REVIEW ARTICLE

A Cellular Approach to the Study of Complex Natural Behavior Patterns in the Ragged Sea Hare (*Bursatella leachii*), a Marine Invertebrate Indigenous to Puerto Rico

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ABSTRACT. The ragged sea hare (*Bursatella leachii*), an aplysiid mollusc indigenous to the tropical waters of Puerto Rico, possesses numerous properties that make it suitable for a neuroethological approach to the study of complex behavior patterns. Field studies spanning three years have established a location and season of *Bursatella* availability on the north coast of the island. In the natural habitat, the *Bursatella* exhibit a daily rhythm of behavior patterns in which feeding-related activities predominate during the day and reproductive behaviors predominate at night. Some aspects of this natural pattern persist in animals held in the laboratory. The *Bursatella* nervous system contains large neurons, some of which appear to be homologous to cells that have been characterized extensively in related species. Following isolation of the nervous system, neural centers associated with feeding movements and locomotion retain extraordinarily robust rhythm generating capabilities. It is proposed that this species offers unique opportunities for deriving general principles governing the regulation and integration of central pattern generator circuits underlying complex natural behavior patterns.

Key words: molluscan behavior, molluscan feeding, aplysiid, molluscan locomotion, central pattern generators.

> *Some of the most fascinating and pressing problems open to science concern the structure and function of the human brain, that exceedingly complex organ believed to be the site of our thoughts, the storeroom of our memories and the source of all human achievements including science itself. A number of avenues are open through which scientists can approach and explore this intricate organ. Perhaps one of the most fruitful is to study equivalent structures in simple primitive invertebrates which live in the tropical waters near the Laboratory of Neurobiology.*
>
> Professor José del Castillo (1)

Most animals, including humans, utilize rhythmic movements in the generation of behaviors related to feeding, locomotion, procreation, and communication. Numerous fundamental principles of brain organization have been derived from studies utilizing certain stereotyped and repetitive behaviors of invertebrates (2-9). Several concepts that were developed in studies using these experimentally favorable 'simpler' systems have proven to be applicable to circuits within the brains of mammals. Our present understanding of the organization of action (10) includes a number of these concepts, including central pattern generators (CPGs), multifunctional neuronal circuits (11-12), command neurons (13), and behavioral hierarchies (3,4).

The purpose of this article is to provide an overview of one research program that is specifically designed to utilize a tropical marine invertebrate as a model neurobiological system for studying the generation and coordination of rhythmic behaviors. The organism that is the focus of these studies is *Bursatella leachii plei* (Fig. 1A,B) commonly known as the 'ragged sea hare' due to the numerous papillae that protrude from its body. *Bursatella* is a large (reaching 10 - 15 cm in length) sea slug that belongs to the subclass Opisthobranchia (rearward gill and reduced or absent shell) of gastropod (belly footed) molluscs. It belongs to the Aplysidae family, and is therefore closely related to one of the most intensively studied and well understood neurobiological systems in the animal kingdom, *Aplysia californica* (14-15).

The overall goal of this research program is to characterize physiological mechanisms that contribute to

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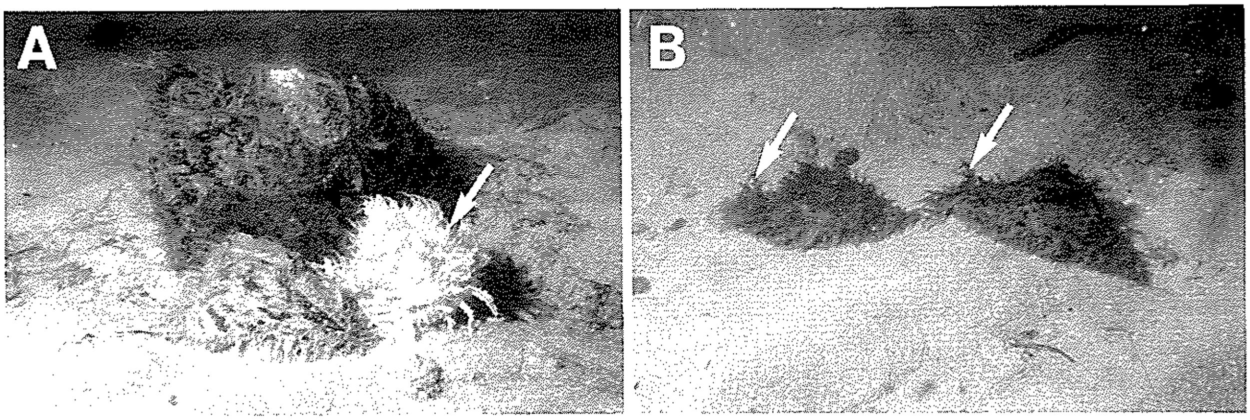

Figure 1. The ragged sea hare in its natural habitat. *Bursatella leachii* were photographed by Dr. Thomas Teyke (Johannes Gutenberg Univ., Mainz, Germany) using a Nikonos V underwater photography system at Escambrón Beach Park. **(A)** A 'pile' of eight *Bursatella*. They are found in such aggregates during the early morning hours (see ref. 22). Note unique pigmentation of individuals, such as the white specimen in the foreground (*arrow*). **(B)** Two individuals photographed while grazing during the afternoon. Animals are oriented toward and locomoting toward the left side of the field (*arrows* pointing to heads).

the generation of *complex natural behavior patterns*. As such, it falls within the field of Neuroethology, a hybrid discipline that is derived from the blending of two distinct traditions: ethology, which emphasizes the study of animal behavior in the native environment, and neurobiology, the study of the structure and function of nervous systems (16-17). Typically, the broad aim of studies in this discipline is to gain an understanding of neuronal circuits that underlie the generation of natural behavior patterns. Historically, certain invertebrate species have been the focus of such investigations, for reasons which will be expanded upon below.

Among the invertebrates, the behavioral biology of opisthobranch molluscs has been a subject of particularly intensive study (14, 15). The nervous systems of these organisms typically consist of a relatively small number of neurons, many of which are large and readily accessible for electrophysiological, biochemical, and molecular analyses. To date, however, the majority of the cellular studies of behavior in marine gastropods have utilized species that are not easily observed in their native habitats (see also 18, 19). It has therefore often been difficult to relate observations made in the laboratory to the natural behavior of the organism (but see 20, 21). Difficulties in obtaining long observation periods in the field may be attributable to the depth, temperature, or turbulence of the waters in which an animal lives, or may arise from the animal's movement into places that are hidden from view. Recently, we and others have begun to investigate opisthobranch molluscs inhabiting environments that favor field observations (22-26). One goal of these studies is to compare behavior patterns that may be mediated by homologous neurons in closely related species. Identification of such circuits may increase our understanding of "which properties of a neural network are most likely to change when behavior is modified by selection pressures" (15, see also 27).

In order to study the neuronal basis of natural behaviors, one would like to identify a species that satisfies the following criteria:

1) Members of the species should exhibit a varied repertoire of easily distinguishable behavior patterns.
2) Behavior patterns should be readily observed and quantified in the natural habitat.
3) A substantial proportion of the animal's time budget should be devoted to the behaviors of interest.
4) Individuals should undergo frequent transitions between behaviors.
5) The general characteristics of these natural behaviors should persist in animals held in captivity.
6) It should be possible to record neuronal activity while specimens are engaged in natural behavior patterns.
7) The general properties of the neural circuits of interest should persist in the isolated nervous system.
8) The nervous system should contain large identifiable neurons that can be shown to regulate specific components of behavior.

The *Bursatella leachii* that inhabit the tropical waters of Puerto Rico exhibit all of these characteristics (22). Furthermore, as indicated above, *Bursatella* is closely related to the North American sea hare *Aplysia californica,* a species for which a wealth of information concerning neural circuits and behavior has been generated during the last 30 years. The nervous systems of these two species are similarly organized, and they contain a number of homologous identifiable neurons. The *Bursatella,* however, exhibit certain properties that should make them particularly useful for analyses of natural behavior patterns. These include a) ease of observation in the field, b) a higher overall activity level, c) more frequent transitioning between behavior patterns, d) persistence of natural behavior patterns in captivity, and e) integrity of certain central pattern generator circuits in the isolated nervous system.

Results

Field Observations. The first requirement for studying a novel species in its native habitat is establishing the location, season, and time of day in which members of that species can be observed. We were introduced to *Bursatella* in September of 1992 by the renowned naturalist Faustino Mackenzie. Quantitative field studies were initiated in 1993 at Escambrón Beach in the Puerta de Tierra district of San Juan. The proximity of this location to the Institute of Neurobiology has enabled us to establish the *Bursatella* 'season' with a resolution that is rarely achieved in studies of this type (Fig.2; cf. 23,28,29). In brief, the *Bursatella* are found at this site between the months of June and November of each year.

On 7 January, 1994, the oil barge Morris C. Berman ran aground directly off Escambrón Point (Fig 3). Estimates of the quantity of heavy No. 6 fuel oil that was discharged over the following weeks ranged from 500,000 to 850,000 barrels. In assessments of oil impact on marine invertebrate populations, it is exceedingly rare to have 'baseline' data with which to compare observations obtained following a catastrophic spill (30). However, such a comparison between pre-spill data from 1993 and post-spill data from 1994 and 1995 (Fig. 2) indicates that the seasonal availability of *Bursatella* was not severely affected by this event. This conclusion is consistent with the observations of others suggesting that the intensive clean-up effort, conducted under the direction of the U.S. Coast Guard in cooperation with numerous Commonwealth and Federal agencies, served to limit the extent of long-term damage to marine life in the area (31).

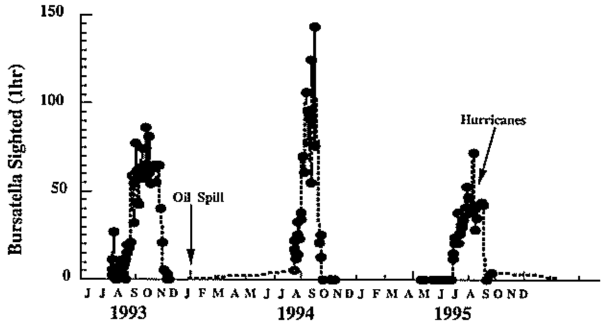

Figure 2. Plot of the number of *Bursatella* sighted during 93 surveys (1 hr each) conducted at Escambrón Beach Park between July, 1993 and September, 1995. Two events discussed in the text, the Morris C. Berman oil spill and the hurricane season of 1995, are indicated by *arrows*. The data from 1993 have been previously published (reference 22).

Figure 3. Oil spill clean-up efforts being conducted at Escambrón Beach Park in January, 1994. The Morris C. Berman can be seen in the background (*arrow*). San Juan Star photo taken by Ingrid Torres.

In 1995, Puerto Rico was threatened by an unusually high number of hurricanes. Although it suffered no 'direct hits', several storms caused severe wave activity and turbulence on the northern (Atlantic) shore of the island. The arrival of the major hurricanes coincided with the peak of the *Bursatella* season (Fig. 4). Following each storm, the number of specimens sighted was markedly diminished. The number of animals observed following

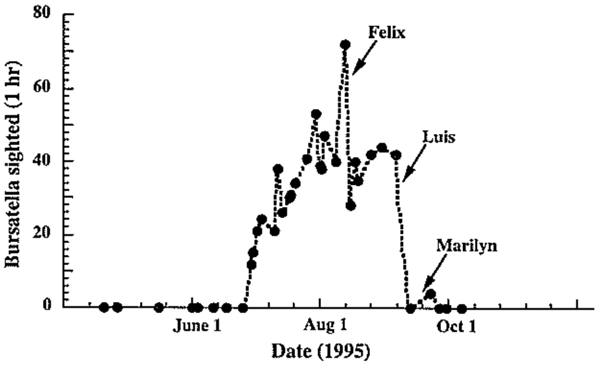

Figure 4. Plot of the number of *Bursatella* sighted during 35 surveys (1 hr each) conducted at Escambrón Beach Park between April and October, 1995. Major hurricanes are indicated by *arrows*.

Hurricane Felix was markedly reduced, but some recruitment or recovery appeared to occur following this storm. However, the *Bursatella* were essentially eliminated by the strongest hurricanes, Luis and Marilyn.

Taken together, our observations to date suggest a fairly predictable and reliable annual period of availability for *Bursatella* on the northern shore of Puerto Rico. Limited observations on the southern (Caribbean) shore at Isla Mata la Gata in La Parguera indicate that the season may be broader in these warmer waters (M.W. Miller, I. Torres, and F. MacKenzie, unpublished).

Natural behavior patterns. During initial field observations, it was apparent that the behavior patterns displayed by the *Bursatella* varied depending upon the time of day (22). During the early morning hours, animals were found in stationary aggregates, ranging in size from 2 to 12 specimens (Fig. 1A). Three to four hours following sunrise, these groups dispersed, and the animals spent the rest of the daylight hours as mobile individuals or pairs (Fig. 1B). During the two or three hours following sunset, the *Bursatella* reassembled (22). Limited observations made to date suggest that they remain assembled through the night.

Reproductive behavior. While aggregated, the *Bursatella* exhibited two forms of reproductive behavior; copulation and egg laying. Since animals remained grouped for some time following sunrise, it was possible to observe single aggregates for extended periods of time. The distinctive colorations of individual specimens within certain aggregates (see Fig. 1A) enabled the observer to record the behavior patterns of each constituent of the group. Initial observations were made on groups of *Bursatella* comprised of three individuals. Raw data were recorded on an underwater slate in a form that enabled subsequent reconstruction of entire bouts of copulatory interactions (Fig. 5). *Bursatella* copulation was not preceeded by any detectable courtship or 'foreplay' rituals, as have been described for other opisthobranchs (20, 21, 32).

During nine observation periods in which group composition remained stable for at least 1 h, individual specimens were noted to copulate numerous times (mean = 5.4 / hour). This high frequency of copulatory behavior enabled us to ask two questions that have not been directly addressed in previous studies:

1) Do individual specimens specialize in one sexual role? (*Bursatella* is a simultaneous hermaphrodite). Our results to date suggest that they do not (Table 1).
2) Do individuals mate preferentially with certain members of their group? Results to date suggest that they do not (Table 2).
Definitive answers to these two questions will enable us to address a third issue:
3) Does egg-laying by one member of an aggregate bias the choice of sexual role or partner by the other members of the group? Preliminary observations suggest that if one member of an aggregate begins to lay eggs, the other two members are (a) biased toward the male role and that they are (b) biased toward copulating with the egg layer (Fig. 6).

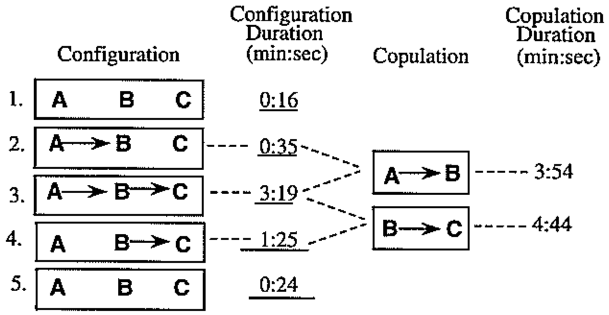

Figure 5. Analysis of *Bursatella* reproductive behavior in the field. A group of three individually identifiable *Bursatella* (denoted *A*, *B*, and *C*) was observed, and the time spent in each configuration was recorded on an underwater slate (first two columns). The animals were observed for over 90 minutes; a period of approximately 6 minutes is shown. *Arrows* indicate copulation (directed toward sperm acceptor). Note that each configuration differs from its predecessor by only a single transition, making it possible to reconstruct the entire sequence of events. During the period shown, two copulations were observed in their entirety. These copulations and their durations are recorded in the last two columns.

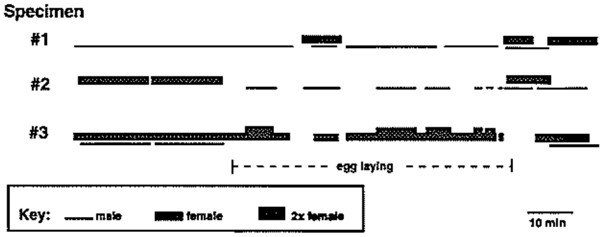

Figure 6. Copulatory interactions within a group of three *Bursatella* specimens in the native environment. The role of each individual is denoted on the time line as denoted in the *Key*. Note that specimen #3 began to lay eggs following an extended period during which it was acting as both a sperm donor and a sperm acceptor. During the period of egg-laying (> 1 hr), specimens #1 and #2 appeared to be biased toward the male behavioral role, and with one exception, mating was directed toward the egg layer (#3). The 2x female role refers to the fact that the intromission of two males can occur simultaneously in an individual female (see Ref. 22).

In summary, the apparent lack of preference regarding sexual roles or partners (Questions 1 and 2) may be considered behaviors that will increase reproductive efficiency and maximize genetic variation in the offspring. The possible adaptive value of biasing individuals toward male behavior and toward copulating with an egg layer

Table 1. Sexual roles of 3 *Bursatella* specimens during a single observation period (90 min) of copulatory activity.

	Copulatory role		
Specimen	Male	Female	Total
A	6	6	12
B	5	10	15
C	9	4	13
Total	20	20	40

- Chi-squared test: P value = 0.16, *df* = 2.
  No significant association between conditions.
- Representive of 3 observation periods in which sufficient data were obtained to permit statistical tests.

(Question 3) may be understood in view of certain characteristics of aplysiid reproductive physiology: (1) these animals are non-self-fertilizing hermaphrodites that store large quantities of exogenous sperm for future use (33) and (2) sperm are transferred between animals in an immature state in which they are not immediately able to fertilize the recipient's oocytes (34). It has been suggested that mating as a female during and shortly after egg deposition will ensure that mature and capacitated sperm will be available to fertilize oocytes produced for subsequent egg-laying events (see 35). In *Aplysia*, it appears that individuals are able to distinguish egg-laying animals from others (36 - 38; 39) and it has been proposed that factors derived from the egg cordon may act as sexual pheromones, attracting conspecifics and inducing mating (20, 35, 37, 38).

Table 2. Mate selection of 3 specimens during a single observation period (80min) of copulatory activity.

	Partner			
Specimen	A	B	C	
A	-	8	9	NS
B	8	-	6	NS
C	9	6	-	NS

- NS: not significant, Binomial tests;
  criterion for significance: $p < 0.05$
- Representative of 3 observation periods in which sufficient data were obtained to permit statistical tests.

Feeding Behavior. During the afternoon, *Bursatella* observed in their natural habitat were rarely grouped in aggregates (22). Rather, they were found as individuals or pairs movng across the substrate (Fig. 1B). Although oral movements were not directly observable, periodic emission of fecal pellets (1 to 3 per minute) were indicative of a high level of digestive activity (see 22). Examination of the digestive system content indicated that the *Bursatella* were, in fact, ingesting the superficial layer of the sandy substratum.

The disposition of locomoting pairs of *Bursatella* was often suggestive of trail-following (Fig. 1B). Limited descriptions of behaviors suggestive of such trail-following have appeared in the literature (40, 41), but questions concerning its stimulus control and function remain unanswered. The *Bursatella* sometimes secrete a mucous trail that is clearly visible, but such trail-following behavior did not appear to require such massive secretion. It is possible that individuals are attracted by a pheromone that is secreted by conspecifics, as has been suggested in other opisthobranchs (39; but see 37, 38). It is difficult to postulate any advantage conferred by this behavior with reference to grazing, but such trail-following may serve to reduce dispersion of animals during the afternoon period of feeding and thereby increase the likelihood of subsequent encounters between potential mates following sunset (see 42).

In summary, our observations of *Bursatella* behavior patterns in the field are indicative of a daily rhythm in which reproductive behaviors dominate nocturnally and feeding-related behaviors predominate throughout the day (cf. 24, 26). It is important to note that, although the transitions between these behaviors appear to be cued or entrained by the setting and rising of the sun, respectively, there is considerable lag time preceding their full expression. Therefore, the *process* of aggregation lasts for several hours following sunset, and aggregates often remain stable for hours following sunrise.

Behavior patterns in captivity. To date, our most systematic analyses of *Bursatella* behavior in captivity have been performed on pairs of animals housed in 20 gallon glass aquaria (22). Paired specimens were moved from a large holding tank to the observation aquaria on the day prior to observation. The observation aquaria were located in a covered outdoor area that received enough sunlight to produce a visible layer of microalgae on their walls. They also contained several pieces of macroalgae (primarily *Chaetomorpha*). Paired animals differed substantially in at least one characteristic (usually color) in order to assure unambiguous identification. Each observation period (2 hours) was divided into 24 sample intervals (5 min each) during which the occurrence of seven operationally defined and easily distinguishable behavior patterns was monitored (Fig. 7). Behaviors were recorded using the one-zero sampling method (43; see 44 and 22 for discussions of the limitations intrinsic to this method of analyzing animal behavior).

In brief, *Bursatella* exhibited an extraordinary level of activity throughout the day (night observations were not

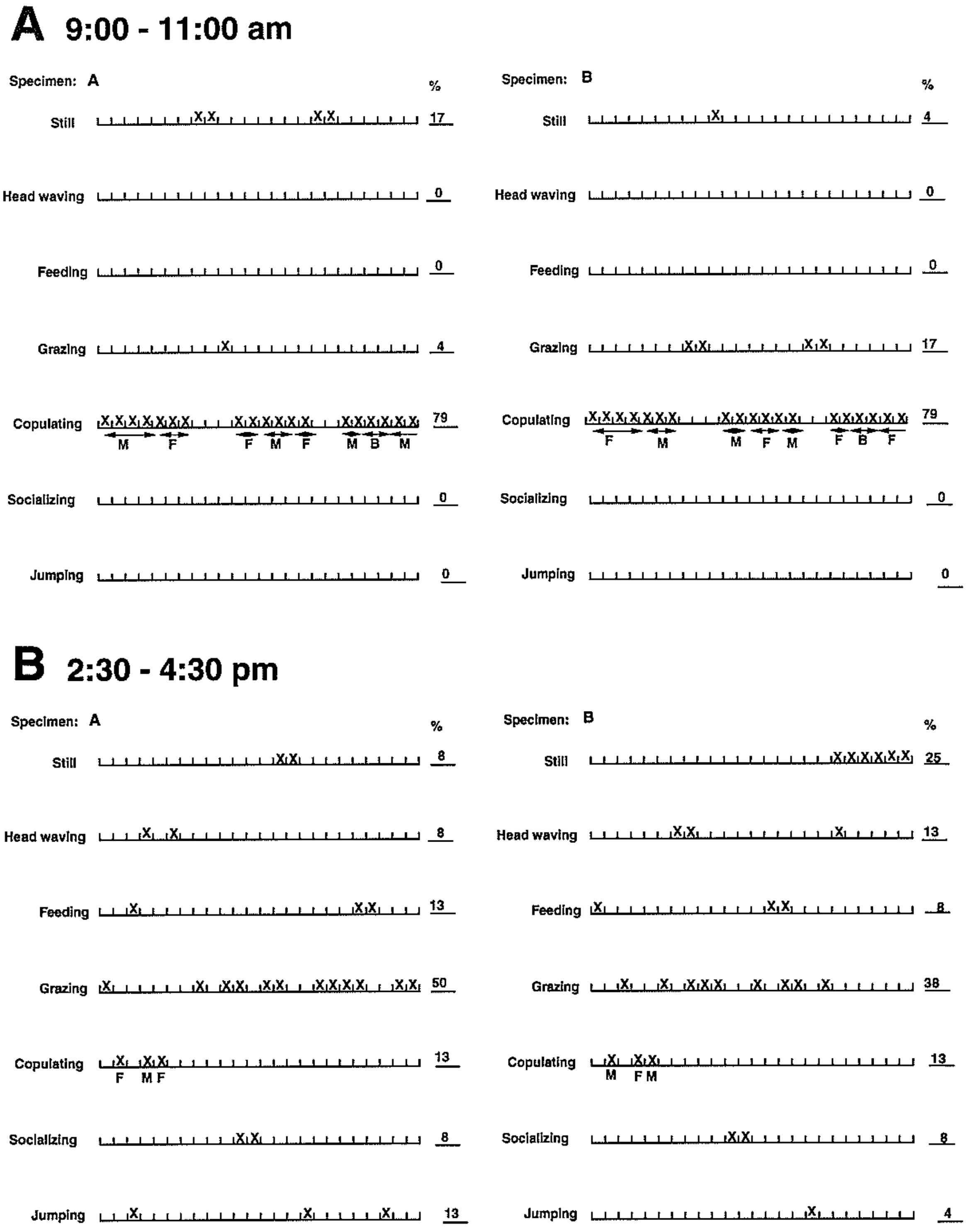

Figure 7. Time budgeting of *Bursatella* under laboratory conditions.. (A) Behaviors of paired animals (*A* and *B*) housed in a 20 gallon aquarium during a representative two hour morning observation period (chosen from over 150 hours; see Ref. 22). Behavior patterns were recorded using one-zero sampling (sample interval: 5 min). Letters beneath the *Copulating* records indicate role; *F*= female, sperm acceptor, *M* = male, sperm donor, *B* = both, simultaneously acting as sperm donor and acceptor. The percentages listed represent the proportion of all samples during which a particular behavior pattern occurred. (B) Time budgeting of two *Bursatella* specimens during an afternoon observation. Note increase in feeding-related behaviors and decrease in copulation, compared to morning observation.

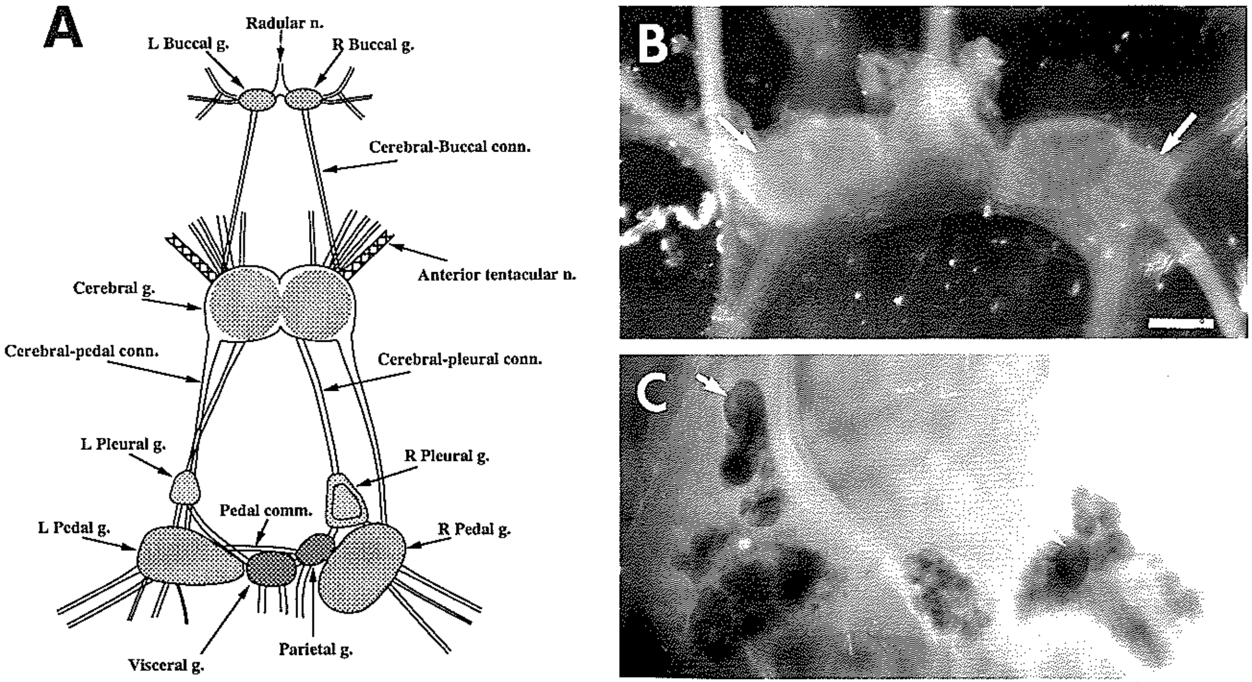

Fig. 8. The major ganglia comprising the central nervous system of *Bursatella leachii*. (A) Schematic drawing of organization of the ganglia (dorsal view). Note asymmetric position (toward the right side) of the abdominal ganglia. Abbreviations: *g.*: ganglion, *n.*: nerve, *R.*: right, *L.*: left, *conn.*: connective, *comm.*: commissure. (B) Caudal surface of paired buccal ganglia. *Arrows* point toward giant lateral peptidergic neurons (see Fig. 9A). (C) Dorsal surface of subesophageal ganglia. *Arrows* indicate giant neurons that are likely to correspond to certain characterized cells (LPl1 and R2) in *Aplysia*. Calibration bar = 400 µm, applies to (B) and (C).

made). The proportion of sample intervals in which specimens exhibited at least one behavior other than the 'still' condition typically ranged from 70 to 80%. Furthermore, individuals generally displayed a range of behavior patterns, with frequent transitions between related behaviors (e.g. copulatory role reversal) or between unrelated behaviors (Fig. 7; note frequent transitions between copulation and grazing).

It was of interest to determine the degree to which the daily rhythm noted in the natural habitat persisted in captive animals. Comparisons between the 'percentage of occurrence' for each behavior were made from morning observations (6 am to 12 n) and afternoon observations (12 n to 6 pm). The only differences reaching statistical significance were a *decrease* in copulation and an *increase* in grazing in the afternoon relative to the morning (22; see also 45). These findings suggest that at least some aspects of the natural daily rhythm persist in *Bursatella* maintained in the laboratory.

Central nervous system: Structure and function. The *Bursatella* central nervous system conforms to the general plan of opisthobranch nervous system organization (see 15, 46, 47). It consists of a pair of buccal ganglia (Fig. 8 A,B; Fig. 9), two fused cerebral (or supraesophageal) ganglia (Fig. 8A), and a subesophageal complex (Fig. 8A, C) comprised of two pleural ganglia, two pedal ganglia, a visceral ganglion, and a parietal ganglion (referred to as the right visceral ganglion by some authors). The major ganglia are euthyneurous (exhibiting little or no crossing of connectives or peripheral nerves) and highly concentrated, features usually associated with higher gastropods (46). The visceral and parietal ganglia exhibit an obvious asymmetry toward the right side of the animal, where they are fused with the right pedal and pleural ganglia (Fig. 8C). The central ganglia of *Bursatella* contain numerous large cell bodies or somata that are located in characteristic positions in all individuals. Some of these cells clearly qualify as "giant" neurons, attaining diameters up to 300 mm in large specimens (Fig. 8B,C). A bilateral pair of such cells, located in the buccal ganglia (Fig. 8B, arrows) are likely to be homologous to giant neurons found in other gastropods (e.g. neurons B1 and B2 of *Aplysia*; see 48 - 51). Consistent with this notion, we have observed immunoreactivity in these cells using antibodies raised against small cardioactive peptide B (SCP_B; Fig. 9A).

Another large neuron, located on the ventral surface of the visceral ganglion is likely to be homologous to neuron R15 of *Aplysia*, an intensively studied autoactive bursting neuron that appears to play a role in reproductive behavior (52-54). In the less centralized *Aplysia* nervous system,

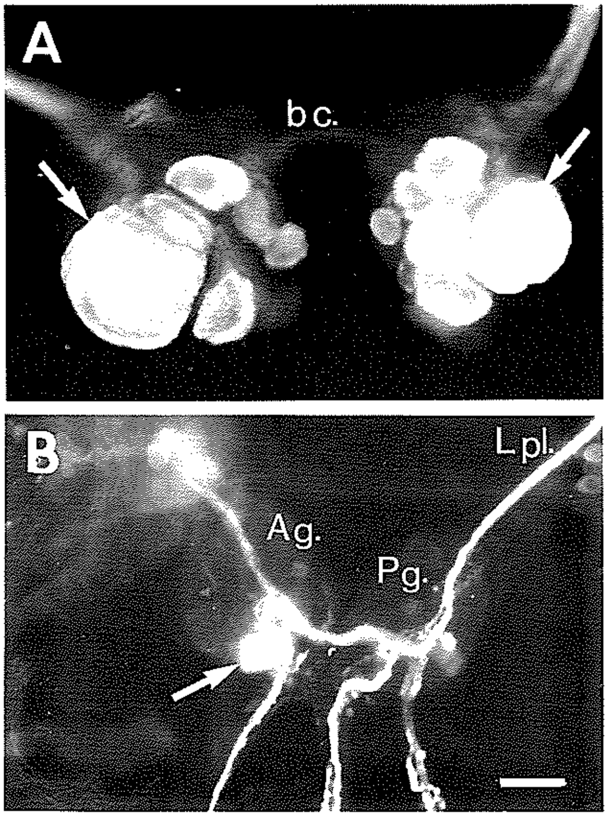

Figure 9. Identifiable peptidergic neurons in the central nervous system of *Bursatella leachii.* **(A)** Paired buccal ganglia stained with antiserum (provided by Dr. K.R. Weiss, Mount Sinai School of Medicine) raised against SCP_B. Several large immunoreactive neurons were located on the caudal surface of the ganglion, including the two giant lateral cells (*arrows*). Abbreviation: *b c.*: buccal commissure. **(B)** A single large cell (*arrow*) in the abdominal ganglion exhibited immunoreactivity to an antiserum (provided by Dr. J. Koester, Columbia University) raised against peptides encoded by the R15 gene of *Aplysia*. The branching pattern of this cell suggests that it is homologous to the *Aplysia* R15. Abbreviations: *A g.*: abdominal ganglion, *P g.*: parietal ganglion, *L pl.*: left pleural ganglion. Calibration bar = 250 μm, applies to **(A)** and **(B)**.

in which the visceral (or abdominal) ganglion is located several centimeters away from the subesophageal complex, electrophysiological experiments indicate that neuron R15 projects a long axon through the pleuroabdominal connective that eventually leaves the CNS via a small nerve of the pedal ganglion (54). Immunohistochemical staining of the *Bursatella* nervous system using an antiserum (Antibody I/II of ref. 55) that reacts with a neuropeptide present in R15 revealed a large neuron in the visceral ganglion that projected a fiber through the parietal ganglion to the left pleural ganglion (Fig. 9B). A large branch of this fiber projected from the left pleural ganglion to the left pedal ganglion and exited the central nervous system via a nerve in the caudal region of the pedal ganglion (not shown). The overall branching pattern of this large R15α2-immunoreactive neuron located in the *Bursatella* visceral ganglion supports its tentative designation as the R15 homolog and is consistent with a conserved role for this neuron in the regulation of some aspect of reproductive behavior (see Discussion).

The grazing behavior exhibited by *Bursatella* places this species within the 'rasper' categorization of Audesirk and Audesirk (56). Raspers are common among the pulmonates, such as the garden slug (*Limax maximus*) and the pond snails (*Lymnaea stagnalis* , *Planorbis corneus*, and *Helisoma trivolvis*)but few opisthobranchs appear to utilize this feeding mode. Certain features of rasping, e.g. its stereotypy, rhythmicity, and dependability tend to facilitate the characterization of underlying CPG circuits (57 - 60). The most intensively studied opisthobranchs, eg. *Aplysia californica*, *Navanax inermis*, *Pleurobranchaea californica*, and *Tritonea diomedea*, are categorized as 'browsers' or 'hunters', behavior patterns that exhibit less stereotypy and dependability than rasping (e.g. 5, 6, 61-63). Although the characterization of CPG circuits underlying such behaviors poses a formidable challenge, recent efforts have resulted in considerable progress, particularly in *Aplysia* (64 - 68).

To graze efficiently, one expects that *Bursatella* must activate and coordinate two distinct behavior patterns, locomotion and biting. Since the microalgae that provides nutrients for the animal are found in a very thin layer on the surface of the sandy substrate, it is essential that the animal be in motion in order to avoid repetitively biting in an area that no longer contains food. Opisthobranch locomotion has been studied at the behavioral and cellular levels in *Aplysia* where it was found to be generated by a central motor program that drives motor neurons located in the pedal ganglion (69). The characterization of this motor program, however, has been hampered by the fact that it does not seem to be spontaneously expressed in the isolated nervous system (Hening et al. elicited motor programs by shocking nerves or applying salt crystals to the tail). In *Bursatella*, however, many pedal neurons exhibit a robust and stereotyped rhythm for hours following isolation of the central ganglia (Fig. 10A). We suggest that subtle differences exist between the organization or regulation of central pattern generator circuits controlling locomotion in grazers versus those of browsers and hunters. Grazing is, in fact, the only feeding mode requiring continuously activated locomotion; a requirement that may be reflected by the reliable and spontaneous expression of this motor program in the isolated nervous system.

Central programs responsible for biting are usually produced by the paired buccal ganglia (Figs. 8B., 9A). These small ganglia are typically considered to be dedicated to behaviors associated with the consummatory phase of feeding, including biting, swallowing, and food rejection (6, 70, 71). Since the isolated buccal ganglia of browsers and hunters rarely produce a robust spontaneous rhythm, coordinated programs in such species are usually evoked by stimulation of certain nerves or 'command' neurons (62, 65, 72, 73). In contrast, the buccal ganglion of the rasping *Bursatella* produces a stereotyped spontaneous rhythm that persists for hours in the isolated ganglion (Fig. 10B). The pattern of impulse activity in the cell shown is representative of the complex rhythm observed in many buccal neurons and from extracellular recordings of buccal nerves. This four-phase rhythm resembles the patterned activity responsible for biting in other molluscs (67, 74; see also 75), but its precise relation to rasping remains to be determined. We suggest that its reliable and spontaneous expression in the isolated ganglion is in some manner reflective of the grazing mode of feeding employed by *Bursatella.*

Future Directions

In his original description of the unique research opportunities available at the Laboratory of Neurobiology (redesignated the Institute of Neurobiology in 1986), Professor Emeritus José del Castillo expressed his vision of establishing a "tropical counterpart of the large marine laboratories of temperate and colder waters, such as those

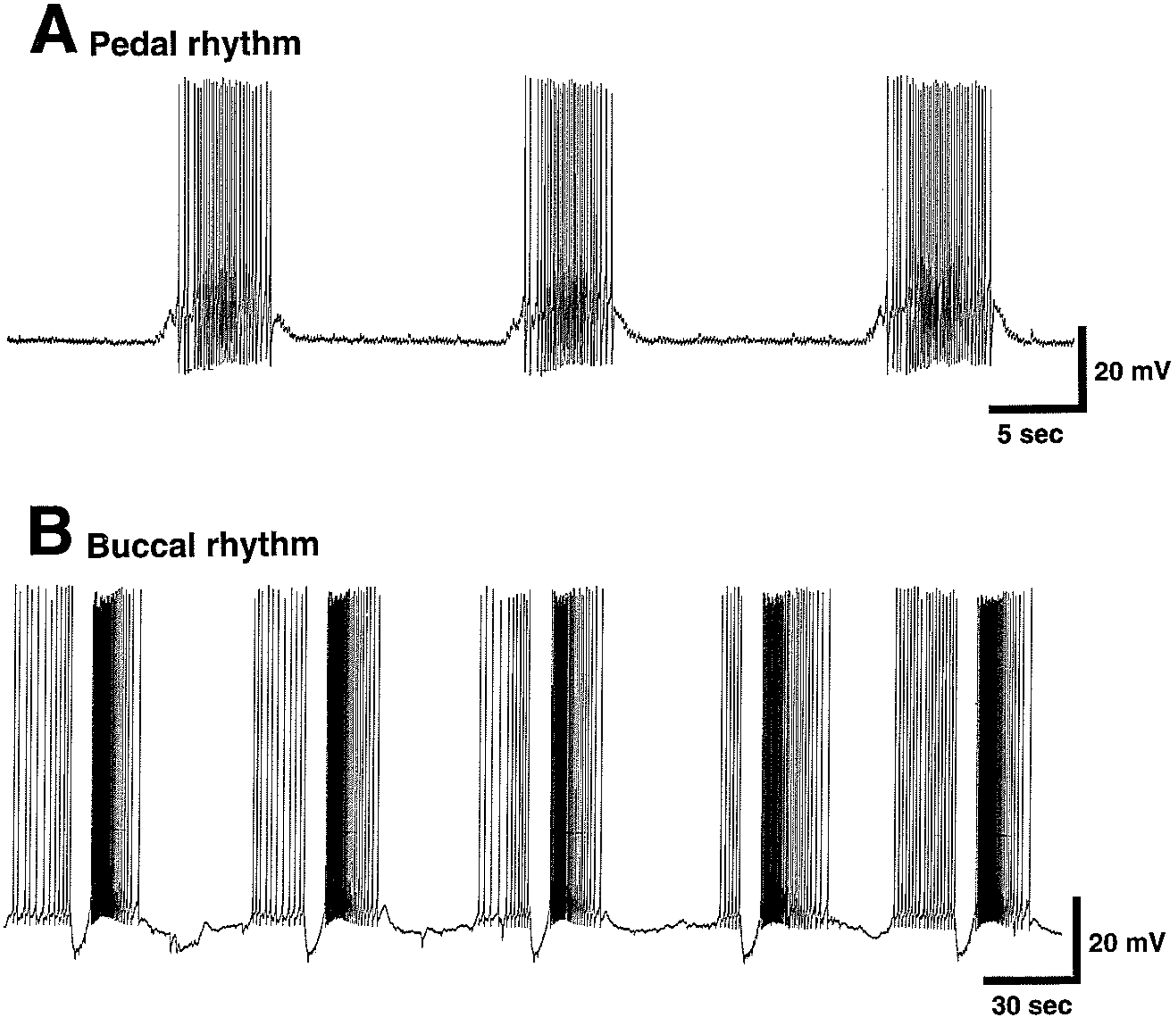

Figure 10. Rhythmic activity in neural centers associated with locomotion and feeding. (A) Intracellular recording from an unidentified neuron in the pedal ganglion. The preparation consisted of the isolated circumesophageal ring. Note simple two phase rhythm, in which bursts of synaptically driven impulses occurred approximately every 20 seconds. (B) Intracellular recording from unidentified neuron in the buccal ganglion. The preparation consisted of the paired buccal and cerebral ganglia. Note complex rhythm (period = approximately 50 sec) in which cell exhibits phases of both low and high frequency impulse activity.

of Naples, Monaco, and Woods Hole" (see Introduction). In a subsequent article that appeared in the *Puerto Rico Health Sciences Journal*, Prof. del Castillo (76) pointed out that : (a) the invertebrates living in tropical waters offer exciting possibilities to experimental biologists since they display *variety and diversity of form even greater than those which inhabit colder seas*, and that (b) in spite of the high interest in tropical marine organisms offer to experimental biologists, *these animals have not been easily available for research, due to the almost complete absence of fully equipped marine biological laboratories on tropical shores.* Since these observations are essentially as true today as when they were written, the tropical species remain a vast untapped resource for the field of Neurobiology in general, and Neuroethology in particular.

Bursatella behavior in the field. Our field studies have established a location and season in which *Bursatella* can be observed in its native habitat. Several features of this habitat favor field observations: 1) the animals live in shallow clear waters, making it possible to conduct observations without SCUBA; 2) the water temperatures permit extended periods of observation; 3) animals spend the entire day and night exposed to direct view.

The overall activity level of *Bursatella* in the field is extraordinary. Their daily rhythm of behavior patterns is consistent with the study of Block and Roberts (45) in which it was found that locomotor activity was predominantly diurnal in the laboratory. Interestingly, in those studies, *Bursatella* exhibited anticipatory locomotor activity (commencing prior to dawn) when placed on 'short day' regime (9 hours light: 15 hours dark). This finding was interpreted as indicating that an endogenous circadian pacemaker influences the locomotor rhythm. The delay or lag time that we note prior to the onset of locomotion following dawn during the 'long' summer days (approximately 14 hours light: 10 hours dark in Puerto Rico; 18° latitude) supports the presence of such an endogenous circadian influence.

A circadian rhythm of afferent electrical activity is present in the eye of *Bursatella* (45). This pacemaker does not appear to control the daily locomotor rhythm (see above), since specimens in which eyes were lesioned continued to exhibit diurnal locomotion. Block and Roberts speculated that the ocular pacemaker could play a role in other rhythmic behaviors. We have observed a daily rhythm in reproduction-related activities (occurring nocturnally and in the early morning), raising the possibility that this class of behaviors is regulated by an endogenous circadian rhythm, e.g. the ocular pacemaker. Interestingly, the peak activity of the *Bursatella* eye occurs about 3 hours prior to dawn, a time at which reproductive behaviors predominate and locomotion is minimal.

Reproductive behavior. Like many gastropod molluscs, *Bursatella* is a simultaneous hermaphrodite in which the capabilities for both male (mating and copulation) and female (mating, copulation, and oviposition) behaviors are present in each mature individual. Due to difficulties encountered in eliciting mating behaviors in the laboratory, this class of molluscan behavior has historically received comparatively little attention from a neuroethological perspective (56). In *Lymnaea stagnalis*, a species in which copulation has been intensively studied, pairs of previously isolated specimens engage in a single bout of mating behavior with an average latency of nearly three hours and a duration of approximately 36 min (77). Such characteristics render the identification of neuronal correlates difficult. The frequency and reliability with which *Bursatella* engage in copulatory behavior in the field and in the laboratory (see 22) should facilitate characterization of its neuronal control.

It is likely that the neural circuits involved in the control of *Bursatella* reproductive behavior are distributed throughout the nervous system and that several neuropeptides are involved in the regulation of each component of this class of complex motivated behaviors. Specific neurons involved in the control of male reproductive behavior have been identified in the pulmonates (77, 78) and *Helix aspersa* (79), and in the opisthobranch *Aplysia* (80). These neurons express several classes of modulatory neuropeptides that may be involved in the regulation male reproductive organs (81, 82). The nervous system of *Bursatella* contains numerous asymmetries (see Fig 8A) that are usually manifested as neurons which are present solely on the right side of the animal. Such asymmetries are usually associated with a reproductive function, since many of the organs involved in reproduction (e.g.the penis and the external genital groove) are located on the right side. Using histological methods, we have identified specific asymmetric cell groups that are immunoreactive to antisera raised against the neuropeptide myomodulin (Giardino and Miller, in preparation). Members of the myomodulin family of neuropeptides are thought to be involved in the regulation of muscles controlling the penis in *Lymnaea stagnalis* (83, 84).

A second class of neuropeptides, encoded by the R15 gene (85), is thought to be involved in the integration of behaviors related to egg-laying in *Aplysia* (86-88). Although the neuron R15 has been intensively studied due to the bursting properties that it exhibits *in vitro* (reviewed in 52), recent evidence indicates that this cell is normally silent *in vivo* (54). It has been suggested that

R15 bursting may occur specifically during egg-laying, or possibly during copulation (54), but this proposal has proven difficult to test in *Aplysia californica*, an animal that does not readily engage in reproductive behaviors in captivity. The presence of numerous large axons from the putative R15 homologue of *Bursatella* in certain peripheral nerves and connectives (Fig. 9B), coupled with this species' proclivity toward reproductive behavior, may facilitate correlating the activity of this cell to specific actions using chronic recording techniques.

Feeding behavior. At a recent symposium entitled "Neurons, Networks, and Motor Behavior" (Tucson AZ, November, 1995) it was noted that the goals of understanding motor function have shifted from determining merely how animals generate behavior to how they produce flexible outputs that respond to environmental variability (89). We propose that the grazing behavior exhibited by *Bursatella*, in which two distinct central pattern generators (biting and locomotion) must be coactive and coordinated, presents exceptional opportunities for addressing such flexibility of motor circuits. For example, in places where algae are plentiful, one may predict that locomotion may be downregulated since it is not necessary, and energetically wasteful, to forage a large area. Conversely, in places where algal growth is sparse, it may be expected that both rhythms will be up-modulated in order to ensure ingestion of sufficient nutrients. Finally, there are likely to be situations in which these programs will be dissociated. For example, locomotion appears to be suppressed during reproductive behaviors, while individuals copulating in the female role often continue to feed if certain types of macroalgae (e.g. *Padina sanctus*) are within reach.

The persistence of spontaneous rhythmic activity in isolated neural centers associated with locomotion should facilitate analyses of forms of motor circuit regulation that are not easily studied in preparations that produce less robust motor patterns. Neural circuits responsible for molluscan swimming have been characterized (90 - 93) but few studies have addressed the CPG circuits responsible for other types of locomotion (but see 69, 94). Motor neurons controlling the type of ciliary locomotion exhibited by *Bursatella* have been described in *Tritonia diomedea* (95, 96). However, the coordination of such motor neurons and the properties of their CPG circuits remain to be explored.

Studies that have directly addressed modulation of opisthobranch feeding have emphasized regulation at the neuromuscular junction (97, 98), sensory integration (99 - 103), and the role of 'command' or modulatory elements that induce or activate responses (64 -66). While each of these levels of integration are likely to be closely associated with CPG elements (see 104), such relationships have proven difficult to characterize in species where isolated ganglia do not sustain spontaneous motor patterns. The stability of the rhythms found in isolated *Bursatella* ganglia should aid in the identification of neurons that modulate the period of the feeding CPG or that modify its phase relationships. Finally, the identification of neurons that suppress or terminate rhythm generation should be facilitated in ganglia that produce such vigorous spontaneous activity (see 105).

Conclusion

It is clear that the rhythms governing the life of the ragged sea hare encompass an enormous range of frequencies, extending from the annual rhythm of the animal's availability (period = 1 year) to the circadian rhythmicity of its major behavior patterns (period = 24 hours), and finally to the motor patterns involved in its feeding and locomotion (periods = tens of seconds). Although many of the studies reviewed in this article are preliminary in nature, they serve to demonstrate that a molluscan species indigenous to the tropical waters of Puerto Rico fulfills numerous criteria (see Introduction) that favor a cellular approach to the production and regulation of complex natural behavior patterns. These observations are intended to stimulate and guide further investigation aimed toward elucidating general principles of nervous system organization that are applicable to all animals, including humans.

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