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Studies on Whole Brain Cathecholamine Levels : 1) In Flagfish (*Jordanella floridae*), Following Injections with Fenitrothion, and 2) In Rainbow Trout (*Salmo gairdneri*) Following Exposure to Cyanide.

Attila Szabo

A Thesis

in

the Department

of

Biology

Presented in Partial Fulfillment of the Requirements
for the Degree of Master of Science at
Concordia University
Montréal, Québec, Canada

March 1989

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ABSTRACT

Studies on Whole Brain Cathecholamine Levels :
1) In Flagfish (*Jordanella floridae*), Following
Injections with Fenitrothion, and 2) In Rainbow Trout
(*Salmo gairdneri*), Following Exposure to Cyanide.

Attila Szabo

A series of experiments examined the whole brain dopamine (DA) and norepinephrine (NE) levels in flagfish (*Jordanella floridae*) and rainbow trout (*Salmo gairdneri*). No sex related differences were found in whole brain DA and NE levels either in flagfish or in rainbow trout under control conditions. Anesthesia with MS222 did not affect whole brain DA and NE levels in a mixed gender group of flagfish, sacrificed within two minutes postanesthesia. Intraperitoneal (ip) injections of fenitrothion (FTN) did not alter whole brain DA and NE levels within 72 hours postinjection in mature female flagfish. A sampling time related decrease of whole brain DA and NE levels in mature female flagfish was attributed to an uncontrolled experimental variable. Two studies have shown that following 12 days of chronic exposure to sublethal (0.01 mg/L water) hydrogen cyanide (HCN) concentration, whole brain DA levels increased more than 21% in sexually mature rainbow trout. A possible mechanism by which CN could affect fish reproduction is presented.

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INTRODUCTION

Cathecholamines (CAs) are monoamines composed of a cathechol nucleus and ethylamine or one of its derivatives. They are identified as three distinct biochemical compounds: 1) dopamine (DA), 2) norepinephrine (NE) and epinephrine (EPI). The CAs differ from one another in two ways; 1) on a chemical basis, in the composition of their side chains (See Appendix A), and 2) on a biological basis, in the activities they perform in the central and peripheral nervous system (Feldman and Quenzer, 1984).

While the majority of CA studies reported in the literature focus on mammalian organisms, CAs also play a significant role in many biological activities of fish (Mazeaud and Mazeaud, 1981). There are two important similarities between mammalian and teleostean central cathecholaminergic systems: 1) the anatomical distribution of CA containing nerve cells is similar in the mammalian and teleostean brain (Parent et al., 1978; Parent and Northcult, 1982), and 2) biosynthesis and catabolism of CAs are identical in mammals and fish (Mazeaud and Mazeaud, 1981). Since the development of high pressure liquid chromatography (HPLC) technique for CAs, there has been an increased enthusiasm, to study the role of these neurotransmitters (NTs) in fish.

In the last two decades a number of studies, using fish as the test organism, have investigated the role of CAs outside the central nervous system (peripheral CAs) on various biological functions. For example, a number of studies have focused on the role of CAs in the circulatory and cardiovascular systems of fish (Ask, Stene-Larsen and Helle, 1980; Bourne and Cossins, 1982; Woodward, 1982; Nikinama, 1983; Baroin et al., 1984; Motais, Garcia-Romeu and Borgese, 1987). Other studies examined the peripheral CAs in respiratory system (Wood, 1975; Bolis and Rankin, 1980; Donald, 1984; Nekvasil and Olson, 1985) and in chromaffin tissue (Abrahahansson, 1979; Jonsson and Nilsson, 1979; Jonsson and Hansson, 1984).

Studies of CAs within the central nervous system (central CAs) of fish are limited. However, there is a major and well-established field of study on the role of hypothalamo-pituitary CAs in endocrine functions in relation to reproduction. For example, it has been shown that DA has an inhibitory action on the release of prolactin from the pituitary of rainbow trout (McKeown, Jenks and Overbeeke, 1980). Later, James and Wigham (1984), demonstrated that prolactin secretory cells are controlled (at least in part) by both the inhibitory action of DA and the stimulatory action of serotonin. Still other studies suggest that DA is inhibitory not only on prolactin cell activity, but on gonadotropins (GTH) as

well. Treatment with pimozide (a DA antagonist) increased serum GTH levels in goldfish (Chang and Peter, 1983a; Sokolowska et al., 1984) and in salmonids (Billard et al., 1984; Van Der Kraak, Donaldson and Chang, 1986). In contrast, treatment with DA or apomorphine (a DA agonist) resulted in decreased serum GTH levels (Chang and Peter, 1983b). It was suggested that DA has a specific GTH inhibitory action on the pituitary gonadotrops (Chang et al., 1984).

Various physiological and environmental factors have been shown to influence CAs in fish. A circadian rhytmicity was found in goldfish brain, with a fivefold difference between minimal and maximal CA levels (Saurbier and Meyer, 1977). In eel hypothalamus CA levels differed more than 50% from low to high points of the diurnal cycle (Popek, Sokolowska and Bieniarz, 1981). Locomotor activity was found proportional to whole brain CA levels in goldfish (Deliége, Cession-Fossion and Vandermeulen, 1969), and inversely proportional to hypothalamic CA content of the eels (Popek, Sokolowska and Bieniarz, 1981). Lower whole brain NE and higher DA levels were associated with aggression in rainbow trout (McIntyre, Healy and Saari, 1979). Both, diet and hypoxia, have been shown to influence CAs in various brain structures of rainbow trout (Pouliot, De La Noue and Roberge, 1988). Light or dark background can also alter pituitary CA

levels in fish. The former has been associated with low, while the latter with high CA levels in eels (Fremberg and Meurling, 1975). Forced exercise may also influence CA levels as well as cathecholaminergic response to stress in rainbow trout (Woodward and Smith, 1985). Acclimation to salt water significantly increased NE and EPI levels in trout (Hegab and Hanke, 1983). Stress, in general, is associated with CA release, which in turn increases ionic transfer at the gills resulting in water or ionic overload, depending upon the water medium (Mazeaud and Mazeaud, 1981).

In addition to physiological factors, toxic agents may also alter CA balance of an organism. Few experiments, using fish as the test organism, have analysed the effects of toxicants on central CAs (Sloley et al., 1986).

Recently the organophosphorus (OP) insecticide, fenitrothion (FTN), received special attention in the literature. This compound is widely used in Canada and elsewhere to control the spruce budworm in coniferous forests (NRCC, 1984). Similar to other OP compounds, FTN is an inhibitor of cholinesterase activity (Klaverkamp and Hobden, 1980; Busby et al., 1983; Kobayashi et al., 1983; NRCC, 1984). Aerial field application of FTN may reach the water ecosystems, which in turn may have adverse effects on the inhabitants of these environments (Verma, Bansal and Dalela, 1978).

A number of studies on aquatic organisms, with special focus on fish, have demonstrated negative consequences of FTN application on various physiological functions. For example, Mani and Saxena (1985), reported decreased ovarian weight, retarded maturation of previtellogenic oocytes and increased number of atretic oocytes in freshwater murrel (Channa punctatus) following exposure to 1.5 ppm FTN from 30 to 120 days. It has been suggested that a decline in GTH levels may result following FTN treatment (Kapur, Kamaldeep and Torr, 1978; Sreenivasula, Ghagyalakshmi and Ramamurthi, 1983; Mani and Saxena, 1985). Since DA has a specific GTH release inhibitory action (Chang et al., 1984), it is possible that FTN alters central DA levels, which in turn may affect GTH release from the pituitary.

Fenitrothion may affect directly or indirectly DA and/or NE levels. As mentioned earlier, FTN is an acethylcholinesterese (AchE) inhibitor (Klaverkamp and Hobden, 1980; Busby et al., 1983; Kobayashi et al., 1983; NRCC, 1984). Vijayalakshmi (1980), found that FTN exerted its maximal AchE inhibitory action at the brain level in fish, suggesting a target tissue specificity of this compound. Brain AchE inhibition results in increased acethylcholine (Ach) levels. Accumulation of Ach triggers the release of CAs in the nervous system (Brzezinski, 1969).

Flagfish as test organism has been used in a number of toxicological investigations that focused on the effects of an insecticide on serotonin levels and/or reproduction (Holdway and Dixon, 1985; Holdway and Dixon, 1986a; Holdway and Dixon, 1986b; Holdway et al., 1986). A recent study (Holdway et al., 1988), investigated brain CA levels in male and female flagfish following selection on behavioral bases associated with reproductive status (males: dominant, subordinate, solitary and resting and females: laying and resting). The study revealed significant differences in whole brain DA levels between resting males and resting females, resting males and subordinate males, and NE levels between dominant males and solitary males, dominant males and laying females and dominant males and resting females. These results indicate a behavior related CA fluctuation in flagfish brain. However, the study does not clarify whether CA levels differ in the two sexes in a random sample.

Apart from gender, another variable needs to be considered in studies on CAs. In many toxicological and physiological studies it is necessary to administer anesthesia. Epple, Vogel and Nibbio (1982) found that MS222 (a frequently used anesthetic in studies with teleosts) altered blood CA levels of the eel after 20 minutes of exposure. However, Sloley et al. (1986), reported that anesthesia of rainbow trout with MS222 or 2-

phenoxyethanol had no effect on brain DA or NE levels if the sampling procedure lasted not longer than 2 minutes. Although this finding indicates that under rapid sampling procedure anesthesia may not affect brain CA levels in rainbow trout, the effects of anesthesia remain to be determined in flagfish.

The first part of the studies reported in this manuscript was designed to examine whole brain DA and NE levels in flagfish. Experiment I was undertaken to assess any gender related differences in whole brain CA levels of flagfish in a random sample. Experiment II examined the effects of anesthesia (under rapid sampling conditions) on whole brain CA levels of male and female flagfish. Experiment III was performed to find a dose of FTN, via intraperitoneal (ip) route, that would be able to produce subtle changes (if any) in brain DA and NE levels, but would not be lethal to the fish during the time course of the study. Experiment IV evaluated whether FTN affects brain CA levels in female flagfish and whether this effect (if any) was time related.

The importance of CA studies in toxicopharmacology has been previously outlined. Fenitrothion is only one of the many toxicants that may alter central CAs in fish. One of the most widely studied aquatic toxicants is hydrogen cyanide (HCN) (Leduc, Pierce and McCracken, 1982). This compound may also alter central CAs in fish.

Cyanide toxicity is attributed to the inhibition of terminal oxidase (cytochrome aa3) in the mitochondrial respiratory chain (Solomonson, 1981). Upon the penetration of cell membrane first, then the mitochondrial membrane, HCN reaches the target cytochrome oxidase ligand binding site, where it binds to the reduced form of the enzyme. The latter is then oxidized to form the more stable cytochrome aa3-CN complex. The complex halts aerobic respiration by blocking the electron transport chain (Solomonson, 1981; Naqui et al., 1984).

Brain cytochrome oxidase is presumed to be the most sensitive to HCN toxicity because of the relative inability of the brain to metabolize HCN (Piantadosi and Sylvia, 1984) its limited anaerobic metabolic capacity and its high energy requirements (Johnson, Meisenheimer and Isom, 1986). Depleted ATP sources in the brain, as a result of respiratory inhibition by HCN, inactivates the active transport system, such as the Ca/Mg pump. The net result is intracellular Ca2+ accumulation, lowered transmembrane potential and hyperexcitability (Johnson et al., 1986). This sequence is confirmed by hyper electroencephalographic activity signaling the occurence of convulsions (Johnson et al., 1986). The final consequence of the events may bring about changes in whole brain NT levels (Borowitz, Born and Isom, 1988).

Information from mammalian studies (with rats) reveal

that acute NaCN intoxication affects the central CAs at distinct brain regions. For example, in striatum of the brain, a dose-dependent decrease in DA levels was observed following NaCN treatment. Norepinephrine levels of the same region were unaffected. Interestingly, in the olfactory tubercle both DA and NE levels increased as a result of NaCN administration (Cassel and Persson, 1985). In another study, rats were injected with NaCN and NSD10015. The latter compound is an inhibitor of aromatic amino acid decarboxylase (AAAD), the enzyme that converts dopa to DA. Striatal l-dopa (precursor of DA) levels increased in both, NaCN and NSD10015 treated rats (Cassel, Karlsson and Persson, 1985). Due to its similar effect to decarboxylase inhibitors, it was suggested that NaCN may inhibit the biosynthesis of DA.

Studies on fish (rainbow trout) have demonstrated that exposure to sublethal levels of HCN produced 60-80% of cytochrome oxidase inhibition within the first 24 hours of treatment (Raymond, Leduc and Kornblatt, 1986), suggesting the sensitivity of this species to HCN. Impairment of reproduction in salmonids, as a result of HCN exposure was clearly established (Ruby, Dixon and Leduc, 1979; Lesniak and Ruby, 1982; Ruby, Idler and So, 1986; Ruby, Idler and So, 1987). The mechanism by which HCN affects reproduction is not fully understood, however evidence indicates that GTH plays an important role. Since

DA regulates GTH at the hypothalamo-pititary axis (Chang et al., 1984), it is possible that HCN alters DA levels, which in turn may modify GTH regulation. Up to date, there are no published reports on the effects of HCN on central CAs in fish. Such information may provide a better understanding of HCN toxicity.

Two identical experiments, one performed in July/1988 the other in August/1988, were designed to investigate the chronic effects of sublethal (0.01 mg/L) HCN exposure, for 12 consecutive days, on the whole brain DA and NE levels of sexually maturing male and female rainbow trout.

MATERIALS AND METHODS

1) MATERIALS

Test Organisms

a) Flagfish (*Jordanella floridae*), used in the first set of studies, were obtained from Florida Fish Farm Co., Tampa, Florida. Their average size was approximately 3.5 cm and average weight 1.15 g. Air transportation lasted not longer than 24 hours. During this time, the fish were placed in oxygenated plastic bags containing approximately 7-10 liters of water. Each bag contained 40-50 fish and was enclosed in a 2 cm thick styrofoam box to maintain ambient temperature during transportation.

Upon arrival at the Water Pollution Research Laboratory, Concordia University, the temperature in the plastic bags (+21°C) was equilibrated to the laboratory water (+25°C) and fish were subsequently placed in holding tanks. This ensured gradual temperature changes and reduced the risk of mortality.

Fish were acclimated to laboratory conditions for a minimum period of three months. They were fed *ad libitum* once a day with either tropical fish foodflakes (Nutra Fin, Staple Food, Rolf C. Hagen Inc., Montreal, Quebec) or frozen brine shrimp (Nutra Fin). The nutritional composition of these products are shown in Table 1.

Table 1: The composition of flagfish diet. *

COMPONENT	BRINE SHRIMP	STAPLE FOOD
Crude protein (min.)	5.02%	46%
Crude fat (min.)	0.24%	5%
Crude fiber (max.)	29%	2%
Moisture (max.)	90%	8%

* This analysis was provided by Nutra Fin on the commercial package.

b) Rainbow trout (*Salmo gairdneri*) served as test organism for an ecotoxicological study. The fish were purchased from La Pisciculture Mont Sutton, Sutton, Québec. Fish were held at the Water Pollution Research Laboratory for 15 months prior to the experiments. Mean fish weight was 299.53 grams (SEM=±12.32). All fish were between their second and third year of life. During the holding period, they were fed *ad libitum* four times a week with trout chow (Table 2). During the experiments, and preexperimental acclimation period, fish were fed daily at 1% of their body weight (BW) with the same diet.

Apparatus

The holding tanks in the studies conducted on flagfish were the same as the experimental tanks. Each tank was made from a white polyethylene material, was cylindrical in shape and was covered with a white polymer top. The holding capacity of each tank was 60 liters (L).

A main water source supplied 25 ^{O}C (heated) dechlorinated (activated charcoal filter) City of Montreal water (Table 3) to a 7.5 cm diameter and 250 cm long poly vinyl chloride (PVC) pipe. The latter was horizontally fixed 30 cm above and parallel to the tanks and supplied 10 tanks simultaneously. The apparatus was designed to ensure equal pressure and flow rate (Figure 1).

The water flow to individual tanks was adjusted to 0.5

Table 2: The composition of rainbow trout diet. *

COMPONENT	QUANTITY
Crude protein (min.)	50%
Crude fat (min.)	18%
Crude fiber (max.)	1%
Sodium	0.48%
Calcium	1%
Phosphorus	0.98%
Vitamin A	6000 IU/Kg
Vitamin D	3748 IU/Kg
Vitamin E	135 IU/Kg
Vitamin C	600 mg/Kg

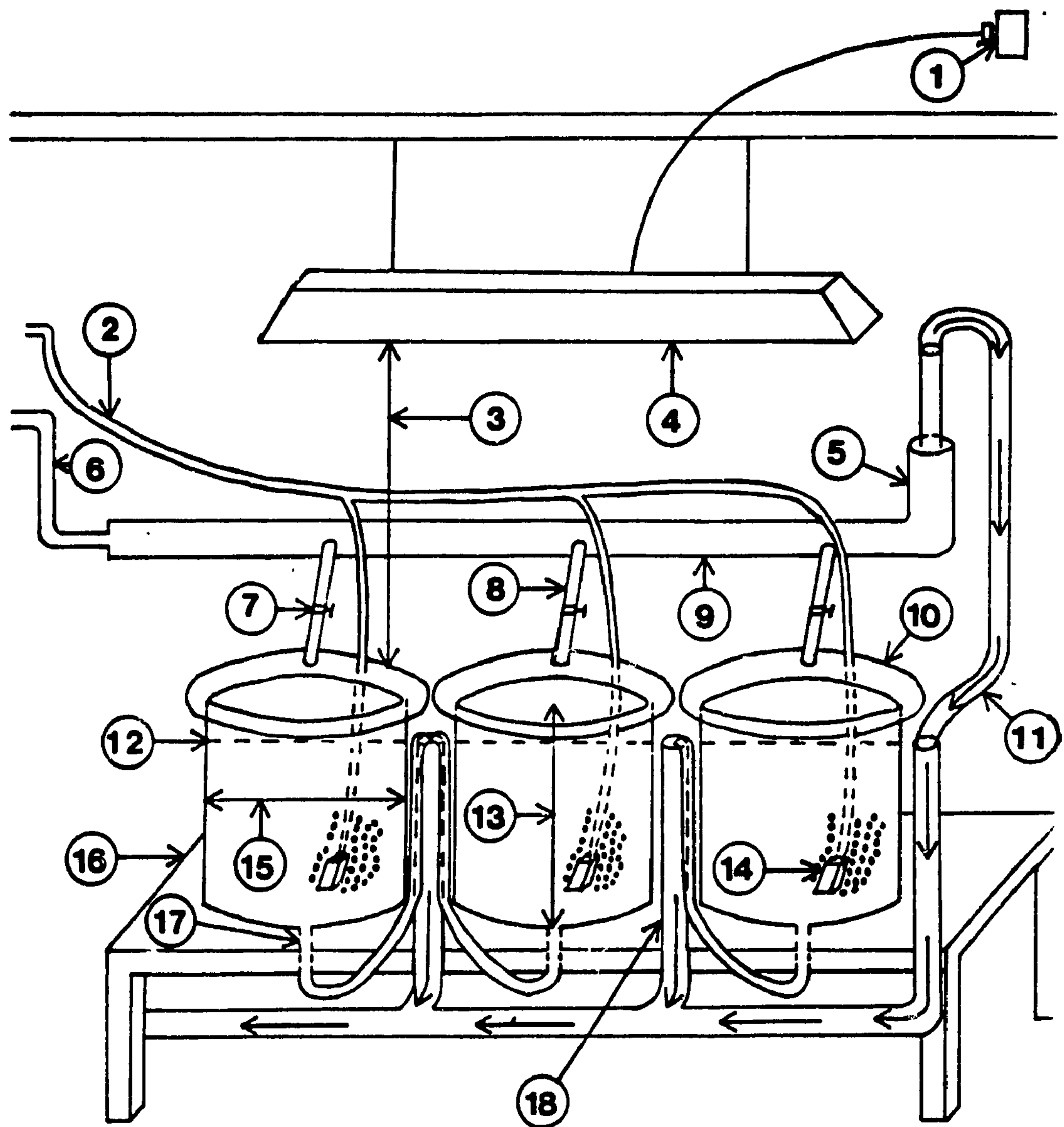
* This analysis was provided by Nutribec Co.

Table 3: Chemical characteristics of laboratory water (Total Hardnes (TH), Alkalinity (Alk) Total Organic Carbon (TOC) and pH) used during the experiments with flagfish (FF) and rainbow trout (RT). All the presented chemical parameters were provided by the City of Montreal Public Works Department.

EXPERIMENTAL PERIOD	TH*	Alk*	TOC**	pH
June/1987 Experiment I (FF)	135	91	2.10	7.89
November/1987 Experiment II (FF)	132	89	2.22	7.90
April/1988 Experiment III (FF)	122	82	2.45	7.87
June/1988 Experiment IV (FF)	129	89	1.94	7.87
July/1988 Experiment I (RT)	128	87	1.83	7.83
August/1988 Experiment II (RT)	121	81	1.74	7.78

* Expressed in $CaCO_3$ (ppm)
** Expressed in mg/l

Figure 1: Diagram of the experimental apparatus used for flagfish studies: 1) automatic light switch, 2) air tube, 3) light source was 60 cm above the tanks, 4) fluorescent lights (40 WW), 5) vertical PVC pipe equilibrated the pressure in the horizontal pipe, 6) main water source, 7) water flow control clip, 8) water tube to tanks, 9) horizontal PVC pipe, 10) plastic tank cover, 11) excess water drain, 12) water level in tanks, 13) tank height = 47 cm, 14) airstone, 15) tank width = 52.5 cm, 16) table for tank suspension, 17) tank water drainage tube, 18) U system water level control.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L/min. This provided 90% water replacement in 4 hours (h) and 30 minutes (min) (Sprague, 1973). If more than 30 fish were placed in a holding tank (maximum 50 fish/tank) the water flow rate was adjusted accordingly to a maximum potential flow of 1 L/min. In addition, each tank was supplied with an airstone, except the experimental tanks where no more than 10 fish were placed at any given time. Oxygen never fell below 80% saturation in any of the tanks.

Illumination was provided by fluorescent lights (F40 WW Mainlighter, General Electric Co.) placed about 60 cm above and to the center of the tanks. An automatic time-switch controlled the 15 h light and 9 h dark photoperiod.

Water temperature was recorded on a daily basis with a FISHERbrand thermometer (Model 15-21B), and was found to be consistent at $25^{O}C \pm 1^{O}C$ for the tropical fish and 11.5 $^{O}C \pm 1.5$ C for the rainbow trout. Bi-daily pH measurements were taken with a Fisher Calomel reference electrode (Model 7292) and a Fisher Universel Glass electrode (Model 7006) attached to pH meter (Model STC 7, Concordia University, Science Technical Center). The registered mean value was 7.8 throughout the studies reported here. The experimental area was enclosed with a black plastic curtain to minimize external disturbance.

The rainbow trout were held in 620 L rectangular green fiberglass tanks. These tanks were supplied with

dechlorinated City of Montreal water. The water flow was adjusted to 6 L/min. This provided 90% water replacement in 4h and 30 min (Sprague, 1973). In addition, compressed air, through airstones, was delivered to each of the tanks. Each tank was covered with a green plastic top to minimize external disturbance. Automatically controlled lourescent

fluorescent lights above the tanks were set for 12 h light and 12 h dark photoperiod. Temperature and water flow rate were monitored on a daily basis.

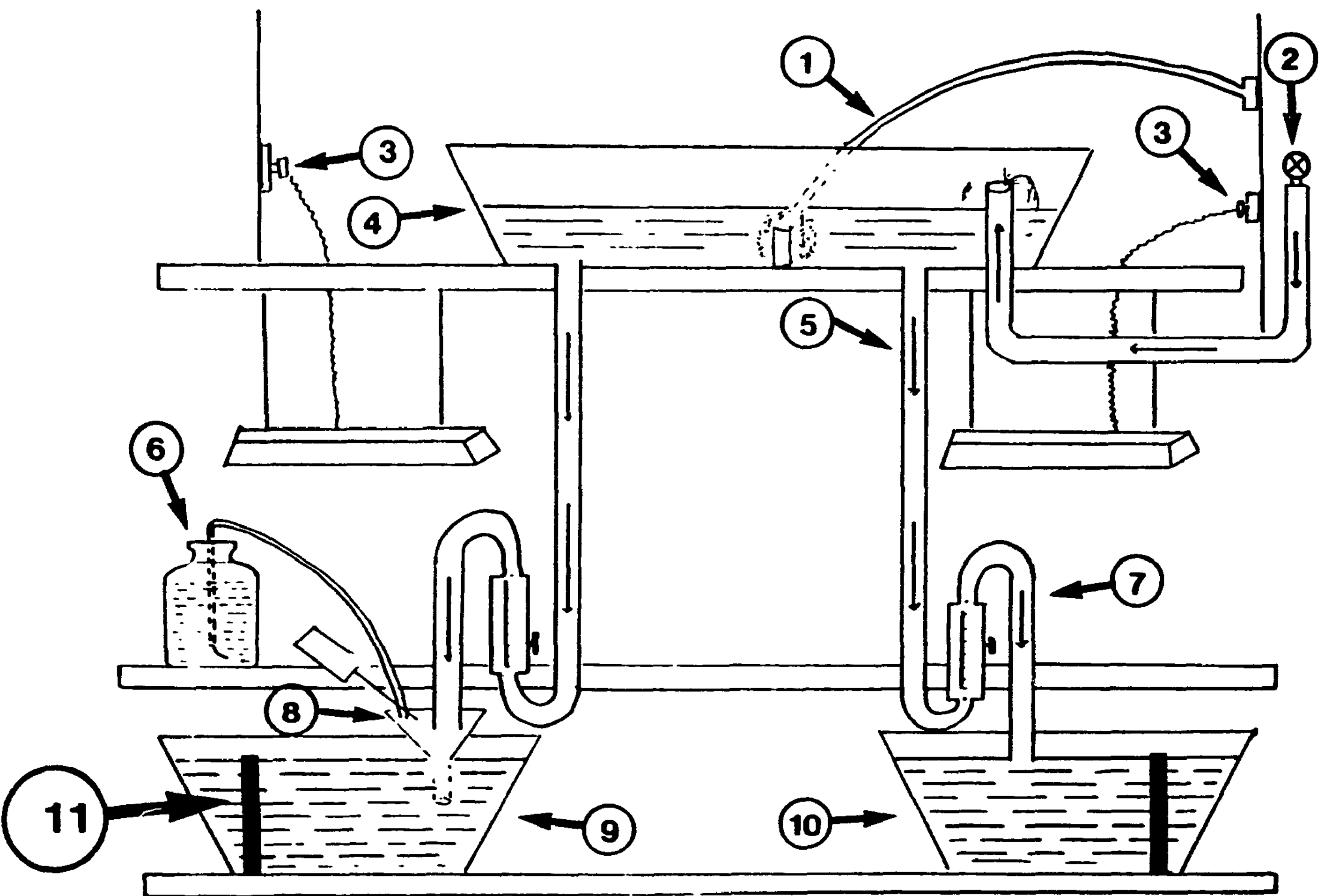
The experimental tanks for rainbow trout studies consisted of four 243 L capacity oval fiberglass tanks. A plastic cover was placed on each of the experimental tanks. These tanks were supplied from the same water source as the holding tanks. However, the water was first delivered to a headtank where aeration, with compressed air through airstones, was performed. From the headtank the water passed through PVC pipes to a flowmeter (Monostat Co.) which permitted the regulation of the flow rate. The latter was adjusted to 2 L/min, providing about 90% replacement water in 4 h and 30 min (Sprague, 1973). Water was drained from the tanks through a centrally located PVC standpipe.

Cyanide delivered to the experimental tanks through Mariotte bottles (Leduc, 1966) was mixed with inflowing water in a funnel placed firmly into a 1 cm diameter hole in the plastic cover. The ´a priori´ mixing allowed the

achievement of the desired toxicant concentration. Fluorescent lights aligned above the tanks were controlled by an automatic switch adjusted to 12 h light and 12 h dark photoperiod (Figure 2). Temperature, water flow, pH, toxicant inflow and toxicant concentration were monitored on a daily basis (Table 4). A minimum of one week acclimation period to the experimental tanks, following removal from the holding tanks, was imposed before the start of each experiment.

In all the studies reported here, brain cathecholamine levels were determined using high pressure liquid chromatography (HPLC) at the Center for Studies in Behavioral Neurobiology, Concordia University. The samples were injected into a uBondapak C18 reversed-phase stainless steel column (30 x 3.9 cm, 10 um particle size) using a WISP, (Model 710B) automated sample processor (Waters Associates Inc.). The aqueous mobile phase contained (per liter) 13.6 g KH2PO4, 100 mg EDTA, 100 mg octane sulfonic acid and 5 ml of acetonitrile. The solution was adjusted to a pH of 2.8 with phosphoric acid and it was pumped at a flow rate of 1.8 mL/min by a Waters Associates Chromatography pump, (Model 6000-A). Electrochemical detection was performed with an LC-4B amperometric detector (P/N 99898) synchrinized with a TL-5 tube glassy carbon electrode (both from Bioanalytical Systems Inc.). The detector potential was set at + 0.7 V

Figure 2: Diagram of the experimental apparatus used for rainbow trout studies: 1) compressed air tube, 2) water source to head tank, 3) automatic light switch, 4) head tank, 5) PVC pipe from head tank to flow meter, 6) Mariotte bottle, 7) flow meter controlled water inflow to the tanks, 8) water-toxicant mixing funnel, 9) experimental tank, 10) control tank, 11) water overflow outlet.


1
2
3
3
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6
7
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9
10
11

Table 4: Average temperature (TEMP), flow rate (WFR), pH and cyanide concentartion (HCN CONC) of test water during the rainbow trout studies.

EXPERIMENTAL PERIOD	TEMP	WFR	pH	HCN CONC
July/1988	12.5 ^{O}C	2 L/min	7.83	0.012 mg/L
August/1988	10.7 ^{O}C	2 L/min	7.78	0.011 mg/L

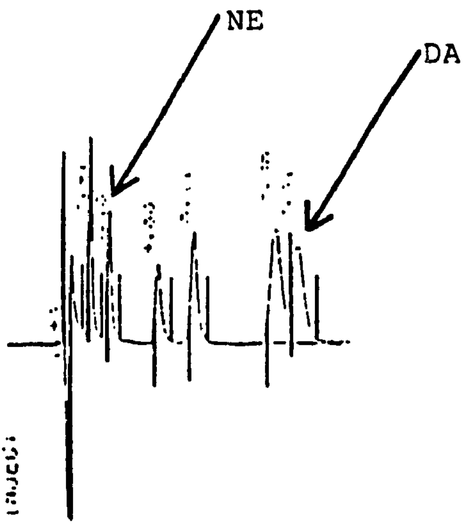
with respect to the Ag/AgCl reference electrode. The HPLC elution profile was simultaneously recorded by two units; 1) Data Module (Model 730, Waters Associates Inc.), and 2) Watanabe (Model: Servocorder 6255).

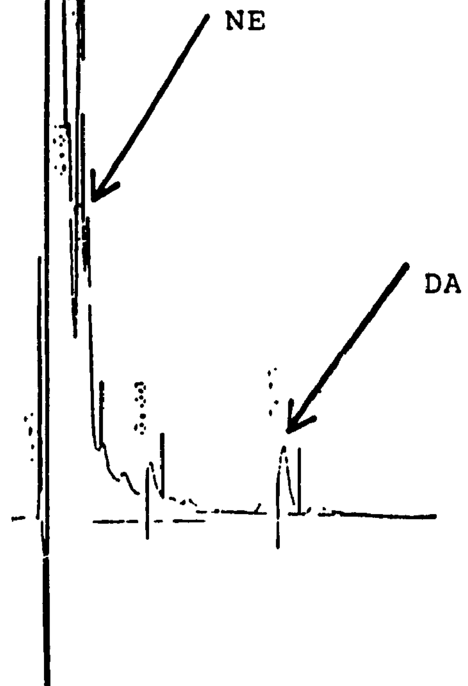
2) **METHODS**

Cathecholamine Determination

Brain CA determination followed a similar pattern across the studies reported in this manuscript. The isolated brain tissue was homogenized in 0.1 M HClO4 (Baker Chemical Co.), using a Sonifier Cell Distruptor (Model W185, Branson Sonic Power Co.) at 40 W for 45 seconds. The homogenate was centrifuged using a Fisher Microcentrifuge (Model 235A, Fisher Scientific Co.), for 5 min at 10000 RPM. The CA containing supernatant was separated from the sediment. The latter was stored for protein assays. Prior to HPLC analysis, a number of external standards, including NE and DA, were made up in a known concentration (1 ng/10 uL). All standards were purchased from Sigma Chemical Co.. These standards were injected into the column while various parameter calibrations were performed. The two recorders connected to the amperometric detector were set to different sensitivities. Both recording units yielded a good elution profile of both CAs (a sample from Data Module is shown in Figure 3). This double-recorder set up permitted

Figure 3: The HPLC elution profile of a standard (1) and a sample from rainbow trout brain (2), obtained under the following conditions: Both samples were injected into a uBondapak C18 reversed-phase column (particle size = 10 um) using a WISP automated sample processor. The mobile phase was pumped at a flow rate of 1.8 ml/min by a Waters Associates Chromatography pump. Electrochemical detection was performed with an LC-4B amperometric detector (TL-5 glassy carbon electrode). Detector potential was set at +0.7 V with respect to the Ag/AgCl reference electrode. Standard injection volume was 10 ul, while sample injection volume was 40 ul. The numbers not clearly visible above the peaks indicate the time of elution.


1
NE
DA
No. 747C1


2
NE
DA

a functional back-up control unit for each sample.

Preliminary studies, using both internal (3,4-dihydroxybenzylamine, (DHBA)) and external standards indicated that the HPLC elution profile was consistent in both cases. All calculations were based on the external standards. The percentage of error in CA content determination was less than 5% using this method. Spiking was performed periodically to ensure the identity of the measured NT. The samples were injected in identical volumes for each experiment. Sample CA concentrations were expressed in ng /100 ug protein. For this purpose the sedimented layer of the brain homogenate was assayed for protein according to the method described by Lowry et al. (1951). Literature on mammalian organisms suggests that this technique is very reliable for small amounts of tissues (Palkovits and Brownstein, 1983). The calculation method is presented in Figure 4.

Flagfish Studies

Experiment I assessed whether gender related differences existed in brain CA levels in flagfish. The study was performed in June/1987. Following more than three months acclimation period to laboratory conditions, ten male and ten female flagfish were randomly removed from the tanks (one at a time). Randomization followed the systematic sampling methods described by Minium and

Figure 4: Calculation of the CA concentrations in samples on the basis of HPLC elution profile of the sample and external standard.

LET

ESH = External Standard Height
ESC = External Standard Concentration
SH = Sample Height
SIV = Sample Injection Volume
STV = Sample Total Volume
STC = Sample Total Concentration
SPC = Sample Protein Concentration
X = STC/100 ug Protein

THEN

STC = ((SH/ESH)(ESC)(STV/SIV))
X = (STC)(100)/(SPC)

Clarke (1982), serial for the tanks and alternate for the sexes. (Appendix B). This technique was used throughout all the experiments.

Upon removal from the tanks, fish were anesthetized in 1g/L MS222 (3-Aminobenzoic acid ethyl ester, Sigma Chemical Co.). The time to effective anesthesia (cessation of opercular movements) lasted from 30 to 45 seconds.

The surgical procedure was performed under a disecting microscope (ausJENA, Model 475615, Germany). The spinal cord was severed, proximal and posterior to the eyes, by a sagital cut that was followed by a posterior-anterior longitudinal cut in the cranium. The two dorsolateral portions of the cranium, containing the eyes, were folded back to expose the brain. The optic and spinal nerve projections were severed from the brain using a sharp glass instrument. The freed brain was removed by suction with the aid of a modified Pasteur pipette and frozen in liquid nitrogen at about -195.8^{O}C. The surgical procedure, including removal of the fish from the anesthetic to freezing of the brain, lasted not longer than 60 seconds. Cathecolamine levels were determined as previously described.

Experiment II examined the effects of anesthesia on brain CA levels of flagfish. Ten fish, regardless of gender, were removed from the tanks and placed into an anesthetic solution (1g of 3-aminobenzoic acid ethyl

ester (MS222) /liter water) prior to sacrifice. Ten other fish represented the control group and received no anesthesia prior to sacrifice. Brain disection and CA determination followed a similar pattern to that described for experiment I.

Experiment III with flagfish assessed the relative toxicity of FTN. This study was performed in order to determine an injection dose of FTN that would permit subtle changes in brain cathecholaminergic activity (if any) without being lethal to the fish during the time course of the experiment (Please note that this was not an LD50 study). Fenitrothion was a gift from Chemagro Co. (Mississuaga, Ontario) a subdivision of Bayer Pharmaceuticals, Germany. Its purity was 99.6%. Dilutions were made with sesame oil (Sigma Chemical Co.) in 1:1 ratio. Due to limited amount of FTN available for this study, only 5 fish/group was possible. A total of 30 fish were subdivided into 6 groups and each group received one of the following treatments: 1) 24 g/Kg body weight (BW) sesame oil (corresponding to the highest volume of FTN injection dose), 2) 1 g/Kg BW FTN injection, 3) 4 g/Kg BW FTN injection, 4) 8 g/Kg BW FTN injection, 5) 10 g/Kg BW FTN injection and 6) 12 g/Kg BW FTN injection. The range of injection dose was selected on the basis of the ip LD50 of FTN for brook trout, found to be 8g/Kg BW (Gauthier, Audet and Chevalier, 1983). Following

anesthesia the fish were weighed, the injection volume was determined and introduced intraperitoneally (ip) anterior to the pelvic fin. Needle position was 45 degree perpendicular to the anterior-proximal portion of the pelvic fin in a posterior-ventral anterior-dorsal direction. Post injection, the fish were placed in the tanks and the time of death was recorded over a period of ten days. The fish were monitored hourly for the first 12 h postinjection and three times a day for the next consecutive 9 days.

Experiment IV using flagfish, was designed to assess whether FTN elicits modifications in whole brain NE and DA levels, and whether these modifications are time dependent. Only female fish were used in this experiment. On the basis of the results from experiment III, two doses of FTN were utilized; 2g/Kg BW and 4g/Kg BW. The experimental design is shown in Table 5.

This study contained 13 groups, with 10 fish/group. The first group, or time 0 control group was removed from the tanks and sampled immediately on day 0, prior to injections of the other groups. The sampling procedure was identical to the previous studies, except that no anesthesia was used. In addition to time 0 sampling, the design included three sampling times; 4 hours post treatment, 24 hours post treatment and 72 hours post treatment. There were four groups at each sampling time;

Table 5: Design for experiment IV with flagfish. The time of sampling is indicated in the first column. The rows corresponding to each sampling time indicate the number of fish used in each group.

TIME OF SAMP-LING	TREATMENT GROUPS			
	CONTROL	SESAME OIL	2g/Kg BW FTN	4g/Kg BW FTN
0 h	n=10			
4 h	n=10	n=10	n=10	n=10
24 h	n=10	n=10	n=10	n=10
72 h	n=10	n=10	n=10	n=10

1) control, 2) 8 g/Kg BW sesame oil (vehicle) injection, 3) 2 g/Kg BW FTN injection and 4) 4 g/Kg BW FTN injection. The no treatment control groups were simply removed from the tanks and transferred to the designated experimental tanks. The experimental tanks were identical to the each other and to the holding tanks.

All treatments were performed in the morning to control for diurnal rhythms in CA levels (Saurbier and Meyer, 1977; Popek, Sokolowska and Bieniarz, 1981). Pretreatment, the fish were removed from the holding tanks, anesthetized, weighed and injected with the calculated volume of toxicant or sesame oil. The injections were given ip, as described for experiment III. The injection of 10 fish (i.e. a group) lasted not longer than 15 minutes. Following the injection, the fish were placed in highly oxygenated water for 15-30 seconds. This ensured fast and complete recovery prior to placement in the experimental tanks.

The first sampling occured four hours post treatment and proceeded as follows: The fish were removed from the experimental tanks and immediately exposed to spinal cord severing (no anesthesia). The whole brains were disected, frozen in liquid nitrogen and stored for CA determination. The same procedure was repeated at 24 h and at 72 h. The sampling times were choosen on the basis of previous studies with teleost (Kanazawa, 1975; MIyamoto, 1977; St-

Louis, Van Coillie and Lebrun, 1987; Takimoto, Ohshima and Miyamoto, 1987). These reports reveal that around 4h there is an active absorbance phase for FTN, at 24h a maximal absorbance phase and at around 72h there is an elimination phase. Disturbance was limited during the study while diet, temperature, pH and photoperiod remained constant.

Rainbow Trout Studies

Two identical studies were performed using adult rainbow trout. The first was conducted in July/1988 and the second in August/1988. Mean fish weight was 299.53 g. In both experiments fish were exposed to sublethal (0.01 mg/L) HCN concentration in water for 12 consecutive days.

Freshly prepared HCN stock solution was made up from sodium cyanide (Baker Chemical Co.). The HCN content of the stock solution was determined by titration with silver nitrate, according to the method described by Epstein (1947). Fish were acclimated for at least one week to the experimental tanks prior to exposure to toxicant. Cyanide was introduced in the test tanks through Mariotte bottles, following the method outlined by Leduc (1966). Cyanide concentration of test water was monitored on a daily basis using the modified König reaction (Lambert et al., 1975).

An equal number of fish in the control group and HCN exposed group were sampled after 12 days of exposure. In July a total of 12 fish were utilized (6 control and 6 HCN

exposed), while in August a total of 20 fish were sampled (10 control and 10 HCN exposed). Following removal from the tanks, fish were anesthetized with 3-aminobenzoic acid ethyl ester (2 g/5 L of water). The time to effective anesthesia was 60-90 seconds. Fish were subsequently dried with paper towel and weighed. The head was severed with a single dorso-ventral sagital cut proximal-posterior to the eyes. The head was subsectioned into three parts to access the brain. The latter was removed with medical forceps, isolated at its tip with multiple layers of parafilm. The brain was introduced in a 13 x 75 mm sterile plastic tube (Fisher Scientific Co.) and frozen in liquid nitrogen. Cathecholamine determination proceeded as described for flagfish studies, except that the brain was homogenized in a proportionally higher volume (3 mL) of 0.1 M $HClO_4$ due to its larger size.

Statistical Analysis

Data were expressed as a mean, +/- standard error of the mean (SEM), throughout the studies reported here. Descriptive statistics for all the data were analysed with the aid of statistical software packages (Concordia University) writen in basic language for Apple II personal computer (PC). Group comparisons were performed using multivariate analysis of variance (MANOVA) and ANOVA with the aid of the SPSS-X computerized statistical program (on

Vax 2) at the Computer Center, Concordia University. Non parametric tests were performed manually, on the basis of the techniques outlined by Minium and Clarke (1982). The level of significance selected was $p<0.05$.

RESULTS

A) Whole brain CA levels in male and female flagfish

Experiment I was undertaken to assess whether gender related differences exist in whole brain DA and NE levels in flagfish. The study was performed in June/1987 using 10 male and 10 female fish randomly selected from among a larger pool of available stock. Fish were anesthetized before sacrifice. Brain samples were analysed, for DA, NE and protein, within 3 weeks following sampling. The CA concentrations were expressed in ng/100 ug protein.

Whole brain DA and NE levels were not significantly different between male and female flagfish (MANOVA, $F(2,17)=1.34$, $p>.05$). Mean DA levels were found to be 0.103 (SEM=±0.008, n=10) for males and 0.121 (SEM=±0.016, n=10) for females (Figure 5). Regardless of gender, it was appearent that flagfish whole brain contains about ten times more NE than DA. For instance, mean whole brain NE levels were 1.326 (SEM=±0.138, n=10) for males and 1.223 (SEM=±0.133, n=10) for females (Figure 6).

B) The effect of anesthesia on flagfish brain CAs

Experiment II was undertaken to evaluate whether anesthesia significantly affects whole brain DA and NE levels in flagfish. The study was performed in November 1987. Ten fish, that were anesthetized prior to sacrifice,

Figure 5: Whole brain DA levels in male and female flagfish sampled in June/1987. Each bar represents the mean (+/- SEM) for ten fish. (Experiment I).

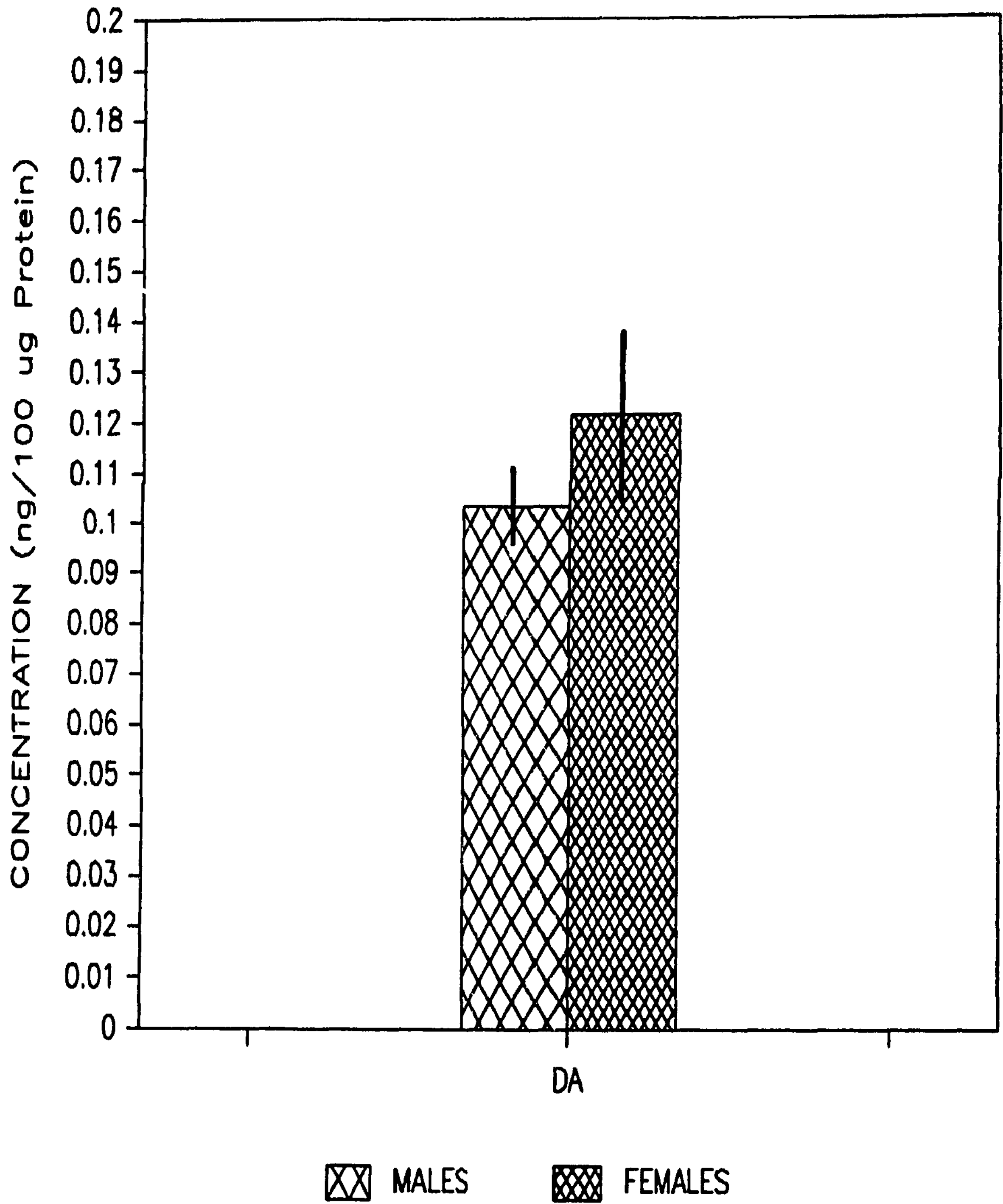

CONCENTRATION (ng/100 ug Protein)
0.2
0.19
0.18
0.17
0.16
0.15
0.14
0.13
0.12
0.11
0.1
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
DA
MALES
FEMALES

Figure 6: Whole brain NE levels in male and female flagfish sampled in June/1987. Each bar represents the mean (+/- SEM) for ten fish. (Experiment I).

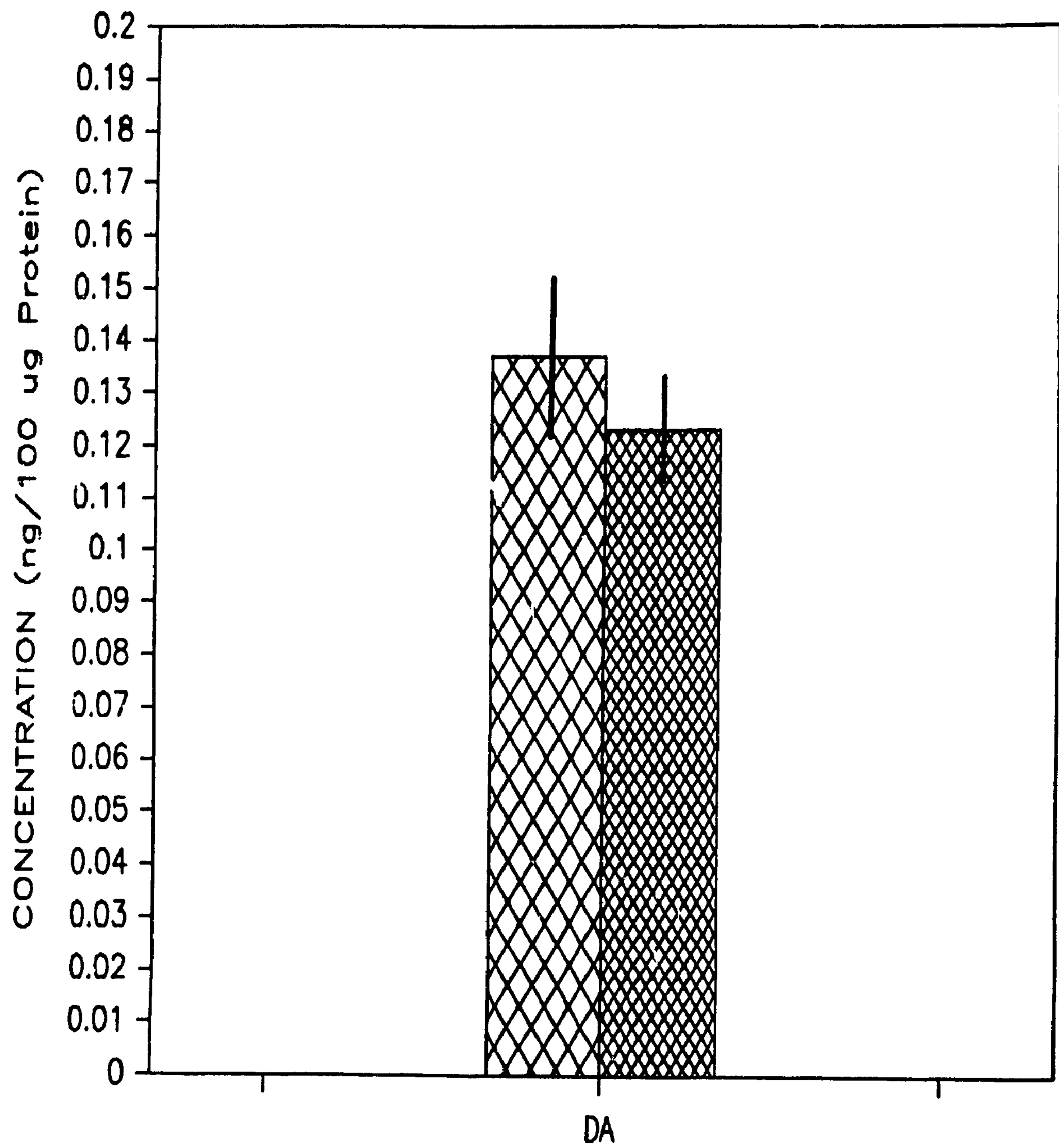
represented the experimental group and ten fish, that were sacrificed without anesthesia by spinal cord severing, served as controls. Both males and females were used for this experiment to ensure an adequate supply of females for subsequent studies. Brain samples were analysed, for DA, NE and protein, within two weeks following sampling. The CA levels were expressed in ng/100 ug protein.

Anesthesia with 3-aminobenzoic acid ethyl ester (1g/L water) did not alter whole brain DA and NE levels in a mixed gender group of flagfish (MANOVA, $F(2,17)=1.45$, $p>.05$). Mean DA values were 0.137 (SEM=±0.016, n=10) for the anesthetized fish and 0.123 (SEM=±0.010, n=10) for controls (Figure 7). As Figure 8 reveals, mean NE levels were 32% higher in the anesthesia group (1.297, SEM=±0.238, n=10) than in the control group (0.876, SEM=±0.056, n=10), but this observation was due to a high degree of variability in the anesthesia group, and hence it was not significant.

C) Determination of a FTN injection dose for experiment IV

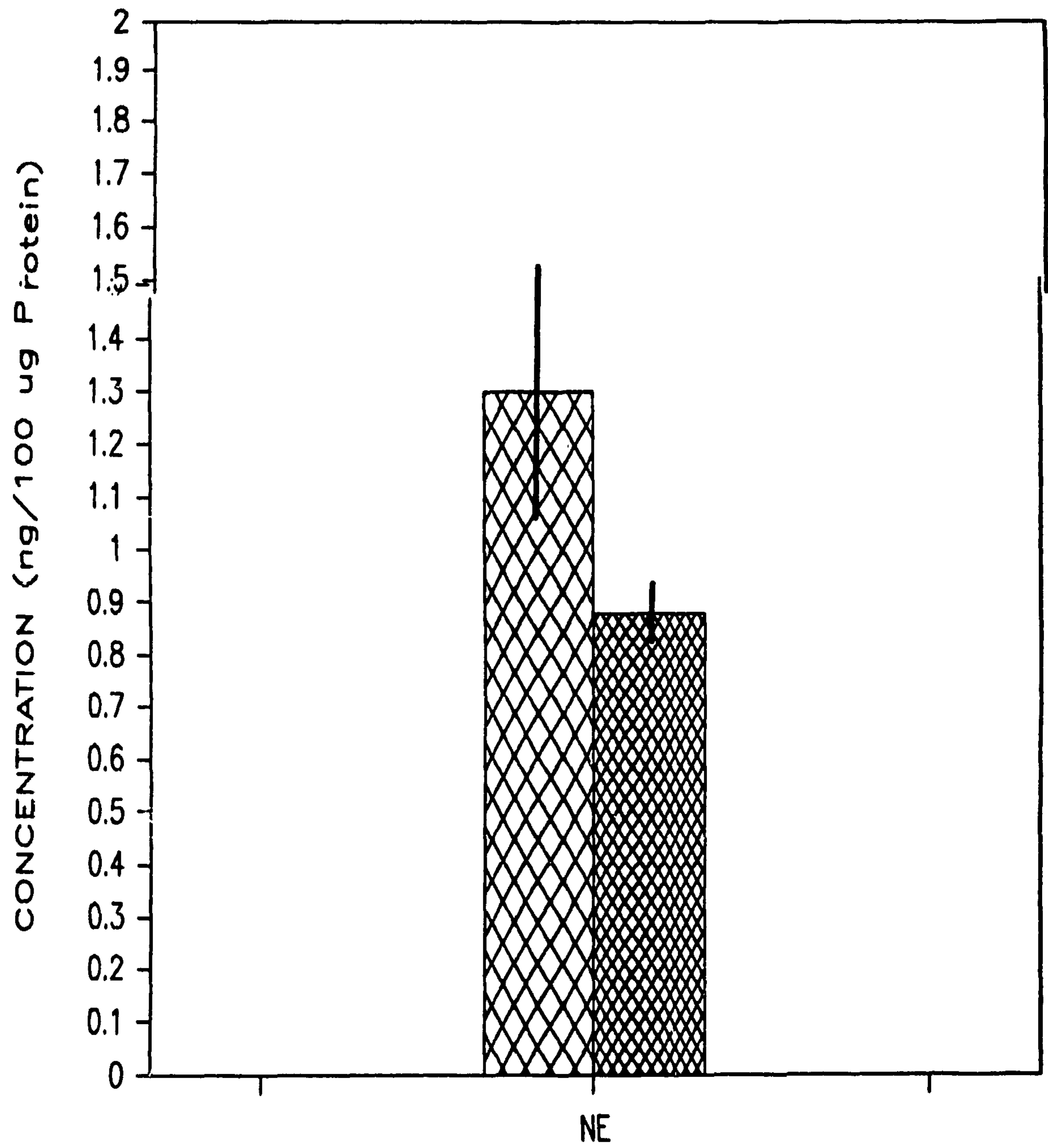
Experiment III was performed to find a FTN injection dose (ip) that was potent enough to elicit subtle changes in brain CA levels (if any), but was not lethal to the fish during the time course of the study. The experiment was performed in April/1988, using female flagfish only. Five fish per group were injected with either sesame oil,

Figure 7: Whole brain DA levels in anesthetized (1g/L water, 3-aminobenzoic acid ethyl ester) and control flagfish (mixed gender) sampled in November/1987. Each bar represents a mean (+/- SEM) for ten fish. (Experiment II).



0.2
0.19
0.18
0.17
0.16
0.15
0.14
0.13
0.12
0.11
0.1
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
CONCENTRATION (ng/100 ug Protein)
DA
ANESTHESIA
CONTROL

Figure 8: Whole brain NE levels in anesthetized (1g/L water, 3-aminobenzoic acid ethyl ester) and control flagfish (mixed gender) sampled in November/1987. Each bar represents a mean (+/- SEM) for ten fish. (Experiment II).


2
1.9
1.8
1.7
1.6
1.5
1.4
1.3
1.2
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
CONCENTRATION (ng/100 ug Protein)
NE
ANESTHESIA
CONTROL

1g/Kg BW FTN, 4g/Kg BW FTN, 8g/Kg BW FTN, 10g/Kg BW FTN or 12g/Kg BW FTN respectively. Survival time was recorded for ten days. The sesame oil injected, and the 1g/Kg BW FTN injected groups, survived beyond the ten days of observations. Fish exposed to 8g/Kg BW FTN started to die after seven days following injections. All the fish in this group were dead at the end of the 8th day. Yet, 4 out of 5 fish, subjected to half of this dose (4g/Kg BW FTN), died on day 4 and only one fish survived up to day 5 postinjection. Fish receiving 10 g/Kg BW FTN and 12 g/Kg BW FTN started dying within 24 hours. All fish in these groups were dead by day 5 postinjection (Table 6).

On the basis of this study, it was decided that two doses, 4g/Kg BW FTN and 2g/Kg BW FTN, would be used for the next experiment. A dose of 4g/Kg BW FTN was not likely to cause death within 72h postinjection, which was the planned duration of the subsequent study.

D) The effects of two doses of FTN on brain CA levels of flagfish at three different sampling times

Experiment IV was undertaken to assess whether FTN alters whole brain DA and NE levels in flagfish. Only female flagfish were used for this study. The study was performed in June/1988. A control group (time 0 controls, n=10) was sampled prior to injections to detect possible changes from experimental manipulation. Apart from this

Table 6: Dose versus time to death following injections of flagfish with 1,4,8,10 and 12 g/Kg BW FTN. The numbers in the cells indicate the number of dead fish. The right column shows the mean survival time (days) at selected doses.

	DAY OF MORTALITY FOLLOWING INJECTIONS										
DOSE	1	2	3	4	5	6	7	8	9	10	MEAN**
0*											
1g/Kg											
4g/Kg				4	1						4.2
8g/Kg							1	4			7.8
10g/Kg	1		2		2						3.4
12g/Kg	1	1	2	1							2.6

* VEHICLE (SESAME OIL) INJECTED CONTROL GROUP

** MEAN SURVIVAL TIME (DAYS)

group, the design included 12 groups with 10 subjects/ group (Table 5). There were four experimental conditions; 1) control 2) sesame oil injection, 3) 2g/Kg BW FTN injection and 4g/Kg BW FTN injection. A group from each experimental condition was sampled at 4h, 24h and 72h (4 conditions x 3 sampling times). Two samples from the 4h control group were lost due to technical problems during HPLC analysis. Three fish were lost from among 24h 2g/Kg BW FTN injected group and two from both 72h 2g/Kg BW FTN and 4h 4g/Kg BW FTN injected groups. These losses occured early in the study and may have been due to accidental puncture of a vital organ during injections.

The data collection included whole brain DA, NE and protein concentrations. All samples were analysed within three weeks following sampling.

Fenitrothion or sesame oil injections did not produce any significant changes in whole brain DA and NE levels in female flagfish (MANOVA, $F(6,198)=1.17$, $p>.05$). In the 4g /Kg BW FTN injected group, NE levels declined from 4h to 24h and sharply increased from 24h to 72h, but these observations were not significant (MANOVA, $F(12,198)=1.41$, $p>.05$).

Whole brain CA levels decreased from 4h to 72h, in all groups (MANOVA, $F(4,198)=7.8$, $p<.05$). Examination of univariate ANOVA (Norusis, 1988) revealed that the finding was due to changes in DA levels (ANOVA, $F(2,99)=16.69$,

$p<.05$), and not in NE levels (ANOVA, $F(2,99)=0.78$, $p>.05$).

Comparison of the four control groups (time 0, 4h, 24h and 72h) revealed an overall decrease in both, DA and NE levels (MANOVA, $F(6,68)=4.28$, $p<.05$). The time 0 control group had significantly higher whole brain DA and NE levels than all other groups (Scheffe, $p<.05$). Figures 9 and 10 summarize the results of this experiment.

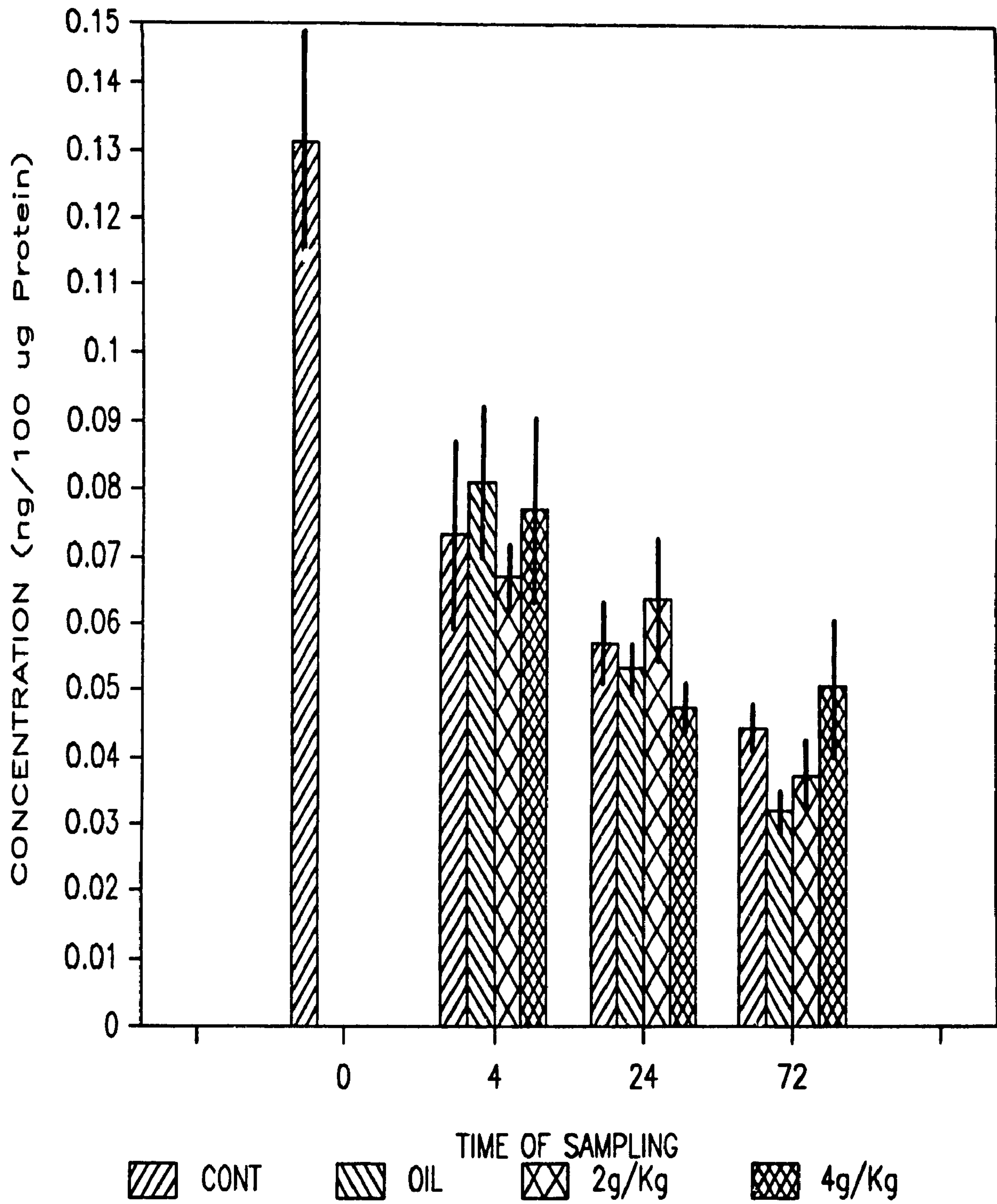
Although not part of this study, a decrease in fish locomotor activity was observed in all groups. This behavioral change started 6-7 hours after the beginning of the experiment and persisted throughout the study.

E) Rainbow Trout Studies

Two identical studies were conducted on the effects of 12 day sublethal (0.01 mg/L) HCN exposure on brain CA levels of rainbow trout. Both male and female fish were used for the studies. One sample from the July experiment (control group) was lost during the sonification process. Another four samples (three from HCN treated group and one from control group) were lost from the August study due to technical problems during HPLC analysis.

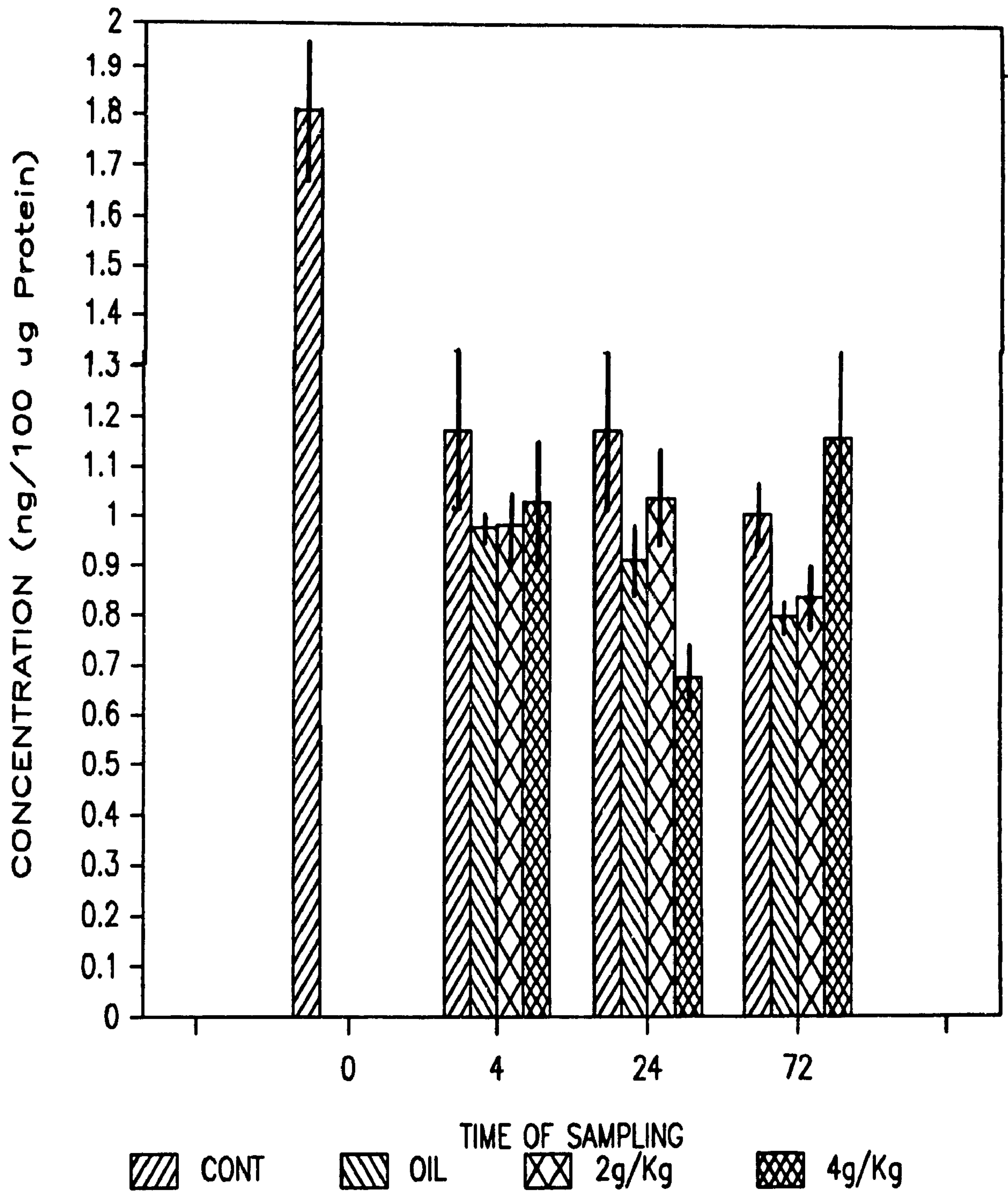
Fish exposed to sublethal (0.01 mg/L water) HCN concentration, for 12 days, showed higher whole brain CA levels than controls. A factorial MANOVA, with time (July and August) on one axis and treatment (control and HCN exposed) on the other axis, suggested that these findings

Figure 9: Whole brain DA levels in female flagfish (mean body weight = 1.15 g) under four experimental conditions: 1) control, 2) sesame oil injection (8g/Kg BW), 3) 2g/Kg BW FTN injection and 4) 4g/Kg BW FTN injection. Fish were sampled at time 0 (start) and at 4, 24 and 72h following injections in June/1988. Each bar represents a mean (+/- SEM) for ten fish, except for the following groups: 4h-CONT (n=8), 24h-2g/Kg (n=7), 72h-2g/Kg (n=8), 4h-4g/Kg (n=8). An overall decrease with time in DA levels, was found significant (MANOVA, $p<.05$). The time 0 control group had significantly higher DA levels than all other controls (ANOVA, Scheffe, $p<.05$). (Experiment IV).



CONCENTRATION (ng/100 ug Protein)
0.15
0.14
0.13
0.12
0.11
0.1
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
0
4
24
72
TIME OF SAMPLING
CONT
OIL
2g/Kg
4g/Kg

Figure 10: Whole brain NE levels in female flagfish (mean body weight = 1.15 g) under four experimental conditions: 1) control, 2) sesame oil injection (8g/Kg BW), 3) 2g/Kg BW FTN injection and 4) 4g/Kg BW FTN injection. Fish were sampled at time 0 (start) and at 4, 24 and 72h following injections in June/1988. Each bar represents a mean (+/- SEM) for ten fish, except for the following groups: 24h-2g/Kg (n=7), 72h-2g/Kg (n=8), 4h-4g/Kg (n=8). The time 0 control group had significantly higher NE levels than all other controls (ANOVA, Scheffe, $p<.05$). (Experiment IV).


CONCENTRATION (ng/100 ug Protein)
2
1.9
1.8
1.7
1.6
1.5
1.4
1.3
1.2
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
4
24
72
TIME OF SAMPLING
CONT
OIL
2g/Kg
4g/Kg

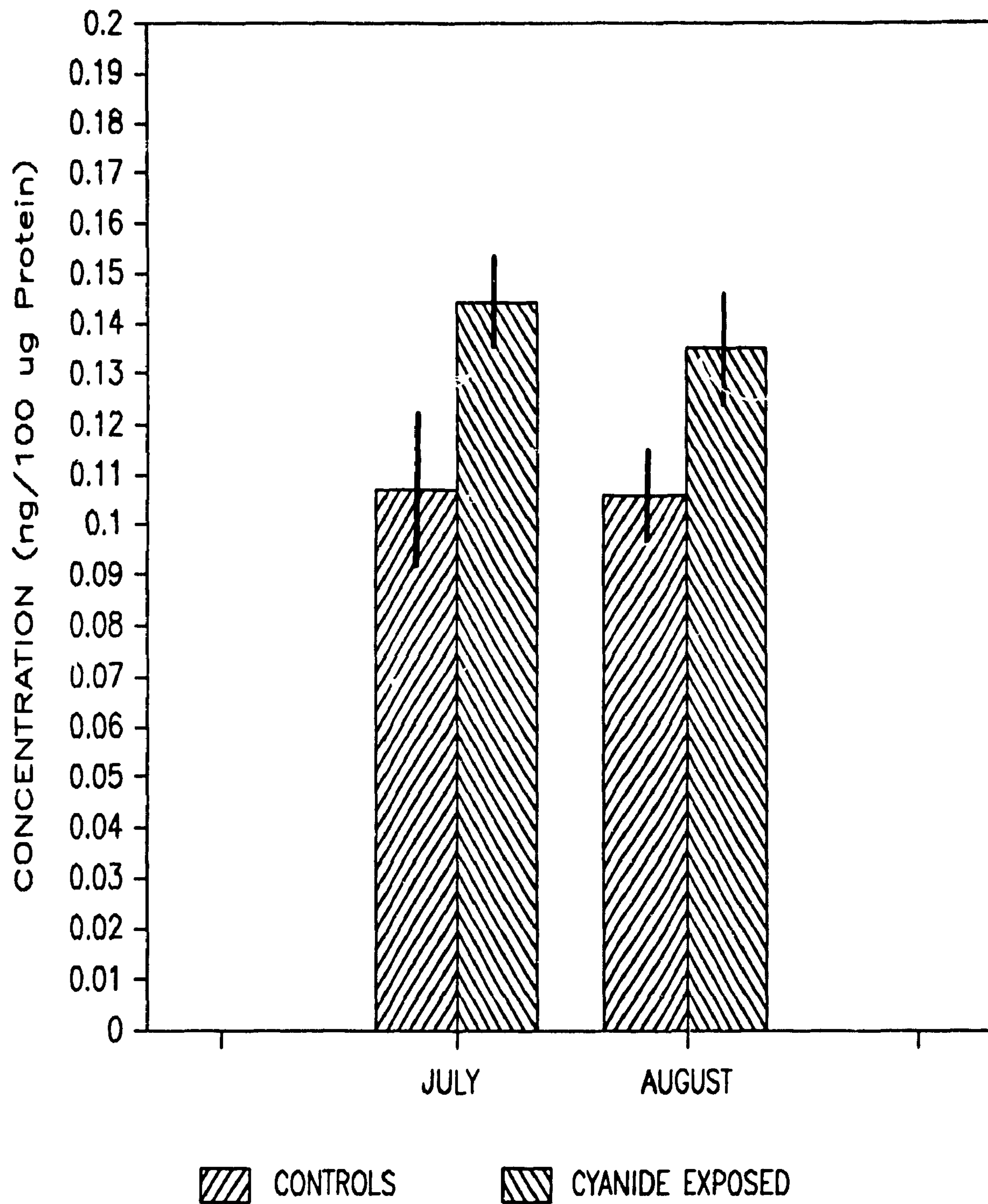
were significant ($F(2,22)=4.84$, $p<.05$). Univariate F-tests (Norusis, 1988) revealed that DA, but not NE, levels were significantly different between HCN treated and control groups (ANOVA ($F(1,23)=8.8$ $p<.05$). Mean whole brain DA levels of the control fish (expressed in ng/100 ug protein) were 0.107 (SEM=±0.016) in July and 0.106 (SEM=±0.009) in August, while for CN treated fish were 0.144 (SEM=±0.009) in July and 0.135 (SEM=±0.011) in August. The time of experiment (July or August) did not affect the results in this study ($F(2,22)=0.31$, $p>.05$). Figure 11 illustrates the differences in DA levels between CN treated and control fish. Figure 12 presents the whole brain NE levels in rainbow trout brain during the two experiments.

Brain DA and NE levels were directly proportional (Figure 13) to one another in control fish ($r = + 0.81$). This finding was significant (df=12, two-tailed test, $p<.05$). As Figure 14 reveals, in the cyanide treated fish this relationship was not observable, probably because of changes in brain DA levels in these groups ($r = + 0.28$, df=11, $p>.05$).

Since the time of the experiment (July or August) did not affect brain CA levels, the data for controls (n=7 for both sexes) was pooled and analysed for possible gender related differences in brain DA and NE levels. Figures 15 and 16 illustrate the mean (+/- SEM) whole

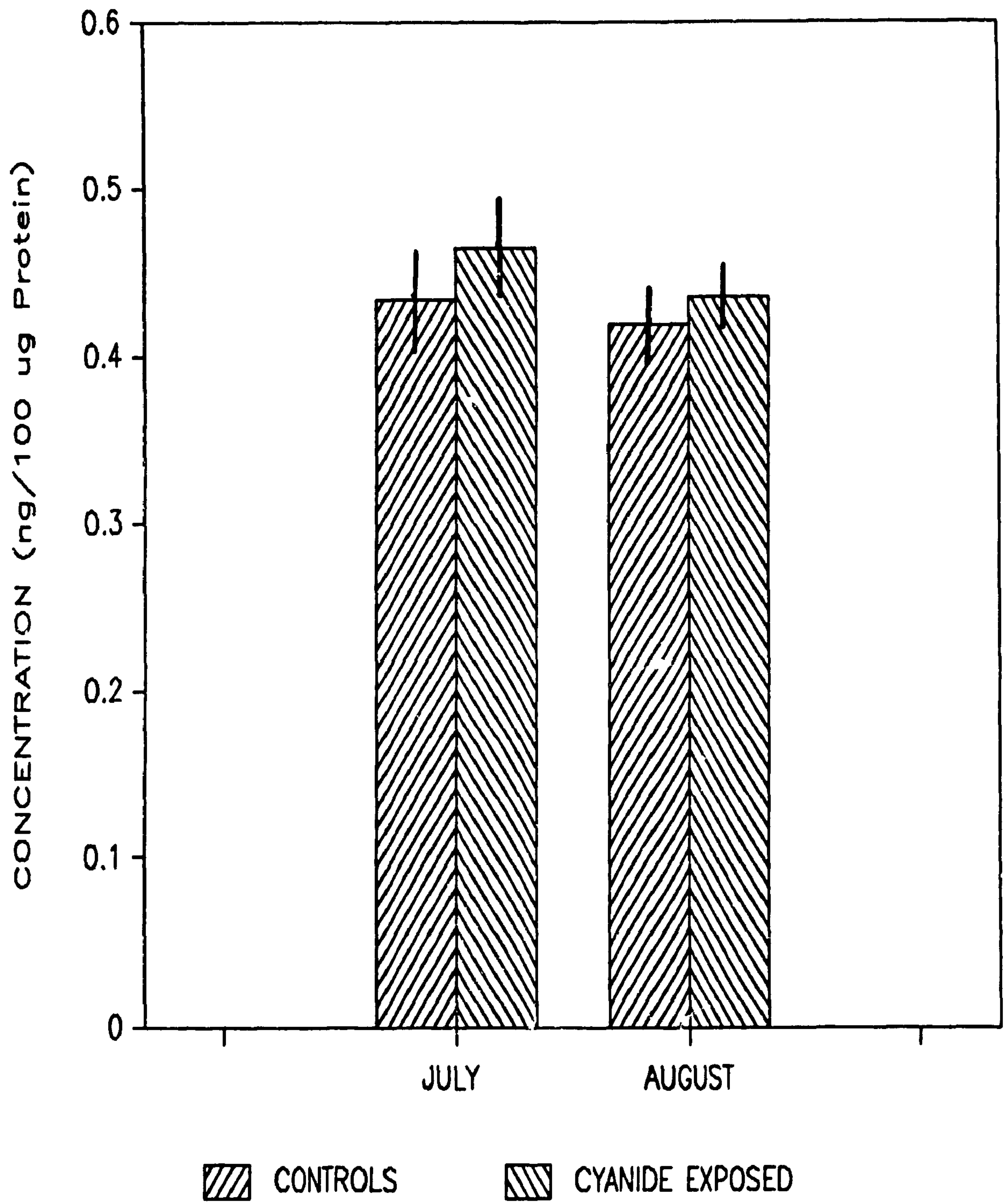
brain DA and NE levels in male and female rainbow trout. The two sexes had no significantly different whole brain DA (ANOVA, $F(1,12)=0.026$, $p>.05$) or NE levels (ANOVA, $F(1,12)=1.926$, $p>.05$).

Figure 11: Whole brain DA levels in control and HCN exposed (0.01 mg HCN/L H20, for 12 days) sexually maturing rainbow trout (mean body weight = 299.53 g) sampled in July/1988 and in August/1988. Control-HCN exposed fish ratio (n) was 5/6 in July and 9/7 in August. Each bar represents a mean (+/- SEM). The HCN treated fish had significantly higher DA levels than controls (ANOVA, $p<.05$).



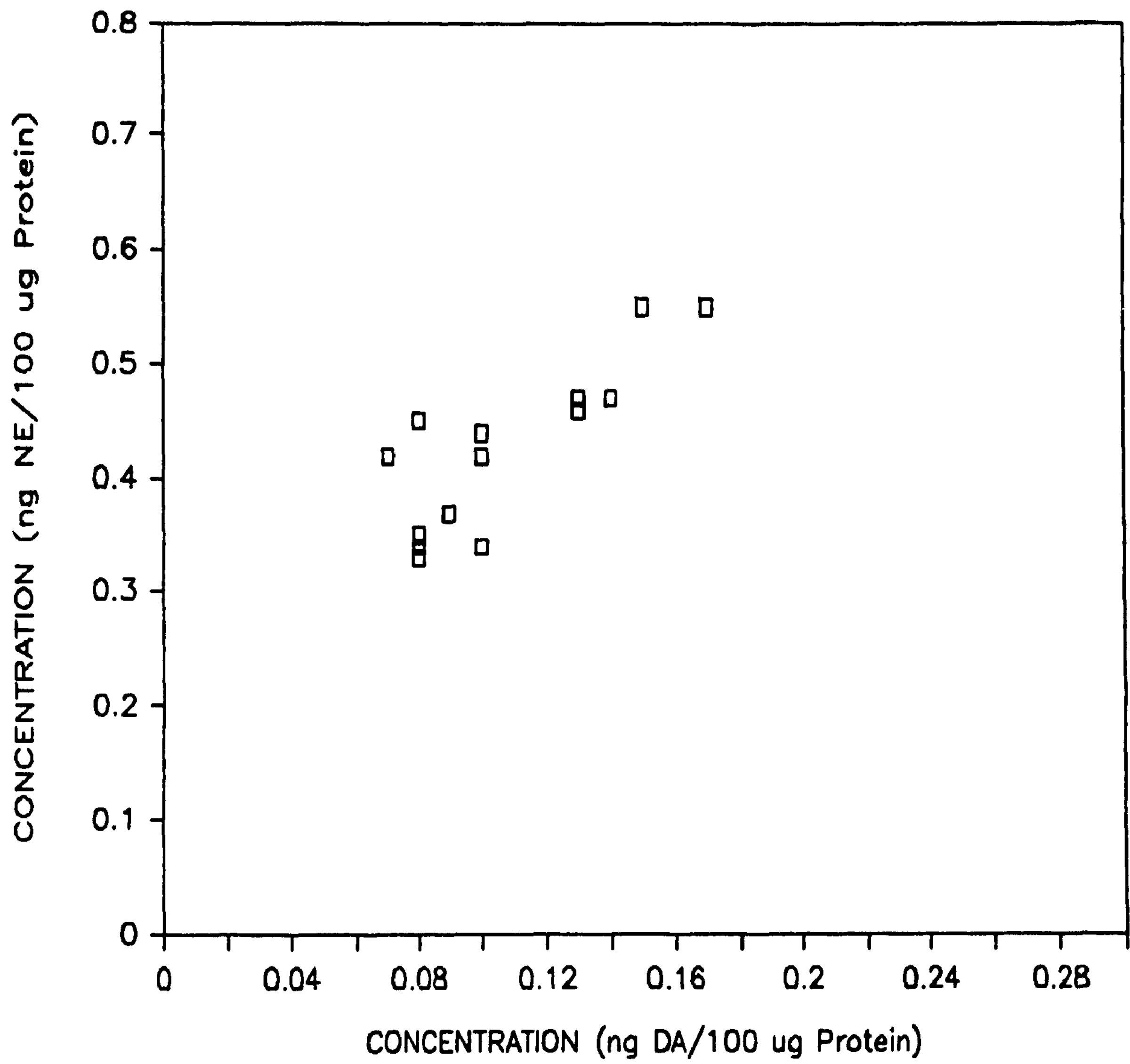
0.2
0.19
0.18
0.17
0.16
0.15
0.14
0.13
0.12
0.11
0.1
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
CONCENTRATION (ng/100 ug Protein)
JULY
AUGUST
CONTROLS
CYANIDE EXPOSED

Figure 12: Whole brain NE levels in control and HCN exposed (0.01 mg HCN/L H2O, for 12 days) sexually maturing rainbow trout (mean body weight = 299.53 g) sampled in July/1988 and in August/1988. Control-HCN exposed fish ratio (n) was 5/6 in July and 9/7 in August. Each bar represents a mean (+/- SEM).



CONCENTRATION (ng/100 ug Protein)
0.6
0.5
0.4
0.3
0.2
0.1
0
JULY
AUGUST
CONTROLS
CYANIDE EXPOSED

Figure 13: Relationship between whole brain DA and NE levels in sexually maturing control rainbow trout (mean body weight = 299.53 g) sampled in July/1988 and in August/ 1988. The correlation was significant ($r = + 0.81$, $df=12$, $p<.05$).



CONCENTRATION (ng NE/100 ug Protein)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
0.04
0.08
0.12
0.16
0.2
0.24
0.28
CONCENTRATION (ng DA/100 ug Protein)

Figure 14: Relationship between whole brain DA and NE levels in sexually maturing HCN exposed (0.01 mg/l H20, for 12 days) rainbow trout (mean body weight = 299.53 g) sampled in July/1988 and in August/ 1988. The correlation was found not significant ($r = +0.28$, $df=11$, $p>.05$).

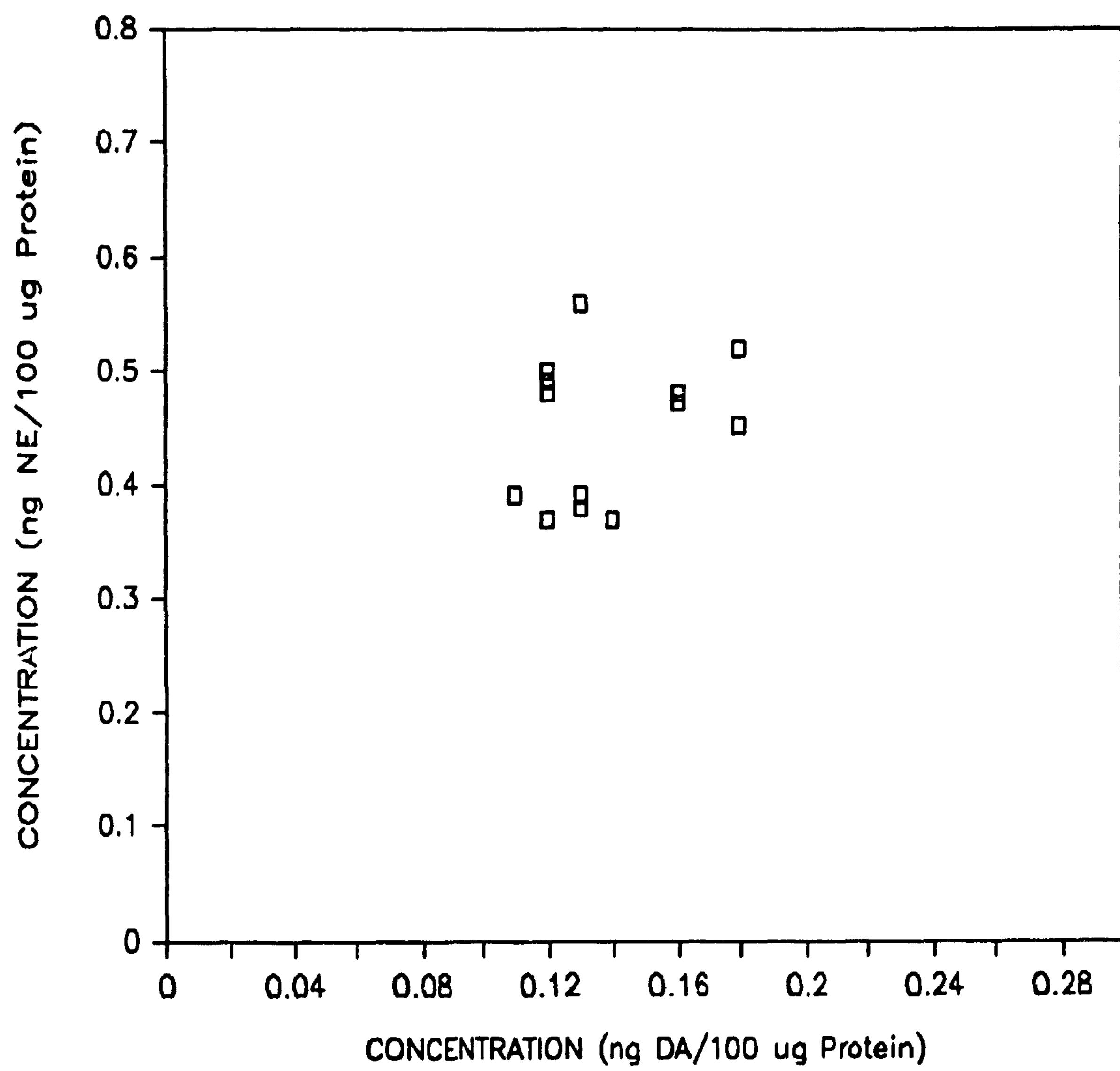
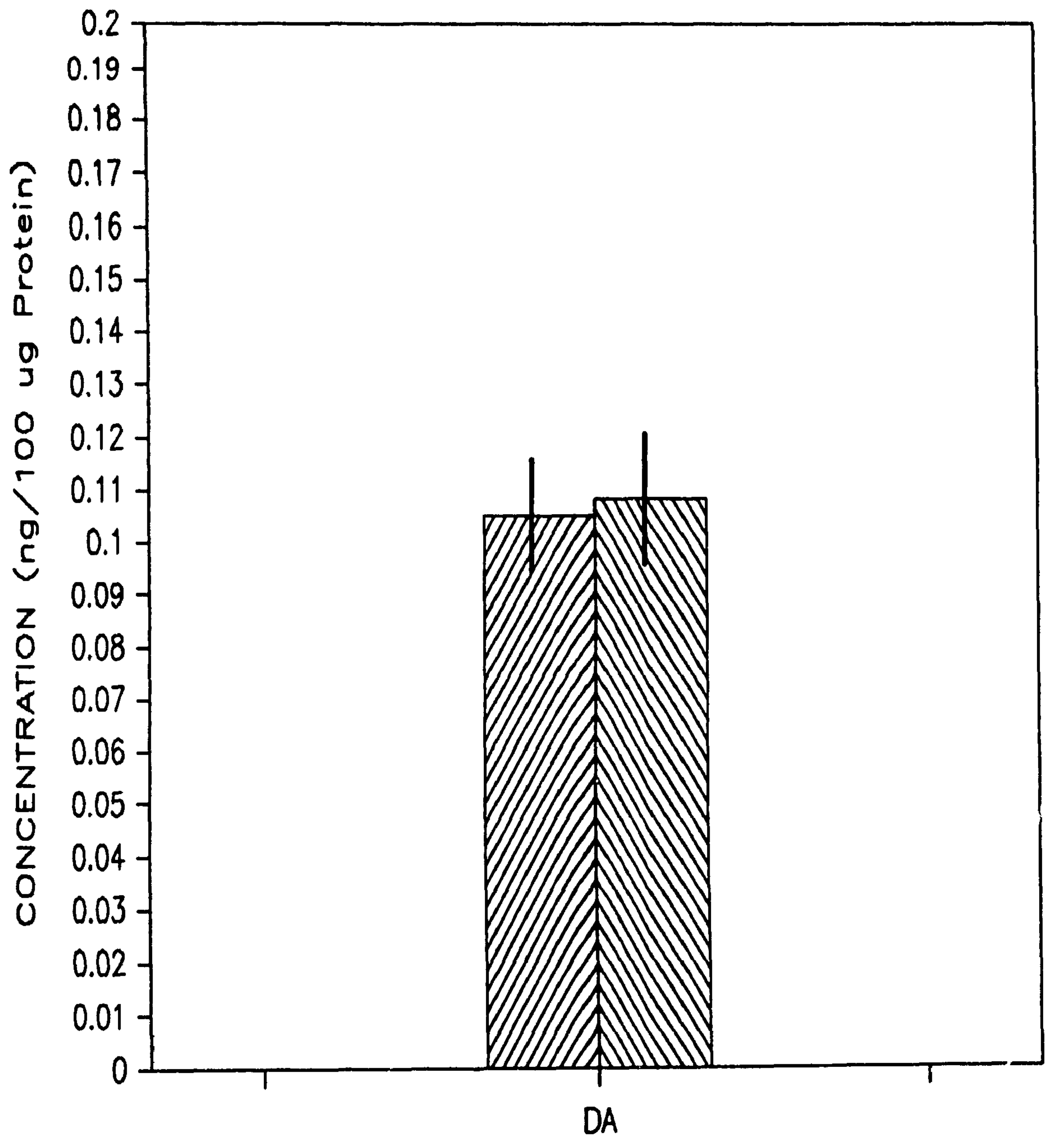
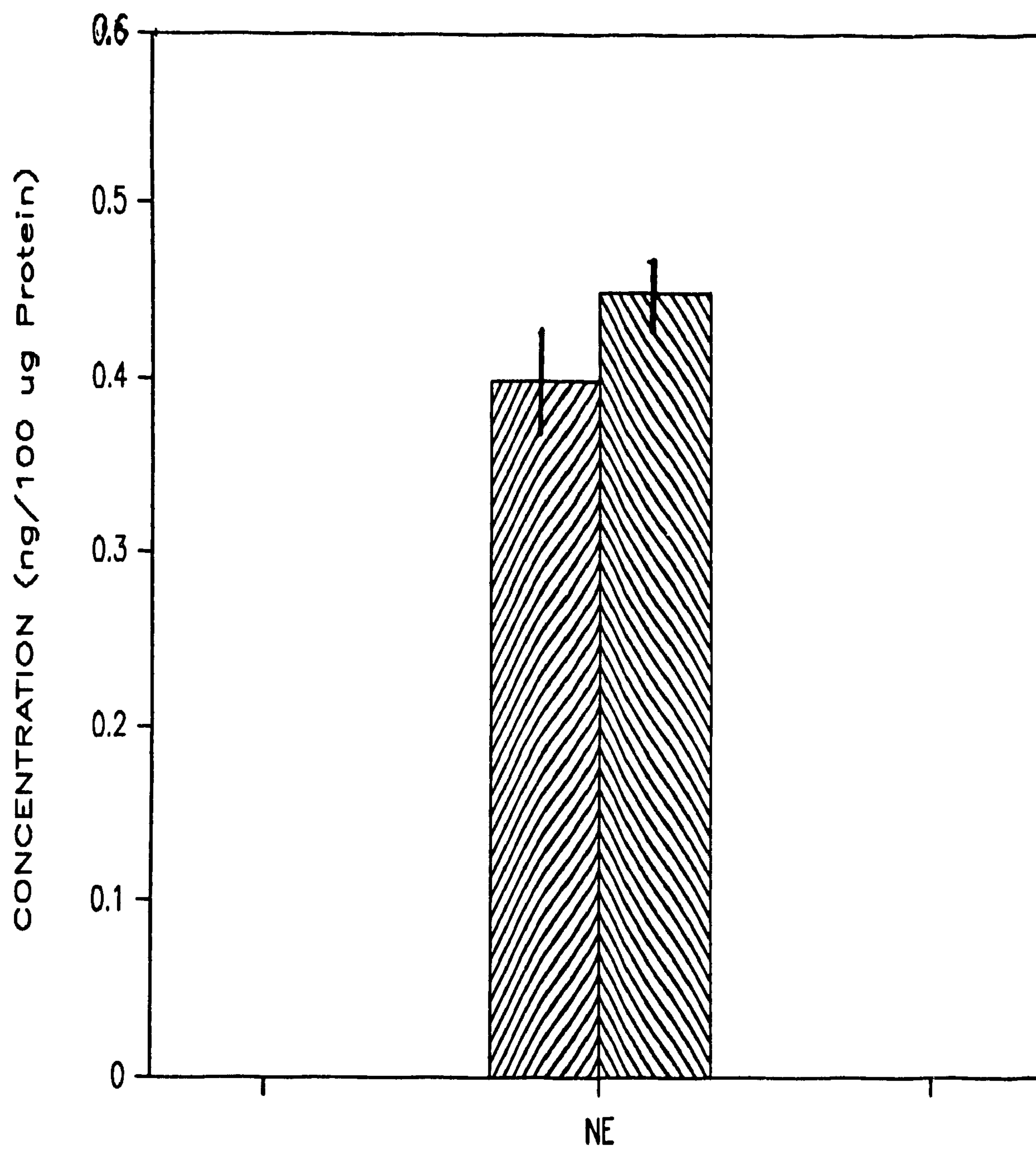

0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
CONCENTRATION (ng NE/100 ug Protein)
0
0.04
0.08
0.12
0.16
0.2
0.24
0.28
CONCENTRATION (ng DA/100 ug Protein)

Figure 15: Whole brain DA levels in male and female rainbow trout (control, mean body weight = 299.53 g) sampled in July/1988 and in August/1988. Each bar represents a mean (+/- SEM) for seven fish.



CONCENTRATION (ng/100 ug Protein)
0.2
0.19
0.18
0.17
0.16
0.15
0.14
0.13
0.12
0.11
0.1
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
DA
MALES
FEMALES

Figure 16: Whole brain NE levels in male and female rainbow trout (control, mean body weight = 299.53 g) sampled in July/1988 and in August/1988. Each bar represents a mean (+/- SEM) for seven fish.



CONCENTRATION (ng/100 ug Protein)
0.6
0.5
0.4
0.3
0.2
0.1
0
NE
MALES
FEMALES

DISCUSSION

The results of experiment I clearly indicate that whole brain DA and NE levels do not differ in male and female flagfish in a random sample. These results are difficult to compare with the only study reported in the literature on flagfish whole brain DA and NE levels (Holdway et al., 1988), for two reasons. First, Holdway et al. (1988) selected their subjects on the basis of behavioral traits associated with reproductive status (i.e. dominant, subordinate, solitary or resting males and laying or resting females). In contrast, the study reported here employed randomization (Minium and Clarke, 1982) with no attempt of selection on any basis. Second, aspects of the experimental conditions, such as water characteristics, diet and photoperiod were different in the two studies. Although little is known about the effects of these variables, it is possible that they could alter central CAs in fish. For example, Pouliot, DE La Noue and Roberge (1988) have demonstrated that diet influences CA levels in various brain structures of the rainbow trout.

Similar findings to that in experiment I emerged from studies on rainbow trout. They have demonstrated no gender related differences in whole brain DA and NE levels in this species.

Given that in a random sample of male and female flagfish and/or rainbow trout no differences in whole brain DA and NE levels exist, it is safe to assume that both sexes of both species can be used for studies on central CAs, without being overly concerned about gender related variations in the measurements.

Experiment II demonstrated that anesthesia with 3-aminobenzoicacid ethyl ester (1g/l water) does not alter whole brain DA and NE levels in flagfish (mixed gender) given that : 1) exposure to the prescribed dose of anesthetic agent is less than 60 seconds and 2) sampling procedure is performed within two minutes following anesthesia. These findings are in accordance with another study by Sloley et al. (1986), which demonstrated that anesthesia with MS222 had no effect on brain DA and NE levels of rainbow trout if sampling procedure lasted not longer than two minutes. This form of anesthesia is widely used in many studies with fish, because it facilitates handling and decreases the stress response of the organism (Mazeaud and Mazeaud, 1981). However, to date there are only two studies (including the present) that report the effects of MS222 on teleostean brain CAs. It should be pointed out that in the present study a ten times higher concentration of anesthetic was used than in the study by Sloley et al. (1986). The insensitivity of brain CAs in flagfish to this relatively high dose

suggests that MS222 may be the method of choice for studies that require rapid anesthesia prior to sampling.

Experiment III provided a reasonable guidline for the determination of two injection doses of FTN that were not lethal to the fish during the subsequent experiment. Considering the variability in biological organisms, the small sample size (n=5) for each group presented a major limitation. This variability was the most evident in the group injected with the highest dose of toxicant (12g/Kg BW FTN). Four out of five fish in this group died on a different day. Surprising was the finding that the 8 g/Kg BW FTN injected fish survived beyond the fish injected with half of this dose. However, the use of small sample size in toxicity asessment increases the probability that two doses of toxic agents will manifest their effects in a reversed order, in such a way that the weaker will appear to be the stronger (Trevan, 1927). It is possible that such reversal had occurred, between the 4g/Kg BW FTN and 8g/Kg BW FTN injected groups, in the present study. The implications of these findings to other studies is that no attempt of toxicity testing should be made using small sample sizes.

Experiment IV revealed no evidence for changes in whole brain DA and NE levels as a result of FTN treatment in female flagfish. The signs of intoxication particular to OP compounds (in fish), such as refusal of diet and

decreased locomotor activity (Walsh and Ribelin, 1975), were present throughout the study. These signs have indicated the presence of FTN in the organism and that the doses injected were potent enough to produce intoxication. It appears that a single injection, of relatively high FTN dose, does not alter brain CA levels in flagfish within 72 hours following injection.

A decrease in whole brain DA and NE levels in flagfish, from time 0 to the 4 h sampling time, indicates that an initially unidentified experimental variable has been introduced within this time interval. Since the findings were apparent in all groups, including the control group, these changes in CA levels were independent of FTN or sesame oil injections. The only variable that was introduced at this point, and previously ignored, was the change in social environment. When fish were removed from the holding tanks they were placed in identical experimental tanks. The only difference between the two tanks was fish density and gender. The density of holding tanks was approximately 30-40 fish/tank and included both male and female flagfish, while the density of experimental tanks was 10 fish/tank and included only female flagfish. Given that peripheral CAs increase in response to stress (Nakano and Tomlinson, 1967; Mazeaud and Mazeaud, 1981), it would be expected that removal of fish from a more crowded environment alleviates a number

of stress factors hence decreasing CA levels. However, this may not be the case for central CAs. In contrast to peripheral CAs, central CAs seem to decrease in response to stress (Mazeaud and Mazeaud, 1981; Popek, Sokolowska and Bieniarz, 1981). In the present study, the only stress factor, introduced between time 0 and 4h, was the removal of fish from the holding tank, prior to placing them in the experimental tank. However, the duration of this type of stress is not expected to last longer than a few hours (Mazeaud and Mazeaud, 1981). Since decrease in brain CA levels persisted up to 72h, in the present study, other factors, such as change in fish density and/or gender distribution, from here on referred to as social environment, may have contributed to decline in CA levels. Two important consequences of the change in social environment were: 1) larger available space per fish, with subsequent decrease in territorial competition, or 2) absence of males, with a subsequent absence of aggression toward females. Either or both of these factors may have contributed to changes in brain CA levels in female flagfish.

A decline in locomotor activity was also observed in experiment IV, six to seven hours after the fish were placed into the lower density experimental tanks. Deliége, Cession-Fossion and Vandermeulen (1969), have observed that decreased locomotor activity was associated with

lower whole brain CA levels in goldfish. It is apparent that in the present study, the decrease in locomotor activity was related to lower whole brain CA levels in female flagfish.

Results obtained from experiment IV, suggest that fish density and/or gender distribution may be important variables which should be controlled in experiments that involve determination of brain CA levels. The present study also supports the observation by Deliége, Cession-Fossion and Vandermeulen, (1969), that lower locomotor activity is associated with decline in central CA levels in fish. This overtly observable behavioral trait may provide a valuable, however robust, indication of changes in whole brain CA levels in teleost. If these variables are overlooked, in studies on central CAs, they may result in significant error in measurements.

Two experiments, performed during the Summer 1988, suggest that chronic exposure to sublethal levels of HCN (0.01 mg/L) for 12 consecutive days may increase whole brain DA levels in sexually maturing male and female rainbow trout. However, before such conclusions can be made it would be important to replicate the study using a time 0 control group to account for possible changes in brain CA levels during the time course of the experiment. Since HCN is converted to thiocyanate (Westley, 1981) in the organism and thiocyanate accumulation increases with

the duration of exposure to HCN (Raymond, Leduc and Kornblatt, 1986) it is possible that thiocyanate may have contributed to the changes in brain DA levels in rainbow trout. To clearly identify whether HCN or thiocyanate (or both) affects brain DA levels, it would be important to assess the effects of thiocyanate on DA levels. However, to date there is no reported evidence that a mechanism for thiocyanate uptake exists in the central neurons. A third factor that should not be overlooked is the possible interference of HCN and/or thiocyanate with MS222 when anesthesia is used prior to sampling. While the short period of time (i.e. from the placement in MS222 to sampling that lasts not longer than 90 seconds) would not be expected to result in changes in brain DA levels due to interaction of MS222 with HCN or thiocyanate, this probability cannot be ruled out and therefore it merits future consideration.

A positive correlation was found between whole brain DA and NE levels in control rainbow trout, but in HCN treated fish this relationship was no longer observable probably due to the effects of HCN on DA levels in this group. At the moment there are no other reports in the literature that have investigated the effects of cyanide on central CAs in fish. Most of the present knowledge, about the effects of -CN on CNS, comes from mammalian studies.

In the CNS the effects of -CN are similar to that of anoxia (Reempts, 1984, Benabid et al., 1987). The most prominent of these effects is intraneuronal Ca2+ accumulation. This is a consequence of impairment in the energy dependent Ca2+ regulatory mechanisms (Johnson, Meisenheimer and Isom, 1986). For example, KCN administration resulted in significant whole brain Ca2+ increase in mice (Johnson, Meisenheimer and Isom, 1986). Since the release of NTs is Ca2+ dependent (Feldman and Quenzer, 1984), it is likely that intraneuronal Ca2+ accumulation results in increased NT release (Borowitz, Born and Isom, 1988). Consequently, it would be expected that -CN induced changes in Ca2+ levels result in NT depletion (Borowitz, Born and Isom, 1988).

Decreased brain NT levels, following NaCN treatment, were observed in mammalian studies. In rats injected with NaCN, and sacrificed one minute later, a decrease in DA levels in the striatal brain region has been demonstrated (Carlsson and Persson, 1985; Persson, Cassel and Sellstrom, 1985).

On the basis of mammalian studies the following questions can be raised: 1) Is there a significant intracellular Ca2+ accumulation in fish brain in response to -CN, similar to that observed in mammals ?, and if the answer is yes then 2) what are the relationships between various doses of HCN, length of exposure and Ca2+

accumulation in fish ? It is also feasible to ask the question whether enzymes in the CA catabolic pathway are affected by chronic HCN exposure in fish ? If these enzymes are impaired, an increase in NT levels could be expected. Last, but not least, it would be important to assess how thiocyanate accumulation following chronic exposure to HCN is related to these parameters. The answers to these questions would provide a better understanding of -CN neurotoxicity in fish. Since changes in brain CA levels could result in alteration of numerous physiological processes, a better understanding of -CN and/or thiocyanate neurotoxicity would be an asset for research in this domain.

An important physiological mechanism that could be altered by changes in DA levels, as a result of HCN treatment, is fish reproduction. Given that the hypothalamic region has the largest CA content in fish brain (Mazeaud and Mazeaud, 1981), and that hypothalamic DA processess regulate pituitary GTH (Ball, 1981; Chang et al, 1984), it is possible that increased DA levels at hypothalamo-pituitary axis impose a strong inhibition on GTH release. While this study does not provide direct evidence for this hypothesis, measurements of pitutary DA levels in HCN exposed vs. control fish, along with measurements of plasma GTH, would clarify this issue.

In summary, the series of studies reported in this manuscript have demonstrated that :

1) There is no difference in whole brain DA and NE levels between male and female flagfish in a random sample.

2) Anesthesia with 3-aminobenzoic ethyl ester (MS222) (1 g/L) does not produce measurable changes in whole brain DA and NE levels in flagfish, given that: a) the time of exposure to the prescribed dose of anesthetic is less than 60 seconds, and b) the sampling procedure lasts not longer than 2 minutes.

3) Intraperitoneal injections with FTN (2g/Kg BW and 4g/Kg BW) do not result in observable changes in whole brain DA and NE levels in female flagfish within 72 hours following treatment.

4) Chronic exposure to sublethal levels of HCN in water (0.01 mg/L) may result in increased whole brain DA levels in sexually maturing male and female rainbow trout.

5) There are no differences in whole brain DA and NE levels between sexually maturing male and female rainbow trout (controls).

6) Dopamine and NE are directly proportional to each other in rainbow trout brain under control conditions, but this relationship is absent in HCN treated fish.

7) Rainbow trout brain contains about four times more NE than DA, while flagfish brain contains about ten times more NE than DA.

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APPENDIX A

Cathecholamine synthesis and catabolism

Major pathways in the biosynthesis and catabolism of cathecholamines. The full names of abbreviations are presented below. The metabolism of norepinephrine and epinephrine are not shown due to their complexity. The dark (large) shaded arrows indicate the sequence in the pathway, the closed unshaded arrows indicate the locus of DA and NE in the pathway, while the small dark shaded arrows indicate the enzyme and/or cofactor that are needed for the pointed step. The identity of various compounds is indicated by small dark shaded arrow pointing in the direction of the corresponding chemical structure. Note that the enzymes are represented with lower case letters !

List of Abbreviations

comt - cathechol-O-methyltransferase

dbh - dopamine-beta-hydroxylase

DA - dopamine, (3,4-dihydroxyphenylethylamine)

DOPAC - 3,4-dihydroxyphenylacetic acid

DHPA - 3,4-dihydroxyphenylacetaldehyde

EPI - epinephrine, (3,4-dihydroxyphenyl-N-methylethanolamine)

HVA - homovanillic acid

mao - monoamine oxidase

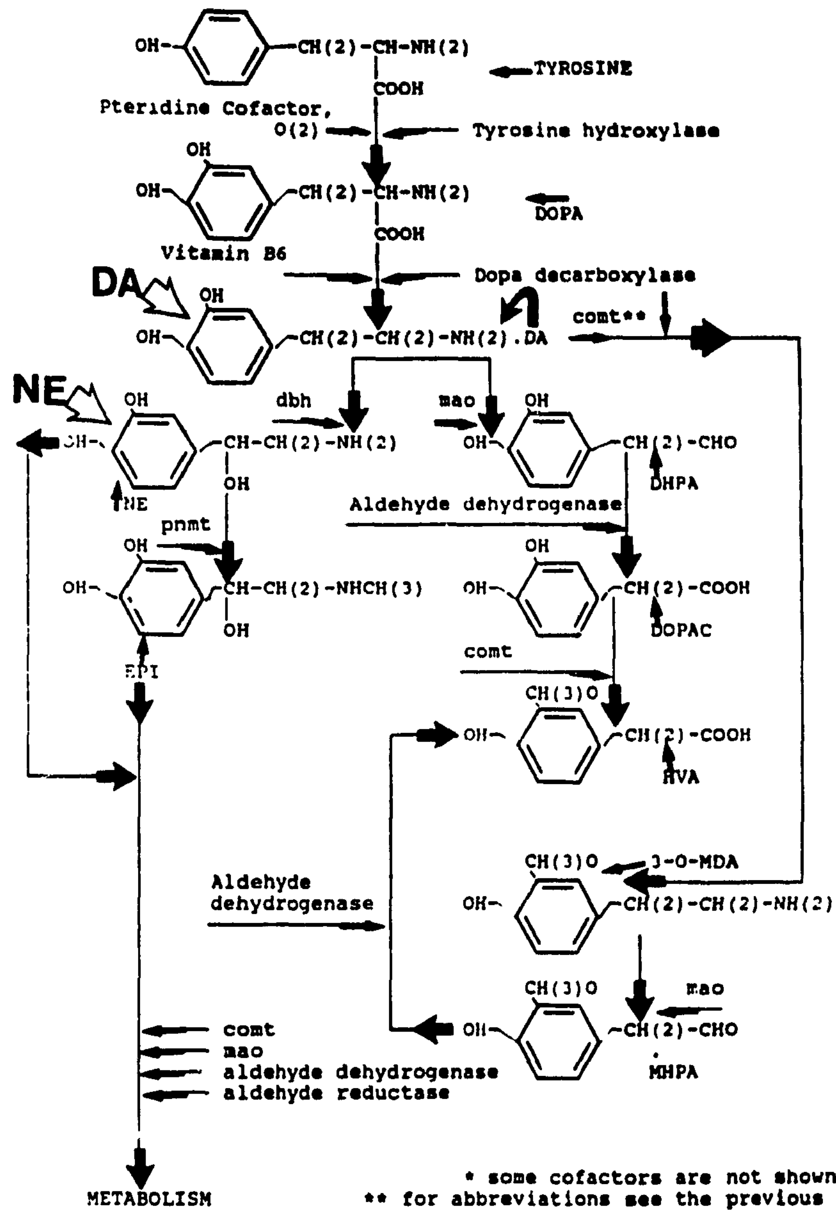
MHPA - 3-methoxy-4-hydroxyphenylacetaldehyde

NE - norepinephrine, (3,4-dihydroxyphenylethanol-amine)

3-O-MDA - 3-O-methyldopamine

pnmt - phenylethanolamine-N-methyltransferase

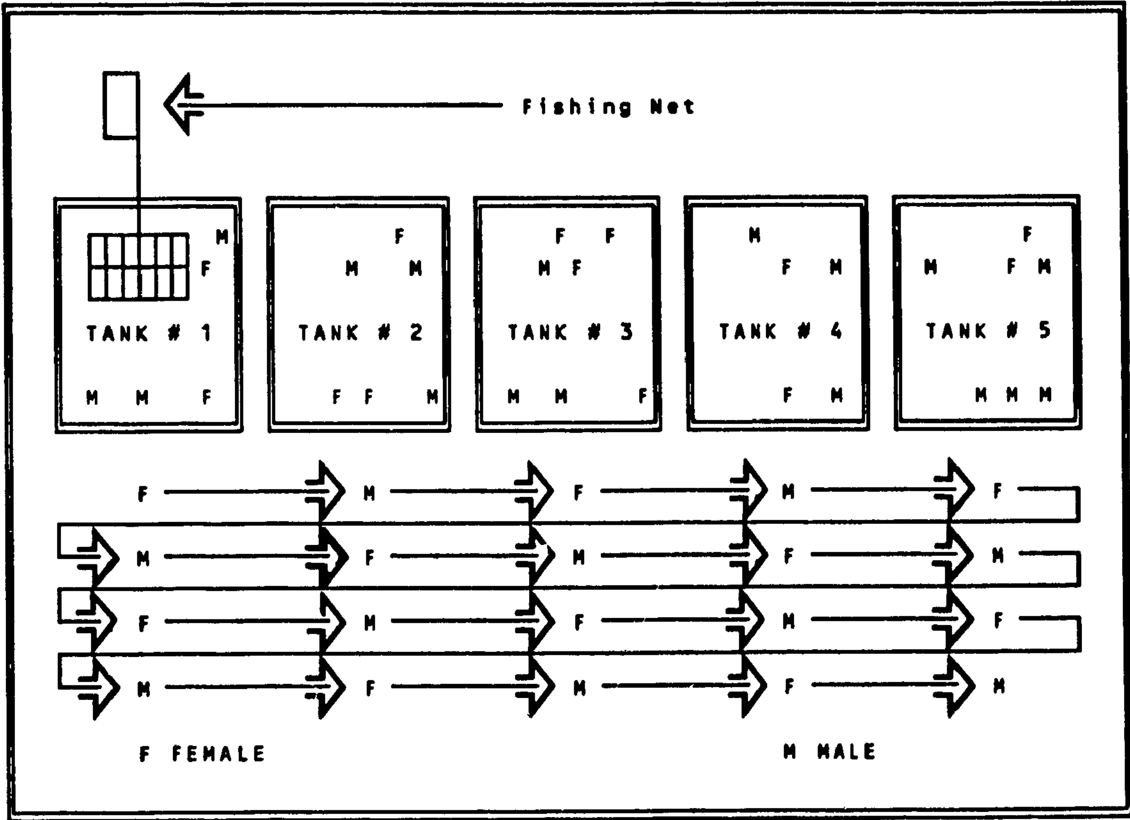
CATHECHOLAMINE SYNTHESIS AND CATABOLISM*



APPENDIX B

Systematic randomization method

Systematic randomization (Minium and Clarke, 1982) as applied to the present experiments. Serial for tanks, implies that one fish a time was removed form each and every tank, starting from tank 1 to tank 5. Alternate for sexes, implies that every second fish removed was of the same sex. The fishing nets were placed on the bottom of the tank and every third fish that entered the net was removed (only identical sex fish were counted). While randomization will not provide a truly random sample, the results obtained through this method are very close to the results that would be obtained using a truly random sample. In fact under laboratory conditions randomization is the only method of choice for obtaining a sample comparable to a truly random sample (for further details see Minium and Clarke, 1982).



Fishing Net
TANK # 1
TANK # 2
TANK # 3
TANK # 4
TANK # 5
F FEMALE
M MALE

APPENDIX C

Frequently used abbreviations

AAAD = aromatic amino acid decarboxylase
Ach = acetylcholine
AchE = acetylcholinesterase
Alk = alkalinity
ANOVA = univariate analysis of variance
ATP = adenosine triphosphate
BW = body weight
CA = cathecholamine
cm = centimeter
-CN = cyanide group (from a donor)
CN CONC = cyanide concentration
CNS = central nervous system
DA = dopamine
EDTA = (ethylenedinitrilo) tetraacetic acid
EPI = epinephrine
FF = flagfish
FTN = fenitrothion
g = gram
GTH = gonadotropin hormone
h = hour
HPLC = high pressure liquid chromatography
ip = intraperitoneal
IU = international units
kg = kilogram
L = liter
LD50 = median lethal dose
M = molar
MANOVA = multivariate analysis of variance
MAO = monoamine oxidase
max. = maximum
mg = miligram
min = minutes
min. = minimum
mL = mililiter
mm = milimeter
n = sample number
NE = norepinephrine
ng = nanogram
NRCC = National Research Council of Canada
NT = neurotransmitter
OP = organophosphorus
ppm = parts per milion
PVC = poly vinyl chloride
RPM = revolution per minute
RT = rainbow trout
SEM = standard error of the mean
SPSS = statistical package for social sciences
TEMP = temperature
TH = total hardness
TOC = total organic carbon
ug = microgram
uL = microliter
um = micrometer
V = volts
WISP = Waters Intelligent Sample Processor