Identification of a Novel Secreted Metabolite Cyclo(Phenylalanyl-Prolyl) from Batrachochytrium Dendrobatidis and its effect on Galleria Mellonella.

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Research Article

Keywords: Fungi, Secondary Metabolite, Wax Moth Larvae, Mass Spectrometry

Posted Date: December 1st, 2021

DOI: https://doi.org/10.21203/rs.3.rs-1099327/v1

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Abstract

The fungus, *Batrachochytrium dendrobatidis,* is the causative agent of chytridiomycosis and a leading cause of global decline in amphibian populations. The first stages of chytridiomycosis include: inflammation, hyperkeratosis, lethargy, loss of righting reflex, and disruption of internal electrolyte levels leading to eventual death of the host. Previous work indicates that *B. dendrobatidis* can produce immunomodulatory compounds and other secreted molecules that regulate the growth of the fungus. In this study, filtrates of the fungus grown in media and water were subjected to ultra performance liquid chromatography-mass spectrometry and analyzed using Compound Discoverer 3.0. Identification of cyclo(phenylalanyl-prolyl), chitobiose, and S-adenosylmethionine were verified by their retention times and fragmentation patterns from *B. dendrobatidis* supernatants. Previous studies have analyzed the effects of *B. dendrobatidis* on amphibian models, *in vitro,* or in cell culture. We studied the effects of live *B. dendrobatidis* cells, spent culture filtrates containing secreted metabolites, and cyclo(pheylalanyl-prolyl) on wax moth larvae (*Galleria mellonella)*. Concentrated filtrates caused melanization within 24 hours, while live *B. dendrobatidis* caused melanization within 48 hours. Our results indicate *B. dendrobatidis* produces secreted metabolites previously unreported. These findings provide another alternative for the use of a non-amphibian model system to study pathogenicity traits in this fungus.

Key Words

Fungi, Secondary Metabolite, Wax Moth Larvae, Mass Spectrometry

Introduction

Amphibians, including frogs, toads, salamanders, newts, and caecilians, have been declining at alarming rates since the 1970s [1]. A major contributor to global amphibian decline in the 1990s was determined to be the chytrid fungus *Batrachochytrium dendrobatidis*, and in 2010 a decline in salamanders was attributed to *Batrachochytrium salamandrivorans* [2-4]. *Batrachochytrium* species are currently known to infect between 50-55% of frogs, toads, salamanders, and newts globally and 29% of caecilian species [5]. *Batrachochytrium dendrobatidis* exists in two life stages, the motile zoospore and the sessile, reproductive zoosporangium. The motile zoospore can produce a germ tube and enzymes to degrade intracellular junctions, skin components, and amphibian anti-microbial peptides [6-8]. The zoospore matures into a zoosporangium, which is known to produce metabolites to inhibit B and T cell proliferation [9-11]. *In vitro* the zoosporangium also produces tryptophol, a quorum sensing compound, that is associated with aggregated growth [12]. Infected amphibians have symptoms of hyperkeratosis and slough skin to reduce infection loads. Severely infected individuals present neurological symptoms including loss of righting reflex and succumb to cardiac arrest [13, 14]. The reason for loss of righting reflex is unknown.

Fungi are known to produce primary metabolites, essential for growth and reproduction, and secondary metabolites, compounds not essential for growth or reproduction, but instead used to aid in survival. Secondary metabolites of fungal origin that play roles in colonization and establishment of infections have been shown to contribute to cell death and neurological symptoms, and possess anti-microbial, anti-nematode, and anti-protozoan activity [15-19]. In the context of amphibian infections, we currently understand that such molecules may play roles in quorum sensing, interference with immunological responses, and establishment in the host [9, 10, 12, 20]. Proteins responsible for secondary metabolite synthesis may be encoded in gene

clusters within the genome [21]. These metabolites have been identified through a variety of methods, but most success has been accomplished through use of liquid chromatography in tandem with mass spectrometry techniques [22]. *Batrachochytrium dendrobatidis* may possess genes that encode proteins responsible for secondary metabolite production [23].

Parasitic fungi have been studied using a variety of "non-primary hosts." Non-amphibian model systems that have been used to study *B. dendrobatidis* infections are *Xenopus laevis* amphibian skin and cell lines (i.e. splenocytes, leukocytes, and macrophages), nematodes, zebra fish, and daphnia [9, 24-28]. Another model system used to study virulence mechanisms of fungi is the wax moth larvae (*Galleria mellonella*) [29, 30]. Here we show, for the first time, that *B. dendrobatidis* can produce cyclo(phenylalanyl-prolyl) (cPP) during *in vitro* cell culture and this molecule can cause melanization in *G. mellonella* in a manner similar to live *B. dendrobatidis.* The selection of *G. mellonella* over a previously identified non-amphibian host was to explore the use of another animal model not previously utilized with *B. dendrobatidis*.

Materials and Methods

Growth Conditions for Fungus

Batrachochytrium dendrobatidis VM1 isolate was grown in 50% H-broth with lactose (HBL) (0.5% tryptone, 0.16% glucose, 2% lactose, w/v for all) with shaking in dark for 4 d until cell density of the culture grew to 10^7 cells mL^{-1} as quantified by hemacytometer for all experiments. Media controls with HBL only and ~0.5 ml sterile 0.5 mm diameter glass beads were used to mimic volume of growing *B. dendrobatidis*. Cultures were centrifuged at 1,865 x g for 10 min. Cell culture pellets were washed twice with 2 mL HyClone Pure cell culture water. To determine if cPP was a media artifact, samples of 50 HBL with glass beads and samples with

86 living *B. dendrobatidis* cells were heated at 60ºC for 10 min. To confirm heat treating killed *B.*
87 *dendrobatidis* cells, 10 µl aliquots were grown on H-agar for 5 d. Heat-treated cells or beads
88 were washed as described above. Tubes were shaken (0.03-0.1 x g) on a rotary shaker in the dark
89 for 24 hrs. The cultures were centrifuged at 1,865 x g for 10 m and filtered with a 0.22 µm
90 polyvinylidene difluoride (PDVF) filter to remove cells/glass beads. All samples were treated in
91 triplicate.

92 *Ultra-Performance Liquid Chromatography-Mass Spectrometry (UPLC-MS/MS)*

93 All conditions, *B. dendrobatidis* supernatant, cPP standards, and media and water
94 controls, had 5 µl analyzed by UPLC-MS/MS using Q Exactive™ HF Hybrid Quadrupole-
95 Orbitrap™mass spectrometer (Thermo Scientific, San Jose, CA). Standards of cyclo(L-Phe-
96 L-Pro), cyclo(L-Phe-D-Pro), cyclo(D-Phe-L-Pro), and cyclo(D-Phe-D-Pro) were each dissolved
97 at a concentration of 1 mM in water (Sigma-Aldrich and Santa Cruz Biotechnology). The
98 chromatographic separation of *B. dendrobatidis* supernatant, cPP standards, and media and water
99 controls were performed on Vanquish UPLC system using an Acquity UPLC column (2.1 x 150
100 mm, HSS T3 C18, 1.8µm). A 15-minute gradient was used for separation using solvents A
101 (water, 0.1% formic acid (FA) and B (methanol, 0.1% FA). The gradient profile started with
102 0.5% solvent B, followed by an increase to 90% B in 9 minutes and then maintained at 90% B
103 for another 1.5 min and finally decreased to 99.5% A for the equilibration of the column. The
104 flow rate was kept at 0.45 µl min^{-1} during the run and column temperature was set at 55°C
105 throughout the run.

106 Data-dependent acquisition mode with two scan events was employed for MS/MS
107 analysis. The first scan event was a full MS scan of 80–500 *m/z* at a mass resolution of 120,000.
108 In the second scan event, the five most intense ions detected in the first scan event were selected

to perform higher-energy C-trap dissociation (HCD) MS/MS with resolution of 15,000. An elevated normalized collision energy (NCE) from 20-60% was applied to obtain the MS/MS spectra. The dynamic exclusion was set to have repeat count of 2 and repeat duration of 6 s.

Mass Spectrometry Data Analysis

Metabolite structure search and predictions were done using Compound Discoverer 3.0 (Thermo Fisher Scientific) based on molecular weight, retention time, and fragmentation pattern for each feature. Features from each data file were selected and aligned by retention time and molecular weight (mass tolerance of 5 ppm, intensity tolerance of 30%, min. peak intensity of 100,000). Common adducts of each feature were removed. Compound names were assigned to each feature with a similar match in the ChemSpider database or an elemental composition was assigned with Predict Compositions processing node. To identify more unknown features, mzLogic processing node compared MS2 data to the mzCloud database for similar fragmentation. For those files with missing features, Fill Gap Nodes was applied to allow for statistical analysis. The identified compounds were then mapped to KEGG, Metabolika, and BioCyc Pathways.

Features were narrowed down to those that included ddMS2 data, had exact compound names, matched to the mzCloud database, had a p-value <0.01, $-2 < \log_2\text{fold} > 2$, and no repeat compound names. Annotated feature intensities were normalized between *B. dendrobatidis*-water and media-water samples and area under the curve for each feature was compared using t-tests. Metaboanlayst 5.1 software will be utilized for over-representation analysis and pathway analysis modules [31].

Wax Moth Larvae Bioassay

The use of wax moth larvae, *Galleria mellonella*, is an accepted model to study microbial infections [29, 30]. Larvae were purchased from Carolina Biological (Burlington, NC), allowed to rest 24 hrs in empty Petri dishes, and then treated as described below. All worms were used within 7 d of receipt. Supernatants or whole cells of *B. dendrobatidis* at a concentration of 10^7 ml^{-1} in 1X PBS were used in these experiments. Solutions of cyclo(L-Phe-L-Pro) (Sigma-Aldrich), cyclo(L-Phe-D-Pro), cyclo(D-Phe-L-Pro), and cyclo(D-Phe-D-Pro) (Santa Cruz Biotechnology) at a concentration of 1 mM were dissolved in water for these experiments. In all cases larvae were injected into the left lower-proleg of each larvae using sterile disposable syringes and sterile 25.5-gauge needles in 20 µl volumes into each of 10 worms in 3 independent replicates. Larvae not injected and those injected with only 1X PBS and water were used as controls. Worms were kept in sterile glass petri dishes and photographed every 24 hr for 48 hr and observed for the occurrence of melanization at 24ºC. After 2 d, worms were frozen for 4 d and autoclaved. Melanization characteristics of each larva was scored on a scale of 0-4 as previously described [32]. Photos of each group of larvae were scored blind based on melanization levels (health index score) to reduce bias (Fig. 1). The melanization curve was analyzed with a linear regression and two-way ANOVA with post-hoc Tukey's analysis.

Thirty larvae were injected with *B. dendrobatidis* re-suspended in 1X PBS and 1X PBS and incubated at 24ºC for 48 hrs. After incubation, the larvae were transferred to 4ºC for 15 min to slow down movements and hemolymph extracted for analysis. Hemolymph was extracted by puncturing the lower abdomen with a sterile 25.5-gauge needle and outflowing hemolymph was collected in a 1.5 mL microcentrifuge tube. Hemolymph was centrifuged at 200 x g for 5 min at 4ºC to remove hemocytes, and the supernatant was centrifuged at 20,000 x g for 15 min at 4ºC to remove cellular debris. The degree of melanization of hemocyte-free hemolymph from each set

of 10 injected larvae was measured as optical density (O.D.) at 600 nm. A Student's t-test was performed to determine statistical significance.

Results

Identification of unique secreted metabolites produced by Batrachochytrium dendrobatidis in vitro

There were 2,285 compounds identified in the supernatant of *B. dendrobatidis* incubated in water compared to the media-water control. The list was narrowed by selecting compounds that had MS2 data, had an exact compound name, had a match to the mzCloud database, p-value <0.01, and $-2 < \log_2$fold change >2. Any repeat compound names were removed. There were 113 remaining compounds after the selection criteria (Supplementary Table 1). Four novel metabolites and previously known secreted metabolites from *B. dendrobatidis,* including methylthioadenosine, kynurenine, tryptophan, and tryptophol were observed in this study and are highlighted in Table 1. Using the enrichment metabolite set enrichment analysis (MSEA) module in Metaboanalyst 5.1, 92 of 113 compound names matched under the KEGG, PubChem, and Human Metabolome Database (HMDB). These 92 compounds were observed to be significantly enriched for amino acids and peptides, purines, indoles, pyridinecarboxylic acids, imidazoles, heterocyclic compounds, and pyridines ($p<0.05$, FDR <0.05) (Fig. 2). The pathway analysis module in Metaboanalyst 5.1 was also utilized with the following parameters: enrichment utilizing Fisher's Exact Test, topology utilizing the relative-betweenness centrality, and the *Saccharomyces cerevisiae* KEGG pathway. The metabolic pathways these 92 compounds most affected were: beta-alanine, tryptophan, nicotinate/nicotinamide, vitamin B6, biotin, and lysine degradation (impact factor>0.2) (Fig. 3).

We detected unique peaks with m/z of 245.12, 399.14, 424.17, and 236.14. Further analysis with Compound Discoverer, identified the 245.12 peak as cyclo(phenylalanyl-prolyl) which was significantly increased in area under the peak in the *B. dendrobatidis*-water (*B. dendrobatidis* cultured in HBL for 4 d, washed in water, and further incubated in water for 24 hrs) versus water (glass beads cultured in HBL for 4 d, washed in water, and further incubated in water for 24 hrs) (Log_2Fold Change = 2.97, $P<0.000926$). Standards of cyclo(L-Phe-L-Pro), cyclo(L-Phe-D-Pro), cyclo(D-Phe-L-Pro), and cyclo(D-Phe-D-Pro) were compared by mass spectrometry with those observed in the *B. dendrobatidis* sample at a concentration of 1 mM. The comparisons indicate that the smaller peak (5.4 min) is the cyclo(L-Phe-D-Pro) or cyclo(D-Phe-L-Pro) conformation, and the larger peak (5.6 min) is the cyclo(L-Phe-L-Pro) or cyclo(D-Phe-D-Pro) conformation (Fig. 4). The 236.12 peak showed a compound similar to isopropyl aminoethylmethyl phosphonite (QL compound) or possesses a D-carnitine side chain following analysis with Compound Discoverer 3.0. The fraction containing the 236.12 peak was collected 20 times with 20 µl injections and used for bioassays. The fraction containing the 424.16 peak was identified as chitobiose and the 399.14 peak as S-adenosylmethionine.

Melanization occurs in Galleria mellonella after injections with Batrachochytrium dendrobatidis cells and culture supernatants

The health score index based on melanization of wax moth larvae was significantly reduced when exposed to 10^7 *B. dendrobatidis* cells (cultured in modified H-Broth 4 d, washed, and re-suspend in 1X PBS) after 48 hrs of exposure (Fig. 5a, $P<0.01$) [33]. Analysis of hemocyte free hemolymph, showed significant melanization in the *B. dendrobatidis*- injected larvae compared to the 1X PBS injected larvae (Fig. 5b, $P<0.0001$). A significant reduction of health score index was observed within 24 hrs after injection with concentrated water supernatant from

B. dendrobatidis (Fig. 6, P<0.0001). Use of pure cyclo(L-Phe-D-Pro), cyclo(D-Phe-L-Pro), and cyclo(D-Phe-D-Pro) conformations in our wax moth larvae model significantly decreased the health score index of the larvae within 48 hrs (Fig. 7, P<0.0001, P<0.0001, and P<0.01, respectively). Injection of larvae with the fraction containing the 236 analyte (QL reagent or D-carnitine containing molecule) resulted in no significant melanization (data not shown).

Discussion

Fungi are known to produce a broad range of primary and secondary metabolites [34]. The primary metabolites are required for growth [35]. Secondary metabolites are important for pathogenesis, survival in the environment, and can regulate reproduction [36-38]. We identified growth and immunomodulatory fungal metabolites produced by *B. dendrobatidis*. These included the immunomodulatory compounds methylthioadenosine, tryptophan, and kynurenine and the growth modulatory compound, tryptophol. A novel small cyclic dipeptide, cyclo(phenylalanyl-prolyl), QL compound related to carnitine, S-adenosylmethionine, and chitobiose were also identified. Identification of metabolites secreted by *B. dendrobatidis* could help with the development of vaccine targets in the prevention of infection [39].

Small cyclic dipeptides are known to have a variety of functions in organisms. These include anti-bacterial and anti-fungal activities, moderation of gene expression, cell growth and apoptosis, interference of toxin production, altering auxin related gene activity, and quorum sensing [40-47]. Cyclic dipeptides are capable of inhibiting calcium channels [48]. Calcium channels are required for basic neurological responses, as calcium allows for the fusion of neurotransmitter membrane packets to enter neurons. We postulate that with the calcium channels inhibited during infection with *B. dendrobatidis*, the amphibian would no longer be able to release neurotransmitters. Furthermore, D-amino acids such as the cPP molecules

identified in this study play a role in immune suppression and central nervous system health and disease [49]. This finding may explain why infected amphibians often experience the loss of the righting reflex.

The biosynthesis of cPP by non-ribosomal peptide synthetases (NRPS) is well-documented [50-53]. *Bacillus brevis* is known to produce tyrocidine, of which cPP is a precursor molecule, through a NRPS (*TycA)*[54]. The NRPS amino acid sequence in *B. brevis* was used as a probe for NRPS sequences in *B. dendrobatidis*. We found that *B. dendrobatidis* encodes proteins with high similarity to the *B. brevis* non-ribosomal peptide synthetase (20 potential matches, e value <9E-06) (Table 2). While the cyclo(L-Phe-L-Pro) conformation of cPP is known to be produced by eukaryotes, we show *B. dendrobatidis* can potentially make all four conformations of the cyclic dipeptide (Fig. 4). The cyclo(L-Phe-D-Pro), cyclo(D-Phe-L-Pro), and cyclo(D-Phe-D-Pro) conformations of cPP produced a significant decrease in the health score index (significant increase in melanization of the larvae) in our wax moth larvae model system. Prophenoloxidase, an enzyme that is activated during the innate immune response of insects, causes melanization to occur [55, 56]. This enzyme level is directly linked to the calcium concentrations in the hemolymph of the wax moth larvae. In high concentrations of calcium, this enzyme slows the activation of the prophenoloxidase system and prevents coagulation and healing to occur in the wax moth larvae [57, 58]. Our findings suggest that the presence of the D-amino acid in cPP plays a role in the induction of an immune response in the wax moth larvae, as evidenced by the production of melanization when the insects were exposed to the cyclo(L-Phe-D-Pro), cyclo(D-Phe-L-Pro), and cyclo(D-Phe-D-Pro) conformations. Additionally, the conformations that contain the D-isomers may contribute to a loss of the righting reflex seen in chytridiomycosis, since D-amino acids are largely found in

neurotransmitters and the melanization was not inhibited during infection indicating lower calcium levels [59]. We interpret the above results to suggest that the cPP compound produced by *B. dendrobatidis* may aid in the loss of righting reflex seen in amphibians by inhibiting calcium channels and competing with receptors for neurotransmitters.

Since cPP is present in modified H-Broth, *B. dendrobatidis* and media-glass beads were heat-killed prior to 24 hr water incubation. Samples were processed through mass spectrometry and there was a significant increase in cPP in the *B. dendrobatidis*-water sample compared to heat killed *B. dendrobatidis,* media-water, or heat-killed media-water (Table 3). Heat-killed *B. dendrobatidis* did not significantly change +/- 1 log fold change compared to controls. These additional experiments, in conjunction with *B. dendrobatidis*-water and pure cPP causing melanization while media-water has a lack of melanization in the wax moth larvae, support our contention that cPP is being produced by *B. dendrobatidis* and is not solely an artifact from the media.

Our unknown compound with a mass of 235.12 shows high similarity to molecules possessing a carnitine side chain. Carnitine in fungi is known to aid in the utilization of alternative carbon sources and to promote growth [60]. This compound could be used in conjunction with tryptophol to regulate growth. This compound also shows high similarity to isopropyl aminoethylmethyl phosphonite (QL compound) and procainamide. Isopropyl aminoethylmethyl phosphonite is a precursor molecule to a nerve agent known as VX (an organophosphorus compound that is an acetylcholinesterase inhibitor) [61]. Procainamide is a medication that is used to correct arrhythmias by blocking sodium channels on heart muscle [62]. The injection of the 235.12 compound into the wax moth larvae model system, did not show a significant decrease in health score index.

S-adenosylmethionine is a co-factor molecule required for growth in all organisms including fungi [63], and can be a precursor metabolite for the production of methylthioadenosine and spermidine, which inhibit amphibian lymphocyte proliferation [20, 64]. Chitobiose is a type of N-glycan and is used in the production of chitin, which is found in the cell wall of *B. dendrobatidis* zoosporangium. The production of these compounds and their identification in *B. dendrobatidis* allows us to understand the basic biology of the fungus.

In addition to the identified novel compounds secreted by *B. dendrobatidis*, 99 more compounds were identified. Of these 99 compounds, 92 were able to be assessed for their impacts on compound class and metabolic pathways. A large portion of these metabolites were enriched in amino acids and peptides, purines, indoles, pyridinecarboxylic acids, imidazole, heterocyclic compounds, and pyridines. Amino acids and peptides are known to be important in immunomodulatory regulation for *B. dendrobatidis*, as shown by tryptophan and cPP [10]. Purines and imidazole compounds are produced by fungi and have been shown to have antiviral, antibacterial, analgesic, antipyretic, and anti-inflammatory properties [65]. Indole by-products, such as tryptophan and kynurenine, are known to also be immunomodulatory [10]. Pyridinecarboxylic acids and heterocyclic compounds are known to be produced by *B. dendrobatidis* and other fungi. For example, spermidine from *B. dendrobatidis* can inhibit B and T cell proliferation, while tryptophol from the chytrid encourages cell aggregation through quorum sensing [20, 66]. Other compounds linked in these pathways may be of importance to understanding functions of other metabolites secreted by *B. dendrobatidis.* The top 5 metabolic pathways most impacted by these 92 molecules are beta-alanine, tryptophan, nicotinate/nicotinamide, vitamin B6, and biotin. The beta-alanine pathway is important for fungal virulence because beta alanine is a precursor for polyamines (such as spermidine) and

291 could contribute to altering the immune response of amphibians [20, 67]. The
292 nicotinate/nicotinamide pathways are important in dimorphic fungi, such as *Botrytis cinerea* for
293 the formation of sclerotia and can be scavenged from the tryptophan pathway [68, 69].
294 *Batrachochytrium dendrobatidis* could utilize this pathway for aiding in transition from the
295 zoospore to the zoosporangium stage. The tryptophan biosynthetic pathway is already known to
296 be important for production of immunomodulatory compounds from *B. dendrobatidis.* These
297 compounds include tryptophan, kynurenine, methylthioadenosine, and tryptophol [9, 70].
298 Vitamin biosynthesis, especially vitamin B mechanisms, could be potential antifungal targets
299 [71]. Vitamin B6 (pyridoxine) could contribute to *B. dendrobatidis* fungal virulence by altering
300 iron homeostasis. In *Aspergillus fumigatus* and *Aspergillus nidulans,* knockouts of pyridoxine
301 genes resulted in reduced virulence compared to wild-type in murine pulmonary and systemic
302 models, and was linked to interference with iron homeostasis [72, 73]. Vitamin B7 (biotin) can
303 increase the production of germ tubes in *Candida albicans* [74]. When biotin genetic knock-outs
304 were created in *Histoplasma capsulatum,* no reduction in virulence was observed [75]. Future
305 analysis of vitamin B6 and B7 pathways in *B. dendrobatidis* could yield interesting results into
306 understanding more pathogenic traits of *B. dendrobatidis.*

307 *Galleria mellonella,* possesses a complex immune response in the form of anti-microbial
308 peptides, lysozyme, and phagocytic cells [76]. When *G. mellonella* is infected with pathogenic
309 fungi, similar responses to vertebrate immune responses are observed [77, 78]. The
310 transcriptome of *G. mellonella* is also well-documented and allows for the better understanding
311 of the immune responses in this organism when faced with infection [79]. When *G. mellonella* is
312 infected with an external agent, prophenoloxidase is up-regulated, intracellular calcium levels are
313 decreased, and melanization is observed [80]. We show that secretions of *B. dendrobatidis* cause

314 significant melanization in the wax moth larvae. Together with our observation that intact *B.*
315 *dendrobatidis* cells cause melanization and death in *G. mellonella,* we propose the establishment
316 of *G. mellonella* as a model organism for future studies of this pathogen.

317 **Acknowledgments**

318 We thank A. Moss for her review of the manuscript, P. Mirzaei for her assistance in mass
319 spectrometry work, and members of San Francisco Laboratory for comments and discussion.

320 **Statements and Declarations**
321
322 *Funding*

323 Partial financial support was received for this research from the Texas Tech Association of
324 Biologists research mini-grant and the Texas Tech Graduate Student Research Support Award.

325 *Authors Contribution*

326 AMS, MSF, MZM: Development of methodology, AMS, MZM: planning and executing the
327 experiments, AMS: data analysis, AMS: writing original manuscript.

328 **Compliance with Ethical Standards**

329 *Competing Interests*

330 The authors have no relevant financial or non-financial interests to disclose.

331 *Ethics approval*

332 This study did not require ethical approval.

333 *Informed Consent*

This study did not use human subjects or materials gathered from human subjects. No informed consent was necessary.

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Figure Captions

Fig 1 Visualization of the health index score to rank the level of melanization in wax moth larvae. A score of 4 is no melanization with a score of 0 being complete melanization and death of the larvae.

Fig 2 Over-representation analysis utilizing Metaboanalyst 5.1 of 92 metabolites secreted from *Batrachochytrium dendrobatidis* from water-*B. dendrobatidis* as compared to water (N=3). Metabolite chemical structures were categorized by main class of compound and based on fold enrichment (number of hits/expected). * indicates those classes of compounds with enrichment values $p<0.05$ and FDR <0.05

Fig 3 Metabolite pathway analysis utilizing Metaboanalyst 5.1 of 92 metabolites secreted from *Batrachochytrium dendrobatidis* from water-*B. dendrobatidis* as compared to water. *Saccharomyces cerevisiae* KEGG pathway used a base for analysis and based on impact factor (# metabolites from enrichment/centrality)

Fig 4 Mass Spectrometry results of 245.12 m/z peak from a) *B. dendrobatidis* water, b) cyclo(L-Phe-L-Pro), c) cyclo(L-Phe-D-Pro), d) cyclo(D-Phe-L-Pro), and e) cyclo(D-Phe-D-Pro)

Fig 5 A) Health index score (degree of melanization) of wax moth larvae injected with *B. dendrobatidis* in 1X PBS and 1X PBS over 48 hrs of observations N=10, ** $P<0.01$, 3 replicates, and B) O.D. of hemocyte free hemolymph from larvae injected with *B. dendrobatidis* and 1X PBS, N=10, 3 replicates, **** $P<0.0001$

Fig 6 Health index score (degree of melanization) of wax moth larvae injected with *B. dendrobatidis* water 10X concentrated (BWC) and water control 10X (WC) over 48 hrs of observations N=10, **** P<0.0001, 3 replicates

Fig 7 Health index score (degree of melanization) of wax moth larvae injected with 1 mM cyclo(L-Phe-L-Pro) (L-L), cyclo(L-Phe-D-Pro) (L-D), cyclo(D-Phe-L-Pro) (D-L), cyclo(D-Phe-D-Pro) (D-D), and control water (Water) over 48 hrs of observation. N=10, **** P<0.0001, **P<0.01, N=3

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Table 1 Identity of selected compounds secreted from water-*B. dendrobatidis* as compared to water. Known compounds produced from *B. dendrobatidis* verify results (*) and data showing the presence of unique compounds by water- *B. dendrobatidis* (**) (N=3)

Compound Name	Molecular Weight	RT (min)	Log_2Fold	P-Value
Unknown (QL or Similar to D-Carnitine)**	235.1415	0.826	15.26	2.23E-06
S-Adenosylmethionine**	398.1365	0.631	13.96	1.61E-05
5'-S-Methyl-5'-thioadenosine*	297.0891	0.599	12.35	9.27E-05
Chitobiose, di-N-acetyl**	424.1685	0.813	11.52	1.79E-06
Tryptophol*	161.0839	5.299	8.21	9.46E-05
Cyclo(phenylalanyl-prolyl)**	244.1209	5.633	2.97	0.000926
DL-Tryptophan*	204.0897	3.312	2.88	0.039086
Cyclo(phenylalanyl-prolyl)**	244.1209	5.481	2.87	0.000346
Kynurenine*	208.0846	3.043	2.38	0.003395

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Table 2 Comparison of tyrodicine encoding genes in *Bacillus brevis* compared to *Batrachochytrium dendrobatidis* (*Bd*) amino acid sequences to determine putative tyrodicine-like molecule-encoding genes using NCBI BLAST

Protein in *Bacillus brevis*	Encoded function	*Bd* match E-value	*Bd* Encoded function
TycA (WP_137029047.1)	Peptide Synthetase	2e-86	Peptide Synthetase
TycB (WP_137029049.1)	Peptide Synthetase	3e-95	Peptide Synthetase
TycC (WP_137029046.1)	Peptide Synthetase	6e-92	Peptide Synthetase
TycD (AAC45931.1)	ABC Transporter	9e-83	ABC Transporter
TycE (AAC45932.1)	ABC Transporter	3e-73	ABC Transporter
TycF (RAT94638.1)	Thioesterase	0.25	Ubiquitin-protein ligase

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Table 3 Peak area comparisons of compound m/z peak 245.12 identified as cyclo(phenylalanyl-prolyl) utilizing Compound Discoverer 3.0 software to calculate the log2fold change differences between controls (Heated *B. dendrobatidis,* Heated Media Water, and Media Water) and sample of interest (*B. dendrobatidis* Water) and their respective p-values

Comparisons	Log_2Fold Change	P-Value
B. dendrobatidis Water/ Heated *B. dendrobatidis*	3.21	7.26E-06
B. dendrobatidis Water / Media Water	1.82	0.00357
B. dendrobatidis Water / Heated Media Water	4.27	7.48E-07
Heated *B. dendrobatidis* Water/ Media Water	-1.39	0.003829
B. dendrobatidis Water / Heated *B. dendrobatidis* Water	1.06	0.011177

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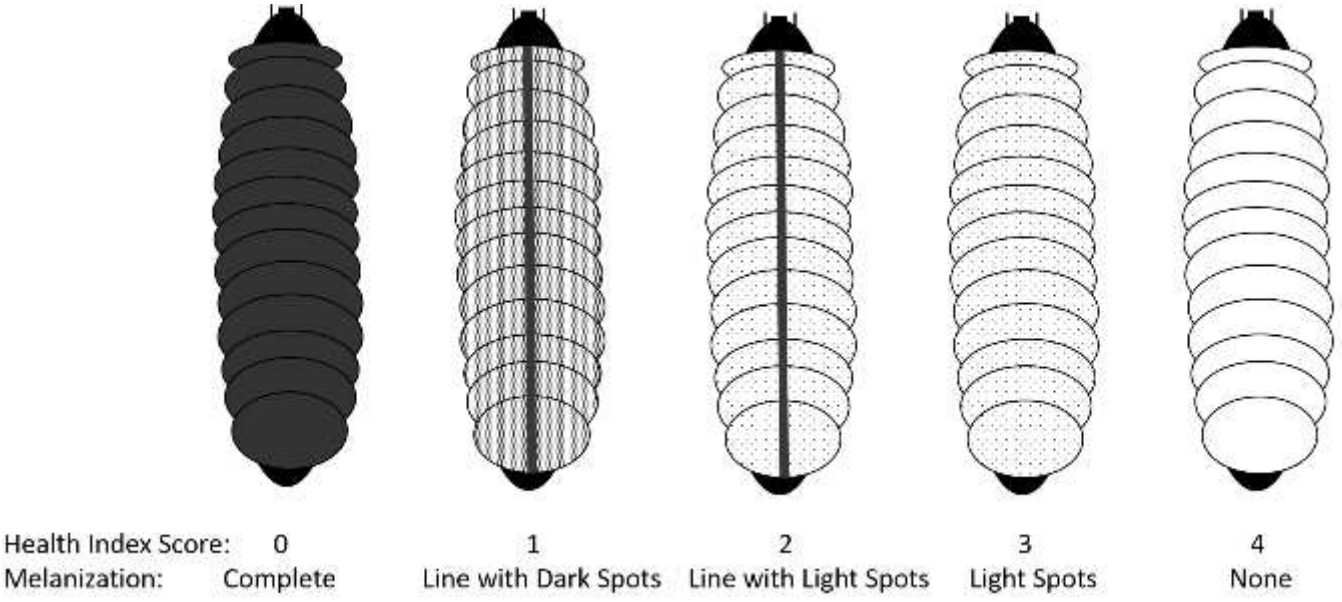
Figures



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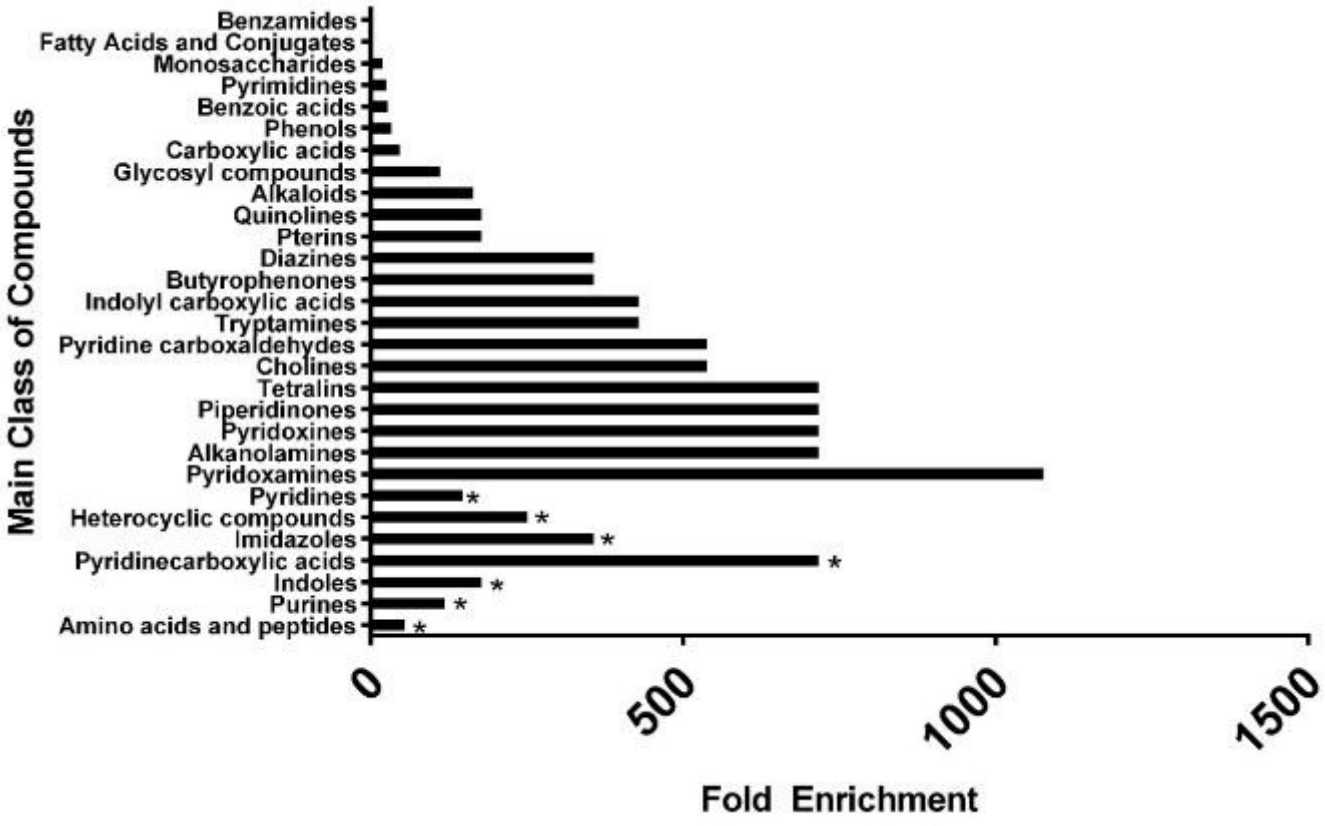

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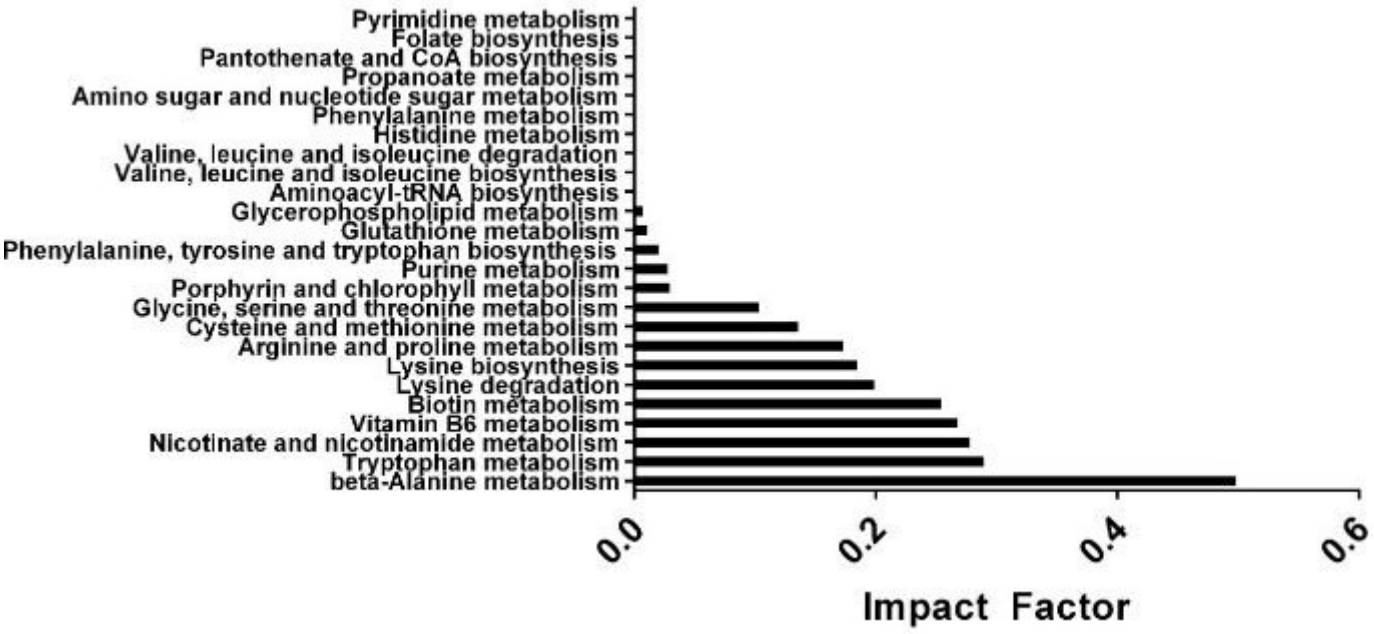

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Metabolite pathway analysis utilizing Metaboanalyst 5.1 of 92 metabolites secreted from Batrachochytrium dendrobatidis from water-B. dendrobatidis as compared to water. Saccharomyces cerevisiae KEGG pathway used a base for analysis and based on impact factor (# metabolites from enrichment/centrality)

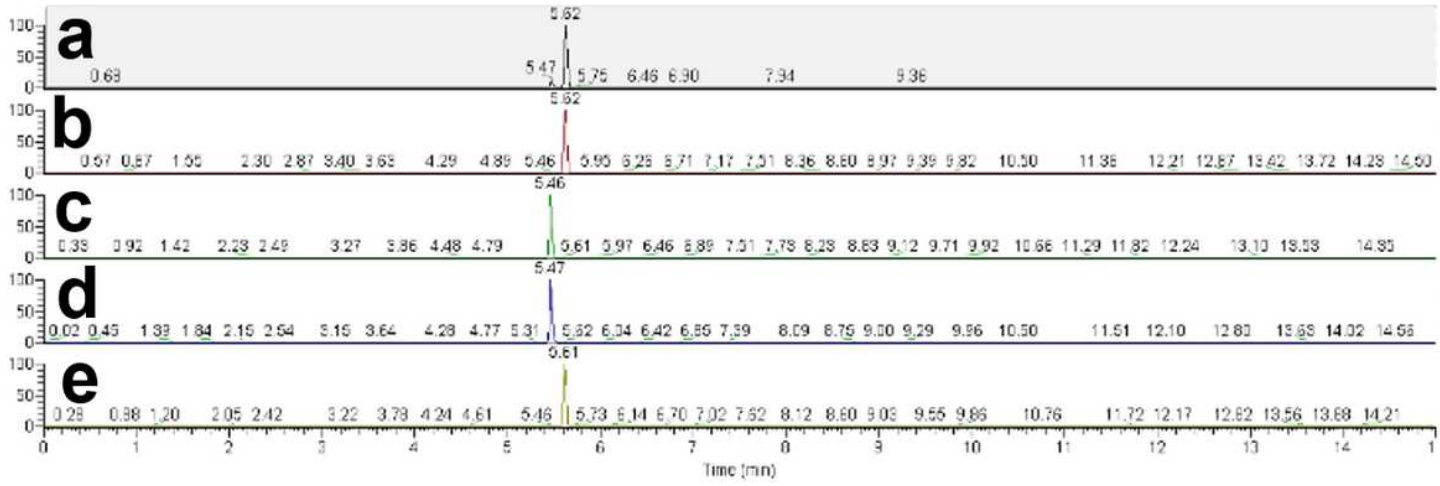


Figure 4

Mass Spectrometry results of 245.12 m/z peak from a) B. dendrobatidis water, b) cyclo(L-Phe-L-Pro), c) cyclo(L-Phe-D-Pro), d) cyclo(D-Phe-L-Pro), and e) cyclo(D-Phe-D-Pro)

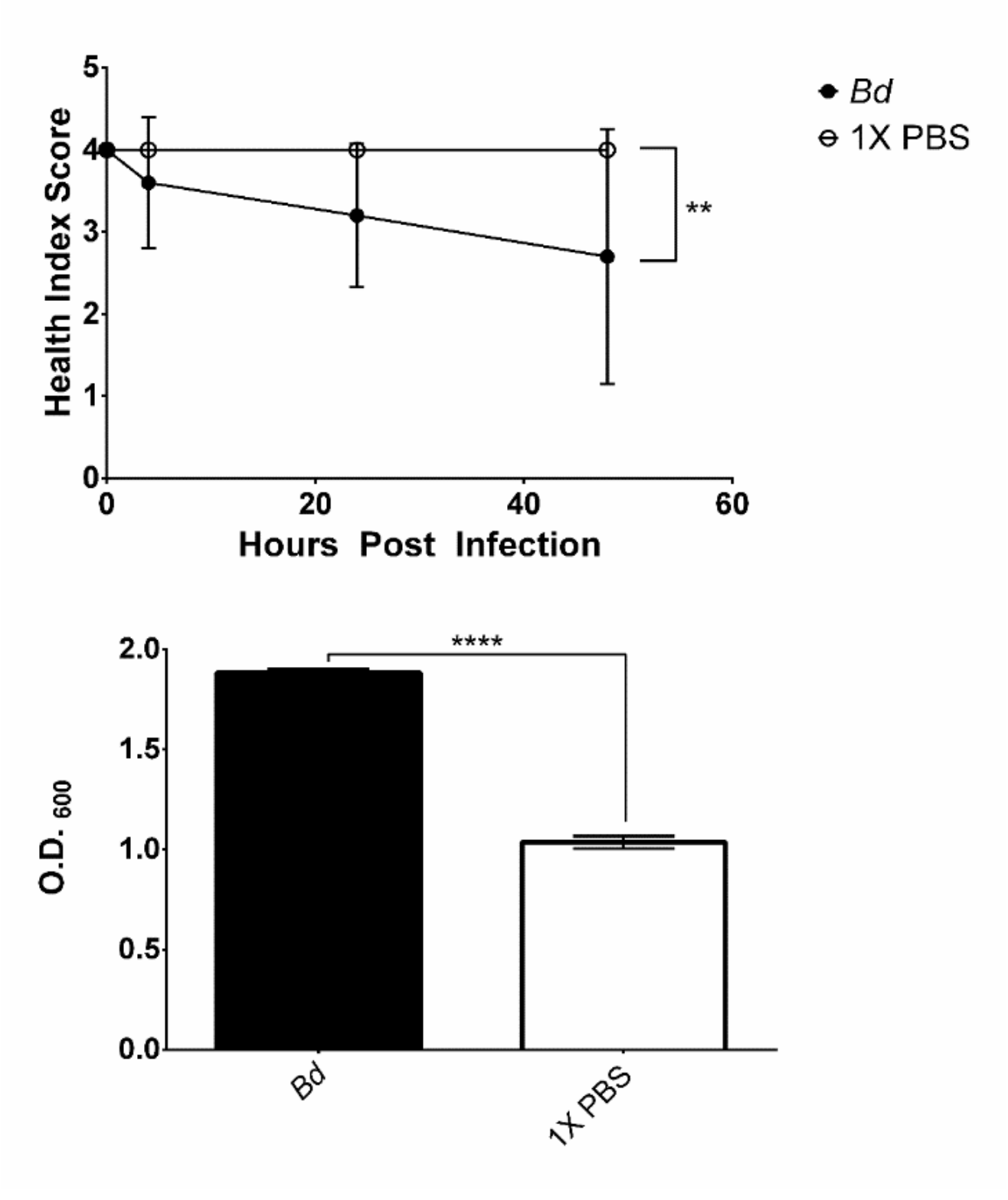
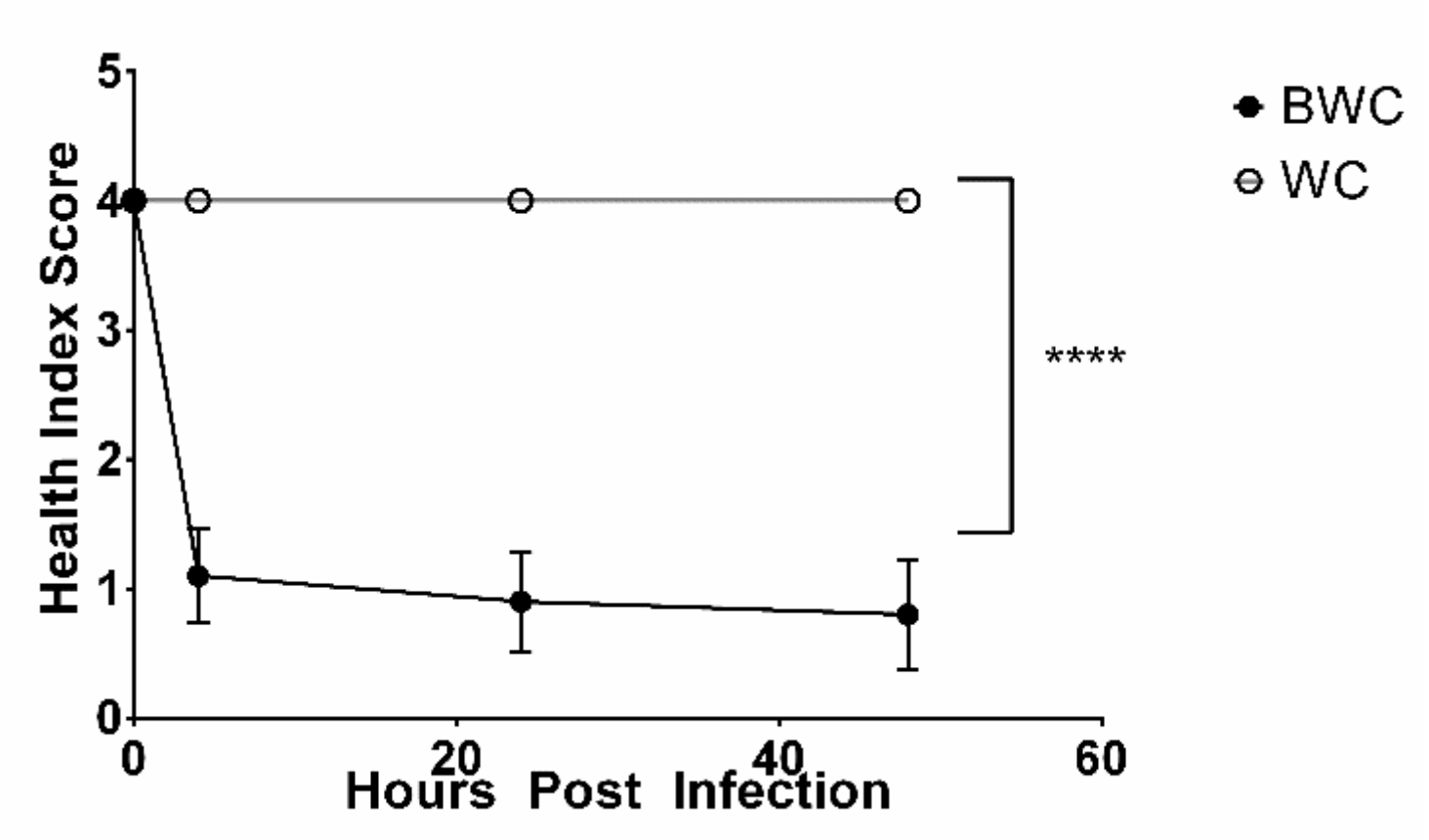


Figure 5

A) Health index score (degree of melanization) of wax moth larvae injected with B. dendrobatidis in 1X PBS and 1X PBS over 48 hrs of observations N=10, ** P<0.01, 3 replicates, and B) O.D. of hemocyte free hemolymph from larvae injected with B. dendrobatidis and 1X PBS, N=10, 3 replicates, **** P<0.0001



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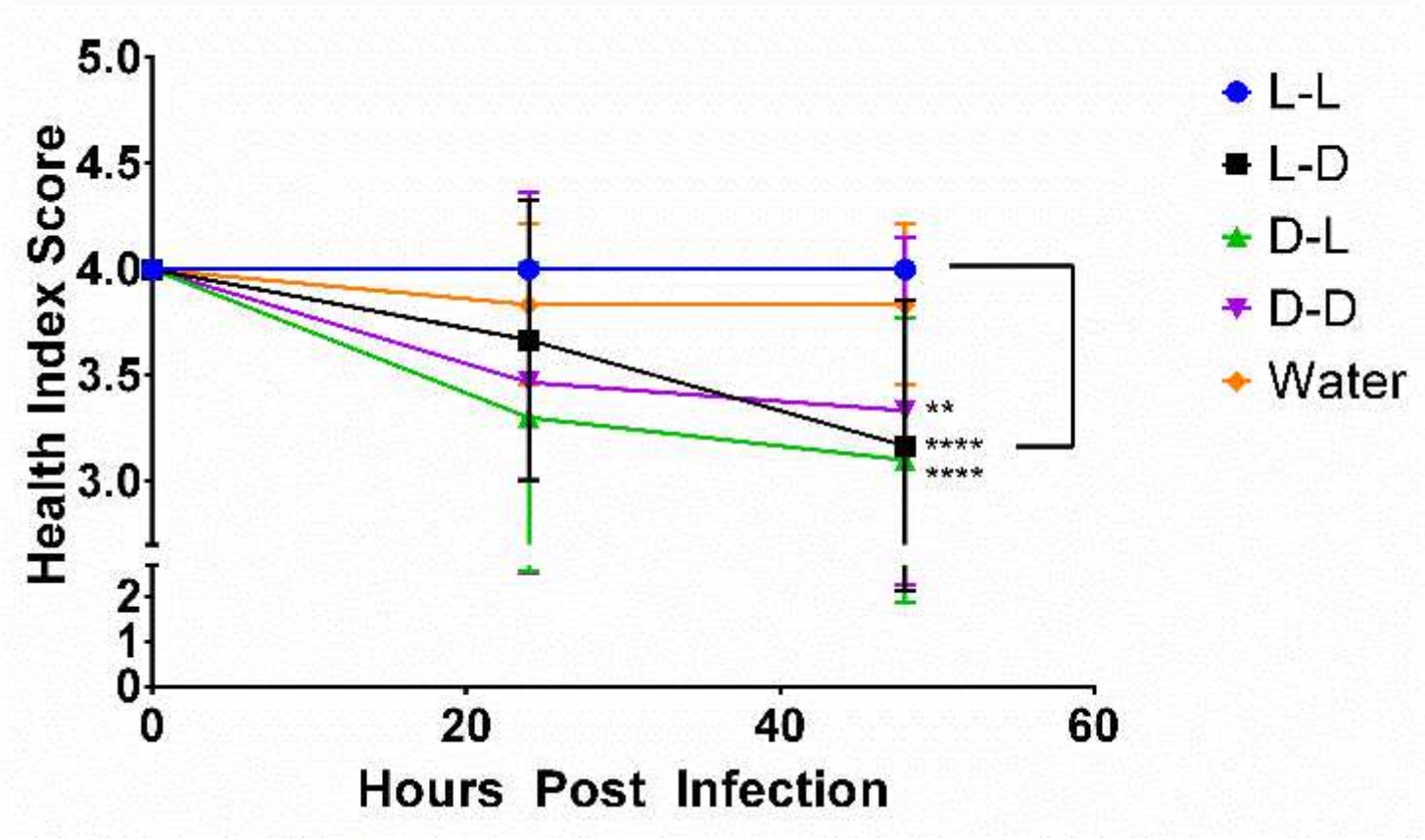

Figure 7

Health index score (degree of melanization) of wax moth larvae injected with 1 mM cyclo(L-Phe-L-Pro) (L-L), cyclo(L-Phe-D-Pro) (L-D), cyclo(D-Phe-L-Pro) (D-L), cyclo(D-Phe- D-Pro) (D-D), and control water (Water) over 48 hrs of observation. N=10, **** P<0.0001, **P<0.01, N=3

Supplementary Files

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