

Article

Change of Leaf Trait Asymmetry Type in *Tilia cordata* Mill. and *Betula pendula* Roth under Air Pollution

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Received: 30 March 2020; Accepted: 15 April 2020; Published: 3 May 2020



Abstract: Leaf fluctuating asymmetry (FA) is widely used as an environmental stress index, including pollution. Besides FA, leaf bilateral traits can have directional asymmetry (DA) and antisymmetry (AS), which are considered hereditary. Leaf FA transitioning to DA/AS or mixed asymmetry, under air pollution, has been insufficiently investigated. This study analysed leaf asymmetry types in *Tilia cordata* Mill. and *Betula pendula* Roth under traffic air pollution over several years. In addition, the relations of such transitions to pollution, and their effect on FA-integrated index, were studied. The asymmetry types of all studied leaf traits varied with air pollution increase, as well as in control trees in different years. *T. cordata* most often had FA transition to DA/mixed asymmetry, while *B. pendula* rarely had a mixed asymmetry and FA transitions to DA/AS were observed with the same frequency. Air pollution impacted FA transitions to other asymmetry types. In most cases their frequency changed non-monotonically that corresponded to hormesis and paradoxical effects. However, FA integrated index in studied trees did not depend on change of leaf asymmetry type. Thus, DA and AS in studied plants were not exclusively hereditary. Hence, the changes of leaf asymmetry type should be considered when using leaf FA in environment assessment.

Keywords: *Tilia cordata* Mill.; *Betula pendula* Roth; leaf; fluctuating asymmetry; directional asymmetry; antisymmetry; mixed asymmetry; air pollution; hormesis; paradoxical effect

1. Introduction

Developmental stability characterizes the ability of an organism to maintain the trajectory of development within certain bounds [1,2]. Fluctuating asymmetry (FA) is widely used as an index of developmental instability of bilateral morphological structures in plants. Random insignificant deviations from the symmetrical state [3–5], due to the stochastic nature of molecular processes, provide the expression of genes (developmental noise) [6]. FA has a mostly non-hereditary nature, but it is often observed in the background of hereditary types of asymmetry, such as antisymmetry (AS) and directional asymmetry (DA) [2]. FA is characterized by a normal distribution of R - L differences with a mean of zero [1].

DA is the consistent difference between a pair of morphological structures, i.e. the larger structure in the pair occurs consistently on one side [7]. DA reflects a consistent bias of a trait within a species towards greater development on one side of the body than on the other; the coiling and associated anatomical asymmetry of gastropods or the flatfish asymmetry are typical examples. DA has normally distributed R - L differences around a mean that is significantly different from zero [1].

AS is the larger development of any morphological structure in a pair (the left or the right structure) [2]. AS is distinguished by a platykurtic (broad peaked) or bimodal distribution of R - L differences around a mean of zero. In male fiddler crabs, for example, the oversized signalling claw,

which is much larger than the opposing one, occurs with approximately equal frequency on both, the right and left sides in nearly all species [1].

It is known that FA increases under the influence of many environmental stress factors [4,8–11]. Therefore, the FA of the leaves of various plant species is widely used to estimate the level of environmental stress (for bioindication) induced by anthropogenic [12–14], abiotic [10,15–19] and biotic [11,20–22] stressors.

Researchers usually determine the bilateral asymmetry type of morphological structure of a plant leaf only once. It is implied that the environment cannot change the asymmetry type as AS and DA have a significant genetic basis and FA is mostly non-hereditary [3].

However, several experimental studies demonstrated a dynamical interrelationship between the three types of asymmetry in different animal species under stress conditions [23–25]. For instance, a high concentration of benzole (10,000 mg / kg) in the nutrient medium caused a transition from FA to DA for the number of bristles in *D. melanogaster*, and lower concentrations simply increased FA [24]. The insecticide metoxychlor induced the transition from FA to DA in mice for morphological traits of skull bones [25]. Graham et al. [23] reported that environmental stress caused a change of morphogen concentration and a transition from FA to AS in a model of morphogenesis.

Similar results were also achieved for natural animal populations. Lens and Van Dongen [26] showed that FA of tarsus traits was mixed with DA in natural bird populations under increasing habitat disturbance. It was hypothesized that increased developmental instability was reflected in a transition from FA to DA and/or AS [26]. These facts allowed us to hypothesize that similar shifts in leaf asymmetry may also occur in plant leaf traits used for environmental quality assessment.

Therefore, the study of leaf FA transition to other types of asymmetry under environmental pollution has both, practical implications for bio-indication and theoretical importance for understanding the patterns of leaf asymmetry development under stressful environments.

Road transport is a major source of air pollution and soil contamination in most megacities [27,28]. Linden (*Tilia cordata* Mill.) and birch (*Betula pendula* Roth) often grow in roadside forest strips in Russian cities. Notably, these species are bioindicators and their leaf FA is often used for environmental quality assessment [29–31]. Additionally, *B. pendula*, compared to *T. cordata*, is also more sensitive to certain gaseous air pollutants of urban roadside territories, such as sulphur and nitrogen oxides [32,33], that allows comparison of their responses to the same levels of pollution. This is the reason these species of woody plants have been selected for this study.

We previously evaluated leaf FA in these species, over a number of years, which was reported in a publication for *B. pendula* [34]. At the same time, we did not analyse FA transition to other asymmetry types in *T. cordata* and *B. pendula* under different levels of traffic pollution.

The application of plant and animal responses for environmental quality assessment is based on the idea that an increasing level of pollution always causes an increase in disturbance of organism parameters, that is, a dose-response relationship is always monotonic (with no extrema) [35,36].

However, it is known that non-monotonic dose-response relations (with maximums and/or minimums) which include hormesis [37–39] and paradoxical effects [40,41] are frequently found for different plant species and parameters, including various pollutant exposures [42–47]. Hence, an increase in the pollution level will not always be accompanied by plant index deteriorations.

The hormetic curve has two phases, and is characterized by an improvement of parameter at low doses, and its impairment at high doses compared to the control level [38,48,49]. Paradoxical effects, include both biphasic and multiphase non-monotonic responses, and are characterized by a decrease in the damaging effect with an increase in the toxicant dose [40,41,43,50]. In fact, all non-monotonic dose-response relations, except hormesis, are paradoxical effects. At the same time, pollutant ability to cause hormesis and paradoxical effects in plants for leaf FA transitions to other types of asymmetry remains unexplored.

Therefore, this study analysed the change of leaf trait asymmetry type in *T. cordata* and *B. pendula* with traffic air pollution in a wide range of values in an urbanized area (with the example of Nizhny

Novgorod in Russia) using long-term data. In addition, relations of leaf FA transitions to other types of asymmetry to air pollution levels and their effect on FA integrated index were studied.

2. Material and Methods

2.1. Study Area and Study Plots

We carried out this research in Nizhny Novgorod which is the fifth largest city in Russia with a population of 1,250,619. Nizhny Novgorod is located in European Russia, about 400 km east of Moscow at the confluence of the Oka and the Volga Rivers. The Oka River flows into the Volga and divides the city into two parts. The upland part of Nizhny Novgorod is located on the high eastern bank of the Oka and the lowland part of Nizhny Novgorod occupies the low western bank of the Oka. The main stationary sources of pollution (industrial plants, thermoelectric power station) are located in the lowland part. At the same time, a major source of air pollution in upland part of the city is the road transport, therefore, the study was performed in this area.

For the study, we used primary data obtained during 3 years for *T. cordata* (2010–2011 and 2013) and five years for *B. pendula* (2007–2008 and 2010–2012). The trees grew in 8–9 model areas (plots) of tree stands planted along roadsides in the upland part of Nizhny Novgorod. It was impossible to study exactly the same set of polluted plots in all years of observation because of the long periods of road repair work. Thus, the set of polluted plots varied slightly in some years. However, we tried, as far as possible, to maintain approximately the same range of traffic intensity in the study sites and plot number.

The control plots were far from pollution sources near the village of Kiselikha, 20 km north of Nizhny Novgorod (*B. pendula*), and in Forest Park Shchelkovsky (*T. cordata*) situated in the upland part of the city.

The studied polluted plots were located at a distance of 1–3 meters from the road and had similar soil conditions, as well as control plots (sod-podzolic soils with mixed upper horizons, normal moisture regime). The soil type is known to be the same in the upland part of the city [51].

2.2. Estimation of Traffic Air Pollution

We previously demonstrated that traffic intensity had a high correlation with the content of the main pollutants (oxides of sulphur, nitrogen and carbon as well as benzene, kerosene, benzo[a]pyrene, and formaldehyde) in the air along roads in Nizhny Novgorod ($r = 0.8–0.9$; $p < 0.05$). We also revealed the dependences of different plant parameters such as seed production indices, phenological traits, photosynthetic pigment content, leaf FA in *B. pendula* [34] and lipid peroxidation rate in *B. pendula* and *T. cordata* leaf [52] on traffic intensity. These facts confirm that the traffic intensity is an objective indicator of air pollution levels.

Therefore, air pollution was estimated by the traffic intensity (vehicles per hour). The traffic intensity was the median of vehicles per hour, counted thrice a weekday: in the morning (from 8 until 10); in the afternoon (from 12 until 15); and in the evening (from 17 until 19). Plot location was chosen so that traffic intensity varied within a wide range, with the minimum and maximum values differing by a factor of several dozens.

2.3. Leaf Trait Selection and Estimation of FA Integrated Index

The sun-lit leaves of *T. cordata* and *B. pendula* were collected from unshaded areas of the tree crown facing the road.

The stages of plant development and leaf growth were also considered during the study, and only parameters of mature reproductive plants (g2) were analyzed. Tree leaves were collected in the second half of July. During this period, most leaves reach the full size but do not yet start to senesce. Therefore, the influence of growth and senescence processes on studied leaf traits can be excluded [34].

We collected leaf samples at a height of 1–3 m. Leaves were sampled outside the branches. In most cases we collected leaves from the same trees every year. We sometimes used the other trees on the same plots, due to tree cutting and pruning. After scanning the leaves, we measured morphological traits of leaf electronic images (three repeated measurements) using a blind method (the operator had no information about the origin of samples) [53].

We measured four leaf traits in *T. cordata* (Figure 1, traits 1–4) and five leaf traits in *B. pendula* (Figure 2). In 2013, the number of *T. cordata* leaf traits was increased to seven (Figure 1, traits 5–7) to assess the influence of the trait number on the studied patterns.

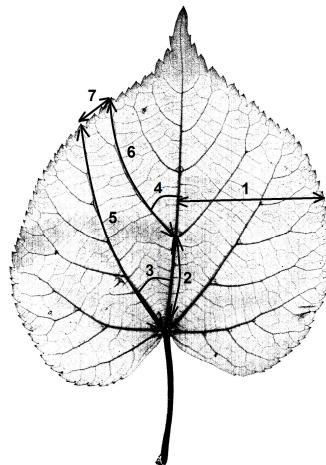


Figure 1. Traits of *T. cordata* leaf: 1. $1/2$ of the width of the leaf in the area of $1/2$ of the length of the central vein; 2. Distance between the bases of the first and the second from the bottom veins of the second order; 3. Angle between the central vein and the first from the bottom vein of the second order; 4. Angle between the central vein and the second from the bottom vein of the second order; 5. Length of the first from the bottom vein of the second order; 6. Length of the second from the bottom vein of the second order; 7. Distance between the ends of the first and the second from the bottom veins of the second order.

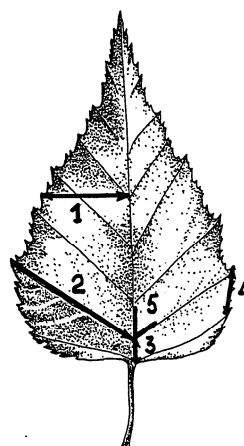


Figure 2. Traits of *B. pendula* leaf: 1. $1/2$ of the width of the leaf in the area of $1/2$ of the length of the central vein; 2. Length of the second from the bottom vein of the second order; 3. Distance between the bases of the first and the second from the bottom veins of the second order; 4. Distance between the ends of the first and the second from the bottom veins of the second order; 5. Angle between the central vein and the second from the bottom vein of the second order.

Many of studied leaf traits are widely used to estimate the level of leaf FA in trees, including *T. cordata* [30,31] and *B. pendula* [4,5].

In our study, the statistical unit was the leaf because different types of asymmetry were observed even in the leaves of the same tree. To estimate leaf asymmetry in *T. cordata* and *B. pendula*, ten leaves from each of ten trees growing in the plot (10 leaves $\times$ 10 trees, $n = 100$ /per plot) were collected. To assess the influence of sample sizes on identifying different types of asymmetry, the sample size of *T. cordata* leaves was increased to 200 for each plot in 2013 (20 leaves $\times$ 10 trees, $n = 200$ /per plot).

We calculated FA integrated index for each leaf sample using the normalized difference algorithm which eliminates the effect of leaf size on the value of this index [4]:

$$\bar{A} = \frac{1}{m \cdot n} \sum_{i=1}^m \sum_{j=1}^n \frac{|L_{ij} - R_{ij}|}{(L_{ij} + R_{ij})} \quad (1)$$

where L_{ij} and R_{ij} are the values of the j th trait on the right and left sides of the i th leaf, respectively; m —number of studied morphological traits; n —sample size (number of leaves).

Regression analysis (Statistica 10.0) was used to evaluate the dependence of FA integrated index on traffic intensity. Shapiro–Wilk’s test (Statistica 10.0) was applied to check data normality. We believed that regression model adequately described the relationship between the test parameters if p -value for the regression equation did not exceed 0.05 and the determination coefficient R^2 was greater than 0.5. Points outside the 95% confidence interval for values were excluded from regression analysis [54]. Sample medians were used for graphical data presentation. Kruskal–Wallis and nonparametric Newman–Keuls tests (Primer of Biostatistics 4.03) were applied for multiple comparisons of leaf asymmetry integrated index. The least significant difference was used for multiple comparison at the $p < 0.05$ level between impact and control medians.

2.4. Analysis of Leaf Trait Asymmetry Types

To reveal different types of leaf asymmetry, we used the Palmer and Strobeck (1992) criteria (Table 1).

Table 1. Criteria for different forms of bilateral asymmetry (by Palmer and Strobeck (1992) with our changes).

Form of Asymmetry	Median (L-R)	Shape of L-R Distribution	Differences between the Left (L) and Right (R) Traits
Ideal fluctuating asymmetry (FA)	0	Normal	$L = R$
Directional asymmetry (DA)	$\neq 0$	Normal	$L \neq R$
Antisymmetry (AS)	0	Platykurtic or bimodal (negative kurtosis)	$L = R$

Ideal FA has a normal L-R distribution and an L-R median (or mean) of 0 ($L = R$) (Table 1). To check for significant differences in L-R distribution from the normal curve, we calculated the skewness and kurtosis of the L-R distribution (Statistica 10.0) and compared them to critical values at $\alpha = 0.05$. We tested whether L-R median = 0 using the Mann–Whitney U test and carried out an analysis of the differences between the left and right value of leaf traits using the Wilcoxon matched pairs test (Statistica 10.0).

DA has a normal L-R distribution, the L-R median $\neq 0$ and the right and left trait values have significant differences ($L \neq R$). AS has a platykurtic or bimodal L-R distribution (negative kurtosis) and the L-R median = 0 ($R = L$) (Table 1).

We performed a mixture analysis of asymmetry, which allows the representation of an admixture of asymmetry types as a combination of normal distributions with different means and/or variances, and verification of the assumption of normality of their components [24,55]. For model selection we first determined the number of normal components that best described the distribution of the data (L-R). For this purpose, we used a generalized expectation maximization (EM) cluster analysis

(Statistica 10.0). After that, we tested if means of the selected components significantly differed from zero (i.e. DA) (Mann–Whitney U test, Statistica 10.0). If means of two directional components had opposite signs and significantly differed from each other (Mann–Whitney U test, Statistica 10.0) we attributed these components to AS. If the cluster had fewer than 10 values ($n < 10$) it was excluded from the analysis as an artifact.

The average proportion of leaves with FA for the studied leaf traits in each of the studied species (*T. cordata* and *B. pendula*) was calculated, in order to study the impact of anthropogenic load on FA transition with other types of asymmetry, including identifying the dependence of FA occurrence in the studied leaf traits on traffic intensity. To do this, at each plot in each year of observation, the proportion of leaves with FA for each studied leaf trait was found, and then the proportions were summed and divided by the number of leaf traits. Regression analysis (Statistica 10) was used to determine whether the average FA proportion depended on traffic intensity in each year of observation both in *T. cordata* and in *B. pendula*.

In addition, regression analysis was applied to determine the effect of FA transition to other types of asymmetry on FA integrated index, i.e. to study the relation of FA integrated index to the average proportion of leaves with FA in *T. cordata* and *B. pendula* in each year of observation.

The Chi-square test (Statistica 10) with Bonferroni correction in the case of multiple pairwise comparisons was applied to compare the average proportion of leaves with FA in the control and polluted plots.

3. Results

3.1. Analysis of Leaf Trait Asymmetry Type in *T. cordata* under Air Pollution

In 2010, leaf traits 1–4 had FA in most cases, but sometimes their asymmetry type changed under different traffic intensities. Trait 4 had DA in plots 2 and 5 and FA in the other plots. Traits 1–3 also had DA in some plots (plot 5, trait 1; plot 10, trait 2) (Table 2). We did not find AS in its pure form, but it existed in one leaf sample together with FA and DA (0.50FA, 0.11DA, 0.39AS), i.e., in this sample, the same trait could have different types of asymmetry in different leaves at the same pollution level (Table 2).

Table 2. Asymmetry forms of *T. cordata* leaf traits in 2010. FA—an ideal fluctuating asymmetry; DA—a directional asymmetry; AS—an antisymmetry. Asymmetry forms which were not FA given in bold.

Studied Plots, Their Numbers and Traffic Intensity	Trait Number			
	1	2	3	4
1. Forest Park Shchelkovsky (control plot) (0 veh/h)	FA	FA	FA	FA
2. Nizhni Novgorod Kremlin (63 veh/h)	FA	FA	DA	DA
3. Nevzorovyyh Street (375 veh/h)	FA	0.39AS; 0.11DA; FA 0.50FA		FA
4. Meditsinskaya Street (690 veh/h)	FA	FA	FA	FA
5. Timiryazeva Street (1302 veh/h)	DA	FA	FA	DA
6. Genkinoy Street (1233 veh/h)	FA	FA	FA	FA
7. Belinskogo Street (no data)	–	–	–	–
8. Gagarina Prospect (Lebedeva Street bus stop) (3768 veh/h)	DA	FA	FA	FA
9. Gagarina Prospect (University bus stop) (4050 veh/h)	FA	DA	FA	FA
10. Lyadov Square (5586 veh/h)	FA	DA	FA	FA

In 2011, different deviations from FA were revealed more frequently than in 2010. Besides FA, trait 1–4 had DA, AS or mixed FA and DA/AS (Table 3). In 2011, leaf traits 1–3 of trees in the control area had FA. However, trait 4 had DA (Table 3). Deviations from FA were observed quite often in polluted plots. At the same time, in addition to DA, AS was detected in one case (trait 3 in plot 10), as well as various types of mixed asymmetry, which were much more common than in 2010 (Table 2). In some cases, the type of asymmetry could not be identified, apparently due to the fact that there was a small admixture of leaves in the samples, in which this trait had a type of asymmetry differing from FA (Table 3).

Table 3. Asymmetry forms of *T. cordata* leaf traits in 2011. FA—an ideal fluctuating asymmetry; DA—a directional asymmetry; AS—an antisymmetry. ?—the asymmetry form has not been identified. Asymmetry forms which were not FA given in bold.

Studied Plots, Their Numbers and Traffic Intensity	Trait Number			
	1	2	3	4
1. Forest Park Shchelkovsky (control plot) (0 veh/h)	FA	FA	FA	DA
2. Nizhni Novgorod Kremlin (60 veh/h)	FA	FA	DA	DA
3. Nevzorovyh Street (291 veh/h)	FA	?	?	?
4. Meditsinskaya Street (801 veh/h)	FA	FA	FA	DA
5. Timiryazeva Street (1167 veh/h)	FA	0.88FA; 0.12DA	FA	FA
6. Genkinoy Street (1221 veh/h)	FA	0.54FA; 0.46DA	FA	FA
7. Belinskogo Street (2103 veh/h)	FA	FA	FA	0.76FA; 0.24DA
8. Gagarina Prospect (Lebedeva Street bus stop) (3552 veh/h)	FA	0.84FA; 0.16DA	0.69FA; 0.28DA	DA
9. Gagarina Prospect (University bus stop) (4455 veh/h)	DA	FA	DA	FA
10. Lyadova Square (5082 veh/h)	DA	FA	AS	DA

In 2013, an increase in the sample size from 100 to 200 leaves and in the trait number from four to seven did not change the situation significantly. Deviations from FA were observed quite frequently (Table 4). In the control plot, trees had a deviation from FA for traits 1 (DA) and 2 (0.77 FA; 0.23 AS). In case of road pollution, the transition from FA to DA or to various types of mixed asymmetry was detected for all studied traits in certain plots (Table 4). In 2013, we did not find AS in its pure form as in 2010.

Thus, for all studied leaf traits of *T. cordata* FA transitions to other types of asymmetry (mostly to DA) or mixed asymmetry were detected. Such transitions were observed both in the trees of polluted sites and in the control plot.

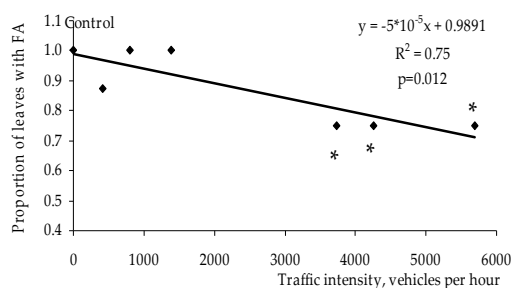
For leaf traits 1–4 (Figure 1), studied in all the years of research, it was shown that their asymmetry type in the same trees on both the control and polluted sites varied in different years of observation (Tables 2–4).

Table 4. Asymmetry forms of *T. cordata* leaf traits in 2013. FA—an ideal fluctuating asymmetry; DA—a directional asymmetry; AS—an antisymmetry. ?—the asymmetry form has not been identified. Asymmetry forms which were not FA given in bold.

Studied Plots, Their Numbers and Traffic Intensity	Trait Number						
	1	2	3	4	5	6	7
1. Forest Park Shchelkovsky (control plot) (0 veh/h)	DA	0.77FA 0.23AS	FA	FA	FA	FA	FA
2. Nizhni Novgorod Kremlin (89 veh/h)	?	FA	FA	DA	0.83DA 0.17AS	DA	FA
3. Nevzorovyh Street (336 veh/h)	FA	FA	FA	FA	DA	FA	FA
4. Meditsinskaya Street (750 veh/h)	FA	FA	FA	DA	FA	FA	FA
5. Timiryazeva Street (876 veh/h)	FA	0.72FA 0.28AS	FA	FA	FA	FA	DA
6. Genkinoy Street (1410 veh/h)	DA	0.92FA 0.08DA	DA	0.91FA 0.09DA	AS	0.60DA 0.40AS	FA
7. Belinskogo Street (2145 veh/h)	FA	0.58FA 0.42AS	DA	DA	FA	0.90FA 0.01AS	0.60DA 0.40AS
8. Gagarina Prospect (Lebedeva Street bus stop) (3642 veh/h)	FA	0.93FA 0.07DA	0.22DA 0.78AS	DA	DA	0.45DA 0.55AS	0.81FA 0.19AS
9. Gagarina Prospect (University bus stop) (4869 veh/h)	FA	0.60FA 0.40AS	FA	DA	FA	FA	DA
10. Lyadov Square (3888 veh/h)	?	?	FA	FA	AS	FA	FA

Regression analysis revealed the dependence of the average proportion of leaves with FA for the studied leaf traits on traffic intensity in all years of observation (Figure 3), that is, the impact of anthropogenic load on this index. At the same time, the functional forms of dependence differed in different years. In 2010, the proportion of FA decreased linearly with increase in pollution level (Figure 3), that corresponded to data from other authors [25]. However, in 2011, this indicator first increased and then decreased relative to control value. In 2013, on the contrary, the proportion of FA first decreased, and then increased almost to the control level (Figure 3).

Regression analysis showed dependence of FA integrated index on traffic intensity only in 2011 and 2013. An increase in traffic intensity caused a decrease in this parameter relative to the control (Figure 4) which contradicted the idea of increasing leaf FA with stressful environments [4]. At the same time, *T. cordata* FA integrated index did not depend on average proportion of leaves with FA ($R^2 < 0.50$; $p > 0.05$) in all years of observation, that is, on FA transitions to other types of asymmetry (data are not shown in Figures).



(a)

Figure 3. Cont.

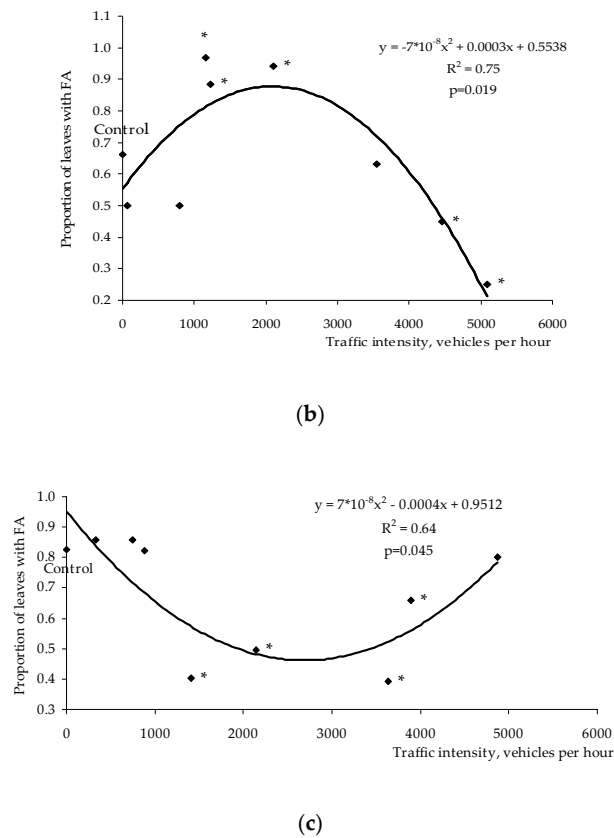


Figure 3. Dependence of the average proportion of *T. cordata* leaves with FA in the studied traits on traffic intensity in 2010 (a), 2011 (b) and 2013 (c). *—indicates significant differences between trees in control and polluted plots at $p < 0.05$.

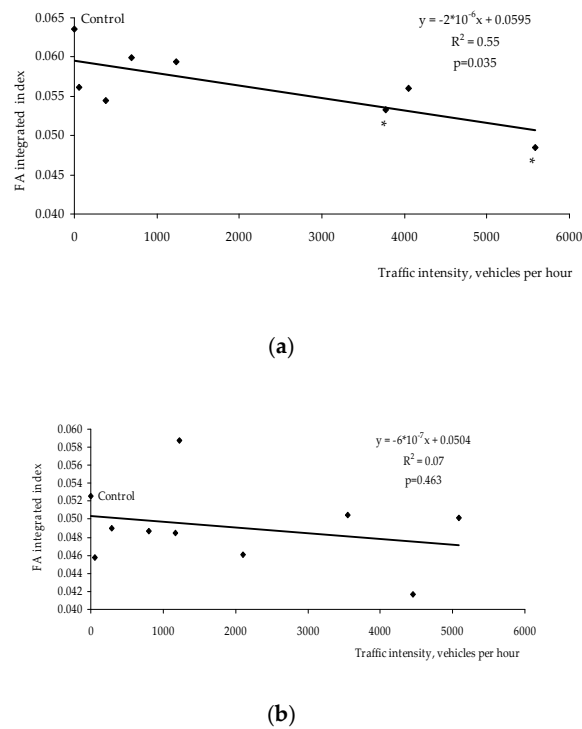
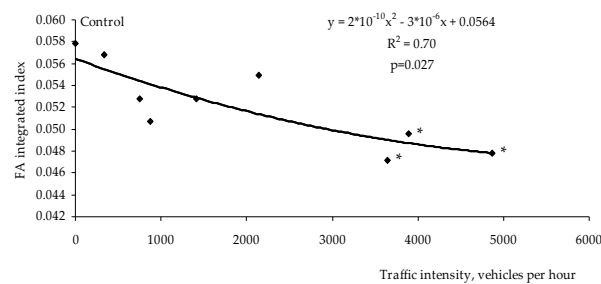


Figure 4. Cont.



(c)

Figure 4. Dependence of *T. cordata* FA integrated index on traffic intensity in 2010 (a), 2011 (b) and 2013 (c). *—indicates significant differences between trees in control and polluted plots at $p < 0.05$.

3.2. Analysis of Leaf Trait Asymmetry Type in *B. pendula* under Air Pollution

Similar to *T. cordata*, *B. pendula* had FA transitions to other types of asymmetry or to mixed types both in the control and polluted plots during all years of observation for all studied leaf traits (Tables 5–9). In addition, asymmetry types of studied leaf traits in the same trees varied in different years of observation (Tables 5–9).

Table 5. Asymmetry forms of *B. pendula* leaf traits in 2007. FA—an ideal fluctuating asymmetry; DA—a directional asymmetry; AS—an antisymmetry. Asymmetry forms which were not FA given in bold.

Studied Plots, Their Numbers and Traffic Intensity	Trait Number				
	1	2	3	4	5
1. Kiselikha Village (control) (0 veh/h)	FA	AS	AS	AS	FA
2. Krilova Street (59 veh/h)	DA	FA	FA	FA	0.9FA 0.1DA
3. Nizhni Novgorod Kremlin (108 veh/h)	0.71FA 0.29DA	FA	AS	AS	FA
4. Lomonosova Street (137 veh/h)	DA	FA	FA	FA	FA
5. Nesterova Street (303 veh/h)	FA	DA	FA	DA	DA
6. Nartova Street (399 veh/h)	FA	FA	FA	FA	0.73FA 0.27DA
7. Meditsinskaya Street (973 veh/h)	FA	FA	FA	FA	FA
8. Belinskogo Street (2204 veh/h)	FA	0.77FA 0.23AS	FA	FA	FA
9. Gagarina Prospect (Lebedeva bus stop) (3564 veh/h)	FA	FA	FA	FA	DA
10. Gagarina Prospect (University bus stop) (3964 veh/h)	FA	FA	FA	AS	FA

Table 6. Asymmetry forms of *B. pendula* leaf traits in 2008. FA—an ideal fluctuating asymmetry; DA—a directional asymmetry; AS—an antisymmetry. ?—the asymmetry form has not been identified. Asymmetry forms which were not FA given in bold.

Studied Plots, Their Numbers and Traffic Intensity	Trait Number				
	1	2	3	4	5
1. Kiselikha Village (control) (0 veh/h)	DA	FA	FA	FA	DA
2. Melnikova-Pecherskogo Street (4 veh/h)	FA	FA	FA	?	FA
3. Nizhni Novgorod Kremlin (72 veh/h)	FA	FA	FA	FA	DA
4. Lomonosova Street (162 veh/h)	FA	FA	FA	FA	DA
5. Hevzorovyh Street (293 veh/h)	DA	DA	FA	FA	DA
6. Nartova Street (477 veh/h)	FA	FA	DA	FA	DA
7. Meditsinskaya Street (989 veh/h)	FA	FA	DA	DA	DA
8. Timiryazeva Street (1434 veh/h)	?	DA	FA	FA	DA
8. Belinskogo Street (2333 veh/h)	FA	FA	?	FA	FA
9. Gagarina Prospect (Lebedeva bus stop) (3135 veh/h)	FA	FA	FA	FA	DA
10. Gagarina Prospect (University bus stop) (3784 veh/h)	DA	DA	FA	FA	DA

Table 7. Asymmetry forms of *B. pendula* leaf traits in 2010. FA—an ideal fluctuating asymmetry; DA—a directional asymmetry; AS—an antisymmetry. Asymmetry forms which were not FA given in bold.

Studied Plots, Their Numbers and Traffic Intensity	Trait Number				
	1	2	3	4	5
1. Kiselikha Village (control) (0 veh/h)	FA	FA	AS	AS	FA
2. Melnikova-Pecherskogo Street (51 veh/h)	FA	FA	AS	AS	FA
3. Nizhni Novgorod Kremlin (63 veh/h)	DA	AS	FA	FA	DA
4. Lomonosova Street (153 veh/h)	AS	DA	AS	FA	FA
5. Nevzorovih Street (282 veh/h)	FA	FA	0.33 FA 0.67 DA	FA	FA
6. Nartova Street (573 veh/h)	ϕA	AC	AC	AC	ϕA
7. Meditsinskaya Street (618 veh/h)	DA	FA	FA	DA	DA
8. Timiryazeva Street (1239 veh/h)	AS	FA	FA	DA	DA
8. Belinskogo Street (2706 veh/h)	FA	FA	FA	FA	AS
9. Gagarina Prospect (Lebedeva bus stop) (3291 veh/h)	AS	FA	AS	FA	AS
10. Gagarina Prospect (University bus stop) (3990 veh/h)	FA	FA	FA	DA	FA

Table 8. Asymmetry forms of *B. pendula* leaf traits in 2011. FA—an ideal fluctuating asymmetry; DA—a directional asymmetry; AS—an antisymmetry. Asymmetry forms which were not FA given in bold.

Studied Plots, Their Numbers and Traffic Intensity	Trait Number				
	1	2	3	4	5
1. Kiselikha Village (control) (0 veh/h)	FA	FA	AS	AS	FA
2. Nizhni Novgorod Kremlin (60 veh/h)	FA	DA	FA	AS	FA
3. Melnikova-Pecherskogo Street (84 veh/h)	FA	FA	FA	DA	FA
4. Lomonosova Street (120 veh/h)	FA	DA	DA	DA	AS
5. Nevzorovih Street (186 veh/h)	FA	DA	DA	DA	AS
6. Nartova Street (591 veh/h)	DA	FA	FA	FA	AS
7. Meditsinskaya Street (915 veh/h)	DA	AS	AS	AS	FA
8. Tmiryazeva Street (1167 veh/h)	DA	FA	FA	FA	DA
8. Belinskogo Street (2103 veh/h)	FA	0.81 FA; 0.19 DA	0.80 FA; 0.20 DA	FA	FA
9. Gagarina Prospect (Lebedeva bus stop) (3552 veh/h)	FA	DA	FA	FA	AS
10. Gagarina Prospect (University bus stop) (4455 veh/h)	FA	FA	DA	FA	FA

Table 9. Asymmetry forms of *B. pendula* leaf traits in 2012. FA—an ideal fluctuating asymmetry; DA—a directional asymmetry; AS—an antisymmetry. ?—the asymmetry form has not been identified. Asymmetry forms which were not FA given in bold.

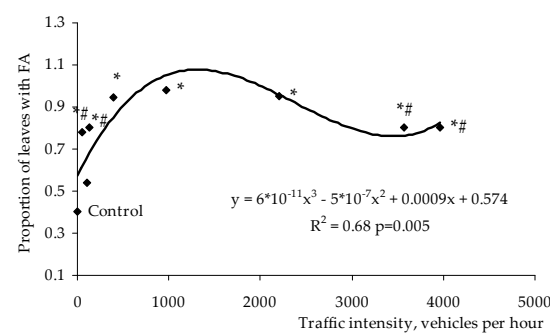
Studied Plots, Their Numbers and Traffic Intensity	Trait Number				
	1	2	3	4	5
1. Kiselikha Village (control) (0 veh/h)	DA	DA	?	FA	DA
2. Campus of Lobachevski University (6 veh/h)	DA	AS	FA	DA	DA
3. Nizhni Novgorod Kremlin (102 veh/h)	0.60 FA 0.40 DA	AS	FA	FA	DA
4. Nevzorovih Street (285 veh/h)	FA	DA	FA	FA	DA
5. Nartova Street (663 veh/h)	FA	AS	FA	FA	FA
6. Meditsinskaya Street (915 veh/h)	DA	DA	FA	FA	DA
7. Tmiryazeva Street (1308 veh/h)	FA	FA	FA	FA	DA
8. Belinskogo Street (2487 veh/h)	0.71 FA 0.29 DA	FA	FA	FA	DA
9. Gagarina Prospect (Lebedeva bus stop) (3762 veh/h)	?	FA	DA	FA	0.80 FA 0.20 DA
10. Gagarina Prospect (University bus stop) (4245 veh/h)	FA	DA	DA	FA	0.90 FA 0.10 DA

We compared a frequency of different asymmetry types in *T. cordata* and *B. pendula* for the same leaf traits using data obtained in the same years (2010–2011, total data for all plots). Proportions of FA and DA in these species were the same (Table 10). However, AS was more often observed in *B. pendula*, and mixed asymmetry was detected more often in *T. cordata* (Table 10).

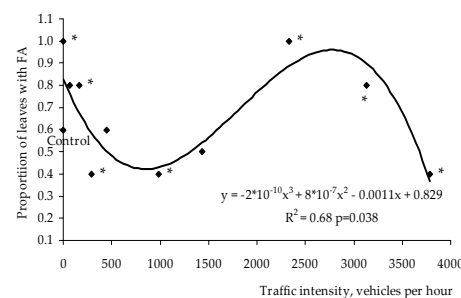
Regression analysis showed the dependence of the average proportion of leaves with FA for the studied leaf traits on traffic intensity in 2007, 2008 and 2012 (Figure 5), that is, the impact of anthropogenic load on the frequency of FA transitions to other types of asymmetry. All revealed dependencies were non-monotonic (Figure 5). In 2010–2011, dependencies were not observed ($R^2 < 0.50$; $p > 0.05$) (data not shown in Figures).

Table 10. Frequency of different leaf asymmetry types in *B. pendula* and *T. cordata* in the same years of observation (2010–2011). FA—an ideal fluctuating asymmetry; DA—a directional asymmetry; AS—an antisymmetry. For each type of asymmetry, the frequency is calculated using the total data of all plots and traits in 2010–2011. * Indicates significant differences between *T. cordata* and *B. pendula* at $p < 0.05$.

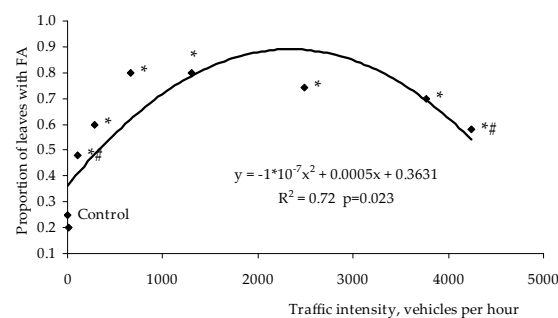
Plant Species, Sample Size	Asymmetry Type				Any Deviations from FA
	FA	DA	AS	Any Types of Mixed Asymmetry	
<i>B. pendula</i> (n = 65)	0.56 ± 0.06	0.17 ± 0.05	0.25 ± 0.05	0.03 ± 0.02	0.45 ± 0.06
<i>T. cordata</i> (n = 56)	0.68 ± 0.06	0.20 ± 0.05	0 *	0.13 ± 0.05	0.32 ± 0.06



(a)



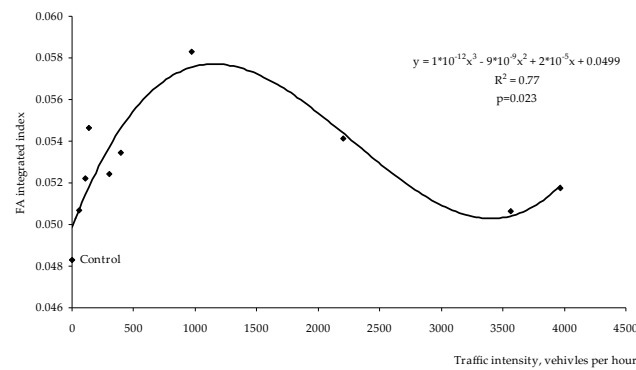
(b)



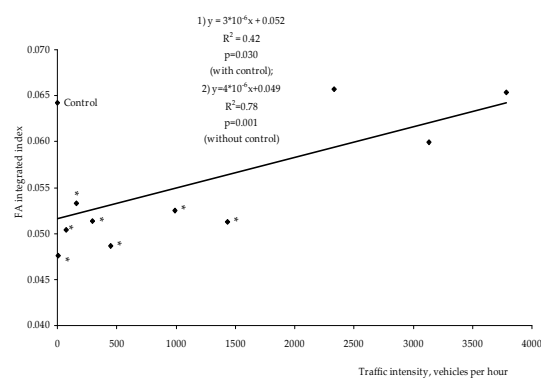
(c)

Figure 5. Dependence of the average proportion of *B. pendula* leaves with FA in the studied traits on traffic intensity in 2007 (a), 2008 (b) and 2012 (c). *—indicates significant differences between trees in control and polluted plots at $p < 0.05$; #—indicates significant differences in relation to the maximum of the dependence.

Regression analysis revealed a dependence of *B. pendula* FA-integrated index on traffic intensity in all observation years. Unlike *T. cordata*, this parameter for *B. pendula* increased with air pollution in most cases (Figures 6 and 7) that corresponded to the idea of increasing leaf FA under stressful environments [4]. Similar to *T. cordata*, *B. pendula* FA integrated index did not depend on average proportion of leaves with FA ($R^2 < 0.50$; $p > 0.05$) in all years of observation, that is, on FA transitions to other forms of asymmetry (data not shown in Figures).

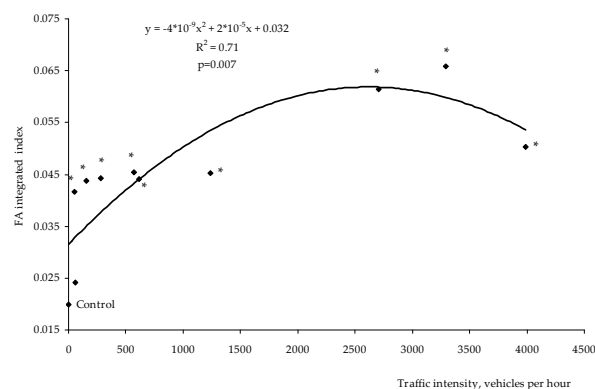


(a)



(b)

Figure 6. Dependence of *B. pendula* FA integrated index on traffic intensity in 2007 (a) and in 2008 (b) ([34], with changes). *—indicates significant differences between trees in control and polluted plots at $p < 0.05$.



(a)

Figure 7. Cont.

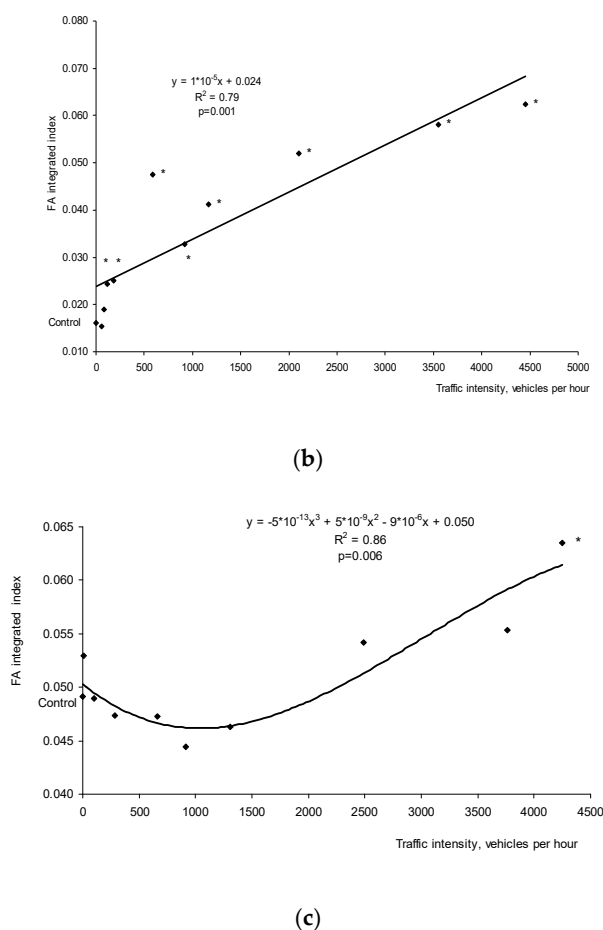


Figure 7. Dependence of *B. pendula* FA integrated index on traffic intensity in 2010 (a), 2011 (b) and 2012 (c) ([34], with changes). *—indicates significant differences between trees in control and polluted plots at $p < 0.05$.

4. Discussion

In the present work, we showed that all the studied leaf traits of *T. cordata* and *B. pendula* changed the asymmetry type both in trees that grew in polluted plots and control ones, although previously FA for these leaf traits was reported by other authors who determined the leaf asymmetry once [4,30,31]. Our results indicate that leaf asymmetry type needs to be evaluated multiple times. Apparently, FA transition to other asymmetry types was due, not only anthropogenic load, but also to weather variation in the control plots in different years. It was observed that adverse weather conditions impacted the leaf bilateral asymmetry [17,18]. This fact was also considered in detail in our previous studies [34,56].

Moreover, FA transitions to other asymmetry types were species specific, despite the fact that the total percentage of deviations from FA was the same for two species. *T. cordata* most often had an equally likely transition from FA to DA and various types of mixed asymmetry, which corresponded to results of other studies for animals [23–25] and plants [26]. *B. pendula*, in contrast, rarely had a mixed asymmetry, and FA transitions to DA and AS were observed with the same frequency.

For the most years of observations, the high possibility of traffic air pollution impact on FA transitions to other asymmetry types in *T. cordata* and *B. pendula* was established. Despite this, an increase in anthropogenic load did not always lead to an increase in the probability of FA switching to DA and/or AS, which is believed to be observed under the exposure to environmental stressors [25]. *T. cordata* had both a linear decrease in FA proportion in the studied leaf traits with an increase in traffic intensity (in 2010), and a non-monotonic changes of this parameter (in 2011 and 2013). In *B.*

pendula, similar dependencies were only non-monotonic. Some of the non-monotonic responses of *T. cordata* (in 2011) and *B. pendula* (in 2007 and 2012) corresponded to hormesis, whereby low pollution levels caused an increase in FA proportion, while high pollution reduced it relative to the control level. Other dependencies were typical two-phase or multi-phase paradoxical effects (*T. cordata* in 2013; *B. pendula* in 2008). Apparently, only strong external stressors caused a linear decrease in the proportion of leaves with FA. It was reported that significant stress led to a switch from FA to DA and/or AS [25]. For instance, in 2010, similar change of this *T. cordata* parameter was induced by combination of traffic air pollution and drought during the leaf growth period (in May–June of this year) [34]. *B. pendula* was less demanding of soil moisture compared to *T. cordata*, therefore, linear relationships were not observed even under drought conditions.

The high frequency of non-monotonic dose-effect relationships was revealed for various plant indices under stressful environments [37–39], including the effects of chemical pollutants on physiological and biochemical parameters [43,44,57], as well as on leaf FA [44,58]. In this study, we first found non-monotonic responses in development of leaf bilateral asymmetry type, that is, for leaf morphogenesis.

Our results indicated that DA and AS of *T. cordata* and *B. pendula* leaf traits were not exclusively hereditary and their development depended on environmental conditions. Apparently, the morphogenesis of leaf bilateral asymmetry is more plastic than it is considered, which may be due to the plasticity of epigenetic regulation of leaf structure development. It is currently known that epigenetic regulation, such as DNA methylation, histone modification, histone variants, chromatin remodeling, and small RNAs play crucial role as linkers between the environment and the genome in plants. They are involved in the regulation of leaf development [59–61] and its responses to abiotic stresses [62,63], including the development of leaf bilateral asymmetry [61]. Therefore, we assume that epigenetic mechanisms of leaf morphogenesis can be very plastic and the bilateral asymmetry type of the same leaf trait can change rather quickly under different environmental conditions.

We showed that the FA-integrated index of two plant species reacted differently to the increase in pollution level. In *B. pendula*, which was more sensitive to exhaust pollutants [32,33], there was a typical stressful increase in this parameter relative to the control over all years of observations, which indicated a disruption of leaf developmental stability. In *T. cordata*, which was more resistant to air pollution, by contrast, this index did not change (in 2010), and even decreased relative to control in 2011 and 2013, i.e., traffic pollution increased leaf development stability. Apparently, this was due to the stimulating effect in the first phase of hormesis.

At the same time, FA integrated index of *T. cordata* and *B. pendula* leaf did not depend on the frequency of FA transitions to other asymmetry types. Apparently, this was caused by insignificant leaf asymmetry under DA and AS. Perhaps, a significant asymmetry disrupted normal leaf functioning, and such leaves were eliminated by natural selection. It was reported that woody plants, including *B. pendula* and *T. cordata*, lost some amount of leaves in summer [64–67].

Therefore, in our study, we demonstrated the need for a long-term assessment of leaf trait asymmetry type in plants used in bioindication. We also emphasize the need to study the non-monotonic response role in the development of leaf asymmetry with pollution impact, and the identification of molecular mechanisms underlying FA transition to other types of asymmetry.

Author Contributions: E.A.E. and B.N.Y. performed research, statistical analysis, and wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Conflicts of Interest: The authors declare no conflict of interest.

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