

Universidade de Lisboa
Faculdade de Ciências
Departamento de Biologia Animal



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Batrachochytrium dendrobatidis AFFECTS THE
TADPOLES OF THE COMMON TOAD, *Bufo bufo*,
UNDER DIFFERENT STRESSES?**

Pedro Miguel Brilha Patrício

Dissertação de Mestrado em Biologia da Conservação

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Professor Doutor Rui Miguel Borges Sampaio e Rebelo

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HOW DOES EXPOSURE TO THE FUNGUS *Batrachochytrium dendrobatidis* AFFECTS THE TADPOLES OF THE COMMON TOAD, *Bufo bufo*, UNDER DIFFERENT STRESSES?

Resumo

Os anfíbios enfrentam uma crise de extinção em massa e são atualmente considerados um dos grupos mais ameaçados de vertebrados. O atual declínio global deste grupo é causado por múltiplos fatores, e um dos mais importantes é a propagação do fungo *Batrachochytrium dendrobatidis* (*Bd*), que já foi documentado como agente infeccioso em 350 espécies de anfíbios. Genericamente a sobrevivência das larvas de anfíbios é afetada por fatores bióticos e abióticos. Como fatores bióticos podemos considerar: a densidade intra e interespecífica, a competição, a pressão de predação, a estrutura da comunidade, e a doença ou a pressão parasitária. Pouco se sabe sobre o potencial de interação entre os fatores bióticos e doenças, mas é aceite que o aumento de densidade intraespecífica e a pressão induzida por predadores pode potenciar a vulnerabilidade à infecção. Os efeitos das pressões de cada stresse individualmente têm sido amplamente estudados e documentados em formas larvares de anuros, mas estudos que testem a resposta a vários stresses simultâneos são escassos.

Neste estudo testou-se o efeito do impacto da exposição ambiental ao agente patogénico *Bd* em girinos de *Bufo bufo* (sapo comum), quando sujeitos a diferentes fatores indutores de stresse, nomeadamente, a competição intraespecífica e a existência de sinais químicos e visuais que indicam a presença de um predador, numa experiência fatorial que testou todas as combinações possíveis dos três agentes de indução de stress considerados.

Todas as experiências foram realizados no biotério da Faculdade de Ciências da Universidade de Lisboa. O biotério foi dividido em duas seções, separadas fisicamente para evitar a contaminação: uma das seções incluiu todos os tratamentos expostos a *Bd*, e a outra os tratamentos não expostos.

Para a realização das experiências procedeu-se à recolha de 6 posturas / casais em amplexo de *B. bufo* em Sintra, Portugal, assim como de exemplares de um dos seus

predadores naturais (ninfas de libélula – *Aeshna cyanea*). Foi colocado em todos os aquários um recipiente transparente com orifícios. Para testar os efeitos do stress provocado por predador (tratamento P) foi colocada uma ninfa de libélula nos recipientes. Para testar os efeitos do stress causado por conspecíficos (tratamento C) foi diminuído o volume de água nos aquários para 1/5 do volume dos outros tratamentos. Nos primeiros 4 dias da experiência procedeu-se à inoculação dos tratamentos expostos a *Bd* (tratamento Bd). Para tal utilizámos uma estirpe do fungo *Bd* altamente virulenta (BdGPL). Cada inoculação continha 50.000 zoósporos. O mesmo procedimento foi realizado com os girinos não expostos, mas inoculando apenas meio de cultura de *Bd*, (sem zoósporos).

Quando os girinos atingiram o estágio de Gosner 25, foram distribuídos aleatoriamente por 8 tratamentos distintos com diferentes pressões associadas (sete combinações de tratamentos diferentes e um grupo não exposto a nenhum dos agentes causadores de stress). Cada tratamento tinha 6 replicados (aquários), com 6 girinos em cada aquário, perfazendo 36 animais por tratamento e 288 no total, em 54 unidades experimentais.

Como variáveis de resposta, foram realizados vários tipos de registo ao longo e no fim da experiência. Em 8 ocasiões ao longo da experiência foram registados 5 tipos de comportamento dos girinos (número de girinos ativos, número de girinos a tocar no recipiente do predador, localização dentro do aquário, número de girinos inativos à superfície do aquário e número de girinos inativos no fundo do aquário). Os períodos de observação consistiram em seis ocasiões consecutivas separadas por 15 minutos.

De modo a determinar e quantificar a prevalência de *Bd* nos girinos expostos, um girino por aquário foi eutanasiado com uma overdose de MS222 no dia 39 da experiência.

No dia 47 da experiência todos os girinos foram analisados para verificar o seu estágio de desenvolvimento (Gosner, 1960).

Quando os girinos remanescentes atingiram o estágio de desenvolvimento Gosner 46 (fim da metamorfose), a experiência foi concluída. Cada recém-metamorfoseado foi pesado e fizeram-se ensaios de capacidade de salto. Realizou-se também a avaliação da duração do período larvar e da taxa de sobrevivência de todos os girinos.

Após eutanásia, procedeu-se à extração das peças bucais e dos membros posteriores de todos os girinos e juvenis. O material recolhido foi analisado por *quantitative real-time polymerase chain reaction protocol (qPCR) - TaqMan assay* - a única técnica capaz de quantificar com exatidão o número de zoósporos de *Bd* presentes numa amostra, permitindo obter informação sobre o nível da infecção. As análises de *qPCR* foram realizadas no Centro de Investigação Interdisciplinar em Sanidade Animal da Faculdade de Medicina Veterinária da Universidade Técnica de Lisboa.

Para analisar as diferenças de comportamento entre tratamentos foram utilizados Modelos lineares generalizados mistos (GLMM) com uma distribuição de erro binomial e uma função de ligação *logit* (as várias observações do mesmo aquário foram incluídas como medidas repetidas). Estes foram seguidos por testes emparelhados para cada GLMM significativo. Para analisar as diferenças noutros parâmetros (massa à metamorfose, período larvar e capacidade de salto) foram utilizados Modelos lineares generalizados (GLM), considerando-se as distribuições de erro normais e funções de ligação *logit*, e foram realizados testes emparelhados para cada GLM significativo. Todas as análises estatísticas foram realizadas utilizando o *software* IBM SPSS Statistics versão 22.0.0.0.

Nenhum dos girinos expostos a *Bd* recolhidos no dia 39 da experiência e no final da mesma estavam infectados com o fungo, assim como nenhum animal não exposto a *Bd*.

A taxa de mortalidade dos girinos foi muito baixa e não se verificaram diferenças significativas na mortalidade entre todos os girinos expostos a *Bd* vs todos os não expostos..

A nível comportamental verificou-se uma interação significativa entre os três stresses estudados, originando uma diminuição da atividade dos girinos.

A localização dos animais dentro do aquário foi fortemente influenciada pela interação entre a competição e *Bd*, na qual a exposição ao fungo aumentou significativamente o número de girinos do lado do predador. Esta mesma interação teve o mesmo efeito positivo no número de animais que tocavam no recipiente do predador. Outro parâmetro comportamental em que houve influência dos stresses analisados foi número de girinos inativos à superfície do aquário. Aqui, verificaram-se várias interações significativas: i) entre C e P, que aumentou o valor; ii) entre C e *Bd*, com o mesmo efeito, e iii) somente *Bd*, em que o número de girinos à superfície

também aumentou. O último parâmetro de comportamento foi o número de girinos inativos no fundo do aquário, em que houve interação significativa entre C e *Bd*, na qual o *Bd* aumentou o valor, e também efeito significativo de P, cuja presença originou uma diminuição.

A análise da fase de desenvolvimento dos girinos ao dia 47 da experiência revelou que os stresses C e *Bd* tiveram efeitos significativos mas opostos sobre este parâmetro: a competição teve um efeito negativo no desenvolvimento dos girinos de *B. bufo*, enquanto que o *Bd* o acelerou.

Em termos do período larvar dos girinos de *B. bufo*, encontrou-se uma interação significativa entre C, P e *Bd*, na qual a exposição simultânea a P e *Bd* diminuiu significativamente o período larvar. No entanto, não se verificaram quaisquer efeitos na massa à metamorfose.

A distância máxima de salto máximo foi também afectada pela interação simultânea dos três stresses em estudo. Houve efeitos na média dos três saltos mais longos, com uma interação significativa entre C, P e *Bd*, na qual os recém-metamorfoseados previamente expostos a C e *Bd* apresentaram saltos menores.

Apesar de não se ter verificado infecção por *Bd* em nenhum girino, os nossos resultados demonstram que o *Bd* pode atuar como um fator de stresse e tem impactos sobre a história de vida dos girinos de *B. bufo*. As respostas que encontramos incluem níveis de atividade reduzida, redução do período larvar, capacidade de salto após a metamorfose reduzida, e uso diferencial do aquário. Assim, o fungo não afeta diretamente a sobrevivência de *B. bufo*, mas influencia parâmetros relacionados com comportamento, crescimento e desenvolvimento.

O estudo não suporta a hipótese de que a exposição a pressões naturais resultantes da predação e / ou a competição aumenta a susceptibilidade a *B. dendrobatidis*, e mostra que uma ligação entre a exposição ao stresse e susceptibilidade à doença não pode ser assumida em todos os casos.

HOW DOES EXPOSURE TO THE FUNGUS *Batrachochytrium dendrobatidis* AFFECTS THE TADPOLES OF THE COMMON TOAD, *Bufo bufo*, UNDER DIFFERENT STRESSES?

Abstract

The current global decline of amphibians is caused by multiple factors, and one of the most important is the spread of the fungus *Batrachochytrium dendrobatidis* (*Bd*) which is directly responsible for the infection of 350 amphibian species.

The factors that influence susceptibility to infection by *Bd* are not completely known, but it is known that factors such as density, intra- and inter-specific competition, and predation may have consequences for the development and possibly the immune system of larval amphibians.

The effects of single stresses on anurans have been widely studied and documented, but studies examining the extent to which anurans can respond to multiple stresses are scarce.

We tested the effect of stress-inducing factors, namely intraspecific competition, the existence of chemical and visual signals indicating the presence of a predator, and environmental exposure to *Bd*, in tadpoles of *Bufo bufo* (common toad), in a factorial experiment that tested all combinations of the three stress-inducing agents considered. Tadpole mortality was very low and not related with infection by *Bd*. In fact, all the tadpoles were cleared of *Bd* at the end of the experiment. However, our results demonstrated that the non-lethal infection by *Bd* can be a stressor and have impacts on larval life-history traits. The responses we found included reduced activity levels, shortened larval period, reduced ability to jump after metamorphosis, and differential use of the aquarium. So, the fungus does not directly affect *B. bufo* survivorship, but influences traits related to behavior, growth and development.

Our study did not support the hypothesis that the exposure to natural stresses resulting from predation and/or competition increases susceptibility to *B. dendrobatidis*, and shows that a link between stress exposure and disease susceptibility cannot be assumed in all cases.

Keywords: Amphibia; Anura; Amphibian decline; Chytridiomycosis,

Batrachochytrium dendrobatidis, *Bd*, *Bufo bufo*, common toad, stress, competition, predation

Table of Contents

Agradecimientos	i
Resumo	iii
Abstract	vii
Introduction	1
Material and Methods	5
Animal collection and maintenance	5
Experimental Setup / Procedure	6
Response variables	9
Statistical analysis	10
Results	11
Infection by Bd	11
Effects on Behavior	11
Effects on life-history parameters	15
Tadpoles.....	15
Metamorphs (froglets).....	16
Discussion	22
Competition	22
Predator	23
Interaction between C and P	24
Bd	24
Literature cited	27
Annex Tables	35

Introduction

Amphibians face a crisis of mass extinction and are now considered one of the most threatened groups of vertebrates (Stuart et al., 2004). Of the 6370 species of amphibians assessed in the IUCN red list, about 30% are listed as vulnerable, endangered or critically endangered (IUCN, 2012), with population declines recorded in 43% of the species (Gascon et al., 2007).

This global decline of amphibians is caused by multiple factors (Medina et al., 2012), being among the most important the loss of habitat, global warming, invasive non-native species, over-exploitation and outbreaks of infectious diseases.

It is now widely recognized that the emergence of a new infectious disease, chytridiomycosis, caused by the aquatic pathogenic fungus *Batrachochytrium dendrobatidis* (Longcore, 1999) (*Bd*) (Fisher et al., 2009; Bosch et al., 2001; Parris et al., 2006; Parris & Beaudoin, 2004), is leading to the rapid decline of amphibians (Berger et al., 1998; Stuart et al., 2004; Fisher et al., 2009; Olson & Ronnenberg, 2010; Daszak et al., 2003; Morehouse et al., 2003) of all orders on all continents where amphibians are present (all except Antarctica) (Olson & Ronnenberg, 2010). This infection has already been documented in over 350 species (Fisher et al., 2009), it is able to reduce the populations to very low levels (Bosch et al., 2001; Daszak et al., 1999) and may even be able to lead them to extinction (Daszak et al., 1999). Skerratt et al. (2007) consider that the impact of chytridiomycosis on frogs is the most spectacular loss of vertebrate biodiversity due to disease in recorded history.

The fungus infects the keratinized areas of amphibians, including the mouthparts of the larvae (leading to loss of sections of the mouthparts) (Fellers et al., 2001; Knapp & Morgan, 2006) and the skin of adults (leading to proliferation of keratin cells and hyperkeratosis), which causes the disruption of skin integrity and tissue function, and subsequent loss of homeostasis (Berger et al., 1998; Kilpatrick et al., 2010; Voyles et al., 2009).

B. dendrobatidis has several features that enable it to have catastrophic effects on their hosts (Bosch et al., 2001; Daszak et al., 1999). These include: its low host specificity (Fisher et al., 2009), its ability to infect hosts at various stages of their life cycle (Blaustein et al., 2005) and the potential to remain viable in the environment for

up to seven weeks without host (Parris et al., 2006; Johnson & Speare, 2003; Tobler & Schmidt, 2010). At the moment no other environmental reservoirs are known, except amphibians (Longcore, 1999; Parris et al., 2006; Pessier et al., 1999).

The most serious outbreaks of chytridiomycosis have been described from the American Neotropics, where the disease is reported to have been responsible for the extinction of about 67% (110 species) of *Atelopus* spp. frogs throughout the distribution range of the genus (La Marca et al., 2005) and caused rapid loss of biodiversity of amphibians in eight families of frogs and salamanders in Panama (Lips et al., 2006). However, severe impacts of chytridiomycosis were also registered in different environments: tropical, temperate and mountain, including the Caribbean, South America, Australia, North America and Europe (Fisher et al., 2009; Catenazzi et al., 2011).

In Europe, the presence of *Bd* was first recorded causing mass mortality of amphibians during the summer of 1997 (Bosch et al., 2001). The fungus was detected in Portugal in 2005 and until then it had been restricted to just three European countries: Spain, Germany and Italy (Garner et al., 2005). The common midwife toad, *Alytes obstetricans* (Laurenti, 1768), was the first species in Europe with massive die-offs caused by the appearance of *Bd* (Bosch et al., 2001; Govindarajulu & Anholt, 2006), resulting in sharp declines throughout its distribution (IUCN, 2012). The best documented case of amphibian declines in Europe occurred in the Natural Park of Peñalara (PNPL - Sierra del Guadarrama, Central Spain) between 1997 and 1999 (Bosch et al., 2001). In 1999, *A. obstetricans* had disappeared in 86% of ponds which occupied only a few years before and the estimated larval density decreased markedly in three of the five ponds where the larvae were still found, being now almost completely extinct (Bosch et al., 2001; Bosch & Rincon, 2008).

Susceptibility to chytridiomycosis varies widely among the various species and populations of amphibians, but the causes of this variation are not well understood (Tobler & Schmidt, 2010; Belden & Harris, 2007; Medina et al., 2012). Parris et al. (2006) showed that tadpoles of *R. pipiens* infected with *Bd*, did not die, but presented a longer larval period and lower body mass at metamorphosis, which often reduces the fitness in terrestrial stages. Thus, infection was associated with reduced rates of growth, indicating that chytridiomycosis has a cost for their amphibians hosts, even if they do not die from it (Parris & Beaudoin, 2004).

Susceptibility to disease may also be affected by other factors affecting survival and/or growth. Biotic factors, such as intra and inter-specific competition, predation pressure, community structure, and disease or parasite pressure have been shown in different occasions to affect these parameters (Govindarajulu & Anholt, 2006).

In the work of Teplitsky and Laurila, (2007) with tadpoles of *Rana temporaria* (Linnaeus, 1758), it was found that tadpole body mass was adversely affected with increasing intraspecific density, and a similar pattern was found in the work of Rachowicz and Briggs (2007), with *Rana mucosa* (Camp, 1917). Bardsley et al. (2001), demonstrated that *Bufo calamita* reduced mean tadpole size, growth and survival under intraspecific competition conditions. On the other hand, Relyea (2002) refers that intraspecific competition increased activity in *Rana sylvatica* (LeConte, 1825), but lead to tail length becoming shorter and also caused smaller and less muscular tails, larger bodies, and wider mouths. Relyea (2002) also refers that larval anurans commonly experience reduced growth under high densities.

Predation is an important selective force that can influence life-history decisions such as the time and the size at which key life-history switch points occur, which can strongly influence individual fitness (Nunes et al., 2014). In the presence of a predator, tadpoles may experience reduced activity level, growth, development, body condition, and immune function (Haislip et al., 2012; Blaustein et al., 2012; Nunes et al., 2014). A decrease in activity level is one of the most common and effective behavioral antipredator responses that reduces vulnerability to predation (Parris et al., 2006). Spatial avoidance of predators also acts as an antipredator defense by reducing the rate of predator-prey encounters and, consequently, predation risk (Relyea, 2001) (Nunes et al., 2014).

Empirical studies showed that competitors and predators often have interactive effects on the fitness of the individuals (Teplitsky & Laurila, 2007). Relyea (2002) demonstrated that responses to competition are antagonistic to those triggered by predation. Gurevitch et al (2000) combined the results of 20 articles to compare the average effects of competition and predation and concluded that the effects of competition in the presence of predators were less than in the absence of predators. Also, the removal of competitors had much more positive effects on organisms' growth and mass than did exclusion of predators.

It is accepted that increased intraspecific density and pressure induced by predators may both increase vulnerability to infection (Parris & Beaudoin, 2004). It was shown that *Hyla chrysoscelis* (Cope, 1880) tadpoles metamorphosed 12% more slowly in environments with predators and *Bd*, and showed a 34% decrease in metamorphic body mass. This may lead to a decrease in juvenile survival, first reproduction at an older age and smaller body mass / size, and smaller reproductive success in the terrestrial environment (Parris & Beaudoin, 2004). In the study by Parris et al., (2006), with tadpoles of *Rana pipiens* (Schreber, 1782) it was demonstrated the complexity of the effects of *Bd* infection in the response to anti-predator behavior, causing lower susceptibility to predation, lower activity and greater distance to predators in infected individuals. This study suggested that the infection by the fungus can change the behavior of host and, as a consequence, its survival, thereby benefiting *Bd* by creating opportunities for transmission (Parris et al., 2006).

In spite of previous works, still little is known about the potential interaction between biotic factors and diseases, despite the importance of this information for understanding the disease progression, and although evidence shows that virulence and patterns of infection are context-dependent (Parris & Beaudoin, 2004; Blaustein et al., 2005; Garner et al., 2009)

The aim of our study was to understand if exposure to *Batrachochytrium dendrobatidis* simultaneously with two natural different stresses (intraspecific competition and exposure to a predator) affects the development and behavior of common toad, *Bufo bufo* (Laurenti, 1768) tadpoles.

We decided to use *B. bufo* to perform this study because this species is sensitive to *Bd*, has suffered mass mortalities due to chytridiomycosis in Spain, in Peñalara Natural Park, Spain (Garner et al., 2009) and is a species with a vast distribution range that still maintains abundant populations.

Several studies on the impacts of different stressors have already been developed with *B. bufo*. For example, Richter-Boix et al., (2004), refer that body mass of tadpoles of *B. bufo* subject to intraspecific competition was negatively affected. Tadpole activity level was also affected by the increase in density: time dedicated to foraging and swimming increased, and resting time decreased.

In the study of A. Nunes, et al., (2011), *B. bufo* showed antipredator responses in the presence of a native dragonfly by strongly decreasing his activity level (Nunes et al., 2014). Also, the tadpoles decreased both growth rate and time to metamorphosis, which resulted in a smaller mass at metamorphosis (Nunes et al., 2014).

We performed a laboratory experiment in which we combined exposure to *Bd* with exposure to intraspecific competition and to the chemical stimulus of a predator. To our knowledge, no study compared of the effects of the three stresses simultaneously.

We predict that the exposure to *Bd* will amplify the already described negative effects of competition and predation, thus affecting the development and survival of the *B. bufo* tadpoles.

Material and Methods

Animal collection and maintenance

Several egg strings of *Bufo bufo* were collected from water bodies and small rivers / dams in the natural park of Sintra-Cascais, specifically in the Parque dos Lagos and Parque de Monserrate (Figure 1, Annex - Table II). Couples in amplex were captured and taken into laboratory, were they were kept in aquarium to collect the egg strings immediately after they were laid and fertilized. Both egg strings and couples were collected on 6th of March of 2013.



Figure 1. Location of the collection site, in Sintra, Portugal

Aeshna cyanea nymphs were captured in the same locations with dip-nets, as both species co-occur naturally in this area. *Aeshna cyanea* nymphs are voracious native predators of tadpoles, and are abundant in the same habitats.

All the experiments were performed in the biotherium of the Faculty of Sciences of the University of Lisbon, from March 26th to July 10th of 2013 (106 days).

Six egg strings were kept in separated and identified aquariums filled with one week aged tap water, until tadpoles reached Gosner stage 25 (Gosner, 1960).

The temperature of the biotherium was controlled at 18°C, and the photoperiod was set to 12L: 12D. Throughout the experiment the water was changed every three days, with one week aged tap water and the tadpoles were fed with commercial fish food (commercial granulated fish food (Sera®)) *ad libitum* every three days (Martins et al., 2013), in the day before changing water.

Aeshna cyanea nymphs were transferred to the laboratory and kept individually in 0,1L plastic boxes. They were fed with Diptera, Hemiptera (*Noctonecta* sp), Coleoptera (*Dytiscus* sp) and Ephemeroptera larvae.

All the unnecessary eggs and tadpoles were returned back to the locations where they had been collected.

Experimental Setup / Procedure

Our experimental design consisted in a full factorial experiment to test all combinations of the three stress-inducing agents considered, namely intraspecific competition, the existence of chemical and visual signals indicating the presence of a predator, and environmental exposure to *Batrachochytrium dendrobatidis*, resulting in eight different treatments. Each combination was replicated six times in each of the six blocks. This originated a total of 54 experimental units, each consisting of a plastic aquarium (9 × 16 × 16 cm) filled with 1L of water (except for the competition treatment, where the water volume was 0,2L).

The competition treatment consisted in reducing the volume of water in the aquarium to 200 ml. By doing so, encounters between tadpoles were promoted, including during feeding times.

The predation treatment consisted in one *A. cyanea* nymph placed inside a transparent and perforated cage in the aquarium, allowing the tadpoles to sense and see the predator, but blocking effective predation.

Exposure to *Bd* was accomplished by inoculating the aquarium with live *Bd* zoospores raised in a culture medium, prepared according to Garner et al. (2009).

In the first day (day zero – time point 1 (Annex – Table I)) one individual at developmental stage 25 (Gosner, 1960) of each of the six selected egg strings was randomly placed in each aquarium. Each aquarium thus contained one tadpole of each family, totaling six tadpoles; 288 tadpoles were used in the whole experiment.

One predator cage was placed in each aquarium. In the aquarium subjected to the predator treatments one randomly assigned *A. cyanea* nymph was placed inside the cage. The cages consisted in transparent falcon tubes perforated with 3 mm holes all-around in order to allow both visual and chemical cues emitted by the predator to reach the tadpoles, and were submersed in the same position (one corner) in all aquariums. The dragonfly nymphs were starved before the experiment for 4 days, and were placed in the cages in the same day as the tadpoles were placed in the aquaria (day zero). *Aeshna cyanea* nymphs were not fed during the experiment, so they were replaced every two weeks by others that were starved for at least 72 hours. While out of the aquarium, dragonfly nymphs were fed as explained above.

To infect tadpoles with *Bd*, we used a highly virulent isolate of the chytrid fungus (BdGPL) generated from a dead *Alytes obstetricans* metamorph collected at the Pyrenees, Spain (isolate IA 2011). Zoospore concentration of *Bd* cultures was assayed prior to infection using a haemocytometer and by counting only visibly active zoospores. Fungal inoculum was prepared according to Garner *et al.* (2009).

We inoculated four times, in each of four consecutive days (time point 1 – 4: days 1 to 4 of the experiment: 26th to 29th March (Annex – Table I)), with 50.000 zoospores per day. The tadpoles were kept in the inoculum bath for a period of 6 hours, after which were returned to their aquarium. The same procedure was realized with the uninfected tadpoles, which were kept in a bath with same volume but only of culture media (without *Bd* zoospores) and for the same period (and also during four consecutive days).

As a positive control for *Bd* infectivity, a group of 6 *Alytes obstetricans* tadpoles (a highly susceptible species: (Bosch et al., 2001; Rosa et al., 2013) was also exposed. For these tadpoles we obtained a prevalence of *Bd* of 83.3% (5/6).

The biotherium was divided in two sections, physically separated to avoid contamination: one of the sections contained all the four treatments infected with *Bd*, and the other contained the four non infected.

Each section had the same four treatments: predation (P / Bd+P), conspecific competition (C / Bd+C), predation and conspecific competition simultaneously (C+P / Bd+C+P) and simple exposure to *Bd* (Bd- / Bd+).

Table 1. Identification of the treatments and respective codes

	Exposed to the culture medium	Code	Exposed to <i>Bd</i> + culture medium	Code
Treatment	No <i>Bd</i>	Bd-	<i>Bd</i>	Bd+
	Intraspecific competition	C	<i>Bd</i> and Intraspecific competition	Bd+C
	Predator	P	<i>Bd</i> and Predator	Bd+P
	Intraspecific competition and Predator	P	<i>Bd</i> and Intraspecific competition and Predator	Bd+C+P

There was no rotation of tadpoles between aquariums, to avoid contaminations.

Two sets of material were used to handle the animals: one for animals not experimentally infected with *Bd* and another for those infected. Virkon (10 mg/l / 10 min) and sodium hypochlorite (120 ml/15l / 1 min) were used to disinfect the material, and then thiosulfate (36g / 15l / 30 sec) to remove all the sodium hypochlorite. Animals were handled only with disposable gloves.

In order to determine and quantify the prevalence of *Bd* in the tadpoles, one tadpole per aquarium was euthanized with an overdose of MS222 (tricaine methanesulfonate) in day 39 of the experiment (time point 5 – 4th May, 2014 (Annex – Table I)) and then transferred to Eppendorf tubes with 1 ml of 70% ethanol which acts as a preservative of DNA (Hyatt et al., 2007).

When the remaining tadpoles reached development stage 46 (Gosner, 1960) (end of the experiment – time point 7 (Annex – Table I) date of metamorphosis) they were euthanized, using the same procedure described above. All the samples were stored and preserved in 70% ethanol at -80°C until DNA extraction (Hyatt et al., 2007).

Larval mouthparts and hind limbs of dead metamorphs were all extracted according to Boyle et al. (2004). The material collected was analyzed by quantitative real - time polymerase chain reaction protocol (qPCR) - TaqMan assay - the only technique able to accurately quantify the number of *Bd* zoospores present in a sample (Hyatt 2007, Boyle et al., 2004). Extractions were diluted 1/10 before real time PCR amplification, performed in duplicate, and with *Bd* genomic equivalent (GE) standards of 100, 10, 1 and 0.1 GE (Garner et al., 2009).

Response variables

Mortality was monitored daily. Dead tadpoles were removed from the aquaria and preserved in 70% alcohol.

Tadpole behavior was recorded since the beginning of the experiment until the tadpoles reached stage 42 (Gosner, 1960), in a total of eight periods (Table 2).

Table 2. Date of behavioral records

Period	Date	Experiment day
1	16-Apr	21
2	19-Apr	24
3	22-Apr	27
4	25-Apr	30
5	28-Apr	33
6	04-May	39
7	07-May	42
8	10-May	45

Behavior was assessed in each aquarium as:

- tadpole activity, determined by counting the number of active tadpoles – swimming or feeding - and the number of tadpoles resting at each point count;
- number of tadpoles touching the predator cage (physical contact with the cage).

The observation periods consisted in six repeated and consecutive occasions between 15:00 and 17:00h, separated by at least 15 minutes.

On day 47 of the experiment (time point 6: 12th May (Annex – Table I)) all tadpoles were analyzed to verify their development stage (Gosner, 1960).

When both front limbs emerged (Gosner stage 42), the tadpole was removed from the aquarium and transferred into an individual flask with 1 mm deep aged water. Once the tadpoles reached Gosner stage 46 (complete tail reabsorption), their mass at metamorphosis was calculated by quickly blotting each toadlet in laboratory paper and then weighed it in a digital balance to the nearest 0.01 mg. The larval period was defined as the number of days between the beginning of the experiment - Gosner stage 25, and the day when each tadpole started metamorphosis (Gosner stage 42).

Each froglet was individually placed into a plastic box (100 x 40 x 10 cm) lined with a milimetric scale and persuaded to jump. A series of ten sequential jumps was measured for each froglet. The length of all jumps length was recorded. For each froglet we recorded the longest jump, and calculated the average length of the three highest jumps.

Statistical analysis

We used a Generalized Linear Mixed Model (GLMM) with a binomial error distribution and a logit link function to analyze the behavioral differences throughout treatments (the several observations of the same aquarium were included as repeated measures). These were followed by pairwise tests for every significant GLMM.

To analyze the differences in the development of other parameters (mass at metamorphosis, time to metamorphosis and jump capacity) we used Generalized Linear Models (GLM), considering normal error distributions and logit link functions, and we conducted pairwise tests on every significant GLM.

A *t*-test was used to test for significant differences in mortality between all tadpoles exposed to *Bd* vs all unexposed.

All the statistical analyses were performed using IBM SPSS Statistics Version 22.0.0.0.

Results

Infection by Bd

None of the exposed tadpoles collected on day 39 and in the end of the experiment (after metamorphosis) were infected with *Bd*. Also, no animal from the disease free treatments was infected.

There were no significant differences in mortality between all *Bd*+ versus all *Bd*- treatments ($t_{(6)} = 1.868$, $p > 0,05$).

Table 3. Number of tadpoles that died during the experiment in each treatment (out of a total of 36).

Treatment	Deaths
Bd-	3
Bd-C	3
Bd-P	5
Bd-CP	4
Bd+	0
Bd+C	0
Bd+P	0
Bd+CP	5

Effects on Behavior

There was a significant three-way interaction term between competition, predator and *Bd* affecting the activity level of tadpoles (GLMM, $F_{1,376} = 4.781$, $p = 0,029$) (Fig. 2).

We found a significant difference between treatments C+P+Bd- and C+P+Bd+ ($P = 0.004$) and between treatments C-P-Bd- and C-P-Bd+ ($P < 0,001$), with a decrease in activity level when *Bd* is present in both cases. Between treatments C-P+Bd- and C-

P+Bd+ activity also decreased marginally ($P=0,052$), and again the effect of *Bd* was to decrease activity.

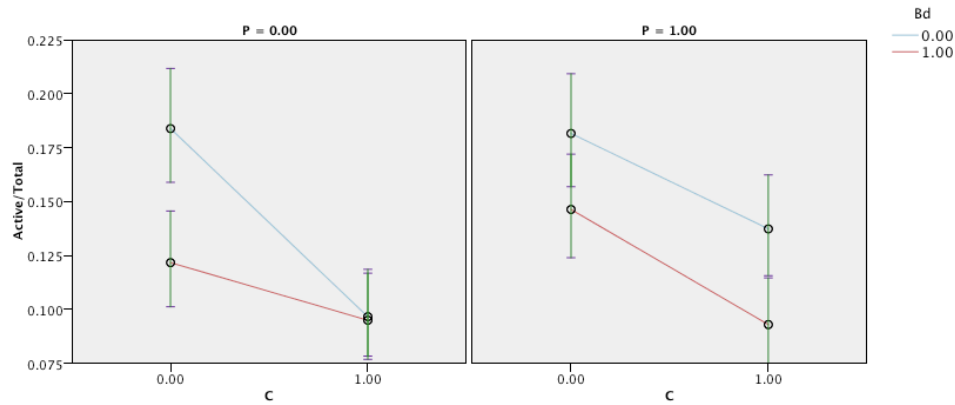


Figure 2. Estimated Means of the proportion of active tadpoles in all the combinations between C, P and *Bd* treatments.

Relatively to the spatial use of the aquarium, there was a significant two-way interaction between C and *Bd* (GLMM, $F_{1,376} = 14.892$, $p < 0,001$) (Fig. 3).

Pairwise contrast detected differences between treatments C+Bd- and C+Bd+ ($P < 0.001$), meaning that exposure to *Bd* increased the number of tadpoles on the predator side in the C treatment.

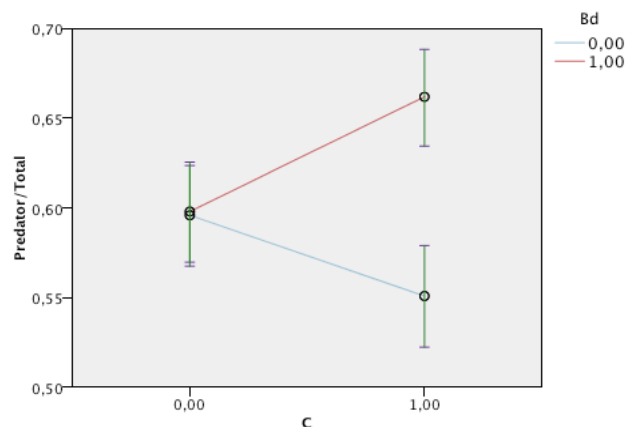


Figure 3. Estimated Means of the proportion of tadpoles on the *A. cyanea* side in the combinations between C and *Bd* treatments.

A significant interaction between C and *Bd* (GLMM, $F_{1,376} = 8.837$, $p = 0,003$) was found affecting the number of tadpoles that were registered touching the *A. cyanea*

cage (Fig. 4). Pairwise contrasts detected differences between treatments C-Bd- and C-Bd+ ($P = 0.030$), and between C+Bd- and C+Bd+ ($P < 0,001$), indicating that *Bd* increased the number of *B. bufo* touching the cage in all treatments, except in combination with P.

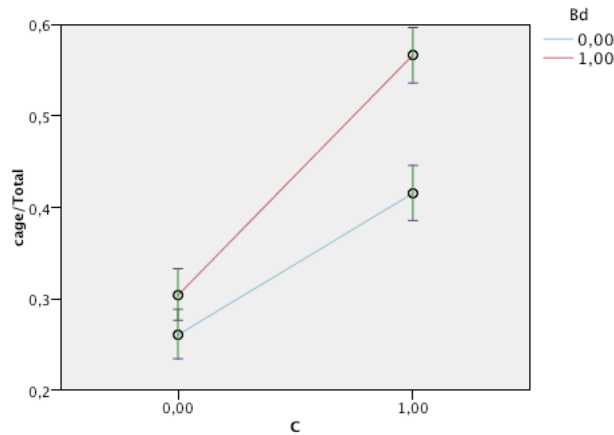


Figure 4. Estimated Means of the proportion of tadpoles touching the *A. cyanea* cage in the combinations between C and *Bd* treatments.

The number of animals resting at the water surface was significantly different when both stresses C and P were present (GLMM, $F_{1,376} = 7.203$, $p = 0,008$) (Fig. 5). *Bd* also affected this parameter, but only when interacting with competition, ($F_{1,376}=16,042$, $P < 0,001$) (Fig. 6).

Pairwise contrast detected differences between treatment C-P+ and C+P+ ($P < 0,001$), with C increasing the number of tadpoles at surface in the P treatment, between C+Bd- and C+Bd+ ($P = 0,007$), with *Bd* increasing the number of tadpoles at surface in the treatment C, and between C-Bd- and C-Bd+ ($P = 0,003$), with *Bd* decreasing the number of tadpoles at surface.

The tadpoles were differently affected by *Bd*, depending on the number of stresses they were subjected to. So, chytrid fungus decreased significantly the number of animals resting close to the surface when they were subject to *Bd* only, but when tadpoles faced *Bd* and C, the number increased significantly.

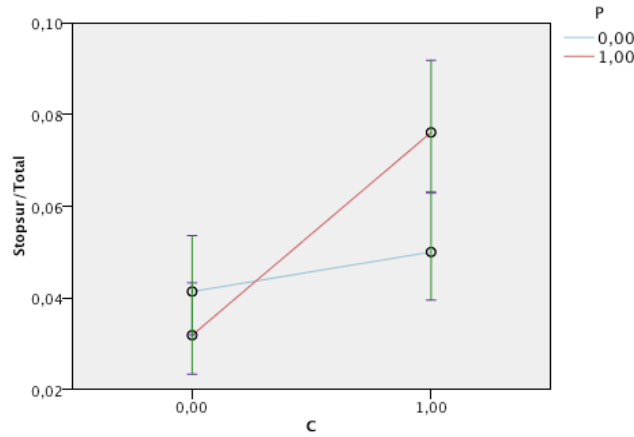


Figure 5. Estimated Means of the proportion of tadpoles resting at the water surface in the combinations between C and P treatments.

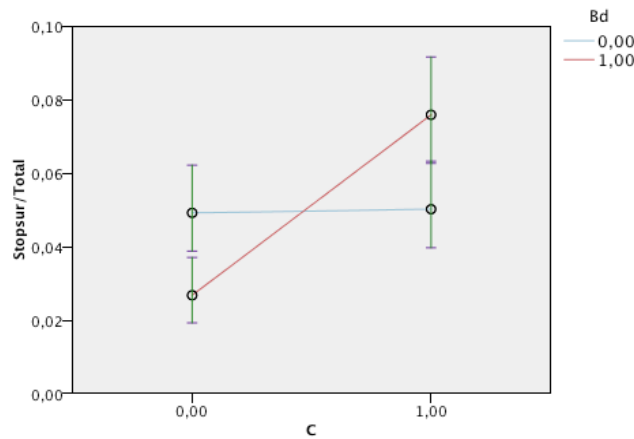


Figure 6. Estimated Means of the proportion of tadpoles resting at the water surface in the combinations between C and *Bd* treatments.

There was a significant two-way interaction term between C and Bd (GLMM, $F_{1,376} = 12.251$, $P < 0,001$) affecting the number of animals resting at bottom (Fig. 7). A significant effect of P was also found (GLMM, $F_{1,376} = 4.124$, $P=0.043$) (Fig. 8). Pairwise contrast detected differences between treatment C-Bd- and C-Bd+ ($P < 0,001$), indicating that *Bd* increased the number of animals resting at the bottom of the aquarium, and also between treatments P- and P+ ($P = 0.043$), when the effect was the opposite.

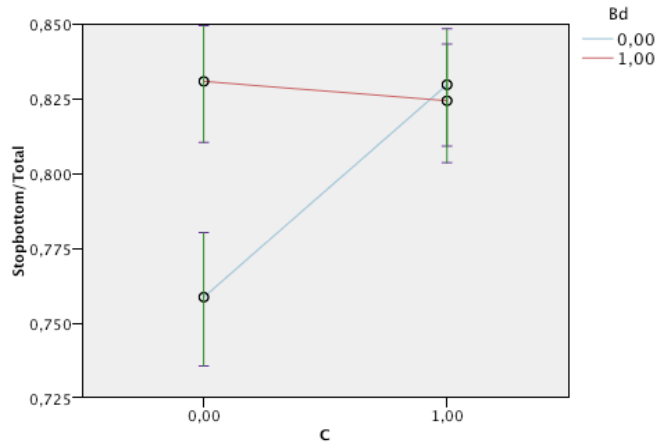


Figure 7. Estimated Means of the proportion of tadpoles resting at bottom of the aquarium in the combinations between C and *Bd* treatments.

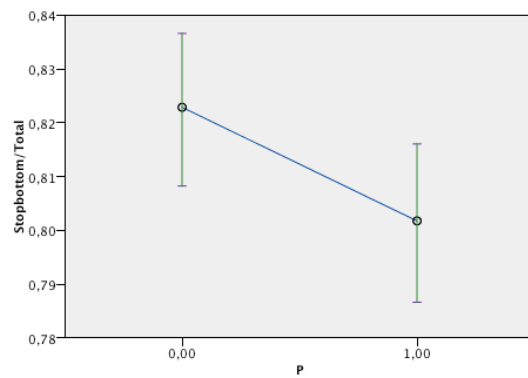


Figure 8. Estimated Means of the proportion of tadpoles resting at bottom of the aquarium on the whole P- and P+ treatments.

Effects on life-history parameters

Tadpoles

A significant effect of single stresses C and *Bd* was detected (GLM, $F_{1,232} = 4,049$, and GLM, $F_{1,232} = 42.759$, respectively) (Figs. 9 and 10) on the developmental stage of tadpoles on day 47.

Competition delayed tadpole development. On the other hand, *Bd* had the opposite effect, increasing the growth rate, so the *Bd*-exposed tadpoles presented a higher development stage compared to the ones not exposed to *Bd*.

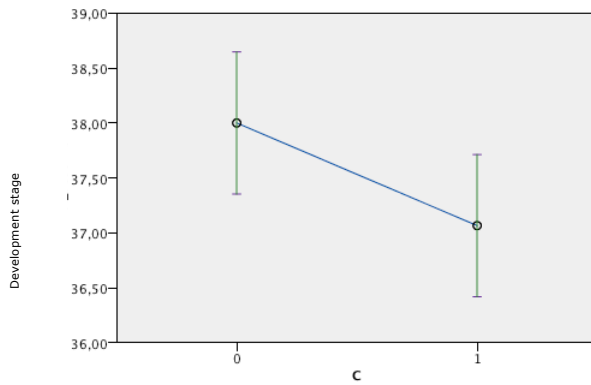


Figure 9. Estimated Means of the development stage (Gosner, 1960) at day 47 of the experience on the whole C- and C+ treatments.

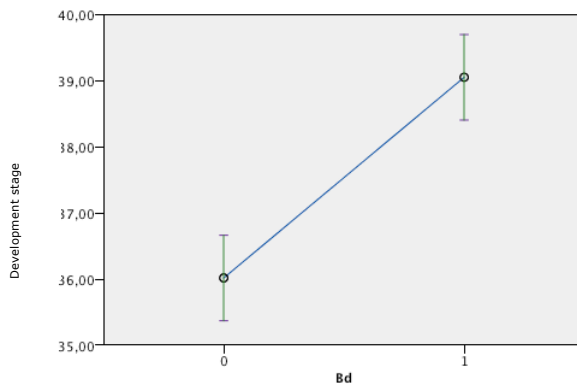


Figure 10. Estimated Means on the effect of *Bd* on development stage (Gosner, 1960) at day 47 of the experience

Metamorphs (froglets)

We found a significant three-way interaction term between Predation, Competition and *Bd*, affecting larval period (GLM, $F_{1,215} = 5.468$, $P = 0.020$) (Fig. 11).

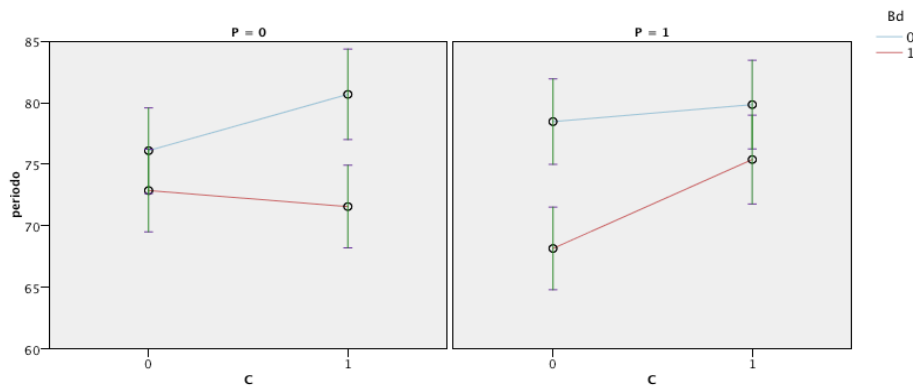


Figure 11. Estimated Means on the 3-way interaction between C, P and *Bd* for the duration of the larval period of *Bufo bufo*

Pairwise contrasts detected differences between treatment P-C+Bd- and P-C+Bd+, ($P < 0.001$), and between P+C-Bd- and P+C-Bd+ ($P < 0,001$), indicating that simultaneous exposure to *Bd* and P shortened the larval period. No significant effect on the tadpoles body mass at metamorphosis was detected among all treatments (all analyses $P > 0,05$).

The longest jump was affected by the exposure to all the stressors simultaneously (GLM, $F_{1,212} = 4.256$, $P=0,040$) (Fig. 12).

However, the pairwise contrasts did not reveal differences among any group.

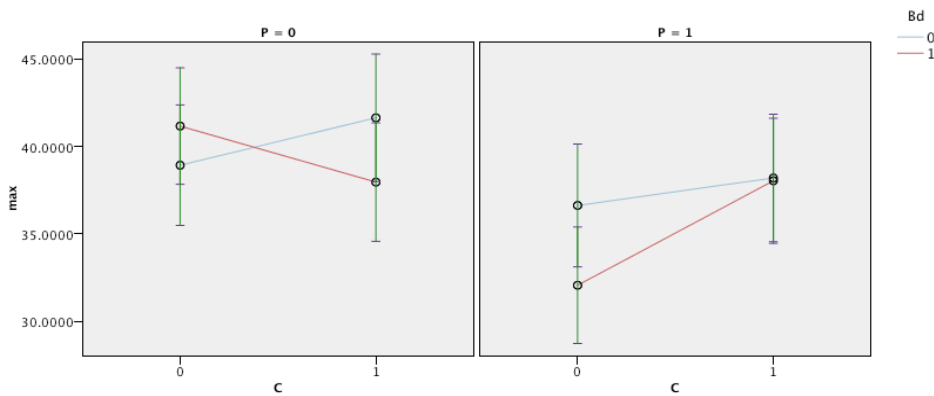


Figure 12. Estimated Means on the 3-way interaction between C, P and *Bd* for the ability to jump longer of *Bufo bufo* metamorphs

The selected measure to evaluate the fitness of the young metamorphs was the average length of the three highest jumps. A significant three-way interaction was found to affect the average length of the three longest jumps (GLM, $F_{1,211} = 7.578$) (Fig. 13).

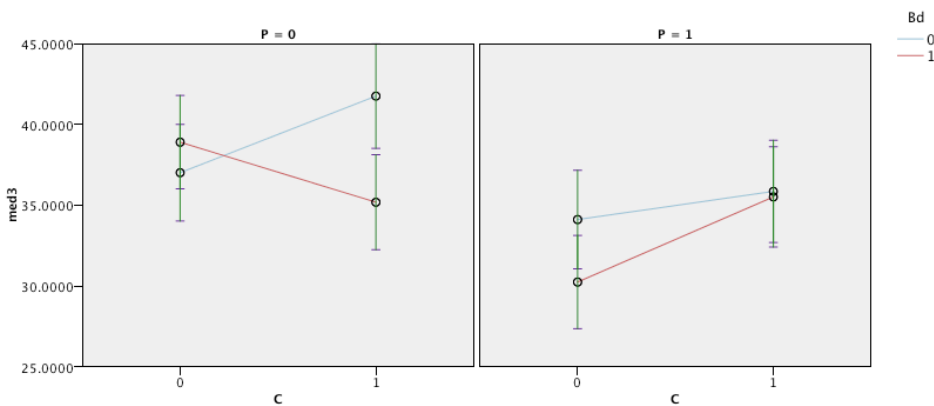


Figure 13. Estimated Means on the 3-way interaction between C, P and *Bd* for the average length of the three longest jumps of *Bufo bufo* metamorphs

Pairwise contrasts detected differences between treatment C+P-Bd- and C+P-Bd+ ($p = 0,003$); *B. bufo* metamorphs exposed to *Bd* and competition showed shorter jumps.

Table 4. Analysis of larval behavioral traits (activity level, spatial use of the aquaria, touching the predator cage, resting at the surface, resting at the bottom) of *B. bufo* tadpoles. Results (p- values) of the GLMMs performed for each trait (df = 1,376) are shown on the right hand side of each column. P-values < 0.05 are marked in boldface

Variables	Treatments	F	p-value
Activity level	C	36,617	<0,001
	P	3,716	0,055
	Bd	16,416	<0,001
	C*P	0,353	0,553
	C*Bd	0,92	0,338
	P*Bd	0,424	0,515
	C*P*Bd	4,781	0,029
Spatial use of the aquaria	C	0,58	0,447
	P	0,114	0,736
	Bd	16,028	<0,001
	C*P	1,664	0,198
	C*Bd	14,892	<0,001
	P*Bd	0,307	0,58
Touching the predator cage	C	182,697	<0,001
	P	0,006	0,941
	Bd	38,401	<0,001
	C*P	1,113	0,292
	C*Bd	8,837	0,003
	P*Bd	0,001	0,978
Resting at the surface	C	17,344	<0,001
	P	0,434	0,511
	Bd	0,507	0,477
	C*P	7,203	0,008
	C*Bd	16,042	<0,001
	P*Bd	0,145	0,703
Resting at the bottom	C	8,28	0,004
	P	4,124	0,043
	Bd	8,958	0,003
	C*P	2,81	0,095
	C*Bd	12,551	<0,001
	P*Bd	0	0,987
C*P*Bd	0,707	0,401	

Table 5. Analysis of pairwise contrasts of GLMMs of larval behavioral traits (activity level, spatial use of the aquaria, touching the predator cage, resting at the surface, resting at the bottom) of *B. bufo* tadpoles. Results (p- values) of the pairwise contrasts performed for each trait are shown on the right hand side of each column. P-values < 0.05 are marked in boldface

Variables	Treatments			Mean	Pairwise contrasts	Adj. Sig.
	C	P	Bd			
Activity level	0	0	0	0,184	0,062	<0,001
			1	0,122	-0,062	<0,001
	1	0	0	0,182	0,035	0,052
			1	0,146	-0,035	0,052
	1	0	0	0,097	0,002	0,909
			1	0,095	-0,002	0,909
		1	0	0,137	0,044	0,004
			1	0,093	-0,044	0,004
Spatial use of the aquaria	0	0	0	0,596	-0,002	0,918
			1	0,598	0,002	0,918
	1	0	0	0,551	-0,111	<0,001
			1	0,662	0,111	<0,001
Touching the predator cage	0	0	0	0,261	-0,043	0,003
			1	0,304	0,043	0,003
	1	0	0	0,416	-0,151	<0,001
			1	0,567	0,151	<0,001
Resting at the surface	0	0	0	0,049	0,022	0,003
			1	0,027	-0,022	0,003
	1	0	0	0,050	-0,026	0,007
			1	0,076	0,026	0,007
	0	0	0	0,041	-0,009	0,287
			1	0,032	0,009	0,287
		1	0	0,050	-0,044	<0,001
			1	0,076	0,044	<0,001
Resting at the bottom	0	0	0	0,759	-0,072	<0,001
			1	0,831	0,072	<0,001
	1	0	0	0,830	0,005	0,705
			1	0,824	-0,005	0,705
	0	0	0	0,823	0,021	0,043
			1	0,802	-0,021	0,043

Table 6. Analysis of GLM of development on day 47 (Development), larval period (Period), mass at metamorphosis (Mass), maximum jumps distance (Max) and average length of the three highest jumps (Ave3) of *B. bufo* tadpoles. Results (p- values) of the GLMs performed for each trait (df = 1,232) are shown on the right hand side of each column. P-values < 0.05 are marked in boldface

Variables	Treatment	F	p-value
Development	C	4,049	0,045
	P	0,373	0,542
	Bd	42,759	<0,001
	C*P	0,466	0,496
	C*Bd	0,516	0,473
	P*Bd	0,105	0,747
	C*P*Bd	2,17	0,142
	Period	C	5,622
P		0,016	0,898
Bd		29,318	<0,001
C*P		1,132	0,289
C*Bd		0,000	0,994
P*Bd		0,231	0,631
C*P*Bd		5,468	0,020
Mass		C	0,015
	P	2,104	0,148
	Bd	0,079	0,324
	C*P	0,310	0,579
	C*Bd	0,842	0,360
	P*Bd	1,763	0,186
	C*P*Bd	0,075	0,785
	Max	C	1,990
P		8,723	0,003
Bd		1,519	0,219
C*P		2,581	0,110
C*Bd		0,091	0,763
P*Bd		0,433	0,511
C*P*Bd		4,256	0,040
Ave3		C	3,407
	P	15,481	<0,001
	Bd	4,168	0,042
	C*P	1,893	0,170
	C*Bd	1,282	0,259
	P*Bd	0,012	0,914
	C*P*Bd	7,578	0,006

Table 7. Analysis of pairwise contrasts of GLMs Pairwise contrasts of GLM of development on day 47 (Development), larval period (Period), mass at metamorphosis (Mass), maximum jump distance (Max), average length of the three highest jumps (Ave3) of *B. bufo* tadpoles. Results (P- values) of the pairwise contrasts performed for each trait are shown on the right hand side of each column. P-values < 0.05 are marked in boldface

Variables	Treatment			Mean	Pairwise contrasts	Adj. Sig.
	C	P	Bd			
Development			0	36,017	-3,033	<0,001
			1	39,050	3,033	<0,001
	0			38,000	0,933	0,045
	1			37,067	-0,933	0,045
Period	0	0	0	76,107	3,240	0,187
			1	72,867	-3,240	0,187
		1	0	78,464	10,298	<0,001
			1	68,167	-10,298	<0,001
	1	0	0	80,680	9,113	<0,001
			1	71,567	-9,113	<0,001
		1	0	79,846	4,462	0,086
			1	75,385	-4,462	0,086
Max	0	0	0	38,929	-2,238	0,358
			1	41,167	2,238	0,358
		1	0	36,630	4,563	0,064
			1	32,067	-4,563	0,064
	1	0	0	41,640	3,674	0,147
			1	37,966	-3,674	0,147
		1	0	38,200	0,162	0,950
			1	38,038	-0,162	0,950
Ave3	0	0	0	37,012	-1,888	0,372
			1	38,900	1,888	0,372
		1	0	34,111	3,867	0,071
			1	30,244	-3,867	0,071
	1	0	0	41,750	6,566	0,003
			1	35,184	-6,566	0,003
		1	0	35,853	0,341	0,880
			1	35,513	-0,341	0,880

Discussion

Our study demonstrated that *Bd* can act as a stressor and have impacts on larval life-history traits related to fitness. The responses we found included reduced activity levels, shortened larval period, reduced ability to jump, and differential use of the aquarium. However, no significant effect of *Bd* on survival was encountered. So, the fungus does not directly affect *B. bufo* survivorship, but influences traits related to behavior, growth and development.

Our experiment is one of the first that tests the influence of the interactions of three different stresses on the larval development of an amphibian. In doing so, we approach the ecological reality of what happens in natural habitats.

Several studies have focused on the separate roles of stresses, such as predation, competition and pathogens, or the combination of two factors, in development and behavior of amphibian larvae (Relya, 2001; Bardsley & Beebee, 2001; Laurila et al., 2002; Katzmann et al., 2003; Parris & Beaudoin, 2004; Teplitsky & Laurila, 2007; Searle et al., 2014). However, the interactions between several and simultaneous stresses, such as environment, stress effects and infectious diseases are complex and not well understood. The role of physiological stress in altering disease dynamics appears to be more complex than can be explained simply by adding single stress measurements.

Competition

Competition affected the tadpoles, decreasing their level of activity and increasing the number of animals resting at the bottom. However, other studies have found the opposite effect (Relyea, 2002), or no effect at all (Katzmann et al., 2003). The reduced water volume in aquaria subject to this treatment (200 ml) might have been a cause for the decrease of the level of activity of the tadpoles, by limiting the area available.

Tadpoles also reached metamorphosis with a lighter weight, which can result into a lower terrestrial performance (Parris & Cornelius, 2004; Parris et al., 2006) or even lead to mortality after metamorphosis (Garner et al., 2009). Previous studies have also

found negative effects of crowding on growth rate in *Rana pipens* tadpoles (Glennemeier & Denver, 2002), in *Rana mucosa* (Rachowicz & Briggs, 2007) and in *Rana sylvatica* (LeConte, 1825) (Relyea, 2002), probably by decreasing the level of resources available.

Predator

Exposure to *Aeshna cyanea* nymphs also had a depressing effect on tadpoles, delaying their development and, therefore, increasing the larval period. However, in natural habitats where predators are present it is an ecological advantage to leave the water as early as possible, to escape predation (Katzmann et al., 2003). A non-adaptive response similar to what was found in our experiment (an increase in the larval period) has been found when larval amphibians react to an unknown stress, such as exotic predators (Almeida et al., 2011).

After metamorphosis, these *B. bufo* juveniles exposed to *A. cyanea* nymphs were able to jump longer than the ones not exposed to the predator, probably because the extra time spent as tadpole allowed a better development of the muscles of the hind limbs.

Other effect of the exposure to *Aeshna cyanea* was that the tadpoles spent more time closer to the cages containing the nymphs.

Aeshna cyanea nymphs used in the study were collected in the same location of the egg strings and amplexing adults of *B. bufo*. The nymphs of *Aeshna* sp. are usually described as a natural predator to *B. bufo* tadpoles (Nunes et al., 2014). However, in a separate experiment (*unpubl. data*), we found that the *Aeshna cyanea* specimens didn't predate over *B. bufo* tadpoles, even avoiding to touch them. This fact was only realized after the start of the experiment. So, it is possible that in our study area *A. cyanea* nymphs do not prey over *B. bufo* tadpoles, which could explain the tadpole behavior. Also, during the experience, we noticed the development of algae on the walls of the cages with *A. cyanea*, and not on the ones without predator. So, most probably, the tadpoles were foraging on the wall, and so spending more time on the *A. cyanea* side.

Interaction between C and P

For the majority of the parameters measured, the simultaneous exposure to competition and predator resulted in responses similar to those of competition alone. Metamorph weight was lower in the tadpoles subjected to both treatments and the effects previously found for predator alone in the duration of the larval period or in the ability to jump were not detected in this experimental group.

Bd

Garner et al., 2009, describes mass die-offs of *B. bufo*, in Peñalara National Park, Spain, in 2004 and 2005 but only in two of the three ponds within the *Bd* zone of infection (Garner et al., 2009).

Contrary to our expectations, we found that no animals were infected at the middle of the experiment, as well as after metamorphosis. Furthermore, we found no association between exposure to *Bd* and tadpole mortality, as described in previous studies (Parris et al., 2004). The results of our infection protocol differed from those of previous studies that were able to produce infection on *B. bufo* tadpoles (Garner et al., 2009). This was probably because these authors used higher concentrations of *Bd* and longer exposure durations. However, each of these studies used slightly different rearing and exposure conditions, indicating that infection may be context - dependent (Searle et al., 2014).

Other studies with experimental exposures of larval amphibians to *Bd* also did not achieve infection (Venesky et al., 2010; Searle et al., 2011b; Gervasi et al., 2013). Among *plethodontid* species, susceptibility to clinical chytridiomycosis varies greatly, and field studies of other amphibian species have reported low prevalence or absence of detectable infection in species that show seasonal fluctuations in prevalence (Pasmans et al., 2013). Since the tadpoles did not become infected, one plausible hypothesis is that its skin must contain highly effective fungicidal properties that prevent invasion of amphibian skin by the pathogen. Factors present in the skin secretions that account for the observed *Bd* killing need further identification but probably include antimicrobial peptides (AMP) and/or bacterially produced metabolites (Pasmans et al., 2013; Medina et al., 2012). Other possibility is that the immune system of the tadpoles might have been able to fight *Bd* right at the start, and

so prevented the infection (Garner et al., 2009). This may be due to the ability of tadpoles to reject infective agents over time (Searl et al., 2013), so it can be considered the possibility that the tadpoles became infected when they were exposed to *Bd*, and then lost the infection during their development.

Recent evidence suggests that variable temperatures may be most favorable for *Bd* driven declines, probably due to temperature drop induced zoospore release (Venesky et al., 2012). Since the laboratory temperature was constant (at 18°C), this may have affected the *Bd* capacity to produce infection.

B. bufo tadpoles exposed to *Bd* reached metamorphosis faster than unexposed individuals. One of the known effects of the *Bd* on tadpoles is the disturbance of the mouthparts integrity, and so the reduction of the feeding ability, even if it does not increase mortality, will have implications for growth and development rate by reducing feeding efficiency (Venesky et al., 2010, Parris et al., 2004). Clearly this did not happen with our *Bd*-exposed tadpoles. In spite of the possible damage to the mouthparts, the deleterious effect of a lower feeding ability may not be noticed if food is provided *ad libitum* (as in our study). It is known that amphibian larvae can adapt developmental rates in response to many stress factors; Relyea, 2001). Reducing time to metamorphosis in order to exit stress-inducing habitats locations may be an adaptive response, that may be elicited when pathogens such as *Bd* are present (Searle et al., 2014).

Tadpoles exposed to *Bd* showed a lower activity level and spent more time on the *Aeshna cyanea* side than non-exposed tadpoles. In the paragraph concerning predation we discussed a probable reason why *B. bufo* tadpoles spent more time on the *A. cyanea* side, and even touching the cage. Other explanation to this fact could be that the *Bd* zoospores might have aggregated on the walls of the cage and even on the *A. cyanea* body, allowing the development of bacterial or algal films on the cage, which would attract the tadpoles.

Also, *Bd*-exposed animals spent more time at the surface. The effect was unique to the stressor *Bd*, and can be disadvantageous in nature, because the tadpoles become more visible to predators, such as birds, increasing predation risk. Therefore, this microhabitat change may indicate a stress response. As far as we know, this effect has not been described before.

In competition treatments, the addition of *Bd* did not cause any significant change; the changes in the weight at metamorphosis and the activity levels were the same as those for the competition-only treatments.

In our experiment, *Bd* exposed animals reached metamorphosis faster than non-exposed, in contrast to other experiments, where it increased larval period (Parris et al., 2004) or where had no effect on the duration of the larval period (Garner et al., 2009), even when combined with the stress of predator, overcoming the effect of the predator. This could indicate that animals exposed to *Bd* experience higher levels of stress compared to treatments without *Bd*, which is manifested in faster development.

Other effect of *Bd* in the combined *Bd* x Predator treatment was to cancel the effect of the predator in producing *B. bufo* juveniles with higher ability to jump. This maybe due to the shorter larval period, that doesn't allow a longer development time for the muscles and the hind limbs.

Overall, we found no evidence of summation of stressful effects; tadpoles seem to support well the impact of three stress agents simultaneously. However, there were interesting interactions and we registered an unique behavior in the presence of *Bd*: animals exposed spend more time at the surface. Also, *Bd* overcame the effects of some stresses, namely P, by decreasing the larval period and producing froglets with lower capacity to jump.

Our study did not support the hypothesis that the exposure to natural stresses of a predator and competition increases susceptibility to *B. dendrobatidis*, but demonstrated that a link between stress exposure and disease susceptibility cannot be assumed in all cases, as demonstrated previously by other studies (e.g., Haislip et al., 2012; Reeve et al., 2013); (Searle et al., 2014). Also, it demonstrated that *Bd* has effects in *Bufo bufo*, even when infection is not detected.

Literature cited

Literature cited

- Almeida, E., Alves, S., Rebelo, R., Guerreiro, C., Nunes, A., & Andrade, P. (2011). Antipredator responses of two anurans towards native and exotic predators. *Amphibia-Reptilia*, 32(3), 341–350.
- Belden, L. K. & Harris, R. N. (2007). Infectious diseases in wildlife: the community ecology context. *Frontiers in Ecology and the Environment*, 5(10), 533–539.
- Berger, L., Speare, R., Daszak, P., Green, D. E., Cunningham, A. A., Goggin, C. L., Slocombe, R., Ragan, M. A., Hyati, A. D., McDonald, K. R., Hines, H. B., Lips, K. R., Marantelli, G. & Parkes, H. (1998). Chytridiomycosis causes amphibian mortality associated with population declines in the rain forests of Australia and Central America. *Proceedings of the National Academy of Sciences of the United States of America*, 95(15), 9031-9036.
- Blaustein, A. R., Romansic, J. M., Scheessele, E. A., Han, B. A., Pressier, A. P. & Longcore, J. E. (2005). Interspecific variation in susceptibility of frog tadpoles to the pathogenic fungus *Batrachochytrium dendrobatidis*. *Conservation Biology*, 19(5), 1460-1468.
- Blaustein, A. R., Gervasi, S. S., Johnson, P. T. J., Hoverman, J. T., Belden, L. K., Bradley, P. W. & Xie, G. Y. (2012). Ecophysiology meets conservation: understanding the role of disease in amphibian population declines. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 367(1596), 1688–707.
- Boyle, D. G., Boyle, D. B., Olsen, V., Morgan, J. T., & Hyatt, D. (2004). Rapid quantitative detection of chytridiomycosis (*Batrachochytrium dendrobatidis*) in amphibian samples using real-time Taqman PCR assay. *Diseases of Aquatic Organisms*, 60(2), 141–8.
- Bosch, J., Martinez-Solano, I., & Garcia-Paris, M. (2001). Evidence of a chytrid fungus infection involved in the decline of the common midwife toad (*Alytes*

- obstetricans*) in protected areas of Central Spain. *Biological Conservation*, 97(3), 331-337.
- Bosch, J., & Rincon, P. A. (2008). Chytridiomycosis-mediated expansion of *Bufo bufo* in a montane area of Central Spain: an indirect effect of disease. *Diversity and Distributions*, 14(4), 637-643.
- Bardsley, L. & Beebee, T. J. C. (2001). Strength and mechanisms of competition between common and endangered anurans *Ecological Applications*, 11(2), 453–463.
- Carey, C., Bruzgul, J. E., Livo, L. J., Walling, M. L., Kuehl, K. A., Dixon, B. F., Pessier, A. P., Alford, R. A. & Rogers, K.B. (2006). Experimental exposures of boreal toads (*Bufo boreas*) to a pathogenic chytrid fungus (*Batrachochytrium dendrobatidis*). *EcoHealth*, 3(1), 5–21.
- Catenazzi, A., Lehr, E., Rodriguez, L. O. & Vredenburg, V. T. (2011). *Batrachochytrium dendrobatidis* and the collapse of anuran species richness and abundance in the upper Manu National Park, southeastern Peru. *Conservation Biology*, 25(2), 382-391.
- Daszak, P., Berger, L., Cunningham, A. A., & Hyatt, A. D. (1999). Emerging infectious diseases and amphibian population declines. *Emerging Infectious Diseases*, 5(6), 735-748.
- Daszak P, Cunningham AA, Hyatt AD (2003). Infectious disease and amphibian population declines. *Diversity and Distributions* 9(2), 141-150
- Fellers, G. M., Green, D. E. & Longcore, J. E. (2001). Oral chytridiomycosis in the mountain yellow-legged frog (*Rana muscosa*). *Copeia*, 4, 945-953.
- Fisher, M. C., Garner, T. W. J., & Walker, S. F. (2009). Global emergence of *Batrachochytrium dendrobatidis* and amphibian chytridiomycosis in space, time, and host. *Annual Review of Microbiology*, 63, 291-310.

- Gascon, C., Collins, J. P., Moore, R. D., Church, D. R., McKay, J. E., & Mendelson, J. R., eds (2007). Amphibian Conservation Action Plan, IUCN/SSC Amphibian Specialist Group.
- Garner, T. W., Walker, S., Bosch, J., Hyatt, A. D., Cunningham, A. A. & Fisher, M. C. (2005). Chytrid fungus in Europe. *Emerging Infectious Diseases*, 11(10), 1639-1641.
- Garner, T. W. J., Walker, S., Bosch, J., Leech, S., Marcus Rowcliffe, J., Cunningham, A. A., & Fisher, M. C. (2009). Life history tradeoffs influence mortality associated with the amphibian pathogen *Batrachochytrium dendrobatidis*. *Oikos*, 118(5), 783–791.
- Gervasi, S., Gondhalekar, C., Olson, D. H., Blaustein, A. R. (2013). Host identity matters in the amphibian - *Batrachochytrium dendrobatidis* system: fine scale patterns of variation in response to a multihost pathogen. *PLoS One*, 8(1), e54490.
- Glennemeier, K. A., & Denver, R. J. (2002). Role for corticoids in mediating the response of *Rana pipiens* tadpoles to intraspecific competition. *The Journal of Experimental Zoology*, 292(1), 32–40.
- Gosner, K. L., & Sep, N. (1960). A simplified table for staging Anuran embryos and larvae with notes on identification, *Herpetologica*, 16(3), 183–190.
- Govindarajulu, P. P., & Anholt, B. R. (2006). Interaction between biotic and abiotic factors determines tadpole survival rate under natural conditions. *Ecoscience*, 13(3), 413-421.
- Gurevitch, J., J. A. Morrison, and L. A. Hedges. (2000). The interaction between competition and predation: a meta- analysis of field experiments. *American Naturalist*, 155(4): 435–453
- Haislip, N. A., Hoverman, J. T., Miller, D. L., & Gray, M. J. (2012). Natural stressors and disease risk: does the threat of predation increase amphibian susceptibility to ranavirus? *Canadian Journal of Zoology*, 90(7), 893–902.

- Hyatt, A. D., Boyle, D. G., Olsen, V., Boyle, D. B., Berger, L., Obendorf, D. & Coiling, A. (2007). Diagnostic assays and sampling protocols for the detection of *Batrachochytrium dendrobatidis*. *Diseases of Aquatic Organisms*, 73(3), 175–92.
- Harris, R. N., James, T. Y., Lauer, A., Simon, M. A. & Patel, A. (2006). Amphibian pathogen *Batrachochytrium dendrobatidis* is inhibited by the cutaneous bacteria of amphibian species. *EcoHealth* 3(1):53–56.
- IUCN (2014). The IUCN Red List of Threatened Species. Version 2012.1. <<http://www.iucnredlist.org>>. Downloaded on 25 September 2014.
- Johnson, M. L., & Speare, R. (2003). Survival of *Batrachochytrium dendrobatidis* in water: quarantine and disease control implications. *Emerging Infectious Diseases*, 9(8), 922-925.
- Katzmann, S., Waringer-Ischenkohl, A., & Waringer, J. A. (2003). Effects of inter- and intraspecific competition on growth and development of *Bufo viridis* and *Bufo bufo* tadpoles, *Limnologica*, 33(2), 122–130.
- Kilpatrick, A.M., Briggs, C.J. & Daszak, P. (2010). The ecology and impact of chytridiomycosis: an emerging disease of amphibians. *Trends in Ecology and Evolution*, 25(2), 109-118.
- Knapp, R.A. & Morgan, J.A.T. (2006). Tadpole mouthpart depigmentation as an accurate indicator of chytridiomycosis, an emerging disease of amphibians. *Copeia*, 2, 188- 197.
- Laurila, A., Pakkasmaa, S., Crochet, P.-A., & Merilä, J. (2002). Predator-induced plasticity in early life history and morphology in two anuran amphibians. *Oecologia*, 132(4), 524–530.
- La Marca, E. Lips, K. R., Lötters, S., Puschendorf, R., Ibáñez, R., Rueda-Almonacid, J. V., Schulte, R., Marty, C., Castro, F., Manzanilla-Puppo, J., García-Pérez, J. E., Bolaños, F., Chaves, G., Pounds, J. A., Toral, E. & Young, B. E. (2005). Catastrophic population declines and extinction in neotropical harlequin frogs (Bufonidae: *Atelopus*). *Biotropica*, 37(2), 190-201.

- Lips, K. R., Brem, F., Brenes, R., Reeve, J. D., Alford, R. A., Voyles, J., Carey, C., Livo, L., Pessier, A. P. & Collins, J. P. (2006). Emerging infectious disease and the loss of biodiversity in a neotropical amphibian community. *Proceedings of the National Academy of Sciences of the United States of America* 103(9), 3165-3170.
- Longcore, J. E. (1999). *Batrachochytrium dendrobatidis* gen et sp nov, a chytrid pathogenic to amphibians. *Mycologia*, 91(2), 219-227.
- Marantelli, G., Berger, L., Speare, R. & Keegan, L. (2004). Distribution of the amphibian chytrid *Batrachochytrium dendrobatidis* and keratin during tadpole development. *Pacific Conservation Biology*, 10(3), 173–179.
- Martins, F. M. S., Oom, M. D. M., Rebelo, R., & Rosa, G. M. (2013). Differential effects of dietary protein on early life-history and morphological traits in natterjack toad (*Epidalea calamita*) tadpoles reared in captivity. *Zoo Biology*, 32(4), 457–62.
- Medina, E. M., Restrepo, S., Flechas, S. V, Sarmiento, C., & Ca, M. E. (2012). Surviving Chytridiomycosis: Differential anti-*Batrachochytrium dendrobatidis* activity in bacterial isolates from three lowland species of Atelopus, 7(9), e44832.
- Morehouse, E. A., James, T. Y., Ganley, A. R. D., Vilgalys, R., erBerger, L., Murphy, P. J. & Longcore, J. E. (2003). Multilocus sequence typing suggests the chytrid pathogen of amphibians is a recently emerged clone. *Molecular Ecology*, 12(2), 395-403
- Nunes, A. L., Orizaola, G., Laurila, A., & Rebelo, R. (2014). Morphological and life-history responses of anurans to predation by an invasive crayfish: an integrative approach. *Ecology and Evolution*, 4(8), 1491–503.
- Olson, D., & Ronnenberg, K. (2010). Global Bd mapping project: update July 2010. U.S. Department of Agriculture Forest Services, Pacific Northwest Research Station, Corvallis, Oregon. <<http://www.parcplace.org>>. Downloaded on 20 July 2014.

- Parris, M. J., & Beaudoin, J. G. (2004). Chytridiomycosis impacts predator-prey interactions in larval amphibian communities. *Oecologia*, 140(4), 626–32.
- Parris, M. J., & Cornelius, T. O. (2004). Fungal pathogen causes competitive and development stress in larval amphibian communities. *Ecology*, 85(12), 3385–3395.
- Parris, M. J., Reese, E., & Storfer, A. (2006). Antipredator behavior of chytridiomycosis-infected northern leopard frog (*Rana pipiens*) tadpoles. *Canadian Journal of Zoology*, 84(1), 58–65.
- Pasmans, F., Van Rooij, P., Blooi, M., Tessa, G., Bogaerts, S., Sotgiu, G., Salvidio, S. (2013). Resistance to chytridiomycosis in European plethodontid salamanders of the genus *Speleomantes*. *PloS One*, 8(5), e63639.
- Pessier, A. P., Nichols, D. K., Longcore, J. E. & Fuller, M. S. (1999). Cutaneous chytridiomycosis in poison dart frogs (*Dendrobates* spp.) and White's tree frogs (*Litoria caerulea*). *Journal of Veterinary Diagnostic Investigation*, 11(2), 194–199.
- Rachowicz, L. J., & Briggs, C. J. (2007). Quantifying the disease transmission function: effects of density on *Batrachochytrium dendrobatidis* transmission in the mountain yellow-legged frog *Rana muscosa*. *The Journal of Animal Ecology*, 76(4), 711–21.
- Reeve, B. C., Crespi, E. J., Whipps, C. M., & Brunner, J. L. (2013). Natural stressors and ranavirus susceptibility in larval wood frogs (*Rana sylvatica*). *EcoHealth*, 10(2), 190–200.
- Relyea, R. A. (2001). Morphological and behavioral plasticity of larval anurans in response to different predators. *Ecology*, 82(2), 523–540.
- Relyea, R. A. (2002). Competitor-induced plasticity in tadpoles: consequences, cues, and connections to predator-induced plasticity. *Ecological Monographs* 72(4),523–540.

- Richter-Boix, A., Llorente, G. A. & Montori, A. (2004). Responses to competition effects of two anuran tadpoles according to life-history traits. *Oikos*, 106(1), 39–50.
- Rosa, G. M., Anza, I., Moreira, P. L., Conde, J., Martins, F., Fisher, M. C., & Bosch, J. (2013). Evidence of chytrid-mediated population declines in common midwife toad in Serra da Estrela, Portugal. *Animal Conservation*, 16(3), 306–315.
- Searle, C. L., Gervasi, S. S., Hua, J., Hammond, J. I., Relyea, R. A., Olson, D. H. & Blaustein, A. R. (2011). Differential host susceptibility to *Batrachochytrium dendrobatidis*, an emerging amphibian pathogen. *Conservation Biology*, 25(5), 965–974.
- Searle, C. L., Xie, G.Y. & Blaustein, A. R. (2013). Development and infectious disease in hosts with complex life cycles. *PLoS One*, 8(4), e60920.
- Searle, C. L., Belden, L. K., Du, P. & Blaustein, A. R. (2014). Stress and chytridiomycosis : exogenous exposure to corticosterone does not alter amphibian susceptibility to a fungal pathogen. *Journal of Experimental Zoology Part A: Ecological Genetics and Physiology*, 321(5), 243-253.
- Semlitsch, R. & Wilbur, H. (1988). Effects of pond drying time on metamorphosis and survival in the salamander, *Ambystoma talpoideum*. *Copeia*, 4, 978–983.
- Skerratt, L. F., Berger, L., Speare, R., Cashins, S., McDonald, K. R., Phillott, A. D. & Kenyon, N. (2007). Spread of chytridiomycosis has caused the rapid global decline and extinction of frogs. *EcoHealth*, 4(2), 125–134.
- Stuart, S.N., Chanson, J.S., Cox, N.A. Young, B.E., Rodrigues, A.S.L., Fischman, D.L. & Waller, R.W. (2004). Status and trends of amphibian declines and extinctions worldwide. *Science* 306(5702), 1783-1786.
- Teplitsky, C., & Laurila, A., (2007). Flexible defense strategies: completion modifies investment in behavioral vs. morphological defenses. *Ecology*, 88(7), 1641-1646.

- Tobler, U. & Schmidt, B. R. (2010). Within- and among-population variation in chytridiomycosis-induced mortality in the toad *Alytes obstetricans*. *PloS One* 5(6), e10927.
- Venesky, M. D., Parris, M. J. & Storfer, A. (2010). Impacts of *Batrachochytrium dendrobatidis* infection on tadpole foraging performance. *EcoHealth*, 6(4), 565–575.
- Voyles, J., Young, S., Berger, L., Campbell, C., Voyles, W.F., Dinudom, A., Cook, D., Webb, R., Alford, R.A., Skerratt, L.F. & Speare, R. (2009). Pathogenesis of chytridiomycosis, a cause of catastrophic amphibian declines. *Science*, 326(5952), 582-585.
- Warne, R. W., Crespi, E. J., & Brunner, J. L. (2011). Escape from the pond: stress and developmental responses to ranavirus infection in wood frog tadpoles. *Functional Ecology*, 25(1), 139–146.
- Werner, E. (1986). Amphibian metamorphosis: growth rate, predation risk, and the optimal size at transformation. *American Naturalist*, 128(3), 319–341.

Annex Tables

Table I. Time points of the experience

		Day	Time point
Beginning of experiment	26-Mar	0	1
1 st exposure	26-Mar	0	1
2 nd exposure	27-Mar	1	2
3 rd exposure	28-Mar	2	3
4 th exposure	29-Mar	3	4
Removal of 1 tadpole	04-May	39	5
Evaluation of Gosner stage	12-May	47	6
End of experiment (Metamorphosis)	variable	variable	7

Table II. Number of cluches / amplex couples collected, collection site, date of their collection

Eggs	Clutch	Amplex couple	Posture date	Collection site	Coordinates	Collection date
A		✓	6 th March	Parque dos Lagos (Sintra)	30°47'18,26''N 9°23'33.97''W	6 th March
B	✓		--	Parque dos Lagos (Sintra)	30°47'18,26''N 9°23'33.97''W	6 th March
C	✓		--	Parque dos Lagos (Sintra)	30°47'18,26''N 9°23'33.97''W	6 th March
D		✓	7 th March	Parque dos Lagos (Sintra)	30°47'18,26''N 9°23'33.97''W	6 th March
E		✓	8 th March	Parque dos Lagos (Sintra)	30°47'18,26''N 9°23'33.97''W	6 th March
F	✓		--	Parque dos Lagos (Sintra)	30°47'18,26''N 9°23'33.97''W	6 th March