Serotonin immunoreactivity in the nervous system of the free-swimming larvae and sessile adult females of *Stephanoceros fimbriatus* (Rotifera: Gnesiotrocha)

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Abstract. Unlike most rotifers (Rotifera), which are planktonic and direct developers, many gnesiotrochan rotifers (Monogononta: Gnesiotrocha) are sessile and have indirect development. Few details exist on larval metamorphosis in most gnesiotrochans, and considering the drastic transformation that takes place at metamorphosis—the replacement of the ciliated corona with a new head that bears ciliated tentacles (the infundibulum)—it is perhaps surprising that there are limited data on the process. Here, we document part of this metamorphosis by examining the presence and distribution of neurons with serotonin immunoreactivity in the nervous system of both planktonic larvae and sessile adult females. Using antibodies against serotonin combined with confocal laser-scanning microscopy (CLSM) and 3D reconstruction software, we mapped the immunoreactive cell bodies and neurites in both life stages and found that relatively few changes occurred during metamorphosis. The larvae possessed a total of eight perikarya with serotonergic immunoreactivity (5HT-IR) in the brain, with at least two pairs of perikarya outside the brain in the region of the corona. Cells with 5HT-IR in the brain innervated the larval corona and also sent neurites to the trunk via the nerve cords. During metamorphosis, the corona was replaced by the infundibulum, which emerged from the larval mouth to become the new functional head. This change led to a posterior displacement of the brain and also involved the loss of 5HT-IR in the lateral brain perikarya and the gain of two perikarya with 5HT-IR in the anterior brain region. The innervation of the anterior end was retained in the adult; neurites that extended anteriorly to the mouth of the larva formed a distinct neural ring that encircled the infundibulum after metamorphosis. Significantly, there was no innervation of the infundibular tentacles by neurites with 5HT-IR, which suggests that ciliary control is unlikely to be modulated by serotonin within the tentacles themselves.

Additional key words: indirect development, confocal, ontogeny, freshwater

It has been argued that metamorphosis is a time of new beginnings, when a larval stage undergoes an often dramatic morphological change that involves a subsequent transition to a new and profoundly different environment (Young 2006). In most cases, the morphological remodeling of internal and external anatomy is extreme, as evidenced in cases of metamorphic transformation of the nervous system in species of ascidians (e.g., Meinertzhagen & Okamura 2001), brachiopods (Altenberger & Wanninger 2010), ectoprocts (Wanninger et al. 2005), entoprocts (Fuchs & Wanninger 2008), and phoronids (Santagata 2002), to name a few. Most studies of invertebrate metamorphosis are necessarily focused on marine invertebrates, many of which go through maximally indirect development (Brusca & Brusca 2003). Significantly, at least for the purposes of observation and experimentation, these animals can be cultured and often produce dozens or more embryos that can be followed through metamorphosis, making many species particularly tractable for studies of development. In the case of rotifers, where indirect development is comparatively rare and only leads to the production of a few embryos that hatch into short-lived larvae, few studies have made detailed observations of metamorphosis (e.g., Kutikova 1995). The presence of a free-living larval stage is an exceptional phenomenon among rotifers and one strongly correlated with the sessile adult lifestyle. In
fact, most of the approximately 2000 described species of rotifers are direct developers, with only select members of the Gnesiotrocha (Monogononta) undergoing indirect development (Wallace et al. 2015). To date, indirect development has been documented from several species of both gnesiotrochan subclades, Collothecaceae, and Flosculariaceae, but details on the structure of the larvae and their transition (metamorphosis) to adulthood remains only partly known for a few species (Montgomery 1903; Fontaneto et al. 2003; Hochberg et al. 2010). Two relatively recent studies highlight some of the details of larval development in members of both subclades. Fontaneto et al. (2003) used scanning electron microscopy to study the morphology of the sessile tube-dweller Floscularia ringens (Linnaeus 1758) and followed the larva-to-adult transition. They revealed that neonates possess many adult features at hatching (e.g., lateral antennae, dorsal hooks, sublabium, etc.), but their squat and vermiform body shape, underdeveloped corona, apparent lack of feeding, and planktonic habitat clearly differentiates them from the adults. As these larvae develop in the plankton over a period of 24 h, their corona gradually expands, their body lengthens, and after settlement on submerged vegetation they secrete a protective tube to house their body (Fontaneto et al. 2003). In the collothecacean Acyculus inquietus Leidy 1882, which lives parasitically in the colonial rotifer Sinantherina socialis (Linnaeus 1758), the larvae have a vermiform appearance, and while their time in the plankton is considerably shorter than for other species (<24 h; unpubl. data), further development is delayed until they locate their new host. Once settled, the larvae attach with their foot, secrete a tube, and begin a drastic metamorphosis that involves the replacement of the larval corona with the adult infundibulum (i.e., head) and a reconfiguration of the somatic musculature that supplies the head (Hochberg et al. 2010).

The infundibulum of collothecacean rotifers is a unique morphological feature and one that is a priori to the clade. While the infundibulum is structurally variable throughout the Collothecaceae, it is generally bowl-shaped with a central mouth surrounded by a wavy or tentaculate rim. According to observations on Collotheca ambiguа (Hudson 1883) by Montgomery (1903), the infundibulum is derived from the larval alimentary tract and not from the (external) larval corona. How the infundibulum moves from internal to external has never been recorded, nor has the fate of the larval corona, despite observations on several species (Kutikova 1995). In species of Stephanoceros, the infundibulum bears five ciliated tentacles that appear to emerge through the larval mouth at settlement (unpubl. data). While the morphogenetic dynamics of this phenomenon are largely unknown, they would appear to necessitate dramatic changes in both muscle supply and innervation of the head because behaviors of both the larval corona and post-metamorphic infundibulum involve control of muscles and cilia. In this study, we follow the larva-to-adult transition in Stephanoceros fimbriatus (Golfuβ 1820) and attempt to determine if any changes occur in the structure of the nervous system during metamorphosis. We focus on immunoreactivity to serotonin in the nervous system for two reasons: (1) serotonin is a modulator of ciliary activity in invertebrates (Weiger 1997; Hay-Schmidt 2000) and (2) the large anatomical differences between the ciliated, larval corona and the ciliated, adult, tentaculate infundibulum would appear to demand a change in innervation. To date, there are few detailed studies of the nervous systems of any gnesiotrochan rotifer (see earlier studies of Vallentin [1890], Gast [1900], & Montgomery [1903]; also Hochberg [2007] & Hochberg & Lilley [2010]), and no knowledge of how the nervous system might be affected by larval metamorphosis.

Methods
Adults of Stephanoceros fimbriatus were collected from submerged grass and species of Urticularia (bladderwort) in Mascuppic Pond (42°40′42.15″N, 71°24′07.64″W) and Flint Pond (42°40′30.45″N, 71°25′32.71″W), Massachusetts during the summers of 2009–2013. Plant material containing adult animals was placed in glass dishes containing 30–50 mL of pond water and observed with a Zeiss stereomicroscope. Animals were isolated on small pieces of vegetation and placed in 5 mL embryo dishes with 64 µm filtered pond water and observed for the production of parthenogenetic eggs. Most animals produced 3–6 eggs over the course of 7 d when kept at room temperature (ca. 21–23°C) on a 12 h light/dark cycle. After hatching, larvae were observed to swim around the dish and could be identified by the presence of red ocelli and a birefringent crystal (Wallace 1993). Larvae were removed from the dish using a micropipette and placed on a glass microscope slide with filtered pond water and a coverslip. In some cases, larvae were relaxed with the dropwise addition of 1% MgCl2 or 1% neosynephrine to the slide (wicked beneath the coverslip) until the larvae stopped swimming. These two anesthetics were the only two that worked on more than 30% of the specimens. Other anesthetics,
including bupivacaine and cocaine, had no observable effects, or stimulated the larvae to contract and remain contracted prior to fixation. Photographs and digital video of all stages of development (free-swimming larvae and adults) were obtained with a Sony Handycam digital camera mounted on a Zeiss A1 compound microscope. Measurements of individual specimens were performed using an ocular micrometer on mostly relaxed specimens.

Several specimens were fixed in place on microscope slides by placing a drop of 4% paraformaldehyde in 0.1 mol L⁻¹ phosphate buffer (PB, pH 7.3) next to the coverslip and then drawing it beneath the coverslip by wicking away water from the opposite side of the coverslip. After a minimum of 1 h fixation, specimens were transferred via micropipette to a small dish and rinsed with PB, followed by transfer to a microcentrifuge tube containing PB with 1% bovine serum albumen (PB-BSA) for 1 h on a rotator. The labeling of neural antigens by a series of different antibodies (e.g., anti-FMRFamide, anti-GABA, anti-serotonin, and anti-tubulin) was attempted on larval and adult specimens; however, only anti-serotonin antibodies consistently labeled neural structures. No labeling was observed when these antibodies were preabsorbed with serotonin, and labeling was also eliminated in control experiments in which the primary antibody was not included in the processing steps. For labeling, serotonin-immunoreactive neurons, relaxed specimens (n=6 larvae, n=10 adults) were processed in microcentrifuge tubes on a rotator at 4°C. Subsequent processing steps were performed in the following order: PB plus 1% Triton X-100 (PBT) for 24 h; primary antibody (rabbit anti-serotonin, 1:2000 dilution, Immunostar Inc.) in PBT for 12 h; PBT for 12 h; secondary antibody (goat anti-rabbit conjugated to CY3 [550 nm excitation, 570 nm emission], 1:500, Life Technologies) in PBT for 12 h; and PBT for 12 h. One adult and one larva (contracted) were processed in an identical fashion as controls but incubation in primary antibody was omitted. Two of the experimental specimens of each stage were placed in 1 ml of NucGreen Dead 488 Ready Probes Reagent (488 nm excitation, 513 nm emission, Life Technologies) for 30 min to stain cell nuclei. All specimens were mounted in Fluoromount-G (Southern Biotechnology Associates, Birmingham, AL, USA) on glass slides and kept at −20°C for several days prior to examination.

Neurons with serotonin immunoreactivity (5HT-IR) were observed using an Olympus FV300 confocal laser-scanning microscope. A green helium neon laser (543 nm) was used to excite the samples, and Olympus software was used to capture the images. Confocal z-stacks were collected and processed in Olympus software as .TIF and .MOV video files. Files were processed with Volocity (Perkin Elmer) to generate z-projections in TIF format and to generate QTVR videos. Photoshop CS5.1 (Adobe) was used to crop TIF files, increase brightness and/or contrast, and in four greyscale images to invert image brightness or colors to reveal fine neurites that did not show up well against a black background. All original data files and their videos may be requested for viewing by contacting the corresponding author.

Results

Cultured adult females (Fig. 1A) each produced 2–3 amictic eggs over the course of 1 week. Eggs were deposited within the gelatinous tube that surrounds the adult (Fig. 1A,B). Approximately 48–72 h later, the larvae hatched from their egg capsules and slowly squirmed their way out of the tube, often aided by longitudinal contractions of the adult (Fig. 1B). Larvae were highly contractile and often withdrew their foot into their trunk (Fig. 1C). After ~2–3 h, the developing infundibulum could be observed within the larval body, close to the anterior end (Fig. 1C). The adult infundibulum did not emerge until the larva had settled permanently on a substrate. No metamorphosing larvae were successfully fixed or stained.

Larvae

Serotonin-immunoreactive (5HT-IR) cell bodies and neurites were present in all examined larvae, although not all specimens were labeled with equal intensity or were in the appropriate orientation for visualization. Some specimens were also partly contracted along the anterior-posterior axis, with the head and/or foot partially withdrawn, thereby making neurites bend and loop in what appeared to be unnatural orientations. Serotonin immunoreactivity was distributed throughout the central nervous system including the cerebral ganglion and paired nerve cords (Fig. 2A,B). The cerebral ganglion was demarcated by a large cluster of nuclei at the anterior end and could be distinguished from the remaining cell population by the level of NucGreen staining (not shown) and its co-localization with 5HT-IR. The brain was bilaterally symmetrical and contained approximately eight 5HT-IR perikarya (Figs. 2, 3). A cluster of four unipolar perikarya (BP₁₋₄) was centrally positioned in the posterior side of the brain.
The two outermost perikarya (BP_{p1,4}) were largest (~8–9 μm long×6 μm wide) and had an anteriorly directed neurite that curved beneath the neuropil and bent ventrolaterally, eventually projecting away from the brain. The innermost perikarya (BP_{p2,3}) were more spindle-shaped and smaller (5–6 μm long×3–4 μm wide); each had an anteriorly directed neurite that joined or synapsed on the thick, pretzel-shaped, neuropil that was also innervated by the nerve cords (described below). A pair of spindle-shaped perikarya (BP_{L}) was present close to the lateral border on either side of the brain (Fig. 2E). These perikarya were ~7 μm long and appeared to be unipolar with a laterally directed neurite, but 5HT-IR in the neurite was too weak to follow its path. Toward the anterior end of the brain was a single pair of 5HT-IR perikarya (BP_{an}) that bent medially toward the ventral side of the brain (Figs. 2B,D, 3B); their specific site of innervation could not be determined. Also outside of the brain in a region just posterior of the coronal cilia was a diffuse region of 5HT-IR (dIR, Fig. 2A,D). This diffuse staining occurred around the ciliary cushions of the corona and obscured visualization of most 5HT-IR cells in all but one specimen. In this single specimen, a large pair of perikarya was observed (Fig. 2B,C). These perikarya (CP), each ~13–15 μm long, were centrally located and appeared to be the origins of a pair of CP_{an} neurites, although their connections were never confirmed. These neurites (CP_{an}) extended posteriorly and then bent medially to form a U-shaped arc ventral to the brain neuropil (Fig. 2B).
Immunoreactive components of the neuropil had a peculiar pretzel-like shape that lay flat in the dorsoventral plane (Figs. 2B, 3). A pair of nerve cords (~1–1.5 μm wide) fed into the neuropil at its most anterolateral aspect (Figs. 2B,E, 3), thereby forming the anterior surface (top) of each pretzel “loop.” The thickened neurites (~1.5–2.5 μm) of each loop came together medially to form a central cord that extended down the midline of the neuropil (Fig. 3A). Posteriorly, the cord split and each branch bent laterally again to form the posterior surface of the pretzel loop. These branches then projected anteriorly, but instead of coalescing on the anterior surface (to form a cohesive pretzel shape), the projections crossed the anterior end and connected to the BP_{apn} (see Fig. 3).
Adults

Interpretation of staining in adults was complicated by the flattening of the infundibulum under the cover slip, which compressed the anterior end into unnatural shapes. Nonetheless, many of the 5HT-IR pathways were followed at least partway, with a complete reconstruction of all elements being based on examination of several specimens that each showed only some of the details. All 5HT-IR perikarya and neurites in the adult that were found to be of similar size and position to those in the larvae were considered to be the same.

The cerebral ganglion was centrally located and positioned posterior to the dorsal infundibular tentacle (dt); the brain appeared as a large cluster of cell nuclei in preparations stained with NucGreen (Fig. 4A). Both the cerebral ganglion (Fig. 4B–D) and nerve cords (Fig. 4E) displayed strong 5HT-IR. Many of the 5HT-IR perikarya of the cerebral ganglion appeared to be in a similar position to those in the larvae, although in most cases the orientation of the brain and corresponding cell positions were affected by contraction of the specimen. Four perikarya (BP_{p1,a}) with 5HT-IR were centrally located behind the neuropil within the borders of the brain (Figs. 4C, 5). The largest perikarya (BP_{p1,a}: 8–10 μm long) were on the outside and the smaller perikarya (BP_{p2,3}: 5-6 μm long) were medial in position. Anteriorly directed neurites from these perikarya appeared to follow a similar path to those in the larvae (Figs. 4D, 5); however, the precise connections of some neurites (BP_{p2,3}) to the neuropil could not be confirmed. A large pair of perikarya (BP_{a}: 10 μm long) was positioned just anterior of the neuropil within the borders of the brain (Figs. 4C,D, 5). In some specimens, these perikarya appeared to be unipolar with only short, posterior neurites directed toward the neuropil. In other specimens, a short anterior neurite appeared to be present, but the IR signal was extremely weak and the pathway could not be followed. A pair of large (30 μm long), weakly immunoreactive perikarya (AP, only confirmed in one specimen; Figs. 4C, 5) was present close to the anterior side of the brain. These perikarya were spindle-shaped and positioned with their termini directed laterally. There were no obvious neurites extending from these cells; however, in one specimen, the lateral termini did appear to be projecting toward some neurites (CP_{n} described below) but a connection between the two was not obvious (see Fig. 5). No lateral brain perikarya (e.g., BP_{L}) with 5HT-IR were detected in any adult specimens.

The structure of the neuropil was not obvious in adults due to the intense immunoreactivity (fluorescence), so the pretzel-like shape present in the larvae

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could not be confirmed in the adult (Fig. 4C,D). A pair of nerve cords (1–1.5 µm wide) projected laterally from the neuropil (Fig. 4B,D), bent ventrally, and extended posteriorly, eventually terminating in the foot; no perikarya or commissures were observed in association with these nerve cords, although numerous varicosities were present and

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**Fig. 4.** Nuclear staining and serotonin immunoreactivity (5HT-IR) in the adult female of *Stephanoceros fimbriatus*. A. Nuclear staining in the trunk of an adult, anterior is up and the infundibular tentacles are not in view. The circled area shows the cluster of cells that demarcate the cerebral ganglion. Other nuclei from the epidermis and organ systems are distributed throughout the trunk. Scale bar=60 µm. B. 5HT-IR in a partially contracted adult, inverted gray-scale image, with 5HT-IR cells and neurites shown as white against a darkened background. The circled area includes the 5HT-IR perikarya of the cerebral ganglion. One nerve cord (nc) can be seen. Scale bar=80 µm. C. En-face view of the anterior end of an adult, dorsal is up. The infundibular tentacles have a dashed outline. The large anterior (BPₐ) and posterior (BPₚ₁₋₄) perikarya of the brain can be seen, as can the neurites of the CPₚ that make up the neural ring around the infundibulum. Two large perikarya (AP) with very low IR are present within the cerebral ganglion and artificially outlined. Confocal stack of 81 slices (0.1 µm per slice). Scale bar=20 µm. D. Enlargement of the brain in dorsal view, anterior is down. Confocal stack of 50 slices (0.1 µm per slice). Scale bar=13 µm. E. Enlargement of the nerve cords in the foot. No cell bodies or perikarya were observed. Confocal stack of 50 slices (0.1 µm per slice). Scale bar=10 µm.

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**Fig. 5.** Schematic of serotonin immunoreactivity (5HT-IR) in the adult female of *Stephanoceros fimbriatus* based on analyses of several specimens. The perikarya and neurites are color coded to match their potential homologues in the larva (Fig. 3). The position of specific anatomical landmarks is labeled and includes the infundibular tentacles (dt, dorsal tentacle; la, lateral tentacles; vt, ventral tentacles), infundibular cavity (if), proventriculus (pv), mastax (mx), stomach (st), and gel tube (gt). See text for details.
often appeared as swellings similar in shape to cell bodies (Fig. 4E).

The infundibulum was encircled by a pair of neurites (CPn) that formed a ring around its periphery (Figs. 4C, 5). These neurites appeared to be extensions of a pair of weakly immunoreactive cell bodies (CP) close to the ventral midline (CP, Figs. 4D, 5); it was undetermined if the neurites were extensions of these perikarya or synapsed on them. A short pair of neurites appeared to branch off the neural ring in a region close to the position of the lateral tentacles and then reconnect with the ring a short distance later (arrowhead, Figs. 4C, 5). The branches (CPn2) were similar in morphology to the CPn but highly folded in some cases.

A single pair of perikarya (LP, Fig. 5) was present on either side of the infundibulum but not contained within the brain itself. These perikarya each gave rise to a single neurite that extended medially toward the center of the infundibulum where they appeared to coalesce or at least terminate close to one another.

**Discussion**

Our studies of metamorphosis and nervous system architecture in the sessile rotifer, Stephanoceros fimbriatus, reveal for the first time the distribution of neurons expressing serotonin-like immunoreactivity (5HT-IR) in both larvae and adults, and show that metamorphosis has little effect on the structure of these components of the nervous system. In fact, despite the drastic morphological transformation that occurs after settlement of the larva—when the larval corona is replaced by the adult infundibulum—there is little evidence to suggest that neuronal pathways are altered in any drastic way. The larval nervous system consists of twelve distinct perikarya showing 5HT-IR, eight of which are in the cerebral ganglion and four of which are peripheral. In the adult, there are at least twelve 5HT-IR perikarya, eight of which are in the cerebral ganglion and at least four that are peripheral. Significantly, one pair of cerebral perikarya (BP₁) in the larvae could not be detected in the adults, and a pair of large perikarya in the adults (AP) could not be detected in the larvae. Whether or not these cells are truly absent from their respective life stages or were not observed due to low immunoreactivity remains to be determined.

In both the larval and adult stages, one pair of 5HT-IR neurons (CP) appears to innervate a region around the corona and infundibulum, respectively. The cell bodies appear close to the mouth in the larva (though the larval mouth is difficult to see) and remain in a similar position in the adult, which is just ventral to the infundibulum approximately midway between the ventral tentacles. A single neurite projects laterally from each cell and appears to extend along the periphery of the corona in the larva and the infundibulum in the adult (Fig. 5). In the adult, this neural ring appears to branch in a symmetrical fashion approximately one-third the way around the infundibulum, only to unite several micrometers further along the ring. In both the larva and adult, the ring terminates in the cerebral ganglion, although the precise site of innervation could not be determined. A similar immunoreactive nerve ring, although without any perikarya or bifurcations, is also present in other rotifers, including species of Conochilus (Gnesiotrocha: Hochberg 2006) and to some degree Platias patulus (Muller 1786) (Ploima: Kotikova et al. 2005). While the larval corona and adult infundibulum of S. fimbriatus are drastically different from each other, and from the coronae of other species, the presence of a neural ring suggests a conserved pattern of innervation in Rotifera. It has been hypothesized that the neural ring might play a role in modulating ciliary activity of the corona (Kotikova et al. 2005; Hochberg 2007). The position of the neural ring in the larvae of S. fimbriatus supports this hypothesis, while its position in the adult is a little less convincing. The reason for this is that there is no serotonin immunoreactivity in the tentacles where the cilia are present. However, this does not rule out the possibility of serotonin-immunoreactive neurites in the region of the infundibulum depolarizing the syncytial epithelia of the tentacles, thereby stimulating ciliary movement. Still another possibility exists that serotonin-immunoreactive neurites stimulate individual muscle fibers that supply the tentacles; in rotifers, muscles are known to insert on ciliary rootlets and thereby produce ciliary beating (Clément & Wurdak 1991).

Other anatomical sites of serotonergic innervation in S. fimbriatus are difficult to ascertain. According to Montgomery (1903), who has performed histological research on S. fimbriatus, five sets of neurons project from the cerebral ganglion, three of which innervate sense organs: one pair innervates the dorsal sense organ; one pair innervates the lateral sense organ; and one unpaired median neuron innervates the hypodermis posterior of the dorsal sense organ. Our results could not confirm these observations, but in some respects this is not surprising since serotonergic innervation of sensory organs has only rarely been documented in rotifers (e.g., Hochberg
2009; Hochberg & Lilley 2010). Our observations do indicate that at least two of the neurons detected by Montgomery (1903) are likely to correspond to a pair of 5HT-IR neurites we detected in both larval and adult specimens. He describes two thick nerves that pass from the brain and ventral to the proventriculus (see Fig. 5); we hypothesize that these nerves are the nerve cords, which contain at least one 5HT-IR neurite each extending from the cerebral ganglion. This is a common condition throughout Rotifera, having been documented in both Bdelloidea (Leasi et al. 2009) and numerous species of Monogononta (Kotikova et al. 2005; Hochberg 2006, 2007). Perhaps surprisingly, Montgomery (1903) does not document any cerebral nerves innervating the mastax, although more modern studies using immunohistochemical methods have revealed serotonergic innervation as well as FMRFamidergic innervation (Kotikova et al. 2005). In our studies, there were no obvious cell bodies or neurites expressing 5HT-IR in the region of the mastax of S. fimbriatus.

In conclusion, our study reveals that the larval and adult (female) nervous systems of S. fimbriatus are broadly similar, and that metamorphosis, although dramatic in its restructuring of the head, has little obvious effect on the number or position of neurons expressing 5HT-IR. However, some notable changes do appear to take place after metamorphosis, e.g., the brain becomes positioned farther from the anterior end, there is an apparent loss of the larval BP1, the addition of the CN2 and AP in adults, and the apparent repositioning and changing innervation of the BPn in adults. The importance of these changes remains to be determined, and we think that only through additional studies of metamorphosis in other indirectly developing gnetrotrochans (e.g., species of Acyclus, Cupelopagis, and Collotheca) using antibodies to serotonin and other neurotransmitters will we truly appreciate how ontogeny may restructure the rotifer nervous system.

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