TESTING FOR *BATRACHOCHYTRIUM DENDROBATIDIS* ("Bd")

The purpose of this tutorial is to provide protocols and pertinent information for those professionals who are in the field collecting samples and would like to test their samples for potential Bd infection. It is assumed that these individuals will be either sending their samples out to another facility or will be handing them over to a qualified colleague who will perform the tests for them. This tutorial is NOT intended to teach folks how to identify the pathogen, nor is it intended to teach the readers how to do wet mounts, histology or run PCR analyses. It is our hope that this guide will provide standardization of collection procedures and meet a perceived need for information on how to properly collect, preserve and store samples destined to be tested for Bd.

Introduction

There are four methods currently available to test amphibians for Bd infection. Due to the recent recognition and identification of this pathogen (Laurance et al. 1996; Longcore et al. 1999), diagnostic methods are still under investigation to ascertain which of these methods is most reliable in detecting infection in amphibians and which methods are most appropriate for the variety of specific hypotheses being tested (see entire lit cited). There are advantages and disadvantages to each method. For example, wet mount preparations of skin scrapes is fastest, cheapest and easiest; however you cannot use this method with anuran larval samples AND you must have a qualified, highly experienced scientist who can prepare the wet-mount slides and identify the Bd cells. DNA testing can be very expensive depending on the number of samples you have, but it is much faster than histology. So as you can see, there are trade-offs regardless of which testing method you opt for.

At this time, methods to diagnose Bd consist of: skin scrapes for microscopic examination, histological serial sectioning of tissue, PCR analysis and real-time ("quantitative") PCR analysis of either tissue samples or cell samples collected on cotton swabs. The difference between PCR and qPCR is that qPCR (Boyle et al. 2004; Kriger et

al. 2006a; Kriger et al. 2006b; Hyatt et al. 2007; Kriger et al. 2007; Smith 2007) will quantify the number of zoospores in a sample, whereas regular PCR (Annis et al. 2004) will diagnose the presence or absence of the pathogen in a sample and does not attempt to quantify zoospores in a sample. Again there is some disagreement between geneticists regarding the sensitivity of PCR and qPCR as Dr. Alex Hyatt states that qPCR is more sensitive than regular PCR, whereas Dr. John Wood (who does both types of PCR in his lab) asserts that there is no difference in sensitivity between methods.

The method of choice is primarily based on three factors: 1) the amount of funding available for the diagnostic work; 2) the equipment and laboratories available to the investigator; and 3) the availability of scientists qualified to identify the pathogen in *situ*. Other considerations when choosing a diagnostic method include the purpose of the investigation; whether it is to determine overall presence or absence of the pathogen or to quantify the prevalence of the pathogen within a demographic sector, a population or a community. In addition, the lifestage and taxonomic group of the amphibian tested will influence the method chosen.

If the samples you are dealing with are archived museum specimens, the only choice for Bd diagnosis is histology. The vast majority of museum specimens were originally (and may still be) preserved in formalin. Formalin-preserved specimens cannot be tested through DNA methods (PCR and qPCR) as formalin damages the DNA in a sample, potentially giving false negative PCR results; therefore histology is the only option to diagnose formalin-preserved or formalin-fixed samples.

It is best if the method of testing has already been decided before going in the field, as this will impact how samples can be collected, preserved and stored. If the method of testing has not been decided, or there is a potential that DNA testing may be done on the tissues at some future date, then follow the protocol for collection and preservation of samples for PCR (i.e. DNA) testing. The protocol is the same whether PCR or qPCR will be used. It is vital that you prepare ahead of time for sampling to ensure that you have the right supplies. Using whatever you have on hand, at best, may constrain the type of testing that can be done; at worst, it can result in false negative or false positive results; it is even possible that no results can be obtained. So please prepare by ensuring you have the appropriate supplies as outlined below.

SUPPLIES

This is a complete list of all supplies for all methods of sampling for Bd. If you are not going to be collecting whole animals, then you can omit the containers for whole animals from your list. If you are planning to send tissue samples for testing- you can omit the cotton swabs and skin scrape sticks from your supply list. Similarly, if you plan to submit skin scrapes you can omit the swabs and the whole animal containers.

***Gloves (lots of them)*:** Figure out before hand how many you may need so you are not caught short. It is better to have too many and bring some back, than to run out before you are done with your sampling. If you wear two gloves each time and plan to collect 100 animals with 2 people working- you will need a minimum of 500 gloves. (Gloves tear easily!)

When you remove the gloves, be sure to pull the first glove off by pulling it from the outside and inverting it; then with your ungloved hand, slide your thumb inside the cuff of the other glove and tug it down to invert and envelope the first glove. Touching only the inside of the gloves, dispose of them inside a plastic bag.

***Cotton swabs on 2mm-diameter wood sticks without adhesive*:** The swabs do not need to be sterile when purchased, but you do need to ensure that the cotton is NOT attached to the wood with adhesive. If glue is present it can contain PCR inhibitory compounds and render a false negative result**.** Swabs such as Puritan Cotton-Tipped Applicators are great for swab samples, but they will be too long to fit in the vials. Therefore you will need to cut them down before you begin sampling. Cut the wood stick to lengths (approximately 3-cm) that will fit into your 1.5-2-ml vials. Large scissors work fine for this.

✦One really important concern is the **storage** of your swab sticks prior to using them. When you receive them, you will find that they are shipped in bulk and packaged in paper. It is crucial to re-package them immediately into Ziploc baggies (or any air-tight, water-tight holder) to prevent moisture from collecting. Any moisture getting into

the packaging will facilitate the growth of a variety of molds and fungi on the cotton tip that may not be detectable with the human eye. Molds and fungi can destroy or damage the Bd DNA- rendering yet again, a false negative result.

There are many companies that make cotton swabs; nonetheless we are providing the link to the VWR page as wading through pages on the web can be discouraging. This link will take you directly to the page at VWR to order swabs:

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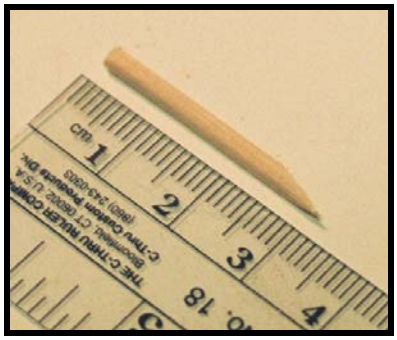
http://www.vwrsp.com/psearch/ControllerServlet.do?D=10806-005&CurSel=Ntt&Ntt=10806-005&Ntk=All&spage=header&Ntx=mode%2bmatchpartialmax&Np=2&N=0&Nu=RollupKey&cntry=us&Nty=1

Wooden sticks for scraping skin: Toothpicks work very well for skin scrapes and are inexpensive. If you have cotton swabs that you have cut down, you can use the discarded stick as a skin scraper. Either way, you will have to cut the wood pieces down to a size that will fit in your vials and also create an angled point on one end. The easiest way to do this, is to break or cut the stick or toothpick such that the end has an angle, (see photo below). As with the cotton swabs, you will need to store these in water-tight baggies or containers as mold and fungi will take up residence on the wood if it gets wet. Molds and fungi can destroy or damage the Bd DNA and render a false negative result in the PCR.



***70% DNA-grade non-denatured ethanol (ETOH)*:** We cannot stress enough the importance of the integrity of the ethanol. If you are planning to use ethanol from a

communal lab carboy, first **research** where the ethanol has come from and **verify** that it is **molecular- or histological-grade non-denatured ETOH**. Impurities or trace compounds such as benzene, kerosene, and ketones are frequently found in industrial-grade ethanol and can either degrade the Bd DNA (resulting in a false negative) or can prevent the PCR process from amplifying the Bd target sequence as it should. However, small quantities of methanol or isopropanol in the ethanol do not cause any problems

***Containers to store whole animals*:** You can use whatever types of containers you wish for preserving and storing whole animals, but they should be **leak-proof**. Do not allow any splashing of the ethanol when you place your sample in the container as Bd zoospores may be carried out in the droplets. This will contaminate the outside of the container; which through subsequent handling could potentially contaminate your other samples if you are planning on using PCR methods to diagnose Bd. If you place more than one tadpole in a container, and you plan to do PCR testing, the group will have to be processed together as the samples will likely be cross-contaminated. This is fine if the goal is to test for presence/absence of Bd within a group, but will not work if you are trying to establish prevalence or frequency of Bd within a population. If you are planning to do histology, you can group animals as you wish, but you will not be able to go back at a later time and do PCR once you have grouped animals in a communal container.

***1.5 ml or 2.0 ml screw-cap polypropylene microcentrifuge tubes to hold the oral disc tissue*:** The vial in which the oral disc or tissue samples will be stored is also <u>really important</u>. The vials should have external/male threads and the vial caps should have internal/female threads. The opposite threading - typical of "cryovials" - where the cap has the male threads and the vial has the female threads, result in splashing or leaking of the contents when opened and become a serious problem for the lab, as the splashing not only can contaminate your own samples, but will contaminate within the lab as well. The lab has to go to extra efforts and take extra precautions with these types of vials; which slow down the process and can add to the cost of testing. The lab has had sufficient problems with leaking samples that they are seriously contemplating adding a "leak surcharge" for samples submitted in cryovials.

The vials should be 1.5 ml or 2.0 ml <u>microcentrifuge</u> vials (Cryovials of any sort <u>will not fit</u> into microcentrifuge rotor machines. The samples are placed directly in a

centrifuge in the lab and if the vials are too large or are not centrifuge type, the lab will have to transfer the sample to an appropriate container. This will slow down the processing and can add significantly to the cost of testing. Below is a link to a website page where you can order appropriate vials. There are many companies that make these types of containers, but we have had a tough time wading through catalogue web pages so we are including this here:

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http://www.vwrsp.com/catalog/product/index.cgi?catalog_number=89004-308&inE=1&highlight=89004-308&from_search=1

These vials can be ordered with tops of varying colors that make it easy to differentiate between groups of samples and are also insulated with a silicon O-ring that makes a perfect fit for the cap. ***We have found that only vials with O-rings survive the rigors of shipping without leaking; all the other "leak-proof" vials have a significant failure (leaking) rate.*** We also prefer the flat bottomed vials as they can sit on a flat surface; the curve-bottomed vials must be placed in a tray made specially to hold them, adding to the cost of supplies. In general they are just much more difficult to work with.

Method to label your vials: We use a regular office-type small label making machine. Put the labels on **before** you fill the vials with ETOH. If ETOH is spilled on the outside of the vial- it will melt the adhesive and the label won't stick. Likewise, if you use a marker (even markers guaranteed to be "permanent") the ETOH will remove the ink. Do NOT wrap parafilm around centrifuge vial caps; the centrifuge vials are leak-proof so it isn't necessary. In addition, once again, if you wrap parafilm around the cap, the lab will have to take extra time to unwrap the vial resulting in time delays and potentially extra costs for testing. It also tends to make the lab grumpy!

When sending samples it is best to keep your label as simple as possible. The testing lab doesn't need all the information about the sample; they merely need a method in which to track and report their results to you. This also works in effect as a double-blind research technique.

BATCHING SAMPLES FOR DNA TESTING

A good way to save money on the cost of DNA testing is to batch samples. You can batch samples, cross-reference them to the individual samples and that way if your batch turns up positive, you can send in the individual samples that comprised the batch. There is a bit of disagreement among geneticists as to what constitutes the maximum allowable number of samples in a batch. Hyatt et al. (2007) have found that 5 samples batched gave spurious results, so they recommend 4 samples at most. The lab we work with states that what is important is the VOLUME of tissue in the batched sample, not the actual number of samples in the batch (J. Wood, pers. com). **The maximum total amount of tissue per batched sample you want to send in is approximately 0.25 ml or cc**.

CROSS-CONTAMINATION OF SAMPLES MUST BE AVOIDED!

The primary concern with DNA testing is **cross-contamination,** but it is not a concern for wet-mount slide preparation or histology. The best way to visualize this is to imagine sticking your hand in a bucket of paint and not washing it off. You then go around your daily activities and your neighbor can follow your path by following the paint spots you leave behind on everything you touch. Bd zoospores are microscopic (<10 μm) and so if you touch one infected animal (thus picking up Bd zoospores), and then touch a second uninfected animal, you will leave zoospores on the second animal. This second animal will show positive even though it wasn't actually infected because the zoospores left by you have contaminated the sample. This is cross-contamination.

Cross-contamination is a serious problem because you cannot be 100% certain that you haven't done so. Therefore you must take every precaution throughout the entire process, from field collection to processing in the lab, to ensure that you do not have an opportunity to cross-contaminate. This is also why it is important to follow these standardized procedures. ***Always keep in mind that if Bd zoospores are present in your samples, they will also be present in the solution the samples are stored in***. Any splashing of the solution, particularly on the outside of the storage container will result in Bd zoospores on the outside of the container. If you then pick up the container, you will get zoospores on your hand and they will transfer to the next object you touch. This will

also affect the lab- only on a bigger scale. The lab personnel are processing many samples, not just yours; so there is a potential if you contaminate the outside of your vials, you can affect other's results as well as your own.

The best way to avoid cross-contamination in the field while collecting is to use the "clean team-dirty team" scene described in the decon protocol on this website. Using this technique the clean team holds the container (wearing new gloves for each sample) while the "dirty team" handles the animals ultimately depositing the specimen (either a whole animal, a clipped toe or a cotton swab sample) into the container. This works really well to minimize the potential for cross-contamination. **AND you must change gloves after every single animal!**

If you are not collecting whole animals but rather are collecting toe clips or cotton swab samples with the intention of releasing the animals, you can still use the "dirty team-clean team" scenario. This time you actually have three teams of people. One team consists of the people or person getting wet and capturing animals; they deposit the animal in a bucket. The second team picks up the animal and takes the samples (toe clip or cotton swab) and other pertinent data and then returns the animal to the bucket. The third team records the data and holds the vial out to accept the toe or swab, capping the vial after the toe/swab has been deposited and storing it safely away from the center of action**.** The first team then takes the marked animal and releases it. Each team must change gloves after each animal.

PACKAGING YOUR SAMPLES FOR TESTING

Once you have collected your samples for Bd testing and you have a group of (leak-proof) vials to send, you will want to package them properly. Most samples are sent overnight which means they are traveling by air- this is the reason you do not fill your vials more than half-full with preservative. The easiest method is to group your samples by some method appropriate to your study and place each group in a Ziploc baggie. Place the Baggies in a **box** of appropriate size. You do not need to bubble wrap them or add extra materials to the package. Tape all openings shut as per the U.S. Postal Service standards. Envelopes, even the large ones with bubble wrap insulation, are not suitable as they are squashed during processing at the shipping centers, which means really bad news for you and your samples.

WHERE DO I SEND MY SAMPLES?

We have done exhaustive research into veterinary labs around the U.S. and found that there is only one commercial lab and one national lab that can and will test samples for Bd using DNA analysis. The USGS National Health Center for Wildlife in Madison, Wisconsin can diagnose Bd using any of the methods discussed here, but they are under contract to the federal government and cannot test samples for non-federal personnel or agencies. If you are part of a federal agency or funded by federal monies you will want to submit your samples by following this link: http://www.nwhc.usgs.gov/. Please be aware that because this lab services the entire United States and works on all wildlife not just amphibians, it takes months and in some cases, years, to get your results back.

For the rest of us, there is only one commercial lab available to test for Bd using DNA methods and that is the lab in Boulder, Colorado. The cost for sampling is currently **$23.00** per sample. ***When you send your samples, you will also need to send a spreadsheet electronically so that they can verify the contents of your package***. This will also allow them to be more effective in communicating with you if there are any issues with some of your samples. It is a good idea to give the lab a heads-up that you are shipping samples to them.

Send samples to:

Pisces Molecular

2200 Central Ave, Ste. F

Boulder, CO 80301-2841

jwood@pisces-molecular.com

Telephone: 303-546-9300

AMPHIBIAN LARVAE

ANURANS

In anuran larvae, Bd only occurs on the oral discs.

General Comments: PCR and qPCR analyses will detect Bd DNA in excised tissue samples or cell samples collected via cotton swabs. At this time swabbing of anuran larval mouthparts has been only marginally successful (see Retallick et al. 2006), so the **best methods for detecting Bd infection in anuran larvae are PCR, qPCR or histology**, all of which are performed on the tissues of the **oral disc**. Bd only infects the mouthparts of anuran larval amphibians, so unfortunately; all of these techniques require sacrifice of the organism. Once the tadpoles have been collected in the field, the entire oral disc of the larva is removed in the lab and is either pulverized for PCR or qPCR testing or is serial-sectioned and stained for histologic diagnosis. The commercial lab can remove the oral disc if you choose to send the entire larva or you can do that in your lab when you get back from the field. Be forewarned however, that sending an entire tadpole will result in delays and extra efforts in the lab which will add to the cost of testing as well.

COLLECTION AND PRESERVATION: DNA TESTING (PCR or qPCR)

Anuran Larvae Oral Disc Removal: Once you have collected your samples, you will need to amputate the oral disc, to submit for testing. Removal of the anuran larval oral disc must incorporate a high degree of care to prevent cross-contamination of samples. The easiest method to remove the entire oral disc is to position the tadpole on its dorsal aspect on the curved edge of a glass lens. The curve of the edge follows the natural curve of the tadpole's head, flexes the head region upward and puts the oral disc in a good position for amputation. Then grasp the oral region with a forceps held perpendicular to the head, holding the mouth closed and pulling it slightly forward (Fig.1). You can then use small (i.e. fine) straight scissors to amputate just behind the last toothrow visible in a single clip of the scissors. Use **only instruments** to manipulate each specimen as it is easier to prevent contamination if you keep your hands off of your samples and precludes the need to use gloves.

Fig. 1: Grasp the oral region, pull forward.

Fig. 2: Clip with fine scissors.

Fig. 3: Place oral disc flat side down.

Fig. 4: Bisect into right and left halves.

Fig. 5: Finished sample.

Both instruments (forceps and scissors) need to be **flame-sterilized** after every single sample and the glass dish can be similarly flamed and then wiped of soot. You can use either a candle or a Bunsen burner to sterilize the equipment (Fig 6). Place the entire oral disc in a labeled centrifuge tube or glass vial filled half-way with ethanol and secure the top. Don't forget to label your vials.

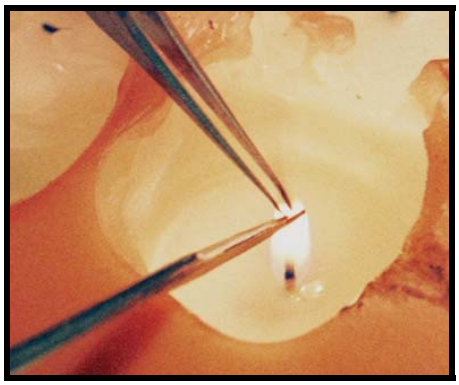
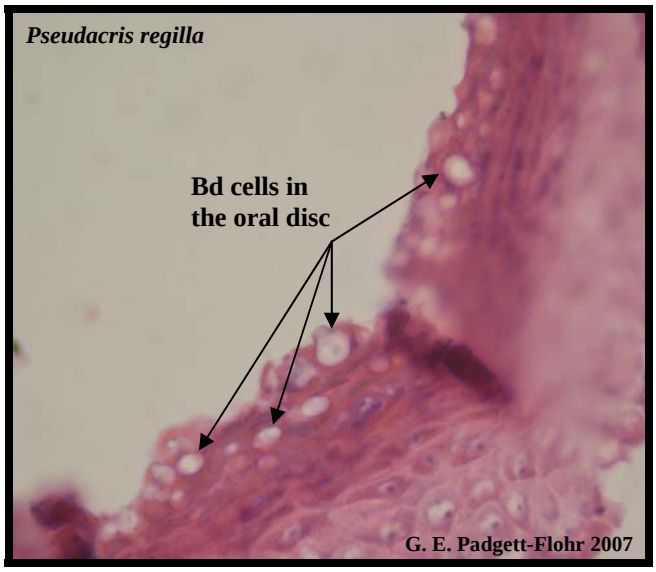


Fig.6: Flame-sterilize your instruments**.**

Batching samples: If you are going to batch anuran larval samples, you will want to cut the oral disc in half and place one half in a "batch" jar and the other half in an individual container. Bisect the oral disc in an antero-posterior direction such that each half contains both upper and lower jaw sheaths and toothrows. Be sure to cross reference your batches with the individual samples so that you can find them if you need to follow up with additional testing on the individuals.

COLLECTION AND PRESERVATION: HISTOLOGY



Serial sectioned and stained tissue of tadpole oral disc- 400x.

Histological serial sectioning is far less expensive than DNA procedures, but it also takes the longest and requires a laboratory that can prepare the slides, as well as a researcher qualified and experienced in diagnosing Bd in histological slides. If you know that histology will be done on your samples, you can use either 70% ethanol or formalin for storing your samples. Formalin is not as desirable a solution because it has a tendency to modify the shape of the Bd cells (they will appear football-shaped rather than round); which can make it tough for less experienced researchers to identify the Bd cells in the tissue.

The important difference between collection for DNA testing and histology is that you do not need to be concerned about cross-contamination of samples during the field collection process. (Of course you will still want to follow good decontamination procedures!) Histologic diagnosis actually views the Bd cells in *situ*; in other words, we are actually looking for Bd cells residing in the tissue that show actual infection. A minimum of 17 histologic sections is required for adequate diagnosis of a sample (Puschendorf and Bolaños 2006); although, based on our own experiences, we recommend viewing the entire sectioned oral disc.

With anuran larvae destined for histological examination, you still need to amputate the oral disc as described above and store in individual vials. However, because

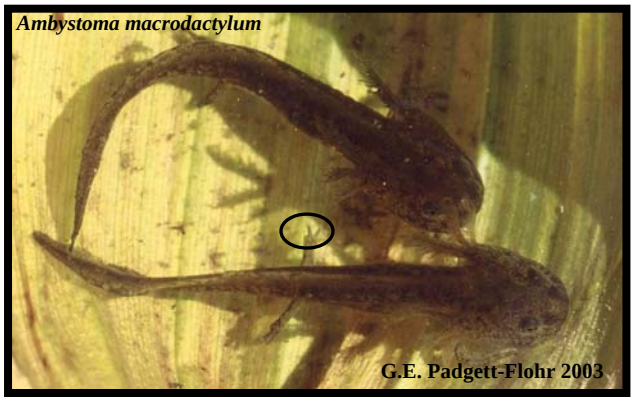
we are not concerned with cross-contamination of samples, you can omit the flame sterilization of the instruments between samples while processing them. Be sure to disinfect your instruments after you are done however.

Store the samples in individual vials that are leak-proof. They do not have to be the more expensive centrifuge tubes used to store samples that will be tested via DNA methods, but they DO NEED to be LEAK-PROOF. The **"cryovial" tubes that many folks have on hand are NOT leak-proof**. If you use cryovial tubes, you will find that within 3-4 weeks the preservative solution has evaporated out of the vial leaving your sample high and very dry. It is really tedious and time-consuming to have to continually monitor and refill all of your vials as the samples are being processed for histology. Histo embedding machines can process a maximum of 110 samples at a time and so there can be substantial time intervals between batches of samples. If your samples are being stored in cryogenic tubes, you will have to check your vials weekly and ensure that the ETOH or formalin has not evaporated out of them. If you only have cryovial tubes available, it helps to parafilm the top- you will still have to add (e.g.) ETOH periodically, but not nearly as often as you would if you do not parafilm the cap. Whatever you decide to do, if you use vials that are not leak-proof, be SURE to check your vials regularly to ensure that your samples are not turning into leather.

If you are going to be shipping your samples for histological diagnosis- please be sure that your vials are leak-proof. You do not need to make their job tougher by forcing the folks doing your samples to babysit your vials to ensure the preservative is not evaporating out! In addition, if you send vials that are not leak-proof using overnight shipping, the air pressure changes during air transport will cause a great deal of leakage in your packaging and the person on the receiving end will have a nice mess on their hands when they open the package.

Don't forget to label your vials. If you are really worried about the label coming off- you can use small pieces of parafilm to wrap around the label. But do NOT use one large piece of parafilm to wrap both the label and the cap; the label may very well come off when the parafilm is removed! If you are going to parafilm the cap and the label, use two small pieces of parafilm and wrap each separately.

CAUDATES



COLLECTION AND PRESERVATION: DNA TESTING (PCR or qPCR)

The majority of research has been focused on anuran larvae but caudate larvae can also be infected with Bd (Padgett-Flohr and Longcore 2003; Padgett-Flohr and Longcore 2007). Caudate larvae have keratin throughout their epidermis but the feet appear to the most common site of initial infection (Padgett-Flohr unpub. data). If you are able to collect the entire larva, you will remove and submit one of the hind feet to the lab. **Follow the same procedures as with anuran larvae**, excepting that you will use hind feet as the tissue sample for submittal to the lab, rather than the mouthparts. We recommend sending the entire foot as this structure is very small and it is just as easy to test the entire foot as opposed to a single toe. You will have a better chance of detecting Bd if you submit the whole foot.

If you cannot collect whole larvae and are constricted to toe clips, try to sample late in larval development near the time of metamorphosis, so that the toes are large and the amount of time the animals have spent in the aquatic environment is maximized. This will increase your ability to detect Bd. You can still use the "dirty team-clean team" scenario. This time you actually have three teams of people. One team consists of the people or person getting wet and capturing animals; they deposit the animal in a bucket. The second team picks up the animal and takes the toe clip and other pertinent data and then returns the animal to the bucket. The third team records the data and holds the vial out to accept the toe, capping the vial after the toe has been deposited and storing it safely away from the center of action**.** The first team then takes the marked animal and releases

it. Each team must change gloves after each animal. Submitting toes can be risky; if the infection is just beginning, only one toe may be infected and if you happen to pick one of the uninfected toes for clipping, you will get a negative result, when in fact, infection was present; just not in the toe you picked.

COLLECTION AND PRESERVATION: HISTOLOGY

You will follow the same procedures as those outlined above for Histology on Anuran Larvae with the following modification:

If you collected whole caudate larvae and plan to have histology done, (or you plan to do your own histology), it is best to submit only the outer epidermis of the dorsal aspect of the foot to your lab. Grab the "ankle" with one forceps to hold and anchor the leg and foot and to provide counter-traction (Fig. 7); and with the other forceps pinch the **only** the outer epidermal layer on the foot-side of the holding forceps (Fig. 8). Then tug downwards, while still anchoring the foot with your holding forceps. The skin will come off in a neat single sheet that will include all the toes as well as the palm. Place this tissue in a vial as described above for anuran oral discs. If you collected toe samples only, you can submit the entire toe for histology.

Fig. 7: Grasp the ankle.

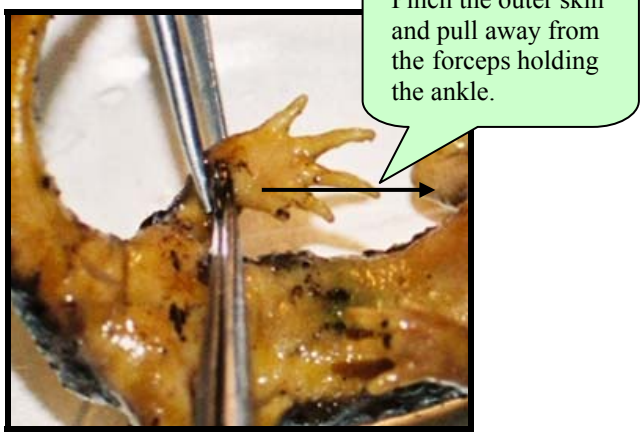


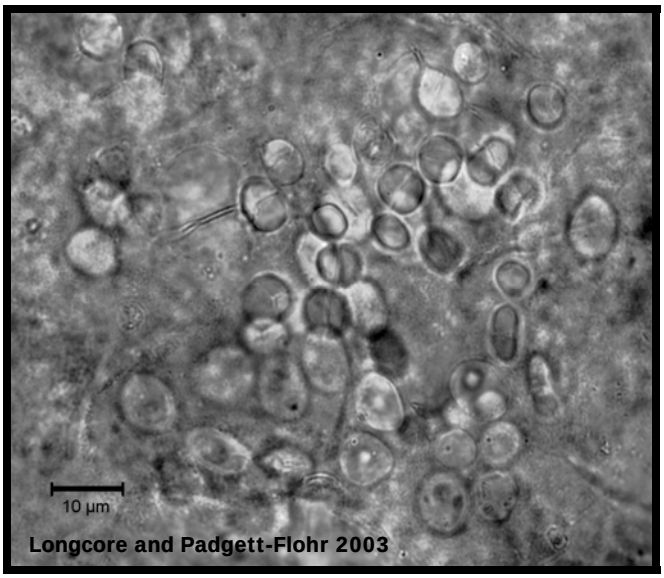
Fig. 8: Grasp the outer skin and pull.

One of the big benefits of histology is that your sample is preserved in "perpetuity". Should there ever be a question as to the accuracy of the diagnosis, you can always go back to the slide.

MICROSCOPIC EXAMINATION-Wet Mount Slides

Another option to diagnose caudate larvae is to make a wet-mount slide of the skin tissue removed from the dorsal aspect of the hind foot (Fig.7 and 8). This is a really fast and cheap method to diagnose Bd, but it requires that the researcher have a high level of skill in identifying Bd cells. **If you are not highly experienced and do not have someone available who is, we do not advise you to do this as a definitive test for Bd**.

The skin is placed on a clean slide and a drop of distilled water is added. The coverslip is then placed over the sample and viewed through a compound microscope.



Wet mount of Bd cells in the hind foot of *Taricha torosa* (California newt).

Once viewed, the tissue can be returned to the container. Wet mounts are not permanent records, but it is possible to preserve your slides, if have a solution that can glue the coverslip in place. Commercial solutions are available for purchase, or you can make your own. Please be aware that it takes a very long time for this "glue" to dry. You need to be able to place your slides on a flat surface and leave them to dry for at least a month before you attempt to catalogue your slides into a slide box. If you pick them up too soon, the slides will stick together and when the glue dries, you will not be able to pry them apart.

Another alternative to preserving the diagnosis record, is to take a photograph of the view through the scope. This requires a fairly expensive set-up and may not be

available to many investigators; however it is a permanent record and these photos can be accessioned into museums as proof of Bd infection.

POST-METAMORPHIC AMPHIBIANS

Post-metamorphic amphibians can be diagnosed via PCR, qPCR, histology or wet-mount slides of skin scrapes.

COLLECTION AND PRESERVATION: DNA TESTING (PCR or qPCR)

★***Obtain the Bd sample prior to doing other procedures on your animals.***★

There are three ways to collect and submit post-metamorphic amphibian samples to test for Bd infection via DNA methods:

1) Collect whole animals in the field and then excise tissue and toes for testing once you are back at the lab.

2) Collect toe clips only and release the animal in the field.

3) Swab the animal with a cotton swab or scrape the skin to collect cells for testing. This can be done in the field or the lab. Keep in mind, that if you collect swabs or scrapes ONLY and release the animal in the field, there is no chance to go back if there is a problem with the swab**.**

SWAB-SAMPLING or SKIN-SCRAPING POST-METAMORPHIC AMPHIBIANS FOR SUBMITTAL TO THE LAB

Use fresh gloves for each sample and be sure that when you place the swab or scrape-stick in the vial, you do NOT touch the outside of the vial. It is best if you follow

the "clean team-dirty team" method and have a second individual to assist who will handle ONLY the vials and record your data.

Normally your swab (or scrape) sticks are grouped together in (e.g.) a Baggie prior to actually sampling the animal; so to avoid contaminating the unused swabs, the second person (i.e. "clean team") should reach in to the Baggie to grab the swab. That person then hands the swab to the animal handler. This should be done carefully so that the two individuals do not contact each other. If the handler has Bd cells on their gloves, they will contaminate the gloves of the clean team and cross contamination of the vial and other swabs may occur. All team members should change gloves between each and every sample. When you remove your gloves be sure to use the glove removal technique described above (PUT IN LINK).

To obtain the sample, hold the animal (using fresh gloves) in one hand, and gently but firmly swab (if using cotton swabs) or scrape (if using skin scrapes) the ventral surface, the feet and toe webbing 25 times with the other hand. Place the swab (cotton-side down) or stick (pointed end down) in the labeled vial.

WHOLE SPECIMENS-
TISSUE REMOVAL FOR SUBMITTAL TO THE LAB

Once you have your whole specimens back in the lab, you will need to remove tissue samples for DNA testing. This process must incorporate a high degree of care to prevent cross-contamination of samples. We recommend submitting **both** the F1 toe AND a small resected section of the groin ("drink") patch for Anurans. You can place both samples in the same vial. The F1 finger is the front digit that is on the medial aspect of the forelimb (this would be your thumb) and the numbers follow sequentially so that the most distal digit (the one furthest away from the body) is F4. These are the two areas that seem to give the most reliable results in Anurans (Berger et al. 2005; Puschendorf and Bolaños 2006). Second choices in descending order consist of the F3 digit, the T2 digit (hind limb, second toe closest to the body) and lastly, the T3 digit. For Caudates submit the T1 digit and a small piece of the venter. Many folks, however, send in toe clips solo and do not include a portion of the ventral skin.

The easiest method to remove a toe without danger of cross-contamination is to leave the animal in its jar and use a forceps to grab the toe you are planning to amputate.

Only the instruments should contact the animal! Clip the toe with a pair of sharp, straight scissors and then drop the toe gently into the vial you have prepared. Fill the vial with ETOH **AFTER** you have placed the toe in it, as that will ensure that you do not splash solution while dropping the toe in the vial. The vial should be filled only to the half-way mark.

If you plan to include the ventral tissue piece, you will have to take the animal out of the storage jar. Use a new pair of gloves for each animal, but still try to touch the animal with instruments ONLY, not your hands. It is easiest to use a glass lens to hold the animal while you excise the ventral skin. Both instruments (forceps and scissors) need to be **flame-sterilized** after every single sample and the glass dish can be similarly flamed and then wiped of soot. You can use either a candle or a Bunsen burner to do this.

Don't forget to label your vials.

BATCHING SAMPLES

Please read the section on Batching Samples for DNA Testing first!

TISSUE SAMPLES: If you only collected toe clips and do not have whole animals to go back to for further sampling, but you still want to batch your samples with an option to follow up on positive results, bisect the toes in the antero-posterior plane into right and left halves (Fig 9 and 10).

Fig. 9: Bisect toe into right and left halves.

Fig. 10: Bisected toe.

This way each half contains tissue from the dorsal and ventral surfaces of the toe and will encompass the toe from the tip to the spot where the toe was amputated from the animal. You can then batch your samples by placing the one-half of the toes in the batch and placing the remaining half into individual vials. Cross reference your batch vials with

the individual vials in your data record so you can easily find them if you need to follow up on positive results from one of your batches.

SWAB SAMPLES: Swab/scrape samples can also be batched and the maximum number of swabs/scrape sticks per vial is 5. Keep in mind, that if you batch your swabs/scrapes you will only be able to follow up on positive batches if you took 2 swabs/scrapes at the time of sampling! If you plan to follow up positive results on batched samples with individual samples, you will need to collect two samples from each animal. One sample goes in the batch vial and the other sample goes into an individual vial. You will then need to cross reference your batched samples with the individual samples, so that you can identify which individuals made up each batch.

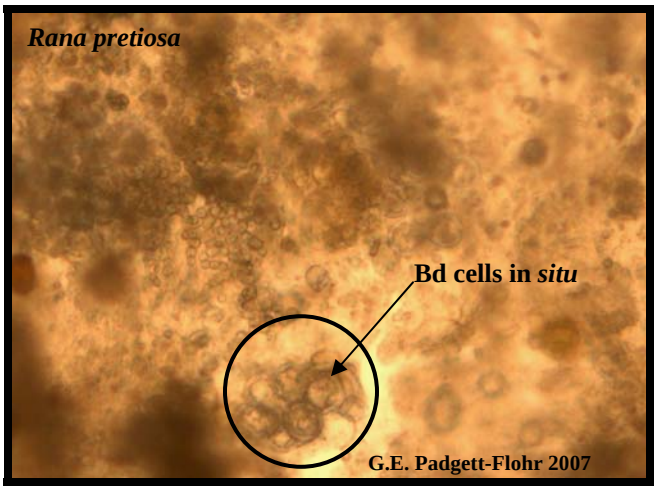
MICROSCOPIC EXAMINATION-Wet Mount Slides

The easiest, cheapest and fastest method available for testing post-metamorphic amphibians is the use of skin scrapes. This must consist of tissue scraped from either live or **very** recently deceased animals because the epidermal layer in dead, unpreserved animals tends to slough and decompose very quickly. If you have an animal that has been dead for longer than 1-2 days it will be better to have either histology or DNA testing done. The longer the animal has been deceased and unpreserved, the less likely a diagnosis can be made from a skin scrape.

To make a skin scrape wet mount slide, skin cells are removed from the animal's ventral groin patch or/and the hind feet (this applies to both Anurans and Caudates) using either a toothpick or a razorblade scraped **gently** across the skin. You do NOT need big hunks of tissues; rather you are scraping sloughing cells from the surface of the animal. This is a relatively innocuous technique that does not require collection or sacrifice of the amphibian. You can even do this in the field if you can lug the supplies and scope with you! BUT it requires that the researcher have a high level of skill in identifying Bd cells. **If you not highly experienced in this, we do not advise you to do this as a definitive test for Bd**.

A wet-mount of the cells is prepared by placing the skin cells on a clean slide and a drop of distilled water is added. The coverslip is then placed over the sample and immediately viewed through a compound microscope. It is possible to preserve your slides, if have a solution that can glue and preserve the coverslip in place. Commercial solutions are available for purchase, or you can make your own. Please be aware that it takes a very long time for this "glue" to dry. You need to be able to place your slides on a flat surface and leave them to dry for at least a month before you attempt to catalogue your slides into a slide box. If you pick them up too soon, the slides will stick together and when the glue dries, you will not be able to pry them apart.

Another alternative to preserving the slide is to take a photograph of the view through the scope. This requires a fairly expensive set-up and may not be available to many investigators; however it is a permanent record and these photos can be accessioned into museums as proof of Bd infection



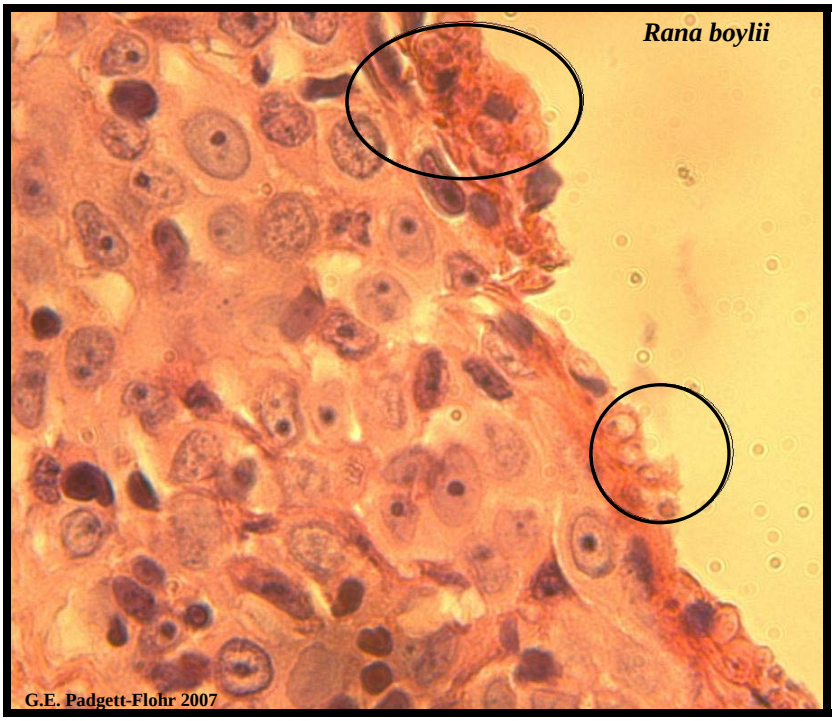
Wet-mount slide preparation magnified 400x.

HISTOLOGY

Histological serial sectioning is far less expensive than DNA procedures, but it also takes the longest and requires a laboratory that can prepare the slides, as well as a researcher qualified and experienced in diagnosing Bd in histological slides. If you know that histology will be done on your samples, you can use either 70% ethanol or formalin. Formalin is not as desirable because it has a tendency to modify the shape of the Bd cells

(they will appear football-shaped rather than round); which can make it tough for less experienced researchers to identify the Bd cells in the tissue- but it CAN be done!

Museum samples can only be diagnosed using histology as most specimens accessioned into museums have been preserved and stored at some point, in formalin. Formalin destroys DNA and thus PCR and qPCR will NOT work on museum or otherwise archived samples.



Bd cells in an archived museum specimen.

The important difference between collection for DNA testing and histology is that you do not need to be concerned about cross-contamination of samples. Histologic diagnosis actually views the Bd cells in *situ*; in other words, we are actually looking for Bd cells residing in the tissue that demonstrate actual infection. A minimum of 17 histologic sections is required for adequate diagnosis of a sample (Puschendorf and Bolaños 2006); although, based on our own experiences, we recommend viewing the entire sectioned tissue sample.

If you have collected and preserved whole animals, we recommend submitting **both** the F1 toe AND a small resected section of the groin ("drink") patch. You can place both samples in the same vial. The F1 toe is the front toe that is on the medial aspect of the forelimb (this would be your thumb). These are the two areas that seem to give the

most reliable results (Berger et al. 2005; Puschendorf and Bolaños 2006). Many folks in the field take toe clips rather than the whole animal; in that case the preferred toe is again the first toe on the front limb that is closest to the body. Second choices in descending order consist of the F3 toe, the T2 toe (hind limb, second toe closest to the body) or T3 toe.

Store the samples in individual vials that are leak-proof. They do not have to be the more expensive centrifuge tubes but they DO NEED to be LEAK-PROOF. The **cryogenic tubes that many folks have on hand are NOT leak-proof**. What will happen is that within 3-4 weeks you will find that the preservative solution has evaporated out of the vial leaving your sample high and very dry. It is really tedious and time-consuming to have to refill all of your vials as the samples are being processed for histology. Histo embedding machines can process a maximum of 110 samples at a time and so there can be substantial time intervals between batches of samples. If your samples are being stored in cryogenic tubes, you will have to check your vials weekly and ensure that the ETOH or formalin does not evaporate out of them. If you only have cryogenic tubes available, it helps to parafilm the top- you will still have to add (e.g.) ETOH periodically, but not nearly as often as you would if you do not parafilm the cap. Whatever you decide to do, if you use vials that are not leak-proof, be SURE to check your vials regularly to ensure that your samples are not turning into leather.

*******If you are sending your samples to someone else for histological diagnosis- please be sure that your vials are leak-proof. You do not need to make their job tougher by forcing them to babysit your vials to ensure the preservative is not evaporating out!**********

Don't forget to label your vials. We use a regular office-type small label making machine. Put the labels on before you fill the vials with ETOH or formalin. If preservative is spilled on the outside of the vial- it will melt the adhesive and the label won't stick. Likewise, if you use a marker (even markers guaranteed to be "permanent") the ETOH will remove the ink. If you are really worried about the label coming off- you can use small pieces of parafilm to wrap around the label. But do NOT use one large piece of parafilm to wrap both the label and the cap; the label may very well come off when the parafilm is removed! If you are going to parafilm the cap and the label, use two small pieces of parafilm and wrap each separately.

Annis SL, Dastoor FP, Ziel H, Daszak P, Longcore JE. 2004. A DNA-based assay identifies *Batrachochytrium dendrobatidis* in amphibians. Journal of Wildlife Diseases, 40(3):420-428.

Berger L, Hyatt AD, Olsen V, Hengstberger SG, Boyle D, Marantelli G, Humphreys K, Longcore JE. 2002. Production of polyclonal antibodies to *Batrachochytrium dendrobatidis* and their use in an immunoperoxidase test for chytridiomycosis in amphibians. Diseases of Aquatic Organisms, 48(3):213-220.

Berger L, Speare R, Skerratt L. 2005. Distribution of *Batrachochytrium dendrobatidis* and pathology in the skin of green tree frogs (*Litoria caerulea*) with severe chytridiomycosis. Diseases of Aquatic Organisms, 68:65-70.

Berger L, Speare R, Kent A. 1999. Diagnosis of chytridiomycosis of amphibians by histological examination. Zoos Print Journal,15:184-190.

Boyle DG, Hyatt AD, Daszak P, Berger L, Longcore JE, Porter D, Hengstberger SG, Olsen V. 2003. Cryo-archiving of *Batrachochytrium dendrobatidis* and other chytridiomycetes. Diseases of Aquatic Organisms, 56:59-64.

Boyle DG, Boyle DB, Olsen V, Morgan JAT, Hyatt AD. 2004. Rapid quantitative detection of chytridiomycosis (*Batrachochytrium dendrobatidis*) in amphibian samples using real-time Taqman PCR assay. Diseases of Aquatic Organisms, 60:133-139.

Briggs C, Burgin S. 2003. A rapid technique to detect chytrid infection in adult frogs. Herpetological Review, 34(2):124-126.

Hyatt AD, Boyle DG, Olsen V, Boyle DB, Berger L, Obendorf D, Dalton A, Kriger K, Hero M, Hines H, Phillott R, Campbell R, Marantelli G, Gleason F, Colling A. 2007. Diagnostic assays and sampling protocols for the detection of *Batrachochytrium dendrobatidis*. Diseases of Aquatic Organisms, 73:175-192.

Kriger KM, Ashton KJ, Hines HB, Hero JM. 2007. On the biological relevance of a single *Batrachochytrium dendrobatidis* zoospore: a reply to Smith (2007). Diseases of Aquatic Organisms, 73:257-260.

Kriger KM, Hines HB, Hyatt AD, Boyle DG, Hero JM. 2006a. Techniques for detecting chytridiomycosis in wild frogs: comparing histology with real-time Taqman PCR. Diseases of Aquatic Organisms, 71:141-148.

Kriger KM, Hero JM, Ashton KJ. 2006b. Cost efficiency in the detection of chytridiomycosis using PCR assay. Aquatic Diseases of Organisms, 71:149-154.

Laurance WF, McDonald KR, Speare R. 1996. Catastrophic declines of Australian rain forest frogs: support for the epidemic disease hypothesis. Conservation Biology, 10:406-4

Longcore JE, Pessier AP, Nichols DK. 1999. *Batrachochytrium dendrobatidis* gen. *et sp. nov.*, a chytrid pathogenic to amphibians. Mycologia, 91:219-227.13.

Padgett-Flohr G, Longcore J. 2005. *Ambystoma californiense* (California Tiger Salamander). Fungal Infection. Herpetological Review, 36(1):50-51

Padgett-Flohr G, Longcore J. 2007. *Taricha torosa* (California Newt). Fungal Infection. Herpetological Review, 38(2):176-177

Puschendorf R, Bolaños F. 2006. Detection of *Batrachochytrium dendrobatidis* in *Eleutherodactylus fitzingeri*: effects of skin sample location and histologic stain. Journal of Wildlife Diseases, 42(2):301-306.

Retallick RW, Miera V, Richards KL, Field KJ, Collins JP. 2006. A non-lethal technique for detecting the chytrid fungus *Batrachochytrium dendrobatidis* on tadpoles. Diseases of Aquatic Organisms, 72(1):77-85.

Smith KG. 2007. Use of quantitative PCR assay for amphibian chytrid detection: comment on Kriger et al. (2006a,b). Diseases of Aquatic Organisms, 73:253-255.