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Treatment of urodelans based on temperature dependent infection dynamics of *Batrachochytrium salamandrivorans*

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The recently emerged chytrid fungus *Batrachochytrium salamandrivorans* currently causes amphibian population declines. We hypothesized that temperature dictates infection dynamics of *B. salamandrivorans*, and that therefore heat treatment may be applied to clear animals from infection. We examined the impact of environmental temperature on *B. salamandrivorans* infection and disease dynamics in fire salamanders (*Salamandra salamandra*). Colonization of salamanders by *B. salamandrivorans* occurred at 15°C and 20°C but not at 25°C, with a significantly faster buildup of infection load and associated earlier mortality at 15°C. Exposing *B. salamandrivorans* infected salamanders to 25°C for 10 days resulted in complete clearance of infection and clinically cured all experimentally infected animals. This treatment protocol was validated in naturally infected wild fire salamanders. In conclusion, we show that *B. salamandrivorans* infection and disease dynamics are significantly dictated by environmental temperature, and that heat treatment is a viable option for clearing *B. salamandrivorans* infections.

In the decades following the identification of *Batrachochytrium dendrobatidis* in 1999[1] it became apparent that this chytrid fungus was one of the biotic drivers of declines and extinctions of hundreds of amphibian species worldwide[2–5]. However, the impact of *B. dendrobatidis* varies regionally from a dramatic decrease of amphibian diversity to a state of host-pathogen equilibrium. In one such region characterized by the co-existence of *B. dendrobatidis* with local amphibian communities[6], another recently described chytrid fungus, *Batrachochytrium salamandrivorans*[7], caused amphibian population declines. The reason for this obvious difference in disease dynamics between both chytrid fungi is not known. Disease dynamics are dictated by pathogen virulence, host factors and environmental determinants. Virulent strains of both chytrids, as well as susceptible host species are present in the affected regions[8–11]. For *B. dendrobatidis*, temperature is considered a key environmental factor[12–14]. One major difference between both chytrids is their different thermal growth characteristics, which is probably due to differences in host spectrum, *B. salamandrivorans* being restricted to urodelan hosts[15]. Knowledge of the infection dynamics of *B. salamandrivorans* at different temperatures may help to develop treatment protocols[16–18]. These are urgently needed as current therapies developed against *B. dendrobatidis*[19] fail to eliminate *B. salamandrivorans* from infected amphibians (unpublished results). We hypothesized that temperature dictates infection and disease dynamics of *B. salamandrivorans* in salamanders, which may be applied to develop a heat treatment protocol to clear infection in animals.

Results & Discussion

Only after exposure at 15°C or 20°C but not at 25°C, the salamanders were colonized by *B. salamandrivorans*. If a 10000 GE infection load per swab is considered indicative for a clinical threshold[20–22], this threshold was reached two times earlier at 15°C than at 20°C (on average 15 ± 4 (SD) days and 31 ± 12 days (SD) respectively, independent *t*-test $p < 0.05$) (Fig. 1). The faster buildup of *B. salamandrivorans* infection loads coincided with earlier mortality at 15°C than at 20°C (22 ± 8 (SD) days and 35 ± 14 (SD) days respectively, Cox regression analysis, $\chi^2 = 3.941$, df = 1, $p < 0.05$) (Fig. 1 and 2). Besides preventing infection of salamanders with *B.*

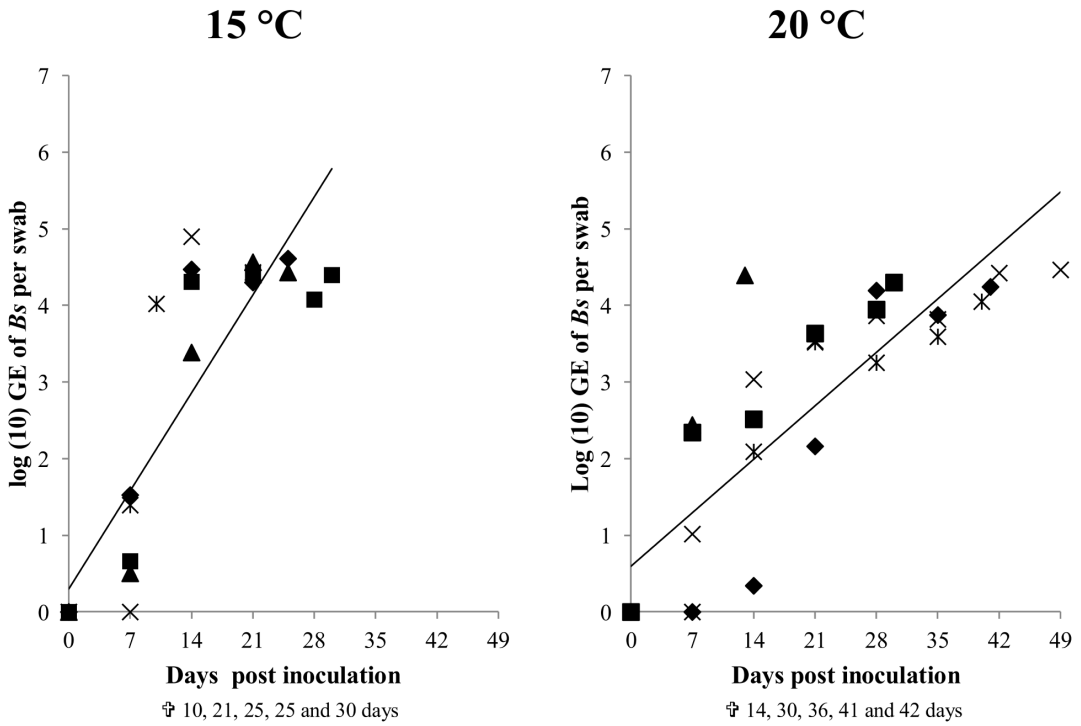

Figure 1 | The course of *Batrachochytrium salamandrivorans* infection in fire salamanders at 15 and 20°C. Each symbol represents the course of infection of an individual animal.Time of death of all animals is depicted beneath the graphs. The line represents the average increase in infection intensity in all tested individuals based on a repeated measure regression analysis.

salamandrivorans, exposure of infected salamanders to a temperature of 25°C during 10 days completely eliminated the infection and resolved *B. salamandrivorans* lesions from all infected animals (Fig. 3 and 4). However, 7 days exposure at 25°C did not result in fungal clearance since recrudescence of infection was observed in all these salamanders within 1–3 weeks after transferring them to an ambient temperature of 15°C (Fig. 4). This is remarkable since cultures of the fungus are killed *in vitro* within 5 days of incubation at 25°C[7] and shows that *B. salamandrivorans* is capable to persist in an urodelan host experiencing temperatures that temporarily surpass the fungal thermal maximum for up to one week. Exposure of the relapsing animals to 25°C for 10 days eliminated the infection. Our results reflect *B. salamandrivorans* growth curves obtained *in vitro*, with an optimal growth range around 15°C[7]. In contrast, the pattern of temperature-dependent growth of *B. dendrobatidis* at 15, 20 and 25°C on frogs was opposite to the pattern of temperature-dependent growth at these temperatures in culture[23], and time until death in frogs infected with *B. dendrobatidis* at 27°C, which is above *B. dendrobatidis'* thermal preference[24], was shorter when compared to time until death of infected frogs kept at lower temperatures[14]. The suitability of raised ambient temperature as treatment option was validated by keeping 30 wild-caught *B. salamandrivorans* infected fire salamanders at 25°C during 10 days. Twenty-six animals were cured of *B. salamandrivorans* infection after this treatment period, 2 died early during treatment, and 2 needed an additional treatment period of 2 days in order to completely clear the infection (Fig. 5). This shows that heat treatment is a viable treatment option for *B. salamandrivorans* infected amphibians when the clinical condition and the thermal tolerance of the animal is taken into account. In order to completely eliminate *B. dendrobatidis* infections higher tempera-

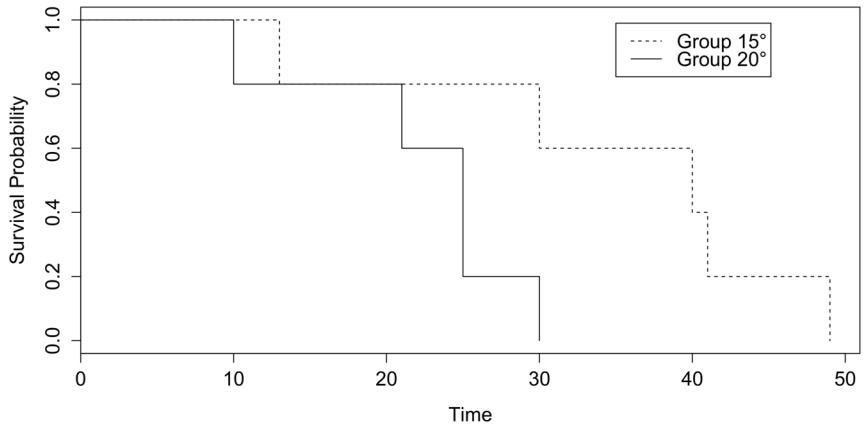

Figure 2 | The probability of survival of salamanders housed at 15 or 20°C after infection with *Batrachochytrium salamandrivorans*. Survival probability was plotted based on a Cox regression analysis ($\chi^2 = 3.981$, df = 1, $p < 0.05$). Time is displayed in days after initial infection. The dotted line represent survival probability of *Batrachochytrium salamandrivorans* infected salamanders housed at 15°C and the full line those housed at 20°C.

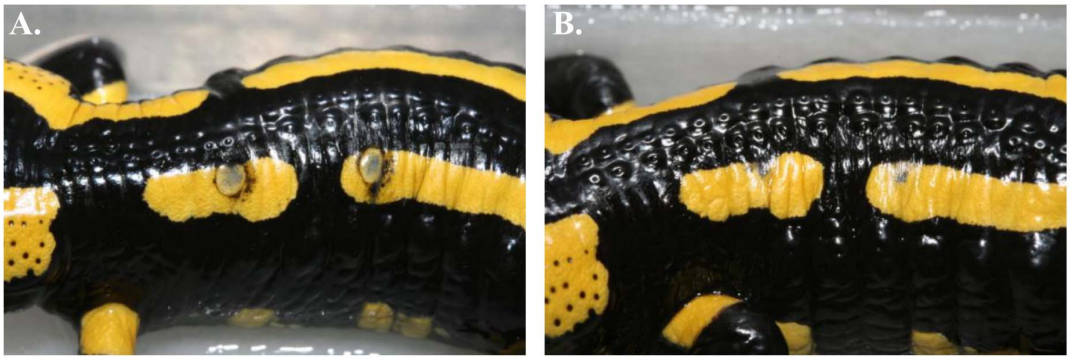

Figure 3 | Heat treatment of amphibians infected with *Batrachochytrium salamandrivorans* clears infection and resolves associated lesions. *Batrachochytrium salamandrivorans* associated skin lesions (A) are clearly reduced after the heat treatment composed of keeping the animals at 25°C during 10 days (B), and will eventually completely resolve.

tures, composed of short exposure to 37°C[16] or extended exposure to 30°C[17] are required. These protocols are not suitable for treating salamanders, as these temperatures surpass the upper thermal limit of most urodelans. The 2 animals that died were in poor clinical condition at the start of the treatment period, and probably died due to thermal shock as *B. salamandrivorans* loads were low at time of death. This points out the narrow margin between the temperature able to eliminate *B. salamandrivorans* and the upper thermal limit most urodelans tolerate. Furthermore, these results show that the course of infection should be carefully monitored since not all animals tested negative for presence of *Bs* DNA after 10 days at 25°C. Although we do not think that this is a result of an active infection but explained by presence of residual *B. salamandrivorans* DNA derived from dead *B. salamandrivorans* cells, this remains uncertain. This could have been further elucidated by transferring the animals back to 15°C after 10 days but we chose to keep them at 25°C until PCR results became negative. Thermal treatment of *B. salamandrivorans* infected amphibians would allow large groups of animals to be treated simultaneously at low costs and lacks the possible downsides linked to drug treatment like toxicity or development of acquired antimicrobial resistance.

In conclusion, these results demonstrate that infection and disease dynamics of *B. salamandrivorans* in urodelans are significantly dictated by environmental temperature. The inability of *B. salamandrivorans* to survive for more than 10 days at 25°C inside its host, renders temperature treatment of infected urodelans a safe, effective and low-cost treatment option, when taking into account the host thermal tolerance.

Methods

All experiments were performed in accordance with the relevant guidelines and regulations. All experiments with experimental animals were carried out with approval of the ethical committee of the Faculty of Veterinary Medicine, Ghent University.

***Batrachochytrium salamandrivorans* strain, culture conditions and experimental inoculation.** The *B. salamandrivorans* type strain was grown in TGhL broth (16 g tryptone, 4 g gelatin hydrolysate, 2 g lactose per liter of distilled water) in 25 cm^3 cell culture flasks and incubated at 15°C. To obtain *B. salamandrivorans* zoospores, a 2 ml aliquot of a 5-day-old culture was inoculated on TGhL agar plates (16 g tryptone, 4 g gelatin hydrolysate, 2 g lactose, 10 g bacteriological agar per liter of distilled water) and incubated at 15°C for 5–7 days. Zoospores were collected by flooding the agar plates with 2 ml of distilled water and subsequent collection of the fluid. A hemocytometer was used to count the number of zoospores present in the suspension and the concentration of the zoospore suspension was adjusted to 5×10^3 zoospores per mL. Animals were inoculated with *B. salamandrivorans* by topically applying one mL of the inoculum on the intact skin.

Animals. *Experimental animals.* Fire salamanders (*Salamandra salamandra*) were experimentally infected with *B. salamandrivorans* to study temperature dependent infection dynamics. The animal experiment was performed with the approval of the ethical committee of the Faculty of Veterinary Medicine (Ghent University, EC2013/87). Twenty-five captive bred fire salamanders were housed individually in plastic containers in a climatized room with an ambient temperature of 15°C. The animals were kept on a moist tissue, with access to a hiding place and water container. Crickets powdered with mineral and vitamin supplement were provided ad libitum as food source. All animals were clinically healthy and free of *B. dendrobatidis* and *B. salamandrivorans* as determined by duplex real-time PCR of skin swabs[20]. An acclimatization period of 1 week was admitted before the start of the experiment.

Field outbreak animals. Heat treatment to clear *B. salamandrivorans* infections in amphibians was validated on 30 wild fire salamanders found to be infected with *B.*

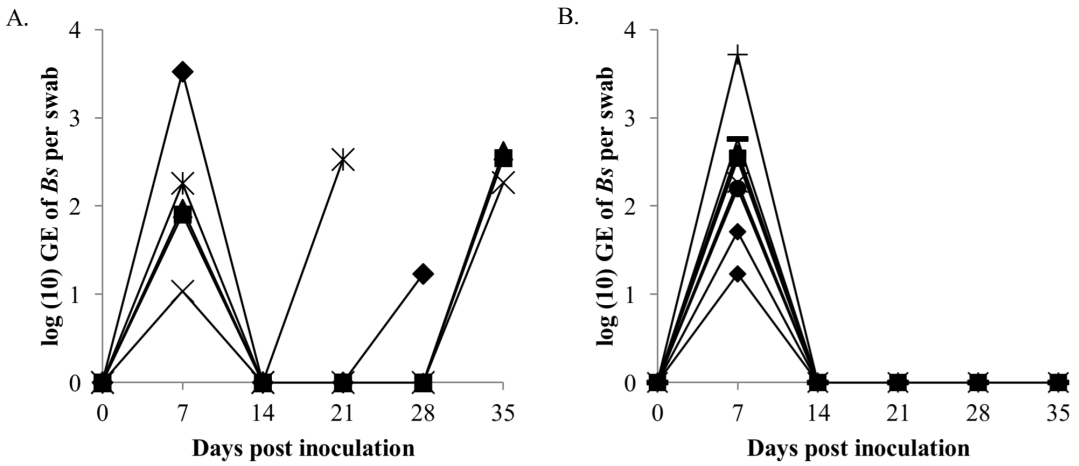

Figure 4 | The effect of exposure to 25°C for 7 and 10 days on the course of *Batrachochytrium salamandrivorans* infection in fire salamanders. After establishment of infection fire salamanders were subjected to an ambient temperature of 25°C for 7 days (A), or 10 days (B). Each symbol represents the course of infection of an individual animal.

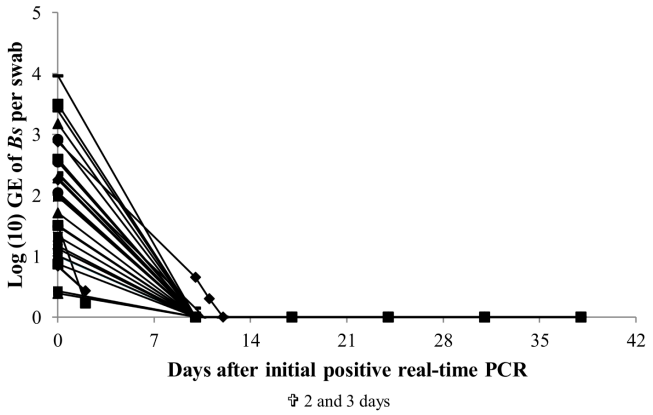

Figure 5 | Heat treatment composed of exposure to 25°C for 10 days of fire salamanders naturally infected with *Batrachochytrium salamandrivorans*. After ascertaining presence of *B. salamandrivorans* in all animals they were subjected to an ambient temperature of 25°C for 10 days. Each symbol represents the course of infection of an individual animal. Time of death for the 2 deceased animals is displayed beneath the graph.

salamandrivorans as determined by real-time PCR. These animals originated from a population in Robertville Belgium (50°29′58.6″N 6°06′21.9″E) undergoing a *B. salamandrivorans* outbreak event and were translocated to the research facility with permission (2014/RS/n°23). Housing conditions of these animals were identical to the conditions described for the experimental animals.

Temperature dependency of *Batrachochytrium salamandrivorans* infection dynamics in salamanders. The experimental animals were randomly assigned to one of the 5 groups (5 animals per group, kept individually). The purpose of the 5 groups was to assess whether *B. salamandrivorans* was able to colonize the animals at different temperatures (groups 1 to 3), and whether temperature could be applied to clear *B. salamandrivorans* from colonized animals (groups 4 and 5). In group 1, animals were inoculated and subsequently kept at 15°C, in group 2 kept at 20°C and in group 3 kept at 25°C (the animals kept at 20 and 25°C were placed in incubators set at the corresponding temperature). The animals in group 4 and 5 were inoculated at 15°C and put at 25°C for 7 or 10 days respectively, after *B. salamandrivorans* infection was established (determined as an increase in infection load between two subsequent samplings). To determine whether the infection would recrudesce after the 25°C exposure, salamanders of groups 4 and 5 were put back at 15°C afterwards and were followed up for another 3 weeks. In case of recrudescence of infection, the animals were put back at 25°C for 10 days. During the experiment, all animals were checked daily for the presence of clinical signs. Skin swabs for *B. salamandrivorans* real-time PCR analysis were collected once every 7 days and/or at the time of death of the animals. An animal was considered negative for *B. salamandrivorans* infection after 4 consecutive negative real-time PCR results. Real-time PCR's were performed on a CFX96 Real Time System (Biorad, Hercules, California, USA) with amplification conditions, primer, and probe concentrations as described elsewhere[20]. Infection loads are presented as genomic equivalents (GE) of *B. salamandrivorans* zoospores. Results were analyzed by means of independent *t*-test and Cox regression analysis using respectively the mass[25] and survival library in R[26]. The censored response variable for the Cox regression analysis was time until death with temperature (15 or 20°C) as explanatory variable.

Thermal treatment of *Batrachochytrium salamandrivorans* infected salamanders. Based on the results of the thermal infection experiments, the *B. salamandrivorans* infected field outbreak animals were treated by putting them at 25°C for 10 days. Skin swabs for *B. salamandrivorans* real-time PCR analysis were collected after 10 days and subsequently every 7 days or at the time of death of the animals. Animals that remained positive after the heat treatment at 25°C during 10 days were kept at 25°C and swabbed daily to follow up remaining infection intensities until the first negative real-time PCR result and subsequently every 7 days. An animal was considered negative for *B. salamandrivorans* infection after 4 consecutive negative real-time PCR results. Real-time PCR's were performed as described above.

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Author contributions

M.B., A.M. and F.P. designed the experiments. M.B. carried out the experiments. M.B., A.M., F.H., F.V., D.B. and F.P. contributed in writing and reviewing the manuscript.

Additional information

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