

## Catecholamines as Potential Sub-lethal Stress Indicators in Great Lakes Macrobenthic Invertebrates

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**ABSTRACT.** Concentrations of the catecholamines [dopamine (D), and norepinephrine (NE)] were measured in representative Great Lakes macrobenthic invertebrates to determine the feasibility of using these compounds as indicators of sub-lethal stress. Epinephrine [E] was not detected in most of these invertebrates. A high performance liquid chromatographic procedure was modified for use with aquatic invertebrates. Chironomids and oligochaetes contained D concentrations that were similar, averaging 1 pmole/mg wet wt. Crustacean D levels were lower, averaging 0.2 pmole/mg wet wt. NE averaged 0.2 pmole/mg wet wt. for chironomids and 0.57 pmole/mg wet wt. for oligochaetes. NE was not detected in the crustaceans. Individual chironomids contained detectable levels of catecholamines; all other invertebrates required pooled homogenates. The chironomid group was targeted for laboratory stress experiments. Cultured midge larvae (*Chironomus tentans*) subjected to acute thermal stress (35°C) exhibited D concentrations significantly lower than controls (23°C). Animals exposed to lindane concentrations 0.5 mg/L - 8.0 mg/L contained D concentrations significantly higher than controls. NE and E were not detected in either stress experiment. Demonstration of dramatic catecholamine concentration changes in response to stressors suggests that these compounds could potentially be indicators of sublethal stress.

**INDEX WORDS:** Catecholamines, chironomids, lindane, bioconcentration.

### INTRODUCTION

Sub-lethal indicators of stress are needed to explore the impact of toxins on the biota of the Great Lakes (Nalepa and Landrum 1988, Giesy *et al.* 1988, Nalepa 1991). Historically, distributions of macrobenthic invertebrates have been used to monitor environmental health in the Great Lakes (Nalepa *et al.* 1985). Determining specific causes of changes in benthic distributions is difficult in field research because many factors, both natural and "human induced," can operate simultaneously to affect the health of benthic organisms. Laboratory studies are needed to investigate the relationship between stress and biochemical changes in the organisms. The controlled conditions of laboratory research allow more precise evaluation of the relative impact of different stressors on organisms. This type of data is needed for comparison with the traditional benthic field surveys. Mixtures of contaminants typically observed in the Great Lakes are complex, their concentrations variable, and factors

controlling their bioavailability poorly understood (Giesy and Hoke 1989). The development of reliable, sensitive biochemical bioassays could potentially circumvent the problem of isolating the proportional effect of one of an array of stressors observed *in situ* (Giesy and Hoke 1989).

Catecholamine concentrations are one of a number of potential indices of sublethal stress. Other biological tests that have been explored in Great Lakes invertebrates include: behavioral modifications in response to contaminant laden sediments in oligochaetes (Keilty *et al.* 1988a, Keilty *et al.* 1988b, White and Keilty 1988, Keilty *et al.* 1988c) and in chironomids (Wentzel *et al.* 1977), and modifications in the swimming behavior of the amphipod *Diporeia* sp. in response to contaminants (Lindstrom and Lindstrom 1980). A substantial effort has been invested in documenting the morphological deformities exhibited by chironomids in polluted regions including: western Lake Erie, near the mouths of the Detroit and Maumee Rivers (Brinkurst *et al.* 1968); Bay of

Quinte, Lake Ontario (Warwick 1980); Georgian Bay, Lake Huron (Hare and Carter 1976); and the Lac St. Louis and Laprairie basins of the St. Lawrence River (Warwick 1990b). In addition, several investigations have specifically explored the utility of chironomid morphological abnormalities as a biomonitoring tool (Warwick 1985, Warwick 1988, Warwick 1989, and Warwick 1990a). A catecholamine concentration bioindex potentially offers an even greater level of sensitivity than these established assays, and may also help to elucidate the modes of action of specific toxicants (Giesy *et al.* 1988).

The functional role of catecholamines in invertebrates is incompletely understood. Most frequently hypothesized is their role as neurotransmitters (Walker 1977, Stone *et al.* 1978, Fingerman and Fingerman 1977, Barker *et al.* 1979, Eloesson *et al.* 1982, Laxmyr 1984, Yoshino and Hisada 1984). It has also been suggested that dopamine and norepinephrine have a role in the sensory modulation of locomotion (Welsh 1972). Although little information is available on the biochemistry of catecholamines in invertebrates, these compounds may be potentially linked with stress responses in invertebrates as has been observed in their vertebrate counterparts. Catecholamine concentrations have been frequently correlated with stress in vertebrates (Lobanova *et al.* 1986, Eremina and Beliakova 1987, Demarest *et al.* 1985, O'Donnel *et al.* 1987, Brokow and Hansen 1987, Nakano and Tomlinson 1967, Mazeaud 1969, Tufts *et al.* 1987). Catecholamine concentrations have been demonstrated to change monotonically with changes in the magnitude of foot shock stress in laboratory rats (Natelson *et al.* 1987). A few studies have indicated a potential correlation between stress and catecholamine levels in invertebrates (Hiripi *et al.* 1985, Zatta 1987). Dopamine and norepinephrine levels varied significantly as a function of duration of hypo-osmotic stress in the crab *Carcinus maenas* (Zatta 1987). Similar changes in catecholamine concentrations may occur in Great Lakes macrobenthic invertebrates in response to stress treatments. This study explores the possibility of using catecholamine concentrations in aquatic invertebrates as a biochemical indicator of stress.

## METHODS

### Sample Preparation and High Pressure Liquid Chromatography Technique

A sensitive high performance liquid chromatography (HPLC) procedure designed to isolate and

measure catecholamines in human plasma (Mitsui *et al.* 1985) was modified for use with aquatic invertebrates. Cation-exchange columns were slurry packed (0.06 mL of BC-X8, 20  $\mu$ m size cation-exchange resin, Benson Co.) into a 2.5 mm I.D. column (Altex Leur adapter drilled and fitted with a frit). Before sample application, each column was washed successively with 0.5 mL KCL elution solution (an aqueous solution of 0.6 M potassium chloride and 0.2 mM potassium ferricyanide mixed with an equal volume of acetonitrile), 1.0 mL of 2.0 M lithium hydroxide, 2 mL deionized water, 0.5 mL of 12 M HCl:ethanol (1:9 v/v), and 1.0 mL of 0.2 M lithium phosphate buffer adjusted to pH 5.8.

Catecholamines were isolated by homogenizing the aquatic invertebrates for 1 minute in 1 mL of cold 0.2 M lithium phosphate buffer using a manual tissue grinder in an ice bath. The buffer was fortified with 1  $\mu$ L of the internal standard isoproterenol (IP). After grinding, the cellular debris was removed from the homogenate by high-speed centrifugation at 14,000 g for 1 min. The supernatant was passed through a cation-exchange column to capture the catecholamines. The column was then washed with 1.5 mL deionized water and 0.5 mL aqueous acetonitrile:water (1:1). The catecholamines were eluted with 0.9 mL KCL elution solution. Three tenths mL 1,2-diphenylethylenediamine (DPE) solution (0.1 M in 0.1 M HCL, pH = 6.7) were added, and the vial was closed and held at 40°C for 40 minutes for derivatization. The vial was placed on ice to stop the reaction, and 0.3 mL of the solution was analyzed by HPLC.

The catecholamines were separated by isocratic reverse-phase chromatography (Varian Micropac MCH-5 column, 300  $\times$  4 mm column or a Beckman 250  $\times$  4.6 mm column [mobile phase = acetonitrile:methanol:water (5:1:4 v/v); flow rate = 1.4 mL $\cdot$ min<sup>-1</sup>] and measured with a Varian Fluorichrom detector (excitation wavelength = 345 nm; emission wavelength = 485 nm).

Primary standards of 1 mM dopamine (D), norepinephrine (NE), epinephrine (E), and IP were prepared in methanol and stored at 4°C. Before analysis, a secondary standard was prepared by adding 0.01 mL of each primary standard to deionized water to make a total volume of 5 mL. The secondary standard was diluted  $\times$  1000 (1  $\mu$ L to 1 mL) with lithium phosphate buffer, and a portion (0.6 mL) of the resulting solution was passed through a prepared cation-exchange column, eluted, and derivatized with DPE in the same manner as the samples. The standard solution and a blank lithium

hydroxide buffer solution were subjected to cation-exchange purification and analysis on each sample-analysis day to compare with the samples based on the ratio of each catecholamine to IP. Any peaks in the blanks were subtracted from both the standard and sample. Catecholamine concentrations were calculated using an internal standard IP by the stock solution method of Yost *et al.* (1980). Stock solutions of the internal standard (IP) and catecholamine standards NE, E, and D were prepared separately and subjected to the identical cleanup and HPLC analysis as the samples; the internal standard was added to the sample solutions in the same concentration as in the stock solution.

#### Field Collection and Handling of Macroenthic Survey Invertebrates

Organisms were collected from Saginaw Bay, Lake Huron, southeastern Lake Michigan offshore of Grand Haven, Michigan, and the western basin of Lake Ontario (Table 1). Station LORAN coordi-

nates, depth, and water temperature were recorded for each site (Table 1). Triplicate Ponar grab samples were collected during October-December 1988; the contents were placed into 33" × 39" plastic bags; lake water added; and the bags were tied at the water surface to eliminate air and prevent organisms from getting caught on an air/water interface during transport. The bags were placed in coolers and covered with ice. Surface water was also collected at each site. Samples were transported to the lab and transferred into separate aquaria for each station. Each aquarium was enclosed with black plastic, covered with a lid to mimic dark field conditions, aerated, and held at 4°C. Additional lake water was added as needed.

*Diporeia* sp. and *Mysis* sp. were maintained in their original aquaria until the day of experimentation. Aquarium sediments were gently sieved (500 µ mesh) to concentrate oligochaetes and chironomids. The oligochaetes and chironomids were identified and counted using a dissection scope with a fiber optic light, to prevent unnecessary heating.

TABLE 1. Great Lakes macroenthic invertebrate survey sampling station characteristics.

Station*	Loran Coord.	Depth (FT)	Bottom °C	Date Collected	Genera** Tested
LH* S-5	x 31318.86 y 49311.832	18	7.7	3/11/88	b,e,f**
LH S-7	x 31344.114 y 49285.741	20	7.7	3/11/88	c
LH S-51	x 30991.503 y 49078.078	90	6.8	3/11/88	a
LH M-57	x 31059.819 y 49054.728	32	7.1	3/11/88	c
LO	y 67968.33 z 51352.96#	105	5.0	20/10/88	a,e,f
LM H-31	x 32545.0 y 49497.40	135	6.7	7/12/88	a,d,e,f
LM H-21	x 32635.02 y 49456.38	300	5.2	31/11/88	g

\*LH = Lake Huron, LM = Lake Michigan, LO = Lake Ontario, station numbers correspond to Nalepa (1987) and Nalepa (unpublished Saginaw Bay macroenthic survey)

\*\*a = *Diporeia* sp.

b = *Chironomus anthracinus*

c = *Chironomus semireductus*

d = *Stylodrilus heringianus* (Oligochaete)

e = *Limnodrilus* sp. (Oligochaete)

f = *Tubifex* sp. (Oligochaete)

g = *Mysis relicta*

# These loran coordinates are the East Coast 9930 chain.

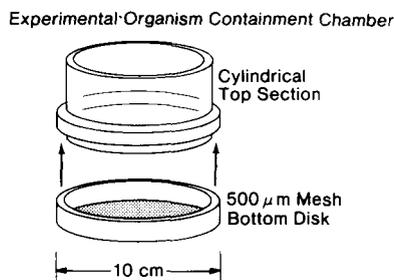


FIG. 1. Experimental organism containment chamber.

They were gently picked from petri dishes cooled with ice, placed in individual 10 cm diameter plexi-glass cylinders covered on one end with 500  $\mu\text{m}$  nytex screen (Fig. 1) and transferred to separate aquaria to minimize potential sampling bias and to reduce handling stress on the day of experimentation. Each cylinder containing approximately 5 cm of sieved station sediments was placed screen side down within a holding aquarium filled with lake water from the collection station. The organisms remained undisturbed within these cylinders until used for experimentation. On the day of the experiment, the cylinder was removed from the aquarium, and organisms were quickly dropped into 4°C commercial club soda to temporarily immobilize them (M. Quigley, personal communication, NOAA-Great Lakes Environmental Research Lab).

Catecholamine measurements represented pooled whole-organism homogenates in all groups except for the chironomids which were analyzed individually.

#### Chironomid Culturing Procedure

*C. tentans* were cultured in reconstituted lake water [20 mg  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 10 mg  $\text{MgSO}_4$ , and 20 mg  $\text{NaHCO}_3$  per liter of distilled, deionized water (Lehman 1980)] (Fig. 2). Water temperature averaged 23°C with a range of  $\pm 2^\circ\text{C}$ ; the light cycle was 16 hr light, 8 hr dark, with onset of light at 6 a.m. The aquaria (approximately 14 L) contained a biological filtration system: water was constantly recycled through a gravel/dolomite bed covered with bacteria (Fig. 2). The dolomite helped to buffer the water; pH was maintained at 7.0 with a range of  $\pm 0.5$ . The gravel bed was covered with 500  $\mu\text{m}$  sand that had been pre-combusted at 500°C for 8 hr to eliminate extraneous food sources. The

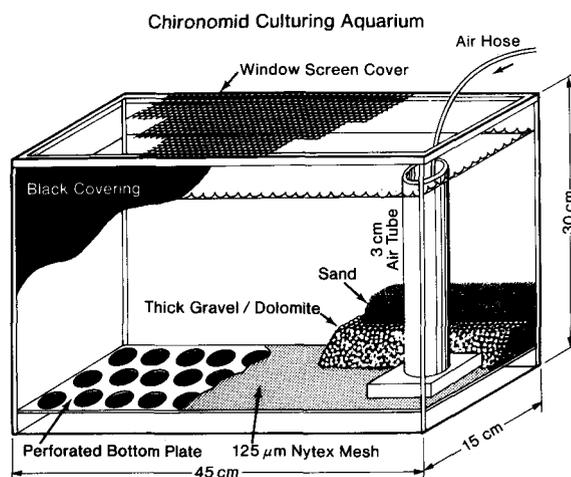


FIG. 2. Chironomid culturing aquarium.

midges were fed approximately 100 mg of Tetra Min brand tropical fish flake food blended in water daily (Mosher 1982). The amount given depended on the number and size of the larvae. If the water was not clear 3-4 hr after feeding, food was considered to be in excess and subsequently reduced. The larval wet/dry weight ratio measured for chironomids from the stock culture was linear with a slope of  $0.185 \pm .003 \text{ SE}$  and  $r$  value of 0.98 ( $n = 49$ ).

Chironomid egg masses hatched 2 or 3 days after deposition. Larval growth occurred in 4 instars of approximately 1 week each and experiments were run on 3rd instar larvae. Adults were aspirated into a 250 mL Erlenmeyer flask each day with approximately 50 mL of the constituted lake water. Egg masses deposited overnight were placed into new aquaria to perpetuate the culture.

#### Temperature Stress Experiment Methodology

Third instar *Chironomus tentans* larvae were wet-weighted 2 days prior to experimentation. Individual midges were pipetted from culture, blotted dry, and wet-weighted. Larvae were immediately returned to culture and allowed to re-acclimate for 48 hr. The pre-weighting reduced the possibility of inflicting extraneous stress to the organisms immediately prior to experimentation. On the day of the experiment, the larvae were removed from culture and placed directly into test beakers. Individual midge larvae were placed in 200-mL beakers (one per beaker) containing 150 mL of reconstituted lake water above a 2.5 cm layer of sterile sand (<500  $\mu\text{m}$ ). Two experimental groups were placed in sepa-

rate incubators at 23°C with a range of  $\pm 1^\circ\text{C}$  (control) and 35°C with a range of  $\pm 1^\circ\text{C}$  under the same light cycle as the culture. Beakers were aerated throughout the experiment maintaining oxygen levels at or near saturation. The larvae were exposed over time intervals ranging from 0-24 hr. At each time point, midges were removed, placed directly in liquid nitrogen, and stored for HPLC analysis (all midges were analyzed within 72 hours).

### Lindane Range-Finding Study Methodology

LC<sub>50</sub> determinations were performed by static exposures of third instar *C. tentans* larvae to five aqueous concentrations of lindane (0.25, 0.75, 1.5, 7.5, and 15 mg/L) for 48 hr in five replicates. A control receiving no chemical, and a solvent control were also tested. A stock acetone solution (15 mg/mL) was diluted with reconstituted lake water (Lehman 1980) to make the exposure solutions. The test was performed in a light tight box, test vessels were maintained at room temperature (21.2°C  $\pm$  0.6) at a pH of 7.0 with a range of  $\pm 0.2$ , and were not aerated. A 16 hr light and 8 hr dark photoperiod was provided. Larvae were not fed during the testing period. Mortality was scored at 24 and 48 hr. The criterion for mortality was failure to move when prodded.

### Lindane Stress Experiment Methodology

The lindane exposure assay for the catecholamine stress experiment followed the procedure described above for the range-finding experiment with the following changes. The wet weight was measured 2 days prior to exposure as described in the temperature stress method above. The chironomids were exposed to 0, 0.5, 1.0, 2.0, 4.0, or 8.0 mg/L lindane plus a solvent control (12  $\mu\text{L}$  acetone/40 mL water) for 24 hr. <sup>14</sup>C Lindane was used as a tracer for the determination of experimental lindane concentrations, as well as body burden and bioconcentration factor (Peter Landrum, personal communication, Great Lakes Environmental Research Laboratory) for each treatment. A mixture of 6.9 mg cold and 1.1 mg <sup>14</sup>C labeled lindane (Sigma >98% pure, specific activity of 9.2 mCi/mmol) was dissolved in 350  $\mu\text{L}$  acetone to yield a stock solution of 8 mg/L for the dilution series. Each test concentration was replicated 10 times using 1 larva per replicate. The dilution series was allowed to equilibrate for 12 hr to allow for <sup>14</sup>C lindane adsorption to the glass

beaker surface. A triplicate set of water samples (2 mL) was collected from each treatment before the introduction of the midges and at 24 hr after the midges were withdrawn. Water samples were counted in 10 mL of scintillation cocktail (RPI 3a70B) to monitor levels of radioactivity in the water over time. Three midge larvae from each treatment were measured for bioconcentration factor at each exposure concentration. Larvae were placed whole in 12 mL of scintillation cocktail, allowed to equilibrate for 72 hr, and counted. No significant changes in disintegrations per minute were observed when the larvae were recounted after an additional 72 hr equilibration time. Samples were corrected for quench using the external standards ratio method after subtracting background. The seven remaining chironomid larvae at each concentration were stored in liquid N<sub>2</sub> until HPLC analysis for catecholamines (all samples were analyzed within 1 week).

## RESULTS AND DISCUSSION

### Macrobenthic Invertebrate Survey

Catecholamine concentrations were measured in: *Diporeia* sp. (Bousfield 1989) [formerly recognized as *Pontoporeia hoyi* (Amphipoda, Gammaridae)]; *Chironomus anthracinus*, *Chironomus semireductus* (Diptera, Chironomidae); *Stylodrilus heringianus*, (Oligochaeta, Lumbriculidae); *Limnodrilus* sp., *Tubifex* sp. (Oligochaeta, Tubificidae); *Mysis relicta* (Mysidacea: Mysidae).

Averaged catecholamine concentrations and the range of concentrations detected in the Great Lakes macrobenthic invertebrate survey are reported in Table 2. A comparison between Great Lakes invertebrate catecholamine concentrations and results for invertebrates in related taxonomic groups is reported in Table 3.

Great Lakes invertebrates exhibited concentration ranges and ratios of catecholamines similar to other reported values for invertebrates. All Great Lakes organisms tested contained one or more of the three primary catecholamines D, NE, and E (Table 2). Dopamine was present in all groups tested at higher concentrations than NE or E (not always present). These results concur with previous investigations of catecholamines; one or more of these compounds have been detected in every invertebrate ever tested (Welsh 1972); and in the great majority of invertebrates that have been examined, D is the dominant catecholamine (Ostlund

**TABLE 2. Catecholamines in the predominant Great Lakes macrobenthic invertebrate groups (pmole/mg wet weight).**

INVERTEBRATE GROUP	compound	MEAN	SE*	RANGE	
				minimum	maximum
Phylum Arthropoda					
Class Crustacea					
Order Amphipoda					
<i>Diporeia</i> sp.	D	0.16	0.01	0.13	0.24
	NE	nd	—	—	—
	E	nd	—	—	—
Class Crustacea					
Order Mysidacea					
<i>Mysis relicta</i>	D	0.19	0.01	0.13	0.37
	NE	nd	—	—	—
	E	nd	—	—	—
Class Insecta					
<i>Chironomus anthracinus</i>	D	1.0	0.02	0.93	1.10
	NE	0.2	0.02	0.13	0.3
	E	0.05	0.02	0	0.14
Phylum Annelida					
Class Oligochaeta					
<i>Stylodrilus heringianus</i>					
<i>Limnodrilus</i> sp.	D	1.1	0.18	0.76	1.48
	NE	0.57	0.07	0.43	0.72
	E	nd	—	—	—

\* = standard error of the mean

D = Dopamine

NE = Norepinephrine

E = Epinephrine

nd = not detected

1954; Murdock 1971, Welsh 1972, Coupland 1979, Evans 1980, and Gardner and Walker 1982).

Dopamine averaged 1 pmoles/mg wet weight and NE averaged 0.2 pmoles/mg wet weight in Great Lakes chironomids; D/NE = 5/1. Both the absolute NE concentrations and D/NE ratio for chironomids closely correlated with previously reported values for catecholamine measurements made on whole organism homogenates for other members of the Class Insecta (catecholamines have not been previously examined in chironomids). Similar concentrations and ratios in insects larvae were reported by Ostlund (1954) and Bjorklund *et al.* (1970).

Dopamine averaged 1.1 pmoles/mg wet weight in Great Lakes oligochaetes; NE was also detected averaging 0.57 pmoles/mg wet weight. Direct comparison of catecholamine concentrations found in this Great Lakes survey with other oligochaetes is

problematic because previous oligochaete studies did not examine catecholamine concentrations in whole organism homogenates. Instead, values were typically reported as  $\mu\text{g}$  catecholamine/g nervous tissue (Gardner and Walker 1982). However, the ratio of D/NE in two previous studies of oligochaetes was 2/1 (Gardner and Cashin 1975, Rude 1969) and agreed with our results.

Concentrations of D were low in the crustaceans, averaging 0.16 pmoles/mg wet weight in *Diporeia* sp. and 0.19 pmoles/mg wet weight in *Mysis relicta*; no NE was detected in either of these organisms (Table 2). Historically, the presence of NE in crustacea was disputed until it was found in the crayfish *Pacifastacus leniusculus* (Eloesson *et al.* 1982) and verified by Laxmyr (1984); many early studies of catecholamines failed to find NE in crustacea (Kerkut *et al.* 1966, Cottrell 1967, and

**TABLE 3.** Catecholamine concentration comparison between Great Lakes and related invertebrates (pmole/mg wet weight).

INVERTEBRATE GROUP	TISSUE	DOPAMINE (D)	NOREPINEPHRINE (NE)	D/NE	Reference
Phylum Arthropoda					
Class Crustacea					
<i>Diporeia</i> sp.	whole	0.16	nd	—	1
<i>Mysis relicta</i>	whole	0.19	nd	—	1
<i>Daphnia magna</i>	protein	25.1	3.6	7/1	6
<i>Carcinus moenas</i>	brain	0.11	.03	4/1	7
<i>Pandalus borealis</i>	eyestalk	0.39	0.25	1.6/1	7
Class Insecta					
<i>Chironomus anthracinus</i>	whole	1.0	0.2	5/1	1
<i>Tenebrio molitor</i> (larvae)	whole	6.5-9.8	1.3	6/1	3
<i>Anabolia nervosa</i>	thorax	24.6	3.3	7/1	5
Phylum Annelida					
Class Oligochaeta					
<i>Limnodrilus</i> sp.	whole	1.1	0.57	2/1	1
<i>Lumbricus terrestris</i>	nerve cord	19.6	8.9	2/1	4
<i>Lumbricus terrestris</i>	ventral nerve cord	12.9	8.2	2/1	2

nd = not detected

1 = this study

2 = Gardner and Cashin (1975)

3 = Ostlund (1954)

4 = Rude (1969)

5 = Bjorklund *et al.* (1970)

6 = Ehrenstrom and Berglund (1988)

7 = Laxmyr (1984)

Barker *et al.* 1979). An unidentified substance reported to interfere with NE and E detection has been reported (Dresse *et al.* 1960, Cottrell 1967). Unidentified chromatography peaks observed for both *Diporeia* sp. and *Mysis relicta* in this study may represent such unidentified compounds.

Epinephrine is rarely found in invertebrates (Osborne 1977, Laxmyr 1984). The chromatograph peaks corresponding to the retention time of E in this survey may be artifacts. Each survey sample of *Chironomus* exhibited peaks corresponding to that of E and could not be ignored. However, later experimental chironomids demonstrated no presence of E. The scattered reports of E concentrations in other invertebrates appeared predominantly in early investigations (Ostlund 1954, Dresse *et al.* 1960, von Euler 1961), although a few more recent papers have also reported the presence of E (Kostowski *et al.* 1975a, Kostowski *et al.* 1975b). The specificity of these analyses for individual catecholamines has been questioned (Evans 1980). More recently developed techniques, such as HPLC with electro-

chemical detection or radioenzymatic assay methods, provide more reliable identifications of individual catecholamines (Evans 1980) yet even studies using these modern methods sporadically detect the presence of a compound corresponding to E. Conclusive evidence that E is present in invertebrates is minimal, and its presence in invertebrates remains an open question.

### Temperature Stress Experiment

Temperature was selected as a general acute stressor. Exposure to temperatures above an organism's normal physiological range of tolerance is well established as an extreme environmental insult to invertebrate organisms (Ashburner and Bonner 1979, Lindquist 1986, Hanazato and Yasuno 1989) potentially causing a wide range of injuries.

The D concentrations of temperature stressed larvae were substantially lower than those of the controls (NE and E were not detected in either treatment group, Table 4). The stress and control

**TABLE 4.** Average dopamine concentrations ( $\pm$  SE) of 3rd instar *Chironomus tentans* in the temperature stress experiment (pmole/mg wet weight).

Time (hrs)	Dopamine 23°C			Dopamine 35°C		
	conc.	n <sup>1</sup>	m <sup>2</sup>	conc.	n	m
0	186 $\pm$ 87	3	0	—	0	0
1	68 $\pm$ 26	2	0	28	1	1
12	109 $\pm$ 2	2	0	11 $\pm$ 7	2	1
18	71	1	1	—	0	1
24	105 $\pm$ 45	3	0	24 $\pm$ 12	2	1
AVG	118 $\pm$ 69			AVG 20 $\pm$ 11		

<sup>1</sup>n = number of replicates

<sup>2</sup>m = mortality

groups were analyzed as two distinct populations; D concentrations for respective 35°C and 23°C groups of chironomids were each averaged. Although D concentrations were highly variable, the average dopamine concentration for the thermally stressed midges at 35°C was 20 pmoles D/mg wet wt., 6X less than the average for the 23°C midges (118 pmole D/mg wet wt.). The difference between the means of the two populations was found to be significant at the 5% level using a weighted student t-test (Snedecor and Cochran 1967).

Catecholamine concentrations are only reported for the chironomids that survived the time exposure in the temperature baths (Table 4). The high number of midge deaths in the high temperature water baths (44% vs. 8% in the 23°C baths) was unexpected as the temperature (35°C) was selected on the basis of previous heat stress experiments with chironomids. In vivo experiments of *Chironomus tentans* protein synthesis have been conducted at temperatures up to 39°C (Vincent and Tanguay 1979). The high mortality observed suggests that those chironomids that did survive experienced near lethal temperatures.

The low D concentrations measured at high temperatures relative to controls may represent chironomid response to acute temperature stress. The low D concentrations observed for heat stressed chironomids suggests that the homeostatic balance of D catabolism and reuptake/resynthesis was disrupted. Temperature shock has been reported to dramatically reduce the synthesis of most proteins (Fink and Zeuthen 1978, Ashburner and Bonner 1979). A gradual decline in total protein synthesis was reported for *Chironomus tentans* after 5-20 minute exposures to 39°C temperatures with almost

complete shutdown after 40 minutes (Vincent and Tanguay 1979). Substantially decreased D levels have been reported in the mussel *Mytilus edulis* after exposure to temperatures higher than environmental temperatures (Stefano 1977). It is possible that the enzymes required for catecholamine synthesis were not synthesized under high temperature conditions.

Results for this experiment were difficult to interpret because D concentrations in the "control" animals were much higher than values observed for "unstressed" field collected chironomids (Table 2) or for D concentrations measured in control animals in the lindane experiment (see below). One explanation of this result is that the temperature experiment "control" organisms were themselves mildly stressed during the experiment, whereas the high temperature chironomids with low D values may have represented severely stressed animals. Catecholamine levels may increase in response to mild stress in invertebrate organisms, but then peak and decrease in response to increased stress. In agreement with this hypothesis, when the midge larvae were placed in the high temperature beakers, they moved rapidly in their characteristic "snapping" motion (a possible indication of increased catecholamine activity). After several minutes of this activity they burrowed into the sand and remained motionless. Presumably the larvae depleted their available supply of D.

#### Lindane Exposure Stress Experiment

Experiments were undertaken to test catecholamine concentration levels in chironomids exposed to lindane. The main site of action for lindane

appears to be at the synapse influencing neurotransmitter release by inhibiting Na<sup>+</sup> - K<sup>+</sup> ATPases. Lindane ( $\gamma$ -hexachlorocyclohexane), an organochlorine pesticide, does not appear to be specific to neurons with a particular neurotransmitter (Smith 1991). Rather lindane has been implemented in neurons employing acetylcholine (Uchida *et al.* 1975a, Uchida *et al.* 1975b); Gamma amino butyric acid (Smith 1991) and the catecholamines, specifically norepinephrine (Smith 1991). Catecholamines are thought to function as neurotransmitters in many invertebrates (Murdock 1971, Gardner and Walker 1982, Laxmyr 1984), and it was hypothesized that concentration levels in cultured 3rd instar *Chironomus tentans* larvae would be affected by the lindane under controlled laboratory conditions.

A range finding experiment was conducted to determine a realistic range of test concentrations to be used in the catecholamine stress experiment. LC50 values were calculated for the 24- and 48-hour exposure intervals using a SAS Probit (SAS Institute 1985) computer program which calculates by means of probit analysis. The LC50 for lindane in *Chironomus tentans* 3rd instar larvae was determined to be 3.2 and 0.2 mg/L for 24 and 48 hours respectively. The 48 hr value concurs with the 0.21 mg/L reported by Macek *et al.* (1976) for 1st instar *Chironomus tentans* at the same temperature and pH.

For the stress experiment, 3rd instar *Chironomus tentans* larvae were exposed to a range of lindane concentrations, and the catecholamine concentration changes were measured in individual midges.

Chironomid mortality in this assay was lower than predicted by the 24 hr LC50 of 3.2 mg/L determined in the range finding study; only 3 of the 60 experimental chironomids were dead at the end of the 24 hr experiment (Table 5).

Two explanations for the unexpectedly low chironomid mortality are possible. First, the actual assay concentrations in the stress experiment may have been less than the calculated concentrations. The experimental lindane concentration calculations assume that the <sup>14</sup>C lindane concentration in the assay water is proportional to the non-labeled "cold" lindane. The crystalline cold lindane may have never completely solubilized, in which case, the <sup>14</sup>C lindane would not be acting as a tracer and exposure concentrations would have been lower. Second, the original LC50 concentrations determined in the range finding study may have been overestimated. The mortality criterion of movement when touched with a probe may have been unreliable. Lindane affects the mobility of chironomid larvae; normal swimming motion is greatly reduced at the onset of toxic symptoms. The effect of the toxicant increases until the larvae lose all ability to move (Estenik and Collins 1979). Chironomids that were tallied as dead may have still been alive, resulting in an overestimation of toxicity.

Dopamine concentrations were dramatically higher in lindane stressed chironomids than in control organisms. Averaged D concentrations for all chironomids at each concentration are reported in Table 5; NE and E were not detected in this experiment. The degree of catecholamine concentration

**TABLE 5. Average dopamine concentrations ( $\pm$  SE) and average bioconcentration factors ( $\pm$  SE) of 3rd instar *Chironomus tentans* after 24 hr lindane exposure.**

LINDANE TREATMENT (mg/L)	BCF <sup>1</sup> [(dpm/g midge)/ (dpm/mL H <sub>2</sub> O)]	DOPAMINE <sup>2</sup> CONC. (pmoles D/ mg wet weight)	MORTALITY <sup>3</sup>
0	0 $\pm$ 0	10 $\pm$ 7	0
0.5	1089 $\pm$ 306	230 $\pm$ 60	0
1.0	966 $\pm$ 114	425 $\pm$ 210	1
2.0	940 $\pm$ 102	175 $\pm$ 60	0
4.0	924 $\pm$ 162	285 $\pm$ 155	1
8.0	758 $\pm$ 131	240 $\pm$ 145	1
	AVG 936 $\pm$ 106		

<sup>1</sup> n = 3

<sup>2</sup> n = 7

<sup>3</sup> n = 10

change in response to lindane stress was similar at all assay concentrations. Consequently, data for all treated organisms were combined and analyzed as a distinct population. In all lindane treatments, a greater than 17X increase in chironomid dopamine concentration compared to the control was observed. The difference between the two population means is significant at the 1% level using a weighted student t-test (Snedecor and Cochran 1967).

The ratio of lindane concentration in the chironomids relative to lindane concentration in the water at equilibrium (bioconcentration factor = BCF) was comparable at all exposure concentrations at the end of the experiment averaging 936 [(DPM/ g dry midge)/(DPM/mL H<sub>2</sub>O)] ± 106 (Table 5). The K<sub>ow</sub> for lindane is 1738 (Leo *et al.* 1971). The measured BCF was 54% of the K<sub>ow</sub>. The ratio is comparable to the regression of 79% reported by Veith and Kosian (1983) for 13 species of freshwater and marine fish and a suite of hydrophobic organic compounds including lindane. Three stock culture 3rd instar *Chironomus tentans* larvae contained lipid levels of 7.5 ± 1.6% lipid levels relative to dry weight using the lipid technique of Gardner *et al.* (1985). This relatively low lipid content may have contributed to the relatively low BCF/K<sub>ow</sub> value observed. These results imply that the radiolabeled lindane was taken up by the chironomids approximately as one would predict by equilibrium uptake kinetics.

Dopamine concentrations in *Chironomus tentans* larvae increased sharply in response to lindane stress. Two interpretations of this response are possible. First, the change may be an "on/off" function. If the larvae are lindane stressed, the catecholamine concentration immediately increases to a relatively high level. In the cockroach *Periplaneta americana*, lindane apparently acts on presynaptic membranes to cause excess release of acetylcholine. The resulting high level of acetylcholine seems to bring about excess after-discharges of acetylcholine in the post-synaptic nerve inducing ataxia and convulsion (Uchida *et al.* 1975a). Lindane may exert an analagous mode of action on the dopaminergic nerve synapses, causing excessive release of D at a threshold concentration lower than 0.5 mg/L, the lowest concentration used in this assay. Second, it is possible that the experimental lindane concentrations were higher than the range where an incremental dose-response change in catecholamine concentration would be observed. Catecholamine concentrations have been demonstrated to change

monotonically with changes in the magnitude of foot shock stress in laboratory rats (Natelson *et al.* 1987). A similar step-wise increase in catecholamine concentrations may occur in *Chironomus tentans* larvae. This is an attractive hypothesis since it implies that the response may be sensitive at extremely low levels of stress.

## SUMMARY

Dopamine concentrations measured in chironomids ranged from a low of 1 ± 0.02 pmole D/mg wet weight in field collected chironomids to a high of 425 ± 210 pmole D/mg wet weight in chironomids exposed to 1.0 mg/L lindane. Dopamine levels dropped significantly in chironomids subjected to temperature stress, whereas they increased in chironomids exposed to lindane. Dopamine concentrations averaged 10 pmole mg/wet weight at 0.5 mg/L lindane and jumped dramatically to an average of 425 pmole mg/ wet weight at 1.0 mg lindane/L. Chironomid D concentrations remained consistently elevated at concentrations up to 8.0 mg lindane/L. The relatively high D concentrations measured in the temperature experiment control organisms (186 ± 87 pmole D/mg wet weight) is inconsistent with the lindane experiment control chironomids (10 ± 7 pmole/mg wet weight and field chironomid (1 ± .02 pmole D/mg wet weight) data, possibly implying that this particular group of organisms may have been "stressed" in the laboratory before the experiment.

## CONCLUSIONS

This study indicates that catecholamine concentrations in aquatic invertebrates could be potential indicators of sub-lethal stress. However, more information is needed to determine whether catecholamines could provide a practical index. The catecholamine response at low lindane levels is promising in terms of developing and testing a potential biomarker response. A laboratory study of chironomid catecholamine response to lindane concentrations <0.5 mg/L would help to elucidate the response observed in this study. Examination of catecholamine levels in field chironomids that have morphological indications of being stressed may be particularly interesting.

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