Article

Experimental Evidence Questions the Relationship between Stress and Fluctuating Asymmetry in Plants

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Abstract: The eco-evolutionary theory of developmental instability predicts that small, non-directional deviations from perfect symmetry in morphological traits (termed fluctuating asymmetry, FA) emerge when an individual is unable to buffer environmental or genetic stress during its development. Consequently, FA is widely used as an index of stress. The goal of the present study was to experimentally test a seemingly trivial prediction derived from the theory of developmental instability—and from previous observational studies—that significant growth retardation (which indicates stress) in plants is accompanied by an increase in FA of their leaves. We induced stress, evidenced by a significant decrease in biomass relative to control, in cucumber (*Cucumis sativus*), sweet pepper (*Capsicum annuum*), and common bean (*Phaseolus vulgaris*) by applying water solutions of copper and nickel to the soil in which plants were grown. Repeated blind measurements of plant leaves revealed that leaf FA did not differ between stressed and control plants. This finding, once again, demonstrated that FA cannot be seen as a universal indicator of environmental stress. We recommend that the use of FA as a stress index is discontinued until the scope of the developmental instability theory is clarified and its applicability limits are identified.

Keywords: confirmation bias; copper; developmental instability; nickel; plant growth rate; research methodology

1. Introduction

The eco-evolutionary theory of developmental instability, which is widely applied in environmental studies, predicts that (1) minor differences in size or shape between the two sides of a bilaterally symmetrical organ or organism emerge when an individual is unable to buffer environmental or genetic stress that occurred during its development; (2) this inability to sustain stress indicates the "low quality" of an organism and results in its low genetic fitness [1–4]. This theory is simple, easily grasped, internally consistent, and, therefore, attractive; that is, it fully meets the conditions which, according to Fagerström [5], define the success of an ecological theory.

Developmental instability is commonly quantified by measurements of fluctuating asymmetry (FA) defined as small, non-directional (at the population level) deviations away from perfect (usually bilateral) symmetry [4,6]. This type of morphological variation was discovered in the 1930s [7], but the first scientific publication that defined and explicitly addressed FA appeared 60 years ago [8]. Since then, FA has been mentioned in over 3600 studies included in the ISI Web of Science core collection (www.webofscience.com; accessed on 20 December 2022). A substantial fraction of these studies used FA as an

indicator of stress, especially in plants, despite no more than half of the conclusive evidence supports the hypothesis that FA increases with an increase in environmental stress [9–12].

Sadly, controlled experiments exploring cause-and-effect relationships between the stressor(s) and FA in living beings remain scarce [13–20], and the wealth of observational studies reporting an increase in FA of plants and animals collected from potentially stressful environments in no way compensates the shortage of experimental data. The goal of the present study was to experimentally test a seemingly trivial prediction, derived from the theory of developmental instability [2,21] and from previous observational studies [22–24], that significant growth retardation (which indicates stress, as defined by Grime [25]) in plants grown in toxic soil is accompanied by an increase in FA of their leaves.

2. Materials and Methods

2.1. Experimental Design

The experiment was carried out in late spring (from 3 May to 2 June) of 2022 in the greenhouse of the Siberian Institute of Plant Physiology and Biochemistry located in Irkutsk, Russia. We applied all possible measures to avoid research biases, including randomization, replication, and blinding. We used three species of cultivated herbaceous plants, which differ in the size and shape of their leaves (Figure 1) and belong to different families: cucumber (*Cucumis sativus* L., Cucurbitaceae), sweet pepper (*Capsicum annuum* L., Solanaceae), and common bean (*Phaseolus vulgaris* L., Fabaceae).
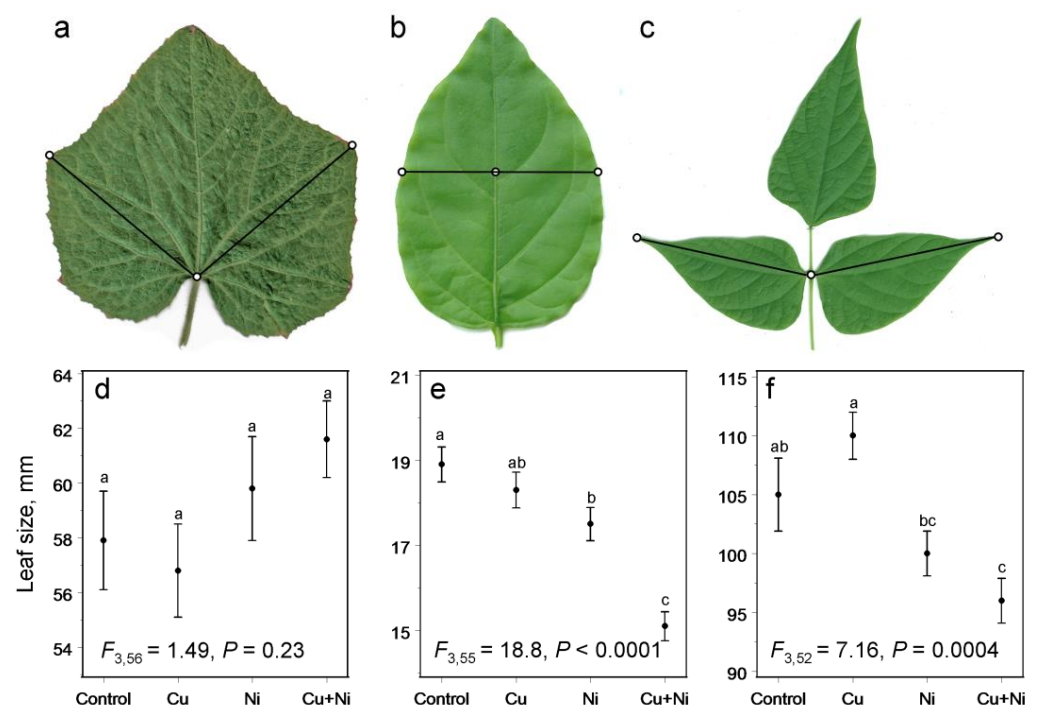

Figure 1. Traits measured for the calculation of leaf fluctuating asymmetry (**a**–**c**) and variation among treatments (mean ± SE, each based on 15 plants, and F/P values) in these traits in (**a**,**d**) cucumber (*Cucumis sativus*), (**b**,**e**) sweet pepper (*Capsicum annuum*), and (**c**,**f**) common bean (*Phaseolus vulgaris*).

Untreated seeds obtained from a local supplier were germinated in the dark, at 25 °C, in Petri dishes containing filter paper moistened with distilled water. After 5 days, 60 healthy seedlings of each species were individually placed in plastic pots each containing 0.9 L of soil (144 g d.w.; 40% fine-grained sand and 60% *Sphagnum*-peat) and 1.25 g of fertilizer (N: 19%; P_2O_5: 6%; K_2O: 20%). For 10 days, all plants were cultivated at 22 °C during the day and 16 °C at night under the natural photoperiod; soil moisture was maintained within 75–100% of the field capacity.

Excess heavy metals affect plant physiology and cause stress symptoms, including impaired growth and reproduction [26,27]. Therefore, we selected copper and nickel to induce stress in experimental plants. Within each species, seedlings were randomly attributed to one of four treatments (15 seedlings in each): control, copper, nickel, and copper plus nickel. Pots with seedlings were interspersed so that their positions within a greenhouse were independent of either species or treatment. Starting from 19 May, metals were added to designated pots with 0.1 L of water solution of $NiSO_4 \times 6H_2O$ and/or $CuSO_4 \times 5H_2O$, so each seedling received 0 or 10 mg of each metal (depending on treatment) per 1000 g of soil (d.w.) each second day. Control seedlings received 0.1 L of pure water. The treatments were discontinued when growth retardation in plants, treated by the combination of nickel and copper, became obvious.

2.2. Sampling and Sample Processing

The three uppermost fully grown leaves of each plant were scanned in colour at 600 dpi (together with a scale) for measurements of FA (Data S1). Then, these leaves were combined with the remaining parts of respective plants for biomass measurement. Roots were manually separated from soil and then washed with water. Plants were dried for 48 h at +105 °C and weighed to the nearest 10 mg (Data S2).

Leaf images were coded (by D.E.G.) so that the measurers (V.Z. and two assistants) were unable to attribute these images to a particular treatment. Images were measured by a standard instrument (ruler) in the Adobe PhotoShop 2020 program to the nearest pixel. In cucumber, we measured the distances from the base of medial vein to the apices of the first lateral veins (Figure 1a). In sweet pepper, we measured the width of the left and right leaf halves at the midpoint between the base and the apex of leaf lamina (Figure 1b). The perpendicularity of the measurement line to the midrib was controlled by instrumentation. In common bean, we measured the distances from the centre of rachis to the apices of lateral leaflets (Figure 1c). All leaves were measured twice by different persons (Data S2). When the two measurements differed by 20 or more pixels (ca. 3% of all leaves), a third measurement was conducted blindly to exclude an occasional error.

2.3. Data Analysis

All analyses were conducted separately for each plant species. Following the protocol suggested by Palmer and Strobeck [28], we used mixed-model ANOVA, with the leaf side (right or left) considered as a fixed factor and the individual plant as a random factor, to check whether our data show the presence of FA and/or directional asymmetry (DA) in leaf shape. We quantified the reproducibility of the measurements by the index $ME5 = (MSi - MSm)/(MSi + MSm)$, where MSi and MSm are the interaction and error mean squares from a side × individual ANOVA for two measurements of each side in each leaf [28]. This index expresses FA variation as a proportion of the total variation between sides, which includes variations due to both FA and measurement error. Additionally, we estimated an average difference between pairs of measurements on one leaf side, $ME1 = \sum abs(M1 - M2)/n$, where M1 and M2 are the independent measurements, and n is the total number of paired measurements [28].

The FA values were calculated as follows: $FA = 2 \times abs(WL - WR)/(WL + WR)$, where WL and WR refer to the measurements of the left and right halves of the same leaf. The use of this size-corrected index is justified by the significant correlation between the absolute difference in the left and right trait measurements and an average trait size (data not shown). The two FA values, based on independent measurements, were averaged for a leaf-specific value. Only at this stage, blind leaf measurements were associated with experimental treatments.

Among treatment variation in plant biomass, the average size of three uppermost leaves and leaf FA (averaged across three leaves for plant-specific values and square-root transformed to achieve the normality of residuals) were explored by ANOVA (SAS GLIM-MIX procedure, type 3 sum of squares). We preferred averaging leaf size and FA from three

measured leaves to the use of leaf-specific data nested within plants to allow direct comparison of these analyses with the analyses of plant biomass, which are based on plant-specific values. Pairwise differences between treatments were revealed by Duncan's Multiple Range Test ($\alpha = 0.05$) [29]. The responses of plant biomass and leaf FA to experimental treatments were compared by mixed-model ANOVA. In this model, the measured trait and treatment were treated as fixed effects, whereas plant individual was treated as a random intercept effect. We adjusted the standard errors and denominator degrees of freedom following [30].

3. Results

Leaf size of cucumber did not vary among treatments (Figure 1d). Application of copper, alone, did not affect leaf size of either sweet paper or common bean; application of nickel, alone, significantly reduced leaf size in sweet pepper only; simultaneous application of copper and nickel significantly reduced leaf size in both sweet pepper and common bean (Figure 1e,f).

Plant biomass varied significantly among treatments (Figure 2a–c). Application of copper, alone, did not affect biomass of any study species; application of nickel, alone, reduced biomass in common bean only; simultaneous application of copper and nickel reduced biomass in all study species (Figure 2a–c).
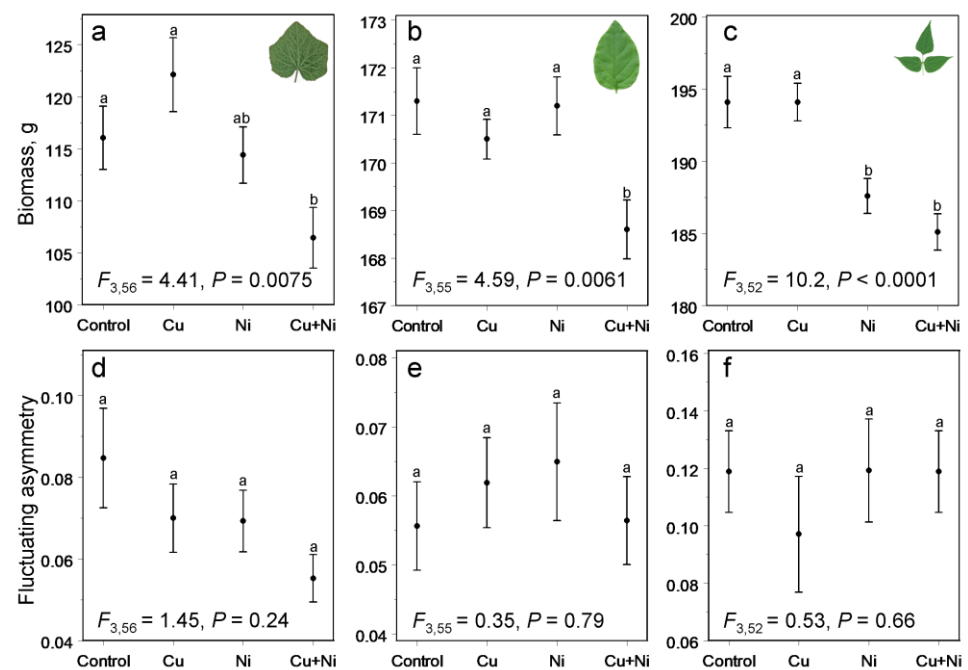

Figure 2. Variation among treatments (mean $\pm$ SE, each based on 15 plants, and F/P values) in (**a–c**) plant biomass and (**d–f**) leaf fluctuating asymmetry of (**a,d**) cucumber (*Cucumis sativus*), (**b,e**) sweet pepper (*Capsicum annuum*), and (**c,f**) common bean (*Phaseolus vulgaris*). Means marked by different letters differ significantly from each other (Duncan's Multiple Range Test, $\alpha = 0.05$).

The analysis of two independent blind measurements revealed the existence of FA in the leaf width in all study species (the side $\times$ individual interaction in Table 1), as well as our ability to precisely quantify this FA using measurements of the given accuracy (repeatability in Table 1). None of the study species demonstrated significant DA in leaf width (the effect of side in Table 1).

Table 1. Basic statistics on repeated blind measurements of the left and right leaf halves of experimental plants.

| Species | DA [1], mm | Source of Variation | | | | | | Reproducibility | |
| | | Side | | | Side × Individual | | | ME1, mm | ME5 |
		d.f.	F	P	d.f.	F	P		
Cucumis sativus	−0.56	1, 178	2.02	0.16	178, 358	147.1	<0.0001	0.50	0.986
Capsicum annuum	−0.08	1, 176	0.57	0.45	176, 354	163.2	<0.0001	0.12	0.988
Phaseolus vulgaris	1.19	1, 164	1.31	0.25	164, 330	1143.1	<0.0001	0.40	0.998

[1] DA, average directional asymmetry.

Leaf FA, in contrast to plant biomass, did not differ statistically among treatments in any study species (Figure 2d–f). In other words, leaf FA in plants stressed by an application of metals (as evident from the decrease in their growth: Figure 2a–c) did not differ from leaf FA in control plants. This difference in responses of plant biomass and leaf FA to experimental treatments was confirmed by mixed-model ANOVA (the trait × treatment interaction; cucumber: $F_{3, 91.1} = 4.67$, $p = 0.0044$; sweet pepper: $F_{3, 93.1} = 4.57$, $p = 0.0050$; common bean: $F_{3, 85.4} = 9.84$, $p < 0.0001$).

4. Discussion

4.1. Does Decrease in Plant Biomass Indicate Plant Stress?

Grime [25] defined plant stress as an external constraint reducing the rate of dry matter production. In our experiment, all plants were sown and harvested in the same dates. Therefore, plant biomass is indicative of plant growth rate and, consequently, of the level of toxic stress caused by application of heavy metals.

Interpretation of growth retardation as a sign of plant stress was questioned by Graham et al. [31]. These authors stated that an optimum size for reproduction in plants is usually smaller under stressful conditions, and therefore, "it is difficult to say with certainty whether smaller size indicates stress, or is simply a way of avoiding stress" ([31], p. 503). Unfortunately, these researchers did not suggest a stress measure other than FA, and they used circular reasoning while discussing relationships between stress, growth, and FA.

We doubt that the decrease in growth rate can increase plant tolerance to heavy metals. A more likely explanation is that, upon sensing toxic stress, plants inhibit protein synthesis, which accounts for a significant part of the energy required for plant growth [32]. By sacrificing growth, plants maximize protective stress responses at the molecular level to ensure survival [33,34]. We, therefore, conclude that plants demonstrating growth impairment in response to the application of heavy metals were stressed.

4.2. Fluctuating Asymmetry and Stress

The first papers reporting increased FA in different organisms collected from disturbed habitats were immediately followed by suggestions to use FA as an index of stress in environmental and conservation projects [21,35–40]. These recommendations, which were produced while ignoring disconfirming findings, are still advertised [4] and uncritically followed by many researchers [9].

Our study adds to the growing body of evidence questioning a consistent causal link between environmental stress and FA. Long ago, Wiener and Rago [41] concluded that "fluctuating asymmetry in adult fishes was . . . insensitive as a potential measure of pH-related stress". At the same time, methodological and interpretive concerns were expressed regarding some studies that have supported developmental instability theory [42]. In controlled experiments, leaf FA of bladder campion (*Silene vulgaris*) stressed by drought, copper, simulated herbivory, nutrient deficiency, and shade was not generally higher than in control plants [17]. Similarly, leaf FA of mountain birch (*Betula pubescens* var. *pumila*) did not increase in plants exposed to heavy metals, drought, and herbivory [18,19]. Leaf FA of common bean was not affected by the application of either lead or cadmium [14]. Further

examples of negative or inconclusive findings can be found in several reviews addressing different aspects of FA-related studies [9,12,31,43,44].

Surprisingly, the obvious inconsistency in outcomes of previous studies did not prevent the growing use of FA in bioindication [9]. In particular, Graham et al. ([31], p. 502), despite agreeing that "fluctuating asymmetry can be a poor indicator of both stress and individual fitness", still concluded that the disconfirming evidence does not invalidate FA as an indicator of stress. We disagree with this conclusion because, so far, no theoretical model is available to predict whether the FA in a particular trait of a given species will change in response to a specific environmental stressor [10]. Consequently, a blind belief that FA is a universal and sensitive indicator of stress imposes a risk of erroneous scientific conclusions and of incorrect management decisions.

4.3. In Asymmetry We Trust? Methodological Implications

Researchers in ecology, evolutionary biology, and environmental sciences generally consider data as "hard facts" that form the objective basis of our knowledge, despite demonstrations that data production, digestion, and interpretation are strongly influenced by theories [5,45]. There are two lines of evidence suggesting that established theories can promote themselves by affecting research data. First, unconscious confirmation bias, defined as the tendency of humans to seek out evidence in a manner that confirms their beliefs and hypotheses [46], compromises data objectivity by making them more consistent with a prevailing theory than they actually are [47]. Second, publication bias, acting in different ways, from the author's decision not to publish disconfirming evidence to a more frequent rejection of manuscripts reporting this kind of evidence, facilitates a preferential accumulation of data that are in agreement with an established theory [48,49]. As a result, the theory obtains increasingly greater support in a positive feedback fashion and, thus, increases its impact on subsequent data collection.

A previous study demonstrated that, when scientists expected to find high FA in some samples, their non-blind measurements confirmed their expectations, whereas blind measurements did not reproduce this result [50]. Despite this discovery, studies based on non-blind measurements continue to dominate this research field because the confirmatory evidence has long been, and still is, published without thorough examination of the methodology used to arrive at the "positive" results [7,11].

The insufficient methodological quality of many studies, exploring different aspects of FA in living beings, compromises this research field. Unfortunately, the analysis of published studies does not allow separation of the solid evidence from evidence distorted by confirmation bias. However, as long as the problem of confirmation bias is spotted, and scientists are informed about its unconscious origin, they can (and should!) apply necessary precautions to assure the quality of their data and reliability of their conclusions. To facilitate this process, we repeat and develop the previously published recommendations addressed to the authors and reviewers of studies involving FA measurements [7,9,11].

1. The samples for FA measurements should be collected either randomly (in the strict statistical sense because haphazard selection is prone of confirmation bias [47]) or blindly with respect to the expected result. In other words, researchers should take precautions against the (unconscious) selection of objects that best fit their expectations.
2. The measurements of FA should be conducted blindly: the measurer should not be aware of the object's origin and/or of the hypothesis being tested. This is easily achieved by the coding of study objects with random numbers prior to measurements.
3. At least a portion of the objects should be measured twice, and the FA should be tested against the measurement error.
4. We recommend that, whenever possible, not only the results of all measurements but also the measured objects (or their images: see our Data S1) are made openly available for reuse in accordance with the FAIR principles [51].

5. We encourage scientists to publish their results obtained using adequate methodology even when these results are "negative" or inconclusive. This is the only way to make the publication portfolio representative of the actual findings.

5. Conclusions

Long ago, Fagerström [5] noted that "many ecological theories are in fact retained—and rightly so—although they are demonstrably wrong". Our study, once again, showed that this statement is valid for the developmental instability theory, at least for its applications in environmental studies. We found that experimental treatments causing stress (*sensu* Grime [25]) in our plants did not induce a predicted increase in their FA. This finding demonstrated, once again, that—contrary to the widespread opinion—FA does not constitute a universal indicator of environmental stress. Even if future experiments, e.g., those imposing greater stress, exposing plants to stress at earlier developmental stages, or measuring FA of organs other than leaves, will discover the expected increase in FA in our study species, our current findings will preclude classifying this effect as universal. Therefore, we suggest that the use of FA as an index of stress is discontinued until the scope of the developmental instability theory is clarified and its applicability limits are identified. To achieve this goal, the methodology of FA-related studies needs to be improved substantially, and these studies should pay greater attention to identifications of conditions, under which FA of living beings correlates with some biological pattern or process.

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