September, 1998

Neurological Effects on Startle Response and Escape from Predation by Medaka Exposed to Organic Chemicals

Steven P. Bradbury
Richard W. Carlson
Robert A. Drummond
Dean E. Hammermeister

ELSEVIER

Aquatic Toxicology 43 (1998) 51–68

AQUATIC TOXICOLOGY

Neurological effects on startle response and escape from predation by medaka exposed to organic chemicals

Richard W. Carlson *, Steven P. Bradbury, Robert A. Drummond, Dean E. Hammermeister

Mid-Continent Ecology Division, National Health and Environmental Effects Research Laboratory, U.S. Environmental Protection Agency, 6201 Congdon Blvd., Duluth, MN 55804, USA

Received 27 June 1997; received in revised form 3 October 1997; accepted 13 October 1997

Abstract

Simultaneous electrophysiological and behavioral studies were performed on 21–32 day old juvenile medaka (*Oryzias latipes*) exposed at sublethal concentrations to organic chemicals representing various modes of action. Non-invasive recordings were made of the electrical impulses generated within giant neuronal Mauthner cells, associated interneurons and motoneurons, and axial musculature, all of which initiate the startle or 'escape' response in fish. Timing in ms between these electrical sequelae was measured for each fish before and after 24 and 48 h exposure to a chemical. Carbaryl and phenol affected Mauthner cell to motoneuron transmission while chlorpyrifos, carbaryl, phenol and 2,4-dinitrophenol (DNP) showed neuromuscular effects. The variety of neurological effects detected at various concentrations of chemicals tested here suggest that different mechanisms may be responsible. Also noted was the number of startle responses to number of stimuli ratio (R/S); this ratio was affected by most chemicals. Medaka generally appeared to be more susceptible to predation after exposure to chlorpyrifos, carbaryl, fenvalerate, endosulfan, phenol, 1-octanol and DNP. The effects threshold for many of the test compounds was found to be consistent for both the neurophysiological and behavioral endpoints. Consequently, electrophysiological responses of Mauthner cell-initiated startle responses provided a measure of neurological injury that is also directly correlated to a definitive and ecologically relevant behavioral endpoint. Published by Elsevier Science B.V.

Keywords: Medaka; Mauthner cell; Neurotoxicity; Startle response; Predation

1. Introduction

It has been estimated that at least 5% of all industrial chemicals, excluding pesticides, are

* Corresponding author. Tel.: +1 218 7205500; fax: +1 218 7205539; e-mail: eparwc@du4500.dul.epa.gov

0166-445X/98/$ - see front matter. Published by Elsevier Science B.V.
PII S0166-445X(97)00097-0

likely to be neurotoxic. Yet, few of the 70000 distinct chemicals in commerce, or the 1000–1600 new chemicals introduced each year in the United States, have been tested for neurotoxicity (National Research Council, 1992) to support human health risk assessments. Since some of these chemicals may find their way into aquatic ecosystems, neurotoxic responses are also a concern in ecological risk assessments. Hence, there is a need to develop valid, sensitive, and reproducible methods to identify neurotoxic potential, to characterize the nature of neurological effects and to determine the mechanism by which a chemical produces neurotoxic effects (Tilson et al., 1996).

The National Research Council (1992) commented that the greatest challenge in developing test methods for neurotoxicology lay in integrating behavioral observation with neuromorpho-logical, neurochemical, and neurophysiological alterations. Because behavioral processes are an integration of biochemical, physiological and morphological processes, behavioral observations also provide a unique link between cellular and subcellular processes and ecological consequences (Little, 1990). Historically, the preponderance of neurotoxicological research has focused on the mammalian and invertebrate nervous systems. To date, many of the associated electrophysiological techniques involve in vitro or surgically invasive approaches with anesthetized or restrained animals. Although these methods yield considerable data regarding cellular and molecular mechanisms of neurotoxic action, disruption of the central nervous system (CNS) can make it difficult to study behavioral correlates to neurological function.

An increasing variety of in vitro and in vivo test methods with fish models have also been developed (Smith, 1984). One experimental approach involves electrophysiological measurements associated with the startle response, which initiates an escape behavior to elude predation (Featherstone et al., 1991). The startle response is initiated by one of two Mauthner cells located in the hindbrain which receive inputs from receptors for audition, gravity, vibration, angular acceleration, water flow, the visual system, and electric fields. The Mauthner cell (M-cell) axon projects down the length of the spinal cord and, when stimulated, an impulse excites primary and secondary motoneurons and interneurons which, in turn, excite the white muscle on the side opposite of stimulus and inhibits motoneurons and interneurons on the same side as the stimulus. The massive excitation of muscle on the side of the active M-cell axon leads to the classic C-start escape behavior (Fetcho and Faber, 1988).

Because the body wall of larval fish offers low electrical resistance, biopotentials due to nerve impulse conduction within the M-cell axon, motoneurons and interneurons, and the white (fast) musculature can be detected by placing the fish in a small chamber mounted on a printed circuit board electrode grid and using sufficient amplification (Featherstone et al., 1991). This is one of the few cases where there exists a causal connection between the activity of a particular cell in the vertebrate nervous system and a defined behavioral response (Eaton and Hackett, 1984). Because the electrical waveforms are easily triggered and recorded, stereotypical and reproducible, Mauthner-initiated escape responses provide the ability to monitor neurophysiological activity noninvasively from identifiable neural sources in captive but intact, alert, and freely moving animals.

The first objective of the research here was to determine the range and variability of measurable in vivo electrophysiological responses associated with the Mauthner-initiated escape reflex in medaka (*Oryzias latipes*) exposed to a series of chemicals representing a variety of toxic mechanisms. The second objective of the study was to compare dose-dependent electrophysiological responses to chemical-specific mortality thresholds and predator avoidance responses to evaluate sublethal and lethal toxic effects.

2. Materials and methods

2.1. Neurotoxicity experiments

Test chemicals were selected based on mode of action as identified previously through fish acute toxicity syndromes (FATS) testing (McKim et al., 1987a; Bradbury et al., 1989a, 1991), behavioral toxicity syndromes (Drummond and Russom,

Table 1
Test chemical characteristics and toxicity

Chemical	CAS number	Chemical class	Mode of action	48 h LC_{50}^{a} (mg l^{-1})
Chlorpyrifos	2921-88-2	Organophosphate	AChE inhibition[b]	0.3
Carbaryl	63-25-2	Phenylcarbamate	AChE inhibition[b]	9.4
Strychnine	60-41-3	Heterocyclic alkaloid	Glycine antagonism[c]	>2.0
Endosulfan	115-29-7	Cyclodiene	Cl^- inhibition in GABA-ergic neurons[d]	0.0015
Fenvalerate	51630-58-1	Type II pyrethroid	Voltage-sensitive Na^+ channel interference[e]	0.0016
Phenol	108-95-2	Benzene ring	Narcosis II[f]	28.0
1-octanol	111-87-5	Alkane alcohol	Narcosis I[g]	21.0
2,4-DNP	51-28-5	Substituted benzene ring	Oxidative phosphorylation uncoupling[h]	11.0

[a] Values estimated from static acute toxicity tests with 24 day old medaka.
[b] Fukuto (1990).
[c] Murphy (1986).
[d] Coats (1990).
[e] Bradbury and Coats (1989).
[f] Bradbury et al. (1989a).
[g] Veith et al. (1983).
[h] Terada (1990).

1990), or the literature (Table 1). Chemical concentrations reported in the text refer to measured values (Table 2).

2.1.1. Animals and toxicity

Eggs obtained from spawning medaka were hatched and the young raised in uncontaminated Lake Superior water (LSW) at the test temperature of 24 ± 1°C, total hardness as $CaCO_3$ between 40–46 mg l^{-1}, alkalinity as $CaCO_3$ of 39–42 mg l^{-1}, and a pH at 7.8 ± 0.2. The juvenile fish were fed live brine shrimp twice daily. Medaka used in electrophysiological experiments averaged 9.2 ± 0.8 mm total length ($n = 463$) and weighed approximately 3.2 mg ($n = 30$). Using a 24-day-old medaka, LC_{50} values were estimated from acute toxicity tests conducted prior to electrophysiological experiments. Range finding tests used 3–4 measured concentrations with ten fish/concentration, each fish placed in an individual chamber containing 100 or 150 ml test solution for 48 h. An estimated LC_{50} value was based on mortality observed at 48 h.

2.1.2. Apparatus and instrumentation

The test apparatus for monitoring electrophysiological events included an electrode chamber similar to that described by Featherstone et al. (1991). The fish chamber was a 2.5 mm W $\times$ 15 mm L opening cut into the center of 3 mm thick plexiglass placed on a printed circuit board that had electrodes etched 0.5 mm apart. The chamber was positioned longitudinally perpendicular to the electrodes and attached to a laboratory stand so that the chamber could be viewed under a 0.7–3.0 $\times$ binocular dissecting scope. Light was supplied to the chamber by a 36 inch fiber optic light pipe attached to a low noise illuminator. Signals detected with the electrodes were connected to preamplifiers with shielded lead wires. Four preamplifiers were built into one chassis with each capable of amplifying the incoming signal 100 $\times$. A D.C. power supply was constructed to provide a regulated, filtered source of 12 V D.C. to power the preamplifiers. The preamplifier unit, microscope, and recording chamber were placed inside a stainless steel Faraday cage to minimize electrical interference from external sources.

Biopotentials of approximately four micro volts and larger were differentially detected from the electrode grid by a pair of electrode leads spaced 1 mm apart and amplified more than 10000 $\times$ in two stages of amplification. This signal was combined in parallel with an identically obtained sig-

Table 2
Effects of chemicals on survival, Mauthner cell characteristics, and consumption times of medaka (*Oryzias latipes*) as prey at 24 h exposure

Chemical	Conc. (mg l^{-1})[a]	*M* (%)	T1 (ms)	T2 (ms)	T3 (ms)	*R*/*S* (%)	CT (s)
Chlorpyrifos	0.0[b]	0	0.53 ± 0.03[c]	0.19 ± 0.03	0.71 ± 0.03	52.4 ± 15.1	30.9 ± 10.5
	0.03	0	0.52 ± 0.02	0.22 ± 0.03**	0.73 ± 0.04	64.2 ± 25.2	21.9 ± 5.5
	0.06	0	0.53 ± 0.04	0.23 ± 0.03**	0.76 ± 0.03**	69.5 ± 18.2	nt
	0.12	0	0.50 ± 0.03	0.28 ± 0.03**	0.77 ± 0.03**	53.8 ± 11.1	24.4 ± 0.6
	0.20	0	0.53 ± 0.05	0.26 ± 0.04**	0.79 ± 0.04**	47.7 ± 25.8	15.4 ± 1.3
	0.27	50	0.52 ± 0.02	0.30 ± 0.02**	0.82 ± 0.03**	31.2 ± 25.5	nt
Carbaryl	0.0	0	0.55 ± 0.02	0.18 ± 0.02	0.73 ± 0.03	60.1 ± 14.8	20.0 ± 7.1
	2.5	0	0.52 ± 0.02	0.19 ± 0.03	0.74 ± 0.04	73.6 ± 13.0	18.0 ± 5.5
	5.1	0	0.55 ± 0.03	0.24 ± 0.04**	0.80 ± 0.03**	70.4 ± 11.0	14.9 ± 3.9
	7.0	10	0.57 ± 0.05	0.24 ± 0.03**	0.81 ± 0.04**	83.9 ± 13.1**	20.1 ± 6.0
	9.4	67	0.61 ± 0.05*	0.26 ± 0.04**	0.87 ± 0.04**	72.2 ± 16.4	33.0 ± 13.8
Strychnine	0.0	0	0.56 ± 0.02	0.18 ± 0.02	0.74 ± 0.03	66.7 ± 16.5	29.5 ± 15.8
	0.4	0	0.59 ± 0.03	0.19 ± 0.02	0.78 ± 0.03**	74.0 ± 18.0	19.5 ± 7.7
	0.8	0	0.59 ± 0.04	0.18 ± 0.04	0.77 ± 0.04	88.4 ± 18.4**	46.9 ± 21.0
	1.6	0	0.60 ± 0.08	0.17 ± 0.05	0.77 ± 0.05	89.8 ± 13.0**	38.2 ± 15.7
Endosulfan	0.0	0	0.53 ± 0.03	0.16 ± 0.02	0.69 ± 0.03	64.1 ± 18.3	19.0 ± 7.6
	0.0005	0	0.55 ± 0.02	0.17 ± 0.02	0.72 ± 0.02	48.1 ± 24.3	16.8 ± 5.8
	0.0010	10	0.52 ± 0.02	0.15 ± 0.02	0.67 ± 0.02	72.8 ± 17.9	17.5 ± 6.9*
	0.0015	20	0.55 ± 0.02	0.16 ± 0.02	0.72 ± 0.03	90.2 ± 10.5**	17.3 ± 6.5
Fenvalerate	0.0	0	0.52 ± 0.02	0.16 ± 0.02	0.68 ± 0.03	60.2 ± 18.2	28.7 ± 9.2
	0.0004	0	0.53 ± 0.02	0.16 ± 0.02	0.69 ± 0.03	44.4 ± 13.8	nt
	0.0009	5	0.53 ± 0.02	0.17 ± 0.02	0.70 ± 0.02	50.9 ± 20.9	22.0 ± 6.2*
	0.0013	0	0.52 ± 0.03	0.16 ± 0.02	0.68 ± 0.04	61.4 ± 17.5	nt
	0.0016	50	0.54 ± 0.01	0.17 ± 0.02	0.71 ± 0.02	60.5 ± 11.1	22.6 ± 5.0
Phenol	0.0	0	0.51 ± 0.04	0.17 ± 0.03	0.67 ± 0.03	50.9 ± 19.3	24.6 ± 9.3
	10.9	0	0.59 ± 0.07	0.19 ± .05	0.78 ± 0.05	nm	nt
	12.9	nt	nt	nt	nt	nt	17.3 ± 6.6
	15.1	0	0.55 ± 0.02*	0.18 ± 0.02	0.73 ± 0.03**	56.5 ± 24.9	nt
	20.6	0	0.62 ± 0.06**	0.16 ± 0.04	0.78 ± 0.05**	26.7 ± 15.2*	nt
	23.6	nt	nt	nt	nt	nt	17.5 ± 3.3
	25.9	20	0.62 ± 0.03**	0.14 ± 0.03**	0.75 ± 0.02**	51.0 ± 22.2	nt
	32.0	40	0.70 ± 0.04**	0.12 ± 0.03*	0.82 ± 0.05**	52.5 ± 21.8	nt
1-octanol	0.0	0	0.53 ± 0.03	0.16 ± 0.02	0.69 ± 0.04	56.7 ± 15.8	22.2 ± 8.4
	3.9	0	0.51 ± 0.04	0.16 ± 0.02	0.67 ± 0.04	61.2 ± 21.9	21.8 ± 10.3
	7.8	0	0.54 ± 0.03	0.14 ± 0.02	0.65 ± 0.05	17.6 ± 19.8**	14.5 ± 3.7*
	17.8	10	ns	ns	ns	0.0**	16.3 ± 5.3*
2,4-DNP	0.0	0	0.49 ± 0.03	0.17 ± 0.03	0.65 ± 0.04	47.9 ± 19.4	24.0 ± 8.1
	7.5	0	0.51 ± 0.03	0.15 ± 0.03	0.66 ± 0.03	51.8 ± 11.9	nt
	10.0	5	0.53 ± 0.05	0.14 ± 0.02**	0.67 ± 0.04	46.8 ± 27.9	17.5 ± 6.9*
	11.2	0	0.51 ± 0.04	0.13 ± 0.04	0.64 ± 0.03	32.8 ± 16.7	nt
	14.5	0	ns	ns	ns	0.0**	nt

M, percent mortality in electrophysiological experiments; T1, time from Mauthner cell peak to motoneuron peak; T2, time from motoneuron peak to start of muscle activity; T3, total time from, Mauthner cell peak to start of muscle activity; *R*/*S*, response to stimuli ratio; CT, bluegill consumption time of medaka prey; nt, not tested; nm, not measured; ns, no measurable signal.
[a] Measured starting (0 h) concentrations.
[b] Controls.
[c] Mean ± S.D.
* Significantly different compared to controls at $P<0.05$;
** $P<0.01$. ANOVA and Dunnett's compared treatments vs grouped controls for T1, T2, T3, and *R*/*S*. A paired difference *t*-test compared CT for exposed medaka vs CT for individual test controls (CT control values in table are average of all control groups per chemical).

nal from electrodes interlaced with the first pair. The resultant signal was led through a line filter to eliminate high frequency noise, and the final output fed into a Tektronix 2221A Digital Storage Oscilloscope (DSO) on two channels. The signal from each channel was internally added by the oscilloscope and displayed. The oscilloscope was set to trigger on the first spike of the electromyographic (EMG) portion of the signal when the fish was stimulated and digitized a 2 Kb record each of electrical events preceding and following this spike. Time measurements from the M-cell peak to the motoneuron peak (T1) and from that point to the onset of EMG activity (T2) were read from the DSO display. Periodically, the stored signal waveform was printed on a Tektronix HC100 plotter which was connected to the DSO via a RS-232-C interface. This was done so that we could examine the signal waveforms for qualitative differences in peak amplitude, which may be influenced by a number of uncontrollable variables such as fish position in the chamber or inconsistent tactile stimulus as described below.

2.1.3. Test procedures

Medaka, 21–25 days old, were selected for electrophysiological testing and transferred via pipette one at a time into the electrode chamber. Excess water was removed and the size of the chamber adjusted so that the animal was allowed some movement and suspended in water just above the electrodes. Fish were not fed during testing. Connecting leads from the preamplifiers were attached to the electrodes nearest the region just posterior to the gills of the fish for maximum signal detection. The fish were stimulated by touching the caudal region with a fine-tipped glass rod, which was done enough times to obtain five baseline recordings of the stereotypic mono- and biphasic biopotentials associated with M-cell, motoneuron, and EMG activity. A count was maintained of the number of stimuli required to obtain five recordings so that a response to stimuli ratio (R/S) was monitored. A response was not analyzed if any component of the waveform was missing, and if a fish failed to respond at least 30% of the time, it was judged unresponsive and discarded. After the initial recordings, the fish was removed from the chamber and placed into an individual exposure container. Except as noted below, each test consisted of ten fish exposed to a toxicant concentration with another group of five serving as controls. Static exposure was continued for 48 h in 100 or 150 ml of test solution or control water. At 24 and 48 h exposure the fish were again pipetted into the recording chamber one at a time and retested for responses in the same sequence as was done to collect baseline values. The 24 h measurements are reported since in preliminary studies this exposure period was adequate for the expression of neurophysiological effects. To minimize potentially confounding responses due to dropping exposure levels with time, 48 h responses were not considered as reliable. However, these measurements were used to evaluate response trends. After the 48-h measurement, the fish was killed and total length measured. The test was repeated for each concentration of test chemical. During the course of experimentation, independent control tests were conducted under conditions identical to exposure experiments, once with five fish and three times with ten fish, to ascertain the stability of baseline values.

2.1.4. Statistical procedures

T3 values were determined by adding T1 and T2 values. The values obtained for T1, T2 and T3 were each grouped for each set of exposed fish and pre- and post-treatment means and standard deviations were calculated ($n = 50$, since there were five measurements/fish and ten fish/concentration). The number of responses (usually five) and number of stimulations required were recorded and a response to stimuli percentage (R/S) was calculated for each fish; these values were then grouped for each set of exposed fish and pre- and post-treatment means and standard deviations were calculated ($n = 10$). Arcsine transformation was performed on values for R/S before statistical analysis. Percentage change from baseline (0 h) values was also calculated for each variable for the 24- and 48-h measurements for each group of exposed fish to permit graphical comparisons. ANOVA and Dunnett's procedure was used to assess significance between treatments

and controls, $\alpha = 0.05$ and 0.01. A paired difference t-test was used to make statistical comparisons between pre- and post-treatment values for each group of fish to verify that the 24 and 48 h values were not only significantly different from control values as determined by ANOVA and Dunnett's procedure, but also significantly different from the baseline (zero h) measurements for each group of exposed fish.

For statistical comparisons, the values for each measurement made with control fish run concurrently with each concentration were grouped for each chemical. Hence, the number of control fish used (15–27) when comparing treatments to controls varied dependent upon the number of control fish used and the number of concentrations tested for a particular chemical. Most tests used five control fish, but some concentrations of chlorpyrifos, phenol and 2,4-dinitrophenol (DNP) were run with only three or four controls. Also, at the end of testing, all control fish from each toxicant test plus independent control tests were grouped ($n = 201$) to provide an estimate of normal values and variability of the various measurements for the entire population.

2.2. Predator/prey experiments

2.2.1. Animals and test apparatus

Apparatus for these experiments consisted of six 15 cm W $\times$ 30 cm L $\times$ 20 cm H glass aquaria, each containing 4.5 l of water and one bluegill (*Lepomis maccrochirus*) as the predator. Each aquarium was shielded on three sides with black foam insulation. Aerated Lake Superior water flowed through each tank at 125 ml min^{-1} to maintain oxygen levels near saturation. Lighting was provided by a single 40 watt fluorescent tube located above and slightly in front of a bank of three aquaria. The bluegill were second year pond-raised juveniles obtained from Rademacher Ponds, Waconia, MN, and fed a minimum ration of dry food and live juvenile medaka. Live medaka were fed to the bluegills in the same manner as that used in the assays. The same bluegills served as predators for all experiments to minimize variability and eliminate training new predator groups to accept prey as described below. At the end of testing, the bluegills averaged 14.6 ± 0.2 cm total length and weighed 48.5 ± 2.4 g.

2.2.2. Test procedures

Juvenile medaka 25–32 days old served as the prey species and were obtained from the same stock of fish used in the neurotoxicity experiments. Test fish averaged 12.6 ± 1.4 mm total length and weighed 15.2 ± 0.5 mg. Ten medaka were placed in each of six control and six exposure containers, which were 1-l beakers filled to capacity. Predator/prey tests were conducted simultaneous to neurotoxicity experiments. Thus, exposure concentrations were the same (Table 2), except for the phenol assay. In this case, the predator/prey tests were run at a later date and the test concentrations differed slightly from those used in neurotoxicity tests. Also, fewer concentrations of chlorpyrifos, fenvalerate, phenol and DNP were used in predator/prey tests.

Medaka were exposed 24 h, consistent with the primary data gathering period in the Mauthner cell assay, and then transferred to smaller beakers containing 150 ml of freshwater. Each test consisted of two feeding rounds to reduce bias. Three of the six individual bluegill predators were each presented with one group of ten control medaka, the other three bluegills were each given one group of ten exposed medaka. The process was reversed for the second round. Each group of medaka was poured quickly from the 150 ml holding beaker into an aquarium where they dispersed rapidly except for severely affected fish. The number of seconds required for each bluegill to consume the group of ten was recorded before the next fish received prey. If a predator did not consume all fish within 100 s, the result was rejected. A t-test was used to determine if consumption times differed from those expected for exposed versus control prey at the 95% level.

2.3. Chemistry methods

2.3.1. Toxicant stock solution preparation

Toxicant exposure solutions were prepared in LSW by using a glass wool saturator, a liquid saturator, a carrier solvent or a slow stir method.

Glass wool saturators (Kahl et al., 1998) were used to generate toxicant super stock solution for fenvalerate (purity = 98%; RS + SR enantiomers = 47.6% and RR + SS enantiomers = 52.4% of the total mixture; obtained from Chem Service, West Chester, PA) and chlorpyrifos (99.7% pure, Dow Chemical, Midland, MI) of 0.090 and 0.850 mg l^{-1} respectively. A liquid saturator (Kahl et al., 1998) was used to produce a stock solution for 1-octanol (99 + %, Aldrich Chemical, Milwaukee, WI) of 500 mg l^{-1}. Stock solutions of carbaryl (99% Technical Grade, Union Carbide, New York, NY), endosulfan (99 + %, alpha = 78.0%, beta = 22.0%, obtained from Cincinnati, OH, as EPA Reference Material 64-1) and strychnine (2:1) hemisulfate (98%, Sigma, St. Louis, MO) were prepared with reagent grade *N*,*N*-dimethylformamide (DMF) as the carrier solvent at 80000, 45.8 and 25000 mg l^{-1}, respectively. DMF concentration did not exceed 200 mg l^{-1} in any exposure solution. Stock solutions of phenol (99 + %, Aldrich Chemical), 2,4-DNP (99%, Aldrich), and DMF were produced at 1000, 1000, and 200 mg l^{-1}, respectively, using the slow stir method. All stock solutions were diluted to the desired exposure concentration with LSW. Phenol and 2,4-DNP solutions required adjustment to pH 7.8 using HCL or NaOH.

2.3.2. Analytical methods

Methods involved organic solvent extraction followed by ECD/GC analysis or direct aqueous injection FID/GC or UV/HPLC. Aliquots of assay water were taken at 0, 24, and 48 h. Spike and duplicate samples represented at least 5% of total sample number. Fenvalerate, chlorpyrifos and endosulfan were extracted with hexane. GC analysis was accomplished using splitless injection on a Hewlett Packard 5890 Series II ECD/GC equipped with a J&W DB-5 fused silica capillary column, 30 M × 0.32 mm ID with 0.25 μm phase thickness; H2 carrier gas at 13 psi and an ECD with N2 makeup at 30 ml min^{-1}. Thermal gradient varied but was from 60 to 280°C employing injector and detector temperatures of 250 and 300°C (340°C for fenvalerate), respectively. 1-octanol was analyzed with a Hewlett Packard 5890 Series II FID/GC. Injector, column and detector temperature were 250, 190 and 300°C, respectively. The carrier gas was N_2 at 30 ml min^{-1} and a 4 M × 2 mm ID glass column packed with 60–80 mesh Tenax GC was used. Carbaryl, strychnine, phenol and 2,4-DNP were analyzed by reverse phase (C18) isocratic HPLC with UV detection wavelengths of 280, 255, 269 and 269 nm, respectively. Carbaryl standards and samples were in 5 mM sodium acetate to retard degradation and eluted with 70/30 methanol/water mobile phase. Strychnine analysis employed a 20 mM potassium dihydrogen phosphate, 16% acetonitrile mobile phase (pH 3.0). Phenol was eluted using a 50/50 methanol/water mixture. Mobile phase for 2,4-DNP was 200 mM sodium acetate, in 22/78 methanol/water (pH 5.8). Spike recovery for all chemicals ranged from 94 to 115%.

3. Results

Electrophysiological results were based on values obtained from measurements made at 24 h into the 48 h exposure period. Except where noted, values at 48 h were similar to those at 24 h.

3.1. Control responses

Control fish displayed stereotypical behaviors. Stimuli elicited activity consisting of strong, rhythmic, side-to-side swimming motions and turning maneuvers. Depending upon the number of stimuli required, about 5–10 min was needed to obtain five suitable recordings of M-cell initiated signal waveforms. Fig. 1 shows normal escape activity in medaka when one of the Mauthner cells fired due to an external stimulus. The baseline, or no activity level, was interrupted by the first large spike representing the C-shaped body bend, which was followed by side-to-side swimming movements shown as repetitive bursts of EMG activity. Increased amplification of electrical activity and extension of the DSO *X*-axis occurring just prior to the escape activity resulted in the typical waveform recorded during these studies (Fig. 2) where (A), (B), and (C) are associated with the Mauthner cell action potential, the

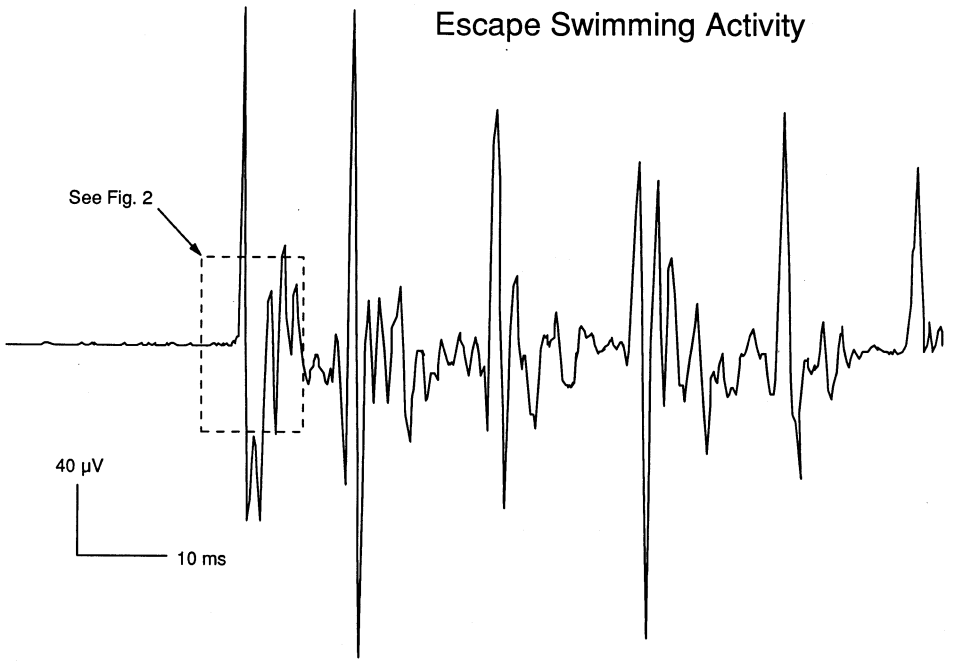

Fig. 1. Non-invasive recording of electrical activity present during escape response after stimulus in medaka (*Oryzias latipes*). Bursts of activity represent swimming movements. Dashed lines delineate area seen in Fig. 2 of typical waveform recorded in these studies.

motoneurons and interneurons compound potentials, and start of EMG activity, respectively. The time measurements (ms) made in these studies were based on the distances between these peaks; T1, T2 and T3 were from peaks A to B, B to C, and A to C, respectively.

For all control fish ($n = 201$), T1 averaged 0.53 ± 0.03 ms at the start of exposures and at 48 h averaged 0.52 ± 0.03 ms. T2 averaged 0.17 ± 0.02 ms in control fish, remaining constant throughout 48 h tests. Total delay time from the M-cell peak to the start of muscular activity (T3) averaged 0.70 ± 0.04 ms. Controls responded to tactile stimuli $62.1 \pm 15.5\%$ of the time at 0 h, and $58.5 \pm 17.2\%$ after 48 h. 0 h measurements showed that approximately 25% of the medaka selected for testing were unresponsive because a Mauthner cell did not fire to initiate an escape response consistently ($R/S < 30\%$).

In the predator/prey tests, the mean consumption time of ten prey in each of 124 control situations was 23.8 ± 9.0 s. All bluegill predators survived to the end of testing, appeared to be in good health, and showed no aberrant behaviors that would suggest toxicant loading due to the consumption of contaminated medaka.

3.2. Chlorpyrifos and carbaryl

Estimated LC_{50}s showed that chlorpyrifos was considerably more toxic to medaka than carbaryl (Table 1). Medaka exposed to these AChE inhibitors were slow to react to handling and displayed rapid body spasms when stimulated. Fish exposed to 0.27 mg l^{-1} chlorpyrifos also displayed pronounced body bends. Recorded signal waveforms showed that chlorpyrifos attenuated both the M-cell and motoneuron peaks in 0.20 mg l^{-1}, while 9.4 mg l^{-1} carbaryl dampened and prolonged the motoneuron peak.

Chlorpyrifos had little effect on T1, whereas carbaryl tended to increase T1 in higher concentrations (Table 2). T1 significantly increased to 0.61 ± 0.05 and 0.73 ± 0.06 ms at 24 and 48 h exposure to 9.4 mg l^{-1} carbaryl, respectively. The AChE inhibitors had a dramatic and significant

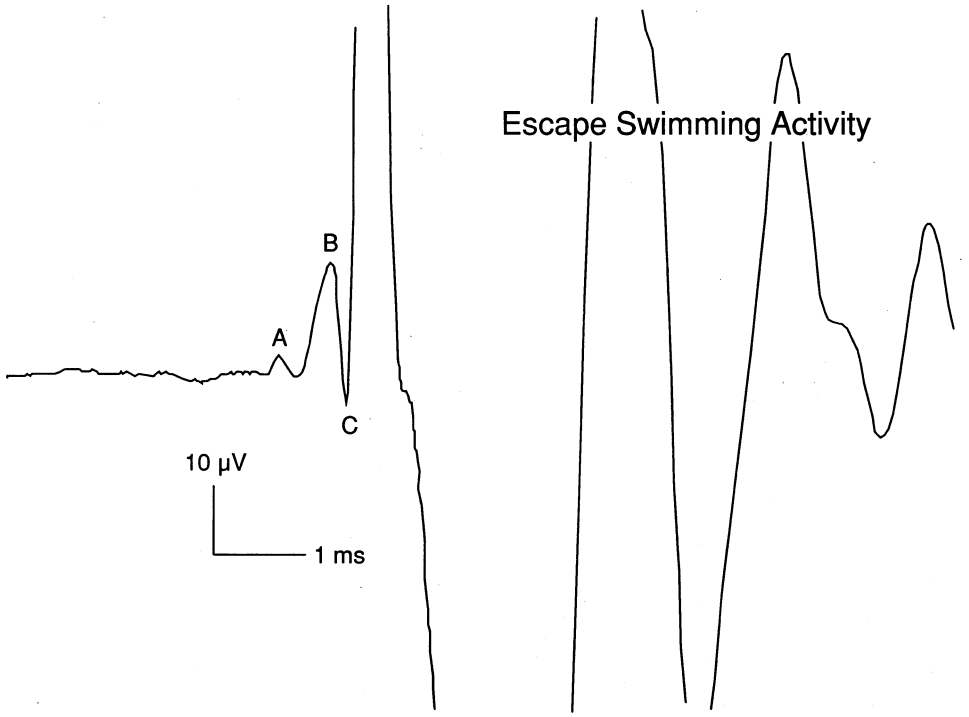

Fig. 2. Detail of electrical activity present during the beginning of startle response and initiation of escape behavior. Labels represent: (A) Mauthner cell action potential, (B) motoneuron compound biopotential, and (C) start of muscle fiber compound biopotentials. Time measurements include: (T1 = A–B) Mauthner cell to motoneuron synaptic delay, (T2 = B–C) neuro-muscular delay, and (T3 = A–C) total time from Mauthner peak to start of swimming activity.

($P < 0.01$) effect on neuro-muscular delay (T2, Fig. 3). After 24 h exposure, chlorpyrifos increased T2 by 24% to 0.22 ± 0.03 ms at the lowest test concentration (0.03 mg l^{-1}) and by 73% to 0.30 ± 0.02 ms at the highest concentration (0.27 mg l^{-1}). Similarly, T2 increased by 33, 30 and 43% at 5.1, 7.0 and 9.4 mg l^{-1} carbaryl, respectively. Both chemicals displayed increases in the M-cell to muscular delay time (T3) proportional to those for T2.

Medaka became significantly more responsive to touch in lower concentrations of chlorpyrifos and carbaryl, and less responsive in near lethal concentrations. The response to stimuli rate (R/S) increased in fish exposed to the three lowest concentrations of both chemicals after 24 h, but significantly so only at 7.0 mg l^{-1} carbaryl (Table 2). After 48 h exposure to chlorpyrifos, R/S was 90, 85 and 70% in 0.03, 0.06 and 0.12 mg l^{-1}, respectively, but decreased to 45 and 41% at 0.20 and 0.27 mg l^{-1}, respectively. The latter were similar to 24 h values (Table 2). Fig. 3 shows these changes as percentage change from baseline control values. For carbaryl, R/S was the same at 24 and 48 h exposure at 2.5 mg l^{-1}, but increased to 70 and 84% at 24 h and to 80 and 86% at 48 h in 5.1 and 7.0 mg l^{-1}, respectively. In 9.4 mg l^{-1}, R/S was 72% at 24 h but decreased to 33% at 48 h.

While not statistically significant, prey consumption time decreased in all test concentrations of chlorpyrifos. Prey exposed to 5.1 mg l^{-1} carbaryl were consumed in 14.9 ± 3.9 s whereas control fish survived 21.2 ± 8.7 s. There were no differences between control groups and exposed prey at 2.5 or 7.0 mg l^{-1} carbaryl. At 9.3 mg l^{-1} carbaryl there was an increase in consumption time.

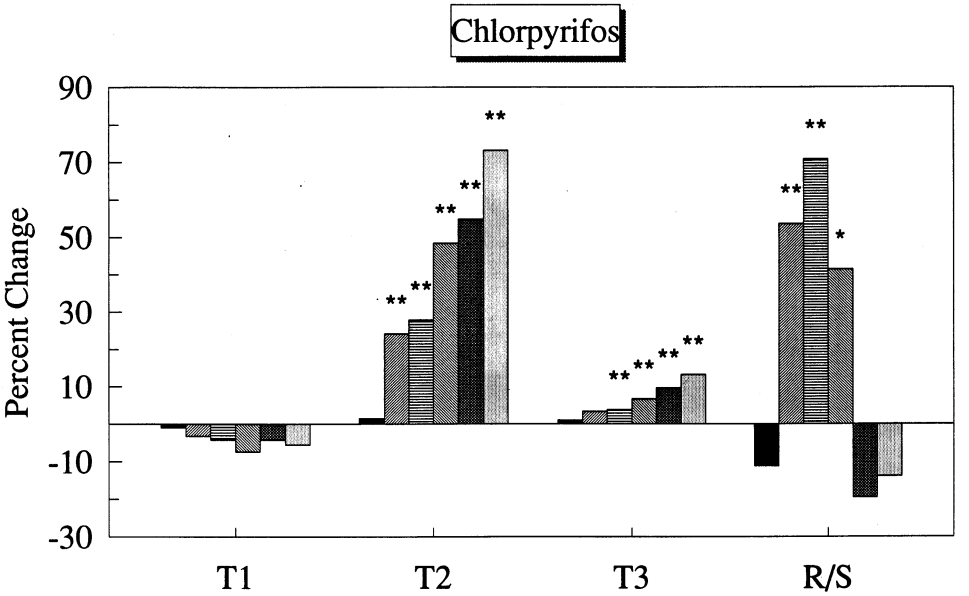

Fig. 3. Percentage change in Mauthner cell to motoneuron synaptic delay (T1), neuro-muscular delay (T2), and total time from Mauthner peak to start of swimming activity (T3) at 24 h exposure, and the response to stimuli ratio (R/S) at 48 h from background (0 h) values for medaka. Concentrations represented by each bar are (left to right): 0.0 (control), 0.03, 0.06, 0.12, 0.20 and 0.27 mg l^{-1} chlorpyrifos. * Significantly different from background values at $P < 0.05$; ** $P < 0.01$.

3.3. Strychnine

Although an LC_{50} value for strychnine was not determined, in acute toxicity tests fish showed signs of imminent death at 2.0 mg l^{-1} strychnine. Most fish at 1.6 mg l^{-1} strychnine demonstrated a total loss of equilibrium and usually laid on their side or upside down in the electrophysiological test chamber. Along with behavioral alterations, higher concentrations of strychnine caused bent backs or swollen areas around the spine in most fish. Strychnine caused convulsions and spasms, but fish demonstrated little ability to move even though electrical activity was recorded. In lower test concentrations strychnine produced very strong motoneuron peaks with enhanced amplitude.

Strychnine increased T1 in all concentrations and T2 in the lowest concentration, but not significantly in either case. However, T3 was significantly increased at the lowest concentration (0.4 mg l^{-1}), but there was no effect on T3 at the two higher concentrations (Table 2). The fish were more responsive in high or near-lethal concentrations and barely affected at low concentrations, with the R/S ratio being similar at both 24 and 48 h exposure (Table 2).

Prey consumption time tended to increase in 0.4 and 0.8 mg l^{-1} strychnine, but was comparable to control fish survival at the highest level (1.6 mg l^{-1}). Consumption times between tests were more variable for strychnine than other chemicals. For exposed versus control fish, consumption times were 19.5 vs 15.0, 46.9 vs 31.6 and 38.2 vs 41.3 s in 0.4, 0.8 and 1.6 mg l^{-1}, respectively. The value in Table 2 for average control consumption time (29.5 ± 15.8 s) reflects this variability.

3.4. Endosulfan

Endosulfan was the most toxic chemical studied (Table 1) and induced a variety of neurological effects. Fish exposed to endosulfan were hyperactive when disturbed, tending to swim rapidly in the exposure container. Rapid body spasms were obvious when stimulated and, similar to strychnine, the signal waveform showed that endosulfan produced very strong motoneuron peaks with en-

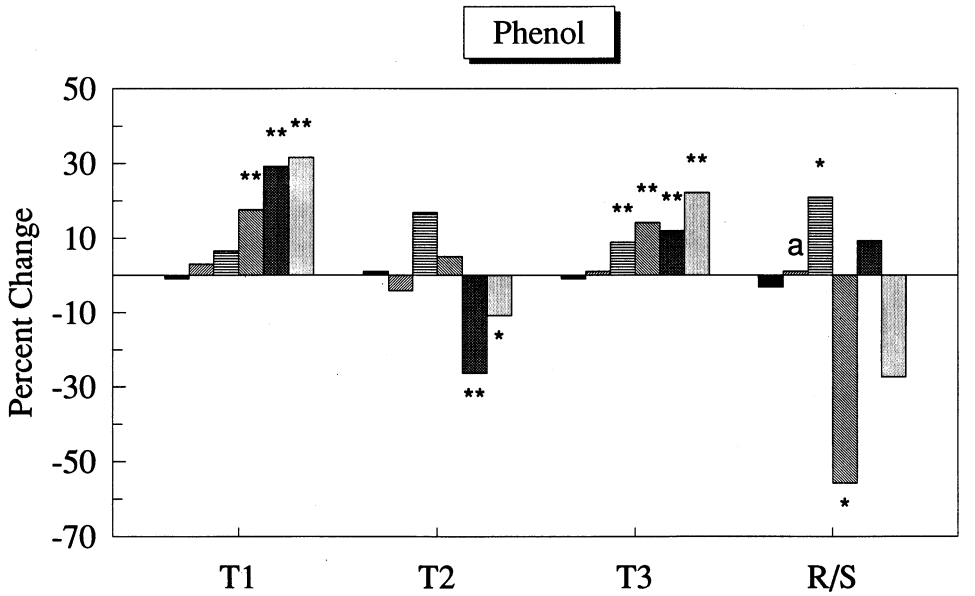

Fig. 4. Percentage change in Mauthner cell to motoneuron synaptic delay (T1), neuro-muscular delay (T2), and total time from Mauthner peak to start of swimming activity (T3) at 24 h exposure, and the response to stimuli ratio (R/S) at 48 h from background (0 h) values for medaka. Concentrations represented by each bar are (left to right): 0.0 (control), 10.9, 15.1, 20.6, 25.9 and 32.0 mg l^{-1} phenol. [a] Not measured. * Significantly different from background values at $P < 0.05$; ** $P < 0.01$.

hanced amplitude, especially in lower concentrations. Despite the increased amplitudes, there were no obvious differences in timing between peaks; T1, T2 and T3 were not affected (Table 2). However, the fish were consistently more responsive to touch throughout the 48 h exposure in high or near-lethal concentrations. R/S increased significantly to 90% at 0.0015 mg l^{-1} endosulfan, but decreased to 48% in the lowest concentration (0.0005 mg l^{-1}) at 24 h exposure.

Prey were consumed slower at 0.0005 mg l^{-1} endosulfan, and significantly more so at 0.0010 mg l^{-1}, than their counterpart controls. However, consumption time was less than controls at the highest concentration (0.0015 mg l^{-1}).

3.5. Fenvalerate

Fenvalerate was similar to endosulfan in toxicity to medaka ($LC_{50} = 0.0016$ mg l^{-1}). Fenvalerate was unique in that few clinical signs of intoxication were noticeable. In near-lethal concentrations, tremor was noted in two fish and some fish displayed convulsions and loss of equilibrium, but not consistently. Electrophysiological recordings showed no effects on T1, T2 or T3 in any fenvalerate concentration. There was also no change in response to touch; R/S remained unchanged from control values (Table 2). There was a significant reduction in prey consumption time for fish in 0.0009 mg l^{-1}, but no change at 0.0017 mg l^{-1}.

3.6. Phenol

Phenol was the least toxic test chemical with an LC_{50} of approximately 28 mg l^{-1}. Phenol exposures were also unremarkable as to observable clinical signs; fish activity patterns seemed normal except for one fish in 15.1 mg l^{-1} that developed some tremor.

Besides carbaryl, phenol was the only other chemical to affect T1. Significant increases in T1 occurred in most concentrations of phenol, ranging from 0.55 ± 0.02 to 0.70 ± 0.04 ms at 24 h exposure (increases of 6–32%) for the concentration range 15.1–32.0 mg l^{-1} (Table 2). Phenol had the opposite effect on T2; high concentrations

shortened T2. Although significant, the decreases were not great, the maximum being −26% in 26 mg l^{-1} phenol. However, the decreases in T2 seen in the two highest concentrations of phenol were offset by the much greater increases in T1 so that the net effect was a significantly increased T3 in the four highest concentrations of phenol (Fig. 4). Phenol had a variable effect on R/S; one lower concentration (15.1 mg l^{-1}) induced significant responsiveness at 48 h exposure and one higher level (20.6 mg l^{-1}) reduced R/S. R/S was affected more at 48 h than 24 h (Fig. 4; Table 2).

Only two phenol concentrations were used for predator/prey tests. Consumption time was reduced for exposed fish in 12.9 and 23.6 mg l^{-1}.

3.7. 1-octanol

The LC_{50} for 1-octanol was 21.0 mg l^{-1}. Medaka in 1-octanol were responsive to touch and showed activity comparable to control fish but failed to initiate an escape response. Qualitative analysis of the signal waveforms showed that 1-octanol attenuated M-cell and motoneuron peaks in all concentrations to the extent that time measurements were not possible at the highest test concentration even though the fish were responsive to touch.

T1 was unaffected by 1-octanol, but T2 declined as concentration increased, although not significantly (Table 2). Similarly, there was no significant effect on T3. The R/S ratio was reduced at high toxicant levels. Unlike all other test chemicals, however, 1-octanol had more effect on R/S at 24 h exposure than 48 h. Medaka responded only 18% of the time in 7.8 mg l^{-1} and not at all in 17.8 mg l^{-1}. At 48 h, R/S equaled 45 and 26% for the same groups of fish, respectively.

There was no effect on prey consumption time in 3.9 mg l^{-1} octanol, but 8.8 and 17.8 mg l^{-1} reduced consumption time significantly.

3.8. 2,4-dinitrophenol

Exposure to DNP depressed locomotor activity, and medaka fatigued rapidly, becoming totally unresponsive as concentration approached the LC_{50} value of 11.0 mg l^{-1}. Fish consistently failed to display any M-cell activity in DNP concentrations of 10.0 mg l^{-1} and higher. Even when the M-cell responses were recorded, the motoneuron peak was dampened and difficult to measure; even active fish at 48 h exposure showed little or no M-cell activity.

T1 was unaffected by DNP, but like 1-octanol, T2 declined as concentration increased and was significantly affected at 10.0 mg l^{-1} DNP. The trend for reduction in T2 paralleled that seen for T3. DNP also made medaka less responsive as toxicant levels rose and, as noted by R/S ratios, the fish were completely unresponsive to touch in 11.2 mg l^{-1} by 24 h exposure (Table 2).

Prey consumption time was significantly reduced in the only DNP concentration tested (10.0 mg l^{-1}).

4. Discussion

4.1. Control responses

Results of recordings obtained here are consistent with and, in several ways, substantiate observations made by previous researchers using a variety of intra- and extra-cellular recording techniques (Prugh et al., 1982; Eaton and DiDomenico, 1986; Fetcho and Faber, 1988; Featherstone et al., 1991, 1993). Qualitatively, the waveforms (Figs. 1 and 2) were virtually identical to those recorded in an untreated fish by Featherstone et al. (1993), and invariably occurred whenever a stimulus provoked one of the M-cells to fire. Results were also quantitatively similar. For instance, the delay time between the Mauthner axon peak and the compound motoneuron peak (T1) was 0.53 ± 0.04 ms for 201 untreated fish in this study compared to 0.55 ± 0.05 ms for 115 untreated medaka in the previous study (Featherstone et al., 1991). Examination of the waveform recordings showed that the measured control value of 0.17 ± 0.03 ms for T2 was consistently between a third and half the distance to the estimated muscle potential peak, or within a reasonable and expected range. Similarly,

signal amplitudes lay within the ranges of 6 ± 2, 15 ± 4 μV, and 1.0 ± 0.10 mV for the M-cell, motoneuron, and muscle potential peaks, respectively, reported by Featherstone et al. (1991).

One measured variable that differed in these studies was R/S. Fish prior to exposure responded 62% of the time to tactile stimuli in these studies, compared to a 70–80% success rate for untreated fish where the stimulus was vibration delivered via a speaker cone that displaced the fish chamber to evoke escape responses (Featherstone et al., 1993). Whereas tactile stimulation is somewhat subjective in that the degree of stimulus and location on the fish is variable, reproducible water movements that impinge upon highly specific receptors are a more objectively applied stimulus. Additionally, fish responsiveness depends in part upon the nature of the pulse producing the vibration; slight modifications of the gating pulse can be used to alter the percentage of successful M-cell firings.

4.2. Mauthner cell response syndromes

Although there exists an extensive database of neuroanatomical and neurophysiological information regarding Mauthner cell form and function (for e.g. Faber and Korn, 1978; Eaton and Hackett, 1984; Fetcho and Faber, 1988), research documenting the effects of chemicals on Mauthner cell electrophysiology and associated neural processes involved in the escape response is scarce. While additional study with a larger set of chemical structures is required, especially with direct neurotoxic modes of action, results with chemicals tested to date are generally consistent with the known neurophysiological and anatomical nature of the M-cell circuitry, as summarized below.

4.2.1. Chlorpyrifos and carbaryl

Signs of poisoning by acetylcholinesterase (AChE) inhibitors include muscular weakness and easy fatigue due to the accumulation of acetylcholine at neuro-muscular junctions along with tremor and convulsions because of acetylcholine buildup in the CNS (Murphy, 1986). Fish exposed to chlorpyrifos and carbaryl in this study exhibited these symptoms as well as involuntary muscle spasms when stimulated, consistent with clinical signs observed in previous studies (McKim et al., 1987c; Drummond and Russom, 1990; Bradbury et al., 1991; Rice et al., 1998). Electrophysiological measurements indicate that the neuromuscular junction as measured by the motoneuron to muscle delay (T2) was particularly sensitive to AChE inhibitors, and effects followed an obvious dose-response relationship for both chemicals (Table 2; Fig. 3). Along with increasing R/S as concentrations and exposure time rose, these responses are consistent with effects due to elevated neurotransmitter (ACh) in the neuromuscular synapses. Lack of similar effects on the M-cell to motoneuron delay (T1), except at the LC_{50} concentration for carbaryl, may indicate that this synapse is non-cholinergic, which is consistent with immunohistochemical analyses reported by Rhodes et al. (1986) and Eddy et al. (1989).

4.2.2. Strychnine

Strychnine caused sensory-invoked spasms and paralysis in medaka in the current study, which was also observed by Rice et al. (1998), and reported in fathead minnows (Drummond and Russom, 1990) and rainbow trout (Bradbury et al., 1991). Convulsions in fish exposed to strychnine, easily elicited by the slightest application of external stimuli, were expected because of the manner in which strychnine blocks control of spinal cord motor cell inhibitory pathways by antagonizing glycine (Murphy, 1986). Previous studies of M-cell soma and dendrites in a number of fish species have documented the role of strychnine in disrupting post-synaptic inhibition due to glycine antagonism (Furukawa et al., 1964; Legendre and Korn, 1994).

4.2.3. Endosulfan

The behavioral and electrophysiological responses elicited by endosulfan (as with the convulsant strychnine, as discussed above) were consistent with their effects on glycine and GABA-mediated synapses. Endosulfan-exposed medaka were hyperactive when disturbed, which was also reported previously in other fish species

(Drummond and Russom, 1990; Bradbury et al., 1991). Consistent with this increased hyperactivity, the results of electrophysiological studies also showed an elevated R/S ratio. Hyperactive and convulsant responses of endosulfan, and other cyclodiene insecticides, have been attributed to their action at the picrotoxinin site in the GABA chloride receptor complex (Coats, 1990) and is consistent with findings that GABA, along with glycine, is an important afferent inhibitory neurotransmitter to the M-cell (Diamond et al., 1973; Faber and Korn, 1988; Seitanidou et al., 1988; Petrov et al., 1991; Lee et al., 1993; Triller et al., 1993).

4.2.4. Fenvalerate

Fenvalerate-exposed medaka displayed few clinical signs of poisoning, but tremor seen in a few fish at near lethal levels is consistent with pyrethroid intoxication observed previously in a variety of species (Bradbury et al., 1985, 1987, 1991). Type II pyrethroids, such as fenvalerate, are thought to specifically affect nerve sodium channels resulting in repetitive discharges which induce tremor progressing to clonic seizures (Bradbury and Coats, 1989; Coats, 1990). The electrophysiological responses quantified in the present study did not show any significant differences to that noted in control fish. However, as discussed below (see Section 4.4.2), fenvalerate has an extremely steep toxicity curve, so the means of detecting a sublethal effect was limited and may have precluded the discrimination of toxicant-induced effects.

4.2.5. Phenol and 1-octanol

Octanol and phenol have been identified as nonpolar (baseline or Narcotic I) and polar (Narcotic II) narcotics, respectively, (McKim et al., 1987b; Bradbury et al., 1989a,b). The generalized depression observed with octanol intoxication in the current study was similar to that observed in numerous other investigations (McKim et al., 1987a; Drummond and Russom, 1990) and was seen as a marked attenuation of the recordings, with signals not detectable at a dose associated with 10% mortality. Consistent with this observation was a marked reduction in the R/S ratio. Decreased R/S may be due to an absence of or a decrease in the M-cell axon potential after an applied stimulus, and may suggest a sensory deficit due to an anesthetic-like effect. It has been demonstrated that anesthetics can completely, but reversibly, inhibit firing activity of isolated neurons in invertebrate preparations (Franks and Lieb, 1990). The trend toward recovery of R/S at 48 h exposure in the current study may have been due to decreases in 1-octanol exposure concentrations at 48 h compared to 24 h.

There was some indication of a reduced T2 with octanol, which was also observed with phenol, and may be associated with the hyperactivity sometimes noted at early stages of intoxication with narcotics (Bradbury et al., 1989b). Phenol did cause a significant decrease in T2, which may be related to reflex stimulation in the spinal cord that has been observed in mammals (Deichmann and Keplinger, 1981). Although the molecular mechanisms of narcosis remain unresolved (Bradbury et al., 1989b; Franks and Lieb, 1990), the electrophysiological results of the current study support previous toxicodynamic and behavioral studies that suggest octanol and phenol may act through different mechanisms or sites of action (Bradbury et al., 1989a,b).

4.2.6. 2,4-dinitrophenol

The oxidative phosphorylation uncoupler DNP was studied to evaluate a compound whose mode of action indirectly affects neurobehavior. As with octanol, there was a slight indication that DNP reduced T2 and the R/S ratio at sublethal doses; however, most notable was the attenuation of peak amplitudes with increasing dose and the lack of a measurable signal at lethal doses. Presumably, DNP exposure results in diminished ATP production due to oxidative phosphorylation uncoupling (Terada, 1990), and the resulting lack of energy to support ion channels may result in failure to maintain appropriate nerve membrane potentials.

4.3. Chemical characterization using M-cell responses

When T1 and T2 are combined with R/S, results of the current studies suggest that unique

response syndromes may be attributed to different modes of action. The response/stimuli ratio (R/S) appears to be a critical indicator as to whether or not the M-cell, interneurons, motoneurons or muscles will fire normally to initiate an effective escape response. Mode of action discrimination may also be improved with inclusion of additional electrophysiological characteristics. For instance, Featherstone et al. (1993) found that phenol lengthened the stimulus to M-cell peak time (onset latency), while strychnine shortened this time. A standardized measure of onset latency would provide data on chemical effect to sensory input, another critical indicator of the integrity of the nervous system. Other variables that should receive more attention in future research include peak amplitudes and the electromyographic activity occurring after the M-cell initiates the escape response. More extensive studies with a larger number of chemical structures, using a standardized stimuli protocol, are in progress to further evaluate the extent to which this in vivo preparation can be used to discriminate neurotoxic responses.

4.4. Ecological effect characterization

4.4.1. Relevancy of electrophysiological responses

While the M-cell assay has potential for discriminating modes of neurotoxic action, it may also have utility in linking sublethal neurophysiological events with associated behavioral deficits. Possessed by most fish species, the Mauthner cell, in association with sensory inputs and motor outputs, is related to several behaviors vital to survival in both larvae and adults (Eaton and Hackett, 1984). These cells trigger abrupt movements to rupture the egg during hatching (Eaton and Nissanov, 1985) and initiate variations of the rapid C-start movement to aid in prey capture (Harper and Blake, 1991; Kaiser, 1992; Canfield and Rose, 1993). However, the primary role of the Mauthner cell-initiated C-start is escape from predation (Eaton and Hackett, 1984).

In the predator/prey studies reported here, the medaka had to rely on rapid predator detection and evasive maneuvers, especially important here since the advantage lay with predators accustomed to the sudden introduction of prey into their environment. As a result, any effect on sensory systems, CNS functions, or motor abilities would result in a reduced ability to escape predation (Little et al., 1993) because of the lack of acclimation time for the prey to inspect their surroundings, seek refuge, disperse into the environment or school (Fuiman and Magurran, 1994). In this context, hyperexcitability and lethargy (hypoactivity) could have significant influences on prey susceptibility. Heightened activity due to minimal stimuli makes a fish more noticeable to potential predators and increased sensitivity to external stimuli may affect timing of the escape response by initiating it too soon (Fuiman and Magurran, 1994). In a consistent manner, a reduction in general locomotor activity levels can be an effective antipredator defense strategy (Weis and Weis, 1995).

In general, response thresholds noted in the M-cell assay were consistent with effect levels noted in the predator/prey assays. We observed decreased survival times at 10, 54, 33, 54, 37, and 89% of the estimated LC_{50} value for fish exposed to chlorpyrifos, carbaryl, endosulfan, phenol, 1-octanol, and DNP, respectively. Except for endosulfan, these effects were noted at the lowest concentrations that also significantly affected one or more of the electrophysiological variables monitored in the M-cell assay (Table 2). Consistent with the points raised above, medaka exposed to chlorpyrifos and carbaryl may have been more susceptible to predation due to reduced efficiency in the C-start, as reflected by effects on T1 and T2, as well as increased activity as shown by increases in R/S. Strychnine, however, resulted in increased survival times for medaka. This also occurred in the highest concentrations of endosulfan and carbaryl. The tetanic nature of strychnine and lethargy in near-lethal concentrations of other chemicals rendered exposed medaka completely inactive and, presumably, less visible to the bluegill, at least for a time. While refinement of current techniques along with additional types of predator/prey studies are required to more quantitatively relate predation susceptibility to electrophysiological responses in the M-cell assay, current findings suggest that R/S may be a sensi-

tive measure of chemically-induced hypo- or hyperactivity, which are important behavioral measures responsible for survival.

4.4.2. Lethal vs sublethal dose-response relationships

An examination of the effects thresholds for the variables measured in these studies showed that electrophysiological and behavioral effects associated with some modes of action occurred well below acutely lethal concentrations. Although additional chemicals with various modes of action need to be tested, these data suggest that the techniques used here may provide the means to systematically identify modes of action for which sublethal neurotoxic effects following acute exposures should be expected.

For example, chlorpyrifos elicited distinct thresholds for T2, T3, and R/S, and reduced prey survival time with effects occurring at approximately one tenth of the LC_{50} (0.03 mg l^{-1}). The lowest sublethal effect concentration for carbaryl was 54% of the estimated LC_{50}. These findings are consistent with threshold effects of a variety of AChE inhibitors on feeding behavior in rainbow trout (Little et al., 1993). Strychnine affected T1, T3, and increased R/S and prey survival at <20–40% of a minimally lethal concentration. Susceptibility to predation and increases in T3 occurred at approximately 50% of the phenol LC_{50}. Although both 1-octanol and DNP reduced the effectiveness of the M-cell and significantly increased susceptibility to predation, effects on R/S were seen at a much lower level for octanol; 37% of the LC_{50} for octanol, but close to 90% of the DNP LC_{50}. As with DNP, sublethal and lethal effects of endosulfan and fenvalerate occurred at similar exposure concentrations. With fenvalerate, there was no observed mortality or distinct electrophysiological effects at 1.3 μg l^{-1} compared to an LC_{50} value of 1.6 μg l^{-1}. As a consequence, the dose range for detecting sublethal effects was extremely narrow.

5. Conclusions

These results demonstrate that the M-cell assay may serve as an effective tool in neurotoxicity assessments and satisfy many of the criteria considered central to several proposals for behavioral indices of neurotoxicity. The variables obtained from electrophysiological recordings serve as measures of effect and injury critical to survival and differences in electrophysiological responses may aid in toxicant identification along mechanistic lines. With further development, the assay could be used in a tiered approach for identifying chemical-induced neurotoxicity since it uses functional endpoints that are indicators of the net sensory, motor and integrative processes occurring in the central and peripheral nervous system (Tilson, 1990). Further research using chemicals with mechanistically similar actions is necessary to validate these results and substantiate observed relationships between lethality and the neurophysiological basis of behavioral correlates associated with the startle response.

Acknowledgements

We thank Chris Russom for her assistance in preparing the research proposal. Thanks also to Alex Hoffman, Colleen Elonen, Greg Tufts and Rick Kolanczyk for their assistance with chemical analytical support, and to Carol Shreve and Tom Beeman for performing routine water chemistries and data entry. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

References

Bradbury, S.P., Coats, J.R., 1989. Comparative toxicology of the pyrethroid insecticides. Rev. Environ. Contam. Toxicol. 108, 134–177.

Bradbury, S.P., Coats, J.R., McKim, J.M., 1985. Differential toxicity and uptake of two fenvalerate formulations in fathead minnows (*Pimephales promelas*). Environ. Toxicol. Chem. 4, 533–541.

Bradbury, S.P., Symonik, D.M., Coats, J.R., Atchison, G.J., 1987. Toxicity of fenvalerate and its constituent isomers to the fathead minnow, *Pimephales promelas*, and bluegill *Lepomis macrochirus*. Bull. Environ. Contam. Toxicol. 38, 727–735.

Bradbury, S.P., Henry, T.R., Niemi, G.J., Carlson, R.W., Snarski, V.M., 1989a. Use of respiratory-cardiovascular

responses of rainbow trout (*Salmo gairdneri*) in identifying acute toxicity syndromes in fish: Part 3. Polar narcotics. Environ. Toxicol. Chem. 8, 247–261.

Bradbury, S.P., Carlson, R.W., Henry, T.R., 1989b. Polar narcosis in aquatic organisms. In: Cowgill U.M., Williams, L.R. (Eds.), Aquatic Toxicology and Hazard Assessment, vol. 12. ASTM STP 1027. American Society for Testing and Materials, Philadelphia, pp. 59–73.

Bradbury, S.P., Carlson, R.W., Niemi, G.J., Henry, T.R., 1991. Use of respiratory-cardiovascular responses of rainbow trout (*Oncorhynchus mykiss*) in identifying acute toxicity syndromes in fish: Part 4. Central nervous system seizure agents. Environ. Toxicol. Chem. 10, 115–131.

Canfield, J.G., Rose, G.J., 1993. Activation of Mauthner neurons during prey capture. J. Comp. Physiol. A 172, 611–618.

Coats, J.R., 1990. Mechanisms of toxic action and structure-activity relationships for organochlorine and synthetic pyrethroid insecticides. Environ. Health Perspect. 87, 255–262.

Deichmann, W.B., Keplinger, M.L., 1981. Phenols and phenolic compounds. In: Clayton G.D., Clayton, F.E. (Eds.), Patty's Industrial Hygiene and Toxicology, 3rd. edn., vol. IIA. Wiley, New York, pp. 2567–2627.

Diamond, J., Roper, S., Yasargil, G.M., 1973. The membrane effects, and sensitivity to strychnine, of neural inhibition of the Mauthner cell, and its inhibition by glycine and GABA. J. Physiol. London 232, 87–111.

Drummond, R.A., Russom, C.L., 1990. Behavioral toxicity syndromes: A promising tool for assessing toxicity mechanisms in juvenile fathead minnows. Environ. Toxicol. Chem. 9, 37–46.

Eaton, R.C., DiDomenico, R., 1986. Role of the teleost escape response during development. Trans. Am. Fish. Soc. 115, 128–142.

Eaton, R.C., Hackett, J.T., 1984. The role of the Mauthner cell in fast-starts involving escape in teleost fishes. In: Eaton, R.C. (Ed.), Neural Mechanisms of Startle Behavior. Plenum, New York, pp. 213–266.

Eaton, R.C., Nissanov, J., 1985. A review of Mauthner-initiated behavior and its possible role in hatching in the immature zebrafish *Brachydanio rerio*. Environ. Biol. Fish. 12, 265–279.

Eddy, P.L., Godfrey, D.A., Parli, J.A., Ross, C.D., 1989. Quantitative measurement of the choline acetyltransferase activity of the Mauthner cell axon. Proc. Oklahoma Acad. Sci. 69, 1–4.

Faber, D., Korn, H. (Eds.), 1978. Neurobiology of the Mauthner Cell. Raven, New York, p. 290.

Faber, D.S., Korn, H., 1988. Unitary conductance changes at teleost Mauthner cell glycinergic synapses: A voltage-clamp and pharmacologic analysis. J. Neurophysiol. 60, 1982–1999.

Featherstone, D., Drewes, C.D., Coats, J.R., 1991. Noninvasive detection of electrical events during the startle response in larval medaka. J. Exp. Biol. 158, 583–589.

Featherstone, D., Drewes, C.D., Coats, J.R., Bradbury, S.P., 1993. A non-invasive neurotoxicity assay using larval medaka. In: Gorsuch, J.W., Dwyer, F.J., Ingersoll, C.G., LaPointe, T.W., (Eds.), Environmental Toxicology and Risk Assessment, vol. 2, ASTM STP 1216. American Society for Testing and Materials, Philadelphia, pp. 275–288.

Fetcho, J.R., Faber, D.S., 1988. Identification of motoneurons and interneurons in the spinal network for escapes initiated by the Mauthner cell in goldfish. J. Neurosci. 8, 4192–4213.

Franks, N.P., Lieb, W.R., 1990. Mechanisms of general anaesthesia. Environ. Health Perspect. 87, 199–205.

Fuiman, L.A., Magurran, A.E., 1994. Development of predator defenses in fishes. Rev. Fish Biol. Fish. 4, 145–183.

Fukuto, T.R., 1990. Mechanism of action of organophosphorus and carbamate insecticides. Environ. Health Perspect. 87, 245–254.

Furukawa, T., Fukami, Y., Asada, Y., 1964. Effects of strychnine and procaine on collateral inhibition of the Mauthner cell of goldfish. Jpn. J. Physiol. 14, 386–399.

Harper, D.G., Blake, R.W., 1991. Prey capture and the fast-start performance of northern pike *Esox lucius*. J. Exp. Biol. 155, 175–192.

Kahl, M., Russom, C.L., DeFoe, D.L., Hammermeister, D.E., 1998. Saturator units for generating chemical stock solutions at near water solubility for use in aquatic bioassays. in press.

Kaiser, M.J., 1992. The ontogeny of predatory mechanisms in the fifteen-spined stickleback *Spinachia spinachia* (L.). J. Fish Biol. 40, 485–487.

Lee, R.K.K., Finger, T.E., Eaton, R.C., 1993. GABAergic innervation of the Mauthner cell and other reticulospinal neurons in the goldfish. J. Comp. Neurol. 338, 601–611.

Legendre, P., Korn, H., 1994. Glycinergic inhibitory synaptic currents and related receptor channels in the zebrafish brain. Eur. J. Neurosci. 6, 1544–1557.

Little, E.E., 1990. Behavioral toxicology: Stimulating challenges for a growing discipline. Environ. Toxicol. Chem. 9, 1–2.

Little, E.E., Fairchild, J.F., DeLonay, A.J., 1993. Behavioral methods for assessing impacts of contaminants on early life stage fishes. In: Fuiman, L.A. (Ed.), Water Quality and the Early Life Stages of Fishes, American Fisheries Society Symposium, vol. 14. American Fisheries Society, Bethesda, MD, pp. 67–76.

McKim, J.M., Bradbury, S.P., Niemi, G.J., 1987a. Fish acute toxicity syndromes and their use in the QSAR approach to hazard assessment. Environ. Health Perspect. 71, 171–186.

McKim, J.M., Schmieder, P.K., Carlson, R.W., Hunt, E.P., Niemi, G.J., 1987b. Use of respiratory-cardiovascular responses of rainbow trout (*Salmo gairdneri*) in identifying acute toxicity syndromes in fish: Part 1. Pentachlorophenol, 2,4-dinitrophenol, tricaine methanesulfonate and 1-octanol. Environ. Toxicol. Chem. 6, 295–312.

McKim, J.M., Schmieder, P.K., Niemi, G.J., Carlson, R.W., Henry, T.R., 1987c. Use of respiratory-cardiovascular re-

sponses of rainbow trout (*Salmo gairdneri*) in identifying acute toxicity syndromes in fish: Part 2. Malathion, carbaryl, acrolein and benzaldehyde. Environ. Toxicol. Chem. 6, 313–328.

Murphy, S.D., 1986. Toxic effects of pesticides. In: Klaassen, C.D., Amdur, M.O., Doull, J. (Eds.), Casarett and Doull's Toxicology: The Basic Science of Poisons, 3rd ed. Macmillan, New York, pp. 519–581.

National Research Council, 1992. Committee on Neurotoxicology and Models for Assessing Risk, Environmental Neurotoxicology. National Academy Press, Washington, D.C.

Petrov, T., Seitanidou, T., Triller, A., Korn, H., 1991. Differential distribution of GABA- and serotonin-containing afferents on an identified central neuron. Brain Res. 559, 75–81.

Prugh, J.I.P., Kimmel, C.B., Metcalfe, W.K., 1982. Noninvasive recording of the Mauthner neurone action potential in larval zebrafish. J. Exp. Biol. 101, 83–92.

Rhodes, K.J., Zottoli, S.J., Mufson, E.J., 1986. Choline acetyltransferase immunohistochemical staining in the goldfish (*Carassius auratus*) brain: evidence that the Mauthner cell does not contain choline acetyltransferase. Brain Res. 381, 215–224.

Rice, P.J., Drewes, C.D., Klubertanz, T.M., Coats, J.R., Bradbury, S.P., 1998. Acute toxicity and behavioral effects of chlorpyrifos, permethrin, phenol, strychnine, and 2,4-dinitrophenol to 30-day old Japanese medaka (*Oryzias latipes*). Environ. Toxicol. Chem., in press.

Seitanidou, T., Triller, A., Korn, H., 1988. Distribution of glycine receptors on the membrane of a central neuron: an immunoelectron microscopy study. J. Neurosci. 8, 4319–4333.

Smith, J.R., 1984. Fish Neurotoxicology. In: Weber, L.J. (Ed.), Aquatic Toxicology, vol. 2. Raven, New York, pp. 107–151.

Terada, H., 1990. Uncouplers of oxidative phosphorylation. Environ. Health Perspect. 87, 213–218.

Tilson, H.A., 1990. Behavioral indices of toxicity. Toxicol. Pathol. 18, 96–104.

Tilson, H.A., MacPhail, R.C., Crofton, K.M., 1996. Setting exposure standards: a decision process. Environ. Health Perspect. 104 (Suppl 2), 401–405.

Triller, A., Sur, C., Korn, H., 1993. Heterogeneous distribution of glycinergic and GABAergic afferents on an identified central neuron. J. Comp. Neurol. 338, 83–96.

Veith, G.D., Call, D.J., Brooke, L.T., 1983. Structure-toxicity relationships for the fathead minnow, *Pimephales promelas*: narcotic industrial chemicals. Can. J. Fish. Aquat. Sci. 40, 743–748.

Weis, J.S., Weis, P., 1995. Effects of embryonic exposure to methylmercury on larval prey-capture ability in mummichog *Fundulus heteroclitus*. Environ. Toxicol. Chem. 14, 153–156.