Molecular Analysis of the Effects of Atrazine on *Xenopus Laevis* Frogs

Ronald Edward Celestine  
*Western Michigan University*

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MOLECULAR ANALYSIS OF THE EFFECTS OF ATRAZINE ON
XENOPUS LAEVIS FROGS

by

Ronald Edward Celestine

A Dissertation
Submitted to the
Faculty of The Graduate College
in partial fulfillment of the
requirements for the
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Department of Biological Sciences
Dr. Charles Ide, Advisor

Western Michigan University
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MOLECULAR ANALYSIS OF THE EFFECTS OF ATRAZINE ON *XENOPUS LAEVIS* FROGS

Ronald Edward Celestine, Ph.D.
Western Michigan University, 2006

Atrazine, the most commonly used herbicide in the U.S., has been implicated as one of the factors that may be causing the worldwide decline of amphibians. First, I examined effects of atrazine on laboratory raised *Xenopus laevis* tadpoles, treated from 5 days post fertilization to NF stage 65/66 with a concentration of 25 ppb of atrazine. This study showed no significant difference in body weight, length and metamorphic rate.

Second, I used DNA microarrays to examine mRNA expression levels of genes in atrazine treated and control *Xenopus laevis* tadpoles. I found significant changes in gene expression levels of 18 genes. These 18 genes in the final dataset were categorized into biologically functional groups. These were genes associated with immunity and defense (6 genes), protein degradation (2 genes), DNA binding and repair (3 genes), and signal transduction (2 genes). I used quantitative RT-PCR to validate 2 candidate genes: Proteasome beta and Calbindin D28K. One was not significant, while Calbindin D28K was near significant (p=0.072).

Third, I spawned and reared *Xenopus laevis* tadpoles in atrazine at the sub-lethal concentration of 400 ppb after 5 days post fertilization to NF stage 62. I found
significant difference in metamorphic rate, growth rate (weight and length), and fat body development of the atrazine treated tadpoles compared to untreated tadpoles.

Next, I utilized DNA microarrays to identify gene expression that may relate to phenotypic changes observed. The results showed altered expression of 49 genes. These were mainly concentrated in the digestive system (10 genes), blood and plasma function (8 genes), and cell adhesion (12 genes). In order to validate the DNA microarray expression data, qRT-PCR was used on 4 candidate genes: pancreatic trypsin (upregulated), chitinase (upregulated), chitobiase (downregulated) and CYP-P450 (downregulated). Results showed 3 out of 4 of the transcripts were significant. This research indicates that atrazine may have a metabolic effect on treated tadpoles.
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Ronald Edward Celestine
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CHAPTER 1

INTRODUCTION

Global loss of amphibians was first recognized in 1989 (Collins and Storfer 2003, Wake 1991). Over 500 species of amphibians were reported on the decline by 1993 (Collins and Storfer 2003). This discovery was of great concern as amphibians are considered vital environmental indicators (Kloas et al. 1999, Venturino et al. 2003).

Amphibians because of their life history are able to live in both aquatic and terrestrial environments. They possess permeable and moist skins, with large unshelled eggs and free living larvae (Duellman and Trueb 1986). These factors enable frogs to be used for aquatic toxicology studies with great accuracy (Kiesecker et al. 2001). Frogs are also important predators for both aquatic and terrestrial ecosystems and serve as prey for other organisms (Kats and Ferrer 2003). Worldwide decline of this class of organisms could prove to have a deleterious effect on these ecosystems (Kloas et al. 1999).

There has been much debate as to the cause of the decline of amphibians. Some of the main reasons postulated are the introduction of predatory species (Kats and Ferrer 2003), habitat fragmentation, wetland losses, Ultra Violet-B (UV-B) radiation from ozone depletion (Ankely et al. 1998), increased incidence of infectious diseases (Carey 2000), and environmental chemicals such as pesticides and herbicides (Allran and

In this modern age many areas that were once wetlands and were breeding grounds for amphibians are quickly being depleted. Deforestation and draining of wetlands to expand human habitats, or to increase farmlands have been on the rise, decreasing the aquatic niche that frogs need in order to successfully reproduce (Collins et al. 2003, Collins and Storfer 2003). In addition, climate changes may also have an effect on amphibian populations. Increasing temperatures may cause evaporation of aquatic areas leading to high amphibian mortality rates (McDiarmid and Altig 2000).

As the Green House Effect depletes the ozone layer, organisms are being exposed to higher levels of UV-B radiation. This radiation is detrimental to amphibian eggs because they are unshelled and therefore exposed to the elements (Blaustein et al. 2003, Blaustein and Wake 1990, Davidson et al. 2001, McDiarmid and Altig 2000). Deforestation also increases the incidence of sunlight exposure (Collins and Storfer 2003).

Another theory is the introduction of competitive species, for example, mosquito fish (Gambusia affinis), bullfrogs (Rana Catesbiana), and crayfish (Astacidea) are major contributors to tadpole decline and extinctions (Daszak et al. 1999, Kats and Ferrer 2003, Kiesecker et al. 2001).

Aquatic contamination by household and industrial chemicals is also a factor that contributes to amphibian decline (Christin et al. 2004, Collins and Storfer 2003). The permeable skins of the larval and adult frogs allow penetration of the harmful
chemicals, which disrupt physiological processes. Pollutants found in industrial waste, household cleaners, effluents and sewage are being produced in increasing quantities. Increased pollution by these and other contaminants cause waterways to become infested with harmful, chemicals, bacteria and other organisms (Daszak et al. 1999). Many of these substances are endocrine disruptors that affect the organisms normal hormonal function (Blaustein et al. 2003, Blaustein and Wake 1990).

**Atrazine**

Atrazine (2-cholo-4-ethylamine-6-isopropylamino-S-triazine) is a white, odorless, crystalline solid from the symmetrical triazine chemical class that was first registered in the US in 1958. Atrazine has a molecular weight of 215.7, solubility of 33 mg/L at 25° C in water and a melting point of 173-175 degrees Celsius (Hunter et al. 1985). It has been classified by the EPA (United States Environmental Protection Agency) as a Restricted Use Pesticide (RUP) because of its ability to leach through the soil and contaminate ground water (Cox 2001, Hayes et al. 2003). Atrazine runoff can pollute surface water in close proximity to sprayed areas and can be transported by air currents polluting distant waterways. Concentrations greater than 40 ppb have been found in precipitation, and over 500 ppb have been found in surface waters (Storrs and Kiesecker 2004).

Atrazine is commonly used in the US to control broad leaf and grassy weeds in such crops as corn (86% of total US domestic usage), sugarcane (3% usage), pineapples and sorghum (10% usage) (Coady et al. 2005, Narotsky et al. 2001). It is a pre- and
post-emergence herbicide. It is mainly utilized in early spring (Coady et al. 2005, Hayes et al. 2002). Application quantity varies depending on soil type. High clay or organic soils require greater quantities. It is applied using hand-held sprayers, tractor-drawn spreaders and crop dusting aircraft. Atrazine is active in the soil up to 7 months after spraying, and persists longest under dry and cold conditions. It can be slightly broken down by microbes and sunlight and is slightly volatile in high temperatures. The main metabolites of atrazine are desethylatrazine, deisopropylatrazine, didealkylatrazine and hydroxyatrazine (Abel et al. 2004). The harmful biological effects of atrazine metabolites may be equal to the parent compound.

More than 74 million pounds of atrazine is being used annually and more than 64 million acres were treated with this chemical in the US alone (Abel et al. 2004, Hayes et al. 2002). Atrazine's mode of action in plants is inhibition of photosynthesis, which renders the plant unable to produce carbohydrates and other substances necessary for plant growth (Stoker et al. 2000, Tavera-Mendoza et al. 2002). Resistant plants and certain animals are able to metabolize atrazine using glutathione S-transferase conjugation, which makes it more water-soluble. In vertebrates it is sent through the gall bladder out into the feces or through the kidneys to be excreted in urine. The EPA considers 3 ppb to be an acceptable level of atrazine in human drinking water supplies and 12 ppb to be an acceptable chronic exposure level for aquatic life.
Atrazine Toxicology

There have been many recent studies on atrazine and its effects on organisms including mammals, amphibians, and birds (Cummings et al. 2000a, Hanioka et al. 1999, Hayes et al. 2002, Hayes et al. 2003, Murphy et al. 2005, Stoker et al. 2000, Storrs and Kiesecker 2004, Tavera-Mendoza et al. 2002). Many European countries such as Denmark, Sweden, Germany, France and Norway have banned the use of atrazine because of its prolific contamination of ground water used by the population.

Chronic sub lethal effects in mammals include effects on the hypothalamic pituitary axis in rats causing inhibition of lutenizing hormone (LH) surges and delayed puberty. Previous studies have also demonstrated hermaphroditism in cricket frogs (Acris crepitans) (Cooper et al. 2000, Reeder et al. 1998), ovarian dysfunction (Cooper et al. 2000, Stoker et al. 2000), reduced testicular volume, and reduced spermatogonia in rat testes (Tavera-Mendoza et al. 2002). The mode of action appears to be an alteration of the secretion of steroids and related changes in development of the reproductive tract (Stoker et al. 2000). Atrazine may inhibit the ovulatory surge of prolactin, apparently via a neuroendocrine mechanism (Cooper et al. 2000, Narotsky et al. 2001). Atrazine and some of its metabolites are considered to be equally potent.

Atrazine Effects on Xenopus laevis and Other Amphibian Species

There are two opposing positions on the effects of atrazine on amphibians. Dr. Tyrone Hayes, a professor in endocrinology at the University of Berkley, California, created a firestorm with his initial laboratory based study on the effects of atrazine on
Xenopus laevis tadpoles. His objective was to test whether atrazine had an effect on metamorphic rate, mortality, growth rate (length and weight), testicular morphology, laryngeal muscle size (regulated by testosterone), plasma testosterone and aromatase levels of tadpoles (Hayes et al. 2002). He studied these using nominal exposure concentrations that ranged from 0.01 ppb to 200 ppb of atrazine. The tadpoles were treated from 96 hours post hatching to Nieuwkoop and Faber (NF) stage 66. They were maintained at a density of 27 tadpoles per liter in plastic containers and atrazine was dissolved in ethanol to make the stock solutions. Results demonstrated that atrazine concentration over 0.1 ppb had gonadal effects on 16-20% of the tadpoles (Hayes et al. 2002). Gonadal abnormalities included multiple testes and ovaries in one tadpole, and discontinuous gonads. He also reported that the laryngeal size of the dilator muscles of the male frogs were significantly reduced when compared to the control tadpoles (Hayes et al. 2002, Hayes et al. 2003). He reported that these abnormalities were not seen in control tadpoles. The study showed that over 80% of the exposed tadpoles had reduced laryngeal muscle size. Because of these findings, he postulated that endogenous estrogen levels were being increased in the male tadpoles, thus, causing the males to become more feminized (Hayes et al. 2002). In order to support his claim that atrazine caused an increase in aromatase activity resulting in increased estrogen levels in males, he treated adult Xenopus laevis frogs with 25 ppb of atrazine for 46 days and measured the plasma testosterone concentrations. He found a 10 fold reduction of testosterone levels as compared to untreated males (Hayes et al. 2002). He postulated that testosterone levels were being reduced, while estrogen levels increased. He,
however, had no data to show estrogen levels in the adult male frogs (Hayes et al. 2002). He also did not measure aromatase levels in neither treated nor control frogs. This study demonstrated that atrazine might be a potential endocrine disrupter at a very low ecologically relevant concentration. If this study is correct, it implies that atrazine has an effect on amphibians at a level 30 times lower than previously thought and that it could account for one of the factors contributing to the worldwide decline of amphibian. In order to investigate this phenomenon in the field on native amphibian species he identified areas considered atrazine contaminated and areas considered clean. Water samples were collected from each of the areas to quantify the amount of atrazine present in the water. For this study he used the native leopard frog (R. pipiens). The study showed that 7 of the eight sites tested contained frogs with testicular oocytes. Hayes found ovotestis and hermaphroditism as high as 92% (Hayes et al. 2002, Hayes et al. 2003). In one site, however, where no atrazine was found there was still an 18% incidence of testicular oogenesis. Although the study corroborated, somewhat, the laboratory findings, there were no reports on other chemicals present in the environment and ages of tested frogs were omitted. However, these studies led Hayes to conclude that atrazine is a potent endocrine disruptor which is affecting amphibian populations, not only in the laboratory but in the wild (Hayes et al. 2002, Hayes et al. 2003). No previous study had reported that atrazine might have such a profound effect on amphibians at such a low concentration.

In order to test Hayes’ findings, other researchers attempted to repeat his study (Carr et al. 2003, Goldman and Carr 2003, Tavera-Mendoza et al. 2002). Goldman and
Carr (2003) found no effects on weight, time to metamorphosis and laryngeal muscle size, but they found a significantly increased number of intersex tadpoles exposed to 25 ppb when compared to control animals. However, this significant increase was small (5%) and there were no effects at lower concentrations (Carr et al. 2003). Studies done by Tavera-Mendoza et al (2002) observing testicular development also found that testes size, spermatogonia nests, and nurse cells were decreased after exposure to 21 ppb atrazine. When he studied the female tadpoles he noticed that primary oogonia, secondary oogonia and atresia were increased. These findings were significant because the length of exposure was only 48 hours when the tadpoles were at NF stage 56 (Nieuwkoop and Faber 1994).

It was also interesting that no other researcher was able to replicate the findings of Hayes et al (2002) at 0.1 ppb concentration of atrazine. Other researchers trying to repeat the findings of Hayes found no difference between control and treated animals at 25 ppb atrazine (Hecker et al. 2005a).

Giesy et al (2004) found no differences in metamorphosis, weight, length and testicular abnormalities between atrazine treated and control tadpoles. He also found no change in brain or testicular aromatase levels. Plasma testosterone levels were not significantly different at 25 ppb atrazine, but showed significantly decreased levels at 250 ppb of atrazine. This led to the conclusion that atrazine does not affect amphibians at such low concentrations as reported by Hayes.

In addition to Hayes et al. (2002b) other field and microcosm studies were attempted to assess the effects of atrazine exposure on amphibians. Dupreez et al
(2003) attempted a microcosm study where he took animals from a reference site in South Africa and induced them to spawn in the laboratory. He then divided the 4-day-old spawning into treatment groups (0, 1, 10, 25 ppb atrazine). However, atrazine was still detected in the reference tank (0-0.1 ppb). Larvae were exposed to the varying concentrations until they reached NF stage 66. These tadpoles were then examined for growth rate (length and weight), time to metamorphosis and gonadal abnormalities. The authors found that when gross morphology was analyzed there were discontinuous testis at low percentages (1.3%, 0.7% and 3.3%) of all the groups examined (DuPreez et al. 2003). The authors also found that the untreated male and female frogs were larger (weight and length) than the treated tadpoles.

Because *Xenopus laevis* is not native to the U.S several experiments were conducted in South Africa (where Xenopus is native). Giesy et al. (2003) conducted studies to observe metamorphic rate, testicular morphology and sex steroid concentration in Xenopus frogs at reference sites (free or very little atrazine present) to atrazine-affected areas. Five experimental and 3 reference sites were identified. Frogs from each area were collected over a period of 6 months. The authors concluded that there were no differences in the lengths and weight of males or female frogs collected. They found that tadpoles from experimental areas had testes weights equivalent to those in reference areas. Surprisingly tadpoles which showed testicular oocytes were found in 3% of the reference area but 2% in the experimental sites. They found the males that were in experimental areas had lower plasma testosterone levels than reference site animals. Aromatase concentration in testes was not analyzed. These data, therefore,
suggested that atrazine may affect testosterone levels in the wild, however, because of the time difference of collecting the frogs, and the method of capture the results have been deemed unreliable by reviewers (Steeger and Tietge 2003).

In addition to Xenopus frogs, a number of native frogs were used in field studies to determine the effects of atrazine on several endpoints (Crabtree et al. 2003, Jones et al. 2003, Sepulveda and Gross 2003). Some of the native species studied were *R. clamitans, R. Pipiens, R. catesbiana, Bufo marinus* and *B. terrestris*. These studies suggested that atrazine did not significantly affect gonadal function of *R. clamitans*. Thus, a review of the literature on atrazine’s effects on amphibians reveals widely varied findings with regard to atrazine induced gonadal abnormalities and related changes in hormone concentrations.

Gonadal Differentiation

Sexual determination in *Xenopus laevis* is genetically controlled. Unlike mammals, males carry the ZZ genotype and the females carry the ZW genotype. Stage 52 (Nieuwkoop and Faber 1994) marks the point where the indifferent gonads begin to differentiate into pre-testis and pre-ovaries. The testis produces Anti-Mullerian Hormone (AMH) which causes the regression of the mullerian duct and causes aromatase (the enzyme that converts testosterone to estrogen) to be inhibited. The testes also produce large quantities of testosterone, which further causes the differentiation of the Wolffian duct into male structures. AMH production does not occur in the females (considered to be the default gender). Males can be sex reversed
using estrogen during early stages of gonadal development (stage 54 to stage 66) while females cannot be sex reversed using testosterone. The SRY genes are the principle initiators of the male cascade, for example SOX9 causes sertoli cells to differentiate when upregulated by SRY and SF1. Wilms’ tumor 1 (WT1), SOX9 and GATA-4, control AMH transcription. The mullerian ducts form the female urinogenital system, the default structures that differentiate in the absence of high levels of testosterone. This duct degenerates, and disappears in males when the sertoli cells secrete AMH. Testosterone, produced in the Leydig cells, causes the wolffian ducts to differentiate into vas deferens, epididymis and seminal vesicles.

*Xenopus laevis* Tadpole Development

*Xenopus laevis* tadpoles are extremely specialized microphagous obligatory filter feeders and obligatory air-breathers, which means that they must emerge to the surface for breathing (Tinsley and Kobel 1996). The tadpoles do not possess keratinized external mouthparts. They possess two symmetrically arranged spiracles on either side at their caudal end. Spiracles are apertures through which air and water is admitted and expelled.

Tadpoles draw water into their mouths via a buccal pump. Tinsley and Kobel (1996) explain that the moving parts (piston) of this mechanism are the ceratohyal cartilage found on each side of the buccal floor. When the orbitohyoiodyl muscle contract it causes a drop in the medial portion of the buccal floor. This causes the mouth to open and water to rush in. In most other tadpoles a transverse flap called the
ventral velum is present to prevent the indrawn water from returning by separating the pharynx and the buccal cavity. When the cavity is filled the floor is elevated. This pressure change causes water to flow into the pharynx, pressure changes in the spiracle and water is ejected. In the case of Xenopus there is no ventral velum to control one-way movement. Instead this action is maintained by the margins of their spiracles acting as a non-return valve. It is also reported that unlike other tadpoles Xenopus possess secretory ridges on their buccopharyngeal floor homologous with the bronchial food traps. While these structures are confined to the pharyngeal region of other tadpoles it covers the whole buccopharyngeal floor in Xenopus. Mucus from these structures is secreted to trap food particles. Cilia are present along the alimentary tract to move the food particles, together with the mucus, towards the esophagus and into the intestine. This mechanism and adaptation has made Xenopus an extremely efficient filter feeder compared to other tadpoles. Depending on the concentration of suspended food in the water tadpoles may reduce or increase the volume of water entering its mouth. This is accomplished by varying the amplitude or frequency, of buccal pump activity. If concentration of food is low tadpoles may change the frequency of buccal pumping activity and/or change the amount of mucus produced in the secretory beds in the bronchial food traps. When Xenopus tadpoles feed they orient themselves at a 45° angle to the bottom of the pond or container with their heads facing downward. Their tails oscillate back and forth to move the water current and food particles toward their mouth.
Tinsley and Kobel (1996) also reported that *Xenopus laevis* tadpoles because of the lack of musculoskeletal mechanism needed to compress the lungs are unable to increase pressure in the pleuroperitoneal cavity so that they have no visceral functions. This means that tadpoles are unable to forcefully move substances out of their alimentary tract. Vomiting, laying eggs, or ejaculating is impossible. Vomiting is important to most vertebrates as this allows the organism to eject toxic substances that it may have ingested. Defecation is accomplished by a steady stream of fecal material going through the alimentary canal and very weak contractions of the colon and rectal area. Peristalsis is not present in the foregut. It is not known how the nervous system affects visceral regulation of the tadpoles.

Wassersug (1989) postulated a number of theories that correlates ecological and endocrinological regulation. Research has shown that hormones play a major part in metamorphosis; some hormones correlate the interaction of the environment with metamorphic rate alterations. Wassersug (1989) postulated that there might be a metamorphic inhibitor present in the mucus that traps the food and is swallowed by the tadpoles. In prometamorphosis the tadpoles feed rapidly and when there is much food present. The tadpole swallows the inhibitor and metamorphosis is slowed. When there is less food, less of the inhibitor is swallowed. Also as the tadpoles size increase the surface area of the bronchial food traps decreases so that less of the inhibitor is present.

Another theory postulated is that protein content in the diet of the tadpoles may be an important factor that regulates metamorphic rate and weight at metamorphic climax.
Metamorphosis of *Xenopus laevis* tadpoles can be divided into 3 major stages: premetamorphosis, prometamorphosis and metamorphic climax. Changes in morphology are controlled by 3 endocrine glands, that is, the thyroid, pituitary and hypothalamus (McDiarmid and Altig 2000). The premetamorphic stage is characterized by embryogenesis and growth with no dramatic morphological changes from tadpole to frog. Premetamorphosis occurs from NF stage 1 to NF stage 53. The thyroid develops during this stage, but is not active.

The prometamorphic stage begins approximately at NF stage 54. This stage is characterized by rapid growth, hind limb development and thyroid hormone initial activity. Thyroxine (T4) and 3, 5, 3’ triiodothyronine (T3) are released from the thyroid glands at this time. At this stage the tadpole reaches its maximum size (Tinsley and Kobel 1996).

The emergence of forelimbs signals the onset of metamorphic climax. This begins at approximately NF stage 58/59. Within this stage T4 and T3 reach their peak concentration. Thyroid hormone receptors also increase in target tissues. In addition to forelimb emergence, many morphological changes and physiological changes occur. Some of these are tail and gill resorption, and craniofacial restructuring, gut remodeling (herbivory to carnivory), ocular muscle development, larval hemoglobin changing to the adult form, and ammonia excretion changing to urea excretion (McDiarmid and Altig 2000, Tinsley and Kobel 1996). At the onset of metamorphic climax, tadpoles stop feeding until gut remodeling is completed at approximately stage 62/63 (Schreiber et al.)
Energy for these stressful activities is obtained by the intense feeding of tadpoles during premetamorphic and prometamorphic periods. The immune system of the tadpoles is also suppressed during remodeling to avoid autoimmune responses. Tadpoles begin feeding again at NF stage 63.

In order for metamorphosis to begin, the tadpole must reach an optimal weight and stage of development (McDiarmid and Altig 2000). Factors such as temperature, predator presence, feeding, water levels, and chemicals may have effects on the rate at which metamorphosis takes place (Shi 2000). Stressors can impact on the central nervous system causing the hypothalamus to produce Corticotropin Releasing Factor (CRF) (Crespi and Denver 2005). This factor stimulates the pituitary gland to produce Thyroid Stimulating Hormone and adrenocorticotropic hormone (ACTH) (Shi 2000, Shi and Hayes 1994). TSH stimulates the thyroid gland to produce thyroid hormones. The main thyroid hormone produced by the thyroid gland is tetraiodothyronine (T4) with a smaller concentration of triiodothyronine (T3). These two hormones circulate throughout the organism’s body as free molecules or bound to serum albumin or transthryetin. Free hormones bind to thyroid receptors on target tissues, which then trigger the morphological and physiological changes seen at metamorphic climax (Shi 2000). Increased TSH may act on the hypothalamus and pituitary as negative feedback. In addition to this route, ACTH produced by the pituitary, may stimulate interrenal glands, to produce corticosteroids, which may also act upon thyroid glands and regulate metamorphic rate.
Intestinal Development of *Xenopus laevis*

Shi and Hayes (1993) stated that intestinal development in *Xenopus laevis* contains two phases. The first is the larval or tadpole phase and the second is a more complex adult-like intestinal phase. Shi and Hayes (1993) used intestinal fatty acid binding protein (IFABP), whose gene expression is restricted to intestinal epithelial cells, to develop a profile of intestinal development of *Xenopus laevis*. They found that intestinal epithelial cells begin differentiation at stage NF 33/34. This is interesting as feeding of the tadpole begins at NF stage 45. The premetamorphic tadpole’s duodenum is a thin tube with large involution (Schreiber et al. 2005). Nieuwkoop and Faber (1994) reported that by stage 20 the embryonic archenteron has developed into foregut, midgut and hindgut. By NF stage 40 the intestines have lengthened, narrowed and formed coils. At stage 45, when feeding begins, the intestine has a spiral shape (Nieuwkoop and Faber 1994). Many studies show that thyroid hormone is responsible for the activation of early response genes, which bring about the changes that underlie intestinal metamorphosis (Schreiber et al. 2005). At stage 59, larval intestinal epithelial cells begin to undergo apoptosis. This loss of cells continues until stage NF 62 in the proximal intestine, and until NF 63 in the distal parts of the small intestine. As larval epithelial cells die, adult primordial cells are detected and rapidly begin to proliferate. By stage NF 62/63, morphogenesis of the intestine is almost complete, and larval epithelial cells are replaced by adult ones (Ishizuya-Oka et al. 1997). From NF stage 58/59 to stage 62, a duration of eight days, the tadpole intestine has shortened by 75% (Schreiber et al. 2005). Tadpoles at NF stage 62, also have intestinal epithelial cells
that heap into as many as eight cell layers compared to one layer before metamorphosis. This is attributed to increased contraction of the mucosa of the intestine (Schreiber et al. 2005). This process causes disruption of cells in the mucosal region and sloughing of dead epithelial cells into the lumen. Dead cells are excreted out of the animal through the anus (Schreiber et al. 2005).

Summary of Dissertation Research

In this thesis, I carried out research to better define the relationship between the potential environmental contaminant, atrazine, and the health of aquatic organisms. First, I examined effects of atrazine on *Xenopus laevis* tadpoles treated from 5 days post fertilization to NF stage 65/66. In order to accomplish this, I exposed laboratory spawned tadpoles to atrazine at a concentration of 0 ppb (control) and 25 ppb. These were then collected and analyzed for metamorphic rate, and growth (length and weight). This study showed that there were no significant difference in body weight, length and metamorphic rate after treatment with 25 ppb atrazine.

Second, I used DNA microarrays to examine mRNA expression levels in 25 ppb atrazine treated and untreated tadpoles. This was done because subtle genetic changes may have occurred that may have an effect on the fitness of the adult, although there was no significant overt alteration in larval stages. I found significant changes in gene expressions levels of 18 genes. These genes were obtained by first removing all absent calls in the raw dataset of DNA microarray analysis and using the MannWhitney nonparametric t-test to obtain significantly different transcripts, treated compared to
control tadpoles. Only significantly different genes with a p value of 0.05 and less were considered. Further filtering was done by setting a limit of +1.5 fold change and above for upregulated genes and -1.5 fold change and below for downregulated genes. Expressed sequence Tags (EST’s) were removed, and only named genes were considered. These 18 genes in the final dataset were divided into biologically functional groups, that were related to immunity and defense (5 genes), protein degradation (2 genes), DNA binding and repair (3 genes), and signal transduction (2 genes). I used quantitative RT-PCR to validate 2 candidate genes from the microarray dataset: Proteasome beta and Calbindin D28K. One was found to be not significant while Calbindin D28K was near significant (p=0.072).

Third, I observed the effects of atrazine at a higher concentration on *Xenopus laevis* tadpoles. Laboratory tadpoles were again spawned and reared in atrazine at the sublethal concentration of 400 ppb after 5 days post fertilization to NF stage 62 (Nieuwkoop and Faber 1994). As in the 25 ppb atrazine exposure study, endpoints assayed were metamorphic rate, growth (length and weight), and gene expression changes. At 400 ppb concentration of atrazine, I found significant differences in metamorphic rate, growth rate (weight and length), and fat body development of the treated tadpoles as compared to untreated tadpoles.

In order to identify gene expression differences underlying the phenotypic effects of atrazine treated tadpoles compared to control tadpoles, I utilized DNA microarray gene expression analysis. Raw data were filtered as mentioned previously. Tadpoles treated with 400 ppb atrazine had 49 genes with significantly altered gene expression.
These included genes involved in digestive system function (10 genes), blood and plasma function (8 genes), immunity (3 genes), cell adhesion (12 genes), transcription and chromosomal activity (5 genes), ion transport (2 genes), cell cycle (1 gene), thyroid related (1 gene), neuroprotection (2 genes), signal transduction (3 genes) and cytoskeleton (1 gene).

In order to validate the DNA microarray expression data, qRT-PCR was used on 4 candidate genes from the microarray dataset: pancreatic trypsin (upregulated), chitinase (upregulated), chitobiase (downregulated) and CYP-P450 (downregulated). These were chosen based on their level of signal intensity, p-values, strength of fold change, and variability of expression among individuals in the study. Three out of 4 of the transcripts chosen for qRT-PCR were congruent with DNA microarray analysis.

In summary, I found that exposure of tadpoles to 25 ppb atrazine produced no significant changes in metamorphic rate, and growth rate (length and weight). When exposed to an atrazine concentration of 400 ppb, tadpoles showed significant differences in weight, length, metamorphic rate and fat body development. Slower metamorphic rate and a smaller body size may be factors that affect the fitness of tadpoles in terms of reproduction and ability to find food and escape predators.

Interestingly, microarray data showed statistically significant differences in expression of 10 transcripts related to carbohydrate and protein metabolism. (e.g., trypsin, amylase, elastase, and chitinase). This is consistent with phenotypic observations of atrazine induced reductions in weight and fat body size.
On a broader perspective, the work in this thesis demonstrates how DNA microarrays and qRT-PCR can be used as a tool to quantitatively define how anthropogenic substances alter gene activity patterns that underlie organismal health. It also demonstrates how these techniques can be used to create physiological roadmaps, which can be used to identify and, and in the future, treat health effects induced by exposure to chemicals found in the environment.
CHAPTER 2

MOLECULAR ANALYSIS OF TADPOLES TREATED WITH 25 PPB ATRAZINE USING DNA MICROARRAY AND QRT-PCR GENE EXPRESSION TECHNIQUES

Abstract

Atrazine, the most common herbicide used in the United States, has been implicated as a factor in the worldwide decline of amphibians. Affymetrix DNA microarrays were used to measure changes in gene expression of *Xenopus laevis* tadpoles treated with a concentration of 25 ppb atrazine from 5 days post fertilization to NF stage 66. There were no significant changes in metamorphic rate and weight when treated tadpoles were compared to controls. Statistically significant changes in expression of 18 genes (15 downregulated and 3 upregulated) occurred in atrazine treated *Xenopus laevis* tadpoles when compared to control tadpoles. The downregulated genes were associated with biological functions as immunity and defense, protein degradation, DNA binding and repair, protein binding and transport and glucose metabolism. Of the 3 upregulated genes 2 were related to defense and 1 to DNA binding. Two genes from the final microarray dataset were chosen to undergo qRT-PCR validation (proteosome beta, Fold Change $-1.52$; $p=0.024$; and calbindin D28K, Fold Change, $-1.94$; $p=0.012$). The proteasome gene was not significantly different while the Calbindin D28K gene was near significant ($t$-test, $p=0.07$). These

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data indicate that atrazine treated tadpoles show gene expression changes at a low ecologically relevant concentration.

Introduction

A growing body of literature has implicated atrazine as one of the factors responsible for the worldwide decline of amphibians (Hayes et al. 2002, Hayes et al. 2003, Sullivan and Spence 2002, Tavera-Mendoza et al. 2002). While some laboratory studies have reported changes in testicular morphology, laryngeal muscle sizes and plasma testosterone levels at concentrations of 25 ppb and lower (Goldman and Carr 2003, Hayes et al. 2002, Hayes et al. 2003, Kinyamu et al. 2005) others have found no overt effects at these concentrations (Coady et al. 2005, DuPreez et al. 2003, Hecker et al. 2005b). In all of these studies, however, there were no changes in metamorphic rate, mortality, weight, and sex ratios.

Organismal responses to anthropogenic substances such as atrazine can be subtle and involve changes in physiology that are not readily detectable using traditional toxicological methods. Rather, these substances may cause subtle changes that have major impacts over the life cycle. For example, endocrine disruptors do not always harm organisms in an acute manner, yet may limit their reproductive capability such that species fitness is reduced. Since frog populations are in worldwide decline, understanding the basis of subtle long-term problems in adapting to anthropogenic chemicals is important. Use of DNA microarrays to assay global gene expression patterns will help define the frog’s adaptive responses to atrazine. Using this method,
specific changes in gene pathways that control specific physiological processes can be easily detected. Changes in the expression of specific genes may underlie subtle physiological effects of low levels of atrazine, which may provide new molecular tools for risk assessment related to atrazine in the environment.

This study provides a roadmap of atrazine-induced changes in gene expression in male *Xenopus laevis* frogs reared in 25 ppb atrazine from 5 days post fertilization (NF stage 43) to metamorphosis (NF stage 65/66). There were 18 genes in the final filtered affymetrix dataset. There were 15 downregulated and 3 upregulated genes. Gene expression data were grouped according to physiological functions. The majority of atrazine-induced changes in gene expression occurred in the following groups: structural support, transport, protein degradation and modification, DNA repair, binding and transcription, translation and protein synthesis and metabolism.

**Materials and Methods**

**Animals**

*Xenopus laevis* tadpoles were obtained by injecting adult mating pairs (Western Michigan University Animal Facility) with human chorionic gonadotropin hormone (Sigma, St. Louis, MO), and raised in spring water (Absorpure, Plymouth, MO). Tadpoles were staged once a week, using a Wild Heerbrugg Apochromat dissecting microscope, according to Nieuwkoop and Faber (1994). Tadpoles were fed daily ad libitum with Nasco Brittle tadpole chow (Fort Atkinson, WI). Animals were treated in
accordance with an animal use protocol approved by the Institution Animal Care and Use Committee at Western Michigan University. Kalamazoo, MI, USA (Appendix).

Chemical Exposure

Tadpoles were exposed to technical grade atrazine (Sigma, St. Louis, MO) starting 5 days post fertilization (stage 43, according to Nieuwkoop and Faber, 1994) through stage 65/66 (total tail resorption). Experimental treatment bowls contained four tadpoles placed into each of 7 glass dishes containing 1L of water and 25 ppb atrazine concentration (n= 28 tadpoles). The concentration of atrazine was verified by Kar Laboratory (Kalamazoo, MI; project # 044162; method: EPA 8000). Control treatment bowls contained four tadpoles placed into each of 7 glass dishes containing 1L of water (n=28). Each tadpole was collected when it reached stage 65/66. Water temperature and tadpole stages were determined before every change of rearing solution. Tadpoles that survived to NF stage 65/66 were euthanized using tricaine methanesulfonate (MS222, 1:2000 dilution), snap frozen in liquid nitrogen and stored in the -80°C freezer.

Gross Morphology

Assessment of gross morphology was performed on all tadpoles that were collected at NF stages 65/66 (Nieuwkoop and Faber 1994) by direct visual inspection. This method is described in the methods section of Chapter 3.
Metamorphic Rate Analysis

Metamorphic rate was calculated by comparing the mean time (in days) to complete metamorphosis (stage 65/66) for all the tadpoles reaching metamorphosis from both the atrazine treated (n= 20) and control tadpole (n=25) groups. Mean values were analyzed using Fisher’s t-test (SAS).

Weight and Length Analysis

Male control (n=9) and treated (n= 11) tadpoles reaching NF stage 65/66 were euthanized and measured for weight and length. Length was measured from the tip of the snout to the end of the tail. Fisher’s t-test (SAS) was used to calculate statistical differences between control and atrazine treated tadpoles.

RNA Isolation

Total RNA was isolated from whole tadpoles using Qiazol reagent and Qiagen RNeasy Maxi kits (Qiagen. Velencia, CA) following the manufacturer’s instructions. RNA was isolated from 5 tadpoles from each treatment group. The RNA concentration in the solution was determined using a GeneQuant Pro Spectrophotometer (Amersham Pharmacia Biotech, Piscataway, NJ). The total RNA was concentrated using an ethanol precipitation method.
Microarray Analysis

Samples were prepared for microarray analysis according to Affymetrix GeneChip® protocols. The protocol for preparing RNA for microarray analysis and the method used for filtering and analyzing the microarray dataset is described in Chapter 4 of this document.

Taqman qRT-PCR Procedure and Analysis

Two genes chosen from the final Affymetrix dataset were validated using Taqman qRT-PCR. The genes were chosen based on the fold change difference and signal intensity (expression level) derived from the microarray analysis. The 2 genes were: calbindin D28K and proteasome beta. The qRT-PCR protocol and data analysis are described in the methods section of Chapter 4 of this document.

Results

Phenotypic Analysis of 25 ppb Atrazine Treated Tadpoles

Stage, length, weight of frogs and days to reach stage 65/66 were measured and recorded for both control and atrazine treated groups. In the control group there were 9 male and 16 female control tadpoles collected. Three mortalities were recorded. In the atrazine treated group there were 11 males and 9 females; 8 mortalities recorded (Table 1). There were no obvious changes in external or gonadal morphology between the atrazine treated tadpole group and the control group. Stages between treated and

26
control tadpoles were not significantly different. Treated tadpoles were not significantly heavier than controls.

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th># Males</th>
<th># Females</th>
<th>Mortality</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>9</td>
<td>16</td>
<td>3</td>
<td>28</td>
</tr>
<tr>
<td>25 ppb</td>
<td>11</td>
<td>9</td>
<td>8</td>
<td>28</td>
</tr>
</tbody>
</table>

Table 1: Total number of male and female tadpoles collected, including number of mortalities, from the total number of control (n=28) and treated (n=28) tadpoles in the study.

Global Gene Expression

The total number of genes significantly altered in atrazine treated tadpoles when compared to controls was 1,034 out of the 15,500 transcripts on the Affymetrix *Xenopus laevis* gene chip (Table 2). After filtering, using criteria described in the methods and materials section of chapter 4 of this document, there were 18 transcripts that were significantly altered. As shown in Table 3, a high number of the 18 genes were downregulated (15 genes) while only 3 genes were upregulated. Genes may have multiple roles in an organism, but in this study each gene was placed in one functional group. Altered genes were grouped into 5 major functional groups. These were immunity/defense (5 genes), DNA binding or repair (3 genes), protein binding/transport (4 genes) and metabolism (1 gene), protein degradation (2 genes). There were two genes with unknown function.
<table>
<thead>
<tr>
<th>Treatment Group</th>
<th># of genes after initial statistical analysis (No filtering)</th>
<th># of genes upregulated in final dataset</th>
<th># of genes downregulated in final dataset</th>
</tr>
</thead>
<tbody>
<tr>
<td>25 ppb atrazine</td>
<td>1034</td>
<td>3</td>
<td>15</td>
</tr>
</tbody>
</table>

Table 2: Number of transcripts, in initial dataset after DNA microarrays analysis, and the number of transcripts remaining in the final dataset after filtering with fold change cut off values of +1.5 or -1.5, and removal of ESTs.

Proteasome Beta and Calbindin D28K mRNA Levels from 25 ppb Atrazine Treated Tadpoles Were Not Significantly Different Than Those from Control Tadpoles

Proteasome beta (Fisher’s t-test, p= 0.59, n=5) and Calbindin D28K (Fisher’s t-test, p= 0.07, n=5) mRNA expression levels measured using qRT-PCR were not significantly different to controls in atrazine treated tadpoles when compared to control tadpoles. However, Calbindin D28K was near significant. Both these mRNA levels were significantly downregulated in the microarray array dataset (Table 3).
<table>
<thead>
<tr>
<th>Affymetrix Code</th>
<th>Gene Name</th>
<th>Fold Change</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Immunity/Defense</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>XL21890.1.S1_at</td>
<td>MHC class II beta-chain</td>
<td>-2.04</td>
<td>0.048</td>
</tr>
<tr>
<td>XL4728.2.S1_a_at</td>
<td>glutathione S-transferase</td>
<td>-1.84</td>
<td>0.043</td>
</tr>
<tr>
<td>XL16323.1.S1_at</td>
<td>pre-xenoxin-1</td>
<td>+1.54</td>
<td>0.024</td>
</tr>
<tr>
<td>XL21625.1.A1_at</td>
<td>mucin B.1</td>
<td>+1.78</td>
<td>0.044</td>
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<tr>
<td>XL46.1.S1_at</td>
<td>tyrosine phosphatase</td>
<td>-2.01</td>
<td>0.029</td>
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<tr>
<td><strong>Protein Degradation</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>XL440.1.S1_at</td>
<td>proteasome beta</td>
<td>-1.52</td>
<td>0.024</td>
</tr>
<tr>
<td>XL21618.1.S1_x_at</td>
<td>proteasome subunit Y</td>
<td>-1.51</td>
<td>0.044</td>
</tr>
<tr>
<td><strong>DNA Binding/DNA Repair/transcription</strong></td>
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<td></td>
<td></td>
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<td>XL8230.1.S1_at</td>
<td>leucine zipper</td>
<td>-1.66</td>
<td>0.043</td>
</tr>
<tr>
<td>XL5475.1.S1_at</td>
<td>flap endonuclease 1</td>
<td>-1.52</td>
<td>0.043</td>
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<tr>
<td>XL3152.1.S1_at</td>
<td>FD-4 protein</td>
<td>+1.52</td>
<td>0.024</td>
</tr>
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<td><strong>Binding proteins/Adaptor/Transport</strong></td>
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<td></td>
<td></td>
</tr>
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<td>XL137.1.S1_at</td>
<td>calbindin D28k</td>
<td>-1.94</td>
<td>0.012</td>
</tr>
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<td>XL17371.1.S1_at</td>
<td>apolipoprotein A-I</td>
<td>-1.97</td>
<td>0.012</td>
</tr>
<tr>
<td>XL1201.1.S1_at</td>
<td>galectin family xgalectin-IIa</td>
<td>-1.88</td>
<td>0.012</td>
</tr>
<tr>
<td>XL513.1.S1_at</td>
<td>T-cell factor XTCF-3</td>
<td>-1.6</td>
<td>0.012</td>
</tr>
<tr>
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Table 3: Genes in the final dataset, organized by biological functions. Fold changes and p-values for each gene are depicted. Negative sign (-) in front of fold change represents gene expressions which were downregulated. Fold changes with positive sign (+) represent upregulated gene expression.
Discussion

Phenotypic Analysis of 25 ppb Atrazine Treated Tadpoles

The phenotypic endpoints of this study were gonadal abnormalities, metamorphic rate, weight, and length. There was no observable difference in gonadal morphology when treated tadpoles were compared to control tadpoles. There were no metamorphic rate, weight or length differences between treated and control tadpoles. This result is in keeping with published data (Coady et al. 2005, Hayes et al. 2003, Nieuwkoop and Faber 1994). As discussed in Chapter 1 of this document there is an ongoing debate as to atrazine’s effects on gonadal differentiation of amphibians (Coady et al. 2005, Hayes et al. 2002, Hayes et al. 2003, Nieuwkoop and Faber 1994, Smith et al. 2003, Spano et al. 2004). There are several possible factors that could cause abnormalities in some groups and not others.

First, the difference in strains of Xenopus laevis tadpoles might underlie differences in susceptibility to atrazine. This has been known to happen in rats where one strain, Sprague Dawley (SD) rats treated with atrazine showed early onset mammary gland tumors while another strain, Fischer 344, did not (Wetzel et al. 1994).

Secondly, Hayes used plastic shoeboxes to rear the tadpoles while others, including this laboratory, used glass. Plastics may leach chemicals that may cause endocrine disruption to occur, for example, phthalates are added to polyvinyl chloride (PVC) plastics to make them more pliable, while Bisphenol A is used in the manufacture of polycarbonate and plastics including baby bottles and tableware. Its
mode of action mimics the action of estrogen. This has caused serious concerns in the scientific community, especially in relation to males (Latini et al. 2004). The synergistic action of both atrazine and estrogenic compounds that may leach out from the plastics may feminize males.

Another factor may be that different laboratories use varying grades of atrazine in their experiments. Also, inert substances present in the atrazine mixture may be toxic to tadpoles (Cox 2001). Hayes, in his review, highlights poor rearing conditions such as overcrowding of tadpoles, poor feeding and rearing water changing practices as factors which may have caused the negative results seen in other studies (Hayes 2004). In this laboratory precautions were taken to have a density of 4 tadpoles per liter of water, 100 percent water was changed every two days and tadpoles were fed daily. This is in keeping with EPA standards of husbandry of *Xenopus laevis* tadpoles.

Atrazine Induced Changes in Global Gene Expression in Male *Xenopus laevis* Frogs

This is the first study to use DNA microarray analysis to characterize the global effects of atrazine on *Xenopus laevis* tadpoles at NF stage 65/66. The expression of 18 genes was altered by 25 ppb atrazine treatment. Analysis showed that genes were concentrated in 5 basic physiological areas. These were immunity, protein degradation, DNA binding and repair, and metabolism. It must be noted that tadpoles were collected at NF stage 65 so that these results reflect gene expression changes at this stage. Other significant genes may have not been expressed at this stage, but in an earlier stage.
Atrazine Induced Downregulation of Genes Involved in Immunity and Defense

Of the 18 genes that were significantly altered by atrazine, 3 genes were involved in immunity (MHC class II beta, glutathione S-transferase, and tyrosine phosphatase). All 3 genes were downregulated.

There is a growing body of evidence that suggests that atrazine may be involved in suppressing the immune system of amphibians by altering lymphocyte proliferation and phagocyte activity (Forson and Storfer 2006). Atrazine treated amphibians show increased susceptibility to bacterial and parasitic infections. This was highlighted dramatically in studies where atrazine treated tadpoles were more susceptible to trematode infection, which in turn, caused limb deformities in the animals (Kiesecher 2002).

Major Histocompatibility Complex II is a heterodimeric cell surface receptor that is involved in antigen presentation of peptide fragments to T-Cells that are responsible for cell-mediated immune responses to extracellular pathogens (Sherwood 2001). This stimulates the B-cell development and activation of inflammatory T-Cells. Downregulation of this complex may compromise immune function, making the amphibian susceptible to pathogens (Kiesecher 2002). This suggests that atrazine could be causing suppression of the immune system by downregulating the activity of this complex even at the low concentration of 25 ppb.

Tyrosine phosphatase plays an integral role in cellular processes such as signal transduction, cell proliferation, differentiation, and transport. It is also involved in the immune response (Mizuno and Yakura 2005). Tyrosine phosphatase is necessary for
signaling to occur in T-Lymphocytes, B-Lymphocytes and Natural killer cells (Mustelin et al. 2005). It works in concert with MHC complex II for antigen presentation to T helper cells (Kuhn et al. 1998).

Mucin B1 which is a glycoprotein that is secreted to protect epithelial cells from the external environment (Joba and Hoffman 1997, Strous and Dekker 2003) was upregulated in this dataset. This could suggest a protective measure to decrease atrazine effects.

Gluthathione S-Transferase is active in atrazine resistant crops and in some vertebrates, helping detoxification and excretion of atrazine (Andrews et al. 1997, Kelner et al. 2000). It does this by catalyzing the conjugation of glutathione to the electronegative atrazine by either nucleophilic subtraction or sulphoxide splitting. In vertebrates, atrazine is excreted out of the liver through the bile duct into faeces, or through the kidneys where it is urinated out of the body. In plants, its activity is mostly concentrated in the leaves of the plant (Anderson and Gronwald 1991, Favaloro et al. 2000, Wiegand et al. 2000). In the present study, there was a decrease of Gluthathione S-transferase activity in atrazine treated tadpoles. Decreased activity may increase susceptibility to other toxic compounds.

Atrazine Induced Downregulation of Gene Expression Involved in Proteasome Function

The 26S proteasome multienzyme complex is expressed in the cytoplasm and nucleus of all eukaryotic cells (Adams 2003). It is made of two 19S Regulatory complexes that act as caps on either ends of the 20S catalytic core complex, which is a
barrel shaped structure that possess two inner rows of alpha rings each containing 7 subunits, and two outer beta rings of seven subunits each (Adams 2003, Groll and Clausen 2003). The main function of this complex is to degrade and recycle damaged or misfolded proteins. Proteins to be degraded are first tagged by ubiquitin enzymes, attached to the 19S Regulatory Cap, and then degraded in the 20S. This system plays an essential role in the degradation of proteins with short half lives, playing a role in cellular functions that include cell cycling, cell surface receptors, ion channels, and antigen presentation (Adams 2003, Groll and Clausen 2003, Ikezoe et al. 2004). Recent studies have shown that proteasomes are not only involved in degradation of proteins, but also in protein trafficking and transcriptional elongation. It was also demonstrated that proteasome activity is required for transcription and nuclear translocation of the androgen receptor (Hui-Kuan et al. 2002). Hui-Kuan et al (2002) found that when a proteasome inhibitor, MG 132, was administered to COS-1 cells also treated with DHT (5 alpha- dihydrotestosterone, a testosterone metabolite), translocation of AR from the cytoplasm to the nucleus was significantly suppressed and transactivation was inhibited. The study also demonstrated that interaction between the AR and its coregulators was significantly reduced with proteasome inhibition, Thus, the AR may not be able to efficiently bind to its promoter site on a gene target, reducing AR induced transcription (Hui-Kuan et al. 2002, Tanner et al. 2004). Since atrazine downregulates proteasome subunits this may have a potential effect on testosterone levels.
Atrazine Induced Downregulation of Binding Proteins

The present study demonstrated that Calbindin D28K mRNA was downregulated by atrazine treatment of male tadpoles. Calbindin is a calcium binding protein, which is found throughout the central nervous system and is important for this system’s development and function. This protein acts as a buffer to intracellular calcium and plays a critical role in neuroprotection by inhibiting apoptosis (Perez and Kelley 1997). Previous studies have reported that calbindin expression is sexually dimorphic in the medial basal hypothalamus and preoptic area (MBH-POA) of the rat brain, an area involved in reproductive behavior and endocrine function. It also plays a role in neurogenesis of sexually dimorphic structures (Lephart 1996, Stuart et al. 2001).

The male sexually dimorphic nuclei (SDN) in the MBH-POA is larger than the female because of the presence of calbindin which protects the cells from apoptotic degradation. If these nuclei degenerate in males, then feminine development occurs. Monitoring changes in size of this area of the brain might serve as a biomarker for the effects of endocrine disrupters, because its size is affected by androgen or estrogen exposure (Scallet et al. 2004). In western blot studies, flutamide (androgen receptor blocker)-treated Sprague-Dawley rats were tested for Calbindin D28K levels in the sexually dimorphic region. The males’ fetal MBH-POA calbindin level was significantly decreased. This did not occur in the females. In testosterone treated only animals, there was no significant change in calbindin levels in the male animals (Scallet et al. 2004). This demonstrates that the AR is important for Calbindin D28K functioning in the MBH-POA.
Apolipoprotein A1, manufactured in the liver, is secreted into plasma where it transports lipids, phospholipids and cholesterol, which are usually insoluble (Tachibana et al. 2003). This protein promotes the removal of lipids from tissues and transports it to the liver where it is processed (Chan et al. 1993). A deficiency in apolipoprotein A1 causes increased cholesterol and lipid levels that lead to an increased risk of atherosclerosis (Zou et al. 2003). Apolipoprotein A1 was downregulated in the present study.

**Atrazine Induced Downregulation of Genes Involved in Signal Transduction**

The 14-3-3 protein is an adaptor protein implicated in a wide range of biological processes. It is involved in signal transduction, cell division, apoptosis and transmitter release. It is involved in transactivation of nuclear proteins such as Glucocorticoid receptor and androgen receptor (Huang et al. 2004).

Glucocorticoid receptor proteins are involved in glucose metabolism because they mobilize glucose and other nutrients from storage during the stress response. Glucocorticoid receptors serve as transcription factors because they bind to glucocorticoid response elements (Aguilar-Valles et al. 2005). In the present, study there was a decrease in glucocorticoid receptor mRNA. Absence of glucocorticoid receptor activity has been linked to numerous metabolic abnormalities including weight irregularities, growth retardation, and immune suppression.

It is interesting to note that there were no overt phenotypic changes in 25 ppb atrazine treated tadpoles when compared to controls.
ATRAZINE INDUCED PHENOTYPIC EFFECTS ON MALE *XENOPUS LAEVIS* TADPOLES EXPOSED IN THE LABORATORY TO 400 PPB OF ATRAZINE MOLECULAR

Abstract

Atrazine is an herbicide that has been implicated in endocrine disruption, which may be one of the factors that is contributing to the worldwide decline of amphibians (Forson and Storfer 2006). The concentration at which atrazine causes health effects on amphibians has been vigorously debated (Carr et al. 2003, Coady et al. 2005, Hayes et al. 2002, Hayes et al. 2003). In the present study, tadpoles were exposed to a 400 ppb concentration of atrazine from 5 days post-fertilization and allowed to develop until stage 62 (Nieuwkoop and Faber 1994). Exposure to this concentration of atrazine caused significantly reduced growth (weight, \( p=0.0001 \); length, \( p=0.0005 \)), reduced fat body development (\( p=0.0001 \)), and reduced metamorphic rate (\( p=0.0165 \)) when compared to controls. The adverse phenotypic effects associated with exposure of tadpoles to 400 ppb atrazine during development serve as useful endpoints for correlated molecular based analysis presented in the next chapter.
Introduction

Atrazine has been implicated in affecting amphibians at low ecologically relevant concentrations. A number of studies have demonstrated that atrazine adversely affects *Xenopus laevis* tadpoles at concentrations as low as 0.1 ppb (Hayes et al. 2002, Hayes et al. 2003). This concentration is 30 times lower than the concentration designated as safe by the US EPA. However, other studies have failed to show any adverse effects at this very low concentration (Carr et al. 2003, Coady et al. 2005), but have reported changes in physiology after exposure to higher concentrations.

In the study presented in the previous chapter, 25 ppb atrazine produced no observable phenotypic differences between control and treated tadpoles. Because few changes were observed in the 25 ppb atrazine studies, a higher concentration of 400 ppb was selected as a basis for further studies.

Atrazine runoff concentrations as high as 480 ppb to 500 ppb have been reported in several agricultural areas (Allran and Karasov 2001, deNoyelles et al. 1982, Larson et al. 1998). Rohr et al. (2003) demonstrated that amphibians (*Ambystoma barbouri*) treated with 400 ppb of atrazine, significantly increased motile activity, as compared to controls, when the glass of their tank was tapped, suggesting an underlying nervous malfunction. Treated tadpoles also exhibited decreased foraging behavior because of lethargy caused by atrazine.

The main objective of the present study was to define phenotypic effects of 400 ppb atrazine on tadpoles treated from stage NF stage 43 (5 days post fertilization) to stage 62. Significant differences occurred in metamorphic rate, growth (weight and
length), and fat body size. These findings suggest that there may be an underlying metabolic dysfunction in the atrazine treated tadpoles.

Materials and Methods

Animals

*Xenopus laevis* tadpoles were obtained by injecting mating pairs (Western Michigan University Animal Facility) with human chorionic gonadotropin (Sigma, St. Louis, MO). Tadpoles were reared in purified tap water and staged once a week according to Nieuwkoop and Faber (1994). Tadpoles were fed daily with commercially available purina rabbit chow (Purina Mills LLC, MO) ad libitum and changed into fresh water every 48 hours. Water temperature and tadpole stage were determined before every change of rearing water. Animals were treated in accordance with an animal use protocol approved by the Institution Animal Care and Use Committee at Western Michigan University.

Chemical Exposure

Tadpoles were exposed to technical grade atrazine (99.4% purity, Sigma, St. Louis, MO) from five days post fertilization (stage 43, according to Nieuwkoop and Faber, 1994) through NF stage 62. Four tadpoles were placed into each of 15 dishes (n= 60 tadpoles) with rearing solution containing an atrazine concentration of 400 ppb and, into 20 control dishes with 4 tadpoles each (n= 80 tadpoles). Of the 80 control
animals, 36 were frozen for molecular analysis, 20 were euthanized for histological analysis and 24 were found dead. Of the 60 treated tadpoles, 24 were collected for molecular analysis, 12 for histological analysis, and 24 were found dead. Because the rate of metamorphosis can be affected by tadpole density, 4 tadpoles were kept in each bowl at all times. Stage data collection for statistical analysis was discontinued from the first day of tadpole collection so that density was not a factor in analysis of metamorphic rate. Metamorphic rate data were collected from day 17 to day 47. At stage 62, tadpoles were euthanized using tricaine methanesulfonate (MS222, 1:2000 dilution), snap frozen in liquid nitrogen and stored in a -80°C freezer. Twelve males, at NF stage 62, were chosen from among the frozen tadpoles (6 controls and 6 atrazine treated) to be used in microarrays analysis.

Gross Morphology

Gross morphology was assessed by direct inspection of each of the tadpoles at collection. Gender was determined by analyzing the presence of either testes or ovaries in each tadpole. In order to assess this, tadpoles were euthanized with MS222, pinned to a dissecting dish of hardened paraffin wax, and placed under a dissecting microscope. Forceps were used to lift the skin between the hind legs of the tadpole, and a pair of scissors was used to cut along the centre of the body from the cloaca to the thorax. The skin was then cut towards the side of each leg. The body cavity was opened by cutting away the abdominal muscles and breastbone. The intestines were displaced away from the body cavity displaying the kidneys in the retroperitoneal
region. Testes were slender, transparent, unpigmented and unlobulated strips of tissue lying medially on each kidney. Ovaries were longer than testis (running the length of the kidneys) pigmented and lobular, lying in the same position as the testes. Fat bodies are yellow finger like projections situated anteriorly on both sides of the kidneys.

**Statistical Analysis**

**Mortality Rate Analysis**

The difference in mortality rate between control animals and those raised in 400 ppb atrazine was tested using the accelerated failure time model implemented by SAS™. The developmental NF stage at death or sacrifice was used as a covariate in the model. The lognormal distribution was used as the error distribution in the model, because the log of the natural death times fit the normal distribution for both groups of animals. The Shapiro-Wilkes test was used to test the hypothesis of normality. For both groups the hypothesis of normality was not rejected at the 5% level. This test was done using Proc Univariate in the SAS™ software.

**Growth (Weight and Length) and Fat Body Content**

Male control (n=16) and treated (n=17) tadpoles reaching NF stage 62 were euthanized and measured for weight and length. Length was measured from the tip of the nose to the end of the tail. Fisher's t-test was used to calculate statistical differences between control and atrazine treated tadpoles. Fat body areas were measured for control (n= 23) and treated (n= 33) tadpoles. The Metamorph and Image J 1.34s
computer programs were used to photograph and measure the area of the fat bodies of tadpoles, from the group collected for histological analysis, at a magnification of 2X. Fisher’s t-test was used to analyze statistical significance between control and atrazine treated tadpoles.

**Metamorphic Rate Analysis**

Data for metamorphic rate analysis was recorded using NF measurements. These were collected once a week for all tadpoles. In order to determine if there was a statistically significant difference in developmental stages between the control and atrazine treated tadpoles, a common slope cumulative logistic regression model was used (SAS™, PROC Logistic). This model takes into account any potential effects that may be due to differences in rearing bowls.

**Results**

**Exposure to 400 ppb Atrazine Had No Effect on Mortality**

There was a mortality of 26 tadpoles in the control group (n=80) and 25 tadpoles in the atrazine treated group (n=60). The accelerated failure time model showed no significant difference in mortality rates between the two groups (p= 0.23).
400 ppb Atrazine Treated Tadpoles Showed Metamorphic Rate Differences when Compared to Control Tadpoles

Metamorphic rate of tadpoles treated with 400 ppb atrazine (n=80) was significantly slower (p=0.0165) than control tadpoles (n=60).

Atrazine Treated Tadpoles Have Smaller Terminal Weights Than Control Tadpoles at Stage 62

Tadpoles collected at NF stage 62 were weighed and measured prior to gonadal determination. Control tadpoles (n=17) were significantly heavier than 400 ppb atrazine treated tadpoles (Figure 1, p<0.0001, n=16).

Figure 1: Mean weight of atrazine treated tadpoles vs. control tadpoles (Fisher’s t-test, *p=0.0001).
Atrazine Treated Tadpoles Showed Significantly Reduced Length When Compared to Control Tadpoles

When tadpoles were treated with 400 ppb atrazine and their whole body length (tip of snout to end of tail) measured at NF stage 62, control males (n=17) were significantly longer than treated males (n=16) (Figure 2, p=0.0005, Fisher’s t-test).

Figure 2: Mean length in mm for *Xenopus laevis* tadpoles treated with atrazine and control tadpoles. Statistically significant decreases (Fisher’s t-test, *p* = 0.0005) occurred in treated tadpoles (n = 16) compared to control tadpoles (n=17).

400ppb Atrazine Treatment Causes Underdevelopment of Fat Bodies in *Xenopus laevis* Frogs

Control males (n=23) possessed large fat bodies, which appeared to cover a large portion of the kidney surface (Figure 3A). Fat bodies of the 400 ppb atrazine treated tadpoles (n=33) were extremely underdeveloped (Figure 3B). The mean area of fat bodies was significantly smaller in atrazine treated tadpoles than control tadpoles (Figure 4, *p*=0.0001, Fisher’s t-test).
Figure 3: Fat bodies (FB), gonads (T) and kidneys (K) of atrazine treated and untreated males of *Xenopus laevis* tadpoles. Dramatic differences in fat body size occur between control and atrazine treated tadpoles. A) Control male. B) Male atrazine treated tadpoles with underdeveloped fat bodies.
Figure 4: Mean fat body areas in mm$^2$ of control *Xenopus laevis* tadpoles and atrazine exposed tadpoles. Statistically significant decreases occurred in atrazine treated tadpoles compared to control tadpoles (Fisher's t-test, *p* = 0.0001)

**Discussion**

**Exposure to 400 ppb Atrazine Had No Effect on Mortality**

In this study there was no significant difference in rate of mortality between the control and atrazine treated tadpoles. In general, increased mortality can occur at high doses from the toxic effects of atrazine on vital organs such as liver, and kidneys in an attempt to detoxify and excrete atrazine (Morgan et al. 1996, Pechmann et al. 1991, Rohr et al. 2003, Rohr et al. 2006, Rowe et al. 1998). Atrazine at high doses have been known to cause death in rats and other vertebrates with associated respiratory distress and paralysis (Roberge et al. 2004, Rohr et al. 2003, Rohr et al. 2006, Santa-Maria et al. 1987). There are also reported structural changes and hemorrhaging in brain, heart, liver, ovaries and other endocrine glands (Cox 2001). It should also be noted that the *Lowest Observable Effect Concentration* (LOAEC) for *Xenopus laevis* tadpoles is 1.1...
mg atrazine/L (1.1 ppm), which is higher than the concentration used in this study. The No Observed Effect Concentration (NOEC) in *Rana piliens* tadpoles is 200 ppb. The LC$_{50}$ toxicity test with amphibians ranges from 15.8 to 126 mg atrazine/L (15.8 to 126 ppm) (Carr et al. 2003). This suggests that a 400 ppb concentration of atrazine used in the present study is below concentrations known to influence mortality.

400 ppb Atrazine Treated Tadpoles Have Significantly Different Metamorphic Rate When Compared to Control

Results from this present study, demonstrated that *Xenopus laevis* tadpoles treated with 400 ppb atrazine showed significantly reduced metamorphic rate when compared to control tadpoles. This finding is in agreement with other studies conducted with amphibians (Forson and Storfer 2006, Larson et al. 1998, Sullivan and Spence 2002). The fact that metamorphic rate can be influenced by temperature (Warkentin 1992a), tadpole density, food availability (Anholt and Werner 1995), food quality and as a response to stress (Alford and Harris 1988, Kupferberg 1997) was taken into account in the present experiment as temperature was monitored each time the water was changed, food portions were equivalent for all tadpoles, and tadpole density in each bowl was kept equivalent for both groups.

Crump (1981) demonstrated that timing of metamorphosis might be dependent on energy accumulation. The observed delay in metamorphosis, in this present study, could have allowed the tadpole time to accumulate the necessary energy threshold needed to begin metamorphic climax. The conclusion that atrazine could be diverting the energy supply of the tadpole can also be illustrated in the fact that usually tadpoles
which take a longer time to complete metamorphosis are heavier than those that take a shorter time to completion (Crespi and Denver 2005, Larson et al. 1998). However, in this present study, the tadpoles were metamorphically slower and weighed less than their control conspecifics. Tadpoles that complete metamorphosis at a reduced weight have a lower survival rate and decreased reproductive abilities (McDiarmid and Altig 2000). Another factor that could affect the timing of metamorphosis is the stage at which the tadpoles begin to be affected by atrazine. Tadpoles at the premetamorphic stage have nonfunctional thyroid glands that do not produce thyroid hormone in the presence of stress. Added stress may cause them to utilize the maternal energy stores which impedes growth and lengthens the time for metamorphosis (McDiarmid and Altig 2000, Tinsley and Kobel 1996)

400 ppb Atrazine Treated Tadpoles Have Significantly Decreased Growth Rate (Weight and Length) When Compared to Control Tadpoles

The present study demonstrated that tadpoles treated with 400 ppb atrazine weighed significantly less and were significantly reduced in length when compared to control tadpoles. These findings are in agreement with a number of studies which have demonstrated significant growth reduction in various organisms, such as dogs, rats, rabbits and frogs, when treated with atrazine (Cummings et al. 2000a, Laws et al. 2000, Rayner et al. 2005, Trentacosta et al. 2001). Atrazine may affect appetite systems in the hypothalamus which in turn leads to appetite suppression and weight loss (Kandori et al. 2005). The hypothalamus possesses two lateral regions that regulate appetite (McDiarmid and Altig 2000, Nieuwkoop and Faber 1994, Sherwood 2001, Shi 2000)
The paraventricular nucleus of the hypothalamus controls feeding behavior, while the hypothalamic arcuate nucleus controls ingestion (Sherwood 2001). When the lateral region of the hypothalamus is stimulated over eating occurs. When the cells in these areas are destroyed reduced eating occurs. On the other hand, if the ventromedial area (arcuate) (satiety center) is stimulated, reduced feeding occurs and if the area is destroyed, over eating and obesity occurs (Sherwood 2001). There is a growing body of evidence that suggests that atrazine may affect the hypothalamus of various organisms, causing endocrine disrupting effects (Shafer et al. 1999, Smith et al. 2003, Stoker et al. 2000, Tinsley and Kobel 1996, Tran et al. 1996, Trentacosta et al. 2001). While many studies have demonstrated weight loss and food intake reduction in various organisms, the mechanism of atrazine’s effect on food intake has not been elucidated.

Atrazine induced reduction in weight observed in the present study may be related to the metabolic cost of detoxification and excretion of atrazine (Srinivas et al. 1991). In order to survive, tadpoles incur maintenance and developmental metabolic costs (Beck and Congdon 2003, Crespi and Denver 2005, Pandian and Marian 1985). The added load of metabolizing and excreting atrazine may cause a deficit in metabolic energy causing increased energy utilization from stored nutrients and decreased weight.

Other studies have highlighted the point that tadpoles and other organisms treated with atrazine became lethargic (Rohr et al. 2003). Because *Xenopus laevis* tadpoles are obligate suspension feeders, food intake can be affected by the frequency at which the suction pumping movement of the mouth occurs and the position and movement of their bodies and tails (Tinsley and Kobel 1996). If the frequency of buccal pumping
action is decreased, the amount of food ingested is also decreased causing decreased food intake resulting in metabolic dysfunction similar to what is seen in this present study.

General toxicity of atrazine may also cause a decrease in body weight by causing damage to essential organs. Some contaminants, including atrazine, bioaccumulate in specific organs especially organs such as liver, kidney, pancreas and gall bladder which are important for the metabolic function (Cox 2001, Edginton and Rouleau 2005, Klassen 2001, Santa-Maria et al. 1987, Wiegand et al. 2001). If these organs are damaged by atrazine, weight loss or even death may occur (Klassen 2001, Santa-Maria et al. 1987).

400ppb Atrazine Treated Tadpoles Have Significantly Underdeveloped Fat Bodies Compared to Control Tadpoles

In the present study there was a significant reduction in fat body size in the 400 ppb atrazine treated tadpoles compared to the control tadpoles. Fat bodies are finger like structures located at the anterior end of the gonad, lateral to the kidney (Zancanaro et al. 1996). They are typically yellow or light orange in color, indicating the presence of stored lipids.

Fat bodies possess the same embryonic origin as the gonads and may even possess the ability to produce and metabolize sex steroids (Girish and Saidapur 2000, Zancanaro et al. 1996, Lupo et al. 1971). They are suspected to play a role in spermatogenesis, as ablation of the fat bodies affect spermatogenesis, and causes gonadal atrophy in a number of anuran species, such as *R. esculenta, Rana hexadactyla*
and Rana nigromaculata (Girish and Saidapur 2000, Lupo et al. 1971). They also play a part in vitellogenin transfer to the ovaries (Lupo et al. 1971, Wiegand et al. 2001).

The fact that severely reduced fat body development coincided with reduced growth rate and metamorphic rate is not surprising as lipids are very important for the regulation of numerous cellular activities (Roberts 2002). They are the main component of cell membranes and are crucial for the normal functioning of receptors, enzymes and transporters that utilize cell membranes for their signal transduction pathways (Roberts 2002, Srinivas et al. 1991). Depletion of lipids causes severe effects on the normal physiological functioning of an organism. As tadpoles metamorphose, they need lipids for the production of many hormones.

There is also a great need for energy reserves in tadpoles at the climax of metamorphosis. As mentioned earlier, tadpoles spend most of their premetamorphic and prometamorphic stages feeding to reach the largest body size possible by stage NF stage 58/59 (McDiarmid and Altig 2000). Most of this energy is stored in fat bodies. When tadpoles begin metamorphic climax (from NF stage 58/59 to stage 66) feeding stops until the alimentary tract is remodeled (NF stage 62/63) (Tinsley and Kobel 1996). This means that the only energy stores available during this period in the tadpoles, are stored fat and glycogen that are utilized for maintenance metabolism and organ remodeling. Tadpoles that enter this period of development with a reduced energy reserve may be smaller in size after metamorphic climax. This is a disadvantage to the juvenile and adult frogs as body weight is correlated with the ability of the animal to survive (McDiarmid and Altig 2000).
Another theory that may account for the reduced fat bodies observed in the treated tadpoles could be chemical stress imposed on the tadpole by the presence of atrazine. This stress may cause the activation of the hypothalamic-pituitary-adrenal axis (stress axis) increasing release of corticotrophin-releasing factor (CRF) (Srinivas et al. 1991). This may have a two-fold effect on fat body size. First, a corticosterone increase causes lipids to be mobilized from the fat bodies and into the bloodstream so that the demands for extra energy are met (McDiarmid and Altig 2000). The other factor is that CRF is associated with reduced food intake causing stress-induced anorexia, causing the body again to draw on stored energy supplies from the fat bodies (Crespi and Denver 2005). Further studies measuring the levels of lipids in the bloodstream could be done, to measure the release of lipids from the fat bodies.

Studies by Santa Maria et al, (1987) demonstrated that subacute atrazine treatment in rats, caused lipid droplet accumulation in the liver, associated with a proliferation and degeneration of smooth endoplasmic reticulum. This indicated that atrazine may induce fatty liver and is associated with the inability of the liver to produce lipoproteins. Lipoproteins play an important role in the absorption of lipid molecules into the body (Sherwood 2001). This effect of atrazine was accompanied by hypoglycemia. They concluded that hyperlipemia in the liver with hypoglycemia and body weight reduction indicates a metabolic dysfunction provoked by atrazine. It was interesting to note that there were no observed histological changes in the liver tissues but damage was done to cellular ultrastructures such as the smooth endoplasmic reticulum, and the mitochondria. Tadpoles in studies in this laboratory showed reduced body weights,
which agree with Santa Maria et al (1987). Further investigations using electron microscopy to study organelles of the liver would help to elucidate if atrazine has a similar effect on frogs.

Summary

In the present study, *Xenopus laevis* tadpoles were treated with 400 ppb atrazine to determine health effects of this herbicide on amphibians. Significant changes in growth rate (weight and length), metamorphic rate and fat body development were found. Similar findings have been reported in other vertebrates and have been attributed to several possible actions of atrazine. Atrazine may cause these effects by reducing food intake, affecting the satiety centers in the hypothalamus, and producing malabsorption of nutrients, chemical stress, and/or liver dysfunction. Further studies are required in earlier tadpole stages to determine atrazine’s mechanism of action.
CHAPTER 4

MOLECULAR ANALYSIS OF TADPOLES TREATED WITH 400 PPB ATRAZINE USING DNA MICROARRAY AND QRT-PCR GENE EXPRESSION TECHNIQUES

Abstract

Tadpoles were exposed to 400 ppb atrazine from 5 days post fertilization to NF stage 62. Gene expression from 6 control and 6 atrazine treated male tadpoles was analyzed using Affymetrix DNA microarrays and qRT-PCR methods. Use of DNA microarrays to assay global gene expression patterns underlying tadpoles’ adaptative responses to atrazine is a powerful method for determining specific changes in gene pathways that correlate with phenotypic changes induced by atrazine. Microarray results showed the expression of 49 genes was altered in atrazine treated tadpoles compared to control tadpoles. The statistically significant genes were grouped according to physiological function and were concentrated in the following groups: digestion, blood and plasma function, cell adhesion, and immunity. The following four genes from the final microarray dataset were validated using qRT-PCR: chitinase, chitobiase, pancreatic trypsin and Cyp-P450. Expression levels of three of the four genes were consistent with the microarray results while one gene was not significantly different. This study supports the conclusion that atrazine has major impacts on gene expression underlying the function of digestive, blood, and immune systems of exposed tadpoles.
Introduction

Previous work, carried out as part of this thesis, demonstrated that exposure to 400 ppb atrazine affects growth (length and weight), metamorphic rate, and fat body development of tadpoles. The work presented in this chapter uses a genomics based approach to define atrazine induced changes in gene expression that may underlie atrazine induced changes in frog physiology.

Since frog populations are in worldwide decline (Ankely et al. 1998, Dalton 2002, Duellman and Trueb 1986, Storrs and Kiesecker 2004) understanding the basis of long-term problems in adapting to anthropogenic chemicals is paramount to protecting the role frogs play in aquatic ecosystems. Use of DNA microarrays to assay global gene expression patterns characteristic of frog’s adaptive responses to atrazine is a novel and powerful method for determining specific changes in gene pathways that control physiological processes. This research produced a roadmap of atrazine-induced changes in gene expression in male frogs. Gene expression data are grouped by physiological function including digestive, circulatory and immune systems. The roadmap will provide targets for studies regarding atrazine-induced changes in specific protein expression and related function.

One objective of this research was to identify gene expression levels that are altered by treatment of tadpoles (from NF stage 43 to NF stage 62) with atrazine at a concentration of 400 ppb. It must be noted at this point that gene expression levels vary according to the developmental stage of the tadpoles. Gene expression changes that occurred early in tadpole development may not always be identified at higher stages.
since expression of particular genes may have diminished after physiological tasks were accomplished at earlier stages. For example, aromatase, the enzyme that converts testosterone to estrogen may be upregulated by atrazine causing feminization of the tadpoles (Hayes et al. 2003). However, *Xenopus laevis* tadpoles gonadal differentiation is completed by NF stage 56 (Nieuwkoop and Faber 1994). This means that aromatase mRNA levels might be upregulated prior to this period in order to affect the formation of gonadal structure. If tadpoles are collected after stage 56 no alteration in gene expression for aromatase may be identified. This argument applies to other genes that may play a role in normal development. This is important for this study, as tadpoles were collected for microarray analyses only at NF stage 62.

The Affymetrix DNA microarray technique generates a large volume of data, which display alterations in mRNA expression for each individual gene in the genome of the organism being studied. The *Xenopus laevis* gene chip contains over 15,500 gene probes, each representing an individual transcript in the genome of the organism. This technology allows researchers to survey thousands of genes, using RNA expression values, in a short time as opposed to a few genes as is done in RT-PCR and Northern Blotting.

For this research, I employed DNA microarrays as an environmental risk assessment tool to determine mRNA expression levels of the 15,500 genes in the genome of *Xenopus laevis* tadpoles as they relate to the health effects of atrazine exposure. I extracted total RNA from six control male tadpoles and six 400 ppb atrazine treated tadpoles at NF stage 62 and used these RNA samples to conduct DNA
microarray experiments. Only male tadpoles were chosen for this study as a large body of literature highlights the point that atrazine may be affecting male tadpoles differently than females (Hayes 2004, Hayes et al. 2002, Hayes et al. 2003, Srinivas et al. 1991). I used the final dataset obtained from these experiments to categorize genes that showed significantly different expression between control and atrazine treated tadpoles, into physiological groups to produce a roadmap of physiological functions that are affected by atrazine. Results obtained demonstrated that atrazine affected mainly genes associated with digestion, blood and plasma function, cell adhesion and the immune system. These groups of altered genes can be related to observed phenotypic changes, which occurred in the tadpoles.

In order to validate the microarray data, I used total RNA samples remaining from the microarray analysis to do a two-step qRT-PCR analysis of chosen candidate genes (chitinase, chitobiase, CYP-P450 and pancreatic trypsin). These genes were chosen from the final microarray dataset after it was filtered to reflect only named genes that were significantly different using the MannWhitney statistical test (Affymetrix Data Mining Tools (DMT) program (p ≤ 0.05) and that had a fold change value of 1.5 and greater for upregulated genes and -1.5 and less for downregulated genes. Two of the genes (chitinase and pancreatic trypsin) were upregulated in the microarray dataset and two were downregulated (chitobiase and CYP-P450). qRT-PCR validated the microarray results for 3 out of 4 of the candidate genes. The CYP-P450 gene was not significantly different as originally indicated by the gene chip data but was downregulated.
Microarray Technology Overview

Figure 5. Schematic diagram showing the process of DNA microarray technology from extracted total RNA template to scanning and measurement. cRNA is biotinylated (B) to facilitate the binding of streptavidin, which provides amplification of the fluorescent signal before scanning (adapted from Affymetrix website {www.affymetrix.com}).

Material and Methods

Animals

Six control and six treated male tadpoles (all at NF stage 62) were collected and frozen in the phenotypic study described in chapter 3.
RNA Extraction

Total RNA was extracted from tadpoles using a Qiagen RNeasy Maxi Kit® (Valencia, CA) for animal tissues, and concentrated using an RNeasy MinElute Cleanup Kit® according to manufacturer’s protocol. Briefly, a whole tadpole was euthanized, then ground in liquid nitrogen, homogenized with Qiazol lysis reagent (Valencia, CA) and eluted with RNase free water. Quantity and purity of extracted RNA was determined using GeneQuant Pro spectrophotometer (Pharmacia Biotech, Cambridge, England).

DNA Microarray

Sample preparation for microarray analysis was carried out according to the affymetrix microarray protocol. Briefly, an aliquot of total RNA (7.5ug) from individual tadpoles was used as a template to synthesize double stranded complementary DNA (cDNA) using an Invitrogen SuperScript Choice System and a T7-oligo-dT primer (Affymetrix, Santa Clara, CA). The cDNA sample was converted to biotin- labeled cRNA by in vitro transcription using an Affymetrix IVT labeling kit (Affymetrix). The biotin labeled cRNA was fragmented to an average size of 35-200. An aliquot of the fragmented cRNA was hybridized onto individual gene chips for 16 hours at 45°C with rotation at 60 RPMs. Each chip was then washed and stained (streptavidin) in an Affymetrix Fluidics Station (400) according to Affymetrix Technical Manual (Affymetrix, Santa, Clara, CA). Chips were scanned using a laser.
Scanner (Gene Array Laser Scanner, Agilent Technologies). The data was imported into the Affymetrix Microarray Suite Software program.

DNA Microarray Statistical Analysis

The Affymetrix *Xenopus laevis* Gene Chip contains approximately 15,500 transcripts and expressed tags (ESTs). Each gene is represented by 16 pairs of 25mer sequence probes. Each probe pair consists of a perfectly matched probe (PM) and a mismatched probe (MM), the mismatched probe being identical to the perfect matched probe except for an alteration of the 13th base pair. The MM probe controls for non-specific hybridization and background signal intensity. Affymetrix Microarray Suite quantifies the expression of each by subtracting the signal intensity value of the MM from the PM probes.

After gene chips are scanned, raw data is generated as a data file (dat file). These files are obtained via Affymetrix Microarray suite and can be converted into chip files. All probe sets were scaled to a mean target intensity of 500. A preliminary dataset was obtained by analyzing this raw data using a nonparametric t-test (MannWhitney, SAS) using a p-value cutoff of 0.05, which generated a primary dataset. This primary dataset was imported into the Affymetrix Data Mining Tools software for further filtering, using detection calls {Present (P), Marginal (M), and Absent (A)}. In this study only genes with Present and/or Marginal calls for all 6 tadpoles in either the control or treated datasets were included in the final dataset. The dataset was further filtered using fold changes. Only genes with fold changes of 1.5 and above (upregulated) or -1.5
(downregulated) and less were utilized. Information concerning gene function was obtained using web based bioinformatics databases such as NetAffx, GeneCards and PubMed, and scientific papers obtained from journals found online or from libraries.

**Taqman Real-Time RT-PCR**

Four genes chosen from the final Affymetrix dataset were chosen for validation using Taqman qRT-PCR (Applied Biosystems). These were chitinase, chitobiase, pancreatic trypsin and CYP-P450. Total RNA samples for each tadpole were used as template to generate complementary DNA strand (cDNA) according to manufacturer’s protocol. Each sample utilized 3μg of total RNA sample. cDNA synthesis was performed using the High Capacity cDNA Archive Kit according to manufacturer’s instructions. Twenty-five ng of yeast (Clontech, Palo Alto, CA) mRNA was added to each synthesis reaction to provide an exogenous control for the qRT-PCR. Yeast was not added to the Affymetrix experiment to prevent competitive binding with the yeast controls that are present on the Affymetrix gene chips. The reaction was run at 25°C for 10 minutes and 2 hours at 37°C.

cDNA was used as the template in the polymerase chain reaction. Taqman Universal® PCR Master Mix reagent kit (Applied Biosystems) was used according to manufacturer’s instructions. For each gene a four point relative standard curve was included on each 96 well PCR plate. Standard curves were run in duplicates using serial dilutions of cDNA (0.5, 1, 2, 4 ng) of all genes using a reference sample of pooled control cDNA samples. Standard curve generation is a method used to quantify
mRNA levels for each target gene (Jelaso et al. 2003). Standard curve template cDNA values were chosen that generated correlation coefficients of 0.99 and had slope of $-3.3 \pm 0.3$, an indication of PCR efficiency. A no template control was utilized. The reactions were run in an ABI Prism® 7700 Sequence Detection System. The thermal cycling parameters set for all PCR runs were 10 minutes at 95°C, then 40 cycles of 95°C for 15 seconds decreasing to 60°C for 1 minute. Primers and probes are listed in Table 4.

RT-PCR Data Analysis

In order to quantify the mRNA levels for each target gene the relative standard curve method was used (Applied Biosystems). First, the Cycle threshold value ($C_T$) was set manually in the exponential growth phase of the PCR reaction. This value represents the cycle at which a statistically significant increase of signal intensity occurs when compared to the baseline. The four point standard curve (plot of the $C_T$ value versus the log of the amount of cDNA template) was used to relate $C_T$ values using linear regression analysis. These standard curves and unknowns were run in duplicates. The relative expression level for each gene was calculated by dividing the target gene value by the yeast actin value (exogenous control) (Jelaso et al. 2003). The mRNA values were then expressed as a ratio of the untreated pooled reference samples. The mRNA values for each treatment group were expressed as a ratio of the pooled reference control group expression value. This data was used to do statistical analysis.
of the experimental group compared to the control group (Statview; SAS Institute, Cary, NC).

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Sequence</th>
<th>Amount Used</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chitinase</td>
<td>GCCTTTGTACTCACCTGATCTATGC</td>
<td>300nM</td>
</tr>
<tr>
<td>Forward primer</td>
<td>TGTACAAGTAACATCATTCATTCAAT</td>
<td>300nM</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>GCTCCCTCAATCCGTGTATCTCT</td>
<td>200nM</td>
</tr>
<tr>
<td>Probe</td>
<td>6FAM-CCGGAATGTCCAATACCCAGATACCA-TAMRA</td>
<td></td>
</tr>
<tr>
<td>Chitobiase</td>
<td>GCAAGTTACATCAAGTTGGGTATAGGA</td>
<td>300nM</td>
</tr>
<tr>
<td>Forward primer</td>
<td>TGCCCTCTCAATCCGTGTATCTCT</td>
<td>300nM</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>GCTCCCTCAATCCGTGTATCTCT</td>
<td>200nM</td>
</tr>
<tr>
<td>Probe</td>
<td>6FAM-CCGGAATGTCCAATACCCAGATACCA-TAMRA</td>
<td></td>
</tr>
<tr>
<td>CYP-P450</td>
<td>TCTCCCTTCCCTGTATCTCTG</td>
<td>300nM</td>
</tr>
<tr>
<td>Forward primer</td>
<td>TGATAGTTGGGAACATATTCACAA</td>
<td>300nM</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>GCTCCCTTCCCTGTATCTCTG</td>
<td>200nM</td>
</tr>
<tr>
<td>Probe</td>
<td>6FAM-CCGGAATGTCCAATACCCAGATACCA-TAMRA</td>
<td></td>
</tr>
<tr>
<td>Pancreatic</td>
<td>GCAATGACATTATGCTGATCAAGCT</td>
<td>300nM</td>
</tr>
<tr>
<td>Trypsin</td>
<td>GCAGTGCGCCAGGTATTGACA</td>
<td>300nM</td>
</tr>
<tr>
<td>Forward primer</td>
<td>GCTCCCTTCCCTGTATCTCTG</td>
<td>200nM</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>GCTCCCTTCCCTGTATCTCTG</td>
<td></td>
</tr>
<tr>
<td>Probe</td>
<td>6FAM-CCGGAATGTCCAATACCCAGATACCA-TAMRA</td>
<td></td>
</tr>
<tr>
<td>Yeast Actin</td>
<td>CGCCCTTGGACCTCGAAACAG</td>
<td>300nM</td>
</tr>
<tr>
<td>Forward primer</td>
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<td>300nM</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>TCACCATCTGGAAGTCGTAGGA</td>
<td>200nM</td>
</tr>
<tr>
<td>Probe</td>
<td>6FAM-CCGGAATGTCCAATACCCAGATACCA-TAMRA</td>
<td></td>
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</tbody>
</table>

Table 4. Primer and probe sequences and volumes used for QRT-PCR. Primer and probe concentrations were 10μM. Sequences were designed using Primer Express v1.2. Sequences used are publicly available (NCBI). Primers and probes were designed using *Xenopus laevis* cDNA sequence publicly available at GenBank.

Results

Global Gene Expression Analysis

Initial analysis of raw data for 6 control and 6 atrazine treated tadpoles was carried out using the nonparametric MannWhitney t-test (SAS, p ≤ 0.05), selecting genes characterized by Present and Marginal calls in either all treated or all control tadpoles. This analysis produced 1812 transcripts that were significantly altered; 911
transcripts were upregulated and 901 were downregulated. Other filtering restrictions mentioned in the methods section of this chapter caused the number of transcripts to decrease to 49 transcripts (Table 5).

Two tables (Tables 6 and 7) were generated, one table with upregulated genes placed into biologically functional groups and the second with downregulated genes placed into functional groups. The number of genes in each biologically functional group is depicted in Figures 6 and 7. There were 34 genes that were upregulated and 15 that were downregulated. The groups with the largest concentration of genes in the upregulated group were associated with the digestive system, cell adhesion and blood and plasma. In the downregulated group the two highest concentrations were cell adhesion and blood and plasma related (Figures 6 and 7).

A heatmap is depicted in Figure 8 that shows the signal intensities of individual control tadpoles (n=6; first 6 columns) and 400 ppb atrazine treated tadpoles (n=6; second 6 columns). In the heatmap (Figure 8), each colored bar (row) indicates a single gene and each column represents the response of a single tadpole. The colors indicate fold change intensities. The bars are grouped into nodes (arms on left) with shorter arms representing clusters of genes with similar expression profiles and the longer arms representing clusters of genes with disparate expression profiles. The color bar to the right of the graph is a scale with the lowest gene expression intensities being blue and the highest being red. The heatmap visually delineates genes that are downregulated from upregulated and shows variability in signal intensities for specific genes between
individual frogs from each group. Heatmaps showing intensities averaged for both control and atrazine treated groups are shown in figure 9.

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th># Transcripts Upregulated</th>
<th># Transcripts Downregulated</th>
<th># Transcripts with p ≤ 0.5 and 1.5 FC</th>
</tr>
</thead>
<tbody>
<tr>
<td>400 ppb atrazine treated</td>
<td>911</td>
<td>901</td>
<td>49</td>
</tr>
</tbody>
</table>

Table 5: Number of transcripts which were upregulated and downregulated from the primary dataset after DNA microarray analysis, and the number of transcripts remaining in the final dataset after filtering with fold change cut off values, P and M calls, and removal of ESTs.

400 ppb Atrazine Treatment Caused Significant Changes in Expression of Genes Related to the Digestive System

There were 10 genes associated with the digestive system that showed significant gene expression alteration. Eight were upregulated (Figure 6) and 2 downregulated (Figure 7). Fold changes ranged from 7.6 to 1.5 with pancreatic amylase having the highest fold change of 7.65. Three genes are expressed in the pancreas, i.e., pancreatic trypsin, pancreatic amylase and pancreatic elastase. Pancreatic lipase was not significantly different. Two of the genes were chitinolytic enzymes. Chitinase was upregulated (Table 6) while chitobiase was downregulated (Table 7). In addition to these enzymes there were 5 hormones involved in digestive processes. Alpha-1 microglobulinkinin precursor was downregulated (Fold Change -1.51), and Neuropeptide Y (Fold Change 1.66), preprocholecystokinin (Fold Change 1.5); Gastric H, K-ATPase beta (Fold Change 2.87) and Furin (Fold Change 1.58) were all upregulated.
<table>
<thead>
<tr>
<th>Affy ID</th>
<th>Gene Name</th>
<th>foldchange</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>XI.21603.1.S1_at</td>
<td>pancreatic alpha-amylase</td>
<td>7.65</td>
<td>0.013</td>
</tr>
<tr>
<td>XI.119.1.S1_at</td>
<td>pancreatic trypsin</td>
<td>6.86</td>
<td>0.013</td>
</tr>
<tr>
<td>XI.241.1.S1_at</td>
<td>gastric H, K-ATPase beta subunit</td>
<td>2.87</td>
<td>0.004</td>
</tr>
<tr>
<td>XI.21574.1.S1_at</td>
<td>chitinase</td>
<td>3.12</td>
<td>0.013</td>
</tr>
<tr>
<td>XI.3275.1.S1_at</td>
<td>pancreatic elastase 1</td>
<td>2.07</td>
<td>0.047</td>
</tr>
<tr>
<td>XI.1146.1.S1_at</td>
<td>preprocholecystokinin</td>
<td>1.91</td>
<td>0.002</td>
</tr>
<tr>
<td>XI.831.1.S1_s_at</td>
<td>neuropeptide Y</td>
<td>1.66</td>
<td>0.002</td>
</tr>
<tr>
<td>XI.6944.1.S1_at</td>
<td>furin</td>
<td>1.72</td>
<td>0.013</td>
</tr>
</tbody>
</table>

**Blood/ Plasma Related**

<table>
<thead>
<tr>
<th>Affy ID</th>
<th>Gene Name</th>
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<th>p-value</th>
</tr>
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<tbody>
<tr>
<td>XI.1126.1.S1_at</td>
<td>alpha-globin (alpha T4)</td>
<td>3.55</td>
<td>0.013</td>
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<tr>
<td>XI.1135.1.S1_at</td>
<td>larval beta I globin</td>
<td>2.81</td>
<td>0.033</td>
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<tr>
<td>XI.24530.1.S1_at</td>
<td>larval beta II globin</td>
<td>2.47</td>
<td>0.033</td>
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<tr>
<td>XI.1125.1.S1_at</td>
<td>alpha-globin (alpha T3)</td>
<td>2.46</td>
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**Immunity**

<table>
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<th>p-value</th>
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<tr>
<td>XI.25172.1.Al_x_at</td>
<td>immunoglobulin light chain variable region.</td>
<td>1.641378</td>
<td>0.0465</td>
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**Cell Adhesion**

<table>
<thead>
<tr>
<th>Affy ID</th>
<th>Gene Name</th>
<th>foldchange</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>XI.21637.1.S1_at</td>
<td>metavinculin</td>
<td>1.60</td>
<td>0.021</td>
</tr>
<tr>
<td>XI.185.1.S2_at</td>
<td>integrin alphaV</td>
<td>1.59</td>
<td>0.047</td>
</tr>
<tr>
<td>XI.315.1.S1_at</td>
<td>NF-protocadherin</td>
<td>1.53</td>
<td>0.033</td>
</tr>
<tr>
<td>XI.1589.1.S1_at</td>
<td>cement gland-specific</td>
<td>2.29</td>
<td>0.004</td>
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<tr>
<td>XI.22006.1.S1_at</td>
<td>caveolin-1</td>
<td>1.71</td>
<td>0.047</td>
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<tr>
<td>XI.24460.1.S2_at</td>
<td>rac GTPase</td>
<td>1.60</td>
<td>0.021</td>
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</table>

**Transcription/Chromosomal**

<table>
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<th>Gene Name</th>
<th>foldchange</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>XI.23196.1.S1_at</td>
<td>HMG-14</td>
<td>1.82</td>
<td>0.008</td>
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<tr>
<td>XI.21459.1.S1_at</td>
<td>helix-loop-helix transcription factor</td>
<td>1.65</td>
<td>0.013</td>
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<tr>
<td>XI.7817.1.S2_at</td>
<td>centrin</td>
<td>1.53</td>
<td>0.013</td>
</tr>
<tr>
<td>XI.21515.1.S1_at</td>
<td>c-fos proto-oncogene</td>
<td>1.52</td>
<td>0.033</td>
</tr>
<tr>
<td>XI.110.1.S1_at</td>
<td>serum response factor</td>
<td>1.66</td>
<td>0.008</td>
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</table>

Table 6: Functional groupings of genes significantly upregulated by 400 ppb atrazine treatment.
Table 6—Continued

<table>
<thead>
<tr>
<th>Affy ID</th>
<th>Gene Name</th>
<th>foldchange</th>
<th>p-value</th>
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</thead>
<tbody>
<tr>
<td>Ion Transport</td>
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</tr>
<tr>
<td>XI.1109.1.S1_at</td>
<td>alpha-1 gap junction protein.</td>
<td>1.762665</td>
<td>0.0325</td>
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<tr>
<td>XI.11973.1.S1_at</td>
<td>progressive ankylosis-like protein</td>
<td>1.570277</td>
<td>0.0022</td>
</tr>
<tr>
<td>Cell Cycling</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>XI.21086.1.S1_at</td>
<td>cyclin E2</td>
<td>1.590757</td>
<td>0.0465</td>
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<tr>
<td>Apoptosis</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>XI.977.1.S1_at</td>
<td>caspase-6</td>
<td>1.545498</td>
<td>0.0076</td>
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<tr>
<td>Thyroid Related</td>
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<tr>
<td>XI.21638.1.S1_at</td>
<td>type II deiodinase</td>
<td>1.517877</td>
<td>0.0325</td>
</tr>
<tr>
<td>Neuroprotection</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>XI.199.1.S1_s_at</td>
<td>myelin proteolipid protein</td>
<td>1.604829</td>
<td>0.0043</td>
</tr>
<tr>
<td>XI.9576.1.S1_at</td>
<td>carbonic anhydrase II</td>
<td>1.610134</td>
<td>0.0465</td>
</tr>
<tr>
<td>Signal Transduction</td>
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<td></td>
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</tr>
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<td>SMAD3</td>
<td>1.554411</td>
<td>0.0076</td>
</tr>
<tr>
<td>XI.11962.1.S1_at</td>
<td>gonadotropin-releasing hormone</td>
<td>1.532588</td>
<td>0.0206</td>
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<tr>
<td></td>
<td>receptor type II</td>
<td></td>
<td></td>
</tr>
<tr>
<td>XI.11965.1.S1_s_at</td>
<td>sprouty2delta</td>
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Table 7: Functional groupings of genes significantly downregulated by 400 ppb atrazine treatment

400 ppb Atrazine Treatment Caused Significant Changes in Expression of Genes Related to Blood and Plasma Function

Three fibrinogen subunits were significantly reduced in the atrazine treated tadpoles (Table 7). These represent all the subunits of fibrinogen in the amphibian. These were fibrinogen beta (p= 0.006; Fold Change, -1.5) alpha (p=0.001; Fold Change, -1.6) and gamma (p= 0.003; Fold Change, -1.5) (Table 4). Serum albumin was downregulated in the treated tadpoles (Fold Change, -2.6). There were 4 globin genes
that were upregulated by atrazine treatment. These are two forms of alpha-globin subunits and 2 beta globin subunit molecules (Table 6).

400 ppb Atrazine Treatment Caused Significant Changes in Expression of Genes Related to Transcription Regulation

There were 4 genes that were upregulated by atrazine treatment of *Xenopus laevis* tadpoles that related to either transcription or chromosome regulation. These were HMG-144 (p=0.012; Fold Change 1.82). Helix loop helix transcription factor (Fold Change, 1.8), centrin and c-fos proto-oncogene.

![Bar graph showing number of upregulated genes per biological function](image)

Figure 6: Number of upregulated genes from final dataset grouped according to biological functions.
Figure 7: Number of downregulated genes from final dataset grouped according to biological functions.
Figure 8: Heatmap (generated using Genespring software v7.2) showing the signal intensity of individual 400 ppb atrazine treated tadpoles (n=6; First 6 columns) and control tadpoles (n=6; second 6 columns) for transcripts significantly altered by atrazine treatment. Red color is highest intensity and blue is lowest intensity.
Figure 9: Heatmap (generated using Genespring software v7.2) showing the signal intensity of mean 400 ppb atrazine treated tadpoles (n=6: second columns) and mean control tadpoles (n=6, first column) for transcripts significantly altered by atrazine treatment. Red color is highest intensity and blue is lowest intensity.
400 ppb Atrazine Treatment Caused Significant Changes in Gene Expression Related to Cell Adhesion

A high concentration of genes both upregulated (6) and downregulated (4) were associated with cell adhesion. Genes that were upregulated were metavinculin, integrin, protocadherin, cement gland specific, caveolin 1 and rac GTPase. Downregulated genes were desmoplakin-related protein, galectin, pentraxin fusion protein and lactose binding protein (Tables 6 and 7). The fold change of these genes ranged between 1.5 and 2.8.

400 ppb Atrazine Treatment Caused Significant Changes in Expression of Genes Related to the Immune System

Two downregulated genes associated with the tadpole immune defense were MHC Class II (p=0.034, Fold Change −1.9) and Cytochrome P-450 (p= 0.012, Fold Change −2.05). One gene was upregulated (immunoglobulin light chain; Fold Change 1.6; p-value= 0.046).

Additional Genes Significantly Upregulated by Atrazine Treatment

Other groups with upregulated genes were placed into the following functional groups: ion transport, cell cycle, apoptosis, thyroid related, neuroprotective, and signal transduction (Table 6). There were two genes associated with ion transport. These were alpha-1 gap junction protein and progressive ankylosis-like protein. Cell cycling, apoptosis and thyroid system functional groups each possessed one gene each. The
group associated with neuroprotection included 2 genes, myelin proteolipid protein (Fold Change 1.6, p=0.004) and carbon anhydrase (Fold Change 1.6, p=0.046). Type II deiodinase (Fold Change 1.55, p=0.032) was placed in the thyroid system functional group.

There were 3 genes associated with signal transduction. These were SMAD3 (Fold Change 1.55, p= 0.0076), GRHRII (Fold Change 1.53; p=0.02) and sprouty 2 delta (Fold Change 1.52, p=0.013).

**Chitobiase mRNA Levels from 400 ppb Atrazine Treated Tadpoles Were Significantly Lower Than Those from Control Tadpoles**

qRT-PCR analysis showed chitobiase mRNA expression levels from tadpoles treated with 400ppb atrazine were significantly decreased (Figure 10, t-test, p=0.021, n=5) when compared to control tadpoles (n=5). This was in agreement with the microarray experiment, which showed a fold change of −3.08 and a P-value of 0.001 (atrazine treated tadpoles compared with control tadpoles).
Figure 10: qRT-PCR was used to validate the reduction of chitobiase mRNA levels as measured in the microarray dataset. (t-test, *p=0.021, n=5).

Chitinase mRNA Levels from 400 ppb Atrazine Treated Tadpoles Were Significantly Higher Than Those from Control Tadpoles

Chitinase mRNA expression levels measured using qRT-PCR were significantly increased in atrazine treated tadpoles (Figure 11, t-test, p=0.05, n=5) when compared to control tadpoles (n=5).
Figure 11. qRT-PCR was used to validate the upregulation of chitinase mRNA levels as measured in the microarray dataset. (t-test, *p=0.05, n=5).

Pancreatic Trypsin mRNA Levels from 400 ppb Atrazine Treated Tadpoles Were Significantly Higher Than Those from Control Tadpoles

Pancreatic trypsin mRNA levels measured using qRT-PCR of 400 ppb atrazine treated tadpoles (n=5) were significantly increased when compared with control tadpoles (n=5; t-test, p=0.017).
Figure 12. qRT-PCR was used to validate the upregulation of pancreatic trypsin mRNA levels as measured in the microarray dataset. (Fisher’s t-test, *p=0.017, n=5).

CYP-P450 mRNA Levels from 400 ppb Atrazine Treated Tadpoles Were Not Significantly Higher Than Those from Control Tadpoles

CYP-P450 mRNA levels measured using qRT-PCR from 400 ppb atrazine treated tadpoles (n=5; Fisher’s t-test, p=0.81) were not different from levels measured in control tadpoles (n=5). This differed from the microarray dataset, which indicated that CYP-P450 was downregulated with a fold change of -2.05. The mean signal intensity for treated tadpoles used in the microarray study was 606.82 for the treated and 1245.9 for the control with a p= 0.012. This was the only one of the four genes chosen to validate the microarray genechip that did not show significance but the signal was downregulated in the qRT-PCR data in agreement with the microarray data.
Figure 13. qRT-PCR was used to validate the downregulation of CYP-P450 mRNA levels as measured in the microarray dataset. qRT-PCR results were not statistically significant.

Discussion

Global Gene Expression Changes in Tadpoles Treated with 400 ppb Atrazine

Affymetrix DNA microarrays were used to determine gene expression changes due to exposure of *Xenopus laevis* tadpoles to atrazine from 5 days post fertilization to NF stage 62. After filtering the initial dataset according to p-value (p=0.05), fold changes (≥ +1.5 for upregulated genes and ≤ -1.5 for downregulated genes) and using genes which only showed Present and Marginal calls in either all control tadpoles or all treated tadpoles, there were 49 genes present in the final dataset. Genes that were significantly altered in the treated tadpoles when compared to controls were separated into groups according to biological functions. The analysis showed that these genes were mainly concentrated in groups associated with digestive system, blood and plasma...
function, cell adhesion, and the immune system. This is the first time that microarray
gene expression technology has been used to characterize global gene expression
changes in atrazine treated tadpoles.

400 ppb Atrazine Treatment Caused Significant Changes in Expression of Genes
Related to the Digestive System

The digestive system is vital for the health and survival of an organism
(McDiarmid and Altig 2000, Sherwood 2001, Tinsley and Kobel 1996). This
physiological system is involved in delivery of enzymes, nutrients and hormones to the
lumen of the intestines, and for their absorption into the blood stream (Sherwood 2001,
Tinsley and Kobel 1996) providing nutrients and energy to keep the organisms’
metabolic needs adequately supplied. Dysfunction of this system, as seen in this study,
may cause complications that may affect a variety of body structures and functions
(Anholt and Werner 1995, Beck and Congdon 2003, Bossola et al. 2005, Crespi and
Denver 2005, Crump 1981, Dodd and Dodd 1976, Duellman and Trueb 1986,

Phenotypic observations in a previous experiment demonstrated that atrazine
treated tadpoles had significant reductions in growth (weight and length), fat body
development and metamorphic rate when compared to control male tadpoles. Gene
expression changes described in chapter 4 may help explain phenotypic observations.
A large body of evidence has associated reduction in growth rate and metamorphic rate
with malnutrition (Anholt and Werner 1995, Kupferberg 1997, McDiarmid and Altig
2000, Pandian and Marian 1985). There were 10 genes associated with the digestive
system whose expression levels were altered by atrazine treatment and these changes may indicate a digestive system efficiency deficit. Three genes representing pancreatic enzyme expression were upregulated with the highest fold changes recorded in the study. Two of these, are proteolytic enzymes (trypsin and elastase). In normal development at metamorphic climax, these enzymes are upregulated to play a role in the destruction and resorption of tissues and organs that are tadpole specific (Dodd and Dodd 1976). However, their expression levels are controlled by inhibitors (Alpha-1-microglobulinbikunin precursor for example). Over production of catabolic enzymes, or downregulation of inhibitors, may affect the viability of new cells and cause autodigestion of pancreatic tissues (Akira et al. 1997). Increased activity of these enzymes may cause protein wasting and weight loss (Kupferberg 1997).

Results in the present study also indicated that there were enterohormones that were altered by atrazine. Furin and preprocystokinokinin were upregulated. Preprocystokinin (a precursor to CCK) plays a role in stimulating release of proteolytic enzymes from the pancreas, and serves to reduce food intake (Konturek et al. 2003, Sherwood 2001). Furin functions to fold and activate zymogens to their active forms in the small intestine (Bissonnett et al. 2004, Lo’pez-deCicco et al. 2004). NPY a powerful orexogenic hormone produced in the brain acts to increase food intake (Konturek et al. 2003, Konturek et al. 2004). Upregulation of these genes may indicate that tadpoles may be attempting to counteract the anorexic effect of the atrazine.

Alpha1-microglobulinbikunin (AMBP), which was downregulated in the dataset, is an enzyme found in the small intestine that acts to downregulate trypsin (Rouet et al. 2004).
The altered activity of these hormones in the small intestines further supports the phenotypic findings of this study. These gene expression changes may be as a direct result of atrazine, or as a secondary result of atrazine’s effect on food intake causing an anorexic result.

It should also be noted that tadpoles were collected at NF stage 62 for microarray genechip analysis. At this stage the tadpole gut is being remodeled from a herbivorous orientation to a carnivorous one (Schreiber et al. 2005). Tadpoles do not eat at the time of remodeling. Atrazine treated tadpoles, because they showed reduced weight and depleted fat bodies entering metamorphic climax, appeared to be at risk for surviving metamorphosis. (McDiarmid and Altig 2000). With this situation increased digestive enzyme activity would increase the efficiency at which energy is produced, thereby stemming the effects of undernourishment. It is unclear if metabolism during this period involves digestion of food stores ingested earlier, and/or energy produced through catabolic breakdown of tissues during remodeling. In this regard, protein can be obtained internally during this period, for example, from the epithelial cells from the mucosal lining of the intestines (Sherwood 2001). This is interesting because reports show that during metamorphic climax sloughing of intestinal cells increases (Hourdry et al. 1996, Schreiber et al. 2005). It is also reported that atrazine and its metabolites mainly accumulates in the liver, gall bladder and intestines of *Xenopus laevis* tadpoles (Edginton and Rouleau 2005). This may be one of the reasons for the increase of pancreatic trypsin activity. It is possible that atrazine may be causing increased
irritation of the mucosal lining of the small intestines causing increased sloughing which stimulates the production of pancreatic trypsin.

The theory that atrazine may be affecting the mucosal lining of the small intestine can also explain the inability of the treated tadpoles to obtain necessary nutrients in order to accumulate energy storage. Damage to the mucosal lining of this structure could cause malabsorption of nutrients by damaging the intestinal villi. Constant irritation may cause villi to become flattened and reduced in number. This reduces the surface area of the small intestines and absorption of nutrients is impaired (Sherwood 2001).

In response to external stress such as the presence of a harmful chemical in the environment such as atrazine the stress hormone corticosteroid is produced (Crespi and Denver 2005, Sapolsky et al. 2000). This hormone acts to mobilize stored fuels to serve as energy in an attempt to counteract the metabolic effects of the stressors (Crespi and Denver 2005, Dodd and Dodd 1976). In order to obtain necessary energy to maintain physiological and structural function in a malnourished animal, the body first metabolizes available glucose (glycogen), the most readily available energy source. If this source is not in sufficient quantity for the body’s metabolic needs, lipid stores are accessed and metabolized. In amphibians, including Xenopus laevis tadpoles, lipids are mainly stored in the fat bodies (Girish and Saidapur 2000), which were decreased in size in treated tadpoles in this study. The last main energy source that is utilized is protein. If the malnutrition condition persists, then the animal eventually dies. This is consistent with the phenotypic findings of this research where the tadpoles are thought
to be malnourished and attempting to provide nutrients for maintenance and developmental metabolism by increasing digestive enzyme activity.

Metamorphic rate is also highly affected by nutrition. Tadpoles fed a high protein diet were larger and metamorphosed faster than controls (Eales 1988). This is compatible with the reduced metamorphic rate seen in the present study. Increased proteolytic enzyme activity may act to increase protein availability.

Eales (1988) also reported that tadpoles with food restrictions or starved animals showed decreased levels of T3 and T4, decreased production of TSH in the hypothalamus, decreased T3 receptor binding capacity and decreased Type II Diodinase levels. All of these factors act in concert to reduce metamorphic rate.

Type II Diodinase was significantly increased in the present study by exposure to atrazine. This enzyme converts T4 into the active T3, possible indicating that, by stage 62, the tadpoles were adapting to atrazine’s effects and metamorphic rate was being increased.

This present study also demonstrated alteration in two chitinolytic enzymes. Chitin is second in abundance in nature only to cellulose (Jackson et al. 1992). This consists of the polymers of aminosugar N-acetylglucosamine. It is found complexed with polysaccharide and proteins (Jackson et al. 1992). It is the main component of the cell wall of fungi (Ram et al. 2004), and the exoskeleton of arthropods including insects. Chitin degradation requires chitinase (poly-β-N-acetyl-D-glucosaminidase) and chitobiase (β-N-acetyl-D-glucosaminidase). Chitinase is able to hydrolyze chitin into trimers (Chitotriose) and dimers (Chitobiose) of N-acetyl-D-glucosamine (NAG)
(Jackson et al. 1992). These trimers and dimers are then hydrolyzed to the monomers of NAG by chitobiase.

Chitobiase is a lysosomal glycosidase which splits GlcNAc beta D- (1, 4) GlcNAc moiety in Asn-linked glycoprotein (Aronson and Kuranda 1989). Aronson and Kuranda (1989) showed that glycoprotein disassembly to free amino acids and sugar is an ordered bidirectional pathway where chitobiase catalyses the last step during digestion of the protein oligosaccharide linkage region. These hormones are produced in the liver of vertebrates.

Zou (1998) demonstrated that xeno-estrogens Arochlor 1242, DES, Endosulfan and PCB 29, all were able to significantly inhibit chitobiase activity in the epidermis and/or hepatopancreas of the fiddler crab, U. pugilator. This impacts on the organism as molting depends on the ability of chitobiase to partially digest the crab’s chitinous exoskeleton. Inhibition of chitobiase may account for the slower molting process that occurs in crustaceans when treated with these xenobiotic chemicals. Molting in crustaceans is under the control of steroid hormones called ecdysteroids (Zou 2005). These steroids are thought to regulate chitobiase, whose expression correlates with the profile of ecdysteroids activity. This fact establishes a connection between chitobiase activity and steroid hormones activity. This has not been previously found in frogs but atrazine is thought to have endocrine disrupting effects through altering of steroid hormone levels in vertebrates (Hayes et al. 2003).

Hydrolytic products N-acetyl-D-glucosamine and glucosamine are structurally similar to glucose. In the case of nutrition the organism must be able to have high
activity in both chitinase and chitobiase. If the organism has a high chitinase activity but low chitobiase activity, as seen in the dataset, it may suggest that the organism is not using chitin as an energy source but as a protection against bacterial and fungal invasion (Jackson et al. 1992).

Jackson (1992) demonstrated that juvenile chickens fed chitin and chitosan supplements gained mass at a lower rate than control chickens, and that the growth rate of rainbow trout fed chitin and chitosan was also retarded when compared to controls. This may have occurred because of the energy cost of the organism to digest the chitin. Studies have shown that amino-sugars like NAG and Gln are respired at a less rapid rate than D-glucose, which could also mean that when the end product is absorbed, a high quantity of energy is not obtained.

In this present study *Xenopus laevis* tadpoles were fed ground Purina Rabbit chow. This food source contains *A. Aspergillus*, a fungus whose cell wall is composed of a high level of chitin (Ram et al. 2004). Four hundred ppb atrazine treated male tadpoles showed an increased mRNA expression of chitinase and a decreased activity of chitobiase. This means that the chitin cell walls of *A. Aspergillus* can be broken down into chitobiose and chitotriose in *Xenopus laevis*. The rate-limiting step in the digestion of this fungus is chitobiase activity, which is downregulated in atrazine treated tadpoles.

Chitin as a food source could provide both protein and glucose products (Jackson et al. 1992). Studies have shown that chitin may have lipid chelating properties and is used to absorb free fat molecules from the intestines of an organism blocking the absorption of fat into the body. Atrazine’s upregulation of trypsin may have caused an
increased activation of chitinase, which may have caused the partially digested molecules to absorb fat molecules, thus inhibiting its absorption by the body of the tadpole. This is purely speculative, however, as only few animal studies have demonstrated chitin, and its derivative chitosan to absorb fat from the intestines of an organism.

Interestingly, plasmodium the parasite which causes malaria, invades the Anopheles mosquito blood stream by utilizing chitinase activity (Shen and Jacobs-Lorena 1997). As the mosquito has a meal of infected blood, the plasmodium produces ookinetes. These ookinetes then secrete a pro-chitinase that is activated by trypsin in the gut of the insect (Shen and Jacobs-Lorena 1997). This activated chitinase then destroys the peritrophic matrix of the insect, which is a barrier on the walls of the gut that prevents pathogenic invasion. This mechanism of trypsin to activate chitinase could serve as a partial explanation for the upregulation of chitinase in the treated tadpoles.

400 ppb Atrazine Treatment of *Xenopus laevis* Tadpoles Caused Significant Expression Changes of Genes Related to Blood and Plasma

In this present study gene expression related to blood and plasma function was altered by atrazine (fibrinogen alpha, beta and gamma subunits, alpha globin and rac GTPase). Fibrinogen downregulation is indicative of several physiological malfunctions. Low levels of fibrinogen in an organism may cause excessive bleeding. This condition can also signify that the organism may be suffering from chronic liver dysfunction or malnutrition. Therefore, decreased fibrinogen levels is in keeping with the theory that atrazine may be causing metabolic deficits in *Xenopus laevis* tadpoles,
resulting in reduced energy storage in liver cells, and thus, causing malnutrition. Liver
dysfunction has been reported as a possible outcome of atrazine’s effect on an organism
(Santa-Maria et al. 1987). Further studies must be performed to elucidate this
possibility.

Serum albumin also showed decreased mRNA expression in the treated tadpoles.
Serum albumin acts to keep the plasma in the blood at the correct osmotic pressure. It
acts to transport many important small molecules like thyroid hormones, bilirubin,
calcium and progesterone (Manson 2004). Downregulation of this protein (like
fibrinogen) signifies liver or kidney dysfunction. It also occurs because of malnutrition
or some form of protein deficiency in the diet (Pagana 1998).

Decreased serum albumin may also partially explain the decrease in metamorphic
rate, as it is one of the transporter molecules for thyroid hormones, T3 and T4. These
hormones must be transported to target tissues before their effects can be achieved.
Decreased serum albumin could, therefore, decrease this occurrence. Another thyroid
hormone binding protein (transthyretin) was also downregulated by atrazine.
Transthyretin also helps to deliver thyroid hormones to target tissue (Prapunpoj et al.
2000).

This study also demonstrated an upregulation of 4 globin subunits. These are
alpha globin (T4), alpha globin (T3), beta larval beta globin I, and larval beta globin II.
These all are heme-containing proteins that are responsible for the transport of oxygen
in vertebrates (Sherwood 2001). A stressful environment, including chemical stress,
may have several responses. Some of these are accelerated heart rate, increased basal
metabolic rate and increased oxygen consumption (Burchfield 1979). As previously mentioned increased stress affects the hypothalamic-pituitary-adrenal axis causing corticosteroids to be produced, with in turn increases metabolic rate. In order for the increased activity to occur oxygen consumption is increased. This maybe reflected in the increased globin subunits. Oxygen stress may also cause fatigue, poor digestion and a suppressed immune system, all of which may have occurred to some decree in atrazine treated tadpoles.

400ppb Atrazine Treatment of *Xenopus laevis* Tadpoles Caused Significant Expression Changes of Genes Related to Immunity

Genes involved in immunity were affected by atrazine treatment (Major Histocompatibility Complex Class II beta (MHC IIB), CYP-P450 and pentraxin fusion protein). MHC IIB is a molecule that presents antigens to T-lymphocyte cells, which are responsible for cell-mediated immune responses. T-cells can then stimulate B cells which launch an immune response against antigens (Penn and Potts 1999). Expression of this gene was downregulated in the final dataset. This agrees with previous studies on rats (Filipov et al. 2005).

CYP-P450 is involved in the detoxification of xenobiotic compounds (Klassen 2001). This class of compounds is important for the degradation of potentially dangerous xenobiotics by making them more water-soluble (Klassen 2001). These compounds may then be excreted in the urine of the organism. This iron-containing compound can detoxify substances by various mechanisms (Shiota et al. 2000). Some of these are epoxidation, N-dealkylation, S-oxidation and hydroxylation. It is also

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involved in production of cholesterol and steroids (Klassen 2001). If this compound's ability to function is affected, then the organism may become more susceptible to toxic effects of compounds, which can cause damage to organs and tissues. In the microarray dataset, CYP-P450 mRNA expression decreased. Although, using QRT-PCR, no significant difference between gene expression levels of treated and control tadpoles was demonstrated.

The exact mechanism of atrazine's detoxification in amphibians is not totally known. Pentraxin fusion protein binds to necrotic cells and prevents autoimmunity. Expression of this gene was decreased in atrazine treated tadpoles. Down regulation of these genes suggests that the immune system of the atrazine treated tadpoles may be compromised making the tadpole more susceptible to disease, infection and toxic chemicals. It should also be noted that in normal development the immune system of the tadpole is suppressed in metamorphic climax so that the larval immune system does not attack the adult tissues that are being formed. Still, atrazine treatment lowered expression of several immune system genes compared to control tadpoles. A growing body of evidence shows that atrazine along with other herbicides and pesticides may cause immunosuppression in amphibians. This may leave the amphibians open to parasitic and bacterial infections, by alteration of lymphocyte proliferation and suppression of phagocytic activities (Forson and Storfer 2006, Gilbertson et al. 2003, Marie-Soliel et al. 2003).
400ppb Atrazine Treatment of *Xenopus laevis* Tadpoles Caused Significant Expression Changes of Genes Involved in Cell Adhesion

Results of this study demonstrated that when treated with atrazine *Xenopus laevis* tadpoles showed altered gene expression changes in 12 genes associated with cell adhesion, and with transport and storage of ions and molecules. Six were upregulated and 6 downregulated (Figure 11). During metamorphic remodeling, larval tissues and organs are degraded and new organs and tissues are formed. Cell adhesion genes are present to allow binding of cells to other cells or to the extracellular matrix.

However, altered expression of these molecules may be indicative of factors other than metamorphic climax. For example protocadherin is a tumor suppressor for epithelial cells in liver, kidney and colon tissues (Okazaki et al. 2002). Blood vessels associated with tumors express elevated levels of integrin alpha (Gasparini et al. 1998). Caviolin-1 in addition to its many other functions is over expressed in adenocarcinoma and colon carcinoma (Patlolla et al. 2004). Rac ATPase to regulate integrin cell to cell adhesions (delPozo et al. 2000). Desmoplakin is related to cardiomyopathy and galectin altered expression can be a marker for breast cancer (Zubieta et al. 2006). Alteration of these adhesion molecules may, then, be an indicator of impaired cell growth that may be caused by atrazine.

**Summary**

Phenotypic studies on *Xenopus laevis* tadpoles treated with a 400 ppb concentration resulted in decreased metamorphic rate, decreased growth (weight and length), and decreased fat body development. In order to identify gene expression
changes underlying these results, DNA microarrays and qRT-PCR were utilized to measure the abundance of over 15,000 transcripts. In an initial list, approximately 1800 genes, showed significantly altered expression in atrazine-exposed tadpoles. This initial dataset was filtered using a p-value cutoff point of 0.05 and smaller and a fold change cutoff point of 1.5 and greater for upregulated genes and −1.5 and less for downregulated genes. This generated a final dataset of 49 genes. These genes were grouped according to physiological function. Regulated genes were concentrated in groups associated mainly with the digestive system, cell adhesion, immunity, and blood and plasma function.

Because treated tadpoles showed deficits in weight, length, and metamorphic rate, atrazine may have induced metabolic dysfunction during development. Upregulation of digestive system genes may be an attempt to mitigate atrazine’s metabolic effects. This may be a form of adaptation to atrazine, since other genes, NPY, for example, cause increased food intake.

This study demonstrated that DNA microarray technology could be used to identify changes in gene expression related to phenotypic changes induced by treatment of tadpoles with 400 ppb atrazine. Further work using DNA microarrays on earlier stage tadpoles might elucidate how atrazine causes metabolic dysfunction to occur.

Conclusion

This thesis is the first to characterize global gene expression underlying observed phenotypic effects of atrazine on *Xenopus laevis* tadpoles. We propose that chronic
exposure of tadpoles to 400 ppb atrazine causes decreases in growth, metamorphic rate, and fat body size the following manner:

1. Atrazine causes decrease in food intake by affecting the hypothalamus satiety centers in the brain. Reduced food intake has been reported in many atrazine treated vertebrates.

2. The metabolic costs (stress) of detoxification of atrazine utilize stored energy needed for growth and development.

3. Atrazine causes malabsorption of nutrients from the intestines.

4. Atrazine alters the digestion of chitin that leads to its blocking the absorption of fats into the body.

The final gene expression dataset included transcripts that were highly concentrated in functional groups associated with the digestive system, blood and plasma, and cell adhesion. Upregulation of digestive system genes correlated with significant reductions in weight and length may indicate a response to atrazine induced malnutrition and protein wasting. Other atrazine regulated transcripts were involved in immunity, transcription, ion transport and signal transduction.

Reduced growth and metamorphic rate may reduce frog fitness through the following:

- (A) Increased susceptibility to predation.
- (B) Increased susceptibility to disease and parasites.
- (C) Decreased ability to compete with conspecifics for food.
- (D) Increased time to sexual maturity that leads to decreased mating success.
APPENDIX

IACUC Approval Letters
PROJECT OR COURSE TITLE: Molecular Analysis Of Atrazine-Treated Xenopus Laevis Frogs

IACUC Protocol Number: 05D2-02 Date of Last Approval: 04-25-06
Date of Review Request: 04/06/06

Purpose of project (select one): Teaching Research Other (specify): Research

PRINCIPAL INVESTIGATOR OR ADVISOR
Name: Charles Ide, PhD Title: Professor
Department: Electronic Mail Address: charles.ide@wmich.edu

CO-PRINCIPAL OR STUDENT INVESTIGATOR
Name: Anna Jetasso Title: Co-Principal Investigator
Department: ENV Electronic Mail Address: anna.jetasso@wmich.edu

1. The research, as approved by the IACUC, is completed: Yes [ ] No [ ]

If the answer to any of the following questions (items 2-4) is "Yes," please provide a detailed explanation on an attached sheet of paper. Include details of any modifications made to the protocol based on new findings or publications, adverse events or mortalities.

2. Have there been any changes in Principal or Co-Principal Investigators? Yes [ ] No [ ]

3. Have there been any new findings or publications relative to this research? Yes [ ] No [ ]

Describe the sources used to determine the availability of new findings or publications:

Animal Welfare Information Center (AWIC) [ ]
Search of literature databases (select all applicable) [ ]
AGRICOLA [ ]
Current Research Information Service (CRIS) [ ]
Biological Abstracts [ ]
Medline [ ]
Other (please specify): [ ]
Date of search: 04/25/06; Years covered by the search: 1970-Present[ ]

Key words: [ ]

4. Are there any adverse events, in terms of animal well being, or mortalities to report as a result of this research? Yes [ ] No [ ]

Cumulative number of mortalities: 40 [ ]

5. Animal usage: Number of animals used during this quarter (3 months): 0
Cumulative number of animals used to date: 200

Principal Investigator/Faculty Advisor Signature Date 4/25/06
Co-Principal or Student Investigator Signature Date 4/25/06

IACUC REVIEW AND APPROVAL
Upon review of the relevant information regarding this protocol, the IACUC approval for this project has been extended for one year from the date of this signature.

Revised 10/01 WMU IACUC
All other copies obsolete.
Date: April 25, 2005

To: Charles Ide, Principal Investigator
    Anna Jelaso, Co-Principal Investigator

From: Robert Eversole, Chair

Re: IACUC Protocol No. 05-02-02

Your protocol entitled "Molecular Analysis of Atrazine-treated *Xenopus laevis* Frogs" has received approval from the Institutional Animal Care and Use Committee. The conditions and duration of this approval are specified in the Policies of Western Michigan University. You may now begin to implement the research as described in the application.

The Board wishes you success in the pursuit of your research goals.

Approval Termination: April 25, 2006
BIBLIOGRAPHY


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