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CONTAMINANTS PROGRAM

Determination of Pesticide Aerial Drift and Associated Effects to the Endangered Wyoming Toad (*Bufo baxteri*) at Mortenson and Hutton National Wildlife Refuges and Potential Reintroduction Sites

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Abstract

The endangered Wyoming toad is confined to Mortenson National Wildlife Refuge (NWR) in southeast Wyoming. Reasons for the decline of the toad are unknown, but it is suspected that pesticide aerial drift from mosquito control activities on lands adjacent to the refuge may be partly responsible. We used pesticide indicator strips and spray cards to determine the extent of malathion entering Mortenson NWR and potential reintroduction sites. Spray cards from three sampling sites had detectable malathion concentrations (0.298 - 28.8 µg/g). To determine exposure of the Wyoming toad to malathion aerial drift, we used percent survival and measured acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) activity in blood and brain samples of caged surrogate Woodhouse's toads. Survival was 100% and mean AChE and BChE activity in brain and plasma did not differ significantly among sampling sites. Righting trials were conducted on surrogates before and after spraying to determine if predatory avoidance behavior was affected. There was no significant difference ($p < 0.05$) among sites. We did abundance estimates on terrestrial and aquatic insects prior to and after the spray event to determine if the toads' food source was affected. Terrestrial invertebrate abundance results were inconclusive. Aquatic invertebrate abundance was not significantly different ($p < 0.05$) before and after spraying at any sites except the reference site and Meeboer Lake. No malathion residues were detected in the aquatic invertebrates. Our results indicate that, although some drift of malathion is occurring, the toads were not exposed to concentrations great enough to reduce adult survival, affect predator avoidance behavior, or reduce their food source. The data also provide needed information on ChE activities in amphibians.

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INTRODUCTION

Wyoming toads (*Bufo baxteri*), confined to wetlands and irrigated meadows in the Laramie Plains of Albany County, were abundant prior to the 1960's. By the 1970's, the population had declined drastically and was confined to Mortenson National Wildlife Refuge (NWR) near Laramie. In 1984 the species was listed as endangered under the Endangered Species Act (Lewis et al. 1985) and the last few remaining individuals were taken into captivity. Because of a successful captive breeding program, the endangered Wyoming toad was reintroduced to both Mortenson NWR and the nearby Hutton Lake NWR in June 1995.

Reasons for the decline of the Wyoming toad and other amphibians are numerous and varied but represent a world-wide problem (Blaustein and Wake 1990; Griffiths and Beebee 1992; Corn 1994; Vertucci and Corn 1996). A monitoring study was conducted by the U.S. Fish and Wildlife Service (Service) in 1989, 1990, and 1991 (Ramirez 1992) to investigate trace element concentrations at Mortenson NWR. Concentrations of trace elements in water, sediment, and biota from several sites within Mortenson NWR were below levels determined to be harmful to amphibians. Another study investigated predation, habitat modification, and soil and water conditions; but, none of these parameters was documented as posing serious threats to the Wyoming toad population at Mortenson NWR refuge (Stone 1991).

Both the bacterial disease redleg caused by *Aeromonas hydrophila* and the recently discovered chytrid fungus (*Batrachochytrium dendrobatidis*) have been confirmed in the Wyoming toad population. Redleg can result in death to amphibians, although the bacteria can be common in aquatic environments. The chytrid fungus is very lethal to amphibians but little is known about how the fungus is transmitted and why suddenly it is affecting amphibian populations. It is unclear whether either redleg or chytrid is responsible for toad mortality and the resulting drastic decline of the Wyoming toad. A confounding factor such as pesticides exposure may be weakening the immune system causing the amphibians to become more susceptible to these agents.

The pesticide malathion is used in the Laramie Plains for mosquito control but aerial drift and potential contamination into habitats used by the toads on Mortenson NWR from drift has not been addressed. Malathion is an organophosphate insecticide and considered non-persistent, but it is water soluble and very toxic to fish, amphibians, and aquatic invertebrates (Briggs 1992). Malathion is aerially sprayed on fields adjacent to Mortenson NWR and along the Laramie River at 21 mg/m^2 (3 oz. active ingredient / acre) as an ultra low volume (ULV) spray with no carriers (97% malathion / 3% impurities). Label requirements prohibit the application of malathion to open water areas and malathion can be applied only when the weather is favorable. Even so, one study indicates that ULV applications can yield up to a 10-fold increase in off-target drift even when a pesticide is applied correctly as compared to applications with solid stream nozzles that produce a coarser spray (Bird et. al 1996).

The application of malathion occurs during late June in the early morning and malathion may be sprayed a second time in July if the mosquito outbreak is particularly severe. The application period is of particular concern because it coincides with the period when most of the growth and development of the Wyoming toad is occurring. Absorption of malathion can result in death or cause indirect effects such as intoxication, physical lethargy, or paralysis which increase the toads' risk of

predation (Monanty-Hejmadi and Dutta 1981, Grue et al. 1991, Cowman and Mazanti 2000). Additionally during this early summer period, the toads are actively foraging for arthropods (Stone 1991) increasing their risk of pesticide exposure dermally and from ingestion.

Malathion breaks down quickly in the environment (half-life in soil is ranges from 1-2.5 days and in water the half-life at pH 7 is 6.21 days and at pH 9 the half-life is 12 hours) (www.epa.gov/pesticides/op/malathion.efedrra.pdf) and does not readily bioconcentrate in aquatic organisms (Howard 1991). Typical pH of water at Mortenson Lake is 8.5. Therefore, linking cause and effect in organisms exposed to malathion in the field is difficult. One method for documenting exposure to organophosphate is to measure cholinesterase (ChE) activity in animals. A significant depression of ChE activity usually results in death (Grue et al. 1991).

Cholinesterase inhibition can be measured in brain, liver, and blood tissues. For example, acetylcholinesterase (AChE) is an enzyme that is essential for normal nerve function within the peripheral and central nervous systems. AChE serves to break down acetylcholine which transmits impulses across synapses from nerve to nerve or muscle. When an animal is exposed to an organophosphate pesticide, AChE is inhibited allowing an accumulation of acetylcholine that subsequently causes an excessive stimulation of the nerves. This results in muscle spasms, paralysis, and subsequent respiratory failure and death (Grue et al. 1991). Inhibition $\geq$ 50% is considered indicative of organophosphate poisoning, although laboratory studies suggest that inhibition of about 20% below the control mean in a species is indicative of exposure (Grue et al. 1991). Additionally, organophosphates will bind to other cholinesterases such as butyrylcholinesterase (BChE), which can also be used to indicate organophosphate exposure (Walker and Thompson 1991).

In addition to direct exposure through pesticide aerial drift, the Wyoming toad's food source may be reduced. A report by the Agricultural Experiment Station of the University of Wyoming (Pfadt et. al 1985), in which a field dose of malathion was applied at 56 mg/m^2 (8 oz. active ingredient / acre) for the control of grasshoppers, showed that ants (an important food of the Wyoming toad) were very susceptible to the toxic effects of malathion. An inventory of aquatic insects, which may also be an important food sources of the Wyoming toad, showed that Odonata populations did not recover for a year in areas sprayed with malathion and populations of *Hyallela azteca* appeared eradicated (Pfadt et al. 1985). This potential eradication of food source may affect the Wyoming toad's survival.

The objectives of this study were to determine: 1) the extent of malathion entering Mortenson NWR or potential reintroduction sites by aerial drift; 2) if malathion is entering Mortenson NWR via water from the Laramie River; 3) the effect of malathion aerial drift on predator avoidance behavior in surrogate Woodhouse's toads (*B. woodhousii*); 4) the exposure of the Wyoming toad to malathion through aerial drift using percent survivability and cholinesterase activity in surrogate Woodhouses toads; and, 5) if malathion aerial drift is affecting the food source of Wyoming toads.

METHODS

Study Sites

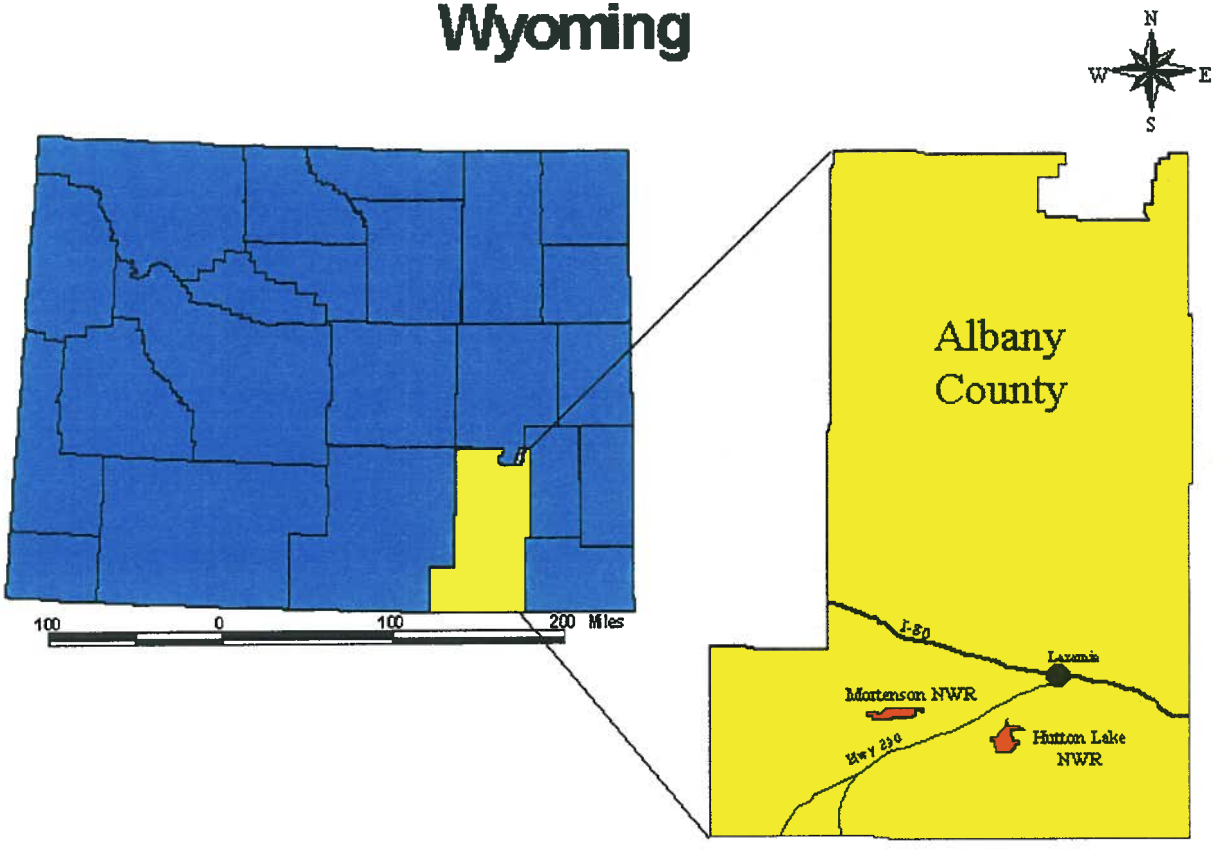
The Laramie Plains in Albany County, Wyoming (elevation 2,135 to 2,288 m) are semi-arid and consists of wetlands, ponds, seepage lakes, and irrigated meadows with shortgrass prairie and sagebrush in the uplands.(Figure 1). Study sites (Figure 2) included Mortenson Lake and Gibbs Pond on Mortenson NWR; Meeboer and Gelatt Lakes, located on Wyoming State Land adjacent to Mortenson NWR; two sites near the Laramie River at 37 Mile Road and 7 mile Road; and private property (White's Place) bordering Mortenson NWR to the West. Water seeps from Mortenson NWR into Meeboer Lake via a small ditch, which makes it possible for Wyoming toads to migrate to Meeboer Lake. Gelatt Lake is considered a potential reintroduction site for the Wyoming toad because of its similar wetland qualities to Mortenson Lake.

Mosquito control activities occur on private property surrounding Mortenson NWR, Meeboer Lake, and Gelatt Lake. Mosquito control activities also occur along the Laramie River, which feeds these three lakes. Hutton Lake NWR served as the reference site because no mosquito control activities occur on or adjacent to this refuge.

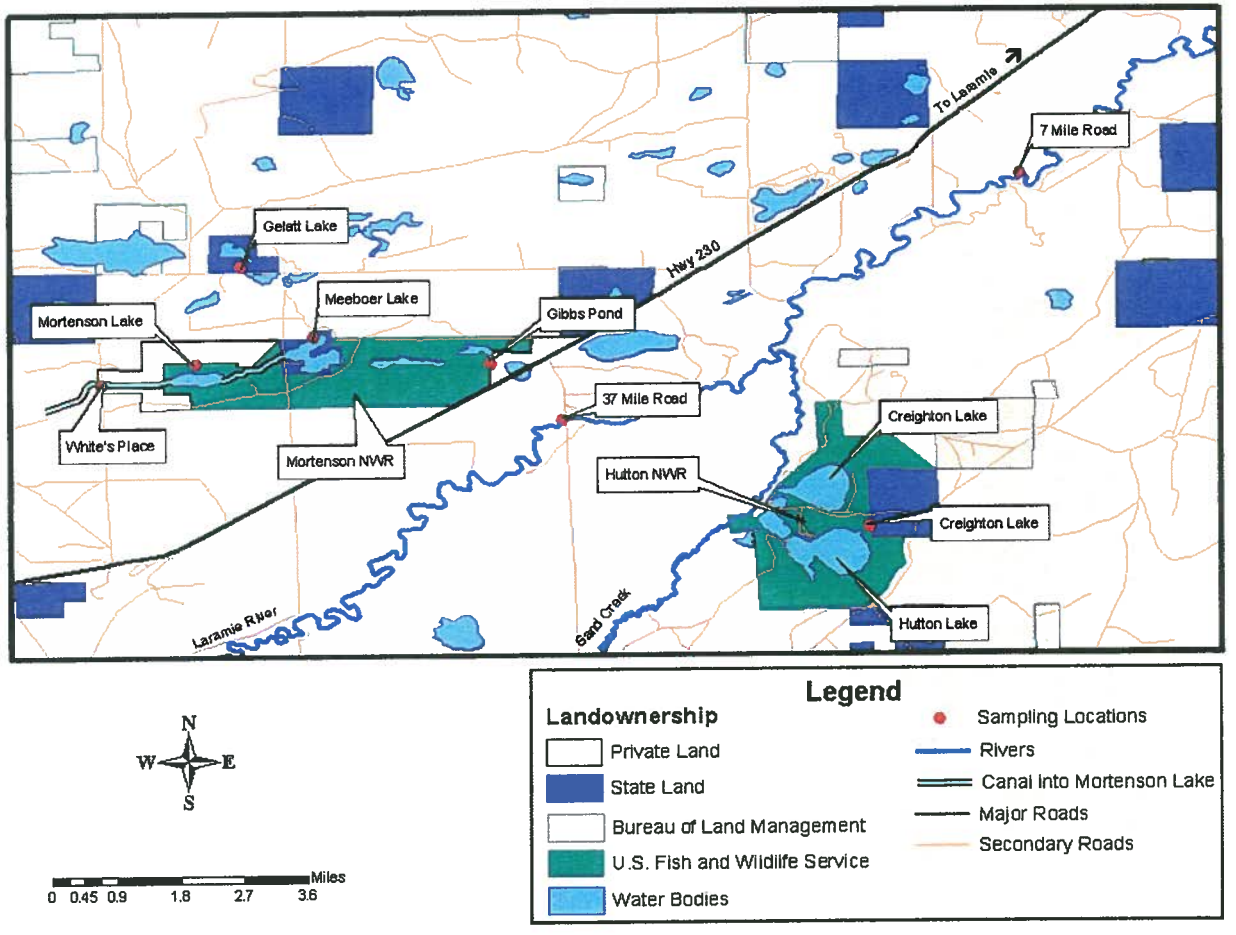
Spray Application and Detection

The endangered status of the Wyoming toad requires that the local mosquito control district notify the U.S. Fish and Wildlife Service (USFWS) 48 hours prior to mosquito spraying. This allowed us the opportunity to place pesticide indicator strips and pesticide spray cards at the study sites prior to spraying. Pesticide indicator strips, manufactured by Neogen Corporation, Lansing, Michigan, turn blue to indicate the absence of organophosphorus pesticides or white to indicate the presence of such pesticides (detected in the part per billion range). Spray cards (filter paper disks taped to index cards) were used to determine an actual concentration of pesticide drift at the site. Ten indicator strips were attached every 30 m to fence posts. Three or four pesticide spray cards were also attached to fence posts, alternating with pesticide strips. Strips and spray cards were placed near Mortenson Lake, Meeboer Lake, Gibbs Pond, Gelatt Lake, the canal at White's Place, Creighton Lake at Hutton Lake NWR, and along the Laramie River at 37 Mile Road and 7 Mile Road.

Spraying occurred on the morning of June 30, 1998 near Mortenson Lake. Mosquito control activities concluded the following morning near the Laramie River. Immediately after spraying, we retrieved the pesticide strips and spray cards from the fence posts. We dipped fresh pesticides strips in the water at each site. Pesticide strips were tested for indications of organophosphates. Filter paper disks were removed from the spray cards, preserved individually in glass jars with methylene chloride, and submitted to Patuxent Analytical Control Facility (PACF) in Laurel, Maryland for analyses of malathion.



ure 1. General location of study area, Albany County, Wyoming.



igure 2. Locations of sampling sites in the Laramie Plains, Wyoming.

Surrogate Toad Exposures

Surrogate Woodhouse's toads were used to determine actual exposure to malathion at selected field sites. Surrogate animals were raised at the Wyoming Game and Fish Sybille Wildlife Research Unit. This facility also raises Wyoming toads for reintroduction purposes. The Woodhouse's toads were one year of age to correlate with the age of the Wyoming toads at Mortenson and Hutton NWRs. At the time of the study, most of the reintroduced Wyoming toads were 1-2 years old (Mitch Bock, pers. comm., 1/21/97).

Prior to spraying, righting trials were conducted on unexposed toads. Righting is the measure of time it takes the toad to right itself after being placed on its back. Righting is standard method to determine if the pesticide affects the behavior of the animal. Under normal conditions, toads will be able to right themselves within 10 seconds of being placed on their back (Deborah Cowman, Texas A&M, pers. comm.1/6/98).

Toads were then placed in stainless steel mesh (1.91 cm^2) enclosures (30.5 cm x 20.3 cm x 15.25 cm). Five toads were placed in each enclosure and two enclosures were placed at each of the following sites: Gibbs Pond, Gelatt Lake, Meeboer Lake, the canal near White's Place, the Laramie River at 7 Mile Road, and the Creighton Lake at Hutton Lake NWR. Enclosures were positioned next to the fence posts with pesticide strips and spray cards at the edge of the water at each site. Woodhouse's toads were not placed inside Mortenson NWR to prevent disease transmittal to the Wyoming toads.

Immediately after spraying was completed, we retrieved the surrogate toads. Some toads escaped from their cages during spraying (3 from White's Place, 5 from Meeboer Lake, and 2 each from Gibbs Pond, Gelatt Lake, and 7 Mile Road) and we were unable to recapture them. Righting trials were repeated on the remaining toads to determine if predator avoidance response was affected by potential pesticide exposure. Blood samples were drawn with heparinized 1-ml disposable syringes with 27½ gauge needle via heart puncture, placed in sterilized collection vials , and allowed to clot. The toads were placed in sterile plastic bags. Both the blood samples and toads were frozen in an alcohol / dry ice slurry and shipped overnight to Dr. Michael J. Hooper at Texas Tech University for analysis of cholinesterase inhibition (AChE and BChE) in brain and plasma. To remove any possible bias in the analysis, Dr. Hooper was not informed as to which samples came from the reference site.

Insect Collections

We used insect pitfall traps to collect terrestrial insects (ants and beetles) as described by (Catangui et al. 1996; Riddick and Mills 1996). Ten pitfall traps were placed at each sampling site for two consecutive nights prior to and after the spray event. Insects were collected the morning following each trap night.

We collected aquatic invertebrates from the water column using the modified Gerking device as described by Kaminski and Murkin (1981) before and after the spray event. Species and quantity of both aquatic invertebrates and terrestrial invertebrates from pitfall traps were recorded for abundance estimates before and after spraying. Invertebrates were placed in chemically-cleaned glass vials and frozen immediately. Samples were submitted to PACF for malathion analyses. Study sites

were observed for dead and dying terrestrial insects and aquatic invertebrates before and after the spray event.

Cholinesterase Analysis

Toads were thawed, skin and tissue of the neck removed, and the junction of the skull and spinal cord was exposed. A longitudinal cut through the skull was made following upward along each side of the spinal chord to the front of the cranium. The skull was then reflected forward to expose the brain. The brain was excised, cutting all neuronal connections consistently and severing the spinal cord at the base of the medulla oblongata where it exited the skull. Because of the extremely small size of the toad brains and the importance of obtaining identical brain regions for each sample, every effort was made to ensure that the brain excision was consistent between all animals. However, we were unable to get an adequate brain sample from one toad at each of the following sites: Gibbs Pond, Meeboer Lake, Gelatt Lake, and 7 Mile Road.

Brain samples were weighed and homogenized (25-fold dilution, w/v, in 0.05 M tris buffer, pH 7.4) in a glass Wheaton homogenizing tube equipped with a Teflon-coated pestle attached to an overhead stirrer. Approximately 10 strokes were used to complete the homogenization. The samples were then diluted another 4-fold with the same buffer, giving a 100-fold overall dilution.

Blood samples were thawed and samples were carefully transferred to a refrigerated centrifuge (Heraeus Biofuge 15R), avoiding mixing of the clot and serum sample. Tubes were spun for 10 minutes at 3000 rpm and the resulting serum was removed, diluted five-fold in the 0.05 M tris buffer (pH 7.4), and assayed. One plasma sample was lost during preparation from Gelatt Lake and four plasma samples were lost during preparation from Gibbs Pond.

ChE activities were measured in brain and serum samples using the method of Ellman et al. (1961) as modified by Gard and Hooper (1993). The method was modified for use on a SPECTROmax 96-well spectrophotometric plate reader (Molecular Devices Corporation, Palo Alto, California), which was used in conjunction with a computer equipped with Softmax software (Molecular Devices Corporation). The spectrophotometer was set in a kinetic mode and measured absorption at 412 nm for three minutes with readings taken at thirteen second intervals with a zero second lag phase. AChE was differentiated from BChE by a five-minute pre-incubation with the specific BChE inhibitor, iso-tetraisopropyl pyrophosoramide (OMPA) (1×10^{-4} M final concentration [FC] in the assay). BChE activity was calculated as the difference between total ChE and AChE activity. Components of the assay were as follows: 0.05 M tris buffer (pH 8.0), 150 µmL; dithio[bis-2-nitrobenzoic acid] (DTNB), 20 µL, 3.23×10^{-4} M FC; diluted enzyme source, 30 µmL; iso-OMPA (or buffer for total ChE), 20 µmL, 1×10^{-4} M FC; and AThCh, 30 µmL, 1×10^{-3} M FC. Final volume was 250 µL/well. In blank wells, buffer replaced enzyme volume. Samples were run in triplicate at 25°C. ChE activities were converted from optical density units/minute to µmoles AThCh hydrolyzed/minute (or "units") per ml plasma or g brain using the extinction coefficient, 13,600 $cm^{-1} M^{-1}$.

Diluted brain samples were divided into three 500 µL aliquots. One of the two aliquots was assayed immediately for absolute ChE activity and maintained on ice. The other two were used for 2-PAM (2-pyridinealdoxime methochloride) reactivation, which tests for the presence of cholinesterase - inhibited ChEs. One of the aliquots was spiked with 2-PAM (FC = 2×10^{-4} M) and the other with an equal volume of deionized water. These samples were incubated in a water bath

at 25°C. Sub-samples were removed from the incubating material at 1 hour and assayed for ChE activity. An upper-tailed Student's t-test was then used to compare mean activities, from the values of samples run in triplicate, to determine if there was a significant activity increase in the 2-PAM incubated sample over the non-2-PAM incubated sample at any time point. Those samples found to have a significant increase in activity of at least 5 percent after 2-PAM incubation were considered to contain OP-inhibited ChE and, thus, had most likely come from individuals exposed to an organophosphate.

Statistical Analysis

All statistical analyses were performed using Systat 8.0 statistical software (SPSS Corporation 1996) at alpha = 0.05 unless otherwise noted. Because of non-random sampling for pre-spray righting trial data, quantitative statistics could not be used to determine if there was a difference in right trial times of toads between pre-spray and post-spray trials. We were able to compare the righting trial post-spray times between the reference site and each study site using a Fisher's exact test.

AChE and BChE activity levels conformed to a normal distribution. We used general linear models to determine if there was significant interaction between the weight of the toads and the site because brain and plasma ChE means were lower at the reference site than at most of the sites adjacent to spraying activities. In the case of brain AChE and BChE, we also examined if there was a significant interaction between the brain weight of the toads and the site. If there was no significant interaction between the covariate (weight or brain weight) and the site, we used an analysis of covariance (ANCOVA) to determine if there were differences in ChE activities among sites adjusted for weight or brain weight. In some cases, the coefficient for the covariate was not significant. Therefore, we used a one-way analysis of variance (ANOVA) to determine if there were differences in ChE activities among sites to avoid the possibility that the ANCOVA may be taking away a degree of freedom without reducing the mean-square error (SPSS Corporation 1996).

Aquatic invertebrate abundance sample data were transformed by $\log_{10}$ to correct for skewness prior to statistical analysis. The pre-spray and post-spray means were compared for each site using a paired t-test and are presented with 95% confidence intervals.

RESULTS

Spray Application and Detection

Pesticide strips - Locations where pesticide strips tested negative for pesticides occurred at Mortenson Lake (10), Gibbs Pond (8), Meeboer Lake (9), White's Place (10), and Hutton Lake NWR (9). The number of strips retrieved from each site are in parentheses. Locations where pesticide strips tested positive for pesticides occurred at 37 Mile Road (6/9), Gelatt Lake (2/8), and 7 Mile Road (3/7). The number of strips testing positive / number of strips retrieved from the site are in parentheses. Pesticide strips tested negative for malathion in water from all study sites.

Spray cards - Malathion was detected on the filter paper from spray cards at Meeboer Lake, Gelatt Lake, and 37 Mile Road. Detectable malathion concentrations in two of the three filter papers from Meeboer Lake were 0.298 and 0.354 µg/g wet weight (ww), although the pesticide strips tested negative. The three filter papers from spray cards at Gelatt Lake had detectable malathion concentrations of 7.86, 21.4, and 5.97 µg/g ww. The four filter papers from 37 Mile Road had the highest malathion drift concentrations of 28.8, 22.8, 16.3, and 0.595 µg/g ww. These filter papers indicated the amount of drift that may be entering the Laramie River, although pesticide strips used to detect the presence of organophosphate pesticides in the river water tested negative. At 7 Mile Road, filter papers (n=3) had no detectable malathion (<0.00200 µg/g ww) even though pesticide strips tested positive for pesticides. It is possible that the strips detected an organophosphate other than malathion. Malathion was below the detection limit (<0.00200 µg/g ww) for the remaining sites (Appendix 1).

We extrapolated from the malathion concentrations measured on the filter paper spray cards for comparison to the label application rate of 21 mg/m^2. Concentrations were less than the label application rate indicating drift rather than direct spray at these sites (Table 1).

Surrogate Toads

Righting trials - There was no significant difference (Fisher's exact test; $p > 0.05$) between the post-spray ratios of those toads that could right in 10 seconds or less at the reference site and those toads at each site where nearby spraying occurred (Table 2, Appendix 2). This indicates that if the toads were exposed to malathion aerial drift, the concentration was not great enough to affect the toads' behavior when compared to the reference site. However, Meeboer Lake and 7 Mile Road were significantly different from the reference site (Fisher's exact test; $p = 0.095$ and $p = 0.069$, respectively) at $p < 0.10$.

Table 1. Extrapolated concentrations of malathion at each site for comparison with the label application rate of 21 mg/m^2 (3 oz. active ingredient / acre).

Site	Malathion concentration on filter paper (µg/g)	Malathion concentration per area of filter paper (µg/cm^2)	Malathion concentration per m^2 (µg/m^2)	Malathion concentration / acre (g/acre)	Geometric mean** of Malathion concentration / acre (g/acre)
37 Mile Road	28.8	0.239	2390	9.67	3.00
	0.595	4.39×10^{-3}	49.39	0.200	
	16.3	0.135	1354	5.48	
	22.8	0.189	1893	7.66	
Gelatt Lake	7.86	0.065	653.0	2.64	3.37
	21.4	0.178	1777	7.19	
	5.97	0.050	495.8	2.01	
Meeboer Lake	0.298	2.48×10^{-3}	24.75	0.100	0.110 ** average
	0.354	2.94×10^{-3}	29.38	0.119	

Table 2. Results of Fisher's exact test for toads that could right themselves in 10 seconds or less from each site to those that could not at each site when compared to the reference site (n=10). The probability level determining significance was alpha ≤ 0.05.

Site	n	p-value
Meeboer Lake	5	0.095
White's Place	7	0.467
Gibbs Pond	8	0.444
Gelatt	8	0.444
7 Mile Road	8	0.069

Cholinesterase inhibition - Mean brain AChE and BChE activities in Woodhouse's toads were higher from spray sites (except at Gelatt Lake) than AChE and BChE activities in surrogates from the reference site, although the differences among sites were not significant (Table 3). None of the brain samples exhibited an increase in either AChE or BChE activity after the addition of 2-PAM (Appendix 3).

Similar to the brain ChE results, mean plasma AChE and BChE activities in Woodhouse's toads from spray sites were higher than from toads at the reference site (Table 4). None of the plasma samples exhibited an increase in either AChE or BChE activity after the addition of 2-PAM (Appendix 3).

Invertebrates

Terrestrial - Pitfall traps were not successful for collecting terrestrial invertebrate samples. The limited quantity of invertebrates collected was insufficient for malathion residue analysis or to make statistical comparisons before and after spraying occurred. No dead or dying terrestrial invertebrates were observed in the sampling areas after spraying occurred.

Aquatic - There were no differences in numbers of aquatic invertebrates collected before or after the spraying occurred except at Creighton lake of Hutton Lake NWR ($p = 0.002$) and Meeboer Lake ($p = 0.017$) (Table 5). More aquatic invertebrates were collected prior to spraying than after spraying at the reference site but the opposite was true for the number of invertebrates collected at Meeboer lake. Malathion concentrations in all invertebrates samples from all sites were below the detection limit (Appendix 4).

ɔle 3. Mean (± 1 SE) brain AChE and BChE activities in Woodhouse's toads collected following malathion spraying.
ɛ probability level determining significance was alpha ≤ 0.05.

Mean Brain Acetylcholinesterase, µmol/min/g				Mean Brain Butyrylcholinesterase, µmol/min/g			
Site	**Reference Site**	**d / c % [1]**	**Anova p-value**	**Site**	**Reference Site**	**d / c %**	**Anova p-value**
Gibbs Pond: 5.710 ± 0.583 n=8, (-14.3) [2]		114.3		Gibbs Pond: 0.462 ± 0.124 n=8, (-39.6)		139.6	
Gelatt Lake: 4.957 ± 0.577 n=7, (0.78)	4.996 ± 0.329 (n=10)	99.22	0.069	Gelatt Lake: 0.330 ± 0.078 n=7, (0.31)	0.331 ± 0.053 (n=10)	99.69	0.363
7 Mile Road: 5.813 ± 0.294 n=7, (-16.4)		116.4		7 Mile Road: 0.389 ± 0.061 n=7, (-17.5)		117.5	
Meeboer Lake: 6.200 ± 0.442 n=4, (-24.1)		124.1		Meeboer Lake: 0.510 ± 0.107 n=4, (-54.1)		154.1	
White's Place: 6.567 ± 0.327 n=7, (-31.4)		131.4		White's Place: 0.557 ± 0.103 n=7, (-68.3)		168.3	

/ c % is ChE activity as % of control.
ımbers in parentheses indicate % inhibition of dosed / controls (d / c).

ıble 4. Mean (± 1 SE) plasma AChE and BChE activities in Woodhouse's toads collected following malathion spraying.
ıe probability level determining significance was alpha ≤ 0.05.

Mean Plasma Acetylcholinesterase, µmol/min/ml				Mean Plasma Butyrylcholinesterase, µmol/min/ml			
Site	**Reference Site**	**d / c % [1]**	**Ancova p-value**	**Site**	**Reference Site**	**d / c %**	**Ancova p-value**
Gibbs Pond: 0.067 ± 0.010 n=5, (-11.7)		111.7		Gibbs Pond: 0.232 ± 0.050 n=5, (-25.4)		125.4	
Gelatt Lake: 0.070 ± 0.005 n=7, (-16.7)	0.060 ± 0.005 (n=10)	116.7	0.311	Gelatt Lake: 0.248 ± 0.090 n=7, (-34.1)	0.185 ± 0.029 (n=10)	134.1	0.308
7 Mile Road: 0.074 ± 0.006 n=8, (-23.3)		123.3		7 Mile Road: 0.192 ± 0.041 n=8, (-3.8)		103.8	
Meeboer Lake: 0.084 ± 0.006 n=5, (-40.0)		140.0		Meeboer Lake: 0.192 ± 0.075 n=5, (-3.8)		103.8	
White's Place: 0.069 ± 0.006 n=7, (-15.0)		115.0		White's Place: 0.199 ± 0.042 n=7, (-7.6)		107.6	

) / c % is ChE activity as % of control.
Jumbers in parentheses indicate % inhibition of dosed/controls (d / c).

Table 5. Quantification of aquatic invertebrates using a modified Gerking Device pre- and post-spraying for mosquito control, Laramie Plains, Wyoming, 1998.

# of Trials conducted / pre- or post-Spray	Site and date collected	Common names of invertebrates collected	# of invertebrates collected in each trial	Two sample t-test p value
10 / Pre-spray	7 Mile Road 6/10	Odonates, Chironomids, Dipterans, Amphipods	10, 17, 28, 27, 53, 40, 20, 33, 20, 52	0.495
10 / Post-spray	7 Mile Road 7/2	Waterboatmen, Odonates	20, 117, 16, 4, 3, 12, 10, 14, 10, 12	
10 / Pre-spray	Meeboer Lake 6/11	Amphipods, Odonates	14, 34, 42, 36, 12, 21, 6, 44, 29, 54	0.068
10 / Post-spray	Meeboer Lake 7/2	Odonates, Amphipods, Waterboatmen	27, 45, 38, 31, 19, 69, 63, 68, 48, 163	
10 / Pre-spray	Mortenson Lake 6/11	Odonates, Chironomids, Amphipods	5, 30, 55, 32, 12, 44, 20, 8, 26, 4	0.872
10 / Post-spray	Mortenson Lake 7/2	Odonates, Amphipods	17, 6, 11, 11, 1, 14, 9, 10, 119, 18	
10 / Pre-spray	Gelatt Lake 6/10	Amphipods, Odonates	42, 12, 26, 20, 34, 25, 12, 8, 38, 11	0.389
10 / Post-spray	Gelatt Lake 7/2	Chironomids, Amphipods, Odonates, Waterboatmen	6, 14, 13, 29, 23, 12, 13, 20, 21, 35	
10 / Pre-spray	Gibbs Pond 6/25	Waterboatmen, Odonates Chironomids, Amphipods,	42, 65, 36, 12, 41, 56, 28, 31, 25, 101	0.082
10 / Post-spray	Gibbs Pond 7/2	Amphipods,, Waterboatmen, Backswimmers, Chironomids	27, 5, 24, 18, 17, 22, 54, 47, 16, 34	
10 / Pre-spray	Hutton Lake NWR 6/15	Amphipods, Odonates	11, 17, 25, 50, 46, 74, 32, 23, 18, 56	0.002
10 / Post-spray	Hutton Lake NWR 7/2	Backswimmers, Amphipods	4, 2, 15, 12, 5, 10, 9, 8, 7, 6,	

DISCUSSION

There was no evidence of cholinesterase inhibition in Woodhouse's toad at any of the study sites. However, the finding of lower cholinesterase activity at the reference site compared to the spray sites led to an investigation to determine if another factor such as brain weight or body weight was influencing cholinesterase activity. There was no significant interaction between the weight of the toads and the sites for either brain AChE ($p = 0.070$) or brain BChE ($p = 0.701$) activities based on a general linear model. Brain AChE, adjusted for weight using an ANCOVA, was not significantly different ($p = 0.111$) among sites. The coefficient for the covariate was also not significant ($F = 0.160$, $p = 0.692$), indicating that weight as a covariate is not correlated with AChE activities and was not helpful in explaining why AChE values at the reference site were lower than sites adjacent to spraying. Similarly, brain BChE, adjusted for weight using an ANCOVA, was not significantly different ($p = 0.407$) among sites and the coefficient for weight was also not significantly different ($F = 0.005$, $p = 0.945$).

Furthermore, there was no significant interaction between the brain weight of the toads and the sites for either brain AChE ($p = 0.775$) or brain BChE ($p = 0.746$) activities. Brain AChE, adjusted for brain weight using an ANCOVA, was not significantly different ($p = 0.076$) among sites. The coefficient for the covariate was also not significant ($F = 0.238$, $p = 0.629$) indicating that using brain weight as a covariate was not helpful in explaining why AChE values at the reference site were lower than sites adjacent to spraying. Similarly, brain BChE, adjusted for brain weight using an ANCOVA, was not significantly different ($p = 0.374$) among sites and the coefficient for brain weight was also not significantly different ($F = 0.039$, $p = 0.845$).

We also examined the possibility that the weight of the toads may explain the differences in mean plasma AChE and BChE activities. There was no significant interaction between the toad body weight and the sites for either plasma AChE ($p = 0.595$) or plasma BChE ($p = 0.139$) activities. The coefficient for the covariate weight was significant ($F = 4.963$, $p = 0.032$) for plasma AChE indicating that weight was correlated with plasma AChE activities; but, mean plasma AChE adjusted for weight using an ANCOVA was not significantly different ($p = 0.311$) among sites. Similarly, the coefficient for weight was significant ($F = 27.511$, $p \leq 0.0005$) for plasma BChE; but, mean plasma BChE adjusted for weight using an ANCOVA was not significantly different ($p = 0.308$) among sites.

Physiological factors did not clarify the generally lower ChE activities at the reference site. Therefore, it is possible that airborne particles of malathion may have entered Hutton Lake NWR from mosquito spraying activities occurring near Sand Creek located approximately 1.6 kilometers southwest of the refuge. The wind direction in the Laramie Region is primarily from the southwest and southeast (National Weather Service Forecast Office, Cheyenne, Wyoming). One study conducted for the Boll Weevil Eradication Program at Penn State (1993), showed deposition of aerially applied ULV malathion up to 21.0, 11.5, 2.9, and 0.7% at 100, 200, 500, 1000 m downwind. Nevertheless, spray cards and aquatic invertebrates from Hutton Lake NWR had no detectable malathion residue.

Of the three sites where malathion was detected on the spray cards, only the mean brain ChE activities in toads from Gelatt Lake were lower than at the reference site, but differences were not significant. The behavior of the toads was not affected and malathion residues were not detected in aquatic invertebrates or water from Gelatt Lake. Although there is very limited information on ChE

activities in adult amphibians, it is possible that the differences in ChE activities are due to natural variation rather than from malathion exposure. Typically, a 50% depression in ChE activities in birds from the reference mean is the threshold for indicating pesticide poisoning (Hill and Fleming 1982) though some laboratory studies suggest an inhibition of 20% below the control mean in a species is indicative of exposure (Grue et al. 1991). Further study is warranted to determine a more realistic dose/response relationship. In addition, analysis of malathion residues in body tissues of the Woodhouse's toads may have been helpful for indicating very recent exposure to malathion (Sparling 2001), particularly because it has been suggested that amphibians are resistant to some degree to organophosphorus insecticides and can bioaccumulate relatively high concentrations (Powell et al. 1982).

We were unable to evaluate the potential exposure of terrestrial invertebrates as dietary items of the Wyoming toad to malathion because of considerable uncertainties associated with the data. Setting up additional pitfall traps for more than two consecutive nights may have allowed us to obtain the necessary information, while reducing variable factors such as temperature, meteorological conditions, or episodic insect hatching events that influence invertebrate activity or numbers. Differences in aquatic invertebrate relative abundance before or after spraying were not significant among sites except for Meeboer Lake and the reference site. At Meeboer Lake, more aquatic invertebrates were collected after spraying than before, even though malathion residues were detected on spray cards. Conversely, fewer aquatic invertebrates were collected after spraying than before at the reference site. No malathion residue was detected in the tissue samples or spray cards indicating that the decrease in numbers was likely due to other factors.

During our study, we did not look at malathion spray drift concentrations that might affect Wyoming toad tadpoles because no natural reproduction at Mortenson NWR had occurred at the time of the study (personal communication with Mitch Bock, Wyoming Game & Fish, Laramie, WY, January 1997). Since the time of the study, natural reproduction has occurred. Further investigation into the possibility of Wyoming toad tadpole exposure to malathion is reasonable because very low concentrations of malathion in water are reported to adversely affect amphibian tadpoles and sublethal exposures of malathion have been shown to retard larval development, cause abnormalities in larvae, and behavioral aberrations (Pawar et al. 1983, de Llamas et al 1985, Rosenbaum et al 1988). For example, the LC50 for *B. woodhousii fowleri* tadpoles (4 to 5 weeks old) exposed to malathion at 24 hour = 1.9 mg/L, at 48 hour = 0.5 mg/L; and, at 96 hr = 0.2 mg/L in static acute toxicity tests (Sanders 1970). A study by Mohanty-Hejmadi and Dutta (1981) showed that malathion (in concentrations lower than those recommended for field application) affected the growth of the Indian bullfrog (*Rana tigerina*) by prolonging the tadpole stage. Of those bullfrogs that did metamorphose, test animals were significantly smaller than control animals.

Additionally, our study did not investigate the potential for other effects (such as immune suppression) to occur in toads after exposure to sublethal concentrations of malathion aerial drift. A laboratory study conducted at the University of Wyoming showed that adult male Woodhouse's toads were more susceptible to the disease redleg and died at a higher rate when exposed to high or low sublethal doses (11.0 or 1.1 µg malathion/g toad, respectively) of field grade ULV malathion (the same type as aerially sprayed in the Laramie Plains) than when just dosed with the red leg causing bacterium alone (Taylor et al. 1999). Because the redleg bacteria and chytrid fungus are found at Mortenson NWR, any challenge (such as pesticide exposure) to the immune system of the Wyoming toad could jeopardize their survival.

Management Implications

Pesticide drift was not detected at Mortenson NWR, but was detected at potential reintroduction sites. The detected malathion concentrations were not acutely toxic to the toad and were lower that the application concentration indicating that the sites did not receive any direct spray. However, the presence of drift is an important consideration to the refuge manager when considering pesticide use on lands bordering Mortenson NWR. Current label restrictions, including the width of the buffer zone which is 0.5 mile for aerial application, may not be adequate for protecting the toad at Mortenson NWR.

Additionally, the presence of drift is also an important consideration to the Wyoming Toad Recovery Team members when they select reintroduction site(s). Reintroduction sites may need to be located in areas where mosquito control activities are carefully monitored. Reintroduction sites that are located in areas where larvaciding is the primary mosquito control method may be the best option as larvicides such as Bti are specific to mosquito larvae unlike malathion which can affect numerous non-target species.

SUMMARY

The purpose of this study was to determine if the survival of the Wyoming toad is detrimentally affected by aerial drift of malathion. We used surrogate Woodhouse's toads and endpoints were defined as mortality, removal of food source, and reduced predatory avoidance. The data demonstrated that drift from mosquito control activities does not directly enter Mortenson NWR but drift does enter potential reintroduction sites. Although the malathion drift concentrations detected at the reintroduction sites are lower than the application concentration, the data are important for making future management decisions regarding pesticide use on lands bordering Mortenson NWR and for continuing reintroduction efforts of the Wyoming toad.

Our data also indicate that any exposure to the pesticide was not great enough to affect predator avoidance behavior, ChE activities, or survival of adult Wyoming toads; but the data does provide some much needed information on ChE activities. Tissue analysis for malathion residues may have been helpful in determining if the small differences in ChE activities observed were the result of malathion exposure or from natural variation. Exposure of the Wyoming toad to malathion drift concentrations greater than those we observed, repeated applications from drift, or to direct spray may be detrimental for the survival of the species. Additional research on the potential effects of spray drift on immune suppression and tadpole survivorship is needed.

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Appendices
Analytical Results

Appendix 1. Malathion concentrations (µg/g wet weight) on filter papers from aerial drift of mosquito control activities in the Laramie Plains, Wyoming, 1998.

Sample ID	Site	Malathion (µg/g) wet weight
TPFP3701	37 Mile Road	28.8
TPFP3702	37 Mile Road	0.595
TPFP3703	37 Mile Road	16.3
TPFP3704	37 Mile Road	22.8
TPFP7M01	7 Mile Road	<0.00200
TPFP7M02	7 Mile Road	<0.00200
TPFP7M03	7 Mile Road	<0.00200
TPFPGB01	Gibbs Pond	<0.00200
TPFPGB02	Gibbs Pond	<0.00200
TPFPGB03	Gibbs Pond	<0.00200
TPFPGL01	Gelatt Lake	7.86
TPFPGL02	Gelatt Lake	21.4
TPFPGL03	Gelatt Lake	5.97
TPFPHL01	Hutton Lake	<0.00200
TPFPHL02	Hutton Lake	<0.00200
TPFPMB01	Meeboer Lake	<0.00200
TPFPMB02	Meeboer Lake	0.298
TPFPMB03	Meeboer Lake	0.354
TPFPMT01	Mortenson Lake	<0.00200
TPFPMT02	Mortenson Lake	<0.00200
TPFPMT03	Mortenson Lake	<0.00200
TPFPWH01	White's Place	<0.00200
TPFPWH02	White's Place	<0.00200
TPFPWH03	White's Place	<0.00200

Date	# of trials that occurred at a given time	Time (seconds)
6/9/1998	52	1
	10	2
	9	3
	5	4
	4	5
	2	7
	4	9
	2	10
	1	11
	1	17
	1	20
	1	26
	1	29
	6	≥30
	Mean time	4.97
	% trials that occurred ≤ 10 seconds	89%
	% trials that occurred > 10 seconds	11%

Date	Site	# of trials that occurred at a given time	Time (seconds)
6/30/1998	Meeboer	3	1
		3	2
		1	4
		1	9
		1	15
		1	≥30
		Mean time (seconds)	6.97
		% trials that occurred ≤10 seconds	80%
		% trials that occurred >10 seconds	20%
6/30/1998	Gibb's	10	1
		2	2
		2	9
		1	16
		1	19
		2	≥30
		Mean time (seconds)	7.06
		% trials that occurred ≤10 seconds	78%
		% trials that occurred >10 seconds	22%
6/30/1998	Gelatt	9	1
		3	2
		3	3
		1	21
		Mean time (seconds)	2.81
		% trials that occurred ≤10 seconds	94%
		% trials that occurred >10 seconds	6%

Date	Site	# of Trials that occurred at a given time	Time (seconds)
6/30/1998	White's	10	1
		1	2
		1	5
		1	6
		1	12
		Mean time (seconds)	2.50
		% trials that occurred ≤10 seconds	93%
		% trials that occurred >10 seconds	7%
7/01/1998	7-Mile	14	1
		2	2
		1	3
		1	4
		1	11
		1	12
		4	≥30
		Mean time (seconds)	7.00
		% trials that occurred ≤10 seconds	83%
		% trials that occurred >10 seconds	17%
6/30/1998	Hutton	10	1
		1	2
		1	4
		1	5
		2	6
		1	7
		2	≥30
		Mean time (seconds)	5.56
		% trials that occurred ≤10 seconds	89%
		% trials that occurred >10 seconds	11%

ppendix 3. Brain and plasma ChE activities in Woodhouse's toads collected following malathion spraying in adjacent fields.

Sample ID	Site	Animal Weight (g)	Sex	Brain Weight (g)	Brain		Plasma	
					AChE	BChE	AChE	BChE
GB 01	Gibbs Pond	26	F	0.0439	7.49	0.85	NA	NA
GB 02		65	M	0.0452	6.95	0.76	NA	NA
GB 03		67	M	0.0854	5.42	0.42	0.063	0.208
GB 04		65	M	SL	NA	NA	0.060	0.104
GB 05		59	M	0.0587	5.61	0.51	0.064	0.168
GB 06		38	F	0.035	7.34	0.12	0.103	0.393
GB 07		55	M	0.0461	6.21	0.89	NA	NA
GB 08		39	F	0.0234	3.42	0.00	NA	NA
GB 09		45	M	0.0296	3.24	0.15	0.046	0.285
				Min	3.24	0.00	0.05	0.10
				Max	7.49	0.89	0.10	0.39
				Mean	5.47	0.42	0.065	0.210
				Median	5.91	0.47	0.063	0.208
GL 01	Gelatt Lake	59	M	0.0393	4.87	0.50	0.062	0.140
GL 02		88	F	0.0651	5.42	0.33	0.064	0.147
GL 03		43	M	0.0262	2.96	-0.01	0.078	0.727
GL 04		40	F	0.0267	3.32	0.31	NA	NA
GL 05		46	M	SL	NA	NA	0.082	0.385
GL 06		68	F	0.116	4.56	0.3	0.053	0.136
GL 07		72	F	0.0503	7.15	0.65	0.057	0.021
GL 08		88	F	0.0593	6.42	0.23	0.091	0.178
				Min	2.96	-0.01	0.05	0.02
				Max	7.15	0.65	0.09	0.73
				Mean	4.75	0.36	0.068	0.162
				Median	4.87	0.31	0.06	0.15

.ppendix 3. cont.

Sample ID	Site	Animal Weight (g)	Sex	Brain Weight (g)	Brain		Plasma	
					AChE	BChE	AChE	BChE
HL 01	Hutton Lake NWR	27	F	0.0229	5.43	0.18	0.076	0.280
HL 02		82	F	0.0624	5.39	0.28	0.046	0.165
HL 03		37	F	0.0488	5.71	0.68	0.085	0.331
HL 04		70	F	0.0504	5.64	0.39	0.060	0.234
HL 05		81	F	0.0403	3.49	0.34	0.041	0.190
HL 06		83	F	0.0500	5.85	0.06	0.052	0.024
HL 07		93	F	0.0764	4.35	0.44	0.063	0.124
HL 08		84	F	0.0509	4.11	0.25	0.044	0.104
HL 09		69	F	0.0705	3.53	0.26	0.063	0.148
HL 10		39	F	0.0507	6.46	0.43	0.071	0.252
				Min	3.49	0.06	0.04	0.02
				Max	6.46	0.68	0.09	0.33
				Mean	4.89	0.28	0.06	0.15
				Median	5.41	0.31	0.06	0.18
MB 01	Meeboer Lake	51	M	SL	NA	NA	0.079	0.395
MB 02		41	F	0.0437	7.50	0.82	0.078	0.354
MB 03		63	M	0.0712	5.78	0.38	0.093	0.036
MB 04		51	M	0.0508	5.97	0.36	0.104	0.083
MB 05		53	M	0.0336	5.55	0.48	0.067	0.091
				Min	5.55	0.36	0.07	0.04
				Max	7.50	0.82	0.10	0.39
				Mean	6.16	0.48	0.083	0.131
				Median	5.88	0.43	0.08	0.09

ppendix 3. cont.

Sample ID	Site	Animal Weight (g)	Sex	Brain Weight (g)	Brain		Plasma	
					AChE	BChE	AChE	BChE
7M 01	7 Mile Road	71	M	0.0711	6.56	0.57	0.051	0.113
7M 02		88	F	0.0737	4.19	0.39	0.082	0.128
7M 03		45	F	SL	NA	NA	0.087	0.126
7M 04		79	F	0.410	6.31	0.36	0.087	0.066
7M 05		29	F	0.0273	5.82	0.38	0.067	0.206
7M 06		55	F	0.0397	6.13	0.42	0.047	0.172
7M 07		36	F	0.0223	6.04	0.07	0.087	0.401
7M 08		28	M	0.0333	5.64	0.53	0.085	0.323
				Min	4.19	0.07	0.050	0.070
				Max	6.56	0.57	0.090	0.400
				Mean	5.76	0.34	0.072	0.165
				Median	6.04	0.39	0.080	0.150
WH 01	White's Place	69	M	0.0548	6.23	0.53	0.080	0.209
WH 02		64	M	0.0612	5.13	0.49	0.047	0.091
WH 03		37	F	0.0425	6.65	0.40	0.095	0.354
WH 04		48	M	0.0433	6.97	0.80	0.057	0.280
WH 05		52	M	0.032	7.33	0.47	0.067	0.272
WH 06		69	M	0.0659	7.67	0.19	0.068	0.047
WH 07		61	M	0.0533	5.99	1.02	0.066	0.138
				Min	5.13	0.19	0.05	0.05
				Max	7.67	1.02	0.10	0.35
				Mean	6.52	0.50	0.07	0.16
				Median	6.65	0.49	0.07	0.21

. - Sample lost in preparation.

A - Not Analyzed if brain not available or if serum not recoverable.

Site	Invertebrate Sample Type	Malathion (µg/g) wet weight
7 Mile Road	Mayfly Larvae (Ephemeroptera)	<0.0500
7 Mile Road	Dragonfly / Damselfly Larvae (Odonata)	<0.0500
Gibbs Pond	Waterboatmen (Heteroptera)	<0.0500
Gibbs Pond	Waterboatmen (Heteroptera)	<0.0500
Gelatt Lake	Water Fleas (Cladocera)	<0.0500
Gelatt Lake	Water Fleas (Cladocera)	<0.0500
Hutton Lake NWR	Dragonfly / Damselfly Larvae (Odonata)	<0.0500
Hutton Lake NWR	Amphipods (Amphipoda)	<0.0500
Hutton Lake NWR	Backswimmers (Heteroptera)	<0.0500
Meeboer Lake	Dragonfly / Damselfly Larvae (Odonata)	<0.0500
Meeboer Lake	Dragonfly / Damselfly Larvae (Odonata)	<0.0500
Mortenson Lake	Dragonfly / Damselfly Larvae (Odonata)	<0.0500