

October 20, 2003

## Lecture 12 Reproductive Toxicity

- I. Overview of Reproductive Physiology in Invertebrates and Vertebrates
  - A. Chemical signals (i.e., hormones) direct reproduction and development; thus these two physiological systems can be extraordinarily sensitive to environmental and contaminant perturbations. (ideas adopted from Palmer, B. D. 2000. Aspects of reptilian anatomy and physiology. pp. 111-139 in "Ecotoxicology of Amphibians and Reptiles". D. W. Sparling, G. Linder, and C. A. Bishop (ed.) SETAC Technical Publications Series, Society of Environmental Toxicology & Chemistry, Pensacola, FL)
    1. In fully formed adults, disruptions from contaminants can lead to **activational effects** that are temporary in nature.
      - a. In other words, a physiological system is disrupted in a dose-dependent manner, and a threshold effect is operational. However, the tissues are already developed, but their normal functioning can be adversely affected.
    2. For developing embryos, chemical signals direct the formation of anatomical systems and establish physiological set points.
      - a. Signal disruption due to toxicants during development can lead to permanent **organizational level effects**.
        1. For example, anatomical defects
        2. Permanent physiological imbalances
  - B. Toxicological effects of contaminants on reproduction of animals can include: (ideas based on Henry, P. F. P. 2000. Aspects of amphibian anatomy and physiology. pp. 71-110 in "Ecotoxicology of Amphibians and Reptiles". D. W. Sparling, G. Linder, and C. A. Bishop (ed.) SETAC Technical Publications Series, Society of Environmental Toxicology & Chemistry, Pensacola, FL (<http://www.setac.org>)
    1. Overt effects on survivorship of breeding adults
    2. Adult's physiological ability to successfully fertilize eggs
    3. Egg viability, hatchability, and/or success in development of embryo
    4. Effects on mating displays and male/female attraction to opposite sex
  - C. Physiology of reproduction includes (based on Henry 2000):
    1. Timing and type of breeding behavior
    2. Fecundity (how many eggs are produced) and fertility (how many eggs successfully hatch or number of live young produced)
    3. Parental care
    4. Embryonic development
    5. Gender determination
    6. Hatchling or offspring growth
    7. Reproductive success (successful mating and fertilization)
  - D. Aspects of Reproduction in Daphnia
    1. Daphnia spp., which are crustaceans, serve as excellent environmental biomonitoring organisms, because of their aquatic habitat (which makes is easy to bioconcentrate contaminants), and their important role in the aquatic food web. They are filter feeders, but they also serve as food for fish predators or predacious insects.
    2. Daphnia spp. have "both sexual and asexual phases. In most environments, the population consists entirely of females that reproduce asexually. Under

optimum conditions, a female may produce more than 100 eggs per brood, repeating every 3 days. A female may have as many as 25 broods in its lifetime, but the average is about 6. The female will start to reproduce at about 4 days old with a brood size of 4 to 22 eggs. Under adverse conditions, males are produced, and sexual reproduction begins. The result is the laying of resting eggs, just like the brine shrimp. Factors that can trigger this are a lack of food, low oxygen supply, a high population density, or low temperatures.”

(Information quoted from the following URL:

<http://www.ee.pdx.edu/~davidr/discus/articles/daphnia.html>

- E. Aspects of Amphibian Reproductive Physiology (based on Henry 2000)
1. In temperate climates breeding is cyclic, gametogenesis is seasonal and adult gametes mature uniformly.
    - a. Breeding activity is controlled by endogenous neuroendocrine cycling coupled with extrinsic, seasonally derived triggers, including temperature, photoperiod, and precipitation.
  2. In tropical climates or under adverse environmental conditions (e.g., drought), gametogenesis is continuous; gametes at different stages of development provide flexibility (or greater potential) for successful breeding at any time to take advantage of favorable conditions.
  3. Depending on the amphibian group, fertilization may be internal or external.
    - a. Anurans (includes frogs and toads): eggs are laid in water and fertilization is external
    - b. Gymnophiona (includes caecilians): reproduce biennially; internal fertilization
    - c. Urodela (includes newts and salamanders): 90% of the species fertilize eggs internally (Henry 2000)
  4. Influences on anuran egg survival:
    - a. Oxygen levels in water
    - b. Ph
    - c. Alkalinity
    - d. Temperature
    - e. Surface-to-volume ratio of deposited egg mass to water
    - f. UV radiation
    - g. Presence of symbiotic green algae
    - h. Energy sources
  5. Amphibians show extremely diverse reproductive strategies
    - a. Oviparity
    - b. Viviparity
    - c. Ovoviviparity
  6. Gonadal development
    - a. In caecilians and salamanders, gonads develop at the larval stage
    - b. In anurans anurans, testes differentiate throughout development depending on the species; ovaries develop post-metamorphosis
  7. Egg development—pre-fertilization
    - a. Lipid-storing fat bodies are associated anatomically with the ovaries and provide nutrition for the developing ova

- b. Ova are transported in Mullerian ducts and released into the oviduct
  - 1. In the oviduct, they accumulate a proteinaceous semipermeable vitelline membrane capsule
  - 2. Ova are stored until deposited or fertilized
  - 3. Once deposited, perivitelline chamber increases in volume as a result of the uptake of surrounding water and accumulation of waste products
  - 4. Deposited eggs are covered with a gelatinous substance
- 8. Annual fecundity can range from 1 to >80,000 viable hatchlings per “clutch”
  - a. Generally, <5% of eggs survive to metamorphosis
- 9. Amphibians exhibit various degrees of parental care
  - a. Rare in caecilians
  - b. Present in some anurans
  - c. Present in the majority of urodeles
    - 1. Attending eggs
    - 2. Transporting eggs or young
    - 3. Feeding young
  - d. Parental care improves rate of hatching and survival
- 10. Growth and initial activity of the young are environmentally determined
  - a. Thus, larvae that transform at a larger size survive better and are more fit
  - b. More susceptible to predation and risk of pond drying
    - 1. Thus, delays (or slow down) in growth can influence survival
- 11. Life span variable among species: <1 year to more than 30 years
- F. Reproductive Physiology: Fish (information from Heath, A. G. 1995. Reproduction. Chapter 13, pp. 299-323 in "Water Pollution and Fish Physiology". Lewis Publishers, Boca Raton, FL.)
  - 1. Reproduction involves the following processes (note that these are more or less common to all organisms)
    - a. Formation of gametes (egg and sperm)
    - b. Laying or release of eggs
      - 1. In some fish species, the eggs are retained internally)
    - c. Fertilization, which is often associated with complex spawning behavior
    - d. Embryonic development in the egg
    - e. Hatching and larval development
  - 2. Reproductive physiology and behavior is cued to particular seasons and are under hormonal control.
    - a. Control of reproduction is modulated by environmental factors.
      - 1. Photoperiod and water temperature are the most important environmental cues for reproductive functions (temperature is the most dominant).
  - 3. Reproductive strategies: (Note that reproductive strategy will influence the extent to which the eggs and larvae come in contact with certain pollutants)
    - a. Most teleost fish species are seasonal breeders
      - 1. In the Northern hemisphere, temperate zone warm-water species are often late spring and early summer spawners.
      - 2. Salmonids and other cold-water species are often late summer and autumn spawners.
    - b. Parental behavior (three basic groups)

1. Those that do not guard their eggs after laying or shedding into water column;
2. Those that do guard eggs;
3. Those that are bearers who carry them in brood chambers or that bear live young.
4. Hormonal controls
  - a. Formation of eggs and sperm (gametogenesis) is cued by environmental factors, which stimulate the hypothalamus in the brain.
  - b. Hypothalamus releases gonadotropin-releasing hormones (GnRH), stimulating the pituitary to release gonadotropins I & II (GtH I & II).
  - c. GtH stimulates gametogenesis in the sex organs.
    1. GtH I is the dominant gonadotropin. It is similar to follicle stimulating hormone of mammals is dominant in fish undergoing active growth and gametogenesis.
    2. GtH II is similar to luteinizing hormone in mammals and predominates during final maturation of the gonads and at spawning.
  - d. Ova develop in an ovarian follicle that is composed of the central ovum surrounded by follicular and thecal layers of cells that help with yolk formation. These cells also secrete the ovarian sex steroid hormones.
    1. Yolk formation is called vitellogenesis.
      - a. Vitellogenin, which is a lipophosphoprotein, is synthesized in the liver and transported by the blood to the ovary.
      - b. It is taken up into the oocytes.
      - c. Estrogenic hormones from the ovary stimulate vitellogenin production and its uptake into the ovum.
        1. Hormones are produced upon stimulation of tissue by GtH I.
    2. The sex steroid hormones affect gametogenesis via both positive and negative feedback on the hypothalamus and pituitary.
      - a. In seasonally immature fish, the sex steroids generally promote GnRH and GtH release through positive feedback.
      - b. Negative feedback predominates in sexually mature fish.
  - e. Germ cells of the testes develop as spermatogonia; they are either in synchronous or variable stages of development.
    1. Spermatogonia are surrounded by Sertoli and Leydig cells that provide support and regulation of spermatogenesis.
      - a. Leydig cells produce primarily the androgens testosterone, ketotestosterone, and androstenodione.
      - b. Note that estrogen (estradiol) is also produced, but at lower levels than found in females; also testosterone is present in female, but at lower levels than in male.
  - f. Secondary sexual characteristics (i.e., morphology at reproductive maturity) in under hormonal control, but may be under temperature control (i.e., sex reversal at certain developmental temperatures)
    1. Steroid hormones influence sexual behavior (such as spawning behavior).
    2. Sex pheromones are secreted externally and affect opposite sex.

- a. Sex pheromones are detected by olfactory glands that may be in the nose or line the body.
  - g. Pelagic swimmers (open water habitats) tend to lay a lot more eggs than fish confined to protected habitats.
  - h. Eggs are enclosed by a tough membrane called the chorion.
    - 1. Sperm enters through a small opening called the micropyle, that closes up after fertilization.
    - 2. Chorion then swells by water imbibition, but remains permeable to dissolved substances.
      - a. Imbibed water forms the perivitelline fluid that is retained between the chorion and vitelline membrane surrounding the egg
  - i. The time between fertilization and hatching is usually shorter in pelagic fish than in those that provide protection for the eggs. For example, anchovy and striped bass hatch out in 2-4 days, but salmon may require up to 5 months of embryonic development while buried in a gravelly substrate.
    - 1. After eggs hatch, young quickly disperse. They may be free living or remain attached to the yolk sac during further development (for salmon, this stage is termed alevins or sac-fry).
- G. Unique Aspects of Avian Reproduction (All information from <http://www.biology.eku.edu/RITCHISO/avianreproduction.html>)
- 1. Females (see Figure 1 below). In most birds, only the left ovary and oviduct persist. The ovary enlarges greatly during the breeding season.
    - a. Active ovaries resemble bunches of tiny grapes -- the developing follicles. The oviduct opens medially to it in a funnel-shaped ostium.
    - b. Ovulation results in the release of an egg from a mature follicle on the surface of the ovary.
  - 2. Fertilization takes place in the infundibulum or upper region of the tubular-like oviduct.
    - a. As with mammals, fertilization of the egg takes place in the oviduct, usually by sperm that have been stored in the lower end of the tract and squeezed out by the passage of an egg.
      - 1. They then swim up the tract and meet the oncoming next egg.
      - 2. Contractions of the muscular oviduct and body movements help shove the egg along.
  - 3. The egg, with extensive food reserves in the form of concentric layers of yolk, is picked up by the ostium and ciliary currents carry it into the **magnum** region.
    - a. Over about three hours the egg receives a coating of albumen. the egg then passes into the **isthmus** , where the shell membranes are deposited. This takes about one hour. The egg then moves to the **uterine region, or shell gland**, where the calcareous shell is added and, in some birds, pigment is added in characteristic patterns. The egg then passes into the vagina and cloaca for laying.
  - 4. Male birds (see Figure 2 below) have paired abdominal testes lying cranioventral to the first kidney lobe. Testes increase dramatically in size

during the breeding season. The vas deferens emerges medially and passes caudally to the cloaca where it has a common opening with the ureter in the Urodeum. The terminal vas deferens is swollen as a storage organ: the seminal vesicle. (Information from

<http://www.biology.eku.edu/RITCHISO/avianreproduction.html>)

- a. As in mammals, sperm formation is temperature sensitive, and maturation is assisted by nocturnal drops in temperature, or by the development of scrotal-like external thermoregulatory swellings holding the seminal vesicles.
- b. In addition, male birds tend to have relatively low extragonadal sperm reserves and sperm are ejaculated soon after production in the testes.

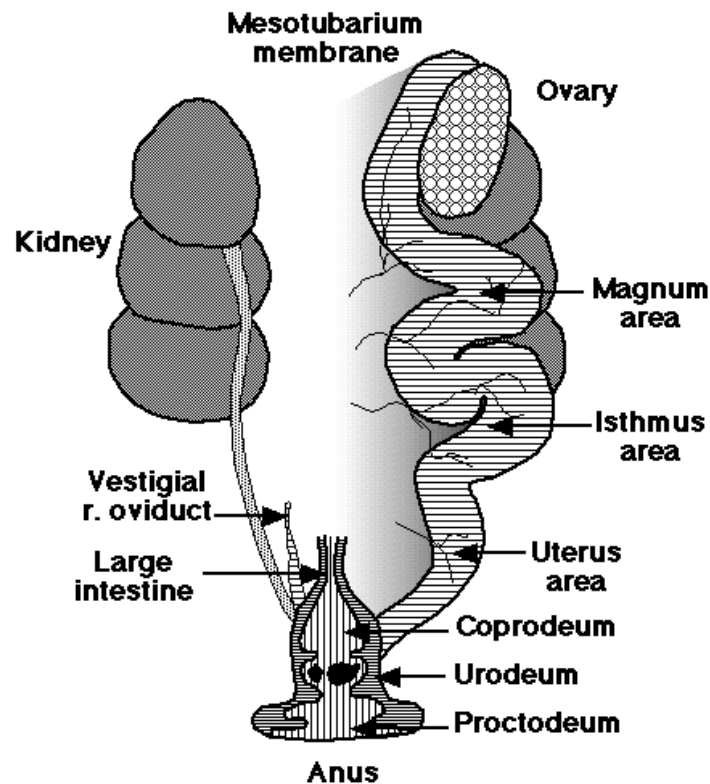


Figure 1. Avian Female reproductive tract Source:  
<http://wwwvet.murdoch.edu.au/Anatomy/avian/fig5.2.GIF>

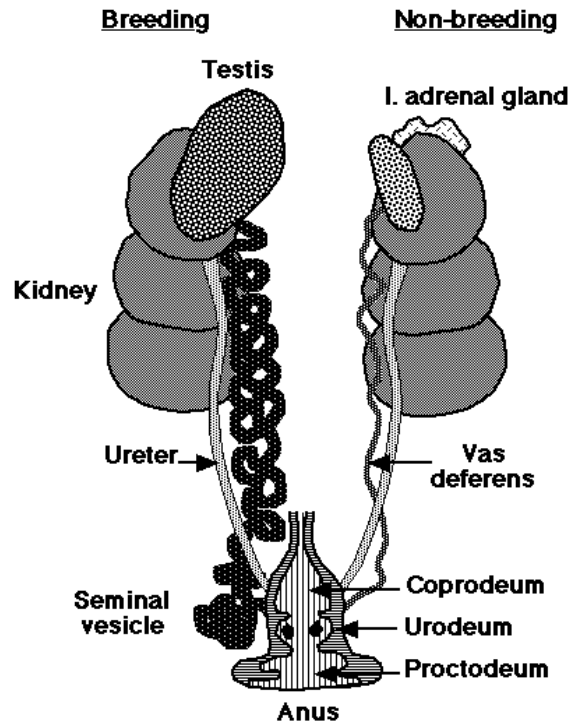
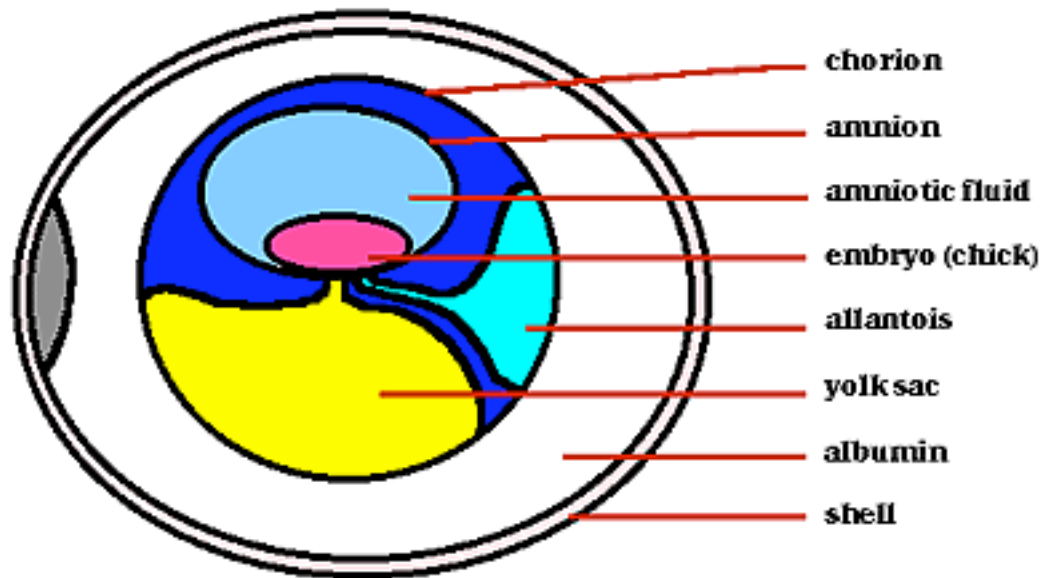


Figure 2. Avian male reproductive tract. Source: <http://wwwvet.murdoch.edu.au/Anatomy/avian/fig5.1.GIF>

5. For most birds, copulation involves a 'cloacal kiss', with the male on the female's back & twisting his tail under the female's
6. Egg morphology (information from <http://www.biology.eku.edu/RITCHISO/avianreproduction.html>)



- a. Amnion
  1. Surrounds only the embryo

2. Inner layer of cells secretes amniotic fluid in which the embryo floats; fluid keeps the embryo from drying out and protects it
- b. Chorion - surrounds all embryonic structures & serves as a protective membrane
- c. Allantois (or allantoic sac)
  1. Grows larger as embryo grows, fuses with the chorion & is called the chorio-allantoic membrane
  2. Works together with chorion to permit respiration (exchange of oxygen and carbon dioxide) and excretion
  3. Important in storage of nitrogenous wastes (uric acid)
- d. The egg consists of three components
  1. Yolk (provides nutrients)
    - a. 21-36% lipid; 16-22% protein; remainder is water
  2. Albumen (provides water to prevent dehydration but also acts as shock absorber; also insulates eggs from temperature extremes)
    - a. 90% water; 10% protein
  3. Shell (see Figure 3)
    - a. Protects embryo
    - b. Contains thousands of pores permitting gas exchange

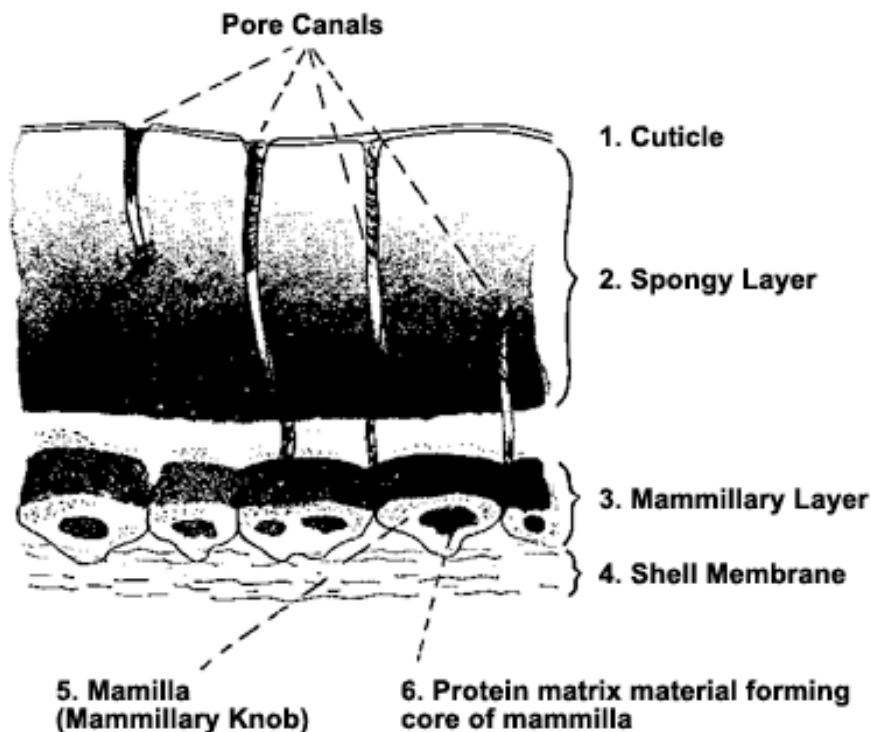


Figure 3. Diagram from <http://www.biology.eku.edu/RITCHISO/avianreproduction.html>

## II. Reproductive toxicity of contaminants

### A. Laboratory Based Studies

1. The procedures for conducting mammalian reproductive toxicity studies chronic invertebrate studies (using *Daphnia* spp. and Mysid shrimp) , and



avian reproductive toxicity studies are proscribed for ecological risk assessments of pesticides by the EPA Office of Prevention, Pesticides, and Toxic Substances.)

- B. Note that many laboratory based studies do show effects on reproductive success, or at least on hormonal levels, tissue morphology, and fecundity/fertility if not survivability of embryos and young directly.
- C. Field Based Studies
  - 1. Field based studies do not always support laboratory observations of adverse effects of contaminants
    - a. One of the problems is knowing the exact level of exposure and the timing of the exposure during development.

### III. Case Studies (To be elaborated during class)

- A. Atrazine effects on male amphibian development of reproductive tissue
  - 1. Research by T. Hayes
    - a. Hayes, T. B., A. Collins, M. Lee, M. Mendoza, N. Noriega, A. A. Stuart, and A. Vonk. 2002. Hermaphroditic, demasculinized frogs after exposure to the herbicide atrazine at low ecologically relevant doses. *Proc. National Acad. Sci.* 99(8):5476-5480.
      - 1. Hermaphroditism rate of 16-20% at concentration greater than or equal to 0.1 ppb. Does not give specific percentage for each dose, but narrative implies 16% at 0.1 ppb. Does not indicate length of exposure. Water was replenished with fresh atrazine every three days. Voice box (larynx) reduced at 1 ppb or above (the threshold). Hypothesis is that aromatase is induced, thus speeding up transformation of testosterone to estrogen. This same phenomenon (i.e., aromatase induction) was noted in alligators.
    - b. Hayes, T., K. Haston, M. Tsui, A. Hoang, C. Haeffele, and A. Vonk. 2003. Atrazine-induced hermaphroditism at 0.1 ppb in American leopard frogs (*Rana pipiens*): laboratory and field evidence. *Environmental Health Perspectives* 111:568-575.
      - 1. Abstract from the paper: Atrazine is the most commonly used herbicide in the United States and probably the world. Atrazine contamination is widespread and can be present in excess of 1.0 ppb even in precipitation and in areas where it is not used. In the current study, we showed that atrazine exposure (0.1 ppb) resulted in retarded gonadal development (gonadal dysgenesis) and testicular oogenesis (hermaphroditism) in leopard frogs (*Rana pipiens*). Slower developing males even experienced oocyte growth (vitellogenesis). Furthermore, we observed gonadal dysgenesis and hermaphroditism in animals collected from atrazine-contaminated sites across the United States. These coordinated laboratory and field studies revealed the potential biological impact of atrazine contamination in the environment. Combined with reported similar effects in *Xenopus laevis*, the current data raise concern about the effects of atrazine on amphibians in general and the potential role of atrazine and other

- endocrine-disrupting pesticides in amphibian declines. *Key words:* amphibian, atrazine, endocrine disruption, hermaphrodite.
- c. Hayes, T., K. Haston, M. Tsui, A. Hoang, C. Haeffele, and A. Vonk. 2002. Feminization of male frogs in the wild. *Nature* 419(31 October):895-896.
    1. Found testicular abnormalities in field collected populations of frogs, but the correlation with atrazine levels was poor.
    2. Furthermore, Hayes noted that there were plenty of frogs around the collection areas.
      - a. Although not stated by Hayes, his observation suggests that reproduction was not affected.
  2. An alternative perspective that suggests under field conditions (at least at the expected residue levels of atrazine) that atrazine is unlikely to adversely affect reproductive physiology of frogs.
    - a. Carr, J. A., A. Gentles, E. E. Smith, W. L. Goleman, L. J. Urquidi, K. Thuett, R. J. Kendall, J. P. Giesy, T. S. Gross, K. R. Solomon, and G. Van Der Kraak. 2003. Response of larval *Xenopus laevis* to atrazine: assessment of growth, metamorphosis, and gonadal and laryngeal morphology. *Environmental Toxicology and Chemistry*: Vol. 22, No. 2, pp. 396-405.
    - b. Abstract from the paper: Larval *Xenopus laevis* were exposed to one of four concentrations of atrazine (0, 1, 10, or 25 g/L, 11 replicate tanks per treatment, 60\_65 larvae per replicate) dissolved in an artificial pond water (frog embryo teratogenesis assay- *Xenopus* [FETAX]) medium beginning 48 h after hatching until the completion of metamorphosis. Separate groups of larvae (six replicate tanks per treatment, 60\_65 larvae per replicate) were exposed to estradiol (100 g/L), dihydrotestosterone (100 g/L), or ethanol vehicle control dissolved in FETAX medium. None of the treatments affected posthatch mortality, larval growth, or metamorphosis. There were no treatment effects on sex ratios except for estradiol, which produced a greater percentage of female offspring. Exposure to either estradiol or 25 g atrazine/L increased the incidence of intersex animals based on assessment of gonadal morphology. Atrazine did not reduce the size of the laryngeal dilator muscle, a sexually dimorphic muscle in this species. We conclude that environmentally relevant concentrations of atrazine do not influence metamorphosis or sex ratios and do not inhibit sexually dimorphic larynx growth in *X. laevis*. The incidence of atrazine-induced intersex animals was small (<5%) and occurred only at the greatest concentration of atrazine tested, a concentration that is rarely observed in surface waters in the United States.
- B. DDE and the eggshell thinning hypothesis as a cause of declining bird populations
1. The crux of this hypothesis is the correlation between eggshell thickness and the levels of DDE in the eggs.
    - a. The hypothesis centered on failure to hatch owing to easily breakable eggshells that were too thin to survive environmental stresses.

- b. Thus, reproductive potential was lowered significantly enough to effect breeding success and therefore population stability.
2. Note that all eggshell measurements of extant egg collections were compared to average thicknesses that had been measured in museum specimens.
  - a. Many of the specimens in museums had been collected pre-DDT commercialization.
    1. For example: Anderson, D. W. and J. J. Hickey. 1982. Oological data on egg and breeding characteristics of brown pelicans. *Wilson bulletin* 82:14-28.
  - b. One peculiar aspect of the eggshell thinning hypothesis was the quick recovery in some bird populations shortly after DDT was banned in 1973.
    1. For example: Anderson, D. W., J. R. Jr. Jehl, R W. Risebrough, L. A. Jr. Woods, L. R. Deweese, and W. G. Edgecomb. 1975. Brown pelicans: improved reproduction off the southern California coast. *Science* 190:806-808.
      - a. Attributed recovery of population to the lowering of total DDT residues in anchovies (food source) and correlation with lower amounts in eggs.
  - c. A better measure of the effects of eggshell morphological structure on hatching success is probably the parameter known as breaking strength.
    1. Henny, C. J. and J. K. Bennett. 1990. Comparison of breaking strength and shell thickness as evaluators of white-faced ibis eggshell quality. *Environ. Toxicol. Chem.* 9:797-805.
      - a. Breaking strength was better correlated to hatching success in controlled exposure of ibis than was eggshell thickness.