

Temporal patterns in immunity, infection load and disease susceptibility: understanding the drivers of host responses in the amphibian-chytrid fungus system

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Summary

1. Many pathogens infect a wide range of host species. However, variation in the outcome of infection often exists amongst hosts and is shaped by intrinsic host traits. For example, contact with pathogens may trigger changes in hosts directed toward preventing, fighting, or tolerating infection. Host responses to infection are dynamic; they change over time and vary depending on health, condition and within the context of the environment.

2. Immunological defences are an important class of responses that mediate host-pathogen dynamics. Here, we examined temporal patterns of immunity in two amphibian species, Pacific tree frogs (*Pseudacris regilla*) and Cascades frogs (*Rana cascadae*), exposed to control conditions or experimental inoculation with the emerging infectious fungal pathogen, *Batrachochytrium dendrobatidis* (Bd). For each species, we compared bacterial killing ability of blood and differential white blood cell counts at four different time-points after pathogen inoculation. We also quantified infection load over time and monitored survival.

3. We detected qualitative and quantitative differences in species responses to Bd. *Pseudacris regilla* exhibited an increase in infection load over time and 16% of Bd-exposed animals died during the experiment. Tree frogs lacked robust treatment differences in immune responses, but Bd-exposed *P. regilla* tended to display weaker bacterial killing responses than unexposed control animals. Neutrophil counts did not vary consistently with treatment and lymphocytes tended to be less abundant in Bd-exposed animals at the later sampling time-points.

4. In contrast, Bd-exposed *R. cascadae* exhibited a decrease in infection load over time and no mortality occurred in the Bd treatment. Bd-exposed Cascades frogs showed stronger bacterial killing responses and an elevated number of neutrophils in blood when compared with control animals, and both responses were upregulated within 48 h of pathogen exposure. Lymphocyte counts did not vary significantly with treatment.

5. Although only statistically significant in Cascades frogs, neutrophil:lymphocyte ratios showed a trend of being elevated in Bd-exposed animals of both species and are indicative of pathogen-induced physiological stress.

6. Our results suggest that variation in systemic immunological responses of two syntopic amphibian species is associated with and may contribute to differential patterns of survival and infection load during exposure to the chytrid fungus. Species variation in immunological responses as soon as 48 h after pathogen exposure suggests that initial host–pathogen interactions may set the stage for subsequent infection and disease progression. Variation in host responses can drive disease dynamics and comparative studies of host responses to pathogens are critical for making predictions about pathogen emergence, spread and persistence.

Key-words: Batrachochytrium dendrobatidis, immunocompetence, multi-host, resistance, tolerance

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Introduction

Understanding host-pathogen dynamics has become an urgent priority as emerging infectious diseases increase in abundance and impact and contribute to losses of biodiversity (Harvell et al. 1999; Daszak, Cunningham & Hyatt 2000; Keesing et al. 2010; Cunningham, Dobson & Hudson 2012; Fisher et al. 2012; McCallum 2012). For example, global amphibian population declines have been linked to the emerging infectious fungus Batrachochytrium dendrobatidis (Bd) (Stuart et al. 2004; Skerratt et al. 2007; Fisher, Garner & Walker 2009; Olson et al. 2013). While not all amphibian populations decline when Bd is present (Briggs et al. 2005; Briggs, Knapp & Vredenburg 2010; Vredenburg et al. 2010; Brannelly, Chatfield & Richards-Zawacki 2012), the pathogen has been associated with mass mortality of amphibians throughout the world (e.g. Berger et al. 1998; Bosch, Martinez-Solano & Garcia-Paris 2001: Green, Converse & Schrader 2006: Lips et al. 2006: Mendelson et al. 2006; Skerratt et al. 2007; Crawford, Lips & Bermingham 2010).

Whereas some species die rapidly after exposure to Bd (Blaustein et al. 2005; Carey et al. 2006; Gahl, Longcore & Houlahan 2011; Searle et al. 2011; McMahon et al. 2013), others can persist with infection (Daszak et al. 2004; Brannelly, Chatfield & Richards-Zawacki 2012; Woodhams, Bigler & Marschang 2012; Gervasi et al. 2013). Some species fall within the middle of the 'disease susceptibility continuum', displaying intermediate levels of mortality and infection load (Searle et al. 2011; Gervasi et al. 2013). Susceptibility to Bd may be influenced by abiotic factors such as temperature, season, resource availability or biotic interactions (Berger et al. 2008; Bielby et al. 2008; Murray et al. 2011; Warne, Crespi & Brunner 2011; Murray & Skerratt 2012: Raffel et al. 2012). Life history traits such as lifespan, social or breeding status and behaviour may also correlate with susceptibility to Bd (Rowley & Alford 2007; Richards-Zawacki 2010; Bancroft et al. 2011). Although Bd occurs on every continent where amphibians exist (Fisher, Garner & Walker 2009) we know little about what drives interspecific variation in susceptibility and how this variation affects the spread and persistence of Bd (reviewed in Blaustein et al. 2011, 2012).

Bd zoospores infect keratinized epidermal tissue of postmetamorphic amphibians (Longcore, Pessier & Nichols 1999; Berger *et al.* 2005). Host–pathogen contact begins when zoospores encyst on the surface of the epidermis (Berger *et al.* 2005). Infection progresses with cellular contents of zoospores being transferred into keratin-containing host cells and formation of an intracellular zoosporangium. New zoospores are produced asexually within the zoosporangium and are subsequently discharged from skin cells into the environment (Berger *et al.* 2005; Greenspan, Longcore & Calhoun 2012). Infection can lead to disruption of the epidermal cell cycle and molting, hyperkeratosis and hyperplasia of skin cells and erosions and ulcerations of the skin (Nichols *et al.* 2001; Berger et al. 2005; Greenspan, Longcore & Calhoun 2012). Impairment of osmoregulatory function due to infection can be lethal (Voyles et al. 2009). Infection can occur within 12 h (Greenspan, Longcore & Calhoun 2012), which is relevant with regard to experimental studies showing mortality in Bd-exposed animals as soon as 24 h postexposure (Gahl, Longcore & Houlahan 2011; Searle et al. 2011). In addition to zoospore invasion of host cells, Brutyn et al. (2012) observed disruption of intercellular junctions, necrosis and apoptosis of amphibian epidermal cells exposed to proteolytic secreted factors of Bd zoospores. Taken together, these studies provide evidence that Bd infection is an active process that has the potential to disrupt host homeostasis and trigger host responses aimed at preventing, fighting or tolerating the pathogen.

Interspecific variation in immunological responses may drive differences in infection and disease progression in amphibians exposed to Bd. Amphibians possess both innate and adaptive immune responses and maintain a diverse repertoire of cellular and humoral defenses, enabling them to respond to a broad range of pathogens (Richmond et al. 2009). Innate immune responses are rapid, non-specific, and include cellular and humoral mechanisms for killing pathogens (Janeway et al. 2005). Cellular effectors such as neutrophils kill pathogens by releasing toxic and reactive compounds extracellularly or during the process of phagocytosis. Humoral effectors, such as the blood complement system, coat pathogens and enhance phagocytosis or destroy pathogens directly by lysis (Janeway et al. 2005). It is currently unknown how Bd is directly or indirectly recognized by the immune system (Richmond et al. 2009). The invasion of epidermal layers by zoospores and host exposure to proteolytic secreted factors from zoospores (Rosenblum et al. 2008; Brutyn et al. 2012) suggests the possibility of pathogen cues that signal general, non-specific 'pathogen invasion' or 'damage' to hosts (Matzinger 2002).

Innate immune responses have been the most studied aspects of immunity against Bd in amphibians (reviewed in Rollins-Smith *et al.* 2009, 2011). For example, antimicrobial peptides (AMPs) secreted from the glands of amphibian skin may be critical in preventing initial colonization of the skin by Bd (Rollins-Smith 2009). There is little support for a memory response to the pathogen (Ribas *et al.* 2009; Rollins-Smith *et al.* 2009; Cashins *et al.* 2013). Savage & Zamudio (2011) found that genetic variation in host immune genes (heterozygosity in MHC genotype) was associated with susceptibility to Bd and represented a significant predictor of survival across different amphibian populations of the same species (Savage & Zamudio 2011).

We experimentally examined mortality, infection load and immunological responses in two amphibian species exposed to Bd or control conditions. Our study is unique in comparing changes in immune factors in circulating blood (vs. skin responses) across multiple time-points during pathogen exposure and infection. In particular, this study addresses early host-pathogen contact, as soon as 24 and 48 h after experimental inoculation, which may set the stage for patterns in infection and disease progression. We also examined later time-points, 5 and 15 days postinoculation. Changes in responses over time provide relevant insight that might be missed if we were to take a snapshot of host-responses at a single time-point. We employed a bacterial killing ability assay and examined differences in leucocyte profiles in Bd-exposed when compared with non-exposed control animals. The bacterial killing assay represents a functional immune challenge that depends on multiple integrated humoral and cellular innate mechanisms for killing microbes, including complement, AMPs, lysozyme, soluble acute phase proteins, neutrophils, monocytes and natural killer cells (Tizard 2004). This assay has been used in birds (Matson, Tieleman & Klasing 2006; Liebl & Martin 2009), mammals (Martin, Weil & Nelson 2007; Allen et al. 2009) and some ecotherms (Sparkmann & Palacio 2009) including amphibians (Venesky et al. 2012). Leucocyte numbers (neutrohil and lymphocyte counts) give an indication of the concentration of cells recruited toward innate and adaptive immunity. We also examined the ratio of neutrophils to lymphocytes (N : L ratio) in blood, which has been used as an indicator of physiological 'stress' in amphibians and other taxa (reviewed in Davis, Maney & Maerz 2008). An increase in circulating glucocorticoid stress hormones acts to increase neutrophils in the blood and results in a larger N : L ratio (Davis, Maney & Maerz 2008). Our measures of immunity were examined in conjunction with individual mortality and quantitative infection load.

Materials and methods

We chose to compare Pacific tree frogs (*Pseudacris regilla*) and Cascades frogs (*Rana cascadae*) because previous work showed variation in susceptibility and infection load in these species (Gervasi *et al.* 2013) and because these two species are syntopic in the Oregon Cascades range. Limited information is available about natural susceptibility and quantitative infection status of these two species, although both species have been found positive for Bd in the wild (www.bd-maps.net/). Quantitative infection load data on tree frogs from California suggest that this species is a potential reservoir host for chytrid fungus (Reeder, Pessier & Vredenburg 2012).

Because of the complexities associated with immune function and infection in the natural environment, we collected amphibian eggs and raised them through the larval stage to metamorphosis in semi-natural mesocosm environments (to preclude previous Bd-infection). Animals were c. 10–11 months post-metamorphosis when they were used in experiments. We used controlled laboratory experiments to rule out the effect of external factors and examine intrinsic host responses to infection. See Data S1 (Supporting information) for full 'Animal Husbandry and Rearing' protocols.

We controlled for the context of infection (inoculation dose, timing, duration of pathogen exposure) and standardized protocol between species, so that we could focus on variation in the progression of infection and responses to infection as a function of intrinsic host traits, only. Animals received a single 50 000 zoospore inoculation with *Batrachochytrium dendrobatidis* isolate, JEL 274 on the first day of the experiment and subsets of animals were randomly sampled for quantitative infection load and immunity 24, 48 h, 5 and 15 day post-inoculation. See Data S1 (Supporting information) for full 'Pathogen Preparation, Exposure and Quantification' protocols.

We used gentamicin-resistant *Escherichia coli* strain Dh5a for our bacterial killing assays. We randomly euthanized 12 control and 12 Bd-exposed experimental animals at each time-point for the immune assay and to make haematological smears. For the killing assay, a standardized dilution of *E. coli* was mixed with amphibian blood and amphibian ringer solution and plated for 24 h before bacterial colony counts were made. Control tubes contained bacteria and ringer only. Please see Data S1 (Supporting information) for full 'Bacterial Killing Assay' protocol.

STATISTICS

We examined differences in initial mass amongst time-points with a one-way ANOVA and compared initial body mass between treatments (all Bd-exposed animals vs. control animals) and between species with two sample *t*-tests. Because we observed no mortality in the pathogen-exposed treatment for one of the two species (R. cascadae), we did not examine survival statistically. We logtransformed infection load (q-PCR) data and compared log genome equivalents amongst time-points within each species with a one-way ANOVA. We examined differences in infection load between the two species with two sample t-tests for each timepoint. We examined the relationship between mass and infection load (by species and within the Bd treatment), mass and bacterial killing ability (by species and within both control and Bd-exposed treatments) and bacterial killing ability and infection load (by species and within the Bd treatment) with a general linear regression models. For immune responses we used a full linear regression model to examine whether species identity, time-point (as a continuous variable), treatment (control or Bd-exposed) or all interactions between these variables predicted immunity. Because a different subset of animals was randomly selected for leucocyte counts we did not include all immune response variables in a single regression model. Our immunological regression models were checked to ensure that residuals were normally distributed, had a mean at zero for all values of X and had constant variance. We were primarily interested in whether immune responses of animals differed between the control and Bd-exposed treatments at each independent time-point. We compared killing ability and cellular responses between animals in the control and Bd-exposed treatments within each sampling time-point using two sample t-tests or nonparametric Mann-Whitney Rank Sum tests, if our assumptions of normality were not met. We also calculated effect sizes for treatment differences at each time-point for each species using Cohen's d (Cohen 1988). Cohen's d was calculated as the difference in the means of the two groups (experimental-control) over the pooled standard deviation. This value indicated how many standard deviations separate the two groups. By convention, a Cohen's d value above 0.8 indicates a 'strong' or 'large' effect of the treatment, a d value of 0.5-0.8 indicates a 'medium' or 'large' effect, and a value equal to or lower than 0.2 indicates a weak effect (Cohen 1988). All statistical analyses were run in TIBCO Spotfire S+ Version 8.1 for Windows (Somerville, MA, USA). and figures were created in s+ and SigmaPlot version 12.0 for Windows (Systat Software Inc., San Jose, CA, USA).

Results

MASS, SURVIVAL, AND INFECTION LOAD

There were no differences in initial mass amongst timepoints or treatment in *R. cascadae* ($F_{7,88} = 0.812$; P = 0.579 and $t_{94} = 0.614$; P = 0.541) or in *P. regilla* ($F_{7,100} =$ 0.708; P = 0.665 and $t_{106} = 0.954$; P = 0.342) although R. cascadae metamorphs were on an average heavier than *P. regilla* metamorphs ($t_{202} = 18.05$; P < 0.0001). No mortality occurred in Bd-exposed Rana cascadae and a single control individual died over the duration of the experiment. Conversely, 16% of Bd-exposed Pseudacris regilla metamorphs died during the experiment and all mortality occurred between days 7 and 15. No control P. regilla died. Infection load in R. cascadae tended to decrease over time. Although the difference in quantitative infection load amongst 24, 48 h and 5 day was not significant (one-way ANOVA P = 0.571), it was noteworthy that all R. cascadae sampled for infection (n = 7) on day 15 tested negative for infection in all three replicate wells (Fig. 1a). In comparison, infection loads in P. regilla increased significantly over time (P = 0.023); infection load was significantly greater on day 15 than at the 48 h time-point (Tukey's Honestly Significant Difference P < 0.05). (Fig. 1b). Differences in infection load between species were marginally significant at the 24 and 48 h time-points (two-sample *t*-tests; P = 0.054; P = 0.061). We did not compare infection load between species on day 15 as no R. cascadae tested positive for infection at this time-point. Mass predicted infection load in P. regilla (with smaller animals having higher loads P = 0.026, $R^2 = 0.160$). Mass was a marginally significant predictor of infection load for R. cascadae (P = 0.062, $R^2 = 0.150$) with heavier animals tending to have higher infection loads (Fig. 2a,b).

IMMUNITY

Bacterial killing ability of blood was greater in Bd-exposed *R. cascadae* when compared with control animals (Fig. 3a). Differences in killing responses between the control and Bd-exposed treatments were significantly dif-

ferent at 48 h ($t_{22} = 2.5$; P = 0.020), 5 day (Mann-Whitney U = 41; P = 0.043) and 15 day ($t_{21} = 2.19$; P = 0.040). Effect sizes for Bd-exposed vs. Controls at the 48 h. 5 and 15 day time-points were also medium or large (all >0.7; Table 1). In P. regilla, control animals tended to show greater bacterial killing ability than Bd exposed animals (Fig. 3b). However, treatment differences were less pronounced in this species and were significant only on day 5 $(t_{22} = 2.90; P = 0.008)$. Differences in killing ability also changed over time in the control treatment for both species (Kruskal–Wallis test on ranks; R. cascadae $H_3 = 15.26$ P = 0.002; P. regilla: $H_3 = 17.26$; P = 0.001) and in the Bd-exposed treatment but only for P. regilla (Kruskal-Wallis test on ranks; $H_3 = 14.6$; P = 0.001). However, a full linear regression model including species identity, treatment and time-point as predictors for bacterial killing ability showed that treatment (P = 0.048), species (P = 0.020) and the interaction between treatment and species identity (P = 0.0002) were significant explanatory predictors, but not time-point (P = 0.216). There was no relationship between bacterial killing ability and mass or bacterial killing ability and infection load within the control or Bd-exposed treatments in either species.

Number of neutrophils per 2000 counted red blood cells varied with treatment and the treatment effect was different depending on species (Fig. 4a,b). In *R. cascadae*, neutrophil numbers were greater in blood of Bd-exposed animals when compared with control animals, and this difference was significant at the 48 h (Mann–Whitney U = 8.5; P = 0.010) and 5 day (Mann–Whitney U = 7.5; P = 0.007) time-points (Fig. 4a,b; Table 1). Neutrophil numbers were greatest in Bd-exposed animals after 48 h of exposure to the pathogen and then declined. In *P. regilla*, neutrophil numbers were not influenced by treatment. Although treatment-level differences were not significant at



Fig. 1. (a, b) Infection load (log genome equivalents) over time for *Rana cascadae* and *Pseudacris regilla*. We randomly sampled six to seven Bd-exposed animals at each time-point for each species for quantitative infection load using qPCR. In *R. cascadae*, infection load decreased over time, but there was no significant difference in infection load amongst the 24, 48 h and 5 day time-points (P = 0.571). However, no *R. cascadae* tested positive for infection on day 15 (n = 7). In *P. regilla*, infection load increased over time (P = 0.023) and individuals had significantly higher infection loads on day 15 than at the 48 h time-point (P < 0.05). We did not include animals testing negative for infection (R. cascadae, 15 d only) in the figure. Lines in the boxes represent the 'mean' infection load. Bars are one SE of the mean (\pm) and outliers are represented by black dots.



Fig. 2. (a, b) Relationship between initial mass and infection load in *Rana cascadae* and *Pseudacris regilla*. Linear regression indicates that mass is a marginally significant predictor of infection load in *R. cascadae* (heavier animals also had higher average infection loads; P = 0.062), and a significant predictor of infection load in *P. regilla* (heavier animals tended to have lower average infection loads; (P = 0.026). We did not include infection negative animals (*R. cascadae*, 15 d, only) in the figure.



Fig. 3. (a, b) Bacterial killing ability (proportion of bacterial colonies killed) for *R. cascadae* and *Pseudacris regilla* in Bd-exposed or control treatments. In *R. cascadae*, bacterial killing ability tended to be elevated in Bd-exposed animals. The difference in killing ability between Bd-exposed and control animals was significant at all time points (P < 0.05) except 24 h. For *P. regilla*, killing ability of blood tended to be reduced in Bd-exposed animals but was only significant on day 5 of the experiment (P < 0.05). We sampled 11–12 animals in each treatment and at each time-point for each species. Dots represent average killing ability and bars represent standard error of the mean.

any of the time-points, effect sizes for treatment differences were large (c. 0.8 or above) for the 48 h and 15 day timepoints (Table 1). Pseudacris regilla tended to have greater neutrophil counts when they were in the Bd-exposed treatment at the 48 h mark but lower neutrophil counts in the Bd-exposed group when compared with controls at the 15 day sampling time-point. In our full regression model for the response 'neutrophil counts', there were no significant main effects; however, there was a marginally signifibetween cant interaction treatment and time (P = 0.053). The number of lymphocytes per 2000 red blood cells did not vary between treatments in R. cascadae at any of the time-points (Fig. 5a). However, lymphocyte counts were higher in control P. regilla when compared with Bd-exposed animals (Fig. 5b), but only significantly

at the 5 day sampling point ($t_{14} = 2.15$; P = 0.050). The effect size for treatment differences in lymphocytes was large at both 5 day and 15 day for *P. regilla* (Table 1). In our full regression model for lymphocyte counts, we did not find any significant main effects or interaction terms between explanatory variables. Numbers of eosinophils, monocytes, basophils and thrombocytes were not analysed statistically because of low counts. Neutrophil to lymphocyte ratios were generally greater in Bd-exposed when compared with control animals across species however, differences were only significant in *R. cascadae* at the 48 h (Mann–Whitney U = 9.5; P = 0.015) and 5 day ($t_{14} = 3.15$; P = 0.007) time-points. Effect sizes for treatment differences in N : L ratios were large for both species at some time-points. (Table 1) at several time-points. In

R. cascadae, Cohen's *d* was >0.6 for all time-points. In *P. regilla*, Cohen's *d* was >0.6 at the 48 h time-point and >0.8 at the 5 day time-point. In a full linear regression model with neutrophil : lymphocyte ratios as the response variable we found that both treatment (P = 0.052) and species (P = 0.036) were significant explanatory predictors.

Discussion

We observed temporal and treatment-level differences in patterns of mortality, infection load and several measures of immunity in two amphibian species exposed to the chytrid fungus. In *R. cascadae*, we observed no mortality and decreasing infection loads in Bd-exposed animals. These responses were associated with elevated immune responses (bacterial killing ability and neutrophil counts) in pathogen-exposed animals at most sampling time-points. In *P. regilla*,

Table 1. Effect sizes for immunological responses in Pseudacris regilla and Rana cascada. For each species an effect size represented by Cohen's d value is given for each treatment (Bd-exposed vs. Control) comparison at each time-point (24, 48 h, 5 and 15 day). Cohen's d is calculated as the difference in the means of the two groups (experimental-control) over the pooled standard deviation. This value indicates how many standard deviations separate the two groups.

Species	Response	Time-point	Effect size (Cohen's <i>d</i> for Bd-exposed vs. control treatments)
P. regilla	Bacterial	24 h	0.334
	killing ability	48 h	0.195
		5 day	1.195
		15 day	0.221
R. cascadae		24 h	0.237
		48 h	1.020
		5 day	0.710
		15 day	0.956
P. regilla	Neutrophils	24 h	0.131
		48 h	0.836
		5 day	0.019
		15 day	0.762
R. cascadae		24 h	0.177
		48 h	1.208
		5 day	1.525
		15 day	0.200
P. regilla	Lymphocytes	24 h	0.219
		48 h	0.287
		5 day	1.101
		15 day	0.886
R. cascadae		24 h	0.815
		48 h	0.311
		5 day	0.367
		15 day	0.390
P. regilla	N : L ratio	24 h	0.073
		48 h	0.630
		5 day	1.131
		15 day	0.414
R. cascadae		24 h	0.604
		48 h	1.385
		5 day	1.627
		15 day	0.714

Cohen's d value is in bold if the effect size is large (>0.8).

we observed pathogen-induced mortality (16% of animals), increasing quantitative infection loads and modest treatment-level differences in immunological responses. Although zoospore invasion of host cells can occur within 12 h (Gahl, Longcore & Houlahan 2011), we note caution in inferring 'infection' at the earliest, 24 and 48 h timepoints (Hyatt et al. 2007). Live and dead pathogen DNA persisting in the housing enclosures of experimental animals after inoculation could have been sampled in addition to any DNA encytsted on or embedded in amphibian skin. As all animals were treated equally in our experiment, we assumed that differences in 'infection loads' at early timepoints were relevant comparatively, but not in an absolute sense. Most notable were species differences in infection load at the later time-points of our study. In P. regilla infection load was highest on day 15, whereas in R. cascadae, all animals tested for infection were Bd-negative.

The temporal trend in immunity in R. cascadae implies that activation of general, non-specific, host responses to Bd-exposure may start to occur soon after 24 h after exposure and significantly so after 48 h of pathogen exposure. This rapid initiation of innate responses in the blood may correspond to pathogen-induced disruption of skin structure and function (Greenspan, Longcore & Calhoun 2012), and rapid innate responses shortly after host-pathogen contact has also been known to occur in the skin [e.g. AMPs; (Rollins-Smith et al. 2009)] and other immune organs (Rosenblum et al. 2009). The relationship between robust treatment differences in bacterial killing ability and neutrophil counts in R. cascadae coupled with the trend for infection load to decrease to zero over time in Bd-exposed animals suggests that pathogen contact may trigger resistance mechanisms that allow R. cascadae to fight infection (Raberg, Sim & Read 2007; Read, Graham & Raberg 2008). Innate effectors like complement, AMPs, as well as neutrophils and macrophages and their associated toxic products are all likely to be functioning in our pathogen challenge assay. These effectors represent efficient mechanisms for killing invading pathogens quickly, but have the potential to lead to tissue and cell damage when they are chronically upregulated (Janeway et al. 2005). Lymphocytes, important components of the adaptive immune system, are comparatively slow to respond (e.g. proliferate and infiltrate tissues) during infection (Janeway et al. 2005). We did not observe significant differences in lymphocyte responses in R. cascadae. However, as lymphocyte responses may be slower to occur, we suggest that the 5 and 15 day time-points might be most relevant in this experiment. At both of these time-points, R. cascadae showed a trend of increased lymphocytes in pathogen-exposed animals when compared with controls. Interestingly, the effect size of treatment on lymphocyte numbers at 24 h was high (Cohen's d = 0.815) but low (<0.4) at all other time-points for this species. Although R. cascadae show elevated bacterial killing and neutrophil responses in the Bd-exposed treatment and associated lower infection loads, and 100% survival, the patterns in



Fig. 4. (a, b) Neutrophil counts for *Rana cascadae* and *Pseudacris regilla* in control vs. Bd-exposed treatments. There were no significant treatment differences in total neutrophils for *P. regilla* at any of the sampling time-points. However, neutrophil counts differed between treatments in *R. cascadae* at the 48 h (P = 0.010) and 5 day (P = 0.007) time-points. We sampled eight animals in each treatment and at each time-point for each species. Dots represent average neutrophil counts and bars represent standard error of the mean.



Fig. 5. (a, b) Lymphocyte counts (total lymphocytes in 2000 red blood cells) for *Rana cascadae* and *P. regilla* in control vs. Bd-exposed treatments. There were no significant treatment differences in total lymphocyte counts in *R. cascadae* at any of the sampling time-points although lymphocyte counts tended to be higher in Bd-exposed animals at the later time-points. In *P. regilla*, lymphocyte counts tended to be lower in Bd-exposed animals at the later time-points and this trend was significant at the 5 day sampling time-point (P = 0.050). We sampled eight animals in each treatment and at each time-point for each species. Dots represent average lymphocyte counts and bars represent standard error of the mean.

neutrophil: lymphocyte ratios we observed indicated increased physiological 'stress' in pathogen-exposed animals (Fig. 6b; Table 1). This cellular ratio has been shown to change with glucocorticoid levels; as stress hormones increase in circulating blood, the number of neutrophils generally increases compared to lymphocytes (Davis, Maney & Maerz 2008). If this is the case, then *R. cascadae* may be experiencing a physiological 'cost' of resistance.

In *P. regilla*, the relationship between infection load and immunity differed from *R. cascadae*. Infection load in tree frogs increased over time, and bacterial killing ability was generally, although not always significantly, lower in Bd-exposed animals when compared with controls. Blood complement plays an important role in the bacterial killing assay we employed, and failure of this pathway to become 'activated' during host–pathogen interaction could have contributed to the functional responses we saw in our immunological challenge assay. Further, the lack of a robust elevation in innate responses in blood, in particular blood complement response and cellular response (i.e. neutrophil numbers), might directly relate to infection load by allowing the pathogen to get a foothold and replicate, unchecked, in the skin. Secondary or opportunistic infections could be particularly damaging in P. regilla if immunity is downregulated or non-activated during infection with Bd. However, dampening of innate immune responses may also limit self-damage caused by these inflammatory responses (Matzinger 2002) and could allow P. regilla to tolerate initial infection. Alternatively, this species may be unable to respond with resistance mechanisms to limit pathogen growth in the skin (Rollins-Smith et al. 2009). The effect size for the treatment difference in N : L ratios



Fig. 6. (a, b) Neutrophil: Lymphocyte counts for *Rana cascadae* and *Pseudacris regilla* in control vs. Bd-exposed treatments. N : L ratio generally tended to be greater in Bd-exposed animals when compared with control animals of both species and at most time-points indicating potentially greater 'physiological stress' in pathogen-exposed animals in the study. However, differences in the ratio were only significant different in *R. cascadae* at the 48 h (P = 0.015) and 5 day (P = 0.007) time-points. We sampled eight animals in each treatment and at each time-point for each species. Dots represent average N : L ratios and bars represent standard error of the mean.

was high at the 5 day time-point in tree frogs (Table 1). So, although innate immune responses were minimal, exposure to Bd and associated pathogen-invasion signals might be 'stressful' in this species. We saw a significant decrease in lymphocyte numbers in P. regilla at the 5 day time-point (the same time-point associated with lower bacterial killing ability in this species), and a large effect size of treatment for lymphocyte counts at both 5 and 15 day for this species (Table 1), indicating the potential influence of Bd infection on other (i.e. adaptive) components of the immune system. At both 'later' time-points, lymphocytes tended to be reduced in pathogen exposed animals when compared with controls and this response was the opposite of what was observed in R. cascadae. The Australian Green Tree frog, Litoria caerulea, exhibited patterns in leucocyte profiles not seen in either of our species (Peterson et al. 2013). Animals showing clinical signs of disease had higher neutrophil counts and lower lymphocyte numbers than animals that did not show signs of chytridiomycosis (Peterson et al. 2013). Combined with our findings, this highlights that the species-specific nature of host immune responses to this single pathogen.

The immune responses we detected in circulating blood underscore the high degree of integration and crosstalk in the vertebrate immune system (Janeway et al. 2005). Both humoral and cell-mediated factors in the blood can be initiated during infection that occurs first in the skin. For example, disruption of cell junctions (Brutyn et al. 2012; Greenspan, Longcore & Calhoun 2012) and skin function (Voyles et al. 2009, 2012; Voyles, Rosenblum & Berger 2011) may serve as a 'danger' signal (Matzinger 2002), and cause the recruitment of cellular components of the innate arm of the immune system. Due to highly synchronized cytokine signalling in response to pathogen cues, a continuous feedback may occur between disruption of normal function in the skin and systemic-wide responses (Janeway et al. 2005). Initial skin responses are likely to activate other components of the immune system, especially pro-inflammatory and anti-inflammatory mediators (e.g. cytokines) which could determine the level of pathology, progression to and severity of infection, and the outcome of disease on host mortality and morbidity. As infection progresses, further disruption to homeostasis (Voyles, Rosenblum & Berger 2011; Voyles *et al.* 2012) could potentially compromise other basic functions and interact with immunity in complex and context dependent ways.

The relationship between infection load and body mass varied between species. We tended to see heavier animals with higher infection loads in *R. cascadae* but this trend was not significant (Fig. 2a). This could be explained by heavier animals also being longer, resulting in a greater body area for pathogen colonization (Kuris, Blaustein & Alio 1980). However, the trend may have also resulted if heavier animals were in better condition, overall, and tolerated higher infection loads. In *P. regilla*, mass was a significant predictor of infection load (Fig. 2b). Body mass had no association with bacterial killing ability in control or Bd-exposed animals of either species.

Although we predicted that temporal differences in bacterial killing ability of Bd-exposed animals would change over time, we also observed temporal variation in bacterial killing ability of control animals. Laboratory conditions including our feeding regime may have contributed to this observation. For example, animals were fed crickets on days 4, 8 and 12 of the experiment. Almost every animal, regardless of treatment, was observed to eat all crickets in their enclosures within minutes. Metabolic and energetic changes associated with digestion could have influenced immunity and could potentially affect host–pathogen dynamics.

Understanding the contribution of different hosts to multi-host pathogen dynamics is critical. Species that succumb to mortality during infection, maintain low levels of infection, or clear infection may contribute to pathogen fade-outs, whereas species that survive with high levels of infection over time (e.g. reservoir hosts) may facilitate pathogen dispersal, maintenance in the environment (Keesing, Holt & Ostfeld 2006) and contribute to the extinction or population declines of more susceptible species (McCallum 2012).

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Supporting Information

Additional Supporting information may be found in the online version of this article:

Data S1. Methods.