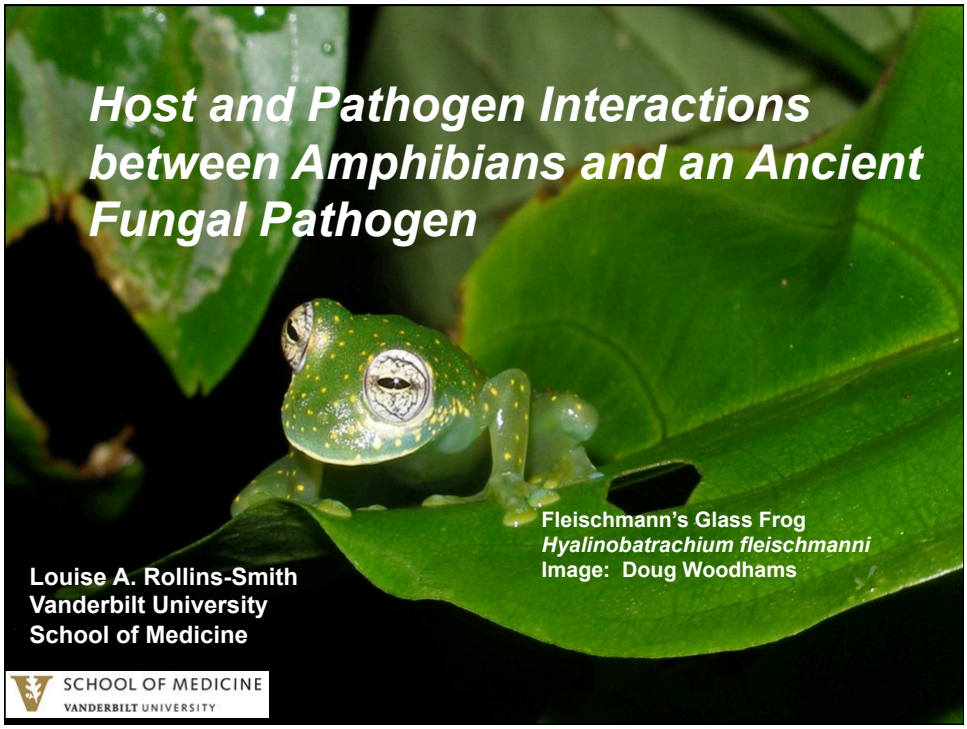
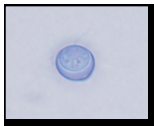
Host and Pathogen Interactions between Amphibians and an Ancient Fungal Pathogen



Fleischmann's Glass Frog
Hyalinobatrachium fleischmanni
Image: Doug Woodhams

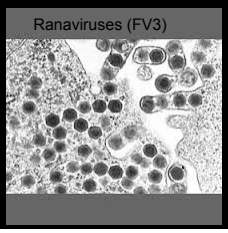
Louise A. Rollins-Smith
Vanderbilt University
School of Medicine

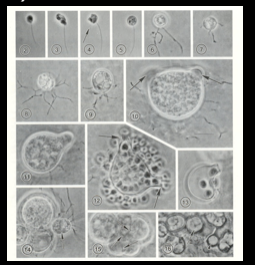
SCHOOL OF MEDICINE
VANDERBILT UNIVERSITY

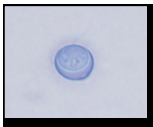


Introduction

- For at least four decades, amphibian populations have been declining.
- Although the causes are complex, most agree that emerging diseases are one of the most important causes.
- Disease agents linked to amphibian declines are
  - Ranaviruses
  - *Batrachochytrium dendrobatidis (Bd)*
  - *Batrachochytrium salamandrivorans (Bsal)*







Introduction

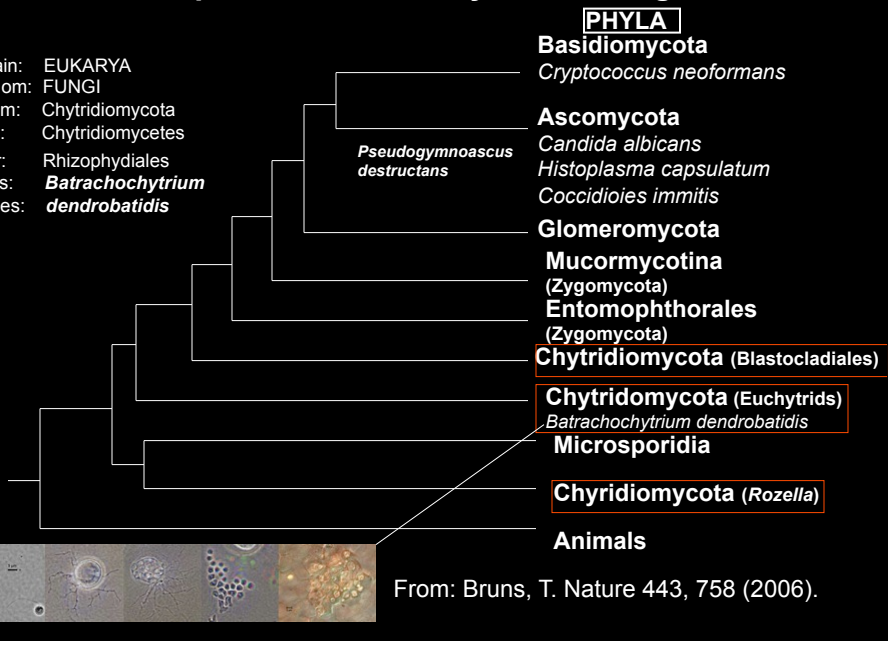
- My lab has focused our research on *Batrachochytrium dendrobatidis (Bd)*, a chytrid fungal pathogen that causes the skin disease chytridiomycosis.
- Until recently, *Bd* was the only known chytrid pathogenic to vertebrates.
- In 2013 a new species, *Batrachochytrium salmandrivorans* (*Bsal*) was described.
  - Likely originated in Asia
  - Lethal to European salamanders
  - Significant threat to North American salamanders. USA is "hot spot" of salamander diversity.

Yellow-flecked Glassfrog (Cochranella albomaculata)

Fire salamander
(*Salamandra salamandra*)

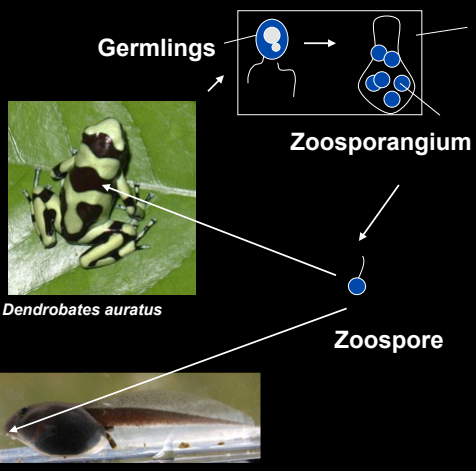
Simplified evolutionary tree of fungi

Domain: EUKARYA
Kingdom: FUNGI
Phylum: Chytridiomycota
Class: Chytridiomycetes
Order: Rhizophydiales
Genus: ***Batrachochytrium***
Species: ***dendrobatidis***

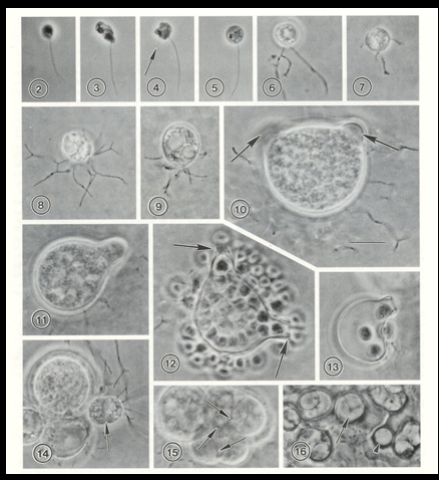


From: Bruns, T. Nature 443, 758 (2006).

Life Cycle of Batrachochytrium dendrobatidis (Bd)



[Stages that occur within frog skin cells]



Duration of life cycle is 4-5 days at 22ºC

J.E. Longcore et al. 1999. Mycologia 91:219

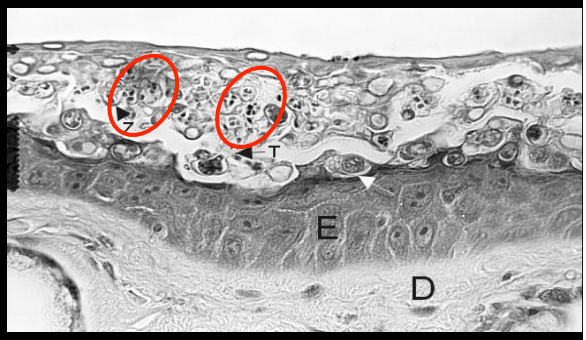
Bd appears to kill by disturbance of skin functions

- Electrolyte transport across ventral skin is impaired in diseased frogs.
- Plasma sodium and potassium concentrations are significantly reduced.

Voyles et al. 2009.** Science **326: 582-585.

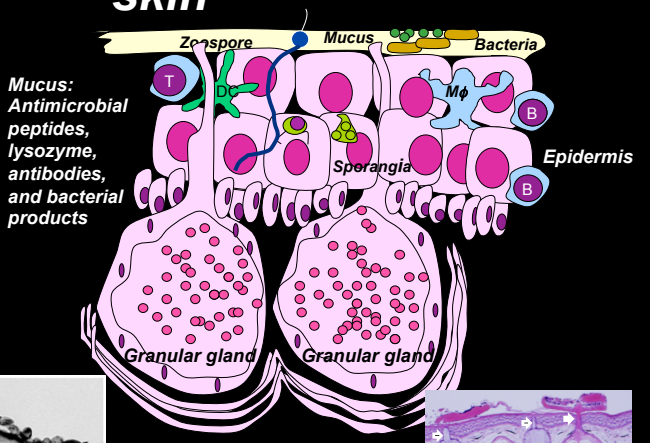
Chytridiomycosis is a disease of the skin epidermis

- Unlike other fungal diseases, this pathogen does not migrate to other organs.
- Therefore, to understand immune defenses against chytridiomycosis, we need to understand the skin defenses.



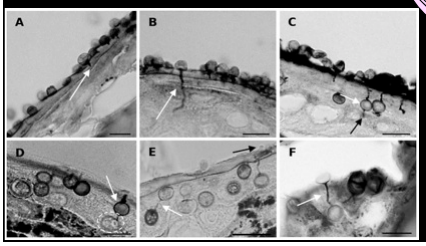
From Berger, L. et al., 1998. *Proc. Natl. Acad. Sci.* USA 95:9031-9036

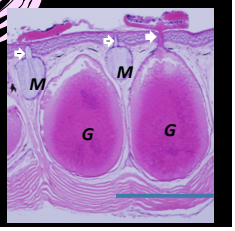
Model of immune defenses in the skin



Mucus: Antimicrobial peptides, lysozyme, antibodies, and bacterial products

Van Rooij P. et al. 2012 PLoS One 7(7): e41481



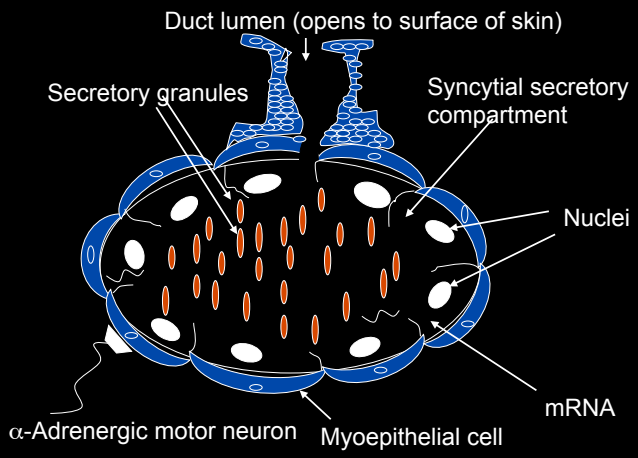


Gammill W. et al. 2012. *Dev. Comp. Immunol.* 37: 19-27

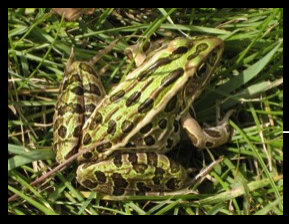
The dermis of the skin of many amphibians is rich in granular glands which produce and store antimicrobial peptides (AMPs)

AMPs

- 10-50 amino acid residues
- Cationic, hydrophobic and usually adopt an a-helical conformation
- Act by binding to charged residues or through hydrophobic interactions with target cells and disruption of membrane function



Are AMPs present on the skin of resting and active frogs and do they inhibit Bd?

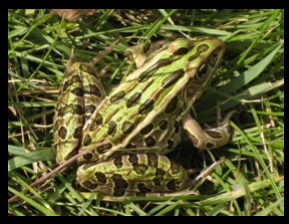


First Aid

Antibiotic Mantle?

First Aid Kit?

Peptide collection and MALDI-TOF



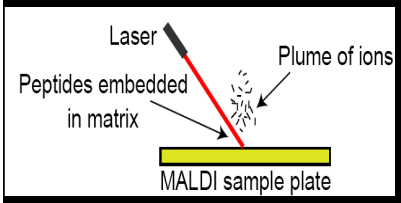
Apply stimulus

Place frog in collection buffer for 15 mins

Add 1%TFA to inactivate proteases

Enrich peptides over C18 Sep-paks

Determine peptide concentration by microBCA

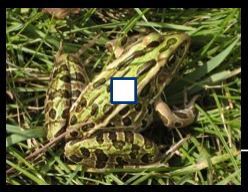


Determine peptide profile by Mass spectrometry

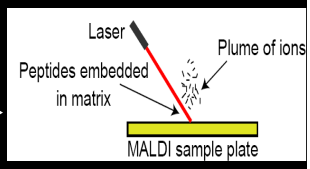
Direct sampling of skin secretions for MALDI-TOF MS

Carbon embedded film

Activate in 100% methanol

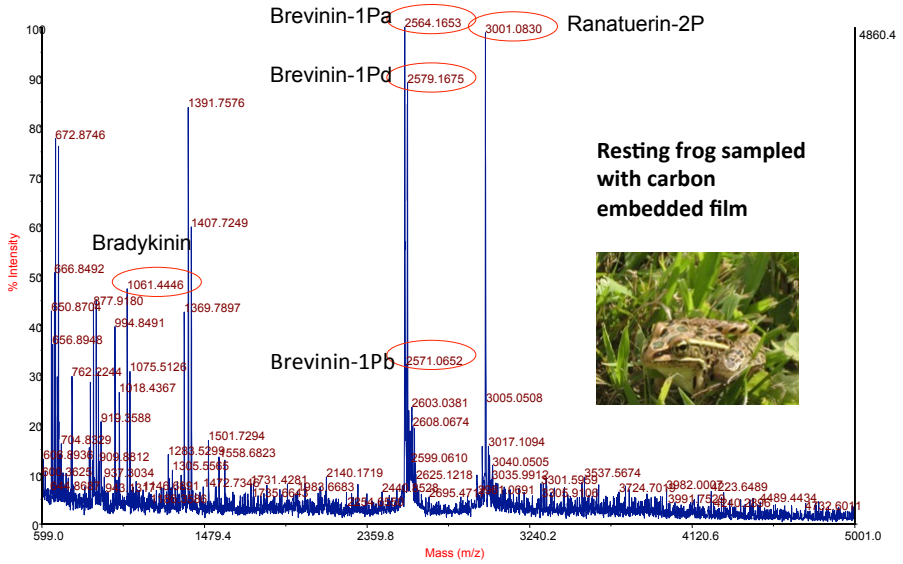


Apply directly to frog

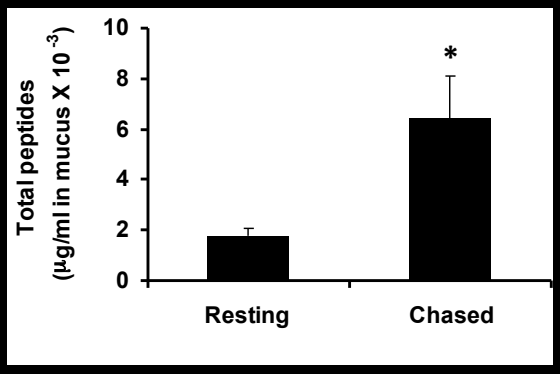


Perform MALDI-TOF

MALDI-TOF profile of skin AMPs by direct sampling

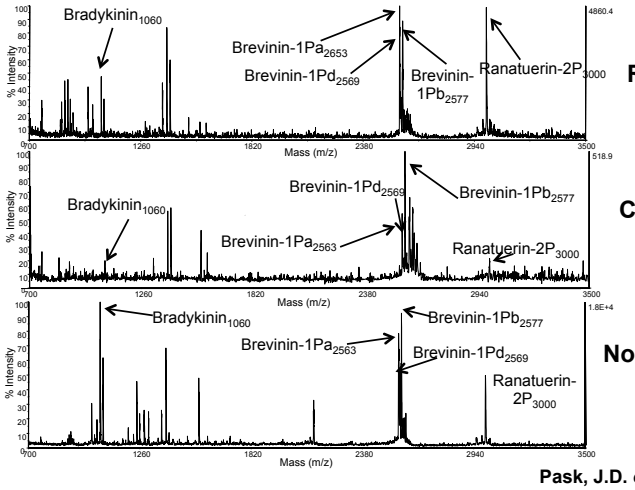


Chased frogs release more skin peptides



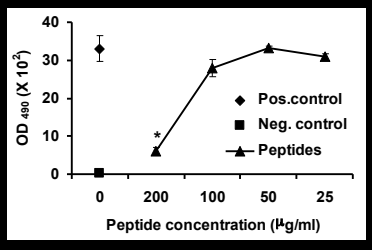
Pask, J.D. *et al.* 2012. *Global Change Biol.* 18:1231-1238

MALDI-TOF analysis shows AMPs in resting and chased frogs

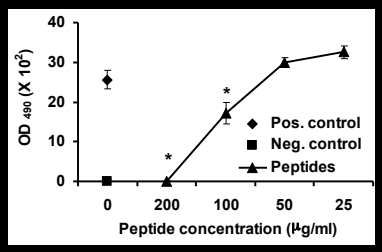


Pask, J.D. *et al.* 2012. *Global Change Biol.* 18:1231-1238.

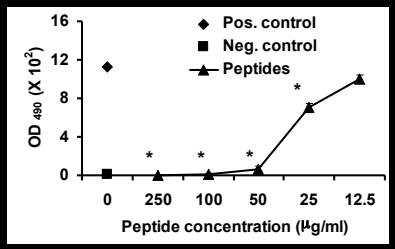
Peptides collected from resting and chased frogs have antimicrobial activity



Resting



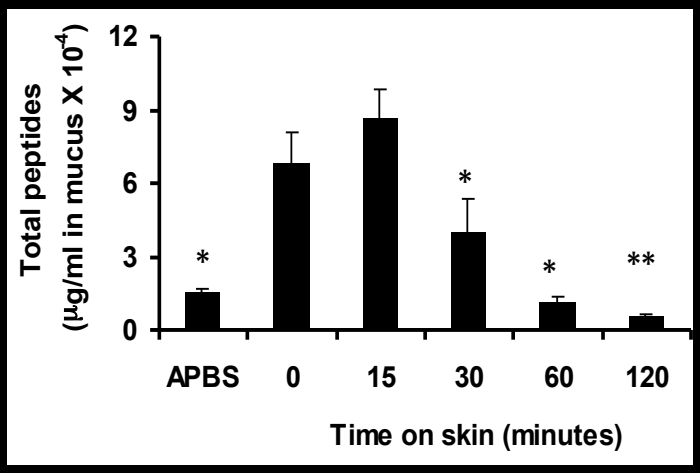
Chased



Norepinephrine-induced

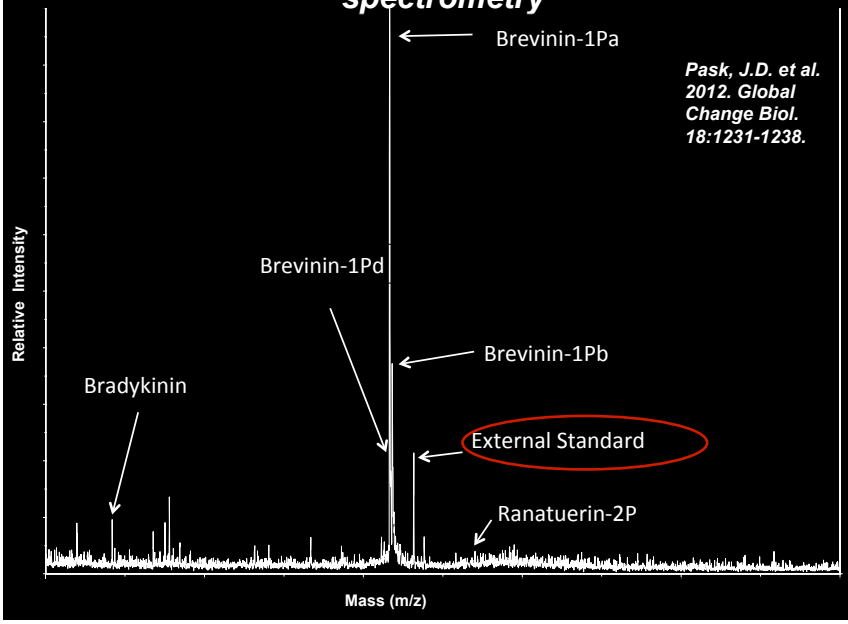
Pask, J.D. *et al.* 2012. *Global Change Biol.* 18:1231-1238.

Following secretion, how long are the skin peptides detectable?

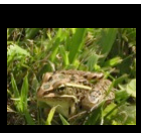


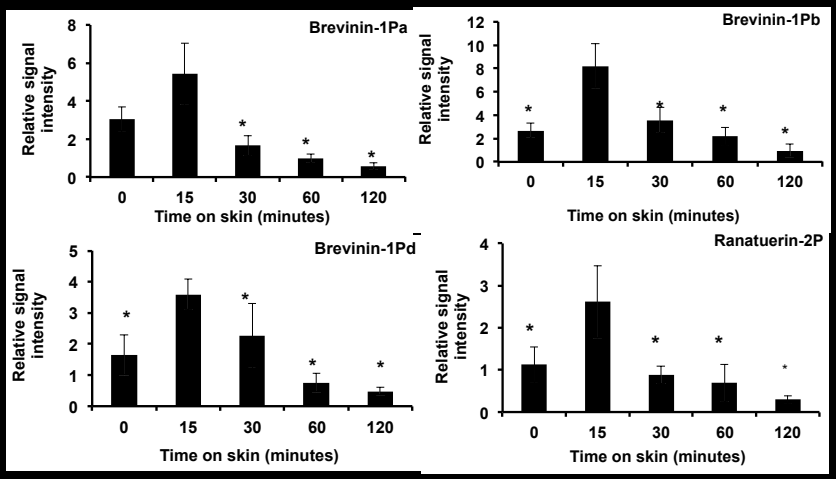
Pask, J.D. *et al.* 2012. *Global Change Biol.* 18:1231-1238.

Use of an external standard allows us to determine relative concentrations of AMPs on the skin by mass spectrometry



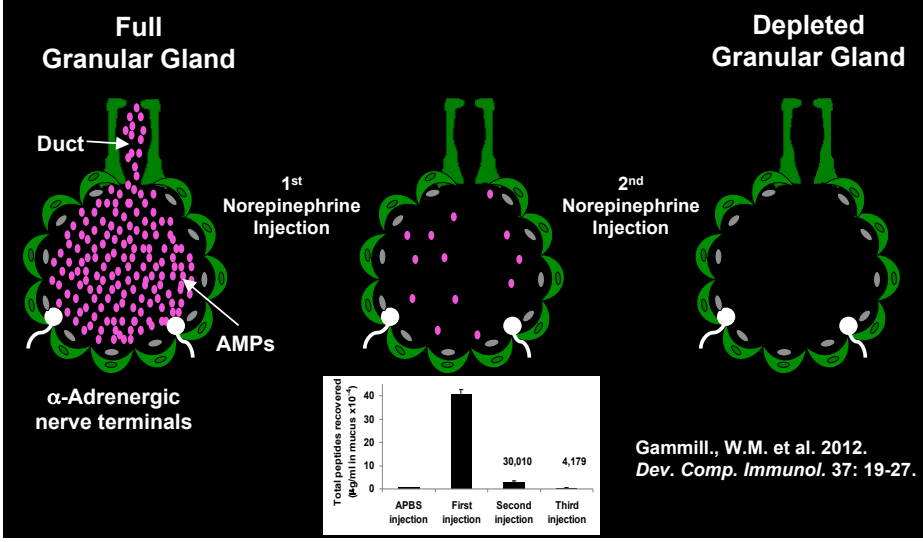
Use of an external standard shows that individual AMPs persist for about 60 min





Pask, J.D. et al. 2012. Global Change Biol. 18:1231-1238.

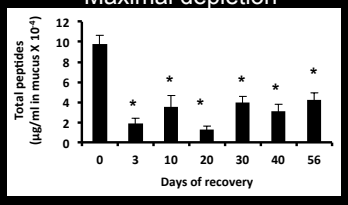
Can we deplete and exhaust skin peptide stores?



Gammill., W.M. et al. 2012. *Dev. Comp. Immunol.* 37: 19-27.

After depletion, peptide renewal is slow

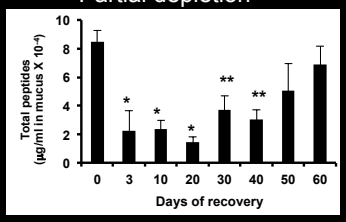
Maximal depletion

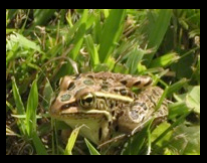


Before depletion

After depletion

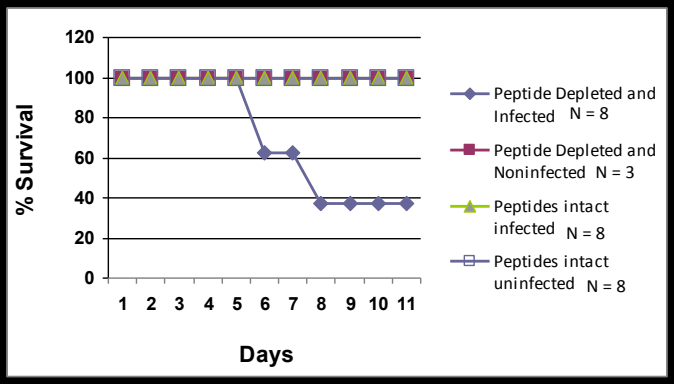
Partial depletion





Pask et al. 2013. J. Exp. Biol. 216: 2908-2916.

Depletion of skin peptides in Rana pipiens juveniles results in greater susceptibility to Bd



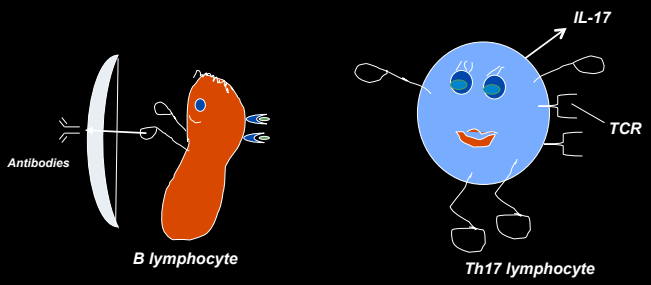
Pask et al. 2013. *J. Exp. Biol.* 216: 2908-2916.

Summary of AMP studies

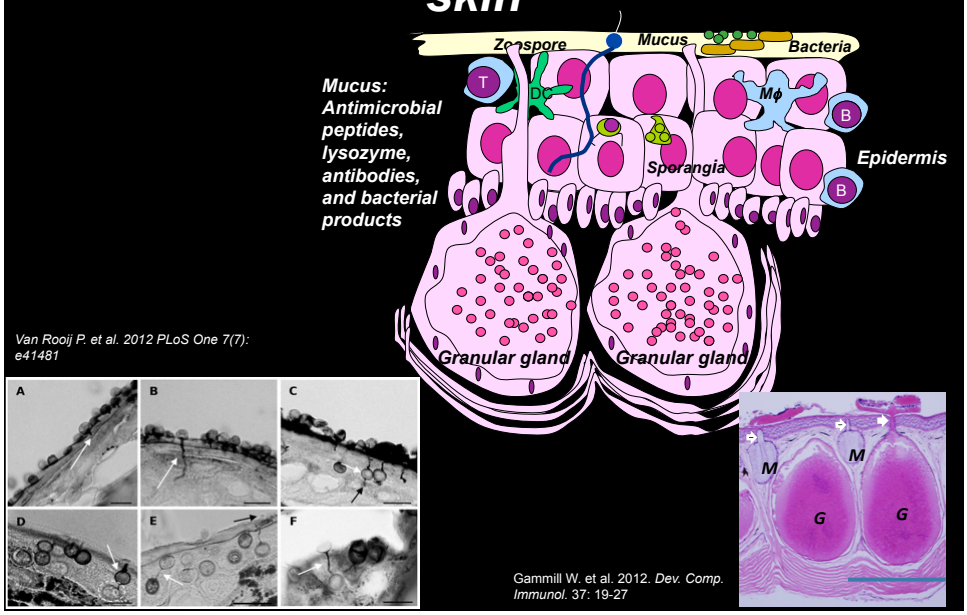
- Frogs constitutively release low amounts of AMPs that inhibit *Bd*, and AMP defenses are elevated following a simulated predator attack.
- AMPs are effective inhibitors of *Bd* at these low constitutive concentrations but degrade within two hours, protecting the integrity of the skin and commensal bacteria.
- Depletion of AMP responses increases susceptibility to *Bd*.

What is the role of the adaptive immune system in control of Bd?

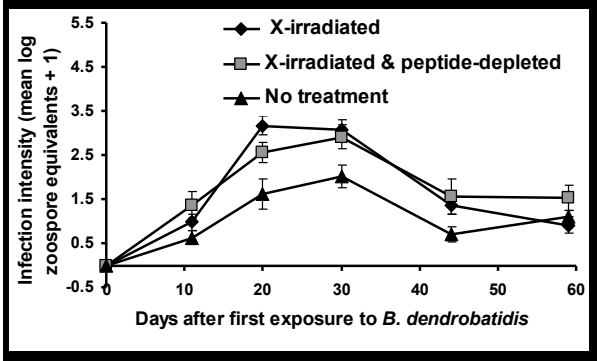
- Is there a role for B lymphocytes?
- What is the role for T lymphocytes?
- Does *Bd* evade adaptive immune defenses?



Model of immune defenses in the skin

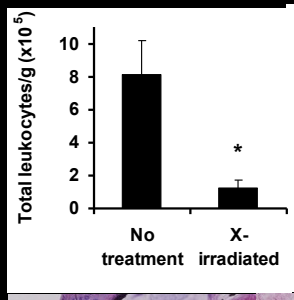


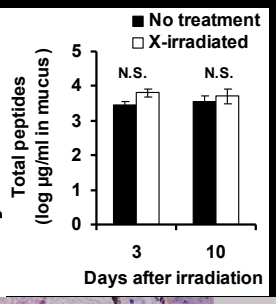
Sublethal X-irradiation reduced resistance to Bd infection



Ramsey, J. et al. 2010. *Infect. Immun.* 78:3981-3992

X-irradiation reduced lymphocyte numbers but did not impair function of granular glands





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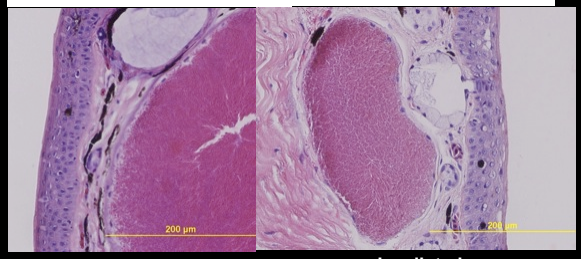
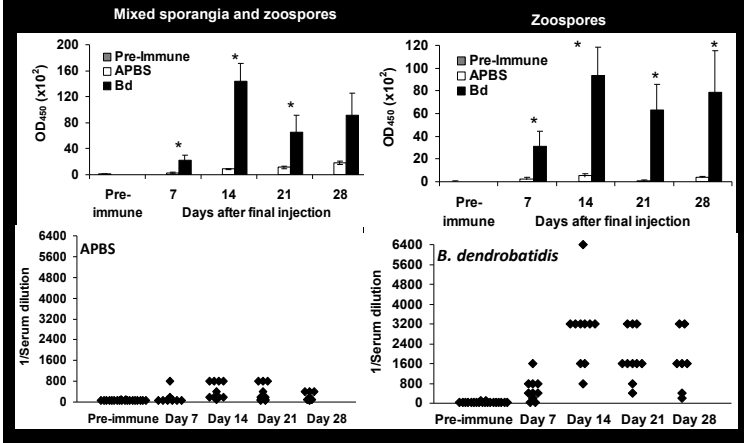


Not irradiated Irradiated

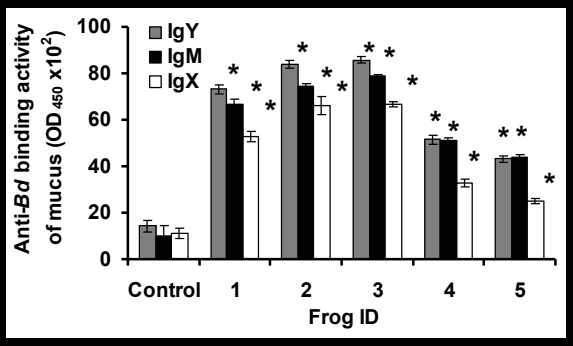
Immunization against Bd resulted in development of high-titer antibody responses

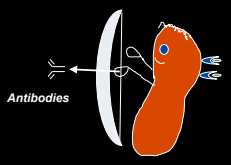
IgY antibody responses



Ramsey, J. et al. 2010. *Infect. Immun.* 78:3981-3992

Skin mucus of Bd exposed frogs contains antibodies of three classes that can bind specifically to Bd



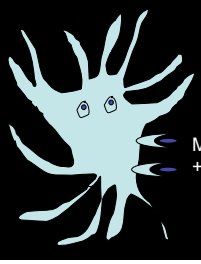


Ramsey, J. et al. 2010. *Infect. Immun.* 78:3981-3992

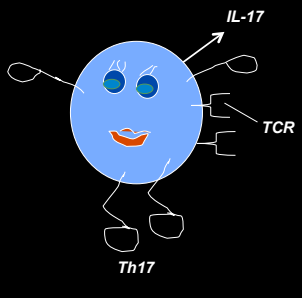
- Collectively, these studies suggest that both innate defenses (antimicrobial peptides) and conventional lymphocyte-mediated immune responses help to protect frogs from lethal *Bd* infections.
- If the immune system is capable of recognizing this pathogen, why are many amphibian species not protected?

How does Bd escape immune surveillance?

- Successful immunity against fungal pathogens begins with recognition by phagocytic cells, which begin to control the infection.
- The phagocytic cells then recruit lymphocyte effectors.
- The lymphocytes amplify the response and recruit more phagocytic cells to clear the infection.



MHC Class II
+ Peptide

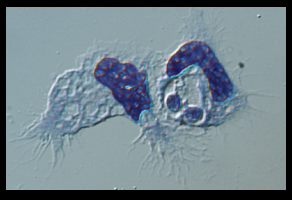


Macrophages and neutrophils can phagocytose Bd

Developing thallus with rhizoids

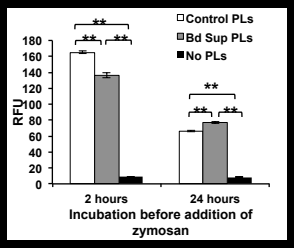
Maturing zoosporangium

Frog neutrophil has engulfed *Bd* cells

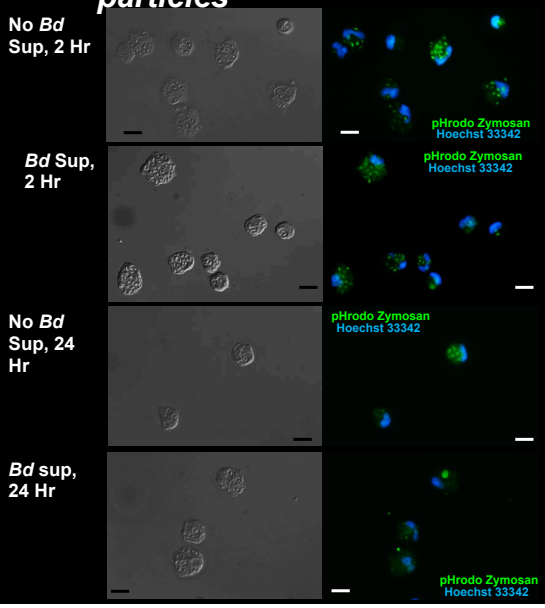


Frog macrophage has engulfed *Bd* cells

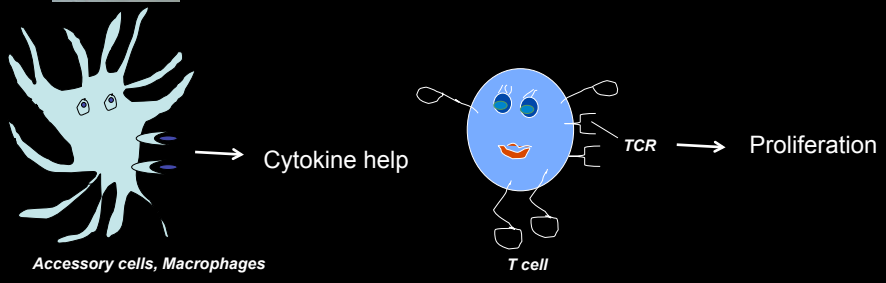
Bd culture supernatants have a minimal effect on the ability of peritoneal leukocytes to engulf zymosan particles



Zymosan is a protein-carbohydrate complex prepared from yeast cells. It binds to TLR 2 on macrophages to induce proinflammatory responses.

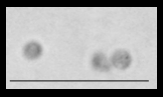


Overnight treatment of peritoneal leukocytes with Bd Sup does not impair accessory function

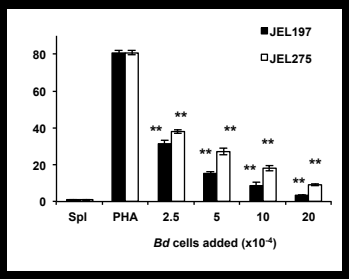


Fites, J.S. et al. 2013. *Science* 342:366-369.

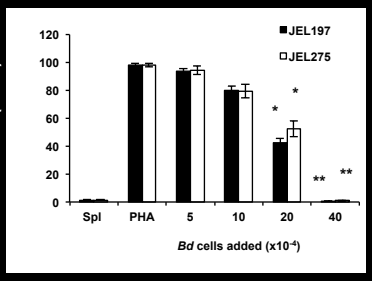
Live or heat killed Bd cells inhibit T lymphocyte proliferation



Live *Bd* co-cultured *with* T cells

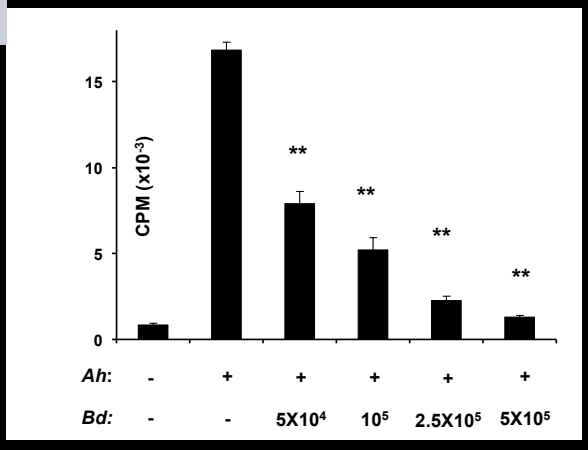


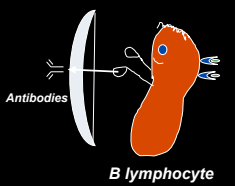
Killed *Bd* co-cultured with T cells



Fites, J.S. et al. 2013. *Science* 342:366-369.

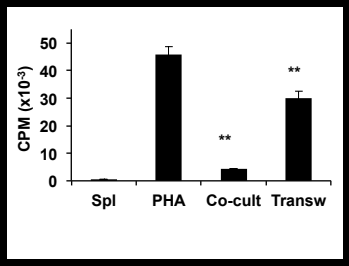
Live Bd cells also inhibit B lymphocyte proliferation

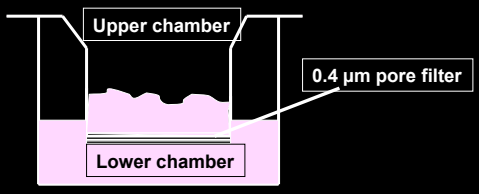


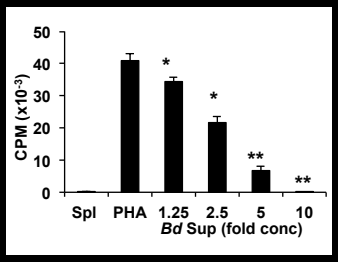


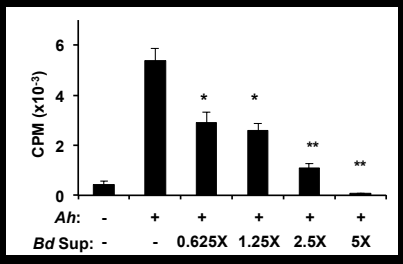
Fites, J.S. et al. 2013. *Science* 342:366-369.

Inhibition of lymphocyte proliferation occurs even when Bd cells are separated from lymphocytes by a 0.4 µm membrane or replaced by Bd supernatants



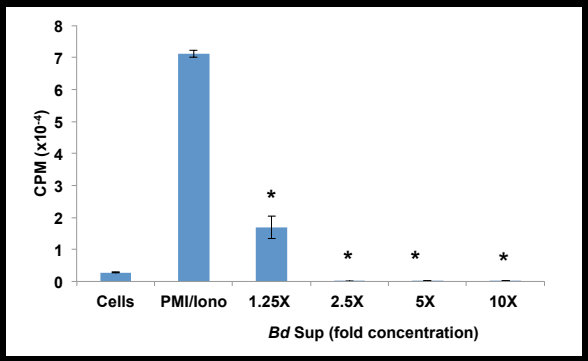






Fites, J.S. et al. 2013. *Science* 342:366-369.

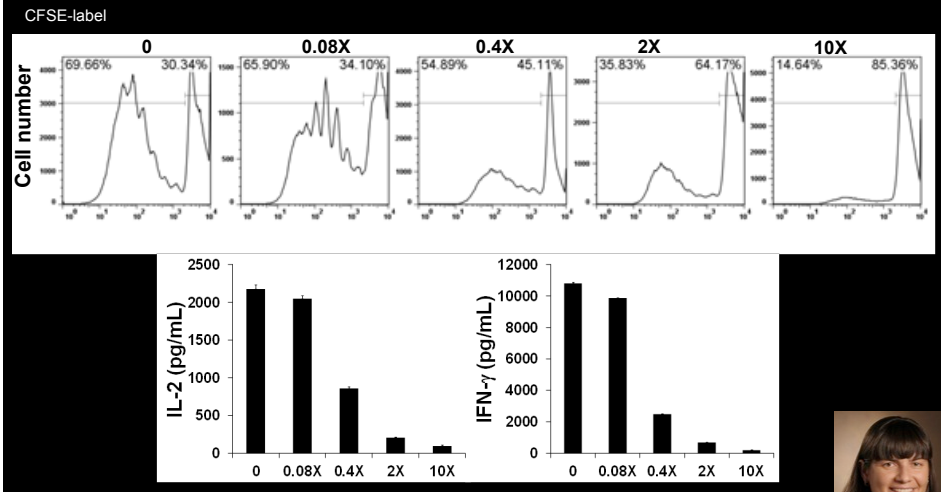
Bd-induced inhibition of lymphocytes is not limited to frogs

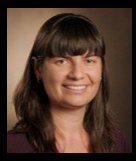


Therefore, the lymphotoxic factors target a vulnerability shared by lymphocytes of amphibians and mammals.

With Sarah Parker Collier and Tom Aune

Bd factors also inhibit proliferation and cytokine production by human T cells

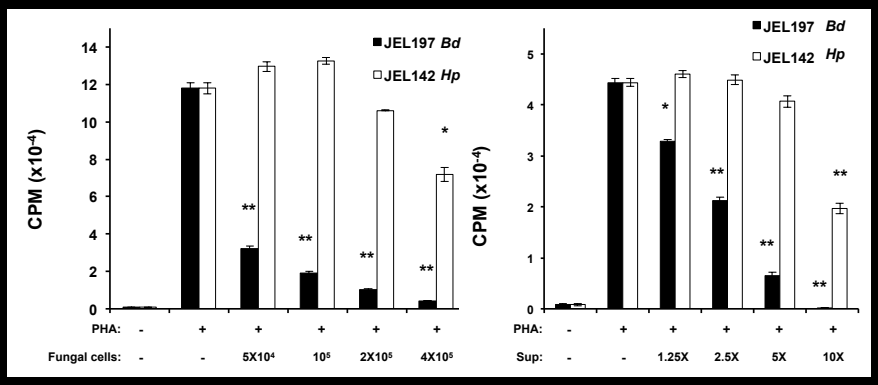




Proliferation of human T cells stimulated with anti-CD3 and anti-CD28 was impaired by *Bd* supernatants. Supernatants from *Bd* cultures inhibited secretion of IL-2 and IFN-γ by purified human CD4+ T cells

With Kyra Richter

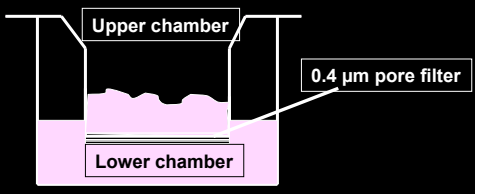
A nonpathogenic chytrid Homolaphlyctis polyrhiza does not inhibit lymphocyte proliferation

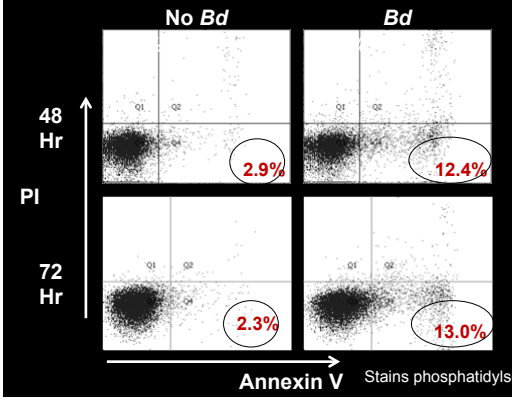


JEL142 = nonpathogenic chytrid, Homolaphlyctis polyrhiza

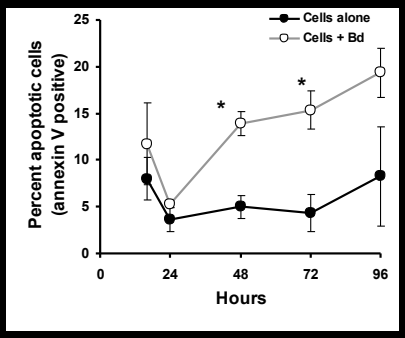
Fites, J.S. et al. 2013. *Science* 342:366-369.

One mechanism of inhibition is the induction of lymphocyte apoptosis by killed Bd



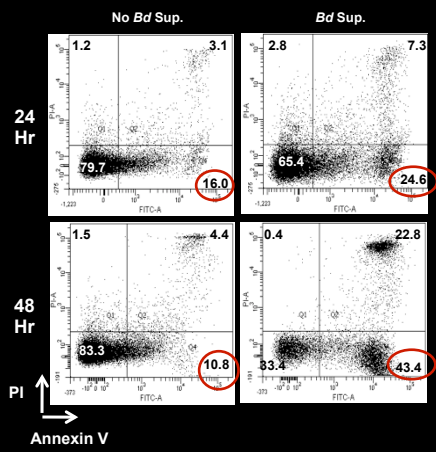


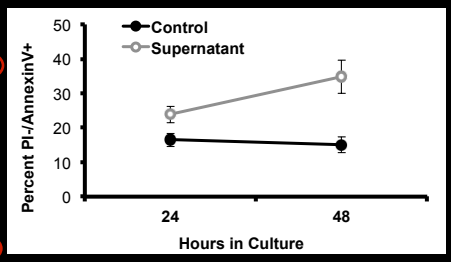
Stains phosphatidylserine



Fites, J.S. et al. 2013. *Science* 342:366-369.

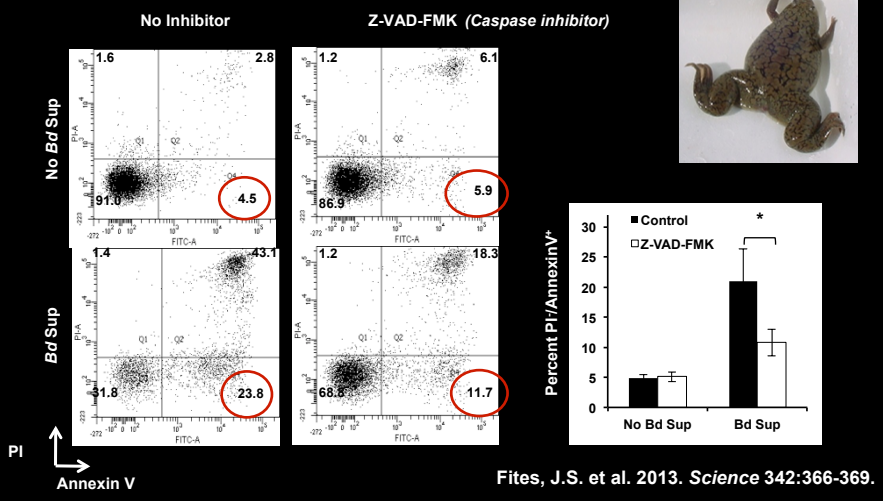
Lymphocyte apoptosis is also induced by Bd supernatants





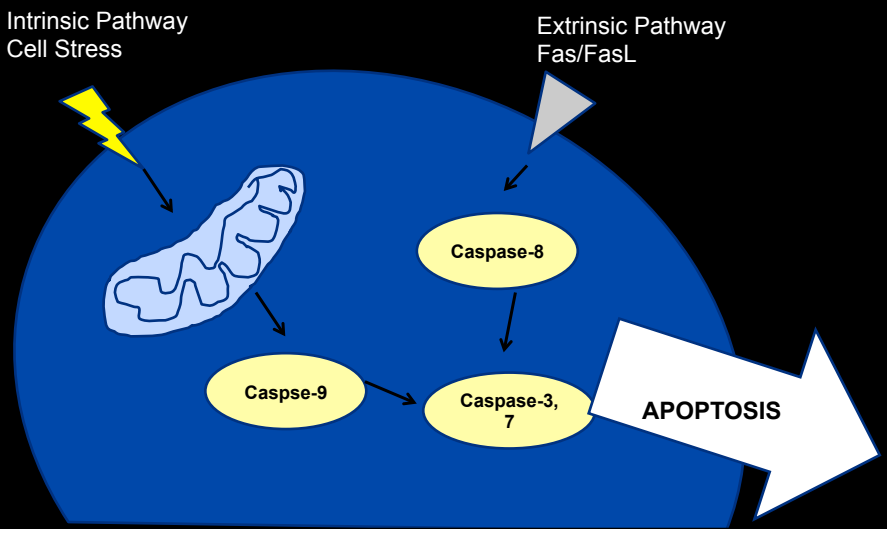
Fites, J.S. et al. 2013. *Science* 342:366-369.

Lymphocyte apoptosis induced by Bd supernatant is diminished in the presence of a pan caspase inhibitor

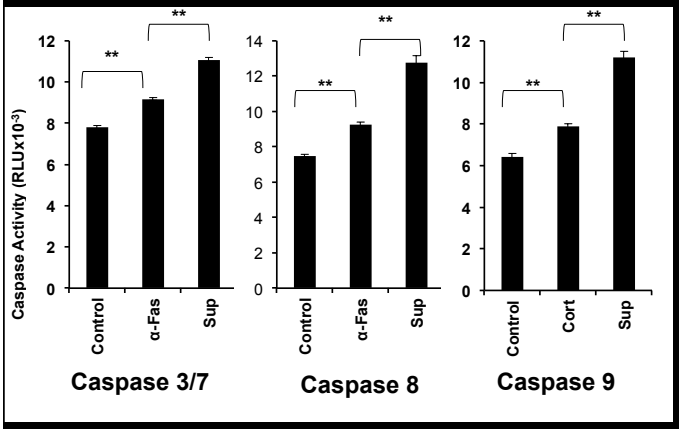


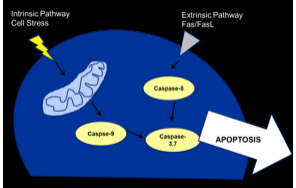
Fites, J.S. et al. 2013. *Science* 342:366-369.

Pathways of Apoptosis



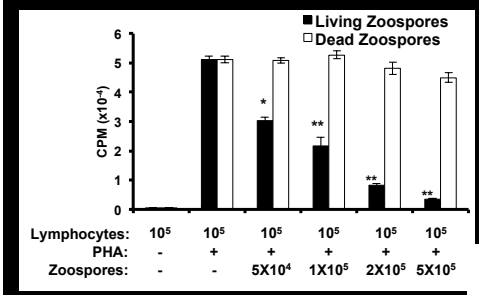
Bd supernatants appear to induce apoptosis via both the intrinsic and extrinsic pathway

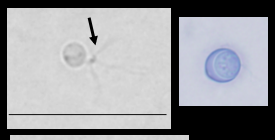




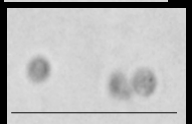
Fites, J.S. et al. 2013. *Science* 342:366-369.

Zoospores can inhibit only if they mature in culture; zoospore supernatants do not inhibit

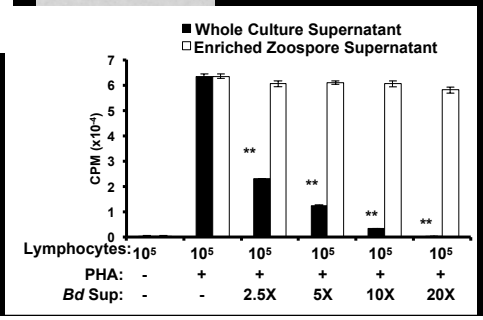




Live



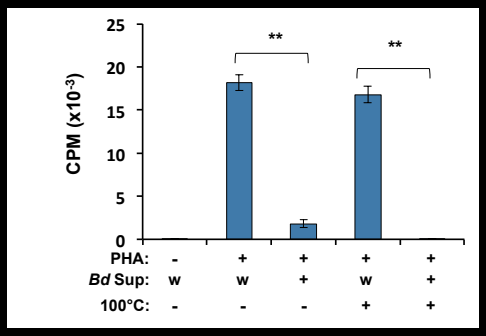
Heat-killed

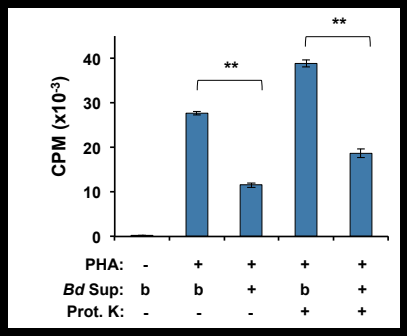


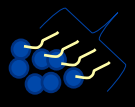
Fites, J.S. et al. 2013. *Science* 342:366-369.

What is the nature of the immunotoxic factor or factors?

***Bd* supernatants retained their capacity to inhibit lymphocyte proliferation following treatment with high heat or proteinase K**





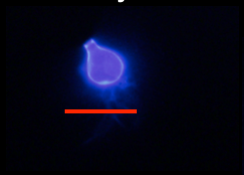


Proteinase K

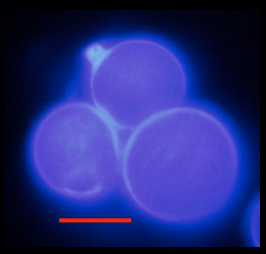
Fites, J.S. et al. 2013. Science ***342:366-369.***

Production of the Bd factors is impaired by treatment with agents that inhibit cell wall synthesis

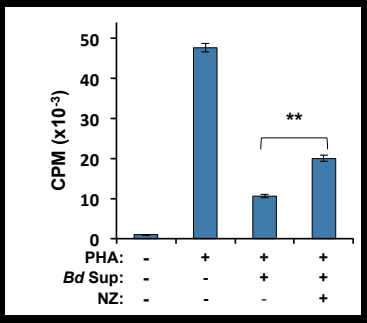
No nikkomycin Z



3.13 µg/ml nikkomycin Z



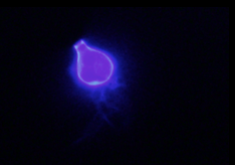
Supernatants from nikkomycin Z-treated cells

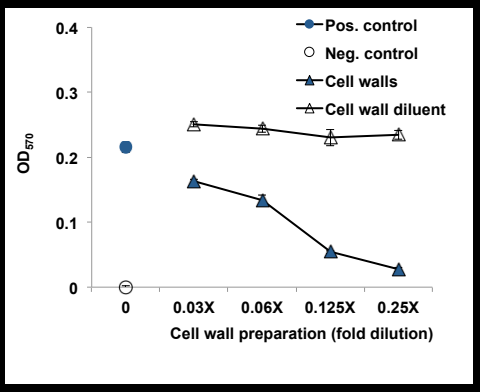


Therefore, the inhibitory factors may be components of the cell wall.

Fites, J.S. et al. 2013. Science 342:366-369.

Isolated cell walls have significant inhibitory activity against T cells

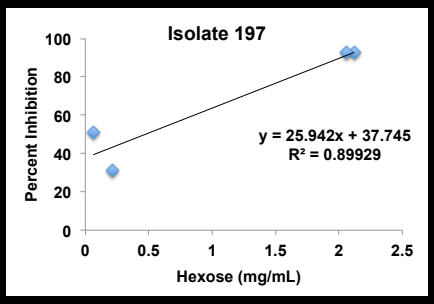


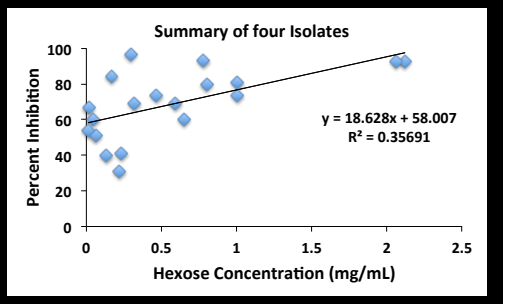


Therefore, some inhibitory factors are enriched in the cell wall.

Jack Lee

Inhibition of T cells is positively correlated with carbohydrate concentration of supernatants



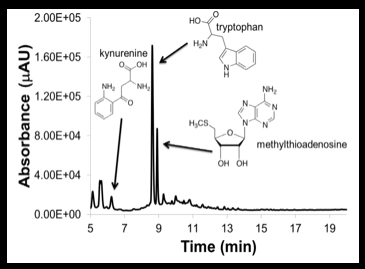


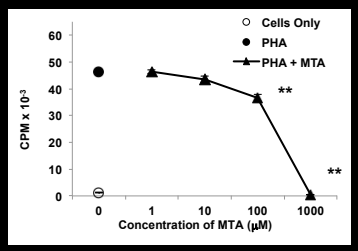
two-tailed t-test, p = 0.0069.

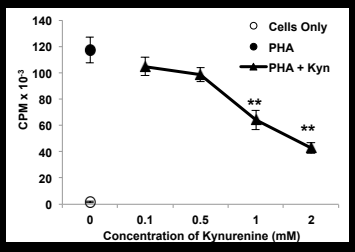
Therefore, the some inhibitory factors may be soluble carbohydrates associated with the cell wall.

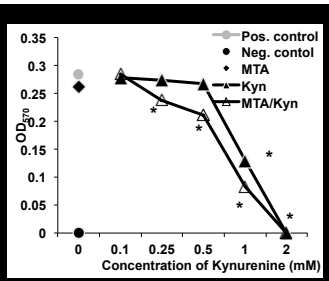
Tim Chappell

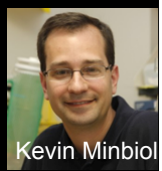
HPLC analysis of cell-free Bd supernatants show the presence of tryptophan, kynurenine, and MTA (methylthioadenosine)











Kevin Minbiol

Thomas Umile

Summary of immune evasion by Bd

- ***Bd*** **factors inhibit T and B cells by induction of apoptosis.**
- **The inhibitory factors are water soluble and can cross a cell-impermeable barrier.**
- **The inhibitory factors are heat-resistant and protease-resistant, suggesting that they are not proteins or peptides.**
- ***Bd*** **factors produced after treatment with the chitin synthase inhibitor, nikkomycin Z, have reduced activity, suggesting that they may be cell-wall components.**


Agalychnis callidryas

Summary of immune evasion by Bd

- **Enriched cell-wall preparations alone inhibit T cells**
- **Inhibitory activity correlates with carbohydrate content.**
- ***Bd*** **also releases small metabolites, MTA and kynurenine, which may also induce T-regulatory cells or otherwise inhibit lymphocytes.**


Dendrosophus ebraccatus

Summary of immune evasion by Bd

- **Taken together, these results suggest that *Bd* has evolved strategies to resist immune surveillance in order to survive in amphibian skin.**
- **Ongoing studies aim to identify additional fungal immunotoxic factors and define their mechanism of action.**

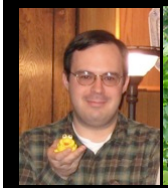
Litoria xanthomera
Image D. Woodhams

Acknowledgements

Rollins-Smith Lab
- **J. Scott Fites**
- **Laura K. Reinert**
- **Whitney Holden**
- **Timothy Chappell**
- **David Qu**
- **Julia Quinn**
- **Jack Lee**
- **Andrea Shiakolas**

2014

2015



Jeremy Ramsey **Doug Woodhams** **Jim Pask**

Support: NSF IOS-0520847, IOS-0619536, and IOS-0843207, IOS-1121758