THE EFFECTS OF AGRICULTURAL EFFLUENT ON RED-LEGGED FROGS (*Rana aurora aurora*) AND THREESPINE STICKLEBACK (*Gasterosteus aculeatus*) IN THE ELK CREEK AND SUMAS PRAIRIE WATERSHEDS, BRITISH COLUMBIA, CANADA

By

Albert Ryan Loveridge

B.Sc., Simon Fraser University, British Columbia, 1998

THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

In the Department

of

Biological Sciences

SIMON FRASER UNIVERSITY

Your file Votre référence
ISBN: 0-612-81870-5
Our file Notre référence
ISBN: 0-612-81870-5

Abstract

The effects of exposure to agricultural effluent on amphibians were investigated with red-legged frogs (*Rana aurora aurora*) in the Elk Creek Watershed, British Columbia, Canada. In 1999, an egg and tadpole *in situ* site exposure design was created along a gradient of agricultural activity (upper Elk Creek to lower Elk Creek) to determine if effects related to water quality. Significant effects on *R. aurora aurora* survivorship and biochemical stress indicators (plasma glucose, plasma lactate, haematocrit, and leucocrit) were observed at downstream sites adjacent to agricultural activity, but were confounded by site water temperature. An unexplained mortality event and inhibited metamorphosis, most likely as a result of cool-late season temperatures, were also observed. Similar results were observed in Threespine stickleback (*Gasterosteus aculeatus*) exposed in mesocosms along Elk Creek. The following year, a laboratory, temperature-controlled (10°C, 15°C, and 20°C) design was conducted to determine the modulating effect of temperature on the *R. aurora aurora* exposure effects. The effects on survivorship and biochemical indicators of stress were not replicated in each temperature regime, but a significant temperature effect on the biochemical effects measured was observed. *G. aculeatus* mesocosm exposure results were also not replicated in 2000. Additional studies demonstrated that whole agricultural effluent did not significantly affect plasma thyroid hormone levels, and organochlorine pesticide and polychlorinated biphenyl tissue residues were not detected at levels in adult

green frogs (*R. clamitans*) likely to elicit toxic effects. These results suggest that the agricultural effluent in Elk Creek may not be consistent from year to year and that ambient temperature is an important factor in assessing the effects of the agricultural effluent in Elk Creek. The temperature of Elk Creek, which did not reach levels required for metamorphosis, may prohibit this site as suitable aquatic habitat for *R. aurora aurora*.

Acknowledgements

I gratefully acknowledge the following for their assistance with laboratory analyses and field activities: Bev Anderson and the Ministry of Environment, Lands, and Parks' Lower Mainland Region office, Mark Sekala and Stephanie Sylvestor, the National Wildlife Research Centre, Mike Blouin, Tony Williams, Karen Petitt, and Natasha De Sousa. This study was undertaken under the Georgia Basin Ecosystem Initiative, and partially funded by the Toxic Substances Research Initiative. All methods used in this research were cared for in accordance with the recommendations of the Canadian Council on animal care, and the regulations and policies of Simon Fraser University.

Table of Contents

List of Tables

List of Figures

Chapter I: Introduction and Literature Review

Amphibians can be exposed to large quantities of agricultural chemicals in terrestrial and aquatic environments. Each year, three million tons of pesticides, 72 million tons of nitrogen-based fertilizers, and an undetermined quantity of nitrogen as ammonia from the livestock industry are added to agricultural lands worldwide (Hough 1998; Rouse et al. 1999). In North America, amphibian-breeding periods coincide with the highest pesticide application rates and run-off losses. Consequently, early-life stages considered the most sensitive to contaminants (Vitt et al. 1990) develop in the sink for these contaminants (Semlitsch 2000). Therefore, agricultural chemical loading may be an important factor contributing to declines in amphibian population sizes and occurrences (Berger 1989; Bishop 1992; Bonin et al. 1997).

Exposure-related effects of agricultural chemicals are not well documented in the wild, however. Berger (1989) noted the declines of several amphibian species in Poland's farmland, and attributed them to the use of nitrogen fertilizers. Bonin et al. (1997) observed mutagenic, teratogenic, and pathogenic effects in tadpoles, juveniles, and adults in heavily farmed areas of southern Quebec, and similarly attributed them to the use of pesticides. Using an *in situ* exposure technique, Bishop et al. (1999) studied an intensive vegetable growing area in Ontario, Canada, and concluded that both habitat loss and possibly elevated nitrate levels in wetlands affected amphibian survival and species diversity.

The exposure effect typically observed in the wild is lethality. In this situation, exposure-related effects may be successfully identified because concentrations in abiotic or biotic samples may be comparable to published toxicological values, since the toxicological values are primarily based on lethal effects of short-term exposures on a few model organisms (Pauli et al. 2000). Sublethal effects are less likely to be observed in the wild. If agricultural contamination is suspected to cause sublethal effects in a population, the current toxicological values have a limited application for comparison to the concentrations observed at lower and more environmentally realistic levels. More research is needed that describes sublethal effects in whole effluent or multi-chemical exposures at environmentally realistic concentrations and with exposure responses of diverse amphibian groups for this purpose to be protective of native populations.

At lower and more realistic concentrations, agricultural chemicals can disrupt growth, development, reproduction, physiology, and behavior (Bishop 1992; Fioramonti et al. 1997). Sublethal effects for the major agricultural chemical groups are described in several recent reviews. Common sublethal effects associated with organochlorine pesticides (cyclodienes such as endrin, toxaphene, and dieldrin; and DDT and its metabolites) include deformities, skins discolorations, depressed white and red blood cell counts, and altered stress responses in amphibians (Sparling 2000). Organophosphorous insecticide exposure at low levels may cause developmental (teratogenic effects and other

deformities) and behavioral (paralysis and hyperactivity) effects that are commonly associated with cholinesterase inhibition, in addition to measurable endpoints such as reduced hatching success, decreased size at metamorphosis, and physiological stress (Cowman and Mazanti 2000). However, since exposure effects are often dependent on species or life-stage and environmental conditions, comprehensive prediction of toxicity is difficult.

Aquatic concentrations of nitrogen compounds are commonly observed in agricultural landscapes that can be toxic to native species (Rouse et al. 1999). Reduced survivorship, increased deformity, and delayed growth and development were observed in leopard frogs (*Rana pipiens*), green frogs (*R. clamitans*), and American toads (*Bufo americanus*) (Jofre and Karasov 1999) at ammonia concentrations between 0.6 and 1.5 mg/L (Canadian Water Quality Guidelines [CWQG; CCME 1999] set for the protection of aquatic life for ammonia are pH dependent, but range from 1.37-2.2 mg/L). U.S Environmental Protection Agency recommended limits of nitrite for warm water fishes (5 mg/L) were lethal to *R. pretiosa, R. aurora, Hyla regilla*, and *Ambysotma gracile* larvae (Marco et al. 1999). Nitrate levels at the recommended limits for warm water fishes (90 mg/L) were also lethal to *R. pretiosa* and *A. gracile* larvae. Decreased *R. clamitans* tadpole survivorship and reduced numbers reaching metamorphosis were also observed at ammonium nitrate concentrations of 10 mg/L, levels which are often exceeded in agricultural areas (Hecnar 1995).

Very little is known about other agricultural contaminants such as hormones, antibiotics, retinoids, and the new insect juvenile growth regulators (Sparling 2000), and effects such as endocrine disruption. Evidence suggesting endocrine disruption in amphibians includes snout deformities from DDT exposure that are similar to a corticosterone exposure effect (Cooke 1970, 1972, 1979; Hayes 1997) and production of vitellogenin, the major egg yolk protein, in males exposed to toxaphene and dieldrin (Palmer et al 1998). Hayes (2000), in a review of the endocrine disruptor chemical toxicological literature, cited the need for models to measurable physiological endpoints as the first step in this investigation.

Effects from multi-chemical exposures are more applicable to wild populations because several chemicals would be present in an agricultural area. For example, the effects of atrazine and alachlor, two pesticides widely used in the Midwestern United States, combined were shown to be additive, resulting in a lower LC50 with northern leopard frogs and American toads than either chemical singularly (Howe et al., 1998). In contrast, atrazine and nitrate did not interact to affect development, metamorphosis, and hematocrit, at environmentally realistic concentrations, despite speculation that they may disrupt the oxygen-carrying capacity of larval blood (Allran and Karsov 2000).

The most realistic exposure scenario is with whole effluent, however. Agricultural effluent is a mixture of chemicals and nutrients, whose toxicity may be difficult to describe solely on chemical and physical parameters and

standardized acute toxicity tests of individual chemicals (Adams 1990; Vindimian et al. 1993; Wong and Dixon 1995). Consequently, an assessment of the toxicity based on measurements of sublethal effects in native species chronically exposed to whole effluent, with the toxicity testing including chemical synergisms and the stresses of the normal environment, may be the most useful to investigations into local populations (Wong and Dixon 1995). For instance, while laboratory studies predicted reduced mass and survival at environmentally realistic concentrations, field experiments in cattle ponds and experimental wetlands indicated that carbaryl had a stimulatory effect (Boone et al. 2001). Carbaryl was suggested to stimulate stress hormones as well as act through the food chain to affect metamorphosis.

Observing negative impacts *in situ*, for example, with caged animals and water quality data, will isolate water quality exposure from sediment and food exposure, and may facilitate identification of harmful aquatic chemicals and synergistic effects (Cooke 1981). There are no standardized protocols, however, although Cooke (1981) suggested characteristic deformities in tadpoles were generally indicative of pesticide exposure. Recent studies by Bishop et al. (1999), Harris et al. (1998), and Harris et al. (2000) have demonstrated a readily employable *in situ* nylon cage technique for monitoring amphibian exposure in the wild, which provided a more accurate assessment of the impact of contamination in their study areas. Bishop and Martinovic (2000) also provide a

review of *in situ* techniques and some considerations involved in employing this strategy.

For the application to native species, research is needed in several areas. The confidence or margins of safety in applications of toxicity values based on *X. laevis* or *R. pipiens* to an untested group must be defined with comparative testing. There is wide variation in the sensitivity to pesticides, fertilizers and nitrogen compounds among aquatic animal groups. Significant differences have been shown among developmental stages (Power et al. 1989; Harris et al. 1998), populations (Hecnar 1995), strains (Fioramonti et al. 1997), species (Power et al. 1989) and other aquatic taxa (Power et al. 1989; Allran and Karasov 2000; Rouse et al. 1999). Thus, it may be unsuitable to apply these toxicity values to management strategies for a native species.

The level of protection provided by water quality guidelines and the possibility of increased chemical resistance in a species or populations are important management concerns that arise from sensitivity differences among groups. The current water quality guidelines set for the protection of aquatic life are based on toxicity values for other aquatic taxa (primarily fish), even though amphibian embryo-larval stages may be more sensitive (Birge et al. 2000). Revised water quality guidelines, which could incorporate lower amphibian toxicity values, still may not provide adequate protection for native species because of the variation among taxa. In addition, species with a higher resistance to the toxicity of a chemical have a selective advantage in agricultural

areas, and may be able to out-compete more sensitive groups (Marco et al. 1999), which are often the intended targets of protective strategies. Increased resistance to certain classes of chemicals, which has been proposed for organochlorines and organophoshorous insecticides in amphibians (Cooke 1973; Schuytema 1994), may also pose a major bioaccumulation risk to higher trophic levels.

In summary, traditional laboratory studies to determine lethal toxicity are inadequate for predicting effects in the wild and the development of strategies to protect native species threatened by agricultural contamination. Whole effluent and multichemical chronic field studies, using a diversity of amphibian groups (preferably native species), with measurements of sublethal effects, are necessary to provide a realistic scenario and practical research for watershed management strategies and amphibian recovery plans in areas of intensive agriculture. Well-designed field studies are needed to understand the cumulative adverse effects of individual pesticides on susceptible species in localized environments (de Solla et al. 2002b).

Chapter II: The Effects of Agricultural Effluent on Red-legged Frogs (*Rana aurora aurora*). Part I – *In Situ* Exposure in the Elk Creek and Sumas Prairie Watersheds with Effects Comparisons to a Threespine Stickleback (*Gasterosteus aculeatus*) in a Mesocosm Exposure

Introduction

Run-off from agricultural areas adds significant quantities of chemicals (pesticides, fertilizers, and nutrients) to aquatic ecosystems (Hough 1998; Rouse et al. 1999), creating non-point sources of pollution (US EPA 1987). Typically, the effluent is a mixture of chemicals and nutrients, whose toxicity to aquatic biota may be difficult to describe with chemical and physical parameters and standardized acute toxicity tests of individual chemicals (Adams 1990; Vindimian et al. 1993; Wong and Dixon 1995). A more accurate assessment of the toxicity, which includes effects from chemical synergism and the stresses of the normal environment, is based on measurements of sublethal effects in native species chronically exposed to whole effluent (Wong and Dixon 1995). For amphibians, whole effluent toxicity data may be especially useful in local investigations of declines in population size and occurrence and individual deformities observed in agricultural settings.

In southern British Columbia, Canada, much of the Fraser River lowlands in the Georgia Basin have been converted from valley marshes to intensive agriculture land and is designated as an Agricultural Land Reserve (ALR). Declines in native amphibian population sizes and occurrences have been

observed in this low elevation ecosystem (Orchard 1992) and de Solla et al. (2002a; 2002b) recently demonstrated reduced *R. aurora aurora* hatching success in agricultural effluent from the Sumas Prairie watershed. Under the auspices of the Georgia Basin Ecosystem Initiative, several government and community agencies are interested in the Elk Creek watershed as a management paradigm for the Agricultural Land Reserve (ALR).

The objective of this study is to determine if negative effects on the early life-stages of red-legged frogs result from exposure to agricultural effluent from the Elk Creek watershed and relate to water quality. Biochemical stress indicators (plasma glucose, plasma lactate, haematocrit, and leucocrit) were measured in addition to the typical amphibian sublethal (growth) and lethal (survivorship) effects because biochemical indicators have a rapid response time, provide a physiological basis to the amphibian endpoints (Thomas 1990), and may be a response to pesticide exposure (Cowman and Mazanti 2000). These biochemical indicators are secondary stress parameters, and may increase or decrease when an animal is stressed (Wedemeyer et al. 1992). The red-legged frog is listed as a species of special concern in Canada (COSEWIC 2002), and has disappeared from a large portion of their historic range in Oregon and California for uncertain reasons (Hayes and Jennings 1986; Blaustein et al. 1996). For effects comparisons, *R. aurora aurora* was also exposed to reference sites in the recently studied Sumas Prairie watershed (de Solla et al. 2002a; de Solla et al. 2002b), and Threespine stickleback (*Gasterosteus aculeatus*) were

exposed in mesocosms in the Elk Creek watershed. Mesocosms and *in situ* techniques were used here and have frequently been used by other studies to determine the overall impact of environmental degradation on aquatic biota exposed to non-point sources (Marsh 1993) of pesticides (see review by Rand and Petrocelli 1985; Stay and Jarvinen 1995). Threespine stickleback were chosen as a comparison species because they are found abundantly in Elk Creek and concurrent reproductive experiments were performed in Elk Creek with this species (unpublished data).

Materials and Methods

Study Area

Elk Creek, a tributary of the Fraser River, originates in the Skagit Mountain range before flowing east into the lower Fraser Valley. Intensive agricultural activity dominates land-use in the lowlands of this watershed (dairy and poultry farming, field crops, greenhouses, and nurseries), and the area is part of the ALR. Six sequential study sites were chosen in Elk Creek that represent a gradient of increasing agricultural activity (Figure 1). The first two upstream study sites (1-R and 2-R) are considered reference sites (R). The area surrounding these sites is relatively free of agricultural development, although urban residential development is planned in the surrounding lower slopes. The next four downstream sites (3-A, 4-A, 5-A, and 6-A) are considered sites impacted by agricultural activity (A), as they occur in the ALR.

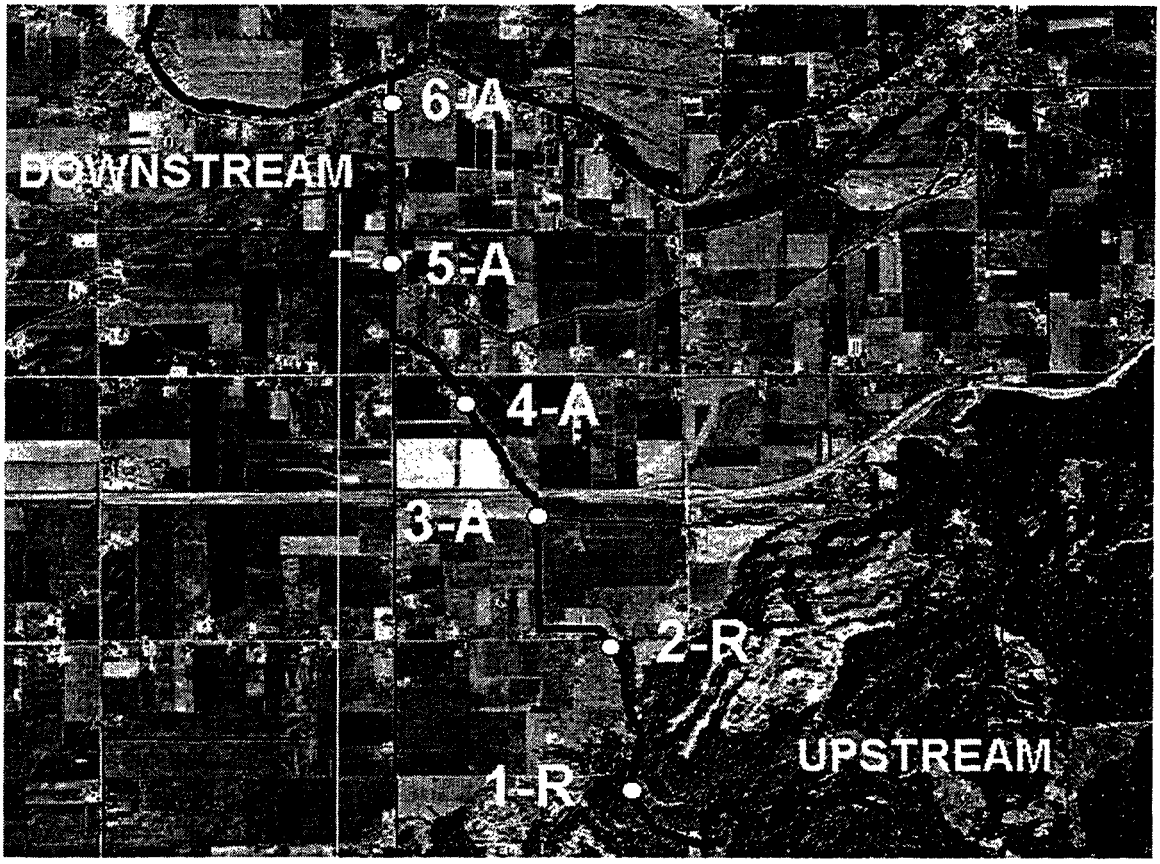


Figure 1: Aerial photo of Elk Creek study sites. The 1-R and 2-R sites are reference sites while sites 3-A to 6-A are adjacent to agricultural activity. Scale is approximately 1:30,000. Photo courtesy of Environment Canada Aquatics Section.

Water Quality

The British Columbia Ministry of Water, Air, and Land Protection performed biweekly water quality measurements at sites 4-A and 6-A from March 25 to September 29, 1999, as part of their watershed monitoring plan. The Pacific Environmental Science Centre in Vancouver, British Columbia analyzed whole water samples following standard methods (Environment Canada 1997a, b, c, d, e, f, g, h) for chloride, major ions (Mg, Ca, Na, and K), trace metals (Ni, Pb, Zn, Cr, Cu, Al), total phosphorous, nitrate + nitrite, ammonia, and total Kjeldahl nitrogen. The method detection limits for calcium, magnesium, sodium, and potassium were 0.01, 0.002, 0.03, and 0.06 mg/L. The method detection limit for chloride was 0.06 mg/L. The reporting limits for metals ranged from 0.00001 to 0.05 mg/L. On June 24, 1999, BCMELP also collected water samples for pesticide measurements. The Pacific Environmental Science Centre in Vancouver, British Columbia analyzed the whole water samples for 2,4-D, 2,4,5-T, azinphos-methyl, chlorpyrifos, demeton, diazinon, dicamba, dichlorprop, dimethoate, dinoseb, ethion, malathion, methidathion, mevinphos, naled, parathion, picloram, triclopyr, atrazine, and metolachlor following standard methods (Environment Canada 1992).

R. aurora aurora

R. aurora aurora egg masses were collected in March of 1999 at the negative reference site. Only egg masses deposited within the prior 24 hours

were collected. Egg masses were transported on ice to SFU in separate unsealed ziploc bags rinsed with site water, where they were staged according to Gosner (1960) with a dissecting microscope. Eggs were not de-jellied so as to provide a realistic exposure scenario. In total, five egg masses in the earliest stages of development (range 8-11) were sorted into groups of 30 with ethanol rinsed stainless steel tweezers in steel trays. Once sorted, eggs were transported on ice to the field sites. To eliminate individual bias, genetic differentiation among egg masses (breeding pairs) was verified through amplification of a mitochondrial gene unique to *R. aurora* at the Zoology Department of Oregon State University (Blouin, personal communication).

Five cages (replicates) were placed at each of the 6 sequential sites in Elk Creek (1-R to 6-A) (Figure 1). The *in situ* cage design and maintenance followed Harris and Bogart (1997) and Bishop et al. (2000). Egg groups (30 eggs) from each of 5 egg masses were placed inside each of five cages in floating baskets. The floating baskets consisted of a modified plastic strainer with foam fittings and bridal tulle material covering. In addition, 30 eggs consisting of 6 eggs from each of the 5 egg masses were placed in cages at the positive and negative reference sites. After the eggs hatched and the jelly was consumed, free-swimming tadpoles were released from the floating baskets into the outer cage. Tadpoles were fed store-bought green leaf lettuce that had been boiled for three hours. Every two days, fresh food was added to cages, any remaining old food was removed, and the number of individuals was counted. Maximum and minimum

surface water was recorded every two days with a submerged maximum/minimum thermometer.

In mid- to late-May, high mortality was observed at all of the upstream sites over a 2-week period. Since 100% tadpole mortality was observed at the 2-R site, tadpoles hatched in dechlorinated tapwater from different egg masses at the Simon Fraser University aquatic facilities were added to the cages described above. Subsequent observations for this group are reported as 2-R(b). Tadpoles were not replaced at any other sites.

The *R. aurora aurora* endpoints that were measured included hatching success, snout-to-vent length, and survivorship through to complete metamorphosis or upon experiment termination. Tadpole snout-to-vent length was measured weekly from hatching to Gosner (1960) stage 31. Accumulated thermal units (cumulative number of degrees above 0°C) were determined for the exposure duration by adding the doubled average of the maximum and minimum temperatures (recorded maximum and minimum temperatures were for the previous two days). Upon completion of the experiment, animals were examined for deformities and blood samples were collected. Blood samples were collected using heparinized micro-capillary tubes following cardiac puncture. Care was taken to avoid lymph fluid contamination in blood collection. Blood samples were centrifuged immediately upon collection in a microhematocrit centrifuge for 5 min at 13,000 xg. The hematocrit, or packed blood cell volume, and leucocrit, or white blood cell volume, are expressed as percentages of the total column.

The plasma was then frozen at -80°C until analysis. Plasma samples were analyzed for glucose and lactate levels with kits purchased from Sigma-Aldrich (St. Louis, MO).

G. aculeatus

G. aculeatus ranging in weight from 1.3 to 2.5 g were captured at Marion Lake with minnow traps in June of 1999. Marion Lake is a protected lake in the University of British Columbia Research Forest, Maple Ridge, BC, Canada, and is considered uncontaminated other than by atmospheric deposition.

G. aculeatus were placed in eight 25L aquaria (3 per aquaria) at the three sites (1-R, 4-A, 6-A) in mesocosms created by the Environment Canada Aquatics Section, and exposed from July 01 to Sep 30. Water from Elk Creek continuously flowed through the aquaria, which were housed inside streamside mesocosms. Photoperiod was adjusted weekly to match natural light cycles (16 hours light and 8 hours dark in mid-summer to 12 hours light and 12 hours dark in early autumn). Fish were fed bloodworms *ad libitum.* Fresh food was provided and any remaining old food was removed every 2 days. Any dead fish were removed and examined for parasites or infection.

G. aculeatus were given a gross morphological examination for parasites and infection, weighed, and measured for fork length upon completion of the three-month exposure. Care was taken to remove fish from tanks without stress. Blood samples were collected from the caudal vein using heparinized micro-

capillary tubes after severing the caudal peduncle. Care was taken to avoid lymph fluid contamination in blood collection. Blood samples were centrifuged immediately upon collection in a microhematocrit centrifuge for 5 min at 13,000 xg. The hematocrit, or packed blood cell volume, and leucocrit, or white blood cell volume, are expressed as percentages of the total column. The plasma was then frozen at -80°C until analysis. Plasma samples were analyzed for glucose and lactate levels with kits purchased from Sigma-Aldrich (St. Louis, MO).

Statistics

Data were analyzed using SAS Version 8.0 (Cary, NC). Data were tested for normality and homogeneity of variance prior to analysis. The null hypothesis of normality was not rejected for all water quality, amphibian, and fish data except the number of hatched larvae at site 3-A. No transformation of hatching data achieved normality, however. The null hypothesis of homogeneity of variance was not rejected for all data. Water quality data comparisons between the two collection sites were analyzed using t-tests. Amphibian and fish Least-squares means comparisons were made using one-way analysis of variance (ANOVA) and Tukey-Kramer multiple comparisons, with random effects assigned to variables. For amphibians, all measurements (averages of each cage) except weekly total length were compared among Elk Creek sites with a one-way ANOVA. Weekly total length measurements among sites could not be compared because of temperature differences. The number hatched, total length and

survivorship was compared among all sites. Measurements at stage 31 were used for the reference sites since tadpoles at Elk Creek sites did not undergo metamorphosis. Statistical comparisons of biochemical parameters were not made between tadpoles from the Elk Creek sites and newly transformed juveniles from the reference sites. Comparisons of post-transformation survivorship and SVL and biochemical parameters were not made between reference sites because only one cage was placed at each site. Temperature became a confounding factor in the *R. aurora aurora* experiment. Biochemical endpoints were significantly correlated to ATU, however ATU was not a covariate in the model y = site because only one temperature is available for each site. Correlation of endpoints to water quality parameters was not performed because only two downstream sites (4-A and 6-A) had water quality data. For fish, all data were compared using a one-way ANOVA. Mesocosm exposures took place in summer, when water temperatures were warmer, and the confounding effect of temperature was expected to be less. Differences among sites were considered significant at $p \leq 0.05$, and are indicated on graphs and tables with repeated letters.

Results

Water Quality

Water quality measurements were performed biweekly by the British Columbia Ministry of Water, Air, and Land Protection during the study period. Data from the two sites (4-A and 6-A) are presented here. Water quality parameters only rarely exceeded Canadian Water Quality Guidelines set for the protection of aquatic life (CCME 1999; Table 1) at any time during the sampling period. Significant differences between the two sites were observed for arsenic ($p = 0.0007$), barium ($p = 0.0067$), fecal coliform bacteria (0.0045), magnesium ($p = 0.0068$), manganese ($p < 0.0001$), orthophosphate ($p = 0.0001$), silicon, and total phosphorous ($p = 0.0011$). Organic carbon exceeded 3 mg/L infrequently at all sites (twice at 6-A and once at 4-A). Pesticides were not detected in water samples taken on June 24 (Table 2).

R. aurora aurora

R. aurora aurora eggs and tadpoles were exposed *in situ* to a gradient of input from March to September in 1999. Site water did not have a significant effect on hatching success ($F = 0.59$, $p = 0.2529$; Figure 2). Hatching success at the 2-R site, for the 2-R(a) group prior to the mortality event was 79%. Hatching successes at the 3-A, 4-A, 5-A and 6-A sites were 59%, 80%, 75%, and 77%,

Table 1: Results of biweekly water quality measurements at sites 4-A and 6-A. All units are mg/L except pH and Fecals (CFU/100 mL). Non detects are included as one-half the detection limit. Bold numbers exceed Canadian Water Quality Guidelines (CWQG) set for the protection of aquatic life (CCME 1999). Repeated letters represent $p \leq 0.05$. Data courtesy of the British Columbia Ministry of Water, Air, and Land Protection.

		4-A					6-A				
Analyte	CWQG	N	Mean	Standard Deviation	Minimum	Maximum	N	Mean	Standard Deviation	Minimum	Maximum
Aluminum	0.1*	13	**0.13**	0.14	0.020	**0.54**	12	**0.10**	0.13	0.017	**0.49**
Antimony	NA	13	0.00017	0.000090	0.000050	0.00030	12	0.00044	0.00024	0.00010	0.00080
Arsenic	0.005	13	0.000039 [a]	0.000032	0.0000025	0.000090	12	0.000032 [a]	0.000031	0.0000025	0.000080
Barium	NA	13	0.022 [b]	0.0065	0.014	0.036	12	0.033 [b]	0.013	0.018	0.058
Beryllium	NA	13	0.00014	0.00013	0.0000010	0.00025	12	0.00013	0.00013	0.0000010	0.00025
Bismuth	NA	13	0.00014	0.00012	0.000010	0.00025	12	0.00013	0.00013	0.000010	0.00025
Boron	NA	13	0.014	0.0030	0.0090	0.018	12	0.014	0.0033	0.0090	0.019
Cadmium	0.000029 (4-A)*; 0.000032 (6-A)*	13	**0.000033**	0.000019	**0.0000050**	**0.000070**	12	0.000030	0.000018	0.000010	**0.000070**
Calcium	NA	13	33	9.6	17	47	12	36	10	18	50
Chromium	0.001**	13	**0.0012**	0.0016	0.000050	**0.0043**	12	**0.0015**	0.0020	0.000050	**0.0051**
Cobalt	NA	13	0.00034	0.00033	0.000050	0.0011	12	0.00028	0.00022	0.00010	0.00090
Copper	0.002*	13	0.00072	0.00039	0.00029	0.0014	12	0.0069	0.021	0.00032	0.072
Fecal Coliform	NA	12	175 [c]	226	0.50	640	12	527 [c]	770	0.50	2500
Hardness	NA	7	86	22	50	116	6	96	24	60	125
Iron	0.30	13	**0.33**	0.20	0.12	**0.75**	12	**0.62**	0.26	**0.33**	**1.2**
Lead	0.002*	13	0.000083	0.00010	0.000010	0.00039	12	0.0012	0.0037	0.0000050	0.013
Lithium	NA	13	0.0012	0.00037	0.00050	0.0020	12	0.0012	0.00028	0.0010	0.0020
Magnesium	NA	13	2.5 [d]	0.67	1.4	3.6	12	3.5 [d]	1.1	1.8	5.0
Manganese	NA	13	0.020 [e]	0.010	0.0072	0.046	12	0.089 [e]	0.047	0.031	0.16
Mercury	0.0001	7	0.0000086	0.0000038	0.0000050	0.000015	6	0.0000083	0.0000041	0.0000050	0.000015
Molybdenum	0.073	13	0.0011	0.00052	0.00020	0.0019	12	0.0012	0.00048	0.00032	0.0017
N/Ammonia	0.715-1.04*	13	0.0091	0.0071	0.0025	0.025	13	0.037	0.082	0.0025	0.31
Nickel	0.065*	13	0.00094	0.00060	0.000050	0.0017	12	0.0012	0.00071	0.00005	0.0019

Analyte	CWQG	4-A					6-A				
		N	Mean	Standard Deviation	Minimum	Maximum	N	Mean	Standard Deviation	Minimum	Maximum
Nitrite and Nitrate	NA	13	0.33	0.13	0.13	0.57	13	0.37	0.14	0.17	0.64
Orthophosphate	NA	12	0.0013 [f]	0.0007	0.00050	0.0020	12	0.0047 [f]	0.0025	0.0020	0.011
pH	6.5-9	13	7.8	0.19	7.5	8.1	13	7.8	0.20	7.4	8.0
Potassium	NA	13	0.75	0.29	0.30	1.0	12	0.93	0.26	0.40	1.5
Selenium	0.001	13	**0.0012**	0.00060	0.00040	**0.0020**	12	0.00079	0.00037	0.00010	**0.0012**
Silicon	NA	13	3.8 [g]	0.49	2.8	4.5	12	4.9 [g]	0.96	3.2	6.2
Silver	0.0001	13	0.0000066	0.0000030	0.0000010	0.000010	12	0.0000075	0.0000026	0.0000050	0.000010
Sodium	NA	13	2.8	0.86	1.0	4.0	12	3.0	1.0	1.0	4.3
Strontium	NA	13	0.26	0.078	0.12	0.37	12	0.26	0.064	0.12	0.33
Thallium	NA	13	0.000016	0.000011	0.0000010	0.000025	12	0.000016	0.000011	0.0000010	0.000025
Tin	NA	13	0.000029	0.000023	0.0000050	0.000050	12	0.00021	0.00063	0.0000050	0.0022
Titanium	NA	13	0.011	0.012	0.0010	0.043	12	0.0095	0.012	0.0010	0.046
Total Inorganic Carbon	NA	13	16	4.9	8.1	23	13	20	6.2	9.6	32
Total Organic Carbon	NA	13	1.4	1.0	0.25	3.2	13	1.9	1.4	0.25	5.7
Total Phosphorous	NA	13	0.012 [h]	0.0050	0.0030	0.022	13	0.026 [h]	0.013	0.0090	0.055
Uranium	NA	13	0.000045	0.000023	0.000020	0.000090	12	0.000054	0.000022	0.000030	0.000090
Vanadium	NA	13	0.00067	0.00046	0.00020	0.0015	12	0.00072	0.00051	0.00020	0.0016
Zinc	0.3	13	0.0023	0.0019	0.00050	0.0060	12	0.0079	0.020	0.00064	0.071

* Adjusted with average site-specific parameters following CWQG recommendations (CCME 1999)

** Criteria is for chromium VI

NA = Not Available

Table 2: Pesticide levels (μg/L) in whole water samples from sites 4-A and 6-A. All levels were below detection limits. Data courtesy of the British Columbia Ministry of Water, Air, and Land Protection.

Analyte	4-A	6-A
2,4 – D	<0.1	<0.1
2,4,5 – TP	<0.1	<0.1
2,4,5 – T	<0.1	<0.1
Azinphos-Methyl	NA	<0.1
Chlorpyrifos	NA	<0.5
Demeton	NA	<0.1
Diazinon	NA	<0.5
Dicamba	<0.1	<0.1
Dichlorprop	<0.1	<0.1
Dimethoate	NA	<0.5
Dinoseb	<0.1	<0.1
Ethion	NA	<0.5
Malathion	NA	<0.5
Methidathion	NA	<0.5
Mevinphos	NA	<0.5
Naled	NA	<0.1
Parathion	NA	<0.5
Picloram	<0.1	<0.1
Triclopyr	<0.1	<0.1
Atrazine	NA	<0.1
Metolachlor	NA	<0.2

NA = Not Analyzed.

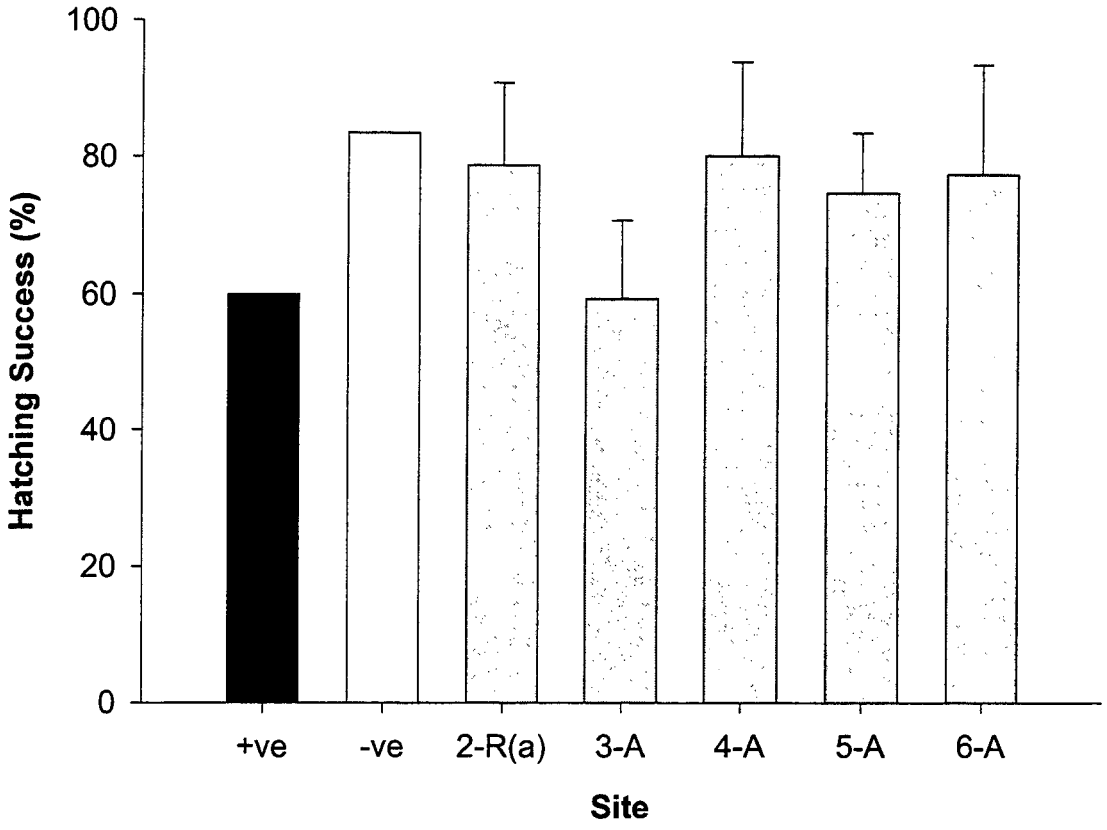


Figure 2: *R. aurora aurora* hatching success at the positive (+ve; black) and negative (-ve; white) reference sites and the Elk Creek sites (gray). Error bars represent ± 2SE.

respectively. Hatching success at the positive and negative reference sites was 60% and 83%, respectively.

In mid- to late-May, high mortality was observed at all of the upstream sites over a 2-week period (1-R 100%, 2-R(a) 100%, 3-A 94%, 4-A 53%, 5-A 38%, and 6-A 28% mortality).This event coincided with an elevated flow period resulting from the spring snowmelt and slight (approximately 2°C) decrease in temperature. Abnormalities were not observed in the dead tadpoles, although decomposition was rapid. Hatched tadpoles were replaced at the 2-R site, with tadpoles hatched in dechlorinated tapwater from different egg masses at the Simon Fraser University aquatic facilities, and subsequent observations for this group are reported as 2-R(b). The temperature in the dechlorinated tapwater was approximately 15°C.

Tadpoles were not replaced at the 1-R site because the water temperature was determined to be too cold. At the time of the mortality event, development was dramatically delayed and hatching had not yet occurred, even though all other sites had completed hatching up to one month earlier and were at Gosner stages 22-23. The average temperature for the first 9 weeks at the 1-R site was approximately 5.6°C with minimum temperatures frequently below 4°C. Licht (1971) lists the lower temperature limit for *R. aurora* embryos as 4°C. Therefore, hatching success data is not available for 1-R (Figure 2), and this site is not represented in further endpoint measurements.

Temperature differences between the sites prevented comparisons of weekly total length and most likely prevented tadpoles in Elk Creek from initiating transformation. Temperatures among sites in Elk Creek varied by as much as 3-5°C in the spring and early summer, with the downstream sites warmer than the upstream sites (site 1-R average temperature for April and May was 5.5°C; while the site 6-A average temperature during the same period was 9.1°C). By late summer, temperatures among sites were more similar (within 1-2°C), but rarely exceeded 15°C (site 1-R average temperature for August and September was 12.4°C; while the site 6-A average temperature during the same period was 12.5°C). At the reference sites, temperatures were above 15°C and tadpoles underwent transformation to juveniles in early summer (the positive reference site average temperature for April and May was 14.2°C). By the end of September, Elk Creek tadpoles had only reached Gosner (1960) stage 31, and were unlikely to transform before freezing winter temperatures. The exposure in Elk Creek was terminated at this point.

Weekly tadpole total length is plotted against degree-days from hatching to Gosner stage 31 (Figure 3). Statistical comparisons were not possible because of ungrouping of length measurements due to significant temperature differences among sites. However, site water had a significant effect on total tadpole length at stage 31 ($F = 106.93$; $p < 0.0001$). Total length was greatest in tadpoles from the 2-R(b) site (6.22 cm) and significantly greater than tadpoles from the negative reference (5.21 cm, $p = 0.0002$), 6-A (5.14 cm; $p < 0.0001$), 5-

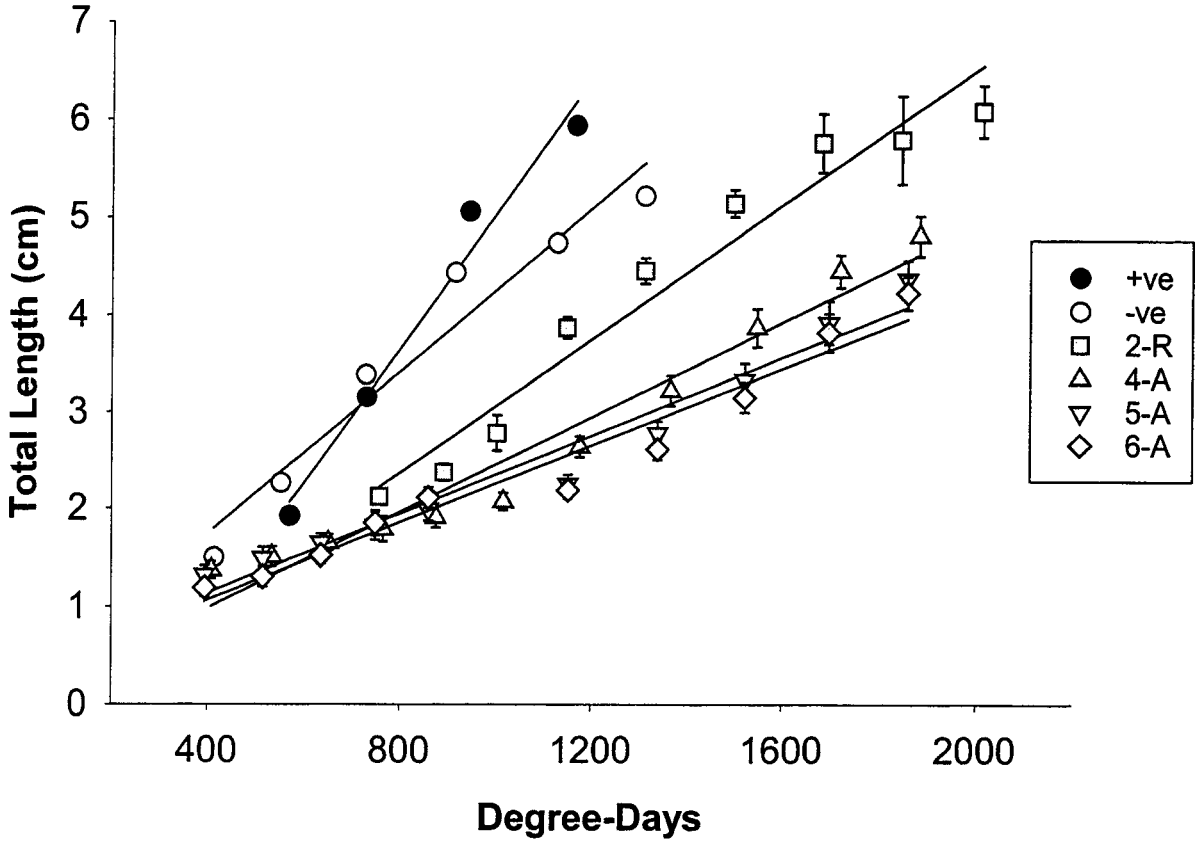


Figure 3: *R. aurora aurora* tadpole growth as total length (cm) versus degree-days from hatching to stage 31 for the positive (+ve) and negative (-ve) reference sites and the Elk Creek sites.

A (5.09 cm; $p < 0.0001$), and 4-A sites (4.90 cm; $p < 0.0001$). Total length of tadpoles was also greater at the positive reference site (5.94 cm) than at the negative reference ($p = 0.0176$), 6-A, ($p = 0.0011$), 5-A ($p = 0.007$), and 4-A sites ($p < 0.0001$.

Site water had a significant effect on survivorship of tadpoles at stage 31 ($F = 28.08$; $p = 0.0008$; Figure 4). Tadpoles at site 4-A (13%) had significantly lower survivorship than at the 2-R(b) (77%; $p < 0.0001$) and negative reference (73%; $p = 0.0021$) sites. Tadpoles at sites 5-A (27%) and 6-A (17%) also had significantly lower survivorship than at the 2-R(b) ($p = 0.004$ and $p < 0.0001$, respectively) and negative reference ($p = 0.0189$ and $p = 0.0041$, respectively) sites. Comparisons with tadpoles at the 2-R(b) site are between tadpoles with different exposure periods, as 2-R(b) tadpoles were not exposed during the embryonic period at the 2-R site, and, therefore, should be examined cautiously.

Elk Creek site water had a significant effect on plasma glucose ($F = 741.11$, $p < 0.001$) and plasma lactate ($F = 23.60$, $p < 0.001$) in tadpoles. In an attempt to quantify the confounding effect of temperature on the biochemical endpoints, plasma glucose and lactate levels were correlated to site ATUs at the end of the experiment. Plasma glucose ($r^2 = -0.61$; $p = 0.0083$) and lactate ($r^2 = -0.85$; $p < 0.0001$) levels were significantly negatively correlated to site ATUs, suggesting a confounding effect of temperature. The correlations are negative because tadpoles at the 2-R site were replaced with larvae hatched in the laboratory at approximately 15°C, and, therefore, the site 2-R ATUs were the

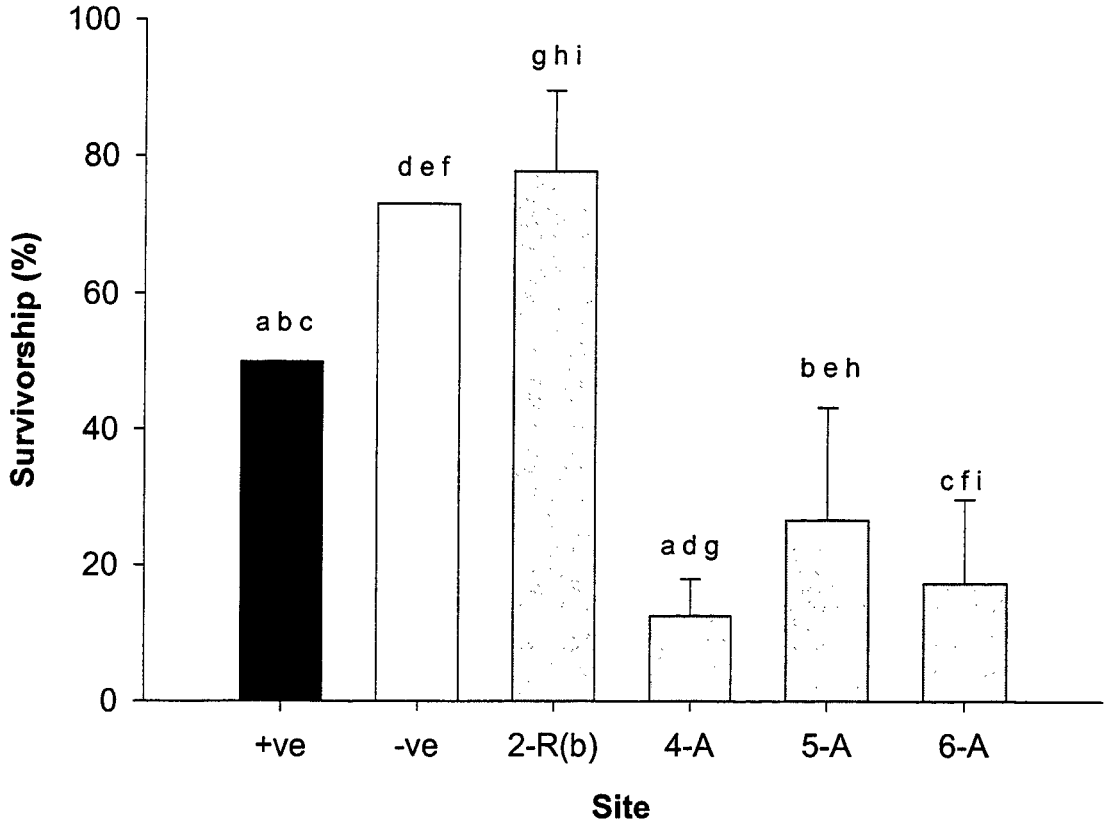


Figure 4: *R. aurora aurora* survivorship for tadpoles in the positive (+ve; black) and negative (-ve; white) reference sites and the Elk Creek sites (gray) at stage 31. Repeated letters represent $p \leq 0.05$. Error bars represent ± 2SE.

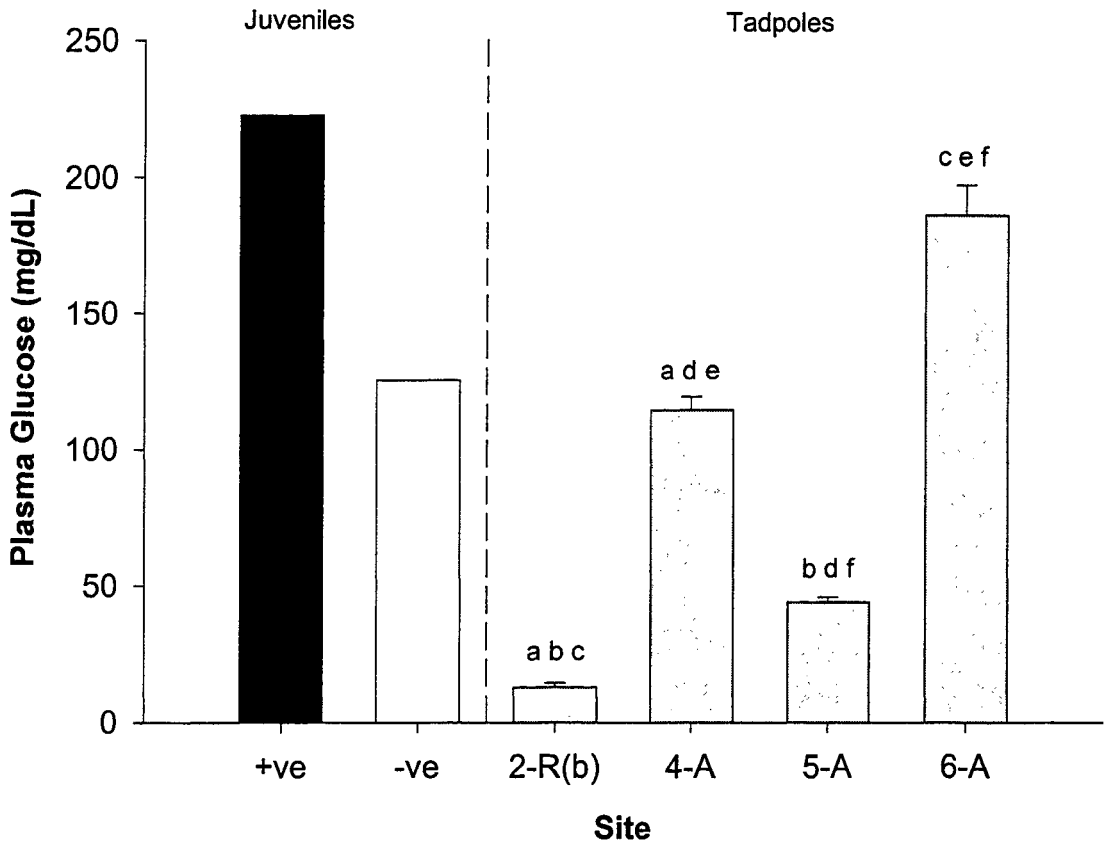


Figure 5: *R. aurora aurora* plasma glucose levels (mg/dL) for juveniles in the positive (+ve; black) and negative (-ve; white) reference sites and for tadpoles at the Elk Creek sites (gray). Repeated letters represent $p \leq 0.05$. Error bars represent ± 2SE.

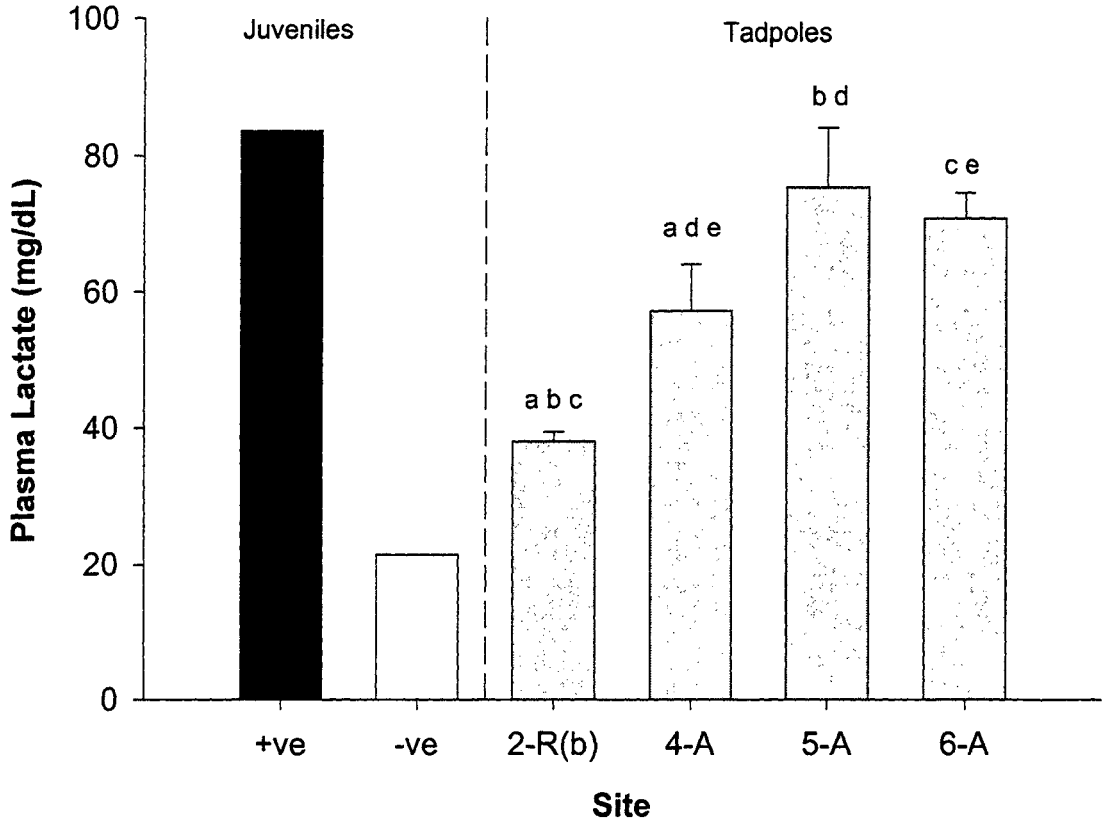


Figure 6: *R. aurora aurora* plasma lactate levels (mg/dL) for juveniles in the positive (+ve; black) and negative (-ve; white) reference sites and for tadpoles at the Elk Creek sites (gray). Repeated letters represent $p \leq 0.05$. Error bars represent ± 2SE.

highest. Significant differences in plasma glucose and lactate levels were observed among tadpole from most Elk Creek sites, with increases observed at in tadpoles from the agricultural sites (Figures 5 and 6) when compared to the upstream sites. For plasma glucose, tadpoles from site 6-A (185.98 mg/dL) had the highest levels, while tadpoles from site 2-R(b) site had the lowest levels (12.97 mg/dL). The differences among tadpoles from all Elk Creek sites were significant at $p < 0.0001$, except for the difference between site 2-R(b) and 5-A ($p = 0.007$). Juveniles from the positive and negative reference sites had levels of 222.62 mg/dL, and 125.42 mg/dL, respectively. For plasma lactate, tadpoles from the 5-A (75.37 mg/dL) and 6-A (70.73 mg/dL) sites had the highest levels, while tadpoles from the 2-R(b) (37.99 mg/dL) site had the lowest levels. All differences among sites were significant at $p < 0.05$, except for the difference between site 5-A and 6-A ($p = 0.7123$). Juveniles from the positive and negative reference sites had levels of 83.64 mg/dL, and 21.49 mg/dL, respectively.

Elk Creek site water also had significant effect on hematocrit values ($F = 42.39$, $p = 0.002$) in tadpoles, with a trend corresponding to the gradient of activity (Figure 7). Lower values were observed at the most downstream Elk Creek and positive reference sites. Juveniles from the positive reference site and tadpoles from the 6-A site had the lowest values (15.26%, 15.95%, respectively), while the highest levels were observed at the negative reference and 2-R(b) sites (22.18%, 24.57%, respectively). Significant differences were observed between the 2-R(b) site and the 4-A ($p = 0.005$), 5-A ($p < 0.0001$), and

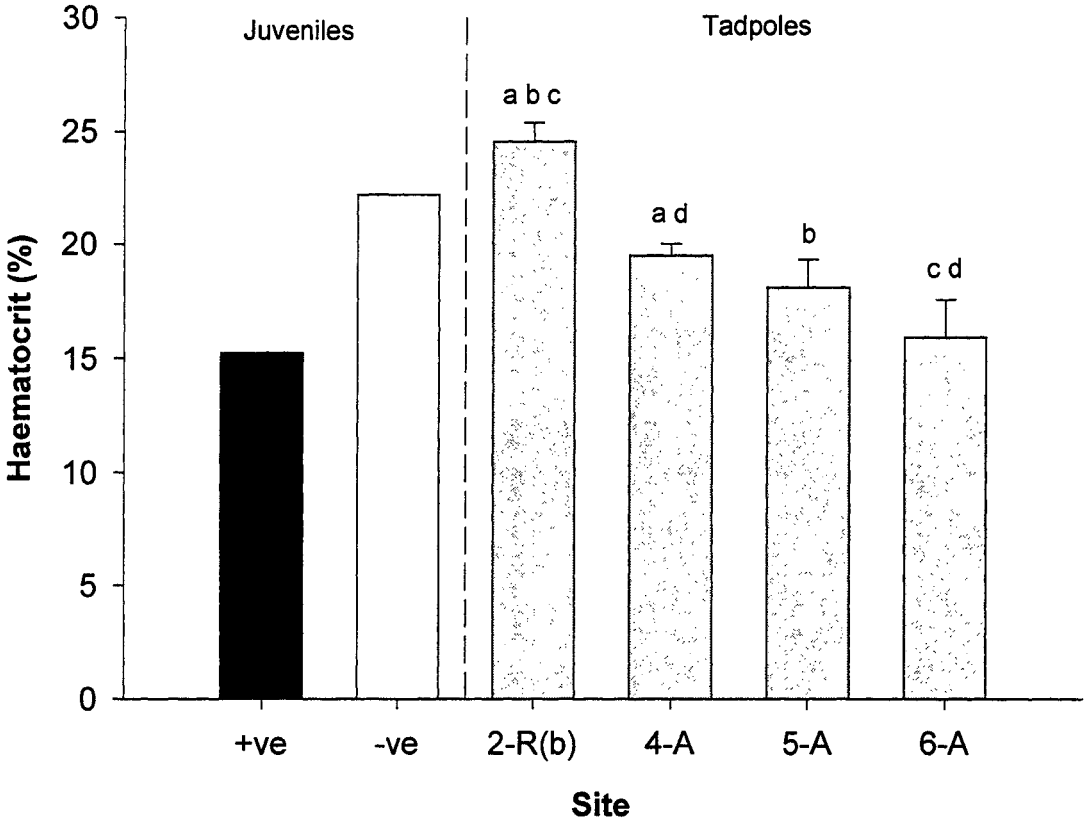


Figure 7: *R. aurora aurora* haematocrit values (%) for juveniles in the positive (+ve; black) and negative (-ve; white) reference sites and for tadpoles at the Elk Creek sites (gray). Repeated letters represent $p \leq 0.05$. Error bars represent ± 2SE.

Elk Creek site water also had a significant effect on leucocrit values ($F = 5383.6$, $p < 0.001$; Figure 8). Juveniles from the positive reference site (2.14%) and tadpoles from the 6-A site (1.25 %) had the highest values, while lower values were observed at the 5-A (0.64%), 4-A (1.05%), 2-R (b) (0.77%), and negative reference (0.87%) sites. Significant differences were observed between the 2-R(b) and 4-A ($p = 0.0469$) and 6-A ($p = 0.0012$) sites, and between the 5-A and 4-A ($p = 0.002$) and 6-A sites ($p < 0.0001$). Leucocrit values were not correlated to ATUs ($r^2 = -0.31$; $p = 0.23$).

G.aculeatus Endpoints

Elk creek site water had a significant effect on survivorship of *G. aculeatus* ($F = 35.45$, $p < 0.0001$). The survivorship (67%) in mesocosms at site 1-R was significantly higher than at sites 4-A (12.5%; $p < 0.0001$) and 6-A (17%; $p < 0.0001$). The difference between mesocosms 4-A and 6-A was not significant ($p = 0.8311$). Site water had a significant effect on plasma glucose ($F = 10.75$; $p = 0.0021$; Figure 10), plasma lactate ($F = 13.38$; $p = 0.0009$; Figure 11), haematocrit ($F = 12.21$; $p = 0.0013$; Figure 12), and leucocrit ($F = 13.14$; $p = 0.0009$; Figure 13). Significantly higher levels of plasma glucose ($p = 0.0031$), lactate ($p = 0.001$), and leucocrit ($p = 0.0014$) were observed in fish at the 6-A site mesocosm (123.72 mg/dL, 93.09 mg/dL, and 1.35%, respectively) when compared to fish in the 1-R site mesocosm (38.79 mg/dL, 54.91 mg/dL, and 0.116 %, respectively). Significantly higher levels of plasma glucose ($p = 0.024$), lactate ($p = 0.0235$), and leucocrit ($p = 0.0135$) were also observed in fish at the

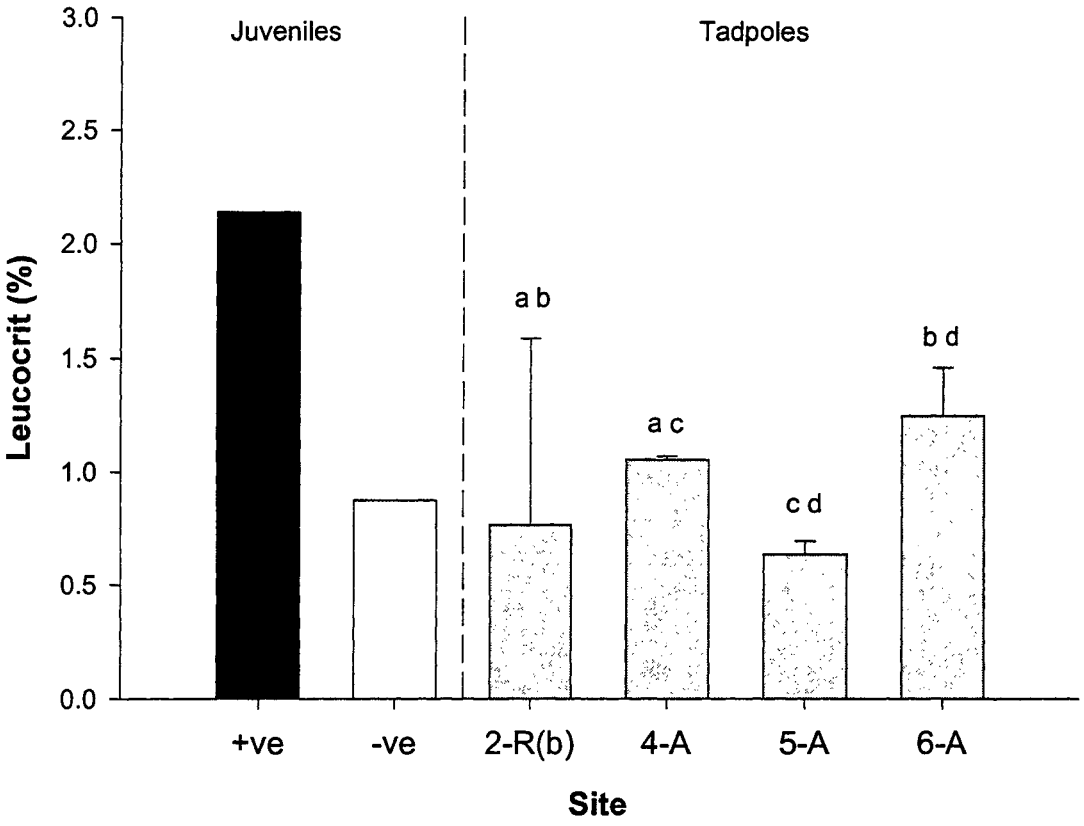


Figure 8: *R. aurora aurora* leucocrit values (%) for juveniles in the positive (+ve; black) and negative (-ve; white) reference sites and for tadpoles at the Elk Creek sites (gray). Repeated letters represent $p \leq 0.05$. Error bars represent ± 2SE.

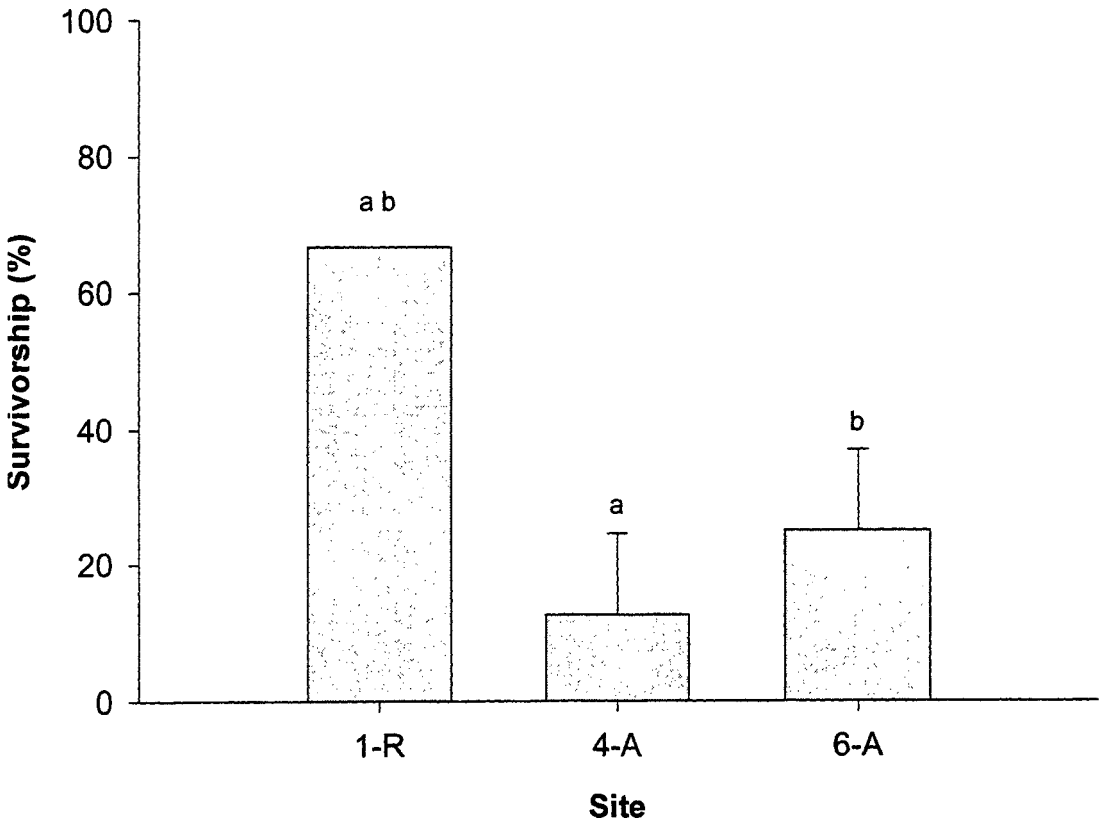


Figure 9: *G. aculeatus* survivorship at the Elk Creek 1-R, 4-A, and 6-A mesocosm sites. Repeated letters represent $p \leq 0.05$. Error bars represent ± 2SE.

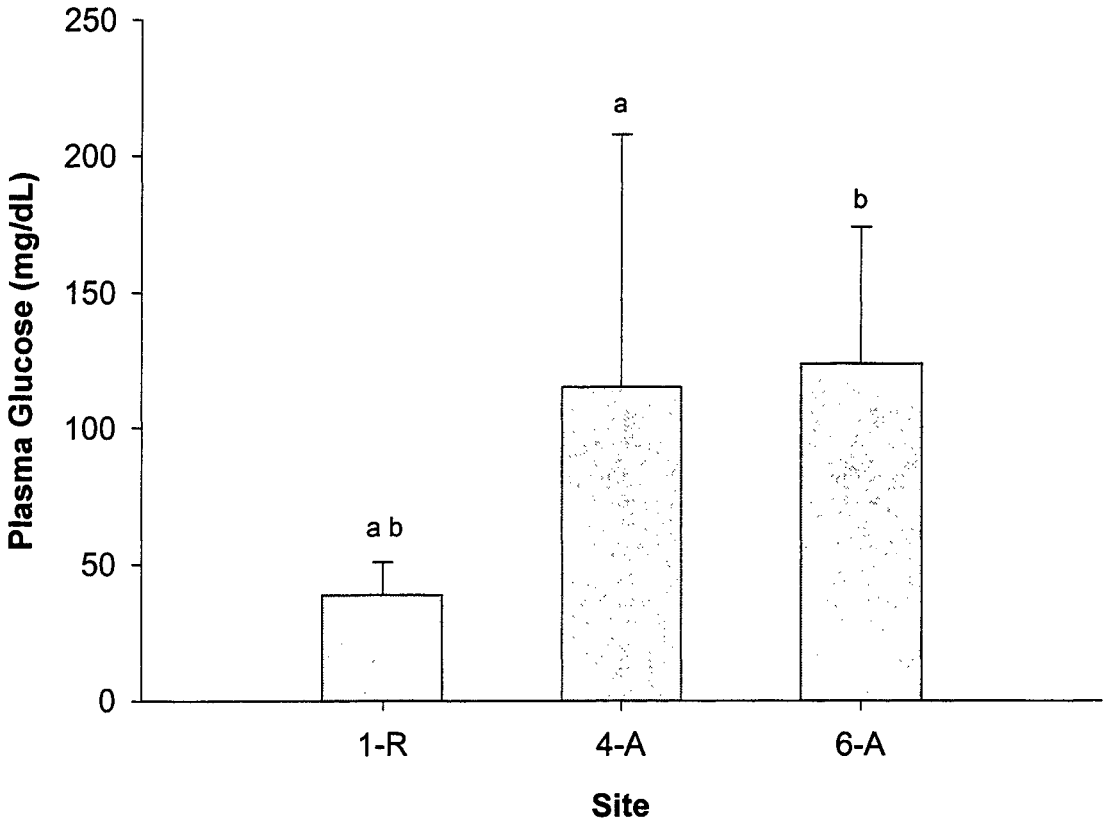


Figure 10: *G. aculeatus* plasma glucose levels (mg/dL) at the Elk Creek 1-R, 4-A, and 6-A mesocosm sites. Repeated letters indicate significant differences at $p \leq 0.05$. Error bars represent ± 2SE.

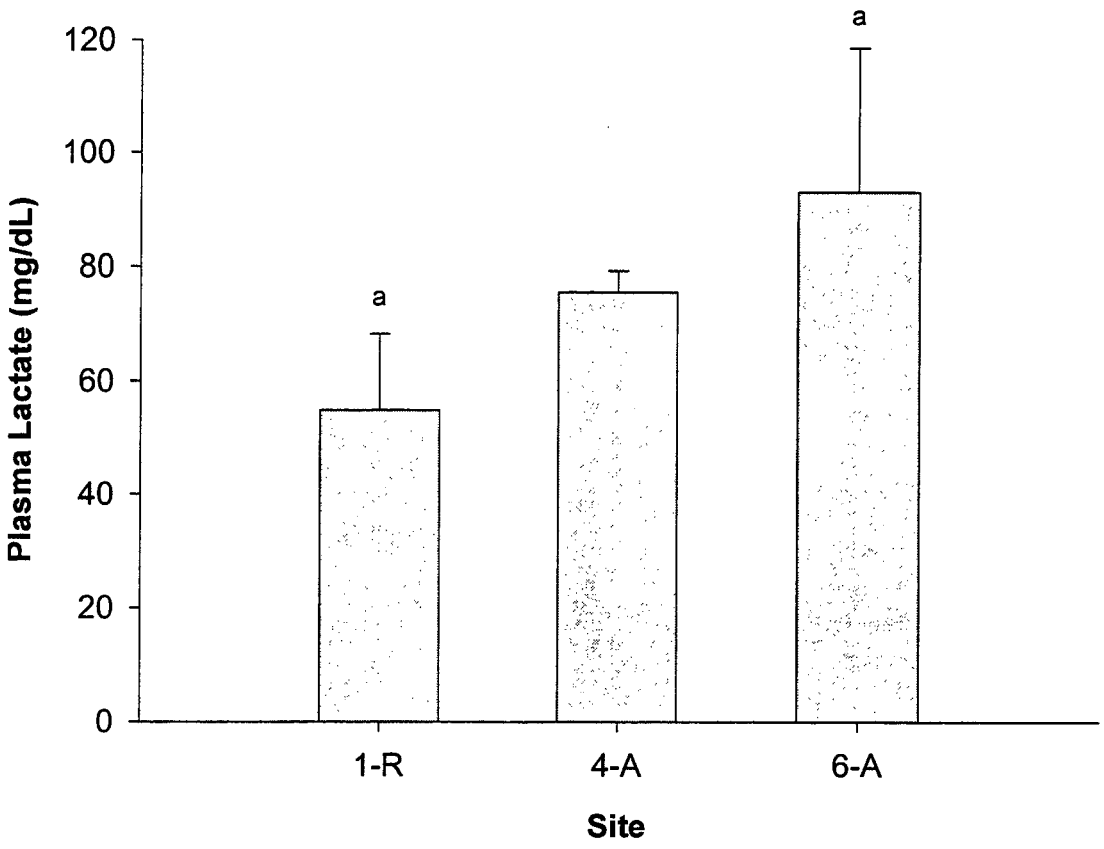


Figure 11: *G. aculeatus* plasma lactate levels (mg/dL) at the Elk Creek 1-R, 4-A, and 6-A mesocosm sites. Repeated letters indicate significant differences at $p \leq 0.05$. Error bars represent ± 2SE.

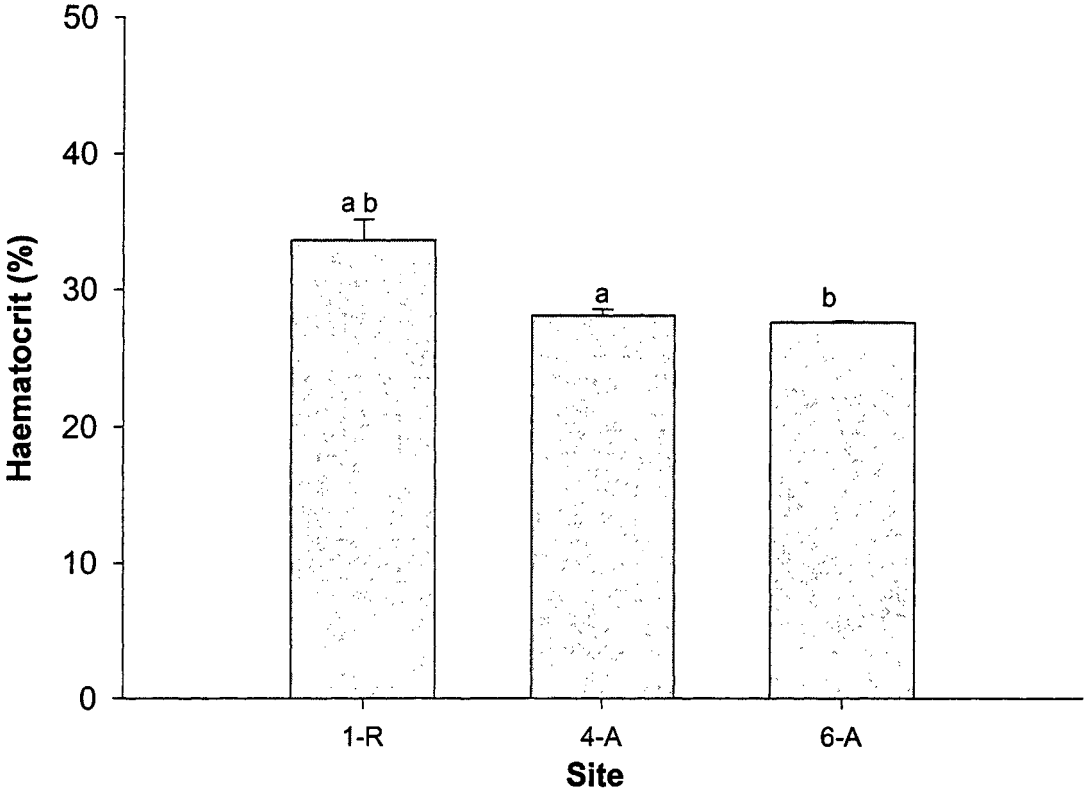


Figure 12: *G. aculeatus* haematocrit values (%) at the Elk Creek 1-R, 4-A, and 6-A mesocosm sites in 1999. Repeated letters indicate significant differences at $p \leq 0.05$. Error bars represent ± 2SE.

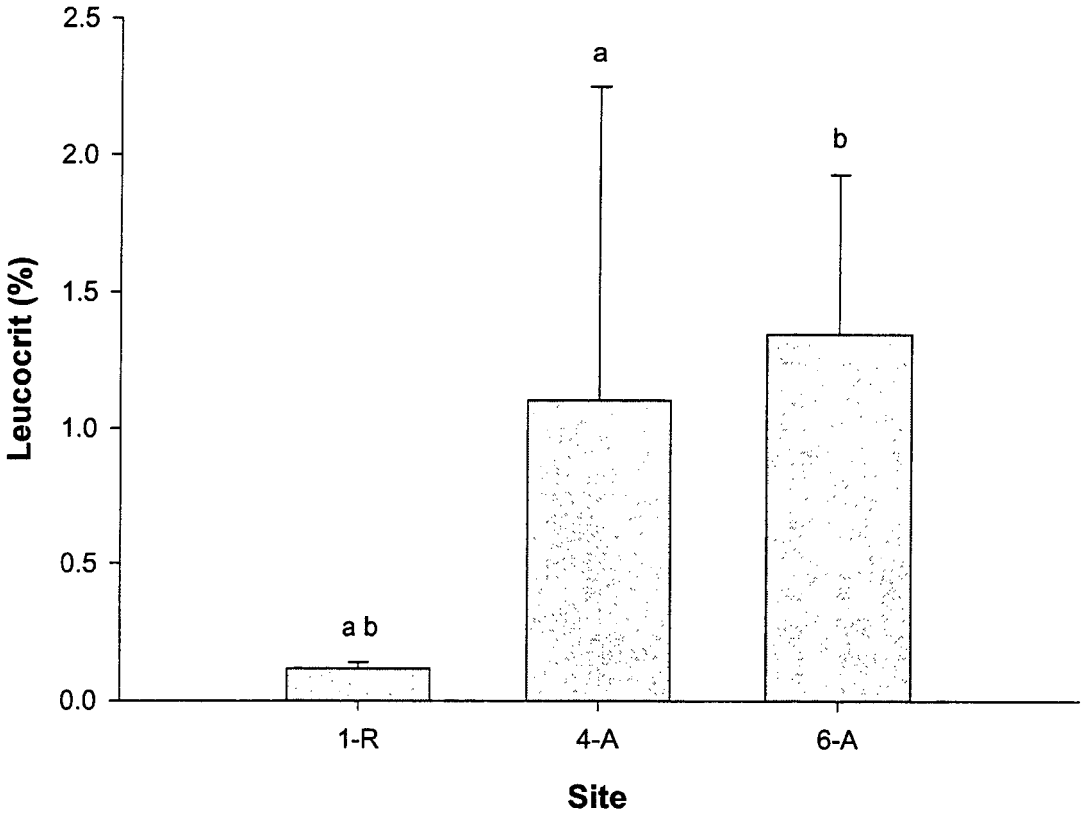


Figure 13: *G. aculeatus* leucocrit values (%) at the Elk Creek 1-R, 4-A, and 6-A mesocosm sites. Repeated letters indicate significant differences at $p \leq 0.05$. Error bars represent ± 2SE.

4-A site mesocosm (115.12 mg/dL, 75.06 mg/dL, and 1.10% respectively) when compared to fish at the 1-R site mesocosm. Haematocrit values were significantly lower in fish at the 6-A (25.6%; p = 0.0015) and 4-A sites (28.1%; *p* = 0.0325), when compared to fish at the 1-R site. There were no significant differences in hematocrit values between fish at the 4-A and 6-A site mesocosms. Temperature was not considered to have a significant effect on the biochemical endpoints measured because the *G. aculeatus* exposure was conducted in the mid- to late- summer, when Elk Creek temperatures were less variable among sites.

Discussion

The objective of this study was to determine if negative effects on the early life-stages of red-legged frogs resulted from exposure to agricultural effluent from the Elk Creek watershed and related to water quality. For effects comparisons, *R. aurora aurora* was also exposed to reference sites in the previously studied Sumas Prairie watershed (de Solla et al. 2002a; de Solla et al. 2002b), and Threespine stickleback were exposed in mesocosms in the Elk Creek watershed. At the Elk Creek sites, measurements of hatching success, length, and survivorship for *R. aurora aurora* were complicated by temperature differences between the sites and an unexplained mortality event. Significant differences were observed among most sites for biochemical indicators of stress (plasma glucose and lactate levels and haematocrit and leucocrit values) that

corresponded to an increase in agricultural activity. However, these effects were also confounded by site water temperature. Similar results were observed for *G. aculeatus* survivorship and biochemical indicators of stress in Elk Creek.

While the downstream water quality of Elk Creek is indicative of human input and agricultural loading (McNeely et al. 1979; Randall et al. 1997), the parameters measured were not at levels considered toxic to amphibians. Mean levels of aluminum, cadmium, chromium, iron and selenium exceeded CWQG benchmarks (CCME 1999) were far below levels considered toxic to amphibians (Power et al. 1989; Pauli et al. 2000). Nitrate and nitrite levels were well below the drinking water quality guidelines of 10 mg/L and levels reported to be toxic to amphibians (Rouse et al. 1999). Since decreased survivorship was observed in *R. aurora aurora* and *G. aculeatus*, decreased hatching success was observed at site 3-A, a large *R. aurora aurora* mortality event was observed, metamorphosis was inhibited in Elk Creek tadpoles, and the trend in biochemical stress measures corresponded to an increase in agricultural activity, other possibilities, in addition to temperature, must be considered to explain the lack of toxicity data.

Several possibilities may explain this inconsistency. First, contaminants may not be present in Elk Creek at levels considered to be toxic, and the observed negative effects were independent of contaminant exposure and related entirely to temperature and other undetermined factors. Second, toxic episodes of measured or unmeasured water quality parameters and pesticides may have been missed in the biweekly water and one-time sampling protocol,

respectively, that produced the observed effects. In complex systems, short-lived contaminants, such as carbaryl, at environmentally realistic concentrations can impact mass, time, and survival to metamorphosis in amphibians (Boone et al. 2001). Third, water quality guidelines may not provide adequate protection for aquatic biota exposed to agricultural effluent in Elk Creek. *R. aurora aurora* might have been sensitive to contamination in Elk Creek at concentrations lower then those designed to protect aquatic life based on non-amphibian test species. In addition, the toxicity of the agricultural effluent might not be determined solely on the singular chemical and physical properties, since, in the normal mixture, synergistic effects and inherent characteristics of Elk Creek and the watershed (such as temperature) may heighten the toxicity. Fourth, *R. aurora aurora* may also have high species-specific sensitivity to the agricultural effluent in Elk Creek, when compared to other tested amphibian species reported in the literature, and few contaminant studies have employed *R. aurora aurora* as a test species. For example, when exposed to the pesticide diuron in static renewal tests, limb malformations were observed in *R. aurora aurora* tadpoles at concentrations greater than 7.6 mg/L in 14-day exposures. This concentration was considered a no-observed-adverse effect-level (NOAEL), and it was lower than NOAELs for *X. laevis* (>29.1 mg/L 14-d), *Hyla regilla* (14.5 mg/L 14-d), and *R. catesbeiana* (7.6 mg/L 21-d) (Schuytema and Nebeker 1998).

The co-occurrence of effects with *R. aurora aurora* and *G. aculeatus* indicates that these species may be useful indicators of the effects in each other.

Although native *G. aculeatus* are abundant in Elk Creek, it is possible that the population in Elk Creek has built-up a resistance to the toxicity of agricultural effluent, as has been documented for mosquito fish and cutthroat trout in selenium contaminated waterbodies (Kennedy et al. 2000). Possible resistance to environmental degradation has been observed in *G. aculeatus* exposed to acid mine drainage in the Upper Sacramento River (Saiki et al. 1995). In addition, elevated tissue concentrations of cadmium and other metals observed above water and sediment concentrations suggest a significant bioaccumulation hazard. Tissue metal concentrations of Elk Creek *G. aculeatus* have not been measured, but may also indicate a bioaccumulation hazard.

Hatching success of *R. aurora aurora* at agricultural Elk Creek and reference sites was not affected by agricultural exposure in this study. In a previous study at the positive reference site (de Solla et al. 2002b), *R. aurora aurora* and *A. gracile* had lower hatching success, possibly as a result of high BOD, phosphorous, and nitrate/nitrite levels. However, in this study hatching success was much higher (60%) at the positive reference site than in the earlier study (0%). The difference between the two studies may a result of variability in the agricultural effluent between years or of subtle differences in experimental conditions. In the current study, eggs were contained in bridal tulle covered floating plastic strainers, while in the previous study egg were contained in modified film canisters. It is possible the exposure to and exchange of oxygenated water was better in the larger and more open containers in this

study. The effectiveness of both methods in minimizing testing bias has not been examined.

The exact cause of the mortality event is unknown. A run-off event is not suspected because less mortality was observed at the downstream sites. Alternately, the cause seemed to originate upstream, with the effects being most severe at the upstream sites. An agricultural contaminant aerially deposited in the spring snow pack may be responsible. Studies have shown pesticides used in the San Joaquin Valley are transported to over 1900 m in the mountains in the Sierra Nevada in California, where they may be impacting high-elevation populations of amphibians (Zabik and Seiber 1993; Drost and Fellers 1996; Datta et al. 1998). Upon melting, a pulse of the contaminant may have been released into Elk Creek during the sensitive early life-stage and been diluted downstream. While pesticide levels were below detectable limits in mid-June, most organophosphorous insecticides have short half-lives, leading to their desirability as pesticides. Unfortunately, local data are not available, but this possibility may warrant further study of this phenomenon in other areas.

Tadpoles in Elk Creek did not undergo metamorphosis, most likely as a result of unusually cool water temperatures. In contrast, tadpoles at the reference sites had completed metamorphosis by late July. *R. aurora aurora* metamorphosis typically begins in July and may last to late September, but the tadpoles do not overwinter, unlike some temperate species (Licht 1974, Corkran and Thoms 1996). Although the thermal requirements for *R. aurora aurora*

tadpoles are not documented, Licht (personal communication) suggests a minimal temperature of about 15°C for initiation of metamorphosis.

Plasma glucose, lactate levels, and haematocrit and leucocrit values are considered measurements of secondary stress parameters. The primary stress response in amphibians is an increase in corticosterone levels; the consequence of this increase is a mobilization of the animal's energy reserves, which results in an increase in plasma glucose (Burggren and Just 1992). When the anaerobic demands in stressful situations are too high, lactic acid will build up, resulting in an increase in plasma lactate (Burggren and Just 1992). Haematocrit values generally represent the oxygen carrying capacity, and in stressful situations the haematocrit may increase to supplement increased metabolism at the tissues (Burggren and Just 1992). Values are dependent on the total blood volume, the erythrocyte number, the intracellular hemoglobin content, the season, sex, age, and the developmental stage of the animal (Harris 1972; Boutilier et al. 1992; Burggren and Just 1992; Bonin et al. 1997). Leucocrit values represent the number of circulating white blood cells. High leucocrit values may suggest an elevated immune response, and possibly a subclinical infection, while low levels may be an indication of a bacterial infection inducing leucotolysis (Goede and Barton 1990) or immunosuppresion from stress (Hontella 1998). Comparisons were not made between tadpoles and juveniles because developmental stages may affect these parameters. For instance, larval anurans have a much lower capacity for glycolysis than adults (Gatten et al. 1992), and generally there is a

slight rise in haematocrit with increasing developmental stage (Burggren and Just 1992).

Biochemical stress measures have only rarely been employed to indicate environmental pollution. In mudpuppies (*Necturus maculosus*), decreased plasma corticosterone levels were observed in animals captured from sites contaminated with PCB's and organochlorine pesticides, most likely as a result of chronic stress (Gendron et al. 1997). Plasma glucose was also measured, but no differences were observed between animals sampled from reference and polluted sites, although mean levels (32.8-64.8 mg/dL of deproteinized plasma) at the reference sites were higher than those observed for *R. aurora aurora* in this study. In fish, the magnitude of the stress response is often dose dependent (Thomas 1990). In goldfish (*Carassius auratus*) exposed to low levels of dieldrin (0.0023 mg/L, ¼ the LC_{50}), for example, blood glucose levels increased by more than 133% of reference levels (Silbergeld 1974).

Lower haematocrit levels corresponding with increasing agricultural activity was an unexpected response. Typically, animal stress increases haematocrit values, as a consequence of the short-term up-regulation of the oxygen carrying capacity of the blood. A low haematocrit may be the result of specific conditions, such as disease (Goede and Barton 1990), or exposure to elevated concentration of nitrate/nitrite or atrazine (Allran and Karasov 2000). Methemoglobinemia, a disruption of the oxygen carrying capacity by nitrite oxidation of iron, is known to result from elevated nitrate/nitrite concentrations

(Huey and Beitinger 1980; Allran and Karasov 2000). Conceivably, a decrease in the haematocrit value could result from short-term exposures to nitrate/nitrite. Exposure to the pesticide atrazine may also decrease circulating erythrocyte numbers (Allran and Karasov 2000). However, nitrate/nitrite levels recorded in Elk Creek were not at levels suggested in the literature to be the result in methemoglobinemia, although sub-chronic and chronic studies have not been published. Atrazine was also not detected in this study. Therefore, the cause of the lower haematocrit levels is unclear.

It is possible that acute stress from handling and observation contributed to the stress response observed in *R. aurora aurora* and *G. aculetus*. However, it is uncertain if this stress was also related to water quality, and confounded the biochemical endpoints measured. At the agricultural sites, water turbidity and electrolyte concentrations were higher. Therefore, awareness of other individuals and predators (outside the cage or aquaria) using chemoreception and visual cues (see review by Alford 1999 and Pough et al. 1992) may have been lower as compared to the upstream sites. If this also resulted in a lower level of stress for the tadpoles and fish at the agricultural sites, then a handling or disruptive observer event may result in greater increase of stress relative to the chronic level. A stress response may have also become exhausted at the upstream sites (Hontella 1998), contributing to the observed results. Unfortunately, normal levels of the biochemical endpoints *in R. aurora aurora* and *G. aculeatus* are not known to support or refute this suggestion.

In summary, negative effects on *R. aurora* early life stage survivorship and biochemical indicators of stress in concordance with effects with *G. aculeatus* were observed in agricultural effluent exposures. The most negative effects were observed at positive reference site in the Sumas prairie. However, the confounding effect of temperature on the observed effects at Elk Creek complicates the interpretation of the results. For instance, the thermal requirements for *R. aurora* embryo-larval development and temperature modulation of the observed effects are not well understood. The cause of the stress response observed in both species is also not clear. Therefore, a duplicate study which controls temperature on the same measured endpoints is necessary for a better understanding of the effects of agricultural effluent for the Elk Creek watershed on early life stages of *R. aurora aurora*. An investigation into the stress response is also needed.

Chapter III: The Effects of Agricultural Effluent on Red-legged Frogs (*Rana aurora aurora*). Part II - Temperature Modulation in a Laboratory Exposure with a Comparison to the Threespine Stickleback (*Gasterosteus aculeatus*) Stress Response in a Mesocosm Exposure

Introduction

Temperature is a primary factor influencing the toxicity of aquatic chemicals. In ectotherms, toxicokinetics (uptake, biotransformation, and excretion) and toxicant-receptor interactions are modulated by temperature (see review by Kennedy 1995; Kennedy and Walsh 1997; Reid et al. 1999). Generally, as temperature increases, metabolism increases exponentially in ectotherms (Burggren and Just 1992), and while uptake increases through changes in ventilation rate and lower oxygen solubility, the rates of enzymatic detoxification, membrane-associated processes, and excretion may also increase (Kennedy 1995). Chemical properties such as solubility, ionic equilibrium (pH, metals, and ammonia, for example), and chemical structural integrity are also modified by temperature (Matsumara 1985; Spear 1991; Beattie et al. 1992; Boone and Bridges 1999).

Toxicity testing of amphibians at more than one temperature may provide a more accurate assessment of the impact to wild populations (Boone and Bridges 1999). Amphibian populations may experience temperature fluctuations through industrial and agricultural input, extremes in marginalized habitat, and

global warming. During larval development, a wide range of temperatures may also be experienced, and at a particular temperature and developmental stage the chemical availability, the toxicological response, and the subsequent lethal and sublethal effects may change. Furthermore, extended larval periods at cooler temperature may make amphibians more vulnerable to acute deleterious conditions resulting from, for example, pesticide applications (Berrill et al. 1993) and acidic conditions (Carey 1993), over a range of seasonal temperatures.

In a previous *in situ* study (Chapter II), the effects of agricultural effluent exposure on early life stages of red-legged frogs were confounded by temperature. Tadpoles failed to undergo metamorphosis in Elk Creek, possibly as a result of a cool late-season, and significant differences were observed in biochemical indicators of stress (plasma glucose and lactate levels, haematocrit, and leucocrit values) at warmer sites adjacent to agricultural activity. The objective of this laboratory study was to investigate the effects of temperature modulation on the effects of whole agricultural effluent exposure to developing *R. aurora aurora* eggs and tadpoles. The temperature range used (10°C, 15°C, and 20°C) was a realistic exposure scenario and brackets the minimum temperature necessary (15°C) for metamorphosis in this species (Licht, personal communication). In addition, the stress response is further investigated by testing the possible impairment of the *G. aculeatus* stress response exposed to agricultural effluent from the Elk Creek watershed. After chronic exposure to contaminated environments, fish may not be able to mount a stress response.

Following continual cortisol output in response to contaminant stress, the interrenal cells of the adrenal cortex may become exhausted and insensitive to adrenocorticotropic hormone induction (Girard et al. 1998). Therefore, an adrenocorticotropic hormone (ACTH) challenge, in which cortisol levels in the blood are measured after ACTH is injected, can be used to test impairment of the stress response.

Materials and Methods

Water Quality

The Environment Canada Aquatics Section performed hourly monitoring of water temperature, conductivity, dissolved oxygen and pH at three sites in Elk Creek during the amphibian exposure period. The first site, 1-R, is a reference site in the Elk Creek watershed. The area surrounding the 1-R site is relatively pristine. The third site, 6-A, is approximately 3 km downstream from the 1-R site, and receives cumulative agriculture input. The 4-A site was mid-way between the 1-R and 6-A sites. See Chapter II for a complete description of this study area and the water collection sites. Every 2 days, from April 11 to June 12, 2000 samples were taken for measurement of ammonia, calcium, hardness, magnesium, nitrate (nitrite + nitrate), nitrite, phosphorous (total and ortho-), potassium, silicon, sodium, total nitrogen (Table 3). The Pacific Environmental Science Centre in Vancouver, British Columbia analyzed whole water samples following standard methods (Environment Canada 1997a, b, c, d). The method

detection limits for calcium, magnesium, sodium, and potassium were 0.01, 0.002, 0.03, and 0.06 mg/L. The method detection limit for total phosphorous ranged from 49 to 100 ug/g.

R. aurora aurora

R. aurora aurora egg masses were collected in March of 2000 at a reference site in the Sumas Prairie, approximately 30 km east of Elk Creek, that is adjacent to agricultural activity but receives mountain run-off. Only egg masses deposited within the prior 24 hours were collected. Egg masses were transported on ice to SFU in separate unsealed ziploc bags rinsed with site water, where they were staged according to Gosner (1960) with a dissecting microscope. Eggs were not de-jellied so as to provide a realistic exposure scenario. In total, three egg masses in the earliest stages of development (range 8-11) were sorted into groups of 0 with ethanol rinsed stainless steel tweezers in steel trays. To eliminate individual bias, genetic differentiation between egg masses (breeding pairs) was verified through amplification of a mitochondrial gene unique to *R. aurora aurora* at the Zoology Department of Oregon State University (Blouin, personal communication).

Amphibians were exposed to water collected from three sites (1-R, 6-A, and a positive [+ve] reference). The positive reference site is in the Sumas Prairie approximately 30 km west of the Elk Creek watershed, but, also, in the ALR and adjacent to intensive agricultural activity. See Chapter II for a complete description of this study site. Amphibians were also exposed to dechlorinated

tap water for a negative (-ve) reference. Water samples were collected weekly in 80 L plastic containers and allowed to acclimate to experimental temperatures.

Eggs were exposed to water from the 3 field sites (1-R, 6-A, and positive reference) and the negative reference at three different temperatures (10°, 15°, and 20°C). The 12 treatments were replicated for each of 3 egg masses from the reference site in Sumas Prairie, resulting in 36 experimental units (25 L aquarium containing 15 eggs).

Water baths in temperature controlled rooms in the aquatics facilities at Simon Fraser University maintained the water temperature in each aquarium within ±1°C. Sediments were resuspended in the water samples prior to weekly water replacements in the aquaria. Eggs were placed in floating baskets and covered with bridal tulle material as in the field studies (see description in Chapter II). After hatching and jelly depletion, tadpoles were released from the floating baskets and fed *ad libitum*. Tadpoles were fed store-bought green leaf lettuce that had been boiled for three hours. Every two days, fresh food was added to each aquaria, any remaining old food was removed, and the number of individuals was counted. All aquaria were slightly aerated constantly and the photoperiod was adjusted once per week to match natural light cycles (12 hours light and 12 hours dark in spring to 16 hours light and 8 hours dark in mid-summer).

The endpoints that were measured were hatching success, tail-to-tip length and survivorship through to complete metamorphosis or upon experiment

termination. Total length of tadpoles was measured weekly from hatching to Gosner (1960) stage 31 over the number of degree-days since the experiment initiation. Degree-days, or accumulated thermal units, are the cumulative number of degrees above 0°C. Upon completion of the experiment, animals were examined for deformity and measured for snout-to-vent length. Blood samples were collected from the caudal vein using heparinized micro-capillary tubes after severing the caudal peduncle. Care was taken to avoid lymph fluid contamination in blood collection. Blood samples were centrifuged immediately upon collection in a microhematocrit centrifuge for 5 min at 13,000 xg. The hematocrit, or packed blood cell volume, and leucocrit, or white blood cell volume, are expressed as percentages of the total column. The plasma was then frozen at -80°C until analysis. Plasma samples were analyzed for glucose and lactate levels with kits purchased from Sigma-Aldrich (St. Louis, MO).

G. aculeatus

G. aculeatus ranging in size from 0.3-1.82 g were captured at Marion Lake with minnow traps. Marion Lake is a protected lake in the University of British Columbia Research Forest, Maple Ridge, BC, Canada, and is considered uncontaminated other than by atmospheric deposition.

G. aculeatus were placed in eight 25L aquaria (3 per aquaria) at three sites (1-R, 4-A, and 6-A) in mesocosms created by the Environment Canada Aquatics Section, and exposed from July 01 to Sep 30 (three month chronic

exposure). Water from Elk Creek continuously flowed through the aquaria, which were housed inside streamside mesocosms. A five-day acute exposure group (5 aquaria per mesocosm, 3 fish per aquaria) was also conducted to compare effects between the chronic and acute exposures to agricultural effluent from the Elk Creek watershed. The acute exposure group of fish was not fed and coincided with the last five days of the chronic exposure. Photoperiod was adjusted weekly to match natural light cycles (16 hours light and 8 hours dark in mid-summer to 12 hours light and 12 hours dark in early autumn). Fish were fed bloodworms *ad libitum.* Fresh food was provided and any remaining old food was removed every 2 days. Any dead fish were removed and examined for parasites or infection.

G. aculeatus were given a gross morphological examination for parasites and infection, weighed, and measured for fork length upon completion of the three-month exposure. Care was taken to remove fish from tanks without stress. Blood samples were collected from the caudal vein using heparinized micro-capillary tubes after severing the caudal peduncle. Blood samples were centrifuged immediately upon collection in a microhematocrit centrifuge for 5 min at 13,000 xg. The hematocrit, or packed blood cell volume, and leucocrit, or white blood cell volume, are expressed as percentages of the total column. The plasma was then frozen at -80°C until analysis.

An ACTH challenge was performed on the chronic exposure group following the procedure by Girard (1998). Briefly, porcine ACTH (Sigma-Aldrich,

St. Louis, MO), dissolved in deoxygenated, and acidified (pH 6) double-distilled water was transported on ice to the mesocosms. Fish were given a slight tricainemethanosulfate anesthetic. The ACTH was dissolved in saline (0.7% NaCl) and then transferred to a 2 μL syringe and kept on ice. Each fish in the chronic exposure group was i.p. injected with a 4 IU ACTH/100 g body mass in 100 μL of 0.7 % saline, and then returned to the aquaria where they recovered from the anesthetic. The acute exposure group was not given the ACTH injection. After 2 hours, fish were euthanized with tricainemethanosulfate and blood samples were collected as above. Haematocrit and leucocrit measurements were made using standard procedures. Plasma cortisol levels were determined using ELISA (Neogen, Lexington, KY). Glucose and lactate assays were not performed in Y2000.

The endpoints that were measured were survivorship, haematocrit, leucocrit, and plasma cortisol levels in the chronic (three-month) exposure group, following ACTH injection, and in the acute (five-day) exposure group, which did not receive an ACTH injection, but for whom a normal stress response is expected because of the short exposure duration, at all the study sites.

Statistics

Data were analyzed using SAS Version 8.0 (Cary, NC). Data were tested for normality and homogeneity of variance prior to analysis. The null hypothesis of normality was not rejected for all data, except tadpole SVL in the 6-A water

sample at 10°C, the number hatched in the 1-R and 6-A water samples at 15°C and the negative reference water sample at 20°C, and juvenile haematocrit values in the 1-R water sample at 15°C. Since no transformation achieved normality, untransformed data were used for comparisons. The null hypothesis of homogeneity of variance was not rejected for all data. Least-square mean comparisons were made using analysis of variance (ANOVA) and Tukey-Kramer multiple comparisons, with random effects assigned to variables. Water quality measurements were compared among the 1-R, 4-A, and 6-A collection and mesocosm sites using a one-way ANOVA. Since tadpoles in the 10°C group did not under metamorphosis, all amphibian endpoints, except hatching, were compared among 15°C and 20°C temperature treatments and water samples using a two-way ANOVA. A one-way ANOVA was used for the 10°C group. Hatching was compared among all groups using a two-way ANOVA. Weekly SVL measurements among water sample exposures among water samples within temperature groups were compared using a one-way repeated measures ANOVA. Fish endpoints were compared using a one-way ANOVA. Fish endpoints were not correlated to levels of water quality parameters because the water-sampling period differed from the fish exposure period. Differences were considered significant at $p \leq 0.05$ and are indicated on graphs and tables with repeated letters.

Results

Water Quality

For most of the parameters measured significant differences were observed between sites over the sampling period (Table 3). The maximum level of ammonia at the 6-A site was 0.319 mg/L. At the 1-R site, the maximum ammonia level was 0.032 mg/L. The maximum nitrite concentration reached at the 6-R site was 0.02 mg/L.

R. aurora aurora

There was a significant main effect of water samples ($F = 3.15$; $p = 0.0433$), but not for temperature ($F = 3.30$; $p = 0.0542$), on the number hatched, with no significant interaction between the two variables ($F = 1.97$; $p = 0.1098$). However, significant differences were not observed between any water samples. A comparison of hatching success among water samples is shown in Figure 14. The lowest hatching success (40%) was observed in the positive reference water samples at 10°C and 15°C, and the highest hatching success (100%) was observed in the positive reference water sample at 20°C.

Weekly total length from hatching to Gosner (1960) stage 31 was only significant at 10°C ($F = 2.54$; $p = 0.0274$; Figure 15), where weekly measurements of tadpoles were consistently lower in the positive reference water sample and highest in the 1-R water sample until the last two sample

Table 3: Means and ± standard deviations of water quality measurements sampled every 2 days from April 11 to June 12, 2002 at the 1-R, 4-A, and 6-A sites. All units are mg/L except pH and conductivity (μS/cm). Significant differences are indicated in the table with repeated letters. Data courtesy of the Environment Canada Aquatics Section.

Analyte	1-R					4-A					6-A				
	N	Mean	Standard Deviation	Minimum	Maximum	N	Mean	Standard Deviation	Minimum	Maximum	N	Mean	Standard Deviation	Minimum	Maximum
Alkalinity	35	44 [a b]	7.0	31	57	35	57 [a c]	7.3	42	68	35	94 [b c]	10	60	107
Ammonia	9	0.014 [d]	0.010	0.0050	0.032	31	0.011 [e]	0.0035	0.006	0.020	34	0.069 [d e]	0.064	0.012	0.32
Calcium	35	21 [f g]	3.6	14	27	35	27 [f h]	3.8	20	33	35	38 [g h]	4.1	24	42
Conductivity	35	131 [i j]	22	87	168	35	170 [i k]	22	127	207	35	245 [j k]	26	171	282
Hardness	35	59 [l m]	10	40	75	35	77 [l n]	11	56	93	35	115 [m n]	13	73	129
Magnesium	35	1.5 [o p]	0.25	1.0	1.9	35	2.1 [o q]	0.30	1.5	2.6	35	5.0 [p q]	0.59	3.1	5.8
Nitrite	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	34	0.011	0.0033	0.0070	0.020
Nitrite and Nitrate	35	0.21 [r s]	0.04	0.13	0.29	35	0.30 [r t]	0.056	0.20	0.41	35	0.67 [s t]	0.12	0.35	1.0
Orthophosphate	9	0.0016	0.0010	0.0010	0.0040	16	0.0019 [u]	0.0011	0.0010	0.0040	35	0.017 [u]	0.025	0.0020	0.11
pH	35	7.7	0.17	7.1	7.9	35	8	0.17	7	8	35	7.7	0.20	7.3	8.1
Potassium	35	0.23 [v]	0.053	0.10	0.30	35	0.32 [w]	0.080	0.10	0.50	35	1.1 [v w]	0.51	0.70	3.0
Silicon	35	3.1 [w y]	0.22	2.7	3.5	35	3.6 [w z]	0.23	3.1	4.1	35	5.1 [y z]	0.42	3.8	5.7
Sodium	35	1.7 [aa bb]	0.23	1.2	2.0	35	2.3 [aa cc]	0.28	1.8	2.8	35	3.4 [bb cc]	0.27	2.7	3.8
Total Dissolved Phosphorous	28	0.0047 [dd]	0.0013	0.0020	0.0070	32	0.006 [ee]	0.0018	0.0020	0.010	35	0.024 [dd ee]	0.027	0.0070	0.13
Total Nitrogen	35	0.28 [ff gg]	0.04	0.19	0.41	35	0.41 [ff hh]	0.064	0.29	0.57	35	1.0 [gg hh]	0.33	0.70	2.1
Total Phosphorous	34	0.0083 [ii]	0.0042	0.0030	0.0230	34	0.015 [ij]	0.015	0.0030	0.096	35	0.053 [ii ij]	0.051	0.016	0.24

NA = Not Analyzed

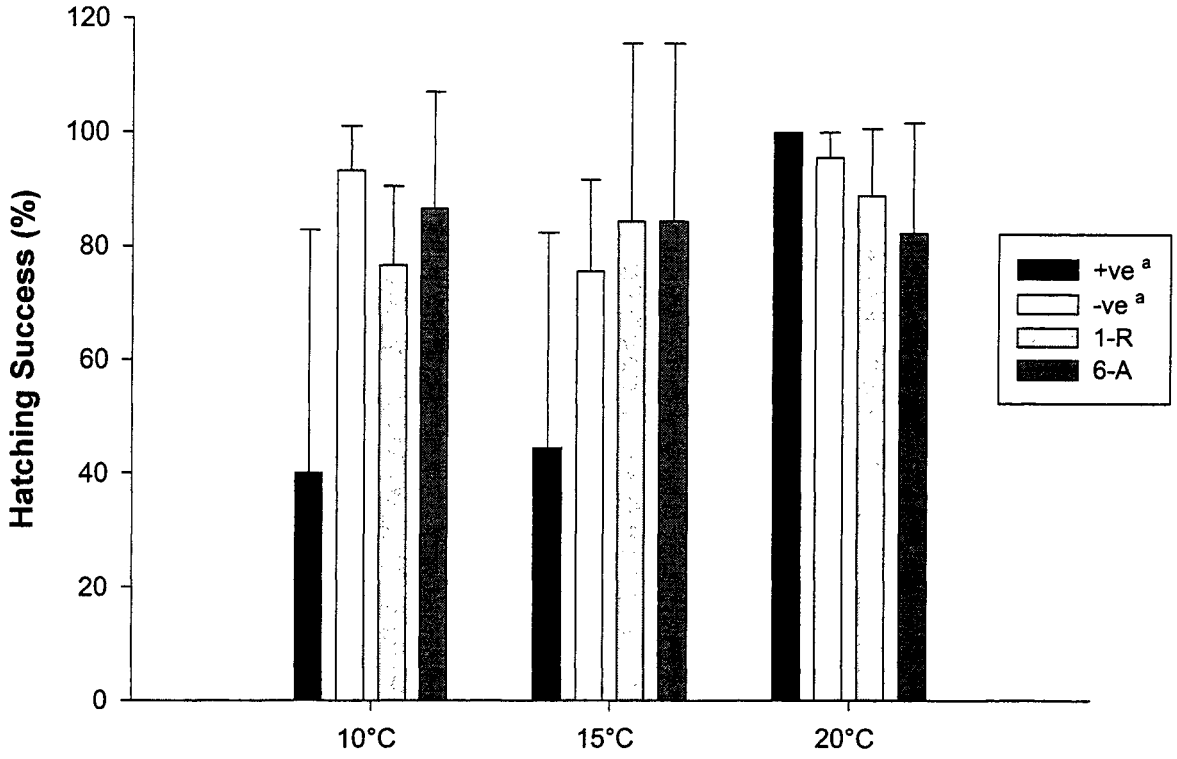


Figure 14: *R. aurora aurora* hatching success (%) in water samples from the positive (+ve; black) and negative (-ve; white) reference and the Elk Creek 1-R and 6-A sites (gray) at three temperatures (10°C, 15°C, 20°C). Repeated letters represent $p \leq 0.05$. Error bars represent ± 2SE.

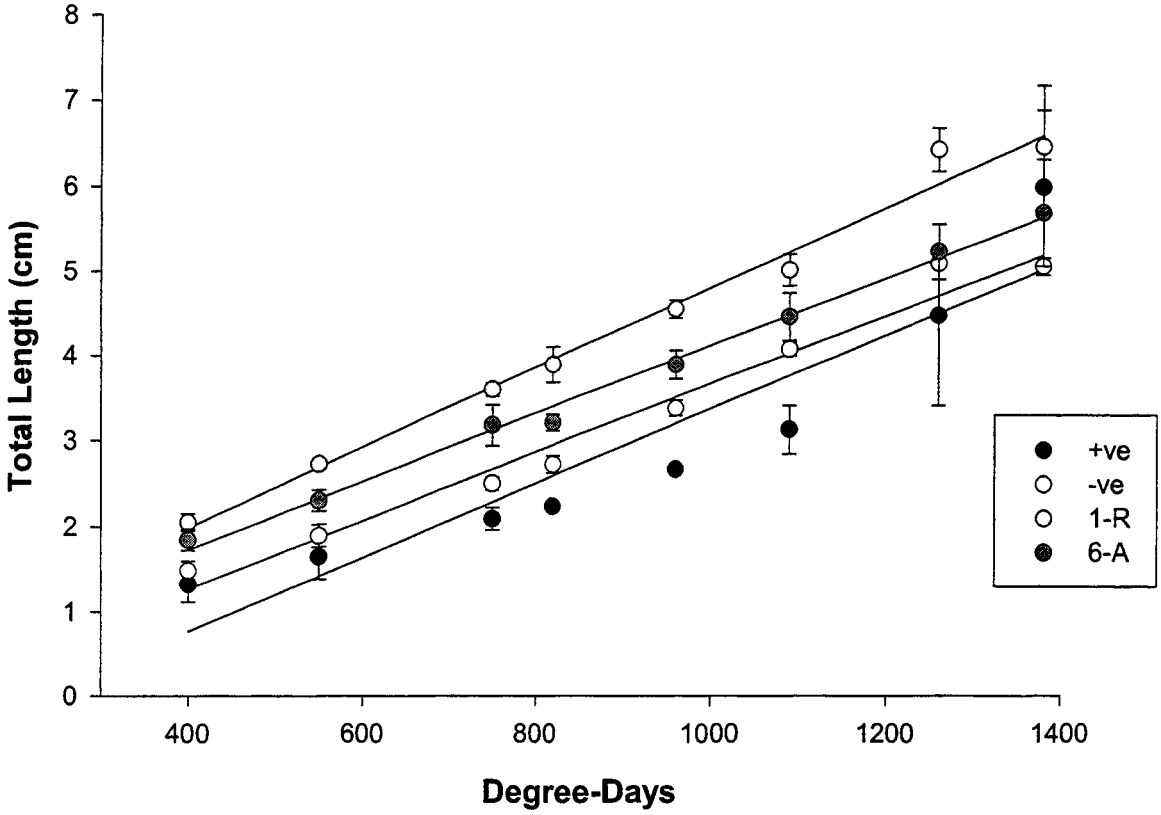


Figure 15: *R. aurora aurora* tadpole growth as total length (cm) versus degree-days from hatching to stage 31, in water samples from the positive (+ve; black) and negative (-ve; white) reference and the Elk Creek 1-R and 6-A sites (gray) at 10°C. All site differences are significant at $p \leq 0.05$. Error bars represent ± 2SE.

dates. On the second last sample day, only tadpoles in the 1-R sample water were significantly longer than all other tadpoles. On the last sample day, there were no significant differences. Significant differences were not observed at 15°C ($F = 1.68$; $p = 0.2846$) and 20°C ($F = 0.81$; $p = 0.5378$). Tadpoles at 10°C did not initiate metamorphosis, while those at 15°C and 20°C completed transformation to juveniles. Water samples also did not have an effect on survivorship and snout-to-vent length (SVL) at the three temperature treatments. For survivorship there was no significant effect of temperature ($F = 0.34$; $p = 0.5654$) or water samples ($F = 0.12$; $p = 0.9452$) at 15°C and 20°C, and there was no significant interaction water sample and temperature interaction ($F = 0.55$; $p = 0.6752$; Figure 15). At 10°C, water sample did not have an effect on survivorship ($F = 1.037$; $p = 0.4305$). For SVL, although at the same stage, water temperature had a significant effect on SVL between juveniles at 15°C and 20°C ($F = 9.73$; $p = 0.0089$), with juveniles at 15°C (2.71 cm) on average larger than juveniles at 20°C (2.44 cm; Figure 16). However, water samples did not have a significant effect on SVL when adjusted for temperature ($F = 0.63$; $p = 0.6101$) at 15°C and 20°C, and there was no significant interaction water sample and temperature interaction ($F = 0.41$; $p = 0.7491$). At 10°C, water sample did not have an effect on SVL ($F = 0.75$; $p = 0.5319$).

Biochemical indicators of stress were compared among water samples at 15°C and 20°C, and among water samples at 10°C. For plasma glucose, there was no significant main effect of temperature ($F = 0.20$; $p = 0.6655$) or water

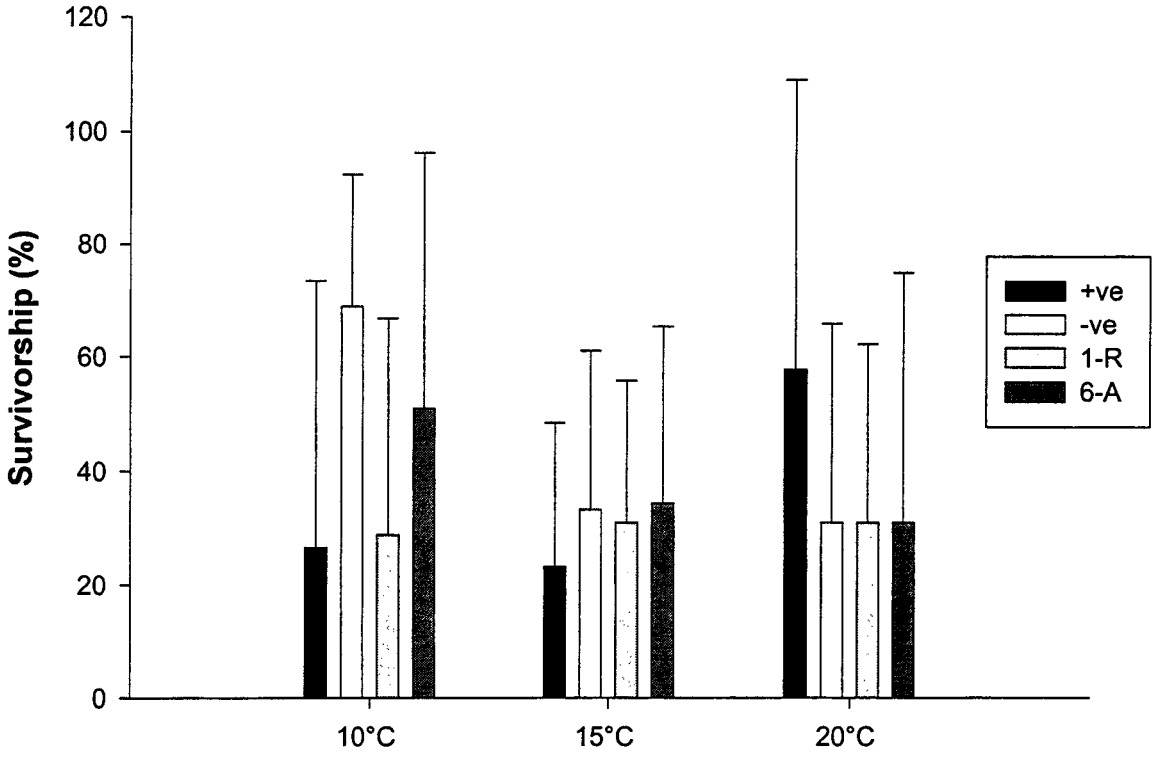


Figure 16: *R. aurora aurora* survivorship (%) in water samples from the positive (+ve; black) and negative (-ve; white) reference and the Elk Creek 1-R and 6-A sites (gray) for tadpoles at 10°C and juveniles at 15°C and 20°C. Error bars represent ± 2SE.

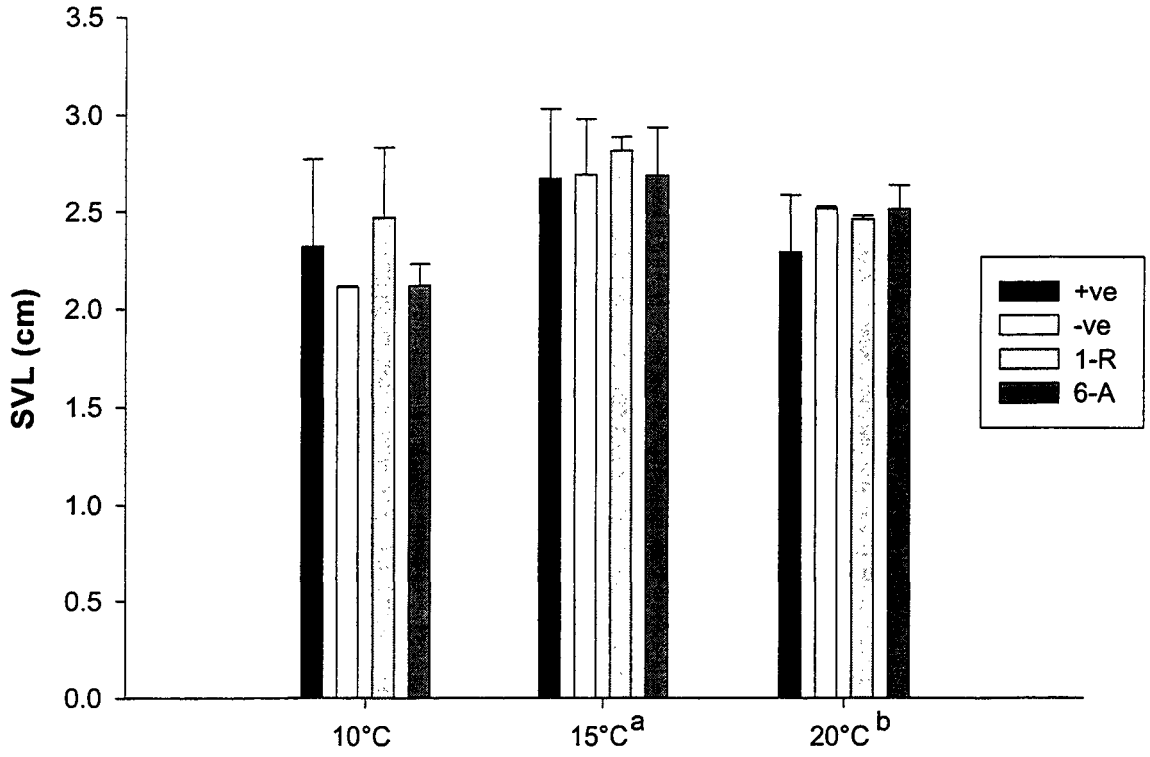


Figure 17 *R. aurora aurora* snout-to-vent length (SVL; cm) in water samples from the positive (+ve; black) and negative (-ve; white) reference and the Elk Creek 1-R and 6-A sites (gray) for tadpoles at 10°C and juveniles at 15°C and 20°C. Repeated letters (small caps within temperature treatments and large caps between temperature treatments) represent $p \leq 0.05$. Error bars represent ± 2SE.

samples (F = 1.30; p = 0.3233) at 15°C and 20°C, and there was no significant interaction water sample and temperature interaction (F = 2.37; p = 0.1259).At 10°C, water samples did not have a significant effect on plasma glucose (F = 0.83; p = 0.5169). Plasma glucose results are plotted in Figure 17.

For plasma lactate, there was a significant main effect of temperature (F = 7.21; p = 0.0212) but not water samples (F = 0.06; p = 0.9809) at 15°C and 20°C (Figure 18). There was also a significant water sample and temperature interaction (F = 3.71; p = 0.046). Between water samples, a significant difference was only observed for 1-R at 15°C and 20°C (p = 0.0252). At 10°C, water samples did not have a significant effect on plasma lactate (F = 2.52; p = 0.1419).

For haematocrit, there was no significant main effect of temperature (F = 1.41; p = 0.2578) or water samples (F = 1.68; p = 0.2250) at 15°C and 20°C, and there was no significant interaction between water samples and temperature (F = 1.40; p = 0.2915; Figure 19). At 10°C, water samples did have a significant effect on haematocrit (F = 8.6; 0.0095). Haematocrit values were significantly higher in tadpoles in water from the 1-R site (9.99%) than in tadpoles from the positive reference (6.00%; p = 0.012) and negative (6.99%; p = 0.0174) sites.

Significant effects on leucocrit were also not observed for temperature (F = 0.73; p = 0.4116) and water samples (F = 3.47; p = 0.0542) at 15°C and 20°C, there was no significant interaction between water samples and temperature (F = 0.16; p = 0.9291; Figure 20). At 10°C, water samples did not have a significant effect on leucocrit (F = 0.96; p = 0.4611).

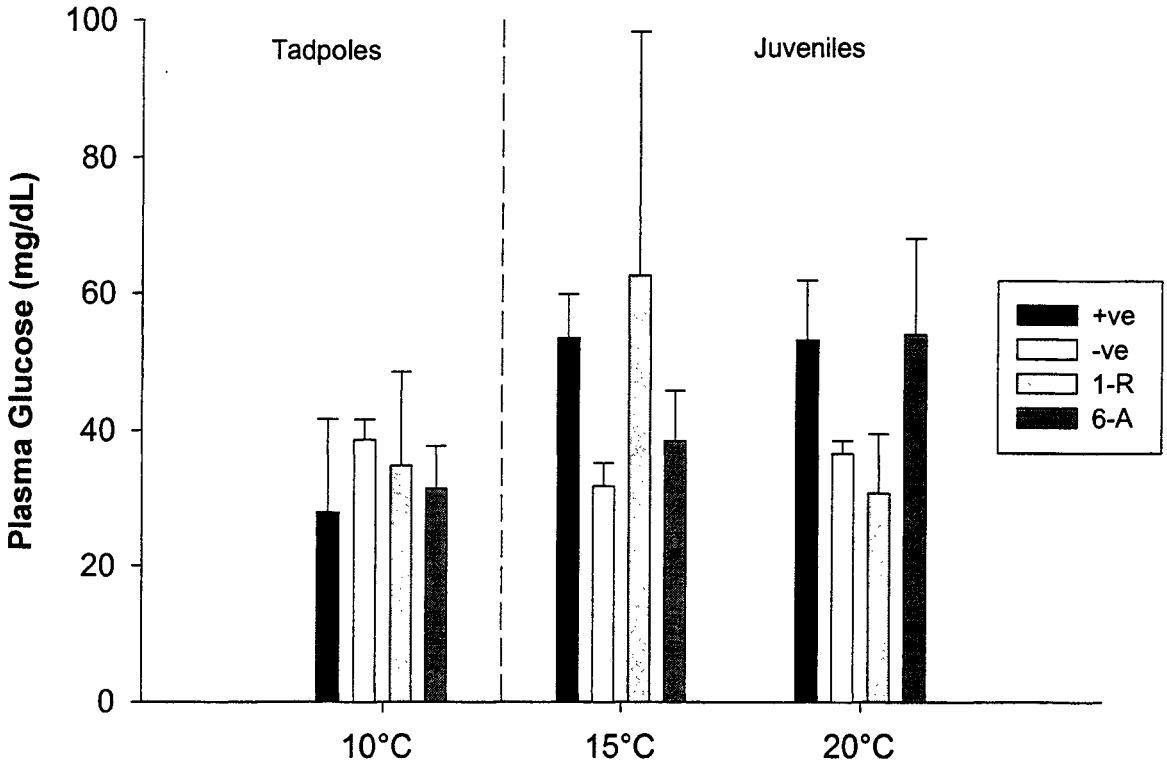


Figure 18: *R. aurora aurora* plasma glucose levels (mg/dL) in water samples from the positive (+ve; black) and negative (-ve; white) reference and the Elk Creek 1-R and 6-A sites (gray) for tadpoles at 10°C and juveniles at 15°C and 20°C. Error bars represent ± 2SE.

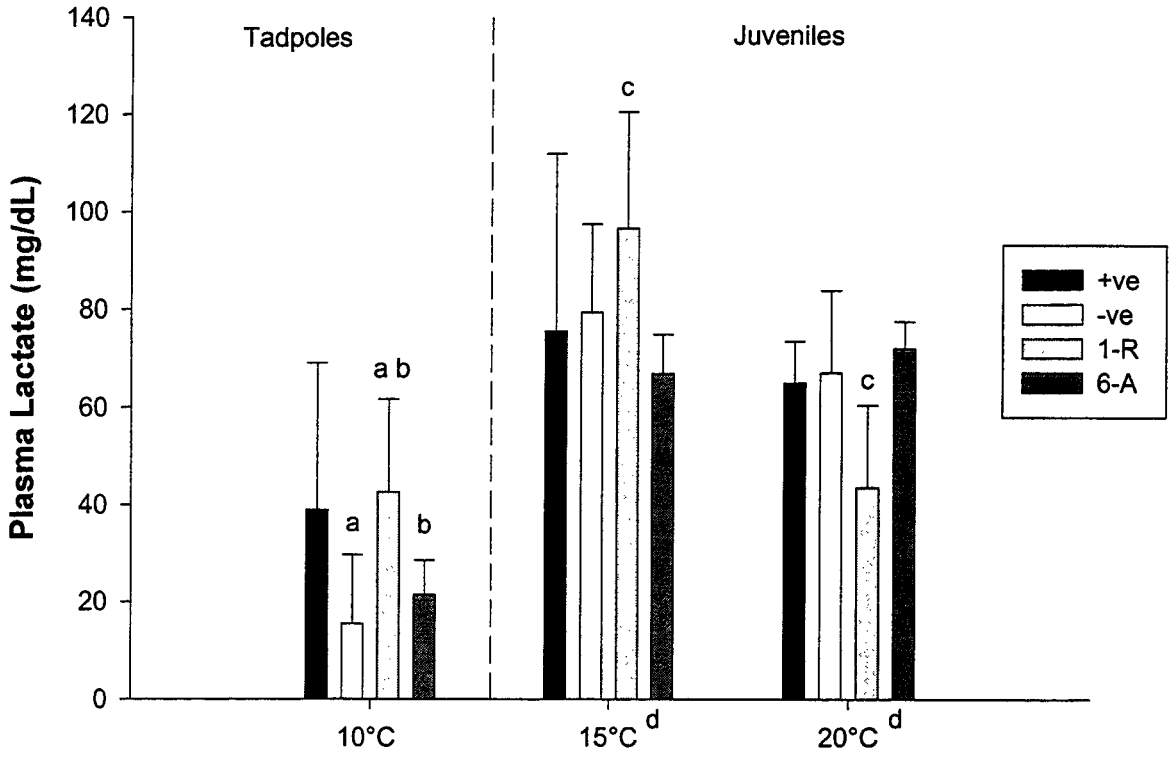


Figure 19: *R. aurora aurora* plasma lactate levels (mg/dL) in water samples from the positive (+ve; black) and negative (-ve; white) reference and the Elk Creek 1-R and 6-A sites (gray) for tadpoles at 10°C and juveniles at 15°C and 20°C. Repeated letters represent $p \leq 0.05$. Error bars represent ± 2SE.

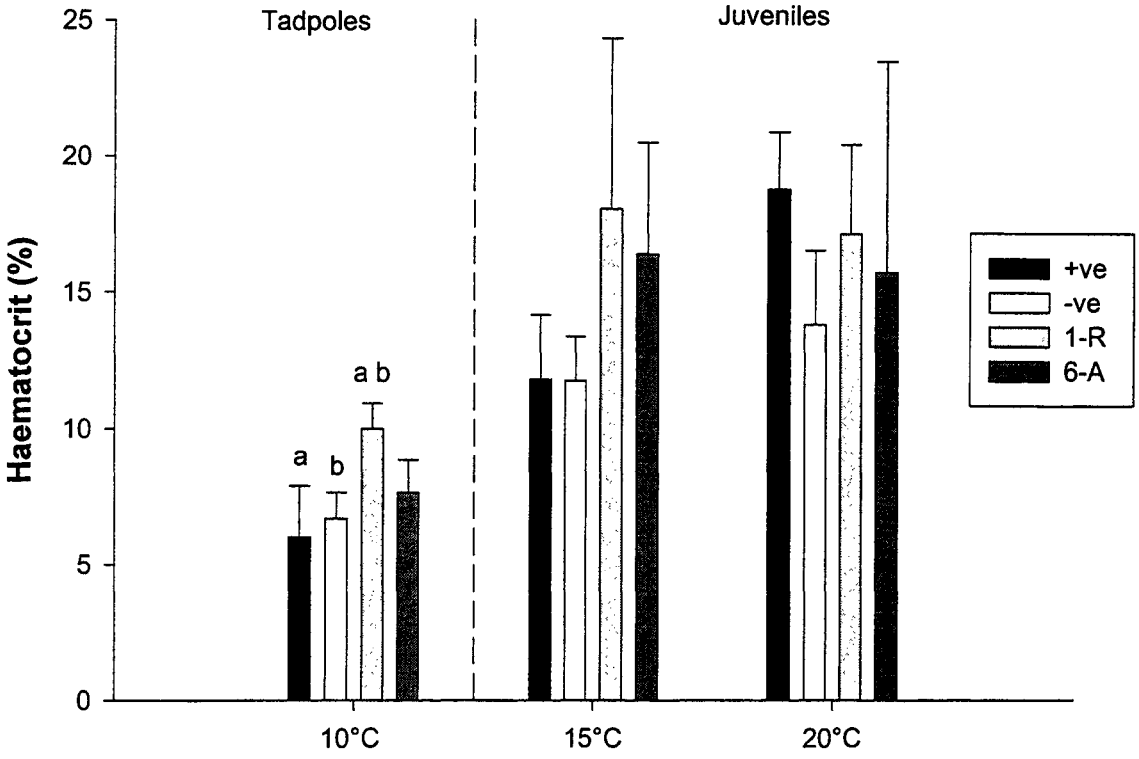


Figure 20: *R. aurora aurora* haematocrit values (%) in water samples from the positive (+ve; black) and negative (-ve; white) reference and the Elk Creek 1-R and 6-A sites (gray) for tadpoles at 10°C and juveniles at 15°C and 20°C. Repeated letters represent $p \leq 0.05$. Error bars represent ± 2SE.

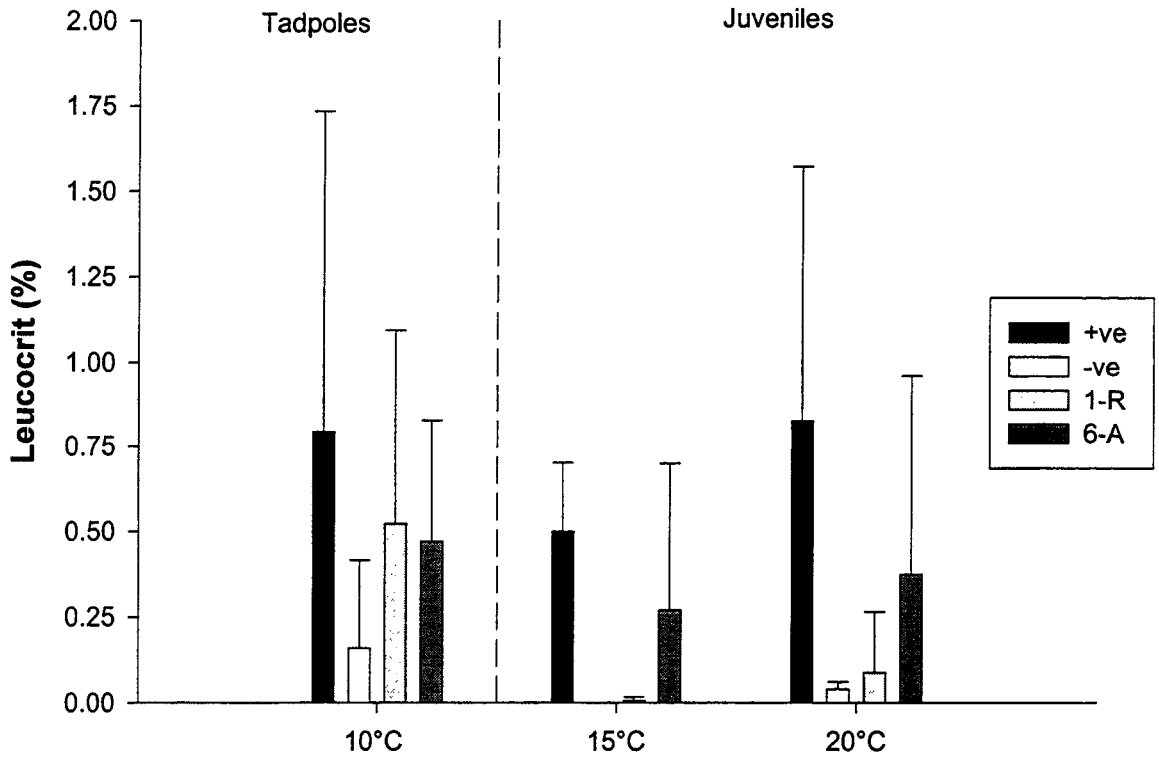


Figure 21: *R. aurora aurora* leucocrit values (%) in water samples from the positive (+ve; black) and negative (-ve; white) reference and the Elk Creek 1-R and 6-A sites (gray) for tadpoles at 10°C and juveniles at 15°C and 20°C. Error bars represent ± 2SE.

G. aculeatus

Mesocosm site water did not have a significant effect on survivorship of *G. aculeatus* (F = 1.14, p = 0.3390; Figure 22). Survivorship at sites 1-R 4-A, and 6-A was 25%, 12.5%, and 17%, respectively.

Measurements of haematocrit and leucocrit values in *G. aculeatus* to Elk Creek site water over a 3-month period did not indicate a stress response related to water samples. Site water did not have a significant effect on haematocrit (F = 0.32; p = 0.7331; Figure 23), and leucocrit (F = 3.36; p = 0.0766; Figure 24). Elk Creek mesocosm water did not have a significant effect on plasma cortisol levels in *G. aculeatus* exposed to agricultural effluent for 3 months (chronic) and 5 days (acute) (F = 0.71; p = 0.6128; Figure 25). As expected, induction with ACTH produced a significantly higher stress response (F = 38.60; p = 0.0008) than the chronic exposure group not given an ACTH injection.

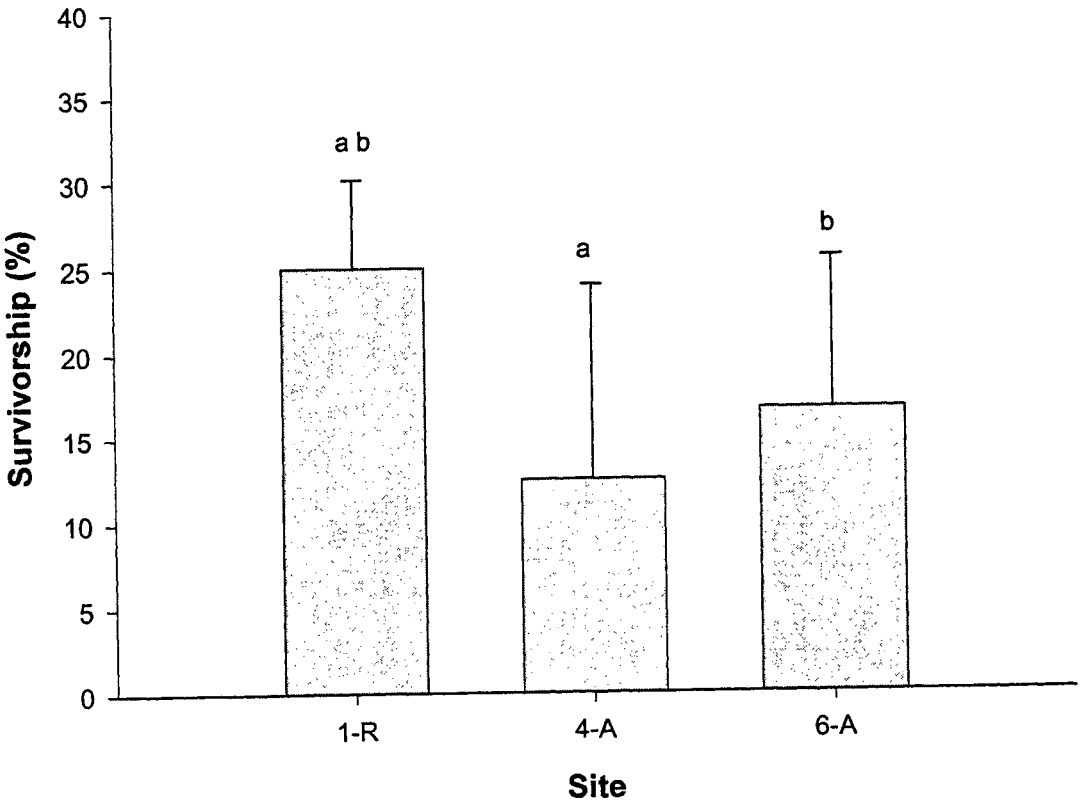


Figure 22: *G. aculeatus* survivorship at the Elk Creek 1-R, 4-A, and 6-A mesocosm sites. Repeated letters represent $p \leq 0.05$. Error bars represent ± 2SE.

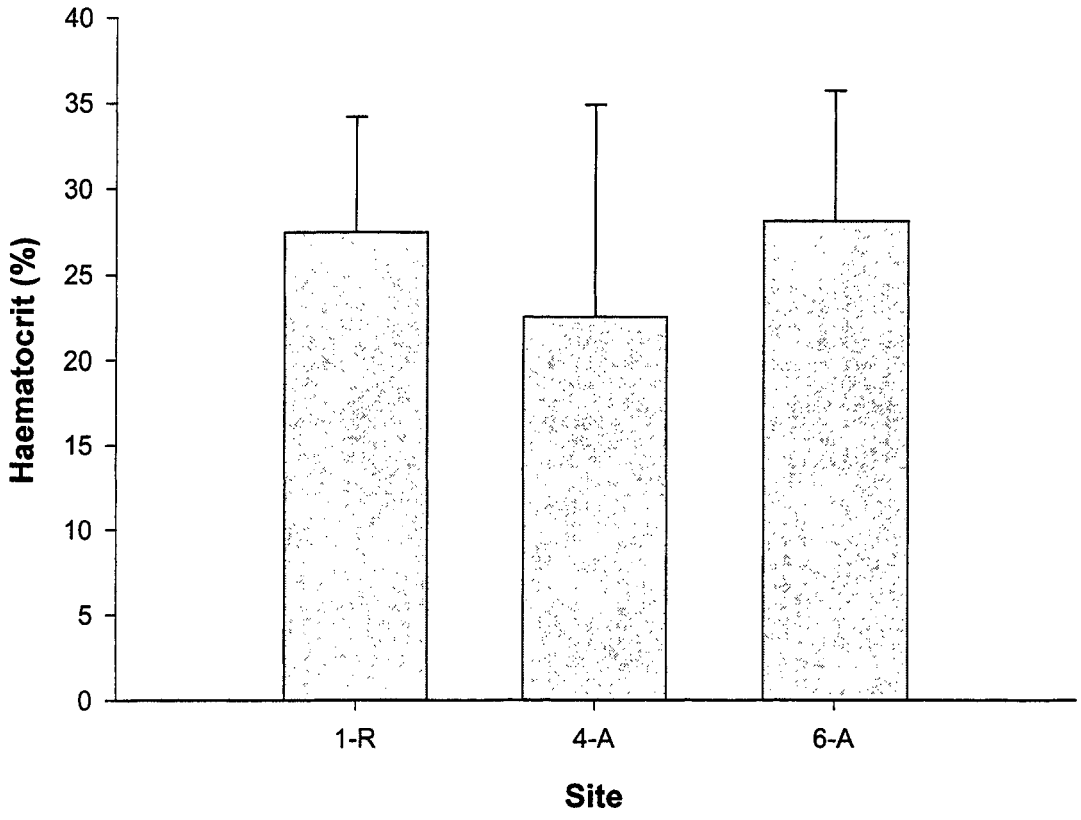


Figure 23: *G. aculeatus* haematocrit values (%) at the Elk Creek 1-R, 4-A, and 6-A mesocosm sites. Repeated letters indicate significant differences at $p \leq 0.05$. Error bars represent ± 2SE.

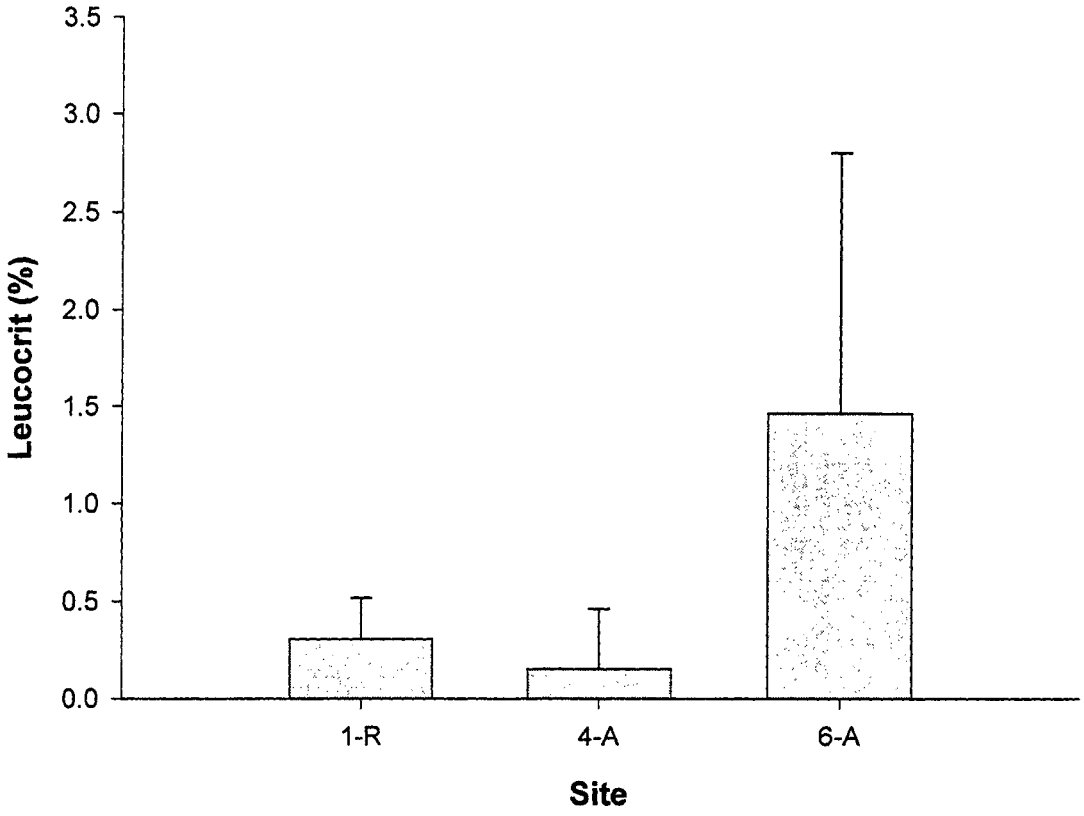


Figure 24: *G. aculeatus* leucocrit values (%) at the Elk Creek 1-R, 4-A, and 6-A mesocosm sites. Repeated letters indicate significant differences at $p \leq 0.05$. Error bars represent ± 2SE.

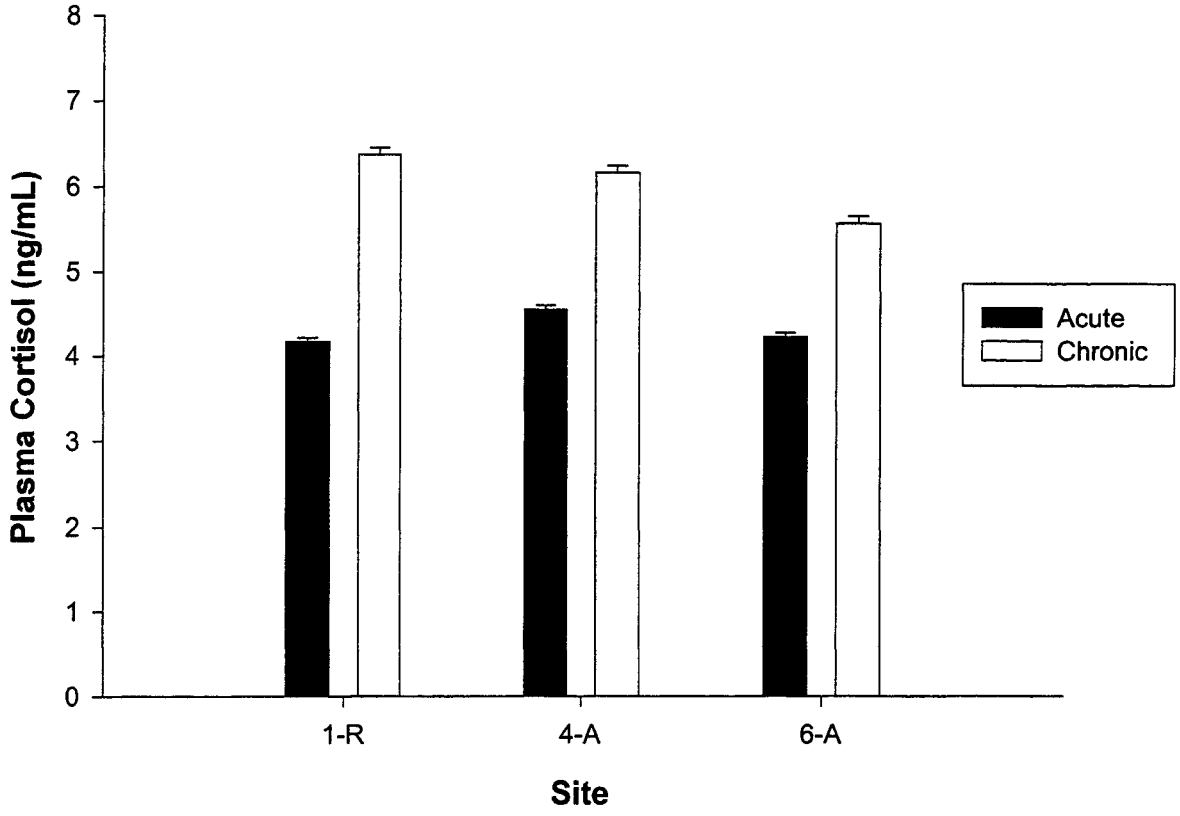


Figure 25: *G. aculeatus* plasma cortisol levels (mg/dL) following acute exposure and chronic exposure after induction with ACTH. Repeated letters indicate significant differences at $p \leq 0.05$. Error bars represent ± 2SE.

Discussion

In an effort to investigate temperature modulation on the effects of whole agricultural effluent exposure to amphibians, *R. aurora aurora* early-life stages were raised at three different temperatures (10°C, 15°C, and 20°C) in water samples collected from reference and agricultural sites. The stress response induced by exposure to agricultural effluent was also investigated with a *G. aculeatus* ACTH challenge. Negative effects related to water treatment were not observed at any temperature in *R. aurora aurora* and *G. aculeatus,* and the plasma cortisol levels of *G. aculeatus* were not inhibited by chronic exposure to agricultural effluent. Baseline water quality measurements also did not indicate toxic conditions.

For amphibians standard acute and chronic toxicity tests have been conducted at a wide range of temperatures (see review by Power et al. 1989), but the effects of the thermal modulation of toxicity are not well described. In exposures of *R. sylvatica* tadpoles to DDT, increased tissue retention of DDT was observed at 15°C compared to 20°C (Licht 1976). After exposures of pyrethroid insecticides, *Rana* spp. tadpoles had faster recovery times at 20°C compared to 15°C (Berrill 1993). At higher pyrethroid concentrations, recovery was only possible at 20°C. In 96-hour exposure studies of carbaryl (Boone and Bridges 1999) and esfenvalerate (Materna et al 1995), temperature increases of 5°C and 4°C, respectively, resulted in increased tadpole mortality. However, the investigators speculated that the mortality was a result of the fatal metabolic

depletion of the energy reserves in the unfed tadpoles at higher temperatures. Increased hatching success was observed in the Jefferson salamander (*A. jeffersonianium*) at 15°C compared to 10°C in acidic conditions (pH 4.5) (Horne and Dunson 1994). The investigators speculated that higher temperatures shortened the development time during debilitating acidic conditions, thereby lessening the toxic effect. Thus, the effect of temperature modulation on xenobiotic exposure may be hard to predict.

The results of the present study emphasizes the importance of performing exposure studies at more than one temperature, especially with endpoints that are affected by temperature. Significant differences among sites were only observed at 10°C for tadpole length, for instance. While a toxic effect on growth was not supported with other results, and there were no differences at the end of the exposure period, a lower temperature may lengthen the development time during which a toxic effect may occur. In addition, plasma lactate levels, for instance, were significantly higher at 15°C than at 20°C. In contrast, Wine and Gatten (1992) observed lower resting levels of total lactate in *R. pipiens* at 15°C than at 20°C. Metabolic and physiological process, such as glycolysis, typically slow at cooler temperatures, and, consequently, tissue requirements for energy and oxygen diminish. Therefore, the biochemical endpoints measured in this study were expected to be lower at cooler temperatures. The most significant result, however, was the absence of effects related to water treatment at controlled temperatures in this study, in comparison to the previous *in situ* study

(Chapter II), in which effects were observed, but at uncontrolled temperatures. This suggests that temperature is a major confounding factor in the interpretation of the results for biochemical endpoints. However, its effect on the toxicity of the agricultural effluent is unclear.

Another possible contributing factor to the difference in results between the two studies is experimental design stress. In the *in situ* study, stress levels (plasma glucose, plasma lactate, and haematocrit) were higher at the agricultural sites than in this study at all temperatures. The laboratory environment of this study, therefore, may have been more stressful than the caged *in situ* environment in Chapter II, and the chronic stress response of animals may have been exhausted (Hontella 1998). However, the ACTH challenge with *G. aculeatus* demonstrates that the mesocosm exposure environment does not affect the chronic stress response, and the mesocosm exposure is probably very similar to the laboratory environment.

An alternate explanation for the difference between results in the *in situ* study and this study is that the variability in the agricultural effluent in 1999 and 2000 produced different effects. During the 1999 exposure period, the toxicity of agricultural effluent may have been greater than in 2000, producing the strong clear trend in the endpoints measured. The suggestion is supported by the absence of a significant post-hatching mortality at the upstream sites as was observed as in the *in situ* study. Differences in tadpole stage sensitivity are not likely, because the tadpoles at 10°C in this experiment were at a similar stage of

development. However, comparable water quality measures (ammonia, calcium, magnesium, nitrite+nitrate, hardness, pH, potassium, silicon, sodium, and total phosphorous) between years indicate no difference in water quality. Significant differences in stickleback survivorship related to the level of agricultural activity were also observed in both years, and no observed change in land-use was observed between years. Therefore, variation in agricultural effluent between years may only be for short time periods.

The predictable effects of temperature modulation on growth and development can be more conclusively applied to a management strategy. In this study, metamorphosis was delayed and individuals were larger at 15°C when compared to individuals at 20°C in all effluent exposure groups. Assuming variables such as density and food availability are kept constant (Newman 1998), a decrease in ambient temperature during the embryo and larval period will result in a longer time to metamorphosis, and a larger size at metamorphosis (Kollros 1961, Smith-Gill and Bervin 1979; see review by Ultsch 1999). This supports the speculation that cool, late-season water temperatures (average temperature of approximately 12°C) in the *in situ* study caused the failure of caged tadpoles to undergo metamorphosis. The average minimum water temperature for initiation of metamorphosis in this population of *R. aurora aurora* may be between 12°C and 15°C. Thus, if thermal requirements are limiting factors for amphibians in Elk Creek, then more marginalized, ephemeral, and protected habitat is needed to support amphibians in the watershed. The current population naturally breeds in

a limited number of field ditches with short hydroperiods (personal observation) that may receive. If the agricultural effluent that enters these field ditches is also toxic for acute durations, then the poor quantity and quantity of this habitat may not be suitable to support a viable population in the watershed.

In summary, ambient temperature is an important factor in assessing the effect of the agricultural effluent in Elk Creek, since temperature may modulate the endpoints measured and the toxicity of the effluent. The agricultural effluent in Elk Creek is most likely not consistent from year to year, and may be toxic for acute durations, as evidenced by periods of high *R. aurora aurora* mortality. Finally, the temperature of Elk Creek, which did not reach levels required for metamorphosis, may prohibit this site as suitable aquatic habitat for *R. aurora aurora*. The lack of suitable of habitat may have a negative impact on amphibians in the Elk Creek watershed.

Chapter IV: The Effect of Agricultural Effluent on Plasma Thyroid Hormone Levels in Red-legged Frogs (*Rana aurora aurora*)

Note: This chapter was written in the "Short Notes" format of the Journal of Herpetology.

Amphibian metamorphosis is a sensitive developmental stage that is dependent on the interaction of several hormones. The thyroid hormones thyroxine (T_4) and triiodothyronine (T_3) are the major hormones involved in metamorphosis and induce many functional and morphological changes, including tail reabsorption, foreleg emergence, sloughing of the opercular membrane, regression of the gills, reorganization of the intestinal tract, and restructuring of the mouth and head (Duellman and Trueb, 1986; Hayes, 1997). Any disruption of the level of these hormones may have fatal or maladaptive consequences to an individual. Therefore, amphibian metamorphosis may be a useful tool for the identification of endocrine disrupting chemicals (EDC's) in areas such as agricultural settings where chemical pesticides may enter the aquatic environment (Wauchope, 1978; Spalding and Snow, 1989)

Several chemicals, including pesticides, are capable of reducing plasma thyroid hormones and inhibiting tail reabsorption during metamorphic climax in *Xenopus laevis* (Fort et al. 1999; Fort et al., 2000). However, the *Xenopus* response to EDC's may differ from other anurans (Hayes, 1999), possibly limiting its application to the assessment of the impact of EDC's on native species. For

other amphibian species, endocrine disruption by pesticides has not been well described. Atrazine can increase plasma thyroid hormones in transforming *A. tigrinum* (Larson et al., 1998), while malathion may decrease circulating thyroid hormone levels in *R. catesbeiana,* as evidenced by delayed metamorphosis (Fordham et al., 2001).

The objective of this research was to compare plasma thyroid hormone concentrations in the blood of prometamorphic *R. aurora aurora* tadpoles that were potentially exposed to endocrine-disrupting chemicals in whole agricultural effluent and dechlorinated tap water. Amphibians were exposed to whole agricultural effluent since wild amphibians are more likely to be exposed to changing mixtures of EDC's whose synergistic (Howe et al. 1998) and environmental interactions may be unpredictable (Wong and Dixon, 1995) and different than single chemical exposures.

Currently, the Sumas Prairie in the lower Fraser Valley, BC, is used primarily for livestock, market crops, and sod (de Solla et al. 2002b). Organochlorine pesticides were used in the area in the 1970s (Finizio et al., 1998; de Solla et al. 2002b), while today organophosphorous insecticide use is widespread (Wan et al., 1994; de Solla et al. 2002b). Agricultural run-off at this site may contribute to diminished *R. aurora aurora* hatching success (de Solla et al. 2002b) and an elevated stress response in newly metamorphosed *R. aurora* juveniles (Chapter II). Organochlorine residues have not been found in

northwestern salamander eggs (de Solla et al. 2002a) and adult green frogs (Chapter V) at levels likely to elicit a toxic effect at this site.

Two *R. aurora aurora* egg masses were collected in March, 2000, from a relatively uncontaminated site, which receives mountain run-off on the periphery of the Sumas Prairie in British Columbia, Canada, and where hatching success is proven to be 85% (de Solla 2002b). Eggs were allowed to hatch in dechlorinated tap water. To eliminate individual bias, genetic differentiation between egg masses was verified through amplification of a mitochondrial gene unique to *R. aurora aurora* (Blouin, M., personal communication). Hatched larvae were then separated into two exposure treatment groups. The reference treatment consisted of weekly renewals of dechlorinated tap water, while the agricultural treatment consisted of weekly renewals of water from a site adjacent to intensive agricultural activity. All aquaria were slightly aerated and photoperiod was adjusted weekly to match natural light cycles (12 hours light and 12 hours dark in spring to 16 hours light and 8 hours dark in mid-summer). Water temperature for all tanks was kept at 20°C ± 1°C. Tadpoles were fed store bought green lettuce boiled for three hours *ad libitum* for the duration of the experiment. Every two days fresh food was added to each aquaria and old food was removed. The exposure was completed when animals were first observed to reach metamorphic climax at Gosner stage 41 (Gosner, 1960), based on daily evaluations, which corresponds to the highest levels of circulating thyroid hormones. Right tibia length measurements were made as an indication of

metamorphic changes and thyroid hormone induced limb development. Blood samples were collected in micro-capillary tubes following cardiac puncture. Blood was immediately centrifuged and the plasma frozen at -80°C until analysis. Plasma samples were analyzed for T_3 levels with ELISA kits (Monobind Inc., Costa Mesa, CA). Data were compared using a student's *t*-test with Jmp IN 3.0 (Cary, NC) statistical software.

Tibia lengths of animals from the reference and agricultural groups were not significantly different (Table 4) and all animals reached Gosner stage 41 (Gosner, 1960) at the same time. No mortality or deformity was observed in either treatment. Agricultural effluent did not significantly affect development or plasma T_3 concentrations in transforming tadpoles. Although T_3 levels were higher at the agricultural site (521 ng/dL ± 213 ng/dL) when compared to the uncontaminated reference (247 ng/mL ± 213 ng/mL), the differences were not significant ($p=0.46$). The observed levels of T_3 were slightly higher than those observed for *R. catesbeiana* tadpoles (Miyauchi et al., 1977; Burggren and Just 1992) and *A. tigrinum* (Larras-Regard et al., 1981; Larson et al., 1998) at metamorphic climax, but similar to those observed for *X. laevis* at metamorphic climax (Schultheiss, 1980; Shi, 2000). Water quality data from these sample locations was not available.

Many factors, including temperature, rearing density, and food availability and quality, have the potential to modulate the speed of metamorphosis (Larson et al., 1998). While laboratory studies, like this one, have the advantage of

Table 4: Plasma T_3 levels and tibia lengths for both groups (n=2; each replicate a mean value of 15 tadpoles per aquarium). Errors represent ± 2 SEM (SEM were calculated from a random effects model with a synthetic denominator). Differences were not significant (plasma T_3 t = 0.9074, p = 0.46; tibia length t = 0.0407, p = 0.97)

Treatment	Plasma T_3 (ng/dL)	Right Tibia Length (cm)
Agricultural Site	521 ± 213	0.57 ± 0.09
Reference	247 ± 213	0.56 ± 0.09

controlling these variables, realistic exposures and conditions may not be achieved. Metamorphic changes occur so quickly that dynamic exposure conditions may be limited by the frequency of water sample collection.

Organophosphorous insecticides, like malathion, have relatively short-half lives following application (Fordham et al., 2001), and, despite their exposure to wild populations, sampling designs may miss toxic concentration spikes. Nevertheless, alterations in plasma T_3 concentrations in *R. aurora* tadpoles are most likely not occurring at this agricultural site.

Chapter V: Organochlorine Pesticides and Polychlorinated Biphenyl Residues in Green Frogs (*Rana clamitans*) From the Elk Creek and Sumas Prairie Watersheds

Introduction

Organochlorine (OC) pesticides and polychlorinated biphenyls (PCBs) have not been used extensively in developed countries since the mid-1970's. However, because of their long half-lives and lipid solubility, they often concentrate in animal tissues (Sparling 2000). In amphibians, lethal and sublethal effects are associated with body burdens of OC pesticides (Cooke 1970, Cooke 1972, Cooke 1973, Cooke 1979, Gendron et al. 1997) and PCBs (Gendron et al. 1997; Huang et al. 1999; Rosenshield et al. 1999; Gutleb et al. 2000; Savage et al. 2002). In addition, the developmental effects associated with body burdens of dichlorodiphenyl-trichloroethane (DDT) and it metabolites, dichlorodiphenyl-dichloroethane (DDD) and dichlorodiphenyl-dichloroethylene (DDE) in amphibians may be a result of endocrine disruption (Hayes 2000).

In previous studies investigating the effects of agricultural effluent in the Sumas Praire and Elk Creek watersheds in the lower Fraser Valley, British Columbia, Canada, significant effects on survivorship and biochemical indicators of stress were observed in red-legged frogs and northwestern salamander early life stages (Chapters II and III; de Solla et 2001a). Historically, OC pesticides have widely been applied in the Fraser Valley in the 1970s (Finizio et al., 1998), and today organophosphorous insecticides use is widespread (Wan et al., 1994).

However, when OC pesticides and PCBs were measured in northwestern salamander and red-legged frog egg at sites in the Sumas Prairie, the concentrations found were unlikely to elicit toxic effects, with little difference between agricultural and reference sites (de Solla et al. 2002a). Endocrine disruption of Red-legged frog metamorphosis, as measured by concentrations of plasma thyroid hormones, by agricultural effluent from the positive reference, was also not observed (Chapter IV).

The objective of this study was to quantify OC pesticides and PCBs in adult amphibians from the site in the Sumas Prairie and the Elk Creek watershed. OC pesticide residues and PCBs have not previously been measured in amphibians from the Elk Creek watershed. In addition these concentrations can be compared to levels reported for green frogs in southern Ontario (Russell et al. 1997). The concentrations of OC pesticides and PCBs measured in amphibians will also be compared to levels likely to elicit a toxic effect in amphibians.

Materials and Methods

Amphibian Collection

Seven green frogs in total were collected from three sites in the lower Fraser River valley, British Columbia. Green frogs were chosen here because they are a very common introduced species in the area and the tissue levels can be compared to similar data from southern Ontario (Russell et al. 1997). Two

sampling sites were in the Elk Creek watershed and the third sampling site was the positive reference in the Sumas watershed (see Chapter II for a complete description of the site). All three sites were ditches adjacent to intensive agriculture potentially receiving high loads of agricultural runoff. Frogs were stored in hexane-rinsed aluminium foil at –20°C until transportation to the laboratory. Samples were pooled from each site for analyses.

Organochlorine pesticide and PCB analysis

The National Wildlife Research Centre in Hull, Quebec, performed OC pesticide and PCB analysis. The samples were thawed to room temperature and extracted with dichloromethane (DCM):hexane (1:1 v/v) after the samples were dehydrated with anhydrous Na_2SO_4. The quantitative analysis of organochlorine compounds was performed using capillary gas chromatography, coupled with a mass selective detector operated in selected ion monitoring mode. Each cleaned sample was injected twice. The first injection was designed to determine the organochlorine compounds by using twenty-one organochlorine standards. The second injection was to determine PCBs by using Aroclors 1242/1254/1260, at a 1:1:1 quantisation standard mixture. The samples were analysed using the HP 5890 GC #1 and HP Mass Selective Detector HP 5971 (Hewlett-Packard, Wilmington, DE). The detection limit was 0.1 ng/g w.w.. The concentration of trace levels was between 0.1 to 0.9 ng/g w.w.. The total PCBs reported is the sum of 59 non-coplanar PCB congeners. The pesticides measured were

hexachlorobenzene (HCB), pentachlorobenzene (PnCB), α-, β-, and γ-hexachlorocyclohexane (HCH), octachlorostyrene (OCS), heptachlor epoxide (HE), oxychlordane, *trans*- and *cis*- chlordane, *trans*- and *cis*-nonachlor, *p,p'*-DDD, *p,p'*-DDE, *p,p'*-DDT, dieldrin, photomirex, and mirex. The percent recovery of $^{13}C_{12}$ labelled internal standard PCBs ranged from 85% to 94.5%, with a mean percent recovery efficiency of 90.5%. The percent recovery of $^{13}C_{12}$ labelled internal standard of tetra-, penta-, and hexa-chlorobenzene ranged from 82.8% to 88.7% with a mean percent recovery of 85.1%. The reported concentrations of pesticides and PCBs were not corrected for the percent recoveries. Lipids were determined by homogenizing 1-2 g of sample with 4 ml of hexane, and the mixture was centrifuged to isolate the hexane later. The hexane was then dehydrated with anhydrous Na_2SO_4. This process was repeated twice, and the hexane extracts were evaporated and the lipids weighed. All concentrations are reported on a lipid weight basis unless otherwise stated.

Results

Most OC pesticides (1,2,4,5-TClBz, 1,2,3,4-TClBz, α-HCH, β-HCH, γ-HCH, OCS, H.E., oxychlordane, trans-chlordane, cis-chlordane, dieldrin, photomirex, and mirex) were not detected in frog tissues. The percent lipid in the egg masses were very low, and ranged from 0.288% to 0.750% (Table 5). The percent moisture ranged from 79.22 % to 79.98 %. At the Elk Creek sites, HCB concentrations ranged from 0.0133 to 0.0388 μg/g lipid weight (l.w.), p,p'-DDE

Table 5: Concentrations of OC pesticides and PCBs (μg/g l.w.; μg/g w.w. italicized in parentheses), % moisture, and % lipid in green frogs.

	Elk Creek Watershed		Sumas Prairie		
	Nevin Road (n=2)	**Banford Road (n=2)**	**Positive Reference (n=3)**	**Positive Reference (northwestern salamander eggs; de Solla et al. 2002a)**	**Southern Ontario (Russell et al. 1997; means of seven sites)**
Moisture %	79.98	78.06	79.22	96.33	
Lipid %	0.750	0.516	0.288	0.39	
Analyte					
Hexachlorobenzene	0.0133 (*0.0002*)	0.0388 (*0.0001*)	0.0694 (*0.0002*)	0.0769	*0.000329*
p,p'-DDE	0.0267 (*0.0009*)	0.116 (*0.0002*)	0.313 (*0.0006*)	0.385	*0.000269*
Sum of PCB Congeners	0.0533 (*0.0028*)	0.0969 (*0.0004*)	0.972 (*0.0005*)	1.231	*0.007514* [a]
Aroclor (1254/1260), (1:1)	0.213 (*0.0028*)	0.310 (*0.0016*)	2.12 (*0.0016*)	0.949	
Aroclor 1260	0.200 (*0.0045*)	0.388 (*0.0015*)	1.56 (*0.0020*)	0.692	

[a] Sum of PCB congeners 99, 101, 105, 118, 138, 146, 153, 171, 180, 183, 194, 201, and 203.

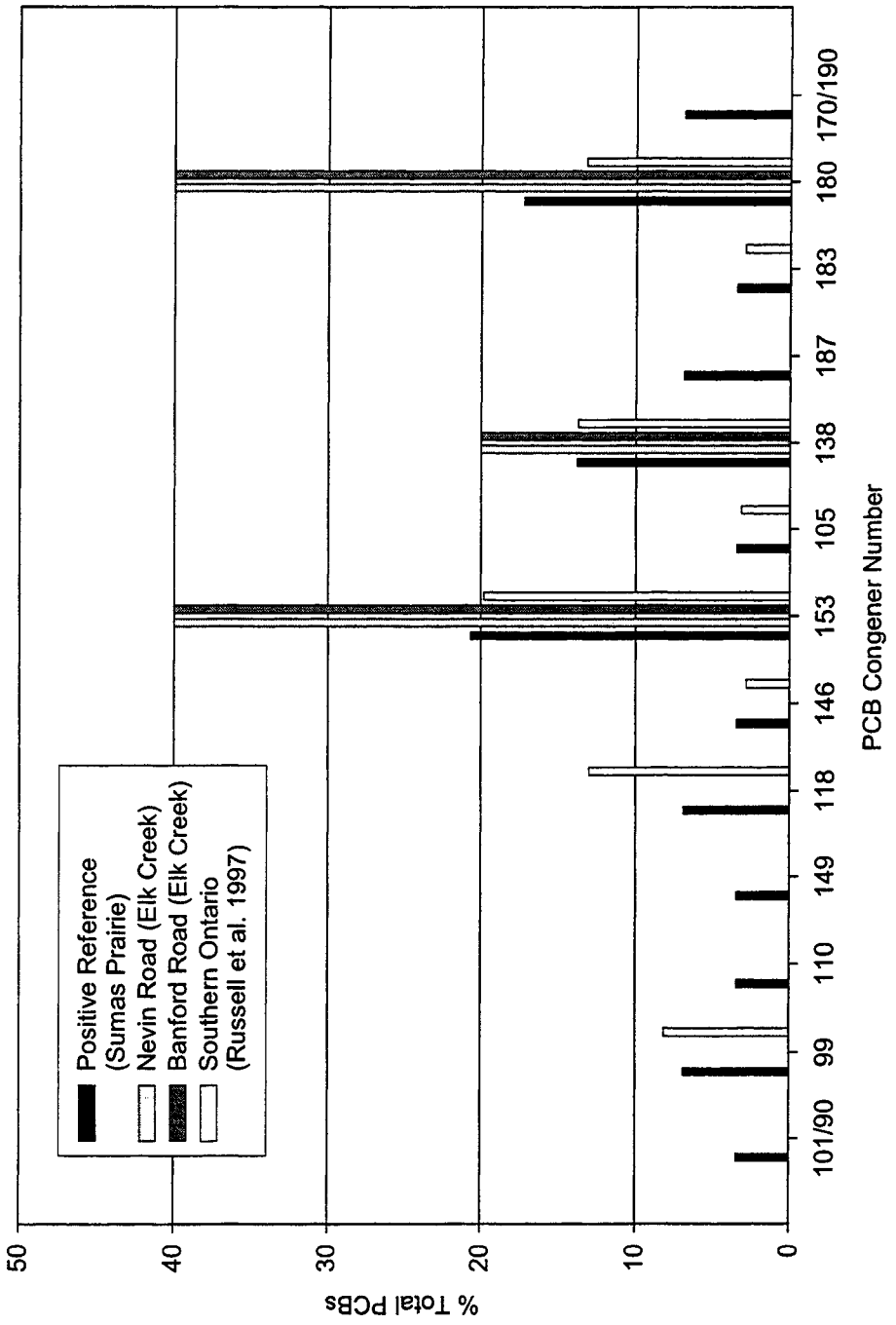
concentrations ranged from 0.0267-0.116 μg/g l.w., while PCBs (sum of PCB congeners) ranged from 0.0969 to 0.0533 μg/g l.w. Both OC pesticides and PCB concentrations were almost an order of magnitude higher in green frogs from the Sumas Prairie when compared to the Elk Creek sites. Concentrations in northwestern salamander eggs in the Sumas Prairie site (de Solla et al. 2002a) had similar concentrations to adult green frogs from the Sumas Prairie. The sum of the PCB congeners was less in green frogs from the Elk Creek and Sumas Prairie watershed in comparison to green frogs from southern Ontario (Russell et al. 1997)

The percent contribution of individual congeners from each site is shown in Figure 26. Only ortho-substituted congeners were detected, and the same general pattern is evident at both watersheds. Major peaks were from congeners 153, 138, and 180/170. The congener profile for green frogs in southern Ontario (Russell et al. 1997) is very similar to the profile for green frogs in the Sumas Prairie. Fewer congeners were detected in the green frogs from the Elk Creek watershed, but this is most likely a function of the low levels detect relative to the method detection limit.

Discussion

The objective of this study was to quantify OC pesticides and PCBs in adult amphibians from two agricultural areas in the Fraser Valley, the Sumas Prairie watershed and the Elk Creek watershed, and compare these concentrations to previously reported levels and levels likely to elicit a toxic effect

Figure 26: Detected PCB congeners in green frogs from two sites in the Elk Creek Watershed and the positive reference site. Congeners are identified according to the IUPAC number.


Positive Reference (Sumas Prairie)
Nevin Road (Elk Creek)
Banford Road (Elk Creek)
Southern Ontario (Russell et al. 1997)
% Total PCBs
0
10
20
30
40
50
101/90
99
110
149
118
146
153
105
138
187
183
180
170/190
PCB Congener Number

in amphibians. Since concentrations in the Sumas Prairie were reported at similar levels in eggs of the northwestern salamander and red-legged frogs (de Solla et al. 2002a) and adult green frogs the reported levels would seem to represent a background level for this area. Similarly, the levels observed in green frogs from two locations in the Elk Creek watershed may also represent a background level. Thus, deposition of OC pesticide and PCB use may have been greater in the Sumas Prairie than in the Elk Creek watershed. The sites sampled by Russell et al. (1997) were not considered highly contaminated in comparison to more heavily contaminated sites in southern Ontario with population-level effects.

The reported concentrations of OC pesticides and PCBs measured in adult green frogs were not at levels likely to elicit negative effects. The lowest reported concentration of DDT that had a toxic effect was 2.4 μg/g wet weight (w.w.) in common frog (*R. temporaria*) tadpoles (Cooke, 1972), and concentrations that were not associated with toxic effects varied from 0.5 to 2.5 μg/g w.w. (Cooke, 1970, 1973, 1979). For PCBs, a whole body concentration of 0.55 μg/g d.w. Aroclor 1260 caused organ damage in tiger salamanders (*A. tigrinum*) (Johnson et al. 1999). Savage et al. (2002) observed decreased activity levels in *R. sylvatica* tadpoles at tissue concentrations of 5.966 μg/g w.w. total PCBs. Mortality was not significantly different from controls at this concentration. The control tadpoles, which probably received PCBs in the laboratory diet, had body burdens of 0.033 μg/g w.w. total PCBs. Huang et al. (1999) found whole

body concentrations did not significant correlate to hepatic ethoxyresorufin-*o*-deethylase levels at whole body tissue concentrations that ranged from 0.023-0.283 μg/g w.w. total PCBs in green frogs

In summary, OC pesticides and PCBs concentrations in adult amphibians from two agricultural areas in the Fraser Valley, the Sumas Prairie watershed and the Elk Creek watershed are present in amphibians at background levels despite discontinued use in the mid-1970's, but not at levels likely to elicit a toxic effect.

Chapter VI: Summary and Conclusions

Agricultural activity can have negative effects on amphibian early-life stages and fish from the run-off of pesticides, fertilizers and nutrients into the aquatic environment. The objective of this study was to determine the effects of whole agricultural effluent from the Elk Creek watershed, BC, on survivorship, growth, and biochemical indicators of stress on early-life stages of red-legged frogs. The Elk Creek watershed is an area of intensive agricultural activity receiving significant management attention in southern British Columbia. For comparison, agricultural effluent exposure effects were also observed in another watershed, the Sumas Prairie, and another species, Threespine stickleback. The major findings of this research were as follows:

(1) Using an *in situ* exposure design, significant effects on *R. aurora aurora* survivorship and biochemical stress indicators (plasma glucose, plasma lactate, haematocrit, and leucocrit) were observed at sites adjacent to agricultural activity. These results were confounded by differences in site water temperature.

(2) An unexplained *R. aurora aurora* mortality event at upstream sites was observed in 1999.

(3) Tadpoles exposed *in situ* at all Elk Creek sites failed to undergo metamorphosis, mostly likely as a result of cool temperatures, in 1999.

(4) Using a mesocosm exposure design in 1999, significant effects were also observed in *G. aculeatus* survivorship and biochemical indicators of stress at sites adjacent to agricultural activity.

(5) Levels of water quality parameters considered toxic were not observed in 1999.

(4) Using a laboratory temperature-controlled (10°C, 15°C, and 20°C) design in 2000, the effects on survivorship and biochemical indicators of stress were not replicated in each temperature regime for *R. aurora aurora*, indicating a significant temperature effect on the biochemical effects measured.

(8) The effects on *G. aculeatus* were also not replicated in 2000, and plasma cortisol levels were also not affected by agricultural activity.

(6) Whole agricultural effluent did not significantly affect plasma thyroid hormone levels in 2000.

(7) Organochlorine pesticide and polychlorinated biphenyl residues were not detected in adult green frogs (*R. clamitans*) at levels likely to elicit toxic effects.

Several conclusions can be suggested from these findings. First, ambient temperature is an important factor in assessing the effect of the agricultural effluent in Elk Creek, since temperature may modulate the endpoints measured and the toxicity of the effluent. Second, the agricultural effluent in Elk Creek is most likely not consistent from year to year, and may be toxic for acute durations, as evidenced by periods of high mortality. Organochlorine pesticides and endocrine disrupters are unlikely contributors to the toxicity of the agricultural effluent. Finally, the temperature of Elk Creek, which did not reach levels required for metamorphosis, may prohibit this site as suitable aquatic habitat for *R. aurora aurora*. The lack of suitable of habitat may have a negative impact on amphibians in the Elk Creek watershed.

References

Adams MJ. 1999. Correlated factors in amphibian decline: exotic species and habitat change in western Washington. *Journal of Wildlife Management*. 63:1162-1171.

Adams SM. 1990. Status and use of biological indicators for evaluating the effects of stress on fish. *American Fisheries Symposium*. 8:1-8.

Alford RA. 1999. Ecology: resource use, competition, and predation. Pp. 240-278 in McDiarmid RW and Altig R, eds. *Tadpoles: The Biology of Anuran Larvae*. University of Chicago Press, Chicago.

Allran JW, Karasov WH. 2000. Effects of atrazine and nitrate on Northern Leopard Frog (*Rana pipiens*) larvae exposed in the laboratory from posthatch through metamorphosis. *Environmental Toxicology and Chemistry*. 19:2850-2855.

Beattie RC, Tyler-Jones R, Baxter MJ. 1992. The effects of pH, aluminum concentration and temperature on the embryonic development of the European common frog, *Rana temporaria*. *Journal of Zoology (London)*. 228:557-570.

Berger L. 1989. Disappearance of amphibian larvae in the agricultural landscape. *Ecology International Bulletin*. 17:65-73.

Berrill M, Bertram S, Wilson A, Louis S, Brigham D, Stromberg C. 1993. Lethal and sublethal impacts of pyrethroid insecticides on amphibian embryos and tadpoles. *Environmental Toxicology and Chemistry.* 12:525-539.

Berrill M, Coulson D, McGillivray L, Pauli B. 1998. Toxicity of endosulfan to aquatic stages of anuran amphibians. *Environmental Toxicology and Chemistry.* 17:1738-1744.

Birge WJ, Westerman AG, Spromberg JA. 2000. In Sparling DW, Linder G, Bishop CA eds. *Ecotoxicology of Amphibians and Reptiles.* Society of Environmental Toxicology and Chemistry. Pensacola, FL. 904 p.

Bishop CA, Mahony NA, Struger J, Ng P, Pettit KE. 1999. Anuran development and diversity in relation to agricultural activity in the Holland River watershed, Ontario, Canada (1990-1992). *Environmental Monograph Assessment.* 57: 21-43.

Bishop CA, Martinovic B. 2000. Guidelines and procedures for toxicological field investigations using amphibians and reptiles. In Sparling DW, Linder G, Bishop

CA eds. *Ecotoxicology of Amphibians and Reptiles*. Society of Environmental Toxicology and Chemistry. Pensacola, FL. 904 p.

Bishop CA, Struger J, Shirose LJ, Dunn L, Campbell GD. 2000. Contamination and wildlife communities in stormwater detention ponds in Guelph and the greater Toronto area, Ontario, 1997 and 1998. Part II – contamination and biological effects of contamination. *Water Quality. Research Journal of Canada*. 35:437-474.

Bishop CA. 1992. The effects of pesticides on amphibians and the implications for determining causes of declines on amphibian populations. In: *Declines in Canadian Amphibian Populations: Designing a National Monitoring Strategy*. Pp. 10-13 in Bishop CA, Pettit KE, eds. Occasional Paper 76, Ottawa, Ontario, Canada: Canadian Wildlife Service.

Blaustein AR, Hoggman PD, Kiesecker JM, Hays JB. 1996. DNA repair activity and resistance to solar UV-B radiation in eggs of the Red-legged frog. *Conservation Biology*. 10:1398-1402.

Bonin J, DesGranges J, Rodrigue J, Oullet M. Anuran species richness in agricultural landscapes of Quebec: foreseeing long-tem results of road call surveys. *Herpetological Conservation*. 1:141-148.

Bonin J, Ouellet M, Rodrigue J, DesGranges, J, Gagne F, Sharbel TF, Lowcock, LA. 1997. Measuring the health of frogs in agricultural habitats subjected to pesticides. *Herpetological Conservation*. 1:246-257.

Boone MD, Bridges CM, Mills NE. 2001. A hierarchical approach in studying the effects of an insecticide on amphibian communities. *Froglog*. No. 48.

Boone MD, Bridges CM. 1999. The effect of temperature on the potency of carbaryl for survival of tadpoles of the Green frog (*Rana clamitans*). *Environmental Toxicology and Chemistry*. 18:1482-1484.

Boutilier, RG, Stiffler DF, Toews DP. 1992. Exchange of respiratory gases, ions, and water in amphibious and aquatic systems. Pp. 81-124 in Feder, MM, and Burggren, WW, eds. *Environmental Physiology of the Amphibians*. University of Chicago Press, Chicago.

Brönmark C, Edenhamn P. 1994. Does the presence of fish affect the distribution of tree frogs (*Hyla arborea*)? *Conservation Biology*. 8:841-845.

Burggren WW, Just JJ. 1992. Developmental changes in physiological systems. Pp. 467-530 in Feder, MM, and Burggren, WW, eds. *Environmental Physiology of the Amphibians.* University of Chicago Press, Chicago.

Calef GW. 1973. Natural mortality of tadpoles in a population of *Rana aurora*. *Ecology*. 54:741-758.

Canadian Council of Ministers of the Environment (CCME). 1999. Canadian Water Quality Guidelines for the Protection of Aquatic Life. In: *Canadian Environmental Quality Guidelines*, 1999, Canadian Council of Ministers of the Environment, Winnipeg.

Carey C. 1993. Hypothesis concerning the causes of the disappearance of the boreal toads form the mountains of Colorado. *Conservation Biology*. 7:355-362.

Chiasson A. 1993. The effect of suspended sediments on ninespine stickleback, *Pungitius pungitius*, and golden shiner, *Notemigonus crysoleucas*, in a current of varying velocity. *Environmental Biology of Fishes*. 37:283-295.

Cooke AS. 1981. Tadpoles as indicators of harmful levels of pollution in the field. *Environmental Pollution Series A.* 25:123-133.

Cooke, AS. 1970. The effect of *pp*'-DDT on tadpoles of the common frog (*Rana temporaria*). *Environmental Pollution*. 1:57-71.

Cooke, AS. 1972. The effects of DDT, dieldrin and 2,4-D on amphibian spawn and tadpoles. *Environmental Pollution*. 3:51-68.

Cooke, AS. 1973. The effects of DDT, when used as a mosquito larvicide, on tadpoles of the frog *Rana temporaria*. *Environmental Pollution*. 5:259-273.

Cooke, AS. 1979. The influence of rearing density on the subsequent response to DDT dosing for tadpoles of the frog *Rana temporaria*. *Bulletin of Environmental Contamination and Toxicology*. 1:837-841.

Corkran CC, Thoms C. 1996. *Amphibians of Oregon, Washington and British Columbia*. Lone Pine. Edmonton, AB.

COSEWIC (Committee on the Status of Endangered Wildlife in Canada). 2002. *Canadian Species at Risk*. 34 pp.

Cowman DF, Mazanti LE. 2000. Ecotoxicology of "new generation" pesticides to amphibians. In Sparling DW, Linder G, Bishop CA eds. *Ecotoxicology of Amphibians and Reptiles*. Society of Environmental Toxicology and Chemistry. Pensacola, FL. 904 p.

Datta S, Hansen L, McConnel L, Baker J, LeNoir J, Seiber JN. 1998. Pesticides and PCB contaminants in fish and tadpoles from the Kaweah River basin, California. *Bulletin of Environmental Contamination and Toxicology*. 60:829-836.

de Solla SR, Bishop CA, Pettit KE, Elliott JE. 2002a. Organochlorine pesticides and polychlorinated biphenyls (PCBs) in eggs of northern Red-legged frogs (*Rana aurora*) and northwestern salamanders (*Ambystoma gracile*) in an agricultural landscape. *Chemosphere.* 46:1027-1032.

de Solla, SR, Bishop, CA, Pettit, KE, Elliott, JE, Cheng, K. 2002b. Effects of agricultural runoff on native amphibians in the lower Fraser River valley, British Columbia, Canada. *Environmental Toxicology and Chemistry*. 21 (2): 353-360.

Drost CA, Fellers, GM. 1996. Collapse of a regional frog fauna in the Yosemite area of the California Sierra Nevada, USA. *Conservation Biology*. 10:414-425.

Duellman WE, Trueb L. 1986. *Biology of Amphibians*. McGraw-Hill, New York, NY.

Environment Canada. 1992. *Manual of Analytical Methods, Volumes 1-3.* The National Laboratory for Environmental Testing, Burlington, Ontario.

Environment Canada. 1997a. *Manual of Analytical Methods, Volume 1, Major Ions and Nutrients; Manual of Analytical Methods.* The National Laboratory for Environmental Testing, Burlington, Ontario. 325 pp.

Environment Canada. 1997b. *Manual of Analytical Methods, Volume 2, Trace Metals.* The National Laboratory for Environmental Testing, Burlington, Ontario. 355 pp.

Environment Canada. 1997c. *Determination of Total Kjeldahl Nitrogen in water, solids and sludges.* Section INW10. Water Technology International Corporation, Burlington, Ontario. 4pp.

Environment Canada. 1997d. *Determination of nitrate in water.* Section INW7. Water Technology International Corporation, Burlington, Ontario. 4pp.

Environment Canada. 1997e. *Determination of nitrite in water.* Section INW7. Water Technology International Corporation, Burlington, Ontario. 4pp.

Environment Canada. 1997g. *Determination of mercury in liquid and solid samples by cold-vapour atomic absorption.* Section INW/ S1. Water Technology International Corporation, Burlington, Ontario. 5pp.

Environment Canada. 1997h. *Determination of metals (total and dissolved) in water by inductively couple plasma (ICP).* Section INW14. Water Technology International Corporation, Burlington, Ontario. 4pp.

Finzio A, Bidleman TF, Szeto SY. 1998. Emission of chiral pesticides from an agricultural soil in the Fraser Valley, British Columbia. *Chemosphere*. 36:345-355.

Fioramonti E, Semlitsch RD, Reyer H, Fent K. 1997. Effects of triphenyltin and pH on the growth and development of *Rana lessonae* and *Rana esculenta* tadpoles. *Environmental Toxicology and Chemistry*. 16:1940:1947.

Fordham CL, Tessari JD, Ramsdell HS, Keefe TJ. 2001. Effects of malathion on survival, growth, development, and equilibrium posture on bullfrog tadpoles (*Rana catesbeiana*). *Environmental Toxicology and Chemistry*. 20:179-184.

Fort DJ, Rogers RL, Morgan LA, Miller MF, Clark PA, White JA, Paul RR, Stover EL. 2000. Preliminary validation of a short-term morphological assay to evaluate adverse effects on amphibian metamorphosis and thyroid function using *Xenopus laevis*. *Journal of Applied Toxicology*. 20: 419-425.

Fort DJ, Rogers R, Copley H, Bruning L, Stover EL, Rapaport D. 1999. Effects of sulfometuron methyl and nicosulfuron and development and metamorphosis in *Xenopus laevis*: Impact of purity. *Environmental Toxicology and Chemistry*. 18: 2934-2940.

Gattem RE Jr., Miller K, Full FR. 1992. Energetics at rest and during locomotion. Pp. 314-377 in Feder, MM, and Burggren, WW, eds. *Environmental Physiology of the Amphibians*. University of Chicago Press, Chicago.

Gendron AD, Bishop CA, Fortin R, Hontella A. 1997. In vivo testing of the functional integrity of the corticosterone-producing axis in mudpuppy (Amphibia) exposed to chlorinated hydrocarbons in the wild. *Environmental Toxicology and Chemistry*. 16:1694-1706.

Girard C, Brodeu JC, Hontela A. 1998. Responsiveness of the interrenal tissue of yellow perch (Perca flavescens) from contaminated sites to an ACTH challenge test. *Canadian Journal of Fisheries and Aquatic Sciences*. 55:438-450.

Goede RW, Barton BA. 1990. Organismic indices and an autopsy-based assessment as indicators of health and condition of fish. *American Fisheries Symposium*. 8:93-108.

Gosner K. 1960. A simplified table for staging anuran embryos and larvae with notes on identification. *Herpetologica*. 16. 183-190.

Gutleb AC, Appelman J, Bronkhorst M, van den Berg JHJ, Murk AJ. 2000. Effects of oral exposure to polychlorinated biphenyls (PCBs) on the development and metamorphosis of two amphibian species (*Xenopus laevis* and *Rana temporaria*). *The Science of the Total Environment*. 262:147-157.

Hall RJ, Henry PFP. 1992. Assessing effects of pesticides on amphibians and reptiles: status and needs. *Herpetological Journal*. 2:65-71.

Harris JA. 1972. Seasonal variation in some hematological characteristics of *Rana pipiens*. *Comparative Biochemical Physiology*. 43:975-989.

Harris ML and Bogart JP. 1997. A cage for evaluation of in situ water quality using frog eggs and tadpoles. *Herpetolgical Review* 28:134.

Harris ML, Bishop CA and McDaniel TV. 2000. Assessment of rates of deformity in wild frog populations using *In Situ* cages: a case study of leopard frogs (*Rana pipiens*) in Ontario, Canada. *Biomarkers*, in press.

Harris ML, Bishop CA, Struger J, Ripley B, Bogart JP. 1998. The funtional integrity of northern leopard frog (*Rana pipiens*) and green frog (*Rana clamitans*) populations in orchard wetlands. II. Effects of pesticides and eutrophic conditions on early life stage development. *Environmental Toxicology and Chemistry*. 17:1351-1363.

Harte J, Hoffman E. 1989. Possible effects of acidic deposition on a Rocky Mountain population of the tiger salamander *Ambystoma trigrinum. Conservation Biology.* 3:149-158.

Hayes MP, Jennings MR. 1986. Decline of ranid species in western North America: are bullfrogs (*Rana catesbeiana*) responsible. *Journal of Herpetology.* 20:490-509.

Hayes TB. 1997. Steroids as potential modulators of thyroid hormone activity in anuran metamorphosis. *American Zoologist.* 37:185-194.

Hayes TB. Endocrine disruption in amphibians. 2000. In Sparling DW, Linder G, Bishop CA eds. *Ecotoxicology of Amphibians and Reptiles.* Society of Environmental Toxicology and Chemistry. Pensacola, FL. 904 p.

Hayes TB. 1997. Steroids as potential modulators of thyroid hormone activity in anuran metamorphosis. *American Zoology*. 37. 185-194.

Hayes TB. 1999. Endocrine disruption in anuran amphibians: a comparative multi-test approach. Abstract. *Society of Environmental Toxicology and Chemistry: 20th Annual Meeting*. Philadelphia, PA.

Hecnar SJ. 1995. Acute and chronic toxicity of ammonium nitrate fertilizer to amphibians from southern Ontario. *Environmental Toxicology and Chemistry*. 14:2131-2137.

Herman CA. 1992. Endocrinology. Pp. 40-54 in Feder, MM, and Burggren, WW, eds. *Environmental Physiology of the Amphibians*. University of Chicago Press, Chicago.

Hontella A. 1998. Interrenal dysfunction in fish from contaminated sites: *in vivo* and *in vitro* assessment. *Environmental Toxicology and Chemistry*. 17. 44-48.

Horne MT, Dunson WA. 1994. Exclusion of the Jefferson salamander, *Ambystoma jeffersonianium*, from some potential breeding ponds in Pennsylvania: effects of pH, temperature, and metals on embryonic

development. *Archives of Environmental Contamination and Toxicology.* 27:323-330.

Hough P. 1998. *The Global Politics of Pesticides.* Earthscan Publications. London, UK.

Howe GE, Gillis R, Mowbray RC. 1998. Effect of chemical synergy and larval stage on the toxicity of atrazine and alachlor to amphibian larvae. *Environmental Toxicology and Chemistry.* 17:519-525.

Howe, G. E., Gillis, R. & Mowbray, R. C. (1998). Effect of chemical synergy and larval stage on the toxicity of atrazine and alachlor to amphibian larvae. *Environmental Toxicology and Chemistry.* 17:519-525.

Huang, YW, WH Karasov, KP Patnode, and CR Jefcoate. 1999. Exposure of northern leopard frogs in the Green Bay ecosystem to polychlorinated biphenyls, polychlorinated dibenzo-*p*-dioxins, and polychlorinated dibenzofurans is measured by direct chemistry but not hepatic ethoxoyresorufin-*o*-deethylase activity. *Environmental Toxicology and Chemistry.* 18:2123-2130.

Huey DW, Beitinger TL. 1980. Hematological responses of larval *Rana catesbeiana* to sublethal nitrite exposures. *Bulletin of Environmental Contamination and Toxicology*. 25:574-577.

Jofre MB, Karasov WH. 1999. Direct effect of ammonia on three species of North American anuran amphibians. *Environmental Toxicology and Chemistry*. 18:1806-1812.

Johnson MS, Franke LS, Lee RB, Holladay SD. 1999. Bioaccumulation of 2,4,6-trinitrotoluene and polychlorinated biphenyls through two routes of exposure in a terrestrial amphibian: is the dermal route significant? *Environmental Toxicology and Chemistry*. 18:873-878.

Kennedy CJ. 1995. Xenobiotics. Pp. 291-312 in Hochachka Pw and Mommsen TP eds. *Biochemistry and Molecular Biology of Fishes*. Elsevier Science BV, Amsterdam.

Kennedy, CJ, McDonald LE, Loveridge R, Strosher ME. 2000. The effects of selenium contamination in adult cutthroat trout (*Oncorhynchus clarki lewisii*) on eggs larvae and fry. *Archives of Environmental Contamination and Toxicology*. 39(1): 46-52.

Kennedy, CJ, Walsh PJ. 1997. Effects of temperature on xenobiotic metabolism. Pp. 303-324 in Wood CM, McDonald DG eds. *Global Warming: Implications for freshwater and marine fish.* Cambridge University Press, Cambridge.

Kollros JJ. 1961. Mechanisms of amphibian metamorphosis: hormones. *American Zoologist.* 1:107-114.

Larras-Regard E, Taurog A, Dorris M. 1981. Plasma T4 and T3 levels in *Ambystoma tigrinum* at various stages of metamorphosis. *General and Comparative Endocrinology.* 43:443-450.

Larson DL, McDonald S, Fivizzani AJ, Newton WE, Hamilton SJ. 1998. Effects of the herbicide atrazine on *Ambystoma tigrinum* metamorphosis: duration, larval growth, and hormonal response. *Physiological Zoology.* 71:671-689.

Licht LE, Grant KP. 1997. The effects of ultraviolet radiation on the biology of amphibians. *American Zoologist.* 37:137-145.

Licht LE. 1974. Survival of embryos, tadpoles, and adults of the frogs *Rana aurora aurora* and *Rana pretiosa pretiosa* sympatric in southwestern British Columbia. *Canadian Journal of Zoology.* 52:613-627.

Licht LE. 1976. Time course, uptake, elimination and tissue levels of [^{14}C]DDT in wood-frog tadpoles. *Canadian Journal of Zoology*. 54:355-360.

Licht, LE. 1971. Breeding habits and embryonic thermal requirements of the frogs, Rana aurora aurora and Rana pretiosa pretiosa, in the Pacific Northwest. *Ecology.* 52:116-124.

Mahaney PA. 1994. Effects of freshwater petroleum contamination an amphibian hatching and metamorphosis. *Environmental Toxicology and Chemistry*. 13:259-265.

Marco A, Quilchano C, Blaustein AR. 1999. Sensitivity to nitrate and nitrite in pond-breeding amphibians from the Pacific Northwest, USA. *Environmental Toxicology and Chemistry.* 18:2836-2389.

Marsh JM. 1993. Assessment of nonpoint source pollution in stormwater runoff in Louisville, (Jefferson County) Kentucky, USA. *Archives of Environmental Contamination and Toxicology*. 25:446-455.

Materna EJ, Rabeni CF, LaPoint TW. 1995. Effects of the synthetic pyrethroid insecticide, esfenvalerate, on larval leopard frogs (*Rana* spp.). *Environmental Toxicology and Chemistry*. 14:613-622.

Matsumara F. 1985. *Toxicology of Insecticides*. Plenum Press. New York.

Mayer FL, Ellersieck MR. 1986. *Manual of Acute Toxicity: Interpretation and Database for 410 Chemicals and 66 Species of Freshwater Animals*. U.S. Department of the Interior Fish and Wildlife Service. Washington, DC.

Mayer FL, Versteeg DJ, McKee MJ, Folmar LC, Graney RL, McMume DC, Rattner BA. 1989. Physiological and nonspecific biomarkers. Pp. 5-86 in *Biomarkers: Biochemical, Physiological, and Histological Markers of Anthropogenic Stress*. Hugget RJ, Kimerle RA, Merhle PM, Bergman HL eds. Lewis Publishers. Chelsea, MI.

McNeely RN, Neimanis VP, Dwyer L. 1979. *Water Quality Sourcebook A Guide to Water Quality Parameters.* Environment Canada, Inland Waters Directorate, Water Quality Branch. Ottawa.

Miyauchi H, La Rochelle FT Jr., Suzuki M, Freeman M, Frieden E. 1977. Studies of thyroid hormones and their binding in bullfrog tadpole plasma during metamorphosis. *General and Comparative Endocrinology*. 33:254-266.

Nebeker AV, Schuytema GS. 2000. Effects of ammonium sulfate on growth of larval northwestern salamander, red-legged and Pacific treefrog tadpoles, and

juvenile fathead minnows. *Bulletin of Environmental Contamination and Toxicology.* 64:271-278.

Newman RA. 1998. Ecological constraints on amphibian metamorphosis: interactions of temperature and larval density with responses to changing food level. *Oecologica.* 115:9-16.

Oldham RS, Latham DM, Hilton-Brown D, Towns M, Cooke AS, Burn A. 1997. The effect of ammonium nitrate fertiliser on frog (*Rana temporaria*) survival. *Agriculture, Ecosystems & Environment* 61:69-74.

Orchard, SA. 1992. Amphibian populations declines in British Columbia. In: *Declines in Canadian Amphibian Populations: Designing a National Monitoring Strategy.* Pp. 10-13 in Bishop CA, Pettit KE, eds. Occasional Paper 76, Ottawa, Ontario, Canada: Canadian Wildlife Service.

Palmer BD, Huth LK, Peito DL, Selcer KW. 1998. Vitellogenin as a biomarker for xenobiotic estrogens in an amphibian model system. *Environmental Toxicology and Chemistry.* 17:30-36

Pauli BD, Perrault JA, Money SL. 2000. *RATL: A Database of Reptile and Amphibian Toxicology Literature.* Technical Report Series No. 357. Canadian Wildlife Services, Headquarters, Hull, Quebec, Canada.

Pechmann JHK, Scott DE, Semlitsch RD, Caldwell JP, Vitt LJ, Gibbons JW. 1991. Declining amphibian populations: the problem of separating human impacts from natural fluctuations. *Science.* 23:892-895.

Pough EH, Magnusson WE, Ryan MJ, Wells KD, Taigen TL. 1992. Behavioral Energetics. Pp. 395-436 in Feder MM, and Burggren WW, eds. *Environmental Physiology of the Amphibians.* University of Chicago Press, Chicago.

Power T, Clark KL, Harfenist A, Peakall DB. 1989. *A Review and Evaluation of the Amphibian Toxicological Literature.* Technical Report No. 61, Canadian Wildlife Service, Headquarters, Hull, Quebec, Canada.

Rand G, and Petrocelli S. 1985. *Fundamentals of Aquatic Toxicology.* Hemisphere. New York.

Randall GW, Huggins DR, Russell MP, Fuchs DJ, Nelson WW, Anderson JL. 1997. Nitrate losses through subsurface tile drainage in conservation reserve

programs, alfalfa, and row crop systems. *Journal of Environmental Quality.* 26:1240-1247.

Reid SD, McDonald DG, Wood CM. 1997. Interactive effects of temperature and pollutant stress. Pp. 325-350. *Global Warming: Implications for Freshwater and Marine Fish*. Cambridge University Press. Cambridge.

Rosenshield ML, Jofre MB, Karasov WH. 1999. Effects of polychlorinated biphenyl 126 on green frog (*Rana clamitans*) and leopard frog (*Rana pipiens*) hatching success, development, and metamorphosis. *Environmental Toxicology and Chemistry.* 11:2478-2486.

Rouse JD, Bishop CA, Struger J. 1999. Nitrogen pollution: an assessment of its threat to amphibian survival. *Environmental Health Perspectives*. 107:799-803.

Russell RW, Gillan KA, Haffner GD. 1997. Polychlorinated biphenyls and chlorinated pesticides in southern Ontario, Canada, green frogs. *Environmental Toxicology and Chemistry.* 16:2258-2263.

Saiki MK, Castleberry DT, May TW, Martin BA, Bullard FN. 1995. Copper, cadmium, and zinc concentrations in aquatic food chains from the Upper

Sacramento River (California) and selected tributaries. *Archives of Environmental Contamination and Toxicology*. 29:484-491.

Savage, WK, FW Quimby, and AP DeCaprio. 2002. Lethal and sublethal effects of polychlorinated biphenyls on *Rana sylvatica* tadpoles. *Environmental Toxicology and Chemistry.* 21:168-174.

Schultheiss H. 1980. Triiodothyronine and thyroxine concentrations during the metamorphosis of *Xenopus laevis* and *Rana esculenta* and in the neotenic Mexican axolotl. *General and Comparative Endocrinology*. 40:372.

Schuytema GS, Nebeker AV, Griffis WL. 1994. Toxicity of Guthion® and Guthion ® 2S to *Xenopus laevis* embryos. *Archives of Environmental Contamination and Toxicology*. 27:250-255.

Schuytema GS, Nebeker AV. 1998. Comparative toxicity of diuron on survival and growth of Pacific treefrog, bullfrog, Red-legged frog, and African clawed frog embryos and tadpoles. *Archives of Environmental Contamination and Toxicology.* 34:370-376.

Semlitsch RD. 2000. Management of aquatic-breeding amphibians. *Journal of Wildlife Management*. 64:615-631.

Shi Y-B. 2000. *Amphibian metamorphosis: from morphology to molecular biology*. John Wiley & Sons.

Silbergeld EK. 1974. Blood glucose: a sensitive indicator of environmental stress in fish. *Bulletin of Environmental Contamination and Toxicology.* 11:20-25.

Smith-Gill SJ, Bervin KA. 1979. Predicting amphibian metamorphosis. *American Naturalist*. 113:563-585.

Spalding RF, Snow DD. 1989. Stream levels of agrochemicals during a spring discharge event. *Chemosphere*. 19:1129-1140.

Sparling DW. 2000. Ecotoxicology of organic contaminants to amphibians. In Sparling DW, Linder G, Bishop CA eds. *Ecotoxicology of Amphibians and Reptiles*. Society of Environmental Toxicology and Chemistry. Pensacola, FL. 904 p.

Spear R. 1991. Recognized and possible exposure to pesticides. Pp. 245-274 in Hayes WJ, and Laws ER, eds. *Handbook of Pesticide Toxicology*. Academic Press. London, UK.

Stay FS, Jarvinen AW. 1995. Use of microcosm and fish toxicity data to select mesocosm treatment conditions. *Archives of Environmental Contamination and Toxicology*. 28:451-458.

Thomas P. 1990. Molecular and biochemical responses of fish to stressors and their potential use in environmental monitoring. *American Fisheries Symposium. 8:9-28.*

Ultsch GR, Bradford DF, Freda J. 1999. Physiology: coping with the environmenta. Pp. 189-214 in McDiarmid RW and Altig R, eds. *Tadpoles: The Biology of Anuran Larvae.* The University Press of Chicago. Chicago.

United States Environmental Protection Agency (US EPA). 1987. *Guidelines for the preparation of the 1988 State Water Quality Assessment Report,* EPA/600/S3-87/001. Office of Water Programs, Water Planning Division. Washington, DC.

Vindimian E, Namour P, Munoz J, Gril J, Migeon B, Garric J. 1993. Ethoxyresorufin-*o*-deethylase induction in fish from a watershed exposed to a non-point source pollution of agricultural origin. *Water Research*. 27:449-455.

Vitt LJ, Caldwell JP, Wilbur HM, Smith DC. 1990. Amphibians as harbingers of decay. *Bioscience* 40:418.

Wan, M.T., Szeto, S., & Price, P (1994). Organophosphorus insecticide residues in farm ditches of the Lower Fraser Valley of British Columbia. *Journal of Environmental Science and Health.* Part B 29, 917-949.

Wan MT, Szeto S, Price P. 1994.Organophosphorus insecticide residues in farm ditches of the Lower Fraser Valley of British Columbia. *Journal of Environmental Science and Health.* Part B 29:917-949

Wauchope RD. 1978. The pesticide content of surface waters draining from agricultural fields – a review. *Journal of Environmental Health Quality.* 7:459-472.

Wedemeyer, GA, Barton BA, McLeay DJ. 1992. Stress and acclimation. Pp. 451-457 in Schreck CB and Moyle PP. eds. *Methods for Fish Biology.* American Fisheries Society.

Wine RN, Gatten RE Jr. 1992. Glycolytic support for locomotion in *Anolis carolinensis* and *Rana pipiens*: simultaneous effects of temperature and duration of exercise. *Physiological Zoology.* 65:724-741.

Wong PTS, Dixon DG. 1995. Bioassessment of water quality. *Environmental Toxicology and Water Quality.* 10:9-17.

Zabik JM, Seiber JN. 1993. Atmospheric transport of organophosphorous insecticides from California's Central Valley to the Sierra Nevada Mountains. *Journal of Environmental. Quality.* 22:80-90.