THE EFFECTS OF ENVIRONMENTAL CONTAMINANTS ON METAMORPHOSIS IN
RANA CATESBEIANA AND SPERM MOTILITY IN XENOPUS LAEVIS

by

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B.Sc., The University of Alberta, 1997

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

In

THE FACULTY OF GRADUATE STUDIES

(Deptartment of Forest Sciences, Faculty of Forestry)

We accept this thesis as conforming
to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

December 2002

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Date December 19, 2002
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The objectives of this research were to observe the effects of specific contaminants on different amphibian species at various times of their life cycles, namely metamorphosis and fertilization. The effects of a non-ionic surfactant, Nonylphenol (NP), on Rana catesbeiana (bullfrog) tadpole metamorphic staging and tail resorption were examined, along with the effects of pH, osmolality and a divalent metal ion, Zinc (Zn$^{2+}$), on Xenopus laevis (African clawed frog) sperm motility and kinematics. I hypothesized that 1) NP would have indirect sub-lethal effects on thyroid hormone function by disrupting metamorphosis in bullfrog tadpoles, and 2) aquatic parameters (pH, osmolality and Zn$^{2+}$ concentration) would have significant effects on percent sperm motility, velocities and kinematics. This latter hypothesis was the basis for the development of the Amphibian Sperm Inhibition Toxicological Test (ASITT) method. To test the effect of NP on metamorphosis, there were 3 NP concentrations used (234, 468 and 936 $\mu$g/L) in both the presence and absence of 3,3',5-triiodothyronine (T$_3$), a well water control, a T$_3$ control and a solvent control for a total of 9 treatments with three replicates each. Tadpoles were exposed for 7 days whereupon tail length, width, and metamorphic staging was measured. NP had a significant effect on tail length and metamorphic staging in the absence of T$_3$, causing an increase in tail length and a decrease in limb development, respectively, with increasing NP concentrations. In the presence of T$_3$, increasing NP caused a significant reduction in cranial transformation. These results suggest an indirect inhibition of T$_3$ during tadpole metamorphosis. For the development of ASITT, first the effects of pH and osmolality on sperm motility had to be determined. The pH 7.0 and 56.625 mosmol/L treatments gave the highest percent motility and the control solution for ASITT was then developed using these solution parameters. Zn$^{2+}$ was used for preliminary validation of ASITT method. It was added to the control solution in concentrations from 0 to 1,417 $\mu$g/L for 7 treatments each with 12 replicates. Increasing Zn$^{2+}$ concentrations caused a significant decrease in percent total motile sperm and progressive sperm. Straight line velocity increased with increasing Zn$^{2+}$ concentrations. These results suggest that Zn$^{2+}$ is inhibiting the sperm at early stages of motility, possibly by preventing calcium influxes into the cell or inhibiting cellular respiration.
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Acknowledgements

This study was supported by Canadian Wildlife Service (Environment Canada), Toxic Substance Research Initiative (TSRI), and Georgia Basin Ecosystem Initiative (GBEI). A HUGE thanks to my supervisor, Dr. John Richardson, and my committee, Dr. Christine Bishop, Dr. John Elliott, Dr. Ken Hall and Dr. Scott Hinch for all their faith in me, their support (financial and emotional), their patience through the more difficult times and the hours of editing it took to get here. I would like to thank Graham van Aggelen for the use of the lab at the Pacific Environmental Science Centre (PESC), as well as Joy Bruno and Michelle Linssen for their support, friendship, sarcasm and laughter. Also, a big thanks to Bruce Pauli for being my "superman". I am grateful to all of you for this wonderful opportunity you have given to me to help me grow in my knowledge, my passion and my career. You have opened so many new doors for me.

I want to give special recognition to Dr. Sharon Mortimer from the Genesis Fertility Clinic, Vancouver, B.C. Her similar passions for successful fertilization (albeit in very different species) were vital to my methodology for ASITT's development. She is the only other woman I know who spent years of graduate school mapping sperm motility by hand in front of a TV monitor, and I respect her for having the patience to do it for years longer than I have.

My family has been so supportive and encouraging through my graduate studies, so I dedicate my thesis to you, Mom and Dad. I couldn’t have done this without your love (and money). Thank you for everything.
And finally, I want to say that I especially could not have finished this thesis without my dear friends I met during my time here. Your friendships are invaluable to me and they made this whole journey worthwhile. Thank you so much.
Chapter I – Introduction

Declines in Amphibian Populations

Declines in amphibian population sizes and occurrences have been reported around the world (Barinaga 1990; Wake 1991; Blaustein et al. 1994). These declines have been attributed to a number of causes, including but not limited to habitat destruction, chemical pollution, acid precipitation, increased UV radiation, introduction of exotic species, pathogens, harvesting by humans, and natural population fluctuations (Berger 1989; Blaustein and Wake 1990; Wake 1991; Blaustein et al. 1994; Marco et al. 1999). Although habitat alteration and destruction seem to be the primary cause of most declines in amphibian population occurrences and size, it does not explain the disappearance or declines in remote areas, including those not impacted directly by human activities (Marco et al. 1999).

Biologists studying in the lowland valleys in western Oregon (Willamette Valley) and Washington have reported significant declines of both the population sizes and occurrences of the Oregon Spotted Frog, *Rana pretiosa*, and the Red-Legged Frog, *R. aurora* (Blaustein and Wake 1990). The agricultural chemicals used in those areas, such as fertilizers and pesticides (Berger 1989), are suspected to have contributed significantly to the near extirpation of *R. pretiosa* in the Willamette Valley (Marco et al. 1999). The Lower Fraser Valley in British Columbia, Canada, which supports a variety of extensive agricultural, pulp mill and urban-related activities, has also noted significant declines in the local amphibian population sizes and occurrences, as seen in the Oregon Spotted Frog (Corkran and Thoms 1996; Cannings et al. 1999). The associated combinations of habitat alteration, agricultural chemical applications, increased sedimentation and untreated waste runoff have been suspected as possible culprits.
affecting native amphibians (Berger 1989; Marco et al. 1999). It has been hypothesized that xenobiotics used for various purposes enter the surrounding waterbodies making them potentially uninhabitable for the aquatic wildlife, including amphibians (Marco et al. 1999). Xenobiotics have the potential to accumulate in the waterbodies that serve as nurseries for larval amphibians, and breeding habitats for adult amphibians (Clark et al. 1998). Therefore their respective concentrations and distributions, acting solely or in synergism with other xenobiotics, could play a significant role in the decline and disappearance of amphibian populations on a local, regional and/or global scale.

The effects of contaminants have been extensively studied on many different portions of the amphibian life cycle, namely fertilization success, hatching success, embryonic development, tadpole growth and development, metamorphosis, sexual maturation, and gametogenesis (Bantle et al. 1990; Nishimura et al 1997; Fort et al. 1999; Kloas et al. 1999; Mann and Bidwell 1999; Harris et al. 2000; Mann and Bidwell 2000, 2001; for review see Sparling et al. 2000). However, effects of contaminants on amphibian sperm motility has not been previously examined. In this document, two components of the amphibian life cycle will be examined in response to chemical contaminants: amphibian metamorphosis and sperm motility. The second chapter examines the effects of a specific chemical, nonylphenol, on bullfrog, *Rana catesbeiana*, metamorphosis and tail resorption. It looks at the possible indirect effects of nonylphenol on the thyroid hormone, 3,3',5-triiodothyronine (T$_3$)$^1$. Chapter three is the development and preliminary validation of a novel toxicity test using African Clawed Frog, *Xenopus laevis*, sperm motility as an endpoint. I tested the effects of pH, osmolality and zinc ion concentration on sperm motility and kinematics. Sperm motility during an external fertilization event is a

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$^1$ See Appendix B for a complete list of acronyms used in this document.
component of the amphibian life cycle that has received very little attention. Chapter four provides general conclusions and recommendations regarding the findings of these studies.
Literature Cited


Chapter II - Effects of Nonylphenol on Tail Resorption and Metamorphic Staging in *Rana catesbeiana* Tadpoles

Introduction

Amphibian metamorphosis is a series of coordinated biochemical and physiological events that cause cell death or transformation in many larval tissues and the generation and growth of new tissues. The timing and specificity of these events are largely under hormonal control. Environmental factors such as crowding, diet, presence of predators, pond desiccation, light availability, and temperature are known to affect the timing, size of the animals and success of metamorphosis (Dent 1968; Dodd and Dodd 1976; Hayes *et al.* 1993; Martínez *et al.* 1996; Denver 1997a,b; Denver *et al.* 1998; Laurila and Kujasalo 1999). Among the possibilities, these factors may function along the neuroendocrine axis and indirectly affect hormone production (Denver and Licht 1989; Denver 1997a,b; Denver *et al.* 1998).

Thyroid hormones (TH), namely 3,3',5,5'-tetraiodothyronine [or thyroxine (T₄)] and 3,3',5-triiodothyronine (T₃), are pivotal in the metamorphic process (Tata 1968; Dodd and Dodd 1976; Bascaglia *et al.* 1985; Galton 1989; Shi and Hayes 1994; Becker *et al.* 1997; Shi 2000). TH is produced in the thyroid gland, which develops in the African Clawed Frog (*Xenopus laevis*) around late embryogenesis (Nieuwkoup and Faber (NF) stages 35/36 – Nieuwkoup and Faber 1956)² and becomes completely functional by NF stage 53 and continues to increase in size until metamorphosis nears completion (NF stage 66).

---

² See Appendix 1 for a comparison of Gosner (1960), Nieuwkoop and Faber (1956), and Taylor-Kollros (1946) amphibian developmental staging systems.
Plasma T₄ levels increase with the size of the thyroid gland (Dodd and Dodd 1976). T₄ is converted to its active form, T₃, late in larval development via 5'-deiodinase (Bascaglia et al. 1985; Galton and St. Germain 1985b; Galton 1988, 1989; St. Germain 1994; Becker et al. 1997). T₃ concentrations are low during premetamorphic stages, increase through prometamorphosis until the beginning of metamorphic climax, and then decrease (Galton and St. Germain 1985a,b; Regard et al. 1978; Shi 2000).

Since amphibian metamorphosis is strongly associated with a complex neuro-endocrine pathway (through the hypothalamus-pituitary-thyroid axis) the introduction of an endocrine disrupting chemical (EDC), such as nonylphenol (Routledge and Sumpter 1996; Christiansen et al. 1998a,b; Kuiper et al. 1998), into the aquatic environment could alter the metamorphic process and change rates of development and tail resorption. Reviews on the effects of sub-lethal concentrations of EDCs on a variety of organisms are available (USEPA 1997; Ankley et al. 1998; Campbell and Hutchinson 1998; Sparling et al. 2000).

Nonylphenol (Figure 1) is a degradation product of a class of chemicals referred to as alkylphenol ethoxylates (APEs). APEs are non-ionic surfactants used in pesticides, spermicides, cleaning agents, textiles, various agricultural chemicals, plastics and paper products (Heinis et al. 1999; Liber et al. 1999a; John et al. 2000; Topp and Starratt 2000). APEs undergo microbial degradation producing metabolites more toxic than the parent compound (Yoshimura 1986; Liber et al. 1999a; John et al. 2000); nonylphenol may be one of the more critical metabolites due to its toxicity, persistence, estrogenic effects, and ability to bioconcentrate, bioaccumulate, and biomagnify in the environment (Liber et al. 1999b; Tsuda et al. 2000; Tsuda et al. 2002).
In British Columbia, nonylphenol can be potentially released to the aquatic environment by the numerous kraft pulp mills located on the Fraser and Columbia River system (Schreier et al. 1991; Bortone and Davis 1994; Bennie et al. 1997), from sewage treatment plants (Schreier et al. 1991; Bennie et al. 1997), and forest and agricultural pesticide use. The use of nonylphenol as a surfactant in herbicides is of concern (Mann and Bidwell 2000) in cases where the application is in proximity to shallow waterbodies is where the aquatic phase of most amphibians takes place (Mann and Bidwell 1999). Stable compounds, like nonylphenol, can potentially accumulate in the sediments of these aquatic systems and may pose an increased threat to aquatic species (Mann and Boddy 2000). Limited surveys for the occurrence of nonylphenol in surface waters, sediments and industrial effluents have been conducted worldwide but few studies exist in Canada (Table 1; for review see Environment Canada 2001). NP is generally found in higher concentrations in aquatic environments near untreated or partially-treated industrial and municipal effluent release sites (Environment Canada 2001).
Table 1. Previous reports of nonylphenol concentrations measured in surface water, sediment and effluents around the world.

<table>
<thead>
<tr>
<th>Country</th>
<th>Source</th>
<th>NP Conc. Range</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Canada (BC)</td>
<td>River sediments</td>
<td>&lt;0.02 to 0.57 µg/g</td>
<td>Brewer et al. 1998</td>
</tr>
<tr>
<td></td>
<td>Municipal wastewater and sludge</td>
<td>Detected, not quantified</td>
<td>Rogers et al. 1986</td>
</tr>
<tr>
<td>Canada (Eastern) and United States</td>
<td>Surface waters</td>
<td>ND - 0.92 µg/L</td>
<td>Bennie et al. 1997</td>
</tr>
<tr>
<td></td>
<td>Sediments</td>
<td>0.17 - 72 µg/g (high concentrations near STP)</td>
<td>Bennie et al. 1997</td>
</tr>
<tr>
<td></td>
<td>Pulp and Paper Mill effluent</td>
<td>0.31 mg/L in a single unbleached white water sample</td>
<td>Sithole and Allen 1989</td>
</tr>
<tr>
<td>United States</td>
<td>River water</td>
<td>21/30 rivers contained &lt;0.1 µg/L</td>
<td>Naylor 1992</td>
</tr>
<tr>
<td>Europe (Switzerland)</td>
<td>Sewage sludge (anaerobically treated)</td>
<td>0.45 – 2.53 g/kg</td>
<td>Giger et al. 1984</td>
</tr>
<tr>
<td></td>
<td>Sewage sludge (aerobically treated)</td>
<td>0.08 – 0.5 g/kg</td>
<td>Giger et al. 1984</td>
</tr>
<tr>
<td>Europe (England and Wales)</td>
<td>River water</td>
<td>&lt;0.2 – 53 µg/L (highest concentrations generally near source)</td>
<td>Blackburn and Waldock 1995</td>
</tr>
<tr>
<td></td>
<td>Sewage effluent</td>
<td>&lt;0.2 – 330 µg/L (total extractable NP)</td>
<td>Blackburn and Waldock 1995</td>
</tr>
<tr>
<td>Japan</td>
<td>River water</td>
<td>Up to 0.30 µg/L</td>
<td>Tsuda et al. 2002</td>
</tr>
<tr>
<td></td>
<td>River water</td>
<td>0.11 to 3.08 µg/L</td>
<td>Tsuda et al. 2000</td>
</tr>
<tr>
<td>Korea (Ulsan Bay)</td>
<td>Sediments</td>
<td>&lt;1.0 – 1,040 ng/g</td>
<td>Khim et al. 2001</td>
</tr>
</tbody>
</table>
Nonylphenol is known to be highly toxic to aquatic organisms, but little information is available for amphibians. Mann and Bidwell (2001) observed mild narcosis upon exposure of various amphibian species to nonylphenol ethoxylates (NPEs) with EC$_{50}$ values between 1.2 and <10.6 mg/L. EC$_{50}$ values for full narcosis ranged from 2.3 to 12.1 mg/L (Mann and Bidwell 2001). Mann and Bidwell (2000) conducted toxicity tests using NPE and calculated a 140-h LC$_{50}$ value of 9.2 mg/L for slender tree frog, *Litoria adelaidensis*, a 134-hr LC$_{50}$ value of 6.4 mg/L for white-throated froglet, *Crinia insignifera*, and a 96-hr LC$_{50}$ values ranging from 3.9-5.4 mg/L for *X.laevis*.

Nonylphenol is an endocrine disrupting chemical (EDC) with estrogen-like properties (Soto *et al.* 1991; Jobling and Sumpter 1993; Routledge and Sumpter 1996; Christiansen *et al.* 1998a,b; Kuiper *et al.* 1998). Concentrations of nonylphenol well below the levels of acute toxicity may cause adverse physiological, histopathological, biochemical and/or reproductive effects. For instance, Jobling and Sumpter (1993) observed significant vitellogenin (VTG) production in male *Oncorhynchus mykiss* following a 4-day exposure to 10 $\mu$M of NP. At 30 $\mu$g/L NP caused an inhibition of testicular growth in this species (Jobling *et al.* 1996). Exposure of *X.laevis* embryos showed induced feminization at 22 $\mu$g/L of NP (Kloas *et al.* 1999). NP mimics 17$\beta$-estradiol by binding to and activating estrogen receptors (ER) in both mammals (White *et al.* 1994) and fish (Yadetie *et al.* 1999), and is suspected to upregulate ER mRNAs (Flouriot *et al.* 1995) and VTG mRNAs *in vitro* in *O. mykiss* hepatocytes (Flouriot *et al.* 1995; Yadetie *et al.* 1999). Most studies of the possible sub-lethal effects of NP use endpoints along the hypothalamic-pituitary-gonadal axis (Jobling *et al.* 1996; Gray and Metcalfe 1997). Less studied are the effects of NP on brain and pituitary tissues where ER also occur (Pakdel *et al.* 1989; Salbert *et al.* 1993).
The function of estrogen in metamorphosis remains unclear, although previous work by Roth (1948) suggested that estrogen might inhibit metamorphosis by acting along the hypothalamic-pituitary-thyroid axis to down-regulate TH levels in the blood. Since previous work suggests nonylphenol has estrogenic properties (Christiansen et al. 1998a,b; Kuiper et al. 1998) it was hypothesized that NP could delay metamorphic development and inhibit tail resorption. However, Fort and Stover (1997) observed a significant increase in tail resorption in prometamorphic X. laevis tadpoles following exposure to 10.0 μg/L of NP, possibly indicative of thyroid hormone mimicry or upregulation.

In this study I hypothesized that NP would affect metamorphic staging and tail resorption in a dose response fashion. I examined metamorphic development by staging the animals using an established developmental staging regime (Taylor and Kollros 1946) and compared the progress of exposed and unexposed animals through these developmental stages. I further hypothesized that NP would affect these endpoints via direct or indirect thyroid hormone disruption, which was tested by adding exogenous T₃ to the NP treatments.

**Methods and Materials**

**Test Organisms**

Early premetamorphic bullfrog (Rana catesbeiana) tadpoles were collected at the beginning of August 2000 from natural ponds, not known to have been previously exposed to nonylphenol, in areas surrounding Victoria, British Columbia. They were held at 15°C for 9 months in 20 litre aquariums filled with well water (at a density of 2 tadpoles per litre) whereupon they were acclimatized to 21°C for one week prior to testing. Tadpoles were fed fish food with calcium supplements and boiled romaine lettuce *ad libitum*. They were kept at approximately 21°C with a light:dark cycle of 16:8 hours for the duration of the experiment. Only tadpoles at stages near
prometamorphosis (Taylor-Kollros (TK) X-XII; Taylor and Kollros 1946) were selected for the experiment. These stages are equivalent to Gosner stages 35 to 37 defined by the development of the hindlimbs, where tadpoles have foot indentations occurring either between the first and second toes, or up to all five toes being separated (Gosner 1960).

**Test Procedure**

Bullfrog tadpoles were randomly assigned to treatments of nonylphenol with and without T₃. Nonylphenol concentrations to examine sub-lethal effects were based on earlier range-finding tests with *Rana aurora* (Red-legged frog) tadpoles. Those tests produced 24-h and 48-h LC₅₀ values of 310 µg/L and 250 µg/L, respectively (Christensen, unpublished data; Appendix C). Mann and Bidwell (2001) demonstrated a size-sensitivity relationship in amphibians exposed to nonylphenol when *X. laevis* data were excluded. Therefore, since *R. catesbeiana* tadpoles are generally larger (smaller surface area: volume ratio) than *R. aurora* tadpoles, exposure concentrations for bullfrogs were set slightly higher, but lower than the lethal level determined during a *R. catesbeiana* preliminary acute toxicity test (NP 24-h LC₅₀ >3.43 mg/L; Christensen, unpublished data). Nonylphenol exposure treatments were therefore set as follows: NP1 = 234 µg/L, NP2 = 468 µg/L, and NP3 = 936 µg/L. Due to the low solubility of NP, methanol (40 µL/L) was added to all treatments. In the T₃ treatments, T₃ was added, alone to one set of exposure vessels and to three other sets of exposure vessels in combination with NP1, NP2 and NP3, respectively, at 1 x 10⁻⁷ M dissolved in dimethyl sulphoxide (DMSO). DMSO remained constant in the NP and T₃ + NP combination treatments at 100 µL/L. The well water control and a solvent control (40 µL/L methanol plus 100 µL/L DMSO) were also included. Thus there were 9 treatments in total with three replicates for each treatment. Exposures occurred in 4 L
plastic buckets with 2 L of aerated water and four tadpoles in each bucket. Tadpoles were exposed for 7 days.

Water was replaced every second day (static-renewal) and temperature, pH and dissolved oxygen (DO) were monitored prior to every water change (Table 2). DO levels prior to the first water change reached 4.0 mg/L in one of the control replicates. It is not certain what caused this drop in DO in this particular replicate and not in others.

Table 2. Temperature, pH and dissolved oxygen readings prior to each water replacement.

<table>
<thead>
<tr>
<th></th>
<th>Minimum Reading</th>
<th>Mean Reading</th>
<th>Maximum Reading</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature (°C)</td>
<td>20.9 (control, solvent, NP1)</td>
<td>21.1</td>
<td>21.2 (NP2, NP3)</td>
</tr>
<tr>
<td>pH</td>
<td>7.05 (control)</td>
<td>7.5</td>
<td>7.64 (solvent)</td>
</tr>
<tr>
<td>Dissolved Oxygen (mg/L)</td>
<td>4.0 (control)</td>
<td>7.8</td>
<td>8.5 (solvent, NP3)</td>
</tr>
</tbody>
</table>

Tail length, tail width and Taylor-Kollros (T-K) developmental stages (Taylor and Kollros 1946) were measured once on Day 0 and again on Day 7 of the experiment. Tail length measurements were made from where the tail began on the dorsal side of the tadpole body to the tip of the tail using a standard ruler to 0.5 mm accuracy (Figure 2). Tail width (also measured with a standard ruler) was the width of the widest part of the tail (Figure 2). T-K stages were subdivided into three categories: limb development, head (cranial) transformation and stage of tail resorption (Figure 3). Limb development was determined by monitoring the development
and growth of the hind- and fore-limbs. Head transformation was measured as the progression of cranial transformation with respect to the shape of the eyes, the angle of the mouth and jaw formation. The stage of tail resorption observed the advancement of tail and tail fin resorption. Percent survival was calculated on Day 7. All values in text are expressed as means ± 1 standard deviation (SD).

![Diagram showing measurement locations for tail length and tail width on a bullfrog tadpole.](image)

**Figure 2. Measurement locations for tail length and tail width on a bullfrog tadpole.**

![Diagram showing measurements for limb development, head transformation and stage of tail resorption.](image)

**Figure 3. Measurements for limb development, head transformation and stage of tail resorption.**

**Statistical Analysis**

Nominal nonylphenol concentrations were used in calculations, since previous work by Mann and Bidwell (unpublished data; see Mann and Bidwell 2000) established loss of surfactant due to degradation or surface adsorption to be consistently less than 10% over a 24-h period.
Statistical analysis was not conducted on the survival data due to lack of variance within and between treatments.

To standardize for individual variation in size and initial metamorphic stage at the beginning of the experiment, ratios were calculated for the endpoint measurements following the 7-d exposures. Ratios were determined as day 7 measurement / day 0 measurement for each replicate. I calculated tail length ratio (TLR), tail width ratio (TWR), limb development ratio (LR), head transformation ratio (HR) and stage of tail resorption ratio (TR) using the following equations, respectively:

\[
\text{TLR} = \frac{\text{tail length day 7}}{\text{tail length day 0}} \\
\text{TWR} = \frac{\text{tail width day 7}}{\text{tail width day 0}} \\
\text{LR} = \frac{\text{stage of limbs day 7}}{\text{stage of limbs day 0}} \\
\text{HR} = \frac{\text{stage of head day 7}}{\text{stage of head day 0}} \\
\text{TR} = \frac{\text{stage of tail resorption day 7}}{\text{stage of tail resorption day 0}}
\]

A general linear model (PROC GLM; SAS software, version 8.2) was used to determine differences in the magnitude of the response among the various treatments. Orthogonal contrasts were used for treatment comparisons. Linear dose-response models were also fitted to the endpoint measurement data (PROC GLM) where possible. A one-way Analysis of Variance (ANOVA) was used to determine if there were significant differences among T-K stage ratios for limb development, head transformation and tail resorption (LR, HR and TR, respectively). Multiple comparisons were made using Bonferroni’s post-hoc test. All tests were considered significant at p < 0.05.
Results

Survival

Mean survival was 100% following the 7-day exposure in the well water control, solvent control, NP1 and NP2 treatments. Mean survival in NP3 was 91.7%. All T3 treatments had lower survival following the 7-day exposure ranging from 58.3% (T3 alone) to 91.7% (NP1). There was no trend between survival and increasing NP concentrations in the T3 + NP combination treatments.

Tail Length Ratios (TLRs)

A TLR greater than 1.0 indicates tail growth and a TLR less than 1.0 indicates tail resorption. When T3 was added to the NP treatments, tail resorption occurred by the end of the 7-day exposure. The mean TLRs in treatments without-T3 (TLRwithoutT3 = 1.04 ± 0.05) were significantly greater (F1,18 = 371.13, P<0.0001) than the TLRs in T3 treatments (TLRwithT3 = 0.64 ± 0.06; Figure 4).

The TLRs for the well water (TLRcont = 0.99 ± 0.01) and solvent (TLRsolv = 1.01 ± 0.01) controls were not significantly different, therefore these data were combined as 0 μg/L NP in a without-
T₃ dose-response regression analysis (Figure 5). Although the TLRs without T₃ addition were greater in the NP1 and NP2 treatments (TLRₙ₃ = 1.04 ± 0.05 and TLRₙ₃ = 1.05 ± 0.01, respectively) they were not significantly different from the well water control. However, NP3 showed a significant difference in TLR (TLRₙ₃ = 1.09 ± 0.05) when compared to the well water control (P<0.05). The overall linear regression model (Figure 5) was significant for the without-T₃ treatments with a positive dose-response relationship (P<0.001, R² = 0.581) between tail length and increasing NP concentrations. There was no significant dose-response for TLRs with increasing nonylphenol concentrations in the presence of T₃ (Figure 5).

Figure 5. Calculated *Rana catesbeiana* Tail Length Ratios (TLRs) following a 7-day exposure to various concentrations of nonylphenol in the absence (●) and presence (○) of T₃. Solid line indicates a significant relationship of tail length ratios with increasing nonylphenol concentrations (Slope=0.0001, R²=0.58, P<0.01). Some data points are hidden due to overlapping.
**Tail Width Ratios (TWRs)**

A TWR greater than 1.0 indicates tail widening and a TWR less than 1.0 indicates tail thinning as expected with metamorphic progression. Almost all tadpoles in all treatments exhibited some level of tail thinning after 7 days (Figure 6). The treatments that contained T₃ had significantly lower TWRs ($F_{1,18} = 113.12, \ P<0.0001$) than the treatments without T₃ ($\text{TWR}_{\text{with T₃}} = 0.51 \pm 0.02$ and $\text{TWR}_{\text{without T₃}} = 0.91 \pm 0.04$). There was no significant nonylphenol dose-response relationship in either the absence or presence of T₃.

![Figure 6. Tail width ratios following a 7-day exposure to various treatments of nonylphenol in the absence (●) and presence (○) of T₃.](image-url)
Taylor-Kollros (TK) Staging

The T₃ exposed tadpoles showed varying developmental stages within the same individuals depending on the trait, therefore the determination of three different ratios (LR, HR and TR) was necessary. For the without-T₃ treatments the limb, head and tail stages were the same within individuals, therefore there is only one regression shown (Figure 7). Metamorphic stages can only increase or remain constant, therefore the LR, HR and TR were 1.0 or greater.

Limb Development Ratio (LR)

The limb development ratio (LR) of T₃-exposed tadpoles (LR_{withT₃} = 1.73 ± 0.05; day 7 from T-K stages XVIII to XXI) was significantly greater (F_{1,18} = 78.42, P<0.0001; Figure 7) than the tadpoles in treatments without T₃ (LR_{withoutT₃} = 1.51 ± 0.08; T-K stages XV to XVIII). There was no significant difference between the well water control (LR_{cont} = 1.45 ± 0.03) and the solvent control (LR_{solv} = 1.49 ± 0.07). As well, there were no differences detected between LRs following exposure to the two lower nonylphenol concentrations (LR_{NP1} = 1.52 ± 0.07; LR_{NP2} = 1.47 ± 0.07) and the well water control. However, the LR from the highest NP exposure (LR_{NP3} = 1.60 ± 0.09) was significantly higher (F_{1,18} = 9.30, P<0.01) from that of the well water control. The range of T-K stages on day 7 was similar among without-T₃ treatments (T-K stages XV to XVIII), however, NP3 had a higher occurrence of later stage tadpoles (T-K stage XVIII; data not shown). There were no significant differences between any of the NP + T₃ treatments. There was no significant dose-response relationship between increasing NP concentrations (with or without T₃) and the ratio of tadpole limb development.
Figure 7. Ratio for Taylor-Kollros developmental stages for limb development, head transformation and stage of tail resorption (LR, HR and TR, respectively) in Rana catesbeiana tadpoles following a 7-day exposure to various treatments of nonylphenol (NP) and triiodothyronine (T₃). The ratio is the stage at day 7 divided by the stage at day 0. • = combined rates for all measures for treatments without T₃, and ▲ = LR, ■ = HR, ● = TR for treatments with T₃. Dashed line denotes a significant regression for the head transformation ratio (HR; Slope= -0.0002, P<0.05). Dotted lines are included to indicate 1 SD about the mean for the control Taylor-Kollros stage ratio for the duration of the exposure. Vertical bars represent 1 SD of the mean.

**Head (Cranial) Transformation Ratio (HR)**

The T₃-exposed tadpoles (HR\textsubscript{with T₃} = 1.90 ± 0.07) had a significantly higher head transformation ratio (HR) (F\textsubscript{1,18} = 249.55, P<0.0001) than the tadpoles in treatments without T₃ (HR\textsubscript{without T₃} = 1.51 ± 0.08). There were no significant differences between the two lower NP+T₃ combination treatments and the T₃ control (Figure 7). However, there was a significant difference between the T₃ control and the NP3+T₃ combination treatment (HR\textsubscript{NP3+T₃} = 1.83 ± 0.040; F\textsubscript{1,18} = 10.28, P<0.005). With the addition of exogenous T₃, increasing NP concentrations produced a significant negative dose-response (slope = -0.0002, P<0.05) in HR. Day 7 tadpoles all reached
T-K stage XXI in the T3 treatments. Without T3, tadpoles reached T-K stages ranging from XV to XVIII based on cranial morphology.

Stage of Tail Resorption Ratio (TR)
The T3-exposed tadpoles (TR\textsubscript{with T3} = 2.04 ± 0.08) had a significantly higher TR (F\textsubscript{1,18} = 377.08, P<0.0001) than the tadpoles in treatments without T3 (TR\textsubscript{without T3} = 1.51 ± 0.08). There was no significant difference between the two lower NP + T3 combination treatments and the T3-alone treatment, however, the NP3 + T3 treatment was significantly lower (F\textsubscript{1,18} = 8.05, P<0.05; Figure 7). There was a downward trend in TR with increasing nonylphenol concentrations, however it was not significant. T3-treated tadpoles reached T-K stages ranging from XXII to XXIII based on tail morphology, while tadpoles not exposed to T3 reached T-K stages XV to XVIII.

Taylor-Kollros Stage Differences Between Tissues
As mentioned above, in the exposure vessels, which did not have exogenous T3 added, individuals exhibited no deviation from the natural metamorphic staging process described by Taylor and Kollros (1946). However, when exogenous T3 was added to the treatments the measured metamorphic staging became uncoordinated and different tissues (limbs, head and tail) had to be described separately due to the different rates at which they were progressing to metamorphic climax. LR showed the lowest ratio of the three tissues monitored (Figure 8) and was significantly different from both the HR (P<0.0001) and the TR (P<0.0001). HR was also significantly different from TR (P<0.001) with the latter having the highest ratio in the presence of T3.
Figure 8. Mean Taylor-Kollros metamorphic stage ratios following a 7 day exposure to nonylphenol with and without T3. LR = limb development ratio, HR = head transformation ratio and TR = stage of tail resorption ratio. Staging is based on Taylor and Kollros (1946) staging for these tissues. Error bars indicate 1 SD of the mean.

Discussion

Nonylphenol did have a significant effect on tail resorption and metamorphic staging in bullfrog tadpoles exposed at T-K stages X to XII. Nonylphenol at the highest concentration tested caused tail growth, which supported my hypothesis. Nonylphenol also caused increased limb development, which was not expected. With the addition of exogenous T3 to the treatments the effect of nonylphenol was mitigated suggesting that nonylphenol had inhibited the effects of T3 in the tadpole.
The addition of T₃ to the treatments produced non-sequenced metamorphic stages that would not normally occur. The cranial and limb tissue did not progress as rapidly to later metamorphic stages as did the tail tissue when exposed to exogenous T₃. The limb, cranial and tail tissues require different levels of T₃ to induce growth, development, transformation or resorption through to metamorphosis. Thyroid hormones also control different gene regulation cascades in varying tissues (Shi 2000). Therefore, it is consistent with literature that these tissues would respond differently when exposed to exogenous T₃. Galton (1992) stated that the extent of response of TH on varying tissues depends on the number of TH receptors present in the target tissue, the receptor binding affinities to TH, and the availability of TH circulating in the cell.

Limb development and cell differentiation are governed by early response genes (Shi 2000). Generally, early response genes will react to T₃ within the first 24 hours in which T₃ will trigger an up-regulation of its own mRNA for TH receptors (Shi 2000). This rapid response within a 7 day exposure is consistent with the quick progression of hindlimbs in the T₃-treated tadpoles in comparison to treatments without exogenous T₃. Limb development in biphasic anurans is TH dependent, however a portion of differentiation occurs in the absence of TH (Elinson 1994). Limb differentiation in *Eleutherodactylus coqui* was not affected by a TH inhibitor, methimazole, indicating that TH is not necessary for limb differentiation (Callery and Elinson 2000). However, they found that the late phase of limb elongation is TH-dependent. Galton (1992, unpublished observations) noted that acceptable hind limb development only occurred at TH levels seen at prometamorphic stages, whereas when TH was administered at higher levels normal tail resorption occurred but hind limb development was impaired. Similarly I found many of the tadpole hindlimbs in the T₃ treatments had little apparent muscle development, no pigment and appeared to be non-functional. Buckbinder and Brown (1992) discovered that connective tissue and muscles in the hindlimbs are dependent on late, rather than early, TH
response genes. Therefore, it becomes necessary for prolonged T₃ exposure to initiate this indirect response for protein production.

The early response genes in the tail tissue encode for the elimination of tail muscle and fin cells (Shi 2000) and an addition of exogenous T₃ would be expected to induce the production of tail TH mRNA and increase TR number causing significant tail resorption and an acceleration of tail stage. This response of increased tail resorption stage by exogenous T₃, as observed with the corresponding increase in tail stage ratio, was demonstrated in this study. The differences in magnitude between the various tissues’ responses could be due to a lower up-regulation of response genes mRNA in the limbs and the head by TH as has been documented for the tail (for review see [Shi 2000]).

The unexpected mortality observed in the T₃ treatments following a brief 7-day exposure appeared related to the uncoordinated development of the tadpoles. The elimination of tail muscle and fin cells caused by the addition of exogenous T₃ would make using the tail for propulsion difficult for the tadpole. At head T-K stage XX, which was reached in the T₃ treatments, the tadpole will also undergo internal structural changes to enable the switch from a herbivorous to a carnivorous life strategy (e.g. intestinal structural changes – Shi and Hayes 1994). At the same stage the tadpole would begin to breathe air rather than to collect oxygen through dermal respiration. The exogenous T₃ induces the production of serum albumin in the liver, which is subsequently released into the blood stream (Tata 1993). Serum albumin is a TH binding protein which 1) picks up T₃ and transports it to the target organs and cells, and 2) its biochemical and biophysical properties enable the metamorph to fulfill the osmotic and extensive oxygen transport needs in connection with terrestrial living (Frieden 1961; 1968). During these latter metamorphic stages hemoglobin will have an increasingly lower affinity for
oxygen. The younger tadpoles require higher affinities of oxygen as they are limited to the dissolved oxygen in the water (Shi 2000). At later metamorphic stages, it may therefore, become necessary for the tadpole to swim to the surface to collect oxygen there. Limb development did apparently progress faster in the T₃ treatments than the treatments without T₃, however many of the tadpole hindlimbs in the T₃ treatments had little muscle development and the limbs were non-functional. Without the use of the tail, which had significantly resorbed, lack of functional limbs for swimming and the inability to obtain enough oxygen from the water, my observations were that the metamorphic tadpole could not get to the surface for air. Callery and Elinson (2000) also documented death in the anuran, Eleutherodactylus coqui, following longer periods of exposure to T₃. The cause of death, however, was not described. Galton (1992) also discussed the uncoordinated development and death that followed exposure to TH at concentrations exceeding the critical upper limit of a particular stage. Concentrations of T₃ employed in most other studies are approximately 10 to 20 times lower than T₃ levels utilized in this study, therefore, it could be that unnaturally high levels of T₃ may lead to tadpole death. Future work on R. catesbeiana should maintain T₃ levels at concentrations below 1 x 10⁻⁷ M.

When NP was added, there was tail growth in all treatments, but only NP at 936 μg/L caused significantly greater TLRs than the controls. However, there was a significant positive TLR dose-response with increasing nonylphenol concentrations, which suggested some inhibition of the thyroid function in the tadpole. NP is a known estrogen mimic and although the purpose of estrogen in metamorphosis is relatively unknown at present (Hayes 1997; Shi 2000), it can inhibit the function of thyroid hormones. Roth (1948) demonstrated that estradiol inhibits T₄ in Rana temporaria.
Sex steroids, like estrogen and testosterone, may not act directly on a metamorphosing organ, but rather through the hypothalamic-pituitary-thyroid axis leading to a down-regulation of TH in the blood or an up-regulation of TH inhibitors, like prolactin (Gray and Janssens 1990; Hayes 1997). Given that nonylphenol has weak estrogen mimic capabilities (300,000X less potent – Soto et al. 1991; 900,000X less potent - Jobling and Sumptor 1993), it may function in the same way as estrogen in inhibiting TH, but to a far lesser extent. Since TH causes an upregulation of its own mRNA (Yaoita and Brown 1990; Rabelo and Tata 1993; Tata 1993), the introduction of nonylphenol should induce tail growth (or a reduced rate of tail resorption) and a deceleration of progression through metamorphic stages. Although, NP did induce significant tail growth in the absence of T₃, metamorphic stage progression was actually accelerated relative to the control. Because cell proliferation and differentiation in the limbs is controlled by TH (Tata 1996), portions of the differentiation can occur in the absence of TH (Elinson 1994). Therefore, the reasons for the acceleration of limb development in the presence of NP, a suspected TH inhibitor, remains unclear.

In support of my hypothesis, NP did alter metamorphosis. NP significantly decelerated the rate of cranial transformation and tail resorption at 936 μg/L in the presence of T₃. The lower developmental stages observed in the absence of T₃, as mentioned above, are defined by limb development, as tail and cranial changes do not occur until later stages when TH levels are at their peak. The deceleration of tail resorption and cranial transformation stages with increasing NP concentrations in the presence of T₃ could be explained by 1) a possible up-regulation of prolactin counteracting the effects of TH in the higher NP concentrations versus the T₃ control, or 2) a down-regulation in TR mRNA transcription in tail and cranial tissues. Since both cranial transformation and tail resorption require near-peak levels of TH, any inhibition of TH in the system or TR mRNA could retard their respective stage progressions. If TH levels are too high,
limb tissue may not be able to respond to TH as seen in the results of this study where in the presence of exogenous T₃ NP did not cause a significant effect on limb staging rates.

Fort and Stover (1997) found opposite responses of NP exposure to *Xenopus laevis* tadpoles (NF stages 60-66, Nieuwkoop and Faber 1956) where increased tail resorption occurred as nonylphenol concentrations increased. The determined No Observed Effects Level (NOEL) and Lowest Observed Effects Level (LOEL) were 5 µg/L and 10 µg/L, respectively. It is uncertain at this time why Fort and Stover (1997) observed a response to nonylphenol opposite to the results of the present study, however it could be that the effects of nonylphenol are species-specific and/or stage-dependent. Fort and Stover (1997) used metamorphosing tadpoles that already had peak TH levels, which would naturally induce tail resorption. Since high levels of TH already exist in the bloodstream at those stages, any indirect inhibition of TH (e.g. TH production) by NP may not be observed.

The acute toxicity of nonylphenol to aquatic organisms is well-documented (McLeese *et al.* 1981; Brooke 1993; Liber *et al.* 1999b; Lussier *et al.* 2000), however, very few studies have been conducted on amphibians (Fort and Stover 1997; Mann and Bidwell 2000). Preliminary studies using technical grade nonylphenol produced 100% mortality at NP >937 µg/L and 75% mortality at NP >468.5 µg/L after only a 12-hour exposure period using *Xenopus laevis* premetamorphic tadpoles (Christensen, unpublished data). As well, 24-hour and 48-hour LC₅₀s for nonylphenol were calculated using *Rana aurora* (red-legged frog) premetamorphic tadpoles at 310 µg/L and 250 µg/L, respectively (Christensen, unpublished; Appendix C). In the present study *Rana catesbeiana* tadpole 7-day survival rate was not affected by the two lower
concentrations of nonylphenol. At 936 μg/L NP overall survival decreased to only 91.7% at day 7 indicating bullfrogs are a less sensitive species under these conditions.

**Conclusion**

The results of this study indicate that NP is probably acting indirectly to inhibit TH in *Rana catesbeiana* tadpoles. Like estrogen, NP could be acting through the hypothalamic-pituitary-thyroid axis to down-regulate TH levels or up-regulate prolactin levels in the blood resulting in an anti-metamorphic effect. At earlier stages (tadpoles in treatments without exogenous T₃) NP caused significant tail growth and an acceleration of limb development. When exogenous T₃ was added to the treatments, staging progressed significantly. Tail tissue was the most sensitive to T₃, cranial tissue was next most sensitive and limb tissue was least sensitive. This may be due to an upregulation of TH mRNA and receptor number in the tail and cranial tissues, which does not occur in the limb tissues. As well, the addition of exogenous T₃ to treatments would cause T₃ levels in the tadpole to reach beyond peak levels, which would naturally induce tail resorption and cranial transformation, while limb development occurs at lower T₃ levels. NP, in the presence of T₃, caused a significant reduction in both tail resorption and cranial transformation stage ratios, but not in the limb development stage ratios because of the possible inhibitory effects of NP on TH.

The concentration of NP which induced a significant effect in the tadpoles was 936 μg/L. This concentration is not commonly found in the environment. However, with the introduction of a safety factor to act as an ‘umbrella’ for the protection of a wider range of species not yet tested, the concentration becomes environmentally relevant.
Literature Cited


Chapter III - Development and Preliminary Validation of the Amphibian Sperm Inhibition Toxicological Test (ASITT) Method

Introduction

Direct sperm motility inhibition tests are a relatively novel concept in toxicology and are just beginning to be developed for a few aquatic organisms. Environment Canada (1992) developed an echinoderm sperm inhibition bioassay in order to determine the effects of contaminants in the marine environment. The tests are conducted by exposing echinoderm sperm cells to the treatment solution for 10-15 minutes prior to the addition of the eggs (Environment Canada 1992). The assessment is based on the presence or absence of the fertilization envelope surrounding the egg, indicating successful fertilization has taken place (Environment Canada 1992). The effects of numerous chemicals have been tested using this assay (Ghirardini et al. 2001).

Teleost sperm inhibition by chemical contaminants has been tested, although it is not yet a standardized method. Thomas et al. (1998) conducted a study with Atlantic croaker (Micropogonias undulatus) sperm and non-steroidal estrogenic chemicals (Kepone, Zearalenone, o,p'-DDE and 2',4',6'-PCB-4-OH). They hypothesized that these chemicals would compete with 17,20β,21-trihydroxy-4-pregnen-3-one (20β-S) at the 20β-S sperm receptor (steroid receptor), thereby affecting sperm motility, velocity, and angular velocity (RCD1). They found significant displacement of 20β-S with as little as 100nM Zearalenone and Kepone. This subsequently caused significant decreases in percent sperm motility at 200μM, sperm velocity at 1000μM and RCD1 at 200μM relative to the control (Thomas et al. 1998). Those significant results showed the sensitivity of sperm given the incubation time was only 2 to 3 minutes.
The toxicological test emerging from this research, the Amphibian Sperm Inhibition Toxicological Test (ASITT) method, is to act as a supplement to previous research and current assays. ASITT addresses contamination in a freshwater environment using sperm from the African Clawed Frog, *Xenopus laevis*. The endpoints used in ASITT, at this stage of the test’s development, are percent motility, velocity and velocity ratios of sperm. Since sperm motility and motility trajectories have been used for many decades in human fertility clinics as an indicator of reproductive health and fertility in males (Mortimer *et al.* 1997), ASITT will indirectly assess the possible reproductive implications to amphibians in the presence of a chemical contaminant. Amphibians in general, are external breeders (Porter 1972), meaning sperm is expelled into the aquatic environment and then swims to the eggs in order to achieve fertilization. Therefore, sperm can be exposed to any contaminants that exist in the water making sperm motility measurements using ASITT ecologically relevant. Therefore, the introduction of a contaminant to a freshwater system could cause an inhibition in motility via a disruption of cellular processes such as ion exchange, ATP conversion, and cellular respiration vital to successful motility. An inhibition in sperm motility and orientation has previously been associated with decreased fertilization success (Mortimer *et al.* 1997).

In this preliminary validation for ASITT, I tested the effects of zinc ion (Zn$^{2+}$) concentration on *Xenopus laevis* percent sperm motility and motility trajectories. Zinc was chosen because it is naturally found in the semen of many species (e.g. human sperm - Henkel *et al.* 1999). It has been suggested that zinc inhibits cellular respiration in the sperm cell resulting in a cessation of motility (Akberali *et al.* 1985). Therefore, zinc as a contaminant introduced into a freshwater environment may have negative effects on sperm motility and possibly fertilization success in amphibians. Zinc can come from mining where levels in soil can be as high as 11,000 μg/L.
Zinc has also been found in urban runoff in concentrations up to 213 μg/L (Hall and Anderson 1988).

The effects of the ionic constituents, osmolality and pH of the aquatic medium on sperm motility has been extensively studied in a variety of organisms, such as mammals (Si and Okuno 1993), marsupials (Johnston et al. 2000) freshwater fish (Morisawa and Suzuki 1980; Morisawa et al. 1983; Takai and Morisawa 1995; Toth et al. 1997; Krasznai et al. 2000), marine fish (Morisawa and Suzuki 1980; Morisawa et al. 1983; Takai and Morisawa 1995; Stoss 1983), and echinoderms (Gray 1928). Those studies, collectively, demonstrate that the effects of the environment on sperm motility are species-specific as well as dependent on mode of fertilization (e.g. internal vs. external; freshwater vs. marine). However, very few studies exist, in this regard, using amphibian sperm (for exceptions see: Hardy and Dent 1986; Bernardini et al. 1988). Therefore, it was necessary in this study, prior to the initiation of zinc exposures, to delineate effects of the water chemistry characteristics of the aquatic medium on the sperm motility of the test organism, *Xenopus laevis*. This knowledge would enable the development of a control medium that would maximize sperm motility and velocity ratios.

This study tested three objectives. I hypothesized that pH and osmolality would have significant effects on *Xenopus laevis* sperm motility and sperm trajectories, therefore, a control test medium for ASITT treatment exposures would be required to control for those effects. I also hypothesized that multiple phases of *X.laevis* sperm motility would exist under those control conditions. Finally, I hypothesized that Zn$^{2+}$, at environmentally relevant concentrations, would elicit a significant effect on *X.laevis* sperm motility, velocities and trajectories.
Methods

*Xenopus laevis* males were purchased from Xenopus One (Detroit, Michigan) and acclimatized to water and air temperatures of 22°C for >2 weeks prior to use. Frogs were anaesthetized with MS-222 (tricaine methyl sulphonate), dissected and testes were surgically removed. Testes were gently pressed between two glass microscope slides to extract the sperm. Sperm was then stored in DeBoer’s solution (DB: 110 mM NaCl, 1.3 mM CaCl₂, 1.3 mM KCl; Vo and Hedrick 2000) on ice prior to same-day use. The sperm solution had a final osmolality of 226.5 mosmol/L (similar to 220 mosmol/kg for Cane Toad, *Bufo marinus*, sperm; Browne et al. 1998) and a pH of 7.0 (similar to 7.2 pH value for *X.laevis* from Vo and Hedrick 2000). Intact sperm were separated from immature, non-motile sperm and testicular tissue using a micropipette.

Sperm density was determined by adding a sub-sample of 10uL concentrated sperm solution to a vial and bringing the volume up to 1mL with well water. The vial was gently shaken for about 1 minute and the bubbles were allowed to clear (Environment Canada 1992). One drop of the diluted sperm solution was added to a Neubauer Hemacytometer and the sperm was allowed to settle. At 40X magnification, the sperm was counted in the diagonal 4 corner squares and then in the centre square (5 squares total). Sperm density was calculated in the concentrated sperm stock using the methods from the echinoderm sperm inhibition bioassay (Environment Canada 1992). Once sperm density was calibrated, this solution was subsequently termed “sperm solution”.

**Test Solutions**

**pH:** Distilled water solutions were made at various pHs (5.5, 6.0, 6.4, 7.0, 7.4 and 7.8). The pH was adjusted using NaHCO₃ and measured with a pH meter. A 10 μL sub-sample of sperm solution was pipetted onto a glass microscope slide followed by 30 μL of one of the pH test solutions. Osmolarities of final solutions were kept constant at approximately 56.63 mosmol/L. There were 3 replicates for each pH solution.

**Osmolality:** Distilled water at a constant pH of 7.0 was added to 10 μL of sperm solution on a glass microscope slide in increments of 10, 20, 30, 40 and 50 μL to obtain the calculated osmolalities of 113.25, 75.50, 56.63, 45.30 and 37.75 mosmol/L, respectively. There were 3 replicates for each osmolality concentration.

**Zinc:** Zinc test solutions were made by creating a 83.33 mg/L Zn²⁺ stock solution using ZnCl₂ followed by serial dilution to obtain nominal concentrations of approximately 52.08, 104.16, 208.33, 416.65, 833.30 and 1666.60 μg/L. There was one blank solution (control) containing distilled water only. Post-hoc water analysis (Susan Harper, Environmental Engineering, UBC) found actual Zn²⁺ concentrations at non-detectable (N.D.) for the control, and 31, 72, 155, 334, 756 and 1417 μg/L, respectively, for the zinc dilution series. These measured values were used in the data analysis, not the nominal values. Based on results of the pH and osmolality tests, the pH of the zinc test solutions was maintained at pH 7.0 and the osmolality at 56.63 mosmol/L. The ZnCl₂ changed the osmolality of the solution, however, the highest nominal concentration (1666.60 μg/L) only increased the calculated osmolality by approximately 0.04 mosmol/L, which was not enough to induce a change in motility of the sperm (data not shown). A 10 μL sub-sample of sperm solution was placed on a glass microscope slide followed by 30 μL of the zinc test solution. There were a total of 12 replicates for each zinc concentration.

The prepared sperm solutions were videotaped under 400X magnification with an Axiovert 135 microscope and the Hitachi KP-C550 CCD Color Camera for four minutes at 30 frames per
second. The initial video playback was conducted on a standard television with a final
magnification of 1718X. However, due to laboratory relocation proceeding the lab work, it was
necessary to analyze the remaining videos (Zinc exposures) on a NextCube computer monitor
where the final magnification was 1375X. The accuracy of the VCR timer was obtained by
observing whether one second of time on the clock/timer corresponded to 30 frames when
played back frame by frame.

**Motility Analysis**

Sperm were assessed as either motile or non-motile for the entire four-minute period. Motile
sperm was further classed as idle, forward progressive (FP), transition phase (TP) and
hyperactivated (HA) phases, which are phases defined for human sperm motility phases
(Mortimer and Swan 1995a,b; Mortimer et al. 1996). FP, TP and HA were used to describe the
*X.laevis* sperm motility phases in this study, since flagellar waves and motility patterns (as
described in Mortimer and Swan 1995a,b; Mortimer et al. 1996) appeared similar following a
preliminary video analysis. Video analysis was based on one observer.

**Sperm Maps and Track Analysis**

For pH and osmolality tests, as many sperm as possible were mapped in the progressive and
hyperactivation phases, while only a maximum of 10 progressive sperm were mapped in the
zinc tests per four-minute video. Once it was determined that there was a one second track of
video of a sperm’s motility, the tape was played frame by frame for 30 frames (one second)
plotting the position of the tip of the sperm head using transparent acetate sheets (pH and
osmolality tests) or NextCube computer-generated x,y co-ordinates (zinc tests). As previously
mentioned, due to a change in lab location it was necessary to analyze the zinc exposure videos
using the NextCube computer. Plots on the acetates were later converted into x,y co-ordinates
using a standard ruler with a precision of 0.5 mm. All methods for calculating velocities and velocity ratios were obtained from Mortimer and Mortimer (1990), except average path velocity (VAP), which was a method developed by the author. Curvilinear velocity (VCL - µm/s) was determined by adding up the distances between each x,y co-ordinate (Figure 9). Straight line velocity (VSL - µm/s) was determined by calculating the straight line distance between the first and last x,y co-ordinate. The average path velocity (VAP - µm/s) was determined by calculating the distance between the first point, 9 intermediate points (the average x,y co-ordinate for groups of three points from points 2-28) and the last point, leaving a total of eleven points. VCL, VSL and VAP values were then corrected for magnifications based on the respective monitor used for tracking. The ratios of linearity [LIN = (VSL/VCL) X 100%], straightness [STR = (VSL/VAP) X 100%] and wobble [WOB = (VAP/VCL) X 100%] were also calculated for each sperm map.
Figure 9. An example of a sperm map at 30 frames per second. Open circles are the x,y co-ordinates determined by frame by frame video analysis. Solid line denotes curvilinear path from time 0 to 1 second. Dashed line represents the straight line path. Dotted line symbolizes the average path.
Statistics

Percent motile was analyzed in the pH and osmolality tests using ANOVA (SAS, version 8.2). Alpha (α) was set at 0.05 for all tests. Differences between treatments were determined using orthogonal contrasts. Linear regression analysis was used to analyze percent motility relative to zinc concentrations. Responses of velocities (VCL, VSL, and VAP) and velocity ratios (LIN, STR, and WOB) to treatments were analyzed using linear regression. The differences between sperm motility phases (FP, TP and HA) in the control solution were analyzed with ANOVA (α<0.05) and orthogonal contrasts. Values in text are displayed as means ± 1 standard deviation (SD).

Results

Effects of pH on sperm motility

The pH significantly affected total percent sperm motility (F_{5,12} = 3.08, P = 0.051; Figure 10). All pH treatments were significantly different from pH 7.0 (P<0.05) which had the highest motility (55.27 ± 8.46 %). The pH did not significantly affect percent progressive or hyperactivated sperm (data not shown). VCL significantly increased as pH increased in a linear dose response (F_{1,10} = 4.22, P = 0.067). However, the other progressive sperm velocities (VSL and VAP) were not significantly affected by pH (Figure 11), nor were the velocity ratios, LIN and STR (Figure 12). WOB decreased significantly with increasing pH (F_{1,10} = 3.59, P = 0.087).
Figure 10. Effect of pH on total motility (%) of Xenopus laevis sperm. Error bars are 1 SD of the mean. Asterisks indicate significant differences (P<0.05) from pH 7.0.
Figure 11. The effect of pH on *X. laevis* sperm velocities. The solid line indicates a significant regression for VCL (slope=8.5, $R^2=0.30$, $P=0.067$). • denotes VCL, ○ depicts VSL, and ▼ represents VAP.
Figure 12. Effect of pH on *X. laevis* sperm velocity ratios. Solid line indicates a significant regression (slope=-6.40, $R^2=0.26$, $P=0.087$) for WOB. • denotes LIN, ○ depicts STR, and ▼ represents WOB.

Effects of osmolality on sperm motility

Percent sperm motility was significantly affected by a change in osmolality ($F_{4,10}=3.04$, $P=0.07$; Figure 13). Total percent motility was highest at 56.63 mosmol/L, however only the mean motilities at 37.75 ($F_{1,10}=5.18$, $P<0.05$) and 113.25 mosmol/L ($F_{1,10}=9.78$, $P<0.05$) were significantly lower than at 56.63 mosmol/L. Progressive motility at 113.25 mosmol/L was significantly different from that at 56.63 mosmol/L ($F_{1,10}=4.54$, $P=0.06$). Hyperactivated sperm was not significantly affected by osmolality (data not shown).
Figure 13. Effects of osmolality on percent sperm motility in *X. laevis*. ● denotes total motility, ○ represent progressive motility. Asterisks indicate significant differences from osmolality 56.63 mosmol/L (P<0.05 for total motility and P=0.06 for progressive motility). Error bars are 1 SD of the mean.

VCL had a significant quadratic response to changes in osmolality (F$_{2,11} = 8.75$, P < 0.01) with the peak at 75.50 mosmol/L (41.42 ± 5.23 µm/s), although the mean VCL at 56.63 mosmol/L was similar at 39.24 ± 5.67 µm/s (Figure 14). The VSL was also significantly affected by osmolality in a quadratic response (F$_{2,11} = 6.13$, P < 0.05) with a predicted peak at approximately 66 mosmol/L. Mean VSL values for 56.63 and 75.50 mosmol/L were 9.68 ± 2.75 and 9.62 ± 1.15 µm/s, respectively. The lowest VSL occurred at 113.25 mosmol/L (4.40 ± 1.68 µm/s). The peak to the significant quadratic response for VAP to osmolality occurred at approximately 66 mosmol/L. The mean VAPs were similar for 56.63 and 75.50 mosmols/L at

48
27.65 ± 5.23 and 27.23 ± 1.22 μm/s, respectively. Again, the lowest VAP occurred at 113.25 mosmol/L (10.06 ± 5.89 μm/s).

Figure 14. Effect of osmolality on *X. laevis* sperm velocities. • denotes VCL, ○ depicts VSL, and ▼ represents VAP. Solid line denotes the significant quadratic response of VCL to osmolality ($R^2=0.61$, $P<0.01$). The significant quadratic response of VSL is represented by the dashed line ($R^2=0.53; P<0.05$). The dotted line signifies a significant quadratic response of VAP to osmolality ($R^2=0.71; P<0.005$).

There was a significant negative linear effect on LIN with osmolality ($F_{1,12} = 11.74$, $P < 0.005$; Figure 15). STR was not significantly affected by osmolality. WOB was highest in the 37.75 mosmol/L treatment (46.53 ± 9.37 %) and lowest in the 113.25 mosmol/L treatment (28.08 ±
6.64%). There was a significant negative linear response of WOB to increasing osmolality ($F_{1,12} = 14.89, P < 0.005$).

![Figure 15](image.png)

**Figure 15.** Effect of osmolality on *X.laevis* sperm velocity ratios. • denotes LIN, ○ depicts STR, and ▼ represents WOB. The solid line denotes a significant negative linear response of LIN to increasing osmolality (slope= -0.09, $R^2=0.49$, $P<0.005$). The dotted line represents the significant linear response of WOB (slope= -0.30, $R^2=0.55$, $P<0.005$).

**Xenopus laevis sperm motility characterization**

Four distinct motility phases existed in *X.laevis* activated sperm in pH 7.0, osmolality 56.63 mosmol/L: idle, forward progressive, transition phase and hyperactivated (Figure 16). Idle sperm (data not shown) are characterized by a vibrating motion with no progression and the flagellum eliciting no propulsion. Idle sperm predominated the proportion of total motile sperm.
in this study (data not shown). Forward progressive sperm moved forward (Figure 16) and had generally lower VCL (55.48 ± 13.10 μm/s), higher VSL (12.53 ± 6.62 μm/s) and lower VAP (20.62 ± 10.15 μm/s; Figure 17). This in turn, made higher LIN (22.16 ± 11.47 %), higher STR (60.07 ± 22.55 %) and lower WOB (36.74 ± 15.13 %; Figure 18). Transition phase sperm progressed forward (Figure 16), but flagellar movement had higher amplitude resulting in greater oscillations of head movement. TP sperm had medium to higher VCL (71.29 ± 18.31 μm/s), lower to medium VAP (12.14 ± 5.31 μm/s) and lower to medium VSL (33.11 ± 10.95 μm/s; Figure 17) generating medium LIN (16.90 ± 5.26 %), medium STR (37.09 ± 12.23 %) and increased WOB (46.77 ± 9.49 %; Figure 18). Forward progressives and transition phase sperm had significantly different VCL (P<0.05), VAP (P<0.01) and STR (P<0.005). Hyperactivated sperm have erratic flagellar movements resulting in erratic head movements that are not in a progressive direction, but rather in a “star-spin” (Mortimer and Swan 1995b; Figure 16). Although increased flagellar motion does not necessarily mean that the head will move, HA sperm generally had high VCL (74.31 ± 27.79 μm/s), very low VSL (9.92 ± 5.64 μm/s) and high VAP (40.74 ± 8.79 μm/s; Figure 17). This resulted in low LIN (14.61 ± 9.73 %), low STR (25.05 ± 15.61 %) and high WOB (57.48 ± 10.21 %; Figure 18). Hyperactivated sperm had significantly different VAP (P<0.05), STR (P<0.05) and WOB (P<0.05). VSL and LIN values remained very low for all three motility phases and were not significantly different between any of the motility phases.
Figure 16. Examples of sperm tracking maps illustrating three motility phases in *Xenopus laevis* sperm. Tracks are based on 30 points over a one second period.
Figure 17. a) Curvilinear velocities, b) Straight line velocities and c) Average path velocities for the various *X.laevis* sperm motility phases (FP=forward progressive; TP=transition phase; HA=hyperactivated). Error bars are 1 SD of the mean.
Figure 18. a) Linearity, b) Straightness and c) Wobble in the three motility phases of *Xenopus laevis* (FP=Forward Progressive; TP=Transition Phase; HA=Hyperactivated). Error bars are 1 SD of the mean.
Preliminary Validation of ASITT using Zinc

$Zn^{2+}$ concentration significantly affected total percent motility in a negative linear dose response ($F_{1,78} = 19.39, P < 0.0001$; Figure 19). In the control solution total percent motility was $57.78 \pm 11.63\%$, decreasing to $36.73 \pm 6.73\%$ in the highest $Zn^{2+}$ treatment.

![Figure 19. The effect of $Zn^{2+}$ on total sperm motility in *Xenopus laevis*. Solid line denotes a significant negative linear regression (Slope= -0.01, $R^2=0.20, P<0.0001$).](image)

The percentage of progressives (arcsin square-root transformed) showed a negative linear dose response ($F_{1,78} = 4.12, P < 0.05$) when exposed to increasing $Zn^{2+}$ concentrations (Figure 20). The highest $Zn^{2+}$ treatment had $19.11 \pm 5.86\%$ progressive motility, while the control solution had $27.94 \pm 7.91\%$. The percent hyperactivated sperm showed no significant difference among varying concentrations of $Zn^{2+}$ (data not shown).
Figure 20. Effect of Zn$^{2+}$ on percent progressive sperm motility in *X.laevis*. Solid line indicates a significant negative linear dose response (Slope= -0.00004, R$^2$=0.05, P<0.05).

Increasing Zn$^{2+}$ concentrations induced a significant positive linear response in VSL (F$_{1,78}$ = 3.99, P < 0.05; Figure 21). Control solutions had a mean VSL of 14.41 ± 3.09 μm/s, while at the highest concentration Zn$^{2+}$ VSL was 15.58 ± 3.71 μm/s. VCL and VAP were not significantly affected by Zn$^{2+}$ concentrations (data not shown). Zn$^{2+}$ concentrations also did not elicit a significant effect on LIN, STR or WOB.
Figure 21. Effect of Zn\(^{2+}\) on the straight line velocity of *Xenopus laevis* sperm. Solid line indicates a significant positive linear response (Slope=0.006, \(R^2=0.05\), \(P<0.05\)).

Discussion

As was hypothesized, both pH and osmolality had significant effects on *X.laevis* sperm motility. The control solution emerging from the pH and osmolality tests was 7.0 pH and 56.63 mosmol/L, which optimized sperm motility. Three *X.laevis* sperm motility phases previously described in human sperm (Mortimer and Swan 1995a,b) were apparent in the control solutions; forward progressive (FP), transition phase (TP) and hyperactivated (HA). These phases had characteristic velocities (VCL and VAP) and velocity ratios (STR and WOB) as was hypothesized (VSL and LIN were not significantly different among motility phases). As was also predicted, Zn\(^{2+}\) concentrations had a significant effect on *X.laevis* sperm motility.
Effects of pH on *X. laevis* Sperm Motility

The low motility observed in the acidic treatments may be due to the H+ influx via the Na+/H+ channels in the sperm membrane. This could result in sperm motility inhibition in *X. laevis*, as has been observed in other work when the cytoplasm acidifies (Schackmann *et al*. 1981). This inhibition of motility has been previously attributed to the inactivation by low pH of the dynein protein on the flagella which normally hydrolyses adenosine triphosphate (ATP) to adenosine diphosphate (ADP) stimulating mitochondrial respiration and sperm motility (Longo 1997). Depending on the species, dynein may be able to function at a range of pHs. In sea urchins, dynein was inactive below a pH of 7.3 (Longo 1997). Hardy and Dent (1986) observed that pH < 7.5 inhibited sperm motility in red spotted newt, *Notophthalmus viridescens*.

*Xenopus laevis* sperm motility also decreased at a high pH. If the pH becomes too alkaline, it may inhibit cyclic guanosine monophosphate (cGMP) synthesis, which deactivates K+ channels causing a membrane depolarization (Cook *et al*. 1994). This would in turn activate Ca2+ channels, causing a Ca2+ influx. It has been proposed that at these higher intracellular Ca2+ concentrations, Ca2+ is exchanged for mitochondrial H+ (Longo 1997). This ion exchange would result in higher mitochondrial levels of Ca2+ and higher levels of cytoplasmic H+, deactivating dynein resulting in a decrease in ATP conversion.

In this study, VCL increased with increasing pH indicating a somewhat stimulatory effect of pH on motility velocity, however an increase in velocity will require greater energy reserves, which are low in acidic pHs where dynein is unable to convert ATP to ADP. This was demonstrated by the low motility observed in the acidic treatments in this study. As pH increases to neutral levels, intracellular Ca2+ concentrations increase, increasing flagellar bending (Brokaw 1987; Boitano and Omoto 1992; Cook *et al*. 1994; Detweiler and Thomas 1998), which would
subsequently increase VCL. As pH continues to increase, Ca$^{2+}$ reaches inhibitory levels leading to cytoplasmic acidification, again deactivating the dynein protein required for continued energy supply to the sperm. Low percent motility was observed in the higher pH treatments lending weight to this theory. Okuno and Morisawa (1989) also found that high concentrations of Ca$^{2+}$ ($10^{-7}$ M) were inhibitory to sperm motility. As well, an inhibition of axonemal bending have been attributed to high concentrations of intracellular Ca$^{2+}$ (Okuno and Morisawa 1989), which may explain why WOB decreased significantly, as well, with increasing pH.

Effects of Osmolality on *X.laevis* Sperm Motility

Dilution of the stock sperm solution, containing Na$^+$, Cl$^-$, Ca$^{2+}$, and K$^+$ ions, in this study was to mimic sperm expulsion into the freshwater aquatic environment in natural systems. Sperm is immotile in a medium that is isotonic with seminal plasma and becomes activated once diluted with hypotonic solution in freshwater species (Morisawa and Suzuki 1980; Morisawa et al. 1983; Takai and Morisawa 1995; Toth et al. 1997; Krasznai et al. 2000). Hypo-osmotic conditions changes the membrane potential of the sperm cell, consequently leading to a change in the intracellular ion concentration (Krasznai et al. 1995). In accordance with previous research, the higher osmolality treatment, which is closest to an isotonic state with the *X.laevis* sperm cells, inhibited sperm motility. As the solution approaches an isotonic state with the sperm cell the osmotic pressure nears zero. Therefore, there would be little to no ion or water exchange across the cell membrane, thereby preventing the activation of sperm motility.

KCl was also included in the stock solution, and the associated K$^+$ ion concentration increase may have contributed to the low motility observed, as it is the K$^+$ efflux, that is thought to be the first trigger of ionic processes associated with motility. High extracellular K$^+$ concentrations have previously been shown to inhibit sperm motility (Gallis et al. 1991; Toth et al. 1997).
It is also possible that external osmolality changes are the first trigger of sperm motility, rather than extracellular K$^+$ concentration since Krasznai et al. (2000) found that using a non-electrolytic isotonic solution could suppress sperm motility in the common carp. Takai and Morisawa (1995) could induce inhibition and activation in sperm using mannitol, a non-electrolyte solution, and dilutions of mannitol, respectively, indicating that osmolality alone is the factor affecting sperm motility initiation in zebrafish (*Brachydanio rerio*).

All velocities were low in high osmolality treatments most likely due to the lack of ion exchange necessary for sperm motility activation. WOB and LIN were lowest in the high osmolality treatments because the ratio between VAP and VCL, and VSL and VCL, respectively decreased. Usually a decrease in WOB, would coincide with an increase in LIN, which did not occur in this study. This suggests that in an isotonic solution motility is due to the inactivation in the preliminary stages, rather than in the cellular or ionic processes that occur to maintain motility and control trajectories.

Since Ca$^{2+}$ and Na$^{2+}$ are necessary for the activation of sperm motility, a very hypotonic solution may not have high enough concentrations of those ions resulting in lower motility, which was observed in this study. Toth et al. (1997) also found that a minimum concentration of ions was necessary for motility in lake sturgeon, *Acipenser fulvescens*, sperm motility. All velocities were low in the hypotonic solution, which could be attributed to an inhibition of a Ca$^{2+}$ influx. Calcium is thought to stimulate the synthesis of cyclic adenosine monophosphate (cAMP), which results in the initiation of flagellar motility (Morisawa and Okuno 1982), so if Ca$^{2+}$ levels are low in the solution, flagellar movement may not occur. Cook et al. (1994) proposed that low Ca$^{2+}$ intracellular concentrations induced linear motility, while high Ca$^{2+}$ induced erratic movement and turns. Since LIN was high in low osmolality treatments, it suggests that
intracellular Ca\(^{2+}\) concentrations are low, most likely attributed to the low availability of Ca\(^{2+}\) in the solution. The high WOB observed in the low osmolality treatments was not expected, but it is attributed to the low velocities, which would cause erratic head movements with very little flagellar movement thereby eliciting low progressive motility. It was observed that low osmolality had low progressive motility lending support to this theory. A hypotonic solution will create high osmotic pressure, which may result in a water influx into the cell causing cellular and flagellar swelling. Both types of swelling were observed in this study (data not shown) and may have affected motility.

**Characterization of X.laevis Sperm Motility Phases**

In the control solution treatments the majority of motility was in the “idle” state, which is just a vibratory motion of the sperm head without any flagellar movement or progressive motility. TP sperm occurred most frequently, because it appears that this phase lasts the longest temporally. HA sperm were least frequent. Human spermatozoa also have only a small proportion of motile sperm in a hyperactivated state at any one time, perhaps because only certain sperm are capable of reaching this state (Mortimer and Swan 1995b). Alternatively, perhaps the increased velocities associated with a hyperactivated state deplete energy resources, so sperm observed in this state will not continue in this state for very long, either becoming immotile or reverting back to a progressive phase. Progressive sperm may enter the HA phase, perhaps due to increasing intracellular Ca\(^{2+}\) concentrations which facilitate flagellar bending, replacing the HA that become immotile or in TP or FP phases.

A previous characterization of *X. laevis* sperm motility noted high VCL, low VSL, lower VAP, very low LIN, and medium percent motility (Van Der Horst et al. 1995). Likewise, I found low LIN values, similar to Van Der Horst *et al.* (1995), who found *X.laevis* sperm LIN to be only
41.3% and attributed it to the corkscrew-like sperm structure. Amphibians with a straight rod-like structure had higher LIN values of 67.6%, as seen in *Bufo* sp. sperm.

There were very few previous studies characterizing *X. laevis* sperm in which to compare this study’s results. Van Der Horst *et al.* (1995) did not report specific VCL or VSL values for *X. laevis*, but the group of species that were identified as “aquatic fertilizers”, which contained *X. laevis*, had VCLs in the range of 20 to 31 μm/s and VSL ranging from 8 to 23 μm/s VSL. They did not report the VAP value for the aquatic fertilizer group, nor for *X. laevis* specifically. The results of this study found VCL values of 50 to 70 μm/s when FP and TP were grouped. The higher VCL in this study may be due to a difference in osmolality of the solution, or differences in pH (the pH used in Van Der Horst *et al.* 1995 was not stated). The two phases of progressive motility were not separated in Van Der Horst *et al.* (1995), and they also did not characterize the hyperactivation motility phase.

**Effects of Zn$^{2+}$ on *X. laevis* Sperm Motility**

Zinc occurs in high concentrations in seminal plasma in some organisms (Akberali *et al.* 1985). Increasing Zn$^{2+}$ caused a significant decrease in percent total motility and progressive motility in this study. Earnshaw *et al.* (1986) also observed a respective decrease in sperm motility in the marine mussel, *Mytilus edulis* (L.) with increasing Zn$^{2+}$ concentrations, with a maximum inhibition at 3mM Zn$^{2+}$ of approximately 45% compared with to the controls. Arver and Eliasson (1980) demonstrated that Zn$^{2+}$ also elicited a significant inhibitory effect on acrosin activity, which suggested the ability of Zn$^{2+}$ to penetrate the sperm membrane. Earnshaw *et al.* (1986) exposed the sperm of the marine mollusc, *Mytilus edulis* (L.), to Zinc [Zn(NO$_3$)$_2$] and found that zinc-treated sperm had a higher incidence of mitochondrial structural damage (in some instances, granules) than in the untreated sperm.
Zinc can inhibit cellular respiration possibly at the level of glycolysis where there is an inhibition of carboxylase activity because Zn\(^{2+}\) binds to the sulphydryl groups (Stoppani et al. 1953) or through inhibition of the electron transport chain of mitochondria (Akberali and Earnshaw 1982). Earnshaw et al. (1986) found a strong positive correlation with his work on sperm motility in the marine mollusc, *M. edulis*, and previous work on cellular respiration, suggesting that inhibition of oxidative phosphorylation by heavy metals reduces the ATP supply available for the contractile process. This parallels this study's results in that VSL significantly increased with increasing Zn\(^{2+}\) concentrations, since flagellar contraction may be inhibited. Zinc can also increase passive permeability in the mitochondria and other organelle membranes to ions in general, with most focus on Ca\(^{2+}\) and K\(^{+}\). Calcium then would more easily flow into the cell and has been suggested as playing a major role in the acrosome reaction and sperm motility (Shi 2000). The treatment of Zn\(^{2+}\) could potentially then facilitate an increased uptake of Ca\(^{2+}\) by the cell stimulating motility initially. Again, once Ca\(^{2+}\) concentrations get too high in the cell, motility is inhibited most probably by the resulting cytoplasmic acidification. This, in addition to Zn\(^{2+}\) having a possible negative effect on cellular respiration, will result in decreased motility, as seen in these results with the decrease in both percent total and progressive motility at higher Zn\(^{2+}\) concentrations. The initial stimulatory effect of Zn\(^{2+}\) on sperm motility may explain why Zn\(^{2+}\) may have less of an inhibitory effect overall (Earnshaw et al. 1986).
**Further Development and Validation of ASITT**

An important process in the development of a novel bioassay is its reproducibility among laboratories. In the case of ASITT, an experiment is currently underway to test observer variability and accuracy in sperm tracking methods employed for this study. Although ASITT needs method adjustment and further validation, this study provides the basis to begin these necessary steps in its development. As well, optimal assay conditions and *X. laevis* sperm motility phases have been identified.

Further research should focus on non-lethal amphibian sperm collection, and the effects of various chemicals on fertilization success, sperm cell membrane integrity, sperm-to-egg binding affinity, and chromosomal damage in amphibians. Further validation will also be necessary with other contaminants. Eventually, the effects of contaminants on sperm motility of other amphibian species may be warranted to address contaminant issues with native species.

**Conclusion**

*Xenopus laevis* sperm are required to swim through the aquatic environment to reach the eggs. Therefore, it is vital that motility of sperm occurs in order for a successful fertilization event. While successful fertilization is easy to identify in amphibian eggs, ASITT could be a key method for identifying the mechanisms when fertilization is not successful. An inhibition of motility may have direct reproductive implications to amphibians. As indicated by the results of this study, the pH and osmolality of the aquatic environment significantly affected percent sperm motility in *X. laevis*, as well as some of the velocities and trajectories. Measurement of physical components of the trajectory of the sperm such as VCL and LIN provided indices of pattern, which may be more robust indicators of the fertilizing capacity (Toth *et al* 1997). These trajectories were used along with percent motility to validate the ASITT method using Zn$^{2+}$. 
Zinc, which caused an increase in VSL, indicated that flagellar movement is being inhibited. This result suggests that ASITT may also help identify disruption of cellular processes via changes in motility trajectories, therefore, it may be easier to predict where the contaminant may be causing a disruption within the cell. This preliminary validation with Zn$^{2+}$, a known sperm motility inhibitor, demonstrated that ASITT’s endpoints are sensitive enough to observe a measurable response.
Literature Cited


Chapter IV – Conclusions

It is necessary to establish dose-response data to assess the health risks associated with the over 2,000 chemicals produced annually to address the continued concern for public safety and wildlife health (NIEHS 1997). Government, industry and scientists continue to seek new methods in toxicity testing that are less costly and time consuming, that incorporate a new understanding of toxic mechanisms, that evaluate important endpoints not previously considered, and that improve prediction of the potential toxic effects of chemicals and other agents (NIEHS 1997). Presently, toxicity tests to assess potential health risks are accomplished largely by using laboratory animals. There is an emphasis in future research for novel toxicity tests that would involve a reduction in the number of animals in a test, a refinement of procedures to make them less painful or stressful, replacement of animals with non-animal systems, or replacement of one animal species with another that is lower on the phylogenetic scale (NIEHS 1997).

This thesis explores the effects of two contaminants on various portions of the life cycle of amphibians: metamorphosis and reproduction. In Chapter II, I examined the effects of nonylphenol on metamorphic staging and tail resorption in *Rana catesbeiana* tadpoles. The exposure period for that study was seven days in which complications could have arisen in amphibian husbandry, bacterial contamination, animal stress/handling and in the frequent water changes in any of the 27 treatment containers. In short, that study was intense, both in the fact that it had a large work effort and that there was a lot of room for experimental complications, which could affect the outcome of the results despite the many attempts one may make to avoid such complications. As well, the 108 tadpoles were exposed to various nonylphenol, methanol, dimethyl sulfoxide, and T₃ treatments where the outcome could have resulted or had resulted in
physiological dysfunction, behavioural changes, growth retardation, developmental retardation, metamorphic inhibition, physical abnormalities, organ dysfunction, abnormal hormone regulation, pain and ultimately death. Many bioassays that are presently used in the field of amphibian toxicology operate in this manner to determine chronic and acute effects of contaminants, both lethal and sub-lethal. This was the main rationale behind the research described in Chapter III – The Amphibian Sperm Inhibition Toxicological Test (ASITT) Method. Therefore, some objectives for the development of ASITT were to develop a bioassay that could minimize the work effort, the experimental error and the suffering of the test organisms. It took one day to collect the data per three to four replicates of all treatment exposures. Manually analyzing the videos was time consuming, however, with the introduction of the computer aided sperm analyzer (CASA) into the methods, ASITT could collect innumerous sperm motility data in a very short time, without observer bias, allowing for the researcher to have many replicates. Unfortunately, I had to sacrifice a total of 9 adult *Xenopus laevis* males to collect all the data in Chapter III. However, in comparison to the 108 potential *Rana catesbeiana* juveniles that were sacrificed in Chapter II, 9 seems diminutive. As well, future research for the further development of ASITT will be focussed on non-lethal amphibian sperm collection creating an entirely non-lethal bioassay. Although it is important to observe the effects of contaminants on different portions of the life cycle, in different species and in a variety of cell types, it may be more important to focus the future research of wildlife toxicology to less harmful methodology while still obtaining valuable data.

It is also interesting to note that toxicity tests that measure cellular damage, caused by a contaminant, normally extrapolate the effects to aspects of the individual’s fitness. This was the case in Chapter II with the tadpoles that were exposed to nonylphenol, which may have indirectly inhibited thyroid hormones and therefore, natural metamorphic progression. In a
natural setting, a disruption in the progression of metamorphosis may mean the difference
between survival and death, with all of the ecological consequences of delayed or accelerated
metamorphosis. In terms of individual fitness being affected, it may be difficult to extrapolate
these effects to the population level. With ASITT, however, the cellular damage incurred by the
sperm cell from the contaminant, is less associated with individual fitness, but rather with
fertilization success, hatching success and recruitment to the population.

Despite the potential value either of these toxicological data provide, it is still important to
remember that extrapolation from a laboratory into natural environments should still be made
with caution.
Literature Cited

Appendix A

Comparison of Gosner (1960 – anurans, 46 stages, fertilization to metamorphic completion) stages to those of Neiuwkoop and Faber (1956 – *Xenopus laevis*, 66 stages, fertilization to metamorphic completion) and Taylor and Kollros (1946 – 25 stages, hind limb bud appearance to metamorphic completion).

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<td>XVIII-XIX</td>
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<td>XXIII-XXIV</td>
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</tr>
</tbody>
</table>
# Appendix B

## Alphabetical list of acronyms used throughout this document.

- **ADP** - Adenosine Diphosphate  
- **ANOVA** - Analysis of Variance  
- **APE** - Alkylphenol Ethoxylate  
- **ASITT** - Amphibian Sperm Inhibition Toxicological Test  
- **ATP** - Adenosine Triphosphate  
- **CASA** - Computer Aided Sperm Analyzer  
- **DMSO** - Dimethyl Sulfoxide  
- **DO** - Dissolved Oxygen  
- **EC₅₀** - Effect Concentration (causing 50% of individuals to express effect)  
- **EDC** - Endocrine Disrupting Chemical  
- **ER** - Estrogen Receptor  
- **FP** - Forward Progressive  
- **GBEI** - Georgia Basin Ecosystem Initiative  
- **GLM** - General Linear Model  
- **HA** - Hyperactivated  
- **HR** - Head (Cranial Transformation) Ratio  
- **LC₅₀** - Lethal Concentration (causing 50% mortality)  
- **LIN** - Linearity  
- **LOEL** - Lowest Observed Effects Level  
- **LR** - Limb (Development) Ratio  
- **ND** - Non-detect  
- **NIEHS** - National Institute of Environmental Health Sciences  
- **NF** - Nieuwkoup and Faber  
- **NOEL** - No Observed Effects Level  
- **NP** - Nonylphenol  
- **NPE** - Nonylphenol Ethoxylate  
- **PESC** - Pacific Environmental Science Centre  
- **PROC** - Procedure  
- **SD** - Standard Deviation (of the mean)  
- **STP** - Sewage Treatment Plant  
- **STR** - Straightness  
- **T₃** - 3,3',5-triiodothyronine  
- **T₄** - 3,3',5,5'-tetraiodothyronine (or thyroxine)  
- **TH** - thyroid hormones  
- **T-K** - Taylor-Kollros  
- **TLR** - Tail Length Ratio  
- **TP** - Transition Phase  
- **TR** - Tail (Resorption) Ratio  
- **TSRI** - Toxic Substances Research Initiative  
- **TWR** - Tail Width Ratio  
- **UBC** - University of British Columbia  
- **USEPA** - United States Environmental Protection Agency  
- **VAP** - Average Path Velocity  
- **VCL** - Curvi-Linear Velocity  
- **VSL** - Straight Line Velocity  
- **VTG** - Vitellogenin  
- **WOB** - Wobble
Appendix C

a) Unpublished 24 hour LC50 data for nonylphenol on Red-Legged Frogs (Rana aurora)

Sample No. Nonylphenol 24 hour without T3  Bioassay Lab
29-May-01

<table>
<thead>
<tr>
<th>Conc. (M)</th>
<th>Number Exposed</th>
<th>Number Dead</th>
<th>% Dead</th>
<th>Binomial Prob (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.782E-5</td>
<td>15</td>
<td>15</td>
<td>100</td>
<td>0.30516E-2</td>
</tr>
<tr>
<td>0.196E-5</td>
<td>15</td>
<td>7</td>
<td>46.6667</td>
<td>50</td>
</tr>
<tr>
<td>0.978E-6</td>
<td>15</td>
<td>3</td>
<td>20</td>
<td>1.75781</td>
</tr>
<tr>
<td>0.489E-6</td>
<td>15</td>
<td>6</td>
<td>40</td>
<td>30.3619</td>
</tr>
</tbody>
</table>

At a confidence level of 95 percent the binomial test shows that the LC50 is below 0.782E-5

An approximate LC50 of 0.208703E-5 is obtained by non-linear interpolation between 0.196E-5 and 0.782E-5

---Results calculated using the moving average method---

<table>
<thead>
<tr>
<th>Span</th>
<th>G</th>
<th>LC50</th>
<th>95 Percent confidence limits</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>0.498757</td>
<td>0.193862E-5</td>
<td>0.966921E-6 0.837104E-5</td>
</tr>
<tr>
<td>2</td>
<td>0.134819</td>
<td>0.198225E-5</td>
<td>0.134624E-5 0.277603E-5</td>
</tr>
</tbody>
</table>

An LC50 calculated using the moving average method may not be a very good estimate if the span is much less than the number of concentrations.

If any higher concentration produces a lower percent dead than a lower concentration, the confidence limits obtained by the moving average method will probably be too close.

---Results calculated using the Probit method---

<table>
<thead>
<tr>
<th>Iterations</th>
<th>G</th>
<th>H</th>
<th>Chi-Square</th>
<th>Probability</th>
</tr>
</thead>
<tbody>
<tr>
<td>11</td>
<td>5.28336</td>
<td>46.1085</td>
<td>92.2171</td>
<td>less than 0.001</td>
</tr>
</tbody>
</table>

Because the probability is less than 0.05, results calculated using the probit method probably should not be used.

Slope = 1.77465
95 Percent Confidence Limits = -2.30448 and 5.85377

LC50 = 0.140992E-5
95 Percent Confidence Limits = 0 and +infinity

Compare results with original data to see if they are reasonable.
b) Unpublished 48 hour LC50 data for nonylphenol on Red-Legged Frogs (Rana aurora)

Sample No. Nonylphenol 48 hour without T3 Bioassay Lab
29-May-01

<table>
<thead>
<tr>
<th>Conc. (M)</th>
<th>Number Exposed</th>
<th>Number Dead</th>
<th>% Dead</th>
<th>Binomial Prob (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.782E-5</td>
<td>15</td>
<td>15</td>
<td>100</td>
<td>0.30516E-2</td>
</tr>
<tr>
<td>0.196E-5</td>
<td>15</td>
<td>9</td>
<td>60</td>
<td>30.3619</td>
</tr>
<tr>
<td>0.978E-6</td>
<td>15</td>
<td>4</td>
<td>26.6667</td>
<td>5.92346</td>
</tr>
<tr>
<td>0.489E-6</td>
<td>15</td>
<td>6</td>
<td>40</td>
<td>30.3619</td>
</tr>
</tbody>
</table>

At a confidence level of 95 percent the binomial test shows that the LC50 is below 0.782E-5

An approximate LC50 of 0.159776E-5 is obtained by non-linear interpolation between 0.978E-6 and 0.196E-5

---Results calculated using the moving average method---

<table>
<thead>
<tr>
<th>Span</th>
<th>G</th>
<th>LC50</th>
<th>95 Percent confidence limits</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>0.436662</td>
<td>0.137662E-5</td>
<td>0.624033E-6 0.3247E-5</td>
</tr>
<tr>
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<td>0.161735E-5</td>
<td>0.986663E-6 0.231277E-5</td>
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<td>1</td>
<td>1.19893</td>
<td>0.159776E-5</td>
<td>0 +infinity</td>
</tr>
</tbody>
</table>

An LC50 calculated using the moving average method may not be a very good estimate if the span is much less than the number of concentrations.

If any higher concentration produces a lower percent dead than a lower concentration, the confidence limits obtained by the moving average method will probably be too close.

---Results calculated using the Probit method---

<table>
<thead>
<tr>
<th>Iterations</th>
<th>G</th>
<th>H</th>
<th>Chi-Square</th>
<th>Probability</th>
</tr>
</thead>
<tbody>
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<td>3.4211</td>
<td>26.4672</td>
<td>52.9343</td>
<td>less than 0.001</td>
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</tbody>
</table>

Because the probability is less than 0.05, results calculated using the probit method probably should not be used.

Slope = 1.8732
95 Percent Confidence Limits = -1.59151 and 5.3379

LC50 = 0.115592E-5
95 Percent Confidence Limits = 0 and +infinity

Compare results with original data to see if they are reasonable.