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Are Brown Bullhead (*Ameiurus nebulosus*) in the Cornwall Area of Concern Exposed to Environmental Estrogens?

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Waters**

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Outline

- Description of Cornwall AOC
- Vitellogenin
- Trial Methods
- Results
- Conclusions



Area of Concern (AOC) Rationale

- “AOCs are locations where environmental quality has been degraded compared to other areas in the Great Lakes and beneficial uses of the aquatic ecosystem are impaired.” - www.ec.gc.ca
- 42 areas met this criteria, 14 of which are in Canada
- Remedial action plans (RAPs) put in place for each area
- Goal- De-list all AOCs after site remediation where appropriate
 - List “Areas in Recovery” where all appropriate measures taken but beneficial use impairments (BUIs) remain



Areas of Concern



Cornwall AOC

- Cornwall AOC listed because of: mercury, PCBs and other contaminants in fish, sediment and water, habitat destruction, bacteria causing beach closures, excessive growth of nuisance aquatic plants, exotic species and wild fish health impacts
- Chemical inputs from the city: municipal wastewater, Al-Can refinery, pulp mill (now closed)
- We divided the Cornwall AOC into three zones- Morrisburg (upstream), International Bridge (exposed) and Gray's Creek (downstream)
- Captured wild fish in each zone- **brown bullhead**, a bottom dwelling fish, and yellow perch, water column fish, by electrofishing



AOC Cornwall- Sites

Electrofishing boat



Cornwall Bridge- Exposed Zone



Gray's Creek- Downstream Zone



Morrisburg- Upstream (Ref) Zone



AOC Fish Health Surveys

- Assess adult fish health: growth, reproduction, and sub-lethal health measures
- Sample 20 adult male and 20 adult female brown bullhead for:
 - Plasma- **Vitellogenin (Vtg)**, T3/T4 hormones, steroid hormones, contaminants of emerging interest and
 - Gill, head kidney, thyroid for histology
 - Liver- EROD analysis, deiodinase, histology and steroid receptor binding chemicals
 - Fin ray- aging
 - Gonad- histology, in vitro assay of steroid hormone production in female gonad, and inter-sex condition
 - Muscle and carcass- contaminant analysis



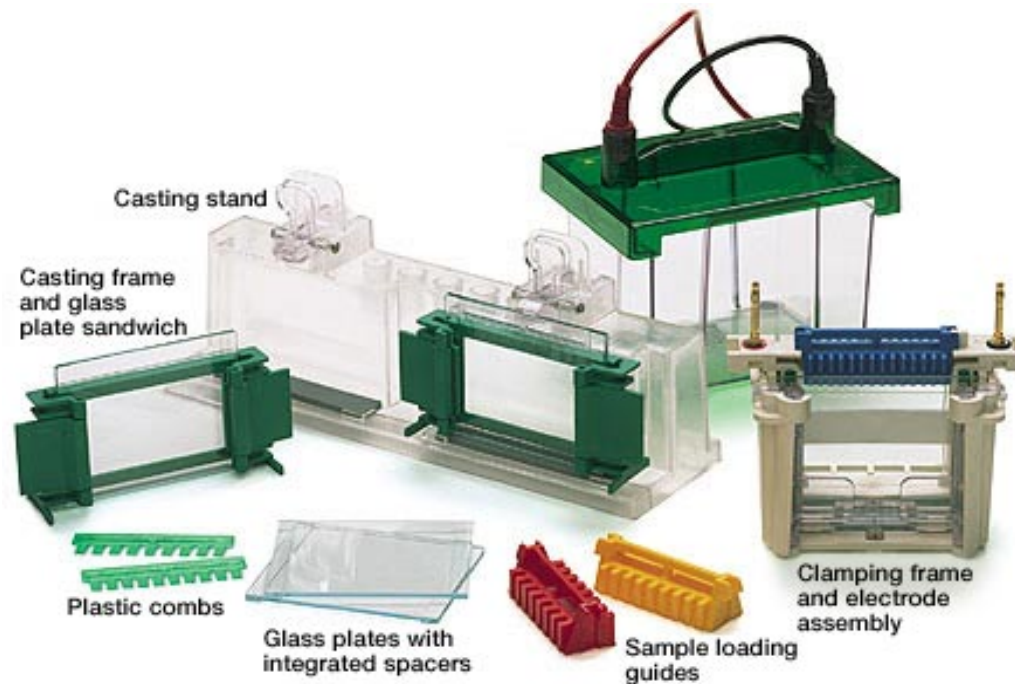
Vitellogenin (Vtg)

- Egg yolk precursor protein
- Produced in the liver, sequestered in the ovaries
- Normally not present in male plasma- production induced by estradiol
- Environmental estrogens:
 - From various sources
 - Can induce weak Vtg production in males
 - Can have anti-estrogenic effects in females, block estrogen receptor



Plasma Analysis- SDS PAGE

- Sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE)
- Separate proteins based on size



SDS-PAGE Continued

- Use 5% acrylamide gel- large pore size, better separation of large proteins (Vtg 200kDa)
- Mix samples with sample buffer- contains SDS, a detergent with negative charge and disrupts cell membranes, and β -mercaptoethanol, helps to linearize proteins
- Voltage constant 20mAmps, proteins move toward positive electrode, can track bromophenol blue dye front to bottom of gel
- Remove gel from glass plates, and stain with silver

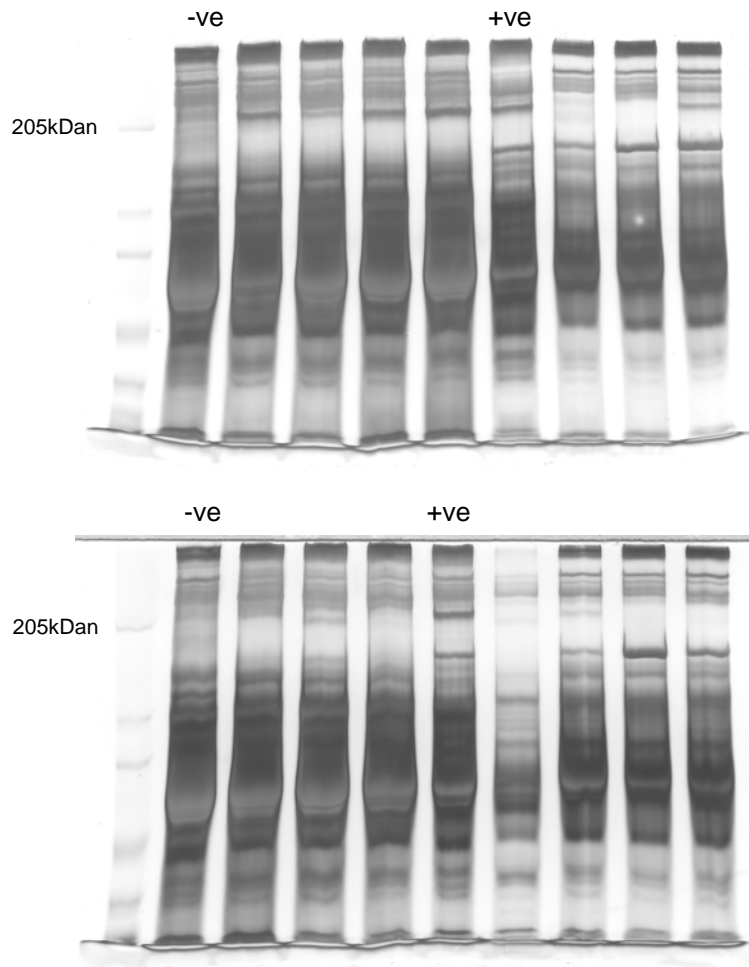


Plasma Analysis- Silver Stain

- Sensitive, but general protein staining technique- detection limit of 0.5ng of protein
- Three main components:
 - Thiosulfate- binds with strong affinity to proteins
 - Silver nitrate- binds anywhere that thiosulfate is bound
 - Sodium carbonate- acts as a reducing agent, precipitate out the silver to create the colour
- Over exposure in thiosulfate leads to high background- takes care to time correctly
- Developing of the gel in sodium carbonate happens rapidly, take care not to overstain



Cornwall AOC Plasma Samples

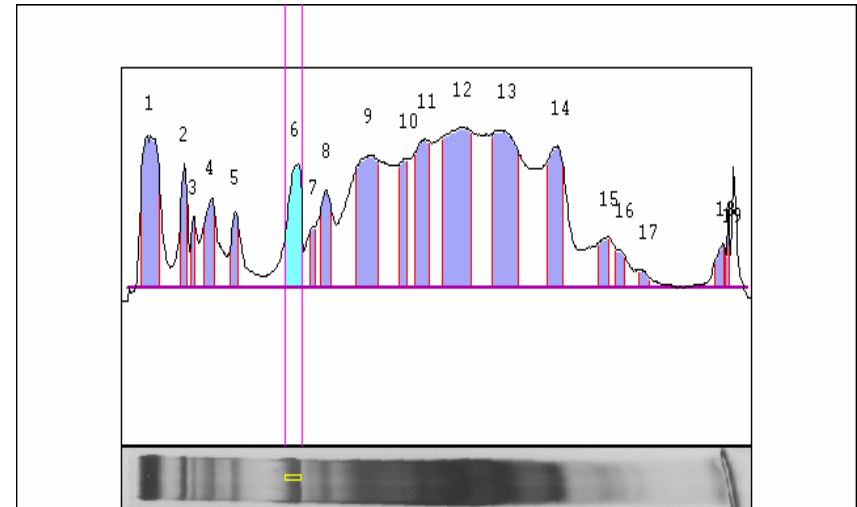


- Each gel contains a molecular weight marker, controls positive (+ve) and negative (-ve)
- All unknown plasma samples are compared to those controls to determine presence of absence Vtg
- All samples with a band matching the size of Vtg are considered to be positive, as it is assumed that band is Vtg because it is female specific protein, confirmed by western blotting in select samples



Vitellogenin Analysis

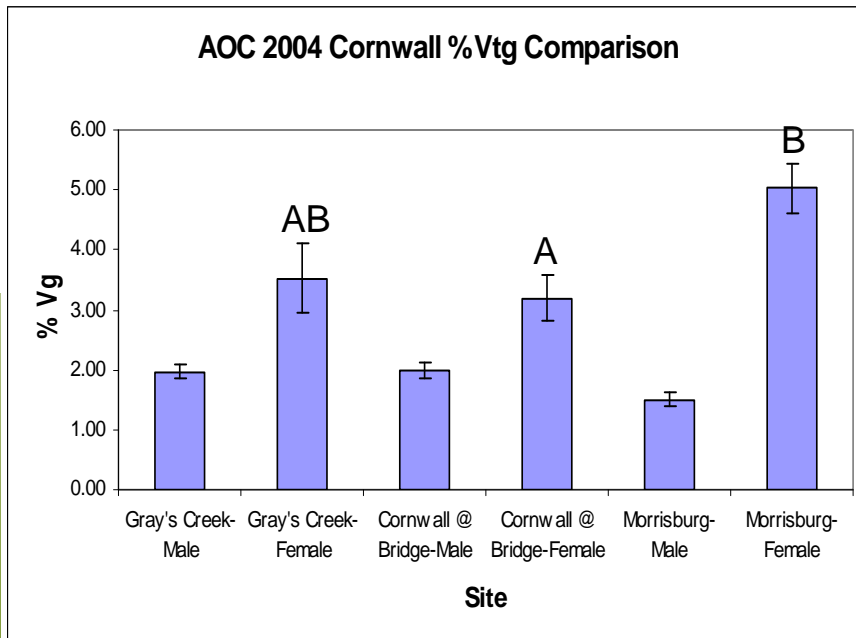
Peak	Area	% Prot.	Band	Pos.	Mol. Wt.	Rf
1	2516	9.7				
2	728	2.8				
3	325	1.3				
4	857	3.3				
5	574	2.2				
6	1709	6.6	4	186	175.54	.278
7	328	1.3				
8	899	3.5				
9	2792	10.8				
10	1145	4.4				
11	2084	8				
12	4382	16.9				
13	3848	14.8				
14	2077	8				
15	529	2				
16	338	1.3				
17	177	0.7				
18	363	1.4				
19	287	1.1				



- One lane of a gel from analysis program Fluorchem
- Outline each peak to get the area and % of overall protein of each band
- Highlighted peak 6 is the Vtg band, data in red font
- Compare the %Vtg across all samples



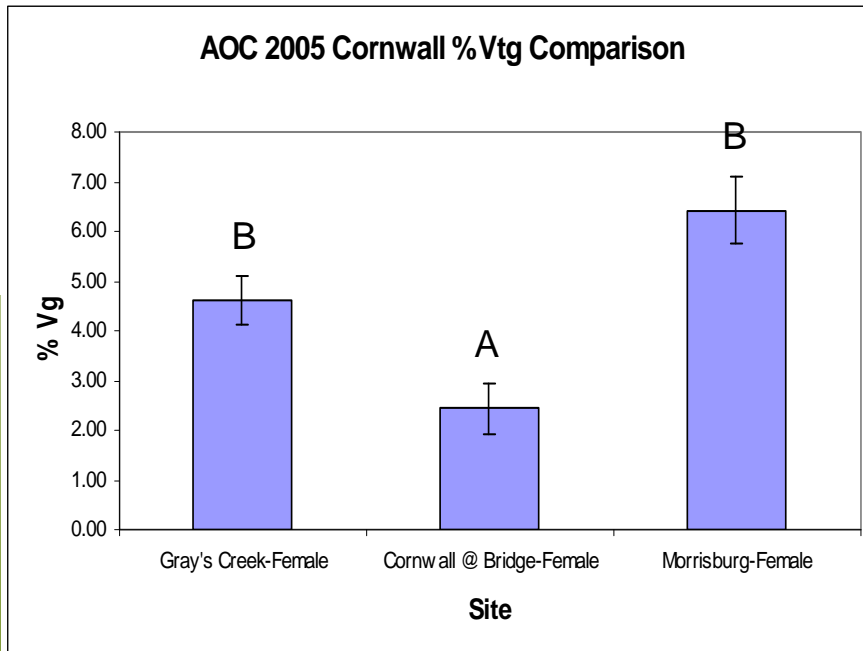
Cornwall 2004 % Vtg Data



- Data is in 2 distinct groups- “A” and “B” which are significantly different from each other ($\alpha=0.05$) for females only
- Bridge zone has lower % Vtg compared to the Morrisburg reference zone in female fish
- There is no statistical comparison between male fish because only 3 individuals were found in each zone- indicates EDC's chemical present



Cornwall 2005 %Vtg Data



- Data in 2 distinct groups “A” and “B” which are significantly different from each other ($\alpha = 0.05$) for females only
- Indicates that Bridge zone has significantly lower % Vtg compared to Morrisburg reference zone and Gray’s Creek downstream zone
- Only one positive male found at the Cornwall Bridge site, none in other zones



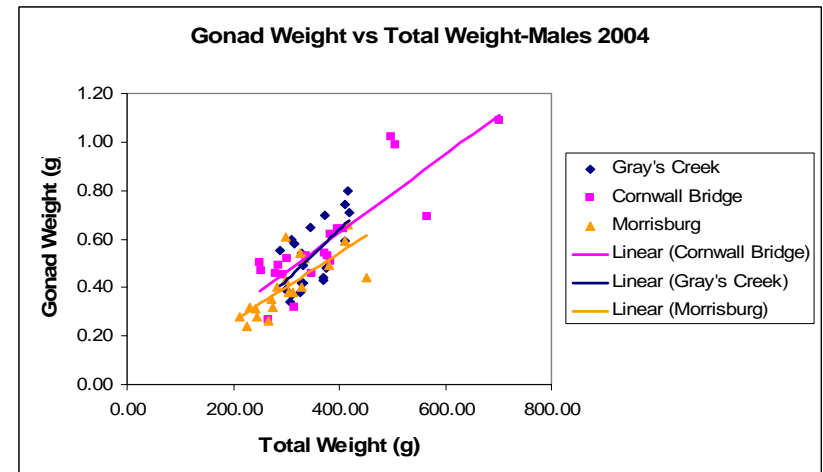
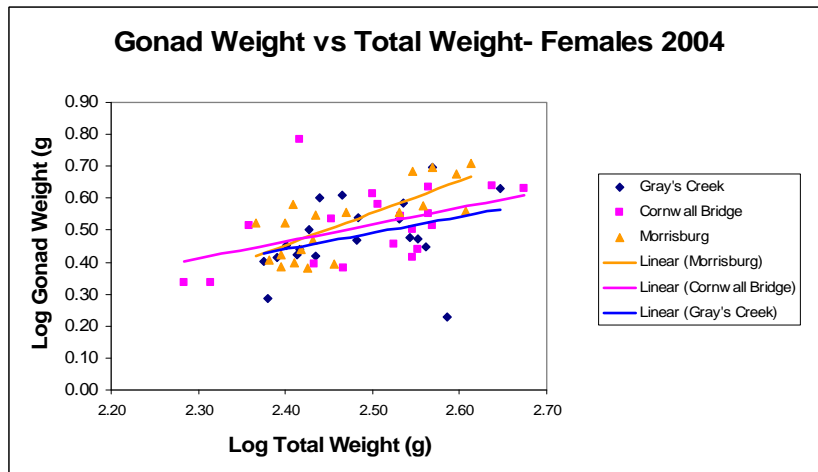
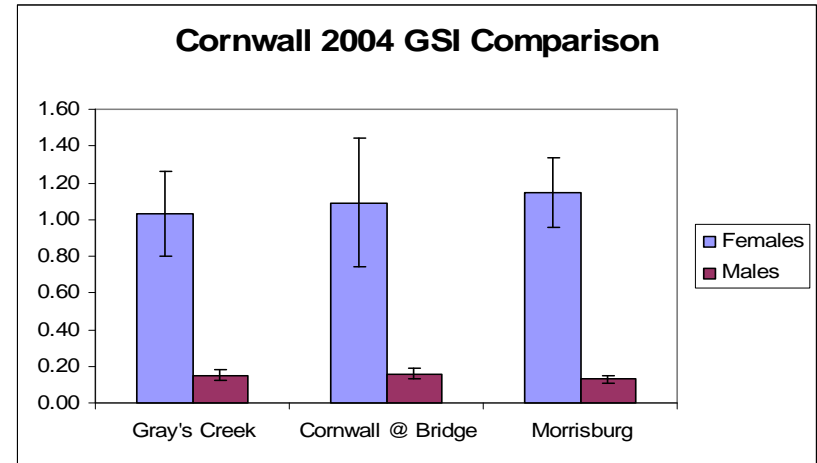
Condition Indices

- Gonad Somatic Index (GSI)- $\text{Gonad weight (g) / Total Weight (g) x 100}$
 - Indicator of amount of energy put into reproduction
- Liver Somatic Index (LSI)- $\text{Liver weight (g) / Total Weight (g) x 100}$
 - Indicator of amount of energy for glycogen storage, also LSI can increase or decrease in response to pollutants
- Condition Factor Index (CFI)- $\text{(Total weight x 10 / Length}^3\text{) x 10}$
 - Indicator of the general health of the fish, eg. undernourished if mass is smaller relative to length



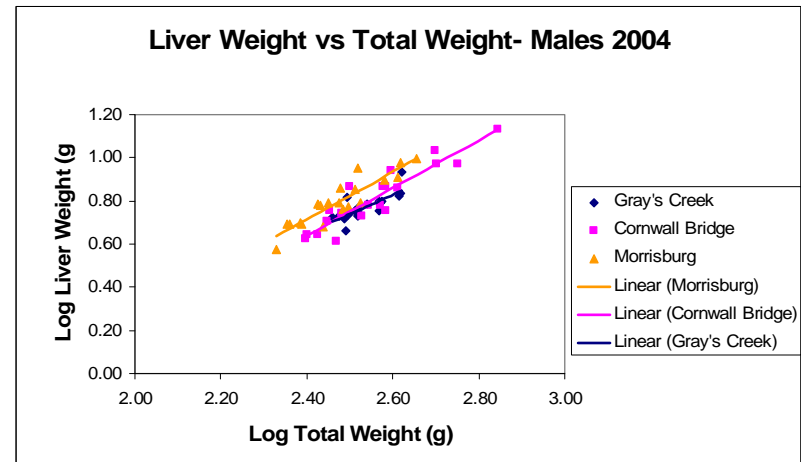
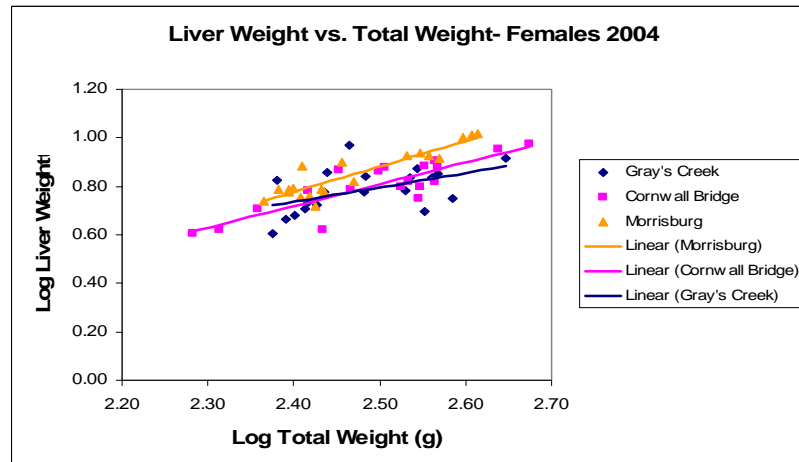
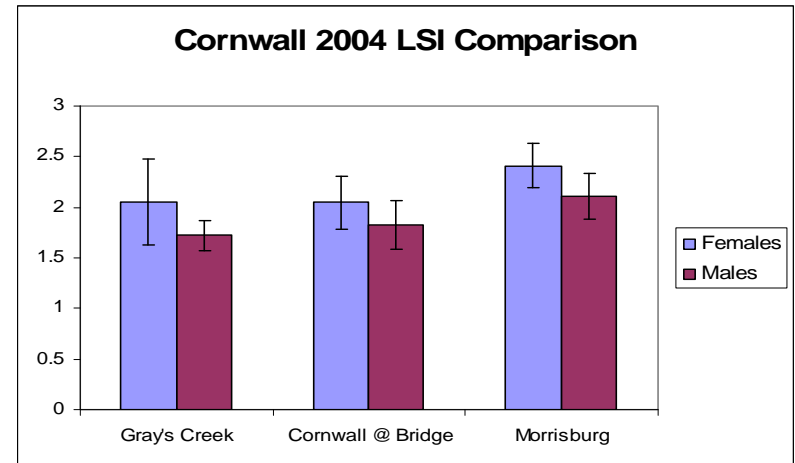
Cornwall 2004 GSI Data

- Data shown in both ratio GSI format and scatter plot format of gonad weight vs. total weight for males and females
- ANCOVA test done to determine significant differences between sites- first look for an interaction between total body weight and site (differences in slope of the regression line)
- If no interaction, can compare the changes in gonad weight while factoring in total weight between site
- Data log transformed for females- no interaction btw site and total weight for males or female; no site effects (Females $P=0.2959$, Males $P=0.1485$)



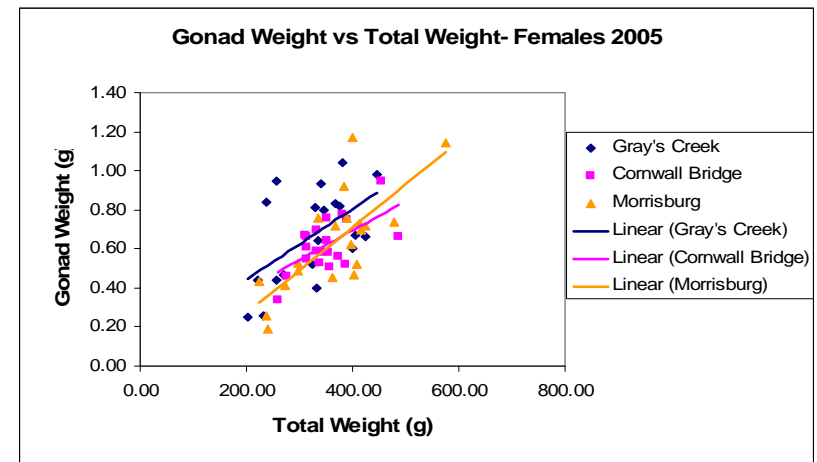
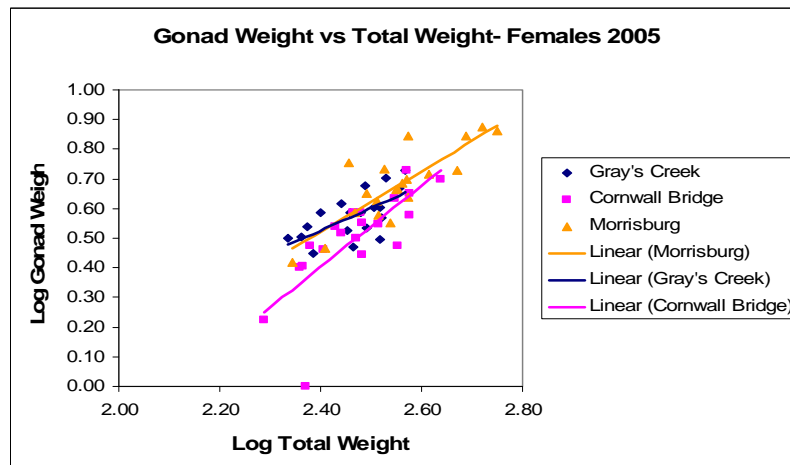
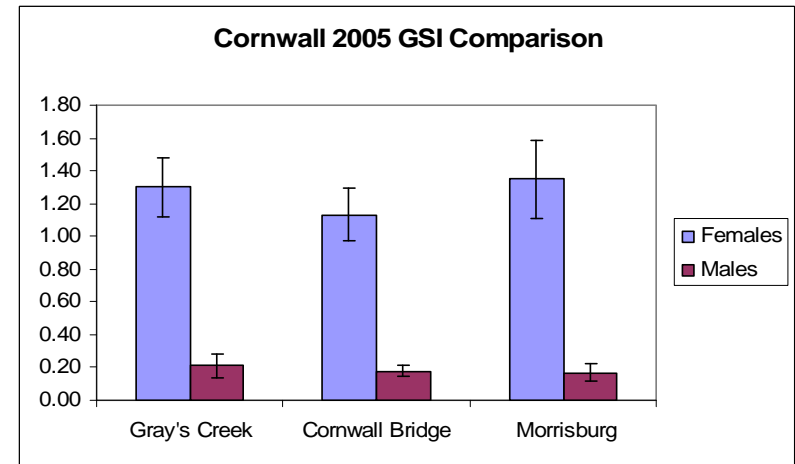
Cornwall 2004 LSI Data

- ANCOVA test done to determine differences between sites
- No interactions between total weight and site for males or females; data log transformed to obtain normal distribution
- Site differences for females- liver weight significantly higher at Morrisburg site compared to Gray's Creek and Cornwall Bridge ($P=0.006$)
- For males- liver weight also significantly higher at Morrisburg reference site ($P<0.001$)



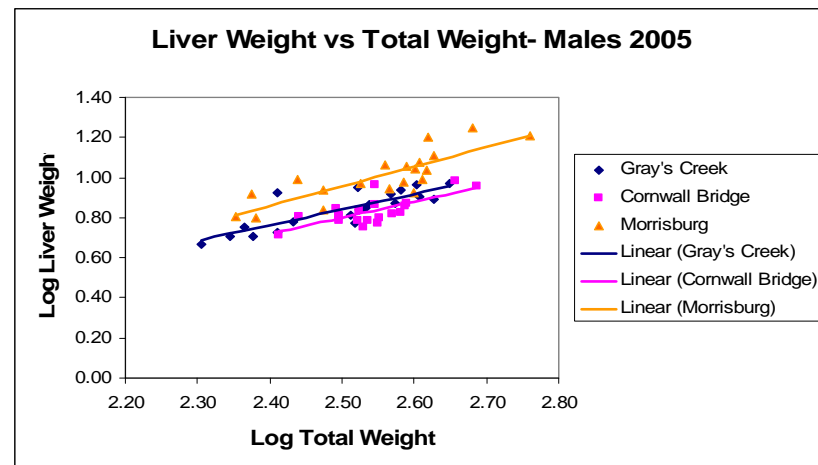
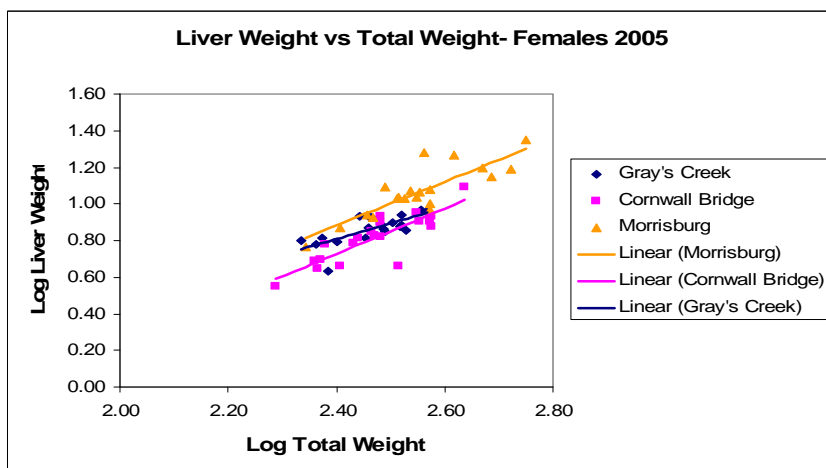
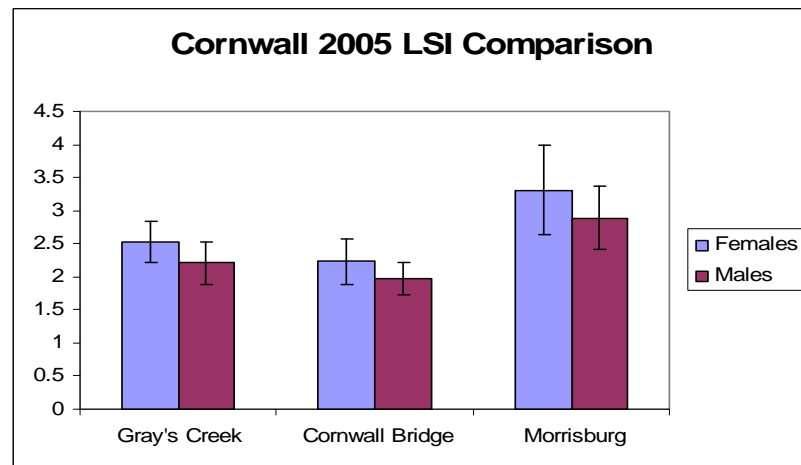
Cornwall 2005 GSI Data

- ANCOVA to determine differences btw sites
- Data log transformed in females, untransformed in males to achieve normal distribution
- No interactions between total weight and site for males or females
- Site effect in females- the exposed Cornwall Bridge site has a significantly lower liver weight (P=0.029)
- No site effects in males (P=0.0820)



Cornwall 2005 LSI Data

- ANCOVA to determine differences between sites
- Data for males and females log transformed to achieve normal distribution
- No interactions between site and total weight for males or females
- Females- site effect- Morrisburg reference site higher liver weight compared to other sites ($P < 0.001$)
- Males- Site effects ($P < 0.001$)- Morrisburg reference site significantly higher liver weight compared to other sites



2004 Summary

- % Vtg depressed in females at the exposed zone
- Liver weight also depressed at exposed site in both males and females, consistent with reduced plasma Vtg
- Males were expressing Vtg, but no apparent differences in numbers or concentrations among zones
- Gonad size and overall condition did not vary among zones- condition factor data not shown



2005 Summary

- % Vtg depressed in females at exposed zone- as in 2004
- Gonad weight and liver weight depressed in females at the exposed zone
- In males, no difference in gonad weight, however the liver weight was significantly depressed at the exposed zone
- Only 1 male showed Vtg- exposed site

Conclusions

- A few males did express Vtg, which could indicate the presence of environmental estrogens, however there were no differences among zones
- The depression in Vtg in females, along with the reduced gonad and liver size could have implications for fish reproduction
- We do not know at this time whether the stressors responsible for the depressed plasma Vtg in females are also impacting gonad and liver size, or if there are other factors at play



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