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Body Length, Activity Level, and Avoidance Learning in Zebrafish Exposed to Nicotine as Embryos

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BODY LENGTH, ACTIVITY LEVEL, AND AVOIDANCE LEARNING IN ZEBRAFISH EXPOSED TO NICOTINE AS EMBRYOS

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BODY LENGTH, ACTIVITY LEVELS, AND AVOIDANCE LEARNING IN ZEBRAFISH EXPOSED TO NICOTINE AS EMBYROS

Tim Lawrence August 2001 40 Pages

Directed by: Joseph Bilotta, Ph.D., Elizabeth Lemerise, Ph.D., and Richard Miller, Ph.D.

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Smoking continues to be a significant health problem in the United States and throughout the world. One of the many aspects of the health risks of smoking that have been investigated is the effect of maternal smoking on developing embryos. In particular, exposure of embryos to nicotine is believed to cause attention-deficit/hyperactivity disorder (ADHD) and related problems in children. Both correlational research with humans and experimental research with animals have supported this belief. However, the mechanisms of the effect of nicotine on developing embryos are not fully understood. The zebrafish offers a useful model of the effects of nicotine on developing organisms because its development is fast, well understood, and easily observable. Also, the embryo can be exposed to nicotine without concern for many of the intermediate factors that are present in research with conventional models (e.g., the rat), such as the effect of nicotine on the placenta. This study was an exploratory attempt to establish the zebrafish as a model for the effects of embryonic nicotine (EN) exposure. Zebrafish eggs were exposed to two levels of nicotine during the first eight hours after fertilization. These subjects and a group of controls were

measured on three variables at different stages of development: body length, activity level, and avoidance learning. Results showed that EN exposure caused a significant decrease in growth and a significant increase in activity level. Thus, the zebrafish responds to EN exposure in a manner similar to that observed in other models and in humans. Further research on the mechanisms of the effect of EN exposure may be possible using the zebrafish.

Chapter 1

Introduction

Effects of Embryonic Nicotine (EN) Exposure

Cigarette smoking has been and continues to be a major public health concern. One specific aspect of this issue is cigarette use by pregnant women and the effects it may have on the developing fetus. While many women who smoke quit upon learning they are pregnant, many do not, especially mothers in their teens or who have not graduated from high school (Stewart & Dunkey, 1985; Streissguth, Darby, Barr, Smith, & Martin, 1983). One of the most studied components of tobacco smoke is nicotine. Nicotine is a drug that stimulates the brain, spinal cord, and peripheral nervous system, as well as the heart, adrenal glands, and other organs (Julien, 1981). Nicotine is the substance in tobacco smoke that is considered to be the most dangerous to the developing fetus. A number of possible risks to the fetus of EN exposure have been identified over the last 30 years. Maternal smoking during pregnancy has been identified as an independent risk factor for sudden infant death syndrome (Alm et al., 1998; Golding 1997; MacDorman, Cnattingius, Hoffman, Kramer, & Haglund, 1997). Brooke, Anderson, Bland, Peacock, and Stewart (1989) found that maternal smoking reduced birth weight more than alcohol consumption, caffeine consumption, socioeconomic factors, and psychosocial stress. Picone, Allen,

Olsen, and Ferris (1982) found lower birth weight for infants of mothers who smoked during pregnancy, even though these women ate more calories than nonsmokers while pregnant. Fried and O'Connell (1987) reported that maternal daily use of nicotine reduced birth weight and head circumference and subsequent weight gain and head growth more than did alcohol, cannabis, or caffeine. Long term behavioral and social effects of EN exposure have also been identified. In children whose mothers smoked during pregnancy, Weismann, Warner, Wickramartne, and Kandel (1999) found a three-fold increased lifetime risk of conduct disorder in males and a five-fold increased lifetime risk of drug abuse or dependence in females. These findings could not be explained by maternal substance abuse during pregnancy, paternal psychiatric diagnosis, or family risk factors such as socioeconomic class.

EN Exposure and Attention-Deficit/Hyperactivity Disorder (ADHD)

ADHD is defined by persistent inattention and hyperactivity or impulsivity manifesting in childhood and severe enough to disrupt functioning in school and other settings (American Psychiatric Association, 2000). This disorder affects 3-7% of school-age children, and is associated with increased risk for other disruptive behavior disorders of childhood and with adult diagnoses such as Antisocial Personality Disorder (American Psychiatric Association, 2000). ADHD is currently a topic of great interest in educational research (Smallwood, 1997). The disorder is considered a major health problem affecting a significant portion of school-age children (Aldridge, Eddows, & Kuby, 1998)

For many years evidence has been reported of a link between maternal cigarette smoking and ADHD. In 1975, Denson, Nanson, and McWatters pointed out that a drastic increase in reported "hyperkinetic syndrome" among children coincided with an increase in smoking among women through the 1950s and 1960s. The symptoms of this syndrome were restlessness and inattention. They investigated the relationship between hyperkinetic syndrome and maternal smoking and found that mothers of hyperkinetic children smoked twice as many cigarettes per day during pregnancy as mothers of dyslexic and control children. Lichtensteiger, Urs, Schlumpf, Odermatt, and Widmer (1988) suggested that the effects of EN exposure on children resemble minimal brain dysfunction, or attention deficit disorder, including such symptoms as short attention span, hyperactivity, decrements in reading ability, some sensory deficits, and social adjustment problems. Milberger and colleagues found ADHD in 22% of children whose mothers smoked during pregnancy, but in only 8% of controls (Milberger, Biederman, Faraone, Chen, & Jones, 1996). This association remained significant after controlling for socioeconomic status, parental IQ, and parental ADHD diagnosis.

Other researchers have identified EN exposure correlates that are associated with ADHD. Kristjansson, Fried, and Watkinson (1989) found that children whose mothers smoked during pregnancy showed deficits on auditory and visual vigilance tasks. They found increased activity levels, as well as more errors of commission, which they attributed to increased impulsiveness. Fried (1989) found a dose-response relationship between embryonic cigarette exposure

and lower language scores and lower cognitive scores, but did not find these deficits to be related to embryonic marijuana exposure. Wakschlag et al. (1997) found that boys of mothers who smoked during pregnancy were more likely to have preadolescent or adolescent diagnoses of conduct disorder, a disorder that is often comorbid with ADHD (American Psychiatric Association, 2000). Orlebeke, Knol, and Verhulst (1997) reported that both girls and boys exposed to EN showed increases in oppositional, aggressive, and overactive behaviors, and that this effect was not mediated by birth weight. Brennan, Grekin, and Mednick (1999) reported a relationship between EN exposure and adult criminal offending. Fergusson, Horwood, and Lynskey (1993) used teacher and parent questionnaires to assess behavior in school-aged children who had been exposed to EN. They found small but significant increases in disruptive behaviors including attention deficit behaviors.

The number and consistency of reports of correlations between EN exposure and behavioral and cognitive problems suggest that the relationship is robust and broad. However, not all reports have supported the strength of this connection. Rantakallio (1983) found that while maternal smoking during pregnancy negatively affected such variables as growth, school performance, and adult employment status, it did not have a greater effect than paternal smoking.

Experimental Data on EN Exposure

Data from humans in studies such as these have several weaknesses. They are necessarily correlational, since it would be unethical to manipulate EN exposure in humans; thus it is difficult to establish that EN causes any of the

symptoms with which it is associated. Measurements of EN consist of retrospective interviews, in which mothers are asked about their smoking habits during pregnancy, which may have been years previous. Such measurement is of low reliability and accuracy. Human studies must cope with an almost inexhaustible number of potential confounds, such as socioeconomic class, parental education level, parental psychiatric diagnoses, and maternal use of other potential teratogens (e.g., alcohol, caffeine, illegal drugs). Also, human studies cannot separate out the effects of EN exposure from the effects of other components of cigarette smoke, such as carbon monoxide. For these reasons, animal models have been used to attempt to confirm and specify a causal relationship between EN exposure and developmental and behavioral deficits.

Research using animal models, typically the rat, has confirmed many of the findings in the human data. For instance, numerous investigations have found that EN exposure decreases fetal weight, pup weight, and pup growth in rats (Martin, Martin, Radow, & Sigman, 1976; Paulson, Shanfeld, Mullet, Cole, & Paulson 1994; Paulson, Shanfeld, Vorhees, et al., 1994; Paulson et al., 1993). Behavioral investigations have strengthened the case that EN exposure is a cause of ADHD. Fung (1988) found that 14-day-old rats that had been exposed to EN showed an increase in spontaneous locomotion. This increase in activity was reduced by the administration of amphetamine, just as ADHD symptoms in children often respond to stimulants. Paulson, Shanfeld, Vorhees, et al. (1994) reported an increase in open field activity in EN-exposed rats during pre- and post- weaning periods.

Research with animal models has also identified cognitive deficits resulting from EN exposure. Levin, Wilkerson, Jones, Christopher and Briggs (1996) found that EN exposure caused radial arm maze performance deficits in rats, though only under pharmacological and behavioral challenges. For instance, testing in an identical maze placed in a different room produced poorer maze performance in EN-exposed rats. The authors suggest that the treated rats may have been more likely to learn the maze using distal (outside the maze) cues. Alternatively, the treated rats may have been more prone to distraction by irrelevant environmental cues. Levin, Briggs, Christopher, and Rose (1993) exposed rats to EN at a dose that did not cause deficits in pup weight gain. These rats did not learn spontaneous alteration of response in a T-maze, while controls did. However, these subjects showed no difference from controls in learning a radial arm maze. Sorenson, Raskin, and Suh (1991) did find that rats exposed to EN showed deficits in learning a radial arm maze. Johns, Louis, Becker, and Means (1982) reported that guinea pigs exposed to EN showed reduced spontaneous alteration and were less likely to enter a novel alley. These effects persisted into adulthood.

Interestingly, Bertolini, Bernardi, and Genedani (1982) reported an increased rate of avoidance learning in rats whose mothers had been exposed to EN. Adult rats that had been exposed to EN required fewer trials than controls to learn to avoid an electric shock in a two-way shuttle box. Geredani, Bernardi, and Bertolini (1983) replicated this effect in female rats, but found the opposite effect in males. Paulson and colleagues did not find an effect of EN on active

avoidance learning in rats (Paulson, Shanfeld, Vorhees, et al., 1994; Paulson et al., 1993). Additional research is needed to confirm whether EN has a different effect on avoidance learning than on other kinds of learning, such as the instrumental learning of radial arm mazes, discussed above. One possible explanation might be that greater activity levels in EN-exposed animals increase the likelihood that the animals will quickly emit a correct operant response, and thus more quickly learn to escape the aversive stimulus; however, this effect would improve any operant learning, not just avoidance learning.

The relationship between the effect of EN exposure and ADHD-like symptoms has been linked theoretically and empirically to various neurotransmitter systems, including nigrostriatal dopaminergic pathways (Fung, 1989; Muneoka et al., 1997, Richardson & Tizabi, 1994), cholinergic pathways (Navarro, Seidler, Eyelers, et al., 1989) and central serotoninergic pathways (Muneoka et al., 1997). While few anatomical studies have been conducted on the effects of EN, Roy and Sabherwal (1994) have reported reductions in cortical thickness and cell size in the somatosensory cortex. They also reported alterations in dendritic arbor and spine density and abnormalities in intracellular anatomy in the somatosensory cortex and the hippocampus (Roy & Sabherwal, 1994, 1998). They have suggested that such anatomical alterations may be involved in behavioral abnormalities observed by other researchers. Navarro, Seidler, Schwartz, et al. (1989) showed that at a dose considerably smaller than commonly used in EN studies, rats showed no decreased viability or growth, and no decreased nervous system growth, but still showed abnormalities in cellular

development and in peripheral noradrenergic projection development. McNerney and Szeto (1993) recorded electrocorticograms in fetal lambs and found that nicotine administration initiated complex changes in cortical activation. The highest doses of nicotine produced a consistent increase in cortical activation, without tolerance. Such changes in fetal cortical activation could have effects on brain development. Ehlers, Somes, Thomas, and Riley (1997) found that neonatal nicotine exposure reduces event-related potentials in the dorsal hippocampus of adult rats without altering background electroencephalograph response. Britos and Orsingher (1991) found that EN exposure increased susceptibility of adult rats to electroconvulsive shock.

All of these experimental findings on the effects of EN exposure suggest strongly that it causes numerous behavioral and physiological changes in animals. However, despite many investigations, the mechanisms of this effect and the relationship between physiological and behavioral changes are not clearly understood. Further research with a simpler animal model has the potential to clarify some of these issues.

Zebrafish as Behavioral Teratogen Model

The rat and other rodents are the most commonly used animal models in the study of EN exposure. However, these models have several disadvantages. For example, the method of nicotine administration is a serious issue. Common routes of administration include adding nicotine to drinking water, giving periodic subcutaneous injections of nicotine to the mother, and implanting subcutaneous osmotic minipumps. Adding nicotine to drinking water causes a bitter taste rats

tend to avoid, thus reducing fluid intake; it may also interfere with nutrient absorption (Murrin, Ferrer, Zeng, & Haley, 1987). Injection is sufficiently stressful to pregnant dams that it affects offspring, which may obscure the effects of nicotine (Muneoka et al., 1997). Subcutaneous minipumps must be purchased and implanted surgically. All these methods may be complicated by variables such as maternal and placental absorption rates. Also, limiting research to a few species weakens external validity; the more species investigated, the more confidently findings can be generalized to humans.

Danio rerio, the zebrafish, is a valuable model for behavioral teratogen research. The zebrafish is a tropical fish, four to five cm in length, originating in Indian and Pakistani rivers (Laale, 1977). This fish produces numerous eggs; one female with one male can produce more than fifty fertilized eggs per spawning cycle of two to three days (Laale, 1977). Thus, many subjects can be conveniently obtained. The embryos develop externally, and the eggs and embryos are transparent. Thus, development can easily be observed and recorded (Westerfield, 1994). These excellent embryology characteristics, combined with a well understood genome, make the zebrafish useful for genetic studies (Barinaga, 1990; Fetcho & Liu, 1998). Behavioral teratogen treatments can be administered at any point during development by adding dilute substances to the water in which the embryos are developing. After a desired period the embryos can then be rinsed and placed in clean water. Thus, it is possible to control the amount and timing of treatment exposure more precisely than with other animal models (Bilotta, Barnett, Hancock, & Saszik, 2000).

The zebrafish is also useful for studies of the nervous system such as phototabulation of genetically or chemically marked neurons in developing embryos, and for noninvasive, laser ablation of specific groups of neurons during development (Fetcho & Liu, 1998). These kinds of studies allow researchers to watch the development of specific neurological systems in teratogen-exposed fish and to selectively damage areas in an attempt to reproduce the deficits caused by teratogens. Thus, it may be possible to identify precisely which parts of the nervous system are affected at which points during development. The range of possibilities for genetic and ablative manipulation and phototabulation during development mean this animal has great potential for use in isolating the anatomical effects of behavioral teratogens.

One study that illustrates the usefulness of the zebrafish as a model for behavioral teratology was done by Bilotta et al. (2000). They conducted research on Fetal Alcohol Syndrome (FAS) using the zebrafish. Subjects were exposed to varying concentrations of ethanol for different lengths of time during the first day after fertilization. Results varied from a mild fetal alcohol effect to a fully expressed FAS. Symptoms were similar to those observed in human FAS: enlarged heart and eyes, increased heart rate.

Purpose

The purpose of this study was to examine the effects of EN exposure on zebrafish. This purpose was accomplished by measuring the effects of EN exposure on activity levels and learning ability. These effects have been suggested by correlational studies of humans and experimental research with

other animal models. This study also measured the effects of EN exposure on physical growth. Effects on growth have been observed in humans and animals. This study also helps establish the zebrafish as a model for the study of exposure to EN and other behavioral teratogens.

It was expected that exposure to nicotine bitartrate during development would cause a decrease in growth (body length) and an increase in activity level (swimming speed). These hypotheses are indicated by the literature on humans and on other animal models. Further, it was expected that exposure to nicotine would cause a decrease in avoidance responses in the aversive learning task. This hypothesis is suggested by the literature cited above on the effects of EN exposure on cognitive abilities and development.

Chapter 2

Method

Participants

Subjects in this experiment were zebrafish, *Danio rerio*, bred in-house (Bilotta, Saszik, DeLorenzo, & Hardesty, 1999) from stocks obtained from local pet stores. Six adult male and six adult female zebrafish were used to produce fertilized eggs. Eggs were collected and divided evenly between control and treatment conditions (about 30 eggs per condition). A random sample of 20 fish from each of the three conditions was used for all measurements. The Institutional Animal Care and Use Committee of Western Kentucky University approved all procedures.

Breeding and Treatment Apparatus and Procedure

A standard five-gallon glass fish tank was used for breeding. Standard 150 ml petri dishes were used to expose the embryos to the appropriate solutions (treatment or control). A cage made of plastic mesh was used to line the breeding tank. The cage bottom was positioned about 3 cm above the bottom of the tank in order to allow eggs to drop beyond the reach of the adults. An air stone was placed inside the tank to aerate the tank water. A nicotine salt, nicotine bitartrate (Sigma Chemical Co., St. Louis, MO, product number N5260), was dissolved in distilled water to create two solutions with concentrations of 2.5

μg and 10 μg of nicotine bitartrate per milliliter of distilled water. This form of nicotine is safer to handle than the free base form and is the form used in most of the animal research cited above. Preliminary work in this lab has shown that a dosage of 10 μg/ml causes some physical abnormalities; for example, after hatching the bodies of the larvae were bent at the midsection, though they straightened out within a few days. This dosage was included in this study because it clearly demonstrated an effect on the zebrafish but did not prevent them from developing. A second dosage was desired for this investigation that did not appear to cause any observable physical abnormalities. The 2.5 μg/ml dose was chosen because it approached the dose that caused observable effects, without causing gross abnormalities. Note that this dose is at least two orders of magnitude higher than the blood nicotine levels present in rats given standard experimental doses of nicotine (Richardson & Tizabi, 1994). This discrepancy is likely the result of the zebrafish chorion's poor permeability to nicotine. Bilotta et al. (2000) found a similar effect of chorion permeability on ethanol absorption; the doses required to produce fetal alcohol effects in zebrafish were at least an order of magnitude higher than blood alcohol levels in intoxicated humans.

The night before breeding, eight to ten adult zebrafish were placed in the plastic cage positioned in the breeding tank. At 8:00 a.m. the following morning the cage was lifted to remove the adults. Twenty milliliters of distilled water was added to a petri dish. Twenty milliliters of the 2.5 μg/ml nicotine solution was

added to a second petri dish, and twenty milliliters of the 10 μg/ml nicotine solution was added to a third. Eggs were siphoned from the bottom of the breeding tank, divided equally, and placed into the three petri dishes. The petri dishes were floated in a five-gallon tank. At eight hours post fertilization (hpf) all eggs were removed from the petri dishes, rinsed twice, and placed in new petri dishes containing distilled water. This period of exposure was used in order to test the effects of brief, early exposure to nicotine. This scenario occurs in humans when women quit smoking after learning that they are pregnant, which may be several weeks after fertilization.

Once all the eggs had hatched the larvae were counted and placed in plastic rearing containers, 6 cm high by 10 cm wide by 10 cm long, holding 400 ml. The rearing containers were floated in 10 gallon glass fish tanks with water heaters set to maintain a temperature of 28.5 degrees Celsius. All rearing containers were filled with distilled water with Instant Ocean aquarium salt added (Aquarium Systems, Mentor, OH) and with the pH adjusted to between 6.8 and 7.2 (Bilotta et al., 1999). The larvae were fed paramecium (Liquifry No1, Interpet Ltd, Dorking, Surry, England) and flake food (TetraMin, Blacksburg, VA). In order to prevent ammonia build up, each day one half of the water in each container was replaced with water from the tank. A mortality count was conducted at seven days post fertilization (dpf).

Body Length Measurement Apparatus and Procedure

At 10 dpf, a sample of 20 fish from each condition was selected for body length measurements. Body length measurements and activity level

measurements were made using a CCTV camera (Javelin Electronics, Torrance, CA, model JE-8142), connected to a Macintosh IIci computer with a frame grabber board. The fish were placed in the well of a microscope slide where they could be viewed under magnification using the video camera. Still pictures of the fish were made, and body length was measured from head to tail in pixels, which were converted to millimeters using a still picture of a ruler as a reference. At this time the larvae were placed in standard 10 gallon fish tanks with air stones. Temperatures in the tanks were maintained at 28.5 degrees Celsius, and pH was maintained at between 6.8 and 7.2.

Activity Level Measurement Apparatus and Procedure

At 45 dpf the activity levels of 12 fish from each condition were measured. Each fish was placed under the video camera (described above), at a distance of 25 cm. A still picture was made of each fish for body length measurement. Then the fish was placed in a plastic tank measuring 13 cm high by 12 cm wide by 19 cm long. The tank was filled with one inch of water. Black paper was taped around the sides of the tank to help prevent the fish from seeing its reflection, since the sight of other fish may alter the behavior of schooling fish. The tank was placed 104 cm from the video camera. Once a fish had been in the tank for one minute, a 10 second movie was recorded, with a frame rate of two per second. A second movie was made after the fish had been in the tank for two minutes. The fish was then returned to its home tank.

The activity levels were obtained by recording the position of the head of the fish in each half-second frame of the 10 second video clips. These position

data were used to calculate the average speed of each fish during the two recorded 10 second intervals. Previous work in this lab with this procedure has shown that average speed correlates highly with average acceleration; therefore, this measure is sensitive to the erratic changes in direction of very active fish.

Avoidance Training Apparatus and Procedure

Subjects were tested in the avoidance training procedure between 55 and 75 dpf. The fish were trained to avoid an aversive stimulus when presented with a light stimulus that was predictive of the aversive stimulus.

Avoidance training was conducted in a two-way shuttle box measuring 3 cm high by 3 cm wide by 6 cm long and divided into two chambers. The sides were lined with aluminum plates such that a mild electric shock could be applied to one chamber of the shuttle box at a time. A function generator (Dynastic Corporation, Chicago, IL, model 3020) provided the electric current. A frame made from 2 cm by 4 cm wooden strips allowed a 5 mm diameter fiber optic cable to be moved back and forth over the two chambers of the shuttle box. The light source was a 150 W xenon arc lamp (Spectral Energy, Westwood, NJ, model LH150). The light stimulus consisted of a 476 nm (blue) light with an irradiance of 100 $\mu W/cm^2$, displayed 6 cm above the top of the water-filled shuttle box. A 476 nm interference filter (Oriel, Stratford, CT, model 340) was used because young zebrafish are very sensitive to this visual stimulus (Saszik, Bilotta & Givin, 1999).

The aversive stimulus consisted of a mild electric shock created by applying a current to one of the two sets of aluminum plates lining the chambers of the shuttle box. Voltage applied to the grids was about 4 V, in the form of a 10

Hz sine wave. Resistance between the grids was approximately 1 MΩ, yielding a current of about 4 mA. Gleason, Weber, and Weber (1977) successfully used a 4 V stimulus to train zebrafish using a two-way shuttle box. However, their training apparatus was considerably larger (holding 3.5 l of water and able to accommodate several fish at once), thus the current used in their study may have been less than 4 mA. Previous work in this lab has shown that a 4 mA level of shock is strong enough to motivate fish to escape, but not strong enough to cause injury.

During the training each fish was placed in the shuttle box and the light stimulus was presented over one chamber of the shuttle box, preceding the shock by two seconds. The light remained on for six seconds after the shock began. The shock remained on for 20 seconds, then was terminated. Two seconds later the light and shock were presented in the opposite chamber of the shuttle box in the same manner. A trial was counted as a correct avoidance response if the fish moved entirely into the opposite chamber of the shuttle box before the shock was applied. The experimenter also noted for each trial whether the fish reentered the opposite chamber of the box before the next light presentation; this premature reentry would cause the fish to receive a shock because the voltage was applied throughout the entire trial, until just before the next light presentation. Any trial in which the fish returned to the opposite chamber of the shuttle box before it was safe to do so was called a premature reentry. A given trial was labeled as both a correct avoidance response and a premature reentry if the fish exhibited both behaviors.

Each training session lasted approximately 25 minutes. When an animal was returned to the home tank after testing, it was carefully monitored for distress. The fish were expected to swim and feed freely within a few minutes of being returned to the tank. Stress Coat (Aquarium Pharmaceuticals, Chalfont, PA, product number 85F) was added to the water to ensure that the fish's protective coating of slime remained intact. It was planned that if a fish did not return to normal patterns of swimming and feeding, or otherwise appeared unhealthy, it would not be used in further training, and standard procedures for care of sick fish would be employed. These procedures are similar to those described by Bilotta and Powers (1991). All fish used in this study appeared healthy throughout and after the data collection; thus, training was completed for all subjects.

A training session consisted of 40 light/shock pairings. Previous work in this lab has shown that normal zebrafish will make a number of avoidance responses when trained in this manner. Figure 1 shows a learning curve obtained by training five zebrafish with this procedure. Note that percent correct responding steadily increased with additional trials. All subjects reached 100 percent correct responding within 120 trials. Originally it was planned that fish would be trained for two consecutive days, for a total of 80 trials. However, after 12 fish (four from each group) had been trained for two days, the performance on the two days was compared. No significant difference was found between the means on the two days, $t(22) = .658$, $p = .517$, and performance on the first day predicted performance on the second day very well, $r = .752$, $p = .005$. Also, the

error variance was actually lower on the first day (3.02) than for both days combined (5.34). Therefore it was concluded that training for two days gave no additional information beyond that obtained on the first day. The remaining subjects were trained for only one session (40 trials), and all data analyses were performed using the data from a single day. This reduction in training had the advantages of being more efficient, subjecting the fish to much less stress, and possibly reducing error variance.

Three to six fish were trained per day. The experimenter was blind to the group membership of the fish at the time of training. A total of eleven fish from each of the three conditions was trained.

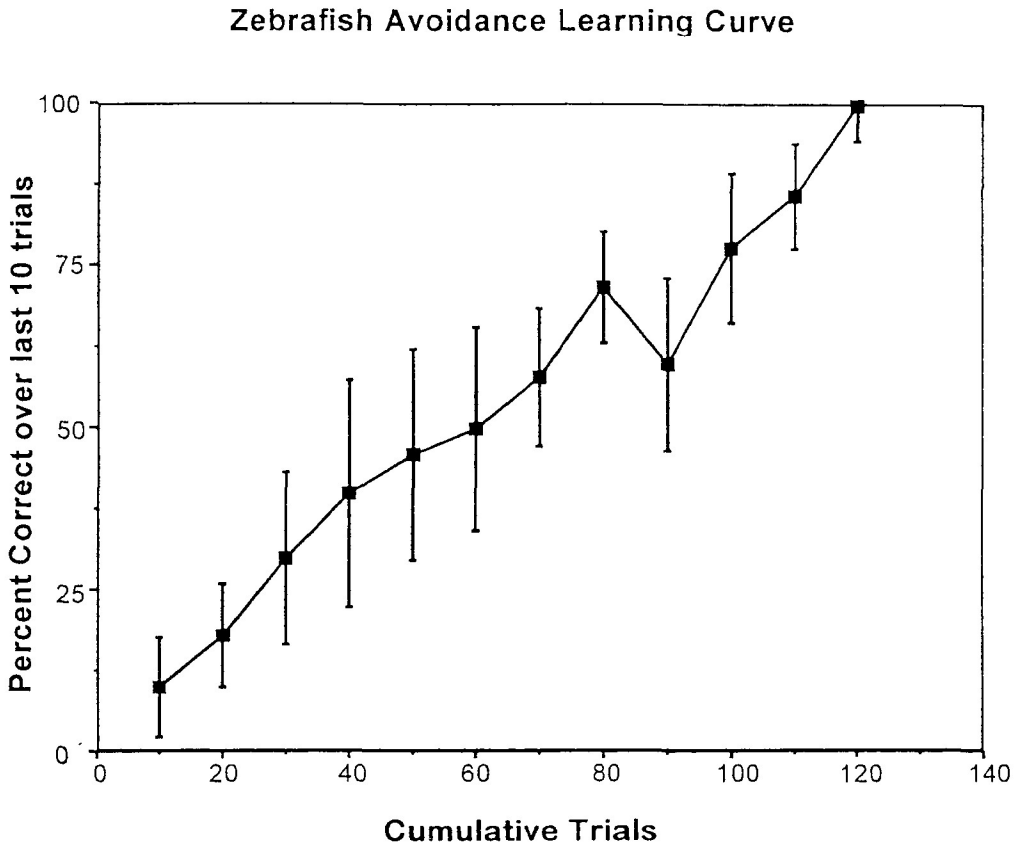

Fig. 1. Learning curve for five zebrafish trained using the avoidance learning procedure. All subjects reached 100% correct responding within 120 trials. Percent correct responses was calculated every 10th trial for the preceding block of 10 trials. Error bars indicate ±1 S.E.M.

Chapter 3

Results

General Characteristics

Eggs in both the nicotine groups were not distinguishable in appearance from those in the control group, and all the eggs in all three groups hatched at approximately the same time (between about 60 and 80 hpf). For 24 hours after hatching, many of the larvae in the two nicotine conditions appeared noticeably bent at the middle. All but three of the 33 larvae in the 10 μg/ml condition displayed this deformity, and at least 10 were so bent that they swam only in tight circles. Fewer of the larvae in the 2.5 μg/ml condition displayed this deformity, though about half of the 36 did appear bent. By the second day after hatching, the larvae in both treatment conditions had straightened out and were indistinguishable from the larvae in the control condition. After this point, the larvae in the nicotine conditions appeared to develop and behave normally. The only casually observable difference among the groups was that of size, the controls being noticeably larger than the fish in the nicotine conditions.

Data Analysis

Analysis of Variance (ANOVA) is the most common data analysis procedure used in experimental designs with discrete, fixed levels of the independent variable (IV); thus one-factor between-subjects ANOVAs were used

in this study to detect differences among the means of the groups on all dependent variables. However, the IV in this study was dosage of nicotine, and although the levels of the IV are discrete, dosage is a continuous variable. Therefore, regression analysis is a more sensitive method for estimating the relationships between EN exposure and the dependent measures being investigated. Pedhazer (1997) recommends regression analysis as superior to ANOVA in all designs with continuous IVs. Thus, regression analysis was used in this study to estimate the relationship between EN exposure and the dependent measures. Because the levels of the IV in this study were fixed, the regression analysis is not intended to be used to predict the values of the dependent variables at levels of the IV other than those used in this study (0, 2.5, & 10 μg/ml).

Experiment-wise alpha level was maintained at .05 by using Bonferroni's correction to adjust the alpha levels for the tests of the three hypotheses. Thus, an alpha level of .017 was required for significance for the tests of body length, activity level (swimming speed) and avoidance learning (number of correct responses). An alpha level of .05 was used for other analyses.

Mortality. A chi-square goodness of fit test was performed on the mortality (percent survival) for each group. Bilotta et al. (2000) found that number of hatched zebrafish larvae and number of larvae surviving at 7 dpf were not significantly different. Therefore, number of hatched larvae at 3 dpf was used as the expected value for each group, and number of surviving larvae at 7 dpf was

used as the observed value. No significant deviation from expected values was found, $\chi^2(2, N = 176) = .481$, $p = .786$.

Body length. Body length in millimeters for the three groups was analyzed with an ANOVA. The result was significant at the alpha level of .017 required by the Bonferroni correction, $F(2, 51) = 11.210$, $p < .001$. A Scheffe's post hoc test revealed significant differences between the control group and the two nicotine conditions ($p < .05$), but did not show a difference between the two nicotine conditions. To estimate the relationship between EN and body length, body length was regressed on dose of nicotine bitartrate in micrograms per milliliter. A correlation of .574 (adjusted $R^2 = .261$) was found between dose and body length, which was significant, $F(1, 52) = 19.702$, $p < .001$. Figure 2 shows the means and standard errors for 54 fish. The regression line is included. Body length was again measured at the time of activity level measurements. A regression of body length on nicotine dose found a correlation of .358, $F(1, 34) = 4.991$, $p = .032$, showing that the effect of dose on body length found at 10 dpf had persisted until 45 dpf.

Activity level. Because of the difference in body length observed among the groups, activity level measurements were made by expressing mean swimming speed in body lengths, rather than in absolute distances. An ANOVA did not reveal a significant difference at the Bonferroni correction level in activity level across the three groups, though there was a trend toward significance, $F(2, 33) = 3.339$, $p = .048$. However, when activity level was regressed on nicotine dose, a correlation of .406 (adjusted $R^2 = 165$) was found between

nicotine dose and swimming speed; this correlation was significant at the corrected alpha level, $F(1, 34) = 6.712$, $p = .014$. Figure 3 shows the means and standard errors for 36 fish. The regression line is also shown. Note that predicted values are quite close to the group means.

Avoidance learning. Mean number of avoidance responses obtained during avoidance training (from 55 dpf to 75 dpf) was compared across groups using an ANOVA. No significant differences were found, $F(2, 30) = .616$, $p = .547$ Number of avoidance responses was regressed on nicotine dose. No significant relationship was found, $F(1, 31) = .780$, $p = .381$. Figure 4 shows the data obtained for 33 subjects. The means are suggestive of a negative correlation between nicotine dose and avoidance learning. However, the error variance is much too large for the relationship to be called significant.

Mean number of premature reentries obtained during avoidance training was also investigated. This measure could potentially lend support to the claim that zebrafish exposed to EN are more hyperactive. However, an ANOVA did not reveal a significant difference among groups, $F(2, 30) = 1.111$, $p = .342$. Also, when number of reentries was regressed on nicotine dose, no significant relationship was found, $F(1, 31) = .165$, $p = .687$.

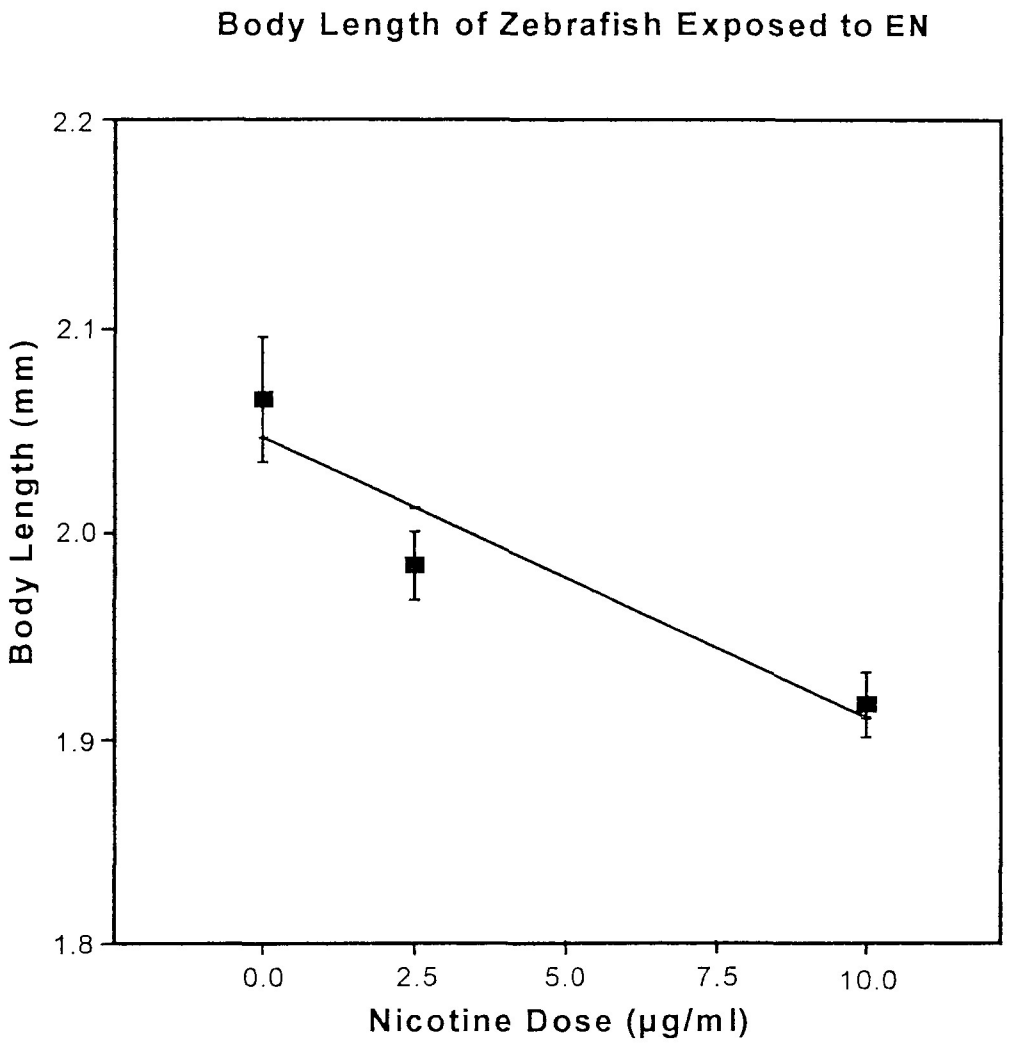

Fig. 2. Mean length in millimeters at 10 dpf (N = 54). The line represents the regression of body length on dose ($\underline{Y}' = 2.0 - .01\underline{X}$). Error bars indicate ±1 S.E.M.

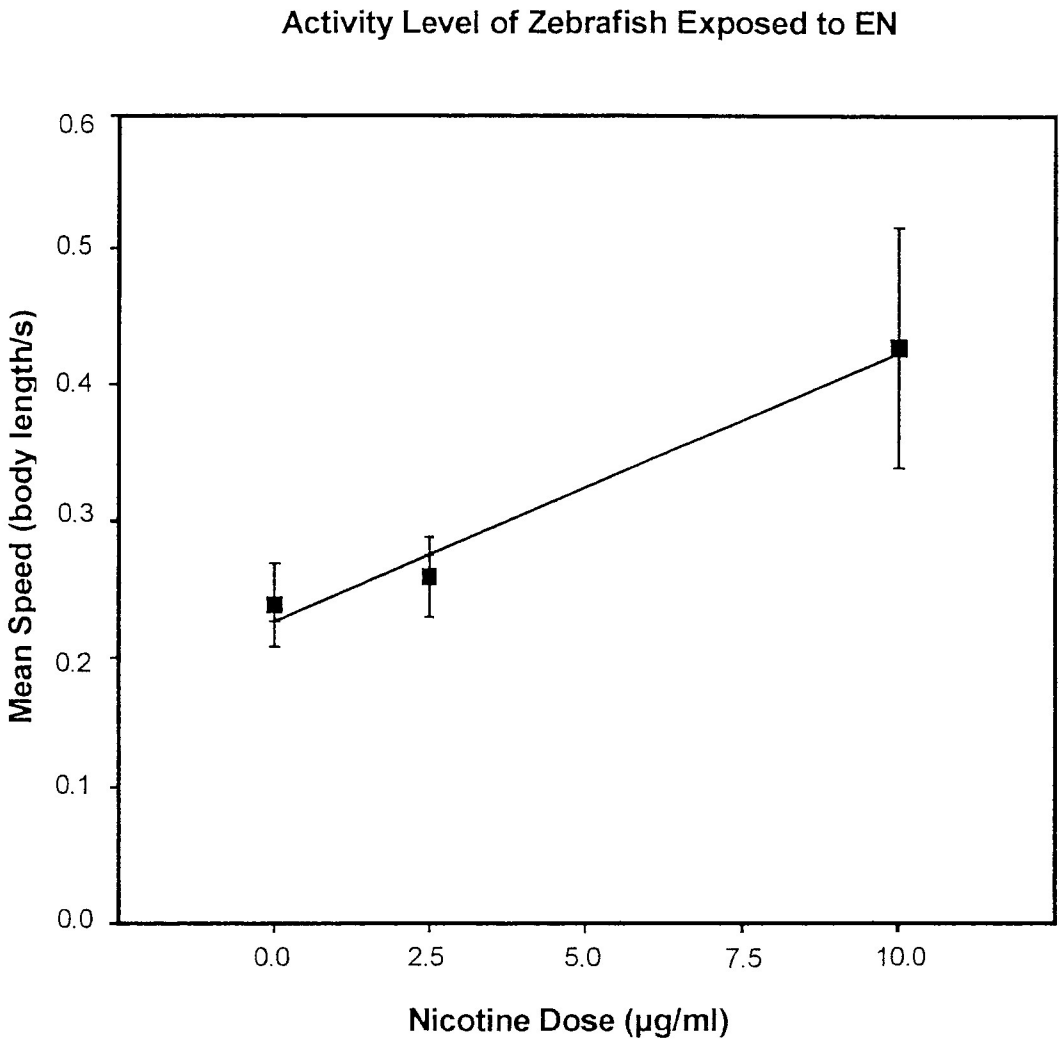

Fig. 3. Activity level operationalized as mean swimming speed per second in fish body lengths (N = 36). The line represents the regression of speed on dose ($\underline{Y}' = .23 - .02\underline{X}$). Error bars indicate ±1 S.E.M.

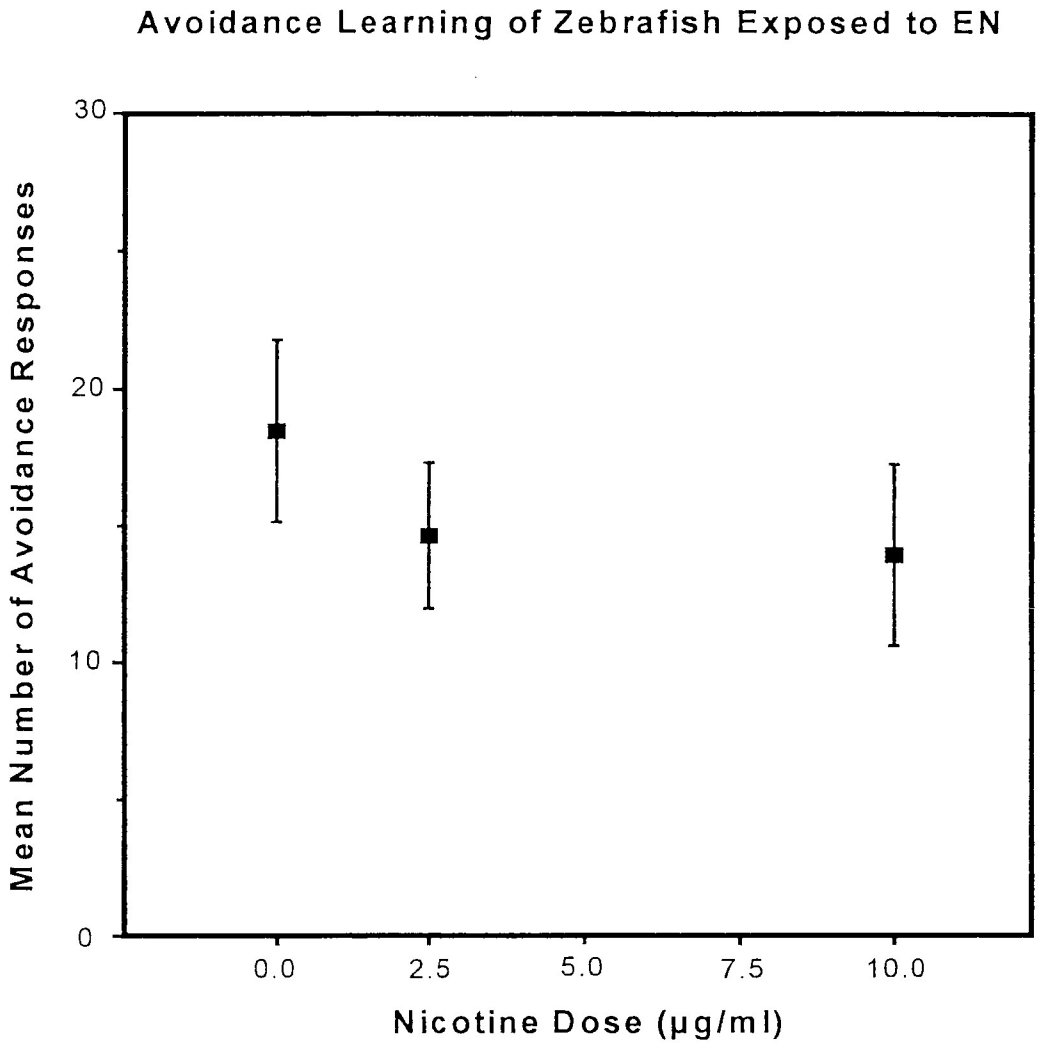

Fig. 4. Mean number of avoidance responses acquired during 40 trials of training (N = 33). Notice that the means are consistent with the hypothesized effect of EN exposure on avoidance learning, but the variance is quite large. Error bars indicate ±1 S.E.M.

Chapter 4

Discussion

The results of the length analyses at 10 and 45 dpf support the hypothesis that EN exposure reduces growth in zebrafish. As discussed above, many researchers have found a relationship between EN exposure and decreased birth weight in humans (Brooke et al., 1989; Picone et al., 1982). Researchers have also found an effect of EN exposure on birth weight and growth in rats (e.g., Martin et al., 1976; Paulson et al., 1993). Thus, the finding that EN exposure decreases body length in zebrafish is consistent with findings in other species.

The regression analyses of the activity level data support the hypothesis that EN exposure causes increased activity levels in zebrafish. As discussed above, EN exposure has been found to be associated with ADHD in humans (Milberger et al., 1996). Also, researchers have found that EN exposure causes increased activity levels in rats (e.g., Fung, 1988). The finding that EN exposure increases activity levels in zebrafish supports the idea that EN exposure is a cause of hyperactivity in animals.

Many researchers have believed that nicotine affects birth weight of humans and rats by altering the function of the placenta. Nicotine is a vasoconstrictor, and it is known to restrict oxygen and blood flow to the developing embryo (U.S. Department of Health, Education and Welfare, 1979).

However, in this study it has been demonstrated that EN exposure also reduces growth in non-mammals. At 10 dpf, nicotine dose accounted for more than one quarter of the variance in body length of the subjects in this study. This finding is important because it shows that nicotine may have a direct effect on the development of embryos. The finding that EN exposure also affects activity levels in zebrafish further supports the idea that the effects of EN exposure on animals cannot be fully explained by reference to EN exposure effects on the placenta. Thus, EN exposure in mammals may affect development through two routes: by affecting the function of the placenta as well as by a more direct route the mechanism of which is not currently known.

The hypothesis that EN exposure reduces avoidance learning in zebrafish was not supported by these data. While the observed mean number of correct avoidance responses was higher in the control group than in the treatment groups, the error variance was too large for the difference to be significant. This hypothesis was the least supported by previous research. As discussed above, researchers have found effects of EN exposure on learning performance, but the findings are inconsistent. For example, some researchers have found EN exposure to affect performance of rats on radial arm mazes (e.g., Sorenson et al., 1991), while others have not (e.g., Levin et al., 1993). In particular, previous findings on the effect of EN exposure on avoidance learning in animals have been mixed, with some researchers even reporting an improvement in performance (e.g., Bertolini et al., 1982). The present study was unable to clarify the issue of the effect of EN exposure on avoidance learning.

There are a number of possible reasons why an effect of EN exposure on avoidance learning may have been obscured if it existed in this sample. First, there is a great deal of variability among normal zebrafish in performance on the task used in this study. Thus, any effect may simply have been too small to be revealed by these methods. Second, previous work in this lab has shown a clear relationship between fish size and reactivity to the aversive stimulus: smaller fish must be given a larger shock in order to provoke an equivalent reaction. Two-way avoidance tasks require an optimal level of the aversive stimulus. Too low a stimulus may not motivate avoidance behaviors. On the other hand, too much shock may cause the animal to be reluctant to escape because it must flee into the side in which it had been shocked during the previous trial. There was a significant relationship between nicotine dose and body length in this sample. Thus nicotine dose may have affected performance on the avoidance task indirectly through its effect on body length. The direction of this effect would have been difficult to predict; it may have obscured any direct effects of dose on avoidance responding. Third, the EN exposure in this sample was brief and early (during the first eight hpf). Previous work in this lab has demonstrated that zebrafish improve dramatically at this task as they mature. The fish in this study were trained between 55 and 75 days after they were exposed to nicotine. It is possible that the fish outgrew any effects of the EN exposure by the time they were tested. This idea is supported by the fact that while the observed mean number of avoidance responses was higher in the control group, the control group performance was not better in the oldest subjects trained. Finally, the

range of ages of the subjects during training may by itself have introduced enough variance to obscure a small effect, because, as mentioned, zebrafish performance on this task varies with age.

A measure of avoidance learning might yet be used to investigate the effects of EN exposure on zebrafish. The procedure used in this study could be improved if fish were trained somewhat earlier, such as at 45 dpf, and if all subjects were trained at close to the same age. Thus, if more than one experimenter trained fish, a sample size of 20 or more per group could be obtained between 45 and 55 dpf. Also, a measure of length should be obtained at the time of training. Thus the effects of length could be partialed out of the correlation between dose and avoidance responding so that the error term would be reduced. Age at training could also be partialed out. The observed difference between the control group and treatment group means in this sample suggests that additional research with an improved procedure is worthwhile.

This study was exploratory in nature. Research on the effects of EN exposure on zebrafish (or other non-mammals) has not been published to date. At the outset it was unclear what dosages should be used. The regression analyses in this study suggest a linear trend in the effects of dose on body length and activity. To verify this finding, higher doses should be used in future research. Also, the timing of exposure should be varied. The timing of the exposure in this study was intended to be analogous to the early exposure a human embryo might receive when the mother quits smoking upon learning that she is pregnant. However, many women still smoke throughout pregnancy,

including during critical brain development periods. The effects of EN exposure on zebrafish for longer periods should be investigated. For instance, exposure during periods of more active brain development (e.g., during the second dpf) may reveal much more dramatic effects on behavior than were found in this study.

Overall, this thesis project has been successful in that it has shown that zebrafish respond to EN exposure in a manner similar to that of other animal models. It has also shown that the effects of EN exposure on animals are not entirely due to nicotine's effects on the placenta. This finding has not been demonstrated previously. The zebrafish has much potential as a model of the effects of exposure to EN and other teratogens.

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