Increased tropospheric ozone levels enhance pathogen infection levels of amphibians

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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.

2. 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 (Cristofanelle et al., 2009, Chen et al., 2017). 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; Lefhon 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 (Jarret et al., 2009; Díaz 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 et al. (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 et al., 2004; Dohm et al., 2001, 2008). 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 defence 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 (Dohne 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

98 interactions with pathogen infections, which are considered to be a major driver of
99 amphibian declines.
100 Global amphibian populations are threatened by an emerging infectious
101 disease, chytridiomycosis, caused by the aquatic generalist fungus
102 *Batrachochytrium dendrobatidis* (hereafter *Bd*) (Fisher et al., 2009). The impacts of
103 this pathogenic fungus are taxonomically, spatially and temporally heterogeneous,
104 depending on different biotic and abiotic factors, some related to climate change
105 parameters (Bosch et al., 2018, Garner et al., 2011). One consistency is that
106 montane species of amphibians have been particularly heavily affected by the
107 disease. *Bd* infects and reproduces in the amphibian skin feeding on keratin, a
108 structural protein found in the mouthparts of larval amphibians and throughout
109 the body of post-metamorphic individuals. *Bd* can cause amphibian mortality by
110 interfering with the multiple physiological processes undertaken by the amphibian
111 skin, such as electrolyte exchange and respiration (Voyles et al., 2009). Immune
112 responses to *Bd* exposure may be also centre on the amphibian skin, with
113 symbiotic bacteria living on the epidermis, either directly inhibiting pathogen
114 growth, or indirectly by priming the amphibian immune system to resist infection.
115 Given our knowledge of how O_3 can reduce individual respiratory activity and
116 suppress immune systems, it seems biologically plausible that tropospheric levels
117 of O_3 could interact with and potentially increase the impacts of pathogens such as
118 *Bd*.
119 Studies on O_3-fungal-pathogen interactions have largely focussed on plants,
120 with the findings being heavily context-dependent. On one hand, O_3 may act as a
121 fungicide and mitigate impacts of pathogens on the host plant (Dohmen et al.,
122 1987), whereas in some host-pathogen systems the pollutant enhances the fungi

123 infection (Tiedemann et al., 1991). The direction of the response is therefore likely
124 to depend on the complex interactions between host and pathogen and their
125 relative O_3-sensitivity. To date, we are aware of no studies investigating the
126 relationship between O_3 and fungal pathogens on wild fauna.
127 The climatic characteristics of the Mediterranean basin favour the
128 photochemical reactions among O_3 precursors and the formation of the pollutant
129 (Millán et al., 1997, Cristofanelli and Bonasoni, 2009). These conditions, such as
130 high solar radiation and temperature, and prevailing stable atmospheric
131 conditions result in some of the highest surface O_3 concentrations in Europe (EEA
132 2011). In the Iberian Peninsula, O_3 levels chronically exceed the current thresholds
133 established for plant ecosystems protection (Ribas and Peñuelas, 2006; Adame and
134 Sole, 2013) and frequently exceeds the thresholds for human health (MITECO,
135 2018). Experimental assays have already demonstrated that these O_3 levels are
136 high enough to reduce crop yield and quality (González-Fernández et al., 2014,
137 2016). On natural vegetation, including forest (Alonso et al., 2013; Marzoulli et al.,
138 2018) and herbaceous species (Sanz et al., 2011; Calvete-Sogo et al., 2014), O_3
139 concentrations affect parameters related to growth and reproductive fitness that
140 may lead to changes in the structure and diversity of communities (Calvete-Sogo et
141 al., 2016).
142 Mediterranean mountains, which are hot spots for biodiversity (Myers et al.,
143 2000) and frequently belong to protected areas like National Parks or Nature 2000
144 Network, are currently suffering extensively from elevated levels of tropospheric
145 ozone (Saavedra et al., 2012; Adame and Sole, 2013; Elvira et al., 2016). In the
146 Sierra de Guadarrama mountains, the O_3 levels recorded during the 2005-2011
147 period indicated that concentrations of this pollutant exceed the thresholds for

148 human health, and can be more than three-fold above the standard values for plant
149 protection, according to the Air Quality Directive EU/50/2008 (Elvira et al., 2016).
150 Thus, a tropospheric O_3 increase should be considered as a stress factor for the
151 health of these ecosystems and their constituent parts. Although there are no
152 standard values for fauna protection, the O_3 seasonal and daily pattern at the
153 highest altitudes, with high background values maintained during the night (Elvira
154 et al., 2016) might increase the potential negative effect for nocturnal fauna like
155 amphibians, which are already experiencing population declines in this region.
156 Chytridiomycosis in the Spanish Central Range negatively affects the
157 population-level dynamics of several amphibian species. The variability of its
158 effects has been associated with water temperature variability (Fernandez-
159 Beaskoetxea et al., 2015) and UV-B exposure (Ortíz-Santaliestra et al., 2011; Hite et
160 al., 2016). However, the relationship between the presence of *Bd* and abiotic
161 factors is not always clear, and the relationship between environmental variables
162 and the prevalence of the infections is weak (Walker et al., 2010). A recent study
163 based on long-term monitoring in the area indicates that the threat posed by
164 chytridiomycosis is ongoing after two decades, and even highlighted a positive
165 effect of climate warming on populations of three out of the nine species present
166 (Bosch et al., 2018). However, to date, there are no studies that incorporate air
167 quality parameters and their interactions with *Bd*, despite the possibility that they
168 may influence *Bd* infection dynamics.
169 In order to study the potential role of the high O_3 levels at Sierra de
170 Guadarrama area in disease-related amphibian declines two different approaches
171 were followed: an experimental assay where *Bd* infected common midwife toad,
172 *Alytes obstretricans*, 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.

3. MATERIALS AND METHODS

3.1. Open-top-chamber experimental study

3.1.1. 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 far from local sources of air pollution and 80 km away from Madrid City. 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

198 control for chamber effect. Ozone treatments were: charcoal filtered air (FA)
199 mirroring the natural preindustrial background levels, non-filtered air (NFA)
200 reproducing ambient levels of the farm and non-filtered air supplemented with 20
201 and 40 nL L^{-1} of O_3 (NFA+ and NFA++ respectively) over an 8-hour period (07:00
202 to 15:00 GTM). Maximum hourly values at NFA++ during the exposure period
203 ranged between 90-110 nL L^{-1} to achieve the sporadically maximum levels
204 observed on the 10-year study of the O_3 levels at Sierra de Guadarrama Mountains
205 (Elvira et al., 2016).
206 Within each OTC, O_3 for the NFA+ and NFA++ treatments was supplied by
207 means of an O_3-generator (Model 16, A2Z Ozone Systems Inc., USA) system fed
208 with pure oxygen. The concentration of O_3 (ML® 9810B, Teledyne, USA), sulphur
209 dioxide (SO_2; ML®9850B UV, Teledyne, USA), and nitrogen oxides (NO_2 and NO;
210 ML®9841, Teledyne, USA) inside each OTC and AA plot were monitored
211 continuously using an automated time-sharing system which sampled each AA plot
212 and OTC for 10 min, thus sampled all the field each 2.5 h. The air temperature and
213 relative humidity within each OTC and AA plot was monitored with a
214 meteorological sensor (HOBO® Pro v2, Onset, USA) and the water temperature of
215 the tadpole containers was also monitored (TMC6-HD HOBO®, Onset, USA). A more
216 detailed description of the facility can be consulted from Calvete et al., (2014).
217 3.1.2. Animal collection and maintenance
218 *Alytes obstetricans* tadpoles at Gosner stage 36 (no, or rudimentary, hind
219 limbs present; Gosner 1960) were captured in April 2016 from Toro, a mid-
220 altitude site (Zamora, Central Spain, 740 m a.s.l.; 41°22'N, 5°26'W), where the
221 prevalence of *Bd* infection in larval stages is known to approach 100% during
222 colder months (Fernández-Beaskoetxea et al., 2015). The oral disc of a subset of 20

223 animals was swabbed to quantify *Bd* infection levels (see methods below), and
224 yielded a 100% prevalence.
225 Individual tadpoles were placed at ground level within a container filled
226 with 2 L of clean spring water, and between six and eight containers were placed in
227 each treatment replicate. Thus, a total of 20-24 individual tadpoles were exposed
228 to each O_3 treatment. Tadpoles were fed *ad libitum* with ground fish food, and
229 water was changed twice a week. Once the forelimbs of a tadpole had emerged the
230 water in the container was reduced by 70% and a piece of plastic mesh was placed
231 inside to provide terrestrial habitat. Individuals were monitored until the
232 completion of metamorphosis.
233 3.1.3. Survival and rate of development
234 Mortality was recorded every day. Rate of development was measured once
235 a week as the proportion of individuals within each O_3 treatments to have reached
236 the phenological stage of Gosner stage 44 and 46 by day 27 from the start of the O_3
237 exposure. Gosner stage 44 (hereafter, forelimbs stage) is reached when forelimbs
238 emerge, mouthparts are restructured for terrestrial foraging (teeth present, mouth
239 fully formed), but the tail stub is still present. Gosner stage 46 is reached when
240 metamorphosis just complete (hereafter, toadlet stage).
241 3.1.4. *Bd* infection
242 *Bd* samples were collected when individuals reached forelimbs stage by
243 swabbing both feet and the belly with a sterile cotton swab (MW 100–100, Medical
244 Wire & Equipment) and at toadlet stage by removing a small portion of tissue of
245 the regressing tail and storing it in 70% ethanol (following Geiger et al. (2013)
246 who found accumulation of *Bd* on this body area of *A. obstetricans* undergoing
247 metamorphosis).

248 DNA extractions from swab and tissue samples were performed using
249 PrepMan Ultra (Applied biosystems) and the amount of *Bd* DNA present in each
250 sample was measured through a CFX96TM Real-Time PCR Detection System (BIO-
251 RAD) with a *Bd*-specific Taqman Assay (Boyle et al., 2004). Each 96-well assay
252 plate included two negative controls and four different standards per duplicate
253 containing DNA from 100, 10, 1 and 0.1 *Bd* genome equivalents (GE). Each sample
254 was performed in duplicate and considered *Bd*-positive when the results of the
255 two replicates were consistent and > 0.1 zoospore genome equivalents. If not, the
256 sample was re-run a third time and considered positive only if another positive
257 result occurred.
258 3.1.5. Statistical analyses
259 Difference in the proportion of individuals to reach forelimbs stage at day
260 27 between the O_3-filtered air treatment (FA) and the rest of non-filtered air O_3
261 treatments was compared with a Fisher's exact test. Differences in *Bd* infection
262 load across O_3 treatments were analysed using a general lineal model analysis on
263 log-transformed infection load data from both swabs (forelimbs stage) and tissue
264 samples (toadlet stage), considering experimental block as a random factor.
265 Normal probability plots and scatter plots of residuals were used to determine
266 whether assumptions regarding the distribution of residuals were validated.
267 Levenne's test was applied to check variance homoscedasticity. Outliers were tested
268 considering the studentized residuals procedure, but only 5 % of the dataset was
269 rejected. When significant differences among treatments were detected ($p<0.05$),
270 those treatments differing significantly from one another were identified using
271 Tukey Honestly Significant Difference test (HSD). Differences among O_3 treatments
272 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: 24h-mean for the 7-days (24M) 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).

3.2. 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%

298 ethanol. All specimens were collected over a two-weeks period every year and had
299 finished their metamorphosis and, therefore were at the stage at which they were
300 reliant on atmospheric air for respiration. Toe clips of 17-20 individuals per year
301 were used for DNA extractions and qPCR analyses were performed as described
302 above.
303 Data from the CIEMAT monitoring station located less than two kilometres
304 away from Laguna de Pájaros at Cotos (1850 m a.s.l., 40°49′ 31″N, 3°57′40″ W,
305 Sierra de Guadarrama National Park, Spain) were used to record daily O_3
306 concentrations (Elvira et al., 2016). Ozone values were registered considering
307 standardized conditions, following the procedure of the air quality networks. The
308 O_3 exposure indexes considered where 24 h mean, and the AOT40 index
309 accumulated for the previous week and for the two weeks preceding the date of
310 death for each of the 175 individuals. We chose a time span of one week to
311 calculate O_3 concentrations because in our experimental setup it took a median of 7
312 days to pass from forelimbs stage to toadlet stage, the breakpoint when usually
313 internal gills are lost. However, we also calculated the average mean air
314 temperatures of the three days, instead a week, preceding the date of death of each
315 metamorphic individual because Fernández-Beaskoetxea et al. (2015) found short-
316 term impacts of temperature on *Bd* load. After *Bd* infection load were log-
317 transformed, we fitted different general linear models using JMP 14 (SAS Inc.) to
318 detect differences in infection intensity among years of collection and across
319 variation on air temperatures, O_3 raw concentrations and AOT40 index calculated.
320 Finally, we ordered all possible models that included at least two explanatory
321 variables according to the corrected AICc. We considered the best explanatory

322 models to be the model with the lowest AICc score, as well as any other models
323 that differed from the top model by < 2 AICc.
324
325 **4. RESULTS**
326 4.1. OTC experimental study
327 Accumulated AOT40 indexes though the whole experiment (48 days) were
328 0, 421, 4.802 and 10.062 nL L^{-1} h for FA, NFA, NFA+ and NFA++ respectively.
329 Considering the 24 h-mean index, the value for the different O3 treatments were
330 13, 26, 33 and 41nL L^{-1} for FA, NFA, NFA+ and NFA++ respectively.
331 4.1.1. Survival and rate of development
332 At the end of the experiment, individual survival at toadlet stage was in the
333 range of 83-100% and no significant differences among treatments were found
334 (p=0.2378). Animals from the FA treatment showed the lowest survival,
335 meanwhile maximum survival was for the AA treatment.
336 From the start of the O_3 exposure, a range of 27 days was necessary for all
337 the individuals to achieve the forelimb stage. Table 1 shows timetable of
338 phenological events during the experiment. As expected, water temperature was
339 lower in the chamberless plots (AA): during May OTC averaged temperature was
340 1.4 ºC higher than AA plots (18.7ºC *vs* 16.9ºC), this difference increased till 1.8ºC
341 during June (22.5ºC *vs* 20.7ºC). It took up to 48 days from initial exposure until all
342 individuals reached forelimbs stage: using the mean values of time until
343 metamorphosis across O_3 treatments, after 21 days of O_3 exposure 6% of the
344 tadpoles reached forelimbs stage; a maximum 35% of the experimental population
345 reached this stage between 27 and 34 days of exposure and a cumulative total of
346 100% 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 (Figure 1).

Ozone affected the phenological pattern of the metamorphosis to reach the toadlet stage (toadlet stage; Figure 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 (Figure 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.

4.1.2. *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 (Figure 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 ($F_{3,37}=3.66$, $p=0.0210$; Figure 2).

When the pattern of the *Bd* infection at the toadlet stage was analyzed (Figure 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 (24h-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).

4.2. *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 (Figure 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.

5. 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 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 6,100 and 30,300 nL L^{-1} h; meaning around 3,050 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 et al., 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, 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_3levels 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

445 better understanding of how the host ecology, genetics, and ontogeny and
446 environment – all of which could be affected by exposure to O_3 over different
447 timescales - shape amphibian skin microbiome (Bates et al., 2018; Jani and Briggs,
448 2018; Griffiths et al., 2018), an important part of amphibian immune function in
449 response to parasites (Bates et al., 2018; Campbell et al., 2019). Further, given the
450 reduced rate of development of O_3-exposed animals, the development and function
451 of the immune system may also be compromised.

452 The complexity of how O_3 could affect multiple elements of amphibian
453 development, infection, and, ultimately, their population status, is highlighted by
454 the non-linear way in which *Bd* infection changes with O_3 exposure level. Our data
455 suggest that *Bd* infection increase in a non-linear way with the level of O_3 exposure.
456 Both were better explained by a quadratic function, rather than a linear one, with
457 lower and higher O_3 concentrations being associated with higher infection levels. It
458 might be possible that, in accordance with results found for the O_3-plant fungal
459 pathogen interactions, the direction of the response depends on their relative O_3
460 sensitivity. Low O_3 levels would allow the spread of the infection; medium levels of
461 the pollutant, without being toxic for the host, could deplete the fungi infection due
462 to its fungicide capacity; meanwhile high ozone levels would be toxic for the host,
463 weakening the host and favouring fungal infection. This may explain why O_3 shows
464 a quadratic relationship with the infection loads of toadlets found dead in the field.
465 While a moderate increment of O_3 values can contribute to reduce *Bd* infection, a
466 strong increment produces the opposite effect. However, given the data in hand, it
467 is not possible to identify a mechanism by which this quadratic relationship
468 between O_3 and *Bd* infection happens, and it would be interesting to use a wider
469 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.

5.1. 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.

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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)

678 **Table 2.** Quadratic and linear relationships between *Bd* infection (dependent
679 variable in log transformed genomic equivalents of zoospores) at the toadlet stage
680 and different O_3 exposure indexes. AOT30 index is calculated as the sum of the
681 differences between hourly concentrations greater than 30 nL L^{-1} and 30 nL L^{-1}
682 over the weekly period (nL L^{-1} h). AOT00 index is the sum of accumulated hourly
683 values over the weekly period (nL L^{-1} h). 24h mean is the O_3 daily average for the
684 week (nL L^{-1}).

685

686 O_3 index		R^2
687	quadratic	
688 AOT30	$y = 5E\text{-}07\ x^2 - 0.001\ x + 1.081$	0.89
689 AOT00	$y = 2E\text{-}07\ x^2 - 0.002\ x + 3.912$	0.71
690 24h mean	$y = 0.006\ x^2 - 0.271\ x + 3.790$	0.72
691	linear	
692 AOT30	$y = 4E\text{-}04\ x + 0.635$	0.50
693 AOT00	$y = 2E\text{-}04\ x + 0.037$	0.33
694 24h 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

716 Figure 1. Proportion of individuals (accumulated values across all replicates)
717 reaching the Gosner stage 44 (all four limbs developed; A) and the toadlet stage (B)
718 per O_3 treatment according to the number of days from the beginning of the
719 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.
721
722 Figure 2. The effect of ozone on *Bd* infection (mean ± SE of log transformed
723 genomic equivalents of zoospores) at the Gosner stage 44 (all four limbs
724 developed; grey bars) and the toadlet stage (black bars) per O_3 treatment. FA,
725 Filtered Air; NFA, Non Filtered Air; NFA+, Non Filtered Air +20 nL L^{-1} of O_3; NFA++,
726 Non Filtered Air +40 nL L^{-1} of O_3; AA, Ambient plots. Different letters indicate
727 statistically significant differences ($p<0.05$).
728
729 Figure 3. Mean *Bd* infection ± SE of log transformed genomic equivalents of
730 zoospores for individuals in the toadlet stage at the different sampling dates and
731 O_3 treatments. FA, Filtered Air; NF, Non-Filtered Air; NFA+, Non-Filtered Air +20
732 nL L^{-1} of O_3; NFA++, Non-Filtered Air +40 nL L^{-1} of O_3; AA, Ambient plots.
733
734 Figure 4. Predicted values (marginal model plots) of *Bd* infection loads of toadlets
735 of spiny common toads found dead between 2004 and 2012 at Sierra de
736 Guadarrama National Park by the three top models relating infection and the
737 averaged values of air temperature, O_3 and AOT40 recorded in the area for the
738 three days (temperature) and the week (ozone values) preceding the date of death.
739