GREAT LAKES FISHERY COMMISSION

1996 Project Completion Report[1]

Sea lamprey olfactory activity: prolarvae and larvae

by:

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Completion Report:

Sea lamprey olfactory activity: prolarvae and larvae

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Summary:

The objective of our study was to characterize olfactory activity in the prolarval and larval stages of the sea lamprey, *Petromyzon marinus*. We found that as early as Piavis stage 17, prolarvae elicited both physiological and behavioral responses to stimulation with odors.

Briefly, our results show:

- Prolarval olfactory mucosal neural responses: large amplitude responses to prolarval nonsiblings, concentration dependent responses to L-arginine and taurocholic acid, and reversible blocking of the L-arginine by N ω nitro L-arginine.
- Prolarvae increased movement in 10^{-12} M taurocholic acid, decreased movement slightly in 10^{-5} M L-arginine.
- Prolarvae abandoned a nest at Piavis stage 17.
- The mouth of stage 18 prolarvae contained putative taste cells.

- Larval electro-olfactogram (EOG) recordings showed concentration dependent responses to taurocholic acid and L-arginine. EOG responses were also present to putative larval settlement cues: γ-aminobutyric acid and serotonin; and relatively low amplitude responses to conditioned water from ammocoetes, prolarvae and rainbow trout.
- Behavioral preference/avoidance observations showed slight preference to L-arginine (10^{-4} M) and avoidance to taurocholic acid (10^{-6} M).

Overall, the responses to L-arginine were intriguing, because they were inhibited by the L-arginine analogue, N ω nitro L-arginine. This is the first example of an odor response that can be chemically reversibly blocked. However, the L-arginine, nitric oxide synthase, nitric oxide pathway is certainly not limited to lampreys, and application of these compounds would likely have transient effects on nontarget organisms. However, amino acids are also commonly used commercially in the sport fishing industry as bait enhancers.

Our results suggest that bile derivatives from conspecifics could be developed to assist with population control. The fact that prolarvae displayed large amplitude responses to nonsibling conspecifics, suggests that the prolarvae use this ability to recognize sites with conspecifics of similar age class during their downstream movement to larval settlement sites.

Summary

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Part 7. Western immunoblot analysis of nitric oxide synthase in prolarval, larval and spawner stages. (E. Wong, A. Zaidi)

Appendix 1. Galley proof: Nitric oxide synthase in the olfactory mucosa of the larval sea lamrpey. J. comp. Neurol.

Appendix 2. 1994: Larval behavioural responses

1. Prolarvae: olfactory mucosal neural responses

Objective: The purpose of these experiments was to determine if the olfactory system in prolarvae responded physiologically to the application of test solutions. The tests included the amino acid L-arginine and its analogues to determine if this potent chemostimulant in larval and adult stages is active during early development. Furthermore, we tested the possibility that the L-arginine response could be blocked by its analogue Nω-nitro-L-arginine, which is also a blocker of the enzyme nitric oxide synthase. We tested the possibility that the prolarvae communicate with conspecifics by the olfactory system, by applying lavages from prolarvae, ammocoetes and by applying the bile acid taurocholic acid.

Technique: Olfactory mucosal neural responses to chemostimulation were recorded at the Lake Huron Biological Station from stage 17 prolarvae (20-25 days after fertilization). Prolarvae were placed onto a PVC ramp in a Petrie dish filled with 0.9% saline (NaCl) in deionized lake water. A recording electrode was applied onto the posterior surface of the nasal cavity, with a ground electrode on the tail of the prolarva. The positioning was visualized with a dissecting micorscope installed onto an overhanging boom. AC recordings using a a Super Z impedence probe were obtained with epoxy-insulated tungsten microelectrodes (Longreach Scientific, Maine). To prevent mechanoreception from interfering with the olfactory responses, test solutions (100 μl) were applied gently from a syringe onto the water surface at the edge of the dish. Test solutions included: L-arginine, L-citrulline, homoarginine, L-serine, L-histidine, Nω-nitro-L-arginine, taurocholic acid, D-arginine, conditioned water from tanks with ammocoetes (approx. 7cm length), conditioned water from sibling prolarvae and conditioned water from nonsibling prolarvae.

Results: The responses were characterized by an increase in spike amplitude (fig.1.1). L-arginine elicited responses, whereas the application of the stereoisomer d-arginine, was followed by a low response magnitude (fig. 1.1A). The neutral amino acid L-serine failed to elicit responses, and there were low magnitude responses to other basic amino acids such as L-histidine. Following application of the nitric oxide synthase inhibitor, N-ω L arginine, the response magnitude to L-arginine decreased (fig. 1.1B). Following this suppression of the

arginine response, the preparation was able to respond to chemostimulation with taurocholic acid, demonstrating that the taurocholic acid was be independent of the arginine response. Concentration dependence was observed both for the arginine series (not shown) and for taurocholic acid (fig. 1.1 C). The response magnitude to conditioned water from nonsibling prolarvae was considerably larger than to conditioned water from sibling conspecifics (fig.1.1 D). Concentration dependence was also demonstrated to water from nonsibling prolarva (fig. 1.1 E). The response to conditioned ammocoete water was of a lower magnitude than to prolarvae, and the activity appeared to have a greater tonic component than the previous tests (fig. 1.1F).

Discussion: In prolarvae, L-arginine was a potent chemostimulant. As previously demonstrated in larvae (Zielinski et al., 1995), the enzyme nitric oxide synthase maybe associated with responses to L-arginine. The response magnitude to L-agrinine decreased following the application of Lω-nitroL-arginine, and did not recover to its full strength. This activity supports the views of Breer and Shepherd (1993) that nitric oxide is a second messenger that amplifies olfactory responses by gating cyclic GMP channels. Alternatively, the blocker may act on the L-arginine receptor site. The blocking effect of Lω-nitroL-arginine was reversible, as the diminished L-arginine response eventually recovered in each preparation. The specific effect of this blocker on decreasing the olfactory response may not be limited to the prolarval lampreys, and may extend to olfactory responses of other animals occupy the same habitat as the prolarvae.

The extreme sensitivity to taurocholic acid suggests that the prolarvae may also respond to application of the unique lamprey bile product petromyzonal sulphate. Unfortunately, we were unable to test this product, but the strong responses to conspecifics suggest that petromyzonol sulphate may be a potent chemostimulant to prolarvae.

The high response magnitude to siblings shows that the prolarvae are able to distinguish siblings from nonsiblings. The low response magnitude to siblings may simply reflect an adaptation and densitization response compared to the nonsibling water. This may be difficult to test, even by recording from prolarvae that had been separated from siblings, because these prolarvae, even if they were kept individually would still be adapting to their own odor.

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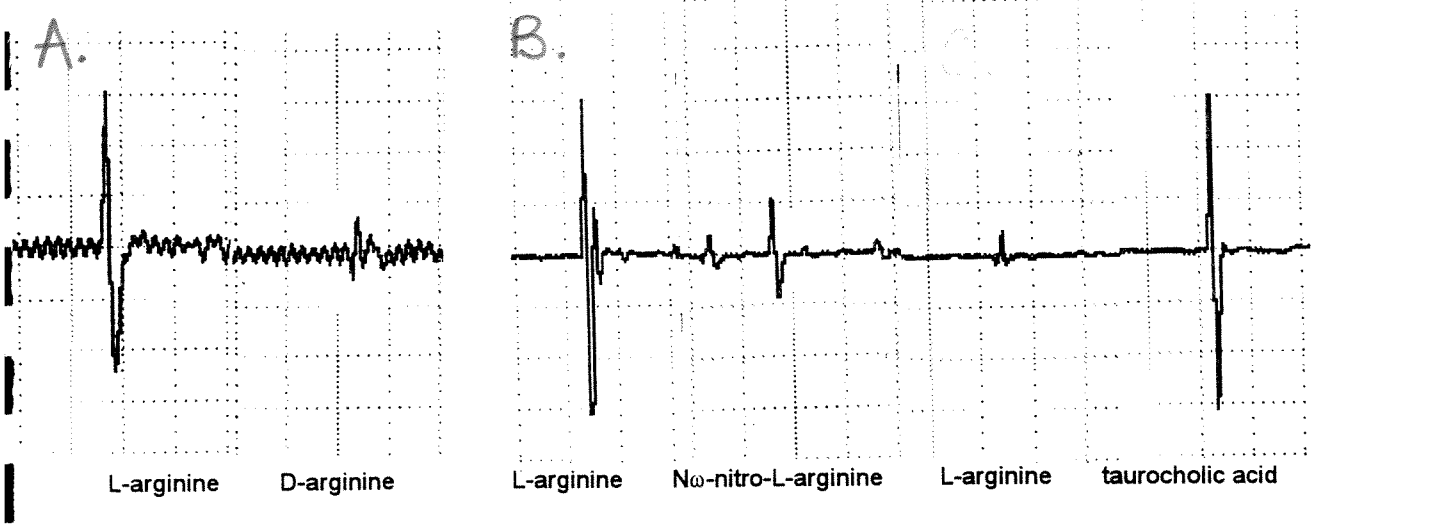
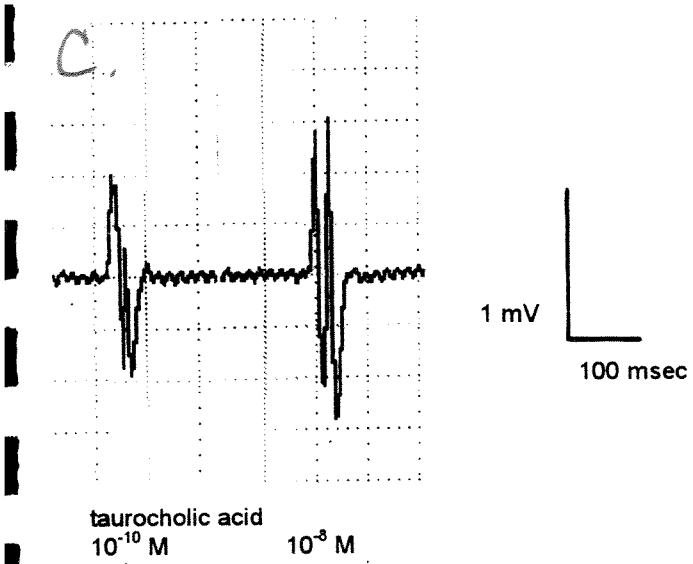
The high magnitude nonsibling response may assist prolarvae that are moving from nests to settlement sites to identify locations with conspecifics of the same age class.

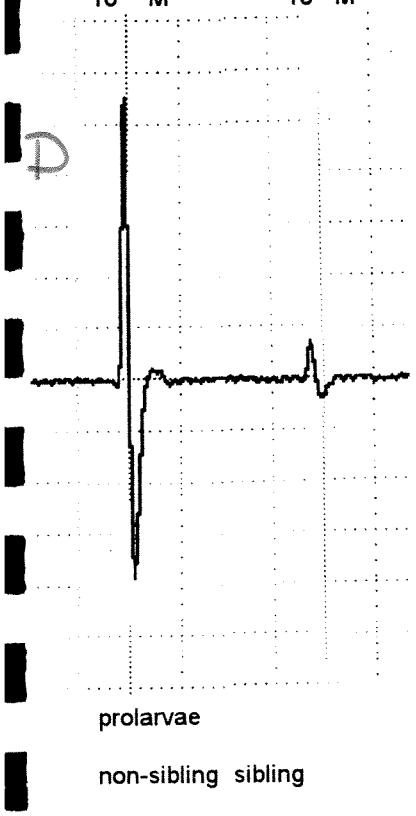
The fact that responses were relatively low to ammocoetes that were at least two years old, supports the view that prolarvae that move downstream to settlement sites, burrow at locations that contain larvae and prolarvae close to their age class.

These results suggest that prolarvae use the olfactory system, and may use chemical cues to identify settlement sites. It may be possible to apply this phenomenon to develop attractants or repellents during the movement of the prolarvae from their nests to settlement sites.


A.
L-arginine
D-arginine
B.
L-arginine
Nω-nitro-L-arginine
L-arginine
taurocholic acid


C.
taurocholic acid
10^{-10} M
10^{-3} M

1 mV
100 msec


D
prolarvae
non-sibling sibling

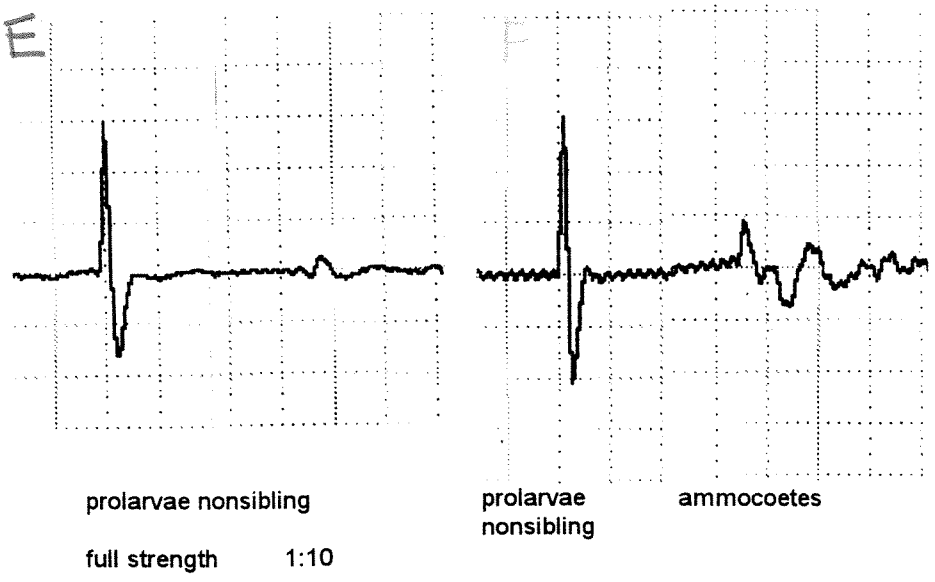

E
prolarvae nonsibling
full strength
1:10
F
prolarvae
nonsibling
ammocoetes

Fig. 1.1 Olfactory mucosal responses from stage 17 prolarvae. Each solution was applied onto the edge of a dish that contained the prolarva, and was diluted approximately 100X upon reaching the olfactory mucosa.

A. The arginine response was stereo-specific for L-arginine.

B. The response to the nitric oxide synthase blocker Nw-nitro-L-arginine was considerably smaller than for L-arginine. Application of L-arginine following Nw-nitro-L-arginine caused a reduced L-arginine response, and the taurocholic acid response was independent of the Arginine-Nw-nitro-L-arginine interaction. The concentration of the solutions that was applied into the dish was 10^{-4} M for L-arginine, D-arginine, Nw-nitro-L-arginine and 10^{-6} M for taurocholic acid.

C. Concentration dependence of taurocholic acid. The response magnitude for 10^{-10} M taurocholic acid is lower than for 10^{-8} M.

D. Concentration dependence of nonsibling prolarval responses. The amplitude of the response to full strength conditioned water was from considerably larger than to 1:10 dilution of the conditioned nonsibling prolarval water.

E. The response to conditioned prolarval nonsibling water was considerably larger than to conditioned ammocoete water.

2. Prolarvae: behavioural responses

Objective: Are there specific attractant or repellent responses that can be observed from prolarvae?

Technique: In this study, increased % movement maybe interpreted as avoidance behavior, while decreased % movement indicates preference behavior. These assumptions are based in part on earlier work in 1994 which demonstrated that when prolarvae were introduced to a plume of test chemical they increased swimming activity to avoid the plume (avoidance response), but decreased swimming activity when remaining in the plume (preference response). The prolarvae used in the present study were approximately at late 16 to early stage 18 according to Piavis (1971). In their natural setting, these prolarvae would be at or near the stage in which they leave their nest and drift to sandy, silty areas where they will live out their ammocoete period (Scott and Crossman, 1973). Therefore, the above interpretation seems reasonable in that these animals are at a stage when they are largely non-active and would move only when displaying avoidance behavior.

In these experiments, the responses of prolarvae to chemostimulation was observed following the application of test solutions into a chamber with a flow-through setup. These were conducted throughout July, August and September, 1995 at the University of Windsor, using prolarvae from eggs fertilized and hatched at the Lake Huron Biological Station.

Behavioural Apparatus: A plexiglass box (15.5 cm X 11 cm X 5 cm) with an outflow and inflow tube, with water level kept at approximately 1.5 cm was used (fig.2.1). Water flowed by gravity from a carboy into the apparatus, and was allowed to drain out into another carboy. Clamps were adjusted on both the inflow and outflow until an appropriate, approximately constant water flow was achieved. The test solution was pumped at a constant flow rate directly into the inflow tubing by a perfusion pump. Video equipment was used to record the reactions of the prolarvae.

Prolarvae were kept in 300 mL beakers containing aerated, dechlorinated water, which was changed approximately daily. Prolarvae used in these tests were at late stage 17 to early stage 18 according to Piavis (1971).

Solutions: Fresh test solutions (L-arginine, D-arginine, taurocholic acid, N-ω-L-arginine; Sigma) were prepared daily in de-ionzed water City of Windsor water. Ammocoete conditioned water was obtained by allowing 10 ammocoete stage lamprey to sit in five litres of dechlorinated water for two hours. Prolarval conditioned water was obtained in a similar manner.

Prolarvae were taken from the coldroom and allowed to acclimate to the temperature of the flow water. When the water flow through the apparatus was constant, the prolarvae was placed generally in the centre of the observation area with a pipette. After initial movement had ceased, a minute of control time was taped. Then the perfusion pump was turned on, delivering the test solution into the flow. After a ten second interval, a minute of test time was taped. This ten second interval was based on informal dye experiments that showed that apparatus would be saturated with the test solution in approximately ten seconds. After this initial ten second interval, if there was a reaction within another ten seconds it was regarded as an "initial reaction." The apparatus was rinsed thoroughly with ethanol (95%) and then dechlorinated water between trials.

Trials with ammocoete and prolarval conditioned water differed slightly in that the test solutions were not administered through the perfusion pump but directly from an alternate carboy. This was done so that the solutions would not be diluted below natural concentrations. Informal dye tests with (0.02%) methylene blue indicated that solutions were diluted by approximately an order of magnitude in the test apparatus. Therefore, the concentrations reported here are the approximate concentrations after dilution in the apparatus.

The mean flow rate of 350.4 $\pm$ 5.48 mL/min (n=10), was determined by measuring volume over time. The temperature of all the solutions was between 21 and 23^0 C.

Tape Analysis: The total time of the control and test segments were determined from the tape counter. Movement in the control and test segment was timed with a stop-watch and rounded to the nearest second. Movement included "twitching" as well as swimming activity. The seconds of movement in the control segment was then divided by the total seconds of the control

segment to determine the percent movement and likewise for the test segment. The relative amount of activity was determined with a grid overlay. The grid was placed over the monitor displaying the tape. A record was made of each time that a prolarva crossed a gridline, and the total number of crossings was totaled. This test was conducted for each treatment. In each there was agreement between the relative activity measurement using both time and crossings methods.

Statistics: Percent movement values were transformed using the arcsine, square root transformation. A paired-comparison test was used to compare % movement in control and test times for each test solution. (Sokal and Rohlf, 1987)

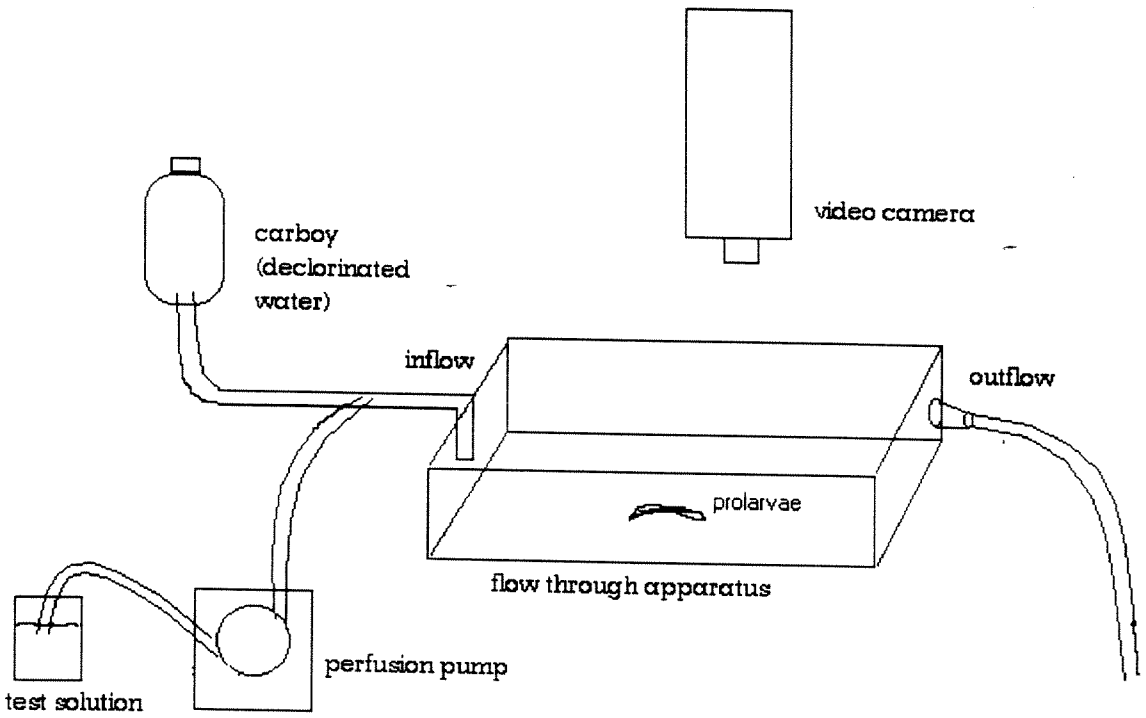


Fig. 1 Flow through apparatus and experimental setup.

Fig. 2.2 An example of behavioural tests in from 1994. Movement of prolarvae kept individually within a Petrie dish was observed following the application of a test solution from the tip of a pipette. The prolarvae remained within the area with the test solution, when 0.01 mM (10^{-5} M) was applied, and moved away following the application of 0.1 mM L-arginine. The results were averaged from 6 trials for each concentration. Tests were conducted on different prolarvae throughout the month of August 1994.

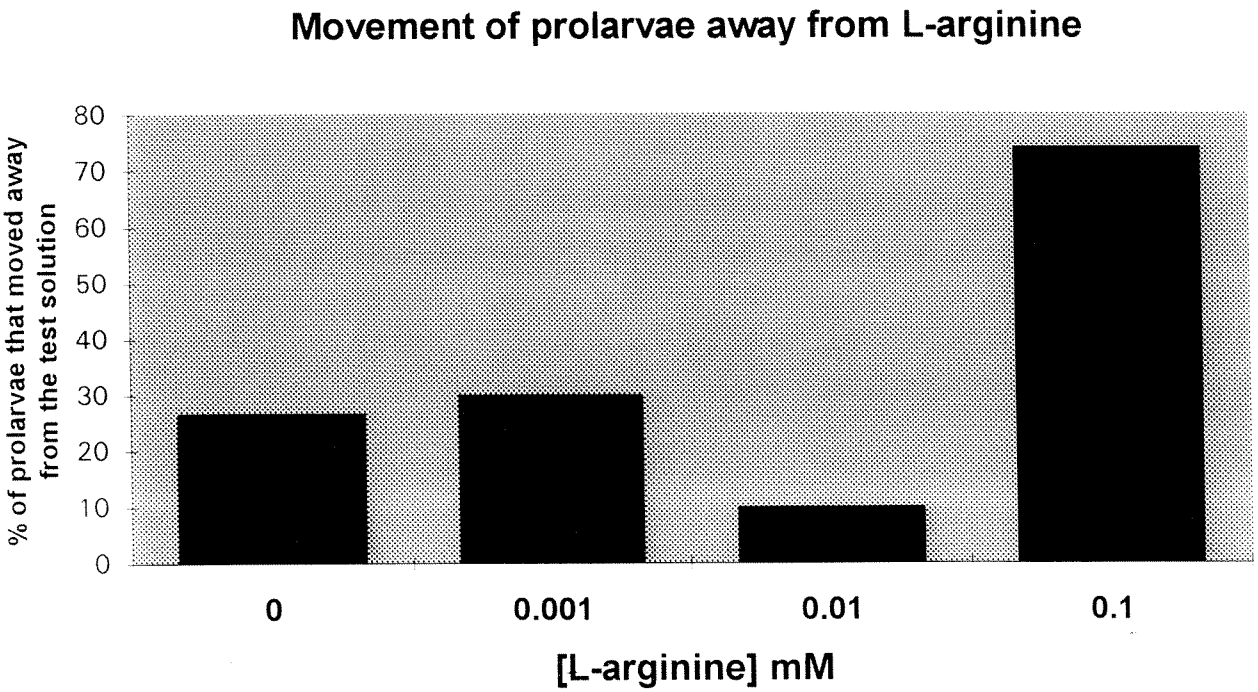


Figure 2.3 Movement of prolarvae during the flow of L-arginine solutions compared to movement in untreated water. There was less movement in test solutions with L-arginine. Least movement was observed in 10^{-5} M L-arginine (0.01 mM).

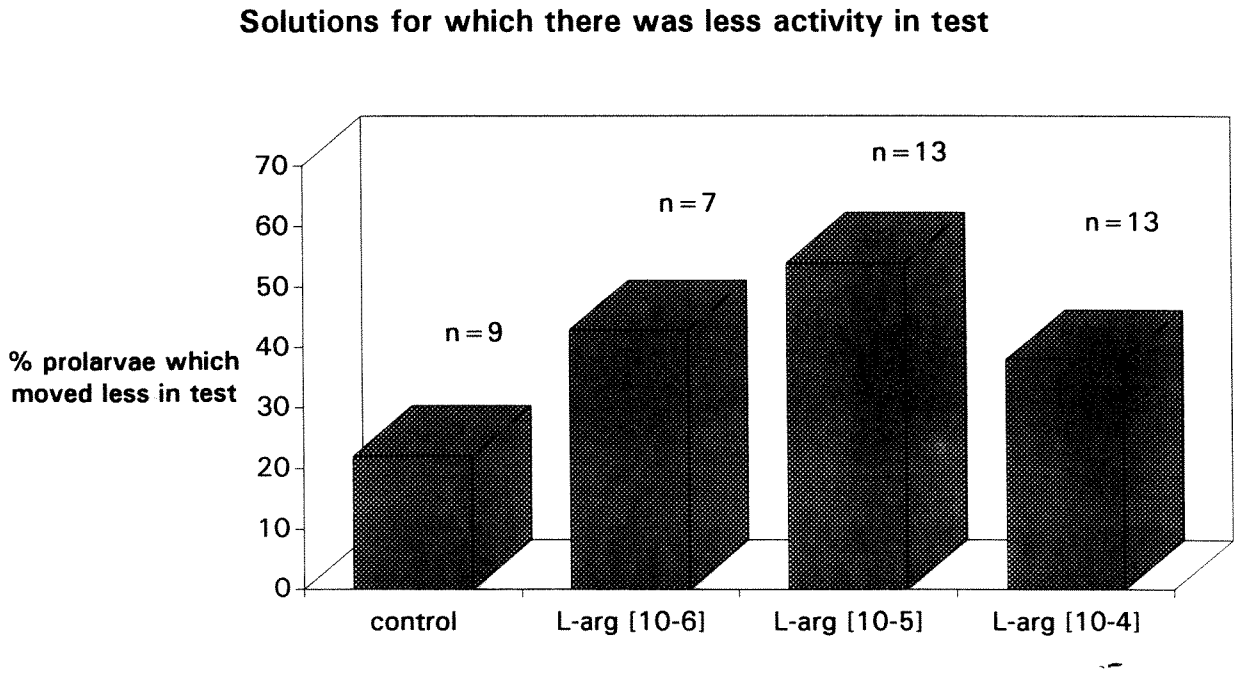


Figure 2.4 Movement of prolarvae during the flow of solutions with taurocholic acid compared to their movement in untreated water. There was significantly more movement when 10^{-12} M taurocholic acid was flowing through the test area than untreated water (control), or higher concentrations of taurocholic acid (10^{-10} M, 10^{-6} M.

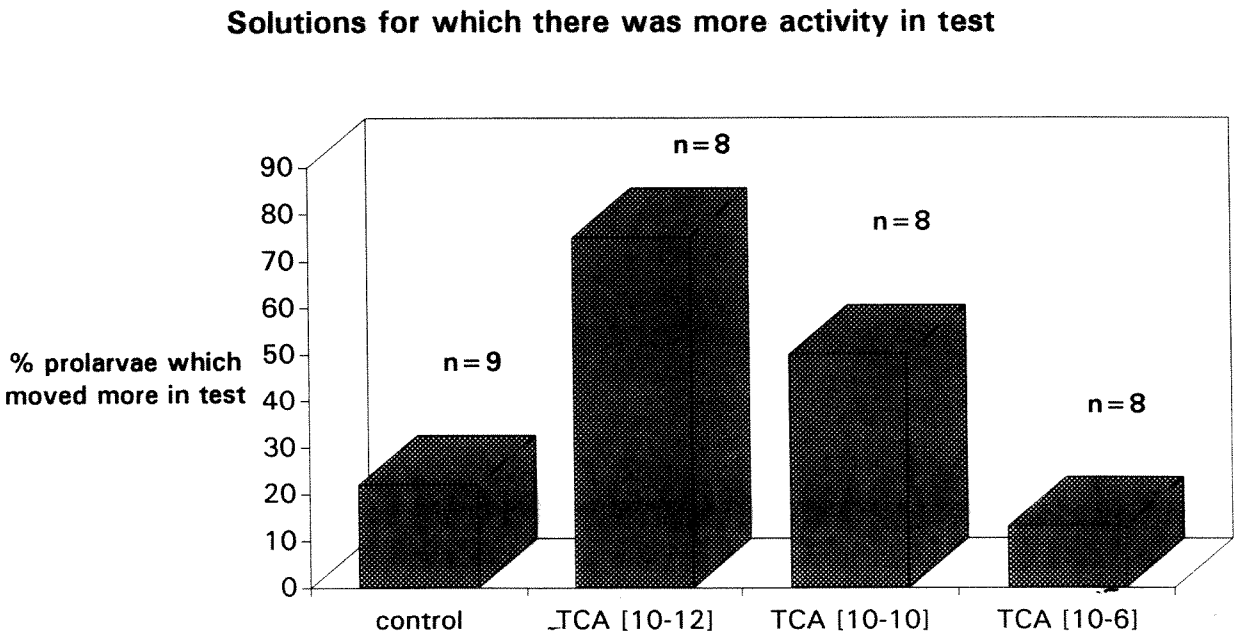


Figure 2.5 Summary of 1995 behaviour trails with prolarvae. TCA, taurocholic acid; L-arg, L-arginine; N-w, N ω-nitro-L-arginine; L-orn, L-ornithine. Concentrations (10^{-12} M to 10^{-4} M).

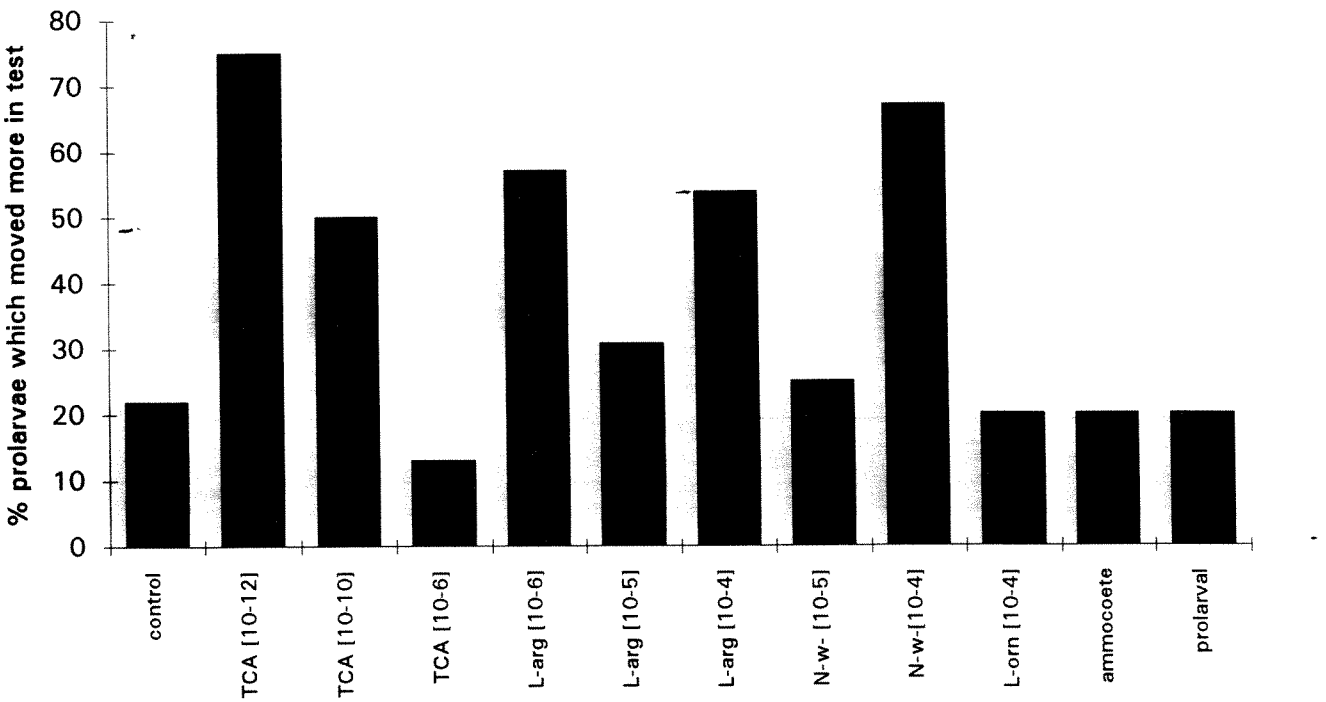


Fig: Percent trials in which movement was greater in test than in control.

Results and discussion: Our results from experiments conducted in 1994 (fig. 2.2) demonstrated that prolarvae tended not to move away when 10^{-5} M L-arginine was applied from a pipette, and moved away from or "avoided" 10^{-4} M L-arginine. In 1995 we re-examined responses by quantitatively comparing movement during the flow of untreated water to movement during the flow of test solutions. The fact that movement was least in 10^{-5} M L-arginine (fig. 2.3), supported our results from 1994. The two tests seem to show that at 10^{-5} M, L-arginine signals prolarvae to move less, and the significance of this is unknown.

The very low concentration of taurocholic acid, (10^{-12} M fig. 2.3), in which significantly more movement was observed ($p<0.05$), is representative of bile acid concentrations found in the ambient environment. This increased activity did not include active swimming to a source with the odor, and can not be conclusively described as either an avoidance or preference response. However, the behavioural observations extend the observations of the physiological sensitivity to taurocholic acid. Olfactory responsiveness to this compound is certainly not limited to lamprey, but these results do suggest that petromyzonal sulphate, a biochemical characterized lamprey bile derivative or other specific lamprey bile compounds may be extremely active to the prolarval olfactory system.

The results presented in Figure 2.5 indicate that a high concentration (10^{-4} M) of the L-arginine analogue, N ω-nitro-L-arginine, like the 10^{-4} M L-arginine was followed by more prolarval movement. These results may reflect nonolfactory, physiological effects that these concentrated products cause.

Our tests of water from ammocoetes and prolarvae showed that these did not elicit either a decrease or increase in movement. However, we were unable to test the effect of nonsibling prolarvae, which have shown high amplitude olfactory mucosal neural responses. The prolarvae used for behavioural testing were pooled together following their shipment from Hammond Bay.

The responses to ammocoetes over two years old, which did not vary from control, untreated water, support our physiological data which showed low, tonic olfactory mucosal neural responses to ammocoete water (fig. 1.1). These results are not surprising; prolarvae may not

settle in areas populated by 2-year old ammocoetes, but may burrow at sites with less competition, that contain larvae (and prolarvae) closer to their age class.

The seemingly high difference in % movement for the control water solution indicates that apparently random variability may be an important factor. Statistically there are problems with using mean values in behavioural testing because individual fish may behave differently. For example, McNicol and Scherer (1991) found that when exposed to the same concentration of cadmium, some individual whitefish were attracted to the cadmium while other individuals were repelled by it. When expressed as a mean, it seemed that whitefish "on average" react only slightly to the cadmium. In the present study, individual responses of prolarvae to 10^{-5} L-arginine varied. However, variation may not be the result of individual choice alone because informal trials indicated that for this solution individuals also behaved differently in multiple trials at the same concentration. It is also interesting to note that for the two solutions for which there was a significant difference in mean % movement between control and test (10^{-12} M taurocholic acid and 10^{-4} M N ω-nitro-L-arginine), all individuals showed the same response.

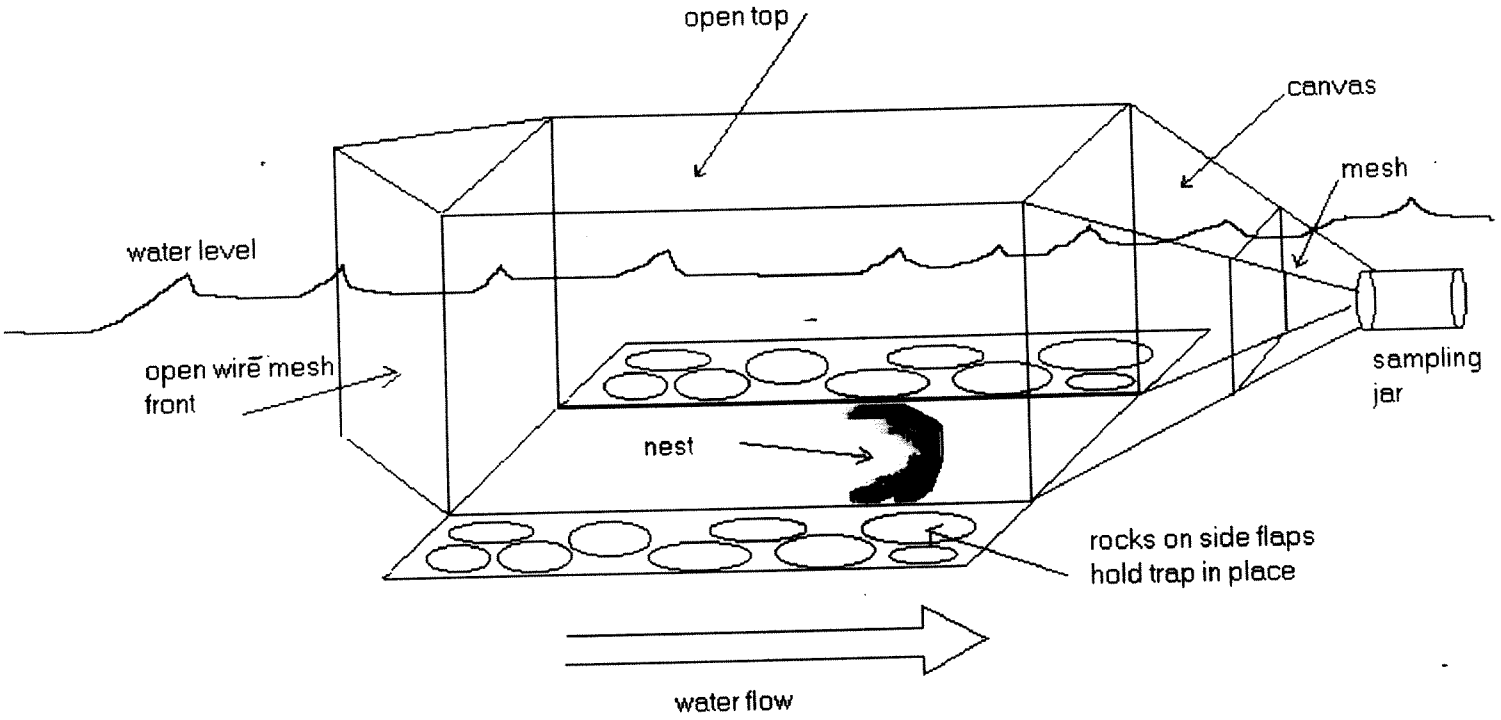
Part Three: Prolarvae: nest abandonment: To determine when prolarvae abandon nests, a field study was conducted in Big Creek, in Delhi Ontario. The prolarvae were trapped leaving from these nests and were prepared for examination by transmission electron microscopy.

Briefly, each lamprey nest consisted of a central circular depression made up of gravel sized sediment and a cresentric lip of larger stones downstream of this depression (Scott and Crossman, 1973). To entrap emerging prolarvae, viable, developing nests were found and emergence traps installed. The construction of the emergence traps (fig. 4-1) was based on a design described by Applegate (1951). It was sturdy and large enough to withstand rapid currents, while forming an undisturbed environment over the nest until the prolarvae emerge. The traps employed the natural flow of the river current to wash and carry the emerging prolarvae into the sampling jar at their base. Once the prolarvae were sampled, their developmental stage was determined under a dissection microscope. The trap was installed on the nest when the prolarvae were at approximately stage 16 according to Piavis (1971), on June 26. The cup of the trap was then checked periodically until emerged prolarvae were found in the sampling cup on July 2. Originally, 5 viable nests were located. Unfortunately however, as a result of high irrigation use, and high temperatures, very low water levels were experienced in early June 1995. Because of these conditions, four of the five nests were either above the water level, or otherwise destroyed. The water temperature over this period ranged from 18^0C to 23^0C (temperature was usually taken before noon). The prolarvae retrieved from the sampling cup were at late stage 17 according to Piavis (1971). Briefly, Piavis defines this stage as 7.5 to 9 mm, has burrowing behavior, and transparent except for yolk-filled gut. The end of this stage is reached when the lumen of the yolk-filled gut is opened. Our examination by electron microscopy demonstrated that these prolarvae contained olfactory receptor cells.

It would be premature to draw conclusions from these results because they are based on one nest in a single river. The temperatures during the summer of 1995 were unusually hot.

Acknowledgments: We are grateful to Mr. Rod McDonald (Sea Lamprey Control Centre, Sault Ste Marie), and Mr. J. Mulholland, Delhi, Ontario for assistance with locating the nests.

Figure 3.1 Diagram of an emergence trap for prolarvae in the nest abandonment experiment.



Part Four: Innervation and sensory cells in the mouth region

Objective: To determine if there are other chemical sensory systems that have developed during the prolarval stage.

Technique: Prolarval specimens that were previously prepared for examination of the development of the olfactory system (e.g. Vandenbossche et al., 1995) were examined by light and electron microscopy. To visualize neuronal projections, crystals of the fluorescent lipophilic Di I (1,1' diocatdecyl-3,3,3',3'-tetramethyl indocarbocyanine perchlorate, *Molecular Probes*, Eugene OR), were applied onto the head region of prolarvae that were fixed briefly in 2% paraformaldehyde in phosphate buffer. The dil was able to enter neuronal cells and follow axonal pathways. Prolarvae were viewed as whole mounts under epifluorescence.

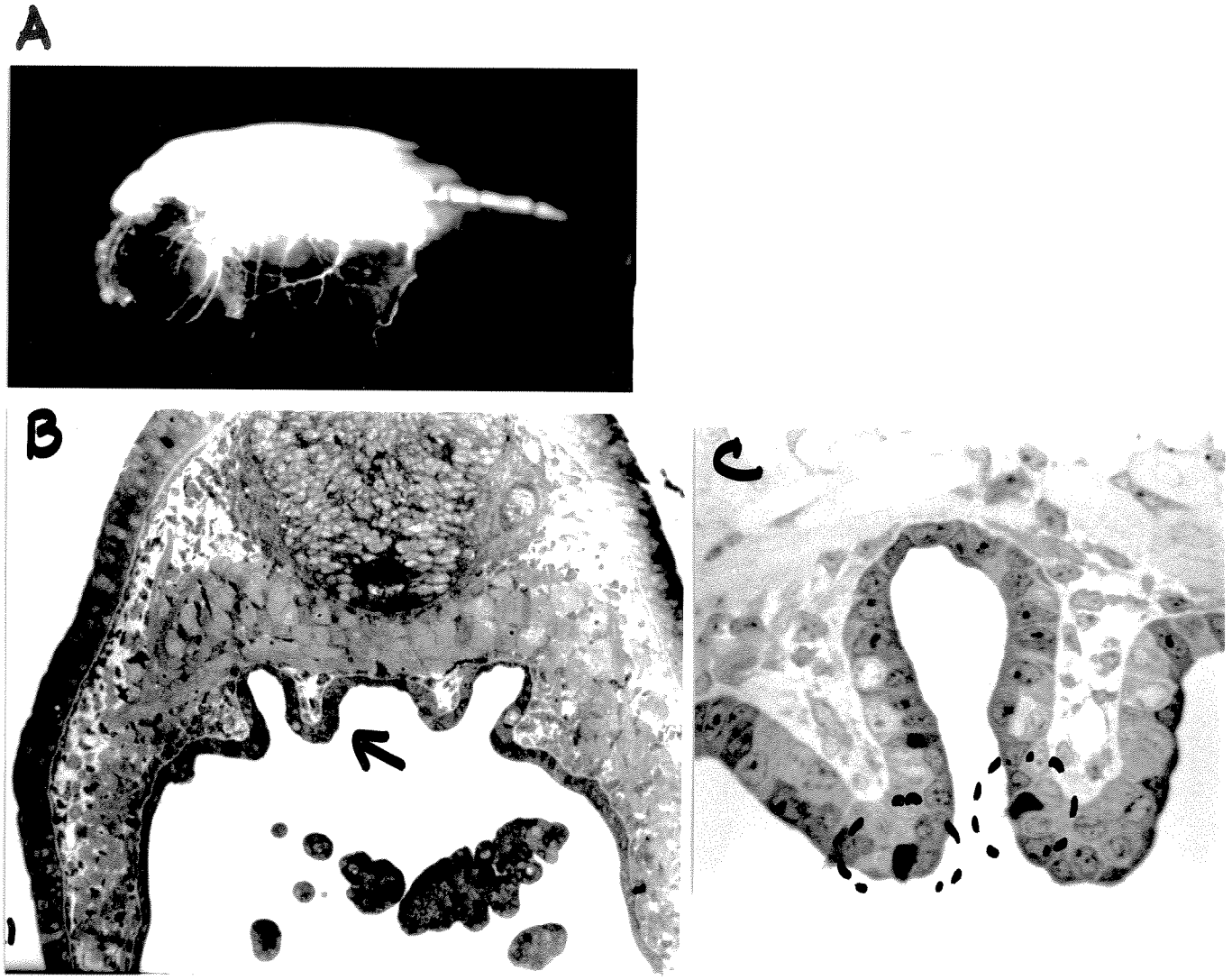
Results and discussion. Extensive innervation of the mouth region was observed by labeling with dil (fig. 4.1A). Undoubtedly some of these were motor fibers to musculature, but some may be innervating sensory cells located in the mouth region. Solitary ciliated cells were observed within the mouth of prolarvae at stage 17. (fig. 4.1B,C). These were similar to previously observed taste cells in pharyngeal terminal buds in larval *Lampetra planeri* (Baastrup, 1983).. These results suggest that prolarvae may have the ability to use gustation to select appropriate food substances.

Figure 4.1 Stage 17 Prolarvae, views of the mouth.

A. A low power view of a whole mount that was injected with dil from the dorsal surface. Nerve fibers extend to various locations on the mouth (arrow).

B. Cross-sectional view of the mouth region viewed in a 1 μm section of a epoxy-embedded tissue. The dorsal surface of the mouth has folds (arrow).

C. A high power image of the folds pointed out in A. Darkly stained cells with cilia-like protrusions are present on these folds.



Part 5: Larvae: Physiological responses to putative attractants or repellents.

Objective: To use physiological experiments, the electro-olfactogram, to investigate putative larval attractants and repellents.

Background: In 1994, we focused on the olfactory activity of amino acids, and found that L-arginine was the potent stimulant, and that the nitric oxide inhibitor, N ω-nitro-L-arginine, was able to competitively inhibit this response. These results have been published in the *Journal of comparative Neurology*, and are included in the appendix of this report.

Technique: In 1995, we continued EOG experiments at the University of Windsor, with larvae that were sent from the Hammond Bay, Michigan. We investigated the olfactory activity of:

1. The sulfated bile acid, taurocholic acid
2. Some compounds that have been shown to stimulate larval settlement in invertebrates: 3,4-dihydroxyphenylalanine (DOPA); γ-aminobutyric acid (GABA) and serotonin
3. Conditioned water:

- from prolarvae
- from ammocoetes of the same age class as the ones that were tested (5 - 10 cm) and from ammocoetes from the same tank that the ones that were tested (adapted)
- from ammocoetes from different tanks than the ones that were tested (nonadapted)
- from nonconspecifics (trout). This was water from a tank of rainbow trout.

Results and discussion: The EOG response magnitudes varied from one preparation to another, presumably on account of differences in the physical characteristics of the EOG capillary electrodes, the dissection, and the responsiveness of individual larvae. To overcome this, in the presentation of the data, we standardized each preparation by initially observing its responsiveness to stimulation with 10^{-5} M L-arginine. If preparations did not respond to L-arginine, they were not tested further. Occasionally, we were unable to observe L-arginine responses. This occurred in July and August, 1995, and in 1 shipment of larvae that we received from Hammond Bay in December, 1995. EOG recordings that are presented in this report were

from Hammond Bay in December, 1995. EOG recordings that are presented in this report were made in the spring and autumn (Sept. to Dec.) of 1995. Each solution was tested on at least 7 larvae.

Of the compounds tested, the sulfated bile acid, taurocholic acid was effective at lowest concentrations (fig. 5.1). The sensitivity to this compound supports the view that some form bile derivative may become effective as a repellent or attractant.. The occurrence of a maximal response magnitude for both taurocholic acid and arginine suggests the EOG activity results from a specific biochemical process, such as a receptor/ligand binding, that is saturated, and then diminishes its activity upon saturation. Furthermore, these results may explain the different behavioural responses that were observed from prolarvae to 10^{-5} M and 10^{-4} M L-arginine. Two compounds that induce larval settlement and metamorphosis is some invertebrates, GABA and serotonin were elicited relatively low magnitude EOG responses. These may be active in inducing behavioural changes, but have not yet been tested. L-DOPA, however, did not elicit EOG responses.

Since taurocholic acid was an effective stimulant of EOG activity, we rationalized that bile compounds present in water conditioned by conspecifics, that presumably contained derivatives of bile from lampreys, would also be chemostimulatory. Water from tanks with ammocoetes displayed concentration-dependent EOG activity (Table 5.1). There was no significant difference between responses to water from a particular tank that held a larva that was being tested (adapted ammocoete conditioned water) to response magnitude values from conditioned water from tanks with other ammocoetes (nonadapted ammocoete conditioned water). However, responses were greater to ammocoete water compared to conditioned water from prolarvae and rainbow trout. These results support the view that lampreys use the olfactory system to communicate between each other at the ammocoete stage.

Figure 5.1 Concentration dependent larval EOG responses to L-arginine, serotonin, GABA and taurocholic acid. L-arginine (L-arg); γ aminobutyric acid (GABA); taurocholic acid (TCA). Response magnitude: 100% is the average of EOG responses to 10^{-5} M L-arginine. Each point represents the response magnitude of an average value from 7 to 30 test recordings.

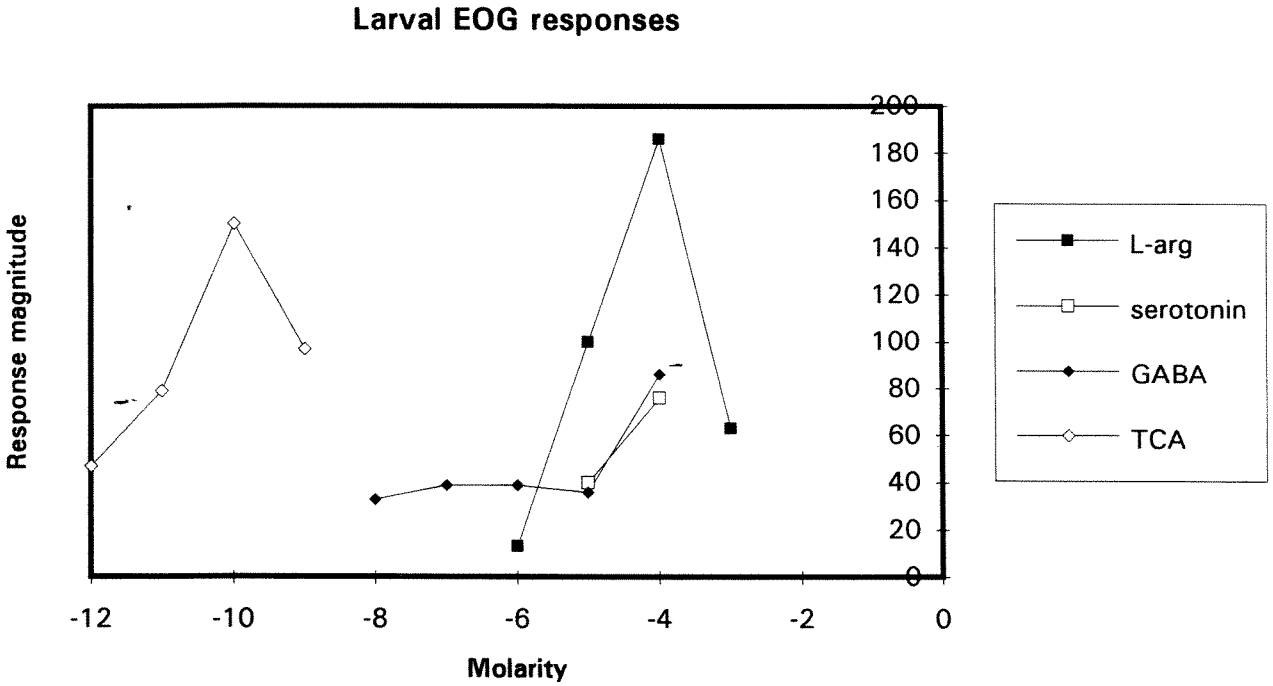


Table 5.1 Larval EOG responses to conditioned water.

Test	Magnitude response
ammocoete adapted*	52
ammocoete adapted: untreated water (1:1)	15
ammocoete nonadapted ++	44
ammocoete nonadapted:untreated water (1:1)	10
prolarval	38
prolarval:untreated water (1:1)	37
rainbow trout **	37
rainbow trout:untreated water (1:1)	37

Response magnitude: 100% is the average of EOG responses to 10^{-5} M L-arginine. Each point represents the response magnitude of an average value from 5 to 10 test recordings.
* water from the same tank as those larvae that were tested
++ water from a tank of larvae, different from larvae that were tested
** water from a tank holding rainbow trout

6. Larvae: behaviour

Our behavioural experiments in 1994 have shown a slight preference to L-arginine (Appendix 2). In 1994, we tested taurocholic acid at concentrations of 10^{-6} M and higher. The larvae consistently avoided the side with taurocholic acid (data not shown). In 1995, we planned to follow these experiments up with 10^{-12} - 10^{-10} M taurocholic acid, which were active physiologically and which would be present in the ambient environment. The design of our simple preference/avoidance trough was not acceptable to test such low concentrations. Preliminary tests in 1995 showed that we were experiencing some mixing in the test compartments. Therefore we made a literature search for flumes, and decided to build a flume with test compartments with no mixing in the test compartments (fig. 6.1, based on Korver, R.M. and J. B. Sprague, 1982, Can. J. Fish. Aquat. Sci. 46: 494-502). We anticipated construction during the autumn by the Division of Technical Support, but did not receive the flume until December, 1995. Consequently, we have not been able to conclude our behavioural testing of the low concentrations of taurocholic acid.

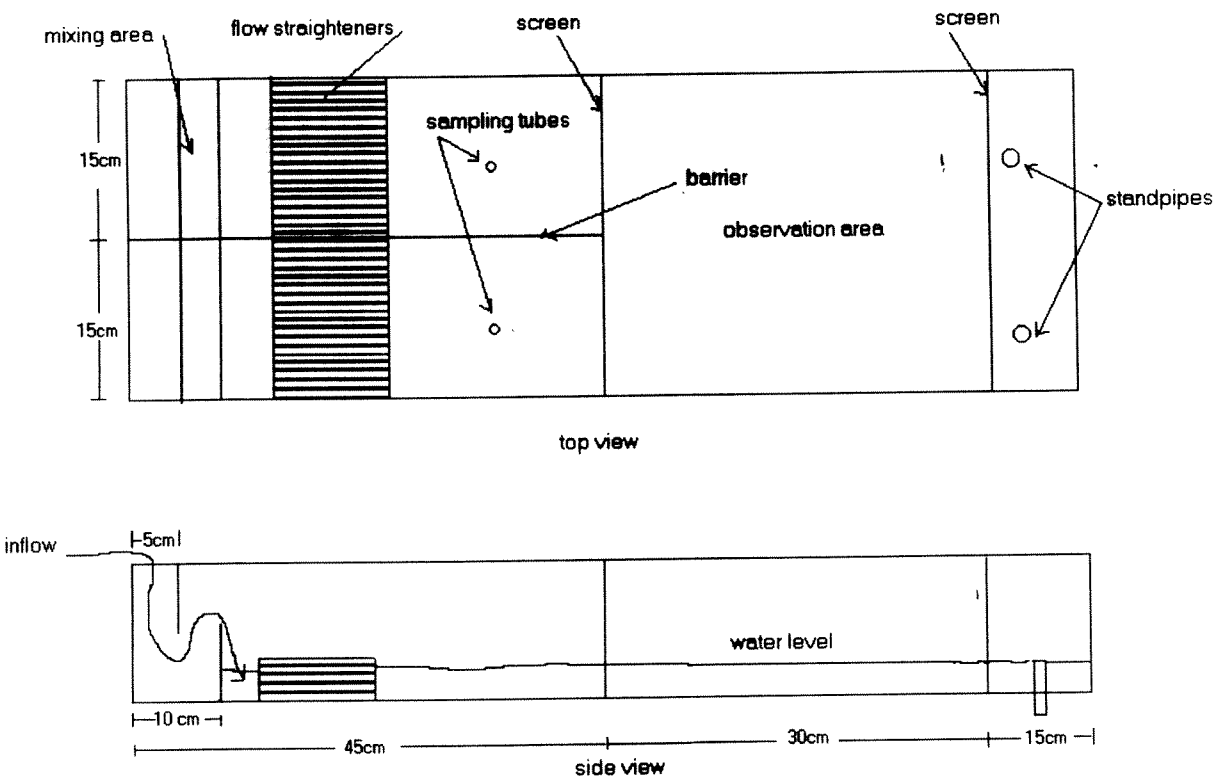


Fig. 6.1 Diagram of the ammocoete behavioral apparatus.

Part 7: Western immunoblot analysis of nitric oxide synthase in prolarval, larval and spawner stages.

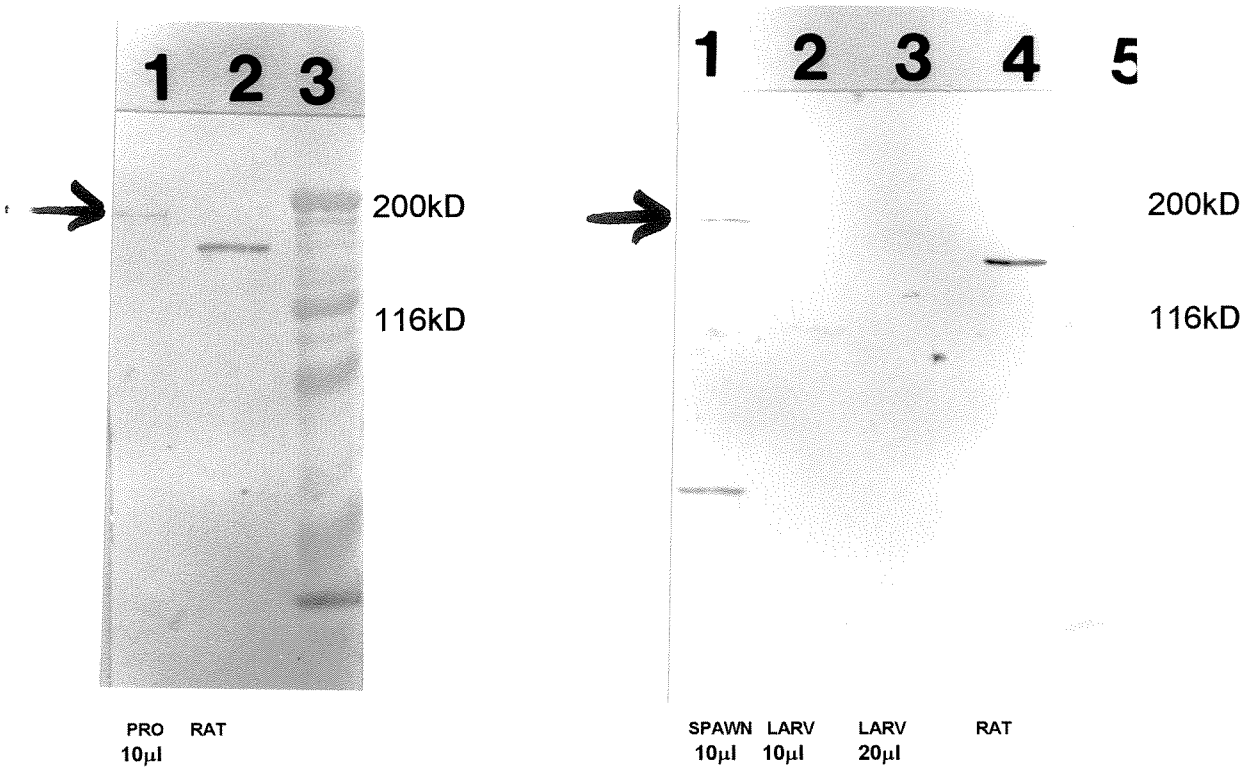
Background: Our physiological experiments of prolarvae and larvae suggested that the blocker of nitric oxide synthase, Nw-nitro-L-arginine, was able to inhibit olfactory responses to L-arginine. This led us to hypothesize that the membrane permeable gas, nitric oxide was active during the olfactory response in lampreys, and that inhibition of this enzyme may inhibit olfactory responses. Histochemical and immunocytochemical studies in larvae supported this hypothesis (Appendix 1).

Objective: To investigate the expression of nitric oxide synthase in prolarval and spawner stages. The use of repellents or attractants at these stages is desirable: to block larval settlement and lamprey spawning respectively.

Techniques: We removed the heads from approximately 100 prolarvae (stage 17), the olfactory organ from 3 larvae, and from 1 spawner (August, 1995, St. Mary's spawner, obtained from Hammond Bay). We made Western immunoblots with a monoclonal antibody that was specific to a mammalian isoform of brain nitric oxide synthase (*Transduction Labs*, Lexington KY); alkaline phosphatase was used as the chromogen.

Results and discussion: Very clear bands were visible in prolarval and spawner samples, close to the 160 kD band of rat brain nitric oxide synthase (fig. 7.1). This suggests that nitric oxide is active as a membrane permeable second messenger. Although bands were not visible in lanes with larval samples, the amount that was loaded may have not been sufficient to visualize the immunoreactivity. The potential of applying the nitric oxide synthase system to lamprey population control is discussed in the Summary section.

Figure 7.1 Western immunoblots of nitric oxide synthase. Heads from stage 17 prolarvae (pro), positive control from rat brain lysate (rat), olfactory organ from spawner (spawn), olfactory organs from larvae (larv).



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Nitric Oxide Synthase in the Olfactory Mucosa of the Larval Sea Lamprey (*Petromyzon marinus*)

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ABSTRACT

The use of nitric oxide, a product of enzymatic reduction of L-arginine by nitric oxide synthase, as a modulator of processes within the olfactory mucosa was investigated in larval sea lampreys, extant fish of ancient vertebrate origin. In the present study, we demonstrated that the sea lamprey olfactory mucosa is specifically sensitive to L-arginine, that the L-arginine responses are inhibited by an inhibitor of nitric oxide synthase, Nω-nitro-L-arginine, and that nitric oxide synthase is present in olfactory receptor cells, sustentacular cells, and basal cells. Electron microscopic examination using NADPH-diaphorase histochemistry revealed intense labeling within secretory vesicles of sustentacular cells and in proximity to mitochondria within olfactory receptor cell dendrites and sustentacular cells. At the base of the olfactory epithelium, NADPH-diaphorase staining was intense in the perinuclear cytoplasm of a subpopulation of basal cells, moderate in sustentacular cell foot processes, and scattered in olfactory receptor cell axons. Throughout axons in the olfactory epithelium and the lamina propria, labeling predominated in axonal profiles with mitochondria. These physiological and ultrastructural studies imply that in sea lamprey larvae, nitric oxide modulates peri-receptor events of L-arginine chemostimulation, olfactory receptor cell axonal activity, and developmental processes. © 1995 Wiley-Liss, Inc.

Indexing terms: L-arginine, electro-olfactogram, electron microscope, NADPH-diaphorase

Sea lampreys are extant representatives of an ancient line of vertebrate evolution. These primitive jawless fish follow a life cycle of morphologically and physiologically distinct phases. The sedentary, microphagous larval (ammocoete) stage is a suspended developmental phase that is followed by metamorphosis to the juvenile stage (Youson, 1980). The larval, juvenile, and upstream migrant stages have a monorhinal olfactory system, with ciliated olfactory receptor cells (ORC) and sustentacular cells (SC; Vandenbossche et al., 1995). Post metamorphic stages of the lamprey *Entosphenus japonica* showed strong physiological responses to L-arginine (Suzuki, 1978), and of all the amino acids tested on sea lamprey juveniles and upstream migrants, L-arginine was the most effective as an olfactory stimulant (Li and Sorensen, 1992). Since L-arginine is a potent chemo-stimulant in post-metamorphic lampreys, its activity would also be anticipated in the larval form of the sea lamprey.

L-arginine is of particular significance as a substrate that is enzymatically reduced by nitric oxide synthase (NOS) in the presence of the hydrogen donor NADPH, and oxygen to form nitric oxide (NO). In the nervous system, NO is a mediator of transduction (Garthwaite, 1991; Bredt and Synder, 1992; Vincent and Hope, 1992) and may confer resistance to excitotoxic damage (Koh and Choi, 1988). In the olfactory system of *Xenopus* and rats, NO stimulated guanylate cyclase, leading to channel modulation by cGMP (Breer et al., 1992; Lischka and Schild, 1993). The fact that NO modulated olfactory interneuron activity in the snail *Helix* (Gelperin, 1994) shows that NO's association with olfactory processes extends to invertebrates.

The enzyme NOS has been demonstrated within cells by the NADPH-diaphorase (NADPH-d) reaction, a simple histochemical technique previously used to stain distinct subsets of neurons in mammals (Vincent et al., 1983; Aimi et al., 1991). In rat brain, neural NADPH-d was shown to be identical to NOS (Hope et al., 1991; Dawson et al., 1991), antibodies against NADPH-d precipitated NOS activity

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from the rat brain tissue extracts (Hope et al., 1991), the NADPH binding domain of NOS has been shown to be responsible for NADPH-d activity (Schmidt et al., 1992a) and NOS-mRNA was colocalized with NADPH-d activity (Bredt et al., 1991). In kidney cells, the transfer of the NOS gene was accompanied by transfer of NADPH-d activity (Dawson et al., 1991). In light of these investigations, the NADPH-d reaction is currently used to indirectly visualize NOS in various systems. Recently NADPH-d activity has been demonstrated in the CNS and the olfactory epithelium of the larval stage of the lamprey, *Lampetra planeri* (Schober et al., 1994), in the olfactory epithelium of rat and catfish (Dellacorte et al., 1994) and in immature and adult opossum (Shnayder et al., 1994). Preliminary immunocytochemical studies of the larval sea lamprey olfactory mucosa, in our lab, have revealed colocalization of NADPH-d activity with immunoreactivity to a neuronal NOS antibody (Hosseini et al., in preparation).

The use of NADPH-d at the electron microscopic level (Vincent and Johansson, 1983; Scott et al., 1987; Loesch et al., 1994) to examine the subcellular distribution of NOS in the olfactory mucosa of sea lamprey larvae is an initial step in the identification of processes that are modulated by NO in one of the most primitive vertebrates.

MATERIALS AND METHODS

Sea lamprey ammocoetes used in this study were obtained from the Hammond Bay Biological Station, National Biological Survey, Hammond Bay, Michigan and were maintained in the Department of Biological Sciences, University of Windsor. In the present study, all experimental procedures were in compliance with guidelines issued by the Canadian Council for Animal Care.

Electrophysiology

Sea lamprey larvae were anesthetized with MS222 and immobilized with Flaxedil. A flow-through apparatus similar to that previously described (Evans and Hara, 1985; Zielinski and Hara, 1988) was used to obtain EOG recordings following the application of 10 μM solutions L-arginine, Nω-nitro-L-arginine, D-arginine, L-serine, and L-leucine (Sigma Chemical Co., St. Louis, MO).

NADPH-d electron microscope histochemistry

The sea lamprey ammocoetes, weighing approximately 1.5 g, were anesthetized with 1% 3-aminobenzoic acid ethyl ester before the dissection. The heads were removed and were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) solution for 2 hours at 4°C. These were cryoprotected through a series of sucrose solutions in 0.1 M phosphate buffer, embedded, vacuum infiltrated, and frozen in OCT. Cryosections passing transversely through the nasal cavity were obtained with a cryomicrotome and collected in 0.1 M Tris buffer (pH 8). For light microscopy, these were approximately 15 μm thick, and for sections that were embedded for transmission electron microscopy, the thickness was 60 μm. Sections with olfactory mucosa were selected for pre-embedding histochemistry of the NADPH-d reaction. The histochemical technique of Vincent et al. (1983) was used, but with Tween-20 omitted from the incubation medium to conserve membranous structures. The free floating sections were incubated in the dark in a 5 ml solution containing 0.1 M Tris buffer (pH 8.0), 50 μl of 1 mM β-NADPH (tetrasodium salt), and 50 μl of 0.2 mM nitro blue tetrazolium (NBT) chloride, at 37°C for 90 minutes. All reagents were obtained from Sigma Chemical Co. (St. Louis, MO). Control incubations, in which β-NADPH was omitted from the incubation medium, were run simultaneously.

The experimental tissue sections became dark blue and control sections remained unstained. The reaction was terminated by rinsing the sections in cold 0.1 M Tris buffer (pH 8). These sections were osmicated in 2% OsO_4 diluted in 0.1 M phosphate buffer, pH 7.4 for 2 hours at 4°C, followed by a wash in distilled water for 5 minutes. The sections were dehydrated in graded ethanol series and embedded into epoxy resin. Semithin plastic sections were cut with glass knives on an RMC ultramicrotome MX6000-XL and examined by light microscopy. In the olfactory mucosa, NADPH-d positive regions were identified by the presence of blue staining, and ultrathin sections were prepared, lightly counterstained with 7% uranyl acetate and viewed with a Philips EM 201 electron microscope. The NADPH-d reaction was identified by the presence of electron dense deposits. The control tissue was unstained at the light microscope level, and did not contain electron dense deposits when viewed by electron microscopy.

There are two reasons for the poor visibility of membranous structures in the electron micrographs. First, to clearly demonstrate the presence of the electron dense formazon deposits, the counterstain with uranyl acetate was very light. Second, to remain consistent with the NADPH-diaphorase protocol, the tissue was fixed with paraformaldehyde rather than glutaraldehyde, which would have improved the fixation and visibility of the membranes.

For conventional electron microscopy, the tissue was fixed in Karnovsky's fixative and prepared as previously described (Vandenbossche et al., 1995).

Brain NOS-like immunocytochemistry

NOS immunocytochemistry was investigated with a polyclonal rabbit antibody against brain NOS (Transduction Labs, Lexington, KY). Cryostat sections were rinsed in 0.1 M phosphate buffered saline (PBS) and treated with 0.3% H_2O_2 in 0.1 M PBS for 30 minutes to inactivate endogenous peroxidases. Nonspecific binding was blocked with diluted normal blocking serum and the sections were incubated in the NOS antibody (1:1,000) in PBS with 0.2% Triton X-100. The immunoreactivity was visualized using a rabbit Vectastain Elite Kit and stained with diaminobenzidine. Staining was absent from negative control sections that were incubated with nonimmunized rabbit serum instead of the brain NOS antibody.

RESULTS

Electro-olfactogram recordings

There was no change in EOG recordings following the application of untreated water and 10 μM solutions of the amino acids L-serine, L-leucine, low response with D-arginine, and a high response following application of L-arginine, into water flowing over the olfactory mucosa (Fig. 1). Application of the competitive inhibitor of NOS, Nω-nitro-L-arginine failed to elicit EOG responses. Following the application of a mixture of 10 μM L-arginine and Nω-Nitro-L-arginine, the EOG response was approximately half of the response elicited by L-arginine, indicat-

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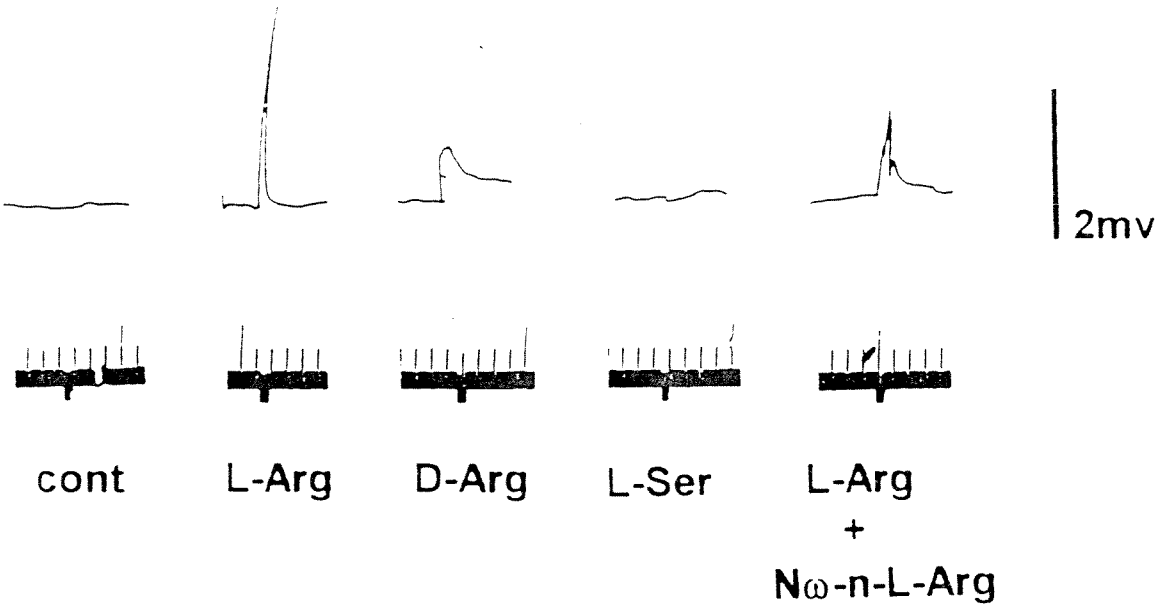

Fig. 1. EOG responses from larval sea lamprey following chemostimulation with water (cont) and 10 μM L-arginine (LArg), D-arginine (D-Arg), L-serine (L-Ser), and a mixture with 10 μM L-arginine and 10 μM Nω-nitro-L-arginine (L-Arg + Nω-n-L-Arg). Responses were recorded on curvilinear graph paper. Time signal, each division = 5 seconds.

ing cross-adaptation competition of the two compounds (Fig. 1).

Light microscopy

Semithin sections of the ammocoete olfactory mucosa revealed the pseudostratified arrangement that is typical of olfactory epithelium, with nuclei populating the lower two thirds of the tissue, and the apical tips of the ORC dendrites forming olfactory knobs (Fig. 2A). The olfactory epithelium had three loosely defined strata: the supranuclear, nuclear, and basal regions. The underlying lamina propria contained nonmyelinated nerve bundles, loose connective tissue, and blood vessels (Vandenbossche et al., 1995). When the 2 μm epoxy NADPH-d sections were viewed without any counterstain, a blue formazon reaction product was detected within the positive cells (Fig. 2B). In the olfactory epithelium, staining was slightly more intense in the supranuclear region than in the nuclear and basal regions (Fig. 2B,C). In the lamina propria, there was labeling in small nerve fascicles directly below the olfactory epithelium and in large nonmyelinated nerve bundles (Fig. 2B). The nuclei of all cell types were unstained. When cryostat sections (10–15 μm) were viewed following either NADPH-d histochemistry (Fig. 2C) or brain-NOS immunoreactivity (Fig. 2D), the staining was heaviest in the supranuclear portion of the olfactory epithelium, with nonmyelinated olfactory nerve fibers labeling moderately.

Ultrastructure of the NADPH-diaphorase staining

In larval sea lampreys, ORC were recognized by the presence of a ciliated olfactory knob and microtubules (Vandenbossche et al., 1995). In unreacted tissue that was prepared for NADPH-d histochemistry, with the omission of β-NADPH, there was a complete absence of electron dense deposits (Fig. 3A) and with the inclusion of β-NADPH, the formazon reaction product was located in the dendritic region below the junctional complex (Fig. 3B). The staining was heaviest in the cytosol of large dendrites that were crowded with mitochondria (Fig. 3B). Dendritic regions with low NADPH-d reactivity contained few mitochondria, had low olfactory knobs and ciliary basal bodies, indicating that they were likely from the periphery of the dendrite and olfactory knob (Fig. 3C).

SC contained the reaction product in the supranuclear region (Fig. 3D). Labeling was intense adjacent to mitochondria and secretory vesicles. Occasionally, the diaphorase reaction product was present in vesicles located in blebs protruding from the apical surface (Fig. 3E).

The basal region of the olfactory epithelium contained olfactory axon aggregates, SC foot processes and basal cells (Fig. 4A). The axons were narrow, but widened in regions that contained mitochondria. The distribution of the diaphorase reaction product in axons was patchy; axonal profiles with mitochondria contained up to three sites of deposits (Fig. 4B). Some basal cells showed heavy cytosolic labeling close to the endoplasmic reticulum and mitochondria (Fig. 4C), whereas others were moderately labeled. There was moderate to strong labeling in the basilar expansions of the sustentacular cells, with the reaction product predominantly localized in the cytosol adjacent to mitochondria and vesicles (Fig. 4D).

In the lamina propria there were numerous small nonmyelinated nerve bundles ensheathed by Schwann cell processes (Fig. 5A). The reaction product was localized in some of the axons within nerve fascicles, with more intense staining of axons with mitochondria (Fig. 5B).

DISCUSSION

The fact that the sea lamprey's responsiveness to amino acid stimulation was limited to L-arginine suggested the activity of a specific biochemical pathway. Inhibition of the larval sea lamprey's stereo-specific response to L-arginine.

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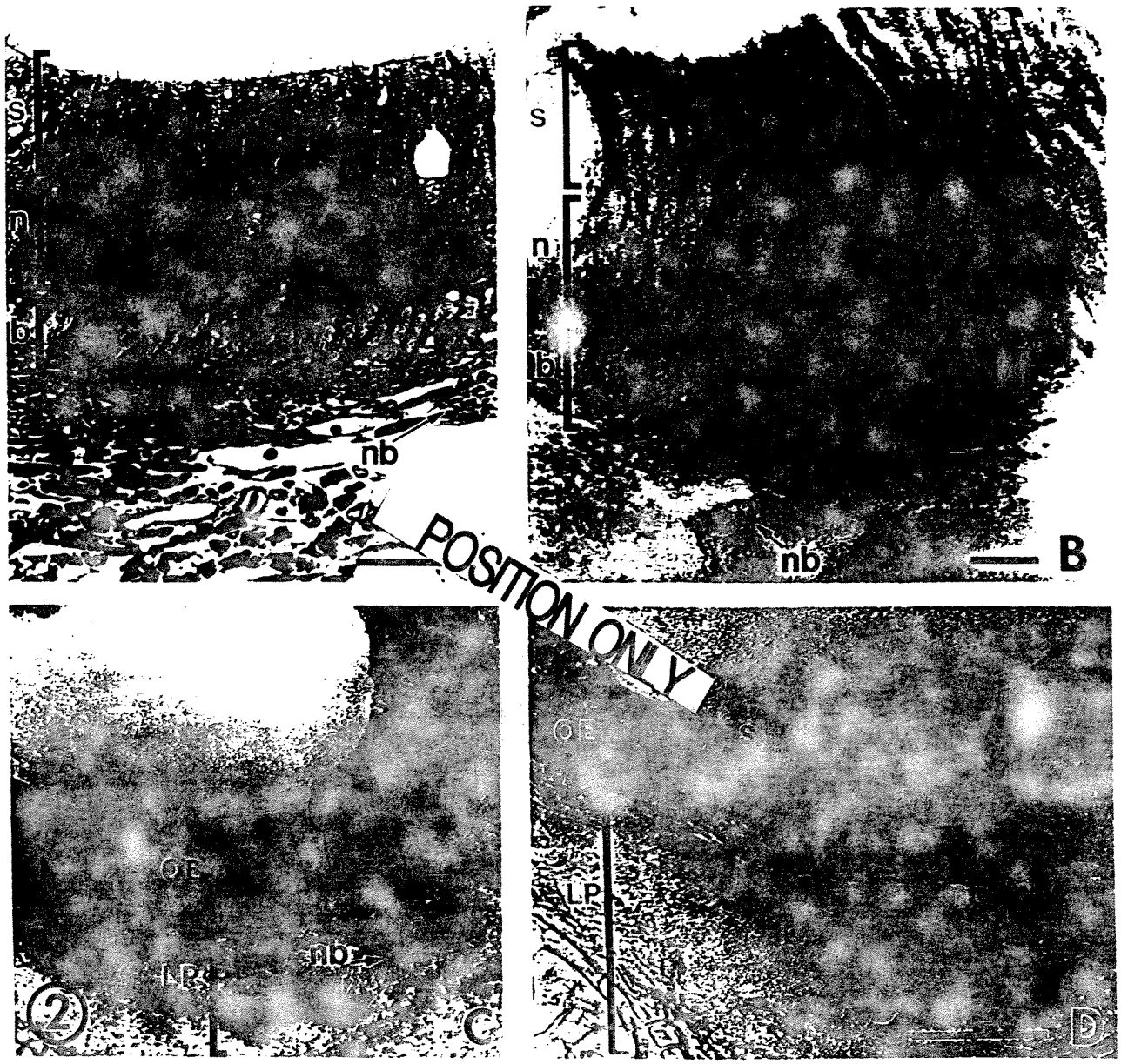

Fig. 2. Light microscopy of the ammocoete olfactory mucosa. A: Modified Richardson's stain of a 1 μm section from epoxy embedded tissue showed the supranuclear (s) region of the olfactory epithelium, the nuclear (n) regions and basal regions. Nerve bundles (nb) are in the lamina propria. The bar is 20 μm. B: NADPH diaphorase staining in a 2 μm epoxy section of the olfactory mucosa. Within the olfactory epithelium, the staining was intense in the supranuclear region (s), and moderate in the perinuclear cytoplasm of the nuclear (n) and basal (b) regions. In the lamina propria, staining was moderate in nerve bundles (nb). Bar is 10 μm. C: NADPH diaphorase staining in a cryostat section of the olfactory mucosa. Within the olfactory epithelium, staining was strong in the supranuclear (s) region, and in the lamina propria (LP), staining was present in nerve bundles. Magnification is the same as in D. D: Brain NOS-like immunoreactivity of the olfactory mucosa. The supranuclear region of the olfactory epithelium stained intensely and in the lamina propria, staining was moderate in the nerve bundles (nb). Bar is 80 μm.

by Nω-nitro-L-arginine a competitive blocker of NOS, implied that peri-receptor events were modulated by NO, and, furthermore, that modulation by NO was an early acquisition during vertebrate evolution. Alternatively, inhibition of L-arginine by Nω-nitro-L-arginine may have been due to the competitive binding of L-arginine molecules to olfactory receptor protein.

Our examination of the NADPH-d histochemistry of the sea lamprey olfactory mucosa by electron microscopy extended previous reports at the light microscope level, of NOS localization in the olfactory system in larvae of the lamprey, *Lampetra planeri* (Schober et al., 1994), and in the olfactory epithelium of catfish (Dellacorte et al., 1994). In the present study, NADPH-d labeling of subcellular compartments followed a definite and consistent pattern. The stain predominated in the cytoplasm surrounding mitochondria and within vesicles, implying that a similar or identical compound reacted at all the locations. It is pos-

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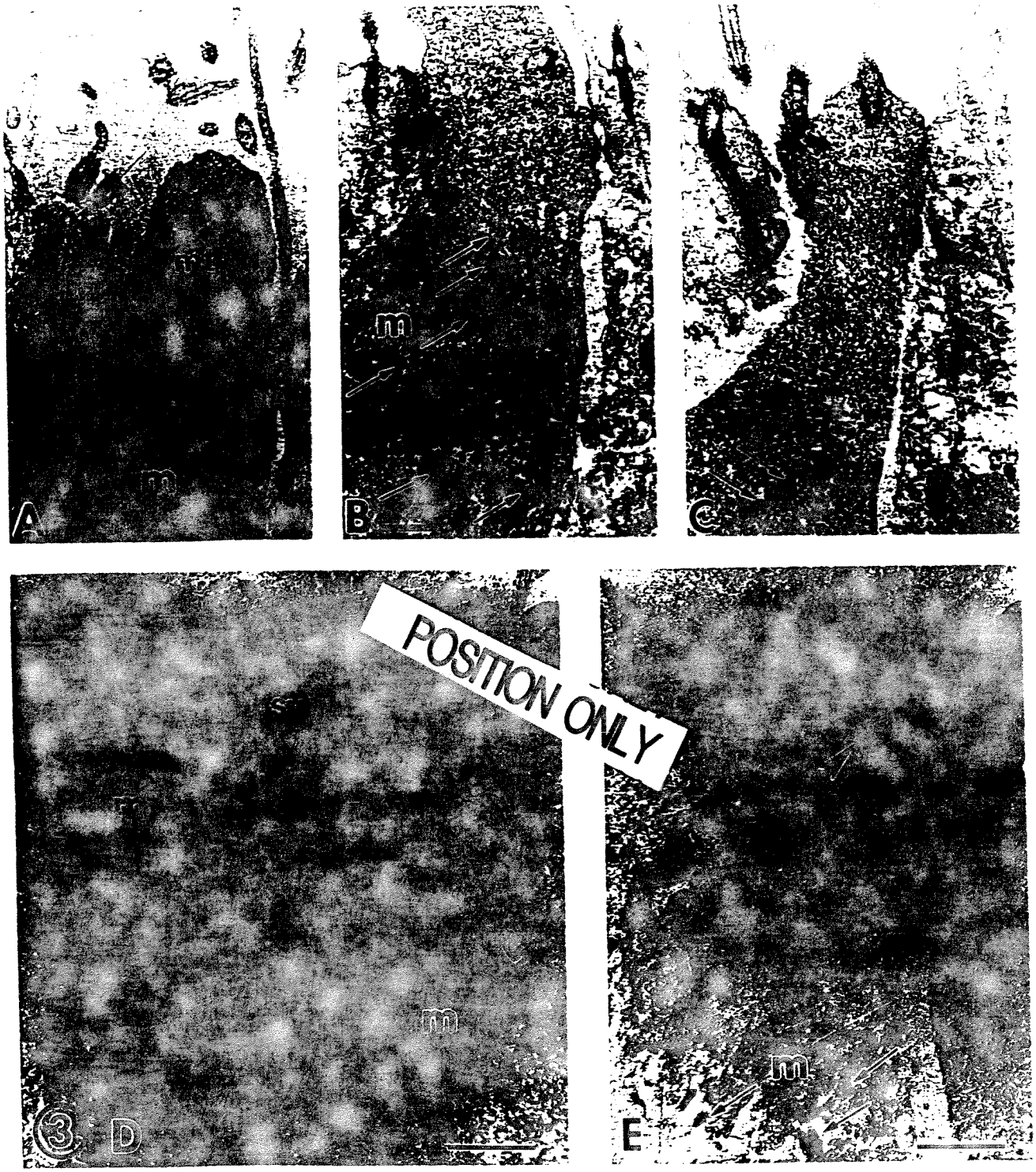

Fig. 3. Electron micrographs of the NADPH-diaphorase reaction product. A: The apical dendritic portion of an olfactory receptor cell (ORC) from the control tissue that was incubated without β-NADPH. Note the rounded olfactory knob, microtubules (mt) and mitochondria (m), and small electron-opaque granules. The magnification is the same in A, C, and E. The micrometer bar, shown in E, is 0.5 μm. B–E: are from tissue that was incubated in the NADPH diaphorase reaction solution. B: The formazon reaction product was heaviest (arrows) in the cytosol of ORC dendrites that were crowded with mitochondria. C: There were few formazon deposits in sections of the ORC dendrites that did not contain mitochondria. D: Sustentacular cells contained numerous deposits of the formazon product. Many are in the vicinity of mitochondria (m) and secretory vesicles (sv). Scale bar is 1 μm. E: Occasionally, secretory vesicles in apical blebs of sustentacular cells contained formazon deposits (arrowhead). Note heavy staining of secretory vesicles below the junctional complex (arrows). Scale bar is 0.5 μm.

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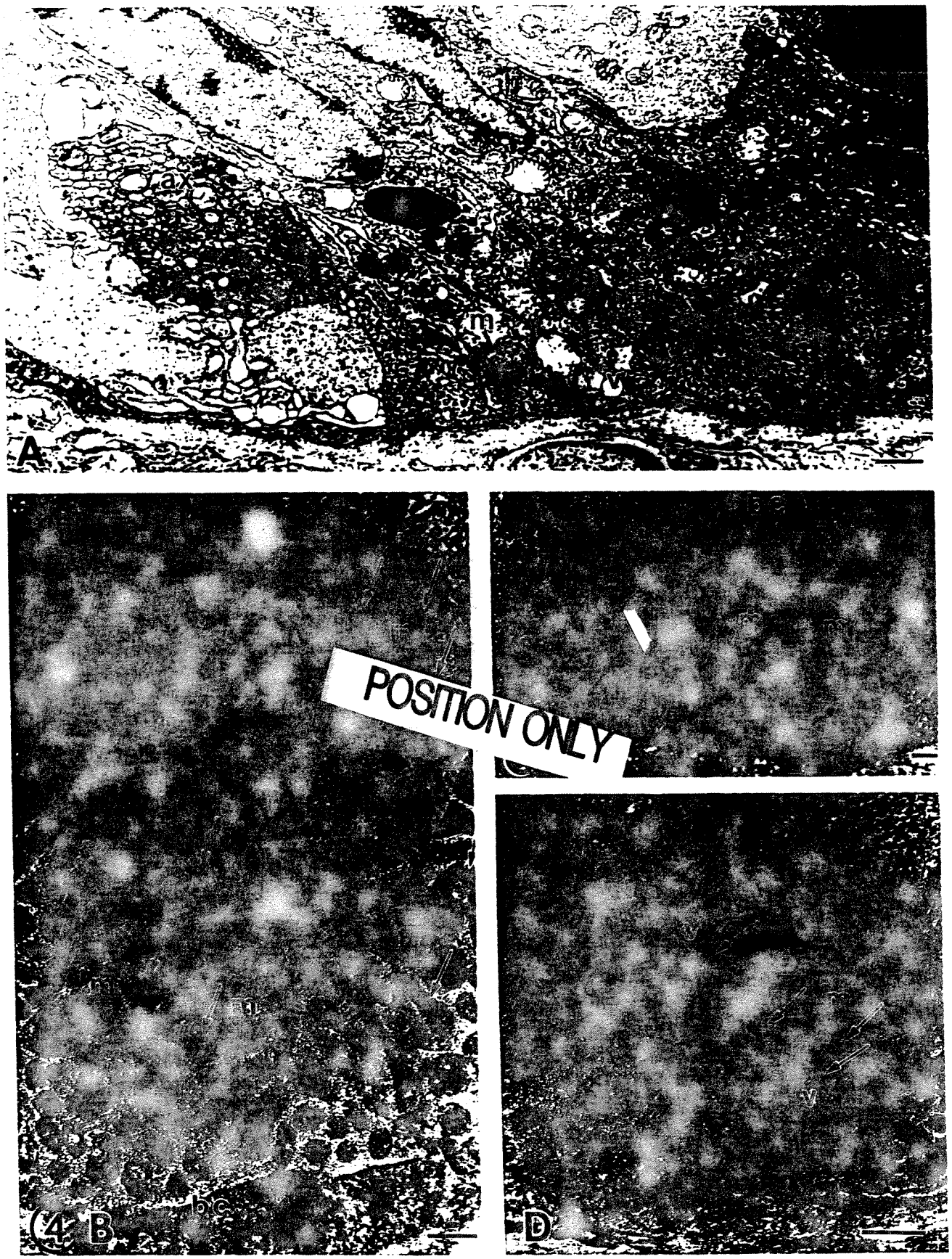

Fig. 4. Electron micrographs from the basal region of the olfactory epithelium. A: Conventional EM sections from the basal region of olfactory epithelium showing olfactory axon aggregates (ax), basal cells (bc) and sustentacular cell foot processes with mitochondria (m), and vesicles (v). B, C, and D are from tissue that was processed for diaphorase staining. B: The reaction product was present in the some axons (arrows). Wider axons with mitochondria (m) showed up to three sites of deposits. The cytoplasm of a basal cell located in the lower portion of the micrograph was heavily labeled. C: Basal cell (bc) contained intense labeling in the cytosol on and around the mitochondria (m). An adjacent foot process also contained labeling beside mitochondria.

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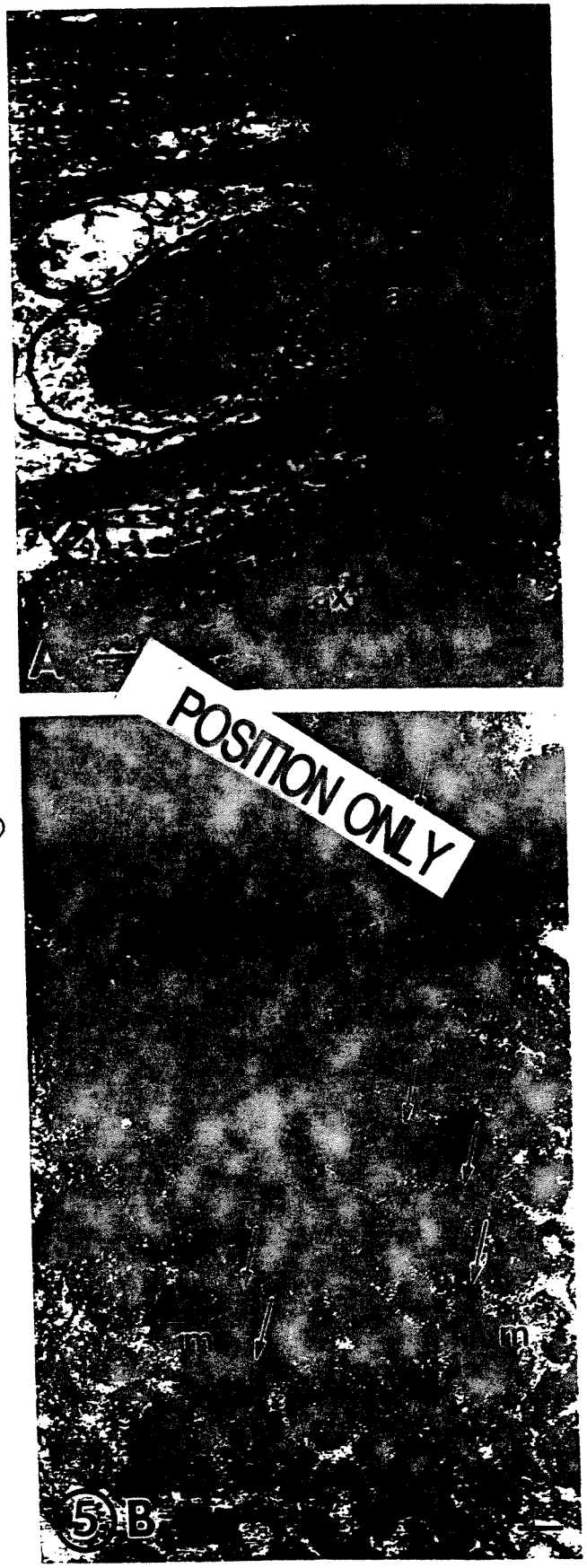

Figure 5 Please supply text

sible, however, that some of NADPH-d positive sites may have no relation to NOS since there are several diaphorases in tissues which are indistinguishable by histochemical procedures used (Hope et al., 1991). In rats and humans, ORN contained NADPH-d activity but not neuronal NOS immunoreactivity (Kulkarni et al., 1995), which in rats was limited to the fetal and early postnatal development and to the period of ORN reconstitution following olfactory bulbectomy (Roskams et al., 1995).

NOS localization in the supranuclear region of the olfactory epithelium supports previous reports of NO modulation of olfactory sensory transduction in rats (Breer et al., 1992) and *Xenopus* (Lischka and Schild, 1993). Although L-arginine is not chemo-stimulatory to the higher vertebrates, they appear to have conserved the use of NO for transduction. There are a number of possibilities for the involvement of NO in peri-receptor events in sea lamprey larvae. L-arginine itself or the arginine-receptor complex may be taken up by ORC or SC and provide a substrate for NOS, with ensuing NO activating guanylate cyclase (Breer and Shepherd, 1993). Uptake of L-arginine into the olfactory epithelium has been suggested from Scatchard analyses of rainbow trout olfactory ciliary preparations (Brown and Hara, 1981) and examples of ligand uptake exist in the nervous system: the internalization of GABA by glial cells (e.g., Orkand and Kravitz, 1971) and dopamine reuptake by pre-synaptic neurons (Kandel and Schwartz, 1985).

The intracellular, cytoplasmic localization of NOS does not preclude the existence of this enzyme on membranous components such as the ciliary membranes of ORC, where NOS may function both as an arginine receptor and a catalyst in the formation of NO. The formazon labeling product compound used in this study, NBT, has a tendency to coalesce into droplets and is not sufficiently osmiophilic and lipophilic to label membranes (Kalina et al., 1972). When electron microscope histochemistry with NBT was used in the rat olfactory bulb (Scott et al., 1987) and striatum (Hope and Vincent, 1989), labeling was absent from membranes. However, labeling with an alternate tetrazolium salt, [2-(2'-benzothiazolyl)-5-styryl-3-(4'-phthalhydrazidyl) tetrazolium chloride (BSPT)], revealed NOS localization on membranes (Hope and Vincent, 1989; Wolf et al., 1992). Biochemical studies of rat brain NOS have shown that it is cytosolic (Ohshima et al., 1992; Matsumoto et al., 1993a,b) and at least a part of it is membrane bound (Kuonen et al., 1988; Matsumoto et al., 1993a,b). Further investigations of lamprey olfactory mucosal NOS must take into account the possible existence of both cytoplasmic and membrane bound forms of the enzyme.

NADPH-d labeling within SC secretory vesicles suggests that NOS is secreted into the mucociliary matrix, where it may catalyze the formation of NO from L-arginine odorant molecules. However, the breakdown of L-arginine by NOS has not been demonstrated; NOS labeling was not observed in the mucociliary matrix in the present study and may have been washed away during specimen preparation. NOS within the SC may also regulate stimulus/secretion coupling: in mammals, there is evidence for NO signaling in the secretory cells (reviewed by Schmidt et al., 1992b).

The patchy NADPH-d labeling of ORC axonal profiles indicates that NOS was located in a subpopulation of axons or, alternatively, that NOS distribution was limited to axoplasm proximal to mitochondria. The scattered axonal labeling is consistent with that previously observed in perivascular nerves of the rat basilar artery (Loesch et al.,

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1994). The fact that the labeled fibers were located in both the olfactory epithelium and the lamina propria confirms that they are ORN axons. In nasal mucosae of rats, however, NADPH-d labeling was present in the extrinsic fibers that innervated structures in the lamina propria (Hanazawa et al., 1994). An additional factor in the patchy axonal NOS labeling may be the larval developmental stage. NOS and ORC development have been previously linked in embryonic rats (Roskams et al., 1994). Examination of the distribution of labeling in the olfactory axons of post-metamorphic lampreys may determine if the axonal labeling is limited to early sea lamprey development.

The NOS labeling of a subpopulation of basal cells, which are probably ORC progenitor cells (e.g., Thornhill, 1970; Graziadei and DeHan, 1973), further supports the observation of Roskams et al. (1994) that NO functions during ORC development. The olfactory organ in larval sea lampreys showed additional characteristics of early development, a simple sac-like shape, and narrow olfactory epithelial cells, compared to the lamellar folds of the olfactory organ and enlarged ORC and SC present following metamorphosis (Vandenbossche et al., 1995). In other fish, such as salmonids, olfactory lamellae form shortly after hatching (Evans et al., 1982). Clearly, further investigation of NO function during ORC development in lampreys is needed.

If NO exerts modulation of peri-receptor events, axonal activity and ORC development in the larval lamprey olfactory epithelium, then our observations imply that there are several subcellular sources for the NO. In our proposed model, chemostimulation by L-arginine is at least partially a consequence of NO which originates either from SC secretions into the mucociliary matrix, or from L-arginine taken up into SC or ORC dendrites. Heavy NOS labeling in a subpopulation of basal cells implies the action of NO during ORC development.

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Appendix 2

1994: Larvae: behavioral experiments

Objective: Do larvae display preference/avoidance behavioural responses to chemostimulation?

Background: Our physiological experiments have shown that L-arginine elicits EOG responses in larvae. We proposed to follow these experiments with behavioural studies based on those conducted on glass eels by Tongiorgi et al., (1986 J. Fish Biol. 28: 501-510).

Techniques: We constructed a preference/avoidance trough previously designed by Tongiorgi et al. 1986. (fig. Ap.2.1)

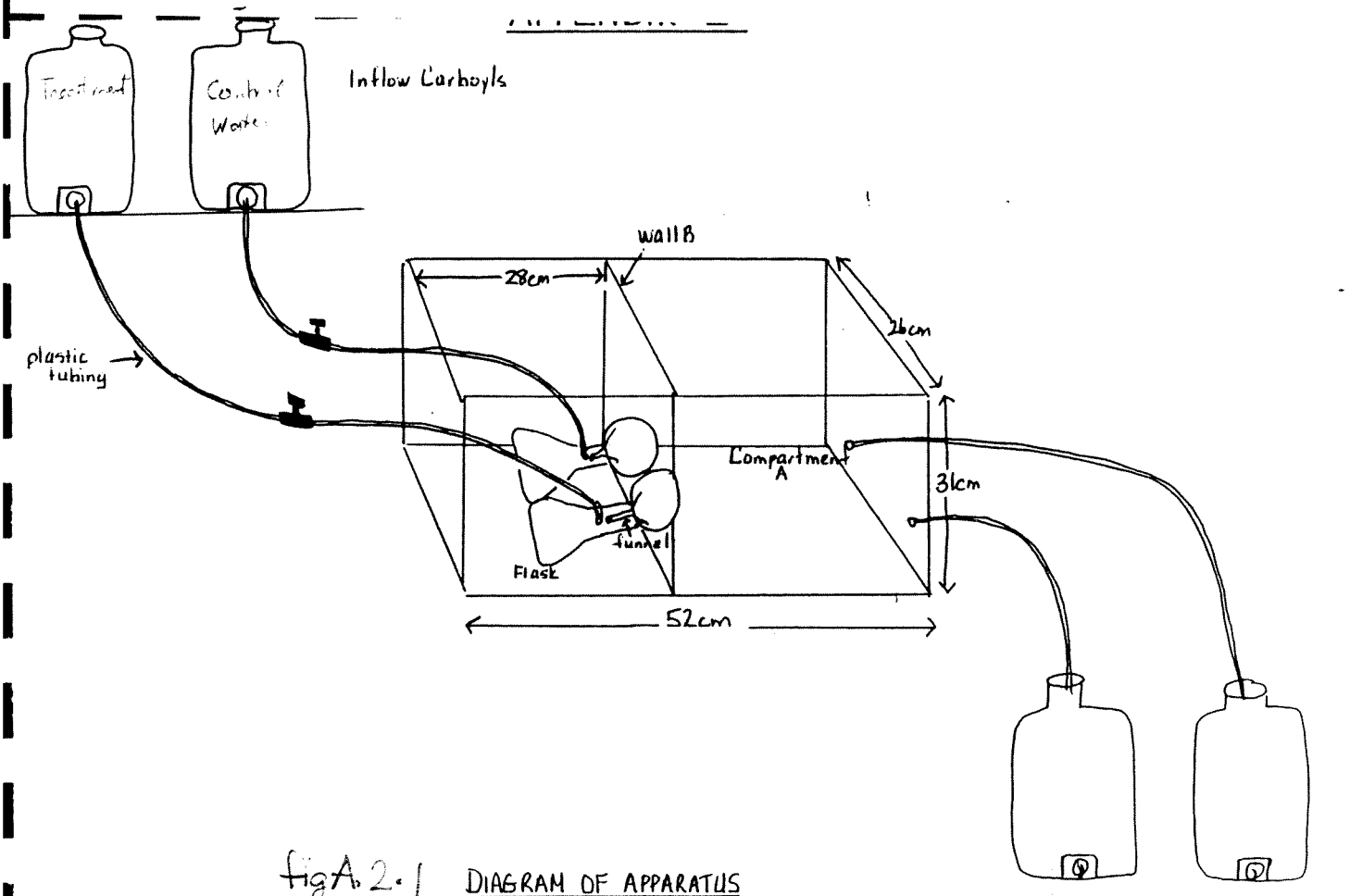


fig A. 2.1 DIAGRAM OF APPARATUS

adapted from Tongiorgi et al. 1986 J Fish Biol 28 501-510.

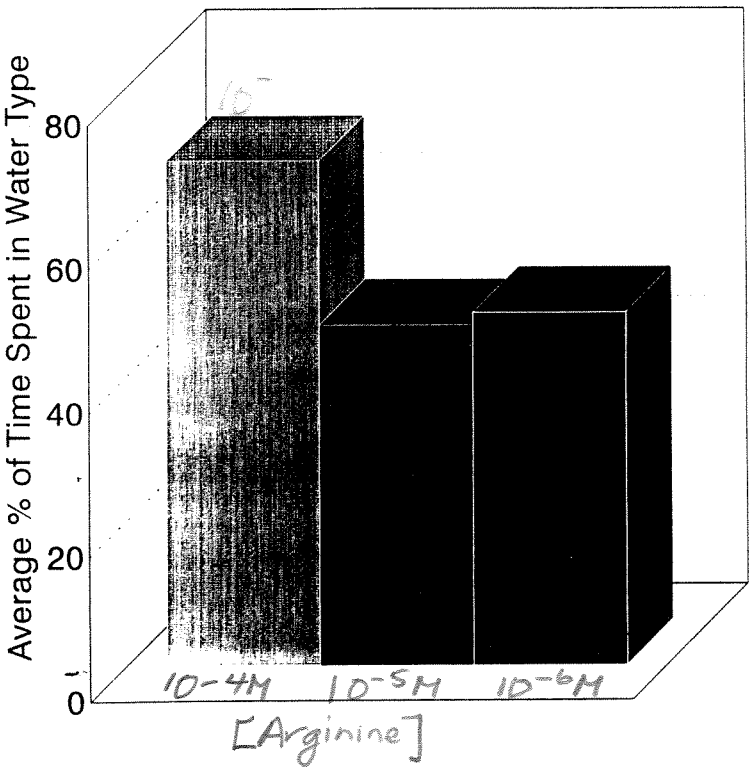
All experiments were conducted at the University of Windsor, either at ambient temperature (21 °C) or at 15 °C, in a dimly lit room. One larva at a time(size 10 cm) was tested. Each larva was initially immersed into the preference/avoidance trough, with untreated, dechlorinated water. After 5 minutes, the flow of water (untreated dechlorinated and test water) through the trough was initiated. The activity of the larva was recorded with a video camera for 5 minutes, and the time that was spent in each compartment was recorded.

We observed that when 10^{-4} M L-arginine was flowing through the test compartment, larvae consistently spent approximately 60% - 70% of the time in the test side. When lower concentrations of L-arginine or untreated water was flowing through the test compartment, larvae spent about 50 % of the time in each compartment. These values were averaged from over 30 trials for each treatment. Some larvae did not show this "average" response.

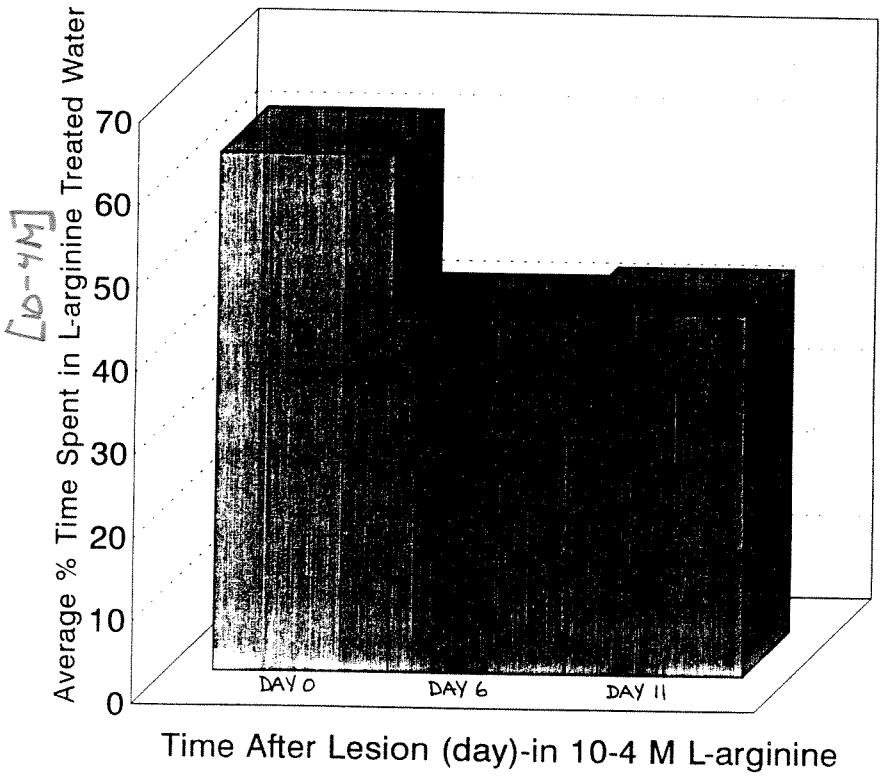
To show that olfactory receptor neurons mediated the slight preference to 10^{-4} M L-arginine, we induced degeneration of these receptor neurons by retrograde degeneration of the olfactory nerve following trans-section of the olfactory nerve. Following the behavioural testing, each larva was sacrificed and prepared for examination of olfactory receptor neurons by light microscopy of 1 μm sections. At two time points (postoperative day 6 and day 11), the slight preference to L-arginine was absent. A decrease in the olfactory receptor neuron density was evident upon morphological examination of the olfactory mucosa from these animals. The density of olfactory receptor neurons dropped from 12 cells/100 μm to 6 cells/100 μm.

These results show that larvae show a slight preference to L-arginine (10^{-4} M).

Our trials with taurocholic acid (10^{-6} M), showed overall avoidance to that bile acid; over 30 larvae were tested.



Time in Water vs Water Type



Time After Lesion (day)-in 10-4 M L-arginine