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Fish Health Studies Associated with the Kingston Fly Ash Spill, Spring 2009 – Fall 2010

S. Marshall Adams

Allison M. Fortner

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S. Marshall Adams
Allison M. Fortner

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Prepared for:
Tennessee Valley Authority
400 West Summit Hill Drive
Knoxville, Tennessee 37902-1499

Prepared by:
Environmental Sciences Division
Oak Ridge National Laboratory
Oak Ridge, Tennessee 37831
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1. INTRODUCTION

On December 22, 2008, over 4 million cubic meters of fly ash slurry was released into the Emory River when a dike surrounding a solid waste containment area at the Tennessee Valley Authority's (TVA) Kingston Fossil Plant ruptured. One component of TVA's response to the spill is a biological monitoring program to assess short- and long-term ecological responses to the ash and associated chemicals, including studies on fish health and contaminant bioaccumulation. These studies were initiated in early Spring 2009 for the purposes of: 1) documenting the levels of fly ash-associated metals in various tissues of representative sentinel fish species in the area of the fly ash spill, 2) determining if exposure to fly ash-associated metals causes short, intermediate, or long-term health effects on these sentinel fish species, 3) assessing if there are causal relationships between exposure to metals and health effects on fish, 4) evaluating, along with information from other ecological and physicochemical studies, the nature and route of contaminant transfer though food chains into higher level consumers, 5) providing important information for the Ecological Risk Assessment (ERA) for the Kingston fly ash project, and 6) serving as an important technology information transfer or model study focused on how to best evaluate the environmental effects of fly ash (and related environmental stressors), not only at the Kingston site, but also at sites on other aquatic systems where coal-fired generating stations are located. This report presents the results of the first two years of the fish health study.

To date, fish health and bioaccumulation studies have been conducted from Spring 2009 though Fall 2011 and includes 6 seasonal studies: Spring 2009, Fall 2009, Spring 2010, Fall 2010, Spring 2011, and Fall 2011. Both the Spring and Fall studies have focused on 3-4 sentinel fish species that represent different feeding habits, behaviors, and home ranges. In addition to fish health and bioaccumulation, the Spring investigations also included reproductive integrity studies on the same fish used for bioaccumulation and fish health. In this report, results of the fish health studies from Spring 2009 through Fall 2010 are presented while an associated report will present the fish reproductive studies conducted during Spring 2009 and Spring 2010. A report on fish bioaccumulation was submitted to TVA in June 2011. The fish health study conducted in conjunction with the bioaccumulation and reproductive study is critical for assessing and evaluating possible causal relationships between contaminant exposure (bioaccumulation) and the response of fish to exposure as reflected by the various measurements of fish health.

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2. APPROACH

Fish health studies were initiated in Spring 2009 and have been conducted in Fall 2009, Spring 2010, Fall 2010, Spring 2011, and Fall 2011 (Spring and Fall 2011 results are not reported here). Spring sampling was conducted during April and May when fish were reproductively mature and developing their gonads. In Spring 2009, largemouth bass, bluegill and white crappie were collected at ERM 8.0 (upstream reference), ERM 3.0, ERM 0.9, CRM 1.5, and CRM 8.0 (where ERM = Emory River mile and CRM = Clinch River mile). The spill occurred near ERM 3.0; sites ERM 0.9 and CRM 1.5 are both downstream from the spill (Figure 1). In Spring 2010, largemouth bass, bluegill, white crappie, and redear sunfish were collected at ERM 8.0 (upstream reference), LERM 2.0 (Little Emory River = upstream reference), ERM 3.0, ERM 0.9, CRM 1.5, and CRM 8.0. Although the CRM 8.0 is upstream of the Emory River-Clinch River confluence, it is influenced by industrial and residential discharges and runoff and is therefore considered a 'positive control' and not a clean reference site. In the Fall of 2009 and 2010 bluegill, largemouth bass, and channel catfish were collected from ERM 8.0, Little Emory River mile 2 (in Fall 2010), ERM 3.0, ERM 0.9, CRM 1.5, and CRM 8.0. Only female fish were collected in the Spring because emphasis was on reproductive integrity while in the Fall of both years a mix of both males and females were collected for analysis. At each site and for each species and sampling period, the goal was to collect eight adult fish of which fish health analyses were conducted on all 8 individuals and metals were analyzed in 6 of these 8 fish. Immediately upon collection by boat electrofishing, a blood sample was taken from the caudal veins of each fish using vacutainers (containing lithium heparin as the anticoagulant), samples were stored on ice, a unique 5 digit identification tag was affixed to each individual, the fish was placed in an aerated live well onboard the boat and transported to the lab alive in large, aerated coolers of water. At the lab, fish were processed for a suite of health indicators, the liver and ovary (Spring only) were harvested for metal analysis, and each individual was filleted for metal analysis of muscle tissue.

Fish health indicators (bioindicators, i.e., Adams 2002a, Adams 2002b) representing different functional response groups and levels of biological organization were measured on individuals of all species collected at all sites including (a) condition indices, (b) bioenergetic and hematological responses, (c) histopathological indicators, (d) indicators of carbohydrate-protein metabolism, (5) organ dysfunction responses, (6) measures of electrolyte homeostasis, and (7) indices of feeding and nutrition including those parameters associated with digestive physiology. Table 1 provides a list of the fish health indicators measured in this study associated with each of these functional groups. Blood chemical parameters were analyzed by a VetScan II clinical analyzer which continuously calibrates itself with internal standards. In addition, periodic internal calibrations were performed using known blood standards provided by the manufacturer (*Abaxis, Inc*).

Fish were returned alive from the field and were processed in the lab for a variety of fish health measurements. In addition to standard measurements of length and weight, all fish were first examined for any external signs of injury including presence of disease, infection, or parasites. Following lethal anesthetization using MS 222, fish were dissected and major organs, including liver, gill, kidney, and ovary, were examined for any anomalies including parasites, necrosis, or discoloration. Ovary tissue was further processed for various measurements of reproductive integrity (Greeley et al. 2012). Sections of gill, liver, and kidney tissue were taken and preserved in 10% buffered formalin for histopathological analysis. Measures of overall health and condition included the liver (LSI), visceral (VSI), and spleno-somatic (SSI) indices, which were calculated as the mass of these respective organs divided by total body mass. The condition factor was calculated as $K=10^2W/L^3$, where W=body mass (g) and L=total length (mm). A Health Assessment Index (Adams et al. 1993) was also determined for each fish based on observation, scoring, and evaluation of a variety of internal and external lesions and anomalies. Bioenergetic

status was assessed by estimating the relative amount of lipid stored in the mesenteries of the viscera (scored 0-4) and the nutritional/feeding level of each fish estimated by the fullness of the stomach and intestine (scored 0-4).

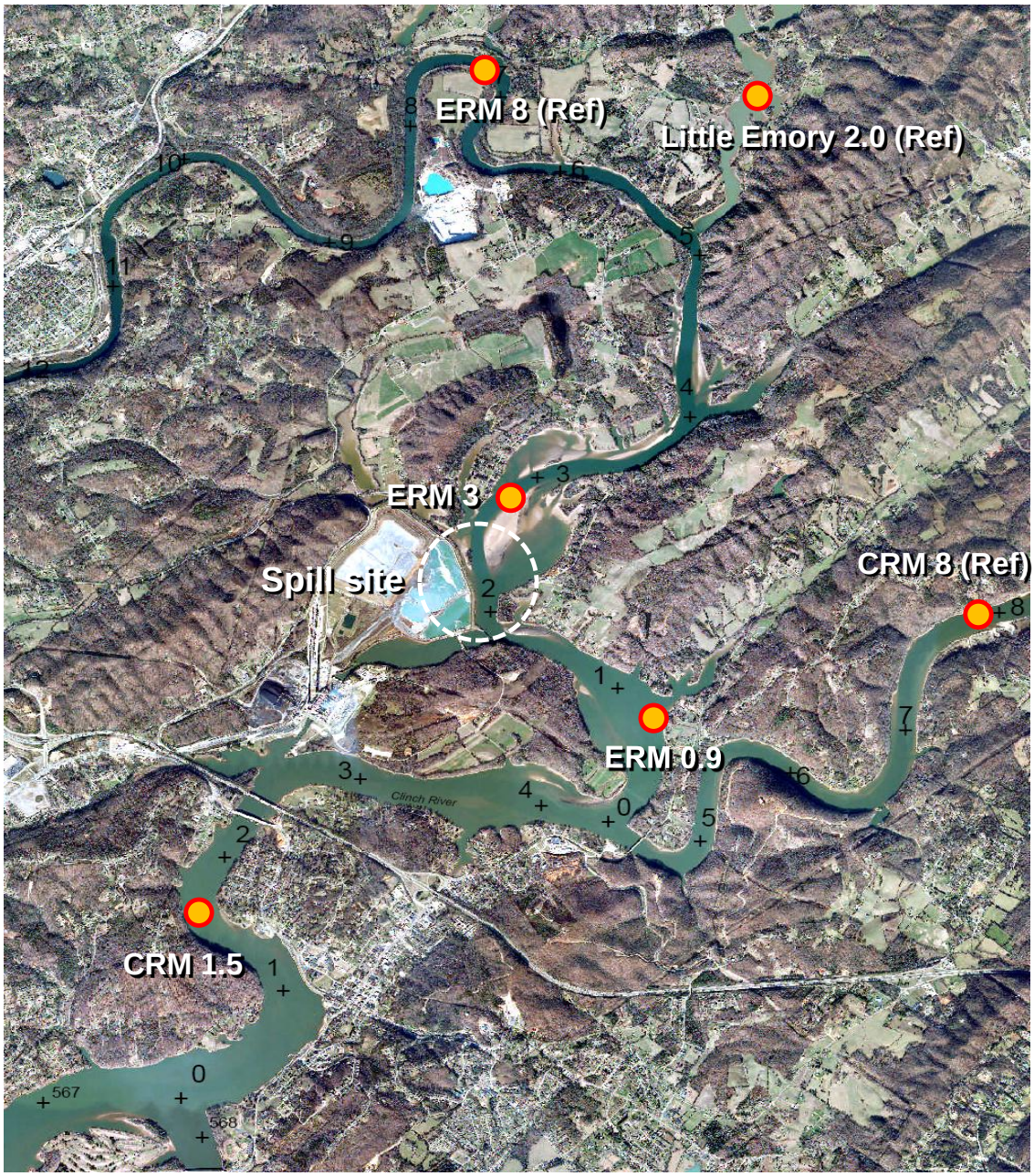


Figure 1. Location of primary sampling sites on the Emory River, Little Emory River, and Clinch River for the fish health study.

Table 1. List of the functional response groups and individual bioindicators measured on the sentinel fish species for the fish health study.		
Functional response group	**Bioindicator**	**Indication**
Condition indices	Condition factor	Low values suggest poor overall condition
	Liver-somatic index	Low values indicate reduced energy storage
Bioenergetics	Visceral-somatic index	Low values suggest below normal lipid storage
	Lipid index	Low values suggest below normal lipid storage
Hematology/immune system	Hematocrit	Low levels associated with anemia and mineral deficiency
	Spleno-somatic index	High values indicate infection/disease
Organ dysfunction	Ananine transaminase	High values indicate liver damage
	Urea nitrogen	High values indicate gill damage
	Creatinine	High values indicate kidney damage
	Total bilirubin	High values indicate liver damage
Histopathology	Liver lesion index	High score indicates liver pathology/disease
	Gill lesion index	High score indicates gill pathology/disease
	Ovary lesion index	High score indicates ovary pathology/disease
Carbohydrate-protein metabolism	Glucose	High levels suggest short-term general stress
	Total protein and albumin	Low levels indicate compromised protein metababolism, kidney/liver function, and immune response
	Globulin	High levels indicate compromised protein metababolism, kidney/liver function, and immune response
Electrolyte homeostasis	Calcium, potassium, and sodium	Depressed levels indicate to a variety of body health malfunctions, poor diet and nutrition, and organ dysfunction
Feeding/nutrition	Stomach fullness	Empty stomachs suggest reduced short-term feeding
	Intestinal fullness	Empty intestines suggest reduced short-term feeding
	Gall bladder color	A high score suggests reduced long-term feeding intensity
	Gall bladder size	Larger than normal size is an indication of reduced long-term feeding intensity

For the histopathology analysis, internal organs such as the liver, gill, and ovary of individual fish were dissected with a clean surgical blade. Tissues were fixed in 10% neutral buffered formalin, and processed samples were shipped to the University of California-Davis toxicopathology lab for analysis. Each fish was assigned a random alpha-numeric identification code, and in addition, each random number was assigned a letter to identify specific tissues (e.g., A=liver and B = ovary). All tissues were routinely paraffin processed and paraffin blocks sectioned at 3 microns thick. Sections were mounted on glass slides and stained with hematoxylin and eosin. Stained tissue sections were screened for lesions and subjected to detailed, semi-quantitative histopathology analysis. The severity of lesions in the liver, gill, kidney and ovarian tissues were scored based on a qualitative scale of 0 = no lesions present, 1 = mild lesions, 2 = moderate lesions, and 3 = severe lesions, with a score of 2 or 3 being considered a significant lesion. A composite lesion score for each species at each site was calculated as the average sum of lesion scores for each organ for that species and site.

The fish health results are presented in this report as both individual responses and integrated (holistic) responses. Individual health responses are presented by functional bioindicator groups for each species including bluegill sunfish and largemouth bass for each of four seasons (Spring 2009 and 2010, Fall 2009 and Fall 2010), channel catfish for Fall 2009 and 2010, white crappie for Spring 2009 and 2010, and redear sunfish for Spring 2010 (redear studies were initiated in Spring 2010). The integrated fish health analysis incorporated all the measured fish health parameters together within a multivariate context to assess the holistic response of fish to environmental conditions at each sample site. For the integrated analysis, temporal changes in overall health among sites for each species were compared between Spring 2009 and Spring 2010 and between Fall 2009 and Fall 2010. Comparisons between Spring and Fall responses were not made because only females were collected in the Spring to meet reproductive study needs, and a mixture of both sexes were collected in the Fall when it is more difficult to discern sex without sacrificing the fish. Temporal and spatial trends were identified subjectively; however, in the future when the 2011 data are included with these data, a more robust statistical analysis of trends will be conducted.

A canonical discriminant analysis procedure was used to assess the integrated health status of bluegill largemouth bass, channel catfish, white crappie, and redear sunfish at each sample site. To examine the integrated health response of each species at each site, the individual bioindicator variables were considered jointly within a multivariate context using this canonical discriminant analysis procedure (Adams et al. 1994). Canonical discriminant analysis generates new sets of variables that are linear combinations of the original bioindicators which account for most of the variation among treatment groups. This procedure provides a reduced set of canonical variables that are the most important and influential for differentiating health responses of fish among sample sites. The greatest difference (or the highest discriminatory ability) among the integrated response over sample sites is represented by the first canonical variate, the next greatest discriminatory ability by the second canonical variate, etc. This multivariate procedure also identifies the canonical variate means that are most closely related among sample sites. The 95% confidence regions (radii) were used to indicate the uncertainty associated with the estimated mean canonical variates of each site. The center of each of the confidence regions is the mean value of the canonical variables. A stepwise variable selection procedure was then used to identify a subset of all the measured bioindicators which were the most influential in causing differentiation or separation among sample sites for each fish species during each season.

3. RESULTS

Individual Health Responses

The individual health responses are presented in this section as a function of fish species and season. For each species, comparisons of individual responses are made on the basis of Spring 2009 versus Spring 2010 and Fall 2009 versus Fall 2010. For each species and seasonal comparison, one or two bioindicators representing each of the eight functional response categories (see Table 1) are presented along a spatial gradient of the Emory and Clinch Rivers. Even though additional bioindicators were typically measured in fish, only about half of the total measured can graphically be presented in this report because of the large possible number of responses, species, and seasonal combinations. Also, only health responses from fish collected in the Spring could be compared to other Spring-collected fish because all fish sampled in the Spring of both years were females (because of the focus on reproduction) while in the Fall a mixture of both sexes was collected. For each species-season comparison in the following section, figures illustrate two representative health indicators from each of seven functional categories including condition indices, bioenergetic-hematological, histopathological, organ dysfunction, carbohydrate-protein metabolism, electrolyte homeostasis, and feeding/nutrition responses. Appendix Table 1 presents the basic statistics (means and standard errors) for each species at each site for each season.

Bluegill - Spring 2009 vs Spring 2010

The two health responses representative of condition indices, the condition factor and the liver-somatic index, are compared on a spatial gradient between Spring 2009 and Spring 2010. The condition factor in 2009 shows no distinct spatial trend relative to the ,location of the ash spill, however, the condition factor in 2010 is somewhat elevated in the Emory and Clinch rivers below the reference sites (Fig. 2). A relatively high liver-somatic index indicates normal storage of glycogen and lipids for energy metabolism. Bluegills in 2009 appear to have low energy reserves compared to the reference while in 2010 this situation greatly improved at downstream sites (Fig. 2). The visceral-somatic index, an indicator of internal lipid storage, exhibited no distinct spatial pattern either year while the hematocrit appeared to be somewhat depressed downstream of the reference during the Spring of both years (Fig. 3). The pathological condition of the liver, indicated by the histopathological composite index score (Fig. 4), was higher downstream of the reference sites during the Spring of both years. There was also some indication that the composite index score of the ovary was higher downstream of the reference site but this index was generally so low and variable among sites that no definitive spatial or temporal trends are obvious. The organ dysfunction enzyme, alanine transaminase (an indicator of liver damage), was elevated downstream of the spill during both seasons (Fig. 5). However, there were no obvious spatial trends either year in levels of blood urea nitrogen, an indicator of gill dysfunction (Fig. 5). The two indicators of carbohydrate-protein metabolism, glucose (low levels are best) and total blood protein (somewhat elevated levels are best) did not show any definitive spatial patterns except possibly for total blood protein during Spring 2009 when levels at the spill site and downstream of the spill were much lower than the reference but increased in Spring 2010 near reference levels (Fig. 6). For calcium and sodium which are indicators of electrolyte homeostasis, levels of calcium were somewhat depressed during Spring 2009 at the spill site and downstream of the spill while in Spring 2010 levels rebounded to reference values (Fig. 7). For sodium there is an indication that values were depressed somewhat in the Clinch River but stabilized closer to reference levels in Spring 2010 (Fig. 7). For gall bladder color, which is an indicator of feeding and nutrition (a high index score is indicative of low feeding intensity over several days), no spatial trend is evident while the index of stomach fullness (an indicator of feeding intensity for the short term, approximately 1-2 days) was higher at some sites downstream of the reference (Fig. 8).

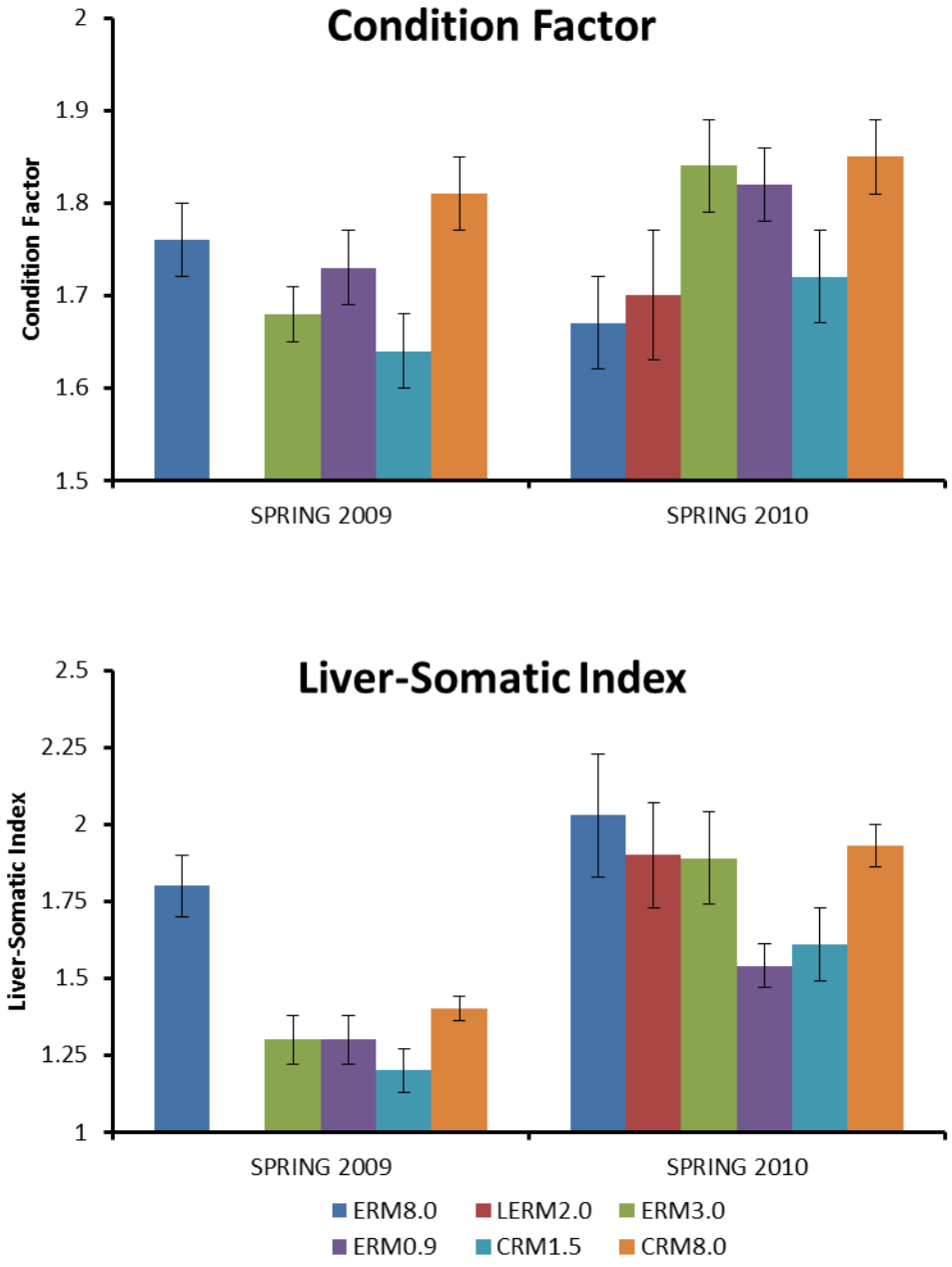


Figure 2. Spatial patterns in condition indices for bluegill sunfish during Spring 2009 and Spring 2010. (From left to right the six sites represented are upstream reference, upstream reference, site of spill, downstream of spill, downstream of spill, and upstream reference.)

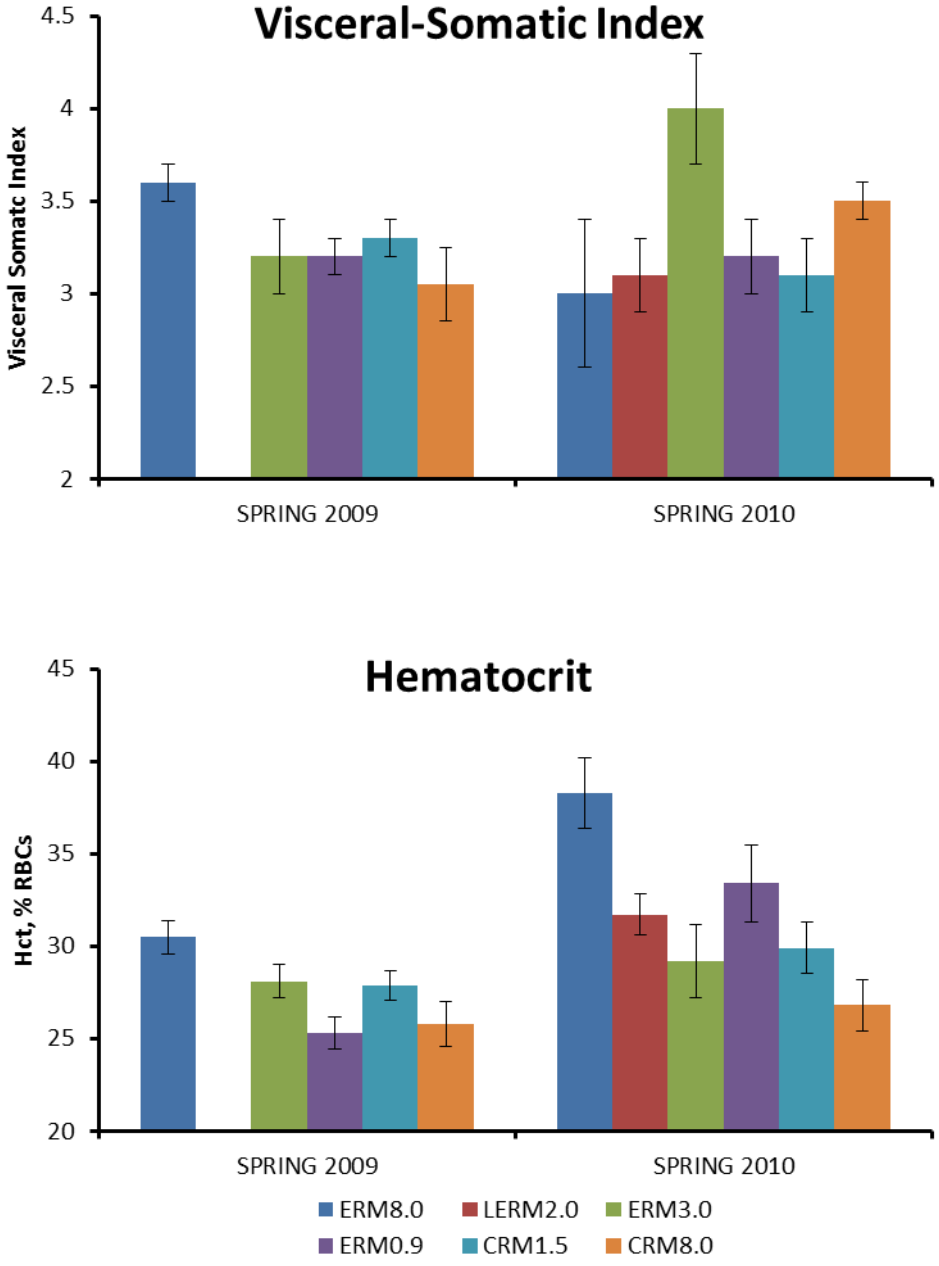
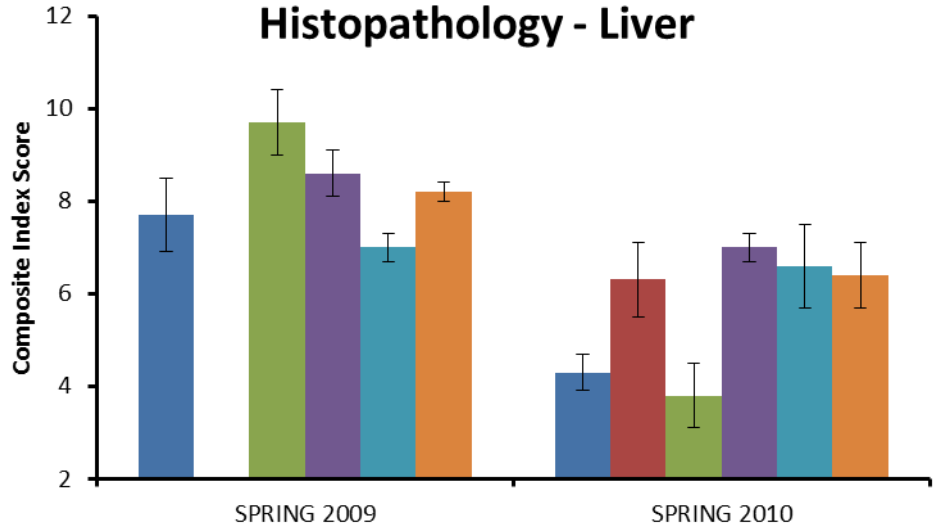


Figure 3. Spatial patterns in bioenergetic and hematological indicators for bluegill sunfish during Spring 2009 and Spring 2010.



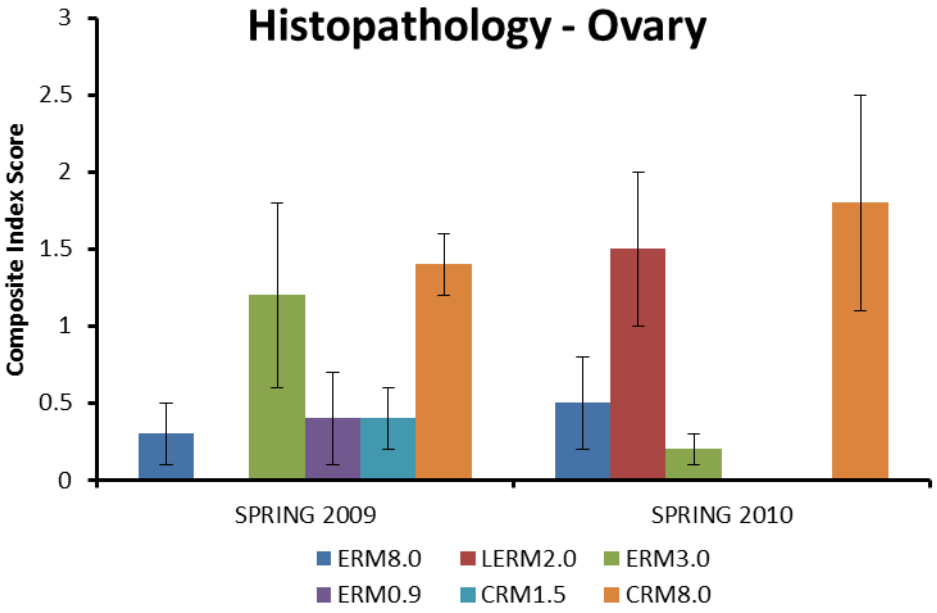


Figure 4. Spatial patterns in histopathological indicators (composite index score) for bluegill sunfish during Spring 2009 and Spring 2010.

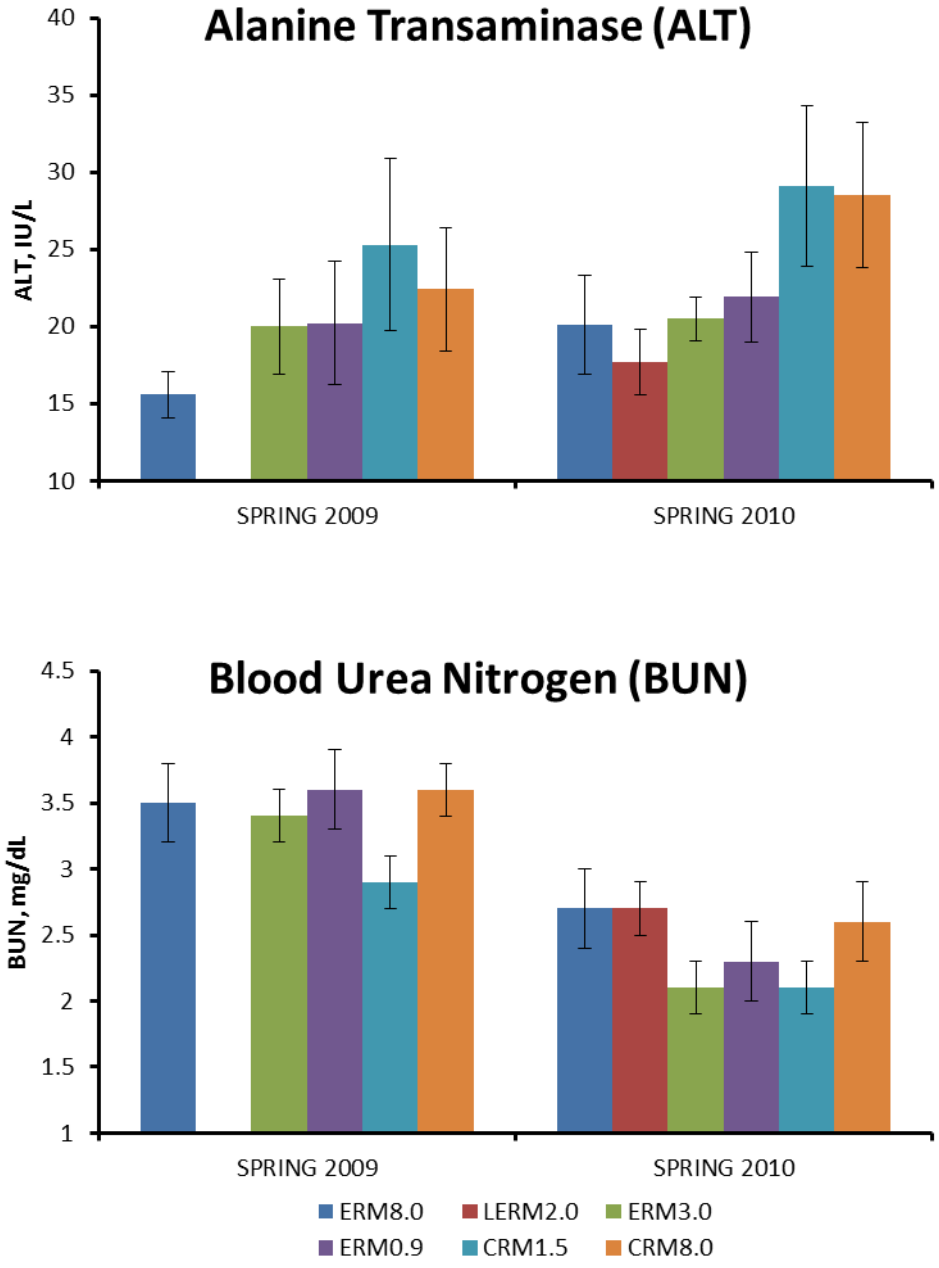


Figure 5. Spatial patterns in organ dysfunction indicators for bluegill sunfish during Spring 2009 and Spring 2010.

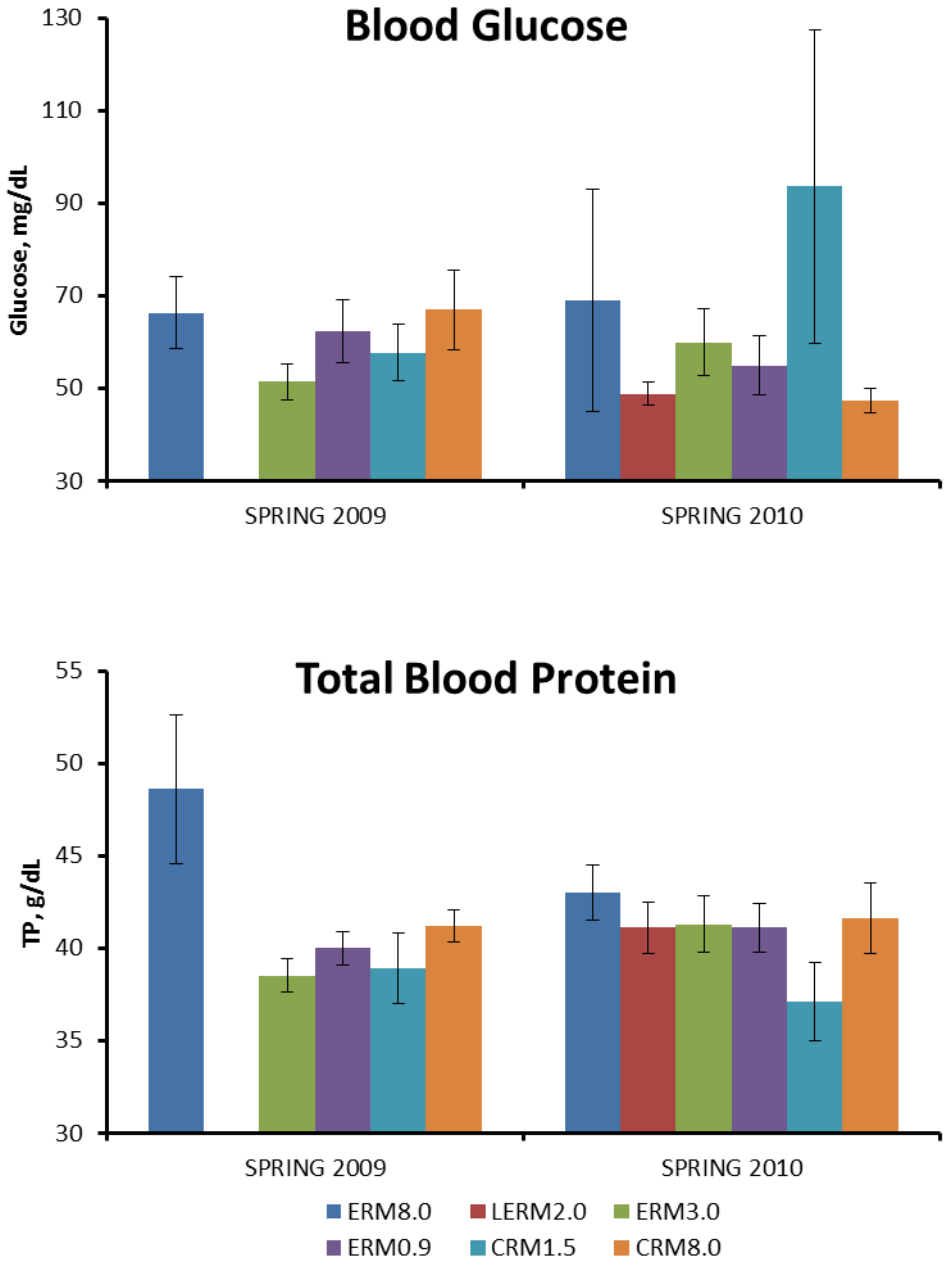


Figure 6. Spatial patterns in carbohydrate-protein metabolism indicators for bluegill sunfish during Spring 2009 and Spring 2010.

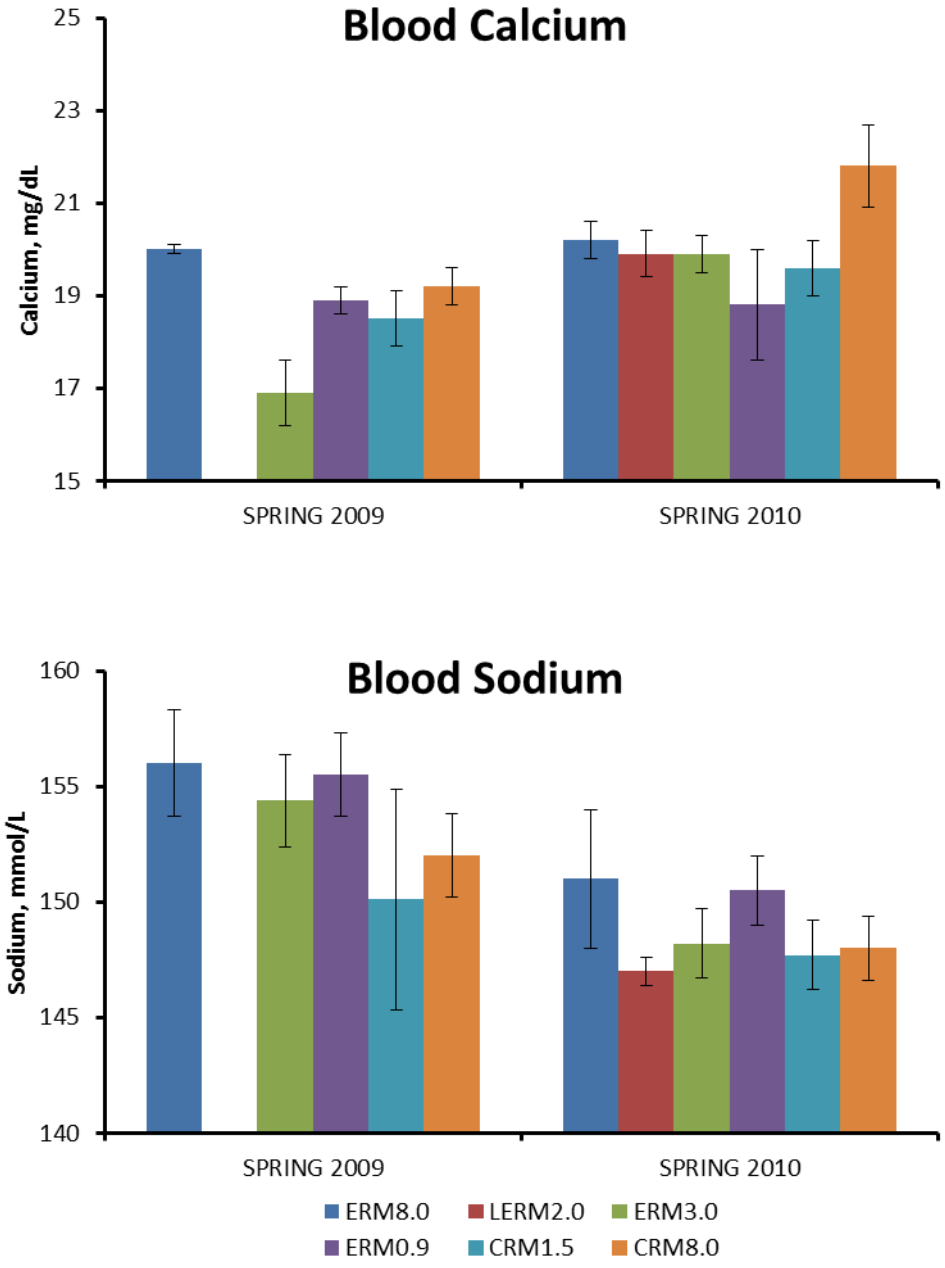


Figure 7. Spatial patterns in electrolyte homeostasis indicators for bluegill sunfish during Spring 2009 and Spring 2010.

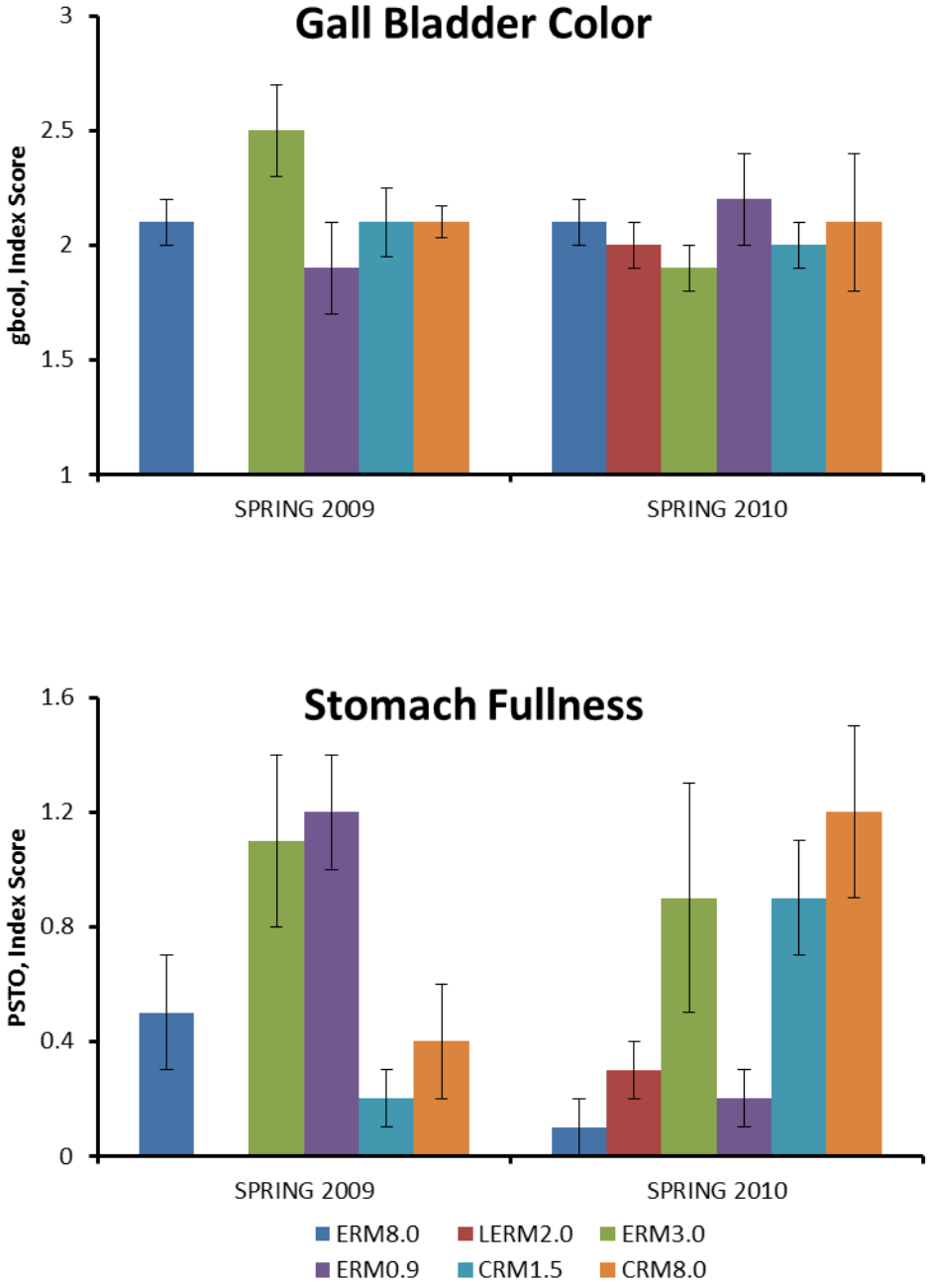


Figure 8. Spatial patterns in feeding and nutrition indicators for bluegill sunfish during Spring 2009 and Spring 2010.

Largemouth bass- Spring 2009 vs Spring 2010

As was also observed for the condition factor of bluegill during the spring of both years, there was no distinct spatial gradient or difference in the condition factor of largemouth bass between years (Fig. 9). The liver-somatic index was higher downstream of the reference sites during both years demonstrating increased "stairstep" levels of energy stores during Spring 2010 (Fig. 9). Even though there was a slight indication that the visceral-somatic index (an indicator of lipid storage) was slightly higher downstream of the reference in Spring 2009, values were very similar at all sites in Spring 2010 (Fig. 10). In Spring 2009, a similar spatial pattern in hematocrit was observed for bass as was seen for bluegill with lower levels downstream of the reference, however in Spring 2010, levels downstream of the references increased sharply (Figs. 3 & 10). Somewhat similar spatial patterns occurred in liver pathology for bass and bluegill during Spring of both years with the composite index scores in 2009 being highly elevated at ERM 3.0 and declining somewhat downstream while in Spring 2010 most sites remained elevated above the reference (Figs. 4 & 11). Except at ERM 3.0 in Spring 2009, the ovarian composite index score was generally lower at sites below the reference and indices were similar to the references at most sites in 2010 (Fig. 11). In Spring 2009 the liver enzyme, alanine transaminase, was generally higher downstream of the reference (except for ERM 3.0) but in 2010 levels were elevated at the references and decreased downstream except for ERM 3.0 (Fig. 12). The physiological response to gill damage, blood urea nitrogen, was consistent across sites in 2009 but increased sharply downstream of the spill site in 2010 (Fig. 12). The short-term stress response indicator, glucose, displayed opposite patterns between Spring 2009 and 2010 with values in 2009 higher upstream and decreasing downstream while in 2010 levels were lowest at the reference sites and increased downstream (Fig. 13). During both seasons, calcium demonstrated a slight increase downstream of the references at most sites while potassium in Spring 2009 was highly elevated above reference levels but returned to near reference values in Spring 2010 (Fig. 14). The gall bladder color, an indirect indicator of longer-term feeding intensity, demonstrated no obvious spatial pattern while stomach fullness, a shorter- term indicator of feeding, was somewhat similar at all sites both seasons considering the relatively large inter-site variability for this parameter (Fig. 15).

Bluegill- Fall 2009 vs Fall 2010

Except for an extremely low condition factor of bluegill at ERM 3.0, levels of this overall well-being indicator were similar at all sites in Fall 2009 (Fig16). Condition indices of bluegill at all sites in Fall 2010 were also similar but much higher than values in Fall 2009. For the liver-somatic index, the two reference sites in Fall 2009 were significantly higher than sites downstream, whereas in Fall 2010 levels were somewhat similar except for a large peak at CRM 1.5 (Fig. 16). Similar spatial trends are evident for the visceral-somatic index for bluegill in Fall 2009 and 2010 with levels being generally higher at the reference sites and lower downstream (Fig. 17). As was also observed for the condition factor and liver-somatic index in Fall 2009, the visceral-somatic index was lowest at ERM 3.0 than at the other sites. The hematocrit was relatively similar and consistent among sites both seasons except for a very low value at one of the reference sites (LERM 2.0) and a very high level at ERM 0.9 in Fall 2009 (Fig. 17). The spatial pattern of liver pathology was similar to that observed for bluegill in the Spring with the lowest composite index values at the reference sites and general higher lesion rates at downstream sites (Fig. 18). However, the spatial pattern in pathology of the gill was somewhat opposite of that for the liver pathology with generally higher gill lesions for reference fish and lower incidences downstream of the references (Fig.18). The higher composite index score for the gill pathology during the fall of both years was due primarily to parasitic lesions which reflect more about the localized habitat of resident fish than exposure to contaminants. The liver dysfunction enzyme, alanine transaminase, was similar at all sites during both seasons except for a very low level at LERM 2.0 and a very high level at ERM 0.9 in Fall 2009 (Fig. 19). This is the same

spatial pattern observed for the hematocrit in Fall 2009 (Fig. 17). For these two seasons, this enzyme did not follow the same spatial patterns observed for bluegill and largemouth bass during the Spring where higher enzyme levels occurred downstream of the reference. Blood urea nitrogen in Fall 2009 demonstrated similar spatial patterns as was seen for bluegill during Spring 2009 except values were generally lower in the Fall (Figs. 5 & 19). In Fall 2010, levels were generally highest at the reference sites (similar to that observed in Spring 2010) and decreased downstream. Blood glucose levels in bluegill during Fall 2009 and 2010 were variable between sites and seasons and did not show the same relatively similarity in sites observed in the Spring of both years (Figs. 6 & 20). Total blood protein was also more variable in Fall 2009 than Spring 2009 with lowest levels upstream and higher values downstream with Fall 2010 being similar among sites (Figs. 6 & 20). Both calcium and potassium demonstrated a relatively distinct spatial gradient in Fall 2009 with lowest levels at the reference sites and increasing values below the ash spill (Fig. 21). Except for a very low value of potassium at ERM 3.0 in Fall 2010, both potassium and calcium had similar spatial patterns among sites. As a general nutrition indicator, stomach fullness was also highly variable among sites both years with gall bladder size (a longer-time indicator of feeding) being relatively consistent among sites in Fall 2009 and displaying increasing size downstream from the references in Fall 2010 (Fig. 22). Such a gradient in this indicator of longer-term feeding suggests that feeding intensity was higher upstream than downstream in Fall 2010.

Largemouth bass- Fall 2009 vs Fall 2010

Except for the positive control site (CRM 8.0) in Fall 2009, the condition factor remained relatively similar among sites during both seasons (Fig. 23). The liver-somatic index was also similar among sites both seasons and demonstrated very little site or seasonal differences compared to the wide spatial variability observed among sites in Spring 2009 and 2010 (Figs. 9 & 23). The spleno-somatic index (an indicator of hematological function and infection or disease) also displayed relatively similar responses among sites and between seasons (except for CRM 1.5 in 2010) (Fig. 24). Spatial differences in hematocrit among sites were more consistent in Fall 2010 than in Spring 2010 but Fall 2009 values were somewhat variable among sites (Fig. 24). Incidences of liver lesions were lowest at the reference sites in Fall 2009 (except for CRM 1.5) and increased downstream while in Fall 2010 liver lesions were slightly elevated at one of the reference sites (Fig. 25). Gill lesions were more similar among sites in Fall 2009 compared to those observed for bluegill during this period, and the spatial gradient of gill lesions in Fall 2010 were similar to Fall 2010 bluegill with slightly higher incidences seen at the ERM 8.0 reference (Figs. 18 & 25). Except for CRM 1.5 in Fall 2009, levels of the liver enzyme alanine transaminase were similar among sites and seasons and were lower than that observed during the two Spring periods for bass (Figs. 12 & 26). Blood urea nitrogen, however, demonstrated a clear downstream gradient in Fall 2009 with levels lowest at the reference sites and increasing downstream while in Fall 2010 values were slightly elevated at the reference sites (Fig. 26). Except for blood glucose and total blood protein at ERM 0.9 during Fall 2009, glucose and protein were not only similar among sites but values were also similar between both Fall periods (Fig. 27). As was also observed for glucose and total protein during Fall 2009, calcium and sodium were also elevated at ERM 0.9 compared to the other sites (Fig. 28). Levels of calcium were relatively similar among sites in Fall 2009 but lower at the references in Fall 2010 where they increased downstream. Sodium levels also were lowest at the reference sites during both Fall periods and increased slightly downstream (Fig. 28). Indicators of feeding and nutrition reveal that feeding intensity was generally higher upstream in Fall 2009 and lower in Fall 2010 with no discernible spatial pattern during either Fall period (Fig. 29).

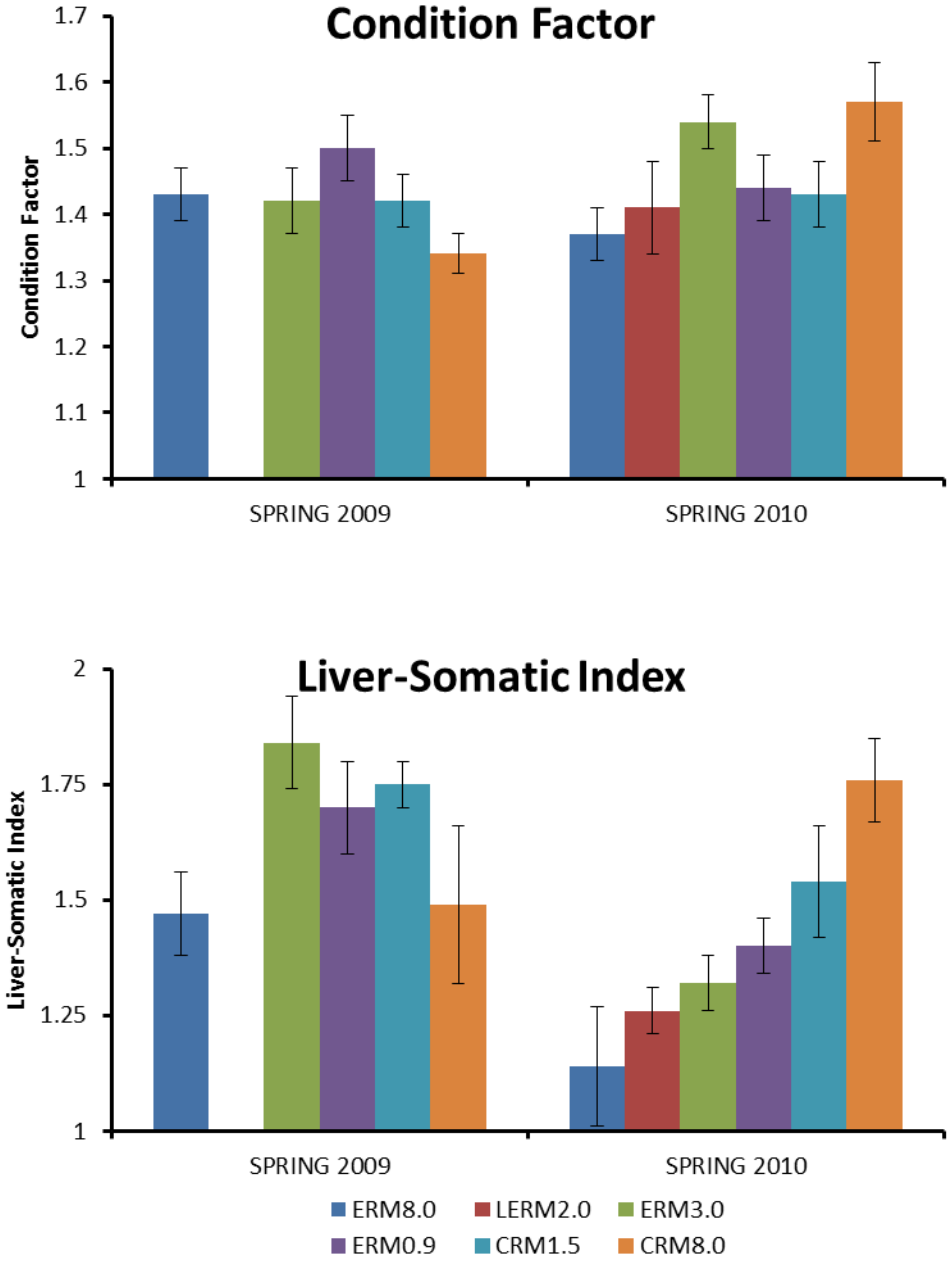


Figure 9. Spatial patterns in condition indices for largemouth bass during Spring 2009 and Spring 2010.

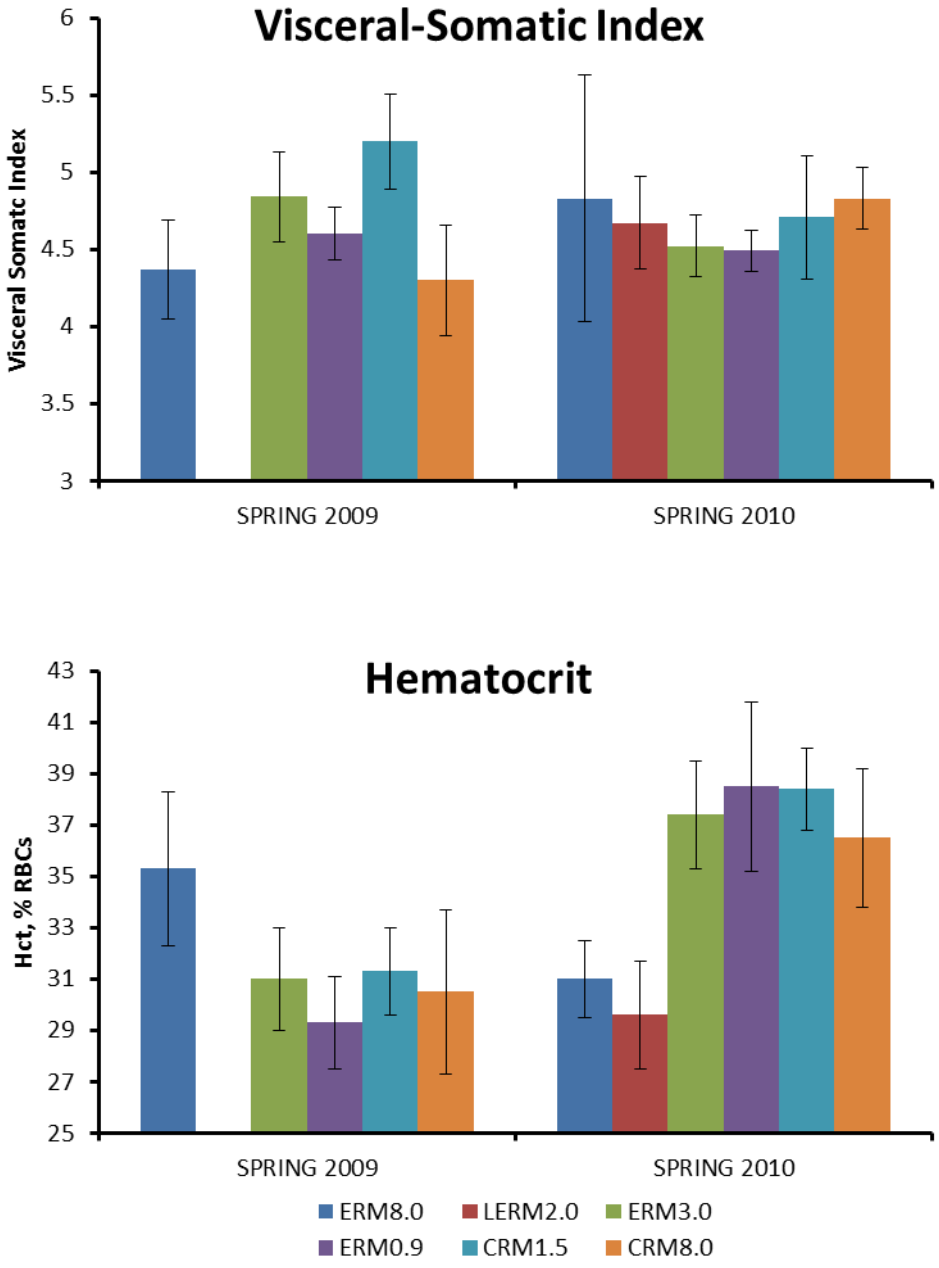
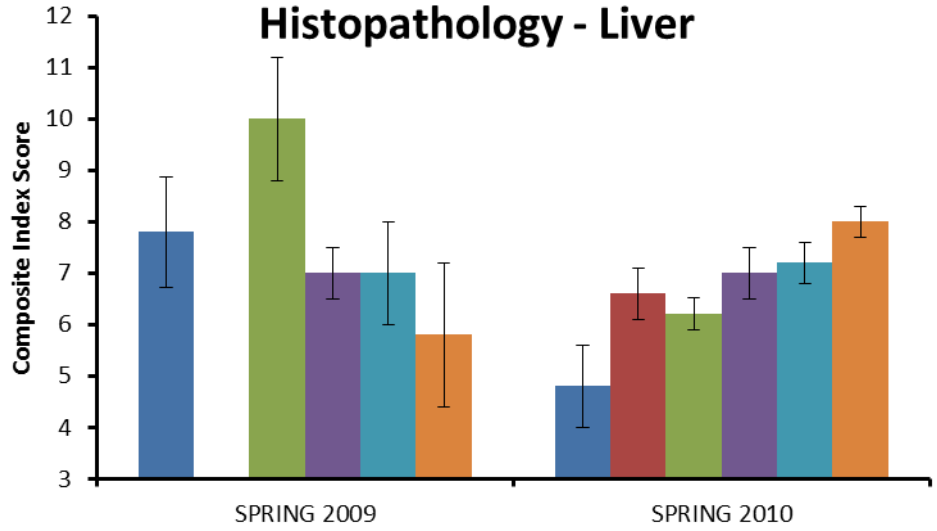


Figure 10. Spatial patterns in bioenergetic and hematological indicators for largemouth bass during Spring 2009 and Spring 2010.



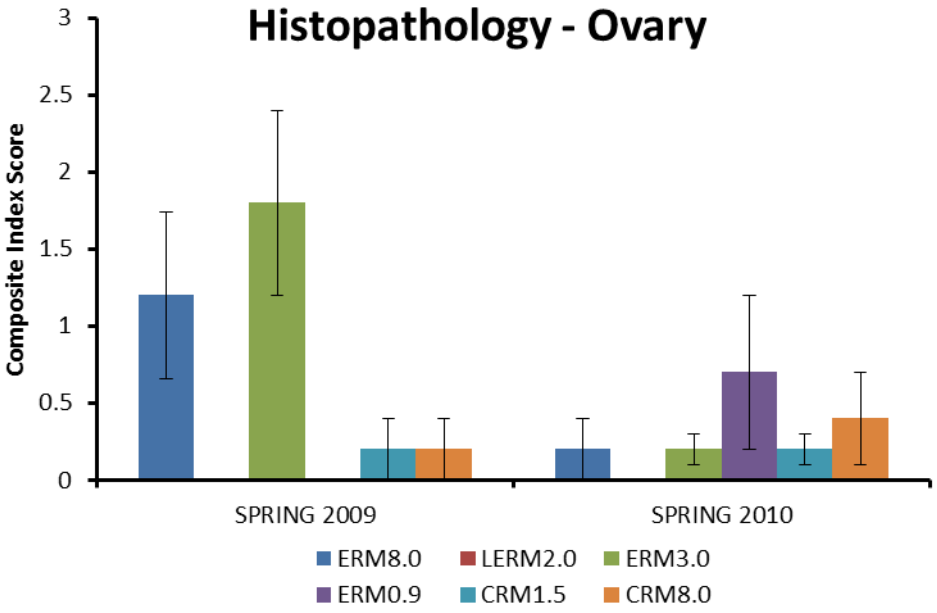


Figure 11. Spatial patterns in histopathological indicators (composite index score) for largemouth bass during Spring 2009 and Spring 2010.

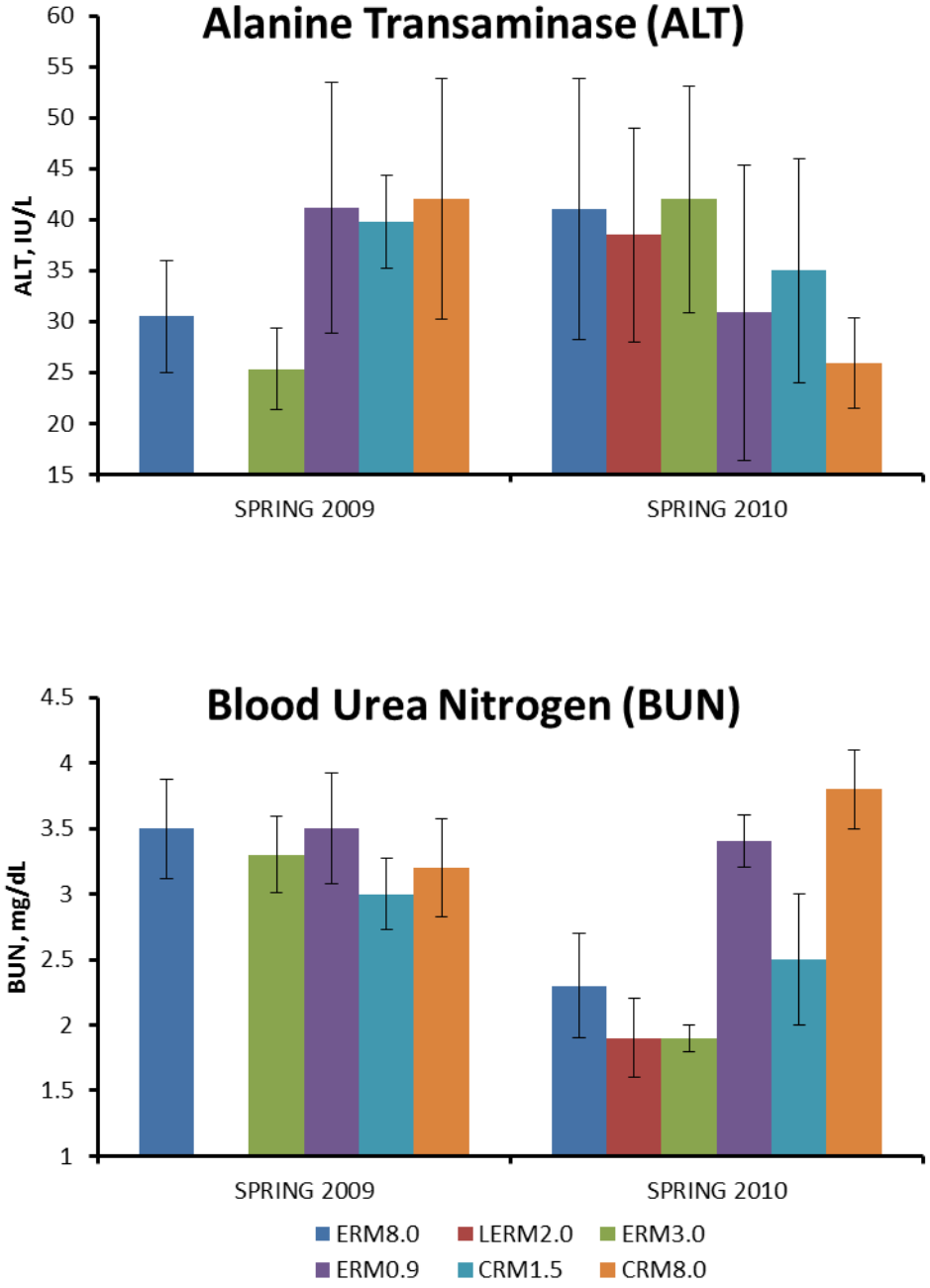


Figure 12. Spatial patterns in organ dysfunction indicators for largemouth bass during Spring 2009 and Spring 2010.

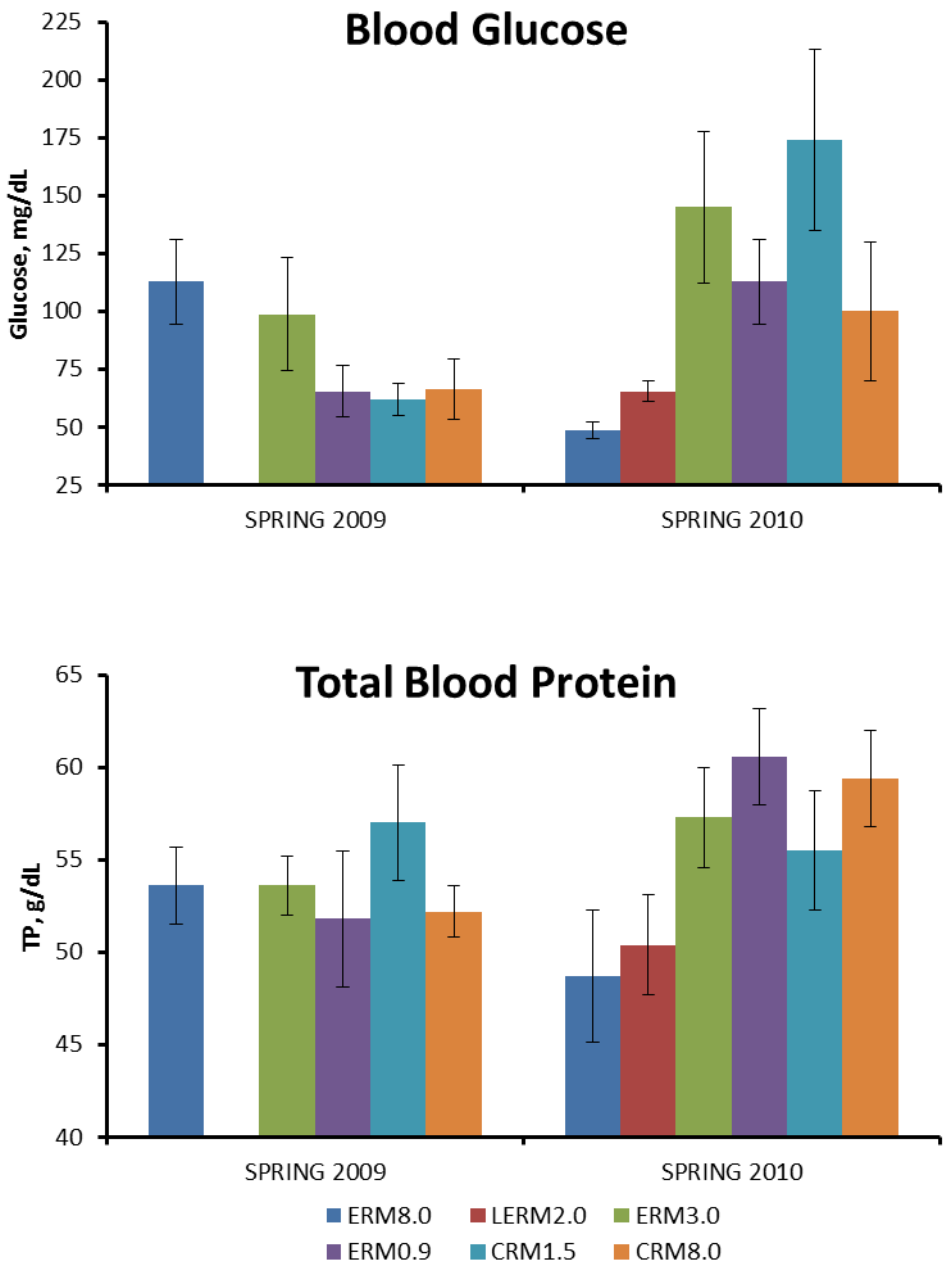


Figure 13. Spatial patterns in carbohydrate-protein metabolism indicators for largemouth bass during Spring 2009 and Spring 2010.

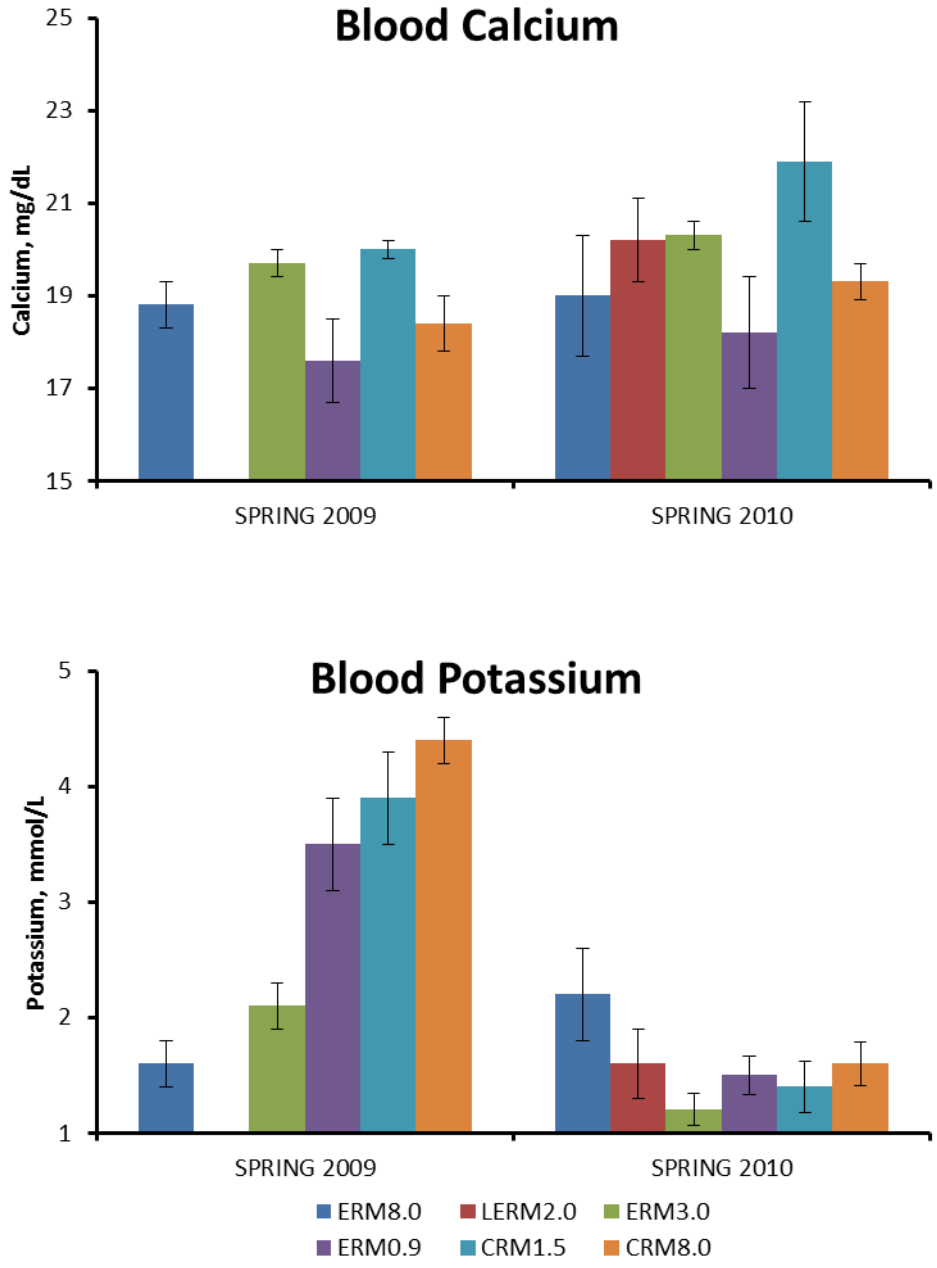


Figure 14. Spatial patterns in electrolyte homeostasis indicators for largemouth bass during Spring 2009 and Spring 2010.

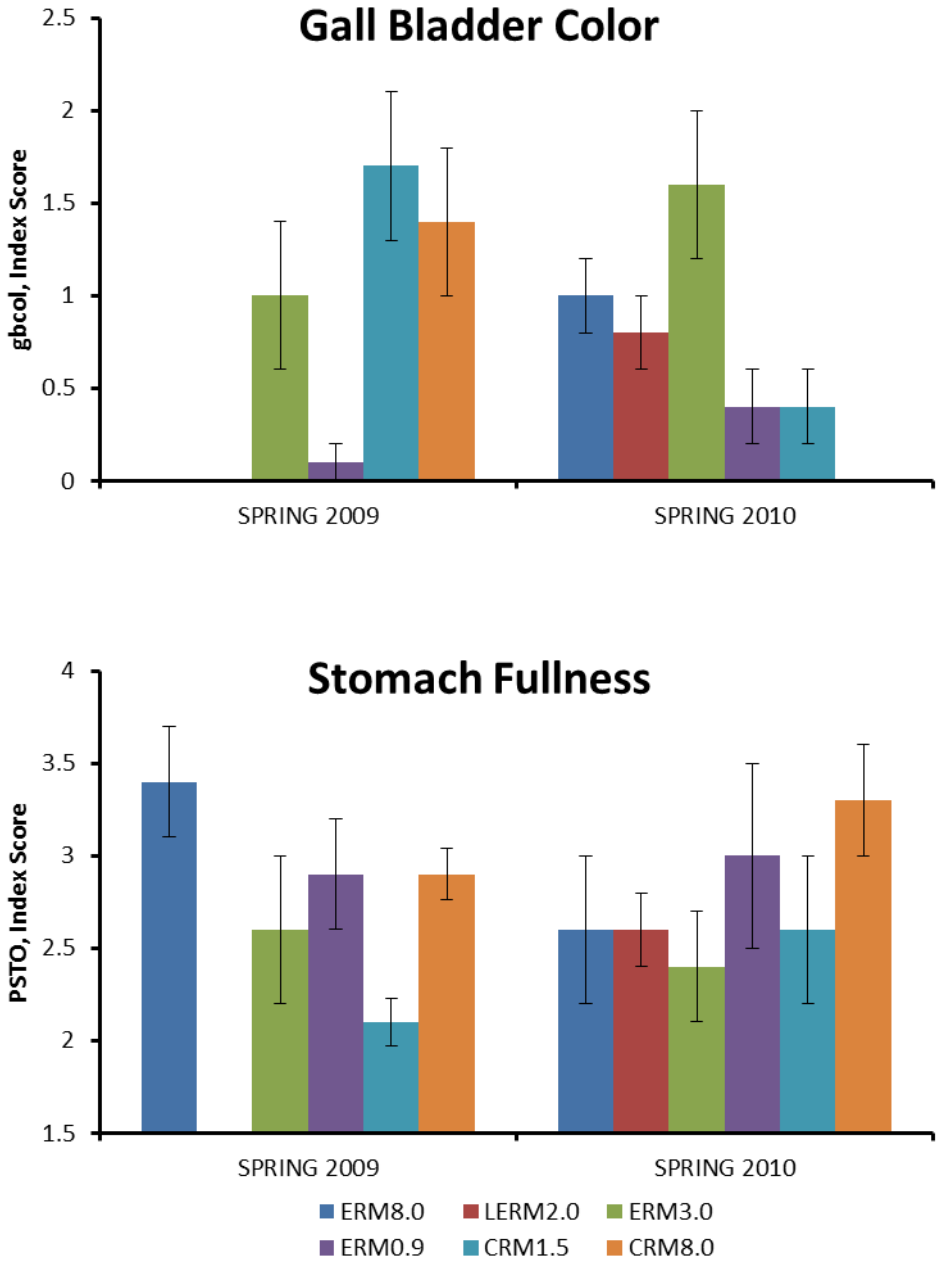


Figure 15. Spatial patterns in feeding and nutrition indicators for largemouth bass during Spring 2009 and Spring 2010.

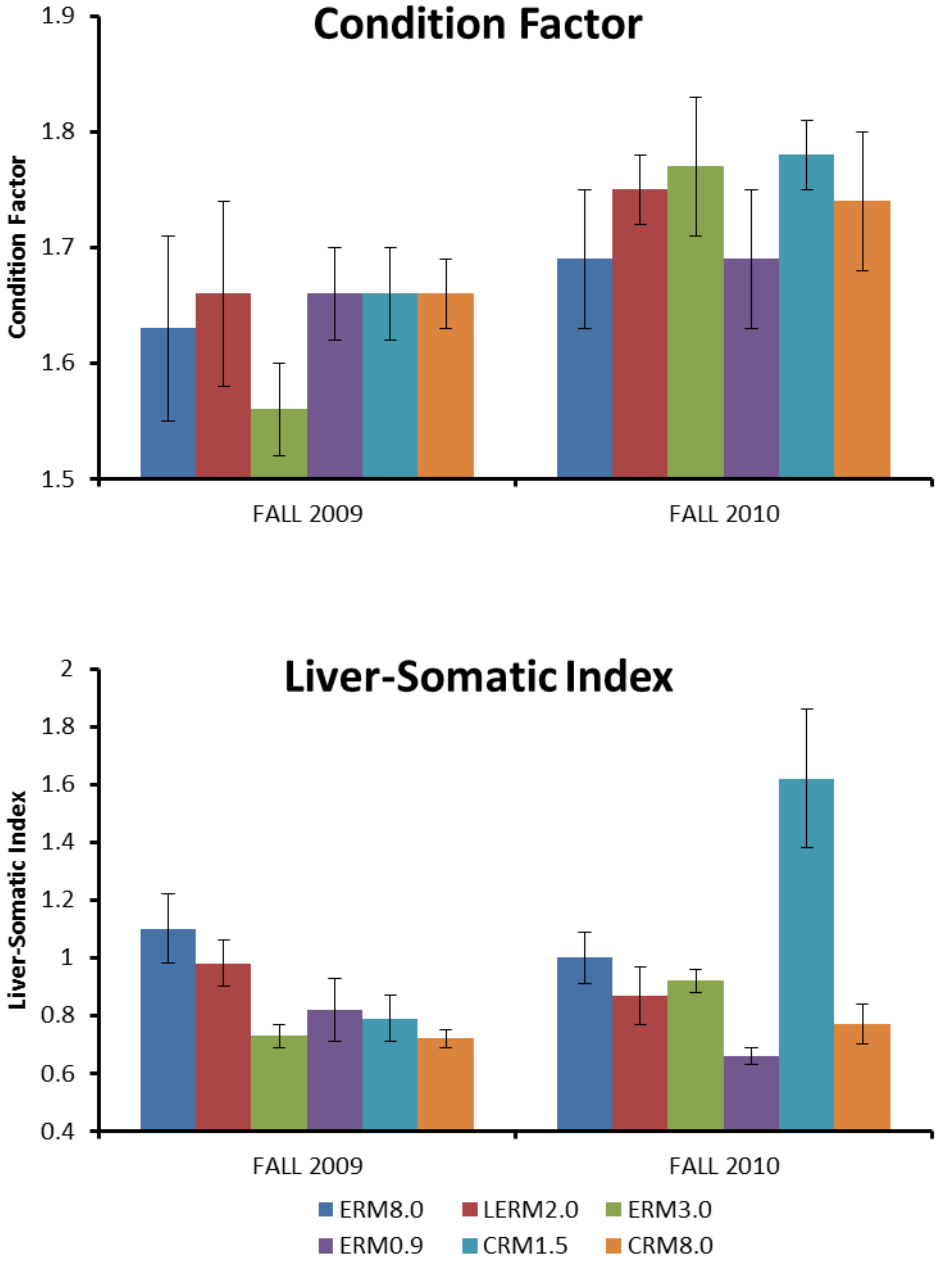


Figure 16. Spatial patterns in condition indices indicators for bluegill sunfish during Fall 2009 and Fall 2010.

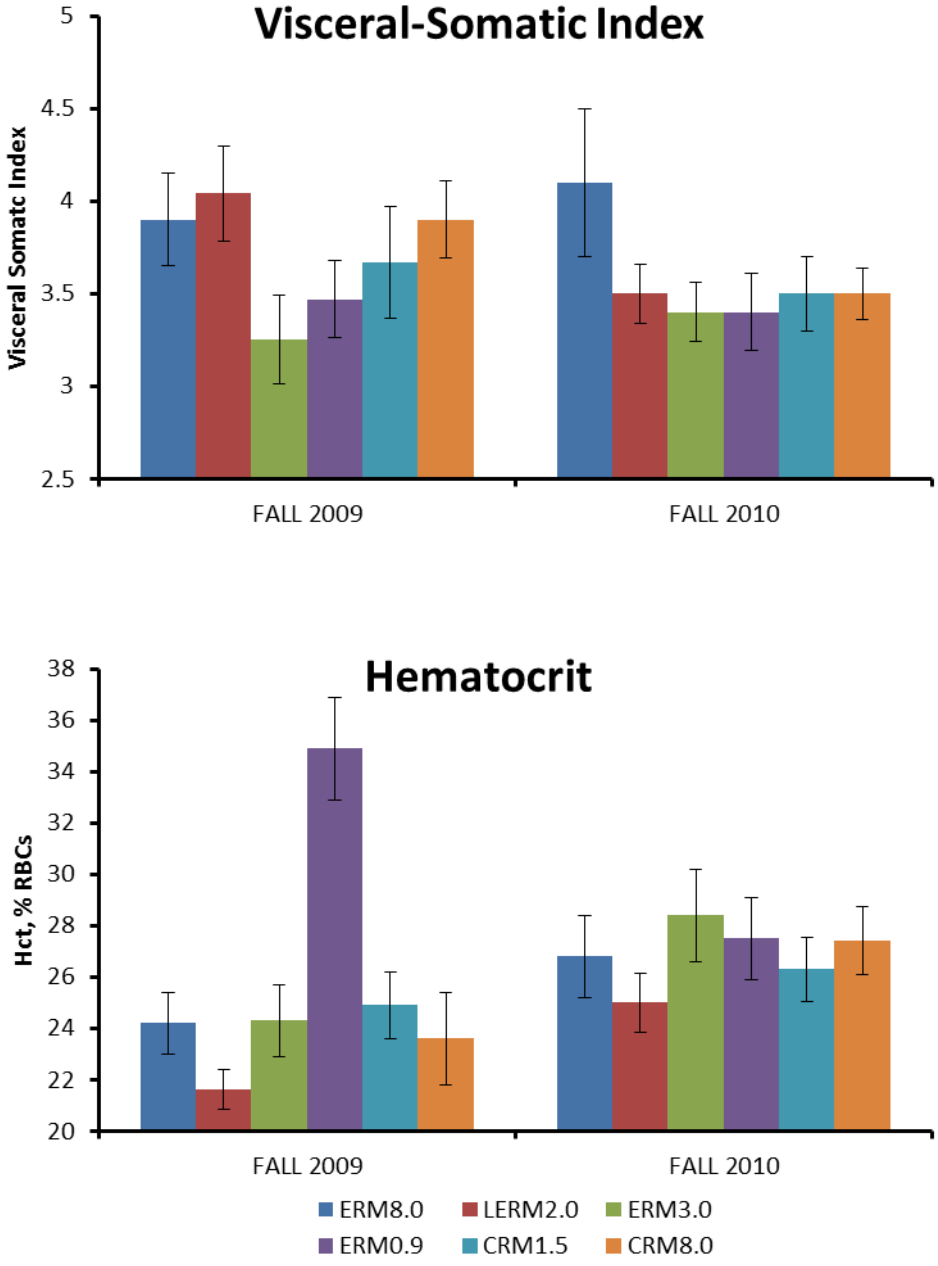


Figure 17. Spatial patterns in bioenergetic and hematological indicators for bluegill sunfish during Fall 2009 and Fall 2010.

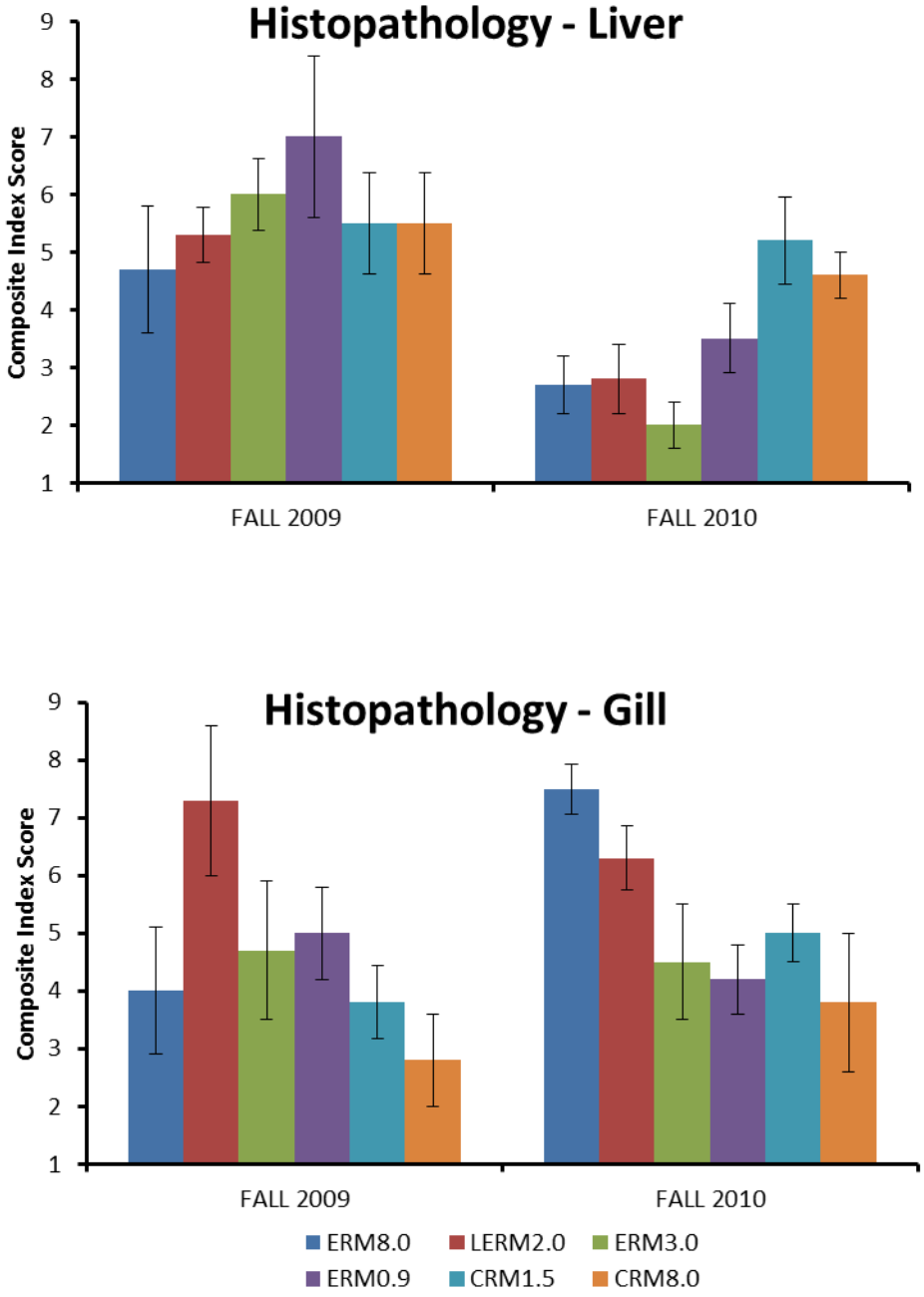


Figure 18. Spatial patterns in histopathological indicators (composite index score) for bluegill sunfish during Fall 2009 and Fall 2010.

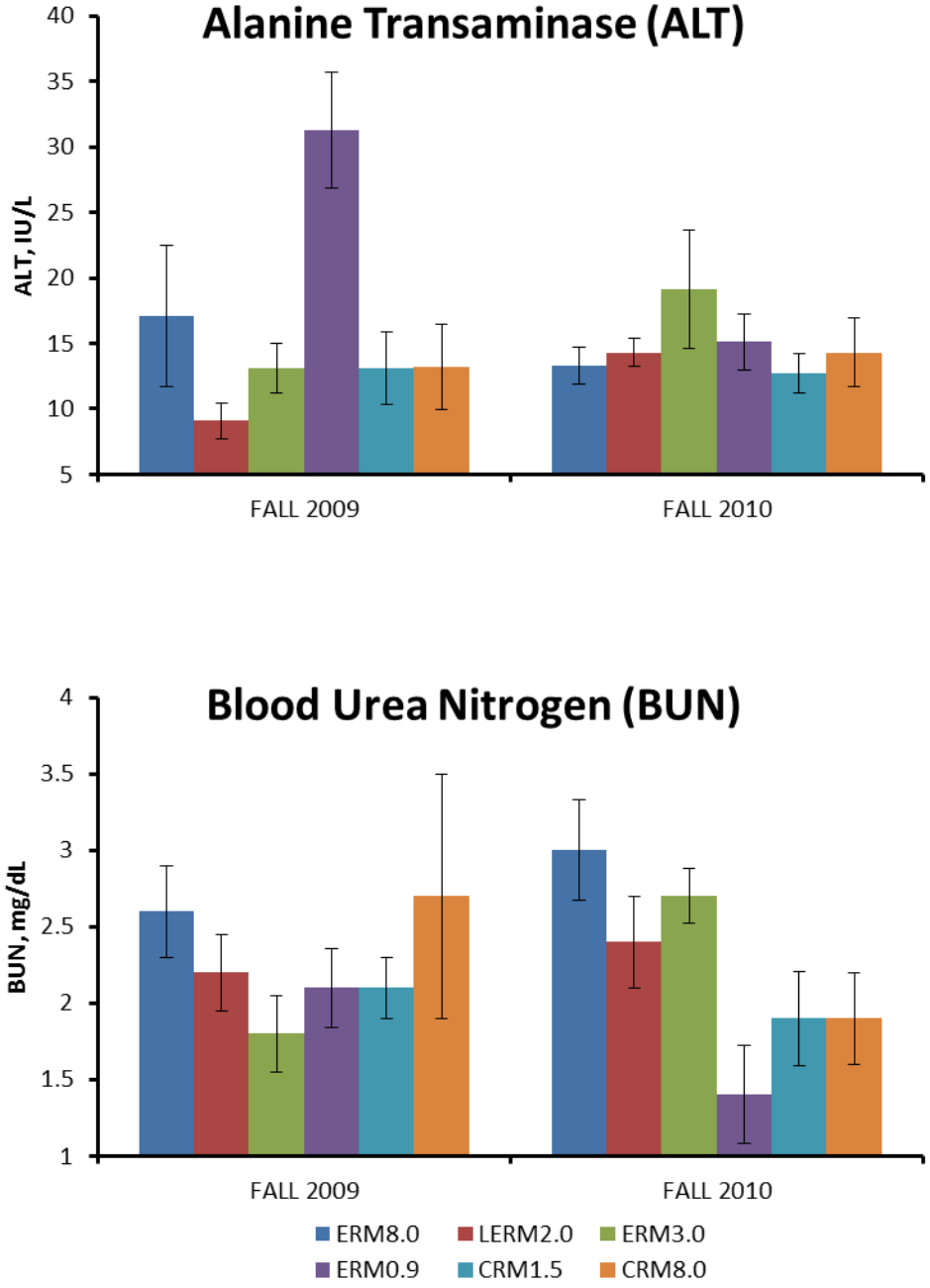


Figure 19. Spatial patterns in organ dysfunction indicators for bluegill sunfish during Fall 2009 and Fall 2010.

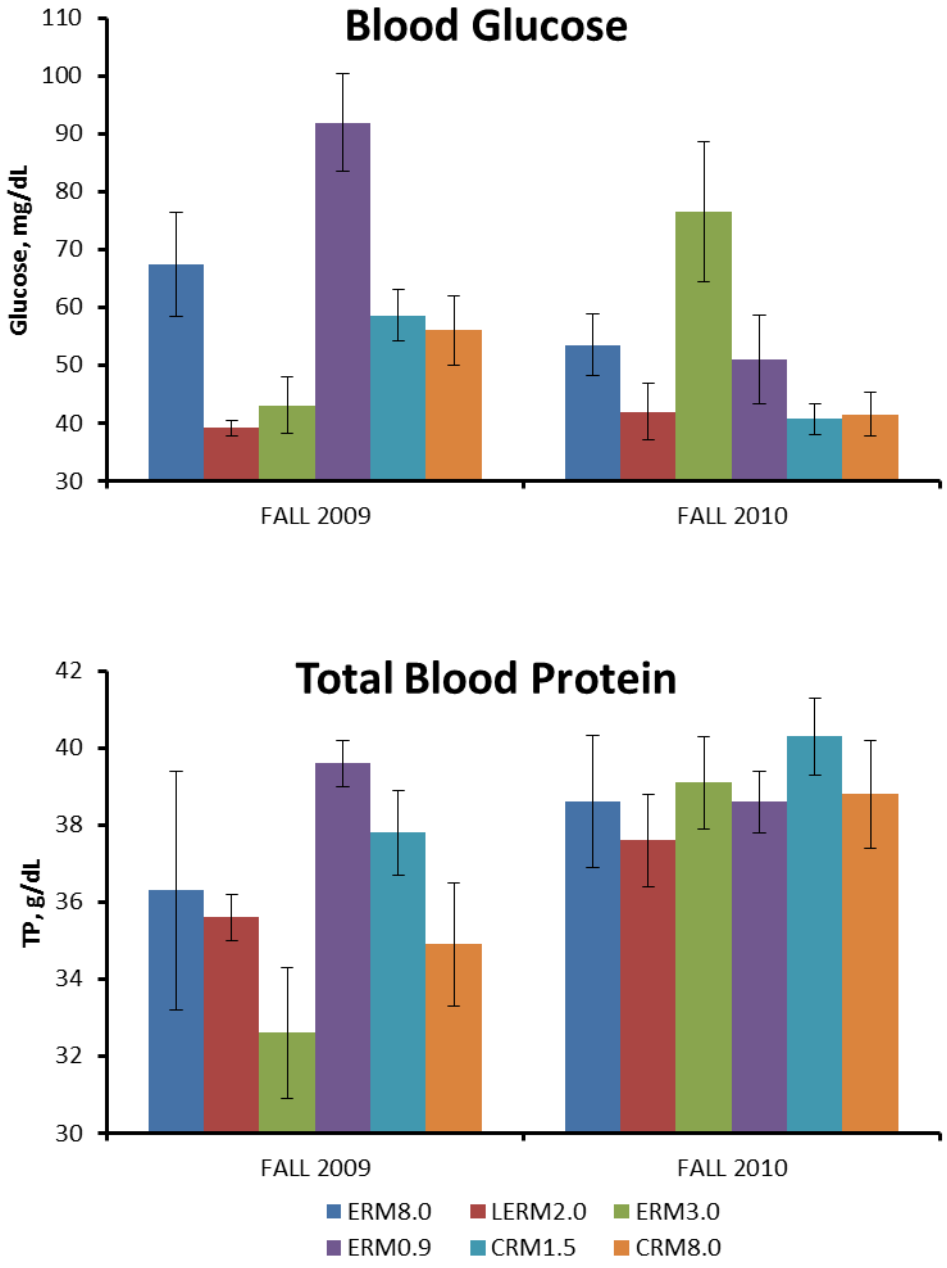


Figure 20. Spatial patterns in carbohydrate-protein metabolism indicators for bluegill sunfish during Fall 2009 and Fall 2010.

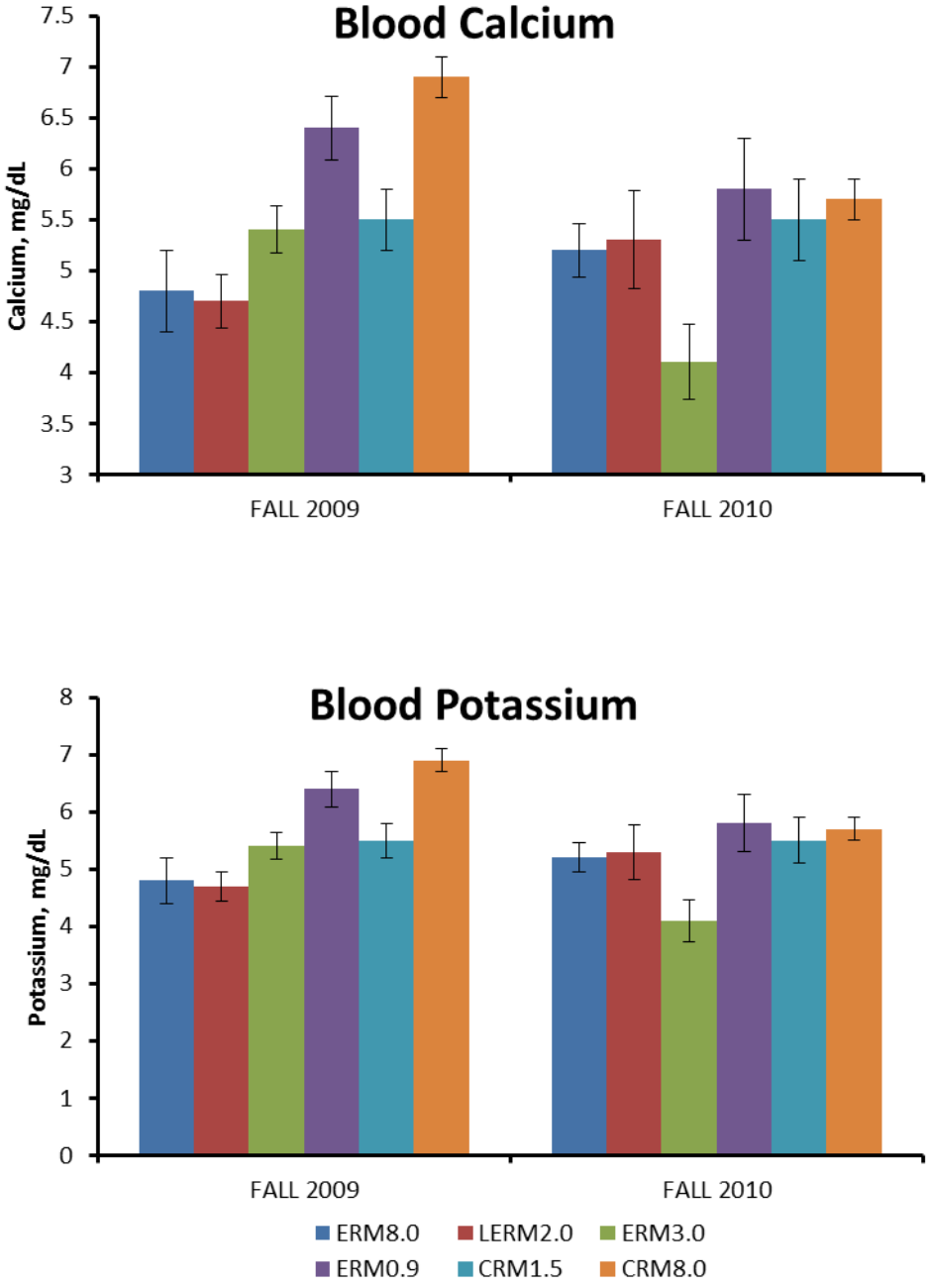


Figure 21. Spatial patterns in electrolyte homeostasis for bluegill sunfish during Fall 2009 and Fall 2010.

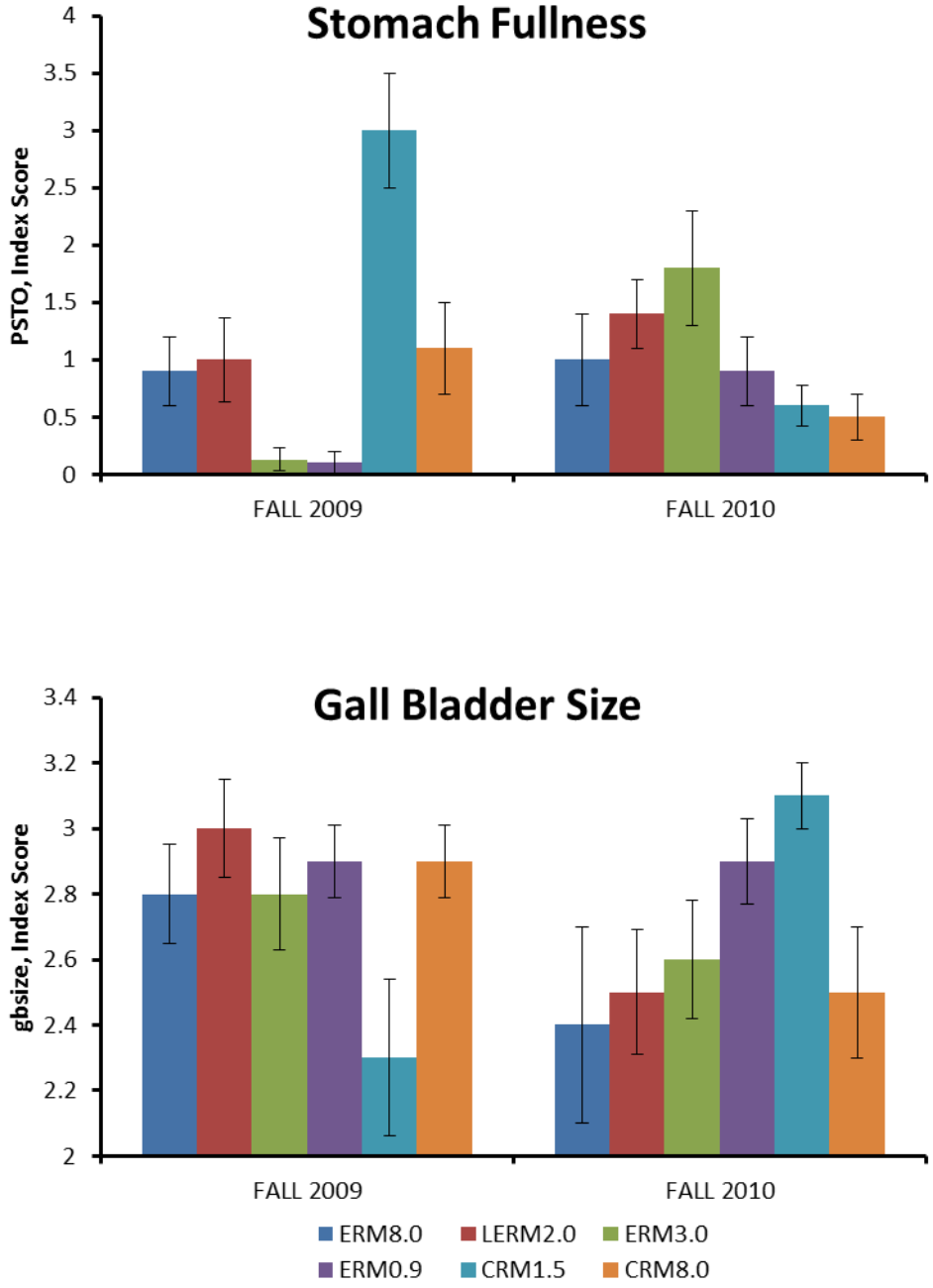


Figure 22. Spatial patterns in feeding and nutrition indicators for bluegill sunfish during Fall 2009 and Fall 2010.

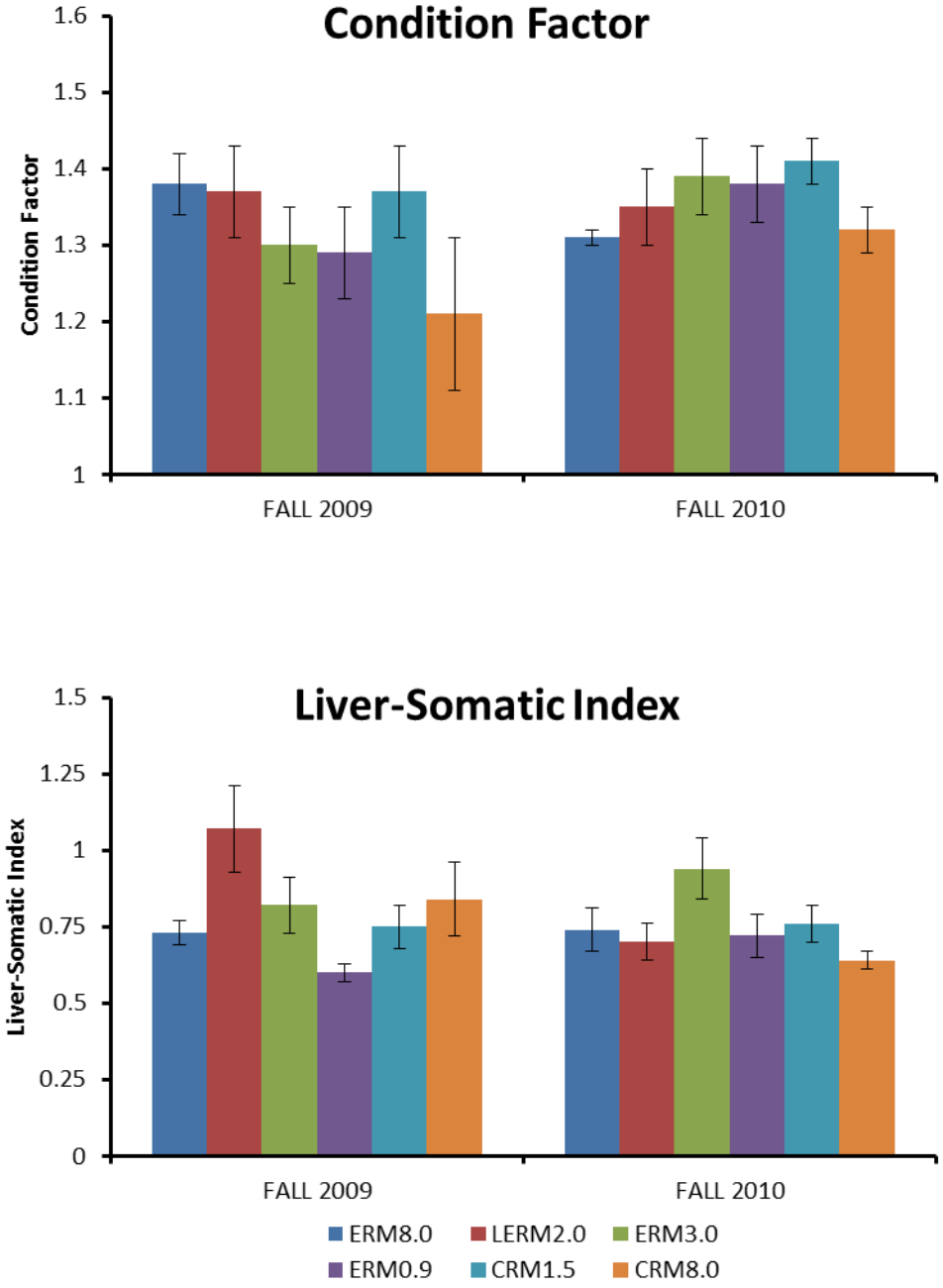


Figure 23. Spatial patterns in condition indices for largemouth bass during Fall 2009 and Fall 2010.

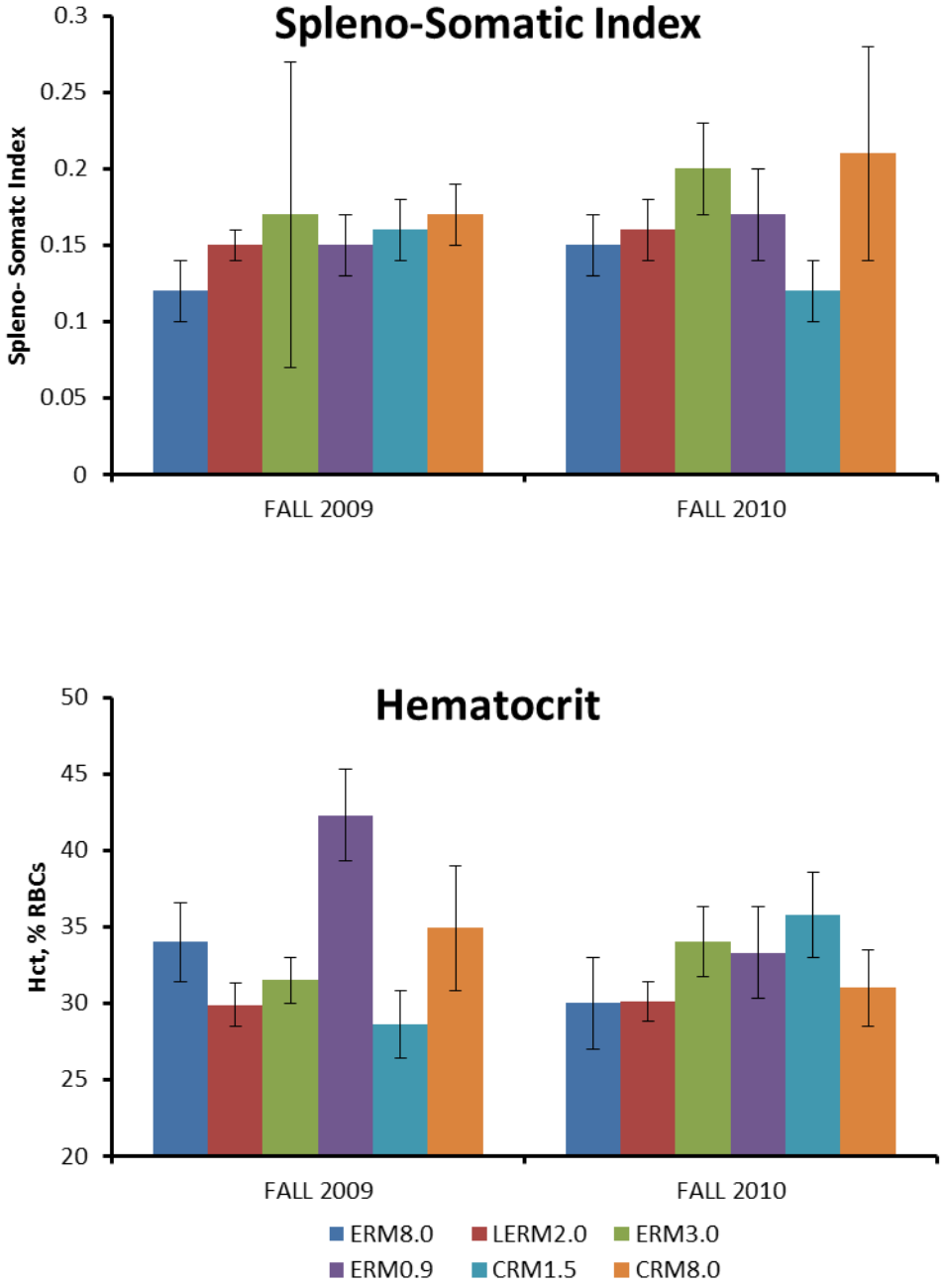


Figure 24. Spatial patterns in hematological indicators for largemouth bass during Fall 2009 and Fall 2010.

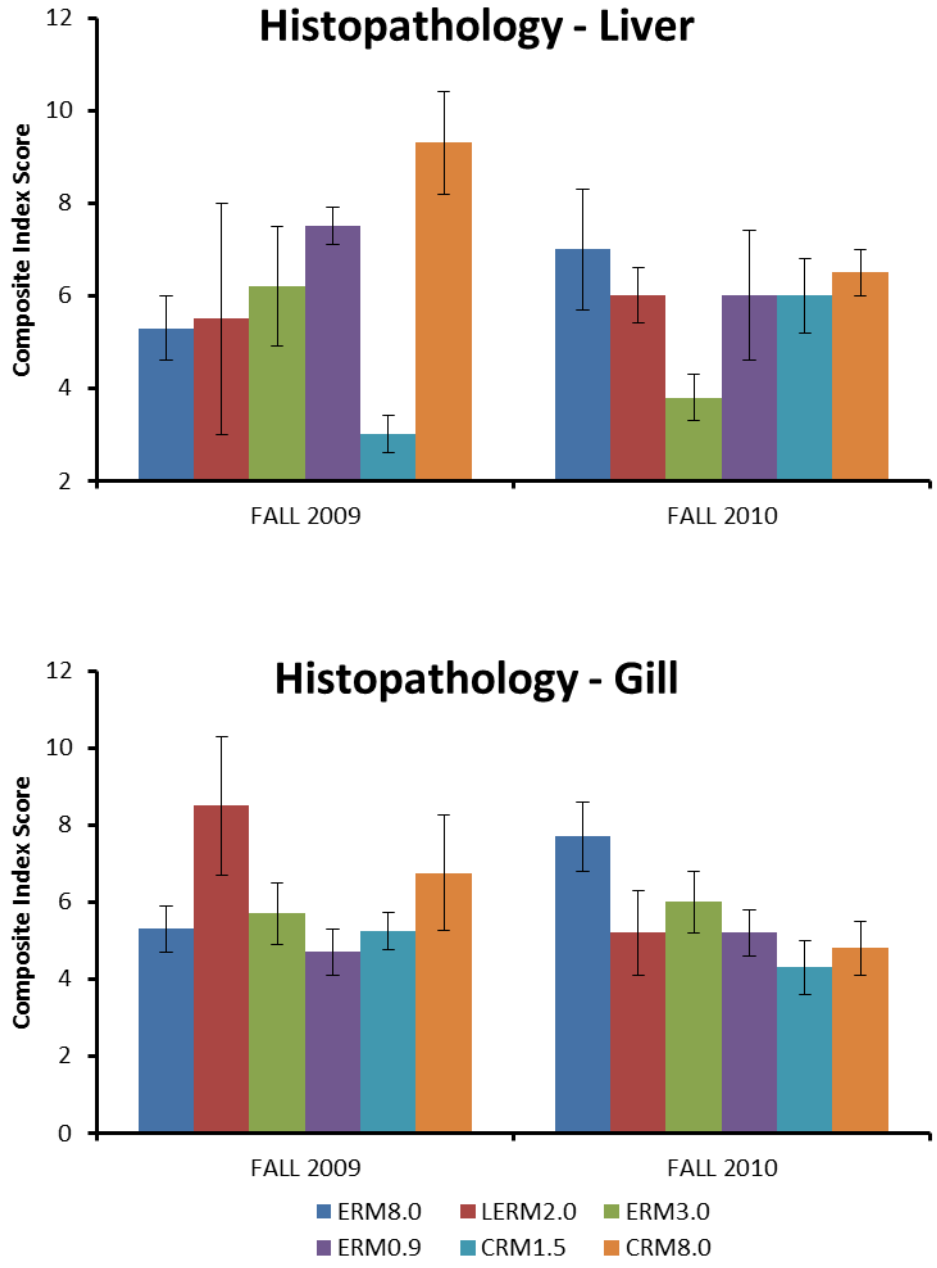


Figure 25. Spatial patterns in histopathological indicators (composite index score) for largemouth bass during Fall 2009 and Fall 2010.

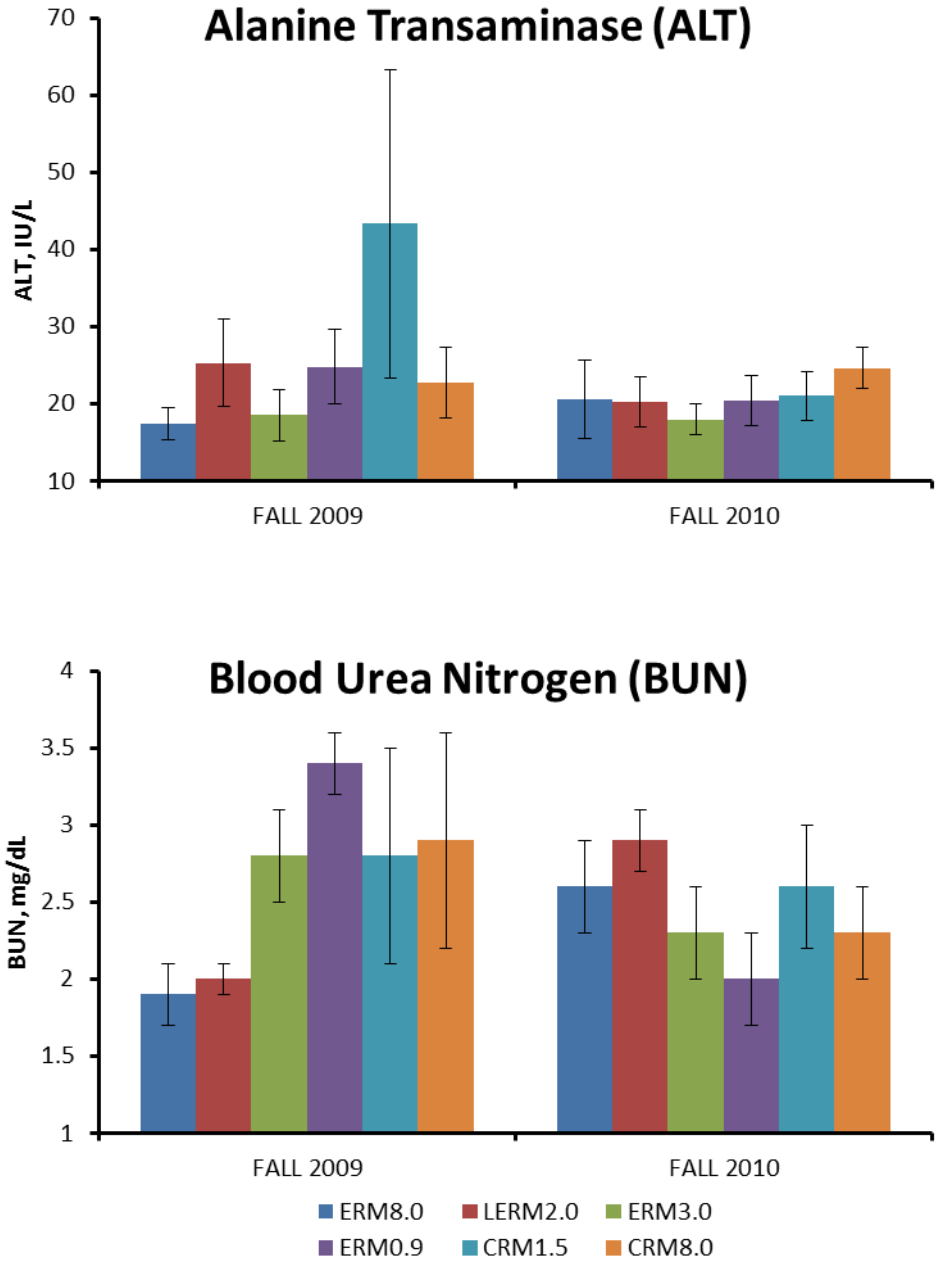


Figure 26. Spatial patterns in organ dysfunction indicators for largemouth bass during Fall 2009 and Fall 2010.

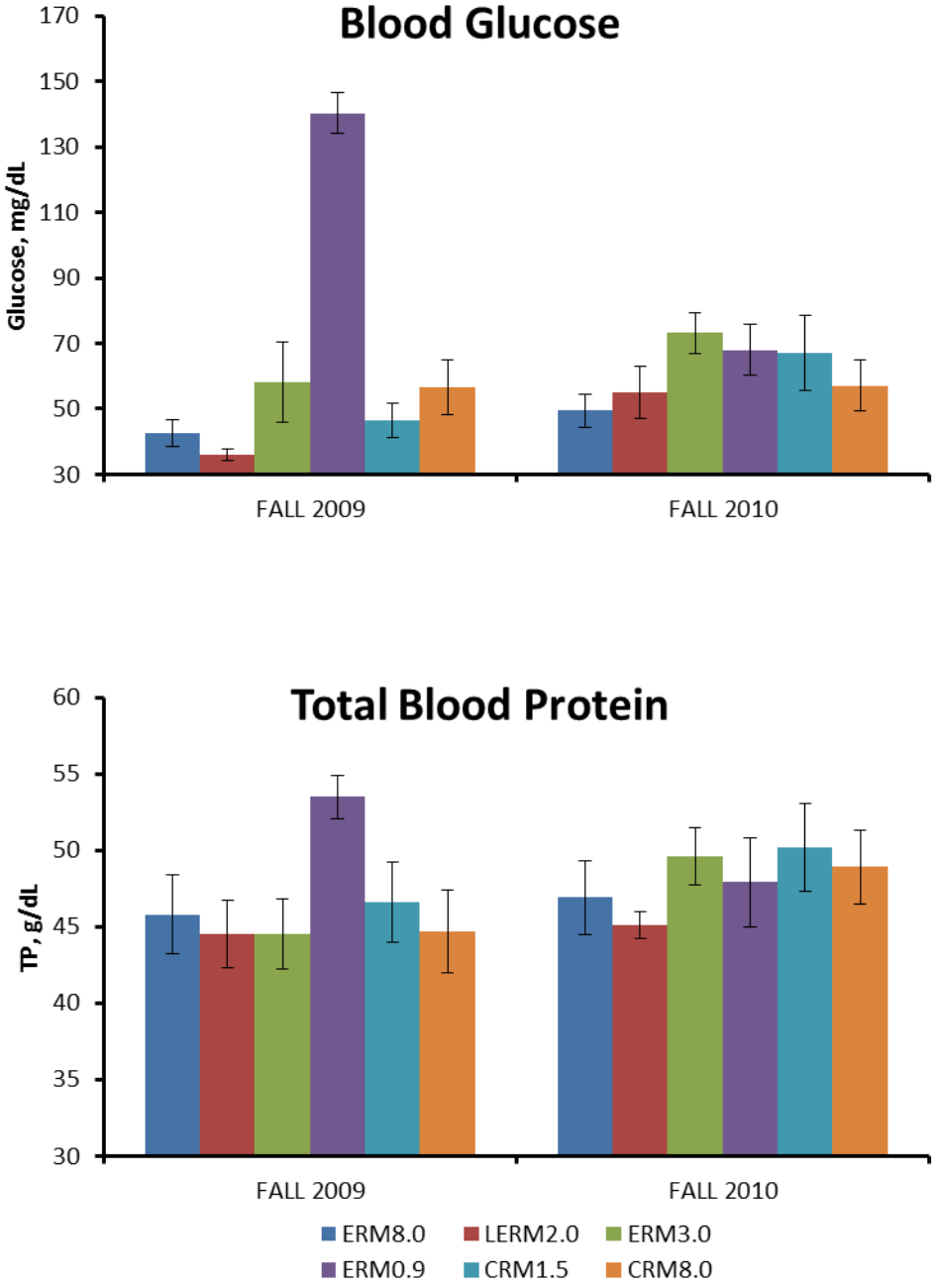


Figure 27. Spatial patterns in carbohydrate-protein indicators for largemouth bass during Fall 2009 and Fall 2010.

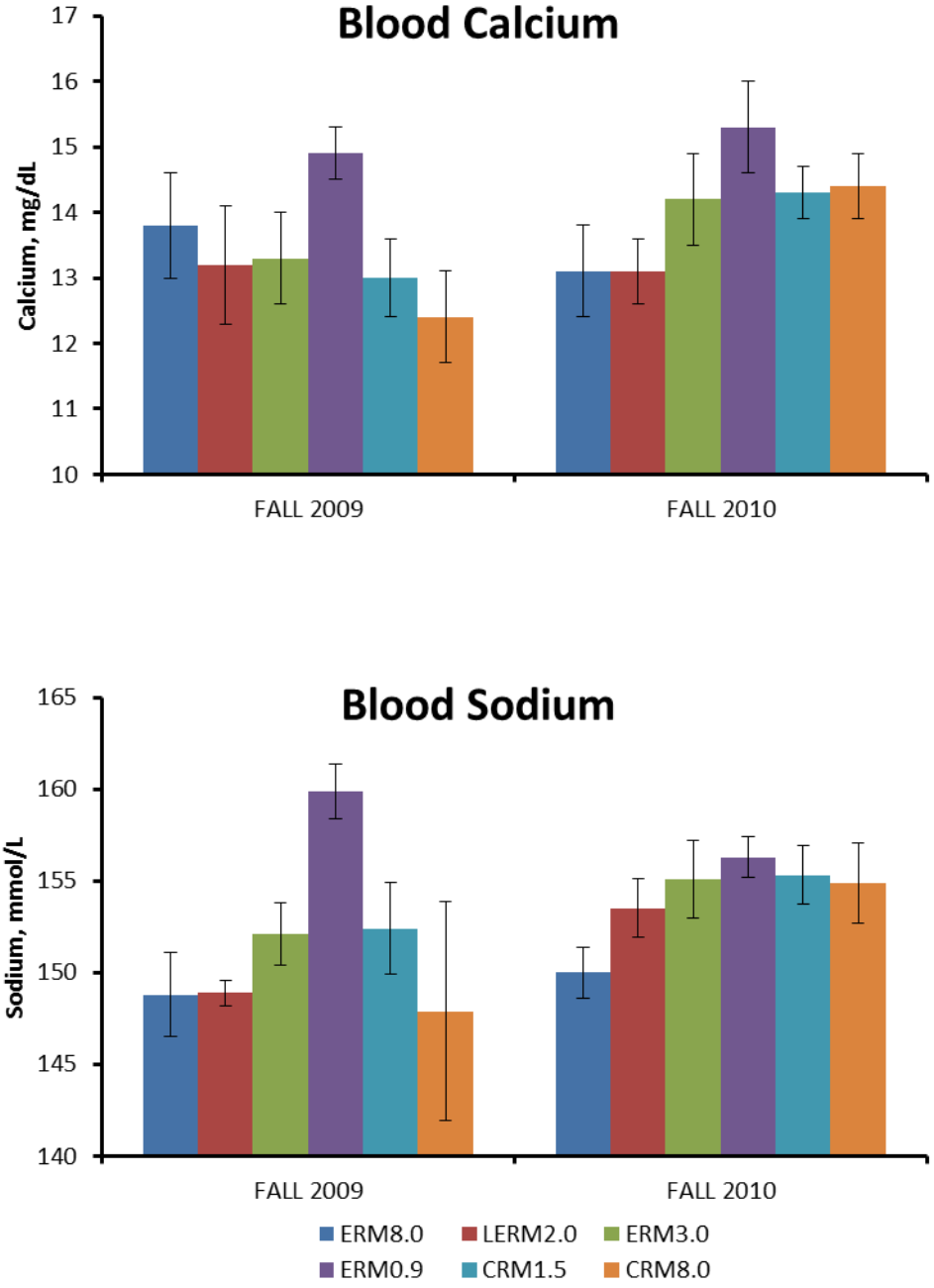


Figure 28. Spatial patterns in electrolyte homeostasis indicators for largemouth bass during Fall 2009 and Fall 2010.

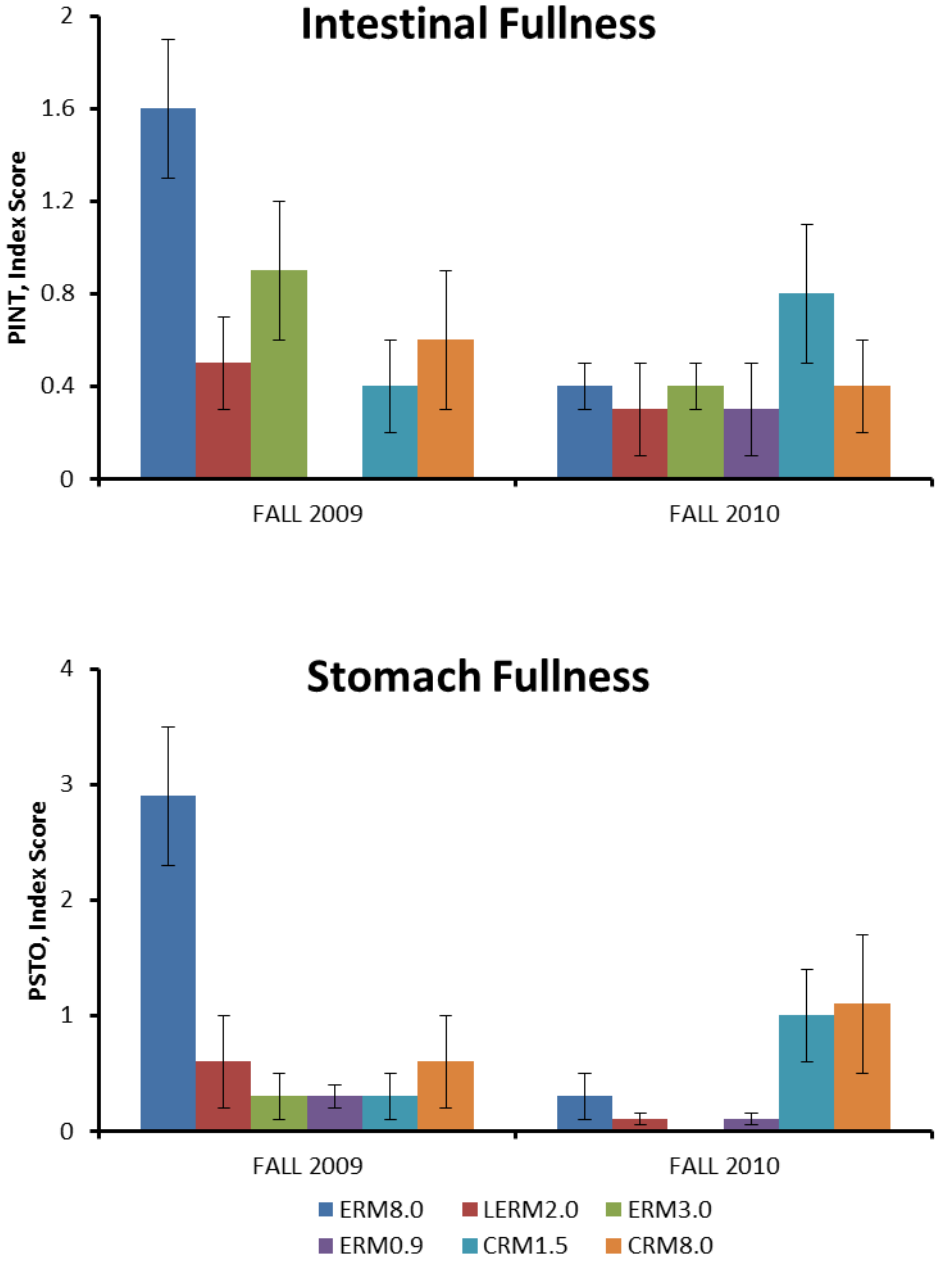


Figure 29. Spatial patterns in feeding and nutritional indicators for largemouth bass during Fall 2009 and Fall 2010.

Channel catfish- Fall 2009 vs Fall 2010

Whereas the spatial response of the condition factor was relatively consistent among sites for largemouth bass in the Spring and Fall of both years and for bluegill in the Fall, the condition factor of channel catfish was highly variable among sites during the Fall of both years (Fig. 30). In Fall 2010, there was also some indication of a spatial gradient with levels being lowest at the references and increasing below the spill. The liver-somatic index was highly variable among sites and seasons with the lower reference levels generally reflecting lower levels of energy storage. The spleno-somatic index was also highly variable among sites during the Fall of both years, however the hematocrit values in Fall 2009 were relatively consistent among sites and levels in Fall 2010 were lower at the references and increased downstream (Fig. 31). Histopathology of the liver displayed somewhat similar spatial patterns both seasons compared to that observed for bluegill and bass with a lower composite lesion index at the reference sites and generally increasing downstream (Figs. 4, 11, 18, 25, 32). Incidences of lesions in the gill also demonstrated a spatial response gradient with lower levels at the references and higher responses downstream (Fig. 32). Albumin, a general indicator of protein metabolism and liver disease under certain conditions, was somewhat similar in Fall 2009 except at ERM 0.9 while in Fall 2010 levels were lowest at the references and increased downstream demonstrating a clear spatial gradient (Fig. 33). The liver enzyme, alanine transaminase, was lowest at the references sites and generally increased downstream. Alkaline phosphatase, an indicator of a variety of diseases and organ functions, was relatively consistent among sites in Fall 2009 but low at the references with increasing downstream levels in Fall 2010. Amylase, a general indicator of the importance of carbohydrates in the diet, was highly variable among sites in Fall 2009 and low at all sites in Fall 2010 except at CRM 8.0 where it demonstrated the highest levels during both Fall periods (Fig. 34). Total protein and globulin had similar spatial patterns among sites in Fall 2009 and in Fall 2010 and also reflected the spatial pattern observed for albumin (Figs. 33 & 35). This is not surprising given that globulin and albumin are both protein-based compounds. During the Fall of both years, a spatial gradient in blood calcium is also evident with lower values at the references and higher levels downstream while sodium levels in Fall 2010 were relatively consistent among sites (Fig. 36).

White Crappie – Spring 2009 vs Spring 2010

The condition factor of white crappie displayed a more distinct spatial pattern both seasons compared to the other species, being generally lower at the upstream reference sites and increasing downstream (Fig. 37). In Spring 2009 the liver-somatic index was similar among sites but displayed a spatial pattern similar to that of the condition factor in Spring 2010 (Fig. 37). Only one white crappie could be collected in Spring 2009 at ERM 3.0 because of the scarcity of preferred habitat for this species in the sample area, therefore, the data point for this site is missing in Figs. 37-42. For both seasons, the visceral-somatic index increased downstream of the reference (except for ERM 3.0 in Spring 2010 which has poor habitat) while the hematocrit also had somewhat of a downstream spatial gradient both seasons being more obvious in Spring 2010 (Fig. 38). No definitive spatial gradients are obvious for the liver and ovarian histopathology index; however, the ovarian composite index was higher both seasons at ERM 8.0 due primarily to parasitic lesions (Fig. 39). The liver enzyme, alanine transaminase, was similar across sites in Spring 2009 but displayed a rather strong downstream gradient in Spring 2010 with this response being very low at the references and peaking at ERM 0.9 and CRM 8.0 (Fig. 40). The indicator of gill dysfunction, BUN, did not show any obvious changes across sites during either Spring period but responses were much lower in Spring 2010 possibly indicating some level of recovery in this organ dysfunction indicator over the year compared to 2009 (Fig. 40). Both glucose and total blood protein demonstrated spatial changes during the Spring of both years with glucose and total protein levels being generally lower at the reference sites and higher downstream (Fig. 41). Relatively slight or moderate spatial patterns are obvious for potassium during both Spring

periods and also for sodium in Spring 2009 where levels were generally lower upstream and increased somewhat at most downstream sites (Fig. 42).

Redear sunfish – Spring 2010

Studies on redear sunfish were initiated in Spring 2010, therefore no temporal or seasonal comparisons are available for this species. There was no obvious spatial pattern in the condition factor or liver-somatic index in Spring 2010 even though there was a large elevation in the liver-somatic index at ERM 3.0 (Fig. 43). There was, however, an obvious downstream response in the visceral-somatic index with highly elevated levels at the two lower Emory River sites and at ERM 1.5 (Fig. 44). Even though the hematocrit was elevated at one of the reference sites, three of the downstream sites were much higher than the other reference, ERM 8.0. Incidences of lesions in the liver and ovary did not display distinct spatial patterns except liver lesions in the Clinch River were much lower than in the Emory River with ovarian lesions being similar among sites except for an elevated index at ERM 1.5 (Fig. 45). No spatial patterns were obvious for albumin (a general indicator of liver dysfunction and also nutritional status) or blood urea nitrogen during the Spring of 2010 (Fig. 46). There was a slight indication that blood glucose increased downstream of the references but because of an elevated level at one of the reference sites and reduced value at ERM 3.0, such a spatial pattern is not definitive (Fig. 47). Likewise, the total protein response was similar among sites except for a slightly depressed level at ERM 3.0 (Fig. 48). Even though calcium levels were elevated somewhat at the two Clinch River sites, no real spatial pattern in this electrolyte exists among sites in the Emory River, however there is some indication that potassium is decreased downstream of the reference sites (Fig. 48).

Integrated health responses

Canonical discriminant analysis provides a simplified graphical presentation of the integrated health status of fish at each site based on using all the individual health measures together in a holistic context. In interpreting the results of the discriminant analysis, the position of each circle (sample site) in relation to the two canonical axes is not important but the relationship or position of the circles to each other serves as a basis for evaluating the integrated health status of fish at each site. In addition, the linear statistical distances between the means of each circle (sample sites) can be measured quantitatively by the Mahalanobis distance which is the linear statistical distance between the midpoints of two canonical responses or circles (for example, dashed line in Fig. 49). Also, the greater the distance between circles, the greater the difference between sample sites in the integrated health responses of fish. The further away the circles or integrated responses are away from each other the more dissimilar fish are in their health status. On a temporal basis, if circles or sample sites move closer to each other over time, then the health response of fish becomes more similar whereas if circles move further apart over time, then fish become less similar relative to their comparative health status.

The integrated health status of fish is presented in this section as five main groups of temporal or seasonal comparisons including 1) bluegill in Spring of 2009 versus bluegill in Spring of 2010, 2) largemouth bass in Spring of 2009 versus Spring 2010, 3) bluegill in Fall of 2009 versus Fall 2010, 4) largemouth bass in Fall of 2009 versus Fall 2010, and 5) channel catfish in Fall of 2009 versus Fall 2010. In addition, the health status of two other species, white crappie and redear sunfish, are each presented on a spatial basis for one season (Spring 2010) because in Spring 2009 white crappie could not be collected at two sites due to scarcity of preferred habitat for this species, and sampling for redear sunfish was not initiated until Spring 2010. Bluegill and largemouth bass collected in the Spring consisted of all females because of the focus on reproductive integrity during this period; however, fish collected in the Spring could not be compared on a temporal basis to fish collected in the Fall because individuals then consisted of a mix of males and females. Therefore, a comparison of all female fish in the spring to a mix of

both sexes in the Fall would not be relevant on a biological basis because of the seasonal differences in physiology between males and females and also differences in reproductive status between seasons.

Bluegill - Spring 2009 vs Spring 2010

In Spring 2009 the health status of bluegill from CRM 1.5 (green circle) was more similar to the reference (red circle) than were fish from the remaining sites including ERM 3.0, ERM 0.9 and CRM 8.0 (Fig. 49, top panel). These latter three sites were about the same distance from the reference (as indicated by the linear statistical distances) and therefore somewhat similar in their health status compared to the reference. One year later in Spring 2010, the health of bluegill from ERM 0.9 was more similar to the references than in 2009 (ERM 0.9 moved closer to the reference as shown by the decrease in the Mahalanobis or linear statistical distance between the integrated site responses) (Fig. 49-bottom panel). In this case the statistical linear distance between the reference and ERM 0.9 was 14 units in Spring 2009 and decreased to 10 units in Spring 2010. Over this period, however, bluegill from CRM 1.5 became less similar in their health status to the reference fish while the overall health of fish from ERM 3.0 and CRM 8.0 did not change appreciably over this period. It is noteworthy that the two reference sites (ERM 8.0 and Little Emory) were very similar in their health status in Spring 2010 which provides credibility that this holistic procedure is an effective and reliable approach for assessing the integrated health status of fish among sites. The specific metrics entered or used in the discriminant analysis procedure are listed on the right side of Fig. 49 and represent indicators of condition indices, hematology, electrolyte homeostasis, organ dysfunction, histopathology, nutrition and feeding, and carbohydrate-protein metabolism. Metrics in red within this list are those responses that were the most influential in discriminating among sample sites based on the results of the stepwise variable selection procedure.

Largemouth bass - Spring 2009 vs Spring 2010

Even though the health of largemouth bass from ERM 3.0 (blue circle) was the most similar to the reference compared to bass from the other three sites (blue circle), relatively long distances separate the other three sites from the reference indicating a large difference in health status compared to the reference condition. These other three sites are approximately equidistant (or equally dissimilar) to the reference with CRM 1.5 being the most dissimilar because of its greater linear distance from the reference (Fig. 50). In Spring 2010 there was a rather large shift in the positions of the sites relative to the reference. Both ERM 0.9 (Spring 09= 20 units and Spring 2010=16 units) and CRM 1.5 (Spring 09=32 units, Spring 10=14 units) moved closer to the reference indicating improvement in fish health while bass at ERM 3.0 moved further away (10 vs 18) reflecting a transition to poorer health over the year for bass collected from this site. As was also observed for bluegill between Spring 09 and 10, health of bass at CRM 8.0, the positive control site, maintained the same health status over the year because it remained approximately equidistant from the reference both years. As was also seen for bluegill during the Spring, the two reference sites were similar to each other. The specific metrics entered or used in the discriminant analysis procedure are listed on the left side of Fig. 50 and represent .indicators of condition indices, hematology, electrolyte homeostasis, organ dysfunction, histopathology, nutrition and feeding, and carbohydrate-protein metabolism. Metrics in red within this list are those responses that were the most influential in discriminating among sample sites using a stepwise regression procedure.

Bluegill - Fall 2009 vs Fall 2010

In Fall 2009 health of bluegill from CRM 1.5 were very similar to the reference sites with fish from the three remaining sites demonstrating a large difference in health status compared to the reference (Fig. 51). Over the year, bluegill from the two sites immediately below the spill (ERM

3.0 and 0.9) moved quite close to the references while bluegill from CRM 1.5 moved further away and those from CRM 8.0 maintained approximately the same distance from the reference as that observed in Spring 2009. In the spring of both years the two reference sites maintained close proximity to each other which would be the expected behavior of those areas not subjected or impacted by the same environmental factors as those sites influence by the ash spill.

Largemouth bass - Fall 2009 vs Fall 2010
As was also observed in Fall 2009 for bluegill, bass from CRM 1.5 had a very similar health status compared to the references (Fig. 52). Another similarity to bluegill in Fall 2009 is that bass from ERM 0.9 are most dissimilar to the references while fish from CRM 8.0 are intermediate in their similarity (i.e., distances from reference sites) to the references. Interestingly, bass from ERM 3.0 were also very similar to the reference fish. In Fall 2010 two major shifts in health status occurred compared to the previous Fall. Even though bass from CRM 1.5 maintained a consistent health status over the year, fish from ERM 0.9 moved closer to the references while those from ERM 3.0 moved further away. As has been observed in the previous comparisons between seasons for both bluegill and bass, the positive control site (CRM 8.0) shifted very little or at all relative to the references during the year.

Channel Catfish - Fall 2009 vs Fall 2010
A very similar spatial pattern in fish health status appears to exist between largemouth bass and channel catfish during Fall 2009. In both cases, fish from CRM 1.5 and ERM 3.0 are very similar to the reference sites while fish sampled from ERM 0.9 are the most dissimilar to the reference and those from CRM 8.0 are intermediate in health status between ERM 0.9 and ERM 3.0 (Fig. 53). Over the period from Fall 2009 to Fall 2010, the health status of catfish collected from all sites did not change appreciably, with all Emory and Clinch River sites being approximately the same distance from the references during both Fall periods. It is interesting to note that compared to bluegill and bass, the health of catfish did not appear to change over a year including both Spring and Fall periods.

White Crappie – Spring 2010
Compared to bluegill, largemouth bass, and channel catfish, only a few white crappie could be collected at some sites in Spring 2009 due to poor availability of preferred habitat for this species. Even though some white crappie were collected in Spring 2009 at all sites, at two sites only 1-2 individuals were captured despite repeated sampling efforts and, therefore, the discriminant analysis could not be performed because of a low sample size. In Spring 2010, however, an adequate number of white crappie were collected at each site to perform the discriminant analysis procedure. On a spatial basis, the health status of the two reference sites were very similar to each other (Fig. 54). Compared to CRM 1.5, CRM 8.0, and ERM 0.9, fish from ERM 3.0 were the most similar to the reference fish. Crappie from ERM 0.9 were the most dissimilar while individuals from CRM 8.0 and CRM 1.5 were approximately equidistant or similar in their health status compared to the reference.

Redear sunfish – Spring 2010
Sampling for redear sunfish was initiated in Spring 2010 therefore no data are available for the Spring 2009 to make temporal comparisons of health status. As opposed to all other species for both sample seasons, the health status of redear at the two references was not similar (Fig. 54). Because of the dissimilarity of the two reference sites, there are no obvious spatial patterns or differences among sites. As will be addressed in the discussion section, redear are specialized feeders consuming primarily mollusks such as snails and small bivalves. The benthic macroinvertebrate bioaccumulation studies have shown that there is a wide difference among sites in the availability of mollusks as food items for redear; therefore, large spatial differences in

preferred food items among sites could affect the nutritional and feeding status of this species and their overall health and condition which may have influenced, therefore the relationship of the two reference sites to each other.

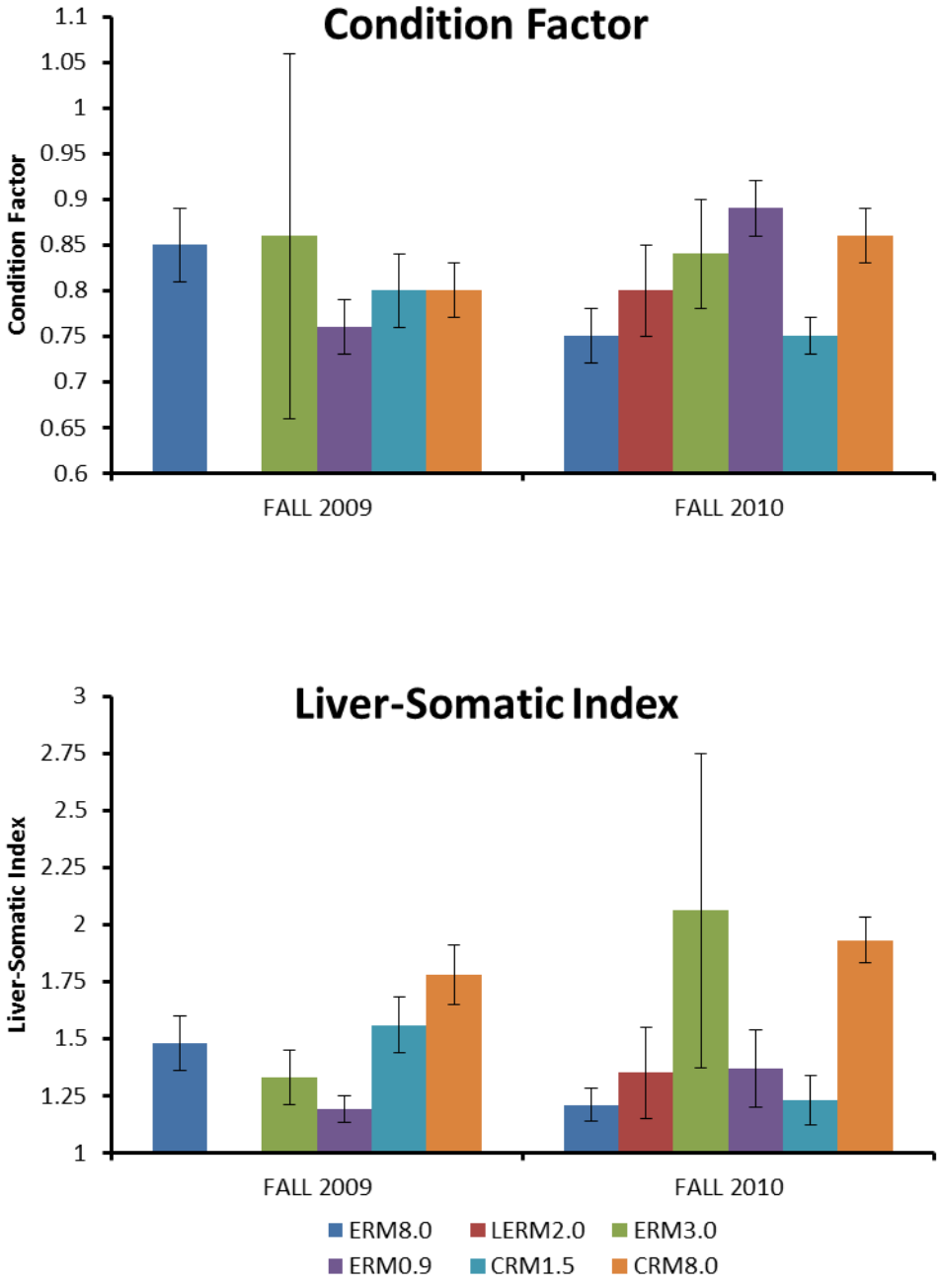


Figure 30. Spatial patterns in condition indices for channel catfish during Fall 2009 and Fall 2010.

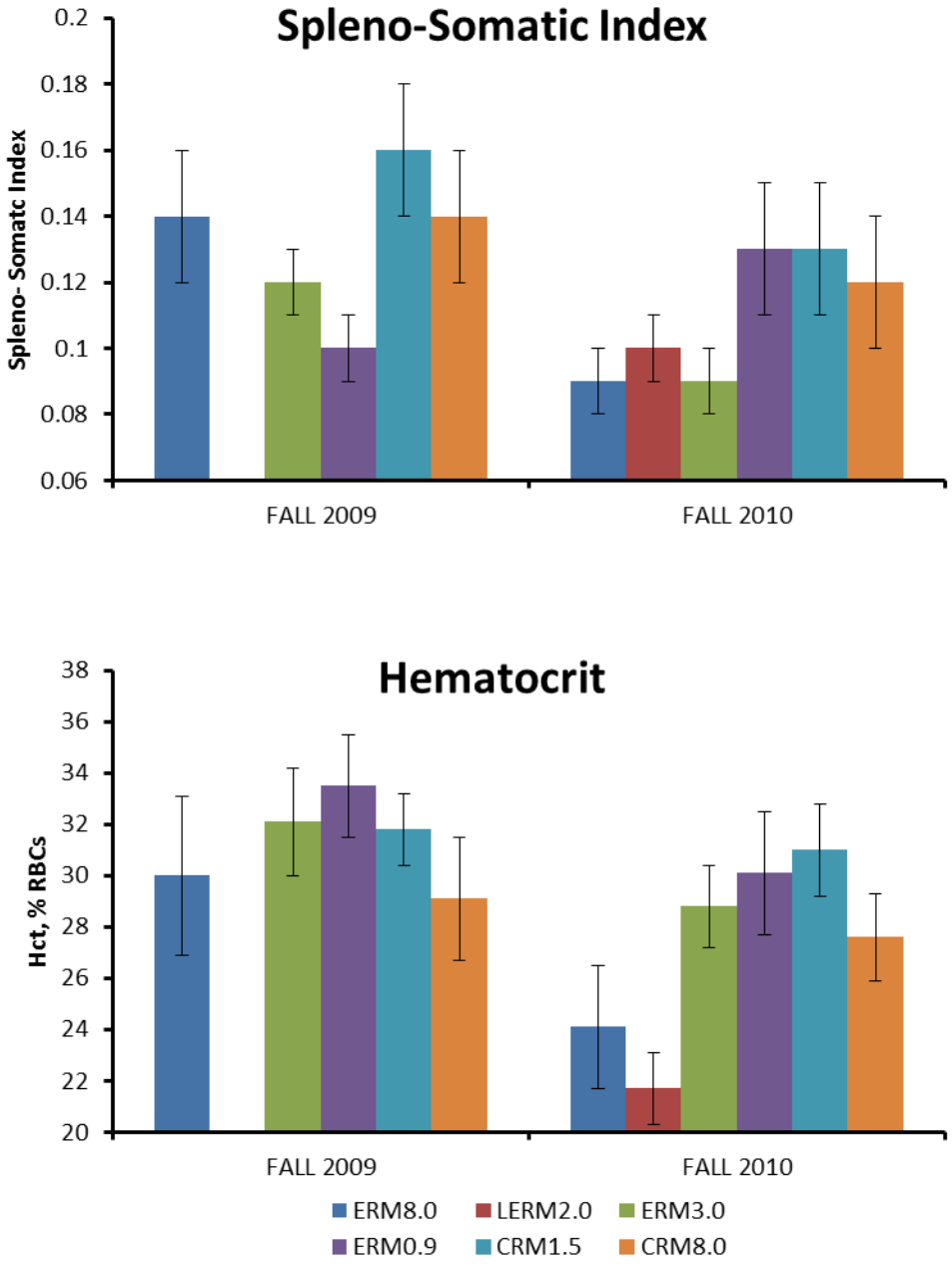


Figure 31. Spatial patterns in hematological indicators for channel catfish during Fall 2009 and Fall 2010.

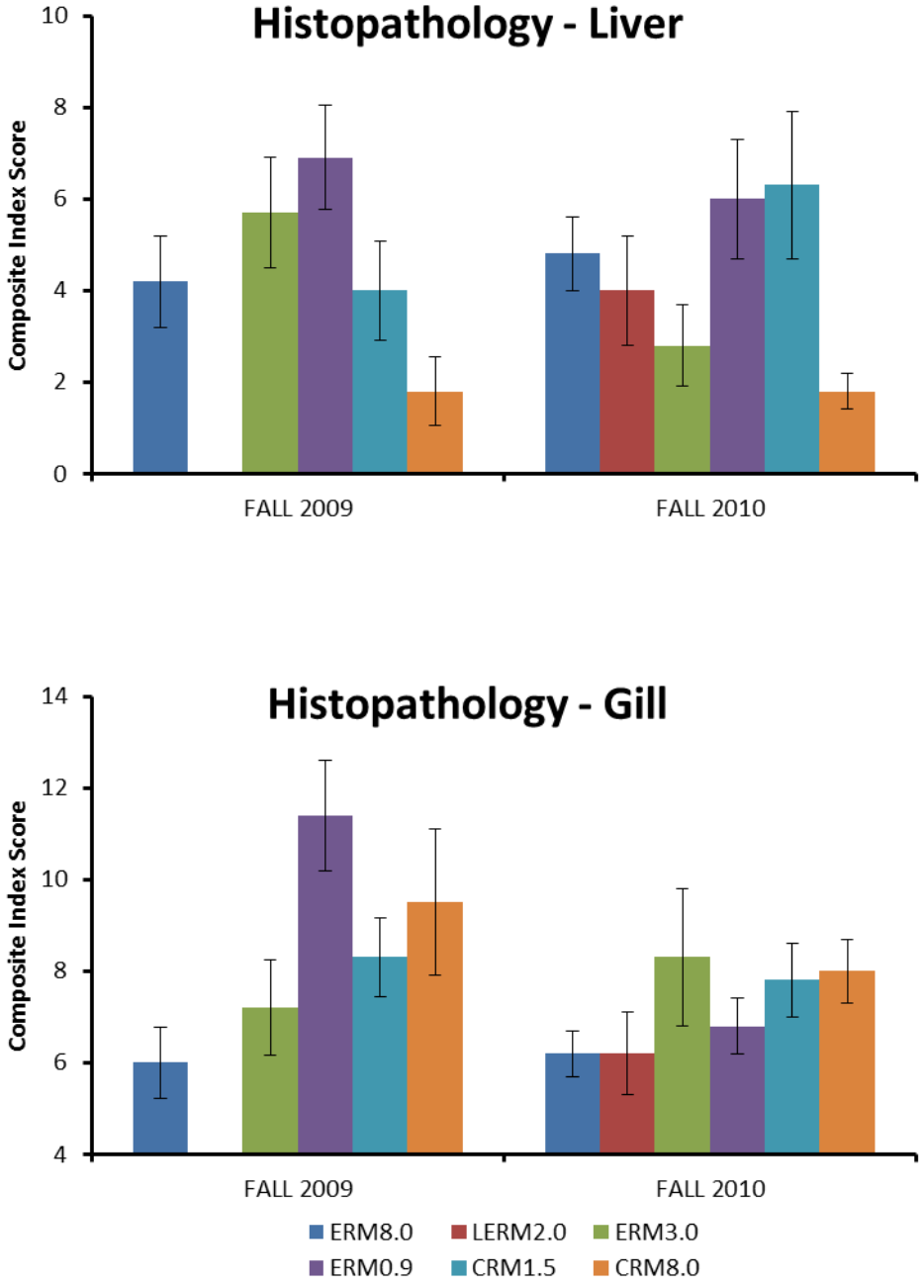


Figure 32. Spatial patterns in histopathological indicators (composite index score) for channel catfish during Fall 2009 and Fall 2010.

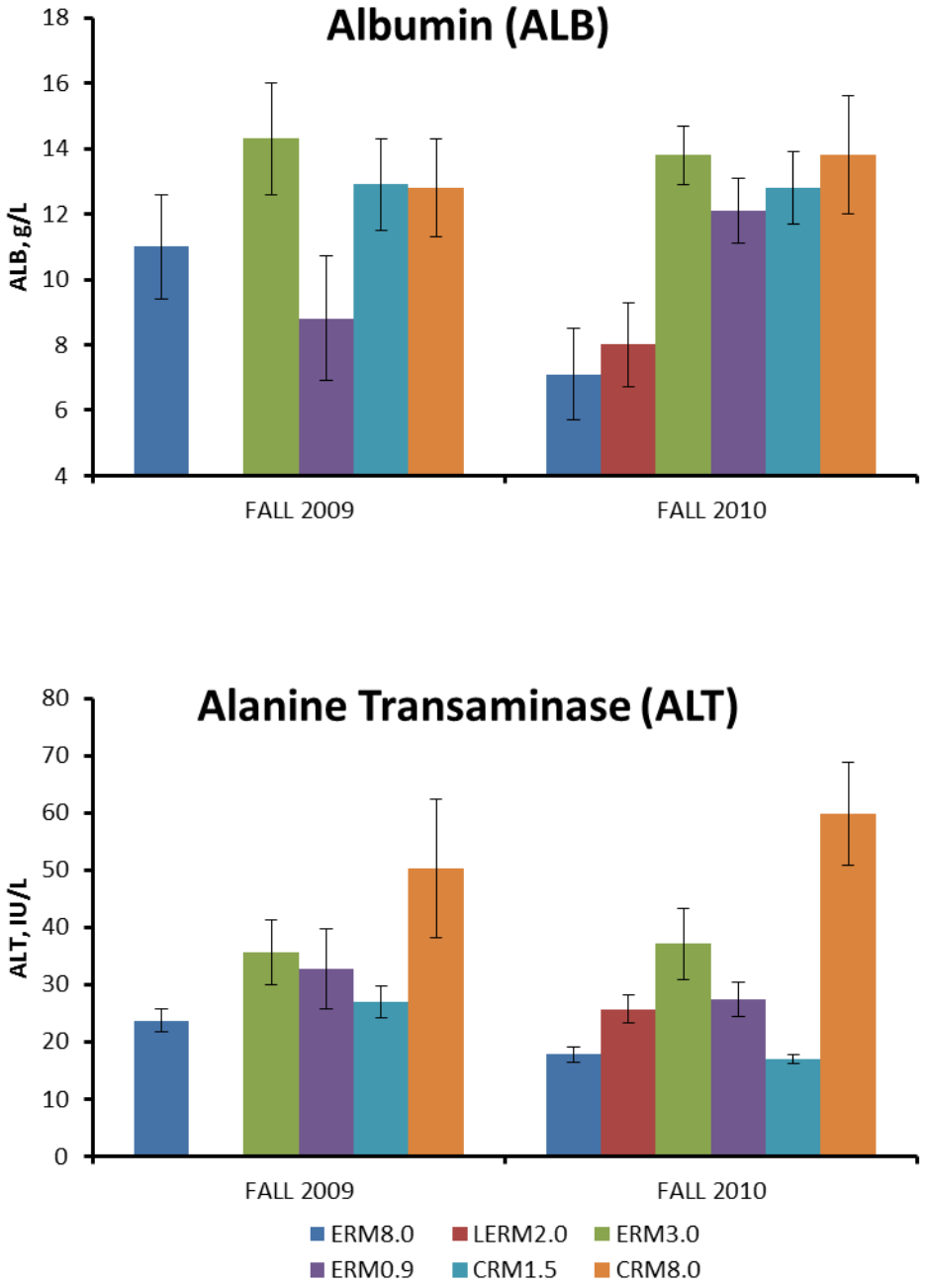


Figure 33. Spatial patterns in organ dysfunction indicators for channel catfish during Fall 2009 and Fall 2010.

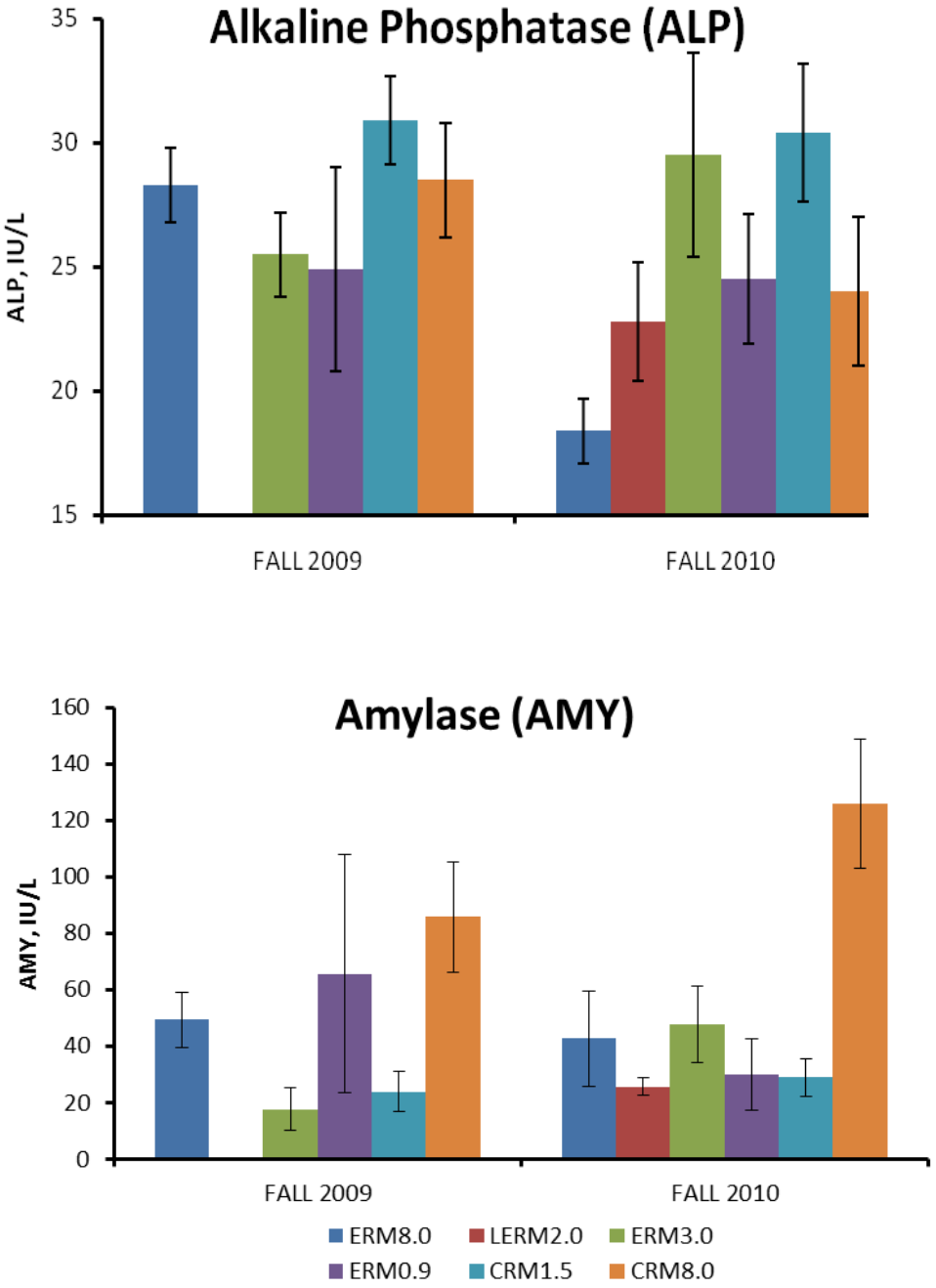


Figure 34. Spatial patterns in general disease and digestive physiology indicators for channel catfish during Fall 2009 and Fall 2010.

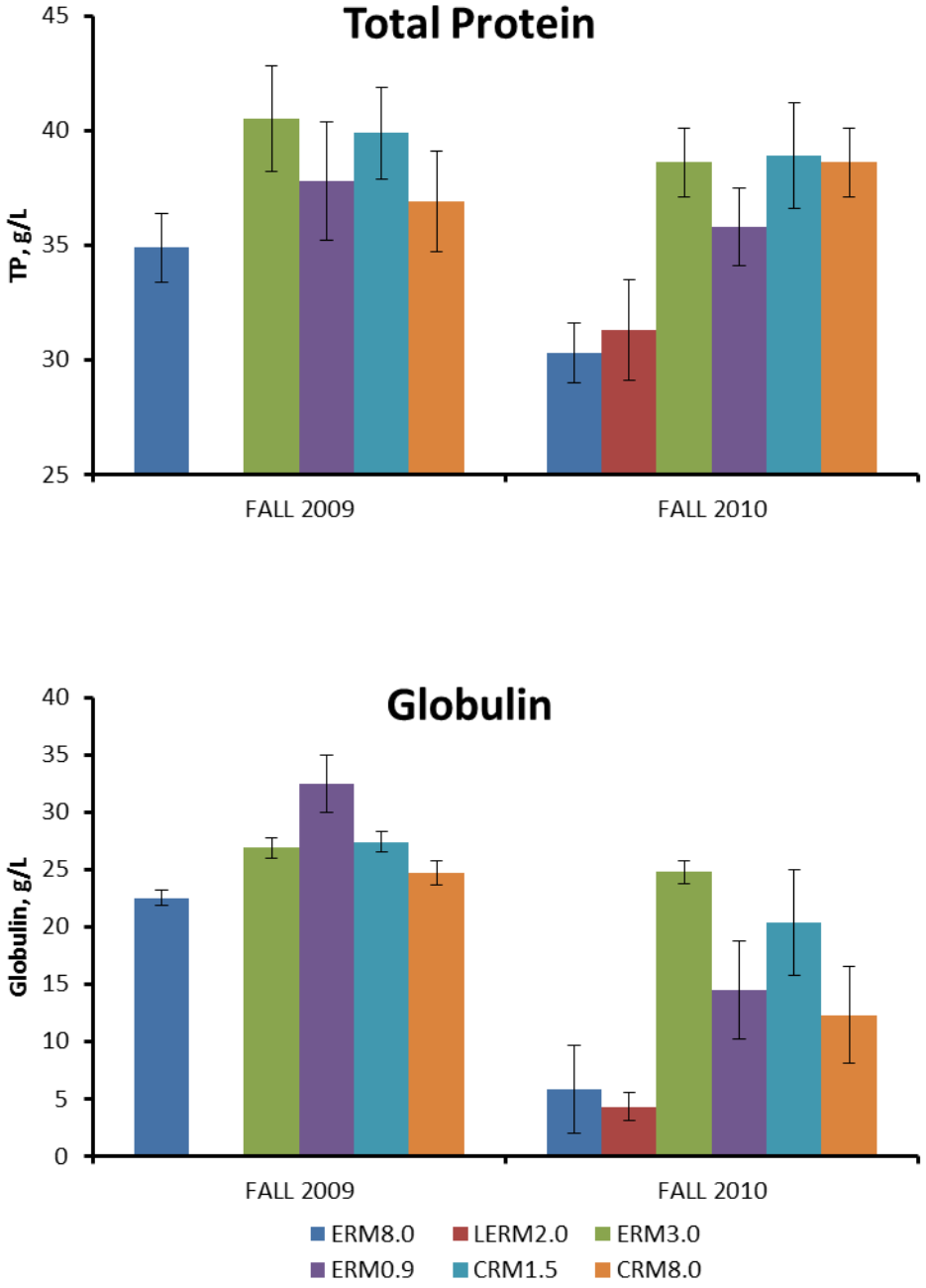


Figure 35. Spatial patterns in protein metabolism indicators for channel catfish during Fall 2009 and Fall 2010.

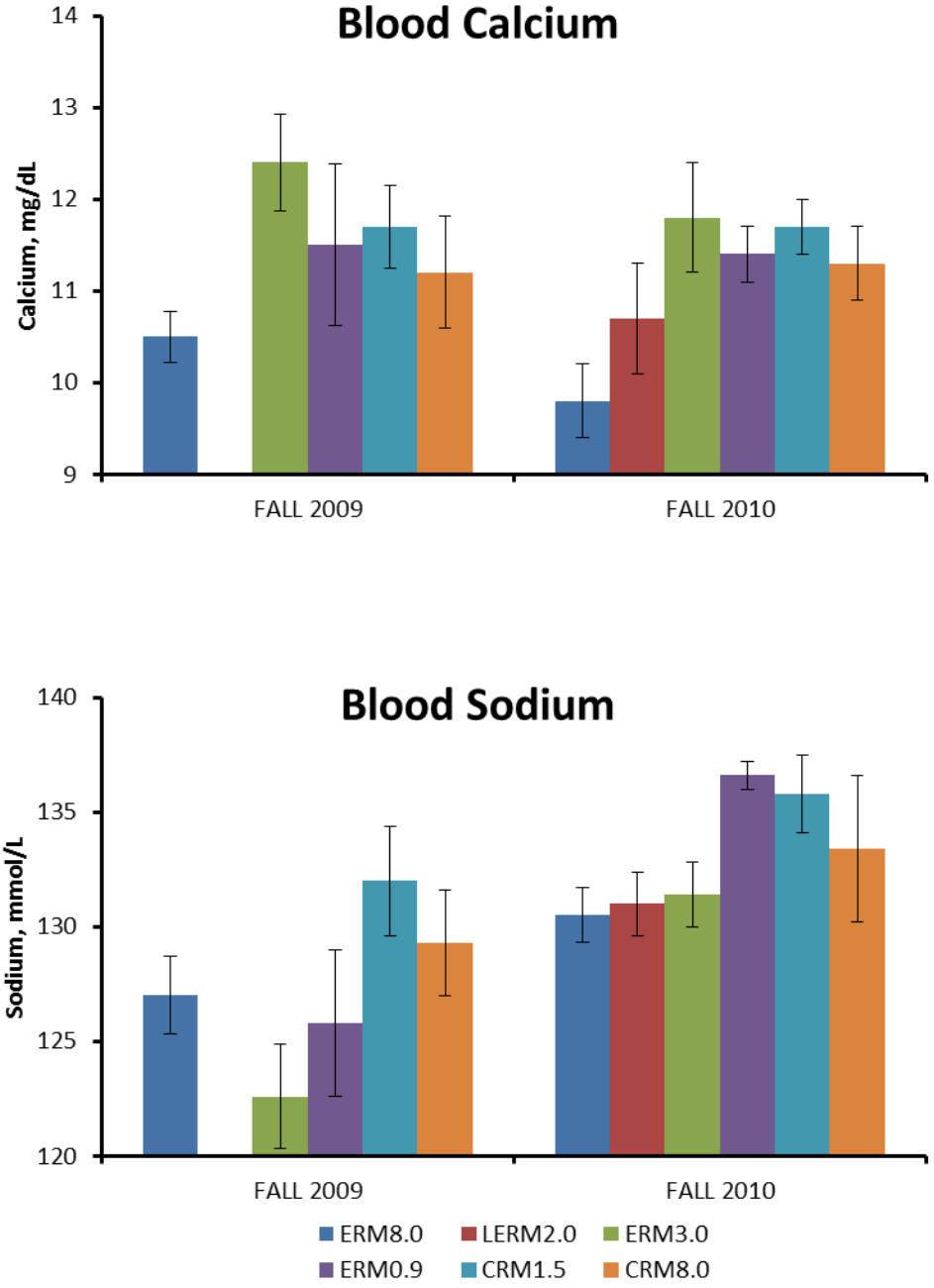


Figure 36. Spatial patterns in electrolyte homeostasis indicators for channel catfish during Fall 2009 and Fall 2010.

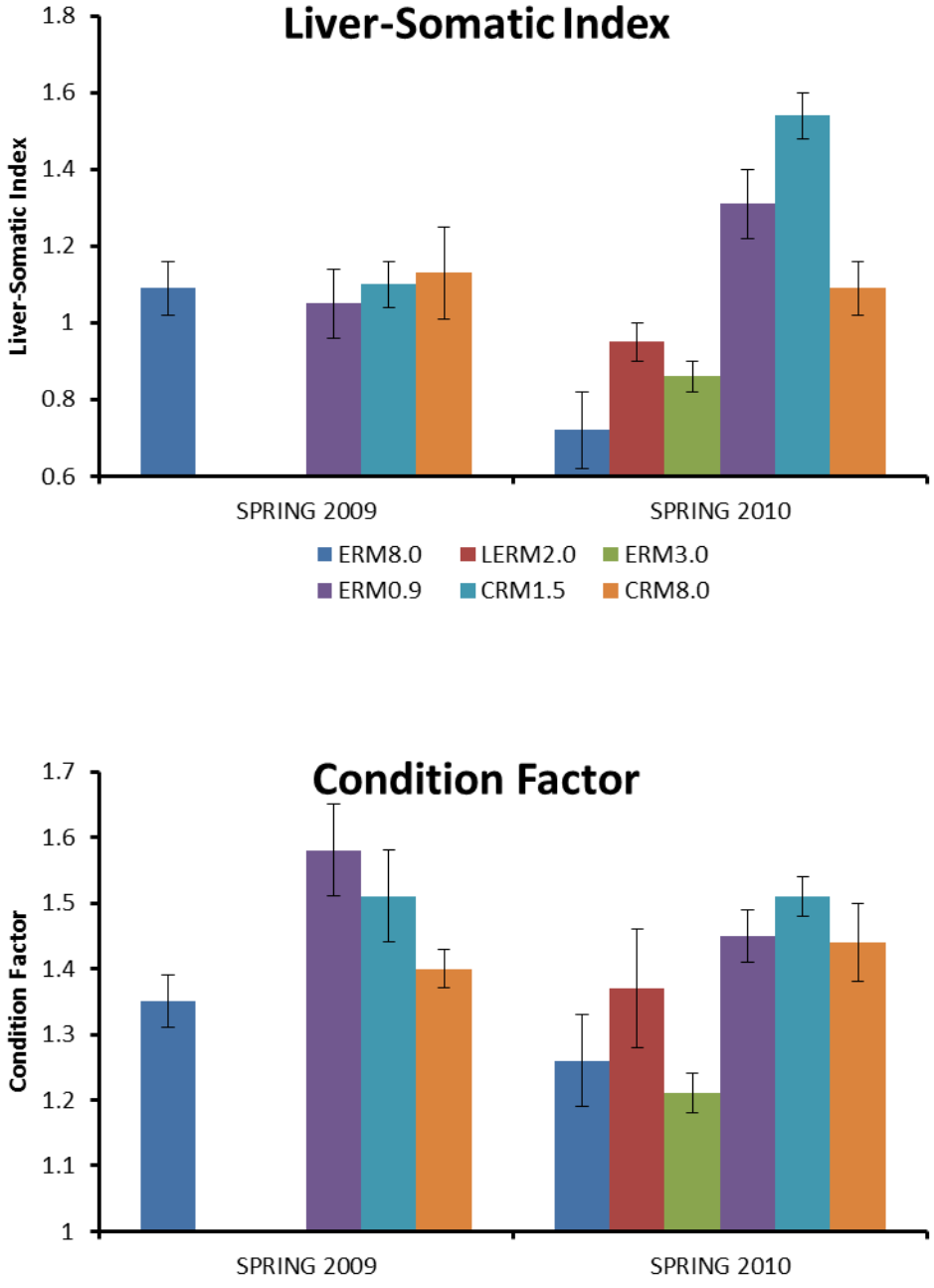


Figure 37. Spatial patterns in condition indices for white crappie during Spring 2009 and Spring 2010.

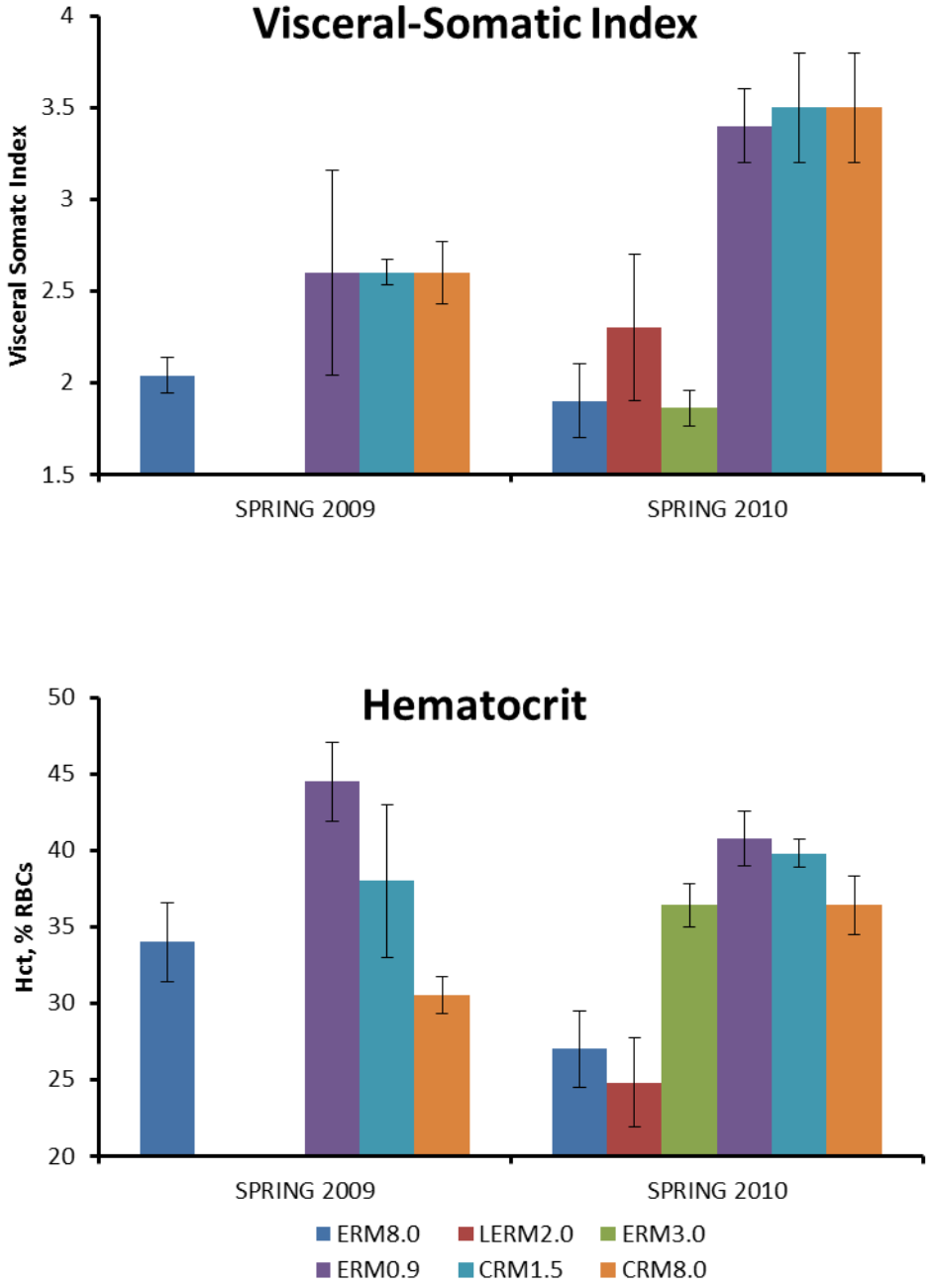


Figure 38. Spatial patterns in bioenergetic and hematological indicators for white crappie during Spring 2009 and Spring 2010.

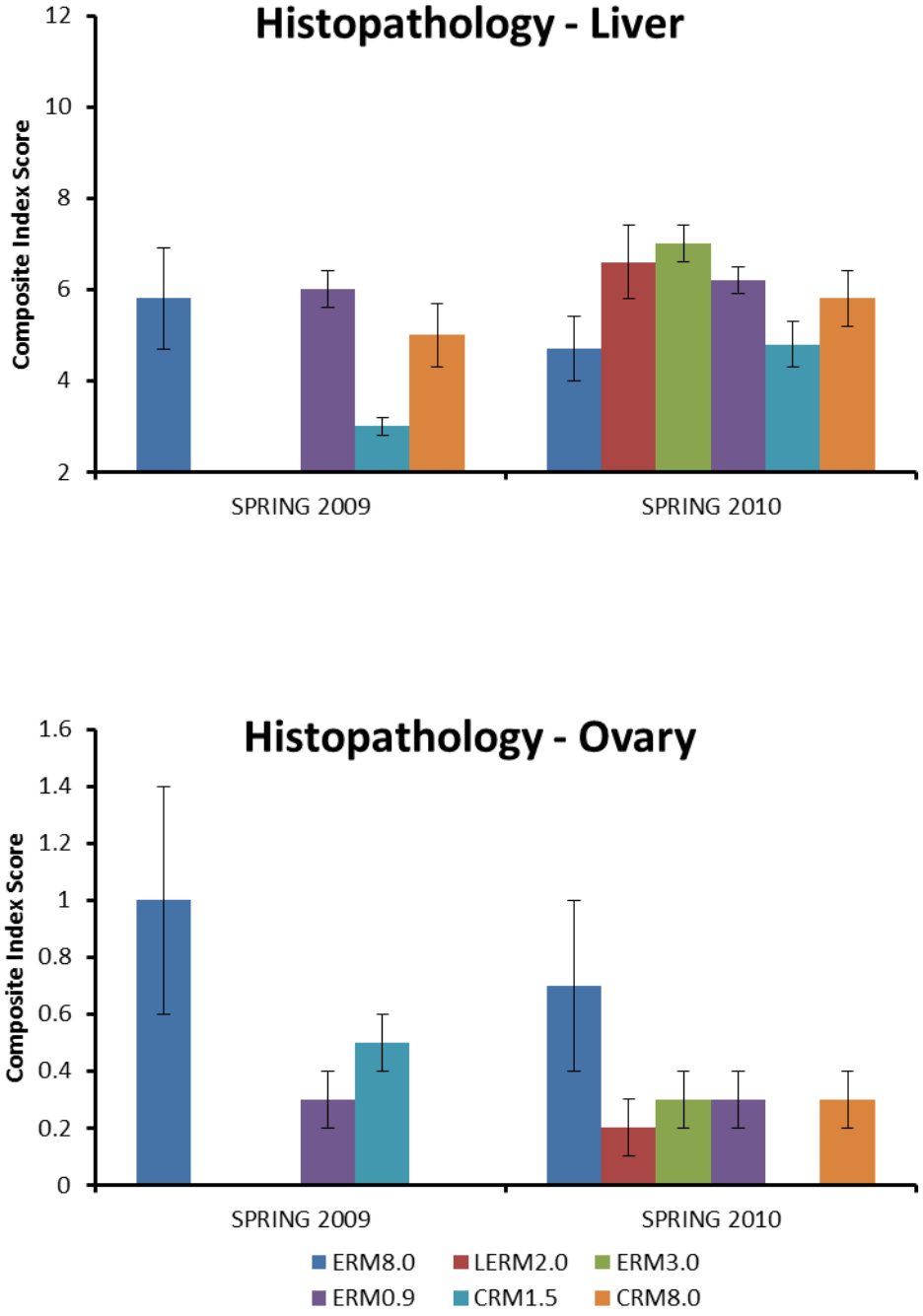


Figure 39. Spatial patterns in histopathological indicators (composite index score) for white crappie during Spring 2009 and Spring 2010.

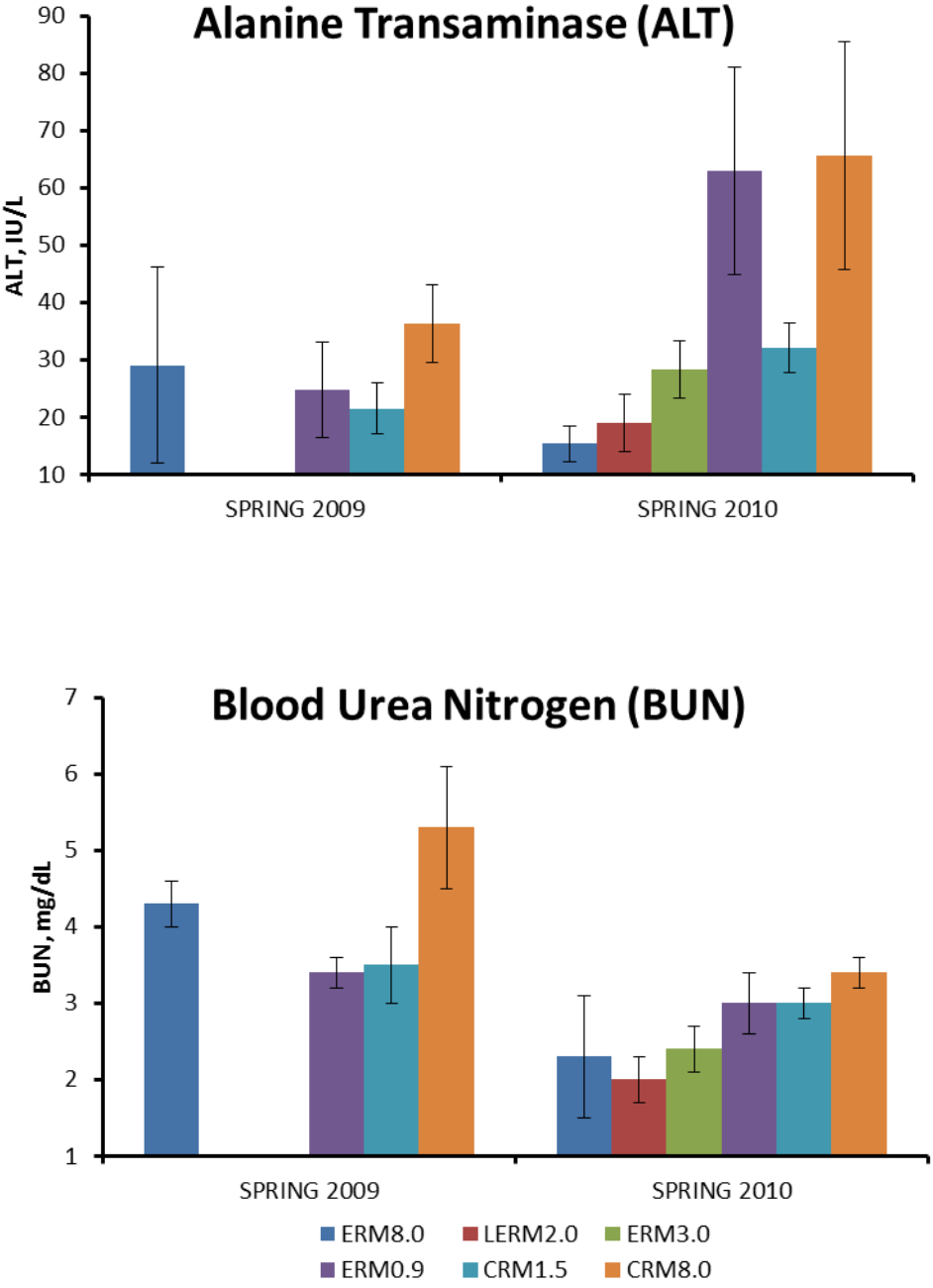


Figure 40. Spatial patterns in organ dysfunction indicators for white crappie during Spring 2009 and Spring 2010.

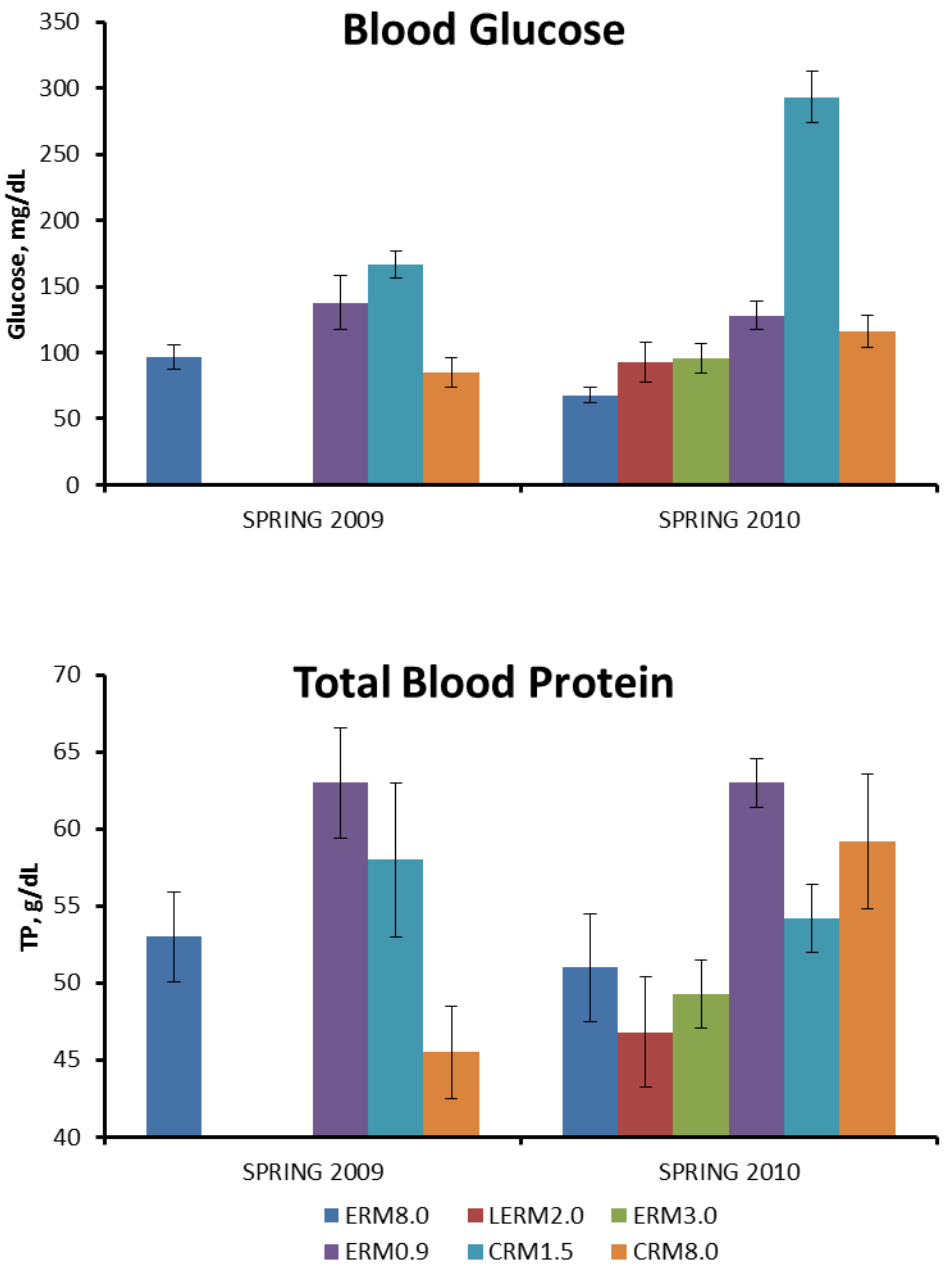


Figure 41. Spatial patterns carbohydrate-protein metabolism indicators for white crappie during Spring 2009 and Spring 2010.

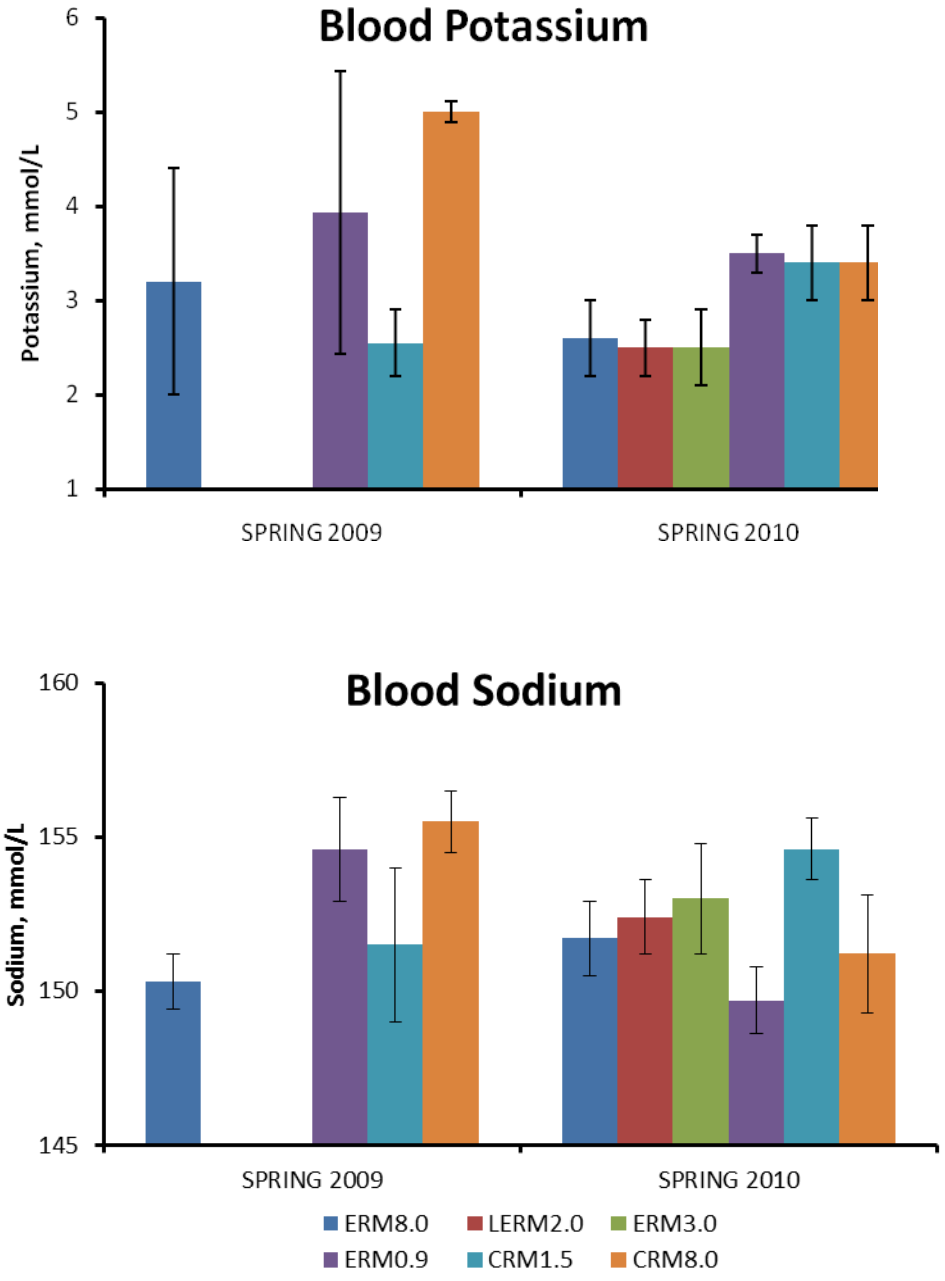


Figure 42. Spatial patterns in electrolyte homeostasis indicators for white crappie during Spring 2009 and Spring 2010.

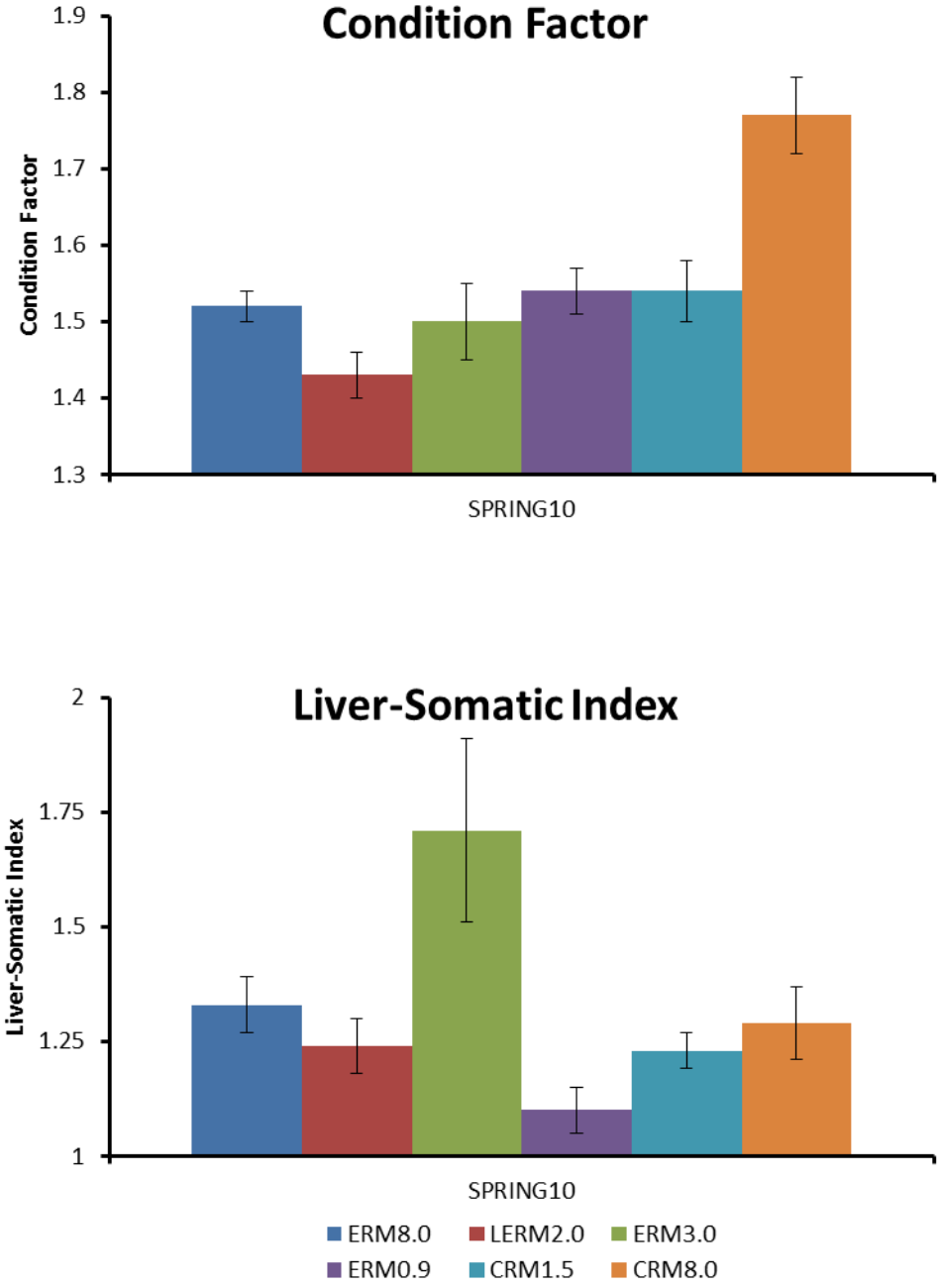


Figure 43. Spatial pattern in condition indices for redear sunfish during Spring 2010.

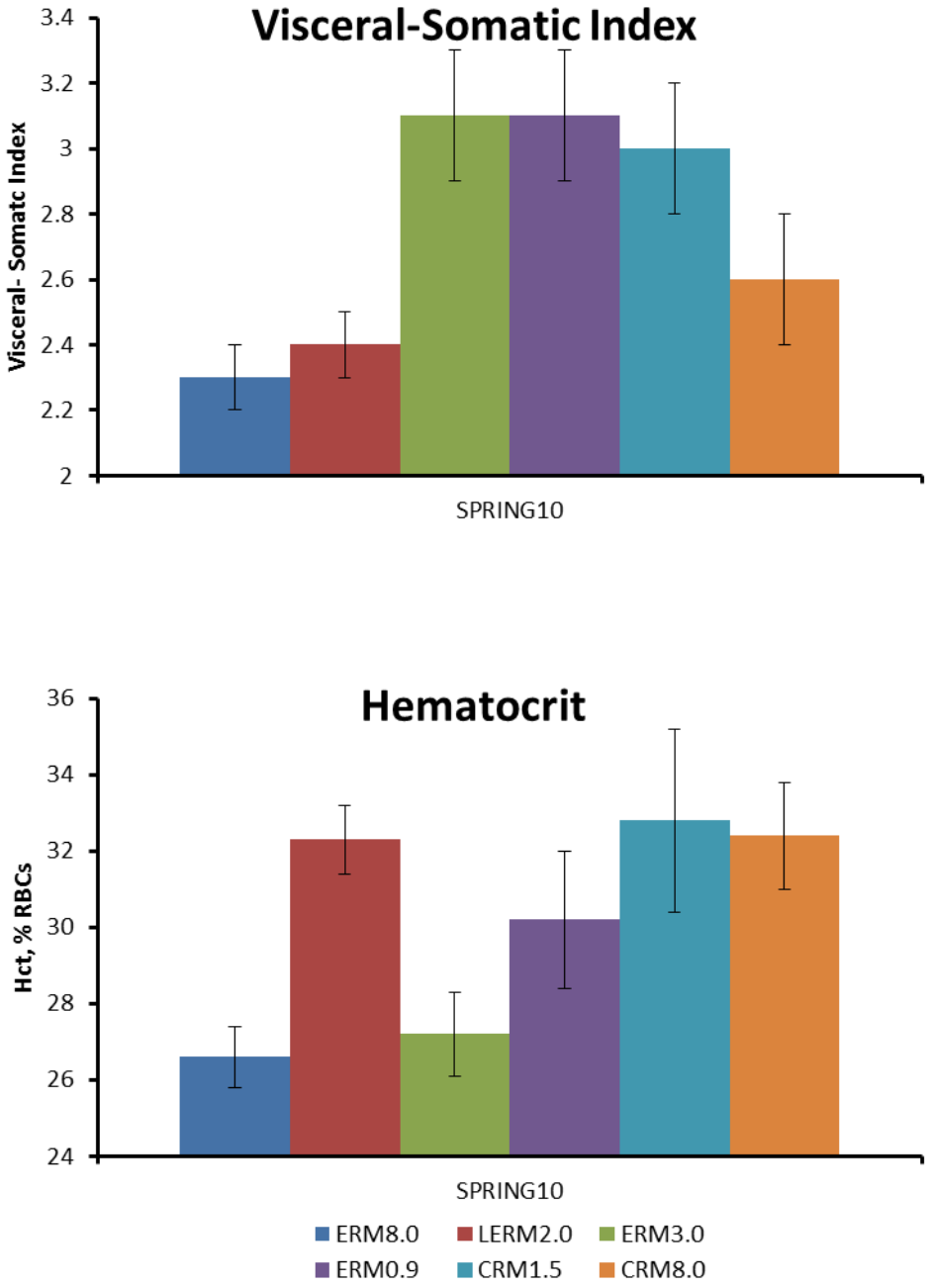


Figure 44. Spatial pattern in bioenergetic and hematological indicators for redear sunfish during Spring 2010.

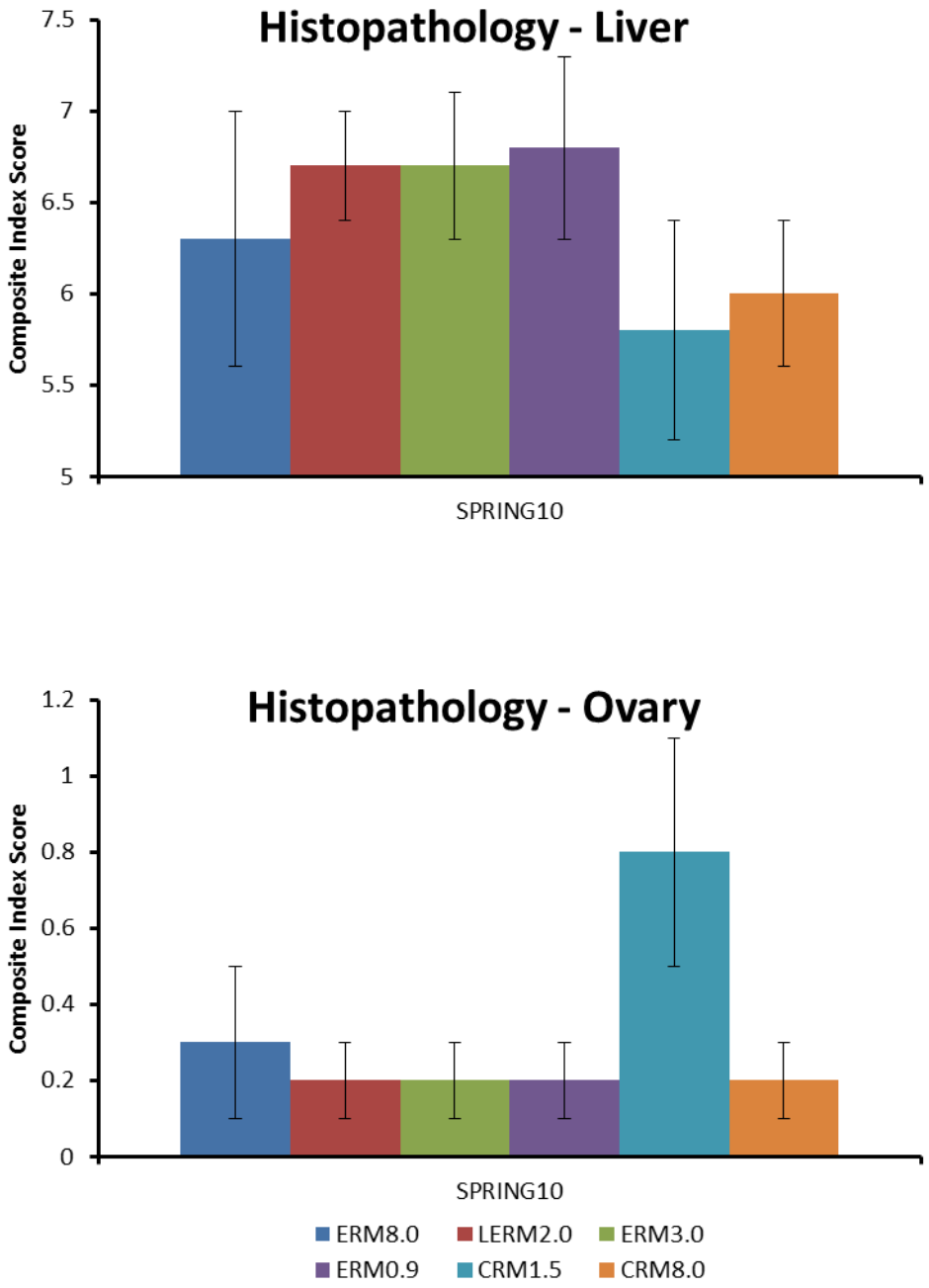


Figure 45. Spatial pattern in histopathological indicators (composite index score) for redear sunfish during Spring 2010.

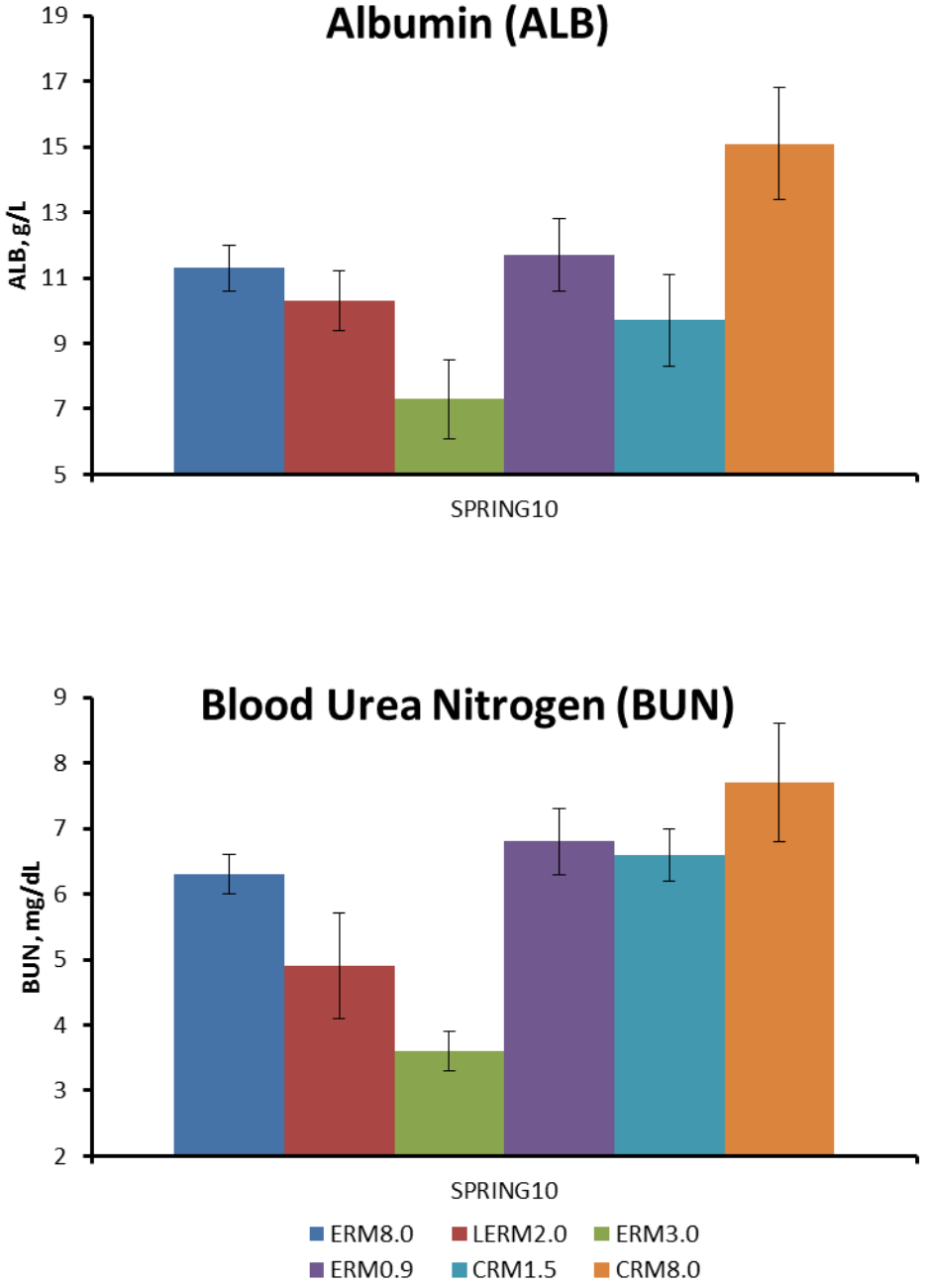


Figure 46. Spatial pattern in organ dysfunction indicators for redear sunfish during Spring 2010.

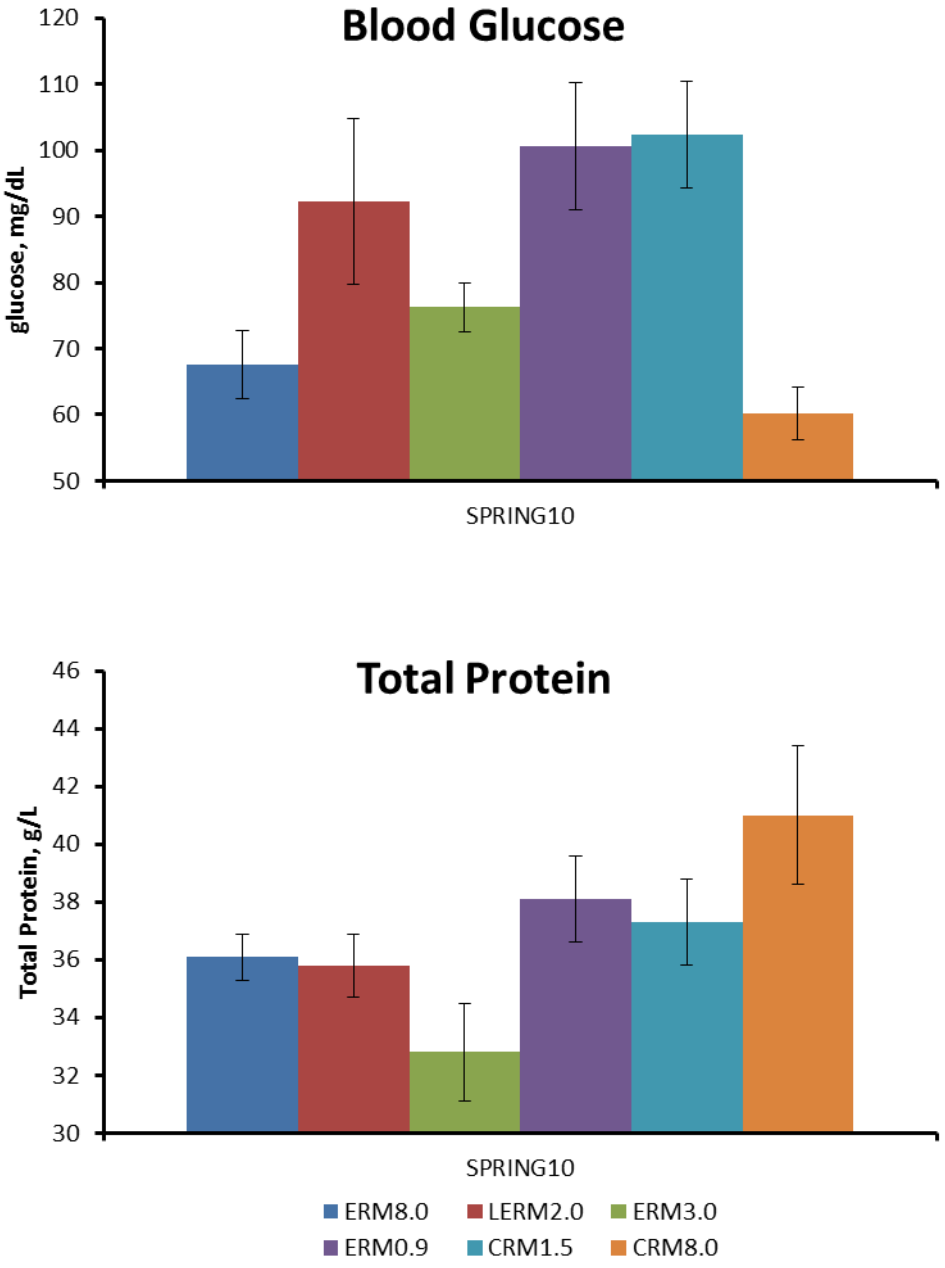


Figure 47. Spatial pattern in carbohydrate-protein indicators for redear sunfish during Spring 2010.

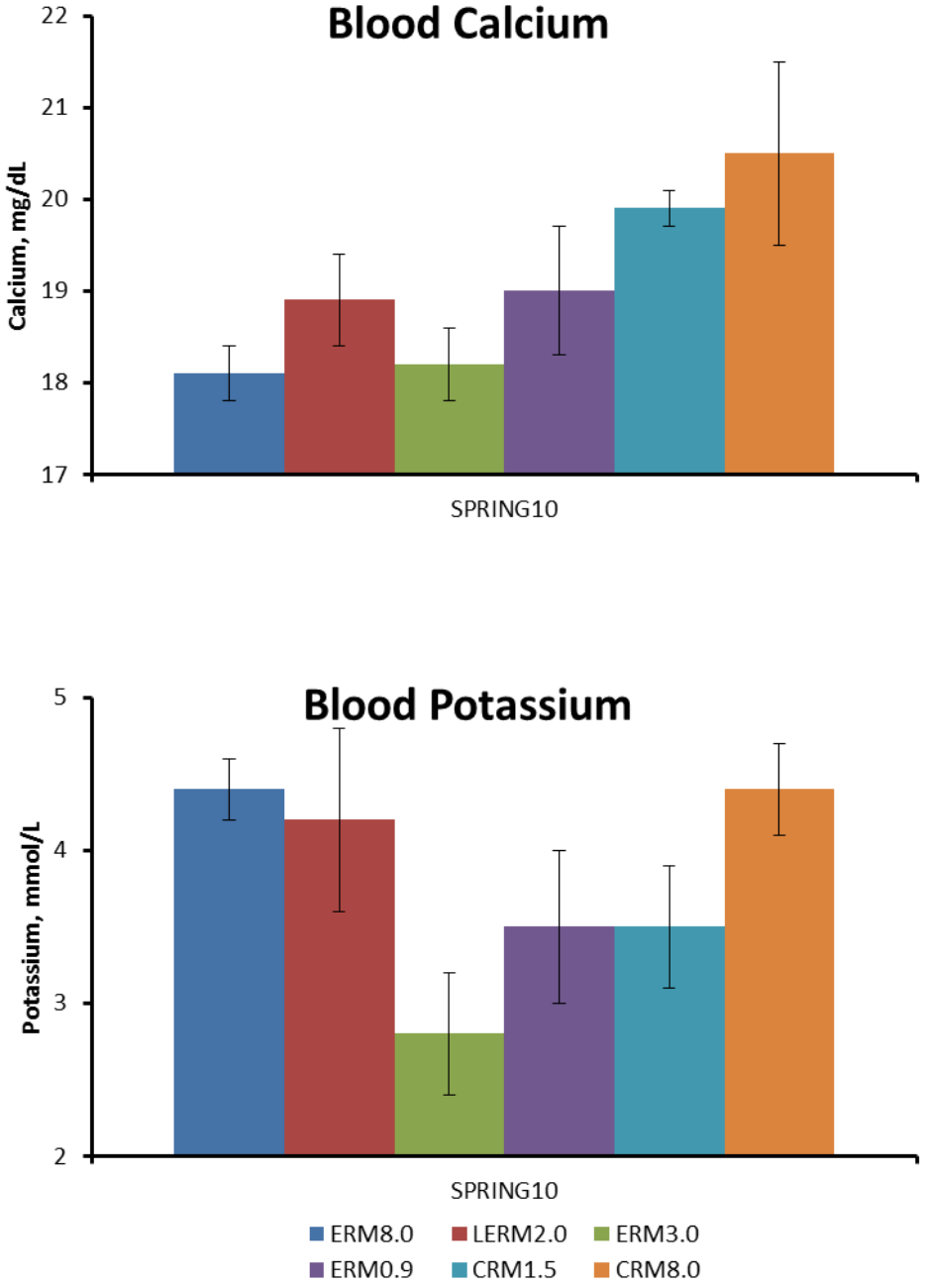


Figure 48. Spatial pattern in electrolyte homeostasis indicators for redear sunfish during Spring 2010.

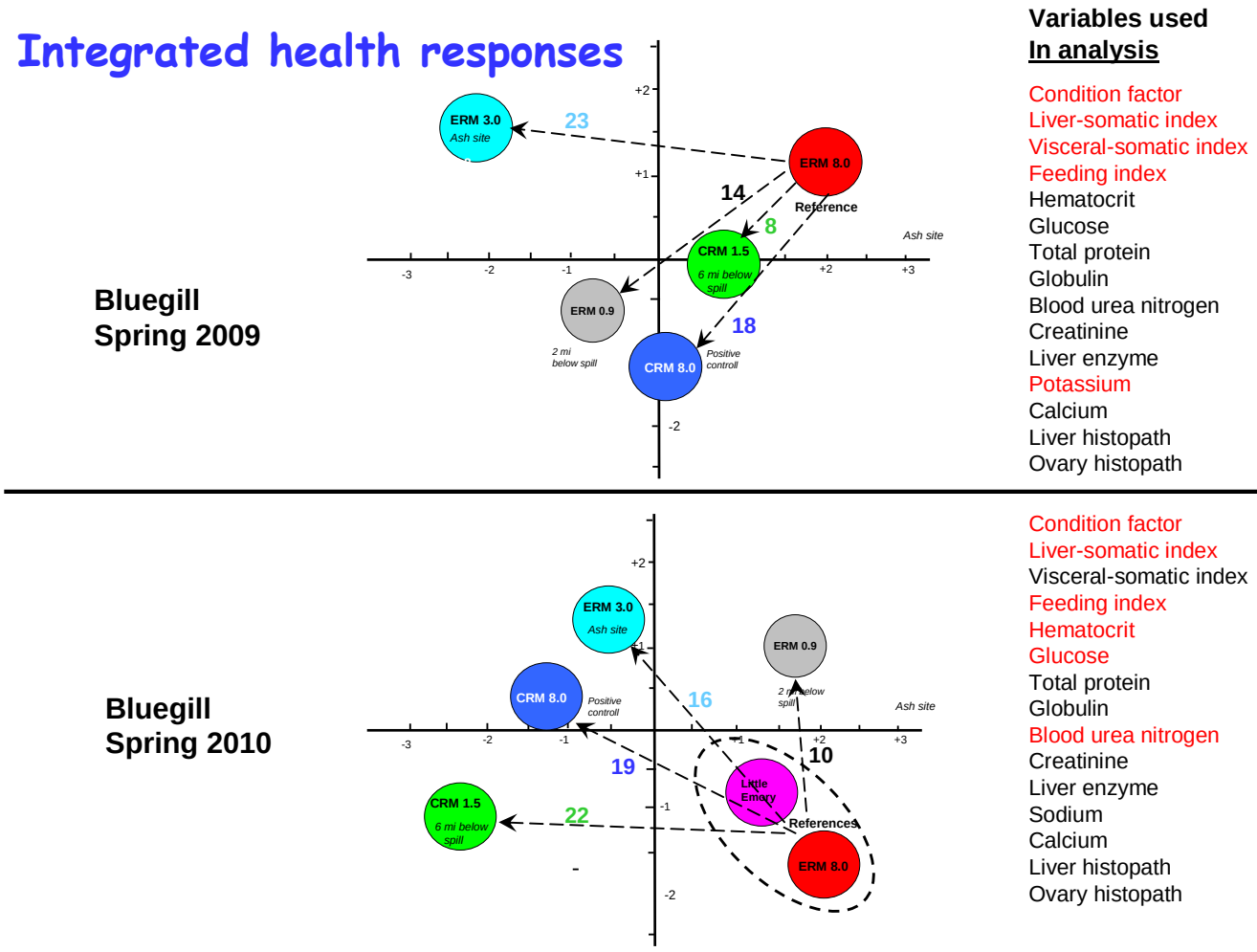


Figure 49. Integrated fish health responses based on canonical discriminant analysis for bluegill sunfish in Spring 2009 and Spring 2010. Circles represent holistic or integrated health response of fish at each sample site. Linear statistical distances between midpoints of circles (sites) are indicated by dashed lines. Variables used or entered into the discriminant analysis are shown on right of figure with those in red being the most influential in discriminating among sites. The closer the circles are to each other, the more similar is the health response.

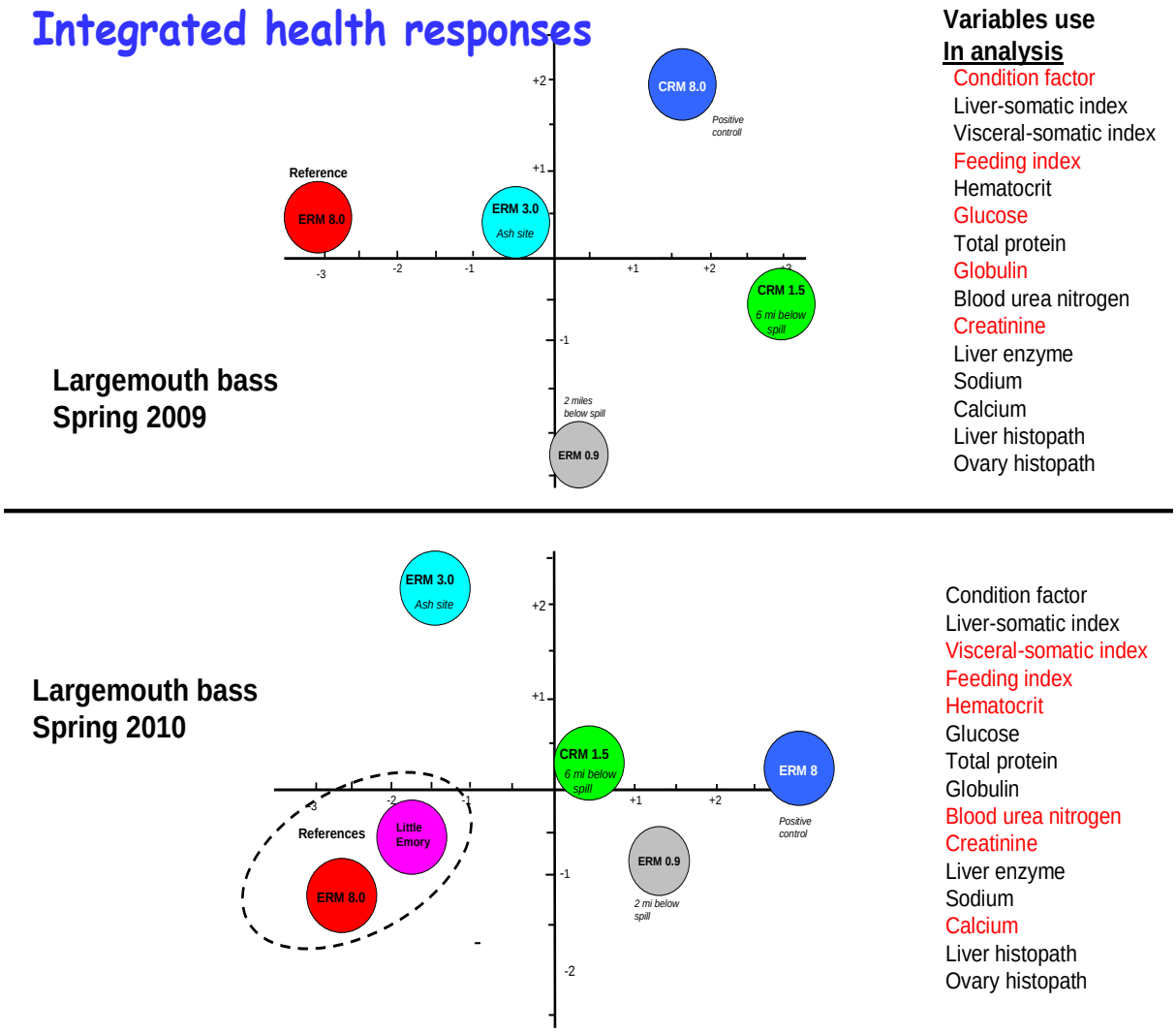


Figure 50. Integrated fish health responses based on canonical discriminant analysis for largemouth bass in Spring 2009 and Spring 2010. Circles represent holistic or integrated health response of fish at each sample site. Linear statistical distances between midpoints of circles (sites) are indicated by dashed lines. Variables used or entered into the discriminant analysis are shown on right of figure with those in red being the most influential in discriminating among sites.

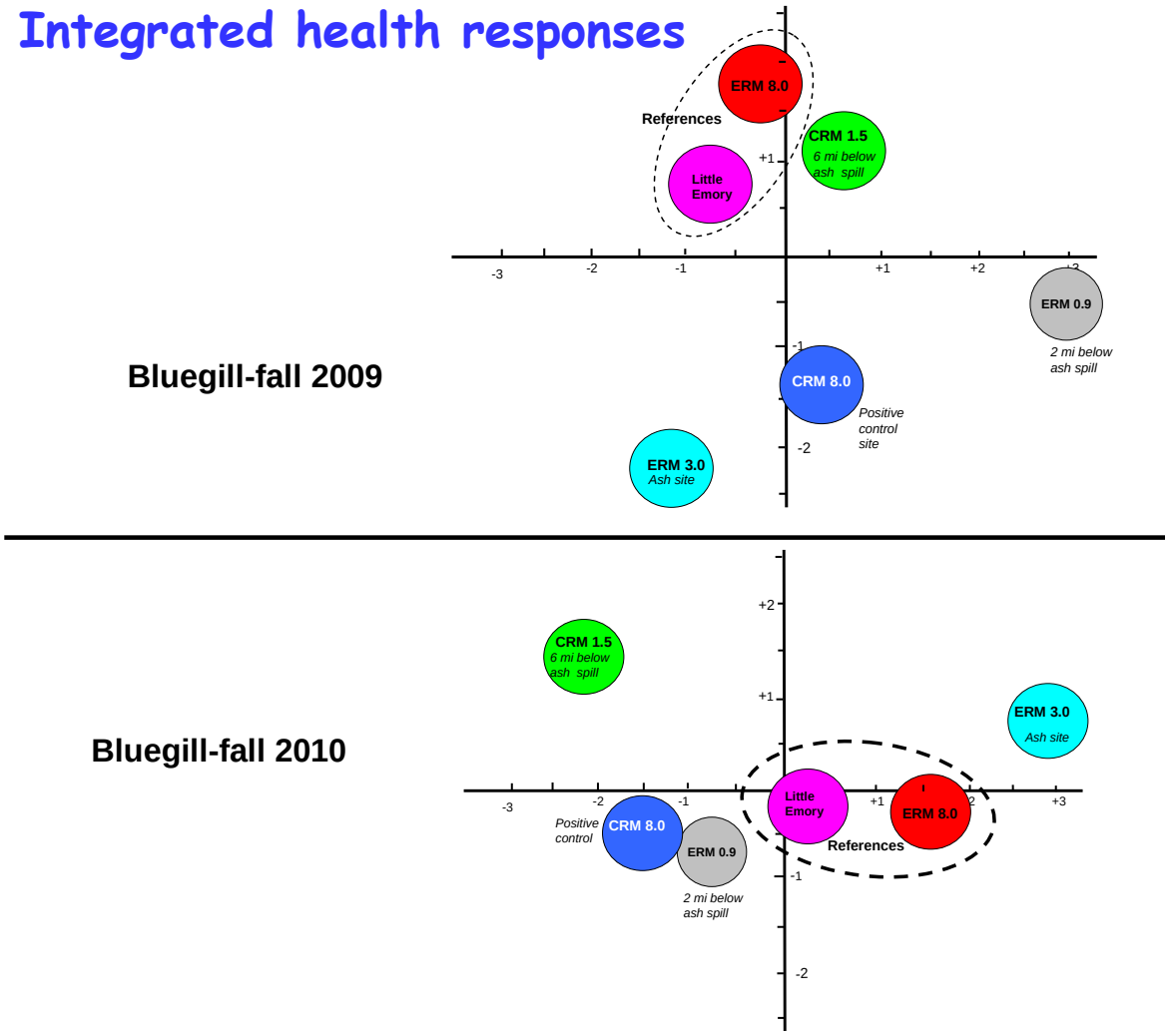


Figure 51. Integrated fish health responses based on canonical discriminant analysis for bluegill sunfish in Fall 2009 and Fall 2010. Circles represent holistic or integrated health response of fish at each sample site. The closer the circles are to each other, the more similar is the health response.

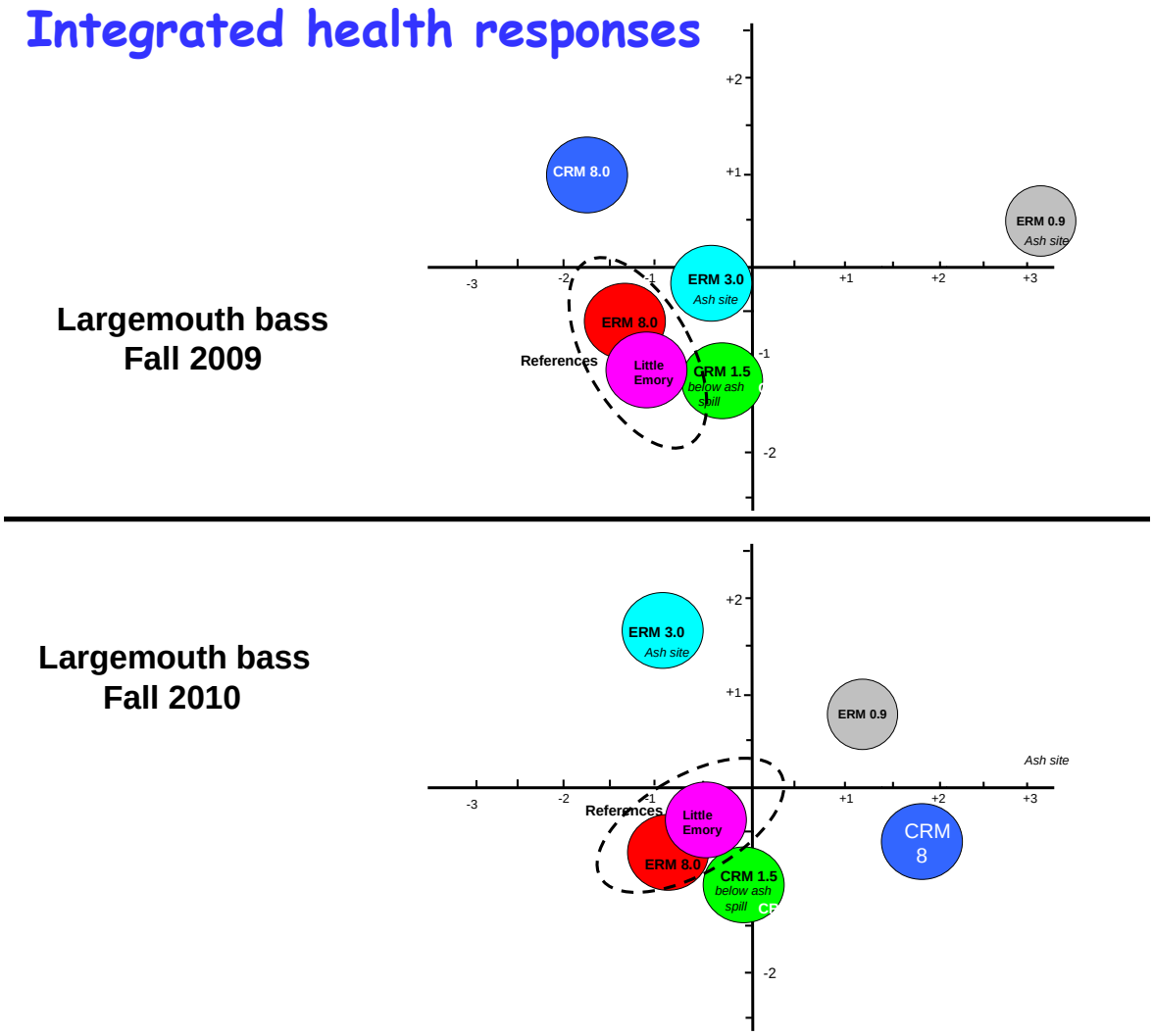


Figure 52. Integrated fish health responses based on canonical discriminant analysis for largemouth bass in Fall 2009 and Fall 2010. Circles represent holistic or integrated health response of fish at each sample site. The closer the circles are to each other, the more similar is the health response.

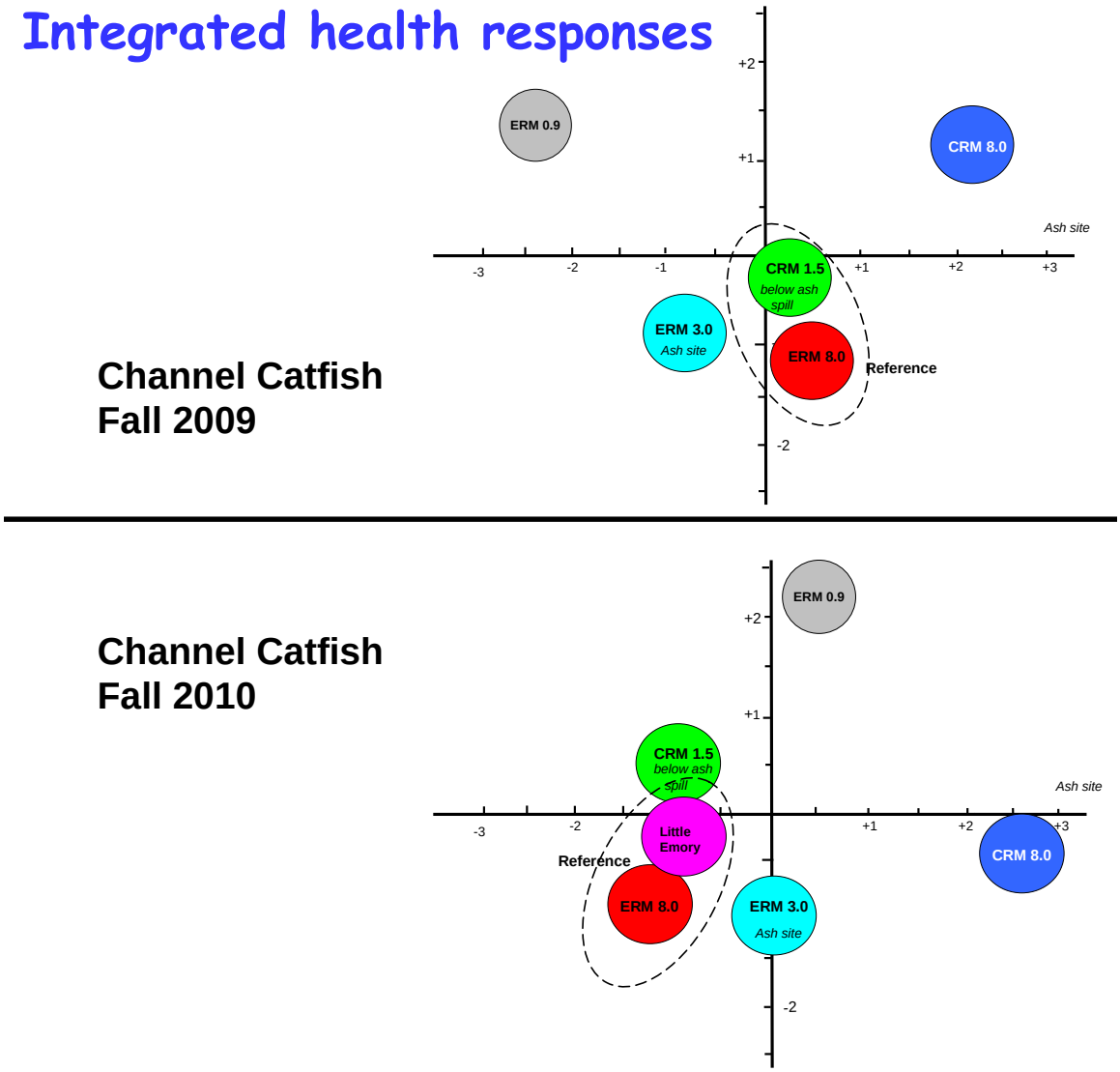


Figure 53. Integrated fish health responses based on canonical discriminant analysis for channel catfish in Fall 2009 and Fall 2010. Circles represent holistic or integrated health response of fish at each sample site. The closer the circles are to each other, the more similar is the health response.

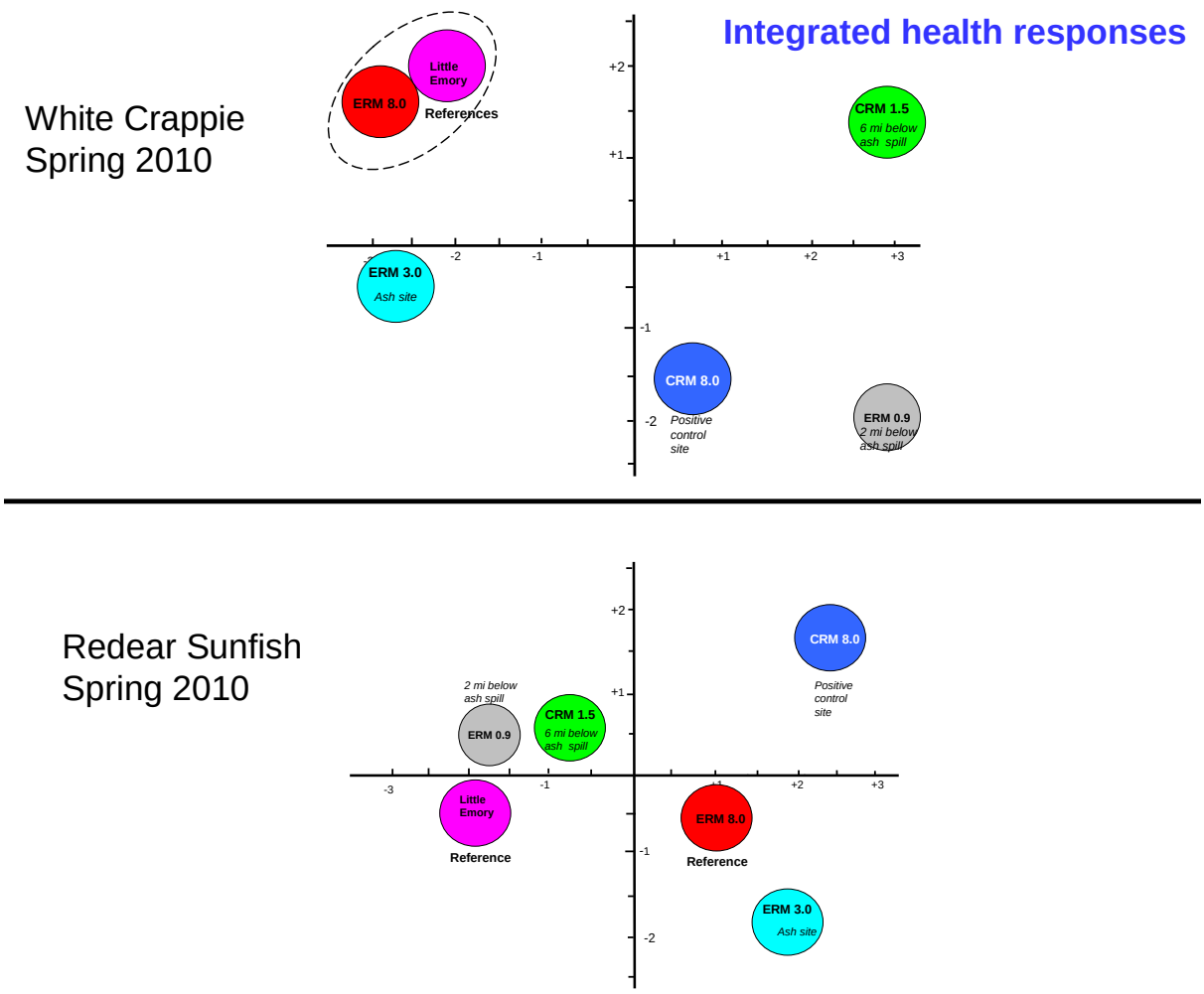


Figure 54. Integrated fish health responses based on canonical discriminant analysis for white crappie and redear sunfish in Spring 2010. Circles represent holistic or integrated health response of fish at each sample site. The closer the circles are to each other, the more similar is the health response.

4. DISCUSSION AND INTERPRETATION

Individual health responses

A suite of bioindicators were used in this study to investigate the possible relationship between exposure of sentinel fish species to fly ash-associated metals and the health response of these fish in the Clinch and Emory River systems. Eight functional groups of fish health responses were measured that reflect different physiological functions and represent varying degrees of sensitivity and specificities to environmental stressors and also reflect different levels of ecological relevance. Results presented in Figs. 2-48 provide examples of two representative bioindicators for each of these seven functional groups including condition indices, bioenergetics, hematology, histopathology, carbohydrate-protein metabolism, organ dysfunction, electrolyte homeostasis, and feeding and nutrition. Comparisons of results between the Spring of 2009 and Spring 2010 and between Fall 2009 and Fall 2010 for four species (bluegill, largemouth bass, channel catfish, and white crappie) in Figs. 2-48 show that is a wide variation in species, seasonal, and site responses for the various measured bioindicators. Because of the large number of possible combinations and comparisons between seasons, species, and site responses for the 14 representative bioindicators presented in these figures, it would be unrealistic to synthesize and evaluate spatial and temporal patterns for all possible combinations of variables. However, some general or overall patterns can be distilled from the plethora of data available from the fish health study. For each of the 14 individual representative bioindicators, there were at least two instances where a spatial gradient was observed when considering results over all species and seasons. For example, for liver histopathology, there were three instances where a distinct spatial gradient in liver lesions could be seen over all species and seasons (Table 2). Also, for liver histopathology, there were two instances where a slight or moderate spatial gradient was observed. The liver enzyme alanine transaminase, which reflects organ dysfunction, was observed to display relatively strong spatial differences among sites in seven instances over both seasons and species (Table 2). The range in number of spatial patterns observed for the suite of bioindicators measured is 2-7 with the mean number per health indicator being approximately 3-4.

For temporal comparisons of individual health responses, the number of spatial gradients observed in 2009 compared to those seen in 2010 was determined considering both bluegill and largemouth bass for all 14 representative bioindicators. For these two species in Spring and Fall 2009, there were 15 apparent spatial gradients and seven instances where a moderate or slight spatial gradient was detected (Table 2). In Spring and Fall 2010, 17 apparent spatial gradients were observed with six moderate level gradients (Table 2). The fish bioaccumulation report submitted to TVA in June 2011 clearly demonstrated that there were spatial patterns in selenium and, to a lesser degree, arsenic in the Emory and Clinch Rivers as a function of the location of the fly ash release. If similar spatial patterns are observed in individual fish health responses along the same downstream gradients of these systems, then the inference is that there could be a causal relationship between fly ash-associated metals and health responses. Therefore, any changes in the spatial patterns of individual health responses over time would suggest the following 1) if the number and/or frequency of spatial trends increases over time after the spill then fish may be incurring increased damage or injury, 2) if the frequency or number of spatial trends decreases over time then some level of recovery or improvement could be occurring, or 3) if the number or frequency of spatial trends doesn't change over time then the sentinel fish species in these systems are neither improving or declining in overall health. The results of this individual fish health analysis suggest that since the number or frequency of spatial trends didn't change appreciably from 2009-2010, then the overall health status of these sentinel fish species, based on assessment of the individual bioindicator responses alone, neither improved nor declined over this study period. However, this analysis is based on the behavior of individual health responses over time which can be highly variable as a function of site, season, and species.

Table 2. Occurrence of downstream spatial gradients demonstrated by a suite of individual bioindicators during the various sample seasons. Yes=a spatial gradient was observed for a particular bioindicator in the Emory and Clinch Rivers downstream of the reference sites; No=no spatial gradient observed; and S=a moderate or slight spatial gradient was observed.

	Species-seasonal Comparisons									
Bioindicator	Bluegill		Bluegill		Largemouth bass		Largemouth bass		Channel catfish	
	Sp'09	Sp'10	Fa'09	Fa'10	Sp'09	Sp'10	Fa'09	Fa'10	Fa'09	Fa'10
Condition factor	No	Yes	No	No	No	No	No	No	No	Yes
Liver-somatic index	No	S	Yes	No	Yes	Yes	No	No	S	Yes
Visceral-somatic or spleno-somatic index	No	No	Yes	No	Yes	No	No	No	No	Yes
Hematocrit	No	Yes	No	No	No	Yes	No	S	No	Yes
Liver histopath	No	S	No	Yes	S	No	Yes	No	Yes	No
Gill or ovary histopath	S	No	Yes	Yes	S	No	No	No	Yes	Yes
Ananine transaminase	Yes	Yes	No	Yes	Yes	No	Yes	No	Yes	Yes
Urea nitrogen	No	S	No	Yes	No	Yes	Yes	No	S	Yes
Glucose	No	No	S	No	No	Yes	S	S	Yes	Yes
Total protein	No	No	No	No	No	Yes	No	No	Yes	Yes
Calcium	No	No	Yes	No	No	No	No	Yes	Yes	Yes
Sodium or potassium	S	No	Yes	No	Yes	No	Yes	Yes	No	No
Stomach fullness	Yes	Yes	No	Yes	No	No	S	No	--	--
Gall bladder color/size	No	No	No	Yes	No	S	No	No	--	--

These multiple sources of variability can also interact in a synergetic fashion resulting in a variance structure that may not reflect a simple linear function for each individual health response. Such potential multiple sources of variability and possible synergistic behavior among variables makes it rather challenging to identify or discern the causes of any temporal changes in fish health such as that due to fly ash exposure. To address the issue of multiple sources of variability, an integrated assessment approach was also taken using all the individual bioindicators together in a holistic context which collapses the multiple sources of variability into one variance structure and provides an overall view of spatial (differences among sample sites) and temporal (differences among seasons and years) changes in fish health.

Integrated health responses

Aquatic organisms in their natural environmental are continually exposed to a variety of environmental factors or stressors which can compromise their health or overall well-being. In

assessing effects of environmental stressors on aquatic biota, use of multiple indicators of exposure and effects (i.e., multiple lines of evidence or weight of evidence) are usually more effective in evaluating the real-world situation than is the use of one or only a few response parameters. Quantitative statistical procedures such as principal component analysis, cluster analysis, and discriminant analysis are useful techniques for combining multiple variables into a single analysis for the purpose of investigating integrated or holistic responses. The canonical discriminant analysis procedure is particularly useful in the case of the assessing fish health because it considers all measured variables or responses jointly within a multivariate context and provides the results as an integrated response which can be easily visualized in graphical form.

The results for the discriminant analysis provide both a spatial and temporal assessment of the integrated fish health responses among seasons and sample sites. There are two main reasons why we are confident that this technique is effective for realistically portraying the integrated health status of fish at each site. First, for those periods when fish from two reference sites were sampled (all seasons except Spring 2009), the integrated health response of fish including bluegill, largemouth bass, and catfish were very similar, being in close proximity to each other. In some cases the circles (indicating the holistic state space of the integrated response) overlapped indicating considerable statistical similarity in their overall response. If, for example, the reference sites were dissimilar or separated by a large distance, then such a spatial difference in integrated response might suggest that this discriminant analysis procedure was somewhat unreliable or ineffective for assessing the integrated health status of fish among sites. In all cases but the Spring 2010 redear spatial comparison, both reference sites were more similar to each other than to any other sites. A possible reason that responses at the two reference sites for redear were very different is related to differences in feeding, nutrition, and availability of preferred prey at these sites. A second reason that this multivariable procedure appears effective for assessing the integrated health status of fish among sites is related to the spatial relationship of the positive control site (CRM 8.0) to the reference sites. CRM 8.0 is considered a positive control site and not a true reference site due to its proximity to legacy contamination sources which historically originated upstream from the Oak Ridge DOE facilities. In all the spatial and temporal comparisons of fish species among sites and seasons (Figs. 49-54), the positive control site always maintained its same relative position to the reference sites (approximate same linear statistical distances between mid-points of circles) by not moving either closer or not moving further away from the references over time (Table 3). In almost all cases, the sites downstream of the ash spill either moved closer or further away from the references as a function of time (Table 3). The positive control site, CRM 8.0, is several miles upstream in the Clinch River above the confluence of the Emory River and therefore not influence by the ash release. Therefore, fish sampled from this site would not be expected to exhibit behavior relative to increasing or decreasing health status over time as a result of the ash spill. The fact that this site remained relatively static in its health status over time compared to sites downstream of the spill is also evidence (along with the similarity of the reference sites to each other) that the discriminant analysis procedure is an effective and reliable approach for assessing the integrated health status of fish among sample sites.

Trends in recovery

The integrated discriminant analysis procedure can be used to assess trends in recovery of fish health over time. For the integrated fish health responses shown in Figs. 49-54, several temporal patterns in recovery in fish health can be observed. Table 3 summarizes these temporal patterns for bluegill, largemouth bass, and channel catfish at each sample site. At the spill site (ERM 3.0) the integrated health status of bluegill improved between Fall 2009 and Fall 2010 and between Spring 2009 and Fall 2010. There was no change in overall health for bluegill from Spring 2009-Spring 2010. Over the 18 month study period (Spring 2009-Fall 2010) there was an indication of

Table 3. Temporal changes in the integrated health status of three sentinel fish species at each of the study sites over seasons. Based on the discriminant analysis, NC=no change in the integrated health status compared to the reference(s) over a particular season, MS=the integrated health status of a species became more similar to reference(s) over a year, LS=the integrated status of a species became less similar to the reference(s) over a season.

Species - Season	Sites				
	ERM 3.0	ERM 0.9	CRM 1.5	CRM 8.0	Reference Sites
Bluegill					
Spring'09 – Spring'10	NC	MS	LS	NC	--
Fall'09 – Fall'10	MS	MS	LS	NC	all similar
Spring'09 – Fall'10	MS	MS	LS	NC	all similar
Largemouth bass					
Spring'09 – Spring'10	LS	MS	MS	NC	--
Fall'09 – Fall'10	LS	MS	NC	NC	all similar
Spring'09 – Fall'10	LS	MS	MS	NC	all similar
Channel catfish					
Fall'09 – Fall'10	NC	NC	NC	NC	all similar

improved health of bluegill at ERM 3.0. At ERM 0.9, two miles below the spill, there appeared to be improvement (recovery) in the health of bluegill over all seasonal comparisons and over the entire 18 month study period (Table 3). At CRM 1.5, approximately 6 miles below the spill, the overall health status of bluegill declined (compared to the reference) over all seasonal comparisons and over the entire 18 month study period. Interestingly at CRM 8.0, the positive control site, there were no temporal changes in health of bluegill compared to the reference. In the seasons when two reference sites were sampled, the reference sites maintained very close similarity to each other illustrating again the reliability of this integrated approach for assessing fish health.

For largemouth bass, the integrated health status declined at ERM 3 between Spring 2009-Spring 2010, between Fall 2009 and Fall 2010, and over the entire study period of Spring 2009-Fall 2010 (Table 3). However, at ERM 0.9, the overall health status improved over all seasonal comparisons and over the 18 month study period. At CRM 1.5, overall health improved from Spring 2009-Spring 2010 and over the entire study period but little improvement was seen over the Fall period. As was observed for bluegill, there was no change in the health status of bass over time at CRM 8.0 compared to the references, and the reference sites maintained very close similarity to each other.

For catfish from all sites over the Fall 2009-Fall 2010 period, there was no change in health status compared to the reference condition. The finding of no temporal trends or changes in health of catfish is most likely related to its large home range which suggests that the health status of catfish is influenced by a wide variety of environmental conditions that occur in the various areas

inhabited by this species which are integrated over the total time period of its large range movements. In other words, because of its low site fidelity, catfish would experience less exposure to the same environmental conditions (i.e., fly ash-associated metals) over time such as that experienced by species with a high site fidelity including bluegill and redear sunfish which may reside for longer periods in the areas influenced by the ash spill (i.e., ERM 3.0, ERM 0.9, CRM 1.5).

Emergent properties resulting from individual and integrated fish health analysis

Long-term monitoring strategies

Analysis of the results of the individual and integrated fish health response has provided insights into two major areas related to the strategy and design of long-term biological monitoring programs. These two areas are 1) selection of the sentinel fish species to monitor, and 2) the most informative and cost-effective metrics to measure for evaluating the effectiveness of remedial actions related to the recovery of sentinel fish populations.

Sentinel species selected to monitor contamination should be those that are characterized by different feeding habitats, trophic levels, and home ranges. By selecting species that reflect a variety of feeding types and trophic levels along with different home ranges, most of the possible exposure and effects pathways operating in aquatic systems are covered or accounted for in the experimental design. More importantly, however, the choice of sentinel organisms should focus on those species with high site fidelity in order that assessment of the effectiveness of remedial actions and recovery can be conducted within an experimental design framework in which the exposure history of the organism to contaminants is largely known and therefore can be accounted for.

The most informative and cost-effective metrics to measure for assessing the overall health status of fish exposed to fly ash associated metals have been identified through the process of applying the discriminant analysis and stepwise variable selection procedure to four species of fish over four collection periods and several sample sites. From a suite of 20-25 response or health-related variables measured in this study and entered into the discriminant analysis procedure, a subset of these variables were selected which were the most influential in discriminating differences in integrated health responses of fish among sites. In most cases, this subset consisted of 5-10 variables that represent different functional responses of the organisms at various levels of biological organization. For example, for bluegill in the Spring 2009 and Spring 2010 analyses, five variables were identified as the most important in Spring 2009 and six in Spring 2010 for distinguishing the integrated health status of fish among sites (Fig. 49). Of these 11 total variables, three were in common between Spring 2009 and Spring 2010 (condition factor, liver-somatic index, and feeding index). These 11 variables represent indicators of overall condition (condition indices), bioenergetic status, feeding and nutritional status, hematological status, carbohydrate metabolism, electrolyte homeostasis, and organ dysfunction. Therefore, in the design of long-term monitoring programs, a narrower suite of bioindicator metrics should be included that reflect different physiological functions and responses of organisms to environmental stress and also represent different levels of biological organization. These levels of biological organization are characterized by varying degrees of response sensitivity (short term vs longer term responses to environmental variables), specificities to stressors (i.e. some indicators are more specific to metal exposure than others), and ecological relevance (i.e., condition indices are more integrative in nature than organ dysfunction responses, for example, and thus might have higher ecological relevance. Inclusion and application of a small but proven (i.e., based on this analysis which demonstrated those sets of metrics which were most informative for assessing fish health over time) suite of fish health indicators in the design of future monitoring programs reduces time and costs and provides a standard and "calibrated" protocol as a basis for evaluating

the effectiveness of remedial actions and assessing causal relationships between exposure and effects on sentinel fish populations in impacted aquatic ecosystems (Adams and Collier 2003, Adams et al. 2005).

Multiple lines of evidence (multiple bioindicators) in assessing fish health and recovery
Responses of organisms to environmental conditions are the integrated result of direct and indirect contaminant exposure, natural environmental stressors such as varying physicochemical regimes, and food and habitat availability. In addition, natural processes operating in food webs, such as interspecific and intraspecific competition, predator-prey relationships, and density-dependent interactions, can influence the nature, magnitude, and final expression of a contaminant response in fish populations. Therefore, because multiple environmental factors can influence how organisms respond to stress, using a weight-of-evidence or multiple-lines-of-evidence approach is critical in attempting to identify causal relationships between environmental variables and fish health.

Biomonitoring and assessment programs whose goals are to assess the effects of environmental stressors on natural resources and to evaluate the effectiveness of remedial action on recovery of natural systems should include in the study design multiple measurements that represent a variety of specificities, sensitivities, and levels of ecological relevance. Multiple measurements or lines of evidence are needed because the mechanisms operating on biological systems and their resultant effects are different at each hierarchal level. For example, environmental stressors may affect each major level of biological organization from the individual to ecosystem, and the consequences and mechanisms of effects or disturbance are different at each hierarchal level. Response analysis of biological systems to environmental stressors at different levels of organization and hierarchal function are vital for understanding the importance of effects on biological systems and the dynamics of the recovery process In assessing recovery, application of suites or multiple bioindicators representing different sensitivities, specificities and levels of ecological relevance should also reduce the risk of false positives (Type I error or falsely concluding that recovery is occurring when it is not) and false negatives (Type II error or falsely concluding that recovery is not occurring when it actually is, or in the case of the Kingston fly ash spill, falsely concluding that that remediation of the fly ash spill has not resulted in recovery of biological systems when, in fact, it actually has). Use of a single or only a few bioindicator or measurement endpoints, however, may not be adequate to reduce the probability of these types of errors. For example, Beliaeff and Burgeot (2002) reported that using a suite of bioindicators instead of a single biomarker could help reduce the risk of false positives and false negatives. Hall and Giddings (2000) also argue for using multiple lines of evidence (measurements at several different response endpoints) for reducing the probability of false positives and false negatives.

Integrative indices such as individual-level responses and population-level attributes provide a description of the recovery of disturbed systems but do not, in themselves, provide information on the underlying basis or cause of the recovery process. Conversely, studies at the suborganismal level can help provide useful information on how stressors interact with target sites, but these sensitive indicators provide little insight relative to the consequences of these effects at ecologically relevant endpoints (Adams 2000). Lower level responses are crucial for elucidating the mechanistic basis of stress and recovery while studies of stress at higher levels of organization are key for understanding the consequences of this stress on ecological relevant endpoints (Maltby 1999). Thus, the importance of organism-level measurements is to provide a pivotal point through which mechanistic understanding and ecological consequences of stress and recovery can be linked (Forbes 1999, Adams et al. 2000). Therefore, the focus on biological monitoring and assessment programs for long-term monitoring should be at the individual

organism level but also include a suite of more sensitive bioindicators or responses at the suborganismal level. Thus, the experimental design of the fish fly ash study uses multiple indicators of response including those at both the suborganismal and organismal levels to 1) determine the holistic or integrative health status of fish exposed to fly-ash associated metals, 2) help assess possible causal relationships between exposure and observed effects, and 3) evaluate the effectiveness of remedial actions on the recovery and health of sentinel fish populations that were potentially exposed to fly ash-associated metals.

Using a multiple lines of evidence approach in environmental studies such as the TVA fly ash spill project helps provide an understanding of the mechanistic processes and causal factors that link environmental stressors, stress responses of biota, and recovery dynamics of natural resources. Such an approach is important in the effective management and restoration of aquatic ecosystems**.** Establishing causal relationships between the recovery process and influential environmental factors is particularly important in environmental management and regulation because of critical decisions that often have to be made regarding remediation, legacy of contaminated sites, role of atmospheric deposition, and other environmental compliance and regulatory issues (Adams and Ham 2011). Identification of those primary actions or environmental variables responsible for recovery should reduce the uncertainty of environmental management and regulatory decisions, resulting in an increased ability to predict the consequences of restoration activities on recovery of disturbed aquatic ecosystems such as the Emory and Clinch River systems.

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5. SUMMARY AND SYNTHESIS

The major summary and synthesis points for the individual and integrated fish health studies are:

Individual fish health

1) Several individual bioindicators demonstrate that the health of fish is generally poorer downstream of the fly ash spill than at the upstream reference areas.
2) Even though the health of fish immediately below the ash spill appears to be compromised to some degree, sublethal effects seem to be localized to a small area and to resident species.
3) Assessment of fish health using a number and variety of individual bioindicators show that there is a wide variation in species, seasonal, and site responses for the various measured bioindicators.
4) Because approximately the same number of spatial patterns (i.e., individual bioindicators either increased or decreased downstream of the reference sites) in individual bioindicators were observed in 2009 compared to 2010, it appears that no definitive conclusions can be reached regarding changes in temporal patterns in individual health responses (and therefore no evidence of temporal changes in fish health) from 2009 through 2010 based on analysis of individual bioindicator responses.

Integrated fish health

1) The integrated fish health analysis incorporates all the measured fish health parameters together within a multivariate context to assess the holistic response of fish to environmental conditions at each sample site.
2) The integrated discriminant analysis procedure was used to assess temporal trends in recovery of overall health for each sentinel species.
3) Validation of the multivariate discriminant analysis procedure as an effective and reliable approach for assessing the integrated health status of fish on both spatial and temporal scales was confirmed based on a) the reference sites were very similar in their integrated response over species and seasons, and b) there were no temporal changes in the integrated response of any species at the positive control site relative to their behavior to the reference sites.
4) From 2009-2010, apparent improvement in health status (recovery) was seen in bluegill and largemouth bass at 2 of the 3 sites (either ERM 3.0, ERM 0.9, or CRM 1.5) downstream of the ash spill. The health status of channel catfish did not change over time.

General and overall summary points

1) To effectively evaluate the health status of fish exposed to environmental stressors, a selected suite of bioindicators should be measured that reflect various physiological functions and represent varying degrees of sensitivity and specificities to environmental stressors and also reflect different levels of ecological relevance.
2) To date, no significant health effects (i.e., obvious damage or injury) have been observed in the sentinel species studied suggesting that: 1) metal exposure is not of sufficient magnitude or duration to elicit observed changes in biological responses, and/or 2) in these species, the rate of biological repair exceeds or equals the rate of damage caused by exposure to contaminants.
3) The choice of sentinel fish species to study in a long term monitoring program should focus on those taxa with a high site fidelity in order that assessment of recovery and the effectiveness of remedial actions can be conducted within an experimental design

framework in which the exposure history of the organism to contaminants and other influential environmental factors is largely known or can be accounted for.

4) Food chain studies in combination with the use of multiple bioindicators provides a multiple-lines-of-evidence approach for evaluating possible causal relationships between exposure to fly ash-associated metals and the health status of sentinel fish populations.

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APPENDIX

Appendix Table 1. Mean and standard error (SE) of representative bioindicators of fish health responses for five fish species studied during four seasons at six sample sites in the Clinch and Emory River systems. In most cases means and SEs are for 6-8 individual fish for each species/season/site combination. Where there are values missing, that particular bioindicator was not measured for that site and/or species. Fish species abbreviations are as follows: BLUGIL = bluegill sunfish; LMBASS = largemouth bass; CHNCAT = channel catfish; and WHCRAP = white crappie. Units of measure for individual bioindicators are: 1) the condition factor, liver-somatic, visceral-somatic, spleno-somatic indices, gall bladder color and size, stomach and intestinal fullness = index score (see Approach section); 2) hematocrit = % red blood cells; 3) ALT, alkaline phosphatase, and amylase=IU/L; 4) urea nitrogen, glucose, and calcium = mg/dL; 5) sodium and potassium=mmol/L; 6) total protein, albumin and globulin = g/L.

BIOENERGETICS & HEMATOLOGY

Visceral-somatic index												
BLUGIL SPR09	3.60	0.10			3.20	0.20	3.20	0.10	3.30	0.10	3.05	0.20
BLUGIL FALL09	3.90	0.25	4.04	0.26	3.25	0.24	3.47	0.21	3.67	0.30	3.90	0.21
LMBASS SPR09	4.37	0.32			4.84	0.29	4.60	0.17	5.20	0.31	4.30	0.36
BLUGIL SPR10	3.00	0.40	3.10	0.20	4.00	0.30	3.20	0.20	3.10	0.20	3.50	0.10
BLUGIL FALL10	4.10	0.40	3.50	0.16	3.40	0.16	3.40	0.21	3.50	0.20	3.50	0.14
LMBASS SPR10	4.83	0.80	4.67	0.30	4.52	0.20	4.49	0.13	4.71	0.40	4.83	0.20
WHCRAP SPR09	2.04	0.10					2.60	0.56	2.60	0.07	2.60	0.17

SITES

	ERM8.0		*LERM2.0*		*ERM3.0*		*ERM0.9*		*CRM1.5*		*CRM8.0*	
CONDITION INDICES												
Condition Factor	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE
BLUGIL SPR09	1.76	0.04			1.68	0.03	1.73	0.04	1.64	0.04	1.81	0.04
BLUGIL FALL09	1.63	0.08	1.66	0.08	1.56	0.04	1.66	0.04	1.66	0.04	1.66	0.03
LMBASS SPR09	1.43	0.04			1.42	0.05	1.50	0.05	1.42	0.04	1.34	0.03
LMBASS FALL09	1.38	0.04	1.37	0.06	1.30	0.05	1.29	0.06	1.37	0.06	1.21	0.10
BLUGIL SPR10	1.67	0.05	1.70	0.07	1.84	0.05	1.82	0.04	1.72	0.05	1.85	0.04
BLUGIL FALL10	1.69	0.06	1.75	0.03	1.77	0.06	1.69	0.06	1.78	0.03	1.74	0.06
LMBASS SPR10	1.37	0.04	1.41	0.07	1.54	0.04	1.44	0.05	1.43	0.05	1.57	0.06
LMBASS FALL10	1.31	0.01	1.35	0.05	1.39	0.05	1.38	0.05	1.41	0.03	1.32	0.03
CHNCAT FALL09	0.85	0.04			0.86	0.20	0.76	0.03	0.80	0.04	0.80	0.03
CHNCAT FALL10	0.75	0.03	0.80	0.05	0.84	0.06	0.89	0.03	0.75	0.02	0.86	0.03
WHCRAP SPR09	1.35	0.04					1.58	0.07	1.51	0.07	1.40	0.03
WHCRAP SPR10	1.26	0.07	1.37	0.09	1.21	0.03	1.45	0.04	1.51	0.03	1.44	0.06
REDEAR SPR10	1.52	0.02	1.43	0.03	1.50	0.05	1.54	0.03	1.54	0.04	1.77	0.05
Liver-somatic index												
BLUGIL SPR09	1.80	0.10			1.30	0.08	1.30	0.08	1.20	0.07	1.40	0.04
BLUGIL FALL09	1.10	0.12	0.98	0.08	0.73	0.04	0.82	0.11	0.79	0.08	0.72	0.03
LMBASS SPR09	1.47	0.09			1.84	0.10	1.70	0.10	1.75	0.05	1.49	0.17
LMBASS FALL09	0.73	0.04	1.07	0.14	0.82	0.09	0.60	0.03	0.75	0.07	0.84	0.12
BLUGIL SPR10	2.03	0.20	1.90	0.17	1.89	0.15	1.54	0.07	1.61	0.12	1.93	0.07
BLUGIL FALL10	1.00	0.09	0.87	0.10	0.92	0.04	0.66	0.03	1.62	0.24	0.77	0.07
LMBASS SPR10	1.14	0.13	1.26	0.05	1.32	0.06	1.40	0.06	1.54	0.12	1.76	0.09
LMBASS FALL10	0.74	0.07	0.70	0.06	0.94	0.10	0.72	0.07	0.76	0.06	0.64	0.03
CHNCAT FALL09	1.48	0.12			1.33	0.12	1.19	0.06	1.56	0.12	1.78	0.13
CHNCAT FALL10	1.21	0.07	1.35	0.20	2.06	0.69	1.37	0.17	1.23	0.11	1.93	0.10
WHCRAP SPR09	1.09	0.07					1.05	0.09	1.10	0.06	1.13	0.12
WHCRAP SPR10	0.72	0.10	0.95	0.05	0.86	0.04	1.31	0.09	1.54	0.06	1.09	0.07
REDEAR SPR10	1.33	0.06	1.24	0.06	1.71	0.20	1.10	0.05	1.23	0.04	1.29	0.08
WHCRAP SPR10	1.90	0.20	2.30	0.40	1.86	0.10	3.40	0.20	3.50	0.30	3.50	0.30

REDEAR SPR10	2.30	0.10	2.40	0.10	3.10	0.20	3.10	0.20	3.00	0.20	2.60	0.20
Spleno-somatic index												
LMBASS FALL09	0.12	0.02	0.15	0.01	0.17	0.10	0.15	0.02	0.16	0.02	0.17	0.02
LMBASS FALL10	0.15	0.02	0.16	0.02	0.20	0.03	0.17	0.03	0.12	0.02	0.21	0.07
CHNCAT FALL09	0.14	0.02			0.12	0.01	0.10	0.01	0.16	0.02	0.14	0.02
CHNCAT FALL10	0.09	0.01	0.10	0.01	0.09	0.01	0.13	0.02	0.13	0.02	0.12	0.02
Hematocrit												
BLUGIL SPR09	30.50	0.90			28.10	0.90	25.30	0.90	27.90	0.80	25.80	1.20
BLUGIL FALL09	24.20	1.20	21.60	0.78	24.30	1.40	34.90	2.00	24.90	1.30	23.60	1.80
LMBASS SPR09	35.30	3.00			31.00	2.00	29.30	1.80	31.30	1.70	30.50	3.20
LMBASS FALL09	34.00	2.60	29.90	1.40	31.50	1.50	42.30	3.00	28.60	2.20	34.90	4.10
BLUGIL SPR10	38.30	1.90	31.70	1.10	29.20	2.00	33.40	2.10	29.90	1.40	26.80	1.40
BLUGIL FALL10	26.80	1.60	25.00	1.15	28.40	1.80	27.50	1.60	26.30	1.25	27.40	1.32
LMBASS SPR10	31.00	1.50	29.60	2.10	37.40	2.10	38.50	3.30	38.40	1.60	36.50	2.70
LMBASS FALL10	30.00	3.00	30.10	1.30	34.00	2.30	33.30	3.00	35.80	2.80	31.00	2.50
CHNCAT FALL09	30.00	3.10			32.10	2.10	33.50	2.00	31.80	1.40	29.10	2.40
CHNCAT FALL10	24.10	2.40	21.70	1.40	28.80	1.60	30.10	2.40	31.00	1.80	27.60	1.70
WHCRAP SPR09	34.00	2.60					44.50	2.60	38.00	5.00	30.50	1.20
WHCRAP SPR10	27.00	2.50	24.80	2.90	36.40	1.40	40.80	1.80	39.80	0.90	36.40	1.90
REDEAR SPR10	26.60	0.80	32.30	0.90	27.20	1.10	30.20	1.80	32.80	2.40	32.40	1.40

HISTOPATHOLOGY

Liver Histopathology												
BLUGIL SPR09	7.70	0.80			9.70	0.70	8.60	0.50	7.00	0.30	8.20	0.20
BLUGIL FALL09	4.70	1.09	5.30	0.48	6.00	0.63	7.00	1.40	5.50	0.87	5.50	0.87
LMBASS SPR09	7.80	1.08			10.00	1.20	7.00	0.50	7.00	1.00	5.80	1.40
LMBASS FALL09	5.30	0.70	5.50	2.50	6.20	1.30	7.50	0.40	3.00	0.40	9.30	1.10
BLUGIL SPR10	4.30	0.40	6.30	0.80	3.80	0.70	7.00	0.30	6.60	0.90	6.40	0.70
BLUGIL FALL10	2.70	0.50	2.80	0.60	2.00	0.40	3.50	0.60	5.20	0.75	4.60	0.40
LMBASS SPR10	4.80	0.80	6.60	0.50	6.20	0.31	7.00	0.50	7.20	0.40	8.00	0.30
LMBASS FALL10	7.00	1.30	6.00	0.60	3.80	0.50	6.00	1.40	6.00	0.80	6.50	0.50
CHNCAT FALL09	4.20	1.00			5.70	1.20	6.90	1.14	4.00	1.08	1.80	0.75
CHNCAT FALL10	4.80	0.80	4.00	1.20	2.80	0.90	6.00	1.30	6.30	1.60	1.80	0.40
WHCRAP SPR09	5.80	1.10					6.00	0.40	3.00	0.20	5.00	0.70
WHCRAP SPR10	4.70	0.70	6.60	0.80	7.00	0.40	6.20	0.30	4.80	0.50	5.80	0.60
REDEAR SPR10	6.30	0.70	6.70	0.30	6.70	0.40	6.80	0.50	5.80	0.60	6.00	0.40
Ovary Histopathology												
BLUGIL SPR09	0.30	0.20			1.20	0.60	0.40	0.30	0.40	0.20	1.40	0.20
LMBASS SPR09	1.20	0.54			1.80	0.60	0.00	0.00	0.20	0.20	0.20	0.20
BLUGIL SPR10	0.50	0.30	1.50	0.50	0.20	0.10	0.00	0.00	0.00	0.00	1.80	0.70
LMBASS SPR10	0.20	0.20	0.00	0.00	0.20	0.10	0.70	0.50	0.20	0.10	0.40	0.30
WHCRAP SPR09	1.00	0.40					0.30	0.10	0.50	0.10	0.00	0.00
WHCRAP SPR10	0.70	0.30	0.20	0.10	0.30	0.10	0.30	0.10	0.00	0.00	0.30	0.10
REDEAR SPR10	0.30	0.20	0.20	0.10	0.20	0.10	0.20	0.10	0.80	0.30	0.20	0.10
Gill Histopathology												
BLUGIL FALL09	4.00	1.10	7.30	1.30	4.70	1.20	5.00	0.80	3.80	0.63	2.80	0.80
LMBASS FALL09	5.30	0.60	8.50	1.80	5.70	0.80	4.70	0.60	5.25	0.48	6.75	1.50
BLUGIL FALL10	7.50	0.43	6.30	0.56	4.50	1.00	4.20	0.60	5.00	0.50	3.80	1.20
LMBASS FALL10	7.70	0.90	5.20	1.10	6.00	0.80	5.20	0.60	4.30	0.70	4.80	0.70
CHNCAT FALL09	6.00	0.77			7.20	1.05	11.40	1.20	8.30	0.85	9.50	1.60
CHNCAT FALL10	6.20	0.50	6.20	0.90	8.30	1.50	6.80	0.60	7.80	0.80	8.00	0.70

ORGAN DYSFUNCTION

Albumin												
CHNCAT FALL09	11.00	1.60			14.30	1.70	8.80	1.90	12.90	1.40	12.80	1.50
CHNCAT FALL10	7.10	1.40	8.00	1.30	13.80	0.90	12.10	1.00	12.80	1.10	13.80	1.80
REDEAR SPR10	11.30	0.70	10.30	0.90	7.30	1.20	11.70	1.10	9.70	1.40	15.10	1.70
Alanine Transaminase												
BLUGIL SPR09	15.60	1.50			20.00	3.10	20.20	4.00	25.30	5.60	22.40	4.00
BLUGIL FALL09	17.10	5.40	9.10	1.35	13.10	1.90	31.30	4.40	13.10	2.80	13.20	3.30
LMBASS SPR09	30.50	5.50			25.30	4.00	41.20	12.30	39.80	4.60	42.00	11.80
LMBASS FALL09	17.40	2.10	25.30	5.60	18.50	3.30	24.80	4.90	43.30	20.00	22.70	4.60
BLUGIL SPR10	20.10	3.20	17.70	2.10	20.50	1.40	21.90	2.90	29.10	5.20	28.50	4.70
BLUGIL FALL10	13.30	1.40	14.30	1.06	19.10	4.50	15.10	2.10	12.70	1.50	14.30	2.60
LMBASS SPR10	41.00	12.80	38.50	10.50	42.00	11.10	30.90	14.50	35.00	11.00	25.90	4.40
LMBASS FALL10	20.50	5.10	20.20	3.20	17.90	2.00	20.40	3.20	21.00	3.20	24.60	2.70
CHNCAT FALL09	23.70	2.00			35.60	5.60	32.80	7.00	27.00	2.80	50.20	12.10
CHNCAT FALL10	17.80	1.30	25.70	2.40	37.10	6.20	27.50	3.00	16.90	0.74	59.80	9.00
WHCRAP SPR09	29.00	17.10					24.80	8.30	21.50	4.50	36.30	6.80
WHCRAP SPR10	15.30	3.20	19.00	5.00	28.30	4.90	63.00	18.10	32.10	4.40	65.60	19.90
Blood Urea Nitrogen												
BLUGIL SPR09	3.50	0.30			3.40	0.20	3.60	0.30	2.90	0.20	3.60	0.20
BLUGIL FALL09	2.60	0.30	2.20	0.25	1.80	0.25	2.10	0.26	2.10	0.20	2.70	0.80
LMBASS SPR09	3.50	0.38			3.30	0.29	3.50	0.42	3.00	0.27	3.20	0.37
LMBASS FALL09	1.90	0.20	2.00	0.10	2.80	0.30	3.40	0.20	2.80	0.70	2.90	0.70
BLUGIL SPR10	2.70	0.30	2.70	0.20	2.10	0.20	2.30	0.30	2.10	0.20	2.60	0.30
BLUGIL FALL10	3.00	0.33	2.40	0.30	2.70	0.18	1.40	0.32	1.90	0.31	1.90	0.30
LMBASS SPR10	2.30	0.40	1.90	0.30	1.90	0.10	3.40	0.20	2.50	0.50	3.80	0.30
LMBASS FALL10	2.60	0.30	2.90	0.20	2.30	0.30	2.00	0.30	2.60	0.40	2.30	0.30
WHCRAP SPR09	4.30	0.30					3.40	0.20	3.50	0.50	5.30	0.80
WHCRAP SPR10	2.30	0.80	2.00	0.30	2.40	0.30	3.00	0.40	3.00	0.20	3.40	0.20
REDEAR SPR10	6.30	0.30	4.90	0.80	3.60	0.30	6.80	0.50	6.60	0.40	7.70	0.90

DIGESTIVE PHYSIOLOGY

Alkaline Phosphatase												
CHNCAT FALL09	28.30	1.50			25.50	1.70	24.90	4.10	30.90	1.80	28.50	2.30
CHNCAT FALL10	18.40	1.30	22.80	2.40	29.50	4.10	24.50	2.60	30.40	2.80	24.00	3.00
REDEAR SPR10	21.20	2.50	25.10	4.70	9.90	0.90	12.90	1.10	23.80	3.80	12.40	1.30
Amylase												
CHNCAT FALL09	49.30	9.90			17.60	7.60	65.60	42.30	23.90	7.00	85.70	19.50
CHNCAT FALL10	42.60	16.70	25.70	3.00	47.80	13.60	30.00	12.80	28.90	6.70	125.80	23.00
REDEAR SPR10	75.80	10.00	43.60	5.10	68.40	8.40	46.80	3.90	45.20	6.80	78.10	11.70

CARBO-PROTEIN METABOLISM

Glucose												
BLUGIL SPR09	66.30	7.70			51.40	3.90	62.30	6.70	57.70	6.00	66.90	8.50
BLUGIL FALL09	67.40	9.00	39.10	1.30	43.00	4.90	91.90	8.50	58.60	4.50	56.00	6.00
LMBASS SPR09	112.80	18.20			98.70	24.30	65.20	11.10	61.90	7.10	66.20	12.90
LMBASS FALL09	42.60	4.00	36.00	1.70	58.10	12.20	140.30	6.20	46.40	5.20	56.60	8.20
BLUGIL SPR10	68.90	24.00	48.80	2.50	59.90	7.20	54.90	6.30	93.60	33.80	47.30	2.60
BLUGIL FALL10	53.50	5.40	41.90	4.90	76.60	12.10	50.90	7.70	40.70	2.70	41.50	3.70
LMBASS SPR10	48.70	3.70	65.30	4.50	145.10	32.80	112.80	18.20	173.80	39.10	100.00	29.90
LMBASS FALL10	49.40	5.10	55.00	8.10	73.10	6.30	68.00	7.70	67.10	11.50	57.00	7.80
WHCRAP SPR09	96.70	9.00					137.80	20.00	166.50	10.50	85.00	11.10
WHCRAP SPR10	67.70	6.00	92.80	15.40	95.70	11.20	127.70	10.70	293.00	19.30	116.40	12.10
REDEAR SPR10	67.60	5.20	92.20	12.50	76.30	3.70	100.60	9.70	102.30	8.10	60.10	4.00
Total Protein												
BLUGIL SPR09	48.60	4.00			38.50	0.90	40.00	0.90	38.90	1.90	41.20	0.90
BLUGIL FALL09	36.30	3.10	35.60	0.60	32.60	1.70	39.60	0.60	37.80	1.10	34.90	1.60
LMBASS SPR09	53.60	2.10			53.60	1.60	51.80	3.70	57.00	3.10	52.20	1.40
LMBASS FALL09	45.80	2.60	44.50	2.20	44.50	2.30	53.50	1.40	46.60	2.60	44.70	2.70
BLUGIL SPR10	43.00	1.50	41.10	1.40	41.30	1.50	41.10	1.30	37.10	2.10	41.60	1.90
BLUGIL FALL10	38.60	1.72	37.60	1.20	39.10	1.20	38.60	0.80	40.30	1.00	38.80	1.40
LMBASS SPR10	48.70	3.60	50.40	2.70	57.30	2.70	60.60	2.60	55.50	3.20	59.40	2.60
LMBASS FALL10	46.90	2.40	45.10	0.90	49.60	1.90	47.90	2.90	50.20	2.90	48.90	2.40
CHNCAT FALL09	34.90	1.50			40.50	2.30	37.80	2.60	39.90	2.00	36.90	2.20
CHNCAT FALL10	30.30	1.30	31.30	2.20	38.60	1.50	35.80	1.70	38.90	2.30	38.60	1.50
WHCRAP SPR09	53.00	2.90					63.00	3.60	58.00	5.00	45.50	3.00
WHCRAP SPR10	51.00	3.50	46.80	3.60	49.30	2.20	63.00	1.60	54.20	2.20	59.20	4.40
REDEAR SPR10	36.10	0.80	35.80	1.10	32.80	1.70	38.10	1.50	37.30	1.50	41.00	2.40

Globulin												
CHNCAT FALL09	22.50	0.65			26.90	0.90	32.50	2.50	27.40	0.90	24.70	1.10
CHNCAT FALL10	5.80	3.80	4.30	1.20	24.80	1.00	14.50	4.30	20.40	4.60	12.30	4.20

ELECTROLYTE HOMEOSTASIS

Calcium											
BLUGIL SPR09	20.00	0.10			16.90	0.70	18.90	0.30	18.50	0.60	19.20	0.40
LMBASS SPR09	18.80	0.50			19.70	0.30	17.60	0.90	20.00	0.20	18.40	0.60
LMBASS FALL09	13.80	0.80	13.20	0.90	13.30	0.70	14.90	0.40	13.00	0.60	12.40	0.70
BLUGIL SPR10	20.20	0.40	19.90	0.50	19.90	0.40	18.80	1.20	19.60	0.60	21.80	0.90
LMBASS SPR10	19.00	1.30	20.20	0.90	20.30	0.30	18.20	1.20	21.90	1.30	19.30	0.40
LMBASS FALL10	13.10	0.70	13.10	0.50	14.20	0.70	15.30	0.70	14.30	0.40	14.40	0.50
CHNCAT FALL09	10.50	0.28			12.40	0.53	11.50	0.88	11.70	0.45	11.20	0.61
CHNCAT FALL10	9.80	0.40	10.70	0.60	11.80	0.60	11.40	0.30	11.70	0.30	11.30	0.40
REDEAR SPR10	18.10	0.30	18.90	0.50	18.20	0.40	19.00	0.70	19.90	0.20	20.50	1.00
Potassium												
BLUGIL FALL09	4.80	0.40	4.70	0.26	5.40	0.23	6.40	0.31	5.50	0.30	6.90	0.20
LMBASS SPR09	1.60	0.20			2.10	0.20	3.50	0.40	3.90	0.40	4.40	0.20
BLUGIL FALL10	5.20	0.26	5.30	0.48	4.10	0.37	5.80	0.50	5.50	0.40	5.70	0.20
LMBASS SPR10	2.20	0.40	1.60	0.30	1.20	0.14	1.50	0.17	1.40	0.22	1.60	0.19
WHCRAP SPR09	3.20	1.20					3.94	1.50	2.55	0.35	5.00	0.11
WHCRAP SPR10	2.60	0.40	2.50	0.30	2.50	0.40	3.50	0.20	3.40	0.40	3.40	0.40
REDEAR SPR10	4.40	0.20	4.20	0.60	2.80	0.40	3.50	0.50	3.50	0.40	4.40	0.30
Sodium												
BLUGIL SPR09	156.00	2.30			154.40	2.00	155.50	1.80	150.10	4.80	152.00	1.80
BLUGIL FALL09	147.00	3.00	149.10	1.50	141.60	4.20	155.30	1.00	153.30	1.70	150.70	1.40
LMBASS FALL09	148.80	2.30	148.90	0.70	152.10	1.70	159.90	1.50	152.40	2.50	147.90	6.00
BLUGIL SPR10	151.00	3.00	147.00	0.60	148.20	1.50	150.50	1.50	147.70	1.50	148.00	1.40
BLUGIL FALL10	155.80	1.70	150.90	2.90	151.90	2.70	152.50	2.30	154.90	1.40	152.60	2.10
LMBASS FALL10	150.00	1.40	153.50	1.60	155.10	2.10	156.30	1.10	155.30	1.60	154.90	2.20
CHNCAT FALL09	127.00	1.70			122.60	2.30	125.80	3.20	132.00	2.40	129.30	2.30
CHNCAT FALL10	130.50	1.20	131.00	1.40	131.40	1.40	136.60	0.60	135.80	1.70	133.40	3.20
WHCRAP SPR09	150.30	0.90					154.60	1.70	151.50	2.50	155.50	1.00
WHCRAP SPR10	151.70	1.20	152.40	1.20	153.00	1.80	149.70	1.10	154.60	1.00	151.20	1.90

FEEDING & NUTRITION

Gall Bladder Color												
BLUGIL SPR09	2.10	0.10			2.50	0.20	1.90	0.20	2.10	0.15	2.10	0.07
LMBASS SPR09	0.00	0.00			1.00	0.40	0.10	0.10	1.70	0.40	1.40	0.40
BLUGIL SPR10	2.10	0.10	2.00	0.10	1.90	0.10	2.20	0.20	2.00	0.10	2.10	0.30
LMBASS SPR10	1.00	0.20	0.80	0.20	1.60	0.40	0.40	0.20	0.40	0.20	0.00	0.00
Gall Bladder Size												
BLUGIL FALL09	2.80	0.15	3.00	0.15	2.80	0.17	2.90	0.11	2.30	0.24	2.90	0.11
BLUGIL FALL10	2.40	0.30	2.50	0.19	2.60	0.18	2.90	0.13	3.10	0.10	2.50	0.20
Stomach Fullness												
BLUGIL SPR09	0.50	0.20			1.10	0.30	1.20	0.20	0.20	0.10	0.40	0.20
BLUGIL FALL09	0.90	0.30	1.00	0.37	0.13	0.10	0.10	0.10	3.00	0.50	1.10	0.40
LMBASS SPR09	3.40	0.30			2.60	0.40	2.90	0.30	2.10	0.13	2.90	0.14
LMBASS FALL09	2.90	0.60	0.60	0.40	0.30	0.20	0.30	0.10	0.30	0.20	0.60	0.40
BLUGIL SPR10	0.10	0.10	0.30	0.10	0.90	0.40	0.20	0.10	0.90	0.20	1.20	0.30
BLUGIL FALL10	1.00	0.40	1.40	0.30	1.80	0.50	0.90	0.30	0.60	0.18	0.50	0.20
LMBASS SPR10	2.60	0.40	2.60	0.20	2.40	0.30	3.00	0.50	2.60	0.40	3.30	0.30
LMBASS FALL10	0.30	0.20	0.10	0.05	0.00	0.00	0.10	0.05	1.00	0.40	1.10	0.60
Intestinal Fullness												
LMBASS FALL09	1.60	0.30	0.50	0.20	0.90	0.30	0.00	0.00	0.40	0.20	0.60	0.30
LMBASS FALL10	0.40	0.10	0.30	0.20	0.40	0.10	0.30	0.20	0.80	0.30	0.40	0.20