September 28, 2005
Lecture 10 Developmental Toxicity

I. Developmental Toxicity—Historical Aspects

A. In the previous lectures on endocrine system toxicity, we learned that hormonal control of metabolism and development can be disrupted by chemicals acting at the level of hormonal receptors or on metabolism of hormones themselves.
   1. The resulting effects may adversely affect reproduction and certainly development.
   2. However, the effects studied thus far in relationship to hormonally active agents largely involved abnormal development of reproductive morphology (i.e., transsexual or intersexual genitalia; or skewed sex ratios; underdeveloped prostate; nipples on males, etc.).
   3. However, in the various "endocrine disrupter" studies it is not clear if the morphological deficits, whether they be on the genitalia or on brain development are actually manifested as reproductive problems in the adult or as a functional deficit.
      a. In some cases, sex specific behaviors have been found to be altered in rats post puberty.

B. In the next two lectures, we will expand the discussion to specific developmental and reproductive toxicological effects.
   1. Note that the mechanism could be disruption at the level of the endocrine or even nervous system, but there are also other mechanism that will be considered.

C. Teratogenesis is historically the term used to describe developmental toxicity resulting in abnormal morphological features, but it is used today for more than just the appearance of abnormal morphology and the term developmental toxicity covers a broader array of effects
   1. The root of teratogenesis is teraton from the Greek, meaning "wonder" and by derivation "monster" (discussion from Francis, B. M. 1994. Toxic Substances in the Environment. Chapter 9, Developmental Toxicity John Wiley & Sons, Inc., New York; pp. 199-229.)

D. Concerns over teratogenesis evolved from human experiences noted in the 1930's (discussion of historical aspects from Francis 1994)
   1. An Australian ophthalmologist noted that an epidemic of congenital cataracts followed the occurrence of an epidemic of rubella (German measles); thus, rubella may be the first identified human teratogen.
      a. The case of rubella is somewhat typical of teratogenesis.
         1. The toxicant or agent is not serious or toxic in adults (i.e., adult may not be symptomatic or adversely affected).
         2. There is a strong correlation between the incidence of malformations in the offspring and the time of exposure during pregnancy (gestation).
            a. For example, women infected with rubella during the first month of gestation had a 50% chance of having an affected child;
            b. Women infected in the second month had only a 30% risk of an affected child;

c. Women exposed in the third and fourth months of pregnancy had a 10% or 5% chance of giving birth to an affected child.

3. The toxic agent must cross the placenta (or somehow reach the developing embryo), which makes the phenomenon of teratogenesis generally applicable to all organisms, including those in which embryonic development occurs outside the mother [e.g., fish].
   a. In the case of congenital cataracts associated with rubella exposure in humans, the chances of the virus crossing the placenta diminished as pregnancy advanced.

2. The only other teratogenic agent known in humans prior to 1950 was radiation.
   a. However, studies with vitamin A deficient diets fed to female pigs (sows) showed high levels of eyelessness (anophthalmia) in piglets.
   b. Similarly, vitamin A excesses led to teratogenic effects.
3. The most famous example of teratogenic effects in humans post 1950 occurred after administration of thalidomide to pregnant women.
   a. When tested in rodents, thalidomide was considered "innocuous".
   b. The drug was used to relieve nausea, tension, or sleeplessness during pregnancy.
   c. It had been used in German, U.K., Australia, and Japan; very little was used in the U.S. owing to disapproval by the FDA for lack of studies on pregnant animals.
      1. The major studies with thalidomide had shown that long-term use in adults could lead to irreversible neuropathy.
         a. However, pregnant women were going to use the drug for only a short while so it had been approved in non-U.S. markets.
         b. Before it was marketed in the U.S., observations by a German pediatrician (W. Lenz) and an Australian physician identified thalidomide as being teratogenic.
            1. Unfortunately, ~8000 infants were born between 1959 and 1962 with severe thalidomide-induced malformations, including no arms, no legs, deformed ears, paralyzed faces, and internal malformations.
   d. The thalidomide history raised tremendous concerns about teratogenicity and the need for adequate testing.
      1. However, the case of thalidomide also illustrates the problem in teratogenicity testing—differential sensitivity among species (see Table 1).

Table 1. Doses of thalidomide eliciting either a response or no response in groups of tested animals. Note that the doses shown are from different studies (data presented in Francis 1994).

Species	Smallest Dose Inducing Malformations (mg/kg/day)	Largest Dose Not Producing Malformations (mg/kg/day)
Human	≤1.0	?
Monkey	5	--

Rabbit	30	50
Mouse	31	4000
Rat	50	4000
Armadillo	100	--
Dog	100	--
Hamster	350	8000
Cat	--	500

2. The differential response among species suggests an intrinsic genetic susceptibility is operational.
3. However, the wide distribution in response within a species is suggestive of an environmental factor.
    a. For example, the fact that some studies show that mice may not react to a dose of 4000 mg/kg, yet others show a dose as low as 31 mg/kg will cause some malformations, suggest that environmental factors (perhaps timing of exposure during pregnancy; stress factors; etc.) may be operational.

II. Basic Principals

A. Developmental toxicity, a phrase that has generally replaced the term teratogenesis, is defined as "any structural or functional alteration, reversible or irreversible, caused by environmental insult, which interferes with homeostasis, normal growth, differentiation, development and/or behavior." (from Tyl, R. W. 2000. Developmental Toxicology. Chapter 53 (pp. 1167-1201) in "General & Applied Toxicology", 2nd ed., vol. 2, B. Ballantyne, T. Marrs, T. Syversen (Eds.), Grove's Dictionaries Inc., NY.)

B. Targets for effects: (Tyl 2000) (Figure 1)
    1. Fertilized egg or zygote prior to implantation (implantation relevant to mammals) or prior to the establishment of the three primary germ layers (ectoderm, mesoderm, endoderm);
    2. Embryo during the period of major organ formation (i.e., organogenesis);
    3. Fetus in the postembryonic period of histogenesis and the neonate or postnatal offspring, occurring or expressed through the postnatal period until sexual maturity.

C. Expressions of developmental toxicity: (Tyl 2000)
    1. Death
    2. Structural malformations
    3. Functional deficits
    4. Developmental delays

D. Vulnerability of the embryo and fetus: (all information from Tyl 2000)
    1. Due to qualitative or quantitative characteristics of both structure and function:
        a. Embryo is composed of a small number of rapidly dividing undifferentiated cells, with limited or nonexistent metabolic capabilities for detoxification of toxicants or repair of damaged cells;

b. Development has a need for proper spatial and temporal sequencing of specific cell numbers and types, as well as specific cell products, for normal differentiation, including programmed cell death;
c. Sensitivities of certain cell types to certain insults may be unique to specific periods of cell movement, induction or differentiation;
d. The immunosuppressive system that recognizes "self" and detects/repairs toxicants or lesions may be absent or undeveloped (immature) in the prenatal or perinatal period.

E. Karnofsky's Law
1. D. A. Karnofsky proposed as "a law, which cannot be disproved, that any drug administered at the proper dosage to embryos of the proper species—and these include both vertebrates and invertebrates—will be effective in causing disturbances in embryonic development." (quoted in Francis 1994)
a. Thus, for teratogenicity there seems to be a threshold effect;
b. However, the threshold will depend on the specific stage of development and the organ system affected.
c. The dose causing a malformation (i.e., the structural threshold dose) may be below, equal to, or above the dose causing acute toxicity (embryolethality) (the threshold dose for lethality).

F. Susceptibility to teratogenesis varies with the developmental stage (Figure 1).

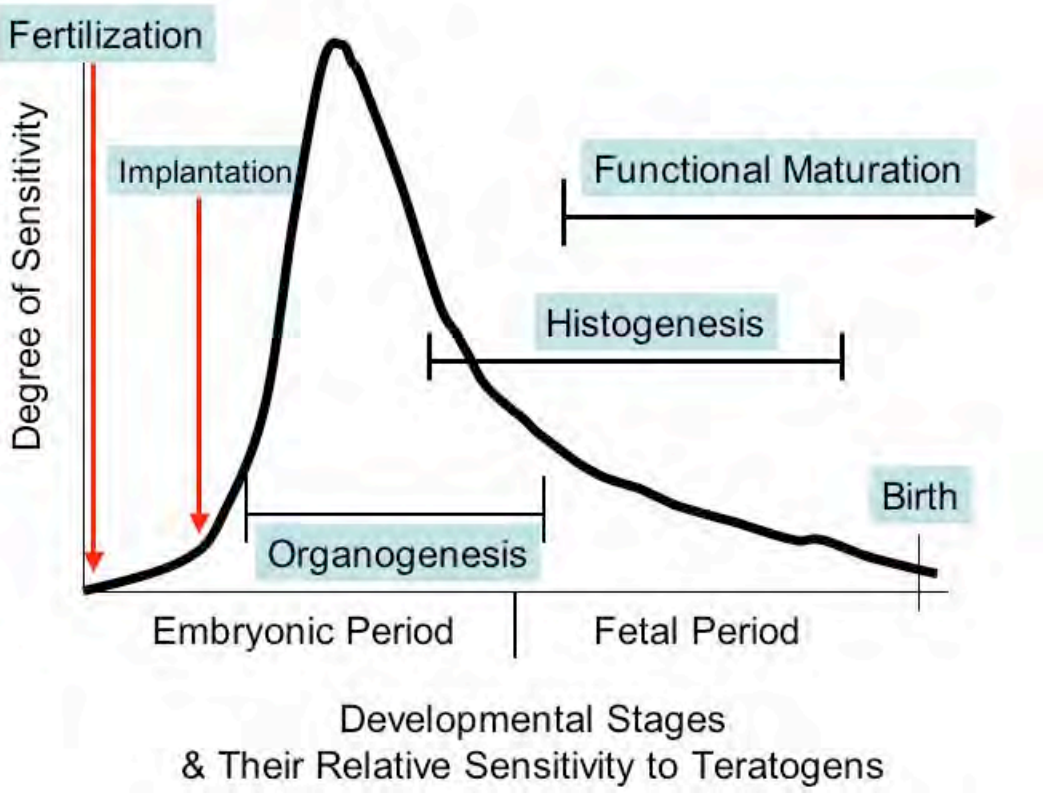
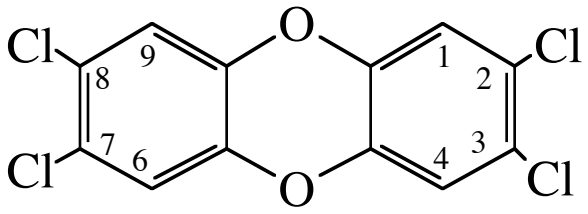


Figure 1. Relationship between development stage of embryo & fetus and sensitivity to teratogenesis (adapted from Tyl 2000).

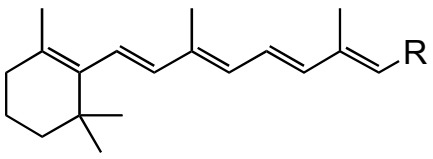
1. During the preimplantation period, the embryo consists of relatively few ells, most of which are still totipotent (i.e., undifferentiated with the capability of turning into any cell type if moved around where they would come under the influence of certain growth factors).
2. Organogenesis and early differentiation are characterized by rapid growth and the differentiation of most organ systems. Period of extremely rapid cell division.
3. The fetal period is marked by growth, maturation, and the functional differentiation of the organs laid down in the preceding stage.
   a. Disruptions during this period can lead to subtle structural malformations and to functional deficits.

G. There is no fully understood mechanism for developmental toxicity resulting in malformations.
   a. Mechanism will be agent specific and involve any conceivable biochemical or cellular process/structure.
   b. Three general mechanisms could explain teratogenic effects (after Francis 1994).
      1. Selective cell death;
      2. Altered biosynthesis leading to structural or functional malformations;
      3. Energy inhibition acting to slow growth.
   c. For some agents, the specific receptors that may be involved in triggering a teratogenic effect are known; for others, a general mechanism is known but not necessarily the specific receptors.
   d. Example of specific receptor involvement
      1. <u>TCDD (2,3,7,8-tetrachloro-*p*-dibenzo dioxin)</u>
         a. TCDD is one of 78 possible congeners for a group of homologous polychlorinated dibenzodioxins known generically as dioxins.
         1. Sometimes TCDD is just called dioxin, but it is the most toxic congener.
         2. Dioxins in general are products of incomplete combustion in the presence of chlorine atoms (synthetic as in PVC, polyvinyl chlorine; or natural as in NaCl, sodium chloride, which is present in all tissues)
            (a) Dioxins were also industrial byproducts from the synthesis of certain chlorinated compounds involving 2,4,5-trichlorphenol (for example, 2,4,5-trichlorophenoxyacetic acid herbicide; hexachlorophene antibacterial agent used as the active ingredient in certain antibacterial soaps).

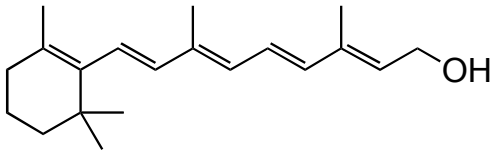


b. The concern over TCDD was generated because of an extremely low LD_{50} in guinea pigs (= 1 μg/kg!!). Fortunately, humans are much less susceptible, and the concerns today are not of acute toxicity but with chronic toxicity, including cancer, endocrine disruption, developmental toxicity, reproductive toxicity, and immunotoxicity.
c. TCDD causes cleft palate in mice, and at higher doses causes hydronephrosis (=swelling of the kidney due to blocked urine flow).
    1. The putative mechanism is the binding of TCDD to certain epidermal growth factor (EGF) receptors, thereby preventing the normal reduction in expression of the EGFs in the medial epithelial cells of the palatal shelves just prior to fusion.
        (a) Therefore, in the presence of TCDD, abnormally high levels of certain EGFs apparently continue to stimulate proliferation and differentiation of the cells normally destined to die. Thus the tissue shelves destined to be palate do not fuse.

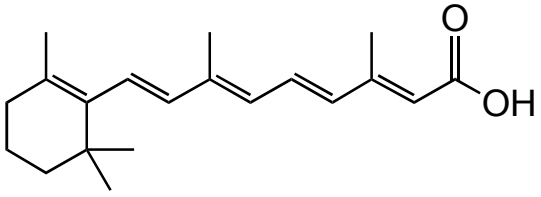
2. <u>Retinoic acid derivatives</u>
    a. Retinoic acid and some of its isomers and derivatives, together with a number of structurally modified retinoids, have been shown to control cell differentiation in many epithelial tissues and to prevent metaplasia (the transformation of one differentiated cell type into another type; an abnormal change in the nature of a tissue).
    b. Retinoic acid isomers are metabolites of Vitamin A;
        1. Certain metabolites of vitamin A, such as *all-trans* and *cis* - isomeric retinoic acids, can perform some, but not all, of the biological functions of vitamin A.



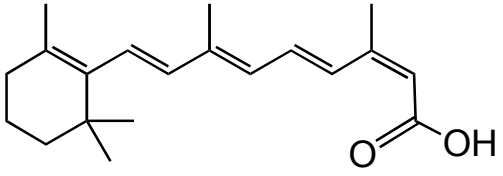
General Structure for Parent Retinoids



retinol (dietary Vitamin A)



retinoic acid



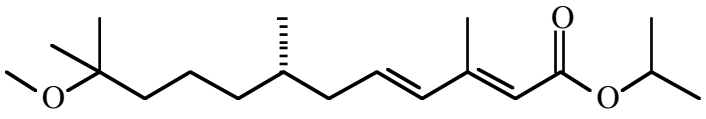
13 cis-retinoic acid (isotretinon)

c. 13-cis-retinoic acid, a vitamin A derivative, also known as **isotretinoin** and marketed as Accutane for treatment of severe acne since 1982, causes structural craniofacial abnormalities (ear, mouth, and palate) and head and limb defects in newborn infants exposed in utero. It is as potent as thalidomide in humans and is also teratogenic in rats in vivo.
d. Proposed mechanism:
   1. Activates the endogenous retinoic acid (RA) receptors, but probably via metabolism to other retinoid derivatives, including retinoic acid.
      (a) RA is an endogenous morphogen with a role in differentiation.
      (b) Binding of RA to the receptor modifies gene expression by affecting transcriptional activation and, therefore, synthesis of gene products.
      (c) RA exhibits selective toxicity to neural crest cells, which play major roles in face, heart, and limb differentiation.
      (d) The mechanism is hypothetically quite similar to other hormonal mimics, where in increased receptor signaling at the wrong time causes developmental abnormalities.

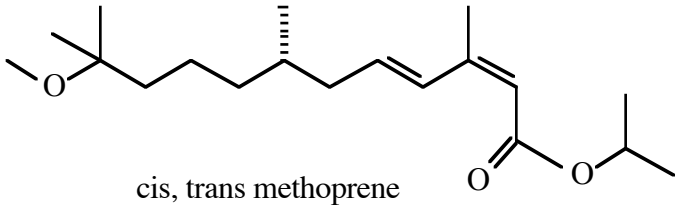
III. Amphibians and Teratogenesis

A. The phenomenon of malformed frogs, especially those with extra limbs and missing eyes, raised quite a few eyebrows when reported in the mid 1990's.
   1. Of course, these kinds of finding are always commonly interpreted as harbingers of adverse effects to come in humans (the sentinel species hypothesis [i.e., canary in the coal mine]).
B. Historically, deformed frogs have always been noted, but in the mid 1990's numerous sightings were made in Minnesota, and soon sightings were reported from most states.
   1. However, it was and still is not clear if the numbers of deformed frogs were related to an increase or just historical levels of malformation but now increased observation through more intense search.
C. Regardless of whether the increase in malformed frog sightings was due to a change from historical levels, the occurrence of the phenomenon was coincidental with observations that amphibian populations worldwide were declining.
D. One interesting report involved frog malformations and exposure to metabolites of the insect growth regulator (which is used as an insecticide for larval mosquito control and for larval flea control).
   1. The metabolites have a retinoic acid type structure, and it was hypothesized that they could take on an analogous spatial configuration to 13-cis retinoic acid (La Clair, J. J., J. A. Bantle, and J. Dumont. 1998. Photoproducts and metabolites of a common insect growth regulator produce development deformities in Xenopus. Environ. Sci. Technol. 32:1453-1461.)
      a. Note below the structure of methoprene (the S-isomer is the most biologically active.)

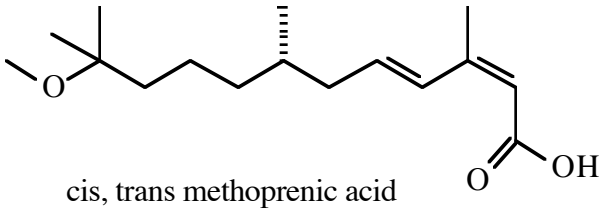
1. In the presence of sunlight, S-methoprene can isomerizes to cis, trans methoprene.
2. Both S-methoprene and cis, trans methoprene can undergo photodegradation to one of several metheprenic acids.



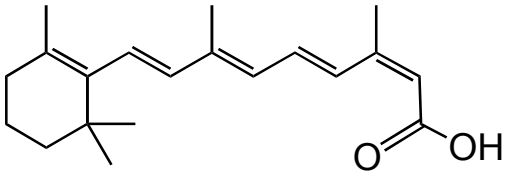
S-methoprene



cis, trans methoprene



cis, trans methoprenic acid



13 cis-retinoic acid (isotretinon)

3. Note the similarity in structural arrangement between the methoprenic acid and the known teratogen 13-cis retinoic acid.

2. La Clair et al. 1998 noted that photodegradative metabolites of methoprene caused frog malformations when tested in a bioassay known as FETAX.
   a. The Frog Embryo Teratogenesis Assay: Xenopus (FETAX) bioassay examines the effects of aqueous agents on *Xenopus* (South African clawed frog, *Xenopus laevis*) embryo development during the first 96 hours of development. The endpoints examined include mortality, malformation rate, and growth inhibition/acceleration as indicated by a change in embryo length and the presence of features indicative of earlier / later stages.
   b. Malformations noted were concentration dependent and included:
      1. Extreme reduction in embryo length
      2. Severe eye defects including lack of pigmentation or severe diffuseness

3. Non-closure of the choroid fissure on the ventral aspect of the eye
4. Mouthparts not readily evident.

3. Another study confirmed the effects of methoprene degradation products on frog teratogenesis but also gave some environmental context to the likelihood of effects in the field (Degitz, S. J. and P. A. K. Gary W. Holcombe, Joseph E. Tietge, Elizabeth J. Durhan and Gerald T. Ankley. 2003. Comparing the effects of stage and duration of retinoic acid exposure on amphibian limb development: Chronic exposure results in mortality, not limb malformations. Toxicological Sciences 74 139-146.)
   a. RA exposure (exposure to stage 8 embryo for 3 days, 9 days, or until tail resorption was complete, which was stage 65) resulted in a concentration dependent increase in mortality and dysmorphogenesis in embryos at concentrations of 0.24 μg/l and above. At concentrations of 0.6 μg/L, 100% of the organisms developed abnormally.
      1. Effects at 0.6 μg/L included the craniofacial region and consisted of micropthalmia, reductions in the prosencephalon, mesencephalon, and edema.
      2. At 2 μg/L the effects were more severe, ranging from holoprosencephaly and anophthalmia to near complete absence of procephalic tissue, and effects on posterior development.
      3. Continuous exposure to RA through metamorphosis resulted in significant mortality.
         a. However, susceptibility was on when exposure was initiated. Short term exposure initiated at stage 8 was more toxic (mortality benchmark) than exposure initiated at stage 48.
   b. However, hind limb development was not affected by early embryo exposure nor by exposure during larval (tadpole) development.
      1. In an earlier paper (Degitz, S. J., P. A. Kosian, E. A. Makynen, K. M. Jensen and G. T. Ankley. 2000. Stage- and species-specific developmental toxicity of all-trans retinoic acid in four native north American ranids and *Xenopus laevis*. Toxicological Sciences 57 (2):264-274.), Degitz et al. did note anterior hind limb deformities in several frog species if they were exposed to much higher concentrations of retinoic acid (i.e., 500-1250 μg/L) at later stages of development (i.e., tadpole stage). However, at earlier stages ("larval" stage), the frogs would have died at these concentrations, and thus from this earlier experiment, Degitz et al. concluded that environmental concentrations of methoprene would be too low to cause limb malformations.
   c. Degitz et al. 2000, 2003 thus concluded it was unlikely that retinoic acid analogs could account for the limb malformations seen in field-collected frogs.

E. Heavy metals have been noted to be teratogenic in humans, rodents and in frogs.
   1. Based on FETAX bioassays, the malformations of frog embryos exposed to certain metals include bent tail, craniofacial deformity, ocular abnormalities, intestinal malrotation and cardiac anomalies.

2. Note that teratogenic effects occurred at much lower doses than toxic effects (Table 2).

Table 2. Teratogenicity of Metals to Xenopus laevis in FETAX bioassay (based on table presented in Sunderman, F. W. 2000. Teratogenicity and Embryotoxicity of Metals. Chapter 54 (pp. 1203-1213), in "General & Applied Toxicology", 2nd ed., vol. 2, B. Ballantyne, T. Marrs, T. Syversen (Eds.), Grove's Dictionaries Inc., NY.)

Metal (Form)	**EC50 (μM)**	**LC50 (μM)**	**Teratogenesis Index ****	**Minimum Concentration (μM) of Metal Added to FETAX Medium that Significantly Inhibited Growth of Embryos**
As (MSMA) *	285	1400	4.9	466
Cd ($CdCl_2$)	3.7	32	8.6	18
Co ($CoCl_2$)	25	10400	416	42
Cu ($CuCl_2$)	2.5	22	8.8	10
Hg ($HgCl_2$)	<0.2	0.3	Not Determined	Not Determined
Ni ($NiCl_2$)	2.5	365	147	5.6
Zn ($ZnCl_2$)	40	850	21	300

* MSMA = monosodium methanearsonate

** Teratogenesis index = LC50/EC50; if index > 1.5, then compounds considered teratogenic to *Xenopus*.

F. The role of parasites in inducing limb malformations
   1. An alternative to the chemical contaminant hypothesis as a causative agent for limb malformations in frogs is the hypothesis that trematode parasites burrow in to developing larval stages and cause hind limb deformities. [Johnson, P. T. J., K. B. Lunde, E. G. Ritchie and A. E. Launer. 1999. The effect of trematode infection on amphibian limb development and survivorship. Science 284 802-804.] [Johnson, P. T. J., K. B. Lunde, R. W. Haight, J. Bowerman and A. R. Blaustein. 2001. *Ribeiroia ondatrae* (Trematoda: Digenea) infection induces severe limb malformations in western toads (*Bufo boreas*). Can. J. Zool. 79 370 -379.]
   2. In a recent publication (Johnson et al. 2004 Ecology Letters), Johnson et al. found an association with eutrophication of pond habitat and the frequency of frog deformities. Excess nutrients were hypothesized to favor proliferation of parasitic trematode populations.