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Temperature and herbivory, but not pollution, affect fluctuating asymmetry of mountain birch leaves: results of 25-year monitoring around the copper-nickel smelter in Monchegorsk, northwestern Russia

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Abstract

Fluctuating asymmetry (FA), which is defined as the magnitude of the random deviations from a symmetrical shape, reflects developmental instability and is commonly assumed to increase under environmental and genetic stress. We monitored the leaf FA of mountain birch, *Betula pubescens* subsp. *czerepanovii*, from 1993–2017 in individually marked trees at 21 sites around the copper-nickel smelter at Monchegorsk, and we then analysed the results with respect to spatial and temporal variation in pollution, climate and background insect herbivory. Responses of leaf FA to different stressors were stressor specific: FA did not correlate with pollution load, it decreased significantly with an increase in June air temperature and it increased slightly but significantly with an increase in the previous-year leaf damage due to defoliating and leafmining insects. Our findings suggest that climate warming is unlikely to impose stress on the explored mountain birch populations, but even small increases in insect herbivory may adversely affect birch trees. However, these conclusions, since they are based on an observational study, should be viewed as tentative until confirmed by controlled experiments. We also demonstrated that the use of non-blinded measurements, which are prone to confirmation bias, was the primary reason for the earlier report of an increase in birch leaf FA near the Monchegorsk smelter. We hope that our findings will promote a wide use of blinded methods in ecological research and that they will contribute to debunking the myth that plant leaf FA consistently increases with increases in environmental pollution.

Keywords: background insect herbivory, climate warming, confirmation bias, developmental stability, ecological indicators, industrial pollution.

1. Introduction

Much of our knowledge about the state of ecological systems and their responses to various disturbances has originated from ecological monitoring. The development of the concept of forest monitoring started in 1970s, in particular due to concerns about potential transboundary pollution effects on forests, which gave the rise to several national and international monitoring programs (Ferretti, 2013). The importance of monitoring disturbances in ecosystem structure and functions is receiving increasing attention, driven by the current intensification of research into natural disturbances due to climate change (Thom and Seidl, 2016) and the emphasis on worldwide recovery of ecosystems from anthropogenic disturbances (Moreno-Mateos et al., 2017).

The existing forest monitoring programs, as a first line of investigation, serve practical goals because ecological management requires timely prediction of emerging environmental problems. This requirement gives special value to long-term data collected on ecological indicators (i.e. on those characteristics of organisms or ecosystems that could be used as early warning systems to signal imminent but not yet apparent problems; Dale and Beyeler, 2001). One such indicator, introduced in the early 1990s, is fluctuating asymmetry (FA), which is defined as small, non-directional deviations from perfect symmetry in morphological characters of plants and animals. FA has long been advertised as a universal and easy to measure stress indicator (Zakharov, 1990; Clarke, 1992; Parsons, 1992; Graham et al., 1993; Freeman et al., 1993). However, to our knowledge, none of the existing forest monitoring programs involves FA measurements in plants. Consequently, long-term data on plant FA, collected from the same study sites and the same plant individuals, are virtually non-existent.

Long ago, one of us (MVK) suggested that the FA of birch leaves could serve as a convenient indicator for rapid assessment of environmental pollution impacts on plant

performance (Kozlov et al., 1996). Based on this hypothesis, in 1993, we established a local monitoring program in multiple sites around the copper-nickel smelter in Monchegorsk in northwestern Russia. However, the results of the first eight years (1993–2000) of monitoring of leaf FA of mountain birch, *Betula pubescens* subsp. *czerepanovii* (Orlova) Hämet-Ahti, surprisingly demonstrated no correlation between FA and heavy metal or sulphur dioxide concentrations around this smelter (Valkama and Kozlov, 2001). At the same time, the birch leaf FA appeared to increase with a decrease in ambient air temperature in June, the month when expansion of birch leaves occurs in our study region (Valkama and Kozlov, 2001). This observation was later supported by Hagen et al. (2008) who found higher leaf FA of mountain birch in northern Norway in the year with lower ambient air temperatures during the period of birch leaf growth.

Keeping in mind the substantial decline in emissions from the Monchegorsk smelter in 1990s and the rapid increase in early summer temperatures in 2000s (Zvereva et al., 2016), we continued to monitor birch leaf FA until 2017 in order to elucidate combined effects of pollution and climate on this potential indicator of environmental quality. Plant FA can also increase in response to herbivory (Zvereva et al., 1997a, b; Møller & Shykoff, 1999; Kozlov, 2005), which, in turn, can be influenced by pollution (Zvereva and Kozlov, 2010). Therefore, starting from 1999, we also measured the percentages of leaves damaged by defoliating and leafmining insects in our monitoring trees by the end of the growth season (Kozlov et al., 2017).

Earlier, we found a significant variation in FA among individual birches within our study plots (Valkama and Kozlov, 2001). This variation suggested that birch genets either responded differently to environmental stress or they demonstrated different degrees of genetic stress. Plant FA increases with hybridisation (Wilsey et al., 1998; Albarran-Lara et al., 2010), which makes the impact of birch genotype on environmentally induced changes in leaf

FA very likely, as birches often form interspecific hybrids (Anamthawat-Jonsson and Thorsson, 2003; Koropachinskii, 2013). In particular, mountain birch is thought to have originated through introgressive hybridisation between the southern *B. pubescens* Ehrh. and the northern *B. nana* L. (Elkington, 1968; Vaarama and Valanne, 1973).

During the past decade, the initial optimism regarding studies of FA disappeared due to steady accumulation of inconclusive and negative results. A large number of field observations (reviewed by Graham et al., 2010; Kozlov, 2017) and controlled experiments (Roy and Stanton, 1999; Andalo et al., 2000; Sandner and Matthies, 2017; Zverev et al., 2018) have failed to detect the expected effects of stress on FA. Nevertheless, scientific questions remain regarding how environmental and genetic factors influence FA (Graham et al., 2010; Klingenberg, 2015), and this gave paramount importance to our uninterrupted, 25-year-long, time series, with data mostly collected from the same plant individuals.

In the present study, we report the spatial and temporal variations in leaf FA of mountain birch around the Monchegorsk smelter, and we explore factors that could explain this variation. We hypothesised that leaf FA: 1) increases with decreasing distance from the smelter due to an increase in pollution load and/or pollution-induced habitat disturbance; 2) decreases with the decreasing annual emission of pollutants due to partial alleviation of pollution-induced stress; 3) decreases during the years with increased ambient air temperature in June due to partial alleviation of climatic stress at the time of leaf expansion; 4) increases with increasing previous-year insect herbivory; and 5) differs among individual trees within the study site, presumably due to different genotypes and/or different sensitivity of individual genotypes to pollution and climatic stress. We tested our hypotheses 1–5 by analysing leaf FA values of 24943 leaves collected during 1993–2017 from 195 mountain birches growing naturally at 21 sites located 1 to 64 km from the Monchegorsk smelter. We also: 6) suggested that imperfect research methodology, primarily the use of non-blinded

measurements, contributed to the earlier conclusion (Kozlov et al., 1996) regarding significant increases in leaf FA of mountain birch near Monchegorsk, and we tested this hypothesis by re-measuring 600 leaves that had served as the basis for that original conclusion.

2. Materials and methods

2.1. Study area and study sites

The monitoring was conducted in the central part of the Kola Peninsula, which is located in the north-west of Russia, next to Finland and Norway, to the north of the Polar Circle (Fig. 1). The nickel–copper smelter in the town of Monchegorsk (67°56' N, 32°49' E), about 150 km south of the tree line, was one of the largest industrial polluters in the Northern hemisphere for decades. The smelter began production in 1937–1938 and had no air-cleaning facilities until 1968. The annual emissions of sulphur dioxide reached a maximum of 278,000 metric tons (t) in 1983, steadily to about 100,000 t by the mid-1990s, dropped to 45,000 t in 1999 and have remained at about this level since then (Table S1). Metal emissions during the 1980s–1990s were 3000–8000 t of nickel and 1000–6000 t of copper annually and then declined in concert with declines in SO₂ (Table S1). For the history of pollution impacts on the study region and the levels of environmental degradation, consult Kozlov and Barcan (2000), Kozlov et al. (2009) and Manninen et al. (2015).

We monitored different characteristics of mature (over 20 years old) individuals of mountain birch growing naturally at 21 sites located in both pristine (unpolluted) subarctic Norway spruce (*Picea abies* (L.) Karst.) forests (Fig. S1) and in forests exhibiting different levels of pollution-induced deterioration (Fig. S2), including industrial barrens (Fig. S3), at distances 1 to 64 km from the smelter (Fig. 1; Table S2). The monitoring sites were selected

along the main roads to allow easy access, but at least 50 m from the roadside to minimise impacts of traffic pollution. In 1993, we established 20 sites and individually tagged five haphazardly selected mature birches per site; 29 of these 100 birches were monitored until 2017. In 1996, we added one more site (5N) to close the gap between the sites located 3 and 8 km north of the smelter; but this site was sampled uninterruptedly only from 2003. In the years 2006–2016, we lost four of our monitoring trees; two of these trees (both from severely polluted plots) died, and two (from a slightly polluted plot) were cut during regular forest clearings.

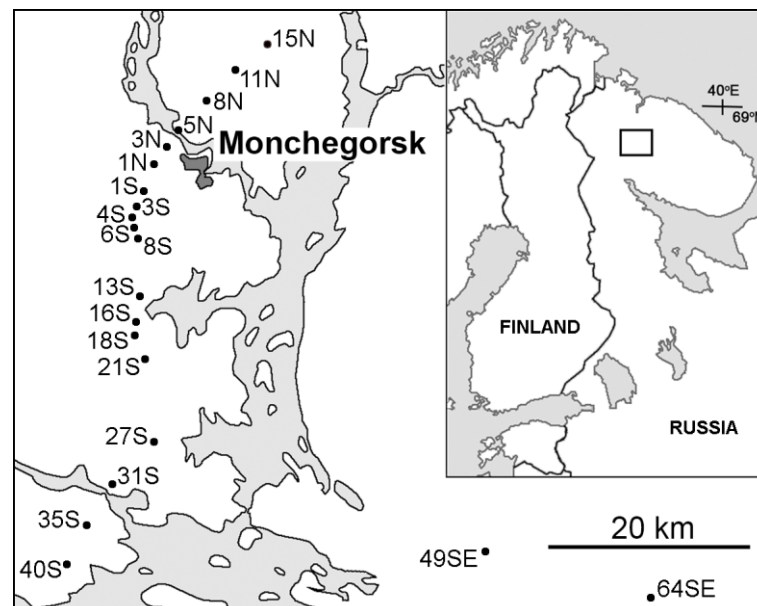


Fig. 1. Location of monitoring sites (dots) in the vicinity of the Monchegorsk nickel–copper smelter, Kola Peninsula. Site codes indicate the approximate distance (km) and direction (to the north, south or south-east) from the smelter. Coordinates of the study sites are provided in Table S2. Insert: position of the study area in Northern Europe.

In 2012, we replaced 58 of our monitoring birches with younger trees growing within 50 m distance of the old ones, because old trees were already too tall for accurate measurements of their height and their lower branches became inaccessible, thereby

hampering leaf sampling and shoot measurements. During two years (2012 and 2013), we monitored both ‘old’ and ‘new’ birch individuals at each site to assure data compatibility; in all other years, we collected leaves for FA measurements from five birches per site. A further nine birches were replaced in 2016.

2.2. Pollution, climate and herbivory data

We quantified pollution load at our study sites by the average concentration of nickel in mountain birch foliage measured in 1991–1993 (Table S2). The substantial decrease in aerial emissions from the Monchegorsk smelter did not affect the spatial distribution of pollutants (Kozlov et al., 2017); therefore, our site rankings based on pollution loads were unchanged throughout the 25-year observation period.

Daily values of temperature (average, minimum and maximum) and precipitation recorded at the meteorological station in Monchegorsk were obtained from the Lapland Biosphere Reserve (1993–2004) and from the web-based archive (www.rp5.ru; 2005–2017). The data for each one-year period preceding the collection of leaves for FA measurements were averaged to four values for each phenological period (Table S3), as follows: summer of the preceding year (July and August), autumn of the preceding year (September and October), winter (November of the preceding year to April of current year), and spring of the current year (May and June).

We explored the relationships between changes in pollution load, climate and leaf FA using principal component analysis (PCA) to combine 16 climate variables (four variables for each of four seasons) into PCA axes w1–w4. Each of these axes had an eigenvalue exceeding 1, and they combined to explain 80.7% of the variation in the climate data. However, only w1 correlated with year ($r = 0.63$, $n = 25$ years, $P = 0.0007$), so w1 is the only PCA axis that

represents climate warming. Similarly, we combined pollution data (3 variables: annual emissions of sulphur dioxide, nickel and copper; Table S1) into a single PCA axis. This axis, with an eigenvalue exceeding 1, explained 87.2% of the variance in deposition of pollutants in the study region. We also correlated year-specific values of birch leaf FA with average ambient air temperature in June, because this variable was found to explain the among-year variations in leaf FA from 1993–2000 (Kozlov and Valkama, 2010).

In this study, the percentage of birch leaves bearing any signs of damage imposed by defoliating and leafmining insects was adopted as an operational index of herbivory. This percentage was assessed from 1999–2017 on the same birch trees, which we used to measure leaf FA. At the end of the growth season, from mid to late August, one twig with approximately 120–150 leaves was haphazardly selected at a height of 0.2–2 m, and leaves bearing feeding marks by insects were counted among the first 100 leaves, starting from the tip of the branch. Over 95% of all censuses were made by the same observer (VZ), which assured uniformity of measurements. The results of these measurements were published by Kozlov et al. (2017).

2.3. Study species

Mountain birch is a deciduous tree-line species and one of the main forest-forming trees in subarctic Europe. This birch is the only woody plant that is still relatively abundant in the extremely contaminated habitats surrounding the smelters at Monchegorsk and Nikel (Kozlov et al., 2009), accounting for up to 99% to the total foliar biomass in the most polluted sites (Manninen et al., 2015). The taxonomic status of the northern birch populations is debatable; although a number of ecological studies refer to mountain birch, *B. pubescens* subsp. *czerepanovii*, some authors suggest that mountain birch is an ecological form of *B. pubescens*,

rather than a distinct taxon (Jonsell, 2000). The high ecological importance of mountain birch in subarctic forests has led to its intensive study in both pristine and disturbed environments (Wiegolaski, 2005; Eränen et al., 2009; Zverev, 2009), and it justified the selection of this species for long-term monitoring.

Birches produce two different kinds of vegetative shoots (Fig. S4). The monopodial short shoots bear two to five (usually three) leaves and an apical bud that includes primordia for leaves flushing in the subsequent year. These shoots grow less than two millimetres per year; their main physiological function is photosynthesis. Growth of branches, and of the whole birch tree, occurs via elongation of sympodial internodes of long shoots. These shoots produce successive single leaves supporting axillary buds. Apical buds of the short shoots usually produce short shoots, whereas axillary buds of long shoots may produce both long and short shoots.

2.4. Sampling and preservation of leaves

During all study years, the leaves for FA measurements were collected and preserved in the same way. Leaves were sampled at least a week after termination of their expansion, between 25 June and 15 July, from vegetative short shoots of tagged birch individuals. These vegetative shoots were haphazardly selected from different sides of the birch crown at a height of 0–2 m. We always collected the largest leaf from the selected shoot. If the largest leaf was damaged (e.g. part of the leaf lamina was removed by insect herbivores) in a manner that prevented accurate measurement of FA, then the shoot was disregarded and another shoot was sampled. We generally sampled 10 leaves per tree, with the exception of years 1995 and 1996 when five leaves were taken per tree. The leaves were packed into plastic bags and

transported to the laboratory, where they were mounted on strong paper using adhesive tape (Fig. S5) and press-dried as ordinary herbarium specimens.

2.5. Measurement of leaves

Following the protocol used in our earlier studies (Kozlov et al., 1996; Valkama and Kozlov, 2001; Zverev et al., 2018), for each leaf we measured the width of the left and right sides from the midrib to the leaf margins (at the midpoint between the base and the tip) perpendicular to the midrib. The measurements were conducted with a ruler to the nearest 0.5 mm; the perpendicularity of the measurement line to the midrib was controlled visually. All measurements were performed twice, by different assistants, who had no knowledge of the hypotheses being tested, the plant origin or the results of previous measurements. Leaves in which the absolute difference between the first and second measurement of the same side was greater than 2.5 mm were remeasured (again, by a different person with no knowledge of the earlier measurements) to exclude an occasional error. The third measurement was used to replace one of the two earlier measurements that was considered erroneous; the detection of the error was usually easy since some two thirds of the errors were equal to 5 or 10 mm.

More than 20 people participated in taking measurements; therefore, individual properties of the observer were unlikely to influence our conclusions. The virtually unavoidable differences in measurement error among measurers (Yezerinac et al., 1992; Helm and Albrecht, 2000) were traded off for a decreased probability of systematic error, which is more likely to arise when the repeated measurements are conducted by the same person.

2.6. Data validation

The FA values were calculated as follows: $FA = 2 \times \text{abs}(WL - WR) / (WL + WR)$, where WL and WR refer to the width of the left and right leaf halves measured at the midpoint between the base and the tip of leaf lamina. This index, labelled FA2 by Palmer and Strobeck (2003), is one of the most frequently used indexes, in spite of several drawbacks. It was applied in many earlier studies of plant leaf FA (e.g. Kozlov et al., 1996; Kryazheva et al., 1996; Wilsey et al., 1998; Ivanov et al., 2015), and its use is justified by the significant positive correlation between the absolute difference in the width between the left and right leaf halves and leaf size ($r = 0.16$, $n = 24943$ leaves, $P < 0.0001$).

In performing the analyses, we followed the most recent and rigorous methodology developed by Palmer and Strobeck (2003), while also accounting for methodological suggestions by Knierim et al. (2007). We conducted a mixed-model ANOVA for data collected in each study year to test for presence of directional asymmetry and FA relative to measurement error. In this analysis, the leaf side was considered a fixed factor and the individual leaf a random factor (procedure MIXED; SAS Institute, 2009). The obtained values of directional asymmetry (DA) (i.e. the signed difference in width between left and right leaf sides) were compared with the FA4a index ($FA4a = 0.798 \sqrt{\text{var}(WR - WL)}$) for each sample, where mixed model ANOVA revealed a significant DA.

Finally, we evaluated the reproducibility of the measurements by calculating the index $ME5 = [MS_i - MS_m] / [MS_i + (n-1) \times MS_m]$, where MS_i and MS_m are the interaction and error mean squares from a sides \times individuals ANOVA (Palmer and Strobeck, 2003). This index expresses FA variation as a proportion of the total variation between leaf sides, which includes variation due to both FA and measurement error.

2.7. Data analysis

Leaf-specific FA values were averaged between two subsequent measurements, square-root transformed to meet a normality assumption, and then analysed with a linear mixed model (procedure GLIMMIX). We considered the year of data collection, pollution at the site of leaf origin (covariate) and their interaction as fixed effects, whereas site of leaf origin and individual tree (nested within a site) were treated as random intercept effects. We facilitated accurate F tests of the fixed effects by adjusting the standard errors and denominator degrees of freedom by the latest version of the method described by Kenward and Roger (2009).

The regularities in spatial and temporal changes in leaf FA were sought using both correlation analysis (for absolute FA values) and meta-analysis (for correlations between FA and pollution load, ambient air temperature or herbivory). We calculated the effect sizes (ES hereafter) by weighing z -transformed correlation coefficients by their sample size using the MetaWin program (version 2.1.4; Rosenberg et al., 2000). These ES were based on Pearson linear correlation coefficients between the tree- or site-specific values of leaf FA and either pollution loads, mean air temperature in June or previous-year percentage of leaves damaged by insects. An effect was considered statistically significant if the 95% confidence interval (CI_{95}) of the mean ES did not include zero. The variation in these ES values, associated with changes in continuous variables (previous-year herbivory, ambient air temperature in June, annual amount of emissions and weather conditions during 12 months preceding measurements of leaf FA) was evaluated by calculating Q_M and the slope of meta-regression; Q_M values were tested against the chi-square distribution. All meta-analyses were performed using random effects models, assuming that the effect sizes differed not only due to sampling error but also due to a random variation.

3. Results

3.1. Data validation

The mixed model ANOVA demonstrated highly significant side×leaf interactions in data (see Appendix S2 in Supporting information) collected in all study years, thereby confirming the existence of FA in mountain birch leaves and our ability to identify this FA using repeated measurements of the given accuracy (Table 1). The width of left and right sides of mountain birch leaves differed significantly in 22 of 25 study years (Table 1). However, the sign of this difference varied among years: the right side was significantly larger than the left side in 15 years and significantly smaller than the left side in 7 years (Table 1).

Table 1. Basic statistics on measurements of differences between the width of left and right sides of leaves of mountain birch, *Betula pubescens* ssp. *czerepanovii*.

Year	Sample size	DA ¹ , mm	Mixed model ANOVA				Reproducibility (ME5)
			Side		Side × Leaf		
			<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	
1993	904	0.10	5.77	0.016	9.89	<0.0001	0.816
1994	212	0.10	1.62	0.204	7.90	<0.0001	0.776
1995	496	0.28	22.6	<0.0001	6.60	<0.0001	0.737
1996	505	0.13	3.93	0.048	8.59	<0.0001	0.792
1997	1000	-0.15	14.0	0.0002	2.34	<0.0001	0.402
1998	989	-0.09	3.94	0.047	5.37	<0.0001	0.687
1999	1050	-0.35	86.9	<0.0001	4.31	<0.0001	0.623
2000	1000	-0.16	17.4	<0.0001	4.10	<0.0001	0.608
2001	1048	-0.13	11.4	0.0008	11.9	<0.0001	0.846

2002	999	-0.12	10.9	0.001	4.10	<0.0001	0.614
2003	1048	-0.17	18.5	<0.0001	6.59	<0.0001	0.736
2004	1050	0.37	58.9	<0.0001	7.49	<0.0001	0.765
2005	1040	-0.13	9.99	0.002	9.01	<0.0001	0.801
2006	1050	-0.18	20.2	<0.0001	3.24	<0.0001	0.528
2007	1050	-0.11	7.57	0.006	2.86	<0.0001	0.481
2008	1048	-0.13	11.1	0.001	5.91	<0.0001	0.711
2009	1047	-0.22	23.5	<0.0001	6.71	<0.0001	0.741
2010	1050	0.36	80.5	<0.0001	5.32	<0.0001	0.683
2011	1050	-0.20	16.2	<0.0001	42.7	<0.0001	0.954
2012	1619	-0.09	7.59	0.006	7.04	<0.0001	0.752
2013	1593	0.07	4.77	0.029	5.05	<0.0001	0.670
2014	1051	-0.09	2.91	0.088	8.22	<0.0001	0.783
2015	1049	-0.02	0.21	0.645	1.98	<0.0001	0.328
2016	1045	-0.20	30.3	<0.0001	2.90	<0.0001	0.487
2017	1000	0.11	7.35	0.007	1.24	<0.0001	0.106

¹DA, directional asymmetry.

Analysis of the signed (WR–WL) difference across all study years confirmed that, on average, the right side of mountain birch leaves was 0.35% wider than the left side (mean DA = -0.055 mm). However, this DA value comprised approximately 5% of the FA4a index, suggesting that the contribution of the DA to the total variation in |WR–WL| values is small and can therefore be neglected. The average reproducibility of FA values was 65.7%.

3.2. Effect of pollution, climate and herbivory on leaf FA

The site-specific level of environmental pollution, quantified by concentrations of nickel in mountain birch foliage, had no overall effect on leaf FA across the 25 study years (Fig. 2a; Table 2). Leaf FA showed no variation among study sites, but differed significantly among individual trees nested within the sites (Table 2).

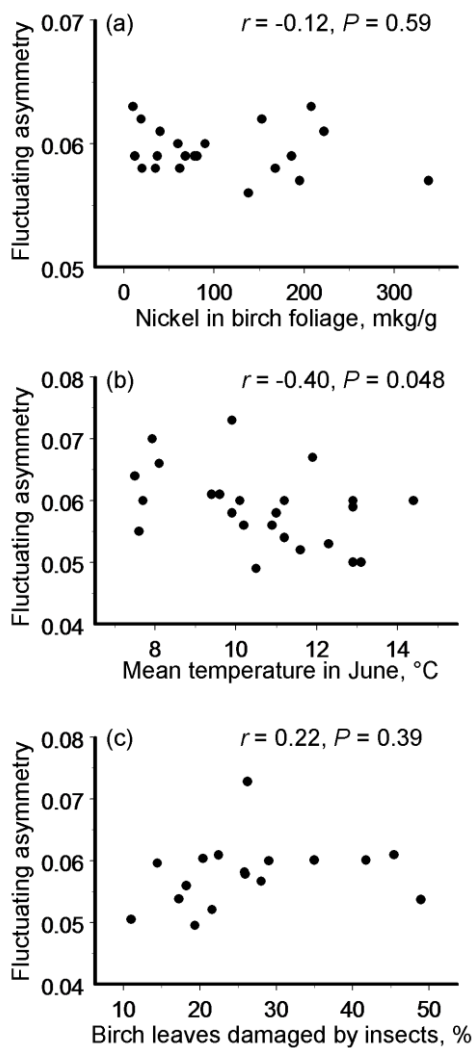


Fig. 2. Sources of variation in fluctuating asymmetry (FA) of mountain birch leaves: correlation with (a) site-specific pollution load (FA values are averaged by study site across 25 years of observations), (b) current-year mean temperature in June (FA values are averaged by study year across 21 study sites), and (c) previous-year mean percentage of birch leaves,

which were damaged by insects by the end of the previous growth season (FA values are averaged by study year across 21 study sites).

Among 29 trees that were monitored uninterruptedly during 25 years, the highest tree-specific FA value (0.078) was 1.6 times as high as the lowest tree-specific FA value (0.049). Four of five trees with the lowest FA values were growing in heavily polluted industrial barrens, and one of five trees with the highest FA values was growing in an unpolluted forest.

The relationships between leaf FA and foliar nickel concentrations varied among study years (Table 2; Fig. 3), from significantly positive (in 1996) to significantly negative (in 1997 and 2017). Nevertheless, meta-analysis based on year-specific correlation coefficients confirmed that, on average, the leaf FA of mountain birch was independent of pollution load (ES = -0.01, CI₉₅ = -0.11 to 0.10, $n = 25$ years). Among-year variation in relationships between leaf FA and foliar nickel concentration was not related to either the amount of aerial emissions from the smelter or the weather characteristics during the 12 months preceding data collection ($Q_M = 0.09-1.90$, $df = 1$, $P = 0.17-0.77$).

Table 2. Sources of variation in leaf fluctuating asymmetry of mountain birch, *Betula pubescens* subsp. *czerepanovii*, around the nickel-copper smelter in Monchegorsk

Type of the effect	Source of variation	Test statistics	P
Fixed	Year	$F_{24,438} = 5.97$	<0.0001
	Pollution	$F_{1,19} = 0.04$	0.84
	Year × Pollution	$F_{24,438} = 1.64$	0.03
Random	Site	$\chi^2_1 = 0.00$	0.99
	Tree (Site)	$\chi^2_1 = 54.8$	<0.0001

The year-specific values of leaf FA did not correlate with either the amount of aerial emissions from the smelter or the weather characteristics of the year preceding data collection (correlations with PCA axes: $R^2 = 0.006\text{--}0.063$, $n = 25$ years, $P = 0.25\text{--}0.71$). However, FA averaged by study year across all birch trees significantly decreased with an increase in the current-year temperature in June (Fig. 2b). At the tree-specific level, among 107 trees which were each monitored from 11–25 years, the correlation between leaf FA and temperature in June varied from significantly negative ($r = -0.58$, $n = 24$ years, $P = 0.003$) to significantly positive ($r = 0.63$, $n = 15$ years, $P = 0.01$). Only 11 of 107 correlation coefficients were significant at $P = 0.05$; ten of these significant correlations were negative and one was positive. Consistently, meta-analysis of these correlations revealed that, on average, leaf FA decreased with the increase in June temperature (ES = -0.17, CI₉₅ = -0.21 to -0.13, $n = 107$ trees), and that the magnitude of this effect was independent of the site-specific pollution load ($Q_M = 0.00$, $df = 1$, $P = 0.97$).

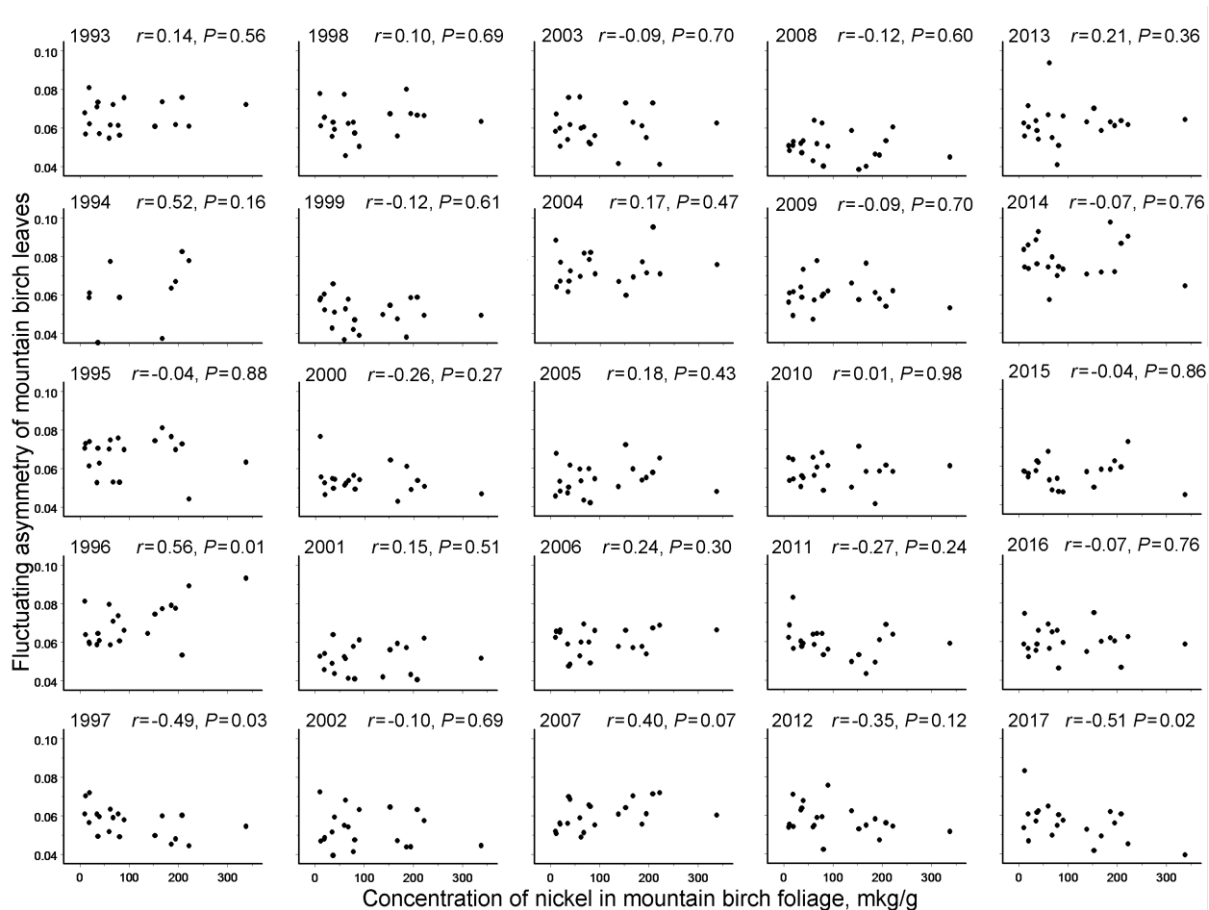


Fig. 3. Year-specific correlations between fluctuating asymmetry (FA) of mountain birch leaves and site-specific pollution load (FA values in each graph are averaged by study site across 5–10 birch trees).

The correlation between leaf FA and the percentage of mountain birch leaves damaged by insects during the previous growth season varied among study years (Fig. 4); this correlation was significantly positive in 2004 only. Meta-analysis based on year-specific correlation coefficients confirmed that, at the spatial scale, the leaf FA of mountain birch increased slightly but significantly with an increase in previous-year insect herbivory ($ES = 0.11$, $CI_{95} = 0.01$ to 0.22 , $n = 18$ years). Among-year variation in relationships between leaf FA and insect herbivory was not explained by either the mean temperature in June or the absolute level of herbivory ($Q_M = 0.01$ and 0.27 , $df = 1$, $P = 0.93$ and 0.60 , respectively). Among-year variation in the absolute values of birch leaf FA did not correlate with the average level of birch damage by insects in the previous year (Fig. 2c).

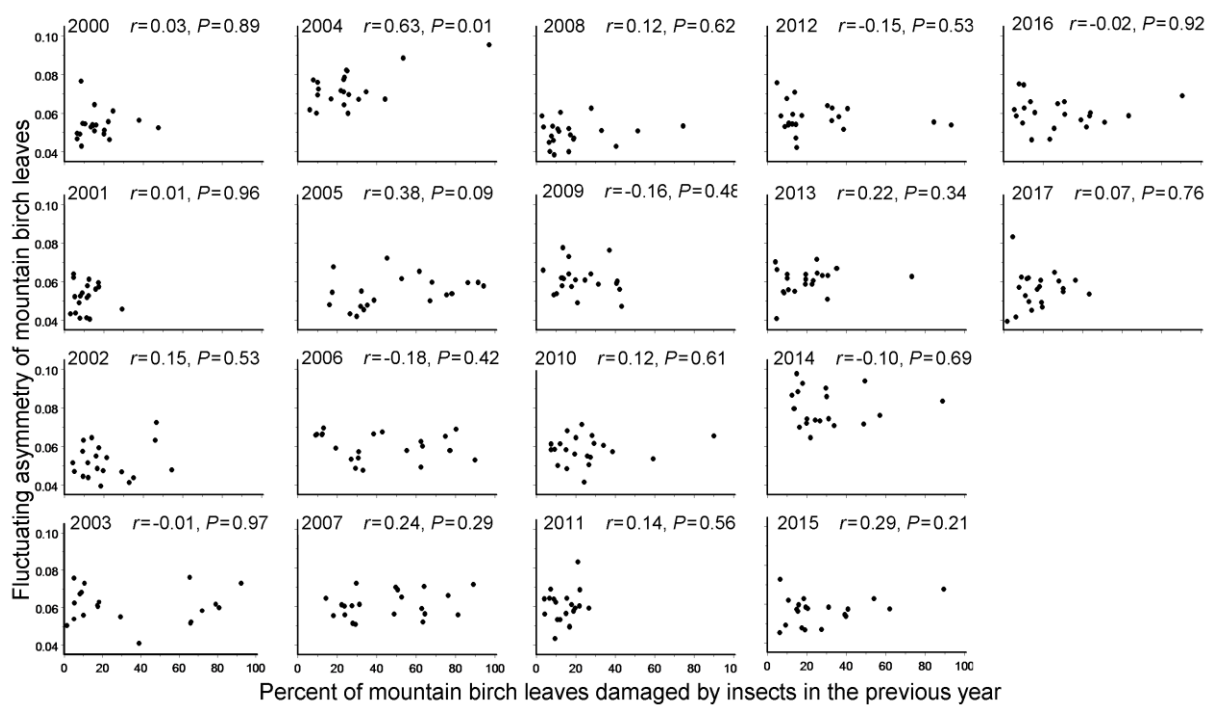


Fig. 4. Year-specific correlations between fluctuating asymmetry (FA) of mountain birch leaves and site-specific percentage of mountain birch leaves, which were damaged by insects by the end of the previous growth season (FA values in each graph are averaged by study site across 5–10 birch trees).

3.3. Re-analysis of data used in the previous publication

The results of blinded measurements of 600 leaves, which were collected in 1994 from haphazardly selected mountain birches (5 leaves from each of 20 trees per each of 6 study sites; Appendix S3) strongly correlated with the results of non-blinded measurements (leaf half-width, by side and measurement: $r = 0.85–0.87$, $n = 600$, $P < 0.0001$). Nevertheless, the site-specific FA based on blinded and non-blinded measurements demonstrated large (although not significant: $z = 1.63$, $P = 0.10$) differences in correlation coefficients between pollution load and leaf FA (Fig. 5): non-blinded measurements yielded a significant positive

correlation, whereas blinded measurements demonstrated no correlation between these variables.

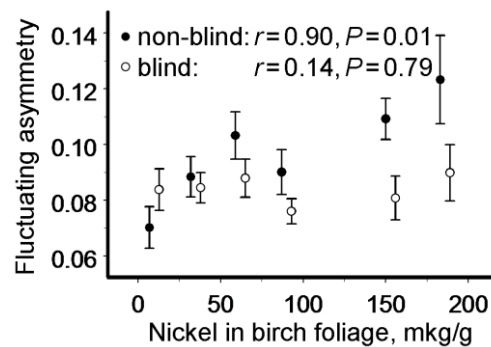


Fig. 5. Correlation between fluctuating asymmetry (FA) of mountain birch leaves and site-specific pollution load based on blind and non-blind measurements of the same set of 600 leaves collected in 1994 (FA values are averaged by study site across 20 birch trees; bars indicate standard errors).

4. Discussion

4.1. Effects of pollution

Our study is the first to monitor plant leaf FA in multiple sites along a steep pollution gradient for a long duration (25 years). Ironically, this study, which had been established to elucidate effects of pollution on plant FA, unequivocally demonstrated that leaf FA in mountain birch is independent of pollution load. Our data provided no support for the hypotheses that FA increases at the spatial scale with increasing proximity to the nickel-copper smelter in Monchegorsk or that it decreases at a temporal scale with decreasing annual emission of pollutants. We honestly hope that this result, which corroborates our earlier findings (Valkama and Kozlov, 2001; Kozlov et al., 2009; Zverev et al., 2018) and the ‘negative’ results obtained by other researchers (e.g. Wuytack et al., 2011; Koroteeva et al.,

2015; Veselkin et al., 2016; Sandner and Matthies, 2017; Wadhwa et al., 2017), will contribute to debunking the myth of a consistent increase in plant leaf FA with an increase in environmental pollution. This myth, which is believed by many researchers, remains rather attractive, so that large amounts of resources have been and continue to be spent in attempts to use plant leaf FA as a bioindicator of environmental pollution (discussed by Kozlov, 2017).

We regret that one of us (MVK) unintentionally contributed to the creation of this myth by reporting, more than 20 years ago, a consistent increase in birch leaf FA with decreasing distance to four industrial polluters in Northern Europe (Kozlov et al., 1996). Fortunately, leaf samples from the surroundings of Monchegorsk used in that study had been preserved, so we were able to re-examine these samples in order to understand the reason behind the discrepancy between the results that were published earlier (Kozlov et al., 1996) and the conclusions of the present study.

Surprisingly, the repeated, blinded measurements of mountain birch leaves collected in 1994 refuted our earlier findings: the results of these measurements showed no correlation with pollution load. On the other hand, we cannot blame the technical quality of leaf measurements performed in 1995, because the results of these earlier measurements were strongly correlated with the results of the later blinded measurements. Thus, the differences between blinded and non-blinded measurements generally do not exceed the accuracy of measurements (0.5 mm). We therefore conclude that non-blinded measurements were prone to confirmation bias; that is, the tendency of humans to seek out evidence in a manner that confirms their hypotheses and beliefs (Rosenthal, 1976; Nickerson, 1998). This bias results primarily from automatic processes occurring unintentionally (Hergovich et al., 2010), and it therefore clearly differs from falsification of research data. We suggest that the possible mechanism behind the detected bias is an unconscious rounding of intermediate values of leaf half-width in the expected direction, a process known as rounding bias (Knott, 1972).

The importance of blinded measurements in studies exploring FA has been stressed only recently. We experimentally demonstrated that when scientists expected to find high FA in some samples, the results of their measurements confirmed their expectations, and we concluded that confirmation bias may considerably influence the outcomes of FA research (Kozlov and Zvereva, 2015). To our knowledge, our current finding represents the first direct demonstration of the distortion of a real set of ecological data by confirmation bias. Blinded methods, such as coding of samples in such a way that the observer cannot link the code with any properties of a sample, are surprisingly rarely used in ecological studies. A recent review demonstrated that, of the articles in ecology, evolution, and behavior that described experiments that could have been influenced by observer bias, only 13.3% reported that experiments were blinded (Kardish et al., 2015). We hope that our finding will stimulate researchers working on FA to always use blinded methods to ensure that the person conducting the measurements is not aware of the origin of the samples being measured.

Intriguingly, the studies published prior to 2000 reported significant increases in plant FA with an increase in pollution, while studies published after 2000, on average, reported no effect of pollution on plant FA (Kozlov et al., 2009). This pattern, at least in part, may reflect overall improvements in research methodology. However, it also hints that development of the hypothesis in the late 1990s and early 2000s regarding a positive association between FA and pollution, in line with the common tendency observed across different ecological hypotheses (Leimu and Koricheva, 2004), has shifted from accumulation of supportive evidence to accumulation of disconfirming evidence. At this second stage, the scientific community looks at the data much more critically than it had done at the first (enthusiastic) stage, which makes it possible to publish results which disagree with the dominating hypothesis. In 1995, we received quite positive reviews for our manuscript (published as Kozlov et al., 1996) that confirmed the influential hypothesis, despite the relatively low

accuracy of our measurements and the absence of statistical tests confirming the presence of FA in our data relative to measurement error. However, a few years later, we were unable to publish our conclusion, obtained using more advanced methodology, that experimentally induced stress of mountain birch seedlings by drought and heavy metals had no effect on leaf FA (Zverev et al., 2018).

Earlier, we suggested that the absence of a correlation between birch leaf FA and environmental contamination, as observed around Monchegorsk and several other industrial polluters, may be due to an evolved tolerance to pollution in the birch trees (Valkama and Kozlov, 2001; Kozlov et al., 2009). However, a controlled experiment demonstrated that seedlings of mountain birch originating from unpolluted sites also showed no increase in leaf FA in response to heavy metal treatment (Zverev et al., 2018). Combined with the among-year variation in the relationships between pollution load and birch leaf FA, this information hints that the observed spatial and temporal patterns resulted from the impacts on FA of other, yet unknown factors.

It could also be suggested that the among-year variation in relationships between pollution load and birch leaf FA represents a random noise. However, two findings contradict this hypothesis. First, we found that individual birch trees differ significantly in their leaf FA, in spite of annual fluctuations in the absolute FA values. Second, we demonstrated that birch leaf FA consistently decreased in our monitoring trees with an increase in ambient air temperature in June, during the period of birch leaf expansion in our study region. Therefore we conclude that leaf FA is, to a certain extent, determined by plant genotype, and it is affected by some (but not all!) environmental factors, weather conditions in particular.

4.2. Effects of temperature

Hundreds, if not thousands, of experimental studies have reported the impacts of air temperatures on different plant performance characteristics, but we are not aware of even a single study that has explored the effects of growth temperature on plant FA under controlled conditions. Consequently, our knowledge of the effects of temperature on plant FA is limited to a handful of observational studies. Within the Kola Peninsula, the leaf FA of mountain birch was reported to decrease with an increase in air temperatures during leaf expansion (Valkama and Kozlov, 2001), whereas the needle FA of Scots pine, *Pinus sylvestris* L., did not vary in response to among-year temperature fluctuations (Kozlov and Niemelä, 2003). At the spatial scale, the leaf FA of both honey locust, *Gleditsia triacanthos* L., and European beech, *Fagus sylvatica* L., showed no correlation with any climatic parameter (Murphy and Lovett-Doust, 2004; Uhl, 2014), whereas leaf FA in mountain birch increased from sea level towards the tree line (Wilsey et al., 1998; Hagen et al., 2008). These studies jointly suggest that the responses of plant FA to ambient temperature are in no way uniform, and that subarctic populations of mountain birch may be particularly sensitive to weather conditions.

Experiments with *Drosophila melanogaster* Meigen demonstrated that the wing FA in these flies increased at both lower and upper extremes of the thermal range (Trotta et al., 2005). Assuming that this regularity is valid for other living beings, we conclude that the air temperatures in June ranging from 7.5 to 14.5°C are much below the upper extreme of the thermal range of mountain birch, because an increase in temperatures resulted in a decrease in leaf FA. Thus, if larger FA reflects greater developmental instability due to environmental or genetic stress (Palmer and Strobeck, 1986; Møller and Shykoff, 1999), our result suggests that the past increase of early summer temperatures (by 2.5–3 °C from 1991–2014: Zvereva et al., 2016) alleviated the climatic stress experienced by mountain birch in our study area, which lies some 150 km south of the tree line. The visual inspection of the figure showing relationships between birch leaf FA and air temperature in June (Fig. 2b) suggests that further

increases in early summer temperatures by 2–3 °C are unlikely to impose adverse effects on mountain birch populations in the central part of the Kola Peninsula. However, the great variation in correlations between leaf FA and temperature in June, observed among individual birch trees, suggests that some birch genotypes may already be suffering from temperature elevation under the current climate.

Importantly, our results demonstrated that only the temperature in June correlated with leaf FA, whereas weather conditions during the 12-month period before leaf sampling, including temperature extremes and precipitation, did not show any relationships with leaf FA. This result may indicate that the FA of birch leaves is primarily affected by weather conditions at the time of leaf growth, and not at the period when leaf primordia are formed. Thus, future studies addressing the mechanisms by which the environment affects leaf FA should concentrate on the period of leaf growth.

In the present study, we correlated data on leaf FA collected from multiple sites located up to 64 km from Monchegorsk with meteorological data recorded in Monchegorsk. However, our earlier findings indicated that our study sites differ greatly in climatic characteristics: the soil thawed earlier in spring in heavily polluted industrial barrens than in unpolluted forests, and the spring and summer soil temperatures were highest in the industrial barrens (Kozlov and Haukioja, 1997). Assuming that birch leaf FA responds to temperature variations at the spatial scale in the same way as it responds to among-year temperature variations, in the absence of a direct pollution effect on leaf FA, we could expect to observe the lowest leaf FA in the most polluted (i.e. the warmest) sites across all study years. However, since this expectation was not met, we conclude that weather conditions alone could not explain the observed variation in FA among our study sites.

4.3. Effects of herbivory

In several study systems, plants that undergo severe defoliation due to insects demonstrated an increase in leaf FA in the year following the damage (Zvereva et al., 1997a; Hagen et al., 2004; Alves-Silva and Del-Claro, 2016), and this effect was directly proportional to the degree of the previous year's herbivory at the scale of individual trees (Díaz et al., 2004). The cause-and-effect relationships between increases in leaf FA and herbivory were confirmed by defoliation experiments with willow, *Salix myrsinifolia* Salisb. (Zvereva et al., 1997b). At the same time, pollution-induced habitat deterioration, associated with decreases in the nutritive quality of polluted soils, can hamper a plant's ability to respond to herbivory by an increase in leaf FA (Zvereva and Kozlov, 2001).

In our study region, insect herbivory on mountain birch from 1991–2016 did not change along the pollution gradient and showed no temporal trend that could be associated with climate warming (Kozlov et al., 2017), so the effects of herbivory on FA could not be confused with effects of air temperatures. Meta-analysis of correlation coefficients demonstrated that leaf FA in mountain birch slightly, but significantly, increased with previous-year insect herbivory. This finding is somewhat surprising, because the losses of birch foliage to insects range from 1–5% in our study area (Kozlov, 2008; Kozlov et al., 2015), and no outbreaks of defoliating insects were recorded in our monitoring sites during the entire observation period (Kozlov et al., 2017). Thus, not only severe defoliation (Zvereva et al., 1997a, b), but also relatively minor (background) insect herbivory, causes measurable changes in leaf FA in mountain birch. This finding is in line with our conclusion that a 2–5% increase in background insect herbivory can potentially have negative impacts on birch growth (Wolf et al., 2008; Zvereva et al., 2012).

5. Conclusions

Our most important finding is that leaf FA in mountain birch could not be seen as a universal, non-specific indicator of stress; instead, the detected changes in leaf FA were stressor-specific. Our long-term data demonstrated that birch leaf FA did not change with pollution, either at spatial or temporal scales, and we hope that this finding will aid in debunking the myth that plant leaf FA consistently increases with increases in environmental contamination. At the same time, leaf FA correlated with ambient temperature and thus could serve as an index of climatic stress. Our findings suggest that climate warming is unlikely to impose stress on subarctic mountain birch populations, whereas even small increases in insect herbivory may adversely affect birch trees.

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Appendices A-C. Supplementary data

Supplementary data to this article – annual atmospheric emissions of principal pollutants from the smelter at Monchegorsk from 1993 to 2017 (Table S1), the characteristics of study sites

(Table S2), seasonal averages of climate variables in Monchegorsk from 1993 to 2017 (Table S3), the habitats where the monitoring was conducted (Figs. S1-S3), two types of annual shoots in mountain birch (Fig. S4), the mode of preservation of mountain birch leaves (Fig. S5), results of measurements of leaves from monitoring trees (Data S1) and of leaves that served the basis for the earlier (Kozlov et al., 1996) publication (Data S2) – can be found online at https://doi.org/10.1016/j.scitotenv.2018.**.***.

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