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Susceptibility of frogs to chytridiomycosis correlates with increased levels of immunomodulatory serotonin in the skin

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Abstract

Chytridiomycosis, caused by the fungus *Batrachochytrium dendrobatidis* (*Bd*), is a skin disease responsible for the global decline of amphibians. Frog species and populations can vary in susceptibility, but this phenomenon remains poorly understood. Here, we investigated serotonin in the skin of infected and uninfected frogs. In more susceptible frog populations, skin serotonin rose with increasing infection intensity, but decreased in later stages of the disease. The more resistant population maintained a basal level of skin serotonin. Serotonin inhibited both *Bd* sporangial growth and Jurkat lymphocyte proliferation *in vitro*. However, serotonin accumulates in skin granular glands, and this compartmentalisation may prevent inhibition of Bd growth *in vivo*. We suggest that skin serotonin increases in susceptible frogs due to pathogen excretion of precursor tryptophan, but that resistant frogs are able to control the levels of serotonin. Overall, the immunosuppressive effects of serotonin may contribute to the susceptibility of frogs to chytridiomycosis.

1 | INTRODUCTION

The fungus *Batrachochytrium dendrobatidis* (*Bd*) causes the disease chytridiomycosis, which has caused the global decline and extinction of numerous amphibian species (Skerratt *et al.*, 2007). Fungal sporangia grow within amphibian epidermal skin cells, and cause hyperplasia, hyperkeratosis, and inhibition of cutaneous ion transport, leading to cardiac arrest (Voyles *et al.*, 2009). Species vary greatly in their susceptibility to *Bd*, ranging from 100% mortality to those that can clear infection, while

others can be resistant reservoirs (Rollins-Smith *et al.*, 2009, Berger *et al.*, 2016, Miaud *et al.*, 2016, Scheele *et al.*, 2016, Brannelly *et al.*, 2018). Recent studies have shown population level differences in susceptibility within species (Bataille *et al.*, 2015). Metabolomic analysis of skin from infected and uninfected alpine tree frogs *Litoria verreauxii alpina* (listed as vulnerable in Australia) from these populations revealed a correlation between serotonin (5'-hydroxytryptamine) identified by gas chromatography-mass spectrometry and the progression of *Bd* infection (Grogan *et al.*, 2018c). Understanding variation in susceptibility is necessary to improve the survival of vulnerable species.

Differential susceptibility (mortality after infection) might correspond to the ability of the host to develop a robust immune response to *Bd* (Bataille *et al.*, 2016, Grogan *et al.*, 2016). Pathogen-induced suppression of lymphocytes is believed to reduce the effectiveness of this response (Fites *et al.*, 2013, Ellison *et al.*, 2014, Young *et al.*, 2014). The immunosuppressive effect of *Bd* is likely due to the secretion of various metabolites, including kynurenine (Kyn), methylthioadenosine (MTA) and spermidine (a biogenic amine), which cause lymphocyte apoptosis *in vitro* (Rollins-Smith *et al.*, 2015a, Rollins-Smith *et al.*, 2019). Kynurenine and its amino acid precursor tryptophan (Trp) were detected in *Bd* supernatants, with Trp being one of the most abundantly excreted molecules (Rollins-Smith *et al.*, 2015a). MTA is a produced from the methionine salvage pathway (Albers, 2009). The fate of pathogen-excreted metabolites, such as Trp, has not been explored *in vivo*.

Frogs must obtain Trp from their diet as they are unable to biosynthesise Trp. Frogs can metabolize Trp into serotonin, 5'-hydroxytryptophan, tryptamine, and 5'-hydroxy 3'-indoleacetic acid (5'HIAA). In contrast, although *Bd* produces Trp (Rollins-Smith *et al.*, 2015a), the pathogen lacks the enzymes to metabolise it into serotonin (Fig S5).

Serotonin occurs within the skin of many vertebrates, including frogs and fish (Fasulo *et al.*, 1993), and has been suggested to have antifungal activities against *Candida albicans* and fungal plant pathogens (Lass-Flörl *et al.*, 2003, Mayr *et al.*, 2005, Du Fall *et al.*, 2013). The role of serotonin in immune function is complex, with conflicting functions proposed in the existing literature (Herr *et al.*, 2017b). Serotonin can be synthesised by mast cells and T-cells, is secreted by platelets during inflammation, and can be taken up by macrophages, dendritic cells, and B-cells (Ahern, 2011, Herr *et al.*, 2017a). The immunomodulatory functions of serotonin include: controlling cytokine secretion by macrophages (Sternberg *et al.*, 1986), inducing proliferation of T-cells (Leon-Ponte *et al.*, 2007), and promoting apoptosis of Burkitt lymphoma cells (Serafeim *et al.*, 2002).

There is a correlation between the levels of *L. v. alpina* skin serotonin and disease progression (Grogan *et al.*, 2018c), however, the mechanism of serotonin's effect on the host-pathogen interaction remains unknown. In this study, we sought to investigate the role of serotonin in frog immunity and its effect on the pathogen, *Bd*. We hypothesized that frogs use serotonin as an immune defence against *Bd*. Our objectives were (i) to determine serotonin levels over the course of *Bd* infection and

correlate this to pathogen load and host susceptibility, (ii) to determine the location and abundance of serotonin in frog skin, (iii) to determine the effects of serotonin and structural analogues on *Bd* and lymphocytes *in vitro*.

2 | RESULTS

2.1 | Serotonin levels change over the course of infection

Firstly, we confirmed the identity of serotonin in the skin of *Bd*-infected frogs (Grogan *et al.*, 2018b, Grogan *et al.*, 2018c) using accurate-mass mass spectrometry (Ultra-Performance Liquid Chromatography-quadrupole-Time of Flight-mass spectrometry; UPLC-qTOF-MS) with comparison to a metabolite reference standard (Figure S1A). The full model including all explanatory variables, treatment effect (infected versus control), population of origin, pathogen burden (infection load), duration of infection (days of the experiment), and their interactions was significant $p < 0.001$ and had an $R^2 = 0.60$. However, of the explanatory variables only infection load was significant, $p < 0.001$. It positively covaried with serotonin levels. When infection load was excluded from the model then only days of the experiment significantly covaried, $p = 0.01$, but $R^2 = 0.31$. Step-wise elimination of non-significant variables and their interactions led to infection load and population of origin as being the only significant explanatory variables, $p < 0.001$ and $p = 0.04$, The estimated marginal means for serotonin levels among frogs from differing populations of origin differed between population E (long exposed), serotonin = 0.021^{a}, and those from population K (longer surviving in infection experiments than G and E, long exposed) and population G (naïve), which were similar,

serotonin = 0.011[a]. Covariates[a] appearing in the model were evaluated at infection load = 6011.85 zoospore equivalents. $R^2 = 0.59$. The lack of fit test for the model was significant $p = 0.01$. This was because the residuals for serotonin levels from the model increased as serotonin levels increased. This likely reflects activation of more biochemical pathways and feedback loops as the infection progresses disrupting the linear relationship. The fit of the model was improved by taking the square root of serotonin levels, $p = 0.4$. However, $R^2 = 0.54$, infection load and population of origin remained significant explanatory variables, $p < 0.001$ and $p = 0.01$, respectively.

Our results demonstrated that there were two types of serotonin responses to *Bd* fungal infection. Serotonin concentrations increased over the course of infection in the more susceptible (higher mortality rates) Populations E and G, demonstrating a 4.8-fold increase in skin serotonin in the first 14 days (Figures 1A and 1B). In contrast, the more resistant (longer surviving) Population K, had no significant change in serotonin levels over the course of the infection ($R^2 = 0.0526$, Figure 1C). We found a positive correlation between subclinical serotonin levels and pathogen burden in the susceptible populations for the first 14 days of infection (population E, $R^2 = 0.4584$; population G, $R^2 = 0.6139$, Figure 2B). The 'baseline' or mean concentration of serotonin for control animals was 0.0091 relative units. The linear models could not be fitted if day 28, when frogs developed clinical signs, was included. This was because serotonin levels decreased dramatically, by three orders of magnitude in conjunction with severe disease and frogs becoming moribund. One-way analysis of variance showed that serotonin levels differed among days post-exposure for infected frogs, $p = 0.028$. A non-

parametric Kruskal-Wallis test was also significant, p = 0.001. Bonferroni post hoc tests suggested that the main difference occurred between days 14 and 28, p = 0.039.

2.2 | Location of serotonin in skin and changes in levels with infection status

Immunohistochemistry showed that serotonin in the skin was concentrated in granular skin glands and the linear mixed model showed that dorsal glands had more serotonin than ventral ($F_{1, 156}$ = 4.365, p = 0.041). There was an accumulation of serotonin in uninfected control frogs at day 35 (Figure 3A), but no serotonin was visible in the frogs moribund with chytridiomycosis ($F_{1, 156}$ = 10.422, p = 0.002) (day 35, Figure 3B). There was no significant interaction between granular gland location and infection status for serotonin ($F_{1, 156}$ = 0.031, p = 0.861).

2.3 | Serotonin inhibits *in vitro* growth of *Bd* sporangia

Serotonin inhibited the growth of *Bd in vitro* (Figure 4A) and decreased the sporangial size (Figures 4A and 5). The average absorbance of Bd cultures also decreased with increasing concentrations of serotonin (Figure 7). Serotonin caused a significant reduction in sporangial size at concentrations equal to and above 0.3125 mM (p < 0.002). At 0.3125 mM and 1.25 mM serotonin, sporangial size was decreased by 30% and 60%, respectively. The minimum inhibitory concentration (where *Bd* was unable to release zoospores to complete its life cycle) of serotonin that inhibited the development of *Bd* sporangia was 1.5-2 mM. Similar results were observed for both strains of *Bd*

tested, where both strains were from the phylogenetically-related global panzootic lineage (O'Hanlon *et al.*, 2018).

In structure-activity relationship assays, serotonin structural analogues were tested for their ability to inhibit *Bd* growth (Figure 4). We tested *Bd*-produced analogues including Trp (Figure S1B) and Kyn; associated frog metabolites such as tryptamine, 5-hydroxy 3-indole acetic acid (5'-HIAA), and 5-hydroxytryptophan; as well as structurally similar histamine (produced from histidine) (Fig S5). Frog metabolites histamine, tryptamine, and 5'-HIAA inhibited *Bd* sporangial growth, while 5-hydroxytryptophan did not. The greatest decrease in sporangial size occurred with tryptamine, which had a similar effect on growth to serotonin (Figure 4A-4B). The endogenous *Bd*-produced metabolite analogues, Trp and Kyn did not affect growth. The addition of either 10 mM Trp or Kyn did not restore *Bd* growth in the presence of increasing serotonin concentrations (Figure 5A). Other biogenic amines were also tested as potential inhibitory agents of *Bd*. Putrescine, spermidine, and spermine (which are metabolically related to *Bd* immunosuppressant MTA) inhibited sporangial growth, while ornithine did not (Figure 4C).

2.4 | Serotonin inhibits *in vitro* growth of Jurkat lymphocytes

The human Jurkat lymphocyte cell line was used as a proxy to test the effect of serotonin on amphibian lymphocytes, as in previous studies (Piovia-Scott *et al.*, 2014). Serotonin inhibited Jurkat lymphocytes at concentrations over 0.5 mM (Fig. 6), similar to the immunosuppressive effects of Kyn (Rollins-Smith *et al.*, 2015a).

3 | DISCUSSION

3.1 | Serotonin levels over the course of infection

We used host skin metabolomics to assess whether survival rates (Bataille *et al.*, 2015) correlated with skin serotonin levels. In populations with higher susceptibility to chytridiomycosis (Populations E and G), skin serotonin levels increased during the subclinical phase of infection, and were correlated to pathogen burden. However, in moribund frogs, serotonin levels dramatically decreased to basal pre-infection levels. In the more resistant Population K, serotonin levels remained at basal levels throughout the course of infection and were not correlated with infection burden.

3.2 | Localization of serotonin in frog skin

Immunohistochemistry indicated that serotonin is localised in granular glands within the frog skin, as shown previously in Marsh frogs (*Rana ridibunda*) (Sengezer-Inceli *et al.*, 2004). The lack of serotonin antibody staining in moribund frogs corresponds to the reduction of serotonin to sub-basal levels in our metabolomic time-course study. Serotonin is expressed in the sweat and sebaceous glands of psoriasis lesions in humans (Huang *et al.*, 2004), and might be excreted by epidermal mast cells to stimulate keratinocyte proliferation (Maurer *et al.*, 1997). Hence, it is possible that the hyperkeratosis observed with chytridiomycosis (Berger *et al.*, 2005) is mediated by increased levels of skin serotonin during the subclinical phase of the disease.

3.3 | The effects of serotonin *in vitro*

Although Trp is a metabolic precursor to serotonin, Trp did not inhibit *Bd* growth *in vitro*. To provide insight into the effect of Trp metabolism to serotonin on *Bd*, we undertook structure-activity relationship assays to determine which structural motifs within serotonin were involved in inhibition of cultured *Bd*. These results indicate that the terminal primary amine and the pyrrole ring in serotonin inhibits normal *Bd* growth, as histamine (which only possesses these motifs) had a similar effect on *Bd* as serotonin. Furthermore, 5-hydroxyindoleacetic acid (5'-HIAA), which does not contain a terminal primary amine, only inhibited sporangia at high concentrations. Given these results, conversion of Trp into tryptamine within the skin of frogs might also inhibit growth of *Bd*, whereas biotransformation to 5'-HIAA and the similar 5'-hydroxytryptophan are unlikely to affect *Bd.* Interestingly, high levels of 5'-HIAA were identified in the skin of moribund frogs (Grogan *et al.*, 2018c), indicating another important fate of *Bd*-secreted Trp in the skin of infected frogs. Other unrelated biogenic amines also inhibited *Bd* growth, including putrescine, spermidine and spermine, which are biosynthetic metabolites of *Bd*-excreted MTA (Rollins-Smith *et al.*, 2015b) . As spermidine affects cell proliferation (Kihara *et al.*, 1957) and controls the membrane potential of potassium, sodium, and calcium ATPases, future work should consider the effect of these biogenic amines on the susceptibility of frogs to chytridiomycosis.

We hypothesised that serotonin might inhibit *Bd* by mimicking metabolites within the Trp/Kyn biosynthetic pathway. Trp analogues inhibited Kyn production (a Trp metabolite) and hyphae development in *Candida albicans* (Bozza *et al.*, 2005).

However, supplementation with 10 mM Kyn or Trp in our study did not restore growth in the presence of increasing serotonin concentrations. Therefore, despite the structural similarities, serotonin does not seem to inhibit Trp or Kyn biosynthetic pathways in *Bd*. It is therefore unlikely that serotonin is mimicking Trp or Kyn precursors to competitively inhibit upstream metabolic enzymes. Despite the inhibition of Bd by serotonin *in vitro*, increasing levels of serotonin do not protect the frogs from Bd *in vivo*. It is possible that localization of serotonin in glands results in compartmentalization away from the pathogen.

Finally, Trp is an essential amino acid that frogs obtain from their diet. It is possible that increased skin serotonin during infection is due to a specific host response or due to increased Trp excreted from the rising burden of Bd, (Priya *et al.*, 2014, Rollins-Smith *et al.*, 2015a). As *Bd* has been shown to alter amphibian skin microbial communities (Jani *et al.*, 2018), Trp from other skin microbes may also contribute, but the relative abundance of Bd likely renders these sources negligible. As *Bd*-infected frogs lose appetite in late stages of disease, this may be an explanation for the final drop in serotonin.

3.4 | Immunosuppressive effects of serotonin

Serotonin inhibited the proliferation of Jurkat lymphocytes with a similar effect to the previously reported Kyn produced by *Bd* (Rollins-Smith *et al.*, 2015a). *Bd* cannot produce serotonin, only its precursor Trp. Frogs, on the other hand, do not possess the necessary biochemistry for the *de novo* synthesis of Trp (Figure S4), so the serotonin

precursor must be introduced exogenously (Table S3). As expected, serotonin was not detected from *Bd* cultures, but the precursor metabolites Trp (Figure S1B) and serine were identified (Grogan *et al.*, 2018c), consistent with previous reports (Rollins-Smith *et al.*, 2015a). Therefore, it is possible that the high levels of Trp secreted by *Bd* are metabolised into serotonin in the infected host, increasing host susceptibility to *Bd* due to immunosuppression. The immunomodulatory role of serotonin as seen in our study has been described in other organisms. Low concentrations of serotonin (50 μM) cause apoptosis and suppression of DNA synthesis in Burkitt lymphoma cells (Serafeim *et al.*, 2002). Serotonin has been shown to be involved in host defence against other fungal pathogens (Lass-Flörl *et al.*, 2003, Mayr *et al.*, 2005, Du Fall *et al.*, 2013). Despite serotonin inhibiting *Bd* growth *in vitro*, there was no correlation of serotonin to increased resistance of frogs to chytridiomycosis. In this host-pathogen interaction, it seems that the effect of elevated skin serotonin is more detrimental to the frog. Overall, these results indicate that increased skin serotonin in susceptible frogs is a response to infection and high levels of pathogen-secreted Trp. However, the ability of more resistant populations to maintain low serotonin levels, despite increasing pathogen burden, could be linked to survival. Our study has brought new insight to the role of serotonin in *Bd* infection. Further investigation is needed to confirm serotonin as detrimental to the host during *Bd* infection, and to understand other effects of this bioactive molecule on pathogenesis. With the overall aim of improving outcomes for frogs with chytridiomycosis, future work could test therapeutics that alter skin serotonin levels to assess survival rates in infected frogs.

3.5 | Conclusions

The contribution of frog metabolites to immune defence against the deadly amphibian fungus *Bd* is not well understood. Here, we determined that serotonin is produced by frog skin in response to *Bd* infection. Despite inhibiting the fungus *in vitro*, the immunosuppressive effects of serotonin likely contribute to the increased susceptibility of frogs to chytridiomycosis. This study improves the understanding of the role of metabolites in the host response to *Bd*, and provides a potential avenue for improving frog survival.

4 | EXPERIMENTAL PROCEDURES

4.1 | Study subjects, exposure experiment, and sampling

Study populations were sourced by D. Hunter in accordance with Scientific Licence number S12848 (Grogan *et al.*, 2018a, Grogan *et al.*, 2018b, Grogan *et al.*, 2018c). Source populations originated in Kosciuszko National Park, New South Wales, Australia, from three sites; Kiandra (Population K), Eucumbene (Population E), and Grey Mare (Population G). Experimental procedures followed approved guidelines under permits issued by James Cook University (A1408) and Taronga Conservation Society (4c/01/10). Adult *L. v. alpina* (n = 44) were inoculated with *Bd* and a further 15 frogs were mock inoculated with dilute salts solution (negative control group) (Bataille *et al.*, 2015). Animals were sampled throughout the course of infection in a random block design (see Table S1 for details). Thirty-six animals were euthanized during the

subclinical phase of infection at 4, 18 and 14 days post exposure (n = 12 at each time point). Fifteen control animals were also sampled (n = 5 at each time point). The remaining eight exposed frogs were sampled from 28 days post exposure when they began to exhibit clinical signs of chytridiomycosis and were hence in the final stages of disease. Frogs were swabbed to quantify *Bd* infection intensity via qPCR, and examined and measured before being euthanized for tissue sample collection (Grogan *et al.*, 2018c). Tissues were collected from ventral abdominal skin, the common site of *Bd* infection (Berger *et al.*, 1998, Berger *et al.*, 2005), and were immediately transferred to 500 µL of 100% methanol (Fisher Scientific), and stored at -80ºC. This study was part of a larger project investigating the effect of *Bd* on host immune responses (Grogan *et al.*, 2018b).

4.2 | Growth of *Bd* by liquid culture

Bd (isolate AbercrombieR-Lbooroolongensis-09-LB1) were cultured at 20 °C on three mTGhL agar plates (8 g L^{-1} tryptone, 2 g L^{-1} gelatin hydrolysate, 4 g L^{-1} lactose, 10 g L^{-1} agar, with the addition of 200 mg L^{-1} penicillin-G and 200-500 mg L^{-1} streptomycin sulfate) before flooding plates with 10 mL dilute salts solution (1.0 mM KH_2PO_4, 0.2 mM $CaCl_2.H_2O$, 0.1 mM $MgCl_2.2H_2O$) for 20 minutes to harvest the sporangia and zoospores. The suspension was then centrifuged at 2,800 x *g* for 10 minutes in a refrigerated Eppendorf Centrifuge 5702 (Eppendorf, USA), the supernatant was removed, and the pellet resuspended in a 2 mL cryotube with 1 mL methanol before storage at -80 ºC.

4.3 | Metabolite isolation from skin tissue and *Bd,* and analysis by GC-MS and UPLC qTOF

Skin metabolites were extracted as previously described (Grogan *et al.*, 2018c), however the metabolite extracts were analysed by 10 µL injection into a UPLCqTOF-MS, and without first drying the extracts. Metabolites from cultured *Bd* were isolated for analysis by GC-MS. *Bd* metabolites were extracted from sporangia (in 1 mL methanol) by sonication for five minutes at ambient temperature, followed by vigorous mixing by vortex to further disrupt the cells. The suspension was then transferred to a 2 mL microcentrifuge tube and 300 µL water added before a further round of cell disruption, as already described. Cell debris were collected by centrifuge at 16,100 *g* for 10 minutes at 4 °C, the supernatant transferred to a fresh tube and 65 µL of 10 $\mu g.mL^{-1}$ $^{13}C_6$-sorbitol standard added. The *Bd* metabolite extracts were dried in preparation for GC-MS analysis as previously described (Gummer *et al*., 2013), by first concentrating the extract in an Eppendorf Concentrator Plus vacuum concentrator, before then submersion in liquid nitrogen and lyophilising to dryness in a Labconco Freezone 2.5 Plus depressurized with a JLT-10 JAVAC high vacuum pump.

For GC-MS analysis, metabolites were derivatised with *N*-Methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA, Sigma-Aldrich). Analysis was using a Shimadzu QP2010 Ultra GC-MS (Kyoto, Japan) as described in (Grogan *et al.*, 2018c). The identity of serotonin and tryptophan were assigned by chromatographic retention time and mass-spectral match, by comparison to an authentic metabolite standard.

Supportive data for the identification of serotonin was by accurate mass measurement using a Waters Synapt G2S quadrupole time-of-flight mass spectrometer operating in positive electrospray ionisation mode and coupled to a Waters Acquity liquid chromagraphy system (UPLC qTOF/MS). Chromatography was conducted using a Kinetex 1.7 µm HILIC column (Phenomenex Inc.). The mobile phases consisted of A; 7.5 mM ammonium formate and B; 90:8:2, acetonitrile:isopropanol:water (7.5 mM ammonium formate). The mobile phase flow of 500 µL min^{-1} was equilibrated and held at 99% B for 2.5 minutes before a linear gradient to 95% B over 12.5 minutes. The ion source was operated with a capillary voltage of 3.0 kV, desolvation gas flow of 1000 L hr^{-1} and at a temperature of 150 °C. MS data were collected in continuum mode, at a mass range of m/z 100 – 1,000, with a scan time of 0.5 seconds and operating at a resolution of 38,000. Mass accuracy was maintained by infusion of leucine encephalin reference. Analyses were conducted using SPSS Statistics Version 22 (IBM). General linear models (univariate) were fitted to serotonin levels measured in skin between days 4 to 14 post-exposure to better understand likely determinants including treatment effects (infected versus control), pathogen burden (infection load as measured in zoospore equivalents), population of origin and duration of infection (days of the experiment). One-way analysis of variance and non-parametric Kruskal-Wallis tests were conducted to compare serotonin levels on different days.

4.4 | Immunohistochemistry

To determine the location of serotonin in *Bd* infected and *Bd* uninfected frog skin, immunohistochemistry (IHC) was performed. Skin tissue from the original, 8-month old *L. v. alpina* used in the exposure experiment was not viable for immunohistochemistry, and as additional animals were not available from this endangered species we changed to an alternative alpine species. We were able to collect fresh skin samples from 3 year old Southern Corroboree frogs (*Pseudophyrne corroboree*) that were euthanized in an unrelated infection experiment. The inoculation and husbandry of *P. corroboree* followed (Brannelly *et al.*, 2015). Skin sections (5 µm thick) were cut from *Bd* infected and *Bd* uninfected *P. corroboree* and embedded in paraffin blocks. Tissue collection followed approved guidelines under James Cook University permit (A2171). Slides were deparaffinised and rehydrated with xylene and ethanol, and rinsed with 1x phosphate-buffered saline (PBS). The slides were incubated overnight at 4 °C with the mouse monoclonal anti-serotonin primary antibody (ThermoFisher Scientific Cat# MA5-12111, RRID: AB_10982728) at a 1:20 dilution, in Tris hydrochloride (Tris-HCL) buffer with 1% bovine serum albumin (BSA). Slides were again rinsed with 1x PBS, and incubated overnight with rabbit anti-mouse IgG secondary antibody, HRP (ThermoFisher Scientific Cat#: PA 1-28568, RRID: AB_10983403) at a 1:1,500 dilution in Tris-HCL buffer 1% BSA. After rinsing with 1x PBS, antibody detection was performed by using 20 µL 3,3'-diaminobenzidine substrate-chromagen (DAB+ Chromagen) in 1 mL DAB+ Substrate Buffer (Dako EnVision+ System-HRP (DAB)) for approximately 10 minutes. Slides were rinsed with distilled water, counterstained with haematoxylin, dehydrated with ethanol and xylene, and mounted.

4.5 | Granular gland fullness

Granular gland fullness was measured in dorsal and ventral skin from 10 infected and 10 uninfected moribund *P. corroboree*. Tissue was prepared as previously described in the section above. Slides were stained with haematoxylin and eosin, dehydrated with ethanol and xylene, and mounted with mounting medium. Photos of granular glands were taken with a microscope, at 20x magnification. Photos of glands were analysed for size and fullness, following Brannelly L. (unpublished data) using ImageJ (Schneider *et al.*, 2012). The protocol for measuring gland fullness consisted of fixing the photo white balance, outlining the gland, adjusting photo saturation and brightness, analysing the area within the outlined gland, and calculating the proportion full. The proportion of gland filled was measured for five granular glands in dorsal and ventral tissues. The proportion filled was not normally distributed, and therefore arcsine transformed. A linear mixed model was performed in SPSS v. 25 where the arcsine transformed proportion filled was the dependent variable, fixed effects were the location of granular glands (dorsal or ventral), infection status (exposed or unexposed), and the interaction of location and status.

4.6 | *Bd* growth assays

The *Bd* isolate “Ethel creek-Lnannotis-2013-LB” (isolated from a Waterfall frog, *Litoria nannotis*, in 2013 from northern Queensland, Australia) was grown on sterile tryptone/gelatin hydrolysate/lactose (TGhL) plates using established methods

(Longcore *et al.*, 1999). Plates were flooded with TGhL media to release zoospores, which were syringe filtered to remove sporangia using a 10 µm isopore filter (Millipore). Chemicals were obtained from the Sigma-Aldrich Corporation. The chemicals tested were serotonin and the structural analogues tryptamine, histamine, 5-hydroxyindole-3-acetic acid, Trp, 5'-hydroxy L-tryptophan, and Kyn, as well as other biogenic amines ornithine, putrescine, spermidine, and spermine at final assay concentrations of 0.156 mM, 0.312 mM, 0.625 mM, 1.25 mM, 2.5 mM, 5 mM, and 10 mM.

Bd zoospores (100 µL, concentration between 2.5×10^5 and 1.0×10^6) were inoculated in a 96 well plate with an equal volume of the chemical of interest in 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) pH 7.4. Sterile deionised water and 20 mM HEPES negative controls were included. Sporangial size was measured at 3-4 days post inoculation, to capture the maximal mature sporangial size of each culture. For each concentration of serotonin a photograph was taken, and the 10 largest sporangia were selected per well and the sporangial diameter was measured, using a light microscope at 20X magnification (Fisher *et al.*, 2009) .

Bd from isolate "Ethel creek-Lnannotis-2013-LB" was grown on a 96 well sterile plate with 20 mM HEPES and serotonin at concentrations of 0.156 mM, 0.312 mM, 0.625 mM, 1.25 mM, 2.5 mM, 5 mM, and 10 mM. Absorbance at 492 nm was measured on a plate reader, and was averaged.

To confirm reproducibility of results, inhibitory experiments were repeated using the isolate "AbercrombieR-Lbooroolongensis-09-LB1", isolated from the Booroolong frog (*Litoria booroolongensis*), from New South Wales, Australia. One-way analysis of

variance was performed in SPSS v.22 using with the sporangia size data obtained from the inhibitory assay. This was used to test the effect of concentration for each chemical.

The minimum inhibitory concentration (MIC) of *Bd* with serotonin was quantified using the isolate "AbercrombieR-LbooroolongensisS-09-LB1". We defined MIC as the lowest concentration that prevented *Bd* from completing its life cycle (release of zoospores from sporangia) (Martel *et al.*, 2011). We observed *Bd* at 0.5 mM, 1.0 mM, 1.25 mM, 1.5 mM and 2 mM concentrations of serotonin for two weeks and noted when sporangia encysted, whether zoospores formed inside sporangia, and when motile zoospores were released (Table S2).

4.7 | Supplementation experiments with Trp and Kyn

To determine whether serotonin inhibits the Trp or Kyn biosynthetic pathways, a supplementation experiment was performed. A 96 well plate was inoculated with 100 µL of *Bd* from the "Ethel creek-Lnannotis-2013-LB" isolate, as above. Fifty microliters of either 10 mM Trp (in 20 mM HEPES), 10 mM Kyn (in 20 mM HEPES), or 20 mM HEPES buffer was added with increasing concentrations of serotonin (0 mM, 0.16 mM, 0.3125 mM, 0.625 mM, 1.25 mM, and 2.5 mM final concentration in 20 mM HEPES buffer). Each condition was tested in triplicate. After day 5, the 10 largest sporangia were measured in each well, using a light microscope at 20x magnification (Fisher *et al.*, 2009).

4.8 | Jurkat growth assays

Jurkat T cells (a commercially available human lymphocyte cell line) were used as a proxy for frog T cells, due to the difficulty of extracting T cells from frogs (Piovia-Scott *et al.*, 2014). Jurkat T cells were warmed at 37 °C for approximately two minutes in a water bath. Warm RPMI medium (ThermoFisher Scientific) supplemented with 10% fetal bovine serum (FBS), 100 IU/mL penicillin, and 100 µg/mL of streptomycin, was added to the cells. Cells were centrifuged at 200 x g for five minutes at 23 °C. Supernatant was removed, and cells were resuspended in fresh media. Twenty-five cm^2 flasks were inoculated with inoculum, and to a final volume of 10 mL with media. Cells were incubated at 37 °C in 5% CO_2 - 95% air and passaged 1:10 with fresh media when 80% confluence was reached (approximately every three days). Ninety µL of Jurkat T cells were inoculated on a 96 well plate in triplicate, with either 10 µL of serotonin suspended in 20 mM HEPES (final concentrations of 0.156 mM, 0.312 mM, 0.625 mM, 1.25 mM, 2.5 mM, 5 mM, and 10 mM), 10 µL L-Kyn suspended in 20 mM HEPES (final concentrations of 0.625 mM, 1.25 mM, 2.5 mM), or 10 µL of 25 µg/mL etoposide. Cells were grown for two days at 37 °C in 5% CO_2 - 95% air.

4.9 | MTT cell proliferation assay

To determine the amount of T cell proliferation when inoculated with varying concentrations of serotonin, a thiazolyl blue tetrazolium bromide (MTT) cell proliferation assay was performed. In a 96 well plate, 100 µL of MTT (500 µg/mL) was added to each Jurkat T cell sample, and incubated for four hours at 37 °C in 5% CO_2-95% air. The plate was spun for 30 minutes at 4,000 x g. Supernatant was removed and 100 µL

of dimethyl sulfoxide (DMSO) was added to each well. The plate was incubated for 10 minutes at 37 °C in 5% CO_2 - 95% air. Absorbance was read in a plate reader at 570 nm after a two minute shake.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

L.F.G, L.F.S., L.B., and A.A.R. designed research. L.F.G. performed the clinical experiment and collected samples. J.P.A.G. performed metabolite extractions and GC-MS analyses. S.C.C., R.J.W., and A.A.R. performed the growth assays and the supplementation experiment. S.C.C. performed the immunohistochemistry. S.C.C. and L.A.B. measured the granular gland fullness. S.C.C. and A.A.R. performed the growth and cell proliferation assays. S.C.C., A.A.R., L.F.G. and J.P.A.G. performed the statistical analyses and wrote the paper. All authors reviewed the manuscript.

FIGURE LEGENDS

FIGURE 1 Skin Serotonin Levels over the Course of Infection in Three Populations of *L. v. alpina.* X's represents the mean. (a-b) Average serotonin concentrations increase over the course of infection in populations Eucumbene (Population E) and Grey Mare (Population G), prior to falling dramatically in moribund animals (Day 28+). (c) Average serotonin concentrations remain low throughout the duration of infection in the Kiandra (Population K) population. Serotonin concentrations are relative to the normalised peak intensities in the dataset. Each box and whiskers plot represents the median and the interquartile range.

FIGURE 2 Infection Intensity and Concentration [Relative Units] of Serotonin within *L. v. alpina* skin. (a) *Bd* burden over the course of infection. (b) Serotonin concentrations correlate positively with infection intensity in Populations E and G, but not in Population K. Baseline represents the mean concentration of serotonin for control animals.

FIGURE 3 Immunohistochemistry of ventral *Pseudophryne corroborree* skin with Serotonin Antibody. (a) In uninfected control skin (day 35), serotonin accumulates within the granular glands (G). (b) In infected frogs showing clinical signs of chytridiomycosis (day 35), serotonin is noticeably absent from granular glands. The arrows in both panels are pointing to granular glands. The chromagen staining is prominent in the granular glands in uninfected animals (panel a, i.e. the dark staining inside the glands), and absent from the granular glands in infected animals (panel b).

FIGURE 4 Sporangial Growth Assays with Serotonin and Structural Analogues Show that an Ethylamine Attached to a Basic Group Reduces *Bd* Sporangia Size. Bolded square corresponds to the inset that shows the data from concentrations of 0-2 mM. (a) Serotonin (produced in frog skin) reduces sporangial growth, while the analogues kynurenine (Kyn) and tryptophan (Trp) (produced by *Bd*) do not affect fungal growth. (b) Inhibition of sporangial growth occurs with histamine and tryptamine, which possess both the terminal primary amine and pyrrole ring of serotonin. At high concentrations (5-10 mM), 5-hydroxyindole-3-acetic acid inhibits growth, while 5-hydroxy-L-tryptophan does not have any effect. (c) Other biogenic amines that contain an aliphatic primary amine, such as spermine, spermidine and putrescine inhibit growth. However, this effect is negated if the ethylamine is attached to an acidic group, as in ornithine.

FIGURE 5 Effect of Supplementation of Cultures. Treatment with 10 mM tryptophan (Trp) or 10 mM kynurenine (Kyn) does not restore the growth of *Bd* with increasing concentrations of serotonin.

FIGURE 6 Effect of Serotonin and Kynurenine (Kyn) on Jurkat Cells. A trend line is shown for the serotonin data points. High absorbance serotonin inhibited Jurkat lymphocytes at concentrations over 500 μM, similar to the effect for Kyn.

FIGURE 7 Average absorbance at 492 nm of *Bd* treated with serotonin. The average absorbance of *Bd* decreased with increased concentrations of serotonin.

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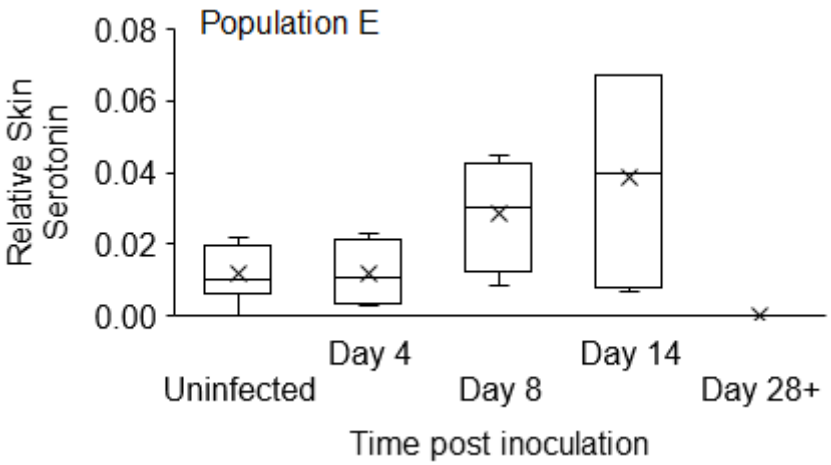

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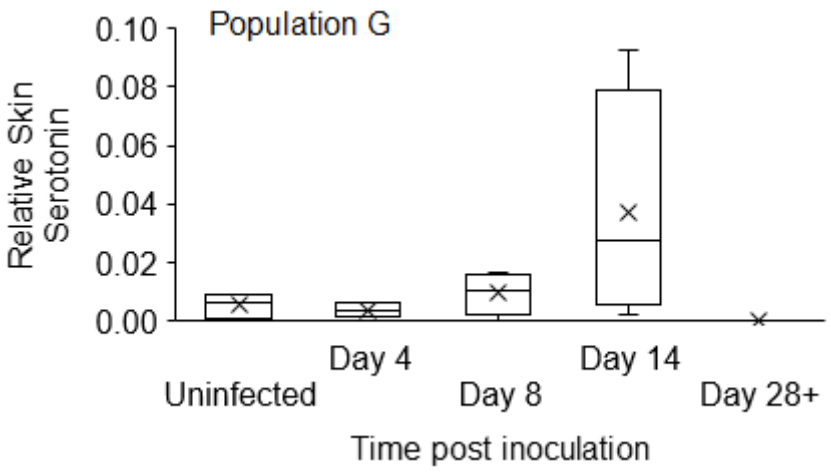

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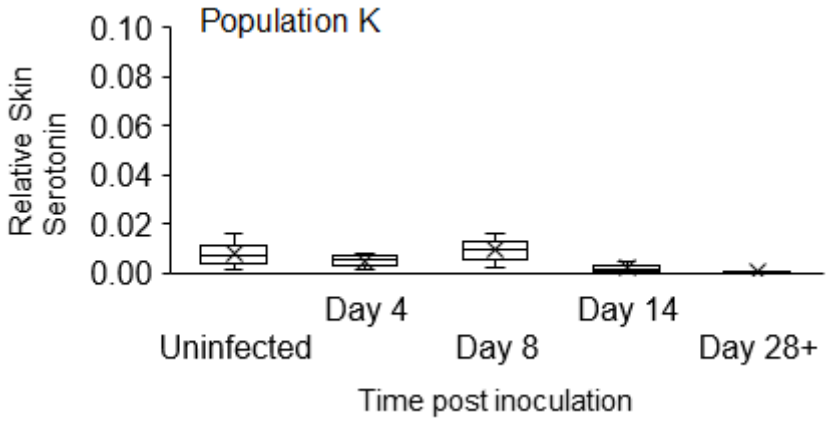

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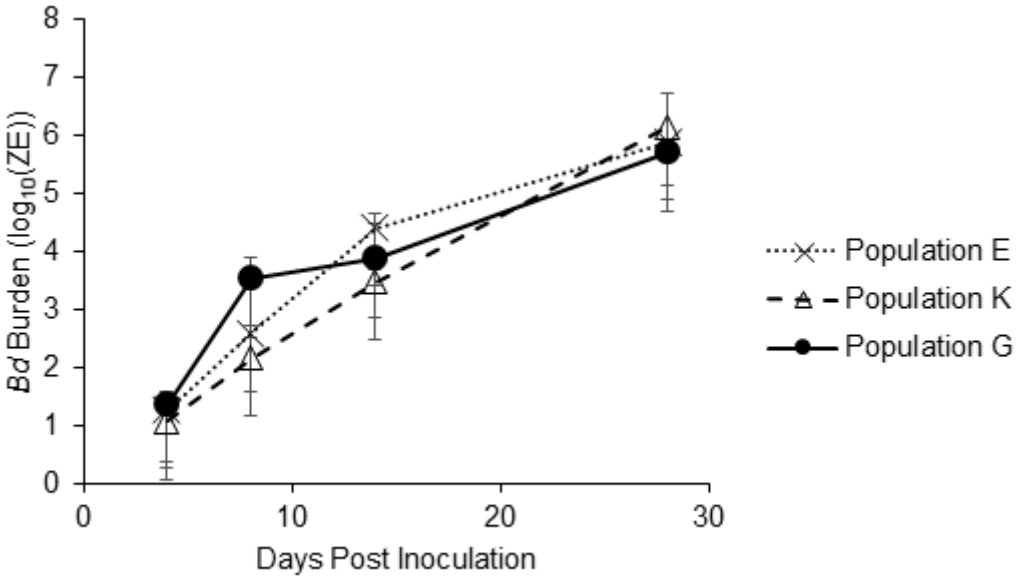

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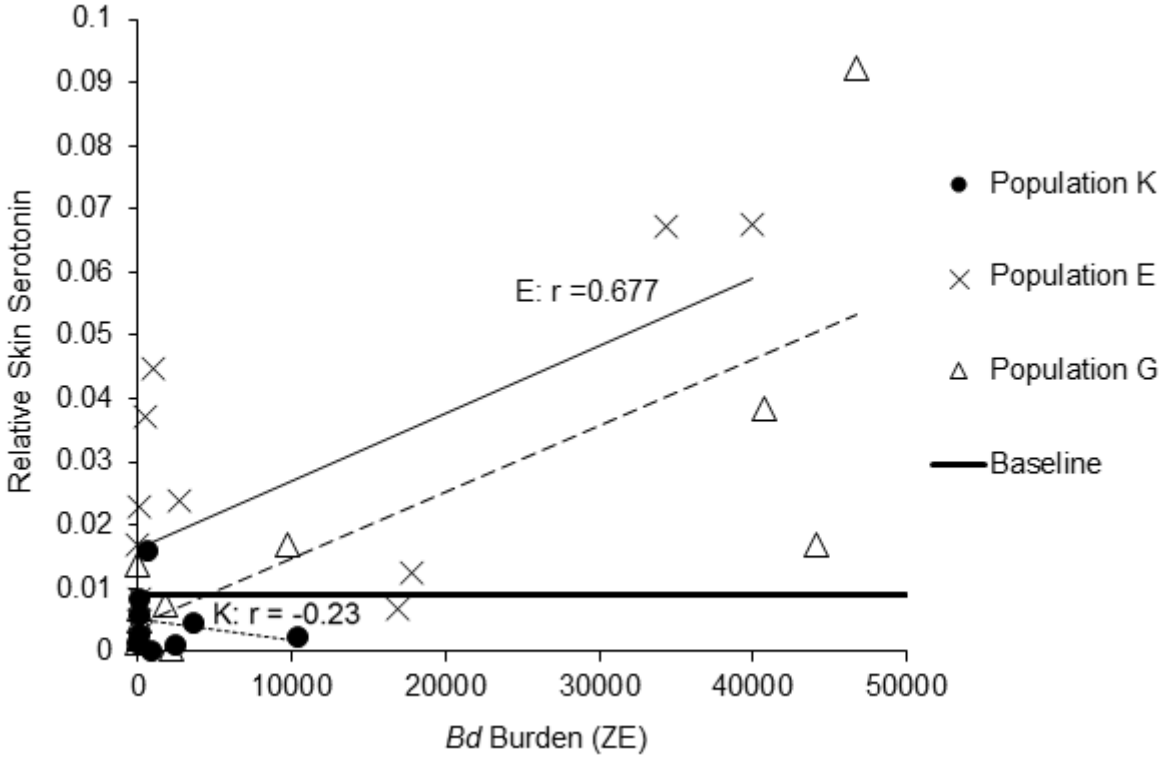

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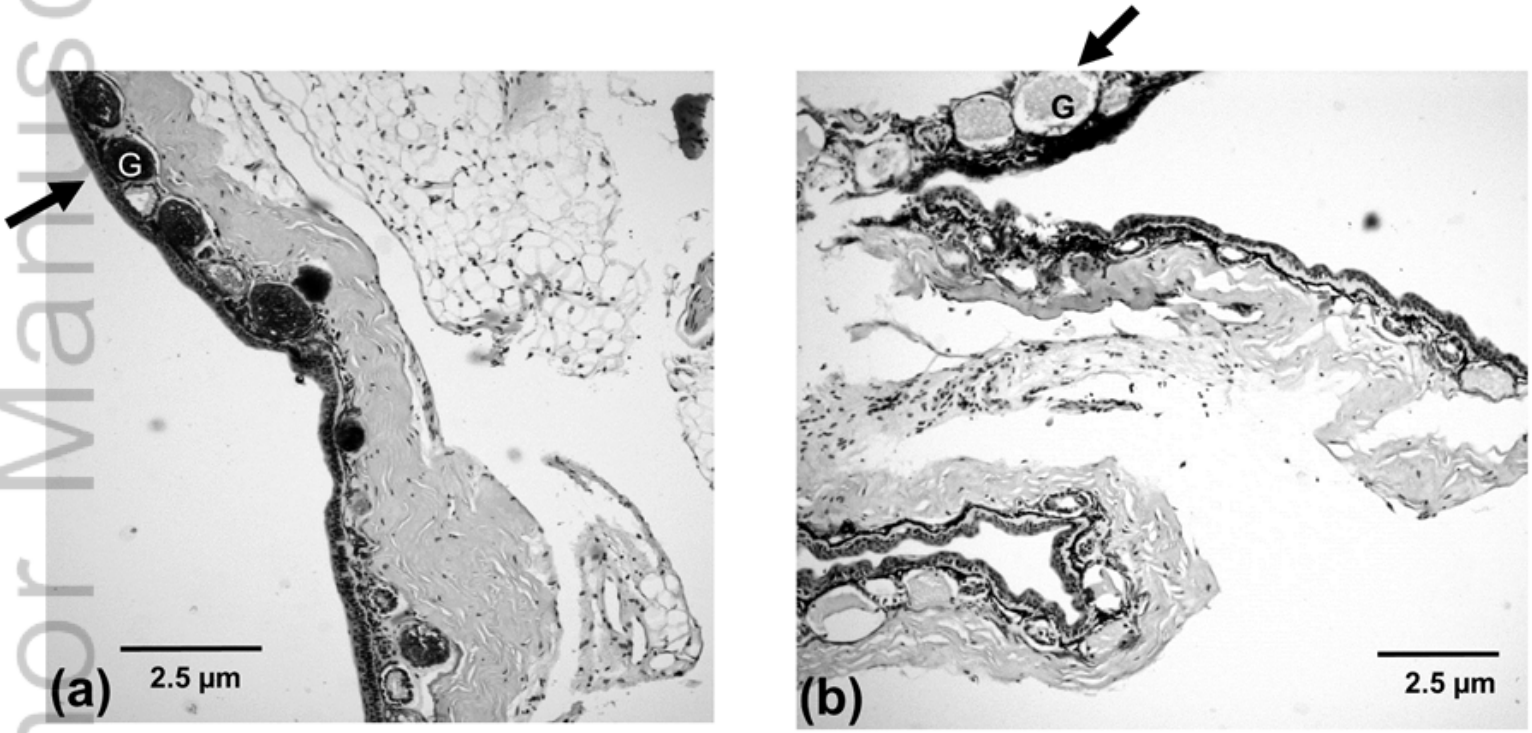

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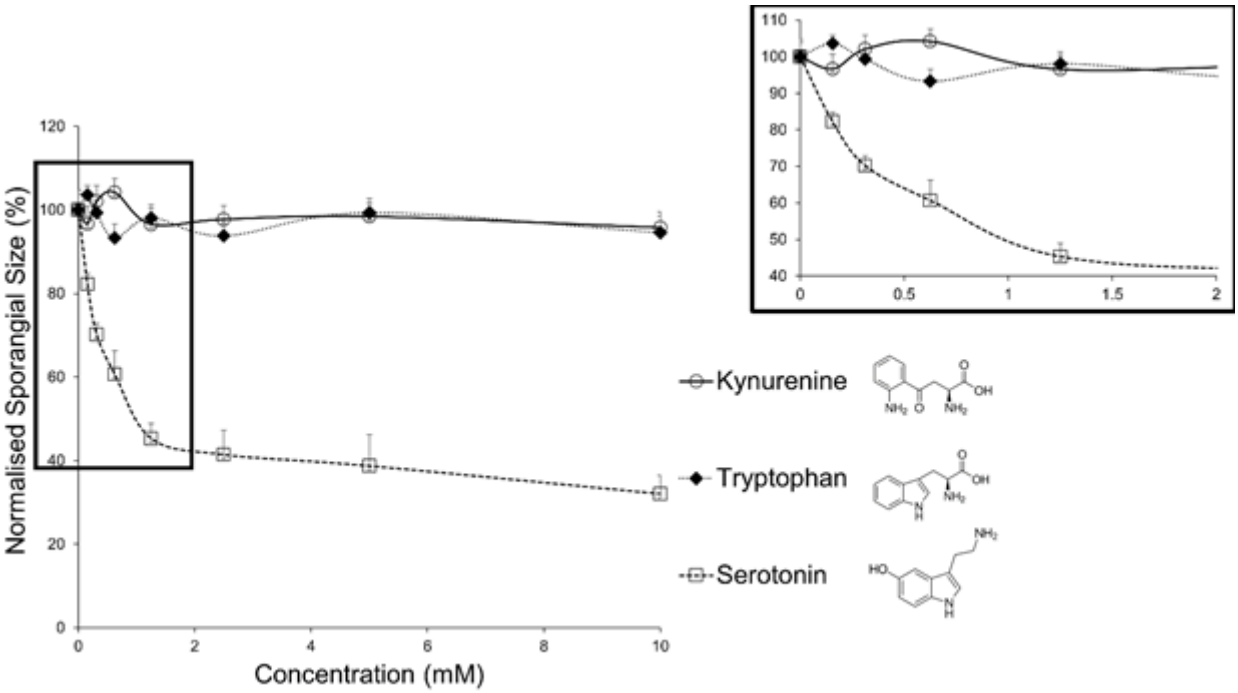

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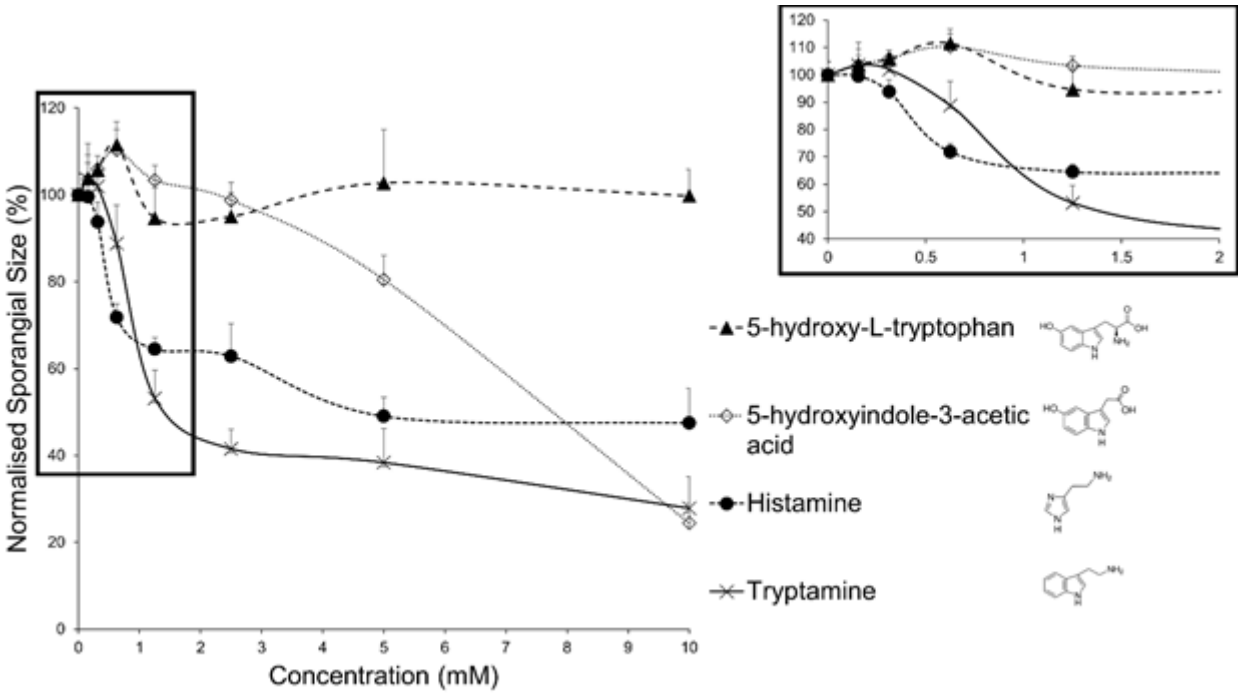

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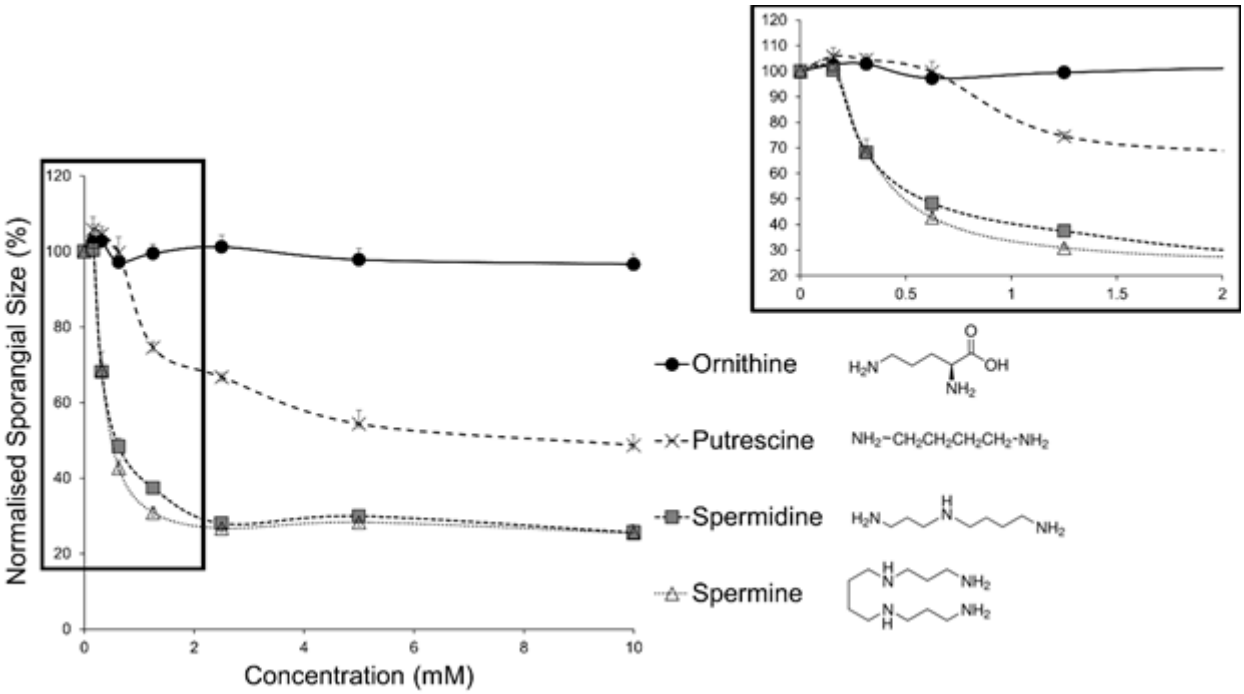

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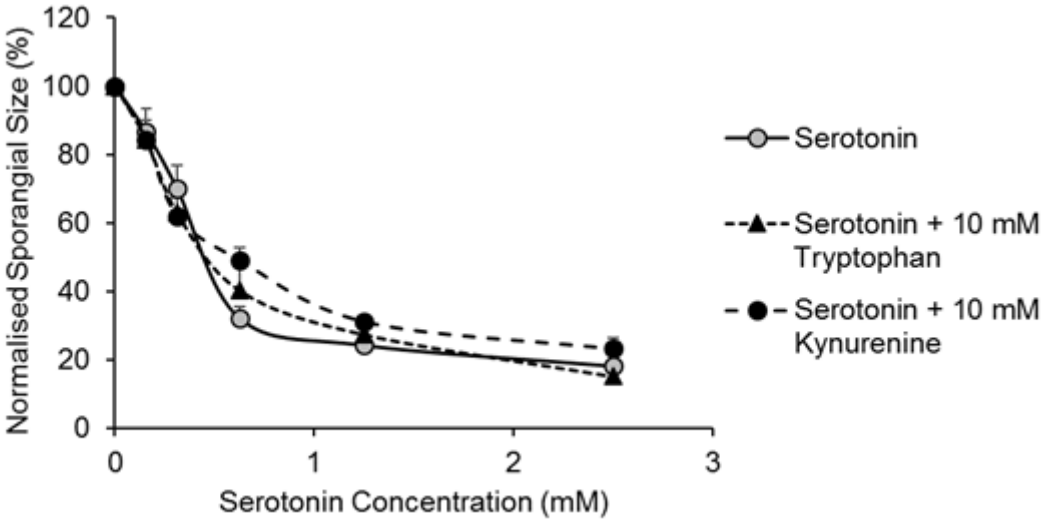

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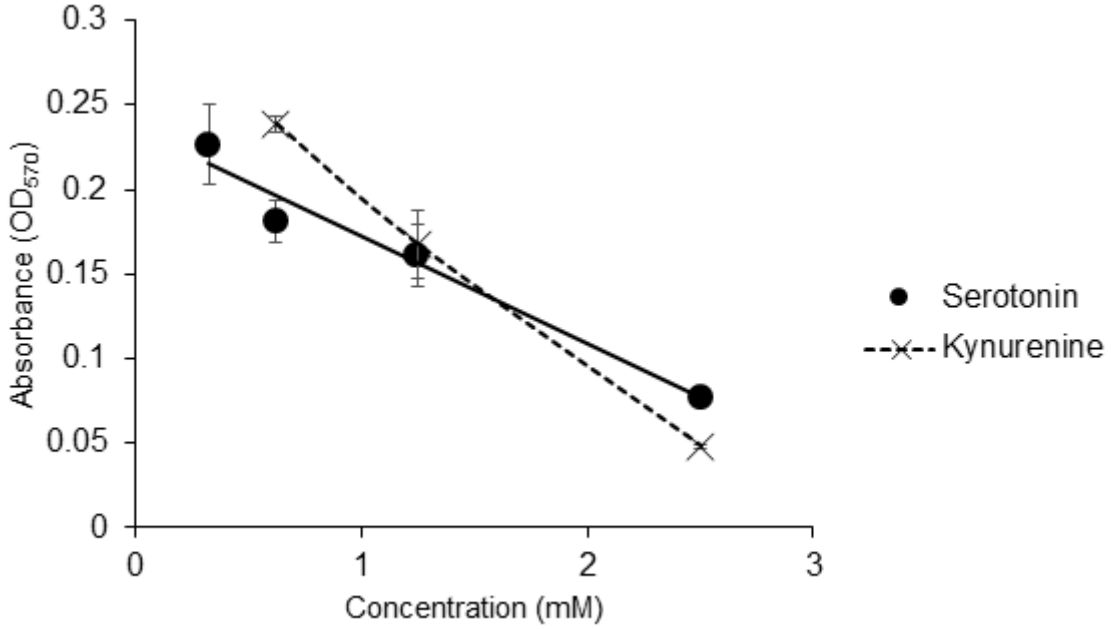

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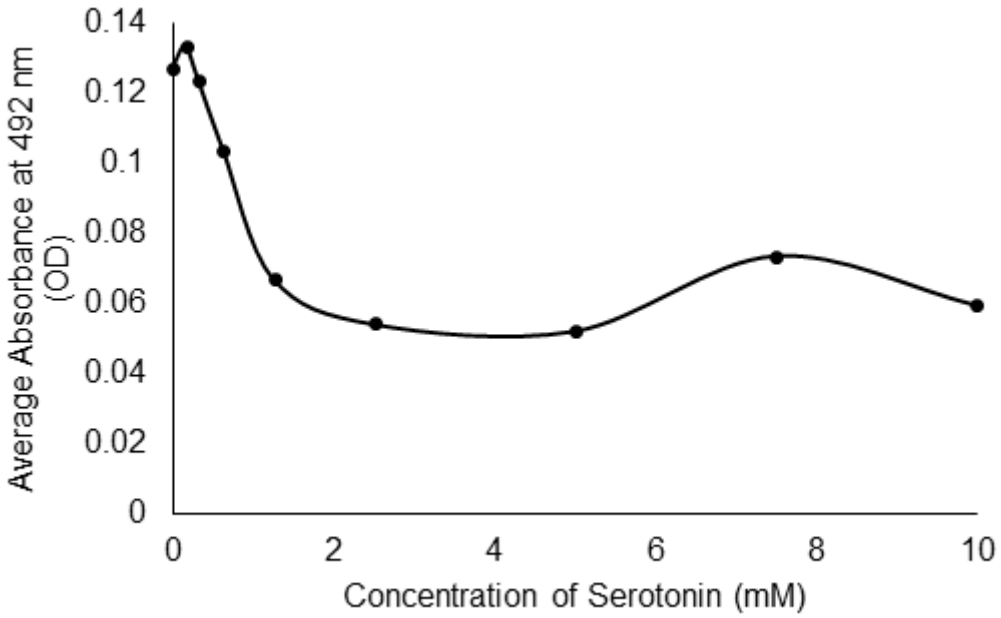

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