# Increased tropospheric ozone levels enhance pathogen infection levels of amphibians

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## HIGHLIGHTS

- Increased tropospheric ozone levels enhance chytrid infection in amphibians.
- Ozone high levels significantly reduce the rate of development of amphibian tadpoles.
- Infection load of dead toadlets in montane areas is related to ozone levels.
- Amphibian declines in montane areas can be mediated by increased tropospheric ozone.

## GRAPHICAL ABSTRACT
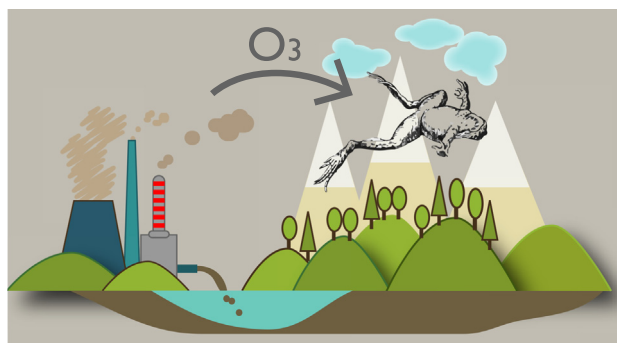
## ABSTRACT

As a result of anthropogenic activities, changes to the chemistry of Earth's atmosphere pose a threat to ecosystem health and biodiversity. One such change is the increase in tropospheric ozone ($O_3$), which is particularly severe in the Mediterranean basin area, where the levels of this pollutant are chronically high during spring and summer time. Within this region, Mediterranean mountain ecosystems are hot spots for biodiversity which may be especially vulnerable to changes in $O_3$ levels. Declines in montane amphibian populations have been recorded worldwide, including the Mediterranean basin. A significant driver of these declines is the emerging infection disease, chytridiomycosis, caused by the aquatic fungus *Batrachochytrium dendrobatidis* (*Bd*). Chytridiomycosis has negatively affected populations of several amphibian species in the Spanish Central Range, including in the Sierra Guadarrama, and interactions with other biotic and abiotic factors are an important part of these declines. However, there is little evidence or knowledge of whether tropospheric $O_3$ levels may be another factor in the outbreaks of this disease. To test the hypothesis that $O_3$ levels are another interactive driver of *Bd* infection dynamics, two different approaches were followed: 1) an experimental study in open top chambers was used to quantify the aspects of how *Bd* infection progressed throughout the metamorphic process under four different $O_3$ levels; and 2) a field epidemiological study was used to analyse the relationship between the *Bd* infection load in the Sierra de Guadarrama and tropospheric $O_3$ levels during a 9 year period. Our results suggest that high $O_3$ levels significantly delayed the rate of development of tadpoles and increased *Bd* infection, providing empirical evidence of two new separate ways that may explain population declines of montane amphibians.

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## 1. Introduction

Air pollution is causing rapid changes to the chemistry of Earth's atmosphere, posing a major threat to our environment. Tropospheric ozone ($O_3$) is a major air pollutant, widely affecting rural and forested areas of the Northern hemisphere, causing harmful impacts on agricultural production, natural ecosystems and loss of the services they provided (Sutton et al., 2011; CLRTAP, 2017). Background $O_3$ levels have been increasing since the 19th century due to the industrial revolution and the increased anthropogenic production of industrial and urban emissions (Young et al., 2013; Nopmongcol et al., 2016; Ainsworth et al., 2020). Ozone precursors (mainly NOx, CO and non-methane volatile organic compounds-NMVOCs) react photochemically to form $O_3$ and can be transported long distances in the atmosphere, enhancing $O_3$ background levels in rural and natural areas. This local and regional transport is accompanied by long-range and intercontinental transport, causing high $O_3$ concentration in regions located far from sources of pollutant emissions (Cristofanelli and Bonasoni, 2009, Chen et al., 2018). Moreover, the link between the $O_3$ problem with the climate change phenomena is widely accepted, considering the future meteorological factors like solar radiation or air temperatures, that enhance atmospheric photochemistry, will also play and important role on the future $O_3$ levels (Colette et al., 2013; Lefohn et al., 2018).

High $O_3$ levels have significant physiological effects on humans and other mammals (Lippmann, 1989; U.S. EPA, 2013; WHO, 2013; Fleming et al., 2018). Ozone exposure induces an oxidative stress at the respiratory tract that affects pulmonary function, bronchial airway reactivity or lung permeability, and depletes the antioxidant defences (Schelegle et al., 2009; Tighe et al., 2015; Brand et al., 2016). Epidemiological studies evaluating chronic long-term effects suggest that daily exposures to $O_3$ increase mortality rates and respiratory morbidity of the European human populations (Jerrett et al., 2009; Diaz et al., 2018). An important effect of $O_3$ on mammalian physiology is related to the alteration of the immune system, and in particular of the function of alveolar macrophages, like decreased phagocytosis of particulate immune complexes, enhanced production of prostaglandin E2 or increased superoxide production (Hollingsworth et al., 2007; Tighe et al., 2015). These functional changes in macrophages are associated with impaired antimicrobial host defense; i.e. the pollutant can enhance pulmonary infections in mice caused by streptococci, which are able to proliferate and more fully express virulence factors after an exposure to the pollutant (Canning et al., 1991; Gilmour et al., 1993). This immunosuppression, expressed as increased sensitivity to bacterial infections, has been considered as a general $O_3$-response of the mammals in the review of Lacroix and Lambre (1998).

Our knowledge of $O_3$ impacts on the health of non-mammalian taxa is scarce, but some studies focussing on amphibians do exist. Toads may exhibit a reduction in lung ventilation and a decline in oxygen consumption after $O_3$ exposure, which is linked to stressful physiological effects (Mautz and Dohm, 2004; Dohm et al., 2001, 2005). Exposure to $O_3$ can also alter the water balance and thermal preferences in anuran amphibians (Dohm et al., 2001, 2005). $O_3$ effects on the immune defense system of amphibians are consistent with those found in mammalian species. In marine toads, $O_3$ can reduce the capacity of the alveolar macrophages to phagocytize foreign particles and microorganisms (Dohm et al., 2005). These results suggest a possible role of oxidant air pollutants, such as $O_3$, in regional declines of amphibian populations, especially considering their potential interactions with pathogen infections, which are considered to be a major driver of amphibian declines.

Global amphibian populations are threatened by an emerging infectious disease, chytridiomycosis, caused by the aquatic generalist fungus *Batrachochytrium dendrobatidis* (hereafter *Bd*) (Fisher et al., 2009). The impacts of this pathogenic fungus are taxonomically, spatially and temporally heterogeneous, depending on different biotic and abiotic factors, some related to climate change parameters (Bosch et al., 2018; Garner et al., 2011). One consistency is that montane species of amphibians have been particularly heavily affected by the disease. *Bd* infects and reproduces in the amphibian skin feeding on keratin, a structural protein found in the mouthparts of larval amphibians and throughout the body of post-metamorphic individuals. *Bd* can cause amphibian mortality by interfering with the multiple physiological processes undertaken by the amphibian skin, such as electrolyte exchange and respiration (Voyles et al., 2009). Immune responses to *Bd* exposure may be also centre on the amphibian skin, with symbiotic bacteria living on the epidermis, either directly inhibiting pathogen growth, or indirectly by priming the amphibian immune system to resist infection. Given our knowledge of how $O_3$ can reduce individual respiratory activity and suppress immune systems, it seems biologically plausible that tropospheric levels of $O_3$ could interact with and potentially increase the impacts of pathogens such as *Bd*.

Studies on $O_3$-fungal-pathogen interactions have largely focussed on plants, with the findings being heavily context-dependent. On one hand, $O_3$ may act as a fungicide and mitigate impacts of pathogens on the host plant (Dohmen, 1987), whereas in some host-pathogen systems the pollutant enhances the fungi infection (Tiedemann et al., 1991). The direction of the response is therefore likely to depend on the complex interactions between host and pathogen and their relative $O_3$-sensitivity. To date, we are aware of no studies investigating the relationship between $O_3$ and fungal pathogens on wild fauna.

The climatic characteristics of the Mediterranean basin favour the photochemical reactions among $O_3$ precursors and the formation of the pollutant (Millán et al., 1997; Cristofanelli and Bonasoni, 2009). These conditions, such as high solar radiation and temperature, and prevailing stable atmospheric conditions result in some of the highest surface $O_3$ concentrations in Europe (EEA, 2011). In the Iberian Peninsula, $O_3$ levels chronically exceed the current thresholds established for plant ecosystems protection (Ribas and Peñuelas, 2006; Adame and Solé, 2013) and frequently exceeds the thresholds for human health (MITECO, 2018). Experimental assays have already demonstrated that these $O_3$ levels are high enough to reduce crop yield and quality (González-Fernández et al., 2014, 2016). On natural vegetation, including forest (Alonso et al., 2014; Marzuoli et al., 2018) and herbaceous species (Sanz et al., 2011; Calvete-Sogo et al., 2014), $O_3$ concentrations affect parameters related to growth and reproductive fitness that may lead to changes in the structure and diversity of communities (Calvete-Sogo et al., 2016).

Mediterranean mountains, which are hot spots for biodiversity (Myers et al., 2000) and frequently belong to protected areas like National Parks or Nature 2000 Network, are currently suffering extensively from elevated levels of tropospheric ozone (Saavedra et al., 2012; Adame and Solé, 2013; Elvira et al., 2016). In the Sierra de Guadarrama mountains, the $O_3$ levels recorded during the 2005–2011 period indicated that concentrations of this pollutant exceed the thresholds for human health, and can be more than three-fold above the standard values for plant protection, according to the Air Quality Directive EU/50/2008 (Elvira et al., 2016). Thus, a tropospheric $O_3$ increase should be considered as a stress factor for the health of these ecosystems and their constituent parts. Although there are no standard values for fauna protection, the $O_3$ seasonal and daily pattern at the highest altitudes, with high background values maintained during the night (Elvira et al., 2016) might increase the potential negative effect for nocturnal fauna like amphibians, which are already experiencing population declines in this region.

Chytridiomycosis in the Spanish Central Range negatively affects the population-level dynamics of several amphibian species. The variability of its effects has been associated with water temperature variability (Fernández-Beaskoetxea et al., 2015) and UV-B exposure (Ortíz-Santaliestra et al., 2011; Hite et al., 2016). However, the relationship between the presence of *Bd* and abiotic factors is not always clear, and the relationship between environmental variables and the prevalence of the infections is weak (Walker et al., 2010). A recent study based on long-term monitoring in the area indicates that the threat posed by

chytridiomycosis is ongoing after two decades, and even highlighted a positive effect of climate warming on populations of three out of the nine species present (Bosch et al., 2018). However, to date, there are no studies that incorporate air quality parameters and their interactions with *Bd*, despite the possibility that they may influence *Bd* infection dynamics.

In order to study the potential role of the high $O_3$ levels at Sierra de Guadarrama area in disease-related amphibian declines two different approaches were followed: an experimental assay where *Bd* infected common midwife toad, *Alytes obstetricans*, tadpoles were exposed to different $O_3$ levels in an Open-Top-Chambers (OTCs) facility; and a field study relating the significance of the $O_3$ factor on the prevalence of *Bd* infection in metamorphs of spiny common toads (*Bufo spinosus*) at Sierra de Guadarrama during a 9 year period. The main hypothesis is that the high oxidative capacity of the pollutant would affect amphibian survival rates by reducing their capacity to combat *Bd* infection, even though the influence of ozone on amphibian survival rates could be moderated by other environment variables which we account for in our analyses.

## 2. Materials and methods

### 2.1. Open-top-chamber experimental study

#### 2.1.1. Experimental design and ozone treatments

The experiment was performed in the CIEMAT Open-Top-Chamber experimental facility (hereafter OTC) located in the Spanish central plateau at Santa Olalla municipality (450 m.a.s.l.; 40°3′N, 4°26′W) at the public research farm La Higueruela (MNCN-CSIC). This location is a rural area 80 km away from Madrid City, the local sources of air pollution. Chronic $O_3$ levels are the only air quality problem in the area. OTC facilities were developed in the 1980s to study the effects of $O_3$ on vegetation and crops (Heck et al., 1982) and they are commonly used to establish exposure and dose-response functions and threshold values for plant protection under international forums like the Air Convention of the United Nations (CLRTAP, 2017).

Twelve National Crop Loss Assessment Network (NCLAN)-type chambers (Heck et al., 1982) with a 3-m diameter, allowed an experimental random block design with four $O_3$ treatments, each replicated three times (three OTCs per $O_3$ treatment). An additional three chamberless ambient plots (AA) were included to control for chamber effect. Ozone treatments were: charcoal filtered air (FA) mirroring the natural preindustrial background levels, non-filtered air (NFA) reproducing ambient levels of the farm and non-filtered air supplemented with 20 and 40 nL L$^{-1}$ of $O_3$ (NFA+ and NFA++ respectively) over an 8-hour period (07:00 to 15:00 GTM). Maximum hourly values at NFA++ during the exposure period ranged between 90 and 110 nL L$^{-1}$ to achieve the sporadically maximum levels observed on the 10-year study of the $O_3$ levels at Sierra de Guadarrama Mountains (Elvira et al., 2016).

Within each OTC, $O_3$ for the NFA+ and NFA++ treatments was supplied by means of an $O_3$-generator (Model 16, A2Z Ozone Systems Inc., USA) system fed with pure oxygen. The concentration of $O_3$ (ML® 9810B, Teledyne, USA), sulphur dioxide (SO$_2$; ML®9850B UV, Teledyne, USA), and nitrogen oxides (NO$_2$ and NO; ML®9841, Teledyne, USA) inside each OTC and AA plot were monitored continuously using an automated time-sharing system which sampled each AA plot and OTC for 10 min, thus sampled all the field each 2.5 h. The air temperature and relative humidity within each OTC and AA plot was monitored with a meteorological sensor (HOBO® Pro v2, Onset, USA) and the water temperature of the tadpole containers was also monitored (TMC6-HD HOBO®, Onset, USA). A more detailed description of the facility can be consulted from Calvete-Sogo et al. (2014).

#### 2.1.2. Animal collection and maintenance

*Alytes obstetricans* tadpoles at Gosner stage 36 (no, or rudimentary, hind limbs present; Gosner, 1960) were captured in April 2016 from

Toro, a mid-altitude site (Zamora, Central Spain, 740 m a.s.l.; 41°22′N, 5°26′W), where the prevalence of *Bd* infection in larval stages is known to approach 100% during colder months (Fernández-Beaskoetxea et al., 2015). The oral disc of a subset of 20 animals was swabbed to quantify *Bd* infection levels (see Methods below), and yielded a 100% prevalence.

Individual tadpoles were placed at ground level within a container filled with 2 L of clean spring water, and between six and eight containers were placed in each treatment replicate. Thus, a total of 20–24 individual tadpoles were exposed to each $O_3$ treatment. Tadpoles were fed ad libitum with ground fish food, and water was changed twice a week. Once the forelimbs of a tadpole had emerged the water in the container was reduced by 70% and a piece of plastic mesh was placed inside to provide terrestrial habitat. Individuals were monitored until the completion of metamorphosis.

#### 2.1.3. Survival and rate of development

Mortality was recorded every day. Rate of development was measured once a week as the proportion of individuals within each $O_3$ treatments to have reached the phenological stage of Gosner stage 44 and 46 by day 27 from the start of the $O_3$ exposure. Gosner stage 44 (hereafter, forelimbs stage) is reached when forelimbs emerge, mouthparts are restructured for terrestrial foraging (teeth present, mouth fully formed), but the tail stub is still present. Gosner stage 46 is reached when metamorphosis just complete (hereafter, toadlet stage).

#### 2.1.4. Bd infection

*Bd* samples were collected when individuals reached forelimbs stage by swabbing both feet and the belly with a sterile cotton swab (MW 100–100, Medical Wire & Equipment) and at toadlet stage by removing a small portion of tissue of the regressing tail and storing it in 70% ethanol (following Geiger et al. (2013) who found accumulation of *Bd* on this body area of *A. obstetricans* undergoing metamorphosis).

DNA extractions from swab and tissue samples were performed using PrepMan Ultra (Applied biosystems) and the amount of *Bd* DNA present in each sample was measured through a CFX96TM Real-Time PCR Detection System (BIO-RAD) with a *Bd*-specific Taqman Assay (Boyle et al., 2004). Each 96-well assay plate included two negative controls and four different standards per duplicate containing DNA from 100, 10, 1 and 0.1 *Bd* genome equivalents (GE). Each sample was performed in duplicate and considered *Bd*-positive when the results of the two replicates were consistent and > 0.1 zoospore genome equivalents. If not, the sample was re-run a third time and considered positive only if another positive result occurred.

#### 2.1.5. Statistical analyses

Difference in the proportion of individuals to reach forelimbs stage at day 27 between the $O_3$-filtered air treatment (FA) and the rest of non-filtered air $O_3$ treatments was compared with a Fisher's exact test. Differences in *Bd* infection load across $O_3$ treatments were analysed using a general lineal model analysis on log-transformed infection load data from both swabs (forelimbs stage) and tissue samples (toadlet stage), considering experimental block as a random factor. Normal probability plots and scatter plots of residuals were used to determine whether assumptions regarding the distribution of residuals were validated. Levenne's test was applied to check variance homoscedasticity. Outliers were tested considering the studentized residuals procedure, but only 5% of the dataset was rejected. When significant differences among treatments were detected ($p < 0.05$), those treatments differing significantly from one another were identified using Tukey Honestly Significant Difference test (HSD). Differences among $O_3$ treatments were also tested with a priori planned comparisons considering linear and quadratic responses. Differences in the proportion of survival toadlets among treatments at the end of the experiment were analysed with a Fisher's exact test.

Ozone exposure indexes to relate $O_3$ levels and effects on wild fauna have not been defined up to now, although for human health or plant damage a complete methodology for risk assessment has been developed in the last decade within the United Nations Air Convention (CLRTAP, 2017) and World Health Organization (WHO, 2013). Thus, for the present study, different $O_3$ exposure indexes weekly calculated were tested: 24 h-mean for the 7-days (24 M) before reaching the forelimbs stage and the toadlet stage, 7-days total accumulated hourly mean values (AOT00), and accumulated hourly mean values above 20, 30 and 40 nL $L^{-1}$ thresholds (AOT20, AOT30, AOT40) for the same 7-days period. The later indexes are calculated as the sum of the differences between hourly concentrations greater than each threshold and the threshold over the considered period (CLRTAP, 2017). Due to the nocturnal activity of toads, accumulated indexes included the whole day period (contrasting with the indexes considered for plants which only considered the daily hours). However, for comparison between the $O_3$ levels during the experiment and previous field data registered at the Sierra de Guadarrama (Elvira et al., 2016), accumulated AOT40 values for diurnal hours thorough the whole experiment (48 days) were also calculated.

Statistical analyses were carried out using Statistica v.11 (StatSoft Inc., USA).

### 2.2. Long term field epidemiological study

We screened 175 toadlets of spiny common toad (*Bufo spinosus*) for *Bd* infection that were found dead at Laguna de Pájaros (Peñalara Massif, Sierra de Guadarrama National Park, Spain) from 2004 to 2012 and preserved in 70% ethanol. All specimens were collected over a two-weeks period every year and had finished their metamorphosis and, therefore were at the stage at which they were reliant on atmospheric air for respiration. Toe clips of 17–20 individuals per year were used for DNA extractions and qPCR analyses were performed as described above.

Data from the CIEMAT monitoring station located less than two kilometres away from Laguna de Pájaros at Cotos (1850 m a.s.l., 40°49′ 31″N, 3°57′40″ W, Sierra de Guadarrama National Park, Spain) were used to record daily $O_3$ concentrations (Elvira et al., 2016). Ozone values were registered considering standardized conditions, following the procedure of the air quality networks. The $O_3$ exposure indexes considered where 24 h mean, and the AOT40 index accumulated for the previous week and for the two weeks preceding the date of death for each of the 175 individuals. We chose a time span of one week to calculate $O_3$ concentrations because in our experimental setup it took a median of 7 days to pass from forelimbs stage to toadlet stage, the breakpoint when usually internal gills are lost. However, we also calculated the average mean air temperatures of the three days, instead a week, preceding the date of death of each metamorphic individual because Fernández-Beaskoetxea et al. (2015) found short-term impacts of temperature on *Bd* load. After *Bd* infection load were log-transformed, we fitted different general linear models using JMP 14 (SAS Inc.) to detect differences in infection intensity among years of collection and across variation on air temperatures, $O_3$ raw concentrations and AOT40 index calculated. Finally, we ordered all possible models that included at least two explanatory variables according to the corrected AICc. We considered the best explanatory models to be the model with the lowest AICc score, as well as any other models that differed from the top model by <2 AICc.

## 3. Results

### 3.1. OTC experimental study

Accumulated AOT40 indexes though the whole experiment (48 days) were 0, 421, 4.802 and 10.062 nL $L^{-1}$ h for FA, NFA, NFA+ and NFA++ respectively. Considering the 24 h-mean index, the value

for the different O3 treatments were 13, 26, 33 and 41 nL $L^{-1}$ for FA, NFA, NFA+ and NFA++ respectively.

### 3.1.1. Survival and rate of development

At the end of the experiment, individual survival at toadlet stage was in the range of 83–100% and no significant differences among treatments were found ($p = 0.2378$). Animals from the FA treatment showed the lowest survival, meanwhile maximum survival was for the AA treatment.

From the start of the $O_3$ exposure, a range of 27 days was necessary for all the individuals to achieve the forelimb stage. Table 1 shows timetable of phenological events during the experiment. As expected, water temperature was lower in the chamberless plots (AA): during May OTC averaged temperature was 1.4 °C higher than AA plots (18.7 °C vs 16.9 °C), this difference increased till 1.8 °C during June (22.5 °C vs 20.7 °C). It took up to 48 days from initial exposure until all individuals reached forelimbs stage: using the mean values of time until metamorphosis across $O_3$ treatments, after 21 days of $O_3$ exposure 6% of the tadpoles reached forelimbs stage; a maximum 35% of the experimental population reached this stage between 27 and 34 days of exposure and a cumulative total of 100% reached forelimbs stage after 48 days of $O_3$ exposure. $O_3$ treatment affected this phenological pattern. The pollutant tended to delay tadpole phenology: tadpoles grown under $O_3$-filtered air arrived earlier at the forelimbs stage compared with the other three $O_3$-treatments ($p = 0.0496$). Consistently with the observed pattern of water temperatures, tadpoles grown in the AA plots were the most delayed (Fig. 1).

Ozone affected the phenological pattern of the metamorphosis to reach the toadlet stage (toadlet stage; Fig. 1). Individuals grown under clean air (FA) reached the toadlet stage earlier. At day 41, when maximum peak of the toadlet stage was observed, 81% of the individuals that completed their metamorphosis were grown under clean atmospheres (FA), while in the other treatments this percentage was 47% ($p = 0.0212$). Considering the cumulate values (Fig. 1), all the individuals in the FA plots completed the toadlet stage at this date, but only 55% of the individuals in the AA plots reached this stage.

### 3.1.2. Bd infection

The *Bd* loads measured prior to the beginning of the experiment were the maximum values found throughout this work, and the interindividual variation was low (mean ± SE of log transformed genomic equivalents of zoospores +1: 4.81 ± 0.12). When tadpoles reached forelimbs stage, *Bd* infection was much lower (Fig. 2). At this stage, although differences between treatments were not significant, the response of the infection to the pollutant showed minimum values in the FA treatment and maximum in the NFA treatment.

At the toadlet stage, ozone effect on the infection intensity was significant when mean values of the *Bd* infection for each $O_3$ treatment was considered: toads developed under NFA++ treatment presented the highest levels of *Bd* infection compared with the other three treatments ($F_{3,37} = 3.66$, $p = 0.0210$; Fig. 2).

When the pattern of the *Bd* infection at the toadlet stage was analysed (Fig. 3), it could be noticed the different pattern of the NFA++ treatment: time range of *Bd* is wider and kept high values at day 34.

Different $O_3$ indexes were tested to express quantitatively the significant $O_3$ effect observed at the toadlet stage (Table 2). The AOT30 accumulated index presented the best correlation with *Bd* infection, considering both quadratic ($R^2 = 0.89$) or linear ($R^2 = 0.50$) relationship, compared with other accumulated indexes based on lower thresholds (AOT20, AOT00) or indexes based in mean values (24 h-mean), showing the importance of $O_3$ values over the $O_3$-preindustrial background on *Bd* spread. However, for quantifying the quadratic relationship the behaviour of all the indexes tested were similar ($R^2$ values in the range 0.71–0.89).

**Table 1**

Timetable of the experimental events and proportion of individuals at the two considered development stages (the cumulative proportion of individual at that stage is given in brackets).

| Event | Date | Days after start exposure | Forelimbs stage (Gosner 44) | Toadlet stage (Gosner 46) |
|---|---|---|---|---|
| Start $O_3$ exposure | 28 April | 1 | 0 | 0 |
| Sampling *Bd* | 19 May | 21 | 0.06 (0.06) | 0 |
| Sampling *Bd* | 25 May | 27 | 0.25 (0.31) | 0.04 (0.04) |
| Sampling *Bd* | 1 June | 34 | 0.34 (0.65) | 0.33 (0.37) |
| Sampling *Bd* | 8 June | 41 | 0.25 (0.90) | 0.42 (0.79) |
| Last sampling *Bd* | 15 June | 48 | 0.10 (1.00) | 0.21 (1.00) |

### 3.2. Long term field epidemiological study

The $O_3$ raw concentrations and the AOT40 index of the week before, as well as the averaged air temperature for the three days preceding the date of death, were related to *Bd* infection loads of animals found dead, as shown by the increase in AICc values and the decrease in model weights in Table 3. All these three variables were included into the top three models that did not differ noticeable among them, while the year of collection was not (Fig. 4). *Bd* infection load has a negative relationship with averaged air temperature for the three days preceding the date of death and with $O_3$ raw concentrations of the week before, while the AOT40 index for the week before has a positive relationship.

### 4. Discussion

Our results suggest that $O_3$ concentration can have a significant effect on both the rate of development in larval amphibians, and on the
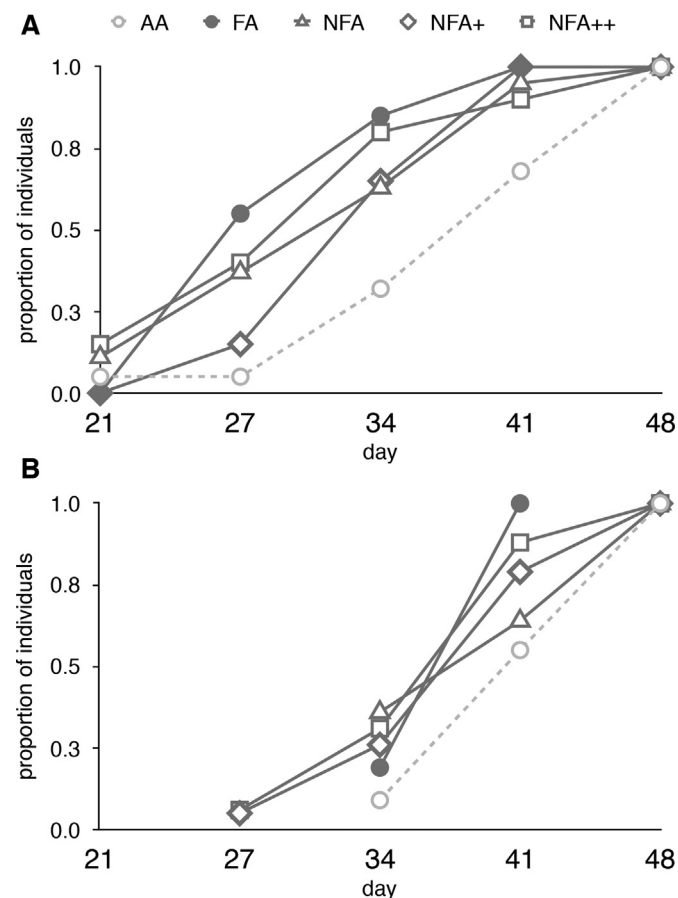


**Fig. 1.** Proportion of individuals (accumulated values across all replicates) reaching the Gosner stage 44 (all four limbs developed; A) and the toadlet stage (B) per $O_3$ treatment according to the number of days from the beginning of the experiment. FA, Filtered Air; NF, Non Filtered Air; NFA+, Non Filtered Air +20 nL $L^{-1}$ of $O_3$; NFA++, Non Filtered Air +40 nL $L^{-1}$ of $O_3$; AA, Ambient plots.

progression of parasite infection within those hosts. Increased levels of $O_3$ delayed the rate of development, and when they did finally metamorphose, those larvae exposed to the highest concentration of $O_3$ had significantly higher levels of *Bd* infection than other treatments. Individually and combined, these results provide empirical evidence of two novel separate mechanisms that may help to explain the high incidence of decline in populations of montane amphibians. Ozone levels at Sierra de Guadarrama show significant interannual variability, with dry years having the highest values (Elvira et al., 2016). The range of the diurnal 3-month AOT40 (May–July) index in the mountains ranged between 6100 and 30,300 nL $L^{-1}$ h; meaning around 3050 and 15,150 nL $L^{-1}$ h for 1.5-month period. These values are also in the range of the 48 days-AOT40 index calculated for the present experiment considering diurnal hours and $O_3$ treatments (NFA+ and NFA++): 4.802–10.062 nL $L^{-1}$ h; even some years the values recorded in the mountain exceed those of the experiment. On the base of this, current $O_3$ levels at the mountains might be enough to produce the observed effects here.

In the experiment, individuals grown under filtered air developed more rapidly than those exposed to increased levels of $O_3$. Individuals in the filtered air were the quickest to develop front limbs (forelimbs stage) and this higher rate was maintained and still evident in the rate at which treatments reached the stage of tail absorption (toadlet stage). $O_3$ is known to have significant negative effects on respiration in a range of taxa, including amphibians. These effects may manifest themselves in pulmonary function, lung permeability and lung ventilation rate (Mautz and Dohm, 2004; Dohm et al., 2001). Metamorphosis is a period in which the methods and mechanisms of respiration of developing amphibians alter radically, via a suite of behavioural, physiological and morphological changes (Duellman and Trueb, 1994). While amphibians are known to increase the rate of development to escape stressful, threatening situations (e.g. the presence of predators,
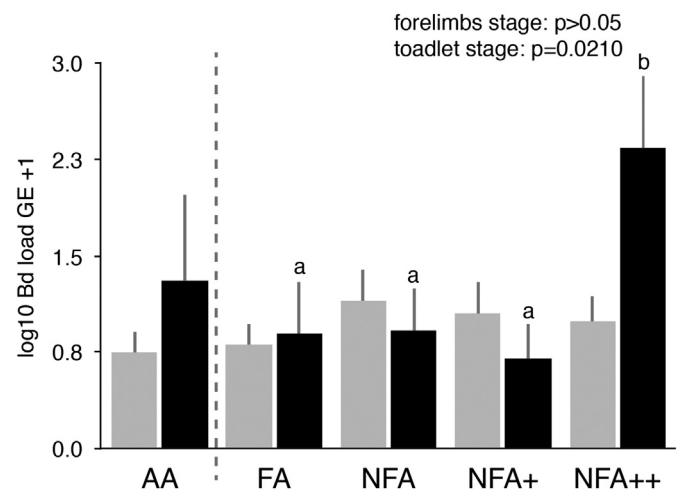


**Fig. 2.** The effect of ozone on *Bd* infection (mean ± SE of log transformed genomic equivalents of zoospores) at the Gosner stage 44 (all four limbs developed; grey bars) and the toadlet stage (black bars) per $O_3$ treatment. FA, Filtered Air; NFA, Non Filtered Air; NFA+, Non Filtered Air +20 nL $L^{-1}$ of $O_3$; NFA++, Non Filtered Air +40 nL $L^{-1}$ of $O_3$; AA, Ambient plots. Different letters indicate statistically significant differences (p < 0.05).
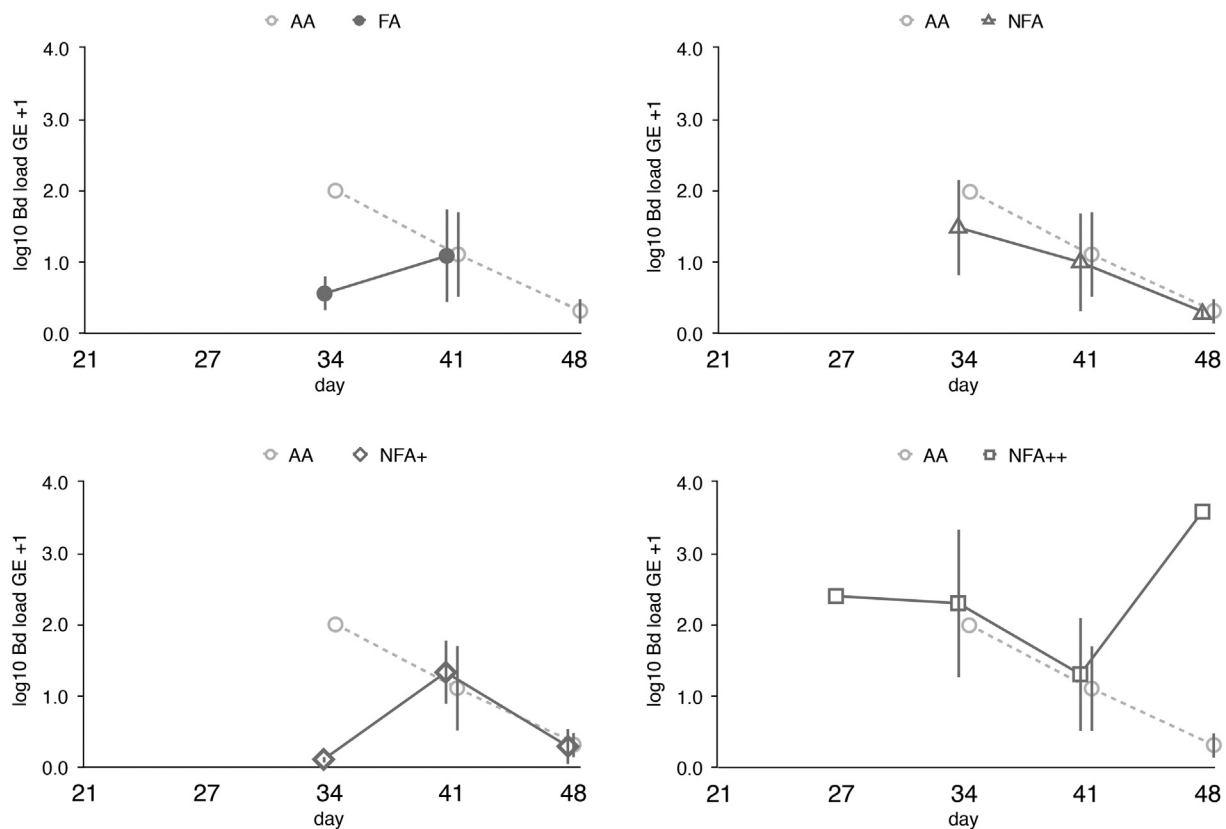
**Fig. 3.** Mean *Bd* infection ± SE of log transformed genomic equivalents of zoospores for individuals in the toadlet stage at the different sampling dates and $O_3$ treatments. FA, Filtered Air; NF, Non-Filtered Air; NFA+, Non-Filtered Air +20 nL L$^{-1}$ of $O_3$; NFA++, Non-Filtered Air +40 nL L$^{-1}$ of $O_3$; AA, Ambient plots.

pollutants or parasites), our data suggest that they may not be able to do so when exposed to increased levels of $O_3$. Alternatively, the observed delay in metamorphosis could be a life-history strategy to maintain the gills for a longer time; at intermediate Gosner stages individuals are respiring via both larval (gills) and metamorphosed (cutaneous and lungs) mechanisms. If cutaneous and lung cells affected by increased $O_3$ levels at these early stage, it may be a viable strategy to delay metamorphosis for an extended period. Finally, perhaps reduced oxygen uptake caused by the exposure to high levels of $O_3$, place a rate-limiting step on amphibian metabolic pathways, thereby dictating the maximum speed on how quickly development can occur.

Our results suggest that exposure to a high level of $O_3$ can also have significant effects on the progression of *Bd* infection; those individuals in the NFA++ treatment had significantly higher infection levels compared to other treatments. $O_3$-driven changes to host-parasite dynamics are likely to be complex and multi-factorial, given the multiple points at which these changes could be caused. The reduced developmental rate

of exposed individuals would result in more contact with other infected individuals and with waterborne *Bd* zoospores, thereby increasing the chances of parasite proliferation and infection progression. Further, there are multiple ways in which amphibian immune functioning could be affected by increased $O_3$ exposure. For example, we are increasingly getting a better understanding of how the host ecology, genetics, and ontogeny and environment – all of which could be affected by exposure to $O_3$ over different timescales - shape amphibian skin microbiome (Bates et al., 2018; Jani and Briggs, 2018; Griffiths et al., 2018), an important part of amphibian immune function in response to parasites (Bates et al., 2018; Campbell et al., 2019). Further, given the reduced rate of development of $O_3$-exposed animals, the development and function of the immune system may also be compromised.

The complexity of how $O_3$ could affect multiple elements of amphibian development, infection, and, ultimately, their population status, is

**Table 2**

Quadratic and linear relationships between *Bd* infection (dependent variable in log transformed genomic equivalents of zoospores) at the toadlet stage and different $O_3$ exposure indexes. AOT30 index is calculated as the sum of the differences between hourly concentrations greater than 30 nL L$^{-1}$ and 30 nL L$^{-1}$ over the weekly period (nL$L^{-1}$h). AOT00 index is the sum of accumulated hourly values over the weekly period (nL L$^{-1}$ h). 24 h mean is the $O_3$ daily average for the week (nL$L^{-1}$).

| $O_3$ index | | $R^2$ |
|---|---|---|
| Quadratic | | |
| AOT30 | y = 5E-07 x$^2$ - 0.001 x + 1.081 | 0.89 |
| AOT00 | y = 2E-07 x$^2$ - 0.002 x + 3.912 | 0.71 |
| 24 h mean | y = 0.006 x$^2$ - 0.271 x + 3.790 | 0.72 |
| Linear | | |
| AOT30 | y = 4E-04 x + 0.635 | 0.50 |
| AOT00 | y = 2E-04 x + 0.037 | 0.33 |
| 24 h mean | y = 0.040 x + 0.026 | 0.35 |

**Table 3**

Candidate general linear models to determine the best predictors of *Bd* infection loads of 175 toadlets of spiny common toads found dead between 2004 and 2012 at Sierra de Guadarrama National Park by the year of collection, the averaged values of air temperature (Temp), $O_3$ raw values and the AOT40 index recorded in the area for the three days (temperature) and the week (ozone values) preceding the date of death of each individual. AOT40 index is calculated as the sum of the differences between hourly concentrations greater than 40 nL L$^{-1}$ and 40 nL L$^{-1}$ over the weekly period (nL L$^{-1}$ h).

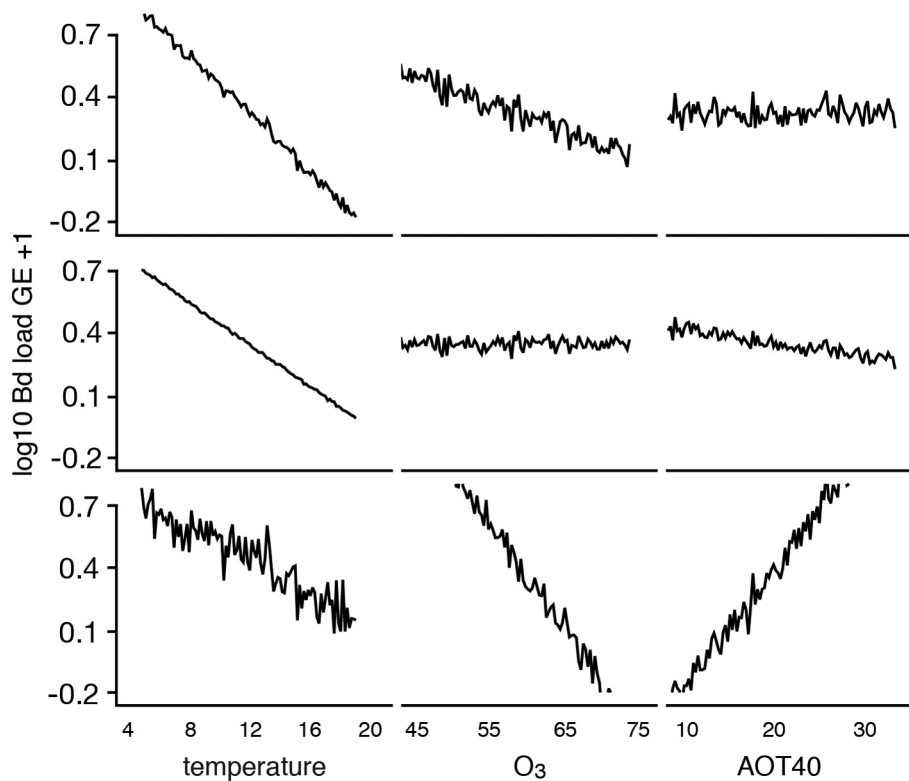| Rank | Model | AICc | $R^2$ | ΔAICc | k | Weight |
|---|---|---|---|---|---|---|
| 1 | $O_3$ + Temp | 385 | 0.26 | 0.0 | 5 | 0.4 |
| 2 | AOT40 + Temp | 385 | 0.26 | 0.1 | 5 | 0.4 |
| 3 | $O_3$ + AOT40 | 386 | 0.23 | 1.6 | 5 | 0.2 |
| 4 | $O_3$ + AOT40 + Temp | 389 | 0.25 | 4.2 | 6 | 0 |
| 5 | year + Temp | 390 | 0.23 | 5.6 | 12 | 0 |
| 6 | year + AOT40 | 391 | 0.22 | 6.4 | 12 | 0 |
| 7 | year + $O_3$ | 391 | 0.23 | 6.8 | 12 | 0 |
| 8 | year + AOT40 + Temp | 395 | 0.23 | 10.8 | 13 | 0 |
| 9 | year + $O_3$ + Temp | 396 | 0.23 | 11.8 | 13 | 0 |
| 10 | year + $O_3$ + AOT40 + Temp | 401 | 0.23 | 16.0 | 14 | 0 |

**Fig. 4.** Predicted values (marginal model plots) of *Bd* infection loads of toadlets of spiny common toads found dead between 2004 and 2012 at Sierra de Guadarrama National Park by the three top models relating infection and the averaged values of air temperature, $O_3$ and AOT40 recorded in the area for the three days (temperature) and the week (ozone values) preceding the date of death.

highlighted by the non-linear way in which *Bd* infection changes with $O_3$ exposure level. Our data suggest that *Bd* infection increase in a non-linear way with the level of $O_3$ exposure. Both were better explained by a quadratic function, rather than a linear one, with lower and higher $O_3$ concentrations being associated with higher infection levels. It might be possible that, in accordance with results found for the $O_3$-plant fungal pathogen interactions, the direction of the response depends on their relative $O_3$ sensitivity. Low $O_3$ levels would allow the spread of the infection; medium levels of the pollutant, without being toxic for the host, could deplete the fungi infection due to its fungicide capacity; meanwhile high ozone levels would be toxic for the host, weakening the host and favouring fungal infection. This may explain why $O_3$ shows a quadratic relationship with the infection loads of toadlets found dead in the field. While a moderate increment of $O_3$ values can contribute to reduce *Bd* infection, a strong increment produces the opposite effect. However, given the data in hand, it is not possible to identify a mechanism by which this quadratic relationship between $O_3$ and *Bd* infection happens, and it would be interesting to use a wider range of concentrations to identify more precisely how infection changes as a function of $O_3$ exposure. Amphibian declines were first observed and recorded in the 1990s, since then multiple drivers of change have been identified and proposed as either 'the' cause of decline or, more realistically in most cases, one of an interactive whole causing amphibian populations to reduce in size and viability. In particular, montane amphibian species and populations have been consistently observed to decline, and a range of factors have been linked to this reduction. Climate change, disease, low genetic variability, and habitat modification have all been proposed as interacting threats to amphibian populations.

### 4.1. Conclusions

Our field and experimental research suggests that the level of $O_3$ is another, perhaps underreported threat to amphibian populations, either alone or in combination with other factors. Understanding better

how $O_3$ levels affect amphibian biology and interact with intrinsic (e.g. development, immune function, breeding biology, genetic diversity) and extrinsic factors (parasite infection, habitat degradation, climate) could provide a more holistic understanding of how amphibian individuals and populations respond to global change and how we may better mitigate these changes with a view to conserving amphibian populations.

### CRediT authorship contribution statement

**Jaime Bosch:** Conceptualization, Methodology, Formal analysis, Investigation, Resources, Data curation, Writing - original draft, Writing - review & editing, Supervision, Funding acquisition. **Susana Elvira:** Conceptualization, Methodology, Formal analysis, Investigation, Resources, Writing - original draft, Writing - review & editing. **Cristina Sausor:** Formal analysis, Investigation, Writing - original draft. **Jon Bielby:** Formal analysis, Writing - original draft, Writing - review & editing. **Ignacio González-Fernández:** Resources, Writing - review & editing. **Rocío Alonso:** Resources, Writing - review & editing. **Victoria Bermejo-Bermejo:** Conceptualization, Methodology, Formal analysis, Investigation, Writing - original draft, Supervision, Funding acquisition.

### Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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