

## Article

# Is Fluctuating Asymmetry a Sufficient Indicator of Stress Level in Two Lizard Species (*Zootoca vivipara* and *Lacerta agilis*) from Alpine Habitats?

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**Abstract:** Alpine habitats are exposed to increasing anthropogenic pressure and climate change. The negative impacts can lead to chronic stress that can affect the survival and reproductive success of individuals and even lead to population extinction. In this study, we analyse different morphological and ecological traits and indices of abiotic and biotic stressors (such as head size and shape, fluctuating asymmetry, body condition index, tail autotomy, and population abundance) in alpine and subalpine populations of two lacertid species (*Zootoca vivipara* and *Lacerta agilis*) from Serbia and North Macedonia. These lizards live under different conditions: allotopy/syntopy, different anthropogenic pressure, and different levels of habitat protection. We found differences between syntopic and allotopic populations in pileus size, body condition index (in both species), pileus shape, fluctuating asymmetry (in *L. agilis*), and abundance (in *Z. vivipara*). Differences between populations under anthropogenic pressure and populations without it were observed in pileus shape, body condition index (in both species), pileus size, fluctuating asymmetry, tail autotomy and abundance (in *L. agilis*). On the basis of our results, it is necessary to include other stress indicators in addition to fluctuating asymmetry to quickly observe and quantify the negative effects of threat factors and apply protective measures.

**Keywords:** sand lizard; viviparous lizard; head shape; body condition; population size; syntopy; allotopy; anthropogenic pressure



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## 1. Introduction

Alpine habitats are very important biodiversity hotspots with many relict and endemic species [1,2]. Alpine species are adapted to specific habitats and environmental conditions (montane meadows and pastures with specific climatic conditions such as short summers and long winters), but they are very sensitive to anthropogenic impacts (mass tourism, afforestation and deforestation, damming and channelisation of alpine rivers, and development of ski centres and road infrastructure) and especially to climate change [3–7]. The influence of negative factors in alpine habitats has been studied mainly on flora [5,8,9], but animals with small home ranges and low dispersal ability, such as amphibians and reptiles, can also be good bioindicators of environmental health [10]. Some of these populations are exposed to anthropogenic factors such as habitat loss and fragmentation, urbanisation, mountain tourism, and natural resource exploitation, in addition to natural stressors (competition within and between species for resources, and presence of predators). Changes in alpine habitats (especially urbanisation) have been shown to lead to changes in abiotic and biotic parameters related to lizard thermoregulation (which is critical to their activities),

as well as increases in predator abundance [11,12]. Adverse effects can lead to chronic stress that can affect the survival and reproductive success of individuals and even lead to population extinction [13–16].

There are numerous ways to determine the presence of stress, such as by examining: morphological structures, fluctuating asymmetry (FA), fitness indices, and population parameters [17–20]. In general, phenotype is influenced by several factors (mostly genotypes and environmental factors). Thus, detailed analysis of morphological structures such as the pileus (dorsal head scalation directly connected to the underlying cranial bones in reptiles) [21] can detect subtle differences between populations living in habitats with different conditions—allotopy/syntopy, presence/absence of environmental stressors, and optimal/suboptimal conditions [22,23]. Populations of closely related species living in syntopy can often undergo morphological divergence due to the character displacement [24–26], phenotypic plasticity, microhabitat shifts, resource availability, or interspecific competition [27–30].

During development, both sides of the body share the same genetic and environmental conditions [31], but deviations can be caused by many stressors [32–34]. Fluctuating asymmetry, as a measure of developmental instability, represents the random deviation from perfect symmetry in bilateral organisms [32] and is often used to determine population stress due to environmental disturbance [35,36]. It is widely used because it is a cost-effective and noninvasive method [37–40]. Another measure, such as body condition index (BCI), is commonly used to assess health and fitness and represents the physical or nutritional status of an individual, and body condition can be influenced by environmental stresses [41]. For example, individuals with a higher BCI have higher reproductive success and a higher chance of surviving during hibernation [42,43]. BCI may be affected by tail autotomy, which is a common antipredator behaviour in lizards [44], and frequencies of individuals with tail autotomy can indicate the level of predation [45]. Tail autotomy can increase the lizard's chances of escape, but may also have negative consequences, such as loss of lipid stores, low reproductive success, altered locomotor performance, lower social status, and reduced territory size [46–48]. Population characteristics such as abundance, density, and sex and age structure can also be indicators of habitat quality and the impact of negative factors [49–51].

The aim of this study is to analyse different morphological and ecological traits and indices of abiotic and biotic stress (such as size and shape of the pileus, FA, BCI, the tail autotomy, and population abundance) in alpine populations of two lacertid species (*Zootoca vivipara* and *Lacerta agilis*) from Serbia and North Macedonia. In this study, we hypothesised that, in populations exposed to higher stress, FA and frequency of tail autotomy will increase, while head size, BCI, and population abundance will decrease. Additionally, we expect differences in pileus shape between stressed and non-stressed populations.

## 2. Materials and Methods

### 2.1. Study Species

We selected two lizard species from alpine habitats: the sand lizard (*Lacerta agilis*) and the viviparous lizard (*Zootoca vivipara*). The first species is slightly larger (average snout-to-vent length (SVL) is 69–89 mm and 73–86 mm, for males and females, respectively), while the second is smaller (average SVL is 48–55 mm and 51–67 mm for males and females, respectively) [52,53]. Both species have a wide Palearctic distribution; in the Balkans, close to their southern distribution limit, they require specific open montane habitats with rich herbaceous cover (mostly above the upper forest limit) and are usually found in small isolated populations [53]. Additionally, both species have small home ranges and low dispersal ability [54,55].

## 2.2. Study Area

The research was conducted within the territory of two national parks (NP): Kopaonik on Kopaonik mountain (Serbia) with five localities (Gobelja, Karaman Greben, Kukavica, Nebeske stolice, and Treska) and Mavrovo on Bistra mountain (North Macedonia). Additional research was conducted at two unprotected sites in Divčibare and Mokra Gora mountains (Serbia). (Figure 1; Table 1).



**Figure 1.** Locality map of the analysed *L. agilis* and *Z. vivipara* populations.

Localities differ in habitat type, elevation, degree of anthropogenic influence, and degree of protection (Table 1). At the Kukavica and Nebeske stolice localities, *L. agilis* and *Z. vivipara* live in syntopy, while, at the Treska, Divčibare, and Bistra localities, *L. agilis* lives in allotopy, and, at the Karaman Greben, Gobelja, and Mokra Gora localities, *Z. vivipara* lives in allotopy.

Adult lizards were captured in July–September 2012–2013 at Bistra, in June 2017 at Divčibare, in June 2020 at Mokra Gora, and in August–September 2019–2022 at Kopaonik NP, totalling 227 *L. agilis* and 172 *Z. vivipara* (Table 1).

## 2.3. Analysis

The lizards were captured by hand, and body weight and length were measured for all individuals. Data on sex and presence of injuries (regenerated tail) were also recorded. Palpation of the abdomen was used to check for the presence of food and the reproductive status of females. Then, the pileus was photographed next to a scale. All photographs were taken with an Olympus 590UZ digital camera (by the first author). The pileus was centred in the optical field of view, and the distance between the camera and specimens, as well as camera settings, was kept constant to minimise the effects of distortion and parallax [56]. All individuals were then returned to the capture site.

**Table 1.** Sample size separated by sex (males—M and females—F) and habitat conditions for analysed species (*L. agilis* and *Z. vivipara*) for each studied locality.

Locality	<i>L. agilis</i>		<i>Z. vivipara</i>		Longitude, Latitude	Elevation (m asl)	Habitat Type and Vegetation	Level of Protection	Risk Factors
	M	F	M	F					
Kopaonik—Kukavica	27	31	10	15	43.329° N, 20.744° E	1606–1701	Alpine pasture mixed with alpine shrubs	1st degree of protection within the Kopaonik NP	No anthropogenic pressure
Kopaonik—Nebeske stolice	5	7	9	20	43.262° N, 20.836° E	1783–1877	Alpine pasture mixed with a rocky outcrop	2nd degree of protection within the Kopaonik NP	Heavy pressure from overcrowded tourists
Kopaonik—Treska	23	31	/	/	43.260° N, 20.785° E	1548–1594	Alpine pasture mixed with rocky outcrops	Just outside the borders of the Kopaonik NP	No anthropogenic pressure
Kopaonik—Gobelja	/	/	21	21	43.317° N, 20.823° E	1809–1917	Meadow mixed (low alpine shrub vegetation at the higher end mixed with spruce at the lower end)	1st degree of protection within the Kopaonik NP	Illegal wild berry picking practices, quad biking
Kopaonik—Karaman greben	/	/	21	33	43.291° N, 20.829° E	1882–1927	A linear habitat of alpine shrub vegetation	3rd degree of protection within the Kopaonik NP	Large pressure (heavy machinery at multiple construction sites, skiing infrastructure), quad biking
Divčibare (Serbia)	18	16	/	/	44.108° N, 19.990° E	960	Subalpine pasture	No protection	Popular touristic destination under considerable pressure due to urbanisation, communal waste pollution, etc.
Mokra Gora (Serbia)	/	/	5	17	42.837° N, 20.361° E	1947	Very fragmented alpine pastures and meadows	No protection	No anthropogenic pressure
Bistra (North Macedonia)	26	43	/	/	41.618° N, 20.660° E	2026	Alpine pasture mixed with rocky outcrops	Part of the Mavrovo NP	No anthropogenic pressure

We used 2D geometric morphometry to analyse the size and shape of the pileus. This method has been shown to be very effective in detecting subtle changes in shape and is especially effective in enabling independent analysis and visualisation of the size and shape of the selected morphological structure [57]. We selected 28 points on the pileus and the constellation of landmarks is shown in Figure S1. Landmarks were digitised using TpsDig2 [58], and landmark recording was repeated twice for each animal (by the first author). We assessed the measurement error from Procrustes ANOVA, by comparing the MS (mean sum of squares) error with the MS individual-by-side interaction [57,59], and measurement error values were several times lower than those for individual-by-side interactions (Table S1). Centroid size (CS), obtained in the Coordgen program, IMP series [60], was used as a measure of size. Differences between sexes and localities in CS were tested by the ANOVA followed by the Tukey HSD test. A principal component analysis (PCA) on covariance matrices of the symmetric component was performed for preliminary analysis of pileus shape. Discriminant analysis (DA) was used to determine differences in shape between sexes and localities (on raw and size-corrected data, residuals from multivariate regression of shape variables on CS). For the comparison of FA between localities, we calculated the FA10a index using the formula  $[(\text{SQRT}(\text{FA MS} - \text{Error MS})) \times 0.798]$  [33] for each population (with pulled sexes). We tested the statistical significance of differences in shape FA scores between populations by Levene's test and Tukey HSD test [61].

BCI was calculated as the residuals from the linear regression between the natural logarithms of body mass and SVL, separately for each species and sex [62]. Differences in BCI between sexes and localities were tested using the ANOVA test. Gravid individuals and individuals without a large portion of the tail were excluded from analyses. In the population of *Z. vivipara* from Mokra Gora, few individuals remained after exclusion of individuals; hence, this population was not included in the body condition index analyses.

Differences in the frequency of individuals with tail autotomy between populations were tested using the chi-square test. The associations among CS, FA scores, and BCI were tested by Pearson's correlation test. CS, FA scores and BCI values were plotted against elevation and latitude, to demonstrate if the patterns between localities with and without anthropogenic pressure change in relation to geographic data.

From 2019 to 2022, we counted all individuals of both lizard species on fixed transects at all localities in Kopaonik NP. We expressed abundance as the number of individuals per researcher per one kilometre (because the length of transects and the number of researchers were not the same at all localities and between years). Unfortunately, transect lengths were not recorded at the localities Bistra, Divčibare, and Mokra Gora; thus, we could not estimate population abundances.

Procrustes superimposition, Procrustes ANOVA, PCA, DA, and multivariate regression (size correction) were performed using MorphoJ software [63], while ANOVA, Levene's test, Tukey HSD test, linear regression (for BCI), Pearson's correlation test, and chi-square test were performed in STATISTICA 10.

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### 3. Results

#### 3.1. Differences in Pileus Size

In *L. agilis* ANOVA showed that there were statistically significant differences in size between the sexes, with males having a larger pileus than females ( $F = 5.99$ ,  $p = 0.015$ ), but no sexual dimorphism in size was observed in *Z. vivipara* ( $F = 1.91$ ,  $p = 0.183$ ). The ANOVA also showed significant differences in size between localities in *L. agilis* ( $F = 8.269$ ,  $p = 0.001$ ;  $F = 6.148$ ,  $p = 0.001$ , for males and females, respectively) (Table 2). The highest values of CS were recorded for *L. agilis* from the Divčibare and Bistra populations, with the lowest recorded for the Kukavica population (Table S2). Populations of *Z. vivipara* differed

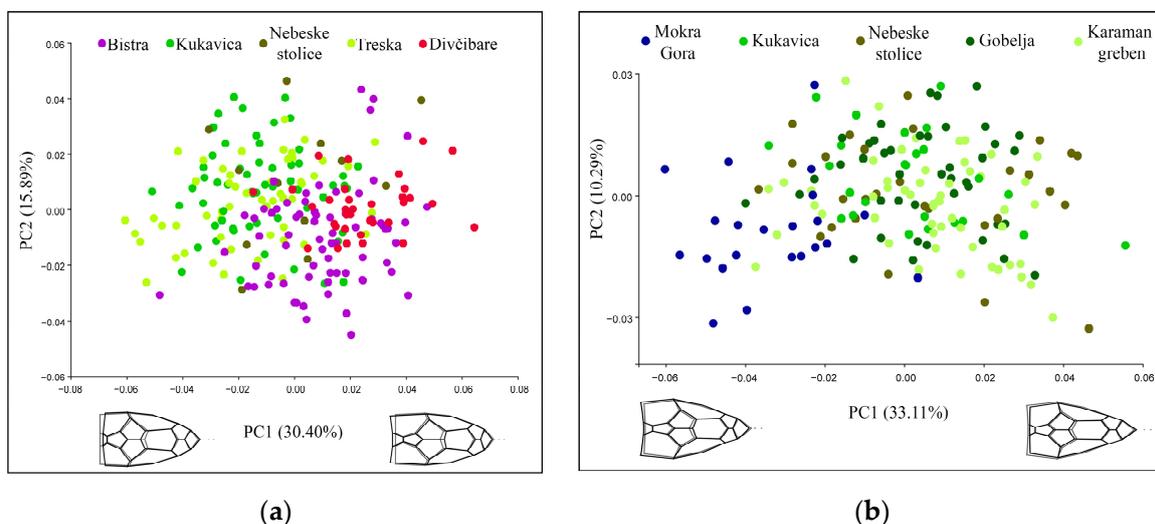
in size ( $F = 6.74$ ,  $p = 0.001$ ) (Table 2), and the highest CS values were recorded at Kukavica, with the lowest values recorded at Mokra Gora (Table S2). Differences in size between syntopic and allotopic populations were observed in *L. agilis* ( $F = 14.326$ ,  $p = 0.001$  and  $F = 11.682$ ,  $p = 0.001$  for males and females respectively), with higher CS values recorded in allotopic populations. In *Z. vivipara*, these differences were recorded only in males ( $F = 5.38$ ,  $p = 0.024$ ), where CS values were also higher in allotopic populations. Differences in pileus size between localities with anthropogenic pressure and localities without anthropogenic pressure were recorded only in *L. agilis* males ( $F = 17.850$ ,  $p = 0.001$ ), with higher CS values in populations with anthropogenic pressure.

**Table 2.** The pairwise comparisons of pileus size between populations (Tukey HSD test) for: (a) *L. agilis* (below the diagonal of the table are the values in females and above the diagonal are the values in males) and (b) *Z. vivipara* (the sexes are pooled because there is no sexual dimorphism in size).

(a)					
Locality	Kukavica	Nebeske stolice	Treska	Bistra	Divčibare
Kukavica		0.2224	0.1747	0.0114	0.0001
Nebeske stolice	0.3291		0.9302	0.9992	0.6655
Treska	0.4621	0.9066		0.8736	0.0084
Bistra	0.0001	0.9340	0.0292		0.0773
Divčibare	0.0362	0.9996	0.5779	0.9324	
(b)					
Locality	Gobelja	Karaman greben	Kukavica	Nebeske stolice	
Karaman greben	0.3835				
Kukavica	0.1658	0.0011			
Nebeske stolice	0.7716	0.9959	0.0165		
Mokra Gora	0.0124	0.3347	0.0001	0.2788	

### 3.2. Differences in Pileus Shape

Discriminant analysis showed that there are significant differences in the pileus shape between the sexes in both species (Procrustes distance—PD = 0.016,  $p = 0.0001$ ; PD = 0.014,  $p = 0.0001$ , in *L. agilis* and *Z. vivipara*, respectively). The effect of size on shape was significant in both species (14.77%,  $p = 0.0001$ ; 3.08%,  $p = 0.0001$  in *L. agilis* and *Z. vivipara*, respectively). After size correction, sexual dimorphism in the pileus shape remained statistically significant in both species (PD = 0.012,  $p = 0.0001$ ; PD = 0.014,  $p = 0.0001$ , in *L. agilis* and *Z. vivipara*, respectively). In the PCA graphs for both species (Figure 2), the populations from Kopaonik NP were grouped together, while the other populations were separated from them along the first principal component axis.



**Figure 2.** Principal component analysis (PCA) shows relationships between populations for (a) *L. agilis* and (b) *Z. vivipara*.

Pairwise comparisons of Procrustes distances between populations confirmed that *L. agilis* populations from Bistra and Divčibare differed in pileus shape from populations from Kopaonik NP. Additionally, differences in pileus shape were found in males between Kukavica and Treska, while Nebeske stolice differed from Treska and Kukavica in females (Table 3). In *Z. vivipara* females, the population from Mokra Gora differed from all other populations; however, in males, the differences between populations were more pronounced (exceptions are the differences between populations from Nebeske stolice and other populations, as well as between Mokra Gora and Kukavica) (Table 3). Discriminant analysis of size-corrected data showed almost the same pattern in *Z. vivipara* and significant differences in pileus shape between all *L. agilis* populations (Table S3). When we compared populations under anthropogenic pressure with populations without such pressure (see Table 1), statistically significant differences were observed in both species (PD = 0.028,  $p = 0.0001$  and PD = 0.022,  $p = 0.0001$  in *L. agilis* and *Z. vivipara*, respectively). Differences between syntopic and allotopic populations were expressed in *L. agilis* (PD = 0.020,  $p = 0.0001$ ), but not in *Z. vivipara* (PD = 0.008,  $p = 0.1600$ ).

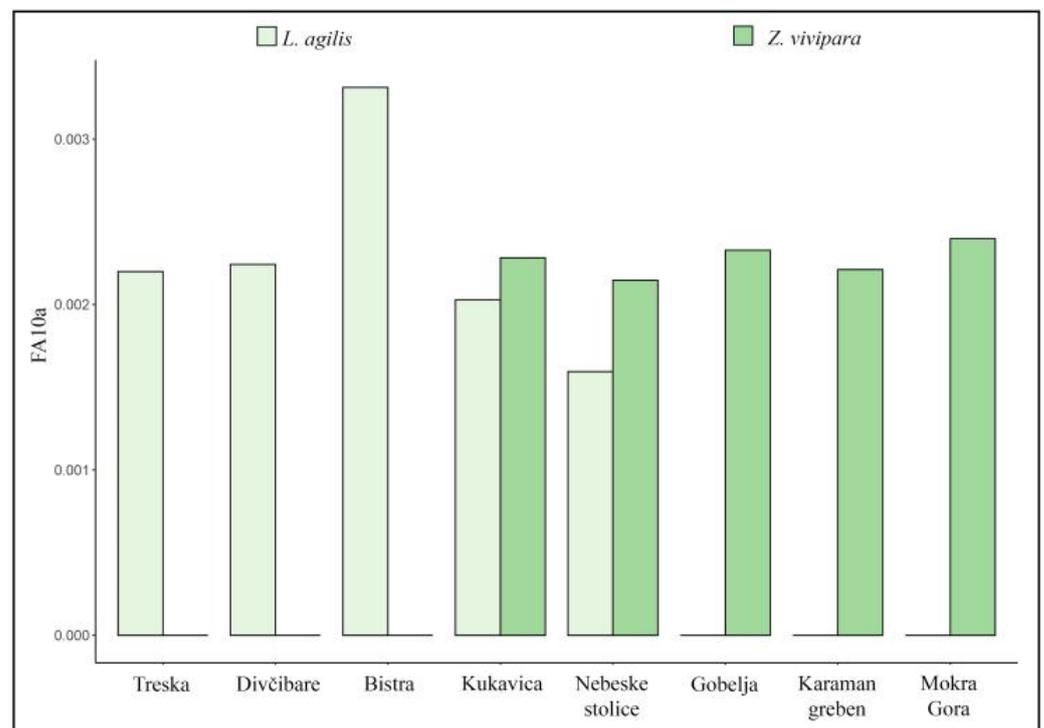
**Table 3.** The values of Procrustes distances (PD) and p values obtained by discriminant analysis in comparisons between the localities (below the diagonal of the table are the values in females and above the diagonal are the values in males) in (a) *L. agilis* and (b) *Z. vivipara*.

(a)					
Locality	Kukavica	Nebeske stolice	Treska	Bistra	Divčibare
Kukavica		0.035/ 0.0001	0.013/ 0.1010	0.038/ 0.0001	0.048/ 0.0001
Nebeske stolice	0.015/ 0.5850		0.032/ 0.0130	0.037/ 0.0030	0.029/ 0.0080
Treska	0.016/ 0.0190	0.018/ 0.2990		0.033/ 0.0001	0.044/ 0.0001
Bistra	0.031/ 0.0001	0.033/ 0.0001	0.035/ 0.0001		0.031/ 0.0001
Divčibare	0.031/ 0.0001	0.033/ 0.0001	0.041/ 0.0001	0.028/ 0.0001	
(b)					
Locality	Gobelja	Karaman greben	Kukavica	Nebeske stolice	Mokra Gora
Gobelja		0.014/ 0.0120	0.021/ 0.0030	0.012/ 0.5290	0.035/ 0.0001
Karaman greben	0.009/ 0.5060		0.021/ 0.0090	0.017/ 0.1690	0.035/ 0.0010
Kukavica	0.015/ 0.1390	0.015/ 0.0700		0.022/ 0.1120	0.023/ 0.1210
Nebeske stolice	0.013/ 0.2360	0.009/ 0.5860	0.018/ 0.0630		0.035/ 0.0380
Mokra Gora	0.040/ 0.0001	0.042/ 0.0001	0.047/ 0.0001	0.044/ 0.0001	

### 3.3. Fluctuating Asymmetry

Levene's test on FA shape scores showed that there were statistical differences in FA levels among *L. agilis* populations ( $F_{4, 222} = 8.169$ ;  $p = 0.001$ ), while the Bistra population had much higher FA values than the other populations (confirmed by Tukey HSD test). There were no differences in FA levels among *Z. vivipara* populations ( $F_{4, 167} = 0.199$ ;  $p = 0.938$ ) (Figure 3).

Differences in FA scores between syntopic and allotopic populations ( $F_{1, 225} = 14.026$ ;  $p = 0.001$ ) and between populations with and without anthropogenic pressure ( $F_{1, 225} = 5.587$ ;  $p = 0.019$ ) were found only in *L. agilis*. Higher FA scores were recorded in allotopic populations compared to syntopic populations and at localities with anthropogenic pressure compared to localities without anthropogenic pressure.



**Figure 3.** FA10a index values for *L. agilis* and *Z. vivipara* populations.

### 3.4. Body Condition Index

ANOVA showed that there was a statistically significant difference in BCI between the sexes ( $F = 71.471$ ,  $p = 0.001$ , and  $F = 113.141$ ,  $p = 0.001$  in *L. agilis* and *Z. vivipara*, respectively); therefore, the comparison between localities was performed separately for both sexes. The statistically significant differences in BCI between localities were observed for both sexes in *L. agilis* ( $F = 6.268$ ,  $p = 0.001$ ,  $F = 5.922$ ,  $p = 0.001$  for males and females, respectively), and in *Z. vivipara* ( $F = 5.266$ ,  $p = 0.003$  and  $F = 3.690$ ,  $p = 0.015$  for males and females, respectively) (Table 4). *Lacerta agilis* populations from Divčibare and Bistra were found to have lower BCI than populations from Kopaonik NP. In *Z. vivipara*, the highest BCI was recorded at Kukavica and the lowest at Gobelja (in females) and Karaman Greben (in males) (Figure S2). ANOVA also showed a statistically significant difference in BCI between syntopic and allotopic populations in *Z. vivipara* ( $F = 12.802$ ,  $p = 0.001$  and  $F = 5.395$ ,  $p = 0.023$  for males and females, respectively) and in *L. agilis* ( $F = 4.353$ ,  $p = 0.039$  for females, but not for males  $F = 1.925$ ,  $p = 1.168$ ). Significant differences in BCI were found in *L. agilis* ( $F = 6.158$ ,  $p = 0.015$ ,  $F = 6.599$ ,  $p = 0.012$  for males and females, respectively) and *Z. vivipara* ( $F = 6.099$ ,  $p = 0.016$  and  $F = 10.804$ ,  $p = 0.002$  for males and females, respectively) between sites with and without anthropogenic impacts. Higher BCI values were recorded in syntopic populations compared to allotopic populations and at localities without anthropogenic influence compared to localities with anthropogenic influence.

The correlation among CS, BCI, and FA was not statistically significant in the analysed species (Table S4). The patterns of distribution of CS, BCI and FA scores between the localities with and without the anthropogenic pressure did not change in relation to elevation or latitude (Figure S3).

**Table 4.** Pairwise comparisons of BCI among populations (Tukey HSD test). Below the diagonal of the table are the values for females and above the diagonal are the values for males, for (a) *L. agilis* and (b) *Z. vivipara*.

(a)					
Locality	Kukavica	Nebeske stolice	Treska	Divčibare	Bistra
Kukavica		0.9971	0.8852	0.0089	0.0495
Nebeske stolice	0.9622		0.9115	0.5000	0.7876
Treska	0.6434	0.5371		0.0008	0.0047
Divčibare	0.0170	0.4928	0.0006		0.8913
Bistra	0.1544	0.9624	0.0037	0.5482	
(b)					
Locality	Gobelja	Kukavica	Nebeske stolice	Karaman greben	
Gobelja		0.0169	0.4636	0.8226	
Kukavica	0.0087		0.4860	0.0031	
Nebeske stolice	0.2007	0.4643		0.1622	
Karaman greben	0.2743	0.2426	0.9843		

### 3.5. Tail Autotomy

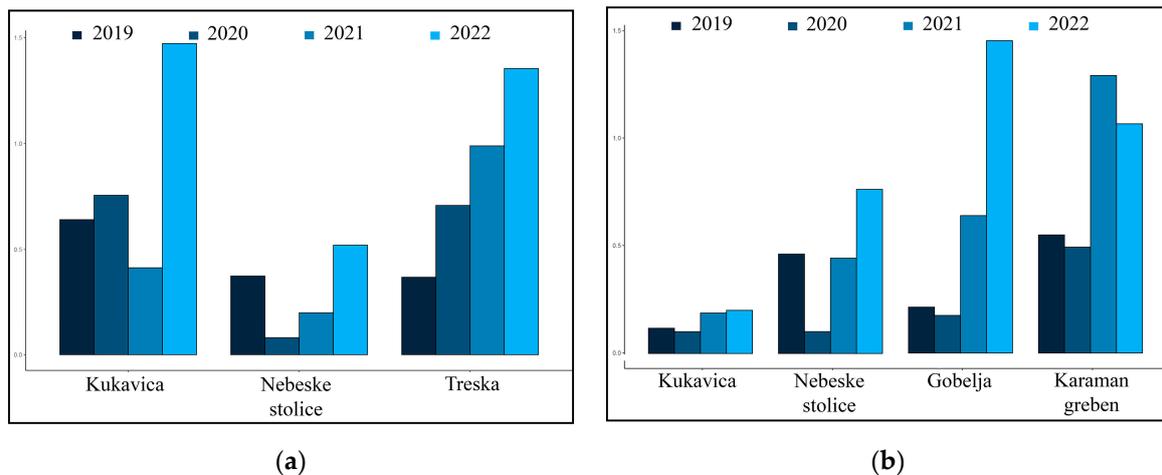
In *L. agilis* populations, the percentage of individuals with a regenerated tail per population varied from 24% to 65% (Table 5), and the chi-square test showed that the frequency of individuals with regenerated tails was significantly higher at Divčibare compared to all other populations except Nebeske stolice. The percentages of individuals with regenerated tails in *Z. vivipara* populations ranged from 40% to 57% (Table 5), and the chi-square test showed that there were no statistically significant differences between populations ( $\chi^2 = 1.836$ ,  $p = 0.766$ ). In *L. agilis*, the frequency of individuals with regenerated tails is higher at localities with anthropogenic pressure than at localities without such pressure ( $\chi^2 = 19.467$ ,  $p = 0.001$ ), but not in *Z. vivipara* ( $\chi^2 = 0.047$ ,  $p = 0.828$ ). Differences in the frequency of tail autotomy were not detected between syntopic and allotopic populations ( $\chi^2 = 0.271$ ,  $p = 0.603$  and  $\chi^2 = 0.930$ ,  $p = 0.335$  in *L. agilis* and *Z. vivipara*, respectively). A higher frequency of tail autotomy was detected in *Z. vivipara* compared to *L. agilis* ( $\chi^2 = 7.2118$ ,  $p = 0.007$ ).

**Table 5.** Percentage and number of individuals with regenerated tails.

Locality	<i>L. agilis</i>	<i>Z. vivipara</i>
Kukavica	25.9% (15/58)	40% (10/25)
Nebeske stolice	54.5% (6/11)	41.4% (12/29)
Treska	24.1% (13/54)	
Gobelja		57.1% (24/42)
Karaman greben		44.4% (24/54)
Divčibare	64.7% (22/34)	
Bistra	28.9% (20/69)	
Mokra Gora		50.0% (11/22)

### 3.6. Abundance

According to the number of individuals, we found that the populations of *L. agilis* at the localities Treska and Kukavica were the most numerous with a growing trend, while the populations of *Z. vivipara* were most numerous at the localities Gobelja and Karaman Greben (Figure 4). Higher abundance was observed in allotopic populations of *Z. vivipara* in populations from Kopaonik NP, while this pattern was not observed in *L. agilis* (Figure 4). Higher abundance was observed at localities without anthropogenic pressure in *L. agilis*, but not in *Z. vivipara* (Figure 4).



**Figure 4.** Number of individuals per researcher per 1 km for (a) *L. agilis* and (b) *Z. vivipara*.

#### 4. Discussion

In this study, we analysed morphological and ecological parameters (such as pileus size and shape, FA, BCI, predation pressure, and abundance) in two lacertid species (*L. agilis* and *Z. vivipara*) from alpine habitats to determine the presence of stress caused by multiple factors (such as different habitat quality, presence of competing species, anthropogenic impacts, and degree of habitat protection).

The size and shape of the head and body can reflect habitat quality, exposure to climatic conditions, food availability, and inter- or intraspecific competition; thus, they have been the subject of numerous studies [64–66]. Body size is often correlated with survival, mating success, and fecundity [67–69]. When habitat conditions are good (preserved natural habitats, favourable climatic conditions, and abundant available food), individuals are expected to be larger and more successful at reproduction than those living in suboptimal conditions. Our results showed that the pileus size differed between localities in both species. In *L. agilis*, individuals were larger in localities with probably more thermophilic habitats (Bistra—the southernmost analysed population and Divčibare—a locality with lower elevation) compared to the other localities, whereas, in *Z. vivipara*, individuals from the southernmost population were the smallest. When we compared syntopic and allotopic populations, size differences were found in both analysed species, with higher values in allotopic populations. It is expected that individuals are larger in allotopic populations since there is no interspecific competition for resources. On the other hand, size differences between populations with and without anthropogenic pressure were found only in *L. agilis* males, with higher values in populations with anthropogenic pressure. The anthropogenic influence is perhaps a selection pressure that leads to an increase in body size, for example, body size in mammals may increase in response to increasing temperatures and urbanisation [70].

As already mentioned, differences in pileus shape may indicate subtle differences between populations living in habitats with different conditions—allotopy/syntopy, presence/absence of environmental stressors, and optimal/suboptimal conditions [22,23]. Significant differences in pileus shape were observed between localities in both species, with populations from Kopaonik NP being more similar to each other in pileus shape than the other populations (for both species). This can be explained by the geographical position of the localities; distant populations differed more in pileus shape compared to geographically close populations. Differences were also observed between localities with or without anthropogenic pressure (for both species), while differences were observed between syntopic and allotopic populations only for *L. agilis*.

Fluctuating asymmetry has often been used as an indicator of stress in lizards [23,37–40,71–73]. Lazić et al. [39,71] found in their research that FA is higher in populations

exposed to urbanisation-induced stress, while Sacchi et al. [72] and Mirč et al. [40] found no association between the level of urbanisation and FA. On the other hand, Vukov et al. [23] showed that the levels of FA may be lower in highly urbanised areas than in periurban and natural habitats. We found statistical differences in FA levels among *L. agilis* populations (population from Bistra had much higher FA values than other populations), but no differences in FA levels among *Z. vivipara* populations. The higher FA values on Bistra could be due to the different climatic conditions during egg incubation (temperature and humidity). It is well known that higher or lower temperatures during incubation can lead to an increase in FA [74,75]. Differences in FA scores between syntopic and allotopic populations and between localities with and without anthropogenic pressure were found only in *L. agilis*. Contrary to our hypothesis, we found higher levels of FA in populations without anthropogenic influence, but a similar result was obtained in *Podarcis muralis*, where individuals from natural populations had higher FA values than individuals from urban populations [40].

Adaptations to new conditions can be physiological without changes in morphology. Therefore, BCI, stress hormones, and reproductive performance should be monitored. Body condition index, as a relatively simple and noninvasive method, has been the subject of numerous studies [41,76–78] and is important for population studies that require rapid and feasible measures of individual quality, such as this study. It is known that the presence of stress can reduce BCI, which is directly related to fitness and reproduction [79]. In our study, higher BCI values were recorded in syntopic populations compared to allotopic populations and at localities without anthropogenic influence compared to localities with such influence. A negative impact on BCI due to anthropogenic influence was also found by other authors [77,78,80]. The population of *L. agilis* at Divčibare is under great anthropogenic pressure due to tourism and is also at a slightly lower elevation; hence, this may explain the lower BCI and higher FA. Since BCI can be considered an indicator of the overall fitness of the animal, greater asymmetry in head shape (i.e., developmental instability) and higher predation exposure can be correlated with lower BCI. High levels of predation negatively affect BCI, as well as population size [81]. Tail autotomy in lizards can increase the chance of escaping predators [82], but it can also have many negative consequences, such as loss of lipid reserves, lower reproductive success, and alteration of locomotor abilities [47,83]. In this study, differences in the frequency of tail autotomy between populations were observed only in *L. agilis* (the frequency of individuals with regenerated tails was significantly higher at the Divčibare compared to all other populations), while no statistically significant differences between populations were observed in *Z. vivipara*. At localities with anthropogenic pressure, we recorded a higher frequency of tail autotomy only in *L. agilis*. Differences in the frequency of tail autotomy were not recorded between syntopic and allotopic populations. The higher frequency of tail autotomy at Divčibare may be due to the lower number of suitable shelters for hiding lizards, the presence of a greater number of predators due to the lower elevation, or the higher degree of urbanisation of this locality (presence of domestic cats). A high percentage of individuals with a regenerated tail in *Z. vivipara* populations could be due to birds preferring smaller lizards that are easier to catch and eat [84]. In addition, tail autotomy may be due to high intraspecific competition [85].

Population abundance can vary due to various factors such as seasonal fluctuations, but stressful conditions may cause populations or individuals to migrate, adapt to new conditions (increase or decrease in abundance), or lead to population extinction [86]. Among the populations from Kopaonik NP, higher abundance was observed in allotopic populations of *Z. vivipara*, while this pattern was not observed in *L. agilis*. Additionally, higher abundance was recorded at localities without anthropogenic pressure in *L. agilis*, but not in *Z. vivipara*. We can see that the population size estimate varied greatly in all these years at Kopaonik NP. Therefore, long-term studies are needed for a more accurate determination of the number and density of populations, while rapid estimates may be prone to error, as the maximum activity of animals could be disturbed by numerous factors.

Although FA is considered a good biomarker for detecting stress [31,87], we can conclude that it is necessary to continuously monitor several parameters for a good assessment of population status and habitat quality. It is unlikely that negative factors will simultaneously affect different morphological structures or fitness of individuals. Therefore, monitoring multiple factors is necessary to determine the effects of threatening factors as soon as possible and to implement conservation plans [35,88]. We think that *L. agilis* and *Z. vivipara* may be good indicators of montane habitat quality because they require specific conditions. This is supported by the fact that ectotherms are more sensitive to changes in environmental conditions compared to endotherms and therefore better reflect environmental stress [35]. Monitoring and protection of bioindicator species, as well as their habitats, can have a much greater impact in conserving biodiversity, the so-called “umbrella species” concept [89]. Europe’s high mountain habitats are included in the European Habitats Directive 92/43/EEC and are under continuous monitoring and habitat conservation [90]. We conclude that populations in syntopy, as well as populations under anthropogenic influence, have more pronounced stress parameters, thus requiring further monitoring and appropriate conservation measures. To protect these species and their habitats, it is necessary to address habitat degradation (deforestation and excessive grazing) and habitat fragmentation (construction of ski centres and forest roads), control the movement of tourists and exploitation of forest products, and prevent and control forest fires.

## 5. Conclusions

The hypothesis was that FA and frequency of tail autotomy would increase, whereas head size, BCI, and population abundance would decrease in populations subjected to higher stress. Furthermore, populations under the influence of stress factors and those without them would differ in the pileus shape. Our results confirmed that stress indicators did not behave the same in both species as expected. In both species, pileus size differed between syntopic and allotopic populations and between populations that were under anthropogenic influence and those that were not, with a larger size expected in allotopic populations as opposed to a larger size in populations under anthropogenic influence. BCI (observed in both species) was higher in populations not under anthropogenic influence, as expected, but not in syntopic populations (due to possible interspecies competition). Abundance was higher in allotopy, as expected, but this was only observed in *Z. vivipara*. The expected higher abundance was also found in populations not under anthropogenic influence, but only for *L. agilis*. The frequency of tail autotomy was expectedly higher in populations under anthropogenic influence, but this was only found in *L. agilis*. Differences in tail autotomy between syntopic and allotopic populations were not observed. The expected differences in pileus shape between localities under anthropogenic influence and those without it were recorded in both species, while differences between syntopic and allotopic populations were observed only in *L. agilis*. Differences in FA between localities under anthropogenic influence and those without it, as well as between syntopic and allotopic populations, were observed only in *L. agilis*.

**Supplementary Materials:** The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/sym15030721/s1>: Figure S1. Constellation of landmarks on the pileus used in geometric morphometric analyses of pileus shape and size; Figure S2. Boxplot of BCI between localities by gender in (a) *L. agilis* and (b) *Z. vivipara*; Figure S3: CS, BCI and FA scores for *L. agilis* plotted against elevation (in meters) and latitude (in decimal degrees). Red dots—localities with anthropogenic pressure. Green dots—localities without anthropogenic pressure; Table S1. Procrustes ANOVA of the shape variation for (a) *L. agilis* and (b) *Z. vivipara*; Table S2. Descriptive statistics (mean  $\pm$  SD) for centroid size by gender and locality for (a) *L. agilis* and (b) *Z. vivipara*; Table S3. Discriminant analysis on size corrected data. The table shows the Procrustes distances and *p*-values among the corresponding localities (below the diagonal of the table are the values for females and above the diagonal are the values for males) in both species; Table S4. Correlation among FA scores, BCI, and Pileus CS in (a) *L. agilis* and (b) *Z. vivipara*.

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