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Research paper

Fluctuating asymmetry of birch leaves did not increase with pollution and drought stress in a controlled experiment

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A R T I C L E I N F O

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A B S T R A C T

Fluctuating asymmetry (FA), defined as small, non-directional deviations from perfect symmetry in morphological characters, is often recommended as a handy indicator of environmental stress. A reliance on observational data to provide empirical evidence for the effects of different stressors on FA, in combination with increasing attempts to use FA in environmental research, underscore the need for careful examination of the relationships between environmental stress and FA. We experimentally tested the hypotheses that (i) heavy metal and drought stress increase the leaf FA in plants, and that (ii) plants persisting in heavily polluted sites possess greater stress tolerance and therefore show smaller increases in leaf FA in response to heavy metals than do plants from unpolluted sites. We collected mountain birch, *Betula pubescens* subsp. *czerepanovii*, seeds from eight polluted and ten unpolluted sites and reared the seedlings in a sophisticated greenhouse (phytotron). We compared leaf FA between control seedlings, seedlings irrigated with water containing copper and nickel sulphates, and seedlings exposed to drought. Leaf FA showed no response to either heavy metals or drought, despite significant impacts of these treatments on seedling height, leaf size and photosynthetic efficiency. This FA result was independent of the level of pollution at the site of seed origin and consistent for FA values based on low and high accuracy measurements of leaf width, as well as for FA values based on measurements of the widths and areas of leaf halves. Our findings add to accumulating evidence regarding inconsistent relationships between FA and abiotic stress, thereby questioning the indicatory value of FA. We strongly recommend that the use of FA as an indicator of environmental stress be limited to study systems for which the existence of cause-and-effect relationship between the stressing impact and the changes in FA is confirmed by controlled, blinded experiments.

1. Introduction

Ecological management requires timely prediction of emerging environmental problems. This requirement gives special value to ecological indicators that could be used as early warning systems to signal problems not yet apparent (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 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). In line with previous studies, one of us (MVK) once suggested that the FA of birch leaves could serve as a convenient indicator for rapid assessment of environmental quality (Kozlov et al., 1996). However, this conclusion was contested a few years later, when an analysis of multiyear data demonstrated no relationship between the leaf FA of mountain birch *Betula pubescens* subsp. *czerepanovii* (Orlova) Hämet-Ahti and heavy metal and sulphur dioxide loads (Valkama and Kozlov, 2001). Further analysis of data collected from multiple plant species in the impact zones of 18 industrial enterprises in the Northern hemisphere also failed to reveal any significant differences in FA between heavily polluted and unpolluted sites (Kozlov et al., 2009), potentially due to a rapid development of evolutionary adaptations to pollution (Kozlov, 2005; Eränen et al., 2009).

The continued accumulation of negative and inconclusive results dampened the initial optimism regarding the use of FA in environmental studies (Lajus et al., 2009), and sceptical reviews (Palmer, 1996; Clarke, 1998; Bjorksten et al., 2000; Rasmuson, 2002) began to point out a general inconsistency in the relationships between FA, stress and fitness. Nevertheless, despite the justified concerns expressed by evolutionary biologists, the number of applied studies substantiating the indicatory value of FA or deriving conclusions on relative levels of environmental disturbance based on FA measurements in plants and animals continues to increase (reviewed by Kozlov, 2017). These

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ongoing attempts to use FA in applied ecological research underscore the need for careful examination of the relationships between environmental stress and FA.

The reliability of FA as a bioindicator can be explored by controlled experiments that focus on the cause-and-effect and dose-and-effect relationships between FA and the stressor(s) of interest. Unfortunately, experiments of this kind remain scarce (e.g. Mal et al., 2002; Savelieva et al., 2017; Kolbas et al., 2014; Nishizaki et al., 2015; Sandner and Matthies, 2017), and the shortage of experimental data is in no way compensated by the wealth of observational studies which demonstrate statistically significant associations between FA and potentially stressing impacts. Furthermore, these correlative studies, due to their great number, outweigh experimental studies in narrative reviews and meta-analyses (e.g. Knierim et al., 2007; Allenbach, 2011; Beasley et al., 2013), thereby leading to an impression of a consistently high indicatory value for FA. For example, Beasley et al. (2013) concluded that FA is a legitimate biomarker of environmental stress.

This shortage of experimental data prompted us to publish the outcomes of an experiment that explicitly addressed the effects of two abiotic stressors, heavy metals and drought, on the leaf FA in mountain birch. This experiment, conducted in 2003, was at that time regarded sceptically by the reviewers, as our conclusion was that stress had no effect on FA. This obliged us to exclude the larger part of the data on FA from our previous manuscript (Eränen et al., 2009) in its final published form. Based on this experience, we strongly agree with the suggestion by Diaz-Gil et al. (2015) that a strong bias may exist in the published literature regarding positive relationships between stress and FA. This gives special importance to the 'negative' results, as publication of these would advance the understanding of the value of FA for environmental ecology and management.

The goal of the present study was to conduct an experimental exploration of the impacts of heavy metals (nickel and copper) and drought on the FA of mountain birch leaves. We tested two specific predictions derived from the theory of developmental stability (Freeman et al., 1993; Møller and Swaddle, 1997) and from earlier observational studies (Kozlov et al., 1996; Hodar, 2002; Kozlov and Niemelä, 2003; Fair and Breshears, 2005; Kozlov, 2005). We predicted that (i) the exposure of growing birches to heavy metals and drought will increase the FA of their leaves, and that (ii) the increases in leaf FA in response to heavy metals will be smaller in birches from heavily polluted sites than from unpolluted sites.

2. Materials and methods

2.1. Study area and study object

The Kola Peninsula is located in the north-western part of European Russia, next to Finland and Norway, to the north of Polar Circle. The mountain ranges and heavy industry in this region create multiple stress gradients, thereby offering unique possibilities for studies on environmental and evolutionary ecology. The presence of two large non-ferrous smelters, in Monchegorsk and Nikel, with similar compositions of pollutants and similar histories of environmental impacts (Kozlov et al., 2009), offers replication of heavily contaminated areas within a similar natural environment (Eränen et al., 2009; Ruotsalainen et al., 2009).

Mountain birch is the tree-line species and one of the main forest-forming trees in subarctic Europe, and it is the only tree species that is still relatively abundant in the extremely contaminated habitats surrounding the smelters at Monchegorsk and Nikel (Kozlov et al., 2009). Its high ecological importance in subarctic forests has led to intensive study of mountain birch in both pristine and disturbed environments (Wiegolaski, 2005; Eränen et al., 2009; Zverev, 2009). Leaf FA of different birch species was reported to increase in industrially polluted areas (Kozlov et al., 1996; Ivanov et al., 2015) and in the years with low early summer temperatures (Valkama and Kozlov, 2001), as well as with altitude of the study site and with birch hybridisation (Wilsey et al., 1998). By contrast, other studies have indicated no change in birch FA after heavy grazing (Berteaux et al., 2007) or in response to nutrient stress (Black-Samuelsson and Andersson, 2003) and no association of birch FA with either leaf growth rate (Kozlov, 2003) or the date of leaf fall (Kozlov, 2004).

2.2. Experimental design

Mountain birch seeds were collected in October 2002 from five mother trees in each of 18 study sites around the Kola Peninsula (for locality data consult Eränen et al., 2009). Eight of these sites were located in heavily polluted areas near the smelters in Monchegorsk and Nikel, and ten sites were located in pristine (unpolluted) habitats. Seeds were stored at +3 °C until 15 January 2003, and then stratified for one month, followed by germination at +21 °C under a 24 h day length. Three weeks after sowing, the seedlings were individually replanted into pots in a mixture of 70% standardized peat soil and 30% perlite and grown at +15 °C until the beginning of the experiments.

The experiment was conducted using a sophisticated greenhouse (phytotron) at the University of Tromsø, Norway. The air temperature was controlled at an accuracy of ± 0.5 °C, and air humidity of ± 5% RH, with a constant water vapour saturated deficit of 530 Pa. We used nine seedlings from each of the 79 mother trees. These seedlings were evenly distributed among three growth chambers with identical climatic characteristics, and among three treatments within each chamber: control, heavy metal stress and drought stress. Thus, each chamber contained three seedlings from each mother tree, and these three seedlings were subjected to different treatments.

The experiment was initiated on 13 May 2003 at +15 °C in natural light conditions. Five days a week, the seedlings in the control and heavy metal stress treatments were watered with 30 mL of tap water, whereas seedlings in the drought stress treatment received 15 mL. The water used to irrigate the seedlings in the heavy metal treatment included added nickel and copper sulphates to give concentrations of 5 mg L^{-1} copper and 10 mg L^{-1} nickel. On the last 2 days of every week, all plants were irrigated with clean tap water in the same quantities as mentioned above. After three months, seedlings exposed to heavy metals and drought demonstrated smaller height, leaf size and leaf photosynthetic efficiency than control seedlings; seedlings from polluted sites showed lower accumulation of foliar nickel and lower stress in heavy metal treatments than seedlings from clean sites (for more details, consult Eränen et al., 2009).

2.3. Leaf sampling and measurements

On 8–9 August 2003, we collected two fully expanded leaves from the top of each seedling, press-dried these leaves between sheets of filter paper, and then mounted them as ordinary herbarium specimens. Some seedlings had died prior to sampling, or had only a single green leaf remaining by the time of sampling, so we obtained a total of 1331 leaves. For each leaf, one author (VZ) measured the width of the left and right halves at the midpoint between the base and the apex of leaf lamina. The measurements were conducted with a ruler to the nearest 0.5 mm (low accuracy measurements hereafter); the perpendicularity of the measurement line to the midrib was controlled visually. This measurement protocol was identical to one used in earlier studies by our team (Kozlov et al., 1996; Valkama and Kozlov, 2001). The measurements were conducted twice, with a two-month interval between measurements.

We excluded the possibility that the low accuracy of our measurements was the primary reason for our failure to detect a stress effect on leaf FA by scanning a random subsample of 150 leaves (50 leaves per treatment) at 600 dpi and re-measuring these leaves in 2016. We used ImageJ and one author (ADL) measured the width of the left and right leaf halves at the midpoint between the base and the apex of leaf lamina