



# Leaf size is more sensitive than leaf fluctuating asymmetry as an indicator of plant stress caused by simulated herbivory

Mikhail V. Kozlov<sup>\*</sup>, Vitali Zverev, Elena L. Zvereva

Department of Biology, University of Turku, 20014 Turku, Finland

## ARTICLE INFO

### Keywords:

Blind data collection  
*Betula pubescens*  
 Fluctuating asymmetry  
 Leaf size  
 Simulated herbivory  
 Environmental stress

## ABSTRACT

Fluctuating asymmetry (FA), defined as small, random departures away from perfect symmetry, is frequently recommended as a sensitive and universal indicator of environmental stress imposed by both abiotic and biotic factors. However, the stress sensitivities of FA and of other morphological traits are rarely compared directly. Here, we tested the hypothesis that leaf FA, rather than leaf size, is more sensitive to simulated herbivory. We performed blind measurements of FA and length of the same leaves of juvenile mountain birches (*Betula pubescens* var. *pumila*) after removal of 0, 2, 4, 8 and 16% of their leaf area during five consequent years. The leaf length in defoliated birches was 88% of that in control birches, indicating that simulated herbivory was stressful for trees. By contrast, leaf FA did not differ significantly between defoliated and control birches, despite a sufficient (91%) power of statistical analysis. Thus, leaf size, rather than leaf FA, was a more sensitive indicator of stress. This low sensitivity of FA to stress, discovered with the use of blind methods, contrasts a large pool of earlier studies, many of which have been likely influenced by confirmation bias. We urge the publication of 'negative' or inconclusive results which currently remain underreported due to publication bias.

## 1. Introduction

Grime (1979) defined plant stress as the external constraints which limit the rate of dry matter production of all or parts of the vegetation. Consequently, any measure of growth (e.g. leaf size, plant height or biomass) can serve as an indicator of plant stress, at least in trees. In addition to these indices, fluctuating asymmetry (FA; i.e. small, random departures away from perfect symmetry) has frequently been recommended as a sensitive and universal indicator of environmental stress imposed on both plants and animals by both abiotic and biotic factors (Valentine and Soulé, 1973; Leary and Allendorf, 1989; Parsons, 1990; Zakharov and Clarke, 1993; Manning and Chamberlain, 1994; Kozlov et al., 2002). However, the rare comparisons of the sensitivity of FA to environmental stress with the sensitivities of other morphological characters, such as size and biomass, have reported a greater sensitivity to stress for growth than for FA (Black-Samuelsson and Andersson, 2003; Fair and Breshears, 2005; Francis and Gilman, 2019). Nevertheless, FA remains widely used as an index of environmental quality (Guo et al., 2017; Klisarić et al., 2019; García-Jain et al., 2022), despite the advantages of FA as an index of stress over the measures of plant growth are not always evident (Zverev and Kozlov, 2020).

Plants damaged by herbivores often demonstrate reduced growth relative to their undamaged neighbours (Nykänen and Koricheva, 2004; Zvereva et al., 2010; Zvereva et al., 2012; Zvereva and Kozlov, 2012); therefore, herbivory fits the definition of a stressor introduced by Grime (1979). Consequently, herbivory has been repeatedly predicted to increase plant FA, but only a handful of studies have tested this prediction experimentally. The outcomes of these experiments are inconsistent, as several studies discovered the expected pattern (Zvereva et al., 1997; Olofsson and Strebom, 2003; Zvereva and Kozlov, 2001; Kozlov et al., 2001; Rautio et al., 2002; Puerta-Piñero et al., 2008) whereas other studies did not detect a statistically significant effect of herbivory on FA. Importantly, Sandner and Matthies (2017) and Shestakov et al. (2020) reported significant effects of herbivory on plant growth but not on FA, whereas Lappalainen et al. (2000) and Gijssman et al. (2021) did not detect any effect of herbivory on either growth or FA. Furthermore, Alados et al. (2002) found a non-linear response of plant FA to grazing, with the lowest FA in the medium-grazed population. Thus, not only the higher stress sensitivity of FA relative to other traits, but even the overall suitability of FA as an index of herbivory-related stress, remains questionable.

The ultimate goal of the present study was to test the hypothesis that

<sup>\*</sup> Corresponding author.

E-mail address: [mikoz@utu.fi](mailto:mikoz@utu.fi) (M.V. Kozlov).

leaf FA, rather than leaf size, is more sensitive to stress caused by long-term simulated insect herbivory in mountain birch (*Betula pubescens* var. *pubmila*). We also asked whether defoliation effects were dose-dependent and whether leaf size (which was predicted to decrease with herbivory) and leaf FA (which was predicted to increase with herbivory) were negatively correlated to each other.

## 2. Materials and methods

### 2.1. Experimental design

The mountain birch, the northern variety of downy birch (*B. pubescens*), is a keystone species in the boreal forests of Eurasia. Due to its ecological and environmental importance, this species has served as a model object for multiple studies of insect herbivory impacts on tree growth and physiology and on the structure and functions of birch-dominated ecosystems (Ruohomäki et al., 1997; Zvereva et al., 2012; Ryde et al., 2021; Meyer et al., 2022; and references therein).

The present experiment was conducted at two northern taiga forest sites located near Apatity (67°36'31" N, 32°38'38" E and 67°32'10" N, 33°56'55" E) in the Murmansk region of Russia. Sixty naturally growing juvenile mountain birches (16–60 cm high) were tagged at each site in June 2005 and randomly assigned to undergo removal of 0, 2, 4, 8 or 16% of their leaf area, implemented annually by punching small (2 to 4.5 mm in diameter) holes in the leaf lamina to mimic insect herbivory. The first half of the damage was applied in early summer (22 June to 12 July) and the second half during the late summer (24 July to 15 August). In our study area, insects usually consume ca. 1% of the birch tree leaf area (Kozlov, 2008); therefore, even our smallest treatment significantly exceeded the regional level of background insect herbivory. For more details, consult Zvereva et al. (2012).

### 2.2. Data collection

Two leaves were haphazardly collected of each of 60 saplings on 29 June – 1 July 2010 (i.e. after five years of defoliation treatments). We randomly selected these 60 saplings from 91 birches which had more than 30 leaves by that date. The person who collected the leaves was not aware of either the hypothesis being tested or the treatments that had been applied to the designated trees; this person was instructed to collect two undamaged leaves of average size from short vegetative shoots of each selected birch. The leaves were press-dried and then mounted on strong paper (Images S1–S4 in supplementary materials).

We used a ruler to measure (to the nearest 0.5 mm) the length of the lamina and the width of its left and right sides from the midrib to the leaf margins (at the midpoint between the base and the tip) perpendicular to the midrib. Each leaf was measured twice by different persons who had no knowledge of either the hypothesis being tested or the leaf origin. When the two measurements differed by 2 or more mm, a third measurement was conducted by a third person to exclude an occasional error. We preferred to make the measurements by ruler rather than to analyse landmark positions because the latter method was previously demonstrated to yield the same conclusions as measurements of leaf half-width (Sandner et al., 2019).

### 2.3. Data analysis

We quantified FA as follows:  $FA = 2 \times \text{abs}(WL - WR)/(WL + WR)$ , where WL and WR are the widths of the left and right halves of leaf lamina. We explored the repeated measurements of the width of leaf halves for the presence of FA and directional asymmetry ( $DA = WL - WR$ ) using mixed-model ANOVA, with the leaf side (right or left) as a fixed factor and the individual leaf as a random factor. We compared the DA value with the FA4a index ( $FA4a = 0.798 \sqrt{\text{var}(WR - WL)}$ ) to assess the potential effect of DA on the analysis of FA, and we assessed

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  $\times$  individual ANOVA for two measurements of WL and WR in each leaf (Palmer and Strobeck, 2003). Finally, we averaged the two measurements of the same leaf to minimize the additive error, and we used SAS UNIVARIATE procedure (SAS SAS Institute, 2009) to test for normality of the distribution of the signed difference in width between left and right leaf halves and to calculate kurtosis of this distribution.

Prior to the analyses of the effect of herbivory on leaf size and FA, we square root-transformed the FA values to normalise the distribution of the residuals and we averaged the leaf length and FA for tree-specific values. We explored the among-treatment variations in these values by one-way ANOVA (SAS GLIMMIX procedure, type 3 tests; SAS SAS Institute, 2009), and we calculated Pearson product-moment correlation coefficients between leaf length and FA across leaves and across trees. The post hoc statistical power of ANOVA was calculated using the online tool at <https://clincalc.com/stats/Power.aspx>.

## 3. Results

We detected the presence of both FA (side  $\times$  individual interaction:  $F_{119, 240} = 3.31, P < 0.0001$ ) and DA (side effect:  $F_{1, 119} = 6.63, P = 0.01$ ) in the width of birch leaves (Data S1 and Images S1–S4 in supplementary materials). Although the DA was significant, its value (0.25 mm) was much smaller than the FA4a index (0.84 mm), suggesting that the contribution of the DA to the total variation has little effect on the analysis of FA. The distribution of the signed difference in width between left and right leaf halves did not differ significantly from a normal one (Shapiro-Wilk statistics = 0.98,  $P = 0.06$ ). Combined with low kurtosis (0.015), this result suggests absence of antisymmetry. The repeatability of our measurements of the widths of leaf halves (0.537) was sufficiently high.

The leaf length in birches that were repeatedly defoliated over five consecutive years was reduced to 88% of the length in the control birches (Fig. 1a;  $F_{1, 58} = 6.28, P = 0.0150$ ). However, this effect did not show any dose dependence: leaf length did not differ either among the four defoliation treatments (Fig. 1b;  $F_{3, 46} = 0.71, P = 0.55$ ) or between the low (2 and

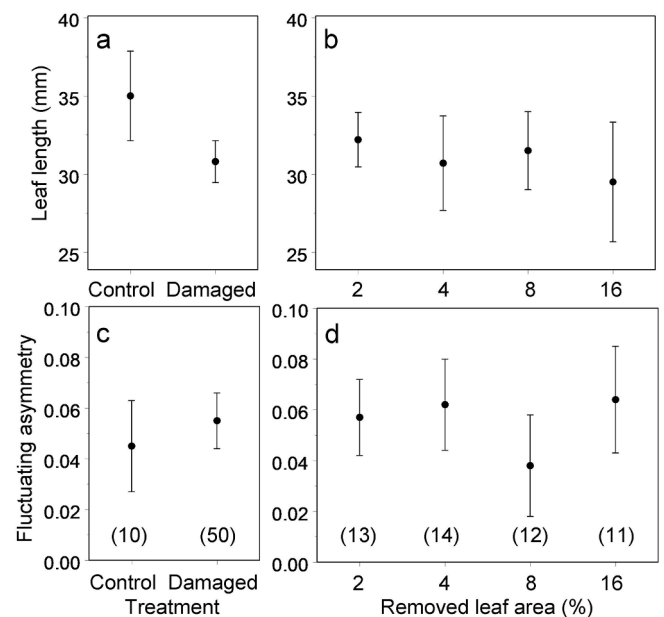


Fig. 1. Effects of simulated herbivory (means and 95% confidence intervals; sample sizes in parentheses) on leaf length (a, b) and the back-transformed values of leaf fluctuating asymmetry (c, d): a, c – contrast between control and damaged trees; b, d – variation among defoliation levels.

4%) and high (8 and 16%) defoliation levels ( $F_{1, 48} = 0.82, P = 0.37$ ).

The FA in birch leaves did not respond to simulated herbivory (Fig. 1c;  $F_{1, 58} = 0.78, P = 0.38$ ); the statistical power of this test was 91%. Similarly, leaf FA did not differ either among the four treatments (Fig. 1d;  $F_{3, 46} = 1.55, P = 0.22$ ) or between low and high defoliation levels ( $F_{1, 48} = 1.05, P = 0.31$ ). FA was independent of leaf length at the levels of both an individual leaf ( $r = -0.10, n = 120$  leaves,  $P = 0.28$ ) and the whole plant ( $r = -0.09, n = 60$  trees,  $P = 0.34$ ).

#### 4. Discussion

Following five years of simulated herbivory, the defoliated birch saplings produced significantly smaller leaves compared to the control saplings. This result, which parallels the observed adverse effects of simulated herbivory on multiple performance indices of our experimental trees, and particularly long shoot growth and number of leaves (described by Zvereva et al., 2012), clearly indicates that the annual removal of 2–16% of the leaf area imposed stress on birches. Nevertheless, the FA measured from the same leaves did not show a statistically significant response to this stress. Importantly, the statistical power of our FA analysis exceeded 80%, i.e. it was sufficient to detect an effect of herbivory on FA if that effect did exist (Cohen, 1988). Thus, our results indicate that leaf size, rather than leaf FA, is a more sensitive indicator of stress caused by simulated herbivory. Keeping in mind that the measurements of leaf size are less laborious and more accurate than the measurements of FA (Zverev and Kozlov, 2020), our results question the advantages of using FA as a stress index over the use of plant growth measurements.

The dominance of confirmatory evidence among the published studies addressing the effect of different stressors on plant FA (Graham et al., 2010; Kozlov, 2017) could be caused by confirmation bias, i.e. the tendency of humans to seek out and interpret information in a way that confirms their pre-existing views and beliefs (Rosenthal, 1976). For example, when scientists believed that birch leaves offered to them for FA measurements had originated from a heavily polluted site, they reported significantly higher values of FA than did scientists who believed that the same leaves had been collected from an unpolluted site (Kozlov and Zvereva, 2015). The current study differs from a vast majority of previously published studies (including our own studies published in 1990s–2000s) in the blind procedure adopted for leaf measurements. The applied protocol, which did not inform the measurers of either the leaf origin or the hypothesis being tested, excluded the impact of confirmation bias on our data. We support the suggestion made by Graham (2021) that all subsequent studies of FA should involve blinding at the sample collection and processing stages. We also repeat our previous suggestion (Zverev and Kozlov, 2021) that researchers exploring FA publish their ‘negative’ and inconclusive results when these are obtained using adequate methodology. Publication of these results, which are currently buried in laboratory drawers (like the results we present above), is the only way to mitigate the impact of publication bias on this research field and to make the publication portfolio more representative of the actual findings.

An accurate estimation of FA requires the use of labour-intensive and high-precision measurements, as well as sophisticated statistical methods (Lajus et al., 2009; Graham, 2021). Furthermore, support for the existence of a causal link between environmental stress and an increase in FA is mixed (Graham et al., 2010; Kozlov, 2017; Sandner and Matthies, 2017; Vandenbussche et al., 2018). We therefore urge researchers who are planning to use FA as an index of stress impact on living beings to consider alternative indices, as the alternatives may provide higher accuracy regarding the impact of a potentially stressful agent on the study organisms and/or may require fewer samples and/or measurements.

#### CRediT authorship contribution statement

**Mikhail V. Kozlov:** Conceptualization, Methodology, Formal analysis, Writing – original draft. **Vitali Zverev:** Methodology, Investigation, Visualization, Writing – review & editing. **Elena L. Zvereva:** Conceptualization, Investigation, Writing – review & editing.

#### Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

#### Acknowledgements

We thank M. Inozemtseva and A. Vassiliev for fieldwork and laboratory assistance. The study was supported by the Academy of Finland (projects 122133 and 316182).

#### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ecolind.2022.108970>.

#### References

- Black-Samuelsson, S., Andersson, S., 2003. The effect of nutrient stress on developmental instability in leaves of *Acer platanoides* (Aceraceae) and *Betula pendula* (Betulaceae). *Am. J. Bot.* 90 (8), 1107–1112.
- Alados, C.L., Giner, M.L., Dehesa, L., Escós, J., Barroso, F.G., Emlen, J.M., Freeman, D.C., 2002. Developmental instability and fitness in *Periploca laevigata* experiencing grazing disturbance. *Int. J. Plant Sci.* 163 (6), 969–978.
- Cohen, J., 1988. *Statistical power analysis for the behavioral sciences*, 2nd ed. Lawrence Erlbaum, New Jersey.
- Fair, J.M., Breshears, D.D., 2005. Drought stress and fluctuating asymmetry in *Quercus undulata* leaves: confounding effects of absolute and relative amounts of stress? *J. Arid Environ.* 62 (2), 235–249.
- Francis, B., Gilman, R.T., 2019. Light intensity affects leaf morphology in a wild population of *Adenostyles alliariae* (Asteraceae). *Italian Botanist* 8, 35–45.
- García-Jain, S.E., Maldonado-López, Y., Oyama, K., Fagundes, M., de Faria, M.L., Espírito-Santo, M.M., Cuevas-Reyes, P., 2022. Effects of forest fragmentation on plant quality, leaf morphology and herbivory of *Quercus deserticola*: is fluctuating asymmetry a good indicator of environmental stress? *Trees* 36, 553–567.
- Gijssman, F., González, Y., Guevara, M., Amador-Vargas, S., 2021. Short-term plasticity and variation in acacia ant-rewards under different conditions of ant occupancy and herbivory. *Sci. Nat.* 108, 31.
- Graham, J.H., 2021. Fluctuating asymmetry and developmental instability, a guide to best practice. *Symmetry* 13, 9.
- Graham, J.H., Raz, S., Hel-Or, H., Nevo, E., 2010. Fluctuating asymmetry: methods, theory, and applications. *Symmetry* 2, 466–540.
- Grime, J.P., 1979. *Plant strategies and vegetation processes*. J Wiley and Sons, Chichester.
- Guo, R., Zhang, W.Y., Ai, S.W., Ren, L., Zhang, Y.M., 2017. Fluctuating asymmetry rather than oxidative stress in *Bufo raddei* can be an accurate indicator of environmental pollution induced by heavy metals. *Environ. Monitor. Assessm.* 189, 293.
- Klisarić, N.B., Miljković, D., Avramov, S., Zivković, U., Tarasjev, A., 2019. Radial and bilateral fluctuating asymmetry of *Iris pumila* flowers as indicators of environmental stress. *Symmetry* 11, 818.
- Kozlov, M.V., 2008. Losses of birch foliage due to insect herbivory along geographical gradients in Europe: a climate-driven pattern? *Clim. Change* 87 (1–2), 107–117.
- Kozlov, M.V., 2017. Plant studies on fluctuating asymmetry in Russia: mythology and methodology. *Russ. J. Ecol.* 48 (1), 1–9.
- Kozlov, M.V., Zvereva, E.L., 2015. Confirmation bias in studies of fluctuating asymmetry. *Ecol. Ind.* 57, 293–297.
- Kozlov, M.V., Zvereva, E.L., Niemelä, P., 2001. Shoot fluctuating asymmetry: a new and objective stress index in Norway spruce (*Picea abies*). *Can. J. For. Res.* 31 (7), 1289–1291.
- Kozlov, M.V., Niemelä, P., Junttila, J., 2002. Needle fluctuating asymmetry is a sensitive indicator of pollution impact on Scots pine. *Ecol. Indic.* 1, 271–277.
- Lappalainen, J., Martel, J., Lempa, K., Wilsey, B., Ossipov, V., 2000. Effects of resource availability on carbon allocation and developmental instability in cloned birch seedlings. *Int. J. Plant Sci.* 161 (1), 119–125.
- Lajus, D.L., Grem, D.K., Katolikova, M.V., Turtseva, A.O., 2009. Fluctuating asymmetry and random phenotypic variation in population research: history, achievements, problems, and prospects. *Vestn. St. Peterb. Gos. Univ. Ser. Biol.* 3, 98–110 (in Russian).
- Leary, R.F., Allendorf, F.W., 1989. Fluctuating asymmetry as an indicator of stress: implications for conservation biology. *Trends Ecol. Evol.* 4 (7), 214–217.

- Manning, J.T., Chamberlain, A.T., 1994. Fluctuating asymmetry in gorilla canines: A sensitive indicator of environmental stress. *Proc. R Soc. Lond. B* 255, 189–193.
- Meyer, N., Xu, Y., Karjalainen, K., Adamczyk, S., Biasi, C., van Delden, L., Martin, A., Mganga, K., Myller, K., Sietiö, O.-M., Suominen, O., Karhu, K., 2022. Living, dead, and absent trees: how do moth outbreaks shape small-scale patterns of soil organic matter stocks and dynamics at the Subarctic mountain birch treeline? *Glob. Change Biol.* 28, 441–462.
- Nykänen, H., Koricheva, J., 2004. Damage-induced changes in woody plants and their effects on insect herbivore performance: a meta-analysis. *Oikos* 104, 247–268.
- Olofsson, J., Strengbom, J., 2003. Response of galling invertebrates on *Salix lanata* to reindeer herbivory. *Oikos* 91, 493–498.
- Palmer, A.R., Strobeck, C., 2003. Fluctuating asymmetry analyses revisited. In: Polak, M. (Ed.), *Developmental instability: causes and consequences*. Oxford University Press, New York, pp. 279–319.
- Parsons, P.A., 1990. Fluctuating asymmetry and stress intensity. *Trend Ecol. Evol.* 5 (3), 97–98.
- Puerta-Piñero, C., Gómez, J.M., Hódar, J.A., 2008. Shade and herbivory induce fluctuating asymmetry in a mediterranean oak. *Int. J. Plant Sci.* 169, 631–635.
- Rautio, P., Markkola, A., Martel, J., Tuomi, J., Härmä, E., Kuikka, K., Siitonen, A., Riesco, L.L., Roitto, M., 2002. Developmental plasticity in birch leaves: defoliation causes a shift from glandular to nonglandular trichomes. *Oikos* 98 (3), 437–446.
- Rosenthal, R., 1976. *Experimenter effects in behavioral research*. Irvington Publishers, New York.
- Ruohomäki, K., Haukioja, E., Repka, S., Lehtilä, K., 1997. Leaf value: effects of damage to individual leaves on growth and reproduction of mountain birch shoots. *Ecology* 78 (7), 2105–2117.
- Ryde, I., Li, T., Rieksta, J., dos Santos, B.M., Neilson, E.H.J., Gericke, O., Jepsen, J.U., Bork, L.R.H., Holm, H.S., Rinnan, R., 2021. Seasonal and elevational variability in the induction of specialized compounds from mountain birch (*Betula pubescens* var. *pumila*) by winter moth larvae (*Operophtera brumata*). *Tree Physiol.* 41, 1019–1033.
- Sandner, T.M., Matthies, D., 2017. Fluctuating asymmetry of leaves is a poor indicator of environmental stress and genetic stress by inbreeding in *Silene vulgaris*. *Ecol. Ind.* 79, 247–253.
- Sandner, T., Zverev, V., Kozlov, M.V., 2019. Can the use of landmarks improve the suitability of fluctuating asymmetry in plant leaves as an indicator of stress? *Ecol. Ind.* 97, 457–465.
- SAS Institute, 2009. *SAS/Stat. User's guide, version 9.2*. SAS. Institute, Cary, NC.
- Shestakov, A.L., Filippov, B.Yu, Zubrii, N.A., Klemola, T., Zezin, I., Zverev, V., Zvereva, E.L., Kozlov, M.V., 2020. Doubling of biomass production in European boreal forest trees by a four-year suppression of background insect herbivory. *For. Ecol. Manage.* 462.
- Valentine, D.W., Soulé, M., 1973. Effects of p, p'-DDT on developmental stability of pectoral fin rays in the grunion *Leuresthes tenuis*. *Fishery Bull.* 7, 921–926.
- Vandenbussche, P.S.P., Spennato, G., Pierson, P.M., 2018. Assessment of the use of *Oblada melanura* (L. 1758) otolith fluctuating asymmetry as environmental disturbance indicator. *Marine Environ. Res.* 136, 48–53.
- Zakharov, V.M., Clarke, G.M., 1993. *Biotest: an integral assessment of the state of ecosystems and individual species*. Moscow, Biotest (in Russian).
- Zverev, V., Kozlov, M.V., 2020. Variation in leaf size and fluctuating asymmetry of mountain birch (*Betula pubescens* var. *pumila*) in space and time: implications for global change research. *Symmetry* 12 (10), 1703.
- Zverev, V., Kozlov, M.V., 2021. The fluctuating asymmetry of the butterfly wing pattern does not change along an industrial pollution gradient. *Symmetry* 13, 626.
- Zvereva, E.L., Kozlov, M.V., 2001. Effects of pollution induced habitat disturbance on willow response to simulated herbivory. *J. Ecol.* 89, 21–30.
- Zvereva, E.L., Kozlov, M.V., 2012. Sources of variation in plant responses to belowground insect herbivory: a meta-analysis. *Oecologia* 169 (2), 441–452.
- Zvereva, E.L., Kozlov, M.V., Niemelä, P., Haukioja, E., 1997. Delayed induced resistance and increase in leaf fluctuating asymmetry as responses of *Salix borealis* to insect herbivory. *Oecologia* 109 (3), 368–373.
- Zvereva, E.L., Lanta, V., Kozlov, M.V., 2010. Effects of sap-feeding insect herbivores on fitness of woody plants: a meta-analysis of experimental studies. *Oecologia* 163, 949–960.
- Zvereva, E.L., Zverev, V., Kozlov, M.V., 2012. Little strokes fell great oaks: minor but chronic herbivory substantially reduces birch growth. *Oikos* 121 (12), 2036–2043.