



## Research

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**Author for correspondence:**

Bruce Waldman

e-mail: [waldman@snu.ac.kr](mailto:waldman@snu.ac.kr)

<sup>†</sup>Deceased 8 July 2014.

## Animal behaviour

Enhanced call effort in Japanese tree frogs  
infected by amphibian chytrid fungus

Deuknam An<sup>†</sup> and Bruce Waldman

Laboratory of Behavioral and Population Ecology, School of Biological Sciences, Seoul National University,  
Seoul 08826, South Korea

BW, 0000-0003-0006-5333

Some amphibians have evolved resistance to the devastating disease chytridiomycosis, associated with global population declines, but immune defences can be costly. We recorded advertisement calls of male Japanese tree frogs (*Hyla japonica*) in the field. We then assessed whether individuals were infected by *Batrachochytrium dendrobatidis* (Bd), the causal agent of the disease. This allowed us to analyse call properties of males as a function of their infection status. Infected males called more rapidly and produced longer calls than uninfected males. This enhanced call effort may reflect pathogen manipulation of host behaviour to foster disease transmission. Alternatively, increased calling may have resulted from selection on infected males to reproduce earlier because of their shortened expected lifespan. Our results raise the possibility that sublethal effects of Bd alter amphibian life histories, which contributes to long-term population declines.

## 1. Introduction

The pathogenic chytrid fungus *Batrachochytrium dendrobatidis* (denoted Bd) causes chytridiomycosis, a pandemic disease that can kill its amphibian hosts. The pathogen induces physiological changes in susceptible amphibians through multiple pathways. Bd interferes with electrolyte balance and osmoregulation [1,2], effectively causing heart failure in clinically affected individuals. Additionally, Bd disrupts adaptive immune system responses, impairing the proliferation of lymphocytes and inducing apoptosis [3]. Although Bd antigens are recognized, the fungus subverts normal immune responses against it. Bd factors affect many tissues, not just those of the lymphoid system, resulting in lethargy, lack of coordination and other behavioural changes [2,4].

Parasites and pathogens impose costs on hosts that can range from mild to severe. Amphibians have robust immune systems that can clear many infections, presumably by diverting resources from growth and reproduction [5]. Adaptations to tolerate rather than eliminate infection likewise incur costs [6]. Infections activate physiological and behavioural responses of hosts that promote recovery from infection, but pathogens can manipulate these responses to facilitate their own transmission. For example, pathogens sometimes chemically induce their hosts to suppress signs of illness and boost their reproductive efforts [7].

Chytridiomycosis has contributed to global amphibian population declines, decimating populations of some species, sometimes to extinction [8]. Yet, other species, despite themselves harbouring Bd infections, survive in the midst of epizootics affecting amphibian communities. In Asia, enzootic Bd strains commonly are found on amphibians, but hosts typically bear low infection burdens [9]. Asian Bd strains may have had a long historical association with amphibian hosts [10]. Evolutionary responses to the pathogen may be evident in the differential expression of immune genes by frogs resistant to Bd infection [11]. Nonetheless, Bd might inflict sublethal damage on its hosts, even in the absence of clinical signs of disease.

Bioacoustic signalling is the primary mode of mate attraction in anurans, so changes in calling behaviour are likely to result in differential reproductive

success. To examine how Bd affects Japanese tree frogs (*Hyla japonica*) in the wild, we analysed properties of males' advertisement calls as a function of their Bd infection status. *Hyla japonica* is widely distributed in Japan, Korea and eastern and central Asia, and Bd infection prevalence ranges between 10 and 20% in Korean populations [9]. Neither morbidity nor mortality attributable to chytridiomycosis has been reported in this species, suggesting that it is resistant to, or tolerant of, the pathogen.

## 2. Material and methods

We recorded advertisement vocalizations of 59 male *H. japonica* in rice paddies in Namyangju, Gyeonggi Province, South Korea (37°38'15.1" N, 127°13'13.6" E) between 22.00 and midnight throughout their breeding season, from early June to mid-August 2011. We recorded 10 calls from each frog on each of several nights. Recordings were made 20 cm above frogs' calling perches using a Sennheiser ME80 microphone (K3-U power module) with a Marantz PMD661 recorder at 48 kHz. After recording each frog for the first time, we measured its snout-vent length, toe-clipped it for individual identification, and swabbed its ventral skin, limbs and toes 25 times for analyses of Bd infection (MW100 swabs, Medical Wire and Equipment, Corsham, Wiltshire, UK) [12]. After every recording, we also obtained the temperature and relative humidity at the frog's calling perch. Air rather than water temperature was measured as males called on the ground or from vegetation.

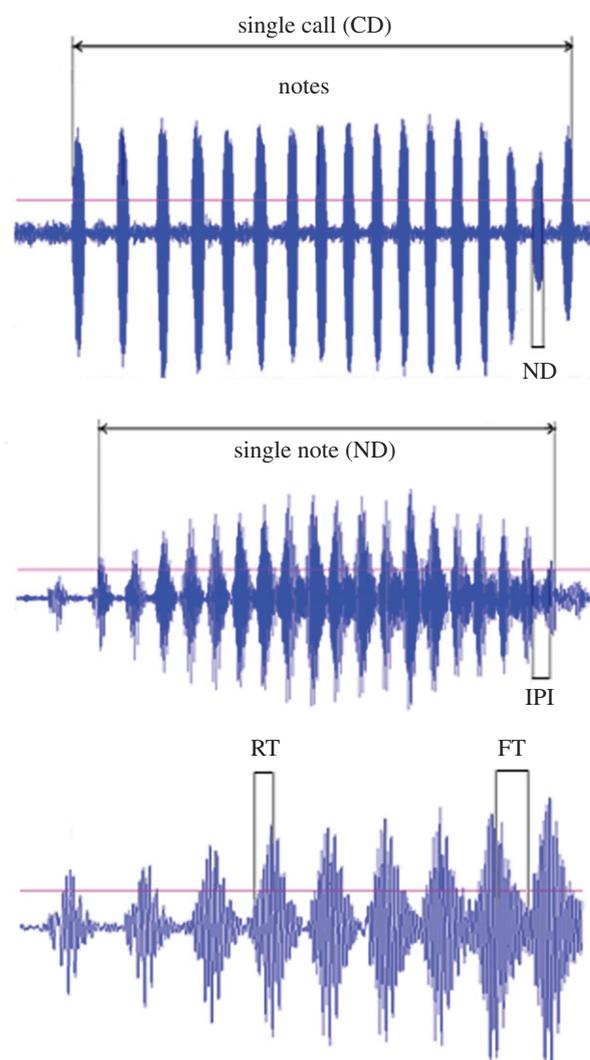
*Hyla japonica* advertisement vocalizations consist of bouts of calls, each composed of a train of notes that includes multiple pulses. We analysed 10 call parameters using RAVEN v. 1.4 (Cornell Laboratory of Ornithology, Ithaca, NY, USA): number of pulses per note (PN), pulse repetition rate (PRR), inter-pulse interval (IPI), number of notes (NN), note duration (ND), note repetition rate (NRR), rise time (RT), fall time (FT), call duration (CD) and dominant frequency (DF) (figure 1). To control for unequal numbers of pulses and notes, means of these parameters were first determined for each call. *Hyla japonica* typically calls in dense, loud choruses, so only those recordings in which individual calls could be clearly identified were used. This reduced our sample size to 42 calling males.

We analysed each call parameter as the dependent variable in separate repeated-measures general linear models with Bd infection status as a fixed factor together with snout-vent length, temperature, relative humidity and their interactions. To confirm results, when call parameters were significantly affected by temperature and snout-vent length, their values were adjusted to the mean temperature of 21.4°C or mean snout-vent length of 39.4 mm by interpolation using regression coefficients. Statistical analyses were run using the SAS MIXED and TTEST procedures (SAS v. 9.2, SAS Institute, Cary, NC, USA).

DNA was extracted from swabs using 50 µl PrepMan Ultra (Applied Biosystems, Carlsbad, CA, USA) and tested for Bd infection using nested PCR targeting 5.8S rDNA of the pathogen [13]. Samples were run in duplicate, with Bd DNA as a positive control, and were considered as positive for Bd infection when an amplified band of approximately 300 bp was visualized by ethidium bromide staining. Bd infection intensity subsequently was determined in positive samples by qPCR [12].

## 3. Results

Infected males were found throughout the breeding season, with 9 of 42 males testing Bd-positive. They were slightly, but not significantly, larger than uninfected males ( $40.17 \pm 0.74$  versus

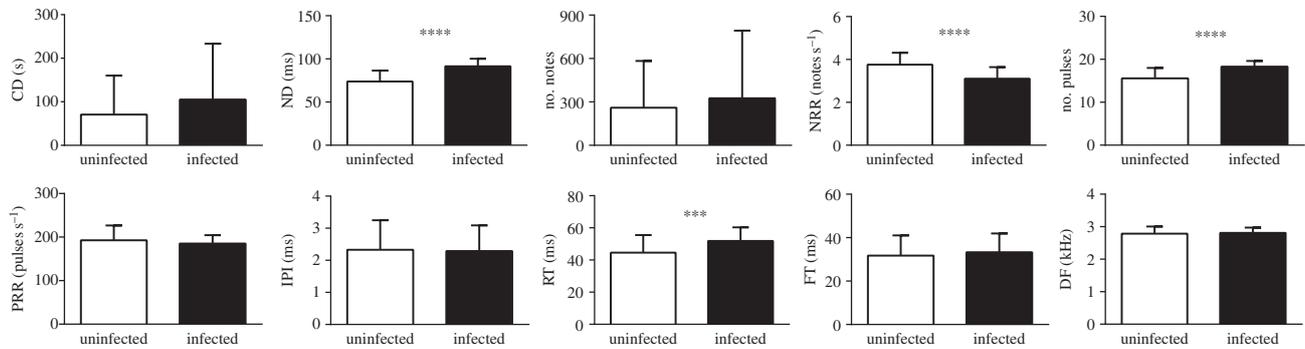


**Figure 1.** *Hyla japonica* advertisement call. Each call consists of a series of notes which include many pulses. Temporal call parameters were compared between uninfected and infected callers. These include: call duration (CD); and within calls, note duration (ND), number of notes, note repetition rate; and within notes, number of pulses, pulse repetition rate, inter-pulse interval (IPI), rise time (RT) and fall time (FT). (Online version in colour.)

$39.24 \pm 0.51$  mm;  $\bar{x} \pm$  s.e.) ( $t_{39} = 0.88$ ,  $p = 0.38$ , two-tailed). Infection loads varied between 15 and 43 zoospore genomic equivalents, similar to those found in other Korean *H. japonica* populations [9].

Calls of Bd-infected males contained more PN ( $F_{1,242} = 16.73$ ,  $p < 0.0001$ ), longer ND ( $F_{1,242} = 15.97$ ,  $p < 0.0001$ ) and longer RT ( $F_{1,242} = 13.74$ ,  $p = 0.0003$ ) than those of uninfected frogs (figure 2). They also tended to have more notes and longer call duration but these parameters were highly variable so did not significantly differ between groups (NN,  $F_{1,242} = 0.42$ ,  $p = 0.51$ ; CD,  $F_{1,242} = 0.91$ ,  $p = 0.34$ ). Mirroring their shorter notes, uninfected frogs repeated notes more frequently than infected ones (NRR,  $F_{1,242} = 21.40$ ,  $p < 0.0001$ ; figure 2). Dominant frequency did not differ between infected and uninfected frogs (DF,  $F_{1,242} = 0.00$ ,  $p = 0.95$ ). Also, no differences were apparent in PRR, IPI or FT ( $F_{1,242} = 0.01$ ,  $p = 0.92$ ;  $F_{1,242} = 0.01$ ,  $p = 0.94$ ;  $F_{1,242} = 1.96$ ,  $p = 0.16$ ; respectively).

Overall, the results suggest, controlling for body size, that infected frogs exerted more calling effort than uninfected, presumably healthier frogs. Aside from Bd infection status, note and pulse repetition rates increased with temperature



**Figure 2.** Call parameters of uninfected and infected males. Call duration (CD), note duration (ND), number of notes, note repetition rate (NRR), number of pulses, pulse repetition rate (PRR), inter-pulse interval (IPI), rise time (RT), fall time (FT) and dominant frequency (DF) (marginal means + s.d.). \*\*\*\* $p < 0.0001$ , \*\*\* $p < 0.001$ .

(NRR:  $F_{1,242} = 27.36$ ,  $p < 0.001$ ; PRR:  $F_{1,242} = 4.02$ ,  $p = 0.05$ ) and other call properties also were affected (IPI:  $F_{1,242} = 11.49$ ,  $p = 0.0008$ ; ND:  $F_{1,242} = 76.24$ ,  $p < 0.0001$ ; RT:  $F_{1,242} = 11.63$ ,  $p = 0.0008$ ; FT:  $F_{1,242} = 68.33$ ,  $p < 0.0001$ ; DF:  $F_{1,242} = 13.99$ ,  $p = 0.0002$ ). DF decreased as body length increased ( $F_{1,242} = 38.35$ ,  $p < 0.0001$ ). Body length also affected PRR ( $F_{1,242} = 15.01$ ,  $p = 0.0001$ ), NN ( $F_{1,242} = 5.21$ ,  $p = 0.02$ ), FT ( $F_{1,242} = 5.60$ ,  $p = 0.02$ ) and CD ( $F_{1,242} = 5.29$ ,  $p = 0.02$ ). Significant interaction effects between body size and infection status on call properties (PN:  $F_{1,242} = 8.61$ ,  $p = 0.004$ ; NN:  $F_{1,242} = 7.59$ ,  $p = 0.006$ ; CD:  $F_{1,242} = 6.58$ ,  $p = 0.01$ ) suggest that larger individuals were more likely than smaller ones to change their call structure when they become infected.

## 4. Discussion

Males infected by Bd appeared to invest more in calling behaviour than their uninfected competitors. As female anurans generally prefer males that produce longer calls with higher call rates [14], infected males thus should benefit from increased reproductive success, at least over the short term. Immunological responses to parasites and pathogens impose metabolic costs on hosts that decrease resources available for other physiological activities. Calling is energetically expensive [15], and thus, infected males might be expected to produce fewer or weaker calls. For example, the calling rate of *Hypsiboas prasinus* tree frogs decreases as their helminth parasite load increases [16]. Yet, paradoxically, male *H. japonica* not only compensate for any costs of infection but also increase their reproductive effort.

Although we never have observed *H. japonica* showing clinical signs of chytridiomycosis, infected individuals are not uncommon in populations [9]. Possibly, frogs, when they are infected, suffer sublethal effects that result in earlier reproductive senescence. In such circumstances, infected frogs might be selected to increase calling effort at the expense of growth, survival or other life-history traits to ensure reproductive success [17]. Indeed, previous studies have demonstrated that testes of Bd-infected frogs are larger and contain more sperm than those of uninfected controls [18]. Furthermore, Bd-infected males, when in good condition, are more likely than uninfected males to be callers [19].

Chytridiomycosis is a highly infectious disease that can be socially transmitted [20]. Males that invest more effort in calling might increase their vulnerability to infection owing

to their increased social contacts. If infected males call more, they may attract more females that consequently become infected by Bd. Infection risk may be further heightened during mating, as hormonal changes can induce temporary immunosuppression [21]. While social contact seems unlikely to be the primary mode by which the disease spreads [19], *H. japonica* is a largely terrestrial species so females are unlikely to be exposed to Bd zoospores in standing water except when ovipositing. Whether Bd directly acts on hosts to increase calling behaviour, thereby effectively promoting its own spread, requires further study.

Although chytridiomycosis is believed to be a principal driver of worldwide amphibian population declines, mass mortality events have been reported in limited geographical regions [8]. Rather, population declines often occur gradually, perhaps in response to environmental changes that increase pathogen effects. Estimates of anuran population sizes typically are based on call surveys [22] but these might not reliably indicate population health. As frogs call to attract mates, enhanced call effort should immediately increase infected males' reproductive success, yet negatively affect population viability over the longer term.

Korean frogs are unlikely to be naive to Bd. Recent research suggests that Bd has been infecting amphibians in Korea for at least the last century [10], sufficient time for endemic Bd to evolve into numerous strains [9]. Over time, frogs presumably have adjusted their life-history strategies to compensate for effects of the pathogen. Achieving higher reproductive success when infected presumably comes at the cost of future survival or reproduction. The enhanced calling effort demonstrated by infected individuals represents only one component of the evolutionary response of amphibians to the pathogenic fungus. Long-term field studies of effects of Bd on amphibian life histories and population dynamics are needed to fully understand the threat that the pathogen poses to its amphibian hosts.

**Ethics.** Experimental protocols were approved by the Institutional Animal Care and Use Committee, Seoul National University (SNU-120710-1).

**Data accessibility.** Data have been deposited in the Dryad repository: <http://dx.doi.org/10.5061/dryad.fp573>.

**Authors' contributions.** B.W. designed the study and wrote the paper. D.A. conducted the fieldwork, laboratory and data analyses.

**Competing interests.** We have no competing interests.

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