).

Whole heart

Species sensitivity

My data were pooled with data collected by Grace Bukowski-Thall (Bukowski-Thall, 2020), and Alexandra Miller (data unpublished). *C. opilio* responded to 11/12 neuromodulators, *L. emarginata* responded to 6/12 neuromodulators, and *P. producta* responded to 8/12 neuromodulators (Table 2). None of the species tested responded to RPCH. Only *C. opilio* responded to G-SIFamide, HIGSLYRamide, and ACP. *L. emarginata* did not respond to CabTRP or proctolin, but *P. producta* did. Conversely, *P. producta* did not respond to HIGSLYRamide, while *L. emarginata* did. Overall, *C. opilio* responded to the most

neuromodulators, followed by *P. producta,* and finally *L. emarginata*. The neuromodulatory capacity of the heart did not correlate to dietary diversity.

CabTRP

CabTRP caused significant responses in *C. opilio* and *P. producta*, but not *L. emarginata* (Table 2). In *C. opilio*, CabTRP decreased cycle period (Figure 13A), increased width at ½ max (Figure 13B), increased duty cycle (Figure 13C), decreased beat duration (Figure 13D), increased peak force (Figure 13E), increased minimum force (Figure 13F), increased area (Figure 13G), and increased time to peak (Figure 13H). In *P. producta*, CabTRP decreased cycle period (Figure 13A), increased width at ½ max (Figure 13B), increased duty cycle (Figure 13C), increased peak force (Figure 13E), and increased area (Figure 13G).

CCAP

CCAP caused significant responses in *C. opilio, L. emarginata*, and *P. producta* (Table 2). In *C. opilio*, CCAP decreased period (Figure 14A), increased width at ½ max (Figure 14B), increased duty cycle (Figure 14C), decreased beat duration (Figure 14D), increased peak force (Figure 14E), increased minimum force (Figure 14F), and increased area (Figure 14G). In *L. emarginata,* CCAP decreased period (Figure 14A), increased duty cycle (Figure 14C), increased peak force (Figure 14E), and increased area (Figure 14G). In *P. producta*, CCAP decreased cycle period (Figure 14A), increased width at ½ max (Figure 14B), increased duty cycle (Figure 14C), increased peak force (Figure 14E), increased minimum force (Figure 14F), and increased area (Figure 14G).

Dopamine

Dopamine caused significant responses in *C. opilio, L. emarginata*, and *P. producta* (Table 2). In *C. opilio*, dopamine decreased cycle period (Figure 15A), increased duty cycle (Figure 15C), decreased beat duration (Figure 15D), increased peak force (Figure 15E), increased minimum force (Figure 15F), and increased area (Figure 15G). In *L. emarginata,* dopamine decreased period (Figure 15A), increased duty cycle (Figure 15C), increased beat duration (Figure 15D), and increased peak force (Figure 15E). In *P. producta*, dopamine decreased cycle period (Figure 15A), increased width at ½ max (Figure 15B), increased duty cycle (Figure 15C), increased peak force (Figure 15E), increased area (Figure 15G), and increased time to peak (Figure 15H).

Myosuppressin

Myosuppressin caused significant responses in *C. opilio* and *P. producta,* but not *L. emarginata* (Table 2). In *C. opilio*, myosuppressin decreased cycle period (Figure 16A), increased beat duration (Figure 16D) and increased peak force (Figure 16E). In *P. producta*, myosuppressin decreased cycle period (Figure 16A), increased width at ½ max (Figure 16B), increased duty cycle (Figure 16C), increased peak force (Figure 16E), increased minimum force (Figure 16F), and increased area (Figure 16G).

Oxotremorine

Oxotremorine caused significant responses in all three species (Table 2). In *C. opilio*, dopamine increased cycle period (Figure 17A), increased duty cycle (Figure 17C), decreased beat duration (Figure 17D), decreased peak force (Figure 17E), and increased time to peak (Figure 17H). In *L. emarginata,* oxotremorine increased duty cycle (Figure 17C) and increased

time to peak (Figure 17H). In *P. producta*, oxotremorine decreased cycle period (Figure 17A), increased width at ½ max (Figure 17B), increased duty cycle (Figure 17C), increased peak force (Figure 17E), and increased area (Figure 17G).

Proctolin

Proctolin caused significant responses in *C. opilio* and *P. producta* but not *L. emarginata* (Table 2). In *C. opilio*, proctolin increased duty cycle (figure 18C), increased peak force (Figure 18E), and increased area (Figure 18G). In *P. producta*, proctolin increased cycle period (Figure 18A), increased duty cycle (Figure 18C), increased peak force (Figure 18E), and increased area (Figure 18G).

RPCH

RPCH caused no significant responses in any species (Figure 19). It appears not to affect cardiac function.

CLDH

CLDH caused significant responses in *C. opilio* and to a lesser extent *L. emarginata,* but not *P. producta* (Table 2). In *C. opilio*, CLDH decreased period, (Figure 20A), decreased duty cycle (Figure 20C) and decreased beat duration (Figure 20D). In *L. emarginata,* dopamine decreased cycle period (Figure 20A).

HIGSLYRamide

HIGSLYRamide caused significant responses in *C. opilio* and *L. emarginata,* but not *P. producta* (Table 2). In *C. opilio*, HIGSLYRamide caused a significant increase in width at ½ max and minimum force (Figure 21B, 21F). In *L. emarginata*, HIGSLYRamide decreased beat duration (Figure 21D).

ACP

ACP caused significant responses in *C. opilio,* but did not affect *L. emarginata* or *P. producta*. In *C. opilio*, ACP decreased width at ½ max (Figure 22B) and minimum force (Figure 22F).

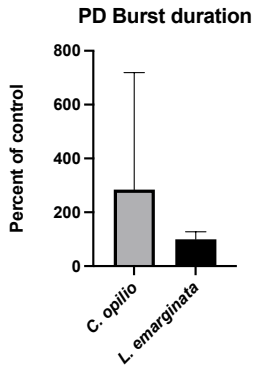
G-SIFamide

G-SIFamide caused a significant response in *C. opilio,* but not *L. emarginata* or *P. producta* (Table 2). In *C. opilio*, G-SIFamide caused a significant increase in minimum force (Figure 23F) and area (Figure 23G).

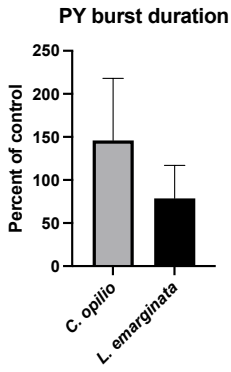
NRNFLRFamide

NRNLRFamide caused significant responses in *C. opilio*, but did not change *L. emarginata* or *P. producta*. In *C. opilio*, NRNFLRFamide increased peak force (Figure 24E) and area (24G).

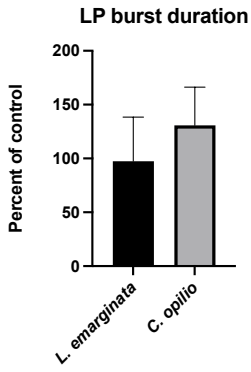
A.


PD Burst duration
Percent of control
800
600
400
200
0
C. opilio
L. emarginata

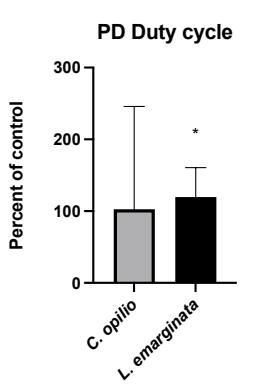
B.


PY burst duration
Percent of control
250
200
150
100
50
0
C. opilio
L. emarginata

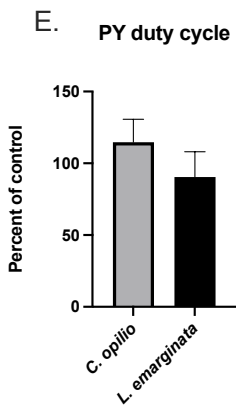
C.


LP burst duration
Percent of control
200
150
100
50
0
L. emarginata
C. opilio

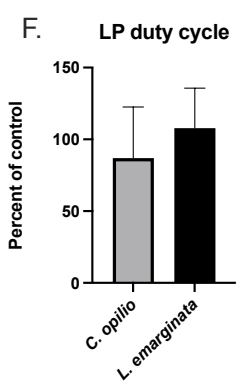
D.


PD Duty cycle
Percent of control
300
200
100
0
*
C. opilio
L. emarginata

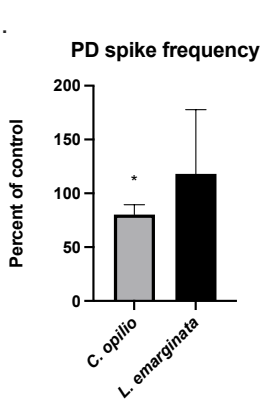
E.


PY duty cycle
Percent of control
150
100
50
0
C. opilio
L. emarginata

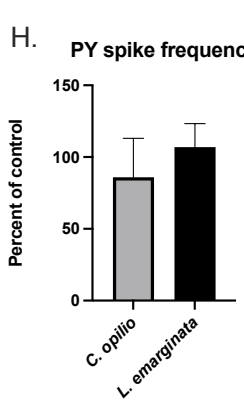
F.


LP duty cycle
Percent of control
150
100
50
0
C. opilio
L. emarginata

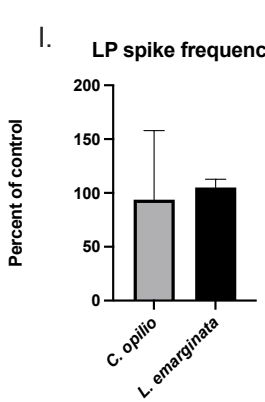
G.


PD spike frequency
Percent of control
200
150
100
50
0
*
C. opilio
L. emarginata

H.


PY spike frequency
Percent of control
150
100
50
0
C. opilio
L. emarginata

I.


LP spike frequency
Percent of control
200
150
100
50
0
C. opilio
L. emarginata

J.

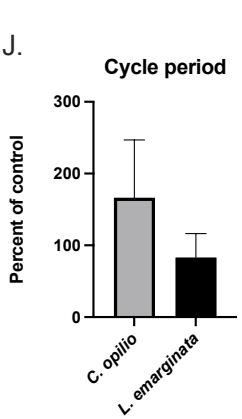

Cycle period
Percent of control
300
200
100
0
C. opilio
L. emarginata

Figure 3. CabTRP causes limited but significant changes in the pyloric rhythm of *C. opilio* and *L. emarginata.* Mean percent of control and standard error in the presence of 10^{-6} M CabTRP of the PD, PY, and LP neurons of of *C. opilio* (n = 10) and *L. emarginata* (n = 4) measured for four different parameters are pictured. Significance of results was assessed with a two-tailed paired *t*-test ($p < 0.05$). **A)** The burst duration of the PD neurons was not significantly different than control for either species, but trended towards increasing in *C. opilio.* **B)** The PY burst duration did not change significantly for *C. opilio*, but did decrease significantly for *L. emarginata* ($p < 0.05$). **C)** The LP burst duration of was not significantly changed for either species. **D)** The PD spike frequency did not change significantly for *L. emarginata*, but did decrease significantly for *C. opilio*. **E)** The PY duty cycle did not change significantly for either species, but did trend towards decreasing for *L. emarginat*a. **F)** The LP duty cycle was not significantly changes for either species. **G)** The PY spike frequency increased significantly for both species ($p < 0.05$). **H)** No significant difference was found in the duty cycle of the LP neurons either species. **I)** No significant difference was found in the LP spike frequency of either species. **J)** Cycle period was not significantly different than control for either species.

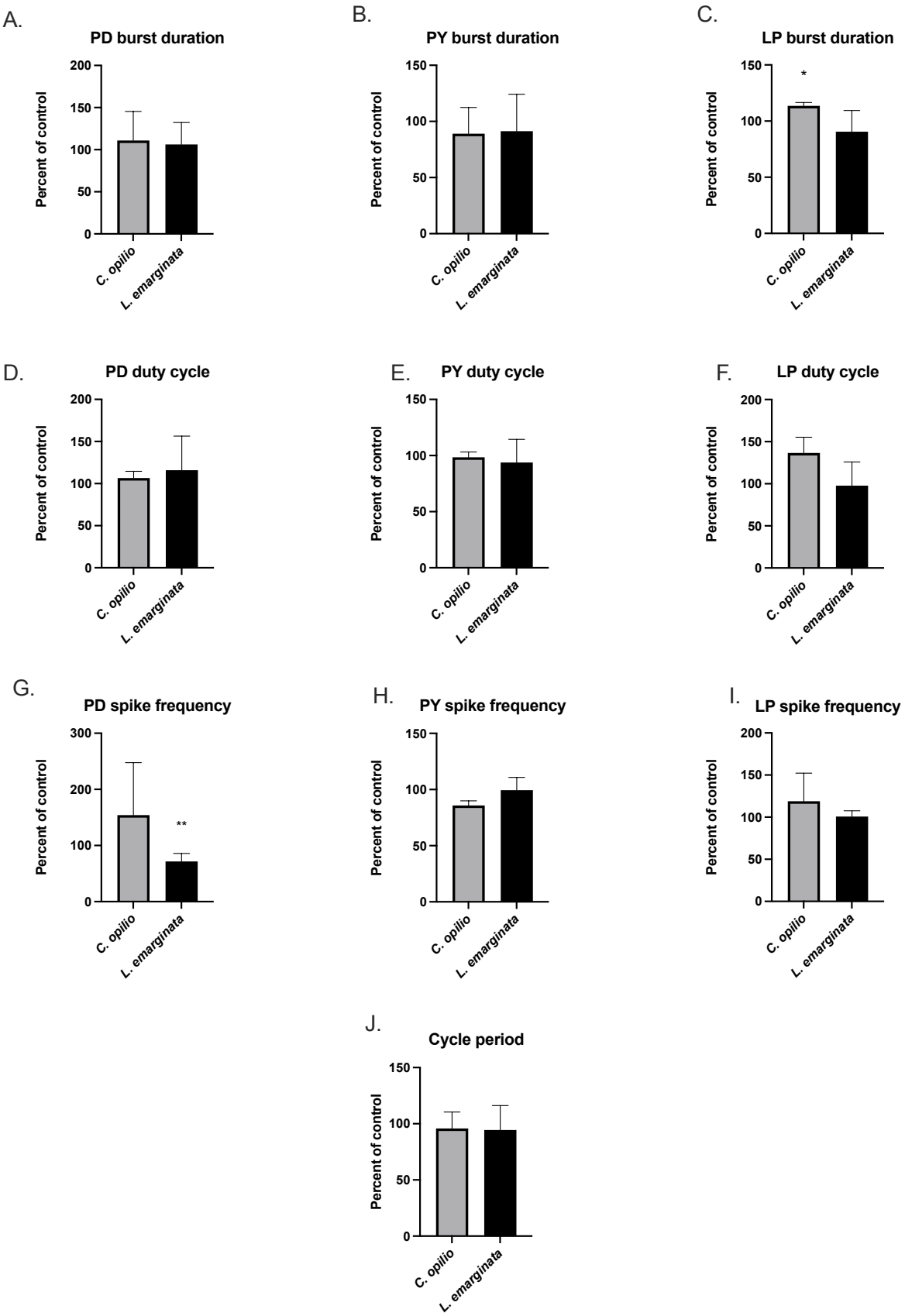

A.
PD burst duration
Percent of control
200
150
100
50
0
C. opilio
L. emarginata
B.
PY burst duration
Percent of control
150
100
50
0
C. opilio
L. emarginata
C.
LP burst duration
*
Percent of control
150
100
50
0
C. opilio
L. emarginata
D.
PD duty cycle
Percent of control
200
150
100
50
0
C. opilio
L. emarginata
E.
PY duty cycle
Percent of control
150
100
50
0
C. opilio
L. emarginata
F.
LP duty cycle
Percent of control
200
150
100
50
0
C. opilio
L. emarginata
G.
PD spike frequency
**
Percent of control
300
200
100
0
C. opilio
L. emarginata
H.
PY spike frequency
Percent of control
150
100
50
0
C. opilio
L. emarginata
I.
LP spike frequency
Percent of control
200
150
100
50
0
C. opilio
L. emarginata
J.
Cycle period
Percent of control
150
100
50
0
C. opilio
L. emarginata

Figure 4. CCAP causes limited but significant changes in the pyloric rhythm of *C. opilio* and *L. emarginata.* Mean percent of control and standard error in the presence of 10^{-6} M CabTRP of the PD, PY, and LP neurons of of *C. opilio* (n = 16) and *L. emarginata* (n = 5) measured for four different parameters are pictured. Significance of results was assessed with a paired two-tailed t-test ($p < 0.05$). **A)** The burst duration of the PD neurons was not significantly different than control. **B)** The duty cycle (burst duration/cycle period) of PD neurons was not significantly affected, although it trended towards increasing for *L. emarginata*. **C)** The spike frequency of the PD neurons of *C. opilio* was not significantly different than control, but trended towards increasing. The spike frequency of the PD neurons of *L. emarginata* was significantly increased ($p < 0.05$). **D)** No significant difference was found in the burst duration of the PY neurons compared to control. **E)** No significant difference was found in the duty cycle of the PY neurons compared to control. **F)** No significant difference was found in the spike frequency of the PY neurons compared to control. **G)** The burst duration of the LP neurons of *C. opilio* was significantly greater than control ($p < 0.05$). No significant difference was found between the burst duration of the LP neurons of *L. emarginata* and control. **H)** No significant difference was found in the duty cycle of the LP neurons compared to control. **I)** No significant difference was found in the spike frequency of the LP neurons compared to control. **J)** Cycle period was not significantly different than control.

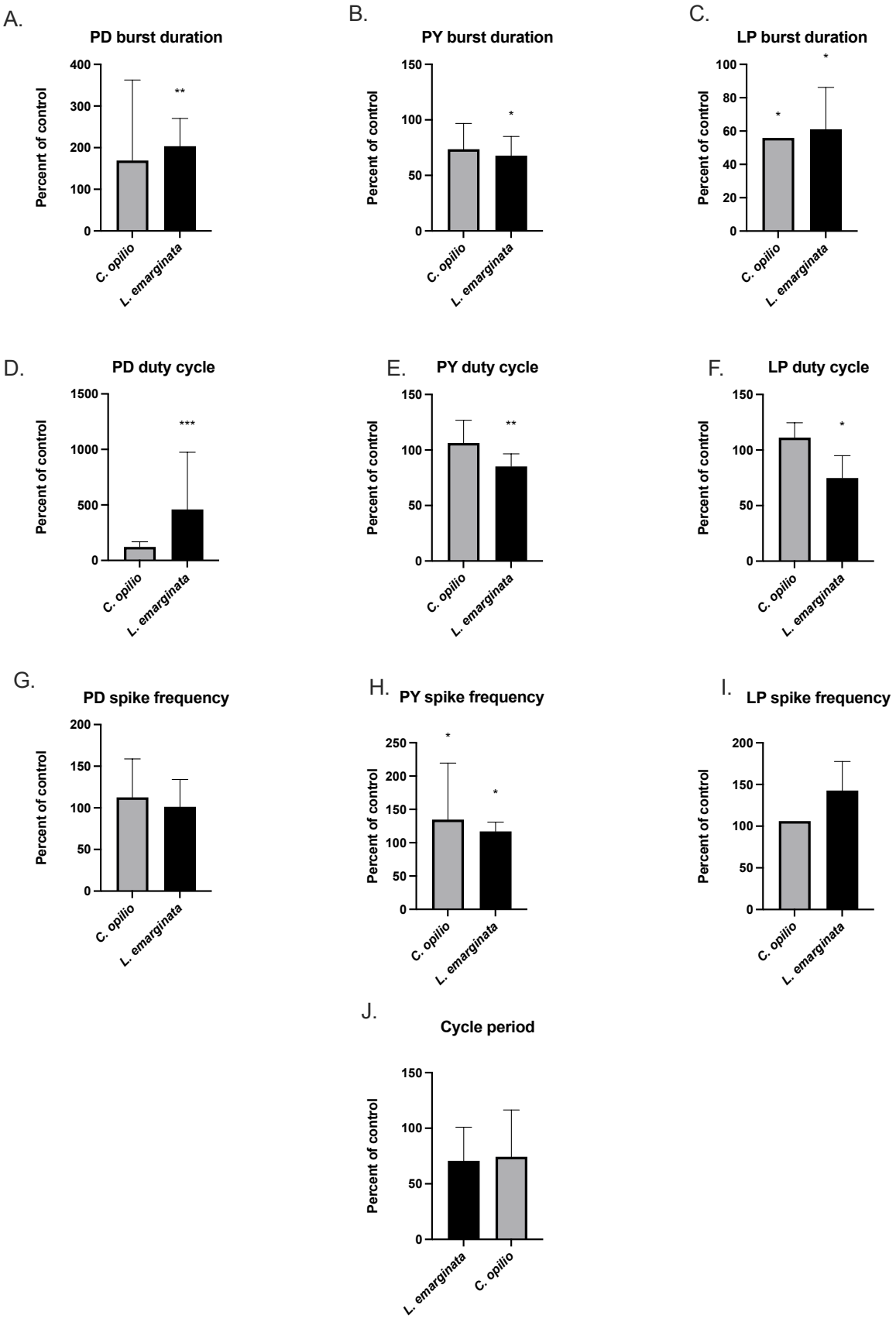

A.
PD burst duration
Percenrt of control
400
300
200
100
0
**
C. opilio
L. emarginata
B.
PY burst duration
Percent of control
150
100
50
0
*
C. opilio
L. emarginata
C.
LP burst duration
Percent of control
100
80
60
40
20
0
*
*
C. opilio
L. emarginata
D.
PD duty cycle
Percent of control
1500
1000
500
0
***
C. opilio
L. emarginata
E.
PY duty cycle
Percent of control
150
100
50
0
**
C. opilio
L. emarginata
F.
LP duty cycle
Percent of control
150
100
50
0
*
C. opilio
L. emarginata
G.
PD spike frequency
Percent of control
200
150
100
50
0
C. opilio
L. emarginata
H.
PY spike frequency
Percent of control
250
200
150
100
50
0
*
*
C. opilio
L. emarginata
I.
LP spike frequency
Percent of control
200
150
100
50
0
C. opilio
L. emarginata
J.
Cycle period
Percent of control
150
100
50
0
L. emarginata
C. opilio

Figure 5. Dopamine elicits limited but significant changes in the pyloric rhythm of *C. opilio,* whereas it causes more extensive alterations to the pyloric rhythm in in *L. emarginata.* Mean percent of control and standard error in the presence of 10^{-6} M dopamine of the PD, PY, and LP neurons of *C. opilio* (n = 14) and *L. emarginata* (n = 4) measured for four different parameters are pictured. **A)** The burst duration of the PD neurons was not significantly different than control for *C. opilio.* The PD burst duration for *L. emarginata* was significantly increased (paired t-test, $p < 0.01$). **B)** The duty cycle (burst duration/cycle period) for PD of *C.* opilio did not change significantly. The PD duty cycle for *L. emarginata* significantly increased (paired t-test, $p < 0.001$). **C)** The PD spike frequency did not change significantly. **D)** The PY burst duration did not change significantly for *C. opilio*, but did decrease significantly for *L. emarginata* (paired t-test, $p < 0.01$). **E)** The PY duty cycle did not change significantly for *C. opilio*, but did decrease significantly for *L. emarginata* (paired t-test, $p < 0.01$). **F)** The PY spike frequency increased significantly for both species (paired t-test, $p < 0.05$). **G)** The LP burst duration of *C. opilio* and *L. emarginata* was significantly decreased for both species (paired t-test, $p < 0.05$). **H)** No significant difference was found in the duty cycle of the LP neurons of *C. opilio,* but the duty cycle of *L. emarginata* decreased significantly (paired t-test, $p < 0.05$). **I)** No significant difference was found in the spike frequency of the PY neurons compared to control, but *L. emarginata*'s spike frequency trended towards increasing as compared to control. **J)** Cycle period was not significantly different than control for either species.

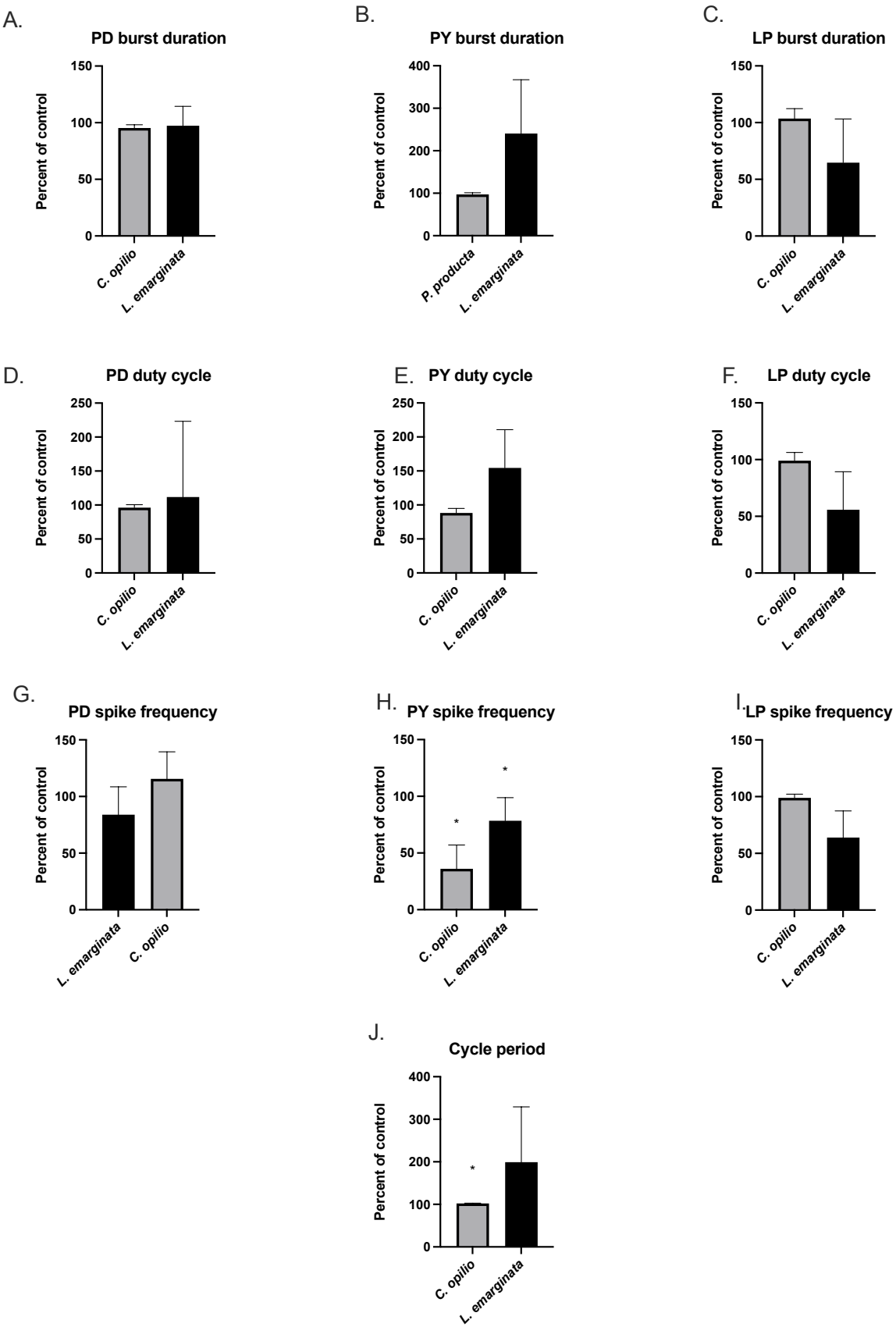

A.
PD burst duration
Percent of control
150
100
50
0
C. opilio
L. emarginata
B.
PY burst duration
Percent of control
400
300
200
100
0
P. producta
L. emarginata
C.
LP burst duration
Percent of control
150
100
50
0
C. opilio
L. emarginata
D.
PD duty cycle
Percent of control
250
200
150
100
50
0
C. opilio
L. emarginata
E.
PY duty cycle
Percent of control
250
200
150
100
50
0
C. opilio
L. emarginata
F.
LP duty cycle
Percent of control
150
100
50
0
C. opilio
L. emarginata
G.
PD spike frequency
Percent of control
150
100
50
0
L. emarginata
C. opilio
H.
PY spike frequency
Percent of control
150
100
50
0
*
*
C. opilio
L. emarginata
I.
LP spike frequency
Percent of control
150
100
50
0
C. opilio
L. emarginata
J.
Cycle period
Percent of control
400
300
200
100
0
*
C. opilio
L. emarginata

Figure 6. Myosuppressin caused limited but significant changes in the pyloric rhythm of both species. Mean percent of control and standard error in the presence of 10^{-6} M CabTRP of the PD, PY, and LP neurons of of *C. opilio* (n = 12) and *L. emarginata* (n = 5) measured for four different parameters are pictured. **A)** The burst duration of the PD neurons was not significantly different than control for either species. **B)** The PD duty cycle (burst duration/cycle period) did not change significantly for either species. **C)** The PD spike frequency did not change significantly for either species. **D)** The PY burst duration did not change significantly for either species, but did trend towards increasing for *L. emarginata*. **E)** The PY duty cycle did not change significantly for either species, but did trend towards increasing for *L. emarginat*a. **F)** The PY spike frequency decreased significantly for both species (paired t-test, $p < 0.05$). **G)** The LP burst duration of was not significantly changed for either species, but trended towards decreasing for *L. emarginata*. **H)** No significant difference was found in the duty cycle of the LP neurons of either species, but trended towards decreasing for *L. emarginata*. **I)** No significant difference was found in the LP spike frequency of either species, but trended towards decreasing for *L. emarginata.* **J)** Cycle period of *C. opilio* was significantly increased in comparison to the control (paired t-test, $p < 0.05$). *L. emarginata* did not have a significant change in cycle period.

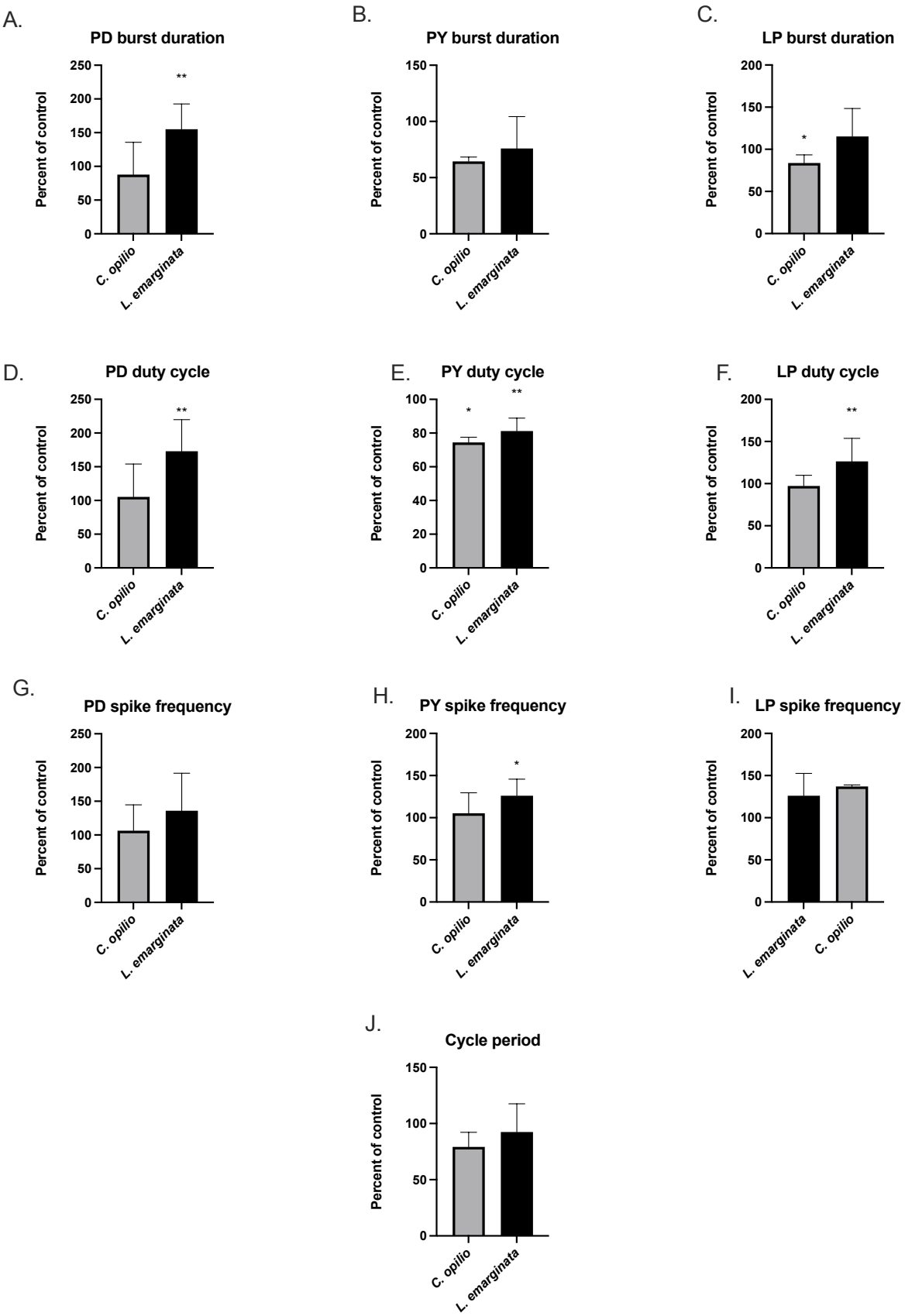

A.
PD burst duration
Percent of control
250
200
150
100
50
0
**
C. opilio
L. emarginata
B.
PY burst duration
Percent of control
150
100
50
0
C. opilio
L. emarginata
C.
LP burst duration
Percent of control
200
150
100
50
0
*
C. opilio
L. emarginata
D.
PD duty cycle
Percent of control
250
200
150
100
50
0
**
C. opilio
L. emarginata
E.
PY duty cycle
Percent of control
100
80
60
40
20
0
*
**
C. opilio
L. emarginata
F.
LP duty cycle
Percent of control
200
150
100
50
0
**
C. opilio
L. emarginata
G.
PD spike frequency
Percent of control
250
200
150
100
50
0
C. opilio
L. emarginata
H.
PY spike frequency
Percent of control
200
150
100
50
0
*
C. opilio
L. emarginata
I.
LP spike frequency
Percent of control
200
150
100
50
0
L. emarginata
C. opilio
J.
Cycle period
Percent of control
150
100
50
0
C. opilio
L. emarginata

Figure 7. Oxotremorine more extensive changes in the pyloric rhythm of *L. emarginata* than *C. opilio,* but both species show significant response to oxotremorine. Mean percent of control and standard error in the presence of 10^{-6} M CabTRP of the PD, PY, and LP neurons of of *C. opilio* (n = 12) and *L. emarginata* (n = 5) measured for four different parameters are pictured. **A)** The burst duration of the PD neurons was not significantly changed for *C. opilio,* but increased significantly for *L. emarginata (*paired t-test, $p < 0.01$). **B)** The PD duty cycle did not change significantly for *C. opilio,* but did increase significantly for *L. emarginata (*paired t-test, $p < 0.01$). **C)** The PD spike frequency did not change significantly for either species. **D)** The PY burst duration did not change significantly for either species. **E)** The PY duty cycle decreased significantly for both species (paired t-test; *C. opilio*: $p < 0.05$; *L. emarginata*: $p < 0.01$). **F)** The PY spike frequency did not change significantly for *C. opilio,* but increased significantly for *L. emarginata* (paired t-test, $p < 0.05$). **G)** The LP burst duration decreased significantly for *C. opilio* ($p < 0.05$), but did not change significantly for *L. emarginata.* **H)** The LP duty cycle did not change significantly for *C. opilio,* but did increase significantly for *L. emarginata* (paired t-test, $p < 0.05$*)*. **I)** No significant difference was found in the LP spike frequency of either species, but trended towards increasing for both. **J)** Cycle period of neither species was significantly changed, but trended towards decreasing for *C. opilio.*

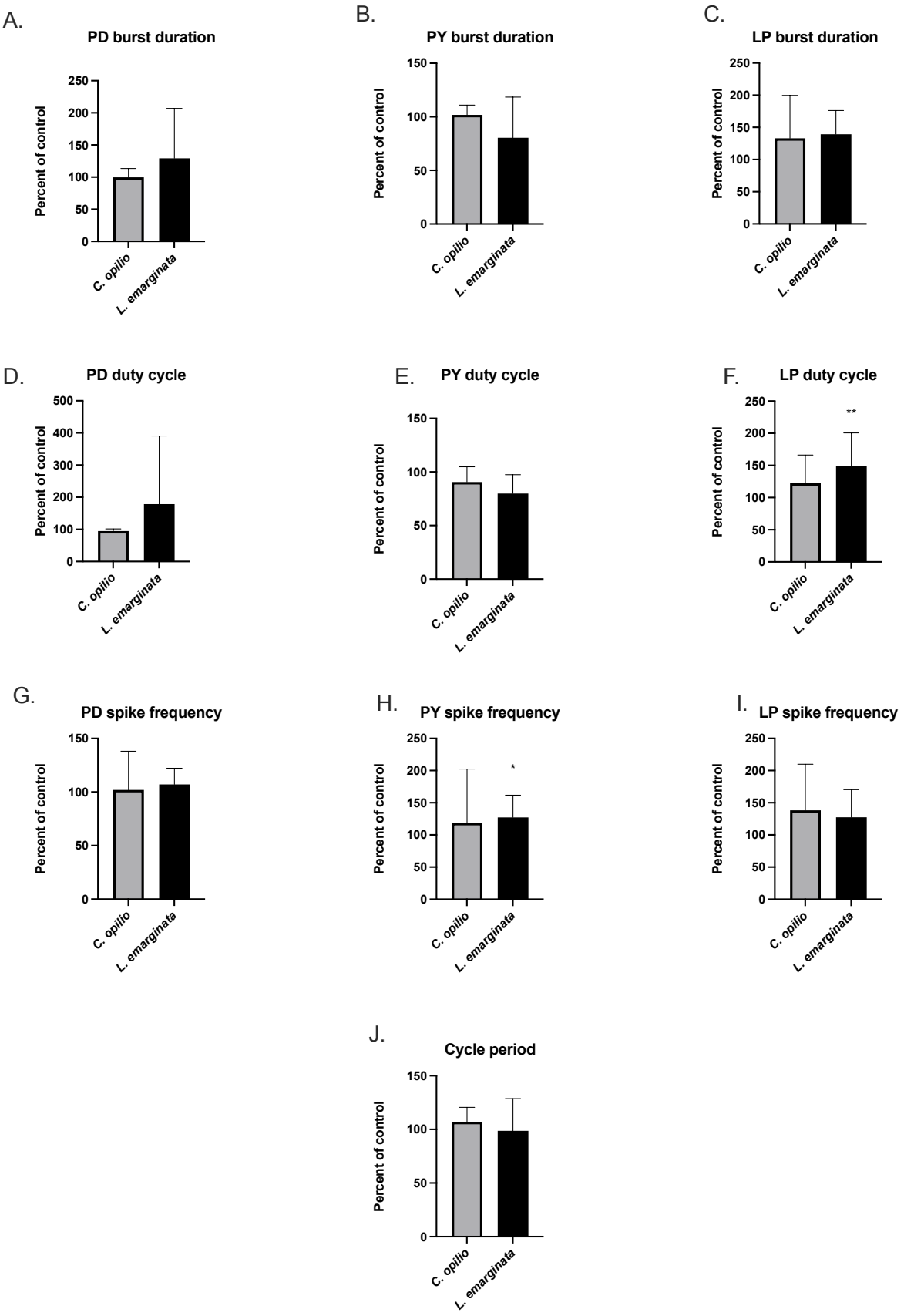

A.
PD burst duration
Percent of control
250
200
150
100
50
0
C. opilio
L. emarginata
B.
PY burst duration
Percent of control
150
100
50
0
C. opilio
L. emarginata
C.
LP burst duration
Percent of control
250
200
150
100
50
0
C. opilio
L. emarginata
D.
PD duty cycle
Percent of control
500
400
300
200
100
0
C. opilio
L. emarginata
E.
PY duty cycle
Percent of control
150
100
50
0
C. opilio
L. emarginata
F.
LP duty cycle
Percent of control
**
250
200
150
100
50
0
C. opilio
L. emarginata
G.
PD spike frequency
Percent of control
150
100
50
0
C. opilio
L. emarginata
H.
PY spike frequency
Percent of control
*
250
200
150
100
50
0
C. opilio
L. emarginata
I.
LP spike frequency
Percent of control
250
200
150
100
50
0
C. opilio
L. emarginata
J.
Cycle period
Percent of control
150
100
50
0
C. opilio
L. emarginata

Figure 8. Proctolin causes limited but significant changes in the pyloric rhythm of *L. emarginata,* but does not affect *C. opilio* pyloric rhythm. Mean percent of control and standard error in the presence of 10^{-6} M CabTRP of the PD, PY, and LP neurons of *C. opilio* (n = 18) and *L. emarginata* (n = 5) measured for four different parameters are pictured. **A)** The burst duration of the PD neurons was not significantly changed for either species but trended toward increasing for *L. emarginata.* **B)** The PD duty cycle did not change significantly for either species, but trended towards increasing for *L. emarginata.* **C)** The PD spike frequency did not change significantly for either species. **D)** The PY burst duration did not change significantly for either species. **E)** The PY duty cycle did not change significantly for either species. **F)** The PY spike frequency did not change significantly for *C. opilio,* but increased significantly for *L. emarginata* (paired t-test, $p < 0.05$). **G)** The LP burst duration but did not change significantly for either species, but trended towards increasing for both. **H)** The LP duty cycle did not change significantly for *C. opilio,* but did increase significantly for *L. emarginata* (paired t-test, $p < 0.01$). **I)** No significant difference was found in the LP spike frequency of either species, but trended towards increasing for both. **J)** Cycle period of neither species was not significantly changed

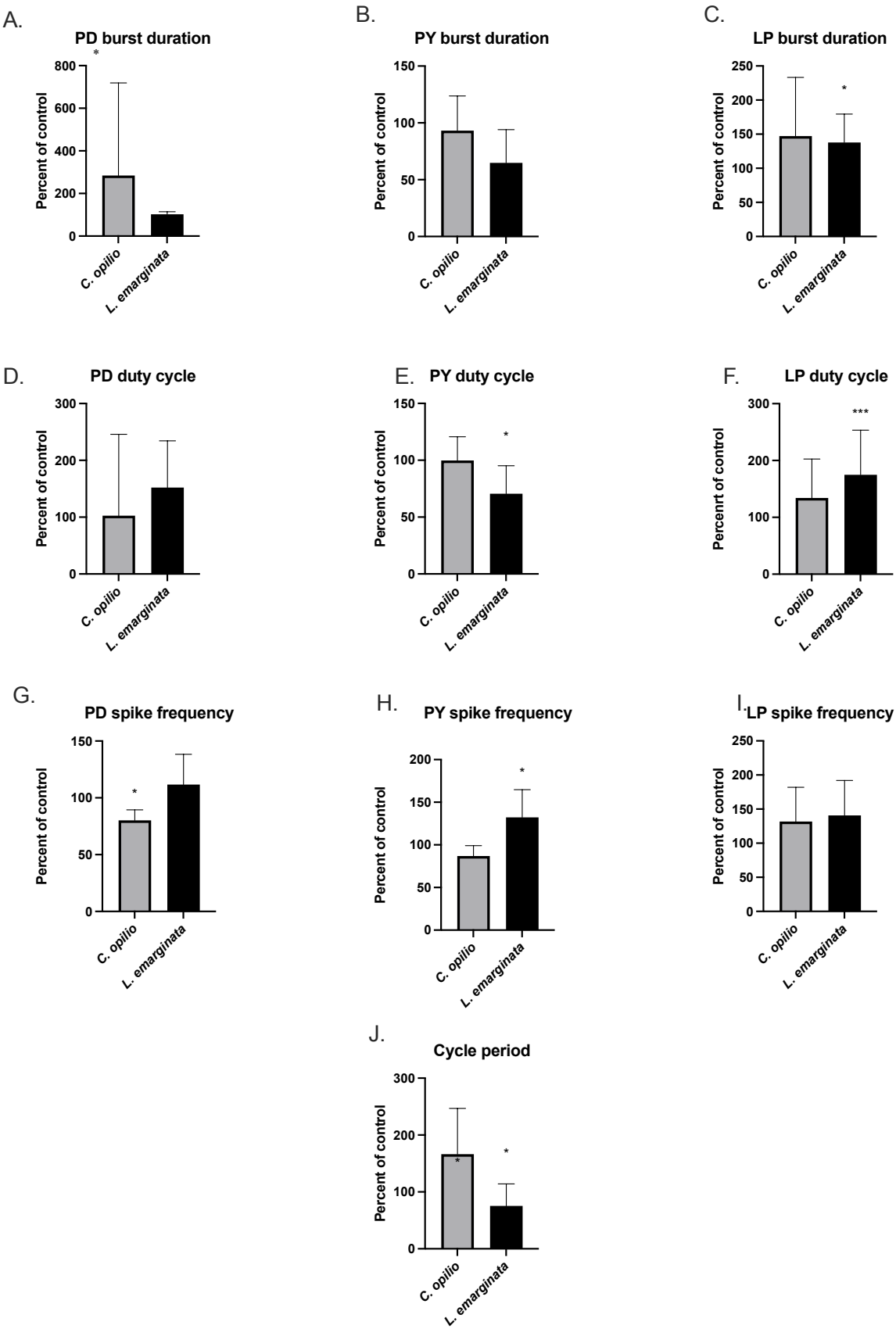

A.
PD burst duration
Percent of control
C. opilio
L. emarginata
B.
PY burst duration
Percent of control
C. opilio
L. emarginata
C.
LP burst duration
Percent of control
C. opilio
L. emarginata
D.
PD duty cycle
Percent of control
C. opilio
L. emarginata
E.
PY duty cycle
Percent of control
C. opilio
L. emarginata
F.
LP duty cycle
Percenrt of control
C. opilio
L. emarginata
G.
PD spike frequency
Percent of control
C. opilio
L. emarginata
H.
PY spike frequency
Percent of control
C. opilio
L. emarginata
I.
LP spike frequency
Percent of control
C. opilio
L. emarginata
J.
Cycle period
Percent of control
C. opilio
L. emarginata

Figure 9. RPCH causes significant changes in the pyloric rhythm of both species, but more extensive effects in the pyloric rhythm of *L. emarginata.* Mean percent of control and standard error in the presence of 10^{-6} M CabTRP of the PD, PY, and LP neurons of *C. opilio* (n = 16) and *L. emarginata* (n = 4) measured for four different parameters are pictured. **A)** The burst duration of the PD neurons was not significantly changed for either species but trended towards increasing for *C. opilio.* **B)** The PD duty cycle decreased significantly for *C. opilio* (paired t-test, $p < 0.05$)*,* but did not change significantly for *L. emarginata.* **C)** The PD spike frequency did not change significantly for either species, but trended towards decreasing for *L. emarginata*. **D)** The PY burst duration did not change significantly for *C. opilio*, but decreased significantly for *L. emarginata*. **E)** The PY duty cycle decreased significantly for both species (paired t-test; *C. opilio*: $p < 0.05$; *L. emarginata*: $p < 0.01$). **F)** The PY spike frequency did not change significantly for *C. opilio,* but increased significantly for *L. emarginata* (paired t-test, $p < 0.05$). **G)** The LP burst duration trended towards increasing for *C. opilio* and significantly increased for *L. emarginata* (paired t-test, $p < 0.05$). **H)** The LP duty cycle did not change significantly for *C. opilio,* but did increase significantly for *L. emarginata* (paired t-test, $p < 0.001$*)*. **I)** No significant difference was found in the LP spike frequency of either species, but trended towards increasing for both. **J)** Cycle period of increased significantly for *C. opilio* (paired t-test, $p < 0.05$) and decreased significantly for *L. emarginata* (paired t-test, $p < 0.05$).

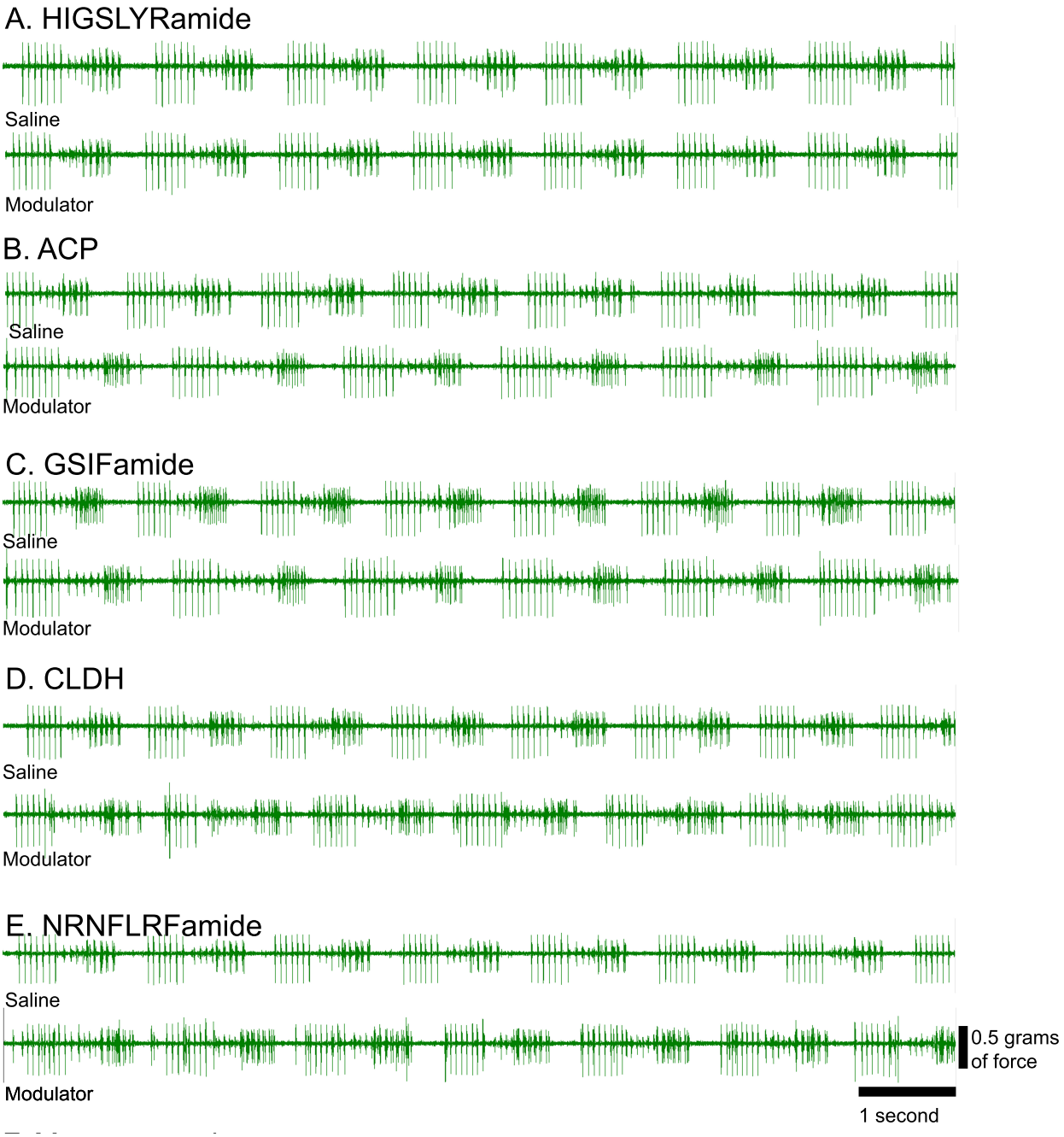


Figure 10. All neuromodulators except for HIGSLYRamide cause visible changes in STNS recordings of *C. opilio* as compared to control. Pictured are recordings of the *lvn* in *C. opilio* before and in the presence of 10^{-6} M neuromodulator. **A)** HIGSLYRamide (n = 8) does not affect the pyloric rhythm; control is identical to neuromodulator application. **B)** ACP (n = 8) increases

total cycle period and also appears to increase the burst duration of the PY neuron. **C)** G-SIFamide (n = 8) appears to cause an increase in the total cycle period and in the burst duration of LP and PY as compared to control. **D)** CLDH (n = 8) causes an increase in total cycle period and in the buration of the PY neuron. **E)** NRNFLRFamide (n = 5) appears to cause some disorganization in the rhythm. It also appears to increase the cycle period slightly and the burst duration of the PD neuron.

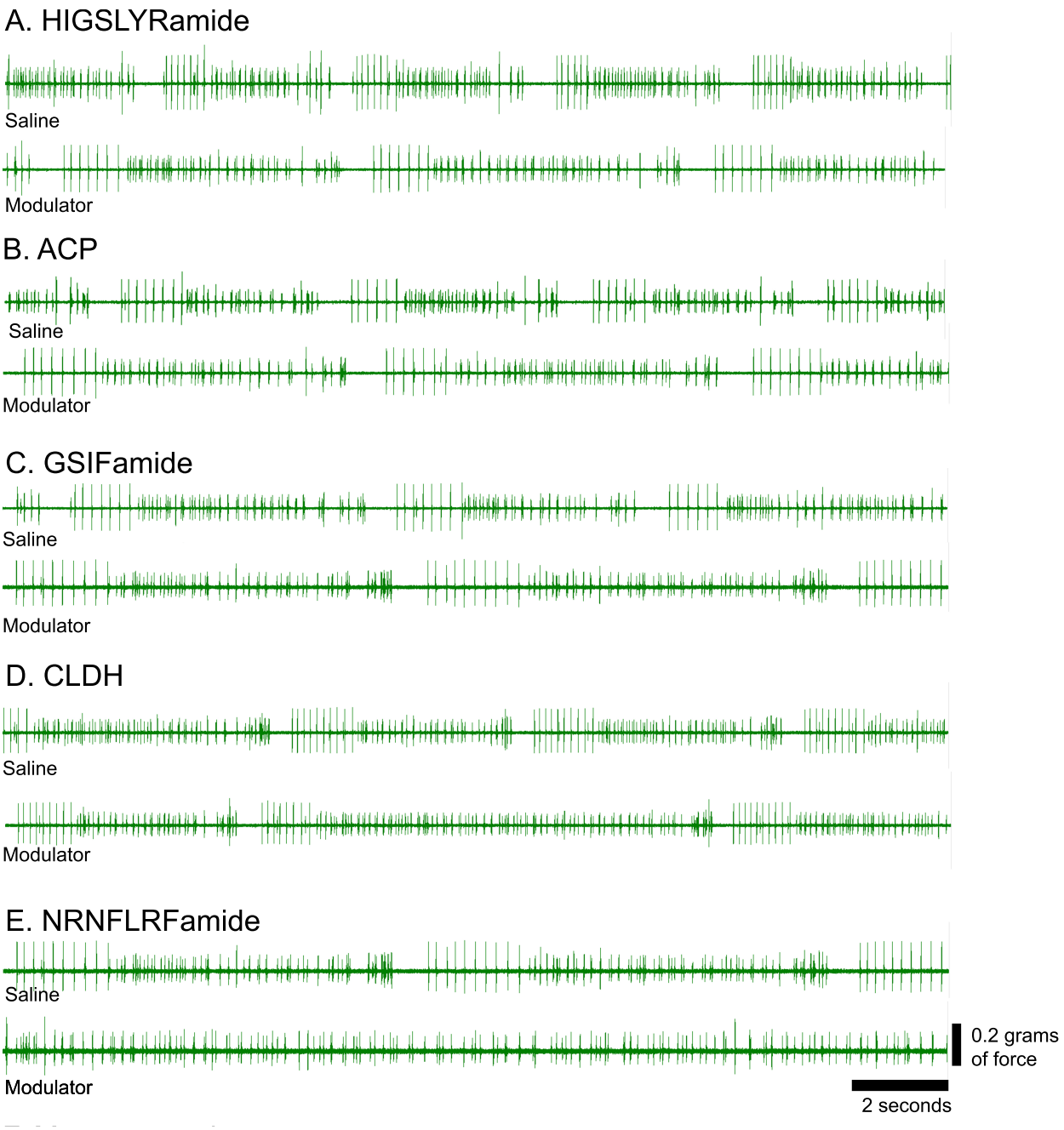


Figure 11. All neuromodulators cause visible changes in STNS recordings of *L. emarginata* as compared to control. Pictured are recordings of the *lvn* in *L. emarginata* before and in the presence of 10^{-6} M neuromodulator. **A)** HIGSLYRamide (n = 4) appears to increase the total cycle period of the pyloric rhythm, increase burst duration of PD, LP, and PY, and causes decreased spike frequency for all neurons as well. **B)** ACP (n = 5) appears to cause the same

effects as HIGSLYRamide: increasing cycle period and burst duration or all neurons, and decreasing spike frequency for all neurons. **C)** G-SIFamide (n = 4) appears to cause an increase in cycle period and in the burst duration of the LP neuron and PY neuron as compared to control. **D)** CLDH (n = 5) appears to cause a large increase in cycle period and a large increase in burst duration of the PY neuron as compared to control. **E)** NRNFLRFamide (n = 5) seems to disrupt the rhythm completely. PY burst duration is so long that cycle period is not visible.

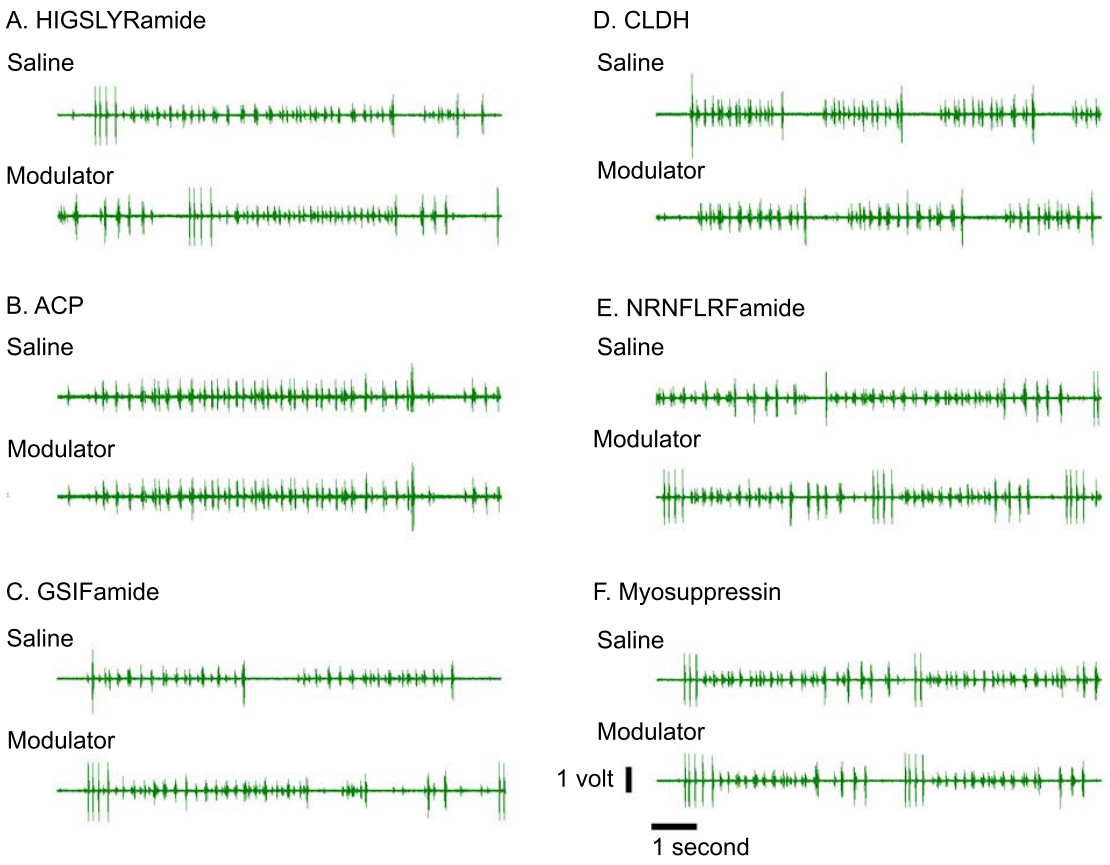


Figure 12. All neuromodulators but ACP cause visible changes in STNS recordings of *P. producta* as compared to control. Pictured are recordings of the *lvn* in *P. producta* (n = 5) before and in the presence of 10^{-6} M neuromodulator. **A)** HIGSLYRamide appears to increase the cycle period and the burst duration of the PY neuron as compared to control. **B)** ACP does not have a visible effect as compared to control. **C)** G-SIFamide appears to cause an increase in cycle period and in the burst duration of the LP neuron and PY neuron as compared to control. **D)** CLDH appears to cause a slight increase in cycle period and a slight increase in cycle period of the PY neuron as compared to control. **E)** NRNFLRFamide appears to cause a decrease in cycle period, a decrease in burst duration of the PY neuron, and an increase in burst duration of the LP

neuron. **F)** Myosuppressin appears to cause a decrease in cycle period, an increase in burst duration of the LP neuron, and a decrease in burst duration of the PY neuron.

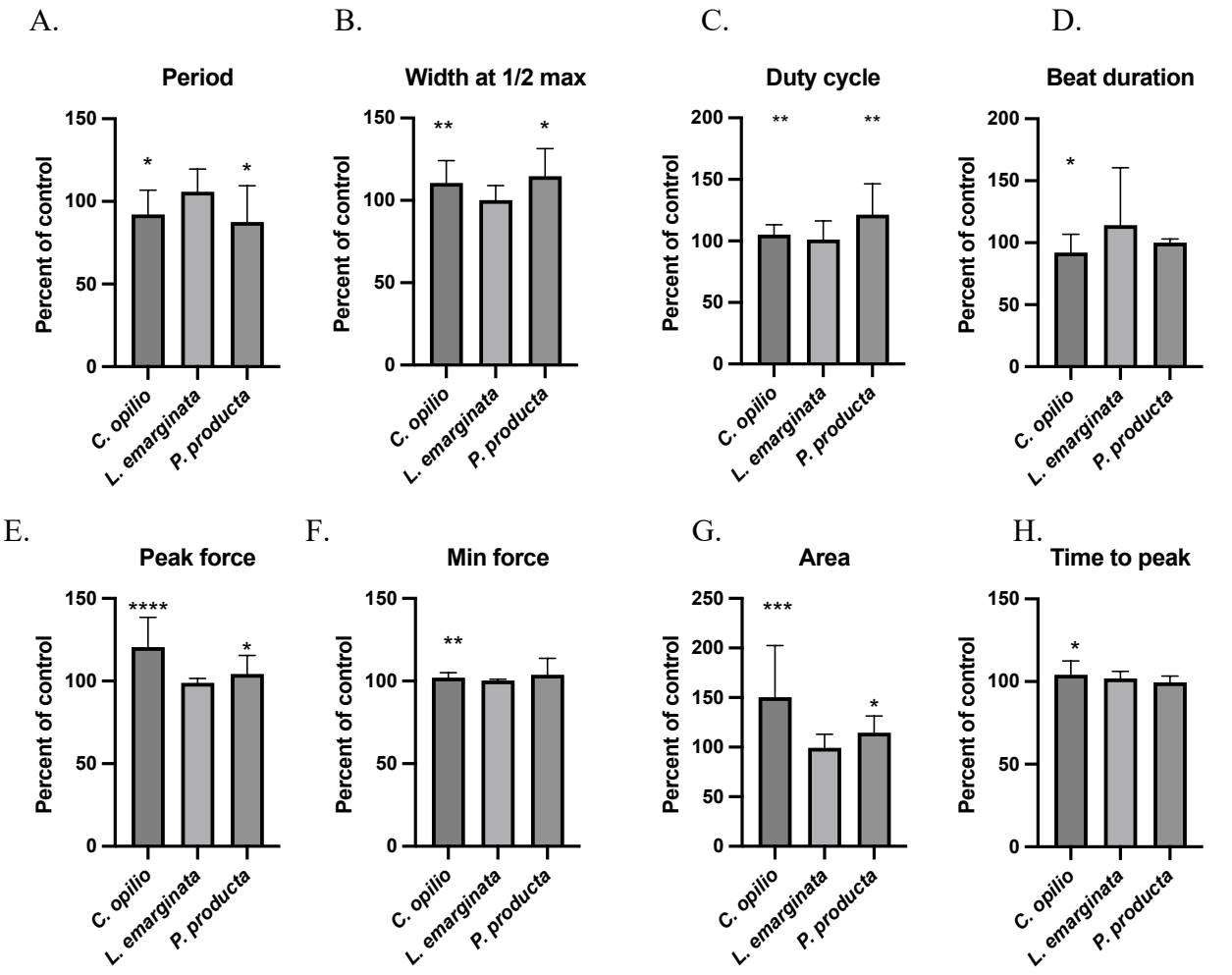


Figure 13. CabTRP causes significant changes in nearly all parameters of the heartbeat of *C. opilio* and *P. producta,* but not *L. emarginata.* Pictured are mean percent of control and standard error for eight parameters during CabTRP application in *C. opilio* (n = 24)*, L. emarginata* (n = 14) and *P. producta* (n = 15). **A)** Cycle period was significantly decreased for *C. opilio* and *P. producta* (paired t-test, $p < 0.05$), but not significantly changed for *L. emarginata.* **B)** Width at ½ max was significantly increased for *C. opilio* (paired t-test, $p < 0.01$) and *P. producta* (paired t-test, $p < 0.05$), but not significantly changed for *L. emarginata.* **C)** Duty cycle was significantly increased for *C. opilio* and *P. producta* (paired t-test, $p < 0.01$), but not significantly changed for *L. emarginata.* **D)** Beat duration was significantly decreased for *C. opilio*, but unchanged for *L. emarginata* and *P. producta.* **E)** Peak force was significantly increased for *C. opilio* (paired t-

test, $p < 0.0001$) and *P. producta* (paired t-test, $p < 0.05$), but unchanged for *L. emarginata*. **F)** Minimum force was significantly increased for *C. opilio* (paired t-test, $p < 0.05$). Minimum force was unchanged for *L. emarginata* and *P. producta*. **G)** Area was significantly increased for *C. opilio* (paired t-test, $p < 0.001$) and *P. producta* (paired t-test, $p < 0.05$), but unchanged for *L. emarginata*. **H)** Time to peak was significantly changed for *C. opilio* (paired t-test, $p < 0.05$), but results were inconsistent between individuals. Time to peak was not significantly different for *L. emarginata* or *P. producta*.

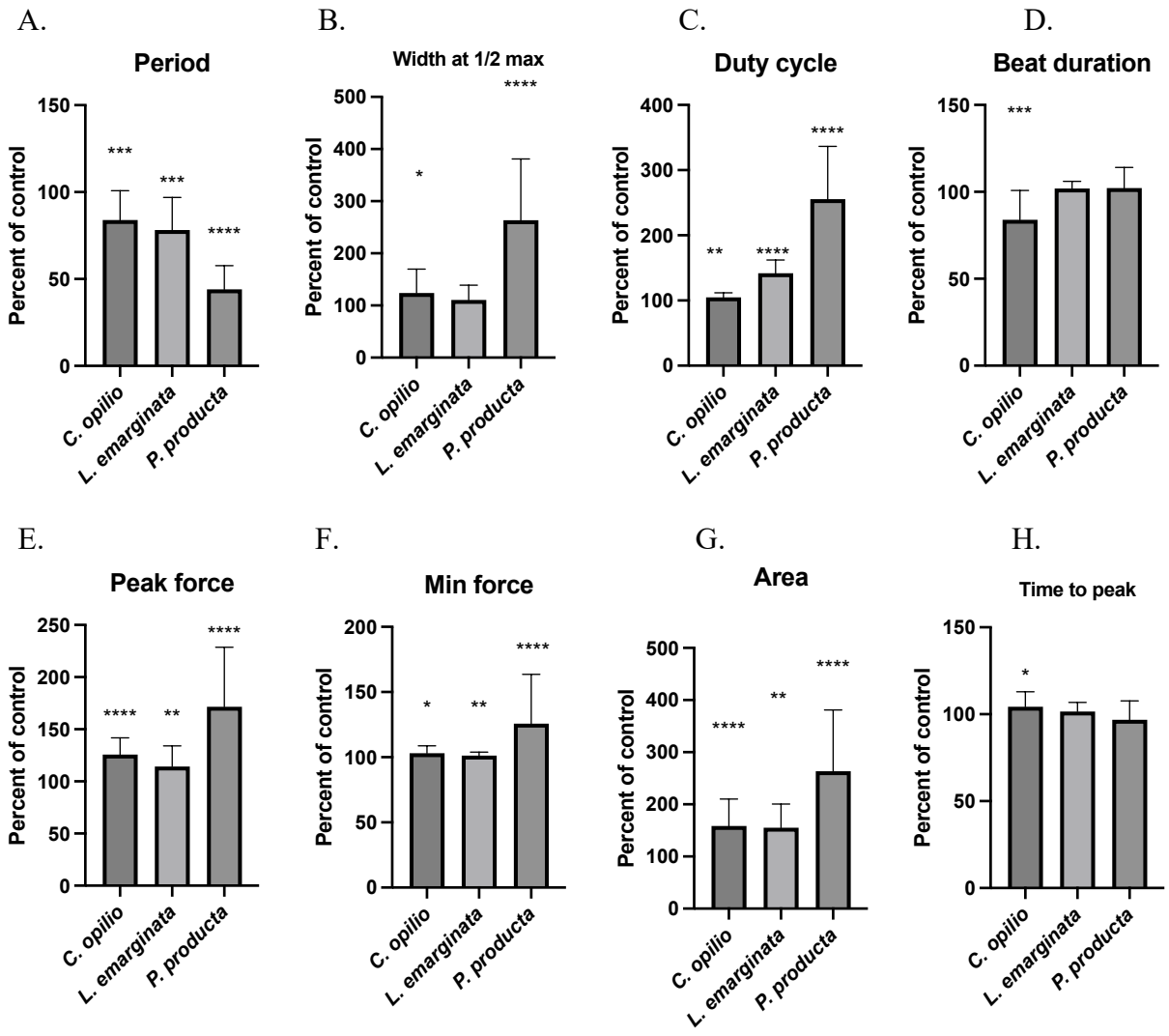


Figure 14. CCAP causes significant changes in the heartbeat of all three species. Pictured are mean percent of control and standard error for eight parameters during CCAP application in *C. opilio* (n = 24)*, L. emarginata* (n = 14) and *P. producta* (n = 15). **A)** Cycle period was significantly decreased for *C. opilio* (paired t-test, $p < 0.001$), *L emarginata* (paired t-test, $p < 0.001$), and *P. producta* (paired t-test, $p < 0.0001$). **B)** Width at ½ max was significantly increased for *C. opilio* (paired t-test, $p < 0.05$) and *P. producta* (paired t-test, $p < 0.0001$), but not significantly changed for *L. emarginata.* **C)** Duty cycle was significantly increased for *C. opilio* (paired t-test, $p < 0.01$), *L. emarginata* (paired t-test, $p < 0.0001$) and *P. producta* (paired t-test, $p < 0.0001$). **D)** Beat duration was significantly decreased for *C. opilio* (paired t-test, $p < 0.001$),

but unchanged for *L. emarginata* and *P. producta.* **E)** Peak force was significantly increased for *C. opilio* (paired t-test, $p < 0.0001$), *L. emarginata* (paired t-test, $p < 0.01$) and *P. producta* (paired t-test, $p < 0.0001$). **F)** Minimum force was significantly increased for *C. opilio* (paired t-test, $p < 0.05$) and decreased for *L. emarginata* (paired t-test, $p < 0.01$). Minimum force significantly increased for *P. producta* (paired t-test, $p < 0.0001$). **G)** Area was significantly increased for *C. opilio* (paired t-test, $p < 0.0001$), *L. emarginata* (paired t-test, $p < 0.01$), and *P. producta* (paired t-test, $p < 0.0001$). **H)** Time to peak was significantly increased for *C. opilio* (paired t-test, $p < 0.05$). Minimum force was unchanged for *L. emarginata* and *P. producta*.

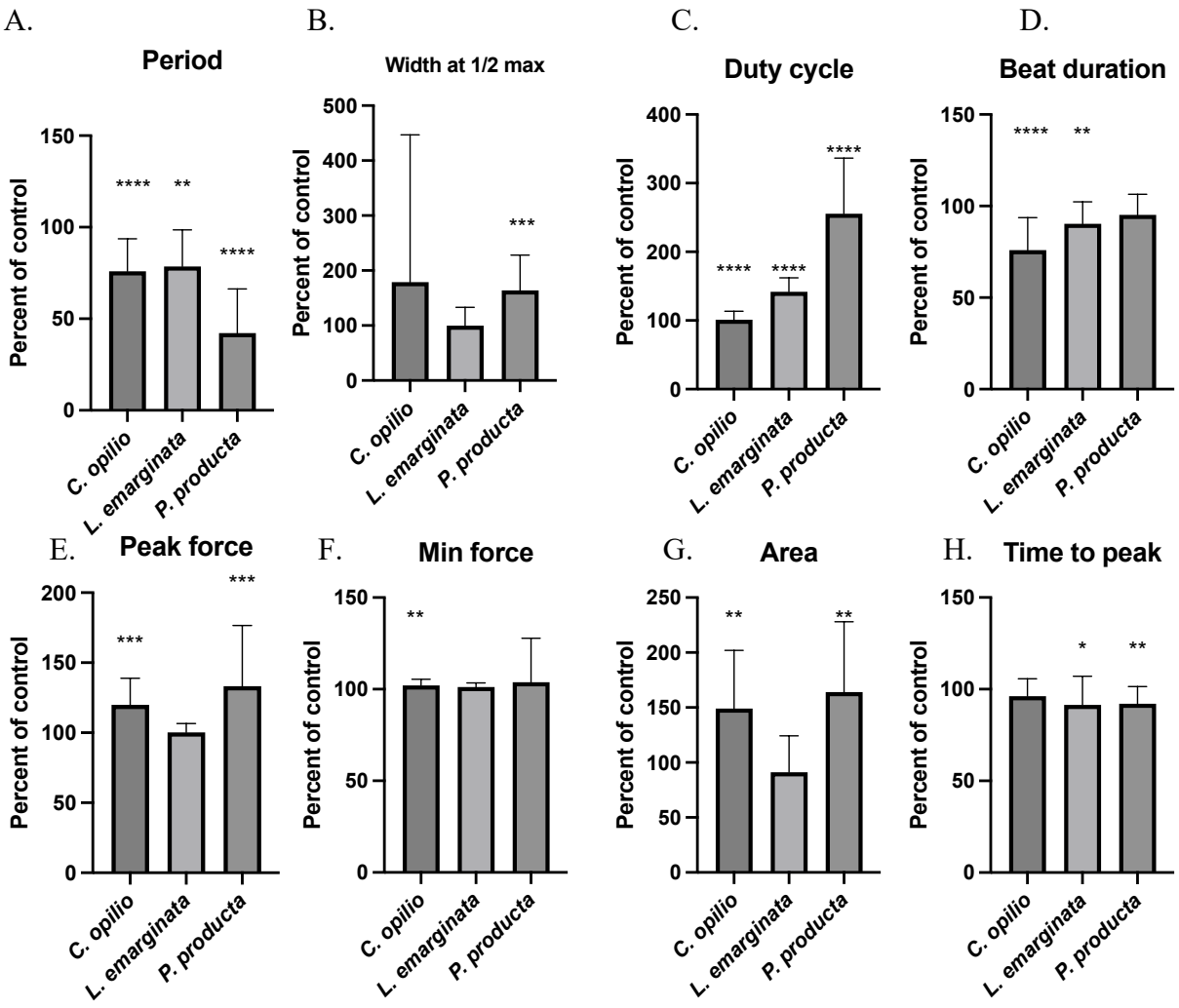


Figure 15. Dopamine caused significant changes to the heartbeat of all three species. Pictured are mean percent of control and standard error for eight parameters during dopamine application in *C. opilio* (n = 24)*, L. emarginata* (n = 14) and *P. producta* (n = 15). **A)** Cycle period was significantly decreased for *C. opilio* (paired t-test, $p < 0.0001$), *L emarginata* (paired t-test, $p < 0.01$), and *P. producta* (paired t-test, $p < 0.0001$). **B)** Width at ½ max was significantly increased in *P. producta* (paired t-test, $p < 0.001$), but not significantly changed for *C. opilio* or *L. emarginata.* **C)** Duty cycle was significantly decreased for *C. opilio* (paired t-test, $p < 0.0001$) and significantly increased for *L. emarginata* (paired t-test, $p < 0.0001$) and *P. producta* (paired t-test, $p < 0.0001$). **D)** Beat duration was significantly decreased for *C. opilio* (paired t-test, $p <$

0.0001) and *L. emarginata* (paired t-test, $p < 0.0$. **E)** Peak force was significantly increased for *C. opilio* (paired t-test, $p < 0.001$), *P. producta* (paired t-test, $p < 0.001$), but unchanged for *L. emarginata*. **F)** Minimum force was significantly increased for *C. opilio* (paired t-test, $p < 0.05$) but unchanged in *L. emarginata* and *P. producta*. **G)** Area was significantly increased for *C. opilio* (paired t-test, $p < 0.01$) and *P. producta* (paired t-test, $p < 0.01$), but unchanged for *L. emarginata*. **H)** Time to peak was significantly decreased for *L. emarginata* (paired t-test, $p < 0.05$) and *P. producta* (paired t-test, $p < 0.01$), but unchanged for *C. opilio.*

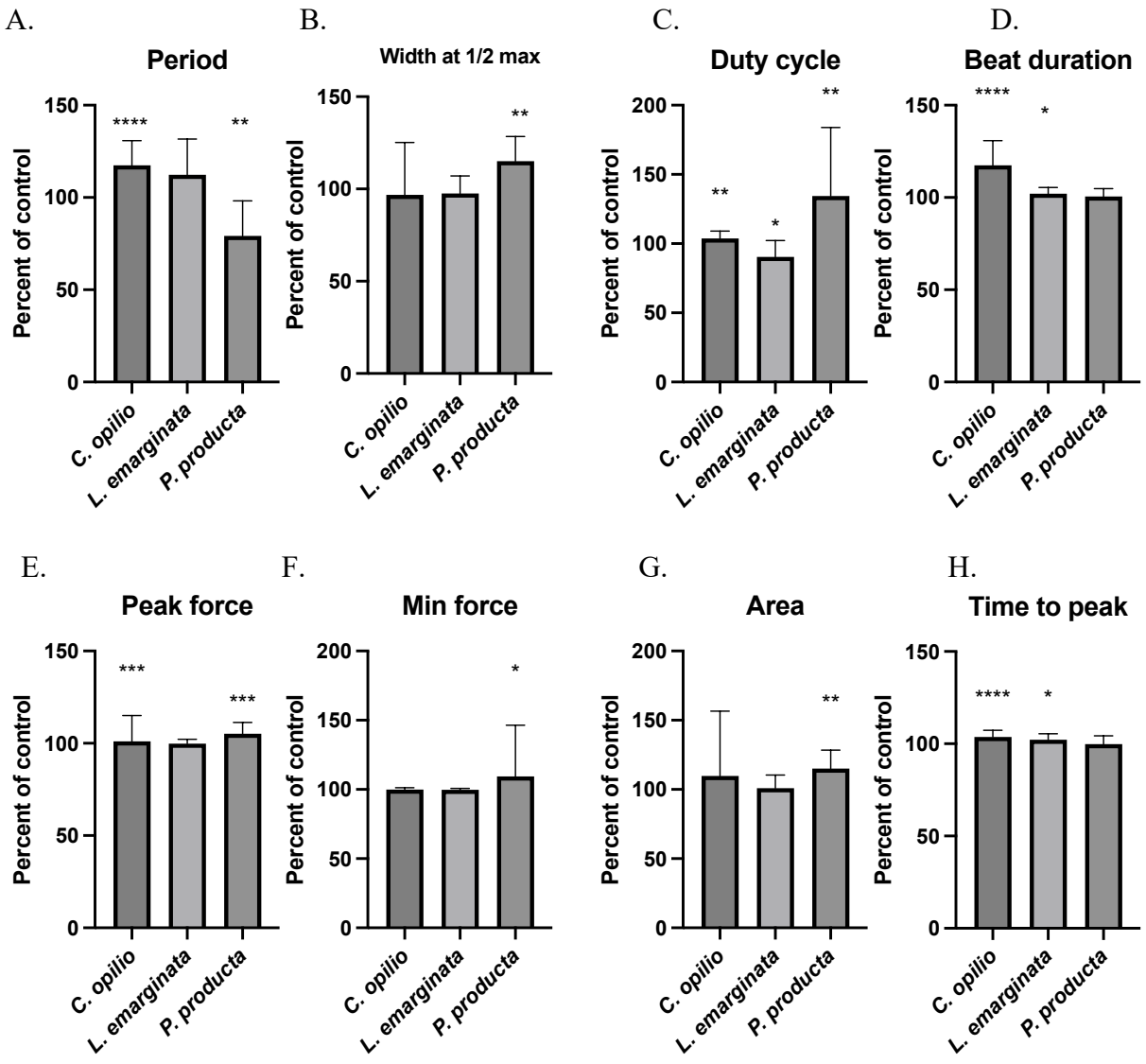


Figure 16. Myosuppressin elicited widespread changes in the heartbeat of *C. opilio* and *P. producta* but elicited less pronounced changes in the heartbeat of *L. emarginata.* Pictured are mean percent of control and standard error for eight parameters during myosuppressin application in *C. opilio* (n = 24)*, L. emarginata* (n = 14) and *P. producta* (n = 15). **A)** Cycle period was significantly increased for *C. opilio (*paired t-test, $p < 0.0001$) and *P. producta* (paired t-test, $p < 0.01$), but not significantly changed for *L. emarginata.* **B)** Width at ½ max was significantly *P. producta* (paired t-test, $p < 0.01$), but not significantly changed for *C. opilio* or *L. emarginata.* **C)** Duty cycle was significantly changed for *C. opilio* although the type of change

was unclear (paired t-test, $p < 0.001$), significantly decreased for *L. emarginata* (paired t-test, $p < 0.01$), and significantly increased for *P. producta* (paired t-test, $p < 0.01$). **D)** Beat duration was significantly increased for *C. opilio* (paired t-test, $p < 0.0001$) and decreased for *L. emarginata* (paired t-test, $p < 0.05$), but unchanged for *P. producta.* **E)** Peak force was significantly decreased for *C. opilio* (paired t-test, $p < 0.001$) and *P. producta* (paired t-test, $p < 0.001$), but unchanged for *L. emarginata*. The type of change was unclear. **F)** Minimum force was significantly increased for *P. producta* (paired t-test, $p < 0.05$), but it was unchanged for *L. emarginata* and *P. producta*. **G)** Area was significantly increased for *P. producta* (paired t-test, $p < 0.01$), but unchanged for *C. opilio* and L*. emarginata*. **H)** Time to peak was significantly changed for *C. opilio* (paired t-test, $p < 0.0001$) and *L. emarginata* (paired t-test, $p < 0.05$), but results were inconsistent between individuals. Minimum force was unchanged for *P. producta*.

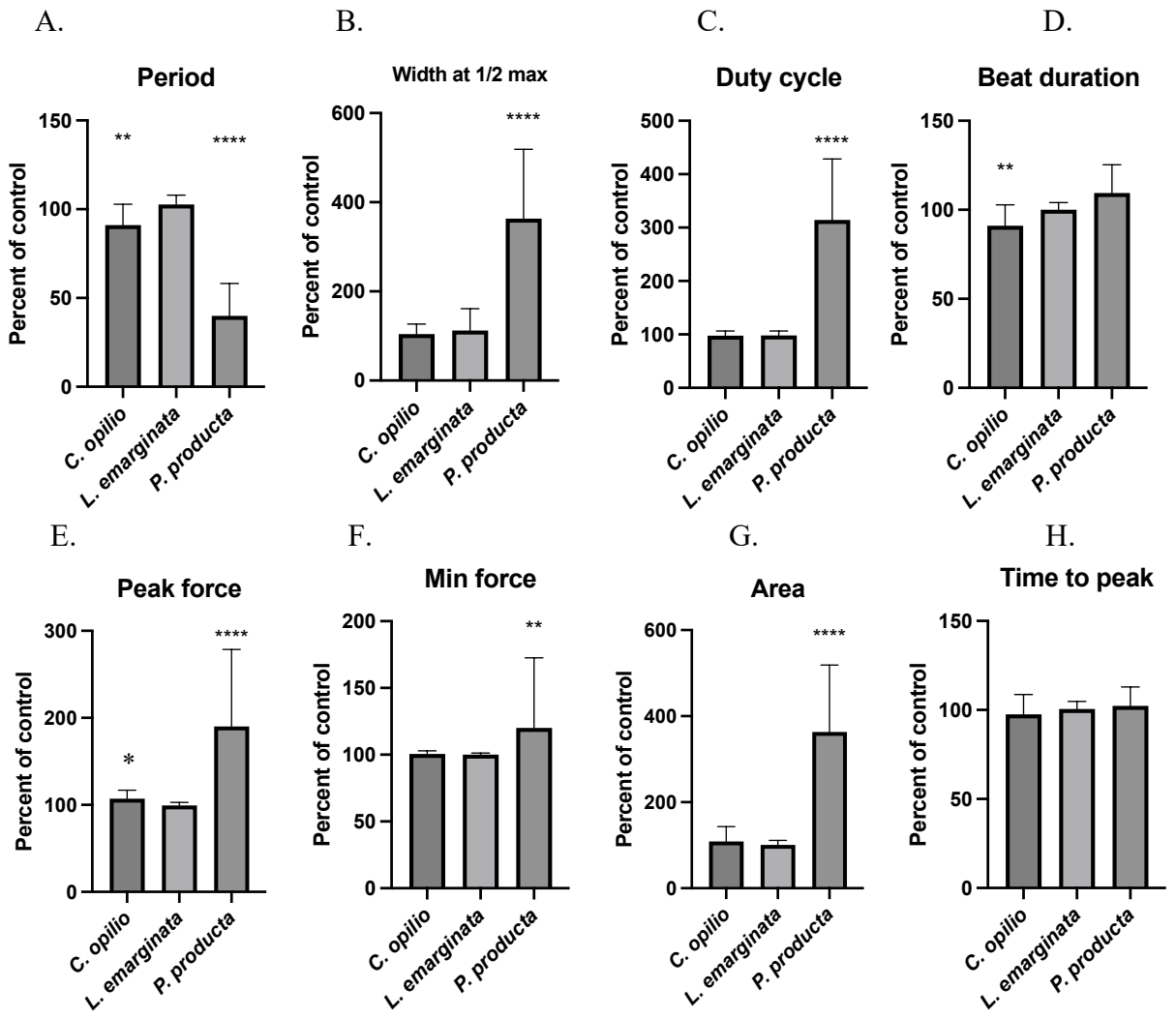


Figure 17. Oxotremorine elicited widespread changes in the heartbeat of *P. producta* but caused less pronounced changes in the heartbeat of *C. opilio* and no changes in the heartbeat of *L. emarginata.* Pictured are mean percent of control and standard error for eight parameters during oxotremorine application in *C. opilio* (n = 24)*, L. emarginata* (n = 14) and *P. producta* (n = 15)*.* **A)** Cycle period was significantly decreased for *C. opilio* (paired t-test, $p < 0.01$) and *P. producta* (paired t-test, $p < 0.0001$), but not *L emarginata.* **B)** Width at ½ max was significantly increased in *P. producta* (paired t-test, $p < 0.0001$), but not significantly changed for *C. opilio* or *L. emarginata.* **C)** Duty cycle was significantly increased in *P. producta* (paired t-test, $p < 0.0001$), but not significantly changed for *C. opilio* or *L. emarginata.* **D)** Beat duration was significantly

decreased for *C. opilio* (paired t-test, $p < 0.01$), but unchanged for *L. emarginata* and *C. opilio.* **E)** Peak force was significantly increased for *C. opilio* (paired t-test, $p < 0.05$) and increased for *P. producta* (paired t-test, $p < 0.0001$), but unchanged for *L. emarginata*. **F)** Minimum force was significantly increased in *P. producta (*paired t-test, $p < 0.01$), but unchanged for *C. opilio* and *L. emarginata*. **G)** Area was significantly increased for *P. producta* (paired t-test, $p < 0.0001$), but unchanged for *C. opilio* and *L. emarginata*. **H)** Time to peak was unchanged for all three species.

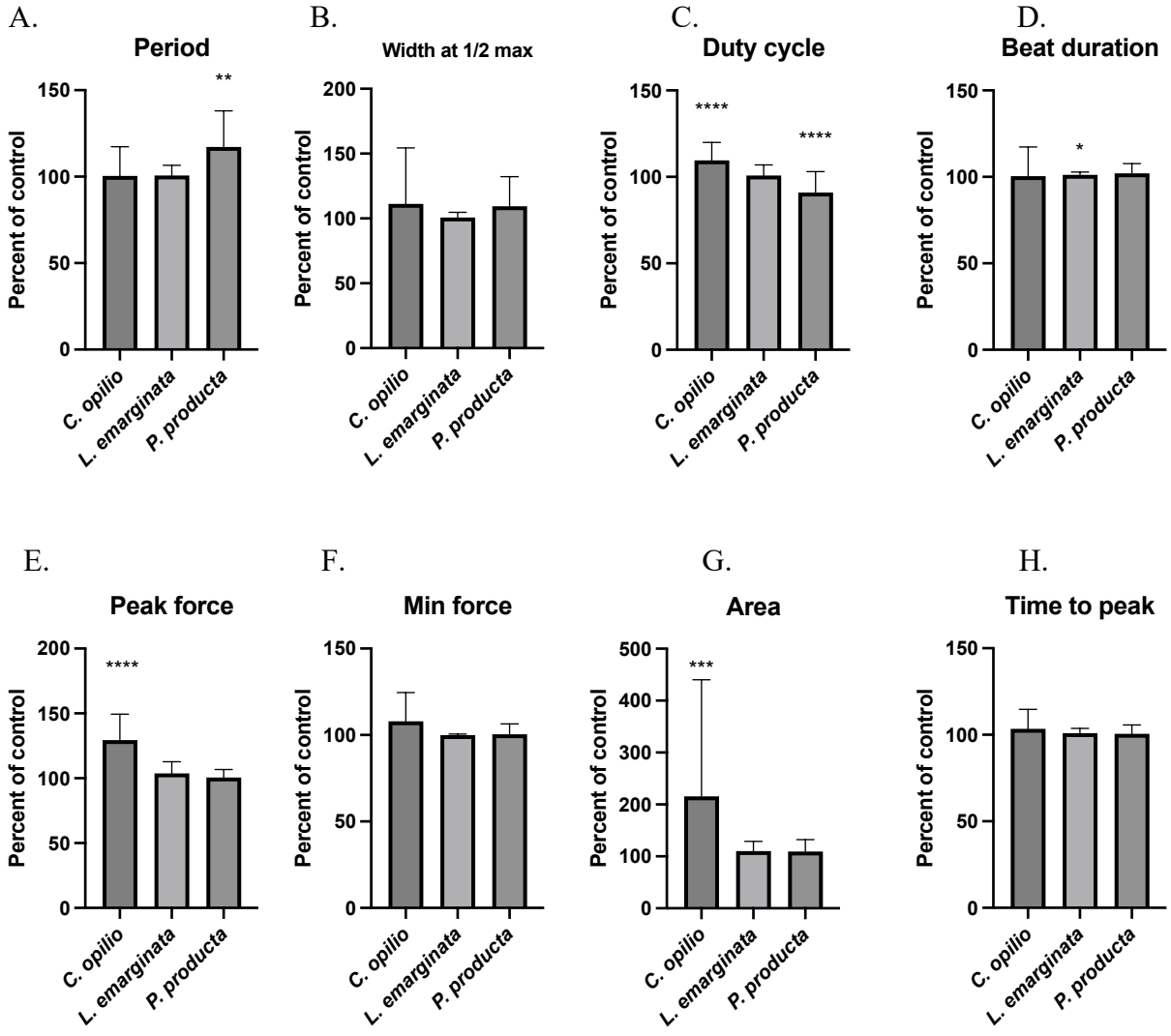


Figure 18. Proctolin caused changes in the heartbeat of all three species. Pictured are mean percent of control and standard error for eight parameters during proctolin application in *C. opilio* (n = 24), *L. emarginata* (n = 14) and *P. producta* (n = 15). **A)** Cycle period was significantly decreased for *P. producta* (paired t-test, $p < 0.01$), but not *L emarginata* or *C. opilio*. **B)** Width at ½ max was not significantly increased for any species. **C)** Duty cycle was significantly increased in *C. opilio* (paired t-test, $p < 0.0001$) and P. *producta* (paired t-test, $p < 0.0001$), but not *L. emarginata*. **D)** Beat duration was significantly changed for *L. emarginata* although the type of change was unclear (paired t-test, $p < 0.05$), but unchanged for *L.*

emarginata and *C. opilio.* **E)** Peak force was significantly increased for *C. opilio* (paired t-test, $p < 0.0001$) but unchanged for *L. emarginata* and *P. producta*. **F)** Minimum force was unchanged in all species. **G)** Area was significantly increased for *C. opilio* (paired t-test, $p < 0.0001$), but unchanged for *P. producta* and *L. emarginata*. **H)** Time to peak was unchanged for all three species.

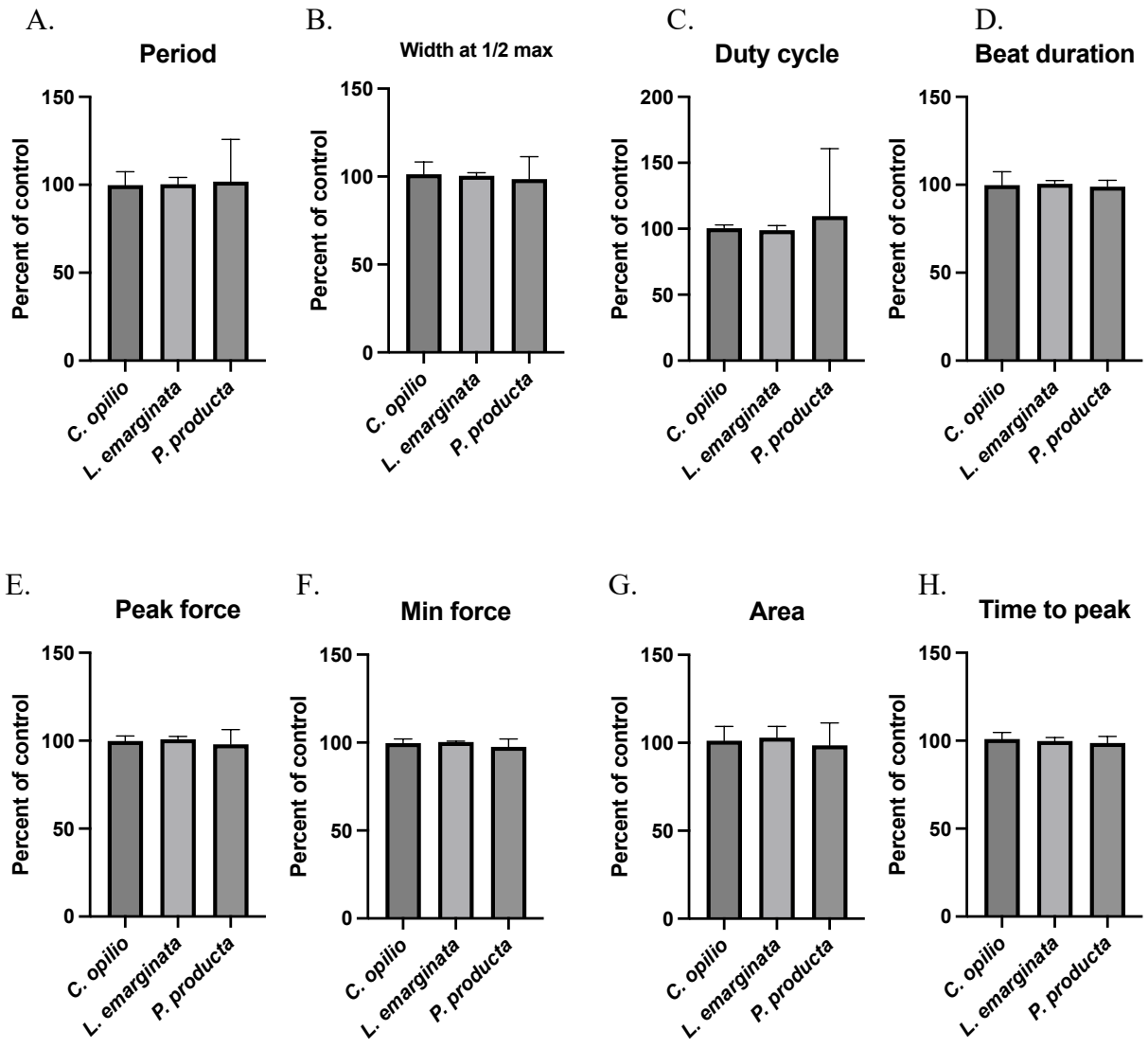


Figure 19. RPCH did not appear to have any effect on cardiac function. Pictured are mean percent of control and standard error for eight parameters during RPCH application in *C. opilio* (n = 24), *L. emarginata* (n = 14) and *P. producta* (n = 15). **A)** Cycle period was not significantly changed for any species. **B)** Width at ½ max was not significantly changed for any species. **C)** Duty cycle was was not significantly changed for any species. **D)** Beat duration was not significantly changed for any species **E)** Peak force was not significantly changed for any species. **F)** Minimum force was not significantly changed for any species. **G)** Area was not

significantly changed for any species. **H)** Time to peak was not significantly changed for any species.

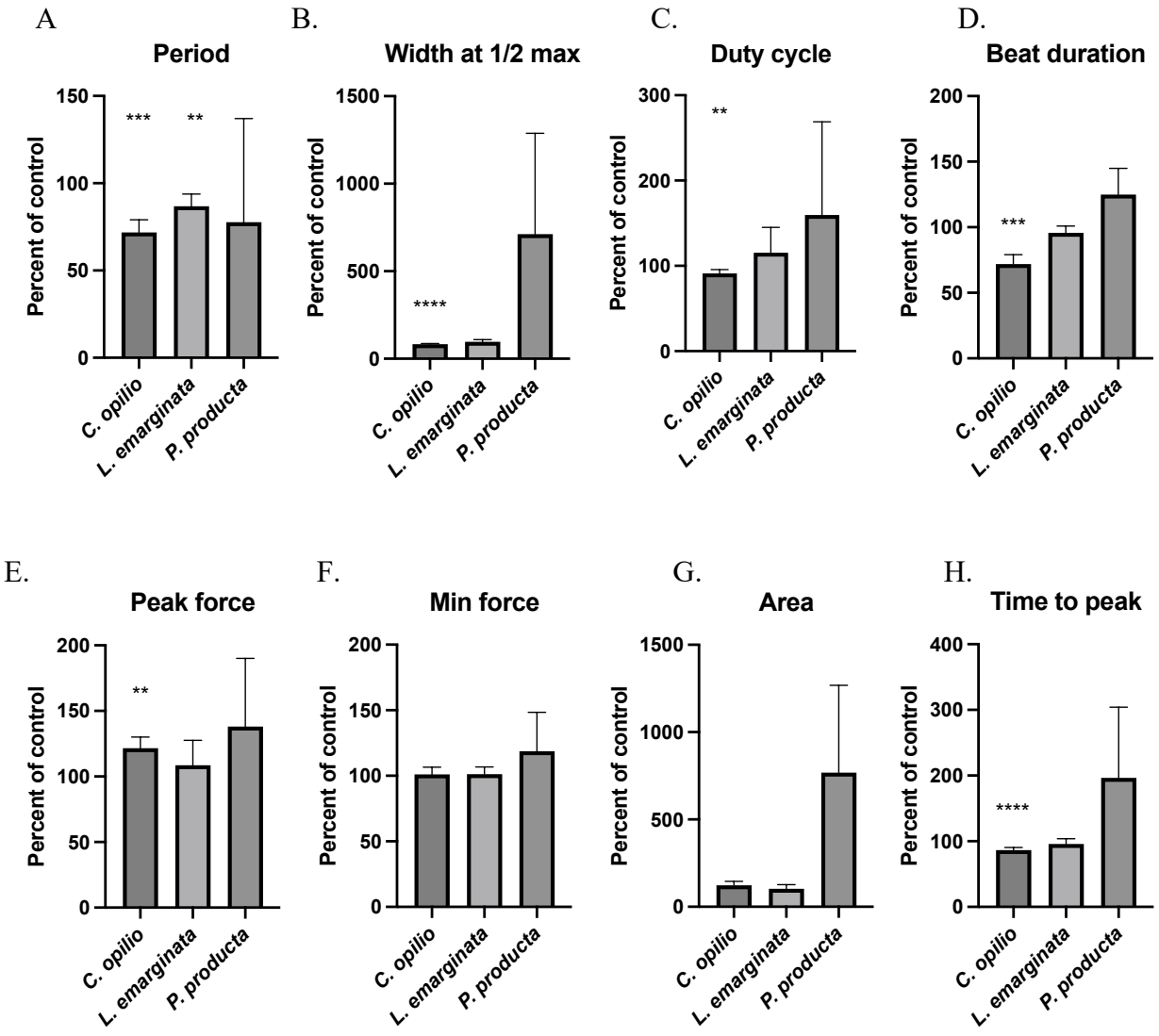


Figure 20. CLDH elicited widespread changes in the heartbeat of *C. opilio* and limited changes in the heartbeat of *L. emarginata*, but did not affect *P. producta.* Pictured are mean percent of control and standard error for eight parameters during CLDH application in *C. opilio* (n = 8)*, L. emarginata* (n = 9) and *P. producta* (n = 4). **A)** Cycle period was significantly decreased for *C. opilio* (paired t-test, $p < 0.001$) and *L emarginata* (paired t-test, $p < 0.01$) but unchanged for *C. opilio.* **B)** Width at ½ max was significantly decreased for *C. opilio* (paired t-test, $p < 0.0001$), but unchanged for the other two species. **C)** Duty cycle was significantly decreased in *C. opilio*

(paired t-test, $p < 0.01$) but not the other two species. **D)** Beat duration was significantly decreased for *C. opilio* (paired t-test, $p < 0.001$), but unchanged for the other two species. **E)** Peak force was significantly increased for *C. opilio* (paired t-test, $p < 0.01$) but unchanged for *L. emarginata* and *P. producta*. **F)** Minimum force was unchanged in all species. **G)** Area was not significantly different for any species, although it is likely that there was an outlier in *P. producta* that caused mean area to be so high. **H)** Time to peak was significantly decreased for *C. opilio* (paired t-test, $p < 0.0001$), but unchanged for the other two species. Again, it is likely that there is an outlier in *P. producta* that caused mean time to peak to be so high.

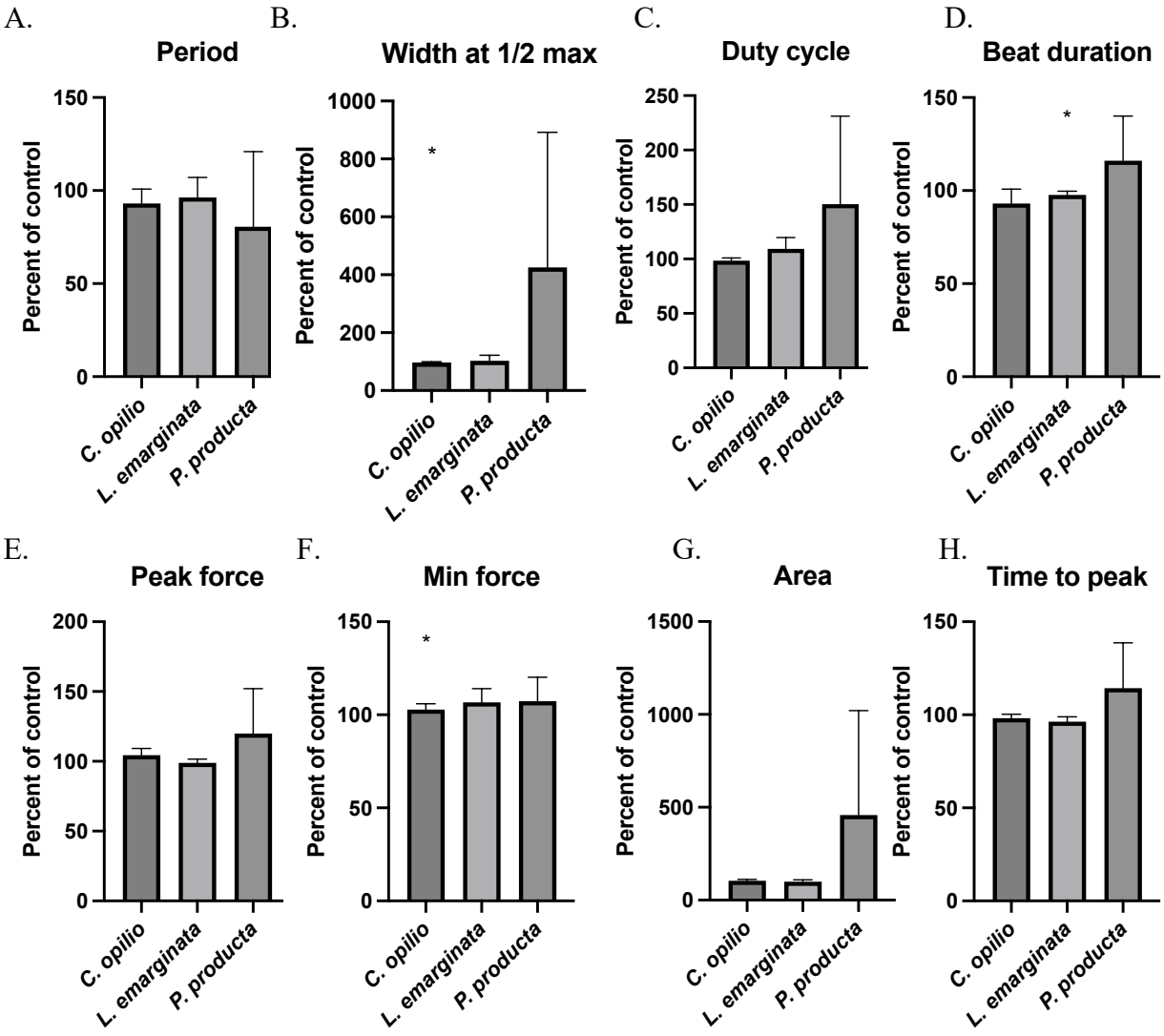


Figure 21. HIGSLYRamide caused limited changes in the heartbeat of *C, opilio* and *L. emarginata*, but did not affect cardiac function of *P. producta.* Pictured are mean percent of control and standard error for eight parameters during HIGSLYRamide application in *C. opilio* (n = 8)*, L. emarginata* (n = 9) and *P. producta* (n = 4). **A)** Cycle period was not significantly changed for any species. **B)** Width at ½ max was significantly decreased for *C. opilio* (paired t-test, $p < 0.05$), but not significantly different for the other two species. **C)** Duty cycle was not significantly changed for any species. **D)** Beat duration was significantly decreased for *L. emarginata* (paired t-test, $p < 0.05$), but unchanged for *C. opilio* and *P. producta.* **E)** Peak force

was not significantly different in any species. **F)** Minimum force was significantly greater for *C. opilio* (paired t-test, $p < 0.05$) but not significantly different for the other two species. **G)** Area was not significantly different for any species, although it is likely that there was an outlier in *P. producta* that caused mean area to be so high. **H)** Time to peak was not significantly different for any species.

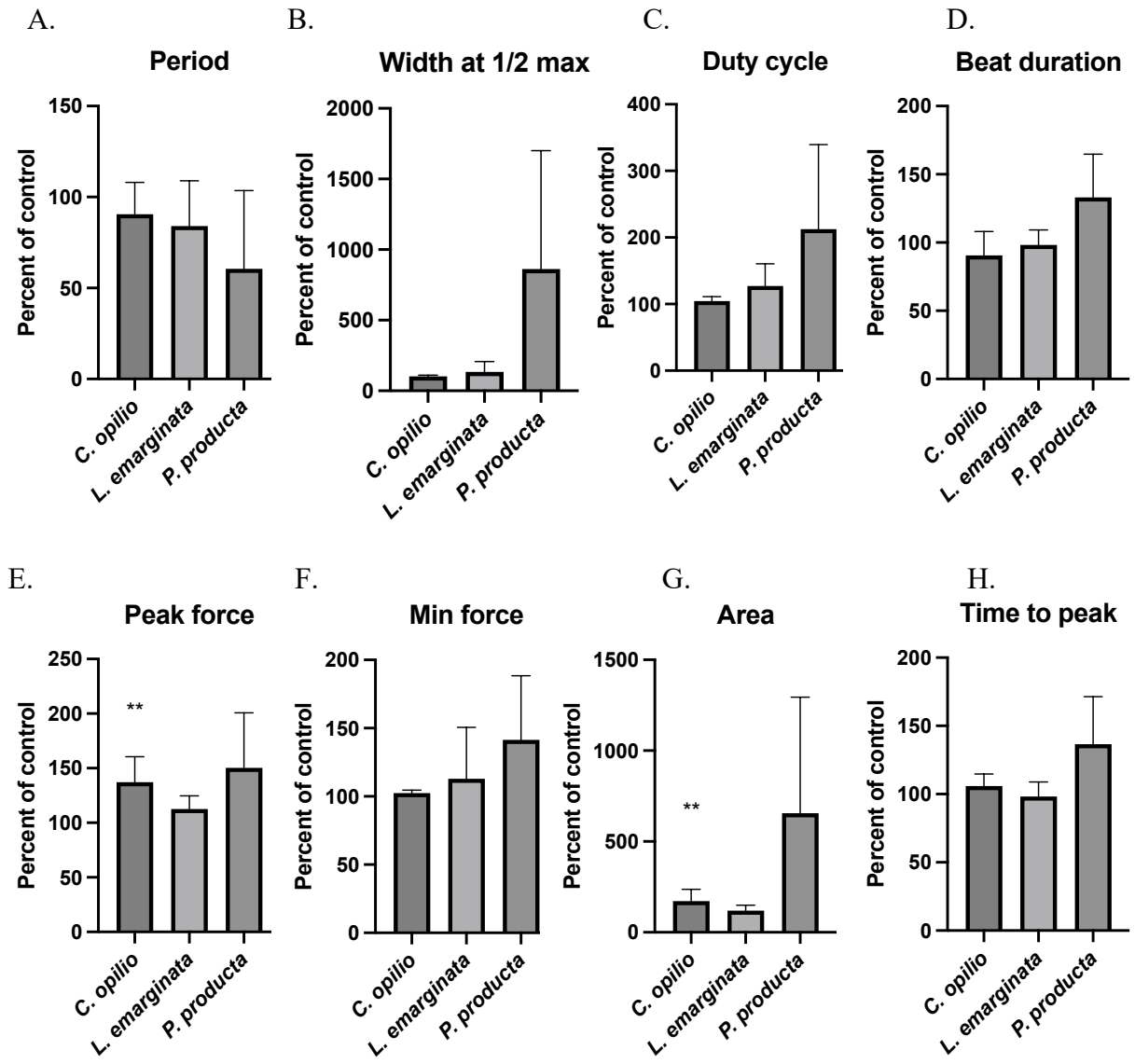


Figure 22. ACP had a limited effect on the heartbeat of *C. opilio,* but did not affect the other two species. Pictured are mean percent of control and standard error for eight parameters during ACP application in *C. opilio* (n = 8)*, L. emarginata* (n = 9) and *P. producta* (n = 4). **A)** Cycle period was not significantly changed for any species. **B)** Width at ½ max was not significantly changed for any species, although it is likely that there was an outlier in *P. producta* that caused mean area to be so high. **C)** Duty cycle was was not significantly changed for any species, although it is likely that there was an outlier in *P. producta* that caused mean area to be so high. **D)** Beat duration was not significantly changed for any species **E)** Peak force was significantly increased

in *C. opilio* (paired t-test, $p < 0.01$), but not significantly different in the other two species. **F)** Minimum force was not significantly changed for any species. **G)** Area was significantly increased in *C. opilio* (paired t-test, $p < 0.01$). Again, it is likely that there was an outlier in *P. producta* that caused mean area to be so high. **H)** Time to peak was not significantly changed for any species.

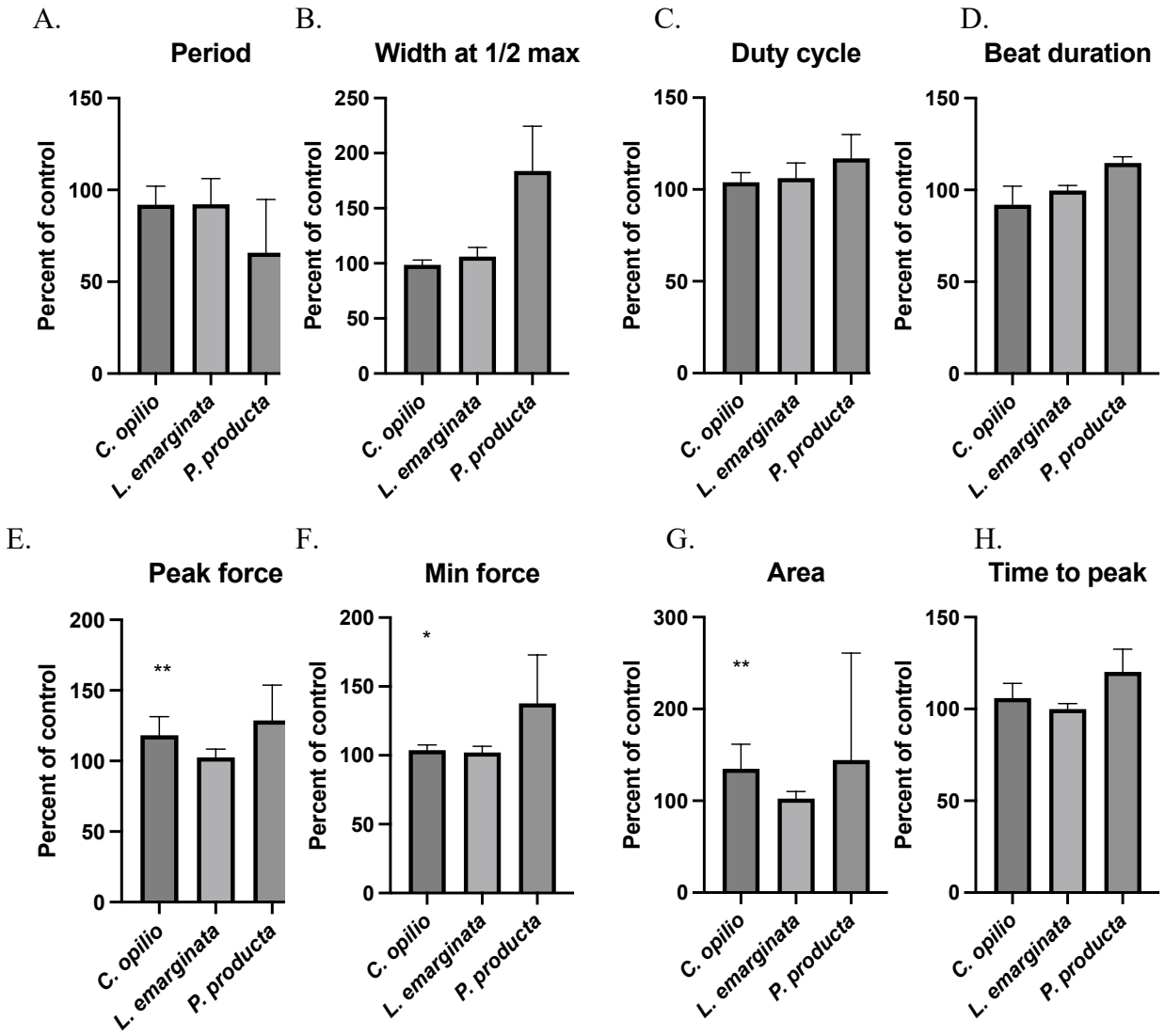


Figure 23. G-SIFamide had a limited effect on the heartbeat of *C. opilio,* but did not affect the other two species. Pictured are mean percent of control and standard error for eight parameters during G-SIFamide application in *C. opilio* (n = 8), L. *emarginata* (n = 9) and *P. producta* (n = 4). **A)** Cycle period was not significantly changed for any species. **B)** Width at ½ max was not significantly changed for any species. **C)** Duty cycle was not significantly changed for any species. **D)** Beat duration was not significantly changed for any species **E)** Peak force was significantly increased for *C. opilio* (*paired t-test,* $p < 0.01$), but not significantly different for the

other two species. **F)** Peak force was significantly changed for *C. opilio* (*paired t-test,* $p < 0.01$) although the type of change is unclear, but not significantly different for the other two species. **G)** Peak force was significantly increased for *C. opilio* (*paired t-test,* $p < 0.01$), but not significantly different for the other two species. **H)** Time to peak was not significantly changed for any species.

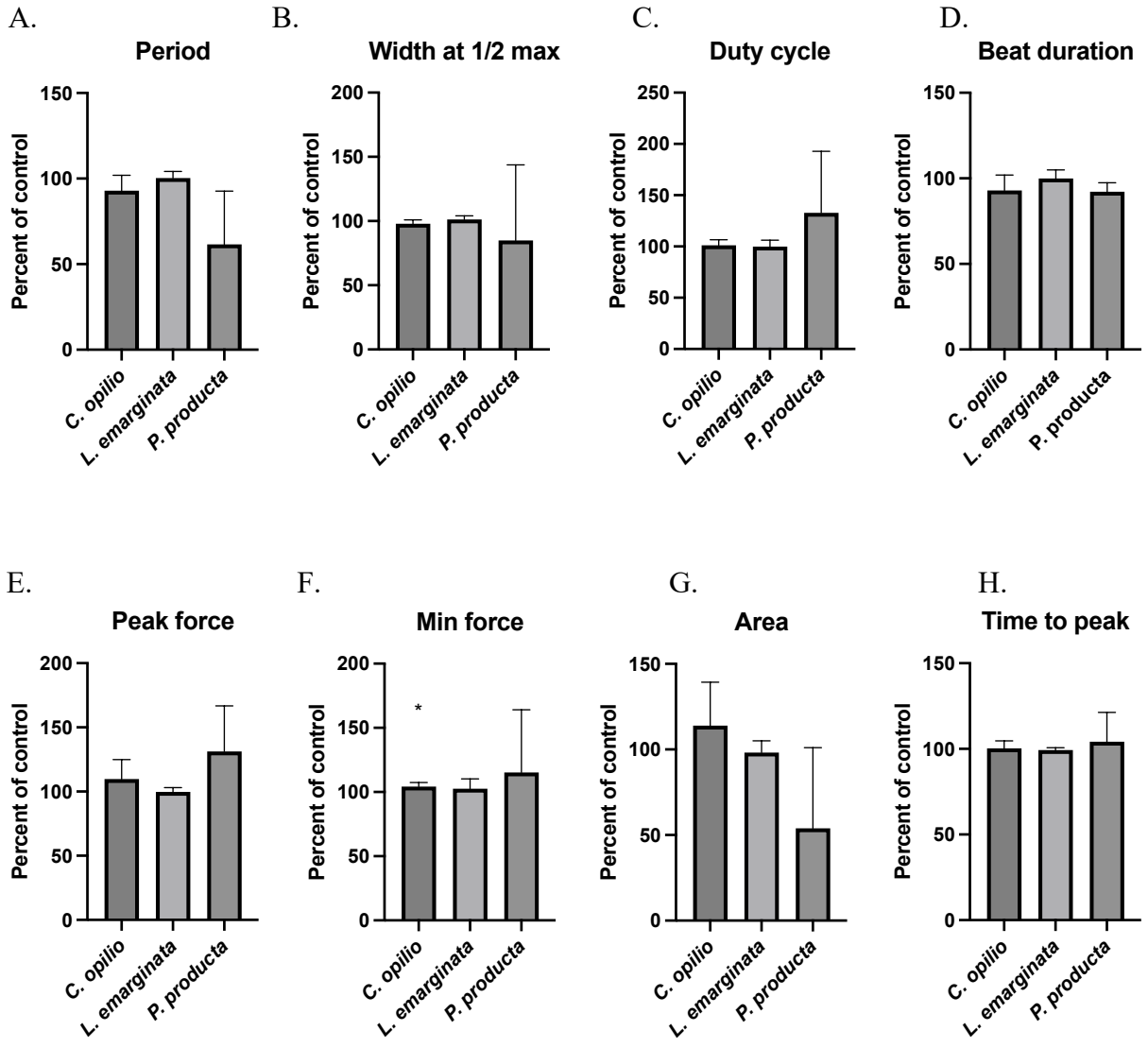


Figure 24. NRNFLRFamide had a very limited effect on the heartbeat of *C. opilio,* but did not affect the other two species. Pictured are mean percent of control and standard error for eight parameters during NRNFLRFamide application in *C. opilio* (n = 8), L. *emarginata* (n = 9) and *P. producta* (n = 4). **A)** Cycle period was not significantly changed for any species. **B)** Width at ½ max was not significantly changed for any species. **C)** Duty cycle was not significantly changed for any species. **D)** Beat duration was not significantly changed for any species **E)** Peak force was not significantly different in any of the species. **F)** Minimum force was significantly changed for *C. opilio* (paired t-test, $p < 0.05$) although the type of change is unclear, but not

significantly different for the other two species. **G)** Area was not significantly changed for any species. **H)** Time to peak was not significantly changed for any species.

Table 1. The STNS of *P. producta* responds to fewer neuromodulators than the whole hearts of *C. opilio* and *L. emarginata*. A paired two-tailed *t*-test was used to identify parameters with a significant difference between modulator and control ($p < 0.05$). (+) represents neuromodulators that have at least one parameter that is significantly different than control values for a given neuromodulator. (-) represents neuromodulators that do not have at least one parameter that is significantly different than control values for a given neuromodulator. Results with an asterisk are observational. *C. opilio* responded to 11/12 neuromodulators, *L. emarginata* responded to 10/12 neuromodulators, and *P. producta* responded to 8/12 neuromodulators.

STNS	***C. opilio***	***L. emarginata***	***P. producta***
CabTRP	+ ($n = 16$)	- ($n = 5$)	- ($n = 6$)
CCAP	+ ($n = 14$)	+ ($n = 4$)	- ($n = 7$)
Dopamine	+ ($n = 10$)	+ ($n = 4$)	+ ($n = 7$)
Myosuppressin	+ ($n = 12$)	+ ($n = 5$)	+ ($n = 5$)
Oxotremorine	+ ($n = 17$)	+ ($n = 5$)	+ ($n = 9$)
Proctolin	- ($n = 18$)	+ ($n = 5$)	+ ($n = 5$)
RPCH	+ ($n = 16$)	+ ($n = 4$)	- ($n = 5$)
HIGSLYRamide	-* ($n = 7$)	+* ($n = 4$)	+* ($n = 4$)
ACP	+* ($n = 7$)	+* ($n = 5$)	-* ($n = 4$)
G-SIFamide	+* $n = 7$)	+* ($n = 4$)	+* ($n = 5$)
CLDH	+* ($n = 7$)	+* ($n = 5$)	+* ($n = 4$)
NRNFLRFamide	+* ($n = 7$)	+* ($n = 5$)	+* ($n = 4$)

Table 2. The whole heart of *L. emarginata* responds to fewer neuromodulators than the whole hearts of *C. opilio* and *P. producta*. A 2-tailed *t*-test was used to identify parameters with a significant difference between modulator and control ($p < 0.05$). (+) represents species that have at least one parameter that is significantly different than control values for a given neuromodulator. (-) represents species that do not have at least one parameter that is significantly different than control values for a given neuromodulator. *C. opilio* responded to 11/12 neuromodulators, *L. emarginata* responded to 5/12 neuromodulators, and *P. producta* responded to 7/12 neuromodulators.

Whole heart	***C. opilio***	***L. emarginata***	***P. producta***
CabTRP	+ (*n* = 24)	- (*n* = 14)	+ (*n* = 15)
CCAP	+ (*n* = 24)	+ (*n* = 14)	+ (*n* = 15)
Dopamine	+ (*n* = 24)	+ (*n* = 14)	+ (*n* = 15)
Myosuppressin	+ (*n* = 24)	- (*n* = 14)	+ (*n* = 15)
Oxotremorine	+ (*n* = 24)	+ (*n* = 14)	+ (*n* = 15)
Proctolin	+ (*n* = 24)	- (*n* = 14)	+ (*n* = 15)
RPCH	- (*n* = 8)	- (*n* = 14)	- (*n* = 15)
HIGSLYRamide	+ (*n* = 8)	+ (*n* = 9)	- (*n* = 5)
ACP	+ (*n* = 8)	- (*n* = 9)	- (*n* = 5)
G-SIFamide	+ (*n* = 8)	- (*n* = 9)	- (*n* = 5)
CLDH	+ (*n* = 8)	+ (*n* = 9)	+ (*n* = 5)
NRNFLRFamide	+ (*n* = 8)	- (*n* = 9)	- (*n* = 5)

DISCUSSION

It was hypothesized that the variability of motor output required of the STNS was correlated to the sensitivity of the system to neuromodulators. The variability of a species' diet was assumed to represent a species' need for flexibility of motor output from the STNS. Therefore, it was expected that the STNS of *P. producta*, the exclusive feeder, would respond to fewer neuromodulators than the STNS of the opportunistic feeders *C. opilio* and *L. emarginata*, both of which presumably require highly variable STNS motor output to digest the variety of fauna that these species are known to consume. Regarding the whole heart, it was hypothesized that the observed sensitivity of motor output to neuromodulators of the *majoid* species' cardiac systems would not correlate with diversity of diet.

The former hypothesis concerning the relationship between the variability of a species' diet and the neuromodulatory capacity of the STNS was supported by the data. The STNS of the exclusive feeder, *P. producta,* responded to fewer neuromodulators than the STNS of the opportunistic feeders, *C. opilio and L. emarginata.* Altogether, these results support the hypothesis that the relative sensitivity of the STNS to neuromodulators would correlate with the variability of the species diet, suggesting an evolutionary link between neuromodulatory capacity and the need for variable motor output.

It was also hypothesized that the neuromodulatory capacity of the whole heart would not correlate with the species' diet. It was found that the whole hearts of *C. opilio* and *P. producta* were sensitive to more neuromodulators than the whole heart of, *L. emarginata*, even though *L. emarginata* eats a wide variety of food types. Altogether, these results support the hypothesis that the relative sensitivity of the whole heart to neuromodulators would not correlate with species' diet.

Overall, the hypothesis that the neuromodulatory capacity of the STNS correlates with dietary diversity was supported. The results detailed in this study lend credence to the idea that the evolutionary basis for neuromodulatory capacity of a system is related to the behavioral demands on that system.

Presence of neuromodulators versus presence of neuromodulator receptors

As previously stated, it was found that "vestigial" neuromodulators that had no effect on the motor output of the STNS were still present in STNS tissue of *P. producta* (Dickinson et al., 2008). Although mass-spectrometry analysis has not been conducted on the opportunistic feeders used in this study, it is quite possible that nonfunctional neuromodulators are also present in the relevant local tissue of these species. The presence of certain neuromodulators in the tissues of organs they do not appear to modulate suggests that the limiting factor for neuromodulation in such systems is not the synthesis, or lack thereof, of certain neuromodulators, but the presence or absence of localized receptors for neuromodulators. In regard to *P. producta*, this might mean that *P. producta* had an opportunistically-feeding ancestor that progressively limited its diet to kelp. Hypothetically, as this ancestor evolved into the kelp crab, it continued to produce all the same neuromodulators in its pericardial organ and X-organ sinus gland, but neurons local to the STNS stopped expressing receptors for select neuromodulators over time, limiting their effect on digestive output. Data confirming the presence or non-presence of receptors for "vestigial" neuromodulators in *P. producta, C. opilio,* and *L. emarginata* is necessary to further understand why some neuromodulators are ineffectual in the STNS and whole heart systems, and how neuromodulatory sensitivity evolves. Thus far, a transcriptome for each species has been

generated, but identification and confirmation of the presence of putative receptors has not been carried out yet.

Neuromodulatory redundancy

Another consideration to be addressed is that multiple neuromodulators may evoke the same functional output in a system, creating redundancy of neuromodulation. For example, CabTRP and CCAP elicited nearly identical responses in both the STNS (Figure 2, figure 3) and heart (Figure 10, figure 11) of *C. opilio.* If CabTRP and CCAP cause the same effects in the STNS and the heart, then increased neuromodulatory capacity of the system does not, in the cases of CCAP and CabTRP, correlate to increased diversity of motor output. In *C. borealis*, the effects of proctolin on the STNS were analyzed via patch-clamp electrophysiology and it was found that six other neuropeptides acted on the same voltage-dependent current and showed "proctolin-like" current-voltage relationships, demonstrating redundancy of neuromodulatory effect like the redundancy observed in this study (Swensen & Marder, 2000). However, it is also worth noting that the receptors for the proctolin-like neuropeptides are in different anatomical locations, so the functional effect of each neuropeptide may be different.

These observations contradict our evolutionary hypothesis that neuromodulatory capacity evolves in response to the required diversity in functionality of the system. However, the possibility of comodulation by multiple neuromodulators helps to rescue our evolutionary hypothesis. Computer modelling of neuromodulatory scenarios has shown that the effects of one direct neuromodulator may alter conductances in such a way that when a second comodulator is introduced, there may be a completely different output than when either neuromodulator is applied on its own (Goldman et al., 2001). Additionally, it has been shown that the application of

one neuromodulator may change the response to another (Dickinson et al., 1997). In the spiny lobster *P. interruptus,* the cardiac sac motor pattern was activated in the presence of RPCH but not proctolin. However, when proctolin was applied shortly after RPCH, it was able to activate the cardiac sac motor pattern. Therefore, it is plausible that the neuromodulators that appear to have redundant effects on the target system when applied alone might have unique effects when applied in concert with another neuromodulator, thereby increasing diversity of neural output. For example, in the STNS, CCAP and CabTRP are comodulators that have distinct but converging effects on the same voltage channel, producing a different output when they are applied together than when either is applied on its own (DeLong et al., 2009). Therefore, neuromodulators that seem to have redundant effects may in fact still contribute to a motor system's functional diversity.

Limitations

This study was subject to a number of limitations. Firstly, the possibility of comodulation presents a confounding factor in this study. We made a concerted effort to remove preexisting modulatory effects in a preparation by "washing" the preparation with saline for one hour before applying any neuromodulators. This means that some of the "silent" neuromodulators that were applied may be dependent comodulators that would in fact evoke diverse patterns of neural output in the presence of a different neuromodulator.

Secondly is the possibility of state-dependent modulation in our organisms. Neurons may respond to the same inputs differently based on the physiological state of a network, a phenomenon known as state-dependent neuromodulation (Nadim et al., 2008; Nusbaum & Marder, 1989). While I did my best to keep the internal states of our organisms consistent with

one another throughout the course of the experiment and also washed out endogenous neuromodulators during the wash period before any exogenous neuromodulators were applied, we still observed a high degree of variation in neuromodulatory sensitivity between individual organisms. This intraspecies variability can at least in part be chalked up to a degree of variation in the internal states of our organisms and resultant state-dependent modulation by the neuromodulators I applied.

Thirdly, the whole heart including the muscle was used to assess the heart's neuromodulatory capacity, whereas for the STNS, I used an isolated nervous system preparation with all other tissue removed. Because I measured the muscle output of the heart, not the neural activity in the CG, I can conclude that the application of various neuromodulators does in fact alter the function of the heart. However, because I measured the neural output of the STNS without verifying that the neural output has a functional correlation (eg. causes the muscles of the stomach to contract), I cannot necessarily assume that a significant change in the pattern of neural firing in the STNS is an accurate proxy for the functional motor output of the stomach.

Additionally, it is known that in the STNS some neuromodulators, including dopamine, proctolin, RPCH, and CCAP, operate at the level of the muscle/neuromuscular junction as well as the isolated nervous system, evoking measurable changes in nerve-evoked contractions in the muscles of the gastric mill (Jorge-Rivera & Marder, 1996; Jorge-Rivera et al., 1998). Neuromodulators may act on the muscle/neuromuscular junction in the heart as well (Stevens et al., 2009; Oleisky et al., 2021). The STNS preparation did not allow me to assess neuromodulatory effects at the neuromuscular junction, but because I measured the muscular output of the whole heart, the whole heart data does include neuromodulatory effects on the

neuromuscular junction and muscle itself. Thus, I cannot draw a direct comparison between neuromodulatory capacity of the whole heart, and neuromodulatory capacity of the STNS.

Whole heart adaptive sensitivity

The data showed that whole heart of *L. emarginata* had reduced neuromodulatory capacity in comparison to *C. opilio* and *P. producta,* although *P. producta* responded to fewer neuromodulators than *C. opilio*. As previously stated, the most obvious distinction between *L. emarginata* and the other two crabs is that*,* while *L. emarginata* is found along the coast from Nova Scotia to the Gulf of Mexico, we sourced our specimens from the warm waters of Florida (Nizinski, 2003). In contrast, the *C. opilio* specimens were sourced from the frigid waters of Halifax, Canada, and *P. producta* specimens were sourced from the San Juan Archipelago off the coast of Washington State, where the water is slightly warmer. It is possible the colder the water is, the greater demands are placed on the heart to vary its functional output, correlating to greater neuromodulatory capacity. Why the heart might need more neuromodulation in cold waters is not entirely clear. It is known that crustaceans display heart rate hysteresis in response to temperature: the heart rate speeds up during heating, and slows down during cooling, demonstrating their ability to physiologically regulate their hearts in a similar manner to ectothermic ("cold-blooded") reptiles (Goudkamp et al., 2004; Worden et al., 2006). Perhaps cold-water species require a higher degree of temperature-dependent regulation than warm-water species to maintain homeostasis, and greater neuromodulatory capacity helps the organism to achieve greater regulation.

It is also possible that the neuromodulatory capacity of the heart is related to each species' mode of locomotion. Unlike most crabs, *L. emarginata* moves forward 80% of the time, instead of sideways (Vidal-Gadea & Belanger, 2009). *C. opilio's* and *P. producta's* preferred modes of

locomotion have not been studied and no specimens are currently available for observation, but anecdotally, I recall that these two species move sideways. Crustaceans that move forward instead of sideways have evolved a variety of physiological and neurological adaptations to go along with their unusual gait (Vidal-Gadea et al., 2008; Vidal-Gadea & Belanger, 2013). For example, in *L. emarginata,* proximal motor neurons are more numerous than distal motor neurons as compared to sideways-walking crabs (Vidal-Gadea & Belanger, 2013). As the supplier of blood and therefore oxygen to the muscles, the cardiac system must evolve to support the energy demands of a crab's preferred mode of locomotion, and the energy demands of forward locomotion and sideways locomotion are likely different. In this light, it is not far-fetched to suggest that some of the physiological and neurological adaptations of forward-walking crabs involve reducing neuromodulatory capacity in the heart.

Finally, *P. producta* and *L. emarginata* are more closely related to each other than either species is to *C. opilio* (Figure 1). Thus, it is possible that the lesser neuromodulatory capacity of the heart in *L. emarginata* is due to its phylogenetic closeness to *P. producta*, and is not necessarily functionally correlated.

Future directions

Due to difficulty in obtaining certain crab species and the time limitations of an honors project, we did not gather sufficient *n* for some neuromodulators. It is important to bolster our sample size to overcome intra-species differences and the confounding factor of possible state dependency in some organisms. Experiments will continue into the summer with the aim of achieving sufficient sample sizes as soon as possible. Transcriptomes for different tissues from

each species, including the STNS and heart, should be generated and receptor expression levels should be quantified to elucidate the mechanism underlying neuromodulatory capacity.

Additionally, although this project dealt primarily with the evolutionary implications of neuromodulatory capacity in the stomach, it raised intriguing questions about the neuromodulatory capacity in the heart of different crab species. I have advanced a few hypotheses to explain why the hearts of *L. emarginata* had reduced neuromodulatory capacity as compared to *C. opilio* and *P. pugettia*, but further investigation is needed to explain our cardiac data. One possible experiment would be to gather *L. emarginata* specimens from Florida and from Connecticut to test whether the hearts of the warm-water and cold-water populations have differing neuromodulatory capacities. This could potentially tell us whether the neuromodulatory capacity of the heart is related to temperature, as opposed to mode of locomotion or other unknown factors.

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