Short Note

Effect of temperature on subclinical infection by *Batrachochytrium dendrobatidis* in three species of plethodontid salamanders

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Abstract. We assessed the effect of temperature on subclinical *Batrachochytrium dendrobatidis* (*Bd*) infection in three species of plethodontids. Collected individuals were tested for *Bd* in the field during the dry and rainy season and randomly assigned to 15° and 18°C treatments for 30 days. We collected 129 salamanders, of these nine individuals tested positive for *Bd* in the field. At the two temperature trials, 12 individuals that were *Bd*-negative in the field, tested positive for *Bd*. Near the end of the temperature trials, 18 of 21 *Bd*-positive individuals tested negative for *Bd*. Three individuals that were *Bd* positive in the field and assigned to the 18°C trial died during the experiment. Our results suggest that at 15°C the most of plethodontids present subclinical infection, whereas at higher temperatures, *Bd* infection can increase to detectable levels. The processes underlying plethodontids’s recovery from *Bd* infection warrants further study.

Keywords: Chytridiomycosis, prevalence, subclinical infection, terrestrial caudates.

Chytridiomycosis is an emerging infectious disease caused by *Batrachochytrium dendrobatidis* (*Bd*) and *Batrachochytrium salamandrirans* (*Bsal*). *Bd* is a fungus that has generated lethal epizootic outbreaks since the 1980s (Crump et al., 1992; Stuart, 2004) and that has been detected in more than 500 amphibian species distributed worldwide (Olson et al., 2013; Scheele et al., 2019). This fungal species is markedly sensitive to temperature, requiring relatively cool temperatures (17-25°C) to be able to survive and reproduce (Piotrowski et al., 2004). Environmental temperatures also affect ectothermic host species, influencing their immune system efficiency and their susceptibility to *Bd* infection (Stevenson et al., 2014).

Despite records of *Bd* infection in Mexican plethodontids as early as the 1970s (Rovito et al., 2009; Cheng et al., 2011), information about infection patterns and the effects of temperature on the levels of *Bd* infection in this group of salamanders is limited. López (2014) reported that *Bd* prevalence of *Pseudoeurycea leprosa* in La Malinche National Park ranged from 7.69 to 21.09%. For this species, Mendoza-Almeralla et al. (2016) found that 20% of individuals that were *Bd*-negative at the time of collection tested positive for *Bd* in the course of temperature treatments. These authors conclude that *P. leprosa* presented subclinical levels of *Bd* infection in the field (false negative results), and after exposure to the experimental temperatures *Bd* load increased to detection levels. For amphibian conservation, the region of trans-Mexican
volcanic belt is important, therefore, it is relevant to measure the prevalence and Bd load in other populations and species of plethodontids in that area to determine if these vertebrates can effectively maintain and transmit the infection.

To assess Bd subclinical infection levels and the response of Bd load to environmental temperature in Mexican plethodontids, we selected three species from the trans-Mexican volcanic belt at three different sites located in central Mexico, including El Chico National Park (ECNP), Río Frío de Juárez (RFJ) in Mexico state, and Santa Rita Tlahuapan (SRT) in Puebla state (supplementary fig. S1). We selected these localities because they exhibit a similar average ambient temperature in the range of 14.6 to 18°C (ECNP between 12 to 18°C, RFJ 1°C and SRT 14.6°C) and because they have different populations of plethodontids (CONANP, 2005; CONANP, 2013). We conducted a field and an experimental study to evaluate how Bd infection responded to two different temperature regimes. This study had the following objectives: 1) to identify Bd prevalence between seasons, sites and host species, 2) to assess differences in Bd load between populations of Aquiloeurycea cephalica, Chiropterotriton dimidiatus and P. leprosa, and 3) to evaluate if constant exposure to 15°C (temperature lower than the optimal range for Bd growth, Piotrowski et al., 2004) and 18°C (within the range for optimal Bd growth [17-25°C], Piotrowski et al., 2004) of individuals of the three salamander species with subclinical Bd infection resulted in detectable Bd infection levels.

In 2017 we conducted two field surveys at each of the three studied sites during the dry (February and March) and rainy seasons (July and August) (supplementary fig. S1). Wearing surgical gloves we collected and swabbed salamanders with a sterile swab (Deltalab, 01-310253.1) following the Chytrid Swabbing Protocol (Vredenburg and Briggs, 2009), and each collected individual was placed in a plastic bag to prevent cross-contamination. After collection, all individuals were transported to the Disease Ecology and One Health Laboratory (Facultad de Medicina Veterinaria y Zootecnia, Universidad Nacional Autónoma de México).

In the laboratory, each specimen was placed in individual plastic containers with a moistened paper towel laid on the bottom. Half the number of individuals from each species and sampled site was randomly assigned to one of two temperature treatments (15° or 18°C). 24 individuals of A. cephalica, 12 of C. dimidiatus and 28 of P. leprosa experienced 15°C treatment; while 26 individuals of A. cephalica, 12 of C. dimidiatus and 27 of P. leprosa were exposed to 18°C treatment. We used chambers of controlled temperature (Haier chiller HVTM12DABB for the 15°C treatment, and two General Electric chillers GW8XDBB for the 18°C treatment). The temperature and humidity in both treatments were recorded twice a day, using a thermometer-hygrometer (Radio-Shack TM, 6300699; with a precision of ± 1.5% of the reading). The average variation was 0.5 degrees for the 15°C treatment and 0.6°C for the 18°C treatment; while the humidity in both treatments was in the range of 55 to 65%. During the experiment, the salamanders were fed with crickets twice a week and the substrate was changed every three days. The experiment lasted for 30 days and each salamander was swabbed once per week. After the experimental study, salamanders were euthanized and deposited at the Herpetological Collection of the Instituto de Biología, Universidad Nacional Autónoma de México.

DNA was extracted from the salamander skin swabs using the PrepMan protocol (Applied Biosystems). After DNA extraction, Real-Time PCR assays were conducted using the protocol of Boyle et al. (2004). We analyzed each sample in duplicate using 5 μl of the diluted DNA extract, the primers ITS1-3 and 5.8S, the Chytrid MGB2 probe, standards for triplicate (0.1, 1, 10, 100 and 1000 zoospore genomic equivalents
(ZGEs), a positive control and a negative control. A positive result was considered when the values were equal to or greater than 0.1 ZGEs.

The prevalence of infection was estimated by dividing the number of infected salamanders by the total number of specimens swabbed in each site, species and season. To test whether Bd infection load differed between populations, we performed a nested ANOVA using STATISTICA 10 software (Statsoft). In this analysis, the response variable was the zoospore load, which was nested in the following order: times of the year (dry and rainy seasons), sites and species. We run the chi-square test with Yate’s correction (Sokal and Rohlf, 1995) to assess differences in the number of infected individuals between treatments (15°C and 18°C).

A total of 129 individuals of the three salamander species were collected in the three localities during the dry (61) and rainy (68) seasons (table 1). We detected nine Bd-positive individuals (4 at ECNP, 1 at RFJ and 4 at SRT) (table 1). Infected individuals were registered only in the dry season (prevalence of 0.14) in all sampling sites. In ECNP, total prevalence was 0.13; A. cephalica’s prevalence was 0.13 and for C. dimidatus prevalence was 0.15 (table 1). In RFJ, the only species registered was P. leprosa, with a prevalence of 0.50. In SRT, total prevalence was 0.14; 0.1 for A. cephalica and 0.16 for P. leprosa (table 1). Although, overall Bd load ranged in the wild from 16 in P. leprosa to 30 480 in C. dimidiatu (ZGEs), we did not detect differences in the infection load among seasons, localities, and species (ANOVA $F_{11,117} = 1.5554; P = 0.1212$). The highest Bd load was registered at ECNP in an individual of C. dimidiatu, and the lowest at SRT in an individual of P. leprosa (table 1).

Of the 64 individuals assigned to the 15°C treatment, none exhibited Bd infection in the field. During the experiment, one individual of A. cephalica was positive to Bd at the end of the first week and another individual for the same species was positive at the end of the third week (table 2). Similarly, one individual of C. dimidiatu...
dimidiatus was Bd-positive at the end of the first and second week (table 2). All 64 experimental individuals survived and tested negative for Bd at the end of the trial, including the three salamanders that exhibited detectable levels of Bd in the course of the experiment (table 2).

Of 65 individuals assigned to the 18°C treatment, 9 tested positive for Bd at the time of collection (4 P. cephalica, 1 C. dimidiatus and 4 P. leprosa). In this treatment, three of these individuals showed an increase in the degree of infection to lethal levels and six exhibited a decrease in the degree of infection (table 2). During the experiment, nine individuals (4 A. cephalica, 2 C. dimidiatus and 3 P. leprosa) tested positive during the first or second week (table 2). By the third week, all the infected individuals tested negative to Bd (table 2). Additionally, two individuals of A. cephalica died in the third week of this trial, whereas one C. dimidiatus died at the end of the first week. At the time of death, the infection loads of the two A. cephalica individuals were 43 120 and 57 360 ZGEs. At the time of collection, intensity of infection for these two individuals was 50.08 and 133.6 ZGEs, respectively. For the C. dimidiatus individual, infection load in the field was 30 480 ZGEs and died within the first 24 hours after initiation of the 18°C trial.

The total count from collection until the end of the experiment across both temperature treatments was: 108 (40 P. cephalica, 20 C. dimidiatus, 48 P. leprosa) individuals tested negative to Bd, ten individuals of A. cephalica, four of C. dimidiatus and seven of P. leprosa tested positive to infection, of which eight individuals of A. cephalica, three of C. dimidiatus and seven of P. leprosa showed decreased infection load; while two individuals of A. cephalica and one of C. dimidiatus showed increased infection load until death (table 2).

The number of individuals that became positive all along the experiment did not differ between the treatments of 15 and 18°C ($X^2 = 2.1, P > 0.5$). The fact that some individuals that at time of collection tested negative for Bd and at a later date, in the course of the experiments, presented detectable Bd infection loads, suggests that, initially these salamanders had subclinical levels of infection, which subsequently increased in the course of the experiments to a level detected by the qPCR. This could be due to the fact that the temperature of 18°C is affecting the response of the innate immune system, as occurs in other amphibians when they remain at temperatures that are not optimal for their physiological performance (Raffel et al., 2006; Ribas et al., 2009).

Levels of Bd prevalence have been considered low when 2.1 to 14% individuals are infected, and high when prevalence values are greater than 14% (Kriger and Hero, 2007; Guayasamin et al., 2014). Following these criteria, in general we detected low infection prevalence (13-14%) in the field at all sampled sites with the exception of RFJ that showed high prevalence (50%). It is important to mention that this prevalence may be overestimated because one of the two individuals collected presented the infection. The low number of individuals found in RFJ could be caused by the loss of habitats or due to higher Bd infections for these vertebrates (CONANP, 2013). Prevalence was low for the three salamander species (A. cephalica 8.0%, C dimidiatus 4.1%, P. leprosa 7.2%). In the field, infected individuals were registered only in the dry season. It has been suggested that Bd markedly respond to seasonality, with greater detectability during the dry season (Bletz et al., 2015).

Our results showed that the 18°C treatment presented a higher percentage (16% vs. 4.6% in the 15°C treatment) of individuals that had been Bd-negative at the time of collection, developed detectable levels of Bd load in the course of the trials. Additionally, three (33%) individuals that were Bd positive in the field and that were assigned to this trial died during the experiment. These results are probably because this temperature (18°C) falls within the temperature range of optimal growth for Bd (Piotrowski et al., 2004).
Table 2. Comparison of number of infected individuals and *Bd* load in the wild and the trials. ZGEs is the infection load in zoospores genomic equivalents. Week is the week in which *Bd* was detected. The NA = Not applicable and * means that the organism died before of the next week. The ID = name of individual, found in dry (d) or wet (w) season.

| Species                     | Temperature treatment | Number of infected individuals in the wild (ID) | ZGEs [wild] | ZGEs Week 1 | ZGEs Week 2 | ZGEs Week 3 | ZGEs Week 4 | Number of infected individuals during trial (ID) | ZGEs [wild] | ZGEs Week 1 | ZGEs Week 2 | ZGEs Week 3 | ZGEs Week 4 |
|-----------------------------|-----------------------|-----------------------------------------------|-------------|-------------|-------------|-------------|-------------|-----------------------------------------------|-------------|-------------|-------------|-------------|-------------|
| *Aquiloeurycea cephalica*   | 15°C                  | 0                                             | NA          | NA          | NA          | NA          | NA          | 1 (Caudate 10,w)                              | 0           | 50.88       | 0           | 0           | 0           |
|                            | 18°C                  |                                               |             |             |             |             |             | 2 (Caudate 11,w)                              | 0           | 0           | 0           | 74.48       | 0           |
| *Chiropterotriton dimidiatus* | 15°C                  | 0                                             | NA          | NA          | NA          | NA          | NA          | 1 (Caudate 16,w)                              | 0           | 113.6       | 47.12       | 0           | 0           |
|                            | 18°C                  |                                               |             |             |             |             |             | 1 (Caudate 17,d)                              | 0           | 0           | 51.92       | 0           | 0           |
| *Pseudoeurycea leprosa*    | 15°C                  | 0                                             | NA          | NA          | NA          | NA          | NA          | 2 (Caudate 18,d)                              | 0           | 0           | 16.32       | 0           | 0           |
|                            | 18°C                  |                                               |             |             |             |             |             | 4 (Caudate 19,d)                              | 0           | 0           | 16.48       | 0           | 0           |
These results suggest (but statistical analyses cannot confirm) that at low temperatures (<18°C) the three studied salamander species can be reservoirs of Bd in subclinical conditions, whereas at higher temperatures (18°C in the study), more individuals with previously subclinical levels present eventual manifestation of Bd clinical signs and that some individuals succumb to Bd infection. Additionally, the immunological responses of these three species of plethodontids to Bd infection should be analyzed since our results showed that some individuals can recover within a short period of time (30 days). Although not evaluated in our study, the presence of peptides and bacterial communities in the plethodontids’ skin may play an important role in the Bd inhibition associated with these recovery events (Hol et al., 2015; Longo et al., 2015).

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