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***Lehardyia alleithoros*, sp. nov. (Turbellaria, Kalyptorhynchia) from the Coast of North Carolina, USA**

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Abstract

As with other high-energy beaches, those of North Carolina harbor a diverse fauna of kalyptorhynch turbellarians, and most appear to be new to science. Here, we describe *Lehardyia alleithoros*, a new kalyptorhynch turbellarian of the Karkiorhynchidae, from 3 high-energy beach sites in North Carolina. We also report an apparent range extension for *Carcharodorhynchus flavidus* Brunet, 1967. These observations bring the total number of kalyptorhynch turbellarians reported from the marine interstitial environment of North Carolina to five.

Introduction

Although interstitial sand fauna is both speciose and widely distributed, it has been noted that the distribution of known species often depends more strongly on the distribution of taxonomists than on ecological factors within the meiobenthos (e.g., Curini-Galletti 2001). Among Kalyptorhynchia, an important order of predatory flatworms, only 14 of the approximately 530 known species are described or reported from the entire southeastern United States (collectively, the coasts of North Carolina, South Carolina, Georgia, and Florida—data compiled from Tyler et al. 2010). Hence, we have begun an intensive study of the interstitial kalyptorhynch fauna from four sites along the coast of North Carolina. This paper contributes one new species and extends the range of an existing species formerly known only from the coast of France.

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Authors' Contributions

AW carried out studies of the living specimens, prepared material for and imaged all specimens of *L. alleithoros* for CLSM, made the initial measurements of organ size and position, executed preliminary reconstruction drawings, and rough-drafted the manuscript. JSIII prepared and imaged specimens of *C. flavidus* for CLSM, prepared serial sections, checked organ positions and sizes using FIJI/TrakEM2, executed the final reconstruction drawings and made final edits on the manuscript. MKL sequenced material preserved in ethanol. All three authors edited drafts and approved the final manuscript before submission.

Materials and Methods

Samples were collected from the lower part of the open beach (“swash” or “otoplanid” zone, terminology following Giere 2009) at Emerald Isle, NC, at Long Beach, NC, and from the shallow (<0.5 m) subtidal zone in Lockwoods Folly Inlet, NC. GPS waypoints (Garmin eTREX Legend H) were used to mark collection sites. Magnesium chloride anesthesia/decantation was used to extract the animals from the sediment (Hulings and Gray 1971). Individual specimens were selected using a Wild M5 stereomicroscope, anesthetized with $MgCl_2$, and examined, drawn and photographed using Nikon or Olympus microscopes equipped with DIC optics and digital cameras.

Specimens for confocal laser scanning microscopy (CLSM) were anesthetized as above, fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) with 10% sucrose, rinsed thoroughly in PBS with 0.1% bovine serum albumin, permeabilized for 1 hour in BSA-2T (PBS, 1% BSA, 0.2% Triton X-100), rinsed thoroughly as above, and stained overnight in 1:500 diluted Hoechst 33242 and 1:200 diluted Alexa 488/phalloidin (Invitrogen), then mounted in 9:1 Glycerin:10x PBS. Coverslips were supported by double layers of aluminum foil, and attached to the slide using nail polish. Two specimens were additionally stained for acetylated tubulin and for mitotic cells, using primary antibodies diluted 1:200 (anti-Acetylated Tubulin—Sigma; anti-PhosphoH3—Upstate) in 1x PBS, with appropriate secondary antibodies also diluted 1:200 (Alexa568-labeled Goat anti-rabbit—Invitrogen; Cy5-labeled Donkey anti-mouse—Jackson). CLSM stacks were collected on an Olympus Fluoview FV1000. Specimens for serial sectioning were fixed in hot (60°C) Bouin’s fluid, and embedded, sectioned, and stained according to Smith and Tyler (1984). Two of the whole-mounted specimens used for CLSM were removed from the slide, dehydrated, and prepared as permanent whole-mounts in epoxy resin.

A single specimen from our collection site on Bogue Banks, NC (see below) was preserved in absolute ethanol for sequencing. Total genomic DNA was extracted according to the protocol supplied with the DNEasy Kit (Qiagen Inc., Valencia, CA). PCR conditions followed Litvaitis et al. (1994), and primer sequences targeting the D1-D2 expansion segment of the 28S rDNA gene can be found in Sonnenberg et al. (2007). The amplicon was gel-purified and sequenced in both directions at the University of New Hampshire Hubbard Center for Genome Studies. Trace files were edited using FinchTV (vers. 1.4; Geospiza Inc.) and the sequence was deposited in GenBank under Accession No. JF340473.

Digital images were combined where necessary by 2D stitching (Preibisch et al. 1999); single images of serial sections were assembled into stacks within FIJI, registered using SIFT (rigid transform) and measured in FIJI. TrakEM2 (Cardona et al. 2010) was used to segment organ positions and generate 3D reconstructions which were used to guide the production of drawings. All images for final plates were contrast- and brightness-adjusted in Adobe Photoshop (CS5). Except as noted, measurements of *L. alleithoros* were made on six specimens (two whole-mounts, two sets of serial sections, and two anesthetized squeezed living specimens); adhesive papillae were counted only on the two serially-sectioned specimens; standard deviations are listed after the mean. In the descriptions below, positions

of anatomical structures are given as percentage of total body length in the format “U#”, where “U0” is the anterior tip and “U100” is the posterior tip.

***Lehardyia alleithoros*, n. sp**

Taxonomy: Phylum Platyhelminthes Minot, 1876; Order Kalyptorhynchia Graff, 1905; Suborder Schizorhynchia Meixner, 1928; Family Karkinorhynchidae Meixner, 1928; Subfamily Karkinorhynchinae Schilke, 1970; Genus *Lehardyia* Karling, 1983; *Lehardyia alleithoros* sp. nov.

Etymology: The specific epithet *alleithoros* is latinized from the Greek ἀλλήθωρος (cross-eyed) and refers to the closely spaced pigment-cup eyes that appear to form an “X” in the anterior end of the living animal.

Type Material: Deposited at the National Museum of Natural History (NMNH) Smithsonian Institution, Washington, DC. Holotype, a whole-mounted specimen from NC (NMNH XXXX); paratypes two sectioned specimens and a whole-mounted specimen from NC (NMNH XXXX–XXXX) and one whole-mounted specimen from FL (NMNH XXXX).

Occurrence: Type Locality. Swash zone at low tide on Oak Island, NC, USA, within 50 m, parallel to the waterline, of the location 33°54′46″N; 78°13′05″W. Other: Shiny zone at low tide near Emerald Isle on Bogue Banks, NC, USA (34°38′41″N; 77°5′23″W) and outer beach at Pepper Park, FL (28°50′N; 80°45′W).

Material examined: Eleven specimens examined and photographed alive in squeeze preparation; eleven specimens examined as whole-mounts by CLSM (two subsequently prepared as permanent whole-mounts); 9 specimens serially sectioned and stained; 7 whole-mounted specimens, photographs, and CLSM stacks of material collected at Pepper Park, FL, by Dr. Rick Hochberg; unpublished sketch by R.M. Rieger, showing “Karkino augi” from the lower beach at Bogue Banks, NC, dated 1970.

Description

External Anatomy: Under a stereomicroscope using transmitted light, the animal appeared translucent light brown in color, but was not pigmented except for a pair of closely-spaced pigment-cup eyes that appeared to form an “X” shape posterior to the proboscis. The large pharynx and paired adhesive belts were easily discernable. The animal tapered to a blunt point anteriorly and was broadly rounded posteriorly. From about the level of the brain to the posterior end, the body was cylindrical. In the extraction dishes, *L. alleithoros* was found either swimming or attached by the posterior adhesive belt along the side of the dish. Animals used both adhesive belts when crawling and when attempts were made to dislodge them from the dish with a jet of water from the pipette. One specimen was observed feeding on a piece of detritus with the pharynx protruded from the body. The average body length was $1294 \pm 249 \mu\text{m}$ and the average width was $217 \pm 28 \mu\text{m}$. (Figs. 1A, 2A, 2C).

Body wall: The entire epidermis of *L. alleithoros* was ciliated, possessing intraepithelial nuclei and small rounded epitheliosomes that were slightly eosinophilic in sectioned

material. There were two belts of adhesive pads—one, located behind the pharynx at U52 (n=2) with 10 papillae and another, with six to eight papillae, near the posterior end at U96 (n=2). The epidermis was underlain by circular and longitudinal muscles. Crossed-helical diagonal muscles occurred in the pre-cerebral part of the body (Fig. 2B). Anti-phosH3 staining for mitotic cells was observed in the testes and in occasional dividing cells underneath the body wall (data not shown).

Proboscis apparatus: *L. alleithoros* had a small, split proboscis, opening at the anterior tip of the body. The proboscis comprised two muscular lobes (average length in living specimens and wholemounts $59 \pm 7 \mu\text{m}$), each armed with a pair of hooks. Each hook averaged, in living specimens and wholemounts, $31 \pm 2 \mu\text{m}$, and was armed with a single denticle (Figs. 1B–D). Divaricator muscles occurred along the outside edge of the proboscis lobes (Fig. 1B). At the base of the proboscis, a postrostral bulb, surrounded by a weak layer of circular muscle, contained the lateral glands (Fig. 2B). Paired ventral and lateral proboscis retractors inserted on the proboscis through the post-rostral bulb and ended on the ventral and lateral body wall, respectively, at about the same level as the insertion of the post-cerebral septum. An unpaired dorsal proboscis retractor arose on either side of the dorsal proboscis divaricator muscle and ran posteriorly inside the post-rostral bulb, exiting the bulb about one-third along its length. At the anterior margin of the brain, the dorsal proboscis retractor split, and left and right branches inserted on the dorsal body wall at about the level of the eyes. A net-like post-cerebral septum comprising pseudostriated muscle was present (Fig. 2B). Paired dorso-lateral and ventro-lateral anterior retractor muscles arose on the lateral body wall just behind the mouth and inserted on the body wall at the anterior tip (Fig. 2B).

Pharynx: The elongate pharynx, located in the anterior one-half of the body, averaged $294 \pm 23 \mu\text{m}$ long and $109 \pm 23 \mu\text{m}$ wide, possessed a prominent anterior glandular (“grasping”) margin, and was located from U20 to U43 (Figs. 1A, 2D). The pharynx was connected by a relatively short pharyngeal cavity to the mouth, located at U15. The pharyngeal cavity was folded when the animal was not feeding, everted when the pharynx was protruded from the body, and possessed stout internal circular and somewhat weaker longitudinal muscles (Fig. 2B). The epithelium lining the pharyngeal cavity was thin and ciliated. The pharynx was of complex construction, regionated among the distal grasping fold, the middle, and the proximal portion (Fig. 2E). Muscle layering comprised outer longitudinal, outer circular, pseudostriated radial muscles (thickest in the middle), inner circular (thickest in the middle) interspersed with inner longitudinal (Fig. 2E).

Nervous System: The proboscis pore at the anterior tip was bordered by four tufts of sensory cilia (Fig 1A). The brain was dorsally located in the anterior half of the body and was immediately between the proboscis and the pharynx. The eyes were located within the brain at U10, possessed lens-like spherical aggregations of photoreceptors, and the pigmented portions of the eyes were extremely close together (Fig. 1A). Attempts to stain the nervous system immunocytochemically with anti-acetylated tubulin were unsuccessful, although all body ciliation was stained strongly with this antibody.

Male reproductive system: The reproductive system was in the posterior half of the body on the ventral side. The 6 testes, arranged as two bilateral pairs of 3 testes, extended laterally and dorsally behind the pharynx beginning at U52 (Figs. 1A, 2C). In two of the serially-sectioned specimens, the three testes on one side appeared to have degenerated. Fine ducts proceeded from the testes to form 2 muscular seminal vesicles, located posteriorly and ventrally to the testes (Figs. 2C, E). The vesicles were always found full of sperm and were surrounded by helically-arranged broad bands of muscles (Figs. 2C, E, F). The copulatory bulb was elongated, pear-shaped, contained two types of glands whose secretions filled the region around the ejaculatory duct, and possessed outer circular and inner longitudinal muscles (Figs. 1E, 2F, 3A). The very short muscular penis was armed internally with a short “cuticular” stylet in the form of a slightly curved, laterally-flattened, truncated cone, averaging, in three living specimens, $13.6 \pm 1.7 \mu\text{m}$ long and $14 \pm 0.9 \mu\text{m}$ wide at the base (Figs. 1E, 2F, 3A). The common genital atrium arose from the gonopore at U67, was underlain by strong circular muscles along the first one-third of its length, and was otherwise surrounded by fine longitudinal muscles (Figs. 2E, F). About midway along the atrium, a prominent crown-shaped glandular region occurred; this region possessed basophilic granular gland cells (Fig. 1E, 2F). Proximally to this glandular region, a small opening led anteriorly to the vagina interna (Figs. 2C, F). Surrounding the upper part of and opening into the atrium were eosinophilic gland cells (Figs. 1E, 2F).

Female reproductive system: The vagina interna arose on the anterior face of the common genital atrium as noted above and widened greatly as it proceeded posteriorly on the left side of the body (Figs. 2C, E, F). The vagina interna, which appeared to be sparsely ciliated internally in living material (Fig. 1E), possessed internal longitudinal and external circular muscles and ended near the ventral body wall in a small vesicle with weak musculature that was filled with foreign sperm (Fig. 2F). At the juncture between the vagina interna and the vesicle, two short muscular canals led to the bursa (Figs. 2E, F). The bursa varied widely in size among our specimens. In many specimens, multiple bursa-like vesicles containing foreign sperm were found throughout the bursal tissue (Fig. 1E). Except for being embedded in the bursal tissue, these possessed no discernable connection to the remainder of the female genital system. The single germarium was embedded in the bursal tissue in the dorsal mid-line, close behind the glandular region of the common genital atrium (Figs. 1E, 2E, 2F). With the exception of a single CLSM stack, no sperm duct connecting the bursa to the germarium was visible in our material. From the germarium, a germo(vitello-?)duct led anteriorly to open into the common genital atrium through a small pore in the upper glandular region (Fig. 2F); this region was equipped with a sphincter muscle and a radiating corona of longitudinally-oriented muscles. The vitellaria were located ventrally on either side of the mid-line from the posterior end of the testes nearly to the posterior end of the body; no ducts leading from the vitellaria to the germoviteloduct region described above were discernable in our material.

Discussion

Taxonomic considerations: The recent revision of Karkinhynchidae by Karling (1983) requires placement of *L. alleithoros* in the Karkinhynchinae primarily because of the presence of eyes (see below concerning lateral gland sacs). Generic placement in *Leyhardyia*

is warranted by proboscis hooks with denticles and a penis armed with a stylet, not a cirrus (Karling, 1983). The pharynx of *L. alleithoros* is similar in its regionation to that of *L. megalopharynx* (L'Hardy 1966), and possesses a glandular grasping margin like that figured for *L. tetragnathus* (Ax and Schilke 1971). In addition, the ducts belonging to the female system are identical in their basic layout to that found in *Lehardyia tetragnathus* (Ax & Schilke, 1971). However, the possession of six testes located posterior to the pharynx in *L. alleithoros* differs from the condition in the two congeners, and requires a modification of the diagnosis, as follows:

Genus *Lehardyia* Karling, 1983 (emended): “Two pairs of proboscis hooks with branches or denticles, male copulatory organ with cuticular stylet. Two, four, or six testes, located ahead of, beside, or behind the cylindrical pharynx.”

Functional considerations: To date, all known Karkinorhynchidae have testes located in the anterior half of the body, although the testes may be located beside the pharynx in some species (see discussion in Karling 1983). The testes in *L. alleithoros* are not only numerous (six) but located in the posterior half of the body. It seems possible that this is because of the large relative size of the pharynx in *L. alleithoros* (23% of the body length and approximately 50% of the resting body diameter) and its likely eversion during feeding. We would predict that other karkinorhynchids having a large, eversible pharynx and a relatively large volume of testicular tissue may show a trend toward posterior displacement of the testes with respect to the pharynx.

Our species appears to be the only karkinorhynchid in which pseudostriation of the muscles in the post-cerebral septum has been noted. Although this character is easily seen with fluorescent labeling, it is easily missed in conventionally-stained sectioned material where the muscles are thin. As summarized elsewhere, pseudostriation of muscles in flatworms is generally an indicator of increased contraction velocity (Rieger et al. 1991). The presumed function of the post-cerebral septum is hydrostatic isolation of the anterior tip of the body, allowing more rapid proboscis eversion.

Of taxonomic concern, our species appears to have the lateral gland sacs incorporated into a post-rostral bulb, at least by Karling's (1961) definition, and this would exclude it from the subfamily Karkinorhynchinae as defined by Karling (1983). However, the increased anatomical detail obtained by fluorescent phalloidin staining of muscles combined with CLSM (as already illustrated by Hochberg 2004a, b), suggests that there is much more to be discovered about the specific muscular arrangements around the internal organs of many kalyptorhynchids. In the present case, these extremely fine muscles could be missed in living animals and probably also in serial sections, and it thus remains to be seen just how far the character “post-rostral bulb” extends in the Kalyptorhynchia. Ecologically, karkinorhynchid kalyptorhynchids are most probably tactile-ambush predators with the anterior end of the body specialized for rapid hydrostatic eversion of the proboscis, and the perhaps concomitant need to eject glandular secretions into or onto their prey (Karling 1961). The circular muscles around the lateral glands in this species would certainly provide that capability.

***Carcharodorhynchus c.f. flavidus* Brunet, 1967**

Locality: Lockwoods Folly Inlet, NC. shallow (approx 40cm) subtidal.

Material examined: Two specimens examined and photographed in squeeze preparation. Two specimens fixed, fluorescently stained, and mounted as described above, and studied by LSCM for muscle positions and by interference-contrast transmitted-light microscopy for proboscis dentition.

Description: Our specimens appear to fit well within the range of morphologies described by Brunet (1967), and possesses the characteristic “basal gap” in the proboscis denticles (Figs. 3B, C; see Schilke 1970). The cirrus (Figs. 3D, E) is identical in structure and, at 19 & 22µm long, within the lower size range reported by Brunet for his species. The copulatory bulb, at 89 & 75 µm, is again within the range reported by Brunet for his specimens. The golden-yellow granules in the epidermis appear to be flavonoids—they are brilliantly fluorescent under 405nm illumination. We were unable (even with confocal microscopy of Alexa-phalloidin stained specimens) to discern a connection between the bursa and the germarium; in fact, it appears in our specimens that the entire posterior end is filled with foreign sperm (see Brunet 1967, p. 149) and perhaps comprises bursal tissue in which the paired germaria are embedded. In addition to the specimens described above, we have found at least two other morphological types of closely-similar *Carcharodorhynchus* in our samples, each of which possesses brown-to-yellow pigmentation, an elongate-oval copulatory bulb (with three types of glandular granules arranged in a similar pattern) equipped with a small (approximately 20 µm-long) tubular twisted cirrus, and an equal-lobed proboscis measuring 120 µm – 250 µm overall. One of these has a proboscis dentition that is completely different from that in *C. flavidus*. Given this, and similar (unpublished) observations by the late R.M. Rieger on *Carcharodorhynchus* “flavidus” from Bermuda and the Carolinas coasts, we prefer to refer our species to that of Brunet and leave the other undescribed species to a much-needed (future) revision of the Genus *Carcharodorhynchus*. In short, given these observations and the range of sizes reported in Brunet (1967), it seems possible that *Carcharodorhynchus flavidus* as described by Brunet is actually a species-group. For now, we would follow Schilke (1970), and suggest that only the species with the characteristic basal gap in the proboscis dentition be included under this name.

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Figure 1.

A: Ventral view of live animal, slightly squeezed. Proboscis (pr), mouth opening (mo), pharynx (ph) with anterior glandular margin (gm), testes (t) and two belts of adhesive papillae (ad) can be seen. B: Lateral view of anterior end of preserved animal, showing pigment-cup eye (e), muscular proboscis lobes (pl) and attached hooks (h). Each “hook” is actually a pair of left and right hooks superimposed on each other in plane of the photograph.

C and D: Camera lucida drawings of the proboscis hooks in largely lateral view (c) and oblique ventral view (d), from resin-embedded wholemounts. Dorsal (d) and ventral (v) proboscis lobes are labeled. Anterior end is to the top right; E: Genital region of mature specimen, heavily squeezed. Muscular penis with internal tubular stylet (ps) protrudes into common genital atrium (ga), which is surrounded by a prominent glandular ring (gr) about halfway along its length. Eosinophilic glands (eg) are located in the parenchyma dorsally to and posterior to the common genital atrium. Also visible: Granular secretions inside copulatory bulb (cb), weakly-ciliated vagina interna (vi), unpaired germarium (ge) and bursa with foreign sperm (b). Anterior to the top.

Figure 2.

A: Habitus, from freehand sketch; B: Partial CLSM stack of the anterior end, lateral view of specimen rolled slightly to the dorsal side, stained for actin to show musculature and for DNA to show nuclei. Anterior end to the right. Musculature around mouth (mo) and pharyngeal cavity (pc) are shown. Note fine striated muscle fibers making up post-cerebral septum (arrows), striated diagonal musculature in body wall (dm), fine circular muscles of post-rostral bulb (prb) and dorsolateral (dl) and ventrolateral (vl) anterior retractor muscles.; C: Dorsal reconstruction of whole specimen showing position of major organs. Note copulatory bulb (cb), and that bursa (b), vagina interna (vi) and germarium (ge) are embedded in bursal tissue (bt). Other abbreviations as in previous figures; D: Sagittal section through pharynx; anterior end to the left. Ciliated pharyngeal cavity (pc) enters from left; pharyngeal lumen (lu) at center. Note regionation of pharynx (marked by arrows), characterized by changes in the diameter of the inner circular muscles (icm) and width and spacing of the radial muscle bundles (rm); E: Partial CLSM stack, slightly oblique ventral view with anterior end to the left. Ventral mid-line toward the bottom of the figure. Note band-like muscle fibers surrounding seminal vesicles (sv—right seminal vesicle shown in two places) and copulatory bulb (cb). Genital atrium (ga) possesses circular muscle in its distal-most region (right-hand line) and longitudinal muscle alone more proximally (left-hand line). Vagina interna (vi) arises at anterior face of genital atrium (ga—arrow) and continues posteriorly (lines—ventral-most portion not in figure), ultimately giving rise to paired muscular canals (arrows) that connect to bursa; F: Reconstruction of the genital

apparatus as viewed from the left side; anterior end to the left. Bursa (b) not shown covering germarium (ge) for clarity. Note two types of glands opening into genital atrium (ga): eosinophilic glands (eg) open into the upper part; basophilic granular glands form a glandular ring (gr) below the opening of the germovitelloduct (gvd). Note narrowing of vagina interna (vi) as it proceeds posteriorly, where it gives rise to small vesicle (v) ventrally and to paired muscular canals (ca) that connect to the bursa dorsally. Ductus spermaticus (dsp) was visible only as a tenuous muscular connection between the germarium (ge) and the bursa (b) in a single confocal stack.

Figure 3.

A: Schematic reconstruction of the penis stylet in *L. alleithoros*, anterior to left, showing two schematic cross-sections of the stylet. From serial sections. B: *C. cf. flavidus*, oblique ventral view of proboscis in a wholemount for confocal microscopy; camera lucida drawing. Note that proboscis halves are relatively equal in size, and that denticles on ventral proboscis lobe are only shown completely on one side. As these denticles are being viewed through the thickness of the lobe, they are less clear on the other side; the dotted arrow indicates that the denticles also appeared continue to just short of the nodus on this side as well (although they could not be individually counted beyond the point shown). Note the characteristic gap in the proboscis denticles at the nodus (arrow). C: *C. cf. flavidus*, view of proboscis in squeezed specimen, anterior to left. A marginal array of denticles occurs along the outer edge of upper and lower proboscis lobes (pl). Note gap in denticle rows at the junction of the two proboscis lobes (arrow); D, E: *C. cf. flavidus*, two planes of focus from the same squeezed specimen, showing and cirrus (ci) and distal part of copulatory bulb (cb)