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# The pyloric neural circuit of the herbivorous crab *Pugettia producta* shows limited sensitivity to several neuromodulators that elicit robust effects in more opportunistically feeding decapods

Patsy S. Dickinson[1,2,*] Elizabeth A. Stemmler[3] and Andrew E. Christie[4,5]

[1]Department of Biology, Bowdoin College, 6500 College Station, Brunswick, ME 04011, USA, [2]Friday Harbor Laboratories, University of Washington, 620 University Road, Friday Harbor, WA 98250, USA, [3]Department of Chemistry, Bowdoin College, 6600 College Station, Brunswick, ME 04011, USA, [4]Department of Biology, University of Washington, Box 351800, Seattle, WA 98195-1800, USA and [5]Mount Desert Island Biological Laboratory, PO Box 35, Old Bar Harbor Road, Salisbury Cove, ME 04672, USA

*Author for correspondence (e-mail: pdickins@bowdoin.edu)

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## SUMMARY

**Modulation of neural circuits in the crustacean stomatogastric nervous system (STNS) allows flexibility in the movements of the foregut musculature. The extensive repertoire of such resulting motor patterns in dietary generalists is hypothesized to permit these animals to process varied foods. The foregut and STNS of *Pugettia producta* are similar to those of other decapods, but its diet is more uniform, consisting primarily of kelp. We investigated the distribution of highly conserved neuromodulators in the stomatogastric ganglion (STG) and neuroendocrine organs of *Pugettia*, and documented their effects on its pyloric rhythm. Using immunohistochemistry, we found that the distributions of *Cancer borealis* tachykinin-related peptide I (CabTRP I), crustacean cardioactive peptide (CCAP), proctolin, red pigment concentrating hormone (RPCH) and tyrosine hydroxylase (dopamine) were similar to those of other decapods. For all peptides except proctolin, the isoforms responsible for the immunoreactivity were confirmed by mass spectrometry to be the authentic peptides. Only two modulators had physiological effects on the pyloric circuit similar to those seen in other species. In non-rhythmic preparations, proctolin and the muscarinic acetylcholine agonist oxotremorine consistently initiated a full pyloric rhythm. Dopamine usually activated a pyloric rhythm, but this pattern was highly variable. In only about 25% of preparations, RPCH activated a pyloric rhythm similar to that seen in other species. CCAP and CabTRP I had no effect on the pyloric rhythm. Thus, whereas *Pugettia* possesses all the neuromodulators investigated, its pyloric rhythm, when compared with other decapods, appears less sensitive to many of them, perhaps because of its limited diet.**

Key words: stomatogastric nervous system, neurohormone, neuropeptide, amine, feeding.

## INTRODUCTION

Neural circuits contained within the crustacean stomatogastric nervous system (STNS) control the rhythmic movement of muscles in the esophageal, cardiac sac, gastric mill and pyloric regions of the foregut (Selverston and Moulins, 1987; Harris-Warrick et al., 1992; Nusbaum and Beenhakker, 2002; Marder and Bucher, 2007). Working in concert, these rhythmic movements allow food items to be ingested, chewed, and ultimately filtered from this portion of the digestive tract (Selverston and Moulins, 1987; Harris-Warrick et al., 1992; Nusbaum and Beenhakker, 2002; Marder and Bucher, 2007). In the decapod species examined thus far (i.e. the crab *Cancer borealis*, the chelate lobsters *Homarus americanus* and *Homarus gammarus* and the spiny lobster *Panulirus interruptus*), the STNS neural circuits are extensively modulated both by locally released and circulating substances (Selverston and Moulins, 1987; Harris-Warrick et al., 1992; Nusbaum and Beenhakker, 2002; Marder and Bucher, 2007). The result of this modulation is the expression of a large suite of distinct motor patterns that is hypothesized to allow these highly opportunistic feeders to process multiple food types.

Unlike most decapod species, the Northern kelp crab *Pugettia producta* is reported to be a dietary specialist, feeding almost exclusively on kelp (Hines, 1982). If the extensive STNS modulation previously reported for generalist feeders is an evolutionary consequence of a need to process highly variable food types, then this system in *P. producta* may need less modulatory control to process its relatively uniform diet. To test this hypothesis, we investigated the distribution of a number of well-known and highly conserved crustacean neuromodulators in the STNS and neuroendocrine organs (i.e. the sinus gland and the pericardial organ) of *P. producta* and tested their physiological actions on the pyloric motor pattern of this species. Some of these data have appeared previously in abstract form (Dickinson et al., 2004).

## MATERIALS AND METHODS

### Animals

Northern kelp crabs, *Pugettia producta* Randall, were collected by hand from dock pilings and in kelp beds in the greater Puget Sound area and San Juan archipelago of Washington State (USA). Animals were maintained in either flow-through natural seawater tanks (Friday Harbor Laboratories, Friday Harbor, WA, USA; ambient water temperature 8–12°C) or aerated natural seawater aquaria chilled to 10°C (Department of Biology, University of Washington, Seattle, WA, USA and Department of Biology, Bowdoin College, Brunswick, ME, USA). Animals for most experiments were collected in the summer months, and fed kelp in the holding tanks. For the experiments using 'winter animals', crabs were collected from dock pilings in late December and early January, a time at which no kelp is available in the Puget Sound area and the animals

from this population are reported to feed on barnacles and small bivalves. These animals were fed chopped mussels in our aquaria.

### Tissue collection

For the collection of tissues, crabs were anesthetized by packing in ice for 30–60 min. After anesthetization, the dorsal carapace was removed and the foregut was dissected free. The eyestalks and lateral walls of the pericardial chamber were also isolated at this time. To obtain the STNS (Fig. 1), which includes the paired commissural ganglia (CoG), the esophageal ganglion (OG), the stomatogastric ganglion (STG), as well as a number of interconnecting and the motor nerves, the foregut was flattened by making a longitudinal cut from the esophagus to the pylorus on its ventral side, followed by a pair of medial cuts directed along the ossicles of the cardiac sac/gastric mill. The teeth of the gastric mill were then removed and the flattened foregut was pinned, inside down, in a wax- or Sylgard-lined Pyrex dish containing chilled (10°C) physiological 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). The STNS was dissected free in chilled physiological saline. To obtain the sinus gland, the carapace encasing an eyestalk was split both dorsally and ventrally and one half of the split shell was gently teased away from the other half. The remaining half of the eyestalk was then pinned in a wax-lined Pyrex dish filled with chilled physiological saline and the eyestalk ganglia, to which the sinus gland is affixed, were subsequently isolated. To obtain the pericardial organ, the isolated walls of the pericardial chamber were pinned in a wax-lined dish filled with chilled physiological saline and the nerve roots constituting this endocrine site were dissected free from the surrounding connective tissue.

### Anatomy

#### Whole-mount immunocytochemistry

Immunohistochemistry was done on whole-mounts using methods and antibodies described in detail by Fu et al. (Fu et al., 2005). As a general marker for regions of synaptic neuropil, a mouse monoclonal antibody generated against a glutathione S-transferase fusion protein that included a portion of a *Drosophila* synapsin homolog (code SYNORF; kindly provided by Dr E. Buchner, Universität Würzburg, Würzburg, Germany) (Klagges et al., 1996) was used at a final dilution of 1:100. To assay tissues for the presence of *Cancer borealis* tachykinin-related peptide I (CabTRP I), a rat monoclonal antibody generated against substance P (clone NC1/34 HL; Abcam Incorporated, Cambridge, MA, USA; catalog no. ab6338) (Cuello et al., 1979) was used at a final dilution of 1:300. To assay tissues for the presence of crustacean cardioactive peptide (CCAP), a rabbit polyclonal antibody generated against this peptide (Dircksen and Keller, 1988; Stangier et al., 1988) (kindly provided by Dr H. Dircksen, Stockholm University, Stockholm, Sweden) was used at a final dilution of 1:500. To assay tissues for the presence of the peptide proctolin, a rabbit polyclonal antibody to proctolin (code K9832/13; kindly provided by Dr D. Nässel, Stockholm University) (Johnson et al., 2003) was used at a final dilution of 1:500. To assay tissues for the presence of red pigment concentrating hormone (RPCH), a rabbit polyclonal antibody generated against this peptide (Madsen et al., 1985) (kindly provided by Dr R. Elde, University of Minnesota, Minneapolis, MN, USA) was used at a final dilution of 1:300. To assay tissues for the amine dopamine, a mouse monoclonal antibody generated against tyrosine hydroxylase (Immunostar Inc., Hudson, WI, USA; catalog no. 22941), the biosynthetic enzyme of dopamine was used at a final dilution of 1:1000. The secondary antibodies used in our experiments were donkey anti-rabbit IgG conjugated to Alexa Fluor 488 (Molecular Probes, Eugene, OR, USA; catalog no. A-21206) or Alexa Fluor 594 (Molecular Probes; catalog no. A-21207); donkey anti-mouse IgG conjugated to Alexa Fluor 488 (Molecular Probes; catalog no. A-21202) or Alexa Fluor 594 (Molecular Probes; catalog no. A-21203) or donkey anti-rat IgG conjugated to Alexa Fluor 488 (Molecular Probes; catalog no. A-21208) or Alexa Fluor 594 (Molecular Probes; catalog no. A-21209).

#### Confocal and epifluorescence microscopy

After immunolabeling, preparations were viewed and data collected using one of two Bio-Rad MRC 600 laser scanning confocal microscopes (Bio-Rad Microscience Ltd, Hemel Hempstead, UK), a Bio-Rad Radiance 2000 laser scanning confocal microscope or a Nikon Eclipse E600 epifluorescence microscope. Descriptions of the hardware and software used for imaging on these systems have been extensively described in previous publications (Christie et al., 1997a; Messinger et al., 2005; Christie et al., 2007).

### Matrix-assisted laser desorption/ionization Fourier transform mass spectrometry

For direct tissue matrix-assisted laser desorption/ionization-Fourier transform mass spectrometry (MALDI-FTMS), STGs and sinus glands were analyzed as freshly dissected tissue samples; pericardial organs were stored in acidified water and frozen prior to analysis. STGs or sinus glands were isolated as described earlier, removed from the saline with fine forceps, rinsed sequentially in two 25 μl droplets of 0.75 mol $l^{-1}$ fructose (Sigma-Aldrich, St Louis, MO, USA) and then placed on a face of a ten-faceted probe tip, minimizing co-transfers of solution. STGs were left intact (with the exception of removal of the ganglionic sheath), as were sinus glands. Pericardial organs were thawed, rinsed with fructose, and cut into pieces before being applied to one face of a ten-faceted probe. Once on the probe, the tissue was sliced 10–20 times with a 0.2 mm needle; the macerated tissue was then gathered together and covered with a 0.5 μl droplet of 1.0 mol $l^{-1}$ 2,5-dihydroxybenzoic acid (DHB; SigmaAldrich; 98%, sublimed prior to use), prepared in 1:1 acetonitrile (Fisher Scientific, Pittsburgh, PA, USA; HPLC grade) and water containing 0.1% (v/v) trifluoroacetic acid (SigmaAldrich, 99%). All samples were analyzed using a HiResMALDI Fourier transform mass spectrometer (IonSpec, Lake Forest, CA, USA) equipped with a 4.7 Tesla actively shielded superconducting magnet (Cryomagnetics, Oak Ridge, TN, USA) as described previously (Christie et al., 2006).

### Electrophysiology

For physiological recordings, the STNS was dissected and pinned out in a Sylgard-lined Petri dish as described above, with the motor nerves and the nerves interconnecting the ganglia left intact. In addition, the sheath over the STG was removed to provide access to the cell bodies of neurons contained within the ganglion, and a section of sheath around the stomatogastric nerve (*stn*) was removed so that action potential conduction could be reversibly blocked using isotonic (750 mmol $l^{-1}$) sucrose in a petroleum jelly well surrounding this desheathed area of nerve. This sucrose block eliminated all modulatory inputs to the STG, since the *stn* is the only nerve that carries inputs from the CoGs and OG to the STG. During recordings, the dish containing the STNS was constantly superfused with chilled (10–12°C) physiological saline at a rate of 2–3 ml $min^{-1}$. It should be noted that a number of different saline compositions, based on those used in other species, were tested in preliminary experiments. The saline used here was based on *Cancer borealis* saline (Hooper

et al., 1986), and was chosen because it gave the most robust activity and was the one in which recovery from *stn* block was quickest and most complete. Modulators were made up immediately before use, and were added *via* a manual switching port to the superfusion system. The peptides CabTRP I (APSGFLGMRamide; synthesized by the Cancer Research Center of the University of Pennsylvania School of Medicine and kindly provided by Dr M. Nusbaum, Department of Neuroscience, University of Pennsylvania School of Medicine, Philadelphia, PA, USA), CCAP (PFCNAFTGCamide; Bachem AG, King of Prussia, PA, USA; catalog no. H-6745), proctolin (RYLPT; Sigma-Aldrich; catalog no. P4280) and RPCH [pELNFSPGWamide; Bachem Biosciences, Inc., King of Prussia, PA, USA; catalog no. H-6750 (dissolved first in 7% dimethyl sulfoxide)] were each reconstituted and stored frozen as stock solutions at $10^{-3}$ mol $l^{-1}$, then diluted. Oxotremorine (Sigma-Aldrich; catalog no. O-9126) and dopamine (Sigma-Aldrich; catalog no. H-8502) were dissolved directly in the saline.

Neuronal activity was recorded extracellularly using standard electrophysiological techniques. Specifically, activity on the motor nerves was recorded *via* A-M Systems Model 1700AC amplifiers (A-M Systems, Inc., Carlsborg, WA, USA) using stainless steel pin electrodes, which were isolated from the bath with petroleum jelly wells. All electrical activity was further processed with a Brownlee 410 instrumentation amplifier (Brownlee Precision Co., San Jose, CA, USA) and recorded directly onto a PC computer *via* a Micro 1401 board and Spike 2 software (Cambridge Electronic Design, Cambridge, UK). Data were processed using Spike 2 and further analyzed with Prism4 (GraphPad Software, Inc., San Diego, CA, USA).

## RESULTS

### Location of putative synaptic neuropil in the stomatogastric nervous system

Previous studies have shown that an antibody to the synaptic vesicle-associated protein synapsin labels regions of synaptic neuropil in the crustacean STNS (Skiebe and Ganeshina, 2000; Skiebe and Wollenschlager, 2002). Using this antibody, we mapped the distribution of putative synaptic neuropil in the STNS of *P. producta* and found it to be essentially identical to that reported previously for other brachyuran species (Skiebe and Ganeshina, 2000; Skiebe and Wollenschlager, 2002). Specifically, synapsin labeling was routinely seen in the CoGs and the STG, but not in the OG. In the CoGs and STG, all staining was confined to the neuropil, with little or no label seen in or around the intrinsic somata. In the CoGs, the synapsin label appeared to fill the entire volume of the neuropilar region with only a few sites of apparent avoidance. Within the STG, synapsin staining was confined primarily to the peripheral portion of the neuropil, with the central core of the structure relatively devoid of immunoreactivity. Extraganglionic patches of synapsin labeling were also present in each superior esophageal nerve (*son*; near the junction of the dorso-posterior esophageal nerve; *dpon*), at the junction of the *son*s, esophageal nerve (*on*) and the stomatogastric nerve (*stn*), as well as in the anterior portion of the *stn* proper. Regardless of location, the labeling was found in small blob-like varicosities. The distribution of synapsin-like labeling in the *P. producta* STNS is shown schematically in Fig. 1.

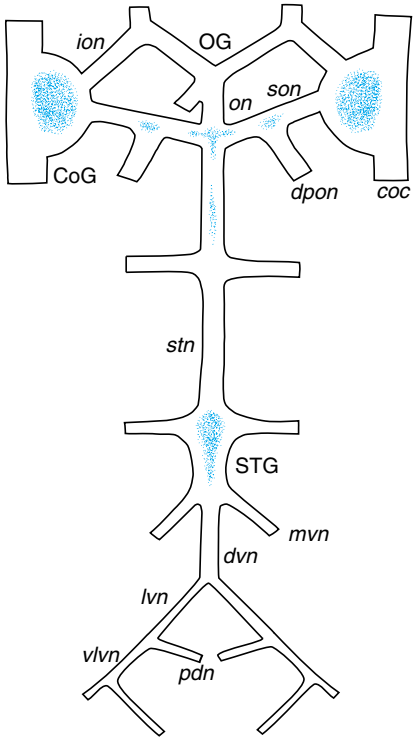


Fig. 1. Schematic representation of the stomatogastric nervous system (STNS) of *Pugettia producta*, including the locations (blue stippling) of putative synaptic neuropils, as indicated by the presence of synapsin-like immunoreactivity, within the STNS. *coc*, circumesophageal connective, CoG, commissural ganglion; *dpon*, dorso-posterior esophageal nerve; *dvn*, dorsal ventricular nerve; *ion*, inferior esophageal nerve; *lvn*, lateral ventricular nerve; *mvn*, medial ventricular nerve; OG, esophageal ganglion; *on*, esophageal nerve; *pdn*, pyloric dilator nerve; *son*, superior esophageal nerve; STG, stomatogastric ganglion; *stn*, stomatogastric nerve; *vlvn*, ventro-lateral ventricular nerve.

### Immunohistochemical survey for putative neuromodulators in the stomatogastric ganglion and neuroendocrine organs

#### The stomatogastric ganglion

The results of our synapsin immunostaining showed that among the putative synaptic neuropils of the *P. producta* STNS is one located within the STG. In other decapod species, local release of substances from the STG neuropil modulates the output of the pyloric motor pattern, which is produced by a neural circuit contained within the ganglion (Selverston and Moulins, 1987; Harris-Warrick et al., 1992; Nusbaum and Beenhakker, 2002; Marder and Bucher, 2007). To determine if several well-known and highly conserved neuromodulators were present in the STG of *P. producta* (i.e. the amine dopamine and the peptides CabTRP I, CCAP, proctolin and RPCH), we immunolabeled the STNS of this species with antibodies to these substances or to their biosynthetic enzyme. Although each of these antibodies produced labeling within the STNS, only those used to detect CabTRP I, proctolin and RPCH labeled the STG neuropil (Table 1). For each of these antibodies, labeling in the ganglion appeared to originate from input axons descending from the anterior ganglia (i.e. the CoGs and/or OG).

#### Neuroendocrine organs

In addition to locally released neuroactive substances, the output of circuits within the STG is also known to be modulated by hormones released from several neuroendocrine organs located outside the STNS, specifically the sinus gland of the eyestalk and the pericardial organ that surrounds the heart (Christie et al., 1995; Marder et al., 1995; Skiebe, 2001). To determine whether any of the above mentioned compounds might reach the *P. producta* STG neuropil *via* a hormonal route, we immunolabeled both the sinus gland and

Table 1. Immunohistochemical detection of putative neuromodulators in the stomatogastric ganglion and neuroendocrine organs of *Pugettia producta*

| Neuromodulator | Tissue | | |
|---|---|---|---|
| | STG | SG | PO |
| Amines | | | |
| Dopamine* | – | – | + |
| Peptides | | | |
| CabTRP | + | – | – |
| CCAP | – | – | + |
| Proctolin | + | + | + |
| RPCH | + | + | + |

*The presence of dopamine was assessed using an antibody to its biosynthetic enzyme tyrosine hydroxylase.
STG, stomatogastric ganglion; SG, sinus gland; PO, pericardial organ; CabTRP, *Cancer borealis* tachykinin-related peptide; CCAP, crustacean cardioactive peptide; RPCH, red pigment concentrating hormone; +, immunoreactivity present; –, immunoreactivity absent.
$N \geqslant 3$ preparations for each antibody in each tissue.

the pericardial organ of this species for each of the substances. In the sinus gland, only the proctolin and the RPCH antibodies stained putative endocrine release terminals (Table 1). Within the pericardial organ, the CCAP, proctolin, RPCH and TH antibodies each immunolabeled an extensive set of release sites (Table 1). No CabTRP I-like labeling was found in either the sinus gland or pericardial organ (Table 1).

### Mass spectrometric analysis of native peptides

The results of our immunohistochemical surveys suggested the presence of CabTRP I, proctolin and RPCH in the STG, proctolin and RPCH in the sinus gland, and CCAP, proctolin and RPCH in the pericardial organ. To confirm the presence of authentic peptide in these tissues, we conducted direct tissue MALDI-FTMS analyses on isolated tissue samples from each of these structures. We found peaks corresponding to the $[M+Na]^+$ ion of authentic RPCH (Stemmler et al., 2006) in the sinus gland, as well as $[M+H]^+$ ions corresponding to authentic CabTRP I and authentic CCAP in the STG and the pericardial organ, respectively (Fig. 2; Table 2). By contrast, and in spite of the fact that we saw proctolin-like immunoreactivity in the STG, sinus gland and pericardial organ, we did not find a peak corresponding to the mass of authentic proctolin in any of the tissue samples we tested from those regions.

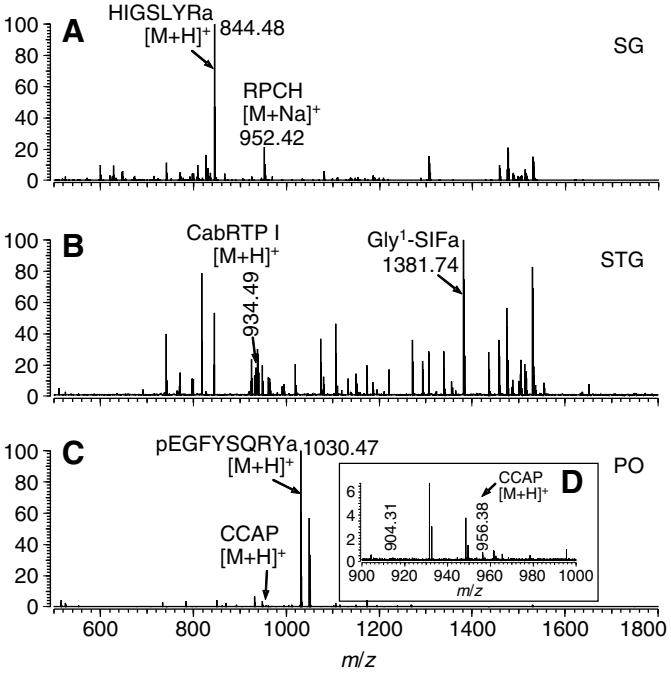


Fig. 2. MALDI-FTMS identification of native *Pugettia producta* neuropeptides. (A–C) Representative direct tissue spectra of the (A) sinus gland (SG), (B) stomatogastric ganglion (STG) and (C) pericardial organ (PO). In the SG, a peak corresponding to the $[M+Na]^+$ ion of pELNFSPGWamide (red pigment concentrating hormone; RPCH) was routinely detected. In the STG, the $[M+H]^+$ ion corresponding to APSGFLGMRamide (authentic *Cancer borealis* tachykinin-related peptide I; CabTRP I) was present in most spectra. In PO samples, the $[M+H]^+$ ion of PFCNAFTGCamide (crustacean cardioactive peptide; CCAP) was commonly seen. In addition, peaks corresponding to other peptides were also detected in these tissues, some of which are labeled in the spectra. *m/z*, mass/charge; Gly$^1$-SIFa, GYRKPPFNGSIFamide.

### Expression of the pyloric motor pattern is dependent on modulatory input from anteriorly located sources

The pyloric motor pattern in *P. producta* strongly resembled that recorded in other decapod species (Fig. 3). The core pyloric pattern consisted of bursts in the two pyloric dilator (PD) neurons, followed by a brief silent period before bursts in the lateral pyloric (LP) neuron, then pyloric (PY) neurons. Bursts in the inferior cardiac (IC) neuron began soon after the end of the PD burst, and overlapped the LP burst, whereas those in the ventricular dilator (VD) neuron began at the end of each PY burst, and overlapped the next PD burst. However, the burst period in this species was considerably longer than has been reported for other decapods: average burst period in *P. producta* was 2.9±0.32 s ($N$=15), whereas it is approximately 1 s in the spiny lobster *Panulirus interruptus* (Selverston et al., 1976) and in the crab *Cancer borealis* (Hooper et al., 1986), and about 1.5 s in the lobsters *Homarus americanus* and *Homarus gammarus* (Meyrand et al., 1991; Richards et al., 1999; Mizrahi et al., 2001). In addition, we noted that the pattern in *P. producta* was highly dependent on input from the anterior ganglia. Blocking the *stn* with isotonic sucrose eliminated all pyloric bursting within 15 min in all but two preparations ($N$=22). With the exception

Table 2. Exact mass measurements for peptides detected in the direct analysis of *P. producta* tissues using MALDI-FTMS

| | Measured *m/z** (error, p.p.m.) | | | |
|---|---|---|---|---|
| | CabTRP Ia $[M+H]^+$ | CCAP $[M+H]^+$ | RPCH $[M+Na]^+$ | Proctolin $[M+H]^+$ |
| Standard: | *m/z* 934.4927 | *m/z* 956.3753 | *m/z* 952.4288 | *m/z* 649.3668 |
| Tissue | | | | |
| SG | ND | ND | 952.4289 (0.2) | ND |
| STG | 934.4944 (1.8) | ND | ND | ND |
| PO | ND | 956.3759 (0.6) | ND | ND |

CabTRP 1a, APSGFLGMRamide; CCAP, PFCNAFTGCa; RPCH, pELNFSPGWa; proctolin, RYLPT; G$^1$-SIF, GYRKPPFNGSIFamide; SG, sinus gland; STG, stomatogastric ganglion; PO, pericardial organ; ND, not detected.
*Internal calibration with polypropylene glycol.

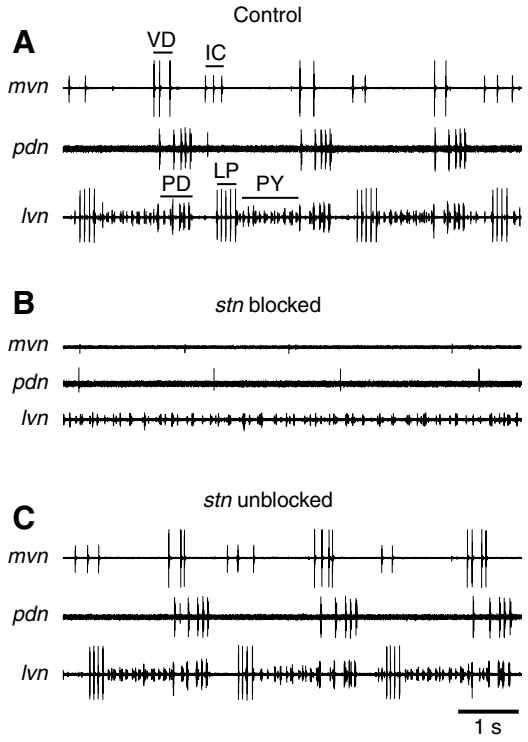


Fig. 3. The pyloric motor pattern of *Pugettia producta* is similar to that of other decapod species, though its expression is more highly dependent on the presence of modulatory inputs from anteriorly located sources. (A) In *P. producta*, the pyloric motor pattern strongly resembled that recorded in other decapod species, and consisted of alternating bursts of action potentials in the three core pyloric neuronal types: the pyloric dilator (PD), lateral pyloric (LP) and pyloric (PY) neurons, recorded on the lateral ventricular nerve (*lvn*). The ventricular dilator (VD) and inferior cardiac (IC) neurons, recorded on the medial ventricular nerve (*mvn*), fired weaker bursts that were more or less in phase with the bursts in the LP and PY neurons, respectively. (B) Blocking the stomatogastric nerve (*stn*), which provides the only neuronal input to the STG, with isotonic sucrose eliminated all pyloric bursting within 15 min in most preparations, as shown here. (C) When the sucrose was replaced with saline, so that normal conduction was restored in the *stn*, the complete pyloric pattern recovered within 2–3 min. Nerves: *mvn*, medial ventricular nerve (recording action potentials of the VD and IC neurons); *pdn*, pyloric dilator nerve (recording action potentials of the PD neurons); *lvn*, lateral ventricular nerve (recording action potentials of the PD, LP and PY neurons).

of the PY neurons, which fired tonically in sucrose block, the pyloric neurons were largely silent, although there were occasional spikes in many of the neurons (e.g. in the PD neurons in the preparation shown in Fig. 3). This effect was fully reversible, and the complete pyloric pattern was restored within 2–3 min after the sucrose was replaced with saline (Fig. 3). We considered the possibility that this level of dependence on modulatory input was an artifact of the saline used in the experiments, and so tried a number of saline formulations, based on other decapod salines as well as on the measured concentrations of ions in *P. producta* hemolymph (Cornell, 1979). A physiological saline formulation based on one routinely used for *C. borealis* (Hooper et al., 1986), another brachyuran crab, was used in all physiological experiments as it gave the fastest recovery from sucrose block.

Because the modulator complement of the *P. producta* STG and neuroendocrine organs appeared to be similar to those of other decapod species that have been studied, we tested the physiological effects of a number of modulators on the pyloric pattern. The goal of these experiments was to determine whether or not they functioned as neuromodulators in this system. To eliminate the confounding effects of modulatory substances spontaneously released from axons projecting from the somata present in anterior ganglia, and to preclude false negatives due to a ceiling effect (if a preparation was already maximally active), we tested each putative modulator on both intact and *stn*-blocked preparations. However, as we were primarily interested in whether or not the modulators were able to modulate the pyloric pattern, we did not fully characterize the effects of each modulatory transmitter, although we did determine its effects on cycle period, phase of the pyloric neurons, burst durations, and spike frequencies in each group of neurons. In order to avoid damaging neurons with microelectrodes, all neuronal activity was recorded extracellularly. Thus, the measurements of spike frequency represent a composite of all the neurons firing, and as such are useful only as a point of comparison between conditions in this species.

### Oxotremorine and proctolin are strong and consistent modulators of the pyloric rhythm

When modulatory inputs from the anterior ganglia (CoGs and OG) were eliminated by the application of a sucrose block to the *stn*, only three of the modulators assayed in these experiments, oxotremorine, proctolin and dopamine, modulated the pyloric pattern in most preparations tested. Of these, only oxotremorine and proctolin consistently activated a complete triphasic pyloric pattern.

#### Oxotremorine

At a concentration of $10^{-6}$ mol $l^{-1}$, the muscarinic agonist oxotremorine routinely activated the pyloric pattern in blocked preparations (seven of seven), in which there was no previously ongoing pyloric activity (Fig. 4), and enhanced it in unblocked preparations (seven of eight) with already active patterns (Fig. 5). In the blocked preparations, only the core pyloric pattern was activated, with firing in the PD, LP and PY neurons, but not in the IC or VD neurons. Moreover, the cycle period in the presence of oxotremorine, with the *stn* blocked, was 4.5 s, somewhat slower than the control frequency of less than 3 s in unblocked preparations ($P$=0.06, unpaired $t$-test, $N$=15 control, 4 in oxotremorine). In unblocked preparations, as can be seen in Fig. 5, oxotremorine led to a significant decrease in pyloric cycle period (Fig. 5D, paired $t$-test, $P$<0.05), which was largely due to a decrease in the PY burst duration (Fig. 5F, paired $t$-test, $P$<0.05).

#### Proctolin

The pentapeptide proctolin, like oxotremorine, consistently modulated the pyloric pattern when it was bath applied at a concentration of $10^{-6}$ mol $l^{-1}$ ($N$=14 of 14). In addition, in 55% of preparations with the *stn* blocked (Fig. 6; $N$=6 of 13), proctolin activated the core pyloric pattern (PD–LP–PY); however, it induced bursting in the VD or IC neurons in only half of these preparations. Moreover, cycle period was significantly longer (mean 15.4±2.3 s) than that recorded in the same preparations with the *stn* intact (mean 2.6±0.3 s; paired $t$-test, $P$<0.01, $N$=6). In these preparations, the PY neurons predominated, as can be seen in the phase diagram in Fig. 6D, as well as in the graphs of burst duration (Fig. 6E).

When the anterior inputs were intact (Fig. 7) and the ongoing pyloric pattern was active, proctolin did not cause significant changes in cycle period (Fig. 7D), but it did cause significant changes in the relative phases of the pyloric pattern. In such unblocked preparations,

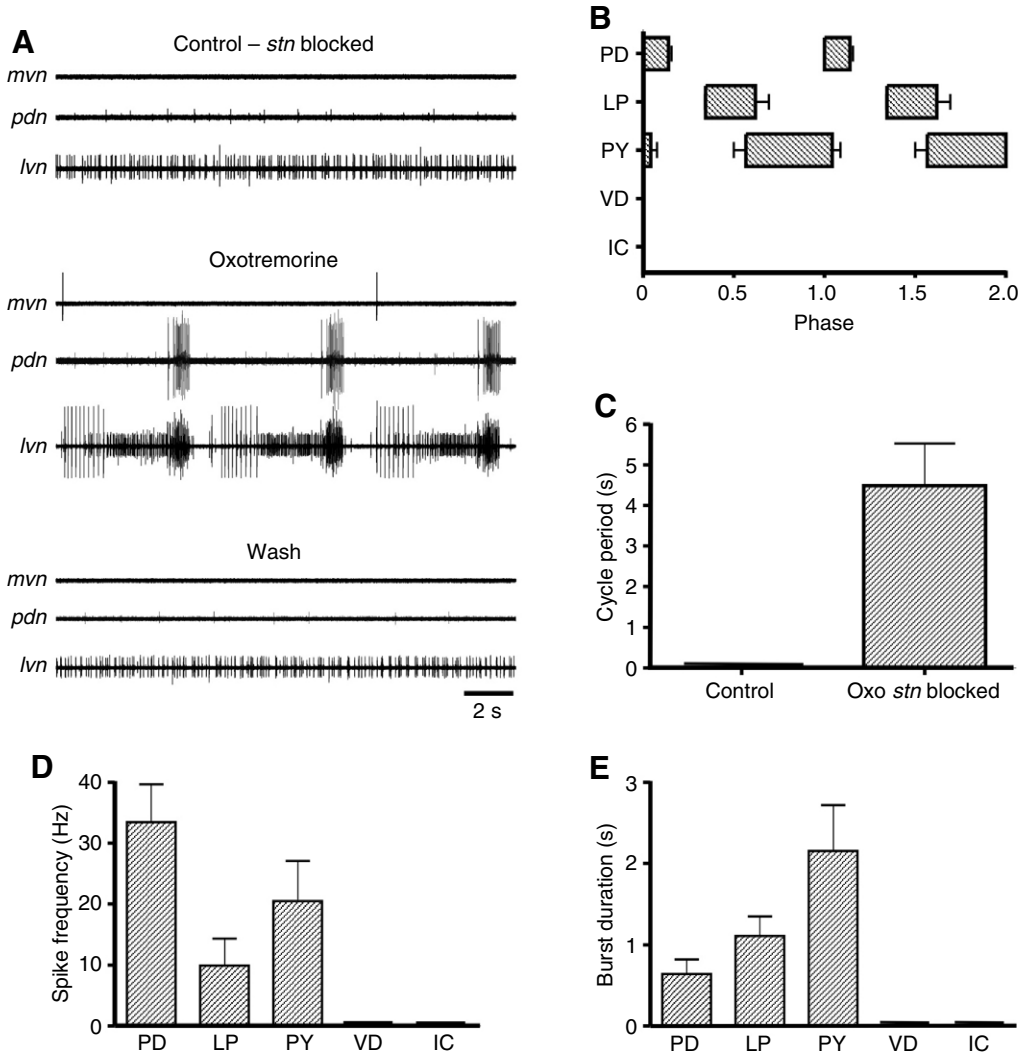


Fig. 4. The muscarinic acetylcholine agonist oxotremorine routinely activated the pyloric pattern in preparations in which the *stn* was blocked with isotonic sucrose, and there thus was no ongoing pyloric pattern. (A) Representative recordings of pyloric activity in a preparation with conduction in the *stn* blocked with isotonic sucrose and superfused with normal saline, then superfused with oxotremorine ($10^{-7}$ mol $l^{-1}$), followed by a wash in normal saline. A complete core pyloric pattern, with intense firing in all three neuronal types (PD, LP, and PY) was induced by the oxotremorine (seen in *pdn*, PD, and *lvn*, PD, LP and PY, recordings); in contrast, regular bursting was not initiated in the VD and IC neurons (*mvn*). (B) Phase plot, showing two cycles of the pyloric pattern recorded in oxotremorine, taken from four preparations, showing that oxotremorine activated the core pyloric pattern, but not the VD and IC neurons. (C) Graph of average cycle period: in control saline there was no activity, while cycle period was approximately 4 s in the presence of oxotremorine (Oxo); this is somewhat longer than cycle period in control saline when the *stn* is not blocked (approximately 3.3 s in Fig. 3, for example.) (D,E) Graphs of the spike frequency during bursts and burst duration in each neuronal type during oxotremorine superfusion with the *stn* blocked. Because there was no rhythmic activity, and therefore no bursts in any of the neurons, values during saline superfusion are not shown. *N*=4 for all graphs. Bars indicate standard deviations. Nerves: *mvn*, medial ventricular nerve (recording action potentials of the VD and IC neurons); *pdn*, pyloric dilator nerve (recording action potentials of the PD neurons); *lvn*, lateral ventricular nerve (recording action potentials of the PD, LP and PY neurons).

the most prominent change was an increase in burst duration in the LP neuron (paired *t*-test, $P<0.05$, $N=4$), as has been reported in other species in response to proctolin superfusion, and in the IC neuron (paired *t*-test, $P<0.05$, $N=4$), as can be seen in both the phase diagram and the graph of burst durations (Fig. 7D and E).

### Dopamine regularly modulates the pyloric pattern, but is inconsistent in its effects

Although most preparations were modulated by dopamine (seven of seven *stn*-blocked preparations and five of seven *stn*-intact preparations), the preparation-to-preparation variability in the qualitative effects seen in both conditions was high, as can be seen in the two examples shown in Figs 8 and 9. This variability effectively precluded pooled quantification of the patterns. In all cases, however, dopamine enhanced at least one aspect of the pyloric pattern.

### RPCH is capable of modulating the pyloric rhythm but rarely does so

RPCH generally had no effect on the pyloric rhythm in preparations with the *stn* intact and an ongoing pyloric pattern. In four of four such preparations, cycle frequency, burst durations and spike frequencies and phase relationships remained unchanged in the presence of $10^{-6}$ mol $l^{-1}$ RPCH (Fig. 10). It was clear, however, that

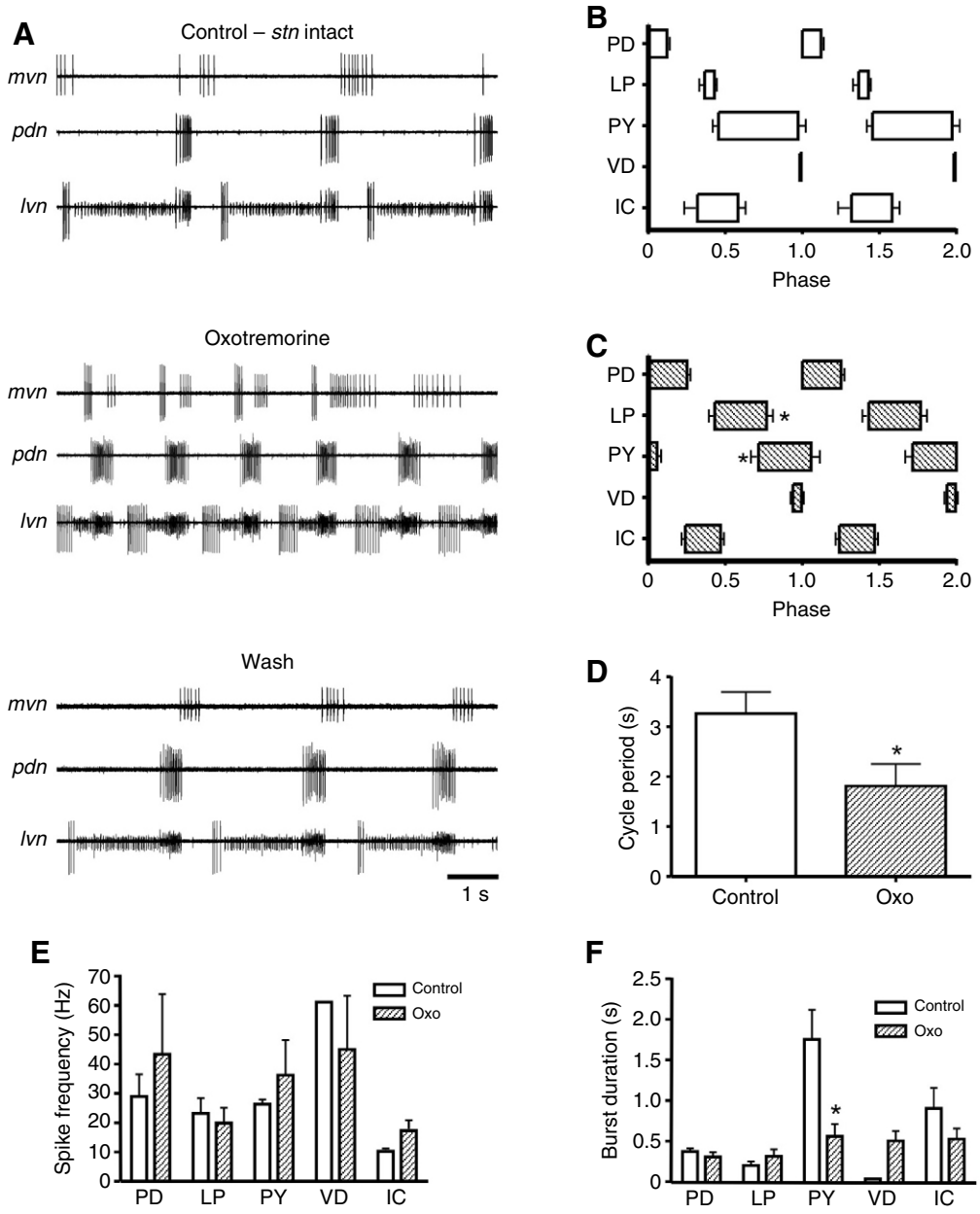


Fig. 5. In preparations in which the *stn* was intact, the muscarinic acetylcholine agonist oxotremorine enhanced ongoing pyloric activity. (A) Recordings of pyloric activity in a representative preparation in control saline, during superfusion of $10^{-6}$ mol $l^{-1}$ oxotremorine, and during the wash with normal saline. Oxotremorine enhanced the overall pyloric pattern, seen most clearly here as an increase in pyloric cycle frequency and as increased activity in the PD (*pdn* and *lvn*) and VD (*mvn*) neurons. (B) Phase diagram, showing two cycles of the activity of the pyloric pattern in control saline. (C) Phase diagram of the pyloric pattern when the STG was superfused with $10^{-6}$ mol $l^{-1}$ oxotremorine. (D) Plot of the cycle period in both control saline and oxotremorine, showing the decreased cycle period during oxotremorine application. (E,F) Graphs of spike frequency during bursts and burst duration, respectively, in each of the pyloric neuronal types in control saline and when the STG was being superfused with oxotremorine. *N*=5 for all graphs. Bars indicate standard deviations. Asterisks indicate a value significantly different from control ($P$<0.05). Nerves: *mvn*, medial ventricular nerve (recording action potentials of the VD and IC neurons); *pdn*, pyloric dilator nerve (recording action potentials of the PD neurons); *lvn*, lateral ventricular nerve (recording action potentials of the PD, LP and PY neurons).

RPCH is able to affect the pyloric pattern, as it did so in 22% of preparations (two of nine) with the *stn* blocked (Fig. 11). In both of these cases, there was a weak pyloric pattern even in the presence of the sucrose block, suggesting the possibility that RPCH weakly activates the pattern, but does not do so strongly enough to bring any of the pyloric neurons in a completely inactive pattern to threshold.

### The pyloric rhythm is not activated by either CabTRP I or CCAP

Two peptides that are present in the *P. producta* stomatogastric nervous system and/or neuroendocrine organs, and that consistently activate the pyloric pattern in other decapod species, CabTRP I and CCAP, had no effect on the pyloric pattern in either *stn*-blocked (*N*=4 for both peptides) or *stn*-intact (*N*=5 for both peptides)

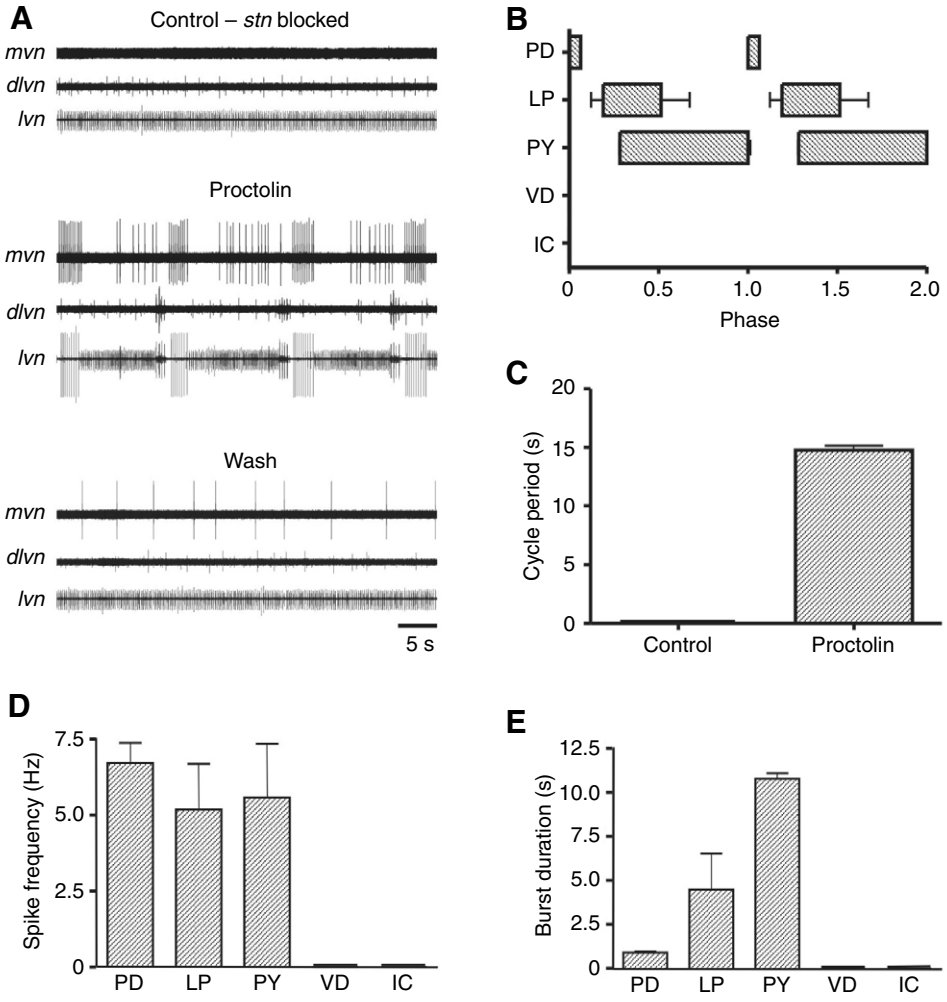


Fig. 6. (A) When axonal conduction in the *stn* was blocked with isotonic sucrose, and there was no ongoing pyloric rhythm, superfusion of the pentapeptide proctolin ($10^{-6}$ mol $l^{-1}$) induced rhythmic pyloric activity in 55% of preparations, including the one shown here. (B) Phase plot, showing two cycles of the pattern induced by proctolin in those preparations (*N*=6) that were activated. Phases of firing in the VD and IC neurons are not shown, because they were active in only three preparations (including the one shown in A). (C) Graph of average cycle period. In control saline there was no activity, whereas cycle period was nearly 15 s in the presence of proctolin; this is considerably longer than cycle period in control saline when the *stn* is not blocked (approximately 3.3 s in Fig. 3, for example.) (D,E) Graphs of spike frequency during bursts and of burst duration in each of the core pyloric neurons (the PD, LP and PY neurons) that were regularly activated during proctolin superfusion with the *stn* blocked. Because there was no rhythmic activity, and therefore no bursts in any of the neurons, values during saline superfusion are not shown. Nerves: *mvn*, medial ventricular nerve (recording action potentials of the VD and IC neurons); *dlvn*, dorso-lateral ventricular nerve (recording action potentials of the PD neurons); *lvn*, lateral ventricular nerve (recording action potentials of the PD, LP and PY neurons).

preparations. As can be seen in Fig. 12 (CabTRP I, *N*=5) and Fig. 13 (CCAP, *N*=5), there were no changes in cycle period, burst duration or spike frequency of any of the pyloric neurons, nor were there changes in the phase relationships of the pyloric pattern in actively cycling preparations. Neither peptide induced any rhythmicity in any preparation when the *stn* was blocked (*N*=4 for each peptide).

Because kelp is available only seasonally in the northern portion of the range of *P. producta* (including the Puget Sound region where the animals used here were collected), we considered the possibility that these peptides might exert their effects only in the winter, when *P. producta* is reported by some to eat a more varied diet. We therefore tested the effects of both CabTRP I and CCAP on crabs collected in late December and fed on mussels. As in *P. producta* collected during the summer and fed kelp, there was no effect of either peptide in either *stn*-intact or *stn*-blocked preparations (CabTRP I, *N*=4; CCAP, *N*=3; data not shown).

## DISCUSSION

For nearly four decades, the neural circuits contained within the crustacean STNS have been used as models for understanding the modulatory control of rhythmic motor patterns (Selverston and Moulins, 1987; Harris-Warrick et al., 1992; Nusbaum and Beenhakker, 2002; Marder and Bucher, 2007). Work on a number of species has shown that these neural circuits are extensively influenced both by locally released and circulating substances, which results in the expression of a large number of distinct motor patterns. Given that the species commonly used for study have all been highly opportunistic feeders, this extensive modulation has been hypothesized to be an evolutionary response to the need to process multiple food types. In this report, we have investigated the

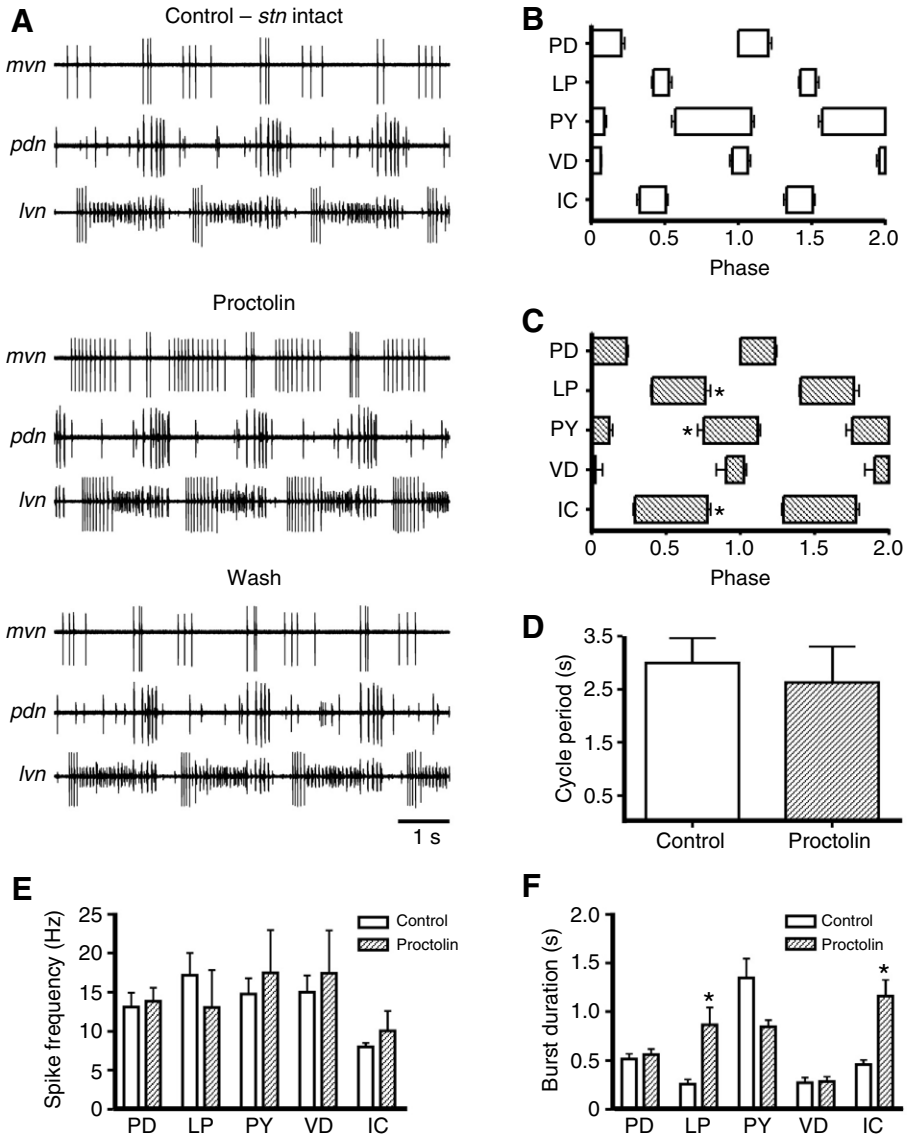


Fig. 7. When the anterior inputs were intact and the ongoing pyloric pattern was active, proctolin did not cause significant changes in cycle period, but it did cause significant changes in the relative phases of the pyloric pattern and in the burst duration of some neurons. (A) Recordings of control activity and activity when $10^{-6}$ mol $l^{-1}$ proctolin was bath-applied to the STG. (B,C) Phase plots, showing two cycles of the pyloric pattern in control saline (B) and in the presence of proctolin (C). The LP and IC neuron bursts were each prolonged, starting at about the same phase, but continuing longer, in proctolin than in control saline. The burst of PY neurons started sooner in proctolin than in control saline. (D) Cycle period was not significantly changed by proctolin when the *stn* was intact. (E,F) Graphs of the spike frequency during bursts (E) and of burst duration (F) in control saline and in proctolin, showing that most parameters were not altered, but both LP and IC neuron bursts increased in duration. *N*=5 for all graphs. Bars indicate standard deviations. Nerves: *mvn*, medial ventricular nerve (recording action potentials of the VD and IC neurons); *pdn*, pyloric dilator nerve (recording action potentials of the PD neurons); *lvn*, lateral ventricular nerve (recording action potentials of the PD, LP and PY neurons).

neuromodulatory control of the *P. producta* STNS, which, unlike previously studied species, is a dietary specialist, consuming a relatively uniform diet consisting primarily of kelp and other brown algae (Hines, 1982).

In terms of general organization, we found that the overall structure of the *P. producta* STNS is essentially identical to that of opportunistically feeding brachyurans (i.e. it consists of the same four ganglia, which are interconnected by a complement of nerves that are similar in location and innervation patterns to those of the other species thus far investigated). Likewise, we found that within the system, the location and organization of putative synaptic regions are conserved between *P. producta* and the other species. Our immunohistochemical survey of putative transmitters in the STG and neuroendocrine organs of *P. producta* suggested that a number of bioactive compounds are readily available to function as locally and/or hormonally delivered modulators in its STG. In fact, the distribution of each of the investigated substances is essentially identical to that seen in the highly modulated and opportunistically feeding crabs *Cancer borealis* and/or *Cancer productus* (Marder et al., 1986; Goldberg et al., 1988; Blitz et al., 1995; Christie et al., 1995; Christie et al., 1997a; Christie et al., 1997b; Fu et al., 2005). Thus, in terms of gross structure and the availability of modulators, there appeared to be few, if any, large-scale differences between the STNS of the

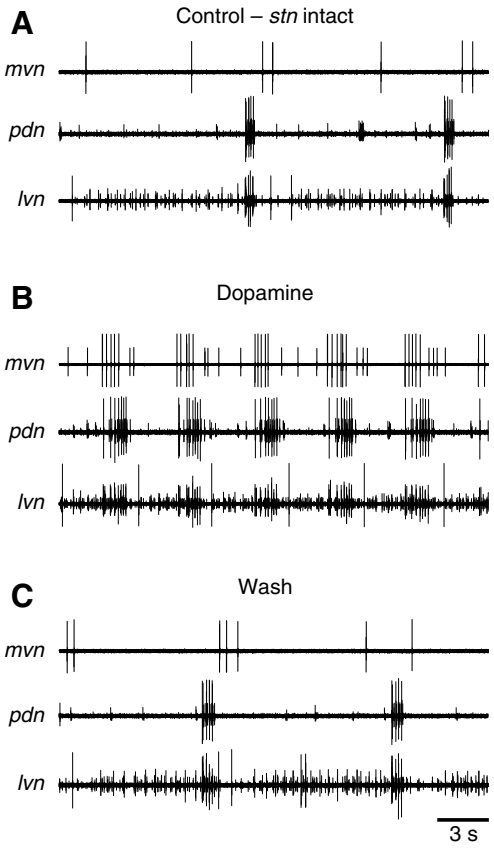


Fig. 8. In preparations with relatively weak baseline firing, dopamine was capable of activating a typical pyloric pattern, as was the case in the preparation shown here. Recordings of the pyloric pattern in (A) control, (B) when dopamine ($10^{-4}$ mol $l^{-1}$) was superfused over the STG, and (C) in wash. Nerves: *mvn*, medial ventricular nerve (recording action potentials of the VD and IC neurons); *pdn*, pyloric dilator nerve (recording action potentials of the PD neurons); *lvn*, lateral ventricular nerve (recording action potentials of the PD, LP and PY neurons).

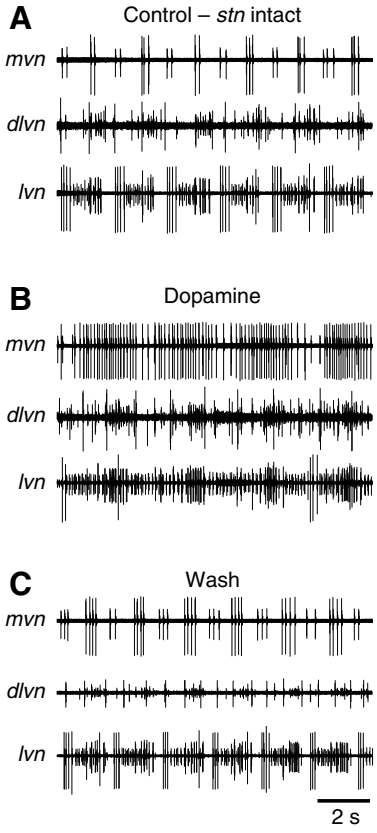


Fig. 9. In preparations with stronger ongoing pyloric activity, dopamine did not alter cycle frequency, but did cause changes within the pattern, notably a strong increase in activity in the VD neuron (*mvn*) and a decrease in activity in the LP neuron (large spike on *lvn*). Recordings of the pyloric pattern in (A) control saline, (B) in the presence of dopamine ($10^{-4}$ mol $l^{-1}$), and (C) in wash. Nerves: *mvn*, medial ventricular nerve (recording action potentials of the VD and IC neurons); *dlvn*, dorso-lateral ventricular nerve (recording action potentials of the PD neurons); *lvn*, lateral ventricular nerve (recording action potentials of the PD, LP and PY neurons).

dietary specialist *P. producta* and those of more opportunistically feeding crabs.

With respect to the motor output produced by the *P. producta* STG circuit, we found that its gross pyloric motor pattern is similar to those described from other brachyurans [i.e. the core pyloric pattern is triphasic (PD, LP, PY), with bursts in the VD and IC neurons more or less in phase with those in the PD and LP or PY neurons, respectively (Nusbaum and Beenhakker, 2002)]. As in other species, the expression of the *P. producta* pyloric rhythm appears to be dependent on modulatory influences provided by descending inputs from the CoGs and OG, as blocking impulse activity in the *stn* (the sole route of input to the STG from these ganglia) always diminished or stopped production of this motor pattern. In fact, the extent to which the pyloric rhythm was suppressed by *stn* blockade suggested that it might have a stronger dependence on input from these anterior ganglia than do the opportunistic feeders, such as *C. borealis*. Given these results, we were quite surprised to find that many of the modulators we localized to the STG neuropil and/or identified as putative hormones in *P. producta* exerted little or no modulatory action on the pyloric rhythm in this species (i.e. the amine dopamine and the peptides CabTRP I, CCAP and RPCH), despite their strong modulatory influence on this motor pattern in all other decapods thus far investigated. In fact, only the muscarinic acetylcholine receptor agonist oxotremorine and the peptide proctolin showed strong modulatory effects on the system that were similar to those seen in other decapod species. Thus, whereas *P. producta* possesses a number of neuromodulators known to influence the output of the stomatogastric circuit in many opportunistically feeding crustaceans, our results show that it is relatively insensitive to many of them, perhaps as it needs only a limited repertoire of motor outputs to process the relatively uniform food types it commonly ingests.

### What is responsible for the decreased modulation in *Pugettia producta*

The two most likely differences between *P. producta* and other decapods that might account for the decreased modulation in this species are changes in the modulatory environment or changes in the receptors to those modulators. We found that the modulators we examined all appear to be present in *P. producta* in locations similar to those in other species. Moreover, even the amino acid sequences of the native peptide isoforms we examined were identical to those reported in other crab species, with the possible exception of proctolin. Ironically, proctolin was the peptide with effects that most strongly resembled those seen in other species, but was the only peptide that was not detected in the STNS or in the

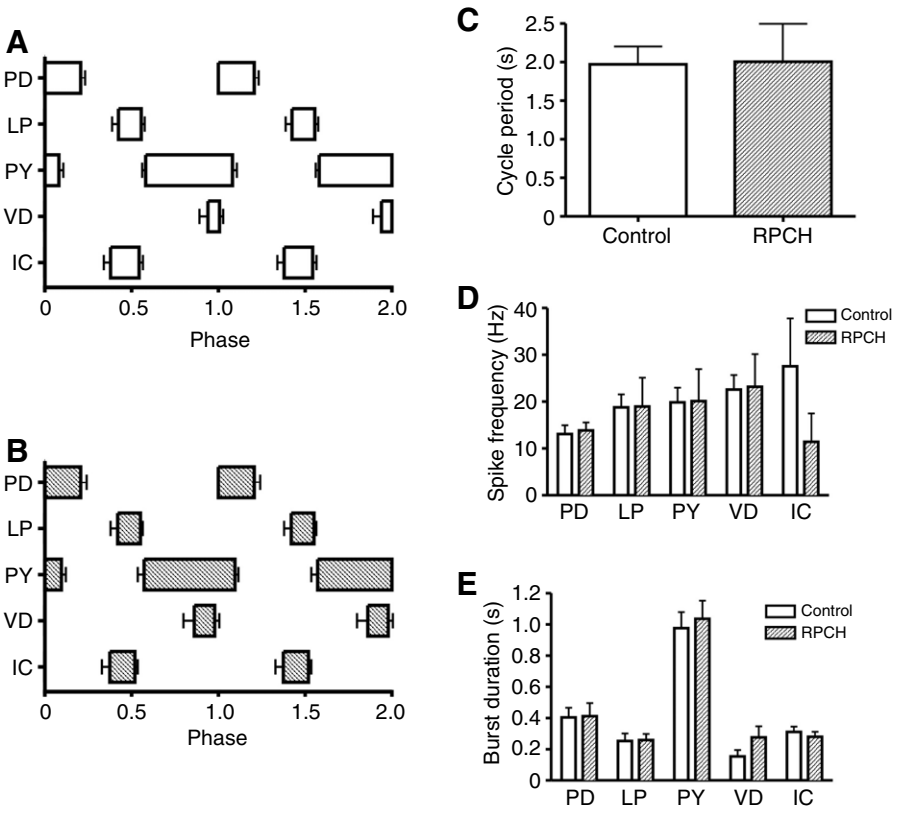


Fig. 10. The peptide red pigment concentrating hormone (RPCH) did not affect the pyloric pattern when the *stn* was intact and an ongoing pyloric pattern was active. (A,B) Phase plots, showing two cycles of the pyloric pattern in control saline (A) and in the presence of $10^{-6}$ mol $l^{-1}$ RPCH (B), showing that the pattern itself remained virtually unchanged by RPCH. (C–E) Cycle frequency (C), spike frequencies during bursts (D) and burst durations (E) were all unchanged. *N*=4 preparations. Error bars represent standard deviations.

neuroendocrine organs we examined using direct tissue MALDI-FTMS. In contrast to the MALDI results, our immunohistochemistry experiments indicate that either proctolin itself, or a proctolin-like peptide is present in the STG, the CoGs, the pericardial organ and the sinus gland. One explanation is that the proctolin concentrations are below the level of detection by MALDI or that it does not ionize under the conditions we used. We are, however, consistently able to detect proctolin in the sinus gland of other brachyuran crabs. Another intriguing possibility is that the amino acid sequence of the native proctolin isoform differs from that of other species, as has been suggested to be the case in the Colorado potato beetle (Spittaels et al., 1995).

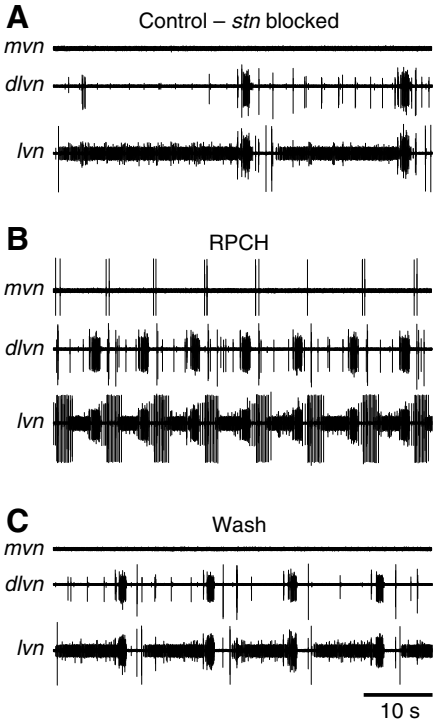


Fig. 11. Red pigment concentrating hormone (RPCH) is able to affect the pyloric pattern, shown by the fact that it did so in two of nine preparations with the *stn* blocked, one of which is shown here. Recordings of the pyloric pattern in (A) control saline, (B) during RPCH ($10^{-6}$ mol $l^{-1}$) bath application and (C) when washed with control saline. Note that, in contrast to the vast majority of preparations, the pyloric pattern continued even when the *stn* was blocked in this preparation. To ensure that this was not due to an incomplete block of condition in the *stn*, the *stn* was later cut, which did not alter the pattern of activity recorded. RPCH strongly activated the complete core pyloric pattern [bursting in the PD, LP, PY neurons seen on the *lvn* and the *dlvn* (PD), as well as weak bursting in the IC neuron]. Nerves: *mvn*, medial ventricular nerve (recording action potentials of the VD and IC neurons); *dlvn*, dorso-lateral ventricular nerve (recording action potentials of the PD neurons); *lvn*, lateral ventricular nerve (recording action potentials of the PD, LP and PY neurons).

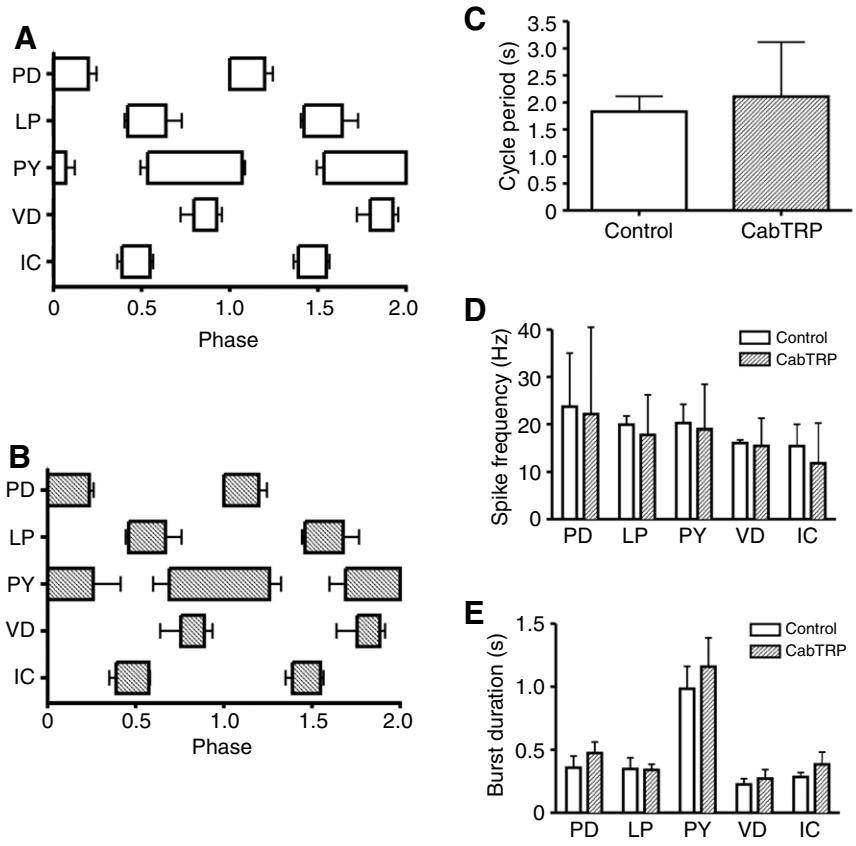


Fig. 12. In the presence of *Cancer borealis* tachykinin-related peptide I (CabTRP I), there were no changes in the ongoing pyloric activity in preparations with the anterior inputs intact (A–E), nor was there any activation of the pattern in preparations with the *stn* blocked (not shown). (A,B) Phase plots, showing two cycles of the pyloric pattern in control saline (A) and when CabTRP I ($10^{-6}$ mol $l^{-1}$) was bath-applied to the STG (B). No differences are apparent. (C–E) There were no changes in cycle period (C), spike frequency within bursts (D) or burst duration (E) in any of the pyloric neurons when CabTRP I was bath applied to the STG. *N*=3. Error bars indicate standard deviations.

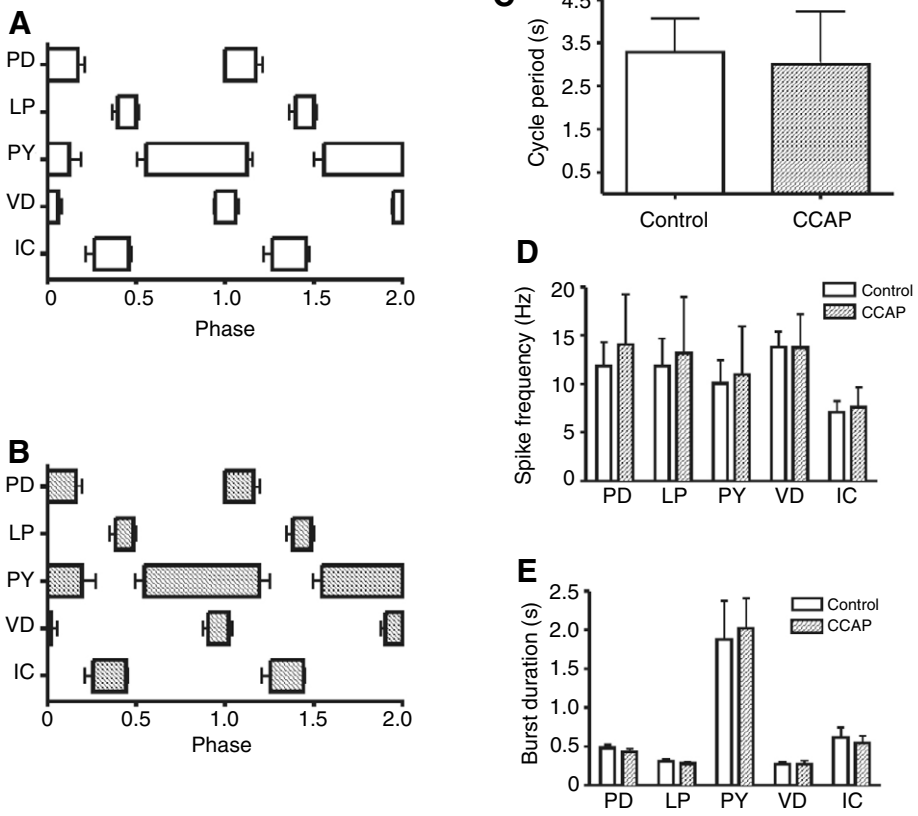


Fig. 13. The neuropeptide crustacean cardioactive peptide (CCAP) did not affect the pyloric pattern in preparations in which the *stn* was blocked (not shown) or in preparations with the *stn* intact. (A,B) Phase plots, showing two cycles of pyloric pattern in control saline (A) and during bath application of CCAP ($10^{-6}$ mol $l^{-1}$) to the STG (B). (C–E) There were no changes in cycle period (C), spike frequency within bursts (D) or burst duration (E) in any of the pyloric neurons when CCAP was bath applied to the STG. *N*=4. Error bars indicate standard deviations.

With respect to CabTRP I, one additional possibility is that, as has recently been reported for several *Cancer* species (Stemmler et al., 2007), there is a second TRP (tachykinin-related peptide) isoform present in *P. producta*. This peptide, TPSGFLGMRamide, is present in *P. producta* in the highest proportion relative to CabTRP I of any of the Brachyuran species we have examined (E.A.S., unpublished observations). Virtually 50% of the TRP in *P. producta* is TPSGFLGMRamide (CabTRP II), compared to approximately 15% in *C. borealis* and *C. productus*, and 30% in *C. irroratus*. In the only species in which it has been tested, *C. borealis*, the effects of CabTRP I and CabTRP II are identical (Stemmler et al., 2007). This suggested the possibility that the active TRP in *P. producta* was CabTRP II rather than CabTRP I. However, in preliminary experiments, CabTRP II, like CabTRP I, had no effect on the pyloric pattern in *P. producta* (P.S.D., unpublished observations).

Changes in receptors to the modulators could also account for the lack of effect seen with many of the modulators we tested. Although we could not directly test this hypothesis, it is interesting to consider that kelp is available only seasonally in the waters in which *P. producta* were collected. Thus, in the winter, *P. producta* from the Puget Sound area may become opportunistic feeders. To test the possibility that *P. producta* seasonally express receptors for the inactive peptides, thus increasing their modulatory repertoire in the winter when they are eating a more varied diet, we collected animals in late December, after the kelp had been gone for over 2 months and *P. producta* were feeding opportunistically, and tested the two peptides that had no effect in the summer. Neither CCAP nor CabTRP I caused any effect in these animals, suggesting that the receptors to CCAP and CabTRP I in the neurons of the pyloric circuit may have been evolutionarily lost in this species.

### Why maintain superfluous neuromodulators?

The data presented in our study raises the question 'Is the expression of many well-known neuromodulators in the *P. producta* STG truly superfluous?'. Clearly, there are many possible answers to this question, the most likely of which is that they are not, in fact, superfluous. We have examined the effects of these neuropeptides on only one target, the neurons of the pyloric central pattern generator, and they undoubtedly have other targets. Although we did not examine the distribution of the modulators in other parts of the nervous system, CabTRP I, proctolin, RPCH and dopamine are widely distributed in the brain and thoracic ganglia in other species, and the similarity of neuromodulator distributions within the tissues we examined suggests that they are likewise present in other parts of the nervous system in *P. producta*, where they could still be exerting their effects. It is also possible that these modulators do alter the expression of the pyloric pattern, but do so only under certain conditions, which we may not have tested. The effects of many neuromodulators and modulatory neurons on the stomatogastric system are known to be state dependent (Nagy and Dickinson, 1983; Nusbaum and Marder, 1989a; Nusbaum and Marder, 1989b). Moreover, other modulators require the presence or recent presence of another modulator in order to exert a given effect, as is seen with the activation of the cardiac sac pattern by the peptide proctolin; proctolin activates the cardiac sac pattern in an isolated STG only if superfused with or shortly after superfusion with RPCH (Dickinson et al., 1997). We did not test combinations of modulators in this study, but the possibility remains that the inactive peptides could modulate the pyloric pattern when applied in appropriate combinations.

## LIST OF ABBREVIATIONS

| | |
|---|---|
| CabTRP I | *Cancer borealis* tachykinin-related peptide I |
| CabTRP II | *Cancer borealis* tachykinin-related peptide II |
| CCAP | crustacean cardioactive peptide |
| CoG | commissural ganglion |
| DHB | 2,5-dihydroxybenzoic acid |
| *dpon* | dorso-posterior esophageal nerve |
| IC | inferior cardiac neuron |
| LP | lateral pyloric neuron |
| MALDI-FTMS | matrix assisted laser desorption/ionization Fourier transform mass spectrometry |
| OG | esophageal ganglion |
| *on* | esophageal nerve |
| PD | pyloric dilator neuron |
| PY | pyloric neuron |
| RPCH | red pigment concentrating hormone |
| *son* | superior esophageal nerve |
| STG | stomatogastric ganglion |
| *stn* | stomatogastric nerve |
| STNS | stomatogastric nervous system |
| VD | ventricular dilator neuron |

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