Early exposure to *Batrachochytrium dendrobatidis* causes profound immunosuppression in amphibians

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Abstract

Fungal pathogens have evolved a broad suite of strategies aiming at evading the host immune response. Amphibians are globally-infected by the panzootic chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*) and, while robust innate immune defences have been characterised, there is little evidence for the existence of effective adaptive immunity. We determine the immune response of the common midwife toad following challenge by *Bd* as larvae. Immune function was described for both the cell-mediated and antibody-mediated immune responses following infectious challenge as larval amphibians. While there were no significant differences in the ratio of neutrophils/lymphocytes between infected and uninfected individuals, early exposure of tadpoles to *Bd* significantly dampened the levels of circulating immunoglobulins (IgM and IgY) in the serum of juveniles after metamorphosis. Our results show that *Bd* immunosuppresses amphibians when infection occurs as larvae with potentially broad effects on the remodelling of immunity during metamorphosis.

Introduction

The vertebrate immune system comprises both an innate and an adaptive component that interact with each other to protect organisms against infections. This functional division is found in amphibians which have an immune system similar to that of mammals (Rollins-Smith 2001; Robert and Ohta 2009). The amphibian innate immune system consists of granulocytic leukocytes, phagocytic cells, natural killer cells, complement proteins and antimicrobial peptides, and serves as the rapid first line of defence against challenge by pathogens (Robert and Ohta 2009; Rollins-Smith and Woodhams 2012). Conversely, the amphibian adaptive immune system consists of antigen-presenting cells, the major histocompatibility complex (MHC), T and B-lymphocytes, and immunoglobulins, however is slower in its response to pathogen assault (Robert and Ohta 2009; Rollins-Smith and Woodhams 2012).

An important trait of the amphibian immune system is that it develops in two different phases that are separated by metamorphosis (Rollins-Smith 1998; Rollins-Smith 2001). During metamorphosis, amphibian larvae undergo a series of physiological processes, which entail the complete remodelling of their immune system and of all its components (Rollins-Smith 1998; Rollins-Smith 2001).

The main organ responsible for the immune changes during amphibian metamorphosis is the hypothalamus-pituitary-interrenal (HPI) axis that acts by increasing levels of plasma corticosteroids (Rollins-Smith 1998; Rollins-Smith 2001; Gervasi and Foufopoulos 2008; Kindermann et al. 2012). This sharp increase in corticosteroid levels, however, results in a transient immune impairment during metamorphosis (Flajnik et al. 1987; Rollins-Smith 1998, 2001). While this is a physiological process, the lymphocyte breakdown and temporary immunosuppression that happens during the metamorphosis climax, leaves amphibians more prone to infections caused by

pathogens such as the chytrid fungus *Batrachochytrium dendrobatidis* (hereafter, *Bd*) (Rollins-Smith 2001; Gervasi and Foufopoulos 2008). Infection by *Bd* is also known to directly influence circulating levels of glucocorticoids and may reinforce temporary immunosuppression during metamorphosis. For instance, in some amphibians levels of corticosteroids are dictated by the intensity of their infection (Kindermann et al. 2012, Gabor et al. 2013), suggesting that infection by *Bd* may indirectly immunosuppress amphibian hosts by stimulating the HPI axis. However, there are very few studies that examine the role of the adaptive (lymphocyte-mediated) components of the immune system on host resistance to *Bd*.

In this study we determine the immune response of the common midwife toad (*Alytes obstetricans*) following challenge by *Bd* as larvae. Cell-mediated response mounted by *A. obstetricans* tadpoles and juveniles was described through differential white blood cells counts and the humoral immune response was assessed by measuring circulating levels of immunoglobulins (IgM and IgY) present in the serum of juveniles after being exposed to *Bd* during their larval development.

Materials and Methods

Tadpoles were wild caught from infected and non-infected populations of the Provinces of Teruel and Zamora (north and central Spain). *Alytes obstetricans* is an amphibian species highly susceptible to *Bd* with an extremely long larval stage characterised by high prevalences and fungal loads upon metamorphic climax (Fernández-Beaskoetxea et al. 2015). To determine the leukocyte profiles, tadpoles were raised in captivity until metamorphosis and blood samples were obtained by heart puncture from tadpoles and juveniles prior to terminal anaesthesia. Animals were swabbed immediately after taking blood samples (the oral disc for larvae and the lower ventral surface and hind-limbs of

juveniles) with sterile rayon-tipped swabs (MW100-100; Medical Wire & Equipment Co, Corsham, UK), then DNA was extracted with PrepMan Ultra and quantitative PCR analyses were carried out following Boyle et al. (2004).

Standard blood smears were made on clean microscope slides that were air-dried, fixed with pure alcohol, and stained with the Diff-Quick staining technique. Haematological smears were read under 40X magnification using a compound microscope. Cell counts were performed until at least 100 leukocytes were enumerated. White blood cells were identified and the proportion of each cell type was afterwards determined.

To determine humoral immune response to *Bd*, overwintering (OW) and non-overwintering (NOW) tadpoles were captured for performing the following experiment. A subsample of tadpoles (n = 20) of every capture session was analysed by quantitative PCR as described before to determine their infection status. NOW tadpoles tested negative for *Bd* even when the whole oral disc was used for DNA extractions. 180 NOW experimental larvae were singly housed with either one OW *Bd*-negative or one OW *Bd*-positive tadpole (early contact phase). After 50 days NOW experimental tadpoles were removed and treated with either the antifungal itraconazole (a first generation systemic triazole antifungal drug) at 0.01% for seven days (Forzan et al. 2008), or with elevated temperature (30ºC) for 10 days (Geiger et al. 2011) (intermediate treatment phase). Finally, every NOW experimental tadpole was re-housed for 50 days with one *Bd*+ or *Bd*- OW tadpole (late contact phase). Not every possible combination of levels across temporal phases was used, resulting in nine different experimental groups with 20 replicates each (Fig. 1). OW tadpoles were tested before each contact phase to confirm their infection status.

NOW tadpoles were allowed to complete metamorphosis singly housed and surviving animals (n = 77) were sampled to obtain blood samples through heart puncture and then

euthanased with an overdose of MS222 (Sigma-Aldrich, Saint-Louise, Missouri, USA). Indirect enzyme-linked immunosorbent assays were carried out in order to determine circulating levels of immunoglobulins. *Bd* antigen-coated plates were prepared (96-well; Nunc Maxisorp, Rochester, NY) by adding 5 x 10^4 cells at 50µL/well as *Bd* zoospores alone or as mixtures of zoospores and sporangia. These cells were fixed by the addition of a fixing solution (Amphibian Phosphate Buffered Saline-APBS + 0.25% glutaraldehyde) then plates centrifuged (200 x g) and stored overnight at 4ºC. Following removal of the fixative, a blocking buffer was added and the plates were stored for two hours at 37ºC. Subsequently, the plates were washed with a washing solution (APBS + 0.05% Tween20) and the *A. obstetricans* serum samples diluted 1/1000 in ABT (APBS + 0.5% Bovine Serum Albumin-BSA + 0.1% Tween20) were added to the wells. *Bd*-specific antibodies were detected using anti-*Xenopus* monoclonal antibodies specific for IgM (10A9) and for IgY (11D5), at a concentration of 1/1000, followed by horseradish peroxidase-conjugated goat anti-mouse antibodies (Invitrogen Corporation, Camarillo, California). The reactions were visualized with 3,3',5,5'-tetramethylbenzidine (TMB) substrate (Sigma). Reactions were stopped after 60 minutes by adding 2M H_2SO_4 and plates were read at 450 nm (OD450).

We used a general linear model to determine whether there was an association between the ratio of neutrophils/lymphocytes and *Bd* infection load of tadpoles, with mass and Gosner stage included as covariates. Another general linear model was used to look for differences on IgM and IgY levels across groups of tadpoles in the experiment. For this second analysis, three different fixed factors were considered: early contact with *Bd* (yes/no), type of *Bd* treatment (none, itraconazole, elevated temperature), and late contact with *Bd* (yes/no). Days to metamorphosis was included into the model as a covariate to control for any biases related to differences in development rates among

animals. Only the interaction between the first and the third factor was considered because not every level of the *Bd* treatment factor was replicated across the other two factors. For statistical analyses, variables were transformed using Box-Cox or Johnson normalizing transformations and residuals of general linear models did not deviate from the canonical assumptions.

All animal experiments were conducted in compliance of the Directive 2010/63/EU for the protection of animals used for scientific purposes in facilities of the regional government and under permit of the competent authorities (permits 10/032921.9/12 and 10/071126.9/13, Consejería de Medio Ambiente of Madrid).

Results

The ratio of neutrophils/lymphocytes was not related to *Bd* infection load in tadpoles after controlling for mass and Gosner stage ($F_{3,29}$=0.6427, p=0.5945). The infection intensity of tadpoles ranged between 270 and 8690 *Bd* genome equivalents, which is in accordance with values found in previous studies (Fernández-Beaskoetxea et al. 2015). Also, the proportion of monocytes, eosinophils and basophils were very similar in infected and non-infected animals (Table 1), suggesting that prior infection by *Bd* does not observably impact cell populations that are involved in cell-mediated immunity. Levels of IgM did not differ across *Bd* treatments ($F_{2,70}$=0.905, p=0.409), among animals being in contact or not with *Bd* during the late contact phase of the experiment ($F_{1,70}$=0.238, p=0.626), nor of the interaction between *Bd* contact during the early and the late phases ($F_{1,70}$=0.630, p=0.430; Fig. 1). On the other hand, there was a strong effect upon levels of IgM following contact with *Bd* during the first contact phase ($F_{1,70}$=321.67, p=0.0001). Similar results were obtained for IgY, showing a very

significant effect of being in contact with *Bd* during the early contact phase of the experiment ($F_{1,70}$=308.47, p=0.0001).

Discussion

Our results show that humoral immunity of *Bd*-infected *A. obstetricans* tadpoles was highly affected, with levels of circulating IgM and IgY antibodies being profoundly down-regulated. Further, we found no significant effects on the cell-mediated immunity for leukocyte profiles or the neutrophils/lymphocytes ratio, although the small sample size of non-infected tadpoles (*n*=4) may compromise the strenght of this observation. However, we caution that our work did not take into account the skin microbial community, which many authors have identified as part of the amphibian innate immune system (Harris et al. 2009) and may increase the effectiveness of the innate defences against *Bd* (Woodhams et al. 2007).

Bd appears to be capable of inhibiting the normal development of an adaptive immune response, and perhaps even evading the amphibian immune response (Rollins-Smith 2001; Rosenblum et al. 2009). Work by Ribas et al. (2009) showed a generalised down regulation in spleen markers of adaptive immunity in *Silurana tropicalis* frogs infected with *Bd*, even when housed at host-optimal temperatures (26ºC). The mechanism by which *Bd* evades host immune response may include soluble toxic factors from cell wall components that inhibit lymphocyte proliferation and induce apoptosis, perhaps explaining in part why *Bd* can be lethal to species lacking a robust innate immune defence (Rollins-Smith et al. 2009; Fites et al. 2013). Thus, when it comes to amphibians fighting against challenge by *Bd*, it appears that survival of infected amphibians is more correlated with the host innate immune response rather than being

associated with the adaptive immunity (Ribas et al. 2009; Stice and Briggs 2010; Savage et al. 2016).

Studies have attempted to determine how repeated exposures to *Bd* influence survival rates, immunity or infection intensities. Wood frogs (*Osteopilus septentrionalis*) are able to increase cellular immunity (spleen lymphocytes) after a number of repeated cycles of exposure to *Bd* and clearance of their infection through heat (McMahon et al. 2014). In Shaw et al. (2010) a few individuals of Archey's frog (*Leiopelma archeyi*) showed low *Bd* infection intensities when reinfected, with some animals eventually clearing their infection. Yet another study showed that Boreal toads (*Anaxyrus boreas*) that were previously exposed to *Bd* lived for a longer period of time after reinfection and suffered from a lower infection intensity than *Bd*-naïve individuals (Murphy et al. 2011). Together, these studies may suggest the possible presence of a *Bd*-specific acquired immune response. On the other hand, effects of previous immunization with heat-killed *Bd* have also been studied in the highly susceptible species *Rana muscosa* (Stice and Briggs 2010). In this case, no significant differences between control and immunised individuals in the infection's proportion, fungal loads and mortality was observed. Cashins et al. (2013) also showed that prior infection and treatment was ineffective in increasing protective immunity in *Litoria booroolongensis*.

Clearly, these contradicting studies show that the relationship infection by *Bd* and the generation of adaptive immunity is not straightforward and more research is needed to determine why amphibian species vary in their ability to generate a humoral immune response. Amphibian immunological memory is known to occur (DuPasquier and Haimovich 1976; Rollins-Smith 1998), but we do not know how this persists through the metamorphic period when combined with an infectious challenge by *Bd*. Many aspects of the immunological response of amphibians when facing *Bd* remain

unresolved and further work on the levels of antibodies expressed by the larval stages, as well as the humoral immune response in adults previously exposed as tadpoles, is needed.

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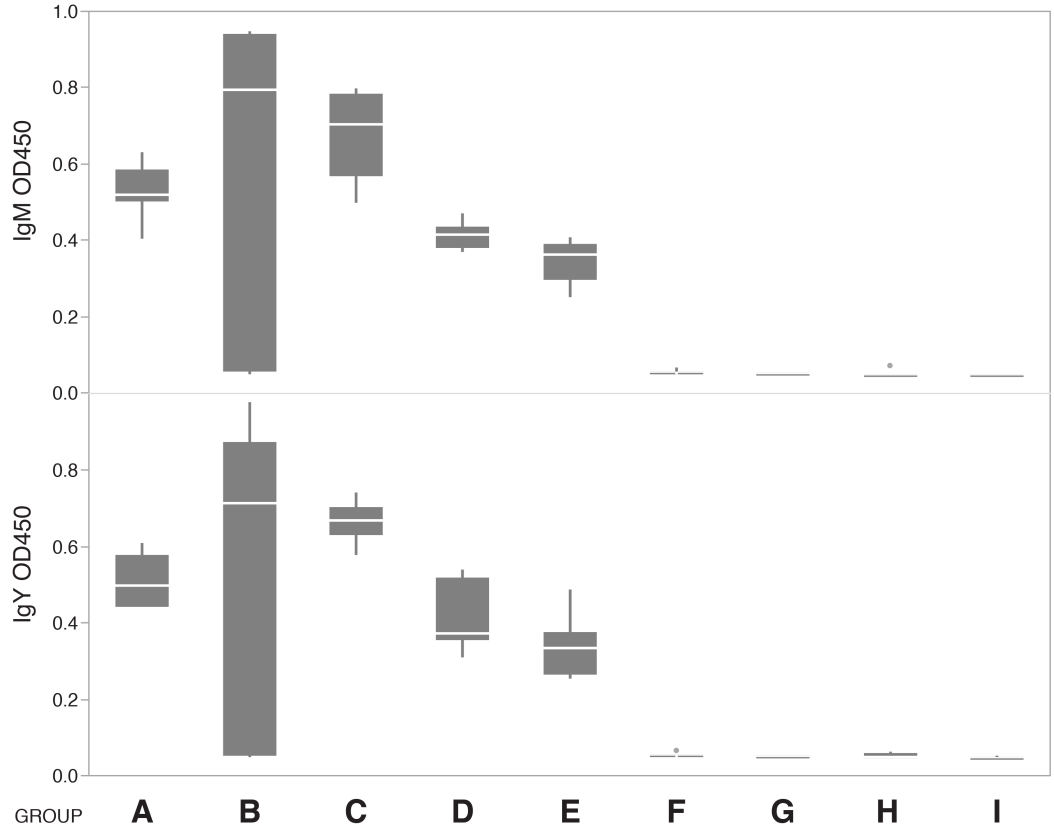
Gosner stage	N	Neutrophils	Lymphocytes	Monocytes	Eosinophils	Basophils
Bd-Infected tadpoles						
26-30	16	23.2	74.8	1.8	0.1	0.1
31-34	6	17.0	79.7	3.3	0.0	0.0
35-40	1	8.0	92.0	0.0	0.0	0.0
41-42	3	17.7	79.3	2.3	0.7	0.0
	Averaged	20.5	77.1	2.1	0.2	0.1
Non-Infected tadpoles						
26-30	3	15.3	82.1	1.3	0.0	1.3
35-40	1	10.0	85.0	0.0	0.0	5.0
	Averaged	14.0	82.7	1.0	0.0	2.3
Juveniles (Non-Infected)						
-	15	31.2	64.8	2.5	0.3	1.2

Table 1. Average percentages for each cell type in each development group.

Figure 1. Humoral immune response (IgM and IgY OD levels) of experimental larvae on nine different experimental groups (A-I) belonging 3 consecutive phases. 1) Early contact phase: non overwintering (NOW) experimental larvae being in contact with *Bd*- (groups A-E) or *Bd*+ (groups F-I) overwintering larvae (OW); 2) Intermediate treatment phase: NOW experimental larvae were treated with itraconazole (ITZ; groups B, D, F and H), elevated temperature (Temp; groups C, E, G and I), or not treated (NA; group A); and 3) Late contact phase: NOW experimental larvae being in contact again with *Bd*- (groups A-C and F-G) or *Bd*+ (groups D-E, and H-I) OW larvae. Sample size of each experimental group at the end of the experiment was, respectively, 8, 10, 6, 7, 10, 10, 8, 7 and 11.



1. Early contact phase	OW *Bd*-	OW *Bd*-	OW *Bd*-	OW *Bd*-	OW *Bd*-	OW *Bd*+	OW *Bd*+	OW *Bd*+	OW *Bd*+
2. Intermediate treatment phase	NA	ITZ	Temp	ITZ	Temp	ITZ	Temp	ITZ	Temp
3. Late contact phase	OW *Bd*-	OW *Bd*-	OW *Bd*-	OW *Bd*+	OW *Bd*+	OW *Bd*-	OW *Bd*-	OW *Bd*+	OW *Bd*+