EFFECT OF MERCURY CONCENTRATION ON ASYMMETRY IN FISH SKULLS

by

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(Under the Direction of Charles H. Jagoe)

ABSTRACT

Largemouth bass (*Micropterus salmoides*) from L Lake, Par Pond, and Pond B located on the Savannah River Site in SC, were analyzed for mercury concentration, length, weight, and age. Fish skull asymmetries were assessed using geometric morphometrics by landmarking dorsal and ventral images. Mercury was positively correlated with fish length, weight, and age for all populations, but not with age for Par Pond. Directional asymmetry was significant in each population but not different among populations. Each population had significant levels of fluctuating asymmetry (FA), but the only difference was that Par Pond had greater FA than Pond B for the dorsal view of the skull. Correlations between individual overall asymmetry values and Hg for either view were not significant for any population or for populations considered together. Patterns of variation in asymmetry and mercury concentrations among locations were not consistent.

INDEX WORDS: Directional asymmetry, Fish skull, Fluctuating asymmetry. Geometric morphometrics, Mercury, Landmarking, Largemouth bass, Symmetric shape variation, *Micropterus salmoides*, Overall asymmetry

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DEDICATION

My thesis is dedicated to my wonderful family.

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INTRODUCTION

Mercury (Hg) in fish has been a matter of concern worldwide, following the Minamata Bay episode of mass human poisoning. Mercury is released to the atmosphere largely by burning fossil fuels. Most Hg in the atmosphere is in the form of elemental vapor. It is transferred to water and soil via precipitation. Hg occurs in water as an inorganic salt or as organic methylmercury (MeHg). Inorganic salts of Hg are transformed to MeHg by bacteria that are ingested by small aquatic organisms. Phytoplankton may also concentrate MeHg from water. MeHg is almost completely absorbed in the digestive tract of organisms (Bloom, 1992) and is very slowly excreted. Thus, MeHg biomagnifies, or increases with trophic position in foodwebs (Gilmour et al., 1992; Fitzgerald et al., 1998). Humans eating fish containing Hg can be exposed to toxic levels of Hg. Health advisories on eating contaminated fish have been issued to minimize neurotoxic and teratogenic effects of Hg on humans (National Research Council, 1978)

Fish living in waters receiving Hg inputs where the potential for methylation is high often have Hg concentrations higher than EPA risk-based guidelines for human consumption (EPA, 2000). These guidelines suggest consumption of no more than 1 meal per month of fish with muscle Hg concentrations above 0.47 mg/kg. Mercury concentrations tend to increase with trophic level, and higher concentrations are typically found in predatory fish (Kidd et al., 1995; Neumann and Ward, 1999; Bowles et al., 2001; Snodgrass et al., 2000). Adult largemouth bass (*Micropterus salmoides*) are largely piscivorous (Howick and O’Brien, 1983), and so tend to accumulate higher concentrations of mercury in their bodies. In addition to trophic position, mercury content of fish is positively related to fish length (Scott and Armstrong, 1972; Weiner et al., 1990; Lange et al., 1993) and age (MacCrimmon et al., 1983; Guntnmann et al., 1992), and may vary between sexes (Nicoletto and Hendricks, 1988)

Organisms are genetically predisposed to attain a particular form, defined by size, shape and symmetry. Fish and other vertebrates are bilaterally symmetrical; presumably, the same genes code for development of structures on both the left and right sides. Variation in the development (instability) of various structures and the tendency for developmental stability interact to produce the adult form of an organism. Developmental instability tends to increase with environmental stress. Thus, stress can disrupt precise development, and may result in individuals with an asymmetric shape. A variety of stresses, including pollutant exposure, may affect growth and level of morphological asymmetry (Ames et al., 1979; Jagoe and Haines, 1985; Oleksyk et al., 2004). The effects of stressors including heavy metals on asymmetry are still somewhat equivocal (reviews by Leary and Allendorf, 1989; Clarke, 1992 and 1993; Graham et al., 1993; Sommer, 1996).

There are three types of asymmetry including directional asymmetry (DA), antisymmetry, and fluctuating asymmetry (FA). DA is present when one side (right or left) of the body is consistently bigger than the other. Antisymmetry is present when the side which is bigger varies among individuals creating a bimodal distribution for the differences. FA is a pattern of bilateral variation where the mean difference between sides for a population is zero, and the variation is normally distributed about zero (Palmer, 1994). FA is frequently used to assess developmental stability due to environmental stress.

Canalization is the ability of a structure to develop along an ideal developmental trajectory under a variety of different environmental conditions (Waddington, 1940). FA is a result of the organism's inability to completely canalize its development. FA can be quantified as the variance of the difference between right and left sides (R - L) corrected for measurement error. Several studies have been conducted to measure FA and DA for morphological characters in fish (Ames et al., 1979; Felley, 1980; Jagoe and Haines, 1985; Allenbach et al., 1999). Mouse skeletons have also been used to estimate FA and DA and establish their genetic basis and heritability (Leamy, 1997 and 1999 Leamy et al., 2000). Geometric morphometrics extend the traditional approach of measuring left and right side traits to quantify individual variation and asymmetry in geometric shape of paired structures. This approach consists of landmarking photographic images of each specimen and creating mirror images of the right and left sides to form a consensus figure. Differences between landmarked points and consensus points are used to calculate Procrustes residuals as a measure of asymmetry for all landmarks allowing shape variation to be partitioned into symmetric shape and asymmetry (Klingenberg et al., 2002). Environmental stressors increase asymmetry in a variety of organisms (Møller and Swaddle, 1997; pp. 134 - 153). Geometric morphometrics have also been used to support this generalization (Oleksyk et al., 2004) and to study the relationship of asymmetry to other factors like population density (Novak, 2003). However, some studies have failed to find a relationship between pollutants and asymmetry (e. g., Rabitsch, 1997; Vollestad et al., 1998). The use of FA as an inexpensive reliable indicator of environmental stress is still an area of active research.

My primary objective was to investigate relationships among Hg, asymmetry in skulls, body length, weight, and age of largemouth bass (*Micropterus salmoides*) from three sites on the Savannah River Site in South Carolina. Secondly, the effects of location on symmetric shape variation, DA and FA in skulls were evaluated. Finally, I tested for relationships between overall skull asymmetry and Hg concentrations in individual fish.

MATERIALS AND METHODS

Sampling

Adult largemouth bass were collected from three sites at the U.S. Department of Energy's Savannah River Site in South Carolina (Fig 1). The sites, Pond B, Par Pond, and L Lake, are former reactor cooling reservoirs located in Aiken and Barnwell Counties. These sites are impoundments of streams that drain into the Savannah River and support aquatic communities typical of southeastern reservoirs (Paller et al., 1992; Paller, 1997).

Fish were collected by angling during all seasons in the years of 1998 to 2002. Fish wet weight and total length were recorded, and whole fish were and frozen until analysis. Muscle mercury concentrations were measured as described below. Random numbers were assigned to each individual in a list ordered by their Hg concentrations. The list was divided into three groups, the highest, middle, and lowest thirds. Approximately equal numbers of fish were selected from each group using the random numbers. The number of fish was approximately equal for each group and also for each location..

Mercury Analysis

Skinless muscle tissue was taken adjacent to the dorsal fin of each fish. The muscle tissue was weighed and freeze dried to determine moisture content. The dried tissue was analyzed for Hg concentration using a modification of EPA method 7473 (EPA, 1998) using a DMA-80A Analyzer, (Milestone Inc, Monroe, CT). This method uses automated thermal decomposition, preconcentration onto a gold trap, thermal desorption, and measurement of Hg in the vapor phase by a dual-path atomic adsorption spectrophotometer. Approximately 50 mg of muscle tissue was analyzed for each individual. For QA/QC purposes, analyses were carried out in batches of ten, with each batch including a blank, a sample replicate, and a tissue standard certified for Hg concentration (DORM-2, dogfish muscle, or TORT-2, lobster hepatopancreas, purchased from the National Research Council of Canada, Ottawa). Differences between replicates averaged 3.4 %, and concentration values for all standard materials fell within the certified range.

Asymmetry measurements

Frozen fish were thawed at room temperature, and heads were cut from the bodies and individually boiled in water for 20 to 40 min to partially clean each skull. Skulls were then soaked in strong detergent solution for a few hours, cleaned thoroughly with a brush and left to air dry. Skulls were labeled and stored. Opercular bones from the skulls were collected and used to determine age as in Peles *et al*. (2000). Annuli on both opercular bones were counted by two investigators (MS and NY) and were rechecked when inconsistencies were noted.

Images of the skulls were recorded using a Fuji Fine Pix 4900 digital camera. Individual skulls were placed on clay adjacent to a ruler, which was used as a scale for every image. A bubble-level was placed at the anterior end of the skull for both the dorsal and the ventral images to level the skull. The camera was attached to a copy stand facing downward at a height of about 50 cm above the skull, which allowed the skull to fill most of the image frame. Lighting from incandescent bulbs was supplied from both sides of the copy stand. The camera was also leveled using the bubble-level centered on its upper side. Duplicate images of the dorsal and ventral sides were recorded with skulls selected in a random order. Thus, each skull had four pictures taken; two each for the dorsal and ventral surfaces. The images were downloaded to the computer from the camera via a USB port. Each dorsal image was digitized using TPSDIG

(Rohlf, 2001) for a series of 35 landmarks including 17 pairs with points on each side of the skull and one on the midline (Fig. 2a). Each ventral image was digitized for a series of 10 pairs of landmarks using the same program (Fig, 2b). There were no points located on the anterior end of the ventral image, because of disarticulation of part of the skull in this region in some specimens. Each image was then digitized again with images placed in a random order. As a result there were two sets of landmarked images for each dorsal and ventral view.

Data analyses

Analyses of FA were performed using a geometric morphometric protocol (Adams *et al*., 2004) similar to that used in Oleksyk *et al*. (2004). The shape of the skull is defined by the set of paired and unpaired landmarks and the symmetry only by the paired landmarks. RelWarp, a software package provided by Rohlf (2003) was used to conduct generalized least-squares Procrustes fit of the landmark data for each image (Rohlf and Slice, 1990, Goodall, 1991; Dryden and Mardia, 1998). A mixed model Procrustes ANOVA (Klingenberg and McIntyre 1998, Klingenberg et al., 2002) was used to examine the relative amounts of symmetric variation and FA. SAS was used to conduct a multivariate ANOVA (SAS Institute Inc., 1999). The SAS code is given in Appendix A.

Estimates of overall asymmetry for individuals were calculated as in Oleksyk *et al*. (2004) using aligned landmark coordinates of the left and right sides of the skull. Statistica 6.0 (StatSoft Inc., 2001) was used to perform linear and multiple regressions and generate scatter plots. Estimates of FA and mean values of DA were also calculated for each population (Oleksyk *et al*., 2004). Mean squares for the effects of side (DA), individuals (shape), and side*individual (FA) for each population were used to calculate *F* ratios comparing two populations at a time. Sequential Bonferroni corrections were applied to adjust for Type 1 errors

(Palmer, 1994). Antisymmetry was evaluated by examining the distributions of the Procrustes residuals of the aligned coordinates of the landmarks for deviations from normality.

The univariate relationships between Hg concentrations with age, total length, and total weight for fish in each population were calculated using Statistica 6.0. Duncan's Multiple Range tests were used to compare population means. Shapiro-Wilks' tests for normality of the distributions of Hg concentration, age, total length, and total weight for fish in each population were conducted using SAS. Multivariate analyses of the relationships between Hg concentration and total length, total weight, age, population, and the interactions of the independent variables were conducted using PROC GLM in SAS. Population was treated as a categorical variable with three levels in the model. Statistical significance was indicated when $p \leq 0.05$.

RESULTS

The distributions of Hg concentrations, total weight, total length, and age for individual populations did not depart from normality as indicated by Shapiro-Wilks' tests. The means for these variables, FA, DA, and the results of Duncan's tests comparing them are given in Table 1. Par Pond and Pond B fish had similar mean values for Hg, but that for L Lake was significantly lower than the other two (Table 1). Hg concentrations in bass ranged from 0.55 mg/kg to 5.64 mg/kg dry weight, 1.33 to 7.82 mg/kg, and 1.6 to 7.47 mg/kg in L Lake, Pond B, and Par Pond, respectively. The means of total weight and total length were significantly less in fish from Pond B than those from L Lake and Par Pond, while those from the latter two sites were not different. Mean ages of fish among the three populations were not significantly different.

There were positive linear relationships between Hg concentrations and total weight (Fig 3). The linear relationships were also positive between Hg concentration and total length for each population. A significant positive linear relationship between Hg and age was found for fish from L Lake and Pond B but not from Par Pond (Fig. 3). Slope of the relationship between Hg and total length was significantly greater for Pond B fish compared to that for fish from L Lake but not from Par Pond. Slopes for L Lake and Par Pond were not significantly different. The slopes of the relationship between Hg and total weight were not different among the populations either (Appendix B).

Multiple regression analyses were carried out for each location, using Hg as the dependent variable and total weight, total length, and age as the independent variables. Hg was positively related to total length in Par Pond (p = 0.006) and Pond B (p = 0.008), while it was positively related to total weight only in Pond B fish ($p < 0.001$). There were no significant relationships between Hg and any variable for L Lake fish. Multicollinearity due to interactions between the variables occurred. Hg concentration was not related to any of the variables when all three or four way interactions were included in the model. When the three and four way interactions were dropped, total length, total weight and age were found to be significant predictors of Hg concentration in fish from all populations ($R^2 = 0.78$, $p < 0.001$, < 0.001, and 0.034, respectively). All two-way interactions were significant except that between total length and location ($p = 0.27$).

Skull shape and asymmetry

There was no evidence for antisymmetry in any population. There were significant levels of DA (p varied from 0.002 to 0.013) and FA ($p < 0.001$), and variation in symmetric shape ($p < 0.001$) for both views in each population. Appendix C provides ANOVA tables and pairwise comparisons of asymmetry in fish skulls between populations. Mean symmetric shape of the dorsal view was significantly different among locations with fish from L Lake having greater values than those from Pond B ($p < 0.001$), and Par Pond ($p < 0.001$), but shape did not differ between the latter two locations ($p = 0.07$). Fish skulls from Par Pond were more variable in symmetric shape of the ventral view than those from Pond B ($p < 0.001$) or from L Lake ($p = 0.02$), but those from the latter two sites were not different ($p = 0.23$).

DA was significantly greater than zero for both dorsal and ventral views of the skulls for all locations ($p \leq 0.01$). There were no significant differences between populations for the DA of either dorsal or ventral views ($p > 0.1$). FA values were significantly greater than zero for both views in each population. Pond B fish had the lowest FAs both for dorsal and ventral views of the skulls. Pair-wise differences of FAs were significant only for the dorsal view between Pond B and Par Pond skulls ($p = 0.01$).

Relationships between individual overall asymmetry values for either view and Hg concentrations were not significant as revealed by linear regressions for each population (Figs. 4 and 5; p ranged from 0.1 to 0.9 and r^2 from 0.001 to 0.16 for ventral and dorsal views). The results were also non-significant when the analyses were done for individuals from the three populations considered together for dorsal as well as ventral views (Figs. 6a and 6b; $p = 0.08$ and 0.34; $r^2 = 0.056$ and 0.017, respectively). Relationships between overall asymmetry and age were not significant for either view of the skull (Appendix D).

DISCUSSION

All largemouth bass in this study had detectable levels of mercury as has been reported for Savannah River Site bass (Cummins et al.,1990, 1991; Arnett 1992; Jagoe et al., 1996). The source of this Hg is probably atmospheric deposition along with industrial discharges into the Savannah River upstream from the Savannah River Site (Kvartek et al., 1994). There were no significant differences in the mean ages of fish collected from the three locations, but Pond B bass were significantly lighter and shorter than those from the two other locations (Table 1). Hg concentrations in fish sampled from L Lake were significantly lower than those from the two other sites, which had comparable levels (Table 1). Jagoe et al. (1996) reported much higher values of Hg in muscle for L Lake bass than our values for this population, a difference that may reflect different analytical methods, small samples, or temporal variation.

Hg is positively correlated with length and body mass in fresh water and estuarine fishes (Scott and Armstrong, 1972 ; Weiner et al., 1990 ; Lange et al., 1993; Davis, 1997) as was found in this study (Fig. 3). Health advisories have been issued informing people not to eat larger fish based on an understanding of this relationship. Hg concentrations and the fish's age were positively related in L Lake and Pond B but not Par Pond (Fig. 3). Largemouth bass, being long lived and functioning at a high trophic level should accumulate Hg as they grow in size and age. There is a larger scatter of the data for the fish from Par Pond than from the other two locations, and there were few young fish sampled from this location.

Mercury concentrations in Pond B fish tended to increase at a greater rate with increasing length and weight than did those in L Lake and Par Pond fish. The water chemistry of Par Pond and L Lake are similar, but Pond B has softer and more acidic water than the other reservoirs (DOE, 1997; Peles et al., 2000). The Pond B fish were smaller in length and weight than those from the other two sites, and this may be due to a lack of nutrients in this reservoir. This stunting of growth is different from the thin bass phenomenon observed in Par Pond (Gibbons et al., 1978). The latter is caused by overall food limitation among larger bass leading to the loss of weight. Thin bass may have contributed to the scatter of the Par Pond data.

Analyses indicated that the independent variables age, total length, total weight, and population were highly correlated with each other when data for fish from all populations were considered together. The expected increase in Hg concentration in fish depends on age, total length, total weight, and on location. The significant interactions between variables indicate that Hg concentrations change differently depending upon which variables are involved in the interactions. Hg levels in fish are clearly influenced by a complex set of interactions, which are not completely understood at this time.

It is important to understand how pollutants including metals act as stressors that affect an organism's development. FA has been used as an indicator of developmental stability in populations. Lower pH was found to elevate FA in fish species (Zakharov, 1981; Jagoe and Haines, 1985). Polluted waters are associated with increased asymmetry in grey seal skulls (Zakharov, 1990). Oleksyk et al. (2004) reported higher levels of FA in yellow-necked mouse skulls collected from radioactively contaminated populations around Chornobyl in Ukraine. FA is generally elevated in stressed populations (Møller and Swaddle, 1997).

Hg may affect asymmetry in certain fish species (Ames et al., 1979) but not in others (Vollestad et al., 1998). Significant DA was found in each population of largemouth bass in this study. These results are similar to those of Oleksyk et al. (2004) where significant DA was present in the yellow-necked mouse skulls collected from reference as well as contaminated sites. This result implies that certain features on one side of the skull must be larger than on the other in each fish population. There was no difference in DA, but there was in Hg levels among sampling sites (Table 1) implying no relationship between DA and Hg. FAs for both views were significant in each population. Comparisons of FA for the ventral view showed no significant differences among populations. This result might be partially due to the lack of landmarks on the anterior portions of the ventral view of the skull. The dorsal views did show a significant difference in FA between Par Pond and Pond B skulls. However, these results do not correspond with differences in mean Hg concentrations in fish from these reservoirs (Table 1). At this level of analysis, Hg concentrations appear unrelated to asymmetry in the skulls of largemouth bass.

A study of developmental instability in grayling exposed to methylmercury during embryogenesis showed an increased proportion of phenodeviants in individuals exposed to the highest concentration of MeHg during early development (Vollestad et al., 1998). However, after three years of development, the effect on morphological variability and FA was non-significant, although exposed fish had significantly reduced feeding ability (Fjeld et al., 1998). Other sub-lethal effects may be present after a few years, but FA was apparently reduced by growth correcting the differences between the left and right sides. There were some older individuals in our study that had low levels of overall asymmetry. This could be due to adults correcting asymmetries during their development. Alternatively, some of them may not have survived as well as others leading to their elimination by selection. The lack of significant relationships between overall asymmetry and age in any population does not support the idea of asymmetries being corrected as the fish age (Appendix E). We have no data for survivorship of `bass with different asymmetries. However, Allenbach et al. (1999) studied two fish species and found that the least symmetric individuals do not survive as well as the more symmetrical ones when exposed to pesticides. The survivorship hypothesis needs further support if it is to be applied to largemouth bass.

Overall asymmetry values of individual skulls include asymmetry due to shape, DA, FA and measurement error. Uniform measurement error and equal amounts of isotropic variation in landmarks were assumed. The regression of individual overall asymmetry values against individual Hg levels in the fish for each population showed a lot of scatter (Fig. 5). However, the relationship between overall asymmetry and Hg was close to being significant for the dorsal view, when data from all fish were considered together (p = 0.08; Fig. 6a). The lack of significant relationship between overall asymmetry and Hg suggests that Hg is probably not acting as a stressor on developmental stability of bass skulls at the contaminant concentrations observed. My data are consistent with a general lack of convincing evidence for Hg having an effect on asymmetry in the literature for fish.

LITERATURE CITED

Adams DC, Rohlf FJ, Slice DE (2004) Geometric morphometrics: ten years of progress following the 'revolution'. Ital J Zool 71:5-16.

Allenbach DM, Sullivan KB, Lydy MJ (1999) Higher fluctuating asymmetry as a measure of susceptibility to pesticides in fishes. Environ Toxicol Chem 18:899-905.

Ames LJ, Felley JD, Smith MH (1979) Amounts of asymmetry in Centrarchid fish inhabiting heated and nonheated reservoirs. Trans Am Fish Soc 108:489-495.

Arnett MW, Karapatakis, LK, Mamatey AR, Todd JL (1992) Savannah River Site Environmental Report for 1991, WSRC-TR-92-186. Westinghouse Savannah River Company, Environmental Monitoring Section, Aiken, SC.

Bloom N (1992) On the chemical form of mercury in edible fish and marine invertebrate tissue. Can J Fish Aquat Sci. 49:1010-1017.

Bowles KC, Apte SC, Maher WA, Kawei M, Smith R (2001) Bioaccumulation and biomagnification of mercury in Lake Murray, Papua New Guinea. Can J Fish Aquat Sci. 58:888-897.

Clarke GM (1992) Fluctuating asymmetry: a technique for measuring developmental stress of genetic and environmental origin. Acta Zoologica Fennici 191:31-35.

Clarke GM (1993) Fluctuating asymmetry of invertebrate populations as a biological indicator of environmental quality. Environ Poll 82:207-211.

Cummins CL, Martin DK, Todd JL (1990) Savannah River Site Environmental Report for 1989, WSRC-IM-90-60. Westinghouse Savannah River Company, Environmental Monitoring Section, Aiken, SC.

Cummins CL, Martin DK, Todd JL (1991) Savannah River Site Environmental Report for 1990, WSRC-IM-91-28. Westinghouse Savannah River Company, Environmental Monitoring Section, Aiken, SC.

Davis JA (1997) Contaminant concentrations in fish from San Francisco Bay. San Francisco Estuarine Institute, Richmond, California.

DOE (U.S. Department of Energy) (1997) Final Environmental Impact Statement, shutdown of the river water system at the Savannah River Site, DOE/EIS-0268, Savannah River Operations Office, Aiken, South Carolina.

Dryden IL, Mardia KV (1998) Statistical Shape Analysis. John Wiley and Sons, New York, NY, USA.

EPA (U.S. Environmental Protection Agency) (1998) Mercury in solids and solution by thermal decomposition, amalgamation, and atomic absorption spectroscopy. EPA Method 7473, Revision 0. Washington, D.C.

EPA (U.S. Environmental Protection Agency) (2000) Guidance for Assessing Chemical Contaminant Data for Use in Fish Advisories, Volume 2: Risk Assessment and Fish Consumption Limits, Third Edition, EPA-823-B-00-008. Office of Water, Washington, D.C.

Felly J (1980) Analysis of morphology and asymmetry in bluegill sunfish (*Lepomis macrochirus*) in the Southeastern United States. Copeia 1980(1):18-29.

Fjeld E, Haugen TO, Vollestad LA (1998) Permanent impairment in the feeding behavior of grayling (*Thymallus thymallus*) exposed to methylmercury during embryogenesis, The Science of the Total Environment 213:247-254.

Fitzgerald WF, Engstrom DR, Mason RP, Nater EA (1998) The case for atmospheric mercury contamination in remote areas. Environ Sci Technol 32:1-7.

Gibbons JW, Bennett DH, Esch GW, Hazen TC (1978) Effects of thermal effluent on body condition of largemouth bass. Nature 274:470-471.

Gilmour CC, Henry EA, Mitchell R (1992) Sulfate stimulation of mercury methylation in freshwater sediments. Environ Sci Technol 26:2281-2287.

Goodall CR (1991) Procrustes methods in the statistical analysis of shape. J Roy Stat Soc 53:285-339.

Graham JH, Roe KE, West TB (1993) Effects of lead and benzene on developmental stability of *Drosophilia melanogaster*. Ecotoxicology 2:185-195.

Guntenmann WH, Ebel JG, Kuntz HT, Yourstone KS, Lisk DJ (1992) Residues of p,p'-DDE and mercury in lake trout as a function of age. Arch Environ Contam Toxicol 22:452-455.

Howick GL, O'Brien WJ (1983) Piscivorous feeding behavior in largemouth bass: An experimental analysis. Trans Amer Fish Soc 112:508-516.

Jagoe CH, Haines TA (1985) Fluctuating asymmetry in fishes inhabiting acidified and unacidified lakes. Can J Fish Mgmt 15:156-158.

Jagoe CH, Shaw-Allen PL, Brundage S (1996) Gill Na^{+}, K^{+}-ATPase activity in largemouth bass (*Micropterus salmoides*) from three reservoirs with different levels of mercury contamination. Aquat Toxicol 36:161-176.

Kidd KA, Hesslein RH, Fudge RJP, Hallard KA (1995) The influence of trophic level as measured by delta 15N on mercury concentrations in freshwater organisms. Water, Air Soil Poll 80:1011-1015

Klingenberg CP, Barluenga M, Meyer A (2002) Shape analysis of symmetric structures: quantifying variations among individuals and asymmetry. Evolution 56:1909-1920.

Klingenberg CP, McIntyre GS (1998) Geometric morphometrics of developmental instability: Analyzing patterns of fluctuating asymmetry with Procrustes methods. Evolution 52:1363-1375.

Kvartek EJ, Carlton MH, Denham L, Eldridge L, Newman MC (1994) Assessment of mercury in the Savannah River Site environment. WSRC-TR-94-0218ET. Westinghouse Savannah River Company, Aiken, SC.

Lange TR, Royals HE, Connor LL (1993) Influence of water chemistry on mercury concentration in largemouth bass from Florida lakes. Trans Amer Fish Soc 122:74-84.

Leamy L (1997) Genetic analysis of fluctuating asymmetry for skeletal characters in mice. J Hered 88:85-92.

Leamy L, (1999) Heritability of directional and fluctuating asymmetry for mandibular characters in random-bred mice. J evol Biol 12:146-155.

Leamy LJ, Pomp D, Eisen EJ, Cheverud JM (2000) Quantitative trait loci for directional but not fluctuating asymmetry of mandible characters in mice. Genet Res 76:27-40.

Leary RF, Allenrorf FW (1989) Fluctuating Asymmetry as an indicator of stress: implications for conservation biology. Trends Ecol Evol 4:214-217

MacCrimmon HR, Wren CD, Gots BL (1983) Mercury uptake by lake trout *Salvelinus namaycush* relative to age, growth and diet in Tadenac lake with comparative data from other Precambrian shield lakes. Can J Fish Aquat Sci 40:114-120.

Møller AP, Swaddle JP (1997) Asymmetry, Developmental Stability, and Evolution (In Oxford series in Ecology and Evolution). Oxford University Press Oxford, UK.

National Research Council (1978) An Assessment of Mercury in the Environment. National Academy of Sciences, Washington, DC.

Neumann RM, Ward SM (1999) Bioaccumulation and biomagnification of mercury in two warmwater fish communities. J Freshwater Ecol 14:487-497.

Nicoletto PF, Hendrix AC (1988) Sexual differences in accumulation of mercury in four species of centrarchid fishes. Can J Zool 66:944-949

Novak JM (2003) Scale and organismal form: an ecological genetic perspective. PhD Dissertation, Univ. Georgia, Athens, GA.

Oleksyk TK, Novak JM, Purdue JR, Gashchak SP, Smith MH (2004) High levels of fluctuating asymmetry in populations of *Apodemus flavicollis* from the most contaminated areas in Chornobyl. J. Environ. Radioactivity 73:1-20.

Paller MH, Gladden JB, Heuer JH (1992) Development of the fish community in a new South Carolina reservoir. American Midland Naturalist, 128:95-114.

Paller MH (1997) Recovery of a reservoir fish community from drawdown related impacts, N Am J Fish Mgmt, 17:726-733.

Palmer AR. 1994. Fluctuating asymmetry analyses: A primer. In Markow RA, ed, Developmental Instability: Its Origins and Evolutionary Implications. Kluwer, Dordrecht, The Netherlands, 335-364.

Peles JD, Philippi T, Smith MH, Brisbin Jr. IL, Gibbons JW (2000) Seasonal variation in radiocesium levels of largemouth bass (*Micropterus salmoides*): Implications for humans and sensitive wildlife species. Environ Toxicol Chem 19:1830-1836.

Rabitsch WB (1997) Levels of asymmetry in *Formica pratensis* Retz. (Hymenoptera, Insecta) from a chronic metal-contaminated site. Environ Toxicol Chem 16:1433-1440.

Rohlf FJ, Slice D (1990) Extension of the Procrustes method for the optical superimposition of landmarks. Syst Zool 39:40-59.

Rohlf FJ (2001) TPS DIG: digitizing software. Department of Ecology and Evolution, State University of New York, Stony Brook. Stony Brook, NY.

Rohlf FJ (2003) TpsRelW: A Program for Relative Warp Analysis. Department of Ecology and Evolution, State University of New York, Stony Brook. Stony Brook, NY.

SAS Institute (1999) SAS/STAT® User's Guide, Ver. 8. SAS Institute Inc., Cary, NC.

Scott DP, Armstrong FA (1972) Mercury concentrations in relation to size in several species of freshwater fishes from Manitoba and northwestern Ontario. J Fish Res Bd Canada 29:1685-1690.

Snodgress JW, Jagoe CH, Bryan Jr. AL, Brant HA, Burger J (2000) Effects of trophic status and wetland morphology, hydroperiod, and water chemistry on mercury concentrations in fish. Can J Fish Aquat Sci 57:171-180.

Sommer C (1996) Ecotoxicology and developmental stability as an *in situ* monitor of adaptation. Ambio 25:375-376.

StatSoft Inc. (2001) STATISTICA (data analysis software system), version 6.

Vollestad LA, Fjeld E, Haugen T, Oxnevad SA (1998) Developmental instability in grayling (*Thymallus thymallus*) exposed to methylmercury during embryogenesis. Environ Poll 101:349-354.

Waddington CH (1940) Organizers and genes. Cambridge Univ. Pr. Cambridge, UK.

Wiener JG, Martini RE, Sheffy TB, Glass GB (1990) Factors influencing mercury concentrations in walleyes in northern Wisconsin lakes. Trans Amer Fish Soc. 119:862-870.

Zakharov VM (1981) Fluctuating asymmetry as an index of developmental homeostasis. Genetica 13:241-256.

Zakharov VM, Yablokov AV (1990) Skull asymmetry in the Baltic grey seal: effects of environmental pollution. Ambio 19: 266-269.

Table1. Mercury concentrations for and characteristics of largemouth bass taken from three locations on the Savannah River Site*. Fluctuating asymmetry (FA) and directional asymmetry (DA) are given for both ventral and dorsal views of the skulls.

Variable	**L Lake**	**Par Pond**	**Pond B**
Mean Hg (mg/kg dry weight)**	2.66±0.63[a]	4.53±0.77[b]	4.06±0.79[b]
Mean Total Length (mm)**	419.3±34.03[b]	443.5±33.60[b]	307.1±17.25[a]
Mean Total Wet Weight (g)**	1034±173[b]	1160±256[b]	329±57[a]
Mean Age (yrs)**	4.70±0.87[a]	4.94±0.80[a]	4.77±0.77[a]
FA (ventral view)+	1.21E-06[a]	1.34E-06[a]	7.51E-07[a]
FA (dorsal view)+	6.37E-07[a,b]	7.48E-07[a]	2.47E-07[b]
DA (ventral view)+	9.25E-06[a]	9.81E-06[a]	3.19E-06[a]
DA(dorsal view)+	6.94E-07[a]	6.67E-07[a]	3.82E-07[a]

*N = 19 for L Lake; N =17 for Par Pond; and N = 22 for Pond B. N occasionally varies by one or two within a location for certain tests because of missing data

**Means are given with 95% confidence intervals

[a,b]Values not sharing the same superscript for a variable are significantly different at p 0.05

+Unitless values based on Procrustes analyses

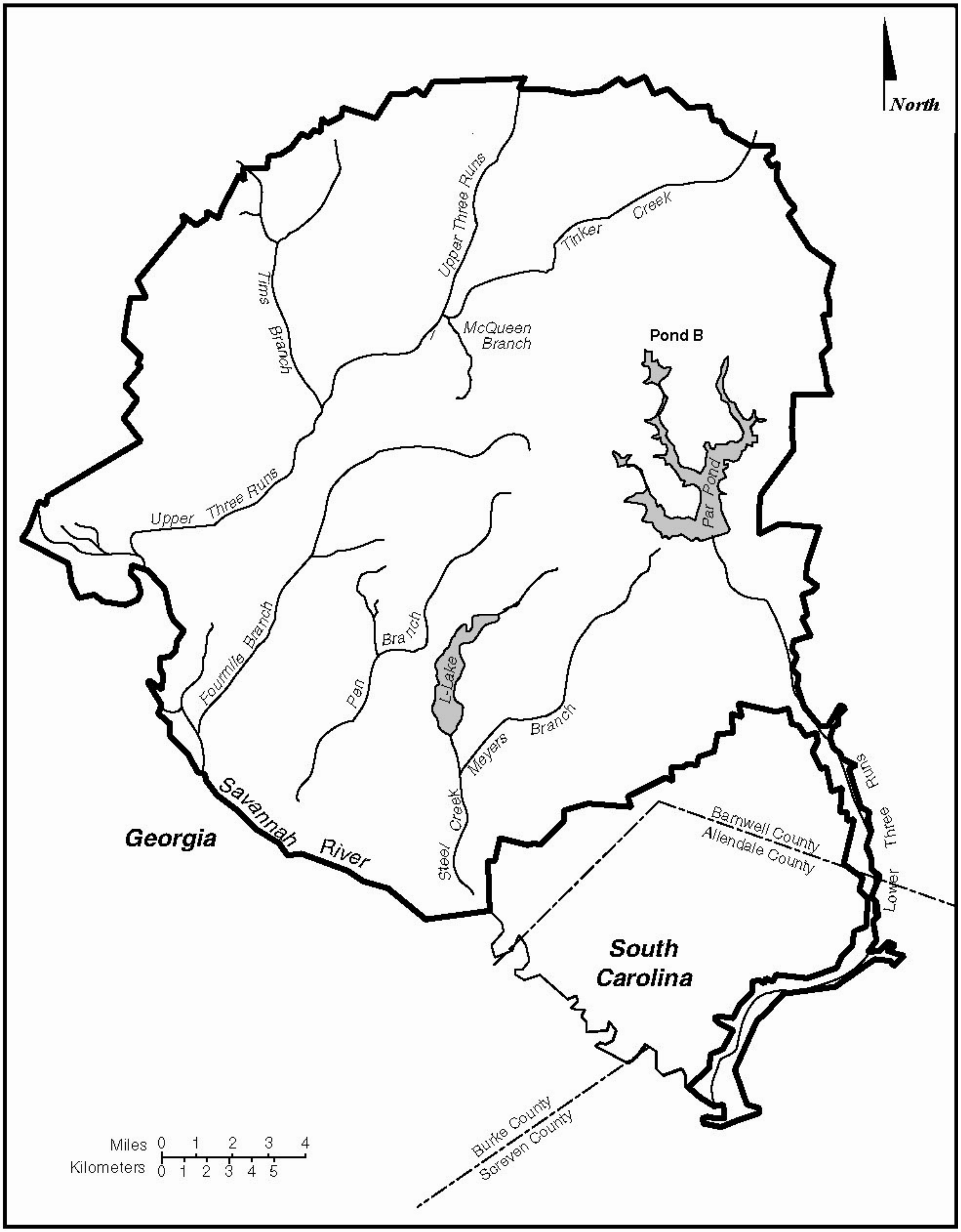


Fig 1. Savannah River Site (outlined in bold) with the three sampling sites L Lake, Par Pond, and Pond B identified.

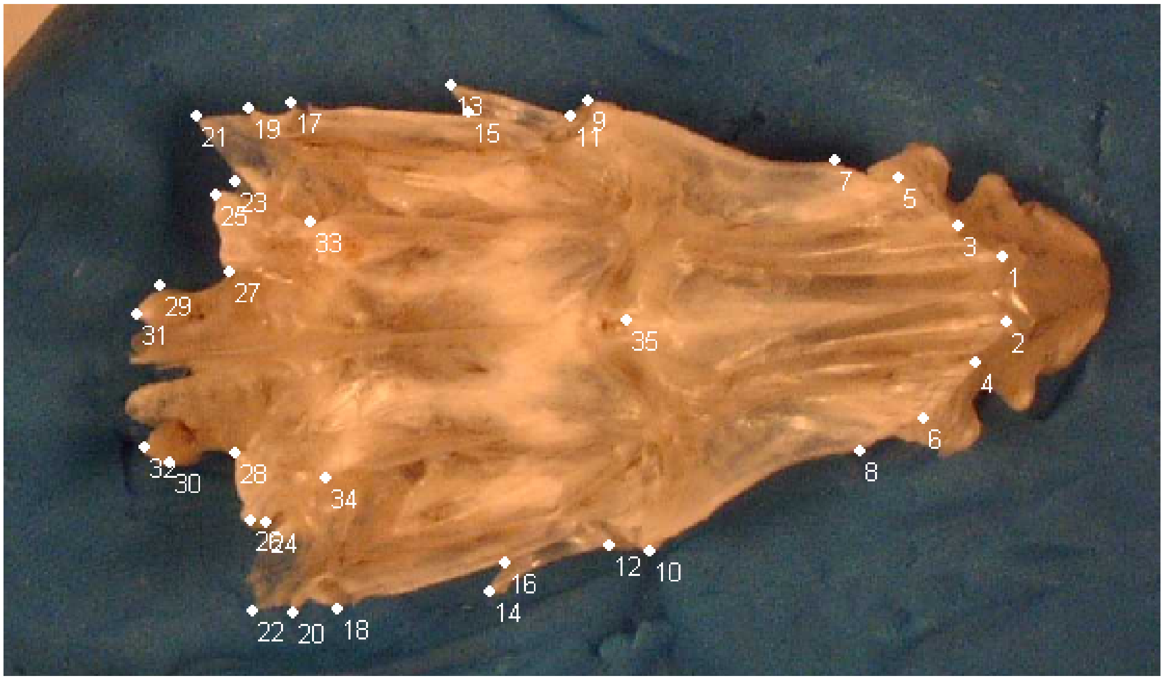


Fig. 2a. Locations of landmarks on the dorsal image of a largemouth bass skull.

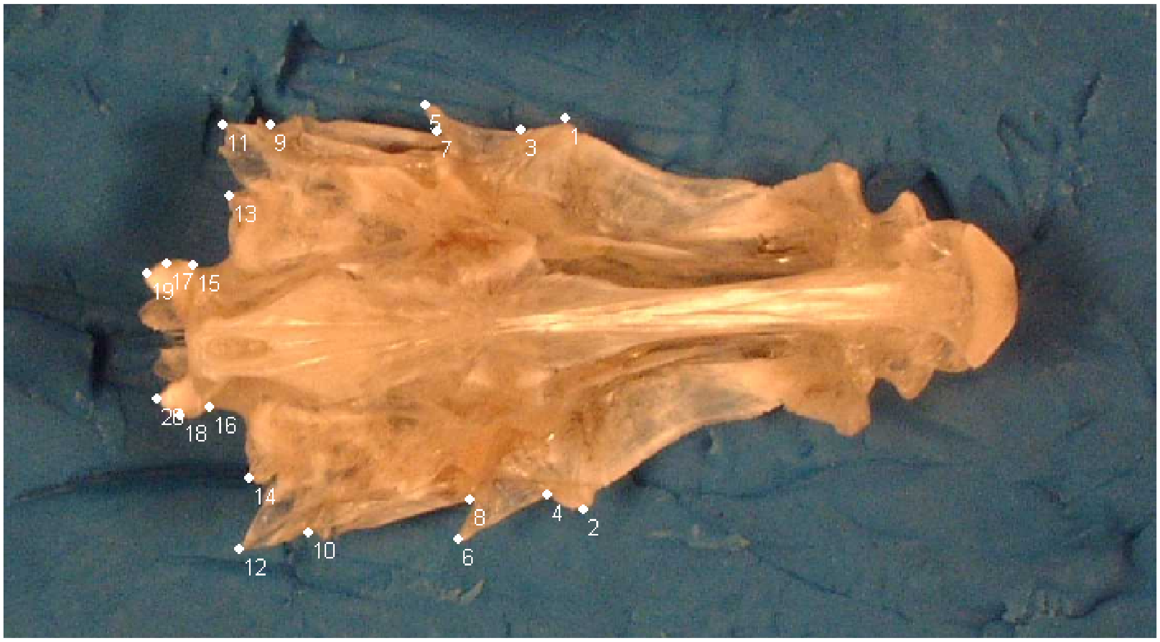
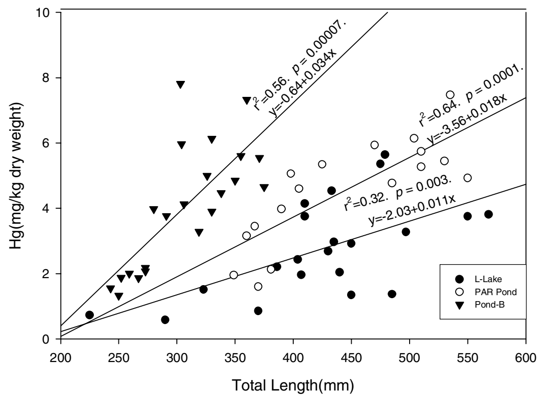
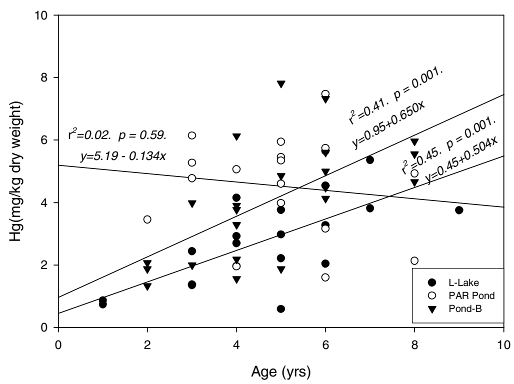


Fig. 2b. Locations of landmarks on the ventral image of a largemouth bass skull.





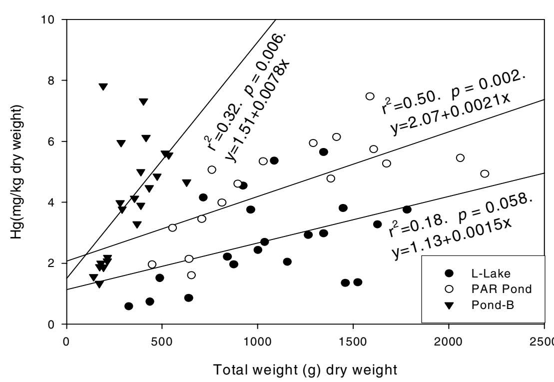


Fig. 3. Regression equations and coefficients of determination for the linear relationships between Hg concentrations with total length, age, and total weight of fish from three sites. Confidence intervals for the slopes and intercepts are given in Appendix D.

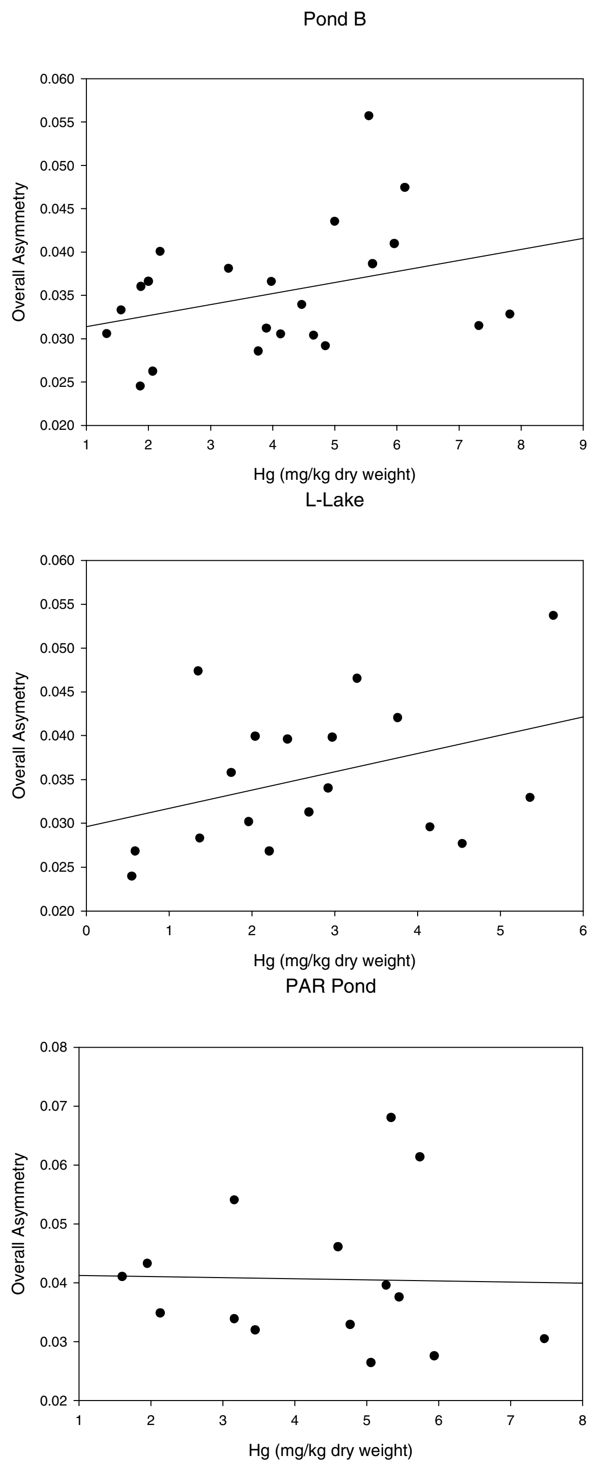


Fig 4. Linear regression plots of Hg (mg/kg dry weight) vs overall asymmetry (which includes asymmetry due to shape, DA, FA, and measurement error) for dorsal views of fish from the three locations. No significant relationships were found.

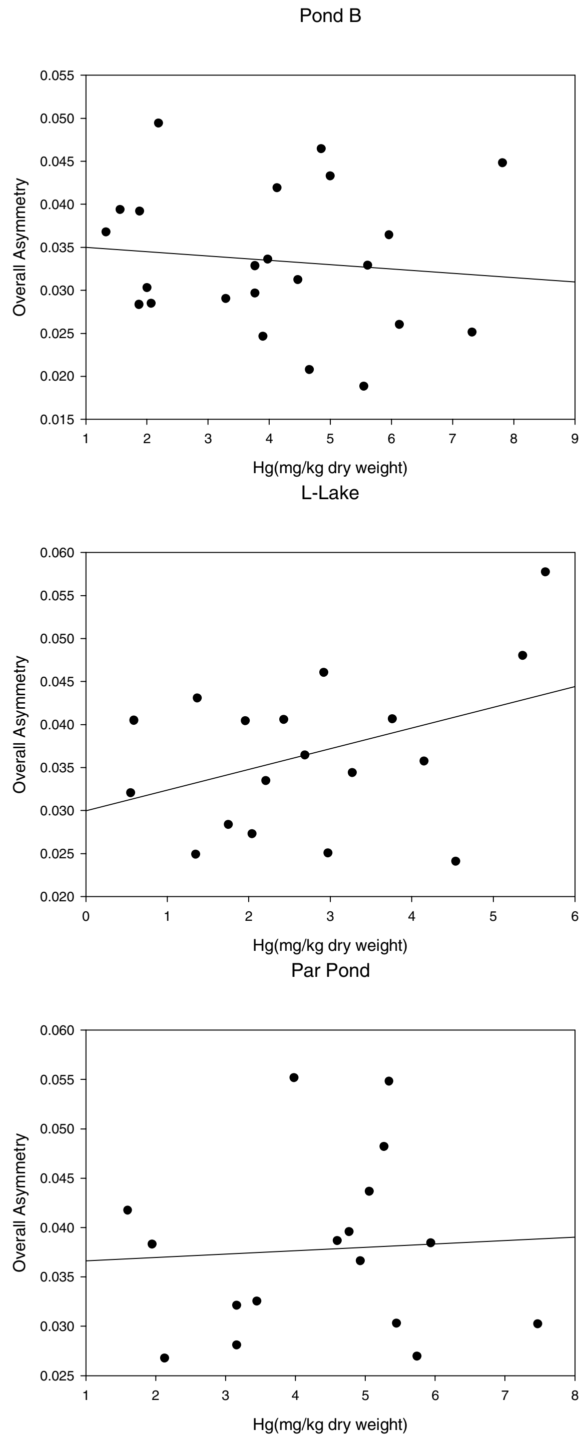


Fig 5. Linear regression plots of overall asymmetry (which includes asymmetry due to shape, DA, FA, and measurement error) for ventral views of fish from the three locations vs Hg (mg/kg dry weight). No significant relationships were found.

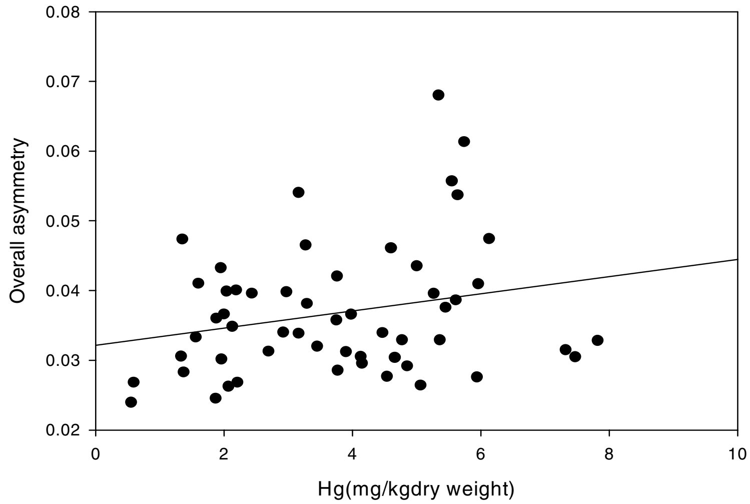


Fig. 6a. Linear regression of overall asymmetry (which includes asymmetry due to shape, DA, FA, and measurement error) for dorsal views of fish from the three locations considered together vs Hg (mg/kg dry weight).

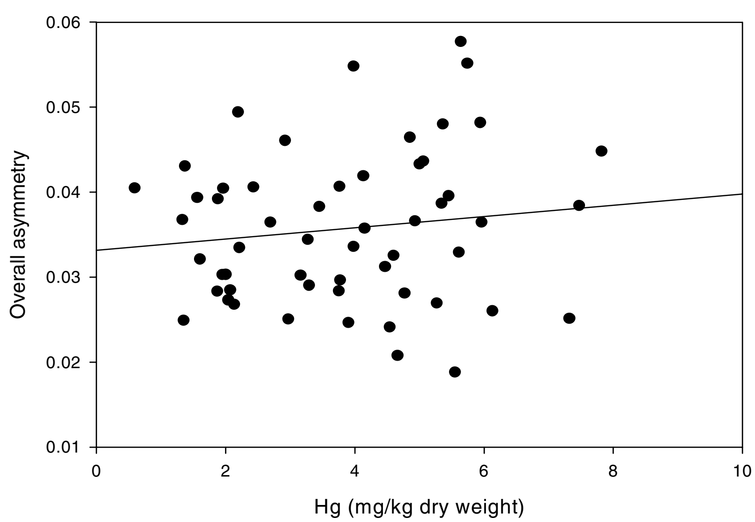


Fig. 6b. Linear regression of overall asymmetry (which includes asymmetry due to shape, DA, and FA) for ventral views of fish from the three locations considered together vs Hg (mg/kg dry weight).

APPENDIX A

SAS CODE FOR PROCRUSTES MULTIVARIATE ANOVA

Dorsals SAS code

```
PROC IMPORT OUT= WORK.dorsals
            DATAFILE= "C:\Neera\Dorsalsall.xls"
            DBMS=EXCEL2000 REPLACE;
     GETNAMES=YES;
RUN;

Data in;Set work.dorsals;
run;

Proc sort data=in;by POPID;
run;

Proc GLM Outstat=Stats Noprint; by POPID;
Class Side ID2;
Model X1-X35 Y1-Y35=Side ID2 Side*ID2/SS3;
Random ID2 Side*ID2/Test;
Run;

Proc Print Data=stats;
Var POPID _NAME_ _SOURCE_ _TYPE_ DF SS;
Run;
quit;
```

Ventral SAS code

```
PROC IMPORT OUT= WORK.ventrals
            DATAFILE= "C:\Neera\SASventrals.xls"
            DBMS=EXCEL2000 REPLACE;
     GETNAMES=YES;
RUN;

Data in;Set work.ventrals;
run;

Proc sort data=in;by POPID;
run;

Proc GLM outstat=stats Noprint;
by POPID;
Class Side ID2;
Model X1-X20 Y1-Y20=Side ID2 Side*ID2/SS3;
Random ID2 Side*ID2/Test;
Run;

Proc Print data=Stats;
Var POPID _NAME_ _SOURCE_ _TYPE_ DF SS;
Run;
quit;
```

APPENDIX B

CONFIDENCE INTERVALS FOR SLOPES AND INTERCEPTS FOR LINEAR REGRESSIONS FOR MERCURY DATA

Mercury (mg/kg dry weight) vs total length (mm)

L Lake

	Coefficients	Standard Error	t Stat	P value	Lower 95%	Upper 95%
Intercept	-2.03	1.45	-1.40	0.18	-5.06	0.997
Slope	0.011	0.0034	3.36	0.0033	0.0043	0.018

Par Pond

	Coefficients	Standard Error	t Stat	P value	Lower 95%	Upper 95%
Intercept	-3.56	1.58	-2.25	0.040	-6.94	-0.19
Slope	0.018	0.0035	5.17	0.0001	0.011	0.026

Pond B

	Coefficients	Standard Error	t Stat	P value	Lower 95%	Upper 95%
Intercept	-6.42	2.11	-3.04	0.0065	-10.8	-2.01
Slope	0.034	0.0068	5.01	0.0001	0.02	0.048

Mercury (mg/kg dry weight) vs total weight (g)

L Lake

	Coefficients	Standard Error	t Stat	P value	Lower 95%	Upper 95%
Intercept	1.13	0.86	1.32	0.202	-0.66	2.93
Slope	0.0015	0.0008	2.02	0.058	-0.0001	0.0031

Par Pond

	Coefficients	Standard Error	t Stat	P value	Lower 95%	Upper 95%
Intercept	2.07	0.700	2.97	0.0096	0.58	3.55
Slope	0.0021	0.0005	3.87	0.0015	0.0010	0.0033

Pond B

	Coefficients	Standard Error	t Stat	P value	Lower 95%	Upper 95%
Intercept	1.51	0.904	1.67	0.111	-0.377	3.39
Slope	0.0078	0.0025	3.05	0.0063	0.0025	0.0131

Mercury (mg/kg dry weight) vs age (years)

L Lake

	Coefficients	Standard Error	t Stat	P value	Lower 95%	Upper 95%
Intercept	0.452	0.661	0.684	0.503	-0.937	1.84
Slope	0.504	0.130	3.87	0.0011	0.230	0.777

Par Pond

	Coefficients	Standard Error	t Stat	P value	Lower 95%	Upper 95%
Intercept	5.19	1.28	4.06	0.0010	2.47	7.92
Slope	-0.134	0.246	-0.547	0.593	-0.659	0.390

Pond B

	Coefficients	Standard Error	t Stat	P value	Lower 95%	Upper 95%
Intercept	0.958	0.899	1.07	0.299	-0.918	2.83
Slope	0.650	0.176	3.69	0.0014	0.283	1.018

APPENDIX C

ANOVA TABLE AND PAIRWISE COMPARISON OF ASYMMETRY IN FISH SKULLS BETWEEN POPULATIONS

Dorsal View

	Pond B			L Lake			Par Pond		
	df*	SS	MS	df	SS	MS	df	SS	MS
Side	33	0.00279	8.4E-05	33	0.004	1E-04	33	0.004	0.00010685
Ind	726	0.17326	0.00024	594	0.19	3E-04	495	0.1	0.000202004
Side* Ind	726	0.03746	5.2E-05	594	0.035	6E-05	495	0.032	6.41769E-05
Error	8316	0.13834	1.7E-05	7524	0.037	5E-06	6336	0.052	8.13823E-06

Note: Side MS estimates DA; Individual (Ind) MS estimates Symmetric Shape; Side*Ind estimates FA; Error estimates measurement error

	Pond B vs L lake			Pond B vs Par Pond			L lake vs Par Pond		
	F	*P*	c*P*[a]	*F*	*P*	c*P*[a]	*F*	*P*	c*P*[a]
DA	1.31	0.2182	0.6547	1.265	0.2516	0.7547	1.039	0.4566	1.3697
Shape	1.34	0.0001	0.0002	1.181	0.0225	0.0675	1.59	0.0000	0.0000
FA	1.130	0.0587	0.1761	1.244	0.0038	0.0115	1.10	0.1315	0.3946

[a]c*P* = Bonferroni corrected *p*-value

Ventral View

	Pond B			L Lake			Par Pond		
	df*	SS	MS	df	SS	MS	df	SS	MS
Side	18	0.006994	0.000389	18	0.01459	0.000811	18	0.013856	0.00077
Ind	414	0.180687	0.000436	324	0.1642	0.000507	288	0.194155	0.000674
Side* Ind	414	0.036834	8.9E-05	324	0.03484	0.000108	288	0.031456	0.000109
Error	5040	0.047623	9.45E-06	4104	0.03126	7.62E-06	3672	0.021887	5.96E-06

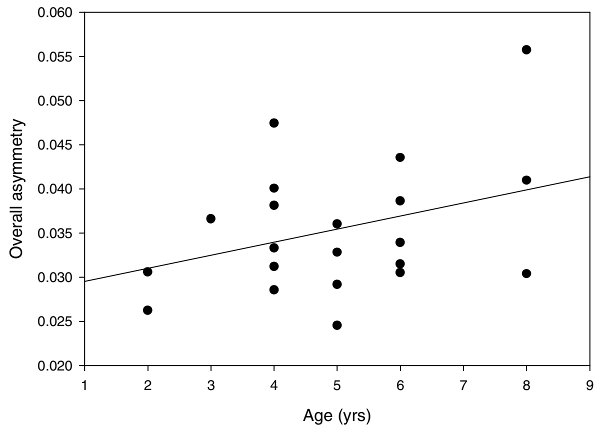
Note: Side MS estimates DA; Individual (Ind) MS estimates Symmetric Shape; Side*Ind estimates FA; Error estimates measurement error

	Pond B vs L Lake			Pond B vs Par Pond			L Lake vs Par Pond		
	F	*P*	c*P*[a]	*F*	*P*	c*P*[a]	*F*	*P*	c*P*[a]
DA	2.086	0.064	0.19	1.98	0.078	0.23	1.053	0.46	1.37
Shape	1.161	0.076	0.23	1.54	2.59E-05	7.77E-05	1.330	0.0063	0.019
FA	1.209	0.035	0.10	1.23	0.028	0.085	1.016	0.45	1.34

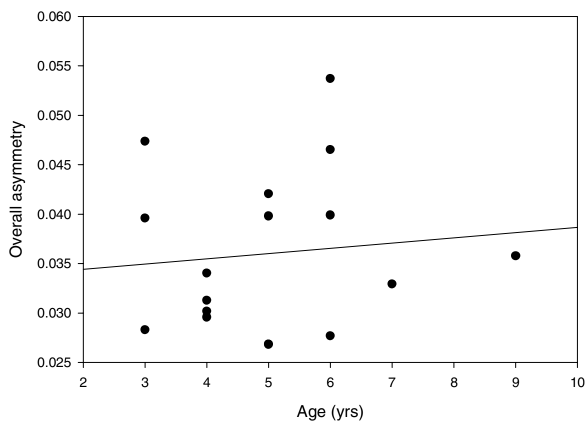
[a]c*P* = Bonferroni corrected *p*-value

APPENDIX D

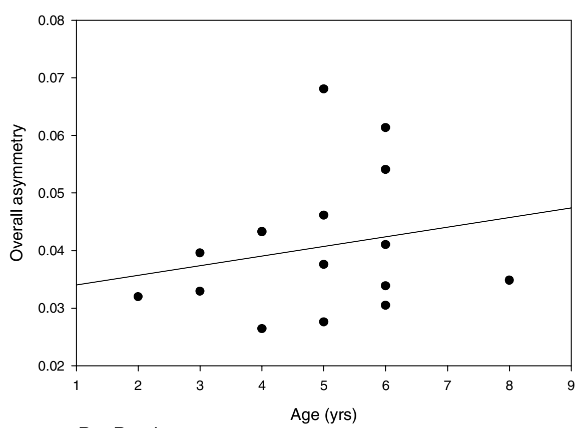
OVERALL ASYMMETRY VS AGE FOR EACH POPULATION AND FOR THE POPULATIONS CONSIDERED TOGETHER



Pond B

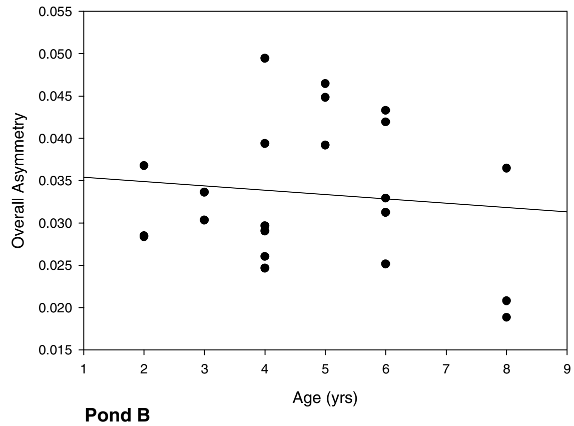


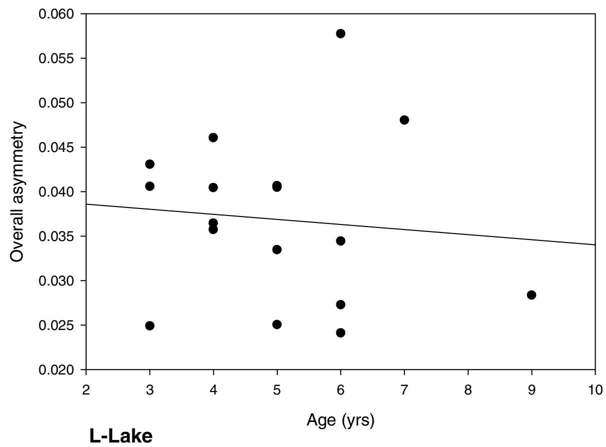
L-Lake



Par Pond

Fig D1. Linear regression plots of overall asymmetry vs age for each population for dorsal view. No significant relationships were found.





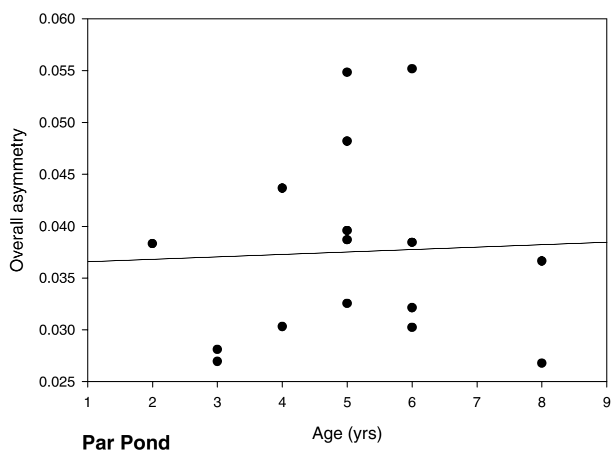


Fig D2. Linear regression plots of overall asymmetry vs age for each population for ventral view. No significant relationships were found.

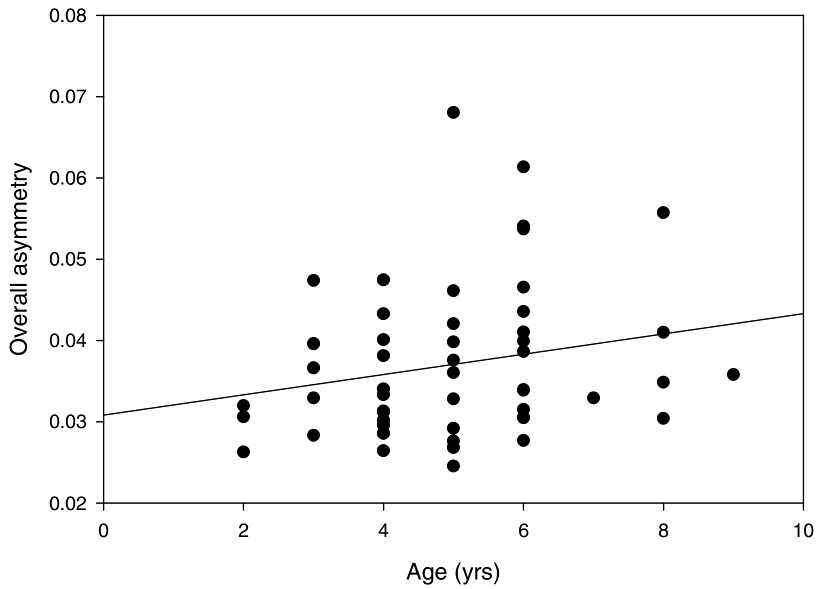


Fig D3. Linear regression plots of overall asymmetry vs age for all populations for dorsal view. No significant relationship was found.

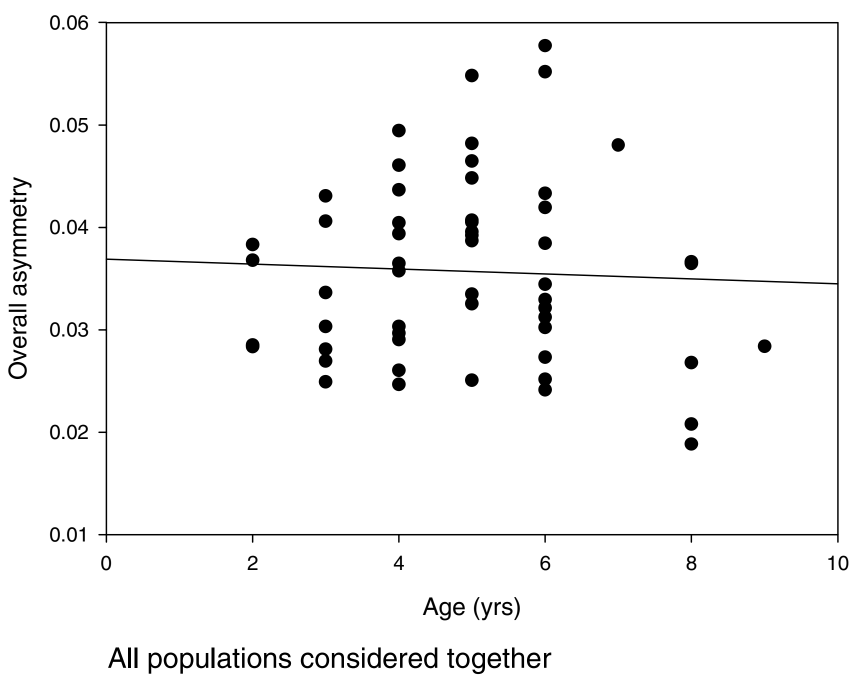


Fig D4. Linear regression plots of overall asymmetry vs age for all populations for ventral view. No significant relationship was found.