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Effects of *Batrachochytrium dendrobatidis* infection on ion concentrations in the boreal toad *Anaxyrus* (*Bufo*) *boreas boreas*

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ABSTRACT: *Batrachochytrium dendrobatidis* causes mortality in various amphibian species including the boreal toad *Anaxyrus* (*Bufo*) *boreas boreas*. The purpose of this study was to determine the physiological effects of this pathogen on experimentally infected boreal toads. Plasma osmolality, sodium, and potassium concentrations were analyzed to evaluate the differences between diseased and non-exposed animals. Infected animals with clinical signs of chytridiomycosis had significantly lower plasma osmolality, sodium, and potassium levels than non-infected animals ($p < 0.06$). On average, clinically infected animals housed in an aquatic environment had sodium and potassium levels of 60.1 (SE = 9.7) and 2.06 (SE = 0.32) mmol l^{-1}, respectively. These ion levels were significantly lower than the negative controls (sodium = 115.0 mmol l^{-1}, potassium = 3.7 mmol l^{-1}) and consistent with the clinical signs observed in affected animals. We propose that infection with *B. dendrobatidis* results in an electrolyte disorder in boreal toads.

KEY WORDS: *Batrachochytrium dendrobatidis* · Boreal toad · *Bufo boreas* · Osmoregulation · Chytridiomycosis

INTRODUCTION

Batrachochytrium dendrobatidis is an aquatic, fungal pathogen that infects the skin of amphibians (Berger et al. 2005) and has been associated with mortalities and population declines worldwide (Lips 1998, Skerratt et al. 2007). Zoospores infect amphibian keratinocytes and develop into sporangia (Nichols et al. 2001). Clinical signs associated with chytridiomycosis, the disease caused by *B. dendrobatidis*, can include excessive shedding of epidermal cells, lethargy, abnormal posturing, loss of the righting reflex, seizures, and death (Nichols et al. 2001, Carey et al. 2006).

It has been hypothesized that mortality from *Batrachochytrium dendrobatidis* is caused by damage to the skin, which interferes with osmoregulaton (Berger et al. 2005, Voyles et al. 2007). Voyles et al. (2007, 2009) reported decreased blood pH, low plasma osmolality and reduced concentrations of sodium, potassium, chloride, and magnesium in *Litoria caerulea*, the green tree frog.

Batrachochytrium dendrobatidis has been reported in boreal toads *Anaxyrus* (*Bufo*) *boreas boreas* in the Rocky Mountains of Colorado, Wyoming, and Montana, USA (Muths et al. 2008, Murphy et al. 2009). This species of amphibian resides in a predominantly low humidity woodland habitat above 1800 m, and spends extensive time feeding, basking, and resting in dry microenvironments (Carey 1978). It is unknown whether this pathogen affects osmoregulation in this species of amphibian. The objective of this study was to determine the effects of *B. dendrobatidis* on plasma osmolality, sodium, and potassium concentrations in this species.

MATERIALS AND METHODS

Fifty-two toads from the Colorado Division of Wildlife Native Aquatic Species Restoration Facility (Alamosa, CO, USA) were housed in individual 5 gallon

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(ca. 18.9 l) glass aquaria. Toad weight ranged from 5 to 33 g (mean ± 1 SE: 17.9 g ± 1.0). The animals were part of another study to evaluate the role of previous exposure to *Batrachochytrium dendrobatidis,* temperature, and moisture on the manifestation of chytridiomycosis, the results of which are reported elsewhere. In brief, *B. dendrobatidis* was grown on TGhL (tryptone, hydrolysed gelatin, lactose) plates at 24ºC for 48 h. Zoospores were scraped from the plates and placed in TGhL broth. Inoculation of 42 toads was done with 1 ml of *B. dendrobatidis* in TGhL broth at a concentration of 10^6 zoospores ml^{-1} dripped directly onto the toad for 3 consecutive days. The toads were housed in 700 ml Tupperware with 40 ml of water, which was changed each day during the inoculation period. The isolate used for this study was strain JEL 275 obtained from J. Longcore (Orono, ME, USA). Half of the animals received a second exposure of *B. dendrobatidis* after 64 d. For negative controls, 10 animals were inoculated with sterile TGhL broth. Infection was verified by examination of skin sheds by light microscopy and PCR at time of death. PCR samples were sent to Pisces Molecular for PCR confirmation of *B. dendrobatidis* using the specific PCR assay protocol described in Annis et al. (2004).

Morbidity associated with *Batrachochytrium dendrobatidis* started 2 wk after the second exposure, with toads showing lethargy, abnormal posturing, and loss of the righting reflex. Half the animals in the study were provided with small platforms to allow them to escape their aquatic environment. The remaining toads were maintained in an aquatic environment. Toads were maintained at room temperature (~15°C) in 8 l aquariums. Tanks were cleaned and toads were fed 2 to 3 crickets every other day. The protocol for this study was approved by the Idaho State University Animal Care Committee.

Moribund animals were euthanized in a 1% tricaine methanesulfonate (MS-222) bath solution and the spinal cord was severed immediately after death. Blood (45 to 100 µl) from the incision site (posterior to the cranium) was collected in heparinzed capillary tubes (StatSpin Technologies) and spun in a microcentrifuge (CritSpin, IRIS Int.) for 5 min. Plasma was separated from formed elements and stored at –20ºC for subsequent analysis. At the end of the experiment (115 d post exposure to *Batrachochytrium dendrobatidis*), all remaining animals were euthanized, including negative controls, and blood was collected as described above.

Skin samples from the toads' entire abdominal area were taken from exposed and negative control animals at the time of blood collection. The samples were fixed in 10% buffered formalin. Tissues were processed for histological evaluation by the Oregon State University Veterinary Diagnostic Laboratory and slides were stained using hematoxylin and eosin. Each sample was assessed for damage to the epidermis.

In total, we collected 37 blood and 27 tissue samples. Not all animals in this study were sampled because 10 died before they could be euthanized. Only animals that were showing signs of disease associated with *Batrachochytrium dendrobatidis* and negative controls were included in our analyses.

Plasma volumes <90 µl were diluted 1:1 with 290 mmol l^{-1} sodium chloride solution (Wescor). The osmolality of each sample was measured in triplicate using vapor pressure osmometry (Model 5520, Wescor). The average of the 3 measurements was taken and recorded. For samples that were diluted, the following equation was used to determine the actual osmolality:

$$\text{Plasma osmolality (osmol kg}^{-1}) = 2\,(\text{sample osmolality average} - 1/2\ \text{dilutant osmolality}) \quad \text{Eq. (1)}$$

Plasma samples were assayed for sodium and potassium concentrations by the Portneuf Medical Center (Pocatello, ID, USA) using ion-selective electrodes (Cobas c 501, Roche Diagnostics).

We compared plasma osmolality and ion concentrations between negative controls and clinically infected animals (i.e. showing signs of disease) using an analysis of variance test, and because of our small sample sizes, we also confirmed our results with a Kruskal-Wallis non-parametric test. Due to a loss of plasma samples by the laboratory during processing, we only had sufficient sample sizes to compare ion concentrations for animals housed in aquatic (wet) environments. Nonetheless we graphically presented the data from infected animals housed in a dry environment for illustration purposes. However, we had sufficient data to statistically evaluate whether platforms affected plasma osmolality levels. This was done using a general linear model. All statistical analyses were conducted in Minitab (v 15.1).

RESULTS

Of the 42 toads infected with *Batrachochytrium dendrobatidis*, 32 developed chytridiomycosis and were either euthanized or died of the disease. Infection status of all the animals was confirmed throughout the course of the study by microscopic examination of their skin sheds (Fig. 1) and at the end of the study by PCR. All animals with skin sheds positive for *B. dendrobatidis* also tested positive by PCR. Four other exposed toads died due to husbandry issues. These animals were excluded from the analyses as no blood could be collected after death. None of the 10 controls were

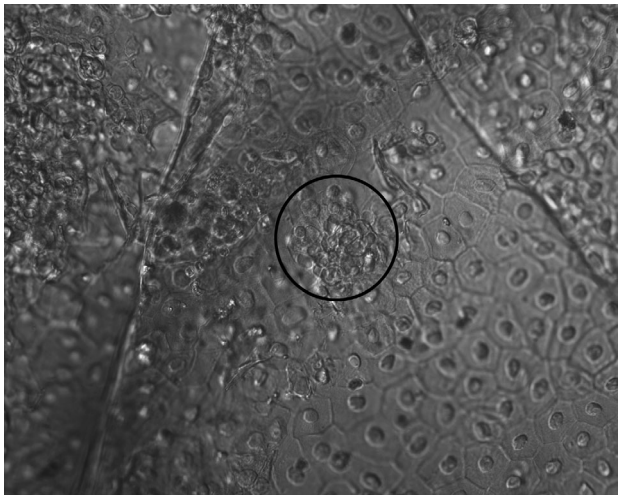

Fig. 1. *Batrachochytrium dendrobatidis* infecting *Anaxyrus* (*Bufo*) *boreas boreas*. Wet mount of a skin shed from a *B. dendrobatidis*-infected boreal toad at 400× magnification. Circle: cluster of *B. dendrobatidis* sporangia

infected with *B. dendrobatidis* as determined by examination of shed skin over the course of the study and by PCR.

There was no apparent skin damage in the 10 toads that were not exposed to *Batrachochytrium dendrobatidis* (Fig. 2a). In contrast, skin damage including sloughing of the epidermis and lymphocytic infiltration was observed by histology in 11 out of the 17 infected toads that were sampled in this study (Fig. 2b) and *B. dendrobatidis* was observed in the skin sheds of all 17 animals sampled (Fig. 1).

Uninfected and infected toads housed in an aquatic environment had an average osmolality of 254 (SE = 4.46) and 144 (SE = 8.35) osmol kg^{-1} H_2O, respectively. The osmolality was slightly higher for animals housed in a dry environment (Fig. 3a, but providing animals with platforms did not significantly affect the osmolality ($p = 0.536$). Toads held both in an aquatic and a dry environment with severe chytridiomycosis had significantly lower plasma osmolality than their negative counterparts (Kruskal-Wallis ratio, $H_{dry} = 6.82$, $p = 0.009$; $H_{wet} = 9.7$, $p = 0.002$)

Animals housed in an aquatic environment and showing severe clinical signs of chytridiomycosis had significantly lower sodium levels than the negative controls ($H_{wet} = 7.29$, $p = 0.007$), with mean levels of 60.1 (SE = 9.7) and 115.0 (SE = 3.5) mmol l^{-1}, respectively (Fig. 3b). This difference was statistically significant using a parameteric test ($F_{1,9} = 11.17$; $p = 0.009$), but not statistically significant using a non-parametric test ($H_{wet} = 3.38$, $p = 0.06$).

Potassium levels also varied significantly between negative controls and infected toads housed in an aquatic environment ($H_{wet} = 4.69$, $p = 0.03$,). Uninfected and clinically infected toads had potassium concentrations of 3.73 (SE = 0.32) and 2.06 (SE = 0.32) mmol l^{-1}, respectively (Fig. 2c).

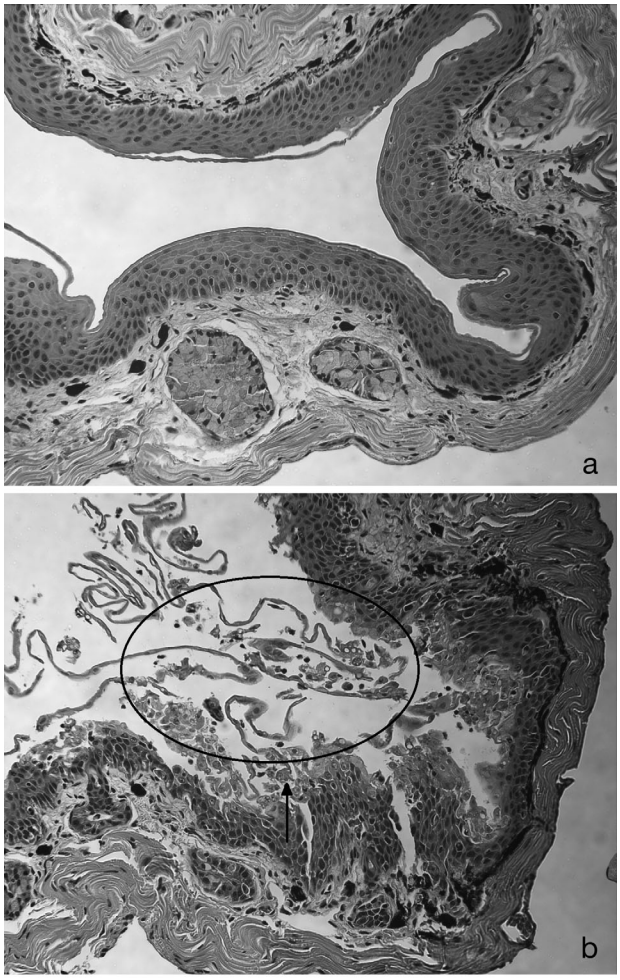

Fig. 2. *Batrachochytrium dendrobatidis* infecting *Anaxyrus* (*Bufo*) *boreas boreas*. Boreal toad skin from the abdomen of (a) a negative control animal and (b) an infected toad, magnified 200× and hematoxylin and eosin (H&E) stained. Arrow: example of lymphocytic infiltration (dark cells) and clear vacuoles typical of *B. dendrobatidis*. Circle encompasses epithelial sloughing

DISCUSSION

We detected significant differences in the plasma osmolality, and sodium and potassium ion concentrations in animals clinically infected with *Batrachochytrium dendrobatidis* and those not exposed to the pathogen (Fig. 3). The levels of sodium and potassium ions measured in clinically infected animals was well below the range reported in *Bufo viridis* (osmolality = 270 osmol kg^{-1} H_2O, sodium = 113 mmol l^{-1}, potas-

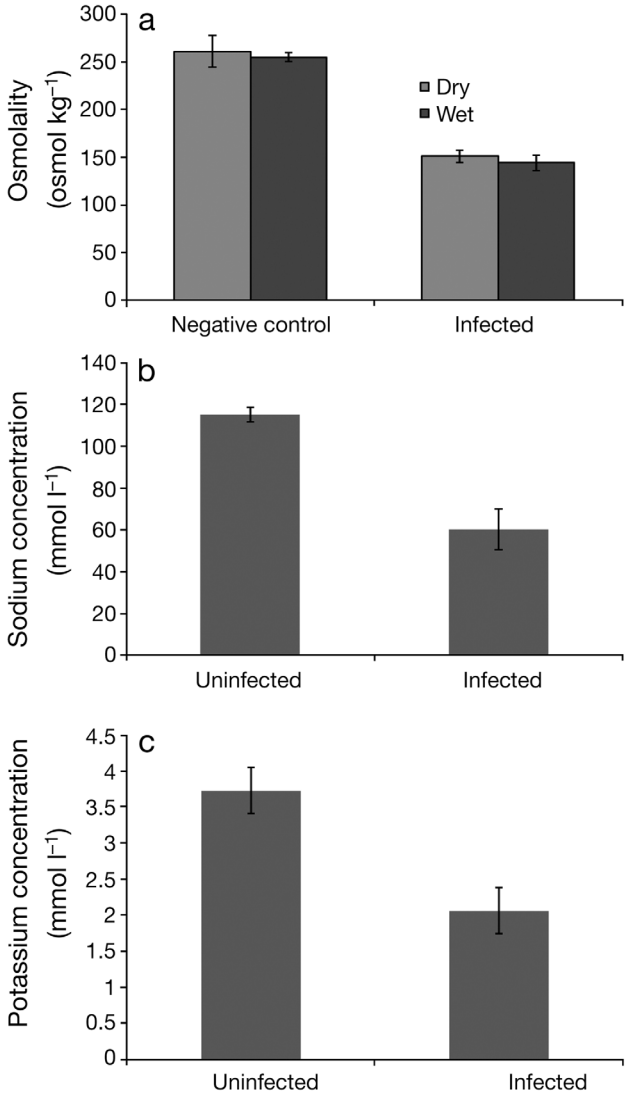

Fig. 3. *Batrachochytrium dendrobatidis* infecting *Anaxyrus* (*Bufo*) *boreas boreas*. (a) Osmolality for negative control and infected boreal toads housed with (dry) or without (wet) platforms. (b,c) Ion concentrations for negative control and infected toads housed without platforms. Bars: SE of mean

sium = 5 mmol l^{-1}) (Shoemaker et al. 1992) and below the levels found in our negative control animals. The results of our study are also consistent with those reported by Voyles et al. (2007, 2009) for diseased and normal green tree frogs. They reported slightly lower normal levels for osmolality (217.2 osmol kg^{-1} H_2O), sodium (102.8 mmol l^{-1}) and potassium (2.74 mmol l^{-1}) than our controls.

The skin plays a critical osmoregulatory role in amphibians and is involved in the active transport of sodium (Boutilier et al.1992). Presumably, damage to the skin could interfere with this process and may contribute to low extracellular sodium concentrations. Further, damaged skin may permit the loss of ions to the environment. Using histology, we observed severe damage to the epidermis in 65% of the diseased animals, specifically the outer epithelial layer (Fig. 2b). Although not measured in the current study, it is possible that skin damage impaired the active uptake of sodium by the epithelial cells and increased the loss of both sodium and potassium.

Sodium is the most abundant ion in the extracellular fluid and is the main determinant of plasma osmolality. Low sodium imbalance referred to as hyponatremia results in cellular overhydration and can prevent the propagation of action potentials (Eckert et al. 1988) and ultimately limit neuromuscular activity. Hyponatremia is therefore associated with flaccid paralysis and lethargy, both signs observed in toads with chytridiomycosis. Severe hyponatremia can also result in impaired cardiac function and death (Fraser et al. 1991).

Potassium ion deficiency or hypokalemia results in skeletal muscle weakness, reduced muscle tone, and cardiac arrhythmias (Fraser et al. 1991). These signs are once again consistent with those observed in toads with chytridiomycosis. We cannot confirm whether this hypokalemia was due to increased loss of potassium through the skin of boreal toads or possibly increased entry of potassium into cells. The latter has been noted in other animal species after elevated β-adrenergic activity due to stress (Gardner et al. 1973, Furukawa et al. 1980, Bourne & Cossins 1982).

The results of low osmolality and plasma sodium and potassium concentrations appear to explain the disease state and mortality associated with *Batrachochytrium dendrobatidis* in the boreal toad. Specifically, damage caused by *B. dendrobatidis* results in insufficient levels of sodium and potassium in the plasma, possibly due to loss of extracellular sodium and potassium through damaged skin and reduced active uptake of sodium.

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