Use of Respiratory-Cardiovascular Responses of Rainbow Trout (Oncorhynchus Mykiss) in Identifying Acute Toxicity Syndromes in Fish: Part 4. Central Nervous System Seizure Agents

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USE OF RESPIRATORY-CARDIOVASCULAR RESPONSES OF RAINBOW TROUT (ONCORHYNCHUS MYKISS) IN IDENTIFYING ACUTE TOXICITY SYNDROMES IN FISH: PART 4. CENTRAL NERVOUS SYSTEM SEIZURE AGENTS

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Abstract—The respiratory-cardiovascular responses of spinally transected rainbow trout to acutely lethal concentrations of chlorpyrifos, cypermethrin, fenvalerate, endosulfan, endrin, and strychnine were examined. Common to all six toxicants, the most striking change in respiratory-cardiovascular parameters was an increased cough frequency. Ventilation frequency and volume dropped in strychnine-exposed trout, but both remained near predose levels (frequency) or elevated (volume) in the pyrethroid- and cyclodiene-exposed trout. In chlorpyrifos-intoxicated trout, ventilation frequency decreased while volume increased. Oxygen consumption remained near predose levels in the chlorpyrifos-, pyrethroid-, and strychnine-exposed trout, but increased dramatically in the cyclodiene-exposed trout. Arterial oxygen, carbon dioxide, and pH declined in all the toxicant groups. In the pyrethroid- and strychnine-exposed trout, hematocrit and hemoglobin levels tended to increase or remain constant during intoxication. Conversely, in the chlorpyrifos- and cyclodiene-exposed trout, values for these parameters decreased. The responses for these pesticides (N = 23 fish) were combined with five fish acute toxicity syndromes (FATS) (N = 52 fish) previously described (nonpolar narcosis syndrome, polar narcosis syndrome, acetylcholinesterase inhibitor syndrome, respiratory uncoupler syndrome, and respiratory irritant syndrome) and assessed using discriminant function analyses. The final analysis resulted in 93% correct classification of the trout.

Keywords—Cyclodiene insecticides Pyrethroid insecticides Strychnine Rainbow trout Respiratory-cardiovascular responses

INTRODUCTION

The U.S. Environmental Protection Agency (EPA), under a variety of legislation, is charged with the responsibility of assessing the hazard of chemicals to human health and the environment. In some instances, EPA incorporates predictive techniques in its decision-making process [1]. Predictive toxicological methods are often employed as cost-effective components in an overall strategy for prioritizing chemicals for in-depth investigation. Predictive approaches are also used where empirical toxicological data are either unavailable or not required under a specific statute. For example, under Section 5 of the Toxic Substances Control Act (TSCA) the EPA Office of Toxic Substances must review and assess the potential hazard of a new industrial chemical within 90 d, generally with little accompanying information beyond the compound's structure [1]. The implementation of TSCA illustrates the need to establish reliable predictive techniques because laboratory resources are limited and the potential number of compounds for study is large.

In the field of environmental toxicology, and
especially aquatic toxicology, quantitative structure-activity relationships (QSARs) have developed as scientifically defensible tools for predicting the toxicity of xenobiotics. As has been discussed previously [2,3], the proper application of predictive techniques requires that QSAR models be generated for specific modes of toxic action and that methods be developed to systematically assign chemicals to the appropriate QSAR. Thus, a fundamental understanding of both toxic mechanisms and the critical structural characteristics and properties of a chemical that governs its action by a specific mechanism are required.

Current efforts in developing a knowledge base for an expert system designed to predict toxic mechanism from structure involve information acquisition from leading scientists [3] and the generation of an empirically derived database. In the latter case, approaches to defining common modes of action for groups of xenobiotics include application of joint toxicity theory for chemical mixtures [4,5] and the assessment of fish acute toxicity syndromes (FATS). FATS are distinct sets of in vivo rainbow trout (Oncorhynchus mykiss) toxic responses. By measuring a number of respiratory-cardiovascular variables, FATS associated with nonpolar narcotics, polar narcotics, oxidative phosphorylation uncouplers, respiratory membrane irritants, and acetylcholinesterase (AChE) inhibitors [6-8] have been described.

Thus far, the development of FATS, with the exception of AChE inhibitors, has been applied to toxicants representative of industrial chemicals whose action is not initiated by an interaction with a specific or well-defined receptor. Protonophoric oxidative phosphorylation uncouplers have very specific and potent effects (i.e., the ability to transport H+ through H+-impermeable membranes), but they do not have a specific receptor site in the mitochondrial membrane [9]. The respiratory membrane irritants studied thus far are direct-acting electrophiles whose effect is elicited by covalent binding to nucleophiles incorporated in gill membranes, which are the first nucleophiles available with an aqueous exposure [10]. Finally, receptor sites for compounds classified as narcotics are probably quite variable, especially as the dose and internal distribution vary with increasing length of exposure to elicit truly anesthetic as well as toxic effects [11].

To further assess the FATS techniques for resolving modes of toxic action, a study was initiated to determine whether pesticides known to act as peripheral and central nervous system (CNS) toxicants, but due to different molecular mechanisms and sites of action, could be resolved with the set of respiratory-cardiovascular parameters currently monitored. The studies by McKim et al. [7] and Bradbury et al. [8] indicated that the AChE inhibitors malathion (diethyl mercaptocarbamate, S-ester with O,O-dimethyl phosphorodithioate) and carbaryl (1-naphthyl-N-methylcarbamate) could be resolved from nonpolar and polar narcotics, uncouplers, and irritants. Earlier work with fenvalerate ([R,S]-α-cyano-3-phenoxybenzyl [R,S]-2-(4-chlorophenyl)-3-methylbutyrate), a pyrethroid insecticide, provided preliminary evidence that a convulsant can cause a response distinct from the original FATS [12]. At present, however, there are insufficient data to fully assess whether responses elicited by different classes of neurotoxicants can be resolved from responses elicited by other classes of toxicants. It is also not clear if convulsants with different mechanisms can be differentiated.

The compounds selected for investigation and analysis in this study included an additional AChE inhibitor, chlorpyrifos (O,O-diethyl O-(3,5,6-trichloro-2-pyridyl) phosphorothioate); the pyrethroid insecticides, fenvalerate and cypermethrin ([R,S]-α-cyano-3-phenoxybenzyl [R,S]-cis, trans-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropylcarboxylate); the cyclodiene insecticides, endrin (1,2,3,4,10,10-hexachloro-6,7-epoxy-1,4,4a,5,6,7,8,8a-octahydro-1,4-endao-endo-5,8-dimethanonaphthalene) and endosulfan (6,7,8,9,10,10-hexachloro-1,5,5a,6,9,9a-hexahydro-6,9-methano-2,4,3-benzodioxathiepin-3-oxide); and the rodenticide strychnine (strychidin-10-one). The selected convulsants have distinct mechanisms of action. The pyrethroid insecticides are thought to have their primary effect on the sodium gate in nerve axons. Consistent with Type II pyrethroids, fenvalerate and cypermethrin cause a depolarization of nerve membranes and impulse conduction block, due to an extremely prolonged sodium current [13,14]. It has been proposed that cyclodiene insecticides act at the picrotoxinin site in the GABA-receptor-ionophore complex leading to an inhibition of chloride influx into the nerve [15]. Finally, strychnine is thought to block inhibitory pathways mediated by Renshaw cells in the spinal cord by acting as a glycine antagonist [16-18].

Insecticide toxicology, and toxicology in general, careful examination of both behavioral and physiological signs of intoxication can provide insights into a toxicant’s site and mechanism of action. All the selected compounds elicit convulsions in mammals and/or insects [14,19] and can be discriminated.
criminates to some degree based on signs of intoxication [19]. The ability to discriminate these convulsants in fish, by examining respiratory-cardiovascular responses, was assessed in the present study.

**METHODS**

The methods used were basically the same as those described by McKim et al. [6,7] and Bradbury et al. [8]. The data for fenvalerate were obtained from a previous study [12] that was performed at the same laboratory under the exact conditions described by McKim et al. [6,7].

**Fish preparation and physiological monitoring**

Rainbow trout, weighing 600 to 900 g, were maintained at the U.S. EPA Environmental Research Laboratory in Duluth, Minnesota several months before use and were acclimated to a temperature of 11 to 12°C. Four rainbow trout were exposed to each chemical. Because exposures to these chemicals occurred over a two-year period, separate control groups of four trout each were used for the endosulfan and strychnine, and for the endrin and cypermethrin, experiments.

Spinally transected trout were surgically prepared as described previously [6,8] and fitted with copper-wire heart electrodes, indwelling dorsal aortic cannulae, and latex-rubber oral membranes to separate inspired and expired water. After surgery, the fish were placed in individual respirometer-metabolism chambers, and physiological measurements were initiated approximately 24 h later. The trout were placed in their respective chambers within 2 h of one another.

All four fish for a given experiment were exposed simultaneously, using a specially designed flow-through exposure unit that provided automated data acquisition and measurement control [8,20]. During each experiment, physiological measurements were made of 11 variables. Ventilation volume (Vg) and dissolved oxygen (DO) measurements were automatically made by the exposure system. Oxygen uptake efficiency (Ue) and oxygen consumption (VO2) were derived from inspired and expired DO concentrations as outlined previously [6]. Vg, VO2, and Ue were measured every 15 min. Heart frequency (fH), ventilation frequency (fV), and cough frequency (fC) were monitored every 30 to 60 min and were recorded on a Narco Physiograph rectilinear strip-chart recorder (Narco Biosystems, Houston, TX). Blood chemistry variables were measured in arterial blood samples two to five times during an exposure.

Samples were analyzed for pH (pHå), total carbon dioxide (TåCO2), total oxygen (TåO2), hemoglobin (Hb), and hematocrit (Hct), as described by McKim et al. [6].

Before exposure, all variables were measured in each fish to provide predose (control) values. Twenty-eight predose measurements of Vg, Ue, and VO2, seven measurements of fH, fV, and fC, and one set of blood-chemistry variables were collected.

**Toxicant preparation and exposure**

Preliminary range-finding tests, using transected trout held in the respirometer-metabolism chambers, were run to select optimal aqueous exposure concentrations, i.e., 24-h LC100s. Exposure concentrations employed in the experiments are provided in Table 1. Due to specific dose-response curves and time to death profiles, the selection of a 24-h LC100 was not always possible and mean trout survival times for the five chemicals ranged from 10.8 to 44.8 h (Table 1).

Strychnine hemisulfate was purchased from Sigma Chemical Company (St. Louis, MO). Technical-grade endosulfan (94–96% purity; 70% α-endosulfan and 30% β-endosulfan) and technical-grade cypermethrin (88% purity) were generously provided by FMC Corporation, Agricultural Chemical Company (Princeton, NJ). Technical endrin (96% purity) was provided as a gift from J. R. Coats, Department of Entomology, Iowa State University (Ames, IA). Using dimethylformamide (DMF) as an aid in dissolution, aqueous stock solutions of these chemicals were prepared in 19-liter glass bottles by stirring a preweighed amount of chemical into 18 liters of distilled water. The final nominal concentration of DMF in the exposure chambers was 13 mg/L; concentrations up to 200

<table>
<thead>
<tr>
<th>Chemical name</th>
<th>Lethal aqueous concn. (mg/L)</th>
<th>Mean survival time (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cypermethrin</td>
<td>0.129 ± 0.075</td>
<td>34.3 ± 6.1</td>
</tr>
<tr>
<td>Fenvalerate*</td>
<td>0.412 ± 0.050</td>
<td>10.9 ± 1.5</td>
</tr>
<tr>
<td>Endosulfan</td>
<td>0.0079 ± 0.0014</td>
<td>44.8 ± 9.9</td>
</tr>
<tr>
<td>Endrin</td>
<td>0.029 ± 0.012</td>
<td>35.6 ± 7.2</td>
</tr>
<tr>
<td>Strychnine</td>
<td>4.75 ± 0.46</td>
<td>12.8 ± 6.6</td>
</tr>
<tr>
<td>Chlorpyrifos</td>
<td>0.208 ± 0.024</td>
<td>43.8 ± 7.3</td>
</tr>
</tbody>
</table>

Values are means ± SD (N = 4 trout for each chemical except for the endosulfan group (3)).

*Data from Bradbury et al. [12].
mg/L DMF do not have an effect on the monitored physiological parameters (unpublished data). Stock bottles were covered with black plastic to prevent photodegradation of the chemicals. A pre-adjusted direct-current FMI metering pump (Fluid Metering Inc., Oyster Bay, NY) was used to deliver stock solutions to the exposure unit mixing chamber, where they were diluted with filtered Lake Superior water. A granular formulation of chlorpyrifos, consisting of sand coated with 1% active ingredient (Clarke Granular Larvicide®, obtained from the Clarke Outdoor Spraying Company, Roselle, IL), was packed into glass columns to construct saturators. Lake Superior water was pumped through the saturators and the output fed to the mixing chamber of the exposure unit. The toxicant was maintained at 11.0 to 11.5°C. Overall means and standard deviations (alkalinity, hardness, DO, pH, and temperature) were measured before and during each exposure. The temperature was maintained at 11.0 ± 0.7°C and 44.9 ± 1.7°C as CaCO₃, respectively. DO ranged from 10.5 to 11.0 mg/L (96–100% saturation). The mean pH (N = 14) was 7.64 ± 0.24.

Toxicant analysis

Toxicant concentrations in the inspired water of each chamber were monitored at least four times per exposure period. For chlorpyrifos, cypermethrin, endosulfan, and endrin analyses 10 (to which was added 165 ml of distilled water), 180, 175, and 10 ml (to which was added 30 ml of distilled water), respectively, of the inspired water were collected and extracted with 20 to 25 ml hexane by vigorous stirring for 45 min. In the case of strychnine, 1.5 ml of a saturated NaCO₃ aqueous solution was added to 225 ml of inspired water prior to extraction with toluene/hexane (5:1).

Concentrations of the toxicants in solvent extracts were determined using a Hewlett-Packard 5710A gas chromatograph (Hewlett-Packard, Avondale, PA) equipped with either an electron capture detector (chlorpyrifos, cypermethrin, endosulfan, and endrin) or a flame ionization detector (strychnine). Glass-coiled columns (all 1.8 m x 2 mm i.d., except for the cypermethrin analysis, which was 1.2 m x 4 mm i.d.) were used and packed with 3% OV-7 on Gas Chrom Q (Applied Science Lab, Inc., State College, PA), 1.5% SP2250/1.95% SP2401 on Supelcoport (Supelco Inc., Bellefonte, PA), and 3% OV-1 on Chromosorb W-HP (Applied Science Lab, Inc.) for cypermethrin; chlorpyrifos, endosulfan, and endrin; and strychnine analyses, respectively. Column temperatures were maintained at 200, 240, 220, 250, and 285°C, respectively, for the chlorpyrifos, cypermethrin, endosulfan, endrin, and strychnine analyses. Injector and detector ovens were held at 250°C and 300°C, respectively, for all the analyses. Either nitrogen or argon with 5% methane (chlorpyrifos, cypermethrin, endosulfan, and endrin) was used as a carrier gas at a flow rate of 30 ml/min. Extraction of water spiked with chlorpyrifos, cypermethrin, endosulfan, endrin, and strychnine resulted in recoveries of 95.2 ± 6.7% (N = 5), 108.3 ± 7.7% (N = 3), 103.0 ± 6.6% (N = 5), 100.3 ± 1.8% (N = 3), and 92.8 ± 15.0% (N = 6), respectively.

Data analyses

The data were handled as previously described [6–8]. Each fish was treated as an experimental unit. The mean percentage of change in the physiological parameters from their predose means was determined for the 25 to 75% survival time interval for each fish. Measurements within this interval were used in the analysis to minimize inclusion of responses due to initial stress and death itself. Mean percentage changes for the control fish were determined in an identical manner. A total of 12 control fish were used. Four control fish are from the earlier study with fenvalerate [12] and are the same controls reported by McKim et al. [6,7]. A second group of four controls were monitored for the chlorpyrifos, endosulfan, and strychnine experiments; these are the same controls reported by Bradbury et al. [8]. A final group of four additional control trout were used during the cypermethrin and endrin experiments. The responses of these 12 fish were pooled in subsequent analyses (see Results). Mean percentage changes during the specified survival time interval for each toxicant group were compared to the mean response of the control group using the nonparametric Wilcoxon two-sample test [21].

As reported previously [6–8], principal components analysis (PCA) [22] and discriminant function analysis (DFA) [23] were used as exploratory/descriptive procedures to assess the responses of the intoxicated fish. Prior to analysis, each of the variables was logarithmically transformed to stabilize the variance and reduce the effects of skewed distributions. All analyses were performed using BMDP [24] and SPSS [25] statistical software. The responses of the toxicant-exposed trout were compared to those of the control fish (N = 16, 12 control and 12 intoxicated trout) using ANOVA and the least significant difference test. A two-way ANOVA was performed on physiological parameters that showed a significant (p < 0.05) interaction with condition and time, to further evaluate the specific response of the trout to the toxicants. The time to inhibit cholinesterase (AChE) activity was determined for each fish; this is the response of the toxicant-exposed trout to the toxicants as new chemical agents or as new toxic mechanisms.

Visible syndrome

Rats and trout exposed to the toxicants of cypermethrin, endosulfan, and strychnine showed 44.8, 30.1, and 42.8% mortality, respectively (N = 16). The toxicity of intoxication increased with increasing time. The time to inhibit cholinesterase (AChE) activity was determined for each fish; this is the response of the toxicant-exposed trout to the toxicants as new chemical agents or as new toxic mechanisms.

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chlorpyrifos, a known AChE inhibitor, was added to the dataset associated with the previously defined FATS (N = 52 trout; narcosis Type I or nonpolar narcosis syndrome, narcosis Type II or polar narcosis syndrome, respiratory uncoupler syndrome, respiratory membrane irritant syndrome, and the AChE inhibitor syndrome [6-8]). In the initial analysis, responses of the fish exposed to the five convulsants were treated as unknowns to examine whether the DFA model was sensitive in recognizing potentially new FATS. Based on these results the DFA was repeated with the appropriate response sets identified as new FATS for the different convulsant mechanisms. Interpretation of discriminant functions (DFs) was based on standardized DF coefficients.

**RESULTS**

**Visible signs of intoxication**

Rainbow trout exposed to lethal concentrations of cypermethrin, fenvalerate, endosulfan, endrin, strychnine, and chlorpyrifos died after 34.3, 10.9, 44.8, 35.6, 12.8, and 43.8 h of exposure, respectively (Table 1).

Fish exposed to chlorpyrifos showed few signs of intoxication early in the exposures except that \( f_c \) started to increase and ventilatory movements became more pronounced. Between 25 to 75% survival time, the trout were somewhat hyperresponsive and displayed occasional body shudders and headshakes as well as eye twitches, but did not exhibit convulsive activity. Increased defecation and bile loss from the anal opening were observed with all fish during this period.

Visible signs of intoxication common to the remaining five toxicants included tremors and seizures. Shortly after exposure to the pyrethroids (10% survival time), \( f_c \) seemed to increase, which was confirmed through evaluation of ventilatory traces (Fig. 1). Visual examination of expired gill water indicated that an elevated secretion of mucus was associated with increased \( f_c \). Except for these effects, pyrethroid-exposed trout seemed normal, compared to control fish and predose appearance, through approximately 20% survival time, at which point fine tremors were observed. Tremors progressed to periods of violent head shaking and twisting that culminated in episodes in which the head was held at a 30% angle from the body for several seconds. During these seizures, the opercula were flared and in a state of tetany. Between seizures trout were intensely ventilating. The intensity and severity of the seizures increased through approximately 70 to 75% survival time, after which they subsided and the fish became inactive and expired.

The cyclodiene-exposed fish seemed generally unchanged through 25% survival time, except for occasional branchial tremors and intensified ventilatory movements. From approximately 20 to 40% survival time, \( f_c \) and ventilation activity seemed to increase; again these observations were confirmed after analysis of the ventilatory traces (Fig. 1). Branchial tremors also intensified at this time and progressed to increasingly severe seizures that consisted of head shudders similar to, but not as exaggerated as, those observed in pyrethroid-exposed trout. Most notable in cyclodiene-exposed trout were increased pectoral fin movement and eventual tetany; they were first observed at approximately 25% survival time and intensified as the exposures continued. These signs of intoxication became less severe and occurred with less frequency after 70 to 80% survival time. One endosulfan-exposed trout died 10 h after exposure and exhibited signs of intoxication similar to those observed in nonpolar narcotic-exposed fish [6]. This fish also did not recover well from surgery. The rapid time to death and unusual toxic responses were attributed to some other factor influencing this trout; e.g., an anomalous response to the surgical preparation. Due to these abnormalities, this trout was excluded from subsequent statistical analyses.

Trout reacted rapidly to strychnine. By 10% survival time increased coughing, as well as whole-body spasms that included tail-arching, were observed. The spasms were similar in appearance to those observed in polar narcotic-exposed trout [8]. Strychnine-exposed fish also exhibited elevated responsiveness to outside stimuli, which was not observed in other intoxicated trout. Through approximately 50 to 60% survival time these signs of intoxication continued and intensified with the fish becoming hyperresponsive; a gentle tap on the chamber would result in a violent seizure in the animal. From 60 to 75% survival time ventilation became uncoordinated and the fish were less re-
Fig. 1. Mean respiratory-cardiovascular responses \( (f_C, f_V, f_H, V_C, V_O_2, U_E) \) of rainbow trout exposed to lethal aqueous concentrations of cypermethrin, fenvalerate, endosulfan, endrin, strychnine, and chlorpyrifos. Each measurement represents the mean response of four trout (except for endosulfan, where \( N = 3 \)). The shaded area represents the range of the mean response of 12 control fish.

Physiological responses

Predose values for the respiratory-cardiovascular and blood-chemistry variables monitored in the study are listed in Table 2. As additional FATS studies were conducted, the data were pooled to provide a more robust statistical analysis. The control fish in each group were exposed to the following concentrations:

- Cypermethrin: 100 ppm
- Fenvalerate: 500 ppm
- Endosulfan: 150 ppm
- Endrin: 150 ppm
- Strychnine: 200 ppm
- Chlorpyrifos: 150 ppm

In general, the trout exposed to lethal concentrations of these pesticides exhibited a decrease in respiratory frequency \( (f_V) \) and heart rate \( (f_H) \), along with a decrease in blood oxygen saturation \( (V_O_2) \). The effects were more pronounced at lower concentrations, with a 10% decrease in oxygen saturation observed at concentrations as low as 20% in the endosulfan-exposed group. The blood chemistry parameters also showed corresponding changes.

Presentation changes in the respiratory-cardiovascular responses of rainbow trout exposed to lethal concentrations of cypermethrin, fenvalerate, endosulfan, endrin, strychnine, and chlorpyrifos. Each measurement represents the mean response of four trout (except for endosulfan, where \( N = 3 \)). The shaded area represents the range of the mean response of 12 control fish.

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studies are performed, control values are being pooled to provide better estimates of the physiological status of transected trout. To date, the 12 control fish represent data from three years of testing. In general, the predose variables were similar between the control fish and the toxicant-exposed fish, although $T_{aO_2}$ was somewhat lower in the endosulfan group. Mean postdose percentage changes in the control group are generally within 10% of predose levels (Table 3). $T_{aO_2}$ dropped 20% in the control fish, which is expected with serial blood sampling and the resulting loss of red blood cells (Hb and Hct decreases of 11 and 14%, respectively).

Marked changes in respiratory-cardiovascular status were observed in all the toxicant-exposed trout (Table 3, Fig. 1). One of the most noticeable changes occurred with $f_V$; increases of 56.6, $(p = 0.01), 107$ $(p = 0.03), 205$ $(p = 0.02), 139$, and 518% $(p = 0.01)$ were recorded for the endosulfan-, endrin-, cypermethrin-, fenvalerate-, strychnine-, and chlorpyrifos-exposed fish, respectively. In the cypermethrin group, $f_V$ increased through about 80% survival time and then declined (mean increase of 48.5%, $p = 0.01$). Strychnine-exposed trout exhibited a steady and consistent decline in $f_V$ throughout the exposure period (mean decrease of 32.6%, $p = 0.03$). In the chlorpyrifos-exposure group, $f_V$ declined through the 25% survival time and then stabilized (mean decrease of 31.9%; $p = 0.01$). $f_V$ was essentially unchanged in the cyclodiene-exposed trout. Trout in the cyclodiene- and pyrethroid-exposure groups generally exhibited $f_V$ near predose levels through 75% survival time. Fish exposed to strychnine showed a steady decline in $f_V$ during intoxication, while trout exposed to chlorpyrifos exhibited a large initial reduction.

Except for the trout in the strychnine-exposure group, increased $V_G$ was observed during intoxication. $V_G$ generally peaked between 50 and 75% survival time with significant increases of 76.2 $(p = 0.01), 40.4$ $(p = 0.01)$, and 78.1% $(p = 0.01)$ observed in the endosulfan-, endrin-, and chlorpyrifos-exposed trout. After an initial increase, strychnine-exposed trout exhibited a dramatic decline in $V_G$. In general, changes in $V_O_2$ and $U_E$ were consistent with the changes observed in $V_G$. In the pyrethroid-exposed trout, a moderate drop in $U_E$ was associated with the increase in $V_G$ and resulted in essentially no overall change in $V_O_2$. Responses of the chlorpyrifos-exposed trout were similar in that $U_E$ dropped 18.1% $(p = 0.01)$, along with the noted compensatory increase in $V_G$. $V_O_2$, as a result, remained near predose levels. In the strychnine-exposed trout, the drop in $V_G$ led to an increase in $U_E$; this increase was seemingly sufficient to maintain $V_O_2$ near predose levels. Surprisingly, the large increase in $V_G$ noted in the cyclodiene-exposed trout was not associated with a resulting drop in $U_E$, and instead efficiencies remained near predose levels. As a consequence, large increases in $V_O_2$ of 70.3 $(p = 0.01)$ and 50.5% $(p = 0.01)$ were observed in the endosulfan- and endrin-exposed trout, respectively.

Changes in several of the blood-chemistry parameters were also observed in the toxicant-exposed trout (Table 3, Fig. 2). In both the cypermethrin- and fenvalerate-exposed trout, $T_{aO_2}$ showed an initial increase, followed by a dramatic drop, while in the strychnine-exposed fish $T_{aO_2}$ generally showed a steady decline. $T_{aO_2}$ in the cyclodiene-exposed fish tended to remain fairly constant throughout the exposure periods, with some decline noted during the later sampling periods. Exposure to chlorpyrifos resulted in a steady decline in $T_{aO_2}$ through 40% survival time, after which it slowly rose to about predose levels. $T_{aCO_2}$ in the intoxicated trout generally decreased during exposure. Significant decreases of 12.2 $(p = 0.04), 19.9$ $(p = 0.02)$, and 12.2% $(p = 0.02)$ were observed for the cypermethrin, fenvalerate, and endrin groups, respectively. Moderate decreases in $pH_a$ were generally observed in all the toxicant groups. The largest mean drops in $pH_a$ were recorded in the strychnine (5.7%, $p = 0.01$) and cypermethrin (4.4%, $p = 0.01$) groups. Hct tended to increase in the cypermethrin- (20.7%, $p = 0.01$), fenvalerate- (9.1%, $p = 0.04$), and strychnine- (59.5%, $p = 0.01$) exposed trout. These increases were associated with either steady or increased Hb. In the cyclodiene and chlorpyrifos groups, Hct and Hb changes were somewhat more similar to the responses noted in the control group, except that in the endrin-exposed trout Hct increased 1.1% $(p = 0.02)$.

**Principal components analyses**

PCA was used primarily as a graphical tool to help establish that the responses of the intoxicated trout were different from the controls; typically those differences could be discerned along PC1 and occasionally PC2 (Fig. 3). The PCA of each of the toxicants showed high correlations among the 11 respiratory-cardiovascular variables, with PC1, PC2, and PC3 accounting for between 73 and 82% of the variation. In many of the PCAs, the percentage changes in variables that were highly correlated with the PC axes were also significantly
Table 2. Physiological status of rainbow trout prior to exposure to acutely lethal concentrations of cypermethrin, fenvalerate, endosulfan, endrin, strychnine, and chlorpyrifos (absolute predose values)

<table>
<thead>
<tr>
<th>Physiological variables</th>
<th>Control</th>
<th>Cypermethrin</th>
<th>Fenvalerate</th>
<th>Endosulfan</th>
<th>Endrin</th>
<th>Strychnine</th>
<th>Chlorpyrifos</th>
</tr>
</thead>
<tbody>
<tr>
<td>$f_c$ (no./min)</td>
<td>0.8 ± 0.4</td>
<td>1.2 ± 0.7</td>
<td>1.0 ± 1.0</td>
<td>1.0 ± 0.4</td>
<td>0.8 ± 0.2</td>
<td>0.8 ± 0.3</td>
<td>1.0 ± 0.4</td>
</tr>
<tr>
<td>$f_V$ (no./min)</td>
<td>71.0 ± 5.2</td>
<td>64.8 ± 6.1</td>
<td>69.3 ± 4.7</td>
<td>65.0 ± 11.5</td>
<td>64.0 ± 5.4</td>
<td>66.0 ± 3.9</td>
<td>73.0 ± 5.7</td>
</tr>
<tr>
<td>$V_O_2$ (ml/min)</td>
<td>129.3 ± 40.2</td>
<td>234.0 ± 103.0</td>
<td>152.6 ± 64.7</td>
<td>96.3 ± 24.1</td>
<td>155.2 ± 66.6</td>
<td>146.8 ± 43.4</td>
<td>176.2 ± 107.4</td>
</tr>
<tr>
<td>$V_O_2$ (mg/kg/h)</td>
<td>56.7 ± 9.3</td>
<td>81.8 ± 12.0</td>
<td>76.5 ± 16.2</td>
<td>48.6 ± 7.9</td>
<td>64.9 ± 13.5</td>
<td>62.0 ± 7.1</td>
<td>69.2 ± 13.8</td>
</tr>
<tr>
<td>$U_e$ (%)</td>
<td>5.0 ± 0.4</td>
<td>46.0 ± 13.5</td>
<td>54.8 ± 7.0</td>
<td>68.4 ± 13.2</td>
<td>46.9 ± 7.0</td>
<td>54.2 ± 13.9</td>
<td>62.7 ± 17.5</td>
</tr>
<tr>
<td>$f_H$ (no./min)</td>
<td>54.0 ± 5.3</td>
<td>53.8 ± 5.9</td>
<td>42.0 ± 7.4</td>
<td>4.4 ± 0.8</td>
<td>8.6 ± 0.8</td>
<td>5.0 ± 0.9</td>
<td>7.0 ± 0.4</td>
</tr>
<tr>
<td>$T_{O_2}$ (g/100 ml)</td>
<td>9.2 ± 2.7</td>
<td>8.4 ± 0.7</td>
<td>5.7 ± 2.9</td>
<td>11.3 ± 2.0</td>
<td>9.6 ± 1.3</td>
<td>7.6 ± 0.5</td>
<td>8.3 ± 2.1</td>
</tr>
<tr>
<td>$T_{CO_2}$ (mmol/L)</td>
<td>9.4 ± 2.7</td>
<td>7.5 ± 0.1</td>
<td>9.1 ± 3.0</td>
<td>11.3 ± 2.0</td>
<td>9.6 ± 1.3</td>
<td>7.6 ± 0.5</td>
<td>8.3 ± 2.1</td>
</tr>
<tr>
<td>$pH_s$ (pH units)</td>
<td>8.1 ± 0.2</td>
<td>8.20 ± 0.10</td>
<td>7.99 ± 0.10</td>
<td>8.1 ± 0.02</td>
<td>8.11 ± 0.06</td>
<td>8.14 ± 0.02</td>
<td>8.14 ± 0.02</td>
</tr>
<tr>
<td>Hct (%)</td>
<td>26.3 ± 9.0</td>
<td>22.2 ± 1.9</td>
<td>22.4 ± 1.8</td>
<td>21.0 ± 1.7</td>
<td>22.2 ± 2.6</td>
<td>23.0 ± 5.3</td>
<td>23.8 ± 1.3</td>
</tr>
<tr>
<td>Hb (g/100 ml)</td>
<td>7.8 ± 1.7</td>
<td>7.2 ± 0.6</td>
<td>7.7 ± 1.2</td>
<td>5.8 ± 0.4</td>
<td>6.8 ± 1.0</td>
<td>6.5 ± 1.2</td>
<td>7.5 ± 0.7</td>
</tr>
</tbody>
</table>

Values are means ±SD ($N = 4$ trout for each chemical except for the endosulfan (3) and control groups (12)).

Table 3. Physiological response (postdose percentage change) of rainbow trout during exposure to acutely lethal concentrations of cypermethrin, fenvalerate, endosulfan, endrin, strychnine, and chlorpyrifos

<table>
<thead>
<tr>
<th>Physiological variables</th>
<th>Control</th>
<th>Cypermethrin</th>
<th>Fenvalerate</th>
<th>Endosulfan</th>
<th>Endrin</th>
<th>Strychnine</th>
<th>Chlorpyrifos</th>
</tr>
</thead>
<tbody>
<tr>
<td>$f_c$ (no./min)</td>
<td>1.0 ± 21.5</td>
<td>107 ± 55</td>
<td>206 ± 166</td>
<td>56 ± 28</td>
<td>66 ± 44</td>
<td>139 ± 146</td>
<td>518 ± 73</td>
</tr>
<tr>
<td>$f_V$ (no./min)</td>
<td>-4.7 ± 6.1</td>
<td>48.5 ± 34.2</td>
<td>3.7 ± 13.3</td>
<td>6.4 ± 12.9</td>
<td>6.1 ± 15.1</td>
<td>-32.6 ± 22.0</td>
<td>-32.4 ± 4.7</td>
</tr>
<tr>
<td>$V_O_2$ (ml/min)</td>
<td>-8.7 ± 15.5</td>
<td>12.7 ± 24.0</td>
<td>40.4 ± 61.6</td>
<td>76.2 ± 14.1</td>
<td>40.4 ± 29.3</td>
<td>-23.6 ± 43.3</td>
<td>78.1 ± 60.2</td>
</tr>
<tr>
<td>$V_O_2$ (mg/kg/h)</td>
<td>-1.3 ± 12.6</td>
<td>-0.6 ± 9.2</td>
<td>4.1 ± 28.7</td>
<td>70.3 ± 46.1</td>
<td>50.5 ± 18.4</td>
<td>-9.7 ± 43.1</td>
<td>8.8 ± 22.7</td>
</tr>
<tr>
<td>$U_e$ (%)</td>
<td>3.0 ± 2.6</td>
<td>-7.4 ± 19.6</td>
<td>-16.5 ± 18.6</td>
<td>-3.8 ± 17.4</td>
<td>9.2 ± 13.3</td>
<td>24.2 ± 39.1</td>
<td>-33.2 ± 17.8</td>
</tr>
<tr>
<td>$f_H$ (no./min)</td>
<td>-11.3 ± 11.1</td>
<td>6.5 ± 5.5</td>
<td>-3.3 ± 21.4</td>
<td>1.7 ± 3.3</td>
<td>1.5 ± 6.3</td>
<td>-21.6 ± 24.7</td>
<td>-18.1 ± 3.7</td>
</tr>
<tr>
<td>$T_{O_2}$ (g/100 ml)</td>
<td>-20.0 ± 12.7</td>
<td>-23.7 ± 19.1</td>
<td>11.1 ± 36.8</td>
<td>2.2 ± 8.1</td>
<td>-25.9 ± 12.1</td>
<td>-41.3 ± 33.8</td>
<td>-17.2 ± 30.3</td>
</tr>
<tr>
<td>$T_{CO_2}$ (mmol/L)</td>
<td>12.0 ± 13.9</td>
<td>-9.1 ± 11.0c</td>
<td>-19.9 ± 15.6c</td>
<td>-2.0 ± 3.3c</td>
<td>-12.2 ± 13.4c</td>
<td>-15.0 ± 20.3c</td>
<td>-2.3 ± 15.2</td>
</tr>
<tr>
<td>pH_s (pH units)</td>
<td>-0.8 ± 1.0</td>
<td>-4.4 ± 0.8c</td>
<td>-0.6 ± 3.6</td>
<td>-0.06 ± 0.12</td>
<td>-2.0 ± 0.6</td>
<td>-5.7 ± 2.8c</td>
<td>-0.4 ± 1.6</td>
</tr>
<tr>
<td>Hct (%)</td>
<td>-14.0 ± 6.2</td>
<td>20.7 ± 12.5c</td>
<td>9.1 ± 16.5c</td>
<td>-21.1 ± 17.3</td>
<td>1.1 ± 9.5c</td>
<td>59.5 ± 35.5c</td>
<td>-10.5 ± 16.1</td>
</tr>
<tr>
<td>Hb (%)</td>
<td>11.0 ± 14.1</td>
<td>-1.5 ± 11.3</td>
<td>6.0 ± 10.3</td>
<td>-18.6 ± 19.8</td>
<td>-6.9 ± 12.8</td>
<td>19.6 ± 23.1</td>
<td>-11.0 ± 8.0</td>
</tr>
</tbody>
</table>

Values are means ±SD ($N = 4$ trout for each chemical except for the endosulfan (3) and control groups (12)).

*Percentage change from predose mean; postdose absolute value is derived from the mean of the measurements between 25 and 75% survival time.

bData from Bradbury et al. [12].

cSignificantly different ($p = 0.05$) from control postdose percentage change using the Wilcoxon two-sample test.
different using the Wilcoxon two-sample test (Table 3, Fig. 3). The spread in PC scores in the control group used for this study is somewhat larger than that noted in earlier studies, due in part to the larger number of fish (N = 12) in the current control group relative to previous analyses (N = 4) [6–8].

Responses of the chlorpyrifos-exposed trout compared to the control fish were markedly different along PC1, in which the exposed trout had in-
Fig. 3. Two-dimensional ordinations of the first two principal components (PCs) from the principal components analyses of 11 physiological response variables in rainbow trout exposed to acutely lethal concentrations of cypermethrin, fenvalerate, endosulfan, endrin, strychnine, and chlorpyrifos. Only variables with a correlation of greater than 0.70 \((p < 0.05)\) with the PCs are included in the interpretation of the axes. Asterisks denote those variables for which the mean percentage change for intoxicated fish was significantly different \((p < 0.05)\) from that for the control fish, using the Wilcoxon two-sample test (see Table 3). Each point represents an individual trout.
increased $f_c$ and $V_O$ and decreased $U_E$ and $f_V$. The responses of the cyclodiene-exposed trout were similar in that increases in $f_V$, $V_O$, $V_O$, and $f_H$ were highly correlated with PC1 and the PC1 axis distinguished the control and intoxicated fish. The PCAs with the pyrethroid-exposed trout also indicated a distinct response to that observed in the control group along PC1. Increasing Hct, $f_H$, and $V_O$, which were correlated with PC1, were common responses noted with fenvalerate- and cypermethrin-exposed trout. In the strychnine PCA, differences along both the PC1 and PC2 axes were important. Decreasing $T_aO_2$, $T_aCO_2$, pH, $f_V$, $f_c$, $f_H$, $V_O$, and $V_O$ were highly correlated with PC1, while increasing Hb, Hct, and $f_c$ were correlated with PC2. The PC1 and PC2 axes together showed that the strychnine-exposed fish had much different responses in those parameters than the control fish. Although consistent trends along the PC axes were generally noted for toxicants within a class (i.e., the cyclodienes and pyrethroids), there were differences between classes. The differences were most notably reflected by class-specific responses in $V_O$, $V_O$, $f_V$, Hb, and Hct.

**Discriminant function analyses**

DFA was used to determine whether responses associated with the three specific mechanisms of convulsant activity were consistently different from those responses incorporated in the previously developed five-FATS model [8]. Evidence for suspecting that the responses elicited by a chemical classified as an unknown do not fit a FATS already included in the analysis is described by Niemi et al. [26]. Briefly, the determination is derived in one of three ways: (a) large Mahalanobis distances to the FATS centroid for fish exposed to the unknown chemical, relative to the "known" fish classified within that group; (b) an inconsistent pattern in classification of the unknowns (e.g., unknowns being classified into two or more FATS groups), often accompanied with large Mahalanobis distances; or (c) a distinct pattern of response for the unknowns such as tight grouping or consistent orientation of a group with respect to other FATS groups (e.g., see Bradbury et al. [8] with regard to the polar narcosis FATS analysis and Niemi et al. [26] for illustrations).

In the initial DFA, chlorpyrifos was included as an additional AChE inhibitor with malathion and carbaryl [7], while cypermethrin, fenvalerate, endosulfan, endrin, and strychnine were treated as unknowns. Consistent with observed similarities in toxic responses for chlorpyrifos, malathion, and carbaryl (see Discussion), the compounds were readily classified into a common and distinct FATS (i.e., the AChE inhibitor FATS). Results with the trout classified as unknowns suggested that the toxic responses associated with three of the chemicals were dissimilar to the five original FATS. Cypermethrin, fenvalerate, and strychnine met the conditions described in the above paragraph, especially with regard to the second criterion (Table 4). In contrast, endrin- and endosulfan-exposed trout were consistently classified with the uncoupler FATS, and the Mahalanobis distances for those individuals were within the range for fish previously classified with that group (Table 4). The data would suggest that the strychnine group and the pyrethroid-exposed trout be analyzed as two separate FATS groups, while endrin- and endosulfan-exposed trout be combined with the fish in the uncoupler FATS. Hence, there appear to be some limitations in the ability of the DFA to recognize a potentially new FATS associated with the cyclodiene insecticides.

Despite this limitation, and because endrin and endosulfan are not oxidative phosphorylation uncouplers (see Discussion), a final DFA was run in which the cyclodiene insecticide-exposed trout were identified as a FATS group, as were the strychnine- and pyrethroid-exposed fish. The purpose of this DFA was to assess the overall discriminating ability of the measured variables when the mechanisms are identified a priori.

The final DFA with eight FATS resulted in the correct discrimination of 93% of the fish (70 of 75) and included nine variables (Table 5, Fig. 4). The first five variables ($pH_a$, $f_c$, $f_V$, $U_E$, and $V_O$) discriminated 64 of the 75 fish (85%). Although $f_H$ and Hct seemingly did not directly contribute to the discrimination of additional fish, those variables, in conjunction with $T_aO_2$ and $T_aCO_2$, were required to classify over 90% of the individuals correctly. In this analysis all fish exposed to non-polar narcotics (narcotic Type I compounds), respiratory uncouplers, respiratory irritants, and cyclodiene insecticides were correctly classified. Hence, despite the lack of evidence in the previous DFA that would suggest the cyclodiene response set was different from the uncoupler FATS, when the cyclodiene-exposed trout were explicitly identified, the respiratory-cardiovascular variables could correctly discriminate those individuals. Trout that were misclassified (5 out of 75) included two pyrethroid-exposed fish (a cypermethrin-exposed trout assigned to the strychnine FATS and a
Table 4. Summary of the discriminant function analysis when treating cyclodiene-, pyrethroid-, and strychnine-exposed trout as unknowns and using five fish acute toxicity syndromes (FATS) previously identified as known groups (narcosis Type I, narcosis Type II, respiratory irritant, AChE inhibitor, and respiratory uncoupler syndromes [7,8]).

<table>
<thead>
<tr>
<th>Chemical</th>
<th>N</th>
<th>FATS classification</th>
<th>Range of Mahalanobis distances for original fish in FATS to which specific trout was classified</th>
<th>Mahalanobis distance of specific trout to respective FATS centroid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endosulfan</td>
<td>3</td>
<td>UNCP</td>
<td>0.9-5.6</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>UNCP</td>
<td>0.9-5.6</td>
<td>2.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>UNCP</td>
<td>0.9-5.6</td>
<td>3.2</td>
</tr>
<tr>
<td>Endrin</td>
<td>4</td>
<td>UNCP</td>
<td>0.9-5.6</td>
<td>2.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>UNCP</td>
<td>0.9-5.6</td>
<td>2.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>UNCP</td>
<td>0.9-5.6</td>
<td>3.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>UNCP</td>
<td>0.9-5.6</td>
<td>4.3</td>
</tr>
<tr>
<td>Cypermethrin</td>
<td>4</td>
<td>NARCI</td>
<td>1.5-11.5</td>
<td>5.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NARCI</td>
<td>1.5-11.5</td>
<td>8.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NARCI</td>
<td>1.5-11.5</td>
<td>19.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>UNCP</td>
<td>0.9-5.6</td>
<td>12.0</td>
</tr>
<tr>
<td>Fenvalerate</td>
<td>4</td>
<td>AChE</td>
<td>1.8-17.6</td>
<td>4.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>UNCP</td>
<td>0.9-5.6</td>
<td>6.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IRRI</td>
<td>1.2-6.5</td>
<td>3.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NARCI</td>
<td>1.6-18.6</td>
<td>20.4</td>
</tr>
<tr>
<td>Strychnine</td>
<td>4</td>
<td>AChE</td>
<td>1.8-17.6</td>
<td>32.3</td>
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<td></td>
<td></td>
<td>IRRI</td>
<td>1.2-6.5</td>
<td>14.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>UNCP</td>
<td>0.9-5.6</td>
<td>4.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NARCI</td>
<td>1.6-18.6</td>
<td>11.3</td>
</tr>
</tbody>
</table>

NARCI = narcosis Type I (nonpolar narcosis) syndrome, NARCI = narcosis Type II (polar narcosis) syndrome, IRRI = respiratory irritant syndrome, AChE = AChE inhibitor syndrome, and UNCP = respiratory uncoupler syndrome.

Table 5. Summary statistics of the stepwise discriminant function analysis for eight fish acute toxicity syndromes (FATS) (narcosis Type I, narcosis Type II, respiratory irritant, AChE inhibitor, respiratory uncoupler, pyrethroid insecticide, cyclodiene insecticide, and strychnine syndromes).

<table>
<thead>
<tr>
<th>Step</th>
<th>Variable</th>
<th>Univariate $F$</th>
<th>Standardized discriminant function coefficients</th>
<th>Percentage of trout correctly classified</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>pH&lt;sub&gt;a&lt;/sub&gt;</td>
<td>14.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.48 0.07 0.09</td>
<td>32</td>
</tr>
<tr>
<td>2</td>
<td>$f_C$</td>
<td>13.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-0.62 0.16 -0.07</td>
<td>49</td>
</tr>
<tr>
<td>3</td>
<td>$f_D$</td>
<td>10.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-0.40 -0.04 0.77</td>
<td>67</td>
</tr>
<tr>
<td>4</td>
<td>$U_C$</td>
<td>8.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.53 0.57 0.62</td>
<td>75</td>
</tr>
<tr>
<td>5</td>
<td>$V_O_2$</td>
<td>9.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-0.39 -0.98 -0.16</td>
<td>85</td>
</tr>
<tr>
<td>6</td>
<td>$f_H$</td>
<td>3.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.18 0.50 0.33</td>
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<tr>
<td>7</td>
<td>Hct</td>
<td>4.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.07 0.27 -0.49</td>
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</tr>
<tr>
<td>8</td>
<td>$T_O_2$</td>
<td>5.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.32 -0.11 0.03</td>
<td>89</td>
</tr>
<tr>
<td>9</td>
<td>$T_CO_2$</td>
<td>6.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.28 0.15 -0.17</td>
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</tr>
</tbody>
</table>

Eigenvalue 3.20 1.46 1.20
Wilks lambda 0.05 0.13 0.30
$x^2$ of Wilks lambda 190.1<sup>a</sup> 131.2<sup>a</sup> 79.4<sup>a</sup>

$N = 75$ fish.

<sup>a</sup>$p < 0.01.$
Trout physiological toxicity syndromes: Part 4

Pyrethroid and cyclodiene intoxication was associated with seizures anterior to the site of spinal transection; however, seizures were more intense during pyrethroid intoxication. The visible signs of pyrethroid intoxication were similar to those previously reported in fish and consistent with reports for higher vertebrates [14]. In addition to the involvement of the head and opercula in seizures, cyclodiene intoxication resulted in tremors and tetany of the pectoral fins, which were not observed in the other toxicant groups. Clonic convulsions, recurrent tremors, rapid pectoral movements, and tonic paralysis before death were consistent with the clinical signs associated with other observations of cyclodiene intoxication in mammals [27] and fish [28,29].

Seizures elicited by strychnine were unique to the compounds tested in this study. Spasms involved the entire body, which indicated involvement of the CNS posterior to the spinal transection site. Seizures were also associated with heightened reflex excitability; seizure frequency and severity were increased by sensory stimulation. This seizure response was distinct from that noted in trout exposed to polar narcotics, which also elicited whole body convulsions [8]. The strychnine-induced responses were consistent with effects observed in other fish [30] and vertebrates [31,32], and with the generally accepted hypothesis that strychnine, by acting as a glycine antagonist [16,33], lowers the threshold for stimulation of spinal reflexes by blocking inhibitory pathways mediated by Renshaw cells [18].

**Physiological responses**

Except for increased $f_c$, responses of the respiratory-cardiovascular parameters were generally distinct for each neurotoxicant group. Increased $f_c$ with pyrethroid, cyclodiene, and strychnine intoxication was typically associated with the onset and frequency of seizures. Because seizures are typically stimulus-dependent in hypersensitized fish (especially critical early in an exposure), it seems reasonable to assume that neurotoxicant-induced coughs could trigger convulsions. The cough response itself could be a CNS-mediated component in a seizure syndrome or possibly a side effect due to interactions with sensory receptors in the pharynx and gill arches [34] or direct irritation of gill tissue. Fenvalerate and permethrin (3-phenoxybenzyl $[R,S]$-cis,trans-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate) have been shown to cause gill damage consistent with irritation.

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**DISCUSSION**

Visible signs of intoxication

The visible signs of intoxication elicited by chemicals within a neurotoxicant group were generally distinct. Trout exposed to chlorpyrifos showed few overt signs of intoxication, which was similar to previous observations in experiments with malathion and carbaryl [7]. Increased defecation and bile loss were observed in the chlorpyrifos-intoxicated trout, which is consistent with observations in mammalian studies and presumably is related to the muscarinic effects of AChE inhibition [19].

fenvalerate-exposed fish assigned to the AChE inhibitor FATS, two narcosis Type II-exposed fish (one assigned to the cyclodiene FATS and the other to the respiratory irritant FATS), and one strychnine-exposed fish (assigned to the pyrethroid FATS). Despite some misclassifications, the majority (≥75%) of exposed fish for any one FATS were correctly classified and in no case would it be concluded that a FATS was not resolvable with the current suite of respiratory-cardiovascular variables.

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Fig. 4. Two-dimensional plot of the first two discriminant functions (DFs) that separate eight fish acute toxicity syndromes (FATS): narcosis Type I syndrome (I), narcosis Type II syndrome (II), respiratory irritant syndrome (III), AChE inhibitor syndrome (IV), respiratory uncoupler syndrome (V), cyclodiene syndrome (C), pyrethroid syndrome (P), and strychnine syndrome (S). Interpretation of the axes was based on the standardized DF coefficients (see Table 5 and text). Each symbol represents the centroid of a FATS. Data associated with FATS I, II, III, IV, and V were obtained from McKim et al. [6,7] and Bradury et al. [8].
Cycloidiene insecticides also have been previously reported to promote coughing [36] and are known to cause gill damage [37].

Increased $f_c$ in chlorpyrifos-intoxicated trout was probably due to an effect unrelated to the primary mechanism of AChE inhibition. In fish, lethal aqueous exposures to AChE inhibitors do not consistently increase $f_c$, which suggests that some other mechanism is responsible for coughing [38]. Klaverkamp et al. [38] reported that fenitrothion ($O, O$-dimethyl $O$-(4-nitro-$m$-tolyl) phosphorothioate) increased coughing in rainbow trout, whereas acephate ($O,S$-dimethyl acetylphosphoramidodithioate) did not. McKim et al. [7] did not report a cough response in trout exposed to malathion or carbaryl. The ventilatory patterns of chlorpyrifos-exposed trout were similar to those elicited by the gill irritants benzaldehyde and acrolein [7] and suggest a strong irritant effect in addition to AChE-related effects. Lunn et al. [36] also attributed increased coughing in trout exposed to carbaryl to affected respiratory surfaces.

Responses of the remaining respiratory-cardiovascular parameters were generally distinctive for each class of neurotoxicant. Except for increased $f_c$ (see above paragraph), the physiological responses associated with chlorpyrifos intoxication (e.g., decreased $f_m$ and $U_e$ and increased $V_G$) were comparable to those reported previously by McKim et al. [7] for malathion and carbaryl. Effects of AChE inhibitors on cholinergic neural–muscular junctions in the gills and heart have been implicated as being responsible for those responses [7].

The physiological response to pyrethroid intoxication was consistent with the increased muscular activity associated with intense seizures. As is typical for trout [39], the increased oxygen demand to support this activity was associated with an increase in $V_G$. Interestingly, the cypermethrin-exposed trout showed a rise in $f_c$ until near death. This response may have been an artifact due to the seizures or perhaps a chemical-specific response. Due to a drop in $U_E$, $V_O2$ did not significantly increase, which seemingly resulted in a shift to anaerobic metabolism as reflected by dropping $T_{O2}$, $T_aCO_2$, and $pH_a$. Erythrocyte swelling, as a response to increased lactic acid levels (decreasing $pH_a$) and decreased $T_{O2}$ [40,41], was the most likely cause of increased Hct. Convulsions in mammals also result in similar changes in physiological status and have been associated with the CNS activity of the Type II pyrethroids [14].

The physiological response of trout to cyclodiene intoxication was distinct in that seizure activity was seemingly not associated with a shift toward anaerobic metabolism. Steady $U_E$ with increased $V_G$ resulted in large increases in $V_O2$. As a consequence, $T_{O2}$ remained near predose levels until late into the exposures. In addition, only a slight decline in $pH_a$ was noted, which was associated with steady or declining Hct. The responses of the cyclodiene-intoxicated trout were similar to those observed by McKim et al. [7] for trout exposed to the oxidative phosphorylation uncouplers, pentachlorphenol and 2,4-dinitrophenol. Although it is becoming increasingly accepted that the cyclodiene act specifically at the GABA-receptor–ionophore complex [15], previous in vitro and in vivo research led to an earlier hypothesis that ionoimbalances, and resulting neuronal dysfunction, were the result of impaired energy metabolism through the selective inhibition of oligomycin-sensitive Mg$^{2+}$-ATPase [42]. This ATPase is an energy-regulating enzyme that is associated with oxidative phosphorylation; its inhibition by certain organochlorine insecticides, including the cyclodiene, results in decreased oxidative phosphorylation efficiency [42]. Any impairment of oxidative phosphorylation, by either classic uncouplers that act via a protonophoric mechanism [9] or compounds that directly inhibit a critical enzyme in the respiratory chain, would likely elicit the same physiological response to reduced ATP production. It is possible that the elevated $V_G$ (the typical response of trout to the increased oxygen demand imposed by uncouplers [7]) and $V_O2$ noted in the cyclodiene-exposed trout are due to a side effect associated with those insecticides at high exposure concentrations. In addition to signs manifested by neurotoxic activity, nonsalmonid species show greatly elevated $f_V$ when exposed to aldrin [43] and chlordane [44]. Increased $f_V$ in nonsalmonid species is a typical response to increased oxygen demand [39], and the observations noted with aldrin and chlordane may also be symptomatic of the possible secondary oxidative phosphorylation effect due to acute cyclodiene exposure.

The physiological responses of strychnine intoxication were also distinct. During the initial stages of intoxication, $V_G$ increased, presumably as a response to an increased oxygen demand due to the muscular activity associated with seizures. After this initial response, however, $V_G$ declined with a resulting increase in $U_E$ and a moderate drop in $V_O2$. Associated with this decline in respiration was the development of a hypoxic condition, as evidenced by decreasing $T_{O2}$ and $pH_a$ and increasing Hct. Except for the continued seizure activity and gill damage, the effects of strychnine intoxication were similar to those reported for other Class I and II neurotoxicants. Increased scores for increased $f_m$ on cholinergic neural–muscular junctions. Except for increased $AChE$ inhibitors on cholinergic neural–muscular junctions, this activity was associated with an increase in $pH_a$ (e.g., decreased $f_r$ and $U_e$) for malathion and carbaryl. Effects of non-neurotoxic activity, nonsalmonid species show greatly elevated $f_V$ when exposed to aldrin [43] and chlordane [44]. Increased $f_V$ in nonsalmonid species is a typical response to increased oxygen demand [39], and the observations noted with aldrin and chlordane may also be symptomatic of the possible secondary oxidative phosphorylation effect due to acute cyclodiene exposure.

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and associated cough response, the stages of respiratory collapse with strychnine intoxication are similar to physiological responses elicited by narcotics [7, 8]. Declining respiration is a common response noted in strychnine-intoxicated mammals as well. Hypoxia, thought to result from periods of impaired respiration during convulsions, eventually leads to medullary paralysis and death [31, 32].

**FATS discrimination**

Based on the toxic responses, DFA was used to determine whether the fish exposed to known CNS convulsants could be separated into FATS that were distinct from those previously identified [8]. The results of the DFA, in which the five convulsants were treated as unknowns, were mixed. The pyrethroid- and strychnine-intoxicated trout exhibited respiratory-cardiovascular responses that were not readily classifiable into any of the original FATS. The results indicated that the parameters used in the five-FATS model [8] were sufficient to discriminate between the responses associated with those convulsants and the responses elicited by chemicals representing the other five modes of action. Interestingly, the cyclodiene-intoxicated trout were all classified as oxidative phosphorylation uncouplers, primarily due to the similarity of the responses of the cyclodiene-exposed trout in $V_0$, $V_O$, and $T_nO_2$ to those elicited by pentachlorophenol and 2,4-dinitrophenol [7]. This classification is consistent with the observation that a possible side effect of the cyclodiienes may be increased respiration due to inhibition of oligomycin-sensitive Mg$^{2+}$-ATPase (see previous discussion). As would be expected, compounds capable of impeding oxidative phosphorylation at different sites (or steps) produced similar in vivo responses related to respiration.

In the final DFA, in which the five convulsants were identified as three distinct FATS, over 90% of the fish were correctly classified. Because the ratio of variables to sample size is relatively high (9 variables and a total of 75 fish), the results should be viewed with caution. The results of this analysis indicated that the 11 respiratory-cardiovascular parameters could correctly differentiate the three convulant modes of action and the original five modes of action into eight FATS. The responses of the strychnine-intoxicated trout were similar to the responses of the nonpolar narcotic-intoxicated fish. A similarity in responses for the pyrethroid- and respiratory irritant–exposed trout was also observed (Fig. 4). These patterns were consistent with the respiratory depression associated with strychnine-induced seizures and the gill irritation associated with pyrethroid exposures, previously described. Although the results of the first DFA did not substantiate that the uncoupler- and cyclodiene-intoxicated trout were distinct, the results of the final DFA indicated that when identified a priori, the model could correctly differentiate the cyclodiene-intoxicated trout from the uncoupler FATS. This differentiation is based on greater increases in $T_nO_2$ and $V_O$ in the uncoupler-exposed trout and the elevated $f_C$ (associated with seizures) in the cyclodiene-exposed trout. When the cyclodiene-exposed trout were not identified as a distinct FATS a priori, those variables were not weighted sufficiently in the DFA to discriminate the fish from the uncoupler FATS.

The ultimate objective of this research is to better define chemical structures associated with specific toxic mechanisms. Through this understanding more robust QSAR models for hazard assessments can be developed. A major application of this effort is in the assessment of new and existing industrial chemicals under TSCA. In a context where the regulated chemicals are not overtly designed to have biological activity and the sites and mechanisms of action are either unknown or poorly defined, the use of whole organism bioassays and physiological studies is certainly effective in identifying potentially common modes of action [4–8]. As more specific modes of action are encountered, these approaches will be useful as initial screens to determine the extent and type of in vitro testing needed to define mechanistic classifications more accurately. The results of this investigation, using model toxicants and associated mechanisms, illustrated the ability of in vivo physiological techniques to identify potential CNS seizure agents within the industrial chemical inventory.

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**REFERENCES**


