NADPH-Diaphorase Activity Changes During Gangliogenesis and Metamorphosis in the Gastropod Mollusc *Ilyanassa obsoleta*

By: Miao-Fang Lin and Esther M. Leise

Lin, M.-F. and Leise, E.M. (1996) NADPH-diaphorase activity changes during gangliogenesis and metamorphosis in the gastropod mollusc *Ilyanassa obsoleta. Journal of Comparative Neurology* 374(2):194-203.

Abstract:

Gaseous nitric oxide (NO) is produced through the action of the enzyme nitric oxide synthase (NOS) and acts as a neurotransmitter (Jacklet and Gruhn, 1994b; Elphick et al., 1995a; Jacklet, 1995) in the nervous systems of adult gastropod molluscs. By comparison, little or no information appears to exist about the ontogeny of molluscan NOS-containing neurons. NADPH-diaphorase (NADPHd) has been determined biochemically and histochemically to colocalize with NOS immunoreactivity in neurons; NOS is an isoform of NADPHd (Dawson et al., 1991; Hope et al., 1991). We used NADPHd histochemistry to map the distribution of NOS activity in the nervous systems of larvae, including metamorphosing individuals, and juveniles of the marine snail *Ilyanassa obsoleta.* Several ganglionic neuropils displayed reaction product throughout development. The most intense NADPHd staining occurred in the neuropil of the apical ganglion, a specialized larval structure. Intermediate staining levels occurred in neuropils of the cerebral, pedal, and pleural ganglia. Larval buccal and intestinal ganglia showed little reaction product, with slight increases arising in metamorphically competent larvae. NADPHd activity conspicuously decreased in the central nervous systems of metamorphosing larvae. The osphradial ganglion, which was present in young larvae, showed only weak NADPHd activity. Our results provide evidence for the existence of a nitrergic signalling system in molluscan larvae and juveniles.
Indexing terms: caenogastropod, nitric oxide, neuroanatomy, prosobranch, veliger

Abbreviations

A	apical ganglion
B	buccal ganglion
C	cerebral ganglion
CC	cerebral commissure
E	eyespot
ES	esophagus
G	gut
LB	left buccal ganglion
LC	left cerebral ganglion
LP	left pleural ganglion
O	osphradial ganglion
P	pigment
PC	pedal commissure
PD	pedal ganglion
PL	pleural ganglion
PP	propodial ganglion
R	radula
RB	right buccal ganglion
RC	right cerebral ganglion
RP	right pleural ganglion
S	statocyst
SB	subintestinal ganglion
SP	supraintestinal ganglion
VI	visceral ganglion

Article:

Nitric oxide (NO) is active in trans-synaptic communication in both vertebrates (Snyder, 1992; Bredt and Snyder, 1992; Lowenstein and Snyder, 1992; Bruhwyler et al., 1993; Zhuo et al., 1993; Dawson and Snyder, 1994; Schuman and Madison, 1994; Vincent, 1994) and invertebrates (Moroz and Park, 1993; Moroz et al., 1993a,b, 1994a; Gelperin, 1994a,b; Jacklet and Gruhn, 1994b; Miller and Bicker, 1994; Robertson et al., 1994;

Elphick et al., 1995a; Jacklet, 1995; Sawada et al., 1995). NO is produced from L-arginine by the activity of nitric oxide synthase (NOS), which is a form of NADPH-diaphorase (NADPHd), as demonstrated histochemically, immunocytochemically, and biochemically (Hope and Vincent, 1989; Dawson et al., 1991; Hope et al., 1991). In NOS-containing neurons, NADPHd can reduce nitroblue tetrazolium and produce a formazan precipitate, yielding a morphological marker for NOS activity, again, in both vertebrate (Hope and Vincent, 1989; Bredt and Snyder, 1992; Lowenstein and Snyder, 1992; Snyder, 1992; Bruhwyler et al., 1993) and invertebrate nervous systems (Elofsson et al., 1993; Moroz et al., 1993c; Mailer and Buchner, 1993).

NO-producing neurons in invertebrates are located in both their central nervous systems (CNSs) and peripheral tissues (Elofsson et al., 1993; Elphick et al., 1993, 1995a,b; Moroz et al., 1993c, 1994a,b; Moroz and Roylance, 1993; Mailer and Buchner, 1993; Chichery and Chichery, 1994; Cooke et al., 1994; Gelperin, 1994a; Jacklet and Gruhn, 1994a; Johansson and Carlberg, 1994; Muller, 1994; Mailer and Bicker, 1994; Nezlin et al., 1994; Sanchez-Alvarez et al., 1994; Bicker and Hahnlein, 1995; Sawada et al., 1995; Talavera et al., 1995). The numerous studies using NADPHd histochemistry cited above, biochemical measurements of NOS activity in tissue extracts (Elphick et al., 1993, 1995b; Elofsson et al., 1993; Muller, 1994; Willer and Bicker, 1994), electrophysiological (Moroz and Park, 1993; Moroz et al., 1993a,b; Gelperin, 1994a,b; Jacklet and Gruhn, 1994b; Elphick et al., 1995a; Jacklet, 1995; Sawada et al., 1995), and behavioral studies (Robertson et al., 1994, 1995; Elphick et al., 1995a) all support the idea that NO is an intercellular messenger in nervous systems of the more evolutionarily advanced invertebrates. In arthropods, such as the fruit fly, *Drosophila melanogaster,* the honey bee, *Apis mellifera,* the locust, *Schistocerca gregaria,* and the crayfish, *Pacifastacus leniusculus* and *Cambarellus montezumae,* the greatest NOS activity has been detected in the glomeruli and somata of the antennal lobes where olfactory receptor neurons make synaptic connections with local and relay interneurons (Müller and Buchner, 1993; Johansson and Carlberg, 1994; Meyer, 1994; Miller, 1994; Müller and Bicker, 1994; Bicker and Hahnlein, 1995; Elphick et al., 1995; Talavera et al., 1995). Analogous olfactory structures in gastropod molluscs also display significant NADPHd activity. In the slug, *Limax maximus* (Gelperin, 1994a), and the pulmonate snail, *Helix apersa* (Cooke et al., 1994; Sanchez-Alvarez et al., 1994), neurons in the procerebral lobes, the major sites for odor processing, contain significant NADPHd activity. *Helix aspersa* and other freshwater gastropods, including *Lymnaea stagnalis, Helisoma trivolvis,* and *Biomphalaria sp.,* also manifest intense NADPHd staining in their buccal ganglia and peripherally in their osphradia and esophagi (Elofsson et al, 1993; Moroz et al., 1993a,b,c; 1994a,b; Cooke et al., 1994; Nezlin et al., 1994).

Physiologically speaking, our knowledge of molluscan nitrergic functions is beginning to take shape, although it is still derived from a limited number of preparations. Work done on the feeding circuitry of *Lymnaea* has shown that NO acts as a neurotransmitter mediating sensory input into the central pattern generator (CPG) that underlies this behavior (Moroz et al., 1993a,b; Elphick et al., 1995a). NO is also active as a neuromodulator of olfactory processing in *Limax* (Gelperin, 1994a,b) and affects membrane conductances in neurons of two species of *Aplysia,* presumably through the actions of the second messenger cGMP (Jacklet and Gruhn, 1994b; Sawada et al., 1995). Although the behavioral relevance of this latter study is still being clarified, Gelperin (1994b) and Robertson et al. (1994; 1995) have also shown that NO inhibitors can block specific types of learning in *Limax* and *Octopus,* respectively.

Thus, while it is clear that NO is necessary for the normal functioning of adult neural circuitry, little is known about its activity during molluscan development. We used NADPHd histochemistry to map the development of NOS activity in larvae and post-metamorphic juveniles of the prosobranch mollusc *Ilyanassa obsoleta* as a first step towards determining possible roles for NO during organogenesis and metamorphosis. Some of the data presented here have been published in abstract form (Lin and Leise, 1994).

MATERIALS AND METHODS

Larval culture methods and specimen preparation were described in the preceding paper (Lin and Leise, 1996). NADPHd histochemistry was performed as follows: anesthetized, whole larval or juvenile *Ilyanassa obsoleta* were fixed at 4°C for 1.5 hours in chilled 4% paraformaldehyde in 0.1 M phosphate buffer solution (PBS) at pH 7.6 and then rinsed once with 0.5 M PBS (Eloffson et al., 1993 and K. Lukowiak, personal communication).

After being rinsed with PBS, animals were immersed in the dark at room temperature for 3 hours in the incubation solution, which contained 0.5 mM Nitroblue tetrazolium, 0.1 mM dicumoral, 0.25% Triton X-100, and 1 mM β-NADPH in a 0.5 M Tris-HC1 buffer solution (TBS) at pH 8.0. The incubation solution was freshly prepared 20-30 minutes before use.

Control solutions were prepared without NADPH or with the replacement of β-NADPH by β-NADH. NADPHd requires β-NADPH; β-NADH produces a reaction product non-specifically if NADH-diaphorase (which is not NOS) is present.

After incubation, specimens were rinsed in TBS three times followed by dehydration in a graded ethanol series. Some whole mounts were then cleared and viewed in methyl salicylate. Fading is rapid and excessive if specimens remain immersed in methyl salicylate. Preparations in absolute ethanol fade slowly. To best preserve staining, specimens were stored in 100% ethanol until the embedding procedure. Storage times between fixation and embed-ding were never more than 2 days. No antemedium such as acetone was used to dissolve the plastic resin. Embedding, sectioning, and photographic procedures were as previously described (Lin and Leise, 1996).

RESULTS

The nervous system of juvenile *Ilyanassa* contains paired cerebral, pedal, pleural, buccal, and intestinal ganglia, and unpaired apical, osphradial, and visceral ganglia (Lin and Leise, 1996). NADPHd reaction product occurred as scattered punctae in the ganglionic neuropils of both larvae and juveniles. We found no NADPHd-positive neuronal somata. All of the neuropils displayed similar nonspecific back-ground staining whose density varied with the lengths of the fixation and β-NADPH incubation steps. β-NADH, when used in control solutions, caused intense staining throughout the neuropils of the CNS in juveniles, but far less staining in competent larvae (Fig. 1). No staining occurred in animals incubated without β-NADPH (Fig. 1).

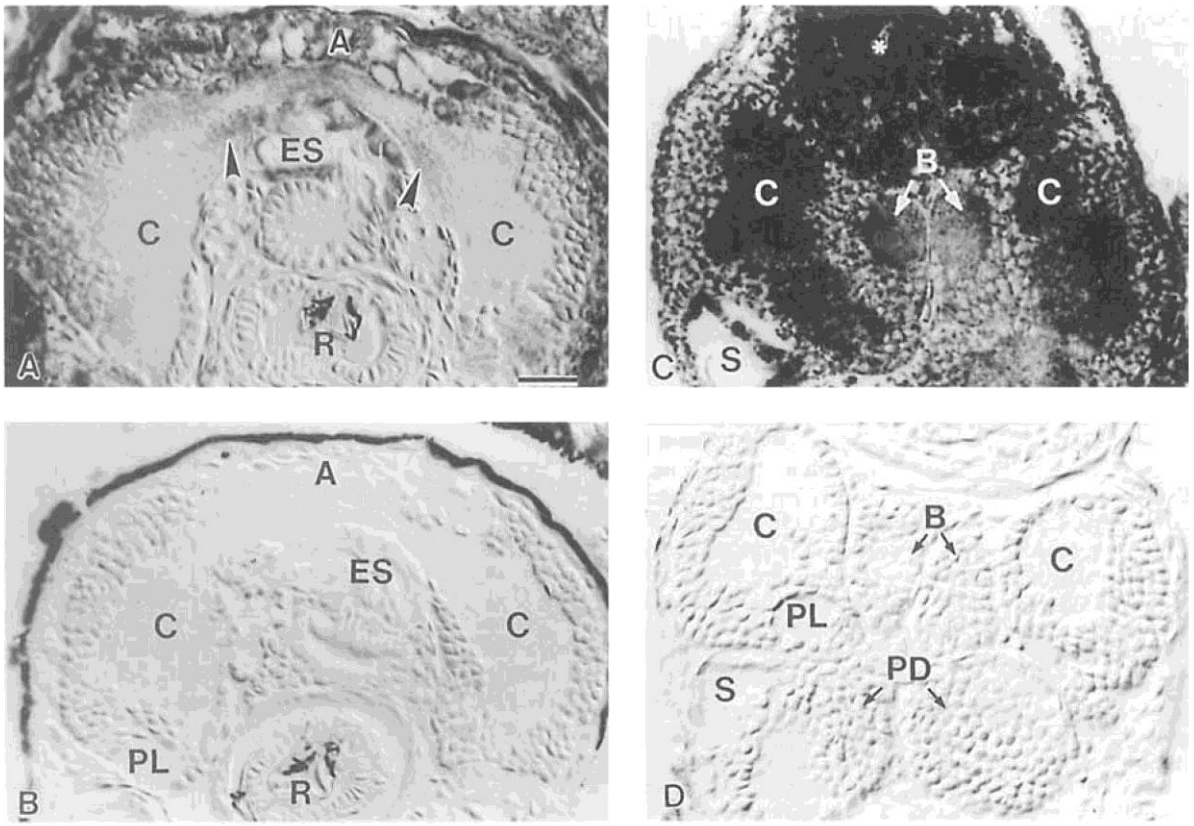

Fig. 1. Transverse sections of controls for NADPHd staining. **A,B:** Competent larvae. **C,D:** Advanced juveniles. A: Staining with β-NADH causes peripheral darkening and some staining in apical and cerebral ganglia (arrowheads). B: Lack of β-NADPH results in no staining in the central nervous system (CNS). C: Note strong staining with β-NADH in all ganglia and in proboscis (asterisk). D: Lack of β-NADPH results in no staining. Scale bar = 20 μm.

Throughout the larval phase of the life cycle, the neuropil of the apical ganglion displayed the greatest NADPHd activity. Staining was especially intense in larvae at 12 days after hatching and in competent larvae

(Fig. 2C,D). At the beginning of metamorphosis, the width of the neuropil of the apical ganglion decreased and the neuropil began to lose NADPHd activity (Fig. 2E). In young juveniles, the apical ganglion, which had shifted caudally towards the visceral mass, was nearly devoid of NADPHd activity (Fig. 2F).

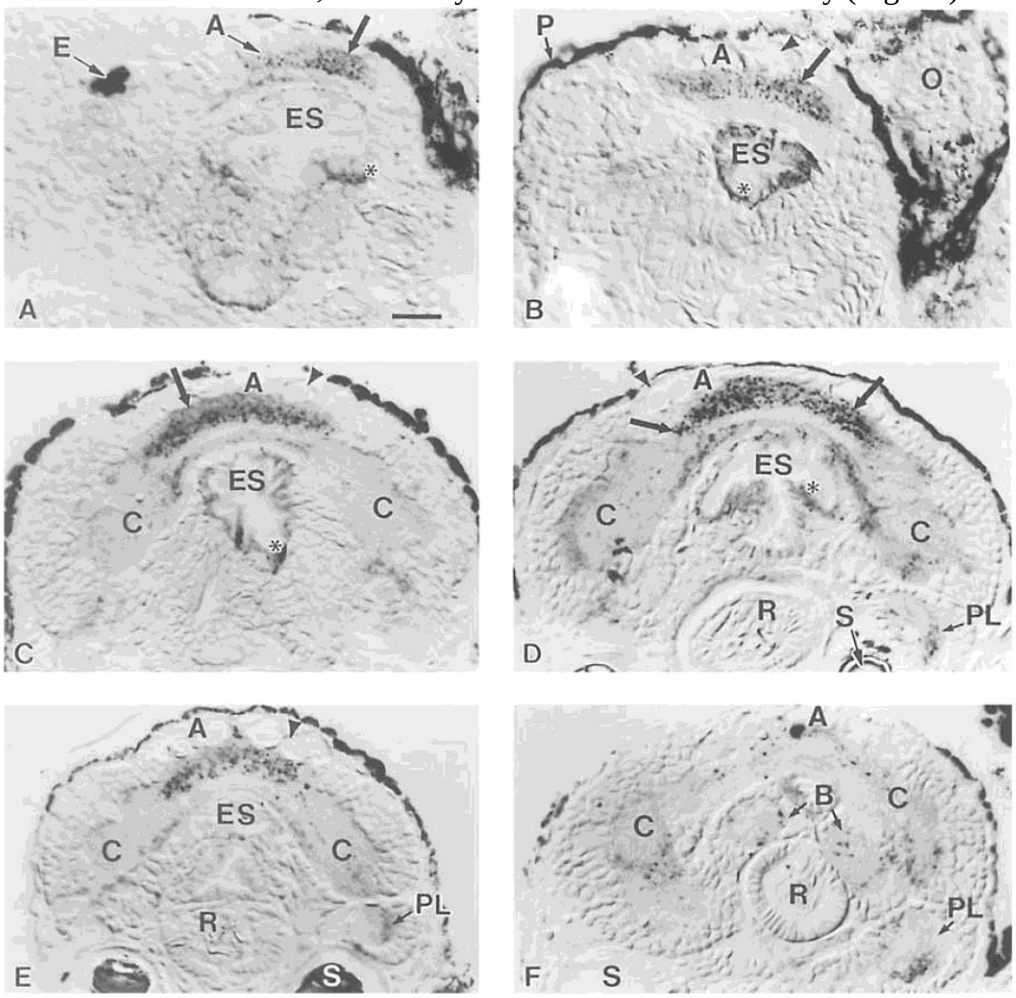

Fig. 2. Transverse sections through the apical ganglion showing NADPHd activity. **A:** 6 days. **B:** 8 days. **C:** 12 days after hatching. **D:** Competent larva. **E:** Metamorphosing larva. **F:** Newly metamorphosed juvenile. Note the large somata (arrowheads in B–E) above the stained neuropil of the apical ganglion and the increasing NADPHd activity (arrows in A–D) during larval development. Staining intensity decreases in metamorphosing larvae (E) and is virtually absent in young juveniles (F). Esophageal epithelium also displays NADPHd activity (asterisks in A–D). Scale bar = 20 μm.

The neuropils of the cerebral ganglia contained much punctate staining for NADPHd (Fig. 3) and at 6 days and 8 days after hatching, regions of these neuropils displayed localized staining intensities similar to those of the apical ganglion (Fig. 3A,B). In older larvae, NADPHd staining was predominately located at the margins of the neuropils, with relatively less staining occurring medially (Fig. 3B,C). Staining on the interior margin of the neuropil was generally darker than that of the exterior margin (data not shown). As in the apical ganglion, staining intensity in the neuropils of the cerebral ganglia decreased during metamorphosis (Fig. 3D). In newly metamorphosed juveniles, NADPHd staining in the cerebral ganglia was similar to that of competent larvae. However, in advanced juveniles, NADPHd staining intensity is enhanced and restricted to the anterolateral margin of these neuropils (Fig. 3F,H).

NADPHd reaction product in the pedal ganglia was first detectable 8 days after hatching (Fig. 3A). In older larvae and in juveniles, punctate staining in the pedal ganglia was scattered throughout the neuropil (Figs. 3F, 4). Staining intensity in the pedal ganglia was typically less dense than in the apical ganglion.

The pleural ganglia showed no NADPHd activity in young animals (Fig. 5A). By 12 days, each pleural ganglion manifested a unique staining pattern (Fig. 5B), with the left pleural ganglion staining intensely in its ventromedial neuropil and the right one staining dorsomedially. This pattern was retained in all subsequent stages (Fig. 5B—E). In competent larvae, the staining intensity was stronger and the stained region was

relatively large. During metamorphosis, NADPHd activity was reduced in the pleural ganglia, but increased to its pre-metamorphic level in newly metamorphosed juveniles (Fig. 5D,E). In advanced juveniles, the left pleural displayed NADPHd activity ventrally, near the left cerebral ganglion, just dorsal to the statocysts (Fig. 3G).

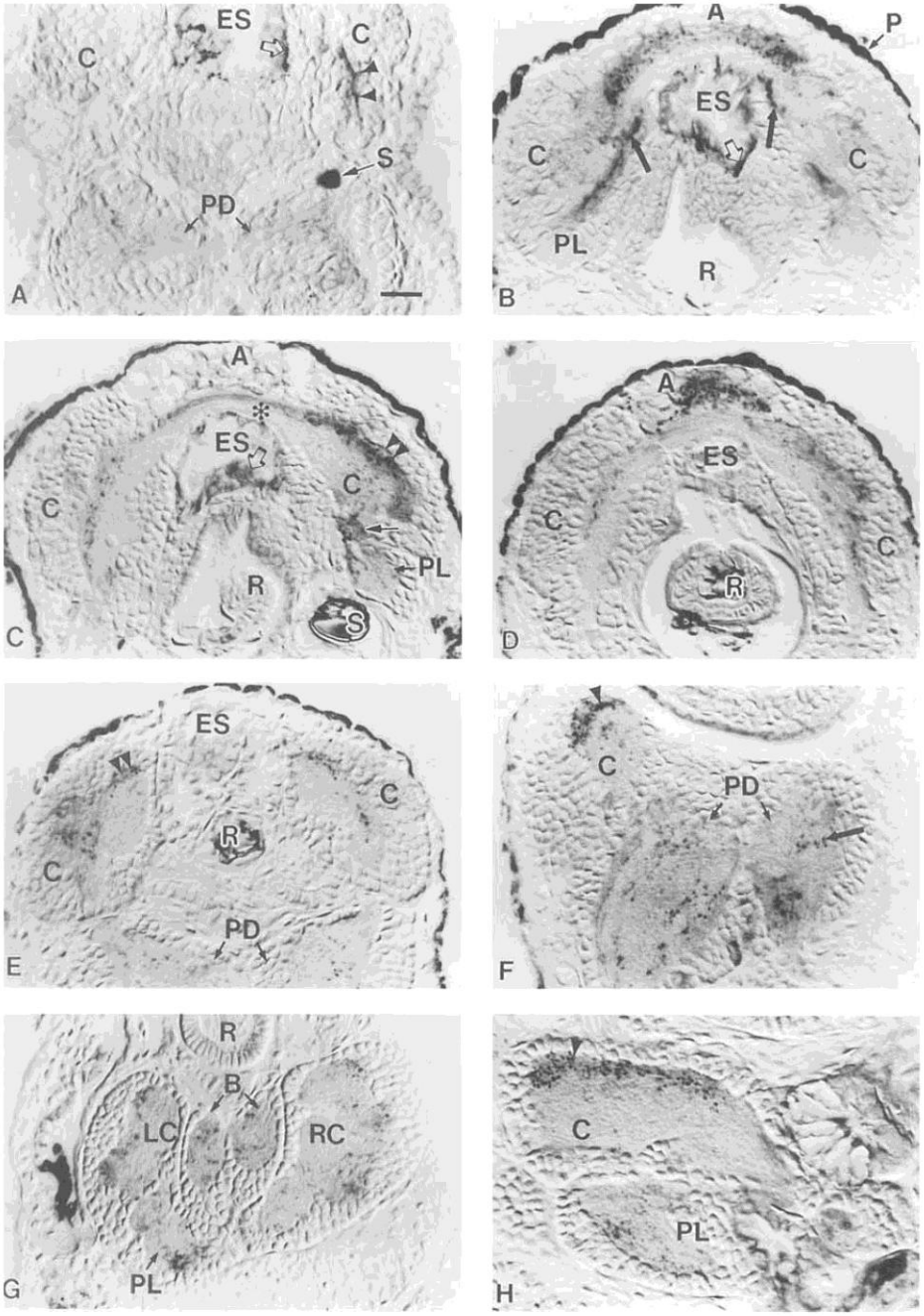

Fig. 3. Transverse (**A–G**) and sagittal (**H**) sections through the cerebral and pedal ganglia in larvae and post-metamorphic juveniles. F: Slightly oblique section. A: NADPHd activity (arrowheads) in the cerebral ganglia at 8 days after hatching occurs between neuronal somata in the developing neuropils. Staining in the pedal neuropils is inconspicuous. B: At 12 days after hatching, staining in the cerebral ganglia occurs on their interior neuropilar margins (arrows). C: Little NADPHd activity occurs in the cerebral commissure (asterisk), even in competent larvae. NADPHd activity in the cerebral ganglia now occurs at their exterior margins (arrowheads) and at the junction with the pleural ganglia (arrow). NADPHd activity also occurs in the esophageal epithelium (open arrows in A–C). D: The cerebral ganglia lose much staining during metamorphosis. E: NADPHd activity in the cerebral ganglia of newly metamorphosed juveniles remains relatively low and occurs along their exterior margins (arrowheads). F,H: Staining in the cerebral ganglia of advanced juveniles is mostly anterior and is scattered along their exterior margins (arrowheads). F: NADPHd activity in the pedal ganglia occurs scattered throughout the neuropils (arrow). G. The buccal ganglia of advanced juveniles also display scattered NADPHd activity. Scale bar = 20 μm.

Before metamorphosis, the buccal ganglia displayed little (Fig. 5C) or no staining (Fig. 5A,B) for NADPHd. In contrast, juveniles exhibited scattered NADPHd activity throughout the buccal neuropils (Fig. 3G).

At 8 days after hatching, the subintestinal ganglion expressed little NADPHd activity (Fig. 5A). In competent larvae, staining was detected at the junction between the subintestinal and the right pleural ganglion (Fig. 5C).

Throughout all larval stages the supraintestinal ganglion exhibited only weak staining (Fig. 6). Staining levels in both intestinal ganglia increased in juveniles (Figs. 5E,F, 6E,F).
The osphradial ganglion exhibited only a small amount of staining in competent larvae (Figs. 5C, 6C), with no staining occurring in younger animals. In advanced juveniles, the osphradial neuropil displayed significant amounts of staining (Fig. 6F). The visceral ganglion also showed some staining ventrally in advanced juveniles (Fig. 5F).

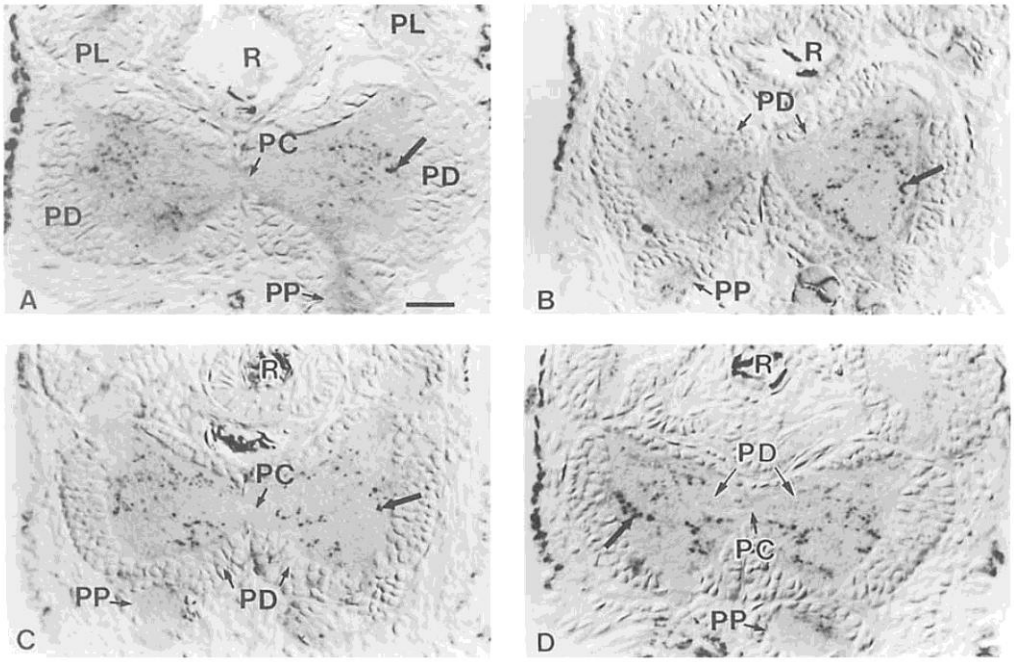

Fig. 4. Transverse sections through the pedal ganglia. Note scattered punctate staining (arrows) throughout their neuropils in all stages shown. The propodial ganglia show only minor NADPHd activity. **A:** 12 days after hatching. **B:** Competent larva. **C:** Metamorphosing larva. **D:** Newly metamorphosed juvenile. Scale bar = 20 μm.

DISCUSSION

Throughout the planktonic phase, specific NADPHd staining patterns occurred in different ganglia in a consistent fashion (Table 1). In larvae, the greatest NADPHd activity occurred in the apical ganglion; intermediate levels of activity arose in the neuropils of the paired cerebral, pedal, and pleural ganglia. The least amount of activity existed in the buccal, intestinal, and unpaired osphradial ganglia. The youngest group we examined, larvae 6 days after hatching, had NADPHd-positive reaction product in only three positions: in the neuropils of the apical and paired cerebral ganglia. In juveniles, staining levels remained relatively constant in the cerebral, pedal, and pleural ganglia but showed marked increases in the buccal, osphradial, and subintestinal ganglia.

Reports of NADPHd staining in other molluscs include descriptions of reactive somata and neuropilar processes (Eloffson et al., 1993; Cooke et al., 1994; Jacklet and Gruhn, 1994a; Moroz et al., 1994a,b; Nezlin et al., 1994; Sanchez-Alvarez et al., 1994). Cooke et al. (1994) accentuated the staining of somata by crushing various nerves and connectives, a method which is not amenable to our small specimens. As Elphick et al. (1995a) reported for adult *Lymnaea*, we consistently found no NADPHd activity in neuronal cell bodies in either larval or juvenile ganglia. NOS in young molluscs appeared to be contained mostly within synaptic neuropilar processes.

Synaptic glomeruli are common structures in olfactory systems in vertebrates, arthropods, and molluscs (Leise, 1990; Chase and Tolloczko, 1993). As an example, in pulmonate gastropods they occur in the ganglia that lie at the bases of the olfactory tentacles (Chase, 1986). As yet, these glomeruli have not been reported to possess NO activity. On the contrary, the lateral terminus, a large synaptic glomerulus that occurs in the visual pathway of *Aplysia*, displays strong NADPHd staining (Jacklet and Gruhn, 1994). NADPHd staining in larval and juvenile *Ilyanassa* revealed no evidence of glomeruli or other internal organization in any ganglionic neuropils, not even in the apical, cerebral or osphradial ganglia, which are the ones most likely to participate in any olfactory

processing. Larval and juvenile *Ilyanassa* probably respond to a relatively small number of odorants as compared to the strongly chemoreceptive adults (Carr, 1967a,b; Gurin and Carr, 1971; Atema and Burd, 1975; Hurd, 1985) and so may have experienced little selection pressure for the evolution of complex modular organization within their neuropils. An understanding of internal neuropilar organization within *Ilyanassa* larvae and juveniles awaits further neuroanatomical studies.

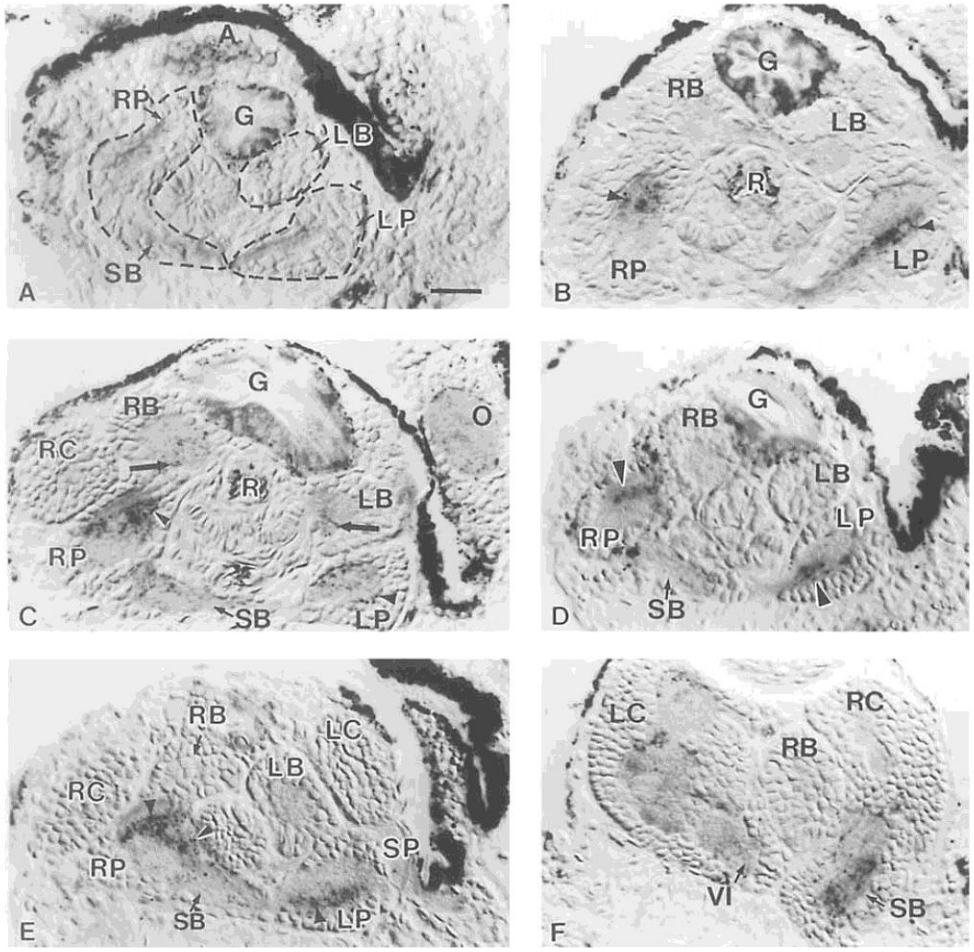

Fig. 5. Transverse sections through the pleural, buccal, and subintestinal ganglia. **A:** 8 days. **B:** 12 days after hatching. **C:** Competent larva. **D:** Metamorphosing larva. **E:** Newly metamorphosed juvenile. **F:** Advanced juvenile. A,B: The buccal ganglia display little staining in young larvae. C: NADPHd activity occurs in both buccal ganglia (arrows) and weakly in the osphradial ganglion in competent larvae. B–E: Each pleural ganglion has its own staining pattern (arrowheads): left, ventromedially and right, dorsomedially. C–F: The subintestinal ganglion shows weak NADPHd activity earlier, but stains intensely in advanced juveniles. Scale bar = 20 μm.

The functions of NO in *Ilyanassa* larvae are as yet unknown, but the staining patterns we observed suggest a number of possibilities. NADPHd staining was significantly reduced in most ganglia of metamorphosing larvae and in the apical ganglion of young juveniles. This ganglion is lost in advanced juveniles (Lin and Leise, 1996). Whether this occurs by apoptotic or necrotic cell death related to NO release or by the translocation of these cells into other ganglia is unknown. In the abalone *Haliotis rufescens*, cerebral serotonergic neurons that innervate the velum are most likely lost by cell death (Barlow and Truman, 1992). In mammals, changes in NOS activity correlate with synaptogenesis and cell death (Bredt and Snyder, 1994; Williams et al., 1994; Ogilvie et al., 1995), two processes common to developing nervous systems. Edelman and Gally (1992) have suggested that NO could function to coordinate the development of groups of interactive neurons, but further experiments would be necessary to document this phenomenon in developing *Ilyanassa.*

Perhaps more intriguing is the differential pattern of NADPHd staining we observed between competent, metamorphosing larvae, and juvenile *Ilyanassa,* particularly in the apical ganglion. Our results suggest that NO is involved somewhere in the pathway that leads to metamorphosis and are consistent with three alternative hypotheses: NO could be active either in metamorphic inhibition or initiation, or NO may have no metamorphic function per se, but modulate distinctive activities in larvae and juveniles. We are currently testing these hypotheses with pharmacological methods.

The strong NADPHd staining we observed in the apical and cerebral ganglia of larval *Ilyanassa* further suggests that NO may function in larvae in ways that resemble NO activities in adult molluscs. NO is active in feeding (Elphick et al., 1995a) and olfactory (Gelperin, 1994a,b) circuits in adult gastropods. For example, chemosensory stimulation of the lips of *Lymnaea stagnalis* appears to activate NO-producing bipolar sensory neurons that synapse upon the cerebral giant cells (CGCs), the command neurons that activate the CPG underlying feeding movements (Elphick et al., 1995a). These synapses are located in the neuropil of the cerebral ganglion. Inhibition of NOS has no effect on CGC activation of the feeding motor program, but does inhibit sensory input to these neurons (Elphick et al., 1995a). In another gastropod, the slug *Limax maximus,* normal oscillatory electrical activity of the procerebral lobe is related to behavioral odor selectivity and learning, and is dependent upon the release of NO (Gelperin, 1994a,b).

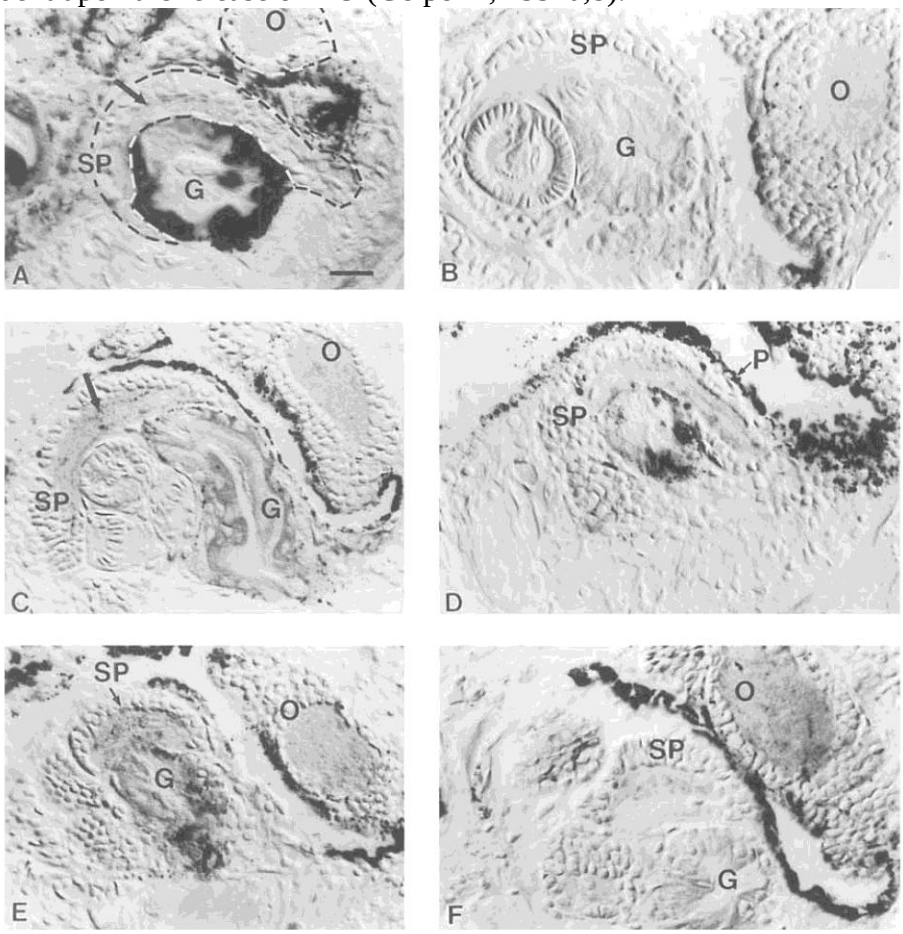

Fig. 6. Transverse sections through the supraintestinal and osphradial ganglia. **A:** 8 days. **B:** 12 days after hatching. **C:** Competent larva. **D:** Metamorphosing larva. **E:** Newly metamorphosed juvenile. **F:** Advanced juvenile. A,B: Note weak staining in supraintestinal ganglion (arrow) in young larvae. C–E: Some reaction product is visible in competent larvae (arrow) and juveniles. E,F: The osphradial ganglion displays significant NADPHd activity only after metamorphosis. Scale bar = 20 μm.

When competent, larval *Ilyanassa* settle and metamorphose in response to a small organic molecule that occurs in the natural juvenile habitat (Scheltema, 1961; Levantine and Bonar, 1986). The chemosensory neurons that mediate this response are as yet unidentified but are likely to be either in the apical ganglion or the foot (reviewed in Lin and Leise, 1996). The cerebral, pedal, and apical ganglia all displayed NADPHd staining, suggesting that NO could be involved in the processing of chemical signals in any of these ganglionic neuropils.

Little is known about the neuronal control of feeding in larval molluscs. In larvae, the velum, mouth, and foregut are the major organs used in opposed band feeding (Carter, 1926; Thompson, 1959; Fretter and Graham, 1962). The compound cilia of the pre-oral band move water and food particles into the food groove on the edge of the velum, which then transports trapped food particles to the mouth. In gastropod larvae, the velum and foregut are innervated by the apical and cerebral ganglia (Carter, 1926; Mackie et al., 1976; Arkett et al., 1987; Arkett, 1988; Marois and Carew, 1990; Barlow and Truman, 1992), both of which exhibited positive staining for NADPHd *Ilyanassa.* This staining pattern again suggests that NO could be active either in circuits that integrate information about environmental odorants or that control ciliary beating leading to particle acquisition.

Cessation of ciliary beating is under neuronal control (Mackie et al., 1976; Arkett et al., 1987), but changes in ciliary beating or velar posture that directly relate to particle capture are unexplored. Baldwin (1995) has shown that selective particle retention, presumably based on chemosensory information derived from com-pounds released by the available algal cells, occurs in bivalve larvae. Whether or not such selection occurs in our gastropod larvae is unknown, but the strong NADPHd staining in the apical and cerebral ganglia, and the existence of neurons there that innervate the velum, suggest that these ideas deserve further investigation.

TABLE 1. Relative Staining Intensities for NADPHd Activity in Ganglia at Different Developmental Stages[1]

Ganglion	6 days after hatching	8 days after hatching	12 days after hatching	Competent larva	Metamorphosing larva	Young juvenile	Advanced juvenile
Apical	+++	+++	++++	+++++	++++	+	a
Cerebral	a	++	+++	+++	++	+++	++++
Pedal	a	+	+++	+++	++	+++	+++
Pleural	a	–	++	+++	+	+++	+++
Buccal	a	–	–	+	–	+	++
Osphradial	–	–	–	+	–	+	++
Subintestinal	a	–	–	+	–	+	+++
Supraintestinal	a	–	–	+	+	++	++
Visceral	a	a	a	+	–	+	+

[1]a, ganglion has not yet developed or has disappeared after metamorphosis; –, staining absent; ++++ indicates most intense staining.

In adult *Lymnaea*, NO indirectly activates the buccal motoneurons of the feeding CPG through synaptic interactions in the cerebral ganglia (Moroz et al., 1993a,b; Elphick et al., 1995a). However, NADPHd-positive staining and immunoreactivity to NOS have also been reported for its buccal ganglia (Moroz and Roylance, 1993; Moroz et al., 1994a,b). How or if these neurons affect the activity of the feeding circuitry is unknown. The buccal ganglia of larval *Ilyanassa* showed little NADPHd staining. However, in juveniles the buccal ganglia exhibited significant amounts of staining which temporally overlap with the transition to radular feeding (Lin and Leise, 1996). NADPHd reaction product in the buccal ganglia of juvenile *Ilyanassa* suggests that in these animals and perhaps in adults, the feeding circuitry could be under nitrergic modulation.

LITERATURE CITED

Arkett, S.A. (1988) Development and senescence of control of ciliary locomotion in a gastropod veliger. J. Neurobiol. /9:612-623.

Arkett, S.A., G.O. Mackie, and C.L. Singla (1987) Neuronal control of ciliary locomotion in a gastropod veliger *(Calliostoma).* Biol. Bull. / 73:513-526.

Atema, J., and G.D. Burd (1975) A field study of chemotactic responses of the marine mud snail, *Nassarius obsoletus.* J. Chem. Ecol. /:243-251.

Baldwin, B.S. (1995) Selective particle ingestion by oyster larvae *(Grassostrea virginica)* feeding on natural seston and cultured algae. Mar. Biol. *123:95-107* .

Barlow, L.A., and J.W. Truman (1992) Patterns of serotonin and SCP immunoreactivity during metamorphosis of the nervous system of the red abalone, *Haliotm rufuscens.* J. Neurobiol. 23:829-844.

Bicker, G., and I. Halnlein (1995) NADPH-diaphorase expression in neurones and glial cells of the locust brain. NeuroReport 6:325-328.

Bredt, D.S., and S.H. Snyder (1992) Nitric oxide, a novel neuronal messenger. Neuron *8:3-11.*

Bredt, H.S., and S.H. Snyder (1994) Transient nitric oxide synthase neurons in embryonic cerebral cortical plate, sensmy ganglia, and olfactory epithelium. Neuron /3:301-313.

Bruhwyler, J., E. Chleide, J.F. Liegeois, and F. Carreer (1993) Nitric oxide: A new messenger in the brain. Neurosci. Behav. Rev. / 7:373-384.

Carr, W.E.S. (1967a) Chemoreception in the mud snail, *Nassarius obsoletus. I.* Properties of stimulatory substances extracted from shrimp. Biol. Bull. /32:90-105.

Carr, W.E.S. (1967b) Chemoreception in the mud snail, *Nassarius obsoletus. II.* Identification of stimulatory substances. Biol. Bull. *133:106-127* . Carter, G.S. (1926) On the nervous control of the velar cilia of the nudibranch veliger. J. Exp. Biol. *4:1-26.*

Chase, R. (1986) Lessons from snail tentacles. Chem. Sens. 11 :411-420. Chase, R., and B. Tolloczko (1993) Tracing neural pathways in snail olfaction: From the tip of the tentacles to the brain and beyond. Micros.

Res. Tech. 24:214-230. Chichery, R. and M.-P. Chichery (1994) NADPH-diaphorase in a cephalopod brain *(Sepia):* Presence in an analogue of the cerebrellum. NeuroReport 5:1273-1276.
Cooke, I.R.C., S.L. Edwards, and C.R. Anderson (1994) The distribution of NADPH diaphorase activity and immunoreactivity to nitric oxide synthase in the nervous system of the pulmonate mollusc *Helix aspersa.* Cell Tiss. Res. 277:565-572.
Dawson, T.M., and S.H. Snyder (1994) Gases as biological messengers: Nitric oxide and carbon monoxide in the brain. J. Neurosci. 14:5147-5159.
Dawson, T.M., D.S. Bredt, M. Fotuhi, P.M. Hwang, and S.H. Snyder (1991) Nitric oxide synthase and neuronal NADPH diaphorase are identical in brain and peripherial tissue. Proc. Natl. Acad. Sci. U.S.A. 88:7797-7801.
Edelman, G.M., and J.A. Gally (1992) Nitric oxide: linking space and time in the brain. Proc. Natl. Acad. Sci. U.S.A. 89:11651-11652.
Elofsson, R., M. Carlberg, L. Moroz, L. Nezfin, and D. Sakharov (1993) Is nitric oxide (NO) produced by invertebrate neurons? NeuroReport 4:279-282.
Elphick, M.R., I.C. Green, and M. O'Shea (1993) Nitric oxide synthesis and action in an invertebrate brain. Brain Res. 61 9:344-346.
Elphick, M.R., G. Kemenes, K. Staras, and M. O'Shea (1995a) Behavioral role for nitric oxide in chemosensory activation of feeding in a Mollusc. J. Neurosci. *15:7653-7664.*
Elphick, M.R., R.C. Rayne, V. Riveros-Moreno, S. Moncada, and M. O'Shea (1995b) Nitric oxide synthesis in locust olfactory interneurones. J. Exp. Biol. /98:821-829.
Fretter, V., and A. Graham (1962) British Prosobranch Molluscs. London: Royal Society.
Gelperin, A. (1994a) Nitric oxide mediates network oscillations of olfactory interneurons in a terrestrial mollusc. Nature 369:61-63.
Gelperin, A. (1994b) Nitric oxide, odour processing and plasticity. Neth. J. Zool. *44:159-169.*
Gurin, S., and W.E.S. Carr (1971) Chemoreception in *Nassarius obsoletus:* The role of specific stimulatory proteins. Science *174:293-295.*
Hope, B.T., G.J. Michael, K.M. Knigge, and S.R. Vincent (1991) Neuronal NADPH diaphorase is a nitric oxide synthase. Proc. Natl. Acad. Sci. U.S.A. 88:2811-2814.
Hope, B.T., and S.R. Vincent (1989) Histochemical characterization of neuronal NADPH-diaphorase. J. Histochem. Cytochem. 37:653-661.
Hurd, L. E. (1985) On the importance of carrion to reproduction in an omnivorous estuarine neogastropod, *Ilyanassa obsoleta* (Say). Oecologia 65:513-515.
Jacklet, J.W. (1995) Nitric oxide is used as an orthograde cotransmitter at identified histaminergic synapses. J. Neurophys. 74:891-895.
Jacklet, J.W., and M. Gruhn (1994a) Co-localization of NADPH-diaphorase and myomodulin in synaptic glomeruli of *Aplysia.* NeuroReport *5:1841-* 1844.
Jacklet, J.W., and M. Gruhn (1994b) Nitric oxide as putative transmitter in *Aplysia:* Neural circuits and membrane effects. Neth. J. Zool. 44:524- 534.
Johansson, K.U.I., and M. Carlberg (1994) NADPH-diaphorase histochemistry and nitric oxide synthase activity in deutocerebrum of the crayfish, *Pacifastacus leniusculus* (Crustacea, Decapoda). Brain Res. 649:36-42.
Leise, E.M. (1990) Modular construction of nervous systems: A basic principle of design for invertebrates and vertebrates. Brain Res. Rev. *15:1-23.*
Levantine, P.L., and D.B. Bonar (1986) Metamorphosis of *Ilyanassa obsoleta:* Natural and artificial inducers. Am. Zool. *26:14A.*
Lin, M.-F., and E.M. Leise (1996) Gangliogenesis in the Prosobranch Gastropod *ilyanassa obsoleta.* J. Comp. Neurol. *374:180-193.*
Lin, M.-F., and E.M. Leise (1994) NADPH-diaphorase staining in the nervous systems of larval and juveniles of the prosobranch mollusc *Ilyanassa obsoleta.* Am. Zool. *34:100A.*
Lowenstein, C.J., and S.H. Snyder (1992) Nitric oxide, a novel biologic messenger. Cell *70:705-707* .
Mackie, G.O., C.L. Singla, and C. Thiriot-Quievreux (1976) Nervous control of ciliary activity in gastropod larvae. Biol. Bull. *151:182-199.*

Marois, R., and T.J. Carew (1990) The gastropod nervous system in metamorphosis. J. Neurobiol. *21:1053-1071.*
Meyer, W. (1994) NADPH diaphorase (nitric oxide synthase) in the central nervous system of spiders (Arachnia: Araneida). Neurosci. Lett. /65:105- 108.
Moroz, L.L. A.G.M. Bulloch, K. Lukowiak, and N.I. Syed (1994a) Putative NO-synthesizing neurons of *Lymnaea* in vivo and in vitro. Neth. J. Zool. *44:535-549.*
Moroz, L.L., and J.-H. Park, (1993) Nitric oxide modulates the central respiratory patterns in *Lymnaea stagnalis. J. Physiol. 473:188P.*
Moroz, L.L., J.-H. Park, and W. Winlow (1993a) Nitric oxide activates buccal motor patterns in *Lymnaea stagnalis.* NeuroReport 4:643-646.
Moroz, L.L., J.-H. Park, and W. Winlow (1993b) Nitric oxide activates the central feeding programme in Lymnaea stagnalis. J. Physiol. 47,3:27P.
Moroz, L.L., I. Roger, N.I. Syed, A.G. M. Bulloch, and K. Lukowiak (1993c) Abundance of putative NO-synthesizing cells in the oesophagus of gastropod molluscs. J. Physiol. 473:247P.
Moroz, L.L., and A.J. Roylance (1993) Antibodies against cerebellar nitric oxide (NO) synthase label cells in the CNS and peripheral tissues of the freshwater snail, *Lymnaea stagnalis.* J. Physiol. 473:28P.
Moroz, L.L., W. Winlow, R.W. Turner, A.G.M. Bulloch, K. Lukowiak, and N. Syed (1994b) Nitric oxide synthase-immunoreactive cells in the CNS and periphery of *Lymnaea.* NeuroReport 5:1277-1280.
Muller, U. (1994) Ca.2-+ /calmodulin dependent nitric oxide synthase in *Apis mellifera* and *Drosophila melanogaster*. Eur. J. Neurosci. 6:1362-1370.
Muller, U., and G. Bicker (1994) Calcium-activated release of nitric oxide and cellular distribution of nitric oxide-synthesing neurons in the nervous system of the locust. J. Neurosci. *14:7 521-7528.*
Muller, U., and E. Buchner (1993) Histochemical localization of NADPH-diaphorase in adult *Drosophila brain:* Is nitric oxide a neuronal messenger also in insects? Naturwissenschaften 80:524-526.
Nezlin, L.P., R. Elofsson, and D.A. Sakharov (1994) Transmitter-specific subsets of sensory elements in the prosobranch osphradium. Biol. Bull. /87:174-184.
Ogilvie, P., K. Schilling, M.L. Billingsley, and H.H. Schmidt (1995) Induction and variants of neuronal nitric oxide synthase type I during synaptogenesis. FASEB J. 9:799-806.
Robertson, J.D., J. Bonaventura, and A.P. Kohm (1994) Nitric oxide is required for tactile learning in *Octopus vulgaris.* Proc. R. Soc. Lond. B 256:269-273.
Robertson, J.D., J. Bonaventura, and A. Kohm (1995) Nitric oxide synthase inhibition blocks octopus touch learning without producing sensory or motor dysfunction. Proc. R. Soc. Lond. B. *261:167-172.*
Sanchez-Alvarez, M., M. Leon-Olea, E. Talavera, F. Pellicer, E. Sanchez-Islas, and G. Martinez-Lorenzana (1994) Distribution of NADPH-diaphorase in the perioesophageal ganglia of the snail, *Helix aspersa.* Neurosci. Lett. /69:51-55.
Sawada, M,, M. Ichinose, and N. Hara (1995) Nitric oxide induces an increased Na+ conductance in identified neurons of *Aplysia.* Brain Res. 670:284-256.
Scheltema, R.S. (1961) Metamorphosis of the veliger larvae of *Nassarius obsoletus* (Gastropoda) in response to bottom sediment. Biol. Bull. /20:92-109.
Schuman, E.M., and D.V. Madison (1994) Nitric oxide and synaptic function. Annu. Rev. Neurosci. / 7:153-183.
Snyder, S.H. (1992) Nitric oxide: First in a new class of neurotransmitters? Science 257:494-496.
Talavera, E., G. Martinez-Lorenzana, M. Leon-Olea, M. Sanchez-Alvarez, E. Sanchez-Islas, and F. Pellicer (1995) Histochemical distribution of NADPH-diaphorase in the cerebral ganglion of the crayfish *Cambarellus montezumae.* Neurosci. Lett. /87:177-180.
Thompson, T.E. (1959) Feeding in nudibranch larvae. J. Mar. Biol. Assoc. U.K. 38:239-248.
Vincent, S.R. (1994) Nitric oxide: A radical neurotransmitter in the central nervous system. Prog. Neurobiology *42:129-160.*
Williams, C.V., D. Nordquist, and S.C. McLoon (1994) Correlation of nitric oxide synthase expression with changing patterns of axonal projections in the developing visual system. J. Neurosci. *14:1746-1755.*
Zhuo, M., S,A. Small, E.R. Kande', and R.D. Hawkins (1993) Nitric oxide and carbon monoxide produce activity-dependent long-term synaptic enhance-ment in hippocampus. Science *260:1946-1950.*