U.S. Fish & Wildlife Service

Fish Technology Center

eDNA: a tool for inventory and monitoring of aquatic invasive species

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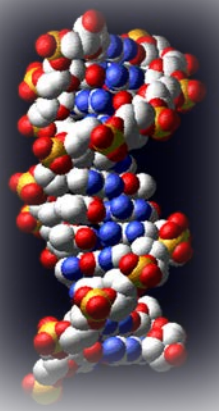

Acknowledgements

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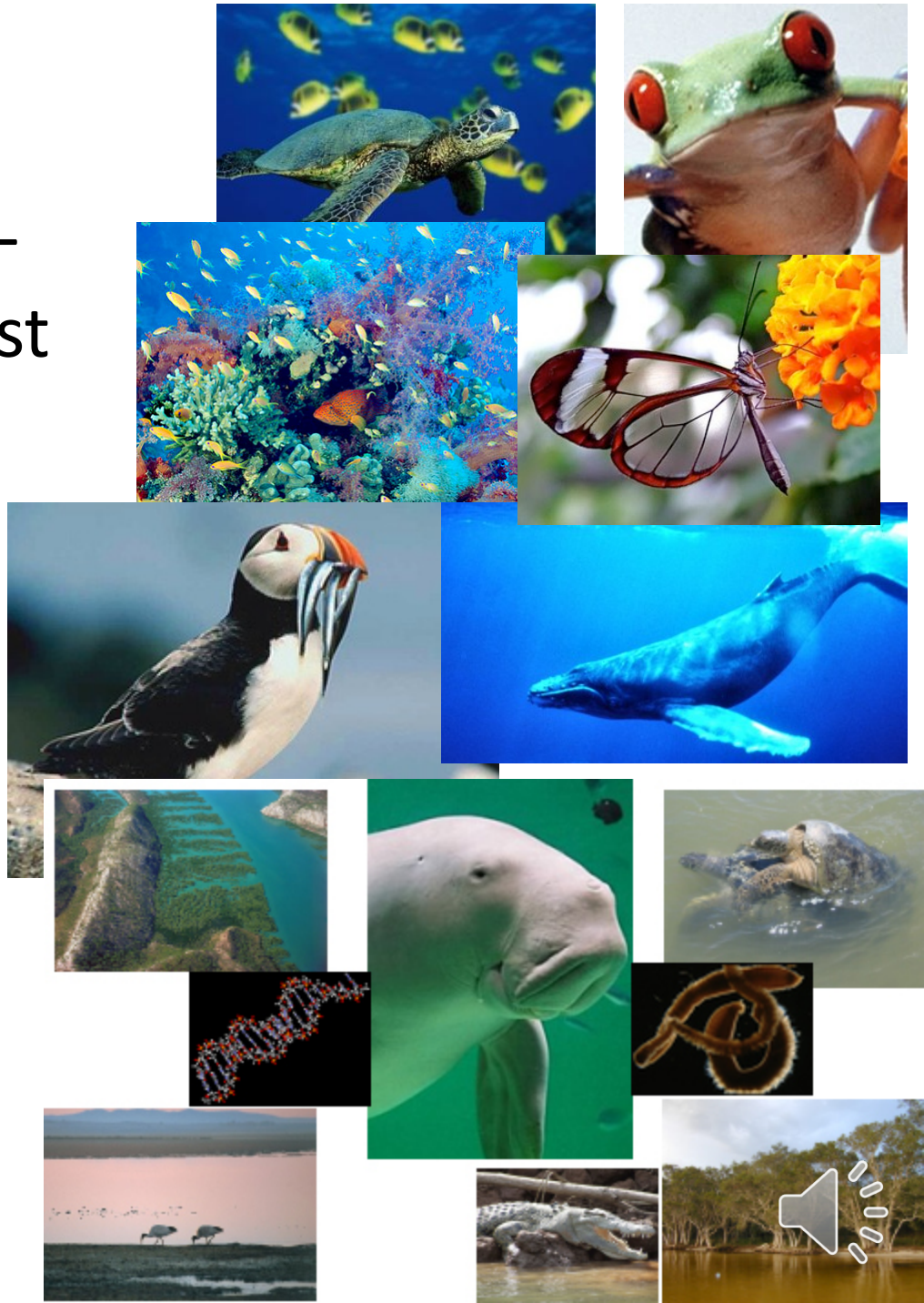
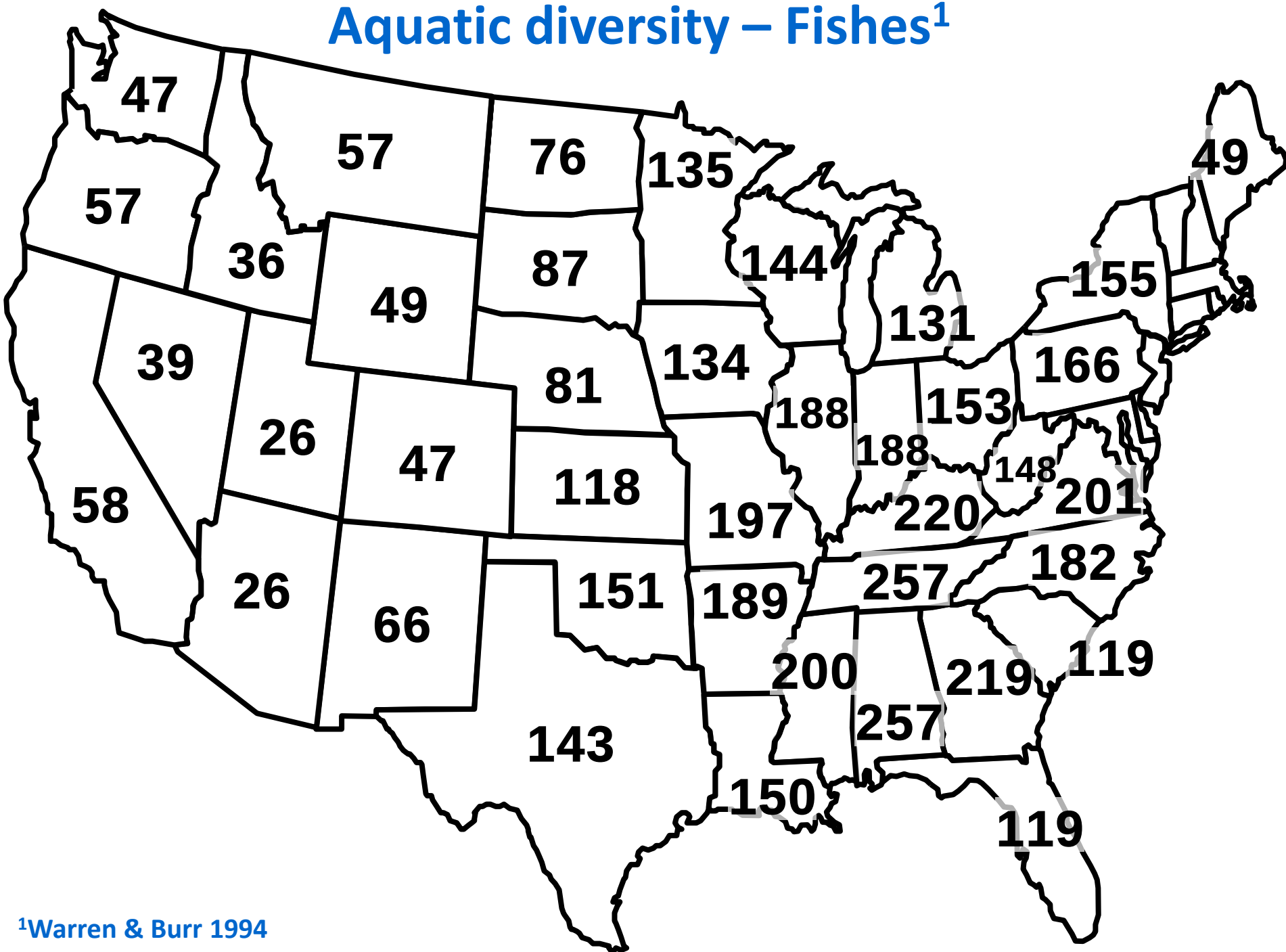
- B. Hickson and A. Williams - lab support
- Permits
  - Federal Fish and Wildlife permit number MA57272A-0,
  - NWR System Research and Monitoring Special Use Permit number S7-WBH-12-19
  - Florida Fish and Wildlife Conservation Commission Conditional/Prohibited/Nonnative Species Permit number EXOT-12-67

Problem

- Biologists estimate 10–200 million species exist on Earth
- Humans are rapidly pushing many species toward extinction
- There is a need to **<u>protect biodiversity</u>**



Aquatic diversity – Fishes[1]



[1]Warren & Burr 1994

Percent of imperilment – fishes[1]

Bluemask darter (*Etheostoma akatulo***)**

Smoky madtom (*Noturus baileyi***)**

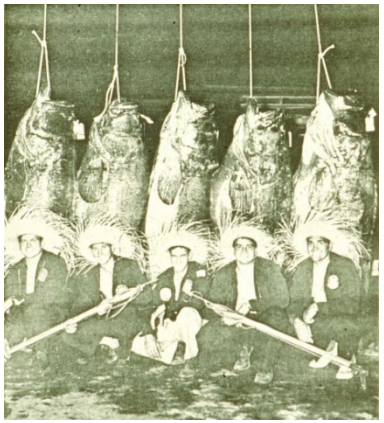
Pallid sturgeon (*Scaphirhynchus albus***)**

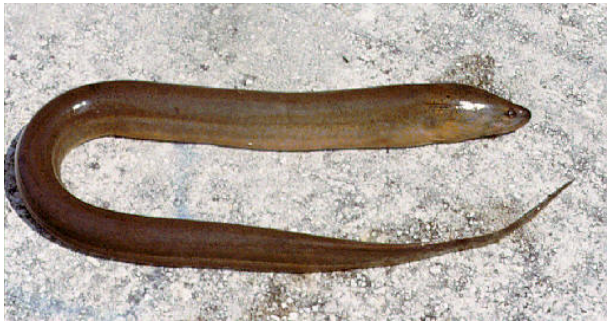
[1]Warren et al. 1997

Demography and extinction

- Deterministic factors
  - Habitat loss
    - SE has highest # impoundments km^{-2}
  - Over-exploitation
  - Invasive species

Tellico Dam

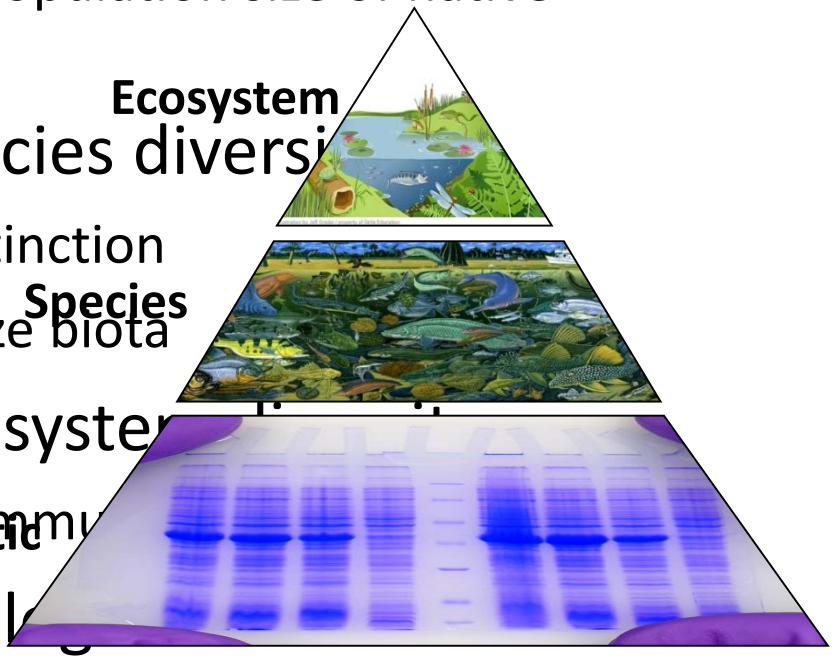




Asian swap eel (*Monopterus albus*)

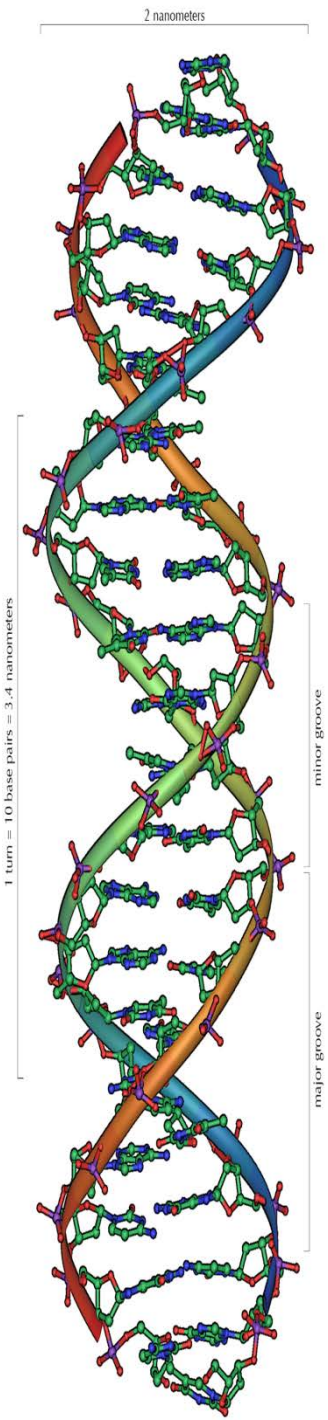
The problem of invasive species

- Reduce genetic diversity
  - Decrease population size of native taxa
- Reduce species diversi
  - Species extinction
  - Homogenize biota
- Reduce ecosyste
  - Change commu
- Reduce ecol
  economic value of ecosystems



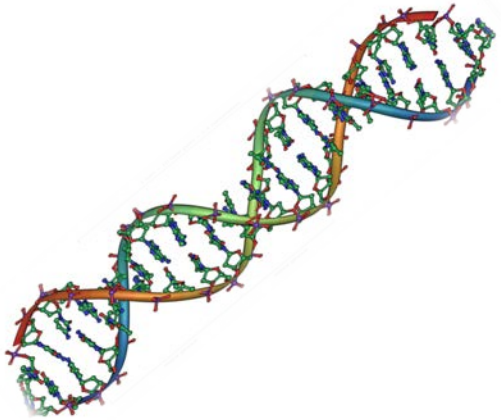
-Todays talk-

- What is environmental (e) DNA
- How can it be used for inventory and monitoring
  - early detection and rapid response of AIS
- Methodological details
- Associated costs
- Problems and concerns
- Ongoing research



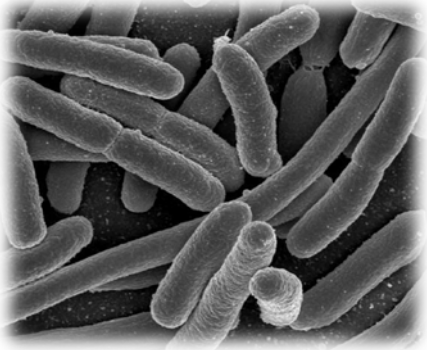
Environmental DNA

- Definition
  - Detection of target taxon's genetic material (cellular or extracellular) from its environment without seeing/capturing the taxon.



Environmental DNA

- History of eDNA
  - **1987** - Bacterial detection from sediments
  - **2005** – Eukaryote detection of fecal contamination in rivers
  - **2008** – AIS monitoring
  - **2010** – AIS monitoring in US rivers

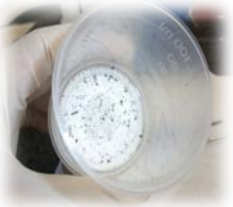


eDNA applications

- Early detection, surveillance and determine routes of invasion of AIS into natural systems
- Identification and monitoring of endangered species and declined populations
- Assess ecosystem health
- Biodiversity assessment and inventory.
- Environmental impact and risk assessment.
- Trophic interactions
- Change in species distribution- climate change & niche stability

Early detection of AIS

- How does eDNA work?



Early detection of AIS

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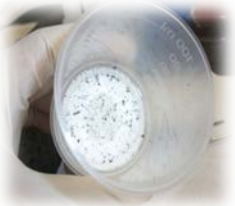
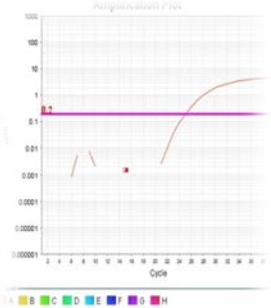
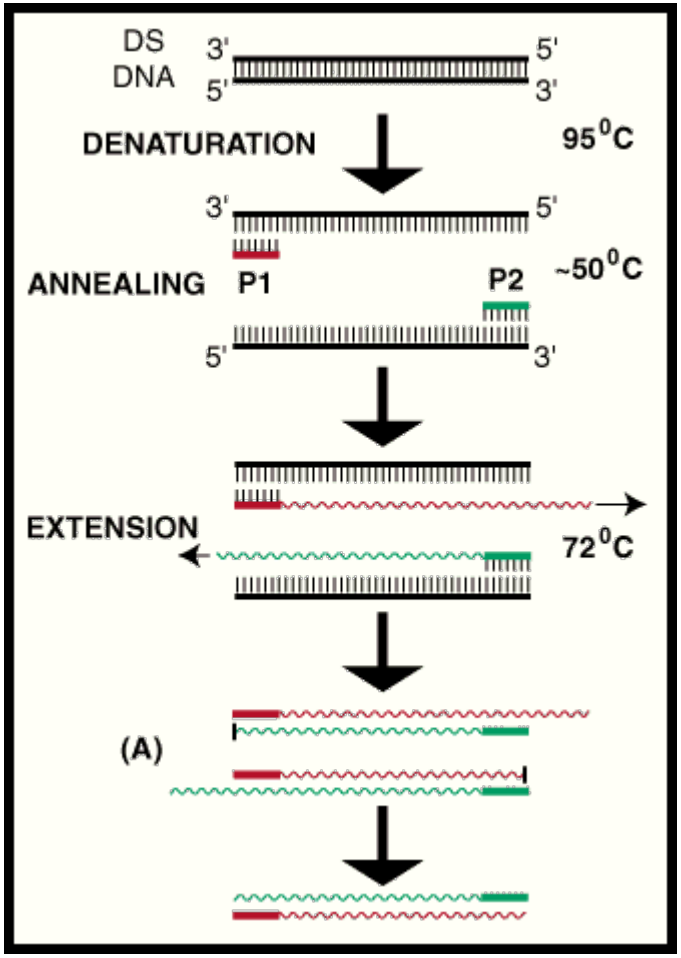




Methodological details

- PCR Primer design
  - Short 20bp sections of DNA
  - Only attach to target sequence!
  - Rely on available sequence data or generate new data
  - Takes time to develop and test for cross species amplification



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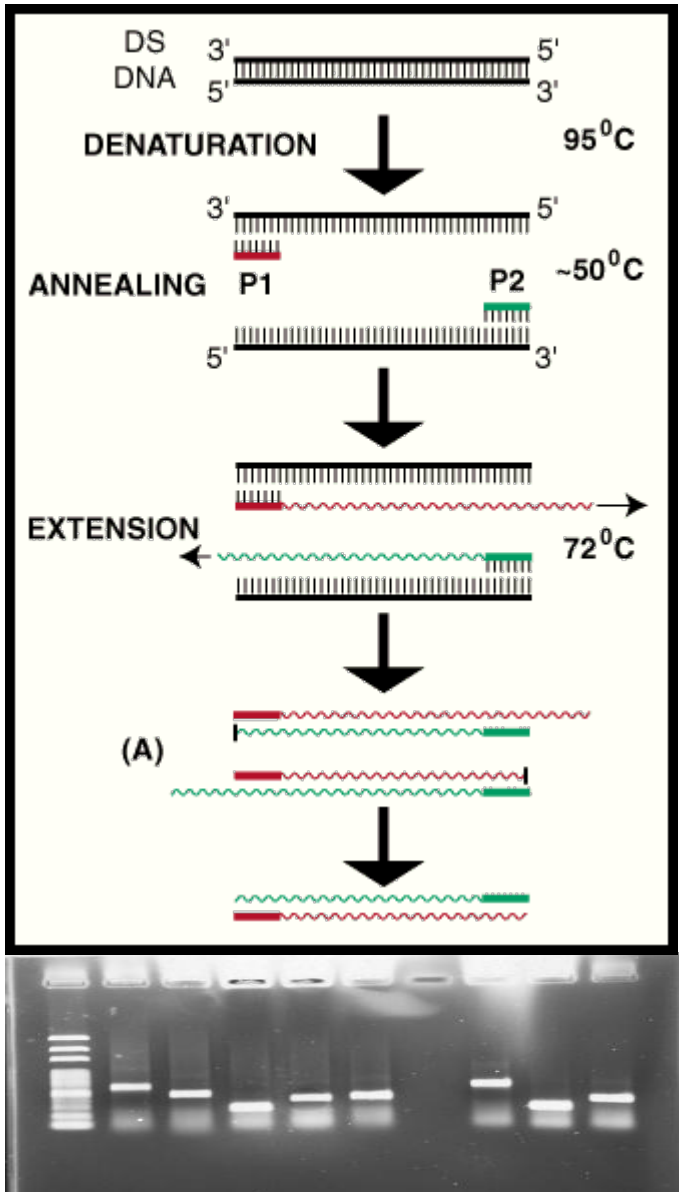


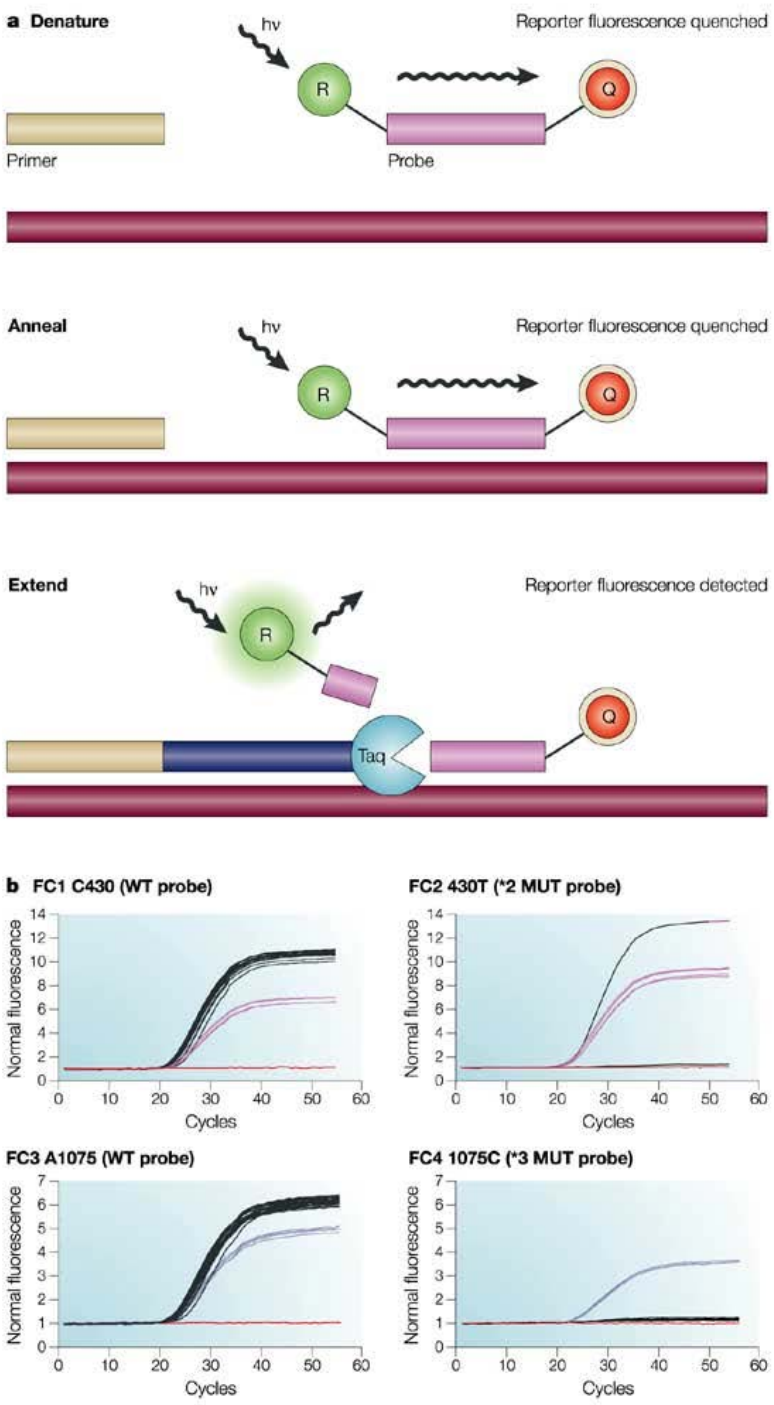
Methodological details

- Problems with detection via agarose?

		Replicate (PCR)			Replicate (qPCR)			Replicate (seq. conf.)		
Day	No. fish/treatment	I	II	III	I	II	III	I	II	III
3	0	-	-	-	-	-	-	-	-	-
	1	-	+	-	-	-	-	-	-	-
	3	+	+	-	+	+	+	+	+	+
	6	+	+	-	+	+	+	+	+	+
5	0	-	-	-	-	-	-	-	-	-
	1	-	+	-	-	-	-	-	-	-
	3	+	+	-	+	+	+	+	+	+
	6	+	+	+	+	+	+	+	+	+
7	0	-	-	-	-	-	-	-	-	-
	1	-	-	-	-	-	-	-	-	-
	3	+	+	-	+	+	+	+	+	+
	6	+	+	+	+	+	+	+	+	+

Methodological details

- qPCR primer design
  - Short 20bp sections of DNA
  - Only attach to target sequence!
  - Also has a specific probe
  - Detection of specific amplicon as it accumulates during PCR cycles
  - Takes time to develop and test for cross species amplification



Application of eDNA for AIS monitoring

- Strategy
  - Develop suite of primer/probes for regional AIS needs (10-20)
    - can run 3-4 primer/probes in one cocktail
  - Collect a water sample
    - perhaps train volunteers to cut costs
  - Test DNA of sample for eDNA of target taxa

Cost- Depends on methods used and various factory discounts/overhead etc . . .

Category	catalog #	Item	Vendor price	Price/sample
Taqman assays				
	N8010560	MicroAmp 96 well reaction plates	$62.75	$0.07
	4306311	MicroAmp clear adhesive film	$115.00	$0.01
	450056	Primers (qPCR)	$105.00	$0.18
	450025	Taqman Tamra probe	$255.00	$0.40
	N8080228	TaqMan PCR Core Reagents	$498.00	$2.49
	GPL200F	tips	$91.85	$1.20
			Total	**$4.35**
DNA extraction				
	14810-50-NF	Rapid Water DNA Isolation Kit-NF	$704.00	$7.04
	14800-100-WF	Water Filters (100 * 45micron)	$688.00	$6.88
	GPL200F	tips	$91.85	$0.91
	16120-962	Nalgene bottles	$147.00	$6.13
			Total	**$20.96**
PCR				
	PAM8298	Flexi taq DNA polymerase	$1,136.80	$0.23
	N8080007	GeneAmp dNTPs	$57.00	$0.43
	N8010560	MicroAmp 96 well reaction plates	$62.75	$0.07
	4306311	MicroAmp clear adhesive film	$115.00	$0.01
	450056	Primers	$105.00	$0.11
	GPL200F	tips	$91.85	$0.72
			Total	**$1.56**
qPCR calibration				
	4362201	7500 Fast Real-Time PCR Systems Spectral Calibration Kit II	$396.00	
	4360788	7500 Fast Real-Time PCR Systems Spectral Calibration Kit I	$876.00	
	4351979	TaqMan® RNase P Fast 96-Well Instrument Verification Plate	$566.00	
			Total	$1,838.00

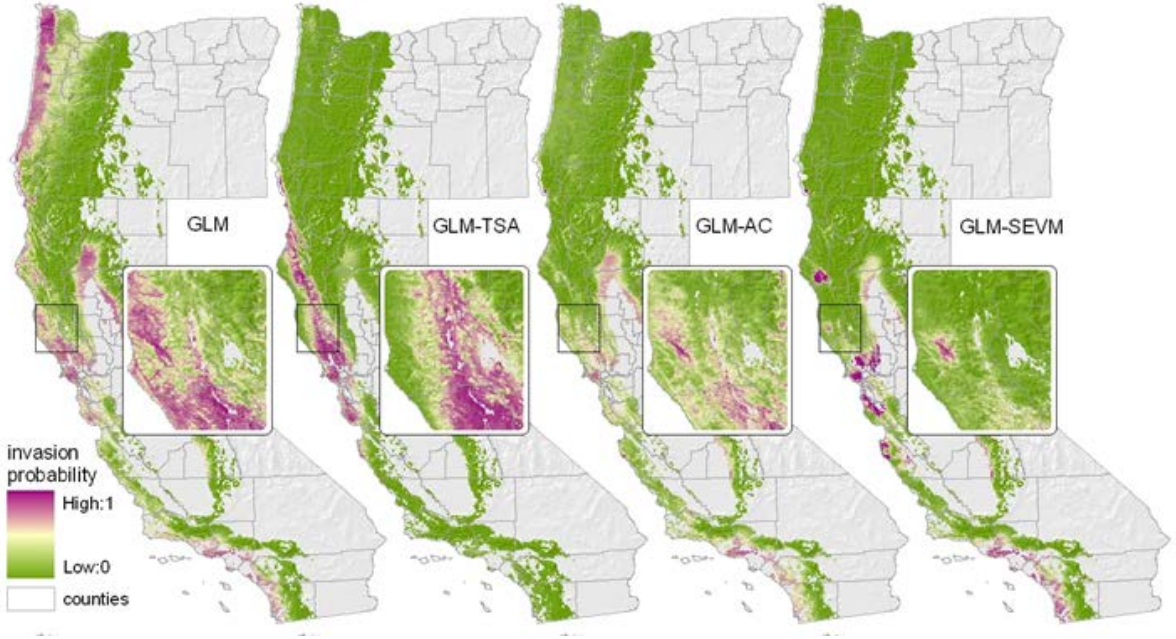
Problems and concerns

- Lab
  - Primer and probe design
  - Trace amounts of DNA
    - Corps QAPP is a good first step
  - Confirmation of positive qPCR identifications via sequencing
- Field
  - Lentic vs. lotic systems
  - How much water?
  - Where to take sample?

Problems and concerns

- Potential court issues
  - Daubert challenge
    - Are the findings based on sound science?
      - Published methods
      - Published databases
      - Subject to peer-review
  - Evidence handling and SOPs
  - USFWS Forensics Lab (http://www.lab.fws.gov/)

Moving forward



- AIS goal?
  - Early detection and rapid response
    - Predictive modeling using GIS
      - Incorporation of urban growth and climate change forecasts
    - eDNA sampling
      - Still some major hurdles
    - Establishment of eDNA community of practice
    - Legal issues

Ongoing research

- National Wildlife Refuge System inventory and monitoring
  - Loxahatchee
    - Bullseye snakehead
    - African jewelfish
  - Savannah
    - Lionfish
    - Mayan cichlid
    - Asian swamp eel

- eDNA detection in a controlled lentic system

Development of eDNA tools for Loxahatchee NWR

Loxahatchee Objectives

- Develop primer and probes for bullseyes snakehead and African jewel fish
- Establish water preservation method
- Estimate theoretical detection threshold level for qPCR using known controls of DNA
- Estimate detection probabilities for each eDNA method using density trials
- Assess influence of abiotic factors and time on eDNA persistence

Loxahatchee Methods

- Phase 1
  - Gene selection and primer design from tissue samples of *C. marulius & H. letourneuxi*
    - PCR / sequencing target gene (mtDNA *COI*).
    - qPCR primer and probe design
  - Optimization of qPCR reactions from tissue samples.
  - Optimization of DNA extraction and preservation from water samples.

Loxahatchee Methods

- Phase 2
  - Aquaria trials: proof-of-concept
  - Evaluation of eDNA persistence and abiotic factors and
- Phase 3
  - Field trials

Loxahatchee Phase 1

- Water preservation experiment
  - 1L samples collected from pond (n = 12)
  - Samples ‘spiked’ with lyophilized tissue of target taxon
  - 6 samples treated with
    - 3M sodium acetate (1mL)
    - 95% non denatured ethanol (33mL)
  - 6 remaining samples went untreated
  - Samples held at 4 or 25C for 9 and 18 days
  - PCR testing for presence of target DNA

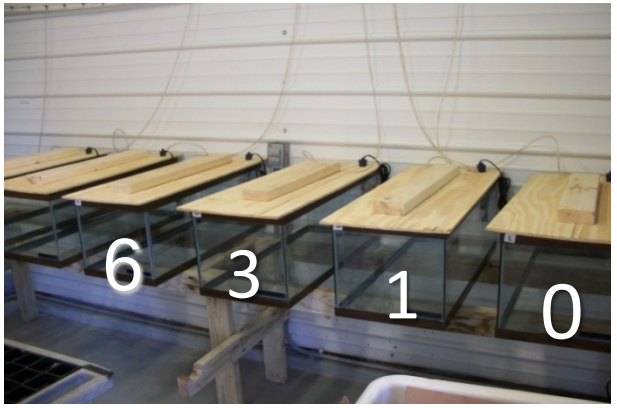
Loxahatchee Phase 1

- Results

Treatment	Time (Days)	Temp (˚C)	PCR conf.
NaOAc + EtOH - distilled water control	9	25	+
NaOAc + EtOH - pond water	9	4	+
NaOAc + EtOH - pond water	18	25	+
NaOAc + EtOH - pond water	18	4	+
No preservative - distilled water control	9	25	+
No preservative - pond water	9	4	-
No preservative - pond water	18	25	-
No preservative - pond water	18	4	-

Loxahatchee Phase 2

- Aquaria trials - triplicate
  - Densities = 0, 1, 3, 6 fish
    - 5.5, 15.8, 30.71g – jewelfish
    - 44.5, 97.9, 197.9g - snakehead
  - Water sample (1L) taken on days 3, 5, 7
  - detection via PCR and qPCR, sequence confirmation
  - Test factors influencing eDNA detection and persistence (GLR or ANOVA)



Loxahatchee Phase 2

- Results – African jewelfish
  - Both methods failed to detect eDNA at lowest density
  - No sig. diff ($P = 0.38$) b/w time and detection
  - Sig. and positive diff. ($P > 0.01$) b/w density and detection
  - Detection probabilities
    - PCR = 0.32, 0.54, and 0.82 (1, 3, 6 density treatments)
    - qPCR = 0.00, 1.00, 1.00

Loxahatchee Phase 2

- Results – Bullseye snakehead
  - Both methods detected eDNA at lowest density
  - No sig. diff ($P < 0.18$) b/w time and detection
  - Sig. and positive diff. ($P > 0.03$) b/w density and detection
  - Detection probabilities
    - PCR = 0.42, 0.53, and 0.63 (1, 3, 6 density treatments)
    - qPCR = 0.46, 0.59, and 0.76**

eDNA persistence:
the influence of abiotic factors

- Completion of tank trials (African jewelfish)
  - Removed all fish
    - removed 1L of water
      - 7, 14, 24, 31 days (for densities 3 and 6)
    - Measured [DNA] and ODs
  - Removal of 500ml from one tank (density = 3; n=12)
    - Four temps (8°C, 15°C, 25°C and 33°C)
    - On days 0, 4 and 8 measured water quality parameters
      - pH, ammonia nitrogen, nitrate, nitrogen, alkalinity, carbon dioxide, chlorine, hardness, and dissolved oxygen
      - Measured [DNA] and ODs

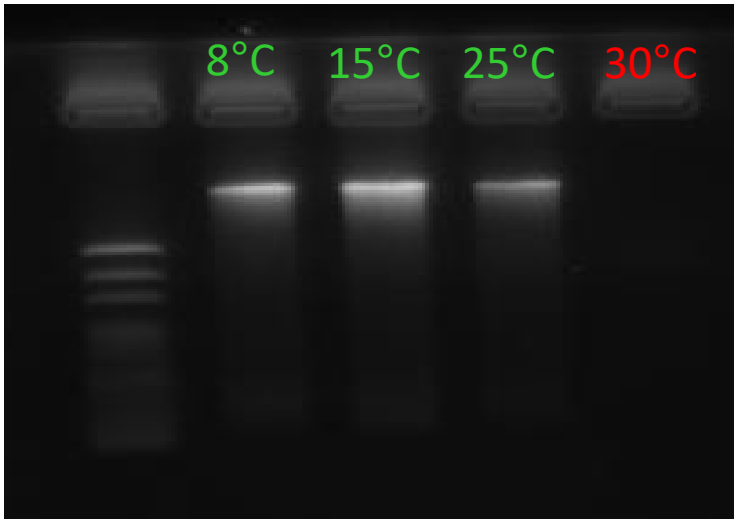
eDNA persistence:
the influence of abiotic factors

- Results
  - Post removal of fish
    - eDNA persisted for up to 24 days post removal
      - Temp ~ 23C
    - 24-31 day saw [DNA] and OD readings decrease
    - Negative and significant relationship b/w
      - [DNA] and time ($P = 0.002$)
      - OD and time ($P = 0.006$)
  - Abiotic factors
    - Temperature was only significant ($P = 0.03$) factor influencing DNA persistence

eDNA persistence:

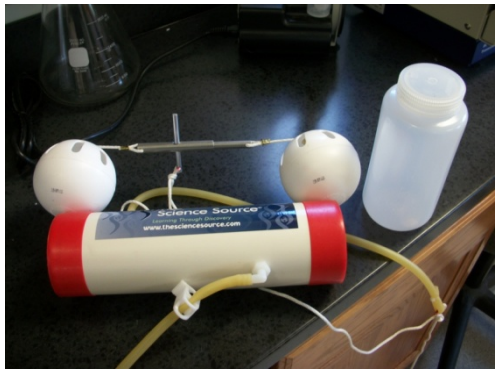
the influence of abiotic factors

Factor	F Value	Pr(F)
time	97.8153	0.064151
temp	452.4894	0.029906
ph	77.9409	0.071804
ammonia	19.464	0.141902
alkalinity	52.1592	0.087592
CO2	3.418	0.315652
Chlorine	2.69	0.348568
Hardness	43.8338	0.095434
O2	52.5775	0.087247
OD	19.0961	0.143217
Residuals	4.881	4.881



Loxahatchee Phase 3

- Field trials: African jewelfish
  - 3 x 3 grid across water column
  - Sampled
    - Loxahatchee NWR (n = 9)
    - Adjacent Hillsboro canal (n = 9)



  - Filter, DNA extraction, qPCR, sequencing
  - Results
    - eDNA detected from one sample (Surface_Hillsboro)
    - Sequencing confirmed it was African jewelfish DNA
      - 99% similar to sequences found on Genbank

Loxahatchee discussion

- Question raised
  - Able to take one water sample and detect eDNA from African jewelfish at a density of 3 fish/22L water
    - A 18m x 7.5m x 1.4m pond = 25,773 fish!
  - How many water samples will it take to detect at lower densities?

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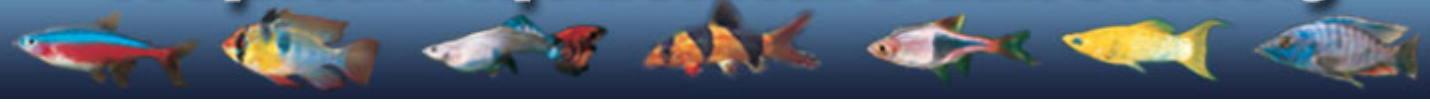
University of Florida
School of Forest Resources & Conservation
Program in Fisheries and Aquatic Sciences

Tropical Aquaculture Laboratory



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eDNA detection probabilities

lentic systems - ongoing research

Objectives

- Estimate the probability of detecting *H. letourneuxi* in a field setting

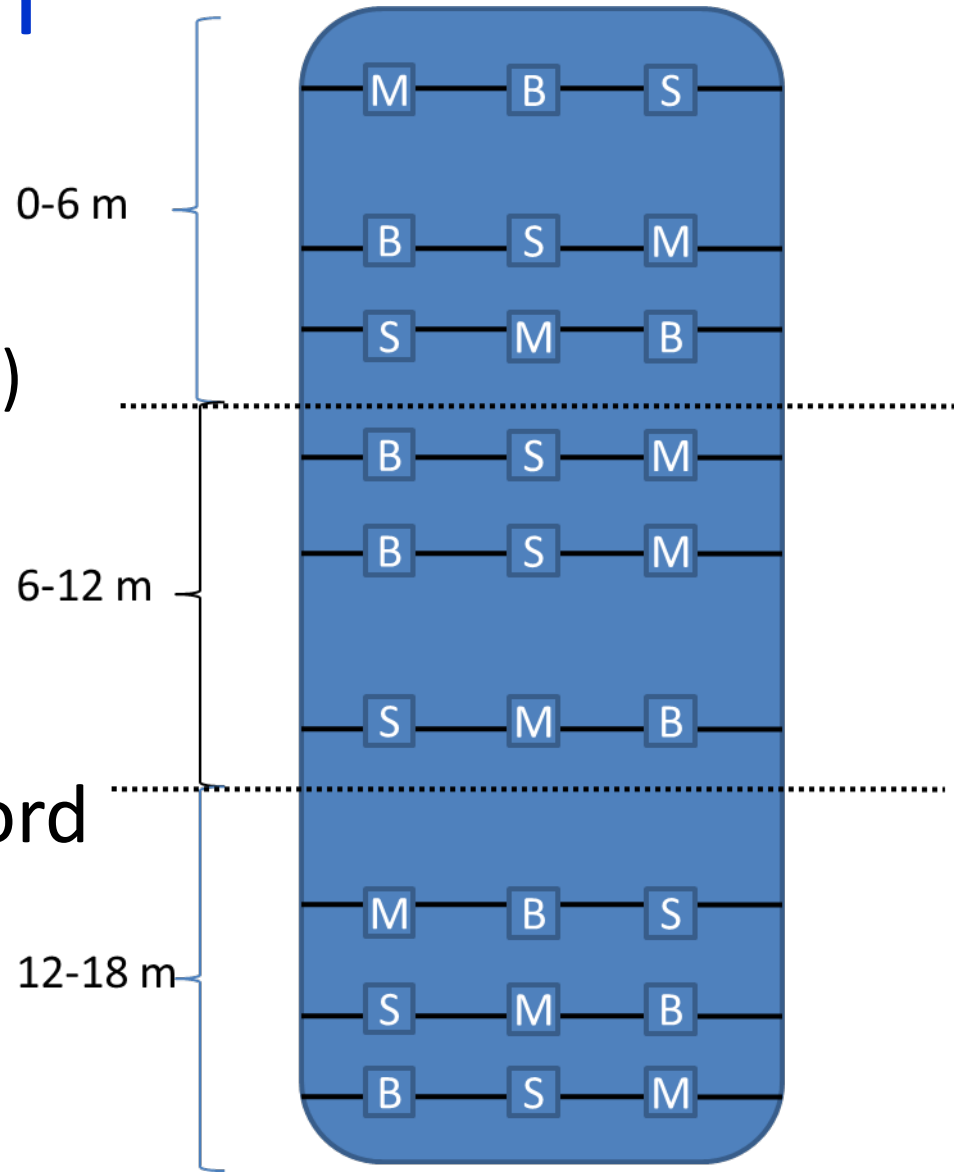
- Estimate the influence of pond- (fish density, pond area) and sample-specific temperature) factors on the detection of eDNA.

- Evaluate whether the probability of detection varies substantially through time at a given location.

- Use modeling results to determine the most effective and efficient sampling strategy for predicting the occurrence of *H. letourneuxi* in lentic systems.

Experimental design

- Four ponds
  - 18m x 7.5m x 1.4m
  - negative control (0 fish)
  - density I (25 fish)
  - density II (330 fish)
  - density III (990 fish)
- HOBO loggers will record temp at each depth
- Conduct sampling on days 1, 5, and 10



Anticipated results

- Determination of sample effort (# water samples) required to be reasonably certain (e.g., 90%) that the species is detected given different densities.