

The Pennsylvania State University  
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RESPONSES OF PLETHODONTID SALAMANDERS  
TO STREAM ACIDIFICATION AND ACID MINE DRAINAGE  
IN THE PENNSYLVANIA CENTRAL APPALACHIANS

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by  
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## ABSTRACT

Low pH and dissolved heavy metals are toxic to amphibians by disrupting ionoregulatory mechanisms that cause loss of body sodium. Stream salamanders of the family Plethodontidae can occupy small, northern Appalachian streams in large numbers and are important vertebrate components. In the Pennsylvania central Appalachians, up to seven species comprise a streambank assemblage. The northern two-lined (*Eurycea b. bislineata*), longtail (*E. longicauda*), northern spring (*Gyrinophilus p. porphyriticus*), and northern red (*Pseudotriton r. ruber*) salamanders (subfamily Plethodontinae) have aquatic egg-laying habits and extended aquatic larval stages (1-4 yr). The mountain dusky (*Desmognathus ochrophaeus*), northern dusky (*D. f. fuscus*), and Appalachian seal (*D. monticola*) salamanders (subfamily Desmognathinae) have terrestrial egg-laying habits and shorter larval stages (3 mo - 1.5 yr). Differences in life history and nesting habits were hypothesized to favor terrestrial nesters in acidified streams because of greater exposure to stream toxicity in more aquatic forms. Three investigations were completed, each focusing on resolving separate but related questions. The effect of acidification on stream salamander abundance and distribution was initially addressed by sampling for stream salamanders along an acid-alkaline gradient. The direct effects of stream toxicity was investigated subsequently by *in situ* exposure of aquatic nesters, assemblage members most likely to be affected. The hypothesized benefits of terrestrial habits and life history were investigated in terrestrial nesters by simulating adverse nesting environments in the laboratory. Investigations subsequent to the field study were intended to compare survival of taxa that exhibited different distribution patterns but

shared similar nesting habits and life histories. Assemblage response in the field (abundance, species and life stage composition) was investigated in 1997-1998 by plot-sampling for salamanders in 14 Pennsylvania Central Appalachians streams representing acid mine drainage-contaminated (AMD), episodically acidified, non-acidic, and non-acidic-high alkalinity conditions. Acidic streams favored the mountain dusky and northern spring salamanders, adult or terrestrial stages, and low abundance. The northern two-lined and northern dusky, aquatic larvae, and higher salamander abundance were favored in non-acidic streams. Ordination by correspondence analysis (CA) grouped the above taxa similarly and along the axis most highly correlated to the acid-alkaline gradient sampled. Removal, by partial canonical correspondence analysis (CCA), of variation tied to stream physical habitat failed to eliminate significant associations with water chemistry. Low abundance of the northern dusky, a terrestrial nester, and persistence of the northern spring, an aquatic nester with an extended aquatic larval stage, in acidic streams, were inconsistent with the proposed general hypothesis that survival favored terrestrial nesting and shorter aquatic larval stages. The direct effects of stream toxicity were investigated by *in situ* exposure of northern spring and northern two-lined salamanders in AMD-contaminated reaches where sampling had revealed low abundance. Survival, changes in body water content and total body sodium were examined as response variables in 1998 and 1999 by 19-d, 48-hr, and 12-hr *in situ* exposures in reaches of varying AMD-contamination. Testing of the northern spring was limited to the 48-hr exposure. Exposures were conducted in southwestern and central Pennsylvania streams using wild-caught larvae and metamorphs. Most northern two-lined

larvae and metamorphs survived the 19-d (weak AMD) and 12-hr (strong AMD) exposures. Survival in the 48-hr exposure favored lower AMD-contamination, larger and older larvae, metamorphs, and northern spring larvae. The proportion of body water was lower after 19-d and 48-hr exposures, relative to controls, but increased initially during the 12-hr exposure. Total body sodium was lost in all exposures. The northern dusky and mountain dusky are both terrestrial nesters. In acidic streams, the northern dusky declined, whereas the mountain dusky persisted. Absence of an aquatic larval stage, as reported in the early literature, was hypothesized to favor survival in acidic streams. Survival in hatchlings of both species was examined in the laboratory by exposure to treatments simulating acidic, dry, and normal nesting environments. Hatchlings were collected in nests from southwestern and central Pennsylvania. Early transformation was observed in only 2 (<1%) of the original 302 northern dusky hatchlings collected and before the experiment began. Hatchling survival was lowest in acidic and dry treatments. Hatchling size and their origins also affected survival. A single mountain dusky nest was found; all 10 hatchlings transformed within weeks of hatching and survived the 51-d exposure irrespective of container treatment. Based on the findings of the three investigations, reduced abundance and distribution of stream salamanders is attributed to the direct effects of acidification. Persistence in acidic streams by the life-history route was possible by early transformation, the bypassing of the aquatic stage. Natural acid tolerance aided survival in one aquatic nester. The impact of stream acidification was anticipated to be considerable in stream salamanders lacking either mechanism.

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## PREFACE

Considerable evidence exists on the effects of acidification on amphibians, likewise for fishes occupying smaller acidic headwaters. The present investigation sought to contribute to our understanding of the responses to acidification by an abundant, stream-dwelling vertebrate, the lungless stream plethodontids, by addressing several questions: are their assemblages shaped by episodic or chronic acidification? Are responses consistent with the degree of terrestriality and stream independence afforded by their life histories? If responses exist, are these attributed to the direct effects of stream toxicity? Does greater terrestriality favor survival and persistence? The dissertation consists of a brief introductory chapter, three chapters describing the core investigations, and a fifth chapter synthesizing the findings. The middle chapters were prepared as stand-alone journal papers. The investigation described in Chapter 1 uses the same data set presented in an EPA final report submitted in 2000 and co-authored by R.P. Brooks. Field and laboratory work was completed between April 1997 - December 1999. I am deeply indebted to my Thesis Advisor and Chair of Committee, R. P. Brooks, and my Committee, R.F. Carline, W.M. Tzilkowski, and A.H. Taylor for their patience during this lengthy “gestation period”, the time from completing the investigation to the preparation of the present document.

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## Chapter 1

### INTRODUCTION

Lungless stream salamanders (family Plethodontidae) are an ubiquitous and abundant vertebrate component of small, often fish-less Appalachian headwaters, and serve the dual function of invertebrate predator and vertebrate prey (Coker 1931, Bennett and Bellis 1969, Burton and Likens 1975, Krzysik 1979, Davic 1983, Davic and Welch 2004). Not surprisingly, the investigation of factors determining plethodontid assemblage composition and structure were the focus for numerous investigations (Hairston 1987 and Petranka 1998 offer broad reviews).

The impact of low pH and heavy metals on amphibians was studied extensively in recent years, originally in response to the concern for acidic precipitation, and subsequently in reaction to reports of worldwide declines (Pough 1976, Pierce 1985, 1993, Dunson et al., 1992, Wake 1991, see reviews by Freda, 1991a, 1991b). Numerous investigations have demonstrated the lethal and sublethal effects of elevated  $H^+$ , Al, and other metals on amphibians in relation to species (Freda and Dunson 1984), life stage (Freda 1986, Pierce et al. 1984, Freda and McDonald 1990), other water chemistry variables (Saber and Dunson 1978, Horne and Dunson 1995a, 1995b, Freda et al. 1990), and aquatic and terrestrial habitats (Wyman and Jancola 1992, Wyman 1988, Horne and Dunson 1994). At lethal concentrations, low pH and metals become toxic to amphibians by several mechanisms, depending on life stage. Acidic and metal bearing waters also have an indirect impact on amphibian recruitment and community structure by inhibiting

growth, adversely affecting lower trophic levels and predator-prey interactions (Freda et al. 1991). Curiously, many of the investigations described above was focused on vernal pool-breeding amphibians.

The abundance of stream plethodontids and the relative ease to sample them, reports of widespread episodic and chronic stream acidification in the region, and the dearth of information at the intersection of these very wide research topics captured my interest. Broadly defined, the objective of the present investigation was to resolve how stream acidification affected stream salamanders and document their responses.

In the first (Chapter 2) of the three separate investigations completed, the effect on assemblages and their responses to stream acidification was examined by sampling stream side salamanders along an acid-alkaline gradient. For this purpose, 14 small watersheds in the Pennsylvania Central Appalachians were chosen for their water chemistry and intensively sampled in 1997 and 1998. Episodically acidified and acid mine drainage (AMD) impacted stream reaches provided the acidic end of the gradient. Alkaline watersheds on ridges and valleys represented the opposite end. In this region, as many as seven species of stream salamanders can occupy streambanks (Shaffer 1992). Four species, the northern two-lined (*Eurycea bislineata*), longtail (*E. longicauda*), northern spring (*Gyrinophilus p. porphyriticus*), and northern red (*Pseudotriton r. ruber*) salamanders are tightly bound to the aquatic portion of streambanks by virtue of their life histories and aquatic egg-laying habits, and aquatic larval stages that require up to 4 years



before transformation (metamorphosis) can occur. The mountain dusky (*Desmognathus ochrophaeus*), northern dusky (*D. f. fuscus*), and Appalachian seal (*D. monticola*) salamanders are terrestrial egg-laying taxa. Unlike the former, grape-like clusters of eggs suspended under rocks are deposited and guarded in shallow, moist, terrestrial hollows (Bishop 1941). Soon after hatching the young move to water and continue development as aquatic larvae for periods of 8-12 months (Pfungsten and Downs 1989).

Differences in life histories and greater sensitivity in early amphibian life stages (eggs, embryos, and recent hatchlings) were hypothesized to favor terrestrial egg-laying taxa. If true, stream acidification was anticipated to shape stream salamanders in favor of the more terrestrial forms. Salamander abundance, and species and life stage composition were the response variables examined.

The second investigation (Chapter 3) was aimed at confirming the direct effects of low pH and dissolved heavy metals, resulting from AMD contamination, on larval and metamorphic life stages of two aquatic-nesting taxa, the northern two-lined and northern spring salamanders. Hence, *in situ* stream exposures were performed where previous field sampling had revealed their absence or depressed abundance. Survival and changes in body water and total body sodium were examined as response variables. The three experiments completed in 1998 and 1999 tested the hypotheses that stream water toxicity was the direct cause for the depressed aquatic stages of the northern two-lined and northern spring salamanders and that the former was less acid tolerant than the latter.

The third investigation (Chapter 4) was completed in the laboratory in fall 1998, to observe hatchling northern dusky and mountain dusky salamanders survival and transformation in treatments simulating dry, acidic, and normal (control) nesting environments. According to early literature accounts (Bishop 1941, Noble and Evans 1932), both taxa can bypass the aquatic life stage, a trait that in acidic environments was hypothesized to be advantageous where adjacent terrestrial surfaces serve as refugia. The response variables examined were hatchling survival and evidence of transformation. The results of the *in situ* and laboratory bioassays were anticipated to provide insight on the field sampling results.

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## Chapter 2

### ACIDIFICATION AS AN ASSEMBLAGE-SHAPING MECHANISM: ABUNDANCE AND DISTRIBUTION OF STREAM PLETHODONTIDS IN AN ACID-ALKALINE GRADIENT IN THE PENNSYLVANIA CENTRAL APPALACHIANS

Abstract --The effects of low pH and dissolved heavy metals from episodic acidification and acid mine drainage (AMD) were suspected to adversely affect stream salamanders of the family Plethodontidae, a taxa that can occupy small Appalachian headwaters in high numbers. In the Pennsylvania Central Appalachians, assemblages can comprise up to seven species. The northern two-lined (*Eurycea b. bislineata*), longtail (*E. longicauda*), northern spring (*Gyrinophilus p. porphyriticus*), and northern red (*Pseudotriton r. ruber*) salamanders (subfamily Plethodontinae) have aquatic egg-laying habits and extended aquatic larval stages (1-4 yr). The mountain dusky (*Desmognathus ochrophaeus*), northern dusky (*D. f. fuscus*), and Appalachian seal (*D. monticola*) salamanders (subfamily Desmognathinae), have terrestrial egg-laying habits and shorter larval stages (0 - 1.5 yr). Differences in life history and nesting habits were hypothesized to favor terrestrial nesters in acidified streams in response to direct effects of stream toxicity. Assemblage response (abundance, species and life stage composition) was investigated in 1997-98 by plot-sampling for stream salamanders in 14 Pennsylvania Central Appalachians streams representing an acid-alkaline gradient: AMD-contaminated, episodically acidified, non-acidic, non-acidic-high alkalinity. Species associations and species - environment associations were examined by ordination (correspondence analysis (CA), canonical correspondence analysis (CCA)). A total of 305 plots (586 person-hours) were sampled from 8 tributary and 53 mainstem transects. A total of 3,560 salamanders were captured, ranging 20-700 per stream and 0 - 117 per plot. Almost 87% were captured in non-acidic streams. Median plot salamander density (salamanders/m<sup>2</sup>) was 0.5 in AMD, 0.75 in episodic, 3.12 in non-acidic, 4.5 in non-acidic-high alkalinity.

Of the 7 species observed, only 4 were widespread and commonly encountered: the northern two-lined and *Eurycea* larvae composed 58%. Larvae and metamorphs were the most common (64.9%) life stage. Acidic streams containing salamanders favored the mountain dusky and northern spring salamanders, and adult, or terrestrial stages and low abundance. Non-acidic streams favored the northern two-lined and northern dusky, aquatic larvae, and high abundance. Ordination by CA grouped the above taxa similarly and along the axis most highly correlated to water chemistry, the acid-alkaline gradient sampled. Fitting of covariates tied to stream physical habitat by partial CCA failed to eliminate the significant associations with water chemistry. Low abundance of the northern dusky, a terrestrial nester, and persistence of the northern spring, an aquatic nester with an extended aquatic larval stage, in acidic streams were inconsistent with the proposed hypothesis that independence from the stream to complete the life cycle favored survival. Persistence of the mountain dusky was attributed to the absence of an aquatic stage; in the northern spring, it was linked to natural acid tolerance.



## Introduction

Acidification of surface waters is a significant stressor to aquatic biota. Among the amphibians, osmoregulatory failure and the subsequent loss of body sodium are cited as the principal mechanisms for physiologic stress and toxicity (Freda and Dunson 1984). Early life stages (eggs, embryos, and hatchlings) are the most sensitive and interspecific differences in tolerance to low pH and metal toxicity were confirmed in past studies (Saber and Dunson 1978, Dunson and Connell 1982, Pierce et al. 1984, Freda 1986, Freda and Dunson, 1986, Freda and McDonald 1990, Freda et al. 1990, Freda 1991, Horne and Dunson 1994, 1995a, 1995b). Beyond lethal or physiological effects, acidification has the potential to disrupt amphibian community structure and composition through ecological interactions (Kucken et al. 1994, Sadinski and Dunson 1992). Surprisingly much of what is known today on the impact of acidification on amphibians was learned from the investigation of vernal pool and lentic biota.

In the northern Appalachians, small headwaters often comprising as much as 60 - 75% of a riverine network and watershed area (Herlihy et al. 2000), provide habitat to a diverse assemblage of lungless salamanders in the family Plethodontidae (Petranka 1998). Yet, the effect of acidification on stream-dwelling salamanders has received comparatively little attention. Poorly-buffered headwaters in the northern Appalachians appear to be the most vulnerable to acidification. Atmospheric deposition from Ohio Valley powerplants and acid mine drainage (AMD) has degraded thousands of stream

kilometers. The potential for impact to stream salamanders by acidification from the single or combined action of episodic acidification or AMD would seem to be significant.

Episodic acidification of streams is the lowering of pH and alkalinity with increasing stream flow, a phenomenon typically observed in small, poorly buffered headwaters. In addition to elevated  $H^+$ , stream organisms must cope with high, often toxic Al concentrations (DeWalle 1990, Sharpe 1990, Van Sickle et al. 1996). Numerous studies document the responses of fish and benthic macro invertebrates to episodic acidification (Sharpe et al. 1983, Sharpe et al. 1984, Gagen and Sharpe 1987, Tietge et al. 1988, Gagen et al. 1994, DeWalle et al. 1995, Van Sickle et al. 1996). The impact of terrestrial acidification as may occur from acidic deposition in plethodontid salamanders was investigated by Wyman (1988), Frisbie and Wyman (1991), and Wyman and Jancola (1992).

Depending on the volume and chemistry of the discharge and buffering capacity of receiving waters, acid mine drainage (AMD) can have severe effects on aquatic life forms, including amphibians (Porter and Hakanson 1976, Boyer and Wismer 1995). Huckabee et al. (1975), Mathews and Morgan (1982), and Kucken et al. (1994) document the early and long-term adverse effects of sulfate and metal contamination on fish and stream salamanders following the use of pyritic rocks in the construction of a highway stream crossing. The contamination eliminated aquatic stream salamander species by direct toxicity and indirectly altered community structure and composition through

competitive exclusion (Jaeger 1971). Spring salamander museum records are absent from southeastern Ohio, presumably, due to extensive degradation of streams from coal mining (Pfungsten and Down 1989). Gore (1983) found northern dusky salamander larvae in only 16 of 78 low order streams impacted by AMD and noted that acidic waters combined with high metal concentrations excluded larvae more effectively than low pH alone. Within the Appalachian Coal Region, which includes eight eastern states, approximately 5,000 stream km are degraded by AMD. In Pennsylvania, it is considered the leading cause of stream pollution and approximately 2,500 stream km are affected (Arway 1995, Boyer and Wismer 1995).

In Pennsylvania, up to seven species representing two subfamilies, can compose a streambank salamander assemblage (Shaffer 1992, Hulse et al. 2001). Four species, the northern two-lined (*Eurycea b. bislineata*), longtail (*E. longicauda*), northern spring (*Gyrinophilus p. porphyriticus*), and northern red (*Pseudotriton r. ruber*) salamanders belong to the subfamily Plethodontinae. These species have aquatic egg-laying habits and aquatic larval stages that may last up to 4 years. Egg masses are typically attached to the underside of submerged rocks that may be guarded by one or more females. The mountain dusky (*Desmognathus ochrophaeus*), northern dusky (*D. f. fuscus*), and Appalachian seal (*D. monticola*) salamanders belong to the Desmognathinae, a terrestrial egg-laying group. Unlike the Plethodontinae, grape-like clusters of eggs suspended under rocks are deposited and guarded in shallow, moist, terrestrial hollows (Bishop 1943). Soon after hatching the young move to water and continue development as aquatic larvae

for periods of 3-15 months (Pfungsten and Downs 1989, Petranka 1998). Life history, breeding habits, and natural history, even among northern Appalachian stream salamanders, are highly variable and range from terrestrial-nesting with short or no aquatic stage to aquatic-nesting with aquatic larvae requiring up to 4 years to transform (metamorphosis).

In the present study, stream acidification was hypothesized to be an important factor shaping salamander assemblages. In consideration of the variable life histories and nesting habits, terrestrial nesters were predicted to have an advantage over aquatic nesters and those with extended aquatic larval life stages. Sampling salamanders along an acid - alkaline gradient was anticipated to provide insight on distribution of salamanders in response to acidification and as a function of life history. Hence, what's the impact of stream acidification on stream plethodontids and is life history a reliable predictor of the response to acidification?

In order to complete the study, 14 small Central Appalachian headwaters providing an acid-alkaline gradient were sampled for stream salamanders (Appendix 2.1). Species composition, abundance, and age-structure were the response variables of interest, as were salamander association to environmental correlates relevant to water chemistry and stream physical habitat. The latter were anticipated to play an important, yet secondary role relative to water chemistry in shaping the assemblage of interest.

## Methods

### Sampling Design and Rationale

The acid-alkaline gradient sought for this study was represented by four stream condition categories: chronic acidification (AMD), episodic acidification, non-acid-circumneutral, and non-acid-high alkalinity. Initial selection of study watersheds was guided by previous investigations confirming the conditions of interest, e.g., identification of episodic acidification required intensive water sampling, particularly after heavy storm events. Candidate watersheds were further evaluated by considering landscape settings, land cover, land use, stream physical attributes, and geographic vicinity to other study streams. Presence of other non-acid related stressors was minimized to the extent possible. Streams in the high alkaline category were originally chosen to represent moderate levels of agricultural disturbance (Rocco and Brooks 2000). Preference was given to first and second order watersheds. All study sites drained to the Ohio or Susquehanna Rivers and were located in the Pennsylvania portion of the Appalachian Plateau physiographic province.

A nested design was chosen for sampling. Stream reaches within a watershed representing one of four stream condition categories were sampled by five, 4 m<sup>2</sup> plots (Rocco and Brooks 2000). Thus, plots were nested in transects, in individual watersheds, and in stream condition categories. Transects were 100 - 150 m in length and spanned portions of a mainstem or tributary. Their selection within a watershed was dependent on the availability of suitable plot sampling sites and stream reach water chemistry. Streams

or stream segments possessing mostly sandy substrates, large boulders (> 2m), bedrock bottom, or numerous waterfalls were excluded from consideration because of the lack of habitat or sampling difficulties they presented. Mainstem transects, as their namesake implies, were at trunk portions of a stream. A first order stream reach draining to a mainstem was considered a tributary.

There were five, 2-m by 2-m rectangular plots to each transect. The use of plots to sample stream salamanders originated from Davic's (1997) 10-m transect method. Plots were flagged 1 m on both sides of the land-water interface to include both terrestrial and aquatic stream bank habitat. Only riffle and run areas were sampled to minimize substrate and stream bank variability. All rocks, logs, and debris within plots were temporarily removed and substrate raked by hand to search for concealed and buried salamanders on land and water (Figure 2.1). Plots were searched by two persons simultaneously with each assigned to the wet or dry portion of the bank. Adult salamanders and larvae were captured by hand or dipnet. Headlamps illuminated search areas when overhead canopy reduced ambient light. Sampling was terminated when no additional animals were found and the entire plot area was searched. The search was timed to the minute. A minimum of 30 min were spent sampling each plot. Searches were time consuming and labor intensive, but effective in keeping surveyors close to the ground. Plot salamander counts and specimen attributes were the basis for determining abundance, and species and life stage composition.

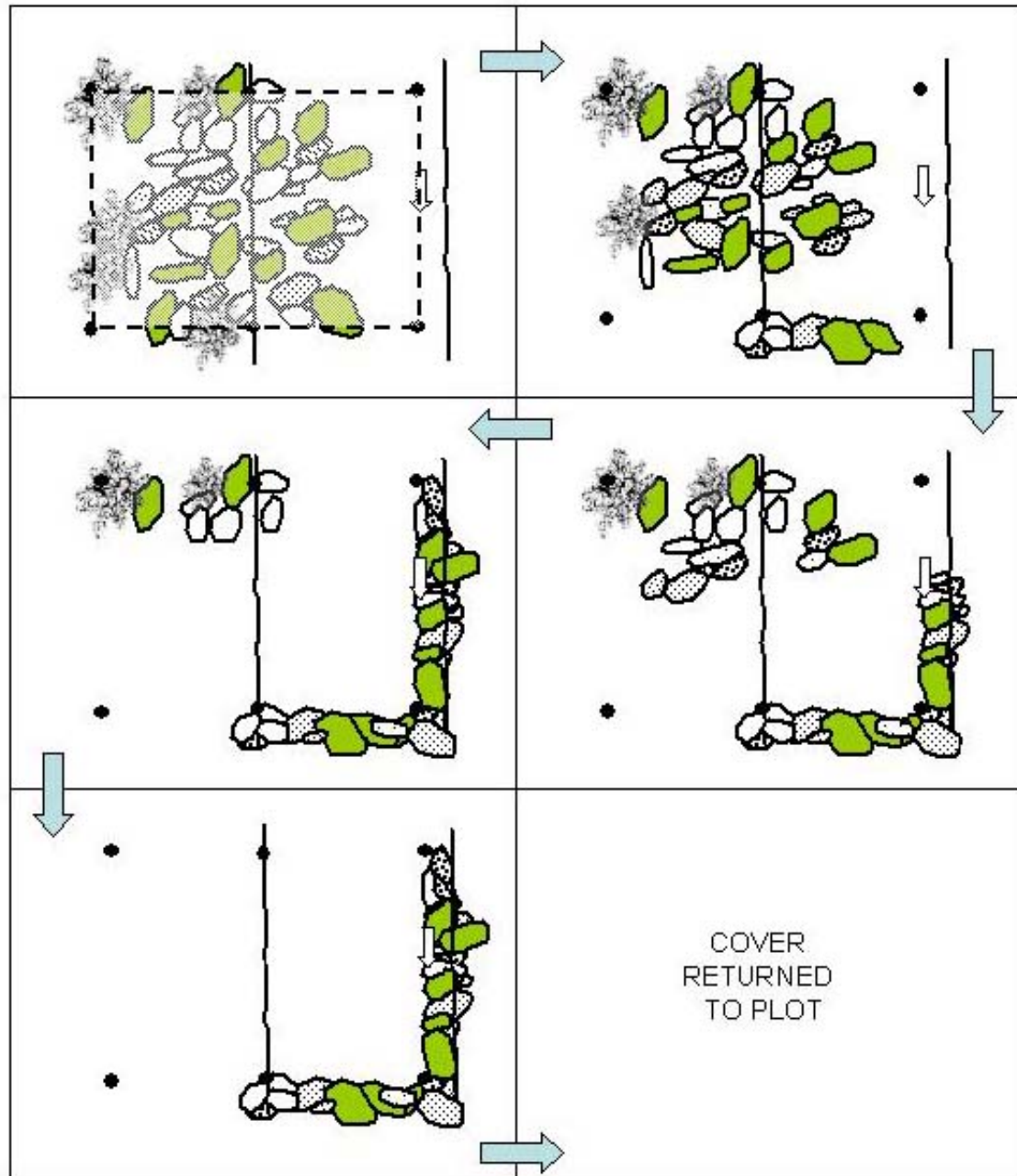


Figure 2.1. Positioning of the 4-m square rectangular plot in the stream bank and sampling sequence implemented to sample 14 small Pennsylvania watersheds for salamanders in 1997-1998. Removal of cover began at the downstream end of the plot and proceeded upstream. Wet and dry bank areas of the plot were sampled by 2 persons simultaneously. Positioning of removed cover along the outer edge of the wetted portion of the plot improved visibility. All cover was returned to the plot upon sampling completion.

## Specimen / Voucher Processing

Specimens were temporarily held in labeled plastic bags and transported back to the field station on ice. Plastic bags were partly filled with moist humus or stream water (depending on life stage), inflated with air, and sealed with rubber bands. Specimens were grouped by size to avoid cannibalism. Vouchers were euthanized by immersion in warm 15-25% ethyl alcohol solution (Heyer et al. 1994). Vouchers were subsequently rinsed in water and transferred to shallow trays for positioning and initial hardening in 10% formalin (30% formaldehyde). The latter facilitated future specimen examination, identification, dissection, and measurement. Final voucher storage was in 70% ethyl alcohol solution. In this study, early larvae of the genus *Eurycea* spp. were not identified to species. All other taxa were identified to species regardless of life stage. Designation of life stage was based on the presence (larvae) or absence of gills. Non-gilled specimens were classified as juveniles or adults based on snout-vent length (SVL) measurements reported in the literature (Pfungsten and Downs 1989) or presence of yolked follicles.

## Environmental Measurements

### Microclimatic Variables and Water Chemistry

Variables related to microclimate were measured before each search. These included ambient, surface, and soil temperature, and ambient and surface relative humidity. Stream water temperature, pH, conductivity, and dissolved oxygen concentration and saturation were determined in the field. A Fisher Accumet 1003 pH meter measured water temperature and pH. Onset ® temperature loggers enclosed in



submersible cases were installed in 4 Westmoreland county streams to monitor water temperature at 2.5-h intervals. These monitored streams represented fragmented (n = 2), reference (n=1), and episodic (n=1) stream condition categories and were within 6.25 km radius of each other. HACH® pH kits measured pH calorimetrically. Specific conductance was measured in microsiemen ( $\mu\text{S}$ ) units with an Hanna HI 8733 conductivity meter. A YSI Model 55 Hand held Dissolved Oxygen System measured dissolved oxygen. Equipment was calibrated with standard buffers and solutions.

Grab samples were collected periodically at several locations in each of the 14 study watersheds. Samples were transported on ice within 48 hr of sample collection and analyzed for the following attributes: pH, alkalinity ( $\text{CaCO}_3$ ), acid neutralizing capacity (ANC), nitrate ( $\text{NO}_3\text{-N}$ ), sulfate ( $\text{SO}_4$ ), dissolved organic content (DOC), Al, Mn, and Fe. All analyses were carried out at the Penn State Water Quality Laboratory, an EPA approved facility. In 1998, samples collected for metals concentrations were filtered through a 0.45  $\mu\text{m}$  filters within 48 hr and prior to acidification. Samples were not filtered in 1997 prior to acidification, consequently, reported metal concentrations may be higher than actual from previously suspended particulates. Analyses for ANC, DOC,  $\text{SO}_4$ , and Mn were only performed in 1998. Grab samples supplemented field measurements and were used to evaluate the range of water chemistry during the study period and during high flows.

## Stream Bank Physical Habitat and Watershed Land Cover Measurement

Stream bank physical attributes were determined by plot and stream reach measurements. A diagonally-placed metric tape aided the estimation of physical and vegetative cover in sampling plots. This method, similar to a modified strip transect, was highly correlated to plot photographs (G. Rocco and M. Wise pers. obs. 1999). It also caused minimal disturbance to plots before the sampling of salamanders. Other physical habitat measurements recorded were stream depth, stream width, stream depth, and bank full width among others suggested by Davic (1997). Land cover and associated landscape metrics (e.g. diversity, dominance, contagion, cover type, patch frequency and mean size) for study watersheds were computed from the 1990s-developed Multi-Resolution Land Cover (MRLC) data sets for USEPA's Region 3 (Miller et al. 1997).

## Data Analysis

Associations between assemblage attributes (species composition, abundance, life stage) and environmental factors (water chemistry, stream physical habitat, etc.) were examined at two spatial scales: watershed and transect. Both offered advantages and disadvantages. Unlike transects drained by the same channel, populations of salamanders in different watersheds represent truly discrete, independent "replicates" of the units of interest, namely, stream condition. Environmental heterogeneity, however, can be considerable even in small watersheds (< 3 km<sup>2</sup>), e.g., non-acidic tributaries and seeps may exist in strongly AMD-degraded watersheds. Transects, by comparison, are not truly independent of their upstream or downstream counterparts.

Stream salamanders are territorial, generally confined to home ranges measured in square meters (Pfungsten and Downs 1989, Petranka, 1998), and are relatively site-specific organisms. The investigation of stream salamander response to acidification was focused at the transect level, a spatial scale believed to be most relevant to these organisms. Results by watershed are nevertheless presented as an overview of the findings.

ANOVA (Model 1) was used in multiple comparisons of assemblage and environmental attributes among the four stream condition categories. Post-hoc Tukey's pairwise comparisons typically followed to identify significant group(s). Variability in salamander abundance was analyzed by nested ANOVA to decompose variance by the 4 nested sampling levels. Thus, in this analysis, stream condition, stream, transect, and plot, were the 4 sequentially, fully nested factors. Normality and homoscedasticity were tested by Shapiro-Wilks and Bartlett's tests. Non-parametric comparisons were performed by Mann-Whitney Test (2 groups) or Kruskal-Wallis Test (more than 2 groups). Testing of tabular, frequency data were by Chi-square.

Ordination is an effective method to investigate species associations and species-environment associations (Gauch 1982). Correspondence Analysis (CA) and Canonical Correspondence Analysis (CCA) are two widely-used forms of indirect and direct gradient ordination, respectively. In the former, the ordination proceeds entirely independent of any environmental variables; the outcome is strictly driven by the

proportional abundance of species comprising the assemblage under investigation. In CCA, the input requires two data sets: a species by site matrix and an environmental variable - site matrix. Hence, the proportional abundance of organisms and environmental data drive the outcome and the process extracts only the variance attributable to the environment (constrained or direct), whereas in the former, extraction of the variance proceeds entirely unconstrained (indirect).

CA was chosen to reveal the arrangement of taxa and their life stages in an ordination space created totally independent of environmental data. Species-environment relationships were investigated subsequently by examining associations between CA axes scores and predictors of interest (Waite et. al. 2000) and through partial CCA (ter Braak and Smilauer 1998). In the latter, association strength was tested on the residual variance, the variation remaining after the fitting of covariables unrelated to water chemistry. In other words, did the association to water chemistry persist after adjusting the response to stream physical habitat? Marginal effects, a variable's fit in the absence of other predictors, were computed on the total and residual variance. In this last step, the goal was to present marginal effects both before and after the fitting of covariables.

Ordinations (CA, CCA) were performed by CANOCO 4.0 (Software for Canonical Community Ordination, version 4.0, ter Braak and Smilauer 1998). Biplot scaling with focus on inter-species distances were chosen to optimize the display of dissimilarities between species occurrence patterns. In performing CCA, levels of

significance as indicated by P and F-values, were computed by a Monte Carlo permutation test, selecting the default reduced model option. Because the study design was unbalanced, permutations were model - based, and the default number of permutations (499) were specified.

Abundance data were square-root transformed before analysis. Some species were made supplementary by assigning them a weight of zero; this prevented their influencing the ordination but not the computation of their ordination axis scores for plotting alongside contributory sites and species (ter Braak and Smilauer 1998). The need for transformation, prior to CCA, was determined by visual inspection of histograms. Logarithmic, square root, or arcsine square root for percentages, were applied accordingly. Values for environmental variables measured at plots were the medians of 5 measurements. Plot salamander counts were averaged, divided by 4 to obtain unit area density, and further divided by average person-hrs to adjust for search time (salamanders / m<sup>2</sup> /person-hrs). The resulting units were square-root transformed to minimize skewness.

## Results

### Study Sites

The 14 study watersheds consisted of 3 AMD-impacted (AMD), 3 episodically acidified (episodic), 4 non-acid-circumneutral (reference), and 4 non-acid-high alkalinity (fragmented) streams. Parenthetical names are introduced here as short hand for the four stream conditions. Circumneutral streams were “reference” because of their minimally

disturbed, forested nature; the term “fragmented” reflected the broken forest and agricultural components existing in high alkalinity, valley watersheds.

Ten watersheds were located in Westmoreland county in southwestern Pennsylvania; 4 were in Blair, Cambria, Centre, and Clearfield counties (Figure 2.2). Most watersheds (n = 10) drained to the Ohio River; 3 to the Suquehanna River. Watersheds ranged from 155.3 to 858.7 ha. Elevation ranged from 359 to 894 m. All watersheds, except for Muddy Run, were first or second order stream reaches. Stream length and gradient, respectively, ranged from 1.5 to 3.4 km and 27 to 63 m/km. In general, reference and episodic streams occupied higher elevations and were steeper. Fragmented watersheds were smaller and at the lowest elevations. Such disparity was unavoidable and reflected geologic and human development patterns in southwestern Pennsylvania. Fragmented landscapes coincided with lower-laying valleys and softer, carbonate-bearing geologies. Mostly forested headwaters drained steeper, higher ground underlying harder, carbonate-poor, acidic formations. Acidic sandstones and shales of the Pottsville and Allegheny Group were present in all study watersheds. Reference watersheds had greater exposure to the Mauch Chunk Formation, a limestone-bearing formation. Fragmented watersheds were underlain by the Casselman Formation, a thick geological unit consisting of freshwater and marine limestones. Watersheds with minimal exposure to carbonate-bearing formations were more susceptible to natural and anthropogenic acidification. Additional information on study watersheds are provided in Appendix 2.1.

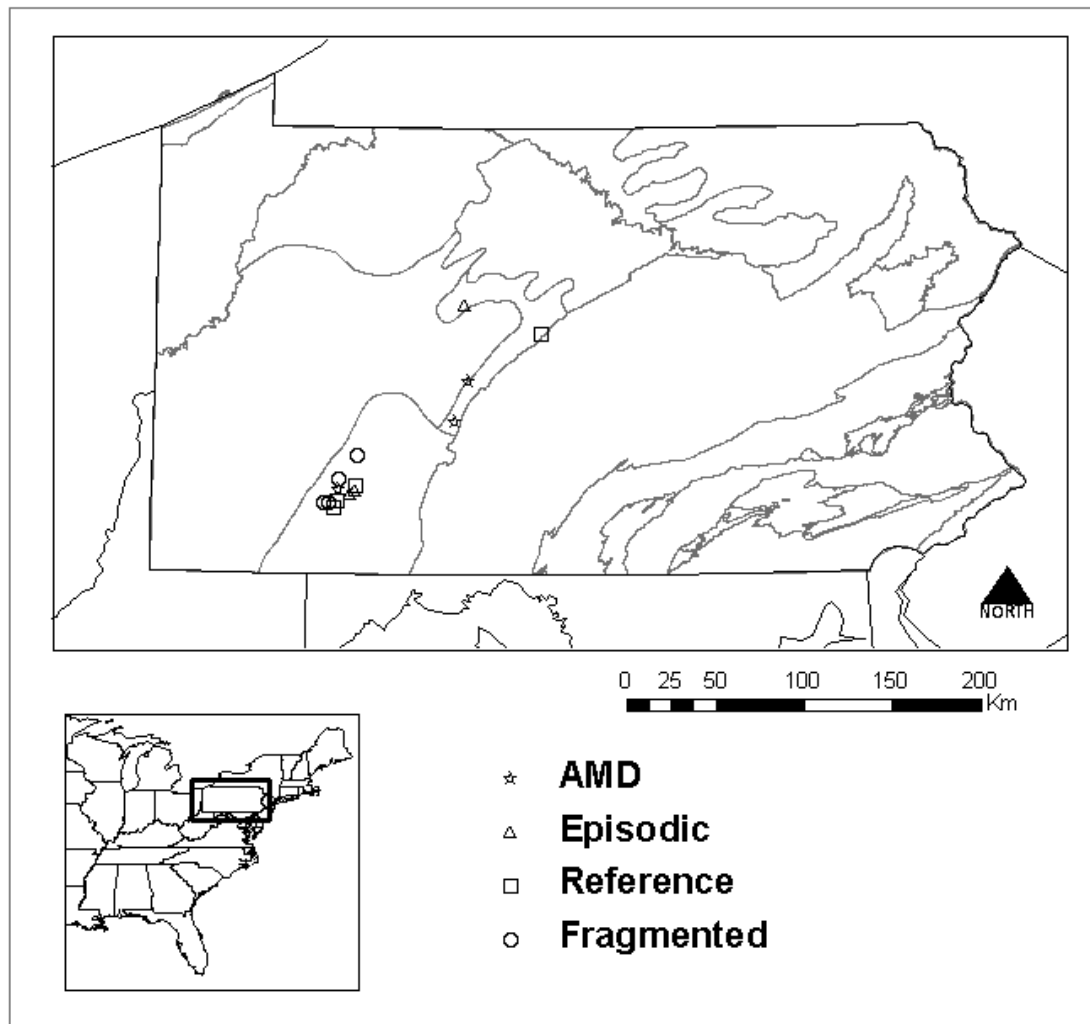


Figure 2.2. Location of the 14 small Pennsylvania watersheds in the Central Appalachians physiographic province sampled for salamanders in 1997-1998 and representing the acid-alkaline gradient. AMD = chronic, acid mine drainage-contaminated, Episodic = episodically acidified, Reference = non-acidic, and Fragmented = highly alkaline valley streams.

## Water Chemistry

Stream water chemistry was largely consistent with geologic settings and previous investigations of study streams. Based on the attributes pH, alkalinity, ANC,  $\text{SO}_4$ , and to some extent, metals contamination, the four stream classes provided a broad acid-alkaline gradient (Table 2.1). One-way ANOVA comparisons for laboratory-analyzed water samples revealed differences among AMD, episodic, reference, and fragmented watersheds for mean pH ( $F_{3,119} = 309.7$ , p-value < 0.0001), alkalinity ( $F_{3,111} = 92.64$ , p-value < 0.0001), and nitrate,  $\text{NO}_3\text{-N}$ , ( $F_{3,105} = 43.49$ , p-value < 0.0001). Tukey *a posteriori* pairwise comparisons revealed differences in pH between all 4 categories (Tukey C.I.<sub>0.05</sub> did not include zero), and no differences in alkalinity between reference and episodic (Tukey C.I.<sub>0.05</sub> = -1.55, 23.99) and no differences in nitrates between reference and fragmented streams (Tukey C.I.<sub>0.05</sub> = -0.1813, 0.089).

In 1998, water samples were filtered and analyzed for ANC, DOC, and  $\text{SO}_4$ . One-way ANOVA comparisons with available water samples (variable n) for each attribute revealed differences among AMD, episodic, reference, and fragmented watersheds for mean ANC ( $F_{3,58} = 53.32$ , p-value < 0.0001), mean  $\text{SO}_4$  ( $F_{3,58} = 7.11$ , p-value < 0.0001), mean dissolved Fe ( $F_{3,50} = 3.97$ , p-value < 0.013), mean dissolved Mn ( $F_{3,50} = 5.26$ , p-value < 0.003), and mean dissolved Al ( $F_{3,58} = 6.38$ , p-value < 0.001) and no differences for mean DOC. Tukey *a posteriori* pairwise comparisons identified differences in mean  $\text{SO}_4$  and mean dissolved toxic metals to be attributable to the AMD category. Similar comparisons showed no differences in mean ANC between reference and episodic



Table 2.1. Stream water chemistry by stream condition category for the 14 Pennsylvania watersheds sampled in 1997-1998. Cell contents include mean and standard error ( $O \pm SE$ ), and number of samples (**n**). Significant differences across categories of stream condition are based on one-way ANOVAs with Tukey's test for multiple comparisons. Within rows, cells with different letters are significantly different at  $p$ -value  $> 0.05$ . AMD= Acid mine drainage-contaminated.

Attribute	AMD High acid (Watersheds = 3)	Episodic Acidification Low acid (Watersheds = 3)	Reference Non-acidic (Watersheds = 4)	Fragmented High alkalinity (Watersheds = 4)	p-value <
Temperature (C)	13.37 $\pm$ 0.72, <b>25 AB</b>	12.43 $\pm$ 0.79, <b>15 AB</b>	11.75 $\pm$ 0.60, <b>27 A</b>	14.68 $\pm$ 0.69, <b>39 B</b>	0.015
pH	4.32 $\pm$ 0.16, <b>27 C</b>	4.83 $\pm$ 0.11, <b>24 B</b>	6.91 $\pm$ 0.07, <b>29 A</b>	7.63 $\pm$ 0.03, <b>40 D</b>	0.000
CaCO <sub>3</sub> mg/l	0.27 $\pm$ 0.12, <b>27 B</b>	0.06 $\pm$ 0.01, <b>16 A</b>	11.29 $\pm$ 1.22, <b>29 A</b>	55.51 $\pm$ 4.04, <b>40 C</b>	0.000
NO <sub>3</sub> -N mg/l	0.21 $\pm$ 0.02, <b>25 B</b>	0.12 $\pm$ 0.02, <b>22 B</b>	0.57 $\pm$ 0.05, <b>25 A</b>	0.62 $\pm$ 0.04, <b>34 A</b>	0.000
ANC mg/l <sup>1998</sup>	-326.1 $\pm$ 116.67, <b>16 B</b>	-6.6 $\pm$ 2.19, <b>14 A</b>	180.7 $\pm$ 39.44, <b>13 A</b>	1052.2 $\pm$ 95.5, <b>16 C</b>	0.000
DOC mg/l <sup>1998</sup>	2.5 $\pm$ 0.22, <b>16</b>	1.91 $\pm$ 0.23, <b>14</b>	2.17 $\pm$ 0.36, <b>13</b>	2.83 $\pm$ 0.24, <b>16</b>	NS
SO <sub>4</sub> mg/l <sup>1998</sup>	248.7 $\pm$ 80.55, <b>16 B</b>	8.5 $\pm$ 0.27, <b>14 A</b>	7.1 $\pm$ 0.64, <b>13 A</b>	47.6 $\pm$ 9.05, <b>16 A</b>	0.000
Fe mg/l <sup>1997</sup>	0.18 $\pm$ 0.05, <b>9 A</b>	0.32 $\pm$ 0.13, <b>8 B</b>	0.05 $\pm$ 0.01, <b>12 A</b>	0.18 $\pm$ 0.04, <b>18 A</b>	0.04
Fe mg/l <sup>1998</sup>	1.17 $\pm$ 0.47, <b>16 B</b>	0.10 $\pm$ 0.03, <b>6 AB</b>	0.06 $\pm$ 0.01, <b>13 A</b>	0.06 $\pm$ 0.01, <b>16 A</b>	0.013
Mn mg/l <sup>1998</sup>	7.06 $\pm$ 2.6, <b>16 B</b>	0.05 $\pm$ 0.01, <b>6 AB</b>	0.00 $\pm$ 0.00, <b>13 A</b>	0.02 $\pm$ 0.02, <b>16 A</b>	0.003
Al mg/l <sup>1997</sup>	0.45 $\pm$ 0.23, <b>9 B</b>	0.51 $\pm$ 0.06, <b>8 B</b>	0.04 $\pm$ .01, <b>12 A</b>	0.05 $\pm$ .01, <b>18 A</b>	0.001
Al mg/l <sup>1998</sup>	15.13 $\pm$ 5.61, <b>16 B</b>	0.22 $\pm$ 0.03, <b>14 A</b>	0.03 $\pm$ 0.00, <b>13 A</b>	0.04 $\pm$ 0.00, <b>16 A</b>	0.001

streams (Tukey C.I.<sub>0.05</sub> = -142, 516), and with respect to Mn and Fe concentrations between episodic and AMD.

One-way ANOVA comparisons for the 1997 unfiltered samples revealed differences among AMD, episodic, reference, and fragmented watersheds for mean Fe and Al ( $F_{3,46} = 3.01$ , p-value < 0.04;  $F_{3,46} = 7.10$ , p-value < 0.001, respectively). Only mean Fe for episodic samples were different to all other categories. In Al, acidic categories were different from non-acidic categories. These conflicting results may be linked to the analysis of unfiltered grab samples if suspended particulates, dissolved by the acidification step, caused spurious Fe and Al concentrations.

Stream temperature differed among categories ( $F_{3,105} = 3.68$ , p-value < 0.015). Grab sample locations in fragmented watersheds were on average warmer than at sampling stations in reference streams (Tukey C.I.<sub>0.05</sub> did not include zero). Comparison of stream temperatures from June - September, 1998, measured at 2.5 h intervals by the in-stream temperature loggers in the four streams representing fragmented (n = 2), reference (n = 1), and episodic (n = 1) conditions revealed similar trends. Stream temperatures in fragmented watersheds were on average higher and more variable than in the latter two streams ( $F_{3,4671} = 1425.2$ , p-value < 0.0001; Levene's Test = 110.97, p-value < 0.0001). In addition to differences in land use, Golfcourse Run and Jefferson Run, (fragmented) were at lower elevations than Linn Run or Roaring Run, the reference and episodic streams, respectively.

Reference and episodic watersheds were > 99% forested. Paved or gravel roads and hunting or recreational camps accounted for the remaining non-forested portions. Forest covered 84 - 98% in AMD watersheds. Agricultural and rural development spanned most of the difference in these watersheds. Tracts of barren land, claiming up to 14% as in Muddy Run, were typically associated with former coal-strip mines. Forest cover ranged from 44 - 64% among fragmented watersheds. Agriculture (corn, wheat, pasture) and recreation (golf course, trailer park, amusement grounds) claimed most of the remaining watershed surface. Small rural towns or small commercial centers accounted for urban cover.

### Sampling Effort

The 305 plots positioned in the 8 tributary and 53 mainstem transects required a total sampling effort of 586 person-hours (Table 2.2). Most watersheds were sampled with 3 - 4 mainstem transects. Laurel Run (AMD) was sampled with 6 mainstem transects to ensure that the low densities of salamanders observed were not limited to areas initially sampled. Total sampling effort by watershed ranged from 28.6 - 53.3 person-hours (mean =  $41.85 \pm 2.17$ ). Individual plots required 0.7 - 4.5 person-hours (mean  $1.92 \pm 0.03$ ) to complete, including two plots terminated prematurely because of a severe thunderstorm. Generally, the sampling of plots in non-acidic watersheds required more effort because of the greater number of salamanders found in non-acidic plots. Searches in acidified watersheds were frequently terminated after the minimum 30 min

Table 2.2. Salamander sampling effort at the 14 Pennsylvania watersheds sampled in 1997-1998. There were five plots per transect. Sampling effort was search time multiplied by two persons. Watersheds within categories are listed in order of increasing median pH.

Condition	Stream	Year	No. Plots	Transect Type		Sampling Effort (in person-hrs)		
				Mainstem	Tributary	Total	Plot mean ( $O \pm SE$ )	
							stream	condition
Acid Mine Drainage (AMD)	Muddy	1998	20	3	1	28.5	1.42 $\pm$ 0.07	1.49 $\pm$ 0.04
	Glenwhite	1998	20	3	1	30.0	1.5 $\pm$ 0.07	
	Laurel	1997	35	6	1	53.3	1.5 $\pm$ 0.06	
Episodic Acidification	McGinnis	1997	25	4	1	36.8	1.47 $\pm$ 0.07	1.69 $\pm$ 0.05
	Linn	1997	20	3	1	33.5	1.68 $\pm$ 0.08	
	Stone	1998	20	4	0	39.4	1.97 $\pm$ 0.09	
Reference	Wallace	1998	20	3	1	48.2	2.41 $\pm$ 0.1	2.20 $\pm$ 0.06
	Wildcat	1997	20	4	0	38.6	1.93 $\pm$ 0.13	
	Camp	1997	20	4	0	46.4	2.32 $\pm$ 0.12	
	Roaring	1998	20	4	0	43.2	2.16 $\pm$ 0.08	
Fragmented	Jefferson	1998	20	3	1	50.6	2.53 $\pm$ 0.11	2.21 $\pm$ 0.07
	Minnow	1997	20	4	0	38.4	1.92 $\pm$ 0.12	
	Golfcourse	1998	20	4	0	53.1	2.66 $\pm$ 0.14	
	Olive	1997	25	4	1	45.9	1.83 $\pm$ 0.09	
Total			305	53	8	586.0	1.92 $\pm$ 0.03	

had elapsed (1 person-hour) if no additional salamanders were observed and the entire plot was searched.

### Abundance

A total of 3,560 stream salamanders were captured. Abundance ranged from 20 - 700 per watershed and 0 - 117 per plot (Table 2.3). Almost 87% of the total were captured in non-acidic watersheds. The latter proportion would be higher if salamanders from non-acidic tributaries in acidic watersheds were included. Median plot density, the total number of stream salamanders in a plot divided by 4 m<sup>2</sup>, ranged from 0 salamanders/m<sup>2</sup> in Muddy Run (AMD) to 6.88 salamanders/m<sup>2</sup> in Golfcourse Run (Fragmented). Plot density differed among streams (Kruskal - Wallis  $H_{13,304} = 204.5$ , p-value < 0.0001) and stream condition categories (Kruskal - Wallis  $H_{3,13} = 160.7$ , p-value < 0.0001). AMD and Episodic watershed categories yielded the lowest median plot densities; 0.5 and 0.75 salamanders/m<sup>2</sup>, respectively. The median plot densities in Reference and Fragmented streams were 3.12 and 4.5 salamanders/m<sup>2</sup>, respectively. Abundance distributions were strongly left-skewed in most streams (Skewness = 0.4 - 2.8). The median plot density in mainstem and tributary transects was 1.8 and 1.2 salamanders/m<sup>2</sup>, respectively. Tributaries were typically first order stream reaches.

Completing the nested ANOVA on salamander abundance by sampling levels (stream condition, stream, transect, plot) required reducing the data set to 12 streams (3 streams per condition category; streams without tributary data were eliminated) and 3

Table 2.3. Total number and median salamander density at the 14 Pennsylvania watersheds sampled in 1997-1998. All life stages are included. Watersheds within categories are listed in order of increasing median pH.

Condition	Stream	Total No. of Salamanders	Median Plot Salamander Density (plot abundance/m <sup>2</sup> )	
			Stream	Condition
Acid Mine Drainage (AMD)	Muddy	20	0	0.5
	Glenwhite	38	0.37	
	Laurel	117	0.5	
Episodic Acidification	McGinnis	51	0.5	0.75
	Linn	60	0.5	
	Stone	185	1.87	
Reference	Wallace	345	4.0	3.12
	Wildcat	186	1.75	
	Camp	440	4.37	
	Roaring	170	2.0	
Fragmented	Jefferson	491	5.87	4.5
	Minnow	422	4.12	
	Golfcourse	700	6.88	
	Olive	335	2.25	
Total		3,560	1.5	

transects/stream (only transects in upper, middle, and lower reaches were retained) to achieve a balanced design. Stream condition was a significant factor ( $F_{3,179} = 10.47$ , p-value  $< 0.004$ ) and accounted for almost 58% of the total variability (Table 2.4). Plots were the next most variable (23.1%), followed by stream (15.6%) and transect (3.4%). The nested ANOVA confirmed that stream condition, the four classes representing the acid-alkaline gradient, had a significant effect on salamander density notwithstanding other sources of variability related to sampling scale, i.e., differences in the response variable between streams, between transects, or between plots, were not greater than between condition.

### Species Composition

Of the 7 stream salamander species observed, only 4 were widespread and relatively abundant. The northern two-lined salamander and *Eurycea* larvae composed almost 58 % of all captured individuals. The mountain dusky, northern dusky, and northern spring salamander accounted for 17.9 %, 13.5 %, and 7.4 % of the total, respectively. The remaining 3 species, the Appalachian seal salamander, northern red, and longtail totaled less than 3.5 %. Species richness varied from 1- 5 in acidic to 4 - 6 in non-acidic watersheds (Table 2.5). Frequency counts of the mountain dusky, northern dusky, northern two-lined, and northern spring were strongly associated with stream condition category ( $\chi^2 = 1201.06$ , DF = 9, p-value  $< 0.0001$ ). Ranking of counts by species and watershed in descending order provided further insight on the associations. The mountain dusky and northern spring ranked higher (had lower values), in acidic than

Table 2.4. Results of nested ANOVA on log-transformed salamander density (salamanders/m<sup>2</sup>) by sampling level. All salamander life stages were included. Salamanders were captured at the 14 Pennsylvania watersheds sampled in 1997-1998. Condition category (n = 4), stream (n = 12), transect (n = 36), and plot (n = 180) were the sequentially, fully nested factors.

Source	DF	SS	MS	F	P-value <
Condition	3	6.61	2.20	10.47	0.004
Stream	8	1.68	0.21	6.85	0.000
Transect	24	0.74	0.03	1.74	0.025
Plot	144	2.55	0.01		
Total	179	11.58			
Variance Components					
Source	Variance Components		Percent of Total		Standard Deviation
Condition	0.044		57.85		0.21
Stream	0.012		15.65		0.109
Transect	0.003		3.41		0.051
Plot	0.018		23.09		0.133
Total	0.077				0.277



Table 2.5. Salamander species composition at the 14 Pennsylvania watersheds sampled in 1997-1998. Cell values are count ranks within watersheds i.e. highest count = 1. Number of salamanders are in parentheses. Watersheds within categories are listed in order of increasing median pH. MTD = mountain dusky, NRD = northern dusky, APS = Appalachian seal, LNT = long-tailed, TWL = northern two-lined, RED = northern red, SPR = northern spring.

Condition	Stream	No. Taxa	aquatic dependence						
			LOW			HIGH			
			Terrestrial Nesting			Aquatic Nesting			
			MTD	NRD	APS	LNT	TWL	RED	SPR
Acid Mine Drainage (AMD)	Muddy	1	1 (20)						
	Glenwhite	4	1(26)	3(3)			4(2)		2(7)
	Laurel	4	1(90)	2(22)				4(1)	3(4)
Episodic Acidification	McGinnis	3	1(35)	3(4)					2(12)
	Linn	4	1(37)		4(1)		3(3)	4(1)	2(18)
	Stone	5	3(28)	4(3)			1(96)	5(1)	2(57)
Reference	Wallace	4	3(47)	4(33)			1(200)		2(65)
	Wildcat	5	2(48)	3(42)	5(6)		1(54)		4(36)
	Camp	6	2(65)	4(27)	3(66)		1(54)	6(2)	5(24)
	Roaring	4	1(110)	3(42)			2(190)		4(30)
Fragmented	Jefferson	6	3(20)	2(148)	5(3)	6(1)	1(303)	4(16)	
	Minnow	5	3(25)	2(34)		4(7)	1(354)	5(1)	5(1)
	Golfcourse	5	3(37)	2(60)	5(1)		1(598)	4(4)	
	Olive	6	3(50)	2(62)	4(12)		1(201)	6(1)	5(9)
Mean Rank			2.0	2.8	4.3	5.0	1.5	4.8	3.3

in non-acidic watersheds whereas the opposite was observed in the other two taxa, the northern two-lined and northern dusky. For example, the average rank for the mountain dusky in AMD, Episodic, Reference, and Fragmented watersheds was 1, 1.7, 2, and 3, respectively, whereas for the similarly-ordered categories, the northern two-lined ranked 4, 2, 1.25 and 1, respectively. The most notable aspect of these associations, however, is that co-occurring species do not necessarily share similar life histories and nesting habits.

#### Life Stage Composition

Of the total salamander count, 2,312 (64.9 %) were larvae or metamorphs, 665 (18.7 %) were juveniles, and 583 (16.4 %) were adults (Table 2.6). Life stage composition differed among taxa. Gilled or gill-stubbed forms of the mountain dusky were never encountered; yet post-metamorphic, gill-less specimens were observed and always encountered on the terrestrial half of plots. Larvae of the northern dusky and Appalachian seal were observed, albeit in relatively smaller numbers than their older life stages. In contrast, the northern two-lined, northern red, and northern spring were found overwhelmingly as larvae (91 - 98%). Life stage counts were significantly associated with the 4 stream condition categories ( $\chi^2 = 397.9$ ,  $DF = 6$ ,  $p\text{-value} < 0.001$ ). Ranking of counts by life stage and watershed identified adults as the highest ranking in AMD and Episodic watersheds. Larvae were overwhelmingly the most numerous life stage, but ranked highest only in non-acidic watershed types. By these measures, acidic watersheds favored adult stream salamanders over the larval, water-dependent forms and suggested higher reproduction in non-acidic than acidic watersheds.

Table 2.6. Salamander life stage composition at the 14 Pennsylvania watersheds sampled in 1997-1998. Cell values are count ranks within watersheds i.e. highest count = 1. Number of salamanders are in parentheses. Watersheds within categories are listed in order of increasing median pH.

Condition	Stream	No. Species	Life Stage		
			Adult	Juvenile	Larva
Acid Mine Drainage (AMD)	Muddy	1	1(11)	2(9)	3(0)
	Glenwhite	4	1(25)	3(4)	2(9)
	Laurel	4	1(63)	2(48)	3(6)
Episodic Acidification	McGinnis	3	2(18)	1(22)	3(11)
	Linn	4	1(25)	3(13)	2(22)
	Stone	5	2(23)	3(11)	1(151)
Reference	Wallace	4	3(38)	2(46)	1(261)
	Wildcat	5	3(42)	2(50)	1(94)
	Camp	6	3(40)	2(163)	1(237)
	Roaring	4	2(60)	3(32)	1(78)
Fragmented	Jefferson	6	3(76)	2(110)	1(305)
	Minnow	5	2(49)	3(34)	1(339)
	Golfcourse	5	2(57)	3(42)	1(601)
	Olive	6	3(56)	2(81)	1(198)
Mean Rank			2.1	2.4	1.6

## Ordination

Only 58 transects were used in the CA ordinations because no salamanders were captured in 3 Muddy Run transects. Adding a constant or use of a pseudo-species representing “no salamanders observed” produced unsatisfactory results i.e. the first axis corresponded to the 3 transects without salamanders.

## Presence - absence Matrix

The CA of these data produced a total inertia of 0.802 and eigenvalues of 0.266 (33.2 %) and 0.148 (18.4 %) for the first and second axes, respectively. The 4 axes combined claimed 73.4 % of the total inertia. Gradient length, the range of site scores along CA axis 1, was 4.157. An ordination by species alone, without life stages, produced a total inertia of 0.501, an eigenvalue 37.5 % smaller than what was generated from the CA by species and life stage.

Plotting the 13 species life stages by the first 2 CA axes revealed an irregular-shaped constellation of points in the center of the graph with several smaller clusters within (Figure 2.3, above). Squares representing the terrestrial nesters, the mountain dusky, northern dusky and Appalachian seal, trended toward the upper left quadrant, whereas aquatic nesters, the northern two-lined, northern red, and northern spring, symbolized by circles, gravitated toward the lower right quadrant. In fact, the centroid for terrestrial nesters lies to the left and above (-0.08, 0.47) of that for aquatic nesters (0.47, -

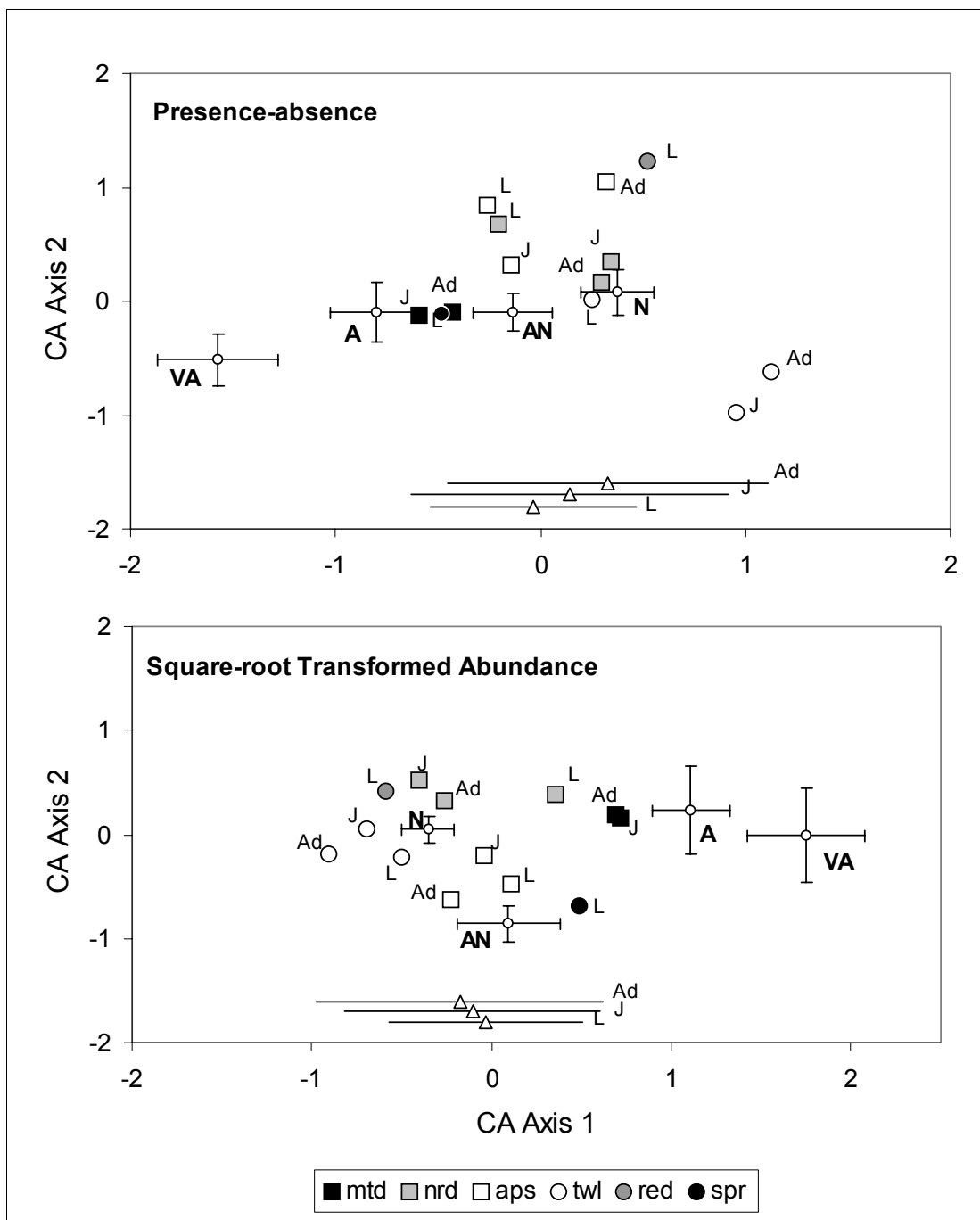


Figure 2.3. Plots from correspondence analysis (CA) of salamander presence-absence and abundance at the 14 small Pennsylvania watersheds sampled in 1997-1998. Squares and circles symbolize terrestrial and aquatic nesters, respectively. Error bars are 1SE of the mean for site scores corresponding to very acid (VA), acid (A), acid-neutral (AN), and neutral (N) stream transects (n=58). Lines and triangles at bottom of plots are the mean and range for salamander centroids by life stage for CA axis 1. mtd=mountain dusky; nrd=northern dusky; aps=Appalachian seal; twl=northern two-lined; red=northern red; spr=northern spring; Ad=adult; J=juvenile; L=larva.

0.09). Closer inspection revealed adult and juvenile mountain dusky centroids coinciding with the mostly larval northern spring. All other centroids plotted to the right.

Notable was the proximity in ordination space of the mountain dusky and northern spring to each other and to centroids representing the more acidic transects. Centroids and their standard errors (SE of the mean) were computed and plotted for this purpose from ordination scores of transects with very acid (pH < 4.5), acid (pH < 5.5), acid neutral (pH < 6.5), and neutral (pH > 6.5) stream water, determined from the mean pH of laboratory-analyzed water samples. The above stream acidity classes differed significantly in their axis 1 scores (Kruskal Wallis  $H = 19.51$ ,  $df = 3$ ,  $p\text{-value} < 0.0001$ ) but not along axes 2 - 4. Separation of these 4 stream transect types along axis 1 is evident in the ordination plot. CA axis 2, in contrast, provided minimal separation.

Life stages of the same species did not necessarily group together and were not consistent to the watershed-scaled responses. Centroids for juvenile (second from the right) and adult (first from the right) northern two-lined life stages clustered together, but were distant and trended to the right, the more alkaline side, of larvae, a pattern that was repeated in the northern dusky and to some degree, by the Appalachian seal salamander. The remaining taxa were found overwhelmingly as juveniles or adults (mountain dusky) or larvae (northern spring, northern red), rendering life stage comparison for these taxa impossible.

Response to CA axis 1 by life stages for the entire assemblage is presented graphically in Figure 2.3 (above) irrespective of the absence of life stages for some species. In this graphic, the mean (triangle) and range (line) of centroids along CA 1 axis are plotted by life stage. The wide range, denoted by line length, and their overlap is evident. Relative to adults, younger life stages plotted towards the acidic side. Larvae, however, exhibited the shortest gradient length. These patterns were consistent with “species tolerance” values, the root mean squared deviation for assemblage elements output by CANOCO (ter Braak and Smilauer, 1998). By this measure of niche breadth, the mean for adults ( $n = 4$ ), juveniles (4), and larvae ( $n = 5$ ) was 0.77, 0.84, and 0.69, respectively. These differences were not significant (Kruskal - Wallis  $H = 1.40$ ,  $df = 2$ ,  $p$ -value = 0.495). In summary, younger life stages trended to the acidic side relative to their older counterparts. This result was counter-intuitive because amphibian early life stages are the most sensitive to acidification (Freda 1991) and were less common in acidic watersheds.

Ordination axes scores were tested against 24 environmental variables (Appendix 2.2). CA axis 1 was significantly correlated ( $p$ -value < 0.05) to 14 water chemistry variables, plot and stream physical habitat and other watershed attributes (Table 2.7, left column). Ordination axis 1 corresponded to increasing water temperature, pH, alkalinity, nitrate, and total plot vegetation cover and decreasing Al, stream gradient, elevation, stream depth, canopy cover and watershed in forest. CA axis 2 was positively correlated

Table 2.7. Pearson product-moment correlation coefficients for 17 environmental variables vs. axis 1 site scores (58 transects) computed from the correspondence analysis (CA) of salamander presence-absence (left column) and abundance (right column) in the 14 Pennsylvania watersheds sampled in 1997-1998. Coefficients for variables tested with p-value < 0.05 are either not shown or denoted ns. (\*\* p-value < 0.0001, \* p-value < 0.001).

Variable	Presence / absence Matrix	Abundance Matrix
Water chemistry		
Stream temperature (C)	0.431*	-0.448*
pH - laboratory	0.663**	-0.739**
pH - <i>in situ</i>	0.664**	-0.744**
Alkalinity (CaCO <sub>3</sub> mg/l)	0.638**	-0.68**
NO <sub>3</sub> -N (mg/l)	0.400	-0.48**
Electrical conductivity (μS/m)	0.388	-0.314
Fe (mg/l)	ns	0.312
Al (mg/l)	-0.402	0.432*
Stream Physical / Plot Cover / Watershed		
Plot cobble cover (%)	ns	-0.383
Stream gradient (m/km)	-0.510**	0.434*
Elevation (m)	-0.636**	0.609**
Stream depth (cm)	-0.331	0.343
Plot vegetation cover (%)	0.371	-0.378
Plot canopy closure (%)	-0.361	0.335
Watershed in forest (%)	-0.646**	0.623**
Transect drainage area (ha)	ns	0.404
Sampling effort (person-hr)	0.350	-0.444**



to plot vegetation whilst inversely correlated to stream length. Both ordination axes were positively correlated to plot search effort.

### Abundance Matrix

The CA of the square-root transformed, search effort-adjusted, salamander density (individuals / m<sup>2</sup>) matrix produced a total inertia of 0.782, a smaller, albeit comparable value to that yielded by the presence-absence data set. Eigenvalues for CA axis 1 and 2 were 0.314 and 0.121, and accounted for 40.2 % and 15.5 % of the total inertia, respectively. The four axes combined claimed 76 % of the total inertia. Gradient length for CA axis 1 was 3.901.

The spatial relationship of transects and species life stages remained similar to that observed in the first ordination plot, however, low scores for CA axis 1 corresponded to alkaline transects and *vice versa* (Figure 2.3, below). Thus, centroids representing acidic sites lie to the right of the more alkaline ones and the centroid for terrestrial nesters lies in the upper right quadrant (0.12, 0.03) and in the lower left quadrant for aquatic nesters (-0.44, -0.12). Individual species life stages arranged correspondingly: along CA axis 1, three of the 4 aquatic nesters lie to the left of terrestrial nesters. The exception, as in the first ordination, is the northern spring. Horizontally, (CA axis 1) its centroid lies close to the mountain dusky life stages with all other centroids clustering to its left, the direction inferring increasing alkalinity. Unique to the second ordination plot is the amount of vertical separation between the northern spring and the mountain dusky as well

as other aquatic nesters. Other notable differences is the tighter clustering of life stages of the same species and greater visual separation of the acid neutral (AN) transect class along CA axis 2. In spite of the latter, separation of the 4 classes of stream transects was only achieved along CA axis 1 (Kruskal Wallis  $H = 25.5$ ,  $df = 3$ ,  $p\text{-value} < 0.0001$ ).

As in the first ordination, the range for centroids by life stage along CA axis 1 was wide and exhibited considerable overlap (Figure 2.3 below). Early life stages were the more acid-trending. Centroids for larvae spanned the shortest gradient length.

Bubble plots reflecting the density of the more commonly encountered species, the mountain dusky, northern dusky, northern two-lined, and northern spring for each of the 58 transects reveals their distribution in ordination space (Figure 2.4). Even by this crude approach, affinities and differences among taxa and transect types discussed earlier, are evident. Similar trends resulted when examining response curves along CA axis 1 for 5 species (Figure 2.5). In the latter, the northern red, an aquatic nester with a prolonged larval period, was added to the graphic. Curves were constructed from 2<sup>nd</sup> and 3<sup>rd</sup> order polynomial regression of square-root transformed, sampling effort-adjusted, salamander density versus CA axis 1. Most evident is that only one, perhaps two taxa, exhibit a unimodal response along the inferred acid-alkaline gradient. For these taxa, the optima lies on the acidic side (A, VA), whereas for the remaining three, the northern two-lined, the northern dusky, and the northern red, the optima lies to the left of the graph near the more alkaline transects (N, AN). The lack of consistency in curve shapes, when examined

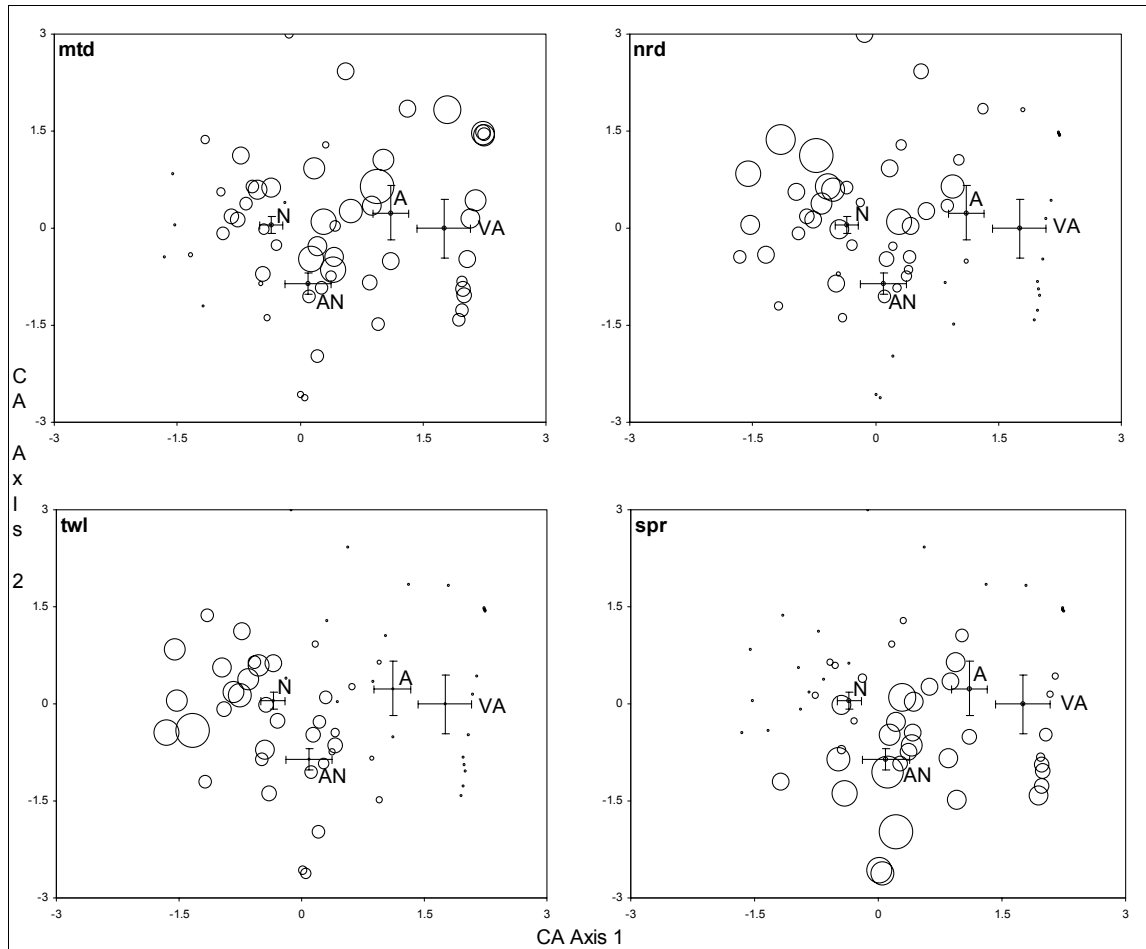


Figure 2.4. Bubble plots in correspondence analysis (CA) space from mountain dusky (mtd), northern dusky (nrd), northern two-lined (twl), and northern spring (spr) salamander abundance at the 14 small Pennsylvania watersheds sampled in 1997-1998. Bubble size in each plot is proportional to search effort-adjusted, square-root transformed salamander density (individuals/m<sup>2</sup>). Error bars represent 1SE of the mean for site scores corresponding to very acid (VA), acid (A), acid-neutral (AN), and neutral (N) stream transects (n=58).

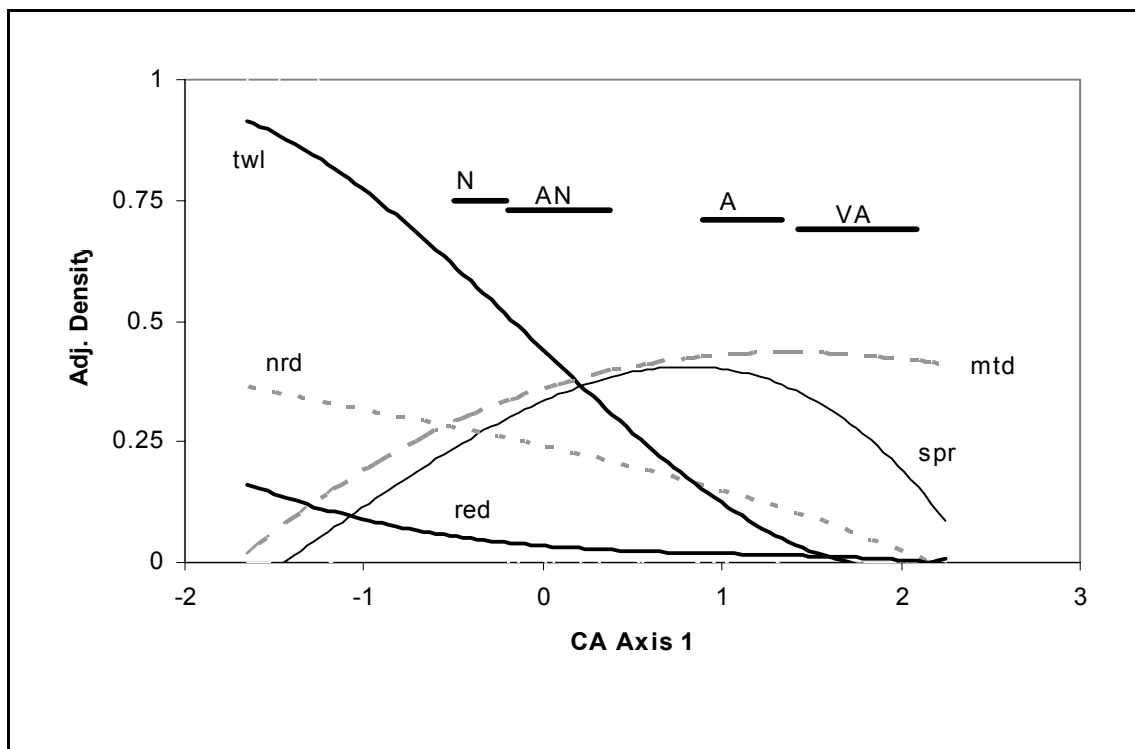


Figure 2.5. Response curves for correspondence analysis (CA) axis 1 scores for the mountain dusky (mtd), northern dusky (nrd), northern two-lined (twl), northern spring (spr), and northern red (red) salamanders. The second and third order polynomial lines reflect square-root transformed, search effort-adjusted salamander density (individuals /m<sup>2</sup>) in the 14 small Pennsylvania watersheds sampled in 1997-1998. Error bars represent 1 SE of the mean for CA axis 1 site scores corresponding to very acid (VA), acid (A), acid-neutral (AN), and neutral (N) stream transects (n=58).

by nesting type, is the other striking aspect of the graphic. The northern spring, the northern two-lined, and northern red are aquatic nesters, but only the last two have similar curves. In fact, the curve for the northern spring is most similar to the mountain dusky, a terrestrial nester with the most terrestrial habits and life histories among dusky salamanders in the northern Appalachians. The northern dusky and mountain dusky, both terrestrial nesters, have disparate curves.

The abundance matrix-based CA ordination produced axes that inferred similar gradients as those described for the analysis using only presence absence data (Table 2.7; right column). In comparing coefficients from this table, however, it is important to remember that low pH environments in the first and second ordination were plotted on opposite ends of the primary ordination axis. Thus, CA axis 1 arranged sites and species along decreasing gradients of water temperature, pH, alkalinity, conductivity, cobble, and percent total vegetation, and increasing Fe and Al concentration, stream gradient, elevation, stream depth, drainage area, canopy cover, and percent watershed in forest.

CA axis 2 inferred a gradient of decreasing stream length (-0.308, p-value < 0.019), stream width (-0.333, p-value < 0.011), moss cover (-0.275, p-value < 0.036), drainage area (-0.448, p-value < 0.0001) and percent watershed in forest (-0.312, p-value < 0.017). The last two variables, and Fe concentration (0.300, p-value < 0.022), were the only variables significantly correlated to both axes. The species matrix used in the second ordination was constructed from salamander densities adjusted for sampling effort.

Despite such adjustment, CA axis 1 continued to be highly correlated to sampling effort (-0.444,  $p$ -value < 0.0001) and CA axis 2 showed similar, albeit non-significant trends ( $p$ -value = 0.057).

#### Partial CCA: Marginal Effects Before and After fitting of Covariables

Only the abundance matrix was analyzed by CCA. Of 24 environmental variables tested, 20 variables had significant marginal effects when they alone were entered as predictors and without covariates (Appendix 2.2). The fit ( $\lambda_1$ ) for significant variables ranged from 0.19 (stream water pH, measured in the field) to 0.04 (watershed drainage area at each transect) of a total inertia of 0.782. The top five variables were related to water chemistry (3) and watershed cover (2). The fit for average plot sampling effort was significant and 7<sup>th</sup> in magnitude ( $\lambda_1 = 0.11$ ,  $p$ -value < 0.002). This finding was surprising because salamander densities in the abundance matrix was already adjusted for search effort. Marginal effects for water chemistry-related variables were all significant ( $p$ -value < 0.05).

Partial CCA was performed with 8 covariables, namely, average sampling effort, overhead canopy, stream width, stream depth, cobble cover, total plot vegetation, ambient temperature and relative humidity (Table 2.8). Not all covariables were significant as individual predictors, but all covariables were related to stream and stream bank physical habitat, sampling effort, or microclimate. The cumulative eigenvalue for all 8 covariables was 0.294, or 37.6 % of the total inertia. The marginal effects of all water chemistry

Table 2.8. Marginal effects computed by canonical correspondence analysis (CCA) for stream water chemistry variables after fitting 8 covariables related to stream physical habitat and microclimate recorded at the 14 Pennsylvania watersheds sampled for salamanders in 1997-1998. The CCA was performed on effort-adjusted, square-root transformed salamanders abundance in 58 stream transects. Columns under marginal effects show fit ( $\lambda_1$ ), or eigenvalue, and corresponding F and p-values for each variable, when the latter are entered as single predictors and in combination with the 8 covariables listed below. Levels of significance, as indicated by F and p-values, were calculated by a Monte Carlo permutation test, reduced model, default number (499) of unrestricted permutations.

Covariables			
Sampling effort (person-hr), canopy closure (%), Stream width (cm), stream depth (cm), Plot cobble cover (%), plot vegetation cover (%), ambient temperature (C), relative humidity (%)			
Total inertia			0.782
Sum of all unconstrained eigenvalues			0.488
Cumulative eigenvalue for all covariables			0.294
Marginal Effect for Water Chemistry Related Variables			
Variable	$\lambda_1$	F-value	p-value
Electrical Conductivity ( $\mu\text{S/m}$ )	0.057	6.292	0.002
pH - laboratory	0.056	6.222	0.002
pH - <i>in situ</i>	0.055	6.159	0.002
Alkalinity ( $\text{CaCO}_3$ mg/l)	0.052	5.715	0.002
$\text{NO}_3\text{-N}$ (mg/l)	0.034	3.581	0.002
Water temperature (C)	0.026	2.752	0.026
Al (mg/l)	0.023	2.367	0.04
Fe (mg/l)	0.017	1.708	ns

variables were reduced by the prior fitting of the 8 covariables, however, all but Fe, remained significantly associated to the residual variance. Among significant variables, fits varied from 0.057 for conductivity to 0.023 for Al. These results confirm the strength of the association between stream salamander response and gradients related to water chemistry. Weak or non-significant relationships for Al and Fe, primary contributors to the toxicity of acid waters, may be attributed to the disparate method for determining their concentrations during the study (total vs. dissolved Al and Fe) and perhaps, the omission of strongly AMD-contaminated transects lacking salamanders.

### Discussion

The objectives of the study were to sample acidic and alkaline watersheds to observe the response of stream salamander assemblages to acidification, and the impact of life history in this context. There is no doubt that the 14 small headwaters, containing the 61 transects sampled, provided the desired gradient. Field measurements and laboratory analyses of periodic grab samples confirmed severe acidification and elevated toxic metal concentrations at multiple stream salamander sampling locations (transects). On the opposite end of the gradient, valley stream transects provided modestly high alkalinity and circumneutral conditions. In this regard, the first set of objectives were met: to conduct sampling along a broad acid - alkaline gradient.

Sampling was completed in a manner that sought to minimize the effects of hydrology (Davic 1997) and physical habitat on salamander counts. For this purpose,



sampling was restricted to 4 m<sup>2</sup> plots containing both aquatic and terrestrial habitat, positioned in riffle and run portions of stream reaches. The positioning of plots in locations favoring timed hand and dip-net sampling was probably effective in reducing salamander count variability that might otherwise arise from haphazard sampling in all habitats. Physical habitat varied appreciably nonetheless as evidenced by the range of values presented for several stream bank attributes (Appendix 2.2). For example, average cobble cover in transects ranged from 0 to 60%; its average was 41% with almost 70 % (1 SD) of transects averaging between 28 % - 54 %. Davic and Orr (1987) described the relationship between the number of salamanders and the amount of cobble as being positively curvilinear. Substrate particle size are important determinants to stream salamander assemblage composition and structure (Krzysik 1979, Southerland 1985, Davic and Welch 2004).

Plot salamander searches were presumed to be complete counts of animals in plots at the time of the search (Jung et al. 2000, Southerland et al. 2004). A complete count is comparable to a census (Thompson et al. 1998). Several considerations lend support to this assumption. Searches were limited to a few square meters of stream bank and were performed simultaneously by two persons. Dip nets and headlights facilitated sampling. All cover in the plot was temporarily removed and smaller substrates hand-raked to seek or flush hidden salamanders. Removal of plot cover was systematic and began at its downstream end. Searches were not time-constrained and continued until no more salamanders were observed in the plot. These protocols brought surveyors close to the

ground where even the smallest terrestrial or aquatic individuals were visible. It is probable that only a few animals were missed during the search. For example, large boulders or rocks deeply imbedded in the bank were sometimes left undisturbed intentionally to avoid significant stream bank disturbance. These few undetected salamanders are unlikely to have changed the findings. Not surprisingly, results based on presence-absence data were similar to counts.

No alternative methods to estimate population size, i.e., capture-mark-recapture (CAR) or removal methods, were attempted. The former was logistically impractical because of the intensity and breadth of the proposed sampling: more than 300 plots from 14 different streams were sampled during the 2-year field study. The removal method was implemented by Southerland et al. (2004) to obtain salamander density estimates for 2 m by 15 m transects but not for their smaller 4 m<sup>2</sup> plot searches because these were assumed to yield complete salamander counts. Dodd and Dorazio (2004) adapted a technique from Royle (2004) to simultaneously estimate abundance and detection function probabilities from plot salamander counts. Application of their technique to these data may reveal the importance of having considered ( $p$ ), the probability of detection, in these analyses. However, it is important to realize that their estimator was applied to incomplete counts from 30 x 40 m plots, sampling areas 300 times larger than the 4 m<sup>2</sup> plots used in this study. Their plots contained mostly upland terrestrial environment and averaged only 12% of stream side habitats. In that situation, searches are unlikely to yield anything close to complete counts.

Stream acidification was hypothesized to be an assemblage-shaping factor for stream bank salamanders. The findings from this field sampling study support the latter hypothesis conclusively: assemblage attributes at one end of the gradient differed dramatically to those on the opposite end. Abundance was among the more obvious and striking. Most salamanders (85%) in this study were captured from non-acidic transects, yet 46% of all plots were in acidic watersheds. Median plot salamander density in Fragmented watersheds was 4.5 - 14 times greater than Laurel Run, the AMD watershed with the highest median plot density and the most intensively sampled of all study watersheds. Acidification, with its associated contaminants, was in no uncertain way associated with a reduction of total numbers of stream salamanders. This response was perhaps the most obvious.

Taxonomic diversity generally declined with increasing acidification. Muddy Run and Jefferson Run exemplified these trends at the watershed scale: a single species was observed in the former, the most acidic watershed, whereas 6 species were confirmed in the latter, a Fragmented watershed sampled with the same number of plots (20). Median diversity was positively related to stream pH.

Acidic streambanks were not only more likely to have fewer species, but those present differed from those most commonly found in non-acidic sites. The best case in point is the northern two-lined. This species, found mostly as an aquatic larvae, ranked highest (1) in abundance in non-acidic environments, but was replaced by the mountain

dusky in acidic settings. Interestingly, the contender for second place in acidic sites was the northern spring salamander, an aquatic nester with an extended larval life stage and whose presence was mostly known from its aquatic larvae. These assemblage composition patterns were also reflected in ordination plots, bubble plots, and response curves.

Acidic sites favored adults. Non-acidic transects were overwhelmingly occupied by aquatic larvae. While true, these patterns were driven by mostly 2, sometimes 3 species - the northern two-lined in non-acidic sites and the mountain dusky, and to some extent, the northern dusky, in acidic streams. When larvae appeared in acidic sites, these were usually of the northern spring, a taxon showing greater tolerance than the northern two-lined (Chapter 3). Stone Run was an exception in many respects, including in the number of northern two-lined it supported and consequently, the number of larvae present. This exception is attributed to the presence of several alkaline feeder streams believed to be mitigating an otherwise acidic mainstem (Dolte 1998).

Acidification was strongly associated with changes in stream salamander assemblages. The reduction in assemblage complexity resulted from decreased abundance, reduced taxonomic and life stage diversity, and species replacement. A vigorous response was also evident from the ordination results. Both data sets, the presence - absence and abundance matrices, produced a relatively small amount of variance and correspondingly short gradient lengths. Yet, a sizeable portion of the total

“noise” generated was tied to the first and second axes. In both CA ordinations, the first two axes claimed over 50% of the total variance. The first axis alone, in the abundance matrix-based set, captured 40%. Furthermore, since the axis claiming the highest proportion of the variance was highly associated with water chemistry, among other stream bank attributes examined (Appendix 2.2), it linked the assemblage response back to acidification. The partial CCA revealed that the association was strong, persisting even after elimination of the effect of physical stream habitat. In other words, an amount of the explained variance was unique rather than shared with stream physical habitat and climatic variables suspected to co-influence salamander presence and capture.

The ordination also helped visualize how individual species and their life stages responded to acidification. Highly acidic streambanks were unlikely to support stream salamanders. Among the first to be encountered along this acid - alkaline gradient were the mountain dusky and northern spring. The northern dusky and Appalachian seal salamanders followed. Common encounters of the northern two-lined and northern red occurred in alkaline stream banks. The aforementioned ordering is based on the location of centroids along the first ordination axis. Not infrequently, however, species and life stages were found in stream banks contrary to what centroids locations might be inferring e.g, juvenile northern dusky salamanders were occasionally encountered in acidic watersheds even though their centroid trended on the alkaline side of the ordination biplot. Hence, the ordination is best viewed as a simple, albeit useful model of how stream salamanders and their life stages generally responded to acidification.

Sites were ordinated by species and their life stages rather than by species alone. Several factors drove this decision. The most immediate one was the desire to extract as much information possible from this species-poor assemblage. As noted in the tabular data, life stage composition in acidic stream banks differed from that in non-acidic sites. Furthermore, the ordination by life stages generated almost 40% more inertia (variation) than by species alone. Ordination by life stage also offered the opportunity to examine how aquatic vs. terrestrial forms of the same taxon, each bound to the aquatic medium differently, responded to acidification. Was this approach worthwhile, or at least informative from the latter perspective? The ordination positioned early life stages closer to acidic sites than older ones within a species. This trend was not unique to the northern spring larvae. It was evident among the northern two-lined, northern dusky, and Appalachian seal salamanders, the only taxa to be commonly found in their larval, juvenile, and adult forms. The watershed-scaled results suggested the opposite; larvae ranked first in all but acidic watersheds. Furthermore, early amphibian life stages are supposed to be the less tolerant, most sensitive to acidification. Why these discrepancies?

The ordination was based on transects, a scale of observation providing fine resolution to the association between assemblage attributes and environment. The ordination may be revealing signals lost in the watershed-scaled perspective. Secondly, older members of an assemblage can be viewed analogously as the fraction of a sample that passed through several filters. In this respect, and relative to older cohorts, larvae and juveniles may be found across a greater variety of environments, including less than

ideal ones, because factors preventing permanency (toxicity, stress, higher predation, etc.) in such inferior environments have yet to eliminate them. The tendency for early amphibian life stages to disperse widely is consistent with the latter interpretation.

Life tables may offer additional support. In amphibians with larval stages, mortality is most severe during a cohort's initial years, a survivorship curve known as Type III. Some Plethodontids exhibit constant death rates over time, a survivorship curve known as Type II (Zug et al. 2001). In either pattern, and more so in the former, focusing on older life stages may be providing a stronger, clearer signal on the outcome of occupying acidic stream banks. Thus, centroids representing adult salamanders, although physiologically more tolerant than larvae, trend on the alkaline side more than their younger life stages. Linking survivorship curves to the adult response assumes that mortality was directly or indirectly influenced by acidification. The narrower niche breadth exhibited by larvae is consistent with their greater physiologic sensitivity and aquatic-dependence.

In developing the Stream Salamander Biotic Index (SSIBI), Southerland et al. (2004) found the metric "number of adult stream salamanders" to be equally effective as its larval-based equivalent, a "counterintuitive response" given the well known greater sensitivity of early life stages to stressors in general. Stohlgren et al. (1998) describe comparable patterns among tree life-stages. In their ordination plot, earlier life-stages (seedlings), rather than older forms (mature trees) trended toward less optimal habitats.

In conclusion, adult salamanders may be indicative of stressors that earlier life stages may appear to be ignoring because of their greater mobility in the first years of life, e.g., larvae captured while drifting passively or actively (Petranka 1998).

In consideration of the preceding life stage discussion, were responses at the taxonomic level consistent with predictions based on life history? In acidic stream banks, terrestrial nesters with reduced larval periods were predicted to have an advantage over aquatic nesters with multi-year larval periods. The results of the present study indicate that life history and nesting habits are unreliable predictors of survival in acidic streams. The northern spring salamander, being the most dependent on the aquatic environment, and anticipated to be the most adversely affected in acidic streams, turned out to be surprisingly persistent. Why?

Perhaps the most convenient explanation is that the northern spring salamander may be naturally acid-tolerant. Physiological as well as behavioral adaptations may contribute to its appearance in acidic streams. In this respect, the northern spring salamander may be an ecological equivalent of other headwater-dwelling, acid-tolerant taxa such as the brook trout (*Salvelinus fontinalis*) and to some extent, mottled and slimy sculpins (*Cottus bairdi*, *C. cognatus*) (Dunson et al. 1977, Gagen 1991).

Behavior may also play a crucial role in minimizing exposure to episodic or chronic acidification. Brook trout are physiologically acid-tolerant (Dunson and Martin



1973, Daye and Garside 1975), yet survival of individuals and populations in several Central Appalachian episodically acidified streams was attributed to the availability and exploitation of alkaline refugia, microhabitats maintained by alkaline inflows and seeps (Gagen 1991). It is not known if alkaline refugia were used by the northern spring salamander in situations where grab samples of surrounding water confirmed extreme channel acidification. Most larvae in this study, regardless of water chemistry, were hand-captured under submerged and often times larger, partly buried rocks and boulders, observations fairly consistent with Bruce (1980), where he noted that larvae "...reside in subterranean haunts far below the surface of streambeds". Seeking refuge in the hyporheic zone may minimize extended exposure to harmful stream water.

Amphibian larvae respond to pH gradients (Freda and Taylor 1992), hence locating subterranean alkaline sources may be within the sensory capabilities of the northern spring and other stream plethodontid taxa. Salamanders encountered in toxic stream water may have been drifting or dispersing, experiencing only short-term exposure. Ultimately, the species may have high physiologic tolerance to low pH, a trait found in several other amphibian species. The Pine Barrens treefrog (*Hyla andersonii*) breeds in acidic blackwaters of the New Jersey Pine Barrens and is extremely low pH-tolerant (Pehek 1995).

Other distribution patterns inconsistent with life history revolved around terrestrial nesters that clustered with aquatic nesters on the alkaline end of the ordination

space. The northern dusky is the best case in point: it has a life history similar to the mountain dusky, but its low abundance in acidic waters mimics aquatic nesters rather than its hardy congener. Perhaps, exposure to acidic waters is incompatible with an early aquatic larval stage (hatchlings, aquatic larvae) even if relatively short (8-9 mo). In other words, survival in acid streams in the absence of acid-tolerance may not be possible unless the aquatic stage is omitted.

Presence in acidic streams was observed consistently in only two species of stream bank salamanders. Strategies facilitating their survival are hypothesized to be entirely different. In the mountain dusky, complete avoidance of acidic water is hypothesized to be the mechanism enabling survival because an early aquatic life stage may not exist. In the northern spring, survival is hypothesized to depend on physiological and behavioral adaptations to increase acid-tolerance. All other members of the assemblage are largely excluded from acid waters. Invariably, these salamanders have early life stages tying them to the stream for variable lengths of time; their absence in acidic waters suggests they lack adaptations to render them acid-tolerant.

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## Chapter 3

*IN SITU* EXPOSURE OF NORTHERN TWO-LINED AND  
NORTHERN SPRING SALAMANDER LARVAE IN ACID  
MINE DRAINAGE-IMPACTED STREAMS

Abstract -- Toxicity and physiologic stress was suspected to be the primary cause for the decreased abundance and distribution of several stream plethodontids in episodically acidified and acid mine drainage-impacted (AMD) streams. The northern two-lined salamander (*Eurycea b. bislineata*) was among the species most affected; the northern spring salamander (*Gyrinophilus p. porphyriticus*) persisted, except in the more acidic reaches. Both taxa have extended aquatic larval stages. It was hypothesized that persistence in the northern spring was due to greater acid tolerance. Survival, and changes in body water content and total body sodium were examined in both species in 1998 and 1999 following 19-d, 48-hr, and 12-hr *in situ* exposures in reaches of varying AMD-contamination. Limited availability of the northern spring restricted testing of this species to the 48-hr exposure. Exposures were conducted in southwestern and north central Pennsylvania streams using wild-caught larval and metamorphic northern two-lined and larval northern spring salamanders. Most northern two-lined larvae and metamorphs survived the 19-d (weak-AMD) and 12-hr (strong-AMD) exposures. Survival in the 48-hr exposure favored less acidic reaches, larger, older animals, metamorphs, and northern spring larvae. The proportion of body water was lower in 19-d and 48-hr exposures, relative to controls, but increased initially during the 12-hr exposure. Total body sodium was lost in all exposures. Body mass, life stage, water chemistry, species and exposure time (12-hr) and their interactions were significant terms in models.

## Introduction

In 1997-1998, field investigation of 14 small streams in the central Appalachians revealed depressed densities as well as absence of most stream salamanders (family Plethodontidae) in episodically acidified and acid mine drainage (AMD) impacted streams (Chapter 2). Toxicity and physiologic stress were suspected to be the primary cause for the observed distribution patterns in acidic streams.

The northern two-lined salamander (*Eurycea b. bislineata*) and the northern spring salamander (*Gyrinophilus p. porphyriticus*) are two commonly encountered streamside plethodontids in small, northern Appalachian streams. In the field study described in Chapter 2, larval northern two-lined were the first to become scarce or absent in acidified stream reaches. Oviposition in the northern two-lined takes place on the underside of submerged rocks. Hatching occurs after 30-70 d and aquatic larvae transform approximately 1.5 - 2.5 yr later at snout vent length (SVL) 23 - 32 mm (Petranka 1998).

The northern spring salamander is highly dependent on the aquatic portion of its riparian environment. Oviposition takes place in submerged sites and aquatic larvae transform after 3-4 yr at SVL ranging from 55 - 70 mm (Petranka 1998). Unlike its smaller, more common counterpart, the northern spring salamander was more common in acidified waters and was occasionally encountered in extremely acidic, toxic metal-bearing waters (Chp. 2). On August 1, 1998, a single larva (42.5 mm SVL) was captured at a sampling location (Glenwhite Run, GW-T2-10) where grab samples collected several

minutes later revealed surprisingly low pH and high dissolved toxic metal concentrations (pH = 3.86, Alkalinity CaCO<sub>3</sub> mg/l = Not detectable, ANC = -220.8, SO<sub>4</sub> = 119.2 mg/l, DOC = 2.31 mg/l, NO<sub>3</sub>-N = 0.22 mg/l, Fe = 0.34 mg/l, Mn = 1.07 mg/l, Al = 7.06 mg/l).

Regardless of potential differences in tolerance between these two taxa, stream water toxicity and physiologic stress were hypothesized to be the causes for the depressed abundance or absence of both species in the field. As part of a larger study aimed at investigating the effects of low pH and acid mine drainage (AMD) on stream plethodontid assemblages, three *in situ* exposure experiments were designed to examine the survival and physiologic effect of AMD-impacted waters on the aquatic larvae of these two plethodontids.

The first experiment was a prolonged, 19-d exposure of larval and metamorphic northern two-lined to weakly AMD-contaminated waters to ascertain survival and degree of physiologic stress. In the second experiment, larvae and metamorphic northern two-lined and larval northern spring salamanders were exposed to different levels of AMD contamination for 48-hr to assess relative tolerance. A 12-hr exposure of larval northern two-lined in a stream reach identified from the 48-hr exposure to be lethal, served as a high-resolution, hour by hour study of physiologic response. Exposure to non-contaminated water served as a control in all three experiments. Most importantly, all *in situ* exposures, whether in non-acid or acid waters, were completed in stream reaches where presence and abundance of free-ranging test subjects was known from previous

sampling (Chp. 2). Mortality, changes in the proportion of body water content, and total body sodium concentration served as response variables.

## Methods

### Collection of Larvae and Pre-Exposure Husbandry

In June 1998 aquatic larvae and metamorphic northern two-lined were collected from several streams in the vicinity of the Powdermill Biological Station and Reserve, Carnegie Museum, in Westmoreland County, Pennsylvania. In August 1999, in addition to northern two-lined, northern spring salamander larvae were collected from streams in Rothrock State Forest and Black Moshannon State Forest in the vicinity of State College, Centre County, Pennsylvania. On both years, animals were housed in modified polyethylene shoe boxes of various lengths (200 - 400 mm) in anticipation of tests. Short strips of 15 mm diameter polyvinyl chloride (PVC) tubes hot-glued together served as refugia. In 1998, a continuous supply of stream water (Powdermill Run, Powdermill Biological Station and Reserve) fed containers holding test specimens by a pump and gravity feed system. A fine mesh was not used to trap incoming sediments, therefore potential prey were not purposely introduced to feed test animals during the several week pre-exposure period. The entire flow-through system consisting of an array of PVC tubes and 4 greenhouse fiberglass tanks (2 m long) was housed in a wooden shed at the edge of the stream. In 1999, the same flow-through system was used, except that water was pumped from a well and the fluvarium was housed in a large building formerly used by the Pennsylvania Fish and Boat Commission for aquaculture at the Fisherman Paradise

Hatchery, Bellefonte, PA. Mosquito larvae (*Culex*) and amphipods (*Gammarus*) were introduced in holding tanks as prey during the several week-long pre-exposure period.

On the day of the test, specimens were captured by dipnet and individually transferred to plastic bags. Animals were assigned to treatment groups systematically to avoid biasing by originating watershed. Plastic bags were subsequently filled with air, sealed, and transported in large coolers over ice. Once on site, larvae were transferred to containers by gently pouring the contents of each bag in the partly submerged enclosures. The latter were typically located in shallow run portions of the stream and secured to the bottom by large rocks. PVC tubes were left partly above the water surface in containers holding metamorphic salamanders. Containers were constructed from acrylic tubes 75 mm in diameter and 450 mm in length. Plastic caps fitted with fine mesh on each end allowed the flow of water and fine particulate matter. Larger square polyethylene containers fitted with fine hardware cloth on at least two sides held the larger northern spring salamander larvae. Short lengths of 15 mm diameter PVC tubes hot-glued together served as refugia in test containers.

Specimens were transferred to zip lock bags filled with test site water and transported on ice upon termination of *in situ* exposures. In 1998, animals were frozen within 2-3 hr of removal from exposure sites by placing zip lock bags in a deep freezer. In 1999, dry ice was used to flash freeze specimens in the field. Dead animals, when present, were separated from live ones in the field and stored separately.



## *In situ* Exposures

### 19-d Exposure

There were 4 *in situ* exposure sites (transects); two in each of Camp Run (Control) and Laurel Run (AMD-contaminated). Only larval and metamorphic northern two-lined salamanders were used in the experiment. There were 45 small larvae (SVL = 9 - 19.5 mm) and 6-12 large larvae and metamorphs (SVL = 20-31 mm) per exposure transect (total n = 216). The exposure began on July 20 and was terminated on August 7, 1998, 19 d later. The increase in mortality of test subjects largely determined the duration of the exposure. Stream water grab samples were collected at each location on exposure begin and end dates.

### 48-hr Exposure

There were 7 *in situ* exposure sites; 2 in Wallace Run, 3 in Glenwhite Run and 2 in Muddy Run. Different levels of AMD-contamination existed in Glenwhite Run and Muddy Run sites. Wallace Run and one Glenwhite Run site served as controls. Larval and metamorphic northern two-lined and larval northern spring salamanders served as test subjects. No metamorphic northern spring salamanders were available for testing because none were found. Exposures began on August 4 and were terminated on August 6, 1999, 48-hr later. Stream water grab samples were collected at each test site on both days.

## 12-hr Exposure

The *in situ* exposure for this experiment was performed at a single location, Glenwhite Run T2, a stream reach with relatively high AMD-contamination as determined from past chemical and stream salamander surveys and the earlier 48-hr exposure experiment. Only larval northern two-lined were tested. The exposure began on August 10, 1999 at 0800 hr and was terminated 12-hr later at 2000 hr.

Experimental animals were transferred to zip lock bags on the evening before the exposure (August 9) and left under ice inside coolers. That same evening, 5 first year larvae were set aside and flash frozen to serve as Laboratory Control subjects. At 0800 hours of the next day, subsequent to the installation of all containers in the test stream, a second group of specimens ( $n = 7$ ; 5 first year larvae, 2 second year larvae), representing the Field Control, were flash frozen. Every hour thereafter, two containers, one containing 5 first year larvae and one containing two second year larvae were removed from the stream and flash frozen. There were a total of 70 first year larvae and 26 second year larvae. Grab samples were collected every 4-hr. Stream temperature was recorded at 15-min intervals with an Onset<sup>®</sup> Hobo temperature logger placed in a submersible case.

## Specimen Processing

Only animals surviving exposures were processed further to avoid the spurious effects of decay and autolysis on the response variables of interest (W. Sharpe, pers. comm.). The snout vent length (SVL), wet mass (WM), dry mass (DM), and proportion of body water content [ $PBWC = (WM - DM) / WM$ ] were measured for each animal.

Specimens were oven dried at 60 degrees C for 6 d. Dry mass was digested by HNO<sub>3</sub>. Digested samples were subsequently analyzed for total body Na (BNA) by atomic adsorption (AA) at the Penn State Water Quality Laboratory.

### Data Analysis

The effects of stream exposure on the response variables PBWC and body Na were examined graphically and by multivariate linear regression. Wet mass (WM), dry mass (DM), and exposure time (TM), the latter applicable only to the 12-hr exposure, were treated as continuous variables. Water chemistry (WCHM; alkaline *vs.* acid), species (SPCS; northern two-lined *vs.* northern spring), and life stage (LF; larva *vs.* metamorph) were treated as categorical variables and coded 0 or 1, respectively. Interactions between continuous and categorical variables were also tested. Regressions were developed for body Na concentration ( $\mu\text{moles Na/ml}$ ) rather than by its specimen weight-adjusted form,  $\mu\text{moles Na/WM}$  or  $\mu\text{moles Na/DM}$ . This approach, while admittedly not the most intuitive, was chosen because the predictors, WM or DM were required in all models to account for the effect of body mass and it avoided regressing a ratio by its denominator (Pendleton et al. 1979). BNA, WM and DM were natural log-transformed prior to analysis; PBWC was arcsine transformed (Zar 1984). Multivariate regression was chosen because of the wide range of body size in test subjects.

Model development and selection was aided by Minitab's best subset and forward selection regression procedures (Minitab Ver. 13, 2000). Parsimonious models were

preferred and evaluated by their R-value and C-p criterion, the total mean squared error. Weighted regression was used where necessary to lessen the impact of unequal variance. Weights for the latter were computed from the reciprocal of the squared fits obtained by regressing the absolute value of the residuals against the original unweighted fits (Neter et al. 1996).

## Results

### Water Chemistry at Exposure Sites

AMD contamination varied considerably among exposure sites (Table 3.1). Median pH ranged from 6.3 - 7.0 in control sites and from 2.9 - 5.18 in AMD-affected reaches. Alkalinity was  $< 0.05$  mg CaCO<sub>3</sub>/l in the latter to 6.3 - 45.6 mg CaCO<sub>3</sub>/l in control sites. ANC was not measured in Glenwhite and Muddy Run, but in other acidic sites ranged from -2.7 to -107  $\mu$ eq/l for Laurel Run and the lowermost Muddy Run reach. Control sites ranged in ANC from 137-297  $\mu$ eq/l. Concentration of dissolved organic carbon (DOC) was relatively similar across all stream reaches, ranging from 1.22 - 5.25 mg/l. There was a slight overlap in sulfate concentration in control and AMD sites. The median range in the former was 8.1 - 82.1 mg/l; in the latter it was 37.8 - 1000 mg/l. Concentration of sulfate in Glenwhite Run and Muddy Run exposure sites were above 157 mg/l. There appeared to be no discernible differences in NO<sub>3</sub> concentration; Camp Run samples measured the highest at 0.77 mg/l. Concentrations of dissolved heavy

Table 3.1. Grab sample water chemistry at the 11 Pennsylvania *in situ* stream exposure sites in 1998 and 1999. Water chemistry on August 10, 1999 are medians of 4 grab samples. Water chemistry on other dates are from grab samples collected on exposure begin and end dates (n=2). Metals are dissolved amounts. ANC = acid neutralizing capacity, DOC = dissolved organic carbon.

Stream & Exposure Site	Condition	pH	Alkalinity mg CaCO <sub>3</sub> /l	ANC ueq/l	DOC mg/l	SO <sub>4</sub> mg/l	NO <sub>3</sub> -N mg/l	Fe mg/l	Mn mg/l	Al mg/l
19-d Exposure (July 20-August 7, 1998)										
Camp Run, T51/52	No AMD/ Control	7.03	13.02	282.25	1.22	8.07	0.77	0.07	n/d	0.02
Laurel Run, T40	AMD/Acid	4.90	<0.05	-13.03	1.93	38.54	0.31	0.09	0.24	0.24
Laurel Run, T30	AMD/Acid	5.18	<0.05	-2.70	2.24	37.81	0.29	0.1	0.33	0.20
48-hr Exposure (August 4-6, 1999)										
Wallace Run, T1	No AMD/ Control	6.3	6.3	115.8	2.8	25.56	0.219	0.167	0.933	0.075
Wallace Run, T3	No AMD/ Control	6.6	7.8	137	5.25	5.16	0.184	0.037	0.055	0.087
Glenwhite Run, T1	No AMD/ Control	6.5	45.6	297	3.63	82.12	0.219	0.046	0.0445	0.073
Glenwhite Run, T2	AMD/Acid	3.7	<0.05	na	3.62	176.7	0.18	0.37	1.48	0.337
Glenwhite Run, T4	AMD/Acid	3.6	<0.05	na	3.17	197.7	0.132	1.43	1.561	0.385
Muddy Run, T1	AMD/Acid	2.9	<0.05	na	3.01	1000.2	0.14	5.41	44.455	0.901
Muddy Run, T4	AMD/Acid	4.1	<0.05	-106.7	2.38	157.55	0.145	0.152	4.867	0.259
12-hr Exposure (August 10, 1999)										
Glenwhite Run, T2	AMD/Acid	3.74	<0.05	na	2.75	175.8	1.73	0.39	1.52	0.41

metals were highest in acidic AMD-impacted reaches. From greatest to least, Muddy Run T1, Muddy Run T4, and Glenwhite Run T4, and Glenwhite Run T2 exhibited the highest concentrations in dissolved Fe (0.152 - 5.41 mg/l), Mn (1.48 - 44.45 mg/l), and Al (0.259 - 0.901 mg/l). The greatest concentration for Fe, Mn, and Al in control exposure sites was 0.167 mg/l, 0.933 mg/l, 0.087 mg/l, respectively.

Severe storm events have the potential to affect stream base flow water chemistry. During the period between July 20 - August 7, 1998, the NCDC weather observation station in Rector, PA, located 8 mi and 12.8 km northeast of Laurel Run and Camp Run, respectively, recorded a total of 36.2 mm of precipitation on July 22 - 24, and 31 (NCDC 1998). The greatest amounts of precipitation were recorded on July 22 (13.9 mm) and July 31 (19.6 mm).

Minimal precipitation was recorded during the 48-hr exposure. Rain gauges at Muddy Run exposure sites T1 and T4 recorded 2.7 mm and 3.8 mm of precipitation. No measurable precipitation was recorded at Glenwhite Run and Wallace Run. The NCDC weather observation stations in Altoona (Glenwhite Run), Prince Gallitzin State Park (Muddy Run), and State College (Wallace Run) recorded 0 mm, 1.5 mm, and 53.3 mm of precipitation, respectively (NCDC 1999). Light rain toward the late afternoon on August 10, the day of the 12-hr exposure, did not result in measurable amounts of precipitation. Not surprisingly, the 4 grab samples collected during this period showed minimal variation in water chemistry and water temperature.

## Survival

### 19-d Exposure

A surprisingly high proportion (84 - 98%) of larval northern two-lined salamanders survived the 19-d exposure (Table 3.2). Differences in survival between Camp Run (98%) and Laurel Run (84-91%) are evident, but not widely so. Large larvae and metamorph survival was 100% in all 4 exposure sites irrespective of test site water chemistry

### 48-hr Exposure

Survival following the 48-hr exposure differed widely depending on level of AMD contamination, life stage, and species. Almost all control subjects, animals in Wallace Run and Glenwhite Run T1, survived. One animal injured accidentally during installation of a control container in Wallace Run was found dead at the end of the exposure period. Missing larvae were assumed to have escaped. Among first year, second year, and metamorphic northern two-lined salamanders, the median survival in the four AMD-contaminated reaches was 0% (0%, 0%, 0%, 89.5%), 17 % (0%, 4.8%, 28.6%, 100%) and 100% (50%, 100%, 100%, 100%), respectively. In northern spring salamander larvae, the median survival for small and large larvae in the 4 acid reaches was 91.5% (0%, 83%, 100%, 100%) and 100% (0%, 100%, 100%, 100%), respectively.

Only 3 metamorphic northern two-lined salamanders survived exposure to the highly toxic Muddy Run T1 test site; all other test subjects perished, resulting in a

Table 3.2. Survival (%) of northern two-lined and northern spring salamanders (larval and metamorphic) following 19-d, 48-hr, and 12-hr *in situ* stream exposures at 11 Pennsylvania acid mine drainage-contaminated (AMD) and control stream sites in 1998-1999. Number of salamanders are in parentheses. Toxicity Index = ranking of 4 AMD transects (48-hr), where 1= most toxic; 4 least toxic.

Stream & Exposure Site	Condition and Toxicity Index	Northern two-lined			Northern spring	
		First Year Larvae	Second Year Larvae	Metamorphs	Small Larvae	Large Larvae
19-d Exposure (July 20-August 7, 1998)						
Camp Run, T51,T52	No AMD/ Control	97.8 (90)	100 (8)	100 (10)	n	
Laurel Run, T30	AMD/Acid	91.1 (45)	100 (3)	100 (3)		
Laurel Run, T40	AMD/Acid	84.4 (45)	100 (2)	100 (10)		
48-hr Exposure (August 4-6, 1999)						
Wallace Run, T1	No AMD/ Control	100 (20)	100 (20)	100 (7)	100 (4)	100 (4)
Wallace Run, T3	No AMD/ Control	100 (19)	100 (20)	100 (7)	100 (6)	100 (4)
Glenwhite Run, T1	No AMD/ Control	100 (18)	100 (21)	100 (6)	100 (5)	100 (4)
Muddy Run, T1	AMD/Acid - 1.0	0 (20)	0 (21)	50 (6)	0 (6)	0 (4)
Glenwhite Run, T4	AMD/Acid - 2.2	0 (20)	4.8 (21)	100 (6)	83 (6)	100 (4)
Glenwhite Run, T2	AMD/Acid - 3.2	0 (20)	28.6 (21)	100 (6)	100 (6)	100 (4)
Muddy Run, T4	AMD/Acid - 3.6	89.5 (19)	100 (17)	100 (10)	100 (5)	100 (4)
12-hr Exposure (August 10, 1999)						
Glenwhite Run, T2	AMD/Acid - 3.2	100 (70 )	100 (26)	na		



median survival for all test subjects of 0% (0% , 0%, 0%, 0%, 50%). PVC tubing in one of the containers at this site housing 3 metamorphs was found completely submerged. The inability of these metamorphs to crawl out of the acidic water may have affected their survival; the 3 surviving metamorphs mentioned above were able to crawl out of the water and no other metamorphs perished during the 48-hr test. The median percent survival for all test subjects in Glenwhite Run T4 was 83% (0%, 4.8%, 83%, 100%, 100%). The median survival for the remaining two AMD-impacted reaches was 100%. Survival was consistently lower among smaller, younger animals within each species.

In processing specimens after the exposure, 3 survivors thought to be northern spring salamander larvae were identified to be northern red salamander larvae (*Pseudotriton r. ruber*). Two were exposed to control sites and one specimen (SVL = 30.0 mm; WM = 0.69 g) survived exposure to Muddy Run T4. These animals were omitted from the survival discussion above.

#### 12-hr Exposure

None of the first or second year northern two-lined larvae died during the 12-hr exposure at Glenwhite T2. However, a few animals toward the end of the exposure appeared sluggish and less responsive.

## Body Water Relationship

### 19-d Exposure

Percent body water content (PBWC) was higher in smaller, younger animals than in larger, older larvae, or metamorphic animals (Table 3.3, Figure 3.1). Differences were particularly pronounced between the latter group and first year larvae. The relationship is consistent with physiologic changes experienced by amphibian larvae approaching transformation (Feder et al. 1992). Changes in body water content were also exhibited by acid-water exposed subjects.

Changes in body water was modeled by a weighted, multivariate regression consisting of 4 predictors (Table 3.4). Three of the 4 terms were highly significant ( $p$ -value  $< 0.0001$ ). Wet mass (WM) explained almost 85% of the total explained variation whereas water chemistry (WCHM), only 1.5 %. The remaining predictors were interaction terms between WM and life stage (LF). The model explained 73.4 % of the total variation.

The difference in the proportion of body water content between small larvae and older animals was greater than what was observed across all groups in response to acid-exposure. Thus, water chemistry contributed to the model significantly, but explained only a modest amount of the total variation relative to wet mass. Crossing wet mass with water chemistry was not necessary to obtain a satisfactory fit. Hence, fits for acid and non-acid responses appear as parallel lines for similar life staged-animals.

Table 3.3. Mean  $\pm$  1SE for wet mass (WM), dry mass (DM), proportion of body water content (PBWC), and body Na concentration ( $\mu$ moles) by wet mass (BNA/WM) for surviving northern two-lined and northern spring salamanders (larval and metamorphic) following 19-d, 48-hr, and 12-hr *in situ* stream exposures at 11 Pennsylvania acid mine drainage-contaminated (AMD) and control stream sites in 1998-1999. Number of salamanders are in parentheses. LC and FC= laboratory and field control.

Exposure	WM (g)	DM (g)	PBWC (%)	BNA/WM $\mu$ moles Na/WM
Northern two-lined: 19-d exposure (July 20 - August 7, 1998) Camp Run (T51, T52) - Control				
Larvae (96)	0.071 $\pm$ 0.008	0.008 $\pm$ 0.001	0.901 $\pm$ 0.002	29.53 $\pm$ 1.84
Metamorphs (10)	0.333 $\pm$ 0.039	0.046 $\pm$ 0.006	0.862 $\pm$ 0.002	6.95 $\pm$ 0.75
Laurel Run (T30, T40) - AMD				
Larvae (84)	0.067 $\pm$ 0.007	0.007 $\pm$ 0.001	0.899 $\pm$ 0.002	4.49 $\pm$ 0.22
Metamorphs (13)	0.326 $\pm$ 0.036	0.05 $\pm$ 0.007	0.852 $\pm$ 0.005	11.36 $\pm$ 1.95
Northern two-lined and northern spring: 48-hr exposure (August 4-6, 1999) Wallace Run and Glenwhite Run (T1) - Control				
Larvae (118)	0.109 $\pm$ 0.008	0.012 $\pm$ 0.001	0.904 $\pm$ 0.002	35.45 $\pm$ 2.67
Metamorphs (20)	0.218 $\pm$ 0.015	0.032 $\pm$ 0.003	0.858 $\pm$ 0.005	43.2 $\pm$ 3.35
N. spring larvae (27)	2.101 $\pm$ 0.218	0.284 $\pm$ 0.035	0.871 $\pm$ 0.003	7.16 $\pm$ 1.04
Glenwhite Run (T2, T4), Muddy Run (T1, T2) - AMD				
Larvae (41)	0.132 $\pm$ 0.016	0.016 $\pm$ 0.002	0.889 $\pm$ 0.003	15.9 $\pm$ 1.71
Metamorphs (25)	0.199 $\pm$ 0.014	0.030 $\pm$ 0.003	0.85 $\pm$ 0.006	40.15 $\pm$ 3.69
N. spring larvae (28)	2.201 $\pm$ 0.263	0.329 $\pm$ 0.044	0.859 $\pm$ 0.005	9.24 $\pm$ 2.42
Northern two-lined larvae: 12-hr exposure (August 10, 1999) Glenwhite Run (T2) - AMD				
First yr LC (5)	0.021 $\pm$ 0.0015	0.003 $\pm$ 0.0002	0.855 $\pm$ 0.006	27.03 $\pm$ 1.48
First yr FC (5)	0.03 $\pm$ 0.0014	0.004 $\pm$ 0.0002	0.876 $\pm$ 0.002	17.37 $\pm$ 0.63
First yr 1-12 hr (60)	0.026 $\pm$ 0.0005	0.003 $\pm$ 0.0001	0.894 $\pm$ 0.001	9.98 $\pm$ 0.45
Second yr FC (2)	0.265 $\pm$ 0.0031	0.047 $\pm$ 0.0012	0.824 $\pm$ 0.002	26.27 $\pm$ 2.23
Second yr 1-12 hr (24)	0.232 $\pm$ 0.0123	0.036 $\pm$ 0.0023	0.847 $\pm$ 0.002	15.28 $\pm$ 1.07

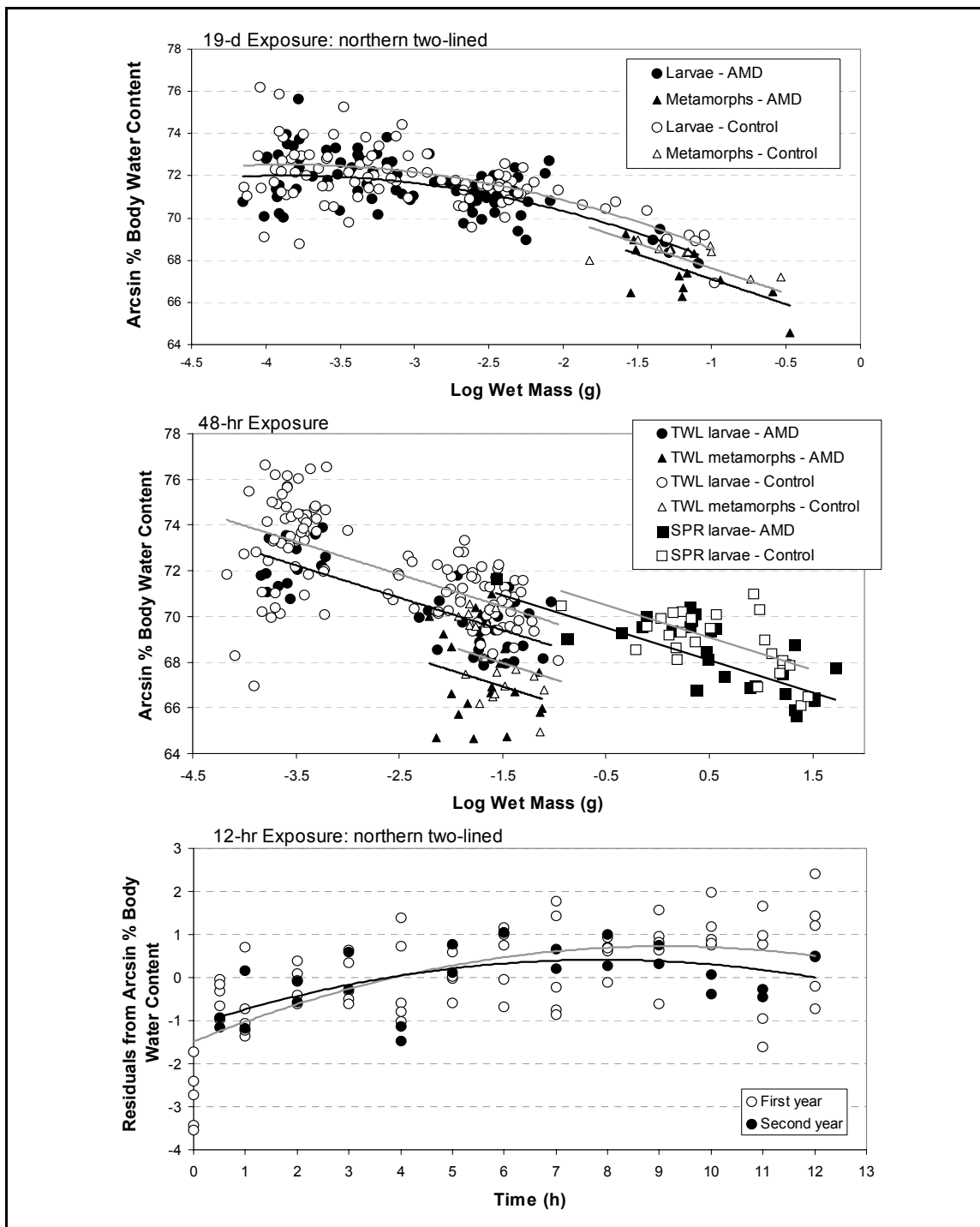


Figure 3.1. Percent body water content in northern two-lined (TWL) and northern spring (SPR) salamanders in 19-d (top), 48-hr (middle), and 12-hr (bottom) *in situ* exposures in Pennsylvania acid-mine drainage contaminated (AMD) and control streams in 1998-1999. Residuals are from regressing percent body water content against dry mass for first and second year larvae. Northern spring larvae were only tested in the 48-hr exposure.

Table 3.4. Results of multivariate regression analyses relating proportion body water content (PBWC) in northern two-lined and northern spring salamanders (larval and metamorphic) following 19-d, 48-hr, and 12-hr *in situ* stream exposures at 11 Pennsylvania acid mine drainage-contaminated (AMD) and control stream sites in 1998-1999. The regressions for the 12-hr exposure were on residuals from regressing PBWC against dry mass. All predictors were significant at P-value < 0.05, \* = p-value < 0.001, WM = Wet mass, WCHM = Water Chemistry, LF = Life stage, SPCS = Species, TM = time (hr).

Response (PBWC)	No. Salamanders	Predictor	Variation Explained	Total Var. Explained
19-d Exposure	204	WM	84.7*	
July 20 - August 7, 1998		WCHM	1.5	
		WM*LF	8.4*	
		WM <sup>2</sup> *LF	5.5*	73.4 *
Equation: 65.6 - 1.95 WM - 0.404 WCHM - 1.50 WM*LF - 0.433 WM <sup>2</sup> *LF				
48-hr Exposure	255	WCHM	20.3 *	
August 4-6, 1999		SPCS	13.7 *	
		LF	37.2 *	
		WM	28.7 *	65.1 *
Equation: 68.3 - 1.03 WCHM + 1.51 SPCS - 2.47 LF - 1.42 WM				
12-hr Exposure - August 10, 1999				
first year	70	TM	80.5 *	
		TM <sup>2</sup>	19.5 *	41.6 *
Equation: 1.48 + 0.486 TM - 0.027 TM <sup>2</sup>				
second year	26	TM	55.5	
		TM <sup>2</sup>	44.5	34.2
Equation: 1.1 + 0.384 TM - 0.024 TM <sup>2</sup>				

In summary, in proportion to their body size, hatchlings contained more body water than older, larger larvae. This pattern persisted among acid water-exposed test subjects. However, acid water-exposed animals contained less body water at the end of the 19-d exposure than their similar-sized and life-staged, control counterparts.

Evidently, prolonged acid water exposure affected the proportion of body water similarly regardless of body size (mass) or age, suggesting that larger, older animals were also at risk and responded to the prolonged exposure in a manner similar to their smaller, younger counterparts.

#### 48-hr Exposure

High mortality of test animals in severely AMD-contaminated stream reaches precluded statistical examination at the reach or stream level. Consequently, test subjects surviving acid ( $\text{pH} < 6.0$ ) or non-acid ( $\text{pH} > 6.0$ ) exposures were coded 0 or 1, respectively. This binary predictor reflecting water chemistry coarsely, allowed model development with a larger sample size.

Similarly to the 19-day exposure, the proportion of body water content (PBWC) was inversely related to wet mass (WM) in both salamander species. Metamorphic northern two-lined exhibited the lowest PBWC (Table 3.3, Figure 3.1). A multivariate regression consisting of 4 terms was a satisfactory model for the observed response (Table 3.4). The model's r-square was 65%. The amount of explained variation for predictors varied from 14% for species (SPCS) to 37% for life stage (LF). All 4 terms

were highly significant ( $p$ -value  $< 0.001$ ). No interaction terms were selected. Marginally better-fitting models included interaction terms between WM and species. However, such models also produced high variance inflation factor (VIF) values. A relatively low fit was anticipated from the use of a simple, binary term to represent exposure to different AMD-contamination levels.

Examination of residuals by AMD-impacted transects among similar species and life stages offered the opportunity to reveal the effectiveness of using a binary predictor to represent multiple treatments i.e., concentration of AMD varied considerably among acid transects. Differences in residuals between northern two-lined exposed to Glenwhite Run transects T2 and T4 (pooled) and Muddy Run T4 were significant (Kruskal -Wallis  $H = 4.79$ ,  $df = 1$ ,  $p$ -value  $< 0.029$ ). On the other hand, residuals did not differ significantly by transect among northern two-lined metamorphs ( $H = 3.69$ ,  $df = 2$ ,  $p$ -value = 0.158) nor northern spring salamanders larvae ( $H = 2.0$ ,  $df = 2$ ,  $p$ -value = 0.368). Different AMD-contamination levels determined survival across similar-aged test subjects. Similarly, the residual analysis suggests that a binary predictor for water chemistry (acid vs. non-acid) was an adequate representation of responses for hardier test subjects (northern two-lined metamorphs and northern spring larvae salamanders) but probably a poor one for the more vulnerable northern two-lined larvae.

To summarize, the response to acid water exposure among survivors was influenced by body size (mass) and age, life stage, and species identity. The proportion of

body water in AMD-exposed subjects was lower than in corresponding control animals. Residual analyses suggest that responses were less varied in older larvae and metamorphic northern two-lined or northern spring salamander larvae, test subjects shown to be more tolerant.

### 12-hr Exposure

Test subjects from the 12-hr exposure represented two distinct size classes or cohorts: 8.0 - 12.5 mm SVL ( $10.44 \pm 0.09$ ) for first year and 19.0 - 25.0 mm SVL ( $21.42 \pm 0.294$ ) for second year larvae. These differences were also evident when examining dry mass, a measure of body size positively correlated to SVL. Among first year larvae, for example, the correlation between SVL and DM was 0.652 (p-value < 0.0001). Test animals represented different cohorts: DM in first year larvae was inversely and significantly related to exposure time = -0.398, p-value = 0.001). Thus, control salamanders and salamanders exposed for the first 4-6 hr were larger than their longer-exposed counterparts.

The above differences were unintentional and were unique to first year larvae. Its effect on the response variable of interest, the proportion of body water content (PBWC), was a concern for two reasons: first, larger larvae were observed to have proportionally less body water (lower PBWC) than smaller ones and, second, data were showing PBWC among 12-hr test subjects to be increasing over time, a response exactly opposite to that observed in 19-d and 48-hr exposures. Was the increase in PBWC during the 12-hr



exposure real or an artifact of decreasing body size, at least among first year test subjects? In consideration of the above, cohorts were analyzed independently and regression models were developed for residuals of the response variable, PBWC. For this purpose, residuals were computed by regressing PBWC against DM to adjust for any body size-related effects.

Adjusting for body size did not reverse the trend: the relationship between PBWC and time (TM) for first and second year larvae remained positively curvilinear (Table 3.4, Figure 3.1). Thus, PBWC increased over time in both groups but leveled beyond the 10<sup>th</sup> hr of acid-exposure. Simple quadratic models were poor-fitting yet significant and representative of the overall response trajectory: r-squared was 42% and 34% for first year and second year cohorts, respectively. Reproducing more faithfully the oscillatory trajectory of the response variable required 6<sup>th</sup> to 8<sup>th</sup> order terms.

The increase in the proportion of body water content (PBWC) over time during the 12-hr exposure period, a response that was directly opposite to the decrease observed following the 19-d and 48-hr exposures was not anticipated. The increase, followed by the apparent leveling, was not unique to either age group, as both cohorts exhibited the response. Of considerable interest was the impact of handling on PBWC response. Evidently, removal from laboratory holding tanks and transport to the study site was sufficient to trigger body water changes well above the baseline represented by flash-frozen laboratory animals.

## Total Body Sodium (Na)

### 19-d Exposure

Body Na (BNA) concentration adjusted for body size in the 96 larvae and the 10 metamorphs northern two-lined salamanders exposed to Camp Run (control) averaged  $29.5 \pm 1.83$  and  $6.95 \pm 0.75$   $\mu\text{moles Na}/\text{WM}$ , respectively (Table 3.3). Larvae were more variable in this attribute; metamorphic salamanders had proportionally lower body Na concentration and were less variable. In acid-exposed survivors (Laurel Run), body Na concentration adjusted for body size in the 84 larvae and the 13 metamorphs averaged  $4.49 \pm 0.22$  and  $11.36 \pm 1.95$   $\mu\text{moles Na}/\text{WM}$ , respectively. The drop in body Na concentration in younger salamanders relative to their control counterparts was striking as well as self-evident (Figure 3.2, upper graph). In this test, body Na concentration in metamorphs, was on average higher and more variable than in control test subjects.

A multivariate regression model consisting of 5, highly significant predictors ( $p < 0.001$ ) was chosen to model body Na concentration (Table 3.5, Figure 3.2, lower graph). Water chemistry (WCHM) and life stage (LF) explained 48 % and 22 % of the variance, respectively. The remaining predictors were related to wet mass (WM) and its interaction with life stage. The model explained 89.5 % of the total variance. Elimination of 3 outliers corresponding to acid-exposed larvae did not greatly improve fitting (points with lowest values). The reader is reminded that the model was for body Na concentration in its non, wet mass adjusted form i.e.,  $\mu\text{moles Na}/\text{ml}$  rather than  $\mu\text{moles Na}/\text{WM}$ .

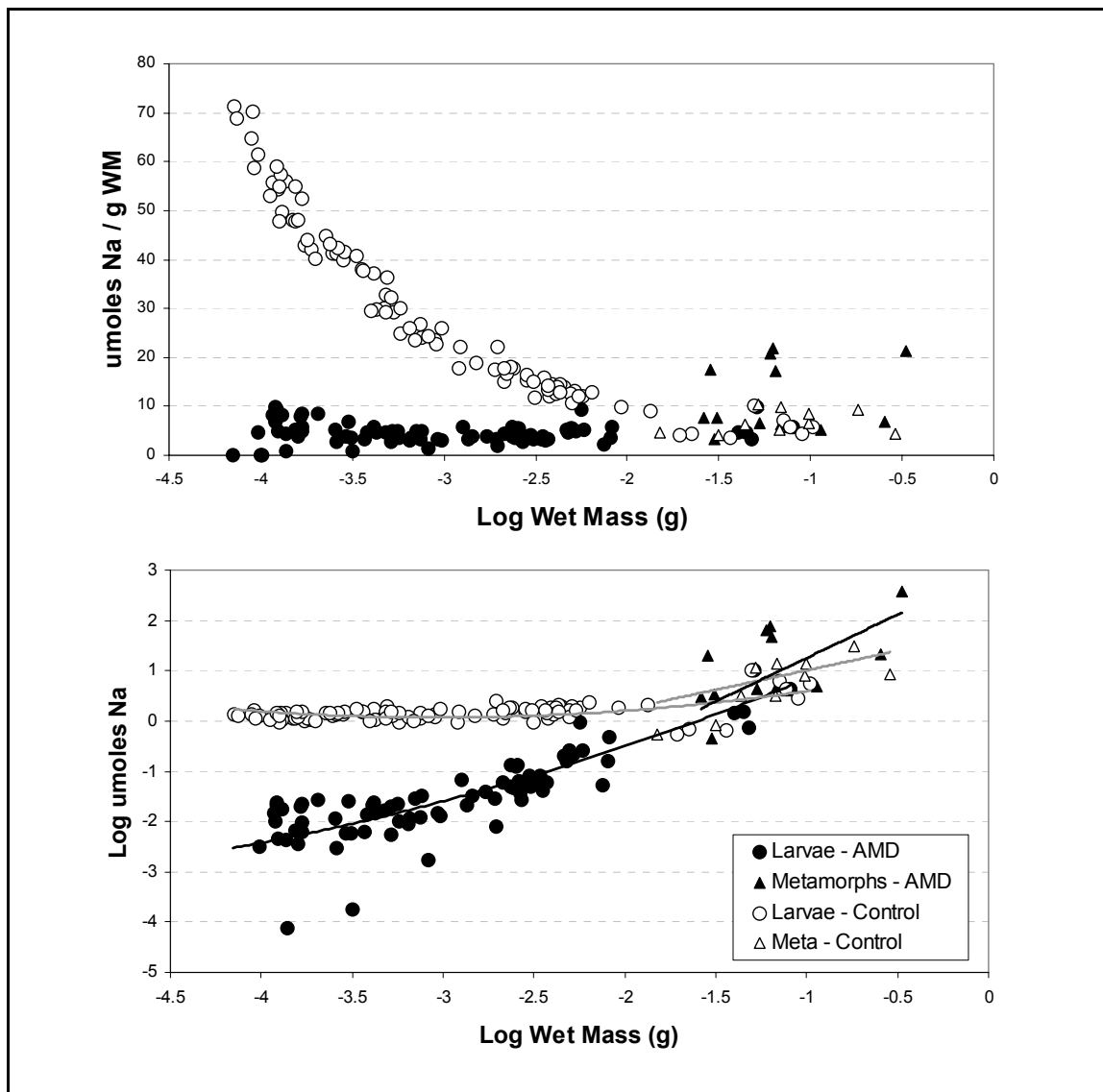


Figure 3.2. Body Na in northern two-lined larvae and metamorphs following 19-d *in situ* stream exposure in a Pennsylvania acid mine drainage-contaminated (AMD) and control stream in 1998. Top graph is body Na concentration adjusted for salamander wet mass (umoles Na/WM); bottom graph is log-transformed body Na in its non-wet mass adjusted form, umoles Na.

Table 3.5. Results of multivariate regression analyses relating body Na (BNA) in northern two-lined and northern spring salamanders (larval and metamorphic) following 19-d, 48-hr, and 12-hr *in situ* stream exposures at 11 Pennsylvania acid mine drainage-contaminated (AMD) and control stream sites in 1998-1999. The regressions for the 12-hr exposure were on residuals from regressing BNA against dry mass. All predictors were significant at P-value < 0.05, \* = p-value < 0.001, WM = Wet mass, WCHM = Water Chemistry, LF = Life stage, SPCS = Species, TM = time (hr).

Response (BNA)	No. Salamanders	Predictor	Variation Explained	Total Var. Explained
19-d Exposure July 20 - August 7, 1998	200	LF	22.2 *	
		WCHM	48.4 *	
		WM	10.4 *	
		WM * LF	17.7 *	
		WM <sup>2</sup> * LF	1.2 *	89.5 *
Equation: 1.78 - 0.542 LF + 1.2 WCHM + 0.776 WM + 0.953 WM*LF + 0.128 WM <sup>2</sup> * LF				
48-hr Exposure August 4-6, 1999	240	SPCS	22.6 *	
		WM	59.4 *	
		WM * SP	2.5	
		WM * LF	4.6 *	
		WM <sup>2</sup> * WCHM	11 *	77.9 *
Equation: 2.66 - 0.54 SPCS + 0.736 WM - 0.422 WM * SP - 0.458 WM * LF - 0.114 WM <sup>2</sup> * WCHM				
12-hr Exposure - August 10, 1999				
First year	70	TM		64.5 *
Equation: 0.415 - 0.074 TM				
Second year	26	TM		40.1 *
Equation: 0.314 - 0.052 TM				

In summary, body Na concentration when adjusted for body size, was generally inversely proportional to size and age of test subjects. Thus, relative to their body size, control hatchlings exhibited the highest body Na concentrations, whereas older larvae and metamorphs had the lowest. Acid-water exposure depressed body Na in younger, smaller larvae conspicuously; older and metamorphic animals appeared to be minimally affected by the same treatment. Most surprising was the survival of young larvae despite their depressed Na content relative to their control counterparts. The model selected confirmed that water chemistry, life stage, and body size were important terms in predicting the total body Na response to acid exposure.

#### 48-hr Exposure

High mortality in severely AMD-contaminated stream reaches reduced sample sizes considerably, especially for northern two-lined. Samples were further reduced, particularly for northern spring salamander larvae when 15 vials containing digested samples were found cracked or shattered before their analysis (PSU Water Quality Testing Laboratory). An additional 3 specimens, as noted earlier, were excluded because they were identified to be northern red salamander larvae<sup>1</sup>. This situation rendered model development at the transect or even stream level undesirable. Consequently, as in

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<sup>1</sup> There were 3 northern red salamanders: the vial containing the digested sample for one of them was among the shattered containers. Body Na concentration for the control (snout vent length (SVL) = 46.0 mm, WM = 2.58 g) and treatment (SVL = 30.0 mm; WM = 0.61 g) specimens were 4.06 and 8.93 umoles Na/WM, respectively.

the analysis for body water content, test animals surviving treatment (pH < 6.0) or control (pH > 6.0) exposures were coded "0" or "1", respectively, to reflect water chemistry.

Body Na concentration adjusted for body mass ( $\mu\text{moles Na}/\text{WM}$ ) among first year (SVL = 9-13 mm), second year (SVL = 16-25 mm), and metamorphic northern two-lined salamanders was  $45.25 \pm 4.85$  (n = 57),  $26.6 \pm 2.28$  (n = 61), and  $43.2 \pm 3.35$  (n = 20), respectively. Exposure to AMD contaminated stream water generally depressed body Na values in larvae ( $15.9 \pm 1.71$   $\mu\text{moles Na}/\text{WM}$  vs.  $34.45 \pm 2.67$   $\mu\text{moles Na}/\text{WM}$  for control salamanders; Table 3.3). Depressed body Na concentration was not as evident in metamorphs or larval northern spring salamander larvae. Medians suggested marginally lower body Na concentrations in most metamorphs ( $36.9$   $\mu\text{moles Na}/\text{WM}$  vs.  $41.2$   $\mu\text{moles Na}/\text{WM}$  for control animals) and northern spring salamander larvae ( $6.3$   $\mu\text{moles Na}/\text{WM}$  vs.  $5.7$   $\mu\text{moles Na}/\text{WM}$  for control animals).

The multivariate regression model selected for these data consisted of 5 predictors, of which 3 were interaction terms (Table 3.5, Figure 3.3, top). Wet mass (WM) and species (SPCS) explained 59.4 % and 22.6 % of the explained variation, respectively. The interaction term between wet mass and water chemistry (WCHM) explained an additional 11% of the variation. The remaining interaction terms were between wet mass and species, and wet mass and life stage (LF). The total variation explained by the model was almost 78%. Four of the 5 predictors were highly significant (p-value < 0.001).

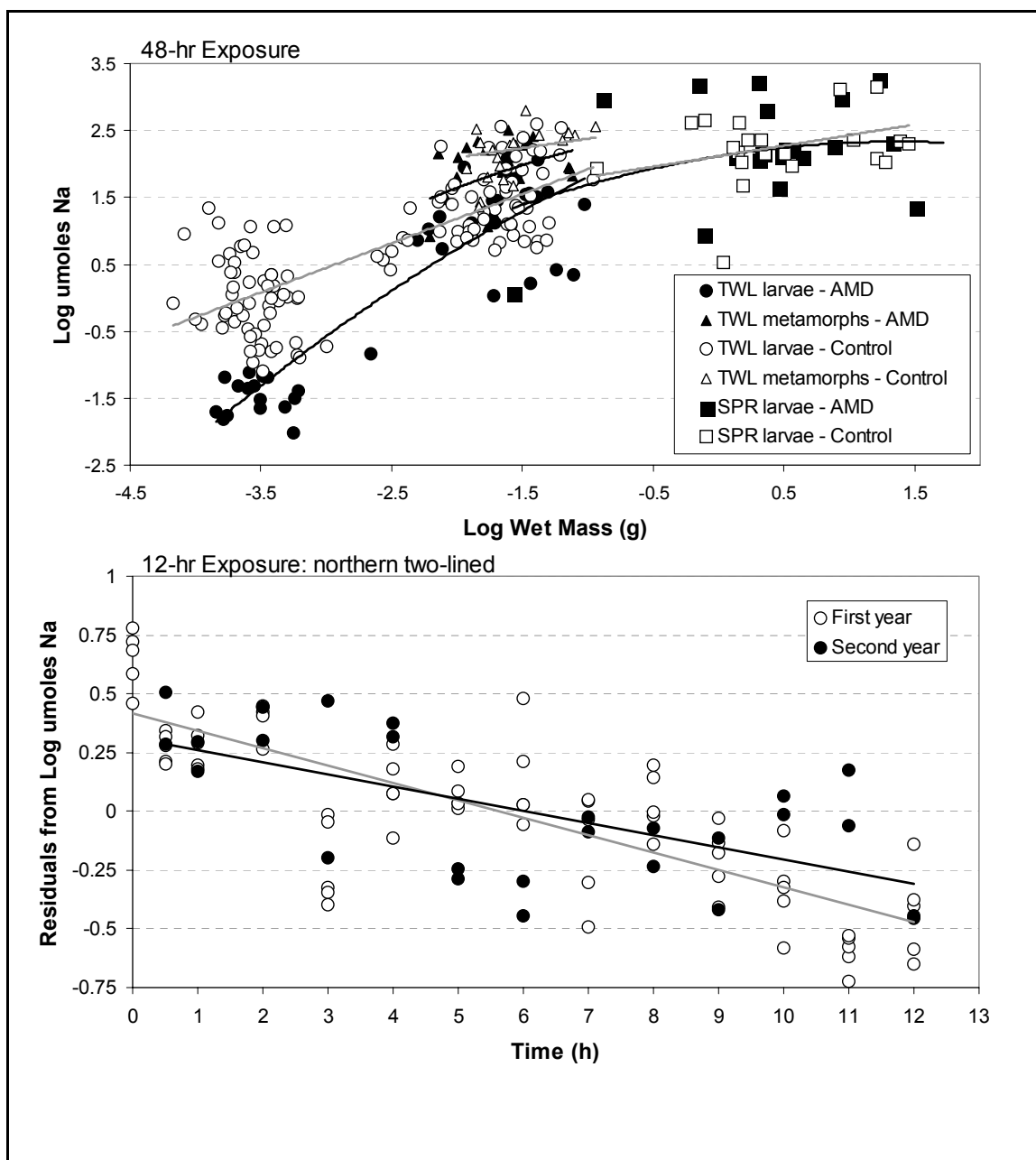


Figure 3.3. Body Na in northern two-lined (TWL) and northern spring (SPR) salamanders in 48-hr (top) and 12-hr (bottom) *in situ* exposures in Pennsylvania acid-mine drainage-contaminated (AMD) and control streams in 1999. Residuals in lower figure are from regressing body Na against dry mass for first and second year larvae. Northern spring salamander larvae were only tested in the 48-hr exposure.

Examining residuals by AMD-impacted transects among similar species and life stages revealed differences in 1, possibly 2 groups. Northern two-lined larvae from Glenwhite Run transects, pooled to increase the number of observations, produced fairly disparate, albeit non-statistically significant residuals to their counterparts exposed to Muddy Run T4 (Kruskal-Wallis  $H = 3.63$ ,  $df = 1$ ,  $p\text{-value} < 0.057$ ). Stronger, clear differences existed among northern spring salamander larvae from different stream reaches (Kruskal-Wallis  $H = 12.08$ ,  $df = 2$ ,  $p\text{-value} < 0.002$ ). In this comparison, residuals were from test animals exposed to 3 different transects that did not require pooling samples. No significant differences existed among metamorphic northern two-lined when comparing residuals from the above-mentioned transects, 2 from Glenwhite Run and 1 from Muddy Run T4 (Kruskal-Wallis  $H = 0.58$ ,  $df = 2$ ,  $p\text{-value} < 0.75$ ). These non-parametric comparisons of residuals suggested that a predictor that preserved the relative strength of the AMD-contamination at each exposure sites may have been more effective than a predictor representing water chemistry as simply acidic vs. non-acidic.

To summarize, as in the 19-d exposure, larval northern two-lined in control sites, exhibited the most variable body Na concentrations. Larval northern two-lined, relative to metamorphs and larval northern spring salamander larvae, were also the most responsive to acid water exposure. These observations were consistent with the greater mortality of test subjects in the same acid transects. The regression model confirmed the importance of body size and age, species identity, water chemistry, and life stage, predictors suspected to influence survival and the body Na response.



## 12-hr Exposure

All experimental subjects lost body Na during the 12-hr exposure period relative to laboratory or field control animals. In fact, body Na loss in both cohorts was surprisingly high. The mean body Na concentration per gram of wet mass (WM) for first year larvae on the 12<sup>th</sup> hour of exposure (6.21  $\mu$ moles Na / WM) was almost 36 % lower than the mean concentration for the 5 field control animals (17.37  $\mu$ moles Na/ WM). This amount does not take into account a difference of close to 36% between laboratory (27  $\mu$ moles Na/ ml / WM) and field control animals. In second year larvae, body Na concentration between field control (26.27  $\mu$ moles Na/ WM) and 12- hour (8.76  $\mu$ moles Na/WM) salamanders differed by 33.3 %. There were no laboratory controls for second year larvae. The above observations were not repeated measures from the same individuals, thus actual losses may have differed. Nonetheless, these results alone suggested that considerable body Na was lost during the 12-hr exposure.

For consistency, analysis of the body Na response by regression duplicated the approach used to examine body water content. Thus, residuals were computed by regressing body Na against dry mass (DM) to adjust for body mass-related effects. Models for first and second year test subjects were subsequently developed from the residuals. As suspected, the relationship between residuals and time (TM) for both cohorts remained an inverse one. A simple linear fit with time as the only predictor was sufficient to capture 64.5% and 40.1% of the total variation for first and second year test subject models, respectively (Table 3.5). While significant and capturing its general

trend, both models failed to reproduce the undulating trajectory of body Na loss over the 12-hr exposure period.

## Discussion

Survival among 19-d in situ test subjects was surprisingly high regardless of test site. Water chemistry from past chemical surveys, the grab samples and the absence of significant storm events during the test period, all suggest that levels of AMD-contamination were relatively low, certainly compared to the other acidic test sites examined in this study. Generally speaking, the outcome attests to the northern two-lined general hardiness and high survival even in sub-optimal stream reaches for a surprisingly long period of time. Higher mortality and evidence of physiologic stress, especially among first year larvae, suggested that extended survival was unlikely. The regression models revealed clear differences in the degree of physiologic stress experienced by acid-exposed relative to control subjects, especially in younger larvae. Among the latter, the difference was graphically alone, obvious. Physiological responses documented here suggest that survival in Laurel Run test transects, and similarly AMD-impacted streams, in particular for small larvae, was probably incompatible with the extended, multi-year aquatic larval period of the northern two-lined salamander.

At the same time, it is important to acknowledge that experimental salamanders were constrained by their enclosures, and thereby did not have the full-suite of behavioral options available to free-swimming larvae facing similar acidic environments. Other

organisms display a wide range of behavioral adaptations to acid exposure when free-ranging. Stream invertebrates drift following experimental acidification (Hopkins et al. 1989, Bernard et al. 1990, and Kratz et al. 1994). In small Pennsylvania streams, brook trout (*Salvelinus fontinalis*) move downstream during acidic episodes and exploit alkaline refugia (Gagen 1996). Anuran tadpoles in fluvariums avoid acidic gradients and their responses correspond to their acid-tolerance (Freda and Taylor 1992). The ventral surfaces and feet of adult toads (*Bufo*) can determine if surfaces provide “osmotically available water” (Hillyard 1999). The onset of transformation in vernal pool-breeding amphibian larvae is triggered by pond drying cues (Wilbur and Collins 1973).

Acute chemosensation, evidently advantageous to terrestrial amphibians striving to maintain water as well as osmotic balance, cannot be ruled out in aquatic larval plethodontids. Drifting, observed in *Eurycea* by several researchers (Petranka 1998), would offer the means to evade stream reaches and or episodes. Thus, the 19-d exposures may represent a highly unlikely scenario if free-ranging larvae actively avoided stressful stream reaches. Nonetheless, even if temporary survival in AMD-contaminated reaches was possible through eventual avoidance or use of alkaline refugia, lower densities or absence of larvae in AMD-contaminated sites confirmed by stream bank surveys (Chp. 2) remains tied to stream water toxicity rather than to other assemblage-shaping mechanisms i.e. predation, competition, etc. Confirming stream toxicity in streams with depressed salamander densities was the foremost purpose of conducting the *in situ* exposure tests.

Survival and physiologic responses of aquatic larvae in AMD-contaminated transects were revealed in the present study to be a function of water chemistry, age/size, and species. Among northern two-lined salamanders, survival favored older, larger animals, especially metamorphs who were the only test subjects to survive almost all acid transects, including Muddy Run T1, the most toxic of all transects. High survival of metamorphs in this study may also have resulted by their avoidance of acid water during exposures; perhaps not coincidentally, the only metamorphs succumbing in Muddy Run T1 were 3 individuals whose PVC-tube shelter was found completely submerged, depriving these salamanders escape from the acidic water.

The impact of stream toxicity on survival was fairly obvious: in second year northern two-lined larvae survival was correlated to test site toxicity and was lowest in the most toxic test site (Muddy Run T1). Similarly with respect to size and age class: within the same test site, among northern two-lined, percent survival increased with size and age. These responses were evident from the survival data.

Species type also appeared to have influenced survival. Species was a significant predictor in modeling changes in body water and body Na during the 48-hr experiment. Of the 39 northern two-lined larvae exposed to acid water, 71.8 % survived (69.5% and 75% for “small” and “large” larvae, respectively). Excluding metamorphs, the proportion for second year northern two-lined was 30% (24 survivors of 80 tested) and for first year it was 21.5% (17 live of 79 tested). With both species exposed to identical conditions,

this empirical evidence suggest that the northern spring salamander was more acid-tolerant than the northern two-lined. The above assertion is also consistent with field sampling results and anecdotal observations. The single free-ranging northern spring salamander larvae captured under a rock on August 1, 1998 was at the same location where all 10 northern spring salamander larvae survived the 48-hr exposure (Glenwhite T2) one year later, on August 1999. Amazingly, the concentration of dissolved Al alone, on that sampling day was almost 21 times higher (7.06 mg/l) than during the 48-hr *in situ* exposure (0.337 mg/l). Considering that mortality of caged brook trout results at Al concentrations in the range of 0.1 - 0.4 mg/l (Baldigo et al. 2005), and in amphibians at similar concentrations, e.g. Horne and Dunson 1994, depending on the duration of the exposure and a multitude of factors related to water chemistry, the August 1998 encounter is quite remarkable. Based on the 48-hr exposure, survival of > 50% of caged northern spring salamanders was observed at 3 AMD-contaminated test sites that ranged in water chemistry as follows: pH (3.6 - 4.2), DOC (2.38 - 3.62 mg/l) Al (0.26 - 38 mg/l), Mn (1.48 to 4.86 mg/l), Fe (0.15 - 1.43 mg/l).

Northern spring salamanders survived low pH and dissolved metals, at least temporarily, more effectively than the northern two-lined. Their larger size alone may impart advantages. The species may also be adapted physiologically to be acid-tolerant. Relative to the northern two-lined, northern spring salamanders and even the few *Pseudotriton r. ruber* tested, exhibited relatively lower body Na concentrations. The latter is notable especially because all test subjects were held under similar conditions

prior to the 48-hr exposure and water irrigating laboratory holding tanks was highly alkaline, allowing test animals to boost body Na levels if so predisposed. Yet, body Na levels varied and that life stages with the proportionally highest body Na concentrations (hatchlings and first year northern two-lined larvae) tended to be the most vulnerable to the exposures. The findings seem consistent with Freda and Dunson (1986) as they noted that total body Na concentration in vernal pool amphibians was inversely related to acid tolerance. In some vernal pool amphibians, greater acid tolerance was achieved by controlling the rate of ion loss (Freda et al. 1991), a mechanism also employed by terrestrial plethodontids held on acidic surfaces (Frisbie and Wyman, unpub. obs., cited in Freda et al. 1991). One factor not considered in these analyses, and relevant to the Freda and Dunson (1986) findings cited above, was specimen capture location and its potential effect on survival and physiologic response as a result of adaptation to local conditions. Low vs. high stream alkalinity and genetic differences in tolerance (Pierce and Wooten 1992) could also have influenced the results presented.

The physiologic responses examined in this study were mostly consistent with literature accounts of acid-exposed amphibians. Body water is lost to maintain depleting body Na at physiologic concentrations (Freda and Dunson 1985, Gonzalez and Dunson 1987). Water balance is presumably maintained by influx or efflux of water through the skin, because “ligation of the gills” does not impact water balance in any measurable way (Baldwin and Bentley 1982). Thus, unlike ion exchange, movement of water does not involve the gills. Body Na is lost to the surrounding aqueous environment when Na ions

from the body (integument, gills) are replaced by  $H^+$  ions at a rate faster than replacement, thus causing a progressive net loss in body Na. The mechanism involves the disruption of tight junctions which essentially serve as gatekeepers for Na and other body ions. Thus transport and uptake of Na are impaired and efflux exceeds influx, resulting in a net loss. The release of intracellular Na can offset the loss of its extracellular form to some extent, but death invariably ensues when animals lose approximately 50% of their total body Na (Freda and Dunson 1984). In the 19-d and 48-hr exposures, these two physiologic responses were generally positively correlated: loss of body Na resulted in loss of body water.

Survival of all northern two-lined subjects through to the 12<sup>th</sup> hour of exposure at Glenwhite T2 suggested that the time of death of their non-surviving, similar-sized counterparts following the 48-hr experiment occurred sometime between the 13<sup>th</sup> and 48<sup>th</sup> hour of exposure, assumes that water chemistry during the 12-hr exposure was comparable to that experienced during the 48-hr exposure. In either situation, dispersal even for first year larvae through fairly contaminated reaches remains plausible; it also offers an explanation for the occasional salamander larvae encounter during stream bank surveys in highly contaminated reaches. At the same time, the sharp changes in body water content and body Na, due to handling alone (differences between laboratory and field control salamanders), evidenced their sensitivity. Loss of body Na from handling-induced stress was also observed during fish *in situ* exposures (Gagen 1996).

Most unexpected in this study was the increase in body water among 12-hr exposure subjects. The analysis of residuals were intended to rule out the effect of other factors, in particular, decreasing body size among first year larvae. Why did northern two-lined exhibit an increase in body water during the 12-hr test period when their similar-sized counterparts exposed to acid water for longer periods showed the reverse, namely body water loss?

One explanation is that uptake of water may be an initial and general response to stress. Unlike marine taxa, freshwater biota are hyperosmotic (hypertonic) to their aqueous, more dilute surroundings. Mechanisms are required to resist water influx and eliminate excess. For example, the aquatic invertebrate *Gammarus* is excluded from Ca-poor waters because of this ion's role in its exoskeleton hardening and impermeability, among other functions. An increase in body water and death from osmotic stress results when ecdysis of the exoskeleton occurs in Ca-poor waters (Zehmer et al. 2002). Body Na loss is accompanied by an increase in body water in *Lepomis gibbosus*, a sunfish not as acid-tolerant as its bog-dwelling, acidic-water adapted cousin, *Enneacanthus obesus* (Gonzalez and Dunson 1987). In the latter species, survival in acid test containers was attributed to adaptations designed to "resist" body Na loss and water uptake, tolerance to low body Na and its swift uptake during favorable conditions. Aquatic amphibians face similar challenges: osmoregulation must resist the flooding of body fluids that would otherwise lead to their dilution and volume increase (Boutilier et al. 1992). In this study, the onset of increased body water content did not require exposure to low pH; stress



associated with removal from laboratory tanks and transport to test sites, was sufficient to trigger this initial response.

Salamanders were not gradually acclimated to test site conditions. Hence, survival and physiological responses pertinent to the 12-hr and perhaps even the 48-hr exposures may not be fully representative of free-swimming salamander survival and physiologic response under similar conditions when given the opportunity to gradually acclimate or adapt to their surroundings. McDonald and Milligan (1988) and Simonin et al (1993) reported reduced mortality of caged brook trout and blacknose dace (*Rhinichthys atratulus*) by acclimation prior to exposure to episodically acidified streams. In the absence of acclimation, hourly removal of control salamanders may have served as a continuous baseline, i.e., responses due to stress from displacement *vs.* responses of stress from displacement and acid exposure. Changes in body water and body Na between laboratory and field control salamanders in the 12-hr exposure confirmed the effect of displacement and handling. Whilst salamander performance may have differed somewhat when allowed to acclimate, results from the *in situ* exposures provide strong evidence that exclusion of stream salamanders from acidic, AMD-contaminated streams is likely to stem largely from physiologic stress and toxicity.

In summary, low pH and AMD-contamination even in seemingly low concentrations impairs survival by inducing physiologic stress in aquatic stream salamanders with prolonged aquatic life stages of the northern two-lined and northern

spring salamander. Stream water toxicity, size and age, life stage, and species identity were determinants of survival and physiologic stress in such environments. The above findings do not rule out the presence of other stream salamander excluding mechanisms. For example, the growth-inhibitory effect of low pH described in Freda and Dunson (1985) may lead to a similar, albeit prolonged outcome by virtue of its deleterious effect on larval growth and size at transformation, attributes deemed crucial to individual and population fitness.

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Chapter 4

SURVIVAL OF NORTHERN DUSKY AND MOUNTAIN DUSKY  
SALAMANDER HATCHLINGS IN LABORATORY-SIMULATED  
ADVERSE ENVIRONMENTS

## Abstract

The northern dusky (*Desmognathus f. fuscus*) and mountain dusky (*D. ochrophaeus*) are common stream salamanders in the northern Appalachians. Both are terrestrial nesters and have similar life histories. In acidic streams, the northern dusky was observed to decline, whereas the mountain dusky persisted. Absence of an aquatic larval stage, as reported in the early literature, was hypothesized to be the reason for the latter's greater survival in acidic streams. Survival in hatchlings of both species was examined in the laboratory by exposure to treatments simulating acidic, dry, and normal nesting environments. Hatchlings were collected in nests from southwestern and north central Pennsylvania. Early transformation was observed in only 2 (<1%) of the original 302 northern dusky hatchlings collected and before the experiment began. In treatment containers, none of the 207 test subjects transformed and only 60 (29%) survived. Survival was lowest in acidic and dry treatments, smaller hatchlings, and among the more northern collection sites. An unidentified ciliate was observed in some hatchling containers. A single mountain dusky nest was found; all 10 hatchlings from this nest transformed within weeks of hatching and survived the 51-d exposure irrespective of treatment. The unidentified ciliate was not observed in mountain dusky containers. Based on these findings, survival of the mountain dusky in acidic streams is attributed to early transformation.

## Introduction

The northern dusky (*Desmognathus f. fuscus*) and mountain dusky (*D. ochrophaeus*) salamanders are common plethodontid assemblage members of northern Appalachian cool forest streams. The northern dusky is larger and more aquatic than the mountain dusky. The muscular, laterally compressed tail and dorsal keel of the former is a notable contrast to the rounded, mouse-like tail of the latter and a hint to their starkly different habits. Both species are terrestrial egg-layers. Oviposition takes place under cover in moist saturated soils commonly associated with seeps and slope wetlands. Nests are guarded by females for the length of the incubation period (6-8 wk). After a brief period in the nest, hatchlings move to water to continue their development as aquatic larvae.

The aquatic larval period in the northern dusky is shorter (2-3 mo) for late winter/early spring hatchlings than for late summer hatchlings (4-8 mo), presumably because overwintering delays metamorphosis (Petranka 1998). In the mountain dusky, the aquatic larval period appears variable among populations. Hatchlings may transform in 2-3 wk or require up to 8 mo (Petranka 1998, Tilley 1973). As with the northern dusky, the month of oviposition appears to affect the time to transformation (Petranka 1998). External factors related to stream environment may also affect their larval period. Rapid gill loss and early transformation by hatchlings held in damp rather than inundated substrates is reported for both taxa (Noble and Evans 1932, Bishop and Chrisp 1933, Bishop 1941).



Kucken et al. (1994) describe the impact of pyritic rocks in the construction of a highway stream crossing on a stream plethodontid assemblage in the southern Appalachians. Of the original assemblage comprising aquatic and terrestrial species, as a result of the highly acidic water, only the pygmy salamander (*D. wrightii*), a small, highly terrestrial desmognathine, and several woodland species (*Plethodon spp.*) populated reaches contaminated by highly acidic water. The field investigation described in Chapter 2 confirmed similar findings: stream plethodontids were affected by acidified streams in a manner mostly consistent with their life history patterns: the abundance and distribution of the mountain dusky was minimally affected in acidic streams, whereas other taxa, including its more aquatic congener, the northern dusky, declined or were absent. Both studies suggest that life history, the degree of dependence on the aquatic portion of their environment, affected survival in acidified streams.

If the mountain dusky and northern dusky are both terrestrial nesters and presumably can bypass the aquatic life stage, why did the northern dusky decline in acidic streams? Do these taxa differ in life history in the region investigated? If total independence from stream water is the primary mechanism by which survival in acidic streams is possible for stream salamanders, I hypothesized that the mountain dusky transformed without the need for an aquatic larval stage, whereas the northern dusky differed in this respect and as a result, survived poorly in acidic environments.

The investigation sought to resolve several questions: Does early transformation exist in these taxa? Are environmental stressors associated with early transformation when it occurs? In the absence of early transformation, is an extended aquatic larval stage (the default) compatible with acidic environments? Conversely, does early transformation favor survival in similar environments?

Observing mountain dusky and northern dusky hatchlings in normal and acidic laboratory settings may have sufficed to test the above hypothesis. However, literature accounts suggest that early transformation in both taxa was triggered by damp rather than inundated nesting surroundings. That possibility was addressed by exposing hatchlings to artificial environments simulating normal (control), chronically acidic, and dry nesting sites. Simulation of dry nesting environments was intended to replicate conditions that led to the original reports of early transformation.

## Methods

### Collection Sites

Hatchlings for the laboratory experiment were obtained from wild nests. From September 5 - 12, 1998, eggs and hatchlings were collected from 20 different locations in central and southwestern Pennsylvania (Appendix 4.1). Camp Run and Roaring Run are in the Youghiogheny River sub-basin. Wildcat Run is in the Kiskiminetas River sub-basin. All three streams are in the Ohio River drainage basin and are high quality, non-acidic watersheds (Lighty et al.1995). Muddy Run and Benner Run flow to the West

Branch Susquehanna and are part of the Susquehanna River drainage basin (Figure 4.1). Acid-mine drainage (AMD) has severely impacted Muddy Run (Chapter 2); the upper reaches of Benner Run drain forest lands and may experience temporary acidification (B. Hollender, pers. comm. 1999). All northern dusky nesting sites were associated with seeps or seepages. Nests were found on rocks (length 24-62 cm, mean = 41 cm), rotting logs, and in non-submerged muck not far from shallow (<10 cm), flowing ground-water. The nearest distance to water for the 20 nesting sites ranged from 7 - 225 cm (mean = 53 cm). Nest height from the water's surface ranged from 5 - 30 cm (mean = 12 cm). Nests consisted of shallow cavities in soil, muck or the combination of live (moss) and decaying plant matter. An attending female was observed at most nests. Despite considerable searching, only one mountain dusky nest was found. The clutch, containing 10 eggs and attended by an adult, was collected from a seep wetland flowing to Muddy Run, Cambria County.

#### Laboratory Incubation

Eggs were incubated in the laboratory at room temperature. Eggs and hatchlings from different clutches and streams were held in separate sealed plastic bags and stored in a vacant classroom, Forest Resources Laboratory, University Park, Pennsylvania, until all eggs hatched. Only one northern dusky clutch failed to hatch; all other eggs hatched by September 17. On September 18 and 19, northern dusky hatchlings (N = 302) were weighed to the nearest 0.1 mg and individually transferred to covered 55-ml plastic containers and labeled. The snout-vent length (SVL) of hatchlings were not measured to

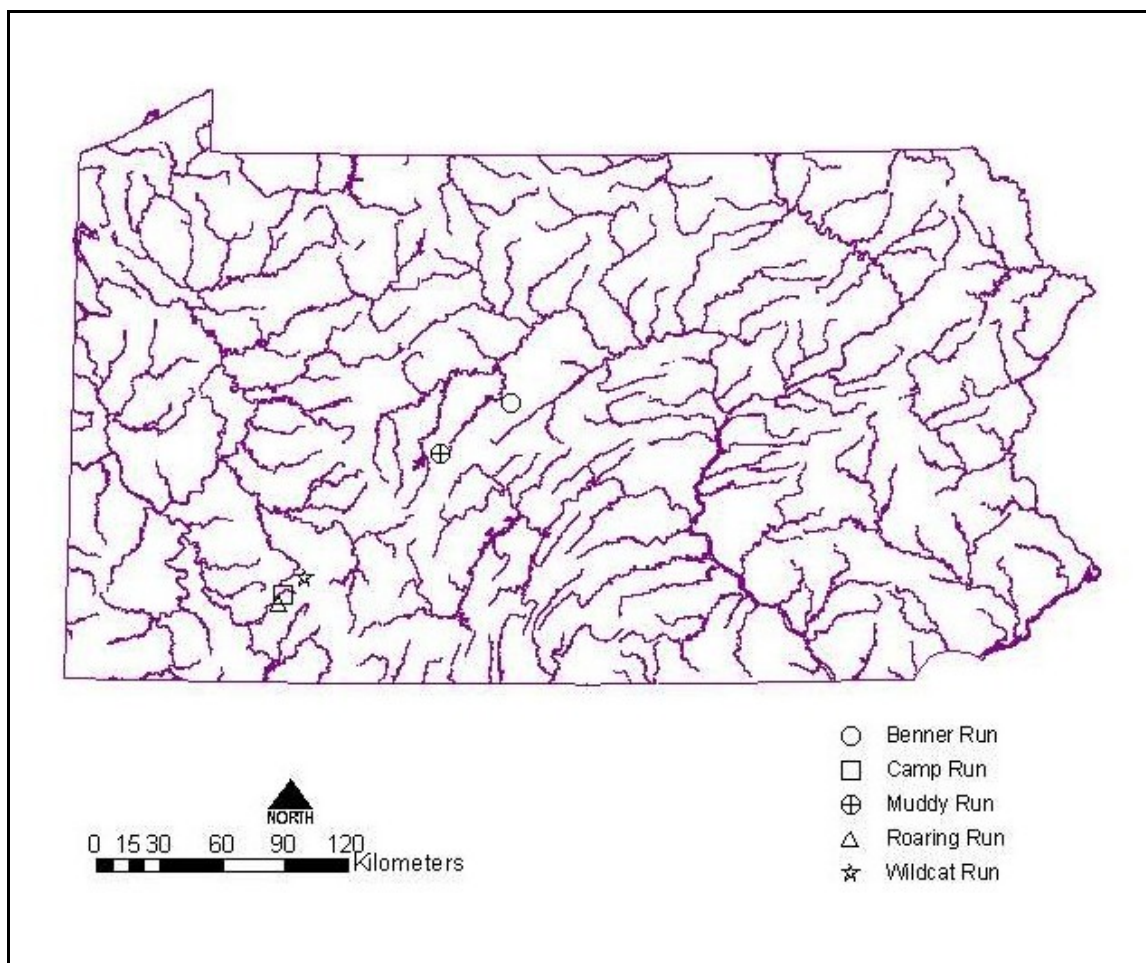


Figure 4.1. Stream locations in Pennsylvania where northern dusky and mountain dusky salamander eggs were collected in September for the 51-d laboratory exposure of hatchlings on October 30-December 20, 1998.

avoid injury to yolk plugs. Unbleached paper towels (120 mm<sup>2</sup>) were hand-crumpled, moistened, and added to containers for cover and moisture. On October 23, a portion of the animals were lost to high room temperature; approximately 220 survived. On October 28, examination of northern dusky hatchlings revealed the absence of gills on two Camp Run specimens; these two individuals were removed from the experiment.

Mountain dusky eggs began hatching on September 17. All 10 eggs hatched successfully shortly thereafter. Gut yolk grossly distended the abdomen of all hatchlings, hence no effort was made to process or transfer hatchlings to individual containers. On October 23, gills could not be observed on some of the specimens. By October 29 the large gut yolks present at hatching had diminished noticeably to allow transfer to individual containers.

On October 30, 207 surviving northern dusky and all 10 mountain dusky hatchlings were assigned to one of three container treatments: normal, acidic, and dry. Hatchlings were assigned to treatments systematically, one clutch at a time. The process assigned 69 northern dusky and 3-4 mountain dusky hatchlings per treatment.

#### Construction of Treatment Containers

Hatchlings were individually housed in 55-ml plastic cups with perforated lids containing platforms and crumpled paper (Figure 4.2). The raised platforms and crumpled paper, intended to simulate the terrestrial bank, provided cover and moisture. Depending

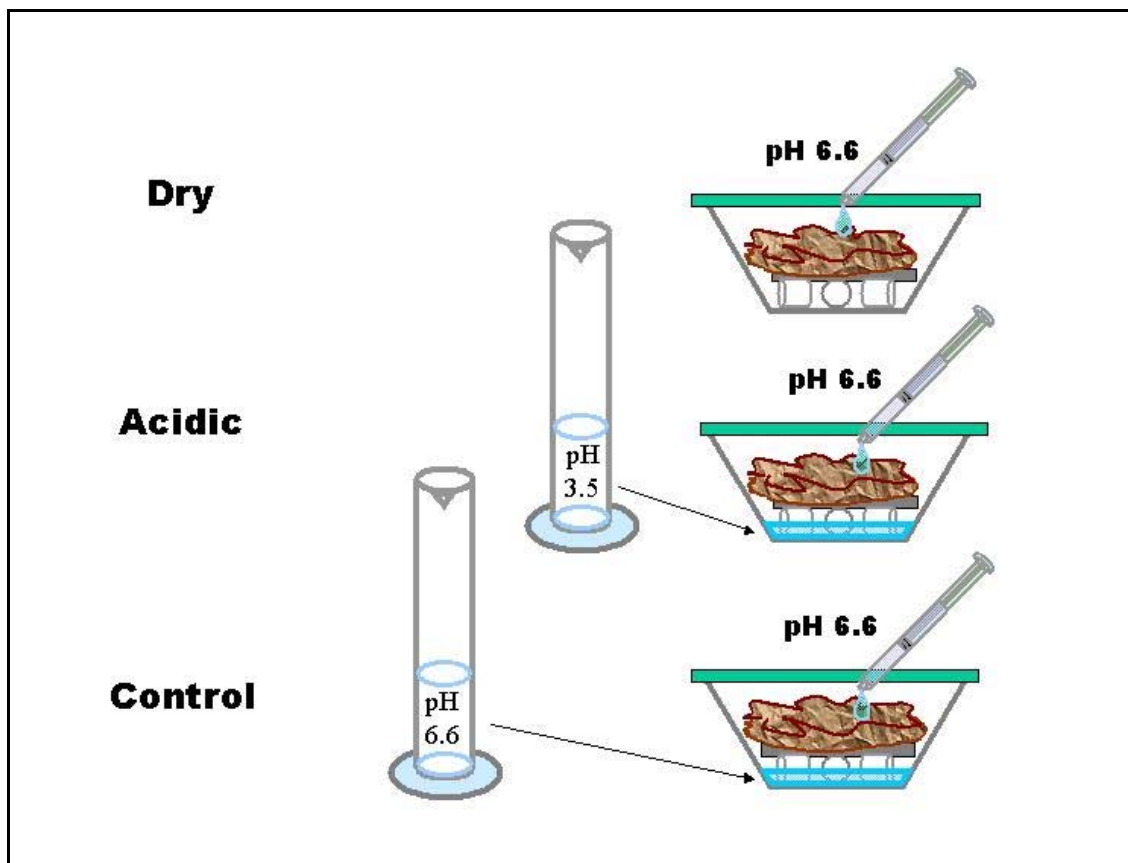


Figure 4.2. Schematic showing components of the three container treatments simulating dry, acidic, and normal (control) nesting environments during the 52-d laboratory exposure of northern dusky and mountain dusky salamander hatchlings on October 30-December 20, 1998. Crumpled, unbleached paper towels were moistened with Bear Spring<sup>®</sup> water in all containers. Only control and acidic containers provided a shallow pool. Bear Spring<sup>®</sup> water was acidified to pH 3.5 in acidic containers.

on the treatment, elevated platforms served to physically separate the crumpled paper from solutions on the bottom of the container. The octagonally-shaped platforms were 40 mm in length and constructed from 2 mm polypropylene sheets. Platform pedestals were made from three strips (5-7 mm) of aquarium airline tubing (10 mm dia.); the latter were hot-glued longitudinally to the bottom of platforms. The height of the assembled platforms from the cup bottom was 12-14 mm. The crumpled paper consisted of 120-mm squares (0.6 g). The above components were assembled by laying platforms in the cups, airline tubing side down, and positioning the crumpled paper atop the platform. The crumpled paper was subsequently moistened with bottled drinking water (Great Bear Natural Spring Water<sup>®</sup>: pH = 6.6; bicarbonate = 5.5 mg/l; Chloride = 1.4 mg/l; Fluoride = ND; Sulfate = 5.3 mg/l; Calcium = 1.3 mg/l; Magnesium = 1.0 mg/l; Potassium = 0.7 mg/l; Na = 1.7 mg/l), hand-squeezed to remove excess water and repositioned if any portion touched the bottom of the container. Control and acidic treatment containers received 9 ml of the bottled spring water. This volume created a 3 - 4 mm deep pool in the cups that was well below the height of the platform or the crumpled paper. Water added to acid treatment containers was previously acidified to pH 3.5 with dilute HSO<sub>4</sub>. The target pH was measured electrometrically. No water was added to containers in dry treatments; specimens in these cups depended on the damp paper for moisture.

Individual containers were color-coded, grouped by simulated stream environment and housed in opaque plastic boxes (350 mm x 250 mm x 120 mm) with lids. For the northern dusky, there were 4 boxes with 15 containers, and 2 boxes with 4 or 5 containers

per simulated hatchling environment (18 boxes; 207 hatchlings total). All 10 mountain dusky containers were placed in a single box. Boxes were stored on shelves in a climate-controlled room (21.6 - 22.2°C; 45-55% relative humidity, 12:12 photoperiod by fluorescent lighting). Containers were attended, cleaned and water replaced, at least once weekly. All hatchlings were fed *ad libitum* laboratory cultured springtails (*Folsonia*). Hatchlings were examined under a dissecting scope without removing them from their container. The experiment began on October 30 and was terminated on December 20 (51 days). At the conclusion of the experiment, surviving hatchlings were immediately frozen and subsequently measured (SVL) with a millimeter marked rule and weighed to the nearest 0.1 mg.

### Behavior

The location of hatchlings in their containers during the 51-d exposure was recorded as one of three places: on the container bottom or underside of the platform (BTM), on moist paper mat above platform (MAT), or on cup wall or lid (WLL). Hatchling locations were noted whenever test specimens containers were serviced, an activity that depended on the schedule of attending technicians.

### Analyses

Hatchling survival and related responses were examined by reporting measures of central tendency, conducting parametric tests (ANOVA, Chi-square), and by logistic regression. The relationship between survival and treatment containers, collection



site(streams), and initial hatchling weight were examined further by binary logistic regression, an effective tool for the analysis of binary responses, such as survival. Binary logistic regression does not require the exact time of death to be known or normally distributed data. Logistic regression allowed the investigation of continuous (covariate) and categorical factors. Simultaneous examination of more than 1 factor (treatment container and stream) was not possible because too few observations existed.

In consideration of these limitations, only two models were tested: Model 1 examined survival in response to the 3 container treatments; Model 2 examined survival by the 5 streams, the nest collecting sites. Arguably, Model 2 contributed minimally to the scope of the present study. Confirming population differences in the same species, after accounting for body mass, would provide evidence that survival was also governed by genetic/population differences. In both models, initial hatchling body mass served as the covariate. The response variable was survival to the 51-day exposure. Non-survivors were coded 0, survivors 1. Model goodness-of-fit was evaluated by the Pearson, Deviance, and Homer-Lemeshow tests. Somers' D, Goodman-Kruskal Gamma, and Kendall's Tau-a evaluated measures of association.

## Results

All mountain dusky hatchlings transformed prior to the start of the experiment. Only two (< 1%) of the originally hatched 302 northern dusky hatchlings lost their gills. As with the mountain dusky, transformation of these two individuals occurred before the

start of the experiment, when specimens were held in cups containing only moistened paper. Evidently, transformation within weeks of hatching and in the absence of an aquatic environment was within reach of both taxa. However, unlike in the mountain dusky, only a fraction of northern dusky observed in the present study exhibited early transformation.

Initial mean weight for northern dusky hatchlings ranged from 28.7 mg  $\pm$  0.5 for Wildcat Run to 36.7 mg  $\pm$  0.3 for Benner Run (mean = 32.2 mg  $\pm$  0.3; Table 4.1). Initial mean weight for hatchlings assigned to normal (32.3 mg), acidic (32.0 mg), and dry (32.4 mg) containers was not significant ( $F = 0.19$ ,  $DF = 2$ ,  $p\text{-value} = 0.831$ ), indicating no bias in hatchling weight at the beginning of the experiment.

Sixty (28.9 %) of the 207 northern dusky and all mountain dusky hatchlings survived the 51-day experiment (Table 4.2). Survival in northern dusky hatchlings by container type differed significantly ( $\chi^2 = 27.6$ ,  $DF = 2$ ,  $p\text{-value} < 0.001$ ), consisting of 36 (52.2 %) in normal, 10 (14.5%) in acidic, and 14 (20.3%) in dry containers.

Survival varied by stream in the northern dusky, ranging from 11.5 % for Muddy Run to 50.8 % for Roaring Run (mean 28.9 % for all watersheds). The allocation of northern dusky hatchlings to treatment containers was not biased by nest location; between 4 to 22 hatchlings (mean 13.8) from any stream were assigned to the same container treatment ( $\chi^2 = 1.784$ ,  $DF = 8$ ,  $p\text{-value} = 0.987$ ).

Table 4.1. Source and initial weight (Mean  $\pm$  1SE) of northern dusky salamander hatchlings that participated in the 51-d laboratory exposure on October 30 - December 20, 1998, by container treatment. Values in parentheses are number of individuals per clutch(s). Initial weight (mg) was measured on September 18.

Watershed Name County Sub-basin Basin (Hatchlings/clutch)	Container Treatments Individuals; Mean Initial Weight $\pm$ 1 SE			
	Control	Acid	Dry	Totals and Grand Mean
Benner Run, Centre Co. Clearfield R., W.B. Susquehanna R. (20)	5 35.7 $\pm$ 0.9	7 37.6 $\pm$ 0.4	8 36.4 $\pm$ 0.3	20 36.7 $\pm$ 0.3
Muddy Run, Cambria Co. Clearfield R, W. B. Susquehanna R. (8,20,24)	19 32.0 $\pm$ 0.6	15 31.2 $\pm$ 0.7	18 31.5 $\pm$ 0.7	52 31.6 $\pm$ 0.4
Camp Run, Westmoreland Co. Youghioghenny R. Ohio R. (2,12)	5 32.4 $\pm$ 1.6	4 32.4 $\pm$ 0.9	5 31.7 $\pm$ 1.6	14 32.2 $\pm$ 0.8
Roaring Run, Westmoreland Co. Youghioghenny R. Ohio R. (6,18,5,28)	18 35.1 $\pm$ 0.7	21 35.2 $\pm$ 0.7	18 35.3 $\pm$ 0.7	57 35.2 $\pm$ 0.4
Wildcat Run, Westmoreland Co. Youghioghenny R., Ohio R. (15,3,11,10,9,16)	22 29.4 $\pm$ 0.7	22 27.5 $\pm$ 0.7	20 29.2 $\pm$ 0.9	64 28.7 $\pm$ 0.5
Total	69 32.3 $\pm$ 0.5	69 32.0 $\pm$ 0.6	69 32.4 $\pm$ 0.5	207 32.2 $\pm$ 0.3

Table 4.2. Survival (%) of northern dusky and mountain dusky salamander hatchlings following the 51-d laboratory exposure on October 30 - December 20, 1998, by container treatment. Initial Weight was measured on September 18. Hatchlings found alive on December 20 were frozen and subsequently weighed to obtain survivor End Weight and Percent Weight Lost. Mean  $\pm$  1SE are shown where applicable. Weight in milligrams (mg).

	Container Treatments			Totals
	Normal	Acid	Dry	
Northern dusky				
<b>Survivors</b>				
No. of individuals	36 (52.2%)	10 (14.5%)	14 (20.3%)	60 (29%)
Benner Run	4	1	1	6 (30.0%)
Muddy Run	6	0	0	6 (11.5%)
Camp Run	3	1	0	4 (28.6%)
Roaring Run	13	7	9	29 (50.8%)
Wildcat Run	10	1	4	15 (23.4%)
Initial weight	34.0 $\pm$ 0.5	37.3 $\pm$ 1.0	36.0 $\pm$ 0.7	
End weight	25.9 $\pm$ 0.6	27.07 $\pm$ 0.8	26.55 $\pm$ 0.9	
Percent weight lost	23.9% $\pm$ 1.4	27.2% $\pm$ 2.2	26.3% $\pm$ 1.6	
<b>Non-survivors</b>				
No. of individuals	33 (47.8%)	59 (85.5%)	55 (79.7%)	147 (71 %)
Initial weight	30.4 $\pm$ 0.7	31.1 $\pm$ 0.6	31.5 $\pm$ 0.5	
Mountain dusky				
<b>Survivors</b>				
No. of individuals	3 (100%)	4 (100%)	3 (100%)	10 (100%)
Muddy Run	3	4	3	10 (100%)
End weight	20.6 $\pm$ 0.9	18.8 $\pm$ 0.6	19.7 $\pm$ 0.6	

The mean initial weight of northern dusky survivors was 34 mg in normal, 36 mg in dry, and 37.3 mg in acidic containers. These figures are 11.7%, 14.3%, and 20.1% greater than the mean initial mass of their non-surviving counterparts. Thus, regardless of container treatment, survival favored heavier hatchlings and survivors in normal, dry, and acidic treatment containers lost an average of 23.9%, 26.3%, and 27.2% of their initial body mass, respectively. Parallel conclusions were reached with comparisons based on the length of survivors at the end of the experiment. Mean SVL of survivors in dry (12.5 mm) and acidic (12.6 mm) containers was on average 0.5 - 0.6 mm longer than for survivors in the control (12.1 mm). An ANOVA on SVL by these 3 groups, followed by Tukey's pairwise comparison ( $\alpha = 0.05$ ), revealed a significant difference ( $F = 6.31$ ,  $DF = 60$ ,  $p\text{-value} < 0.003$ ) among all treatments with only the control differing from the other two treatments. Hence, survival in acidic and dry containers favored heavier, longer hatchlings.

### Binary Logistic Regression

The logit-link function provided a good fit for the data and initial body weight was a significant predictor in both models. Hatchlings with an initial body weight one mg greater than another, had an approximately 1.5 times greater chance of surviving (Tables 4.3 and 4.4). In Model 1, container treatment was a significant predictor ( $\chi^2 = 29.247$ ,  $DF = 2$ ,  $p\text{-value} = 0.0001$ ). After accounting for the effect of initial body mass, hatchlings in control containers were 13.5 times more likely to survive than hatchlings exposed to acidic containers, the treatment with the lowest odds of survival. The odds of surviving

Table 4.3. Binary logistic regression results relating northern dusky hatchling survival by initial body weight and container treatment (Model 1) following the 51-d laboratory exposure on October 30 - December 20, 1998.

Variable	Coefficient	SE Coef	Z	P- value	Odds Ratio	95% CI	
						Lower	Upper
Constant	-14.525	2.351	-6.18	0.000			
Weight	0.372	0.065	5.75	0.000	1.45	1.28	1.65
Treatment							
Control	2.607	0.533	4.89	0.000	13.55	4.77	38.51
Dry	0.531	0.519	1.02	0.306	1.7	0.62	4.7
<b>Tests for terms DF &gt; 1</b>		<b>Statistic</b>		<b>DF</b>	<b>P-value</b>		
Chi-square		29.247		2	< 0.0001		
Log-Likelihood		-85.499		-	-		
Test for slopes = 0		78.238		3	< 0.0001		
<b>Measures of Association (Between Response Variable and Predicted Probabilities)</b>							
<b>Pairs</b>	<b>Number</b>	<b>Percent</b>	<b>Summary Measures</b>				
Concordant	7598	86.1%	Somers'D		0.73		
Discordant	1199	13.6%	Goodman-Kruskal Gamma		0.73		
Ties	23	0.3%	Kendall's Tau-a		0.30		
Total	8820	100.0%					

Table 4.4. Binary logistic regression results relating northern dusky hatchling survival by initial body weight and nest collection sites (Model 2) following the 51-d laboratory exposure on October 30 - December 20, 1998.

Variable	Coefficient	SE Coef	Z	P- value	Odds Ratio	95% CI	
						Lower	Upper
Constant	-15.068	2.812	-5.36	0.000			
Weight	0.3864	0.075	5.16	0.000	1.47	1.27	1.7
Watershed							
Camp	1.474	0.844	1.75	0.081	4.36	0.83	22.84
Muddy	0.349	0.731	0.48	0.633	1.42	0.34	5.94
Roaring	1.497	0.597	2.51	0.012	4.47	1.39	14.38
Wildcat	2.375	0.769	3.09	0.002	10.75	2.38	48.55
<b>Tests for terms DF &gt; 1</b>			<b>Statistic</b>		<b>DF</b>	<b>P-value</b>	
Chi-square			15.310		4	< 0.004	
Log-Likelihood			-94.793		-	-	
Test for slopes = 0			59.652		5	< 0.0001	
<b>Measures of Association (Between Response Variable and Predicted Probabilities)</b>							
<b>Pairs</b>	<b>Number</b>	<b>Percent</b>	<b>Summary Measures</b>				
Concordant	7151	81.1%	Somers'D				0.63
Discordant	1634	18.5%	Goodman-Kruskal Gamma				0.63
Ties	35	0.4%	Kendall's Tau-a				0.26
Total	8820	100.0%					

in dry, relative to acidic containers was 1.7 times greater. This difference was not significant (p-value = 0.306). Of the simulated pairs tested, most were concordant and the model had > 70% chance of predicting responses correctly. In Model 1, hatchlings in dry or acidic containers with a probability of survival greater than 50% had an average initial body mass of approximately 37 mg. In control hatchlings, a probability of survival greater than 50% was observed in smaller hatchlings, with an average initial body weight of approximately 32.5 mg. These results were consistent with earlier comparisons revealing that survivors from dry or acidic containers were larger than survivors from the controls. Model 1 also established that survival favored hatchlings in control containers, relative to other treatments, after accounting for the covariate, initial body weight.

Stream was a significant predictor in Model 2 ( $\chi^2 = 15.31$ , DF = 4, p-value = 0.004). After accounting for the effect of initial body weight, hatchlings from Muddy Run, Camp Run, Roaring Run, and Wildcat Run were 1.4, 4.4, 4.5 and 10.7 times, respectively, more likely to survive than hatchlings from Benner Run, the watershed with the lowest odds of survival. However, at alpha = 0.05, only hatchlings from Roaring Run and Wildcat Run had survival odds significantly greater than hatchlings from Benner Run. Based on the initial group of 302 hatchlings, differences in size between Ohio River basin (32.0 mg  $\pm$  0.3) and West Branch Susquehanna river basin (31.5 mg  $\pm$  0.4) hatchlings were not significant (T = 0.96, DF = 225, p-value = 0.168). Model 2 predicted responses correctly 63% of the time.



In summary, the logistic models revealed that survival favored heavier, larger northern dusky hatchlings, hatchlings from control containers and from southernmost collection sites (Ohio River basin). Alternatively, acidic treatment and hatchlings from northern streams (Susquehanna River basin) fared the worst. The effect of treatment container and stream was significant despite the initial hatchling body weight effect.

### Behavior

The location of the 207 live northern dusky hatchlings in treatment containers was recorded on 1,567 occasions during the 51-day study period (Table 4.5). Fewer observations were recorded from acidic or dry container hatchlings because survival was highest in control environments. Across all treatments, hatchlings were observed mostly on the cup bottom or underside of the platform (56.1%). Relative to the control, hatchlings in dry and acidic containers were observed less frequently on the cup bottom, more frequently on the towel, and at comparable frequencies at all other locations. It is unlikely that the observed ratios were equal ( $\chi^2 = 76.43$ ,  $DF = 4$ ,  $p\text{-value} < 0.0001$ ).

There was no relationship between the location of dead hatchlings and treatment container ( $\chi^2 = 1.36$ ,  $DF = 2$ ,  $p\text{-value} = 0.506$ ; location MAT and WLL were combined to allow analysis), however, the proportion of dead hatchlings on the cup bottom (BTM) coarsely mimicked the locations of live hatchlings (i.e., use of the cup bottom was greatest in control animals, but decreased in the other two environments). The similarity

Table 4.5. Frequency of observation for live and dead northern dusky and mountain dusky hatchlings by container treatments during the 51-d laboratory exposure on October 30 - December 20, 1998. BTM = hatchling on cup bottom or under platform, MAT = hatchling on or in contact with towel, WLL = hatchling on cup wall or cup cover.

Location in Containers	Container Treatments			Total
	Control	Acid	Dry	
Live northern dusky (207 hatchlings)				
BTM	430 (66%)	176 (41.5%)	273 (55.6%)	879
MAT	77 (11.8%)	127 (30%)	108 (22%)	312
WLL	145 (22.2%)	121 (28.5 %)	110 (22.4%)	376
Total	652	424	491	1567
Dead northern dusky (125 hatchlings)				
BTM	16 (51.6%)	19 (38.8%)	21 (46.7%)	56
MAT+WLL	15 (48.4%)	30 (61.2%)	24 (53.3%)	69
Total	31	49	45	125
Live mountain dusky (10 hatchlings)				
BTM	0 (0%)	3 (7.5%)	1 (3.3%)	4
MAT	19 (67.9%)	17 (42.5%)	23 (76.7%)	59
WLL	9 (32.1%)	20 (50%)	6 (20%)	35
Total	28	40	30	98

in location between dead and live hatchlings suggests that handling of containers had minimal effect on live hatchling location at the time of the observation.

In summary, for the northern dusky in control containers, hatchlings were making frequent use of the cup bottom. The cup bottom in controls was the most favorable environment for hatchlings. In containers where the cup bottom was less hospitable (i.e., acidic, dry), hatchlings were observed in the less stressful environment, the moist paper imbibed with non-acidic water.

Mountain dusky hatchlings were observed on only 98 occasions. Of these, only 4 placed the individual on the cup bottom (4%). On all other occasions, hatchling were observed on the moist paper towel or on the cup wall or lid. These proportions were relatively comparable across treatments, especially considering that 4 hatchlings, rather than 3, were assigned to the acidic treatment. In stark contrast to the northern dusky, the mountain dusky was rarely sighted on the cup bottom with or without water.

#### Feeding and Gut Yolk Absorption

Yolk absorption was not monitored quantitatively during the study. Based on notes on the condition of each individual animal, it was evident that northern dusky had depleted its yolk reserves well before the mountain dusky. Many of the former were noted as lacking gut yolk and to be emaciated at the start of the experiment (October 30); hatchlings originating from Muddy Run in particular. Depletion of gut yolk in the

mountain dusky was noted on November 11 and 18, dates also roughly coinciding with the first observed feedings (November 11, 18, and 27). A confirmed feeding occurred when hatchlings were observed snatching collembolan prey. The emaciated appearance of most hatchlings throughout the experimental period coupled with the presence of live or dead prey suggests that the northern dusky was either not feeding or unable to capture prey regularly.

#### Ciliate Infestation

On November 6, I observed numerous tiny, oval, dark organisms moving through the water column in one of the northern dusky containers. Similar sightings were reported subsequently by other technicians tending hatchlings. Examination by light microscope revealed the organism was an undetermined ciliated protozoan, possibly *Balantidium* spp. or *Colpidium* spp. (Rheichenbach -Klinke and Elkan, 1965). Since a definitive identification was not possible, it is unclear whether these organisms were saprophytic or parasitic. By December 20, the ciliate had been observed in 29 live and dead northern dusky hatchlings. Furthermore, 3 of these hatchlings were not part of the study colony and were being held in a separate box in the same room. These “surplus” animals (n = 4) were maintained in control conditions. Overall, the protozoan was found in 13.7 % of all 211 monitored individuals and in 14 of the 19 boxes (18 study boxes + 1 surplus box). The number of known infected hatchlings per box ranged from 1-7. The protozoan was observed in all treatment boxes, but less frequently in the acidic ones; of the 29 infected

individuals, only 5 (17 %) were in acidic treatment boxes; the remaining 13 (45 %) and 11 (38 %) individuals, respectively, were in dry and control treatment boxes.

Infected hatchlings originated from 4 of the 5 collection streams and from 9 (52 %) of the 17 nesting locations/clutches. The ciliate was not observed in any of the 14 Camp Run hatchlings (2 clutches). Hatchlings from 2 Muddy Run clutches (Cambria Co.) and from 5 Wildcat Run clutches (Westmoreland Co.) accounted for 86% of all protozoan sightings. The other infected hatchlings, 2 per stream location, were from Benner Run (Centre Co.) and Roaring Run (Westmoreland Co.). Cross-contamination cannot be excluded; 9 of the 14 infected boxes had at least 1 Camp Run hatchling container. Boxes 14, 11, and 8 had 7, 4, and 2 infected individuals, respectively. Thus, even though the opportunity for contamination in Camp Run containers appears substantial, none were recorded as infected. The proportion of infected individuals in clutches with at least 1 infected hatchling ranged from 7 - 33 %. The highest proportion of infection (20 % - 33 %) was observed in Muddy Run and in 2 of the 5 Wildcat Run clutches. Of the 29 infected hatchlings, 15 (52 %) were alive at time of detection, the others were dead. Among the latter, ciliate density was typically high and often occupied the carcass in large numbers.

In summary, the impact of the ciliate on the survival of hatchlings remains unclear. However, acidic treatments may have provided unexpected benefits to infected animals or at high risk of infection if the ciliate was pathogenic. Collection stream origins

appeared to be a strong determining factor of infection. Wildcat Run accounted for many of the observed infections, yet hatchlings from this stream, across all treatments, had the highest survival odds.

### Discussion

The present study confirmed that early transformation in the absence of an aquatic environment is clearly possible in the mountain dusky. Hatchlings collected in March and held in a jar filled with damp leaves at 68-70 degrees F lost their gills in a few days; larvae from a second clutch held in the same environment with running cold water lost their gills several weeks later (Bishop and Crisp, 1933). Petranka (1998) suggests that gut yolk may nourish hatchlings transforming “a few weeks after hatching” and that feeding may not commence until after transformation. Based on natural history studies in West Virginia, Pauley (pers. comm. 1998) concurred that the mountain dusky may transform before leaving the nest. Early transformation might also explain why gilled, aquatic forms of this species were never observed during the late May to August, 1997 and 1998 intensive sampling of 14 small watersheds in western Pennsylvania (see Chp. 2). The mountain dusky is reported to nest in cryptic subterranean sites (Petranka 1998). The finding of a single nest in this study despite the concerted search effort is consistent. Arguably, the absence of aquatic larvae was an artifact of their cryptic nesting behavior, yet other stream salamanders have similar cryptic nesting habits yet their aquatic larvae were commonly encountered (e.g., *D. monticola*, *Gyrinophilus p. porphyriticus*).

The early transformation of less than 1% of northern dusky hatchlings in the present study suggests that although possible, it is highly unusual. Subsequent exposure to dry or acidic containers failed to trigger early transformation. As observed in the field, the northern dusky is largely tied to the stream as an aquatic larvae before it transforms. Noble and Evans (1932) reported early transformation in northern dusky hatchlings. Discrepancies may be due to population differences, among other factors. Davic (pers. comm. 2004) suggested hybridization with the mountain dusky may explain the early transformation of the 2 northern dusky hatchlings in the present study.

The correspondence between what was observed in the field vs. the laboratory is noteworthy: the species exhibiting early transformation is the same one that was never encountered in the field in its aquatic form; the species remaining as an aquatic larvae in the laboratory (99% of individuals), is commonly encountered as one in the field. This laboratory-to-field correspondence is critical to avoid viewing the laboratory results, and its implications, as an artifact of laboratory conditions.

Did early transformation provide the hypothesized benefits, namely, high survival in dry and acidic containers? And, conversely, did the lack of early transformation lead to poor survival, in acidic and dry containers? The tabulated results speak for themselves. The logistic regression results reinforce the latter. There were no losses among mountain dusky hatchlings, regardless of treatment containers. The high survival of mountain dusky hatchlings in all treatment containers, and in particular the acidic treatments, is attributed

in large part to hatchlings entering the experiment as post-metamorphs rather than larvae, a condition likely to have had profound effects on behavior, feeding, and perhaps tolerance to the laboratory environments. For example, the mountain dusky avoided the bottom of the cup whereas northern dusky hatchlings (larvae) tended to do the exact opposite, a behavior described by Wilder (1913) as a form of “negative heliotropism” and “negative thigmotropism” (positive geotaxis). Post- metamorphs may be simply hardier than larvae, regardless of interspecific differences in tolerance to particular stressors.

Survival in northern dusky hatchlings was generally poor, and even lower in dry or acidic containers. The logistic regression (Model 1) confirmed that the odds of survival were far greater in control than in dry or acid containers, even after adjusting for initial body weight. Survival favored hatchlings from some streams over others (Model 2), in particular, hatchlings from the southwestern portion of the study area (Ohio River Drainage). Despite noteworthy geographic differences, these effects are unlikely to have biased treatment container survival because of the even distribution of hatchlings.

The mountain dusky and northern dusky are both terrestrial nesters. Yet, as observed in the field investigation (Chp. 2), the former was the only taxon persisting in acidic stream reaches. If total independence from stream water is the primary mechanism by which survival in acidic streams is possible for stream salamanders, it was hypothesized that the mountain dusky transformed without the need for an aquatic larval stage, whereas the northern dusky differed in this respect and as a result, survives poorly



in acidic environments. The present study confirms that in spite of similarities in nesting and life histories, these two taxa differ. In contrast to most northern dusky hatchlings, the mountain dusky transformed rapidly and in the absence of an aquatic stage, an attribute demonstrated in the present study to favor survival. Consequently, early transformation in the mountain dusky is suspected to be the primary mechanism for survival in acidic stream reaches. The lack of early transformation in most northern dusky hatchlings, and its poor survival in dry and acidic containers, in particular, is consistent with this taxon's low numbers and absence in acidic streams. These findings, and their implications, were the purpose for the laboratory investigation.

Low soil pH affects the distribution of terrestrial plethodontids and other amphibians (Wyman and Hawksley-Lescault 1987, Wyman 1988, Wyman and Jancola 1992). The disruption of sodium balance caused by acidic substrates is similar to that observed in aquatic salamanders exposed to acidic aqueous solutions (Frisbie and Wyman 1991). Actually, the latter investigators describe forest soils as a "modified type of aquatic environment" where  $H^+$ , Al and other ions in the soil, humus layer, and soil moisture can readily interact with the moist and highly permeable integument of salamanders, a fact that inevitably questions the benefits of early transformation to hatchlings facing acidic environments. In other words, what is the importance of bypassing the aquatic larval stage if acidic soils have a similar impact on terrestrial salamanders?

Frisbie and Wyman (1991) noted a poor correlation between body sodium concentration of field captured red-backed salamanders (*Plethodon cinereus*) and soil pH. Thus, in contrast to their laboratory counterparts housed on uniformly acidic substrates, free-ranging *cinereus* on acidic soils did not have depressed body sodium levels. These seemingly contradictory findings were explained by first recognizing that soil pH can be highly heterogenous even in small areas. Unlike laboratory animals held in uniform substrates, free-ranging salamanders found on acidic soils have the option to move to less stressful sites, a behavior consistent with several studies (Mushinsky and Brodie 1975, Freda and Taylor 1992, Horne and Dunson 1994, Sugalski and Claussen 1997). Second, artificial substrates are unlikely to replicate the complex nature and composition of soils even if they exhibit a similar pH to acidic soils. Calcium, dissolved organic carbon (DOC) and other soil constituents can ameliorate the adverse effects of high H<sup>+</sup> concentration and other toxic ions. From an osmoregulation perspective, if a terrestrial environment is more forgiving than an aquatic one, early access to the terrestrial environment as a post-metamorphic salamander may play a decisive role in determining survival in acidic environments.

There was some evidence of avoidance of acidic water or dry container environments in the northern dusky despite their tendency to move downwards. The lower relative frequency of hatchlings at the bottom of the cup in the acidic containers, compared to the controls, probably reflects avoidance, a behavior consistent with other amphibian studies cited above. As evidenced from the fate of hatchlings in dry treatment

containers, however, avoidance of acidic water by remaining on the moist paper improved the odds of survival marginally. Evidently, the moisture afforded by the moistened paper towels was not compatible with survival. Acid tolerance in amphibians generally increases with age (Freda 1986, Pierce et al. 1984, Freda and McDonald 1990). Early life stages (embryos, recent hatchlings) are particularly vulnerable.

Do harsh nesting environments trigger early transformation, as implied in early reports? When early transformation occurred, hatchlings of both species were housed in artificial environments that with the exception of the platform, were identical to the dry treatments but were intended to simulate natural nests, the shallow, moist cavity lacking inundation where egg incubation and hatching occurs and where hatchlings seek temporary shelter before moving to water. Hatchlings in this study experienced ambient and substrate temperatures above natural nesting sites. The gradual decrease in ambient temperature typical during the autumn in central Pennsylvania was not simulated. Under these conditions all mountain dusky and less than 1% of the original number of northern dusky hatchlings transformed. Was the outcome of the former due to the absence of water and high temperature? If early transformation was triggered by lack of an aquatic environment, since none of the hatchlings were in control containers at the time, why did only a few northern dusky hatchlings transform?

One possibility is that early transformation was under the control of intrinsic factors, i.e., hatchlings that transformed were “pre-wired” to do so independent of

environmental cues. In this study, in contrast to Bishop and Crisp (1933) and Noble and Evans (1932), only 2 specimens from the original 302 northern dusky hatchlings transformed; these individuals were from the same clutch. Of the hatchlings subsequently exposed to normal, dry or acidic containers, none transformed by the end of the 51-day experiment and only 29% survived. Hence, early transformation was evidently not selected for. In contrast, and under identical conditions, all 10 mountain dusky hatchlings transformed (lost their gills) by October 28, 4-5 wk after hatching.

Beachy's (1995) experiment with southern mountain dusky, recently recognized as *D. ocoee* (Tilley and Mahoney 1996), revealed that food, temperature, and the interaction of these two variables can affect, to some degree, metamorphic size as well as the timing of transformation. Whilst no treatment deprived hatchlings of an aquatic environment, all larval periods were within 230 - 255 d, a length of time not comparable to what was observed in this study. In essence, Beachy's study confirmed that within the populations sampled, the larval trajectory remained fairly conservative and incapable of responding to environmental factors and even concomitant body changes resulting from external factors i.e. growth history. He subsequently hypothesized that the disconnection between external factors and metamorphosis may have resulted from the weak selection forces to "detect a deteriorating" environment. The ability to detect and respond to changing conditions might seem highly adaptive to an assemblage facing uncertain environments (Levins 1968, as cited in Beachy 1995). The examples of plasticity and variability in life histories related to larval period and metamorphosis for vernal pool

breeding amphibians given in Wilbur and Collins (1973) in their opening paragraph are especially illuminating.

The cool, forested Appalachian streams occupied by stream plethodontids can generally be regarded as “stable” or at least more “predictable” (Hairston 1987, Davic and Welch 2004), relative to temporary pools. If so, the early transformation of mountain dusky witnessed in this study, also suspected to be the primary mechanism for their survival in AMD-contaminated reaches, may have proceeded quite independently of environmental conditions - it may be an attribute of the life history of the mountain dusky for the particular population sampled. As such, it may be as tenaciously preset as the seemingly aquatic-obligate mountain dusky larvae described by Beachy (1995). If the findings of this laboratory study are interpreted in combination with field sampling results (Chp. 2), it may be reasonable to propose that early transformation in the mountain dusky may occur regularly under natural conditions and independently of its surroundings, at least within the geographic region and populations investigated.

In addition to the impact of life stage, survival in the containers was also influenced by treatment, stream of origin, and initial body weight. Among northern dusky hatchlings, initial body weight was a significant variable in both logistic regression models presented. Depending on the model, with every unit increase in weight (1 mg), the odds of survival increased approximately 1.5 times greater. The relationship between body weight and survival was evident even without the aid of the logistic regression

models: survivors were significantly heavier than their non-surviving counterparts, and the greatest discrepancy exists between survivors and non-survivors in the more stressful dry and acidic containers. Survivors from the latter treatments were also significantly longer (SVL) than survivors in control containers, a finding supporting the assumption that initial body mass was correlated to body size.

Gut yolk is a prominent feature of hatchling desmognathines and serves as an initial source of energy for developing larvae (Wilder 1913, Bishop and Crisp 1933, Burggren and Just 1992). According to Orr and Maple (1978) and Montague (1987), gut yolk absorption is faster in the northern dusky sympatric with mountain dusky than when the former exists in the absence of its smaller congener. Orr and Maple (1978) hypothesized that this form of character displacement minimized interspecific competition for similar-sized prey between northern dusky and mountain dusky by promoting greater disparity in size and allowing feeding to initiate sooner in the larger taxon. Eggs and hatchlings in the present study originated from locations where these taxa coexist.

Hatchlings in the more stressful containers lost a greater proportion of their initial body weight than controls. Montague (1979) noted that feeding can begin before gut yolk resorption is complete. Hatchlings in this study had the opportunity to feed at any time because collembolans were offered *ad libitum* throughout the experimental period to both species and regardless of container treatments. Laboratory-held *Desmognathus* in acidic

containers fed less and lost more weight relative to controls (Roudebush 1988), a finding consistent with the greater loss of weight observed among hatchlings in dry and acidic containers in this study. In Montague (1987), mortality was correlated with yolk depletion and all larvae died by late December, a date close to the loss of nearly 50% of control animals in this study. In summary, initial body weight (size) was important to survival; differences in yolk metabolism may also have played a role in determining earlier depletion and survival probability.

Protozoan parasites have been found on all 21 species of North Carolina salamanders studied, including northern dusky and mountain dusky (Rankin 1937). The diversity of such parasites was higher in the former ( $n = 10$ ) than in the latter ( $n = 2$ ) and in general, if all other parasite types were included, infestation was highest in aquatic, highly variable in semi-aquatic, and lowest in taxa from terrestrial environments. Joy et al. (1993) alluded to a similar pattern in regards to nematode infestation in *D. monticola* and mountain dusky: the smaller, more terrestrial taxon was less commonly infested than its larger more aquatic congener.

In the present study, the ciliate was only observed in the northern dusky, and in predominantly non-acidic container treatments. The acidification of water by dilute  $H_2SO_4$  may have curtailed infection; changes in pH, alkalinity, and solute content are among the recommended treatments for protist ectoparasites (Rheichenbach -Klinke and Elkan, 1965). A large proportion of the infected animals (86%) originated from two

watersheds varying drastically in hatchling survivorship and level of degradation, i.e., Muddy Run, a highly AMD impacted watershed from the West Branch of the Susquehanna River Basin with poorly surviving hatchlings and Wildcat Run, a normally alkaline, headwater in the Ohio River basin with hatchlings exhibiting the highest probability of survival.

There is minimal evidence suggesting that the ciliate in the present study was harmful or compromised survival. Indeed, both Rheichenbach -Klinke and Elkan (1965) and Rankin (1937) indicate that many ciliates associated with amphibians are commensals. Worldwide amphibian declines have triggered numerous investigations to assess amphibian parasites as potential causative agents, among other factors (Wake 1991, Lannoo 1998, see Goater et al. 2001 for review).

Why transform sooner and without an aquatic life stage? The mountain dusky in the northern Appalachians is the smallest, most terrestrial, and most vulnerable to predatory or competitive encounters with other larger, more aquatic salamander species. From the terrestrial side, it faces highly aggressive, territorial forest-floor-dwelling *Plethodon*. Predation and competition, and the complex interactions resulting from these factors feature prominently as likely selective forces shaping speciation as well as life history in demognathines (Hairston 1987). As suggested by Davic (pers. comm., 2004), the mountain dusky may be in a “dynamic steady state in its evolutionary trend to continue its aquatic invasion away from its ancestral terrestrial evolutionary home”. If so,



by further manipulation of gut yolk metabolism or initial volume to allow metamorphosis to occur before feeding, early transformation might be the next step to eliminate competitive feeding, promote greater terrestrial habits, and decreased dependence on lotic environments, whilst solidifying its current standing along the aquatic-terrestrial continuum.

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## Chapter 5

## SYNTHESIS

Stream acidification was hypothesized to be an assemblage-shaping factor for stream bank salamanders. The findings from the field sampling investigation (Chp. 2) corroborates the latter assertion unequivocally: assemblage attributes at one end of the gradient differed dramatically to those on the opposite, alkaline end. Generally, abundance declined, as well as taxonomic and life stage diversity. Alkaline stream reaches, relative to acidic, AMD-contaminated waters, supported densely-populated, species diverse, larval and juvenile-dominated populations. In these environments more than in any other, the aquatic nesting northern two-lined could become disproportionately abundant and widespread. Evidence of reproduction in several taxa was clearly evident. In contrast, strongly acidic, AMD-contaminated reaches rarely supported salamanders. When encountered, these tended to be adult or post-metamorphic juveniles of the mountain dusky, a terrestrial-nester with highly terrestrial habits.

Responses were sufficiently evident that despite the low taxonomic diversity of the target assemblage (7 species at most, usually 4), the acid-to-alkaline gradient was reconstructed by ordination from salamander species and life stage data alone. Furthermore, the explained variation resulting from the ordination was strongly tied to water chemistry, and remained bound to water chemistry even after accounting for the effect of other, non-water chemistry related factors.

Were life history and associated nesting habits useful predictors of acidic stream survival and presence? Mountain dusky distribution patterns noted in the field sampling study (Chapter 2) and high hatchling survival in acidic as well as dry test chambers simulating nesting environments (Chapter 4), match life history-based expectations exceedingly well. Based on this taxon alone, terrestrial habits and nesting, and early transformation appears to confer tremendous advantages to populations and individuals facing acidified streams. The mountain dusky in the present study is Kucken et al.'s (1994) pygmy salamander (*Desmognathus wrighti*), the only stream salamander that survived acidification following the use of iron pyrite-containing rocks as road fill at a stream crossing. Unlike other stream plethodontids present in the above-referenced North Carolina study, pygmy salamanders lack an aquatic larval stage and hatchlings emerge as “miniature adults” (Petranka 1998).

Proverbial flies in the soup appear when the focus is shifted to several other taxa. The northern dusky salamander, a terrestrial nester anticipated to co-persist in acidic stream reaches alongside its congener, is one of them. By most literature accounts, the northern dusky and mountain dusky share very similar life history traits. Yet the northern dusky's distribution in the 14 study streams was more similar to the northern two-lined, a taxon also rarely present in acidic reaches, an aquatic nester with 1.5 - 2.5 yr aquatic larval stage. Their close proximity to each other in ordination space further alluded to their co-occurrence and close association. Hatchling northern dusky survival in the laboratory-simulated dry and acid nesting environments was poor, corresponding to its

distribution in the field. Survival required non-acidic water and only a fraction of all the hatchlings observed transformed earlier.

Particularly incongruent to life history predictions was the persistence of the northern spring salamander in acidic reaches. Despite its extended larval period (up to 4 yr), it matched the taxon with the least similar life history, the mountain dusky. The 48-hr *in situ* bioassay revealed the northern spring to be more acid-tolerant than similar larval northern two-lined. However, it was not immune to physiologic stress or mortality in the highly acidic, AMD-contaminated reaches.

Survival in acidified streams by the life history route for stream salamanders appears possible only when the aquatic life stage is omitted, as in the mountain dusky. As evidenced from the laboratory outcome (Chp. 4) life histories that tie early life stages to an aquatic existence, even if measured in months, were insufficiently beneficial to overcome chronic acidification. Obligated to an extended aquatic life stage, natural acid tolerance appears to have played a crucial role in facilitating occupancy and persistence of the northern spring. Hence, survival was achieved by two very different mechanisms, both providing similar benefits. The two mechanisms enabling persistence explain the close association of the two salamanders with very different life histories.

The associations between the mountain dusky and northern spring salamanders, representing the “acidic guild”, and the northern dusky and northern two-lined, the



“alkaline guild”, described in the present study persisted in a later sampling study of 138 headwater reaches (< 3 km drainage basin) in the Mid-Atlantic Highlands region (Rocco et al. 2004). In this study, sampling was completed along a human disturbance gradient. However, salamander associations consistent with the present study also emerged from a subset of 34 minimally disturbed stream sites. Stream habitat correlates among these minimally disturbed sites revealed that the mountain dusky and northern spring salamanders were closely bound to steeper, cooler, boulder-sized bottomed-streams, whereas the northern dusky and northern two-lined, favored lower gradient, warmer, smaller rock-bottomed reaches. All four species can coexist, thus it is important to realize that the above differences are best viewed as fine-scaled, stream habitat preferences where considerable overlap exists.

Nonetheless, the mountain dusky and northern spring salamanders, relative to the other two taxa, I believe, are the most likely to encounter acidification as a *natural stressor* or to have experienced it as a stressor at the population scale, especially considering the mature forested and geologic settings (steep, acidic sandstone-dominated) in many small central Appalachian headwaters. Hence, whilst the early transformation documented in the mountain dusky may have evolved in response to predatory and competitive interactions with other stream-side taxa (land-water interface) and woodland species (riparian corridor - forest interior interface), acid-tolerance in the northern spring salamander may have resulted to cope specifically with its more acidic-prone lotic environments it must face for an extended period before transforming. In either case, and

by different mechanisms, the mountain dusky and northern spring appear the least affected by stream acidification. The impact to other assemblage members, however, is anticipated to be significant. In consideration of their dual function as predators and prey, the actual cost of such degradation is likely to be underestimated.

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Appendices

Appendix 2.1

Description and tabular information for the 14 study streams sampled in 1997 and 1998

## Study stream narrative

### High acid - acid mine drainage-impacted (AMD)

Laurel, Glenwhite, and Muddy Run are AMD-impacted streams. Laurel Run, a first order stream in Westmoreland County, receives acidic discharges from three abandoned underground mines located at the headwaters and upper midsection (OSM 1995). Near discharge sources, pH values as low as 3.21, and metal concentrations of 2.1 mg/l Fe and 6.2 mg/l Al were recorded and are consistent with other chemical surveys. No fish were present and macroinvertebrate populations were limited (OSM 1995). Laurel Run was mostly forested (85%) and formed part of Powdermill Reserve, Carnegie Museum. Private land holdings, primarily in pasture and cropland, accounted for most of the remaining 14% of land cover. Glenwhite Run was a naturally poorly-buffered second order stream on the Allegheny Front, in Blair County. There appear to be as many as 8 underground and surface mine discharges, with estimated flows of 30 - 200 gpm, pH 3.1 - 4.1, and iron and aluminum concentrations of 1-100 mg/l and 1-20 mg/l, respectively (Spyker 1997). Fish were absent throughout, except for the minimally impaired headwaters. Benthic macro-invertebrate diversity and abundance was highly variable and reflects proximity and severity of AMD discharge points. Glenwhite Run was 98% forested with the remainder in barren land associated with abandoned surface mines and rural development. Muddy Run was a severely AMD impaired stream on the Allegheny Front in Cambria County. Discharges originate from abandoned and reclaimed surface mines which cover about 14% of the total watershed area. Water quality along study sections was generally poor, with pH 2.98 - 4.39, 1 - 3.2 mg/l Fe, 1 - 31.05 mg/l Mn, and 1.4 - 62.82 mg/l Al. No fish were present. Status of macroinvertebrate community was unknown. Approximately 84% of the watershed was in deciduous forest; 1.5% consists of rural development. At its lowest study point, Muddy Run becomes a third order stream. Drainage size and maximum elevation for the three AMD impacted streams was 322 - 858.69 ha and 430 - 752 m, respectively.

### Low Acid - Episodic Acidification

Linn, McGinnis, and Stone Run were poorly buffered, slightly acidic, first and second order streams that flowed primarily through non-calcareous or acidic sandstone and shale of the Allegheny and Pottsville Group (Dinicola 1982). To a greater or lesser extent, all three were subject to episodic acidification in early spring. Episodes of low pH and increases in total Al concentration occurred during storm flows and were thus linked to acid precipitation (Sharpe and Kimmel 1983, Sharpe et al. 1984, Kimmel et al. 1985, Sharpe et al. 1987, Wigington et al. 1996). In general, electroshocking surveys revealed smaller fish populations and lower species diversity than in non-episodically acidified streams (Baker et al 1996, Sharpe et al 1984). Benthic macroinvertebrate density and diversity, as well as rates of leaf litter decomposition were also found to be considerably lower and slower in McGinnis Run, than in Wildcat Run, a reference stream (Kimmel et al. 1985). McGinnis Run and Linn Run drained portions of the Laurel Hill in Westmoreland County. Stone Run lies in north central Pennsylvania in Clearfield County

Drainage size and maximum elevation for these streams ranged from 470 – 677 ha and 497 – 897 m, respectively. Land cover in these watersheds was also > 99% deciduous forest.

#### Non-acid - reference

Camp, Wildcat, Roaring, and Wallace Run were chosen as reference streams for their favorable water quality and aquatic resources, minimal logging or development, and relatively unspoiled condition. The first three streams lie on the west slope of Laurel Hill in Westmoreland County. All were underlain in varying amounts by the Mauch Chunk Formation, a limestone-bearing rock that provides stream alkalinity. Camp Run, a first order stream, was classified by the Pennsylvania Fish and Boat Commission (PFBC) as a Class A brook trout stream. No stocking took place and resident trout were self-reproducing. Benthic macroinvertebrate diversity and biomass were among the highest in Westmoreland County and include many pollution sensitive genera of stoneflies, mayflies, and caddisflies (Lighty 1995). Wild populations of brook trout (*Salvelinus fontinalis*), brown trout (*Salmo gairdneri*) and mottled sculpins (*Cottus bairdi*) of various age classes were noted in Wildcat Run, a second order stream used to represent reference conditions by Sharpe and Kimmel (1983), Sharpe et al. (1984), Sharpe et al. (1987), Kimmel et al. 1985. Roaring Run, a second order stream, is classified by the Pennsylvania Department of Environmental Protection (DEP) as a High Quality - Cold Water Fishes stream and was managed for its wild brook trout population. Following the 1998 stream survey by the PFBC, it was recommended that its designation be upgraded to Exceptional Value (Lorson 1998). Wallace Run was a second order stream situated in the Allegheny Front of Centre County. It was considered high quality, it supports wild brook trout and is included in the Wilderness Trout Stream Program. Drainage size and maximum elevation for the four reference streams ranged from 458 – 632 ha and 455 – 894 m, respectively. Deciduous forest, primarily state owned, covered > 99% of the four watersheds.

#### Non-acid/high alkalinity - Moderate Watershed Fragmentation

Minnow, Olive, Jefferson, and Golfcourse Run are small, first and second order, non-acidic, highly alkaline streams in Westmoreland County that originate at the base of Chestnut Ridge, and flow primarily through the Casselman Formation, a thick geological unit that includes freshwater and marine limestones. All four watersheds, unlike the others, had lower forest cover (44 - 63%) with the remainder in farmland, pasture, recreation or rural development. There were no public lands. Near the headwaters of Minnow Run, a small camp ground, trailer park, and waste water treatment plant were built on previously graded terrain. The effluent is discharged into the stream following pond tertiary treatment. Severe eutrophication during the warm summer months is evident from the large amount of algal growth downstream of the effluent. Benthic macroinvertebrate surveys yielded nine EPT and 14 total taxa (0 mayflies, 1 genus of stonefly). Olive Run originated in pasture and cropland and drained several deeply eroded old fields, an amusement park with golf green and water slides, a forested tract, and an

unfenced horse pasture. Jefferson Run was the least forested (44%) of the four but has no other land uses except pasture, cropland, and some rural development. Golfcourse Run, an unnamed tributary of Hypocrite Creek, is the smallest watershed of the 14 study streams. As its name implies, a golf course covered a large portion of its upper reaches. Other non-forest areas included cropland, pastures, and rural development. Although 63 % of this headwater appeared forested, a substantial portion of the mid to upper reaches of the basin was selectively cut in 1995. No acidic mine discharges exist on any of these streams. Drainage size and maximum elevation for these headwaters was 155 - 353 ha and 359 – 588 m, respectively.

Location and physical characteristics of the 14 study watersheds in the Central Appalachians of Pennsylvania.

Condition	Stream	County	USGS 7.5 Quad.	Location Lat (N) Long (W)	Area (Ha)	Elevation Range (m)	Soil Series	Stream Physical Characteristics		
								Order	Length (km)	Grade m/km
Acid Mine Drainage (AMD) High acid	Laurel	Westmoreland	Stahlstown Seven Springs	40 09 10 79 16 58	321.7	430 703	Gilpin, Cavode, Wharton, Ernest	1	3.2	38
	Glenwhite	Blair	Ashville Cresson	40 30 00 78 30 43	858.7	558 745	Clymer, Hazleton, Laidig, Buchanan, Udifluvents - Dystrochepts Cmplex	2	3.2	32
	Muddy	Cambria	Blandburg	40 42 39 78 25 12	698.3	545 752	Udorthents, Leetonia, Hazleton, Leck Kill, Brinkerton, Cooport- Ernest	3	3.3	31
Episodic Acid. Low acid	McGinnis	Westmoreland	Ligonier	40 08 45 79 10 22	470.2	697 824	Calvin, Dekalb, Cavode, Ernest, Philo	1	1.8	44
	Linn	Westmoreland	Ligonier Bakersville	40 07 55 79 11 52	506.7	697 879	Calvin, Dekalb, Cavode, Ernest	2	2	54
	Stone	Clearfield	Clearfield Huntley	41 05 56 78 26 49	677.1	497 691	Hazleton, Clymer, Cooksport, Ernest	2	2.8	54

Location and physical characteristics of the 14 study watersheds in the Central Appalachians of Pennsylvania. (Cont.)

Condition	Stream	County	USGS 7.5 Quadrangle	Location Lat (N) Long (W)	Area (Ha)	Elevation Range (m)	Soil Series	Stream Physical Characteristics		
								Order	Length (km)	Grade m/km
Reference (non-acidic)	Camp	Westmoreland	Seven Springs	40 05 45 79 17 45	501.8	612 867	Calvin, Dekalb, Ernest	1	2.3	52
	Wildcat	Westmoreland	Ligonier	40 10 19 79 10 26	461.2	606 879	Calvin, Dekalb, Ernest, Philo	2	2.7	63
	Wallace	Centre	Bear Knob	40 57 10 77 56 31	632.2	455 712	Clymer, Hazleton-Dekal, Laidig, Andover	2	3.4	55
	Roaring	Westmoreland	Seven Springs	40 03 25 79 18 43	458.3	648 894	Calvin, Dekalb, Gilpin, Ernest	2	2.8	56
Fragmented (Non-acid- high alkalinity)	Minnow	Westmoreland	Donegal	40 04 55 79 22 48	341.8	479 588	Gilpin, Cavode, Wharton, Ernest, Atkins	2	2.5	27
	Olive	Westmoreland	Seven Springs	40 04 58 79 21 07	230.3	455 588	Gilpin, Cavode, Wharton, Ernest, Philo	1	1.6	38
	Golfcourse	Westmoreland	Stahlstown	40 19 33 79 09 54	155.3	412 503	Gilpin, Cavode, Wharton, Ernest, Philo	2	1.8	36
	Jefferson	Westmoreland	Wilpen	40 12 10 79 16 48	352.7	358 485	Gilpin, Cavode, Wharton, Ernest, Philo	2	1.5	32



## Geology, percent land-cover, and water chemistry at the 14 study watersheds in the Central Appalachians of Pennsylvania.

\* Alkaline tributary

Condition	Stream	Geology	Land Use Cover (Percent watershed area)				Water Chemistry (Min - Max)				
			Forest	Agric./ Rec.	Barren Open Water <sup>†</sup>	Develop.	pH	CaCO <sub>3</sub> mg/l	NO <sub>3</sub> -N mg/l	Fe mg/l	Al mg/l
Acid Mine Drainage (AMD) High acid	Laurel	Glenshaw Fm, Allegheny Gp	99.1	14.7	0.1	< 0.1	2.94 6.00	0.092 3.01	0.015 0.558	0.024 275.6	0.018 28.25
	Glenwhite	Allegheny Gp, Pottsville Gp, Mauch Chunk Fm	98.4	0.2	1.2	0.2	3.79 6.96	< 0.05 24.0*	0.185 0.39	0.042 0.571	0.011 7.062
	Muddy	Allegheny Gp, Pottsville Gp, Mauch Chunk Fm	84.2	0.6	13.9	1.3	2.98 4.39	< 0.05	0.005 0.344	0.045 7.0	1.469 62.82
Episodic Acid. Low acid	McGinnis	Allegheny Gp, Pottsville Gp, Mauch Chunk Fm	99.8	< 0.1	0.2	0	4.02 4.74	< 0.05	0.039 0.188	0.051 1.027	0.276 0.825
	Linn	Allegheny Gp, Pottsville Gp, Mauch Chunk Fm	99.6	0.2	0.3	0	4.66 5.67	< 0.05 1.60	0.219 0.323	0.039 0.2	0.084 0.377
	Stone	Allegheny Gp, Pottsville Gp, Burgoon Ss, Huntley Mountain Fm	99.1	0.2	0.7	< 0.1	5.44 5.82	< 0.05 0.285	0.005 0.127	–	0.02 0.057

## Geology, percent land-cover, and water chemistry at the 14 study watersheds in the Central Appalachians of Pennsylvania (cont.)

\* Alkaline tributary

Condition	Stream	Geology	Land Use Cover (Percent watershed area)				Water Chemistry (Min - Max)				
			Forest	Agric./ Rec.	Barren Open Water <sup>†</sup>	Develop.	pH	CaCO <sub>3</sub> mg/l	NO <sub>3</sub> -N mg/l	Fe mg/l	Al mg/l
Reference (non-acide)	Camp	Burgoon Ss, Mauch Chunk Fm	99.4	0.5	< 0.1	< 0.1	6.59 7.64	8.96 20.29	0.608 0.799	0.024 0.076	0.04 0.061
	Wildcat	Allegheny Gp, Pottsville Gp, Mauch Chunk Fm	99.3	0.2	< 0.1	0.5	6.55 7.00	4.59 23.63	0.526 0.778	0.019 0.098	0.011 0.118
	Wallace	Burgoon Ss, Rockwell Fm, Catskill Fm	99.7	0.3	0	0	6.39 6.8	2.02 3.88	0.005 0.27	0.02 0.162	0.014 0.1
	Roaring	Burgoon Ss, Mauch Chunk Fm Shenango through Oswayo Fm	99.5	0.4	< 0.1	0	7.19 7.44	13.93 23.0	0.705 0.813	0.04 0.076	0.007 0.046
Frag. (Non-acid- high alkalinity)	Minnow	Casselman Fm, Glenshaw Fm, Allegheny Gp	64.5	38.5	< 0.1	1	7.25 8.09	29.61 276.2	0.098 1.105	0.021 0.408	0.012 0.443
	Olive	Casselman Fm, Glenshaw Fm	45.9	49.8	< 0.1	4.3	7.43 7.84	21.25 76.01	0.386 1.856	0.029 0.489	0.021 0.104
	Golfcourse	Monongahela Gp, Casselman Fm	63.3	34.9	0.7 <sup>†</sup>	1	7.37 8.04	29.52 106.71	0.124 0.457	0.023 0.114	0.008 0.082
	Jefferson	Casselman Fm	44.2	55.6	< 0.1	1	7.2 7.8	26.37 79.36	0.47 0.78	0.048 0.076	0.004 0.061

## Appendix 2.2

Central tendency, measures of dispersion, and marginal effects for 24 variables tested against CA ordination scores

Table 1. Central tendency and measures of dispersion for variables tested against ordination scores. Table shows average, standard deviation (SD), and minimum and maximum values for each variable. Values for variables followed by an asterisk are the median of 5 plot measurements. All others represent one or more measurements per transect or watershed. Data only for transects in ordination (58 transects).

Table 2. Marginal effects for 24 environmental variables with no covariables fitted. Columns under marginal effects show fit ( $\lambda_1$ ), or eigenvalue, and corresponding F and P-values for each variable, when the latter are entered as single predictors and in the absence of any covariables. Levels of significance, as indicated by F and P-values, were calculated by a Monte Carlo permutation test (reduced model, default number (499) of unrestricted permutations).

Table 1. Central tendency of 24 environmental variables.

Variable / units	Mean	SD	Min - max
pH plot - <i>in situ</i> *	6.2	1.385	3.79 - 7.84
pH - laboratory	6.17	1.364	3.73 - 8.31
Alkalinity (mg/l CaCO <sub>3</sub> )	18.99	25.108	0.01 - 84.8
NO <sub>3</sub> - N (mg/l)	0.436	0.292	0.051 - 1.421
Fe (mg/l)	0.124	0.12	0.023 - 0.508
Al (mg/l)	0.354	0.834	0.01 - 4.64
Electrical conductivity (uS/m)*	141.26	169.17	22.0 - 765
Ambient temperature (C)*	22.1	2.6	15.0 - 27.0
Stream temperature (C)*	15.7	2.63	9.1 - 22.3
Relative humidity (%)*	0.67	0.095	0.47 - 0.86
Stream width (cm)*	241.3	95.1	77.5 - 504
Stream depth (cm)*	11.0	5.42	3.5 - 30.2
Stream gradient (m/km)	47	23	10.6 - 84.7
Plot cobble cover (%)*	0.41	0.13	0 - 0.6
Plot moss cover (%)*	0.08	0.11	0 - 0.39
Total plot vegetation cover (%)*	0.045	0.065	0 - 0.31
Gravel (% sample weight)*	0.56	0.13	0.01 - 0.79
Plot canopy closure (%)*	0.78	0.13	0.35 - 0.95
Distance to stream source (m)	1,276.9	1,012.4	23.9 - 4,533
Elevation (m)	576	121.4	367 - 794
Watershed in forest (%)	0.84	0.21	0.44 - 0.99
Adjusted watershed in forest (%)	0.83	0.22	0.29 - 1.0
Transect drainage area (ha)	246.4	190.7	2.7 - 759.2
Sampling effort (person-hrs/plot)*	1.95	0.46	1.09 - 3.19

Table 2. Marginal effects for 24 environmental variables with no covariables fitted.

Total inertia			0.782
Variable	$\lambda_1$	F-value	P-value
Ph - <i>in situ</i>	0.19	17.63	< 0.002
Ph - laboratory	0.17	15.80	< 0.002
Watershed in forest (%)	0.16	14.51	< 0.002
Alkalinity (mg/l CaCO <sub>3</sub> )	0.15	13.39	< 0.002
Adj. watershed in forest (%)	0.15	13.31	< 0.002
Elevation (m)	0.15	13.07	< 0.002
Sampling effort (person-hr)	0.11	9.08	< 0.002
Electrical conductivity (uS/m)	0.10	8.68	< 0.002
Water temperature (C)	0.09	6.85	< 0.002
Stream gradient (m/km)	0.08	6.12	< 0.002
NO <sub>3</sub> -N (mg/l)	0.07	5.94	< 0.002
Plot canopy closure (%)	0.07	5.34	< 0.002
T. plot vegetation cover (%)	0.06	4.89	< 0.004
Al (mg/l)	0.06	4.90	< 0.002
Plot moss cover (%)	0.06	4.32	< 0.004
Plot cobble cover (%)	0.05	3.85	< 0.004
Stream depth (cm)	0.05	3.55	< 0.012
Fe (mg/l)	0.04	3.38	< 0.004
Gravel (% sample weight)	0.04	2.69	< 0.026
Transect drainage area (ha)	0.04	2.66	< 0.016
Ambient temp. (C)	0.03	2.21	> 0.05
Distance to stream source (m)	0.03	1.99	> 0.05
Stream width (cm)	0.02	1.38	> 0.05
Relative humidity (%)	0.01	0.77	> 0.05

## Appendix 4.1

Nesting site attributes and body measurements for northern dusky and mountain dusky hatchlings in 1998 laboratory exposures

Collection sites, nest habitat, and attributes of northern dusky and mountain dusky hatchlings collected in western Pennsylvania on September 5-7 and 12, 1998.

Nest ID	Date Nest Found	Stream	County	Nesting Surface, Cover, and Size (cm)	Distance to Water (cm)	Nest Height (cm)	Number of Hatchlings	Date Hatch mm/dd/yy	Initial Weight (mg) on 9/18/98 (Mean $\pm$ 1SE)
Northern dusky									
RO-01-01	9/05/98	Roaring Run	Westmoreland	Soil/moss	15	12	19	9/11/98	39.2 $\pm$ 0.5
RO-02-01				Rock/moss, 62	12	7	24	9/08/98	36.8 $\pm$ 0.3
RO-02-02				Rock/moss, 57	28	17	19	9/08/98	35.1 $\pm$ 0.4
RO-02-03				Rock/moss, 44	105	13	38	9/05/98	33.0 $\pm$ 0.3
CM-01-01		Camp Run		Muck/moss	7	7	9	9/05/98	25.2 $\pm$ 0.4
CM-01-02				Muck/moss	13	7	19	9/11/98	32.8 $\pm$ 0.4
WC-01-01	9/06/98	Wildcat Run		Rotting log/moss	30	30	15	9/06/98	30.4 $\pm$ 0.2
WC-02-01				Rock/moss, 50	60	28	7	9/08/98	21.14 $\pm$ 0.4
WC-02-02				Rock/moss, 32	15	8	15	9/08/98	23.7 $\pm$ 0.3
WC-02-03				Rock/moss, 45	80	12	10	9/06/98	26.6 $\pm$ 0.8
WC-02-04			Muck/rock, 39	25	13	none	na	na	
WC-02-05			Rock/moss, 32	48	12	9	9/06/98	33.8 $\pm$ 0.8	
WC-03-01			Muck/rock, 24	10	6	16	9/08/98	29.8 $\pm$ 0.3	

Collection sites, nest habitat, and attributes of northern dusky and mountain dusky hatchlings collected in western Pennsylvania on September 5-7 and 12, 1998 (cont.)

Nest ID	Date Nest Found	Stream	County	Nesting Surface, Cover, and Size (cm)	Dist. to Water (cm)	Nest Height (cm)	Number of Hatchlings	Date Hatch mm/dd/yy	Initial Weight (mg) on 9/18/98 (Mean $\pm$ 1SE)
Northern dusky									
BN-01-01	9/07/98	Benner Run	Centre	Rotting log/moss	23	23	20	9/7/98	36.7 $\pm$ 0.3
MD-01-01	9/12/98	Muddy Run	Cambria	Rock/moss, 40	22	10	16	9/12/98	28.3 $\pm$ 1.2
MD-01-02				Muck/moss	225	7	23	9/12/98	30.1 $\pm$ 0.6
MD-01-03				Rock/moss, 29	12, 32, 20	5	43	9/12/98	31.0 $\pm$ 0.6
Mountain dusky									
MD-02-01	9/12/98	Muddy Run	Cambria	Rotting log/moss	185	5	10	9/17/98	n



## VITA

I, Gian L. Rocco, was born in Ogbomosh, Nigeria, in 1963 from Manlio and Liliana Rocco, citizens of Udine, Italy. Overseas assignments for my father in the Middle East, Africa, and South America exposed me to many different cultures early in my life. In 1985 I earned a B.S. in Biology from the Interamerican University of Puerto Rico, San German, PR. I completed an MS in Biology in 1988 at Central Michigan University, Mount Pleasant, MI. Shortly thereafter I worked as Herpetologist for Herpetological Associates, Inc., Toms River, NJ. In 1995, I moved to central Pennsylvania to work through The Nature Conservancy as an endangered species biologist for the Pennsylvania Fish and Boat Commission, in Bellefonte. I began to pursue my Ph.D. in Wildlife and Fisheries Science at the School of Forest Resources, Pennsylvania State University, University Park in 1997 and was subsequently hired in 2000 as Research Assistant by the Penn State Cooperative Wetland Center, currently located in the Department of Geography. I became a US Citizen in 1998. I am married to Lori A. Rocco (2002) and have two step-children, Alisa and Jason.