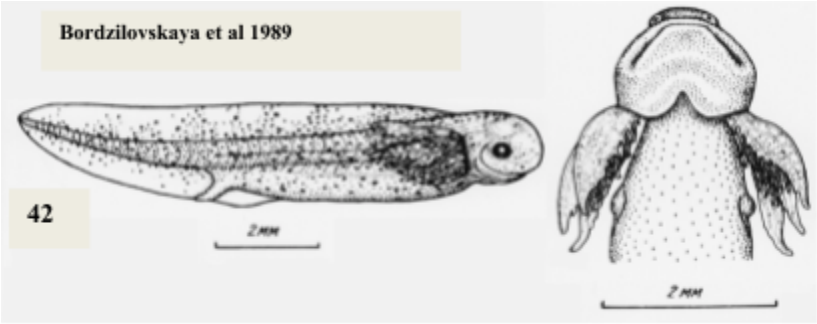
Axolotl

Newsletter of the
Ambystoma Genetic Stock Center
Department of Neuroscience & Spinal Cord and Brain Injury Research Center
University of Kentucky
ambystoma@uky.edu



2020 Issue

Director's Note – Randal Voss

I hope everyone in the community is healthy and safe, the last few months have certainly been trying. The University of Kentucky sent undergraduates home for the semester in March, including several of our student axolotl care workers. Soon after this, all but essential research was halted. Care for the AGSC axolotl population was thankfully deemed essential, and with a reduced workforce, we all pitched in to accomplish axolotl care. I acknowledge our dedicated student workers (Logan Dortsch, Arabella Jackson, Elise Maul, and Micaiah McNabb) and staff (Chris Muzinic, Laura Muzinic, and Grace Zimmerman) for all of their efforts during the pandemic. Thankfully, labs at UK are starting to open up again; we are hopeful that everyone in the community will be back in their labs and classrooms soon. Please let us know how we can best serve your research and educational needs as you start back up.

Last July 2019, approximately 90 investigators attended the 2nd Salamander Models in Cross-Disciplinary Biological Research at Northeastern University in Boston, MA. The meeting brought together a diverse group of researchers from around the world, including PI's, post-docs, graduate students, and undergraduate students. Many thanks to Karen Echeverri, Kate McCusker, James Monaghan, and Jessica Whited for hosting the meeting, and many thanks to Maximina Yun and Tatiana Sandavol Guzman for agreeing to host what will now be the 2021 meeting in Dresden, Germany. More information about this meeting and other relevant salamander information will be made available via email and the **Salamander Research Network,** a Slack Workspace that has been setup to facilitate communication among salamander researchers. If you have not already signed up, please contact Moshe Khurgel for an invitation to join!

Last year, we were surprised to learn that some of our axolotls tested positive by qPCR for the chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*). This fungus can cause chytridiomycosis, a skin disease that is lethal to some amphibians and especially anurans. However, susceptibility to this disease varies within and among species and there have been relatively fewer reports of chytridiomycosis in salamanders. In this issue of *Axolotl*, UK undergraduate Mirindi Kabangu and I detail results from our studies of *Db* in the AGSC. We found that only adults test positive for *Db* and *Db*+ individuals carry very few spores, orders of magnitude less than numbers associated with chytridiomycosis in anurans. Consistent with this, all of our axolotls show no signs of disease and all are in good health. Please see our article below for more details.

The axolotl provides a great model for research activities in the classroom. In this issue, we describe an embryo regeneration assay that we have been using in our research and undergraduate courses at UK. The assay uses embryos to introduce axolotls, chemical screening, and tissue regeneration to students. If you have questions or want help implementing this assay at your school or in your lab, please do not hesitate to ask.

I will close on some good news. First, we updated our website and ordering system last year, and we are now working to build a new relational database for our animal records. Second, we are in the process of installing the second of two new recirculating systems that will increase our ability to fill orders that request relatively large numbers of 3-5 cm and 5-8 cm axolotls. Third, our renewal grant application to continue P40 NIH funding was awarded. We will be able to serve your needs for at least the next 5 years, which at that time will mark 20 years of continuous service at UK.

Finally, please acknowledge the use of AGSC axolotls in your publications by referencing Research Resource Identifiers (RRIDs) for the axolotls that you use and our funding mechanism (P40OD019794). We appreciate the support and look forward to serving your needs and interests into the future.

Community News

Participants at Salamander Models in Cross-Disciplinary Biological Research Meeting in Boston. From the camera of Sergej Nowoshilow.

The second annual Salamanders in Cross Disciplinary Biological Research, otherwise referred to as the “Salamander Summit”, was held in Boston on July 23-25th, 2019. The meeting was built upon the successful first meeting held in Vienna, Austria in 2018. The unique aspect of the second meeting was that it included, for the first time, trainees including undergraduates, graduate students, and postdoctoral researchers. Held on the campus of Northeastern University in the heart of Boston, the meeting was co-organized by Catherine McCusker from University of Massachusetts Boston, Jessica Whited from Harvard University, Karen Echeverri from the Marine Biological Laboratory, and James Monaghan from Northeaastern University.

The conference was well attended, included 88 attendees from 11 countries, highlighting the true international nature of salamander research. The salamander species represented included the axolotl, Eastern Newt, Spanish ribbed newt, spotted salamander, and even lizards made an appearance. Meeting topics ranged from chromosomal organization, genome assembly, lamprush chromosomes, gastrulation, epigenetics, DNA damage, endosymbiosis, and obviously regeneration including the spinal cord, brain, retina, lens, heart, muscle, joint, tail, and limb. Importantly the majority of talks and posters were presented by trainees, the following of which won awards:

Best Graduate Student Trainee Poster Presentation:

1st: Ruben Garcia Vazquez, University of Florida, The role and polarization of axolotl macrophages in wound healing.
2nd: Kaylee Wells, UMass Boston, The molecular basis of size regulation during axolotl limb regeneration.
3rd: Belfran Carbonell, Universidad de Antioquia, DNA damage during axolotl tail regeneration.

Best Postdoctoral Trainee Poster Presentation:

1st: Warren Vieira, UMass Boston, RA-induced pattern formation supports a hierarchical model of patterning in the amphibian limb regeneration.
2nd: Prayag Murawala, Single-cell analysis uncovers convergence of cell identities during axolotl limb regeneration.
3rd: Nour Al Haj Baddar, University of Kentucky, Identification of Axolotl MMP gene family members, including novel salamander-specific MMP paralogs.

Best Trainee Platform Presentation:

1st: Keith Sabin, Marine Biological Labs, AP-1cFos/JunB/miR-200a regulate the pro-regenerative glial cell response during axolotl spinal cord regeneration.
2nd: Konsta Sousounis, Harvard University, DNA damage and the role of Eya2 during axolotl limb regeneration.
3rd: Stephanie Tsai, Harvard University, Midkine regulates wound epidermis function and development in limb regeneration.

After the meeting, PI's met to discuss needs of the overall salamander community as well as needs of specific salamander models. PI's identified the need to accumulate information about axolotl transgenic and knock-out lines that could be brought into the Ambystoma Genetic Stock Center or European labs for distribution. Also, the PI's thought it a great idea to generate a list of needs and research resources that could be presented to funding agencies for consideration of targeted funding. Other needs and resources were identified, including the need to develop cryopreservation methods for salamander sperm, refined genome assemblies, compiled lists and further development of antibodies, molecular probes and pharmaceuticals, and online genomic and bioinformatic knowledgebases for each salamander model.

The meeting was highly interactive and injected a sense of comradery across the salamander research community. The next meeting is scheduled to take place in Dresden, Germany in 2021. Support was provided by the Developmental Systems Hybridoma Bank, Zeiss Microscopy, Iwaki Aquatics, an NIH R13 conference grant, and The Society for Developmental Biology.

NIGMS Research Organisms Workshop

In September 2019, Dr. Voss attended a workshop sponsored by the National Institute of General Medical Sciences (NIGMS) that discussed the need to pursue research of new and under-utilized research organisms. The workshop can be viewed online via this link. It is a long video, but if you have time, please watch the keynote and first session, including Dr. Voss's pitch for axolotl research approximately 1:35 minutes into the video. Please consider submitting future proposals to NIGMS as they showed data indicating no significant difference in their funding for model vs new/under-utilized research organisms over the past couple of years, and this is likely to continue into the future.

Salamander League Seminar Series

Many thanks to Katia Del Rio-Tsonis, Karen Echeverri, and Tatiana Sandoval Guzmán for organizing an online, Zoom Salamander Seminar Series for the community. The first speaker Alfredo Cruz Ramirez, gave a great talk about the Lin28/let-7 circuit and metabolic reprograming during axolotl limb regeneration. Please spread the word and consider being a speaker. You can join the group via this weblink: https://docs.google.com/spreadsheets/d/1q3BYy25QzkQPguejR-98azYgPq43sFxnQG6ySI_kUDM/edit?usp=sharing

NIH Award to Develop Sperm Cryopreservation Method for the Axolotl

Recently, Dr. Terrence Tiersch (Aquatic Germplasm and Genetic Resources Center, Louisiana State University) was awarded a NIH Grant (R24OD028443) to develop a method to cryopreserve axolotl sperm and work with AGSC staff to perform the method and develop an infrastructure for managing frozen stocks. An additional objective of Dr. Tiersch's project is to stablish a comprehensive, centralized unit ("Hub") to integrate activities across NIH-funded stock centers and their communities, and develop approaches and documentation for cryopreservation. This includes development of protocols and pathways, outreach programs, community interaction, standardization, freezing services, and training.

Please Consider Sharing Axolotl Lines With the AGSC

Resource sharing is essential to building a strong axolotl research community. If you have made a transgenic or knock-out line that you feel would be of value to others, please contact the AGSC to discuss import and distribution of your line to more broadly enable community efforts.

Axolotl Embryo Tail Regeneration Assay

S. Randal Voss[1], Larissa Ponomareva[2], and Jon Thorson[2]

[1]Department of Neuroscience, Spinal Cord and Brain Injury Research Center, and AGSC, University of Kentucky, Lexington KY 40536
[2]Center for Pharmaceutical Research and Innovation, Pharmaceutical Sciences, University of Kentucky, Lexington KY 40536

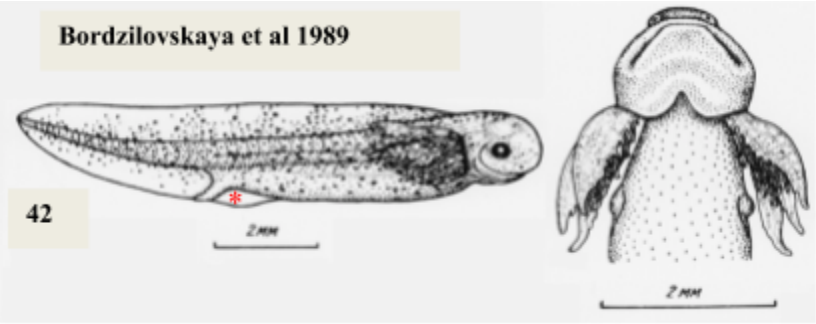
Amphibian embryos provide excellent models for experimental studies. Over the past 4 years we have used an axolotl embryo tail regeneration model to screen thousands of chemical compounds for effects on tissue regeneration (Ponomareva et al 2015). When a chemical is identified as an inhibitor of tail regeneration, it provides a new tool for investigating molecular, cellular, and developmental mechanisms that are required for regeneration. To date, we have identified > 200 chemicals that inhibit regeneration and the results of our work can be searched via a new chemical genetic database at **Sal-Site**. The assay is relatively simple to perform and thus ideal for involving high school and college students in regenerative biology and chemical screening research projects. Below we provide a detailed methodology for performing the assay to motivate interest in performing axolotl chemical screening and data sharing through **Sal-Site.**

Materials

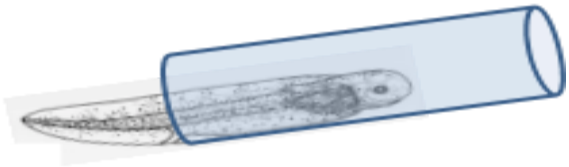
Prior to performing amputations, the following materials are needed:

1) Axolotl Rearing Water (ARW: 1.75 g NaCl, 100 mg $MgSO_4$, 50 mg $CaCl_2$, and 25 mg KCl per liter, buffered with NaHCO3 to ~ pH 7.5).

2) Stage 42 embryos that can be obtained from the Ambystoma Genetic Stock Center. This stage is characterized by a small space in the gut next to the cloaca (see * in the figure below). For more details about axolotl embryo stages see Bordzilovskaya et al (1989).



3) Glass or plastic bowls (1-2 liter) to house embryos.
4) Turkey baster and/or forceps for moving embryos that are encased in egg jelly.
5) Dissecting microscope for visualizing and manipulating embryos.
6) Two forceps (#5) for removing the jelly coat and egg membrane surrounding embryos. One of the forceps is used to hold the egg jelly and egg in place and the other is used to carefully pierce the egg membrane. Both forceps are then moved apart to tear an opening in the egg membrane that allows the embryo to escape. Eggs with little or no jelly can also be held in place by suction from a disposable 3 ml plastic pipette and then the egg membrane can be punctured using forceps. The hydrostatic pressure from the puncture pops the embryo out of the egg capsule.
7) 0.02% benzocaine. Benzocaine is made by dissolving 0.2 g benzocaine in 5 mls 100% EtOH. This solution is quickly stirred into 1 liter of ARW.
8) Disposable plastic droppers (3 ml) used to transfer embryos to and from solutions. The end of the dropper is cut to increase the width so that it can be used to pick up embryos headfirst from solutions or well-saturated paper towels.

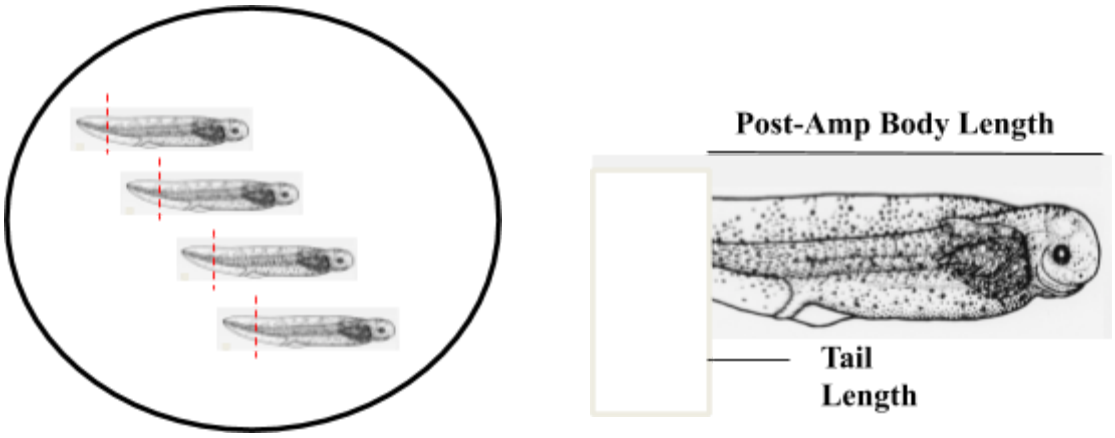


9) Folded paper towels and 1-liter squirt-type reagent bottles filled with ARW. Paper towels are thoroughly saturated prior to aligning embryos for tail amputations.
10) Razor blades or scalpels to perform amputations.
11) Microtiter plates. We typically use 12-well microtiter plates with each well containing 2 mls of solution.
12) Chemical(s) that will be tested. We dissolve chemicals in DMSO and make 10 mM stocks. Further dilutions are made using ARW to make working stocks. Typically, we first test chemicals at 10 uM. This can be done by making enough of a 10 µM solution to fill however many wells that are needed for the experiment. This solution can then be aliquoted using a graduated dropper (2 ml / well) or more efficiently using a repeating pipettor with 50 ml disposable tip. Alternatively, wells of microtiter plates can be filled with ARW first (2 ml / well) and then 2 µl of chemical is added to each well using a precision pipettor. ARW control wells should also include DMS0.

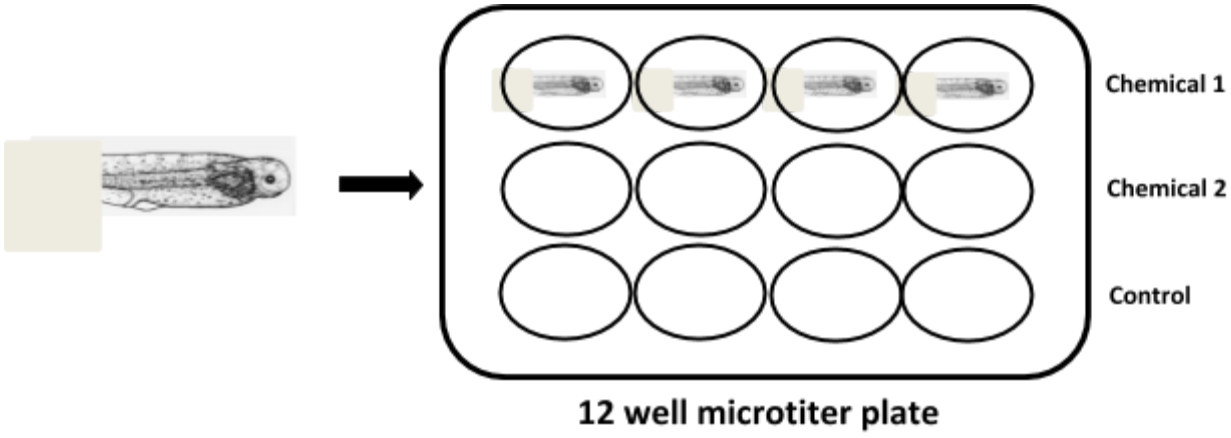
Procedure

Embryos are first anesthetized in a 0.02% benzocaine solution (3-5 minutes). Then they are picked up using a disposable 3 ml plastic dropper and transferred to a container filled with ARW to remove residual benzocaine and EtOH. Embryos are then picked up

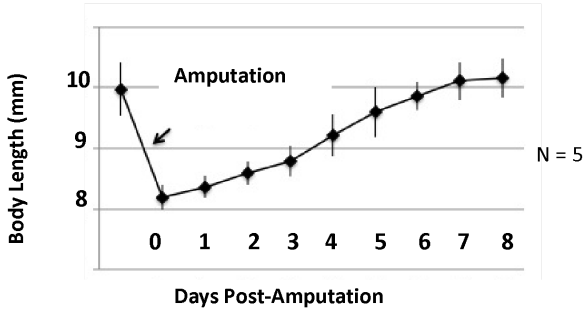
(one at a time) with a 3 ml plastic dropper and oriented on a paper towel that has been thoroughly saturated with axolotl rearing water. Embryos are arranged vertically and staggered to better enable tail amputation surgeries. Under a dissecting microscope, a razor blade is used to make a perpendicular cut through the tail exactly 2 mm from the tail tip. The figure below shows how groups of embryos can be aligned to rapidly administer tail amputations using a dissecting microscope with a 0.5 – 1x objective lens. Embryos can be imaged immediately after amputation to record post-amputation body length and or tail length using the cloaca as a morphological reference.



After amputation, embryos are picked up one at a time with a 3 ml plastic dropper and placed into a well of a microtiter plate. The figure below shows a typical workflow for screening two chemicals and a control using 4 replicate embryos per treatment.



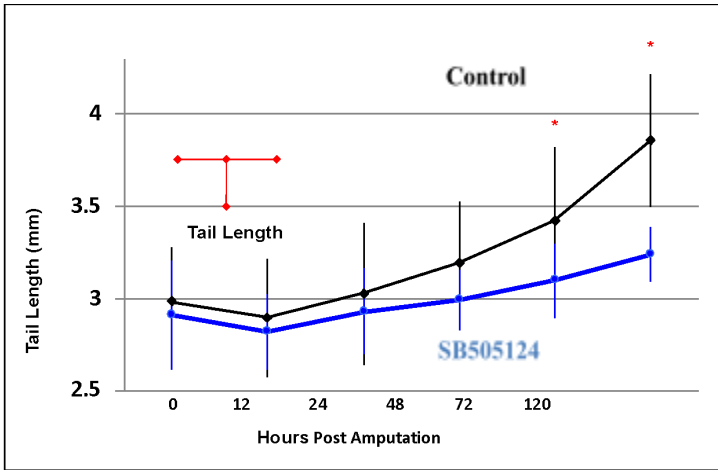
At ~18-20 C, there is sufficient yolk to sustain embryo development and complete tail regeneration in 7 days; at these temperatures, embryos do not typically feed until 12 dpa.



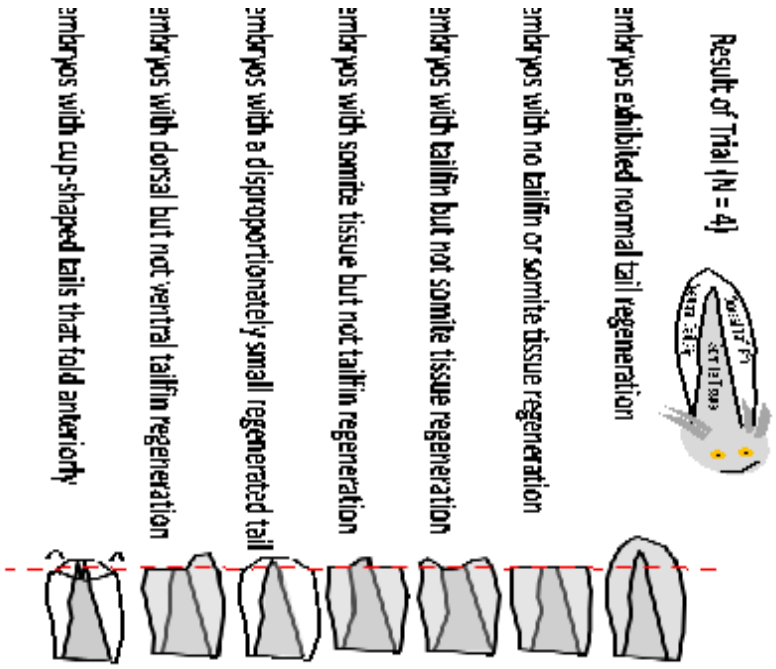
Embryos can be monitored throughout the experiment with a dissecting microscope without removing them from their wells. It is possible to remove and anesthetize embryos before 7 DPA to record changes in tail regeneration or overall body length. The figure below shows changes in tail length for two individuals that were imaged from 0 hours post-amputation (HPA) through 120 HPA (5 DPA). SB505124 is an inhibitor of TGF-β Type 1 receptors and tail regeneration. We do not typically refresh chemicals during the 7-day assay period however this is easy to do if there is concern about chemical stability.

	0 hpa	12 hpa	24 hpa	48 hpa	72 hpa	120 hpa
Control						
SB505124						

After 7 days, embryos are anesthetized with benzocaine and moved to a clear petri-dish with AWR/benzocaine. It is important to remove just enough fluid from the dish so that embryos settle onto their sides and tails become flattened against the bottom of the dish. With a 1 - 0.5x objective lens, it is possible to image 4 embryos in the same field. We use both quantitative and qualitative approaches to characterize the effects of chemicals on regeneration. Four quantitative measures that we have used include 1) proportional change in body length ((7 DPA body length – 0 DPA body length) / 0 DPA body length,) 2) tail length, 3) proportional change in tail length ((7 DPA tail length - 0 DPA tail length) / 0 DPA tail length), and 4) tail area regenerated (7 DPA



regenerated tissue distal to the amputation plane). Results of the embryo tail regeneration assay can also be qualified using a key that we developed from evaluating thousands of chemical effect outcomes. This streamlines the assay because there is no need to obtain a 0 DPA image of embryos after amputation. The key below defines discrete variations of DPA 7 tail morphology.



Summary

The axolotl embryo tail regeneration assay provides an efficient and effective model for involving students in chemical screening and regeneration research. A new searchable database at **Sal-Site** was developed to share results from chemical screening and cross-reference information among animal models that are used in regenerative biology.

References

Baddar NW, Chithrala A, Voss SR. 2018. Amputation-induced ROS signaling is required for axolotl tail regeneration. *Developmental Dynamics* 248:189-196.

Franklin BM, Voss SR, Osborn JL. 2017. Ion channel signaling influences cellular proliferation and phagocyte activity during axolotl tail regeneration. *Mechanisms of Development*, 146:42-54.

Hughes RR, Shaaban KA, Ponomareva LV, Horn J, Zhang C, Zhan C-G, Voss SR, Leggas M, Thorson JS. 2019. OleD Loki as a catalyst for hydroxamate glycosylation. *Chembiochem*, *In press.*

Ponomareva LV, Athippozhy AT, Thorson JS, Voss SR. 2015. Using *Ambystoma mexicanum* (Mexican Axolotl) embryos, chemical genetics, and microarray analysis to identify signaling pathways associated with tissue regeneration. *Comparative Biochemistry and Physiology Part C: Toxicology and Pharmacology* 178:128-35.

Voss SR, Ponomareva LV, Dwaraka VB, Pardue KE, Baddar NW, Rodgers AK, Woodcock MR, Qiu Q, Crowner A, Blichmann D, Khatri S, Thorson JS. 2019. HDAC regulates transcription at the outset of axolotl tail regeneration. *Scientific Reports* 9:6751.

Zhang Y, Ye Q, Ponomareva LV, Cao Y, Liu Y, Cui Z, Van Lanen SG, Voss SR, She Q, Thorson JS. 2019. Total synthesis of griseusins and elucidation of the griseusin mechanism of action. *Chemical Science*, *In press.*

Wang X, Abbas M, Zhang Y, Elshahawi S, Ponomareva LV, Cui Z, Van Lanen S, Sajid I, Voss, SR, Shaaban K, Thorson JS. 2019. Baraphenazines A-G, Divergent Fused Phenazine-based Metabolites from a Himalayan Streptomyces. *Journal of Natural Products*, *In press.*

Wang X, Zhang Y, Ponomareva LV, Qiu Q, Woodcock R, Elshahawi SI, Chen X, Zhou Z, Hatcher BE, Hower JC, Zhan CG, Parkin S, Kharel MK, Voss SR, Shaaban KA, Thorson JS. 2017. Mccrearamycins A-D, Geldanamycin-Derived Cyclopentenone Macrolactams from an Eastern Kentucky Abandoned Coal Mine Microbe. *Angew Chem Int Ed Engl.* 56:2994-2998.

Asymptomatic Chytrid Discovered in the AGSC

Mirindi Kabangu, S. Randal Voss

Department of Neuroscience, Spinal Cord and Brain Injury Research Center, and AGSC, University of Kentucky, Lexington KY 40536

In April 2019, AGSC axolotls were tested for the chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*). This fungus is implicated in the global decline of many amphibian species (Scheele et al 2019) but not the axolotl. All axolotls in the AGSC appear healthy and none present symptoms of chytridiomycosis. AGSC staff skin-swabbed 45 axolotls (Table 1) and the methods of Boyle et al (1994) were followed to isolated DNA and perform qPCR. A *Bd*-specific double-stranded DNA template (146 bp) corresponding to the internal transcribed spacer (ITS1) region between 18S and 5.8S rRNA genes was synthesized by Integrated DNA Technologies (gBlocks gene fragments). This fragment was used to create standard dilutions (1000, 100, 10, 1, 0 copies) for quantifying the absolute number of *Bd*-specific copies from swabs. Two technical replicates for each sample were analyzed and downstream analyses required that both replicates yield comparable results; two samples were excluded based on this criterion.

Only adults tested positive for *Bd* (Table 1). The distribution of *Bd*-positive adults appeared to be random across housing methods (recirculating systems and static bowls on racks) and sex. Half of the positives (5 males, 5 females) were sampled from all AGSC recirculating systems and the other half (4 males, 6 females) were sampled from 5 different racks. The two *Bd*-negative adults were sampled from bowls on racks that yielded *Bd*-positive axolotls. The average number of ITS1 copies among *Bd*-positive axolotls varied greatly (range 0.03-1320), with fewer and less variable copy number estimates obtained for the youngest age class (Figure 1).

Table 1. Results of initial testing for *Db* in the AGSC

	3-5 cm	5-8 cm	8-12 cm	sub-adults	adults
# Tested	**5**	**5**	**6**	**7**	**22**
# Positive	**0**	**0**	**0**	**0**	**20**

PCR was performed on 5 samples to generate DNA sequences for gel electrophoresis and DNA sequencing. Gel electrophoresis confirmed that the primers amplified a single amplicon of the expected size. The 95 bp region flanked by the predicted primer binding sites was 100% identical to Bd strains collected from Ecuador (e.g. MK573624.1).

To investigate longitudinal trends in *Bd* occurrence and load within the AGSC, 12 adults (6 females and 6 males, 3 each from bowls and recirculating systems) and 6 juveniles

(~ 5 months old) were monitored monthly. None of the juveniles tested positive during the 4-month monitoring period. In contrast, 7 of the adults tested positive for all samples. This included 3 males and 3 females from recirculating systems, and 1 female from a bowl. By the 2nd month, adults that initially tested negative, tested positive for *Bd*. We note that a single adult male tested negative at the 4-month period of testing.

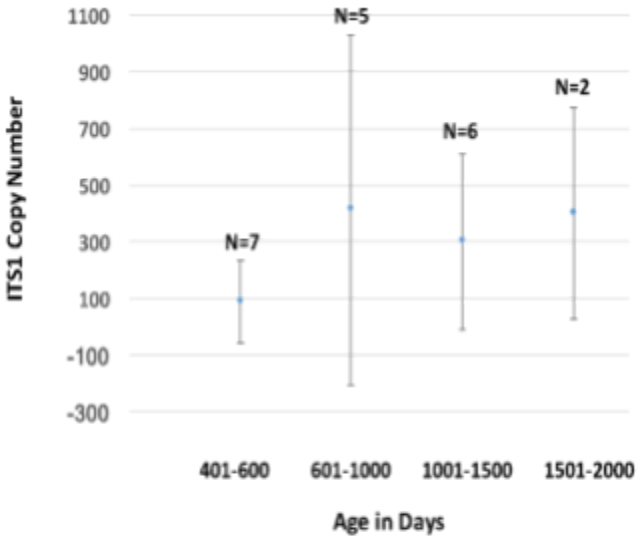


Figure 1. Average ITS1 copy number for adult axolotls that tested positive for *Db*. Error bars are standard deviations of the mean.

We continued to rear the juveniles and tested them at 8, 9, and 13 months of age. None of the juveniles tested positive at 8 months, 1 tested positive at 9 months, and 5 of 6 tested positive at 13 months. These results suggest that axolotls first carry detectable levels of chytrid as they approach and reach maturity (9-12 months).

Conclusions

Our results show that a *Bd* strain is present in the AGSC. It is detected in male and female adults that are maintained in recirculating systems and bowls throughout the facility. Longitudinal sampling of juveniles that transitioned to adults suggests that axolotls first become detectable for chytrid as they approach maturity. *Db*-ITS1 copy number estimates are variable for positive animals with fewer copies observed among younger adults. Using Longo et al (2014) estimates of ITS1 copy number per zoospore across *Bd* strains (10-144), the average number of zoospores per swab is estimated to be 1-110. By way of comparison, the infection threshold for chytridiomycosis in frogs has been estimated to be 10^4-10^6. Low zoospore load does not appear to affect adult health as no axolotl in the AGSC presents symptoms of chytridiomycosis. However, because AGSC axolotl adults carry *Bd,* they pose a transmission risk to susceptible amphibian stocks and fish. Further studies are needed to determine if the *Bd* strain identified in this study is a recent introduction or a commensal strain specific to the laboratory axolotl.

References

Boyle DG, Boyle BD, Olsen V, Morgan JAT, Hyatt AD. 2004. Rapid quantitative detection of chytridiomycosis (*Batrachochytrium dendrobatidis*) in amphibian samples using real-time Taqman PCR assay. Dis Aquat Org. 60:141–148.

Longo AV, Rodriguez D, da Silva Leite D, Toledo LF, Almeralla CM, Burrowes PA, Zamudio KR. 2013. ITS1 copy number varies among *Batrachochytrium dendrobatidis* strains: implications for qPCR estimates of infection intensity from field-collected amphibian skin swabs. PLoS One 8:e59499.

Scheele BC, Pasmans F, Skerratt LF, et al. 2019. Amphibian fungal panzootic causes catastrophic and ongoing loss of biodiversity. Science 363:1459-1463.