

# Field Behavior of the Nudibranch Mollusc *Tritonia diomedea*

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**Abstract.** The nudibranch mollusc *Tritonia diomedea* has been a useful model system for studies of how the brain controls behavior. However, no broad study of *T. diomedea* field behavior exists—an important deficit since laboratory behaviors may differ from what occurs in nature. Here we report analysis of time-lapse video of the slugs in their natural habitat to describe behaviors and their relationships to sensory cues. We found that movements relative to conspecifics, prey, and predators correlated with direction of water flow. These observations lead to three new navigational hypotheses: regardless of the actual heading to the target, *T. diomedea* crawls (1) upstream toward potential mates, (2) upstream toward food, and (3) downstream away from predators. We also describe both the behavior and its sensory context for feeding, escape swims, mating, and egg-laying, among other behaviors. Field behaviors were similar to published descriptions of laboratory behavior. However, the field observations add contextual detail, including preceding and subsequent behaviors and interactions with suites of habitat features not present in the laboratory. For example, the escape swim, previously studied as an isolated behavior in response to a single stimulus, appears to be affected by multiple sensory modalities and coordinated with several other behaviors. Our work will provide a basis for future neuroethological experimentation and also is the first step in the study of navigation in *T. diomedea*.

## Introduction

The goal of neuroethology to link behavior to the underlying neural control requires an understanding of relevant

environmental cues and of behavioral responses to those cues. Information on behaviors and the sensory cues that influence them guides the study of sensory systems, central processing, and motor systems, the elements that constitute the neural control of behavior. The nudibranch mollusc *Tritonia diomedea* Bergh has, over the last 40 years, become a neuroethological model system. In *T. diomedea*, a relatively small number of large, re-identifiable neurons organized in a relatively simple nervous system control behaviors that are robust under the conditions of neurophysiological experimentation. These features have allowed discovery of many aspects of how the nervous system controls behaviors in the laboratory. Work on *T. diomedea* has focused on locomotion (*e.g.*, Audesirk, 1975; Willows *et al.*, 1997; Popescu and Frost, 2002; Wang *et al.*, 2003, 2004; Cain *et al.*, 2005), feeding (*e.g.*, Longley, 1976; Willows, 1978; Audesirk and Audesirk, 1979; Beck, 2000), and escape responses from predators (*e.g.*, Willows and Hoyle, 1969; Getting, 1981; Katz *et al.*, 1994), as well as various sensory systems (*e.g.*, Lohmann *et al.*, 1991; Murray *et al.*, 1992; Wang *et al.*, 2003). However, only two focused experimental studies have been conducted on behaviors in the field (Willows, 1999; Murray, unpubl. data). There is no comprehensive description of behavior in this species. Here, we attempt to describe the behaviors of *T. diomedea* in its natural habitat, as well as the sensory context of those behaviors.

*T. diomedea* adults live, feed, mate, and spawn in the soft coral beds of the northeast Pacific (Thompson, 1971; Murray, unpubl. data). They prey on the pennatulaceans *Ptilosarcus gurneyi*, *Stylatula elongata*, *Virgularia* sp., and *Acanthoptilum* sp. (Thompson, 1971; Birkeland, 1974), and are themselves prey to the sunflower star *Pycnopodia helianthoides* and other sea stars (Mauzey *et al.*, 1968; Birkeland, 1974). Therefore, the sensory systems and behaviors of the adult slugs are presumably adapted to interactions

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with these organisms and the physical environment found in this marine community.

What sensory modalities does *T. diomedea* possess, and therefore what possible cues should be considered in the natural habitat? Mechanosensation of water flows (Field and Macmillan, 1973; Murray *et al.*, 1992; Murray and Willows, 1996; Murray, unpubl. data), chemosensation of food odor (Willows, 1978), and magnetoreception (Lohmann and Willows, 1987; Lohmann *et al.*, 1991; Popescu and Willows, 1999; Wang *et al.*, 2003, 2004) have all been studied in the laboratory. In addition, photoreception (light levels only; Chase, 1974) and balance information from statocysts (see Harrigan *et al.*, 1986) may also be important. Thus, water flow, odors, touch, magnetic fields, body orientation, and light levels are the major candidate cues for control of behavior in this species.

The primary goal of this paper is to describe the behaviors of *T. diomedea* and candidate sensory cues *in situ*. Our goal is not to construct a detailed ethogram (Tinbergen, 1963; Leonard and Lukowiak, 1984), but rather to demonstrate relationships between behaviors and sensory cues in the natural habitat. We use time-lapse underwater video of the slugs in the field to observe behaviors in their natural context. We then seek to answer the following questions for *T. diomedea*: What are the primary behaviors and associated sensory cues in the natural habitat? Do natural behaviors differ from behaviors observed in past experimental conditions? What sensory cues should be considered when designing further behavioral and neuroethological experimentation?

In particular, we focus on navigation. Recent work on *T. diomedea* has targeted both sensory systems (Murray *et al.*, 1992; Murray and Willows, 1996; Wang *et al.*, 2004) and locomotory control (Beck *et al.*, 2000; Popescu and Frost, 2002; Wang *et al.*, 2003; Redondo and Murray, 2005). Progress combining the two into a study of navigation would be advanced by a description of navigation in the native habitat. Accordingly, our goal is to record *T. diomedea* movement patterns relative to environmental guidance cues.

We report several previously unobserved behaviors and document and extend observations on behaviors known from the laboratory. Descriptions of the natural sensory cues associated with those behaviors provide a basis for future neuroethological work on *T. diomedea*. In addition, we report evidence for navigation relative to conspecifics, prey, and predators that relies on direction of water flow, and we generate several hypotheses that can be tested by quantitative analyses of navigational cues and behaviors (Wyeth *et al.*, 2006).

## Materials and Methods

We used two methods to observe behaviors of *Tritonia diomedea* and potential cues in its natural habitat: time-lapse video ("behavior videos") and observation by scuba divers ("diver observation").

### Field sites

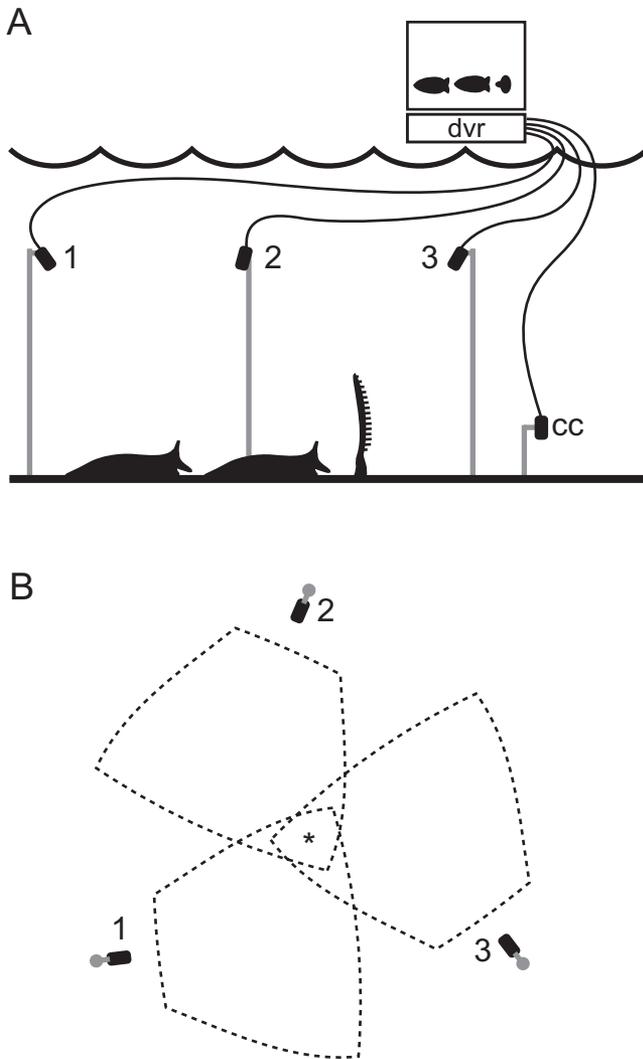
We collected data during five field trips (29 working days) to two sites. MacIntosh Rocks (49°12.60'N, 125°57.45'W), North of Vargas Island, British Columbia, Canada, was visited in October 2000, October 2001, and July 2003. Dash Point (47°19.28'N, 122°25.22'W), in southern Puget Sound, Washington, USA, was visited in April 2001 and October 2002. Both sites have perennially dense beds of the sea pen *Ptilosarcus gurneyi* and, usually, high populations of *T. diomedea* (pers. obs.). No *T. diomedea* individuals were found on the third trip to MacIntosh Rocks, despite an apparently otherwise unchanged *P. gurneyi* bed. Consequently, we imported 48 slugs from Yellow Bank (48°14.07'N, 125°55.16'W, 3.2 km distant) which, despite its high density of *T. diomedea*, was too exposed to wind to be suitable for our behavior camera system.

### Behavior videos

**Behavior camera setup.** At both field sites, our research vessel was anchored in 6 m of water above the *P. gurneyi* bed. We used three submersible black-and-white video cameras (model CVC-320WP, Speco Technologies, Amityville, NY), each attached to a 3-m pole driven perpendicularly into the soft sediment (Fig. 1A). The poles formed a 3-m equilateral triangle (Fig. 1B). At the center of the triangle, we placed a small marker on the substratum to indicate the direction of magnetic north. Each camera's height was determined by seawater clarity, and its field of view was angled down and to the right of the north marker. Thus, each camera covered a largely unique field of view of  $4.7 \pm 1.6 \text{ m}^2$  (mean  $\pm$  st. dev.), except for a slight overlap at the center marker.

**Video recording.** Power and video from all cameras were cabled to the research vessel. In October 2000, video from the three cameras was recorded onto a single VCR tape using a video sequencer (cat no. 49-2511, RadioShack, Fort Worth, TX) and a time-lapse VCR (model no. STR-24N, Samsung, Ridgefield Park, NJ). The video was then digitized to computer hard disk using a VideoBus II (model no. F5U208, Belkin Corp, Compton, CA). For all subsequent field trips, video was written directly to hard disk at 2.5 frames per second at a resolution of  $320 \times 240$  pixels using a PC computer digital video recording system (Novex 2000, Novex (Canada) Ltd., North York, ON, Canada). This frame rate and resolution ensured that no behaviors were distorted by either temporal aliasing or excessive pixilation. Power was provided by a portable generator (model no. EU1000i, Honda, Alpharetta, GA) buffered by an uninterruptible power source (model no. F6C650 USB, Belkin Corp, Compton, CA).

**Current measurements.** We used three methods to determine currents around individuals of *T. diomedea*. First,



**Figure 1.** Schematic of behavior cameras and video recording system (not to scale). (A) Side view, showing the three behavior cameras (1, 2, and 3) recording two individuals of *Tritonia diomedea* and one of *Ptilosarcus gurneyi*, and a current camera (cc) used to record flow patterns near the substratum. Cameras were attached to poles (gray lines) and cabled to the digital video recording (dvr) system on board the research vessel, anchored above (not shown). (B) Top view showing the behavior camera arrangement and the resultant fields of view of each camera (dashed outlines) on the substratum below. The cameras were oriented to just overlap at the center (\*) of the equilateral triangle formed by the poles (gray dots). The combination of a fisheye lens and a  $\sim 35^\circ$  angle from the vertical created the “bulged trapezoidal” field of view for each camera.

natural consequences of flow in the videos, including the motion of drift algae and the orientation and posture of *P. gurneyi* (Best, 1985), were used to estimate relative flow speeds (high, moderate, and low) and absolute flow direction. Second, we deployed fluorescein sources that created dye plumes, allowing us to visualize flow in the behavior cameras. Fluorescein sources consisted of a 1:4:2 (by volume) mixture of fluorescein powder, plaster of Paris powder, and fresh water, hardened overnight. Finally, we sup-

plemented these with flow direction data collected by “current camera” videos of suspended particle movements from two cameras mounted near the substratum (Wyeth and Willows, unpubl. data).

**Daily method.** We deployed the cameras by scuba and oriented the recorded videos by holding a compass under each camera. We then collected nearby individuals of *T. diomedea* and placed them under the behavior cameras. A maximum of 10 slugs (except on 2 days) kept densities comparable to local natural densities ( $1\text{--}3\text{ m}^{-2}$ ; pers. obs.). We video recorded undisturbed slugs when possible ( $n = 44$ ,  $\sim 25\%$  of observed slugs), and found little effect of collection. Collected slugs crawled, fed, and mated similarly to undisturbed slugs. After the slugs had settled and all fluorescein sources had been deployed, the divers surfaced to eliminate observer interference. Thereafter, slugs and fluorescein sources were replenished as needed (about every 3–4 h). On 6 days, we introduced one or two individuals of the predatory sea star *Pycnopodia helianthoides*, either free to crawl or caged under plastic mesh stapled into the sediment. We caged the sea stars because handling them caused them to move quickly beyond the camera fields of view, preventing elicitation and interpretation of slug responses. At the end of each day, we compass-oriented the video again. On most days, we also provided scale by extending a rope marked at 50-cm intervals across the bottom at various orientations under the cameras. Recording was then stopped, and all underwater gear was dismantled and stowed on board the vessel.

### Analysis

**Behavior videos.** All videos were reviewed to track *T. diomedea* individuals within and between behavior cameras. For each slug, we recorded a behavior sequence based on the timing of the following behaviors: *Stationary*: Inactivity. *Crawling*: Forward locomotion without large changes in direction. *Turning*: Forward locomotion with a distinct bending of the body and a consequent change in direction. *Mating*: Contact between two slugs, followed by paired circling, cessation of movement with the right sides of the slugs in close contact, or both. *Egg-laying*: Behaviors occurring while an egg string was laid. *Feeding*: Contact with a sea pen, *P. gurneyi*, which jerks quickly towards the slug and then retracts into its sediment burrow. *Swimming*: Series of dorsal and ventral flexions. *Other*: Activity not included in any of the above.

Behavior sequences were used to determine behavior durations and frequencies. We also noted flow directions; locations of predators, prey, and conspecifics; and any other potential sensory cues. Behavior videos from Dash Point were further analyzed by measuring slug positions, headings, and behaviors every 30 s in all cameras (see Wyeth *et al.*, 2006, for details). These data were used to calculate

behavior frequencies, durations, crawling speeds, and turn rates.

**Calibration.** Each behavior camera video from each day was calibrated for both geomagnetic orientation and distance. For orientation, slug and flow headings were calibrated against the compass image recorded in the same video. For distances, pixel distances for known 50-cm seg-

ments in the images of the scale rope were averaged, and the ratio of mean pixel distance to actual distance was used to scale all distances in the same video.

**Figures from behavior videos.** We used two methods to improve the clarity and depiction of motion in figures based on video sequences. First, we used averages of 3 or 5 frames to sharpen features on the substratum. Second, we enhanced motion contrast by subtracting the mean image of a video sequence from each individual frame (Fig. 2). By varying the mean image interval and sequence frame rate, motion of different speeds was enhanced. We used "motion trails," projections of the mean subtracted video frame stacks, to show the movement of *T. diomedea*, *P. gurneyi*, fluorescein dye plumes, and other moving features as lighter paths on a dark background. Video versions of some figures are available online at <http://www.biolbull.org/supplemental/>.

#### Digital video and photography

For more detailed observation of specific behaviors, scuba divers recorded digital video and still photographs using a Sony PC100 camera (New York, NY) in an underwater housing (Mako, Light & Motion, Monterey, CA).

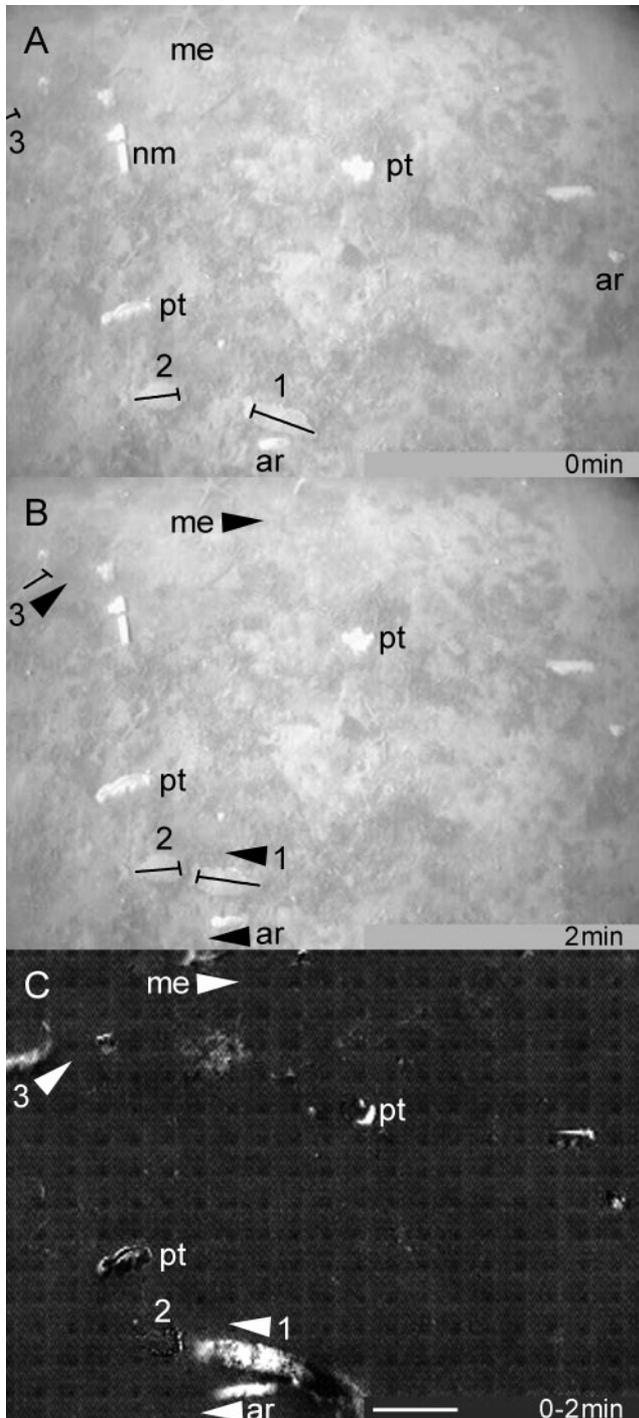
#### Software

Video data were reviewed in Premiere 6.0 (Adobe Systems Inc., San Jose, CA). All analyses were performed in Excel 11.6 (Microsoft, Redmond, WA) and Matlab 6.5 and 7.0 (The Mathworks Inc., Natick, MA).

## Results

#### Summary of behavior data

We collected video data on 29 days; observations included all tidal states: 21 floods (incoming), 27 slacks (changing), and 20 ebbs (outgoing). The total duration of all behavior videos was 510 h (170 h in each camera). We observed 273 slugs in the videos; however, we estimate that



**Figure 2.** Behavior camera recordings show active and inactive *Tridionia diomedea* individuals as well as cohabitants of *Ptilosarcus gurneyi* beds. (A and B) Enhanced single frames, 2 min apart. T-bars are drawn to connect the two rhinophores and tail of each *T. diomedea*; arrowheads indicate direction of motion over the interval between frames A and B. One *T. diomedea* (1) can be seen crawling towards a second, stationary *T. diomedea* (2). A third *T. diomedea* (3) crawls into the field of view between the frames. A total of six *P. gurneyi* (pt) individuals are visible (only two indicated). An *Armina californica* (ar) is visible, crawling roughly parallel to *T. diomedea* 1, and a single sea star, *Mediaster aequalis* (me), is also crawling slowly across the top of the frame. (C) Motion trails over the same 2 min reveal both of the crawling *T. diomedea* (1 and 3) but not the stationary *T. diomedea* (2). In addition, the oscillatory motion of *P. gurneyi* in currents and the crawling of both *A. californica* and *M. aequalis* are all enhanced. North marker (nm). Scale bar: 25 cm.

Table 1

Duration of major activities performed by *Tritonia diomedea* in the behavior videos from Dash Point

Behavior	n	Duration				Total (h)	% of grand total
		Mean (h:min)	St. Dev. (h:min)	Min (h:min)	Max (h:min)		
Stationary	97	0:52	1:00	<0:01	6:14	84.6	45.3
Mating	45	1:07	0:36	0:07	2:13	50.6	27.1
Crawling	200	0:08	0:05	<0:01	0:24	27.6	14.8
Egg-laying	3	5:09	0:28	4:38	5:34	15.5	8.3
Turning	111	0:04	0:04	<0:01	0:32	8.0	4.3
Feeding	13	0:02	0:02	<0:01	0:07	0.5	<1.0
Escape Swimming	2	<0:01	<0:01	<0:01	0:01	<0.01	<1.0

The behavior of each visible slug was coded every 30 s during 55 h of observation over 11 days. The total duration of all coded behaviors for all slugs was 186.8 h.

about 10% of the animals left the field of view and then returned within a single day and about 25% were video recorded on a second day, suggesting that about 180 different animals were observed.

Behavior videos showed *Tritonia diomedea* individuals behaving in their natural habitat. Individual slugs were continuously visible, both when moving and when stationary. We observed many crawling behaviors, including navigation towards mates and prey, as well as predator avoidance. We also observed multiple instances of feeding, mating, egg-laying, and several other specialized behaviors. Other inhabitants of the *Ptilosarcus gurneyi* beds were also visible: *P. gurneyi* (when extended above the sediment), nudibranchs (*Armina californica* and *Tritonia festiva*), a common large cancrid crab (*Cancer magister*), and several sea star species (e.g., *Pycnopodia helianthoides*, *Mediaster aequalis*, and *Solaster stimpsoni*).

Slugs at Dash Point spent about half of daylight hours active (Table 1). *T. diomedea* individuals were visible for  $2.0 \pm 1.9$  h (mean  $\pm$  st. dev.) when tracked across cameras, and  $1.6 \pm 1.2$  h in any one camera. The mean field of view for all three cameras combined on each day was  $14.0 \pm 4.9$  m<sup>2</sup>, which corresponds to a diagonal bottom-left to top-right distance for each camera of  $3.1 \pm 0.6$  m (max = 4.4 m). The mean crawling rate for animals at Dash Point was 10 cm/min (Table 2). Thus, a continuously crawling slug

would spend at most 45 min in the field of view of any one camera. However, the slugs rarely crawled through a field of view without some other behavior being observed. They interspersed relatively short periods of crawling and turns with longer matings or stationary inactivity. Feeding was relatively infrequent (they fed 1/3 as often as they mated) and occupied only a small proportion of their time (Table 1). Egg-laying was even less frequent, though it was the longest recorded behavior, lasting 5 h on average. Swim behaviors to escape predators were rare ( $n = 2$ , at Dash Point) and short in duration.

Behaviors observed in the videos could often be associated with the environment also visible in the videos. Interactions between the slugs and environmental features could then be used to explain both the control and consequences of behaviors.

#### Interactions with the substratum

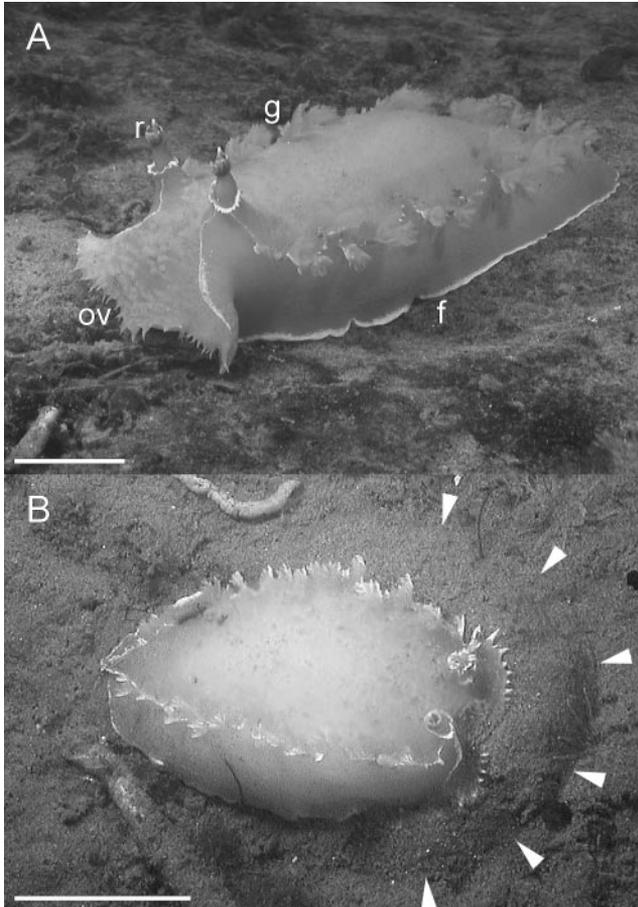
**Crawling and turning.** Active slugs spent much of their time crawling across the soft sediment of *P. gurneyi* beds (Fig. 3A; Table 1). When animals were crawling, their locomotion was always directed forward, never backward. Crawling speeds varied widely (Table 2) but not consistently relative to either preceding or subsequent behaviors. For example, crawling speeds were not consistently higher prior

Table 2

Crawling and turning speeds for *Tritonia diomedea* locomotion in the behavior videos from Dash Point

Behavior	Parameter	Unit	n	Mean	St. Dev.	Min.	Max.
Crawling	Speed	cm min <sup>-1</sup>	127	10.2	3.0	4.0	20.7
	Angular Speed	° min <sup>-1</sup>	199	5.1	5.8	0.0	43.6
Turning	Speed	cm min <sup>-1</sup>	74	8.2	3.6	2.4	17.1
	Angular Speed	° min <sup>-1</sup>	110	27.3	20.9	0.24	148.8

Both behaviors involved forward locomotion. Turns, due to bending the body, had a much higher mean angular velocity than crawling. Speed calculations were limited to 6 days with adequate calibration data.



**Figure 3.** Crawling and stationary *Tritonia diomedea*. (A) A slug crawls over the substratum using cilia on the under surface of its foot (f). The rhinophores (r) and gills (g) are extended, and the oral veil (ov) is spread out with the tips periodically brushing the substratum. (B) A stationary slug, with rhinophores, gills, and oral veil only partially extended. This slug created a small berm by slowly crawling forward and plowing slightly into the sediment (berm edge marked by arrowheads). Scale bars: 5 cm.

to mating. In a crawling animal, the oral veil was normally extended with the tips repeatedly brushing the sediment, often accompanied by slight left and right head movements. This “sniffing” behavior was particularly obvious when approaches toward mates or prey were viewed in time-lapse mode.

Turns seen in the behavior videos were achieved by bending the body; turning without bending the body was also observed by divers. Turning rates varied widely (Table 2). Sharp turns often shortly preceded mating events: 15 animals turned before mating compared to just 6 after mating.

**Berming.** Divers observed slugs actively modifying the substratum during “berming” behaviors (Fig. 3B). As the animal crawled slowly forward, its oral veil bulldozed a berm

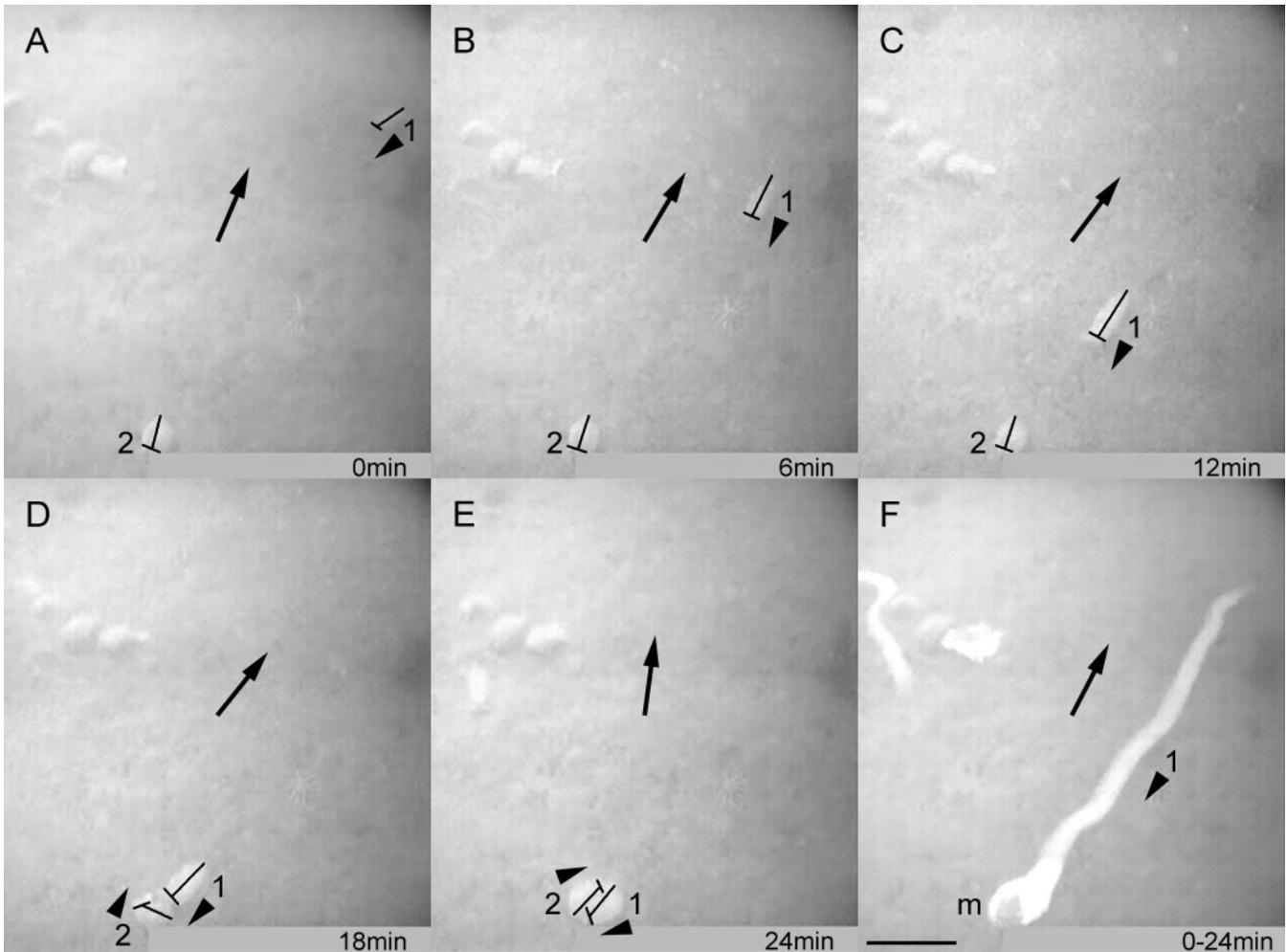
of sand in front of it. Typically the berm was 1 cm higher than the surrounding sediment. Some animals were observed with their dorsum flush with the sediment surface, having moved forward about a body length, raising the berm several centimeters and spilling it along their sides. Although berming was not detectable in the behavior videos, it likely occurred as some small proportion of the slug’s stationary time (Table 1).

#### *Interactions with currents*

Currents in *P. gurneyi* beds were highly variable. Direction and speed varied erratically over the course of minutes and hours, were not consistent across days, and did not appear to strongly correlate with tidal cycle state (Wyeth *et al.*, 2006). Yet slugs’ movements often correlated with both current direction and speed.

**Low and moderate currents: upstream navigation.** Slugs interacted both actively and passively with water flows, depending on current velocity. At slower speeds, the animals appeared to use currents to guide their crawling. They were frequently observed crawling directly upstream (positive rheotaxis). In particular, this seemed to occur when a slug approached another prior to mating (Fig. 4). In 24 of 25 cases with adequately recorded approach behaviors, mating was initiated by one slug crawling toward another and touching its tail or sides (the one exception was a head-to-head initiation). Eleven of these approaches could be qualitatively designated as upstream crawling. Only three counterexamples were observed, in which slug movement did not oppose flow. The remaining 10 approaches occurred with either ambiguous current observations ( $n = 5$ ) or after divers placed slugs close together ( $n = 5$ ). Furthermore, 6 of the 11 instances of positive rheotaxis prior to mating were observed with either a current change or slug movement that led to a new spatial arrangement, with one slug downstream of another slug (Fig. 5). The downstream slug turned as necessary to crawl upstream until it encountered the upstream slug.

**High current: turns, slides, dislodgement, and righting behavior.** At higher current velocities slugs risked being dislodged by the currents. We observed passive slides, when the foot remained in contact with the substratum ( $n = 9$ ), and dislodgement, when animals were swept off the bottom by either bulk flow or oscillating wave motion ( $n = 20$ ). Eleven (55%) of the dislodged animals were transported downstream out of the behavior camera’s field of view. As current speeds increased, the slugs tended to stop crawling and remain stationary. A few examples of slugs turning to face upstream while stationary were observed in higher flows ( $n = 6$ ). Slugs were observed to respond to both brief



**Figure 4.** *Tritonia diomedea* crawls upstream prior to mating. (A–E) Enhanced single frames every 6 min over a 24-min period showing the progress of one slug (1) towards a second, stationary slug (2). Shown are the direction of slug crawling (arrowheads) and the mean current headings (arrows) for the 6-min interval spanning each frame. T-bars are drawn to connect the two rhinophores and tail of each slug. When first visible, slug 1 is  $\sim 1.5$  m away from slug 2, and crawling slightly to the right of upstream (A). Over the next 18 min (B–D), slug 1 crawls almost exactly upstream, until it touches the tail of slug 2, initiating mating. In response, slug 2 turns right (D), and the pair begin the stereotypical clockwise circling behavior of the alignment stage of mating (E). (F) Maximum-projection composite of the motion trail and mean image for the entire 24 min. Slug 1's track (1, arrowhead) is almost exactly anti-parallel to the mean flow direction for the same 24-min interval (arrow). Also visible are the motion artifact created by the initiation turn and alignment stages of mating (m), motion trails of two other moving slugs in the upper left, and a further two stationary slugs also in the upper left. Scale bar: 25 cm.

current “gusts” and some passive slides by flattening the body and spreading out laterally, beyond the typical width during crawling.

After a slug was swept off the bottom, the distance it traveled varied from a few centimeters to several meters or more, though we have no direct observation beyond the behavior cameras' field of view. Slugs transported in the currents tumbled downstream, after which they needed to regain a foothold on the substratum. If the animal came to rest on its ventral surface ( $n = 5$ ), then the foot was spread out laterally, similar to the current gust response. If the animal settled on its dorsum or lateral surfaces ( $n = 4$ ), then

righting behavior was observed. The body was twisted along the anterior-posterior axis, rotating the anterior by about 180 degrees relative to the posterior. With the anterior portion of the foot in contact with the substratum, forward crawling began. The body was progressively untwisted as more of the foot came in contact with the sediment, until the animal resumed its normal posture with the entire foot in contact with the surface.

We observed behaviors subsequent to 9 slides and 8 dislodgements. Four of the 17 slugs crawled a short distance before stopping and remained inactive; 2 of these were facing upstream. Two animals stopped mating after being

separated by the slide, and remained inactive. One slug stopped egg-laying, turned, and crawled upstream to find a mate. The remaining 8 slugs returned to prior activities: inactivity, crawling, mating, crawling on top of a previously mating pair, and egg-laying.

*Interactions with conspecifics*

*Mating.* Slugs mated frequently (Table 1). Mating pairs were observed at all times of day and within minutes of placing the slugs under the cameras. In addition, 10 serial

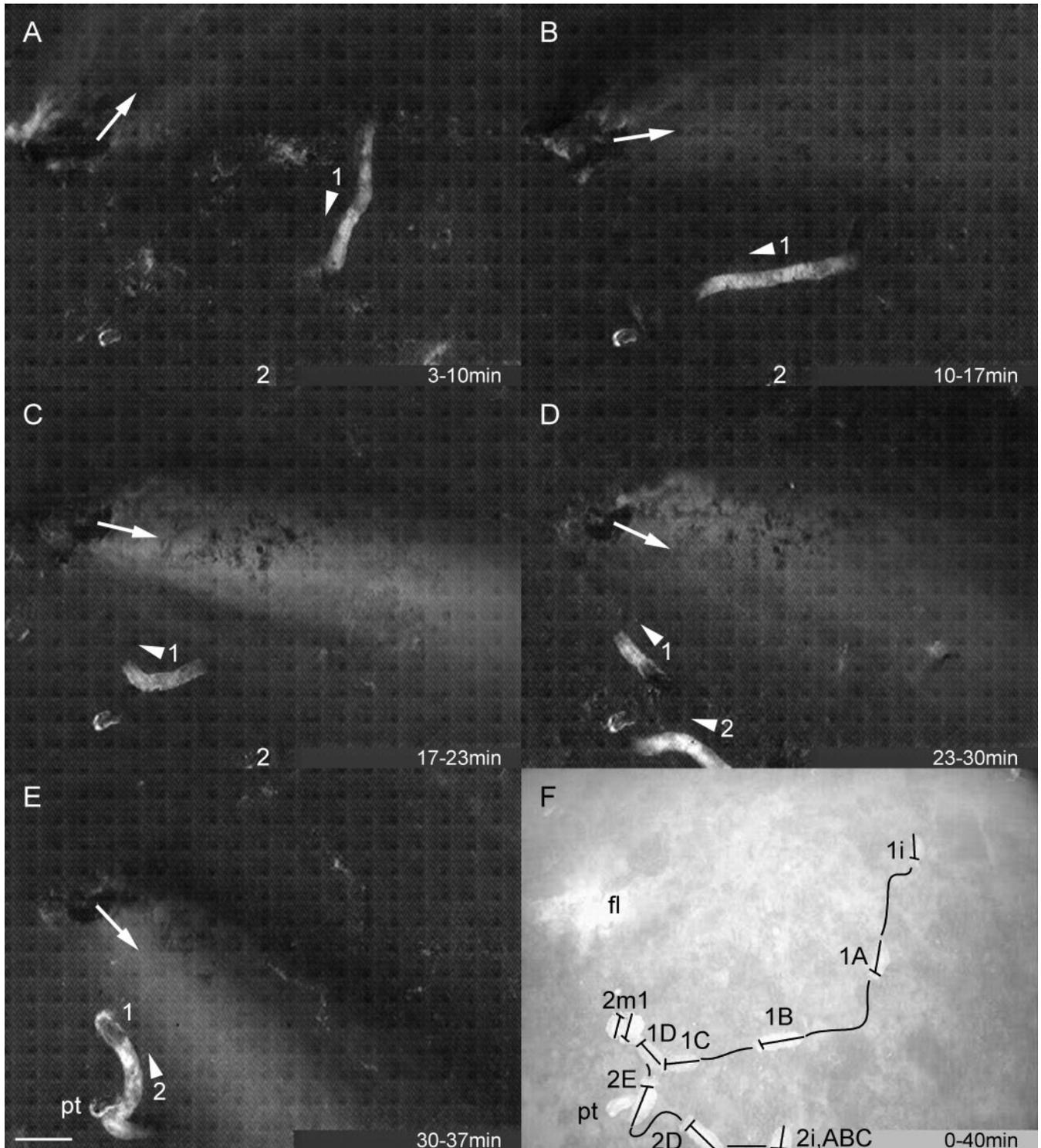


Figure 5

matings were observed, in which a single animal mated consecutively with two or three slugs (either immediately or over the course of the day).

Mating in *T. diomedea* can be separated into three stages: initiation, alignment, and copulation (Fig. 6). Initiation involved one slug (the initiator) touching the other (the initiate) with its oral veil. The initiate (if mating was to occur) performed a right turn. The initiator also proceeded to turn right. The magnitude of these turns varied from 0° (or rarely a very slight left turn) to more than 180°, depending on the relative orientations of the two slugs (Table 3). The paired right turns led to a clockwise circling behavior that brought the right sides of the animals together. This alignment stage continued with the animals circling clockwise for varying amounts of time. While the animals were circling, their oral veils swept the partner's lateral posterior body wall, and the gill tufts on the right side were usually withdrawn. Circling usually then slowed or stopped, and the animals remained stationary for about an hour. Observations in the field and the laboratory suggest that circling behavior is used to align the copulatory organs, and the animals either slow or stop circling during copulation.

Mating usually ended when one ( $n = 11$ , 50% of 22 mating pairs with adequate data after mating) or both ( $n = 8$ , 36%) of the slugs crawled away. Only three instances (14%) were observed where the animals finished copulation, separated by no more than a few centimeters, and then both remained stationary. Initiators, by definition crawling before mating, rarely remained stationary following copulation (2 of 16 with adequate data from both before and after mating). Initiates, rarely crawling before mating (2 of 16), were more evenly split after mating: 9 crawled and 7 remained stationary.

Not all contacts between slugs resulted in mating. Some were apparently aborted attempts to mate on the part of either one or both slugs. In other cases, slugs showed no reaction despite touching each other. No consistent pattern emerged to suggest why certain slug-to-slug contacts led to mating and others did not.

Mating pairs were also attractive to downstream slugs (Fig. 7). A third slug was often ( $n = 12$ ) seen crawling

upstream to find a pair *in copulo*, in similar fashion to mate approaches. The third slug would then repeatedly brush the mating pair with its oral veil, crawling around or even overtop of them. In three cases, this behavior was followed by the departure of one of the previously mating pair, and mating between the third slug and the remaining slug.

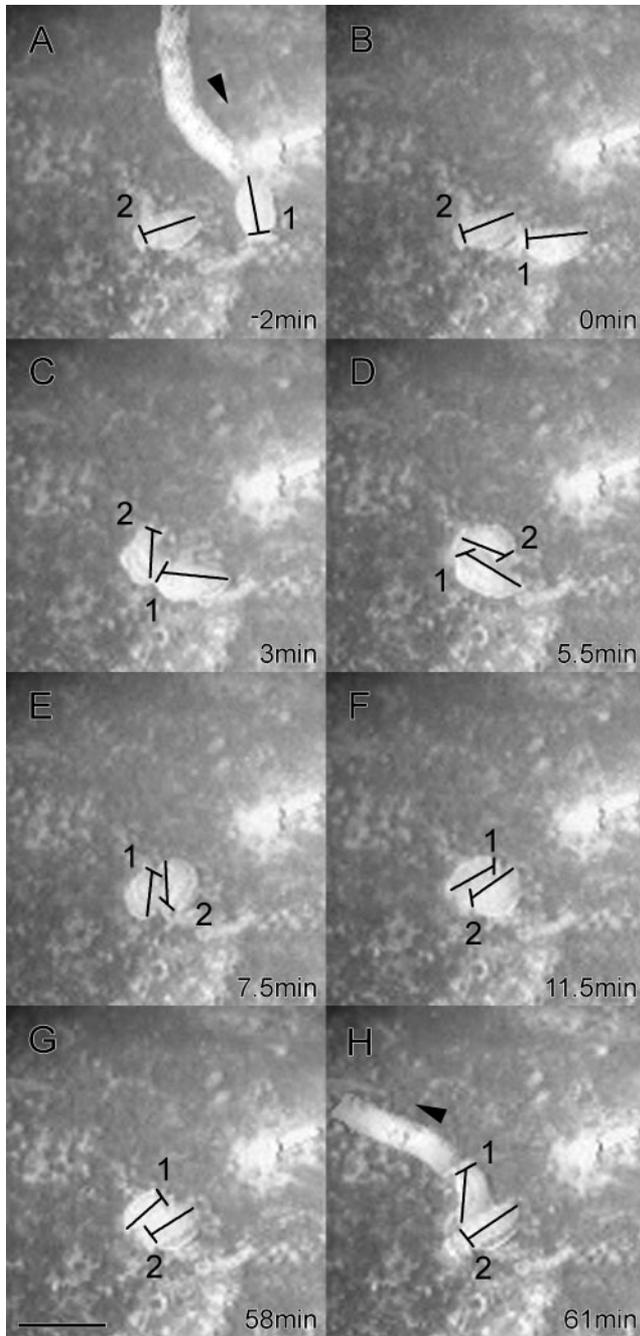
*Egg-laying.* Five examples of egg-laying were observed. The egg string was extruded out of the reproductive openings on the right side of the animal and down onto the substratum. Simultaneously, the animals circled slowly to the left ( $1.6 \pm 1.0$  °/min; cf. regular turning speeds in Table 2), crawling overtop of the egg string already laid down. The result was a loose counterclockwise coil that divers observed to be attached to algae or other debris.

#### *Interactions with prey*

*Bite-strikes.* We observed 13 predation events (bite-strikes, Fig. 8) in the behavior videos. Similar to our observations of slugs approaching conspecifics (see above), bite-strikes were usually preceded by upstream crawling (Fig. 9). Since *P. gurneyi* orients to flow (Best, 1985), it was possible to assign a binary upstream or downstream orientation to each bite-strike, based on the location of the strike on the sea pen (Figs. 8 and 9). In 11 of 13 cases, the slug struck the downstream side, 1 strike was equivocal, and 1 struck from upstream. Whenever ( $n = 8$ ) several sea pens were visible in a behavior camera and consistently oriented as a group (indicating consistent flow direction), bite-strikes were from downstream. In only two cases did *T. diomedea* individuals approach their prey from another direction and then use a sharp turn to strike from downstream.

Diver observations of feeding showed that as a slug neared its prey, locomotion slowed and then ceased prior to the strike. The anterior foot margin was lifted, the oral veil raised and extended towards the sea pen, and the lips protruded (Fig. 8). Only very light contact appeared to be made with the prey before the radula and jaws lunged forward to grasp and cut away a single pinnea ("leaf") from the sea pen. After a bite-strike, most *P. gurneyi* individuals

**Figure 5.** Changing currents result in an upstream-downstream role reversal for two individuals of *Tritonia diomedea* as they crawl prior to mating. (A–E) Motion trails show crawling (arrowheads) by two slugs (1 and 2) over 06:40 (min:s) intervals. Flow direction (arrow) is shown by the motion trail of a fluorescein dye plume. In each panel, the moving slug travels in roughly the opposite direction to flow. Slug 1 initially heads upstream (A) towards slug 2, but the currents change, leading slug 1 astray (B) until it slows and stops crawling (C–E). Meanwhile, slug 2 is stationary (A–C) until the currents change, making slug 1 upstream (D). Slug 2 then turns, heads upstream, avoids a *Ptilosarcus gurneyi* (pt), and eventually contacts slug 1 (E). (F) Composite summary image showing slug positions every 06:40 over the course of the crawling behaviors and subsequent mating (40:00 total). Slug positions correspond to initial positions (1i and 2i, 00:00), the center of each motion trail interval: 06:40, 13:20, 20:00, 26:40, and 33:20 (indicated by the letters A–E beside slug numbers 1 and 2), and final positions while mating (2ml, 40 min). T-bars are drawn to connect the two rhinophores and tail of each slug, and subsequent slug images are connected by lines to help show the path taken by each slug. Fluorescein source and plume (fl). Scale bar: 25 cm. A video version of this figure is available online at <http://www.biolbull.org/supplemental/>.



**Figure 6.** Initiation, alignment, and copulation stages of mating in *Tritonia diomedea*. Cropped single frames; A and G maximum-projected with motion trails. (A) Motion trail shows cross-stream crawling (arrowhead) by a slug (1) over the previous 7 min. A second slug (2) is now just upstream of slug 1. (B) *Initiation*: slug 1, the initiator, has turned upstream and contacts the tail of slug 2. (C) *Initiation*: slug 2, the initiate, turns abruptly right, while slug 1 turns more slowly to the right. (D) *Alignment*: slugs 1 and 2, both still turning right, are now head-to-tail. (E) *Alignment*: both slugs continue circling clockwise. (F) *Copulation*: both slugs remain stationary. (G) *Copulation*: just before mating ends; neither slug has moved in 45 min. (H) *Mating ends*, with the initiating slug (1) crawling away (arrowhead) and the other slug (2) remaining stationary. Motion trails show movement over the 4 min following this frame. T-bars are drawn to connect the two rhinophores and tail of each slug. Scale bar: 25 cm. A video version of this figure is available online at <http://www.biolbull.org/supplemental/>.

**Table 3**

*Duration and rotation of mating stages in Tritonia diomedea*

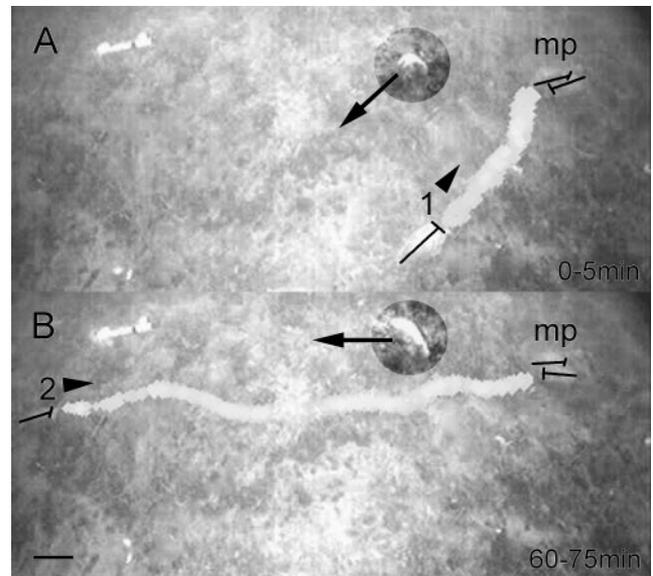
Stage	Duration (min)	Rotation (°)
Initiation	4 ± 2	Initiator: 42 ± 57 Initiate: 147 ± 95
Alignment	9 ± 12	76 ± 130
Copulation	69 ± 30	18 ± 29

Initiation stage: when one slug (the initiator) touched another (the initiate), and they first turned right to create a head-to-tail alignment. Alignment stage: when both slugs continued turning to the right. Copulation stage: when turning slowed or stopped. Rotation measured as the angle swept by the slug's head as it turned. Data are means ± st. dev.  $n = 19$ .

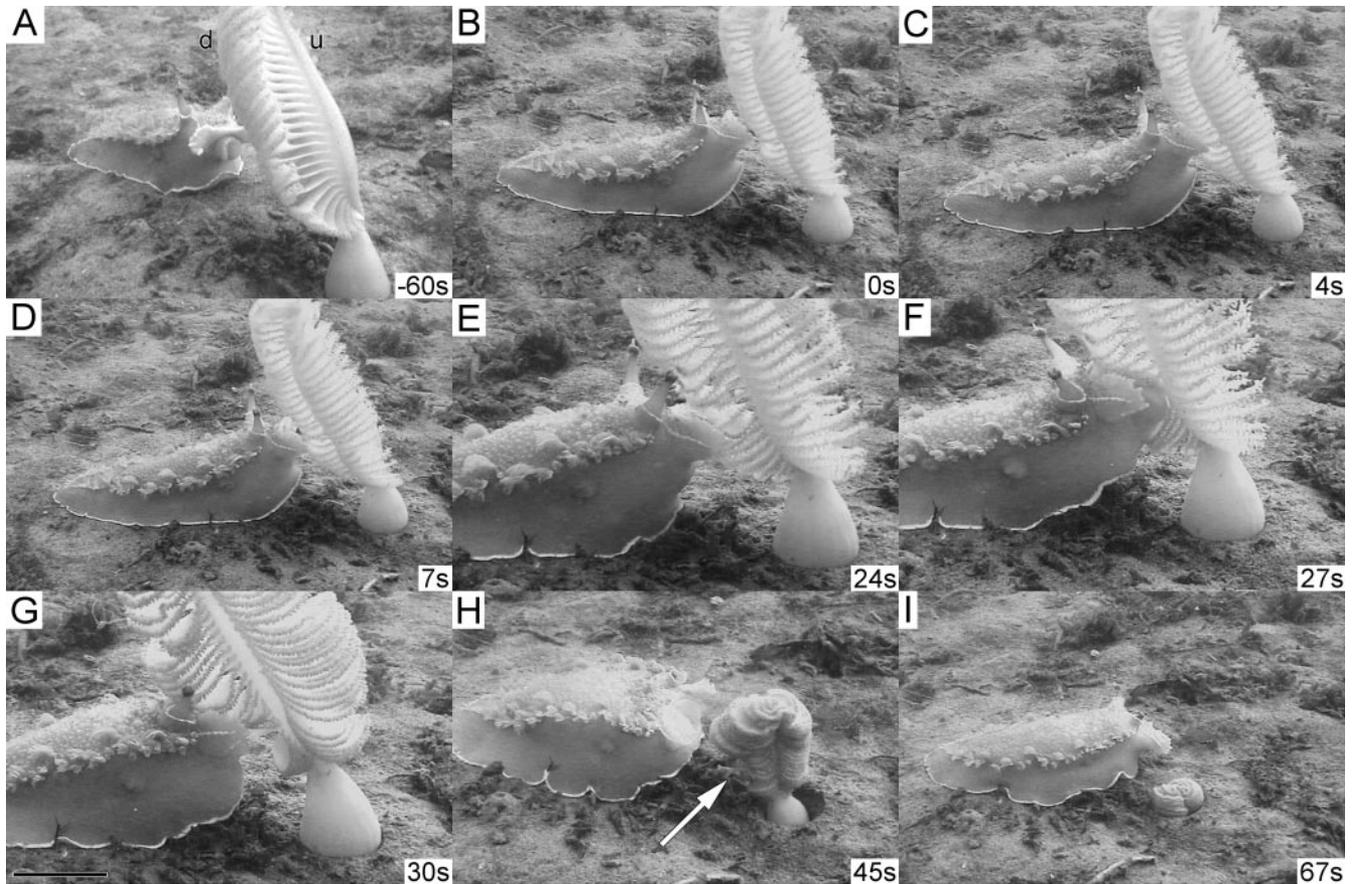
retracted rapidly into the sediment ( $53 \pm 40$  s, mean ± st. dev;  $n = 10$  for complete retraction). Small individuals ( $< \sim 20$  cm when extended) retracted when touched by a slug, while larger ones appeared to respond only after being struck. After a bite-strike, the slug invariably continued crawling.

#### *Interactions with predators*

We observed both escape swims and avoidance crawling in response to the predatory sea star *Pycnopodia heli-anthoides*.



**Figure 7.** *Tritonia diomedea* crawls upstream towards already mating pairs. Cropped single frames maximum-projected with motion trails. (A) One slug crawls (1, arrowhead) directly upstream towards a previously mating pair (mp). Flow direction (arrow) varied little over the 5-min crawling behavior (indicated by the curvature of a *Ptilosarcus gurneyi* in the flow in the enhanced contrast circle). (B) One hour later, after slug 1 has crawled around the mating pair and then left the field of view to the right, a second slug crawls (2, arrowhead) directly toward the still-mating pair (mp). Again, flow direction (arrow), varied little over the 15-min crawling behavior and is indicated by the sea pen. T-bars are drawn to connect the two rhinophores and tail of each slug. Scale bar: 25 cm.



**Figure 8.** *Tritonia diomedea* browsing on a large *Ptilosarcus gurneyi*. (A) A slug approaches from downstream with rhinophores extended, oral veil lifted, and lips protruded. The upstream (u) and downstream (d) sides of the sea pen are indicated. (B) As the slug nears its prey, it slows and then stops crawling, and lifts its head by raising the anterior portion of the foot off the substratum. (C) The oral veil tips make brief contact with the sea pen. (D) The oral veil is immediately pulled back. (E) Strike preparation involves protruding the lips even further and retracting the oral veil. (F) The bite-strike consists of opening the jaws and extending the grasping radula out of the mouth as the entire buccal mass is lunged forward. (G) The radula grasps a pinna and pulls it into the mouth as the sea pen retracts into the sediment. (H) The jaws are used to cut part of the pinna held by the radula, leaving a severed stump (arrow). (I) Crawling resumes after the bite-strike is complete. Relative time (s) is given at the bottom right of each frame. Scale bar: 5 cm. A video version of this figure is available online at <http://www.biolbull.org/supplemental/>.

**Escape swimming.** A single escape swim occurred in the behavior videos after a slug was touched by *P. helianthoides* (Fig. 10). Several apparently spontaneous swims were observed, possibly due to small sea stars obscured from view. All swims resulted in the slugs being transported downstream, in some cases out of the camera's field of view. Once, when the flow was negligible, the swimming slug remained close to the swim initiation point.

**Avoidance crawling.** While focusing on responses to a caged individual of *P. helianthoides*, we observed one slug that turned and crawled away, and another that swam while still a meter downstream from the sea star (Fig. 11). Due to poor visibility with the behavior cameras, we used

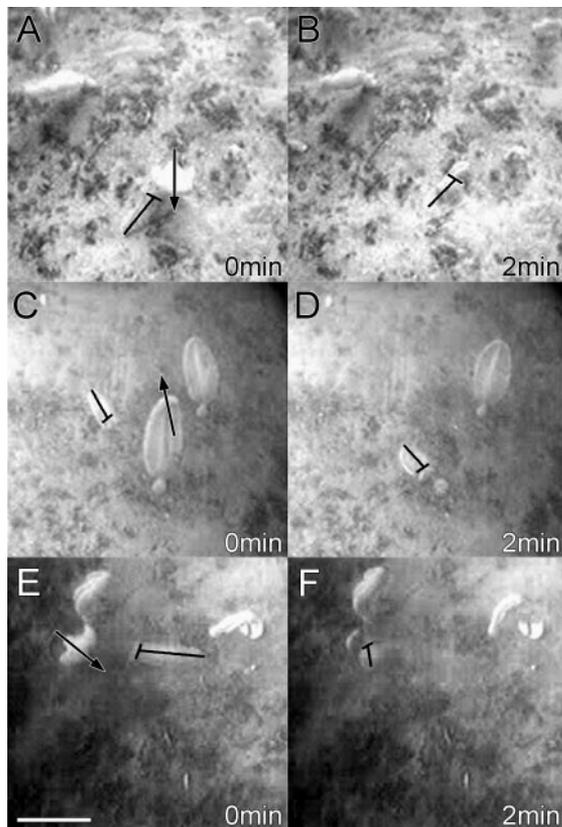
diver observations to further examine *T. diomedea* responses to a distant predator. We coaxed a sea star to crawl cross-stream, between 5 and 50 cm upstream of 10 slugs (tested individually). In 6 trials, the slugs responded by turning and crawling away. In each case, the slug reared its head upwards and lifted the anterior half of the foot off the substrate. The body was bent laterally, 90° or more. The anterior half was then lowered onto the substrate and crawling commenced, distancing the slug from the sea star. In 3 trials, the slugs swam without being touched by the sea star. Initially, the slugs responded with the same rearing turn, but instead of replacing the anterior half of the foot on the substratum, they began an escape swim with a ventral flexion. The remaining slug showed no reaction to the distant sea star.

### Interactions with other organisms

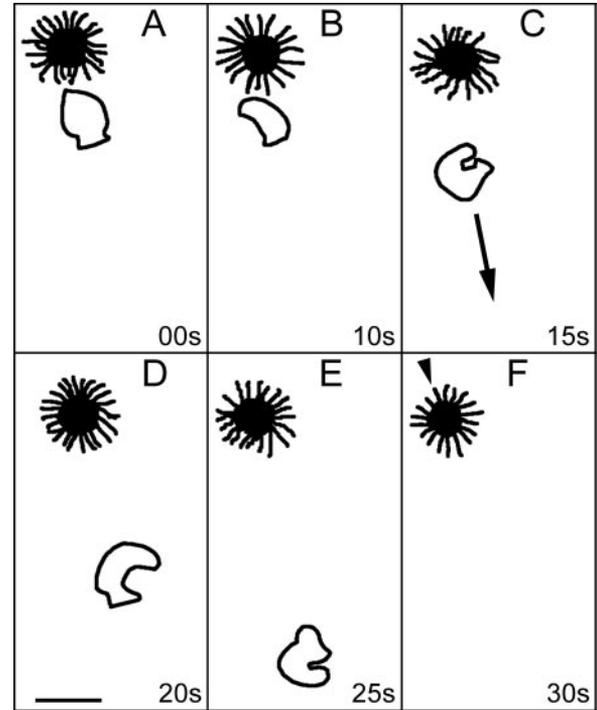
*T. diomedea*'s interactions with other organisms seemed to be limited to treating them as obstacles while crawling. Encounters with drift algae were the most frequent, and slugs were observed crawling around or overtop of algae, which appeared to promote dislodgement. *Cancer* crabs crawled over or into slugs, which responded with temporary gill and oral veil retraction (with the exception of one escape swim). We observed no responses to the other common sea slugs *Armina californica* and *Tritonia festiva* found in *P. gurneyi* beds.

### Discussion

We observed 180 slugs in four years, three seasons, all tidal states, and a wide range of flow rates, in an effort to describe much of the adult behavior of *Tritonia diomedea*. We observed most previously known behaviors for this species, extended observations on several behaviors, and document several behaviors not previously described. In



**Figure 9.** Three examples of *Tritonia diomedea* approaching and striking *Ptilosarcus gurneyi* from downstream. Shown are paired frames 2 min apart, before and after three bite-strikes. Prior to each strike (A, C, E), the slugs are crawling upstream towards a fully extended sea pen that orients to flow, indicating upstream (arrows). The bite-strike occurs on the downstream side of each sea pen between the two frames. After the strike (B, D, F), the sea pen retracts into the sediment. T-bars are drawn to connect the two rhinophores and tail of each slug. Scale bar: 25 cm.



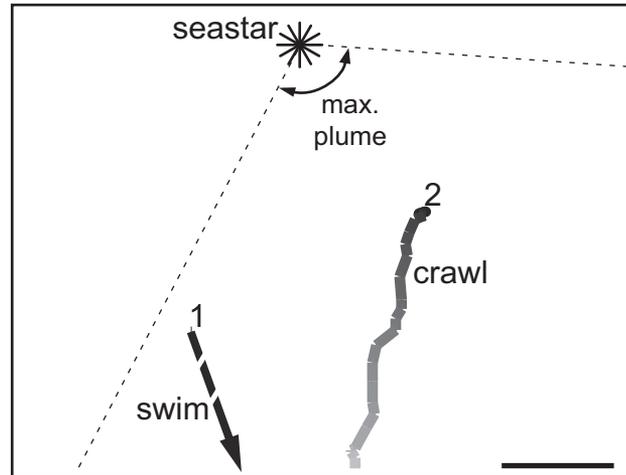
**Figure 10.** *Tritonia diomedea* escape swim in response to contact with *Pycnopodia helianthoides*. Tracings of the slug and sea star made from cropped behavior camera frames spanning a 30-s interval. (A) The sea star approaches the slug. (B) Contact with the sea star causes the slug to begin its escape swim with a ventral flexion. (C) The first dorsal flexion. The combined effects of the current and swimming begin to move the slug away from the sea star (arrow). (D) Second ventral flexion. (E) Second dorsal flexion. (F) The sea star has continued to crawl roughly downstream (arrowhead, cf. panel A). Yet the flow has swept the swimming slug completely out of the field of view, at least 1 m further downstream from the sea star. Elapsed time bottom right. Scale bar: 25 cm. A video version of this figure is available online at <http://www.biolbull.org/supplemental/>.

addition, we describe navigation by *T. diomedea* in its natural habitat.

We observed *T. diomedea* behaviors within their sensory context and can outline possible relationships between different sensory cues and particular behaviors. On average, we observed the activity of a slug for longer than twice the duration of any of the observed behaviors (except for egg-laying; Table 1). We therefore conclude that a single camera field of view is adequate for recording complete behaviors. Similarly, the field of view was also large enough to capture sensory cues (chiefly predators, prey, mates, and flow). Thus, we are able to link the majority of behaviors observed to cues also visible in the videos.

### Interactions with currents: navigational hypotheses

We observed several *T. diomedea* behaviors that involved interactions between water flow and upstream organisms. Previous studies considering flow direction as the only cue reported positive rheotaxis in both the laboratory (Murray



**Figure 11.** *Tritonia diomedea* responses to distant *Pycnopodia helianthoides*. Manual tracking of slug locations (marked every 30 s) relative to a caged sea star over 10 min under a behavior camera. Line segments between consecutive slug positions fade from black to grey over the 10-min interval. Thin dashed lines show the maximal spread of a fluorescein plume created by fluorescein sources amongst the arms of the sea star. Two slugs were clearly within the fluorescein plume, and therefore presumably within any odor plume from the sea star. One slug (1), which had been stationary for at least 4 h beforehand, swam and was swept away downstream (broken arrow) ~ 5 min after the sea star was caged upstream. Slug 2 immediately turned after being introduced downstream of the sea star and crawled quickly downstream until it left the field of view 10 min later. Scale bar: 25 cm.

and Willows, 1996) and the field (Murray, unpubl. data). Here, slug movement patterns in relation to water flow and the locations of conspecifics, prey, and predators suggest three hypotheses about how *T. diomedea* navigates with respect to flow. Crawling prior to most matings was directed upstream (Fig. 4), and this frequently did not occur until after a change in currents or slug position that resulted in a newly detectable upstream conspecific (Fig. 5). This suggests an active choice to crawl upstream towards an upstream conspecific. Prey were also predominately approached by crawling upstream (Fig. 9). In contrast, distant upstream predators elicited downstream crawling (Fig. 11). Thus, we hypothesize that *T. diomedea* uses flow cues to navigate (1) upstream to find potential mates, (2) upstream toward prey, and (3) downstream away from predators. We have now confirmed these hypotheses (Wyeth *et al.*, 2006) and further concluded that orientation to flow depends on odors from upstream mates, prey, and predators (Wyeth and Willows, 2006).

#### *High currents: avoidance, dislodgment, and righting*

*T. diomedea* responds to high flow by reducing drag. When faced with strong current in the field or laboratory (Murray and Willows, 1996), slugs flatten the body laterally, turn upstream, or both. These responses correlate with reduced probability of being dislodged in a laboratory flume (Murray, unpubl. data). Berming may also have similar adaptive value (Willows, 2001). Our observations confirm that in the field there is a risk of dislodgment by either bulk

tidal flow or oscillatory wave-induced flow. Once dislodged, the slugs may not settle again for many meters, with consequences similar to those of the escape-swim behavior (Willows, 2001)—that is, separation from mates or prey.

If a dislodged slug failed to land on its ventral surface, righting behavior occurred. Subsequent behaviors were inconsistent, including those characteristic of high current situations (inactivity, turning to face upstream, or lateral flattening) as well as those that appear to risk further dislodgment (for example, resuming crawling on top of a previously mating pair). Apparently, *T. diomedea* has no fixed response to being moved by the currents and responses are likely to be context-dependent.

#### *Interactions with conspecifics: mating and egg-laying*

Mating was frequently observed (Table 1), and despite its similarly frequent occurrence in the laboratory (pers. obs.), we provide the first description of this behavior for *T. diomedea*. As in some other nudibranchs (Costello, 1938), mating is a long-lasting affair in *T. diomedea*, with a mean duration of more than an hour. Initiation rarely occurred head-to-head, and regardless of initial orientations, the two slugs turned clockwise to achieve head-to-tail orientation (Fig. 6). Head-to-head contact is the norm for other nudibranchs, followed by further movement forward that brings the right sides in contact in a head-to-tail orientation (Costello, 1938; Rutowski, 1983; Longley and Longley, 1984). We have no data to suggest why *T. diomedea* should be different in this regard. Continued clockwise turning

during the alignment stage by *T. diomedea* is also unusual; other species stop moving as the copulatory organs are first aligned. Thereafter, copulation in *T. diomedea* is similar to that in other nudibranchs, with little or no further movement until copulation ends. We observed serial matings between egg-laying events, which is typical of many hermaphroditic gastropods (Baur, 1998). Egg-laying in *T. diomedea* is also similar to that in other nudibranchs (Costello, 1938; Hurst, 1967).

#### *Interactions with prey: bite-strikes*

Predation by *T. diomedea* was rare (Table 1), as in previous observations (Murray, unpubl. data). Our observations of bite-strikes on the sea pen *Ptilosarcus gurneyi* are similar to reports of feeding on *Virgularia* sp. (Willows, 1978; Audesirk and Audesirk, 1979). Differences are presumably due to the differing prey sizes: individual pinnae (leaf-like structure with polyps) are browsed from the larger *P. gurneyi*, whereas a segment of the rachis (stem) is bitten from the smaller *Virgularia* sp.

#### *Interactions with predators: avoidance crawling and escape swims*

We have hypothesized that *T. diomedea* uses avoidance crawling in the presence of a distant upstream predator, the sea star *Pycnopodia helianthoides*. Although this behavior has not previously been described, it is expected given the potential costs of predation. Escape swims, despite their success in avoiding predation (Mauzey *et al.*, 1968), are presumably also costly, chiefly through separation from mates and prey (Willows, 2001). We find support for these costs in the rarity of swims (Table 1), and the current-dependent downstream displacement of swimming slugs (Figs. 10 and 11). Avoidance turns may therefore also reduce the frequency of costly swims.

The behavioral components of swims conformed well to prior laboratory analyses (Willows *et al.*, 1973; Hume *et al.*, 1982). Although most laboratory studies of this behavior have considered only the dorsal and ventral flexions of the swim (except Popescu and Frost, 2002), our observations suggest that parts of the swim behavior are coordinated with other behaviors. The rearing turn used in avoidance crawling also preceded some distant swim responses. This turn is conspicuously similar to the initial dorsal flexion of induced laboratory swims. After an escape swim, the slug crawls at high speed (Audesirk, 1978; Popescu and Frost, 2002), provided it has a grip on the substratum. However, righting behavior is sometimes necessary before the animal can begin crawling. Thus, swims can be coordinated with turns beforehand and both crawling and righting behaviors afterward.

The ability to trigger escape swims at a distance (Fig. 11) suggests that in addition to touch stimuli (Willows *et al.*, 1973; Hume *et al.*, 1982), distance chemosensation can

initiate swimming. Swim behaviors are also inhibited by certain types of dorsal stimulation in the laboratory (Mongeluzi *et al.*, 1998). Thus, the escape swim is part of an integrated response to a potential predator, coordinated with other behaviors and affected by several sensory modalities.

#### *Missing cues and behaviors*

Magnetic fields (Lohmann and Willows, 1987), light levels (Chase, 1974), and presumably gravity are all detectable by *T. diomedea*, but without observable variation in these features, we are unable to interpret behavioral responses to them. We continue to find no evidence for the use of conspecific mucus trails as a navigational cue (Willows, 1978). Long-term crawling patterns (over weeks or months) and nocturnal behaviors are two potentially important groups of behaviors for which we also have no data. Understanding the full extent of navigational cues and behaviors will require further work to document overnight activity. Furthermore, as neuroethology of navigation or other behaviors progresses, observation should return to the natural habitat for more detailed description of the behavior in question.

#### *Neuroethological implications*

A practical conclusion from these behavioral observations is that slug behavior must be observed with appropriate sampling over time. Viewing *T. diomedea* behavior in 10× to 100× time-lapse reveals details difficult for humans to observe in real time. Future behavioral and neuroethological experimentation in the laboratory should take into account the appropriate spatial and temporal scales over which the behaviors occur naturally.

Many of the behaviors recorded here have been described previously from observations in the laboratory. Crawling speeds (Audesirk, 1978), upstream crawling and turns (Murray and Willows, 1996), bite-strikes (Willows, 1978; Audesirk and Audesirk, 1979), and swims (Willows *et al.*, 1973; Hume *et al.*, 1982) are examples. However, several have been missed in the laboratory (navigation relative to conspecifics and predators; Willows, 1978), or have received little attention despite being easily observable (mating and righting behavior). In addition, field observations tend to add details to the context in which the behaviors observed in the laboratory are performed. Thus, bite-strikes are preceded by upstream orientation, and swims can be preceded by avoidance turns and followed by righting behaviors.

Our results suggest new neuroethological experiments. For example, several neurons in the pedal ganglion are hypothesized to be motor neurons that play a role in positive rheotactic turning in *T. diomedea* (Murray *et al.*, 1992; Redondo and Murray, 2005). Recognition that egg-laying and mating behaviors use, respectively, left and right turns provides alternative stimuli for neuroethological tests of

turning. Conversely, turning motor neurons are clearly one starting place for neuroethological exploration of reproductive behaviors in *T. diomedea*, an area with little prior work, yet a dominant part of the slug's field behavior. A second example: neurons involved in the central control of the escape swim have already been linked with control of post-swim crawling (Popescu and Frost, 2002). Do the same neurons also control righting behavior if the animal is inverted?

The recent focus on *T. diomedea* sensory systems (Lohmann *et al.*, 1991; Murray *et al.*, 1992; Murray and Willows, 1996) and the control of locomotion (Popescu and Frost, 2002; Wang *et al.*, 2003; Redondo and Murray, 2005; Cain *et al.*, 2006) has raised the possibility of exploring the neuroethology of navigation in this species. Four other species of opisthobranchs (*Aplysia californica*, *A. fasciata*, *Navanax inermis*, and *Bursatella leachii*) have similar behavioral descriptions or ethograms (Kupfermann and Carew, 1974; Susswein *et al.*, 1984; Leonard and Lukowiak, 1984, 1986; Ramos *et al.*, 1995). Yet for none of these other slugs are detailed, continuous descriptions of navigation available. In *T. diomedea*, we can combine easily recorded navigational behaviors with the neuroethological advantages inherent to gastropod molluscs to consider the problem of how animals navigate. The first step towards understanding navigation in *T. diomedea* has been to describe navigation in the field. The next step is to use quantitative analysis of independent crawling and current data (Wyeth and Willows, unpubl. data) to confirm the importance of water-flow direction in navigation relative to predators, prey, and conspecifics (Wyeth *et al.*, 2006).

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