Axolotl

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

F1 *A. mexicanum* x *A. tigrinum* female used to create new haploid genome assemblies for the axolotl and tiger salamander. See Community News to learn more.

2024 Issue

AGSC Director's Note - Randal Voss

I am happy to report that all AGSC axolotls are in good health and supply. Please let us know how we can help support your research efforts. We had a bit of a scare in 2023 when we experienced a larval mortality event. We necessarily stopped shipping axolotls for approximately three months last summer while we pursued approaches to identify the cause of mortality. As I reported last year to the community, the larval mortality episode resolved as quickly as it appeared, and a causal factor was not identified. In this issue, we provide a case report of the mortality event, detailing approaches that were pursued and lessons that were learned. I hope this information will be useful for those developing disease monitoring and disaster plans for their axolotl facilities.

The NIH P40 funding mechanism that supports the AGSC was recently modified to provide additional funding to animal resource centers for curation and informatics. We submitted a revision application in 2023 to compete for curation and informatics funding to pursue initiatives that will increase efficiency in stock data management, develop new website content to increase the value of axolotl stocks, and create new interfaces to better serve axolotl stakeholders. Thanks for your generous letters of support, we received a Notice of Award and are currently looking to hire two new AGSC positions to facilitate these efforts: 1) Research Software Administrator and 2) Curator/Outreach Coordinator. For the Curator/Outreach Coordinator we are hoping to hire someone with research experience using the axolotl, so please spread the word. For more information about these positions please contact me.

We also received supplemental funding to purchase two new pieces of equipment that may be of interest to others in the community - Calypso automated washing systems by Tecniplast. These systems are specifically designed to wash aquatic animal tanks and accessories. If you would like to learn more about our admittedly limited experience with these systems, please contact us.

The AGSC is funded by the Office of Research Infrastructure Programs at the National Institutes of Health. Please acknowledge use of AGSC stocks, including stocks derived from the AGSC axolotl population, in your publications by citing our grant number (P40-OD019794) and Research Resource Identifiers (RRIDs) for the AGSC (RRID:SCR_006372) and axolotl stocks (e.g. wildtype adult, RRID:AGSC_100A).

Community News

New Axolotl Genome Assembly

The Smith and Voss labs at University of Kentucky, in collaboration with the Vertebrate Genome Project, recently sequenced an *A. mexicanum* x *A. tigrinum* F1 female hybrid and generated two new highly accurate haploid genome assemblies. The axolotl haploid assembly is in queue for gene annotation by NCBI and should become available to the community soon under Bioproject PRJNA1104202. The project was funded by NIH R24OD010435.

Developmental and Regenerative Biology Course: MDI Biological Laboratory

ReBilD: Emerging Leaders in Regenerative Biology and Development
Date: 4-17 August 2024
Venue: MDI Biological Laboratory, Bar Harbor, ME, USA
Course Directors: Romain Madeleine, Iain Drummond, Prayag Murawala
Registration and further information: https://mdibl.org/course/rebild-2024/

Description of the course
The goal of this course is to address the need for research training in developmental and regenerative biology. The long-term goal of the proposed project is to create a network of skilled researchers, with knowledge of the biology and regenerative abilities of diverse model organisms who are successfully performing cross-species studies. This network is crucial to unravel the secrets of tissue regeneration and developmental biology to solve tomorrow's health challenges. This innovative two-week course will feature concurrent experiments in regeneration and development, using an array of models and led by a dedicated cohort of outstanding teaching faculty. Hands-on work in MDI Biological Laboratory's advanced facilities will be complemented by round table discussions and informal dinners with multiple keynote lecturers, seminars, and other training activities. Course participants will be able to present their own research and network with other students and faculty at our coastal Maine campus, minutes from Acadia National Park.

Stem Cells and Regenerative Biology Course: Marine Biological Laboratory

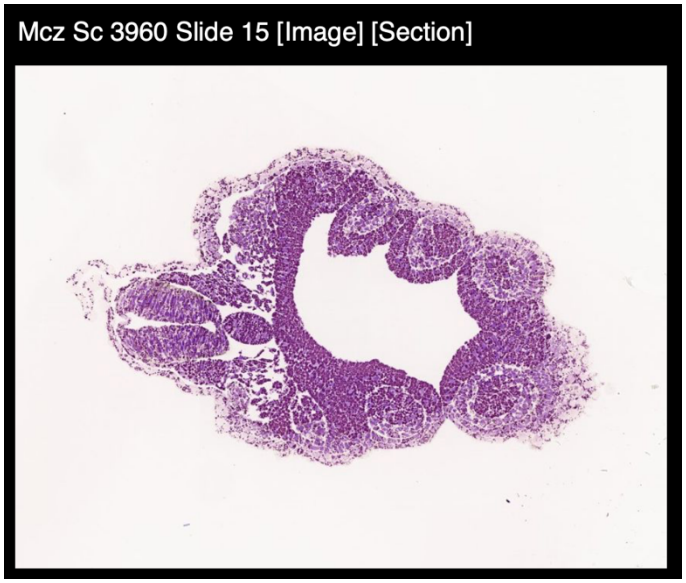
SCARE: Frontiers in Stem Cells & Regeneration
Date: Sept 28 - Oct 7 2024
Venue: Marine Biological Laboratory, Woods Hole, MA, USA
Course Directors: Ina Dobrinski and Charles Easley
Registration and further information: https://www.mbl.edu/education/advanced-research-training-courses/course-offerings/frontiers-stem-cells-regeneration

Description of the course
The Frontiers in Stem Cells and Regeneration Course is a laboratory and lecture-based course that includes a complete array of biological and medical perspectives from fundamental basic biology of "stemness" and mechanisms of regeneration through evaluation of pluripotent stem cells for therapeutic benefit. This dynamic, evolving course features world class lectures from experts in stem cells and regeneration biology, including a keynote Pioneer Lecture delivered by a leading expert. The laboratories explore a variety of timely topics including stem cell derivation, pluripotency, directed differentiation, and spinal cord and limb regeneration, using an array of experimental models ranging from planarians to human stem cells.

Salamander Meeting in Japan

The Salamander Meeting 2024 will be held for the sixth time at the Hiroshima University Amphibian Research Center. Although the Salamander Meeting is a meeting for researchers who study urodele amphibians, we welcome the participation of researchers of frogs or in a wider range of fields, including those who study frogs and caecilians. Let's expand the discussion to a wide range of research fields and make this a highly valuable scientific meeting. We look forward to your participation.
Salamander Meeting 2024 Executive Committee. Toshinori Hayashi
https://sites.google.com/view/salmander2024/home

Histological Slides for Studying Axolotl Development are Available Online



The Harvard Museum of Comparative Zoology recently acquired the entire *R. Glenn Northcutt Collection of Comparative Vertebrate Neuroanatomy and Embryology*, the largest and most taxonomically diverse collection of histological preparations of developing and adult vertebrate brains mounted on glass slides. Among the more than 270 species represented in the huge collection of approximately 33,000 slides are more than 50 slide series of developing axolotls. These slides are now being scanned, and the resulting high-resolution images are freely accessible via two sites on the Internet. Interested users are encouraged to use these materials in their research and teaching.

The axolotl slides were the basis for Prof. Northcutt's pioneering studies of development and evolution of the vertebrate brain, neural crest, cranial nerves and sensory placodes. They comprise slide series of 46 embryonic axolotls of various stages, as well as four series from 1- , 2- and 3-weeks posthatching and one juvenile. There are a total of 1910 slides, which yield a mean number of 37 slides/series. For most series, entire embryos were embedded in plastic matrix, cut into 5 µ-thick serial sections in the transverse plane, and stained with cresyl violet.

The following 12 axolotl slide series have been scanned to date and may be viewed as whole-slide images via MCZbase https://mczbase.mcz.harvard.edu/ and MorphoSource https://www.morphosource.org/ Special Collections (SC) 3954–3961 (8 series), 3978, 3989, 3991 and 3992. Images may be viewed online or individually downloaded for free. Scanning and uploading of additional slide series is ongoing, and we welcome suggestions of particular series to be added to our priority list and of enhancements to the user experience. For additional information, please contact James Hanken hanken@oeb.harvard.edu. Supported by NSF award DBI-2122620 to James Hanken.

Lessons Learned from an Axolotl Disease Outbreak in the Ambystoma Genetic Stock Center

Maddie Thomas[1], Raissa Cecil[1], Cora Bankert[1], James Schwartz[1], Chris Muzinic[1], Jeramiah Smith[2], Randal Voss[1]

Ambystoma Genetic Stock Center and Spinal Cord and Brain Injury Research Center[1], and Department of Biology[2], University of Kentucky, Lexington KY

Mitigating pathogens and disease is essential to ensuring the health and well-being of animals housed in research facilities (Luchans and Langan 2018). Standardized husbandry and sanitation protocols are generally effective in preventing opportunistic and nuisance pathogen outbreaks. However, even with standard protocols in place, unexpected disease outbreaks can occur. In some cases, a disease is diagnosable and strategies are enacted quickly to mitigate spread and treat animals. In other cases, animals may present novel pathologies that are not immediately diagnosable, if diagnosable at all, and large numbers of animals may perish before a treatment is identified. Although devastating, important lessons can be learned from undiagnosed outbreaks to inform future disease prevention plans.

While much is known about mammalian diseases, very little is known about amphibian diseases (Densmore and Green 2007). Excepting chytridiomycosis, which has been intensively studied and associated with global amphibian declines over the last 25 years (Berger et al 1998; Olson et al 2013; Scheele et al 2019), relatively few common diseases are known for frogs and salamanders. Even fewer diagnostic assays are available to test for disease agents. Moreover, histological slides of normal and diseased amphibian tissues are generally lacking, as is expertise to interpret histopathology. When a novel disease suddenly appears in an amphibian housing facility it presents a challenge to identify best strategies for diagnosis and mitigation.

Here, we share information about a recent disease episode that occurred in the AGSC during the spring of 2023. Significant mortality was observed over a 2-month period and then as rapidly as the mortality issue began, it resolved. This case report reviews the chronology of the mortality episode, and actions taken during the crisis to mitigate and potentially identify a disease agent. In the end, a disease agent was not identified but new tools and resources were developed. Lessons were also learned to better guide future courses of action in mitigating axolotl disease.

Case Report

In late April and early May 2023, 100% of newly hatched axolotl larvae from several spawns unexpectedly died. The deaths were first observed in spawns made using transgenic individuals that had been imported into the AGSC in 2022. Soon after, mortality was observed in spawns from well-established AGSC lines (Figure 1). In affected spawns, dead larvae were observed approximately 3-4 days after initiating feeding on newly hatched brine shrimp. Inspection of dead and living larvae revealed small papillae on the gills and on the outer epidermal surfaces of the body (Figure 2). Moribund larvae presented necrotic gills, spinal curvature, swollen cloacae,

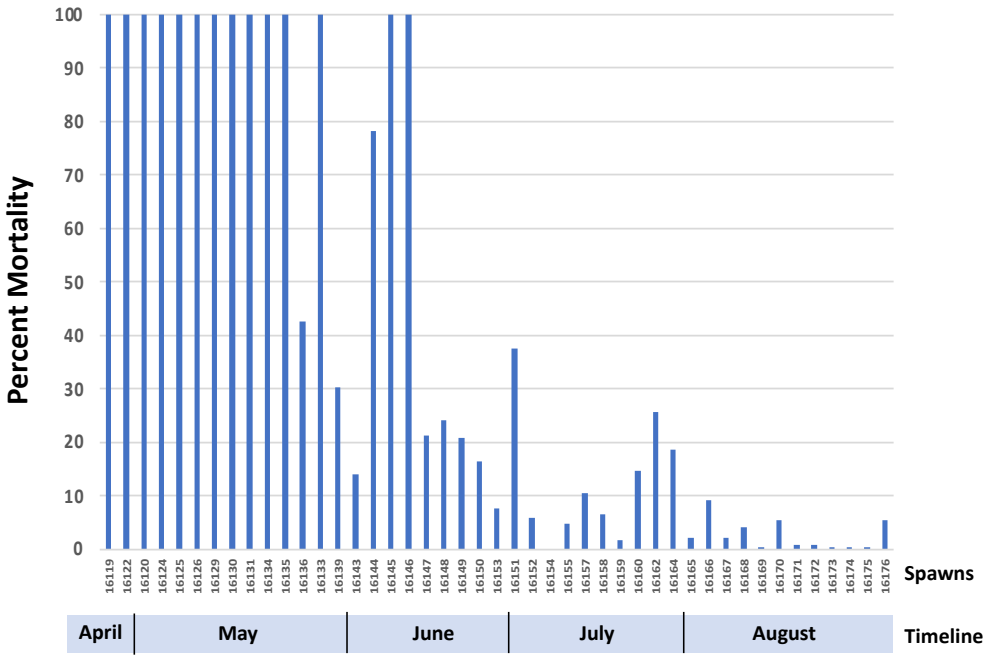


Figure 1. Mortality across spawns during the larval mortality episode of 2023.

loss of righting reflex, abnormal swimming behavior and lethargy. Mortality was not observed for embryos, older larvae, or adults.

AGSC staff learned through email communications with axolotl community members that satellite axolotl facilities were either currently experiencing or had experienced similar epidemics of larval mass mortality during the previous 1-1.5 years. In one case, all attempts to raise larvae in a satellite facility from September 2022 until present had failed and larvae showed similar pathologies described above. Further potential connections were identified: the case above and a second lab

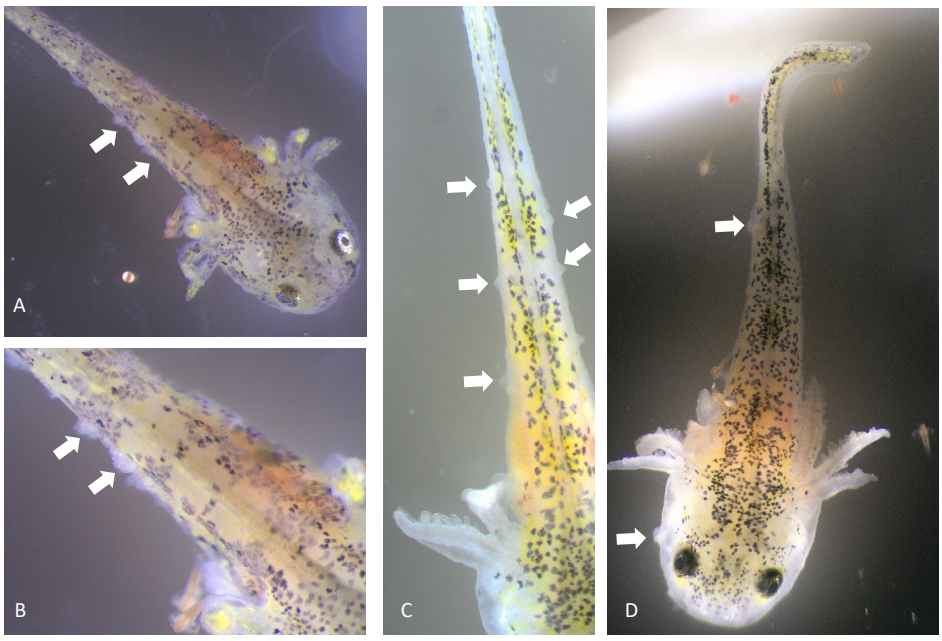


Figure 2. Images of diseased larvae. A) Larva with papillae along the flank (white arrows) and necrosed gills. B) Higher magnification image of larva in panel A. C) Larvae with papillae along the flank (white arrows). D) Larva with papillae along the flank (white arrows), curved gills, and curved spine.

currently experiencing mass mortality had received imported transgenics from the same lab that donated transgenics to the AGSC. Curiously, in reaching out to axolotl pet traders, they too voiced concerns of larval mass mortality episodes in the US and abroad. As the AGSC does not supply axolotls to the pet trade, this suggested that larval mortality may trace to a common husbandry item used by both communities. This could include brine shrimp cysts that are used to generate nauplii for feeding larvae. On May 23, the UK attending veterinarian was contacted and made aware of the larval mortality issue. Actions to mitigate and potentially diagnose the problem were discussed. The epidermal papillae were suggestive of *B. dendrobatidis* and Ranavirus infection. AGSC staff perform quarterly tests for these pathogens, but tests had not been conducted in the last two months. Inhouse testing for these pathogens and also *B. salamanderivorans*, a lethal, salamander-specific chytrid pathogen, was established priority. Additionally, the need to more broadly survey for pathogenic agents was also identified as a priority. To meet this need, samples would be sent to IDEXX, a diagnostic veterinary lab which offers axolotl pathogen testing and histopathology services.

From May 23-26, inhouse PCR testing of water samples and tissues from diseased larvae was performed to detect *B. dendrobatidis, B. salamandrivorans* and Ranavirus species (Appendix). All three tests were negative. On May 25, diseased larvae from 3 different spawns were euthanized and immediately flash frozen to form 4 pooled tissue samples (4 larvae per pool). Additionally, 9 embryos from these same spawns (3 from each spawn) were fixed in 10% formalin. All of the samples were shipped to IDEXX for PCR, bacterial, and histopathology analyses. AGSC staff thoroughly sanitized the facility, focusing on racks and equipment dedicated for larval and transgenic husbandry. Additionally, new husbandry practices were enacted to prevent the spread of pathogens among spawns. Specifically, separate nets were dedicated to racks for cleaning and workers were instructed to sanitize nets in bleach between bowl changes.

The axolotl community was informed on June 7, 2023 that stocks would not be shipped or imported until the larval mortality issue resolved. Also, the community was asked to contact the AGSC if mortality or pathologies described above were observed in their facilities. Three separate labs stated that they had experienced larval mortality issues in the past but did not document disease pathology. While waiting for IDEXX results, PCR assays were developed to detect pathogens that caused similar epidermal lesions in fish and salamanders: *Dermocystidium* (Gonzalez-Hernandez et al 2010) and *Amphibiocystidum* species (Fiegna et al 2017) (Appendix). Both of these PCR assays were negative using tissue obtained from diseased larvae. On June 12, an experiment was initiated to test for the potential of disease transmission from axolotl larvae to adults. Three adults from the AGSC breeding population were randomly assigned to 4-liter plastic (PP) bowls. The first bowl (female) was filled with water taken from diseased larval bowls; the water was filtered to remove brine shrimp prior to use. The second bowl (female) was filed with 100% ARW and supplemented with newly hatched brine shrimp. The third bowl (male) was filed with 100% ARW and 25 diseased larvae. Adults were monitored daily for disease pathologies observed in larvae as well as changes in behavior. Bowls were cleaned after one week and refilled to repeat the treatments. After the second week, the bowls were cleaned, the adults were returned to standard husbandry procedures (pellet diet every other weekday; cleaning every other day), and individuals were monitored daily for two additional weeks. During the experiment, none of the adults presented disease pathologies, including the adult in bowl 3 that consumed all 25 diseased larvae within an hour of initiating treatments on weeks 1 and 2. Although this experiment lacked

replication, the results were consistent with daily health monitoring records of the AGSC adult breeding population during the larval mortality episode. Disease pathologies were restricted to early-stage larvae and never observed in juvenile or adult axolotls.

On June 15, IDEXX made available PCR, microbial, and histopathology reports for larval tissues and specimens that were submitted for analysis 20 days earlier. PCR results were negative for *B. dendrobatidis, B. salamandrivorans* and Ranavirus species, thus confirming AGSC PCR results. Additionally, PCR assays for *Flavobacterium columnare*, *Mycobacterium chelonae*, *Mycobacterium marinum*, *Piscinoodinium pillulare*, and *Salmonella* spp. were also negative. A positive PCR result for *Chilomastix* spp. was obtained for one of the tissue samples but it was not visually confirmed during histology; as *Chilomastix* spp. are not considered pathogenic, this result was not pursued further. Specimens were also negative for common microbial species, including *Aeromonas* species*, Pseudomonas aeruginosa, Saprogenla* species*,* and *Salmonella enterica.* IDEXX sectioned larvae longitudinally and found no internal lesions or anomalies in organ systems of diseased individuals. After asking IDEXX to examine outer epidermal surfaces they reported that papillae did not contain inclusions typical of pathogen infection. The results from IDEXX were reviewed by 3 board certified pathologists and discussed with a DACLAM board certified manager of the IDEXX aquatic species health monitoring team. While the results eliminated common and diagnosable disease agents, a specific or likely agent for axolotl larval mortality was not identified.

With no progress being made to diagnose a disease agent, an experiment was performed to carefully characterize disease onset and progression in bowls with different larval densities. On June 26, 2023, hatchling stage larvae from a single spawn were randomly assigned to 1.5 liter, glass bowls at different densities (11, 20, 40, 60, and 80 per bowl), each containing 1 liter of 100% axolotl rearing water (1.75 g NaCl, 100 mg $MgSO_4$, 50 mg $CaCl_2$, and 25 mg KCl per liter, $NaHCO_3$ added to achieve pH 7.1-7.6). No gill or epidermal papillae were observed at the time larvae were placed into bowls. After this initial inspection, larvae were fed brine shrimp and on the following day bowls were cleaned and new water added. Care was taken not to cross-contaminate bowls during cleaning and feeding. Prior to brine shrimp feeding on days 2-5, 5 individuals were removed from each bowl, anesthetized using 0.02% benzocaine, and the total number of papillae on each larva was recorded under a dissecting microscope. Papillae were observed on all 5 larvae from every treatment on day 2. The number of papillae per larvae ranged from 4-14 and the average number of papillae per larvae was similar across treatments for all days (Figure 3). With respect to mortality, no deaths were recorded from the 11 or 20 density treatments on days 1-5, while dead larvae were recorded on all days for the higher density treatments. However, mortality at day 22 was similarly high across all treatments (Figure 4). These results showed that papillae arose rapidly after larvae initiated feeding on brine shrimp, and although mortality was low under low density conditions for the first 5 days of feeding, the percentage of larvae surviving to day 22 was similar across treatments.

Given the association between the onset of brine shrimp feeding and disease, AGSC staff next considered the possibility that brine shrimp might be the disease source. This seemed reasonable because pet traders that contacted the AGSC were seeing mortality and similar pathologies in

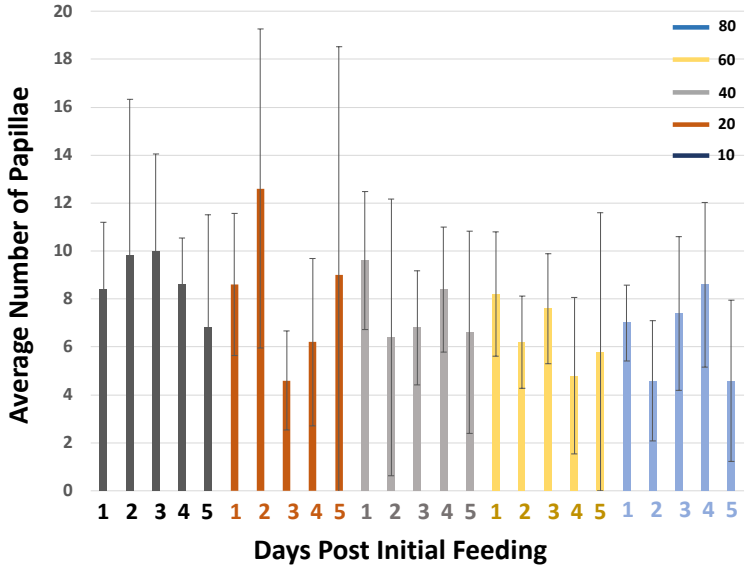


Figure 3. Papillae as a function of time and larval density. On each of the first 5 days of feeding, 5 larvae were sampled from each of 5 density treatments (11, 20 40, 60, 80) and papillae were counted to calculate an average value. The error bars are standard deviations.

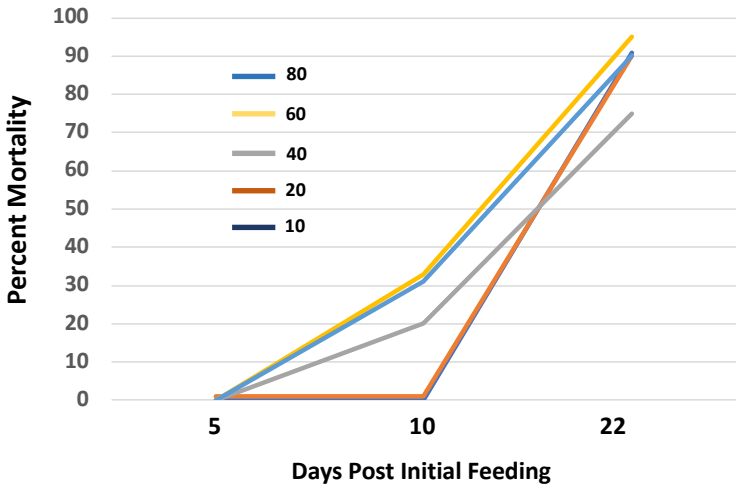


Figure 4. Mortality across different larval densities. Larvae from a single spawn (were reared at different densities for 22 days. Mortality was scored at Days 5, 10 and 22 post-initial feeding.

larvae that were fed brine shrimp. Moreover, most of the brine shrimp cysts that are harvested for culture to generate nauplii come from a common source, the Great Salt Lake (GSL). The GSL has recently experienced historically low lake levels and thus potentially stressful conditions for brine shrimp, possibly increasing the probability of acquiring and transmitting disease. As brine shrimp are known to be vectors for LCDV (Cano et al 2007), a fish pathogen that causes epidermal lesions, AGSC staff performed PCR (Appendix) on June 20 in attempt to detect LCDV in diseased axolotl larvae and brine shrimp. As all tests for LCDV were negative, AGSC staff moved on to perform other brine shrimp experiments. The first experiment considered the possibility that capsulated brine shrimp cysts might be the source of disease. Cysts are sold by commercial vendors with or without a hard outer capsule. The AGSC has long used capsulated cysts because they are

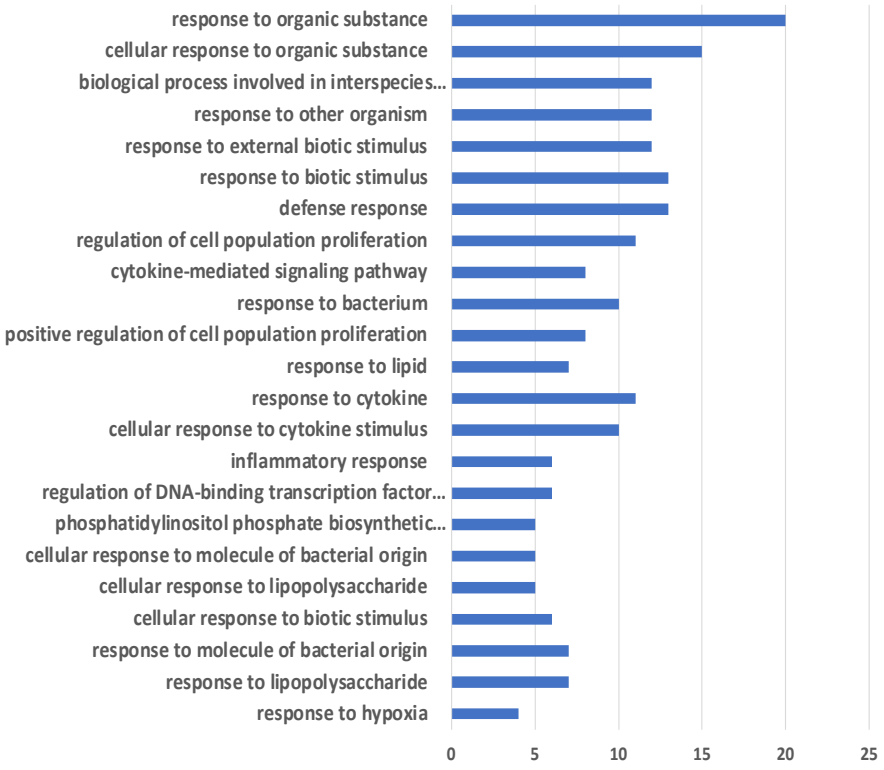
considerably cheaper than decapsulated cysts and it is relatively easy to separate out the outer capsules from nauplii before feeding. Decapsulated cysts are marketed as a healthier alternative because the ingestion of unhatched cysts and cyst shells may damage the GI track. The chlorine treatment process used to dissolve the outer capsule in preparing decapsulated cysts also potentially mitigates pathogens. Indeed, it has become standard practice in fish hatcheries to decapsulate cysts with chlorine prior to feeding (Sais et al 2006).

On July 3, hatching larvae from a single spawn were randomly assigned to two 1.5 liter, glass bowls (60 per bowl), with each bowl containing 1 liter of ARW. One bowl was fed shrimp hatched from the current supply of capsulated cysts and the other bowl was fed with newly purchased decapsulated cysts. The different types of brine shrimp cysts were cultured in separate rooms and were harvested and dispensed using separate equipment. After two days of feeding, papillae were observed on 10 larvae sampled from each bowl. These results showed that disease is observed when feeding brine shrimp hatched from capsulated or decapsulated cysts, at least for GSL sourced cysts.

On July 7, RNA was isolated from the gills and tails of hatchling stage larvae that either presented or did not present papillae after two days of brine shrimp feeding. Presumably these individuals presented two different severities of disease (low vs high) and thus an analysis of genes expressed differently between these samples might provide insight about the disease agent. Gill and tail tissues from each group were pooled to yield four RNA samples that were submitted to Novagene for RNA-Seq to generate approximately 25 million paired reads per sample.

While waiting for the RNA-Seq results, AGSC performed additional brine shrimp studies. With recent declines in water level, the GSL has become increasingly contaminated with heavy metals and toxins are beginning to accumulate in the GSL ecosystem (Wurstbaugh et al 2020). Although there have been no reports of heavy metal toxicity in GSL brine shrimp, a decision was made to switch from GSL cysts to cysts harvested from San Francisco Bay (SFB) and Pacific coast estuaries. On July 12, approximately 60 hatchling larvae from six new spawns were fed SFB brine shrimp, again using 1.5 liter glass bowls and 1 liter of ARW. No papillae were observed on larvae from these spawns and mortality through August 10 declined to less than 11% per spawn. These results suggested that the disease could be prevented, and mortality lessened, by feeding larvae SFB cysts instead of GSL cysts. This implicated GSL cysts and metal toxicity as a potential source of disease. RNA-Seq results obtained on July 17 further supported this hypothesis. For statistical analysis, gill and tail samples for each disease state were treated as replicates and significantly differentially expressed genes were identified between the samples using DESeq2. A total of 384 and 185 differentially expressed genes were identified for the low and high disease states, respectively, using adjusted p-value (prob < 0.05) and log2 fold change (FC > 1.5) thresholds. Differentially expressed genes with annotated gene names (N = 304 low disease state; N = 153 high disease state) that were identified between low and high disease states are typically expressed in response to external cellular stimuli. Using these genes and a Panther statistical over-representation test (Huaiyu et al 2019), the low disease state enriched gene ontology terms associated with responses to organic, biotic and bacterial stimuli, while the high disease state enriched terms associated with response to stress, cell cycle, DNA damage, and DNA repair (Figure 5). Genotoxic gene expression responses are often observed in studies of heavy metal

Low Disease State



High Disease State

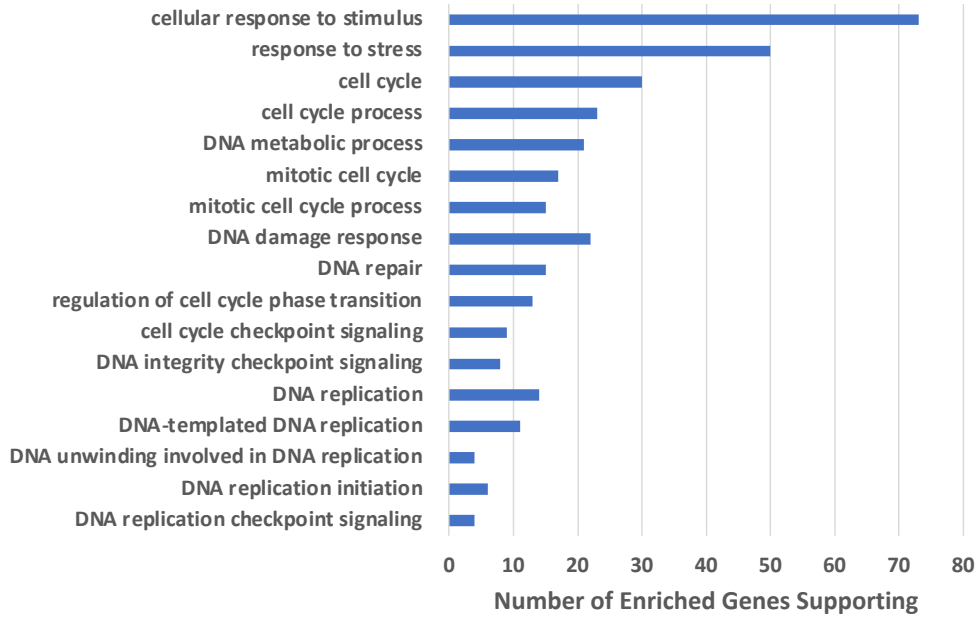


Figure 5. Gene ontologies associated with low and high disease states. Differentially expressed genes between low and high disease states were analyzed using an over-representation statistical test to identify significantly enriched biological process gene ontologies.

toxicity (Beyersmann and Hartwig 2008). To further pursue this hypothesis, GSL and SFB brine shrimp samples were sent to a diagnostics lab at University of Idaho for heavy metal screening by inductively coupled plasma mass spectrometry (ICP-MS).

While waiting for the ICP-MS results, AGSC staff directly compared GSL and SFB cysts in a feeding experiment. On July 28, 120 individuals from each of two spawns were randomly assigned to either a diet of GSL or SFB brine shrimp. In this experiment, 60 individuals were reared per 1.5 liter bowl to yield 2 replicates per spawn. No papillae or mortality was observed over a 7-day period. All individuals were healthy and thriving, and there was no evidence of disease. To further confirm this result, three new spawns were generated on August 1. On August 8, results of the heavy metal analysis were received from the Idaho lab. Neither the GSL nor SFB brine shrimp cyst samples showed elevated levels of heavy metals. On August 12, AGSC staff began feeding the August 1 spawns SFB brine shrimp. No evidence of disease was observed over a 7-day period. On August 22, the AGSC received clearance from the University of Kentucky attending veterinarian to resume shipments of axolotls to the community.

Lessons Learned

AGSC staff expended considerable effort during the larval mortality episode to mitigate the spread of disease and identify a disease agent. AGSC staff worked diligently to identify a disease agent, but in the end, an agent was not identified. Still in hindsight, important lessons were learned.

Animal Monitoring. Daily animal monitoring is key to identifying disease threats within captive populations. It is not unusual to observe mortality during the early larval stage in axolotls. Additionally, transgenic and knockout lines often have lower survival relative to wildtype lines. While the spike in larval mortality observed in May was identified relatively quickly, at the time, AGSC staff were not recording individual deaths of hatchling stage larvae, which are reared at relatively high densities (60-100 per bowl). Accordingly, it was not known if mortality had been incrementally increasing prior to May. Because of the effort required to record daily larval deaths for approximately 500 spawns per year (>50,000 individuals), these data have not been traditionally recorded. However, the recent larval mortality episode clearly established a need to monitor larval deaths as a mechanism to identify infectious disease and more generally safeguard an irreplaceable captive population. Moreover, such data are useful to identify spawns with low viability and potential deleterious alleles, and spawns with high viability from which to select individuals for the breeding population.

Notify stakeholders. Soon after AGSC staff confirmed that hatchlings were dying across multiple spawns, the attending veterinarian was notified, as is required when an adverse event occurs. This initial contact not only met a regulatory requirement, it also established a supportive line of communication to veterinary expertise in how best to deal with a disease outbreak. We quickly identified need to send samples to a diagnostic lab for testing, notify the axolotl community that we were suspending shipments, and enact strict sanitation and quarantine procedures in the AGSC. Throughout the disease episode, UK veterinarians met with AGSC staff to provide guidance and advice about experiments that might be performed to mitigate and potentially diagnose the disease. The partnership that formed helped AGSC staff navigate a very stressful situation. Important lessons were learned about the non-regulatory roles that campus veterinarians play; they are there to support investigators in the shared mission of ensuring animal health and well-being.

Document disease pathology. Several new PCR and qPCR assays were developed during the larval mortality episode that can be used for axolotl disease monitoring (Appendix). Also, disease onset and pathology were carefully detailed, and perhaps most importantly, diseased larvae were imaged. When the AGSC made pathological details and diseased larval images available to the axolotl community, several investigators and pet-dealers communicated that they had observed similar pathologies. However, they did not document cases or take images of diseased animals. It was therefore impossible to verify if the AGSC larval disease was occurring in outside facilities. Given the paucity of information about axolotl diseases, the larval mortality episode clearly revealed a need for investigators to carefully document diseased animals. Moreover, an axolotl disease knowledgebase is needed to document axolotl diseases and catalogue assays that can be implemented by investigators for disease monitoring. Efforts are underway to meet this need.

Be proactive, perform experiments. During the disease event, AGSC staff sent samples to outside service providers for analysis, developed new pathogen monitoring assays, and performed in-house experiments. Although a causal factor was not identified, the pathological data and new assays that were generated may prove valuable in dealing with future disease events. Equally important was the experience gained in performing experiments to test the effects of specific variables on larval pathology and mortality. To operate successfully, an animal resource center needs staff that are both proficient in performing animal husbandry and capable of performing scientific experiments during times of crisis.

Implement a disease disaster plan. The AGSC has a disaster plan in place to mitigate catastrophic events like fire, flood, earthquake, tornado, utility failure and civil disobedience, but the plan does not include actions to mitigate a disease outbreak. A disaster plan generally addresses Preparedness, Response and Recovery. In addition to these responses, we also now include Mitigation as important initial response to a disease outbreak in our revised disaster plan and discuss each of these below:

Mitigation: During this phase, actions are taken to mitigate or reduce the cause, impact, and consequence of disease. As detailed above, the attending veterinarian was informed of the situation, diseased animals were quarantined from all other stocks, and methods of husbandry used during quarantine were enacted. In the case of larval mortality, it was possible to quarantine diseased individuals from other AGSC stocks, however, cases involving larger/older animals might be more difficult to quarantine. Other cases may necessitate euthanizing relatively large numbers of animals. The advice of the attending veterinarian is essential when making decisions about quarantine, euthanasia, medical treatment options, clinical testing, and best paths forward. Seeking out help and expertise in the early phase of a disease outbreak is key to mitigation.

Preparedness: The AGSC is prepared to handle sporadic cases of diagnosable disease. However, in the case of a disease outbreak where many animals are affected simultaneously, initial responses require more effort and resources, and thus a higher level of preparedness. Disease outbreaks are more complicated when dealing with an undiagnosed agent because there are no guidelines for treating animals or how best to contain the agent and prevent transmission. Going forward, the AGSC will maintain a sufficient reserve of animal care supplies to enable quarantine husbandry of 50% of larval, juvenile, and adult stocks. Funds will be budgeted annually to pay

for unanticipated costs that are incurred during a disease outbreak, such as assay testing. Additionally, the AGSC will continue to develop assays for emerging pathogenic threats and staff will meet annually to discuss operations and the disaster plan.

Response: AGSC staff had to quickly adapt to the larval disease outbreak, they had to learn new husbandry protocols, reprioritize job tasks, deal with a business operation shutdown, and interact with disgruntled customers. Experiments were performed to determine the onset and pathology of disease, samples were sent to a diagnostic lab for testing, and assays were developed inhouse to test for potential pathogens. To increase the possibility of disease diagnosis, samples will be sent to multiple labs in attempt to diagnose future diseases. Also, with limited expertise available to diagnose diseases in amphibians, development of inhouse assays will continue to be an AGSC priority.

Recovery: Going forward, it will be important to integrate new methods, assays and protocols developed during the larval disease episode into regular AGSC operations and activities. Although an agent was not identified, higher standards of animal husbandry and sanitation were established, and new assays were developed to monitor pathogens. The only good thing about a disaster is that it provides a crisis, a situational problem that requires new ways of thought and creative action (Beghatto 2020). Lessons learned from the larval disease crisis of 2023 will improve future preparedness and essential functions within the AGSC.

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Appendix. Assays performed in response to the 2023 AGSC larval mortality event. Additional information about assays is available upon request.

Date	Assay	Target	Sample	Result
5/22/23	QPCR	*Batrachochytrium dendrobatidis*	Water Sample from Disease Positive Bowl	Negative
5/25/23	QPCR	*Batrachochytrium salamandrivorans*	Water Sample from Disease Positive Bowl	Negative
	QPCR	*Ranavirus spp.*	Water Sample from Disease Positive Bowl	Negative
5/26/23	QPCR	*Batrachochytrium dendrobatidis*	Diseased Larva 1	Negative
	QPCR	*Batrachochytrium salamandrivorans*	Diseased Larva 1	Negative
	QPCR	*Ranavirus spp.*	Diseased Larva 1	Negative
5/30/23	IDEXX PCR	*Chilomastix spp.*	4 samples, 5 diseased larvae per pool	1 positive, 3 negative
	Analyses	*Batrachochytrium dendrobatidis*	4 samples, 5 diseased larvae per pool	4 negative
		Batrachochytrium salamandrivorans	4 samples, 5 diseased larvae per pool	4 negative
		Flavobacterium columnare	4 samples, 5 diseased larvae per pool	4 negative
		Mycobacterium chelonae	4 samples, 5 diseased larvae per pool	4 negative
		Mycobacterium marinum	4 samples, 5 diseased larvae per pool	4 negative
		Piscinoodinium pillulare	4 samples, 5 diseased larvae per pool	4 negative
		Ranavirus spp.	4 samples, 5 diseased larvae per pool	4 negative
		Salmonella spp.	4 samples, 5 diseased larvae per pool	4 negative
5/30/23	IDEXX Microbiology	*Aeromonas dhakensis*	4 samples, 5 diseased larvae per pool	4 negative
	Analyses	*Aeromonas hydrophila*	4 samples, 5 diseased larvae per pool	4 negative
		Pseudomonas aeruginosa	4 samples, 5 diseased larvae per pool	4 negative
		Salmonella enterica	4 samples, 5 diseased larvae per pool	4 negative
		Saprolegnia spp.	4 samples, 5 diseased larvae per pool	4 negative
		Flavobacterium columnare	4 samples, 5 diseased larvae per pool	4 negative
		Aeromonas dhakensis	4 samples, 5 diseased larvae per pool	4 negative
		Aeromonas hydrophila	4 samples, 5 diseased larvae per pool	4 negative
		Pseudomonas aeruginosa	4 samples, 5 diseased larvae per pool	4 negative
		Salmonella enterica	4 samples, 5 diseased larvae per pool	4 negative
		Saprolegnia spp.	4 samples, 5 diseased larvae per pool	4 negative
5/30/23	IDEXX Histopathology	Longitudinal Sectioning	8 diseased larvae	8 Negative
6/6/23	RNA-Seq	Axolotl and Microbial transcripts	Diseased Larva 3 - Gills	63668668 raw reads
	RNA-Seq	Axolotl and Microbial transcripts	Diseased Larva 3 - Tail	61595390 raw reads
	RNA-Seq	Axolotl and Microbial transcripts	Normal Larva 2 - Gills	62331332 raw reads
	RNA-Seq	Axolotl and Microbial transcripts	Normal Larva 2 - Tail	62805126 raw reads
6/9/23	PCR	*Dermocystidium salmonis*	Diseased Larva 1, Normal Larva 1	Negative
6/13/23	PCR	*Dermocystidium salmonis*	Diseased Larva 4, Normal Larva 1	Negative
6/14/23	PCR	*Amphibiocystidum spp.*	Diseased Larva 5, Normal Larva 1	Negative
6/15/23	QPCR	Lymphocystis Disease Virus	Brine Shrimp 1, Brine Shrimp 2	Negative
7/25/23	U. Idaho ICP-MS	Heavy Metal Screen	Brine Shrimp 3, Brine Shrimp 4	Negative