Axolotl

Newsletter of the
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Director's Note – Randal Voss

What an exciting time for axolotl research! Enormous progress has been made developing axolotl genomic and genetic resources, and cutting edge technologies like single cell sequencing were used recently to address fundamental questions in regenerative biology, questions that we all know are best studied using axolotls and other salamander species. This past July, approximately three dozen PI's came together in Vienna, Austria to discuss opportunities and challenges for the salamander community as we collectively move forward. Ryan Kerney's article in this issue of *Axolotl* nicely summarizes the important outcomes of this meeting and serves as a rallying cry to motivate all researchers that work with salamanders to come together in Boston in 2019 for a meeting that will coincide with the 2019 Society of Developmental Biology Meeting. The coordinators of this meeting, Karen Echeverri, Kate McCusker, James Monaghan, and Jessica Whited, are busy working out logistics and a meeting agenda. Please stay tuned for further information about this meeting and spread the word to others that might be interested in attending. The meeting is meant to bring a diversity of salamander researchers together, not just researchers that work on tissue regeneration.

In this issue, we describe a PCR-based method to genotype *short toes,* a mutant originally described by Rufus Humphrey in 1967. The cover photo of this issue shows siblings that express wildtype and *short toes* phenotypes, the later characterized by ascites and short limbs. The lead author on this work is Caitlin Labianca, currently a senior at Dunbar High School in Lexington, KY. The parent article for this methods paper, which reports the cloning of the *short toes* gene, will be submitted soon for peer review and publication.

This issue also has a highly significant and timely article concerning a pathogen that is decimating natural amphibian populations worldwide – chytrid. Many thanks to Heather Eisthen and Jacquelyn Del Valle for sharing their experience in mitigating an outbreak of *Batrachochytrium dendrobatidis* in the axolotl facility at Michigan State University. This is a must read for all that work with laboratory salamander populations!

We are in the process of updating our website to make the ordering process more seamless. Thanks for your patience! Also, we are in the process of putting together a renewal application to continue P40 NIH funding of the AGSC through 2025. **If you have time to send us a letter of support for our application, please do!** Letters from the community weigh heavily in establishing the need for axolotl stocks provided by the AGSC.

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 (P40-OD019794). We appreciate the support and look forward to serving your needs and interests into the future.

Community News

Community Project – Towards a High-Quality Histological Atlas for the Axolotl

At the recent Salamander PI meeting, the need to develop essential community resources was deemed high priority. This can be accomplished via the submission of collaborative grant proposals and by working together to leverage existing resources. For example, the Tanaka, Voss, and Smith labs are currently working with existing datasets to improve the axolotl genome assembly. As another example of leveraging existing resources, a high-quality histological atlas of the axolotl was identified as a high priority project that could be accomplished as a grass-roots, community effort. Anoop Kavirayani, a histopathologist at the Vienna Biocenter is interested to oversee the project. The current idea is to "crowd source" the community for the funds for performing the work and getting it published. It is estimated to only cost 15,000 euros.
We are now looking for pledges to underwrite the axolotl histology atlas. If you are interested and willing to contribute funds to this endeavor, please let us know by contacting Elly Tanaka.

And the Academy Award Goes to – Axolotl!

Some vintage axolotl motion pictures were recently brought to our attention from colleagues at Indiana University, which hosted the axolotl collection for almost a half century under the leadership of Rufus Humphrey and George Malacinski. One of these films details axolotl color mutants and development and the other film shows how somatic cell nuclear transfer was performed in the early 1960's to create axolotl clones. Many thanks to Kris Klueg, Associate Director of the Drosophila Genomics Resource Center for making us aware of these films, and also to IU Film Archivist Andy Uhrich and Director of University Archives Dina Kellams for the invaluable work that they do in preserving and making available media that connect the history of science to contemporary research.

Participants at the Salamander PI Meeting in Austria

Participants in "Salamander Models in Cross-Disciplinary Research" Vienna, July 2018) Back row: Jeramiah Smith, Jesus Chimal-Monroy, Renee Dickie, Dunja Knapp, Sergej Nowoshilow, Vladimir Soukup, Ryan Kerney, Toshinori Hayashi. Middle Row: Andras Simon, Hans-Georg Simon, Stephane Roy, Jifeng Fei, Moshe Khurgel, Gürkan Ozturk, Kiyokazu Agata, Katia Del Rio-Tsonis, Tatiana Sandoval Guzmán,Ken-Ichi Suzuki (behind Tatiana). Front Row: James Monaghan, Maximina Yun, Alfredo Cruz, Karen Echeverri, Randal Voss, Elly Tanaka, Jessica Whited, Catherine McCusker, James Godwin.

Meeting Report: Salamander Models in Cross-Disciplinary Biological Research

Ryan Kerney

Biology, Gettysburg College, Gettysburg PA

The first "Salamander Models in Cross-Disciplinary Biological Research" meeting was held this July (2018) at the Research Institute of Molecular Pathology (IMP) in Vienna, Austria. The meeting organizers included the Ambystoma Stock Center director Randal Voss, along with Jessica Whited (Harvard), Karen Echeverri (Woods Hole MBL) and our host Elly Tanaka (IMP, Vienna). This was a gathering of principal investigators working on various aspects of salamander regeneration, development, genetics, and genomics. The major goals of the meeting included reviewing recent advances in research tools, along with nuanced tips for employing them, while also establishing a master "to-do" list for the field. Another objective was to lay the groundwork for organizing future salamander meetings intended for the broader community, including postdoctoral and pre-doctoral trainees. The next scheduled meeting will be at Northeastern University in Boston MA on July 22-25th, and will include research symposia and poster sessions.

The Salamanders

Unlike "the" worm or "the" fly, there are multiple salamander models used in molecular studies of development and regeneration. These prominently include the Mexican axolotl (*Ambystoma mexicanum*), the Iberian ribbed newt (*Pleurodeles waltl*), the Japanese newt (*Cynops pyrrhogaster*) and to a lesser extent the North American eastern newt (*Notophthalmus viridescens*) and several species of lungless salamanders (Plethodontidae). Currently the most commonly used model is the axolotl, which likely has the longest captive history of any laboratory animal[1]. This history includes the importation of a founder population to Paris in 1864, some of which contained the mutant "white" phenotype (an *edn3* mutant), and deliberate introgression of an *A. tigrinum* locus found in 1967, which confers albinism through a tyrosinase mutation[4,5]. These salamander species are representatives of three families (Ambystomatidae, Plethodontidae, and Salamandridae – the newts) out of the ten extant salamander families, which likely had extensive limb regenerative abilities at the base of the their clade[6].

Genomics

Our sessions started with a review of the impressive new work in salamander genomics. Recent published genomes for both the Iberian ribbed newt (*Pleurodeles waltl*[7]) and Mexican axolotl (*Ambystoma mexicanum*[8]) provide tremendous new resources for the field. The axolotl genome is roughly ten times the size of the human genome, making it the largest genome to be sequenced and assembled to date (sorry loblolly pine). The publication of both of these genomes promises to help resolve the loci of multiple established mutant lines[5], and offers the opportunity to establish further forward and reverse genetic screens that will improve our mechanistic understanding of regenerative processes. The community identified additional work needed to make consistent annotations, resolving 5' ends of genes, and developing a chromosome scale assembly. The latter was recently accomplished by Jeramiah Smith and co-workers[9]. The lack of

whole-genome sequences in salamanders has been a major impediment to the field, and though the assemblies will still require extensive refinements, having these resources should prove to be enormously beneficial to labs currently working with these species as well as those contemplating it.

Tansgenics and Genome Editing

Genome editing approaches are now available for the axolotl[10,11] , the Iberian newt[12,7] and the Japanese fire-bellied newt *Cynops pyrrhogaster*[13] using TALEN and CRISPR-based approaches. The most recent protocol development by Ji-Feng Fei[14] (now at the South China Normal University) of knock-out and knock-in approaches were reviewed. Current best practice techniques focus on improving knock-in strategies without relying on homology-directed repair. These include targeting introns for knock-in's, screening injected embryo knockouts for efficient guide RNA's prior to knock-ins, and non-homologous end joining approaches with "ORF Baits."

The advent of CRISPR and TALEN approaches to genome editing in *Pleurodeles* was reviewed by Ken-Ichi Suzuki from Hiroshima University. The Suzuki lab is currently working to identify a ROSA-like locus for constitutive expression of knock-in constructs that would also provide a "safe harbor" for exogenous DNA. The intent of this approach is to develop a site that would both be minimally disruptive to normal cellular physiology while experiencing minimal interference from histone modifications in different cell lineages.

A wide range of transgenic and CRISPR edited lines are becoming available, especially in the axolotl. These include constitutive RFP and GFP reporter lines (available from the University of Kentucky's Ambystoma Stock Center - AGSC), a pax7-mcherry muscle satellite cell marker, nerve cell marker, and a brainbow axolotl[15]. Discussions focused on prioritizing existing stocks and their dissemination through the AGSC (primarily in North America), Max Planck Dresden and MPI (in Europe). The need to pursue financial resources to enable more extensive repository functions for salamanders was also addressed. A long-term goal of the community is to secure resource funding for these types of valuable operations, which will be necessary to advance discoveries in regenerative biology through this growing research community.

Temporal control of transgene expression

Many genes implicated in regenerative processes have pleiotrophic roles in early development. These make knock out experiments in studying adult regeneration difficult as they can be embryonic lethal. Therefore, both temporal and spatial control of gene expression is critical for furthering regeneration research.

There are several creative approaches available to address this potential stumbling block. These include inducible cre-lox systems[16], constitutive cas-9 expression in genomic "safe harbors" with drug inducible guide RNA's, and viral delivery of foreign transgenes into regenerative blastemas[17,18,19]. The latter approach has been championed by both Jessica Whited's group at Harvard University and the Tanaka lab at MPI. These pseudotyped retroviruses have tremendous potential for further labeling

and functional studies of the limb blastema, without raising transgenic embryos or modifying the expression of pleiotrophic genes outside the limb.

Open discussions evaluating approaches and avenues for future research allowed individual labs to prioritize their own research agendas towards this critical methodological goal in the field.

Resources needed for the field

The real value of this PI-focused meeting was to allow researchers to champion the resources they found most pressing for the field. Several additional resources were identified in addition to continued improvements to genome annotations and the temporal control of gene expression. These included detailed histological atlases of regeneration, master lists of validated antibodies, and more stable cell lines for in vitro experiments. One of the most obvious resources needed was the continued communication between labs with subsequent salamander research conferences that should continue to strengthen this growing research community.

Coordination and prioritization of a model system

Several research communities have benefitted from the intentional development and promotion of particular model organisms[20]. One-stop repositories of information such as Flybase, Wormbase, ZFinBase and Xenbase have all expanded the research capabilities of participating labs. While many of these labs focus on Developmental Biology, the reach of these model systems includes studies of neuroscience, evolutionary biology, physiology, and ecology. Emulating this deliberate approach will provide vital cohesion and an undoubted boon to investigators studying salamander regeneration and development. While salamanders are a remarkable "model" for regenerative research they are also remarkable organisms for their unique evolutionary histories, ecological roles, life history variation, and conservation biology. Development of tools and resources for the molecular biologists and biochemists working on salamanders will undoubtedly have unintentional spillover benefits into a wider range of research fields.

Outlook

Salamander models continue to be fertile ground for amazing discoveries on regenerative biology, cell differentiation and development. This conference generated a tremendous amount of motivation and inertia in its participants. The 2019 meeting in Massachusetts will be a showcase for these recent developments as well as an iterative checkup on this rapidly growing experimental field.

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A PCR Based Assay to Detect the *Short Toes* Allele in Axolotls

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Short toes is one of the oldest Mendelian mutants in the Ambystoma Genetic Stock Center (Humphrey 1967). The *short toes* mutation is characterized by a reduction in the length of limbs and number of phalanges, and excessive fluid retention in the abdomen (ascites). The stunted limb phenotype suggests a lesion that alters skeletal development. The ascites phenotype is associated with abnormal kidney function that ultimately causes death before reproduction. Interestingly, the timing of ascites presentation and ultimately death varies greatly among *short toes* individuals. For example, some sibs of a spawn present ascites during the early larval phase (2-3 cM total body length, 1 month of age), while other sibs attain late juvenile and early adult body sizes before presenting ascites (9-12 cM body size, 6-8 months of age). The variable timing of *short toes* in affecting kidney function and the pleiotropic effect it has on limb development suggest *short toes* could be a valuable mutant for developmental studies. However, research using this mutant has been hindered because the genetic basis of *short toes* is unknown (Egar and Jarial 1991; Del Rio-Tsonis et al 1992; Washabaugh et al 1993; Gassner and Tassava 1997; Sato and Chernoff 2007).

In the AGSC, *short toes* is maintained in heterozygous carriers, however *short toes* mutants occasionally arise unexpectedly from the mating of individuals that are not thought to carry the *short toes* allele. Generally speaking, recessive alleles can be difficult to manage in populations that use segregating phenotypes to infer genotypes. To more efficiently manage *short toes* alleles and identify the gene for this valuable mutant, we pursued studies to identify the causative gene. A full account of this body of work will be presented in an upcoming peer reviewed paper. In the interim, we detail one aspect of that work to aide researchers in the management of *short toes* in laboratory populations, the development of a PCR assay to genotype individuals that carry the *short toes* allele.

Methods

Two axolotls (13710.R1_female x 14168.R6_male) were mated to generate a spawn (#14981) that segregated wildtype (RRID:AGSC_100E,H,J) and *short toes* (RRID:AGSC_106E**)** phenotypes. The resulting larvae were reared in mass until the time that forelimb development was completed. At this time, individuals were anesthetized in 0.02% benzocaine, examined for short toes phenotypes (abnormal limbs, ascites), and tail-clipped. Fifty-nine of these individuals were assigned to independent bowls and reared for several months to verify short toes and wildtype phenotypes. DNA was isolated from tail tips using a traditional phenol/chloroform

method (Voss 1993). DNA was also isolated from archived tissues of axolotls that were known to be homozygous for wildtype axolotl alleles in this genomic region, *albinos* that were homozygous for tiger salamander alleles in this genomic region, and two presumptive *short toes* individuals that arose unexpectedly in two recent spawns. A PCR primer pair (Forward: 5' CATTCCTGCTGAGGTACTTCTTCT 3'; Reverse: 5' AGAGGAAATGCTGCCACATATAGT 3') was designed using Primer3 plus (Untergasser et al 2007) to amplify a 578 bp DNA fragment from a noncoding region of the *mre11* gene. PCR was performed using standard protocols and a 60 C annealing temperature, and DNA sequencing was performed by Eurofins. DNA sequences were aligned to a genomic scaffold (AMEXG_0030005678) from the axolotl genome assembly (Smith et al *In press*) using the Seqman Pro application in DNAStar. Animal work was performed under Institutional Animal Care and Use protocol #01087L2006 at the University of Kentucky.

Results and Discussion

To identify the most likely position of *short toes* in the axolotl genome, we used a next generation sequencing approach to identify single nucleotide polymorphisms (SNPs) between RNA pools of siblings that segregated wildtype and *short toes* phenotypes. We recently described and used this approach to identify *tnnt2* as the gene for *cardiac* (Smith et al *In press*). We initially mapped *short toes* to Chromosome 7 in the vicinity of *amotl1, fat3, mre11,* and *ankrd49* (Figure 1). This location is not far away from the *tyrosinase* (*tyr*) locus, which we previously identified as the *albino* gene (Woodcock et al 2017). Thus we expected SNP variation in this region might reflect introgressed tiger salamander DNA, a vestige of the hybridization event that brought *albino* into the AGSC axolotl population in 1962. Initial analyses of SNP variation showed that *mre11* harbored the most SNP variation in this genomic region. Thus, we designed PCR primers to screen intron sequences that flanked *mre11* exons for SNPs that could be used to differentiate among wildtype axolotl alleles, tiger salamander DNA, and *short toes* alleles.

For the initial screen, we used DNA isolated from a *short toes* carrier (13199.A3). We successfully PCR amplified and then identified SNPs in a 578 bp PCR amplicon (Ch7:1768806520-1768807097). We reasoned that some of the SNPs might be informative for differentiating wildtype and *short toes* alleles, however the variation might also trace to introgressed tiger salamander DNA. To investigate this further, we isolated DNA from another *short toes* individual, as well as five individuals that were known to be homozygous for wildtype axolotl *tyr* alleles and five individuals that were known to be homozygous for *tyr* tiger salamander alleles. The resulting sequences were used to classify 12 SNPs within the PCR amplicon as either deriving from wildtype axolotl, tiger salamander, or *short toes*. We note that the majority of the SNPs could be attributed to introgressed tiger salamander DNA (Figure 1).

To test the PCR amplicon as a marker for *short toes,* we crossed two individuals that carried *short toes* alleles (s), classified 59 of the resulting offspring as either wildtype or short toes, and then genotyped the parents and offspring for the PCR amplicon. *Short toes* individuals (N = 27) were easily identified by their abnormal limbs. For example, the

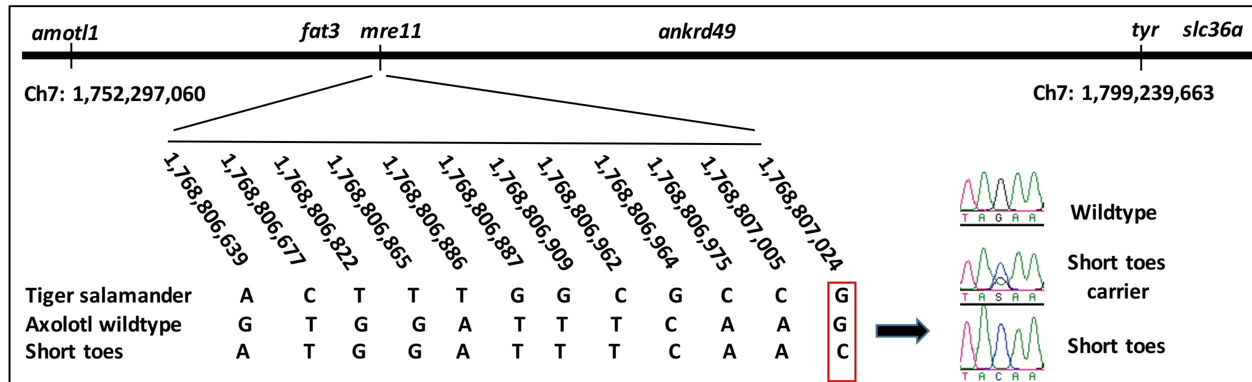

Figure 1. Map showing SNPs within *mre11* that differentiate tiger salamander, axolotl, and *short toes* alleles. The SNP site that is bracketed in red identifies the *short toes* allele to the exclusion of axolotl and tiger salamander alleles.

forelimbs of short toes individuals were typically shorter and presented fewer digits than wild type individuals. Also, four *short toes* individuals presented ascites during the course of the study. When we compared SNPs between the parents, we found that one carried a wildtype axolotl allele (S^{Mex}) while the other carried a tiger salamander allele (S^{Tig}). SNP variation allowed all four of the expected genotypes to be classified among the offspring, including the *short toes* homozygous genotype. The short toes allele differs from wildtype axolotl and tiger salamander alleles at a single SNP site (Ch7:1768807024). Thus, the presence of cytosine and guanine (G/C) nucleotide peaks in an electropherogram at this position can be used to identify individuals that carry *short toes* alleles (Figure 1). We further verified this genotyping assay by sequencing two additional, unrelated *short toes* individuals that arose unexpectedly in the AGSC; in both cases the individuals were homozygous for the cyostine nucleotide at the informative SNP site. We will continue to genotype more individuals and look forward to feedback from the community about the use of this assay for prospective management of axolotl populations.

While we identified a *mre11* SNP that is associated with the *short toes* allele, the causative mutation might associate with a different linked locus. The closest linked gene to *mre11* in the axolotl genome assembly is *fat3*, a large cadherin transmembrane protein that functions in concert with Dachsous family proteins to mediate cell-cell adhesion events that pattern tissues during organ morphogenesis. Consistent with ascites and limb developmental defects observed in *short toes*, FAT4-DCHS1 signaling is essential for nephrogenesis and skeletal development in mammals (Bagherie-Lachidan et al 2015; Mao et al 2015; Kuta et al 2016; Mao et al 2016). Thus, it is possible that axolotl *fat3* is functionally equivalent to mammalian *fat4.* In support of this inference, CRISPR-Cas9 editing of *fat3* in wildtype axolotls yields offspring with ascites, as is seen in *short toes* (Figure 2). We have identified fingerprints based on multiple SNP sites within *fat3* that differentiate among tiger salamander, axolotl, and *short toes* genotypes, but have not yet identified a single SNP that can be used to identify *short toes* alleles to the exclusion of tiger salamander and axolotl alleles. Because Fat

proteins are implicated in Hippo and Wnt signaling cascades, *short toes* may provide a powerful new mutant to investigate mechanisms of kidney development, limb development, limb regeneration, and organ size regulation.

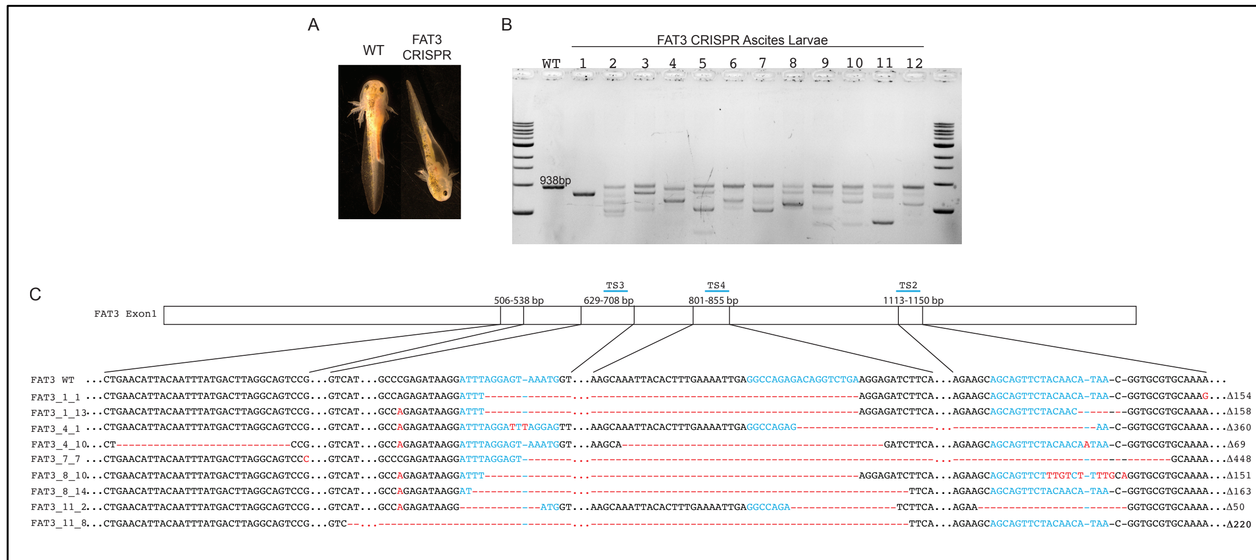

Figure 3. One to two-cell stage axolotl embryos were injected with CRISPR-Cas9 and three gRNAs (TS2, TS3, and TS4) targeting *fat3* exon 1. A) Approximately 15% of injected larvae developed ascites. WT = non-injected wildtype. B) Multiple *fat3* exon 1 PCR products were amplified from 12 ascites larvae, consistent with CRISPR-Cas9 generation of insertion/deletion polymorphisms. C) Representative *fat3* CRISPR-Cas9 alleles from ascites individuals. Blue nucleotides = gRNA targets; red dashes = deletion polymorphisms; red nucleotides = insertion polymorphisms.

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Axolotls Infected with Chytrid?
A How-to for Successful Treatment and Facility Decontamination

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In the past year we learned that the axolotls in our colony were infected with the chytrid fungus (*Batrachochytrium dendrobatidis*, commonly referred to as *Bd*) that is leading to declines in frog populations worldwide. We describe here how we diagnosed the infection, the protocol we followed to eliminate the infection in our animals, and the method we used to decontaminate our facility and equipment. Our hope is to spare other axolotl researchers the trouble of figuring all this out if faced with the same problem in their colony.

For the past few years we have maintained a population of 40-60 adult axolotls of both sexes. In our facility, axolotls are housed in groups of 2-4 individuals in three rows of 30-gal aquaria, with animals segregated by sex and size. Each row constitutes a separate recirculating system. An overhead reservoir above each row feeds the aquaria; water from the surface of each aquarium then drains through a mechanical filter to a sump, where the water is pumped across a biological filter and returned to the reservoir after passing through an ultraviolet sterilizer. Each day, we suction detritus (food particles, fecal material, etc.) off the floor of each aquarium and siphon additional water to ensure that a minimum of 10% of the total water volume within each row is replaced. Each aquarium contains refuges made from PVC pipe. Axolotls are maintained in 100% Holtfreter's solution (Armstrong and Malacinski, 1989) in RO water (pH 7.5) and we test pH, ammonia, nitrite, and nitrate levels biweekly. Each adult is fed 4-5 salmon pellets (Rangen, Buhl, ID) three times per week. The light cycle in our facility is adjusted on the first of every month to match that of animals' native habitat in Mexico City and the temperature is maintained between 19° and 21°C.

In the fall of 2016, our mortality rate suddenly spiked and we lost 4 animals in 6 weeks. No obvious symptoms were noted, and it is entirely possible that these individuals died coincidentally from a variety of causes. Nevertheless, this put us on alert and by spring 2017 we could see that approximately 20% of our animals, particularly in one row, had reddened skin on their legs and toes, or areas of depigmentation along the lateral line and tail tip as well as on the toes. Concerned that our animals might have a bacterial infection, we treated the most significantly affected row by adding tetracycline (1.5 mg/liter) to their tanks for 10 days. The symptoms may have been reduced slightly by this intervention, but the effect was not dramatic. Aerobic cultures of aquaria water samples from our systems and a swab of a toe lesion from an adult male axolotl were analyzed by the Michigan State University Veterinary Diagnostic Laboratory (VDL); the results indicated the presence of *Pseudomonas spp.* and *Shewanella spp.* in our aquaria. However, these microbes are commonly present on amphibians' skin and in their housing, so it was difficult to interpret this result. Over the next few months, we

treated severely affected individuals with enrofloxacin (Baytril; Bayer, Shawnee Mission, KS) based on sensitivity results, at a dose of 10 mg/kg injected into the dorsal musculature along the flanks daily for 7 days. We noticed that animals improve a little with this treatment, but not completely, and symptoms returned within a few weeks or months.

In an attempt to rid our aquaria of the unknown pathogen, in the summer of 2017 we moved all animals into bowls and disinfected all our aquaria with bleach; we hoped that cleaning their housing would provide an improved environment for our animals to clear the infection. The disinfection of their aquaria disrupted the nitrogen cycle (which typically takes 3-6 months to reestablish in our colony) but didn't solve the problem.

In late 2017, IDEXX Bioresearch (Columbia, MO; now IDEXX BioAnalytics) debuted a new Axolotl Microbiology Panel that uses culture assays to test for the presence of the amphibian pathogens *Aeromonas dhakensis*, *A. hydrophila*, *Flavobacterium columnare*, *P. aeruginosa*, *Salmonella enterica*, *Saprolegnia*, and *Serratia marcrescens*. At the same time, IDEXX also debuted an Axolotl Comprehensive PCR Panel that tests for *Batrachochytrium dendrobatidis* (the chytrid fungus that is typically associated with frogs) and *B. salamandrivorans* (the salamander-specific chytrid), *Flavobacterium columnare*, *Mycobacterium marinum*, *Piscinoodinium pillulare*, *Ranavirus*, and *Salmonella*. These tests cost us about $170 and $240, respectively. Technicians from IDEXX told us how to swab our animals and sent us all necessary sampling and shipping materials. We swabbed our animals and shipped the samples to IDEXX on January 24, 2018, and on February 2nd we had our results: all samples were positive for *B. dendrobatidis* but not *B. salamandrivorans* nor any of the other pathogens included in the panels. Follow-up tests carried out by the VDL confirmed the presence of *Bd* in many of our animals, including some that showed no symptoms of any kind. Now we knew why the antibiotics we used had not solved the problem.

We do not know how the fungus entered our animal colony, and anecdotally heard from a colleague who studies *Bd* that they had tried and failed to infect axolotls with the organism. One possibility is that it entered with the people in our lab: many of the students in the lab have amphibians at home and we all participate in animal care as it helps everyone gain an appreciation for the behavior and physiology of our animals. Although we can't rule out this possible route of transmission, no one in the lab reported health problems with their animals at home, all their animals come from well-known breeders and are certified chytrid-free, and we scrub with an antimicrobial soap (BacDown; Decon Labs, King of Prussia, PA) before doing animal care. Another possibility is that the pathogen came from a colony other than the AGSC as we have long had a policy of accepting adults from any researcher who offers them. We generally quarantine new arrivals in separate tanks for a few weeks before mixing them with the existing population and didn't notice any particular health problems in new arrivals over the last few years, but we can't rule out this possibility. Finally, it is possible that our animals were infected by *Bd* that arrived with one or more cohorts of rough-skinned newts (*Taricha granulosa*) that we also keep in our lab. Because the newts are wild-caught, we've always known they had the potential to carry pathogens that could

affect our axolotls. To reduce risk, we keep the axolotls and newts in separate rooms and have protocols in place to ensure that the newts are cared for after the axolotls and that no one touches an axolotl or axolotl tank after working in the newt room. Still, we can't rule out the possibility that we accidentally infected the axolotls from the newts, or that *Bd* spores were deposited on shared equipment, such as taps at sinks, and then transmitted to the axolotls.

In any case, once we knew the source of our problems we had to figure out how to treat the animals and decontaminate the facility. Our axolotl room is cool and wet – perfect for *Bd*, which is highly susceptible to warm temperatures and desiccation. We consulted widely: we talked to researchers who study *Bd,* colleagues who had dealt with *Bd* infections in their amphibian colonies, and veterinarians, particularly those who work at zoos and aquaria as well as other research facilities.

Many studies have documented the efficacy of various *Bd* treatments for frogs but few have addressed treatment in salamanders. Although some researchers are trying to find ways to use antimicrobial peptides to combat *Bd*, the treatments that have well-documented success rates in frogs are heat and an antifungal drug, itraconazole. We briefly considered using heat but could not figure out how to heat up our entire facility to an effective temperature (30 or 37°C for a week or a day, respectively) and were worried about the effects of such temperatures on axolotls, which thrive at much cooler temperatures (Armstrong and Malacinski, 1989). The only study we could find that discussed treatment of ambystomids is anecdotal, describing antifungal treatments used for *A. andersoni*, *A. dumerilii*, and *A. mexicanum* in zoos and private collections in the UK (Michaels et al., 2018). The animals described in this paper had diverse symptoms and the outcomes varied widely; the doses and duration of treatments varied considerably and mortality during treatment was high at some locales. In the end, we treated our axolotls by bathing them for 5 min/day in itraconazole, which has proven effective in some frog species (Pessier and Mendelson, 2017). The most commonly used concentration is 0.01%, although some studies have suggested that such high doses are associated with high mortality and that lower doses can also be effective; we decided to use 0.002% itraconazole, which was the lowest effective dose we found in the literature (Brannelly, 2014). Although the standard treatment for frogs seems to be 11 days, this duration seemed arbitrary and we decided to treat for 10 days. We were advised that powdered itraconazole is poorly soluble in water and that using a commercial formulation would be easier and more reliable, so we used Itrafungol (Elanco Animal Health, Greenfield, IN), which is formulated for oral administration in cats.

Before treating our entire population, we carried out a pilot study on two adult males, one of which had shown symptoms for a year and had been injected with Baytril over the summer of 2017. Seven days after the end of itraconazole treatment we sent swabs from these two animals to the VDL and they came back negative for *Bd*. Recognizing that the pathogen might have been knocked down below the threshold for detection and could then rebound, we tested again 28 days after the end of treatment. When these

second samples came back negative we scaled up to treat our entire colony with itraconazole.

Bd spores can be spread on gloves, paper towels, and bench paper, and returning animals to contaminated bowls after treatment is counterproductive; therefore, we developed the following protocol for treating our axolotls. First, we sterilized the bench top with 70% ethanol, let it sit for a few minutes, and then placed a piece of clean bench paper over the work area. Next, we filled a few clean bowls with Holtfreter's solution and put the bowls and an orbital shaker on the bench paper; we were advised to use an orbital shaker to make sure the animals would be thoroughly coated with the antifungal solution throughout the brief duration of the treatment. We prepared the anti-fungal bath in treatment containers, for which we used small plastic boxes with locking lids that we purchased at a local chain store. For each container, we added 3.25 mL Itrafungol to 1.5 L Holtfreter's solution, which was enough solution to cover the animal without too much excess. The solution was then mixed well by swirling the container manually. We were able to fit four treatment boxes at a time on our shaker through judicious use of a plastic crate and bungee cords. During the treatment and decontamination period, our axolotls were housed in bowls on rolling shelving units; our protocol was to treat the bowls on the top shelf first and then work our way down the shelves to ensure that water from "dirty" animals didn't splash into bowls of just-treated animals. Once the treatment area was prepared, a lab member would put on a pair of nitrile gloves, take one axolotl from its bowl, place it into the treatment container, snap the lid shut, and place the container in the crate on the shaker. The gloves were then discarded, a fresh pair put on, and the next animal was put in a treatment container. Once four animals were in treatment containers, a timer was set for 5 min and the orbital shaker was set to a gentle speed. After 5 min, fresh gloves were put on, the first axolotl was removed from the treatment container to a clean bowl, gloves were changed, another animal was put in another clean bowl, and so on until all four animals were in clean bowls. The bowls the animals had inhabited prior to treatment were then cleaned with a salt scrub, sprayed with 70% ethanol, inverted, and left to dry until needed the following day. Once all animals had been treated for the day, the treatment boxes were similarly cleaned, sprayed with 70% ethanol, inverted, and left to dry. The orbital shaker was sprayed with 70% ethanol and set aside, the bench paper discarded, and the counter was sprayed with 70% ethanol and left to air dry. We suspended feeding the axolotls during the 10-day treatment period. Two of the 46 axolotls died during treatment, which is much lower than the rate reported in some other *Bd* treatment studies. All gloves, paper towels, and bench paper used during treatment were collected in fiber barrels and sent for disposal as hazardous waste.

As with our pilot animals, we tested the axolotls for *Bd* both 7 and 28 days after treatment ended. For these tests, we pooled samples from three sets of four individuals, grouped by the row of aquaria that they had originally inhabited; we also took samples from the internal walls of the sump tank associated with each row. All tests came back negative for *Bd*. In addition, the Michigan Department of Agriculture and Rural Development (MDARD) classifies *Bd* as a reportable animal disease, and positive results for *Bd* in our state are to be reported to MDARD within 24 hours of discovery. An

MDARD quarantine was placed on our animal facility, requiring three consecutive negative PCR tests up to 6 months after treatment before lifting of the quarantine. The quarantine entailed posting signs to indicate the presence of infected animals as well as a prohibition on removing animals from the facility. An additional PCR test was conducted 6 months after the end of treatment and also came back negative. This final negative result allowed MDARD to lift the quarantine on our animal facility.

We decontaminated our facility while the axolotls were housed in individual bowls. First, we discarded and replaced all nets and filter material that had been in contact with our animals or aquarium water. In addition to heating and desiccation, *Bd* can be effectively killed with ethanol, bleach, and Virkon S (Lanxess Corporation; Pittsburgh, PA), an antimicrobial product commonly used in animal facilities (Johnson et al., 2003). We make and store our Holtfreter's solution in 45-gal barrels on rolling dollies. To disinfect these barrels, we added a 5% bleach solution that we left to sit overnight; we then scrubbed the barrels, rinsed them thoroughly with RO water, and allowed them to dry at least overnight. Equipment used in siphoning water from tanks was decontaminated with Virkon. We disinfected our aquaria by adding enough bleach to bring the whole system to a 5% solution, which we left to circulate overnight. (Fun fact: the departmental bookkeeper requested a memo explaining why we ordered 50 gallons of bleach on a federal grant.) We scrubbed all tanks, inside and out, as well as their lids and the PVC hides, with the 5% bleach solution. We then drained the aquaria and refilled them twice with tap water using a garden hose that we attached to our sink with an adapter from a local hardware store. We then drained the aquaria again, refilled them with RO water, and allowed the RO water to circulate overnight. The aquaria were then drained, rinsed with RO water, drained again, and left to dry overnight.

While the axolotls were still housed in bowls, we tried to jump-start the nitrogen cycle in our aquarium systems by refilling with Holtfreter's solution and then adding "Stability" (Seachem; Madison, GA) and enough ammonia to bring all aquaria up to 0.5 – 1 ppm. We waited to return the axolotls to their home aquaria until the 28-day post-treatment test showed the animals to be *Bd*-free as we did not want to have to disinfect the systems again if we learned that the treatment had been ineffective.

We have made some changes to try to prevent future outbreaks of *Bd*. First, we had new RO water taps installed inside our newt room to prevent cross-contamination. We purchased dedicated non-skid, sterilizable, slip-on clogs for each lab member to wear while inside our animal facility and established a location and procedure for changing out of street shoes while in the animal facility; we also purchased disposable shoe covers for veterinary staff and physical plant workers to wear when visiting the facility. We coordinated waste removal with janitorial staff to ensure that they do not need to enter the facility. Perhaps most importantly, we plan to test all new animals that arrive at our facility for the presence of *Bd*, and to treat all affected animals before they are released from quarantine.

We expect that a more formal description of the symptoms and treatment of *Bd* in both our axolotls and newts will be published soon in *Comparative Medicine*.

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