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Glutathione is required for growth and cadmium tolerance in the amphibian chytrid fungus, *Batrachochytrium dendrobatidis*

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ABSTRACT

Batrachochytrium dendrobatidis (*Bd*) is a lethal amphibian pathogen, partly due to its ability to evade the immune system of susceptible frog species. In many pathogenic fungi, the antioxidant glutathione is a virulence factor that helps neutralise oxidative stressors generated from host immune cells, as well as other environmental stressors such as heavy metals. The role of glutathione in stress tolerance in *Bd* has not been investigated. Here, we examine the changes in the glutathione pool after stress exposure and quantify the effect of glutathione depletion on cell growth and stress tolerance. Depletion of glutathione repressed growth and release of zoospores, suggesting that glutathione is essential for life cycle completion in *Bd*. Supplementation with <2 mM exogenous glutathione accelerated zoospore development, but concentrations >2 mM were strongly inhibitory to *Bd* cells. While hydrogen peroxide exposure lowered the total cellular glutathione levels by 42 %, glutathione depletion did not increase the sensitivity to hydrogen peroxide. Exposure to cadmium increased total cellular glutathione levels by 93 %. Glutathione-depleted cells were more sensitive to cadmium, and this effect was attenuated by glutathione supplementation, suggesting that glutathione plays an important role in cadmium tolerance. The effects of heat and salt were exacerbated by the addition of exogenous glutathione. The impact of glutathione levels on *Bd* stress sensitivity may help explain differences in host susceptibility to chytridiomycosis and may provide opportunities for synergistic therapeutics.

1. Introduction

Batrachochytrium dendrobatidis (*Bd*) [1] is a chytridiomycete pathogen that has decimated amphibian species worldwide since its emergence in the 1970s [2]. The motile zoospores infect the host epidermis and develop into zoosporangia, which subsequently produce discharge tubes to release new infective zoospores [3]. The resulting disease, chytridiomycosis, disrupts amphibian skin and causes electrolyte depletion, leading to death of the host by cardiac arrest [4,5]. Chytridiomycosis is responsible for the extinction of at least 90 amphibian species worldwide, and severe population declines in hundreds more [2]. The success of this pathogen is likely due to its ability to evade the host immune system via the production of immunosuppressant molecules [6,7]. However, identification of other virulence factors has been limited by the lack of molecular biology tools developed for this taxa [8]. Confirming virulence factors is an important step in the development of antifungal interventions such as targeted drugs, vaccines and beneficial microbes; all of which currently have limited efficacy against chytridiomycosis [9–11].

One potential source of fungal virulence is the glutathione antioxidant system, as *in silico* studies suggest that *Bd* acquired several glutathione-associated genes via horizontal gene transfer [12]. Glutathione is a thiol tripeptide (containing glutamate, cysteine and glycine) [13], which exists in either a reduced state (GSH) or as an oxidised dimer (glutathione disulfide, GSSG). Glutathione synthesis is a two-step process occurring in the cytosol [14,15]. The first, rate-limiting step is catalysed by glutamate-cysteine ligase (GCL), and is transcriptionally regulated by the transcription factors yAP-1p [16], and Met4 [17]. Excess glutathione creates a feedback loop that inhibits Met4, allowing precise control over glutathione levels [17]. The second synthesis step requires the

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enzyme glutathione synthetase (GS), which forms glutathione by adding glycine to γ-glutamylcysteine [18–20].

Glutathione has antioxidant properties that are not only important for normal cellular metabolism [19], but also for pathogen virulence as it can neutralise oxidative stressors generated by host immune cells [21,22]. Phagocytic immune cells, such as macrophages [23] and neutrophils [24], produce reactive oxygen species (ROS), including hydrogen peroxide, to destroy fungal pathogens within the phagosome [25]. Glutathione can protect some fungal species from this oxidative stress, allowing them to survive phagocytosis [26]. As such, pathogenic fungi often show higher tolerance to oxidative stress compared to non-pathogenic species [27]. Although phagocytosis of *Bd* has not been observed by histology, and infection appears to induce negligible cellular reaction [28], zoosporangia could be exposed to ROS within epidermal cells [29] which may be associated with the apoptosis observed in infected cells [30], or exposed via epidermal mast cells which are linked to increased host resistance [31]. *Bd* may also be exposed to ROS in its free-living phase, for example solar UV causes oxidative stress in other fungi [32]. Investigating how chytrid parasites deal with ROS has been designated as a priority research area [33]. The function of the glutathione system in *Bd* and how it relates to ROS is unknown, but an association with oxidative stress has been suggested by the change in glutathione protein modifications after exposure to hydrogen peroxide [34]. In other fungi, glutathione can neutralise hydrogen peroxide directly [19], or in a reaction catalysed by glutathione peroxidase (GPx) [35]. Both processes result in the oxidation of glutathione, which is then recycled back into the reduced form by glutathione reductase (GR) [36,37]. Disruption or inhibition of GCL, GR, or GPx can reduce oxidative stress tolerance and therefore virulence in a variety of fungal species including *Candida albicans* [38], *Magnaporthe oryzae* [39], and *Alternaria alternata* [40], highlighting the importance of the glutathione system for pathogenicity. Here we aim to determine if *Bd* responds to ROS via a similar process, and whether inhibition of glutathione synthesis has the potential to decrease virulence via a reduction in oxidative stress tolerance.

In addition to combatting host defences, glutathione has also been shown to protect fungi from environmental stressors, including heavy metal, heat, osmotic and chemotherapeutic stress. However, the role of glutathione in *Bd* has not yet been investigated. Glutathione is commonly involved in the sequestration of heavy metals, especially cadmium (Cd) [19]. Glutathione can remove Cd by either forming a direct complex, or by serving as a precursor for a phytochelatin complex, before exportation to a vacuole [41,42]. In various fungi, GCL mutants display increased susceptibility to Cd [43–46], as do fungi exposed to buthionine sulfoximine (BSO), a chemical inhibitor of GCL [47,48]. In many fungi, glutathione can also protect against heat shock [37,49]. The thermal optimum of *Bd* is 17–25 °C [50,51], although *Bd* can survive brief heat pulses of 26–29 °C [52]. It is not known if glutathione is involved in temperature tolerance in *Bd*. In addition to a narrow temperature range, *Bd* is also sensitive to salinity, and only occurs in freshwater environments [53]. Finally, glutathione has also been associated with an increased resistance to antifungal drugs [54]. A wide range of antifungals are active against *Bd*, including terbinafine, voriconazole and itraconazole [55,56].

Glutathione could shield *Bd* from stressors encountered both within and outside the host, contributing to both virulence and environmental persistence. If this is the case, and glutathione is important for stress tolerance in *Bd*, the pathway could be an attractive target for the development of novel therapies for chytridiomycosis. In this study, we use BSO to inhibit glutathione synthesis in *Bd*, and explore the impact of glutathione deficiency on growth as well as tolerance to oxidative, heavy metal, chemotherapeutic, osmotic and heat stress.

2. Methods

2.1. Culture of B. dendrobatidis

Bd cultures (Yanchep-L.moorei-2019-RW and Werribee-L.dumerilli-2021-LB.) were isolated from naturally infected Australian frogs [57,58], and maintained in a tryptone, gelatin hydrolysate and lactose media (TGhL) at 20 °C in tissue culture flasks, as per standard protocols [59]. Pure zoospore suspensions were obtained by removing the media from mature culture flasks and incubating the zoosporangia monolayer with fresh media for 2 h, after which the solutions were filtered with a sterile isopore PC 10 μM filter and concentrated by centrifugation [60].

2.2. Growth and zoospore quantification

Bd growth was quantified using a microbial cell viability assay (BacTitre-Glo kit Promega G8230) measuring ATP [61,62]. Experiments were performed in 96 well plates by adding 5×10^4 zoospores per well with overnight incubation to allow development into zoosporangia before treatment. Cells were lysed and transferred to a white luminescence 96 well plate as per the manufacturer's instructions, and growth was calculated from the blank corrected relative light units (RLU) as relative to the average control value. Zoospore production was quantified by removing a 10 μL aliquot from each replicate well and counting active zoospores on a hemocytometer and calculated relative to the average control value. Zoosporangia size was determined by taking representative images of each well and measuring the area of the 10 biggest zoosporangia per well using image J and calculated relative to the average control value [63].

2.3. Depletion of cellular glutathione by buthionine sulfoximine

To evaluate whether BSO depletes cellular glutathione, 200 μL of actively growing *Bd* culture (containing a mixed population of zoospores and zoosporangia) was added to a 96 well cell culture plate and incubated for 8 h to allow adherence. DL-BSO (Sigma) was prepared as a filter-sterilised 200 mM solution in water. After 8 h, excess TGhL media was removed, 100 μL fresh media added, and 100 μL BSO solution added to produce final concentrations ranging from 2 to 30 mM, with two replicates per concentration. After 20 h BSO exposure, the total glutathione level of the adhered zoosporangia were estimated using a luminescence assay (Promega GSH:GSSG Glo V6611). Cells were lysed and transferred to a white luminescence 96 well plate as following the manufacturer's instructions for adherent cells. The relative amount of total glutathione in the BSO treated wells was calculated compared to the control. BSO at 30 mM resulted in the greatest depletion of total glutathione and was therefore chosen for the remaining experiments.

The duration of glutathione depletion was established by measuring total glutathione after 6, 24 and 48 h of exposure to BSO. Zoospores (100 μL) were added to a 96 well cell culture plate at 5×10^4 zoospores per well and incubated overnight. Excess TGhL media was removed, and cells were incubated with 100 μL of fresh media and 100 μL BSO solution or water. After 6, 24 and 48 h, total glutathione was measured as described earlier with 3 replicates per treatment per time point. Zoosporangia size was also measured to account for differences in total glutathione due to the smaller size of BSO exposed cells. Zoosporangia size at each time point was estimated from the two-dimensional cell surface area in three representative images using Image J [64]. Relative glutathione was

calculated relative to the control for that time point and adjusted for differences in cell growth caused by BSO exposure.

An additional experiment determined if BSO inhibits glutathione synthesis in zoospores. A concentrated zoospore solution was split into 4 sterile 1.5 mL tubes containing 1 million zoospores each. Two replicates were exposed to either 30 mM BSO, or water. After incubation at 20 °C for 10 h, zoospores were pelleted by centrifugation and re-suspended in water, and total glutathione levels were measured using a luminescence assay (Promega GSH:GSSG Glo V6611). Cells were lysed and transferred to a white luminescence 96 well plate as following the manufacturer's instructions for cell suspensions.

2.4. Growth assays

The effect of exogenous glutathione on *Bd* growth and development was monitored by incubating zoosporangia with 0.5 mM–6 mM reduced glutathione, and measuring zoospore production and growth after 2 d. Zoospores (100 μL) were added to a 96 well cell culture plate at 5×10^4 zoospores per well and incubated overnight. Excess TGhL media was removed, and cells were incubated with 100 μL fresh media. Reduced glutathione (Sigma) was prepared as a filter sterilised 100 mM solution in water, and the appropriate amount added to three replicate wells per concentration (0.5, 1, 2, 4, 6 mM).

The effect of BSO mediated glutathione depletion on growth and development was determined by incubating zoospores with 30 mM BSO and measuring zoospore production and zoosporangia size relative to the control after 4 d. A second BSO treatment was included in which 2 mM glutathione was added after zoospore encystation to determine if this would counteract the effect of BSO exposure. Zoospores (85 μL) were added to a 96 well cell culture plate at 5×10^4 zoospores per well, with either 15 μL 200 mM BSO or 15 μL water with 6 replicate wells each. After 24 h, 2 μL of 100 mM glutathione was added to 3 BSO and 3 control wells. Zoosporangia size and zoospore quantity was calculated as above.

2.5. Stress assays

The changes in the glutathione pool in response to stress were monitored in zoosporangia exposed to a variety of stressors (Table 1). Zoospores (100 μL) were added to two 96 well cell culture plates at 5×10^4 zoospores per well and incubated at 20 °C to allow encystation. After 18 h, 3 replicate wells were exposed to each stressor (Table 1). Excess media was removed and replaced with 100 μL fresh TGhL, and 100 μL treatment solution (chemical stressors) or water (control and heat stress). Chemical stressors were freshly prepared in water and filter sterilised before addition to plate 1. Heat stress was applied by incubating plate 2 at 30 °C for 4 h, then returning to 20 °C. Exposure to chemical stressors occurred at T = 0 h, exposure to heat stress occurred at T = 6 h to T = 10 h. To account for differences in glutathione content due to cell size, zoosporangia were photographed at T = 24 h in order to estimate growth from the two-dimensional cell surface area in three representative images using Image J [64]. Reduced and oxidised glutathione was measured at T = 24 h using a luminescence assay (Promega), and calculated as relative to the untreated control, after adjusting for differences in cell growth caused by stress exposure. The ratio of oxidised and reduced glutathione was calculated using the relative light units (RLU) following manufacturer's instructions.

To evaluate the role of glutathione for stress tolerance, the glutathione content was manipulated before stress exposure. Zoospores were added to 96 well plates at 5×10^4 zoospores per well and incubated at 20 °C. After 18 h, cells were treated with either 30 mM BSO for 6 h, or 2 mM glutathione for 1 h prior to stressor exposure. Growth of cells were measured 24 h after stress exposure using the BacTitre-Glo assay as described earlier.

2.6. Statistical analysis

All statistical analyses were conducted using Graph Pad Prism (version 9.5.1). Student's T-test were used to compare the growth of zoosporangia exposed to BSO, GSH or BSO + GSH to the control. Simple linear regressions were used to plot dose dependent and time course decreases in glutathione after BSO exposure. Two-way ANOVA analysis (GraphPad Prism) was used to determine if the interaction between stress tolerance and BSO or GSH exposure was significantly different to the control.

3. Results

3.1. Depletion of cellular glutathione via BSO

GCL can be chemically inhibited using buthionine sulfoximine (BSO) [68]. In other fungi, glutathione synthesis is inhibited using BSO at concentrations ranging from 2 to 6 mM [48,69–71], but its effectiveness in *Bd* was not known. The addition of BSO to growth media caused a dose dependent decrease in total glutathione levels after 20 h. The greatest decrease was seen in cells exposed to 30 mM BSO (70 % decrease in total glutathione) (Fig. 1), and this concentration was chosen for further experiments. The effect of BSO on zoosporangia lasted for at least 48 h (Fig. 1). However, BSO appeared to have only a minor effect on zoospores, with <20 % decrease in total glutathione after 10 h (Fig. 1).

3.2. Glutathione and growth

Exogenous glutathione increased growth and zoospore production at low concentrations but was strongly inhibitory at concentrations above 2 mM (Fig. 2).

BSO mediated glutathione depletion repressed zoosporangia growth and development (Fig. 3). BSO exposed zoospores developed into zoosporangia that were significantly smaller than the control (p=<0.0001) and failed to complete their life cycle. Supplementation with 2 mM glutathione restored the growth of BSO

Table 1
Sub-lethal stressor concentrations used in this study.

Stress	Conditions	Rationale
Oxidative	0.2 mM Hydrogen peroxide (H_2O_2)	Proxy for host immune response [24]
Heavy metal	0.04 mM Cadmium (Cd)	Some heavy metals have been shown to inhibit *Bd* growth [65,66]
Chemotherapeutic	0.1 μg/mL Terbinafine hydrochloride (TBF)	Used as a treatment for chytridiomycosis in amphibians [56]
Osmotic	0.1 M Sodium chloride (NaCl)	*Bd* is sensitive to NaCl [53], and increasing habitat salinity has been suggested to provide refugia from chytridiomycosis [67]
Heat	1 × pulse of 30 °C for 4 h	*Bd* has optimal growth at 15–25 °C, and frogs subjected to daily 4 h pulses of 29 °C can clear infection [52]

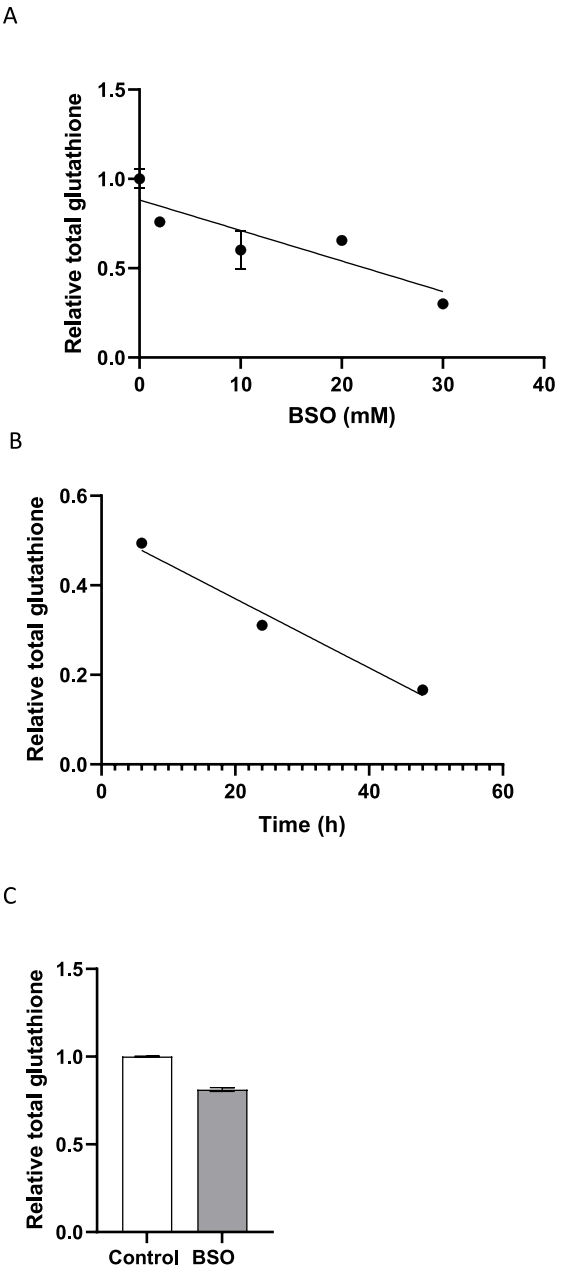

Fig. 1. Total glutathione levels in a mixed stage culture after BSO treatment. **A.** BSO causes a dose-dependent decrease in total glutathione levels in zoosporangia after 20 h incubation. Linear regression analysis, mean and SD shown for 2 replicates per treatment. **B:** Total glutathione levels in zoosporangia after 6, 24 and 48 h treatment with 30 mM BSO. BSO exposed cells had 51 % less total glutathione at 6 h, 69 % less at 24 h, and 84 % less at 48 h compared to the untreated control at the same timepoint, after adjustment for growth. Linear regression analysis, mean and SD shown for 3 replicates per treatment. SD error bars are below the limit for visualisation. **C.** Total glutathione in zoospores after 10 h treatment with 30 mM BSO. Mean and SD shown for 2 replicates per treatment.

exposed cells. Glutathione supplementation increased zoospore production by more than 15 fold, regardless of BSO exposure (GSH $p = 0.0147$, GSH + BSO $p = 0.0261$).

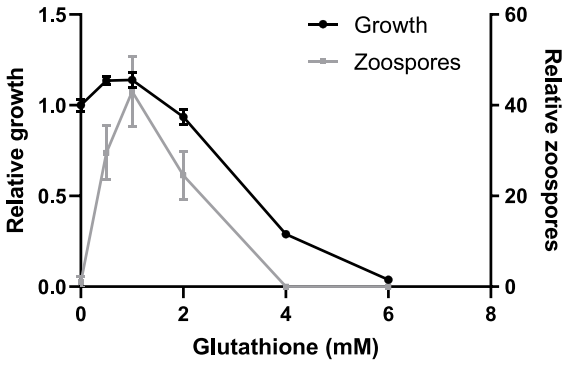

Fig. 2. Effect of glutathione on growth and zoospore production. The addition of 0.5–1 mM exogenous glutathione to young zoosporangia slightly increased growth and dramatically increased zoospore production. Whereas concentrations above 2 mM were strongly inhibitory.

3.3. Glutathione levels in response to stressor exposure

Glutathione levels varied after exposure to different stressors (Fig. 4). Treatment with H_2O_2 decreased total glutathione levels by 42 % ($p < 0.001$), whereas cells exposed to Cd had a 93 % increase in glutathione levels compared to the control ($p = <0.0001$). Exposure to TBF, NaCl and heat caused only slight changes to the total glutathione pool. The ratio of reduced (GSH) and oxidised (GSSG) glutathione also changed depending on the type of stress (Fig. 4). Control untreated zoosporangia had an average GSH:GSSG ratio of 6.44, consistent with a reduced intracellular environment. Cells exposed to heat stress had a significantly lower GSH:GSSG ratio ($p < 0.0001$) whereas cells exposed to H_2O_2, Cd and NaCl had a slight, but significant increase in GSH:GSSG ratio. The GSH:GSSG ratio of cells exposed to TBF fungicide was not significantly different to the control. Unstressed zoospores had a GSH:GSSG ratio of 5.99, which is slightly lower than that of zoosporangia.

3.4. Stress tolerance with glutathione depletion or supplementation

The role of glutathione during stress tolerance was examined under conditions of glutathione depletion (via BSO) or glutathione (GSH) supplementation. Long-term exposure to 30 mM BSO caused some growth inhibition (Fig. 3), but this concentration was necessary for efficient glutathione depletion. Exposure to glutathione at 0.5–1 mM caused an increase in growth, whereas ≥2.5 mM caused immediate growth inhibition (Fig. 2). Therefore, depletion and supplementation experiments were performed with 30 mM BSO and 2 mM GSH (Fig. 5). Cells were incubated with 30 mM BSO for 6 h to ensure total glutathione levels were depleted by ~50 % prior to stress exposure. Supplementation with 2 mM glutathione occurred 1 h before stress exposure as a precautionary measure in case it triggered a reduction in glutathione synthesis via the Met4 feedback loop. Glutathione depletion dramatically increased sensitivity to Cd, with a ~80 % decrease in growth in cells exposed to BSO + Cd vs Cd alone ($p < 0.0001$). Glutathione depletion did not affect tolerance to H_2O_2, TBF, NaCl or heat. Supplementation with exogenous glutathione improved growth under Cd stress ($p < 0.001$), and glutathione also partially reversed the effect of BSO treatment in Cd stressed cells (Fig. S1). Conversely, glutathione supplementation increased sensitivity to H_2O_2 ($p < 0.05$), TBF ($p < 0.01$), NaCl ($p < 0.001$) and heat stress ($p < 0.01$). Assessment of

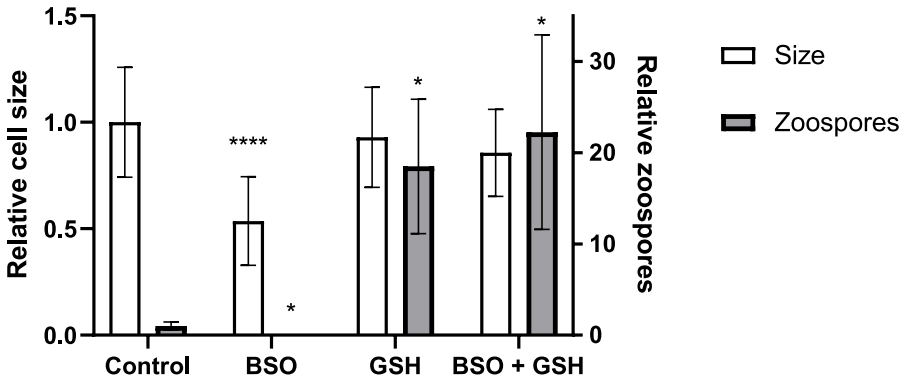

Fig. 3. Zoosporangia size and zoospore production after incubation with BSO and/or glutathione. Cells incubated with BSO for 72 h were significantly smaller and did not produce zoospores during the time they were monitored. Cells supplemented with glutathione were normal in size and displayed increased zoospore production. Glutathione supplementation also mitigated the effects of BSO on growth (BSO + GSH) as well as increasing zoospore production. Mean and SD shown for 3 replicate wells per treatment.

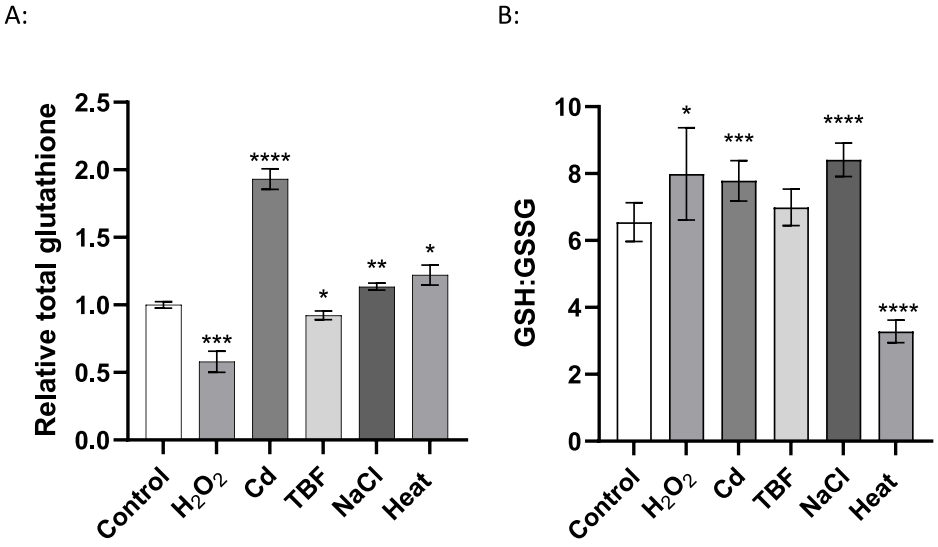

Fig. 4. Total glutathione (A) and GSH:GSSG (B) ratios after stressor exposure. A: Cells exposed to 0.2 mM H_2O_2, had 42 % less total glutathione, whereas cells exposed to 0.04 mM Cd had 93 % more total glutathione. Treatment with 0.1 μg/mL TBF, 0.1 M NaCl and heat slightly altered the total glutathione levels compared to the control. B: Heat exposure reduced the GSH:GSSG ratio by 50 %, whereas treatment with 0.2 mM H_2O_2, 0.04 mM Cd and 0.1 M NaCl slightly increased the GSH:GSSG ratio compared to the control. Mean and SD shown for 3 replicates per treatment.

microscopic cell morphology revealed that the effect of glutathione exacerbation was most obvious in NaCl and heat stressed cells (Fig. S2).

4. Discussion

By inhibiting glutathione synthesis using BSO we show that glutathione is necessary for growth and maturation of *Bd* zoosporangia. By measuring reduced and oxidised glutathione levels after stress exposure we demonstrate that glutathione is involved in the response to Cd, H_2O_2, and acute heat stress in *Bd*.

4.1. Glutathione depletion and supplementation

The GCL inhibitor, BSO, effectively depleted glutathione levels in *Bd* zoosporangia by 84 % after 48 h. However, this required a BSO concentration of 30 mM, much more than the 2–6 mM commonly used in other fungi. BSO was only minimally effective on zoospores, perhaps due to their lack of transcriptional activity [72]. Zoospores exposed to 30 mM BSO were able to encyst and form zoosporangia, however, these grew slowly and failed to develop discharge tubes and produce more zoospores. Hence glutathione appears important for completion of the lifecycle in *Bd* and establishes glutathione biosynthesis as a potential target to decrease virulence.

Supplementing *Bd* cells with exogenous glutathione was beneficial to cell growth at low concentrations, but sharply transitioned to inhibitory at concentrations above 2 mM. Interestingly, adding 0.5–1 mM exogenous glutathione increased zoospore production, in both normal and glutathione-depleted cells. This did not appear to be due to rapid growth, as the zoosporangia did not differ in size to the controls. Rather, glutathione appeared to stimulate the early development and release of zoospores. Future work could investigate how glutathione triggers early zoospore production (e.g. via a more reduced extracellular environment or via changes in glutathionylation of key proteins) and whether there is a trade off with zoospore size or number. In bacterial pathogens, host-derived

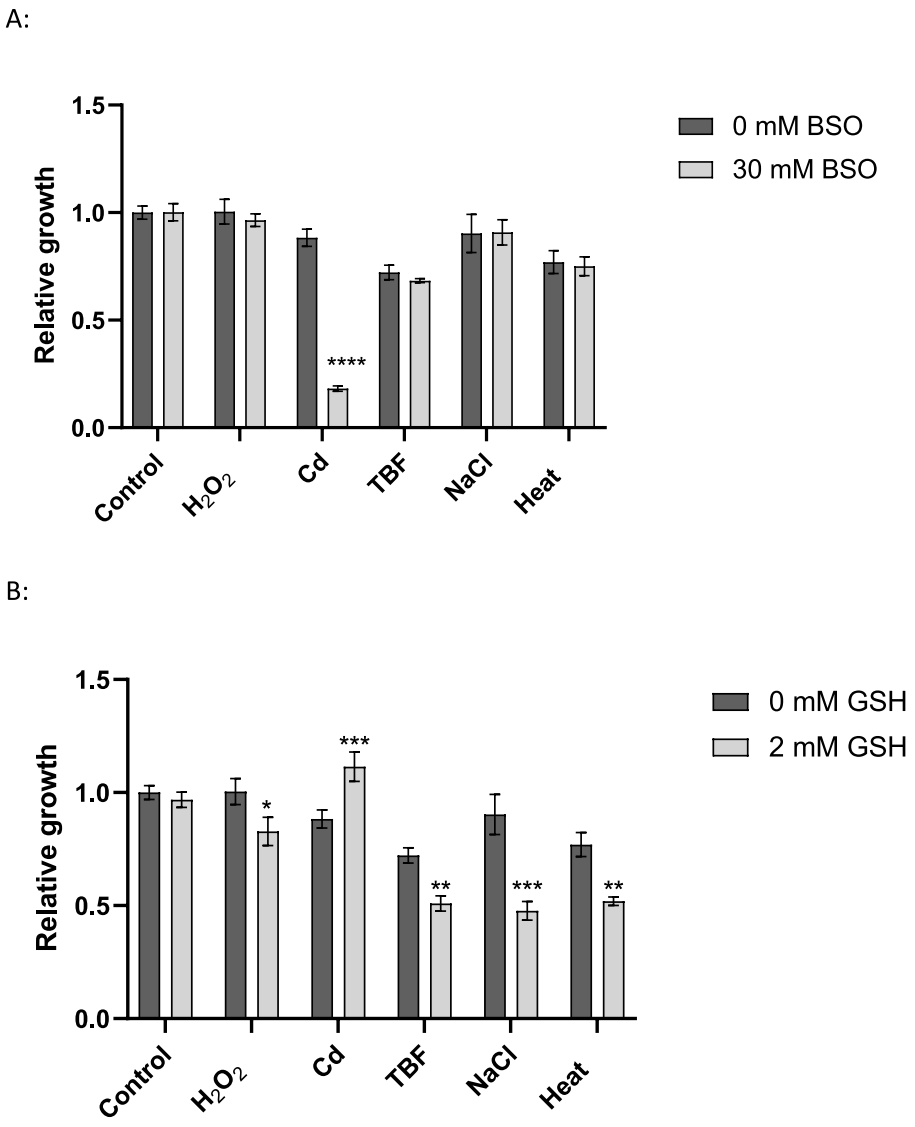

Fig. 5. Relative growth of stressed zoosporangia under either glutathione depletion (via 30 mM BSO) or glutathione (GSH) supplementation. A: Glutathione depletion increased sensitivity to Cd stress, but did not affect tolerance of H_2O_2, TBF, NaCl or heat stress. B: Glutathione supplementation improved the growth under Cd stress, but increased the sensitivity to H_2O_2, TBF, NaCl and heat stress. Growth measured using BacTitre-Glo, and mean and SD shown for 3 replicates per treatment.

glutathione can activate expression of virulence factors as well as the switch to pathogenic life stages, and there is some evidence of glutathione serving as a modulator of virulence in fungi [73]. The accelerated production of infective zoospores following exogenous glutathione exposure raises the possibility that *Bd* may also respond to glutathione in a similar manner.

4.2. Cadmium tolerance

Glutathione is important for Cd resistance in *Bd*. Exposure to Cd doubled the relative total glutathione pool compared to unstressed cells. This response is consistent with that seen in other fungal species, as exposure to Cd increases GCL expression and a corresponding increase in glutathione in *S. cerevisiae* [74] and *Laccaria bicolor* [46]. In *Bd*, glutathione depletion (via BSO) attenuated growth in Cd treated cells, an effect that was reversed by glutathione supplementation. Unlike oxidative stress, Cd detoxification removes GSH from the available pool when the glutathione conjugates irreversibly with Cd and is exported to a vacuole [75], making the process more susceptible to glutathione depletion. Further immunohistochemistry studies could localise Cd in *Bd*, to confirm sequestration within the vacuole [76].

4.3. Hydrogen peroxide tolerance

Total glutathione levels decreased in response to H_2O_2 treatment, suggesting an involvement of glutathione in oxidative stress tolerance in *Bd*. The decrease in total glutathione is consistent with the oxidative stress response of other fungi such as *Candida albicans* [54] and *Ashbya gossypii* [77]. However, in these species, oxidative stress induced a corresponding decrease in the GSH:GSSG ratio, which was not observed in *Bd*. This difference may be due to the limited H_2O_2 concentrations used in this experiment, or could also be due to an increased GR activity, resulting in rapid conversion of GSSG (produced during H_2O_2 neutralisation) back into GSH. In other fungi species, exposure to H_2O_2 has been shown to upregulate GR activity [27] or *GR* expression [78]. Unexpectedly, glutathione depletion (via BSO treatment) did not increase the

sensitivity of *Bd* to 0.2 mM H_2O_2, unlike reports from other fungi [38,79,80]. The experimental design of this experiment included a 6 h BSO incubation prior to stress exposure, which the results of our BSO time course experiments suggest should result in 50 % reduction in total glutathione at the time of stress exposure. It is possible that the remaining glutathione was sufficient for H_2O_2 detoxification, or that the inhibition of GCL by BSO was partially mitigated by the H_2O_2-mediated release of GSH from protein reservoirs, such as enolase [34]. Alternative antioxidant mechanisms, such as superoxide dismutases, catalases, or the thioredoxin system, may also act without glutathione to detoxify H_2O_2 [37,79,81,82]. Future work should aim to distinguish whether *Bd* can operate with minimal glutathione levels, or whether compensatory antioxidant mechanisms are employed for the tolerance of oxidative stress, as this may have implications for *Bd* virulence. In summary, although glutathione is consumed during oxidative stress, the high GSH:GSSH ratio suggests that glutathione may not be utilised in a glutathione peroxidase reaction nor are normal levels of glutathione essential for tolerance of hydrogen peroxide in *Bd*. Further work is required to understand how glutathione is utilised in the oxidative stress response of *Bd*.

4.4. Tolerance of other stressors

In other fungi, heat stress can increase cellular respiration, resulting in ROS accumulation and an oxidative stress response [49,83]. In *Bd*, while heat exposure slightly increased the relative level of total glutathione, it halved the GSH:GSSG ratio. The low GSH:GSSG ratio suggests that GSH oxidation is part of the heat stress response in *Bd*, as also seen in *Aspergillus nidulans* [37]. Exposure to heat pulses can allow frogs to overcome *Bd* infection [52], therefore characterising the role of glutathione in heat stress tolerance could be important for understanding infection dynamics.

NaCl stress slightly increased the GSH:GSSG ratio and the total glutathione levels. This result is consistent with studies in the plant *Arabidopsis*, where NaCl exposure increased glutathione levels [84], and upregulated *GR* in *S. pombe* [85]. However, in *Arabidopsis*, the addition of exogenous glutathione improved NaCl tolerance [84], whereas in our study, supplementation with glutathione increased the sensitivity to NaCl.

Amphibian skin itself contains glutathione and other antioxidants thought to protect against environmental stressors, such as UV [40]. We found that glutathione supplementation increased sensitivity of *Bd* to some stressors such as heat stress. Thus, amphibian-produced cytosolic glutathione might have a protective effect against early *Bd* invasion, especially for amphibians living in warm environments.

In summary, glutathione is an important molecule for normal cell growth and cadmium tolerance in *Bd*. Glutathione depleted cells were unable to grow in the presence of cadmium. Glutathione also appears to be involved in, but not crucial for, the response to oxidative and heat stress. Excess glutathione increased zoospore production in unstressed cells, but inhibited the growth of cells exposed to osmotic and heat stress. These results suggest that glutathione availability has wide ranging implications for *Bd*, as it affects development and zoospore production, and can influence *Bd* growth in adverse conditions.

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Author contributions

RJW and AAR conceived this project, RJW completed the experimental work, all authors (RJW, CR, LB, LFS and AAR) analysed the data and wrote the manuscript.

Declarations of interest

None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biochi.2023.12.002.

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