Gulf and South Atlantic Regional Panel on Invasive Species
Orange Beach, Alabama April 5th-6th 2016

DNR

Developing a qPCR tool to detect the invasive nematode parasite (*Anguillicoloides crassus*).

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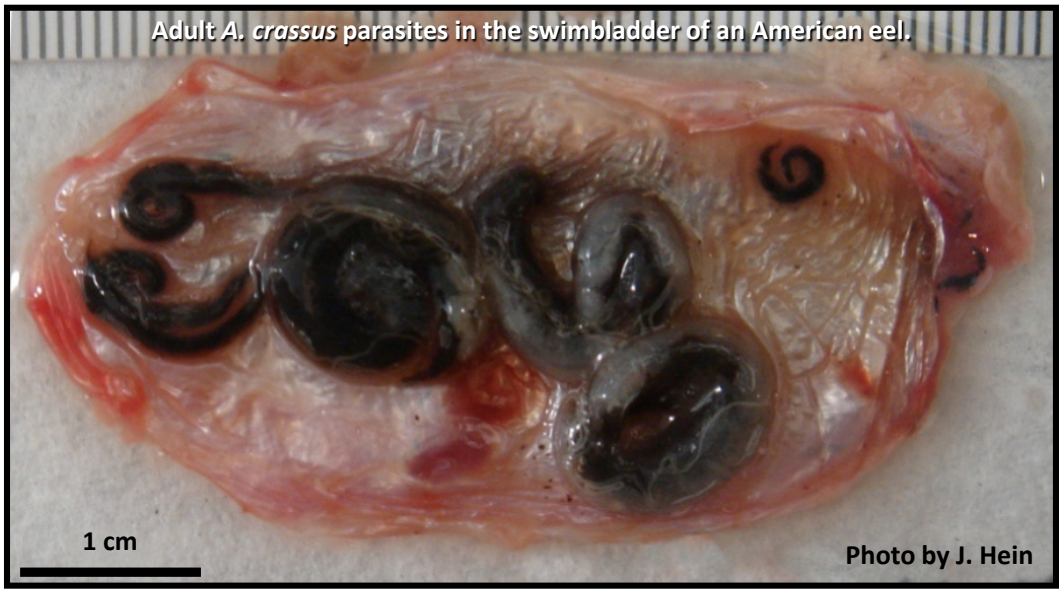
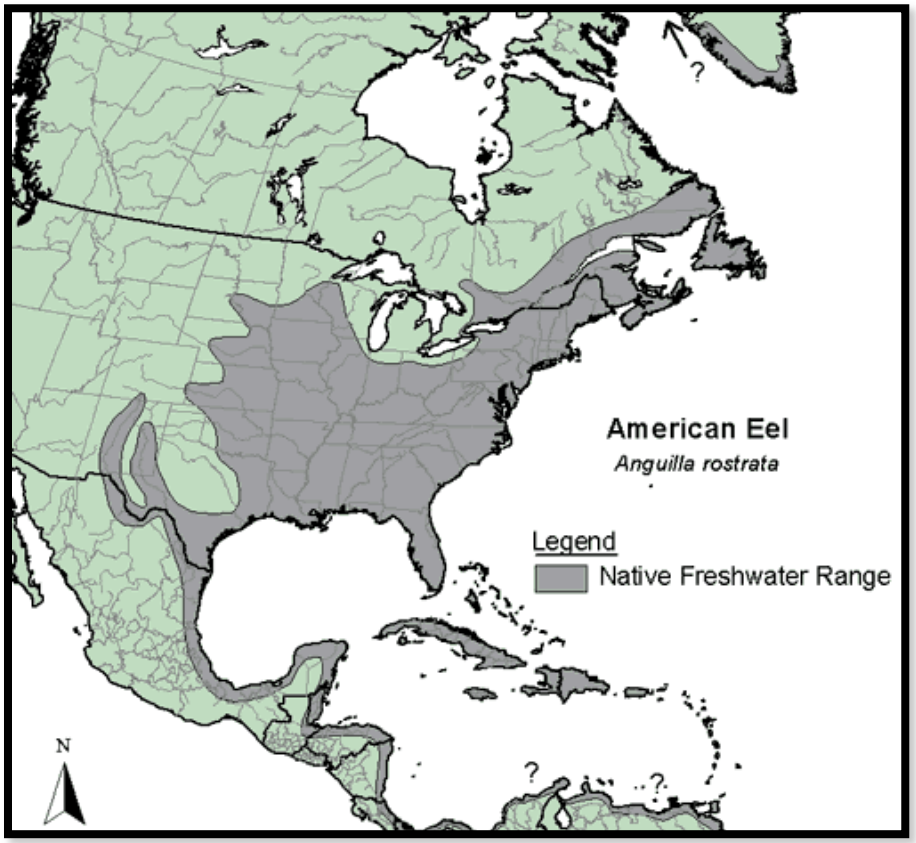
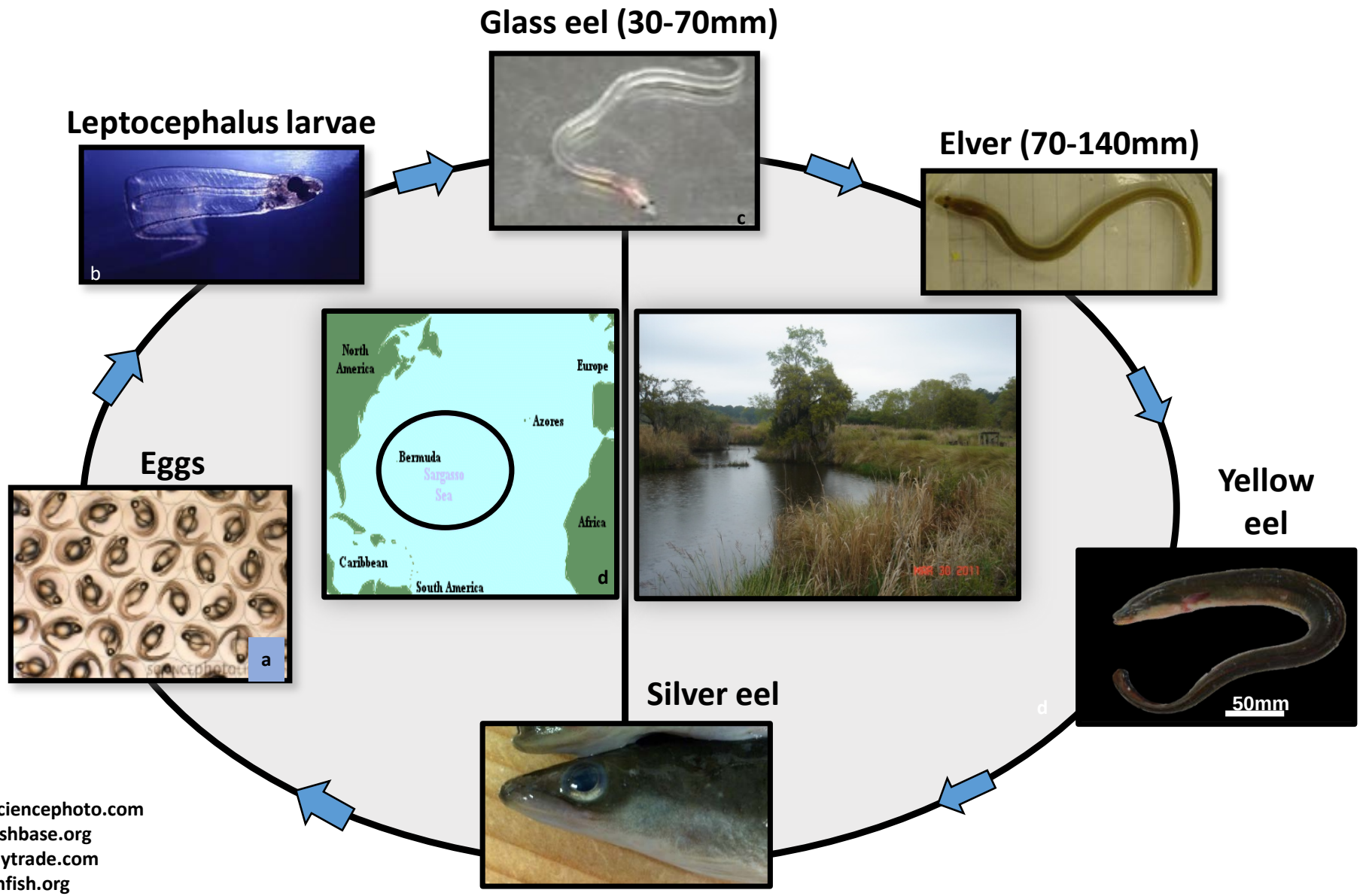


American eel, *Anguilla rostrata*

- Geographic range: Atlantic Coast, Greenland to South America.
- 'Catadromous' – adults spawn in Sargasso Sea and juveniles develop in freshwater, brackish, and estuarine systems.



American Eel Life Cycle



a. Sciencephoto.com
b. fishbase.org
c. diytrade.com
d. tnfish.org

Eel vulnerability and decline

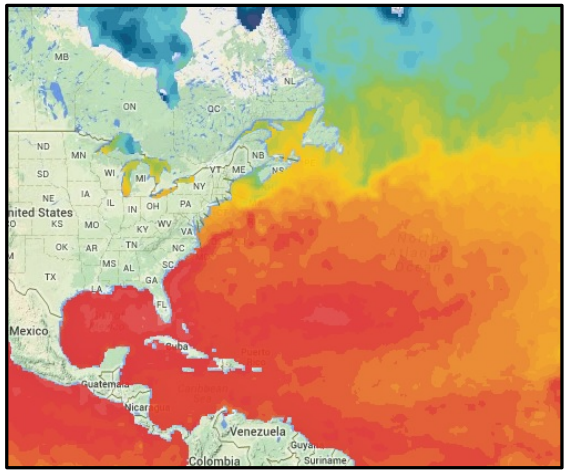
- High age at maturity (~10-30 yrs, varies with latitude), only spawn once in their lifetime.
- American eels are harvested both commercially and recreationally throughout their range.
- Harvest peaked in 1979 at 3.95 million pounds and has been declining since, i.e., for past 35+ years.

Potential threats to eel populations

Harvesting

Barriers to migration

Turbine mortality



Environmental changes

Anguillicoloides crassus

Anguillicoloides crassus

- Nematode parasite, endemic to East Asia, that infects swimbladder lumen of anguillid eels.

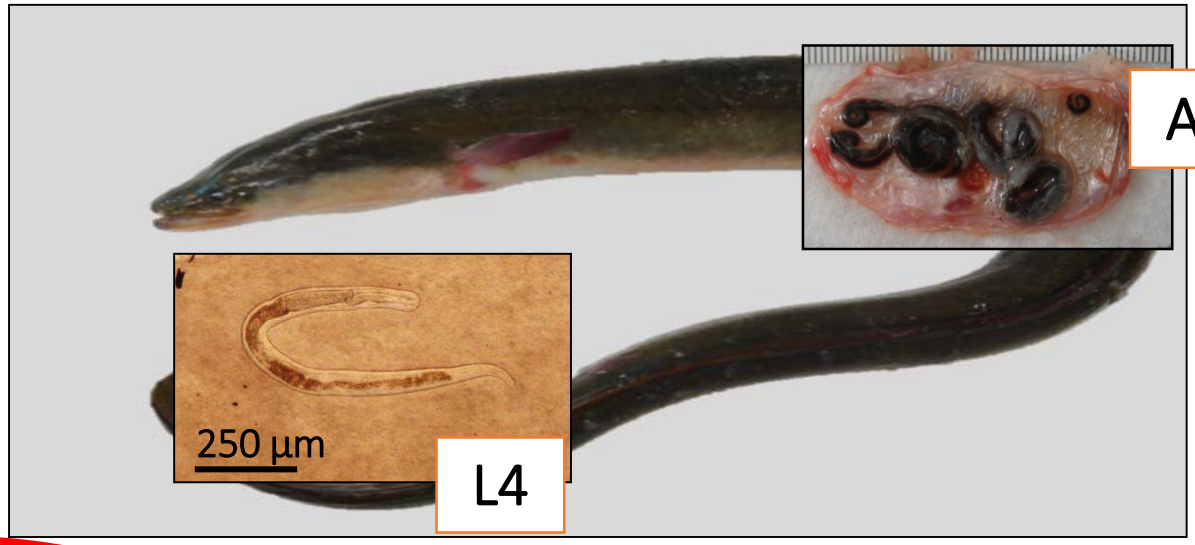
- No serious pathology in *Anguilla japonica* but extremely pathogenic to non-native eel species (e.g., *A. rostrata* and *A. anguilla;* Knopf and Mahnke 2004; Taraschewski 2006).

- *A. crassus* has been spread rapidly beyond its endemci range, infecting eel species in Europe, N. Africa, S. Africa and N. America through the commercial movement of live eels.

- First report of *A. crassus* in wild populations of the American eel *A. rostrata* came from Winyah Bay, South Carolina, USA in 1995 (Fries *et al*. 1996).

A. crassus life cycle

L4 molts to adult parasite in the swimbladder lumen

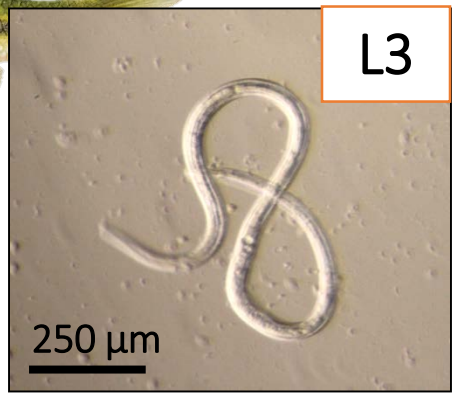


L3 stage molts to L4 stage in the swimbladder wall

Parasite eggs are laid down in the swimbladder lumen, pass through the pneumatic duct, and exit the eel via its vent

[Paratenic host]

L2 crosses gut wall of IH; L2 molts to L3, which are consumed by eel or paratenic host once in body cavity



L2 hatch in the water and are ingested by an intermediate host (IH)

[+ Molluscs, ostracods and others in European studies]

First Record of Paratenic Hosts of the Swimbladder Nematode *Anguillicola crassus* in North America.

Li, W., Arnott, S.A., Jones, K.M., Braicovich, P.E., de Buron, I., Wang, G. & Marcogliese, D.J. (2015).

Journal of Parasitology 101(5):529-535. October 2015.

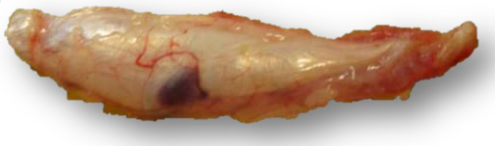
- Tested for larval *A. crassus* in 261 fish specimens from 23 species and 23 orders collected from estuarine habitats in South Carolina (0-9 ppt) and Nova Scotia (10-18 ppt).
- L3 *A. crassus* infections were observed in 35 fish belonging to 5 species and 3 orders.
  - SC: Spot (*Leisostomus xanthurus*)
    Silver perch (*Bairdiella chrysoura*)
    Highfin goby (*Gobionellus oceanicus*)
    Mummichog (*Fundulus heteroclitus*)
  - Nova Scotia Tomcod (*Microgadus tomcod*)
- No L4 or pre-adult worms were found; *A. crassus* arrested at L3 stage, supporting role of these species as paratenic hosts.


Juvenile parasites in eel swimbladder wall.
Photo courtesy of Jen Hein (College of Charleston).

Significance of swimbladder damage

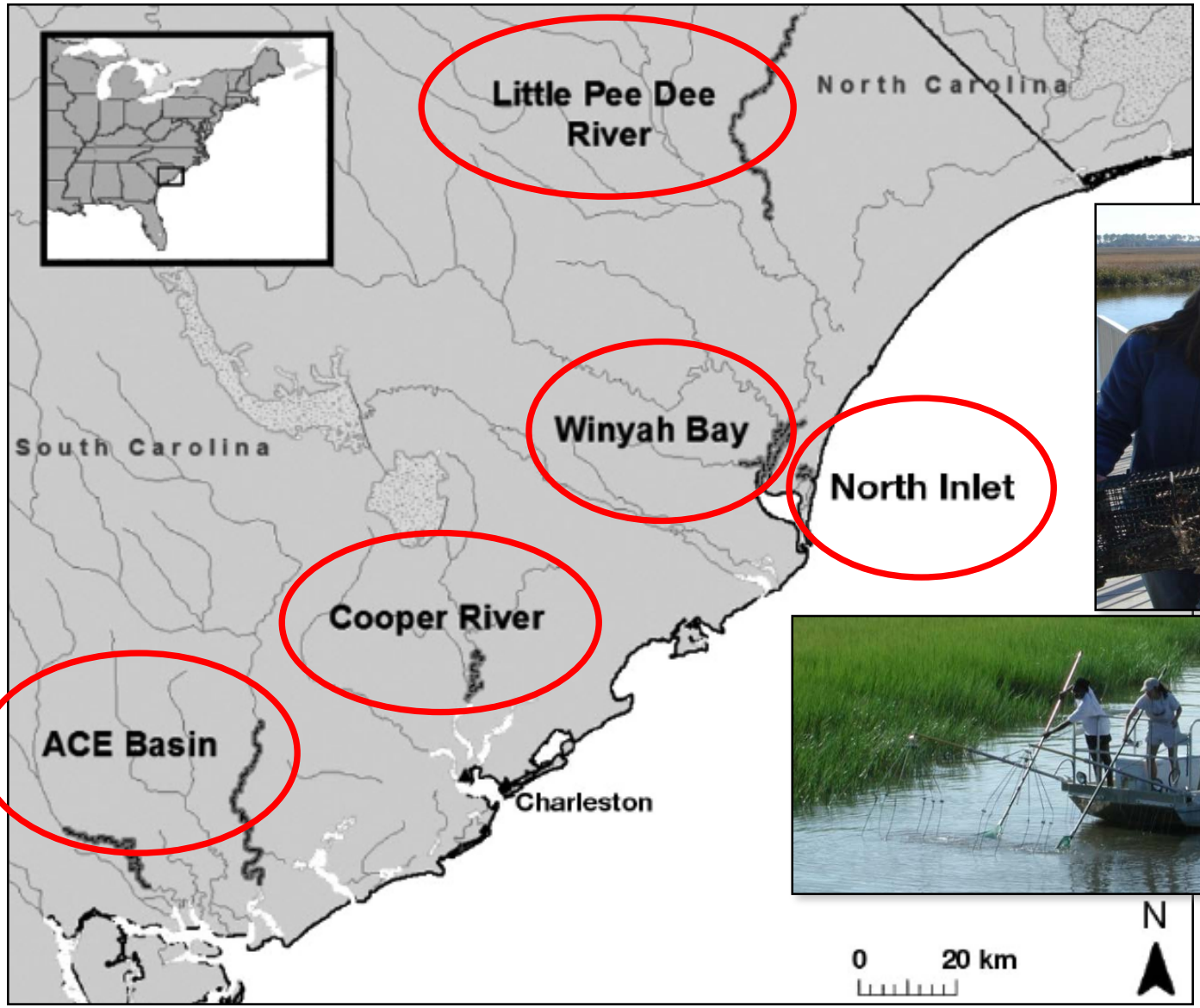
Healthy

Damaged



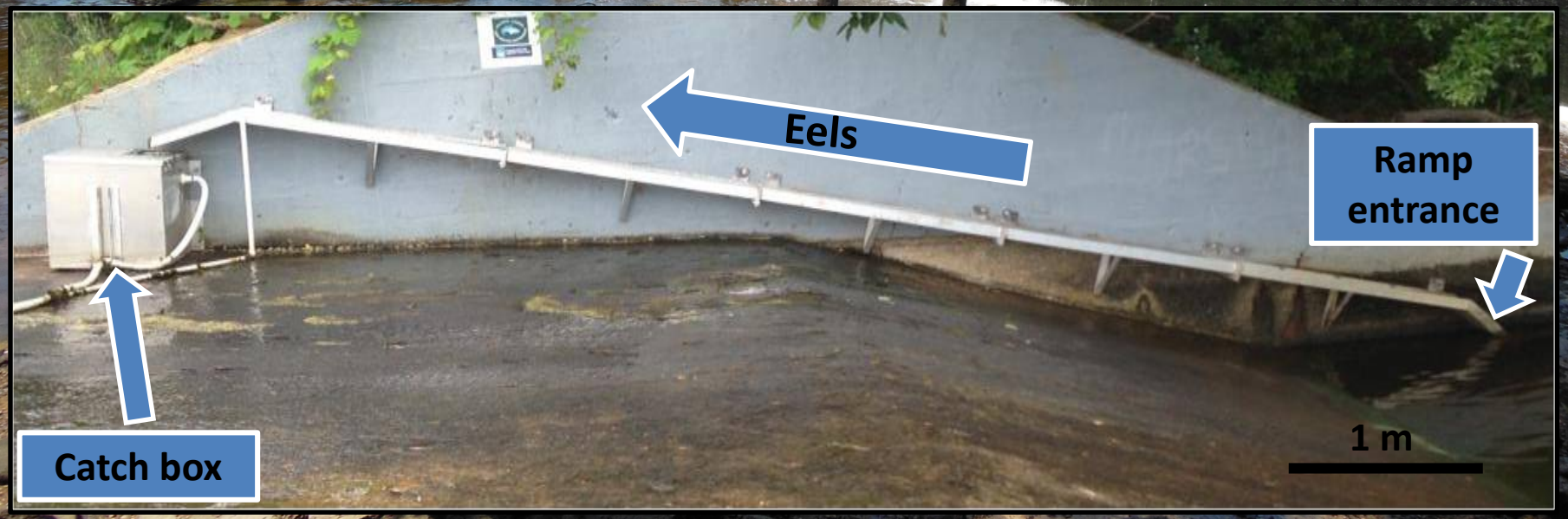
- Damage is irreversible (Molnár *et al.,* 1995)
- Affects buoyancy control (Wurtz *et al*., 1996)
- Compromises swimming efficiency and survival in migrating eels (Molnár *et al.*, 1995; Palstra *et al.,* 2007; Clevestam *et al.,* 2011)
- Leads to mortality under stressful conditions (Molnár *et al.,* 1991; Barus and Prokes 1996; Lefebvre *et al.,* 2002)
- Increased vulnerability to predation (Barse and Secor 1999)
- Migration to Sargasso Sea (Sjoberg *et al.,* 2009); European eels reduce their gut while expanding their swimbladder to enable significant vertical oceanic migrations as they travel to the Sargasso Sea; likely to compromised by parasite.

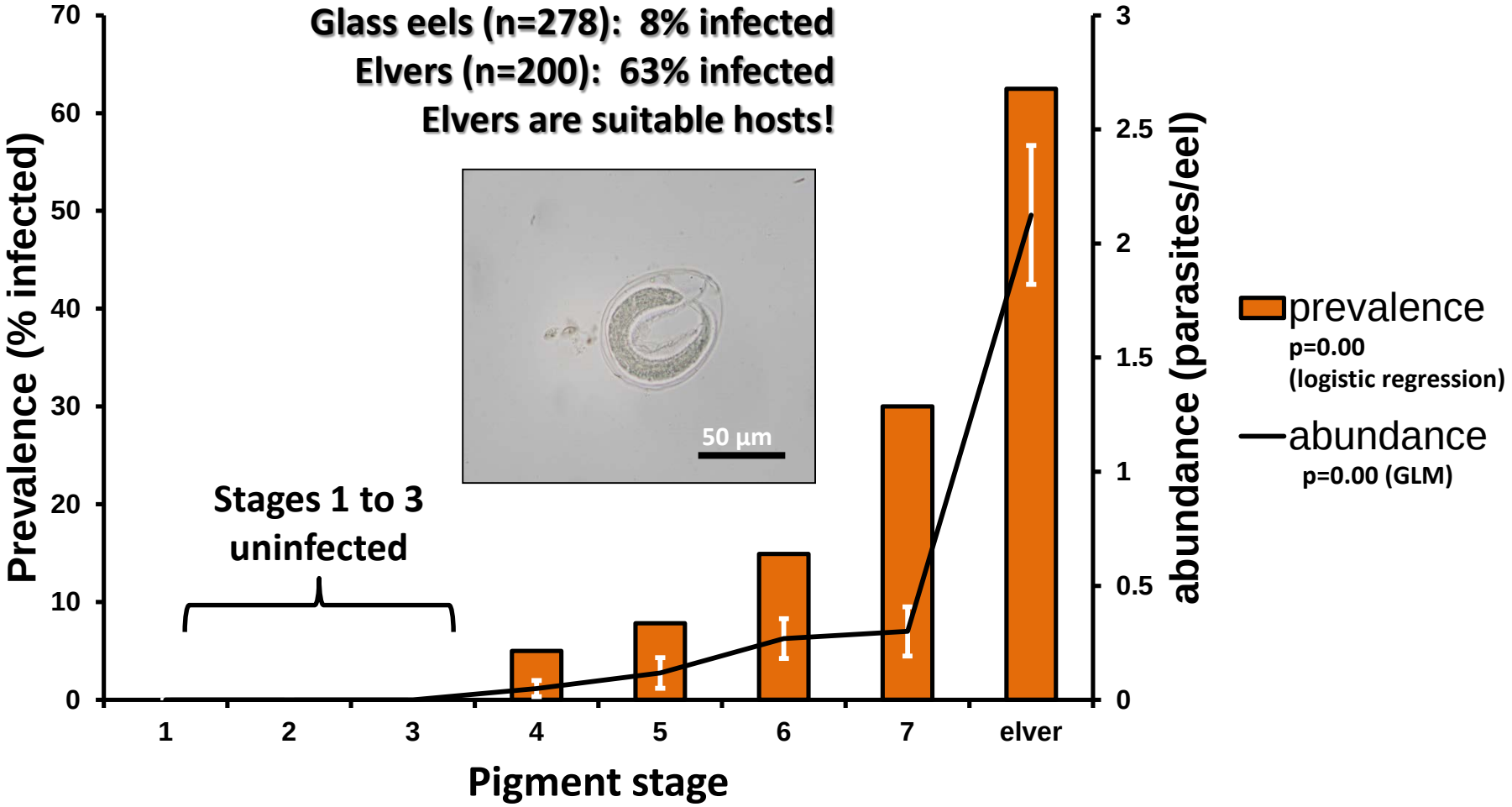
2011-2013 efforts focused on yellow eels



Hein *et al.* (2014). *Dis. Aquat Org* 107:199-209

Dam at Goose Creek Reservoir





- **Infection occurs within months of eel recruitment.**
- **Research attention turned to investigating the potential for detecting the parasite using genetic/molecular tools as an EDRR approach.**

USFWS SOUTHEAST REGIONAL ANS SMALL GRANTS-FUNDED PROJECTS.

2014-2015 PROJECT

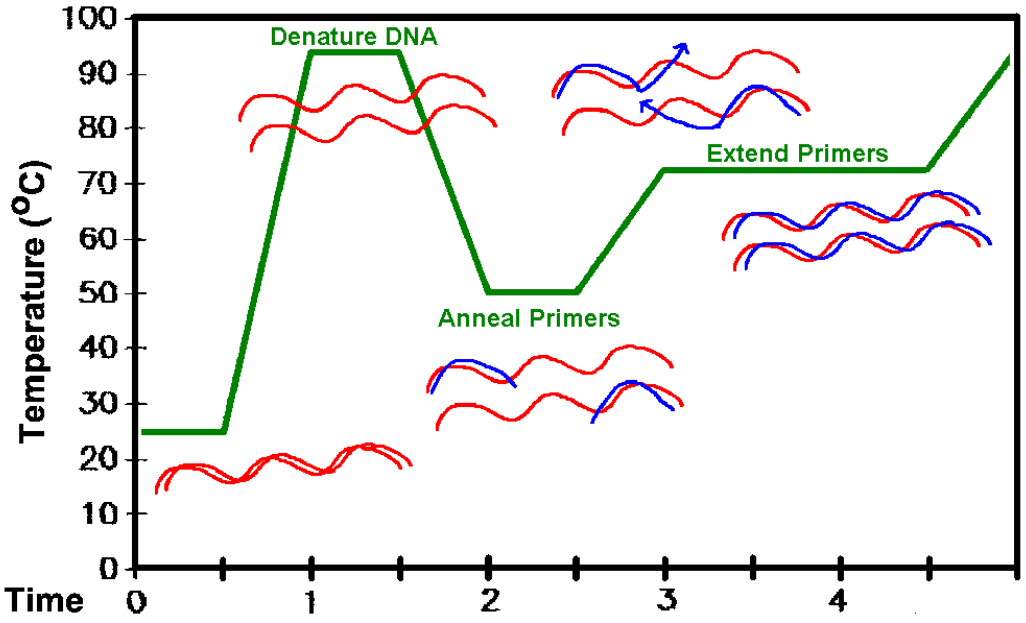
- **Original goal:** Test a qPCR method to detect and quantify the invasive parasite *A. crassus* in the environment.
- Intended method believed to be in the final stages of development based on communication with Canadian colleagues.
- Subsequently learned that the tool was in fact:
  - Not unequivocally species-specific for *A. crassus*;
  - Based on a PCR product that is too large for qPCR;
  - Non-quantitative.
- **New approach:** Develop a species-specific qPCR protocol to detect and quantify *A. crassus*.

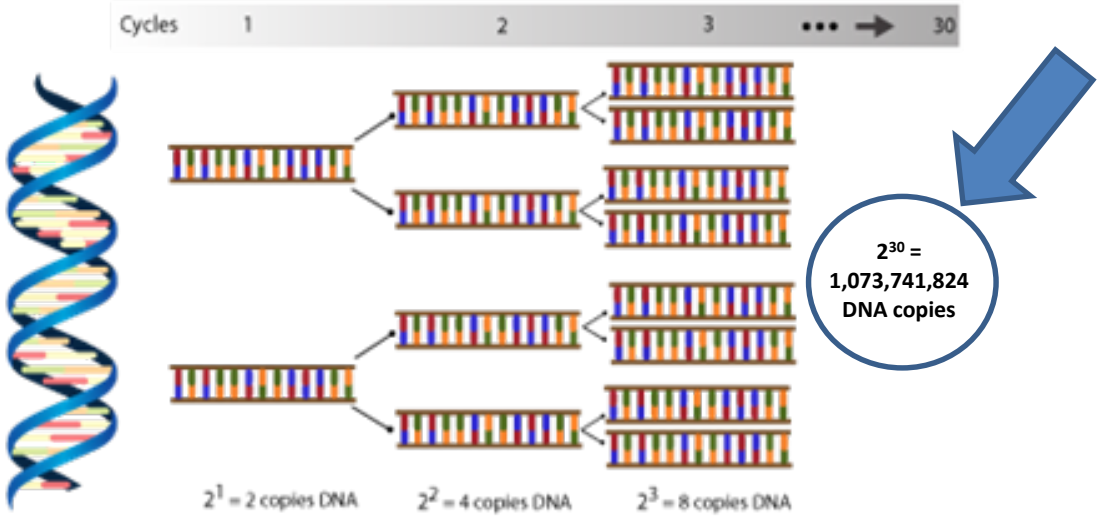
USFWS SOUTHEAST REGIONAL ANS SMALL GRANTS-FUNDED PROJECTS.

2015-2016 PROJECT

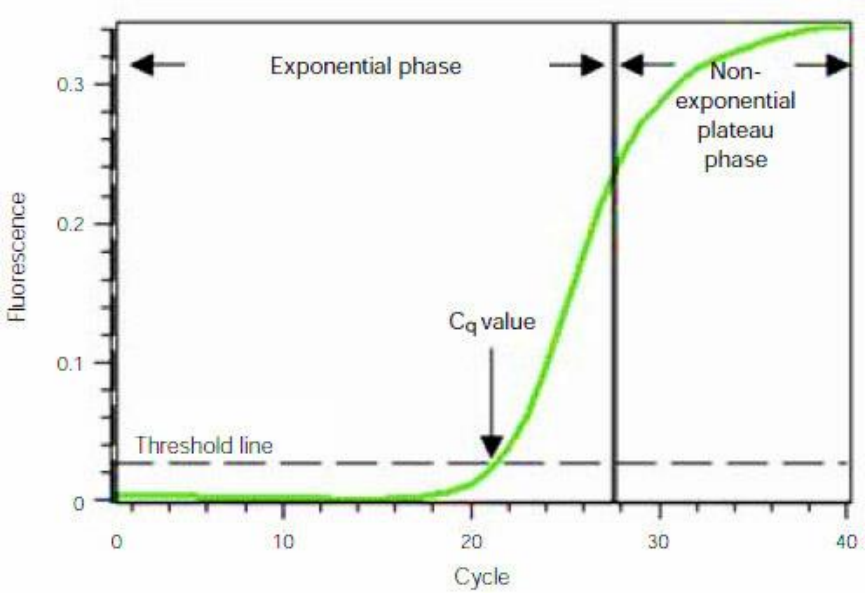
- Use a specific probe to the qPCR protocol to increase its sensitivity and specificity.
- Test for environmental inhibition by spiking field samples with known amounts of *A. crassus* DNA and comparing the qPCR responses to positive laboratory controls.
- Establish limits of detection for the qPCR assay with the infective L_3 larval stage using copepods cultured in the laboratory.
- Test field sampling methods at the Goose Creek Reservoir to validate the field utility of this detection tool.

Polymerase Chain Reaction (PCR)



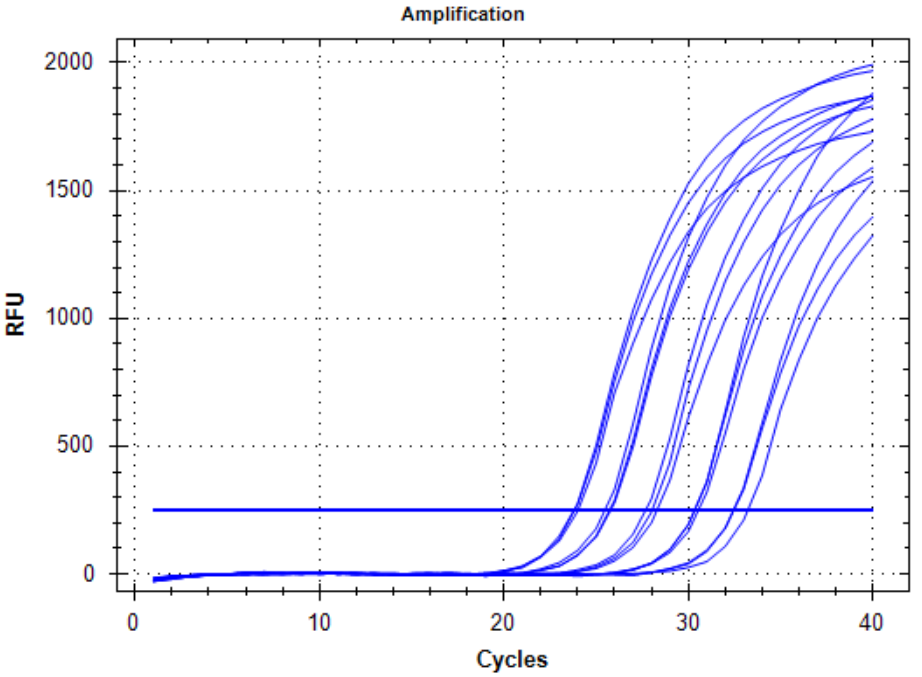


Quantitative Polymerase Chain Reaction (qPCR)

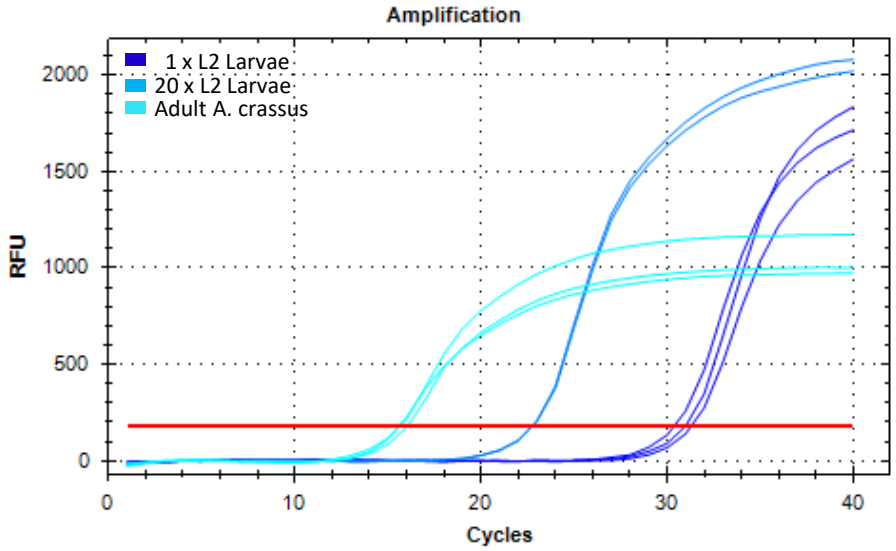


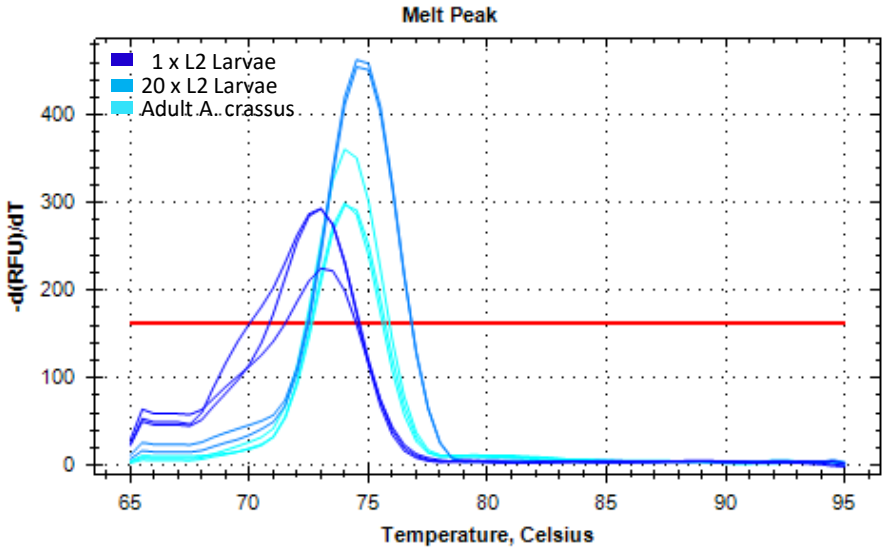
- Lower Cq value corresponds to greater starting quantity of DNA.
- Rule of thumb: desirable for amplification to cross threshold at < 35 cycles.

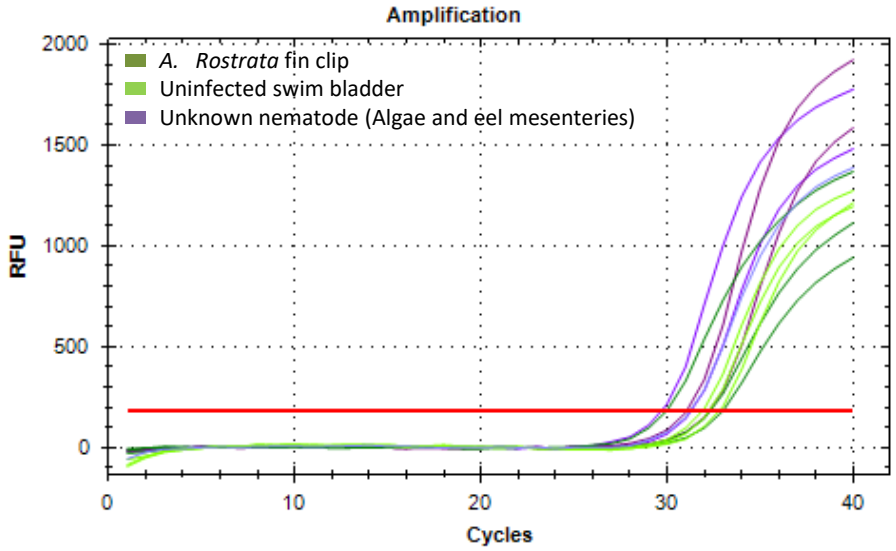
- qPCR is an excellent method for quantifying relative differences in gene expression between species, tissue types, treatments, etc.
- Excellent method for detection of rare DNA in environmental samples (endangered species, invasive species, parasites, etc.)

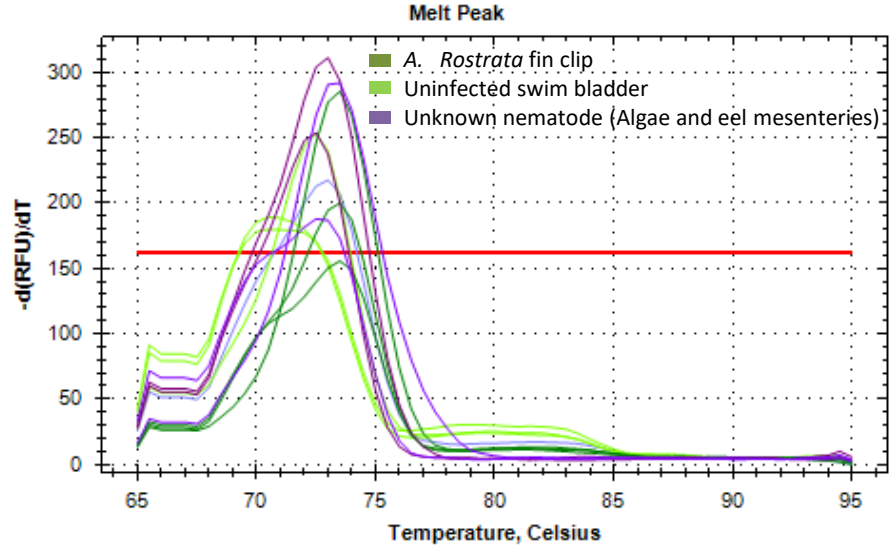


Initial qPCR run @ 58˚C with full primer concentrations based on starting protocol; Amplification of non-target materials, i.e., NOT Species-specific!

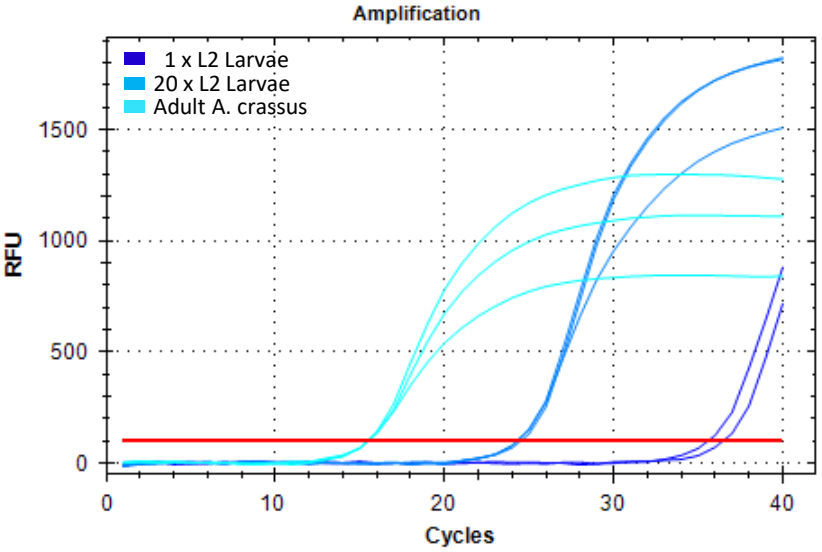


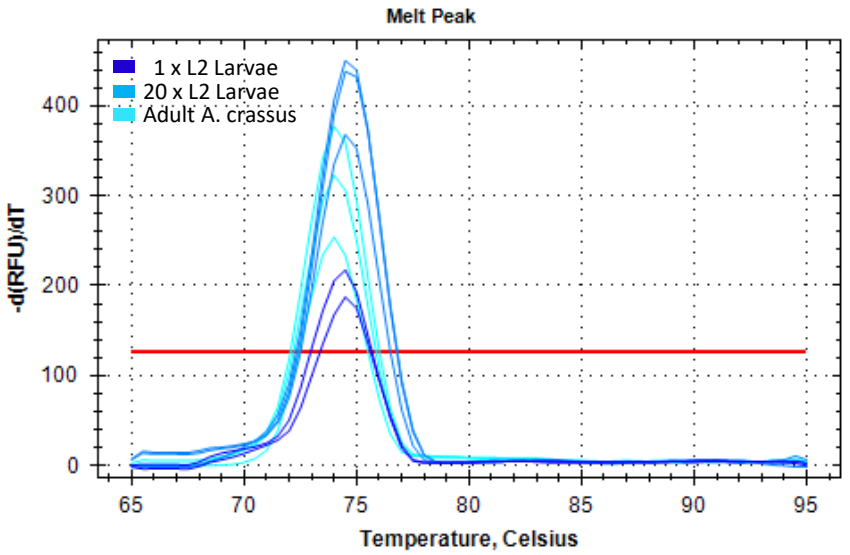


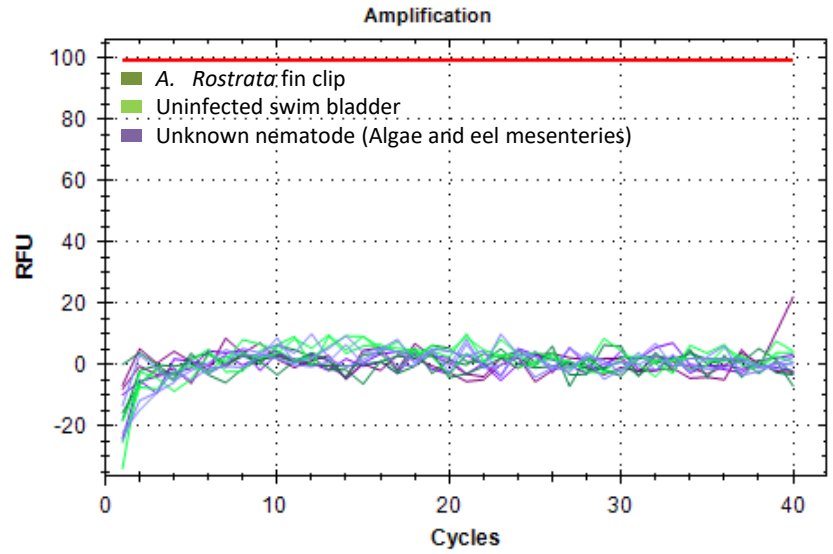


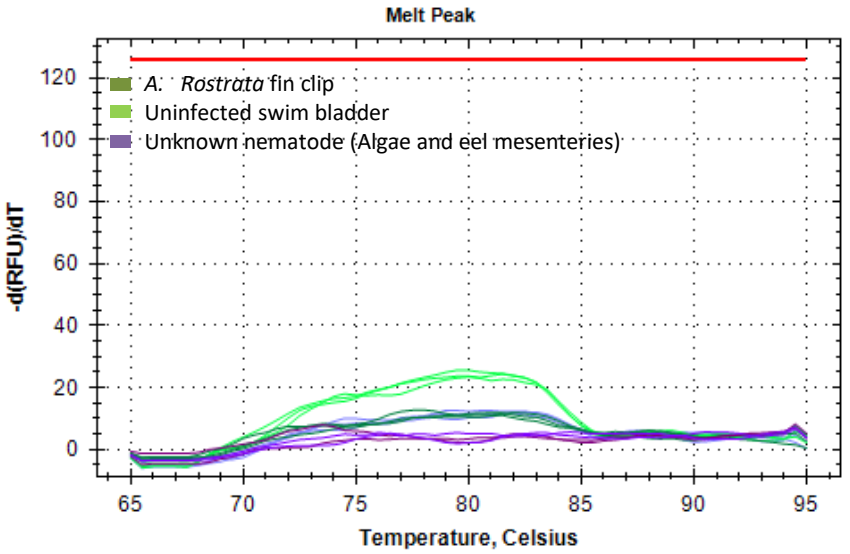


Optimized protocol with ½ Primer concentration and 60˚ annealing temperature - ONLY the *A. crassus* samples amplified.

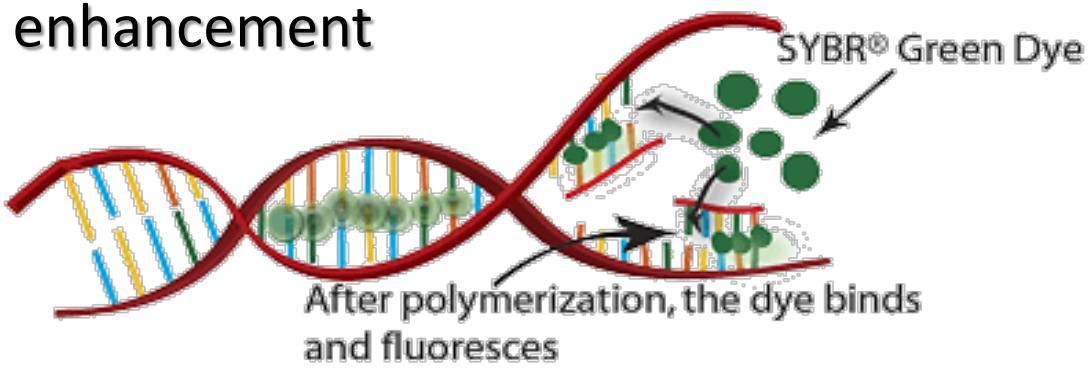




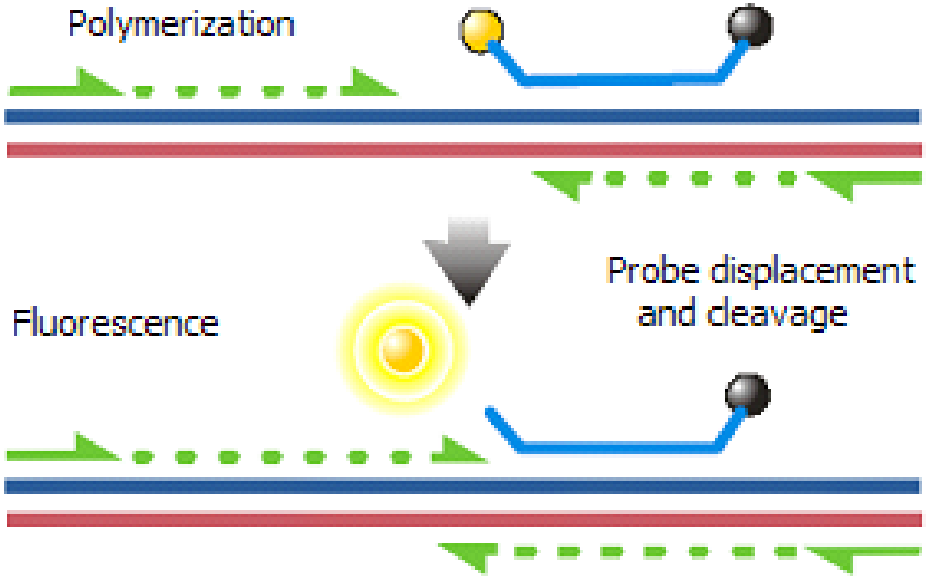




Further protocol enhancement

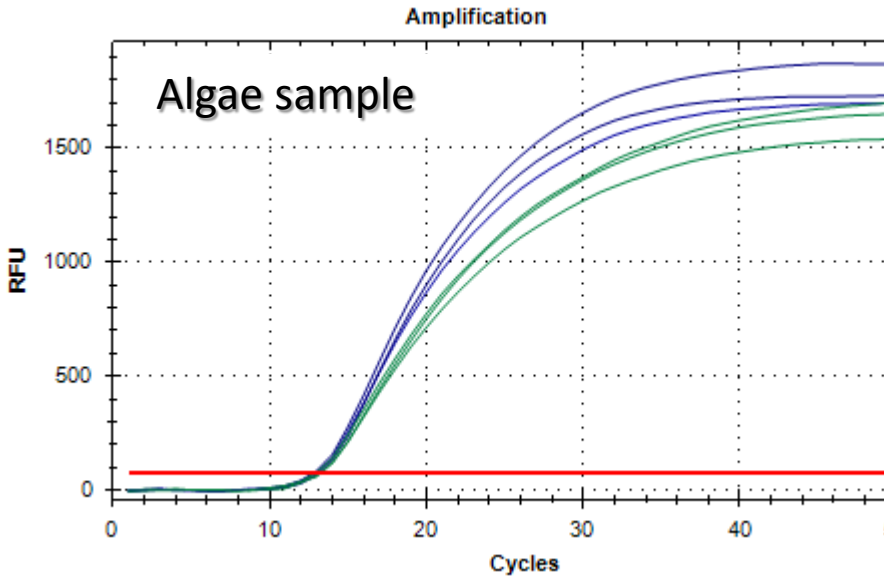


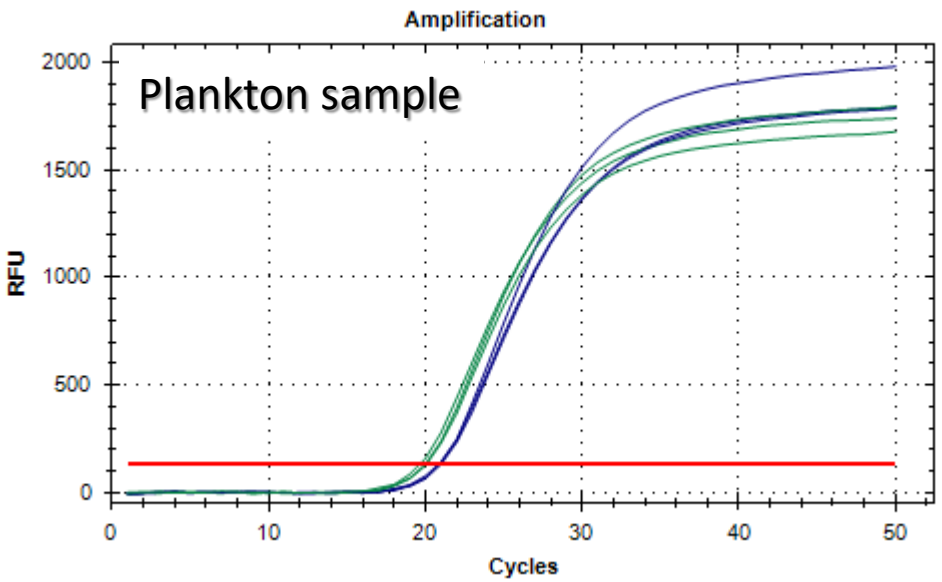
Without the addition of a probe, all double-stranded DNA produced through amplification fluoresces (risk of non-species specific fluorescence).



With the addition of a probe, only the product between the species-specific primers fluoresces, increasingly the specificity of this method.

Testing for reaction inhibition in field samples

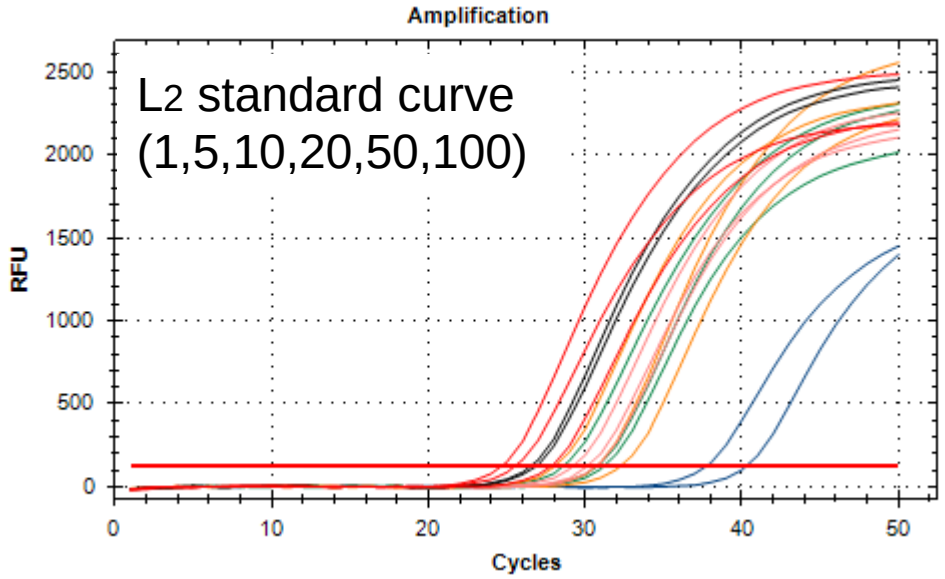


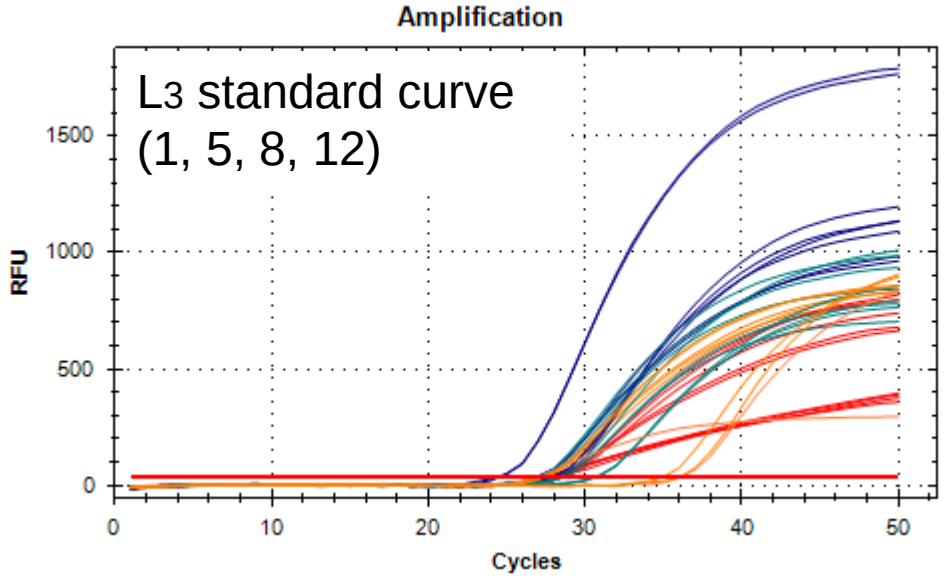


Blue – positive control samples
Green – field samples spiked with *A. crassus* DNA

- No inhibition was seen with any of the field samples.
- When field samples were spiked with *A. crassus* DNA, positive results were seen with similar cycle threshold values to the positive control samples.

Comparing L2 vs L3 standard curves



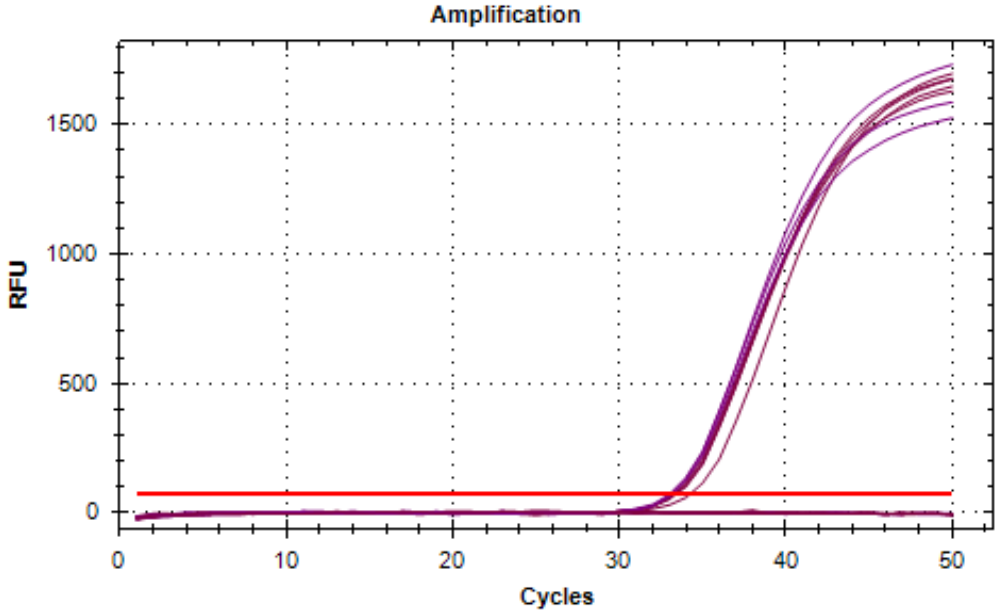


- Overlapping cycle threshold values and ranges of detection.
- Significance: difficult to differentiate which life-stage is present in a sample and inter-individual variation in DNA content appears high.

L_3 individuals (n=80) were successfully extracted from copepods infected with the infective L_2 life stage of *A. crassus*.

Results from 2015 field samples

- Samples collected during known times of infection of *A. rostrata* by *A. crassus* (June-July 2015).
  - 2L filtered water samples – in 500mL replicates
  - Plankton, algae, and soil – (0.5 mL, 1.0 mL, 1.5 mL, 2.0mL)



Positive hits from plankton samples!!

0/0 hits in 0.5mL samples
3/8 hits in 1.0mL samples
5/8 hits in 1.5mL samples
8/8 hits in 2.0mL samples

[No positive hits observed in other sample types.]

- **TAKE HOME MESSAGE**: This newly developed qPCR protocol is species-specific, quantitative and ready to be used for testing field samples to detect the presence of *A. crassus*.

- Future work: Applying the qPCR tool in the field

- Temporal sampling at a site of known infection (GCR):
  - Is *A. crassus* present year-round?

- Spatio-temporal sampling across other habitats in SC:
  - What is the distribution of *A. crassus* in SC?
  - How localized are infectious habitats?
  - Has does prevalence vary by location?

- Retention ponds may serve as "hotspots" for infection that concentrate parasites, intermediate hosts, and eels.

Acknowledgements – People

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SCDNR Bennetts Point Laboratory

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Samantha Suriano (undergraduate)
Isabelle Boersma (undergraduate)
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Dr. Tim Callahan

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DNR

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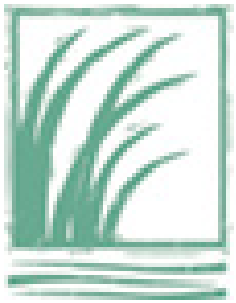
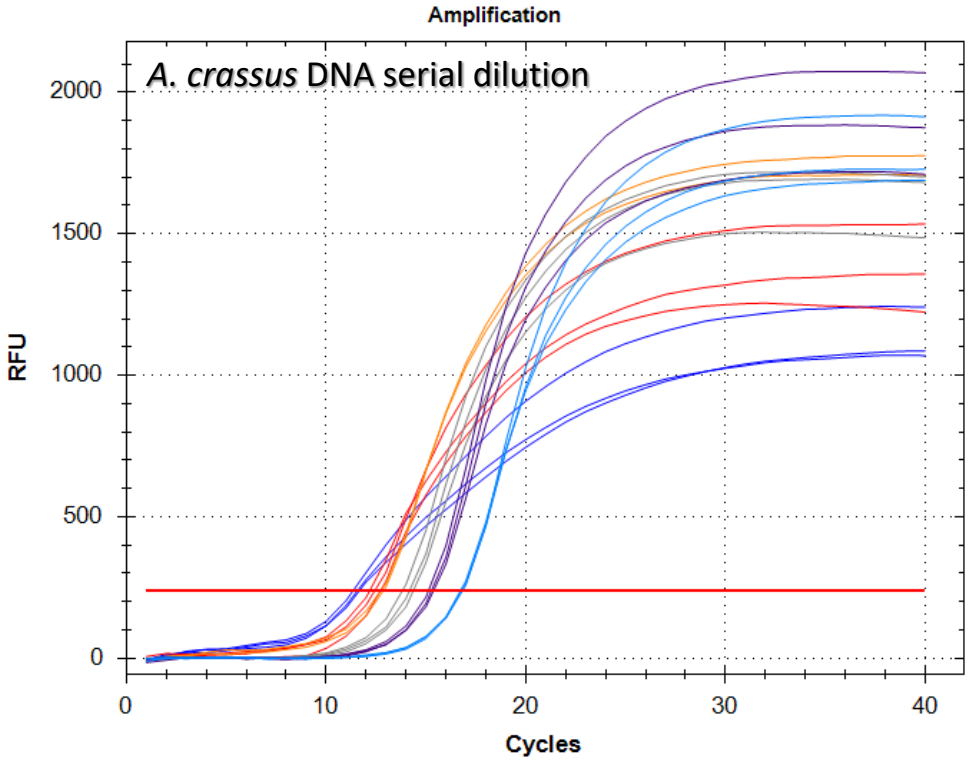
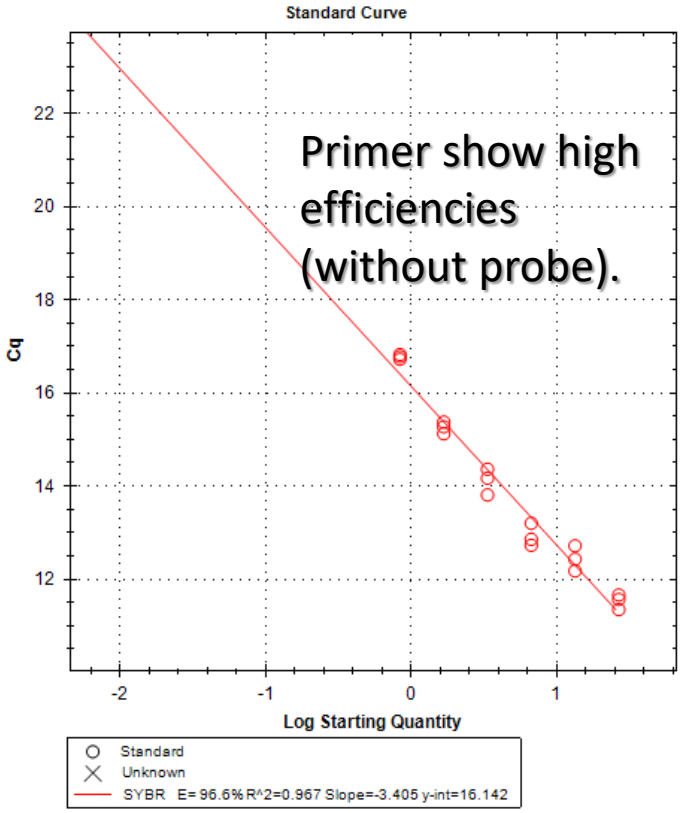
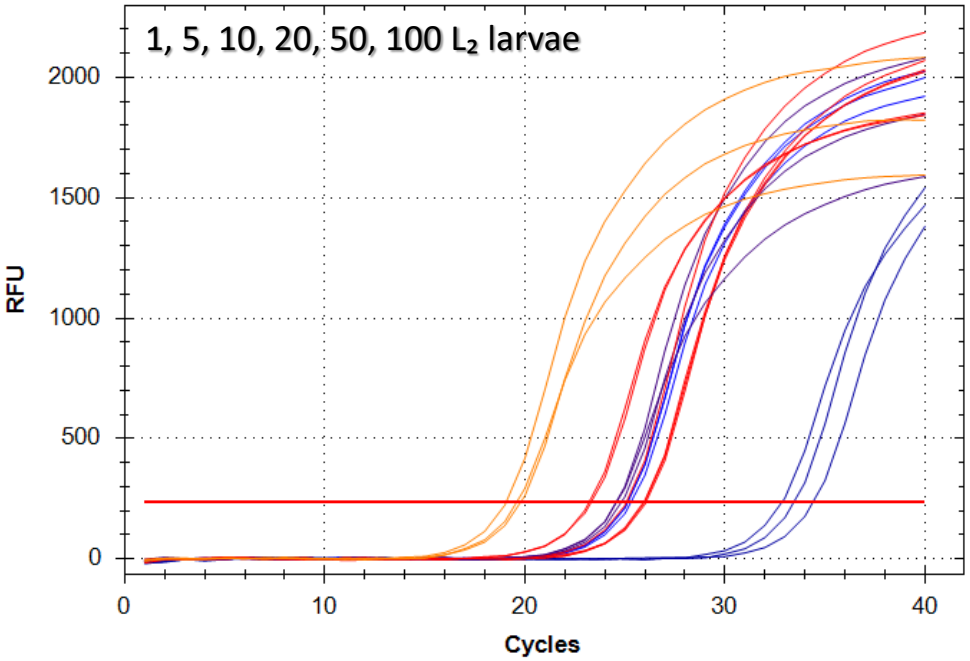


NATIONAL ESTUARINE RESEARCH RESERVE SYSTEM

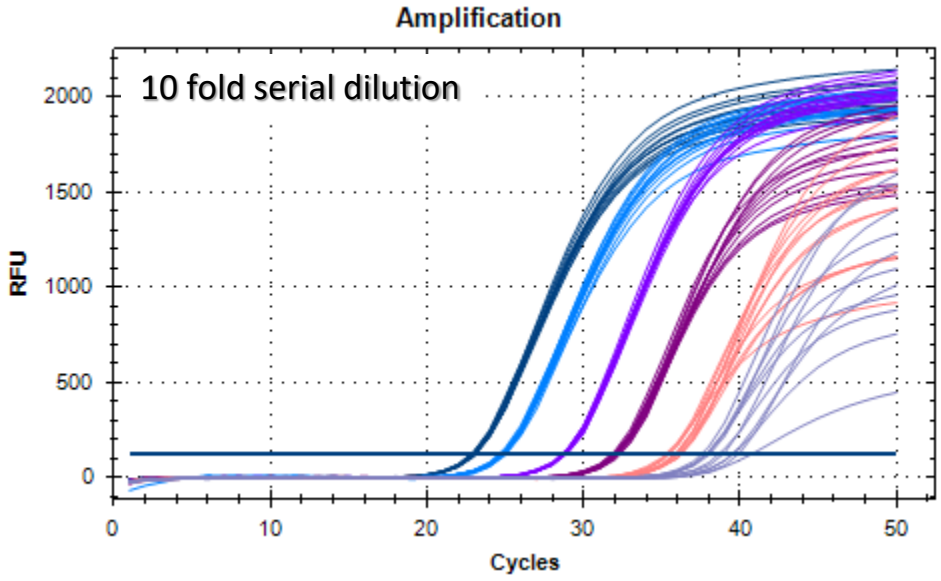
Atlantic Coastal
Fish Habitat Partnership

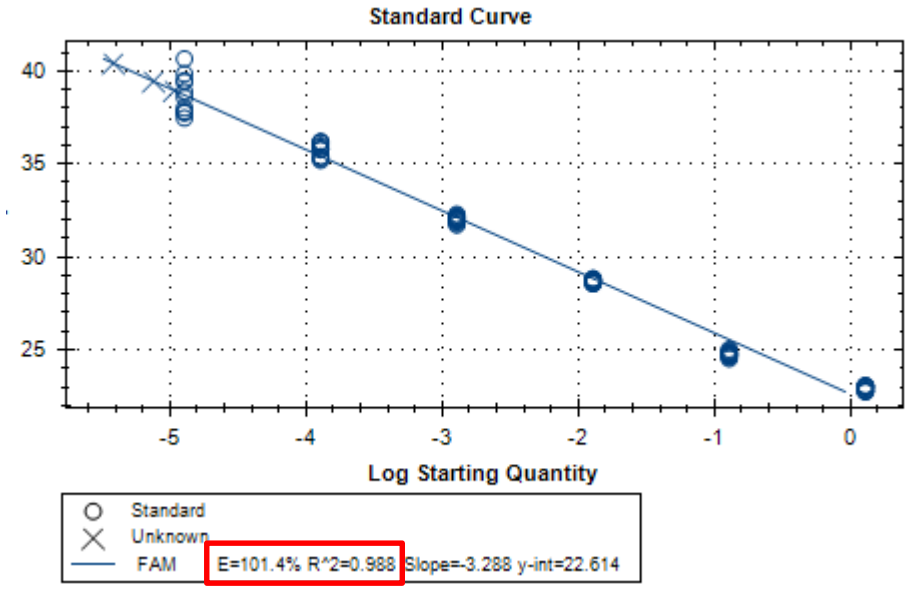

Amplification
A. crassus DNA serial dilution
RFU
2000
1500
1000
500
0
0
10
20
30
40
Cycles


Standard Curve
Primer show high efficiencies (without probe).
Cq
22
20
18
16
14
12
-2
-1
0
1
Log Starting Quantity
Standard
Unknown
SYBR E=96.6% R^2=0.967 Slope=-3.405 y-int=16.142

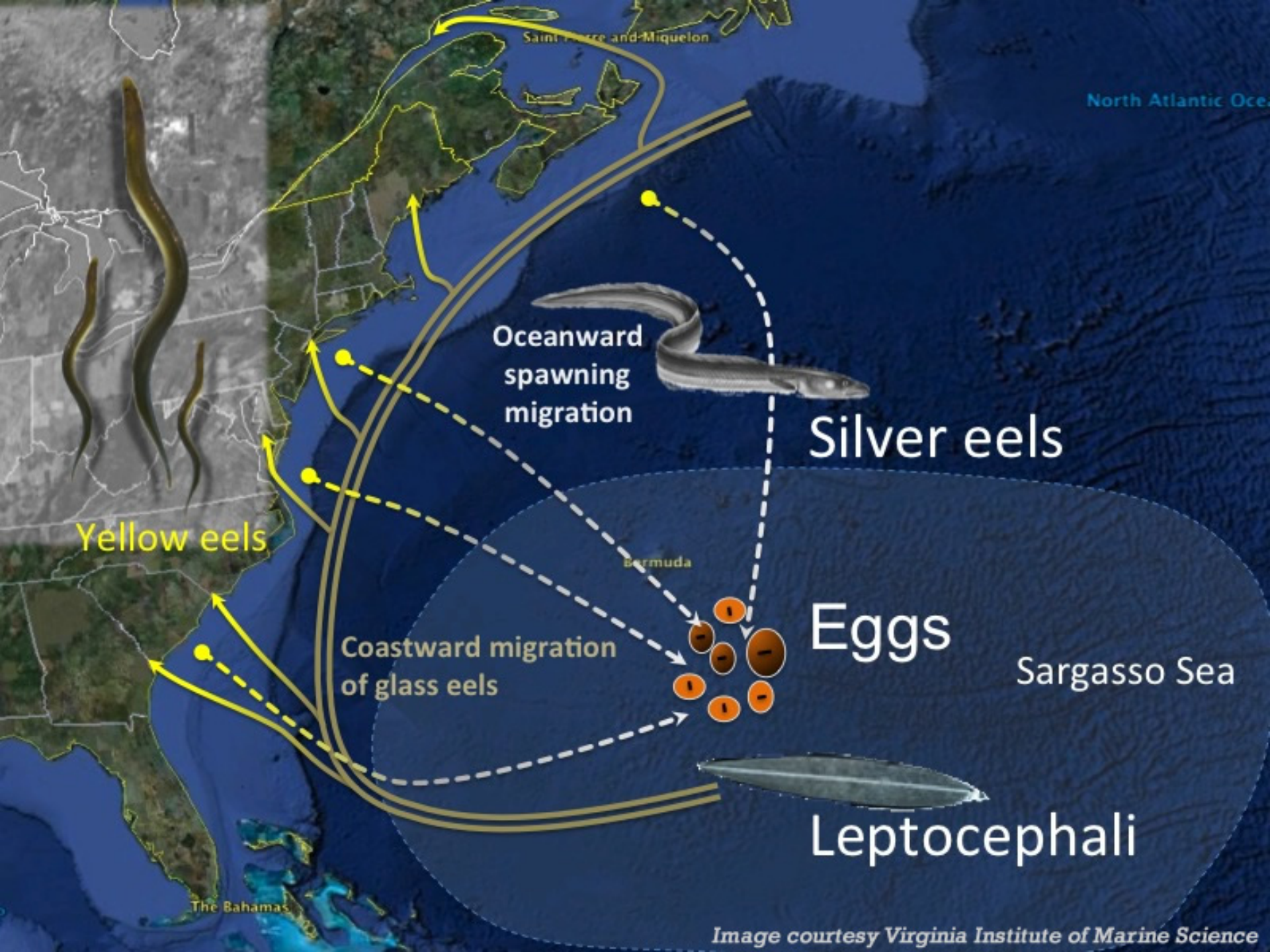

1, 5, 10, 20, 50, 100 L₂ larvae
RFU
2000
1500
1000
500
0
0
10
20
30
40
Cycles

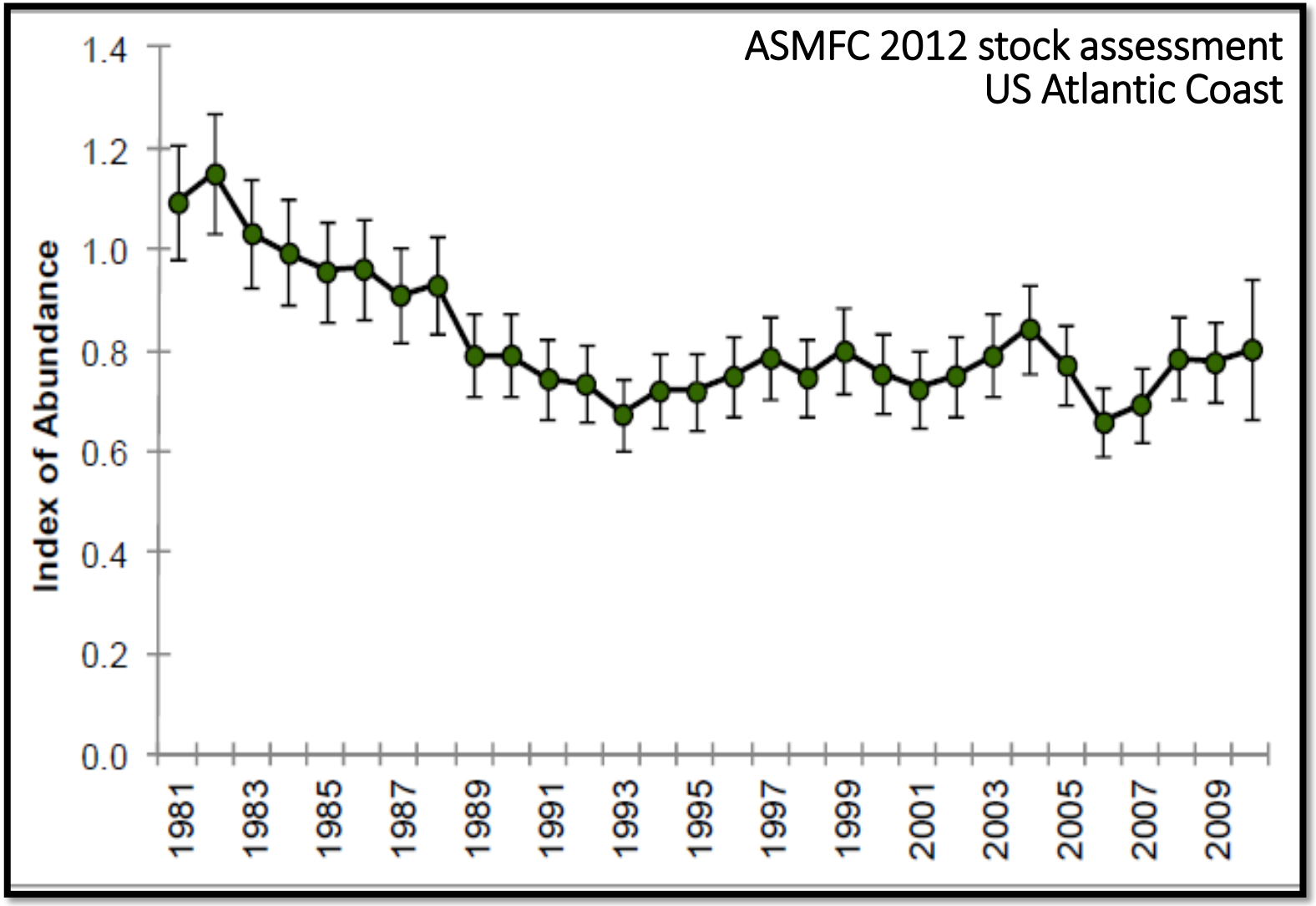
UPDATES FROM 2015-2016 PROJECT: Probe Optimization





- Samples from 2015 were re-run to verify the specificity of the primer-probe pair.
- No amplification was observed from closely related philometrids, uninfected *A. rostrata* swim bladders, or *A. rostrata* fin clips.
- The probe makes the reaction more specific to *A. crassus* enabling differentiation

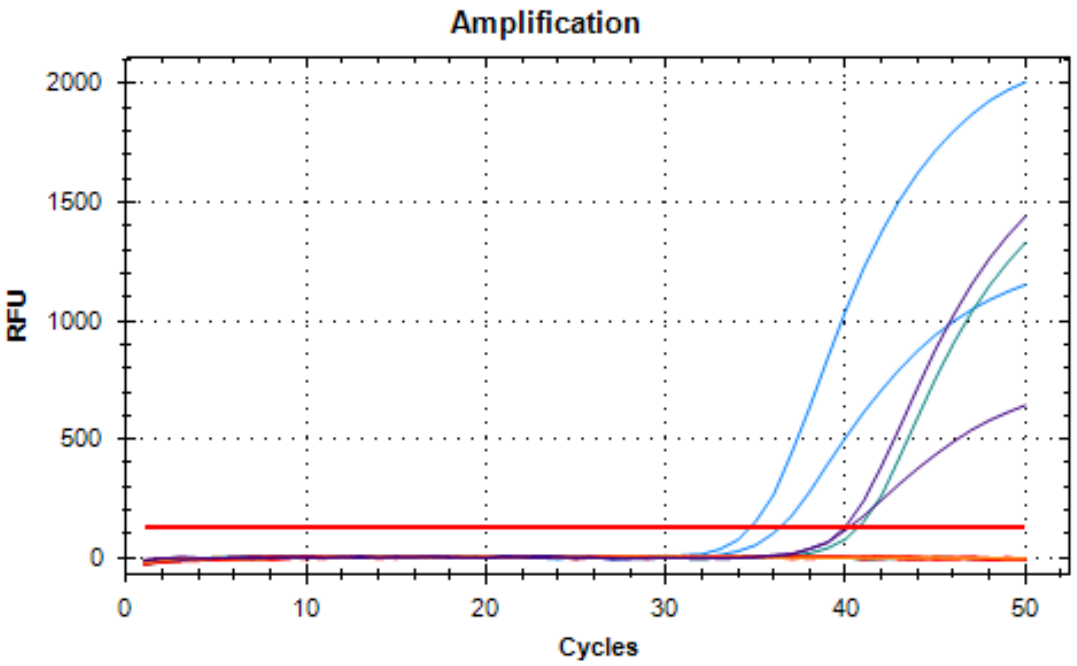

Saint Pierre and Miquelon
North Atlantic Ocea
Oceanward
spawning
migration
Silver eels
Yellow eels
Bermuda
Eggs
Sargasso Sea
Coastward migration
of glass eels
Leptocephali
The Bahamas
Image courtesy Virginia Institute of Marine Science



"Depleted" in US waters (ASMFC 2012 benchmark stock assessment); at or below historically low levels.

Improvement to protocol through addition of probe

- Samples with positive amplification after ambiguous results last year (primer only)



Algal sample - 3
Ambiguities/messy seq at bp 47-49 & 108-119>>>therefore, 90% ID to *A. crassus* (JF805655)

Algal sample - 4
F seq only with ambiguities at bp 48-57>>>therefore, 89% ID to *A. crassus* (JF805655)

Unk. Nematode – eel mesenteries
Messy seq in both directions; 100% ID but only 24% coverage to *A. crassus* (JF805722)

All algal samples from 2015 and samples with questionable results from last year are shown. Three of the samples result in positive detection of *A. crassus* .