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The role of behavioral diversity in determining the extent to which the cardiac ganglion is modulated in three species of crab

Grace Bukowski-Thall
Bowdoin College

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An Honors Paper for the Department of Biology

By Grace Bukowski-Thall

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Table of Contents

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ABSTRACT

Central pattern generators (CPGs) are neural networks that generate the rhythmic outputs that control behaviors such as locomotion, respiration, and chewing. The stomatogastric nervous system (STNS), which contains the CPGs that control foregut movement, and the cardiac ganglion (CG), which is a CPG that controls heartbeat, are two commonly studied systems in decapod crustaceans. Neuromodulators are locally or hormonally released neuropeptides and amines that change the output patterns of CPGs like the STNS and CG to allow behavioral flexibility. We have hypothesized that neuromodulation provides a substrate for the evolution of behavioral flexibility, and as a result, systems exhibiting more behavioral flexibility are modulated to a greater degree. To examine this hypothesis, we evaluated the extent to which the STNS and the CG are modulated in the majoid crab species *Chionoecetes opilio, Libinia emarginata,* and *Pugettia producta*. *C. opilio* and *L. emarginata* are opportunistic feeders, whereas *P. producta* has a highly specialized kelp diet. We predicted that opportunistic feeding crabs that chew and process a wide variety of food types would exhibit greater STNS neuromodulatory capacity than those with a specialized diet. The STNS of *L. emarginata* and *C. opilio* responded to the seven endogenous neuromodulators oxotremorine, dopamine, CabTrp Ia, CCAP, myosuppressin, proctolin, and RPCH, whereas the STNS of *P. producta* only responded to proctolin, oxotremorine, myosuppressin, RPCH (25% of the time), variably to dopamine, and not at all to CabTrp and CCAP. Because *P. producta, L. emarginata,* and *C. opilio* all belong to the Majoidea superfamily, their primary distinctions are their feeding habits. For this reason, we further predicted that there would be no relationship between diet and modulatory capacity in the cardiac ganglion (CG) of the neurogenic heart. This would suggest that a lack of STNS modulatory capacity in *P. producta* relative to *L. emarginata* and *C. opilio* is specific to evolved

foregut function. Whole-heart recordings from *P. producta* indicated that, unlike the STNS, the CG responds to CabTrp and CCAP. *P. producta* hearts also responded to oxotremorine and inconsistently to dopamine and proctolin. The CG of *C. opilio* was modulated by CabTrp, CCAP, dopamine, proctolin, myosuppressin, and oxotremorine, but not RPCH. The CG of *L. emarginata* responded to CCAP, and inconsistently to CabTrp, dopamine, and proctolin, but not to myosuppressin, RPCH, and surprisingly oxotremorine. Although cardiac responses were not identical between species, opportunistic and specialist feeders responded more similarly to the modulators tested in the heart than in the STNS. Notably, *P. producta* responded to each modulator in a similar manner to *C. opilio* and/or *L. emarginata.* However, *L. emarginata's* surprising lack of cardiac response to oxotremorine suggests that phylogenetic closeness may not control for differences in CG and STNS function between species. Nevertheless, sample sizes of all three species were quite small, and individual differences lead to inconsistencies in the data. As a result, sample size must be enlarged to draw firm conclusions.

INTRODUCTION

Central pattern generators

Central pattern generators (CPGs) are neural networks that generate the rhythmic outputs that control behaviors such as breathing, walking, flying, and chewing in both invertebrates and vertebrates. CPGs can continue to function in the absence of other nervous system input (Marder & Calabres). Locally or hormonally released molecules such as neuropeptides and amines can modulate CPGs by modifying action potential firing patterns (Brezina et al., 2010; Jekely et al., 2018; Ma et al., 2008; Marder et al., 2005; Stein, 2009; Taghert et al., 2012). Neuromodulators typically bind to receptors such as G-protein coupled receptors and ionotropic receptors (Gray & Golowasch, 2016; Clark et al., 2008). Modulation of CPGs causes variation in motor pattern, which, for example, may allow an organism to go from a walk to a run, or to perform different kinds of chewing (Nusbaum & Beenhakker, 2002). As a result, neuromodulation gives animals the behavioral flexibility necessary to adapt to changes in both their internal and external environments. Because of their relatively simple central nervous systems, decapod crustaceans, which include species such as lobsters, crabs, shrimp, and crayfish, are good model organisms in which to study the modulation of central pattern generators.

Peptides, which are short, amine-linked chains of alpha-amino acids, are the most abundant type of neuromodulator in the crustacean central nervous system. Neuropeptides are synthesized and released from neurons onto a target. In an autocrine functioning system, peptides target the neuron releasing it; in a paracrine system, peptides target local tissues or neurons; in a hormonal release system, peptides act on distant neurons or tissues. Unlike classical neurotransmitters, a peptide can be released at any point along a neuron, not just at a synapse. In the crustacean central nervous system, which consists of ganglia interconnected through a

longitudinal nerve cord, neuropeptides are released from the neuropil of these ganglia and hormones are released from neuroendocrine cells (Christie et al., 2010).

The stomatogastric nervous system as a model system

The stomatogastric nervous system (STNS) contains several prominent CPGs in the crustacean central nervous system. The STNS is composed of four ganglia containing the CPGs that control the rhythmic movement of muscles in the esophagus, cardiac sac, gastric mill, and pylorus of the foregut (Selverston and Moulins, 1987; Nusbaum & Beenhakker, 2002; Marder & Bucher, 2007; Marder & Weimann, 1992) (Fig. 1A). Most decapod crustaceans rely on foregut movement for chewing; when an animal like a crab eats, large chunks of food travel through the esophagus to the cardiac sac for storage and then to the gastric mill, which is lined with teeth-like ridges that break the food down into more digestible pieces as the foregut moves. The broken-down food is then filtered through the pylorus and transported to the midgut for digestion (Marder & Weimann, 1992, Nusbaum & Beenhakker, 2002). The STNS is modulated by neuromodulators released locally or hormonally. Modulation of the foregut regulates the rhythmic behaviors responsible for foregut motion and flexibility.

The cardiac ganglion as a model system

The cardiac ganglion (CG) is a CPG that controls the crustacean single-chambered neurogenic heart. The CG is located in the lumen of the heart and drives the heartbeat by sending bursts of potential to the heart muscles. The CG is composed of four small pre-motor pacemaker neurons that interact with five larger motor neurons, altogether forming a CPG circuit (Cooke, 2002; Selverston, 2010) (Fig. 1B). The rhythmic activity of the pacemaker neurons is transmitted

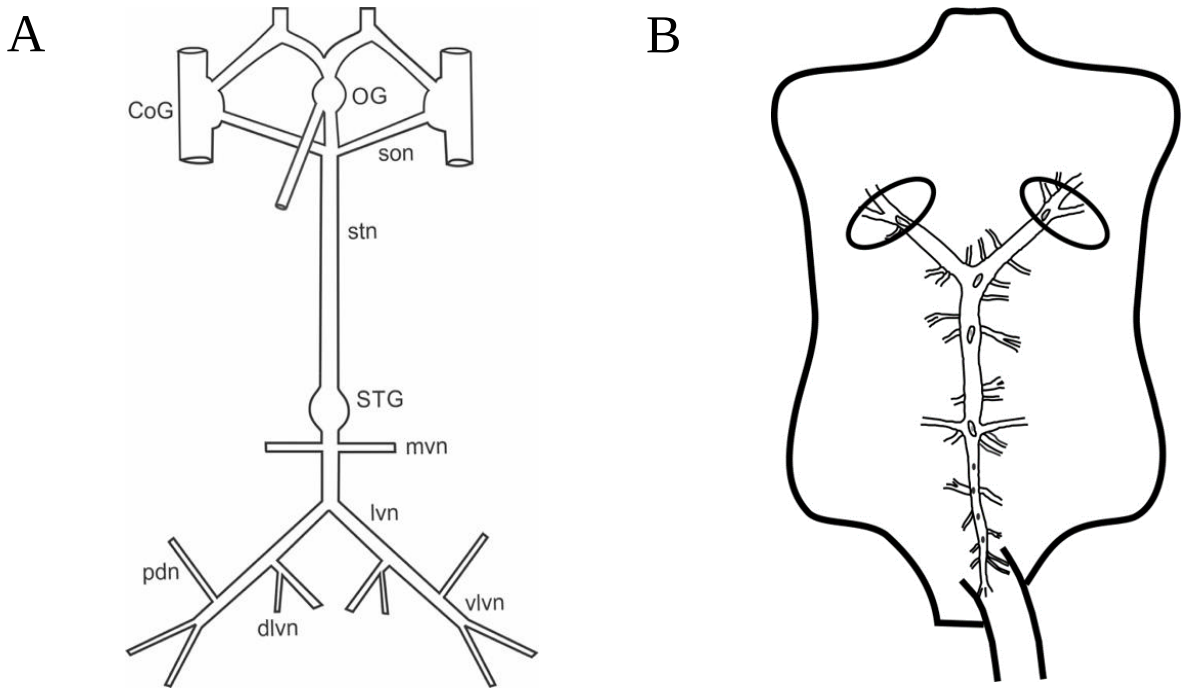

Figure 1. Diagrammatic representation of (A) the stomatogastric nervous system (STNS) and (B) the neurogenic heart and cardiac ganglion (CG). The STNS is composed of four ganglia and control foregut movement. The CG regulates the beating of the neurogenic heart. The CG contains five large motor neurons and four smaller pre-motor pacemaker neurons. Hemolymph flows in the heart through the ostia, and out through the arteries. The ostia are the circles located on top of the diagram. Only the sternal posterior artery is shown.

to the larger motor neurons through both chemical and electrical synapses, allowing the pacemaker and motor neurons to fire synchronously (Selverston, 2010; Cooke, 2002). Synchronous firing of the motor neurons is a unique feature of the crab cardiac system; in lobsters the motor neurons fire in a coordinated manner, but not synchronously (Fort et al., 2004). Additionally, the activity of the CG is regulated by feedback from both nitric oxide, which is produced in the cardiac muscle (Mahadeven et al., 2002) and stretch on the CG neurons (Cooke, 2002; Alexandrowicz, 1932; Garcia-Crescioni et al., 2010).

Neuromodulation of the STNS and the CG not only influences the electrical output of these CPGs, but also the conversion of that electrical output to muscular movements and

contractions. The CG is modulated both by locally released molecules innate to the CG such as dopamine (Fort et al., 2004), and by cardioactive substances from neuroendocrine organs outside the heart (Cooke, 2002; Christie, 2011). At low concentrations, neuromodulators can act peripherally on the neuromuscular junction and the muscle to change the amplitude and frequency of neuromuscular heart contractions (Fort et al., 2007; Dickinson et al., 2018). For this reason, modulation patterns in the CG can be measured with both whole heart neuromuscular recordings as well as CG extracellular recordings. Since the cardiac ganglion is an isolated portion of the nervous system, the whole heart can continue to beat rhythmically for many hours after dissection from the body (Cooke, 2002; Selverston, 2010). The cardiac ganglion can also be extracted and continue to produce rhymical bursts that can be recorded extracellularly.

Neuroendocrine control of the STNS and the CG

The heart pumps hemolymph through a set of arteries into the hemocoel in an open circulatory system (Brusca & Brusca, 2003). Hemolymph flows into the heart through the ostia, and out through the arteries (Fig. 1B). The secretory nerve terminals of neuroendocrine organs are in direct contact with the hemolymph. Decapod crustaceans have two neuroendocrine organs, the X-organ (XO)-sinus gland (SG) system, which is located in the eyestalk ganglia, and the pericardial organ (PO), which is located on the lateral wall of the pericardial chamber surrounding the heart. Both the XO-SG system and the PO are highly conserved and are responsible for releasing most of the neuromodulators that act on the STNS and the CG (Christie, 2011).

Totti. Chionoecetes opilio. 13, April 2019. Wikimedia Commons, the free media repository [Accessed 9, May 2020]. https://upload.wikimedia.org/wikipedia/commons/1/19/Chionoecetes_opilio_Kinosaki.jpg

Nodder RP. William Elford Leach's "A Zoological Mescellany" Vol. 2, 1815. Plate 108: *Libinia emarginata.* https://commons.wikimedia.org/wiki/File:Libinia_emarginata.png

Elliot L. Northern Kelp Crab or Shield-backed Kelp Crab. 2, July 2007. Flickr [Accessed 9, May 2020]. https://www.flickr.com/photos/25980517@N03/4191054695/in/photostream/.jpg

Figure 2. *Chionoecetes opilio* (A), *Libinia emarginata* (B), and *Pugettia producta* (C). *C. opilio* and *L. emarginata* are both opportunist feeders that scavenge for a variety of food types, whereas *P. producta* is a specialist feeder that subsists off only kelp. All three species belong to the Majoidea superfamily. Pictures are not to scale.

An evolutionary basis for neuromodulatory capacity

Although a great deal is known about CPG function, not much is known about the extent to which CPGs are modulated, known as "neuromodulatory capacity." For instance, what evolutionary forces determine the modulatory capacity of a system, and what mechanisms underlie variations in modulatory capacity? Upwards of seventy different types of neuromodulators have been identified in the STNS of decapod crustaceans. However, the specific roles of these neuromodulators as well as the reason for their abundance is undetermined. We have hypothesized that neuromodulation provides a substrate for the evolution of behavioral flexibility. Under this hypothesis, we expect that different modulators work in tandem to allow organisms to adapt to different diets, environmental conditions, life stages, or ecological niches. As a result, CPG systems exhibiting more behavioral flexibility should have greater neuromodulatory capacity than systems exhibiting less behavioral diversity. Nevertheless, it remains unclear why the rhythmic outputs in some species are less influenced by certain neuromodulators; that is, why a CPG in one species may have a lower neuromodulatory capacity than the same CPG in another species.

We evaluated the extent to which different dietary behaviors are correlated with neuromodulatory capacity in the STNS and CG of three species of crab. Like most decapod crustaceans, the majoid crabs *Libinia emarginata* (portly spider crab) and *Chionoecetes opilio* (snow crab) are opportunistic feeders that scavenge for a great variety of food types (Fish & Fish, 1989) (Fig. 2). Studies of *C. opilio* stomach contents have revealed shrimp, gastropods, fish, sea urchins, and other crustaceans. *C. opilio* have even been observed to exhibit cannibalistic behavior (Hubert & Dawe, 2003). Among other things, *L. emarginata* have been found to eat algae, ciliates, brine shrimp (Bigford, 1977) and starfish (Aldrich, 1976). *L. emarginata* are found in the subtidal zones of the North Atlantic from Nova Scotia to the Florida Keys and through the Gulf of Mexico. *C. opilio* live in the northwest Atlantic (Dawe & Colbourne, 2002) and north Pacific. By contrast, *Pugettia producta* (kelp crab), which also belongs to the Majoidea superfamily, has a highly specialized kelp diet (Hines, 1982). *P. producta* are found along the Pacific Northern American coast from southern Alaska to Northern Mexico (Lamb & Hanby, 2005).

Under our hypothesis that modulation provides a substrate for the evolution of behavioral flexibility, we predicted that specialist feeders like *P. producta* would exhibit less STNS neuromodulatory capacity than opportunistic feeders like *L. emarginata* and *C. opilio.* This hypothesis is based on the idea that a species like *P. producta* that consumes fewer food-types will require less foregut movement for chewing than species like *C. opilio* and *L. emarginata* that have more diverse diets. As a result, *P. producta* will have confronted less selective pressure to maintain extensive modulation of the STNS.

Previous data have shown that the STNS of opportunistically feeding *Cancer borealis* is

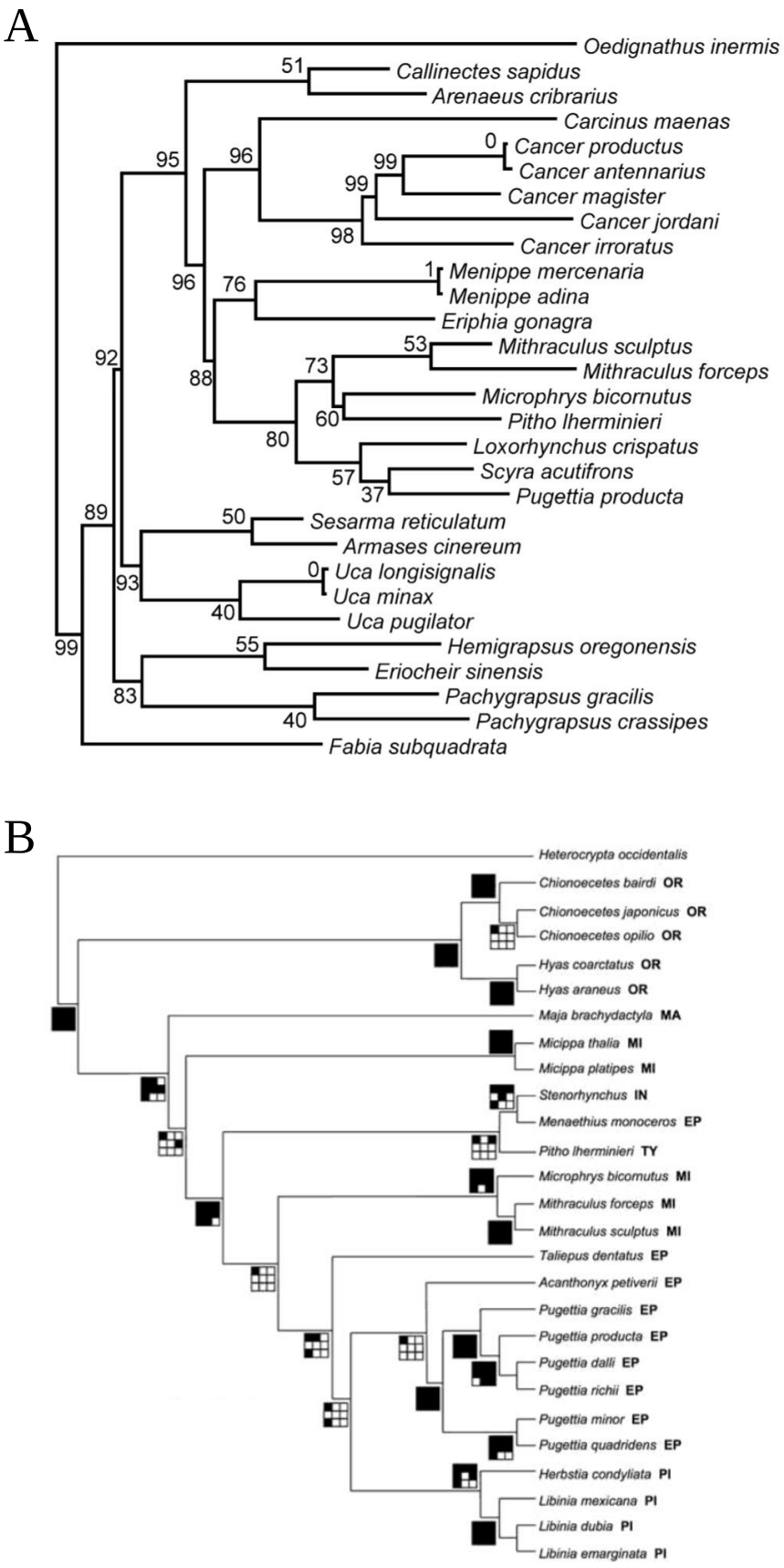

Figure 3. *Pugettia producta* are more closely related to *Chionoecetes opilio* and *Libinia emarginata* than to *Cancer* crabs. (A) *Cancer* crabs belong to the Canceroidea superfamily, whereas *P. producta* are a part of the Majoidea superfamily (From Mahon et al., 2008). (B) *P. producta, L. emarginata,* and *C. opilio* all occupy the Majoidea superfamily. *P. producta* and *L. emarginata* belong to the Epialtidae family and *C. opilio* belongs to the Oregoniidae family. (from Martin et al., 2009).

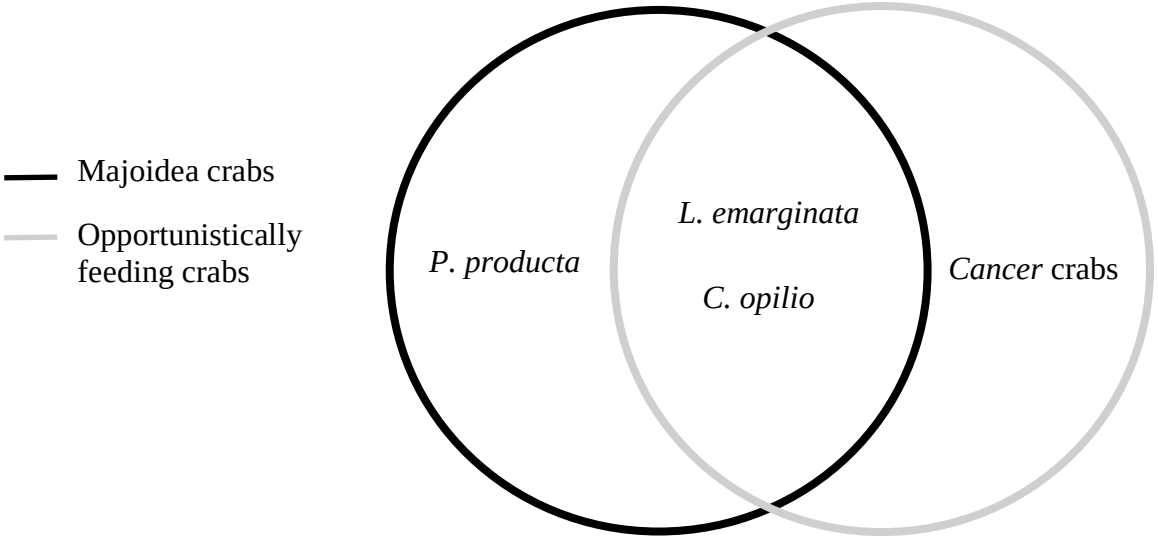

Figure 4. Specialist feeder, *Pugettia producta*, and opportunistic feeders, *Libinia emarginata* and *C. opilio*, all belong to the Majoidea superfamily, whereas opportunistically feedeing *Cancer* crabs belong to the Cancridae family. We compared the neuromodulatory capacity of the STNS and CG of kelp crab *P. producta* to those of opportunistic feeders, *Chionoecetes opilio* and *L. emarginata*, rather than to *Cancer* crabs (as in Dickinsons et al., 2008). Because *P. producta, C. opilio,* and *L. emarginata* all belong to the Majoidea superfamily, we will be able to discern whether differences in modulatory capacity are due to phylogenetic differences (Alexandra Miller Honors Thesis, 2018). Images are not to scale. *Cancer* crab image from nicepng.com.

sensitive to a wider array of neuromodulators than the STNS of *P. producta*. However, these species are not closely related phylogenetically (Fig. 3A). Because *P. producta, L. emarginata,* and *C. opilio* all belong to the Majoidea superfamily, they should not have many differences in biological function due to phylogeny; their primary distinctions are their feeding habits (Fig 3B). For this reason, we further predicted that there would be no relationship between diet and modulatory capacity in the CG of the heart. This would suggest that a lack of STNS modulatory capacity in *P. producta* relative to *L. emarginata* and *C. opilio* is specific to evolved foregut function (Fig. 4). In other words, crabs that digest and chew more food types have likely evolved greater foregut movement and flexibility than crabs with specialized diets; however, the mechanistic and functional organization of neuromodulation in the heart should be unaffected by feeding behavior.

Neuromodulators

Seven highly conserved, endogenous neuromodulators were tested on the STNS and CG of *C. opilio, L. emarginata* and *P. producta* to gauge differences in modulatory capacity between the three species. These neuromodulators included the peptides crustacean cardioactive peptide (CCAP), tachykinin-related peptide Ia (CabTrp Ia), myosuppressin, proctolin, and red pigment concentrating hormone (RPCH), as well as the amine dopamine and the muscarinic acetylcholine agonist, oxotremorine. Each of these neuromodulators has multiple roles in the crustacean central nervous system, and some act on the same CPGs but elicit different response patterns.

Oxotremorine

Oxotremorine is an amine that is an agonist of acetylcholine, which binds to muscarinic receptors that form G protein-coupled receptor complexes in neurons. Oxotremorine has been found to elicit rhythmic activity in the leg ganglia of crayfish (Cattaert et al., 1995), as well as modulate the pyloric pacemaker neurons in the STNS of the lobster species, *Jasus lalandii* and *Palinarus vulgaris* (Nagy & Dickinson, 1983).

Dopamine

Dopamine controls the motor patterns of multiple systems in invertebrates. Dopamine acts as both a neuromodulator and a neurohormone (Sullivan et al., 1977; Barker et al., 1979). Dopamine is secreted by pericardial organs into the hemolymph, which bathes both the STNS and the CG, allowing dopaminergic input to both these CPGs (Clark et al., 2014; Sullivan et al., 1977; Fort et al., 2004). In the blue crab *Callinectes sapidus*, dopaminergic neurons were

found to act both peripherally to control heart muscle contractions and to directly innervate the CG. (Fort et al., 2004). Dopamine receptors primarily belong to the G protein-coupled receptor (GPCR) family (Clark et al., 2014).

CabTrp (Cancer borealis Tachykinin-Related Peptide)

Tachykinin-related peptides like CabTrp (APSGFLGMRamide) are highly conserved in invertebrates. They are called tachykinin-related peptides because their sequences are very similar to the tachykinin neuromodulators that are responsible for controlling gut tissue contraction in vertebrates (Carter & Krause, 1990). In crustaceans, tachykinin-related peptides like CabTrp typically modulate the STNS and the CG (Messinger et al., 2005; Christie et al., 1997; Stemmler et al., 2007; Cruz Bermudez & Marder, 2007; Rehm et al., 2008; Christie et al., 2008; Wood et al., 2000; Blitz et al., 2008; Rehm et al., 2008; Stein et al., 2007). CabTrp Ia is released into the neuropil of the stomatogastric ganglion (STG), where it has been found to excite the pyloric rhythm and increase contraction of the gastric mill (Christie et al., 1997; Thirumalai and Marder, 2002; Messinger et al., 2005; Wood et al., 2000).

CCAP (crustacean cardioactive peptide)

CCAP (PFCNAFTGCamide) was originally identified as a strong modulator of the cardiac ganglion (Stangier et al., 1987), but its presence in the neuroendocrine organs and parts of the central neuropil suggested that it had a more widespread role in the crustacean central nervous system (Christie et al., 2010). CCAP has since been found to modulate the stomatogastric neuromuscular system (DeLong et al., 2009; Kirby & Nusbaum, 2007; Richards & Marder, 2000; Weimann et al., 1997; Jorge Rivera et al., 1998), as well as play a role in

chromatophore pigment dispersion (Nery et al., 1999; Grenato et al., 2004), the changing of retinal light sensitivity (Gaus & Stieve, 1992), and regulation of ecdysis (molting of the cytoskeleton) (Wilckockson & Webster, 2008; Chung & Webster, 2004; Phlippen et al., 2000).

Myosuppressin

Myosuppressin (pQDLDHVFLRFamide) belongs to a subfamily of FMRF-amide-like peptides that share the sequence motif –HVFLRFamide. In decapod crustaceans, myosuppressin is highly conserved (Stemmler et al., 2007). The cardiac neuromuscular system in *Homarus americanus* (American lobster) is highly responsive to myosuppressin; in the isolated CG, myosuppressin hyperpolarizes the resting membrane potential of the cardiac motor neurons and causes a decrease in bursting frequency. In whole-heart preparations, myosuppressin decreases heartbeat frequency, but also increases contraction amplitude, suggesting that myosuppressin acts peripherally on the neuromuscular junction or the muscle, perhaps directly modulating one of the heart's stretch feedback pathways (Stevens et al., 2009; Christie et al., 2010).

Proctolin

Proctolin (RYLPT) is widespread in the decapod crustacean central nervous system. Proctolin functions as both an autocrine/paracrine and as a neurohormone (Schwarz et al., 1984). In crustaceans, proctolin modulates the exoskeletal muscles, neuromuscular junctions, and the cardiac ganglion (Wilkens et al., 1997; Freschi et al., 1989; Miller et al., 1981; Sullivan et al., 1984; Wilkens et al., 2008; Wilkens et al., 2005; Wilkens et al., 2003), the STNS (Dickinson et al., 2008; Jorge-Rivera et al., 1998; Marder et al., 1986; Dickinson et al., 1989; Heinzel, 1988;

Heinzel & Silverston, 1988; Hooper & Marder, 1984; Hooper & Marder, 1987; Rehm et al., 2008), the ventilatory system (Mercier et al., 1985), the CPGs controlling the swimmerets (Acevedo et al.,1994; Mulloney et al., 1987), mechanosensory neurons (el Manira et al., 1991; Pasztor et al., 1987; Pasztor et al., 1989), and the contractions of the hindgut (Mercier et al., 2002).

RPCH (red pigment concentrating hormone)

RPCH (pELNFSPGWamide) is present in many locations of the decapod central nervous system including the neuroendocrine organs (Christie et al., 1995; Chung & Webster, 2004; Fernlund & Josefsson, 1972; Fernlund & Josefsson, 1968), the neuropil of the STG, and the somata of the esophageal ganglion and commissural ganglia in *Cancer borealis* (Nusbaum & Marder, 1988). RPCH has both endocrine and autocrine/paracrine function (Christie et al., 2010). RPCH was originally understood to influence the concentration of erythrophore pigment granules, but has since been identified as a modulator for the STNS in *Cancer borealis* and lobsters (Dickinson & Marder, 1989; Rehm et al., 2008; Nusbaum & Marder, 1988; Dickinson et al., 2001; Dickinson et al., 1993; Dickinson et al., 1990; Thirumalai & Marder, 2002; Thirumalai et al., 2006), and the cardiac ganglion in *Cancer borealis* (Cruz-Bermudez & Marder, 2007).

STNS neuromodulatory capacity

Extracellular recordings from the STNS of *P. producta* and *L. emarginata* supported our prediction that the STNS of specialist feeders exhibits lower modulatory capacity than the STNS of opportunist feeders. The STNS of *L. emarginata* was activated by oxotremorine, dopamine

CabTrp, CCAP, proctolin, myosuppressin, and RPCH (Alexandra Miller Honors Thesis, 2018). This STNS modulatory capacity was like that observed in fellow opportunist feeder, *Cancer borealis,* which is modulated by proctolin (Marder et al., 1986), CabTrp Ia (Christie et al., 1997), RPCH (Nusbaum & Marder, 1988), and CCAP (Christie et al., 1995). However, the pyloric pattern of *P. producta* responded only to proctolin, oxotremorine, myosuppressin, and RPCH (25% of the time), but not to CabTrp and CCAP (Dickinson et al., 2008; Alexandra Miller Honors Thesis, 2018). Dopamine was found to modulate pyloric rhythm of *P. producta*, but elicited a highly variable pattern (Dickinson et al., 2008). Since *C. opilio* is an opportunist feeder, we expect *C. opilio* to also have higher STNS modulatory capacity than *P. producta.* Extracellular recordings from the STNS of *C. opilio* have supported this prediction (Jacob Kazmi Honors Thesis, 2020).

CG neuromodulatory capacity

We used whole-heart neuromuscular recordings to measure the amplitude and frequency of heart contractions. We used percent change in amplitude and frequency to gauge the degree to which the CG was modulated. Whole-heart neuromuscular recordings from specialist feeder *P. producta,* and opportunistic feeders *L. emarginata,* and *Chionoecetes* were expected to reveal how the CG responds to oxotremorine, dopamine, CabTrp, CCAP, proctolin, myosuppressin, and RPCH. We predicted that opportunist and specialist feeders would exhibit more similar neuromodulatory capacity in the cardiac ganglion than in the STNS.

We found that unlike the STNS, the CG of *P. producta* responded to CabTrp and CCAP. *P. producta* also responded to oxotremorine and inconsistently to dopamine and proctolin. The CG of *C. opilio* was modulated by CabTrp, CCAP, dopamine, proctolin, and oxotremorine. The

CG of *L. emarginata* responded to CCAP, and inconsistently to dopamine, and proctolin, but not to myosuppressin, RPCH, and surprisingly oxotremorine. Unlike in *P. producta* and *C. opilio,* heart contraction amplitude and frequency did not increase during CabTrp application, but rather decreased. It was particularly surprising that oxotremorine did not seem to modulate the CG of *L. emarginata* because oxotremorine was cardioactive in *P. producta* and *C. opilio.*

Although cardiac responses were not identical between species, opportunistic and specialist feeders responded more similarly to the modulators tested in the CG than in the STNS. Notably, *P. producta's* response to each modulator was similar to that of *C. opilio* and/or *L. emarginata.* However, *L. emarginata's* unresponsiveness to oxotremorine suggests that phylogenetic closeness may not control for differences in cardiac and STS function between species. Sample sizes of all three species must be enlarged to have conclusive, statistically significant results.

METHODS

Specimen collection and care

Chionoecetes opilio crabs were collected in the Gulf of St. Lawrence in Canada. We purchased them from a commercial seafood distributor, *Fisherman's Market International,* in Halifax, Nova Scotia. *Libinia emarginata* crabs were collected in traps from Niantic Bay, Connecticut, USA and were purchased from Gulf Specimen Marine Laboratory in Panacca, FL. *Pugettia producta* crabs were collected from dock pilings and in kelp beds in the San Juan archipelago of Washington state. We received them from Friday Harbor Laboratory at the University of Washington. Animals were kept in aerated natural seawater aquaria at 7-10ºC for *C. opilio,* 16-21ºC for *L. emarginata,* and 10-15ºC for *P. producta*. *C. opilio* and *L. emarginata* crabs were fed a single faroe island diver scallop once a week. *P. producta* were fed bull kelp *(Nereocystis).*

Dissection

Specimens were anesthetized on ice for about 40 minutes. After anesthetization, the heart was removed from the crab still attached to the dorsal thoracic section of the carapace and pinned in a Sylgard-lined Pyrex dish containing cold (10ºC) physiological crab saline (composition in mmol l^{-1}: NaCl, 440.0; KCl, 11.0; $CaCl_2$, 13.0; $MgCl_2$, 26.0; Trizma base, 12.0; maleic acid, 1.22; pH 7.4–7.5). This physiological saline was originally developed for *Cancer borealis* recordings (Hooper et al., 1986; Dickinson et al., 2008)

Neuromodulator preparation

Neuropeptides proctolin, CabTrp, CCAP, myosuppressin, and RPCH were synthesized by Genscript (Piscataway, New Jersey). Dopamine and the muscarinic acetylcholine agonist, oxotremorine, were ordered from Sigma Aldrich. CabTrp, CCAP, and oxotremorine were stored at 10^{-3} M in deionized H_2O at -20ºC and dissolved in room temperature saline to 10^{-7} M before use. RPCH was stored at 10^{-3} M in 15% DMSO at -20ºC and dissolved in room temperature saline to 10^{-7} M before use. Proctolin was stored at 10^{-5} M at -20ºC and dissolved to 10^{-8} M in room temperature saline before use. Dopamine was dissolved to 10^{-7} M in cold saline immediately before use. The flask containing the dopamine solution was wrapped in foil to reduce light exposure and prevent oxidation. The same neuromodulator concentrations were used for *P. producta*, *L. emarginata*, and *C. opilio*.

Whole heart recordings

The sternal posterior artery of *C. opilio* and *P. producta* hearts was cannulated with a short piece of polyethylene tubing and continuously perfused with temperature-controlled saline (~9.8ºC) at a rate of 2.5 mL/min. We used wider tubing to cannulate the larger arteries of *C. opilio* hearts, and narrower tubing to cannulate the much smaller *P. producta* arteries. We collected data from *L. emarginata* last and found that it worked better to cannulate the ostia on the side of the heart rather than to cannulate the very narrow and hard to find sternal posterior artery. We also determined that bent metal tubing worked better than polyethylene tubing to cannulate the ostia on the side of the heart because it was more rigid and easier to orient. We recommend cannulating *P. producta* the same way for future experiments and data collection. Since *L. emarginata* resides in warmer temperature water, we perfused *L. emarginata* with ~14ºC saline. For three species of crab, second tube perfused cool saline over the heart at the

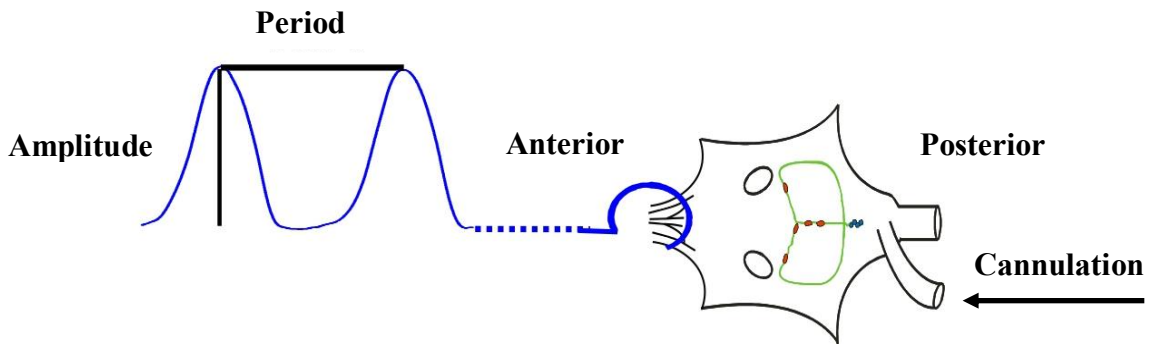

Figure 5. Diagram of the decapod heart showing the CG (green) with large motor neurons in red and pre-motor neurons in blue. The sternal posterior artery of the heart was cannulated and perfused with temperature controlled physiological crab saline. A force transducer tied to the five anterior arteries measured the amplitude and frequency of cardiac neuromuscular contractions (frequency = 1/period).

same rate to maintain temperature. The inflow saline temperature was regulated with a Peltier temperature regulator (Warner Instrument, Hamden, CT). A Rabbit peristaltic pump (Gilson, Middleton, WI) maintained a steady flow of saline in and out of the dish.

To record the amplitude and frequency of cardiac neuromuscular contractions, the five anterior arteries of the heart were tied with 6-0 Suture Silk and connected to a FT03 force transducer (Grass Natus Technologies, Pleasanton, CA) at approximately a 45º angle from the horizontal plane (Fig. 5). To best imitate endogenous conditions, *C. opilio* hearts were stretched to a baseline of about 2.5 grams of force and the much smaller *L. emarginata* and *P. producta* hearts were stretched to about 0.2 grams of force. After being hooked up to the force transducer, hearts were stabilized for 40 minutes.

The signal was amplified with an ETH-250 Bridge/Bio Amplifier and a model 410 Brownlee Precision Instrument amplifier. Neuromuscular heart contractions were recorded with a CED Micro 1401 USB interface and Spike2 version 7.16 (Cambridge Electronic Design Ltd., Cambridge, England) on a PC computer. After control saline was perfused through the heart for a control period of 10 minutes, each neuromodulator was bath applied to the heart through the

cannulated posterior artery for about 10 minutes. The heart was then washed with saline for at least 40 minutes between each neuromodulator. Neuromodulators were applied in a randomized order; however, CCAP, proctolin, and dopamine were typically introduced later in the lineup because they have a more potent effect on the heart and take longer to be washed out.

Data analysis

Heart contraction parameters were analyzed with scripts written on Spike2. The amplitude and frequency of contractions were plotted as a function of time on a macro-enabled spreadsheet in Excel (Microsoft, Redmond, WA). Percent changes in frequency and amplitude were calculated by averaging frequency and amplitude values, respectively, for 50 beats during the control period, 50 beats during the peak effect during neuromodulator application, and 50 beats during the saline wash after return to baseline. I used a Wilcoxon test to analyze my results because my sample size was too small to use a t-test. Because my *P. producta* and *L. emarginata* sample sizes were exceptionally small, I mainly analyzed observed responses to the neuromodulators tested rather than statistical significance. Prism8 (GraphPad Software, La Jolla, CA) was used to generate Wilcoxon tests and graphs. Corel Draw 2020 was used to make figures of recordings.

RESULTS

Chionoecetes opilio

The hearts of *Chionoecetes opilio* were estimated to be about 6 cm long and 2 cm wide (Fig. 6A). Neuromuscular contraction amplitude and frequency in *C. opilio* varied between individuals. We measured baseline amplitudes between 0.6 and 5 grams of force and baseline frequencies between 0.3 and 0.6 Hz. Most *C. opilio* hearts beat vigorously with consistent contraction amplitude and frequency.

We collected data from 8 *Chionoecetes opilio* hearts. Apart from RPCH (Fig. 13A, C, D), all the neuromodulators tested activated the heart of *Chionoecetes opilio*. Depending on the modulator and the individual, cardiac responses varied in pattern and percent change in amplitude and frequency. Oxotremorine (Fig. 7A, D, E), CabTrp (Fig. 9A, D, E), and CCAP (Fig. 10A, D, E) elicited significant increases in both percent change in amplitude and frequency. Dopamine (Fig. 8A, D, E) and proctolin (Fig. 12 A, D, E) caused a significant increase in amplitude but not frequency, although every preparation was observed to undergo an increase in contraction frequency during dopamine application. Myosuppressin elicited a more complex response pattern; after addition of myosuppressin, both the amplitude and frequency of contractions decreased, but amplitude returned to baseline before myosuppressin was removed (Fig. 11A, D, E). During myosuppressin application, we measured "peak effect" as the return to baseline amplitude after the initial decrease in amplitude and frequency. During this time, percent change in frequency remained significantly decreased (Fig. 11E). RPCH did not significantly alter contraction amplitude or frequency (Fig. 13A, D, E), although one individual was observed to undergo an 80% increase in contraction amplitude during RPCH application.

Libinia emarginata

The hearts of *L emarginata* were estimated to be about 2 cm long and 1 cm wide, although we observed variation in the size of the animal and their hearts. The sternal posterior arteries of *L. emarginata* hearts were on the side of the heart and not in line with the anterior arteries as they are in *Homarus americanus* and *C. opilio*. We observed that *L. emarginata* could be either right-handed or left-handed in terms of what side of the heart the sternal posterior artery was on (Fig. 6B). The "handedness" of the sternal posterior artery did not seem to affect heartbeat. The shell of *L. emarginata* was thicker and sturdier than the shells of *C. opilio* and *P. producta* and required a drill for pinning. Baseline amplitude of *L. emarginata* heart contractions ranged from about 0.1 grams to 0.9 grams. Baseline frequency ranged from about 0.4 Hz to 0.6 Hz.

Our recording sample size of *L. emarginata* was quite small (n = 4) because the COVID-19 outbreak prevented further data collection. Because of this, I mainly analyzed my observations rather than statistical significance. Of the *L. emarginata* crabs I recorded from, I observed considerable variation in responses to the modulators tested. Dopamine (Fig. 8 B, D, E) and CCAP (Fig. 10B, D, E) were observed to cause an increase in contraction amplitude and frequency, but inconsistently. I observed that Proctolin inconsistently increased contraction amplitude (Fig 11B, D), but not frequency (Fig. 11E), and that CabTrp decreased both amplitude and frequency (Fig, 9B). I also observed that neither oxotremorine (Fig. 7B, D, E), myosuppressin (Fig. 12B, D, E), nor RPCH (Fig. 13B, D, E) changed amplitude or frequency of the heartbeat.

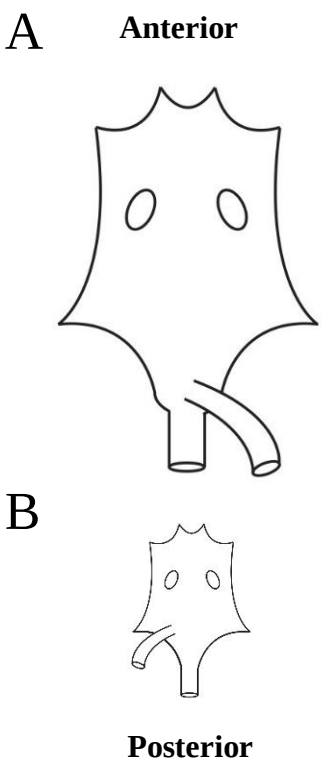

Figure 6. Diagrammatic representation of *Chionoecetes opilio* (A) and both *Libinia emarginata* and *Pugettia producta* heart morphology (B). The hearts of *C. opilio,* which were about 6 cm long and 2 cm wide, were much larger than those of *L. emarginata* and *P. producta,* which were only about 2 cm long and 1 cm wide. The sternal posterior arteries of *L. emarginata* and *P. producta* hearts were located either to the right or to the left and were not in line with the anterior arteries as they were in *C. opilio* hearts. (B) Shows a left-handed heart. Images are not to scale.

Pugettia producta

The hearts of *P. producta* crabs were approximately the same size as *L. emarginata* hearts (estimated to be about 2 cm long and 1 cm wide). *P. producta* hearts could also be either right-handed or left-handed in terms of the sternal posterior artery (Fig. 6A). "Handedness" did not seem to affect heartbeat. It was hard to maintain stretch on the heart because the tissue easily came loose from the carapace. Heart size and contraction patterns also varied between individual. At baseline, *P. producta* heart contraction amplitudes ranged from about 0.05 to 0.3 grams of force and frequencies were generally between 0.2 Hz and 0.4 Hz. These smaller contraction amplitudes and frequencies for *P. producta* and *L. emarginata* hearts were unsurprising given that they are smaller than *C. opilio, C. borealis,* and lobster hearts.

Our sample size for *P. producta* was also quite small (n = 4) because a large crab in the tank murdered most of the smaller crabs. Due to our small sample size, I analyzed observed

responses to the modulators tested rather than statistical significance. I observed that application of oxotremorine (Fig. 7C, D, E), dopamine (Fig 8C, D, E), CabTrp (Fig. 9C, D, E), and CCAP (Fig. 10C, D, E) increased both contraction amplitude and frequency. However, one of the *P. producta* crab hearts that we tested did not respond to dopamine. Proctolin inconsistently increased contraction amplitude, but not frequency (Fig. 12C, D, E). Neither RPCH (Fig. 13C, D, E) nor myosuppressin (Fig. 9B, D) caused any observable changes in *P. producta* heartbeat.

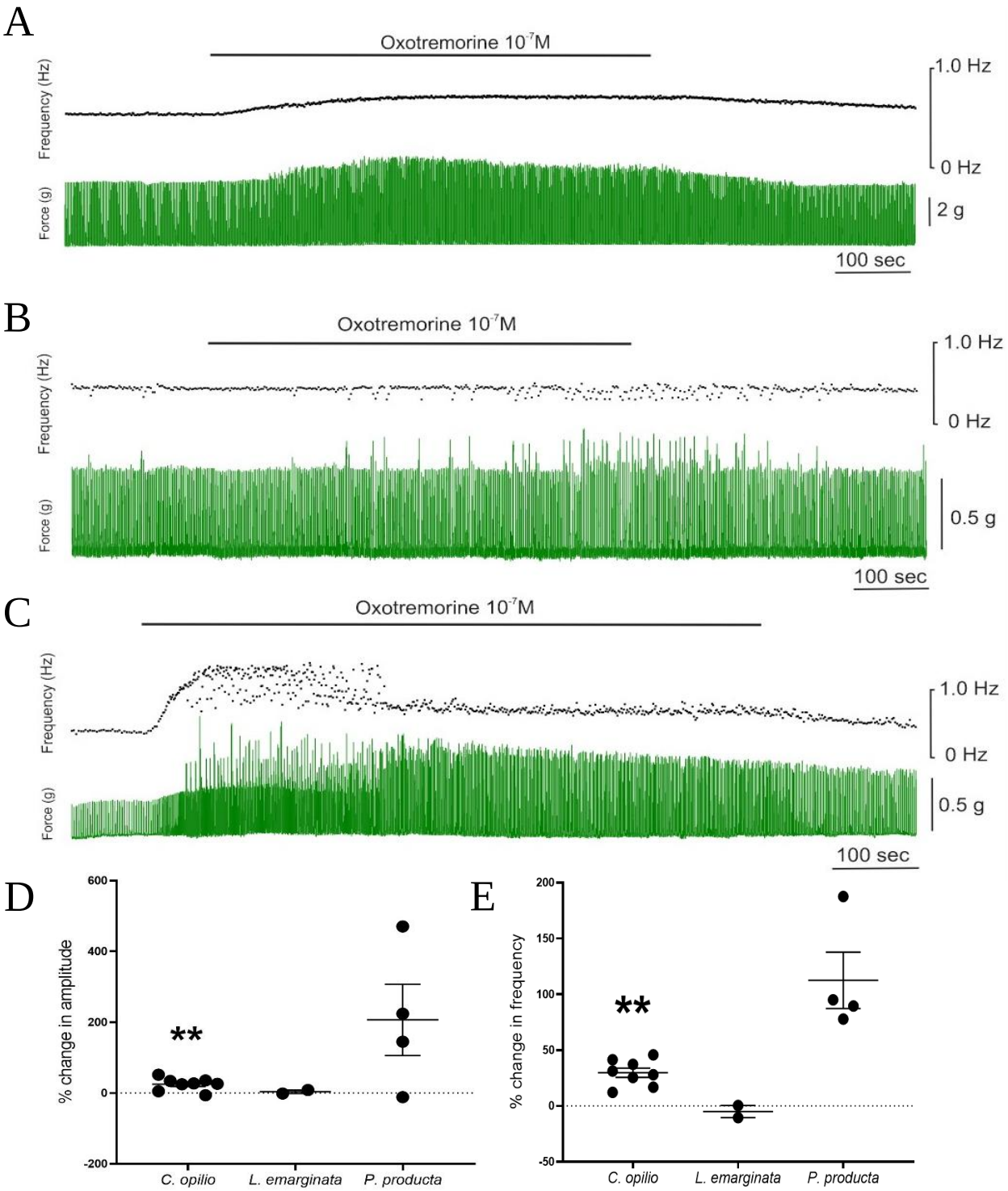

Figure 7. Cardiac response to oxotremorine in *Chionoecetes opilio, Libinia emarginata,* and *Pugettia producta*. Neuromuscular contraction amplitude and frequency as recorded on Spike2 for *C. opilio* (A), *L. emarginata* (B), and *P. producta* (C). Percent change in amplitude (D) and frequency (E) for all three species in response to oxotremorine. *C. opilio* displayed a significant increase in both percent change in amplitude and frequency during application (Wilcoxon test, amplitude $p < 0.05$, frequency $p < 0.008$). *L. emarginata* did not undergo a change in amplitude or frequency during oxotremorine application (Wilcoxon test, too few points). Observations suggest that oxotremorine increased contraction amplitude and frequency in *P. producta* (Wilcoxon test, amplitude $p > 0.2$, frequency $p > 0.1$). Lines represent the mean, and error bars are SEM. Each dot represents a crab preparation (*C. opilio* n = 8, *L. emarginata* n = 2, *P. producta* n = 4).

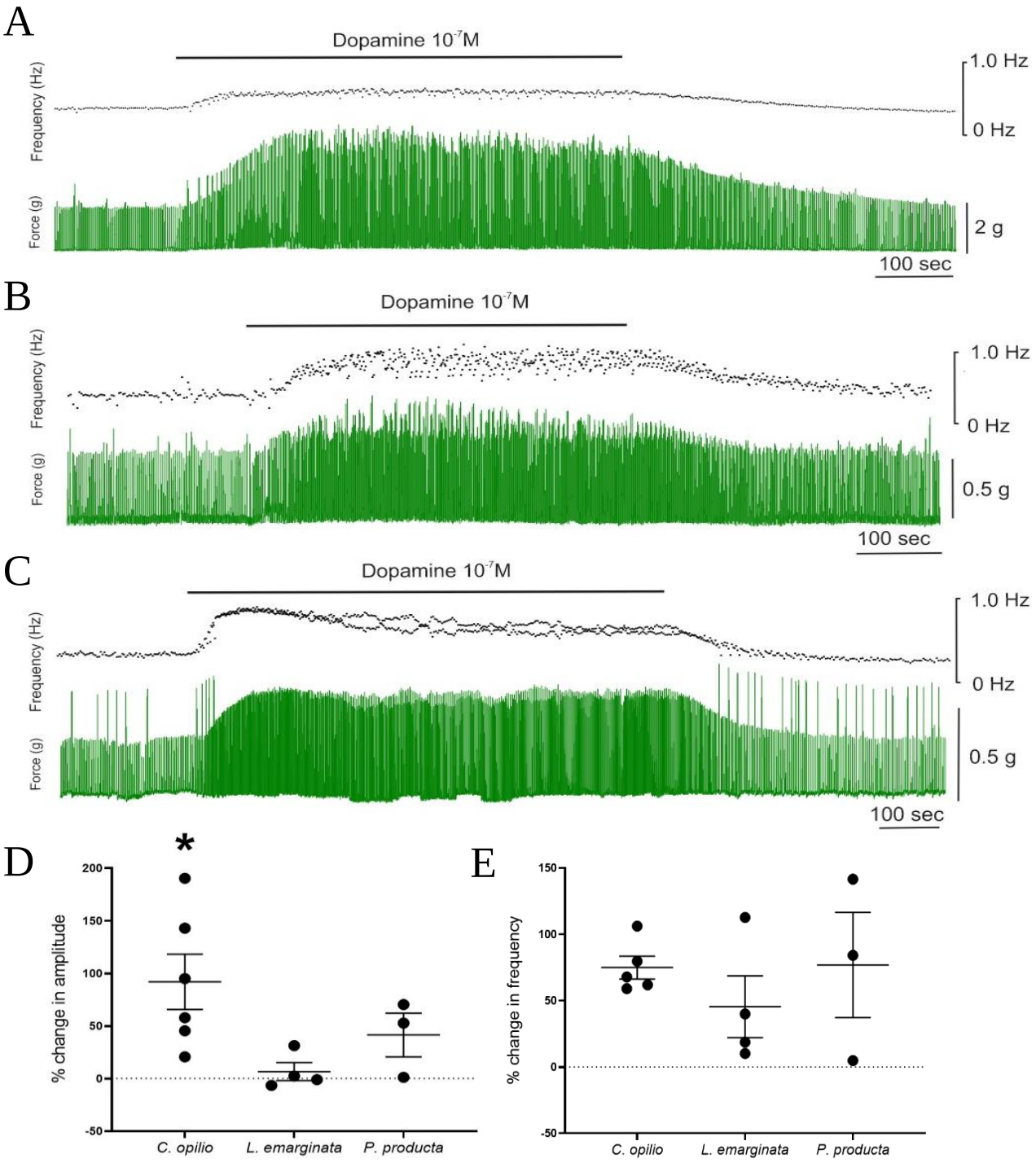

Figure 8. Cardiac response to dopamine (DA) in *Chionoecetes opilio*, *Libinia emarginata*, and *Pugettia producta*. Neuromuscular contraction amplitude and frequency as recorded on Spike2 for *C. opilio* (A), *L. emarginata* (B), and *P. producta* (C). Percent change in amplitude (D) and frequency (E) for all three species in response to DA. *C. opilio* displayed a significant increase in percent change in amplitude but not frequency during application (Wilcoxon test, amplitude $p < 0.05$, frequency $p > 0.06$), although an increase in frequency was observed for all *C. opilio* preparations during DA application. The hearts of some *L. emarginata* individuals were observed to undergo an increase in contraction amplitude and frequency during application. (Wilcoxon test, amplitude $p > 0.8$, frequency $p > 0.1$). Except for one individual, DA increased contraction amplitude and frequency in *P. producta* (Wilcoxon test, amplitude $p > 0.2$, frequency $p > 0.2$). Lines represent the mean, and error bars are SEM. Each dot represents a crab preparation (*C. opilio* n = 8, *L. emarginata* n = 4, *P. producta* n = 3).

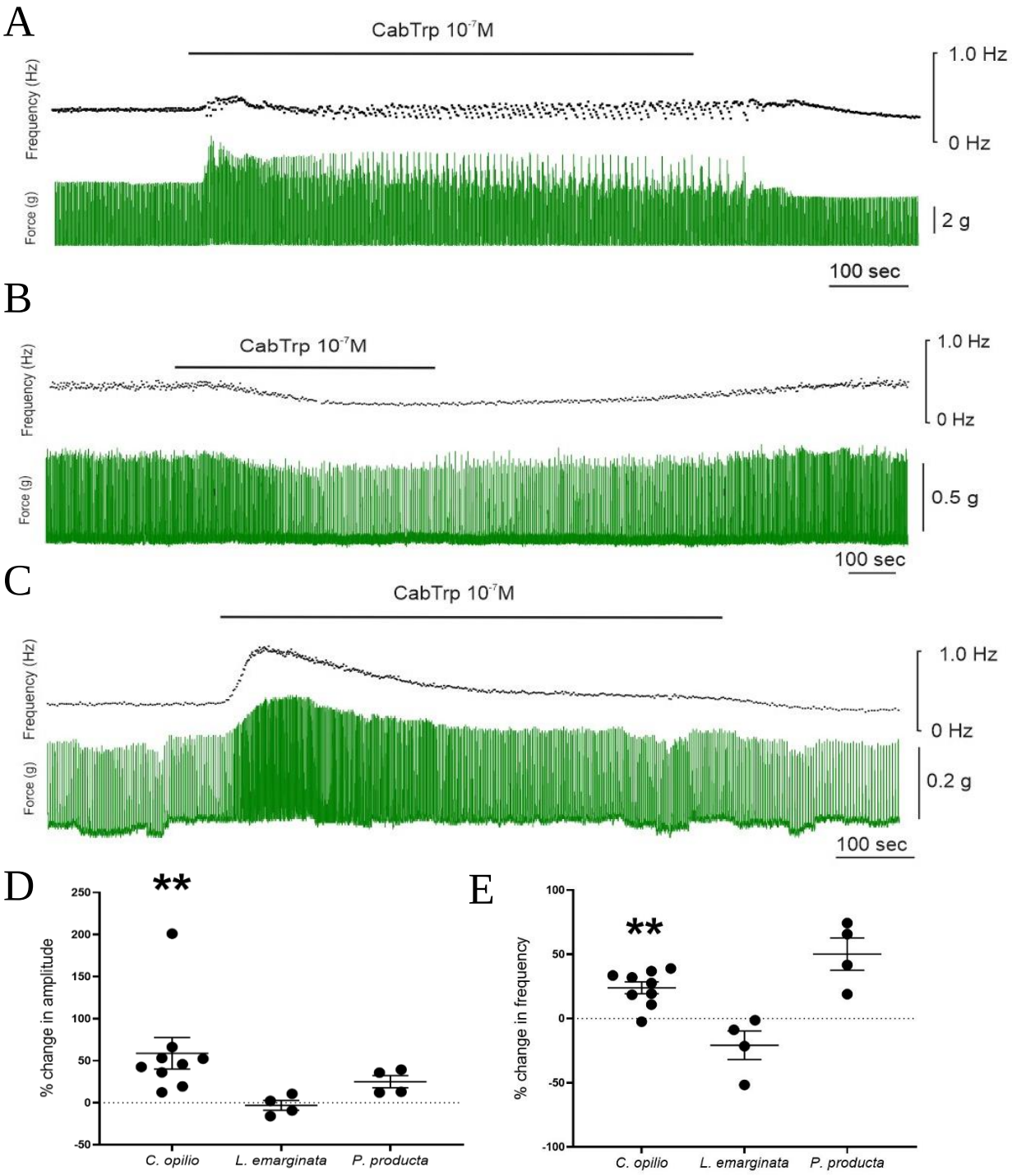

Figure 9. Cardiac response to CabTrp in *Chionoecetes opilio*, *Libinia emarginata*, and *Pugettia producta*. Neuromuscular contraction amplitude and frequency as recorded on Spike2 for *C. opilio* (A), *L. emarginata* (B), and *P. producta* (C). Percent change in amplitude (D) and frequency (E) for all three species in response to CabTrp. *C. opilio* displayed a significant increase in both percent change in amplitude and frequency during application (Wilcoxon test, amplitude $p < 0.004$, frequency $p < 0.008$). *L. emarginata* was inconsistently observed to undergo a decrease in amplitude and frequency during application (Wilcoxon test, amplitude $p > 0.8$, frequency $p > 0.1$) All *P. producta* hearts tested increased in percent change contraction amplitude and frequency during CabTrp application (Wilcoxon test, amplitude $p > 0.1$, frequency $p > 0.1$). Lines represent the mean, and error bars are SEM. Each dot represents a crab preparation (*C. opilio* n = 8, *L. emarginata* n = 4, *P. producta* n = 4).

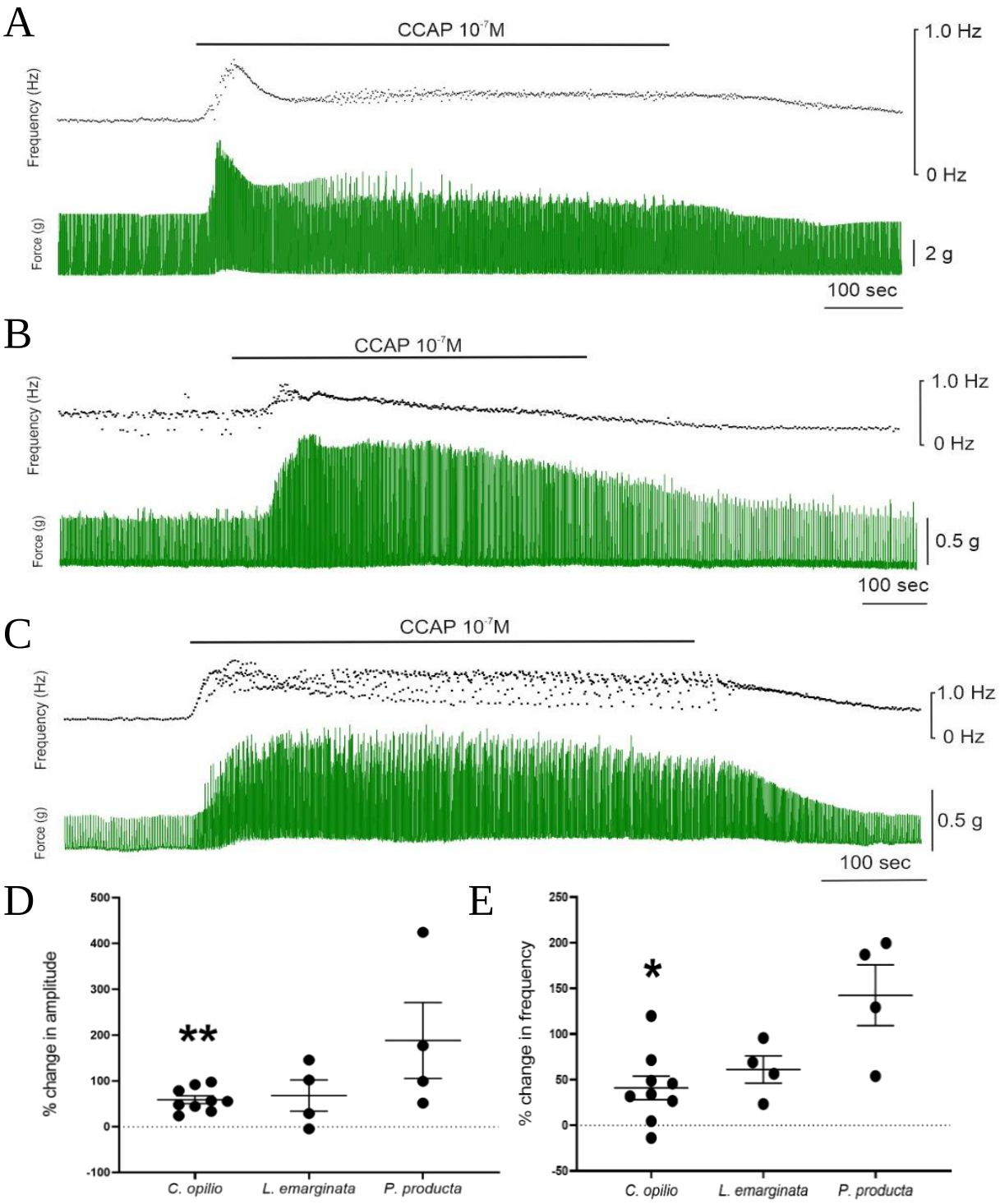

Figure 10. Cardiac response to CCAP in *Chionoecetes opilio, Libinia emarginata,* and *Pugettia producta*. Neuromuscular contraction amplitude and frequency as recorded on Spike2 for *C. opilio* (A), *L. emarginata* (B), and *P. producta* (C). Percent change in amplitude (D) and frequency (E) for all three species in response to CCAP. *C. opilio* displayed a significant increase in both percent change in amplitude and frequency during application (Wilcoxon test, amplitude $p < 0.004$, frequency $p < 0.05$). CCAP inconsistently increased *L. emarginata* heart contraction amplitude and consistently increased heart contraction frequency (Wilcoxon test, amplitude $p > 0.2$, frequency $p > 0.1$). CCAP was observed to consistently increase contraction amplitude and frequency in *P. producta* (Wilcoxon test, amplitude $p > 0.1$, frequency $p > 0.1$). Lines represent the mean, and error bars are SEM. Each dot represents a crab preparation (*C. opilio* n = 8, *L. emarginata* n = 4, *P. producta* n = 4).

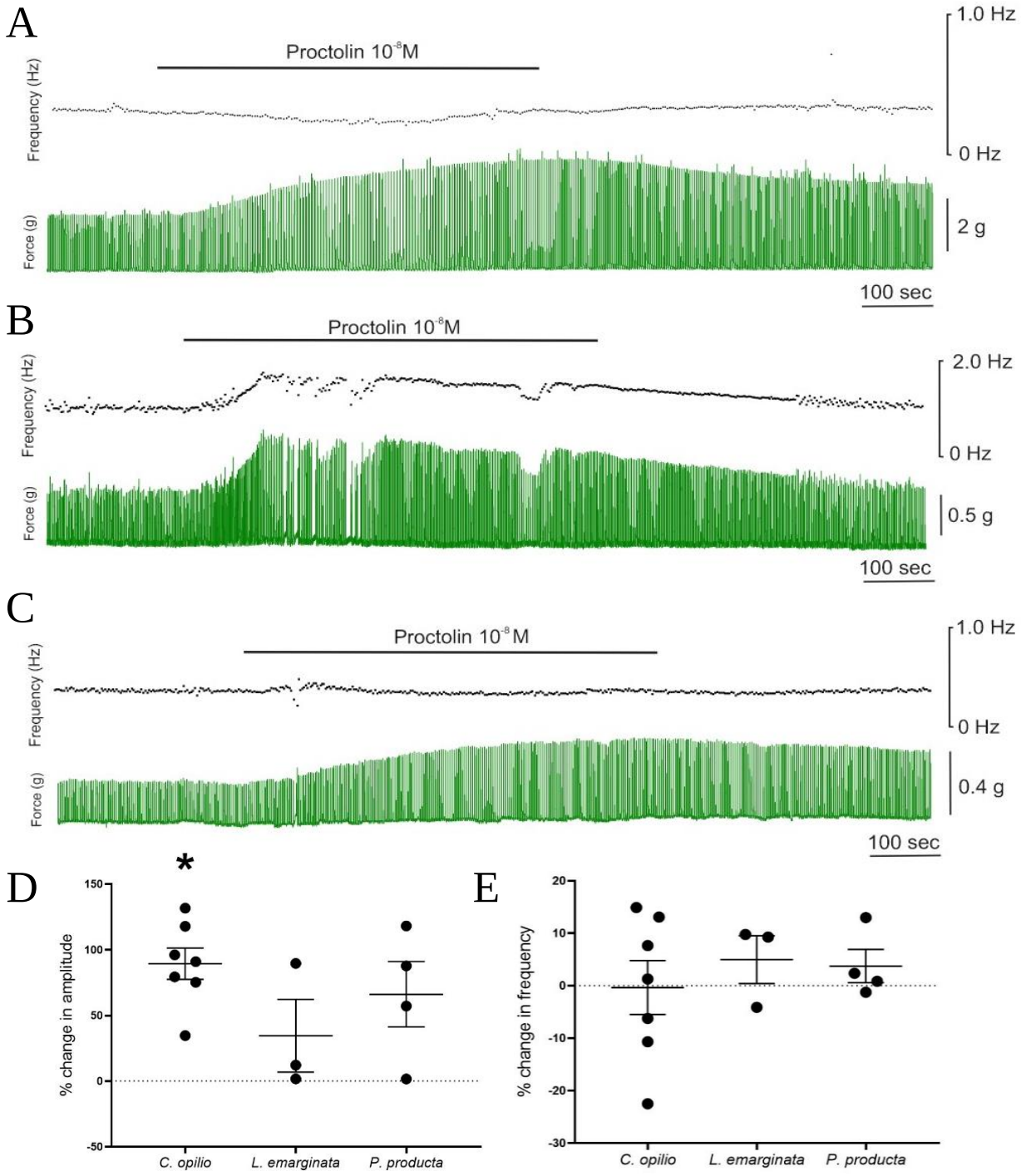

Figure 11. Cardiac neuromodulatory response to proctolin in *Chionoecetes opilio, Libinia emarginata,* and *Pugettia producta*. Neuromuscular contraction amplitude and frequency as recorded on Spike2 for *C. opilio* (A), *L. emarginata* (B), and *P. producta* (C). Percent change in amplitude (D) and frequency (E) for all three species in response to proctolin. *C. opilio* displayed a significant increase in percent change in amplitude but not frequency during application (Wilcoxon test, amplitude $p < 0.02$, frequency $p > 0.9$). *L. emarginata* was inconsistently observed to undergo an increase in amplitude but not frequency during proctolin application (Wilcoxon test, amplitude $p > 0.2$, frequency $p = 0.5$). Proctolin was observed to increase contraction amplitude but not frequency in *P. producta* (Wilcoxon test, amplitude $p > 0.1$, frequency $p > 0.3$). Lines represent the mean, and error bars are SEM. Each dot represents a crab preparation (*C. opilio* n = 8, *L. emarginata* n = 3, *P. producta* n = 4).

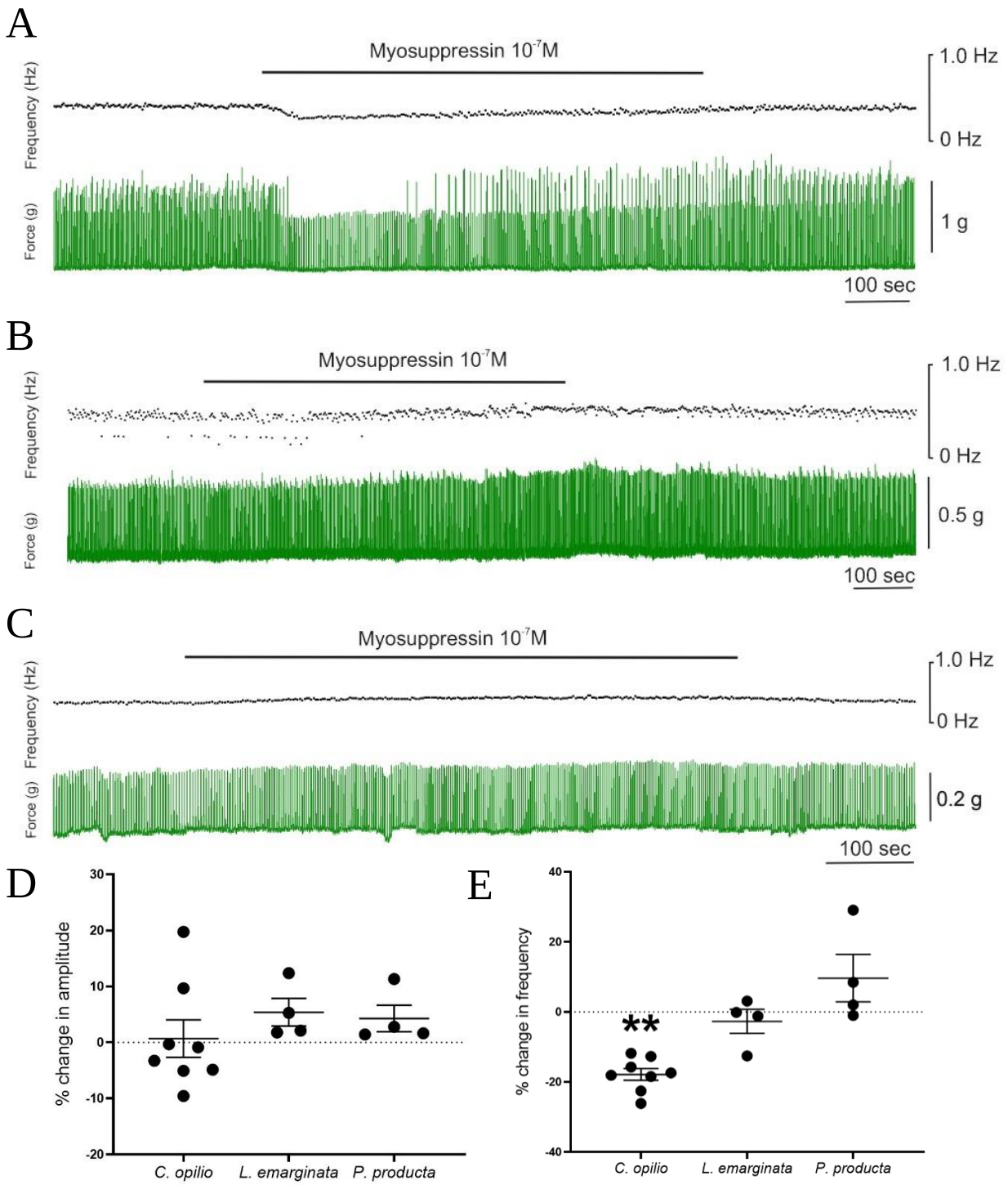

Figure 12. Cardiac neuromodulatory response to myosuppressin in *Chionoecetes opilio, Libinia emarginata,* and *Pugettia producta*. Neuromuscular contraction amplitude and frequency as recorded on Spike2 for *C. opilio* (A), *L. emarginata* (B), and *P. producta* (C). Percent change in amplitude (D) and frequency (E) for all three species in response to myosuppressin. *C. opilio* displayed no significant percent change in amplitude but underwent a significant decrease in percent change in frequency during application (Wilcoxon test, amplitude $p > 0.7$, frequency $p < 0.001$). Myosuppressin was not observed to cause a notable increase in amplitude or frequency in both *L. emarginata* (Wilcoxon test, amplitude $p > 0.1$, frequency $p > 0.6$) and *P. producta* (Wilcoxon test, amplitude $p > 0.1$, frequency $p > 0.2$). Lines represent the mean, and error bars are SEM. Each dot represents a crab preparation (*C. opilio* n = 8, *L. emarginata* n = 4, *P. producta* n = 4).

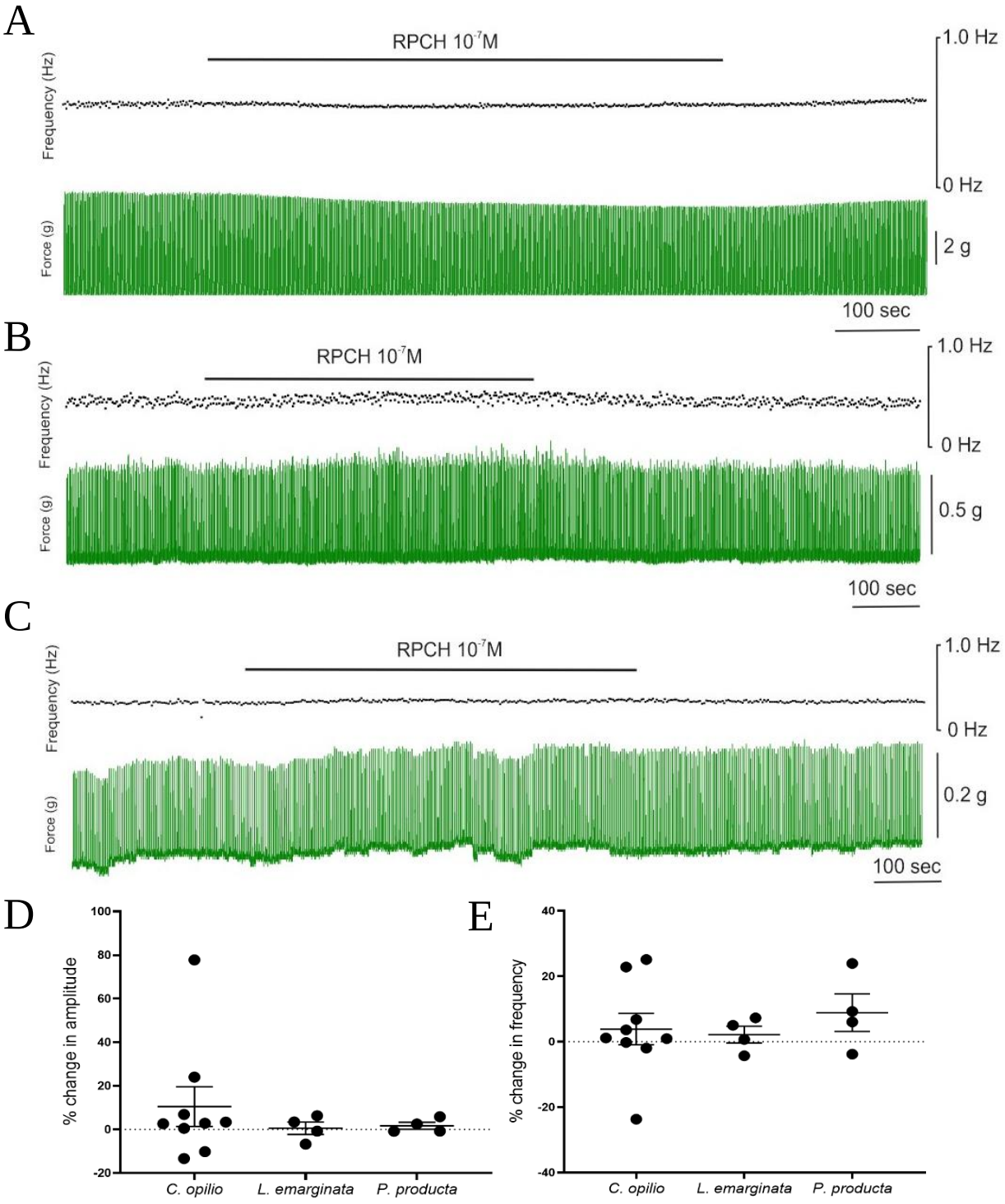

Figure 13. Cardiac neuromodulatory response to RPCH in *Chionoecetes opilio, Libinia emarginata,* and *Pugettia producta*. Neuromuscular contraction amplitude and frequency as recorded on Spike2 for *C. opilio* (A), *L. emarginata* (B), and *P. producta* (C). Percent change in amplitude (D) and frequency (E) for all three species in response to RPCH. *C. opilio* displayed no significant percent change in amplitude or frequency during application (Wilcoxon test, amplitude $p = 0.3008$, frequency $p > 0.3$). RPCH did not elicit any activity in *L. emarginata* (Wilcoxon test, amplitude $p > 0.9$, frequency $p > 0.3$) or *P. producta* (Wilcoxon test, amplitude $p > 0.6$, frequency $p > 0.2$). Lines represent the mean, and error bars are SEM. Each dot represents a crab preparation (*C. opilio* n = 8, *L. emarginata* n = 4, *P. producta* n = 4).

DISCUSSION

Neuromodulators such as peptides and amines regulate the rhythmic outputs of central pattern generators such as the ones in the stomatogastric nervous system, which controls pyloric and gastric motor patterns, and the cardiac ganglion (CG), which controls the muscular contractions of the neurogenic heart. Because changes in pyloric motor pattern by neuromodulation of the STNS are responsible for the chewing and processing of various food-types, animals with a diverse diet are predicted to have greater STNS neuromodulatory capacity than those with a specialized diet. Since neuromodulation of CPGs allows flexibility in behaviors, we have hypothesized that neuromodulation provides a substrate for the evolution of behavioral flexibility. Under this hypothesis, we predicted that the opportunistic feeding crabs *Chionoecetes opilio* and *Libinia emarginata* would have greater neuromodulatory capacity in the STNS than the specialized feeder, *Pugettia producta.* By contrast, we further predicted that *C. opilio, L. emarginata* and *P. producta* would exhibit more similar modulatory capacity in the CG than in the STNS. This is because opportunistic feeders have likely evolved more behavioral flexibility in the STNS to chew a large variety of food types; however, the modulatory capacity of the heart should be unaffected by diet because heart function is conserved between closely related species.

It was found that the STNS of *L. emarginata* is modulated by the neuropeptides proctolin, CabTrp, CCAP, myosuppressin, RPCH, the amine dopamine, and the muscarinic acetylcholine agonist oxotremorine (Alexandra Miller Honors Thesis, 2018). Preliminary data suggest that the STNS of *C. opilio* also responds to all seven neuromodulators (Jacob Kazmi Honors Thesis, 2020). However, the STNS of *P. producta* was only modulated by proctolin, oxotremorine, myosuppressin, RPCH (25% of the time), and dopamine variably, but not by CCAP and CabTrp

(Dickinson et al., 2008; Alexandra Miller Honors Thesis, 2018). These findings support our hypothesis that opportunist feeders such as *L. emarginata* and *C. opilio* have greater neuromodulatory capacity in the STNS than the specialized feeder *P. producta.*

Under our prediction that opportunistic and specialized feeders have similar CG modulatory capacity, we would expect that the same neuromodulators would induce similar responses in the hearts of *C. opilio, L. emarginata,* and *P. producta.* Although all three species did not have identical cardiac responses to the seven neuromodulators tested, we observed more similar modulatory capacity in the CG of opportunistic and specialized feeders than in the STNS. The hearts of *C. opilio* were consistently activated by oxotremorine, dopamine, CabTrp, CCAP, proctolin, and myosuppressin, but not by RPCH. *L. emarginata* hearts inconsistently responded to dopamine, CabTrp, CCAP, and proctolin, but not to oxotremorine, myosuppressin, or RPCH. *P. producta* hearts reliably responded to oxotremorine, CabTrp, and CCAP, inconsistently to proctolin and dopamine, but not to myosuppressin and RPCH.

Oxotremorine

The muscarinic acetylcholine agonist oxotremorine modulated the STNS of *C. opilio* (Jacob Kazmi Honors Thesis, 2020), *L. emarginata* (Alison Miller Honors Thesis, 2018), and *P. producta* (Dickinson et al., 2008). I found that oxotremorine consistently increased both heart contraction amplitude and frequency in *C. opilio* and *P. producta*. Surprisingly, oxotremorine did not elicit a robust response from the CG of *L. emarginata.* On one individual, I tested another muscarinic acetylcholine agonist, pilocarpine, and observed no activity either. Currently, our sample size (n = 2) for oxotremorine is too small to draw any conclusions, but my observations suggest that oxotremorine elicits less activity in the CG of *L. emarginata* than in the CG of *C.*

opilio and *P. producta.* These differences are not likely due to phylogenetic distance because *C. opilio* and *P. producta,* which responded similarly to oxotremorine, are more phylogenetically distant than *L. emarginata* and *P. producta* which both belong to the Epialtidae family (Fig. 3B). Additionally, it has been found that other cholinergic agonists such as pilocarpine and nicotine have excitatory effects on the isolated cardiac ganglion (ICG) of *Cancer borealis* (Cruz-Bermúdez & Marder, 2007), a more phylogenetically distant opportunistic feeder.

I planned to perfuse an *L. emarginata* heart with 9.8ºC saline like I used for *C. opilio* and *P. producta* instead of the usual 14ºC saline to see if temperature differences were affecting neuromodulator responsiveness. However, the lab was unfortunately closed due to COVID-19 and I was unable to do this test before the end of the semester. Nevertheless, it seems unlikely that a difference in perfusion temperature is responsible for *L. emarginata's* lack of responsiveness to oxotremorine; Weiss (2016) reported that oxotremorine increased the frequency of electrophysiological bursts in the CG with increasing temperature in *Cancer borealis.* Although not a perfect comparison due to use of a different species and recording method, these results suggest that increased perfusion temperature should not lessen the effect of oxotremorine on the CG.

Dopamine

The amine dopamine consistently modulated the STNS of *C. opilio* (Jacob Kazmi Honors Thesis, 2020) and *L. emarginata* (Alison Miller Honors Thesis, 2018), but elicited a highly variable pyloric pattern in the STNS of *P. producta* (Dickinson et al., 2008). I found that dopamine consistently increased both the amplitude and frequency of heart neuromuscular contractions in *C. opilio* (Fig. 8A, D, E) and inconsistently in both *P. producta* (Fig. 8C, D, E)

and *L. emarginata* (Fig. 8B, D, E). When dopamine did have an effect, it elicited a similar response pattern in all three species (Fig. 8). These findings support our prediction that the CG of specialized feeder *P. producta* and opportunistic feeders *C. opilio* and *L. emarginata* will be more similarly modulated than in the STNS. Dopamine has also been found to excite the ICG of *Cancer borealis* (Cruz-Bermúdez & Marder, 2007).

CabTrp

The neuropeptide CabTrp Ia has been found to modulate the STNS of *L. emarginata* (Alison Miller Honors Thesis, 2018) and *C. opilio* (Jacob Kazmi Honors Thesis, 2020), but not the STNS of *P. producta* (Dickinson et al., 2008). I found that CabTrp increased both amplitude and frequency of heart contractions in C. opilio (Fig. 9A, D, E) and *P. producta* Fig. 9C, D, E), but decreased contraction amplitude and frequency in *L. emarginata* (Fig. 9C, D, E). CabTrp has been found to excite the ICG of *Cancer borealis* (Cruz-Bermúdez & Marder, 2007) and increase amplitude and frequency of heart contractions in *Homarus americanus* (Christie et al., 2008). The finding that *C. opilio* and *P. producta* responded similarly to CabTrp enforces our hypothesis that dietary behavior does not affect modulatory capacity in the CG. However, it is surprising that CabTrp seemed to decrease contraction amplitude and frequency in *L. emarginata* since it is typically a cardioactive neuropeptide. A reason for this unique response in *L. emarginata* remains unknown.

CCAP

CCAP has been found to modulate the STNS of *L. emarginata* (Alison Miller Honors Thesis, 2018) and *C. opilio* (Jacob Kazmi Honors Thesis, 2020) but not the STNS of *P. producta*

(Dickinson et al., 2008). CCAP increased contraction amplitude and frequency in the hearts of all three species of crab (Fig. 10). Out of all seven neuromodulators tested, CCAP had some of the most consistent effects on neuromuscular contractions. The pattern of response to CCAP was also quite uniform between all three species (Fig. 10A-C). These findings support our hypothesis that *C. opilio*, *L. emarginata*, and *P. producta* will have similar modulatory capacity in the CG. These results are also consistent with other studies that found CCAP to have excitatory effects on the ICG of *Callinectes sapidus* (Fort et al., 2004; Fort et al., 2007) and *Cancer borealis* (Cruz-Bermúdez & Marder, 2007), as well as on the semi-isolated cardiac ganglion of *Carcinus maenas* (Stangier et al., 1987).

Proctolin

The neuropeptide proctolin modulated the STNS of *L. emarginata* (Alison Miller Honors Thesis, 2018), *C. opilio* (Jacob Kazmi Honors Thesis, 2020), and *P. producta* (Dickinson et al., 2008). Proctolin increased amplitude of cardiac contractions in *C. opilio*, and inconsistently in both *L. emarginata* and *P. producta*, but did not affect heart rate either species (Fig. 11). In all three species, when proctolin did have an effect, it was a potent one, and it took longer to wash proctolin out of the heart than other neuropeptides. The fact that all three species of crab responded to proctolin in the CG supports our hypothesis of more similar CG neuromodulator ability between opportunistic and specialized feeders. The excitatory effects of proctolin are consistent with other studies that have also found proctolin to be cardioactive. Proctolin has been found to increase heart contraction amplitude and frequency in many species of crustacean and insect (Lange and Orchard, 2013), and excites the ICG of *Cancer borealis* (Cruz-Bermúdez & Marder, 2007).

Myosuppressin

Myosuppressin modulated the STNS of *L. emarginata* (Alison Miller Honors Thesis, 2018), *C. opilio* (Jacob Kazmi Honors Thesis, 2020), and *P. producta* (Alison Miller Honors Thesis, 2018). I found that myosuppressin did not affect contraction amplitude or frequency in *L. emarginata,* and *P. producta* (Fig. 12), but decreased contraction frequency in *C. opilio* (Fig. 12A, D, E). During myosuppressin application in *C. opilio,* I typically noticed a decrease in amplitude and frequency followed by a return to baseline amplitude but not frequency. This response was not as stark in all *C. opilio* individuals, and with a larger *C. opilio* sample size than *L. emarginata* and *P. producta,* it is hard to make comparisons. The response to myosuppressin observed in *C. opilio* is unlike that of *Homarus americanus,* in which myosuppressin drastically increases the amplitude of heart contractions and decreases heart rate (Stevens et al. 2009). Although observed responses to myosuppressin were different in *C. opilio* compared to both *L. emarginata* and *P. producta,* there is no indication this was a result of different feeding behavior since *L. emarginata* and *P. producta* responded similarly.

RPCH

RPCH modulated the STNS of *L. emarginata* (Alison Miller Honors Thesis, 2018) and *C. opilio* (Jacob Kazmi Honors Thesis, 2020) but only modulated the STNS of *P. producta* 25% of the time (Dickinson et al., 2008). I found that RPCH did not modulate the CG of *C. opilio, L. emarginata,* or *P. producta* (Fig. 13). Overall, these results support our prediction that the CG of opportunistic and specialized feeders would have similar modulatory capacity. However, it was surprising that RPCH did not affect these species of crab since RPCH has been found to excite

the ICG of *Cancer borealis* (Cruz-Bermúdez & Marder, 2007). At first, we thought the lack of responsiveness to RPCH may be due to a problem with the peptide stock, but we made a new stock and continued to see the same results. I have found no reports on the effect of RPCH on the CG of majoid crab species but given that RPCH consistently failed to excite the CG of *C. opilio, L. emarginata,* and *P. producta,* it is possible that a lack of responsiveness to RPCH is specific to the Majoidea superfamily. To test this, we would have to determine whether RPCH activates the CG of other majoid crabs, particularly other *Pugettia, Libinia* and *Chionoecetes* species.

Dissection quality and heartbeat consistency

Throughout my studies, I found that lower quality dissection and cannulation decreased the vigor and consistency of the heartbeat in all three species of crab. As a result, our improved method of using a curved metal tube to cannulate the ostia of *P. producta* and *L. emarginata* hearts instead of the sternal posterior artery may make responses in these species more consistent. I did not have as much difficulty dissecting and cannulating *C. opilio* hearts because they are large enough that the sternal posterior artery is easily identifiable and accessible. *C. opilio* hearts are also similar anatomically to *Homarus americanus* hearts, for which the Dickinson lab has well established dissection and cannulation methods. Because of their small size and unique right or left-handedness, *P. producta* and *L. emarginata* hearts were much harder to cannulate, and as a result, my preparations took longer and often withstood more damage. This potentially explains why I saw more variation in responses to neuromodulators in *P. producta* and *L. emarginata*. However, now that we have determined a better and more efficient method of cannulating *P. producta* and *L. emarginata* hearts, future preparations may yield more consistent results.

Potential neurological differences between species of crab

If we still observe inconsistent responsiveness in *L. emarginata* after increasing sample size, we should investigate possible differences in *L. emarginata* compared to both *P. producta* and *C. opilio* that may cause differential sensitivity. The fact that *L. emarginata* walks forward 80% of the time rather than sideways (Schreiner, 2004) potentially sets it apart neurologically from *P. producta* and *C. opilio.* It has been found that *L. emarginata* has more neurons innervating proximal leg muscles than other brachyurans that walk sideways such as *Carcinus maenas* (Vidal-Galadea & Belanger, 2013). I have found no descriptions of *P. producta* and *C. opilio* walking behavior in the literature but recall that they walk sideways. Unfortunately, I cannot confirm this because I currently have no access to these specimens.

I do not have a proposed mechanism for how the number of neurons innervating proximal leg muscles could affect heart function. However, studies of crab species *Chasmagnathus granulatus* have found that cardiac and locomotor activity are correlated during escape behavior and responses to external stimuli (Burnovicz et al., 2009). For this reason, it is possible that heart function could be connected to walking behavior. On the other hand, if we find that *P. producta* and *C. opilio* also walk forwards, that would showcase a major difference between the crabs in this study and *Cancer borealis.* The Majoidea family contains multiple transitional species since majoid crabs were the first group to evolve sideways walking instead of forward walking as seen in lobster-like ancestors (Rice, 1983; Morrison et al., 2002), setting them apart from other Brachyurans like *Cancer* crabs and potentially explaining differences is responsiveness to modulators such as RPCH.

Transcriptome Assemblies

Many of the neuromodulators that activate the STNS of opportunistic feeders but fail to modulate the STNS of *P. producta* are still present in the neuropil of the stomatogastric ganglion as well as a variety of other tissues (Dickinson et al., 2008). This poses the question of why the STNS of *P. producta* is not responsive to present neuromodulators. In a biological system, it is typically energetically costly to maintain synthesis of protein structures not being. As a result, we predict that the DNA encoding the receptors of unutilized neuromodulators will not be transcribed. If this prediction is correct, the STNS of *P. producta* will express receptors for the modulators it responded to such as RPCH, oxotremorine, proctolin, dopamine, and myosuppressin, but not receptors for CCAP, CabTrp. On the other hand, the STNS of *C. opilio* and *L. emarginata* would express receptors for all seven neuromodulators. We would also expect the receptors for the modulators that activated the heart to be present in the CG. An alternative hypothesis is that differential sensitivity in the STNS is due to alterations in downstream signaling.

To evaluate receptor expression, we can generate transcriptomes from the STNS and CG of *P. producta*, *L. emarginata,* and *C. opilio*. Transcriptomes are a collection of mRNA sequences expressed at the time of extraction from a specific tissue sample. We can then use mass spectrometry and polymerase chain reaction (PCR) to confirm identified RNA sequences (Described in Alexandra Miller Honors Thesis, 2018). If our hypothesis regarding differential receptor expression is correct, we would expect to find fewer mRNA transcripts for receptors in the *P. producta* transcriptome than in *C. opilio* and *L. emarginata* transcriptomes. This would suggest that receptor availability in the STNS is responsible for diet-mandated differences in neuromodulatory capacity. Additionally, if we were to generate transcriptomes from the CG of

C. opilio, *L. emarginata*, and *P. producta*, we would predict to find similar amounts of receptor mRNA transcripts in all three species. Once receptor sequences have been established, we can also use qPCR to measure receptor mRNA expression.

Transcriptomes have been generated from the STG and commissural ganglia of *P. producta*, *L. emarginata*, and *C. opilio*. Brain and eyestalk transcriptomes were also generated because these neural tissues should contain all possible neuropeptides, increasing our chances of identifying receptor sequences. Several modulator receptor sequences have been identified, and we plan to determine their distribution by generating primers for the receptor sequences and using qPCR to establish relative expression levels. No transcriptomes have been generated from the CG of either crab species yet. No transcriptomes have been generated from the CG of either crab species yet.

Future directions

It is important to enlarge sample size in all three species. Individual differences in response to neuromodulators is expected, so we must collect more data get a better idea of what an average response is in a particular species. Additionally, I recommend that whoever works on this project after me test the cardiac response of *L. emarginata* with the same perfusion temperature as *C. opilio* and *P. producta* (~9.8ºC) to ensure that different responses are not a result of temperature. Since the cardiac response of *L. emarginata* to oxotremorine was so surprising, it would also be a good idea to test other muscarinic agonists such as pilocarpine on all three species of crab. Transcriptomes from the CG of all three species of crab should also be generated to access whether receptor expression levels coincide with modulatory capacity.

Conclusions

Although there are points of confusion in my data such as a lack of responsiveness in *L. emarginata* to oxotremorine, my findings support our prediction that specialized feeder *P. producta* and opportunistic feeders *C. opilio* and *L. emarginata* are more similarly modulated in the CG than in the STNS. Notably, my results indicate that *P. producta* responds to CCAP and CabTrp, modulators that did not activate the STNS. For all the seven modulators tested, the hearts of *P. producta* responded similarly to the hearts of *C. opilio* and/or *L. emarginata.* Enlarging the sample sizes of all three species of crab should shed more light on the modulatory capacity of the CG across opportunistic and specialized feeders. However, if we enlarge sample size and continue to see notable differences in responsiveness between the three species, we may have to rework our hypothesis that the phylogenetic closeness will control for any differences in cardiac and STNS function between species.

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