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Convolutriloba macropyga sp. nov., an uncommonly fecund acoel (Acoelomorpha) discovered in tropical aquaria

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Abstract

A new species of *Convolutriloba* Hendelberg & Åkesson, 1988, collected from an aquarium in Marietta, Georgia, USA, and cultured at the University of Georgia comprises exceptionally large individuals, up to 10 mm in length. Like other members of the genus, *Convolutriloba macropyga* **sp. nov.** reproduces asexually and possesses symbiotic zoochlorellae, but it also routinely reproduces sexually, laying relatively large eggs that hatch into aposymbiotic juveniles with a statocyst and frontal organ (which are absent in the adults). *C. macropyga* has a narrow tolerance for extremes of temperature and salinity: it cannot survive outside of a temperature range of 18–28°C and suffers 50% lethality at salinity as low as 24 ppt and as high as 44 ppt. It cannot survive total darkness for longer than 23–26 days, even with prey provided, suggesting an obligate symbiosis with its algal endosymbiont. A method for inducing sexual reproduction in other convolutrilobids is presented, as are suggestions for successful shipping of these acoels.

Key words: Asexual reproduction, sexual reproduction, reverse budding, symbiosis, anterior-posterior axis, toxicity, acoel, flatworm, shipping

Introduction

The genus *Convolutriloba* was erected with the discovery and description of *Convolutriloba retrogemma* Hendelberg & Åkesson, 1988. Hendelberg & Åkesson (1988) hesitated to place the genus in a family due to uncertainties about the maturity of the examined animals. Two years later, Winsor (1990) described *Convolutriloba hastifera* Winsor, 1990, from Australia. Winsor (1990) had mature specimens at hand and, by comparison, confirmed the maturity of specimens of *C. retrogemma* investigated by Hendelberg & Åkesson. On the basis of the male copulatory organ in these species, he assigned the genus to the family Haploposthiidae (Winsor 1990). *Convolutriloba longifissura* Bartolomaeus & Balzer, 1997, is the third and most recently discovered species of the genus. Gschwentner *et al.* (1999) investigated sagittocysts in this species and, weighting the homology of these micro-organs more heavily than that of the male copulatory organ, which appeared to be secondarily reduced, reassigned the genus to the family Sagittiferidae Kostenko & Mamkaev, 1990.

The genus has attracted attention due to its uncommon modes of asexual reproduction. *C. retrogemma* reproduces by reverse budding, a process in which a daughter individual is released at the posterior end of the mother individual, with its anterior-posterior axis reversed 180° to that of the mother individual (Hendelberg & Åkesson 1988; Hendelberg & Åkesson 1991). In *Convolutriloba longifissura* a longitudinal fission, so far the only described case of such fission in bilaterians, occurs in the posterior daughter individual of a transverse fission (Bartolomaeus & Balzer 1997; Åkesson *et al.* 2001).

These species are particularly amenable to study because of the ease with which they can be maintained in the laboratory. In fact, only one species, *Convolutriloba hastifera*, has been described from natural habitats;

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the others were originally discovered in aquaria. Because of their high rate of reproduction through asexual means, all of these species are considered pests in the aquarium trade; they routinely "infest" reef tanks.

Sexual reproduction, on the other hand, has rarely been observed in the genus and has yet to be described. We have discovered a new species in the aquaria of a retail establishment in Marietta, GA, bearing reef organisms collected in the Indo-Pacific. While closely related to *Convolutriloba* species, it reproduces sexually regularly. Its sexually prolific nature, combined with the ease with which it can be cultured under appropriate lighting (for photosynthesis in its algal endosymbiont) and provision of prey, make it an ideal candidate for future studies of development, algal-invertebrate symbioses, and photobiology.

Material and methods

Specimens were collected between May 2006 and February 2007 from a tropical marine aquarium at Cappuccino Bay Aquarium, Marietta, GA, USA, and cultured at the University of Georgia. Animals were maintained in trough-style research aquaria in both a mixed-species population with other convolutrilobids (*C. retrogemma, C. longifissura* & *C. hastifera*) and in a monospecific culture tank separate from other *Convolutriloba* spp. The aquaria were housed in a constant temperature room maintained at 25°C. Artificial seawater (ASW, Instant Ocean®) was maintained at a salinity of 34±1 ppt as measured with an Atago S/Mill hand-held refractometer. The mixed-species tank was illuminated by four Philips 40W 5000°K Ultralume fluorescent lamps providing an average PAR irradiance of ~100 $\mu mole \cdot m^{-2} \cdot s^{-1}$ at the water's surface. Culture tanks were illuminated by two URI Super Actinic and two URI Aquasun-4 VHO 110W fluorescent lamps powered by an IceCap 660 ballast providing ~ 200 $\mu mole \cdot m^{-2} \cdot s^{-1}$. All tanks were maintained on a 14h:10h light-dark cycle. Irradiance measurements were made with an LI-190SA quantum sensor and registered with a Li-Cor Model LI-1400 Data Logger. *Artemia* sp. nauplii were provided daily in superabundance to supplement the acoels' diet of rotifers, copepods, and crustacean larvae already present in the aquaria.

Live animals were viewed with an Olympus SZ40 stereomicroscope and an Olympus CX41 compound microscope and photographed with a DFK 31AF03 fire-wire camera and an Olympus C-5050 digital camera (University of Maine). Live animals were alternatively viewed with a Wild M3Z stereomicroscope and a Zeiss Axioskop-2 phase-contrast compound microscope and photographed with a Sony DSC-P71 digital still camera and a Canon PowerShot A520 digital camera (University of Georgia).

Specimens processed for serial sectioning were relaxed with magnesium sulfate isotonic to seawater and fixed for 1 hour in 4% glutaraldehyde in 0.2 M cacodylate (pH 7.2) containing 0.1 M NaCl and 0.35 M sucrose. Specimens were washed in cacodylate buffer, postfixed in cacodylate-buffered 1% (v/v) osmium tetroxide, dehydrated in acetone, and embedded in EMBed/Araldite epoxy resin. Serial thick sections of 2 µm were made according to Smith and Tyler (1984) using a diamond knife mounted in a Butler trough (Butler 1979) and stained with Heidenhain's hematoxylin according to Smith and Tyler (1984) or toluidine blue.

Musculature was revealed through F-actin staining of whole mounts with fluorescently labeled phalloidin (Alexa 488; Molecular Probes, Eugene, OR) according to Hooge (2001) and examined with a Leica TCS SP2 confocal laser scanning microscope.

Salinity tolerance was determined by subjecting animals to ASW with salinities ranging from 20 to 50 ppt in increments of 1 ppt. Salinity arrays were arranged in Costar (Corning Inc.) 24-well cell-culture trays selecting six randomly chosen wells per salinity (2.5 ml/well). One adult specimen from culture was then added to each well (186 specimens total). Trays were maintained at 25°C on a 14h:10h light-dark cycle with a surface irradiance of 70 $\mu mole \cdot m^{-2} \cdot s^{-1}$ provided by two Philips 5000°K Ultralume and two Philips cool white 40W fluorescent lamps. Surviving numbers for each salinity were recorded every 12 hours concomitant with water changes and removal of asexual progeny. Exposure was continued until the numbers were stable for at least two successive 12-hour periods.

Temperature tolerance was determined in a water tray thermally regulated by a Savant RWC825 constant-temperature circulator. Each experimental trial consisted of 20 adults from culture placed in 15 ml Erlenmeyer flasks immersed in the water tray, one animal per flask. Experimentation commenced at 35°C with successive trials run following an increase or decrease of 1°C. Twenty fresh specimens were tested in each trial. Experimental specimens were maintained in 15 ml ASW at 34 ppt on a 14h:10h light cycle as described for the salinity-tolerance experiments. Numbers surviving were recorded every 24 hours for 3 days at each temperature. Incremental temperature changes continued until any 24-hour exposure resulted in 0% survival.

Release rates of asexual progeny by *C. macropyga* **sp. nov.** were measured under a range of light regimens to determine the effect of irradiance intensity on asexual reproduction. Seventy-two asexually active adult specimens were collected from culture and placed in three 24-well culture plates as previously outlined. Each tray was subjected to one of three light treatments for 15 days: Dark (0 $\mu mole \cdot m^{-2} \cdot s^{-1}$), Low (70 $\mu mole \cdot m^{-2} \cdot s^{-1}$), and High (200 $\mu mole \cdot m^{-2} \cdot s^{-1}$). Low flux was provided from the same system used in the salinity and temperature experiments. High flux was provided from the culture tanks' lighting system. Asexual progeny, i.e., released buds, were counted daily for each individual concomitant with water change, addition of *Artemia* sp. nauplii, and removal of counted progeny. Dark trays were serviced under green light provided from a laptop computer LCD screen (all-green jpeg file viewed in full-screen mode) at <0.01 $\mu mole \cdot m^{-2} \cdot s^{-1}$ in an effort to minimize algal photosynthesis. Presence of egg clusters was also recorded and clusters were removed for observation and measurements of egg and cluster sizes.

Dark-survival experiments were conducted with all four species of *Convolutriloba* for comparative purposes. Twenty-four specimens per species were collected from their respective monospecific culture tanks and randomly placed in 24-well culture plates (one animal per well in 2.5 ml, 34 ppt ASW). Trays were then placed in total darkness. Numbers surviving for each species were recorded daily concomitant with water change, addition of *Artemia* sp. nauplii, and removal of egg clusters and asexual progeny. The maintenance tasks were completed in less than 5 minutes and were conducted under the same green-light conditions as outlined above.

Egg comparisons and general observations were conducted on over 200 egg clusters including those collected in the progeny-release experiments and clusters obtained from *C. retrogemma*, *C. longifissura*, and *C. hastifera*. Adult individuals of these three species possessing visible ovaries and false seminal vesicles were selected from their respective culture tanks and placed in 4½" culture dishes in 200 ml ASW, 10 specimens per dish. Sexual reproduction was induced by subjecting these animals to the lower light regimen of 70 $\mu mole \cdot m^{-2} \cdot s^{-1}$. Egg-laying generally commenced within 24 hours. Twelve egg-clusters per species were collected. Each cluster was placed on a Neubauer brightline hemacytometer (Fisher Scientific) and digitally photographed with a Canon PowerShot A520 on a Zeiss Axioskop-2 phase-contrast compound microscope. Length and width measurements of three randomly selected eggs per cluster were determined in Adobe Photoshop 6.0 using the hemacytometer markings as a linear standard reference.

Permanent cultures of *C. macropyga* **sp. nov.** have been established at the University of Georgia, Athens, GA; the University of Maryland, College Park, MD; and St. Mary's College, St. Mary's City, MD, to ensure availability for future studies.

List of abbreviations

bn, bursal nozzle; **cgc**, gland cell containing cyanophilic vesicles; **cm**, circular muscles; **ds**, digestive syncytium; **ef**, eye field; **fgp**, female gonopore; **fsv**, false seminal vesicle; **g**, ganglion; **lm**, longitudinal muscles; **m**, mouth; **mgp**, male gonopore; **mm**, muscle mantle; **nc**, nerve cord; **o**, oocyte; **pc**, pigment cell; **pg**, prostatoid gland cells; **rh**, rhabdoid gland cell; **sb**, seminal bursa; **sg**, sagittocyte; **sv**, seminal vesicle; **t**, male follicle; **v**, vagina; **ve**, vestibulum; **vg**, vesicula granulorum; **vp**, vacuolated parenchymal cell; **zc**, zoochlorellae.

Results

Family Sagittiferidae Kostenko & Mamkaev, 1990

Genus *Convolutriloba* Hendelberg & Åkesson, 1988

Convolutriloba macropyga sp. nov.

Diagnosis. *Convolutriloba* with sparsely but widely distributed concrements on the dorsal surface; one type of rhabdoid gland cell with 3-µm long rhabdoids. Male copulatory organ consists of paired, lateral, sclerotized canals leading into a seminal vesicle. Seminal vesicle opens into a vesicula granulorum, which is filled with prostate secretion in its proximal part and cyanophilic vesicles in its distal part. Animals have 1 to 3 bursal nozzles. The mouth is positioned at 31 U of total body length (percent, measured from anterior tip to edge of posterior lobe); the female gonopore is at 52 U; the male gonopore is at 75 U.

Type material. Holotype: USNM 1100318, one complete set of 2-µm-thick serial cross sections. Paratypes: USNM 1100329, one partial set of 2-µm-thick serial sagittal sections, USNM 1100330, USNM 1100331, two partial sets of 2-µm-thick serial cross sections.

Type Repository. Smithsonian Natural History Museum, Washington D.C., USA.

Type Locality. Tropical marine aquarium at Cappuccino Bay Aquarium, Marietta, GA, USA.

Etymology. Specific epithet is a derivation of the Greek *macro-* (large) and *pyga* (rump), and reflects the extensive expansion of the posterior region of the body, especially while basking.

Other material examined. Living specimens and eggs in squeeze preparations; 12 whole-mount specimens for fluorescence microscopy; four partial serial sections stained with toluidine blue.

External morphology & behavior. *Convolutriloba macropyga* **sp. nov.** is flat and shield-shaped, its body rounded anteriorly and indented at the anterior tip, broadening to auricular apices set off by a transverse constriction ~2 mm behind the anterior tip, and broadening toward two rounded lateral caudal lobes and a longer, slender median caudal lobe (Figs. 1A–C, 2A). Immature specimens always possess the three caudal lobes (Fig. 1C), but adults often develop multiple median lobes — usually 2 or 3, and up to 9 (Fig. 1A). Individuals are often observed lying stationary with their anterior end erected into the water column in well-illuminated areas. When basking like this, the body is dorso-ventrally flattened to a thickness of 200–360 µm along the lateral margins and 550 µm along the median line. Mature basking specimens are up to 8 mm long and 6 mm wide.

When motile, adult specimens measure up to 10 mm in length and, apart from slight indentations in the lateral margins at the transverse constriction, are uniformly 1.5–2.5 mm wide along the entire length as the body is held tube-like, with the lateral margins curled ventrally. The animal glides by ciliary action.

A sudden increase in light intensity triggers a negatively phototactic, or photophobic, behavior. Mechanical disturbance of specimens in glass culture dishes trigger rapid, forward motion. Similar disturbances in more natural environments cause the animal to move to the shaded undersides of objects or into the substrate.

When food is present, as when *Artemia* sp. are added to the cultures, animals lift the anterior body tip from the substrate and curl the lateral edges and the two ventral flaps (Fig. 2B) anterior to the transverse constriction ventrally, forming a “capturing funnel” (Fig. 1B) *sensu* Hendelberg & Åkesson (1988). The funnel leads to the mouth, which is located medially on the ventral side ~2 mm behind the anterior tip (Figs. 2B, 3A). Though some animals will move in the direction of the prey, most remain relatively motionless with their posterior lateral margins attached to the substrate. When prey moves into the funnel, the animal traps it by pressing down flat against the substrate and moving forward to bring the prey into its mouth.

Body coloration is green, tinged with red, due to symbiotic zoochlorellae and scattered, red rhabdoid gland cells and a diamond-shaped red spot comprised of pigment cells in front of the caudal lobe. The dorsal body surface appears bluish in reflected light due to refractive concrements (Figs. 2A–F).

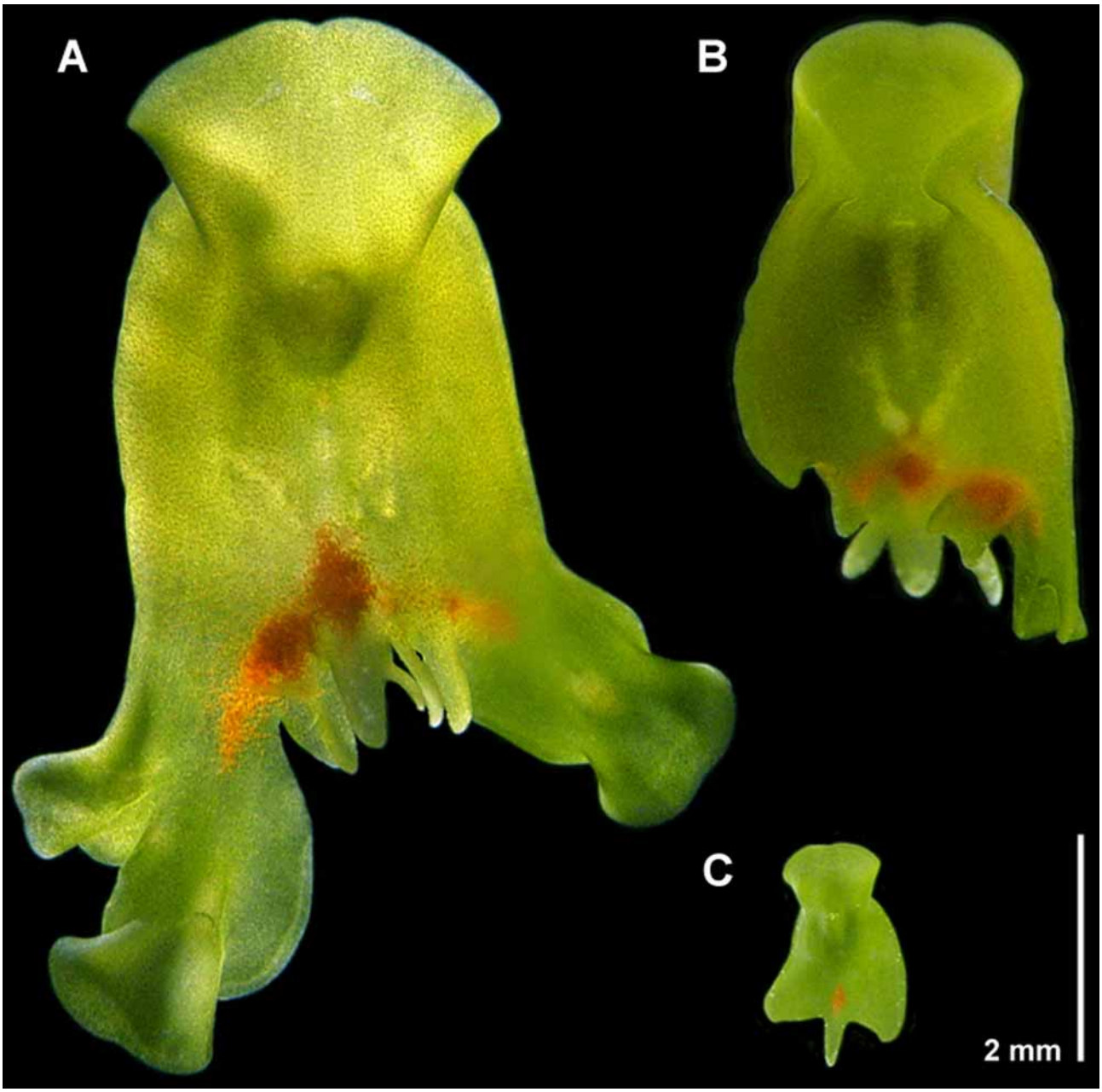

FIGURE 1. *Convolutriloba macropyga* **sp. nov.**; photomicrographs of living, non-anaesthetized, non-squeezed specimens. A. Dorsal view of a large sub-adult with asexual buds and multiple median caudal lobes. B. Ventral view of smaller sub-adult exhibiting the characteristic "capturing funnel" leading to the mouth. Visible are the maturing false seminal vesicles terminating at the male gonopore forward of the central red-pigment spot. C. Dorsal view of an immature, asexually-produced progeny showing the characteristic two rounded lateral caudal lobes and single, longer, slender median caudal lobe.

Epidermis. The epidermis is entirely ciliated. The cilia are commonly ~8 µm long, but can measure up to 12 µm in some areas. On the ventral side of the capturing funnel, the cilia are often sparse or shorter. The epidermal nuclei are sunken beneath the body-wall musculature. Small refractive epidermal concrements occur in large fields. The density of these fields increases gradually from the anterior to the posterior end. The concrements give the surface a bluish sheen under incident light and appear dark purple in transmitted light (Figs. 2C–E). The sheen vanishes when animals are relaxed in magnesium sulfate. Dorsally at the transverse constriction, three to five small white spots of concrements occur in a transverse row. Similar spots occur along the lateral margins in some individuals.

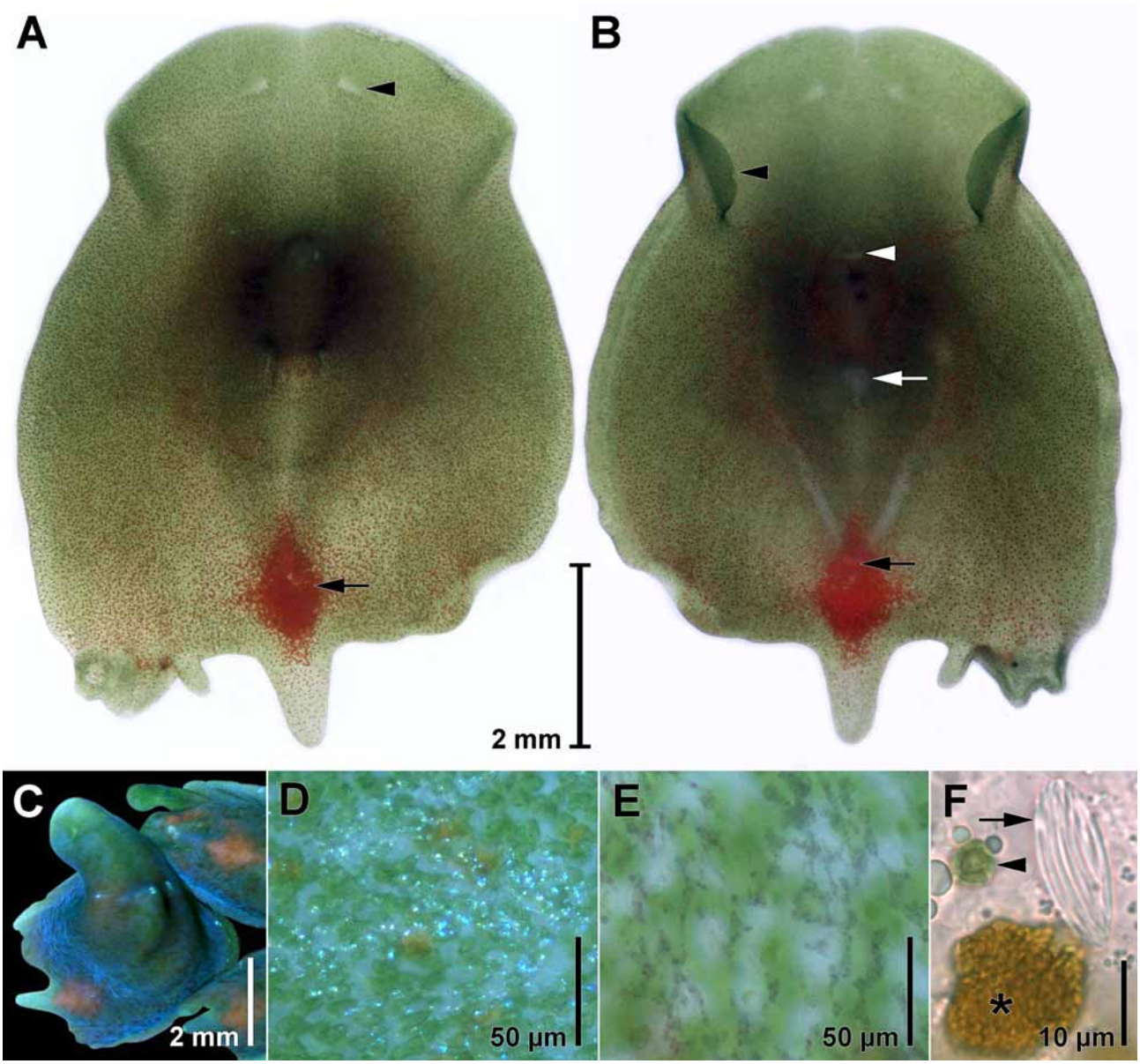

FIGURE 2. *Convolutriloba macropyga* **sp. nov.**; photomicrographs of living specimens. A. Dorsal view of whole anaesthetized specimen. Black arrowhead points to eyespot, black arrow to diamond-shaped spot of pigment cells. B. Ventral view of whole anaesthetized specimen. White arrowhead indicates mouth, white arrow seminal bursa and bursal nozzle tissue, black arrowhead ventral flap, and black arrow male gonopore. C. Dorsal view of cluster of three specimens showing refractive blue sheen. D. Dorsal body surface with blue concrements in incident light. E. Dorsal body surface with concrements in transmitted light. F. Rhabdoid glands of dorsal body wall. Arrow indicates refractile, uncolored rhabdoids; asterisk marks red rhabdoid gland cell; arrowhead indicates symbiotic algal cell.

Sensory organs & nervous system. A pair of eye fields (Fig. 2A) appearing colorless due to the absence of symbiotic algae occurs ~650 µm behind the anterior tip. Paired, insunk ganglia lie ventral to them. The ganglia are connected transversally by a commissure. From each ganglion, two nerve cords run frontally, one runs laterally and one runs latero-caudally. A pair of median longitudinal nerve cords originates at the commissure (Fig. 3A) and can sometimes be seen in live animals as two colorless stripes due to the absence of zoochlorellae. A statocyst is absent in all adult specimens examined, but present in juveniles (Fig. 7A).

Musculature. The body-wall musculature is stronger on the ventral than on the dorsal side. The dorsal musculature consists of outermost circular, diagonal, and longitudinal muscles. The ventral musculature con-

sists of outermost circular muscles (Fig. 5B), a layer of muscles that arc across the body in curves centered on the mouth (Fig. 5C), and an innermost layer of longitudinal muscles and muscles radiating from the mouth (Fig. 5D). Circular muscles near the lateral posterior edge of the mouth bend around its anterior rim in a U-shaped path. Some of these do not bend fully around the mouth but run anteriorly and terminate lateral to it. The next layer inward consists of muscles surrounding the mouth and constituting the wall of the capturing funnel. They bend around the posterior rim of the mouth and run straight and oblique anteriad, crossing each other in front of the mouth (Figs. 4A, 5C). In the posterior half of the body are corresponding longitudinal cross-over muscles (Figs. 4B, 5C). The innermost layer consists of special pore muscles, which fan out from the mouth to the anterior rim and the lateral edges of the capturing funnel (Figs. 4A, 5D), and longitudinal muscles at the posterior end (Figs. 4B, 5D). These longitudinal muscles insert slightly in front of the posterior rim of the capturing funnel (Fig. 4A). Dorso-ventral muscles are abundant, especially laterally.

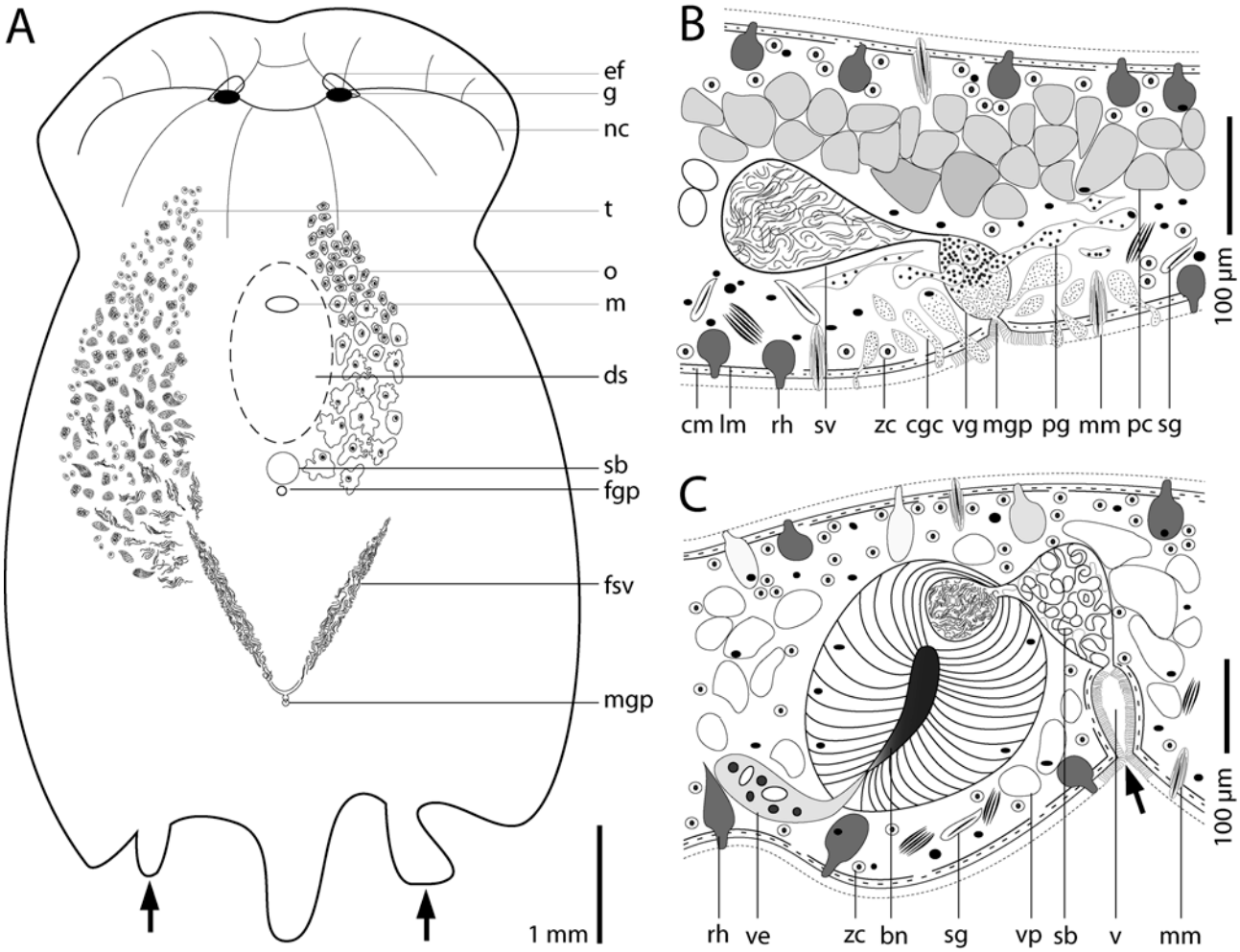

FIGURE 3. *Convolutriloba macropyga* **sp. nov.**; reconstructions to show arrangement of organs. A. Dorsal view. The gonads are paired but for clarity just the left testis and right ovary are shown. Arrows point to buds on lateral caudal lobes, arrowheads to paired ganglia and eyefields. B. Sagittal reconstruction of male copulatory organ. Peripheral parenchyma not shown. C. Sagittal reconstruction of female copulatory organ. Arrow points to female gonopore. Peripheral parenchyma not shown.

Gland cells. Numerous adhesive papillae are distributed along the posterior lateral margin. They comprise the distal tips of glands protruding through the body wall and are ~5 µm long and 2 µm wide.

Two sorts of rhabdoid gland cells, whose cell bodies lie in the parenchyma, protrude on the body surface. The cells of the first type are highly flexible in shape but are commonly ~45 µm long and ~15 µm wide and

contain ~250 rhabdoids measuring 2–3 µm long and ~1 µm wide, the contents of which are reddish-orange (Fig. 2F). Some of these rhabdoid gland cells bear similarly shaped translucent rhabdoids instead. The rhabdoids and the cytoplasm of these cells are cyanophilic. The cells are distributed on the dorsal and ventral side, with the exception of the ventral side of the capturing funnel, including the inner side of the ventral flaps. In non-sexual juvenile specimens the red rhabdoid gland cells are sparsely distributed. As an animal matures, cell densities increase body-wide with higher densities emerging both dorsally and ventrally adjacent to the developing ovaries. In sexually mature adults rhabdoid gland cells are highly numerous along the posterior, lateral margins, the lobes, around the male copulatory organ, and in the region of the gonads on the ventral side (Figs. 2A, B). The second rhabdoid gland cell type occurs solely on the dorsal side, about 20 cells in a specimen. Each cell contains ~18 refractive rods, which are ~20 µm long, 1 µm wide (Fig. 2F), and strongly cyanophilic.

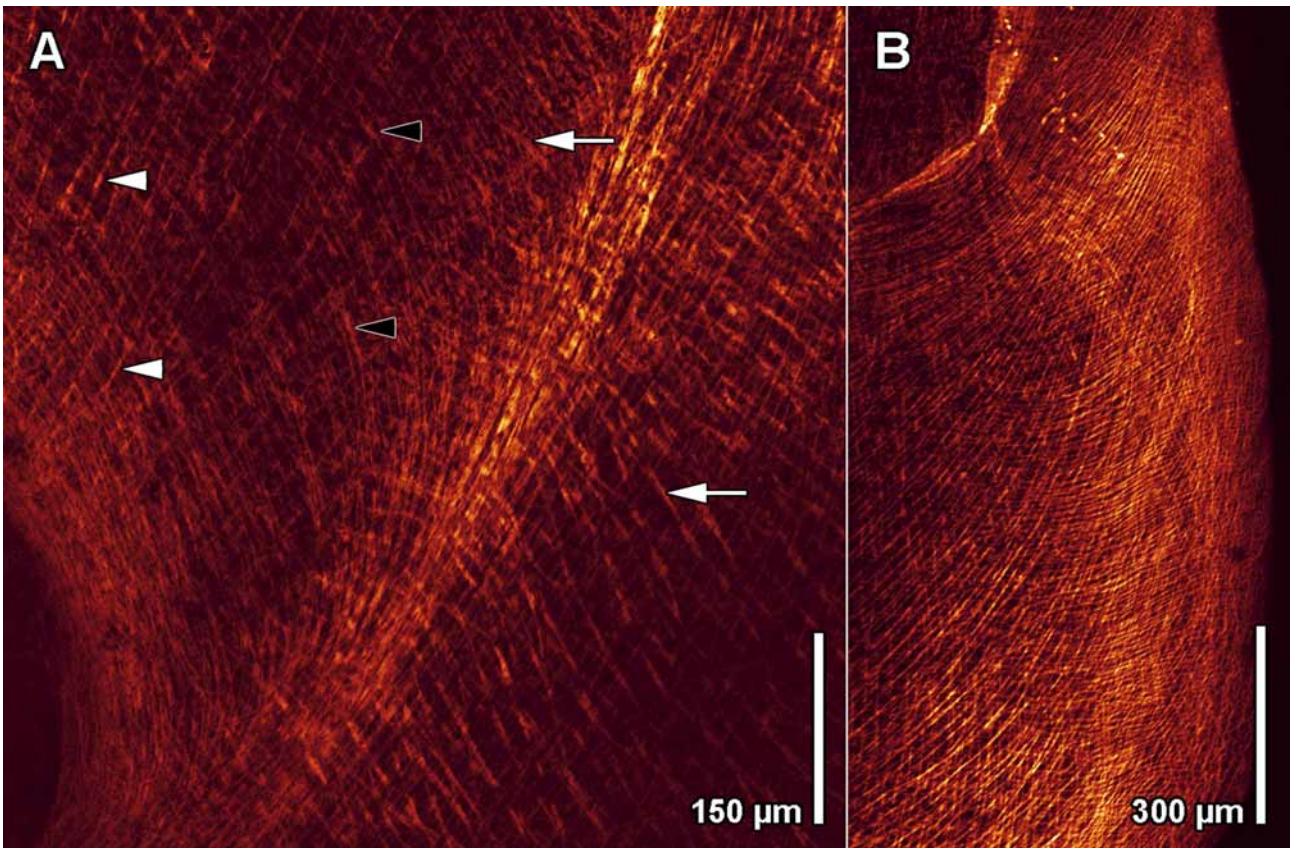

FIGURE 4. *Convolutriloba macropyga* **sp. nov.**; whole mount stained with Alexa-488-labeled phalloidin and viewed with confocal microscopy. A. Optical section of ventral body-wall musculature. Anterior toward upper left corner. White arrows point to longitudinal muscles, white arrowheads to radial muscles, black arrowheads to U-shaped muscles. B. Projection of ventral and lateral body-wall musculature adjacent to the mouth (in upper left corner).

Mucous gland cells are absent. Adults lack a frontal organ, but freshly hatched juveniles have an easily recognized frontal organ with a reservoir, all lying in front of the statocyst (Figs. 7 A, B).

Red pigment cells are densely packed on the dorsal side in a diamond-shaped red spot ~1.4 mm long and ~0.9 mm wide in front of the median caudal lobe (Figs. 1C, 2A, B). The cells lie dorsal to the male copulatory organ and ventral to the body-wall musculature and the rhabdoid gland cells, they do not protrude to the surface, and measure 40–50 µm in diameter (Fig. 3B). In histological sections the cells are filled with a grayish meshwork and their cytoplasm is not stained.

Sagittocysts occur in two sizes. Large sagittocysts occur in abundance and measure ~40 µm long and ~2.5 µm wide. Small sagittocysts, distributed mainly at the anterior lateral margins, measure ~20 µm long and ~1

µm wide. The sagittocysts are formed in sagittocytes which lie ventral to the dorsal body-wall musculature and rhabdoid gland cells. The sagittocytes generally contain a bundle of 8–12 sagittocysts. Each sagittocyst moves towards the distal tip of the sagittocyte, which lies within the body wall. A muscle cell, or muscle mantle, enwraps the sagittocyst within the distal tip of the sagittocyte. This arrangement is connected with a sensory cell and altogether is called an extrusion apparatus *sensu* Gschwentner *et al.* (2002). The muscle mantles enwrapping the small sagittocysts are ~50 µm long and ~6 µm wide, those enwrapping the large sagittocysts are ~75 µm long and ~9 µm wide. The extrusion apparatus are distributed over the entire dorsal surface and the lateral sides of the ventral flaps, with higher densities found on the lateral margins. Ventral distribution is limited to the area between the female gonopore and the caudal end with high densities in a broad region between the gonopores.

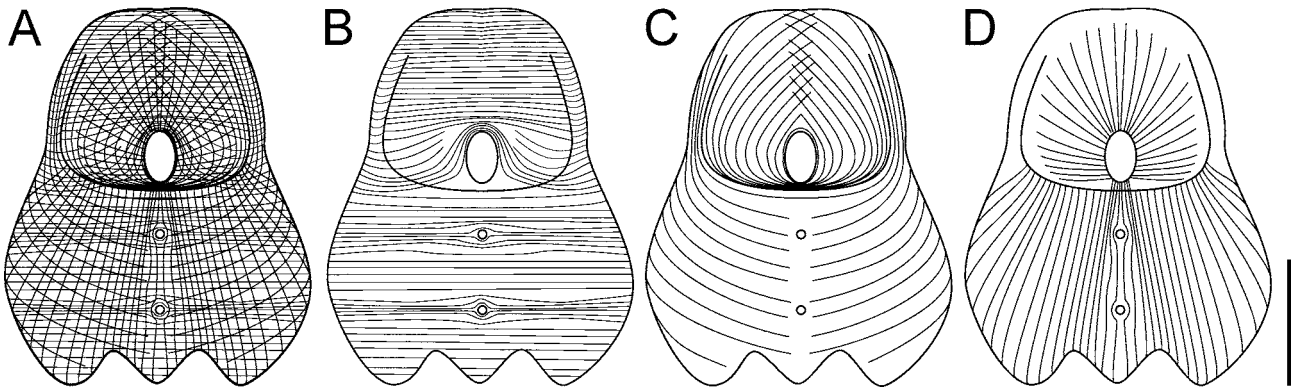

FIGURE 5. *Convolutriloba macropyga* **sp. nov.**; reconstructions to show ventral body-wall musculature. For clarity just a few muscles are shown. Scale bar: 1 mm. A. All muscle components. B. Circular muscles. C. U-shaped muscles and longitudinal cross-over muscles. D. Special pore muscles and longitudinal muscles.

Parenchyma & zoochlorellae. The parenchyma consists of dense peripheral parenchyma and parenchyma cells with large vacuolated spaces. The dense parenchyma occurs primarily in the periphery of the body, but is also found centrally surrounding nervous tissue and often forms extensions into the vacuolated parenchyma. Numerous zoochlorellae, 5–14 µm wide, are distributed throughout the parenchyma. In squeeze preparations and motile specimens, zoochlorellae appear to be arranged in rows, mirroring the overlying musculature (and sometimes the longitudinal nerves, as well); a random distribution is observed in specimens at rest. The algal endosymbiont has been isolated using the CO_2 bubbling method of Boyle & Smith (1975) and cultured in L1 media. We have not yet identified the algal species.

Testes & male reproductive system. The paired testes lie dorsal and lateral to the ovaries. Follicles and sperm pass caudally and sperm accumulate in paired false seminal vesicles, which measure ~170 µm in diameter and converge toward the body-midline (Fig. 3A). Mature spermatozoa measure ~280 µm long, have a thin ~50 µm long tail, and a stepped, ~20 µm long tip.

The male gonopore lies about 1 mm in front of the posterior end, slightly less than 2 mm behind the female gonopore, and opens into a vesicula granulorum. The vesicula granulorum, ~50 µm in diameter and ~75 µm high, lies within a plug of peripheral parenchyma, which is 450 µm long, 300 µm wide, and 150 µm high. Dorso-ventral muscles, sagittocytes, extrusion apparatus, and two types of gland cells are embedded in this plug. The first type of gland cell contains small cyanophilic vesicles with a diameter of 300–500 nm, and protrudes through the body wall around the male gonopore and into the distal part of the vesicula granulorum. The second type, prostate gland cells *sensu* Winsor (1990), produce basophilic vesicles with a diameter of ~1 µm, and protrude exclusively into the proximal part of the vesicula granulorum. A seminal vesicle, measuring ~100 µm in diameter when filled with sperm, opens into the proximal end of the vesicula granulorum (Fig. 3B). One lateral canal on each side connects the seminal vesicle with the caudal end of the corresponding false

seminal vesicle. Each canal is ~ 300 µm long and has a diameter of 22 µm. The paired canals and the seminal vesicle are surrounded by sclerotized tissue and parenchymal musculature.

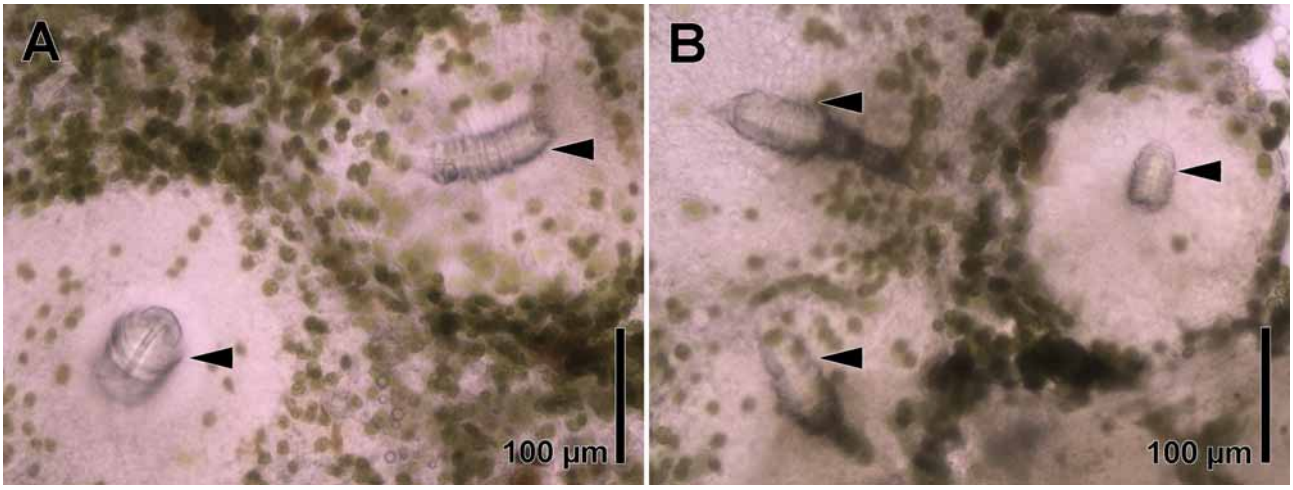

FIGURE 6. *Convolutriloba macropyga* **sp. nov.**; photomicrographs of living specimens. A. Dorsal view of female copulatory organ with two bursal nozzles. Black arrowheads point to bursal nozzles. B. Dorsal view of female copulatory organ with three bursal nozzles. Black arrowheads point to bursal nozzles.

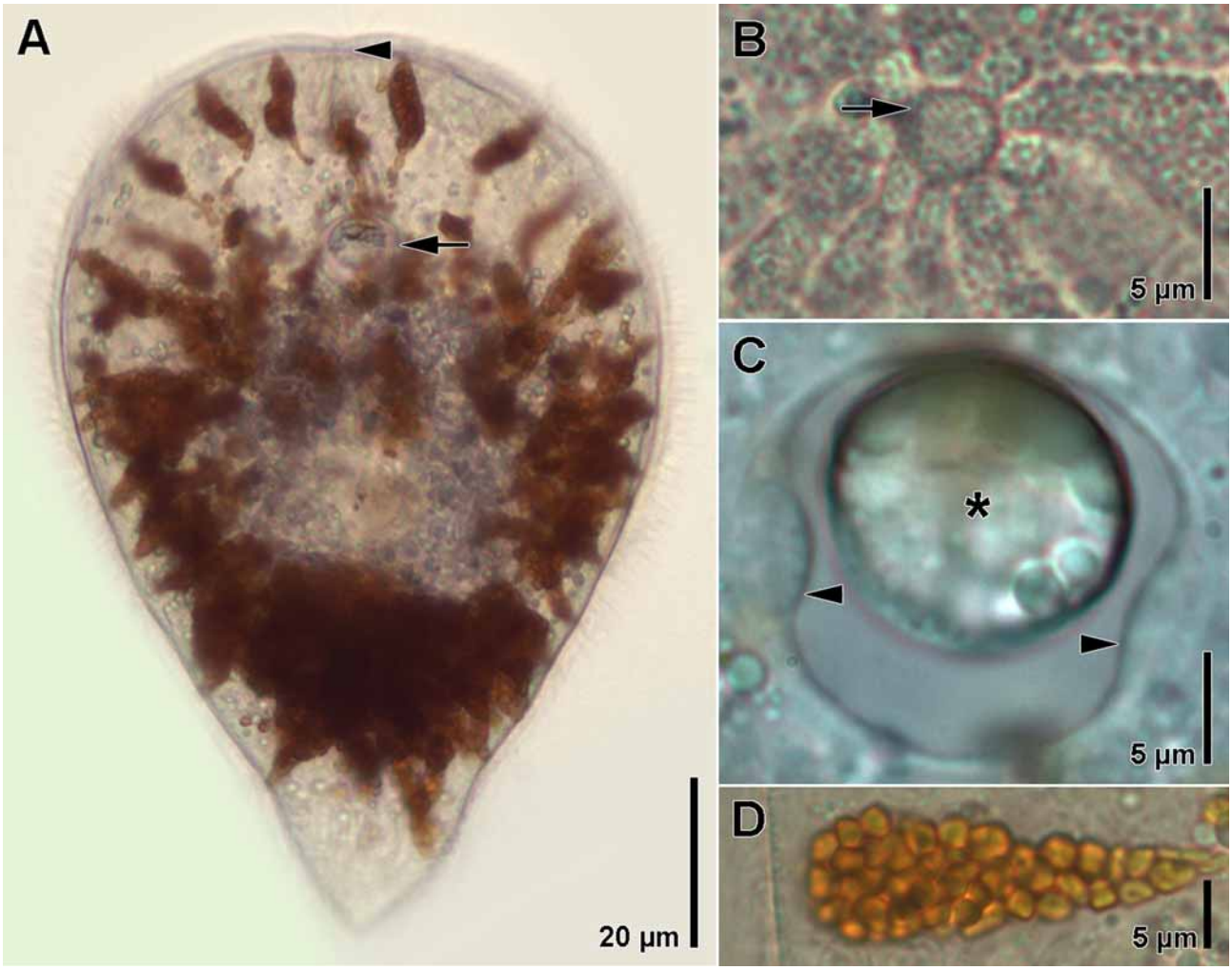

FIGURE 7. *Convolutriloba macropyga* **sp. nov.**; photomicrographs of live juvenile. A. Dorsal view of whole specimen. Arrow points to statocyst, arrowhead to frontal pore. B. Frontal organ. Arrow points to frontal pore. C. Statocyst. Arrowheads point to nuclei of parietal cells; asterisk marks statolith. D. Rhabdoid gland cell.

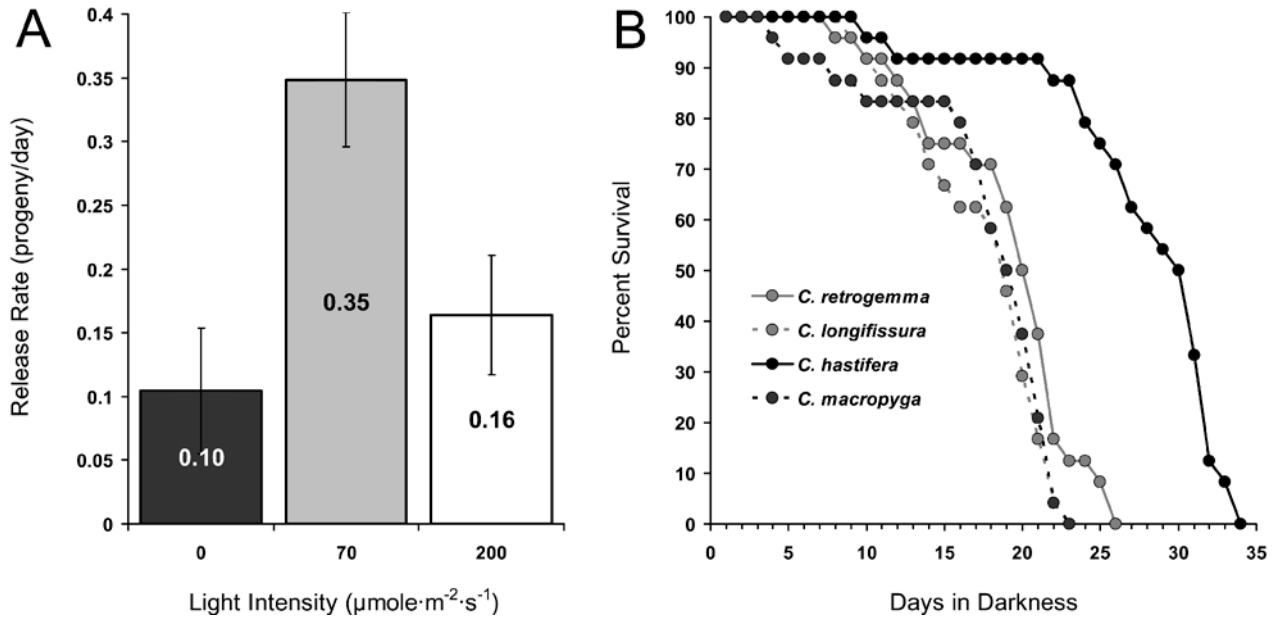

FIGURE 8. Light experiments in *Convolutriloba macropyga* **sp. nov.** and the *Convolutriloba* genus. A. Progeny release rates of *Convolutriloba macropyga* **sp. nov.** in response to light intensity. The experiment involved 24 adult specimens each in one of three light treatments: dark, low light, and high light. Trials ran for 15 days. Results are presented as average number of asexual progeny released per individual per day. Error bars are 95% confidence intervals. B. Comparison of dark-survival of species within the genus. Twenty-four adult animals of each species (*C. retrogemma*, *C. longifissura*, *C. hastifera*, and *C. macropyga*) were subjected to total darkness to determine and compare the extent of the obligate nature of algal symbiosis between the different host species. *Artemia* sp. prey was provided daily in superabundance to minimize the variable of holozoic starvation. Results are presented as percent survival over time.

Ovaries & female reproductive system. The paired ovaries lie ventral and medial to the testes (Fig. 3A). Early oocytes contain numerous translucent granules, have a cell diameter of ~50 µm, a nucleus measuring ~10 µm, and a nucleolus measuring 2–5 µm in diameter. The nucleus is surrounded by dense homogeneous cytoplasm measuring 25 µm in diameter. During cellular growth the size and morphology of the nucleus and nucleolus remain constant as the cell becomes larger and lobulated. In living specimens one can observe the appearance of orange-brown granules in the cytoplasm of oocytes at about the level of the mouth. At the same level, basophilic granules with a diameter of 500–800 nm start to appear in histological sections. As oocytes mature the cytoplasm stains progressively darker.

The female gonopore lies ~1.5 mm behind the mouth (Fig. 3A). The vagina is an invagination of the body wall ~100 µm long, ciliated, and lined with weak musculature (Fig. 3C). The vagina connects with the distal part of the seminal bursa, which is lined with weakly sclerotized tissue and often filled with spongy tissue. The proximal part is surrounded by bursal nozzle tissue. Bursal nozzles range in size from 55 µm to 150 µm and vary in number from 1 to 3 (Figs. 6A, B). Of 26 specimens examined, 15 had one nozzle, 7 had two, and 4 had three. All sectioned animals had two bursal nozzles lying in close proximity, sharing one common seminal bursa. The bursal nozzles are directed antero-ventrally, and curve frontally. The vestibula are extraordinarily large and contain many rounded nuclei, cyanophilic vacuoles, and a weakly stained granular plasma.

Sexual reproduction. Although we have not yet witnessed copulation, sexual reproduction is evident in our populations and sexually mature animals routinely produce eggs. One third of the adults in the *progeny-release* trials laid eggs after being individually isolated at the onset of the experiment. Egg laying was most common in the first five days but continued until the ninth day of isolation. Most animals laid eggs on three or four different days; one animal produced eggs on seven consecutive days.

Eggs are commonly laid in a flat cluster measuring no more than 2 mm in diameter and are suspended in a transparent matrix that adheres the cluster to the substrate. Of fifty clusters collected for egg counts, average

number of eggs per cluster was 78; the smallest cluster contained 31 eggs, and the largest contained 181. Eggs are ovoid in shape with a thin, transparent shell ~170 x 130 µm. Embryos in freshly laid eggs bear a reddish-orange color, have no readily identifiable morphological features, and occupy ~90% of the egg. After 36–48 hours, a darkening, dense, red spot appears within each embryo. The embryos' surfaces are entirely ciliated, and they rotate within the eggs. By the third day the embryos are folded over ventrally, continue to rotate, have fully developed red rhabdoid gland cells concentrated centro-caudally and laterally, and a statocyst. Juveniles begin to hatch on the third day and most have emerged by the end of the fourth. Hatchlings are ~230 µm long and ~120 µm wide, dorso-ventrally flattened, rounded anteriorly and taper caudally to a rounded point (Fig. 7A). They harbor no algal symbionts, but have a frontal organ, a statocyst with a statolith, and approximately 100 red rhabdoid gland cells (Figs. 7A–D). The statocyst is ~22 µm and the statolith ~12 µm wide (Fig. 7C). Four-day-old hatchlings already possess small sagittocysts at the anterior end. Hatchlings glide along the substrate using their cilia; unlike adult animals they routinely swim freely in the water column by cilia. They appear to consume bacteria, as evidenced by large numbers of live bacterial cells sequestered in the central parenchyma, and have been observed preying on small ciliates.

Asexual reproduction. This species reproduces asexually by reverse budding (Hendelberg & Åkesson 1988) whereby the main axis of the progeny is reversed 180° relative to that of the mother. Budding begins as a thickening, slight protrusion anywhere along the caudal margin lateral to the median lobe(s), up to and including the lateral lobes. Concomitant with the thickening is a migration of zoochlorellae to the protrusion rendering it a darker green, and the emergence of red pigment cells medial to, and forward of the protrusion in the mother. Within 24 hours the bud has elongated disto-caudally from the mother and the newly formed red pigment spot has expanded and elongated to span the marginal interface between mother and daughter as can be seen in Figure 1A. Within the following 24–36 hours a head has developed, eye fields are evident, and in some cases the daughter begins feeding holozoically. Shortly thereafter, the bud is not released but torn from the mother when it attaches itself to the substrate and pulls away. At any one time, we have observed upwards of 4–5 daughter individuals in various stages of development on a mother individual.

Released progeny size is directly proportional to the size of the mother animal. Newly released progeny ranges from 1 to 3 mm in length. Larger progeny can produce buds within 24 hours. Sexual maturity is reached within 8–10 days under optimal environmental conditions. Growing buds and newly released progeny exhibit the characteristic refractive blue sheen of their mother. Soon after release, however, the concrement densities decrease to a sparse distribution primarily in the caudal half of the juvenile animal. As the animal matures the concrement distribution expands and becomes denser such that sexually mature adults have the refractive blue sheen over the entirety of their dorsal surface.

Progeny release rates. Rates appear to vary in response to many environmental factors including, but not limited to, prey availability, diversity of prey, water quality, water flow, and light quality. Of the abiotic variables, light intensity appears to have the greatest influence on asexual budding (Fig. 8A). Control animals kept in darkness released about one bud every 10 days; those in high light released one bud every 6.4 days; and those under optimal light conditions released one bud every 2.9 days.

Environmental limitations. Salinity-tolerance experiments on adult specimens revealed 50% lethality concentrations at 24 ppt and 44 ppt over a three-day exposure with an optimal salinity (100% survival) of 34 ppt (Fig. 9A). Temperature experiments showed a 100% survival range of 18–28°C. Unlike the relatively gradual decreases in survival observed in the salinity experiments, abrupt drops to zero-survival occurred immediately outside this range (Fig. 9B).

Comparative data. We found few pronounced differences among species of *Convolutriloba* in their sexual reproduction. Egg size was statistically larger in *C. hastifera* than in the three other species (180 x 125µm in *C. hastifera* vs. 170 x 130 µm; one-way ANOVA with length:width ratio as the independent variable, $\alpha = 0.05$, $p < 0.001$, post-hoc Tukey HSD test verified statistical difference of *C. hastifera* egg size), but there was no difference in embryo size. Embryos developed similarly in all species and hatched within the same 3-to-4-

day window. All species' hatchlings were aposymbiotic and possessed a frontal organ and statocyst. Aposymbiotic hatchlings maintained their size for about one week, then gradually decreased in size and died within two weeks. We do not know how any of the species obtains algal symbionts in the wild, but one-day-old hatchlings of *C. macropyga* were successfully infected with symbionts when algal (previously isolated from *C. macropyga*) culture was added to dishes containing the hatchlings.

Our comparative dark-survival data (Fig. 8B) show that *Convolutriloba macropyga* **sp. nov.**, *C. retrogemma*, and *C. longifissura*, experience 100% mortality after approximately 23–26 days in total darkness with access to prey. *Convolutriloba hastifera*, however, survives for 8 days more. In all species, as zoochlorellae density decreased, density of red rhabdoid gland cells increased. Prior to death, all animals, regardless of species, were primarily red-orange in color and were a fraction of their original size despite having captured and consumed *Artemia* sp.

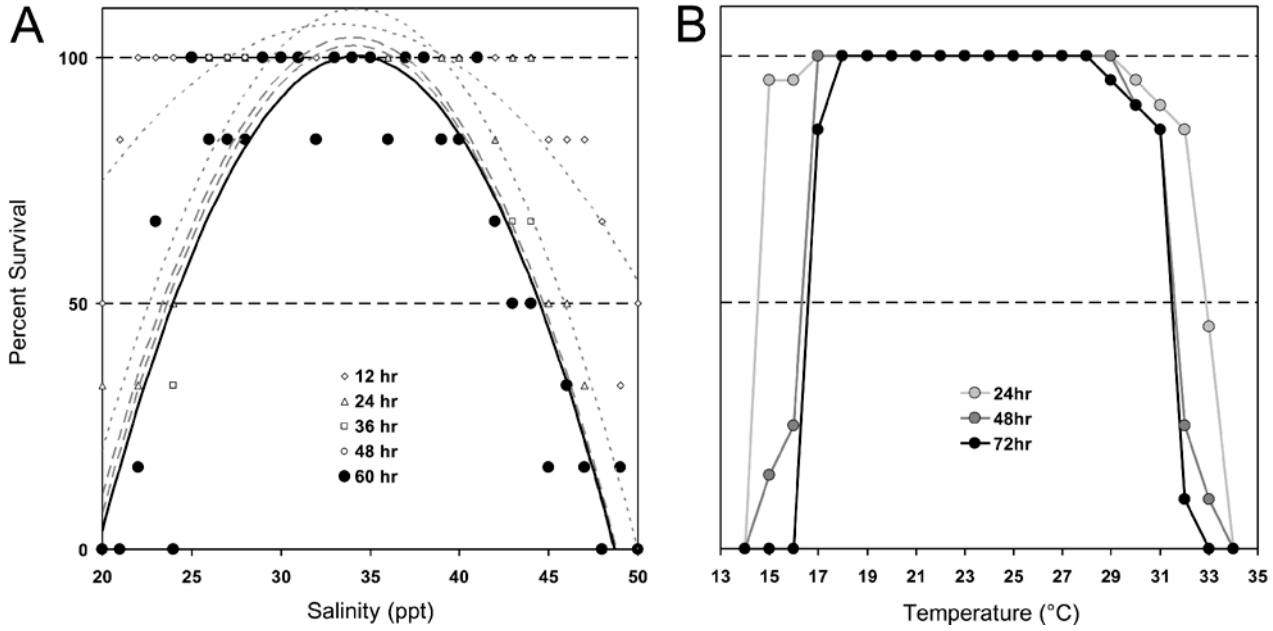

FIGURE 9. Environmental tolerances of *Convolutriloba macropyga* **sp. nov.**; A. Salinity tolerance. Animals were subjected to a range of artificial seawater salinities, $n = 6$ per salinity. Surviving numbers were recorded every 12 hours for three days. No change in survival percentages was noted after 60 hours. Second order polynomial regressions were fit to each data set; $r^2 = 0.81$ for 60-hour regression line. Fifty percent lethality occurred at 24 and 44 ppt. B. Thermal tolerance. Animals were subjected to a range of temperatures, $n = 20$ at each temperature tested. Surviving numbers were recorded every 24 hours for three days.

Taxonomic remarks

One major feature that *Convolutriloba macropyga* and *Convolutriloba retrogemma* have in common is the mode of asexual reproduction by reverse budding, but the number of buds at any one time is generally greater in *C. macropyga*. *Convolutriloba hastifera* reproduces by transversal fission (personal observation of populations in Georgia and Maryland, USA, by Shannon, Sikes, and Hatch, and of populations in Innsbruck, Austria by Achatz, Gärber, and Gschwentner), *Convolutriloba longifissura* by transversal fission with a subsequent longitudinal fission (Åkesson *et al*. 2001).

Secondly, *C. macropyga* and *C. retrogemma* share similarities of the male copulatory organ. Both species possess lateral sclerotized canals leading to a vesicula granulorum ("vesicle filled with granular secretion" *sensu* Hendelberg & Åkesson 1988). In our sections it is evident, especially in Paratype USNM 1100331 that the two canals unite before opening into the vesicula granulorum. In some specimens the appearance of these

canals is different than described above. Instead of a uniform diameter along the entire length, they may have constricted and extended sections, always in a bilaterally symmetrical pattern. This indicates that the canals may transport sperm actively. By contrast, no sclerotized canals are described in *C. hastifera,* and the terminal vesicle is filled with glandular secretions and sperm (Winsor 1990).

The new species shares with *C. hastifera* and *C. longifissura* the distribution of sagittocysts at the anterior end and on the dorsal side.

Like all other species of the genus, *C. macropyga* lacks a statocyst in the adult. As pointed out by Hendelberg & Åkesson (1988) caution should be exercised in declaring the absence of a statocyst since statocysts are absent in asexually produced individuals of *Amphiscolops langerhansi* von Graff, 1882 (Hanson 1960). Though we have found statocysts in the hatchlings of all four convolutrilobids, we have yet to successfully raise a hatchling (and its statocyst) through to adulthood. *Convolutriloba hastifera* and *C. retrogemma* have a frontal organ, which is lacking in *C. macropyga* and *C. longifissura*. Again, *C. macropyga* hatchlings have a frontal organ, though whether it is retained in sexually produced adults is unknown.

Convolutriloba macropyga stands distinct from the other species by having up to three bursal nozzles. The multiplication of bursal nozzles could be an adaptation to the high rate of oocyte production — that is, to facilitate higher rates of fertilization. The variable number of bursal nozzles could indicate that this character has not yet stabilized genetically. Another explanation could be that our population is genetically degraded (see *significance of sex* section below).

Concrement spots, which occur in different patterns over the entire dorsal surface in all other species of the genus, are restricted to a transverse distribution above the mouth and along the lateral margins in the new species. The uniform, scattered distribution of concrement and the blue refractive sheen it affords the animal is exceptional.

Convolutriloba macropyga further differs from the other species by having more pronounced ventral flaps and possessing shorter orange-red rhabdoids (2–3 µm) than the other species (5–6 µm).

The length of spermatozoa varies significantly within the genus, ranging from 280 µm in *C. macropyga* to 200 µm in *C. retrogemma* (Hendelberg & Åkesson 1988), and 130 µm in *C. longifissura* (Åkesson *et al.* 2001).

Yet-unpublished molecular data of 18S-rDNA and COI sequences further corroborate that *C. macropyga* is distinct from all other convolutrilobids (personal communication, James Sikes, University of Maryland).

Body-wall musculature. Our findings on the arrangement of elements in the ventral body-wall musculature differ in various points from the pattern described in *C. longifissura* by Gschwentner *et al.* in 2003. First, circular muscles that bend around the anterior rim of the mouth are absent in *C. longifissura*. Second, the muscles around the mouth are all concentric in *C. longifissura* but in *C. macropyga* just the most inner ones follow this pattern; more eccentrically they cross each other in front of the mouth. In our specimens special pore muscles fan out from the mouth, too, but there are no intermediate muscles between those that run perpendicular to the anterior-posterior axis and those that run caudally. Contrary to the finding of Gschwentner *et al.* (2003) in *C. longifissura,* the posterior longitudinal muscles of *C. macropyga* do not converge towards the mouth but run parallel to the lateral body margin and attach to the body wall in front of the posterior rim of the capturing funnel (compare our figs. 5A–D with figs. 5A–D in Gschwentner *et al.* 2003). The differences found can be explained as modifications due to the larger body size of *C. macropyga*, but it should be mentioned that posterior longitudinal muscles arising at the level of the mouth are described in *Picola renei* Achatz & Hooge, 2006 and *Wulguru cuspidata* Winsor, 1988 (see Hooge 2003), whereas special pore muscles that fan out from the mouth and cover the entire ventral side of the body are unique to *C. longifissura* within the Acoela.

Endemism. Although the natural habitat of the new species is unknown, we can deduce that it is likely indigenous to tropical Pacific or Indian Ocean reefs. *Convolutriloba hastifera* was collected in waters off Magnetic Island, Australia (Winsor 1990). *C. longifissura* came from an aquarium with material from Indonesia (Bartolomaeus & Balzer 1997), and we have collected it in Kaneohe Bay, Hawaii. Most interestingly, Ish-

ikawa & Yamasu (1992) report a species similar to *C. retrogemma* — and even more similar to *C. macropyga* — in Japan. The Indo-Pacific origin is further warranted in that the vast majority of corals (the most common vector responsible for convolutrilobid infestations) in the retail market are imported from this area. No evidence exists, scientific or anecdotal, for a Caribbean *Convolutriloba*. Furthermore, our data show *C. macropyga* to be relatively stenohaline and stenothermal, thereby eliminating all temperate waters and estuaries as potential sources.

Importance of light. Åkesson & Hendelberg (1989) and Bartolomaeus & Balzer (1997) cited the inability of *C. retrogemma* and *C. longifissura* to survive indefinitely in the dark as evidence of the obligate nature of algal symbioses to these acoels. Bartolomaeus & Balzer (1997) determined that *C. longifissura* was more dependent on its algae than *C. retrogemma* in that it survived a mere 4 days in darkness while *C. retrogemma* survived 34–36. Our own experiment on dark-survival included all four species, allowing for comparison under identical conditions (Fig. 8B). The differences between our results and those of Åkesson & Hendelberg (1989) and Bartolomaeus & Balzer (1997), specifically in *C. longifissura* which survived 23 days in our tests, indicate that some other factor(s) may have influenced the experiments (see *Toxicity* section below).

Significance of sex. Sexual reproduction appears to be necessary in this genus to maintain a healthy population. An isolated population of *C. longifissura* in a hobbyist's aquarium yielded "robust" asexual individuals over a one-year span in 2005–2006. We harvested 500–750 individuals every 3 months until June 2006 when only about 100 small specimens could be found. All of these last individuals were similarly deformed in ways affecting the transverse fission phase: both the adult and still-attached daughter usually died, but some simply failed to complete longitudinal fission. Throughout our studies of the convolutrilobids, we have witnessed several crashes in populations from otherwise pristine aquaria. Judging from the case of *C. longifissura*, we surmise that the genetic process known as *Muller's ratchet* (Muller 1932; Gordo & Charlesworth 2000) — the accumulation of deleterious mutations within an asexual population — is responsible for these sudden local extinctions. As in other asexual populations, the process could be reversed through sexual reproduction and genetic recombination.

Toxicity. Hendelberg & Åkesson (1988) and Bartolomaeus & Balzer (1997) both noted a coalesced "secretion" from the red gland cells of squeezed *C. retrogemma*, and *C. longifissura* specimens respectively. We find similarly, in the red rhabdoid gland cells of *C. macropyga*, that the red-orange contents of ruptured rhabdoids not only coalesce into a yellow-orange secretion, but that the secretion diffuses through the animal tissue fueling a chain reaction of further gland cell rupture and even lysis of zoochlorellae. Once a rupture occurs, a rapid disintegration of the entire animal ensues, accompanied by a distinct, bromine-like odor. In studying all four species we found that when multiple animals were maintained in small volumes of water, such as culture dishes, the death of any individual resulted in a discoloration (yellowing) of the water, the bromine-like odor emission, and the subsequent death of the remaining acoels as well as of any live prey (*Artemia* sp.) present. To minimize the impact of this apparent toxic effect, we found it prudent to isolate individuals during experiments. We further found that residual secretions on insufficiently cleaned glassware can affect animals and confound experimental results. We recommend acid cleaning of glassware used to hold Convolutrilobids. Plasticware appears to retain higher residual concentrations and should not be re-used. During experiments in small volumes of water, we find it best to remove released progeny since wounds at the point of separation can leak toxins.

Because of its high fecundity and ability to produce offspring both asexually and sexually, *C. macropyga* could prove to be a good model organism for studying stem cells, reproduction, and development. The basal position that these worms occupy as members of the Acoelomorpha, now widely seen as the most primitive phylum of the Bilateria (Ruiz-Trillo *et al*. 1999, 2004), makes such studies especially significant for understanding the most fundamental aspects of these processes. To encourage use of these animals we recommend the culturing methods described above, and, for shipping animals, we offer the following tips: Thermal extremes are an obvious concern, but death during shipment is more often caused by mechanical agitation.

Through extensive trial and error, we have determined that specimens are best shipped in leak-proof plastic or Nalgene containers of at least 100-ml size, at low density (~10 animals per 100 ml maximum), and with no air bubbles – Containers should be fully immersed in clean seawater as the animals are loaded to ensure all air bubbles are removed from the container and lid prior to sealing it. The container should remain immersed while being sealed. Containers should be packed in Styrofoam shipping-boxes with sufficient insulating Styrofoam to minimize movement.

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