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Dietary immunoassay of pelagic nemerteans by use of cross-reacting polyclonal antibodies: preliminary findings

Robert J. Feller[1], Pamela Roe[2] & Jon L. Norenburg[3]
[1]*Dept. Biological Sciences, Marine Science Program, and Belle W. Baruch Institute, University of South Carolina, Columbia, SC 29208, U.S.A.*
[2]*Dept. Biological Sciences, California State University Stanislaus, Turlock, CA 95382, U.S.A.*
[3]*Dept. Invertebrate Zoology, MRC 163, National Museum of Natural History, Smithsonian Institution, Washington, DC 20560, U.S.A.*

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Abstract

With little data on the diet of pelagic nemerteans, a preliminary immunoassay survey of the gut contents of three species from the Pacific Ocean was performed using non-specific, cross-reacting polyclonal antibodies. Results suggest that *Nectonemertes cf. mirabilis*, *Phallonemertes* cf. *murrayi*, and *Cuneonemertes elongata* contained somewhat different types of prey. Worms elicited strong responses when probed with antibodies to squid-like mollusks and to mysids and shrimp. Heteropods are more likely ingested than pteropods. Additional studies must be done to confirm these highly suggestive results.

Introduction

Most studies of feeding by free-living nemerteans have been conducted on animals collected from intertidal marine habitats in temperate latitudes (Bartsch, 1973, 1975 in McDermott & Roe, 1985; McDermott, 1976a, 1976b, 1984, 1988, 1993; McDermott & Roe, 1985; McDermott & Snyder, 1988; Nordhausen, 1988; Roe, 1970, 1976, 1993). Unless prey are examined very soon after being ingested, visual analysis of nemertean gut contents is likely to reveal only the refractory hard parts of prey, e.g., polychaete jaws, exoskeletons of crustaceans, and perhaps bivalve shell material (Ruppert & Fox, 1988). In the case of suctorial feeders, which includes most of the nemerteans known to feed on crustaceans, no hard parts are eaten (McDermott, 1988).

Several species of pelagic nemerteans are known to be locally abundant (Roe & Norenburg, 1997; J. Childress, P. Herring, A. Rogers, pers. comm.), but there is almost no information on the diet of these potentially high-trophic-level predators. Brinkmann (1917a) reported remains of a free-swimming crustacean in a sectioned specimen of *Nectonemertes mirabilis*, and Coe (1926) observed a small, oval, multicellular organism, that could have been either food or a parasite, in a gut diverticulum of *Pelagonemertes brinkmanni*. Coe (1954) noted that the orange or red color of the lipoid droplets so conspicuous in the digestive tracts of most pelagic nemerteans, when analyzed spectrographically, is closely similar to astaxanthin, a pigment commonly found in crustaceans.

Pelagic nemerteans inhabit mid-water depths in all oceans amidst a suite of potential prey items that typically do not have especially refractory body parts. At present, the simplest working hypothesis for those forms with a gut full of orange lipid droplets, is that they feed on crustaceans and probably do so in a suctorial manner, as do their benthic counterparts. Hence, the stomach contents of pelagic nemerteans reveal a characteristically fluidized mush that may differ in color and consistency among species, but little else can be deduced visually about the identity or composition of the material present.

Several immunoassay techniques using polyclonal antibodies have been used to identify soluble pro-

teins in the gut contents of predators that, like pelagic nemerteans, contain non-particulate prey matter [see reviews by Calver (1984), Yentsch et al. (1988), Ward (1990), and Feller (1992)]. These methods rely on the highly specific binding that occurs between antibodies and their homologous (self) antigens and with heterologous (non-self) antigens having sufficiently similar binding sites as to allow at least partial binding (Hefle, 1995). This latter type of binding with heterologous antigens may be non-specific and is generally referred to as a cross-reaction. When it is desirable to detect a particular type of prey organism (as opposed to a single species of prey), antigens consisting of whole-organism extracts of the target prey can be used to produce polyclonal antibodies to many different immunogenic moieties in the extract. The resultant broadly-specific antiserum, containing many different polyclonal antibodies, can then be used to detect prey types rather than a particular prey species. Such cross-reacting antibodies were exploited successfully by Feller et al. (1985) to examine the fluidized stomach contents of deep-sea predators. This application of trophic immunoassays in the deep-sea was possible because a large battery of antigens and their homologous antibodies, tested for all possible cross-reactions, revealed that immunological similarities among taxa also reflected conventional phylogenetic relationships among them (Feller & Gallagher, 1982).

It is within this same context that an immunological examination of the gut contents of three species of pelagic nemerteans from the Pacific Ocean was undertaken in a very preliminary, exploratory fashion using antibodies that cross-react faithfully within broad taxonomic lines but only weakly across those lines. Our objective was to determine whether any information on diet could be gleaned from specimens that contained visibly different colors of stomach content. Some of the nemerteans collected had distinctly species-characteristic orange, white, or clear gut contents. Our initial hypothesis was that the orange material is derived from ingestion of organisms that contain the same color and live in the same water mass as the nemerteans (e.g., shrimps, calanoid copepods). We tested this hypothesis by using polyclonal antibodies remaining from some other trophic studies to characterize types of prey the nemerteans might be eating.

Methods and materials

Organism collections

In September 1993, February and June 1994, collections of pelagic organisms were made along a transect 160 km west of Point Conception, California (34°N, 121°W) at its southern end, extending north 48–56 km, paralleling the coast 160 km offshore, over a bottom depth of about 4600 m. Most of the June 1994 cruise was in Santa Cruz Basin, south of Santa Cruz Island, southern California, with bottom depths of 1800–2000 m. Animals were collected using a Tucker Trawl modified to bring animals onto shipboard in good condition (Childress et al., 1978). On shipboard, nemerteans and potential prey were identified and nemerteans were measured, then stored in liquid nitrogen until shipped to Feller on dry ice. The diets of *Nectonemertes* cf. *mirabilis*, *Phallonemertes* cf. *murrayi*, and *Cuneonemertes elongata* were examined in this study. Species identifications of *Nectonemertes* and *Phallonemertes* are tentative, pending further taxonomic study (Norenburg & Roe, in prep.).

Immunoassay procedures

The general approach was to test digestive tracts (using whole animals) of a few representative specimens from the collections for the presence of prey taxa representing several major phyla. We used the micro-Ouchterlony double immunodiffusion assay (Feller et al., 1979) in which frozen nemertean guts (actually whole organism macerates) were solubilized in buffered saline. The nemertean/gut content antigen slurry was centrifuged to remove particulates and then 15 ml of the clear slurry was placed in the central well of a template surrounded by antibodies to four different taxa placed in four peripheral wells, one taxon per well. Diffusion of these antigens and antibodies took place through an agarose gel beneath the template. If any of the polyclonal antibodies recognized and combined with antigenic moieties in the gut content mixture, precipitin lines formed between that antibody's peripheral well and the central well. The pattern and numbers of precipitin lines observed characterizes the specificity and strength of the reaction that occurs between the antigenic mixture in the central well and any of the antibodies in peripheral wells. Using a modification of this procedure, wherein the central well contains a polyclonal antibody and peripheral wells contain different antigenic mixtures, it was possible

to determine, through formation of precipitin lines of identity, whether an antibody recognized identical or similar antigens in adjacent peripheral wells.

Initial antibody screen

The initial immunoassays were run on gut contents of *Nectonemertes* cf. *mirabilis* and *Phallonemertes* cf. *murrayi*, collected in September 1993, using polyclonal antibodies to thirteen taxa: a mysid (*Neomysis americana*), a mid-water sergestid shrimp (*Sergestes similis*), the euphausiid *Euphausia pacifica*, an unknown intertidal isopod species, generic 'polychaetes' (several different families, all shallow subtidal), a pelagic squid (sp. unknown), the mid-water fish *Cyclothone* sp., another pelagic shrimp (*Systellaspis* sp.), intertidal meiobenthic ostracods, intertidal meiobenthic harpacticoid copepods, the hard clam *Mercenaria mercenaria*, an intertidal saltmarsh mud snail (*Illyanassa obsoleta*), and the estuarine squid *Lolliguncula brevis*.

We also tested *N.* cf. *mirabilis* and *P.* cf. *murrayi* using two different polyclonal antisera that had been prepared 18 years ago (1977) with antigens from the intertidal predatory nemertean *Paranemertes peregrina* and another unidentified nemertean species (see Feller et al., 1979). This test was designed to reaffirm both the activity (after such a long period of frozen storage) and the phylogenetic fidelity of the antisera.

Our strategy was next to examine additional nemertean gut contents using those antibodies that had the strongest cross-reactions in the initial antibody screen. An additional partial check of antiserum specificity was conducted using whole-organism antigenic extracts of animals collected in mid-water trawls in the same areas and depths as nemerteans were collected.

Results

Direct observations

In shipboard observations of freshly caught, live pelagic nemerteans, one of us (JN) saw a copepod being expelled from the anus of a freshly-collected Hawaiian specimen. PR presented copepods, mysids, euphausids or an amphipod to specimens of *Nectonemertes* cf. *mirabilis* ($n=3$) and *Phallonemertes* cf. *murrayi* ($n=1$), which responded by swimming or by rolling into a ball, or showing no reaction, but they did not capture or feed on any of the potential prey. One serially sectioned specimen of *N.* cf. *mirabilis* had two more-or-less continuous strands of tissue extending through the full length of the foregut lumen (ca. 6 mm), from the anterior of the stomach to the rear of the pylorus (Figure 1). Each strand was about 80 μm in maximum width and consisted of a central lumen lined by a non-ciliated epithelium containing acidophilic and mucoid glandular cells among cells that are not recognizably specialized. This epithelium was surrounded by an outer tissue that lacked recognizable cellular structure but was characterized by numerous muscle fibers appearing to be orientated exclusively along the longitudinal axis of the tissue strands. The lumen was lined by an epithelium reminiscent of cnidarian gastrodermis. The partially digested outer epithelial coat containing muscle fibers was consistent with interpreting these strands as pieces of cnidarian tentacle. Nematocysts within the tissue strand should have been evident, but none were found. A considerable quantity of 'debris' appeared to be associated with these strands, including non-staining, cuticle-like fragments and a long (ca. 500 μm) refractive rod (Figure 1). A single, large ovoid capsule (ca. 8×27 μm) was associated with the debris external to the strands (Figure 1). Although this was superficially similar to a nematocyst, the proportions and internal structure were not consistent with that of nematocysts (R. Mariscal, *in litt.*).

The initial antibody screen

Only a few nemerteans were tested in the initial screen, and none of the gut contents of either nemertean species was tested with all of the antibodies. The strongest reaction (= the greatest number of precipitin lines) was observed with antibodies to mysids, shrimp, and mollusks (Table 1). The molluscan reactions were particularly strong, indicating that the nemerteans were eating something very similar to squid, bivalves, other cephalopods, or gastropods or that nemertean antigens (body tissues of the predator) cross-react quite heavily with the molluscan antibodies. Inspection of Table 2 in Feller & Gallagher (1982) reveals that both of these interpretations of the initial screen results are possible. Their Table 2 data showed that the antibody to *Mercenaria mercenaria* produced between 5 and 7 precipitin lines when tested against extracts of intertidal nemerteans from Puget Sound, WA, whereas the same antibody to the hard clam yielded 6 and 3 lines with *Phallonemertes* cf. *murrayi* and *Nectonemertes* cf. *mirabilis*, respectively (Table 1). The antiserum to the pelagic squid (sp. unknown) produced 9 lines with

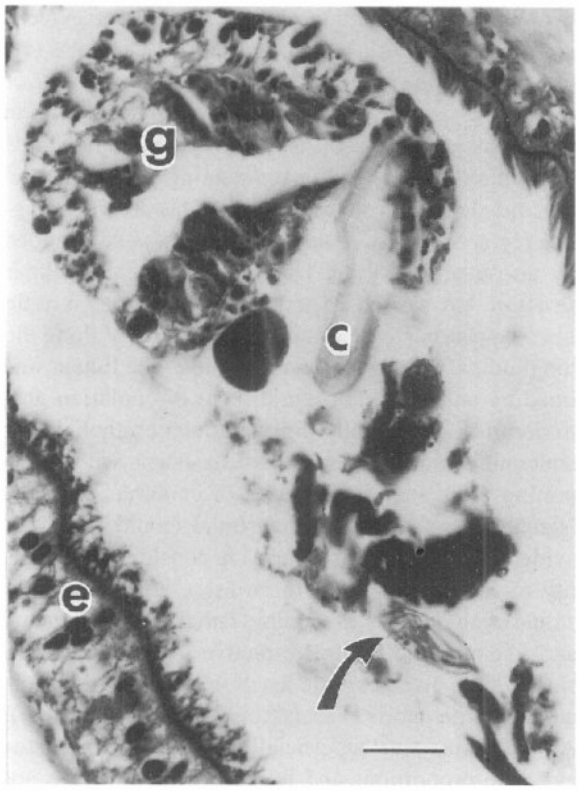

Figure 1. Photomicrograph, 8-μm paraffin transverse section of *Nectonemertes* cf. *mirabilis*, showing gut lumen with strand of putative enidarian tentacle and associated debris, including an unidentified capsular structure (arrow); c, cuticle-like rod; e, gut epithelium; g, putative gastrodermis; scale = 20 μm.

P. cf. *murrayi* (Figure 2a) and 4 with *N.* cf. *mirabilis* in one sample and 3 lines with each nemertean species in another sample (Table 1).

The squid connection

Given that both the pelagic squid (sp. unknown) antibody and the *Lolliguncula brevis* antibody reacted so strongly with the nemerteans (Table 1), we next wanted to see whether this possibly molluscan signal was due to the presence of pteropods or heteropods, the other mollusks besides cephalopods occurring in the pelagos with the nemerteans. This immunoassay revealed that the *L. brevis* antibody produced 15 precipitin lines with its homologous antigen, 11 lines with the pelagic squid (sp. unknown) antigen, 5 with the heteropod antigen, and only 1 line with the pteropod antigen (Figure 2b). Furthermore, there were 3 lines of identity between the pelagic squid (sp. unknown) antibody and the heteropod antigen, indicating that the *L. brevis* antibody

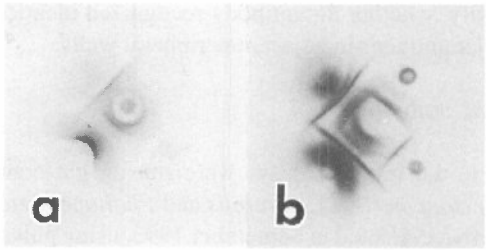

Figure 2. Precipitin lines between wells in micro-Ouchterlony double immunodiffusion assays. a. Cross-reactions between whole body gut content antigens of *Phallonemertes* cf. *murrayi*, in the central well, and polyclonal antibodies to (starting clockwise from upper left well): *Neomysis americana*, *Cyclothone*, *Euphausia pacifica*, and an unidentified species of pelagic squid (the inner circle surrounding the central well is an artifact). b. Cross-reactions between antibody to the estuarine squid *Lolliguncula brevis*, in the central well, and antigens to (starting clockwise from upper left well): pelagic squid, heteropods, pteropods, and *L. brevis* (the self-reaction). Photographic quality in Figures 2 and 3 cannot reproduce details visible to the naked eye.

was recognizing at least 3 identical antigens in both the squid and the heteropod. This result made it highly unlikely that the pelagic squid antibody reaction seen in the initial screen above was due to the presence of pteropods in the nemertean gut contents. The possibility still remained, however, that cephalopods were present or that heteropods may have caused the cross-reaction.

The heteropod connection

We next tested to see whether the two nemertean species tested in the initial screen and a third species, *Cuneonemertes elongata*, would produce precipitin lines with antibody to the estuarine squid, *Lolliguncula brevis*. Antigens were arranged in the immunodiffusion well pattern such that any lines of identity that might form could be seen between a nemertean's gut contents antigens and the estuarine squid's antigens or between the gut contents and heteropod antigens. It was also possible to see identity lines among all three antigens in the peripheral wells, an indication that the antibody to the estuarine squid (*L. brevis*) was recognizing the same antigen(s) in all three peripheral wells. The dominant self-reaction with the squid antigens in the upper left well had 15 precipitin lines, the nemertean's gut contents well (top right) produced 4-6 lines, and the heteropod well (lower right) yielded 5 lines in each case (Figure 3a–c). Two lines of identity were common between the squid and the heteropod for

Table 1. The initial screen. Number of precipitin lines observed in double immunodiffusion assays of whole-organism extracts of *Phallonemertes* cf. *murrayi* (Pm) and *Nectonemertes* cf. *mirabilis* (Nm) with polyclonal antibodies. Blank entries denote that no test was done.

	Sample identification					
	211 2–4	229 T7N2 9-14-93	12-27-93		1-3-94	
Antibodies to:	Pm	Nm	Pm	Nm	Pm	Nm
Crustacea						
N. americana	3	5				
E. pacifica	0	0				
S. similis	0	1				
Systellaspis sp.	4	6				
Isopods	0	1				
Harp. copepods	2	0				
Ostracods	0	0				
Pisces						
Cyclothone sp.	0	0				
Mollusca						
M. mercenaria					6	3
I. obsoleta					3	0
L. brevis					4	7
Pelagic squid (sp. unknown)	9	4			3	3
Polychaeta	1	0				
Nemertea						
P. peregrina			4	5		
nemertea spp.			1	1		

N. cf. *mirabilis* (Figure 3a), but these did not show up in gut contents from the other two nemertean species (Figure 3b, c).

The pteropod connection

Using the same immunoassay setup as for the heteropod connection above, the antibody to *Lolliguncula brevis* again formed 15 lines in the self-reaction, 5 lines with the nemertean gut contents antigens, and up to 4 lines with the pteropod antigen. However, the only identity lines formed were 2 between the *L. brevis* and nemertean gut contents wells. The pteropod antigen, though recognized by the estuarine squid (*L. brevis*) antibody, did not share common antigens with anything in the nemertean gut contents or the estuarine squid for any of the three nemertean species tested. Furthermore, the extent to which gut contents were recognized by the estuarine squid antibody differed greatly, both in numbers and positions of the precipitin lines formed, indicating that the three nemertean species did not contain the same antigens in their guts.

The squid confirmation

As a check of the fidelity of the *Lolliguncula brevis* and the pelagic squid (sp. unknown) antibodies used above, immunoassays were run with antigenic extracts of two squids from the study area, *Bathyteuthis berryi* and *Chiroteuthis calyx*. These assays confirmed that the *L. brevis* antibody recognized both squid species strongly. They shared 4 or 5 common antigens (iden-

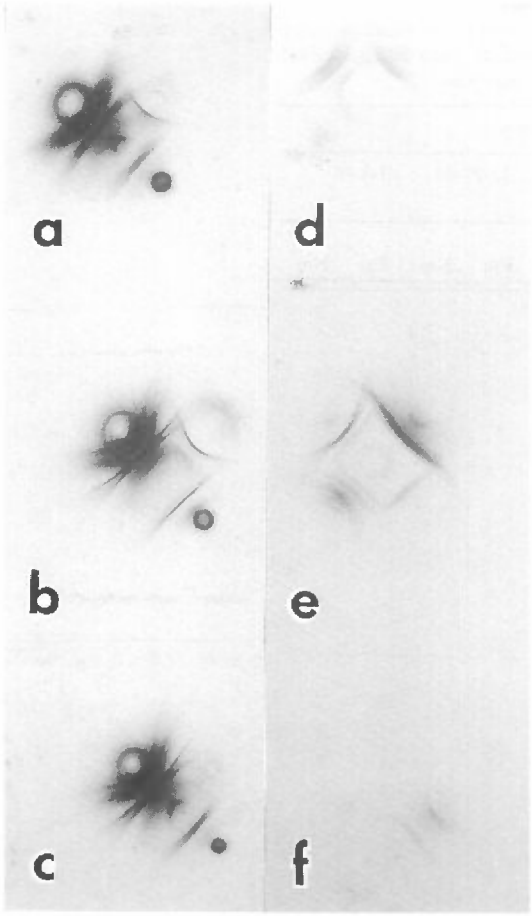

Figure 3. Precipitin lines between wells in micro-Ouchterlony double immunodiffusion assays. a–c. Cross-reactions between antibody to the estuarine squid *Lolliguncula brevis*, in the central well, and antigens from (starting clockwise from the upper left well): *L. brevis* (self-reaction), nemertean whole-organism gut contents (a, *Nectonemertes* cf. *mirabilis*; b, *Phallonemertes* cf. *murrayi*; and c, *Cuneonemertes elongata*.), and heteropods; the lower left well is empty. (d–f) Cross-reactions between nemertean gut-content antigens (d, *N.* cf. *mirabilis*; e, *P.* cf. *murrayi*; and f, *C. elongata*), in the central well, and antibodies to (starting clockwise from upper left): the estuarine mysid *Neomysis americana*, the hard clam *Mercenaria mercenaria*, the Pacific euphausiid *Euphausia pacifica*, and the pelagic shrimp *Systellaspis* sp.

tity lines), and an additional 6 partially identical antigens. Interestingly, the pelagic squid (sp. unknown) antibody used in the initial screen also strongly recognized both squid species but, because no antigen for a self-reaction was included in the assay (an inadvertent error of omission), no lines of identity were formed.

The crustacean connection

Lastly, immunoassays were run on gut contents of different specimens of the three nemertean species using antisera to the following four taxa: the estuarine mysid *Neomysis americana*, the hard clam *Mercenaria mercenaria*, the Pacific euphausiid *Euphausia pacifica*, and the pelagic shrimp *Systellaspis* sp. (Figure 3d–f). The reactions were again strong, indicating that crustaceans were present in both *Nectonemertes* cf. *mirabilis* and *Phallonemertes* cf. *murrayi*, but to a much lesser extent in *Cuneonemertes elongata*. The molluscan signal was again strong in all three species, especially in *P.* cf. *murrayi* (Figure 3e).

Discussion

The initial antibody screen (Table 1) was incomplete because we were only interested in whether there might be any immunological signals worthy of further study. At the time of testing, we had not observed the strands of putative cnidarian tentacle in the sections of a *Nectonemertes* cf. *mirabilis*, and did not test gut content for possible reactions to any cnidarian antibodies. The succeeding immunoassays were similarly incomplete and designed only to see if the signals in the initial screen could be interpreted with any greater phylogenetic certainty, even if by the process of elimination. Another problem was that the two squid antibodies used (sp. unknown and *Systellapsis*) were produced several years after the Feller & Gallagher (1982) study of antigenic similarity; hence, the extent of their cross-reactivity with nemertean tissues was unknown. Therefore, variability seen between the immunoreactivity of different gut contents examined could easily be due to differences in the amounts of material ingested or the amount of time elapsed since it was ingested (= degree of digestion), as well as true differences between meals and/or diets.

A proper examination of the diets of these pelagic nemerteans will require preparation of antibodies specific for the suspected prey and extensive testing to identify any cross-reactions that might give false positive results. The study by Feller et al. (1985) on deep-sea food web connections was limited in the same manner, but in their study antibodies were actually made to several of the organisms from the habitat under study. The latter was not financially feasible for the present preliminary study. There are numerous remaining samples, with which additional work can be done. It is

difficult to make firm conclusions with the data gathered thus far. However, it seems clear that these pelagic nemerteans are predaceous upon crustaceans and possibly upon squid or some other squid-like mollusk. There also seems to be, insofar as the antibodies could detect, some difference between the gut contents of the three species. Finally, it appears that pteropods probably were not consumed by these animals. This is interesting information about the trophic ecology of pelagic nemerteans that we didn't have before, but much remains to be learned about these sometimes abundant predators.

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