Assessing the Role of Long-Lived Reptiles in the Ecology of EEEV

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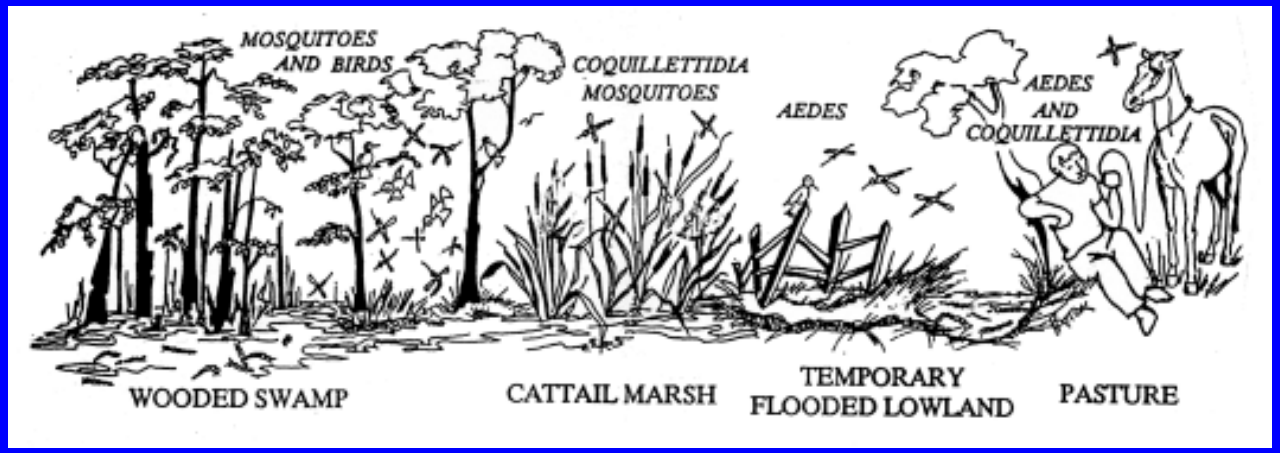


EEEV summer cycle

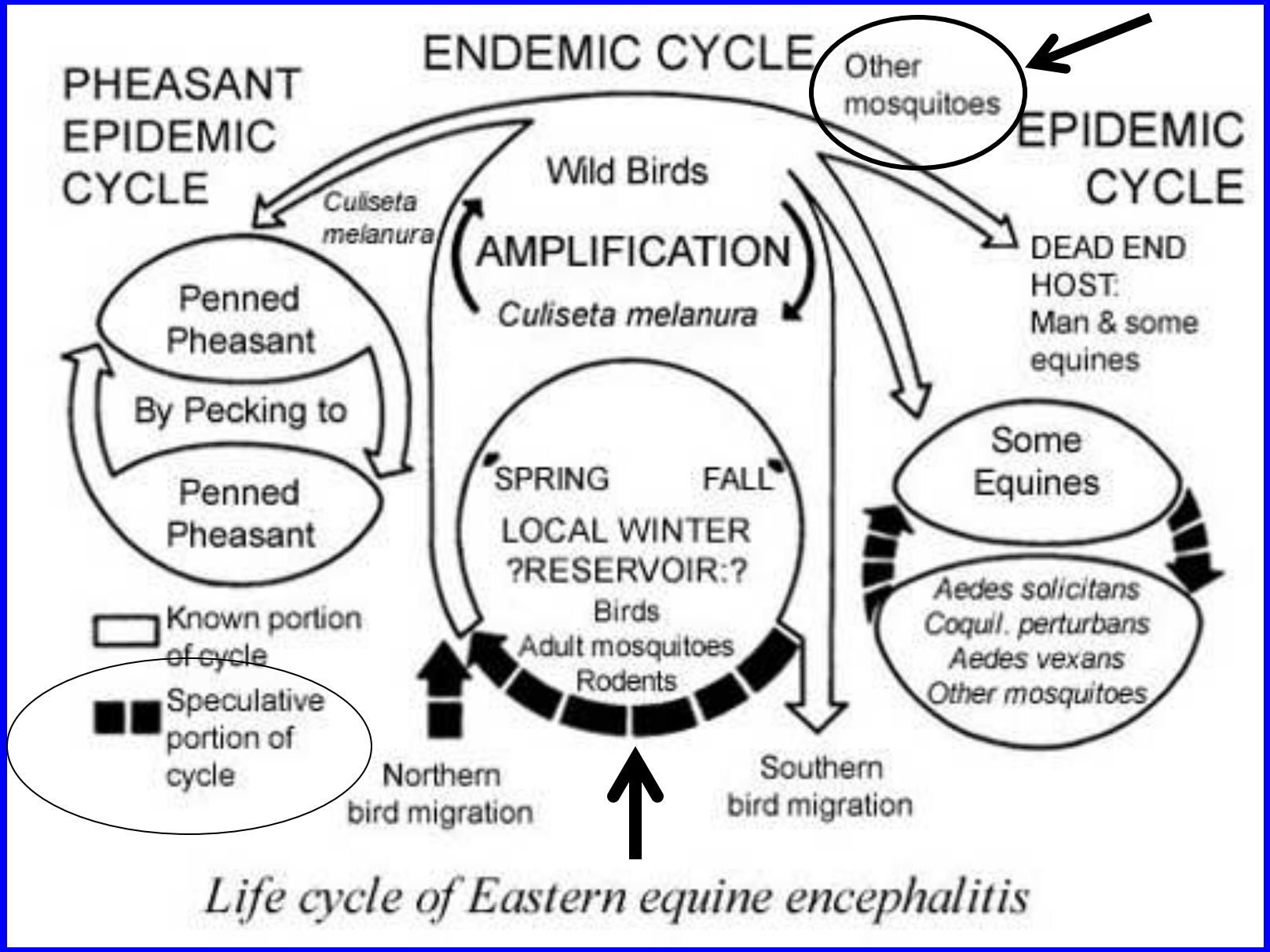
EEEV circulates between bird biting mosquitoes and birds throughout the summer.

Infected mosquito feeds on a person or horse.

Other mosquitoes become infected with EEEV after blood feeding on an infected bird.

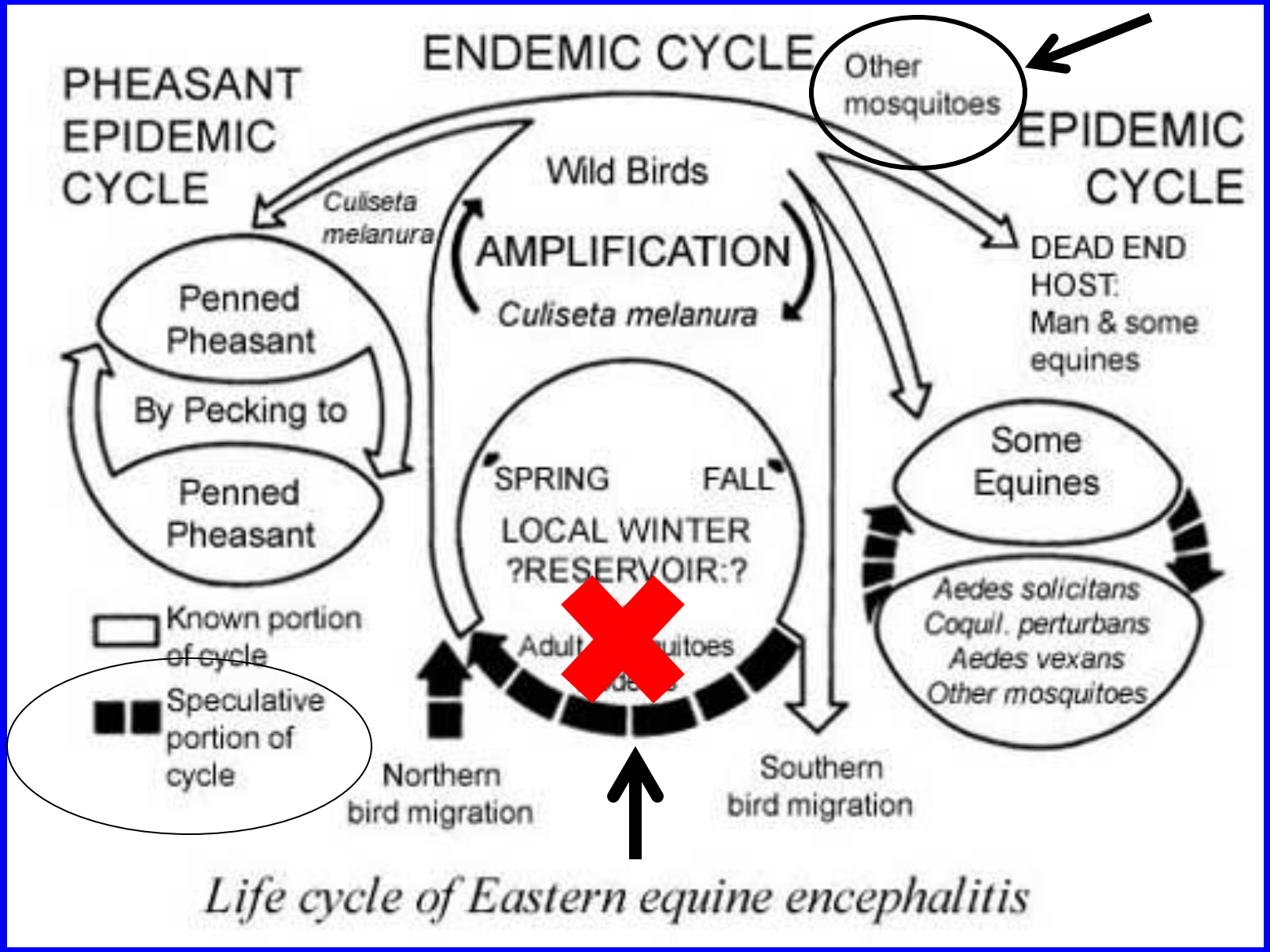
Mosquito vectors

- Isolates from the following: *Ae. vexans*, *An. crucians*, *An. punctipennis*, *An. quadrimaculatus*, *Cs. melanura*, *Cs. minnesotae*, *Cs. morsitans*, *Cq. perturbans*, *Cx. erraticus*, *Cx. nigripalpus*, *Cx. peccator*, *Cx. pipiens*, *Cx. quinquefasciatus*, *Cx. restuans*, *Cx. salinarius*, *Cx. territans*, *Cq. perturbans*, *Oc. atlanticus-tormentor*, *Oc. canadensis*, *Oc. cantator*, *Oc. infirmatus*, *Oc. mitchellae*, *Oc. sollicitans*, *Oc. triseriatus*, *Uranotaenia sapphirina*



Life cycle of Eastern equine encephalitis

Where does EEEV go?



Where does EEEV go?

Role of Reptiles and Amphibians in EEEV Ecology?

- Found to be naturally infected with EEEV
  - (Karstad, 1960)
- Susceptible to experimental infection
  - (Hayes, 1964)
- Maintained high viremia over several months
- Experimentally carry the virus through hibernation

Bridge vectors

- bird ⟶ mammal

  Coquilletidia perturbans

  Aedes sollicitans

- Mosquitoes that feed on reptiles

  Aedes atlanticus – turtle-feeding and mammalophilic spp.

  Aedes canadensis– turtle-feeding and mammalophilic spp.

  Aedes triseriatus– turtle-feeding and mammalophilic spp.

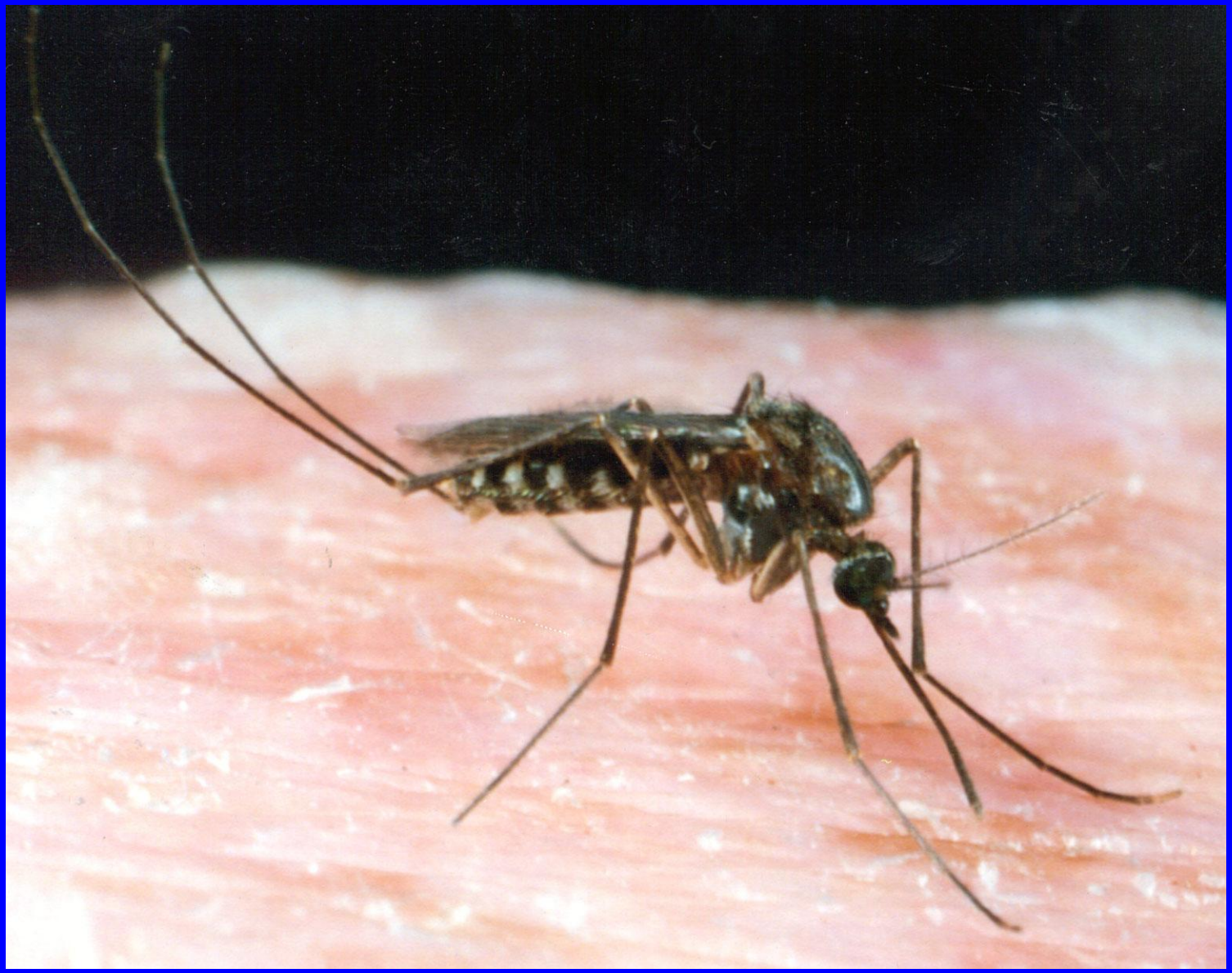
  Culex erraticus – feeds on any terrestrial vertebrate

  Culex peccator - herpetophilic

  Culex territans - herpetophilic

  Culiseta melanura – feeds mostly on birds, ornithophilic

Culex erraticus female



Culex erraticus in the South

- Abundantly in Coastal Plain areas throughout southeastern US
- EEEV isolated from *Culex erraticus* in VA, SC, GA, AL and FL
- *Culex erraticus* feeding patterns:
  - Exhibits unusual opportunism
  - Suggests role as "bridge" vector in EEEV ecology
    - Reptiles to birds?
    - Birds to mammals?

Table 1: Hosts of *Culex erraticus* in North Carolina.

Location	Blood Meals Identified	Mammals	Birds	Amphibians/ Reptiles
Kenansville, NC	13	5 (38%)	4 (31%)	4 (31%)
Raleigh, NC	225	111 (49%)	69 (31%)	45 (20%)

- Mammal blood meal sources included deer (16), dog (5), raccoon (3), cow (2), rodents (2), cat (1), and opossum (1).
- Amphibian blood meal sources included frog (3), and toad (1).
- Reptile blood meal sources included snake (16), turtle (2), and lizard (1).

Culex erraticus collections: 1990s

- CDC light traps and aspirators in 12 counties in Southeastern Georgia
- Vacuum aspiration from beneath bridges at 5 sites in Bulloch Co. and 3 sites in Effingham Co.
- Vacuum aspiration from gopher tortoise burrows and resting boxes at G.L. Smith State Park, Emanuel Co.

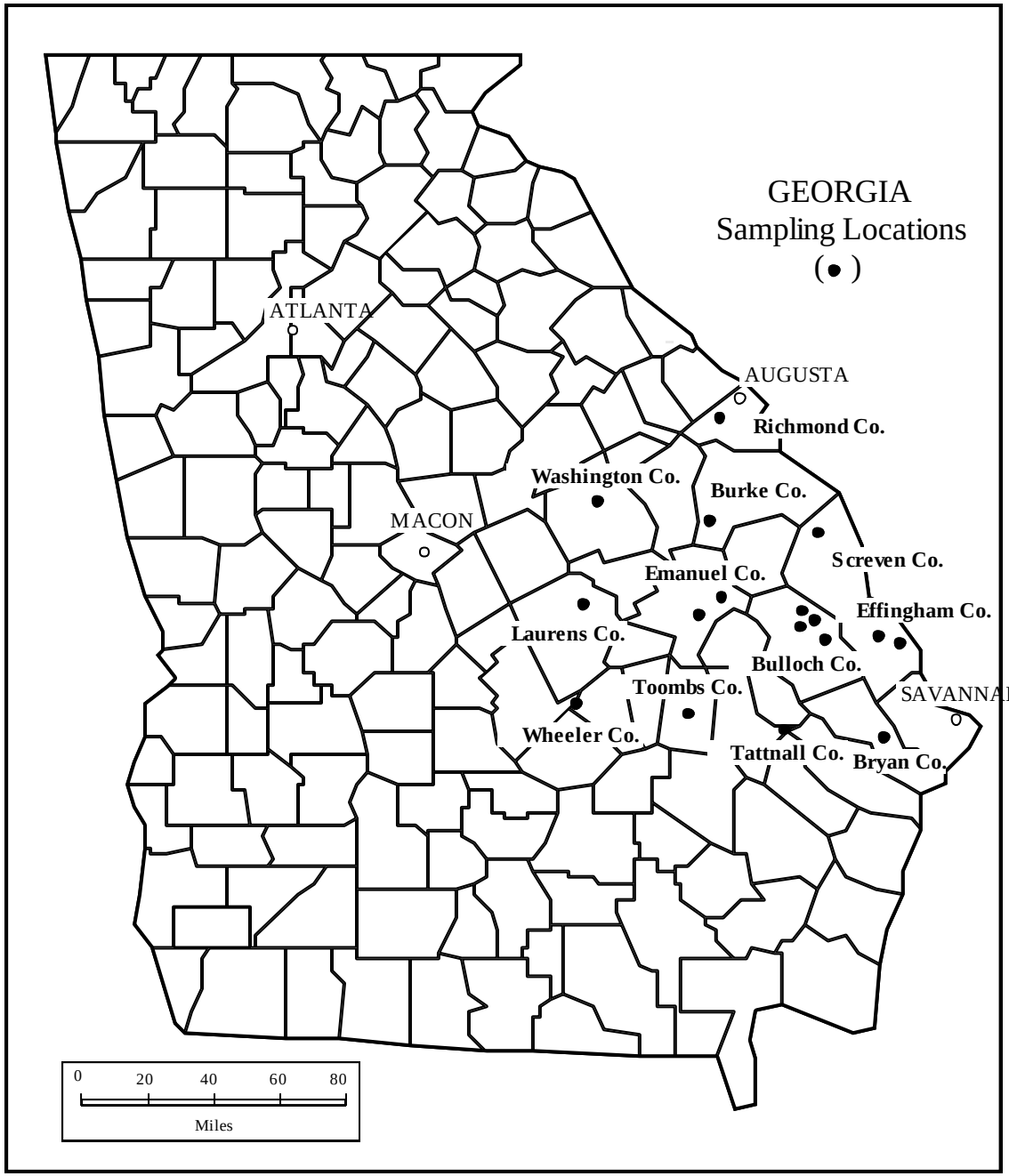

GEORGIA
Sampling Locations
(●)
ATLANTA
AUGUSTA
Richmond Co.
Washington Co.
Burke Co.
MACON
Screven Co.
Emanuel Co.
Effingham Co.
Laurens Co.
Bulloch Co.
Toombs Co.
SAVANNAH
Wheeler Co.
Tattnall Co.
Bryan Co.
0
20
40
60
80
Miles

Table 2: Light trap collections in southeastern Georgia, 1997-1998.

Location	N	Total Collected	No. (%) ***Culex erraticus***	Most Abundant Species	Rank of ***Culex erraticus***
Mosquito Survey, Flood Recovery Program, Georgia Division of Public Health (August-September, 1997)					
Bryan Co.	4	249	5 (2)	*Ae. atlanticus*	T5 of 10
Bulloch Co.	4	417	4 (1)	*Ae. vexans*	9 of 15
Burke Co.	4	243	3 (1)	*Ae. vexans*	4 of 12
Effingham Co.	4	289	81 (28)	***Cx. erraticus***	**1 of 13**
Emanuel Co.	4	121	1 (1)	*Cx. nigripalpus*	T8 of 10
Laurens Co.	4	736	182 (25)	***Cx. erraticus***	**1 of 11**
Richmond Co.	4	43	18 (42)	***Cx. erraticus***	**1 of 7**
Screven Co.	4	229	16 (7)	*Ae. vexans*	4 of 13
Tattnall Co.	4	66	4 (6)	*Cx. nigripalpus*	T5 of 13
Toombs Co.	4	92	2 (2)	*Cx. nigripalpus*	T4 of 9
Washington Co.	4	123	47 (38)	***Cx. erraticus***	**1 of 11**
Wheeler Co.	4	69	27 (39)	***Cx. erraticus***	**1 of 10**
Overall	**48**	**2677**	**390 (15)**	***Cx. erraticus***	**1 of 21**
Georgia Southern University Campus, Bulloch Co. (April-December 1998):					
Herty Preserve	92	1008	5 (0.5)	*An. crucians*	T12 of 23
Raptor Center	63	618	15 (2)	*Ae. vexans*	9 of 18

Table 3: Collections of resting mosquitoes in southeastern Georgia, 1996-1998.

Location	N	Total Collected	No. (%) ***Cx. erraticus***	Most Abundant Species	Rank of ***Cx. erraticus***
Mosquito Survey, Flood Recovery Program, Georgia Division of Public Health (August-September, 1997 from beneath bridges or in culverts within 5 km of light trap sites):					
Bryan Co.	4	23	2 (10)	*Ae. atlanticus*	3 of 3
Bulloch Co.	4	41	28 (68)	***Cx. erraticus***	**1 of 3**
Burke Co.	4	84	1 (1)	*Ps. ferox*	T5 of 9
Effingham Co.	5	119	28 (24)	*Cx. quinquefasciatus*	2 of 4
Emanuel Co.	10	23	8 (35)	***Cx. erraticus***	**1 of 7**
Laurens Co.	4	128	37 (29)	*An. quadrimaculatus*	2 of 6
Richmond Co.	4	3	1 (33)	***Cx. erraticus***	**T1 of 3**
Screven Co.	4	40	1 (2)	*An. quadrimaculatus*	T4 of 6
Tattnall Co.	4	24	18 (75)	***Cx. erraticus***	**1 of 5**
Toombs Co.	4	10	5 (50)	***Cx. erraticus***	**1 of 4**
Washington Co.	8	44	25 (57)	***Cx. erraticus***	**1 of 10**
Wheeler Co.	4	21	15 (71)	***Cx. erraticus***	**1 of 3**
Overall	**59**	**560**	**169 (30)**	***Cx. erraticus***	**1 of 14**
George L. Smith State Park, gopher tortoise burrows, Emanuel Co. (August 1995-July 1996, April-June 1998):					
	~900	223	160 (72)	***Cx. erraticus***	**1 of 7**
Undersides of bridges in Bulloch and Effingham Co., (June 1996-April 1998):					
	~380	8630	4928 (57)	***Cx. erraticus***	**1 of 16**

Mosquitoes collected from beneath bridges in Bulloch Co., GA, 1995-1998.

Mosquito species	Totals (%)	No. (%) Bloodfed	No. (%) Unfed	No. (%) Gravid
Culex erraticus	2673 (68.4)	166 (6.2)	1793 (61.7)	714 (26.7)
Anopheles quadrimaculatus	1091 (27.9)	160 (14.7)	802 (73.5)	129 (11.8)
Others	142 (4.5)			

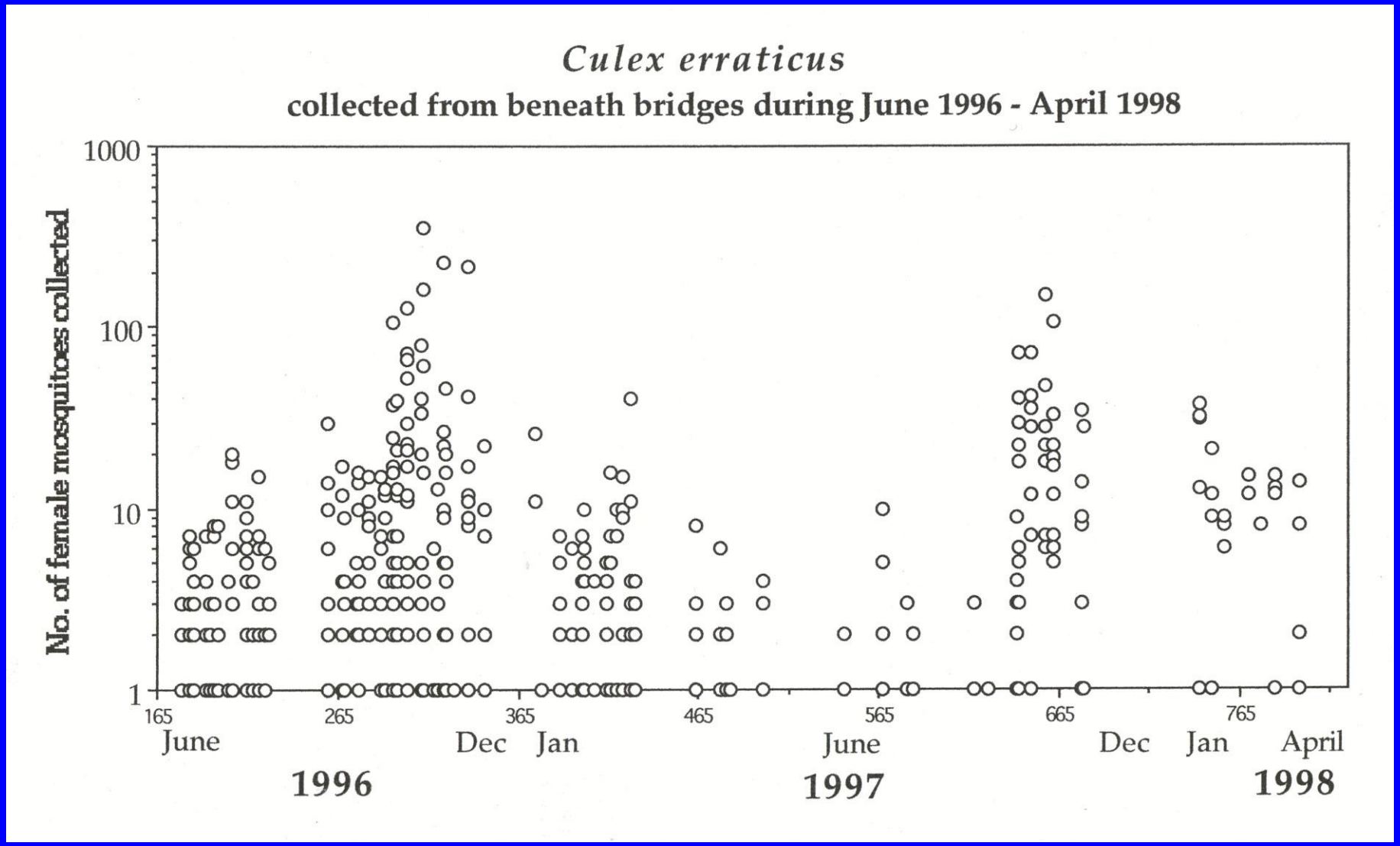
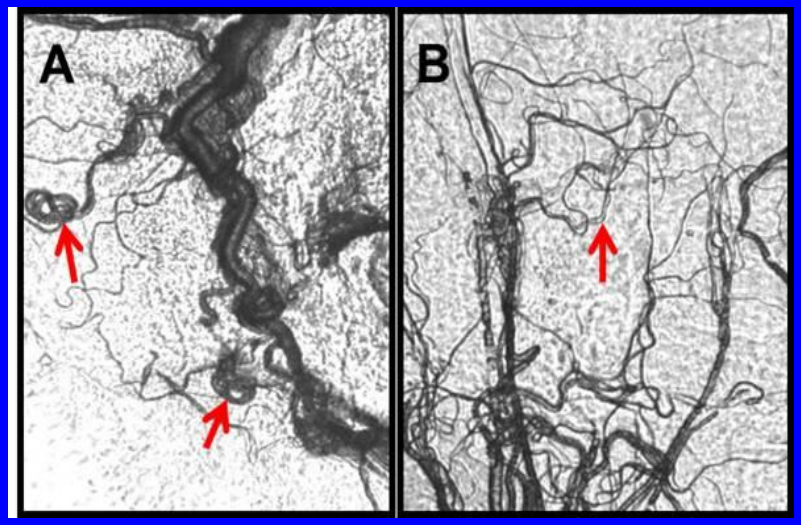

Culex erraticus
collected from beneath bridges during June 1996 - April 1998
No. of female mosquitoes collected
1000
100
10
1
165
265
365
465
565
665
765
June
Dec
Jan
June
Dec
Jan
April
1996
1997
1998

Table 1: Hosts of *Culex erraticus* in North Carolina and Georgia.

Location	Blood Meals Identified	Mammals	Birds	Amphibians/ Reptiles
Kenansville, NC	13	5 (38%)	4 (31%)	4 (31%)
Raleigh, NC	225	111 (49%)	69 (31%)	45 (20%)
Bulloch & Emanuel Co., GA	119	29 (24%)	66 (56%)	24 (20%)

- Mammal blood meal sources included deer (16), dog (5), raccoon (3), cow (2), rodents (2), cat (1), and opossum (1).
- Amphibian blood meal sources included frog (3), and toad (1).
- Reptile blood meal sources included snake (16), turtle (2), and lizard (1).

Nulliparous ovary: tracheolar skeins



Parous ovary

Could virus be overwintering in adult *Culex erraticus*?

- Parity of 250 adult females collected in October-December determined
- All ***nulliparous***

→Overwintering population is inseminated females who have *never taken a blood meal*!

Could a long – lived reptile:

- Serve as an over-wintering host for EEEV?

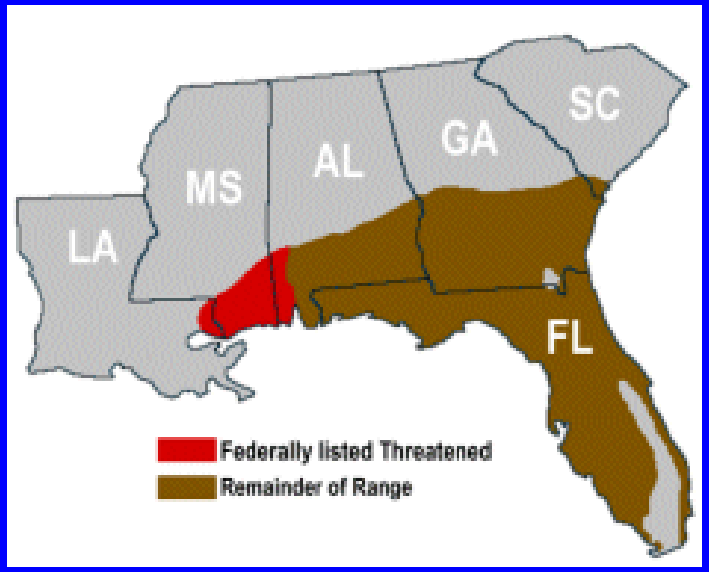
- Serve as a long term maintenance (5-7 years) host for EEEV?

And:

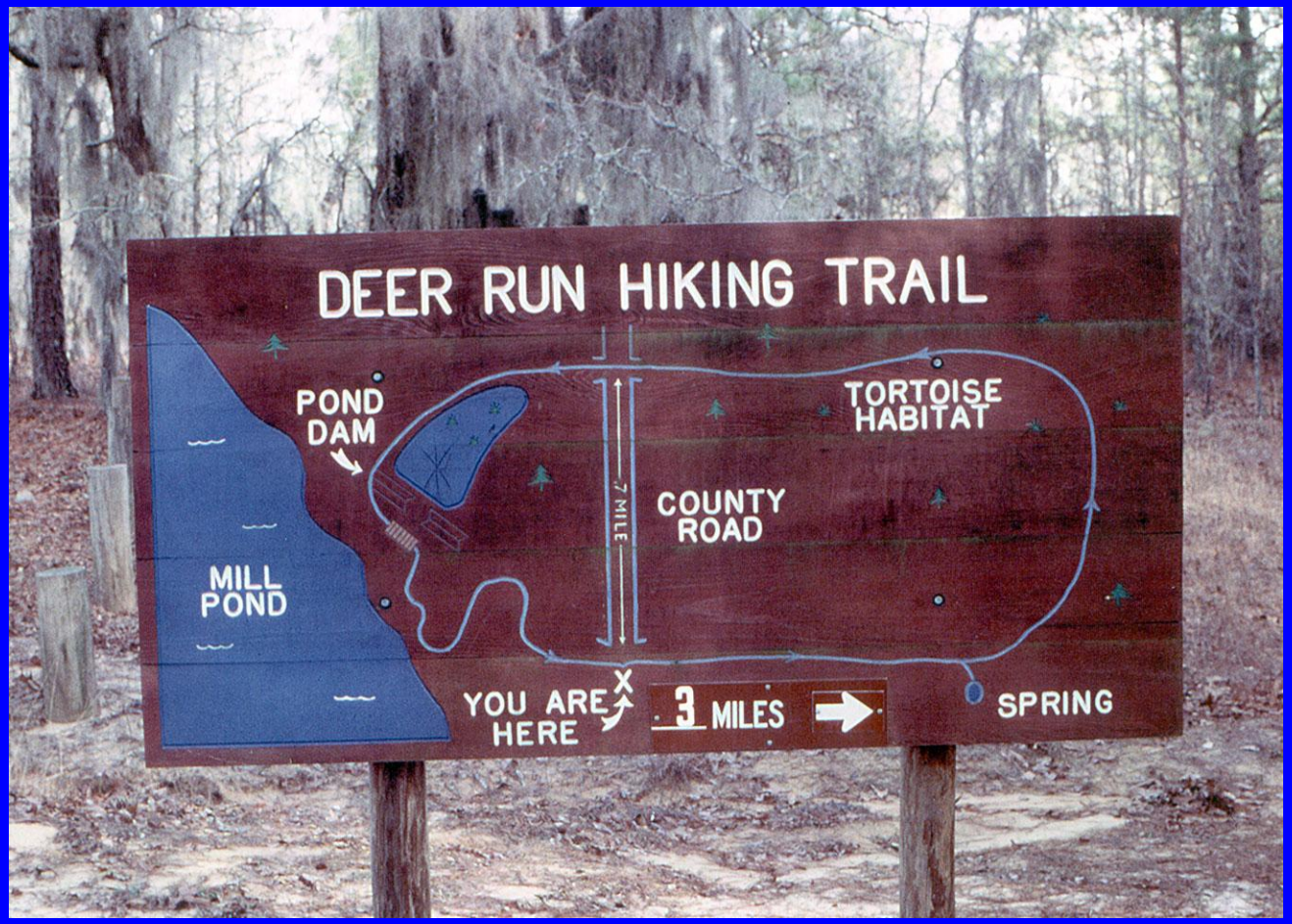
- Does *Culex erraticus* act as a bridge vector from this population?

The gopher tortoise,

Gopherus polyphemus



Gopher tortoise (and mosquito) habitat at G.L. Smith State Park, Emanuel Co., Georgia



"The burrows are just full of mosquitoes!"
-GSU Herpetologist

Burrows of the gopher tortoise (*Gopherus polyphemus*) are prominent landscape features in sand hill habitats

Mosquitoes Associated with Burrows of the Gopher Tortoise at G.L. Smith State Park, Emanuel Co., GA

Mosquito Species	1995-1996 No. (%)	1999-2000 No. (%)	Totals (%)
Anopheles crucians	30 (15)	25 (6)	55 (9)
An. punctipennis	1 (<1)	0	1 (<1)
An. quadrimaculatus	8 (4)	5 (1)	13 (2)
Culex erraticus	160 (78)	367 (90)	527 (86)
Cx. nigripalpus	0	3 (<1)	3 (<1)
Cx. quinquefasciatus	3 (1)	3 (<1)	6 (1)
Cx. salinarius	2 (1)	0	2 (<1)
Uranotaenia sapphirina	2 (1)	0	2 (<1)
Male mosquitoes	17	88	105
Total	223	496	719

Fungus gnats (Diptera: Mycetophilidae) from gopher tortoise burrows

Exechiopsis sp.

Rymosia sp.

Hosts of *Culex erraticus* at G.L. Smith State Park, Emanuel Co., GA (McCoy, 2000)

Host	No. (%) of Bloodmeals
Mouse	29 (37)
Squirrel	12 (15)
Deer	1 (1)
Bird	3 (4)
Toad	4 (5)
Turtle	17 (22)
Lizard	12 (15)

Culex erraticus in Southeastern Georgia

- *Most abundant mosquito* collected in 12 county study on mosquito diversity and distribution
- *Most abundant mosquito* collected in artificial resting shelters (bridges)
- *Most abundant mosquito* associated with burrows of the gopher tortoise
- *Unusually opportunistic* feeding behavior
- Abundance and feeding behavior (and virus detection) suggest *important role* in EEEV ecology

  – *Further studies indicated*

Gopher tortoises, parasite load & EEEV at Ft. Stewart, GA 2000-2002

Objectives

- Measure the seropositivity of gopher tortoises for EEEV in areas where EEEV is endemic
- Determine the presence of blood-borne parasites
- Measure ectoparasite load
- Measure the intestinal parasitic load of fecal samples obtained
- Determine the potential correlation between parasite load and seropositivity

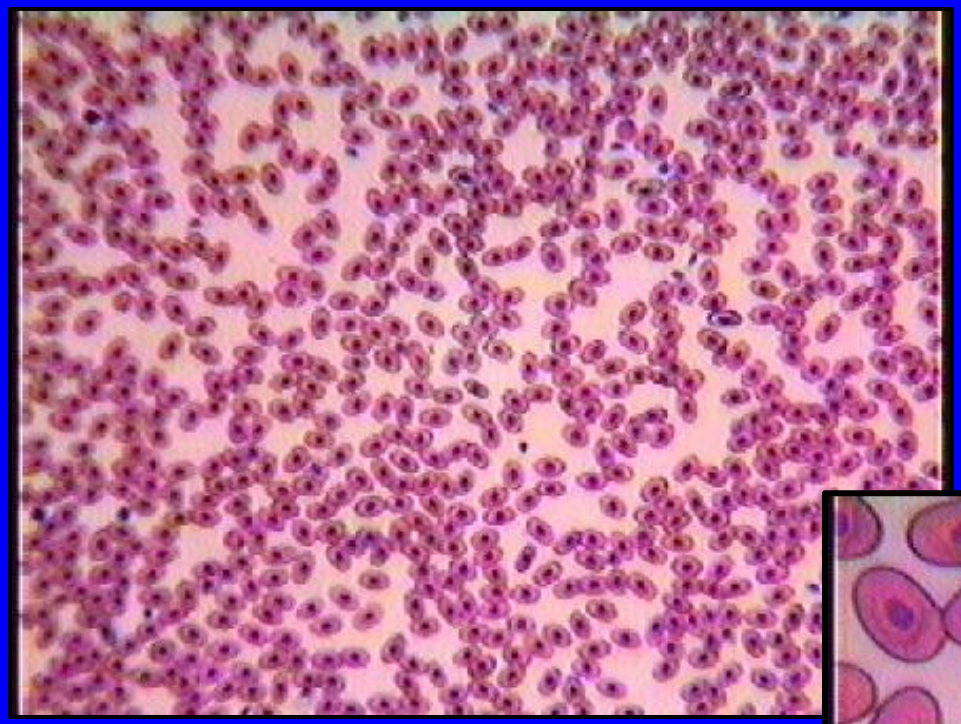
Materials and Methods

- Gopher tortoise sampling (bucket trapping) at Fort Stewart
- Blood sampling for:
  - Antibodies to EEEV
    - Protein LA Indirect ELISA
  - Blood-borne parasites
    - Thin smears with Wright-Giemsa stain
- Tick load
- Intestinal parasite load
  - Quantitative fecal floats (eggs/gram of feces)

Protein LA assay for EEEV

- Indirect ELISA with AP-conjugated protein LA
- Negative controls:
  - normal mouse brain + tortoise test serum
  - EEEV (mouse brain) antigen
    + control tortoise sera* (captive bred tortoises)
  - normal mouse brain + anti-EEEV mouse ascites fluid
  - no antigen + mouse ascites fluid (Mab)
- Positive control
  - EEEV (mouse brain) antigen + mouse ascites fluid
- Test sera: tortoise serum samples at serial 2 fold dilutions starting at 1:100

Indirect ELISA with alkaline phosphatase-conjugated Protein LA



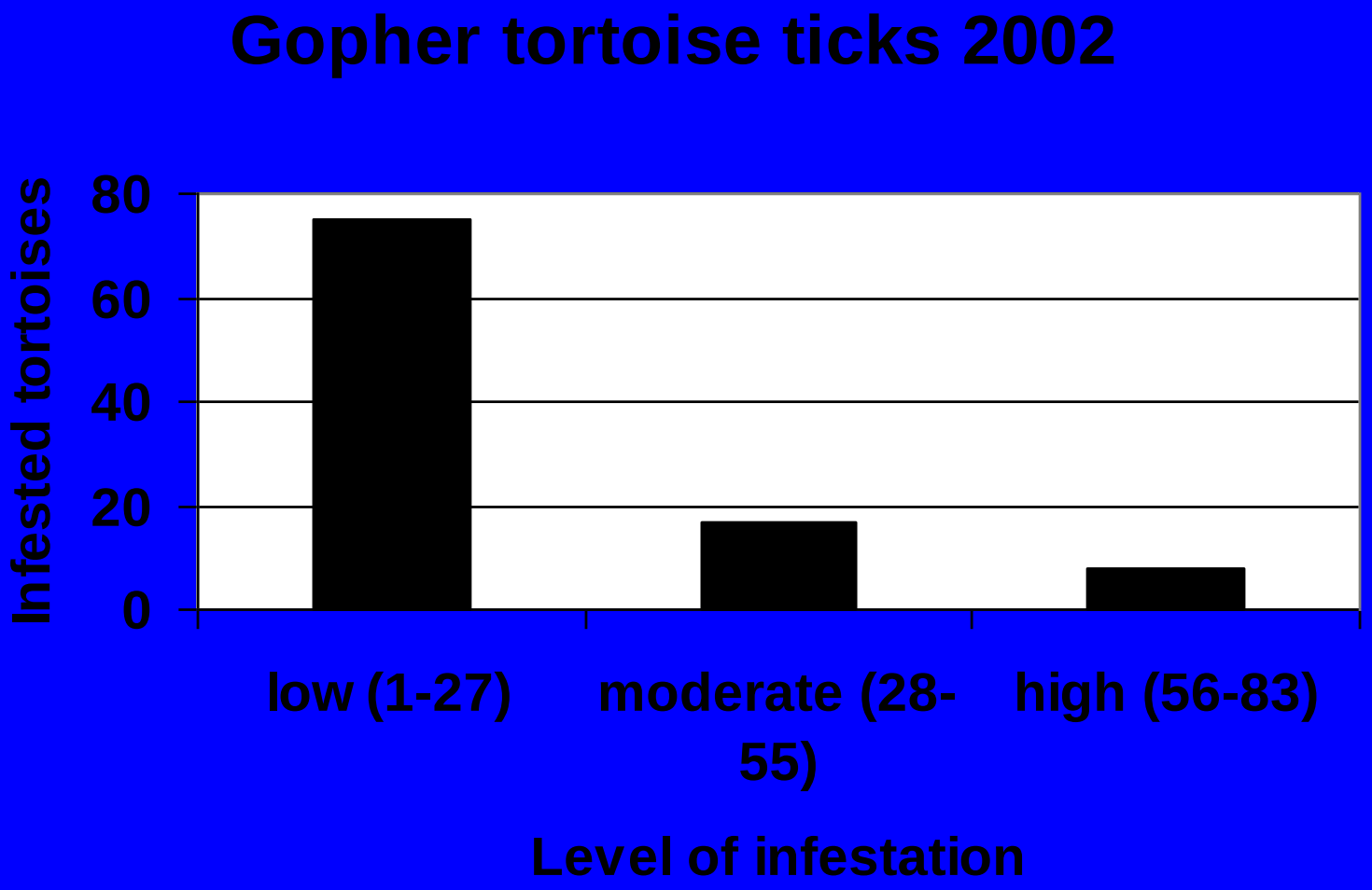
Blood smear (10X)

Blood smear (40X)


Kevin Stohlgren ©2010

Tick Load

- 100 tortoises with 1972 ticks
  (*Amblyomma tuberculatum*)
- 253 male ticks (244 unfed, 9 fed):
  → 2.53/tortoise
- 69 female ticks (52 unfed, 17 fed):
  → 0.69/tortoise
- *1650 nymphs (967 unfed, 683 fed):*
  → 16.5/tortoise



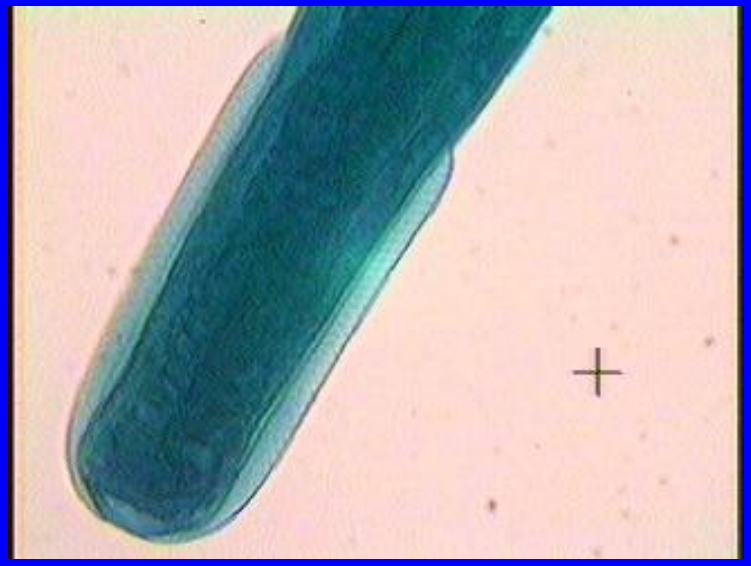
The number of gopher tortoises (*Gopherus polyphemus*) from Fort Stewart Army Reservation infested with the gopher tortoise tick (*Amblyomma tuberculatum*) during the 2002 trapping season.

Intestinal Parasite Load

- Nematode eggs:
  - 93.6-156 μm in length and 52-93.6 μm in width
  - *Found in all tortoises!*
- *Trichostrongylidae*
- Undescribed: *Trichoskrjabinia* n. sp.?
- One road-kill tortoise posted:
  - Adult nematodes found

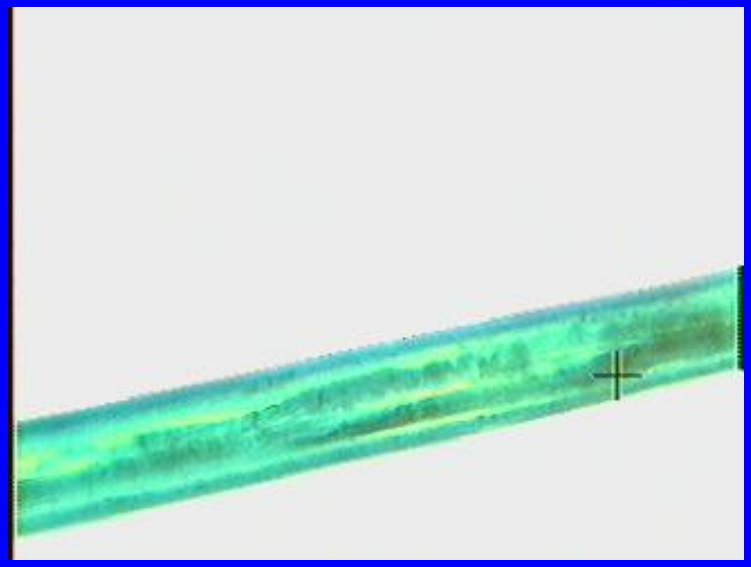
Nematode eggs (10X)

Nematode egg (40X)

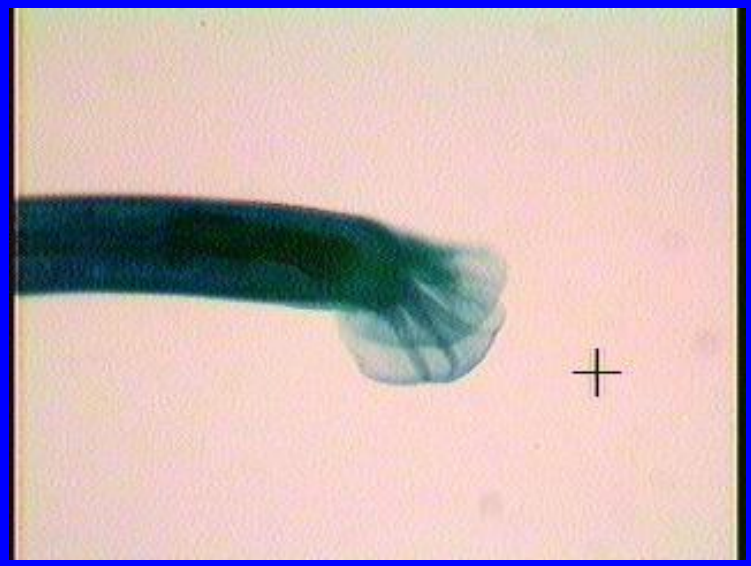


head (with alae)

esophageal bulb

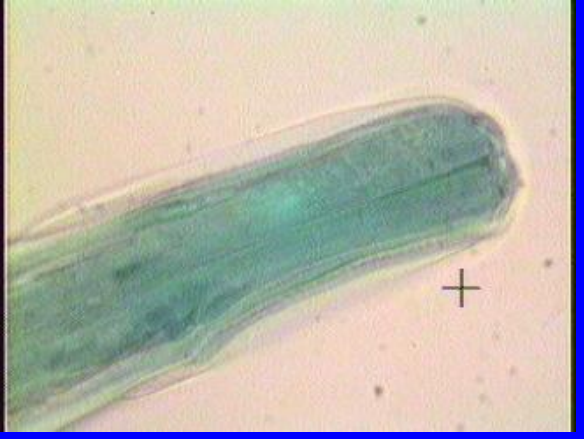


testes

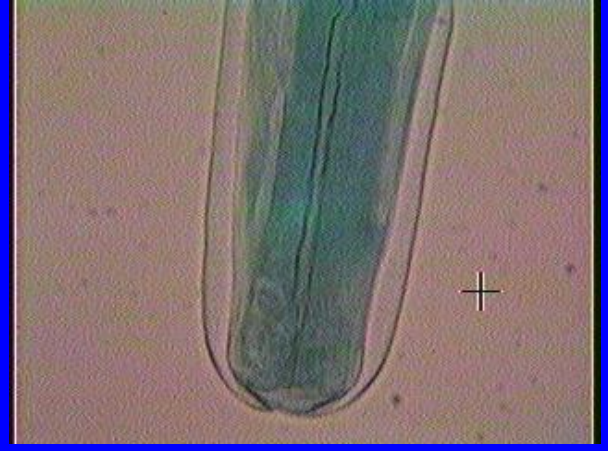


bursa

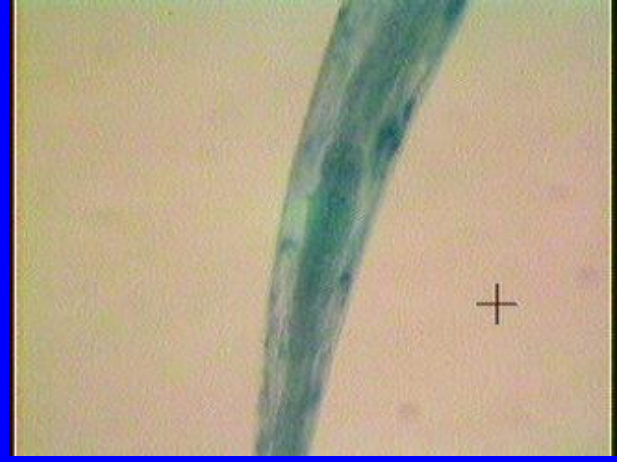
male nematode



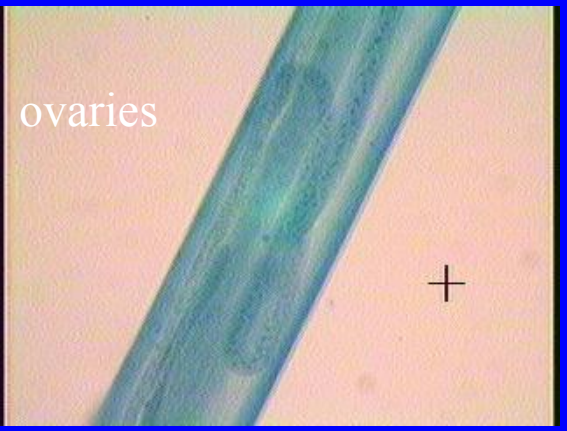
head

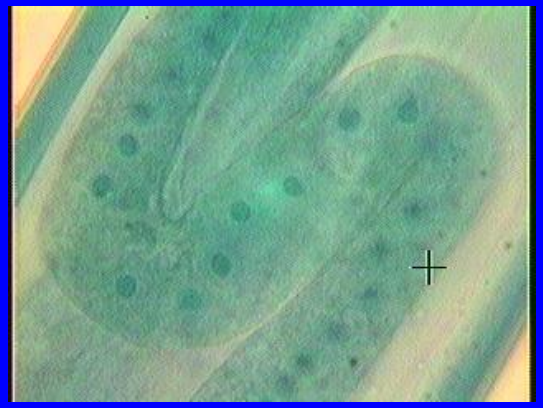


head

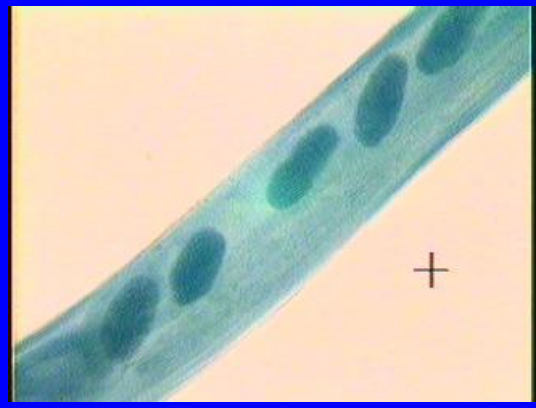


esophageal bulb





ovaries

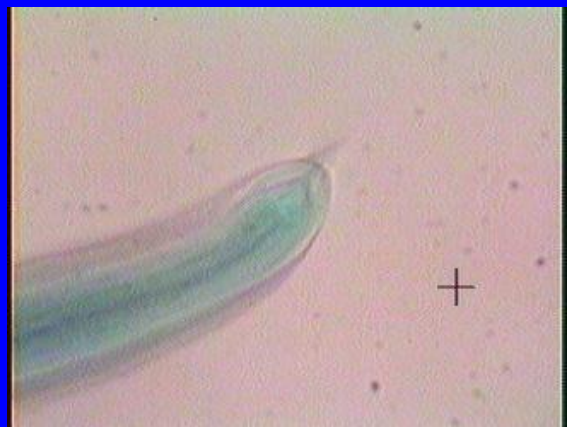


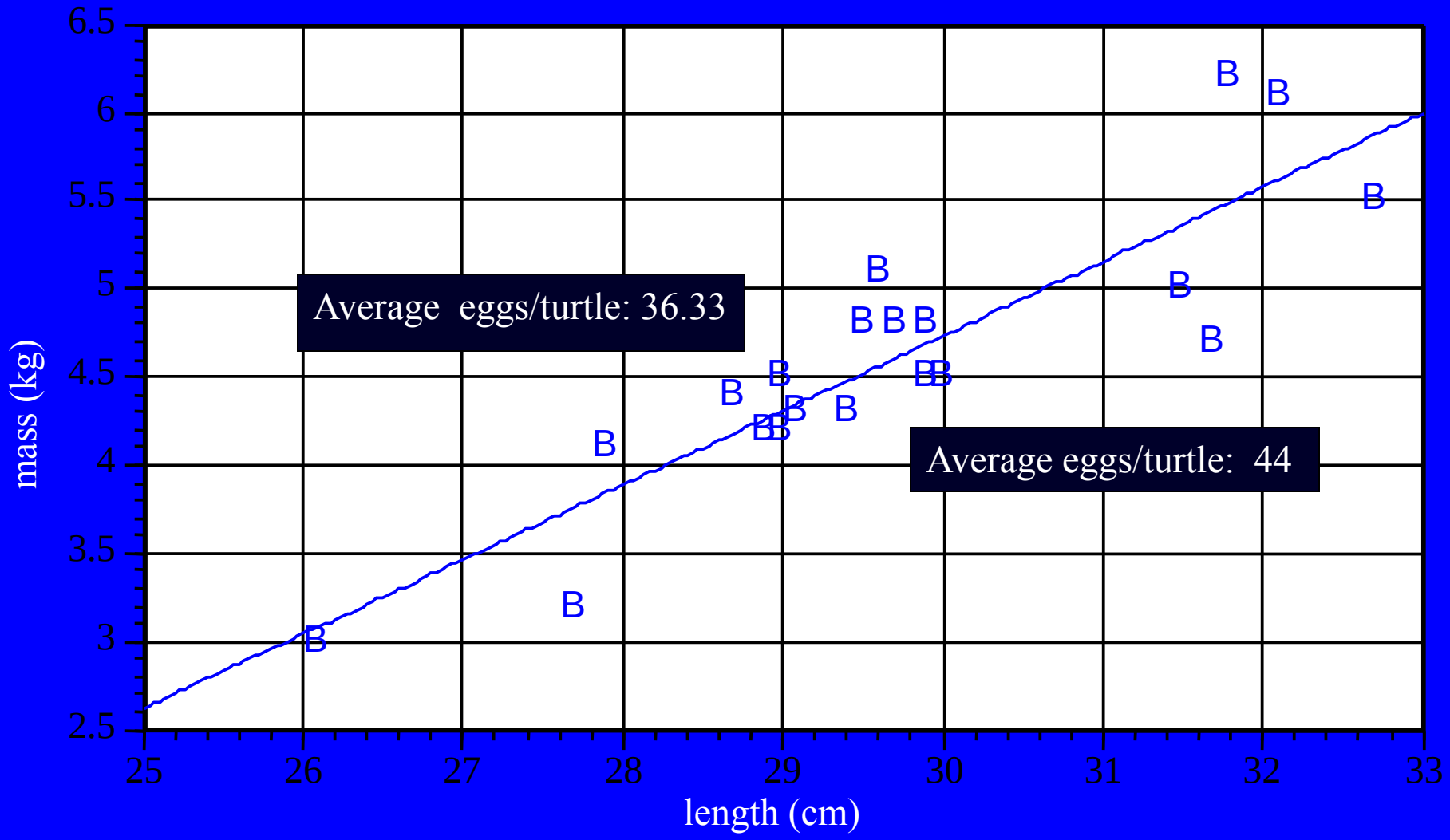
Eggs in utero

female nematode

genital pore

bursa





Regression of mass to straight carapace length of gopher tortoises (*Gopherus polyphemus*) from Fort Stewart Army Reservation in 2002.

→ "Healthier" turtles have fewer nematode eggs (intestinal parasites).

Summary of data collected for viral exposure and parasite infestation of the gopher tortoise.

	Number of samples	Range	Prevalence
Tortoise plasma (EEEV)	152 (2001:50, 2002:102)	n/a	0%
Intestinal parasite load (nematode eggs)	55 (2001:22, 2002:33)	1-230 (2001:2-137, 2002:1-230)	100%
Blood-borne parasite load	101	n/a	0%
Tick load	119	1-80	84.03%

Georgia's State Reptile is not implicated in EEEV ecology!

But another candidate *is* identified:

Cupp, E.W., Zhang, D., Yue, X., Cupp, M.S., Guyer, C., Korves,T., Unnasch, T.R., 2005. Identification of reptilian and amphibian blood meals from mosquitoes in an eastern equine encephalomyelitis virus focus in central Alabama. Am J Trop Med Hyg 71: 272–276.

→ *Culex erraticus*, *Culex peccator*, and *Uranotaenia sapphirina* positive for EEEV in study in Tuskegee National Forest

→ Blood meal IDs show Water Moccasin (*Agkistrodon piscivorus*) is commonly fed on by these mosquitoes, and only common denominator

→Is this long-lived snake an over-wintering reservoir for EEEV?

- mosquito collecting and snake hunting conducted at EEE positive sites in Southeast Georgia during 2010-2011


Water moccasins are well camouflaged!
Eastern Cottonmouth
Agkistrodon piscivorus piscivorus
snakesandfrogs.com

Sampling to date, 2010-11:

- EEE sites in Bulloch, Screven, Jenkins, Emanuel, Candler, Evans, Long, Bryan, Effingham, Tattnall and Chatham Counties
- Over 50 aspirator samples taken from different bridges
- Over 400 hours "hunting" for snakes – no other trapping method

- *Snakes are hard to find when you're looking for them!*

- Snakes encountered/collected:
  - 3 water moccasins
  - 2 brown water snakes
  - 3 banded water snakes
  - 2 red-bellied water snakes
  - 1 eastern king snake
  - 4 black racers
  - 1 yellow rat snake

Jacob Gregory & James Fercilien, sampling near Swainsboro, 2010

Sampling sites in Bulloch & Jenkins County

A red-bellied water snake in Jenkins County

Blood samples are taken from the caudal vein of an intubated snake

About to capture our only water moccasin

Kelly Dabney and Cam Hayes sampling on the Canoochee, Summer 2011

Representative aspirator collections (% of total collected) near EEE sites in Southeastern Georgia during 2010 by county of collection

Species	Bulloch	Chatham	Effingham	Emanuel	Long	Tattnall	Wayne
Cx. erraticus	***62***	***66***	***76***	***20***	***58***	***20***	***75***
An. quadrimaculatus	37		12	20	25	40	18
An. barberi	<1			5			
Ps. ferox	<1	33					
Cx. pilosus				10	8		2
An. crucians			4		8		2
Cx. territans				10			
Ps. columbiae						40	
Cs. melanura				5			
Ur. sapphirina			8				
Cq. perturbans							3
Total collected	272	3	25	20	12	5	60

- *Culex erraticus* is the only species common to all sites
- Mosquitoes are scarce during a drought!

A study in progress:

- Sampling will continue this year and next.

- Blood-fed *Cx. erraticus* will have blood meals identified using sequencing of amplicons of the mitochondrial *cyt b* gene.

- Assay for antibodies to EEEV will be developed using a captive-bred water moccasin for negative control sera.

- Any help locating snakes would be appreciated!