AN ABSTRACT OF THE THESIS OF

Kelly P. Hall for the degree of Bachelor of Science in Bioresource Research, Toxicology Option presented on May 26, 2000. Title: Indole-3-Carbinol and Related Compound Effects on Biochemical Parameters with Respect to Trout Tumorigenesis

Abstract approved: David E. Williams

David E. Williams

Indole-3-carbinol (I3C), a metabolite of glucobrassicin, is found in cruciferous vegetables. It is able to act as an inhibitor or promoter of carcinogenesis depending on the species, the carcinogen and when it is given in relation to the carcinogen. This is probably due to the fact that it reacts in an acid environment, such as the stomach, to form acid condensation products. Two of the many condensation products are Indolo(3,2-b)carbazole (ICZ) and 3,3'-diindolylmethane (I33'). ICZ is a strong agonist for the *A*ryl *h*ydrocarbon *R*eceptor (AhR), but is present to a lesser extent in the reaction mix compared to other acid condensation products. I33' is the major condensation product of I3C, but is a weak AhR agonist. In this study, I3C, ICZ, I33' and Indolplex, a formulation of I33', were compared as to Vitellogenin (Vg) and Cytochrome1A P_{450} (CYP1A) induction as well as AFB1-initiated tumor promotion in rainbow trout. The initiated trout received 12 ppm AFB1 as fry, which is expected to result in a 10 to 15 percent increase in liver tumors. Vg is an egg yolk precursor is a biomarker for estrogenicity. The CYP1A gene belongs to a gene super-family that function in xenobiotic transformation, and its transcription is regulated by the AhR. Initially, trout were treated with 2000 ppm I3C, which was later lowered to 1000 ppm due to trout mortality. The treatment doses of ICZ and I33' coresponded to the percentage found in the normal reaction mix. ICZ treatments contained 0.3 ppm and 3 ppm. The I33' concentrations used were 400 ppm and 1200 ppm. Indolplex™,

used to treat Recurrent Respiratory Papillomatosis (RRP), contained 1200 ppm. Additionally, a comparison of Vg and CYP1A induction was done between this study and another that included compounds known to be tumor promoters. The other study included promoters believed to act through oxidative stress (hydrogen peroxide (H_20_2), t-butyl peroxide and low choline diet), endocrine modulation (β-estradiol (E2), dehydroepiandrosterone (DHEA), perfluorooctanoic acid (PFOA) and through the AhR (β-naphthoflavone (BNF). These treatments were applied for 2 weeks. I3C, ICZ and I33' at the doses used induced Vg to about the same degree after 4 months of treatment in trout. I3C induced CYP 1A more than ICZ or I33'. Both ICZ and I33' seemed to have suppressor characteristics at the higher doses in relation to CYP1A induction. Vg and CYP1A induction did not correlate with tumor induction. Mortality was high throughout the experiment. After a year of treatment, tumor incidence was extremely high. In controls that were not treated with AFB_1, there was an incidence of liver tumors of 8 percent. The normal incidence is 0.1 percent. Indolplex had the highest incidence of tumors, but was also the treatment with the highest survival of fish. More research needs to be done in relation to I3C and its acid condensation products. Also the cause of the high mortality and tumor rates needs to be found.

INDOLE-3-CARBINOL AND RELATED COMPOUND EFFECTS ON BIOCHEMICAL PARAMETERS WITH RESPECT TO TROUT TUMORIGENESIS

by

Kelly P. Hall

A Thesis Submitted

to

Oregon State University

In Partial Fulfillment of
the requirements for the
degree of

Bachelor of Science

Presented May 26, 2000
Commencement June 2000

Bachelor of Science thesis of **Kelly P. Hall** presented on **May 26, 2000**

APPROVED:

David E. Williams

Mentor

George A Darling

Secondary Mentor

Anita Nina Azarenko

Director of Bioresource Research Degree

I understand that my thesis will become apart of the permanent collection of The BioResource Research Program. My signature below authorizes release of my theses to any reader upon request.

Kelly P Hall

Kelly P. Hall, Author

Acknowledgement

I would like to thank Dr. Williams and Dr. Bailey for giving me the opportunity to pursue this degree. Adam Shilling was a great help in learning lab techniques. I could not have accomplished my degree without the generous assistance of Dr. Gayle Orner. I would like to thank Dr. Hendricks and the FTNL staff for help in the tumor study. And thank you to Wanda Crannell, the best advisor at OSU. And to Dr. Azarenko, best degree director a person could have. I also could not have done this without my daughter Laramie French, who at five years old had the patience and understanding of an adult.

TABLE OF CONTENTS

LIST OF FIGURES

INDOLE-3-CARBINOL AND RELATED COMPOUND EFFECTS ON BIOCHEMICAL PARAMETERS WITH RESPECT TO TROUT TUMORIGENESIS

INTRODUCTION

The Dietary Supplement Health and Education Act of 1994 (DSHEA) exempts products (other than tobacco) that are intended to supplement the diet from regulation by the Food and Drug Administration. The labeling includes the disclaimer, "This product is not intended to diagnose, treat, cure or prevent any disease."[1,2] This statement stresses the need for stricter tests and regulations on dietary supplements.

Indole-3-carbinol (I3C) is naturally found in broccoli, cabbage and other cruciferous vegetables. It is sold as a natural supplement under a variety of names over the Internet and in health food stores. I3C has the ability to act as a promoter or an inhibitor of carcinogenesis. This is dependent on the specific carcinogen used to initiate carcinogenesis, when the subject is exposed to the carcinogen in relation to I3C and the species being studied.[3] I3C reacts in the acidic environment of the stomach to form condensation products (Fig. 1). The acid derived products of I3C, but no parent compound, are found in the liver following oral administration of I3C. Products formed are numerous. This investigation focused the two products, indolo(3,2-b)carbazole (ICZ) and 3,3'-diindolylmethane (I33'). ICZ is an agonist for the *A*ryl hydrocarbon *R*eceptor (AhR) to a greater extent, but is present at very low levels compared to I33'. I33' is the major condensation product of I3C.[4] As an agonist of the AhR, ICZ appears to be more biologically active compared to I3C.

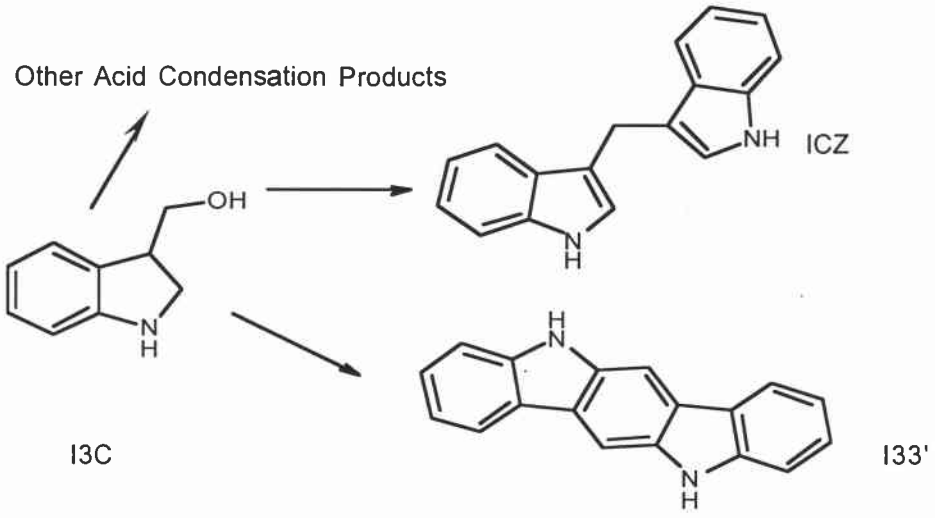


Fig. 1. Structures of I3C and the acid condensation products ICZ and I33'

Vitellogenin (Vg) is a biomarker of estrogenic compounds. It is a high density lipoprotein produced in the liver and is involved in egg production in oviparous vertebrates. Vg is formed as a result of estrogenic compounds binding to a hepatic receptor and causing transcription of the Vg and other genes (Fig. 2). In mature female trout, Vg then enters the blood stream and is absorbed into the oocytes. In immature fish, Vg accumulates in the blood and liver.[5] The salmon ER (rER) has the same specificity as the human ER (hER) in relation to estrogens, but the rER has an affinity constant ten times lower, hence ligands bind less tightly. Considering the similarity between salmon and trout, this a good indication that Vg in the trout model is representative of what occurs in humans.[6]

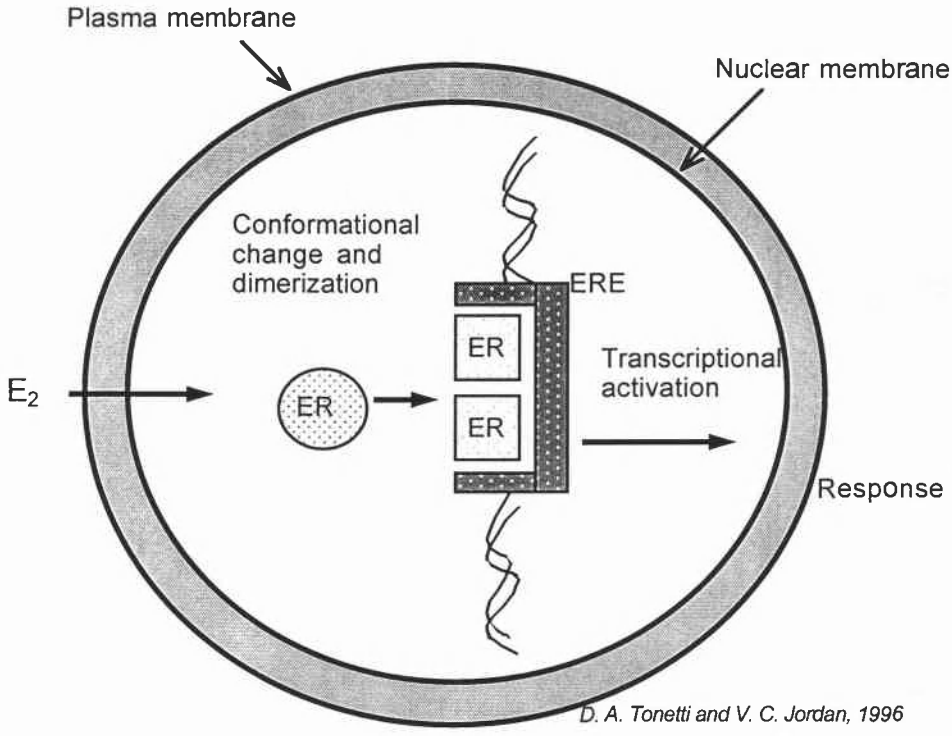


Fig. 2. The Estrogen Receptor (ER). E_2 is β-estradiol, a common estrogen. ERE is the Estrogen Response Element (ERE).

ICZ has high affinity for binding to the AhR compared to the other acid-derived products of I3C. Binding of the ligand to the AhR leads to induction of certain isoforms of cytochrome P_{450} (CYP1A1 and CYP1A2 enzymes) (Fig. 3). Cytochrome P_{450} enzymes are a gene superfamily involved in xenobiotic biotransformation. These enzymes can be used to detoxify xenobiotics, but sometimes result in a more toxic product. The AhR is also called the dioxin receptor because it has the ability to bind with 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD or dioxin). Ah receptor agonists have been shown to exhibit antiestrogenic properties.[7] When a human breast tumor cell line (MCF-7) was treated with ICZ in a tissue culture study, a decrease in the estrogen receptor (ER) and estrogen-responsive element (ERE) binding was observed. There also was a decrease in actual ER levels observed. This shows that ICZ has the ability to be an antiestrogen, possibly decreasing mammary tumor risk. Also ER binding was

observed at high levels of ICZ alone, compared to ICZ plus 17β-estradiol (E_2), indicating a slight pro-estrogenic activity.[8]

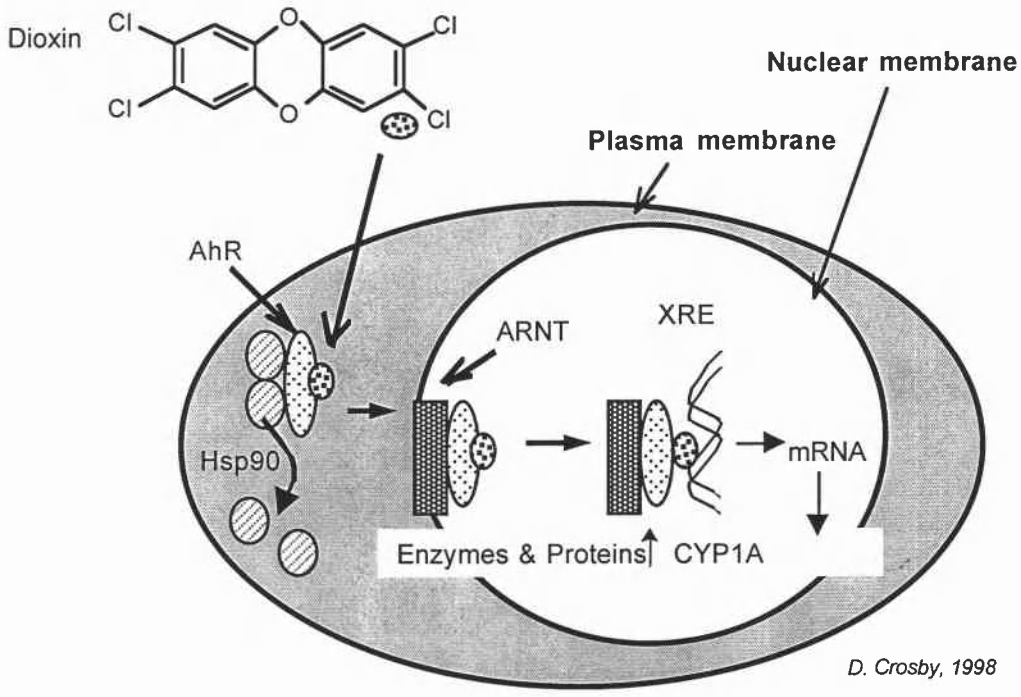


Fig 3. The AhR. Hsp90 stands for heat shock protein 90. ARNT is the Ah Receptor Nuclear Translocator. XRE is the Xenobiotic Response Element.

I33' is a dimer product of I3C. Even though it is not a strong agonist of the AhR compared to ICZ, it does have antiestrogenic properties. This may be due to its interaction with the AhR. Its antiestrogenic properties are weak compared to TCDD, the strongest of the AhR agonists.[9] In DMBA induced rat mammary tumors, there was a dose-dependent decrease in tumors with I33'. For this reason I33' is of interest as a possible chemopreventive agent in preventing certain forms of cancer including breast, since it may have less toxic side effects than other antiestrogens, such as TCDD.[10]

Indolplex™ contains a pure form of I33', as well as other ingredients designed to increase the absorption of the I33' and increase shelf life. It is a commercial dietary supplement. I33' and Indolplex™ are used to treat Recurrent Respiratory

Papillomatosis (RRP),[11] a condition caused by the Human Papilloma Virus (HPV), in which tumor-like lesions grow on the larynx and sometimes the trachea and lungs. RRP is common in children, being more aggressive in children younger than four.[12]

The carcinogen used in the tumor study was Aflatoxin B_1 (AFB_1). It is produced by strains of the fungus, *Aspergillus flavus* and *A. parasiticus*, and is a potent hepatotoxin and hepatocarcinogen in rainbow trout. One of the reactions that occur in the liver involves a cytochrome P450 enzyme resulting in an epoxide[13] (Fig. 4.). An epoxide is highly reactive and can result in cell damage and lead to cancer.

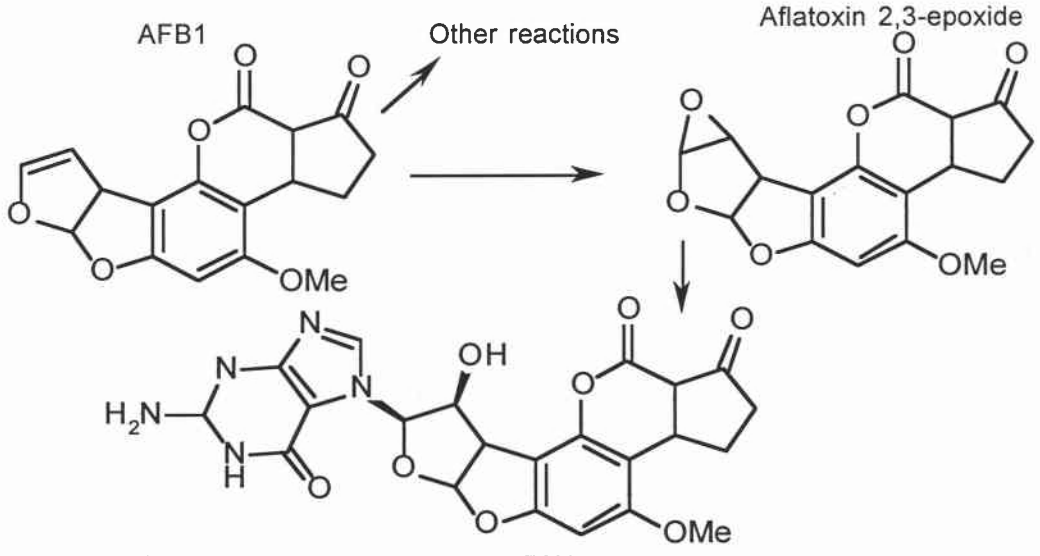


Fig. 4. The reaction of Aflatoxin B_1 (AFB_1) in which an epoxide is formed leading to DNA and/or RNA damage and increasing the rate of cancer.

Effects of I3C, ICZ, I33' and Indolplex™ on CYP1A and Vg induction, and tumor induction in relation to AFB_1 toxicity were determined. Also, a comparison was made between the previously mentioned compounds and some known promoters of carcinogenesis in the rainbow trout (*Oncorhynchus mykiss*) dietary model. These include promoters that act through a variety of mechanisms including those discussed below.

β-Estradiol (E_2) is a potent orally effective estrogen. In male rodents, E_2 causes Leydig cell adenomas, the primary site of testosterone synthesis.[14]

Dehydroepiandrosterone (DHEA) is an adrenal steroid that is the major circulating hormone in human plasma, peaking in early adulthood and decreasing with age. DHEA is a peroxisome proliferator (PP). Like most PP's, DHEA causes liver cancer in susceptible species such as rats and mice. DHEA also is a carcinogen in trout, a species that, like humans, is resistant to PP. DHEA is a precursor of androgens and estrogens.[15]

Perfluorooctanoic acid (PFOA) is used in industry as a plasticizer, lubricant and wetting agent. It is a PP, and in rodents, increases the incidence of Leydig cell adenomas.[16] In trout PFOA is a tumor promoter.[17]

Hydrogen peroxide (H_2O_2) is an oxidant involved in the formation of hydroxyl radicals, when catalyzed by metal ions. Another oxidant, *t*-butyl peroxide is a stable oxidant and a source of free radicals. These compounds are tumor promoters in trout, presumably through induction of oxidative stress.[17]

A low choline diet was another of the treatments investigated. Choline is a dietary nutrient. It is a source of methyl groups and is a precursor of phosphatidylcholine. A low choline diet causes oxidative damage and altered methylation of DNA in the liver.[18]

β-naphthoflavone (BNF) is a synthetic AhR agonist that induces CYP1A1.[19]

MATERIALS AND METHODS

Experiment 1. Known promoters of carcinogenesis:

Materials

I3C was purchased from Aldrich (Milwaukee, WI). E2, DHEA, H_2O_2, t-butyl peroxide and BNF were purchased from Sigma (St. Louis, MO). PFOA was purchased from Fluka (Ronkonkoma, N.Y.).

Animals and Treatments

Rainbow Trout (*Oncorhynchus mykiss*) were provided by the Marine and Freshwater Biomedical Sciences Center (MFBS) at Oregon State University. Animals were kept in 100 gallon circular tanks with 12-14°C continuous flowing well water and alternating 12-h light/dark cycles. Trout were fed Oregon Test Diet (OTD) after spawning. At about six and half months after spawning fish were started on experimental diets, 300 fish per diet. Experimental diets were as follows: 750 or 1500 ppm I3C, 5 ppm E2 (alternated weekly with OTD), 750 ppm DHEA, 5000 ppm PFOA, 3000 ppm H2O2, 5000 ppm *t*-butyl peroxide, low choline diet (no choline supplementation) or 500 ppm BNF. Two weeks after experimental diets were started, three fish from each diet group were sacrificed and the livers removed, placed in liquid nitrogen and kept frozen at -80°C.

Experiment 2. Indole-3-carbinol and condensation products

Materials

I3C was purchased from Sigma (St. Louis, MO). I33' and Indolplex™ were provided by Bio Response (Boulder, CO). ICZ was kindly provided by Dr. Leonard F. Bjeldanes (University of California, Berkeley, CA). Due to the insolubility of ICZ and

I33', they were dissolved in DMSO prior to being added to the oil component of the diet. The other chemicals were water soluble, and were mixed into the water component of the diet during diet preparation.

Animals and Treatments

Rainbow Trout embryos were obtained from Mt. Shasta hatchery, CA. They were then hatched and raised in the Marine and Freshwater Biomedical Research Center, as described above. Approximately 1800 fish were exposed to a 12 ppb AFB1 bath for 30 minutes, as fry 97 days after spawning. This was expected to induce a tumor incidence of 10 -15% after ten months. The sham (control) fish were exposed to the equivalent amount of ethanol (0.055% v/v). They were maintained with approximately 100 fish per tank (both sexes), two tanks per treatment group, of sham and AFB1 treated fish. The treatments were as follows: 2000 ppm I3C, 0.3 or 3 ppm ICZ, 120 or 400 ppm I33', or 1200 ppm Indolplex™. Six and a half months after treatments were started, the amount of I3C was decreased to 1000 ppm due to toxicity. At four months, a subsampling was done, taking three fish per tank. One year after initiation, fish were sacrificed and necropsied. Portions of the liver were frozen in liquid nitrogen and stored at -80^0C. Other portions of the livers were removed and preserved in Bouin's solution for histological analysis.

Preparation of Liver Homogenates and Lowry Assay for Protein

Livers were homogenized by hand, using glass homogenizers, in ice cold phosphate buffer (0.1 M potassium phosphate (pH 7.5), 20% glycerol, 0.1 mM EDTA, 0.1 mM PMSF, 1.9 µg/ml aprotinin) and divided into three equal parts per liver. They were then frozen for Vg ELISA (Vg Enzyme Linked Immunosorbent Assay) and CYP1A analysis at a later date. Protein concentration was determined by methods described by Lowry *et al.*[20]

Vitellogenin ELISA

Vg standards were kindly provided by Dr. Gayle Orner (Pacific Northwest National Lab, Richland, WA.). Reagents for Vg ELISA such as biotinylated donkey anti-rabbit IgG and strepavidin-horseradish peroxidase were purchased through Amersham (Arlington Heights, IL). Rabbit polyclonal antibody against Coho salmon vitellogenin was kindly provided by Dr A. Hara (Hokkaido University, Japan). ELISA's were preformed as described in Donohoe and Curtis, with the exception of using liver homogenates instead of blood plasma.[5]

Electrophoresis and Immunoblotting

CYP1A induction was examined using liver homogenates. Samples were centrifuged in a QIAshredder Mini Column (Qiagen, Santa Clarita, CA) due to the fact that they coagulated when loading into the wells of the gel. Protein concentration in each well equaled 24 μg for each treatment. Proteins were separated by SDS-PAGE on 10% acrylamide gels and electrophoretically transferred onto PVDF or nitrocellulose membranes (Trans-Blot: Bio-Rad, Richmond, CA). Blots were probed with rabbit polyclonal antibody to CYP1A (kindly provided by Dr. Donald Buhler, Oregon State University, Corvallis, OR.) This was followed by a goat anti-rabbit horseradish peroxidase-linked secondary antibody (Bio-Rad, Hercules, CA). Proteins were detected using an Amersham ECL chemiluminescence kit (Amersham, Arlington Heights, IL). Western blots were scanned on a flatbed HP Scanjet IIcx scanner. Densitometry was performed using the public domain software NIH Image v. 1.57 (written by W. Rasband at the US National Institutes of Health).

Necropsy

At the time of sacrifice, the fish were killed by an overdose of tricaine methane sulfonate (MS-22), followed by cutting the gill arches and bleeding. This protocol is

approved by the Oregon State University Institutional Animal Care and Use Committee (IACUC). The fish were weighed, livers removed and weighed and the livers inspected for neoplasms under a dissecting microscope. The size and location of the tumors was recorded and the livers were fixed in Bouin's solution and saved for histological analysis.

Statistics

Data was statistically analyzed on a Macintosh power G3 computer using Statview 5.01. Induction of Vg and CYP1A, and tumor size were statistically analyzed using ANOVA and Fisher's PLSD. Data for Vg induction were log transformed in order to minimize the differences caused by the unequal variances. Tumor multiplicity (number of tumors per fish with tumors) was compared by the nonparametric Kruskal Wallis Test. Statistics on the percentage of tumors was accomplished using the Fisher's Exact Test. A p-value of 0.05 or less was considered significant. Error bars were calculated according to the standard error.

RESULTS

Experiment 1. Known promoters of carcinogenesis:

Both the 750 and 1500 ppm treatments of I3C induced Vg significantly to 10,700 and 11,100 ng/mg in liver homogenate respectively. Induction of Vg by E_2 and DHEA were a little higher and lower compared to the I3C. PFOA induced Vg to the greatest degree and to a level of 142,000 ng/mg. Hydrogen peroxide caused a small, but statistically significant Vg induction of 34.1 ng/mg (Fig. 5).

Only the highest treatment of I3C induced CYP1A significantly to 84.0 pmol/mg. Also hydrogen peroxide treatment caused a slight induction. In the choline deficient

diets, CYP1A was induced quite significantly at 346 pmol/mg. The highest induction was caused by BNF at 673 pmol/mg (Fig. 6).

Experiment 2. Indole-3-carbinol and condensation products:

At four months, the induction of Vg in the control seemed quite elevated (117 ng/mg). I3C treated fish had significantly elevated Vg levels (68,100 ng/mg) compared to the control. The 3 ppm treatment of ICZ induced Vg to 87,096 ng/mg. Both treatments of I33' were induced to 3,590 and 66,700 ng/mg respectively. Indolplex™ treatment also significantly induced Vg to 11,400 ng/mg (Fig. 7).

I3C induced CYP1A to the greatest extent. The lower dose of I33' (120 ppm) also induced CYP1A significantly (Fig. 8).

Effects of I3C and condensation products on AFB_1 initiated carcinogenesis:

Trout in this experiment, including the controls, had a slower rate of growth than what are typical [21] (Fig. 9). Also, the weight range was great within all treatments. The liver somatic Index was high in the 400 ppm I33' and Indolplex™ (Fig. 10 and 11).

High mortality was seen throughout the study. The I3C treatment group had to be lowered from 2000 to 1000 ppm due to toxicity. Certain treatment groups, in particular the Indolplex™ treatment, had much higher survival rates. Overall mortality was 24.3%. The high mortality is believed to be due to an unknown contaminate in the water. Liver tumor incidence was extremely high. There was an incidence in 8.6% uninitiated controls. In the unitiated 400 ppm I33' the percentage of liver tumors was almost significant (Fig. 12). The tumor incidence was higher in the higher treatment of I33' (400 ppm) for both sham and AFB_1 treatments. The highest tumor rate was observed in the Indolplex™ treated with AFB_1, the group that also had the highest survival rate. Indolplex™ had more tumors compared to the I33' treatment (Fig. 13).

The AFB_1 treated groups of I33' (both treatments) and Indolplex™ had tumors that were significantly larger than the control (Fig. 14 and 15). This correlates with the high tumor incidence of the 400 ppm I33' and Indolplex™.

DISCUSSION

Experiment 1. Known promoters of carcinogenesis:

The results for this experiment were as expected with some exceptions. Marked induction of blood plasma Vg, in trout, was seen in diets containing more than 500 ppm I3C. At 1250 ppm I3C, induction of CYP1A was significant. Both of these findings were after 8 months of treatment.[22] Induction of CYP1A1 peaks at 48 hours following the start of the treatment of 2000 ppm I3C and drops to near control level by day seven.[23] This concurs with the current results in relation to induction of Vg and CYP1A. The only significant induction of Vg at two weeks was in the 1500 ppm I3C treatment. I3C induced CYP1A significantly in both the 750 and 1500 ppm treatments. Vg has been reported to be induced by the estrogen, E2 and DHEA.[15] Perfluorooctanoate (C8) causes an increased serum concentration of estradiol in rats,[16] suggesting that PFOA would also act as an estrogen and induce Vg in trout. All three of these compounds induced Vg to approximately the same extent as I3C. Induction by PFOA was slightly higher in comparison to I3C, E2 or DHEA. Small but significant induction of CYP1A was seen in both I3C and H_20_2 treatments. The other treatments did not induce CYP1A significantly, except for the choline deficient diet, in which induction was quite high and BNF in which induction was very high. H_20_2 induction of Vg may be related to redox effects on the ER, cross talk between receptors or both. The results seen in choline deficiency treatment, may reflect the fact that CYP1A is induced in methyl deficiencies such as are seen in folate

deficiency. Methionine and choline are dietary sources of methyl groups and precursors of phosphatidylcholine.[18] BNF is a well-known AhR agonist [19]

Experiment 2. Indole-3-carbinol and related compounds:

I3C and its acid condensation products have shown promise in the fight against cancer as tumor inhibitors or antiestrogens. The compounds, I3C and I33' are sold as natural supplements in various forms and under a variety of names. I3C as been demonstrated to be a tumor inhibitor or promoter depending on the conditions such as species, carcinogen, and co-exposure with I3C. This variation is most likely due to the acid condensation products of I3C. These studies were intended to determine whether tumor promotion by I3C is related to its properties as an AhR agonist or its estrogenic properties. This was assessed by measuring induction of Vg, a biomarker for estrogens, and induction of CYP1A, a transcription product regulated by the AhR. A comparison was made between the effects of I3C and two of its acid condensation products, ICZ and I33' as ER and\or AhR agonists and the ability to promote AFB_1-related heptocarcinogenesis. I3C tumor promotion has been shown to increase when treatment was delayed several weeks to months in relation to AFB_1 exposure.[24] According to our findings tumor promotion does not seem to be related to Vg or CYP1A induction. I3C, the higher treatments of ICZ and I33', and Indolplex™ significantly elevated Vg production. The highest tumor incidence in uninitiated trout was observed in the 400 ppm I33' treatment at a rate that was almost significant . The control in the Vg assay seemed to be elevated also. This could be related to the as yet unexplained recent high tumor incidence and mortality at the MFBS. Liver CYP1A was induced in I3C treated trout. ICZ and I33' exhibited higher induction of CYP1A at the lower treatment levels, possibly an indication of tumor suppressor effects. Indolplex™ did not significantly induce CYP1A. This was not shown in the tumor data in which the higher doses had higher tumor promotion. Indolplex™

promoted the highest in AFB_1 carcinogenisis. This may be due to other ingredients in the compound. Indolplex™ was designed to increase self life and absorption into the body, hence may increase bioavailbility. This could also increase the number of tumors compared to I33'. I3C forms many acid condensation products, which could account for AFB_1 tumor promotion. These acid condensation products have a variety of biological effects including estrogen and AhR agonist and antagonism. The combination of these compounds could have synergistic or antagonistic reactions with each other. CYP1A induction has been demonstrated to play little if any role in the inhibition of AFB1 tumor promotion.[25]

As stated in the introduction, ICZ is structurally similar to the potent AhR agonists, dioxin. Unlike dioxin though, it is halogen free and has a low lipophilicity, making it less persistent in cells. This was shown to be true in murine hepatoma cells.[26] The strong binding to the AhR was supported by evidence from hepatoma Hepa1c1c7 cell lines, as well as evidence that it does not cause DNA damage.[27] According to the four month *in vivo* study, ICZ is quite estrogenic and does not bind to the AhR to any great degree. It should be taken into consideration that both treatments of ICZ were quite low. Given this, the low induction of CYP1A may be due to the low dose. If this were correct, the induction of Vg would be extremely high.

Though not a strong agonist to the AhR, I33' is the major acid condensation product of I3C. It has been shown to be a potent inhibitor of CYP1A reactions in the trout, rat and human. It has been seen to inhibit the catalytic activities of a range of CYP isoforms in the rat, *in vitro*.[28] In the four month study, the high treatment of I33' did not induce as strongly as the low dose, which may indicate an inhibitory effect. All treatments and forms of I33' induced Vg Indolplex™ induced Vg not as greatly as I33', which may be due to the other ingredients it contains.

Obviously, with the strong evidence obtained from the data collected when the fish were sacrificed, there is another variable involved. A major problem in using living

organisms as a model is that they can be highly unpredictable. There is individual and species variability and the fact that the organism is influenced by the many aspects of its environment. Throughout the experiment fish mortality has been abnormally high. Also, the fish are smaller than normal. When the tumor study was completed, abnormally high levels of tumors were found, over 8 percent in the control group versus an historical control incidence of 0.1%. The morphology of the livers was highly varied. These unusual results may be due to a chemical or chemicals in the water, which comes from a shallow unmapped well, near the MFBS. In a study done just prior to this one, ten percent tumors were found in the control group. This is 100 times more than normal. The reasons for the high fish mortalities are unknown and are currently under investigation by OSU researchers, DEQ and EPA.[29, 30] Effects that this presumed environmental toxicant exposure has had on Vg and CYP1A induction are not known.

BIBLIOGRAPHY

1. Commission on Dietary Supplement Labels
http://www.health.gov/

2. FDA/CFSAN Dietary Supplement Health and Education Act of 1994
http://www.fda.gov/

3. Dashwood, R. H. (1998). (Brief Commentary) Indole-3-carbinol: Anticarcinogen or tumor promoter in Brassica vegetables? *Chem-Biol Intract.* **110**, 1-5.

4. Stresser, D. M., Williams, D. E., Griffin, D. A. and Bailey, G. S. (1995). Mechanisms of tumor modulation by Indole-3-carbinol. *Drug Metab. Dispos.* **23**, 965-975.

5. Donohoe, R. M. and Curtis, L. R. (1996). Estrogenic activity of Chlordecone, o,*p'*-DDT and o*p'*-DDE in juvenile rainbow trout: Induction of vitellogenesis and interaction with hepatic estrogen binding sites. *Aquat. Toxicol.* **36**, 31-52.

6. Le Drean, Y., Kern, L., Pakdel, F., and Valotaire, Y. (1995) Rainbow trout estrogen receptor presents an equal specificity but a differential sensitivity for estrogens than human estrogen receptor. *Mol. Cell. Endocrinol.* **109**, 27-35.

7. Whitlock, J. P. Jr. (1994). The Aromatic hydrocarbon receptor, dioxin action, and endocrine homeostasis. *TEM.* **5**, 183-188.

8. Liu, H., Wormke, M., Safe, S. and Bjeldanes, L. (1994). Indolo(3,2-b)carbazole: a dietary-derived factor that exhibits both antiestrogenic and estrogenic activity. *J. Natl. Cancer Inst.* **86**, 1758-1765.

9. Bock, K. W. (1994). Aryl hydrocarbon or dioxin receptor: Biologic and toxic responses. Rev. *Physiol. Biochem. Pharmacol.* **125**, 1-42.

10. Chen, I., McDougal, A., Wang, F., and Safe, S., (1998). Aryl hydrocarbon receptor-mediated antiestrogenic and antitumorigenic activity of diindolylmethane. *Carc.* **19**, 1631-1639.

11. Zeligs, M. A., (1998). Frequently asked questions regarding IndolplexTM/DIM. *The RRP Website.* http://www.rrpwebsite .org/.

12. Gabbott, M., Cossart, Y. E., Kan, A., Konopka, M., Chan, R. and Rowe, B. R. (1997). Human papilloma virus and host variables as predictors of clinical course in patients with juvenile-onset recurrent respiratory papillomatosis. *J. Clin. Microbiol.* **35**, 3098-3103.

13. Nunez, O., Hendricks, J. D. and Fong, A. T. (1990). Inter-relationships among aflatoxin B1 (AFB1) metabolism, DNA-binding, cytotoxicity, and hepatocarcinogenesis in rainbow trout, Oncorhynchus mykiss. *Dis. Aquat. Org.* **9,** 15-23.

14. Pitot, H. C., Fundamentals of oncology. 2nd ed. (1981) M. Dekker, N. Y.

15. Orner, G. A., Hendricks, J. D., Arbogast, D., and Williams, D. E. (1996). Modulation of N-Methyl-N'nitro-nitrosoguanidine multiorgan carcinogenesis by Dehydroepiandrosterone in rainbow trout. *Toxicol. Appl. Pharmacol.* **141**, 548-554.

16. Liu, R. C. M., Hurtt, M. E., Cook, J, C., and Biegel, L. B. (1993). Effect of the peroxisome proliferator, Ammonium Perfluorooctanoate (C8), on hepatic aromatase activity in adult male Crl:CD BR (CD) rats. *Fundam. Appl. Toxicol.* **30**, 220-228.

17. Bailey, G. S., Williams, D. E. and Hendricks, J. D. (1996). Fish models for environmental carcinogenesis: The rainbow trout. *Environ. Health Perspect.* **104**, 5-21.

18. Zhang, J., Henning, S. M., Heber, D., Choi, J., Wang, Y., Swendseid, M. D., and Go, V. L. W. (1997). NADPH-Cytochrome P-450 reductase, Cytochrome P-450 2C11 and P-450 1A1, and the aryl hydrocarbon receptor in livers of rats fed methyl-folate-deficient diets. *Nutr. Cancer.* **28**, 160-164.

19. Takahashi, N., Harttig, U., Williams, D. E., and Bailey, G. S. (1996). The model Ah-receptor agonist β-naphthoflavone inhibits aflatoxin B_1-DNA binding *in vivo* in rainbow trout at dietary levels that do not induce CYP1A enzymes. Carc. **17**, 79-87.

20. Lowry, H. O., Rosebrough, N. J., Far, A. L., and Randall, R. J., (1951). Protein measurements with the Folin phenol reagent. *J. Biol. Chem.* **193**, 265-275.

21. Correspondence with Dr. J. Hendricks, Facility Care Director of the Marine and Freshwater Biomedical Sciences Center (MFBS), Environmental and Molecular Toxicology, Oregon State University, Corvallis, Oregon

22. Oganesian, A., Hendricks, J. D., Pereira, C. B., Orner, G. A., Bailey, G. S., and Williams, D. E. (1999). Potency of dietary indole-3-carbinol as a promoter of aflatoxin B1-initiated hepatocarcinogenesis: results from a 9000 animal tumor study. Carc. **20**,453-458.

23. Takahashi, N., Dashwood, R. H., Bjeldanes, L. F., Bailey, G. S. and Williams, D. E., (1995). Regulation of hepatic cytochrome P4501A by indole-3-carbinol: transient induction with continuous feeding in rainbow trout. Fd. Chem. Toxic. **33**, 111-120.

24. Dashwood, R. H., Fong, A. T., Williams, D. E., Hendricks, J. D. and Bailey, G. S., (1991). Promotion of Aflatoxin B1 carcinogenesis by the natural tumor modulator indole-3-carbinol: influence of dose, duration, and intermittent exposure on indole-3-carbinol promotional potency. Canc. Res. **51**, 2362-2365.

25. Takahashi, N., Stresser, D. M., Williams, D. E. and Bailey, G. S., (1995). Induction of hepatic CYP1A by indole-3-carbinol in protection against aflatoxin B1 hepatocarcinogenesis in rainbow trout. Fd. Chem. Toxic. **33**, 841-850.

26. Chen, Y., Riby, J., Srivastava, P., Bartholomew, J., Denison, M. and Bjeldanes, L. (1995). Regulation of CYP1A1 by indolo[3,2-b] carbazole in murine hepatoma cells. *J. Biol. Chem.* **270**, 22548-22555.

27. Park, J. K., Shigenaga, M. K. and Ames, B. N. (1996). Induction of cytochrome P4501A1 by 2,3,7,8-terachlorodibenzo-p-dioxin or indolo(3,2-b)carbazole is associated with oxidative DNA damage. *Proc. Natl. Acad. Sci.* **93**, 2322-2327.

28. Stresser, D. M., Bjeldanes, L. F., Bailey, G. S. and Williams, D. E. (1995). The anticarcinogen 3,3'-diindolylmethane is an inhibitor of cytochrome P450. *J. Biochem. Toxicology*. **10**, 191-201.

29. Oregon State researchers uncover risk to wells. (March 31, 2000) *Corvallis Gazette Times*.

30. Wells near lab remain a concern. (April 13, 2000) *Corvallis Gazette Times*.

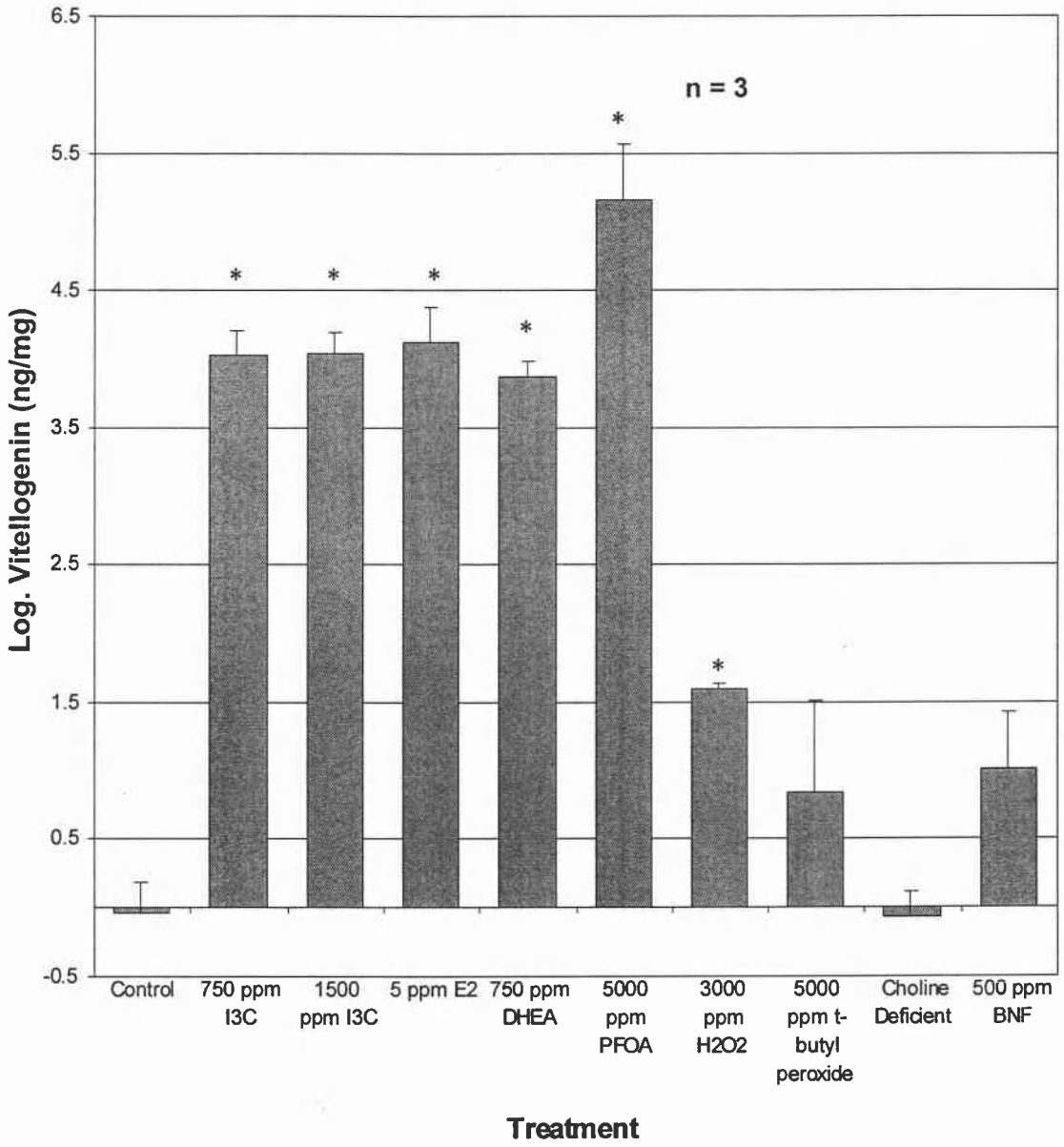


Fig. 5. Induction of Vg after treatment for two weeks

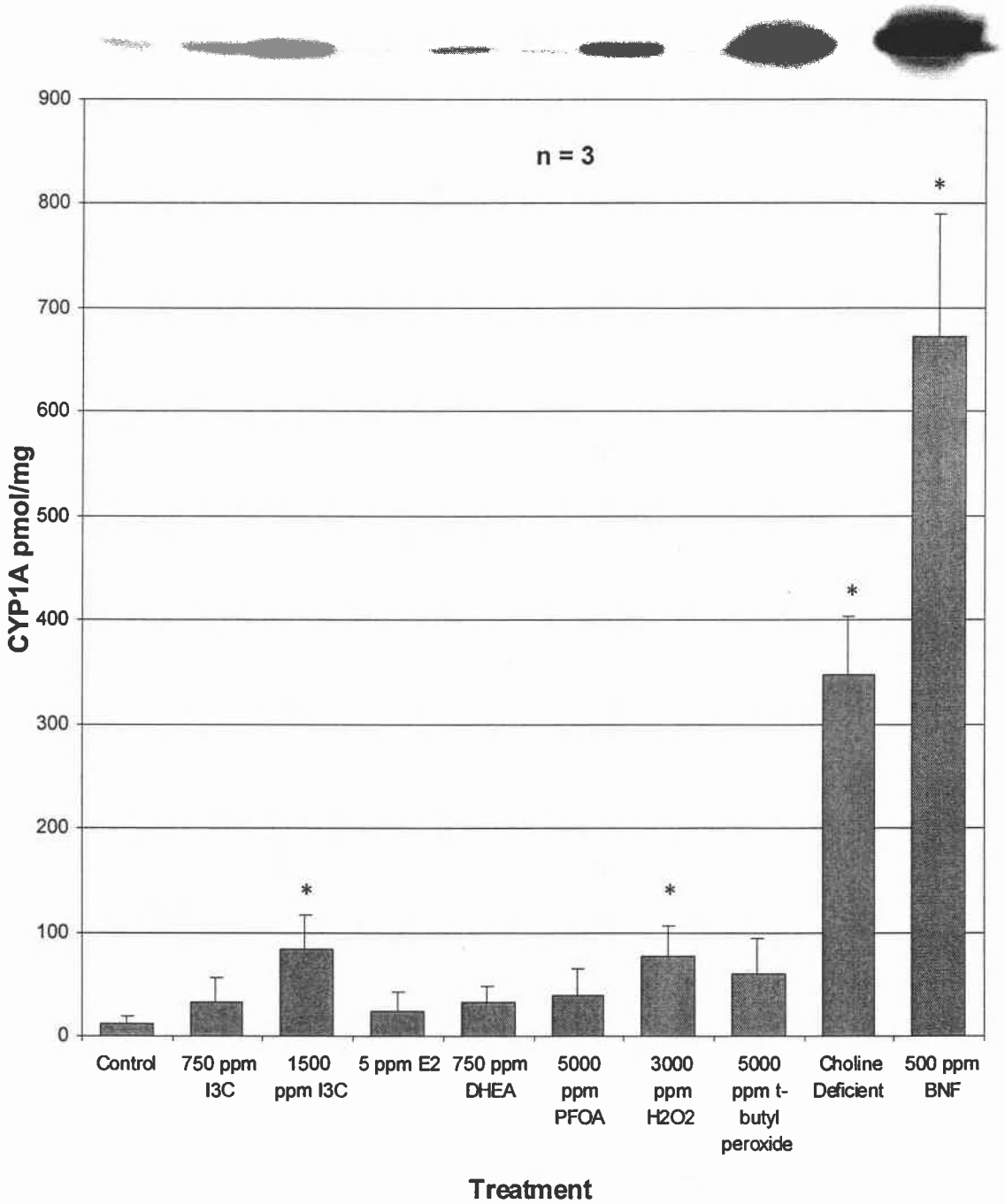


Fig. 6. Induction of CYP1A after treatment for two weeks

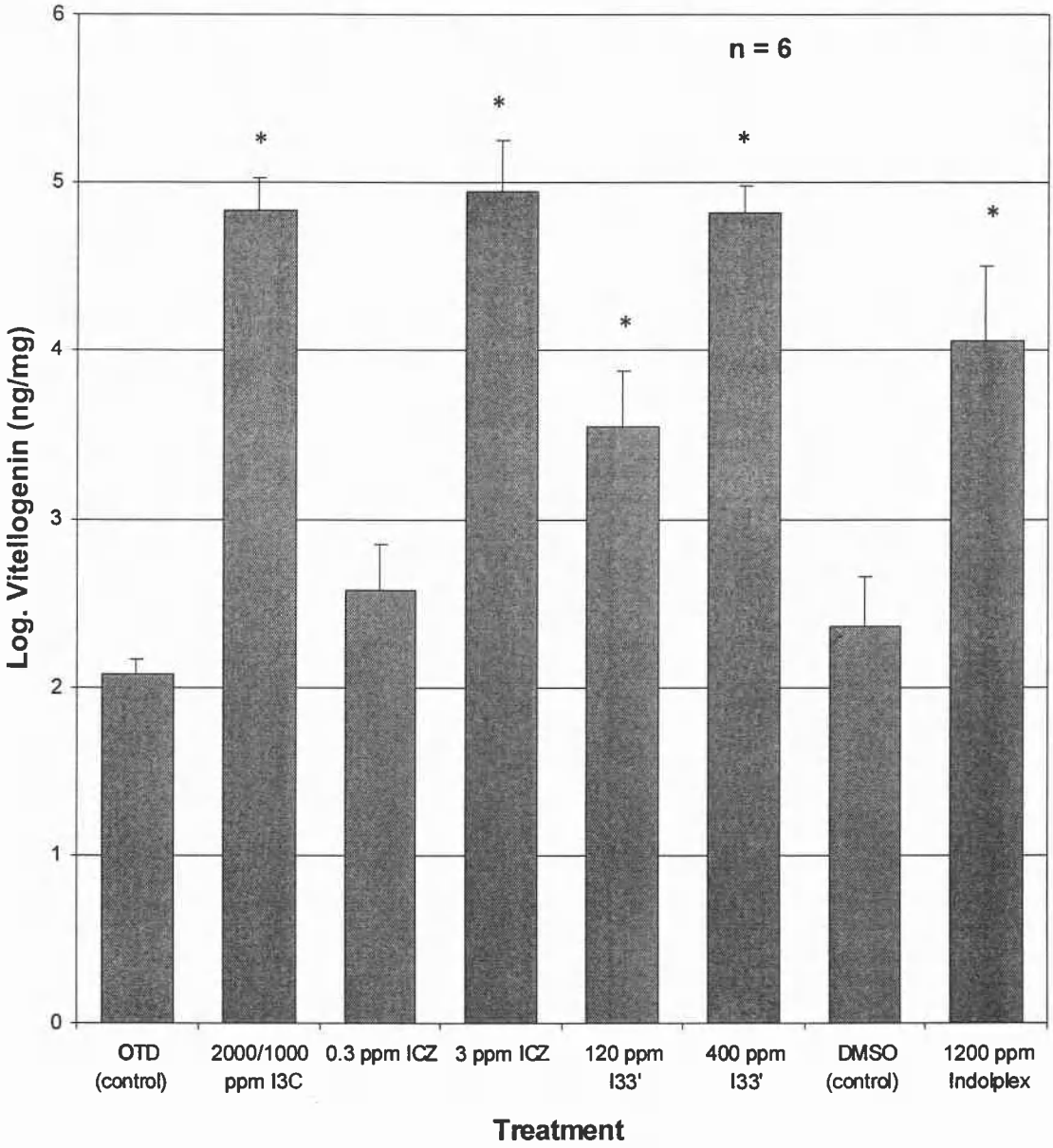


Fig. 7. Induction of Vg after treatment for four months

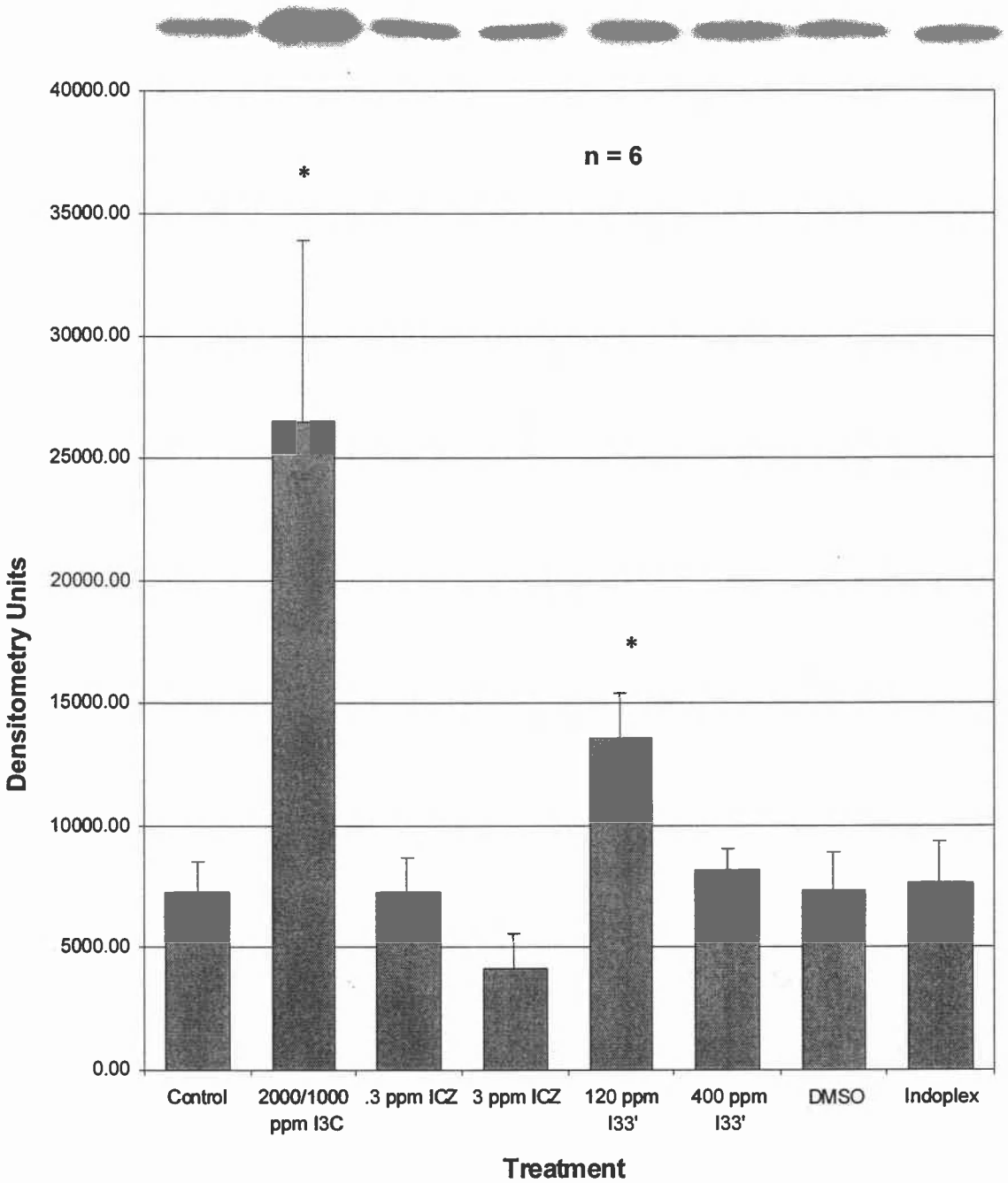


Fig. 8. Induction of CYP1A after treatment for four months

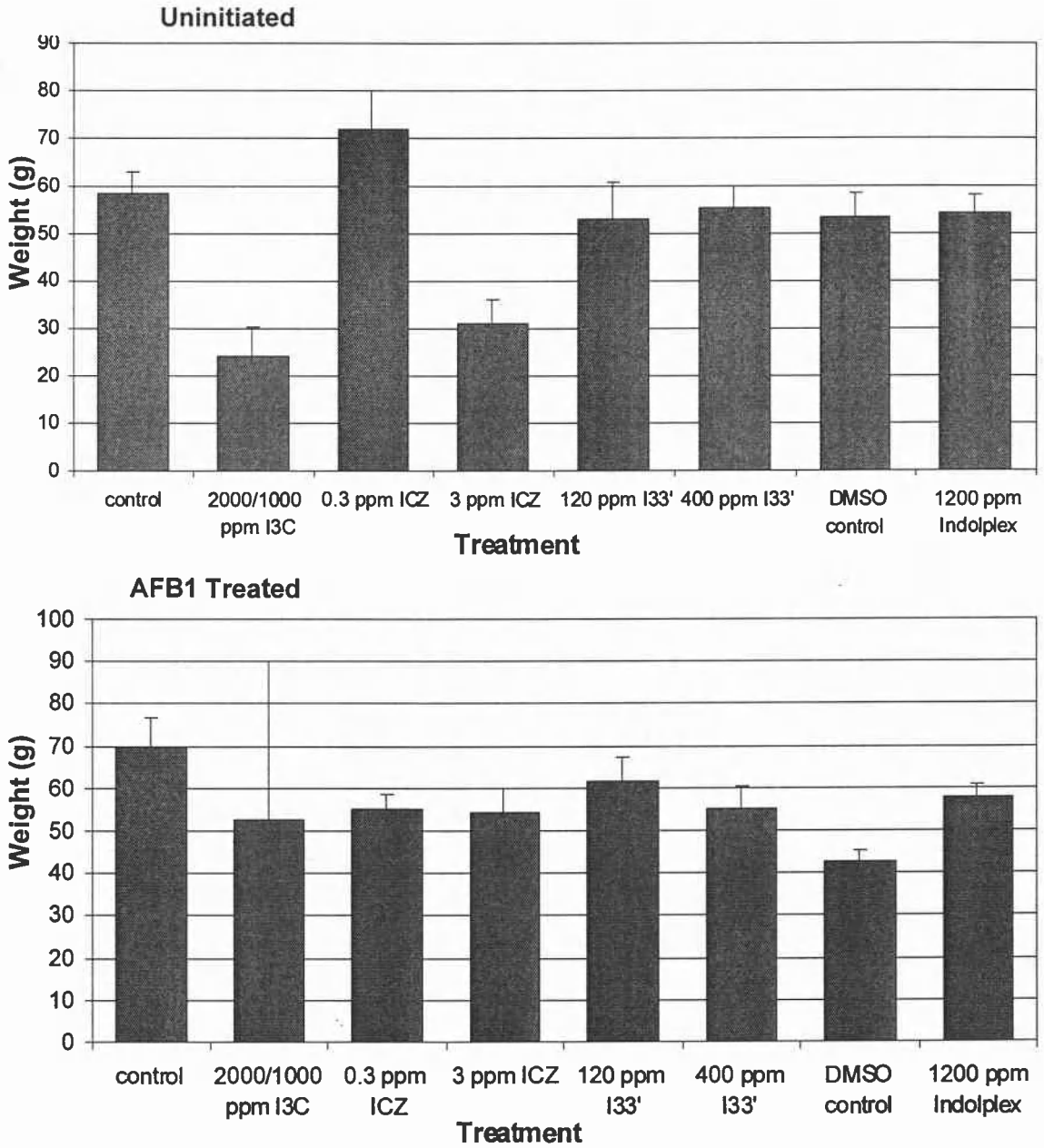


Fig. 9. Weight of trout at sacrifice

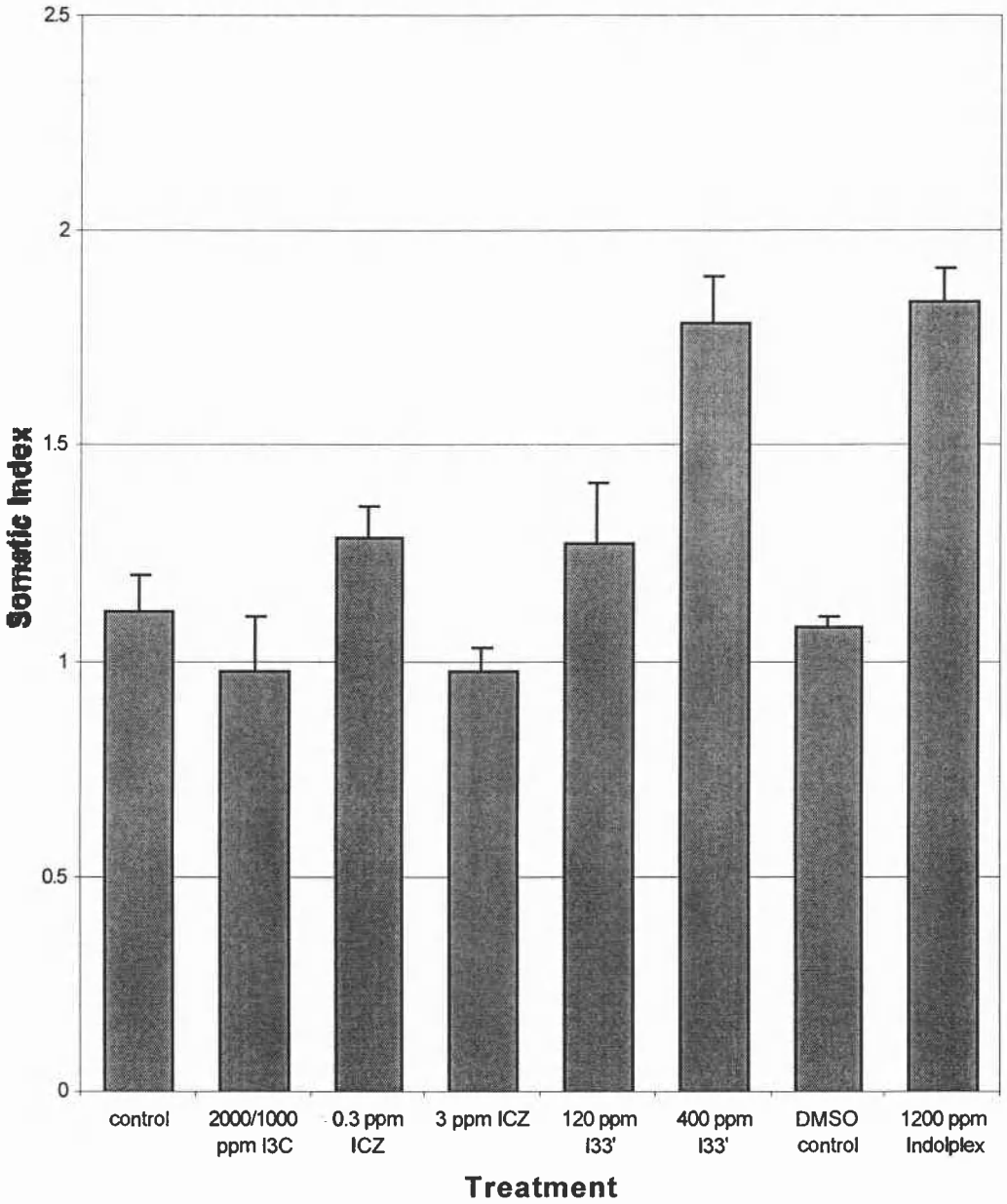


Fig. 10. Somatic Index of uninitiated trout

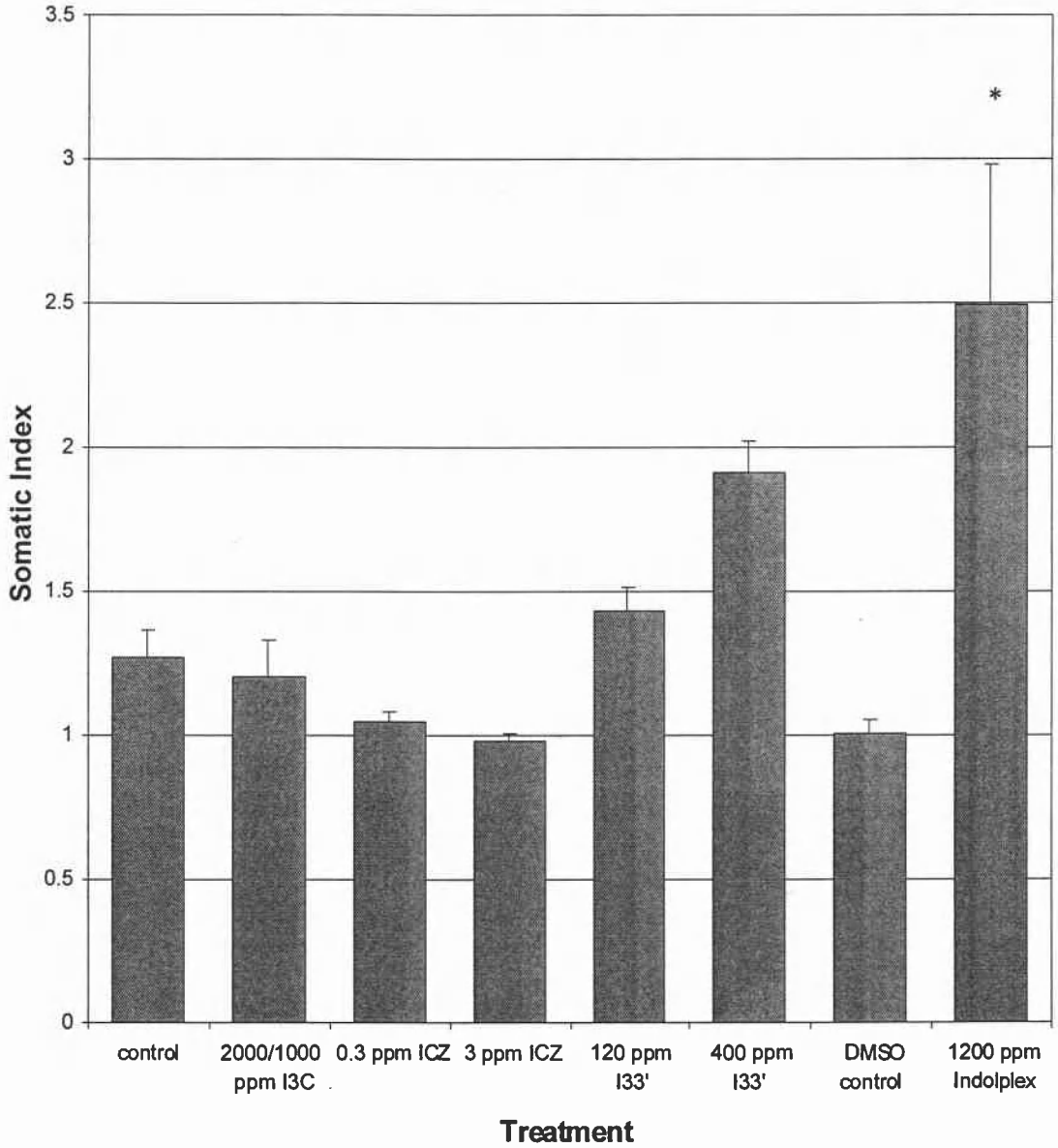


Fig. 11. Somatic Index of AFB1 treated trout

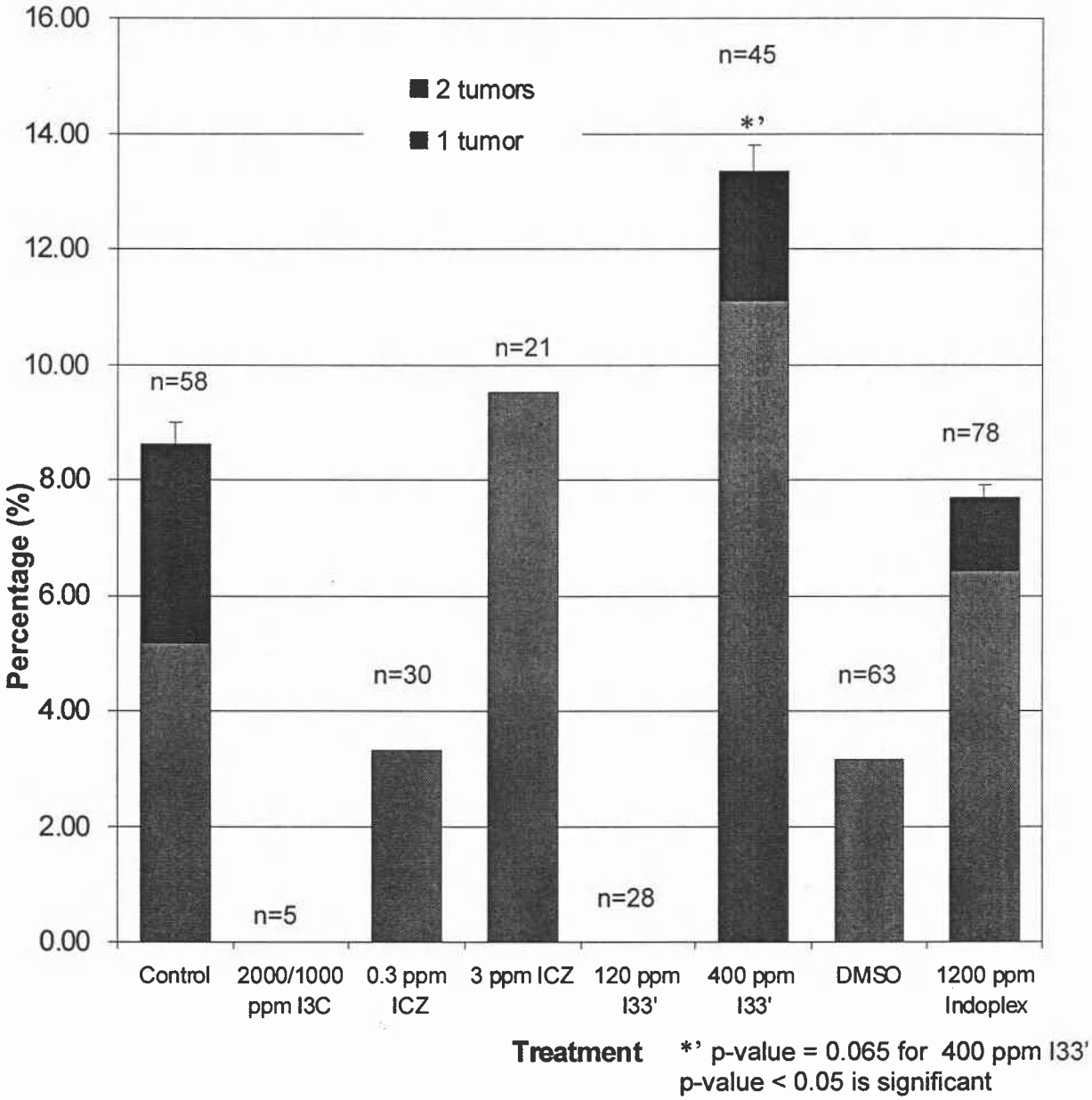
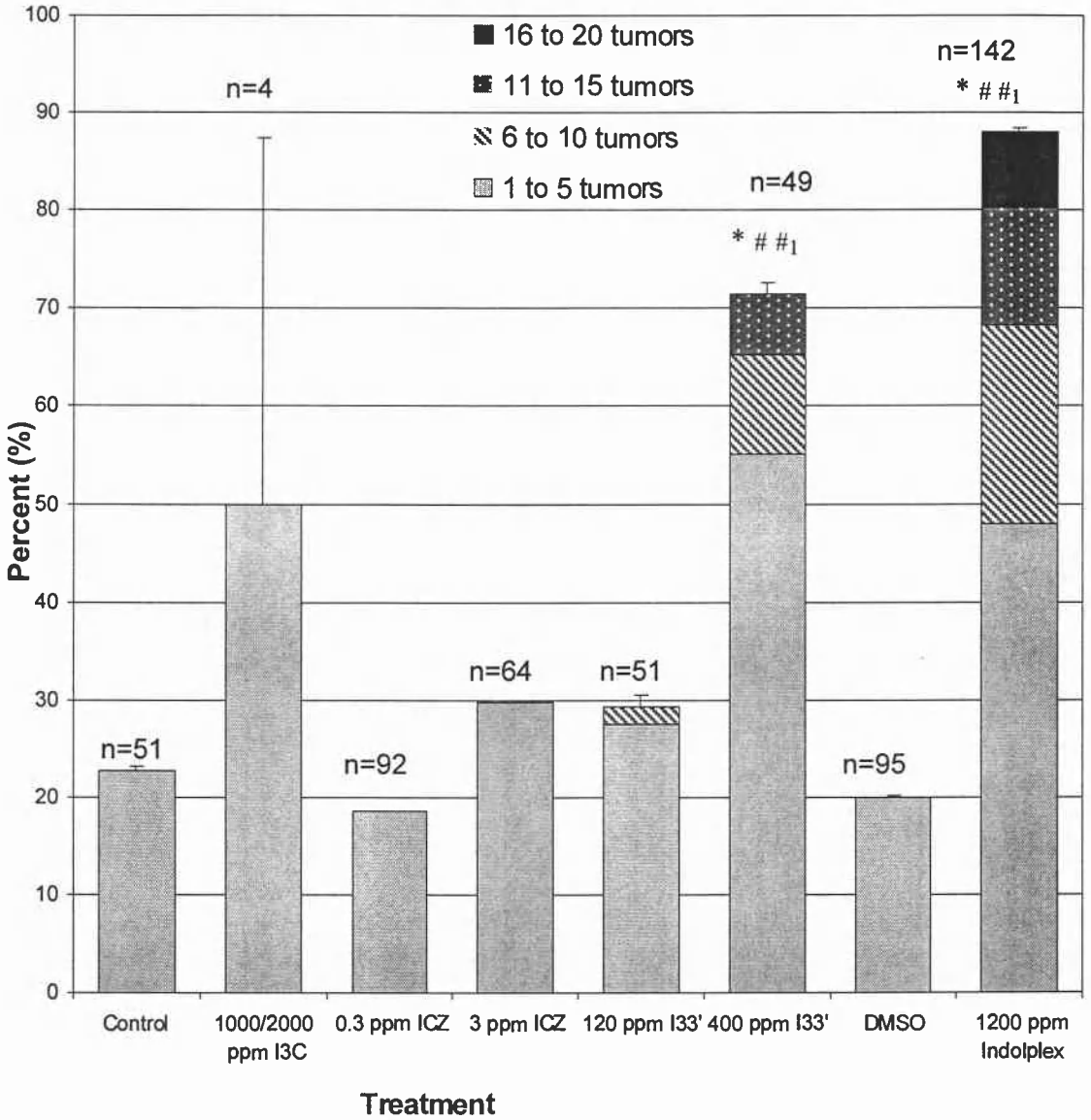


Fig. 12. The percentage of uninitiated trout with tumors



* significantly higher % tumors

significantly more tumors per animal compared to the control or DMSO

#$_1$ significantly more tumors per animal comparing Indolplex to 400ppm I33'

Fig. 13. Percentage of AFB1 treated trout with tumors

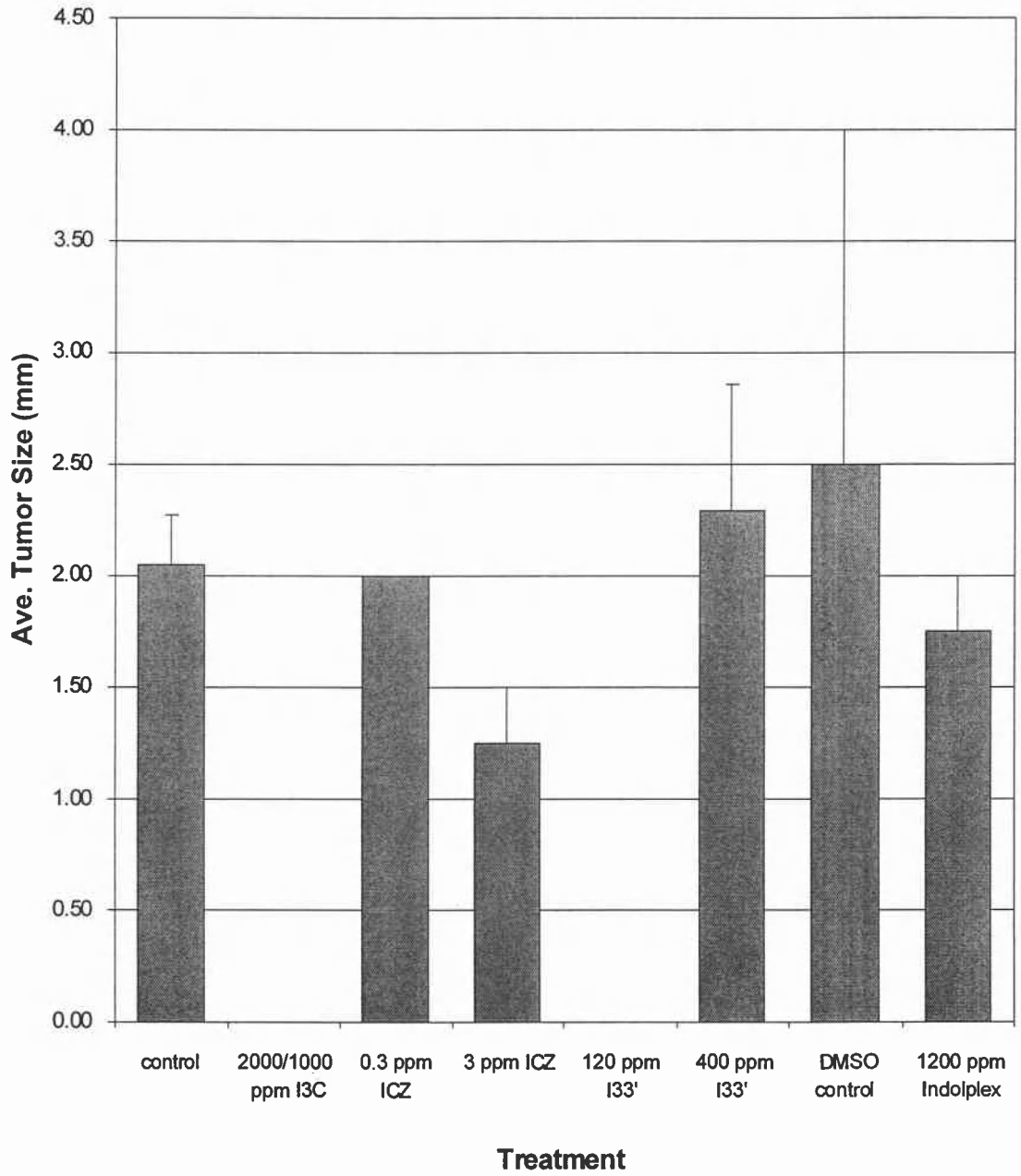


Fig. 14. Average tumor size in uninitiated trout.

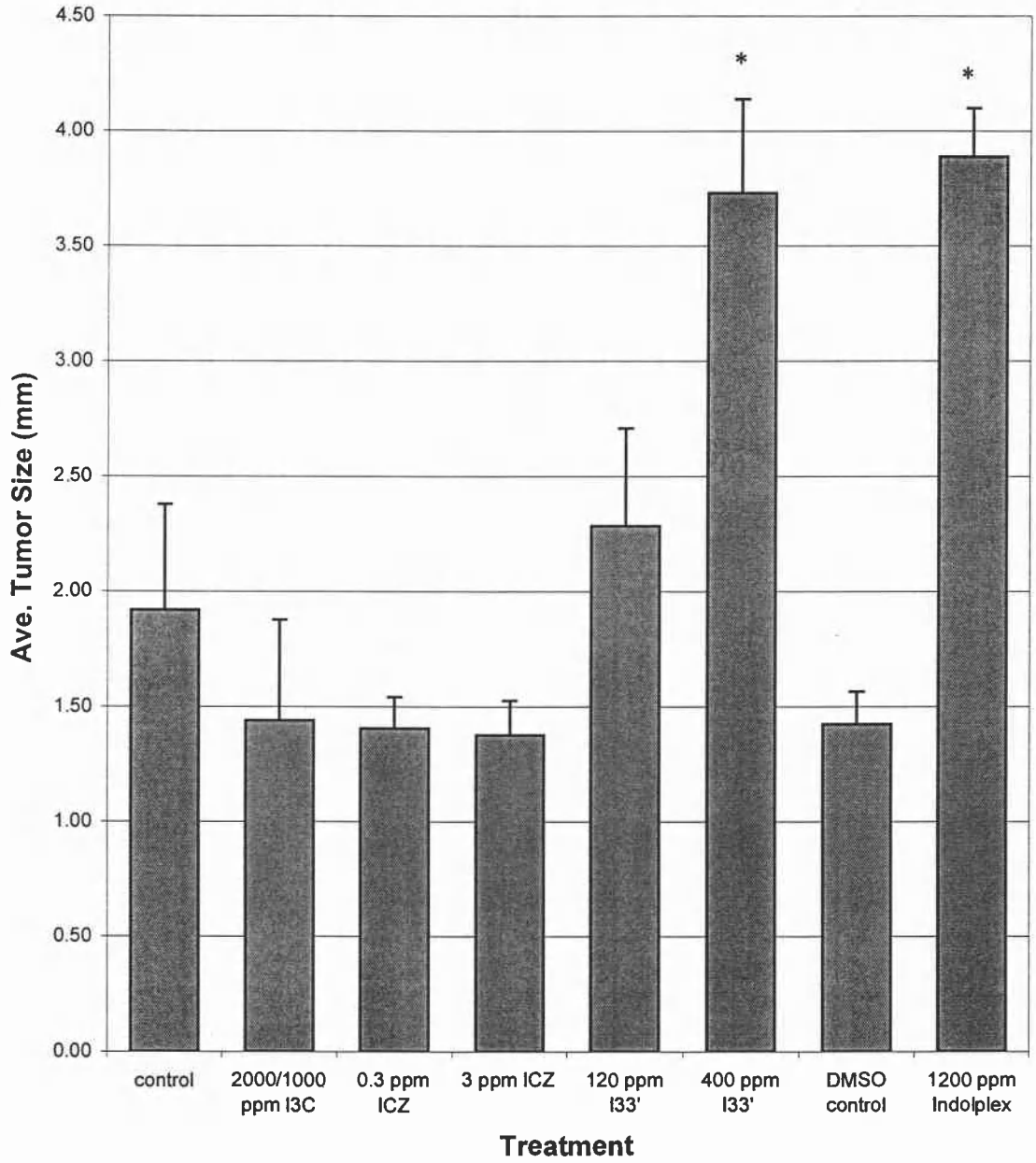


Fig.15. Average size of tumors in AFB1 treated trout