Monitoring of Health of Specific Amphibians

By Cynthia Carey

FINAL REPORT

BOULDER OPEN SPACE AND BOULDER MOUNTAIN PARKS

Monitoring of Health of Specific Amphibians

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ABSTRACT

The phase of the research conducted by Cynthia Carey on this grant from Boulder Open Space was designed to survey two amphibian populations for signs of disease and to assess the immune status of these populations as an indirect, but predictive, way of evaluating whether these populations are under sufficient stress that they could become vulnerable to disease. Since populations of Woodhouse's toad *(Bufo woodhousii)* and tiger salamanders *(Ambystoma tigrinum)* on Boulder Open Space land were either too small for our study or were being studied by other researchers, permission was obtained to work on the Woodhouses's toad and tiger salamander populations at the Boulder Reservoir. This latter site is administered by Boulder Mountain Parks. No diseased salamanders nor toads were found at the Boulder Reservoir. An immunological method was used to determine whether Woodhouse's toads were experiencing significant degrees of stress similar to those observed in laboratory or field observations on other amphibians. The levels of immune function observed in Woodhouse's toad throughout the summer of 1996 do not indicate significant degrees of stress. However, extreme fluctuations in reservoir water level obliterated almost all breeding of Woodhouse's toads and human actions caused mortality in approximately 1/3 of the adult breeding population. Due to factors that will be explained in the report, the tiger salamanders did not breed at the reservoir this year. Therefore all the research on tiger salamanders was conducted elsewhere in Colorado. A method for assaying one immunological parameter in salamander larvae was developed and used in a preliminary study to determine whether ultraviolet-B light (UVB) could cause immunosuppression in salamanders. UVB may have a significant impact on tiger salamander populations if the water is clear enough to allow UVB transmission to the levels at which eggs and larvae exist.

OBJECTIVES

As stated in detail in our original proposal, amphibian populations are declining on the 6 continents on which they occur (Vial and Saylor, 1993). In almost all cases in which habitat destruction is not the cause of the decline, disease is the primary cause of death in instances in which deaths have actually been observed (Bradford 1991, Carey 1993, Taylor et al. 1995, Laurance et al., 1996). The disease vectors are principally bacteria and fungi which should normally be kept in check by the immune systems of the amphibians (Taylor et al. 1995). Because declines in some amphibians in Boulder County have already been documented (Corn et al. 1989; Hammerson 1982; Livo 1984, 1986, 1995), it was the goal of this project to determine 1) whether amphibians on Boulder Open Space land were dying of disease, and 2) whether the animals showed immunological signs of being stressed by one or more unspecified environmental factors in a manner that could make them vulnerable to disease. Specifically, we used an immunological assay that we have found to be an indicator of stress in other studies (Carey et al., 1996a,b and unpubl. data). Figures 1 and 2 indicate that the proliferative responses of certain types of white blood cells (lymphocytes) to chemicals that act like foreign invaders (mitogens) show significantly increased stimulation when the animals are subjected to stresses, such as tailings from a molybdenum mine or low pH for periods of time. It is unknown why these stresses cause immunostimulation and what the link is between immunostimulation and vulnerability to disease. However, we feel this response to stress is reliable enough that it can be used as a predictive biomarker to assess whether populations of amphibians in the field are experiencing stress and on the verge of succumbing to disease.

Because populations of Woodhouse's toads and tiger salamanders on Open Space land were deemed either too small for this study or were being studied by other researchers, populations of these animals were studied at the Boulder Reservoir.

Studies by one research group (Blaustein et al. 1994) have implicated ultraviolet-B light (UVB) as a possible factor in the decline of amphibians. Because of press conferences, appearances on ABC news and The Today Show, his research has received considerable amount of publicity and has led many scientists and lay persons to believe that UVB is the causative factor in the decline of amphibians world-wide. Although this research has received considerable criticism recently (Grant and Licht, 1995, Licht and Grant, 1997), anecdotal information indicates that UVB can cause increased vulnerability to disease in laboratory mammals. Therefore, we wanted to begin to explore what UVB levels salamanders are exposed to in Colorado and whether these exposure levels could cause immunosuppression and increased vulnerability to disease.

Woodhouse's Toad

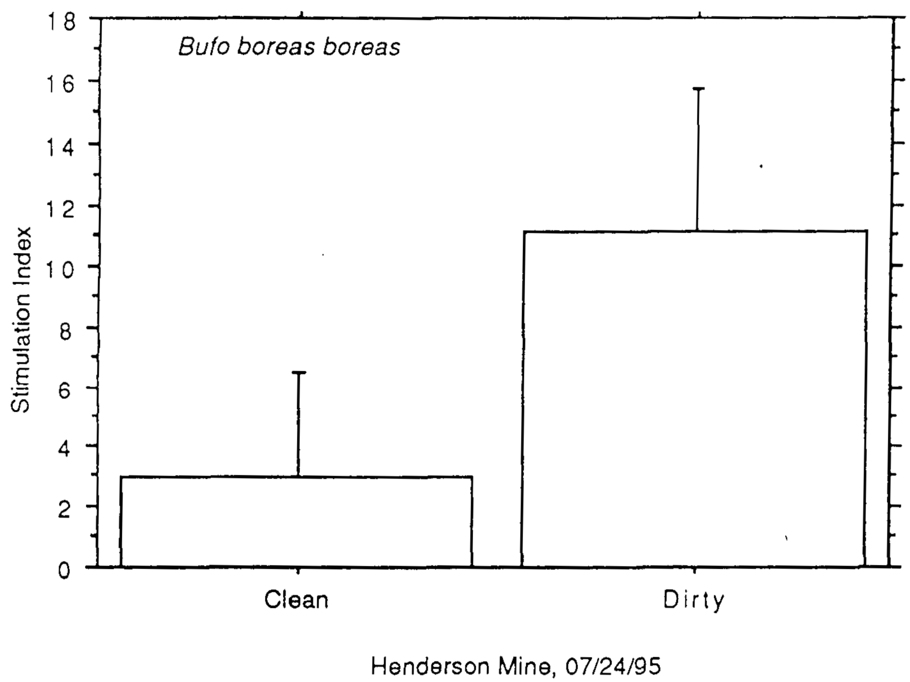
A large population of Woodhouse's toad breeds yearly at the NE end of Boulder Reservoir (40° 5' N, 105° 12' 30" W) near the spillway. Breeding started in late May in 1996, in mid-June in 1995. The large amount of rainfall through May in 1995 resulted in the difference in onset of breeding between the two years. The numbers of adults involved in breeding in 1996 was approximately 20 males and an unknown number of females. Metamorphosis occurred in late July. Both juveniles and adults enter hibernation by early September.

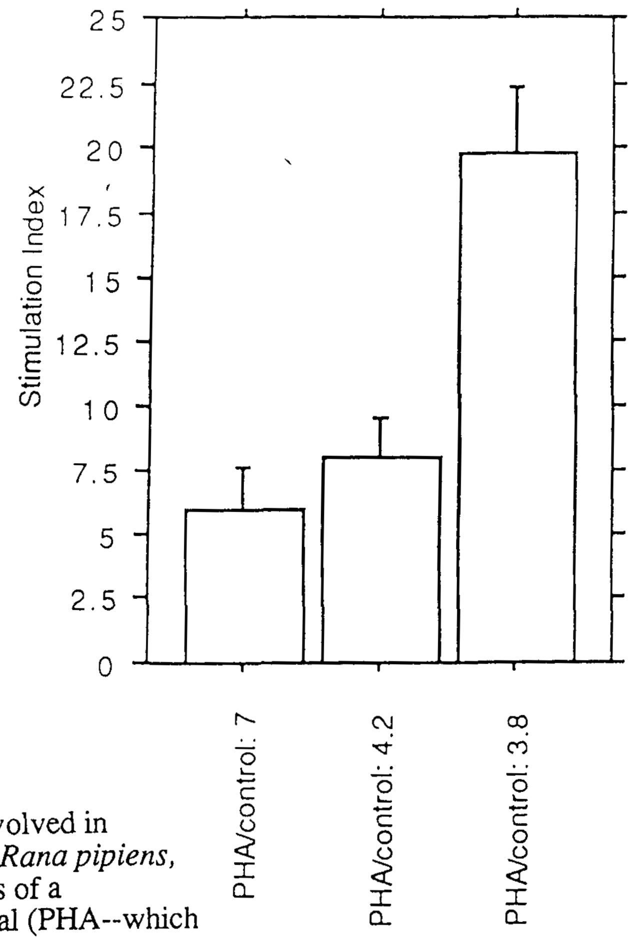
Methods

Five toads were sampled on one night each in May, June, July and August. The toads were caught by hand at the reservoir after 9:30 pm each night. Blood was collected by heart puncture with a 1 ml syringe using a 20 ga needle. The syringes were heparinized with sterile heparin. We have shown in our laboratory work on *Rana pipiens* and *Bufo marinus* that this method results in mortality about 1 out of every 145 blood samples.

The following methods have been developed in my laboratory as modifications of methods on mammalian blood and have served as the basis for other studies on amphibian immune function (Carey et al. 1996a, b). About 0.5 to 0.8 ml blood was placed in sterile nutrient broth (RPMI [Sigma] containing 5 % fetal calf serum). This solution was refrigerated overnight and then taken to the laboratory for analysis. Lymphocytes were separated by centrifugation for 30 min at 1200 rpm on a Percoll density gradient. After three washes in sterile amphibian phosphate buffered saline, cells were counted and the viability of the cells was checked by trypan-blue exclusion. The cells were then reconstituted in a volume of RPMI to reach a final concentration of 5×10^4 cells/ 100 µl . Cells were pipetted into 96-well flat-bottomed plates and were incubated in the presence or absence of 1.25 µg/ml PHA (phytohemagglutinin, a T-cell mitogen) added at the initiation of the culture in 100 µl in complete RPMI. This concentration of PHA was found in preliminary tests to cause the highest amount of stimulation of lymphocytes. Six control and six mitogen wells are run per animal. After incubation for 72 hr at 29 C in a 5% CO_2/95% air environment, the cells were pulsed with ^{3}H- thymidine (0.5 µci/well) for 18 hr and then harvested onto a glass fiber filter using a Harvard cell harvester . ^{3}H-thymidine absorbed by the cells was quantified by liquid-scintillation spectrometry. The average counts per minute of cells stimulated by PHA were averaged for each animal and then divided by the average value of unstimulated control cells for that animal. These averages, comprising the "stimulation index" for each animal, were then averaged to arrive at the mean for each test group. One-way analysis of variance was used to determine if significant differences existed among averages for each test group.

Results





Figures 1 (on left) and 2 (on right)

These data reflect the abilities of lymphocytes (a type of white blood cell involved in immune function) of toads *(Bufo boreas boreas;* Fig. 1) or northern leopard frogs *(Rana pipiens,* Fig. 2) to take up radioactively-labelled thymidine. The data are presented in terms of a stimulation index: the average of counts per minute of cells stimulated by a chemical (PHA--which stimulates cells to divide) divided by the background counts per minute of cells that are not incubated with PHA. The higher the stimulation index, the greater the amount of radioactive label taken up into the cells.

In Fig. 1, the samples came from two populations of boreal toads: one living in a pond contaminated with mine tailings from a molybdenum mine ("dirty"), and the other from an uncontaminated site ("clean"). In Fig. 2, groups of frogs were exposed to one of three different pH's of water: 7.0, 4.2 and 3.8.

The data show that the "stressed" groups (toads in the dirty pond and frogs at pH 3.8) had the highest level of uptake of radioactive label. We believe that this assay can be used successfully as an indicator of environmental stress on a population.

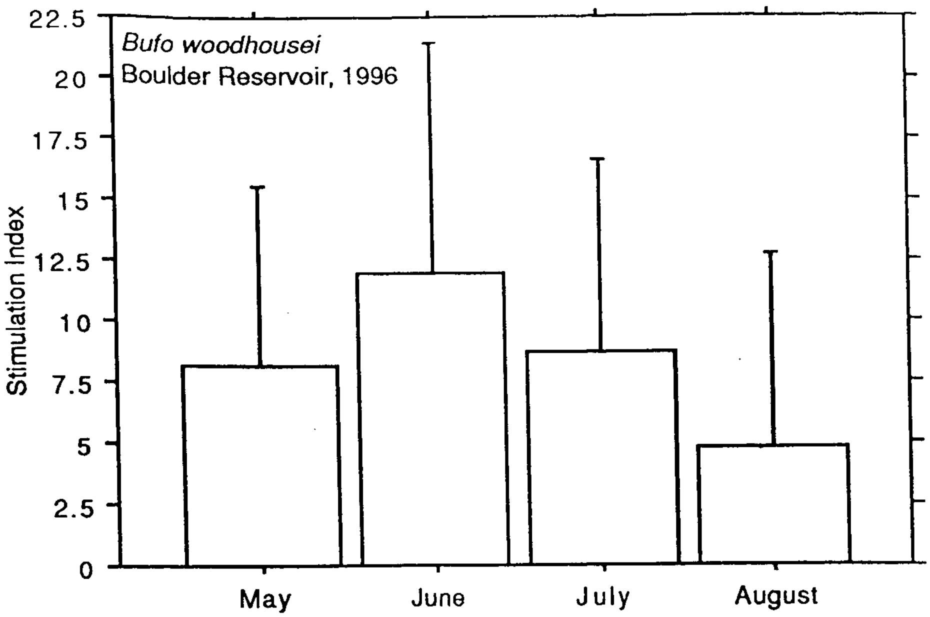


Fig. 3. Mean ± SE stimulation index of lymphocytes of *Bufo woodhousii* measured on animals sampled at Boulder Reservoir during 4 months of 1996. Sample size = 5 for each average. Analysis of variance indicates no significant differences between samples.

The proliferative response of lymphocytes of Woodhouse's toad to the mitogen PHA did not vary significantly (P = 0.34) over the 4-mo sampling period (Fig. 3). Outbred animals in natural populations usually vary considerably in lymphocytic proliferation tests and in other immunological parameters. Since we sampled only 5 animals per night, it is possible that additional samples per night might have resulted in significant differences. However, we did not want risk sampling numerous individuals and possible mortality from the sampling. Since the numbers of Woodhouse's toads sampled were similar to the numbers of individuals sampled in Fig. 1 and 2, we believe that the conclusion that the overall level of lymphocytic proliferation and the lack of seasonal variation indicate that these toads were not highly stressed and that the likelihood of a mass-die off was low during this past summer. No external signs of disease were noted on any animal we handled and no dead animals were observed in the field at the margins of the reservoir or on the banks of ditches or small ponds near the reservoir.

Discussion

As mentioned above, populations of amphibians are declining throughout the world and a few species have completely become extinct. One of the perplexing issues about these declines is that in each general locality in which declines have occurred, some species have been impacted while other species are unharmed. For instance, frogs *(Rana pipiens)* and boreal toads *(Bufo boreas boreas)* populations in the mountains of Colorado experienced severe declines and many complete extinctions in the late 1970's and early 1980's. However, chorus frogs *(Pseudacris triseriata*) and tiger salamanders *(Ambystoma tigrinum)* living and breeding in the same ponds were apparently unaffected by the cataclysmic events that caused population declines in the toads and frogs (Carey, 1993). Frogs living in eastern Boulder County have declined also (Livo, 1984), yet Woodhouse's toad appears to be doing well. Unless we are dealing with a species-specific virus, both toads and frogs should be affected by the same pollutants, toxicants and other man-made airborne and water borne chemicals, atmospheric changes and global climate changes. What is different about species that survive versus those that decline? This study contributes in a small way of the answer to this very large and complex question: Woodhouses's toads at the Boulder Reservoir are certainly exposed to a number of water and airborne man-made chemicals, UVB, and possibly other stresses, yet they are not showing signs of immune disruption and disease. We don't know why but future research could help determine what characteristics they have which make them more resistant to stress than other species.

If environmental stresses were the only factors potentially affecting the toads, our results would suggest no immediate cause for concern. However, two types of events at the reservoir make the future survival of this population a serious concern. First, it was apparent that one or more persons who work either for the reservoir or for the water district and who have authorization to drive on the roads on the top of the dam and to the NE of the dam at night deliberately kill toads by running over them. I ran almost every morning during the summer on those roads and found 12 toads that had been smashed by a vehicle during the previous night. The individual(s) smashing the toads were clearly making an effort to run over the toads, since tire tracks swerved into the center or to the margins of the road in order to smash the toads. These deliberate smashings may have resulted in death for up to 1/3 of the adults in the breeding population. The life history of Woodhouse's toads has not been studied in detail, so the natural rate of population turnover is not known. However, it can be concluded that the man-made loss of breeding adults does not contribute to long-term viability of this population.

Second, breeding in this population in 1995 was successful as judged by large numbers of metamorphic individuals present in late July and numbers of juveniles present just before hibernation in September. In 1996, toads began to breed in May just as the water levels in the reservoir rose. Toads need shallow areas for deposition of eggs to minimize predation and maximize the temperatures at which the eggs and larvae can develop. To my knowledge, no eggs survived this flooding. However, at least one pair took advantage of the rains that occurred in mid-June that flooded the spillway basin below the dam. However, there is no way for toadlets to escape from the spillway basin. We spent over 20 person-hours rescuing toadlets from the spillway and releasing them at the NE corner of the reservoir where eggs are normally laid. Because of predation by birds and bullfrogs, it is unclear that any toadlets survived to hibernation.

Management Suggestions

1. Our immunological survey indicates no immediate cause for concern for the Woodhouse's toads at the Boulder Reservoir to the extent that they show no significant signs of environmentally induced stress.
2. However, human interference may cause the demise of the population. Workers who drive over reservoir roads must be educated about the value of the toads and about the inappropriateness of deliberately killing them.
3. While fluctuations in the water level in the dam undoubtedly reflect important requirements for water management, it would be useful if possible to avoid large fluctuations during mid- to late May if possible.
4. We suggest that this population be monitored every year by visiting the reservoir at night at least nightly during breeding and at least once every one-two weeks during the rest of the summer. Whenever necessary, attempts should be made to improve the breeding success of the population, such as by moving eggs, larvae, or toadlets from areas where they are unlikely to survive to other areas at the NE end of the reservoir, removing bullfrogs that prey on the tadpoles and toadlets, etc. I would be happy to be hired to do this.

Tiger Salamanders

The rains in the spring of 1995 filled the spillway basin below the dam to near capacity. Tiger salamanders apparently bred in the basin in early May. About 40 larvae reached metamorphosis and then disappeared. They may have exited the spillway through a small hole in the middle of the spillway barrier or been eaten by avian predators. The spillway was completely dry this spring until mid-June. Therefore, the salamanders did not breed in the spillway and I was unable to locate any larvae in any of the adjacent ponds west of Coot Lake. Therefore, it was not possible to work on this population this summer.

Boreal toads and northern leopard frogs*(Rana pipiens)* in the Colorado mountains experienced mass die-offs and extinctions in the late 1970's and early 1980's (Carey 1993). Few populations remain of both. Tiger salamanders apparently survived these cataclysmic events, but local (i.e. one breeding site at a time) mass die-offs (all larvae and newly metamorphosed individuals) have been observed in the last 5 years (Carey et al., manuscript in preparation). The proximal cause of death again appears to be bacterial and/or fungal disease because of the presence of skin hemorrhages, fluid accumulation in the abdomen, and other symptoms of the "red leg syndrome" (Taylor et al. 1995). In one case, repeated die-offs of larvae caused population extinction (Carey et al.., manuscript in preparation).

The goals of this part of the study were to determine what levels of UVB tiger salamander eggs and larvae are exposed to during breeding, how these levels correspond with lethal levels of UVB in the lab, and whether sub-lethal exposures in the lab could cause immunosupression in larvae.

Methods

Dr. Ed Little, Dr. David Fabacher and Robin Hurtubise of the Midwest Science Center of the National Biological Service in Columbia, MO and I collaborated on this part of the study. They spent part of June, 1996, in Colorado measuring UVB levels in two breeding locations for salamanders, and then ran exposures of eggs in their facility in Missouri.

UVB levels in the field were measured with a scanner produced by Optronics, Inc. It is comprised of a spherical head which can be placed in either air or water. A computer, run by a 12-volt car battery, scans each wavelength for 15 seconds and then allows total UVB and UVA to be calculated. This device can be calibrated in the field between runs. It can be backpacked to any site if the group is large enough to divide 150 lbs of equipment.

Scans were done last summer at two locations, near Limon, CO (Pond 4 D) (1585 m, 107° 36' W and 39° 34'N), and just south of Nederland, CO (2560 m; 39° 57'N and 105° 31'W) . Scans were made at the surface, just below the surface, and 10 cm deep. Since this species lays its eggs 10-20 cm deep, this latter value indicates that maximum that eggs might be exposed to in their respective locations.

Exposures of eggs collected near Limon were done in Columbia, MO by exposing groups of eggs to 5 hrs daily of graded levels of UVB. The tests were done for 28 days. The experiment with larvae failed since they were all sick upon arrival in MO and died within a week.

Tests of effects of sublethal exposures of UVB on immune function were run in Missouri on groups of salamander larvae captured near Limon. The animals were killed by cervical dislocation and the spleens were removed and homogenized. The removal and homogenization of spleens was done in Missouri and the spleenocytes were shipped overnight to Boulder. The ability of the splenocytes to respond to mitogens was prepared to be measured as described above, but most of the cell solutions arrived contaminated. We hope to repeat this test again sometime this winter.

Results

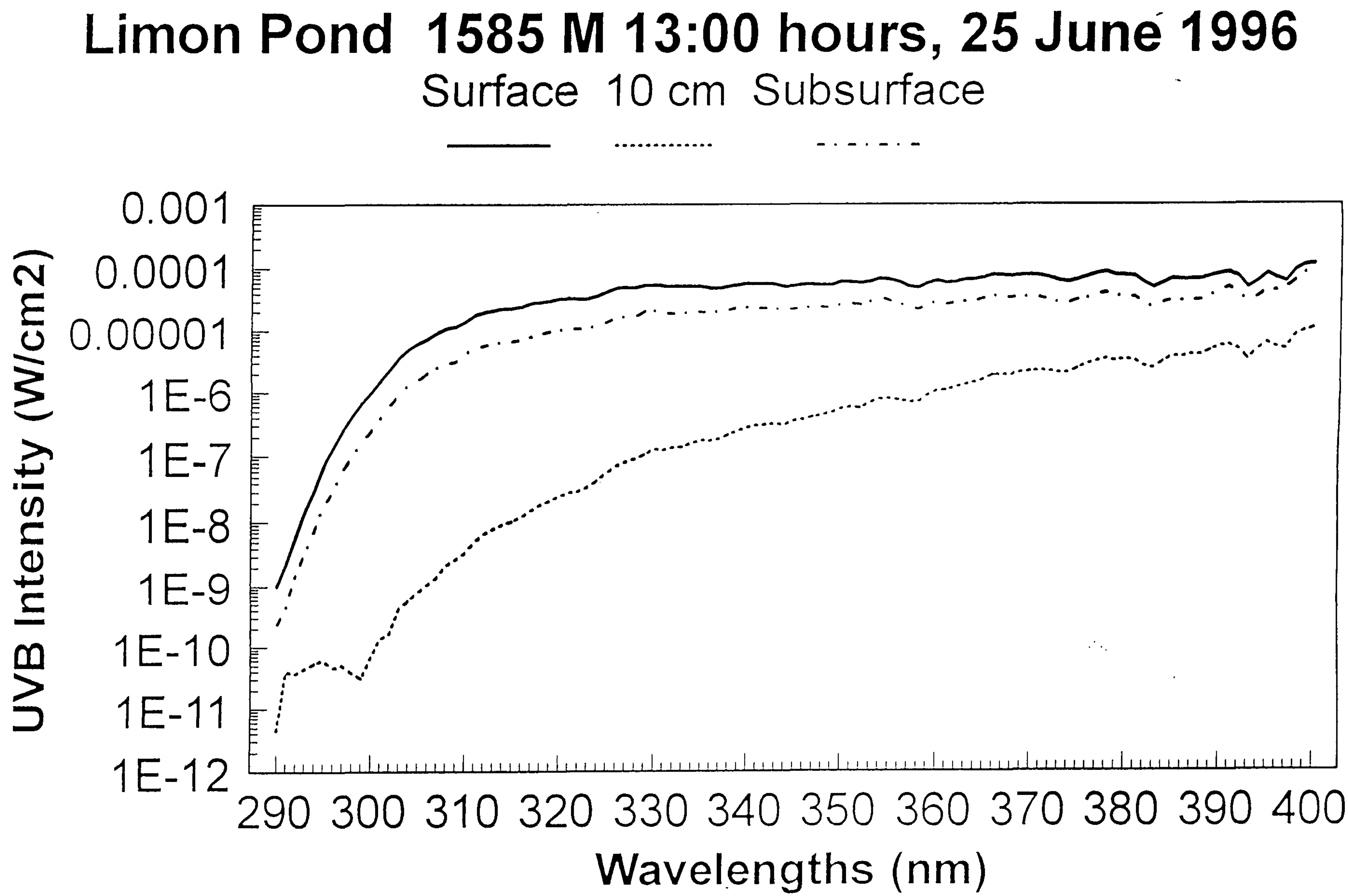
Considerable attenuation of UVB occurs in these two salamander ponds (Fig. 4 and 5). The water is quite murky and high concentrations of humic acid, a product of plant decomposition, occur. Therefore, irradiant levels of UVB at 10 cm, the shallowest depth at which eggs are laid, are quite small (Table 1) compared to levels in air at the same location.

The tolerance of salamander eggs to various exposures of UVB for 5 hrs daily for 28 days is very limited (Table 2), compared to other species that we ran (boreal toad and northern leopard frog). Significant mortality occurred at exposures of 58.5 and 72.7 uW/cm2. It is likely that salamander eggs have been under little or no selection to develop resistance against UVB since they are exposed to such low levels in the field.

Discussion

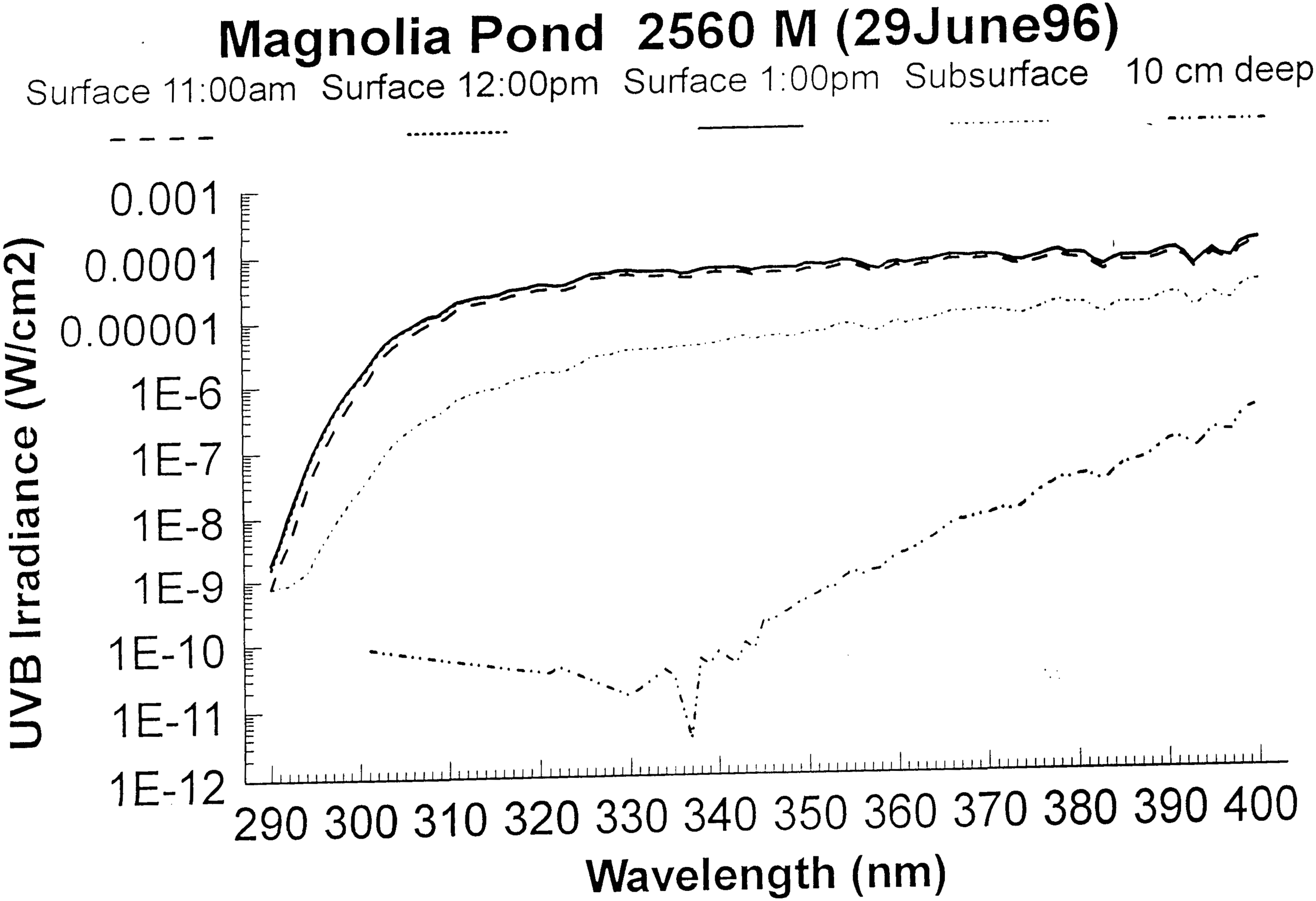
We have little concrete to say about exposure levels and tolerances of Boulder Open Space salamanders, but as long as the water in breeding ponds is similar to that of the two ponds tested thus far, UVB does not appear to be a factor in salamander biology at this point. If water levels drop during development or the water is unusually clear, the eggs would not hatch. The difficulties with our tests on larvae and with sublethal exposure on immune function prevent further speculation.

Fig. 4



Limon Pond 1585 M 13:00 hours, 25 June 1996
Surface 10 cm Subsurface
0.001
0.0001
0.00001
1E-6
1E-7
1E-8
1E-9
1E-10
1E-11
1E-12
UVB Intensity (W/cm2)
290 300 310 320 330 340 350 360 370 380 390 400
Wavelengths (nm)

Fig 5.



Magnolia Pond 2560 M (29June96)
Surface 11:00am
Surface 12:00pm
Surface 1:00pm
Subsurface
10 cm deep
UVB Irradiance (W/cm2)
0.001
0.0001
0.00001
1E-6
1E-7
1E-8
1E-9
1E-10
1E-11
1E-12
290
300
310
320
330
340
350
360
370
380
390
400
Wavelength (nm)

Table 1

UV Irradiance in Limon Pond

1585 Meters *25 June1996*

	Total UVB *u*W/cm2	Total UVA *u*W/cm2
Surface 13:00 hrs	310	5119
Subsurface	90	2360
10 cm depth	13	159

UV Irradiance in Magnolia Pond

2560 Meters *29 June1996*

	Total UVB *u*W/cm2	Total UVA *u*W/cm2
Surface 13:00 hrs	333	4450
Subsurface	11	728
10 cm depth	0.03	728

Table 2

MEAN NUMBER OF DAYS TO DEATH OF
AMBYSTOMA TIGRINUM EGGS
EXPOSED 5 HRS DAILY

UVB (uW/cm2)				
AIR	0.4	4.9	136	169
10 cm	0.17	2.1	58.5	72.7
	26.5±3.0	25.3±2.4	4.4±0.8*	3.1±0.6*

*significantly different from control

Management Suggestions

1. After only two years of observations, it is difficult to know whether water is typically in the basin at Boulder Reservoir at the breeding times of salamanders or toads. If it were possible to fill the basin (by using a water truck) in early May, it may be possible to encourage breeding of salamanders, if the basin is otherwise normally dry in most years. However, if the salamanders breed in the basin, it is unlikely that any later toad breeding would be successful because it is likely that the salamander larvae would eat the larvae. Therefore, if breeding of the two groups is to be "managed", a choice would have to be made about which species would be allowed to breed.

We would recommend the optimal solution for both toads and salamanders: the salamanders breed in the spillway and the toads breed in the reservoir. The former would require that the spillway be filled with water by the end of April if it is not already full, and the latter would require that reservoir levels be kept constant from mid-May until early June. This solution would foster maintenance, if not growth, of both of these populations. Neither species is common on Boulder Open Space or Mountain Park land.

2. Salamander populations on Boulder Open Space lands should continued to be monitored for signs of disease and mass die-offs.

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