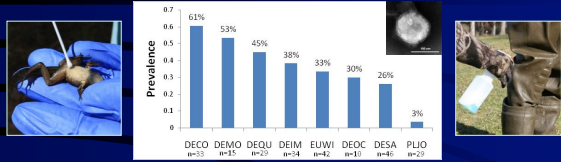**Designing Ranavirus Surveillance Studies, Sample Collection, & Biosecurity**

Matthew J. Gray

University of Tennessee
Center for Wildlife Health
Department of Forestry, Wildlife and Fisheries

Outline

Part One

I. Uses and Biases of Surveillance Data
- What are the limitations?

II. Sample Design and Required Sample Size

Part Two

III. Amphibian Surveillance Example
- Importance of "Aseptic" Sampling

Part Three

IV. Disinfecting and Shipping Procedures

Goal of Surveillance

To <u>detect</u> a pathogen/disease or obtain an unbiased estimate of pathogen/disease <u>prevalence</u> (or incidence) in a population

Pathogen Prevalence

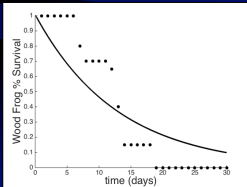An estimate of the proportion of individuals in a population that are infected with a pathogen

Infection ✕ Disease

Challenge Studies: Background Levels

Detection Biases

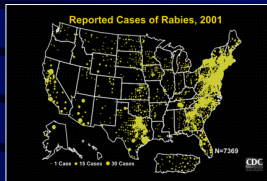Dead vs. Morbid vs. Health

Vegetation

Water Depth

Seasonality

Sample Frequency?

Uses of Surveillance Data

Occurrence and Distribution

GLOBAL RANAVIRUS Reporting System
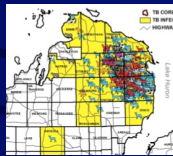
Reported Cases of Rabies, 2001

http://www.bd-maps.net/

Baseline

Evidence of Emergence
Pathogen or disease that is increasing in
distribution, prevalence, or host range

Uses of Surveillance Data

Elevated?

Evidence of Hotspots
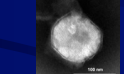
Identification of Mechanisms of Emergence

Population Effects?

$$

Disease Intervention Strategies

Interrupt Host-Pathogen Cycle

Reduce Stressors

1. Novel Pathogen
2. Stressors Present
3. Transmission: Density

OIE: Routes of Entry

Surveillance Designs

Collecting Unbiased, Representative Sample

Random Sampling

Objective?

All individuals or surveillance locations have an equal probability of being sampled

Random Numbers Table or Programs

1) Sample all captured individuals
2) Sample up to *n* captured individuals
3) Randomly select individuals from sample of *n* captured individuals

Avoid Systematic or Haphazard Sampling

Estimating Required Sample Size

Detect a Pathogen

Information Needed

epiR (epi.detectsize)

- Assumed Pathogen Prevalence Level (APPL)
- Estimated Host Population Size
- Confidence in detection (95%)

Population Size	10% APPL	5% APPL	2% APPL
50	20	35	50
100	23	45	75
250	25	50	110
500	26	55	130
2000	27	60	145
>100,000	30	60	150

(Amos 1985, Thoesen 1994)

Estimating Required Sample Size

Precise Estimate of Prevalence

Two Proportions

$Z_{\alpha/2} = 1.96$
(95% confidence)

$$n = p(1-p)\left[\frac{1.96}{d}\right]^2$$

$p =$ Prevalence from a previous study

$d =$ error in estimation

"**Error in Estimation**" is the amount of error you are willing to tolerate in your estimate of prevalence

Error = 5%	Error = 10%	Error = 10%
p = 85%	p = 85%	p = unknown

$$n = (0.85)(0.15)\left[\frac{(1.96)}{0.05}\right]^2 \approx 196$$

$$n = (0.85)(0.15)\left[\frac{(1.96)}{0.10}\right]^2 \approx 49$$

$$n = (0.25)\left[\frac{(1.96)}{0.10}\right]^2 \approx 96$$

What happens if estimation error increases?
What happens if prevalence is near 0.5?

$0.01 < P(1-p) < 0.25$

Wildlife Surveillance:
An Amphibian (Ranavirus) Example

Matthew J. Gray[1] and Debra L. Miller[1,2]

University of Tennessee
[1]Center for Wildlife Health
[2]CVM Department of Pathobiology

The **FUTURE** of Veterinary Medicine

Enclosure (Pipe) Sampling

Count Number of Dips

Dip until No Larvae Captured after 10 dips

Is probability of transmission affected?

Co-housing Animals
Ranavirus Example

10, 20, 40%
X
15, 30, 60 min

What about gloves?

**Mean =
0.8 – 0.9 contacts/min for 40 tadpoles/m²
(10 tadpoles per 5-gal [19 L] bucket)**

Uniform Contact = 9 Minutes

Results: Cohousing

Cohousing Survival Curves

- 10% 15min
- 10% 30min
- 10% 60min
- 20% 15min
- 20% 30min
- 20% 60min
- 40% 15min
- 40% 30min
- 40% 60min

Percent Survival / Day

Results: Glove Changing

Biosecurity Glove Experiment

- No Change, 5%
- No Change, 10%
- No Change, 20%
- No Change, 40%
- Change, 10%
- Change, 40%
- Control

Percent Survival / Day

5
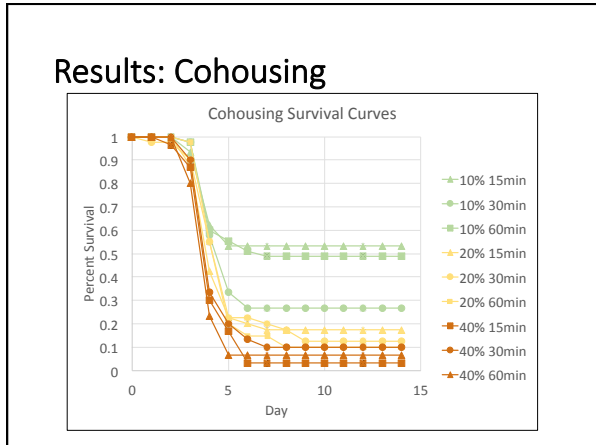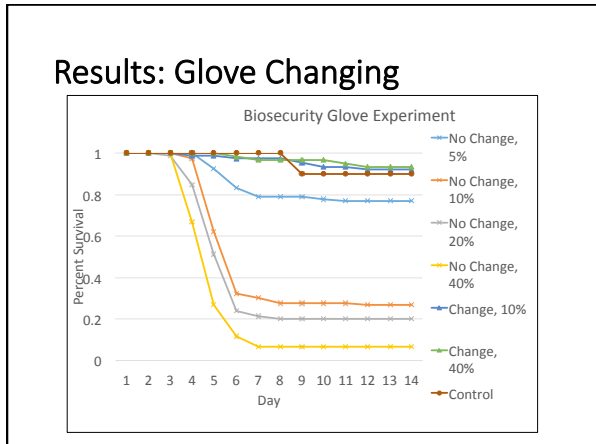
Holding Containers
One Individual per Container

Plastic Bags

1-L or 2-L
Plastic Tubs

Mason Jars

Holding Containers: Larvae
One Individual per Container

Pre-sampling Instructions
Capture Methods and Biosecurity

Area Searches

Search under Cover Objects

Record Time and Observers: **CPU**

Return to Approximate Capture Location

Area Searches
Isolate Individual and Mark Capture Location

Disinfect between Animals
Gloves and Net: 1 min Contact Time & Rinse

1% Nolvasan

Aseptic Processing Station

People that collect do NOT process!!

Aseptic Processing Station

Station 1

ID

Rinsed & Labeled

Aseptic Processing Station

Station 2

Weigh

SVL

Aseptic Processing Station
Station 3: Swab

Bd and *Bsal* Surveillance

Non-lethal Techniques: Brem et al. (2007)
Swabbing Preferred

Adults:
Swab 5 times in 5 locations
- Rear feet (webbing)
- Inner thighs
- Ventral Abdomen

Larvae:
Swab Oral Cavity 5 times

Dry or Store in 70% EtOH

A. Cressler, USGS

A. Cressler, USGS

Video: https://www.youtube.com/watch?v=a5CtPrGOK8c

Aseptic Processing Station
Stations 4: Tail or Toe Clip
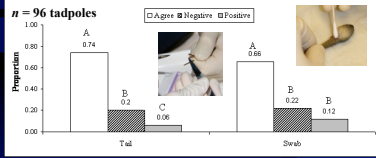
Ranavirus Surveillance

Lethal Collection:
Liver & Kidney Preferred
Miller et al. (2015)

Non-lethal Techniques: Gray et al. (2012): DAO 99:1-6

n = 96 tadpoles

Misclassification Decreases as Disease Progresses
Greer and Collins (2007)

Lethal followed by Tail

Toe Clips
False negative = 7%
False positive = 3%
St-Armour & Lesbarrères (2007)

Aseptic Processing Station
Stations 5: Data Recording
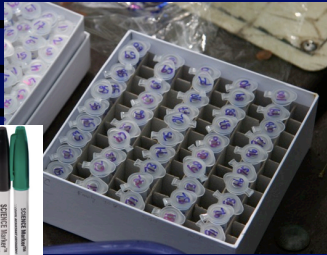
Aseptic Processing Station

Label: EtOH resistant Marker   **Sample Storage**   **Screw Top if Shipping**

70-90% EtOH or Dry Ice

Aseptic Processing Station

Station 6

Near Point of Capture

Releasing or Collecting Individuals

Collecting Additional Data

Water Quality

Vegetation

Apparent Stressors

Take the Opportunity to Instruct

Videos from the Field

Start videos at
3:30 min

http://www.amphibians.org/news/ranavirus-chytrid-video/

https://www.twra.tv/playlist-view/
5640df2f0f59f2f641837976/569baac7367008ef797d7c35

Questions??

| Gray: | mgray11@utk.edu |
| Miller: | dmille42@utk.edu |

Biosecurity Precautions:
Disinfecting Procedures

Matt Gray, Debra Miller, and Amanda Duffus
Diseases, Pathogens and Parasites Task Team

Wear Disposable Gloves

Latex

Vinyl

Gloves Rinsed with Distilled H_2O
Greer et al. (2009)

Nitrile

$12/box of 100

Guidelines for the Safe Use of Disposable Gloves with Amphibian Larvae in Light of Pathogens and Possible Toxic Effects

AMY L. GREER*
Child Health Evaluative Sciences
The Research Institute of the Hospital for Sick Children, 123 Edward Street
Suite 420, Toronto, ON M5G 1E2, Canada
e-mail: amy.greer@sickkids.ca

Disinfecting Equipment

Scrape Mud and Scrub

Back-Country

Disinfecting Equipment

Spray Bottle or Immersion

Johnson et al. (2003), Bryan et al. (2009), Gold et al. (2013)

$75/ bottle

- Bleach $\geq$4%
- EtOH $\geq$70%
- Virkon $\geq$1%
- Nolvasan >0.75%

Amphibian Biosecurity References

Dodd, C. K., editor. 2009. *Amphibian Ecology and Conservation: A handbook of techniques.* Oxford University Press, UK.

ISBN 9780199541188

Matthew J. Gray
V. Gregory Chinchar
Editors

Ranaviruses
Lethal Pathogens of Ectothermic Vertebrates

Miller et al. (2015)

Springer Open

A Manual For Control of Infectious Diseases in Amphibian Survival Assurance Colonies and Reintroduction Programs

Proceedings from a Workshop:
16–18 February 2009
San Diego Zoo

Pessier, A.P. and
J.R. Mendelson
(eds.). 2010.

Questions??

Gray: mgray11@utk.edu
Miller: dmille42@utk.edu
Duffus: aduffus@gordonstate.edu

Shipping Amphibians for Diagnostic Testing

Debra Miller and Matt Gray
Diseases, Pathogens and Parasites Task Team

Southeastern PARC
PARTNERS IN AMPHIBIAN AND REPTILE CONSERVATION

The FUTURE
of Veterinary Medicine

What do you do if you observe diseased amphibians or a die-off?

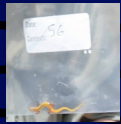**(1) STOP!!**

Are other animals dead?

M. Niemiller

PARC: State Contact List

(2) RECORD
- Number of animals dead
- Number collected animals
- Possible stressors
- Water quality & sample

A. Cressler

A. Cressler

Basic Collection Supplies

(1) Disposable Gloves
(2) Nets
(3) Distilled Water
(4) Sealable Plastic Bags
(5) Permanent Marker
(6) Cooler or EtOH
(7) Disinfectant

Collect Amphibian within 24 hrs of Death

Live Animals Ideal!

Sample Types & Diagnostics

Fresh is Best

Half Frozen, Half Preserved

Table 5
Various specimens used for *Ranavirus* testing, the type of test that can be performed, and the limitations of the test result

Specimen	Test	Limitations
Swab	PCR, virus isolation	False positives (environmental contamination); total DNA may be minimal; no histology
Tail or toe clip	PCR, virus isolation	False positives (environmental contamination); no histology
Whole body or internal organs	PCR, virus isolation, histology, IHC	Dead animals
Fixed tissue	PCR, histology, IHC	No virus isolation, electron microscopy is possible
Blood	PCR, virus isolation	Best obtained from live animals; can be difficult to obtain; often cannot obtain large enough quantity from small individuals
	ELISA if serum separated	
	Differential cell count if blood smear is prepared	

Freezing Prevents Histological Techniques

Miller et al. (2015): Springer

Transporting Amphibians

Most Diagnostic Labs Prefer Fresh Specimens if Possible

Tent Design

Dry Ice

Sample w/ Cushioning Paper Towel

Dry Ice

No Direct Contact with Dry Ice or Ice Packs

Preserving Animals

95% EtOH

10% neutral buffered formalin

50 mL

Separate Containers for Each Specimen! (no glass)

Falcon®

Shipping Animals
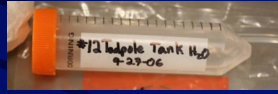
(1) Call the Diagnostic Lab for Specific Instructions

(2) Follow Courier Guidelines

FedEx

UPS

Shipping Specimens

Triple Packaging

First Layer

Label Each Layer!

Shipping Specimens

Do not use Biohazard Bags (unless known to be infected with a BSL-2 agent)

Triple Packaging

Second Layer

Absorbent Paper Towel

Shipping Specimens

Triple Packaging

Third Layer
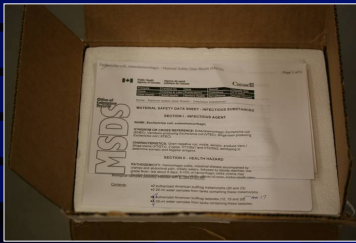

Place Cooler in Cardboard Box

Only Use Dry Ice for Frozen Samples

ThermoSafe* Polar Pack

List of Contents & MSDS if Needed

MSDS Required
•EtOH or Formalin

•Detailed list of all contents
•Description and location of die-off
•Contact information of the shipper

•Requested services
•General Pathological Screening
•Specific Pathogen Testing

Labeling

University of Tennessee *(Matthew J. Gray, Ph.D.)*
Department of Forestry, Wildlife and Fisheries
Institute of Agriculture
274 Ellington Plant Sciences Building
Knoxville, TN 37996-4563 USA

No statement required for environmental samples

Debra L. Miller, D.V.M., Ph.D.
Veterinary Diagnostic Laboratory
The University of Georgia
43 Brighton Road
Tifton, GA   31793-1389

Contents: Exempt Animal Specimen (Refrigerate upon Arrival)
Phone: 229-386-3340
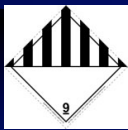
(5 lbs)

Dangerous Goods in Excepted Quantity
(<500 mL, <30 mL Container)

Dangerous Goods Hazardous Quantity
(>1 L or 33.8 oz)

Excellent Example

Questions??

Photo:
A. Balseiro

mgray11@utk.edu
865-974-2740