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RESEARCH ARTICLE

Sources and range of long-term variability of rhythmic motor patterns *in vivo*

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ABSTRACT

The mechanisms of rhythmic motor pattern generation have been studied in detail *in vitro*, but the long-term stability and sources of variability *in vivo* are often not well described. The crab stomatogastric ganglion contains the well-characterized gastric mill (chewing) and pyloric (filtering of food) central pattern generators. *In vitro*, the pyloric rhythm is stereotyped with little variation, but inter-circuit interactions and neuromodulation can alter both rhythm cycle frequency and structure. The range of variation of activity *in vivo* is, with few exceptions, unknown. Curiously, although the pattern-generating circuits *in vivo* are constantly exposed to hormonal and neural modulation, the majority of published data show only the unperturbed canonical motor patterns typically observed *in vitro*. Using long-term extracellular recordings (N=27 animals), we identified the range and sources of variability of the pyloric and gastric mill rhythms recorded continuously over 4 days in freely behaving Jonah crabs (*Cancer borealis*). Although there was no evidence of innate daily rhythmicity, a 12 h light-driven cycle did manifest. The frequency of both rhythms increased modestly, albeit consistently, during the 3 h before and 3 h after the lights changed. This cycle was occluded by sensory stimulation (feeding), which significantly influenced both pyloric cycle frequency and structure. This was the only instance where the structure of the rhythm changed. In unfed animals the structure remained stable, even when the frequency varied substantially. So, although central pattern generating circuits are capable of producing many patterns, *in vivo* outputs typically remain stable in the absence of sensory stimulation.

KEY WORDS: Central pattern generator, Circadian, Crustacean, Motor pattern, Neuromodulation, Stomatogastric ganglion

INTRODUCTION

Neural networks that produce rhythmic motor behaviors are common to all animals. While their interactions with the environment are difficult to study due to the complexity of the involved networks and their sensory feedback, much is known about the mechanisms of motor pattern generation. One of the most striking characteristics is that motor networks show immense plasticity (Marder and Calabrese, 1996; Harris-Warrick, 2011; Marder, 2012; Cropper et al., 2004; Dietz, 2003; Mitchell and Johnson, 2003). In particular, *in vitro* approaches on rhythmic motor networks such as central pattern generators (CPGs) have demonstrated that motor networks are modulated by a multitude of substances and show a variety of distinct outputs in response to such modulation. CPGs lend themselves to *in vitro* studies as they continue to generate rhythmic activity, even in isolation, after being removed from the nervous system. However, much less is known about their modulation *in vivo*.

The CPGs in the stomatogastric ganglion (STG; Fig. 1A) control digestive behavior in decapod crustaceans and have provided substantial insight into the cellular and synaptic actions of neuromodulation (Marder and Bucher, 2007). Many different neuromodulators that act on these CPGs have been identified (Marder and Bucher, 2007; Nusbaum and Beenhakker, 2002; Marder and Eisen, 1984; Christie et al., 1995; Li et al., 2002). They are either released from modulatory neurons or available in the bloodstream. The STG is innervated by descending modulatory projection neurons (Nusbaum and Beenhakker, 2002) and is also located in the ophthalmic artery. This artery supplies the STG with neuromodulatory substances via the bloodstream, from their main source, the pericardial organs (Marder and Bucher, 2007). Accordingly, STG activity should be modulated by neuronal modulation as well as hormonal modulation. Curiously, however, despite stimulation of descending projection neurons both *in vitro* (Nusbaum and Beenhakker, 2002) and *in vivo* (Diehl et al., 2013; Hedrich et al., 2011) resulting in clear changes of CPG activity, most reported *in vivo* data show only the canonical motor patterns typically observed in unperturbed *in vitro* preparations (Hedrich et al., 2011; Massabuau and Meyrand, 1996; Clemens et al., 1998b). This is in spite of there being clear changes in the STG motor output *in vitro* (Marder et al., 2014; Marder and Calabrese, 1996) and *in vivo* (Heinzel, 1988) in response to application of modulators known to be present in the blood (Li et al., 2002; Chen et al., 2009). It is unclear how prevalent *in vivo* modulation actually is, how it affects the long-term activity of the STG motor patterns and if the same variability of motor output reported *in vitro* exists under natural conditions.

Here, we are using the crab pyloric and gastric mill rhythms (Fig. 1B,C) to study the long-term variability of the STG motor patterns and the influence of environmental factors on the rhythmic motor behaviors in freely behaving animals. Both rhythms are produced by distinct, but overlapping CPGs in the STG (Marder and Bucher, 2007). The gastric mill rhythm controls the movement of teeth to produce chewing in the gastric mill and the pyloric rhythm controls filtering of food in the pyloric region of the foregut (Johnson and Hooper, 1992). The continuously active pyloric rhythm has been particularly well characterized *in vitro*. Its canonical activity (e.g. Stein, 2009) consists of a repeating sequence of activity from the LP (lateral pyloric), PY (pyloric constrictor) and PD (pyloric dilator) neurons (Fig. 1B). This phase relationship of the pyloric neurons is exceptionally well maintained *in vitro*, within and between animals (Bucher et al., 2005), as well as between species (Stein and Städele, 2016). Phase constancy is even maintained when the system is perturbed with varying temperatures (Marder et al., 2015; Soofi et al., 2014), despite a dramatic change in pyloric cycle frequency.

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List of abbreviations

CoGs	commissural ganglia
CPG	central pattern generator
CPN2	commissural projection neuron 2
CV	coefficient of variation
DD	constant darkness
LD	12 h light:12 h dark
LG	lateral gastric
LL	constant light
LP	lateral pyloric
lvn	lateral ventricular nerve
MCN1	modulatory commissural neuron 1
PD	pyloric dilator
POC	postoesophageal commissure neuron
PY	pyloric constrictor
STG	stomatogastric ganglion
STNS	stomatogastric nervous system
VCN	ventral cardiac neuron

In contrast to the continuously active pyloric rhythm, the gastric mill rhythm (Fig. 1C) is a slower, episodic rhythm, and only activates under certain neuromodulatory conditions (Nusbaum and Beenhakker, 2002) that are mediated by extrinsic inputs, including descending projection neurons located in the commissural ganglia (Nusbaum and Beenhakker, 2002) and sensory feedback (Stein, 2009). The pyloric and gastric mill circuits overlap with one another, such that certain STG neurons contribute to both rhythms, resulting in gastro-pyloric interactions (Bartos et al., 1999; Weimann et al., 1991; Weimann and Marder, 1994; Dickinson, 1995; for review, see Nusbaum and Beenhakker, 2002; Stein, 2009).

The long-term *in vivo* activity of pyloric and gastric mill rhythms is mostly unknown. In particular, the range of variation in cycle frequency and phase relationships, as well as the range in response to various external and internal influences that these rhythms display is mostly unclear (Heinzel et al., 1993; Hedrich et al., 2011; Diehl et al., 2013; Soofi et al., 2014). For example, the *in vivo* activity patterns described so far for the pyloric rhythm (Soofi et al., 2014; Clemens et al., 1998a,b) reflect the canonical pyloric pattern, but show no signs of the expected plasticity inferred from neuromodulation experiments in the isolated system. This is surprising because in general, animals are continuously exposed to both predictable (e.g. daily cycles) and random (e.g. food acquisition) changes in sensory stimuli. In the STG there is continuous modulation by the pericardial organs (Li et al., 2003; Pulver and Marder, 2002). As such, slow long-term changes in motor patterns that match continuous patterns of variation in the environment, as well as quick changes in modulation, should be present in the pyloric and gastric mill motor patterns.

The purpose of this study was to determine (1) the long-term *in vivo* activity patterns of the pyloric and gastric mill motor patterns, their correlation with sensory input and their intercircuit interactions, and (2) the fast-acting sensory influences capable of altering patterns of rhythmicity.

MATERIALS AND METHODS

Dissection

We performed long-term *in vivo* motor pattern recordings from the lateral ventricular nerve (lvn; similar to Soofi et al., 2014) in the crab *Cancer borealis* Stimpson 1859. We anesthetized the crab on ice for 30–50 min, cut open the dorsal side of the carapace and located the lvn (Fig. 1A). We attached a small tube to the carapace with one end directly pointing at the lvn, inserted a wire (extracellular electrode; Fig. 1A, green arrow) with a hooked end into the tube and carefully placed the lvn onto the hook. We then pulled the nerve inside the tube to electrically insulate it. We secured the wire using beeswax and filled the tube with 'fast-set' silicone to insulate the electrode from outside noise. Finally, we sealed the opening in the carapace using dental cement (Protemp™ Plus temporized material, Neuss, Germany) and Parafilm™, connected the electrode with an amplifier, and returned the crab to its tank. For all experiments, only males were used and the animals were placed in isolation with pre-determined light cycles (see below) for a minimum of 3 days before surgery and allowed between 12 and 24 h of recovery time.

Light cycle experiments

We collected long-term recordings under different light cycles including 12 h light:12 h dark (LD), 24 h dark (DD), and 24 h light (LL). The wavelengths of light used were within the visible range for crustaceans. Under LD conditions, we expected to find some daily changes in activity. We selected the other cycles because if an innate rhythm is present, any daily

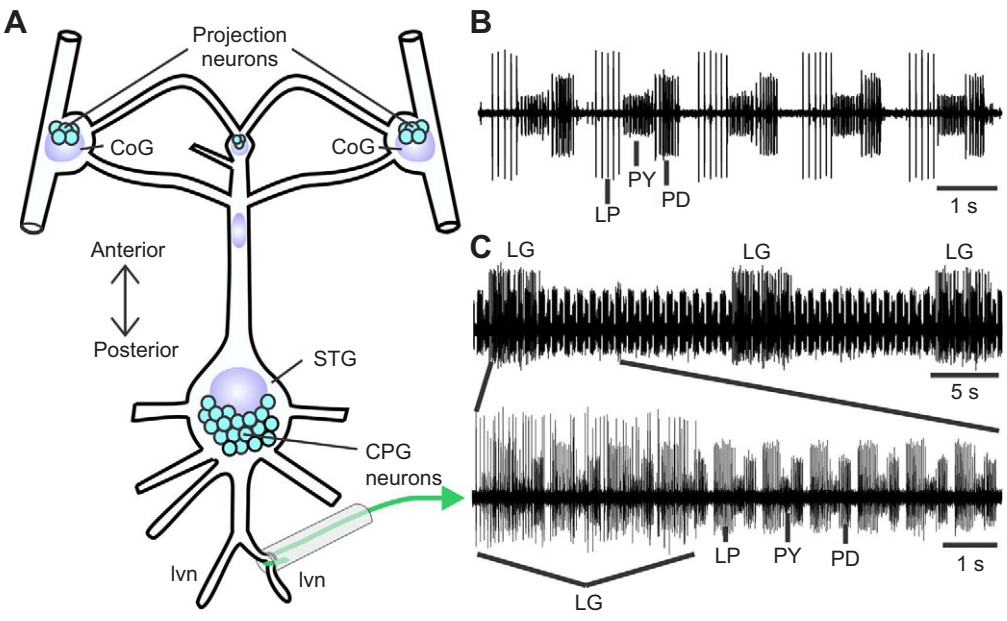

Fig. 1. Pyloric and gastric mill activity in the stomatogastric nervous system (STNS). (A) Schematic diagram showing the STNS of the crab *Cancer borealis*. Commissural ganglia (CoGs) contain modulatory projection neurons that innervate the CPGs in the stomatogastric ganglion (STG) neurons. The activity of the pyloric and gastric mill rhythms can be determined using extracellular recordings from the lateral ventricular nerve (lvn). Green arrow illustrates extracellular electrode placement. (B) Example *in vivo* extracellular recording of the lvn showing the pyloric rhythm. (C) Example *in vivo* extracellular recording of the lvn showing pyloric and gastric mill rhythms. The gastric mill rhythm is represented by the lateral gastric (LG) neuron. Note the differences in time scales. LP, lateral pyloric; PD, pyloric dilator; PY, pyloric constrictor.

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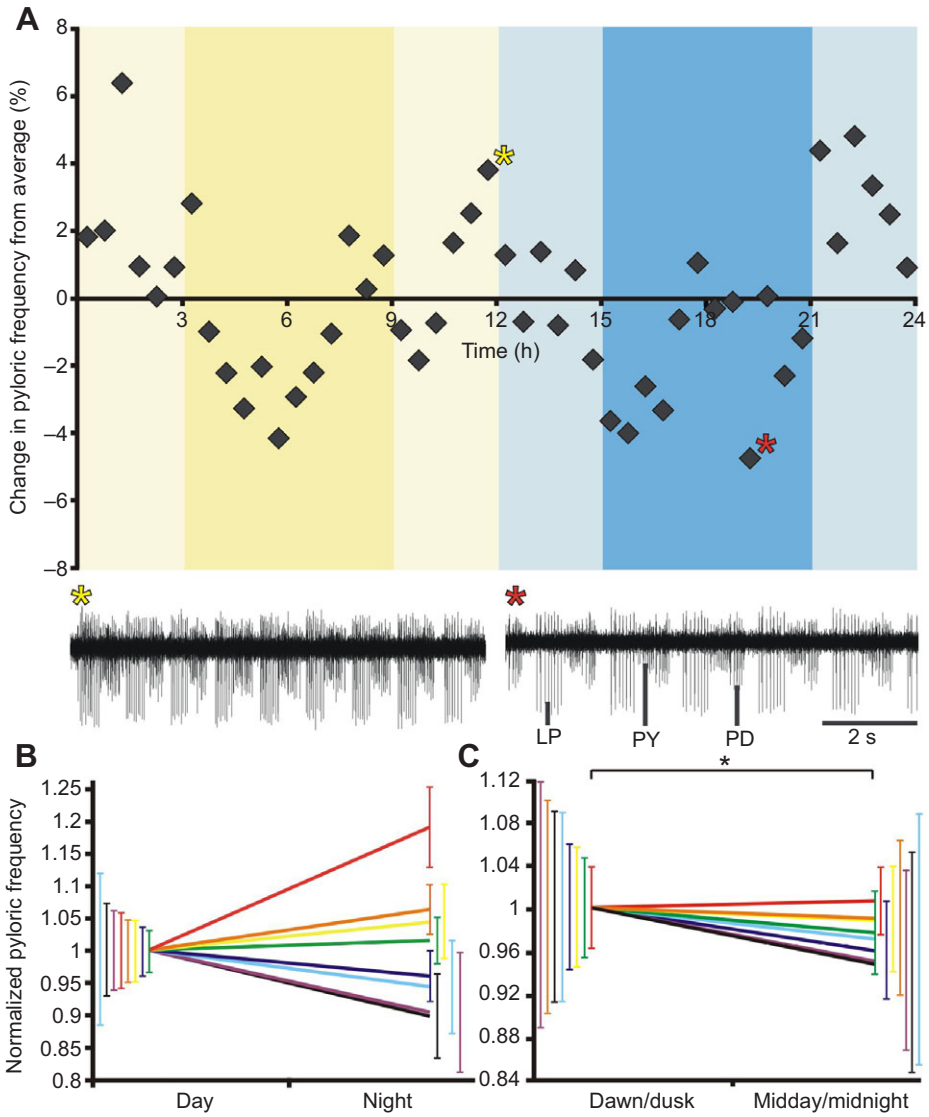

Fig. 2. The pyloric cycle frequency under LD conditions increases during dawn and dusk. (A) Percentage change from average pyloric cycle frequency over 3 days for 8 animals. Error bars were omitted for ease of viewing because variability was high. Yellow indicates day, blue indicates night, pale sections indicate dawn and dusk, and dark sections indicate periods around midday and midnight. Lower traces show example pyloric rhythms at different frequencies, marked with asterisks. These original traces were obtained from a preparation in which period changed quite dramatically at the indicated time points (in this case ~50%). (B) Normalized pyloric cycle frequency during the day and night under LD conditions. Data normalized to 'day'. Paired *t*-test, P=0.809, $\bar{y}_{day}$=0.638±0.151 Hz, $\bar{y}_{night}$=0.643±0.174 Hz; N=8 animals. (C) Normalized pyloric cycle frequency during dawn and dusk compared with midday and midnight under LD conditions. Data normalized to 'dawn/dusk'. Paired *t*-test, P=0.018, $\bar{y}_{dawn/dusk}$=0.647±0.158 Hz, $\bar{y}_{midday/midnight}$=0.633±0.164 Hz; N=8 animals. Different colored lines and error bars represent different animals. Error bars indicate s.d. in this and all subsequent plots.

activity pattern will persist under constant light or darkness (De Mairan, 1729). To determine daily patterns, we averaged each cycle's frequency every half hour over 3 days, so that each animal produced 48 data points representing the average 24 h cycle. We then averaged the 24 h cycles of multiple animals to produce an overall daily pattern (Figs 2 and 5). We also further averaged these values into 12 h bins to compare times of day, including day versus night and dawn/dusk versus midday/midnight. Because the pyloric and gastric mill cycle periods vary, 3 days of recorded activity for one animal included ~100,000–200,000 pyloric cycles and ~1000–20,000 gastric mill cycles.

Recordings

Extracellular nerve activity was recorded, filtered and amplified through an amplifier from AM Systems (Model 1700, Carlsborg, WA, USA). Data were recorded onto a computer using Spike2 and a micro 1401 AD board (CED, Cambridge, UK). Data were analyzed using the Spike2 script language to determine the cycle frequency of the pyloric and gastric mill rhythms and the activity patterns of their motoneurons. Scripts used for pyloric cycle frequency analysis were capable of detecting pyloric cycle period primarily during lateral gastric (LG) inter-burst intervals; however, some cycles during the intra-burst could also be detected. Unless otherwise stated, both inter- and intraburst intervals were included in the analysis. Structural aspects of the pyloric rhythm could only be detected during the LG interburst. Individual analysis scripts can be found at www.neurobiologie.de/spike2.

Sensory manipulations

Since the motor patterns recorded drive food processing, we analyzed the effect of feeding on the pyloric and gastric mill rhythms. The animals in this study were only fed squid, were starved for a minimum of 1 week before surgery and allowed between 12 and 24 h of recovery time. Because we observed feeding to have a long-lasting effect on these circuits, the structure of each animal's pyloric activity was analyzed for 6 h before and 6 h after feeding, and the cycle frequency of the pyloric rhythm was analyzed for an additional 4 days after feeding. Because all crab tanks share the same water, some experiments were performed on animals isolated from the other tanks to ensure that observed variability of activity was not a side effect of the shared water environment. Water temperature, osmolality and pH were all kept constant.

Statistical analysis

We used SAS statistical software. For testing differences between two conditions, such as day and night, a *t*-test was used in cases of normal distribution, and a Wilcoxon signed rank test when data were not normally distributed. For example, the effect of the gastric mill rhythm on pyloric cycle frequency was tested using a Wilcoxon signed rank test, as there are clear control and test conditions (before and during gastric mill rhythm), but no clear post-control because of the slow and discontinuous disappearance of the gastric mill rhythm. For correlations, Pearson's product-moment correlation was used for parametric data and Spearman rank-order

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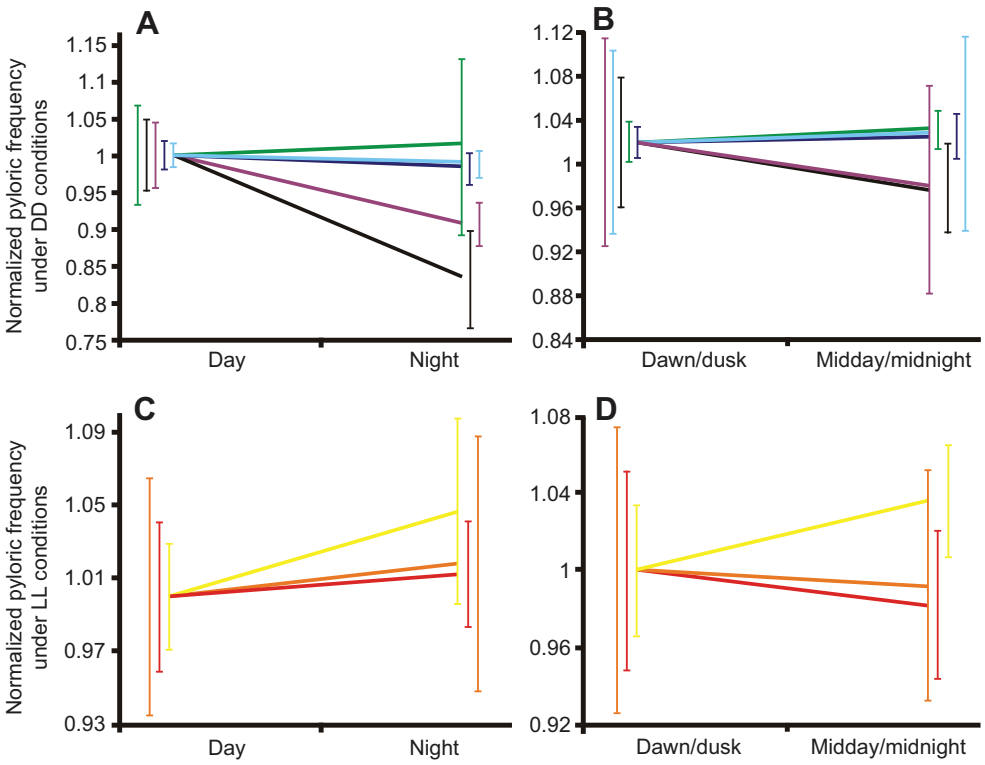

Fig. 3. The pyloric cycle frequency under constant light conditions does not significantly differ between day and night or between dawn/dusk and midday/midnight. (A) Pyloric cycle frequency at different times under DD conditions. Data normalized to 'day'. Paired *t*-test, P=0.113, $\bar{y}_{day}$=0.0.586±0.279, $\bar{y}_{night}$=0.563±0.286; N=5 animals. (B) Pyloric cycle frequency at different times under DD conditions. Data normalized to 'dawn/dusk'. Paired *t*-test, P=0.636, $\bar{y}_{dawn/dusk}$=0.576±0.278, $\bar{y}_{midday/midnight}$=0.573±0.287; N=5 animals. (C) Pyloric cycle frequency at different times under LL conditions. Data normalized to 'day'. Paired *t*-test, P=0.181, $\bar{y}_{day}$=0.656±0.246, $\bar{y}_{night}$ =0.674±0.252; N=3 animals. (D) Pyloric cycle frequency at different times under LL conditions. Data normalized to 'dawn/dusk'. Paired *t*-test, P=0.755, $\bar{y}_{dawn/dusk}$=0.663±0.244, $\bar{y}_{midday/midnight}$=0.667±0.255; N=3 animals. Different colored lines and error bars represent different animals.

correlation was used for non-parametric data. Mean values are reported as $\bar{y}$ and median as M.

RESULTS

To characterize the long-term activity patterns of the pyloric and gastric mill rhythms, we recorded extracellular activity on the lvn (Fig. 1A), which contains axons of several pyloric and gastric mill neurons. We recorded stable motor patterns from both rhythms for as long as 6 weeks (N=27 animals; median duration, 5.25 days; mean, 6.8 days). Like many decapod crustaceans, Jonah crabs are opportunistic predators. They hunt when able, but otherwise are scavengers (Donahue et al., 2009; Ojeda and Dearborn, 1991). They are also prey for birds, predatory fish and larger crustaceans (Good, 1992; Richards, 1992; Novak, 2004). This suggests that their feeding behavior may follow a circadian rhythmicity to both avoid predation and take advantage of the best foraging opportunities. Novak (2004) suggested that Jonah crabs have a diurnal pattern of locomotor activity, indicating that they may display a diurnal foraging pattern. This may be reflected in their digestive behavior (pyloric and gastric mill activity).

We first measured cycle frequency over several days. We placed animals in isolation with pre-determined light cycles (see the Materials and methods). There were no significant differences between day and night phases for the cycle frequency of the pyloric rhythm under LD conditions (0.8% change; Fig. 2A,B). However, cycle frequency increased significantly during the 12 h surrounding dawn and dusk as compared with the 12 h surrounding midday and midnight under LD conditions (2.2% change; Fig. 2A,C).

To determine whether the observed peaks during dawn and dusk were innate or light-driven, we performed the same experiment under DD conditions. There was no significant difference in pyloric cycle frequency between day and night (4.1% change) or between dawn/dusk and midday/midnight (0.5% change) under DD conditions (Fig. 3A,B). This indicates that the pyloric cycle frequency does not display a circadian rhythmicity, but rather follows the light cycle. We also performed the same experiment under LL conditions to confirm the data observed in the DD condition since both LL and DD are stable light conditions. There was no significant difference in pyloric cycle frequency between day and night (2.7% change) or between dawn/dusk and midday/midnight (0.6% change) under LL conditions (Fig. 3C,D).

The structure of the pyloric rhythm remains stable across pyloric cycle frequencies

Cycle frequency has been shown to vary substantially between animals and in response to sensory perturbations such as temperature changes. In contrast, the structure of the rhythm, as assessed by the phasing of the pyloric neurons, tends to be much more stable in response to environmental variations (Soofi et al., 2014). We tested whether the structure of the pyloric motoneurons remained stable over long periods of time and during phases when the cycle frequency of the pyloric rhythm changed considerably (Fig. 4A). We characterized the structure of the pyloric rhythm using aspects of LP activity over long periods of time. We restricted our analysis to the LP neuron, because this neuron has been shown to be the most sensitive to perturbations (Tang et al., 2012; Marder et al., 2014; Soofi et al., 2014). Fig. 4B shows the LP duty cycle over the course of 4 days in LD conditions. Gaps in detection of LP duty cycle were a result of either noise on the recording or gastric mill rhythms overshadowing the pyloric rhythm. The influence of such gastric mill activity on the pyloric rhythm will be discussed below. There was no significant change in LP duty cycle during different times of day. The duty cycle remained stable across multiple days in all animals tested (N=4; Fig. 4D). This was despite the fact that the cycle frequency of the pyloric rhythm changed dramatically within individuals.

The gastric mill rhythm shows a similar daily pattern of activity to the pyloric rhythm

We characterized the gastric mill rhythm, which controls the chewing of food, by extracellular recordings of the lvn. In this case,

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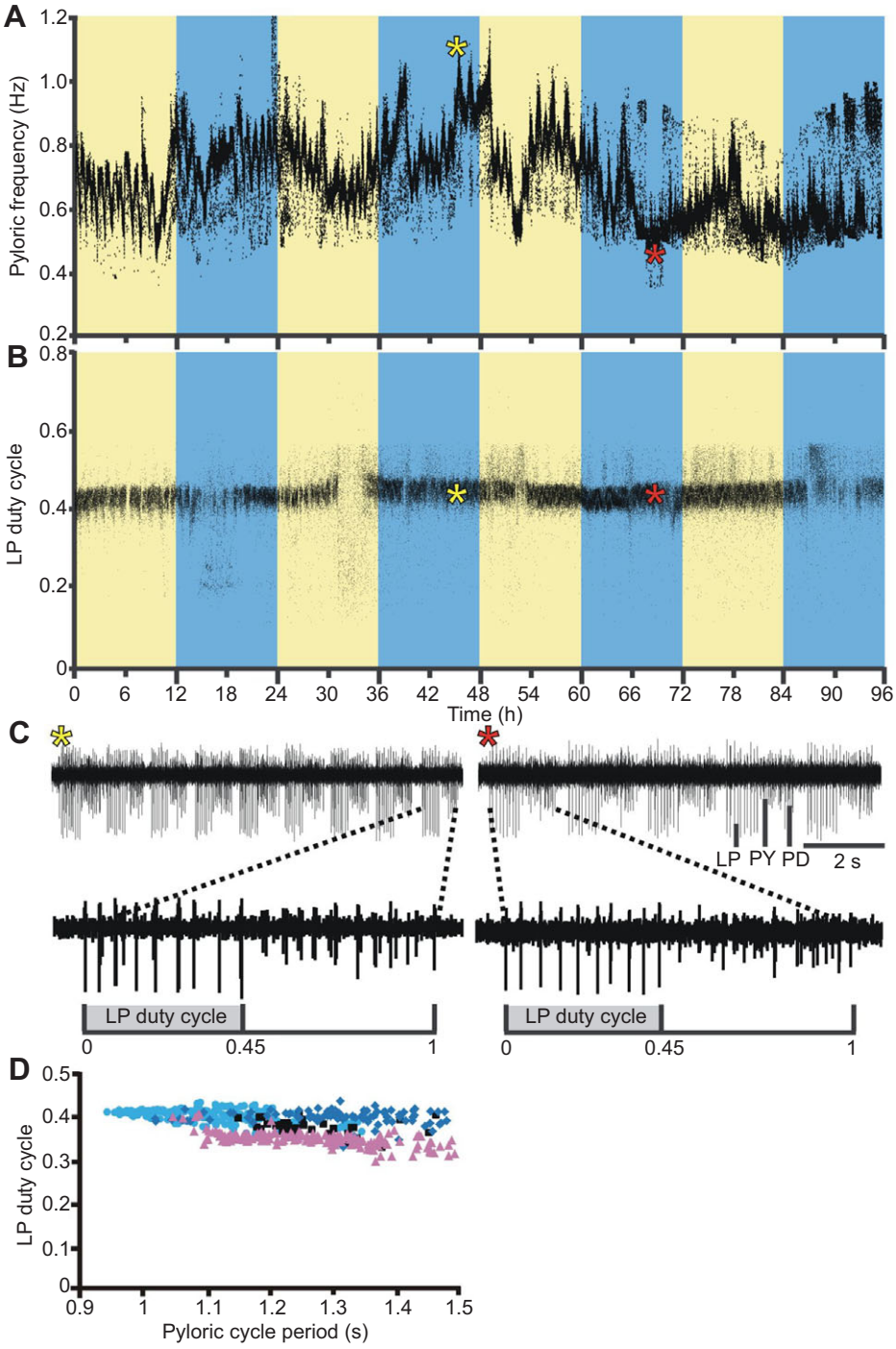

Fig. 4. Example *in vivo* LP duty cycle and pyloric cycle frequency over 4 days under LD light cycle in one animal. (A) Pyloric cycle frequency over 4 days. Yellow area indicates light, blue indicates darkness. Each data point represents the frequency of one pyloric cycle. (B) LP duty cycle over 4 days. Yellow area indicates light, blue indicates darkness. Each data point represents the LP duty cycle of one pyloric cycle. (C) Example *lvn* recordings showing pyloric rhythms with differing frequencies, but equal duty cycles, marked with asterisks in A,B. (D) Pooled animal data showing consistent LP duty cycle regardless of pyloric cycle period. Each data point represents 0.5 h average of the pyloric cycle period plotted against its corresponding average LP duty cycle. Different colors represent different individuals.

we used the activity of the LG neuron to determine gastric mill rhythm occurrence and cycle frequency. We found that the gastric mill rhythm, although only activated by extrinsic inputs, was active ~83% of the time (N=7). The gastric mill cycle frequency showed the same daily rhythmicity as the pyloric cycle frequency under LD conditions, such that the frequency increased during the hours surrounding dawn and dusk (2.9% change; Fig. 5A,C). As with the pyloric rhythm, there was also no significant difference between day and night under LD conditions (0.5% change; Fig. 5A,B). In fact, when we correlated the two rhythms we found that the gastric mill and pyloric cycle frequencies were highly correlated regardless of the light condition (Fig. 6) in all tested animals (N=5), indicating that any long-term influences on the pyloric cycle frequency can also be observed in the gastric mill cycle frequency.

In contrast to the continuously active pyloric rhythm, the gastric mill rhythm was inactive in ~17% of recordings across all experiments. As suggested by previous *in vitro* studies, extrinsic inputs (such as descending modulatory projection neurons or hormones) are necessary to activate this rhythm (summarized in Blitz and Nusbaum, 2011 and Stein, 2009). Since extrinsic inputs also affect the pyloric rhythm, and interactions between gastric mill and pyloric rhythms have been shown *in vitro* (Bartos et al., 1999), the pyloric rhythm should additionally display differences in activity in conjunction with the presence of the gastric mill rhythm

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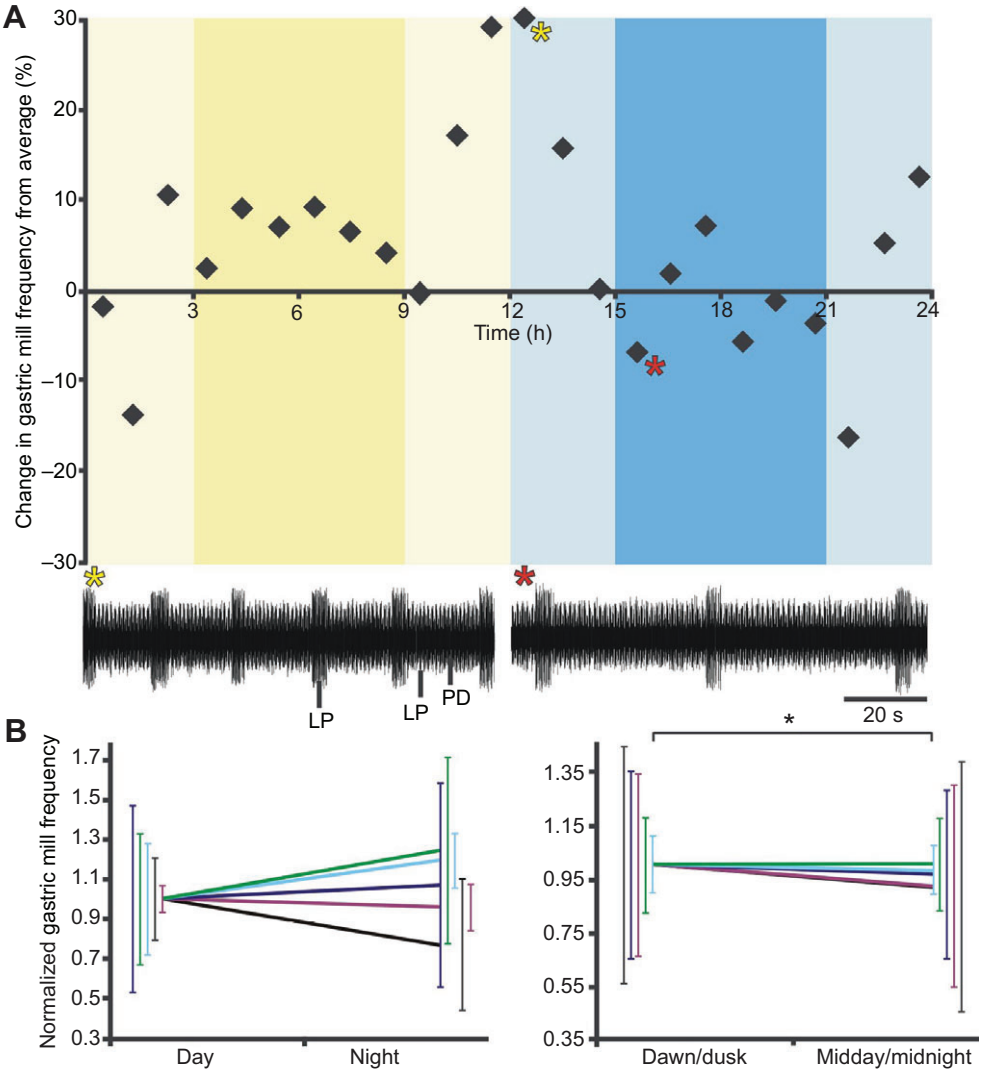

Fig. 5. The gastric mill cycle frequency under LD conditions increases during dawn and dusk. (A) Percentage change from average gastric mill cycle frequency over 3 days for 8 animals. Yellow indicates day, blue indicates night, pale sections indicate dawn and dusk, and dark sections indicate periods around midday and midnight. Lower traces show example gastric mill rhythms at different frequencies, marked with asterisks in A. (B) Normalized gastric mill cycle frequency during the day and night under LD conditions. Data normalized to 'day'. Paired *t*-test, P=0.971, $\bar{y}_{day}$=0.0397±0.018 Hz, $\bar{y}_{night}$=0.0399±0.015 Hz; N=5 animals. (C) Normalized gastric mill cycle frequency during dawn and dusk compared with midday and midnight under LD conditions. Data normalized to 'dawn/dusk'. Paired *t*-test, P=0.035, $\bar{y}_{dawn/dusk}$=0.0412±0.016 Hz, $\bar{y}_{midday/midnight}$=0.040±0.016 Hz; N=5 animals. Different colored lines and error bars represent different animals.

as well as its phasing. We thus examined patterns of pyloric activity during times when the gastric mill rhythm was active and inactive.

In vitro, when the gastric mill rhythm is activated via extrinsic inputs (such as descending projection neurons), the cycle frequency of the pyloric rhythm increases (Coleman et al., 1995; Stein et al., 2007; Stein, 2009). Specifically, when the descending projection neuron, modulatory commissural neuron 1 (MCN1), is selectively activated, the pyloric rhythm speeds up (Stein et al., 2007). Pyloric cycle frequency is additionally modulated with the phasing of the MCN1-activated gastric mill rhythm: pyloric cycle frequency diminishes during LG neuron bursts and speeds up during the LG inter-burst (Coleman et al., 1995). If a similar relationship exists *in vivo*, an LG phase-dependent change of the pyloric cycle frequency should be present. One confounding factor here is that *in vivo*, all sensory influences are still intact and interact with both rhythms, potentially masking these intercircuit interactions.

To determine whether similar intercircuit interactions exist *in vivo*, we compared the pyloric cycle frequency with and without an active gastric mill rhythm, as well as at the different phases within active gastric mill rhythms. When no gastric mill was present, the pyloric cycle frequency was significantly lower than when the gastric mill rhythm was active (Fig. 7A) and the pyloric cycle frequency during LG bursts was significantly lower than during LG inter-bursts (Fig. 7B). This indicates that *in vivo* the gastric mill rhythm interacts with the pyloric rhythm in a similar way as it does *in vitro* (Bartos and Nusbaum, 1997).

We also compared structural aspects of the pyloric rhythm during times with and without gastric mill activity. In contrast to cycle frequency, structural aspects of the pyloric rhythm could only be detected during LG inter-bursts. We found that LP burst duration, spike frequency and duty cycle did not significantly differ between conditions (LP burst duration: P=0.125, 6.7% change; LP spike frequency: P=0.153, 6.3% change; LP duty cycle: P=0.329, 1.7% change; N=4). However, the pyloric cycle frequency coefficient of variation was significantly higher during times when the gastric mill rhythm was active (paired *t*-test, P=0.041, 17% change), indicating that the pyloric rhythm is actually more stable overall during periods of gastric mill quiescence than during gastric mill activity in unfed animals.

Multiple gastric mill rhythms exist *in vivo*

Different projection neurons can elicit distinct types of gastric mill rhythms *in vitro* (Nusbaum and Beenhakker, 2002) and several types of gastric mill rhythms are also elicited by different sensory pathways (Blitz et al., 2004; Hedrich et al., 2009). *In vivo*, two versions have been described to occur after specific sensory

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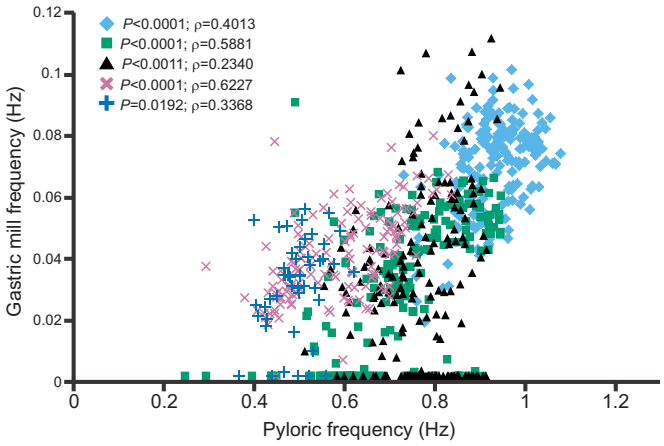

Fig. 6. Gastric mill cycle frequency is correlated with pyloric cycle frequency. Spearman rank order tests revealed significant positive correlations between gastric mill and pyloric cycle frequencies for all animals. Different colors represent different animals. Each data point is the average of 0.5 h of the pyloric and gastric mill cycle frequency.

stimulation (Diehl et al., 2013). In our experiments, we found that at least three distinct types of gastric mill rhythms exist naturally, without artificial stimulation (Fig. 8).

The first two types occurred consistently in unfed animals, whereas the third arose only after feeding. In unfed animals, type 1 rhythms constituted ∼77% of the observed gastric mill rhythms and type 2 rhythms made up the remaining ∼23%. We observed type 1 rhythms in 6 of 7 animals tested and type 2 rhythms in 5 of 7. Type 1 stayed active for very long periods (several days in a row) with its cycle frequency continuously increasing and decreasing every ∼1 h throughout the length of its activity (Fig. 8B, top). Type 2 came on periodically, for ∼20 min at a time, every ∼5 h (Fig. 8B, bottom). During those ∼20 min bouts, cycle frequency sharply increased then decreased again (Fig. 8A, inset). Individual LG bursts of both rhythm types exhibited variable burst durations and overall were not visually distinct from one another. However, type 2 gastric mill rhythms were almost always associated with LP inhibition in the pyloric rhythm (Fig. 8B, bottom), whereas this did not occur for type 1 rhythms.

The third type of gastric mill rhythm (type 3) only occurred after feeding. This type of rhythm stayed active and relatively

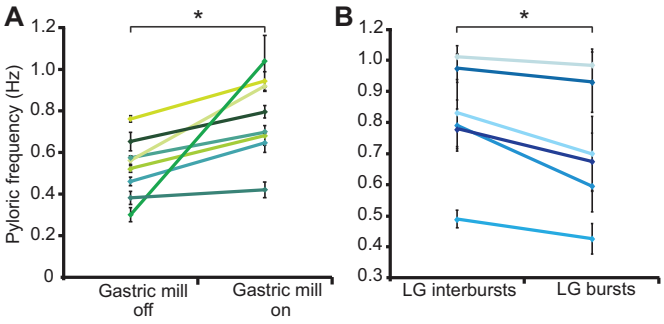

Fig. 7. The pyloric and gastric mill rhythms interact similarly *in vivo* as previously observed *in vitro*. Different colors represent different animals. (A) Pyloric cycle frequency increased when the gastric mill rhythm was active (Wilcoxon signed rank test, $M_{\text{gastric mill off}}$=0.542±0.193, $M_{\text{gastric mill on}}$=0.747±0.267; P=0.008, n=10 h per animal, N=8 animals). Both inter and intra gastric mill burst intervals were included to determine pyloric cycle frequency for gastric mill on. (B) Pyloric cycle frequency decreased during LG bursts (Paired t-test, $\bar{y}_{\text{LG inter-bursts}}$=0.812±0.185 Hz, $\bar{y}_{\text{LG bursts}}$=0.718±0.210; P=0.014, n=15 cycles per animal, N=6).

stable for long periods (multiple days in a row). However, type 3 rhythms also consistently went through periods where the cycle frequency would slow until it stopped (Fig. 8C). These ∼30 min drops and ∼30 min returns to baseline frequency did not follow any obvious pattern of occurrence. Type 3 gastric mill rhythms also often displayed short breaks in LG spiking within individual bursts, i.e. the LG bursts often consisted of several short burstlets (Fig. 8D, arrows).

Feeding immediately influences the activity of the pyloric rhythm and persists for several days

In addition to slow daily influences, motor systems encounter a variety of rapid and unexpected stimuli. Very little is known about how such fast-acting sensory stimuli affect the long-term activity of the pyloric and gastric mill motor patterns, or how they affect and interact with slower daily patterns of activity. We used feeding (a natural, rapidly initiated stimulus for the STNS) to examine the impact of short-term (fast-acting) sensory influences on daily activity patterns. We recorded pyloric rhythm activity (see Materials and methods) for a total duration of 12 h, including 6 h prior to feeding and 6 h after. We also measured cycle frequency of the rhythm for a minimum of 3 days after feeding. We quantified the structure of the pyloric rhythm from these recordings using different aspects of LP neuron activity. There was a significant and long-lasting increase (∼3 days before beginning to decrease) in pyloric cycle frequency immediately after feeding (when food entered the mouth) (Fig. 9A). This increase in pyloric cycle frequency (77.8% change) in response to feeding was observably much larger and quicker than any of the changes in cycle frequency we noticed in unfed animals. Cycle frequency also did not change for long periods and was interspersed with short periods where the frequency dropped to zero and then returned to previous levels (Fig. 9A). There was also a significant decrease in the LP burst duration (53.9% change; Fig. 9B). This, as well as the long-lasting increase in the pyloric cycle frequency, corresponds with previous findings *in vivo* in the lobster (Clemens et al., 1998a), although pyloric cycle frequency in lobsters was only affected for a period of between 24 and 48 h after feeding. This long-lasting change in frequency occluded the previously described 12 h, light-driven rhythmicity observed in unfed animals (Fig. 9A).

Interestingly, feeding not only affected the cycle frequency of the pyloric rhythm, but also its structure. We found that feeding significantly decreased the duty cycle of LP (8.2% change) and also increased LP spike frequency (71.6% change; Fig. 9B). We calculated the variability of the pyloric rhythm cycle frequency using the coefficient of variation (CV) and found that the variability of the pyloric cycle frequency also decreased after feeding (64.6% change), indicating a stabilization of the rhythm in response to food being present in the mouth or gut (Fig. 9B). This was surprising because this was the opposite effect compared with what we observed during spontaneous, unfed gastric mill rhythms (see Discussion). However, the decrease in variability, as well as the effects on duty cycle, LP spike frequency and pyloric cycle frequency is consistent with previous findings in the lobster (Rezer and Moulins, 1983; Clemens et al., 1998a).

Three of the five extracellular recordings from the animals that were fed also had quantifiable LG units. In two of those individuals, the gastric mill rhythm was active before feeding and the onset of feeding then elicited an immediate increase in gastric mill cycle frequency. In the third animal, the gastric mill rhythm was inactive before feeding and the onset of feeding immediately initiated gastric

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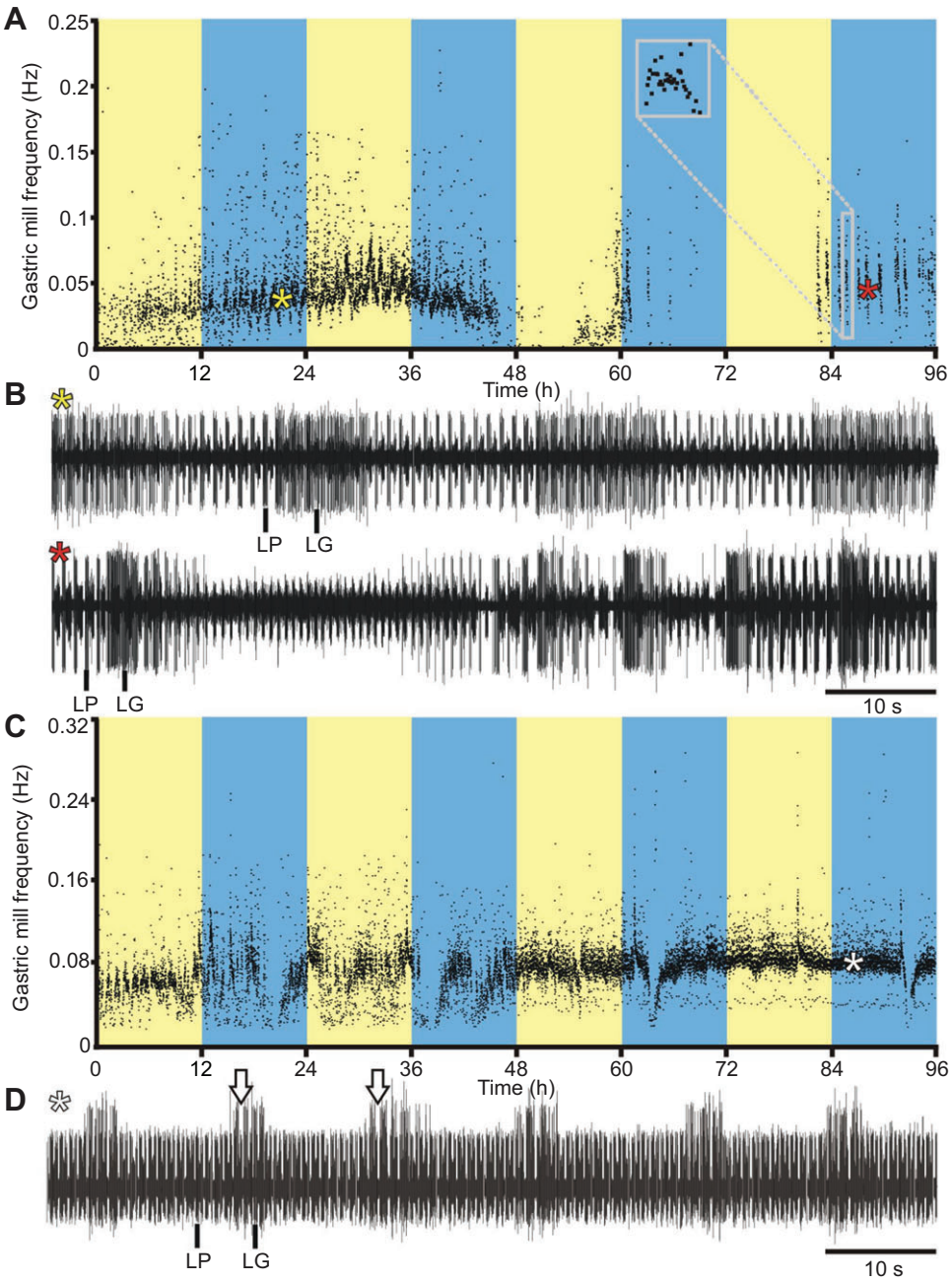

Fig. 8. Three types of gastric mill rhythms from one animal *in vivo*. (A) Gastric mill cycle frequency over 4 days in an unfed animal. Yellow area indicates light, blue indicates darkness. Each data point represents the frequency of one gastric mill cycle. Yellow asterisk indicates type 1: a continuously active gastric mill rhythm. Red asterisk indicates type 2: a sporadically active gastric mill rhythm. Inset shows example bout of gastric mill activity. (B) Example lvn recording during type 1 gastric mill rhythm (top) and type 2 gastric mill rhythm (bottom). (C) Gastric mill cycle frequency over 4 days in a fed animal. Yellow area indicates light, blue indicates darkness. Each data point represents the frequency of one gastric mill cycle. Type 3: a continuously active gastric mill rhythm, post feeding. Cycle frequency does not dramatically change for long periods and is interspersed with short periods where the frequency drops to zero then returns to what it was previously. White asterisk indicates location the of the example recording shown in D. (D) Example lvn recording during type 3 gastric mill rhythm. Arrows indicate example short breaks visible within individual LG bursts.

mill activity. This indicates that feeding also alters the daily rhythmicity of the gastric mill cycle frequency.

DISCUSSION

In this study, we identified the range and sources of variability of the pyloric and gastric mill rhythms *in vivo*. We found that on average a small 12 h light-driven cycle influenced the cycle frequency of both the pyloric and gastric mill rhythms, but that it was masked in individuals by other influences, including feeding. Within individuals there was a large amount of variability in the cycle frequency of the pyloric rhythm, despite the temperature, pH and osmolality all remaining stable throughout the recording time. Some of the observed variability could be accounted for by intercircuit interactions with the gastric mill rhythm, consistent with those previously observed *in vitro*. In contrast to the cycle frequency, the structure of the pyloric rhythm was much more robust and remained stable for multiple consecutive days. The only instance where the structure of the pyloric rhythm did change was in response to feeding.

One of the most striking characteristics of rhythmic motor systems is that they show immense plasticity (Harris-Warrick, 2011) as a result of neuromodulation. There are two major sources of neuromodulation for the pyloric and gastric mill circuits in the STG: descending modulatory projection neurons and hormonal influences, such as those from the pericardial organs (Nusbaum and Beenhakker, 2002; Marder and Bucher, 2007). While it is clear from previous studies that activation of sensory pathways that elicit projection neuron activity and application of modulators found in the hemolymph both have a strong and quick influence on the STG circuits both *in vivo* and *in vitro* (Marder et al., 2014; Marder and Calabrese, 1996; Nusbaum and Beenhakker, 2002; Heinzel et al., 1993; Diehl et al., 2013), until now very little was known about the

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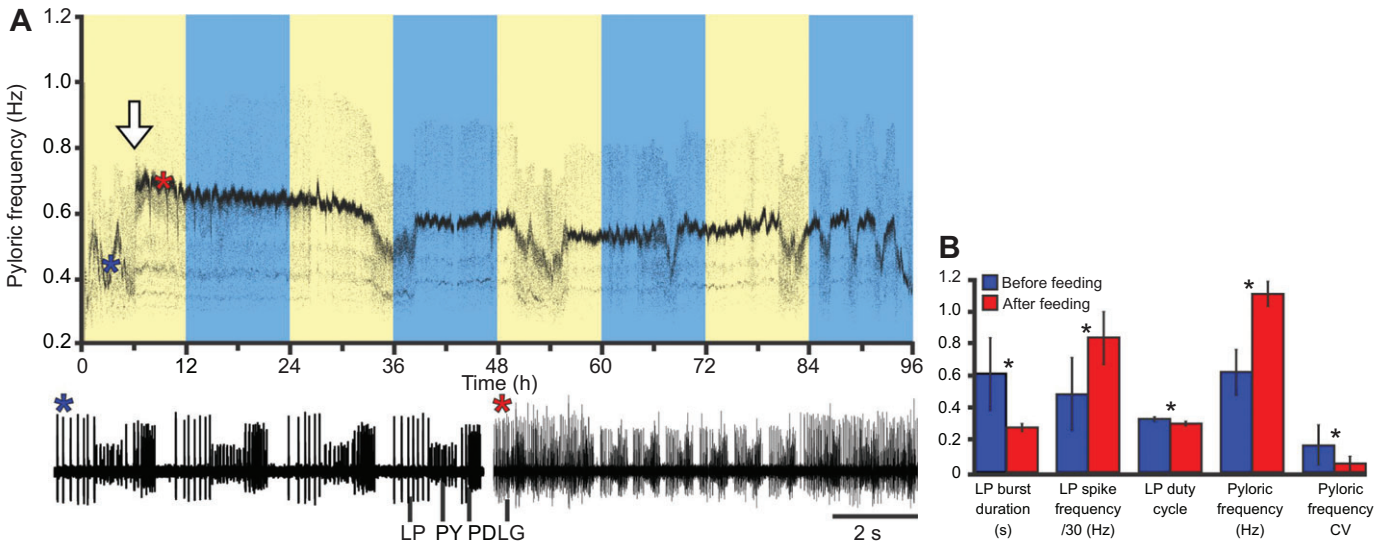

Fig. 9. Pyloric structure and cycle frequency are significantly altered in response to feeding. (A) Example pyloric cycle frequency over 4 days under a LD light cycle in one animal after feeding. Yellow area indicates light, blue indicates dark. Each data point represents the frequency of one pyloric cycle. Red and blue asterisks indicate lower traces which show example pyloric rhythms before and after feeding. White arrow indicates when feeding occurred. (B) Different aspects of the pyloric rhythm during the 6 h before and 6 h after feeding. The units for the *y*-axis depend on the analysis shown in this figure, so appropriate units for each measurement are given below the bar graph. Both inter and intra gastric mill burst intervals were included to determine pyloric cycle frequency and CV; however, all other variables could only be determined from inter gastric mill intervals. *All variables were significantly different before and after feeding. Paired *t*-test results were as follows. LP burst duration: P=0.035, $\bar{y}_{before}$=0.621±0.205 s, $\bar{y}_{after}$=0.286±0.024 s; LP spike frequency: P=0.004, $\bar{y}_{before}$=14.8±6.11 Hz, $\bar{y}_{after}$=25.4±4.47 Hz; LP duty cycle: P=0.018, $\bar{y}_{before}$=0.340±0.015, $\bar{y}_{after}$=0.312±0.014; Pyloric cycle frequency: P<0.001, $\bar{y}_{before}$=0.630±0.145 Hz, $\bar{y}_{after}$ =1.12±0.080 Hz; Pyloric cycle frequency coefficient of variation: P=0.042, $\bar{y}_{before}$=0.175±0.1275, $\bar{y}_{after}$ =0.062±0.047. N=5 animals. Error bars represent s.d.

long-term activity of both motor patterns. We found that in unfed animals, the phase relationships of the pyloric neurons rarely deviated from the canonical patterns, indicating that the pyloric rhythm's structure remains stable. This was despite several studies showing that, *in vivo*, the STG is continuously exposed to both hormonal and neural modulation (Diehl et al., 2013; Hedrich et al., 2011; Behrens et al., 2008; Schmerberg and Li, 2013; Christie et al., 1995), which have the potential to modify the phase relationships of the involved motor neurons (Marder and Weimann, 1992).

Light-driven effects on rhythmicity are occluded by considerable variability within animals

Many tidal crustaceans have innate circatidal or light-driven rhythmicities to their behavior (Akiyama, 2014; Rebach, 1985; Lynch and Rochette, 2007; Williams and Naylor, 1967; Hough and Naylor, 1992), indicating that the neuronal circuits that produce those behaviors show a similar rhythmicity. In the lobster, ablating the eyestalks increases gastric mill activity for many hours (Fleischer, 1981). Conversely, application of eyestalk hormones decreases gastric mill activity, indicating that light sensation may influence the stomatogastric activity patterns. We found a small, but significant increase in gastric mill and pyloric cycle frequencies during the 3 h prior to and 3 h after the lights turned on or off (at dawn and dusk). However, the timing of this change and its small size were not consistent with the effects described by Fleischer (1981). The observed changes in the rhythms in our study lasted only a few hours and included the hours prior to the light change and did not persist under constant light conditions. This indicates that although the rhythm is not innate (circadian), the light changes can be anticipated if they occur regularly each day.

There was also no immediate change in pyloric and gastric mill activity when lighting changed, which would be expected if animals were responding directly to the light input. The light-driven response was also modest, in the order of only a few percentage points, and the variability within individuals (e.g. Fig. 4A) occluded the slower, light-driven pattern found on average (e.g. Figs 2 and 5). This indicates that the light-driven pattern has little influence on the behavior itself and is supported by the substantial variation in pyloric and gastric mill cycle frequencies observed within individual animals. We kept temperature, pH and osmolarity of the water (all of which can affect motor patterns; Soofi et al., 2014; Chesler, 2003; Schwartzkroin et al., 1998) constant, reducing unwanted sensory effects on the motor patterns. And still, pyloric and gastric mill cycle frequencies varied considerably, even within individual animals. This variation may reflect sporadic changes in the modulatory environment or sensory influences not under our control.

One striking observation, however, was that despite the variability of the pyloric cycle frequency, the structure of the rhythm, as measured by the LP duty cycle, remained rather constant. The pyloric rhythm has previously been shown to maintain phase relationships in response to environmental perturbations such as temperature (Soofi et al., 2014; Tang et al., 2012; Bucher et al., 2005), while cycle frequency is much more flexible. Maintaining the stability and robustness of motor output may be important for motor control. Phase maintenance, for example, is responsible for ensuring appropriate functional output of rhythmic motor networks (Hooper, 1997) and has been shown to support adequate behavioral performance, for example, to produce swimming in lamprey, leech or crayfish (Cohen et al., 1992; Smarandache et al., 2009; Kristan et al., 2005; Mullins et al., 2011). However, phase relationships may change depending on the sensory or behavioral conditions. The gastric mill rhythm, for example, has been shown to exhibit various phase relationships, which are associated with different behavioral outputs *in vivo* (Heinzel et al., 1993; Diehl et al., 2013). The behavioral relevance of phase maintenance or phase changes in the pyloric rhythm is not clear, but we did see significant changes when behavioral conditions had changed, i.e. after feeding.

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When applied to the STG, neuromodulators can alter both pyloric cycle frequency and phasing of the motoneurons (Stein, 2009; Marder and Thirumalai, 2002). This might indicate that modulators cause significant changes in the output behavior and/or that modulators allow the circuits to adopt different network states that are required for adequate functioning. Conversely though, we found that phase relationships remained stable throughout the recording time in unfed animals.

Most previous experiments where modulators were shown to have an influence on the structure of the pyloric rhythm were done after desheathing the ganglion and there is recent evidence that the ganglion sheath may significantly affect the response of STG neurons (Goldsmith et al., 2014) or even be selectively permeable for specific modulators. One consequence of the ganglion sheath having a selective permeability could be that modulators in the hemolymph may not affect the activity of the STG motor circuits, or at least not with the expected magnitude. Furthermore, a recent study by Dickinson et al. (2015) showed that although multiple modulators of the same species have similar effects on motor activity, others exhibit isoform-specific selectivity. So, although modulators may be present and permeate through the ganglion sheath, they may not necessarily influence motor output.

In vivo intercircuit interactions

Whereas little information is available about hormonal influences on the *in vivo* motor patterns, the pyloric and gastric mill circuits have long been known to be interconnected and to influence one another *in vitro* (Bartos and Nusbaum, 1997; Marder et al., 2005; Beenhakker et al., 2004). Our data are consistent with the idea that similar mechanisms contribute to the interactions between the gastric and pyloric circuits *in vivo*. *In vitro*, the gastric mill rhythm depends on extrinsic excitation provided by descending projection neurons (Nusbaum and Beenhakker, 2002). The best-studied projection neuron that activates the gastric mill rhythm is modulatory commissural neuron 1 (MCN1; Coleman et al., 1995). MCN1 excites both the gastric mill and the pyloric CPGs. As a result, when MCN1 activates the gastric mill rhythm, the frequencies of both rhythms increase. A hallmark of the timing of excitation that MCN1 provides is that it depends on the motor patterns it controls. When LG is active it presynaptically inhibits the MCN1 terminals, leading to a reduction of MCN1 transmitter release and thus a reduced excitation of both the pyloric and gastric mill CPGs (Bartos and Nusbaum, 1997). Consequently, during LG bursts, the cycle frequency of the pyloric rhythm decreases. During LG inter-bursts, MCN1 is no longer presynaptically inhibited and pyloric excitation is restored, increasing pyloric cycle frequency. Our data are consistent with the idea that similar mechanisms contribute to the gastro-pyloric circuit interactions *in vivo* and these interactions explain at least some of the variability observed in the frequency of the pyloric cycle.

Multiple gastric mill rhythms exist *in vivo*

We also found differences between the gastric mill rhythms observed *in vivo* and those *in vitro*. While the gastric mill rhythm is not always active *in vitro* (45% active; Stein et al., 2005), it is almost always active *in vivo* (82% active). Sensory pathways have been shown to activate the gastric mill rhythm (via the actions of descending modulatory projection neurons) both *in vitro* and *in vivo* (Blitz and Nusbaum, 2012; Beenhakker and Nusbaum, 2004; Blitz et al., 2004; Saideman et al., 2007; Beenhakker et al., 2004; Hedrich et al., 2009, 2011; Diehl et al., 2013). It is reasonable to assume that the increased amount of gastric mill activity *in vivo* is due to additionally activated sensory pathways, as most of them are inactive *in vitro*. Alternatively, the level of excitation could differ *in vivo* and *in vitro* such that gastric mill rhythms are more likely to be activated *in vivo*, as a result of excitatory neuromodulators in the hemolymph or modulatory pathways that may no longer be intact *in vitro*.

We found three different types of gastric mill rhythms *in vivo*. Type 1 was the most common in unfed animals. This type bore similarities to rhythms that had previously been described *in vitro*. This version can be elicited by the mechanosensory ventral cardiac neuron (VCN; Beenhakker and Nusbaum, 2004). During this type of rhythm, LG bursts display continuous spiking. In contrast, when the peptidergic postoesophageal commissure neurons (POCs) are activated, the LG burst firing is interrupted periodically and consists of shorter burstlets (Blitz et al., 2008). The type 1 gastric mill rhythm showed both types of bursts, and seamlessly switched back and forth between them. This indicates that the observed type 1 rhythm could have been elicited via multiple sensory pathways (VCN and POCs), which supports the idea that the rhythms characterized individually *in vitro* may never occur as independent entities *in vivo*. It may also mean that there are no strong attractor states that lock motor patterns into specific states, but that there are rather fluid transitions between distinct motor patterns.

Interestingly, although the majority of observed gastric mill rhythms were type 1, one of the animals did not display any type 1 rhythms. It is unclear why this animal only displayed type 2 rhythms, but it is possible that because of the long activity periods of type 2 rhythms, only this type was observed during the course of the experiment. Type 2 rhythms were very distinct from type 1 in that they were always accompanied by inhibition of the pyloric LP neuron. *In vitro*, such rhythms have not been described, but it is conceivable that other projection neurons could contribute to this type of rhythm. Modulatory commissural neuron 5, for example, causes a strong inhibition of LP *in vitro* (Norris et al., 1996) and has been shown to be affected by sensory stimulation (Beenhakker et al., 2004).

Type 3 only occurred in response to feeding and lasted for several days. The cycle frequency of type 3 gastric mill rhythms did not change for long periods and was interspersed with short episodes where cycle frequency dropped to zero over the course of ∼30 min and then returned to its previous frequency, again over the course of ∼30 min. An interesting aspect of the type 3 gastric mill rhythms was that short breaks were often visible within individual LG bursts. This is reminiscent of the POC-activated gastric mill rhythms observed *in vitro* (Blitz and Nusbaum, 2012).

Feeding

Feeding had a profound impact on the pyloric and gastric mill motor patterns (Fig. 9). The pyloric cycle frequency increased and remained relatively stable for long periods. In all animals, this increase was also interspersed with short periods where cycle frequency dropped to zero then returned to its previous value (Fig. 9A). However, similar changes in cycle frequency were also prevalent in unfed animals, indicating that they may not be directly related to feeding. Pyloric cycle frequency increased immediately after feeding, but its variability decreased. This observation was contrary to what we observed during gastric mill rhythms in unfed animals and suggests that the stability of the pyloric rhythm depends on the type of gastric mill rhythm present. The reduced variability after feeding may indicate that there is an optimal cycle frequency for filtering behavior when food is processed. Without food, cycle frequency may not need to be maintained and is thus not restricted to

a specific range. There are also indications that neuromodulator release from projection neurons, which activate gastric mill rhythms, may influence the variability of the pyloric cycle frequency. This is striking because gastric mill rhythms were always initiated in response to feeding. The link between gastric mill activation and stabilization of pyloric cycle frequency may be explained by the activation of descending modulatory projection neurons such as MCN1. These neurons, which initiate gastric mill rhythms, also directly affect pyloric activity and are known to be activated by food stimuli (Hedrich et al., 2011). MCN1 releases the peptide transmitter proctolin, which supports oscillations in the pyloric circuit (Zhao et al., 2010) and may act to stabilize the pyloric rhythm (Marder and Bucher, 2007).

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Both authors contributed in designing experiments, performing research, analyzing data and writing the manuscript.

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