Vol. 81: 93–97, 2008
doi: 10.3354/dao01953

DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org

Published August 27

Storage of samples at high temperatures reduces the amount of amphibian chytrid fungus *Batrachochytrium dendrobatidis* DNA detectable by PCR assay

M. Van Sluys[1, 2,*], K. M. Kriger[1], A. D. Phillott[3], R. Campbell[3], L. F. Skerratt[3, 4], J.-M. Hero[1]

[1]Centre for Innovative Conservation Strategies, Griffith School of Environment, Griffith University, PMB 50, Gold Coast Mail Centre, Queensland 9726, Australia

[2]Depto. de Ecologia, Instituto de Biologia Roberto Alcântara Gomes, Universidade do Estado do Rio de Janeiro, Rua São Francisco Xavier 524, CEP 20550-013, Rio de Janeiro, RJ, Brazil

[3]Amphibian Disease Ecology Group, School of Veterinary and Biomedical Sciences, James Cook University, Townsville, Queensland 4811, Australia

[4]Biosecurity and Tropical Infectious Diseases Research Group, School of Public Health, Tropical Medicine and Rehabilitation Sciences, James Cook University, Townsville, Queensland 4811, Australia

ABSTRACT: Chytridiomycosis, caused by the amphibian chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*), is an emerging infectious disease responsible for amphibian declines on several continents. In laboratory conditions, optimal temperatures for *Bd* growth and survivorship are between 17 and 25°C. We investigated the effect of different storage temperatures, both in field and laboratory conditions, on detection of *Bd* from swabs stored for 7 d. We sampled 52 wild *Litoria wilcoxii* males for *Bd* by simultaneously running 2 cotton swabs along the skin of the frog. One group of swabs was stored in a freezer within 2 h of sampling and the other was kept in a car in an exposed environment for 7 d before being stored in the freezer. In the laboratory experiment, swabs were inoculated with zoospores of *Bd* and underwent one of 4 treatments: immediate DNA extraction, or storage at 27, 38 or 45°C for 7 d prior to DNA extraction. Swabs from all treatments were analyzed by quantitative (real-time) PCR test. Though prevalence of *Bd* did not differ significantly between swabs that were frozen and those that remained in a car for 7 d (19.2 vs. 17.3%, respectively), the number of *Bd* zoospores detected on car swabs taken from infected frogs was, on average, 67% less than that detected on the corresponding frozen swab. In the laboratory experiment, the number of zoospore equivalents varied significantly with treatment ($F_{3,35}$ = 4.769, p = 0.007), indicating that there was reduced recovery of *Bd* DNA from swabs stored at higher temperatures compared with those stored at lower temperatures or processed immediately. We conclude that failure to store swabs in cool conditions can result in a significant reduction in the amount of *Bd* DNA detected using the PCR assay. Our results have important implications for researchers conducting field sampling of amphibians for *Bd*.

KEY WORDS: Swab storage · Temperature · DNA · Quantitative PCR · Chytrid detection · Chytridiomycosis · Amphibian declines · Field sampling · Laboratory experiments

INTRODUCTION

Chytridiomycosis, caused by the fungus *Batrachochytrium dendrobatidis* (*Bd*) (Chytridiomycetes, Chytridiales), is an emerging infectious disease responsible for amphibian declines and extinctions on several continents (Berger et al. 1998, Lips et al. 2006, Schloegel et al. 2006, Skerratt et al. 2007).

To date, the most sensitive technique for detection of *Bd* on wild amphibians is the swab-PCR assay. In this technique, a cotton swab is run firmly over the amphibian's skin, and then real-time PCR is used to

*Email: vansluys@uerj.br

quantify the number of *Bd* zoospores present on the swab (Boyle et al. 2004, Speare et al. 2005, Kriger et al. 2006a, Hyatt et al. 2007). The analytical sensitivity of the PCR assay is 0.1 of a single *Bd* zoospore (Boyle et al. 2004).

This sensitivity has some drawbacks, however. Swabs taken from wild amphibians are likely to be covered in dirt and microorganisms, and it has been suggested that this may degrade the fungal DNA, resulting in false negatives (Kriger et al. 2006b, Hyatt et al. 2007). The conditions in which swabs are stored prior to PCR analysis may also influence the results obtained in the assay. Previously, the only study on the effect of storage temperature on *Bd* nucleic acid recovery from swabs was from relatively low temperatures, such as those present in the laboratory. Hyatt et al. (2007) found no decrease in nucleic acid recovery from swabs stored at temperatures of <23°C for 18 mo. However, it is often difficult to maintain samples from wild frogs at such temperatures for the duration of a field trip. As research expeditions may take place in remote areas and/or hot, humid regions where no form of refrigeration is available for prolonged periods, it is important to determine the effect of high temperatures on the detection of chytrid DNA on swabs used to sample wild frogs for *Bd*.

We investigated the effect of different storage temperatures, both in field and laboratory conditions, on nucleic acid recovery from swabs stored for 7 d. We demonstrate a significant decrease in the number of *Bd* zoospores detected on swabs exposed to high temperatures, and recommend that researchers store swabs at low temperatures whenever possible.

MATERIALS AND METHODS

Expt 1: Swabs from wild frogs. Field work was carried out on 5 December 2005, along a 100 m transect at Dave's Creek (28.221° S, 153.232° E), in southeast Queensland, Australia. The transect was at an elevation of 200 m above sea level, on a section of the creek that ran through a pasture. We captured 52 adult male stony creek frogs *Litoria wilcoxii* (Anura: Hylidae) in unused plastic bags.

We sampled each frog for *Bd* by simultaneously running 2 cotton swabs (MW100, Medical Wire & Equipment) 10 times over (1) the frog's ventral abdominal surface, (2) each of the frog's lateral sides, from groin to armpit and (3) the ventral surface of each thigh. Additionally, 5 outward strokes of the swabs were employed on the undersides of the frog's feet, for a total of 60 strokes per frog. The 2 swabs were run over each frog's body in such a matter that each swab covered approximately the same locations and amount of surface area on the frog being sampled. Each swab was then replaced in its original container, and then arbitrarily labeled either A or B. All animals were released immediately after being sampled.

All 'A' swabs (hereafter freezer swabs) were stored in a freezer within 1 h of sampling, and all 'B' swabs (hereafter car swabs) were kept in a car for 7 d before being stored in the freezer. The car was used normally during the week and parked in exposed locations. Temperature loggers in both the car and the freezer recorded the temperature regime to which each group of swabs was subjected.

Expt 2: Swabs in the laboratory. We inoculated 39 cotton swabs with approximately 1000 zoospores (10 µl of a 100 000 zoospores ml^{-1} suspension) of *Bd*. We incubated 9 or 10 swabs at each of 3 storage temperatures (27, 38 and 45°C) for 7 d prior to extraction of *Bd* DNA. The storage temperature in each incubator was measured every 30 min by a Thermochron iButton. Nucleic acids from the remaining 10 swabs were extracted immediately.

DNA extraction and analysis. For both sets of data, DNA extraction and quantitative (real-time) PCR analysis followed the procedure of Boyle et al. (2004) and Kriger et al. (2006b). Field samples were initially screened with a single PCR reaction and those that were positive for *Bd* were re-analyzed in triplicate for confirmation of the test result and accurate quantitation of zoospores. The lab samples were analyzed in triplicate. The number of zoospores on each sample was estimated by comparison with a standard curve constructed by control reactions using 100, 10, 1 and 0.1 *Bd* genome $zoospore^{-1}$ equivalents.

We used a paired *t*-test to compare the amount of *Bd* DNA on the car swabs with that on the freezer swabs. The effect of temperature storage in the laboratory was determined by comparing the number of zoospore equivalents from each treatment using a 1-way analysis of variance (ANOVA). Post hoc Tukey tests identified homogeneous sub-sets. Our estimates of the number of zoospores for the field samples represent the mean value of *Bd* genome equivalents detected in the 3 replicates of the triplicate PCR analysis. As the number of zoospores detected on positive swabs varied over several orders of magnitude, data were log-transformed prior to statistical analysis.

The ecological terms of parameters analyzed follow Bush et al. (1997). Prevalence corresponds to the proportion of frogs which tested positive (*Bd* DNA equivalent to at least one *Bd* zoospore was detected), and mean intensity of infection to the total number of zoospore equivalents detected in the population divided by the number of hosts infected.

RESULTS

Expt 1: Swabs from wild frogs

The swabs kept in the freezer were subjected to a mean constant temperature of –19.0°C. The mean air temperature to which the car swabs were subjected during the week was 34.0°C (SD = 7.1°C, min. = 22.5°C, max. = 48.5°C, n = 167; Fig. 1a). Of these 167 measures, 103 were above 30°C, hovering above this point for 14 h on most days. The mean air temperature during these daytime periods was 38.6 °C (± 4.8 °C SD, min. = 30.5, max. = 48.5 °C).

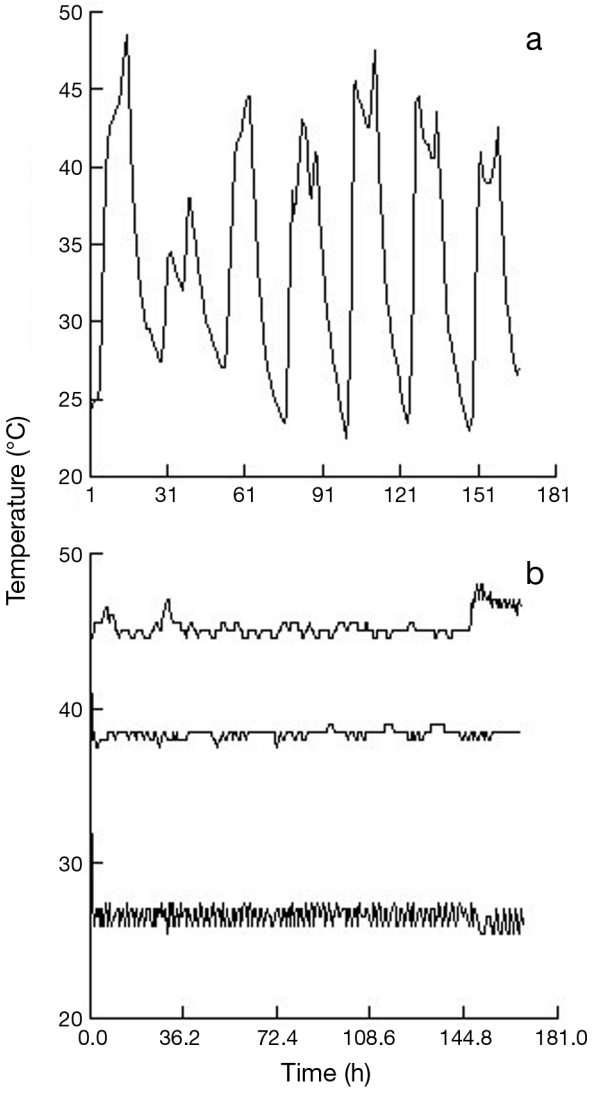

Fig. 1. (a) Thermal regime experienced by swabs stored in a car for 1 wk. (b) Thermal profile of swab laboratory storage conditions over 7 d. Upper line = storage at 45°C for 7 d, middle line = storage at 38°C for 7 d, bottom line = storage at 27°C for 7 d

Bd was detected on 10 of the 52 freezer swabs (prevalence = 19.2%). Of these 10 swabs, 9 of the correspondingly paired car swabs also tested positive (Table 1). All freezer swabs which tested negative also had paired car swabs which tested negative, resulting in a prevalence of *Bd* in the car sample of 17.3%. Prevalence between the 2 groups did not differ significantly (p = 0.80, chi-square = 0.06, df = 1).

The mean intensity of infection was 198.3 zoospore equivalents (± 357.5 SE, min. = 1, max. = 1020) for the freezer swabs, versus only 92.7 zoospore equivalents (± 143.6 SE, min. = 1, max. = 399) for the car swabs. The number of *Bd* zoospores detected on car swabs taken from infected frogs was significantly less than that detected on the corresponding freezer swab (paired *t*-test, $t = 6.58$, df = 9, $p < 0.001$). Counts were consistently lower in the car swabs, and on average there was a 63.4% decrease in the number of zoospore equivalents detected on car swabs as compared with their paired freezer swab (Table 1).

Expt 2: Swabs in the laboratory

The thermal regimes to which the swabs were subjected are shown in Fig. 1b. The number of zoospores recovered from swabs varied significantly among treatments ($F_{3,35} = 4.769$, p = 0.007). Swabs stored at 45°C resulted in significantly fewer zoospores recovered (Fig. 2, Table 2). Post hoc Tukey tests indicated 2 homogenous subsets; subset 1 included immediate extraction and storage at 27 and 38°C for 7 d and subset 2 included storage at 38 and 45°C for 7 d. Regardless of declining DNA recovery after storage at higher temperatures, none of the samples returned a false negative result.

Table 1. *Batrachochytrium dendrobatidis.* Number of zoospore equivalents detected on swabs from infected adult male *Litoria wilcoxii* (n = 52) at Dave's Creek, southeastern Queensland. Swabs were either stored in a freezer directly after sampling or kept in a car for 7 d prior to storage in a freezer

Swab	Zoospore equivalents		Decrease (%)
	Freezer	Car	
1	1020	399	60.9
2	569	250	56.0
3	271	143	47.3
4	87	21	75.9
5	63	8	87.3
6	20	4	80.0
7	14	8	42.9
8	6	1	83.3
9	4	0	100
10	1	1	0

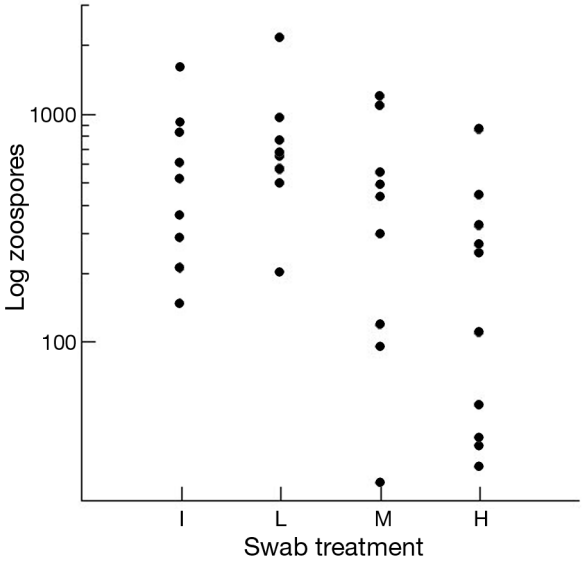

Fig. 2. *Batrachochytrium dendrobatidis.* Influence of immediate or delayed DNA extraction and storage temperature on the number of zoospore equivalents (log transformed). I = immediate extraction, L = storage at 27°C for 7 d, M = storage at 38°C for 7 d, H = storage at 45°C for 7 d

DISCUSSION

While the prevalence of *Bd* in samples that remained in a hot car did not differ significantly from that of samples which were immediately frozen, failure to store swabs in cool conditions consistently resulted in a significant reduction in the number of *Bd* zoospores detected using the PCR assay. The reduction in the number of zoospores detected suggests that degradation of the *Bd* DNA occurred. This decrease in the amount of DNA due to the storage method prior to PCR analysis could produce spurious results, thus potentially making the results of various disease surveys incomparable with each other. Our results from lab experiments agree with those obtained from the field; that is, higher storage temperatures (>27°C) significantly reduced the estimates of *Bd* zoospore equivalents from real-time PCR analyses.

Table 2. *Batrachochytrium dendrobatidis.* Number of zoospores of the chytrid fungus recovered among different storage treatments.

Treatment	N	Mean	SD	Min.	Max.
Immediate extraction	10	556.1	447.3	147	1593
Stored at 27°C for 7 d	10	770.5	523.4	202	2156
Stored at 38°C for 7 d	9	474.7	418	24	1185
Stored at 45°C for 7 d	10	238.1	256.7	28	845

The temperatures to which our car swabs were subjected (max. = 48.5°C) may initially appear to be higher than those temperatures with which the average researcher would have to contend. However, most places that swabs would be stored on a field trip (i.e. car, tent, boxes) are likely to heat up to well above ambient temperature on sunny days. Further, if swabs must be shipped via freight, they are likely to sit in the unventilated compartments of trucks or airplanes, where temperatures are likely to approach those in the current study. As such, we recommend that amphibian disease researchers take precautions to ensure that their samples are not subjected to high temperatures in the interim between sampling and laboratory analysis.

One frog's frozen swab, on which low numbers of zoospores were detected, tested negative on the corresponding car swab. This suggests that poor storage conditions may produce a false negative result on lightly infected individuals, although it is possible that the swab did not pick up *Bd* DNA. Peccoud & Jacob (1996) analyzed DNA amplification and found that a high level of variation is expected for samples with very low initial copy numbers. The method we used for *Bd* analysis (qPCR) can readily detect 0.1 of a *Bd* zoospore (Boyle et al. 2004), and the conventional PCR method described by Annis et al. (2004) sets a lower detection limit of about 10 zoospores. Kriger et al. (2006b) found that nearly 18% (n = 224) of infected frogs from several Australian species tested positive with less than 10 *Bd* zoospore equivalents. This represents a considerable number of frogs whose swabs could potentially yield false negatives if stored in suboptimal conditions, and further supports our suggestion of a precautionary approach to swab storage.

The number of zoospores recovered from swabs in our study was significantly affected by storage temperatures, suggesting that DNA degradation occurred. This could represent a major problem for lightly infected individuals as excessive heat may reduce the number of zoospores to the point where they go completely undetected by the PCR assay. Although there was no strong effect of storage conditions on prevalence levels in this study, the detection of false negatives may significantly underestimate the true prevalence of *Bd* in wild populations, and could cause major problems for mark-recapture studies or work where the detection of a single infected individual has serious implications.

Our laboratory results corroborate our field test. Storing samples at temperatures above 38°C resulted in reduced estimates of chytrid zoospore equivalents from real-time PCR analyses. This could have been due to

nucleic acid degradation by denaturing. No false negative results were obtained in this experiment. However, while high temperatures in our treatment affected only quantitative determination of infection load (as opposed to the qualitative evaluation of infection status), it is conceivable that at lower starting quantities of DNA (i.e. 1 to 100 zoospores), false negative results could be obtained.

Our study demonstrates that storage conditions of swabs affect the long-term stability of zoospores on swabs, and that high temperatures can result in significant decreases in the number of chytrid zoospores detected on swabs. As such, we recommend that field samples be stored at the lowest temperature logistically possible, preferably below 27°C, prior to PCR analysis.

Acknowledgements. This research was supported by The Consortium for Conservation Medicine (New York), the Eppley Foundation for Research, the Australian Government Department of the Environment and Water, and the Endangered Frog Research Group at Griffith University. Albertina Lima helped in the field work and Kevin Ashton and Rebecca Webb assisted in the PCR analyses. During the development of this study, M.V.S. was a visiting lecturer in the Centre for Innovative Conservation Strategies at Griffith University and held a fellowship from CAPES/Brazil (BEX 0570/05-2).

LITERATURE CITED

Annis SL, Dastoor FP, Ziel H, Daszak P, Longcore JE (2004) A DNA-based assay identifies *Batrachochytrium dendrobatidis* in amphibians. J Wildl Dis 40:420–428

Berger L, Speare R, Daszak P, Green DE and others (1998) Chytridiomycosis causes amphibian mortality associated with population declines in the rain forests of Australia and Central America. Proc Natl Acad Sci USA 95: 9031–9036

Boyle DG, Boyle DB, Olsen V, Morgan JAT, Hyatt AD (2004) Rapid quantitative detection of chytridiomycosis (*Batrachochytrium dendrobatidis*) in amphibian samples using real-time Taqman PCR assay. Dis Aquat Org 60:141–148

Bush AO, Lafferty KD, Lotz JM, Shostak AW (1997) Parasitology meets ecology on its own terms: Margolis et al. revisited. J Parasitol 83(4):575–583.

Hyatt AD, Boyle DG, Olsen V, Boyle DB and others (2007) Diagnostic assays and sampling protocols for the detection of *Batrachochytrium dendrobatidis*. Dis Aquat Org 73: 175–192

Kriger KM, Hero JM, Ashton KJ (2006a) Cost efficiency in the detection of chytridiomycosis using PCR assay. Dis Aquat Org 71:149–154

Kriger KM, Hines HB, Hyatt AD, Boyle DG, Hero JM (2006b) Techniques for detecting chytridiomycosis in wild frogs: comparing histology with real-time Taqman PCR. Dis Aquat Org 71:141–148

Lips KR, Brem F, Brenes R, Reeve JD and others (2006) Emerging infectious disease and the loss of biodiversity in a Neotropical amphibian community. Proc Natl Acad Sci USA 103:3165–3170

Peccoud J, Jacob C (1996) Theoretical uncertainty of measurements using quantitative polymerase chain reaction. Biophys J 71:101–108

Schloegel LM, Hero JM, Berger L, Speare R, McDonald K, Daszak P (2006) The decline of the sharp-snouted day frog (*Taudactylus acutirostris*): the first documented case of extinction by infection in a free-ranging wildlife species? EcoHealth 3:35–40

Skerratt LF, Berger L, Speare R, Cashins S and others (2007) The spread of chytridiomycosis has caused the rapid global decline and extinction of frogs. EcoHealth 4: 125–134

Speare R, Skerratt LF, Berger L, Hines H and others (2005) A project that designs and trials a pilot survey to map the distribution of chytridiomycosis (caused by the amphibian chytrid fungus) in Australian frogs. Final report for project ID 44381 (tender 63/2003) to the Australian Government Department of the Environment and Heritage. Department of the Environment and Heritage, Canberra. Available at: www.deh.gov.au/biodiversity/invasive/publications/c-disease/distribution-survey.html (accessed October 29, 2006)

Editorial responsibility: Alex Hyatt, Geelong, Victoria, Australia

Submitted: July 21, 2007; Accepted: February 20, 2008
Proofs received from author(s): July 29, 2008