The role of multiple stressors in ranavirus-caused amphibian mortalities in Acadia National Park wetlands

M.K. Gahl and A.J.K. Calhoun

Abstract: Recent studies suggest that multiple sublethal stressors compromise amphibian immune systems and increase susceptibility to disease. We examined two aspects of multiple stressors and incidence of ranavirus-caused amphibian mortalities in free-living amphibian populations: (1) among-pond differences in physical, chemical, and biological stressors that may exacerbate mortality events, and (2) temporal changes in within-pond stressors that coincide with mortality events. At the among-pond scale, we used principal components analysis and logistic regression followed by Akaike's information criterion ($QAIC_c$) to identify stressors associated with disease incidence. Of the stressors we investigated, aluminum, temperature, and conductivity were most correlated with outbreaks, but it was unclear whether they increased ranavirus-caused mortality events. Sublethal stressors were difficult to isolate in the field and few were significantly associated with ranavirus across all breeding ponds. Our results suggest that each wetland, because of varied physical, biological, and chemical settings, will have its own suite of stressors that sublethally affect amphibians.

Résumé : Des études récentes indiquent que des facteurs de stress sublétaux multiples compromettent le système immunitaire des amphibiens et augmentent leur susceptibilité aux maladies. Nous examinons deux aspects des facteurs multiples de stress et l'incidence de la mortalité d'amphibiens due aux ranavirus dans des populations d'amphibiens libres en nature, soit (1) des différences entre étangs des facteurs physiques, chimiques et biologiques de stress qui exacerbent les cas de mortalité et (2) les changements temporels des facteurs de stress au sein des étangs qui coïncident avec les événements de mortalité. À l'échelle de l'ensemble des étangs, nous utilisons une analyse des composantes principales et une régression logistique ainsi que le critère d'information d'Akaike ($QAIC_c$) afin d'identifier les facteurs de stress associés à l'incidence de maladies. Parmi les facteurs de stress examinés, l'aluminium, la température et la conductivité ont la plus forte corrélation avec les épidémies, mais il n'est pas clair s'ils augmentent les cas de mortalité dus aux ranavirus. Les facteurs sublétaux de stress sont difficiles à isoler en nature et peu d'entre eux sont en corrélation significative avec les ranavirus dans tous les étangs de reproduction. Nos résultats laissent croire que chaque terre humide, à cause de ses conditions physiques, biologiques et chimiques particulières, posséde son propre ensemble de facteurs de stress qui affectent les amphibiens de manière sublétale.

[Traduit par la Rédaction]

Introduction

The prospect of multiple sublethal stressors acting synergistically on amphibians has received more attention as amphibians decline or disappear from pristine and protected areas such as national parks (e.g., Bosch et al. 2001; Muths et al. 2003) and as mortality events increase owing to diseases that have been present for decades (Johnson et al. 2003; Weldon et al. 2004; Ouellet et al. 2005; Longcore et al. 2007). Recent studies have suggested that multiple sublethal stressors may compromise amphibian immune systems and increase susceptibility to disease (Carey et al. 1999; Hatch and Blaustein 2000; Sparling et al. 2003), potentially instigating mortality events in hosts that are not typically killed by a given disease or when combined with diseases that are usually sublethal.

Understanding declines is more problematic when causes are attributed to a combination of sublethal stressors together with disease. Many stressors have been implicated in creating an environment in which amphibians may be more vulnerable to infection: UV-B (Middleton et al. 2001; Blaustein et al. 2003), acidification (Beebee et al. 1990; Grant and Licht 1993), pollution and heavy metals (Beattie et al. 1992; Horne and Dunson 1995; Jung and Jagoe 1995), habitat destruction (Lehtinen et al. 1999; Brook et al. 2003), and climate change (Beattie et al. 1992; Alexander and Eischeid 2001; Carey and Alexander 2003; Bosch et al. 2006). There are a growing number of experimental studies investigating multiple synergistic cofactors (Beattie et al. 1992; Bradford et al. 1994; Horne and Dunson 1995; Long et al. 1995; Boone et al. 2005, 2007), but to our knowledge only a few studies attempt to link sublethal stressors with increased

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amphibian disease susceptibility in an experimental setting (Kiesecker 2002; Forson and Storfer 2006; Romansic et al. 2006); none focus on free-living amphibian populations.

In this study we examined multiple stressors in free-living amphibian populations in Acadia National Park, Maine, USA, where there is a history of ranavirus (family Iridoviridae, genus *Ranavirus*) causing mortality and morbidity in amphibian populations (Gahl 2007; Gahl and Calhoun 2008). We considered two aspects of multiple stressors and incidence of ranavirus-caused amphibian mortalities: (1) among-pond differences in stressors and (2) temporal changes in within-pond stressors that coincide with mortality event onset. Our primary objectives were (*i*) to examine biological, chemical, and physical stressors that may be associated with disease occurrence by analyzing differences among wetlands, and (*ii*) to identify within-pond acute stressors (e.g., aluminum (Al), pH) associated with the onset of a mortality event in a subset of intensively studied sites by analyzing the changes in chemical, biological, and physical stressors over the breeding season.

Materials and methods

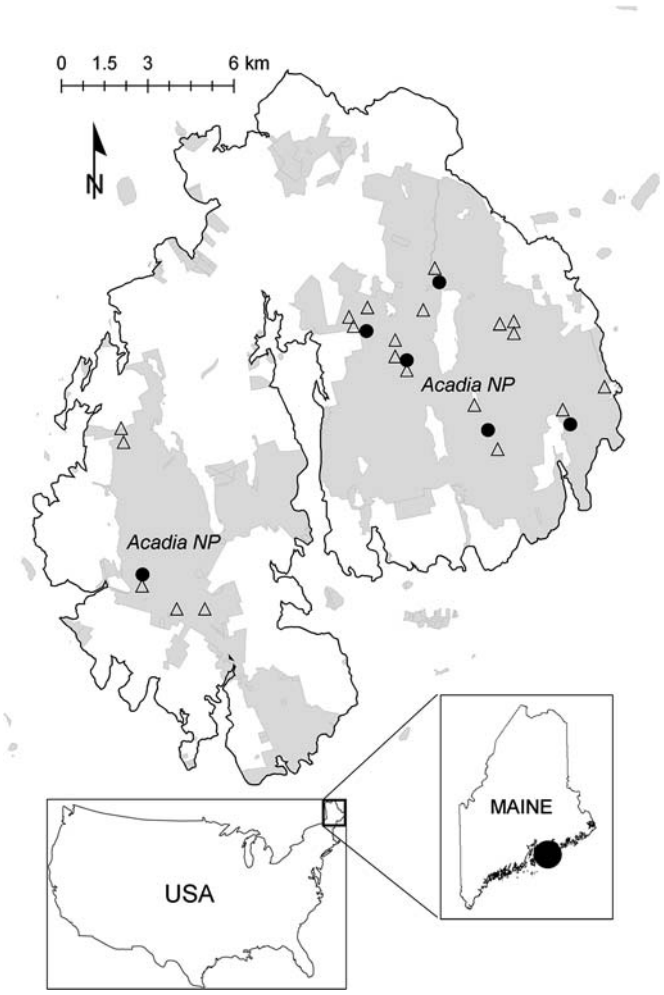
Study sites

We selected 26 wetlands in Acadia National Park (ANP), Maine, USA, of varying hydroperiod, and abundance and diversity of amphibian species for this study (Fig. 1). All wetlands had been characterized previously for amphibian population studies within ANP, and these data provided background for our study-site choices based on amphibian occurrence and disease history (Gahl 2007; Gahl and Calhoun 2008). Of these 26 wetlands, 6 were selected for more intensive study based on previous landscape models that predicted that they were candidates for ranavirus-caused mortality events (Gahl and Calhoun 2008). These 6 primary sites were positioned high within their respective catchments and would all be classified as vernal pools because they hosted relatively abundant populations of Wood Frog (*Lithobates sylvaticus* (LeConte, 1825)) and Spotted Salamander (*Ambystoma maculatum* (Shaw, 1802)) and dried by late summer in most years.

Disease screening

We surveyed for mortality events during the 2003–2005 breeding seasons and used previous data on mortality events (1999–2002; D.E. Green, personal communication). For our in-field surveys, we used visual encounter surveys and dip-netting 3–5 times a week in field health screenings within each wetland to identify morbidity (e.g., lethargy, lack of flee response, extreme sloughing of skin, gross edema, hemorrhaging) and mortality events in all life stages of all amphibian species at each site, focusing on those with a history of ranavirus in ANP: Bronze Frog (*Lithobates clamitans* (Latreille, 1801)), Spring Peeper (*Pseudacris crucifer* (Wied-Neuwied, 1838)), *L. sylvaticus*, and Bullfrog (*Lithobates catesbeianus* (Shaw, 1802) (see Gahl and Calhoun 2008). Fresh carcasses and live animals from sites with mortality events or evident morbidity were sent to the US Geological Survey's National Wildlife Health Center (Madison, Wisconsin, USA) for pathological screening. Screening included dissection, histology of major organs (to look particularly for viral inclusions in liver, parasites in kidneys and muscle tissue, and fungus in skin tissue), and viral (for ranavirus), fungal (for *Batrachochytrium dendrobatidis* Longcore, Pessier & Nichols), and bacterial cultures identified by morphological and molecular methods when appropriate. For statistical analyses, we defined a mortality event as any die-off event in which we observed >10 deaths/day that we could attribute to the same disease by clinical signs, histology, or culture. We pooled mortality events observed at each site across all years (1999–2005).

Fig. 1. Acadia National Park (ANP), Maine, USA, with the 26 study wetlands marked, intensively studied primary sites are designated with a solid circle.



To ensure no disease transmission by researchers among study sites (Jancovich et al. 1997; Harp and Petranka 2006), we disinfected all equipment (e.g., waders, funnel traps) with 10% bleach solution so that we did not transport sediment or water from one site to the next. In addition, at each wetland we had a dip net for use only in that wetland, whereas at all known disease wetlands we dedicated waders to that site.

Wetland-specific stressors

To examine the relationship between potential stressors and ranavirus outbreaks among ponds, we explored a suite of abiotic factors that are potential physical and chemical

stressors, including wetland hydroperiod, potential UV-B, acidification (pH), and water chemistry (anions, cations, Al, dissolved organic carbon (DOC), acid-neutralizing capacity (ANC)) over three breeding seasons (i.e., 2003–2005). In addition, we examined biological parameters that may act as additional stressors for larval amphibians especially when exacerbated with disease (Parris and Beaudoin 2004; Parris and Cornelius 2004), including relative abundance of amphibian larvae and adult amphibian and invertebrate predators (Semlitsch 1987; Relyea 2000; Relyea and Mills 2001).

Physical stressors

Physical predictor variables that sublethally may stress amphibians included mean water temperature (Rojas et al. 2005), standard deviation of water temperature (Raffel et al. 2006), canopy cover, aquatic plant cover, emergent vegetation cover, percent shrub cover, and wetland area (Greer and Collins 2008).

We measured water temperature with HOBO temperature loggers (H01-001-01; Onset Computer Corporation, Bourne, Massachusetts, USA) placed in all primary and secondary wetlands. Temperature loggers were placed in submersible cases and attached to staff gauges 15 cm below the water surface. Ambient water temperature was recorded every 2 h from April through August, the period when amphibian larvae were present. We measured water levels in all wetlands by thrice weekly observation of staff gauges.

We assessed the potential UV-B for each breeding habitat with canopy cover after leaf-out, which coincided with hatch dates and larval period of most pond-breeding amphibians in ANP. We used a densiometer to measure canopy cover at the deepest part of each wetland because as study sites dry each year, water concentrates in the deepest point. In addition, we measured DOC and apparent color (as described in chemical stressor methods) to assess the optical characteristics of the water and resultant UV-B transmittance (Palen et al. 2002; Diamond et al. 2005).

Vegetation type was characterized along two transects in each wetland on north–south and east–west transects. We used a 1 m plot every 5 m along each transect and estimated percent cover of aquatic (herbaceous vegetation that dies back every year), emergent (persistent herbaceous vegetation), and shrub (woody plants <3 m high) vegetation to the nearest tenth percentile. For statistical analyses, we averaged the percent cover over all plots in a wetland.

Wetland area was obtained from wetland polygons corresponding with the area of spring standing water on US National Wetlands Inventory (NWI) maps (1992) using ArcGIS (Environmental Systems Research Institute, Inc., Redlands, California, USA).

Chemical stressors

Chemical variables that may stress amphibians included acidity, conductivity, color, and dissolved oxygen (DO) (Clark and LaZerte 1985; Horne and Dunson 1995; Karraker et al. 2008). We collected biweekly grab samples of water from all wetland sites and analyzed them in the laboratory at ANP for closed-cell pH, specific conductance, and apparent color. Samples were taken from 10 cm below the water surface from the deepest part of the wetland and transported in dark amber plastic bottles (for specific conductance and apparent color) and 60 cc (1 cc = 1 mL) syringes (for closed-cell pH) in chilled coolers..

We measured closed-cell pH with a Ross glass body combination electrode (8104BN; Thermo Orion, Beverly, Massachusetts, USA) and an Orion 230Aplus portable pH meter (Thermo Orion). The electrode was calibrated with standard buffers and enclosed in a closed-cell apparatus. Samples were emptied directly from the syringes into the closed-cell apparatus for pH measurement at room temperature. We measured conductivity at room temperature with a handheld YSI model 30 (YSI Incorporated, Yellow Springs, Ohio, USA) salinity, conductivity, and temperature system calibrated with standard KCl solutions, and standardized measurements to microseimens per centimetre (μS/cm) at 25 °C for relative comparison. Apparent color was determined with an Orbeco-Hellige Aqua Tester (Orbeco Analytical Systems, Inc. Farmingdale, New York, USA) by the same technician to minimize subjective differences inherent in this visual method. We allowed particles in water samples to settle before decanting for color analysis. All samples were temporarily stored in the refrigerator and analyzed within 48 h.

We measured DO every 3 h over the course of 1 day in each breeding site in June 2005. Starting an hour before sunrise, DO and temperature were measured in situ with a handheld portable DO meter (HI9143; Hanna Instruments Inc., Woonsocket, Rhode Island, USA) every three hours until an hour after sunset. In addition, we collected grab samples at each sample time throughout the day and analyzed them for closed-cell pH, conductivity, and apparent color as described above.

Biological stressors

Biological predictor variables that may stress amphibians or increase disease susceptibility included larval density, predator densities (invertebrate and adult amphibian), and amphibian breeding community present (Green et al. 2002; Brunner et al. 2004; Parris and Beaudoin 2004).

We documented the amphibian breeding community in each pond through egg mass counts, funnel trapping, and dip-netting for larvae (see Materials and methods above under Disease screening). We counted egg masses for *L. sylvaticus* and *A. maculatum* by walking the entire area of each wetland once per week from the onset of calling until the number of egg masses ceased to increase.

We approximated relative abundance of amphibian larvae by funnel trapping larval amphibians in all wetlands in early June and again in early July to accommodate different larval species present at both times. We used a stratified random design for placement of funnel traps to standardize for study-site area and habitat. Trapping effort per site was determined by study-site area (Adams et al. 1997), with two traps per 25 m^2 habitat unit and an additional trap each time a habitat area doubled (e.g., for 50 m^2, three traps are required). Standard plastic minnow traps (4.8 mm square mesh, 22 mm entrance; Aquatic Eco-Systems, Inc., Apopka, Florida, USA) were placed near shore or staked near the surface of the water to allow captured larvae to breathe if oxygen levels in the wetland dropped overnight. Traps were checked every 12–18 h. We recorded the number of each species and life stage captured, and amphibians were in-

spected for signs of disease. Unless evidently diseased, we released amphibians at the site of capture.

Invertebrate predators were also captured in funnel traps and family and life stage were recorded. Identified invertebrates were released at the location of capture and unknown specimens were retained and preserved in 70% ethanol for identification in the laboratory.

Because amphibian adults are opportunists and often will eat amphibian larvae (M.K. Gahl, personal observation), we assessed the relative abundance of adult amphibians that are larval predators using visual encounter surveys (as described above under Disease screening). We considered adults of both *L. catesbeianus* and *L. clamitans* to be opportunistic larval predators.

Statistical analysis of potential stressors

We investigated 18 chemical, biological, and physical potential stressors as predictor variables for an exploratory analysis of stressors that may be associated with ranavirus mortality events in the 26 wetlands that we monitored with in-field health screenings. We used principal components analysis (PCA) to derive composite variables representing chemical, biological, and physical stressors. We also identified surrogate variables that had the highest correlation with PCA axes. For our final analysis we retained two composite variables each from chemical and physical stressors and three variables from biological stressors.

Chemical variables included conductivity, acidity, DO, and color. For PCA, we used the mean specific conductivity (μS/cm) and mean closed-cell pH from biweekly sampling over 2004 and 2005 breeding seasons as general measurements of conductivity and pH, respectively, over the larval period. Because apparent color was closely related to DOC in our wetlands (M.K. Gahl, unpublished data), we used the mean maximum apparent color as a measure of DOC. Lastly, we initially included minimum DO measurements from our June 2005 in situ DO measurements throughout the daily sun cycle.

Biological predictor variables included amphibian breeding community, larval relative abundance, and derived indicators of larval predator densities (invertebrate and adult amphibian). Breeding status of *L. sylvaticus* and *L. clamitans* was determined in each wetland during visual encounter surveys and funnel trapping (0, not breeding; 1, breeding). We did not include breeding status for *A. maculatum* or *P. crucifer* in analyses because they bred in most wetlands. In addition, *L. catesbeianus*, Red-Spotted Newt (*Notophthalmus viridescens viridescens* (Rafinesque, 1820)), and Pickerel Frog (*Lithobates palustris* (LeConte, 1825)) were not included because they were observed breeding in only a few monitored wetlands. Relative abundance of amphibian larvae in each wetland was determined from funnel-trapping data in both 2004 and 2005. Because funnel traps were standardized based on wetland size and habitats, we used mean number of larvae per trap for all traps within a wetland over all four sampling periods as the larval density variable. Our invertebrate predator variable was the number of invertebrates per trap that will depredate amphibian larvae (e.g., family Belostomidae, order Hemiptera; family Dytiscidae, order Coleoptera; some suborder Anisoptera, order Odonata), averaged over all four trapping periods. Amphibian adults that depredate larvae were included as a separate variable calculated as the mean number of adult *L. catesbeianus* and *L. clamitans* at each wetland per visual encounter survey during the 2004–2005 field seasons.

Physical predictor variables included wetland area, canopy cover, mean water temperature, and standard deviation of water temperature (April through August in 2004 and 2005), aquatic plant cover, emergent vegetation cover, and percent shrub cover. For variables of aquatic, emergent, and shrub vegetation, we averaged the percent cover for each vegetation type over all plots along two transects in each wetland.

Principal components analysis (PCA) assumes multivariate normality, which is difficult to attain or test (McGarigal et al. 2000). Because this analysis of within-pond characteristics was largely exploratory to determine which predictor variables were worthy of further investigation, we assumed multivariate normality by attaining univariate normality and minimizing multicollinearity between variables. We determined univariate normality for each predictor variable using visual (box plots, dot density plots, normality plots) and nonparametric (Lilliefors) methods. Variables that did not meet the assumptions of normality were transformed with a square root (color, shrub cover, and canopy cover), fourth root (conductivity), or $\log_{10}$ (wetland area) function. Biological variables that were categorical (*L. clamitans* and *L. sylvaticus* breeding, ranavirus occurrence) were used without transformation as dummy variables. Multicollinearity between variables was determined by examining the Pearson correlation matrix and removing one variable of each highly correlated pair. We determined which principal components to retain based on eigenvalues, broken stick criterion, and component loadings (McGarigal et al. 2000).

We analyzed the association of ranavirus mortality events with both the surrogate variable (the highest loaded variable on each retained principal component) and the reduced principal components separately with 25 a priori logistic regression models. We used the binomial response variable of a ranavirus-caused mortality event occurrence for the years prior and during this study (1999–2005). We compared models, including a global model with all variables, using Akaike's information criterion (AIC) corrected for both small sample size and overdispersion, $QAIC_c$ (Burnham and Anderson 2002). Because AIC penalizes more complex models, a priori models were a mix of single stressor and multiple stressor models with up 2–3 stressors included. We used importance weights to assess the relative strength of each predictor variable (Burnham and Anderson 2002; Stoddard and Hayes 2005).

Temporal patterns of within-pond stressors

Because low incidence of mortality events could make finding significant associations with the environmental variables difficult, we also examined the stressors that coincide with a mortality event caused by ranavirus by analyzing the seasonal changes in physical, chemical, and biological variables on a finer scale, before, during, and after a mortality event. We included annual weather patterns (water temperature, precipitation) and collected more complete chemistry data (e.g., cations, anions, Al) from the subset of six primary

Table 1. Mortality events caused by ranavirus in Acadia National Park (ANP) from 1999 to 2005.

Wetland	Years ranavirus was confirmed	Species affected	Mortality rate (%)
29D/C-H02	2000	*Lithobates sylvaticus*, *Pseudacris crucifer*	>90
29A-H02	2002	*Lithobates sylvaticus*, *Pseudacris crucifer*	>90
15A-M01	2002	*Lithobates sylvaticus*	>90
29A-L01	2003, 2004	*Lithobates catesbeianus*, *Lithobates clamitans*	25–50
29D-L01	2004	*Lithobates catesbeianus*	Unknown* (likely <25)
29C/H01	2001	*Lithobates sylvaticus*	Unknown
29A-M01	2001	*Lithobates sylvaticus*, *Ambystoma maculatum*	Unknown
50Aa-H01	2004, 2005	*Lithobates sylvaticus*	>90
50B/Ba-H02	2001	*Lithobates clamitans*	>90

Note: Information for the years 1999–2001 was communicated by D.E. Green for diagnoses, and by M.B. Kolozsvary and B. Connery for field observations.

*Unknown refers to wetlands where mortalities were noted prior to the fieldwork of this study and therefore numbers may not have been recorded.

wetland sites. Field measurements for variables used in the among-pond analysis were the same as described above.

Air temperature and precipitation data were obtained from the nearby National Atmospheric Deposition Program – National Trends Network weather station at McFarland Hill near ANP Headquarters (NADP–NTN site No. ME98). We measured the relative difference in air temperature between catchments by programming a HOBO temperature logger (H01-001-01; Onset Computer Corporation) to record air temperature every 2 h at 1 m above the forest floor under the forest canopy near each of the six primary wetlands. We determined hydroperiod by noting the date of complete wetland drying at a staff gauge located in the deepest part of each wetland.

Three water quality samples per season from each of the six primary sites were analyzed for cations, anions, total Al, ANC, and air-equilibrated pH by the Sawyer Environmental Chemistry Laboratory (University of Maine, Orono, Maine, USA) in 2004 and 2005. Initial grab samples were collected before ice-out in early April. The second sample was collected when *L. sylvaticus* larvae began to hatch in May and the last when a die-off event was detected, or early July if no mortality event occurred.

Results

Disease screening

Of the 26 wetlands included this study, we detected mortality events attributable to ranavirus in 9 wetlands at least once between 1999 and 2005 (Table 1). During our field study period (2003–2005), most wetlands had repeat occurrences of mortality events. We suspect that mortality events may have occurred repeatedly in other wetlands as well (those with mortality events observed prior to this study 1999–2002), but were not detected in the field because of rapid decomposition or predation of carcasses.

Wetland-specific stressors

Conductivity was the most important predictor of ranavirus-affected sites in our modeling, with a greater incidence of ranavirus die-offs in wetlands with low conductivity. Few other biological or physical variables included in our models were strong predictors of ranavirus-caused mortality events.

Of the initial set of potential stressors, we discarded maximum DO and minimum color from further analyses because of their high correlation to minimum DO ($r = 0.794$) and maximum apparent color ($r = 0.742$), respectively. Because these analyses were largely exploratory, other correlated variables were left in the analyses because they addressed different potential stressors and correlations were not as high (Table 2).

Two principal components each for chemical and physical variable groups and three principal components for biological variables were retained (Fig. 2). The two chemical principal components explained 73% of the variation within the water chemistry data. The first was a gradient of acidity (CHEM1), from high pH, high DO, and low apparent color ranging to low pH, low DO, and high apparent color, with apparent color being the heaviest loaded variable. Conductivity (CHEM2) was the second key chemical gradient. Two principal components describing physical characteristics of wetlands explained 66% of the variance. The first was a gradient of canopy cover (PHYS1), the heaviest loaded variable, with canopy and shrub cover lowest when wetland area is greatest. The second was a gradient of temperature (PHYS2), including both standard deviation of temperature (the heaviest weighted variable) and mean temperature. Three principal components retained as biological variables explained 79% of the variance. BIO1 was a gradient from *L. clamitans* breeding and abundant adult amphibians that may act as larval predators to lower predator abundance and no *L. clamitans* breeding. BIO2 was a gradient of larval density and BIO3 was a gradient of invertebrate predator abundance (primarily larvae and adults of Dytiscidae, order Coleoptera, across all ponds).

The most important predictor of ranavirus-affected sites using the relative importance weights for variables from AIC analyses of both the surrogate variable and the principal components was low conductivity or CHEM2 (Tables 3, 4), with a greater incidence of ranavirus die-offs in wetlands with low conductivity. The next two important surrogate variables were standard deviation of temperature and maximum color (Table 4). Using the principal components in the same analyses yielded similar results, though no other principal components were considered relatively important besides CHEM2, which reflected conductivity (Table 4). However, none of the surrogate variables or principal com-

Table 2. Pearson correlation matrix for variables included in principal components analysis.

	LISY breeding	LICL breeding	Larval density	Amph. pred.	Invert. pred.	Mean temp.	SD temp.	Aquat. veg.	Emerg. veg.	Area	Shrub veg.	Canopy cover	Min. DO	Max. color	Mean conduct.	CC pH
LISY breeding	1.000															
LICL breeding	–0.184	1.000														
Larval density	0.451	–0.007	1.000													
Amph. pred.	–0.115	0.525	0.141	1.000												
Invert. pred.	–0.083	0.223	–0.232	0.218	1.000											
Mean temp.	0.057	0.514	0.379	0.607	0.289	1.000										
SD temp.	0.019	–0.022	0.382	0.417	–0.060	0.566	1.000									
Aquat. veg.	0.064	–0.012	0.127	0.076	0.179	0.118	0.197	1.000								
Emerg. veg.	0.145	0.348	–0.057	0.028	0.056	0.089	–0.276	–0.341	1.000							
Area	–0.043	0.522	–0.064	0.515	0.307	0.313	–0.090	–0.288	0.466	1.000						
Shrub veg.	0.140	–0.328	0.047	–0.423	0.050	–0.128	–0.220	0.277	–0.176	–0.433	1.000					
Canopy cover	0.083	–0.585	–0.155	–0.565	–0.187	–0.559	–0.209	0.202	–0.417	–0.721	0.600	1.000				
Min. DO	–0.067	–0.061	0.109	0.087	–0.267	0.388	0.615	–0.221	–0.132	–0.041	–0.235	–0.233	1.000			
Max. color	0.266	–0.165	0.068	–0.056	0.280	0.014	–0.119	0.477	–0.196	–0.233	0.453	0.307	–0.519	1.000		
Mean conduct.	0.341	–0.014	0.047	–0.261	0.200	–0.071	–0.173	–0.108	0.389	0.184	0.133	–0.024	0.039	0.119	1.000	
CC pH	–0.286	0.336	–0.258	0.270	–0.024	0.015	–0.077	–0.585	0.502	0.626	–0.536	–0.495	0.195	–0.622	0.014	1.000

Note: LISY, *Lithobates sylvaticus*; LICL, *Lithobates clamitans*; amph. pred., amphibian predators; invert. pred., invertebrate predators; mean temp., mean temperature; SD temp., standard deviation of temperature; aquat. veg., aquatic vegetation; emerg. veg., emergent vegetation; shrub veg., shrub vegetation; min. DO, minimum dissolved oxygen; max. color, maximum color; mean conduct., mean conductivity; CC pH, closed-cell pH.

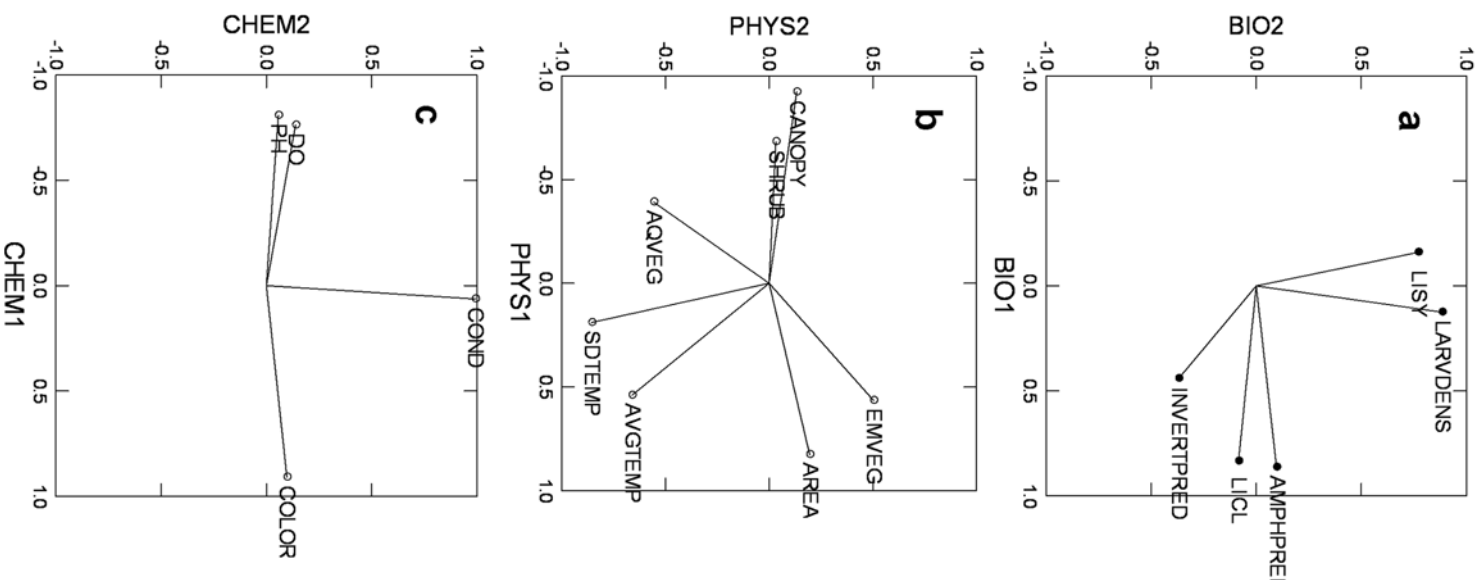


Fig. 2. Plots of factor loadings for principal components of (*a*) biological (AMPHPRED, amphibian predators; INVERTPRED, invertebrate predators; LARVDENS, larval density; LICL, *Lithobates sylvaticus*; LISY, *Lithobates clamitans*), (*b*) physical (AQVEG, aquatic vegetation; AVGTEMP, mean temperature; EMVEG, emergent vegetation; SDTEMP, standard deviation of temperature), and (*c*) chemical (DO, dissolved oxygen; COND, conductivity) variables. Plots illustrate the weighting of each variable on each axis. Only the first two principal components are shown.

Table 3. Highest-ranked logistic regression models correlated with mortality events of amphibian larvae caused by ranavirus in Acadia National Park (ANP) breeding pools.

Candidate model	Log (L)*	$K^{\dagger}$	$QAIC_c$	$\Delta QAIC_c^{\ddagger}$	$w_i^{\S}$	Evidence ratio$^{\|}$
Surrogate variable models						
Conductivity (–)	–13.046	3	12.95	0.00	0.12	1.00
Canopy cover (–)	–14.496	3	13.59	0.64	0.08	1.38
LICL (+)	–14.708	3	13.68	0.73	0.08	1.44
Invertebrate predators	–15.078	3	13.85	0.90	0.07	1.57
Maximum color	–15.422	3	14.00	1.05	0.07	1.69
SD temperature	–15.492	3	14.03	1.08	0.07	1.71
Larval density	–15.672	3	14.11	1.16	0.07	1.78
SD temperature, conductivity	–11.404	4	15.13	2.18	0.04	2.98
Global model	0.00	9	30.86	17.91	<0.01	7 729.75
Principal components models						
CHEM2	–12.634	3	15.97	0.00	0.15	1.00
BIO1	–14.451	3	17.23	1.26	0.08	1.88
BIO2	–14.673	3	17.39	1.42	0.07	2.03
PHYS2	–15.123	3	17.70	1.73	0.06	2.37
CHEM2, BIO2	–11.189	4	17.87	1.90	0.06	2.59
CHEM1	–15.652	3	18.07	2.10	0.05	2.85
CHEM2, BIO3	–11.49	4	18.08	2.11	0.05	2.87
PHYS1	–15.699	3	18.10	2.13	0.05	2.90
BIO3	–15.77	3	18.15	2.18	0.05	2.97
Global model	–5.796	9	34.88	18.91	<0.01	12 768.51

Note: The relationship (negative or positive) of conductivity, canopy cover, and LICL to disease occurrence is shown in parentheses. Models with evidence ratio <3 are shown. LICL, *Lithobates clamitans*; SD, standard deviation; see text for explanation of the principal components models.

*Log-likelihood from the logistic regression for each model.

$^{\dagger}K$ is the number of parameters in a model, including the y intercept (constant).

‡Akaike's information criterion (AIC) difference ($QAIC_c$) is the difference between the model and the highest-ranked model. $QAIC_c$ <2 is considered substantial evidence to be included in the set of best approximating models.

§Akaike weights (w_i) represent the likelihood of a model or model probability.

$^{\|}$Evidence ratio is the relative likelihood of a model compared with the highest-ranked model. Evidence ratio <3 is considered the threshold for inclusion in the set of best-approximating models.

Table 4. Relative importance of individual parameters correlated with ranavirus-caused die-off events in Acadia National Park (ANP) breeding ponds.

Parameter	Σw_i*
Surrogate variables	
Conductivity	0.33
SD temperature	0.21
Maximum color	0.20
LICL breeding	0.18
Invertebrate predators	0.17
Larval density	0.17
Canopy cover	0.17
Principal components	
CHEM2	0.42
PHYS2	0.17
CHEM1	0.18
BIO1	0.20
BIO3	0.15
BIO2	0.22
PHYS1	0.14

Note: Surrogate variables are listed in order by relative importance weight and principal components are listed in the same order as their surrogate variables. SD, standard deviation.

*The importance weight (Σw_i) of a parameter is the sum of Akaike weights from all candidate models which contain that parameter.

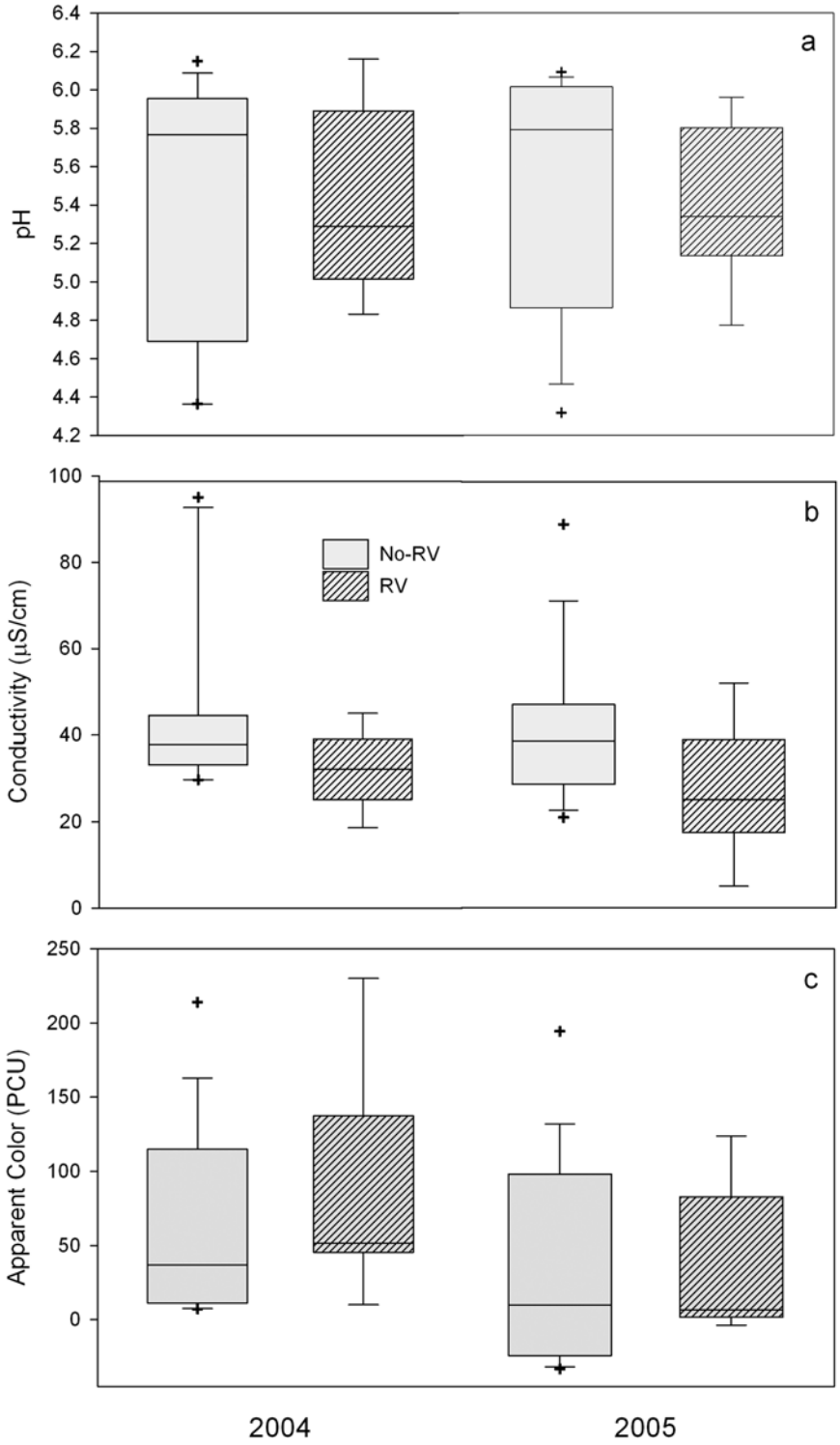
ponents was a good predictor of ranavirus-caused die-off events because all had logistic regression p values >0.05, and only conductivity had a p value <0.10. Therefore, although AIC was able to identify the best approximating models, none of these models was a good predictor of ranavirus-related mortalities.

Temporal patterns of within-pond stressors

Although no statistical differences in wetland chemistry were evident in our modeling, some general patterns were compelling. Sites with high conductivity (>60 μS/cm) were typically nondisease sites, while sites affected by ranavirus tended to have much lower conductivity (Fig. 3*b*). Acidity exhibited the opposite pattern as conductivity (Fig. 3*a*), and sites with pH <4.5 were more likely to be unaffected by ranavirus. Mean water temperature over the breeding season was also higher in sites affected by ranavirus (Fig. 4). We observed no evident patterns of apparent color in ranavirus affected sites (Fig. 3*c*), although overall, ANP wetlands were highly colored (20–400 PCU, where PCU is the platinum–cobalt unit; the higher the PCU value, the darker the water sample).

In intensively studied sites with documented ranavirus, total Al and DOC tended to be higher and SO_4 tended to be lower than in similar unaffected sites (Appendix A, Table A1), although our sample size is too small for this correlation to be statistically significant. Total Al was higher

Fig. 3. Incidence of ranavirus (RV) and (*a*) closed-cell pH, (*b*) conductivity (μS/cm), and (*c*) apparent color (PCU, platinum–cobalt unit) in all breeding wetlands located in Acadia National Park (ANP) during 2004 and 2005. +, outliers.



in ranavirus-affected sites (Fig. 5), sites with pH <5.6, and was correlated with higher DOC. We observed high total Al levels (>200 μg/L) above known sublethal concentrations of monomeric inorganic Al that may stress amphibians (Clark and LaZerte 1985; Andren et al. 1988; Jung and Jagoe 1995) in two wetlands that experienced mortality events caused by ranavirus in 2004 and 2005, though this is not conclusive because we did not examine how much of the total Al measured reflects bioavailable inorganic monomeric Al in these wetlands.

In one primary site, larval mortality events of *L. sylvaticus* and *P. crucifer* were caused by ranavirus in July of 2004 and 2005. Examination of time-series data associated with this site yielded no acute changes in air temperature,

Fig. 4. Mean temperature during the breeding season in ranavirus-affected (RV) and non-ranavirus-affected (NO-RV) wetlands located in Acadia National Park (ANP) during 2003, 2004, and 2005. +, outliers.

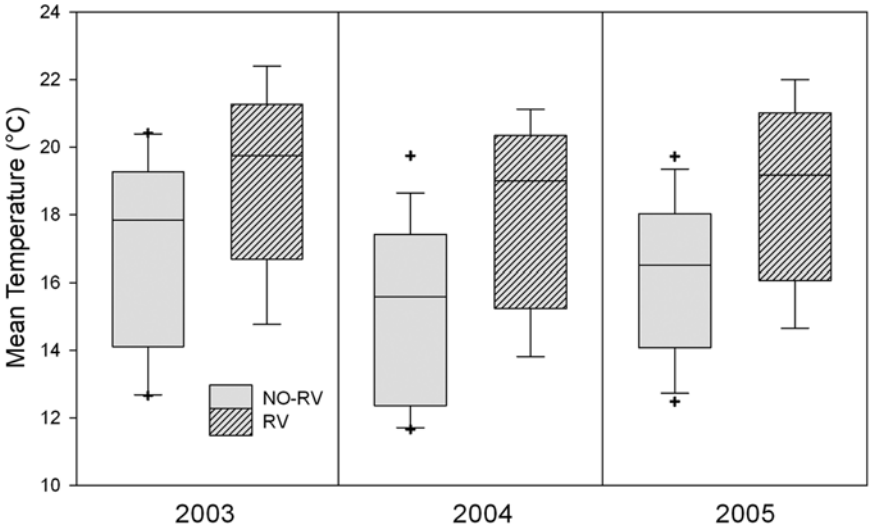
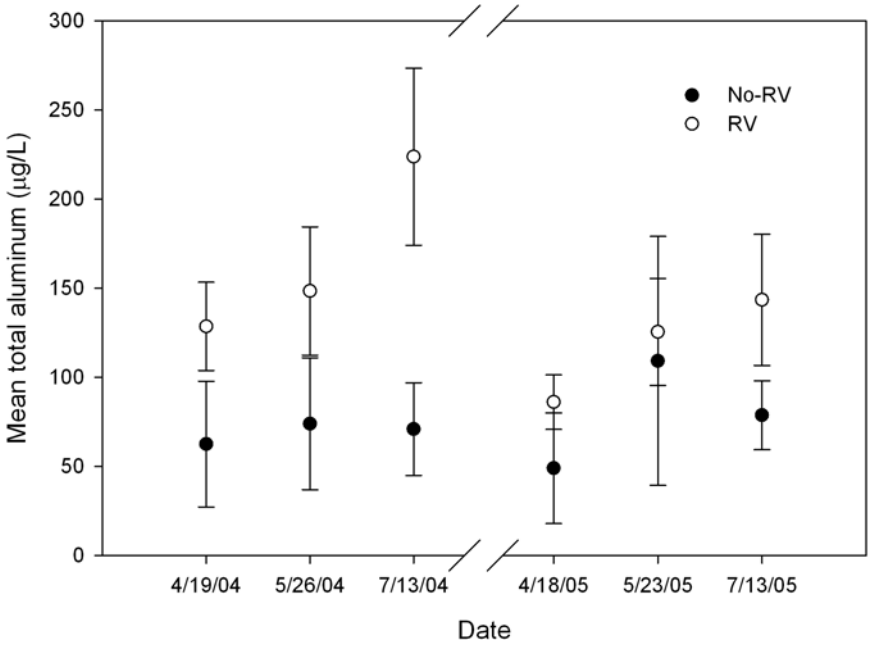


Fig. 5. Total aluminum (Al; μg/L) in the six intensive sites for three sample periods in 2004 and 2005, separated by disease incidence. Mortality events occurred in ranavirus-affected (RV) sites just after the last sampling period of each year (13 July). Date is presented as month/day/year.

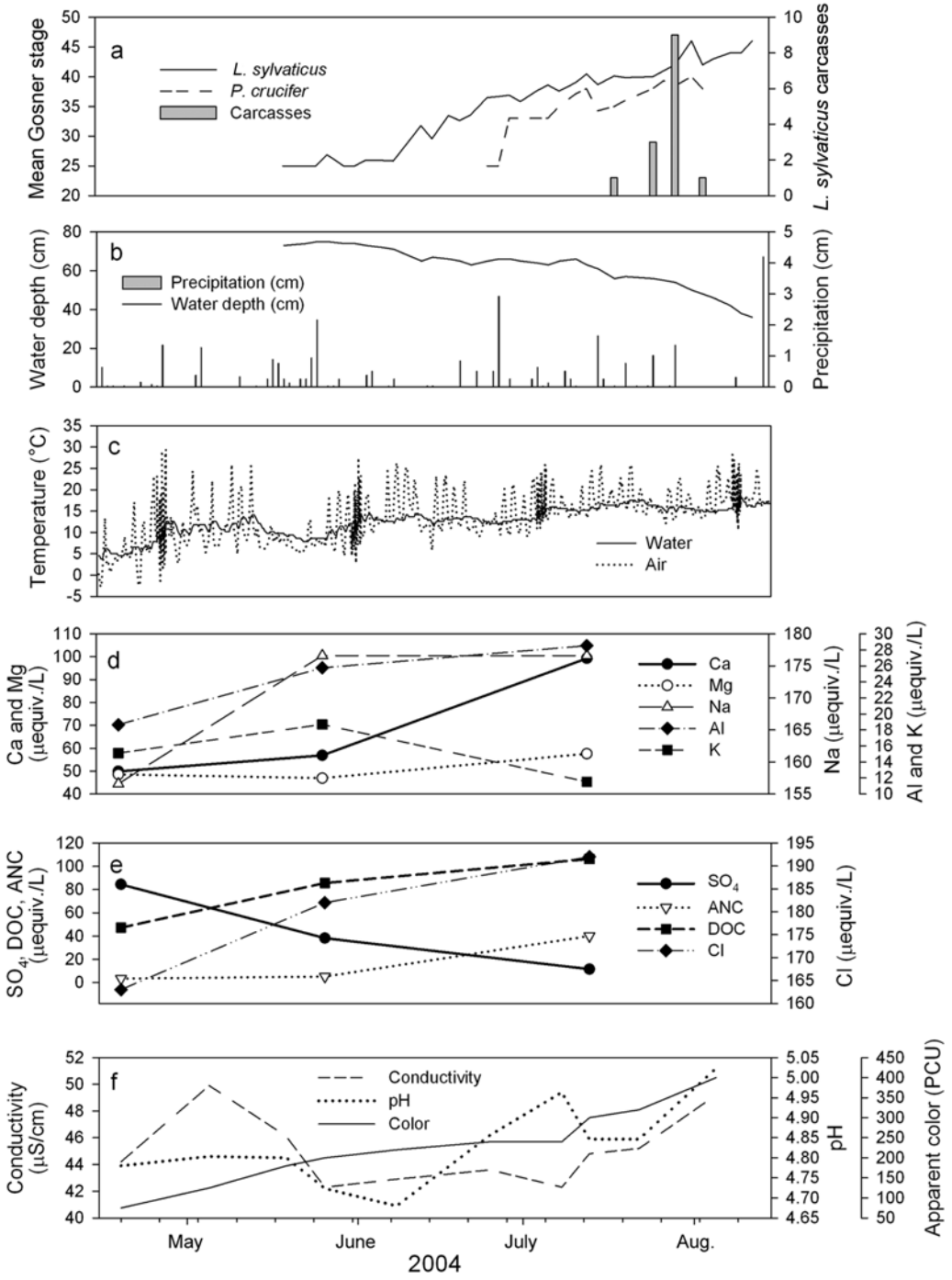


pH, apparent color, or conductivity that might have prompted a mortality event (Figs. 6*a*–6*f*). However, near the time of the mortality events Al, ANC, Na, and DOC had perceptibly risen (Figs. 6*d*, 6*e*) and concentrations of SO_4 had dramatically declined (Fig. 6*e*). These fluctuations were accompanied by wetland dry down, and therefore could be attributed to evapoconcentration and may be common to most sites drying down in that period (Fig. 6*b*). In addition, mortality events were not correlated with hydrology; rather, they occurred in wetlands of all hydroperiods (temporary, permanent, and semi-permanent). Although no strong correlation with chemical or physical data was apparent, these mortality events did occur at later stages of larval development (Fig. 6*a*).

Discussion

In our statistical analyses of potential stressors and ranavirus incidence, no sublethal stressors were strongly correlated with ranavirus-caused mortality events. All variables found relatively important in predicting disease outbreak for ranavirus were unable to successfully predict disease in the logistic regression models. This suggests that other factors may be more important in predicting ranavirus outbreaks and emphasizes the complexity of these systems. This result could be a consequence of the stressors that we chose to include (or not to include), or may indicate that host specificity is also important in determining whether an animal will become infected.

Fig. 6. Pond characteristics through the 2004 season at site 50Aa-H01 in Acadia National Park (ANP) where a mortality event had occurred: (*a*) mean Gosner stages of *L. sylvaticus* and *P. crucifer* and number of fresh carcasses of *L. sylvaticus* collected; (*b*) water depth and precipitation; (*c*) air and water temperature; (*d*) cation (Ca, Mg, Na, Al, K) chemistry; (*e*) anions (SO_4, Cl), dissolved organic carbon (DOC), and acid-neutralizing capacity (ANC); and (*f*) biweekly conductivity, closed-cell pH, and apparent color. Mortality events occurred around 29 July – 3 August 2004.



Our results suggest that amphibian life stage and the species affected may play a key role in determining when ranavirus-caused mortalities occur. In our time-series analysis, ranavirus mortality events, while not strongly associated with potential stressors, always occurred when larvae were in later Gosner stages (>39 Gosner stage; Fig. 6*a*) as reported in other studies (Green et al. 2002). Amphibians in this later larval stage are understood to experience temporary immunosuppression because of immune system reorganization during metamorphosis (Rollins-Smith 1998). Later larval stage amphibians also would be more likely to experience higher densities because of lower water levels and larger animals, and therefore would have more contacts with other conspecifics that may affect the course of an

outbreak (Brunner et al. 2004). In addition, the majority of mortality events occurred in *L. sylvaticus* populations (Table 1). These suggest that amphibian life stage and species may be important predictors of ranavirus mortality events.

Conductivity was the most important factor associated with ranavirus in AIC model comparisons, although it was not a statistically significant predictor of disease. Sites with higher conductivity (>50 μS/cm) did not have ranavirus outbreaks, whereas some sites with lower conductivity (<50 μS/cm) were affected by ranavirus (Fig. 3*b*). The lack of disease occurrence in sites with higher conductivity was unexpected because road salt influxes that cause high conductivity in roadside wetlands are known to create a stressful environment for amphibian larvae (Sanzo and Hecnar 2006; Karraker et al. 2008). However, sites with low conductivity in ANP were distant from roads and were usually high catchment position vernal pools that were suspected in ANP to have higher incidence of ranavirus (Gahl and Calhoun 2008), possibly because of the presence of larval populations of the highly susceptible *L. sylvaticus* (Harp and Petranka 2006).

Temperature may also play a role in ranavirus-caused mortality events. On an acute scale within a wetland, no apparent correlation with temperature was evident during the onset of a mortality event. However, among wetlands, sites that experienced mortality events were markedly warmer the mean values of ANP wetlands (mean 18–20 °C; Fig. 4). This contrasts with the existing literature on iridovirus epidemics caused by the related *Ambystoma tigrinum* virus (ATV) that occur during colder spells, ~10 °C (Jancovich et al. 1997; Bollinger et al. 1999), but is consistent with laboratory experiments with ATV that demonstrated complete mortality of larval salamanders at 18 °C (Rojas et al. 2005). The ranavirus strains found in most anurans could be regulated in part by warmer temperatures in northern climates as suggested by epidemics that occur in mid-late summer warm spells (Green et al. 2002; Cunningham et al. 2007).

Aluminum may be a potentially important stressor in the wetland sites that experienced mortality events in our study. ANP wetlands are naturally low in pH, ideal conditions for speciation to inorganic Al. ANP waters are also naturally low in Ca (<2 mg/L) (Appendix A, Table A1), which can mitigate the effects of toxic inorganic Al in fish (Brown 1983). The temporal changes in Al were particularly important, because small wetlands become much more concentrated with DOC and other solutes as they dry each year (Figs. 6*b*, 6*d*, 6*e*). Highly concentrated solutes, such as inorganic Al, are more susceptible to precipitation because of oversaturation within the water column, which can result in increased bioavailability to aquatic organisms within a drying wetland. This drying in seasonal wetlands occurs at the same time as amphibian larvae reach later Gosner stages toward metamorphosis (Figs. 6*a*, 6*b*), so we speculate that the combination of increased concentrations of Al, low Ca, and a more vulnerable amphibian larval stage may increase mortalities.

Acidity had less of an effect on ranavirus incidence than we initially expected. We expected sites with lower pH (<4.5) to sublethally stress amphibian larvae, but in our field observations these sites did not experience mortality events (Fig. 3*a*). It is possible that the disease organisms are not able to thrive at low pH (<4.5). In addition, many of these sites with low pH host small amphibian breeding populations (<5 *L. sylvaticus* egg masses and <10 *A. maculatum* egg masses in a breeding season), which may influence disease dynamics. Although acidity was not an important factor by itself in influencing amphibian disease in our study, it can affect or indicate other chemical properties of water that may more directly influence amphibian health (e.g., Al, DOC).

The dramatic declines of amphibians around the world, as well as continued mortality events in protected and pristine settings, likely have multiple and complex causes. Of the potential stressors we investigated, higher Al, warmer temperatures, and lower conductivity are the most important to investigate more directly. We provide some evidence that ranavirus is regulated by disease ecology, particularly the most susceptible life stage (>39 Gosner stage) and species-specific vulnerability (particularly *L. sylvaticus*). Sublethal stressors were difficult to identify in the field and, in our study, very few were significantly associated with ranavirus mortality events across all breeding ponds.

Because amphibians are exposed to a suite of sublethal stressors in virtually every environment in which they live, determining a tipping point for when stressors increase susceptibility to disease is difficult to achieve in field studies. However, with newer methods of noninvasive disease sampling (Greer and Collins 2007; St-Amour and Lesbarrères 2007), future studies could begin to address how these sublethal stressors may increase or decrease disease prevalence in the field. In addition, each species may respond differently to each suite of stressors (Blaustein and Kiesecker 2002). In our study, it was unclear whether multiple sublethal stressors encouraged susceptibility to ranavirus mortality events, and we suspect that each wetland, because of varied hydroperiods, landscape settings, and biological communities will have its own suite of stressors that can sublethally affect amphibians.

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Appendix A

Appendix Table A1 is found on the following page.

Table A1. Water chemistry in six primary wetlands in Acadia National Park (ANP) during 2004 and 2005.

	Ranavirus-affected sites		BUBM		ELCR	
	Mean	Range	Mean	Range	Mean	Range
Al (μg/L)	142.62	54.9–257	110.45	80–179	37.65	18–59.4
Ca (mg/L)	1.02	0.31–2.04	1.51	0.78–2.28	17.53	11.1–25.3
Mg (mg/L)	0.38	0.13–0.7	0.49	0.3–0.62	1.52	0.85–2.05
K (mg/L)	0.36	0.09–0.73	0.18	0.12–0.24	0.94	0.25–1.62
Na (mg/L)	3.11	1.85–2.71	3.58	2.18–4.37	83.02	35.8–142
Cl (mg/L)	4.78	2.62–7.73	5.07	2.38–6.81	145.47	63.44–226.21
SO_4 (μequiv./L)	33.23	4.6–84.5	73.35	55–90	91.23	44.5–139
ANC (μequiv./L)	32.00	0–119	69.63	22.8–127	340.83	169–520
DOC (mg/L)	10.39	4.01–26.6	3.04	1.58–4.26	4.87	2.36–7.8
Air-equilibrated pH	5.89	4.68–6.89	6.74	6.24–7.05	7.47	7.28–7.67
Closed-cell pH	5.21	1.06–6.06	5.41	4.86–5.66	6.00	5.52–6.51
Conductivity (μS/cm)	32.0	18.5–46.2	37.2	23.7–45	407.1	70.3–746

Note: Sites BUBM and ELCR did not experience ranavirus-related infections or die-off events from 2000 to 2005. ELCR is presented separately because the site is largely influenced by winter road salting. ANC, acid-neutralizing capacity; DOC, dissolved organic carbon.