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Ancestral chytrid pathogen remains hypervirulent following its long coevolution with amphibian hosts

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Many amphibian species around the world, except in Asia, suffer morbidity and mortality when infected by the emerging infectious pathogen *Batrachochytrium dendrobatidis* (Bd). A lineage of the amphibian chytrid fungus isolated from South Korean amphibians (BdAsia-1) is evolutionarily basal to recombinant global pandemic lineages (BdGPL) associated with worldwide amphibian population declines. In Asia, the Bd pathogen and its amphibian hosts have coevolved over 100 years or more. Thus, resilience of Asian amphibian populations to infection might result from attenuated virulence of endemic Bd lineages, evolved immunity to the pathogen or both. We compared susceptibilities of an Australasian amphibian, *Litoria caerulea*, known to lack resistance to BdGPL, with those of three Korean species, *Bufo gargarizans*, *Bombina orientalis* and *Hyla japonica*, after inoculation with BdAsia-1, BdGPL or a blank solution. Subjects became infected in all experimental treatments but Korean species rapidly cleared themselves of infection, regardless of Bd lineage. They survived with no apparent secondary effects. By contrast, *L. caerulea*, after infection by either BdAsia-1 or BdGPL, suffered deteriorating body condition and carried progressively higher Bd loads over time. Subsequently, most subjects died. Comparing their effects on *L. caerulea*, BdAsia-1 induced more rapid disease progression than BdGPL. The results suggest that genomic recombination with other lineages was not necessary for the ancestral Bd lineage to evolve hypervirulence over its long period of coevolution with amphibian hosts. The pathogen's virulence may have driven strong selection for immune responses in endemic Asian amphibian host species.

1. Introduction

The relentless struggle between pathogens and their hosts is a key factor that shapes global biodiversity. As pathogens evolve mechanisms to exploit their hosts, hosts evolve defences to resist and tolerate infection, driving perpetual cycles of adaptation and counter-adaptation [1]. Steady-state periods of mutual adaptation and persistence can result from reciprocal selection on pathogens and their hosts [2]. Coadaptation may be transient, however, as the evolution of host defences typically lags behind innovations in pathogens' attack strategies [3]. Rather, dramatic declines and extirpations of host populations are expected to occur periodically as their defence strategies catch up. In certain circumstances, pathogens can drive hosts to extinction [4,5]. Rapidly changing environments that disrupt host homeostasis may accentuate these effects [6]. Such appears to be the case with populations of amphibians that are declining in many parts of the world.

The emerging infectious disease chytridiomycosis, caused by pathogenic chytrid fungi, is ravaging amphibian populations in Europe, Australia and the Americas, exacerbated by environmental degradation and climate change [7,8]. Asia accommodates a rich, diverse amphibian fauna, with new species being discovered regularly just as others disappear [9]. Amid the rapid industrialization and

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spewing smokestacks of the continent, amphibians face enormous problems. Yet, epizootics and population extirpations associated with the disease elsewhere in the world have not been witnessed on the continent, nor have clinical signs of amphibian chytridiomycosis been reported. Many Asian amphibians harbour low infection loads of the amphibian chytrid pathogens *Batrachochytrium dendrobatidis* (hereafter denoted Bd) and *Batrachochytrium salamandrivorans* [10–13]. Their effects on hosts, if any, are sublethal; they may affect host life histories [14] and, potentially, long-term population stability [15].

Asian amphibian hosts have been coevolving with chytrid pathogens over the past century if not longer. Chytrid infections have been identified in Korean frogs collected in 1911 [16], in Chinese frogs collected in 1933 [17] and possibly as early as 1902 in Japanese salamanders [18]. Asian endemic Bd lineages are highly diversified when compared with those found on other continents [10,18]. Deep sequence analyses point to four main lineages of the fungus, of which three are distributed globally. The fourth lineage is found only in Korea, on amphibians native to the region, and most closely resembles the common ancestor from which global lineages were derived, probably between 50 and 150 years ago [19]. Substantial genotypic and phenotypic variation, apparent among Bd lineages, may be predictive of their pathogenicity [20,21]. Thus, the study of basal lineages may provide a window into how Bd interacted with its hosts in the past. However, even within lineages, life-history traits of the pathogen are developmentally plastic, which may affect virulence [22].

Korean amphibians are infected largely with endemic Bd lineages, and many populations are infected with high prevalence. Less frequently, native frogs and especially introduced American bullfrogs (*Rana catesbeiana*) bear infections of the hypervirulent global pandemic Bd lineage (BdGPL) [10]. The infection of susceptible hosts by hypervirulent Bd strains fails to trigger effective immune responses [23] and causes the rapid onset of clinical signs of chytridiomycosis, physical disruption of epidermal tight junctions, impairment of osmoregulation and abnormal electrical activity followed by cardiac arrest [24]. Yet Korean amphibian populations fail to show clinical signs of disease and have persisted despite being infected, and sometimes co-infected, by a wide array of Bd lineages. Signatures of recombination between local and more recently derived international lineages are apparent in some Bd genomes, which potentially make these lineages especially virulent to susceptible hosts [25–29].

As Bd has coevolved with its amphibian hosts over many generations, Korean Bd lineages possibly have attenuated in virulence. The infectivity of the pathogen might rise if, for example, infected hosts live longer and engage in more social interactions. Because Korean amphibians are infected largely by endemic lineages, the persistence of their populations might simply be a consequence of reduced pathogen virulence. Alternatively, in their long period of coevolution, Korean amphibians may have evolved resistance to the pathogen. Previous research has demonstrated that both innate and adaptive immunity for resistance to Bd can rapidly evolve [30–32]. In this scenario, recombination may not be a prerequisite for the evolution of hypervirulence. Indeed, endemic Bd lineages may have increased in virulence over time as their hosts evolved resistance [6,33,34].

To discriminate between these possibilities, we infected disease-free subjects of a known Bd-susceptible Australasian species, *Litoria caerulea*, and three Korean native species, *Bufo gargarizans*, *Bombina orientalis* and *Hyla japonica*, with a Korean Bd lineage (BdAsia-1), a BdGPL lineage or a sham inoculate. If Korean Bd lineages have evolved reduced virulence, as commonly expected [35], species such as *L. caerulea* that are susceptible to BdGPL might survive infection by BdAsia-1. A finding that *L. caerulea* is vulnerable to infection by BdAsia-1 would support the alternative hypothesis that the resistance of Asian amphibians to chytridiomycosis is attributable to evolved immune defences. Our study also allows us to assess the specificity of evolved immune defences of amphibians to particular Bd lineages [36], which may be important for planning efficacious disease mitigation strategies [37].

2. Methods

(a) Animal collection and husbandry

Asiatic toads (*Bufo gargarizans*) were collected in February 2017 from Geumsan and Jeonju, South Korea. Oriental fire-bellied toads (*Bombina orientalis*) were collected in Chuncheon, South Korea, during July and August 2017. Japanese tree frogs (*Hyla japonica*) were collected in Seoul (Nakseongdae) and Goyang, South Korea, during July and August 2017. White's tree frogs (*Litoria caerulea*) were collected in New Guinea during November and December 2016 and shipped directly to South Korea. All study subjects were collected in the wild, under permission of relevant statutory authorities.

We swabbed all frogs to test for Bd infection immediately upon collection (Korean species) or on arrival in Korea (*L. caerulea*) (methods below). As expected, based on our previous findings [10], some subjects of the Korean species (less than 20%) were infected by Bd so were excluded from further use. None of the *L. caerulea* imported from New Guinea tested positive for Bd.

All four species are largely terrestrial, so frogs were housed individually in closed plastic containers appropriate to their size (290 × 90 × 200 mm for *L. caerulea*, *B. gargarizans*, *B. orientalis*; 235 × 72 × 165 mm for *H. japonica*) with moistened unbleached paper towels and small open water reservoirs. Water was carbon-filtered and UV-treated to ensure it was pathogen-free. Containers were cleaned and water replaced three times weekly under sterile conditions before the inoculation and twice weekly thereafter. Subjects were fed with mealworms (*Tenebrio molitor* larvae) that had been dusted with amphibian nutrient powder (Superworm, Seoul, South Korea; 20% calcium, 25% crude protein, Vitamin D3 and minerals) ad libitum. Subjects were held in a containment room at 21–22°C on a 12 L : 12 D photoperiod with a relative humidity of 40%.

Prior to beginning treatments, we conducted both nested [18] and real-time PCR [38] on each subject to ensure that it was free of Bd infection (methods below). Any infected subjects were excluded from further experimental work (sample sizes in electronic supplementary material, table S1). We measured the mass and snout–vent length (SVL) of each subject (electronic supplementary material, table S2), and assigned subjects randomly to three treatments: inoculation with BdAsia-1, BdGPL or control.

(b) Infection experiment

We previously isolated BdAsia-1, the basal Bd lineage that emerged to cause worldwide epizootics of chytridiomycosis [19], from *B. orientalis* in South Korea. This culture was cryopreserved upon isolation and revived prior to use with five passages. A second isolate, Abercrombie R-L. booroolongensis, was isolated from frogs in New South Wales, Australia, by Lee Berger in 2009. It was cryopreserved, and underwent seven passages. This isolate is one of the most virulent known to cause clinical signs of

chytridiomycosis [39], and falls within the global pandemic lineage (BdGPL) associated with worldwide epizootics [19].

We cultured BdGPL and BdAsia-1 in TGhL broth (6.4 g tryptone, 1.6 g gelatin hydrolysate, 3.2 g lactose monohydrate, 800 ml distilled water) for 4 or 5 days, and then transferred 1 ml of culture to each TGhL plate (10% agar) [40]. After one week, we flooded each plate with 1–2 ml sterilized water and let the plate stand for 20 min before collecting zoospores from it. We then quantified zoospore activity by counting moving zoospores under a microscope using a haemocytometer.

We inoculated subjects with between 500 000 and 800 000 zoospores, increasing dose with average species mass (electronic supplementary material, table S2). We used larger numbers of zoospores to inoculate the Korean species than *L. caerulea*, which is known to be highly susceptible to Bd. To infect the frogs, we placed subjects into closed containers with prepared inoculum solutions of zoospores in sterilized water. Control groups were treated in the same way but without Bd zoospores. In both experimental and control treatments, inoculum solutions were gently swirled around the subjects to ensure adequate exposure to potential infection. After 24 h, we transferred each individual back into its original container.

After Bd treatment, we monitored subjects for clinical signs of chytridiomycosis, such as lethargy, cutaneous erythema, inappetence and skin sloughing [41], at least once daily. Subjects were considered dead when they failed to show a righting reflex and we could find no evidence of heartbeat. We ended the experiment after three months, when all surviving subjects in infected groups tested negative for Bd infection. These frogs were retained in our laboratory colony.

(c) Bd screening and pathogen loads

Prior to beginning the experiment, all subjects were observed for clinical signs of disease and screened at least twice for Bd infection by nested PCR [18]. To non-invasively detect infection, we swabbed frogs (MW-113 rayon swabs, Medical Wire and Equipment, Corsham, UK) along their ventral skin including legs and feet 10 times using a standardized protocol [42]. We extracted DNA from each swab using 50 µl PrepMan Ultra (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol, and stored it at −20°C. We used Bd18SF1 (5′-TTTGTACACACCGC CCGTCGC-3′) and Bd28SR1 (5′-ATATGCTTAAGTTCAGCGGG-3′) as primers for the initial reaction, and Bd1a (5′-CAGTGTGCCA TATGTCACG-3′) and Bd2a (5′-CATGGTTCATATCTGTCCAG-3′) for the second reaction. The PCR conditions consisted of an initial denaturation at 95°C for 5 min, followed by 30 cycles of 30 s at 94°C, 30 s at 58°C, 30 s at 72°C and a final extension at 72°C for 7 min. PCR was run in a Veriti 96-well thermal cycler (Applied Biosystems, Forest City, CA).

To determine Bd loads carried by infected subjects, we analysed the DNA that we had extracted from swabs by real-time PCR. We used primers ITS1-3 Chytr (5′-CCTTGATATAATACAG TGTGCCATATGTC-3′) and 5.8S Chytr (5′-AGCCAAGAGAT CCGTTGTCAA-3′). Thermocycler conditions consisted of an initial uracil–DNA glycosylase incubation step at 50°C for 2 min, followed by polymerase activation at 95°C for 10 min, then by 50 cycles of 10 s at 95°C and 1 min at 60°C, and lastly a melting curve of 95°C for 15 s, 55°C for 15 s and 95°C for 15 s [38]. Each sample was run in duplicate and infection intensity was estimated using a standard curve prepared by serial dilution of a Bd ITS standards. Negative controls prepared by 1 : 5 dilution of PrepMan Ultra were included on every plate. Real-time PCR was run in an Eco Real-Time PCR system (Illumina, San Diego, CA, USA).

(d) Body condition measurements

We tracked changes in each subject's condition during the course of the experiment. For *L. caerulea*, we compared their mass immediately before infection with that at death or at the conclusion of the experiment. For *B. gargarizans*, *B. orientalis* and *H. japonica*, we measured their mass before infection and then subsequently every week 2 days after feeding. By delaying measurements until after feeding, we were able to obtain more consistent measurements. We gently blotted subjects with unbleached paper towels to remove excess moisture before taking mass measurements.

(e) Histology

We dissected skin from both pelvic and ventral regions of all dead frogs ($n = 28$) that tested Bd-positive by PCR. We fixed the tissue in freshly made 10% neutral buffered formalin (formaldehyde 10 ml, distilled water 90 ml, sodium phosphate monobasic dehydrate 0.65 g, sodium phosphate dibasic anhydrous 0.4 g) for 24 h at 4°C. After 2–6 h washing by running distilled water, the fixed tissue was processed in an automatic tissue processor for dehydration, and then embedded in paraffin, sectioned at 5 µm, and placed onto silane-coated slides for haematoxylin and eosin staining. Stained samples were observed under fluorescence microscopy (Axio Observer Z1, Carl Zeiss, Göttingen, Germany).

(f) Statistics

We used a Cox proportional hazards model [43] to compare survival of experimental and control treatments, treating mass and SVL of subjects measured prior to inoculation as covariates. We analysed differential survival among treatments by the non-parametric Kaplan–Meier procedure. Analyses were conducted with the coxph and survfit functions, respectively, in the R package survival.

We compared change in mass of *L. caerulea* during the course of the experiment among treatments by one-way analyses of variance, after verifying that the data were normally distributed. We then conducted post hoc analyses of the differences by Tukey HSD multiple comparisons of means. R functions aov and Tukey HSD were used for these computations.

We ran semi-parametric regressions using spline smoothing models [44] to examine changes in mass of the three Korean species over the course of the experiment. We compared infection loads of *L. caerulea* and the Korean species after infection until death or the end of the experiment using the same approach. Infection loads were first transformed by adding one and computing base-10 logarithm scores. In each case, we smoothed data with cubic spline models and compared treatments with double hierarchical generalized linear mixed models based on a Gaussian distribution [45]. Species and treatment time were treated as fixed effects and subject variation as a random effect. We did not include the control group for the infection intensity analysis as all subjects tested negative for Bd infection. We used Wald tests to quantify the significance of terms included in the models. Analyses were conducted using the R function dhglmfit [46].

All computations were made with R v. 3.3.1 (R Foundation for Statistical Computing, Vienna, Austria).

3. Results

(a) BdAsia lineage is lethal to *Litoria caerulea*

After 31 days, frogs in the BdAsia-1 treatment group started to die and BdGPL-treated frogs began dying soon thereafter (figure 1). The Bd-inoculated subjects died in significantly larger proportions than the controls (Cox proportional hazards model, $Z = 4.29$, $p < 0.00002$). This result was confirmed by the Kaplan–Meier survival analysis (log-rank test, $\chi^2 = 23.0$, 2 d.f., $p = 0.00001$). The initial size of subjects, measured either in length ($Z = 1.30$, $p = 0.19$) or mass ($Z = 0.54$, $p = 0.59$), did not affect the proportion of survivors.

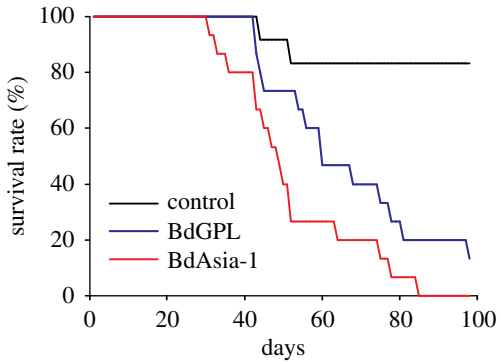

Figure 1. Survival rates among *Litoria caerulea* subjects treated with BdGPL, BdAsia-1 and sham inoculates. More Bd-inoculated subjects than controls died (Cox proportional hazards model, $p < 0.00002$) and BdAsia-1 caused mortality sooner than BdGPL (log-rank test, $p = 0.04$).

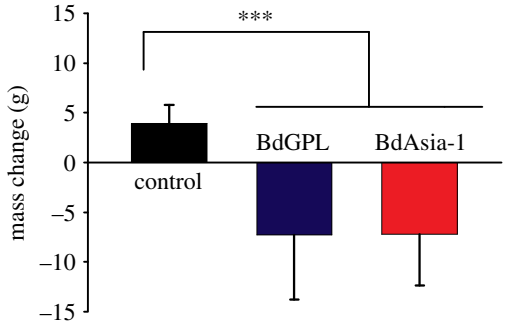

Figure 2. Mass changes of *Litoria caerulea* treated with BdGPL, BdAsia-1 and sham inoculates. Subjects infected by BdAsia-1 and BdGPL lost significantly more mass than those in the control group. Error bars denote s.e.m. ***$p < 0.001$.

BdAsia-1 caused mortality sooner than BdGPL (log-rank test, $\chi^2 = 4.30$, 1 d.f., $p = 0.04$). All BdAsia-1-infected frogs died, while two from the BdGPL infected group survived. Clear clinical signs of chytridiomycosis, including behavioural disorientation, skin sloughing and body wasting, were observed in morbid subjects (electronic supplementary material, figure S1).

Subjects in BdAsia-1 and BdGPL treatment groups lost mass, while those in the control group gained mass during the course of the experiment ($F_{2,28} = 29.79$, $p < 0.00001$) (figure 2). Post hoc analyses demonstrated significant differences in mass change between Bd-treated and control groups ($p < 0.001$), but not between BdGPL and BdAsia-1-treated group ($p = 0.89$).

(b) Pathogen load and histological confirmation in *Litoria caerulea*

Infection intensities, measured as log-transformed zoospore genomic equivalents ($\log_{10}$ZGE), differed between BdGPL and BdAsia-1 treatments when survivors are included ($Z = 4.87$, $p < 0.01$) (figure 3). Censuring the data to exclude survivors, infection loads still trended higher in BdAsia-1 treatment subjects ($Z = 1.94$, $p = 0.053$). We confirmed by histology that Bd-inoculated subjects were infected. In the stratum corneum of infected subjects, we found zoosporangia filled with zoospores as well as empty zoosporangia (electronic supplementary material, figure S2), suggesting that the subjects were infectious.

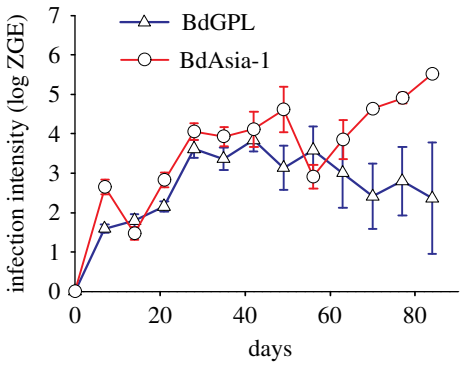

Figure 3. Bd loads in *L. caerulea* following experimental infection. Over the course of the study, infection intensities of subjects infected by BdAsia-1 were higher than those infected by BdGPL ($p < 0.01$). Infection intensities are expressed as base 10 log-transformed zoospore genomic equivalents (+1) (ZGE). Error bars denote s.e.m.

(c) Korean amphibians showed resistance both to BdAsia-1 and BdGPL

Unlike *L. caerulea*, the mass of Bd-inoculated subjects of *B. gargarizans* remained stable, whereas *B. orientalis* and *H. japonica* significantly gained mass during the course of the experiment (figure 4). For each Korean species, mass change did not significantly differ between experimental and control groups (*B. gargarizans*, $Z = 1.77$, $p = 0.08$; *B. orientalis*, $Z = 0.76$, $p = 0.45$; *H. japonica*, $Z = 1.37$, $p = 0.17$).

In Bd-inoculated subjects of each species, Bd loads increased during the first week but then decreased each week subsequently. All *B. gargarizans* and *H. japonica* subjects fully cleared themselves of the pathogen, but most *B. orientalis* retained very low loads at the conclusion of the study. For *B. gargarizans* and *B. orientalis*, infection intensity over time did not differ between subjects inoculated with BdGPL and BdAsia-1 (*B. gargarizans*, $Z = 1.01$, $p = 0.31$; *B. orientalis*, $Z = 1.88$, $p = 0.06$), but for *H. japonica* infection, intensity varied slightly between the two Bd lineages ($Z = 2.11$, $p = 0.04$).

Two *B. orientalis*, one control and one experimental subject inoculated with BdGPL, died during the course of the experiment. Neither subject presented clinical signs of chytridiomycosis and when tested by qPCR, we detected no evidence of Bd infection. For *L. caerulea*, *B. gargarizans* and *H. japonica*, all subjects in experimental treatments became infected with Bd after inoculation. However, 8% (1/13) of *B. orientalis* subjects inoculated with BdGPL and 27% (4/15) of those inoculated with BdAsia-1 never became infected. Taken together, these results show that Korean frogs exhibited resistance to and tolerance of both BdAsia-1 and BdGPL. *Bombina orientalis* especially appears to demonstrate tolerance of the pathogen.

4. Discussion

Our study reciprocally examines the relative virulence of endemic Bd lineages isolated from Asian and Australasian amphibian host species, and the immunological resistance these species present to each lineage. We found that *L. caerulea* is susceptible both to an Australian endemic BdGPL lineage and BdAsia-1, with which it shares no evolutionary history. Yet the three Korean host species that we tested are susceptible to neither Australian BdGPL nor BdAsia-1. By examining how

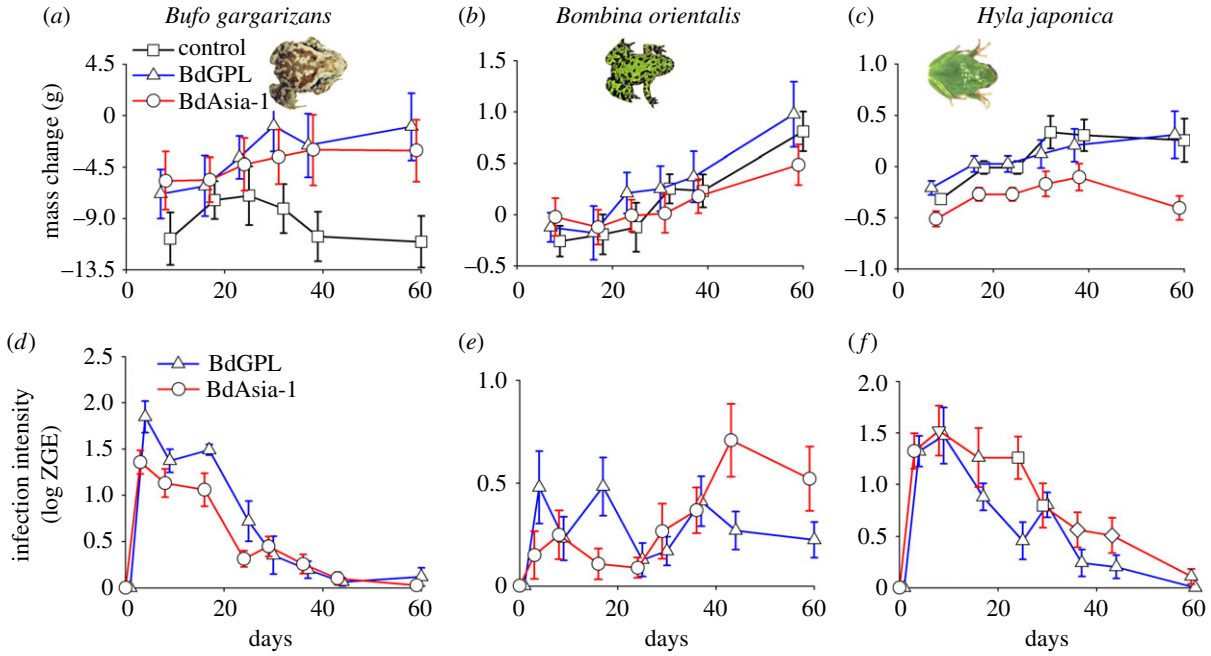

Figure 4. Time course of mass change (*a*–*c*) and Bd loads (*d*–*f*) following experimental infection of *Bufo gargarizans* (*a*,*d*), *Bombina orientalis* (*b*,*e*) and *Hyla japonica* (*c*,*f*). As subjects recovered, *H. japonica* subjects inoculated with BdAsia-1 harboured higher pathogen loads than those inoculated with BdGPL ($p = 0.04$). Infection intensities are expressed as base 10 log-transformed zoospore genomic equivalents (+1) (ZGE). Error bars denote s.e.m.

responses to infection by the amphibian chytrid pathogen vary as a function both of Bd lineage and host species susceptibility, we are able to address why Asian amphibian populations appear resilient to Bd infection even as Australasian species succumb to infection by the same pathogens.

Contrary to the commonly held expectation that pathogens evolve towards benign coexistence with their hosts [35,47], our results provide no evidence that endemic Asian Bd has attenuated in virulence over time. Indeed, the virulence of BdAsia-1 may have dramatically risen over its long evolutionary history with Asian amphibian hosts. While traits associated with Bd virulence may be environmentally influenced [22], climatic conditions in north Asia do not substantially differ from those in some regions of the Nearctic where amphibian populations have been devastated by the pathogen. Our results are consistent with recent findings that amphibians evolve effective physiological responses to tolerate or clear pathogen burdens, as we discuss below. The evolution of immunity to chytridiomycosis may have driven selection for escalated pathogen virulence as host populations increasingly presented resistance to and tolerance of the disease [48–50].

(a) Differences in susceptibility of Asian and Australasian host species to Bd lineages

After inoculation with BdAsia-1, all *L. caerulea* suffered morbidity and mortality. However, two *L. caerulea* inoculated with BdGPL survived through to the end of the experiment. Disease progression was more rapid for subjects infected with BdAsia-1 than for those infected with BdGPL. As their clinical signs became more severe, subjects in both treatments lost mass at comparable rates. However, pathogen loads of subjects infected by BdAsia-1 continued to rise above 10 000 ZGE even as those infected by BdGPL levelled off substantially below this critical threshold level [51]. By all measures, BdAsia-1 caused more consistent pathogenesis in *L. caerulea*, even when compared with responses of Australian *L. caerulea* to BdGPL infection, as previously described [24].

By contrast, all three Korean host species that we tested showed no effects after infection with either BdAsia-1 or BdGPL. Unlike *L. caerulea*, Korean host species gained mass after Bd exposure with the exception of *H. japonica* infected with BdAsia-1, which showed decreases in mass towards the end of the monitoring period. Although we confirmed all subjects inoculated with Bd indeed became infected, peak infection loads, measured just as the experiment began, were very low (less than 70 ZGE). Then, all subjects rapidly cleared themselves of infection, except for *B. orientalis* which appeared to tolerate low pathogen burdens (less than 3 ZGE). Although subjects infected by BdAsia-1 bore higher loads than those infected by BdGPL, these measurements were so low as to be barely detectable.

(b) Virulence varies among Bd lineages

Virulence is best viewed in an ecological context. How pathogens affect hosts may be affected by environmental factors, population structure and community dynamics [6,52]. Even in common garden experiments, where these conditions are held constant, effects of pathogens on hosts can vary dynamically, perhaps owing to differential expression of virulence genes by density-dependent mechanisms such as quorum sensing [53]. Additionally, genomic evolution of traits associated with virulence has been demonstrated in Bd lineages cultured in the laboratory over extremely short time scales, in tens of generations [54]. Thus, comparisons of virulence among lineages with different isolation and passage histories are problematic. Certainly, our data are suggestive of BdAsia-1's higher virulence, given that all *L. caerulea* we infected developed clinical signs of chytridiomycosis and died, but additional studies are needed to confirm our findings.

Recent attempts to assess the relative virulence of Bd lineages have generated conflicting results. At metamorphosis, larvae of North American wood frogs, *Rana sylvatica*, infected

by a Brazilian Bd lineage (BdBrazil) suffered mortality comparable with those infected by BdGPL lineages endemic to their species range, but larvae appeared resistant to a Panamanian BdGPL lineage [55]. Brazilian *Dendropsophus minutes*, some previously Bd-infected when captured as adults, appeared largely Bd-tolerant, but subjects infected with BdGPL or hybrids between BdGPL and BdBrazil carried higher infection burdens than those infected with BdBrazil [25]. One of these hybrid lineages proved most virulent when infecting *Brachycephalus ephippium*, but the parental BdGPL lineage was most virulent when tested on *Ischnocnema parva*. With each host species, BdBrazil caused the least mortality and lowest infection burdens [25].

To compare the virulence of BdAsia-1 with Swiss (BdCH), South African (BdCape) and BdGPL lineages, O'Hanlon *et al*. [19] assessed life-history measures and survival to metamorphosis of British common toads (*Bufo bufo*) inoculated as larvae with each lineage. Subjects inoculated with BdGPL and BdAsia-1 were more likely to become infected and, together with those inoculated with BdCH, were less likely to survive. Metamorphs were smaller than uninfected subjects. However, less than 5% of larvae inoculated with BdCH became infected, and even for the more virulent lineages, most subjects remained uninfected. Previously, we inoculated Korean *Bu. gargarizans* (=*Bufo bufo gargarizans*) larvae with BdGPL, using comparable procedures, but none of the subjects became infected [56]. Inoculated after metamorphosis, BdGPL caused significantly more mortality in *Bufo bufo* than did BdAsia-1 or the other lineages. Taken together, these results suggest that BdAsia-1 is not especially virulent to larval and juvenile British toads, but also that virulence may be modulated by ecological factors.

(c) Coevolution of Asian pathogen and its hosts

Rather than attempting to rank different lineages by their virulence, our main purpose in conducting this study was to establish whether BdAsia-1 became less virulent through coevolution with its hosts over many decades. By comparing responses of Korean and Australasian species to Bd lineages with which they evolved and those to which they are naive, we can conclude that BdAsia-1 is at least as virulent as BdGPL to susceptible hosts. BdAsia-1 is the basal lineage from which hypervirulent BdGPL strains originated, possibly though hybridization and genetic recombination [19]. However, consistent with the substantial genomic variation evident among BdGPL lineages [57], our results demonstrate that the evolution of hypervirulence in this chytrid pathogen is not dependent on recombination events. Rather, we suggest that the emergence of hypervirulence drove the evolution of host resistance, which selected, in turn, for fungal strategies to subvert host immunity [58].

Extant Asian amphibians have overcome fungal counterstrategies so they are immune not only to the endemic strains with which they evolved but also to exotic recombinant strains that are decimating amphibian populations elsewhere in the world. By contrast, *L. caerulea* presented clinical signs of disease not only when infected with BdAsia-1 but also with a Bd lineage that infects hosts in its natural range. *Litoria caerulea* lives in lowland tropical forest in Australia and New Guinea where the climate is not especially favourable to Bd growth or transmission. In these conditions, frogs either are naive to Bd or bear low, subthreshold infection burdens, and so are unaffected by chytridiomycosis. We infected subjects at temperatures optimal for pathogen infectivity rather than host immune response. While Korean amphibians are adapted to this temperature range, tropical species like *L. caerulea* might not be. However, previous studies demonstrate that *L. caerulea*, once acclimatized to these conditions, launch more effective immune responses to Bd than those held in more natural temperature regimens [59]. Thus, we conclude that in the absence of strong selection for resistance or tolerance, *L. caerulea* appears highly susceptible to the pathogen and quickly succumbs to infection by either lineage.

Mass die-offs of amphibians infected by Bd have been reported in Australia, New Zealand, the Americas and parts of Europe. But even amid the carnage caused by chytridiomycosis, some species seem to thrive even as others around them perish. Meanwhile, populations of some threatened species thought to have been extirpated now are slowly recovering [60,61]. And some species feared to be extinct in the wild are beginning to reappear in their former ranges despite the continued presence of virulent Bd lineages harboured by reservoir species [32]. As predicted by laboratory studies [30,31], strong selection for physiological mechanisms that confer resistance to, and tolerance of, the pathogen appears to have rapidly occurred in the wild. As Bd transitions from an epizootic to an enzootic phase, it may pose less of a threat to species that evolve capabilities to cope with it.

(d) Mechanisms of host defence against the pathogen

Variation in susceptibility to Bd among amphibian communities corresponds to interspecific differences in antimicrobial peptides (AMPs), as some lack effectiveness against Bd [62,63]. Symbiotic bacteria also may play a role in innate immunity [64], and their metabolites can exert a synergistic inhibition effect with AMPs to inhibit Bd growth *in vitro* [65]. Possibly, peptides in the skin of *B. orientalis* and *B. gargarizans* have higher antimicrobial activity than those in *L. caerulea* [66–69], but their efficacy against Bd is unknown. Evidence of selection for stronger innate immune responses is accumulating, for example, in descendants of survivors of epizootics in Panamanian rainforests [32].

Adaptive immune responses in some cases also may confer resistance to chytridiomycosis [70]. Comparative studies of Bd-susceptible and resistant amphibians worldwide reveal that resistant species, including *B. orientalis* and *B. gargarizans* that we tested in this study, present similar conformations of the P9 pocket in the MHC class II β1 domain [31]. Within Australian *Litoria verreauxii* populations, only individuals with this MHC conformation survive chytridiomycosis [31]. North American *Rana yavapaiensis* show similar patterns of MHC-based resistance [30,71]. Panamanian *Engystomops pustulosus* are more likely to show such conformations in highland habitats favourable to Bd growth and transmission, as frogs there are most at risk of infection and disease [72]. The resistance mounted by Korean species to Bd in our study thus might be attributable to their evolved innate immunity, adaptive immunity or both.

As amphibians evolved adaptive immune responses against the pathogen, Bd apparently evolved countermeasures to evade host immune responses. Soluble factors released by the pathogen inhibit lymphocyte proliferation and induce apoptosis, although these effects may lessen with repeated infections [58,73,74]. Innate immune responses

to Bd appear to remain unaffected by these factors, as innate leucocyte proliferation remains intact [75,76]. Active suppression of immune function by Bd may make adaptive immune responses costly for susceptible species. Then, paradoxically, rather than conferring resistance, adaptive immunity acts to accelerate disease progression.

5. Conclusion

Pathogen virulence is not static. Infectivity and disease signs vary based on immune responses of hosts, which in turn are modulated by the organism's condition and the social and physical environment in which it lives. Moreover, whether infection culminates either in disease or subclinical effects may depend on interactions between host and pathogen genotypes. Nevertheless, our study clearly demonstrates, for the first time, that Bd has not attenuated in virulence over a long period of coevolution with its amphibian hosts. Indeed, BdAsia-1 appears more virulent than BdGPL when tested on hosts that are naive to it.

Amphibian hosts that have coevolved with endemic Bd express resistance generally to the pathogen in its many forms, including recombinant BdGPL lineages. This finding raises the possibility that effective immunization strategies might be developed, using Bd lineages with low virulence to induce adaptive immune responses to more highly virulent lineages. Asian amphibians are traded internationally and carry endemic lineages, including BdAsia-1, that may threaten amphibian biodiversity. Our research represents a large step forward in resolving the enigma of why Bd infection causes clinical signs of chytridiomycosis in some parts of the world but not others. This, in turn, should assist in the development of management plans for the disease.

Ethics. Experiments were conducted in accordance with regulations of the Institute of Laboratory Animal Resources (permit ILAR-17-04-118) and the Institutional Biosafety Committee (permit SNUIBC-R170502-1) of Seoul National University.

Data accessibility. Data available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.79n5686 [77].

Authors' contributions. B.W. and M.F. conceived and designed the project. M.F. conducted the experimental studies. B.W. and M.F. conducted the statistical analyses. B.W. and M.F. wrote the manuscript.

Competing interests. We declare we have no competing interests.

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