Reference: *Biol. Bull.* **162:** 387–403. (June, 1982)

STYLET FORMATION IN NEMERTEANS

STEPHEN A. STRICKER AND RICHARD A. CLONEY

Department of Zoology, University of Washington, Seattle, WA 98195, and Friday Harbor Laboratories, Friday Harbor WA 98250

Abstract

Stylet formation was examined in nine species of nemerteans by light microscopy. The first stylets produced by larvae are assembled intracellularly over a period of several days within styletocytes of reserve stylet sacs. A reserve stylet is moved to the lumen of the proboscis, apparently by muscular contractions, and placed on the basis to become the central stylet. The stylets of adult nemerteans are also formed in styletocytes of reserve stylet sacs, and, depending on the species, reach full length in two to eight weeks. At the onset of styletogenesis, a membrane-bound vacuole develops in the styletocyte, and an organic matrix is formed at one edge of the vacuole. The calcified cortex of the stylet shaft is subsequently deposited around the organic matrix, and a knob-shaped proximal piece is formed on the shaft. Most adult nemerteans contain at least one developing stylet, and the rate of stylet formation is about the same in starved worms as it is in worms that have recently captured prey. Replacement of the central stylet occurs following prey attack, and occasionally when the worm is not feeding.

Introduction

Nemerteans belonging to the order Hoplonemertea typically possess an eversible proboscis that is armed with a calcareous central stylet. During prey capture, the central stylet is used to inflict wounds, into which paralytic neurotoxins are introduced (Kem, 1973; Stricker and Cloney, 1981). In addition to the central stylet, the proboscis usually contains two to several reserve stylet sacs, in which reserve stylets are formed. Reserve stylets are believed to replace the central stylet, when it becomes damaged or lost (Gibson, 1972).

A fully developed stylet is nail-like in shape and can range in length from about 8 μm in *Carcinonemertes carcinophila* (Humes, 1942) to over 200 μm in species such as *Emplectonema gracile* (Coe, 1901). Each stylet consists of a tapered shaft and a knob-shaped proximal piece. Both regions of the stylet are composed of an inner organic matrix surrounded by an inorganic cortex. The cortex contains mainly calcium, phosphorus, and strontium (Wourms, 1976).

The first extensive studies on the formation of nemertean stylets were conducted by Bürger (1895) and Coe (1905). Recently, Wourms (1976), Stricker (1981), and Stricker and Cloney (1981) have briefly reported on the ultrastructure of styletogenesis.

In this paper, we describe the formation of stylets in nine species of hoplonemerteans. Styletogenesis in adult worms is compared to the formation of the first stylets by larvae. Replacement of the central stylet by a reserve stylet is discussed, and the time required for stylet formation is estimated for three species.

Received 21 December 1981; accepted 24 March 1982.

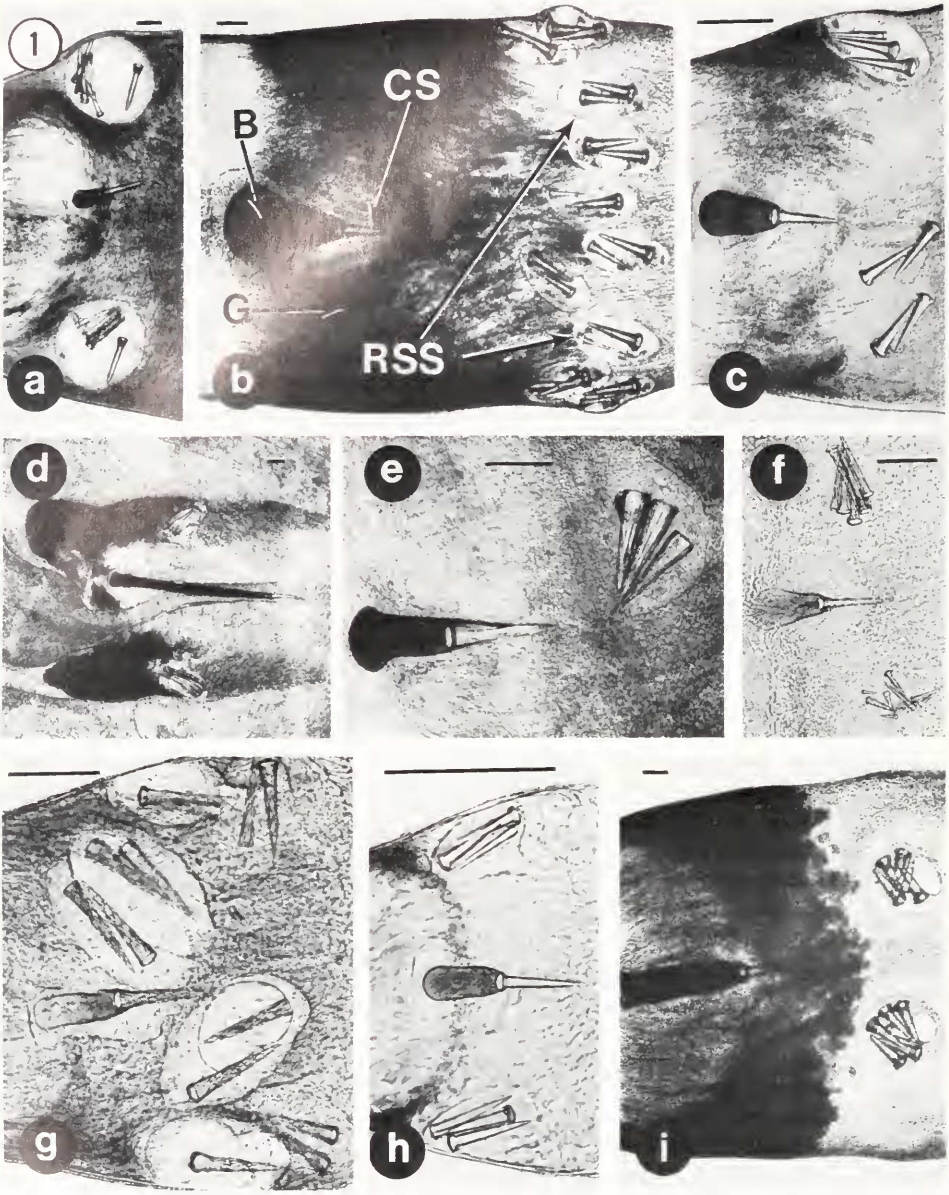

FIGURE 1. Whole mounts of the stylet apparatus in living proboscides removed from adult worms. The central stylet (CS) is attached to a granular basis (B) and surrounded by two to several reserve stylet sacs (RSS), as well as prominent glands (G). Each stylet consists of a knob-shaped proximal piece and an elongate, tapered shaft. Scale bar in 1a–i = 100 μm. a) *Amphiporus bimaculatus*, ×35; b) *A. formidabilis*, ×40; c) *A. imparispinosus*, ×100; d) *Emplectonema gracile*, ×25; e) *E. purpuratum*, ×90; f) *Paranemertes peregrina*, ×80; g) *Paranemertes* sp. ×110; h) *Tetrastemma* sp., ×185; i) *Zygonemertes virescens*, ×40. Note: Figure 1d) depicts the proboscis *in situ*, and 1e) shows only one of the two reserve stylet sacs that are present in *E. purpuratum*.

MATERIALS AND METHODS

The following nine species were examined in this study: *Amphiporus bimaculatus* Coe, 1901; *A. formidabilis* Griffin, 1898; *A. imparispinosus* Griffin, 1898;

Emplectonema gracile (Johnston) Coe, 1901; *E. purpuratum* Coe, 1905; *Paranemertes peregrina* Coe, 1901; *Paranemertes* sp.; *Tetrastemma* sp.; and, *Zygonemertes virescens* (Verrill) Montgomery, 1897. Specimens were collected intertidally on San Juan Island, Washington, or by dredging in adjacent waters. Identifications were based on descriptions presented by Coe (1901, 1905, 1940).

For general studies of styletogenesis in adult worms, whole mounts of living material were examined with a compound microscope. In this paper, the term whole mount always refers to preparations of live, unstained material. The proboscides of thick or pigmented forms were removed in order to observe the stylets, but in relatively translucent specimens, stylets were examined through the body wall.

To study the development of the stylets in larvae, gravid specimens of *Emplectonema gracile* were segregated by sex into bowls containing about 1 liter of filtered sea water, and gametes were obtained following natural spawning or spawning induced by mild shock treatment (30 volts for 5 sec; Powerstat Type 116 voltage generator). The oocytes were washed with filtered sea water and inseminated at various times with diluted suspensions of sperm. Embryos were raised in fingerbowls containing unfiltered sea water at 10–12°C and checked periodically with a compound microscope to monitor the development of the stylets. Egg masses of *Tetrastemma* sp. were collected from the field and reared in the laboratory at 10–12°C. When the stylet apparatus became visible, the worms were removed from their egg mass and observed with a compound microscope.

The rates of stylet formation were studied in three species (*Amphiporus formidabilis*, *Emplectonema gracile*, and *Tetrastemma* sp.), by examining slightly compressed, whole mounts of living, $MgCl_2$-relaxed specimens. The worms were kept in individual containers in the laboratory, and the number of stylets formed by each worm was monitored over a period of several weeks. Some specimens of *Amphiporus formidabilis* and *Tetrastemma* sp. were fed amphipods obtained from the habitat in which the worms were collected; the rest of the experimental worms were not fed during the observation period. In addition, stylets were counted in animals maintained for up to 8 weeks without food, and compared to the number found in worms freshly collected from the field.

The frequency of stylet replacement was studied in *Tetrastemma* sp. following capture of three species of amphipods (*Hyale frequens*, *Paracalliopiella pratti*, and *Aoroides* sp.). The number of reserve stylets was counted in whole mounts of experimental worms prior to prey attack, and the worms were checked within one hour following capture of an amphipod, to see if the central stylet was in the process of being replaced, or if the number of reserve stylets had been reduced.

For histological studies, proboscides removed from $MgCl_2$-relaxed adults, and whole larvae with developing stylets, were fixed, decalcified, and embedded in Epon, according to methods described previously (Stricker and Cloney, 1981; Stricker and Reed, 1981). One micrometer sections were cut with glass knives and stained with a mixture of methylene blue and azure II (Richardson *et al.* 1960), or with a PAS stain, according to the method of Munger (1961).

Results

Comparative morphology of the stylet apparatus

The stylet apparatus of a typical hoplonemertean is located in the mid-proboscis region and consists of a central stylet attached to a basis, and two to several reserve stylet sacs in which reserve stylets are formed (Fig. 1). In most species, the shaft of the central stylet is smooth and straight. Species such as *Emplectonema purpuratum*, *Paranemertes peregrina*, and *Paranemertes* sp., however, have helically-

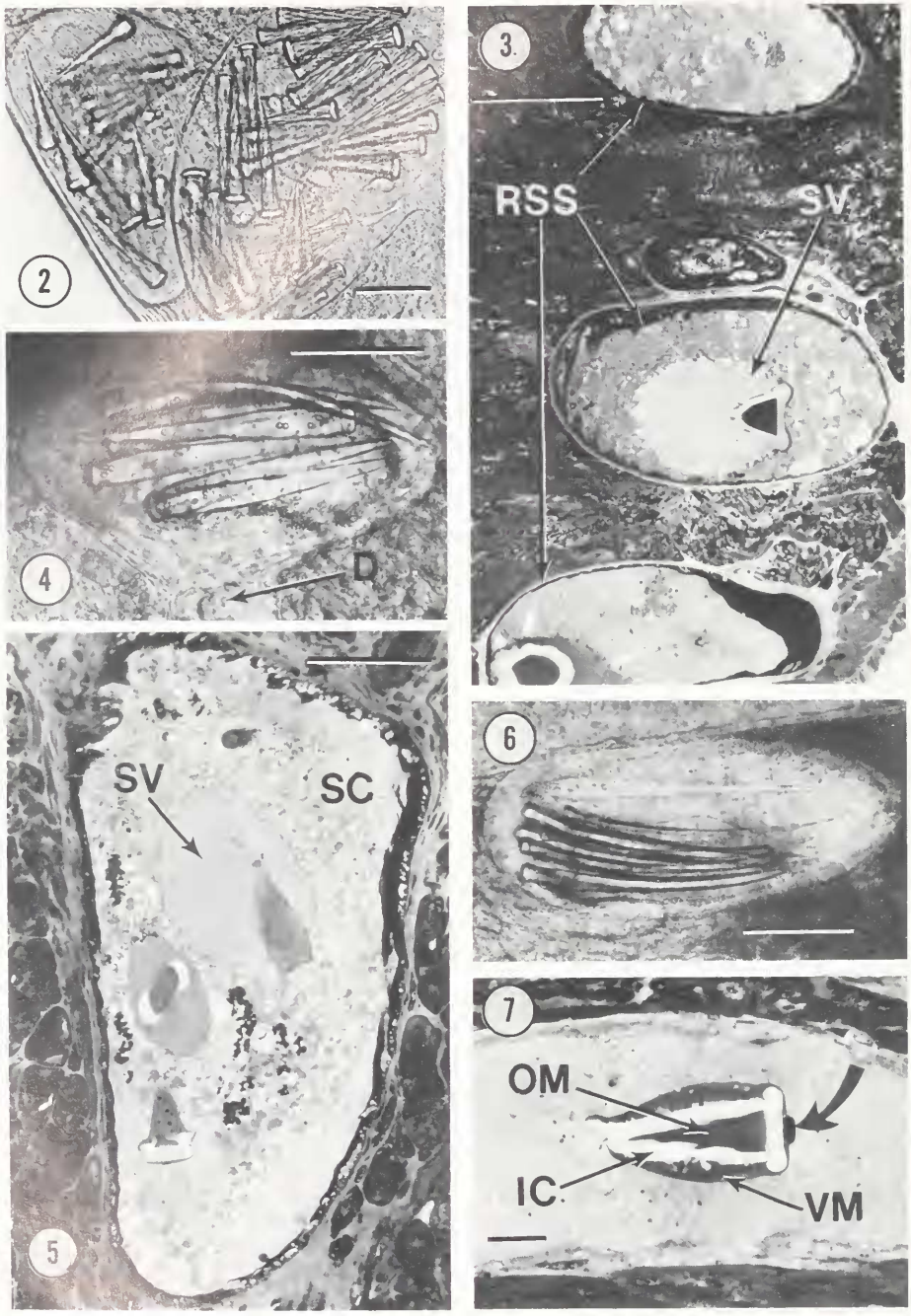

FIGURE 2. Whole mount of two reserve stylet sacs in a specimen of *Paranemertes peregrina* that had an unusually large number of reserve stylets. Reserve stylet sacs typically contain two to several reserve stylets. ×200. Scale bar = 50 μm.

FIGURE 3. Longitudinal section through three reserve stylet sacs (RSS) of *Amphiporus formidabilis*. Each sac consists of a squamous epithelium that envelops a large central cell, called the styletocyte. Stylet vacuoles (SV) are visible within the styletocytes. ×345. Scale bar = 50 μm.

wound grooves along their stylet shafts, and in *E. gracile* the stylet is markedly curved. The basis is an extracellular mass of granules that appear to be partially calcified. It is usually pyriform in shape and equal in length to the central stylet. In *Zygonemertes virescens*, however, the basis is cylindrical with a truncated posterior end, and in *E. gracile* it is much longer than the central stylet.

The reserve stylet sacs are located anterior to, or at the same level as, the central stylet. They usually contain only a few reserve stylets, but on occasion numerous stylets can be seen in a single sac (Fig. 2). The squamous epithelium that comprises the sac surrounds a large central cell, called the styletocyte (Fig. 3), and a duct extends from each reserve stylet sac to provide a pathway for the transfer of reserve stylets from the styletocyte (Fig. 4). One to several stylet vacuoles are located in the styletocyte cytoplasm (Figs. 3, 5). Each vacuole contains a vacuolar matrix that envelops a single developing stylet. Stylets in different stages of development occur in the same styletocyte, and usually do not exhibit any preferred orientation in the styletocyte. An exception occurs in *Emplectonema gracile*, where the stylets tend to be positioned with their proximal pieces facing anteriorly (Fig. 6). Fully formed reserve stylets are identical in structure to central stylets. Central stylets, however, are not located in a cell or enclosed in a vacuole. Each mature reserve stylet has a basophilic organic matrix and an outer inorganic cortex that appears as an unstained area in decalcified preparations of almost all species examined (Fig. 7). In *Amphiporus formidabilis*, however, transverse sections of decalcified stylets often reveal a thin layer of basophilic material that is arranged concentrically around the organic matrix; this layer probably represents organic matrix that is interspersed in the inorganic cortex. In many species the vacuolar matrix of a stylet vacuole contains a basophilic disc of unknown function located next to the proximal piece (Fig. 7, arrow). This disc-like structure has not been observed on any central stylet.

Stylet formation in adults

In all species that we observed, and in those examined by Bürger (1895), stylet formation follows a similar basic pattern in that the stylet shaft develops first and the proximal piece is not formed until the shaft is well developed.On rare occasions, a stylet can be seen with a proximal piece on a very short shaft (Fig. 8). It is not clear in such cases whether the shaft is in the process of actively elongating following the formation of the proximal piece, or if the stylet is fully mature but abnormally short.

During the first stage of stylet formation, a membrane-bound vacuole develops

---

FIGURE 4. Whole mount of a reserve stylet sac of *E. gracile*. A duct (D) arises from each sac and communicates with the region around the central stylet, thus providing a pathway for the transfer of stylets. ×175. Scale bar = 100 μm.

FIGURE 5. Longitudinal section through a reserve stylet sac of *Emplectonema gracile*. The styletocyte (SC) contains a weakly staining ground cytoplasm, in which several stylet vacuoles (SV) are located. ×330. Scale bar = 50 μm.

FIGURE 6. Whole mount of a reserve stylet sac of *Emplectonema gracile*. Stylets in this species tend to have their proximal pieces oriented anteriorly. In all other species examined, no preferred orientation was observed. ×150. Scale bar = 100 μm.

FIGURE 7. Longitudinal section through a styletocyte of *Paranemertes peregrina*. Stylets develop in vacuoles surrounded by a vacuolar matrix (VM). Mature stylets consist of an organic matrix (OM) and an inorganic cortex (IC), that appears as an unstained region in decalcified preparations. In many stylet vacuoles, a basophilic disc of unknown function can be seen next to the proximal piece of the developing stylet (arrow). ×835. Scale bar = 10 μm.

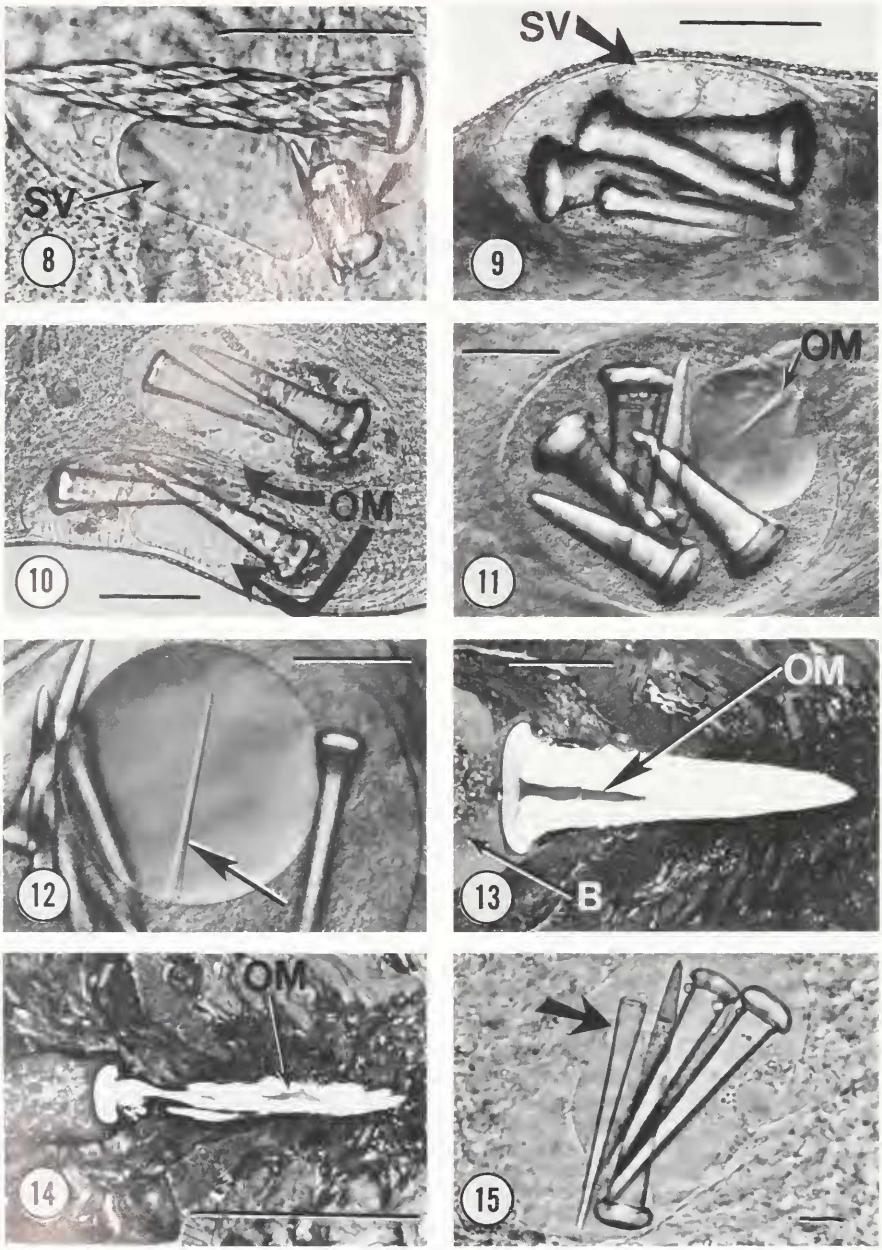

FIGURE 8. Whole mount of a styletocyte of *Paranemertes* sp., showing several reserve stylets and a stylet vacuole (SV). The arrow marks two aberrent stylets that have proximal pieces on their very short shafts. Normally, the stylet shaft develops first, and the proximal piece is not formed until the shaft is well developed. ×500. Scale bar = 50 μm.

FIGURE 9. Whole mount of a reserve stylet sac of *Zygonemertes virescens*. At the onset of stylet formation, a membrane-bound stylet vacuole (SV) forms in the cytoplasm of the styletocyte and becomes filled with a refractile vacuolar matrix, in which a stylet will develop. ×190. Scale bar = 100 μm.

FIGURE 10. Whole mount of two reserve stylet sacs of *Amphiporus formidabilis*. Each sac has a newly formed, conical organic matrix (OM) forming at the edge of the stylet vacuole. ×135. Scale bar = 100 μm.

in the cytoplasm of the styletocyte (Fig. 9). The vacuole is filled with slightly refractile material that is darkly stained in PAS tests. After the vacuole has enlarged, the organic matrix of the stylet shaft begins to develop on the inside of the vacuolar membrane, forming a highly refractile, tapered sliver that is weakly PAS positive (Figs. 10, 11). At an early stage of differentiation, the organic matrix is smooth in most species, but in *Amphiporus bimaculatus* it has fine longitudinal striations (Fig. 12). In fully developed stylets, the organic matrix comprises the core of the shaft and the central part of the proximal piece. The matrix remains visible in proboscides that have been decalcified with E.D.T.A. (6%; pH 7.4), while the rest of the stylet is completely dissolved within several hours. Such decalcified whole mounts as well as reconstructions of serial sections show that the shape of the organic matrix is similar to the general morphology of the cortex (Fig. 13). In *Paranemertes peregrina*, for example, the organic matrix shows helical twists that correspond to the helically twisted grooves and ridges found along the shaft of the stylet (Fig. 14).

After assembly of the organic matrix has begun, the calcified cortex of the shaft becomes deposited around the matrix. The contents of the stylet vacuole diminish as the stylet increases in size (Fig. 15). In well-developed stylets, there is very little vacuolar material left, and the membrane of the stylet vacuole is difficult to detect, except near the junction of the proximal piece and the shaft, as there is a slight indentation in the profile of the stylet in this region (Fig. 16).

The proximal piece is gradually added to a well-developed shaft. Slightly translucent, spherical granules of unknown function are often visible on the shaft in the vicinity of the proximal piece (Fig. 17). As the stylet is being formed in the stylet vacuole, refractile granules that measure about 0.5 to 1.0 μm in diameter can also be seen in the cytoplasm surrounding the stylet vacuoles (Figs. 18, 19). Their abundance varies in the different species examined, and their function is unknown.

In specimens of *Amphiporus formidabilis* and *Emplectonema gracile* monitored over a period of several weeks, stylets were observed to attain full length and have well-developed proximal pieces in 2 to 4 weeks, while in *Tetrastemma* sp. this amount of growth took 3 to 8 weeks (Table I). Additional calcification occurs beyond this point, however, as the overall width of the stylet is increased. The duration of this appositional growth was not determined in this study.

At least one developing stylet was visible in the majority of the specimens examined, regardless of whether the worms had been fed or starved. The rate of

---

FIGURE 11. Whole mount of a reserve stylet sac of *Zygonemertes virescens* that shows a fairly well-developed organic matrix (OM) in a stylet vacuole. The organic matrix is basophilic in sectioned material and remains visible in whole mounts of the styletocyte that have been decalcified with E.D.T.A. Nomarski Differential Interference Contrast (D.I.C.) optics. ×155. Scale bar = 100 μm.

FIGURE 12. Whole mount of a reserve stylet sac of *Amphiporus bimaculatus*, showing a longitudinally striated organic matrix (arrow) within the stylet vacuole. D.I.C. optics. ×155. Scale bar = 100 μm.

FIGURE 13. Longitudinal section of a central stylet in *Amphiporus formidabilis* attached to its basis (B). The shape of the organic matrix (OM) corresponds to that of the cortex. ×280. Scale bar = 50 μm.

FIGURE 14. Longitudinal section through the central stylet and basis of *Paranemertes peregrina*. Decalcified whole mounts and reconstructions of serial sections reveal that the organic matrix (OM) is helically twisted; these helices correspond to the helical grooves and ridges that occur along the shaft. ×605. Scale bar = 100 μm.

FIGURE 15. Whole mount of a reserve stylet sac of an adult *Tetrastemma* sp., showing a developing stylet with an incompletely formed proximal piece (arrow). As stylets increase in size, the size of the stylet vacuole diminishes. ×590. Scale bar = 10 μm.

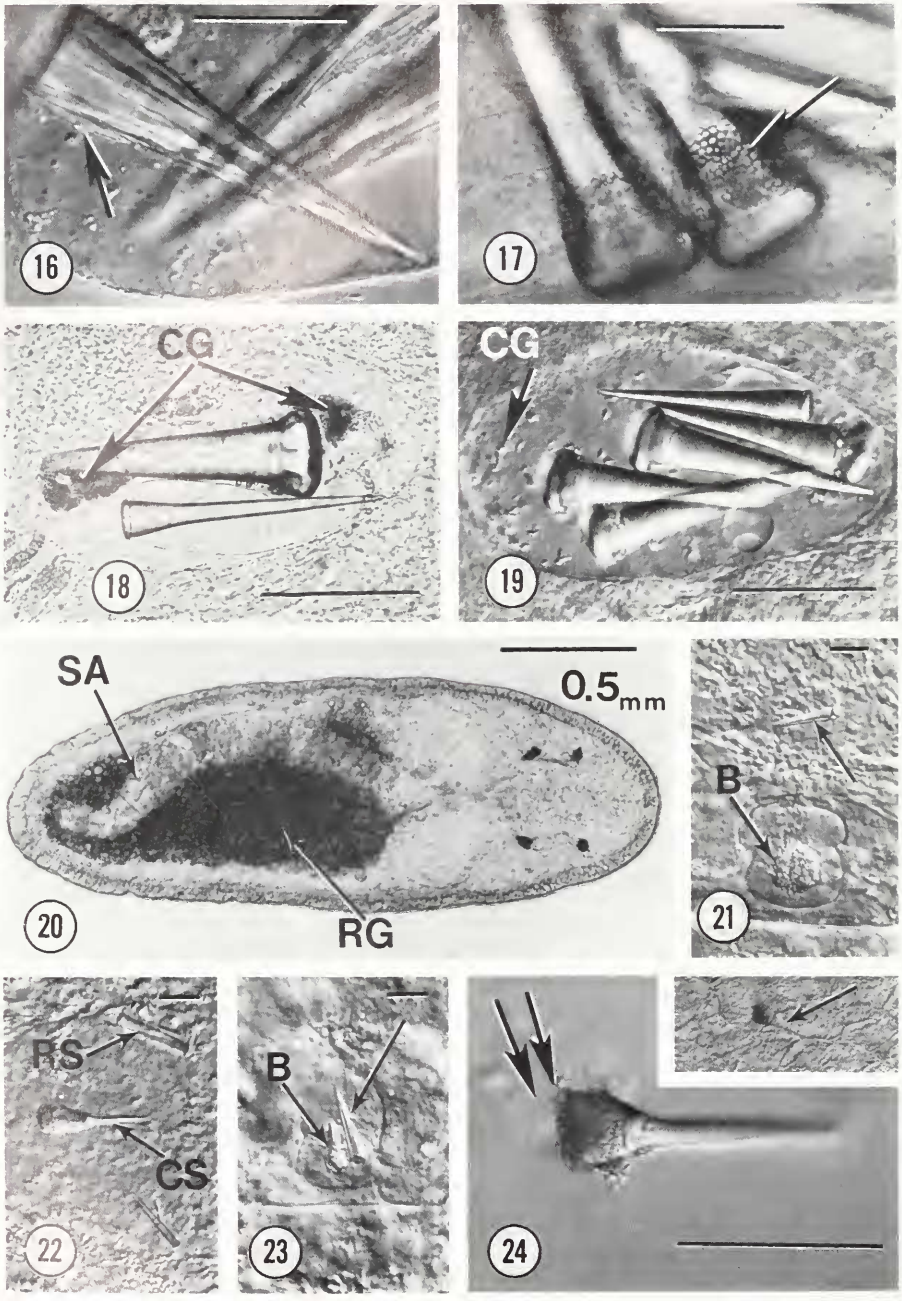

FIGURE 16. Whole mount of a reserve stylet sac of *Emplectonema purpuratum* dissected from the proboscis. The vacuolar membrane is barely discernable near the edge of a well-developed stylet (arrow). This surrounding membrane appears to be left behind in the styletocyte, when the stylet is transferred from the cell to replace the central stylet. D.I.C. optics. ×350. Scale bar = 50 μm.

FIGURE 17. Whole mount of some reserve stylets of *Amphiporus bimaculatus*. Spherical, translucent granules (arrow) of unknown function can be seen at the junction of the proximal piece and shaft in the developing stylets of most species. D.I.C. optics. ×395. Scale bar = 50 μm.

TABLE I

Rates of stylet formation.

Species	Age	Duration of stylet elongation*	Approximate length of mature stylet (μm)
Amphiporus formidabilis	Adult	2–4 weeks	200
Emplectonema gracile	Adult	2–3 weeks	235
	Larva (4 days)	~3 days	15
Tetrastemma sp.	Adult	3–8 weeks	65
	Larva (3 weeks)	4–5 days	12

* Time required for stylet to reach full length and have a proximal piece formed on shaft.

stylet formation did not appear to be significantly different in starved *versus* fed animals. The number of reserve stylet sacs usually remained constant during the several weeks of observation. One small specimen of *Amphiporus formidabilis*, however, increased its number of reserve stylet sacs from 6 to 8 over a period of 2 weeks, while two other specimens lost a sac, presumably during replacement of the central stylet.

Development of the stylet apparatus in larvae

Embryos of *Emplectonema gracile* begin hatching about 1 1/2 days after fertilization, when cultured at 10–12°C, and develop into 200 μm long, free-swimming larvae by the time they are 2 1/2 days old (Table II). Stylets first appear in 4 day old larvae. They can be seen as a pair of small (<5 μm) slivers of refractile material on either side of the developing proboscis. At this stage, there is no sign of the

FIGURE 18. Whole mount of the cytoplasmic granules (CG) in the styletocyte of *Amphiporus formidabilis*. The origin of these granules and their role in stylet formation remain unclear. ×210. Scale bar = 100 μm.

FIGURE 19. Whole mount of the cytoplasmic granules (CG) in the styletocyte of *Amphiporus imparispinosus*. The abundance of these granules varies considerably among different species. D.I.C. optics. ×395. Scale bar = 50 μm.

FIGURE 20. *Tetrastemma* sp.; whole mount of an approximately 4 week old specimen that had been removed from its egg mass. The proboscis is well formed and contains a stylet apparatus (SA), while the rudimentary gut (RG) consists of an undifferentiated mass of yolk. ×35. Scale bar = 500 μm.

FIGURE 21. *Tetrastemma* sp.; whole mount of the stylet apparatus in an approximately 3 1/2 week old specimen that was in the process of attaching its first stylet (arrow) to the basis (B). Reserve stylets begin to form when the worm is about 3 weeks old, and shortly thereafter, they are moved into the lumen of the proboscis, apparently by muscular contractions. D.I.C. optics. ×545. Scale bar = 10 μm.

FIGURE 22. *Tetrastemma* sp.; whole mount of a newly attached central stylet (CS) in a 3 1/2 week old specimen. The central stylet is derived from a reserve stylet (RS) that is made in the reserve stylet sac and transferred to the basis. D.I.C. optics. ×545. Scale bar = 10 μm.

FIGURE 23. *Tetrastemma* sp.; whole mount of an incorrectly positioned central stylet (arrow), found next to the basis (B) in a 4 week old larva. The transfer of reserve stylets to the basis takes several hours to 2 days in this species, and often several reserve stylets are moved into the lumen of the proboscis during the process. D.I.C. optics. ×545. Scale bar = 10 μm.

FIGURE 24. *Tetrastemma* sp.; whole mount of a central stylet in the proboscidial lumen of an adult (inset), as the stylet was being replaced by a new one. The stylet was removed from the proboscis and found to attach to the microscope slide, by way of the adherent basis granules (double arrows). D.I.C. optics. ×540. Scale bar = 50 μm.

TABLE II

Events in the development of the stylet apparatus in Emplectonema gracile *(10–12°C).*

Day	Event
1½	Hatching begins.
2½	200 μm long, free-swimming larva with no stylet apparatus.
4	Reserve stylets present; no basis or central stylet.
5	Basis begins to form.
6	Basis fully formed, surrounded by basis sheath.
7	Reserve stylet moved to basis.
8	Almost all larvae with basis, central stylet, and reserve stylets.

central stylet or basis. The basis becomes visible in larvae that are 5 days old, and is completely formed and surrounded by a basis sheath in larvae that are about 6 days old.

In 7 day old larvae, reserve stylets are moved from the styletocyte and placed on the basis. The entire process takes only a few hours, and it occurs at nearly the same time in almost all the healthy larvae in the culture. Stylets are squeezed from their position at the sides of the proboscis, apparently by contractions of the proboscidial musculature and/or the contractile elements in the epithelial cells surrounding the styletocyte itself. The stylets come to lie anterior to the basis in the lumen of the proboscis, and are moved about by contractions of the body. After the proximal piece is oriented toward the basis, the stylet is moved posteriorly. Contractions of the longitudinal muscles in the proboscis seem to supply the motive force for this movement, and the stylet becomes attached in a slight depression at the anterior end of the basis.

Embryos of *Tetrastemma* sp. reared at 10–12°C develop a proboscis in the region dorsal to their yolk-filled rudimentary gut, and the first stylets form toward the posterior end of the proboscis, when the worms are about three weeks old (Fig. 20). Within 2 to 3 days following the appearance of the reserve stylets, the basis is formed, and shortly thereafter a reserve stylet is transferred to the basis. The entire process of stylet attachment takes several hours to two days, and it occurs at markedly different times in worms that develop in the same egg mass.

The reserve stylet sac epithelium that surrounds the styletocyte appears to be well developed at the onset of stylet transfer. This epithelium seems to contract during expulsion of the stylets and can remain in a wrinkled form, independent of surrounding muscular contractions. In larvae that are attaching a stylet to the basis, one to several stylets can be seen in the lumen of the proboscis (Fig. 21). These stylets are either moved about by contractions of the body musculature or they remain attached to the inner epithelial lining of the proboscis, apparently because of glandular secretions produced by the proboscidial epithelium. On one occasion, the proboscis was everted under the pressure of the coverslip, and the three reserve stylets in the lumen of the proboscis remained attached to the proboscis, even after the proboscis was turned fully inside out.

Following the transfer of the stylets to the lumen of the proboscis, the basis can be seen to extrude a string of granules in the direction of a stylet. The granules were never observed in the process of attaching to the stylet, but in some cases a small amount of material that might have been derived from these granules could be seen adhering to the proximal piece of stylets in the lumen of the proboscis. The final placement of the stylets on the basis appears to be dependent on muscular

contractions that move the stylets toward the basis, and attachment may be aided by granules extruded by the basis (Fig. 22).

Occasionally, a stylet can be seen as it is moved posteriorly to a position behind the correct attachment site on the basis (Fig. 23). It is not clear whether such misplaced stylets can be subsequently maneuvered to their correct position on the basis.

Prey capture and stylet replacement

When amphipods are added to a dish containing adult *Tetrastemma* sp., the worms begin to crawl actively about, and within several minutes most of the nemerteans successfully capture a prey. Prey capture in this species is similar to that described for other suctorial-type feeders (McDermott, 1976). At the onset of attack, the worm rapidly everts its proboscis and coils it around the prey. The central stylet stabs the ventral side of the amphipod several times, and shortly thereafter the prey is immobilized; some prey, however, remain quite active while they are entwined by the proboscis, and on one occasion, an amphipod succeeded in pulling out the proboscis of an attacking nemertean. After the prey is immobilized, the nemertean sucks out the soft tissues of the amphipod and leaves a cleaned exoskeleton behind. The entire feeding episode is usually completed within thirty minutes.

In the thirty feeding encounters that were observed, the loss of a central stylet was seen only twice. In both cases, the central stylet was replaced without the actual attachment of the stylet to the basis being observed. On one occasion, the basis was found lacking a stylet as well as granules in its anterior half, and two stylets were observed in the lumen of the proboscis. In the cytoplasm of one of the styletocytes, there were two membranous structures, each of which comprised the outline of a fully formed stylet. These membranes most likely represented the remains of the stylet vacuoles that were left behind after the stylets had been transferred from the cell.

In addition to this instance of stylet replacement, the central stylet was found to be missing in several specimens of *Amphiporus formidabilis*, *Emplectonema gracile*, and *Tetrastemma* sp. that had been maintained in the laboratory without food. In each case, at least one stylet, and sometimes as many as five stylets, could be seen in the lumen of the proboscis, in front of the basis. The stylets could be seen in the proboscidial lumen of a specimen that was not greatly flattened, and they were sometimes observed up to a centimeter away from the basis; both of these facts tend to refute the possibility that compression of the specimen during examination caused an artifactual dislodging of supernumerary stylets into the lumen.

In one specimen of *Tetrastemma* sp., the stylet in the lumen had numerous refractile granules attached to it. These granules were similar in size and structure to those of the basis, suggesting that the stylet represented the old central stylet that was being replaced. When the coverslip pressure was increased to force the stylet from the proboscis, the stylet became firmly attached to the slide by the adhering granules (Fig. 24). The stylets in the proboscidial lumen of the *A. formidabilis* and *E. gracile* specimens that were observed in the process of replacing their stylets also had granules attached to their proximal pieces. Stylet replacement was not observed to completion in any of these specimens, as movement of the stylet was eventually halted, following the treatment with $MgCl_2$ and the compression of the worm that is required to observe the stylets within the body.

TABLE III

The numbers of reserve stylets in worms freshly collected from the field and worms starved in the laboratory.

Species	Field or starved (length of starvation)	N	Reserve stylet sacs/worm		Reserve stylets/sac		Average no. of stylets/ worm
			Avg.	Range	Avg.	Range	
Amphiporus fomidabilis	Field	10	8.8	7–10	1.7	1–3	15.0
	Starved (3 weeks)	6	8.8	8–11	1.9	1–3	16.7
Emplectonema gracile	Field	10	2.0	2–2	6.9	6–10	13.8
	Starved (3 weeks)	8	2.0	2–2	8.9	8–12	17.8
Paranemertes peregrina	Field	10	2.0	1–3	3.2	2–10	6.4
	Starved (8 weeks)	7	2.3	2–3	4.1	2–6	9.4
Tetrastemma sp.	Field	10	2.0	2–2	3.2	1–5	6.4
	Starved (6 weeks)	17	2.0	2–2	3.8	2–5	7.6

Number of reserve stylets in starved vs *fed worms*

To determine whether the total number of stylets differs in starved *vs* fed populations of worms, stylets were counted in specimens freshly collected from the field and in nemerteans that had been starved in the laboratory for up to 8 weeks. In all species examined, the worms that had been starved in the laboratory had a greater number of stylets than those collected from the field (Table III). This difference was not significant at the $P = 0.05$ level in all species tested (Mann-Whitney U, one-tailed test).

Laboratory experiments were also conducted to monitor the number of stylets in starved and fed groups of *Amphiporus formidabilis*. The experimental worms were kept in individual containers with running sea water, and the number of reserve stylets was monitored in slightly compressed whole mounts on a weekly basis. The increase in the number of reserve stylets tended to be greater in starved specimens than in those that were offered prey (Fig. 25). Starved *A. formidabilis* had an average of 2.6 more stylets at the end of 5 weeks, while worms that were fed amphipods *ad libitum* lost an average of 1.3 stylets. This difference in the number of stylets was found to be statistically significant at the $P = 0.05$ level (Mann-Whitney U, one-tailed test).

DISCUSSION

The nine species examined in this study represent three of the six families that comprise the hoplonemertean suborder Monostilifera (classification of Gibson, 1972). The only other nemerteans with stylets belong to the suborder Polystilifera. Polystiliferous hoplonemerteans comprise a small group of pelagic forms and a few benthic species that are distinguished from monostiliferans by having numerous minute stylets on each basis (Brinkmann, 1917). It is not known if the stylets of these hoplonemerteans have the same composition as those found in monostiliferans, or if stylet formation occurs in a similar manner in the two groups.

Monostiliferans of the genus *Gononemertes* have neither reserve stylets nor a central stylet. Coe (1943) has postulated that the loss of the stylet apparatus in these worms is related to their endosymbiotic life style. Members of the genus

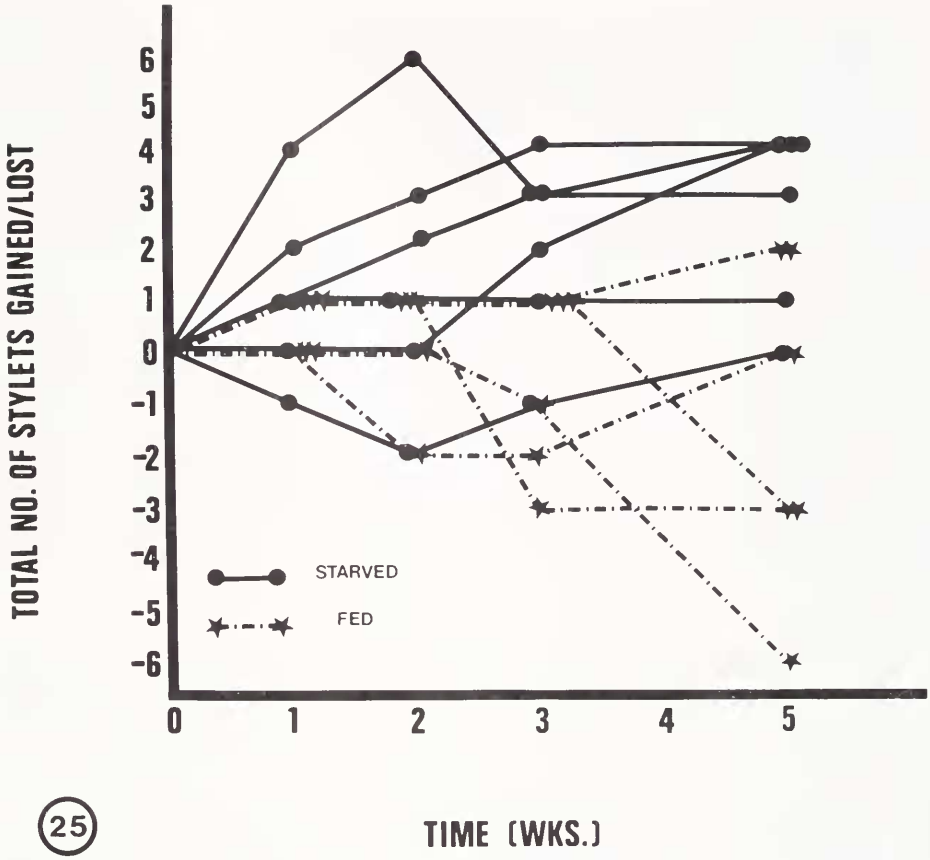

FIGURE 25. The number of reserve stylets gained/lost in starved *vs* fed specimens of *Amphiporus formidabilis* during a 5 week laboratory experiment. Twelve worms, collected from the same locality, were maintained in individual containers with running sea water. Half the worms were starved, while the other half were fed amphipods *ad libitum*. The number of mature reserve stylets was counted in live whole mounts, on a nearly weekly basis. The starved worms had an average of 2.6 more stylets at the end of the 5 week period, while the worms fed amphipods lost an average of 1.3 stylets (statistically significant difference at the $P = 0.05$ level; Mann-Whitney U, one tailed test). Since both groups of worms were observed to form stylets at approximately the same rate, it was concluded that prey capture causes a significant depletion in the number of reserve stylets.

Carcinonemertes (suborder Monostilifera) live on various crabs and eat the eggs of their hosts (Wickham, 1979). Adult worms in this genus possess a central stylet and basis, but they lack reserve stylet sacs (Coe, 1902). Newly hatched larvae of *C. epialti* do not have a central stylet, and an ultrastructural study of the nascent larva failed to reveal any styletogenic cells (Stricker and Reed, 1981). Coe (1943) has suggested that a temporary reserve stylet sac develops and produces the adult central stylet, but further investigations are needed.

Aside from these two aberrent genera, stylet formation in monostiliferous hoplonemerteans occurs in a fairly uniform manner. The following discussion applies to the nine species examined in this study and compares some of the basic features of stylet formation displayed by nemerteans in general.

Site of stylet formation

Although most authors (*e.g.,* Böhmig, 1929; Hyman, 1951; Gibson, 1972) have agreed with the view first presented by Bürger (1895) that stylet formation takes place intracellularly in the reserve stylet sacs, a few have claimed that stylets are formed next to the basis (Montgomery 1894; Coe, 1904, 1905). Montgomery (1894) maintained that the morphologies of the central and reserve stylets are markedly different. Other authors, however, have found the structure of the central and reserve stylets to be identical (Bürger, 1895; Coe, 1904, 1905; Stricker and Cloney, 1981). Apparently, Montogmery did not know that reserve stylets occur within intracellular vacuoles. His drawing of a reserve stylet (Montgomery, 1894) seems to have included the vacuolar matrix in addition to the stylet itself, which may account for his misconception regarding the structure of the two kinds of stylets.

Our observations that the first stylets in larvae develop intracellularly within the prospective reserve stylet sacs, before the basis or central stylet develop, agree with those of Iwata (1960). In the adults of all nine species we examined, stylets were always observed to develop in the styletocyte, and styletogenic cells were not seen at any location other than in reserve stylet sacs, confirming the view proposed by Bürger (1895) that stylet formation in nemerteans occurs intracellularly within the reserve stylet sacs.

Rates of stylet formation

In the adult nemerteans we examined, stylets reached full length and had well-developed proximal pieces in two to eight weeks. It should be emphasized that stylets are not fully formed when they reach this stage. Appositional growth continues for an undetermined length of time, until the stylet attains the width of a central stylet. Thus, the times reported for stylet formation in this study underestimate the minimum time it takes for a stylet to become fully formed. In any case, our observations of stylet formation indicate that styletogenesis in the species we examined takes substantially longer than in other species studied. Sunberg (1979), for example, has shown that in *Tetrastemma laminariae* there is a statistically significant greater number of stylets in specimens that have been starved for 8 days than in those freshly collected from the field, indicating that the assembly of reserve stylets can be accomplished in less than 8 days. According to Bartsch (1973), *Tetrastemma melanocephalum* can regenerate its proboscis within 2 weeks, which implies that stylets can also be formed in this amount of time.

The fact that most of the nemerteans examined in this study contained at least one developing stylet supports Coe's (1943) contention that stylet formation occurs more or less continuously in nemerteans. Reisinger (1926) reported that stylet formation occurs only in specimens that had been fed prey, but we observed developing stylets in worms that had been fed prey as well as in starved specimens.

Although it is probable that stylet formation occurs throughout the life of the worm, whether or not it is fed, there may be an upper limit as to the number of stylets a worm can form. Such a maximum output would account for the fact that most of the specimens that failed to form stylets during the course of this study seemed to be older worms, judging from the size of their bodies and stylets.

Stylet replacement

In nearly all hoplonemerteans, the first stylet apparatus formed is much smaller than that found in the adult. Therefore, the central stylet must be replaced as the

worm increases in size. Coe (1943) has observed a specimen of *Emplectonema gracile* that had a small stylet with an attached basis, apparently in the process of being replaced by a larger stylet and basis. Corrêa (1949, 1954) has made similar observations on other species. According to Gibson (1972), the larvae of some species, in which the adults are quite small (*e.g.*, *Prostoma* and *Tetrastemma*), form a stylet apparatus that can be as large as that found in the adult. These species would not need to replace their stylet except following its loss. The first stylets formed by the *Tetrastemma* sp. that we examined were considerably smaller than the fully formed stylets found in adults; thus, even in this species, the first stylets must be replaced by larger ones during growth of the animal. The only other explanation to account for this increase in stylet size is that further calcification occurs while the stylet is on the basis, but there is no evidence to support this view.

Stylet replacement is also required following loss or damage to the stylet, as occurs during prey attack. According to Reisinger (1926), specimens of *Prostoma rubrum* that were fed oligochaete prey lost their stylet after nearly every feeding episode. In *Paranemertes peregrina*, on the other hand, stylet replacement was not observed in over one hundred specimens examined (Roe, 1970; Stricker and Cloney, 1981). In this study, specimens of *Tetrastemma* sp. that were observed directly following prey attack were only rarely seen to replace their stylets. Starved worms in all four species examined, however, tended to have more reserve stylets than did those that were freshly collected from the field. Since the rates of stylet formation were not seen to differ in the two groups, we conclude that stylet replacement occurs frequently enough in the field following prey attack to cause a depletion in the number of reserve stylets. Although observations of *Tetrastemma* sp. in the laboratory failed to reveal a high frequency of stylet replacement, it is possible that stylets are replaced more frequently in the field, where a greater variety of prey is encountered.

Worms that had been starved for several weeks were also observed in the process of replacing their stylets. In most cases, the stylet that was being replaced was significantly smaller than the new stylet, suggesting that the stylet apparatus was still in the process of growing. On several occasions, the new and old stylets were of equal size, which supports the view held by Reisinger (1926) that stylet replacement occurs throughout the life of the animal, even after growth of the stylet apparatus has ceased. During stylet replacement several stylets were often observed in the proboscidial lumen. Reisinger (1926) also reported that in *Prostoma rubrum*, the entire complement of reserve stylets may be transferred into the lumen during stylet replacement. These observations, coupled with the fact that replacement of the stylet is required at least occasionally following prey attack as well as during times when the animal is not feeding, may account for the relatively large numbers of reserve stylets produced by nemerteans.

The complete sequence of stylet replacement in adult worms was not observed in this study, as the process is apparently terminated by the flattening of the specimen, or the treatment with $MgCl_2$, that is required to observe stylets *in situ*. Reisinger (1926) encountered similar difficulties in observing stylet replacement in *Prostoma rubrum*. The several stages of replacement that we observed indicate that the process occurs in larvae, as well as in adults, in the following manner. One to several stylets are transferred from the styletocyte into the duct of the reserve stylet sac, apparently by contractions of the surrounding muscles and/or the epithelial cells that envelop the styletocyte. This transfer may involve either a partial or complete loss of the contents of a styletocyte, judging from the apparent remains of stylet vacuoles in the styletocyte and the disappearance of an entire stylet sac

following stylet transfer. As shown in *Paranemertes peregrina* (Stricker and Cloney, 1981), the duct of a reserve stylet sac communicates with the proboscidial lumen near the central stylet, so that once the reserve stylets are forced through the duct, they will reach the vicinity of the basis. Stylets in this region of the proboscis are moved about by muscular contractions, and thus oriented so they can be attached to the basis. The ultrastructure of the proximal piece differs from that of the shaft (Stricker, unpublished observations); this difference may increase the chances of the proximal piece being moved toward the basis by muscular contractions. Alternatively, granules may be extruded from the basis and become attached more readily to the proximal piece than the shaft, thus facilitating the correct transfer of the stylet to the basis. The final attachment to the basis appears to be dependent upon the adhesive properties of the basis granules. The ultrastructure of the basis granules in *P. peregrina* suggests that they are adhesive (Stricker and Cloney, 1981), and the fact that an isolated portion of the basis was found to adhere to a microscope slide also supports this view.

Acknowledgments

We are indebted to C. P. Staude for his expert identifications of the amphipods used as prey in this study, and to A. L. Alexander and D. E. Battalia for contributing some of the observations on the development of *Emplectonema gracile*. Throughout the course of this study, G. L. Shinn offered many helpful comments, for which we are grateful. We thank Drs. E. N. Kozloff and A. O. D. Willows of the Friday Harbor Laboratories for making research equipment and facilities available. This study was supported by a Marine/Freshwater Biomedical Core Grant #67-0671, NIH Grant #GM 07270, and a Lerner-Gray Marine Science Research Grant.

LITERATURE CITED

Bartsch, I. 1973. Zur Nahrungsaufnahme von *Tetrastemma melanocephalum* (Nemertini). *Helgoländer Wiss. Meeresunters.* **25:** 326–331.

Böhmig, L. 1929. Nemertini. Pp. 1–110. in Kükenthal, W., and T. Krumbach, Eds. *Handbuch der Zoologie.* Vol **2.** Pt. 1., Walter de Guyter & Co., Berlin.

Brinkmann, A. 1917. Die pelagischen Nemertinen. *Bergens Mus. Skr.* **3:** 1–194.

Bürger, O. 1895. *Fauna und Flora des Golfes von Neapel.* Vol **XXII:** *Nemertinen.* R. Friedländer und Sohn Verlag, Berlin.

Coe, W. R. 1901. Papers from the Harriman Alaska Expedition. XX. The nemerteans. *Proc. Wash. Acad. Sci.* **3:** 1–110.

Coe, W. R. 1902. The nemertean parasites of crabs. *Am. Nat.* **36:** 431–450.

Coe, W. R. 1904. The anatomy and development of the terrestrial nemertean (*Geonemertes agricola*) of Bermuda. *Proc. Boston Soc. Nat. Hist.* **31:** 531–570.

Coe, W. R. 1905. Nemerteans of the west and northwest coasts of North America. *Bull. Mus. Comp. Zool. Harvard.* **47:** 1–318.

Coe, W. R. 1940. Revision of the nemertean fauna of the Pacific coasts of North, Central, and northern South America. *Allan Hancock Pacif. Exped.* **2**(13): 247–323.

Coe, W. R. 1943. Biology of the nemerteans of the Atlantic coast of North America. *Trans. Conn. Acad. Arts Sci.* **35:** 129–328.

Corrêa, D. D. 1949. Ecological study of Brazilian *Ototyphlonemertes. Commun. Zool. Mus. Hist. Nat. Montev.* **3:** 1–7.

Corrêa, D. D. 1954. Nemertinos do litoral Brasileiro. *Concur. Docen-Livr. Cad. Zool. Fac. Filos. Cienc. Univ. S. Paulo* **19:** 1–91.

Gibson, R. 1972. *Nemerteans.* Hutchinson and Co., London.

Humes, A. G. 1942. The morphology, taxonomy, and bionomics of the nemertean genus *Carcinonemertes. Ill. Biol. Monogr.* **18**(4): 1–105.

Hyman, L. 1951. *The Invertebrates*, Vol **II:** *Platyhelminthes and Rhynchocoela.* McGraw-Hill Book Co. New York. Pp. 459–531.

IWATA, F. 1960. Studies on the comparative embryology of nemerteans with special reference to their interrelationships. *Publ. Akkeshi Mar. Biol. St.* **10:** 1–51.

KEM, W. R. 1973. Biochemistry of Nemertine Toxins. Pp. 37–84 in Martin, D. F. and G. M. Padilla, Eds. *Marine Pharmacognosy.* Academic Press. New York.

MCDERMOTT, J. J. 1976. Observations on the food and feeding behavior of estuarine nemertean worms belonging to the order Hoplonemertea. *Biol. Bull.* **150:** 57–68.

MONTGOMERY, T. H. 1894. Über die Stillette der Hoplonemertinen. *Zool. Anz.* **17:** 298–302.

MUNGER, B. L. 1961. Staining methods applicable to sections of osmium-fixed tissue for light microscopy. *J. Biophys. Biochem. Cytol.* **11:** 502–506.

REISINGER, E. 1926. Nemertini. *Biologie Tiere Dtl.* **17:** 7.1–7.24.

RICHARDSON, K. C., L. JARRETT, AND E. H. FINKE. 1960. Embedding in epoxy resin for ultrathin sectioning in electron microscopy. *Stain Technol.* **35:** 313–323.

ROE, P. 1970. The nutrition of *Paranemertes peregrina* (Rhynchocoela: Hoplonemertea). I. Studies on food and feeding behavior. *Biol. Bull.* **139:** 80–91.

STRICKER, S. A. 1981. The ultrastructure of stylet formation in nemerteans. *Amer. Zool.* **21:** 989.

STRICKER, S. A., AND R. A. CLONEY. 1981. The stylet apparatus of the nemertean *Paranemertes peregrina*: its ultrastructure and role in prey capture. *Zoomorphology* **97:** 205–223.

STRICKER, S. A., AND C. G. REED. 1981. Larval morphology of the nemertean *Carcinonemertes epialti* (Nemertea: Hoplonemertea). *J. Morphol.* **169:** 61–70.

SUNBERG, P. 1979. Statistical analysis of variation in characters in *Tetrastemma laminariae* (Nemertini) with a redescription of the species. *J. Zool., Lond.* **189:** 39–56.

WICKHAM, D. E. 1979. Predation by the nemertean *Carcinonemertes errans* on eggs of the Dungeness crab *Cancer magister. Mar. Biol.* **55:** 45–53.

WOURMS, J. P. 1976. Structure, composition, and unicellular origin of nemertean stylets. *Am. Zool.* **16:** 213.