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Fluctuating Asymmetry and Its Relationship to Established Indicators of Environmental Stress.

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Fluctuating Asymmetry and its Relationship to Established Indicators of Environmental Stress

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ABSTRACT

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by

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Fluctuating asymmetry (FA) is commonly thought to be a predictor of environmental stress. However, the relationship between FA and established indicators for environmental stress has received little attention. In this study, 10-38 specimens of the freshwater fish *Rhinichthys atratulus* were collected from 15 natural populations under varying amounts of environmental stress. Asymmetry measurements in three bilateral characters of the specimens were used to investigate the relationship between FA and established indicators of environmental stress. Significant differences in the magnitude of FA were observed between sampling locations. However, the relationship between estimates of FA and established indicators produced varying results. The present study concludes with a discussion on the usefulness of FA as a bioindicator for environmental stress and implications for future studies.

CONTENTS

Page

Chapter

LIST OF TABLES

Table Page

LIST OF FIGURES

Figure Page

CHAPTER 1

INTRODUCTION

Fluctuating asymmetry is the pattern by which morphological characters differ between the right and left sides of individuals within a population of a bilaterally symmetrical species (Palmer 1994). Estimates of FA may be used to estimate the combined effect of developmental noise and developmental stability within a population. The latter includes factors that prevent canalized development of the phenotype and contribute to asymmetric bilateral development. Developmental stability is the set of intrinsic factors that oppose developmental noise, and decrease the magnitude of FA (Palmer 1994). Both environmental stress and genetic stress are thought to be primary contributors to developmental instability (DI) (Van Dongen and Lens 2000). One genetic stressor is a result of decreased genetic variability (Vollestad et al. 1999) and may increase the likelihood that development is influenced by environmental stress (Moller and Swaddle 1997), making it difficult to distinguish between these two contributors to DI. However, Pertoldi et al. (2003) have shown FA to be an unreliable indicator of DI in computer simulated populations under genetic stress.

Fluctuating asymmetry has been widely investigated for its potential use as a bio-indicator for specific environmental stress (toxic stress: Oxnevad et al. 1995; thermal stress: Hogg et al. 2001; climatic stress: Jentzsch et al. 2003). However, there is some controversy regarding the reliability of this method. Meta-analysis by Moller and Swaddle (1997) provides evidence for disruption of developmental stability by a number of specific environmental stressors. While, Hogg et al. (2001) reported that, of 44 experimental studies reviewed, nearly half (43.2%) failed to make a significant connection between FA and a potential stressor. Compiled literature for this study exhibited a result similar to that of Hogg et al. Consequently, some researchers have labeled FA as an unreliable indicator of environmental stress in plants (ex. Waldmann 2002) and animals (ex. Bjorksten 2001; Hogg et al. 2001).

FA has also been examined in relation to factors not directly related to environmental stress. One study has determined that FA may be used to predict the susceptibility of certain fishes to pesticide poisoning (Allenbach et al. 1998). Tornjova et al. (2003) have shown higher levels of FA in humans afflicted with Down syndrome. The relationship between FA and fitness or fitness related characters such as fecundity, are also controversial. A study with wolf spiders indicated a significant negative relationship between FA and clutch mass (Hendrickx et al. 2003). Woods et al. (2002) have shown an insignificant relationship between FA and mean fecundity or development time in the fruit fly. Such findings do not support the common view that FA is a reliable indicator of fitness related characteristics.

In studies where asymmetry is found, significant technical problems are apparent with trait selection, measurement error/technique, size dependence and correction, and interpretation of data. The most appropriate approach to overcome these problems is not yet established. Several of the studies mentioned above (and others) have made comparisons between some environmental or genetic factor and the amount of FA in single traits. Leung et al. (2000) suggest combining the measured FA of multiple traits may provide greater sensitivity to the relationship between stress and FA. This study provides support for the methods used in estimation and correction for size dependence, and data interpretation.

The Tennessee Department of Environment and Conservation (TDEC), in compliance with section 305(b) of the Federal Clean Water Act, must prepare an electronically available assessment of water quality for all major Tennessee waters. This database includes a measure of biological integrity for each stream or water-body and is assigned one of three qualitative measures of support for aquatic life. Bodies of water may be classified as ‘fully supporting’, ‘partially supporting’, or ‘not supporting’. Aquatic life-support classifications are assigned on the basis of several types of assessments, including but not limited to benthos surveys, qualitative habitat assessments, counts of macro-invertebrate families, physical and chemical data, fish tissue data, and studies done by other agencies such as the Tennessee Valley Authority. This methodology is significant because it incorporates assessment of stressors directly (example: dissolved oxygen concentration) and indirectly through known (bio) indicators for environmental stress (example: the number of invertebrate families present). One or more of these methods may be used in determining the aquatic life support score for a water body.

Three common stream assessments are the number of EPT (Ephemeroptera, Plecoptera, and Trichoptera) families present in the stream, total number of invertebrate families, and a checklist-style habitat assessment. These indices are commonly utilized as indicators for habitat quality by the TDEC, are recommended by the EPA in their rapid bioassessment protocol (Barbour et al.1999), and have been used to write legislation and apply penalties. Given that these measures are used to determine the stream’s life support rating, (example: EPT count) and are predictors of environmental stress, it is hypothesized that they may also predict developmental stability and FA. If measures of FA are effective as bioindicators, then significant measurable differences should be detectable among populations living in habitats with different EPT counts, total invertebrate family counts, habitat scores, and life support ratings. This study was designed to investigate the relationship between several measures of FA in fish from streams with available TDEC assessment data.

CHAPTER 2

MATERIALS & METHODS

Species and Sampling

The TDEC 305(b) Assessment Database (available by contacting the TDEC) was used to select 15 sampling locations that vary in life support rating, habitat score, EPT counts, and total invertebrate family counts. Streams rated as "not-supporting" yielded small sample sizes (<10) and were not included because FA cannot be reliably estimated in these cases. The Blacknose Dace (*Rhinichthys atratulus*) is abundant throughout the Tennessee Valley (Etnier and Starnes 1993) and was an ideal species for this study. Fish were captured by sweeping upstream with an electro-fisher while netting stunned fish. Specimens were sacrificed by immersion in a 60mg/L solution of MS-222 and fixed in a 10% formalin solution for later analysis.

Description of Sample Locations

All 15 sample locations were found in eastern Tennessee from the 83rd meridian to the North Carolina/Virginia border. Sampling began in February, 2005 and all samples were taken by October of that year. The number of fish caught at each location ranged from 10 to 38. Indicators for stream quality varied significantly from one location to another (Table 1, columns 6 - 9). However, nearly half of the streams (9) were rated as "Fully Supporting", and the rest (6) "Partially Supporting" with regard to aquatic life.

Table 1 Sample location descriptions

Stream Name	TDEC Acc. # [a]	Latitude[b]	Longitude[b]	Date[c]	EPT[c]	Total[c]	Habitat[c]	Rating[c]
Laurel Fork	601010201301	36˚15.827'	-82˚07.492	2/5/05	23	40	__	Fully
Gap Creek	601010300807	36˚19.640'	-82˚15.325	2/19/05	8	21	152	Fully
Martin Creek	601010801019	36˚08.020'	-82˚25.596	3/5/05	7	15	127	Partial
Dry Creek	601010201203	__	__	3/11/05	12	23	119	Fully
Sinking Creek	601010304610	__	__	8/15/05	12	26	__	Fully
L. Chero. Creek	601010853602	36˚14.083'	-82˚26.439	9/9/05	4	17	74	Partial
L. Lime. Creek	601010851010	36˚17.600'	-82˚28.421	9/14/05	12	25	111	Partial
Brush Creek	601010300910	36˚22.162'	-82˚18.307	9/16/05	4	22	__	Partial
Boones Creek	601010300610	36˚22.926'	-82˚25.045	9/16/05	4	24	102	Partial
Buffalo Creek	601010301110	36˚17.814	-82˚17.902	9/21/05	15	36	__	Fully
Cedar Creek	601010270210	36˚26.280'	-82˚27.188	9/23/05	7	22	113	Fully
Kendrick Creek	601010205710	36˚26.544'	-82˚32.300	9/23/05	7	18	152	Fully
Bradley Creek	601010401105	__	__	10/5/05	14	35	173	Fully
Crockett Creek	6010104004T10	36˚23.872'	-83˚00.821	10/5/05	4	21	137	Partial
Wagner Creek	6010102006T00	36˚29.390'	-82˚23.907	10/28/05	5	17	120	Fully

[a] TDEC accession number is used to locate stream sampling data in the TDEC water quality monitoring database. [b] Longitude and latitude were measured at the time of sampling via handheld Garmin Rino 130 GPS receiver. [c] Codes: Date, date of sampling; EPT, EPT invertebrate count; Total, total invertebrate count; Habitat, habitat score; Rating, TDEC aquatic life support rating.

Pilot Study

A pilot study was used to determine which traits and measurement techniques were appropriate for measurements of asymmetry in this species. Asymmetry in this small fish ranges from 1% to 20% of the trait size (data not shown). Because between-sides variation (R-L) and measurement error are both distributed normally with a mean of zero, they are indistinguishable in a single measurement. For this reason, it is important to determine whether the between sides variation is significantly greater than measurement error. Eye diameter, snout lengths, head lengths, and fin lengths were measured in 20 preserved specimens using both dial calipers and the photographic technique described below. This process was repeated four times for each trait and measurement technique combination. These data were then visually inspected for antisymmetry in a relative frequency histogram. If free of antisymmetry, the data were subjected to a two-way ANOVA (sides – by - individuals). The main effects and interaction terms of this test were utilized according to Palmer and Strobeck (1986) to test for 'ideal FA' (N [0,σ]) and to ensure the between-sides variation was significantly greater than measurement error. Trait and measurement technique combinations that did not meet these requirements were ruled out for use in the larger study.

Measurement error associated with the dial caliper technique was greater than the variation between sides for each trait. Interaction terms of two-way ANOVA's for eye diameter, snout length, head length, and fin length measured with a dial caliper were all insignificant (p = 0.399, 0.088, 0.796, 0.176 respectively). Photographic technique exhibited much less measurement error. Significant interaction terms were found for eye diameter, snout length, and head length measured with this technique (p = 0.006, 0.001, 0.019 respectively). Measurements of fin length (interaction p = 0.522) were excluded, and only the photographic technique was used in the larger study.

Photographic Technique

Steel pins were inserted along both the dorsal-ventral and anterior-posterior axes. The specimen was then placed on a mounting device that angles the specimen such that the trait to be measured lay entirely in the focal plane of a Leica dissecting microscope at 0.65x magnification. The optimum angle was determined by ensuring that both endpoints of the trait to be measured were in sharp focus. Optimum angles for the longitudinal and anterior-posterior axes were ~18˚ and ~11˚ respectively. Right and left side photographs were made by mounting the specimen on separate devices with equal but inverted dimensions for the anterior-posterior axis. Photographs were made using a microscope-mounted 4 megapixel digital camera. Measurements of trait size were made for each photograph using ImageJ 1.31v (Public Domain Imaging Software, National Institutes of Health, http://rsb.info.nih.gov/ij/) and calibrated with a photograph of a stage micrometer (0.005 mm resolution) from the same apparatus. Asymmetry was determined from measurements in snout length, head length, and eye diameter. Body mass and standard lengths were measured for each fish. Asymmetries in all traits were measured three times for each fish from every sample location. Measurements were averaged before assigning an R-L value.

Data Analysis

In order to use measurements of FA in meaningful comparisons, it is essential that the data meet some preliminary assumptions. Size dependence of between-sides variation could cause the data to appear skewed. This factor was assessed by regressing trait asymmetry onto wet mass, standard length, and average trait size ((R+L)/2). This procedure was completed for each trait. Size dependence was indicated if the linear correlation was significant ($p<0.05$). Each trait was found to be significantly correlated with one or more of the three measures for overall size, wet mass, standard length, and average trait size ((R+L)/2) (Table 2). Correction for overall size dependence was carried out by dividing the unsigned response by the average trait size (|R-L|/((R+L)/2)). This form of size correction showed the greatest decrease in correlation coefficients (response vs. overall size measure) (Table 3). Measurements corrected for size dependence are later referred to by "*|R-L|" or "corrected |R-L|".

Table 2 Linear correlation of responses[c] vs. overall size

	Standard Length		Wet Mass		(R+L)/2	
	r[a]	p[b]	r	p	r	p
ED[d]	0.170	0.006	0.173	0.007	0.122	0.052
SL[d]	0.224	<0.001	0.202	0.001	0.363	<0.001
HL[d]	0.141	0.025	0.138	0.028	0.100	0.115

[a] correlation coefficient. [b] p-value. [c] N = 250 for each test. [d]Codes: ED, eye diameter; SL, snout length; HL, head length.

Table 3 Linear correlation of corrected responses[c] vs. overall size

	Standard Length		Wet Mass		(R+L)/2	
	r[a]	p[b]	r	p	r	p
ED[d]	0.032	0.559	0.001	0.854	0.105	0.100
SL[d]	0.179	0.005	0.130	0.041	0.134	<0.037
HL[d]	0.158	0.012	0.122	0.054	0.152	0.016

[a] correlation coefficient. [b] p-value. [c] N = 250 for each test. [d] Codes: ED, eye diameter; SL, snout length; HL, head length.

FA is defined as random variation in between-sides measurements and is assumed to be distributed normally. If their distribution is skewed or bimodal, FA may not be indicated. Both of these types of asymmetry are thought to be influenced by factors that are not present in normally distributed asymmetry. In these cases, between-sides variation may not be an accurate estimator of developmental stability (Palmer 1994). No antisymmetry was observed upon visual inspection of histograms for each of the three responses (Figures 1, 2, and 3)

Raw data (individual right and left side measurements) for each trait were subjected to a two-way ANOVA (sides –by- individuals) according to Palmer and Strobeck (1986). Again, these procedures were used to test for ideal FA (N $[0,\sigma]$) and to ensure between-sides variation was significantly greater than measurement error. Datasets that did not meet these requirements were excluded from the study.

Levene’s test (one-way ANOVA, Palmer 1994) was used to test for differences in the responses (*|R-L|) among sample locations and TDEC life support ratings. Simple linear regression analysis was used to identify the relationship between responses and EPT count, total invertebrate count, and habitat score. Quadratic model regression was used in cases where appropriate. This process was completed for data from each measured trait. The three trait responses were then summed (eye diameter *|R-L| + snout length *|R-L| + head length *|R-L| = *|R-L| sum) for each individual and compared to the environmental quality indicators in an identical manner. A probability level of 0.05 was used to indicate statistical significance

CHAPTER 3

RESULTS

Tests for Ideal FA

The results of the two-way ANOVA's (side –by- individuals; Table 4) indicate that trait measurements within each sampling location varied significantly among individual fish. In all but two cases, no significant difference was observed between right-side and left-side measurements (Table 4). In Little Cherokee Creek ED and Cedar Creek SL measurements a significant difference was observed between right-side and left-side measurements. For each measured trait within each sampling location, the 'side – by - individuals' term was highly significant, indicating the between sides variation was significantly greater than measurement error.

The above results and the absence of antisymmetry indicate that "ideal" fluctuating asymmetry was observed in each location – trait combination with the exception of Little Cherokee Creek ED and Cedar Creek SL due to the possible presence of directional asymmetry (DA). However, after Bonferroni correction, revised p-values were not significant. Given the fact that DA was absent in all other samples and traits, it is likely that these observations are products of statistical type I error. Data from all sampling locations were included in the final data analysis.

Analysis of FA and Stream Quality Indicators

Levene's tests indicated significant variability in corrected (*|R-L|) responses among sampling locations for each trait (ANOVA, Table 5). The highest eye diameter *|R-L| mean was found in the Martin Creek sample at 0.051mm, while the lowest was found in the Bradley Creek sample at 0.016mm (Figure 4). Highest and lowest values for snout length and head length varied (Figures 5 and 6) No significant relationship was found between eye diameter and snout length *|R-L| measurements when data were pooled across all sample locations ($r = 0.118$, $p = 0.063$). However, snout length versus head length and eye diameter versus head length *|R-L| measurements were both highly significant ($r = 0.302$, $p < 0.001$; $r = 0.546$, $p <0.001$ respectively). Levene's test for differences in *|R-L| measurements between 'Fully' and 'Partially' supporting streams was significant ($p = 0.016$; Figure 7) for eye diameter with a 'Fully' mean of 0.0244mm and a 'Partially' mean of 0.0311mm. This difference was insignificant for snout length and head length ($p = 0.431$, 0.328 respectively; Figure 7). However, 'Fully' *|R-L| means were somewhat lower than 'Partially' means for all three traits.

Simple linear regression of *|R-L| responses onto EPT count, total invertebrate family count, and habitat scores yielded mostly insignificant coefficients (Table 6, Figures 8, 10, 12, 13, 15, and 16). However, relationships between snout length vs. EPT count, head length vs. EPT count, and eye diameter vs. total invertebrate family count were significant ($p = 0.014$, 0.010, 0.046 respectively; Figures 9, 11, and 14, Appendix B). The slopes for the first two significant relationships were positive, while the last was negative.

Levene's test for differences between 'Fully' and 'Partially' supporting streams was insignificant for the *|R-L| sums across traits ($p = 0.080$). As before, the 'Fully' *|R-L| mean was somewhat lower than the 'Partially' mean. Regression of these sums onto indicator data yielded a significant result for *|R-L| sums vs. EPT count ($p = 0.032$). However, regressions for *|R-L| vs. total invertebrate family count and habitat score were insignificant ($p = 0.530$, 0.667 respectively).

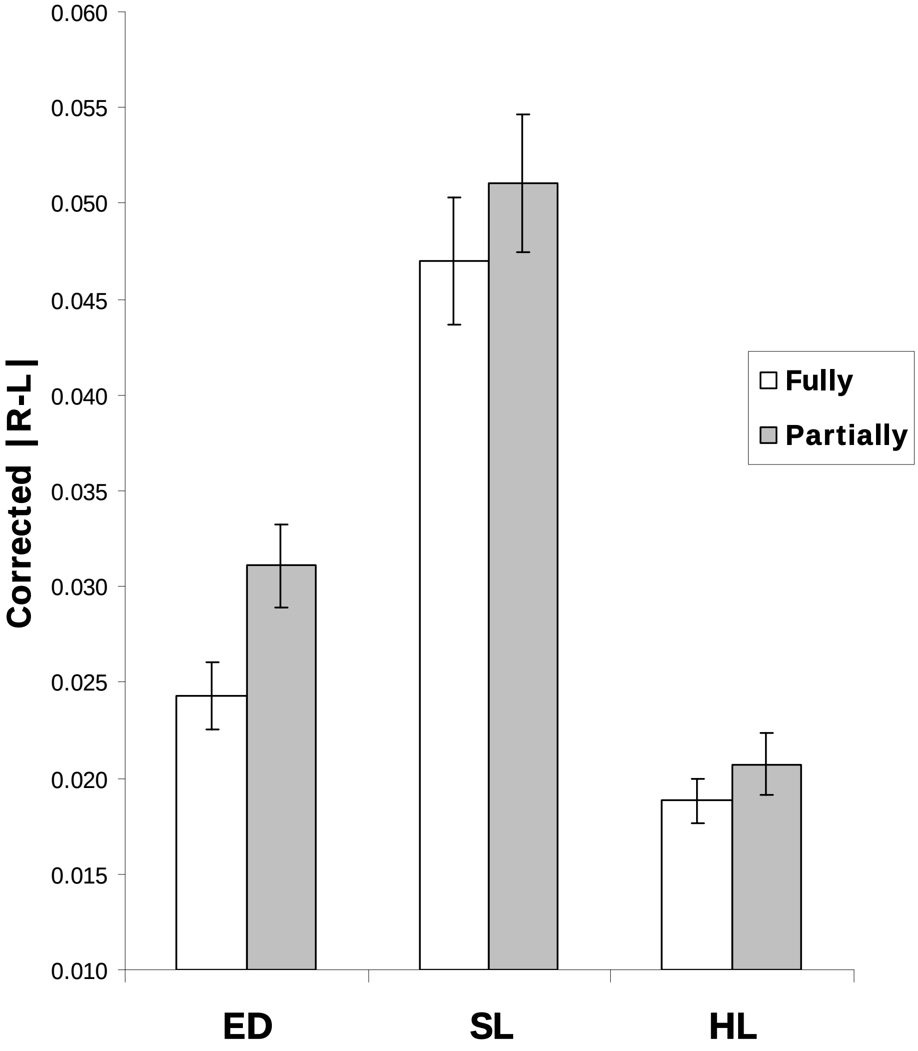

Figure 7 Fully vs. Partially supporting means for each trait.
Codes: ED, eye diameter; SL, snout length; HL, head length; Fully, "Fully" supporting TDEC rating; Partially, "Partially" supporting TDEC Rating. Error bars are SE mean.

*|R-L| data for each trait and their sums were subjected to quadratic model regression. While most tests showed quadratic models were no better than linear models, total invertebrate family counts and their squares proved to be significant predictors for *|R-L| responses for every trait and their sums ($p < 0.01$ for each test and coefficient). In each case the curve was concave up (Figure 17).

CHAPTER 4

DISCUSSION

The results of this study suggest that FA was present and measurable in the samples from each location. It was also apparent that the magnitude of FA varied significantly between samples. These endpoints indicate that some factor, environmental, genetic, or otherwise, has variably influenced the developmental stability and consequently the magnitude of FA in each of these samples.

The most broad indicator, TDEC life support rating was a significant predictor of *|R-L| values in the case of eye diameter. In the cases of snout length, head length, and the *|R-L| sums across traits, this indicator was not a significant predictor. However, as expected, the “Fully” supporting mean was less than the “Partially” supporting mean in each case. *|R-L| sums across traits were modeled by an N (mean, S^2) distribution for both “Fully” and “Partially” supporting groups. When data were simulated using these two models, sample sizes of 850 for each rating group were needed to achieve a significant difference with 95% confidence. While promising, the size of the sample needed to achieve this difference is probably beyond practical limits.

*|R-L| values for each trait were significantly correlated with either EPT count or total invertebrate family count. However, significant correlations with EPT count yielded an unexpected positive slope, while the significant correlation with total invertebrate family count yielded a negative slope. This apparent conflict provides little evidence for the hypothesis of this study. There was no single trait FA that was significantly correlated with both EPT and total invertebrate family count. Furthermore, there was no single indicator that was a significant predictor for every trait FA.

Woods et al. (2002) have suggested that moderately stressful conditions cause high mortality in the most asymmetrical individuals, resulting in lower levels of measured FA in these conditions. A similar argument can be made to suggest that the most asymmetrical individuals are only present in the highest levels of environmental quality, resulting in higher levels of measured FA at these conditions. Under these assumptions, we would expect measured FA to decrease with decreasing environmental quality as the most asymmetric individuals were killed. This trend might continue until the increasing stress caused measured FA in the surviving individuals to increase. In a plot, this scenario would appear quadratic where the magnitude of FA is on the Y-axis, and increasing environmental quality on the X-axis. The quadratic regression in this study of trait and sum *|R-L| values onto total invertebrate family counts are expected under this hypothesis. However, this should not be taken as evidence in support of such a hypothesis because the underlying mechanism is still unknown.

The data in this study support the claim that there are differences in the magnitude of FA among streams with varying levels of environmental quality. However, they fail to confirm a clear connection between FA and established indicators for stream quality. As each of these established indicators is influenced by a variety of environmental conditions, these results suggest that there may be a somewhat smaller or different set of conditions that influence the magnitude of FA. It may be appropriate for future studies of the influence of environmental stress on FA to focus on very specific stressors or perhaps some physiological condition influenced by a specific stressor, such as metal toxicity or hypoxia. This method is likely to decrease the number of confounding factors. The inconsistency of the results in this study suggests that the use of FA as a predictor of environmental stress in this manner is unreliable or impractical at best.

The controversy over the usefulness of FA as an indicator for stress or habitat quality is ongoing. Because publication bias is suspected in reporting FA results (Palmer 1999), it is important that future studies are designed to reveal appropriate applications for FA analysis. There has been some complaint that much of the FA/Stress data have been published in a manner that is not useful for future review and comparison. The present study used standard measures of FA and reporting methods in a manner that will be useful for future fluctuating asymmetry research and metanalysis.

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APPENDICES

APPENDIX A

Additional Tables

Table 4 Results of two-way ANOVA (side X individuals)

Location	N	Trait	Individual[a]	Side[a]	Individual – by – Side[a]
Laurel Fork	20	ED[b]	<0.001	0.966	<0.001
		SL[b]	<0.001	0.210	<0.001
		HL[b]	<0.001	0.441	<0.001
Gap Creek	20	ED	<0.001	0.749	<0.001
		SL	<0.001	0.522	<0.001
		HL	<0.001	0.382	<0.001
Martin Creek	20	ED	<0.001	0.319	<0.001
		SL	<0.001	0.254	<0.001
		HL	<0.001	0.699	<0.001
Sinking Creek	20	ED	<0.001	0.712	<0.001
		SL	<0.001	0.213	<0.001
		HL	<0.001	0.847	<0.001
Little Cherokee Creek	20	ED	<0.001	0.009*	<0.001
		SL	<0.001	0.262	<0.001
		HL	<0.001	0.536	<0.001
Little Limestone Creek	20	ED	<0.001	0.677	<0.001
		SL	<0.001	0.394	<0.001
		HL	<0.001	0.692	<0.001
Brush Creek	20	ED	<0.001	0.205	<0.001
		SL	<0.001	0.843	<0.001
		HL	<0.001	0.457	<0.001
Boones Creek	20	ED	<0.001	0.815	<0.001
		SL	<0.001	0.689	<0.001
		HL	<0.001	0.851	<0.001
Buffalo Creek	20	ED	<0.001	0.833	<0.001
		SL	<0.001	0.351	<0.001
		HL	<0.001	0.166	<0.001
Cedar Creek	20	ED	<0.001	0.052	<0.001
		SL	<0.001	0.041*	<0.001
		HL	<0.001	0.117	<0.001
Dry Creek	10	ED	<0.001	0.300	0.002
		SL	<0.001	0.369	<0.001
		HL	<0.001	0.366	<0.001
Kendrick Creek	10	ED	<0.001	0.052	0.001
		SL	<0.001	0.156	<0.001
		HL	<0.001	0.343	<0.001
Bradley Creek	10	ED	<0.001	0.355	<0.001
		SL	<0.001	0.777	<0.001
		HL	<0.001	0.464	<0.001
Crockett Creek	10	ED	<0.001	0.396	<0.001
		SL	<0.001	0.956	<0.001
		HL	<0.001	0.544	<0.001
Wagner Creek	10	ED	<0.001	0.598	<0.001
		SL	<0.001	0.197	<0.001
		HL	<0.001	0.279	<0.001

[a] p-values for each term in a two-way ANOVA. [b] Codes: ED, eye diameter; SL, snout length; HL, head length.

Table 5 ANOVA tables for Levene's test (Locations[a])

Response Trait	Source	DF[b]	SS[b]	MS[b]	F[b]	p[b]
Eye Diameter	Total	249	0.1197			
	Location	14	0.0185	0.0013	3.08	<0.001
	Error	235	0.1011	0.0004		
Snout Length	Total	248	0.3680			
	Location	14	0.0114	0.0038	2.83	0.001
	Error	234	0.3148	0.0014		
Head Length	Total	248	0.0568			
	Location	14	0.0114	0.0008	4.17	<0.001
	Error	234	0.0455	0.0002		

[a] The factor in each test was sample locations. [b] Codes: DF, degrees of freedom; SS, sum of squares; MS, mean of squares; F, F-ratio; p, p-value.

Table 6 Linear correlation of response traits with stream stress indicators

Response Trait	vs. indicator	Coefficient[a]	p-value[a]
Eye Diameter	EPT[b]	0.032	0.639
	Total[b]	0.127	0.046
	Habitat[b]	0.010	0.930
Snout Length	EPT	0.155	0.014
	Total	0.110	0.080
	Habitat	0.070	0.362
Head Length	EPT	0.161	0.010
	Total	0.055	0.425
	Habitat	0.063	0.433

[a] "Coefficient" and "p-value" are the correlationcoefficient and p-value for a simple linear regression. N = 250. [b] Codes: EPT, EPT family count; Total, total invertebrate family count; Habitat, habitat score.

APPENDIX B
Additional Figures

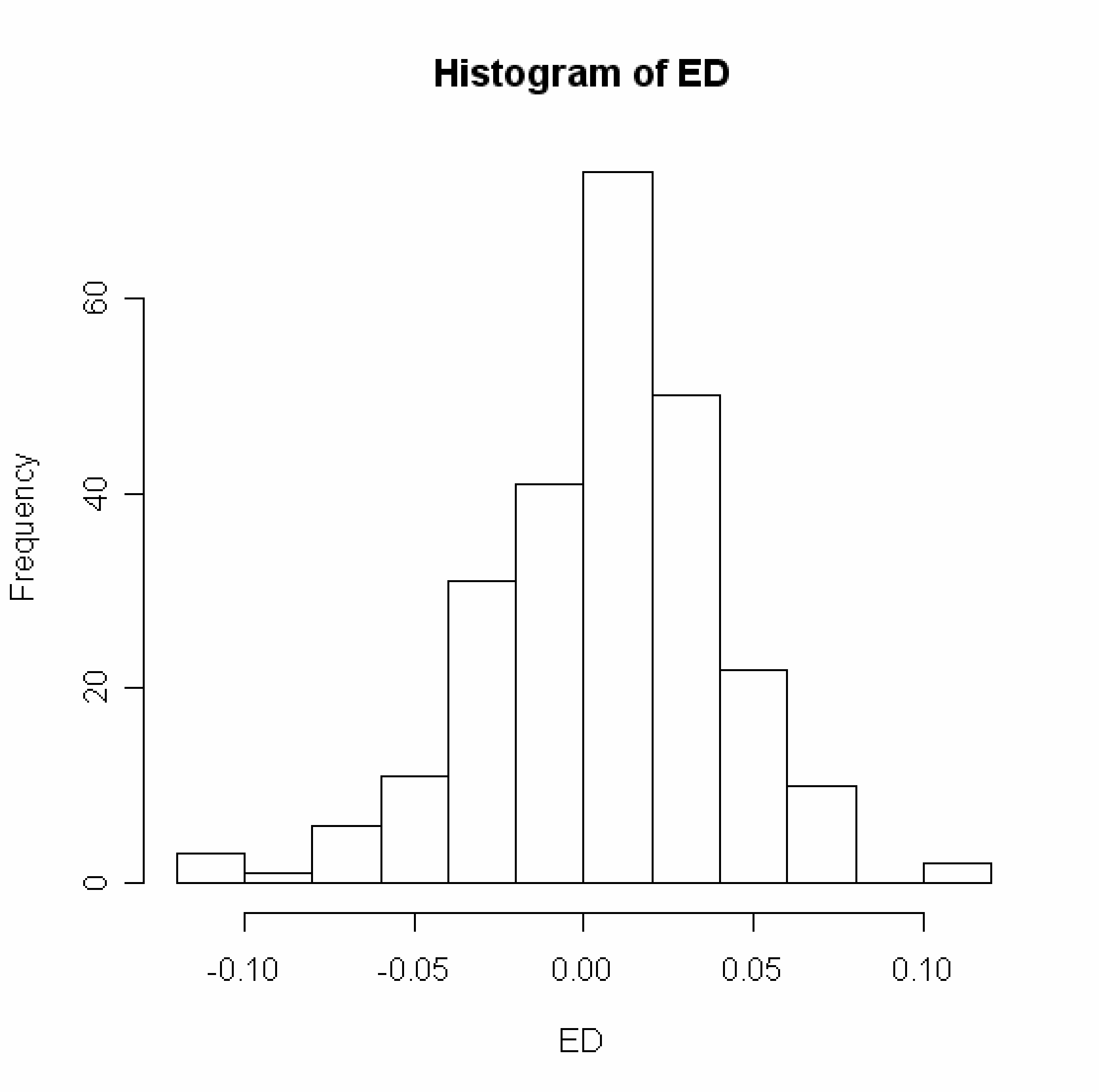

Figure 1 Histogram of eye diameter *(R-L).
Note: histogram appears approximately normal in distribution and free of antisymmetry (bimodality). Trait code: ED, eye diameter. Horizontal axis scale is in millimeters.

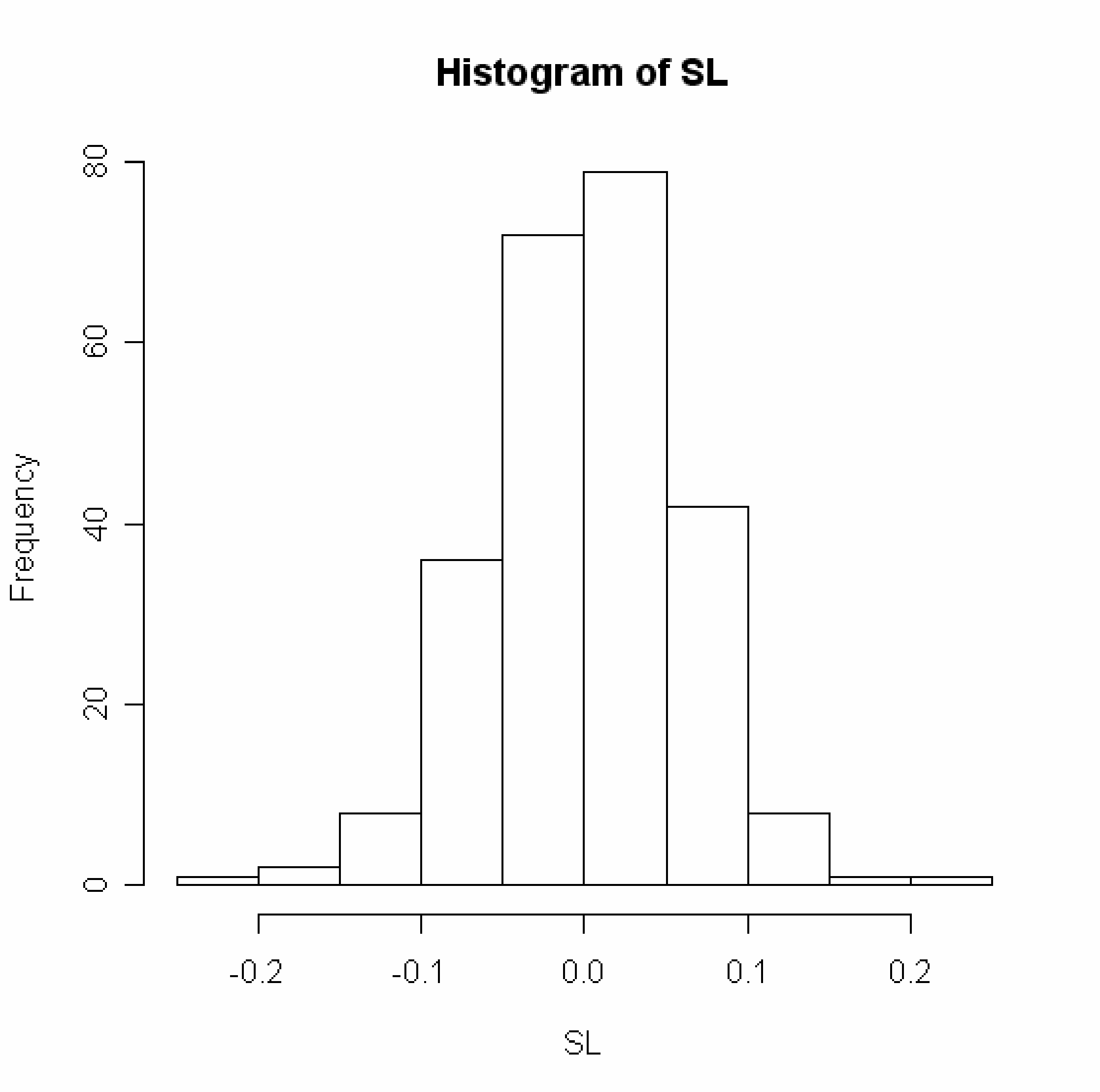

Figure 2 Histogram of snout length *(R-L).
Note: histogram appears approximately normal in distribution and free of antisymmetry (bimodality). Trait code: SL, snout length. Horizontal axis scale is in millimeters.

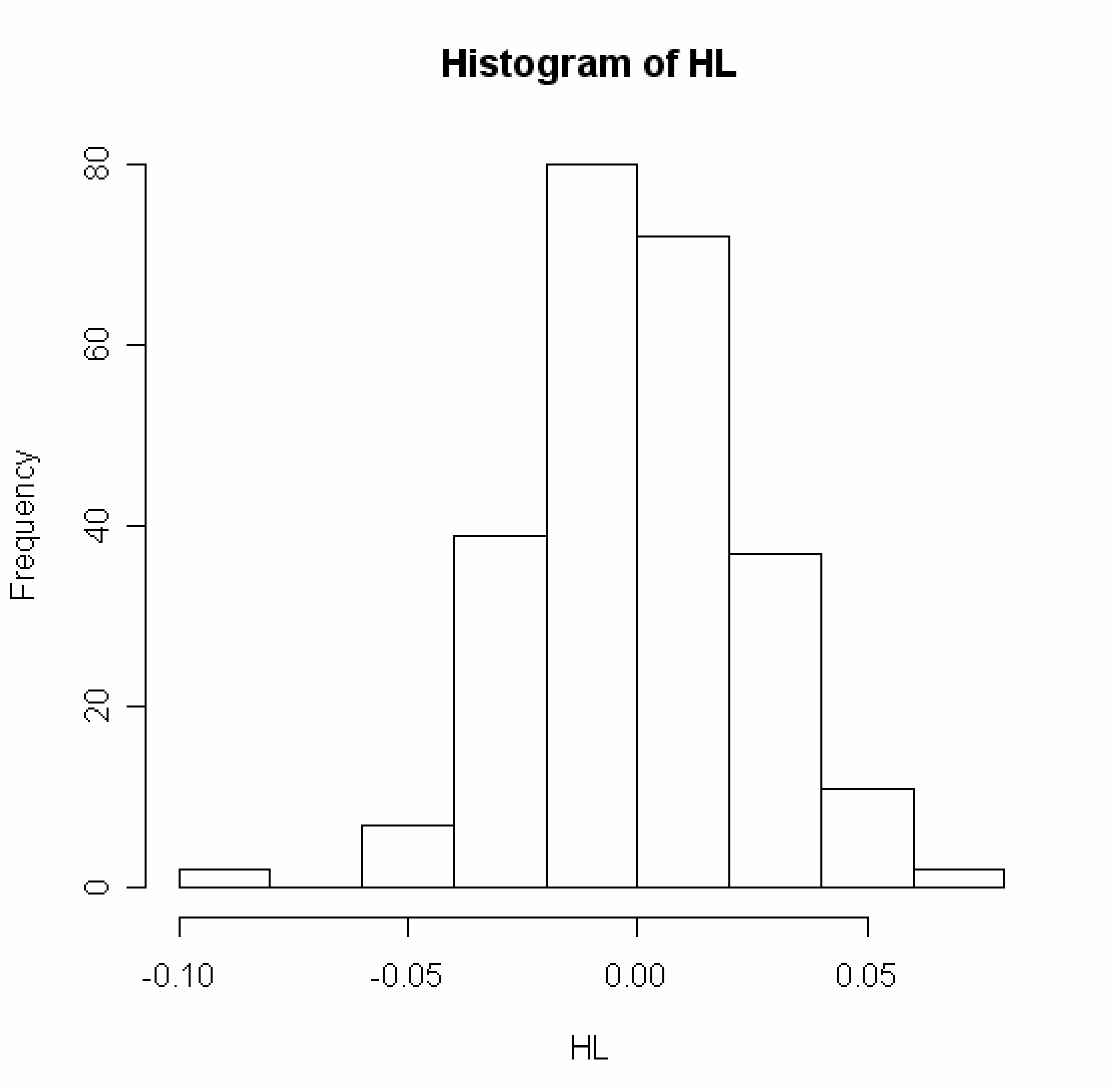

Figure 3 Histogram of head length *(R-L).
Note: histogram appears approximately normal in distribution and free of antisymmetry (bimodality). Trait code: HL, head length. Horizontal axis scale is in millimeters.

```
Location         N    Mean    SD       ---------+---------+---------+---------+

Laurel Fork      20  0.02953  0.02571                (-----*-----)

Gap Creek        20  0.03068  0.02147                (-----*------)

Martin Creek     20  0.05058  0.03024                              (-----*-----)

Dry Creek        10  0.03288  0.01923               (--------*--------)

Sinking Creek    20  0.02673  0.02342              (-----*-----)

L. Chero. Creek  20  0.02419  0.01977            (-----*-----)

L. Lime. Creek   20  0.02551  0.01655             (-----*-----)

Brush Creek      20  0.03317  0.02264                  (-----*-----)

Boones Creek     20  0.02776  0.01899              (------*-----)

Buffalo Creek    20  0.02159  0.02338          (-----*-----)

Cedar Creek      20  0.01612  0.01345       (-----*-----)

Kendrick Creek   10  0.01983  0.00892       (-------*--------)

Bradley Creek    10  0.01453  0.01268   (--------*-------)

Crockett Creek   10  0.01948  0.01110      (--------*--------)

Wagner Creek     10  0.02351  0.01628         (--------*-------)

                                       ---------+---------+---------+---------+

                                          0.015    0.030     0.045     0.060
```

Figure 4 Individual 95% CIs for mean eye diameter *|R-L|.
Scale is in millimeters. This figure was produced in conjunction with a one-way analysis of variance to test for differences in corrected |R-L| responses among sample locations.

```
Location        N   Mean     SD       -------+---------+---------+---------+--

Laurel Fork     20  0.07510  0.06628                        (-----*------)

Gap Creek       20  0.03910  0.03056         (------*-----)

Martin Creek    20  0.07225  0.05287                      (------*-----)

Dry Creek       10  0.03716  0.02960      (--------*--------)

Sinking Creek   20  0.02689  0.01963    (------*-----)

L. Chero. Creek 20  0.04535  0.02852            (-----*------)

L. Lime. Creek  20  0.05244  0.03322               (-----*-----)

Brush Creek     20  0.04490  0.03520           (------*-----)

Boones Creek    19  0.05079  0.03412              (-----*------)

Buffalo Creek   20  0.06436  0.03526                   (------*-----)

Cedar Creek     20  0.04496  0.02883            (-----*-----)

Kendrick Creek  10  0.05614  0.03240             (--------*---------)

Bradley Creek   10  0.03058  0.02085   (--------*--------)

Crockett Creek  10  0.03054  0.02121   (--------*--------)

Wagner Creek    10  0.03339  0.02889    (--------*--------)

                                      -------+---------+---------+---------+--

                                         0.025   0.050      0.075      0.100
```

Figure 5 Individual 95% CIs for mean snout length *|R-L|.
Scale is in millimeters. This figure was produced in conjunction with a one-way analysis of variance to test for differences in corrected |R-L| responses among sample locations.

```
Location          N    Mean    SD       -------+---------+---------+---------+--
Laurel Fork       20   0.02576 0.01850                     (-----*-----)
Gap Creek         20   0.02020 0.01485               (-----*-----)
Martin Creek      20   0.03486 0.02657                              (-----*-----)
Dry Creek         10   0.01973 0.01199            (--------*-------)
Sinking Creek     20   0.00872 0.00767    (-----*-----)
L. Chero. Creek   20   0.01620 0.01106           (-----*-----)
L. Lime. Creek    20   0.02657 0.00966                     (------*-----)
Brush Creek       20   0.01292 0.01313        (-----*-----)
Boones Creek      19   0.01603 0.01161           (-----*-----)
Buffalo Creek     20   0.02438 0.01258                   (-----*------)
Cedar Creek       20   0.01756 0.01334            (------*-----)
Kendrick Creek    10   0.01875 0.01118           (--------*-------)
Bradley Creek     10   0.01858 0.00823           (--------*-------)
Crockett Creek    10   0.01453 0.00638       (--------*-------)
Wagner Creek      10   0.01317 0.00853     (--------*--------)
                                         -------+---------+---------+---------+--
                                           0.010   0.020     0.030     0.040
```

Figure 6 Individual 95% CIs for mean head length *|R-L|.
Scale is in millimeters. This figure was produced in conjunction with a one-way analysis of variance to test for differences in corrected |R-L| responses among sample locations.

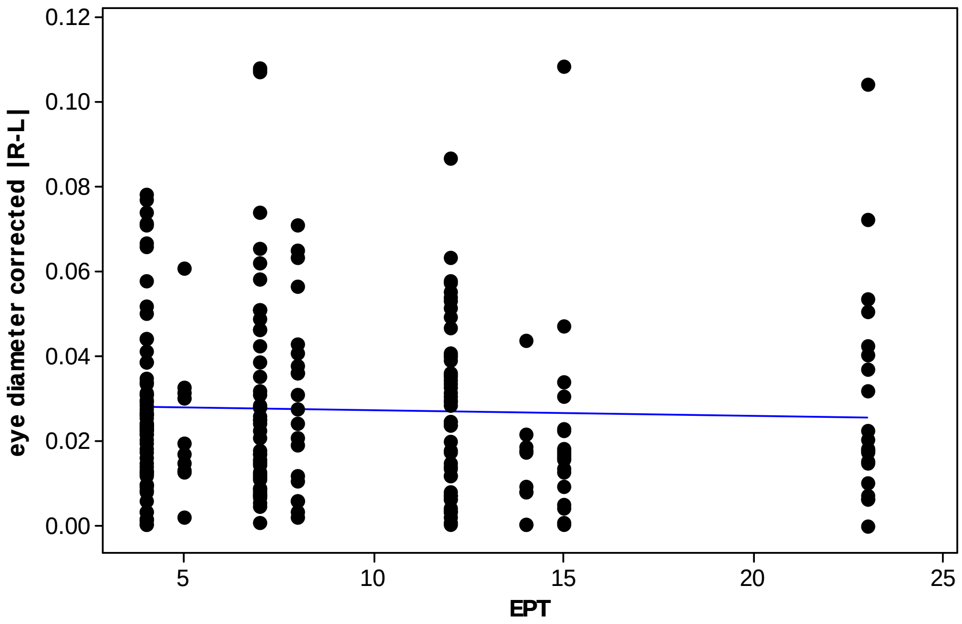

Figure 8 Eye diameter corrected |R-L| vs. EPT count.
(r = 0.032, p = 0.639) "EPT" is the EPT family count.

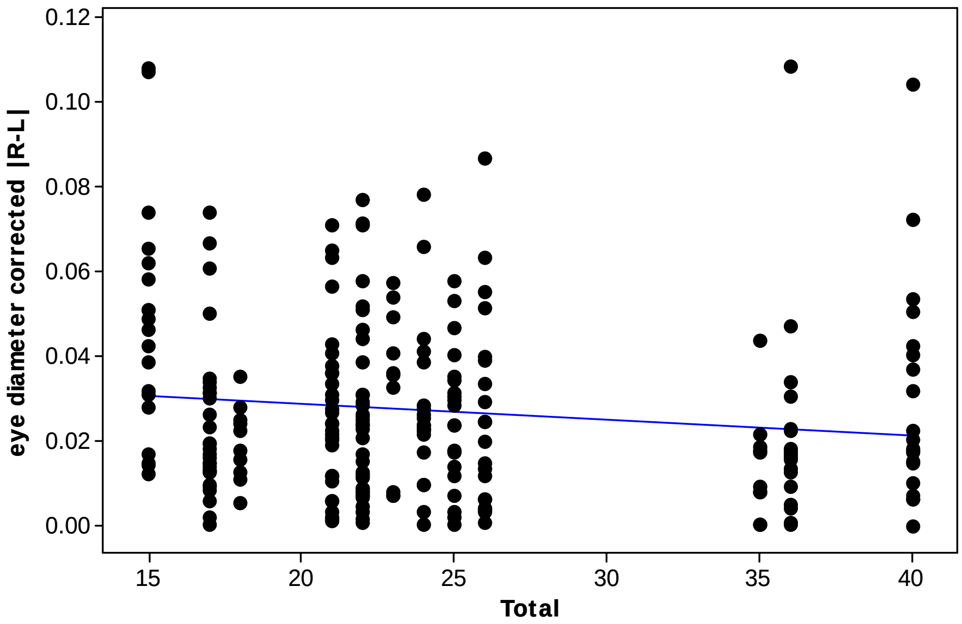

Figure 9 Eye diameter corrected |R-L| vs. total invertebrate family count.
(r = 0.127, p = 0.046) "Total" is the total invertebrate family count.

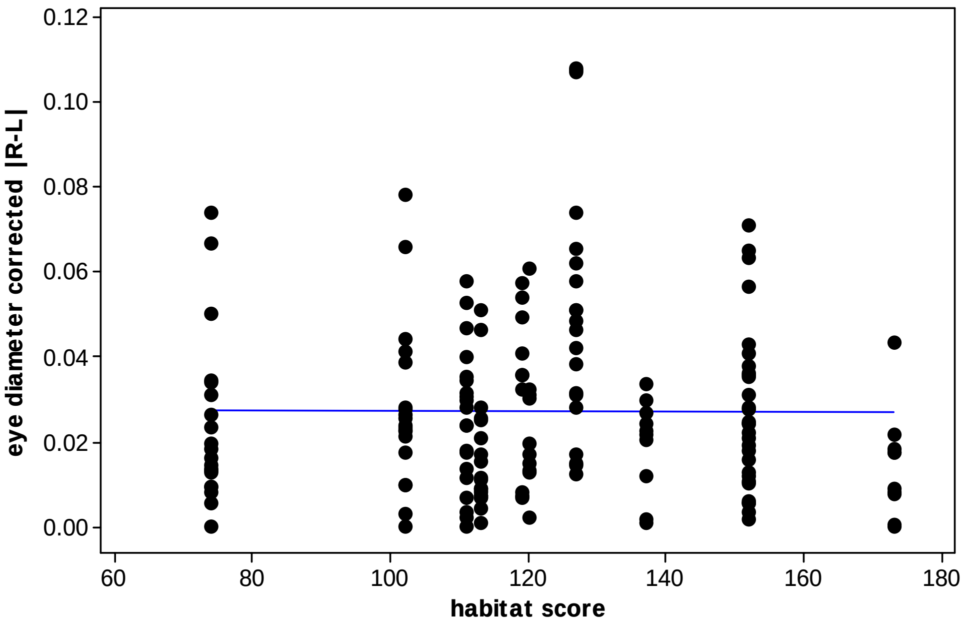

Figure 10 Eye diameter corrected |R-L| vs. habitat score.
(r = 0.010, p = 0.930)

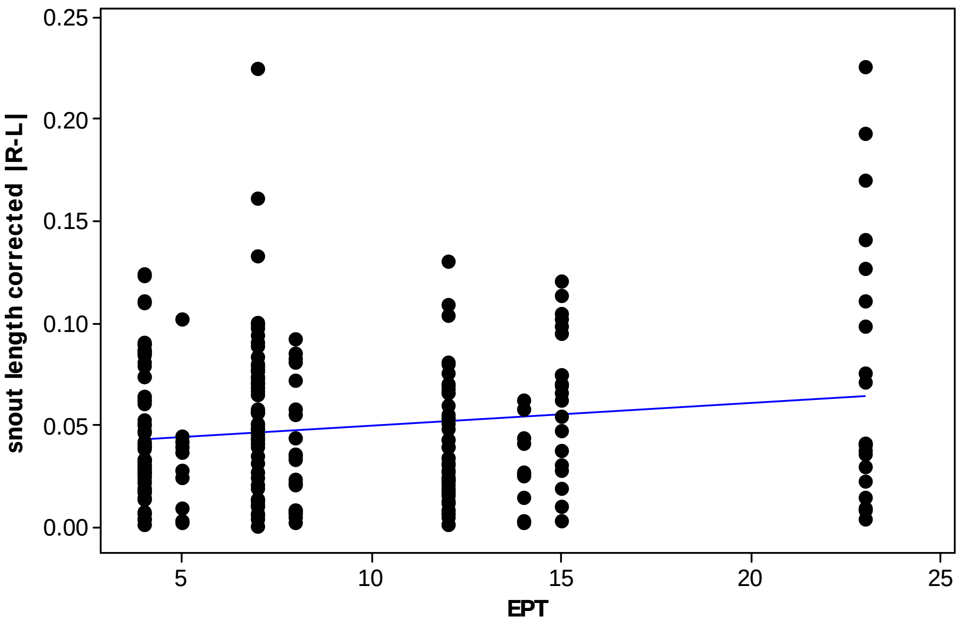

Figure 11 Snout length corrected |R-L| vs. EPT count.
(r = 0.155, p = 0.014) “EPT” is the EPT family count.

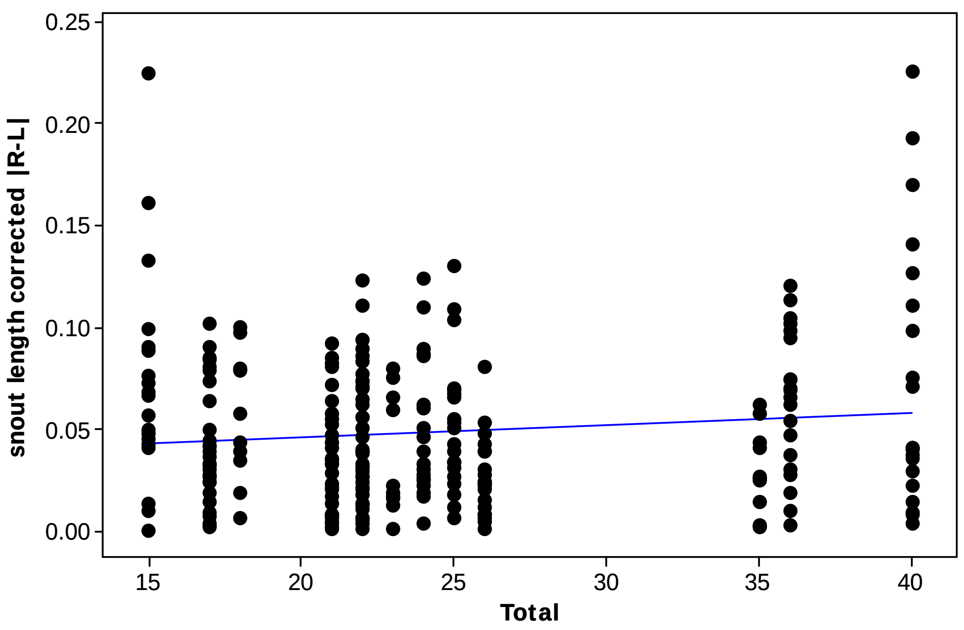

Figure 12 Snout length corrected |R-L| vs. total invertebrate family count. (r = 0.110, p = 0.080) “Total” is the total invertebrate family count.

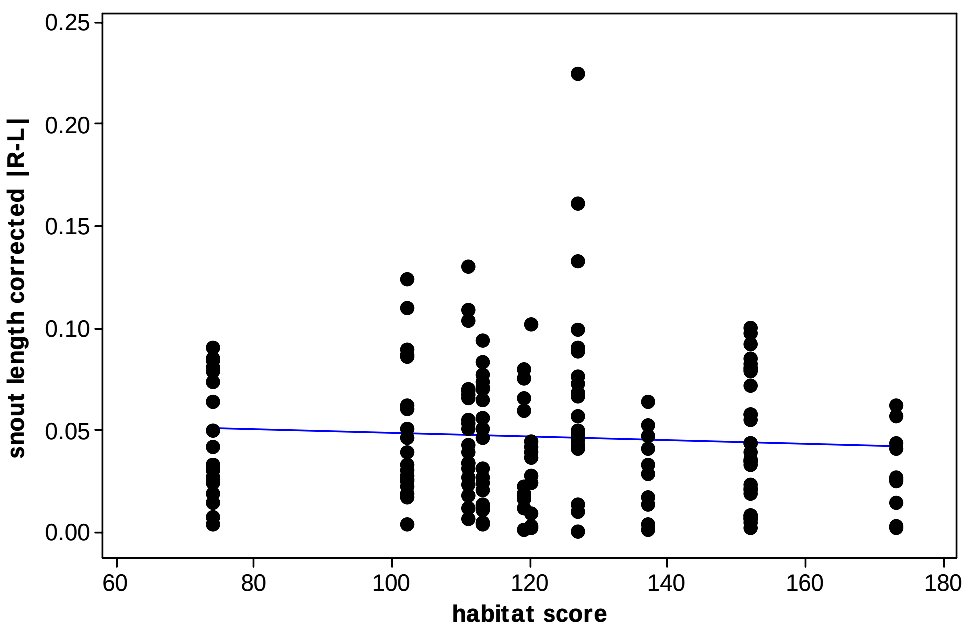

Figure 13 Snout length corrected |R-L| vs. habitat score. (r = 0.070, p = 0.362)

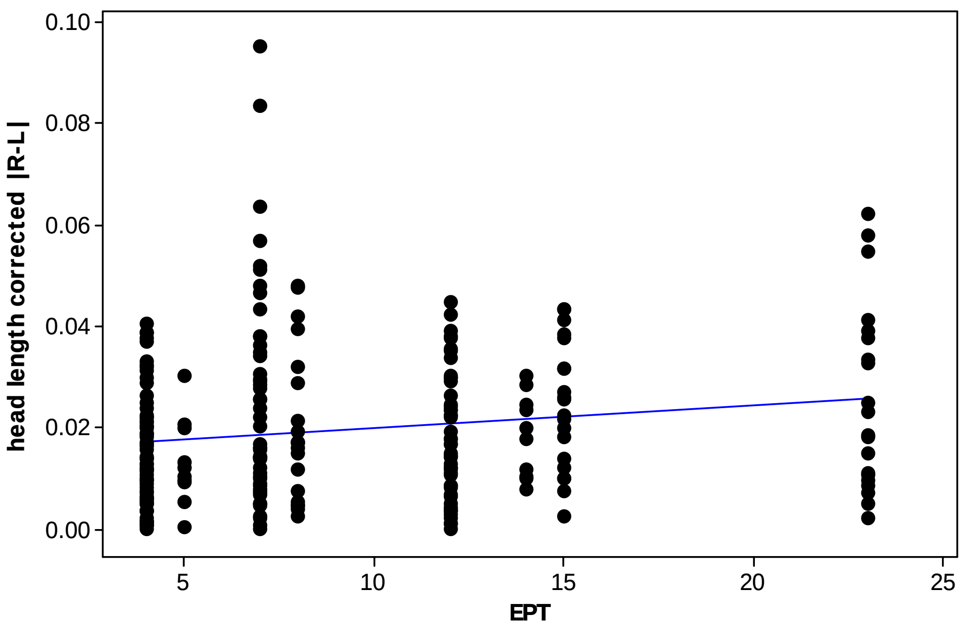

Figure 14 Head length corrected |R-L| vs. EPT count.
(r = 0.161, p = 0.010) "EPT" is the EPT family count.

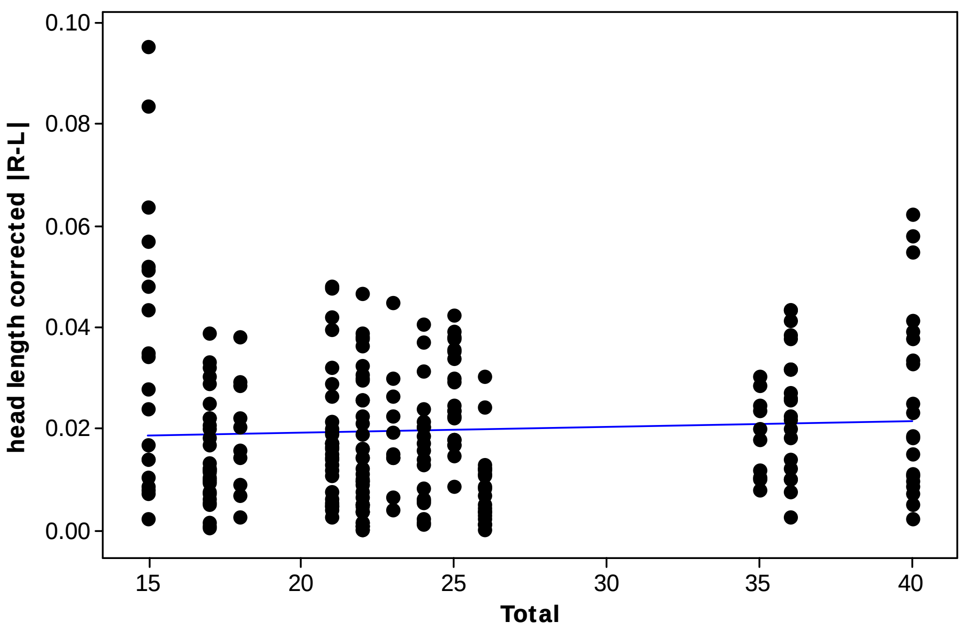

Figure 15 Head length corrected |R-L| vs. total invertebrate family count.
(r = 0.055, p = 0.425) "Total" is the total invertebrate family count.

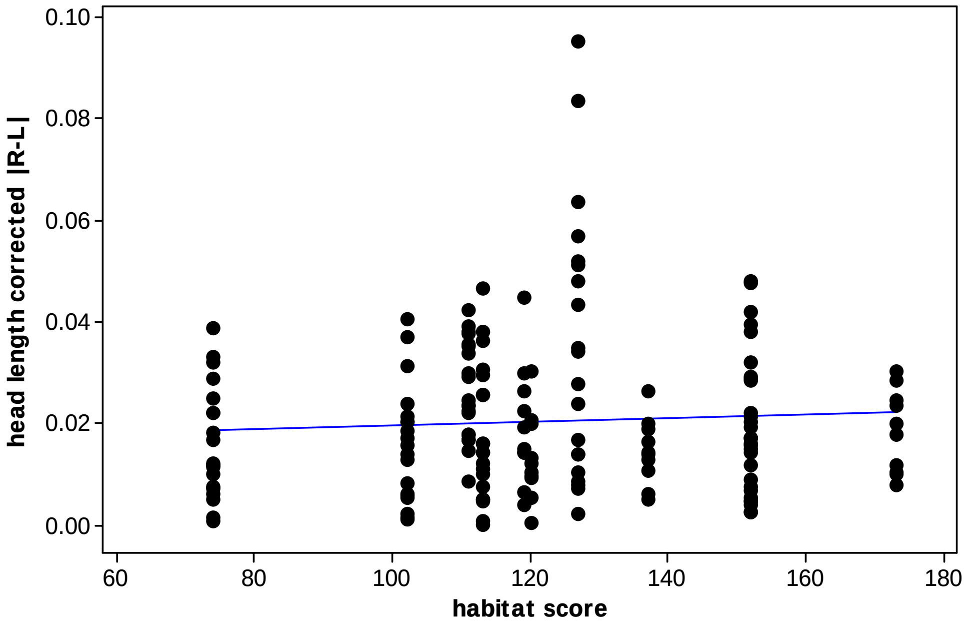

Figure 16 Head length corrected |R-L| vs. habitat score.
(r = 0.063, p = 0.433)

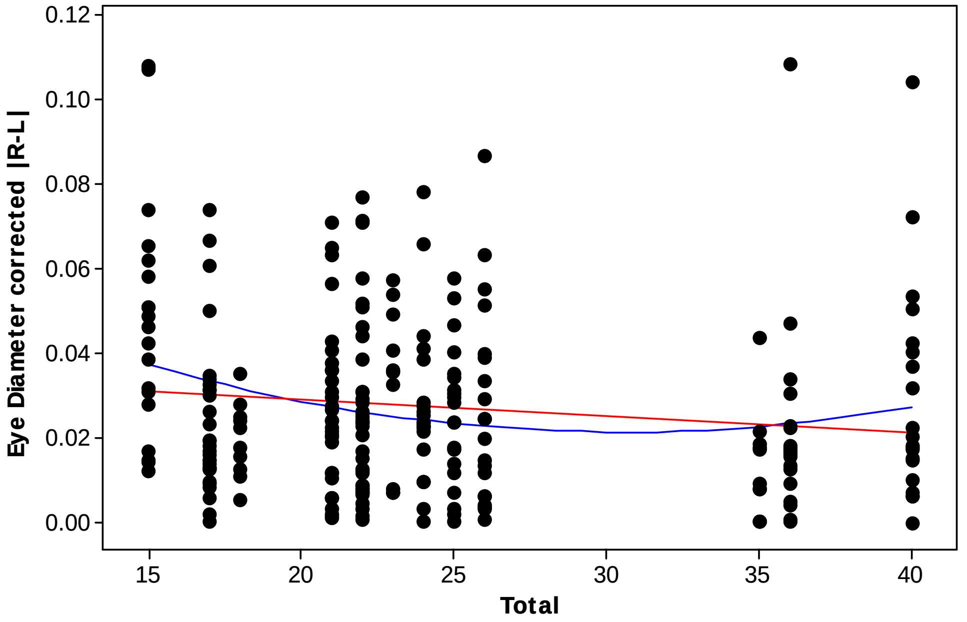

Figure 17 Eye diameter corrected |R-L| vs. total invertebrate family count with linear and quadratic fits.
“Total” is the total invertebrate family count. Y-axis is corrected |R-L| values in millimeters.
(linear model, r = 0.126, p = 0.048) (quadratic model, r = 0.202, p = 0.005).

VITA

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