Quantification of Gastric Mill Network Effects on a Movement Related Parameter of Pyloric Network Output in the Lobster

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Thuma, Jeff B. and Scott L. Hooper. Quantification of gastric mill network effects on a movement related parameter of pyloric network output in the lobster. J Neurophysiol 87: 2372–2384, 2002; 10.1152/jn.00476.2001. It has long been known that gastric mill network activity (cycle period 5–10 s) alters pyloric network output (cycle period approximately 1 s), but these effects have not been quantified. Many pyloric muscles extract gastric mill timed variations in pyloric motor neuron firing, and consequently produce gastric mill timed movements even though no gastric mill neurons innervate them. Determining pyloric behavior therefore requires detailed description of gastric mill effects on pyloric neural output. Pyloric muscle activity correlates well with motor neuron overall spike frequency (OSF, burst spike number divided by cycle period). We quantified OSF variation of all pyloric neurons as a function of time into the gastric mill cycle [as measured from the beginning of Gastric Mill (GM) neuron bursts] in the lobster, Panulirus interruptus. No repeating pattern within individual gastric mill cycles of Lateral Pyloric (LP) and Ventricular Dilator (VD) neuron OSF was visually apparent. Averaged data showed that LP neuron OSF decreased (approximately 0.5 and 1.5 Hz, respectively) at the beginning of each gastric mill cycle. Visually apparent patterns of OSF waxing and waning within each gastric mill cycle were present for the Inferior Cardiac (IC), Pyloric Dilator (PD), and Pyloric (PY) neurons. However, when averaged as a function of phase or delay in the gastric mill cycle, the average changes were smaller than those in individual gastric mill cycles because when the OSF variations occurred varied considerably in different gastric mill cycles. We therefore used a “pattern-based” analysis in which an identifying characteristic of each neuron’s repeating OSF variation pattern was defined as pattern pyloric cycle zero. The pyloric cycles in each repetition of the OSF variation pattern were numbered relative to the zero cycle, and averaged to create an average OSF variation profile. The zero cycle delays relative to GM neuron burst beginning were then averaged to determine when in the gastric mill cycle the profile occurred. This technique preserved the full extent of pyloric neuron OSF changes. Maximum PY neuron OSF occurred within the GM neuron burst, whereas maximum IC and PD neuron OSF occurred during the GM neuron interburst interval. Despite these changes, pyloric muscle did not phase lock with gastric mill activity, nor were an integer number of pyloric cycles present in each gastric mill cycle. In addition to providing data necessary to predict pyloric movement, this work shows how pattern-based analysis can successfully quantify interactions between nonphase-locked networks.

INTRODUCTION

In the 28 years since its introduction into modern neuroscience (Maynard and Dando 1974), the pyloric network of the stomatogastric nervous system of decapod crustacea such as lobsters and crabs has become one of the leading model systems for studying neural network activity (Harris-Warrick et al. 1992). As a result of this extensive work, the neural output pattern the network produces, and many of the cellular and network properties underlying network activity, are well described. However, the movements the network produces remains unknown due to the internal position and small size of the pylorus. In the absence of other information, it was long assumed that the rapid (1 Hz), tri-phasic pyloric neural pattern resulted in a similarly rapid, tri-phasic movement pattern (Kennedy and Marder 1992; Maynard and Selverston 1975; Selverston et al. 1976; Turrigiano and Heinzl 1992).

However, recent work has shown that many pyloric muscles relax much too slowly to accurately follow the pyloric neural pattern (Ellis et al. 1996; Harness et al. 1998; Koehnle et al. 1997; Morris and Hooper 1998a) and that some of these muscles extract low frequency alterations of pyloric motor neuron firing that occur in phase with another stomatogastric neural network, the gastric mill network (Morris et al. 2000). These data indicate that for many pyloric muscles gastric mill timed contractions are a large component of muscle output and thus add a previously unappreciated importance to these internetwork interactions.

Several types of gastric/pyloric interactions have been described. In the crab these interactions are sufficiently strong that neurons switch between the two networks (Weimann et al. 1991), and the networks can become locked such that the pyloric network always produces an integer number of cycles per gastric mill cycle (Bartos et al. 1999; Nadim et al. 1998). Early work by Mulloney (1977) showed that in Panulirus the effects of gastric mill activity on pyloric activity are less pronounced, consisting of only changes in pyloric cycle period and pyloric neuron spiking activity, and that all pyloric neurons continue to cycle with the pyloric network when both networks are simultaneously active. The technical limitations of the period prevented quantitative description of these changes, nor have they been quantified since. We present here a detailed analysis for all pyloric motor neurons of the effects of gastric mill activity on one measure of neuronal firing—overall spike frequency (OSF, burst spike number divided by cycle period)—that strongly correlates with muscle activity (Morris and Hooper 1998a,b).

Lateral Pyloric (LP) and Ventricular Dilator (VD) neuron...
OSF showed no visibly discernable regular variation in individual gastric mill cycles, but averaged data showed that each decreased at the beginning of each gastric mill cycle. Pyloric Dilator (PD), Inferior Cardiac (IC), and Pyloric (PY) neuron OSF showed visually apparent patterns of waxing and waning each gastric mill cycle, with the PD and IC neuron variations occurring in antiphase to those of the PY neurons. Despite these changes, pyloric activity does not phase lock to gastric mill activity, nor are there an integer number of pyloric cycles per gastric mill cycle period (Bartos et al. 1999; Nadim et al. 1998). These data provide quantitative examples of relatively strong, but noncoordinating, interactions among neural networks in a system amenable to analysis on the cellular level.

A preliminary report of these data has appeared in abstract form (Thuma and Hooper 1999).

M E T H O D S

Panulirus interruptus (500–1000 g) were obtained from Don Tomlinson Commercial Fishing (San Diego, CA) and maintained in aquaria with chilled (13–15°C) circulating artificial seawater. Stomachs were dissected in the standard manner (Selverston et al. 1976), and preparations were continuously superfused with 13–15°C *Panulirus* saline. Extracellular nerve recordings of pyloric and gastric mill network activity were made with stainless steel pin electrodes and an A-M Systems amplifier. Intracellular neuronal recordings were made with glass microelectrodes (filled with 0.55 M K$_2$SO$_4$, 0.02 M KCl, resistance 10–20 MΩ) and an Axoclamp 2A or 2B. Data were displayed on a Tektronix oscilloscope, recorded on a MicroData Instruments DT-800 digital data recorder, and transferred to computer using a Cambridge Electronics Design 1401plus interface. OSF was calculated using scripts written in Spike II (Cambridge Electronics Design). Data were plotted using Kaleidagraph (Synergy Software) and figures were prepared in Canvas (Deneba Systems). All error bars are SDs of the mean. Statistical tests were performed with SPSS (SPSS Inc). An α of 0.05 was used in all statistical comparisons.

The somata of all pyloric and gastric mill neurons are located in the stomatogastric ganglion. The activity of the VD, LP, PD, and IC neurons of the pyloric network was monitored either by intracellular recording from the neuron’s soma or by extracellular recordings from the nerves in which their axons run. PY neuron activity was always recorded intracellularly because individual PY neuron activity cannot be determined from extracellular recordings. Gastric mill activity was monitored by extracellular recordings of the Gastric Mill (GM) neurons (Maynard and Dando 1974). The data shown here are from 13 experiments.

R E S U L T S

Figure 1A is a schematic of the lobster stomach and some of its muscles. The inset identifies the four regions of the stomach, the esophagus, cardiac sac, gastric mill, and the pylorus (Maynard and Dando 1974). The gastric mill and the pylorus are adjacent and are believed to have related functions (the pylorus filters the food the gastric mill chews, Selverston et al. 1976). Given the anatomical and functional relationship of these stomach regions, it would not be surprising if the activity of the networks that produce their motions (the gastric mill and pyloric networks) were coordinated, and synaptic interactions (Fig. 1B) between these networks were identified early (Mulloney 1977; Selverston et al. 1976). However, the effects of these interactions on pyloric activity have never been quantitatively characterized.

Figure 2 shows simultaneous intracellular recordings from a
ber per burst divided by pyloric cycle period, and so, for the cycle in which period is marked, the OSF is 3 divided by that period (0.7 s), or 4.3 Hz. The time in the pyloric cycle at which this OSF is defined to occur (e.g., the first spike of the burst, the middle of the spike burst, the middle of the cycle period) is arbitrary; in this paper we plot OSF as occurring at the first spike of the burst, or, in pyloric cycles in which the neuron does not fire (e.g., cycle 3), in the center of the slow wave depolarization. The middle trace is an aln extracellular recording and shows GM neuron activity and one gastric mill cycle period. The bottom plot shows how OSF varies during the gastric mill cycle period for the raw data shown above. IC neuron OSF decreases until approximately the middle of the GM neuron burst and then increases to reach a maximum at approximately the middle of the GM neuron interburst interval, after which it again begins to decline.

To determine if the pyloric neuron OSF variations were significant, the pyloric cycles in each gastric mill cycle were numbered from the beginning of each GM neuron burst (numbers next to data points in bottom plot, Fig. 3), and a univariate general linear analysis was performed with GM cycle number and experiment as fixed factors. This analysis showed that, although a visually apparent pattern of OSF variation was present for only the IC, PD, and PY neurons, the OSF of all pyloric neurons significantly varied with GM cycle number. However, consistent with the OSF variation being visually apparent for only the IC, PD, and PY neurons, the P values of the analyses were orders of magnitude smaller for the IC, PD, and PY neurons than for the LP and VD neurons.

The next step was to average the OSF variations that occur during each gastric mill cycle. Our initial procedure was to transform the data into a gastric mill based reference frame by defining the beginning of each GM neuron burst as time 0, measuring the delay from this reference point to each pyloric burst in the gastric mill cycle period and associating the appropriate OSF with each of these times. Average OSF could then be obtained by binning the data according to delay in the gastric mill cycle period and averaging the OSFs. When analyzed in this manner, the average OSF variations of the IC, PD, and PY neurons were smaller, and the SDs larger, than expected from the variations observed in single gastric mill cycles. Similar attempts to average OSF data according to phase in the gastric mill cycle (pyloric delay divided by gastric mill cycle period) also resulted in smaller OSF variations and larger SDs for these neurons than seemed appropriate from individual gastric mill cycle data.

Examination of the raw data for these neurons revealed two reasons for this difficulty. First, OSF baseline and range were often not stable throughout the experiment. Figure 4A shows a PY neuron’s OSF throughout an entire 600s data acquisition period. Each point on the plot represents the OSF of one pyloric cycle. The areas marked with asterisks are when the cardiac sac network, another stomatogastric network that alters pyloric activity, was active; data from these times were excluded. Baseline OSF was not stable throughout the experiment, but gradually increased from 125 to 325 s; OSF range decreased from 380 to 410 and 500 to 575 s. One way to overcome these difficulties would be to attempt to normalize...
The baseline shifts and range changes thus re- being studied. OSF depends on spike number and cycle period.

Selection does not arise because a derived parameter (OSF) is averaged data, the phenomenon being studied (OSF variation PY neuron spike number) that would tend to obscure, in ing shifts in general neuron activity (here, a slow increase in neuron OSF variation occurred, but it did so at different times. Binning the data relative to delay in gastric mill period would thus result in OSF minima of one gastric mill cycle being averaged with nonminimum data from other gastric mill cycles. Note again that this difficulty does not arise from OSF being the parameter under investigation; this difficulty stems from the change in pyloric neuron activity not always occurring at the same place in the gastric mill cycle and thus would occur for any pyloric activity parameter.

The hypothetical data in Fig. 5 show how this sort of timing variation can lead to substantial averaging distortions and our technique to overcome this difficulty. The top panel in Fig. 5A is OSF; in the absence of gastric mill activity OSF would have a value of 2 Hz. The bottom panel shows pyloric cycle number with cycle zero defined as occurring at the beginning of each GM neuron burst (numbering the same as in Fig. 3, bottom panel). In all cases the OSF variation consists of an identical down-up-down (0–1–0 Hz) pattern from the unperturbed OSF level (2 Hz). The response always occurs around the middle of the gastric mill cycle, but at which pyloric cycle it occurs shows considerable variation. In this data set, if the beginning of the response is defined to be the first drop to 0 Hz, 10% of the responses begin at pyloric cycle 5, 20% at pyloric cycle 4, 40% at pyloric cycle 3, 20% at pyloric cycle 2, and 10% at pyloric cycle 1. Figure 5B shows these five responses plotted against pyloric cycle number in the GM cycle, and Fig. 5C shows the averaged response. The average response has large SDs and, more importantly, fails to accurately capture the response’s characteristic down-up-down pattern or its full amplitude. An alternative approach would be to use the pattern itself as a basis for averaging by defining the first down as each pattern repetition’s cycle zero. Since these data are hypothetical and show no variation, this average would be a perfect down-up-down pattern with zero SD. The next task is correctly placing this pattern in the gastric mill cycle period. This can be accomplished by noting at which pyloric cycle number each of the response patterns begins and then averaging these numbers. Referring to the relative frequencies of pattern beginnings noted above, the pattern’s average position in the gastric mill cycle period is the average of one 5, two 4s, three 3s, two 2s, and one 1, or 3 ± 1.22. Figure 5D shows that the result of this procedure is a much more accurate representation of the data; it correctly captures the fact that there is no variation in the response amplitude pattern, but only a variation in when the response falls in the gastric mill cycle.

IC, PD, and PY neuron OSF showed a visually apparent repeating pattern of OSF variation as a function of gastric mill activity. Figure 4 shows that when in the gastric mill cycle IC neuron OSF variation occurred differed in different gastric mill cycles, and examination of the PD and PY neuron data showed that this was true of these neurons as well. For these neurons we therefore turned to a pattern-based averaging procedure similar to that shown in Fig. 5, in which the OSF variations

![Graph](Image)

**Fig. 4.** Simple data averaging obscures the true extent of gastric mill timed OSF changes. A: OSF baseline and range varies within a single data acquisition episode. Asterisks mark times when the cardiac sac network, another stomatogastric network that affects pyloric activity, was active. Data were only taken from durations with the same OSF baseline and range; the rectangles mark the data chosen for further analysis in this experiment. B: zero cycle (OSF minimum) shows considerable variability relative to GM neuron burst beginning. Two IC neuron response profiles from the same preparation are shown; the minimum of one occurs approximately 1.5 s before GM neuron burst beginning, whereas the other occurs at GM neuron burst beginning.
FIG. 5. Description of “pattern-based” analysis technique. A: hypothetical data in which a perfectly repeating “down-up-down” pattern occurs centered on pyloric cycle 3, but with some timing variability. Choosing the first “down” to define the beginning of the pattern, it begins 10% of the time on pyloric cycle 1, 20% on pyloric cycle 2, 40% on pyloric cycle 3, 20% on pyloric cycle 4, and 10% on pyloric cycle 5. B: plot of OSF variations as a function of pyloric cycle number in gastric mill time. C: average of data in Fig. 5B; note the large SDs and the disruption of OSF variation pattern amplitude and shape. D: average OSF variation profile if the OSF variation patterns are instead averaged using the “first” down to define when each pattern begins; since the variations shown in Fig. 5A are perfectly repeatable, the SDs are zero. The pyloric cycle numbers (one 1, two 2s, three 3s, two 4s, one 5) in gastric mill time of each OSF variation pattern used to create the average OSF variation profile are averaged to place the profile in the gastric mill cycle period (ave, cycle 3 ± 1.22, horizontal error bars). Note that pattern shape is perfectly preserved, and the true locus of variation (when the pattern occurs in gastric mill time) is correctly identified.
themselves defined pyloric response. Figure 6 shows how this procedure was performed. Figure 6A shows an expanded portion of the data shown in Fig. 4A (from the third rectangle, note time axis values). The upper portion of Fig. 6A shows the PY neuron’s OSF variations over four gastric mill cycles. The bottom portion of Fig. 6A shows pyloric cycle number relative to the GM neuron burst beginning, as in Fig. 3. That is, the data point marked with the arrow is the first pyloric cycle after GM neuron burst beginning; the next data point is the second after GM neuron burst beginning, etc. The drop to one after the ninth data point occurs because the next GM neuron burst began between the ninth and tenth data points, and thus each cycle of rise and fall on the bottom portion of Fig. 6A represents one gastric mill cycle.

The experiment shown in Fig. 6A had a very short gastric mill cycle period, and the peaks and valleys of the rhythmic OSF variation have approximately the same durations. However, in experiments in which the gastric mill period was longer, and thus there were more pyloric cycles per gastric mill cycle, the OSF peaks became broader while the valleys remained approximately the same duration. The PY neuron response pattern thus consisted of a variable plateau of high OSF values separated by relatively narrow, constant duration OSF decreases. For the PY neuron, the repeating OSF decrease was therefore the most identifiable characteristic feature of the OSF variation. Similar repeating patterns of OSF variation were present for the IC and PD neurons. A decrease was also the most identifiable characteristic of IC neuron OSF variation, but for the PD neuron a repeating OSF increase was most identifiable.

As will also be seen when the data for the LP and VD neurons are presented (in which no visually identifiable pattern

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**FIG. 6.** Application of “pattern-based” analysis on pyloric neuron data. A: a time expansion of some of the data in Fig. 4A. The OSF variation consists of a rhythmic OSF decrease and increase. Bottom plot shows pyloric cycle number in the gastric mill cycle period (using GM neuron burst beginning to define gastric mill cycle beginning). The OSF minima were defined as pattern response cycle zeros, and the cycles between minima numbered relative to these zero cycles (numbers next to data points). B: OSF variation versus pattern cycle number. C: OSF variation versus delay relative to pattern zero cycles. D: average OSF variation versus delay relative to pattern zero cycles. Rightmost data point was obtained by averaging data in ellipse in Fig. 6C, which corresponds to the +3 cycle data in Fig. 6A. All other data points are similar averages of OSF and delay of data from the same pattern cycle class (−3, −2, etc.) E: OSF variation versus delay relative to GM neuron burst beginning. F: average OSF plot versus GM neuron burst beginning for these data. The OSF minimum occurs approximately 0.75 s before GM neuron burst beginning. The vertical error bars are SDs of the mean OSF. Except for the open circle, the horizontal error bars are the SDs of the mean delay relative to pattern center zero for each pattern cycle number class. The horizontal error bar of the open circle is the SD of the delays relative to GM neuron burst beginning of the zero cycles. See text for detailed explanation.
of OSF variation is apparent), it is essential to stress that it is this presence of patterns of OSF decrease and increase involving several data points that allows a pattern-based analysis to be performed, as it allows the data to be defined and averaged as a function of the characteristic shape of the variation itself. This is not the equivalent of simply identifying the minimum OSF that is present in each gastric mill cycle as representing a repeating pattern, as this would not take into account the OSFs of the data points surrounding this minimum. It is thus not the simple presence of a minimum (or maximum) OSF in each gastric mill cycle that permits a pattern of OSF variation to be identified; the presence of a pattern is instead revealed by the smooth, multi-data point waxing and waning shown in Fig. 6A.

A pyloric cycle number reference frame was constructed by defining pattern minimum (PY, IC neuron) or maximum (PD neuron) OSF to be pyloric cycle zero. The boundaries between pattern repetitions (vertical dashed lines) were defined by dividing the number of pyloric cycles between the two minima in half, and cycles before and after the zero cycle were numbered (numbers next to the data points, Fig. 6A) until the boundary was reached. If an odd number of cycles separated the two minima, the “extra” cycle was always assigned a positive value. For example, seven cycles separated the two minima whose boundary is marked by the dashed line with the asterisk; these cycles were divided such that the first four of the seven belonged to the first profile and the last three of the seven belonged to the second profile.

This procedure allowed each OSF variation profile to be plotted versus pattern cycle number. Figure 6B shows OSF variation versus pattern cycle number for seven gastric mill cycles. At this stage the OSF values for the −4, −3, −2, −1, 0, 1, 2, 3 cycle numbers could be averaged to obtain an average OSF profile versus cycle number. However, cycle number is not the most useful way to categorize the data because pyloric cycle period is not constant within or between experiments. As a result, data that all belong to a single pattern cycle number (e.g., cycle 3 in Fig. 6D, data in ellipse) do not occur exactly the same number of seconds after the pattern cycle zero because of cycle-to-cycle shifts in pyloric period. In a single experiment these variations are small, but pyloric cycle period can show substantial variations between experiments, and thus pattern cycle 3 points would occur approximately 3 s after pattern cycle 0 in an experiment with a pyloric period of 1 s, but approximately 6 s after pattern cycle 0 in an experiment with a pyloric period of 2 s.

We therefore transformed the cycle number reference frame into a pyloric time reference frame (relative to cycle zero) by adding or subtracting the appropriate pyloric cycle periods to or from each data point (Fig. 6C). For instance, if pyloric cycle 1 in gastric mill cycle 1 occurred 0.75 s after pattern cycle 0, then the OSF of pattern cycle number 1 was plotted at 0.75 s in Fig. 6C (note that the ordinate in Fig. 6B is cycle number and the ordinate in Fig. 6C is time). The effects of this transformation can be seen in the data in the ellipse, which are the same data as in the ellipse in Fig. 6B; the small delay variations of these data points in Fig. 6C arise because pyloric cycle period is not absolutely constant even within an experiment. Both OSF, and delay relative to cycle zero, of each cycle number class can now be averaged (Fig. 6D). For instance, the rightmost data point in Fig. 6D was calculated by averaging the OSF (abscissa) and delay (ordinate) values of the +3 pattern cycle number points in Fig. 6C (the points surrounded by the ellipse). Note that there is no horizontal (time) SD for the zero cycle average, as all these points have a delay of 0 s. It is apparent that this averaging procedure well captures the characteristic variation in PY neuron OSF. Figure 6, A–C, shows that, in each OSF pattern repetition, the PY neuron OSF variation consists of a gradual decline, a sharp decline at pattern cycle zero, and a sharp rise in the subsequent two pyloric cycles; Fig. 6D shows precisely this variation shape.

Figure 6E shows both how simple averaging of these data versus time into the gastric mill cycle would distort the OSF variation profile and how the OSF variation profile shown in Fig. 6D is placed in the gastric mill cycle. The lines in Fig. 6E are the same as those in Fig. 6C, but the delay (positive or negative) from the nearest GM neuron burst beginning to each zero cycle pyloric burst (open circles, Fig. 6C) has been added to all the data points associated with that zero point. This figure thus shows how OSF varies as a function of time in the gastric mill cycle period on a gastric mill cycle-by-cycle basis. Note that these data averaged as a function of time into the gastric mill cycle, the OSF variation profile would be considerably different from that shown in Fig. 6D; in particular, the sharp drop at, and sharp rise after, the pattern zero would be replaced with a broader and shallower decline and rise. When in the gastric mill cycle the average OSF variation shown in Fig. 6D occurs can be calculated by noting the delays of the zero cycles (Fig. 6E, open circles) relative to GM neuron burst beginning (0 to −1 s, that is, most zero cycles occurred before the GM neuron burst began). These values were averaged to determine the average delay and SD of pattern cycle zero in gastric mill time.

This average delay in the gastric mill cycle was added to each data point in Fig. 6D to arrive at Fig. 6F. The SD of the average gastric mill delay is plotted as the horizontal error bars on the zero cycle (open circle), since this data point has no pyloric delay SD. The vertical SDs, and the horizontal SDs of all points but the zero cycle, thus show how consistent the pattern of OSF variation is regardless of when in the gastric mill cycles OSF minimum occurs. The horizontal SD of the zero cycle (open circle) indicates the variability of the delay of the OSF minima in the gastric mill cycle.

Figure 7 shows the result of this analysis for the PD, PY, and IC neurons. The top left plot shows the PD neuron data (5 experiments) in the pattern centered reference frame (OSF maximum was used to define pattern cycle zero); in most experiments the increase is relatively small (approximately 1 Hz). The right PD plot shows these data relative to GM neuron burst beginning; the error bars are SDs of pattern delay relative to GM neuron activity. Peak OSF occurred 0–2 s before GM neuron burst beginning.

The middle plots show PY neuron OSF response (7 experiments; OSF minimum was used to define cycle zero). The PY neurons are divided into two classes, the PE and PL neurons (Hartline et al. 1987). Both types showed identical responses to gastric mill activity, and these plots show the activities of both kinds. The left plot shows the data in the pattern centered reference frame. The decreases occur over a relatively narrow cycle number range, with OSF generally returning to control values within two to three cycles of the minima. The right plot shows the data relative to GM neuron burst beginning: the minima occur 0–2 s before GM neuron burst beginning.
The bottom plots show IC neuron OSF response (7 experiments; OSF minimum was used to define cycle zero). The left plot shows the data in the pattern centered reference frame. IC neuron OSF decreases over a broader cycle number range than the PD and PY neurons, often taking the entire pattern to reach maximum OSF. The right plot shows the data relative to GM neuron burst beginning; the minima occur 1–2 s after GM neuron burst beginning.

Although no repeating OSF variation pattern was visually apparent in the LP and VD neurons, statistical analysis of the data showed that the OSF of both neurons changed in a significant manner as a function of time in the gastric mill cycle. Figure 8A shows raw data of a LP neuron OSF variation. The top trace is an aln extracellular recording showing gastric mill activity; the second trace is an extracellular recording of LP neuron activity. Figure 8B shows LP neuron OSF and pyloric cycle number versus time for eight gastric mill cycles. Although there may be a tendency for LP neuron OSF to decrease around the beginning of each gastric mill cycle period, the changes are much too small to visually identify a repeating pattern, and consequently, a pattern-based analysis could not be performed. Figure 8C shows an alternative analysis in which average OSF for each neuron (8 experiments) was plotted versus delay relative to GM neuron burst beginning; no consistent variation in LP neuron OSF is apparent. It was, however, possible that the wide range of mean OSFs in these data were hiding a consistent OSF variation around the mean. We therefore subtracted from each of the experiments in Fig. 8A the mean OSF and plotted ΔOSF versus delay relative to GM neuron burst beginning (Fig. 8D). When the data are plotted in this manner, it is apparent that there is a consistent decline in LP neuron OSF at the beginning of each gastric mill cycle.

Figure 9 shows precisely analogous plots for the VD neuron; panel A is raw data, panel B is VD neuron OSF and pyloric cycle number versus time for eight gastric mill cycles. Although there may be a tendency for LP neuron OSF to decrease around the beginning of each gastric mill cycle
GM neuron. VD neuron OSF shows even less consistent variation as a function of gastric mill activity than the LP neuron, and, although the statistical analysis indicates there is a significant variation in these data, it cannot be discerned here.

The final task was to average the data shown in Figs. 7, 8, and 9 across experiments to obtain an across-experiment average OSF variation profile for all pyloric neurons. This is problematic for two reasons. First, different experiments have different baseline OSF values; for instance, baseline OSF for the PD neuron (Fig. 7, top left plot) ranges from approximately 5 Hz (bottom curve in plot) to approximately 12 Hz (top curve in plot). These differences in baseline OSF have nothing to do with the gastric mill timed OSF changes (note that in all experiments OSF increases at pattern zero), but instead result from the fact pyloric neurons show a wide range of preparation-specific baseline firing frequencies (Hooper 1997). Due to this wide range of baseline OSF, simply averaging across the curves in this plot would result in large SDs that would obscure the point of interest—the change in OSF as a function of gastric mill cycle. We therefore created ΔOSF plots by subtracting from each curve the mean of each experiment’s data (as in Figs. 8 and 9D; Fig. 10A shows an example for two PD neurons.

The second difficulty is that each experiment had different numbers of pyloric cycles per gastric mill cycle, and each experiment’s OSF data points occurred at different times relative to the zero cycle (because both gastric mill and pyloric cycle period varied between experiments). For instance, in Fig. 10A the experiment plotted with circles had eight pyloric cycles per gastric mill cycle and a range of delays relative to the zero cycle of −2.5 to 2.5 s; the experiment plotted with squares had nine pyloric cycles per gastric mill cycle, and a delay range of −4 to 4 s. We used the following procedure to overcome this difficulty. For each experiment’s ΔOSF plot we

**FIG. 8.** LP neuron OSF decreases at the beginning of each gastric mill cycle. A: raw data showing gastric mill (aln) and LP (lpn) neuron activity. B: LP neuron OSF and pyloric cycle number versus time; double-headed arrow shows region corresponding to raw data in A. C: OSF versus delay (relative to GM neuron burst beginning) summary plot. No consistent variation in OSF is apparent. D: ΔOSF versus delay (relative to GM neuron burst beginning) summary plot; OSF is minimum at the beginning of the gastric mill cycles.
linearly interpolated (step size 0.041 s) between each experiment’s data points. We then averaged the ∆OSF’s of all experiments at each interpolation point for which all experiments had data (for the PD neurons, between −2.5 and 2.5 s, see top left plot in Fig. 7) to obtain an across-experiment average ∆OSF profile. Third, we then added the across-experiment average mean OSF (the average of the values that had been subtracted from each experiment’s data to generate the ∆OSF plots) to the across-experiment average ∆OSF profile to obtain across-experiment average OSF plots. For the LP and VD neurons this was sufficient to arrive at an across-experiment average profile (top two traces, Fig. 10B). For the IC, PD, and PY neurons an additional step was necessary because the across-experiment average ∆OSF profiles obtained to this point were still in the pattern zero cycle reference frame. To correctly place the profiles of these neurons in gastric mill time, we added the average delay relative to GM neuron burst beginning (for the PD neuron, the average of the delays of the open circles in the top right plot in Fig. 7) to the times in the zero cycle reference frame.

Figure 10B shows the across experiment average OSF variations of all pyloric neurons. The vertical error bars are OSF SDs of selected interpolation points; the horizontal error bars are the SDs of the times of the zero cycles of the PD, PY, and IC profiles in the gastric mill cycle period. The rectangles at the bottom of the plot represent GM neuron firing. The VD neuron average profile shows a very small decrease, and the LP neuron profile shows a substantial decrease, at the beginning of each gastric mill cycle. PD neuron maximum and PY neuron minimum OSF occur approximately 1 s before, and IC neuron minimum OSF occurs approximately 1.5 s after, GM neuron burst beginning. Statistical comparisons (paired t-test) of delays relative to GM neuron burst beginning showed that the times of the PD neuron maximum and the PY neuron minimum
Fig. 10. Across-experiment average OSF responses. A: ΔOSF plots for
curves a and b in top left plot of Fig. 7. B: across-experiment average OSF
responses of all pyloric neurons. VD neuron OSF decreases slightly, and LP
neuron OSF more substantially, at the beginning of each gastric mill cycle. PD
and IC neuron OSF varies in phase, reaching a maximum in the GM neuron
interburst interval and a minimum during the GM neuron burst. PY neuron
OSF varies in antiphase with that of the PD and IC neurons.

do not differ, but the time of the IC neuron minimum differs
from the times of both the PD neuron maximum and the PY
neuron minimum.

Discussion

We performed a quantitative analysis of the variation of
pyloric neuron OSF as a function of gastric mill activity. The
VD neuron OSF variation, although statistically significant, is
extremely small, and it is questionable whether it has func-
tional significance for pyloric movements, as we have shown
elsewhere that the VD neuron innervated muscle does not show
gastric mill timed variations in its contractions when driven by
physiological VD neuron activity (Morris et al. 1999). The LP
neuron OSF variation is larger. However, it again shows ex-
tremely large variability and is not visibly present as a repeat-
able pattern in either OSF versus time plots (Fig. 8) or in
contractions of LP neuron innervated muscles when driven by
physiological LP neuron activity (Morris et al. 1999). Non-
etheless, a relatively large decrease of LP neuron OSF at gastric
mill pattern beginning is clearly present, and it is thus possible
this change could have functional consequences. One possi-
bility is that, rather than serving to alter pyloric movements, the
changes in LP neuron OSF instead alter pyloric network ac-
tivity through the LP neuron’s synaptic connections in the
network.

The PD, PY, and IC neurons show visibly observable, cycle-
by-cycle, gastric mill timed OSF waxing and waning. PD and
IC neurons reach their maximum OSF approximately 1 s
before, and their minimum OSF approximately 1 s after, GM
neuron burst beginning; PY neuron OSF wanes and waxes in
antiphase with PD and IC neuron OSF. Morris et al. (2000)
have shown that, when pyloric motor neuron activity is used to
drive pyloric muscles, some of the muscles express gastric mill
timed movements; the changes in pyloric motor neuron OSF
described here presumably underlie this process.

Effects of pyloric activity on gastric mill activity

Recent work by Nadim et al. (1998) and Bartos et al. (1999)
has shown that in Cancer borealis an integer number of pyloric
cycles occurs in each gastric mill period. We examined our
data closely for phase or delay locking of the pyloric cycle
nearest to GM neuron burst beginnings and endings, and for
integer numbers of pyloric cycles in gastric mill periods, and
found that neither occur (data not shown). Integer coupling in
Cancer occurs because the Anterior Burster (AB) neuron of the
pyloric network, which fires with the PD neurons, functionally
excites the Lateral Gastric (LG) neuron by inhibiting gastric
mill Interneuron 1 (Int1). During the LG neuron interburst
interval, these pyloric timed excitations bring the LG neuron
progressively closer to threshold. Eventually one of the AB
neuron bursts triggers the LG neuron to burst, and thus, there
are always an integer number of pyloric cycles per gastric mill
cycle.

In Panulirus the PD neurons inhibit Int1 (Fig. 1B), and thus
the synaptic connectivity necessary for integer locking is also
present in our preparation. Whether integer locking failed to
occur in our work because this inter-network connection plays
a different role in the two species, or because gastric mill rhythmicity was arising via a different mechanism in our
preparations, cannot be resolved without further experiments.
With respect to this latter point, it is essential to point out that,
just as with the pyloric network, the gastric mill network can
exist in multiple configurations that produce different output
patterns. All of our work was performed in control saline with
input to the stomatogastric nerve intact; it is thus possible that
under different modulatory conditions (in particular, ones in
which the PD neuron to Int 1 synapse were strengthened) the
integer coupling found in the crab would also occur in the
lobster.

Effects of gastric mill activity on pyloric activity

None of the known gastric mill connections to the pyloric
network (Mulloney 1977; Selverston et al. 1976; Fig. 1B)
appear appropriate to cause the changes in pyloric activity
described here. First, the most strongly affected neurons, the
PY and IC neurons, receive no known input from gastric mill
neurons. Second, although both neurons receive gastric mill
input, the PD neuron shows very little gastric mill timed OSF
variation and the LP neuron shows, on a cycle-by-cycle basis,
consistent variation. Third, the Medial Gastric (MG) neuron
inhibition of the PD neuron is unlikely to explain the PD
neuron activity changes because MG neuron firing is not in
phase with the PD neuron OSF decrease (Selverston et al.
1976). These OSF changes are, however, consistent with the
effects of a descending input to the pyloric network, Modula-
tory Commissural Neuron 1 (MCN1), described by Bartos and
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Nusbaum (1997) in C. borealis, MCN1 is presynaptically inhibited by the LG neuron and has a variety of effects on pyloric neurons. LG neuron activity removes MCN1 input from the pyloric neurons and thus results in pyloric activity varying in gastric mill time. Comparison with the data reported by Bartos and Nusbaum (1997) shows that all the effects shown here are consistent with their data if there is a tonically active MCN1 homologue in Panulirus.

Mechanism of OSF variation

OSF depends on burst spike number and cycle period, and OSF variations can hence arise from changes in either or both of these underlying parameters. Indeed, it might a priori seem it would be more appropriate to analyze these two parameters instead of OSF. We have not done so here for two reasons. The first is the importance of OSF in predicting muscle output and pyloric movement, which is a primary goal of our group. The second is that preliminary efforts show that analyzing spike number and cycle period is much more complex than analyzing OSF and that variations in these parameters play varying roles in controlling OSF in the different neurons.

These issues are well displayed in Fig. 2. First, although the AB/PD neuron ensemble generally acts as the network’s pacemaker, and the rest of the pyloric neurons generally cycle 1 for 1 with this ensemble, nothing prevents a neuron from cycling somewhat faster than the pacemaker for a few cycles, and then somewhat slower than the pacemaker for a few cycles, so long as the neuron’s average cycle period over the entire period equals the pacemaker’s. Given the wide range of spike numbers in the PY and IC neuron traces shown in Fig. 2, it would not be surprising (and preliminary analysis supports this) if pacemaker ensemble and other neuron cycle periods were not tightly coupled on a cycle-by-cycle basis. As such, the confounded effects of changing pacemaker ensemble cycle period, and changes in follower neuron spike number and burst length, must be disentangled.

Second, the changes in cycle period and spike number can, and sometimes do, work at cross-purposes. That is, since OSF equals spike number divided by cycle period, OSF can increase even if spike number decreases if cycle period declines even more, decrease even if spike number increases if cycle period increases even more, or remain the same if spike number and cycle period each change by the same proportion. OSF obviates these difficulties by simply reporting the number of spikes per time that the neuron fires, which, for spike-dependent events such as muscle contraction, would a priori seem the most fundamentally important variable.

Third, the different neurons clearly use different mechanisms to alter OSF. PD neuron spike number is almost invariant within an experiment (unpublished data, but also see Fig. 2), and thus changes in cycle period are the primary determinant of OSF. The PY neuron, alternatively, changes both spike number and cycle period as a function of time into the gastric mill cycle (unpublished data, but see Fig. 2), and thus for this neuron changes in both these parameters control OSF.

Pattern-based analysis

A standard approach to analyzing these data would have been to average OSF as a function of time or phase in the gastric mill cycle. However, when we used this approach, the averages showed smaller OSF variations than were present in the single gastric mill cycles. This difficulty arose because the minima/maxima that mark the OSF variations show considerable variability in GM timing, and thus a gastric mill time or phase-based approach resulted in averaging nonminima/maxima points with pattern minima/maxima. For the PD neurons this difficulty was sufficiently severe that in some preparations, although PD neuron modification by gastric mill activity was apparent both in gastric mill cycle-by-cycle analysis and when PD neuron innervated muscles were driven by the PD neuron activity in question, no correlation was apparent in averaged time or phase-based analyses. These observations suggest that, particularly in cases in which data acquisition and averaging are automated and raw data are not individually examined, non-time and phase-locked correlations could be overlooked.

General implications

A striking aspect of the work described here is the wide range of times in the gastric mill cycle over which OSF minima (in the case of the PD neuron, OSF maxima) occur. This variability may arise in part because of the lack of phase locking of the pyloric rhythm to the gastric mill rhythm. In the absence of phase locking, the pyloric rhythm occurs at all phase relationships with the influence from the gastric mill rhythm that underlies the interaction. In general the effect of an input varies according to when in a cycle it occurs; for instance, a hyperpolarization applied during a pyloric burst has a different effect from the same input applied during an interburst interval. The variation in when, for instance, the IC neuron OSF minima occurs may thus arise, at least in part, because the gastric mill influence that alters IC neuron firing can occur at any time in the IC neuron cycle period. Thus (assuming that the gastric mill influence is inhibitory), in some cases the gastric mill influence will begin just when an IC neuron burst is beginning, in which case that burst’s OSF will be reduced. In other cases the gastric influence will begin in the IC neuron interburst interval, in which case the subsequent IC neuron burst’s OSF will be reduced. Similar effects would be expected whenever a fast rhythm is affected by, but does not phase lock to, a slow rhythm.

This observation raises the question of how frequently rhythms with significantly different cycle periods interact. As noted above, Nadim et al. (1998) and Bartos et al. (1999) have shown that in Cancer the gastric mill and pyloric patterns show integer locking. Larson et al. (1994) showed strong variations of medullary respiratory-related neuron activity during vocalization and swallowing in monkey, and Chrachi and Neil (1993) showed coordination between swimmeret and abdominal positioning movements in crayfish. Other studies of rhythms that often have very different cycle periods include locomotion and respiration in cats (Kawahara and Suzuki 1990; Kawahara et al. 1989), rabbits (Corio et al. 1993), and horses (LaFortuna et al. 1996). However, in this work it was reported that the rhythms did not show interactions until (as a result of various treatments) their cycle periods became similar, at which point they phase-locked one to one. Nonetheless, given the wide period range present in neurobiological rhythms, and the degree of inter-neuronal and inter-network interaction present in nervous systems, it would be surprising if
interactions among rhythmic systems with very different periods did not occur with some frequency. The data presented here show that relatively strong interactions can occur between such systems without phase- or delay-locking occurring, and that phase- or delay-based analyses can fail to adequately describe, and in some cases even identify, such interactions. It is thus possible that systems with very different periods interact more frequently than the literature suggests and that the pattern-based analysis described here may be useful in the identification and description of these interactions.

REFERENCES


