The Dissertation Committee for Maria Del Carmen Alvarez Certifies that this is the approved version of the following dissertation:

SIGNIFICANCE OF ENVIRONMENTALLY REALISTIC LEVELS OF SELECTED CONTAMINANTS TO ECOLOGICAL PERFORMANCE OF FISH LARVAE: EFFECTS OF ATRAZINE, MALATHION, AND METHYLMERCURY.

Committee:

Lee A. Fuiman, Supervisor

G. Joan Holt

B. Scott Nunez

Peter Thomas

David P. Crews

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by

Maria Del Carmen Alvarez, B.S. M.S.

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Dedication

To my husband, Rafael Perez, to whom I owe everything, who has been with me through the “good and the bad”. To my son, Diego T. Perez, and my daughter, Cecilia A. Perez, who have always brightened my day with laughter and love. And to my parents, Alvaro Alvarez and Marie Cecilie d’Otreppe de Bouvette, who, without ever a doubt, have supported me on every decision I have made. Thank you.

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Maria Del Carmen Alvarez, Ph.D.

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Supervisor: Lee A. Fuiman

This study uses a comprehensive approach to assess contaminants and modes of exposure effects on individuals and populations of two species of marine fish, specifically on the ecological performance (growth, behavior, survival potential, and resting respiration rate) of their larvae. Red drum (*Sciaenops ocellatus*) larvae at settlement size (7 mm total length) were given an acute exposure to atrazine (0, 40, and 80 μg l^{-1}) or malathion (0, 1, and 10 μg l^{-1}) in water for 4 days to evaluate the effects on ecologically critical traits. Atrazine significantly reduced growth rate and altered routine behavior (swimming speed, net-to-gross displacement ratio, and activity). Atrazine did not affect escape performance or resting respiration rate. Behavioral effects resulted in higher predicted prey encounter rates, but substantially elevated rates of energy utilization, which together suggest an increased risk of starvation. Atrazine effects on

growth would prolong the larval period, which could reduce the juvenile population by up to 24 %. Malathion exposure at ecologically relevant concentrations did not impair any of the traits tested, suggesting that these levels may be safe for young fishes. However, recent increase in malathion use may elevate environmental levels above those tested here.

In a different experimental approach larvae produced by adult Atlantic croaker (*Micropogonias undulatus*) fed a methylmercury-contaminated diet (0, 0.05, and 0.1 mg kg^{-1} d^{-1}) for one month were screened for effects on routine and escape behaviors. Four developmental stages were studied: (1) end of yolk absorption (yolk), (2) end of oil absorption (oil), (3) 4 days and (4) 11 days after oil absorption (oil+4 and oil+11). MeHg levels in the eggs (0.04 to 4.6 ng g^{-1}) induced a range of stage- and concentration-dependent effects that were more frequent during yolk absorption, suggesting physiological, rather than developmental, effects. Computer simulations applied to predict the ecological relevance of the observed behavioral effects suggested that methylmercury-exposed larvae would have lower survival during the planktonic stage (< 12 mm) compared to unexposed larvae ($\leq$ 96 % reduction). It is demonstrated here that environmentally realistic pollution may substantially reduce fish larvae survival and compromise recruitment to juvenile populations.

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CHAPTER 1: General Introduction

Chemical use has been known to mankind for centuries. At first, man used chemicals extracted from natural sources: mineral, vegetal, or animal. However, the industrial revolution allowed technology to be used in chemistry, thereby increasing knowledge of chemical properties and allowing the synthesis of new compounds. Since Antoine L. Lavoisier (1743-1794) revolutionized the world with his discoveries (importance of oxygen in combustion and the three stages of matter), mankind came to understand the high potential of these chemicals and they have become extensively used. Our society today depends heavily upon chemicals. We use synthetic chemicals to produce, preserve, and cook our food; to make our commodities (cars, appliances, clothing, furniture); to cure diseases; and many other things. This exponential increase in chemical use has led to pollution and, with it, many environmental problems. In the 20th century we became conscious of the health hazards of pollution, both acute and chronic. Industrial and agricultural pollution have been blamed for direct mortality and long-term deleterious effects on organisms including humans.

Many pollutants alter the growth, survival, and reproduction of organisms, which can jeopardize natural populations. Some of the pollutants are endocrine-disrupting chemicals (EDCs), which affect hormone production and/or action. Other chemicals are neurotoxic, affecting neurotransmitter secretion and/or metabolism. Endocrine and neural systems control many physiological and developmental processes in animals and any disturbance to these systems during development could have profound and permanent effects on the organism (reviewed by Colborne et al., 1993; Guillette et al., 1995; Ottinger et al., 2001).

Most species of marine fish larvae have an extremely high natural mortality rate, which is mainly attributable to predation and starvation. Very small changes in larval mortality rates can lead to enormous changes in recruitment (Houde, 2002). Fluctuations in the size of adult fish populations are often the result of variations in recruitment success (Cushing, 1975). Mortality rates diminish as larvae grow and improve their swimming abilities and sensory capabilities, allowing them to avoid predators and find food more effectively. Fish larvae, as free-living and fast-developing organisms, will be exposed to pollutants in the environment. When those pollutants impair sensory or locomotor performance mortality of larvae could increase, thereby having potentially serious consequences for the future population.

Pesticides used to control nuisance animals and unwanted weeds in agricultural and domestic environments enter aquatic nursery habitats through precipitation runoff and groundwater and can either kill fish larvae directly or impair the skills larvae need to avoid death by predation or starvation. Pesticides help mankind maintain sufficient food production to feed the world's growing population. The U.S. Environmental Protection Agency estimates that worldwide expenditures on pesticides between the years 2000 and 2001 totaled about $32 billion with more than $11 billion in the United States (Kiely et al., 2004). In the U.S., 1.2 billion pounds of pesticides were used between 2000 and 2001, of which about 0.55 and 0.12 billion pounds were herbicides and insecticides respectively. The herbicide atrazine and the insecticide malathion were among the ten most used pesticides in the U.S. (Kiely et al., 2004). The Texas Boll Weevil Eradication Program (U.S. Department of Agriculture) accounts for most of the national use of

malathion. Both atrazine and malathion have potential to reach surface waters due to their relatively low sorption coefficients and high solubility in water.

Another class of contaminants in aquatic habitats are heavy metals, which are produced by natural and anthropogenic action. Industrial emissions of metals, such as mercury, have greatly increased total loadings in the environment. Metals from the Earth's crust and from atmospheric deposition reach aquatic environments in an elemental or slightly modified form. In the case of mercury, bacterial action transforms elemental mercury to methylmercury (MeHg) in sediments. Methylmercury is a form more readily absorbed by organisms. Once in the body it can be transferred to the growing oocyte by the formation of liposoluble complexes (mainly with amino acids). Since MeHg bioaccumulates and biomagnifies through the food chain, and levels of MeHg in water are relatively low, exposure of fish larvae to MeHg is most likely through their diet or through maternal transfer (Downs et al, 1998). Of these two routes of exposure, the dietary source probably poses only a small risk to fish larvae because they consume zooplankton, which have little opportunity to biomagnify MeHg. Maternal transfer, however, can result in high exposure for larvae because of the adult fish's higher trophic level and potential body burden.

The fish family Sciaenidae includes commercially and ecologically important species. Atlantic croaker (*Micropogonias undulatus*) and red drum (*Sciaenops ocellatus*) represent two of the most important sciaenid species in recreational fishing in the Gulf of Mexico. Landings in 2003 for Atlantic croaker and red drum were 9.7 and 14.7 million pounds, respectively (National Marine Fisheries Service, 2004). Although adults of red drum and Atlantic croaker have different spawning grounds (coastal water and offshore,

respectively), early larval stages of both species develop in open waters and reach the estuarine waters as larvae. Red drum larvae arrive at the seagrass beds at about 6-8 mm SL while Atlantic croaker larvae arrive at approximately 10-14 mm SL (Rooker et al., 1998).

Aquatic toxicology, the study of contaminants and its effects on aquatic organisms, has dedicated considerable attention to fishes over the past three decades. Many books have reviewed different aspects of fish survival, physiology, development, biochemistry and behavior (for example: Jones, 1964; Sorensen, 1991; Heath, 1995; Lawrence and Hemingway, 2003). Bioassays to establish lethal levels have been traditionally conducted using a variety of organisms including fish larvae and embryos. Typically, 96-h LC_{50} (lethal concentrations for 50 % of the experimental organisms after 96 h exposure) are used as reference to define acute exposure and toxicity levels of a contaminant. This has required international agencies such as the Organization for Economic Co-operation and Development (OECD) to create standard protocols for testing chemicals so that results are comparable and risks of pollution can be assessed (e.g., Solomon et al., 1996).

Although tests of acute exposure are mostly used for regulatory purposes, chronic or sublethal exposure tests are being considered more frequently because increased mortality can also occur when ecological performance is reduced, leading to death by predation, starvation or disease. Since developing embryo and larvae are the most sensitive stages in the teleost life cycle (von Westerhagen, 1988) and changes affecting their survival could have serious implications for the population, it has become imperative to understand sublethal effects of pollutants on the early life stages of fishes.

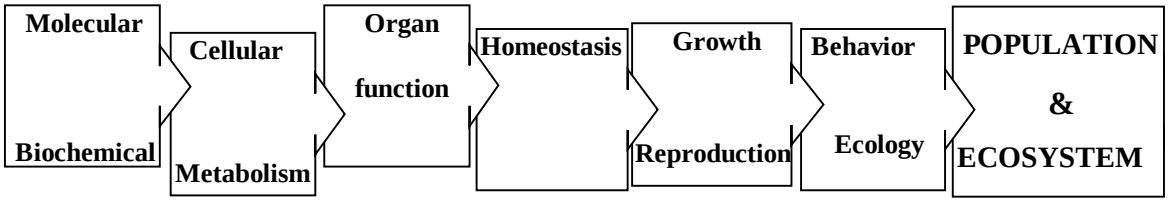
During these early stages, fishes can be exposed to pollutants through parental transfer, water-borne exposure and through diet. Parental transfer occurs when a contaminated female mobilizes contaminants during oogenesis and deposits them in the egg (Niimi, 1983). Therefore, the embryos and larvae are exposed via the yolk and/or oil globule. Malformations are the most evident effects of this kind of exposures (reviewed by Weis and Weis, 1989); however, more subtle effects that could hamper larval survival also occur. For example, Atlantic croaker fed DDT-contaminated food produced larvae with lower average and maximum response speeds to a visual predatory stimulus and had impaired routine swimming activity until oil globule absorption (Faulk et al., 1997). Also Atlantic croaker fed a diet contaminated with the polychlorinated biphenyl Aroclor 1254 produced larvae with delayed growth and impaired escape behavior (McCarthy et al., 2003).

Embryos and larvae can also receive aqueous exposures, since eggs can be deposited over contaminated sediments or in contaminated waters. Experiments have measured a variety of effects of this type of exposures. Göerge and Nagel (1990) exposed fertilized eggs of zebrafish (*Danio rerio*) to several concentrations of lindane, deltamethrin, and atrazine for 35 d and monitored hatching, mortality, and abnormal development. Survival was reduced with increasing contaminant concentration. Also, hatching rate was reduced by deltamethrin exposure (0.8 $\mu g\ l^{-1}$), growth was reduced with lindane exposure (40 $\mu g\ l^{-1}$), and the number of deformations and edema increased with atrazine exposure (1300 $\mu g\ l^{-1}$). Zhou and collegues (1996, 1998, 1999) have studied extensively the effects of aqueous methylmercury (MeHg) exposure on mummichog (*Fundulus heteroclitus*), where exposed embryos and larvae exhibited changes in activity

level, impaired swimming performance, reduced prey capture ability, and alterations in neurotrasmitter levels. Beauvais et al. (2000) found that rainbow trout (*Oncorhynchus mykiss*) larvae exposed to water-borne diazinon or malathion exhibited changes in physiological parameters that correlated with changes in behavior. Both chemicals induced significant decrease in cholinesterase (ChE) activity, which was correlated with changes in swimming speed and distance for both chemicals, and with turning rate for malathion.

Studies of the effects of exposure to pollution through contaminated food on early fish stages are more limited and centered on juvenile stages. Growth, feeding and survival of pink salmon (*Onchorhyncus gorbuscha*) fry were reduced after exposure to oil-contaminated food (Carls et al., 1996). Rockfish (*Sebastes schlegeli*) given food contaminated with copper exhibited reduced growth and increased concentration of some serum enzymes, suggesting liver damage (Kim and Kang, 2004). Bioavailability of petroleum hydrocarbons (after dispersing agents were applied) to larval topsmelt (*Atherinops affinis*) was studied by Wolfe et al. (2001) who showed that dispersants altered uptake and depuration processes. Finally, Hamilton et al. (2002) found that razorback sucker larvae (*Xyrauchen texanus*) fed zooplankton contaminated with selenium reduced larval survival at lower concentrations than water-borne exposure.

Endpoints for assessing sublethal effects of pollutants on any organism can be obtained at almost any level of biological organization: molecular, cellular, tissue organ, etc. (Heath, 1995).



The higher the level affected by a pollutant the more generalized the response and the implications for the general health of the organism, population, and ecosystem. Many studies in aquatic toxicology focus on single endpoints at one level (Heath, 1995; Weis and Weis, 1989; Scott and Sloman, 2004). Moreover, most studies use contaminant levels that do not reflect environmentally realistic concentrations for the organism under study. Little has been done to fully comprehend effects of contaminants at environmentally realistic levels of early life stages of marine fishes. Here, I aim to understand the subtle, sublethal effects of pollution on the ecological performance of sciaenid larvae by concentrating on endpoints that, if impaired, have the potential to affect population numbers and thus ecosystem functioning. The primary objective of this study is to determine the effects on multiple endpoints (survival, growth, metabolism, and behavior) of actual contaminants to early larval stages of sciaenids. This study has two distinct goals: 1) to understand sublethal effects of water-borne exposure of currently used pesticides (atrazine and malahtion) on red drum larvae; and (2) to understand the effects of maternal transfer of MeHg to Atlantic croaker larvae.

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CHAPTER 2: Environmental levels of atrazine and its degradation products impair survival skills and growth of red drum larvae

ABSTRACT

Red drum larvae (*Sciaenops ocellatus*) were exposed to environmentally realistic and sublethal levels of the herbicide atrazine to evaluate its effects on ecologically critical traits: growth, behavior, survival potential, and resting respiration rate. Settlement size larvae (7 mm total length) were given an acute exposure of atrazine at 0, 40, and 80 μg l^{-1} for 4 days. Tests of 96-h survival confirmed that these naturally occurring concentrations were sublethal for red drum larvae. Growth, routine swimming, antipredator responses to artificial and actual predators, and resting respiration rate were monitored 1 and 3 days after onset of exposure. Atrazine exposure significantly reduced growth rate. Atrazine exposed larvae also exhibited significantly higher routine swimming speeds, swam in more convoluted paths, and were hyperactive. Responses to artificial and actual predators were not affected by atrazine exposure nor were resting respiration rates. The higher rate of travel (86% higher in atrazine treated larvae) resulted in higher predicted encounter rates with prey (up to 71%) and slow moving predators (up to 63%). However, hyperactivity and faster active swimming speeds of exposed larvae indicated that naturally occurring sublethal levels of atrazine will result in an elevated rate of energy utilization (doubling the total metabolic rate), which is likely to increase the risk of death by starvation. Finally, atrazine effects on growth will prolong the larval period, which could reduce the juvenile population by as much as 24 %.

INTRODUCTION

Atrazine (2-chloro-4-(ethylamino)-6-(isopropylamino)-s-triazine) is a widely used herbicide in the United States. It was first registered in 1958 and the U.S. Environmental Protection Agency has estimated that between 74 and 80 million pounds of atrazine were used in 2001 (Kiely et al., 2002). Due to its high use and its relatively high mobility in soils, atrazine is frequently detected in surface and ground waters. Atrazine levels in runoff can reach very high levels in the first rain events after application (Southwick et al., 2003). Thurman et al. (1992) showed that atrazine levels in storm runoff could reach levels of 40 $\mu g\ l^{-1}$. Reported levels in South Texas coastal waters have reached 65 $\mu g\ l^{-1}$ (Pennington et al., 2001).

Commercially and ecologically important fish species such as red drum (*Sciaenops ocellatus*) can be affected by contaminated runoffs entering estuaries. Red drum spawn in coastal areas and their larvae reach estuarine areas at a relatively undeveloped stage. Contaminants in the environment can impair growth and development of larvae and ultimately lead to mortality (e.g., Weis and Weis, 1976, 1995; Faulk et al., 1997; Zhou and Weis, 1998; Beg et al., 2001; McCarthy et al., 2003). Although atrazine was developed to inhibit photosynthesis in plants, it has multiple effects on animals. For example, atrazine is a classified endocrine disrupting chemical (EDC) that affects steroidogenesis in alligators and frogs (Crain et al., 1997; Hayes et al., 2002; Goulet and Hontela, 2003) and olfactory-mediated endocrine function in salmon parr (Moore and Lower, 2001). Atrazine exposure has been shown to produce altered behavior. Saglio and

Trijasse (1998) demonstrated that atrazine exposure induced anomalous behavior in goldfish (*Carassius auratus*).

Organisms interact with their environment, and in particular find food and escape from predators, through their behavior. Behavior and growth are both fueled by physiological processes. Deleterious effects on physiology may be reflected in impaired behavior and/or growth. This study assesses the effects of environmentally realistic levels of atrazine on red drum larvae at the size they enter contaminated nursery areas by evaluating ecologically important behaviors, growth and the energetic cost of exposure.

MATERIALS AND METHODS

Experimental animals

Red drum eggs were obtained from three sources: University of Texas Fisheries and Mariculture Laboratory (Port Aransas, Texas), Texas Parks and Wildlife Department CCA/CPL Marine Development Center (Corpus Christi, Texas), and Perry R. Bass Marine Fisheries Research Station (Palacios, Texas). Broodstocks were induced to spawn by manipulating ambient temperature and photoperiod. Eggs were collected within 12 h of spawning and hatched in conical tanks in 20 l of sea water. From day 5 after hatching, the volume was gradually increased to 100 l over approximately 5 d. Larvae were reared with flow-through circulation until experiments were done. Temperature and salinity in the rearing tanks were maintained at about 27 °C and 27 PSU. Larvae were fed 10 rotifers (*Brachionus plicatilis*) ml^{-1} d^{-1} from day 1 until about 10 d posthatching when their diet was gradually shifted to *Artemia* nauplii. At the time of the chemical exposure fish were completely weaned from rotifers and onto nauplii. *Artemia* were enriched overnight with

Algamac 2000 (Aquafauna Bio-Marine, Inc.) and added to rearing tanks in the morning so that they reached a concentration of 5 nauplii ml^{-1}. Fish were fed at approximately 0830 h daily and allowed to feed for about 1 h before moving them to the experimental chambers.

Since the highest levels of atrazine in surface waters are found in estuarine areas, exposures were done on larvae at the size of settlement to estuarine seagrass beds, about 7-8 mm total length (TL) (Rooker and Holt, 1997; Herzka et al., 2002). Red drum larvae reached settlement size within 15-20 days under rearing conditions.

Exposures

Atrazine (with a guaranteed purity of 98%) was purchased from Chem Service, Inc. (West Chester, Pennsylvania). Atrazine (approximately 24 mg) was dissolved in 3 ml of acetone and added with gentle stirring to the trial tanks to the desired concentration. The amount of acetone added to the highest dose group was also added to the control group.

Water samples were taken 5 min and 96 h after the addition of atrazine and sent for analysis to an independent laboratory (Department of Soil and Crop Sciences, Pesticide Fate Research Laboratory, Texas A&M University, College Station, Texas) to characterize the exposures. Concentrations of atrazine and several degradation products (diamino-, deisopropyl-, hydroxy-, and desethyl-atrazine) were measured using high-performance liquid chromatography (HPLC), as described in Senseman et al. (1997).

Survival experiments were performed to evaluate whether environmentally realistic doses were within the sublethal range for settlement size red drum. Groups of 50

settlement size larvae were transferred to 1.5-l exposure watch bowls (20 cm diameter) in a temperature-controlled room. Salinity and temperature were maintained at 27°C and 27 PSU. Atrazine dissolved in acetone or acetone alone was added to the watch bowls 24 h after transfer. Doses tested were 0, 40, 80, and 500 μg l^{-1}. Fish were fed a ration of 5 nauplii ml^{-1} d^{-1}. Survival was recorded 96 h after the herbicide was added to the water. Six replicates for 50 and 500 μg l^{-1} exposures, and 12 for control and 100 μg l^{-1} exposures were performed with larvae from five different spawns. The proportion of surviving larvae from the atrazine-exposed groups was compared to the survival rate of control groups.

Settlement-size red drum larvae (7-8 mm TL) were transferred to six exposure tanks on experimental day -1 at a density of 10 larvae l^{-1} and allowed to recover from handling for 1 d. The six tanks were randomly assigned to the three treatment groups: control, low, and high dose (0, 40, and 80 μg of atrazine l^{-1}) in duplicate. Atrazine was added (experimental day 0) as described above. The 50-l fiberglass tanks were arranged in a water bath to minimize variations in temperatures among tanks. Mean (± S.D.) water temperature and salinity were 28.4 ± 0.67 °C and 27.1 ± 0.14 PSU.

Growth

Ten spawns were used for analysis of growth rates. Growth rates were calculated over a 9-d period. Groups of 20 larvae were sampled from each exposure tank. Samples were taken on the day of transfer (experimental day -1) and on days 1, 3, 6, 7, and 8 after atrazine was added to the water. Fish were anesthetized using tricaine methane sulfonate (MS 222) and digital pictures were taken with the aid of a camera (Sony DCR-TRV350)

attached to a dissecting microscope. Total length (mm) was measured using an image-processing program (ImageJ, National Institutes of Health). Ten replicates from 10 different spawns were done.

Because larval growth rate is size dependent, growth rate was calculated using an exponential growth model to compensate for differences in initial size among trials.

$$TL_t = TL_{-1} \cdot e^{G \cdot t}$$

$$G = (LnTL_t - LnTL_{-1}) / (t+1)$$

where TL_t is total length (mm) on experimental day t, TL_{-1} is total length on experimental day -1 and G is the instantaneous growth rate (d^{-1}).

Behavioral experiments

Two behavioral assays, routine swimming and visual startle response, were conducted on larvae from four spawns (replicates) to assess the effects of atrazine exposure. Routine swimming behavior measured foraging capacity, and the visual startle response measured the ability of larvae to escape from a predatory attack.

Groups of ten larvae were randomly selected from each exposure tank and carefully placed into glass chambers (75 x 70 x 20 mm) containing approximately 50 ml of filtered sea water from their exposure tank. The chambers were placed in a temperature-controlled room and the fish were left to recover from handling for 2-3 h (Fuiman and Ottey, 1993). After this time the chamber was carefully placed above a video camera (Cohu, model 3315-2000/0000) and left undisturbed for 5 min to allow the larvae to acclimate. Routine behavior of the undisturbed larvae was then video-recorded (Panasonic AG-1960) from a remote station for 3 min. After this time, the larvae were

given an artificial predatory visual stimulus and their responses were recorded. The stimulus was a black oval (1 x 1.5 cm) on a white card (7.6 x 12.7 cm) simulating the cross section of a predatory fish approaching. The card was attached at the end of a remotely controlled pendulum held by an electromagnet. By turning off power to the electromagnet the pendulum was released and accelerated towards the chamber containing the larvae and was stopped by a thread attached to the wall just before hitting the chamber (for details see Fuiman and Cowan, 2003). The recorded video was digitalized as AVI files and movements of the larvae were analyzed with the aid of a computerized tracking system (WinAnalyze 2D Software, Version 1.5, Mikromak, Germany).

Analysis of the routine behavior clips was done frame by frame and the paths described by the larvae were tracked throughout 25 s. The behavior was measured for all 10 larvae in the chamber and expressed by four variables: rate of travel (mm s^{-1}), active swimming speed (mm s^{-1}), activity (% time), and net-to-gross displacement ratio (NGDR, dimensionless). Red drum larvae swim in alternating episodes of active swimming and resting. Rate of travel is the average swimming speed (mm s^{-1}), including the resting periods. Active swimming speed (mm s^{-1}) is the average velocity during active time only. Activity is the percentage of time the larva is actively swimming. NGDR expresses the linearity of the path described by a larva. Net displacement is the straight-line distance between the starting point and the ending point of the video segment analyzed. Gross displacement is the actual distance covered by the larva along its swimming path. Therefore, the closer NGDR approaches 1.0, the more linear the swimming path.

Frame-by-frame analysis of the visual response assays began 50 video fields before the time when the pendulum reached its nearest position to the chamber and ended 50 fields after (total: 100 frames or 1.7 s). Responses were characterized by: (1) responsiveness, percentage of larvae responding to the stimulus; (2) maximum response speed (mm s^{-1}); (3) average response speed (mm s^{-1}); (4) latency, elapsed time between the release of the stimulus and the larval response (ms), and (5) time to maximum speed (ms).

Predator exposure

Groups of 20 larvae from each exposure tank were used in four replicate spawns. Experiments were run in duplicate for each spawn. Larvae and predator (50-60 mm gulf killifish, *Fundulus grandis*) were confined at opposite sides of a 45-l aquarium (filled with 15 l of sea water) in transparent plastic cylinders of 4 cm and 15.5 cm diameter, respectively. The video camera was positioned over the cylinder containing the larvae with a field of view of about half of the aquarium (25 x 25 cm). Predator and prey were allowed to acclimate from handling for 2-3 h (Fuiman and Ottey, 1993). Following this, the video recorder was started and the cylinder containing the larvae was raised gently, freeing them into the tank. Immediately afterward the predator was released in the same way. Encounters within the video camera's field of view were recorded for later frame-by-frame analysis until all of the larvae were eaten or disappeared from the field of view. Each attack, response, capture, escape, and secondary attack (see explanation below) was recorded as a binomial variable, given a value of 1 if the event occurred and 0 if it did not. Calculations of responsiveness to an attack, response effectiveness, prey error, and

capture success were made from the binomial variables. Responsiveness was the percentage of fish in a trial responding to a predator attack. Response effectiveness was the number of fish responding to attack but not captured. Some fish initiated a startle response in the proximity of the predator even though they were not attacked. In many cases this behavior made the larva conspicuous to the predator, triggering an attack (secondary attack). Prey error represents the proportion of false alarms triggering a secondary attack. Finally, capture success is the total proportion of attacked larvae captured.

Respiration Rates

The energetic cost of atrazine exposure was evaluated in a specially designed respirometer. The respirometer had four independent chambers, each one with a total volume of 14 ml and consisting of an upper loop (Figure 1) with a length of 36 mm connected by three-way valves to a lower loop of about 30 mm in length containing the fish compartment (30 x 11 mm). These valves controlled whether the system was in recirculating or flow-through mode. A larva was contained within the fish compartment by a 500-µm mesh placed at each end of the compartment. Water used for the experiments was previously autoclaved to minimize bacterial oxygen consumption and then fully oxygenated. The oxygen sensor was a flow-through cell enclosing a fiber-optic oxygen micro-sensor with optical isolation (PreSens GmbH, Germany). Oxygen measurements (% of air saturation) were done with temperature compensation at 23.6 $\pm$ 0.6 ºC at a salinity of 25 PSU.

In the early afternoon of each experimental day, three to four larvae from each treatment were randomly selected and transferred to a glass dish (6 cm diameter) with 20 ml of filtered sea water from their original tanks. Fish were left undisturbed for 6 h to completely evacuate their guts after which a single fish was carefully transferred to the fish compartment. Each trial consisted of four chambers, three containing one experimental fish (one from each treatment) and a fourth chamber was left empty to control for background bacterial respiration. The chambers were then placed in flow-through mode for 2 h to allow the fish to recover from handling, reduce stress-related increase in oxygen consumption and maintain high levels of dissolved oxygen. The chambers were immersed in a water bath to minimize temperature differences. After acclimation the valves were adjusted to place the system into recirculation mode and oxygen measurements were started. Water flow within the chambers was maintained at a speed of approximately 1 mm s^{-1} with a peristaltic pump (Masterflex® L/S™, model 7519-15). Continuous measurements of oxygen content were done on 11 controls and 12 treatment fish (all from separate spawns) for a period of 5 to 7 h each. Experiments were halted when the dissolved oxygen level in the chamber dropped below 70 % of saturation. In order to approximate resting metabolic rates (i.e., minimal activity), trials were done at night and in the dark. Larval behavior in the chambers was videotaped with an infrared-sensitive video camera placed underneath the chambers.

At the end of the experiment, larvae were anesthetized with MS 222 and their TL (mm) measured to estimate their dry weight (DW, mg) using the equation:

$$DW = 0.0005 * TL^{3.466} \qquad (R^2 = 0.984)$$

This equation was derived from 90 red drum larvae of 3.3 to 13.5 mm TL dried at 65°C for 24 h (Appendix 3). Respiration rate was expressed as μg of O_2 min^{-1} mg^{-1} dry weight.

Larval activity within the chambers was quantified for a random sample of 21 fish (7 per treatment). Measurements were done after the 2-h acclimation period. Percent of time active was measured in 30-s video segments by recording the total time spent swimming.

Statistical analyses

Statistical analyses of the data were done using SYSTAT software (version 10.0). All variables were tested for normality and square root, logarithmic or arcsine transformations were applied when necessary as described by Zar (1999). Variability between spawns was accounted for by introducing "spawn" as a blocking variable in all statistical models. Behavioral data were analyzed for each experimental day using one-way analysis of variance (ANOVA). Pairwise comparisons of treatments were done when needed using Tukey-Kramer HSD test. Predator exposure experiments generated binomial data; therefore, comparisons were done using contingency-table analysis and Pearson chi-square statistics. Examinations of growth rates were done on log_e-transformed TL data using analysis of covariance (ANCOVA), with experimental day as the covariate.

RESULTS

Chemical analysis revealed that 4 d after addition of atrazine to the experimental tanks, an average of 17.7 % (± 10.4 SE) of the parent compound had degraded to

desethyl-atrazine (Table 1). No other degradation product was detected 96 h after exposure.

Survival of 7-mm TL red drum larvae at any of the three concentrations of atrazine (40, 80, and 500 µg l^{-1}) for 96 h was not significantly different from control (averaged 77.4 ± 3.2 % SE). Therefore, environmentally realistic doses were within the sublethal range for red drum larvae. Exposure levels chosen for study were 40 and 80 µg l^{-1}.

Growth

Control larvae grew at a significantly faster rate than atrazine-exposed larvae (P = 0.01, Figure 2). Pairwise comparisons indicated that control fish had a significantly different growth rate from the high dose group but not from the low dose group (Table 2).

Behavioral experiments

Three spawns (replicates) were used for behavioral assays. Red drum larvae exposed to either 40 or 80 µg l^{-1} of atrazine for 4 d showed significantly altered performance in all four behavioral traits analyzed compared to controls. Exposed larvae swam significantly faster, with a higher rate of travel (P = 0.001, Figure 3a) and active swimming speed (P = 0.001, Figure 3b). In addition, treated larvae were hyperactive (P = 0.006, Figure 3c) and swam considerably more convoluted paths (i.e. lower NGDR, P = 0.002, Figure 3d) after 4 days of atrazine exposure compared to unexposed larvae. For all the variables studied, significant differences were always observed between treated and control groups, but not between the low and high levels of atrazine exposure.

In contrast to routine behavior assays, no significant atrazine effect was observed for any of the visual startle response traits analyzed. Treated fish were as responsive to the visual stimulus as control fish. Responsiveness averaged 55 ± 0.7 % (± 1 SE) on experimental day 1 and 59 ± 0.004 % on day 3. The magnitude of the responses was also similar in all treatments. Average and maximum response speeds on day 1 and 3 were 58.5 ± 4.2 and 204.4 ± 9.4 mm s^{-1}, respectively. On day 3, average response speeds increased to 78.3 ± 4.3 mm s^{-1}, while maximum response speed remained the same at 182.8 ± 12.9 mm s^{-1} on day 3. Latencies and times to maximum speed averaged 4.4 ± 0.3 and 125.7 ± 9.0 ms for day 1, and 4.4 ± 0.2 and 115.2 ± 7.4 ms for day 3, respectively.

Predator exposure

Atrazine exposure produced no differences in antipredator performance of red drum larvae. Control and treated larvae (both exposure levels) were equally responsive to the attacking predator. Response rates were 69.8 ± 4.3% on day 1 and 84.9 ± 1.7 % on day 3 (± 1 SE). Likewise, response effectiveness was high, 74.2 ± 3.6 % and 82.2 ± 2.1 % for days 1 and 3, respectively. These variables resulted in relatively low capture success for the predators, this being 29.5 ± 2.2% on day 3 and 44 ± 3.6 % on day 1.

Respiration Rates

Analysis of fish activity within the respirometry chambers showed that the fish were active 6.0 ± 2.4 % of the time, with no significant difference among treatments. This level of activity was considerably lower than the routine activity measurements made during the day (35.3 – 57.6 %). This indicates that measurements within the respiration chamber were done on larvae that were essentially at rest. Respiration rates of

atrazine-exposed fish were not statistically different from control on any of the experimental days and averaged 0.024 ± 0.001µg of O_2 min^{-1} mg^{-1} dry weight.

DISCUSSION

Pollutants in the environment can affect physiological processes related to growth, development, and behavior. This is especially true of compounds that disrupt endocrine function, since hormones and neurotransmitters are the signals that guide development. Growth and behavior are crucial traits for larval survival in the environment. Moreover, growth is tightly related to development (Fuiman et al., 1998). Atrazine, at the environmentally-realistic doses used here, significantly reduced red drum larval growth rate by 7.9-9.8 %, thereby increasing the duration of the highly susceptible larval period. Working under the assumptions that (1) atrazine effects on growth rates are permanent, or (2) atrazine exposure levels are constant during the larval period, it is possible to estimate the effects of atrazine exposure on red drum survival to the juvenile stage. Red drum larvae reach the nursery seagrass beds at about 7 mm TL and remain there past the start of the juvenile stage (Rooker and Holt, 1997; Herzka et al., 2002), which is at about 25 mm TL (based upon complete squamation; Fuiman et al., 1998). Atrazine exposure reduced growth rate by about 8.7%. Consequently, the duration of the larval period will be longer when atrazine exposure occurs. Assuming all larvae reach the nursery area at 7 mm TL, an unexposed larval cohort with a growth rate of 0.0693 d^{-1} (control group) will reach the juvenile stage approximately 18 d later, whereas an atrazine-exposed cohort with an average growth rate of 0.0633 d^{-1} (average of low and high dose groups growth rates) will require about 20 d to transform into juveniles.

Rooker and colleagues (1999) estimated that mortality rates in the seagrass beds for settled red drum larvae ranged from 0.134 to 0.139 d^{-1} (average 0.1365 d^{-1}). This implies that a 2-d increase in larval stage duration due to atrazine exposure will increase mortality of the cohort by 24%. Such reductions in the number of juvenile fish produced in contaminated areas could potentially have a profound effect on recruitment and ultimately on the final population numbers.

Behavioral performance is critical to success in dealing with predators and prey. Although no significant effects on larval ability to evade predators were observed, environmentally realistic levels of atrazine induced changes in the routine behavior of red drum larvae. Treated larvae at both doses swam significantly faster and were hyperactive. Also, treated larvae had significantly lower NGDRs than control larvae (i.e., described more convoluted paths; Figure 4). However, since atrazine-treated larvae swam faster than control fish within the small experimental chamber, they could be expected to encounter the chamber's walls about 1.8 times more frequently than control fish. Therefore, the lower NGDRs observed for exposed fish might be an artifact of the confinement in the experimental chambers. Nevertheless, the other effects on routine behavior should affect a larva's likelihood of encountering predators and prey.

Routine behavior effects on rate of travel and its implications for larval survival can be estimated with the equation for encounter rates given by Gerritsen and Strickler (1977) and modified by Bailey and Batty (1983), as done by Cowan et al. (1996). Calculations were done for three species of calanoid copepods that inhabit settlement grounds: *Acartia tonsa*, *Paracalanus parvus* and *Temora turbinata* (newly settled red drum larvae feed mainly on calanoids [Soto et al., 2000]). Encounter rates were also

calculated for five predator species as described by Cowan et al. (1996), including juvenile red drum (cannibalism in red drum has been observed under laboratory conditions [Fuiman, 1994]). Parameters used in the calculations are provided in Table 3.

These calculations show that the apparently contradictory effects of atrazine on rate of travel are predicted to produce substantial increases in encounter rates with prey. Calculations using daily foraging distance (rate of travel · 24 h) showed that exposed larvae have a greater probability of encountering prey (average increase = 71%, Figure 5). In the same way, atrazine-exposed larvae are also more likely to encounter predators (Figure 6), where the outcome is often fatal. Changes in rate of travel have a larger predicted influence on encounter rates with slower swimming predators, such as ctenophores and medusae. The ecological importance of encounters with gelatinous predators in the nursery areas is probably low since red drum larvae will seek cover in the seagrass beds where this type of predator is uncommon and ineffective. However, during the time the larvae are entering the estuary and still in a pelagic stage, an increase in encounters with this kind of predator can have a profound effect on the total number of larvae colonizing the seagrasses. Predatory fish (planktivorous and red drum juveniles) used for these calculations swim much faster than larvae, thus contaminant effects on red drum larvae will not result on detectable changes in encounter rates. However, another consideration that is not included in the encounter-rate calculations is that red drum larvae exposed to atrazine for 4 d were 20 % more active than unexposed larvae. This will make treated larvae more conspicuous to visual predators than controls.

Respiration rates are good estimates of total metabolic rates since fish larvae rely mainly on aerobic metabolism (Finn et al., 1995; Wieser, 1995; Jacob et al., 2002;

Brightman et al., 1997). Measurements of respiration rates were intended to determine whether larvae exposed to atrazine incurred a direct metabolic or energetic cost. Larvae in the respirometry chambers were almost inactive and in a postabsorptive state so that measurements approximated a resting or standard metabolic rate. For unfed animals, total metabolic rate (R_t) is the weighted sum of active (R_a) and standard (R_s) (or resting) metabolic rates:

$$R_t = P \cdot R_a + (1\text{-}P) \cdot R_s$$

where P is the proportion of time active. Active respiration rate (R_a) is primarily a function of swimming speed (u). This relationship is an exponential function when larvae are in an inertial hydrodynamic regime, moving at relatively high Reynolds numbers (Re) where drag is proportional to the square of u [e.g., cyprinid larvae (Kaufmann, 1990)]. In the viscous hydrodynamic regime (low Re), the relationship is linear since drag is directly related to u. Larvae 4 days in the experiment exhibited an average Re of 58. Since viscous effects extend to Re numbers of 300 to 450 (Fuiman and Batty, 1997) a linear relationship is appropriate. Hunt von Herbing and colleagues (2001) described this equation for Atlantic cod (*Gadus morhua*) larvae:

$$R_a = 1.13 + 8.94 \cdot u$$

where, R_a is in mg O_2 g^{-1} h^{-1} and u is in mm s^{-1}. In the current study of red drum larvae, R_s averaged 0.19 mg O_2 g^{-1} h^{-1}. Calculated R_t of treated fish was double that of control fish (Table 4). However, since cod larvae exist at substantially lower water temperatures (about 20ºC lower than red drum in our experiment) and metabolic rates are dependent upon temperature, metabolic rates were adjusted using Q_{10} values of 1.9 for R_a and 2.5

for R_s (Kaufmann and Wieser, 1992). These adjustments did not affect the results; R_t for treated larvae was the double that of control R_t (Table 4).

CONCLUSIONS

Endpoints for assessing sublethal effects of pollutants can be obtained at almost any level of biological organization: molecular, cellular, tissue, organ. The higher the level affected by a pollutant the more generalized the response and the greater the implications for the general health of the organism, population, and ecosystem. Many studies in aquatic toxicology focus on single endpoints at one level (Weis and Weis, 1989; Saglio and Trijasse, 1998; Kazeto et al., 2004). However, few studies analyze effects at different endpoints (Beauvais et al., 2000; Brewer et al., 2001; Zhou et al., 2001). Moreover, studies often use contaminant levels and/or modes of exposure that do not reflect environmentally realistic conditions for the organism under study. Although such studies are important for characterizing the toxicity of a compound, it is difficult to use them to understand the ecological impacts of pollution. Scott and Sloman (2004) recognized the lack of studies evaluating the implications of interrelated behavioral and physiological effects caused by aquatic pollutants for fish populations. In the present study, we used a comprehensive approach that allows us to make detailed predictions for the outcome of ecologically relevant and sublethal exposures to atrazine for red drum larvae.

Environmentally realistic levels of atrazine are not directly lethal for red drum larvae although they could pose a threat to survival through effects on growth and behavior. Reduced growth will increase mortality by prolonging the highly vulnerable

larval period. Alterations of routine behavior caused by atrazine exposure may produce a considerable foraging advantage for treated larvae by increasing encounter rates with prey, but the same effects on routine behavior produce a very high energetic burden (a doubling of R_t). Exposed larvae will need to meet this elevated energetic requirement or suffer reduced growth and possible starvation. Predicted increases in encounter rates with prey averaging 71 % might not be enough to cope with this energetic burden in a patchy and highly variable environment. Moreover, encountering prey does not guarantee ingesting and absorbing the required nutrients. In these experiments, fish were fed relatively high rations, yet an effect on growth was observed. This suggests that one or more processes between encountering prey and growth, such as ingestion, digestion, or protein metabolism, was impaired. Finally, behavioral effects of atrazine exposure also increase the probability of encountering slow moving predators (ctenophores and medusa) which can result in increased mortality during the period larvae are entering estuarine nursery areas.

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Table 1. Atrazine levels in the experimental tanks on experimental days 0 and 3 (± 1 SE) (nd, not detectable).

Treatment	Nominal concentration ($\mu g\ l^{-1}$)	Actual concentration day 0 ($\mu g\ l^{-1}$)	Actual concentration day 3 ($\mu g\ l^{-1}$)
Control	0	nd	nd
Low	40	37.43 ± 5.71	33.33 ± 0.8
High	80	80.51 ± 1.21	58.71 ± 10.99

nd = below the detectable level.

Table 2. Growth rates of red drum larvae over a 9-d period exposed to sublethal levels of atrazine and controls. Asterisk (*) indicates significant difference ($P \leq 0.05$) relative to control. (N = 59)

Treatment	**R^2**	**Growth rate, G (d^{-1})**	**95% confidence interval**
Control (0 µg/l)	0.93	0.069	0.064 - 0.074
Low (40 µg/l)	0.89	0.064	0.059 - 0.070
High (80 µg/l)	0.90	0.063 (*)	0.057 - 0.068

Table 3. Parameters used for calculating encounter rates of red drum larvae with prey and predators. Data for prey obtained from Waggett (2005) and for predators from Cowan et al. (1996). Encounter rates were estimated for a 24-h period in a volume of 200 m^3.

Organism	Swimming speed ($mm\ s^{-1}$)	Average TL (mm)
Prey		
Acartia tonsa	1.42	1.0
Paracalanus parvus	1.83	0.9
Temora turbinata	1.81	1.2
Predators		
Small ctenophore	3.75	15
Small medusa	6.25	25
Large ctenophore	11.25	45
Large medusa	18.75	75
Juvenile red drum	80.9	27
Planktivorous fish	105	35
Red drum larvae		
Control	2.76	8
Atrazine-treated	5.13	8

Table 4. Estimated effects of atrazine exposure on total metabolic rate of red drum larvae ($R_t = P \cdot R_a + (1\text{-}P) \cdot R_s$). Active respiration rate was calculated as a linear function of swimming speed (u) ($R_a = 1.13 + 8.94 \cdot u$; Hunt von Herbing et al., 2001). R_s: resting metabolic rate (mg O_2 g^{-1} h^{-1}); P: proportion of time active; R_a: active metabolic rate: and R_t: total metabolic rate. R_t was adjusted for differences in temperature using Q_{10} values of 1.9 for R_a and 2.5 for R_s (Kaufmann and Weiser, 1992).

	u	**R_a**	**R_s**	**P**	**$P \cdot R_a$**	**$(1\text{-}P) \cdot R_s$**	**R_t**	**Q_{10} corrected R_t**
Control	5.48	50.09	0.19	0.42	20.89	0.11	21.00	77.84
Treatment	8.28	75.19	0.19	0.57	42.82	0.08	42.90	158.85

Figure 1. Diagram of the respirometer used: 1) fish compartment; 2) peristaltic pump; 3) three-way valve; 4) flow through fiber-optic micro oxygen sensor.

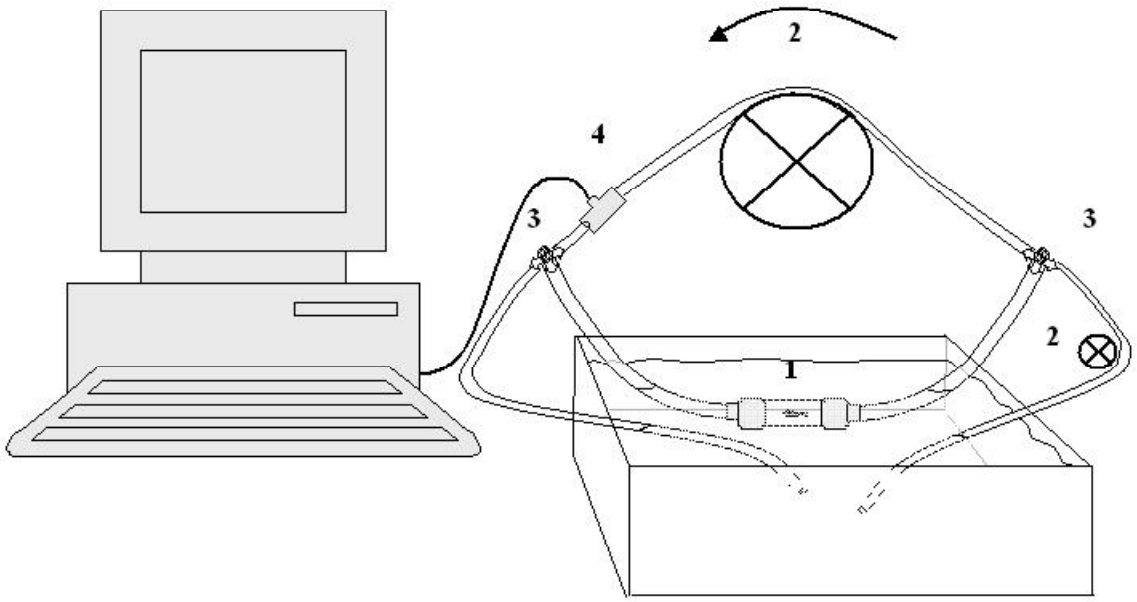
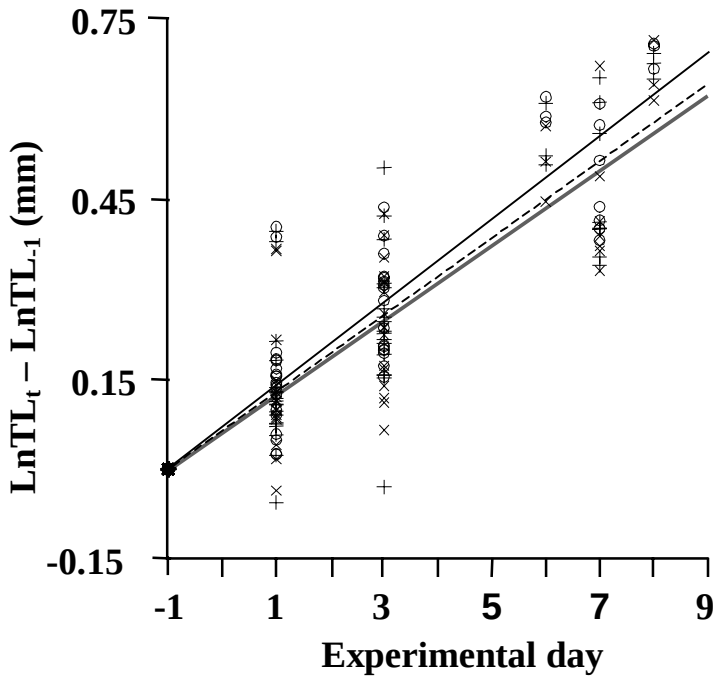


Figure 2. Growth of red drum larvae from a common initial TL over 9 experimental days. Lines represent linear regression fit to the data grouped by treatment. Control group (○ ,—); low dose group (× ,--); high dose group (+ ,—). (n = 59)



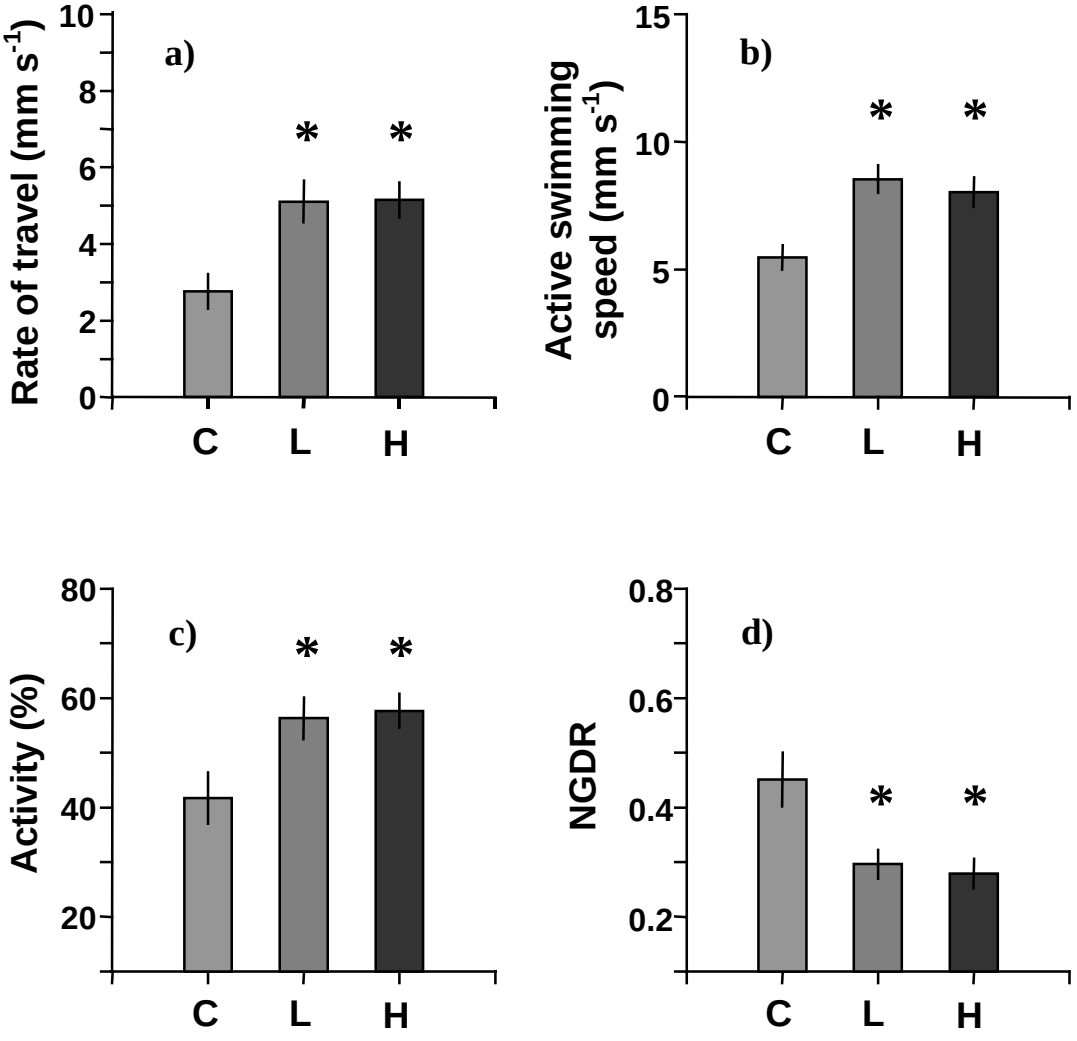
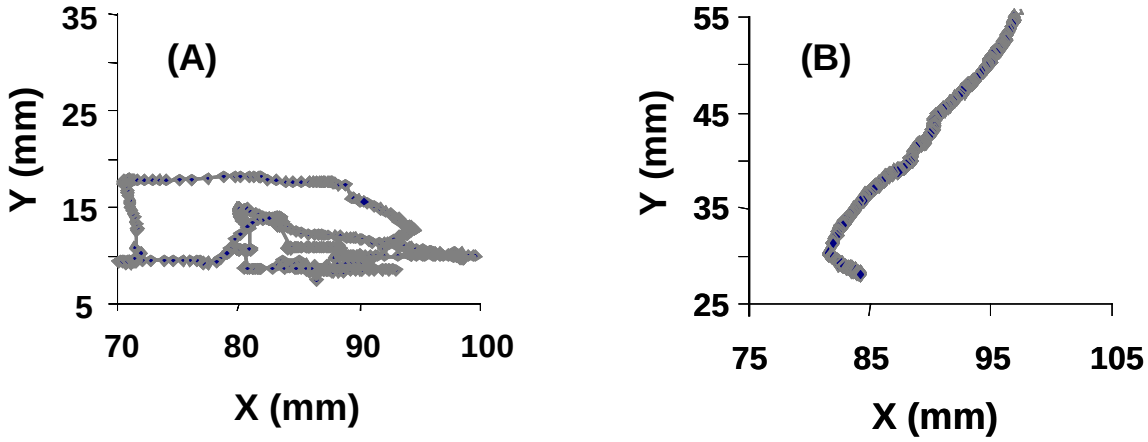


Figure 3. Routine behaviors of red drum larvae treated with 0 (control, C), 40 (low, L), or 80 (high, H) μg l^{-1} of atrazine for 4 days. a) Rate of travel; b) Active swimming speed; c) activity, and d) net-to-gross displacement ratio (NGDR). Values represent means $\pm$ 1 SE. Asterisks (∗) indicate significant differences relative to controls ($P < 0.05$; $n = 36$).

Figure 4. Swimming paths described by a red drum larva treated with atrazine for 4 days (A) and untreated larva (B). Atrazine exposed larvae swam a more erratic path as shown by the low NGDR (= 0.1) compared to control fish (= 0.9).



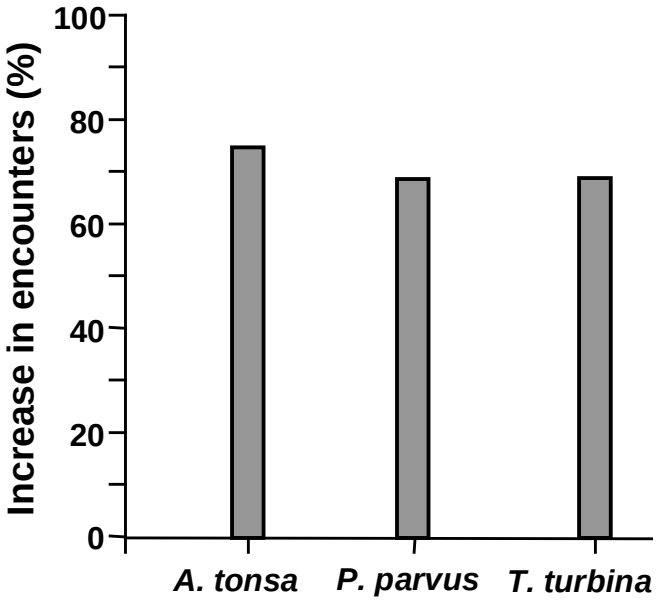


Figure 5. Increase in predicted encounter rates (relative to controls) between red drum larvae and prey as a result of atrazine exposure for 4 days. Prey items are three types of calanoid copepods: *Acartia tonsa*, *Paracalanus parvus*, and *Temora turbinata*. Calanoid copepod speeds were obtained from Waggett (2005).

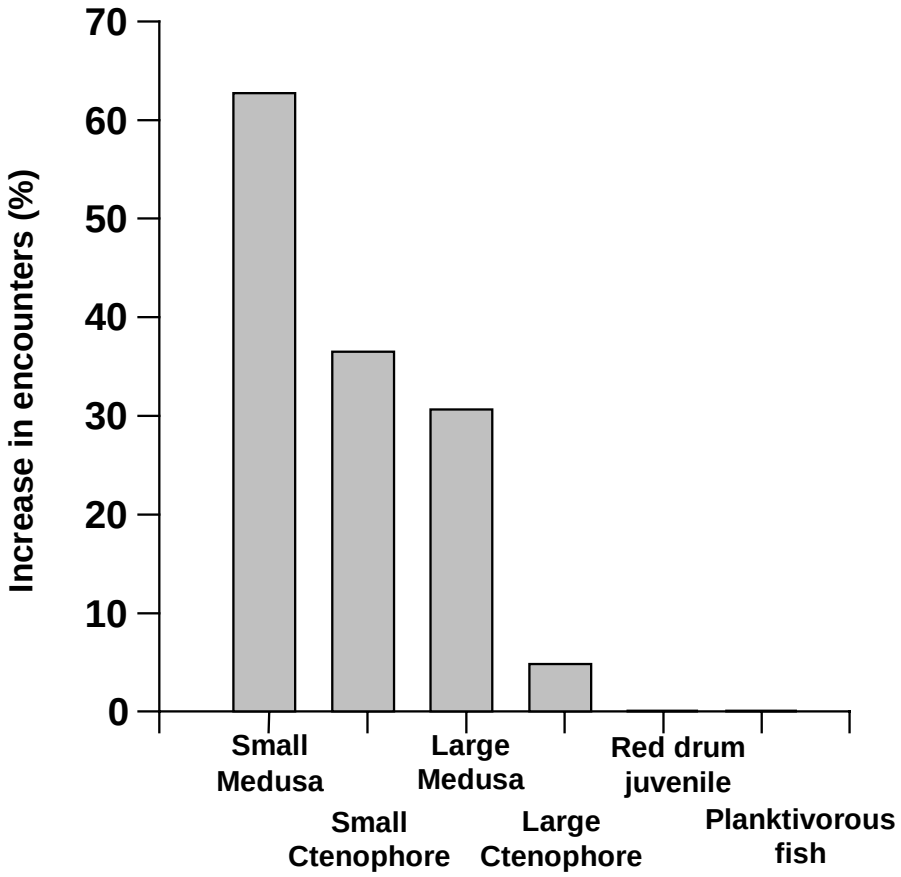


Figure 6. Increase in encounter rates (relative to controls) between red drum larvae and their predators as the result of atrazine exposure for 4 days. Predators are: two sizes of ctenophores (small and large), two sizes of medusa (small and large), 25-mm red drum juvenile, and 35-mm planktivorous fish.

CHAPTER 3: Ecological performance of red drum (*Sciaenops ocellatus*) larvae exposed to environmental levels of the insecticide malathion

ABSTRACT

Malathion is a highly soluble organophosphate insecticide that is widely used in agriculture and mosquito eradication campaigns. Fish species such as red drum (*Sciaenops ocellatus*) that use seagrass beds as nursery areas could be affected by runoff waters contaminated with malathion. In this study I exposed red drum larvae at the size they reach estuarine nursery areas to environmentally realistic and sublethal levels of malathion (0, 1, and 10 μg l^{-1}). The effects of such exposure on ecologically significant behaviors (routine swimming and predator avoidance), growth and resting metabolism were evaluated. Malathion exposure to relatively low but ecologically realistic concentrations did not affect routine behavior, escape behavior, resting metabolic rate or growth, indicating that current environmental levels may be safe for young fishes. However, recent substantial increase in the use of malathion may elevate levels in surface waters above those tested here.

INTRODUCTION

Malathion (o,o-dimethyl-S-(1,2-dicarbethoxy)ethyl phosphorodithioate) is an organophosphate insecticide used in agricultural and non-agricultural settings. Introduced to the market in 1950, it has been widely used for the control of insects, including fruit flies, mosquitos and aphids. Organophosphates inhibit acetylcholinesterase (AChE) activity and impair normal brain functions. Therefore behavioral effects may be expected at sublethal doses (Beauvais et al., 2000). Although malathion was designed to target insect populations, it can affect many other non-target species, including fishes, rats, goats, and humans (Weis and Weis, 1976; Haider and Inbaraj, 1986; Rivett and Potgieter, 1987; Eurich et al., 1995; Tomar et al., 1995; Thangnipon et al., 1995; Akgur et al., 1999; Brewer et al., 2001; Abdel-Rahman et al., 2004). Since the establishment of the USDA sponsored Texas Boll Weevil Eradication Foundation in 1993, the use of this insecticide has increased exponentially, with a maximum use in Texas alone of over 21 million pounds in 1999. Approximately 20 million pounds of malathion were used in the U.S. in 2001. About 90 % of this amount was used in the Boll Weevil Eradication Programs of which over 75 % was applied in Texas (U.S.EPA, 2000; and NASS: Agricultural Chemical Use Database: http://www.pestmanagement.info/nass/).

Malathion has a relatively high solubility and low sorption coefficient, making it extremely mobile and likely to reach surface waters through runoff. Reported levels of malathion in estuaries are few and variable. In the San Francisco Bay estuary during the Medfly Eradication Program rainy season, average malathion concentrations ranged from 1.0 to 7.0 $\mu g\ l^{-1}$, while dry season samples were below the detection limit (Finlayson et

al., 1982). Ward and Amstrong (1992) reported levels of malathion for Galveston Bay and Corpus Christi Bay of 0.46 μg l^{-1} and 0.32 μg l^{-1} respectively, with "hot spots" as high as 1 μg l^{-1} (Ward and Armstrong, 1992; 1997). After aerial application of malathion for medfly eradication in Ventura County (California) in 1994-95, malathion concentration in the outflow site of the treated area averaged 44.2 μg l^{-1}. In the same area, malathion concentrations in runoff reached 11.1 μg l^{-1} 6 days after application (Bradley et al., 1997).

Most published environmental concentrations of malathion are based on single-point-in-time samples. Concentration of this chemical will change over time due to intermittent pulses and loading into surface waters and bays, therefore, single point samples might not reflect the level of contamination in the environment. An example of this was found by Larson et al. (1999), where malathion was one of the most frequently detected pesticide at all sampled sites in surface waters in Las Vegas, Nevada, although malathion concentrations surpassed the U.S. EPA aquatic life criterion of 0.1 μg l^{-1} only in isolated samples. Another example was found by Key et al. (2003) who found that concentrations of short-lived organophosphates, including malathion, were low (in the ng l^{-1} range) at the time of sampling but that organisms in the area showed clear AChE inhibition.

Many commercially and ecologically important fish species, such as red drum (*Sciaenops ocellatus*) use estuaries as nursery areas. Red drum spawns along coastal areas, and eggs and early larval stages enter the estuary and settle on seagrass beds within a few weeks. Larvae reach this nursery area at an average total length (TL) of 7 mm and remain there at least until they reach juvenile stage (25 mm TL; Rooker et al., 1997;

Herzka et al., 2002). It is at this incompletely developed, settlement stage that they will encounter the highest pesticide levels from runoff.

Since mortality rates during the larval stage are naturally high, mainly due to starvation and predation, factors that increase these rates will have a profound impact on the number of recruits to the adult population (Houde, 2002). The aim of this study was to evaluate, in a comprehensive way, the effects of exposure to sublethal and environmentally realistic levels of malathion on the ecological performance and subsequent survival potential of red drum larvae at settlement stage.

MATERIAL AND METHODS

Eggs and larvae

Red drum larvae were raised at the Marine Science Institute of The University of Texas at Austin (Port Aransas, Texas). Eggs were obtained from broodstock induced to spawn by temperature and photoperiod manipulations at the Fisheries and Mariculture Laboratory at the Marine Science Institue and Texas Parks and Wildlife Department CCA/CPL Marine Development Center (Corpus Christi, Texas). Eggs were collected within 12 h of spawning and disinfected with a 0.1-ppm Formalin solution for 30 min, then rinsed and transferred to 150-l conical rearing tanks. Hatching was done in 20 l of sea water, at 27ºC and a salinity matching that of the spawning tank. Beginning 2 days after hatching salinity was gradually adjusted to 27 PSU. From day 1 posthatching until approximately day 10, fish were fed *Brachionus picatilis* at a density of 10 rotifers ml^{-1} daily. From this point on fish were gradually introduced to non-enriched brine shrimp

nauplii (*Artemia* spp.) for a period of 5 days. Once completely weaned, fish were fed nauplii that had been enriched overnight with Algamac 2000 (Aquafauna Bio-marine, Inc.) daily at a density of 5 nauplii ml^{-1}. Experiments were done on fish larvae at settlement size (average 7 mm TL; Rooker and Holt, 1997; Hertzka et al., 2002) when they were feeding on brine shrimp only.

Chemical

On the day of an experiment, malathion (98 % pure, Sigma-Aldrich) was diluted with acetone to a concentration of 1 mg ml^{-1} in a separate container then added to the experimental tank to the desired concentration. The same volume of acetone as used for the high dose group was added to the control group. (Effects of acetone were assessed in a separate experiment, see Appendix 2)

To characterize the exposure over the course of the experiment, water samples were taken 5 min after the addition of malathion to the experimental tanks and again 4 days later. Analyses of malathion levels were done by an independent laboratory (Department of Soil and Crop Sciences, Pesticide Fate Research Laboratory, Texas A&M University, Texas) with high-performance liquid chromatography (HPLC) as described by Senseman et al. (1997).

Survival experiments

Survival trials were performed to evaluate whether environmentally realistic concentrations were within the sublethal range for settlement size red drum. Groups of 50 settlement size larvae were transferred to 1.5-l exposure watch bowls (20 cm diameter) in

a temperature-controlled room. Temperature and salinity were maintained at 27°C and 27 PSU, respectively. Malathion solution or acetone alone was added to the watch bowls 24 h after transfer of the fish. Concentrations of malathion tested were 0, 10, 15, 20, 100, 200, and 2000 μg l^{-1}. Fish were fed a ration of 5 nauplii ml^{-1} d^{-1}. Survival was recorded 96 h after the insecticide was added to the water. Two to 18 replicates (depending upon the dose) were conducted with larvae from seven different spawns.

Exposure tanks

Five hundred settlement size red drum larvae (15- to 20-d old) were transferred to each of six, 50-l fiberglass exposure tanks the day before malathion was added (experimental day -1). The fish were allowed to recover from handling for 1 d. The six tanks were randomly assigned to the three treatment groups: control, low, and high dose (0, 1, and 10 μg of malathion l^{-1}) in duplicate. Malathion was added (experimental day 0) as described above. The exposure tanks were arranged in a water bath to minimize differences in temperatures among tanks. Mean (± S.D.) water temperature and salinity were 26.3 ± 0.14 °C and 28.1 ± 0.55 PSU, respectively. Fish were fed at approximately 0830 h daily and allowed to feed for about 1 h before removing some of them for further experiments.

Growth

Groups of 20 larvae were sampled from each exposure tank. Samples were taken the day of transfer (experimental day -1) and on days 1, 3, and 7 after malathion was added to the water. Fish were anesthetized using tricaine methansulfonate (MS 222) and

digital pictures were taken with the aid of a camera (Sony DCR-TRV350) attached to a dissecting microscope. Total length (mm) was measured using an image-processing program (ImageJ, National Institute of Health). Five replicates were done from five different spawns.

Because larval growth rate is size dependent, growth rate was calculated using an exponential growth model to compensate for differences in initial size among trials.

$$TL_t = TL_{-1} \cdot e^{G \cdot t}$$

$$G = (LnTL_t - LnTL_{-1}) / t$$

where TL_t is total length (mm) on experimental day t, TL_{-1} is total length on experimental day -1, and G is the instantaneous growth rate (d^{-1}).

Behavioral experiments

Two behavioral assays were conducted on four different spawns to assess the effects of malathion exposure: routine swimming and visual startle response. Routine swimming behavior measured foraging capacity and the visual startle response measured the ability to escape from a predatory attack.

Groups of ten larvae were randomly selected from each exposure tank and carefully placed in glass chambers (55 x 60 x 20 mm) containing approximately 50 ml of filtered sea water from their exposure tank. The chambers were placed in a temperature-controlled room and the fish were left to recover from handling for 2-3 h (Fuiman and Ottey, 1993). One chamber was then carefully placed above a video camera (Cohu, model 3315-2000/0000) and left undisturbed for 5 min. Routine behavior of the undisturbed larvae was then video-recorded (Panasonic AG-1960) from a remote station

for 3 min. After this time, the larvae were given a visual stimulus and their responses were recorded. The stimulus was a black oval (1 x 1.5 cm) on a white card, simulating the cross-section of an approaching predatory fish. The card was attached to the end of a remotely controlled pendulum, which was held by an electromagnet. The stimulus was applied by turning off power to the electromagnet. The pendulum then accelerated toward the chamber containing the larvae but was stopped by a tether just before hitting the chamber (for details see Fuiman and Cowan, 2003). The recorded video was digitized as AVI files and movements of larvae were analyzed with the aid of a computerized tracking system (WinAnalyze 2D Software, Version 1.5, Mikromak, Germany).

Analysis of the routine behavior clips was done frame by frame and the paths described by the larvae over a period of 25 s were tracked. Behavior was measured for all ten larvae in the chamber and expressed by four variables: rate of travel (mm s^{-1}), active swimming speed (mm s^{-1}), activity (% time), and net-to-gross displacement ratio (NGDR, dimensionless). Red drum larvae swim in alternating episodes of active swimming and resting. Rate of travel was the average swimming speed (mm s^{-1}) including the resting periods. Active swimming speed (mm s^{-1}) was the average velocity during active periods only. Activity was the percentage of time the larva was actively swimming. NGDR defined the linearity of the path described by a larva. Net displacement was the straight-line distance between the starting point and the ending point of the segment analyzed. Gross displacement was the actual distance covered by the larva along its swimming path. Therefore, the closer NGDR is to 1, the more linear the path.

Frame-by-frame analysis of the visual response assays began 50 video fields before the moment when the pendulum reached its nearest position to the camber and

ended 50 fields after (total: 100 video fields or 1.7 s). Responses were characterized by: (1) responsiveness, percentage of larvae responding to the stimulus; (2) maximum response speed (mm s^{-1}); (3) average response speed (mm s^{-1}); (4) latency, elapsed time (ms) between release of the stimulus and the larval response, and (5) time to maximum speed (ms).

Respiration Rates

The metabolic cost of malathion exposure was evaluated in a specially designed respirometer (Figure 7). The respiration chamber had a total volume of 14 ml and consisted of a 36-mm-long upper loop connected by three-way valves to a 30-mm-long lower loop containing the fish compartment (30 mm long x 11 mm diameter). The valves controlled whether the system was in recirculating or flow-through mode. A larva was contained in the fish compartment by a 500-µm mesh at both ends. Water used for the experiments was previously autoclaved and fully oxygenated. The oxygen sensor was a flow-through cell enclosing a fiber-optic oxygen micro-sensor with optical isolation (PreSens GmbH, Germany). Oxygen measurements (% of air saturation) were done with temperature compensation at 23.6 ± 0.6 ºC at a salinity of 25 PSU.

In the early afternoon of each experimental day, three to four larvae from each treatment were transferred to a glass dish (6 cm diameter) with 20 ml of filtered sea water from their original tanks. Fish were left undisturbed for 6 h to completely evacuate their guts. A single fish was then carefully transferred to the fish compartment. Each trial consisted of four chambers, three containing one experimental fish (one from each treatment) and a fourth chamber was left empty to measure background bacterial

respiration. The chambers were then placed in flow-through mode for 2 h to allow the fish to recover from handling, reduce stress-related increase in oxygen consumption and maintain high levels of dissolved oxygen. The chambers were immersed in a water bath to minimize temperature differences. After acclimation, the valves were adjusted so that the water recirculated through the chamber and measurements were started. Water flow within the chambers was maintained at a linear velocity of approximately 1 mm s^{-1} with a peristaltic pump (Masterflex® L/S™, model 7519-15). Continuous measurements of oxygen content were made on 18 control and 19 treatment fish (from several spawns) for a period of 5 to 7 h. Experiments were halted if oxygen concentration in the camber dropped below 70 % saturation. In order to approximate resting metabolic rates (i.e., minimal activity), trials were done at night and in the dark. Behavior of larvae in the chambers was videotaped with an infrared sensitive video camera and infrared illuminator placed underneath the chambers to confirm minimal activity during the trials.

A random sample of 21 fish (7 per treatment) was taken from the experiments to quantify larval activity within the chambers. Measurements were done after the 2-h acclimation period. Percent of time active was measured in 30-s video segments by recording the total time spent swimming.

At the end of the experiment, larvae were anesthetized with MS 222 and their TL (mm) measured. Dry weight (DW, mg) was estimated using the empirically derived equation:

$$DW = 0.0005 \bullet TL^{3.466} \qquad R^2 = 0.98$$

This equation was derived from 90 red drum larvae of 3.3 to 13.5 mm TL dried at 65°C for 24 h (Appendix 3). Respiration rates were expressed as μg O_2 min^{-1} mg^{-1} dry weight.

Statistical analyses

Statistical analyses of the data were done using SYSTAT software (version 10.0). All variables were tested for normality and square root, logarithmic or arcsine transformations were applied when necessary as described by Zar (1999). Variability due to differences between spawns was accounted for by introducing "spawn" as a blocking variable in all models. Behavioral data were analyzed for each experimental day using one-way analysis of variance (ANOVA). Comparisons among treatments were done when needed using Tukey-Kramer HSD test. Examinations of growth rates were done on log_e-transformed TL data using analysis of covariance (ANCOVA).

RESULTS

Experiments were conducted on settlement size red drum larvae that were 6.4 $\pm$ 0.16 mm TL and 15 to 20 days after hatching. Survival experiments to 96 h showed that levels $\leq$ 15 μg l^{-1} were not lethal for this stage of red drum larvae. Concentrations of 20 μg l^{-1} and above 200 μg l^{-1} significantly reduced larval survival ($P < 0.05$, Figure 8). All larvae died within 24 h at a concentration of 2000 μg l^{-1}. To examine the effects of environmentally realistic exposures, concentrations of 0, 1, and 10 μg l^{-1} were used as control, low, and high exposure levels, respectively. Analysis of the water in the

experimental tanks indicated that actual levels of malathion were approximately 75% that of nominal concentrations on day 0 and decreased sharply after day 3 (Table 5).

Mean growth rate for control larvae was more than twice the mean for either the low or high treatments, but not significantly different (Table 6). Red drum grew at an average rate of 0.089 d^{-1} during the 7-d period.

Malathion exposure also had no effect on routine behavior (Table 7). Rate of travel and active swimming speed were 5.6 $\pm$ 0.34 mm s^{-1} and 1.16 $\pm$ 0.13 mm s^{-1} on day 1 and 6.5 $\pm$ 0.34 and 1.6 $\pm$ 0.21 mm s^{-1} on day 3, respectively. Fish were active 29 $\pm$ 2.29 % and 31.5 $\pm$ 2.9 % of the time, displaying similar degrees of linearity in their swimming paths on both dates, where mean NGDR values were 0.487 $\pm$ 0.016 and 0.483 $\pm$ 0.02 on days 1 and 3, respectively.

Similarly, antipredator behavior was unaffected by malathion exposure (Table 8). Treated fish were as responsive to a visual stimulus as control fish. Responsiveness was high, averaging 63.9 $\pm$ 0.03 % for day 1 and 61.9 $\pm$ 0.04 % for day 3. Mean latency of the response was 1.2 $\pm$ 0.26 ms on day 1 and 2.2 $\pm$ 0.32 ms on day 3. Maximum and average response speeds were 101.2 $\pm$ 6.69 mm s^{-1} and 46.3 $\pm$ 2.92 mm s^{-1} on day 1 and 90.7 $\pm$ 8.69 mm s^{-1} and 41.2 $\pm$ 4.01 mm s^{-1} on day 3, respectively. Time to maximum speed was 114.6 $\pm$ 5.04 ms on day 1 and 122.7 $\pm$ 6.72 ms on day 3.

Finally, treated fish had respiration rates that were not significantly different from those of control fish. Mean oxygen consumption was 0.023 $\pm$ 0.001 $\mu g\ O_2\ min^{-1}\ mg^{-1}$.

DISCUSSION

Organophosphate insecticides such as malathion control insect populations by inhibiting acetylcholinesterase (AChE) activity, an enzyme that is vital to normal nervous function. AChE regulates the function of the neurotransmitter acetylcholine (ACh) in the cholinergic synaptic cleft by metabolizing it to choline and acetic acid. ACh is the intermediary between the nervous and muscular systems, inducing muscular contraction; it also has a major role in learning and memory. Therefore, inhibition of AChE will result in a surplus of ACh in the post-synaptic cleft and an over-stimulation of post-synaptic or muscular target cells. Malathion was developed to target insects, but since the ACh - AChE system occurs in vertebrates and invertebrates alike, behavioral effects are likely to occur in a great variety of organisms exposed to it. Surprisingly, no effects of malathion exposure were observed on any of the diverse behavioral or physiological traits studied here. There are several possible interpretations of this lack of effect. One possibility is that malathion does not affect red drum at all. However, malathion reduced larval survival at concentrations $\geq$ 20 $\mu g\ l^{-1}$ and killed all larvae at a concentration of 2000 $\mu g\ l^{-1}$ (Figure 8). Therefore, red drum larvae are affected by malathion, but only at levels higher than those used here.

Another interpretation of the experimental results is that the behavioral and physiological assays studied here are not sensitive enough to detect sublethal effects. These same assays have been used in several other studies of fish larvae. Maternal transfer of low levels of DDT, DDE, methylmercury and Aroclor 1254 to larval Atlantic croaker (*Micropogonias undulatus*) has been shown to alter a variety of behavioral traits. Larvae with maternally transferred DDT had lower average and maximum response

speeds to a visual predatory stimulus and had impaired routine swimming activity until oil globule absorption (Faulk et al., 1997). Atlantic croaker larvae maternally exposed to Aroclor 1254 had slower growth and reduced responsiveness to a vibratory stimulus (McCarthy et al., 2003). Maternal exposure to DDE decreased linearity of the swimming path (NGDR) of larvae (Sabath, 2001). Maternally transferred methylmercury induced a variety of effects on routine and escape behaviors (Chapter 4). Further, direct exposure of settlement size red drum larvae to atrazine in the water induced delayed growth, increased activity and NGDR and faster rate of travel and active swimming speed (Chapter 2).

To determine whether replication was sufficient to detect a statistically significant effect of malathion, I conducted power analyses for all non-significant variables. Eight of the eleven variables had a statistical power greater than 0.5 and six of these variables had a power greater than 0.8 (Figure 9). Latency and average and maximum response speeds had relatively low power, probably due to the resolution of the video analysis used here (60 video fields s^{-1}). These results lend credence to the general conclusion of no effects of the malathion exposures given to the larvae in this study for at least eight of the eleven variables tested.

Survival of red drum larvae after exposure to environmentally realistic levels of malathion was concentration dependent. Concentrations of 20 and 200 μg l^{-1} had significantly lower 96-h survival than larvae in control groups. The USDA monitoring program as well as EPA estimates of malathion concentrations in the environment agree that, at the levels of usage in programs such as the Texas Boll Weevil Eradication Program, concentrations of malathion in surface waters adjacent to treated areas may

reach acute levels of 226 μg l^{-1} and chronic levels of 21 μg l^{-1} (U.S. EPA, 2000). Chronic levels in estuaries are expected to be lower than those of waters adjacent to application sites due to the short half-life of malathion and the dilution of surface waters entering the estuary. However, acute levels predicted to be more than 10 times higher could create sporadic pulses of malathion-contaminated waters at levels that could affect larval survival.

The degradation rate of malathion mostly depends on the temperature and acidity of the environment (faster at high temperatures and lower pH). The half-life of malathion at 27ºC and pH 8 is about 36 h, increasing to 40 days at 0ºC. The major degradation pathways are acid degradation to mono- and diacids as well as alkaline degradation to diethyl fumarate and ethyl hydrogen fumarate (Wolfe et al., 1977; Miles and Takashima, 1991; Lacorte et al., 1995). At normal environmental conditions for red drum larvae in estuaries, the malathion half-life should be between 1 and 2 days. In this laboratory study less than 20 % of the original amount of malathion applied remained after 96 h.

Contaminant levels in estuarine waters are difficult to measure and peak exposures of contaminants with short lives, such as malathion, are difficult to sample because they are ephemeral. Thus, reported values may be lower than actual values. Recent evaluations of malathion levels in Texas bays have not being carried out even though agricultural use of malathion has increased more than five fold (Figure 10). Average use for 1994-1998 was 2.5 million pounds of malathion per year. Since 1999, an average 13.2 million pounds of malathion have been used per year. This increase is due to the increase in treated acreage by more than 4.5 million acres in the USDA Texas Boll Weevil Eradication Program. Mathematical models or laboratory simulations may be

more useful for estimating environmental concentrations. Given these considerations, it seems likely that sporadic pulses of malathion-contaminated waters at levels high enough to affect larval survival are possible. Malathion does not have sublethal effects on red drum larvae at settlement size at currently reported average environmental levels. However, since current concentrations of malathion in the environment are probably higher than those used in this study, due to the large recent increase in malathion use, it is possible that malathion is already affecting red drum larval survival in nursery areas, but this remains to be demonstrated.

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Table 5. Malathion levels in water from experimental tanks (nd, not detectable).

Treatment	Nominal concentration ($\mu g\ l^{-1}$)	Actual concentration day 0 ($\mu g\ l^{-1}$)	Actual concentration day 3 ($\mu g\ l^{-1}$)
Control (0 $\mu g\ l^{-1}$)	0	nd	nd
Low (1 $\mu g\ l^{-1}$)	1	0.73 $\pm$ 0.12	trace
High (10 $\mu g\ l^{-1}$)	10	7.42 $\pm$ 0.32	1.33 $\pm$ 0.15

nd = below the detectable level.

trace = above the detectable level but below the quantifiable level.

Table 6. Growth rates of red drum larvae over a 9-d period exposed to sublethal levels of malathion and controls. R^2 is the coefficient of determination for the exponential growth curve used to estimate growth rate (G). 95% CI is the 95% confidence interval for G.

Treatment	**R^2**	**Growth rate, G (d^{-1})**	**95% CI**
Control (0 μg l^{-1})	0.84	0.091	0.076 – 0.106
Low (1 μg l^{-1})	0.86	0.087	0.075 – 0.100
High (10 μg l^{-1})	0.88	0.089	0.077 – 0.102

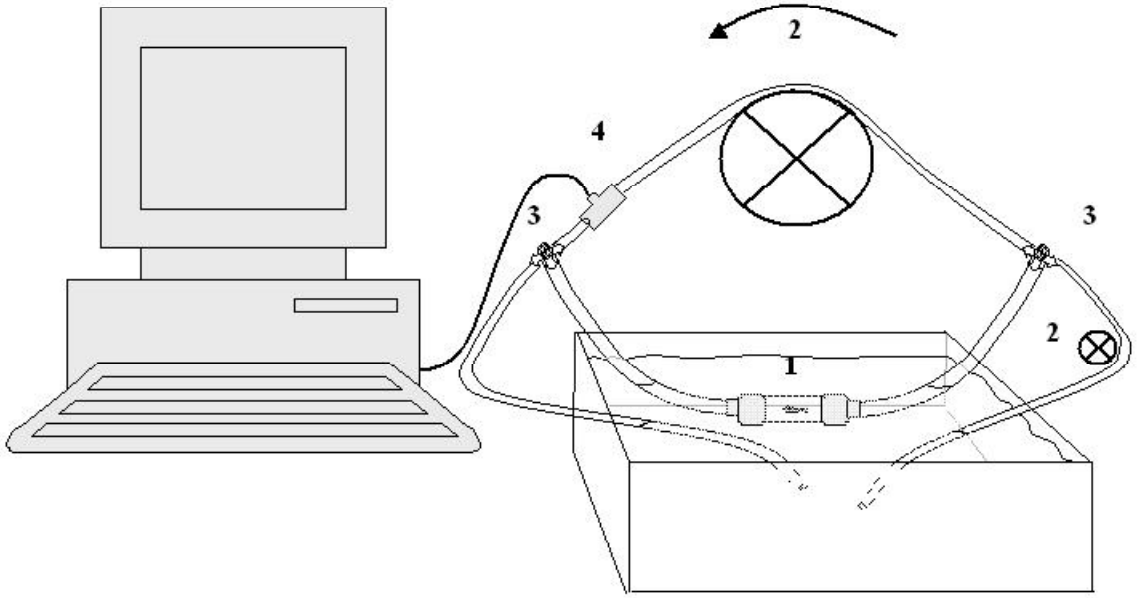
Table 7. Routine behavior trait averages on the two experimental days for control, low, and high (0, 1, 10 μg l^{-1}) malathion exposures. Transformations of original variables to attain normality for statistical tests are shown in parenthesis after each variable. Values represent means (± 1 SE) of the transformed variables.

	Day 1			Day 3		
Variable (statistical transformation)	**Control**	**Low**	**High**	**Control**	**Low**	**High**
Rate of travel	-0.004	-0.120	0.007	0.111	0.096	-0.013
(log_{10})	(± 0.073)	(± 0.084)	(± 0.067)	(± 0.073)	(± 0.083)	(± 0.067)
Active swimming speed (square root)	2.208 (± 0.119)	2.381 (± 0.136)	2.295 (± 0.109)	2.510 (± 0.118)	2.514 (±0.135)	2.397 (± 0.108)
NGDR	0.741	0.765	0.794	0.723	0.799	0.783
(arcsine)	(± 0.029)	(±0.034)	(± 0.027)	(± 0.028)	(± 0.032)	(± 0.026)
Activity	0.476	0.403	0.447	0.498	0.485	0.443
(arcsine)	(± 0.026)	(± 0.030)	(± 0.024)	(± 0.030)	(± 0.034)	(± 0.027)

Table 8. Response to visual predatory stimulus on the two experimental days for control, low, and high (0, 1, 10 μg l^{-1}) malathion exposures. Transformations of original variables to attain normality for statistical tests are shown in parenthesis after each variable. Values represent means (± 1 SE) of the variable transformed to attain normality.

	Day 1			Day 3		
Variable (statistical transformation)	**Control**	**Low**	**High**	**Control**	**Low**	**High**
Responsiveness (arcsine)	1.01 (± 0.06)	0.97 (± 0.06)	0.89 (± 0.06)	0.92 (± 0.07)	0.94 (± 0.08)	0.95 (± 0.07)
Latency	1.69 (± 0.44)	1.25 (± 0.44)	0.61 (±0.46)	2.01 (± 0.56)	2.63 (± 0.57)	1.93 (± 0.56)
Average response speed	44.01 (± 5.10)	44.68 (± 5.10)	50.51 (± 5.28)	37.74 (± 6.50)	45.62 (± 6.2)	43.68 (± 5.9)
Maximum response speed	100.73 (± 11.89)	96.55 (±11.89)	100.693 (± 12.31)	76.05 (± 13.00)	108.26 (± 12.89)	96.19 (± 13.84)
Time to maximum speed	116.05 (± 5.23)	112.91 (± 8.52)	114.85 (± 8.82)	129.27 (± 12.32)	117.18 (± 14.12)	121.6 (± 12.84)

Figure 7. Diagram of the respirometer used: 1) fish compartment; 2) peristaltic pump; 3) three-way valve; 4) flow through fiber-optic micro oxygen sensor. The arrow indicates the direction of the flow.



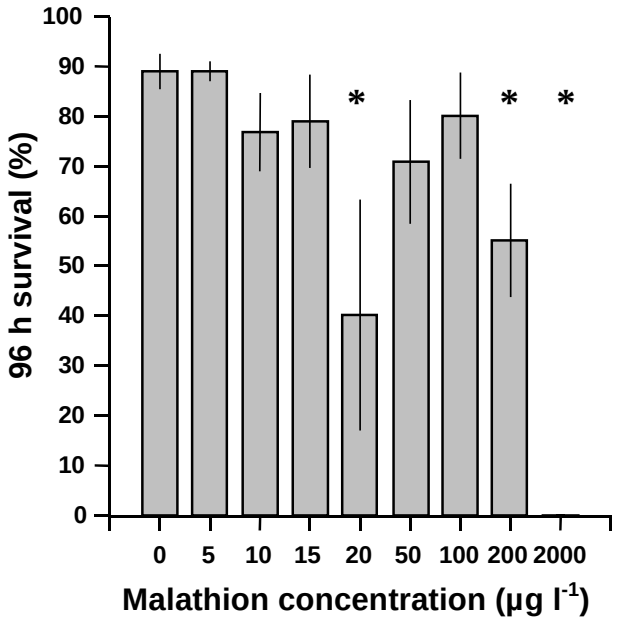


Figure 8. Survival of settlement size red drum larvae to 96-h exposure to various levels of malathion-contaminated water. Asterisks represent values statistically different from control ($P < 0.05$). Values represent means $\pm$ 1 SE.

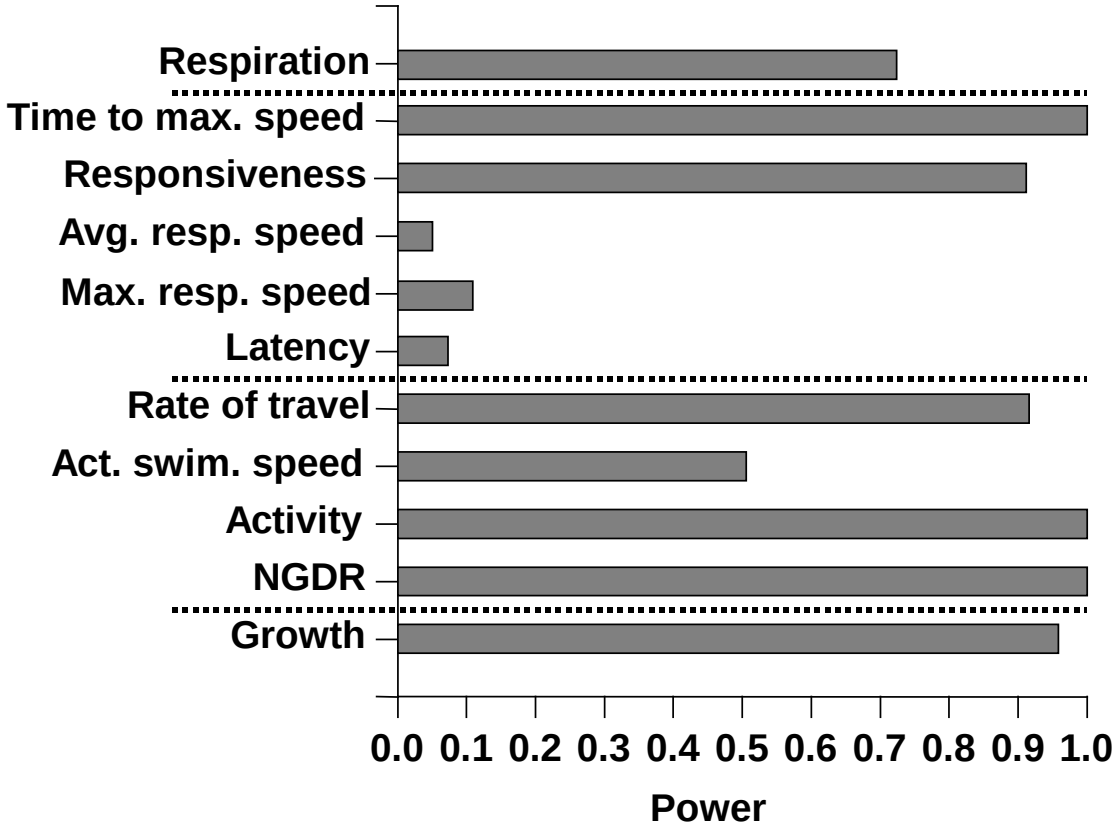


Figure 9. Statistical power of attained results for malathion exposure (control, low and high) of red drum larvae at settlement size.

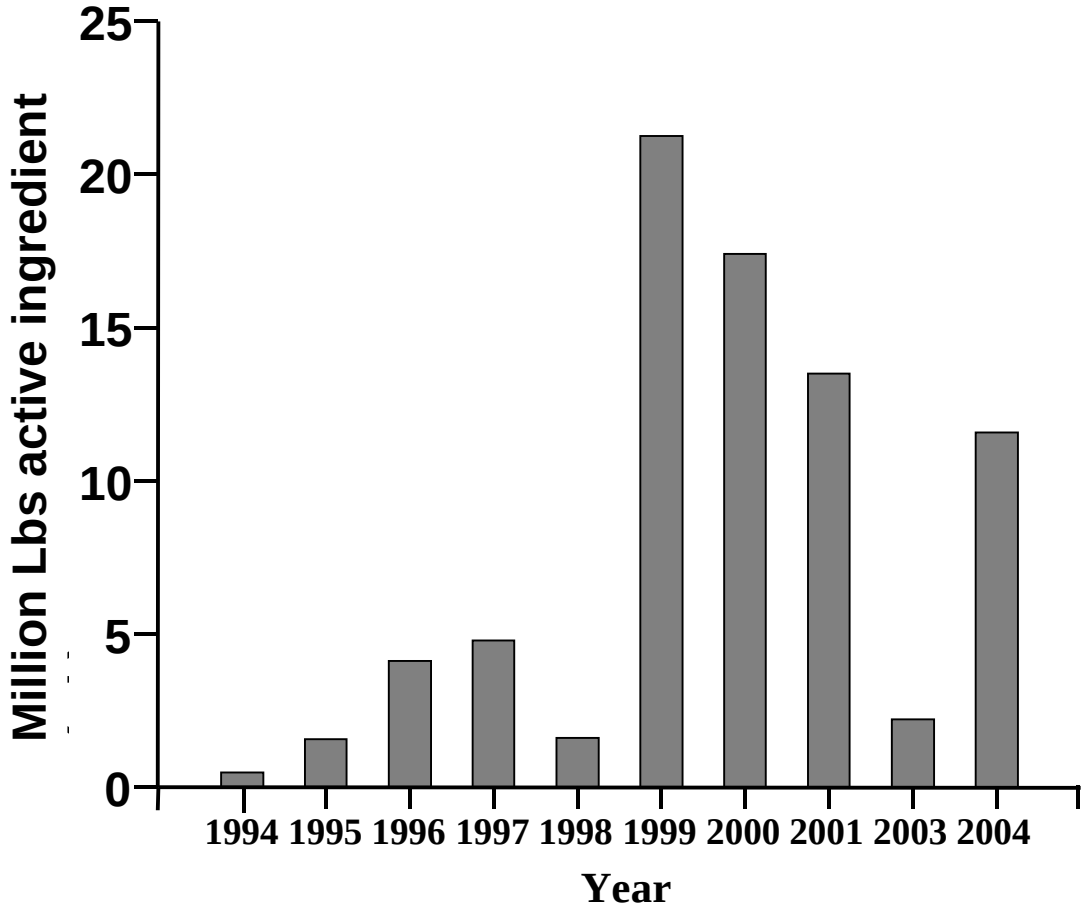


Figure 10. Annual agricultural use of malathion in millions of pounds of active ingredient for Texas from 1994 to 2004 (2002 data not available, 2004 data are until October). About 97% of this amount is used to eradicate the Mexican boll weevil from cotton land (data from NASS: Agricultural Chemical Use Database: http://www.pestmanagement.info/nass/ and USDA Texas Boll Weevil Eradication Program: http://www.txbollweevil.org/).

CHAPTER 4: Maternal body burdens of methylmercury impair survival skills of offspring in Atlantic croaker (*Micropogonias undulatus*)

ABSTRACT

The organic form of mercury, methylmercury (MeHg), bioaccumulates easily through the food chain. Predatory fish can accumulate relatively high levels of MeHg and transfer it to their developing eggs. Here, the effects of maternally derived MeHg on the planktonic larval stage of Atlantic croaker were investigated. Adult Atlantic croaker were fed MeHg-contaminated food at three levels: 0, 0.05, and 0.1 mg kg^{-1} d^{-1} for one month. Fish were then induced to spawn and MeHg levels in the eggs were measured (0.04 to 4.6 ng g^{-1}). Behavioral performance of exposed and control larvae was measured at four developmental stages: end of yolk absorption (yolk), end of oil absorption (oil), and 4 and 11 d after oil absorption (oil+4 and oil+11). Behaviors analyzed included survival skills related to foraging and predator evasion: routine behavior (rate of travel, active swimming speed, net-to-gross displacement ratio, and activity) and startle response to a visual or a vibratory stimulus (reactive distance, response distance, response duration, average response speed, maximum response speed, and responsiveness). Maternally-transferred MeHg induced a range of stage- and concentration-dependent effects. These effects were more frequent at yolk and oil+4 stages, suggesting physiological rather than developmental effects. Mathematical models applied to predict the ecological consequences of the behavioral effects suggested that maternal transfer of MeHg will substantially lower survival of planktonic stage larvae compared to unexposed fish.

INTRODUCTION

Mercury (Hg) is a naturally occurring heavy metal. Natural processes and anthropogenic activities release Hg into the atmosphere as elemental and inorganic Hg where it is readily transported. Anthropogenic sources of Hg are many; however, the largest emissions are from coal and fossil fuel burning (Moore, 2000). Non-point-source deposition occurs mainly in the form of rain, therefore, Hg pollution is widespread (Downs et al., 1998). Hg is transformed into methylmercury (CH_3Hg^{++}, MeHg) mainly by bacterial action within the sediment (Benoit et al., 1998). Organisms readily take up MeHg by direct exposure through the body surface or by ingestion. Then it bioaccumulates and biomagnifies through the food chain (Lawrence et al., 1999). Concentrations of MeHg in water or sediments are generally less than 30% of total Hg, while 80 – 90% of total Hg in fish muscle tissue is in the form of MeHg (Watras and Bloom, 1992).

Atlantic croaker (*Micropogonias undulatus*) supports an important fishery industry in the U.S. (Diamond et al., 1999). Adult fish are demersal and feed mainly on benthic organisms (worms, clams). Spawning occurs along the continental shelf from mid-summer to spring (Miller et al., 2003). Pelagic larvae stay in the open ocean for approximately 30 to 60 days (Nixon and Jones, 1997), arriving at estuarine nursery areas at an average length of 12 mm. Benthic feeding organisms are particularly susceptible to MeHg exposure through ingestion of contaminated food (Hanson and Zdanowicz, 1999) or through the gills as they sift sediments containing MeHg. However, approximately 90% of the MeHg accumulated in fishes is of dietary origin (Spry and Weiner, 1991). Ingested MeHg is quickly absorbed, circulated, and deposited in tissues. In fishes, MeHg

mainly resides in proteins (Mason et al., 1995) and can be transferred from the female to the eggs during oogenesis (Latiff et al., 2001). Although the mechanism of this maternal transfer is unknown, it probably occurs through molecular mimicry of certain amino acids that are easily transported through cell membranes (Ballatori, 2002). MeHg readily forms complexes with amino acids (Ballatori, 2002) deposited in the eggs during oogenesis (Fyhn, 1989). Therefore, female Atlantic croaker inhabiting contaminated areas could transfer part of their body burden of MeHg to their offspring through the yolk.

MeHg is a known endocrine disrupting chemical (EDC) and neurotoxicant (Colborn et al., 1993; Rice, 1995; Zhou et al., 1999; Myers et al., 2000). Endocrine and neural systems control many physiological and developmental processes in animals. Any disturbance to either of these systems during development could have profound and permanent effects on the organism, both in terms of its development and its performance in ecological interactions. Therefore, it is likely that fish larvae, as rapidly developing organisms, will be highly susceptible to MeHg burdens of maternal origin. Faulk et al. (1997) and McCarthy et al. (2003) have shown that exposure to EDCs (o,p'-DDT and Aroclor 1254, respectively) through maternal deposition in the egg can impair the performance of Atlantic croaker larvae in behaviors relevant to foraging and predator evasion. This study evaluates the effects of maternal MeHg exposure on the ecological performance of Atlantic croaker larvae.

MATERIAL AND METHODS

Experimental fish

Sexually mature Atlantic croaker were fed rations contaminated with different amounts of MeHg and the larvae produced were studied for effects on growth and behavior that would forecast reduced probability of survival in their natural environment. Male and female Atlantic croaker were collected with gill nets in early winter 2000 in the Aransas Pass Ship Channel (near Port Aransas, Texas). Fish were maintained in tanks with recirculating water at constant temperature (22°C), 12L:12D photoperiod, and fed a diet of shrimp (3% body weight per day). From December 1 onward, three groups of fish consisting of 16 females and 8 males (average body weight 370 g) were placed into tanks of approximately 10,000 l capacity with recirculating water. The control group was fed only shrimp. The low dose group was fed a diet of blue marlin (*Makaira nigricans*) muscle tissue having a concentration of 0.05 mg kg^{-1} of MeHg. The high dose group was fed marlin supplemented with contaminated shrimp to a final concentration of 0.1 mg kg^{-1} of MeHg. These diets were maintained for 1 month after which one female and one spermiating male were removed from each experimental tank in the afternoon for spawning. The female received a single injection of gonadotropin releasing hormone analog (GnRHa) in fish saline at a concentration of 50 ng g^{-1} of body weight to induce spawning. The next morning, spawned eggs were collected and a sample was frozen for later analysis of MeHg content, using a modification of the digestion procedure in the method described by Dusci and Hackett (1976). In this case the tissue was left to dissolve for one week in 50 ml of HNO_3 solution at room temperature.

Eggs were collected from 6 control, 5 low, and 4 high spawns. Only spawns that were successfully reared until at least 4 d after absorption of the oil globule were used. Therefore, larvae from 3 control, 2 low, and 3 high spawns were analyzed. Upon collection, eggs were disinfected to remove possible parasites by immersing the eggs in a solution of 1 ppm of formalin for 45 min then placing them in glass watch bowls filled with 1.5 l of sea water at a density of 2 eggs ml^{-1} to hatch. The following morning, hatched larvae were transferred to two 150-l conical rearing tanks at a density of 20 larvae l^{-1}. Larvae were reared in constant conditions of photoperiod (12L:12D), salinity (approximately 29.5 PSU), and temperature (23 °C). For the experimental period, larvae were fed rotifers (*Brachionus plicatilis*) enriched with the alga *Isochrysis galbana*. Larvae were sampled for behavioral assays at developmental stages identified by the completion of yolk absorption (referred to as "yolk"), completion of oil absorption ("oil"), and on days 4 ("oil+4") and 11 ("oil+11") after complete oil absorption. The first two stages were chosen to help identify the primary source of MeHg in the larvae and the latter two stages were intended to determine whether effects were physiological (if they were temporary) or developmental (if they persisted).

Growth

Five larvae were sampled from each rearing tank on days 1, 3, 6, 11, and 17 after hatching. Larvae were anesthetized with tricaine methane sulfonate (MS-222, 1% v/v) and their total length (TL, mm) to the nearest μm was measured with the aid of a microscope and computer-assisted measuring system (Measurement TV, Data Crunch software). Growth rates (G) were computed as the slope of the exponential growth model:

$$TL = a \cdot e^{G \cdot t}$$

where t is age in days after hatching.

Routine behavior assays

Twenty larvae for yolk or oil stages and 15 larvae for oil+4 and oil+11 stages were placed in experimental chambers (glass, 2.5 × 7.5 × 2.5 cm) and allowed to acclimate and recover from handling for 2 h (Fuiman and Ottey, 1993) in a temperature-controlled room at 27°C. Then the chambers were carefully placed above an infrared sensitive video camera (Cohu, model 3315-2000/0000) and left undisturbed for 5 min to allow the larvae to recover from handling. Their routine behavior was then video recorded from below using a video recorder (Panasonic, model AG-1960) and indirect infrared light. Video segments were digitized as AVI files and movements of the larvae were analyzed with the aid of a computerized tracking system (WinAnalyze 2D Software, Version 1.5, Mikromak, Germany).

To characterize routine swimming, measurements of rate of travel (mm s^{-1}), active swimming speed (mm s^{-1}), net-to-gross displacement ratio (NGDR), and activity (%) were made from 30-s periods. Atlantic croaker larvae swim in alternating episodes of active swimming and resting. Rate of travel is a measurement of the average swimming speed during the measurement period, including resting periods. Active swimming speed describes the average swimming speed while the fish was actively swimming (excluding the resting periods). NGDR is a measurement of the linearity of the swimming path traveled by a larva, where net displacement is the linear distance between the beginning and the ending points of the measurement period, and gross displacement is the actual

distance covered by the larva along its swimming path. The closer NGDR is to 1, the straighter the path swum by the larva.

Visual startle stimulus

The visual startle assay was conducted immediately after the routine behavior assay. It elicited an evasive response in larvae similar to that used by a larva to escape from a predator (Fuiman and Magurran, 1994). The stimulus was a black oval on a white card, simulating the cross-sectional silhouette of a predatory fish, held at the end of a remotely controlled pendulum. The pendulum was held away from the larvae by an electromagnet. When the pendulum was released the predatory stimulus accelerated toward the larvae but a tether stopped it just before hitting the chamber containing the fish (depicted by Fuiman and Cowan, 2003). The whole procedure was videotaped for later video analysis. Frame-by frame analysis of these video recordings provided data on responsiveness (percentage of larvae responding), reactive distance (distance from the pendulum to a larvae when the response started, in mm), response distance (total distance covered during the response, in mm), response duration (total duration of the response, in ms), average response speed (response distance divided by response duration, in mm s^{-1}), and maximum response speed (largest speed observed during an individual video frame, in mm s^{-1}).

Vibratory startle stimulus

The vibratory startle stimulus consisted of a remotely controlled metal hammer placed at about 5 mm from a metal post upon which a plastic dish containing larvae

rested, as described by Faulk et al. (1997) and McCarthy et al. (2003). Groups of 10 larvae were placed in transparent plastic dishes (50 mm diameter × 12 mm) and allowed to acclimate from handling for 2 h. After this period the dish containing the larvae was placed on top of the metal post and surrounded by a ring of infrared light-emitting diodes. After allowing 5 min for the larvae to recover, the hammer was remotely triggered to strike the metal post and the responses of larvae were recorded with an infrared sensitive video camera (Cohu, model 3315-2000/0000) placed above the chamber. Video recordings were then analyzed using a computerized video measurement system (Measurement TV, Data Crunch software). The percentage of larvae responding to the stimulus (responsiveness) was calculated from the number of visible larvae. For larvae responding to the vibratory stimulus, mean response speed of each larva (mm s^{-1}) was calculated from the response distance (mm) and the response duration (ms).

Statistical analyses

All statistical analyses of the data were done using SYSTAT software (version 10.0). Mean values for each variable were computed for each chamber containing larvae (1 - 4 chambers for each combination of treatment and developmental stage). These means, each based on 1 - 20 larvae (means: 7.6 $\pm$ 0.1 SE larvae for visual startle response, 7.1 $\pm$ 0.1 SE larvae for vibratory startle response and 5.0 $\pm$ 0.1 SE for routine behavior), were used for the statistical comparisons. Variables were screened for normality, and logarithmic or angular (arcsine) transformations were applied when necessary (Zar, 1999).

Measured concentrations of MeHg in the eggs were separated roughly by a factor of ten, therefore, log-transformed MeHg concentrations (larval body burdens) were used in all statistical analysis. Concentrations of MeHg in control eggs were below detection limits (0.001 ng g^{-1}), so a value of 0.0004 was added to values below the detection limit in order to do logarithmic transformation (Table 9). All variables were first examined using analysis of covariance (ANCOVA) with MeHg concentrations in eggs and age as covariates. In the case of a significant concentration × age interaction or a significant concentration effect, linear regressions were computed for each age to detect any concentration-dependent trends.

RESULTS

Since the feeding rate of each adult croaker varied, MeHg concentrations in the eggs varied among spawns within nominal treatment groups. Analysis of the egg samples showed six distinct MeHg levels in the eggs (Table 9).

Growth

There was no significant effect of MeHg exposure on larval growth ($P = 0.267$). Growth rates (G) for control, low, and high dose group were 0.034, 0.032, and 0.033, respectively. The overall mean instantaneous growth rate (all treatments combined), followed the equation:

$$TL = 2.21 \cdot e^{(0.033 \cdot t)}$$

Routine behavior assays

Rate of travel varied significantly with MeHg concentration in the eggs ($P = 0.001$) and with developmental stage ($P = 0.025$), but the concentration × stage interaction was not significant ($P = 0.576$). Within developmental stages, concentration–response relationships were significant for larvae at yolk ($P = 0.033$) and oil+4 ($P = 0.051$) stages. Slopes of the relationships were negative, reflecting the decreasing rate of travel in larvae exposed to MeHg at these stages (Figure 11). Active swimming speed varied significantly with MeHg level ($P = 0.009$) but not among developmental stages, as shown by a non-significant concentration × stage interaction ($P = 0.235$) or developmental stage effect ($P = 0.237$). The only stage to exhibit a significant regression between active swimming speed and MeHg concentration was the oil stage ($P < 0.001$) (Figure 11). There also was a significant effect of MeHg concentration on the activity level of larvae ($P = 0.0004$), although no significant effect of development ($P = 0.067$) or interaction term ($P = 0.129$) was observed. Within stages, activity levels were significantly related to MeHg concentration for only the yolk ($P = 0.006$) and oil+4 ($P = 0.030$) stages, where activity decreased with increasing concentration (Figure 11).

Visual startle stimulus

No significant relationship with MeHg concentration was observed for six of the seven variables computed from the visual startle assay. A significant concentration × stage interaction term ($P = 0.008$) showed that MeHg affected the proportion of larvae responding to the stimulus differently among the four stages studied. Stage-specific analysis showed that responsiveness increased with MeHg concentration in the oil+4

stage (P = 0.008, Figure 12). Larvae from all treatments reacted at similar distance from the stimulus source (P = 0.394), and their responses lasted for comparable amounts of time (P = 0.319) at all ages. MeHg concentration in the eggs did not affect larval response distance (P = 0.195) or the average (P = 0.291) or maximum (P = 0.307) response speeds.

Vibratory startle stimulus

A significant effect of MeHg concentration (P < 0.001) was observed for response duration to the vibratory stimulus. Analysis by developmental stage showed that oil+4 was the only stage of the four to exhibit a strong relation with MeHg body burden (P = 0.017, Figure 13). There was also a significant effect of MeHg concentration on response speed (P < 0.001). Separate analysis of the different stages showed that response speed decreased with increasing MeHg burden in the eggs for the yolk (P = 0.006) and oil+11 (P = 0.010) stages (Figure 13). Response distance and responsiveness of larvae to the vibratory stimulus were not significantly affected by MeHg concentrations in the eggs (P = 0.145 and 0.116, respectively).

DISCUSSION

Mercury contamination is ubiquitous, and Texas coastal waters are no exception. Sager (2004) reported total mercury levels for three important sport fishes from five minimally impacted bays in Texas: southern flounder (*Paralichthys lethostigma*), spotted seatrout (*Cynoscion nebulosus*), and red drum (*Sciaenops ocellatus*). Muscle levels of total mercury ranged between 50 and 250 ng g^{-1}. In another study, Edwards and colleagues (1999) reported total mercury levels in eel (*Anguilla anguilla*) muscle tissue

ranging from 15 to 501 ng g^{-1}, and in roach (*Rutilus rutilus*) muscle tissue ranging from 19 to 121 ng g^{-1}, depending of the river of origin. At least 81 % of this total mercury was in the form of MeHg (Edwards et al., 1999). Latiff et al. (2001) estimated a maternal transfer of MeHg to eggs of 2 % to 11 % of the total mercury in muscle of walleye (*Stizostedion vitreum*) for uncontaminated and contaminated lakes, respectively. Assuming a 2 % transfer, eggs produced by the fish in Texas bays (Sager, 2004) could have MeHg loads between 1 and 5 ng g^{-1}, while fish studied by Edwards et al. (1999) could produce eggs with 0.3 to 2.42 ng g^{-1}. In the current study, Atlantic croaker fed MeHg-contaminated food at levels encountered by benthic feeders in the wild (Locarnini and Presley, 1996), produced eggs with MeHg concentrations of 0.04 to 4.6 ng g^{-1}. These levels are, therefore, environmentally realistic and comparable to levels expected by Sager (2004) and Edwards et al. (1999).

The developmental stages chosen for this study were designed to help determine the role of mobilization of the yolk and oil globule in sublethal effects and whether any observed effects were physiological or developmental. Behavioral effects of maternally derived MeHg exposure were variable and dependent upon the developmental stage and the type of behavior investigated. Atlantic croaker larvae showed a concentration-dependent impairment of routine behavior due to MeHg exposure (Figure 11). Startle response traits also exhibited a concentration-dependent effect for some developmental stages. Surprisingly, MeHg exposure produced a temporary improvement in the overall quality of the response in some stages (Figure 12). This apparent improvement of the response to a visual stimulus disappeared in later stages. Finally, maternally derived

MeHg also affected responses to a vibratory predatory stimulus and these effects were also stage dependent (Figure 13).

Significant behavioral effects were more frequently observed at yolk and oil+4 stages (Figure 14). Of the behavioral effects observed, 30 % occurred in the yolk stage, while 50 % occurred in the oil+4 stage, and only 10 % were observed in the oil and oil+11 stages. This suggests that MeHg primarily affects the developing larvae when it is being mobilized from the yolk and deposited in tissues with the proteins and amino acids of the yolk. The temporal character of the behavioral effects observed (i.e., effect of MeHg on the rate of travel in yolk and oil+4 stages only) suggests that MeHg effects on the larvae are not permanent and therefore unlikely to be developmental; rather, they are likely to be physiological. Such recovery from behavioral impairment has been reported in other species. Zhou et al. (1996) observed recuperation from the behavioral deficits caused by MeHg embryonic exposure on *Fundulus heteroclitus* larvae.

To better understand the actual importance of the various behavioral effects produced by MeHg exposure, data obtained here were applied to an empirical regression tree to predict the probability of a larva surviving attack by a predatory fish based on its rate of travel and visual reactive distance (Fuiman et al., manuscript). Once this probability was obtained, daily growth and mortality rates of the larval groups were followed in computer simulations of encounters with predators and prey as the larvae grew through the four developmental stages (yolk, oil, oil+4, and oil+11 [3 to 12 mm]), using an individual based model (IBM; Rose et al., 2003). For these simulations, experimental results were grouped by dose into control (MeHg in eggs < 0.05 ng g^{-1}), high dose (> 1.0 ng g^{-1}), and low dose (0.05 - 1.0 ng g^{-1}) groups (Table 10). Since the

regression tree was developed for red drum (*Sciaenops ocellatus*) larvae, some adjustments were required. Atlantic croaker's rate of travel and reactive distance to a visual stimulus for control larvae were scaled to match mean values for red drum larvae at each developmental stage and proportional adjustments were made for larvae of low and high groups at each developmental stage. Simulations for each developmental stage and treatment were repeated three times to assess repeatability of the outcomes.

Regression tree results showed that MeHg reduced the probability of surviving a predatory attack in three of the four developmental stages (Table 10). Behavioral effects of maternally transferred MeHg on Atlantic croaker larvae produced higher predicted mortality rates (Z) compared to control larvae. Larvae from eggs in the low dose group exhibited a 35 % higher mortality rate than control group, and larvae from the high dose group had a 29 % higher mortality rate (Table 11). Growth rates were also decreased compared to control group larvae by about 11 % for low dose group and a 21 % for high dose group (Table 11). The combination of a higher mortality rate at this planktonic stage (12 mm) and a slower growth rate (longer stage duration) resulted in a mean reduction in larval survival to the end of the planktonic larval stage of 92 % and 99 % for low dose high dose groups, respectively, compared to control larvae (Table 11). However, the the limitations of these types of mathematical approaches must be considered. Although the regression tree analysis and the individual based model used here are comprehensive methods, they will differ from the real word and conclusions have to be drawn cautiously. Therefore, maternal exposure to MeHg might not exactly induce a 90 – 99 % reduction in survival; however, it has a significant potential for jeopardizing Atlantic croaker populations in the wild.

Maternally derived MeHg induced no effects on growth rates of croaker larvae in the laboratory. However, mathematically estimated (model derived) growth rates were affected by MeHg level and were faster than the rates measured in the laboratory. This discrepancy arises from the fact that the model takes into account natural levels of prey density (artificially elevated in the laboratory), observed stage durations for field collected larvae, and foraging ability of larvae in the field estimated from observed behavioral traits. Therefore, although this study failed to find an effect of MeHg on growth in the laboratory, behavioral effects that relate to foraging are expected to reduce growth rates in the field. This would prolong the larval stage duration during a period when morality is high, thereby reducing survival to the end of the planktonic stage.

This study demonstrated MeHg transfer from food to eggs. Levels in spawned eggs attained in this study were comparable to levels expected in the wild, and this maternally derived MeHg induced adverse effects on behavior that may translate into reduced survival of planktonic larvae, with negative consequences for the population.

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Table 9. Mean concentration in ng of MeHg per g of spawned eggs from each of three treatment groups. Value in brackets represents the nominal dose given to the broodstock through the food.

Spawn number	Control (0 mg kg^{-1} d^{-1})	Low (0.05 mg kg^{-1} d^{-1})	High (0.1 mg kg^{-1} d^{-1})
1	0.0004	-	0.567
3	-	-	4.574
5	0.0004	-	-
7	0.0004	0.639	3.874
8	-	0.294	-

Table 10. Mean rate of travel and visual reactive distance values used in the regression-tree analysis, and the survival from a predator attack of control, low and high MeHg-exposed larval fish. Values in brackets represent the percent difference from control.

Variable	YOLK	OIL	OIL+4	OIL+11
CONTROL				
Rate of travel (mm s^{-1})	0.8	1.2	1.4	0.7
Visual reactive distance (mm)	309.4	201.0	347.4	264.4
Sample size	82	50	37	52
Survival probability	0.508	0.544	0.491	0.506
LOW				
Rate of travel (mm s^{-1})	0.583	0.582	0.521	0.499
Visual reactive distance (mm)	204.7	279.1	287.1	325.1
Sample size	63	29	24	32
Survive (% of control)	0.435 (- 14 %)	0.517 (- 5 %)	0.408 (- 17 %)	0.530 (+ 5 %)
HIGH				
Rate of travel (mm s^{-1})	0.297	0.796	0.570	0.398
Visual reactive distance (mm)	279.8	329.9	291.9	250.9
Sample size	38	34	39	48
Survive (% from control)	0.422 (- 17 %)	0.526 (- 3 %)	0.456 (- 7 %)	0.435 (- 14 %)

Table 11. Calculated growth and mortality rates, and percent survival through the four developmental stages to the end of the planktonic larval stage (12 mm) obtained by the IBM. Values represent the average of three replicates for control, low and high dose groups ± 1 SE.

Treatment	Survival (%)	Growth (mm d^{-1})	Mortality (d^{-1})
Control	1.15 ± 0.02	0.38 ± 0.01	0.17 ± 0.00
Low	0.09 ± 0.01	0.34 ± 0.01	0.23 ± 0.01
High	0.01 ± 0.00	0.30 ± 0.02	0.22 ± 0.02

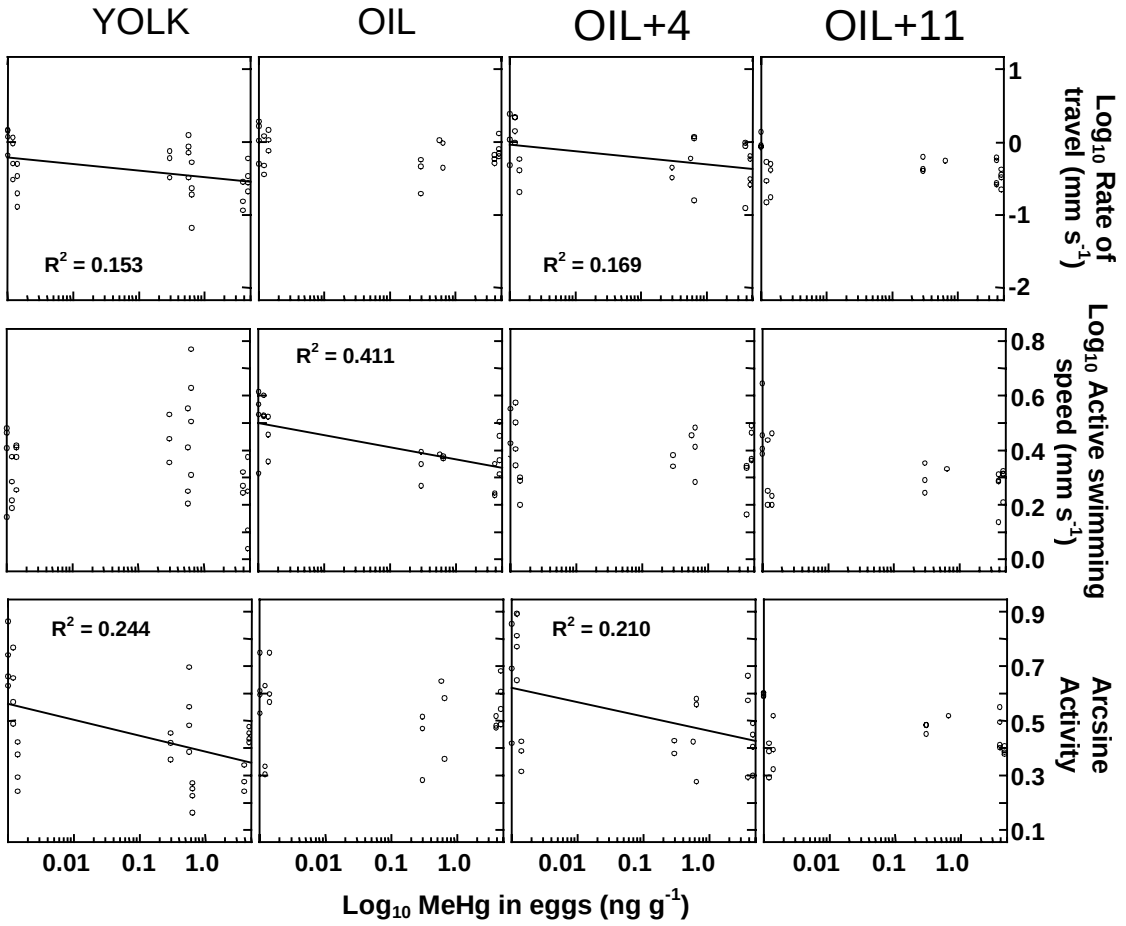

Figure 11. Effect of maternally derived MeHg exposure on routine behavioral traits (rate of travel, active swimming speed, and activity) of Atlantic croaker larvae. Each graph shows a different developmental stage and trait. Traits are shown after statistical transformation to attain normality (logarithmic or arcsine), if needed. Points represent mean values for a dish containing 10 larvae. Only statistically significant regressions ($P < 0.06$) are shown ($N = 98$ dishes).

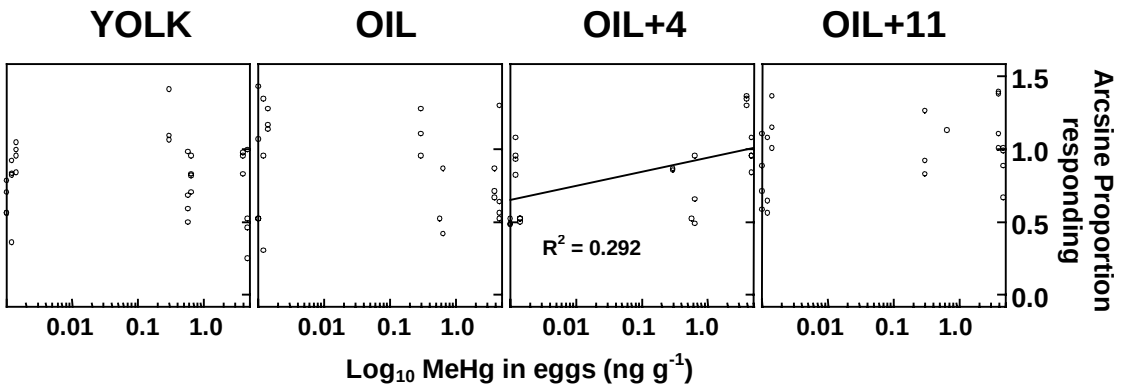

Figure 12. Responsiveness of Atlantic croaker larvae maternally exposed to MeHg to a visual stimulus. Each graph represents a different developmental stage. Arcsine transformation is done to attain normality. Points represent mean values for a dish containing 10 larvae. Only statistically significant regressions ($P < 0.05$) are shown ($N = 98$ dishes).

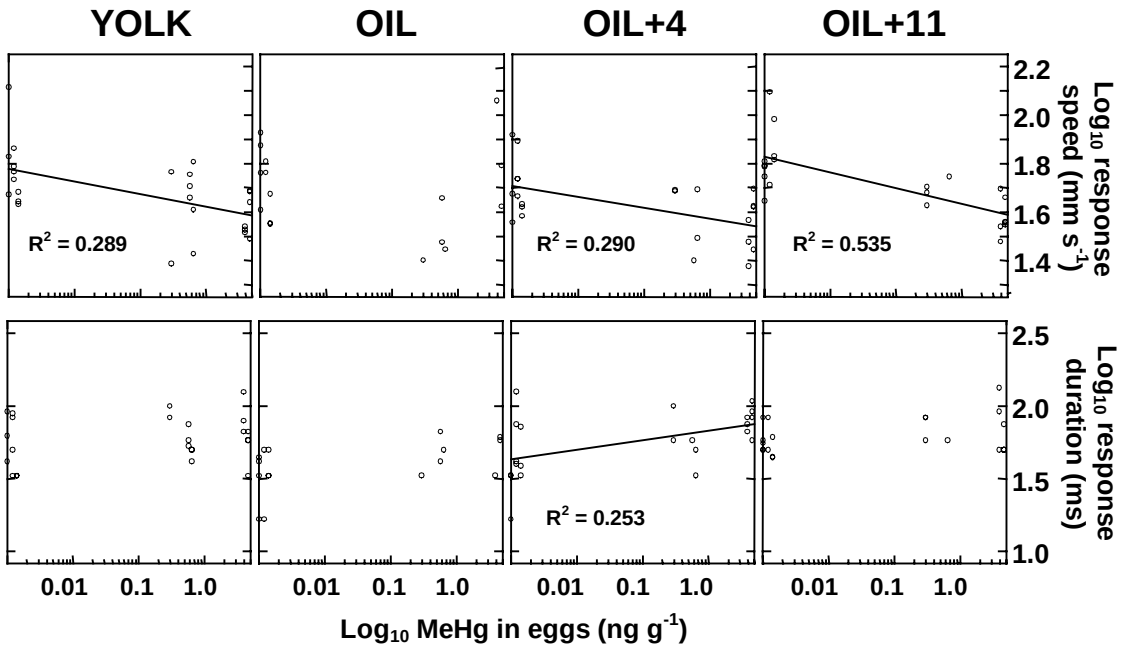

Figure 13. Effect of maternally derived MeHg on response duration and response speed to a vibratory startle stimulus of Atlantic croaker larvae. Each graph represents a different developmental stage. Behavioral traits are logarithmically transformed to attain normality. Points represent mean values for a dish containing 10 larvae. Only statistically significant ($P < 0.05$) regressions are shown (N = 102 dishes).

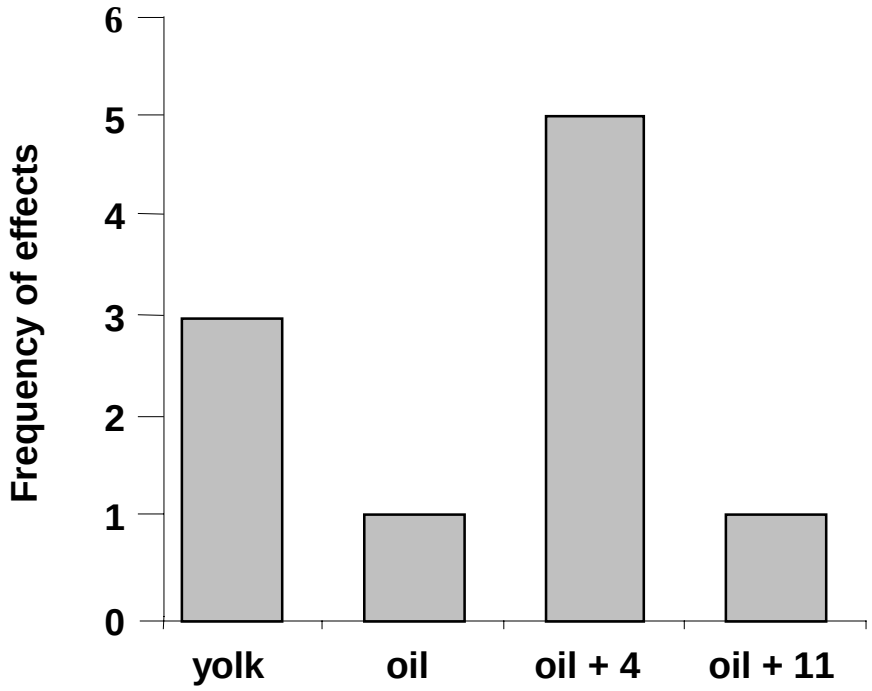

Figure 14. Frequency of significant behavioral effects (of 10 variables with $P \leq 0.05$) observed at the different developmental stages in Atlantic croaker larvae maternally exposed to MeHg.

CHAPTER 5: General conclusions

The common theme in the three studies reported in this dissertation is the use of high-level endpoints to assess the sublethal effects of environmentally realistic levels of contaminants on fish larvae. Results showed that environmentally realistic levels of atrazine are not directly lethal to red drum larvae although they could pose an indirect threat to survival through effects on growth and behavior. Atrazine reduced growth rate, which would increase mortality by prolonging the highly vulnerable larval period. Alterations of routine behavior caused by atrazine exposure could produce a slight foraging advantage for treated larvae by increasing encounter rates with prey, but the same hyperactivity results in a very high energetic burden (a doubling of total metabolic rate). Exposed larvae will need to meet this elevated energetic requirement or suffer reduced growth and possible starvation. Predicted increases in encounter rates with prey of up to 15.7 % might not be enough to meet this additional energetic burden in a patchy and highly variable environment. Moreover, encountering prey does not guarantee ingesting and absorbing the nutrients. In these experiments, fish were fed relatively high rations yet an effect on growth was observed. This suggests that a process between encountering prey and protein growth (e.g., ingestion, digestion, protein metabolism) was altered.

Organophosphate insecticides such as malathion affect normal neuronal function and thus were expected to affect red drum larvae. Unexpectedly, reported levels of malathion did not impair routine behavior, escape behavior, resting metabolic rate, or growth, indicating that current environmental levels may be safe for young fishes. However, malathion reduced larval survival at concentrations $\geq$ 20 $\mu g\ l^{-1}$ and killed all larvae at a concentration of 2000 $\mu g\ l^{-1}$. Therefore, red drum larvae were susceptible to

malathion, but only at levels higher than those used here. However, levels of malathion in surface waters today may be above the levels tested here, since a substantial increase in malathion use has occurred and not many recent reports on malathion levels in surface waters exist.

Maternal transfer of contaminants to offspring is also an important and understudied phenomenon. This study demonstrates that MeHg is transferred from the adult fish diet to the eggs at levels comparable with those expected in the wild. This maternally derived MeHg induced a variety of concentration- and stage-dependent effects on routine and escape behavior that translate into reduced calculated survival of planktonic larvae.

To fully understand the impact of pesticide use on non-target organisms, toxicological studies need to be carried out at environmentally realistic concentrations and include multiple ecologically relevant endpoints. This approach provides better insight into individual- and population-level consequences of exposure than a single or small number of biochemical or physiological endpoints. For this purpose, studies need to include "survival skills," behavioral traits necessary for survival and reproduction (i.e., foraging, antipredator, reproductive, and parental behaviors), together with physiological endpoints such as growth and metabolism. This study uses a comprehensive approach to judge effects of pesticides on organisms and produced clearer ideas of how the contaminants and their modes of exposure would affect individuals and populations in the field.

Appendices

APPENDIX 1: RESPIROMETER LEAKAGE TEST

Tests were done to evaluate the flux of oxygen across the walls and connections of the respiration chambers. Deoxygenated water was prepared by bubbling pure nitrogen gas in 100 ml of deionized water for 40 min. Four respiration chambers were filled up immediately after, immersed in a water bath at 24ºC, and measurements started. Experiments ran for an average of 22.75 ± 2.1 h (± 1 SE). Full (100 %) air saturation was never achieved (Figure 14). Maximum oxygen concentrations reached an average of 90.3 % air saturation (87.4 – 94.2 %) after approximately 19.7 h (Figure 14). The oxygen flux (change in % saturation inside the chamber min^{-1}) was related to the internal oxygen concentration of the chamber (Figure 15). Since measurements of oxygen consumption by larvae (Chapters 2 and 3) were halted when the dissolved oxygen level in the chamber dropped below 70 % of saturation, the flux of oxygen across surfaces of the respiration chambers was considered negligible.

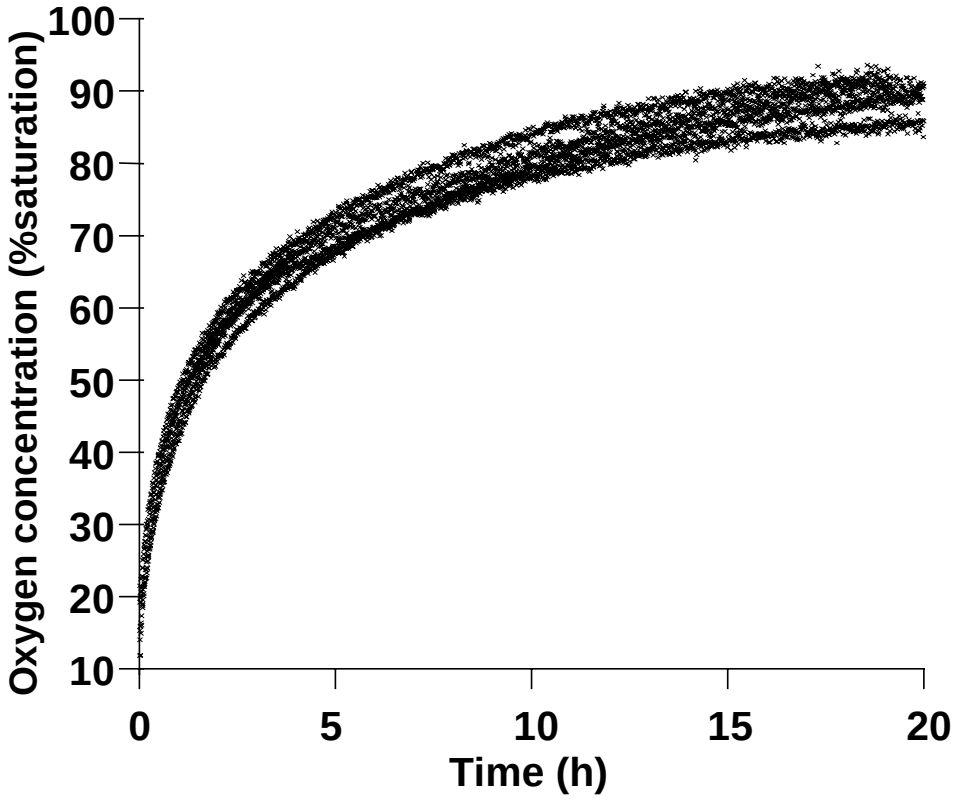

Figure 14. Increase in oxygen concentration (% saturation) within the respiration chambers in a 20-h period. Each data point represents a single measurement. Seven replicate experiments were done, alternating sensors and respiration chambers.

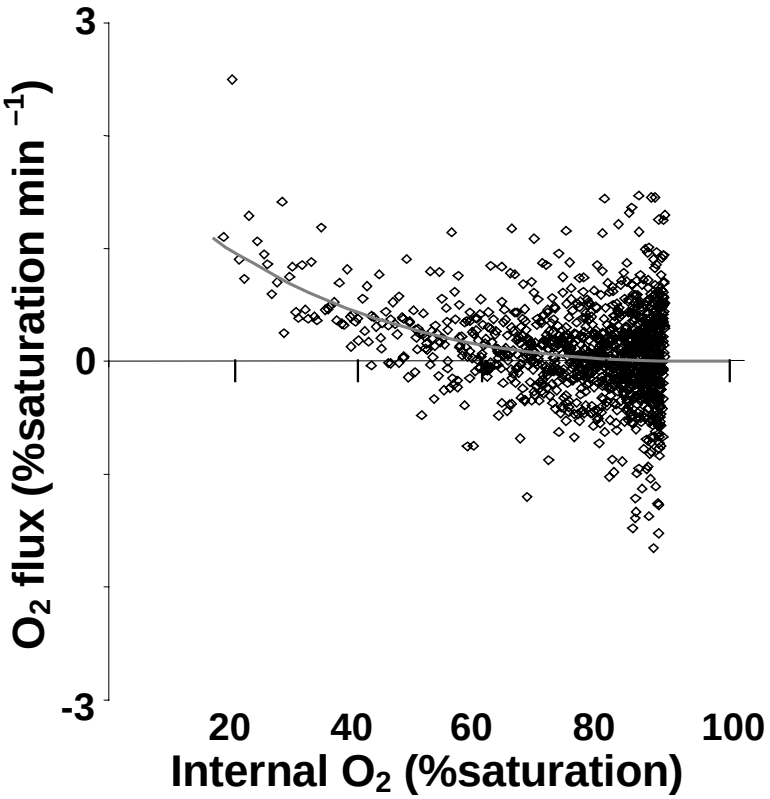

Figure 15. Average oxygen flux (% saturation min^{-1}) in relation to the oxygen concentration in the respiration chamber. Equation for regression line: $Y = -2 \cdot 10^{-06} \cdot X^3 + 0.0005 \cdot X^2 - 0.0524 \cdot X + 1.8384$; ($R^2 = 0.1211$), where Y is oxygen flux and X is oxygen concentration within the chamber. Measurements of oxygen consumption by larvae (Chapters 2 and 3) were halted when the dissolved oxygen level in the chamber dropped below 70 % of saturation.

APPENDIX 2: SURVIVAL AFTER EXPOSURE TO ACETONE

Since acetone was used as carrier for the pesticide in the exposures described in chapters 2 and 3, survival experiments were done to understand whether the levels of acetone used in these experiments were safe for settlement red drum larvae. Groups of 50 settlement size larvae were transferred to 1.5-l exposure watch bowls (20 cm diameter) in a temperature-controlled room. Temperature and salinity were maintained at 27°C and 27 PSU. Acetone was added to the watch bowls 24 h after transfer of the larvae. Acetone concentrations of 0, 8, 10 and 100 µl l^{-1} were tested. Fish were fed a ration of 5 *Atemia* nauplii ml^{-1} d^{-1}. Survival was recorded 96 h after adding acetone and the experiment was done three times. Acetone exposure for 96 h had no effect on survival ($P = 0.89$, Figure 16).

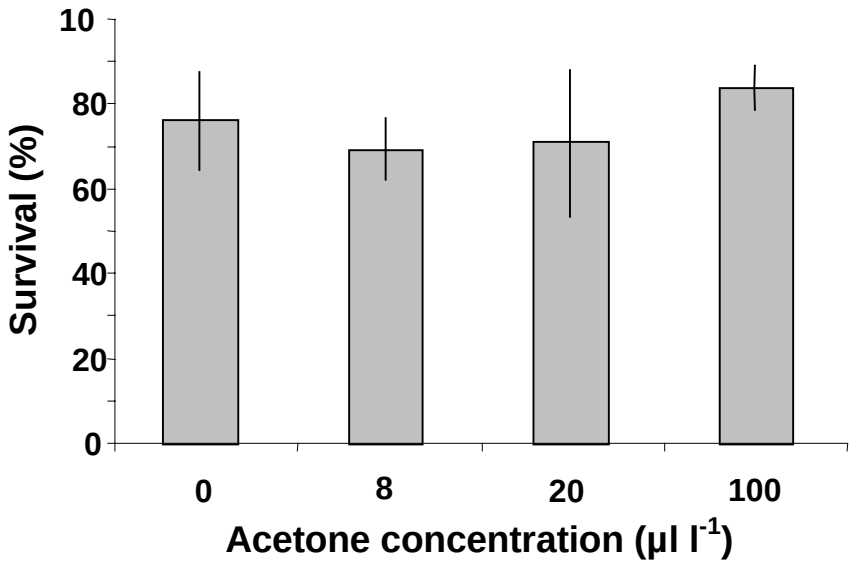

Figure 16. Survival of red drum larvae when exposed to several concentrations of acetone for 96 h. Values represent means $\pm$ 1 SE.

APPENDIX 3: RED DRUM LENGTH-WEIGHT RELATIONSHIP

In order to estimate dry weight of red drum larvae from total length (TL), 90 larvae were measured and dried. Larvae for this experiment (3.3 - 13.5 mm TL) were sampled from rearing tanks in the morning before feeding. Fish were anesthetized using tricaine methansulfonate (MS 222) and digital pictures were taken with the aid of a camera (Sony DCR-TRV350) attached to a dissecting microscope. Total length, to the nearest μm, was measured using an image-processing program (ImageJ, National Institutes of Health). Each larvae was immediately rinsed and placed in an aluminum cups. Cups containing the fish were placed in an oven (65ºC) for 24 h, after which their dry weight was measured to the nearest μg using a precision balance (Mettler Toledo MT5). These data (Figure 17) were described by the equation:

$$DW = 0.0005 \bullet TL^{3.466} \qquad R^2 = 0.9834$$

where DW is dry weight (mg) and TL is total length (mm).

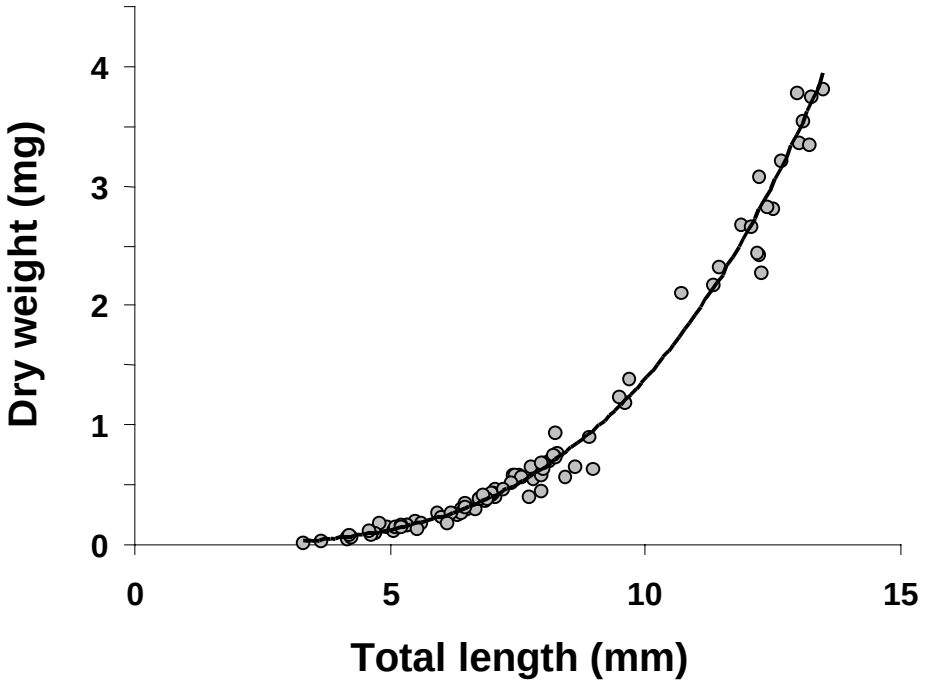

Figure 17. Total length─dry weight relationship for red drum larvae (3.3 – 13.5 mm).

Equation for relationship: $DW = 0.0005 \bullet TL^{3.466}$; $R^2 = 0.9834$.

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Vita

Maria del Carmen Alvarez was born in Quito (Ecuador) on February 16th, 1970, to Alvaro Alvarez and Marie Cecile d'Otreppe de Bouvette. She attended University of Seville (Spain) for her Bachelor's degree where she majored in Biology in September 1993. In April 1998 she received her Master of Science degree form University of Kyoto (Japan), Laboratory of Stock Enhancement Biology, under the supervision of Dr. Masaru Tanaka. She taught multiple laboratories of Introduction to Oceanography for Dr. George Ward in 1999 as a Teaching Assistant for the University of Texas at Austin. In 2004 she taught the laboratory of Biology of Fishes for Dr. Lee Fuiman as a Teaching Assistant for the University of Texas at Austin.

Permanent address: 17 Abades, ESC. A 3-I, 41004 Sevilla, SPAIN

This dissertation was typed by the author.