



***Bradynectes ensifer* n. sp. (Platyhelminthes: Macrostomorpha) from North Carolina, USA**

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Bradynectes ensifer is a new species of Macrostomorpha from shallow subtidal sediment in Bogue Inlet, NC, USA. It differs primarily from all other named members of the genus in the radically different shape of its copulatory stylet. We also consider historical records of currently unnamed specimens with similar stylet morphology collected from the NC coast by the late Dr. Reinhard M. Rieger and his students.

Sediment was collected in July 2011 from detritus-rich sand that had accumulated in the shallow subtidal of Bogue Inlet, NC, USA. Specimens were extracted from the sediment and studied and photographed alive as described in Whitson *et al.* (2011). Additional specimens were anesthetized with isotonic magnesium chloride and fixed with (and then stored in) a phosphate-buffered glutaraldehyde/formaldehyde mixture (Smith & Tyler, 1984, fixative 4). For sectioning, specimens were rinsed in phosphate buffer, post-fixed in 1% osmium tetroxide (90 minutes on ice), rinsed in phosphate buffer, dehydrated in ethanol, cleared in acetone, and embedded in EMBED 812/Araldite (EMS). Serial sections were cut at 1.5 or 2 µm using a Histo-Butler knife (Diatome, Inc), stained with toluidine blue O (0.6% in 1% aqueous sodium borate), and mounted in PolyMount (Polysciences, Inc). One animal was preserved as a wholemount in lactophenol for stylet morphology. Photos were pair-wise stitched in Fiji (<https://fiji.sc>) where necessary (Preibisch *et al.* 2009). Missing areas of background in stitched images were filled in, and contrast, brightness, and color were adjusted in Photoshop (Adobe, Inc.).

***Bradynectes ensifer*, n. sp.**

The specific epithet (L. “sword-bearer”) refers to the curved stylet, as the late Dr. R.M. Rieger’s helping-name for this species was “säbel” (derived from the German word “Säbel” for “saber”). Type material was deposited at the Smithsonian Institution National Museum of Natural History (NMNH) as follows: Holotype—a serially sectioned specimen (USNM 1642033); Paratypes—one compressed specimen mounted in lactophenol for the stylet (USNM 1642031), two sets of serial sections (USNM 1642032 & USNM 1642034); type locality (34.650075, -77.104558). Material examined: three specimens studied alive and photographed (images deposited at <http://macrostomorpha.info> under specimen numbers MTP JS 31, MTP JS 32, and MTP JS 33—the first, mounted in lactophenol as the above-mentioned paratype); three sets of serial sections [images deposited at <http://macrostomorpha.info> under specimen numbers MTP JS 52 (paratype, sagittal), MTP JS 53 (holotype, sagittal) and MTP JS 54 (paratype, frontal)]; drawings by Rieger of specimens collected in Bogue Inlet (June 17, 1974) and at New River Inlet, NC (June 14, 1972); and one wholemount slide prepared by Rieger of the specimen from New River Inlet.

In transmitted light, free-swimming animals were yellowish-brown, 1–1.5 mm long (Fig. 1A) and narrow anteriorly, with a pronounced widening at the level of the pharynx. Animals moved by slow ciliary gliding or by a leech-like alternate attachment of the anterior and posterior ends. The unpaired testis and ovary lay on the right-hand side of the body (Fig. 1B). The brain was penetrated by rhammite-tracts of the frontal glands; the cell-bodies of these lay at about the level of the germinal zone of the testis. The pharynx led into a sac-like gut that was brown to golden-brown in life and extended posteriorly beyond the maturing oocyte (Fig. 1B). The testis was voluminous (Fig. 1B); a short vas deferens led from the testis to a muscular seminal vesicle (Figs. 1A–C), from which an intervesicular duct (Fig. 1C) led to a prostatic vesicle (vesicula granulorum) with two types of secretion (Figs. 1D,E). On the prostatic vesicle was mounted a stylet with a rounded base

and a long, curved, tapering portion ending in a point with a small oblique opening facing the convex side (Figs. 1D–F), and, possibly, a longitudinal slit or a long fold on the convex surface near the tip (Fig. 1F). The stylet (Fig. 1F) had the following dimensions (see also Table 1): base opening = 30 μm , convex side = 95 μm , concave side = 81 μm , tip opening = 4.4 μm (average of three specimens examined in squeeze preparation). A thickened portion of the musculature surrounding the prostatic vesicle inserted on the anterior extension of the base of the stylet (Figs. 1D,E, arrows). The proximal portion of the male antrum was unciliated and received glands with a small granular secretion (Fig. S1A, ag). Distally, the male antrum was ciliated (for more details see deposited images of specimens studied alive). Cement glands (Figs. 1C, S1A,F) were located in an arc surrounding the male gonopore, and opened on the ventral surface. The female tract comprised a single ovary located medial to and near the posterior end of the testis (Fig. 1B). Both male and female gonads originated ventrally. We were unable to detect female ducts in our live material or our sections, nor was there evidence in sections of a sperm-receiving organ or bursa-like tissue in the posterior extension of the gut (as mentioned by Rieger 1971, for *B. sterreri*, p.226; see his figures 4 and 6f). However, both the holotype and one paratype exhibited what may be a bundle of allosperm in the posterior part of the body (Fig. 1H,I); the threadlike material in these bundles is approximately the same diameter of the nucleus in a mature sperm (comp. Fig. 1H & I to J). Sperm released from the seminal vesicle by squeezing measured 60 μm in length (average of seven sperm from one mature specimen), with a nucleus that appeared serpentine in DIC illumination (Fig. 1G).

TABLE 1. Measurements of the stylet and sperm dimensions of the different *Bradynectes* specimens compared in this study (see Fig 1F for the relevant landmarks). Note that the convex and concave side length measures are curves that follow along the stylet boundary. All measurements are in μm .

| Specimen | Stylet dimensions (mean ¹ ; range) | | | | Sperm dimensions |
|---|---|-------------|--------------|-------------|------------------|
| | base opening | convex side | concave side | tip opening | total length |
| this study | 30; 28.7–31.7 | 81; 79–81.8 | 95; 92–96.3 | 4.4; 2–6.4 | 60 ² |
| Bogue Inlet Rieger drawing (Scale on Fig S1) | 16 | 63 | 80 | 6 | 44 |
| Bogue Inlet Rieger drawing (canonical scale) | 20 | 81 | 98 | 8 | 53 |
| New River Inlet Rieger Drawing (see text) | 13 | 25 | 32 | 2 | 23 |
| Tyler 1975 Bogue Inlet Thesis plate 59 | 20 | 58 | 70 | 4 | 36 |

¹ mean of three individuals; ² mean of seven sperm of one individual

Although the layout of the reproductive system in *Bradynectes ensifer* is generally similar to that in the five previously described members of the genus (Tyler *et al* 2006–2016, Janssen *et al* 2015), it differs substantially in the shape of its copulatory stylet, which is a nearly closed, curved tube with a small angled opening directed at the convex side. In contrast, the existing species possess a stylet that was described by Rieger (1971) as resembling “a wide-open boot with no sole”. It would appear that one of these stylet forms could be derived from the other by great elongation and then curving of the shank of the “boot”, to produce a curved tube, along with reduction in the size of the distal opening (or possibly, the closing of the opening into a slit). As they lack female ducts, it is likely that *Bradynectes* spp. copulate by injecting sperm via hypodermic insemination (Janssen *et al* 2015), and the thickened musculature attached to the anterior base of the copulatory stylet in *B. ensifer* could serve to drive the tip of the protracted stylet into the partner when the musculature surrounding the prostatic vesicle contracts. The existence of what may be a bundle of allosperm in the posterior parenchyma of two of our serially-sectioned specimens provides tentative evidence that copulation in this species involves hypodermic sperm injection.

Rieger apparently noted the existence of this species from Bogue Inlet in North Carolina, USA, over 40 years ago. Among his research material stored at the Institute of Zoology, Innsbruck, Austria is a drawing of a *Bradynectes* species with the helping-name “säbel” from Bogue Inlet, N.C. dated June 16, 1974 (Fig. S1). The drawings of stylet and sperm correspond closely in shape to those of our species. The scale-bar on Rieger’s drawing, added in ink at some point after the drawing was made, would make the stylet and sperm of his specimen approximately 20% and 30% smaller, respectively, than the stylet and sperm measured in our specimens (Table 1). However, comparison of the added scale bar with the “canonical” scales for Wild M20 microscopes used in the Rieger lab at that time suggests that the scale added to the drawing

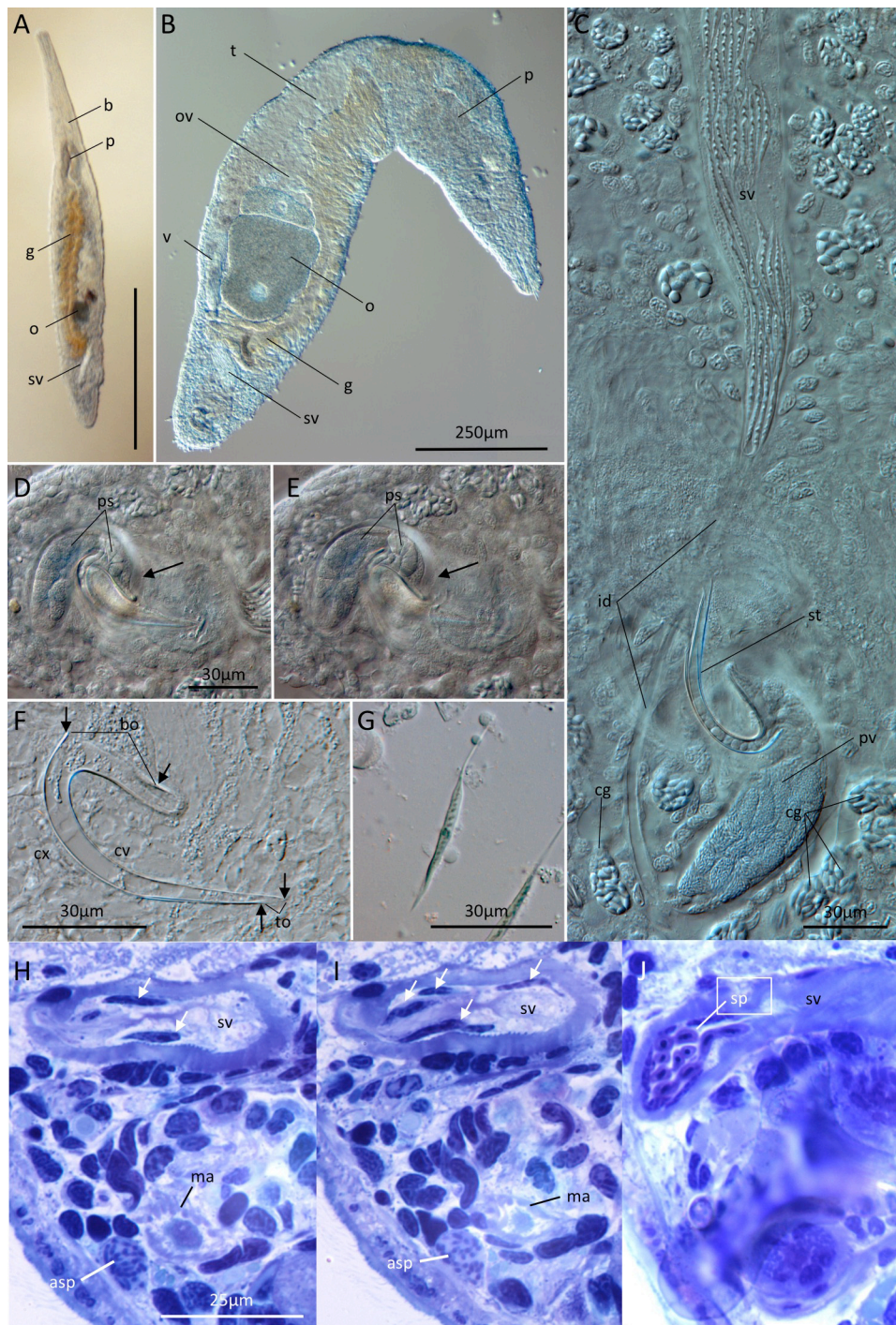


FIGURE 1. Photomicrographs of *Bradynectes ensifer* n. sp. using brightfield (A) and DIC (B–G) illumination. A. Living animal, lightly squeezed, dorsal view: brain (b), pharynx (p), gut (g), maturing oocyte (o), seminal vesicle (sv); scale bar = 0.5mm; B. Squeezed specimen, ventral view. Note pharynx pressed open, extension of gut posteriorly beyond maturing oocyte, anterior extent of ovary (ov) lying medially near the posterior end of testis (t), vas deferens (v) and seminal vesicle (sv); C. High-magnification view of male system, from seminal vesicle with mature sperm leading via the intervesicular duct (id) to the prostatic vesicle (pv) with the stylet (st) and surrounded by cement glands (cg); D,E. Ventrally-focused (D) and more dorsally-focused (E) views of the prostatic vesicle and stylet. Note two types of prostatic secretions (ps) and thick muscle (arrow) of the prostatic vesicle that inserts on the anterior part of the stylet base; F. Stylet, showing base opening (bo) and tip opening (to); arrows indicate endpoints for measurement of convex side (cx) and concave side (cv); G. Sperm, released from the seminal vesicle by squeezing; H,I. Successive 1.5µm sagittal sections of the posterior end, showing seminal vesicle (sv), nuclei of the seminal vesicle epithelium (arrows), cross-sectioned male antrum (ma), and bundle of putative received allosperm (asp); J. Sagittal section, showing cross-sectioned autosperm (sp) in vesicula seminalis. Panels H–J printed at the same magnification.

actually represents 50 μm , instead of the 40 μm indicated by the tick-marks, rendering the size-differences mentioned above insignificant (Table 1). In either case, we are confident that Rieger's drawing of a specimen from the same site as ours corresponds to the species we have described above. Finally, Tyler's figure of the stylet and sperm from his *Bradynectes* sp. (Tyler, 1975) indicate that both of these structures were approximately the same size as those illustrated in Rieger's drawing—stylet: base opening=20 μm , convex side=58 μm , concave side=70 μm , tip opening=4 μm ; sperm 35–38 μm (Table 1).

The likely existence of a second, similar species is posed by an earlier Rieger drawing of a *Bradynectes* species designated with the helping-name "säbel" collected from the New River Inlet, N.C. dated June 14, 1972 (Fig. S2). Although this specimen exhibits a similar stylet morphology to that in *B. ensifer*, notes on the drawing indicate that the animal was only 0.6mm–0.7mm long, or about half the size of our species. A single wholemount slide prepared by Rieger bears the same collection number and helping name, and, as best as can be seen, the stylet in this wholemount is roughly the same size as the stylet in the drawing. Working backward from the canonical scales referred to above, the length of the sperm in this species is 27 μm , close to the 22–23 μm indicated on this drawing. Using the stated length of the sperm (23 μm) for calibration, the drawing of the squeezed specimen using the 20x objective measures 670 μm , exactly in the range given for body length in the notes. Using the same calibration, measurements of the stylet in this drawing (100x objective) give: base opening=13 μm , convex side=25 μm , concave side=32 μm , tip opening=2 μm , so considerably smaller than our specimens (Table 1). Specimens of *Bradynectes* collected from this site and fixed for electron microscopy were used by Doe (1981) in his doctoral thesis work.

It appears that Rieger referred to the New River Inlet species as *Bradynectes* "short-stylet" and to the Bogue Banks species (discovered later) as *Bradynectes* "long-stylet" (S. Tyler, pers. com.). Given the size difference and the fact that this smaller species has not been collected again in the intervening 40+ years, we do not formally assign the specimen depicted in Figure S2 and preserved in Rieger's wholemount or the *Bradynectes* sp. studied by Doe to our new species.

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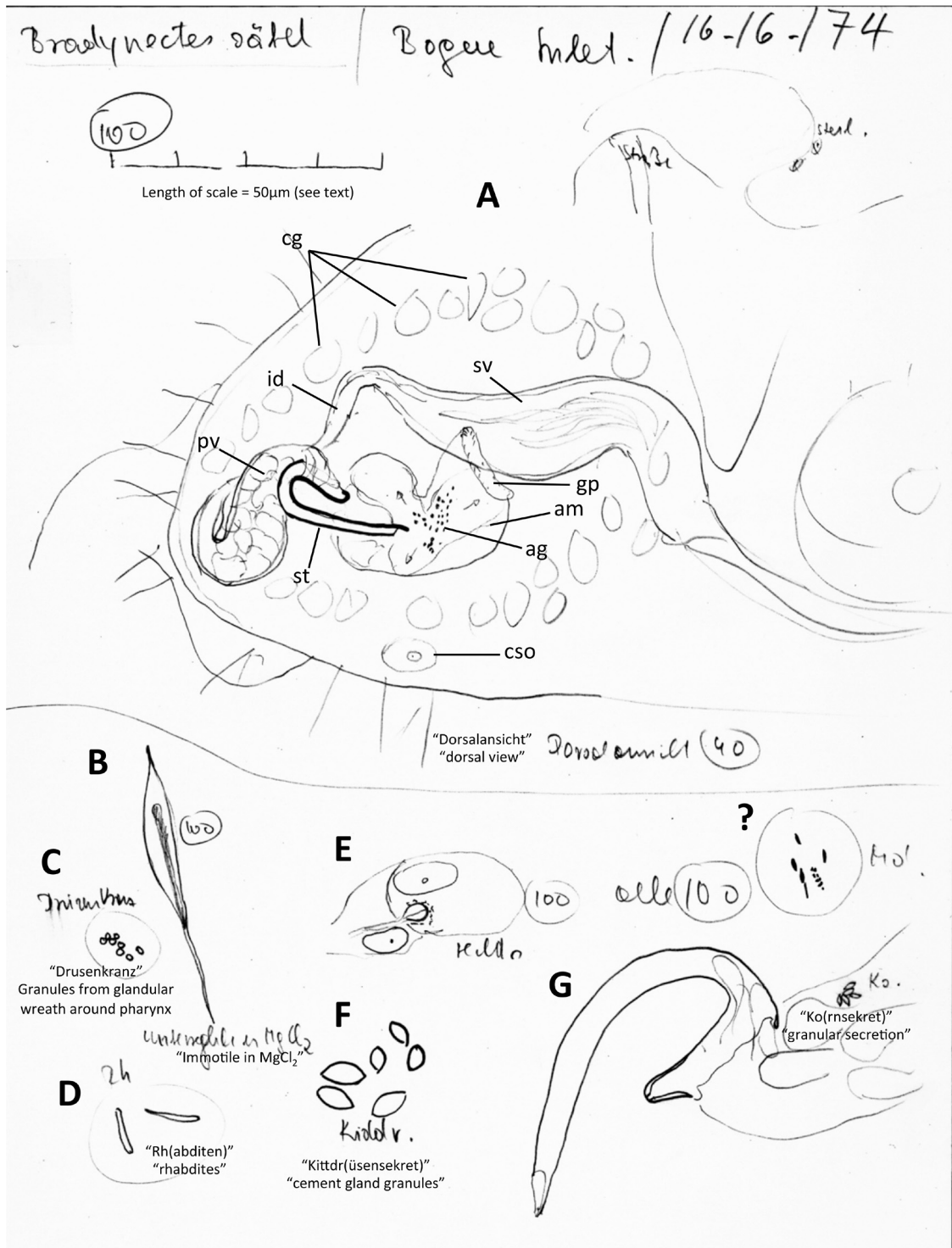


FIGURE S1. Camera-lucida drawings by Reinhard Rieger of a specimen of *Bradynectes ensifer* n. sp. (designated with the helping-name "säbel") collected in Bogue Inlet NC, USA, dated June 16, 1974. Note that the printed lettering was added by us, that we tried to capture and translate as much of the German notes as possible, and that the circled numbers indicate the objective used. A. Dorsal view (40x), showing cement glands (cg), male gonopore (gp), male system: male antrum (am) with proximal fine-granular secretion (ag), stylet (st), prostatic vesicle (pv), intervesicular duct (id), and seminal vesicle (sv); one caudal sensory organ (cso) is also shown. All other drawings are 100x. B. Sperm (possibly not fully mature?); C. pharyngeal gland secretions; D. Rhabdites; E. Caudal sensory organ (see Rieger & Tyler 1974, p. 163); F. Cement-gland granules; G. Stylet and prostatic vesicle; and ?. gland granules possibly located around the mouth or male opening. The length of the scale bar probably represents 50µm, and not 40µm (see main text).

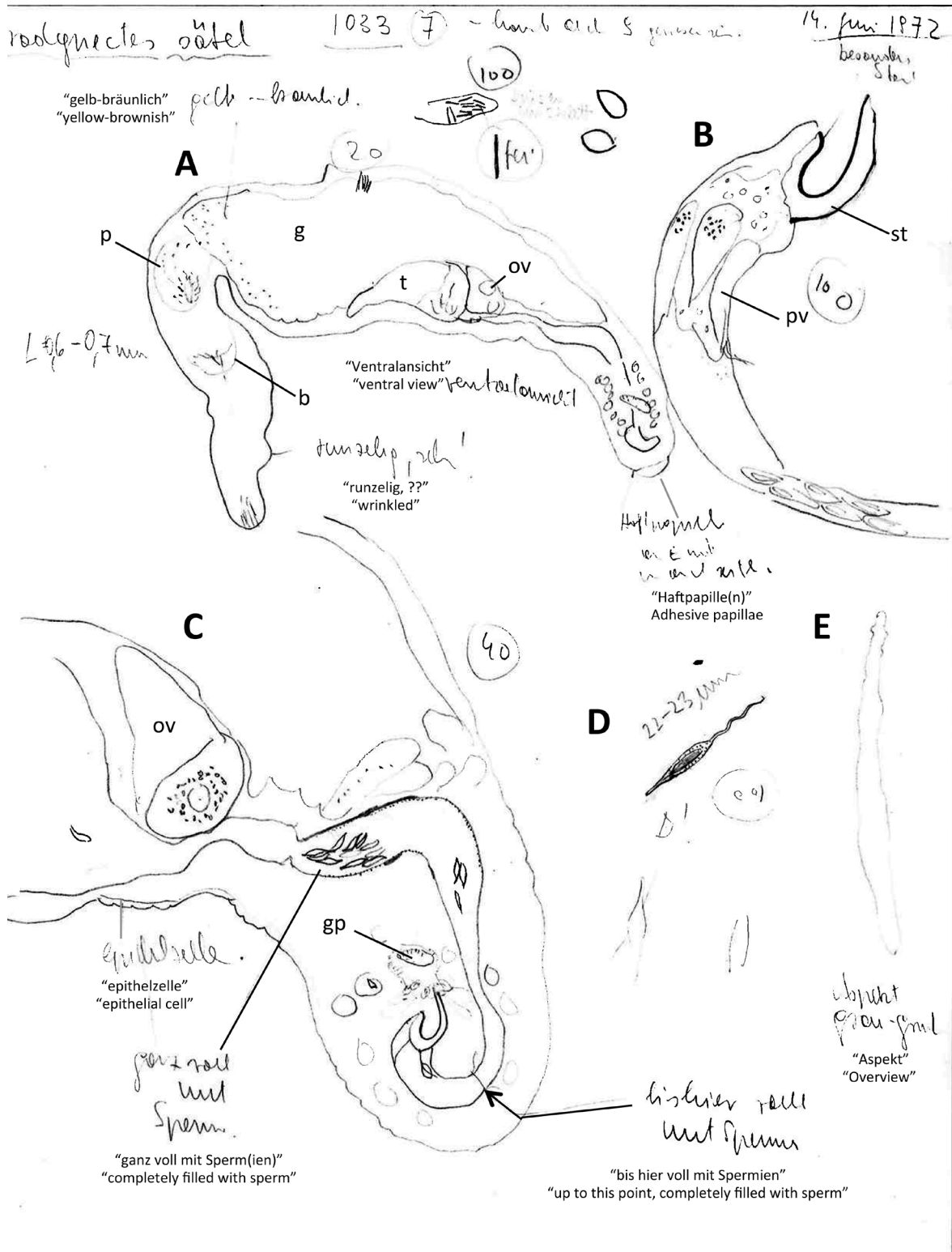


FIGURE S2. Camera-lucida drawings by Reinhard Rieger of a specimen of *Bradynectes* sp. (also originally designated with the helping-name "säbel") collected at New River Inlet, dated June 14, 1972. Note that the printed lettering was added by us, that we tried to capture and translate as much of the German notes as possible, and that the circled numbers indicate the objective used. A. Ventral view (20x), showing brain (b), pharynx (p), gut (g), testis (t), ovary (ov); B. Prostatic vesicle (pv) and stylet (st) (100x); C. Posterior end (40x) showing ovary, male gonopore (gp), and notes indicating position of sperm in seminal vesicle and proximal half of prostatic vesicle. D. Sperm (100x). E. Overview of living specimen, wrinkled due to exposure to magnesium chloride.