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This research was made possible by a grant from the Adrian Tinsley Program (ATP).

Investigating Fluctuating Asymmetry of the Larval Damselfly, *Calopteryx maculata* (Odonata: Calopterigidae)

by Edward Kelliher

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ABSTRACT: Fluctuating asymmetry (FA), or subtle random deviations from perfect bilateral symmetry, has recently become a useful tool in allowing researchers to understand more about an organism's health, fitness, developmental stability and environmental stressors. Ultimately, FA studies can be used as an indirect measurement of the quality of an aquatic system over time. We measured and examined the femur segments of the larval damselfly, *Calopteryx maculata* from sites on the Town, Hockomock, and Salisbury Plain Rivers, of Plymouth County, Massachusetts to determine FA levels. After accounting for measurement error, preliminary results show that variations in symmetry are not correlated to individual trait size. Also, the Hockomock River site showed FA levels thee times higher than the Salisbury Plain river, and twice that of the Town River. Finally, severe femur deformation of some individuals at all sites suggests that other, more serious developmental or environmental factors may be inhibiting normal development. Results from a simple two-way ANOVA of differences in right and left femur segments and a Kolmogorov-Smirnov test for normality strongly suggest that the first femur of *C. maculata* is a useful trait for FA measurement.

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Introduction

Fluctuating asymmetry (FA), or subtle, random deviations from perfect bilateral symmetry (Van Valen, 1962) has become a useful tool in allowing researchers to understand more about an organism's health, developmental stability (Palmer 1996) and genetic or environmental stressors (Leary and Allendorf, 1989; Graham et.al. 1993) as well as the effects of hybridization and adaptation through inheritance (Hochwender and Fritz 1999). The idea of this measurement comes from the expected development of perfect symmetry of a bilateral character (Palmer and Strobeck 1986). Thus, measurement of deviations from perfect bilateral symmetry can be done to infer whether there are factors that affect the morphology of bilateral traits throughout the development of the organism. Ultimately, if one could develop methods that could rule out heritable components and genetic stressors, then fluctuating asymmetry can be used as a measure of how precisely development has occurred in terms of an organism's environment (Palmer 1996).

Bilateral organisms undergoing development are highly homeostatic and capable of buffering for variation due to developmental noise. This is defined as small, completely random accidents or errors in development of a trait that are exclusively environmental in origin and that inhibit an organism's pre-determined genetic path for development (Palmer 1996). Consequently, subtle differences between the left and right measures of a trait in a bilaterally symmetrical organism are the product of opposing forces: one that acts to disrupt development (developmental noise), and one that seeks to counteract the disruptive effects (developmental stability) (Fig. 1) (Palmer 1996). When indices are calculated that express fluctuating asymmetry as a variance or an average absolute value of the difference between right and left measures of bilateral traits (Palmer and Strobeck 1986), the larger the (FA), the lower the developmental stability. Similarly, if (FA) measurement is low, it can be noted that the organism of interest has a high buffering capacity to minimize developmental variations or that there is little developmental noise at play (Fig. 1)(Fuller and Houle 2002; Rowe, et.al 1997).

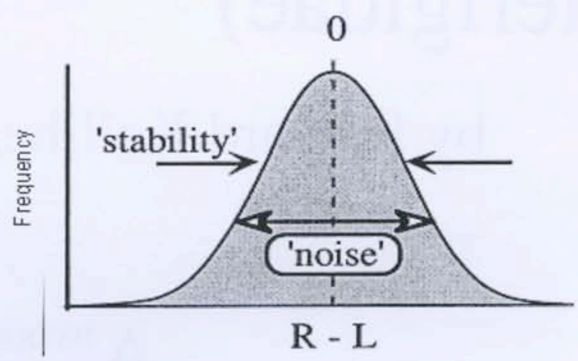

Fig. 1.
FA reflects the opposing processes of developmental noise Vs. developmental stability (Palmer 1993). ' Stability' is the force that decreases FA, thus allowing development to proceed more perfectly (forces the graph inward towards zero). 'Noise' is the force that increases FA, which tends to push the graph outwards away from zero.

There is evidence that FA has a heritable basis (Fuller and Houle 2002; Hochwender and Fritz 1998; Palmer and Strobeck 1986; Palmer 1996). Studies have shown that developmental stability was reduced (or FA increased) through increased homozygosity in hybrids between nominal species and others. Even so, environmental factors such as extreme physical conditions (water temperature), pollutants (heavy metals, nitrates, phosphates, metal salts) or declines in habitat quality will contribute directly to developmental noise (Palmer and Strobeck 1992) and also play a crucial role in the overall condition of individuals and populations.

Therefore, is measurement of (FA) a tool for correlating environmental stressors, genetic stressors or a combination? The question has yet to be answered. The answer most likely is a combination of the two (Clarke, 1995; Fuller and Houle 2002), but to what degree? If fluctuating asymmetry is to be used accurately and effectively, one must somehow be able to measure organisms whose bilateral traits are "well behaved" enough so that underlying factors causing asymmetry are environmental by nature (developmental noise). Then, one must be able to distinguish these traits from "poorly behaved" characters that have asymmetry due to either direct genomic intervention (directional asymmetry, DA) or by factors that create other forms of asymmetry (antisymmetry, non-covariant asymmetry) (Palmer 1996).

Other controversies that surround this measurement for imperfection are measurement error and data interpretation. For bilateral variation in traits to be used as a tool for quantifying a real measure of developmental precision (defined as the accuracy by which genetic programs in the same individual produce the exact same structures) (Palmer 2001), three criteria must be met: 1) between sides variation must be significantly greater than measurement error, 2) variation due to measurement error must be factored out to insure credible measures of asymmetry; this being because FA measures are exceedingly small, close to 1% of the actual trait size 3) the measures taken must meet the statistical criteria for 'ideal' FA having a mean R-L of zero and being normally distributed (Fig. 2) (Palmer 1994). Without these steps, quantitative data for fluctuating asymmetry cannot be interpreted with much confidence (Palmer and Strobeck 1997). Care must also be taken when analyzing graphical interpretations of bilateral variation. With a frequency distribution, two other measures of asymmetry that cannot measure developmental precision or developmental noise can be revealed. Directional asymmetry (DA) defined as repeatable asymmetry with respect to side (Fig 3), and antisymmetry, or repeatable asymmetry that is random with respect to side (Fig. 4) (Palmer and Strobeck 1997) will have distinct qualitative patterns when plotted using a frequency distribution. Directionally asymmetrical graphs will exhibit normal distribution around a mean that is non-zero, as opposed to graphs with a normal distribution that is zero (Fig. 2). Directional asymmetry is often found in nature (the classic example is the larger claw found on fiddler crabs) and can be attributed to that organism's genetic response to its environment. Antisymmetrical graphs will appear as platykurtic or bimodal distributions around a mean of zero. Even though there are arguments that DA and antisymmetry can arise due to some form of developmental noise, not all forms of DA and antisymmetry are signs of reduced fitness (Palmer and Strobeck 1992). By factoring out directional asymmetry and antisymmetry, (that is, by determining normal distribution around mean zero) one can deduce whether FA is present in a population sample.

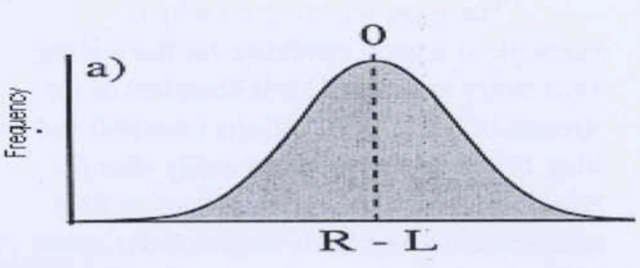

Fig. 2 Example of fluctuating asymmetry (FA) distribution for bilateral organism (Palmer 1994). The majority of organisms measured for FA will appear to be close to zero; in other words, have nearly perfect symmetry.

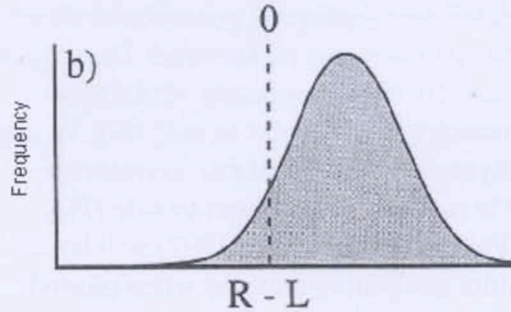

Fig. 3 Example of directional asymmetry (DA) distribution for bilateral organism (Palmer 1994)

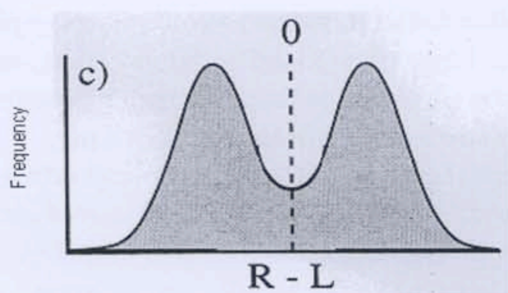

Fig. 4. Example of Antisymmetry (platykurtic or bimodal) Distribution for Bilateral Organism (Palmer 1994)

The focus of this research is to determine whether the bilateral traits of the larval damselfly, *C. maculata*, (specifically the femur segments of three bilateral appendages) exhibit fluctuating asymmetry.

There are four reasons why *C. maculata* is a good candidate for fluctuating asymmetry analysis: 1) it is abundant in the streams of S.E. Massachusetts (Westfall and May 1996), 2) it is the most easily identifiable damselfly larvae when collecting field samples a 3) it is a fairly large aquatic insect with many large bilateral characteristics (femur, tibia, antennae, wing pads) (Westfall and May 1996) and 4) *C. maculata* is a predacious animal that is close to the top of the food chain in its particular ecosystem (Westfall and May 1996) and may be a "sink" for heavy metals acquired though consumption. In this case, the term "sink" (bioaccumulation) refers to an organism that will acquire, over time, contaminants based on what it ingests for food and habitat location.

Materials and Methods

Sample collections. Three sites were chosen with various degrees of anthropogenic influence to compare levels of FA. They were also chosen because the habitat that supports *C. maculata* growth and development (muddy root-filled banks, overhanging vegetation, moderate current) can be found on these three rivers.

Thirty-three male and female *C. maculata* were collected on the south bank of the Town River (TWN029, N 41 degrees 02. 321; W 070 degrees 58.676. Plymouth County, Bridgewater MA.) on June 8, and 9, 2003. Twenty-two C. maculata were collected on the banks of the Hockomock River (N 41 degrees, 00.560; W 070 degrees, 34.199 , Plymouth County, Bridgewater MA.). Thirty-five C. maculata were collected on the banks of the Salisbury Plain River (SLP027: 42 degrees 03'.218N; 71 degrees 00.588W, Plymouth County, Brockton MA.) (fig. 5).

Nymph maturity was assessed in the field using size and wing pads. If wing venation was apparent through the wing pads, the last instar of maturity was inferred. Nymph maturity is important in that developmental processes have allowed for leg segment lengths to reach peak growth. All specimens were captured using aquatic kick nets and placed in sample jars of no more than 6 per jar. Within three hours of acquisition, samples were stored in 70% ETOH at 0 degrees C for 24 hours and then placed at room temperature until measured.

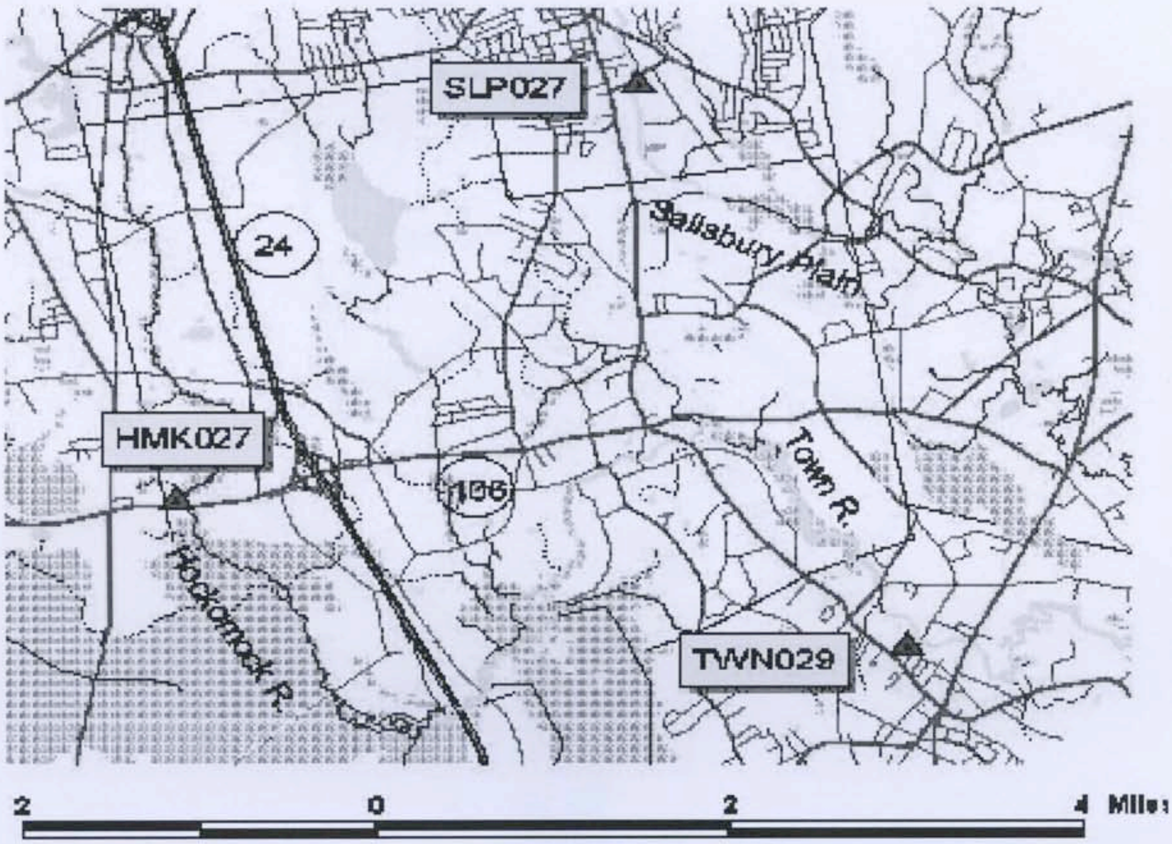

Fig. 5. Map of three sample sites for C. maculata collection, June 2003

Measurement Preparation. Two-dimensional imaging of *C. maculata* was standardized using transparency film and clear microscope slides (fig. 6). Samples were laid ventrally and wholly intact, 8 individuals per transparency sheet (6 femur segments per individual, 48 femur segments per transparency set). Legs of each specimen were pinned flat underneath clear microscope slides that were taped onto the transparency. Seventy percent ethanol was applied to each specimen twice during preparation to ensure that body parts do not shorten due to dessication. 'Wet' specimens were used because resolution of leg sutures became more visible when scanned. Achieving two-dimensional imagery is important in accounting for measurement error when measurement software is used. Each set of specimens was scanned twice at a resolution of 600 dpi using a Hewlett Packard ScanJet 8200C flatbed scanner. At this resolution, all images produced have pixel dimensions of 0.05 x 0.05 mm. All measurements are presented in millimeters. After scanning, samples were catalogued and placed in individual vials of 70% EtOH and kept at room temperature.

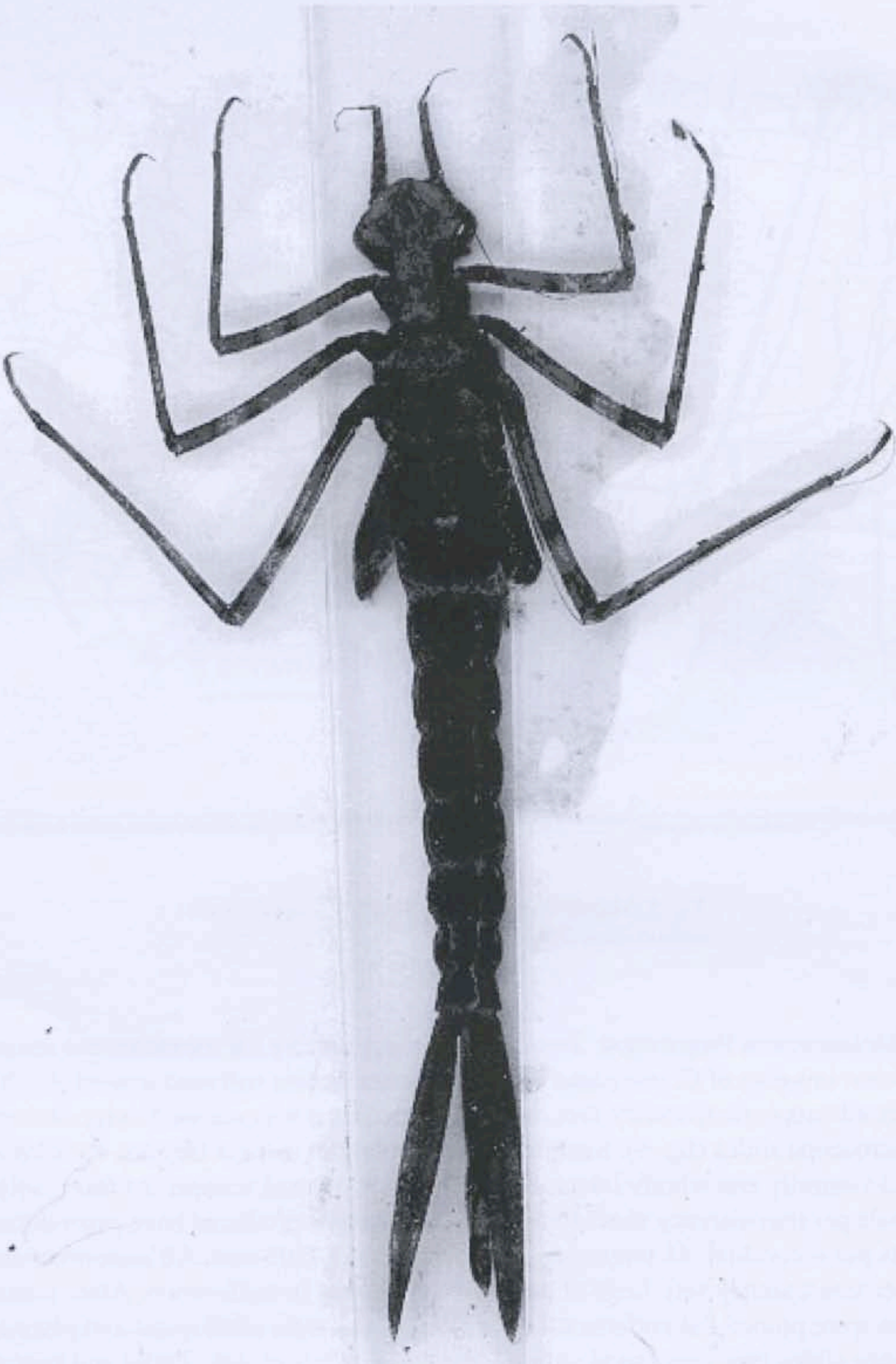

Fig. 6. Scanned image of final instar nymph, *C. maculata*

Measurements.

Scanned images of *C. maculata* from each site were enlarged to 18 times the normal size on the computer screen and measured using a streaming data point-trace tool in Sigma Pro Scan 5.0 image analysis software. A 10mm x 10mm grid was scanned next to each specimen for calibration purposes. Calibration was done at the 2x dimension of Sigma Pro Scan and was set at 25.01996667 pixels/mm based on the average of three calibration measures. Three measurements of each femur segment (6 femur segments, 18 measurements per specimen) were made from the suture connecting the femur to the trochanter joint, to the tip of a hook or bump-like feature located just before the suture that connects femur to the tibia segment (Westfall and May, 1996).

Two measurements were traced following the bottom portion of the scanned image and one taken using the middle of the femur image (care was taken to ensure that the top and bottom of the two-dimensional femur image was kept equidistant while measuring). Sutures were marked with a yellow trace overlay at 27 times normal trait size to ensure accurate location within the image and kept as a permanent overlay. Measurements were taken in red overlay in random order and were erased after each measurement. All measurements were taken on the same image by the same person and exported to an Excel spreadsheet with 5 significant digits.

Two assessments were made to determine if the femur segments of *C. maculata* are suitable to undergo (FA) analysis: 1) determination of whether trait size correlates to deviation from ideal symmetry (mean zero) and 2) if the frequency of deviation around ideal symmetry is normally distributed. Each leg pair was given its own measurement category (R1-L1, R2-L2, R3-L3 respectively) and each pair was treated as an independent trait.

A two-way analysis of variance on femur length data at all sites was done using SPSS 11.5 general linear model; univariate. Femur length was considered the independent variable with sides being the fixed factor and the specimen (individual) treated as the random factor (Palmer 1993). This was done to test the significance of FA in relation to measurement error. Ultimately, if the interaction variance in the ANOVA is not significant, these tests indicate whether or not a trait can be used for FA studies (Palmer 1996). Outliers were removed following Palmer (2001) in order to use C. maculata as an organism whose traits are 'well behaved.'

A Kolmogorov-Smirnov test was also conducted to determine if the R-L data was normally distributed around a mean of zero and not DA or antisymmetry.

Results

Using the SPSS Explore utility tool for outlier analysis and the Kolmogorov-Smirnov test on R-L data, only the first femur pair can be used for FA analyses because it passes the Kolmogorov-Smirnov normality test and the Simple two-way ANOVA test to determine if DA, antisymmetry or non-covariant asymmetry is present (other forms of subtle asymmetry that need to be partitioned out) (Table 1).

K-S Normality Test of R-L pairs			two-way ANOVA of specimens' femur lengths					Variance	
Site stat	df	Signif.	df	Spec	Side	Side x Spec.		df	Var
TWNF1 R-L	0.067	87	0.2	28	0	0.98	0	87	***0.013
TWNF2 R-L	0.085	92	0.104	30	0	0.174	**0.913	92	0.013
TWNF3 R-L	0.101	93	*0.021	29	0	0.705	0	93	0.017
SLPF1 R-L	0.063	83	0.2	27	0	0.365	0	83	***0.023
SLPF2 R-L	0.065	71	0.2	25	0.005	**.034	0	71	0.031
SLPF3 R-L	0.126	73	*0.006	26	0	0.58	0	73	0.016
HMKF1 R-L	0.09	61	0.2	20	0	0.288	0	61	***0.033
HMKF2 R-L	0.103	50	0.2	16	0	0.174	**0.907	50	0.025
HMKF3 R-L	0.156	56	*0.002	18	0	0.346	0	56	0.032

Key
TWN - Town River
SLP - Salisbury Plain River
HMK - Hockomock River
F1, F2, F3 refer to femur pairs
df - Degrees of freedom
Var - Variance
R-L - right minus left
* Does not pass normality test (p<0.05)
** Failed interaction in two-way ANOVA
*** Indicates that this data can be used in FA studies

Table 1. Kolmogorov-Smirnov test for normality, two-way ANOVA and variance data sets for three traits of C. maculata on three rivers in Plymouth County, MA.

The second femur pair passed tests for normality at all three sites (Table 1) but failed to exhibit FA because it failed the 'sides*specimen' interaction from the ANOVA test. Third femur length data sets for all three sites pass the ANOVA test to eliminate DA, antisymmetry or skew, but fail the Kolmogorov-Smirnov test for normality since they are significantly different from a normal distribution (Table 1).

The first femur segment is therefore the best-suited or best "behaved" trait to proceed onto a comparison of variances of the R-L data, since it passes the Kolmogorov-Smirnov test for normality and the ANOVA test (Table 1). Individual graphs depicting normal distribution are given for the right-left differences in first femur lengths (Figs. 7,8,9).

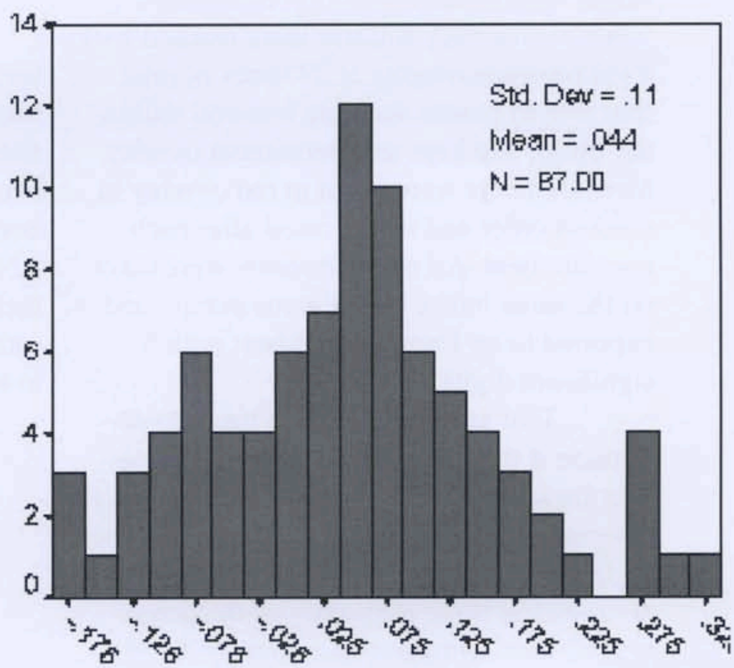

Fig.7. Town River Femur 1 R-L, June 2003

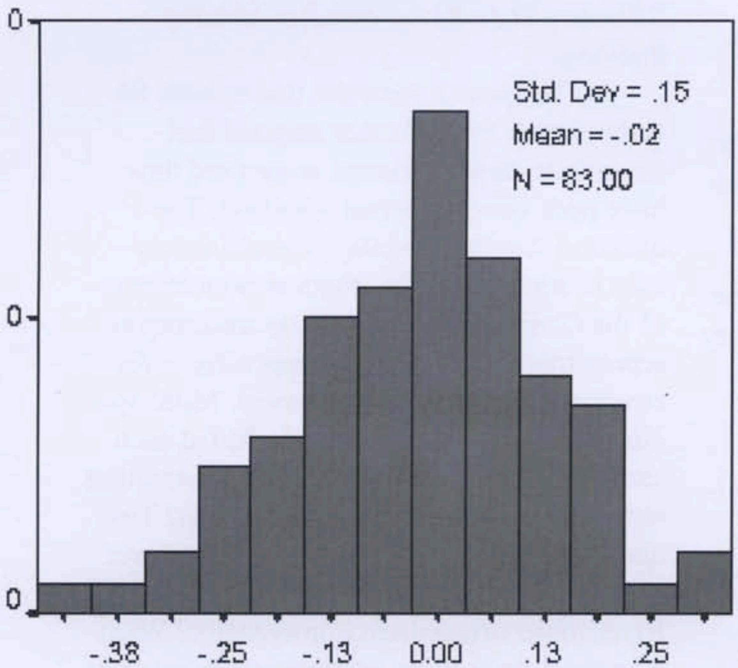

Fig. 8 . Salisbury Plain River Femur 1 R-L, June 2003

The FA index used is the variance of R-L (Palmer 1993, FA #4) because it is easily computed and is the most powerful test for the differences between two samples and is also better for estimating between sides variation. It is also a useful index in that it is not biased by directional asymmetry (Palmer 1993). The variances obtained with the simple two-way ANOVA using the first femur pair showed that the Town River sample to be the lowest (.013), the Salisbury Plain River to be higher (.023) and the Hockomock River sample showing the highest (.033) FA (Table 3).

Discussion

There appears to be a direct correlation to the particular femur mea-

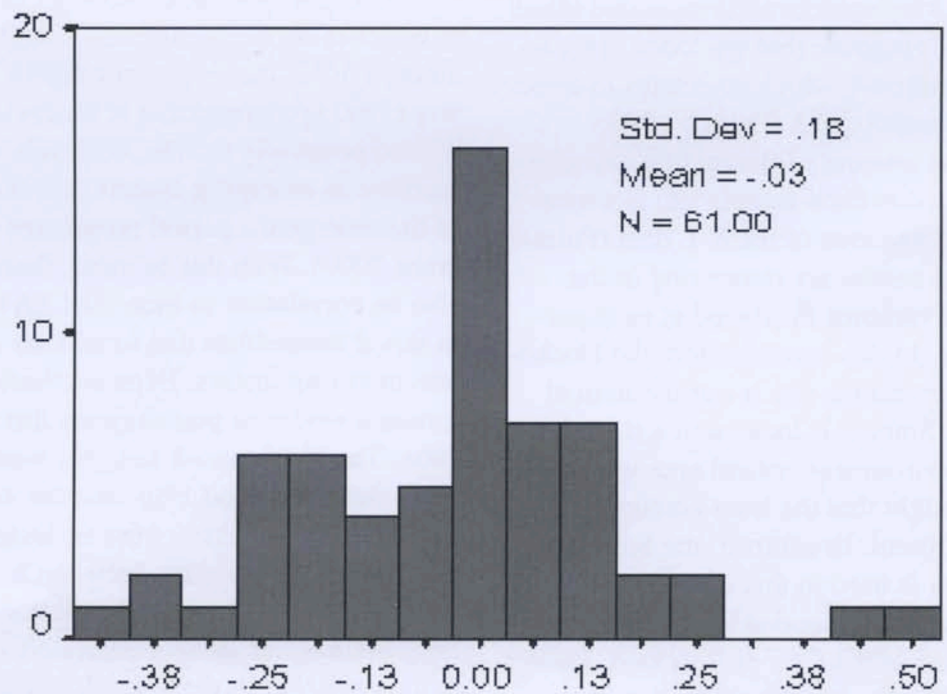

Fig. 9. Hockomock River Femur 1 R-L June 2003

sured and the type of phenomenon that is taking place. All three R-L analyses on the first femur segment had normal distributions and passed the ANOVA tests (Side, Side*Specimen and Specimen) and thus the resulting variances can be useful to FA tests (Palmer 1993). The other two traits measured are not useful to FA investigations, but each femur length R-L failed for similar reasons, (either in the K-S test for Normality or the two-way ANOVA).

The 'Side*Specimen' interaction conducted in the ANOVA test includes all forms of non-directional asymmetry, including FA, antisymmetry and normal-covariant asymmetry (Palmer 1993). If tests are significant for this part of the ANOVA, it indicates that asymmetry is present, but it cannot be deduced that it is fluctuating asymmetry and therefore cannot be used in this investigation. Both the Hockomock and Town river femur length 2 R-L data failed this test (indicated by significant P-values) (Table 2). The Salisbury Plain second femur lengths R-L indicate that the 'Side' interaction is significant, which correlates to directional asymmetry, DA (Palmer 1993).

The amount of fluctuating asymmetry displayed at each sample site is a measure of the variance of the R-L data (Palmer 1993). The results are interesting in the amount of variance displayed at each particular site. In this investigation, the Hockomock River sample site is our theoretical clean site. Since it is located in a state-designated environment critical area wetland, in was thought that the least amount of FA would be found. In contrast, the Salisbury Plain River is used in this investigation as the contaminated site due to its location downstream of the City of Brockton, known in the past for its large tanning factories for shoe manufacturing. However, the data indicates that the Hockomock River site contained *C.maculata* that displayed R-L variances that were higher than that of the Salisbury Plain River, contrary to initial thinking.

The search for traits that exhibit FA is the search for traits that respond inefficiently to developmental noise (and thus have poor developmental stability). The question that this investigation ultimately asks is whether femur length measurements of the *C.maculata* can generate meaningful representations of what is happening in the environment during development. More specifically, are the variances obtained at each sample site giving an accurate representation to the amount of environmental stress? Two questions arise: is the Salisbury Plain River cleaner than expected, or is the Hockomock River more stressed environmentally? Whatever the case may be, more research needs to be done to more accurately determine what is happening. Since aquatic environments are dynamic, so may be the levels of FA that can be measured over time.

There is some correlation to the time at which adult damselflies are caught and the amount of FA that is present. Rowe and Ludwig (1991) proposed that if fitness is correlated positively to size, FA levels should increase as emerging insects decrease in size as the emergence period progresses (Hardersen 2000). With this in mind, there may also be correlation to increased FA in levels in larval damselflies due to smaller sizes of late-maturing instars. Even so, there is only minimal evidence that suggests this phenomenon. The Hockomock samples were collected on June 15th and 19th, and the Town and Salisbury Plain Rivers were collected within three days of each other between June 3rd and the 6th. This being the case, the Salisbury Plain River sample exhibited almost twice the FA as in the Town River sample (Table 3). The importance of this stems from emergence times and the nature of reproductive habits of *C.maculata*. Through field observations and nymph collection tallies, most adults emerged between the first and fourth week of June. This suggests that there is only a small window of time for growth variation due to maturity and egg deposition. Adults that emerge first will copulate first and thus will oviposit early in the summer. The eggs that are laid during the beginning of the summer will have a much longer time to develop than those eggs oviposited during late summer and into the fall. This point may help to explain the size differences observed in final instars of larvae among samples. The significance of the three FA measurements will remain unknown until a more intensive study is conducted in the summer of 2004, following measurement protocol and sample parameters from Palmer (1993) to see if there are changes over time.

From the data gathered, a vast majority of the deviation found in the sample population falls at or below this one percent trait size, but a few specimens from each site exhibited large deviations from perfect symmetry. Some portion of the outliers was obvious measurement error and was not used in any of the tests. Upon closer inspection of the individuals in question, the femur segments were severely underdeveloped or deformed. These asymmetries are by no means subtle, nor do they imply small perturbations that occur accidentally throughout development. They were treated as deformities and were not reported on the graph indicating the frequency distribution.

Whatever caused these deformities to occur is unknown. Severe deformity suggests severe stress and could possibly have both genetic and environmental factors that play into the severity. The question is, to what degree are the deformities genetic or environmental? Another hypothesis that could account for this limb deformation may be that *C. maculata*, in larval development, may be able to regenerate certain segments of its body (say, if the larvae was preyed upon) so that its metamorphosis into adult life as a predacious flying insect will not be compromised. A more thorough investigation into this phenomenon needs to occur in future studies.

There is some question as to whether populations of *C. maculata* between different rivers will mix together to form a heterogeneous population from various areas. This is very important in studies of FA in different populations. If there are different populations mixing together, there will be no way to correlate an FA index with a distinct environmental condition of that area. In this sense, FA measurements cannot be used with much confidence even if there is strong evidence that suggests otherwise. Thus, FA analysis of *C.maculata* will only be useful if they are contained within a well-defined territory (Hardersen 2000). We believe that the populations of *C. maculata* sampled in this study do not mix. Although it was not an observed phenomenon, in *C. maculata* populations, the New Zealand damselfly *X. zealandica* adults do not migrate far from the emergence site (Hardersen 2000). This brings up the question as to how far *C. maculata* travel from their natal origins and the implications it has to biomonitoring studies and FA analyses.

Conclusion

Measurement of the femur segments of *C.maculata* may be good indicators of developmental noise, as the first femur pair held up to a size dependency analysis, a Kolmogorov-Smirnov test for normality and a simple two-way ANOVA. The first femur R-L data from all three rivers met all the criteria for a proper FA measurement and exhibited different amounts of FA. The other two femur pairs, although not normally distributed or exhibiting DA or some other form of asymmetry, may still be strong candidates for FA analysis if larger sample sizes per site are used. If more traits can be recognized as being "well behaved" traits for FA measurement, then the larval damselfly *C. maculata* may be useful as a tool for future biomonitoring studies and may lead to investigations of the rivers that nurtured them.

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