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Influences of Atrazine on Gonadal Differentiation in *Xenopus laevis* Tadpoles During Metamorphosis

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Abstract

Influence of Atrazine on Gonadal Differentiation in *Xenopus laevis* Tadpoles During Metamorphosis.

Luz Elisa Tavera Mendoza

Xenopus laevis tadpoles were exposed for 48, 96 and 144 h during sexual differentiation to 1.8, 19 and 48 µg/L atrazine. Following the exposure, tadpoles were microdissected for histological examination of the gonadal-kidney complex. The experimental tadpoles showed no significant difference in body weight, body length and in their development among the treatments. However, quantitative histological analysis of the gonads revealed a significant reduction of testicular volume, primary spermatogonial cell nests, and nursing cells in males as atrazine concentration and length of exposure increased. Females showed significant declines in primary oogonia cells and a significantly higher frequency of atresia with increasing atrazine concentration and length of exposure. The presence of a Wilms-like tumour was reported for the first time in tadpoles following exposure to atrazine. Qualitative histological analysis of the kidney showed a significant increase in the incidence of Renal Embryonal Adenosarcoma in all atrazine treated tadpoles. Females showed a significantly higher risk from developing tumours and tumour incidence as compared to males. These results suggests that the present non observable effect level (NOEL) for aquatic ecosystem of 20µg/L atrazine should be reconsidered.

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Introduction

Amphibian population declines have been perceived globally over the last few years and extinction has occurred in a few populations (Carey and Bryant, 1995). Canada is no exception, and amphibian losses have been reported in several provinces across Canada including Ontario (Oldham and Weller, 1991; King et al., 1997), and in Quebec, along the Saint Lawrence river valley (Bonin et al., 1997). Declines in amphibian populations in the Saint-Lawrence river valley (Bishop and Pettie, 1992) and the high incidence of deformities reported in frogs (Bonin et al., 1997) have raised concern among Canadian environmentalists for the last decade (Bishop and Pettie, 1992).

Evidence of world wide amphibian population declines have initiated a search for global and anthropogenic causes. Increased UV-B light (290-320 nm), acid rain, disease, global climate change, habitat loss, introduction of exotic species, and the presence of environmental pollutants (Boyer and Grue, 1995; Allran and Karasov, 2000) have all been implicated. There is often a lower species richness and abundance of amphibians at agricultural sites where agrochemicals have been used relative to adjacent, non agricultural habitats (Allran and Karasov, 2000). Herbicides are now the most widely applied chemicals in agriculture both in terms of volume used and area treated (Howe, 1998). Atrazine is the single most heavily used herbicide in North America (Allran and Karasov, 2000) .

Atrazine, (2- chloro-4- ethylamino-6-isopropylamino - s- triazine) is widely used in

corn and sorghum crops to control grass and weeds by inhibiting photosynthesis (DeNoyelles et al., 1982; Shafer et al., 1999; Van Leeuwen et al., 1999). Since it is a widely used herbicide, its environmental distribution is extensive (Taets et al., 1998). A recent survey on the Saint Lawrence river measured the level of atrazine at 21µg/l on the Yamasaka area. Atrazine levels in the water range from 0.04 -30 µg/l with an average of 2 µg/l at the main corn growing area in Quebec located south of Montreal to Saint-Hyacinthe (Berryman and Giroux, 1994).

Atrazine has a moderate solubility (33µg/ml at 22 °C) and K_d of 0.19 to 2.46 and K_m of 25 to 155. This means that the movement of this chemical in the dissolved state from treated soil is favoured towards surface or substrate waters during irrigation or rains. Atrazine is, therefore, not expected to be absorbed into sediments, but remains in the water column. The fate of atrazine once in the water column is determined by the s-triazine ring, which makes this herbicide resistant to microbial attack. Atrazine also has small hydrolysis and aqueous photolysis rates (Solomon et al., 1996). Through its persistence atrazine makes its way into the drinking water supply (Lin et al., 1999). It is in fact, one of the major pesticides found in water supplies (Taets et al., 1998). The environmental half-life has been reported to be 244d at 25 °C and a pH of 4, but atrazine's half-life can be greatly modified by many factors such as pH, salinity, temperature, concentration of atrazine in the water, sediment type and light source (Solomon et al., 1996).

Atrazine concentrations in ponds, streams and water reservoirs usually peak during

May, June and July following spring application (Allran and Karasov, 2001; Solomon et al., 1996). During this three- month period amphibians lay their eggs and subsequent embryonic development occurs (Allran and Karasov, 2001). Several studies have been conducted to show the effects of atrazine on tadpole fitness, that could affect survival and lead to population declines.

Gonadal differentiation is the development of testes or ovaries from indifferent gonadal tissue. Gonadal determination is the mechanism that directs gonadal differentiation. Both are poorly understood in amphibians (Pieau et al., 1999). The best studied amphibian is *Xenopus laevis*, one of the three most widely cited vertebrate animals in the biological literature (Tinsly and Kobel, 1996).

X. laevis embryonic development has been well defined by Nieuwkoop and Faber (1967). There are 66 stages in tadpole development. They range from oocyte fertilization, Stage 1, to completion of metamorphosis, Stage 66 (Nieuwkoop and Faber, 1967). Normally, the developing gonads can first be seen at stage 46 as thickenings of both sides of the dorsal roof of the dorsal mesentery, in the ventral surface of the mesonephric kidney. These thickenings are referred to as the genital ridges (Kelly, 1996). The primordial germ cells (PGC), which are the precursor cells to definitive germ cells (Saunders,1971; Wylie et al., 1985) migrate into the genital ridge by stage 49. PGC entering into the developing gonad quickly become surrounded by epithelial cells coming from the germinal epithelium of the gonad, and the coelomic epithelium bordering the mesonephric kidney (Kelly, 1996; Scherer, 1999; Pieau et al., 1999). These epithelial

cells are called pre-nursing cells in male and pre-granulosa cells in female tadpoles (Pieau et al., 1999). During early development of the gonads, between Stages 46 to 55, ovaries and testes are not distinguishable. Therefore, this gonadal period is referred to as indifferent or bipotent (Villapando and Merchant- Larios, 1990; Scherer, 1999). During the indifferent stage, males and females have two identical paired ducts: the Müllerian ducts and the Wolffian ducts (Parker et al., 1999). Sexual differentiation in *Xenopus* tadpoles occurs at Stage 56 (Villapando and Merchant-Larios, 1990; Kelly 1996). The gonad enlarges, and the interior medulla can be distinguished from the outer cortex. The developing germ cells enter into active mitosis (Kelly, 1996).

Gonadal differentiation in male tadpoles.

In males, the developing germ cells migrate to the medulla of the gonad, and are wrapped by the nursing cells. The germ cells differentiate into primary spermatogonia and enter synchronous mitosis. These synchronously dividing primary spermatogonia wrapped by nursing cells are referred to as primary spermatogonial cell nests (Reed and Stanley, 1972; Kelly, 1996).

Nursing cells, analogous to Sertoli cells in mammals, provide both nourishment and endocrine support for the survival of the primary spermatogonial cell nests (Andrew and Hickman, 1974). During male gonadal differentiation, nursing cells also produce anti-Müllerian hormone, or Müllerian-inhibition substance, AMH or MIS (Parker et al., 1999). AMH causes the regression of the Müllerian ducts. AMH plays a role in the induction of Leydig cells to produce testosterone (Scherer, 1999). It is not clear whether

or not testosterone production is limited to Leydig cells or whether it also occurs in nursing cells (Nagahama, 1987). It is understood, however, that testosterone causes the differentiation of the Wolffian ducts into the epididymis, vas deferential and seminal vesicles. Testosterone is transformed by 4-ene-steroid-5α reductase enzyme into dihydrotestosterone inducing the formation of external male genitalia (Scherer, 1999). Throughout metamorphosis, primary spermatogonia cells do not enter meiosis, remaining at the primary spermatogonia stage until adulthood (Reed and Stanley, 1972).

Gonadal differentiation in female tadpoles.

In female sexual differentiation, the indifferent gonad enlarges and a central cavity forms in the gonad confining the germ cells to the cortex (Kelly 1996; Villapando and Merchant-Larios, 1990). These developing germ cells become surrounded by the granulosa cells, and the germ cells differentiate into primary oogonia. Primary oogonia undergo the first meiotic division and become secondary oogonia. Some of these secondary oogonia enter the second meiotic division to become primary oocytes at around stage 60. At the end of metamorphosis, stage 66, there are essentially only primary oocytes in the ovary. No further development of these cells occurs until the female frog reaches adulthood (Al-Muktar and Webb, 1971).

In the meantime, the granulosa cells of tadpoles produce aromatase. This converts the testosterone made by the thecal cells into estradiol-17 β (Nagahama, 1987). Estradiol-17 β induces the Müllerian ducts to differentiate into oviducts, a uterus and a vagina (Pieau et al., 1999). It is not clear whether it is the absence of testosterone or the presence

of estradiol-17 β which causes the Wolffian ducts to regress and the gonad to develop into an ovary. It is known however, that aromatase, the enzyme that converts testosterone into estradiol-17 β plays a key role in gonadal differentiation and development (Baroiller et al., 1999; Pieau et al., 1999).

PGC and developing germ cells in both males and females, are important in all vertebrates because they represent the reproductive life stock of an individual. Since these cells cannot be replaced if they are removed or damaged and somatic cells cannot be programmed to form germ cells (Saunders, 1971; Wylie et al., 1985), damage to these cells lowers the reproductive potential of the animal.

Gonadal differentiation, that is the development of testes or ovaries from indifferent gonads is regulated by hormones. Ultimately, however, the gonadal determination in an animal, that is the mechanisms that direct gonadal differentiation, are mostly controlled by its genes.

There are 5 sex determining genes in jawed vertebrates : *wt-1*, *sf-1*, *sox-9*, *sry* and *dax-1*. They all code for transcription factors: WT-1, SF-1, SOX-9, SRY, and DAX-1 respectively (Ohno, 1999; Koopman, 1999; Scherer, 1999; Parker et al., 1999).

Gonadal determination.

Gonadal determination starts when *wt-1*, Wilm's tumour suppressor gene, codes for a multi-functional transcription factor regulator WT-1. WT-1 activates *sf-1*, steroidogenic factor coding gene one, to make SF-1. Then, WT-1 and SF-1 form a heterodimer. In males, it is not yet clear exactly how, but this heterodimer together with

SOX-9 and SRY, the testis determination transcription factors, induce the nursing cells to produce AMH (Koopman, 1999; Wallace et al., 1999). SF-1 together with SOX-9 and AMH tightly repress *cyp 19*, the gene that codes for aromatase. With aromatase repressed, only testosterone is produced and male differentiation occurs. In females *dax-1*, the anti-testis gene, codes for DAX-1. It is not know exactly how DAX-1 represses the male determining genes *sox-9* and *sry* (Goodfellow and Camerino, 1999; Pieau et al., 1999). It is not clear either if DAX-1 alone or a heterodimer between DAX-1 and SF-1, but DAX-1 plays a role in blocking the production of AMH (Wallace et al., 1999; Koopman, 1999). In the absence of AMH, SF-1 induces the expression of the aromatase gene. This leads to estradiol-17β production and consequently female differentiation occurs (Parker et al., 1999; Goodfellow and Camerino, 1999).

The mechanisms that regulate these sex determining genes, and the interactions between them are yet to be clarified. It is, however; well documented that *wt-1*, the Wilm's tumour suppressor gene, resides at the top of the hierarchy governing nephric development, of which gonadal development is a part. WT-1 is a single transcription factor that has the capacity of not only regulating many target genes, but it also functions as a tumour suppressor (Kent et al., 1995; Ohno, 1999; Parker et al., 1999; Rauscher, 1993).

The function of WT-1 as a tumour suppressor in the kidney is by repressing the expression of *egr-1*, early growth regulator gene 1. This gene codes for a mitotic inducer factor, EGR-1, which promotes nephroblastic proliferation in the kidney. When there is a

mutation to *wt-1* or damage to WT-1, there is no repression of *egr-1*. This lack of regulation produces an uncontrolled cell proliferation that manifest itself as neoplastic cell growth leading to the formation of Wilm's tumour in the kidney (Rauscher, 1993; Kreidberg et al., 1993; Ohno, 1999).

Wilm's Tumour.

In humans, Wilm's tumour, which is also called embryoma or embryonal adenosarcoma, is one of the most frequent solid tumours in children occurring 1 in about 10,000 live births (Rauscher, 1993). This is a major cancer killer in children, accounting for about 20% of cancers in childhood (Robbins and Kumar, 1987; Anderson and Scotti, 1972). It is a very malignant, fast growing tumour which metastasises rapidly to lymph nodes, lungs, liver, brain, and bone (McSween and Whaley, 1992; McCance and Huether, 1994; Walter and Talbot, 1994). The histological diagnosis rests upon identification of primitive or abortive tubules and glomeruli. These are enclosed in a spindle cell stroma. Additional evidence of this tumour is the presence of striated muscle fibres or fibroblast (Robbins and Kumar; 1987). In addition, collagen fibres and areas of necrotic tissue containing cholesterol crystals and lipid macrophages have been reported (Robbins and Kumar, 1987). There is no association between tumour frequency and gender (McCance and Huether, 1994). Although many adult cancers are associated with environmental agents, childhood tumours are generally not thought to share associations with the environment, because of the length of exposure required for development of cancer.

Early exposures to carcinogens are thought to result in tumour when the child becomes an adult (McCance and Huether, 1994).

Several studies showed no effects on tadpoles following exposure to atrazine at environmentally meaningful concentrations (Allran and Karasov, 2000; Stephen et al., 2000; Larson et al., 1998; Detenbeck et al., 1996). However, the possible effects of atrazine on tadpole gonadal differentiation and its capacity to alter the reproductive potential during its life cycle have never been investigated. An impaired gonadal differentiation could lead to reduced fertility among individuals in a population and to a population decline. The aim of this study was to investigate the potential effects of atrazine at environmentally meaningful concentrations in the gonadal development and differentiation using the amphibian model *Xenopus laevis*.

Materials and Methods

There were two experiments in this project. Experiment I consisted on exposure of *X. laevis* tadpoles to 21µg/L atrazine for 48 h during gonadal differentiation. Endpoints recorded were testicular volume, total number of germ cells, and frequency of nurse cells in male tadpoles while in female tadpole frequency of oogonia were recorded. Experiment II consisted on exposure of *X. laevis* tadpoles to increasing concentrations of atrazine: 1.8, 19, and 48µg/L with increasing exposure length: 0, 48, 96 and 144 h. In addition to gonadal measurements, tadpole body weigh, body length and external rate of development were measured. Incidence of Wilm's-like tumour and the risk of developing Wilm's like tumour were also recorded in experiment II.

Test Organisms for Experiment I

Female and male *X. laevis* tadpoles, stage 53 (Nieukoop,1967), were obtained from Xenopus I (Dexter, Michigan). Tadpoles were acclimated to laboratory conditions for 10 days. They were kept during the acclimation and the experimental period in 100-L glass aquaria filled with dechlorinated aerated City of Montreal water. The experiments were conducted in a static system at 21± 2 ° C in a photoperiod of 12h light- 12h dark.

Test Organisms for Experiment II

Female and male *X. laevis* tadpoles, stage 50 (Nieukoop,1967), were obtained from Xenopus I Co., (Dexter, Michigan). Acclimation and experimental conditions were the same as those outlined for experiment I.

Diet

Tadpoles were fed 0.1 g of a commercial tadpole diet (Boreal laboratories, St. Catherines, ON.) every 96h until stage 54-55. Tadpoles were not fed during the experiments. Atrazine has been shown to interfere with tadpole appetite in *Rana pipiens*, *Rana sylvatica* and *Bufo americanus* (Allran and Karasov, 2001). To avoid this potential effect, control and atrazine treatment tadpoles were not fed throughout the exposure period.

Preparation of Atrazine solution

A stock solution of atrazine was freshly prepared on the evening prior to the 48h pulse exposure. Ninety-nine percent pure atrazine (0.015g) from Sigma Diagnostics, St. Louis, Mo. was weighed and diluted into 1 L of ultra-distilled H_2O (ultra-distilled water is water that has a content of total organic carbon <5 ppb, total dissolved solids <10 ppb, silicates <0.1 ppb, heavy metals <0.1 ppb and resistivity (Megohm-cm at 25 °C) = 18.2). The solution was sonicated for 4 h in a Cole-Parmer 8850 sonicator, in an ice bath. Once the atrazine was in solution, the container was covered with aluminum foil, and kept at 4°C on a stirring plate, to avoid sedimentation of the atrazine.

Exposure For Experiment I

Fifteen tadpoles were placed in each of the six tanks with 15 L of aerated, dechlorinated city water. Stock solution (21.0 ml) was slowly added into the treatment tanks containing 15 L to produce a final concentration of 21µg/L of atrazine in the treatment tanks . There were 2 treatment tanks and 2 control tanks.

Exposure For Experiment II

Thirty tadpoles, at stage 55, prior to gonadal differentiation were placed in each of the tanks with 15 l of aerated, dechlorinated city water. Fresh stock solution of atrazine (15mg/L) was slowly added to the treatment tanks: 2.1 ml, 21 ml and 60 ml of stock solution was added to each of the 3 treatment tanks to produce a nominal concentration of 2.1, 21 and 60 µg/L respectively. There were 2 tanks for each treatment and two control tanks. Twenty tadpoles were sampled at the beginning of the experiment (time 0). Ten tadpoles were sampled from each tank (total of 20 tadpoles per treatment) at 48h, 96h and 144h. The water including the atrazine in each tank was changed every 48h with fresh solutions. Water samples were taken from each treatment and control tanks and sent for analysis of atrazine both at the beginning and end of the exposure (Technitrol Inc., Point-Claire, Quebec).

Sampling Technique

Following exposure, tadpoles were anaesthetized with a 0.5% solution of tricaine methane sulfonate (MS222). A longitudinal incision was made along the peritoneal cavity, and the tadpole was immediately fixed in Bouin's solution (Sigma Diagnostics, St. Louis, Mo.). Tadpoles were weighed and measured. The gonadal-kidney complex was microdissected under a dissecting microscope. The Bouin's fixative was removed from the micro-dissected gonads by several changes of 70% ethanol. Tissues were processed (dehydrated, cleared and infiltrated with paraffin) in a tissuematon (Fisher Scientific). All tissues were embedded in the anterior-longitudinal orientation. Sectioning was done using a rotary microtome set to yield 9 µm sections. Sections were stained using the

routine Hematoxylin (Sigma Diagnostics, St. Louis, Mo.) and Eosin (Sigma Diagnostics, St. Louis, Mo.) procedure and mounted with permount resin (Fisher Scientific).

Photomicrographs were taken in a Leitz binocular microscope with a Leica camera, using a Ektachrome 320T tungsten film (Kodak Rochester, NY).

Measurements

Gonadal Volume.- Due to the shape of the tadpole gonad following gonadal differentiation, the gonadal volume was calculated using the formula of the right circular cone: $V = \frac{1}{3} \pi r^2 h$; where v= volume, h= the length of the largest section, and r = the distance between the first to the largest section.

Total number of Germ Cells.- germ cells were counted in three sections produced from the anterior, middle and posterior regions of the gonad. The number of germ cells over the volume of each of these three sections was recorded. A mean was calculated from these three sections, expressed as the number of primary spermatogonia over the mean volume of the three sections. The total number of germ cells was calculated by multiplying the mean value of primary spermatogonia nest cells by the total gonadal volume divided by the average volume of the three sections. Germ cells are expressed as total number of primary spermatogonia in the testis.

Frequency of Nurse Cells.- A grid was placed in the microscopic field (0.1 mm x 0.01mm) which produced 100 squares. A number was assigned to each square (e.g.1 to 100). Ten random numbers* were selected for each of the three sections each (beginning, middle and end parts of the gonad), and the primary spermatogonia cell nest

corresponding to the number in the grid was examined. If the nurse cells were absent or showed signs of swelling, vacuolation, karyolysis, (nuclei show decreased staining as they undergo dissolution), karyorrhexis, (nuclei break up giving distinct fragments), or pyknosis, (the nuclei shrink and appear as densely staining masses), a score of 0 was assigned. If the nurse cell appeared normal, a score of 1 was assigned. The total scores in each section were added (10) and a score per section was assigned (0 - 10).

Frequency of oogonia.- The same microscopic grid was used for the oogonia estimation. Ten random numbers* where selected and the oogonia were scored. Primary oogonia received a score of 1, secondary oogonia received a score of 2, and atretic oogonia received a score of 3.

**10 Random numbers were needed to have a variance* ($s^2 = \Sigma(X_1 - \bar{x})^2/n-1$) *less than 5% per section.*

Tadpole Body Weight.- Tadpoles were placed on a weighing dish and weighed.

Tadpole length.- It is the distance from head to tail (mm).

External development.- External development has been measured in *X. laevis* as the ratio of the distance between the eyes over the distance from head to tail (Nishimura et al., 1997).

Incidence of Renal Embryonic Adenosarcoma (Wilm's-like tumour incidence) .- Histological characteristics were used to record the incidence of tumours present in male and female tadpoles. Since these tumours have all the histological characteristics of Wilm's Tumour in humans, it will be referred to as Wilm's- like tumour from this point

onwards. The histological features that distinguish this tumour are primitive or abortive glomeruli and tubules, with poorly formed Bowman's spaces, all enclosed in a spindle cell stroma. The incidence of tumours was recorded for each tadpole based on the presence of the 4 histological characteristics. If the tadpole showed them all, a score of 1 was given in the category of Wilm's-like tumour, otherwise a score of zero was assigned.

Risk of developing Wilm's- like tumour.- Frequency of risk of developing tumour was recorded for male and female tadpoles. Tadpoles were considered to be at risk of developing the tumour when one or more histological characteristics were present. If the tadpole was considered at risk from tumour, a score of one was assigned, otherwise a score of zero was assigned to this category.

Statistical Analysis

Data for each group was tested for normality and homogeneity of variances. Primary replicates (averages of each tank) were analysed via one- way ANOVA followed by a Tukey's test. After ensuring that there were no significant differences among replicate tanks and within tadpoles in the same tank, the data were further analysed using the secondary replicates as units, that is, each tadpole as a unit (National Toxicology Program, 1984; Mantel, 1980). For experiment I, the male data: testicular volume, total number of primary spermatogonia and score of nursing cells were analysed using a one-way ANOVA. The female data: frequency of the stages of oogonia was analysed using the non- parametric analog of an un-paired T-test, the Mann Whitney U test. For experiment II, male data were: total number of primary spermatogonia, primary

spermatogonial abundance and score on nursing cells were analysed using a Nested and factorial ANOVA. Female data was: total number of primary oocytes were analysed using Kruskal-Wallis and Nested Anova tests followed by a Tukey test. Tumour incidence in the kidney was calculated by Kruskal-Wallis, Mann-Whitney tests, and regression analysis. For both experiments data were analysed by using SPSS 10.1 statistical software, and the p value was set at 0.05

Results

Experiment I: Males Tadpoles

Total testicular volume decreased from 0.026 in the control testis (Photomicrograph 1) to 0.01 mm^3 in the atrazine treated group (Photomicrograph 2). This represents a 57% decreased in testicular volume among the atrazine exposed tadpoles relative to controls. ($p= 0.004$; S.E± 0.002 df=3, n= 30 F= 5.618. Figure 1).

Primary spermatogonia cell nests decreased in the testis from an average of 242.4 to 72.9 per gonad representing a 70% reduction in spermatogonia cell nests ($p < 0.001$; SE± 29 df=3, n=30, F= 9.38. Figure 2).

Nurse cells declined in atrazine exposed testes from a score of 9.62 out of 10 to 2.35 out of 10. This represents a 74% of the nurse cell integrity on the testis ($p < 0.001$; SE± 0.002 df=3, n=30, F= 40.95. Figure 3).

Experiment I: Female Tadpoles

Distribution of oogonia.- Oogonia cells were located in the outer cortex of the gonad (Photomicrograph 3). During experiment I, both primary and secondary oogonia were present in the ovary (Photomicrograph 4). Following exposure to atrazine 21 µg/L the frequency of occurrence of primary oogonia was 74% in controls and 43.7% in atrazine treated animals. There was an increase in secondary oogonia in atrazine treated animals, from 23% in control to 36% in atrazine treated animals. The rate of atresia also increased in the atrazine treated tadpoles from a frequency of 2% in controls to 20.2% in the atrazine treated animals (Photomicrograph 5). Atresia is the normal process by which the

ovary reabsorbs eggs that are unable to complete normal development. These changes in distribution of primary, secondary and atretic oogonia were all statistically significant (p = 0.001, SE± 0.09, n=30, Z= -9.458; Figure 4).

Experiment II: External Features

Tadpole length.- The distance from the head to the tail showed no significant differences between control tadpoles ($\bar{x}$ = 3.794 cm, S.E = 0.056) and 1.8, 19, and 48 µg/L atrazine treated tadpoles after 48, 96 and 144 h exposure (p= 0.099; Figure 5).

Average body weights.- There was no significant difference between control ($\bar{x}$ = 0.2508 g, S.E =0 .017) and 1.8, 19, and 48 µg/L atrazine treatment tadpoles after 48, 96 and 144 h exposure (p= 0.116; Figure 6).

External development.- There was no significant difference between control ($\bar{x}$ = 5.46 cm, S.E =0 .08) and 1.8, 19, and 48 µg/L atrazine treated tadpoles after 48, 96 and 144 h (p= 0.540; Figure 7).

Experiment II: Male tadpoles

Primary spermatogonia cell nests fluctuated throughout the exposure. After 144 h of exposure to 1.8 µg/L, 19 µg/L and 48 µg/L atrazine, treated tadpoles had a reduction of 57.6% 57.2% and 88% in their primary spermatogonia respectively. The number of primary spermatogonia cell nest differed statistically among treatments (p <0.001, n= 129, df=3, F= 10.77). There was a significant interaction between concentration and time (factorial anova p= 0.036 power= 0.8). Following 144 h, there were significant differences between control and all atrazine concentration exposures : control and 1.9

µg/L (p= 0.021) , control and 18 µg/L (p= 0.039) control and 48 µg/L (p <0.001; Figure 8).

Nurse cells There were significant differences in the scores of Nurse cells for both: dose (p<0.001) and exposure length (p <0.001). There was a significant interaction between time and concentration (p= 0.005). There was a significant difference between control ($\bar{x}$ = 9.52 , S.E = 0.144) and 1.8, 19 and 48 µg/L (p <0.001, n= 129, df=3, F= 120.83 ; Figure 9).

Experiment II: Female tadpoles

Primary oogonia cells.- Tadpoles were younger in this experiment. There were only primary oogonia cells present in the ovary. Numbers of oogonia changed throughout exposure treatment (p= 0.001, df=3, n= 128, $\chi 2$ = 11.344) and time ($\chi 2$ = 9.5,p= 0.009). There was a significant interaction between time and treatment (p= 0.029). Following treatment for 48h an apparent enhancement of oogonial development occurred. This resulted in increasing numbers of primary oogonia from a mean of 178.9 in control ovaries to a mean of 242. It represented a 35% increase on primary oogonia. However, this trend changed after 96 h. Primary oogonia decreased by 40%. This trend continued, and after 144h exposure to 1.8, 19 and 48 µg/L atrazine resulted in declines of 37%, 48.5% and 50.26 % respectively. These declines were significant: 1.8 µg/L (p=0.019), 19 µg/L (p=0.033) and 48 µg/L (p <0.001; Figure 10).

Experiment II: Wilm's-like tumour (Renal Embryonal Adenosarcoma)

Wilm's- like Tumour incidence.- The incidence of this tumour was significantly related

to the dose of atrazine ($p < 0.001$, n= 257, df = 3, $\chi 2 = 73.074$) and length of exposure ($p < 0.001$, df= 3, $\chi 2 = 21.76$). There were no tumours in control animals ($\bar{x} = 0.0$, S.E =0.0). Tumour incidence in 1.9 µg/L group was significant ($\bar{x} = 0.0345$, S.E = .0170, p=0.011). No significant difference between control and 18 µg/L incidence ($\bar{x} = 0.0167$, S.E =0.0117, p=0.078) was observed. There is a significant difference between control and 48 µg/L ($p < 0.001$, S.E =0.0389, $\bar{x} = .2241$; Figure 11, Photomicrograph 6). Incidence of Wilm's-like tumour is significantly higher in females relative to males ($p < 0.001$, Z= -4.86) (Figure 12).

Risk from Wilm's -like tumour.- The risk of developing Wilm's -like tumour significantly increases with increase in atrazine dose ($p < 0.001$,df =3, n =257, $\chi 2 = 110.66$) and time ($p < 0.001$, df =3, $\chi 2 = 34.31$). There were no control animals at risk ($\bar{x} = 0.0$). There was a significant difference in tadpoles at risk between control and 1.8 µg/L ($p < 0.001$ $\bar{x} = 0.069$, S.E = 0.0236,), control and 19 µg/L ($p < 0.001$, $\bar{x} = 0.2333$, S.E =0.0387), and between control and 48 µg/L ($p < 0.001$, $\bar{x} = 0.4310$, S.E =0.0462,) (Figure 13). The incidence of tadpoles being at risk from Wilm's-like tumour following atrazine exposure is also significantly higher in females compared to males ($p < 0.001$, Z = -5.47; Figure 14).

Photomicrograph 1. Experiment I. Primary spermatogonial cell nests (cn) in the testis (T) of control *X. laevis* stage 56 during sexual differentiation (250x).

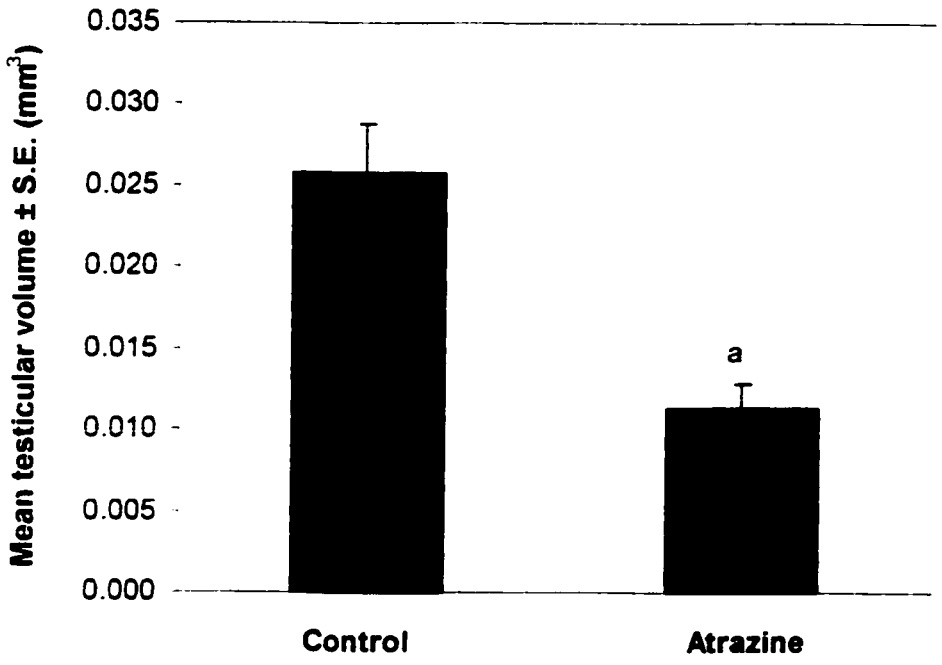
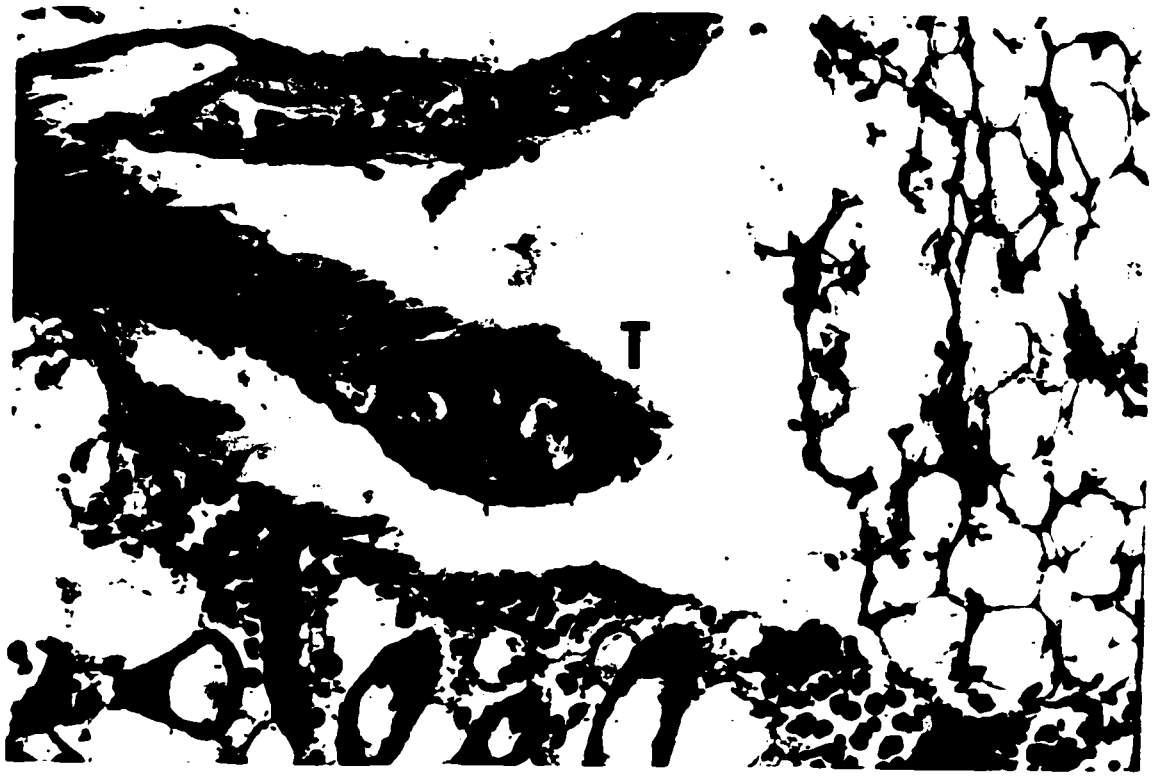


Figure 1.- Experiment I. Mean testicular volume (mm^3) of stage 56 *X. laevis* tadpoles exposed to 21 µg/l atrazine for 48 h. during sexual differentiation. Letter "a" indicates a statistical significance ($p<0.05$).



Photomicrograph 2. Experiment I. Testis (T) from a stage 56 *X. laevis* demonstrating reduction in volume following exposure to 21µg/l atrazine for 48h (250x).

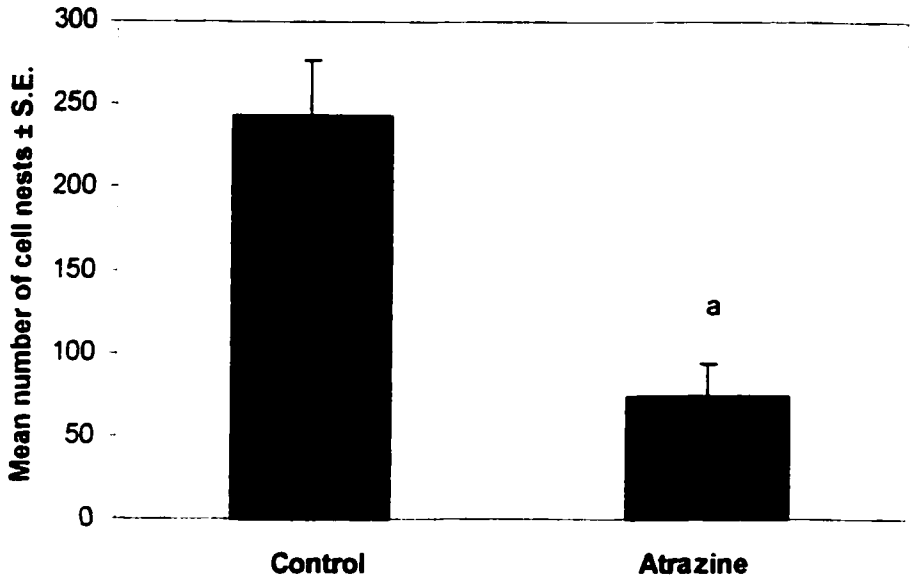


Figure 2.- Experiment I. Mean number of primary spermatogonial cell nests in testes of stage 56 *X. laevis* tadpoles exposed to 21 µg/l atrazine for 48 h during sexual differentiation. Letter "a" indicates significance ($p<0.05$).

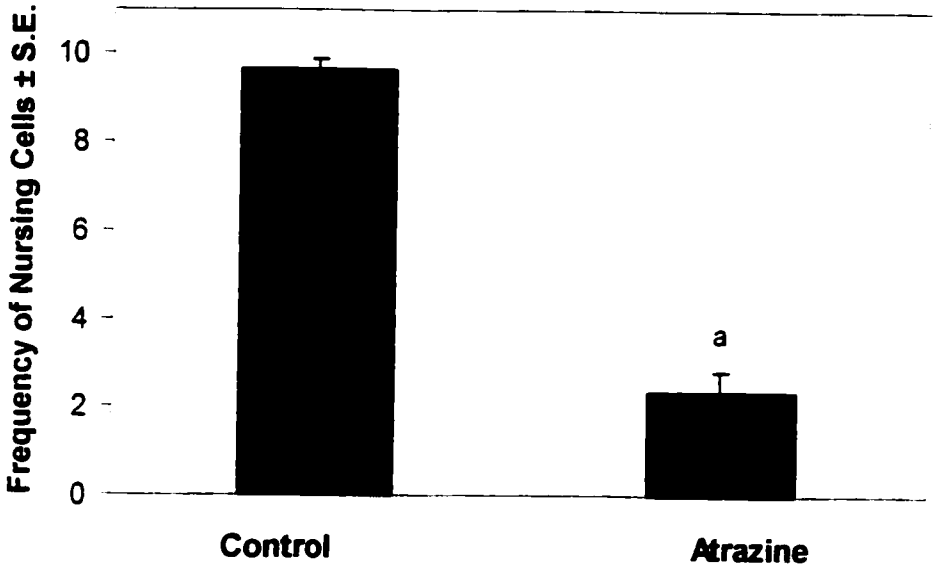
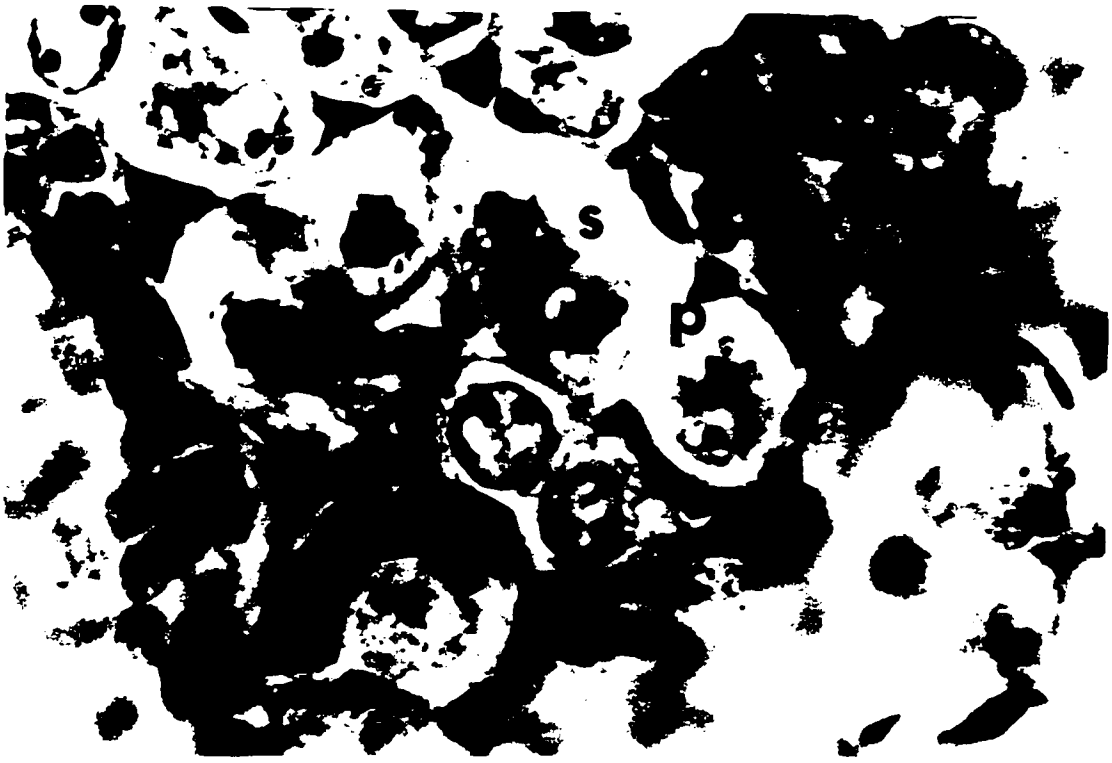


Figure 3.- Experiment I. Frequency of Nursing cells in testes of stage 56 *X. laevis* tadpoles exposed to 21 µg/l atrazine for 48 h during sexual differentiation. Letter "a" indicates a statistical significance ($p<0.05$).

Photomicrograph 3. Experiment I. Ovary (O) from a control *X. laevis* stage 56 showing the oogonia cells in the outer cortex and a cavity (c) in the inner medulla (250x).



Photomicrograph 4. Experiment I. Primary oogonia (p) and secondary oogonia undergoing meiosis (s) in the ovary of a control stage 56 *X. laevis* during sexual differentiation (1000 x).

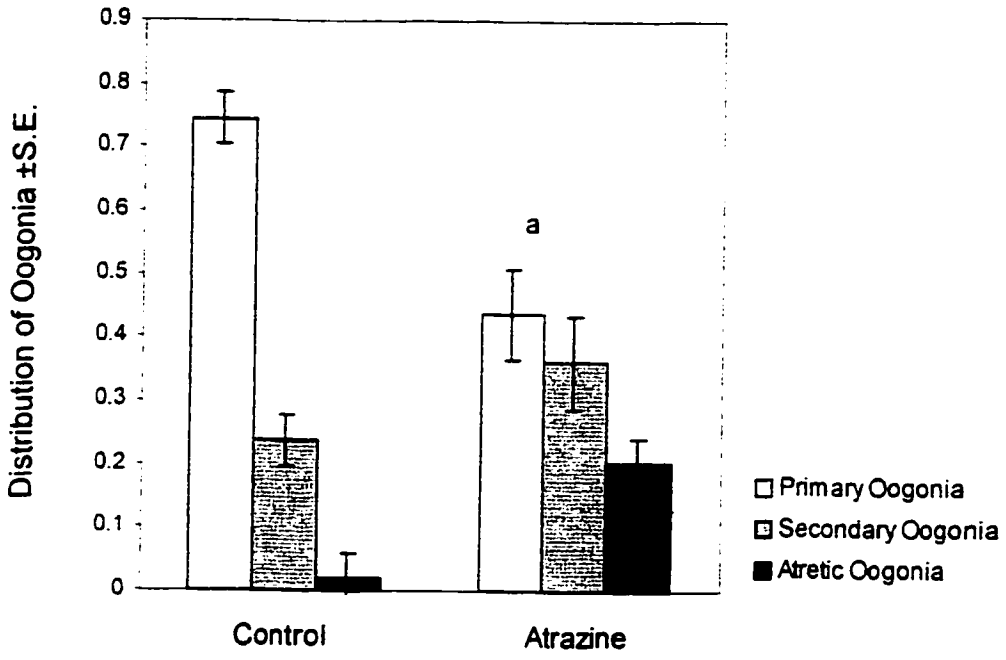


Figure 4.- Experiment I. Distribution of primary, secondary and atretic oogonia in ovaries of stage 56 *X. laevis* tadpoles exposed to 21 µg/l atrazine for 48 h during sexual differentiation. Letter "a" denote a statistical significance ($p<0.05$).

Photomicrograph 5. Experiment I. Atretic oogonia (a) in the ovary of a 21 µg/l atrazine treated *X. laevis* for 48 h (1000 x).

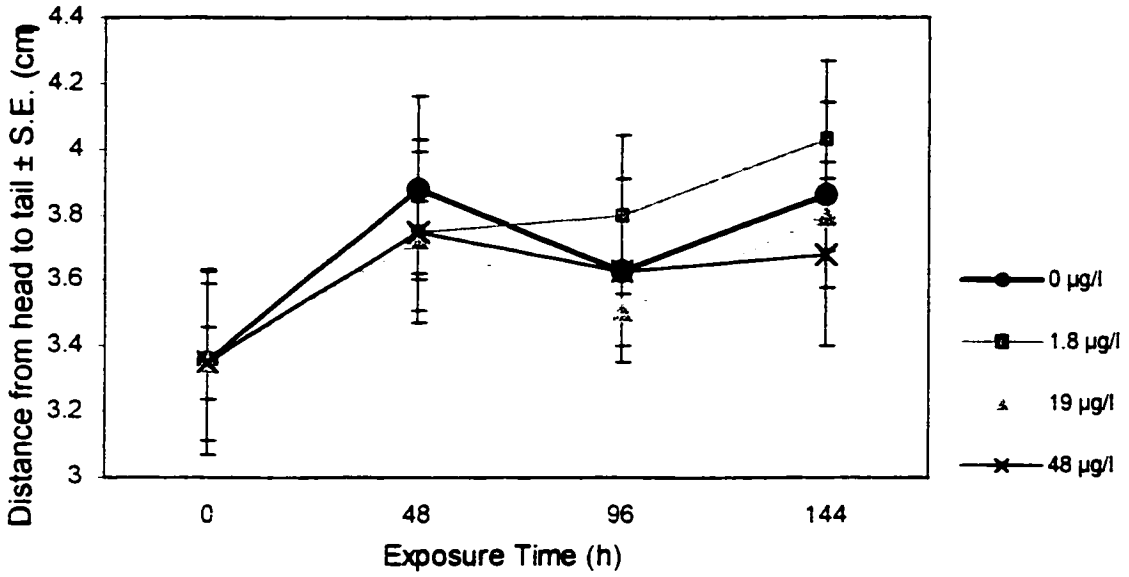


Figure 5.- Experiment II. There were no significant differences on tadpole length (cm) in *X. laevis* exposed to 1.8, 19 and 48 µg/l atrazine treatment for 48, 96 and 144 h during gonadal differentiation (p= 0.099).

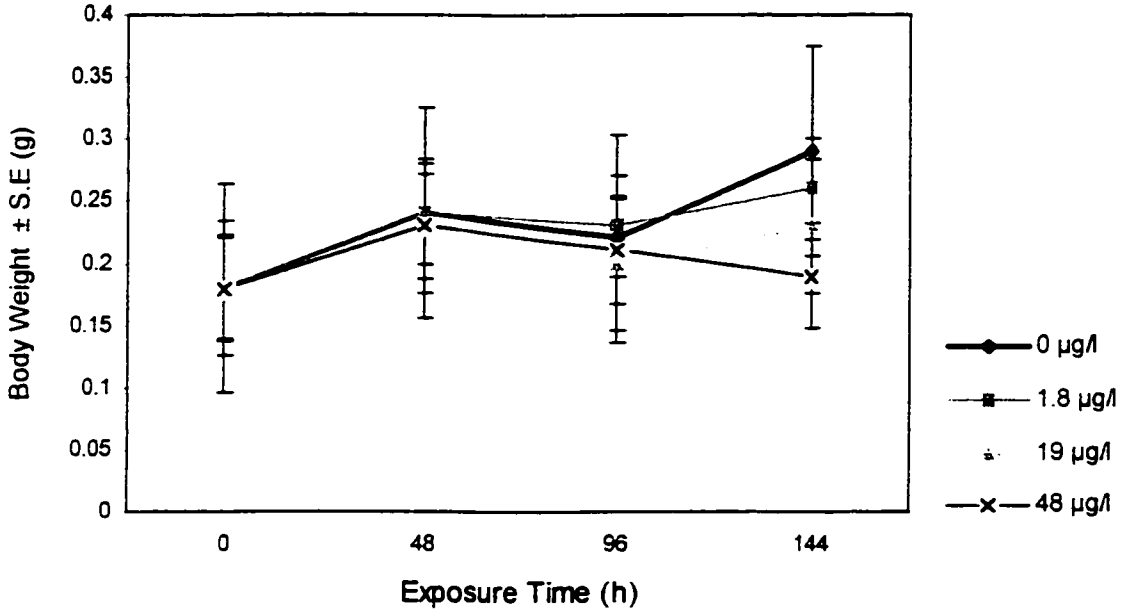


Figure 6.- Experiment II. There were no significant differences on *X. laevis* tadpole body weight (g) following exposure to 1.8, 19 and 48 µg/l atrazine treatment during gonadal differentiation for 48, 96 and 144 h (p=0.116).

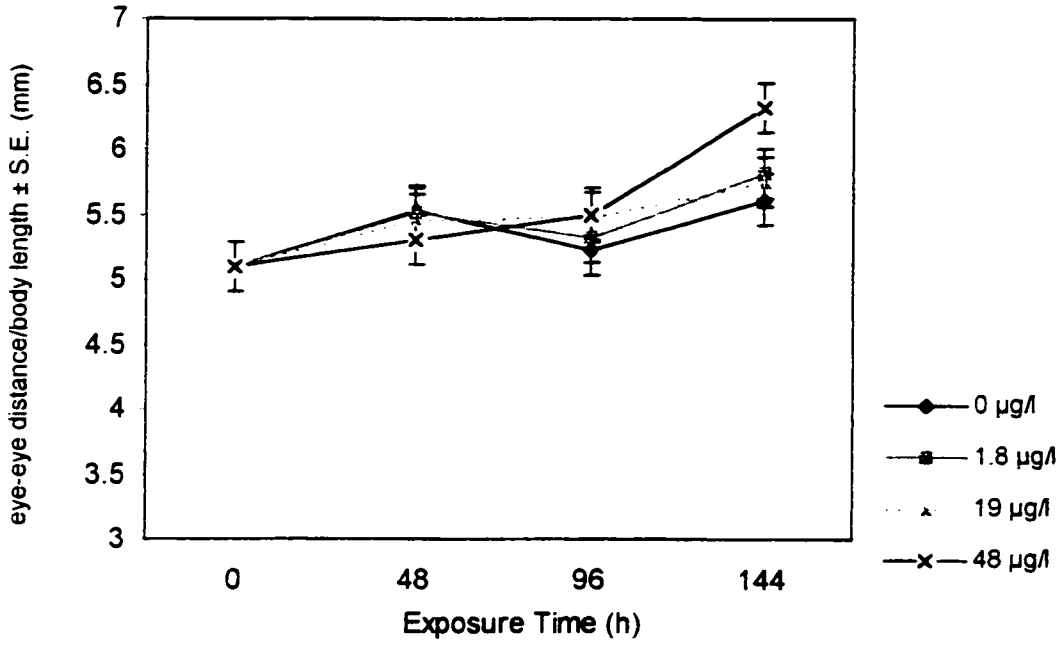


Figure 7.- Experiment II. There were no significant differences on stage 56 *X. laevis* tadpole development (distance from eye to eye/ body length) following treatment to 1.8, 19 and 48 µg/l atrazine treatment during 48, 96 and 144 h ($p = 0.540$).

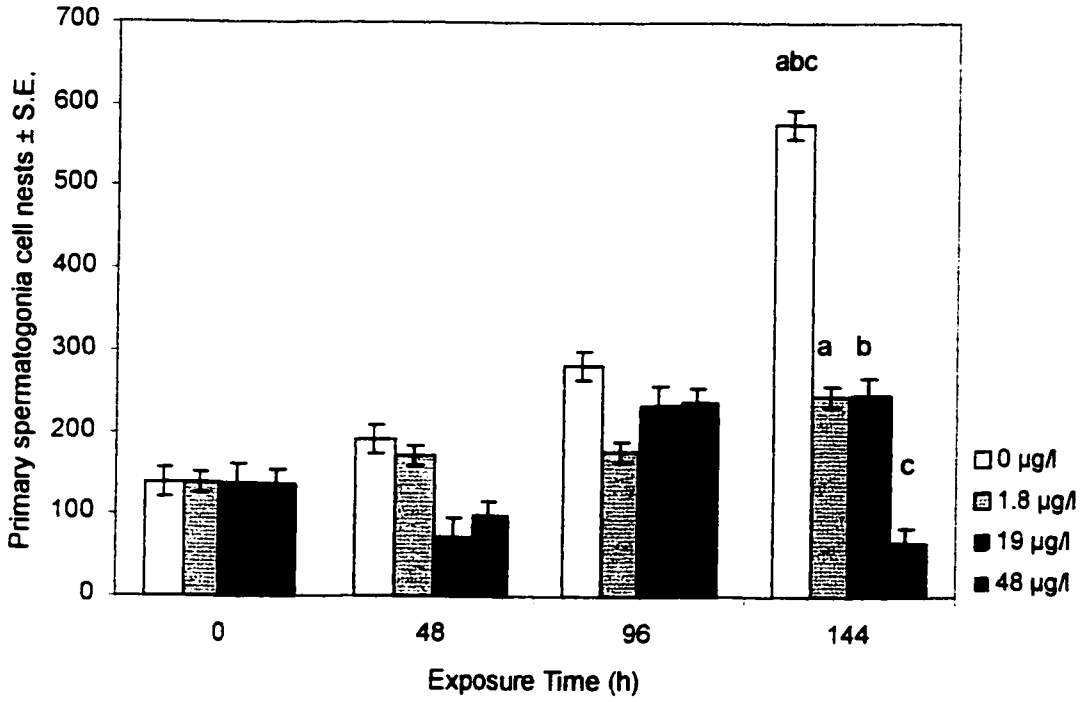


Figure 8.- Experiment II. The overall decrease of primary spermatogonial cell nests in testis of *X. laevis.* tadpoles following treatment to 1.8, 19, and 48 µg/l atrazine for 144 h was statistically significant. ($p = 0.003$). The tendency of control tadpoles to increase their primary spermatogonial cell nests through time was also significant ($p = 0.003$). Letters denote significance ($p < 0.05$), where control (abc) is significantly different from 1.8 (a), 19 (b) and 48 (c)µg/l atrazine.

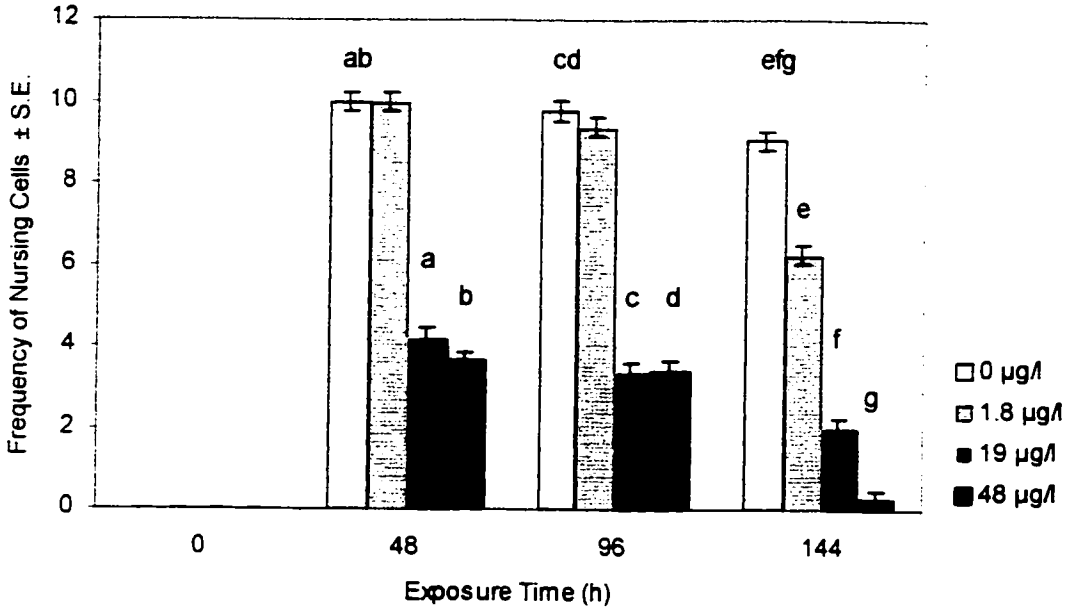


Figure 9.- Experiment II. There was a significant difference in the frequency of nurse cells in *X. laevis* testis following exposure to 1.8, 19 and 48 µg/l atrazine treatment during gonadal differentiation for 48, 96 and 144 h ($p < 0.001$). Letters denote significance ($p < 0.05$) Where ab is significantly different from a, b, and cd from c, d and efg from e, f,g. .

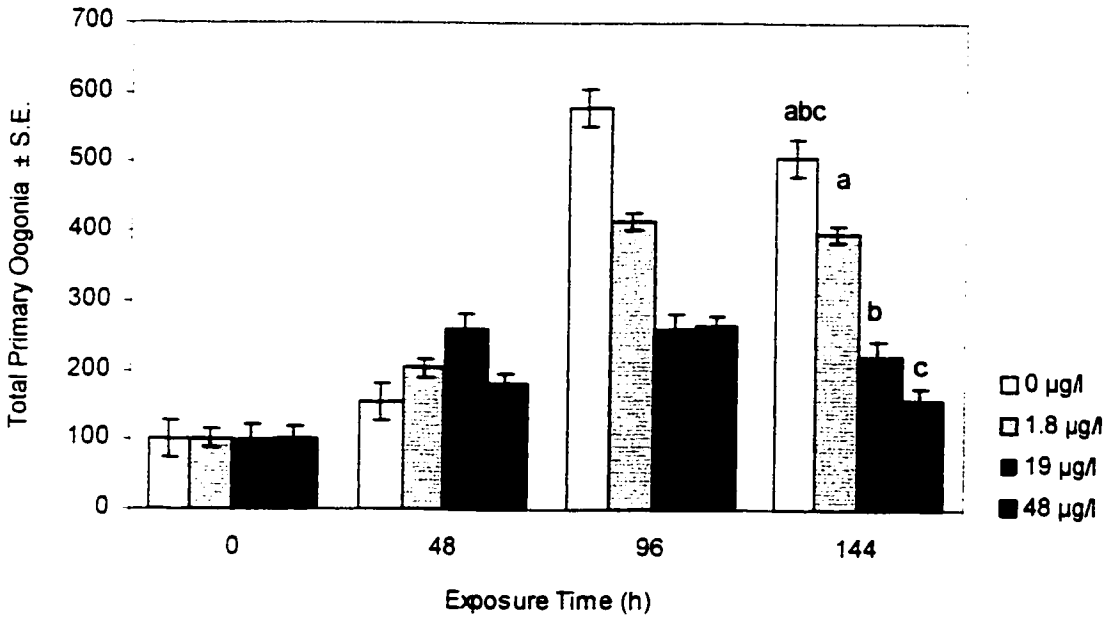
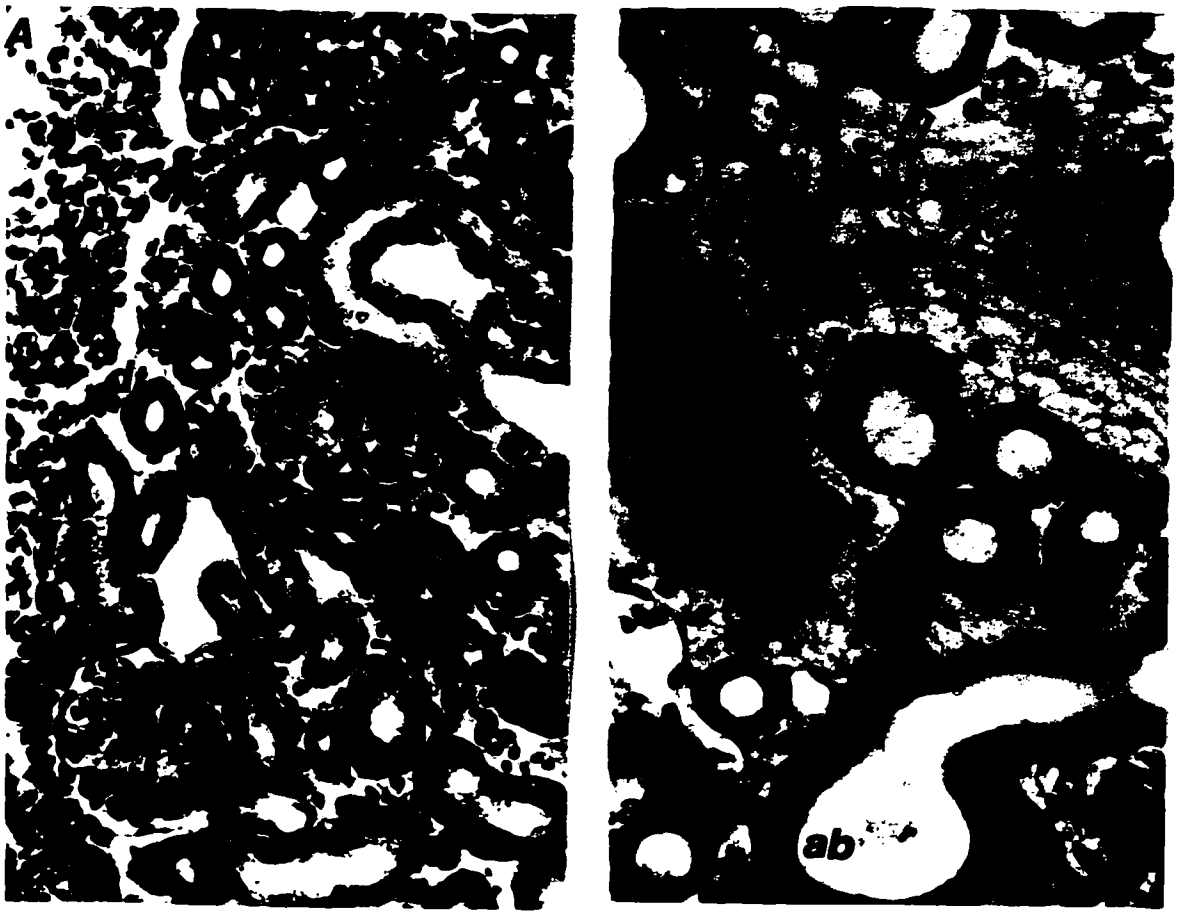


Figure 10.- Experiment II. Numbers of oogonia significantly changed throughout exposure treatment ($p < 0.001$) and time ($p = 0.009$). There is a significant interaction within time and treatment ($p = 0.029$). Letters denote significance ($p < 0.05$), where abc is significantly different from a, b, c.



Photomicrograph 6. Experiment II. a)Mesonephric kidney from control *X. laevis* during gonadal differentiation showing proximal convoluted tubules (pt), distal convoluted tubules (dt), and Bowman's capsule (bc). Arrow shows the clear integrity of endothelium (250 x). b)Wilm's-like tumour in atrazine treated *X. laevis tadpole* demonstrating histological features of Wilm's tumour: abortive tubules (at), abortive Bowman's capsule (ab), enclosed in spindle cell stroma (open arrows). Solid arrow indicates the presence of a fibroblast. Mesenchymal cells (m) and an area of necrotic tissue (n) are also present (250 x).

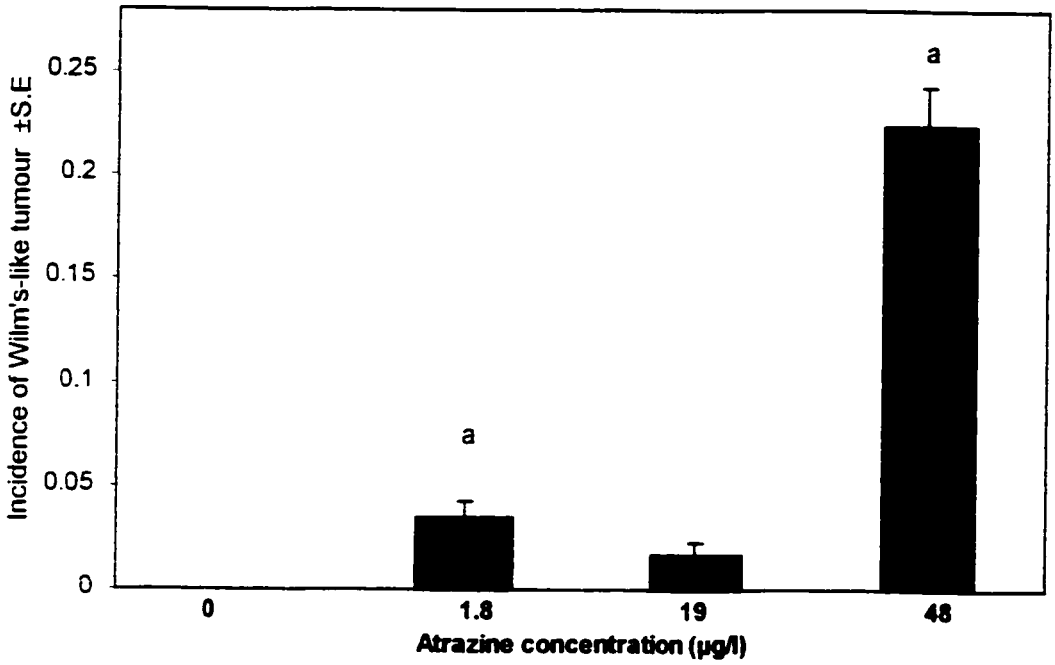


Figure 11.- Experiment II. The incidence of Wilm's-like tumour in *X. laevis* tadpoles following exposure to 1.8, 19 and 48 µg/l during gonadal differentiation for 48, 96 and 144 h was significantly related to dose of atrazine ($p < 0.001$) and length of exposure ($p < 0.001$). Letter "a" indicates significance from control ($p < 0.05$) There were no tumours in control tadpoles.

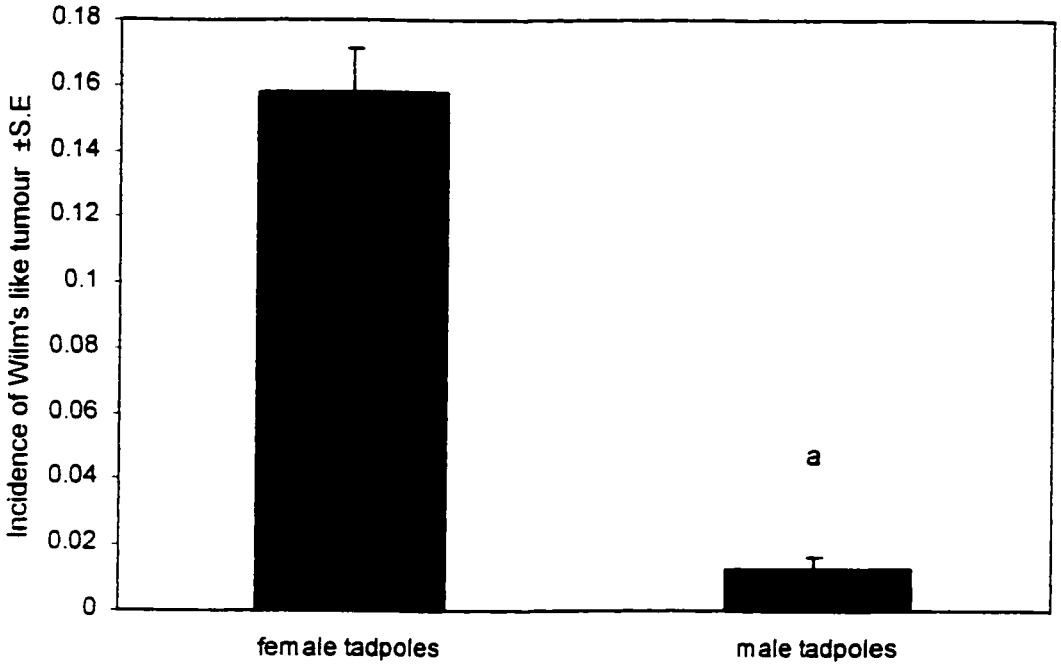


Figure 12.- Experiment II. Incidence of Wilm's-like tumour was significantly higher in females relative to males ($p < 0.001$) in 1.8, 19 and 48 µg/l atrazine treated *X. laevis* tadpoles during gonadal differentiation. There were no tumours in control tadpoles. Letter "a" denotes significance.

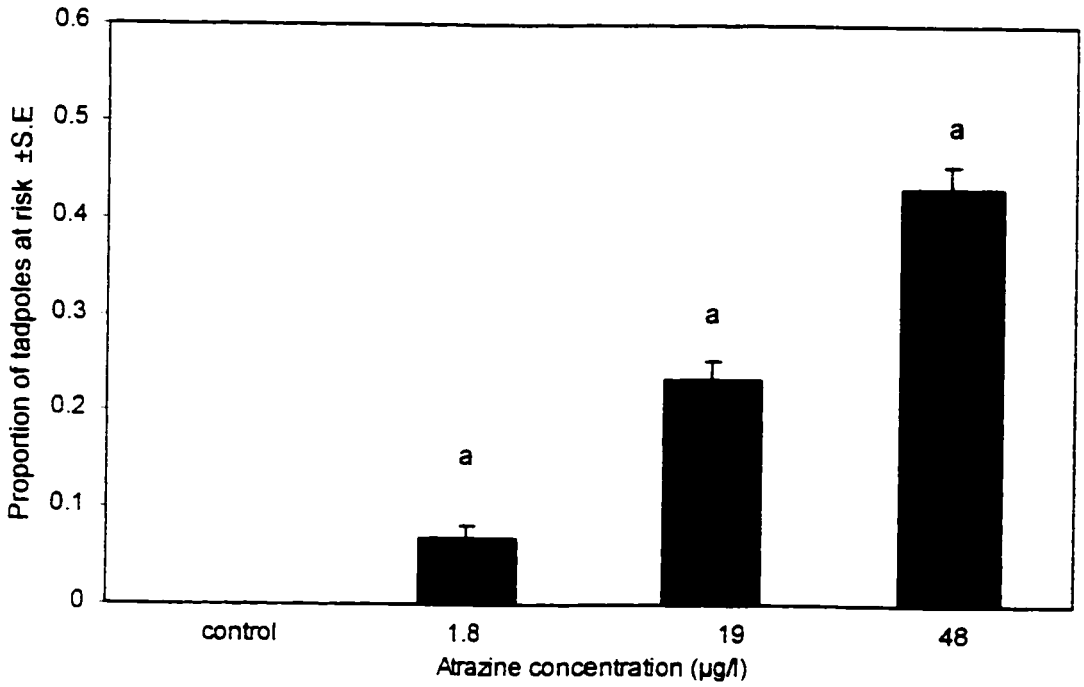


Figure 13.- Experiment II. The proportion of risk of developing Wilm's -like tumour significantly increased in *X. laevis* tadpoles with increasing concentration of atrazine dose ($p < 0.001$) and length of exposure to atrazine ($p < 0.001$). There were no control tadpoles at risk. Letter a indicates significance ($p < 0.05$) from controls.

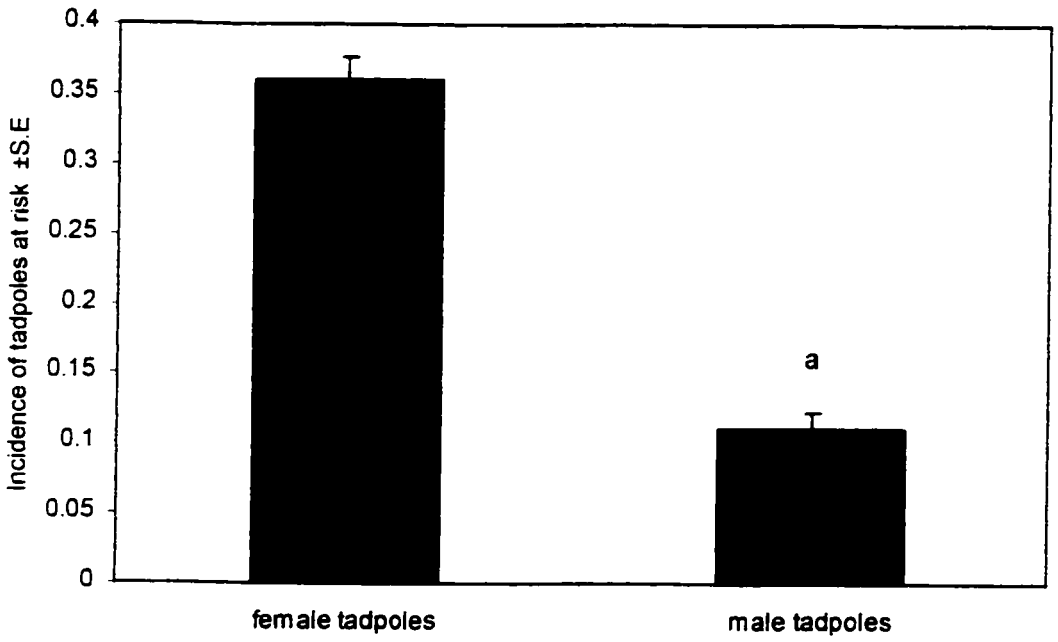


Figure 14.- Experiment II. Incidence of tadpoles at risk from Wilm's-like tumour was significantly higher in females compared to males ($p < 0.001$). There were no control tadpoles at risk.

Discussion

Effects of atrazine on amphibian larvae have been previously studied in laboratory bioassays, microcosm and field mesocosm settings. These studies concluded that atrazine causes effects on amphibians only at concentrations between 200- 2000 µg/l (Allran, 2000; Stephen, 2000; Larson 1998; Detenbeck, 1996). Such concentrations only occur in small streams and emergent marshes vulnerable to agricultural runoff (Detenbeck, 1996; Solomon, 1996). Effects of atrazine in aquatic environments are generally accepted to have direct effects only to its most sensitive components: algae, phytoplankton and macrophytes (Detenbeck, 1996; Solomon 1996). Information on the acute aquatic toxicity of atrazine is available for 26 different species of fresh water fish. They all showed effects from atrazine exposure within a concentration range between 10,000 - 100,000 µg/l (Solomon, 1996). Therefore, effects from atrazine exposures at environmentally effective concentrations on trophic levels higher than primary producers are generally thought to be indirect, secondary to decreases in algae, phytoplankton and macrophyte biomass. When primary producers are reduced by atrazine, this lowers the available food supply and modifies the physical habitat, including dissolved oxygen (Allran, 2001; Stephen 2000).

Aquatic primary producers are usually affected at a mean concentration of approximately 100 µg/l (Stephen, 2000). Rapid recovery is expected as long as atrazine concentrations remain between 50- 100 µg/l (Detenbeck, 1996). Since exposures to 20 µg/l atrazine have not been previously reported to cause any effect in aquatic organisms, the current non-observable effect level (NOEL) of atrazine on aquatic ecosystems has been set at 20 µg/l (Solomon, 1996; Hubert, 1993). Since concentrations of atrazine

rarely exceed 20 µg/l in rivers and streams (Allran, 2000; Solomon, 1996) it has been concluded that atrazine does not pose a significant risk to the aquatic environment (Solomon, 1996). To the best of our knowledge, this is the first time atrazine is shown to have a direct effect on amphibians at environmentally meaningful concentrations. Similarly this is the first report where the reproductive biomarkers of developing germ cells have been used as an end point to assess toxicity of atrazine. Commonly used endpoints for sublethal atrazine effects in aquatic vertebrates have included body weight, body length, and rate of development (Allran, 2000; Stephen, 2000; Larson, 1998; Detenbeck, 1996).

In experiment II, both male and female *Xenopus* tadpoles were exposed to increasing concentrations of atrazine:1.8, 19 and 48 µg/l during increasing exposure times: 48, 96 and 144h.

Tadpole length.

Exposure of *Xenopus* tadpoles to 1.8, 19 and 48 µg/l atrazine for 48, 96 and 144 h. during gonadal differentiation did not have a significant effect on tadpole length. Previous studies also suggest that low concentrations of atrazine have no significant effect upon amphibian larvae length. Northern leopard frog larvae *(Rana pipiens)* and larval tiger salamander *(Ambystoma tigrinum)* exposed to atrazine under laboratory conditions to 20 - 200 µg/l for 138 days and 75 - 250 µg/l for 34 days respectively did not show a significant decrease in length compared to control larvae (Allran and Karasov, 2000; Larson et al., 1998). Similarly, exposure of Northern leopard frog larvae (*Rana pipiens)* to 15 and 25 µg/l atrazine for 14 days in a wetland mesocosm setting showed no

significant effect on tadpole length (Detenbeck et al., 1996). In addition, experiments done on larval gray tree frogs *(Hyla versicolor)* in a microcosm setting exposed to 20 µg/l atrazine for 51 days showed no significant effects in length. However, a significant decrease in tadpole length was reported following exposures to much higher concentrations between 200 and 2000 µg/l (Stephen et al., 2000).

Tadpole development.

Similarly, our results show that exposure of *Xenopus* tadpoles to 1.8, 19 and 48 µg/l atrazine for 48, 96 and 144 h during gonadal differentiation did not have a significant effect on tadpole development. This result is also supported by the literature. Northern leopard frog tadpoles (*Rana pipiens)* exposed to 20 - 200 µg/l atrazine for 138 days in a laboratory setting had no significant effect on development (Allran and Karasov, 2000). Also Northern leopard frog tadpoles (*Rana pipiens*) exposed to 15 and 25 µg/l for 14 days in a wetland mesocosm setting showed no significant effects on development (Detenbeck et al., 1996).

Tadpole body weight

Our results show that exposure of *Xenopus* tadpoles to 1.8, 19 and 48 µg/l atrazine for 48, 96 and 144 h during gonadal differentiation did not have a significant effect on tadpole body weight. These findings are in accordance with the literature. Northern leopard frog larvae *(Rana pipiens)* exposed to atrazine under laboratory conditions from 20- 200 µg/l atrazine for 138 days did not show a significant effect on tadpole weight relative to control tadpoles (Allran and Karasov, 2000). Similarly in another laboratory experiment using larval tiger salamander *(Ambystoma tigrinum)* exposed to 75 µg/l

atrazine for 34 days, there was no significant effect on body weight relative to the control. Significant effect on body weight was obtained only after higher exposure concentration of tadpoles to 250 µg/l atrazine for 34 days (Larson et al., 1998). Similar trends were reported in larval gray tree frogs *(Hyla versicolor)* in a wetland mesocosm setting. Exposure to 20- 200 µg/l atrazine for 51 days showed no significant difference in tadpole body weight relative to controls. Only exposures between 200-2000 µg/l atrazine resulted in a significant effect on body weight (Stephen et al., 2000).

However, exposure of *Xenopus* tadpoles to atrazine for up to 144 h during gonadal differentiation resulted in a significant decrease in testicular volume, primary spermatogonial cell nests and nursing cells in male tadpoles. Similarly, female tadpoles showed significant decline in oogonia cells after 144 h. Our findings suggest, for the first time in aquatic vertebrates, that developing male and female germ cells are highly sensitive to atrazine at environmental concentrations. Developing germ cells cannot be replaced if they are removed or damaged since they represent the permanent source of sperms and eggs in male and female tadpoles respectively (Saunders, 1978; Wylie, 1985). The effect of atrazine are therefore not transitory and recovery is unlikely to occur. Developing germ cells have been shown to be more vulnerable to pollution than somatic cells (Shimada and Egami, 1984). They therefore provide a more sensitive indicator for chronic toxicity. Fish (*Cyprinus carpio)* exposed to 0.14 mg/l alkylphenol during sexual differentiation for 72 h show significantly reduced numbers of primordial germ cells (Gimeno et al., 1997). Pregnant mice treated with 15.6 to 250 mg/kg sobuzoxane and 30 to 50 mg/kg cyclophosphamide resulted in reduced numbers of primordial germ cells in

the fetus (Kato et al., 1996; Johannisson and Hartmut, 1997). Similarly, cynomolgus monkey exposed at much lower dosages of hexachlorobenzene (50mg/kg) that do not produce systemic effects, showed declines in primordial germ cells following exposures (Jarrell et al., 1993). However, the mode of action of these chemicals (anti-neoplasic and estrogenic) is not analogous to atrazine, which will be discussed in further detail in following sections.

Male tadpoles.

Exposure of male *Xenopus* tadpoles to 1.8, 19, and 48 µg/l atrazine during gonadal differentiation resulted in significant declines in both primary spermatogonial cell nests and nursing cells at all concentrations of atrazine.

Primary spermatogonial cell nests in the testis significantly declined to 42.4%, 42.8% and 11.9% following 1.8, 19 and 48 µg/l atrazine treatments respectively after 144h exposure.

While tadpoles were younger in this experiment (stage 54) relative to experiment I (stage 56), the findings validated the results recorded in experiment I. Primary spermatogonial cell nests declined following a 21µg/l atrazine treatment for 48h from a mean of 242.4 cell nests per testis to 72.9 cell nests representing a 70% loss. In this experiment, primary spermatogonial cell nests declined following exposure to 19 µg/l atrazine for 48h from an average of 137.1 cell nests per testis to 53 cell nests representing a 67% reduction. In the first experiment these results were statistically significant. In the second experiment results were not significant. This can be explained by the smaller sample size in experiment II (n=10) relative to the sample size in experiment I (n=24).

Nurse cells, which provide nourishment and endocrine support for the primary spermatogonial cell nests survival, significantly declined to 69%, 22.2% and 2.5% following 1.8, 19 and 48 µg/l atrazine treatment respectively after 144 h exposure. In experiment I, nurse cells declined following exposure to 21 µg/l atrazine for 48 h from a score of 9.62 to 2.35 representing a 74% loss. In experiment II, nurse cells were reduced following exposure to 21 µg/l atrazine for 48 h from 10 to 4 representing a decline of 60%. These differences can be explained by the smaller sample size and by the age difference in the tadpoles used in the two experiments. If we examine the decline at 21µg/l atrazine exposure at 96 h, nurse cells were reduced to 66%. The decline at 21 µg/l atrazine for 144 h was 78%. Tadpoles entered stage 56 sometime between 96 and 144h from the beginning of the experiment. These results could suggest that nursing cells are most sensitive to atrazine at stage 56 . To the best of our knowledge, there is no information in the literature concerning different sensitivity on nurse cells at this particular time, however, the highly significant interaction between time and exposure concentration in nurse cells supports this hypothesis.

Female tadpoles.

Exposure of female *Xenopus* tadpoles to 1.8, 19 and 48 µg/l atrazine during gonadal differentiation resulted in significant declines among primary oogonial cells at all concentrations of atrazine after 144h. Since this experiment, as previously outlined, was performed on younger tadpoles there were only primary oogonia and no secondary oogonia were present. However, the general trends are comparable to those in experiment I. It appears that during the first 48 h of exposure, atrazine caused

stimulation of oogenesis. However this trend changed after 96 h of exposure. Following 144 h of exposure to all concentrations of atrazine resulted in significant declines in oogonia cells. This could be explained if atrazine blocked testosterone production and increased cytochrome P450 aromatase levels. This possibility is discussed in further detail in the following section.

Our findings indicate for the first time that developing germ cells in male and female *Xenopus* tadpoles and somatic nursing cells in male *Xenopus* tadpoles are significantly sensitive to very low concentrations of atrazine. Our results also show that these cells become increasingly damaged as exposure time to atrazine progresses. Trends in oogonia, primary spermatogonia cell nest, and nursing cells from experiment II show that acclimation and recovery in *Xenopus* tadpoles from atrazine exposure during gonadal differentiation is unlikely to occur, and its effects on male and female fertility are permanent.

Suggested mode of action of atrazine in gonadal differentiation

The specific mechanisms by which atrazine alters gonadal differentiation in *Xenopus* are unknown. Mechanisms of sexual differentiation in vertebrates are generally poorly understood (Nagai, 1992), but it is clear that in teleosts fish, reptiles and amphibians that sex steroids play a pivotal role (Pieau et al., 1999; Baroiller et al., 1999; Tata, 1996).

It is unlikely that atrazine produced its effects in these experiments through disruption of the estrogen activity and its receptor at the cellular level. It has been shown for rats both in *vivo* and in *vitro* (Connor et al., 1996) that atrazine does not interfere with

estrogen activity, or its receptor. In *vivo* experiments with rats show however, that exposure to atrazine interferes with normal patterns of ovarian cyclicity in adult females (Cooper et al., 2000).

There is increasing evidence, however, from studies of both mammalian and lower vertebrates in both *vivo* and *vitro*, that atrazine may disrupt sexual differentiation by altering normal testosterone and estrogen metabolism by the enzyme cytochrome P450 aromatase. Cytochrome P450 aromatase is the product of the *cyp 19* gene. Cytochrome P450 aromatase is mostly active in females, and it converts testosterone to estradiol-17β during steroidogenesis (Simpson et al., 1997). Elevated levels of cytochrome P450 aromatase were recorded in *vitro* when gonadal-adrenal-mesonephros (GAM) complexes were excised from neonatal alligators (*Alligator mississippiensis*), following treatment of eggs with a topical solution containing 14 mg/l atrazine (Crain et al., 1997). In *vitro* studies using a human cell line (H295R) have shown an increase in cytochrome P450 aromatase activity correlated to increased *cyp19* induction following exposure to1, 10, and 30 μM atrazine in a dose- dependent fashion (Sanderson et al., 2000). Atrazine can disrupt testicular differentiation in male *Xenopus* by directly blocking testosterone and dihydrotestosterone both through production and at the receptor level. In rats atrazine has been shown both in *vitro* and in *vivo* studies to have anti-testosterone and anti-dihydrotestosterone effects at the receptor level (Danzo, 1997; Babic-Gojmerac et al., 1989). Also in rat studies, atrazine has been shown to inhibit 5α-reductase, the enzyme that converts testosterone to dihydrotestosterone (Babic-Gojmerac et al., 1989). Male rats

exposed to atrazine showed significantly reduced serum and intratesticular testosterone (Trentacoste et al., 2001).

The potential for extrapolation of the results from our experiments with *Xenopus* to other vertebrates is strong. Development and its regulation by hormones is the single most conserved feature through the evolution of vertebrates (Tata, 1996). The mRNA nucleotide sequence for testosterone and estrogen receptors both at the DNA binding domain and ligand binding domains, as well as 5α-reductase and cytochrome P450 aromatase enzymes are well conserved. They show high alignment, >200 alignment score, when compared to amino acid sequences among vertebrates sequenced up to date (http://www.ncbi.nlm.nih.gov/BLAST/). It can be expected that receptors and enzymes with high similarities in composition, structure and function will respond in a similar fashion to atrazine with respect to their mechanism of action. It is also possible that atrazine could have an indirect impact in gonadal development in *X. laevis*. In adult rats, atrazine has been reported to interfere with the hypothalamic-pituitary-gonadal system, and thyroid function. Both Long-Evans hooded and Sprague-Dawley female rats show suppressed LH and prolactin after 3 days of 50-300 mg/kg atrazine treatment (Cooper et al., 2000). To the best of our knowledge, parallel studies in males have not been conducted yet. Also, the level of gonadotropin releasing hormone was suppressed in rats following 100 or 200 mg/kg atrazine treatment (Cooper et al., 1996). In addition, Long-Evans adult females, hypothalamic norepinephrine concentrations were decreased, while dopamine level was increased following exposure to 200mg/kg atrazine within 3 h of treatment (Cooper et al., 1998). Furthermore, exposure of male Wistar rats to 200mg/kg

atrazine resulted in a significant increase of triiodothyronine (T3). No differences were observed in thyroid stimulating hormone (TSH), thyroxine (T4) or in thyroid gland histology (Stoker et al., 2000). Although a specific mechanism(s) of action remains to be identified, it is clear that atrazine can affect reproductive tissues.

Wilm's tumour.-

Our results show that exposure of male and female *Xenopus* tadpoles to 1.8, 19, and 48 µg/l atrazine for up to 144 h during gonadal differentiation significantly increases the risk and incidence of Wilm's-like tumour in the kidney. This study also shows that female tadpoles have a significantly higher incidence of tumour relative to male tadpoles. Histological examination also show that females are at significantly higher risk of developing this tumour than males. Females being at higher risk than males may suggest that females do not develop tumours earlier than males, but that they are generally more sensitive to the tumorogenic effects of atrazine relative to males.

Experimental studies (Sathiakumar and Delzell, 1997) have shown an increased occurrence of tumours in rats and mice particularly among females exposed to atrazine. What these studies however, have not provided is clear evidence that atrazine is a direct acting carcinogen. Life time, high-dose feeding of 400 ppm atrazine in Sprague-Dawley (SD) rats was associated with an earlier development of mammary tumours in female SD rats. However, atrazine did not increase significantly the overall life time occurrence of mammary tumours (Wetzel et al., 1994). Exposure to atrazine has also been shown to increase the incidence of uterine adenocarcinoma, lymphatic and hematopoetic cancers

in female fisher 344/IATI rats (Sathiakumar and Delzell, 1997). Atrazine treatment has also resulted in a higher incidence of lymphomas in both female and male Swiss mice (Sathiakumaand Delzell, 1997). However, the increased occurrence of tumours in these experiments was not significant (Sathiakumar and Delzell, 1997).

There are also epidemiological studies pertaining to the human carcinogenic potential of atrazine. A correlation analysis of pesticide use and cancer incidence rates in California found a significant correlation between atrazine use and higher incidence of brain, testicular and prostate cancer as well as leukemia (Mills, 1998). Another epidemiological case control study done in Kansas and Nebraska showed a positive association between exposure to atrazine and increased incidence of Hodkings lymphoma, non-Hodkings lymphoma and colon cancer. This particular study showed a three fold increase in the risk of non-Hodkings lymphoma among long term, frequent atrazine users, such as agricultural workers (Sathiakumar and Delzell, 1997). Epidemiological data however, does not provide conclusive evidence of an association between atrazine exposure and cancer because correlation does not imply causation (FIFRA, 2000; Mills, 1998).

Since only a few experimental and epidemiological studies have been done, and because each of these had methodological limitations in study design, such as lack of acceptable controls, inadequate randomization, non-significant results, and the choice of a rat strain (SD) that has a normal high incidence of tumours, a causal association between atrazine and neoplasia has not been inferred (Sathiakumar, 1997; FIFRA, 2000).

To the best of our knowledge, this is the first study which provides evidence of atrazine being directly tumorogenic to developing animals. Similarly, this is the first report of increased incidence in renal tumours for aquatic vertebrates following atrazine exposure. However, this is not the first report where the implication of renal distress following atrazine exposure has been made in aquatic animals. Previous studies have reported edema in Northern leopard frog (*Rana pipiens),* wood frog *(Rana sylvatica),* and the American toad (*Bufo americanus)* larvae following 20,000µg/l atrazine treatment for 13 days (Allran and Karasov, 2001). Similarly, abdominal edema has been reported in Northern leopard frog (*Rana pipiens)* and the American toad (*Bufo americanus)* larvae following exposures to sublethal concentrations of atrazine for 96 h (Howe et al., 1998). Edema has also been reported in zebra fish (*Brachydanio rerio)* and in salmon (*Salmo gairdneri*) following exposure of 1300 µg/l atrazine for 35 days and 5 - 40 µg/l atrazine for 28 days respectively (Görge and Nagel; 1990). Furthermore, alteration of renal corpuscles and renal tubules damage was also reported in salmon after the atrazine exposure (Solomon et al., 1996). Abdominal edema is a condition that could indicate renal damage (Robins and Kumar, 1987). It has already been suggested that atrazine may cause renal damage leading to edema (Allran and Karasov; 2001).

A chemical that produces tumours in animals is considered to be a tumorigen. One that has a direct molecular activity which produces DNA damage is considered to be clastogenic or mutagenic. A chemical that is tumorogenic and also mutagenic is generally presumed to be a direct tumorogenic (Mantel, 1980). Flow cytometric analysis has shown atrazine clastogenic effects (Taets et al., 1998). Chinese hamster

ovary (CHO) cells where exposed *in vitro* to 3 and 18µg/l atrazine treatment for 48 h. At both levels tested, atrazine caused chromosomal damage to the CHO cells (Taets et al., 1998). Since atrazine has already been shown to be clastogenic or mutagenic, and because significant tumorogenic effects were observed at such low concentrations of atrazine (1.8µg/l) in our results relative to the lowest observable effect level (LOEL) of atrazine in *Xenopus* (11,000 µg/l) (Allran and Karasov, 2001); we suggest that the tumorogenic effects from atrazine are direct in *Xenopus*.

It is unlikely that the effects of atrazine in *Xenopus* tadpoles are restricted to kidneys and gonads. Preliminary observations have shown that atrazine also has the capacity to disrupt other organs, such as liver, pancreas, heart, blood vessel and lymph node formation in *Xenopus*. These observations are presently too preliminary to make any statement, but the implications of them make more complex the possible mechanism of action of atrazine. At first glance, it seems that atrazine may be causing a toxic systemic effect by disrupting capillaries. This general effect also seems to point specifically to WT-1. WT-1 mutant mice embryos showed massive edema, heart abnormalities, such as very thin myocardium. Hemodynamic failure was suggested as the embryonic cause of death (Kreidberg, 1993). Also, WT-1 has been implicated in regulating endothelium formation (Rauscher, 1993). Further work is needed to assess those endpoints.

Conclusions.

This study has made several contributions to the field of aquatic toxicology. It shows for the first time that atrazine exposure to *Xenopus* tadpoles during gonadal differentiation at environmentally meaningful concentrations, significantly damages primary germ cells in both male and female tadpoles. The implication of these reductions is a permanent fertility loss in *Xenopus*. Since development is the most conserved feature among vertebrate evolution, the possibility of atrazine causing similar effects in aquatic vertebrates is high. This study also shows for the first time that primary germ cell number as a reproductive biomarker, is a more effective endpoint biomarker for atrazine toxicity than currently used endpoints: length, body weight and rate of development. It also suggests that the current atrazine non observable effect level for aquatic ecosystems of 21µg/l should be reconsidered. We provide evidence that atrazine is a direct tumorigen, and females are significantly more sensitive than males to developing tumours. This study also strongly suggests for the first time that effects of atrazine in *Xenopus* are direct. Amphibian population declines that seem to exceed normal population fluctuations have been perceived in North America. It is possible that atrazine, the single most heavily used herbicide in North America can play a role in the decline of Amphibian populations.

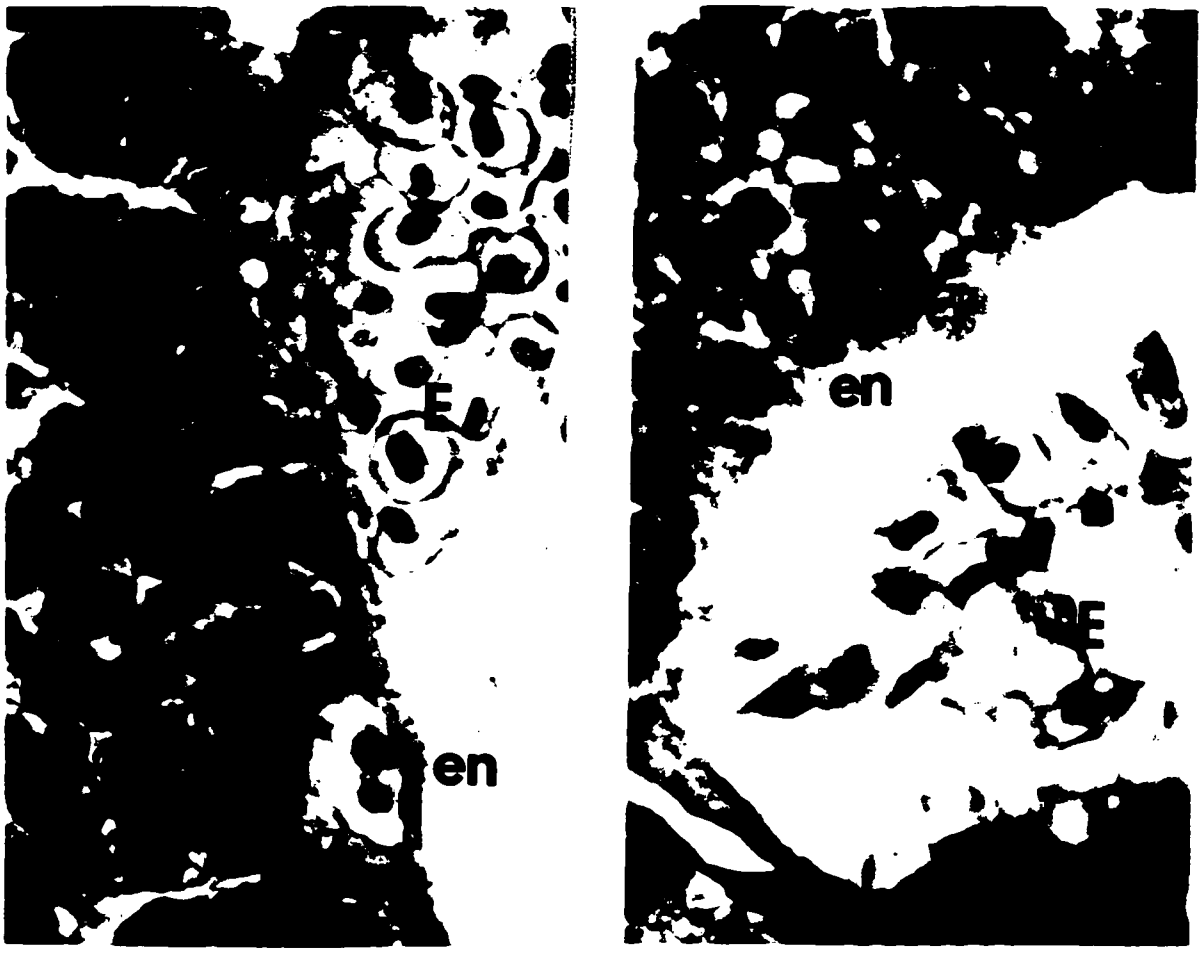
Appendix

Preliminary qualitative observations show that tadpoles exposed to 48µg/l for 144 h show pancreas, liver, and heart damage, as well as defective capillary and lymph node formation.

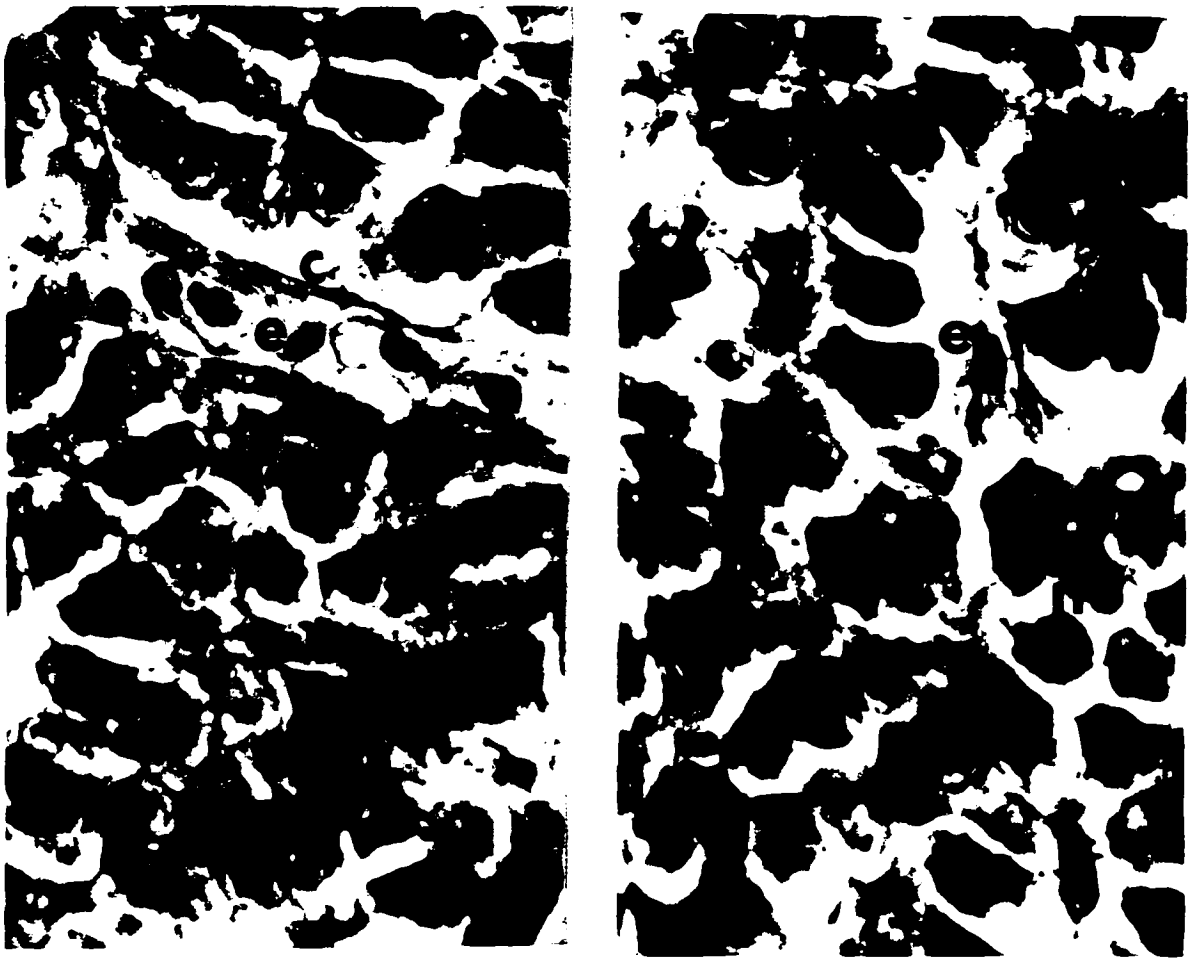
Pancreas.- Developing pancreas from *X. laevis* during gonadal differentiation shows well irrigated and still inactive pancreatic cells. Following exposure to atrazine 48µg/l for 144 h, pancreatic cells appear necrotic. There is severe capillary damaged as well (Photomicrograph 7). There is also an almost complete loss of endothelium in pancreas from atrazine exposed tadpoles (Photomicrograph 8).

Liver.- Developing liver from *X. laevis* during gonadal differentiation show hepatocytes, and defined hepatic sinusoids around hepatic central vein. Liver from atrazine 48µg/l treated tadpoles showed severe haemorrhaging, necrosis and edema. There is also endothelial loss in liver (Photomicrograph 9).

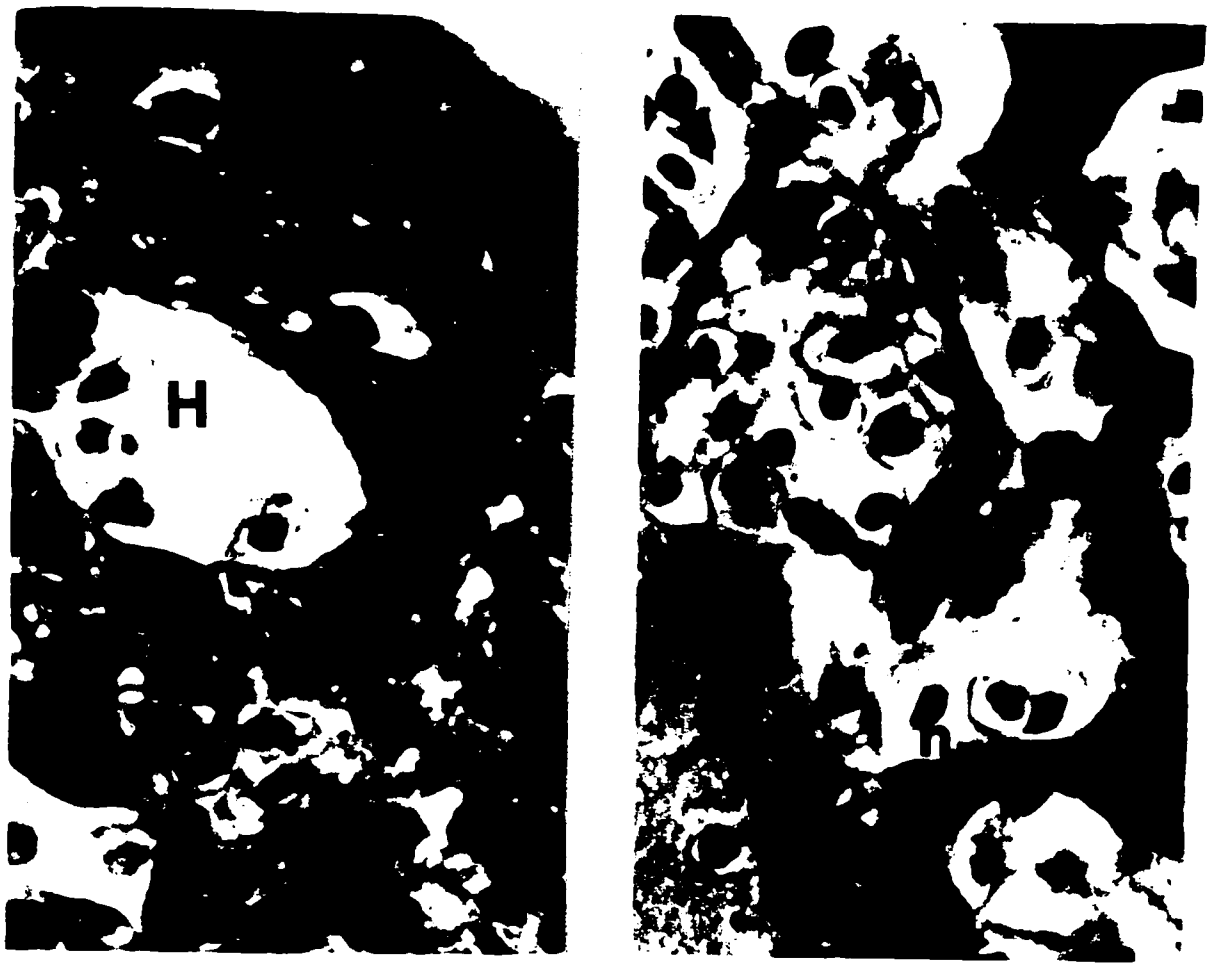
Heart.- Myocardium from 48µg/l atrazine treated tadpoles is considerably thinner relative to control tadpole (Photomicrograph 10).



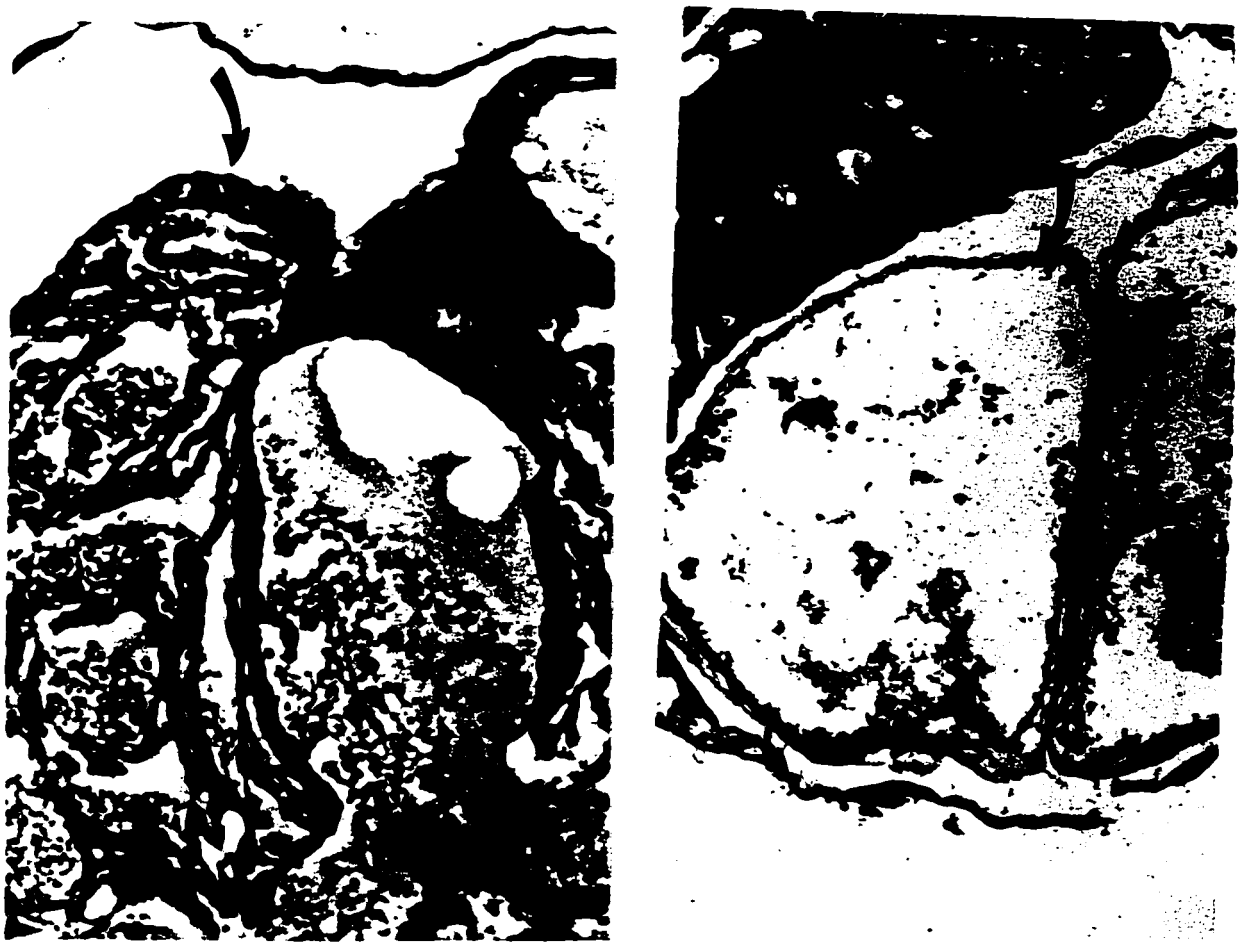
Photomicrograph 7. Experiment II. a) Normal endothelium from control *X. laevis* tadpoles during gonadal differentiation (en). Erythrocytes are also present (E) (1000x). b)Absence of endothelium from a 48µg/l atrazine treated *X. laevis* (en). Damaged erythrocytes are also visible (E) (1000x).



Photomicrograph 8. Experiment II. a) Pancreas from control *X. laevis* during gonadal differentiation, showing a capillary (c), and erythrocytes (e) (1000x). b) Pancreas from 48µg/l atrazine treated *X. laevis* during gonadal differentiation showing necrotic cells (n). Erythrocyte is present (e) in a damaged capillary (1000x).



Photomicrograph 9. Experiment II. a) Liver from control *X. laevis* during gonadal differentiation. Note: hepatic central vein (H), hepatocytes (h) and hepatic sinusoids (s) (1000 x). b)Haemorrhaging, necrotic liver from a 48µg/l atrazine treated *X. laevis*. Necrotic hepatocytes (h), and edema (e) are also present (1000 x).



Photomicrograph 10. Experiment II. a) Longitudinal section of the developing heart at the aorta level from control *X. laevis* during gonadal differentiation. Arrows point to myocardium. b)Longitudinal section of the developing heart at the aorta level from a 48μg/l atrazine- treated *X. laevis* during gonadal differentiation. Arrows point to myocardium (5x).

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