Potential Effects of Tebufenozide on Feeding and Metabolism of Lake Trout (*Salvelinus namaycush*)

H. Volkoff, D. Hamoutene and J.F. Payne

Science Branch
Department of Fisheries and Oceans
P.O. Box 5667
St. John's NL Canada A1C 5X1

2007

Canadian Technical Report of Fisheries and Aquatic Sciences No. 2777

Canadian Technical Report of
Fisheries and Aquatic Sciences 2777

2007

POTENTIAL EFFECTS OF TEBUFENOZIDE ON FEEDING AND METABOLISM OF LAKE TROUT (*SALVELINUS NAMAYCUSH*)

by

H. Volkoff[1], D. Hamoutene[2] and J.F. Payne[2]

[1]Departments of Biology and Biochemistry
Memorial University of Newfoundland
St. John's NL Canada A1B 3X9

[2]Fisheries and Oceans Canada
Science Branch
P.O. Box 5667
St. John's NL A1C 5X1

Cat. No. Fs 97-6/2777E ISSN 0706-6457

Correct citation for this publication:

Volkoff, H., Hamoutene, D. and Payne, J.F. 2007. Potential effects of tebufenozide on feeding and metabolism of lake trout (*Salvelinus namaycush*). Can. Tech. Rep. Fish. Aquat. Sci. 2777: iv + 19 p.

ABSTRACT

Volkoff, H., Hamoutene, D. and Payne, J.F. 2007. Potential effects of tebufenozide on feeding and metabolism of lake trout (*Salvelinus namaycush*). Can. Tech. Rep. Fish. Aquat. Sci. 2777: iv + 19 p.

To assess the effects of the commercial pesticide tebufenozide (Mimic®) on feeding in lake trout, we cloned partial cDNAs encoding the central appetite-controlling factors: neuropeptide Y (NPY), cocaine and amphetamine regulated transcript (CART) and corticotropin-releasing factor (CRF). We examined their brain mRNA expression by semi-quantitative reverse transcription PCR. Levels of metabolites (triglycerides, lactate and proteins) and metabolic enzymes (lipase, aspartate aminotransferase, alanine aminotransferase and catalase) were also assessed in blood and/or tissues. Fish were exposed to one pulse of 0.25 ppm tebufenozide every 3 days for a total of 12 pulses, and sacrificed 4 days after the last pulse. Lake trout NPY, CART and CRF cDNAs showed high sequence identity to other teleost fish pro-peptides. NPY and CRF mRNAs, as well as blood metabolites and tissue enzyme activities were not affected by tebufenozide treatment. CART mRNA expression levels in both telencephalon and hypothalamus were significantly higher in fish exposed to tebufenozide than in control fish. Our results do not provide evidence of a strong effect of tebufenozide on lake trout metabolism. However, the tebufenozide-induced increase in CART expression and the known persistence of this pesticide in the environment calls for long-term studies on effects on fish feeding physiology.

RÉSUMÉ

Volkoff, H., Hamoutene, D. et Payne, J.F. 2007. Les effets du pesticide tebufenozide sur la physiologie alimentaire de la truite du lac (*Salvelinus namaycush*). Can. Tech. Rep. Fish. Aquat. Sci. 2777: iv + 19 p.

Dans le but d'elucider les effets du pesticide tebufenozide (Mimic®) sur la physiologie alimentaire de la truite du lac, nous avons cloné des ADNs complémentaires (cDNAs) pour des facteurs endocriniens centraux contrôlant l'appétit, le NPY (neuropeptide Y), le CART (cocaine and amphétamine regulated transcript) et le CRF (corticotropin-releasing factor). Nous avons examiné l'expression de leurs ARNs messagers dans le cerveau par transcription réverse-PCR semi-quantitative. Nous avons aussi mesuré les taux de métabolites (triglycérides, lactate et protéines) et d'enzymes métaboliques (lipase, aspartate aminotransferase, alanine aminotransferase et catalase) dans le sang et autres tissus. Les poissons ont été exposés à des pulses de 0.25 ppm de tebufenozide tous les trois jours, soit 12 pulses au total, puis sacrifiés 4 jours après le dernier pulse. Les séquences des cDNAS pour le CART, le NPY et le CRF de truite du lac présentent des identités de séquence élevées avec leurs homologues chez d'autres poissons téléostéens. L'expression des ARNs messagers de NPY et CRF dans le cerveau ainsi que les taux sanguins de métabolites et l'activité enzymatique dans les tissues ne sont pas affectés par le tebufenozide. Par contre, l'expression du CART dans le télencéphale et l'hypothalamus est plus élevée chez les poissons exposés au tebufenozide. Nos résultats montrent que le tebufenozide n'a pas d'effets majeurs sur le métabolisme de la truite du lac. Cependant, l'expression génique élevée du CART chez les poissons exposés et la persistance connue de ce pesticide dans le milieu naturel suggèrent la nécessité de futures études sur l'effet à long terme du tebufenozide sur la physiologie alimentaire des poissons.

INTRODUCTION

An increasing number of xenobiotics are released by industry and agriculture and entering aquatic environments, with potential long-term adverse effects on aquatic organisms (Livingstone 1998; Smital et al. 2004). Tebufenozide is a non-steroidal ecdysone agonist that causes premature and incomplete molting in Lepidopteran larvae and that is commonly used as a pesticide. Although very toxic to insects, tebufenozide has been suggested to be safe for land mammals and environmentally benign (Nakagawa 2005). However, nothing is known about the effects of tebufenozide on the physiology of aquatic vertebrates, including fish. Environmental chemicals, including polychlorinated hydrocarbons and pesticides, have previously been shown to affect a number of organs and physiological functions in fish, such as thyroid function (Brown et al. 2004), reproductive function (Jurgella et al. 2006), sensory and digestive organs (Hawkes 1980), oxidative stress and reactive oxygen species (ROS) production (Livingstone,2001), and liver metabolism and physiology (Wolf and Wolfe 2005; Rao 2006; Venkateswara Rao 2006).

In vertebrates, exposure to toxins can trigger behavioral changes known as "sickness behavior" characterized by reduced locomotion and reductions in food intake (Dantzer 2004). In rats, exposure to the pesticide lindane produces a decrease in food intake and body weight gain, 24 h after administration (Camon et al. 1988). In birds, neurotoxic pesticides lead to reduced food consumption (Walker 2003). In fish, exposure to bacterial substances (Volkoff and Peter 2004) or parasites (Chin et al. 2004) causes anorexia. In fish as in mammals, appetite is regulated by the brain, which produces factors that either stimulate (orexigenic) or inhibit (anorexigenic) food intake. Among these central factors, neuropeptide Y (NPY) is a potent appetite stimulator whereas cocaine and amphetamine regulated transcript (CART) and corticotropin -releasing factor (CRF) reduce appetite (Volkoff et al. 2005). In goldfish, treatment with lipopolysaccharide (LPS), a component of the cell wall of gram-negative bacteria commonly used to mimic inflammation, induces a decrease in brain NPY gene expression and an increase in both CART and CRF gene expression (Volkoff and Peter 2004). Xenobiotics have been shown to affect gene expression of these peptides in mammals (Fetissov et al. 2004) but their effects on fish are not known.

The present study was conducted to evaluate the effects of tebufenozide on the feeding physiology and metabolism of lake trout, a native species of Newfoundland and Labrador, by examining (1) the brain mRNA expression of NPY, CART and CRF and (2) levels of metabolites in control fish and fish treated with tebufenozide.

MATERIAL AND METHODS

ANIMALS AND SAMPLING PROCEDURE

Lake trout (*Salvelinus namaycush*) weighing 400-800 g were kept in a circulating system with filtered water and fed dry food pellets every other day. Fish were divided into two groups of 12 fish. One group was exposed to one pulse of Mimic® of 1ppm (0.25 ppm tebufenozide) every 3 days and to a total of 12 pulses. Water flow in both control and exposure tanks was maintained at 3.75 l/min. Doses were such that the theoretical concentrations of tebufenozide in the exposure tank would be 0.15, 0.09, 0.05, 0.03 ppm after 6, 12, 18 and 24 hours respectively. In order to verify these theoretical concentrations, water samples were collected 1, 4 and 24 hours after pulse and analyzed by an Ecology- accredited laboratory (ALS laboratory Group, Edmonton, Alberta) using method number 8151 GC/MS modified. The actual tebufenozide concentration values after obtained GC/MS analysis (0.19, 0.18, 0.051 ppm at 1, 4, 24 hours after pulse) were consistent with our predicted values.

Fork length and total weight were measured for every fish at the beginning of the experiment and on the day of sampling. Sampling was performed 4 days after the last pulse, in 2 consecutive days (6 control + 6 exposed per day). Fish were sacrificed with a blow on the head. Whole brains were dissected and stored at -20°C in RNA*later* (Qiagen) until RNA isolations were performed. Samples of liver and gill tissues were placed in vials and frozen in liquid nitrogen and then stored at –80°C for enzyme and protein assays. Blood samples were taken from fish with heparinized 10ml syringes.

GENE EXPRESSION STUDIES

Preparation of RNA

Total RNA was isolated using a Trizol/chloroform extraction method with Tri-Reagent (BioShop, Burlington, Ontario, Canada) following the manufacturers protocol. Final RNA concentrations were determined by spectrophotometric readings at 260 nm.

Cloning of lake trout NPY, CART, and elongation factor 1 alpha cDNAs

First, strand cDNA synthesis was performed using 2 micrograms of RNA from the brain reverse transcribed with:

- dT-AP (GGCCACGCGTCGACTAGTAC(T) 17)
- Superscipt II Reverse Transcriptase (Invitrogen).

Two rounds of PCR using degenerate sense and anti-sense primers corresponding to the conserved amino acid domains of known fish NPY, CART, and EF1α were used to amplify partial sequences of these peptides. Primers were designed from regions of high homology between several fish NPY (goldfish (Blomqvist et al. 1992), sea bass (Cerda-Reverter et al. 2000), rainbow trout (Doyon et al. 2003), cod (Kehoe and Volkoff, unpubl), and channel catfish (Leonard et al. 2001)) or between cod (Kehoe and Volkoff, unpubl), goldfish (Volkoff and Peter 2001) and zebrafish CART (GenBank accession no. **BQ480503**) or based on rainbow trout CRF (accession no. **AF296672**) or rainbow trout elongation factor alpha (accession no. **AF498320**).

The PCR products were separated on 1.5% agarose gels and visualized by ethidium bromide staining. Bands of expected size were excised from the gel and purified with the GenElute Gel Extraction Kit (Sigma, Canada), cloned using the pGEM-easy vector system (Promega, Madison, WI, USA) and sequenced at the Molecular Biology Facility of MacMaster University (Hamilton, Canada). Alignments and degrees of identity were obtained using the ClustalW software (http://www.ebi.ac.uk/clustalw/)

Expression of mRNAs for NPY, CART and CRF

Semi-quantitative RT-PCR was used to examine the expression of NPY, CART and CRF mRNAs in control and exposed fish. Brain tissue was collected for seven control fish and eight exposed fish. Fresh brain samples were immediately put in RNALater solution (Qiagen) and stored in -20° until use. Whole brains were subsequently dissected into specific brain regions including the olfactory tract, telencephalon, optic tectum, hypothalamus, and cerebellum/medulla. Total RNA was extracted from brain as described previously.

Levels of expression of NPY, CART and CRF mRNAs were first assessed by RT-PCR in different brain regions. Expression was then measured by RT-PCR, optimized for semiquantitative detection, using specific primer pairs and PCR conditions. Five micrograms of total RNA was reverse transcribed using dT-AP and SuperScript II reverse transcriptase (Invitrogen, Burlington, Ontario, Canada). A 30-cycle PCR amplification was performed with the specific primer sets NPY1-/NPY-2, CART-1/CART-2 or CRF 1/CRF2 (Table 1), and the PCR products were run on a 1.5% agarose gel. As an internal control, all cDNA samples were amplified with either primer set EF1α1/EF1α2, designed to amplify trout elongation factor 1 α (Table 1). Elongation factor 1 α was chosen as a reference gene, as its expression has recently been shown to be stable in salmonid fishes (Olsvik et al. 2005; Jorgensen et al. 2006). Negative controls were performed for each primer sets where cDNA was omitted from the PCR reactions. During PCR, one microgram each of first strand cDNA was used as template in all samples. Before performing PCRs with experimental samples,

PCR conditions were optimized in order to check the linearity of the PCR and to determine the optimal amount of cDNA to use for quantification. Based on the results of the linearity check, PCRs of 30 cycles were used for the amplification of NPY, CART and CRF cDNA. 25 cycles were used to amplify EF1α cDNA. The PCR products obtained for each primer pair were sequenced to confirm the target gene was being amplified. To compare the relative amounts of NPY, CART and CRF mRNA between groups, bands on the agarose gel were detected using an EpiChemi Darkroom (UVP, Upland, California, USA) and quantified using LabWorks software (Media Cybernetics, Silver Spring, Maryland, USA). Each sample was expressed as a ratio of NPY/ EF1α or CART/EF1α or CRF/EF1α. PCRs and gels were run in duplicates for all samples.

Table 1. Gene specific primers used for RT-PCR.

Primers	**Sequence (5'-3')**
NPY primers	
NPY-1	CGCAGTGACGCTCATCGTCGT
NPY-2	CTGTGCTCTCCTTCAGGAGC
CART primers	
CART-1	ATGGAGAGCTCCAGGCTATG
CART-2	CAGTTGCTTTTCGTTGGTCA
CRF primers	
CRF-1	GCTCATTGCTTTCTTACCGC
CRF-2	TTCATTTCCCGAAGATCTCC
EF-1 primers	
EF-1α-1	TGACAACGTTGGCTTCAACG
EF-1α-2	ACGGTCTGCCTCATGTCACG

METABOLIC AND ENZYMATIC ASSAYS

Serum analysis

Blood samples were collected into heparin-containing tubes via the tail vein and centrifuged. Serum samples were subsequently frozen until use. Measures of metabolites (proteins, triglycerides, lactate), as well as enzymatic assays (lipase, aspartate aminotransferase, alanine aminotransferase) were

performed on serum samples of both control fish and fish exposed to Mimic®. Analyses were carried out on a Beckman LX automated analyzer at the hematology/biochemistry laboratory of the General Hospital, St John's, Newfoundland. Cortisol levels were assessed using a Unicel Dxl800 Access® immunoassay analyzer with a Cortisol immunoassay kit (Beckman).

Protein levels and catalase activity in liver and gill samples

Tissue (liver and gill) samples were homogenized in 10 volumes of Tris-Buffer (0.05M Tris, pH 7.2). Homogenates were centrifuged at 9000g for 20 min. The supernatant (S9) was retained and stored at -80ºC for further analyses. Protein levels were subsequently measured according to Lowry's assay (Lowry et al. 1951). Catalase activity was measured as previously described (Aebi 1984) by measuring H_2O_2 decomposition at 240 nm.

STATISTICAL ANALYSIS

To compare levels of metabolites and metabolic enzymes and mRNA expression levels between different groups, Student t-tests were used. In gene expression studies, each sample was expressed as a ratio of NPY/EF1α or CART/EF1α or CRF/EF1α. All samples were then expressed as a percentage relative to the control group, which was set at 100%. Significance was considered at $p<0.05$. All data are expressed as mean ± SEM.

RESULTS

Body weight of fish

There were no significant differences in average body weight (BW) between control fish and exposed fish either before (initial BW or BWi) or after tebufenozide exposure (final BW or BWf) (Fig. 1). Within control and exposed fish groups, BWf and BWi were not significantly different.

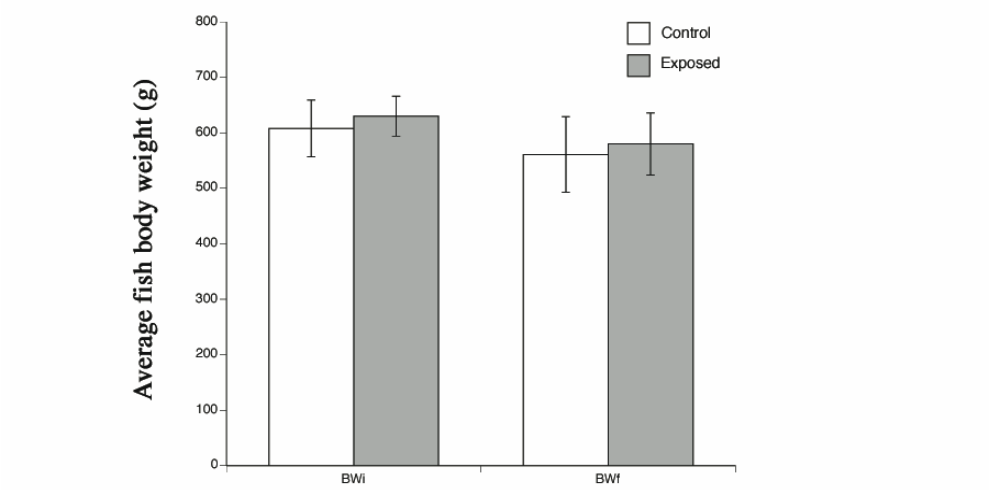


Figure.1. Average body weight of control and exposed lake trout at the start of the experiment (initial body weight BWi) and after exposure to tebufenozide (final body weight, BWf). Data are presented as mean ± SEM.

Changes in NPY, CART and CRF gene expression

Cloning and distribution of NPY, CART and CRF in lake trout brain

We have cloned partial cDNAs encoding for a 96 amino acid fragment of pro-NPY, for a 104 amino acid fragment of pro-CART, for a 155 amino acid fragment of pro-CRF and for a 128 amino acid fragment of EF1α (accessions nos. **DQ836924, DQ836925, DQ836926** and **DQ836928**, respectively). Lake trout NPY has a 60-93% degree of identity with other fish pro-NPY, the highest identity being with rainbow trout (Fig. 2). Lake trout CART has a 55-72% identity with other fish pro-CART, the highest identity being with goldfish CART I. Lake trout CRF has a 56-96-% degree of identity with other fish pro-CRF, the highest identity being with rainbow trout (Fig. 2).

Lake trout NPY was detected in telecephalon, hypothalamus and optic tectum but not in cerebellum (Fig. 3). Highest levels of NPY occur in the telencephalon. CART mRNA has a widespread distribution in the brain and is detected in telecephalon, hypothalamus, optic tectum and cerebellum areas, with highest levels in the telencephalon and hypothalamus (Fig. 3). CRF mRNA was detected in telecephalon, hypothalamus, optic tectum and cerebellum areas, with highest levels in the telencephalon (Fig. 3).

NPY, CART and CRF mRNA expression levels

Following our distribution studies, we examined changes in mRNA expression in forebrain regions in which mRNA was well-expressed, *i.e*. telencephalon and hypothalamus. In the telencephalon, both NPY and CRF

mRNA levels were similar in control fish and fish exposed to tebufenozide (Fig. 4A) whereas CART mRNA levels were higher in fish exposed to tebufenozide than in control fish. In the hypothalamus, CART mRNA levels were higher in fish exposed to tebufenozide than in control fish (Fig. 4B). Fish exposed to tebufenozide had hypothalamic CRF and NPY mRNA levels similar to that of control fish (data not shown).

A

```
Lake trout      -HPNLGRWLGAVTLIVWTCICIGTLAEGYPVKPENPGENAPAEELAKYYSALRHYINLIT
Goldfish        MHPNMKMWTGWAACAFLLFVCLGTLTEGYPTKPDNPGEGAPAEELAKYYSALRHYINLIT
Flounder        MHPNLVSWLGTLGLLLWALLCLSALTEGYPVKPENPGDDAPAEELAKYYSALRHYINLIT
Rainbow trout   MHPNLGTWLGAVTLLVWTFICIGTLAEGYPVKPENPGEDAPTEELAKYYSALRHYINLIT
Seabass         MHPNLVSWLGTLGFLLWALLCLGALTEGYPVKPENPGEDAPAEELAKYYSALRHYINLIT
Zebrafish       MNPNMKMWMSWAACAFLLFVCLGTLTEGYPTKPDNPGEDAPAEELAKYYSALRHYINLIT
                 :**:  * .     .   :*:.:*:****.**:***:.**:*****************

Lake trout      RQRYGKRSSPDTLDSLISELLLKESTDTLPQSRYDEP---
Goldfish        RQRYGKRSS---ADTLISDLLIGET-ESHPQTRYEDQLVW
Flounder        RQRYGKRSSPEILDTLVSELLLKESTDTLPQSRYDPSLW-
Rainbow trout   RQRYGKRSSPDTLDTLISELLLKESTDTLPQSRYDEPSLW
Seabass         RQRYGKRSSPEILDTLVSELLLKESTDQLPQSRYDPSLW-
Zebrafish       RQRYGKRSS---ADTLISDLLIGET-ESRPQTRYEDHLAW
                *********    *:*:*:**: *: :  **:**:
```

B

```
Lake trout      MESSRLWTRAVVCAVLLSIVLSAEIDYLDS--ELDLDTRSVRDFYPKDPNLTNEKQLLGA
Goldfish II     MESSRLKTRMAVCALLICLLTGAKANESEPEIEVELDARAIRDFYPKDPNLTSEKQLLGA
Goldfish I      MESSKLWTTAMACAVLVSCIQGAEMDFDN---ESDLETRALREFYPKDPNLTNEKQLLGA
Zebrafish       MESSSLRMRMAVCALLVCLLTGARANESEPEIEVELDTRAIRDFYPKDPNLNSEKQLLGA
                **** *      ** *                      .* * ********  *******

Lake trout      LHDVLKKLQTKRLPFWEKKFGQVPTCDVGEQCAVRKGARIGKLCDC--------------
Goldfish II     LQEVLEKLQTKRIPPWEKKFGQVPMCDLGEQCAIRKGSRIGKMCDCPRGAFCNFFLLKCL
Goldfish I      LHDVLEKLQSKRISLWEKKFGRVPTCDVGEQCAIRKGSRIGKMCDCPRGAFCNYFLLKCL
Zebrafish       LQEVLEKLQTKRIPPWEKKFGQVPMCDLGEQCAIRKGSRIGKMCDCPRGALCNFFLLKCL
                *::**::**:**:  ****:*:** **:**:**:***:****:***
```

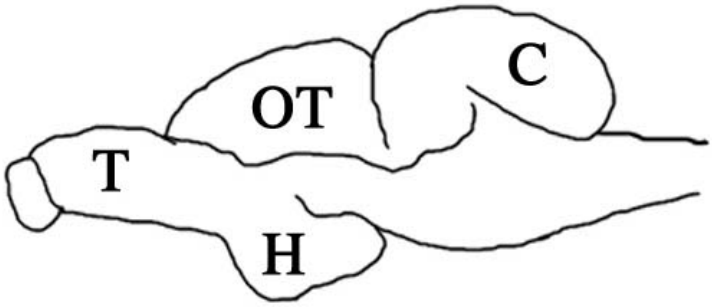
C

```
Lake trout      ------------LIAFLPRYECRAIESPGAVQRATAPHHDAQQQSLPLLTRQGEEYYIRL
Rainbow trout   MKLNFLVTTVVLLIAFLPRYECRAIESPGAVQRATAPHHDAQQQSLPLLTRHGEEYYIRL
Goldfish        MKLNFLVTTVALLVAFPPPYECRAIEGS-SNQPATDP--DGERQSPPVLARLGEEYFIRL
Catfish         MRLNFLVTTMALLVAFPPPYECRAIDSS-SNQPVTDP--DEERQSPAVLARMGEEYFIRL
                            *:** * ******:.. : * .* *  * ::** .:*:* ****:***

Lake trout      GNGNRNSAAPAPKGMYPEGSPAVYNRALQLQLTQRLLQGKVGNISRFVSGFANQLDDSME
Rainbow trout   GNGNRNSVASAPEGMYPEGSPAVYNRALQLQLTQRLLQGKVGNISRFVSGFANQLDDSIE
Goldfish        GNRNQNYLR-SPADSFPETS-QYSKRALQLQLTQRLLEGKVGNIGRLDGNYALRALDSVE
Catfish         GNRNKNSPR-SPPDTYPEAS-QYSKRALQLQLTQRVLEGKVGNVGRWDGNYALRALDSEE
                ** *:*    :* . :** *    :**********:*:*****:.*  ..:* :  ** *

Lake trout      RGRRSDDPPISLDLTFHMLRQMMEMSRAEQLQQQAHSNRKMMEIFGK
Rainbow trout   RGRRSDDPPISLDLTFHMLRQMMEMSRAEQLQQQAHSNRKMMEIFGK
Goldfish        RERRSEEPPISLDLTFHLLREVLEMARAEQMAQQAHSNRKMMEIFGK
Catfish         RERRSEEPPISLDLTFHLLREVLEMARAEQLVQQAHSNRKMMEIFGK
                * ***::**********:**:::**:****: ***************
```

Figure 2. Sequence alignments for NPY (A), CART (B) and CRF (C). Alignments and were obtained using the ClustalW software (http://www.ebi.ac.uk/clustalw/). Lake trout NPY (GenBank accession no. **DQ836924)** was compared to goldfish (Blomqvist et al. 1992), flounder (GenBank accession no. **AB055211**), rainbow trout (GenBank accession no. **AF203902**), sea bass (GenBank accession no. **AJ005381**) and zebrafish (GenBank accession no**. NM131074**) NPY. Lake trout CART (GenBank accession no. **DQ836925)** was compared to goldfish (GenBank accession nos. **AF288811** and **AF288810**) and zebrafish (GenBank accession no. **BQ480503**) CART. Lake trout CRF (GenBank accession no. **DQ836926)** was compared to rainbow trout (GenBank accession no. **AF296672**), goldfish (GenBank accession no. **AF098629**) or catfish CRF (GenBank accession no. **X58784**).



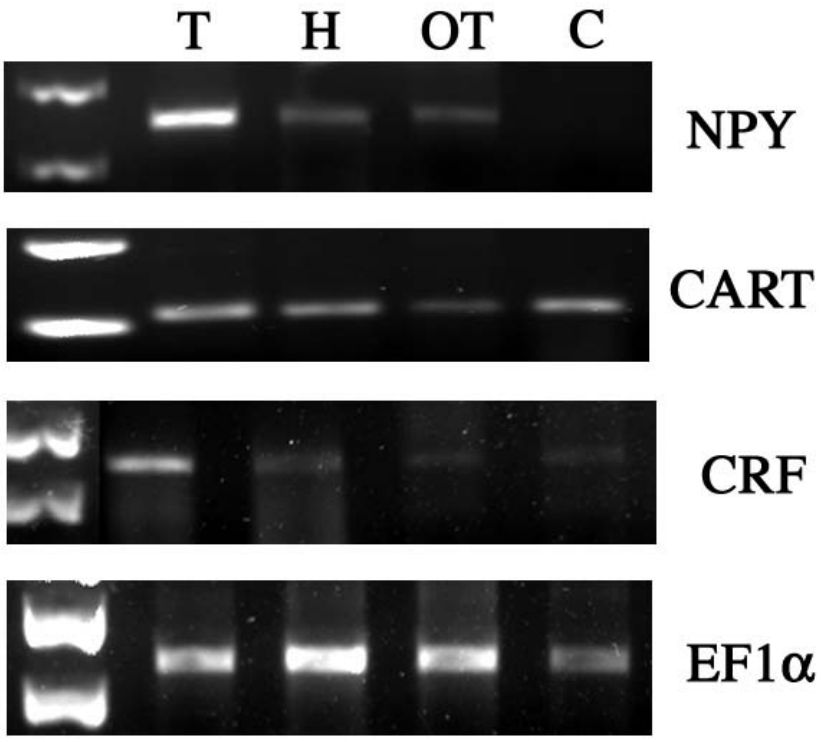


Figure 3. Localization of NPY, CART and CRF mRNA from major brain divisions. The brain divisions are shown in the trout brain diagram as telencephalon (T), hypothalamus (H), optic tectum (OT) and cerebellum (C). Elongation factor 1α (EF1α) was used as a reference gene. RT-PCR products were electrophoresed on a 1.5% agarose gel stained with ethidium bromide. The cDNA templates were amplified by PCR using primers pairs NPY-1/NPY-2, CART-1/CART-2, CRF-1/CRF-2 or EF1α-1/ EF1α-2.

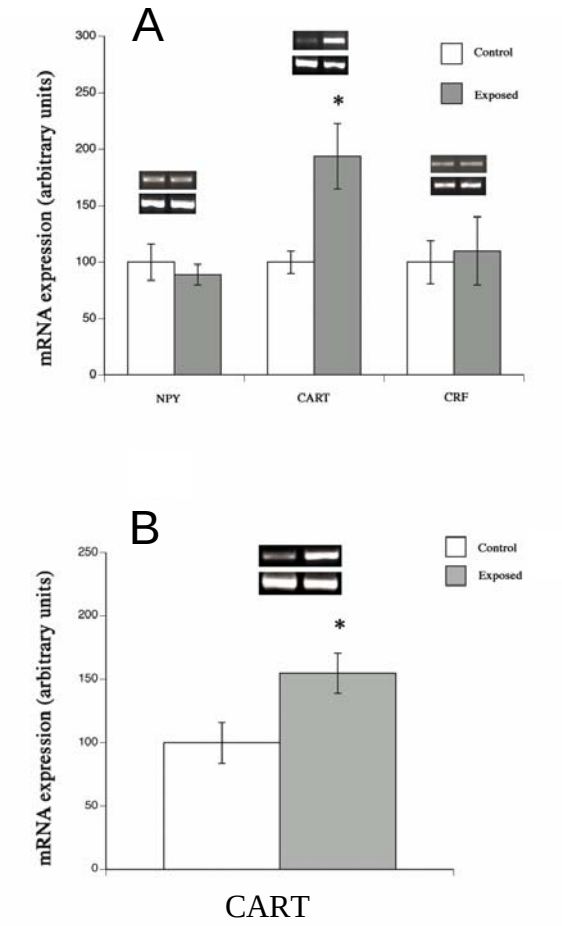


Figure 4. Changes of NPY, CART and CRF mRNA expression within the telencephalon (A) and in CART mRNA expression in the hypothalamus (B) in control fish (white bars) and fish exposed to tebufenozide (shaded bars). NPY, CART and CRF expression levels are expressed as a ratio between NPY, CART or CRF to EF1α. Values for exposed fish were normalized relative to the control group for each gene. Data are expressed as means ± SEM (n = 10-12 for all groups). Significant differences ($p < 0.05$) between groups were detected using a 1-way ANOVA. Exposed groups that differ significantly from control groups are indicated by a star. Insets show representative agarose gels of RT-PCR products for control (left) and exposed (right) fish (upper panel NPY or CART or CRF and lower panel EF1α).

Serum levels of metabolites, cortisol and metabolic enzymes

Serum levels of cortisol, protein, triglycerides, lactate, aspartate aminotransferase, alanine aminotransferase and lipase were not significantly different in control fish and fish exposed to tebufenozide (Table 2).

Table 2. Serum levels of metabolites (protein, triglycerides, lactate), metabolic enzymes (aspartate aminotransferase, alanine aminotransferase, lipase) and cortisol in control fish and fish exposed to tebufenozide. Data are presented as mean ± SEM (n). T-tests were performed on all data. No significant differences were observed between control and exposed fish (n=12 cont, 12 exposed).

Assay	**Control**	**Exposed**
Protein (g/l)	22 ± 2.9 (12)	21 ± 5.3 (12)
Triglycerides (mmol/l)	0.95 ± 0.27 (12)	1.08 ± 0.28 (12)
Lactate (mmol/l)	2.85 ± 1.07 (12)	3.25 ± 1.43 (12)
AST (U/L)	306.9 ± 103.3 (12)	409.417 ± 3.2 (12)
ALT (U/L)	8.4 ± 6.8 (12)	13.8 ± 17.6 (12)
Lipase (U/L)	14.5 ± 1.31(12)	14.25 ± 2.05 (12)
Cortisol (nmol/l)	343.23 ± 46.59 (12)	217.73 ± 45.34 (12)
Cortisol/protein (nmol/g)	15.62 ± 6.41 (12)	6.99 ± 2.21 (12)

Protein and catalase levels in liver and gills

Soluble protein levels in liver (L) and in gills (G) as well as catalase levels in liver (L) were similar in control fish and fish exposed to tebufenozide (Table 3).

Table 3. Soluble protein levels in liver (L) and in gills (G) and catalase levels in liver (L) of control fish and fish exposed to tebufenozide. Data are presented as mean ± SEM (n). T-tests were performed on all data- No significant differences were observed between control and exposed fish.

Assay	**Control**	**Exposed**
Protein L (mg/l)	3.08 ± 0.33 (9)	3.13 ± 0.22 (10)
Protein G (mg/l)	0.66 ± 0.19 (9)	0.72 ± 0.15 (10)
Catalase L (U/mg protein)	1740.52 ± 349.82 (9)	1615.92 ± 294.62 (10)

DISCUSSION

This study investigated the potential effects of short-term exposure to tebufenozide on feeding and metabolism of lake trout. In fish, exposure to environmental contaminants (Bowen et al. 2006) or infections (Chin et al. 2004; Damsgard et al. 2004) often induces anorexia and a decrease in body weight. In our study, tebufenozide treatment did not appear to affect feeding in lake trout. Although we did not quantify food intake, no apparent differences in feeding behavior were seen between control and exposed fish. This is consistent with similar average body weight seen in the two groups following the exposure period.

In order to assess the effects of tebufenozide on feeding-regulating peptides at the molecular level, we conducted gene expression studies. We cloned partial cDNAs encoding for NPY, CART and CRF in lake trout and assessed possible changes in the gene expression of these peptides. Our cloning results show that the amino acid sequences of these three peptides show a high degree of identity (55-96%) with their counterpart in other teleost fish. NPY and CRF mRNA levels in the telencephalon were not affected by tebufenozide treatment whereas CART mRNA levels were increased in both the telencephalon and the hypothalamus of exposed fish. Little data is available regarding the effects of pesticides on gene expression in fish. In European flounder exposed to a cocktail of herbicides, a number of genes related to energy production and general metabolism are differentially expressed in liver, muscle, and gills (Marchand et al. 2006). However, no data is available on the effects of pesticides on brain feeding-related peptides.

Previous studies show that immune challenges can affect appetite and that this effect might be mediated by CART, as administration of lipopolysaccharide (LPS) results in anorexia and in an increase in CART brain mRNA in both mammals (Sergeyev et al. 2001) and goldfish (Volkoff and Peter 2004). As part of this project, we also examined the effects of tebufenozide on lake trout immune response (Hamoutene et al. 2008). Our results show that tebufenozide has an immunostimulating effect. This work may suggest a link between tebufenozide-induced immunostimulation and the observed increase in CART found in exposed fish. On the other hand, our results show that neither NPY nor CRF mRNA expression is affected by tebufenozide. In goldfish, an immune challenge produced by LPS treatment, induces decreases in both NPY and CRF brain gene expression (Volkoff and Peter 2004) and CRF contents in the brain (Pepels et al. 2004). Similarly, carp infected with the protozoan *Trypanoplasma borreli* show a decrease in expression of CRF-binding protein and CRF receptor-1 genes in the gills and in the skin (Mazon et al. 2006).

Our results show that serum levels of protein and lactate, as well as protein levels in liver and gills were not significantly different between control fish

and fish exposed to tebufenozide, suggesting that the overall carbohydrate and protein metabolism of trout are not affected by the pesticide. In contrast to our results, previous studies have shown that protein levels are affected by pesticide treatments in fish. In the gobiid fish, *Glossogobius giuris*, exposed to sublethal concentrations (0.5 ppm) of the pesticide malathion for 96 hours, levels of protein decrease significantly, suggesting a metabolic dysfunction in response to pesticides (Venkataramana et al. 2006). Both Spotted snakehead, *Channa punctatus* (Agrahari et al. 2006) and carp, *C. carpio* (Oruc et al. 2006), display decreased protein levels in several tissues including gills, liver, and muscles after exposure to the insecticide monocrotophos and the organophosphate pesticide Diazinon, respectively. Pesticides have also been shown to affect fish carbohydrate metabolism. Indian catfish (*Clarias batrachus*) exposed to the pesticides such as thiotox, dichlorvos (Verma et al. 1983); or rogor (Begum and Vijayaraghavan, 1999) have increased blood glucose and lactate levels and lower liver and muscle glycogen levels. Carp exposed to pyrethroids show increased lactate dehydrogenase (LDH) activity and lactic acid levels in blood, muscle and liver, as well as decreased muscle and liver glycogen levels (Kamalaveni et al. 2003). In this study, tebufenozide treatment at doses used did not appear to affect protein or carbohydrate metabolism, as protein and lactate levels were not affected.

Our results indicate that tebufenozide has no effect on serum lipid levels or lipase activity in lake trout. Very few studies are available on the effects of contaminants on lipid/triglyceride levels in fish. In white sturgeon (*Acipenser transmontanus*) exposed to contaminant, including pesticides, triglyceride levels are low (Feist et al. 2005). In contrast, in spotted snakehead (*Channa punctatus*), lipid levels increase during exposure to the pesticide monocrotophos, a decrease that might be due to inhibition of lipase activity (Agrahari et al. 2006).

Serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT) were not affected by tebufenozide exposure. AST and ALT are liver specific enzymes that are involved in amino acid catabolism and that are sensitive to hepatotoxicity and histopathology (Tremlett and Oger 2004; Giannini et al. 2005). Blood levels of AST and ALT are indicators of tissue damage. In fish as in mammals, when an organ is damaged, additional AST is released into the bloodstream and increases in AST and ALT might indicate tissue damage in liver, kidney or gill (Oluah 1999). Our results suggest that tebufenozide at moderate doses does not stimulate amino acid catabolism in fish tissues.

Tebufenozide did not affect catalase activity in fish liver. Catalases (CATs) are hematin-containing enzymes that facilitate the removal of hydrogen peroxide (H_2O_2), which is metabolized to oxygen (O_2) and water (Droge 2002). Oxidative stress is an imbalance between the production of reactive oxygen species (ROS), which are atoms or molecules that contain unpaired electrons and form as a natural byproduct of the normal metabolism of oxygen. Many xenobiotics,

such as pesticides, may cause oxidative stress leading to the generation of ROS, such as hydrogen peroxide (H_2O_2) that might cause lipid peroxidation (LPO), DNA damage and protein oxidation, resulting in oxidative stress (Livingstone, 2001; Abdollahi et al. 2004). Changes in catalase activity have been observed in a number of studies using fish exposed to pollutants and pesticides. For example, catalase activity decreases after exposure to pesticides in gills of gilthead seabream *Sparus aurata* (Rosety et al. 2005), in kidney and liver of spotted snakehead (Sayeed et al. 2003), carp and Nile tilapia (Uner et al. 2006). In our study, fish treated with tebufenozide showed no effect on liver catalase activities.

Cortisol is a glucocorticosteroid hormone secreted by interrenal steroidogenic cells in response to pituitary adrenocorticotropin releasing hormone (ACTH), itself controlled by CRF (Bernier 2006). Cortisol plays major physiological roles, including the regulation of hydromineral balance and energy metabolism. Plasma cortisol levels are an indicator of stress in fish (Wendelaar Bonga 1997; Bernier 2006) and fish chronically exposed to pollutants have been shown to exhibit a deficiency in the synthesis of cortisol (Leblond and Hontela 1999). In our study, cortisol levels were not affected by tebufenozide treatment, suggesting that the hypothalamic-pituitary-interrenal (HPI) stress axis was not sensitive to our treatment. Similar cortisol levels between control and exposed fish are consistent with the absence of change in CRF mRNA expression between the two groups.

Fish were exposed to a starting dose (pulse) of 0.25 ppm of tebufenozide; 0.25 ppm is lower than the solubility of tebufenozide (0.83 ppm at 20 ºC) so most of the compound should be dissolved in water. Experiments conducted in water enclosures (worst-case scenarios) showed that 393 days after adding 0.26 ppm of product to water, tebufenozide concentration was still detectable and equal to 0.012 ppm (Sundaram 1997). The initial amount of tebufenozide used in our study is higher than expected values in the field after a spray but potential contamination of streams close to agricultural areas can result in higher concentrations. Our results suggest that exposure to tebufenozide induces little metabolic changes in lake trout. All major pathways of carbohydrate, amino acid, lipid and lactate metabolism do not appear to be significantly altered by tebufenozide treatment. Tebufenozide persistence and bioconcentration (Sundaram 1997) and the discovery that CART can be influenced by exposure to Mimic® seems to warrant further studies on potential effect of this pesticide on fish physiology.

ACKNOWLEDGMENTS

We thank Vincent Soriano for his invaluable help in the processing and analysis of samples. This work was funded by the National Pesticide Fund of Canada and supported in part by a Natural Sciences and Engineering Research Council of Canada (NSERC) Discovery grant to Helene Volkoff.

REFERENCES

Abdollahi, M., Ranjbar, A., Shadnia, S., Nikfar, S., and Rezaiee, A. 2004. Pesticides and oxidative stress: a review. Med. Sci. Monit. 10: RA141-714.

Aebi, H. 1984. Catalase *in vitro*. Methods Enzymol. 105: 121-126.

Agrahari, S., Gopal, K., and Pandley, K.C. 2006. Biomarkers of monocrotophos in a freshwater fish *Channa punctatus* (Bloch). J. Environ. Biol. 27: 453-457.

Begum, G., Vijayaraghavan, S. 1999. Effect of acute exposure of the organophosphate insecticide Rogor on some biochemical aspects of *Clarias batrachus* (Linnaeus). Environ. Res. 80: 80-83.

Bernier, N. J. 2006. The corticotropin-releasing factor system as a mediator of the appetite-suppressing effects of stress in fish. Gen. Comp. Endocrinol. 146: 45-55.

Blomqvist, A.G., Soderberg, C., Lundell, I., Milner, R.J., and Larhammar, D. 1992. Strong evolutionary conservation of neuropeptide Y: sequences of chicken, goldfish, and *Torpedo marmorata* DNA clones. Proc. Natl. Acad. Sci. U.S.A. 89: 2350-2354.

Bowen, L., Werner, I., and Johnson, M.L. 2006. Physiological and behavioral effects of zinc and temperature on coho salmon (*Oncorhynchus kisutch*). Hydrobiologia 559: 161-168.

Brown, S.B., Adams, B.A., Cyr, D.G., and Eales, J.G. 2004. Contaminant effects on the teleost fish thyroid. Environ. Toxicol. Chem. 23: 1680-1701.

Camon, L., Martinez, E., Artigas, F., Sola, C., and Rodriguez-Farre, E. 1988. The ffect of non-convulsant doses of lindane on temperature and body weight. Toxicology 49: 389-394.

Cerda-Reverter, J.M., Anglade, I., Martinez-Rodriguez, G., Mazurais, D., Munoz-Cueto, J.A., Carrillo, M., Kah, O., and Zanuy, S. 2000. Characterization of neuropeptide Y expression in the brain of a perciform fish, the sea bass (*Dicentrarchus labrax*). J. Chem. Neuroanat. 19: 197-210.

Chin, A., Guo, F.C., Bernier, N.J., and Woo, P.T. 2004. Effect of *Cryptobia salmositica*-induced anorexia on feeding behavior and immune response in juvenile rainbow trout *Oncorhynchus mykiss*. Dis. Aquat. Organ. 58: 17-26.

Damsgard, B., Sorum, U., Ugelstad, I., Eliassen, R. A., and Mortensen, A. 2004. Effects of feeding regime on susceptibility of Atlantic salmon (*Salmo salar*) to cold water vibriosis. Aquaculture 239: 37-46.

Dantzer, R. 2004. Cytokine-induced sickness behaviour: a neuroimmune response to activation of innate immunity. Eur. J. Pharmacol. 500: 399-411.

Doyon, C., Gilmour, K.M., Trudeau, V.L., and Moon, T.W. 2003. Corticotropin-releasing factor and neuropeptide Y mRNA levels are elevated in the preoptic area of socially subordinate rainbow trout. Gen. Comp. Endocrinol. 133: 260-271.

Droge, W. 2002. Free radicals in the physiological control of cell function. Physiol Rev 82: 47-95.

Feist, G.W., Webb, M.A., Gundersen, D.T., Foster, E.P., Schreck, C.B., Maule, A.G., and Fitzpatrick, M.S. 2005. Evidence of detrimental effects of environmental contaminants on growth and reproductive physiology of white sturgeon in impounded areas of the Columbia River. Environ. Health Perspect. 113: 1675-1682.

Fetissov, S.O., Huang, P., Zhang, Q., Mimura, J., Fujii-Kuriyama, Y., Rannug, A., Hokfelt, T., and Ceccatelli, S. 2004. Expression of hypothalamic neuropeptides after acute TCDD treatment and distribution of Ah receptor repressor. Regul. Pept. 119: 113-124.

Giannini, E.G., Testa, R., and Savarino, V. 2005. Liver enzyme alteration: a guide for clinicians. CMAJ 172: 367-379.

Hamoutene, D., Payne, J.F. and Volkoff, H. 2008. Effects of Tebufenozide on some aspects of lake trout (*Salvelinus namaycush*) Immune Response. Ecotoxicol. Envir. Saf. 69: 173-179.

Hawkes, J.W. 1980. The effects of xenobiotics on fish tissues: morphological studies. Fed. Proc. 39: 3230-3236.

Jorgensen, S.M., Kleveland, E.J., Grimholt, U., and Gjoen, T. 2006. Validation of Reference Genes for Real-Time Polymerase Chain Reaction Studies in Atlantic Salmon. Mar. Biotechnol. (NY) 8(4): 398-408.

Jurgella, G.F., Marwah, A., Malison, J.A., Peterson, R., and Barry, T.P. 2006. Effects of xenobiotics and steroids on renal and hepatic estrogen metabolism in lake trout. Gen. Comp. Endocrinol. 148(2): 273-81.

Kamalaveni, K., Gopal, V., Sampson, U., and Aruna, D. 2003. Recycling and utilization of metabolic wastes for energy production is an index of biochemical

adaptation of fish under environmental pollution stress. Environ. Monit. Assess 86: 255-264.

Leblond, V. S., and Hontela, A. 1999. Effects of in vitro exposures to cadmium, mercury, zinc, and 1-(2-chlorophenyl)-1-(4-chlorophenyl)-2,2-dichloroethane on steroidogenesis by dispersed interrenal cells of rainbow trout (*Oncorhynchus mykiss*). Toxicol. Appl. Pharmacol. 157: 16-22.

Leonard, J.B., Waldbieser, G.C., and Silverstein, J.T. 2001. Neuropeptide Y sequence and messenger RNA distribution in channel catfish (*Ictalurus punctatus*). Mar. Biotechnol. (NY) 3: 111-118.

Livingstone, D.R. 1998. The fate of organic xenobiotics in aquatic ecosystems: quantitative and qualitative differences in biotransformation by invertebrates and fish. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 120: 43-49.

Livingstone, D.R. 2001. Contaminant-stimulated reactive oxygen species production and oxidative damage in aquatic organisms. Mar. Pollut. Bull. 42: 656-666.

Lowry, O.H., Rosenborough, N.J., Farr, A.L., and Randal, R.J. 1951. Protein measurements with the Folin-phenol reagent. J. Biol. Chem. 193: 265-275.

Marchand, J., Tanguy, A., Charrier, G., Quiniou, L., Plee-Gauthier, E., and Laroche, J. 2006. Molecular identification and expression of differentially regulated genes of the european flounder, *Platichthys flesus*, submitted to pesticide exposure. Mar. Biotechnol. (NY) 8: 275-294.

Mazon, A.F., Verburg-van Kemenade, B.M., Flik, G., and Huising, M.O. 2006. Corticotropin-releasing hormone-receptor 1 (CRH-R1) and CRH-binding protein (CRH-BP) are expressed in the gills and skin of common carp *Cyprinus carpio* L. and respond to acute stress and infection. J. Exp. Biol. 209: 510-517.

Nakagawa, Y. 2005. Nonsteroidal ecdysone agonists. Vitam Horm 73: 131-173.

Olsvik, P.A., Lie, K.K., Jordal, A.E., Nilsen, T.O., and Hordvik, I. 2005. Evaluation of potential reference genes in real-time RT-PCR studies of Atlantic salmon. BMC Mol. Biol. 6: 21.

Oluah, N. S. 1999. Plasma aspartate aminotransferase activity in the catfish *Clarias albopunctatus* exposed to sublethal zinc and mercury. Bull. Environ. Contam. Toxicol. 63: 343-349.

Oruc, E.O., Uner, N., Sevgiler, Y., Usta, D., and Durmaz, H. 2006. Sublethal effects of organophosphate diazinon on the brain of *Cyprinus carpio.* Drug Chem. Toxicol. 29: 57-67.

Pepels, P.P., Bonga, S.E., and Balm, P.H. 2004. Bacterial lipopolysaccharide (LPS) modulates corticotropin-releasing hormone (CRH) content and release in the brain of juvenile and adult tilapia (*Oreochromis mossambicus*; Teleostei). J. Exp. Biol. 207: 4479-4488.

Rao, J.V. 2006. Biochemical alterations in euryhaline fish, *Oreochromis mossambicus* exposed to sub-lethal concentrations of an organophosphorus insecticide, monocrotophos. Chemosphere 65: 1814-1820.

Rosety, M., Rosety-Rodriguez, M., Ordonez, and F.J., Rosety, I. 2005. Time course variations of antioxidant enzyme activities and histopathology of gilthead seabream gills exposed to malathion. Histol. Histopathol. 20: 1017-1020.

Sayeed, I., Parvez, S., Pandey, S., Bin-Hafeez, B., Haque, R., and Raisuddin, S. 2003. Oxidative stress biomarkers of exposure to deltamethrin in freshwater fish, *Channa punctatus* Bloch. Ecotoxicol. Environ. Saf. 56: 295-301.

Sergeyev, V., Broberger, C., and Hokfelt, T. 2001. Effect of LPS administration on the expression of POMC, NPY, galanin, CART and MCH mRNAs in the rat hypothalamus. Mol. Brain Res. 90: 93-100.

Smital, T., Luckenbach, T., Sauerborn, R., Hamdoun, A. M., Vega, R. L., and Epel, D. 2004. Emerging contaminants--pesticides, PPCPs, microbial degradation products and natural substances as inhibitors of multixenobiotic defense in aquatic organisms. Mutat. Res. 552: 101-117.

Sundaram, K.M.S. 1997. Persistence of Tebufenozide in aquatic ecosystems under laboratory and field conditions. Pesticide Sci. 51: 7-20.

Tremlett, H., and Oger, J. 2004. Hepatic injury, liver monitoring and the beta-interferons for multiple sclerosis. J. Neurol. 251: 1297-1303.

Uner, N., Oruc, E., and Sevgiler, Y. 2006. Neurotoxicity evaluation of the organofluorine pesticide etoxazole in the brain of *Oreochromis niloticus*. Drugs Chem.Toxicol. 29: 157-165.

Venkataramana, G.V., Rani, P.N.S., and Murthy, P.S. 2006. Impact of malathion on the biochemical parameters of gobiid fish, *Glossogobius giuris* (Ham). J. Envir. Biol. 27(1): 119-122.

Venkateswara Rao, J. 2006. Sublethal effects of an organophosphorus insecticide (RPR-II) on biochemical parameters of tilapia, *Oreochromis mossambicus*. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 143: 492-498.

Verma, S. R., Rani, S., Tonk, I. P., and Dalela, R. C. 1983. Pesticide-induced dysfunction in carbohydrate metabolism in three freshwater fishes. Environ. Res. 32: 127-133.

Volkoff, H., Peter, R.E. 2001. Characterization of two forms of cocaine- and amphetamine-regulated transcript (CART) peptide precursors in goldfish: molecular cloning and distribution, modulation of expression by nutritional status, and interactions with leptin. Endocrinology 142: 5076-5088.

Volkoff, H., and Peter, R.E. 2004. Effects of lipopolysaccharide treatment on feeding of goldfish: role of appetite-regulating peptides. Brain Res. 998: 139-147.

Volkoff, H., Canosa, L.F., Unniappan, S., Cerda-Reverter, J.M., Bernier, N.J., Kelly, S.P., and Peter, R.E. 2005. Neuropeptides and the control of food intake in fish. Gen. Comp. Endocrinol. 142: 3-19.

Walker, C.H. 2003. Neurotoxic pesticides and behavioural effects upon birds. Ecotoxicology 12: 307-316.

Wendelaar Bonga, S.E. 1997. The stress response in fish. Physiological Reviews 77: 591-625.

Wolf, J.C., and Wolfe, M.J. 2005. A brief overview of nonneoplastic hepatic toxicity in fish. Toxicol. Pathol. 33: 75-85.