



Climate-induced changes in the phenotypic plasticity of the Heath Fritillary, *Melitaea athalia* (Lepidoptera: Nymphalidae)

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Abstract. Recently a large number of studies have reported an increase in the variability in the climate, which affects behavioural and physiological adaptations in a broad range of organisms. Specifically, insects may be especially sensitive to climatic fluctuations, as their physiology and life history traits, like those of other ectotherms, are predominantly affected by environmental factors. Here we aimed to investigate climate-induced changes in several morphometric measures of the Heath Fritillary in North-Eastern Hungary, which is a highly diverse transitional area. During this study we tested the following hypotheses: (i) climate affects genitalia and body size to various degrees (ii) increasing variability in climate induces higher levels of fluctuating asymmetry and variance in all morphological characters. To our knowledge, this study is the first to analyse simultaneously wing size and structure of genitalia of a butterfly in response to variability in climate. Our findings suggest that wing and genital traits may exhibit similar degrees of stability in response to a more variable climate, although the response in terms of forewing size differs from that of other body measurements and the structure of the genitalia. These findings suggest that global climate change may affect lepidopteran body metrics over longer periods of time. Our findings parallel the results of investigations showing that insect morphology might be modified by environmental changes, which is especially the case for those body parts that are phenotypically very variable. However, we found no evidence that increasing variability in climate would induce higher levels of fluctuating asymmetry and greater variability in morphological characters.

INTRODUCTION

Evidence is accumulating that most habitat loss, fragmentation and long-term changes in climate can be attributed to anthropogenic factors (Bellard et al., 2012; Mantyka-Pringle et al., 2012). It is of particular interest to know how different organisms react to changes in climate and weather over long periods of time, especially in terms of their phenology and morphological adaptations. Specifically, insects may be especially sensitive to fluctuations in climate, as their physiology and life history traits are predominantly affected by environmental factors as are those of other small ectotherms (Bale et al., 2002; Wilson et al., 2007). Thus, variation in food availability, predation pressure, temperature, length of the season, photoperiod and humidity can affect the responses of organisms (Hughes et al., 2003, 2007; Karlsson & Van Dyck, 2005). Heterogeneous environments provide opportunities for exploring the role and limits of natural selection in shaping the response of organisms to environmental change (Pigliucci, 2001).

Characteristics of butterfly wings are widely used as model system for studying development or phenotypic plasticity (Dennis & Shreeve, 1989; Berwaerts et al., 1998; Hill et al., 1999; Thomas & Van Dyck, 2006; Talloen et al., 2009; Gibbs et al., 2011). For example, coloration and patterns on the wings of some species of butterflies are known to be very variable and show high levels of phenotypic plasticity in response to different environmental factors (Shapiro, 1976; Brakefield et al., 1996; Brakefield & French, 1999; Talloen et al., 2009) or food quality (Kooi et al., 1996). Specifically, human-induced habitat fragmentation has been shown to drive morphological modifications (Berwaerts et al., 1998). Furthermore, results of experimental studies support the hypothesis that changes in climate drive morphological characters (Gibbs et al., 2011): Xi et al. (2015) indicate that temperature increase per se and its interaction with the direction of temperature change should be primarily responsible for shifts in body size at different times of the year. Therefore, seasonal patterns in temperature in temperate regions could be of gen-

eral importance for predicting changes in animal body size in response to a warmer climate. In contrast, insect genital characteristics differ greatly, even between sibling species, and seem to be only slightly affected by environmental factors (Cesaroni et al., 1994; Dapporto et al., 2009, 2011).

As a result of environmental perturbations, self-regulatory mechanisms might be unable to stabilize development (Van Valen, 1962; Palmer & Strobeck, 1986, 1992). Fluctuating asymmetry, which consists of small random differences between left and right sides in bilaterally symmetrical structures of organisms, is a widely used measure of developmental instability (Van Valen, 1962; Palmer, 1997). However, there is growing evidence from both experimental and non-experimental studies that fluctuating asymmetry does not consistently predict stress or fitness (Lens et al., 2002; Silva et al., 2009).

Owing to their highly variable morphometric characteristics, members of the genus *Melitaea* (Lepidoptera: Nymphalidae) provide opportunities for studying the effects of climate change and variability on lepidopteran morphology. In addition, butterflies are particularly well represented in museum collections, thus morphometric studies can also be carried out on both wings and external genitalia of a number of lepidopterans using historical data.

In the present study, we aim to investigate the effects of changes in climate on the (i) wing size and (ii) genital characteristics of the Heath Fritillary butterfly – *Melitaea athalia* (Rottemburg, 1775), which is a threatened species in Western Europe but not yet endangered in the eastern part of the continent (Van Swaay & Warren, 1999; Van Swaay et al., 2010). For example, this species is a priority species for butterfly conservation in England, where its phenology and population structure have been studied in detail (Warren, 1987a, b, c, 1991; Hodgson et al., 2009). In contrast, this species occurs in an astonishing variety of ecological situations in Hungary. The main object of the present work is to determine whether climate-induced changes occur in several morphometric measures of this butterfly in the Aggtelek Karst area, which is a highly diverse transitional area where continental, mountain and Mediterranean species co-occur within a limited area (Varga, 1997). In addition, this region has long been considered an important site for entomological studies (Varga & Szabó, 1997; Nagy et al., 1999; Pecsénye et al., 2007; Árnys et al., 2009; Bereczki et al., 2011; Bátori et al., 2012a, b), including some on the effect of climate change (Végvári et al., 2014). In our analyses we primarily focused on the relationships between climatic predictors and morphological traits. We choose this approach as relationships between temporal trends in environmental predictors and their effects on morphological metrics is rather complex (Parmesan & Yohe, 2003) and the absence of temporal trends in morphological traits does not exclude interrelationships between morphometry and climatic proxies.

Our study is based on the following hypotheses. Firstly, high temperatures induce longer larval feeding periods, which results in larger body metrics. Secondly, theory predicts that genitalia measurements are under more con-

strained genetic control than body metrics, therefore we expect that changes in temperature will affect genitalia and body measurements differently. Thirdly, we aim to test the hypothesis that with increase in climatic variability there is an increase in fluctuating asymmetry and variance in all morphological characters (Joubert & Bijlsma, 2010).

MATERIALS AND METHODS

Climate data

We obtained meteorological data for 1967–2013 from the nearest meteorological station at Miskolc (48.1°N, 20.4°E), which is 60 km from the study site, and the only station for which there is long-term dataset necessary for determining trends in climate (Végvári et al., 2014). To assess temporal patterns in climatic predictors relevant for larval development of the species studied, we calculated (i) mean temperatures in March, April and May (ii) temperature averaged across March to April and March to May (iii) mean seasonal temperatures (iv) precipitation sums for the above periods, which is when the larvae are feeding. To calculate the same metrics for the pre-diapausal and diapausal periods, we repeated these analyses for July and August to February, respectively (Pöyry et al., 2011). Monthly and seasonal means of climatic proxies are widely used in insect studies (Altermatt, 2010a; Gibbs et al., 2011; Mega, 2014). Although climatic parameters may fluctuate strongly within a month, we were interested in the effects of the overall trend in climate. To detect temporal trends in weather proxies, we fitted linear regressions to these variables as a function of years.

Sample collection

In total, 228 male individuals were analyzed all of which were captured in the Jósvalfö area of the Aggtelek Karst region, East-Hungary. Specimens were collected between 1967–2013 only in May and June and stored in the collection of the University of Debrecen (Table 1). As only male individuals were available in sufficiently large numbers to carry out the analyses and morphometric traits (measure and shape) showed marked sexual dimorphism, we used only the data for male specimens. Further, to increase the robustness of our results, we removed records for years when less than four individuals were caught.

Table 1. Numbers of specimens caught each year that were used in the morphometric analyses.

Year	Number of specimens for wing measurements	Number of specimens for genitalia measurements
1967	22	24
1975	7	15
1976	5	8
1981	16	10
1983	–	1
1984	–	2
1986	1	2
1988	5	6
1999	22	25
2000	12	12
2001	15	26
2007	14	24
2008	21	44
2011	14	15
2012	13	14

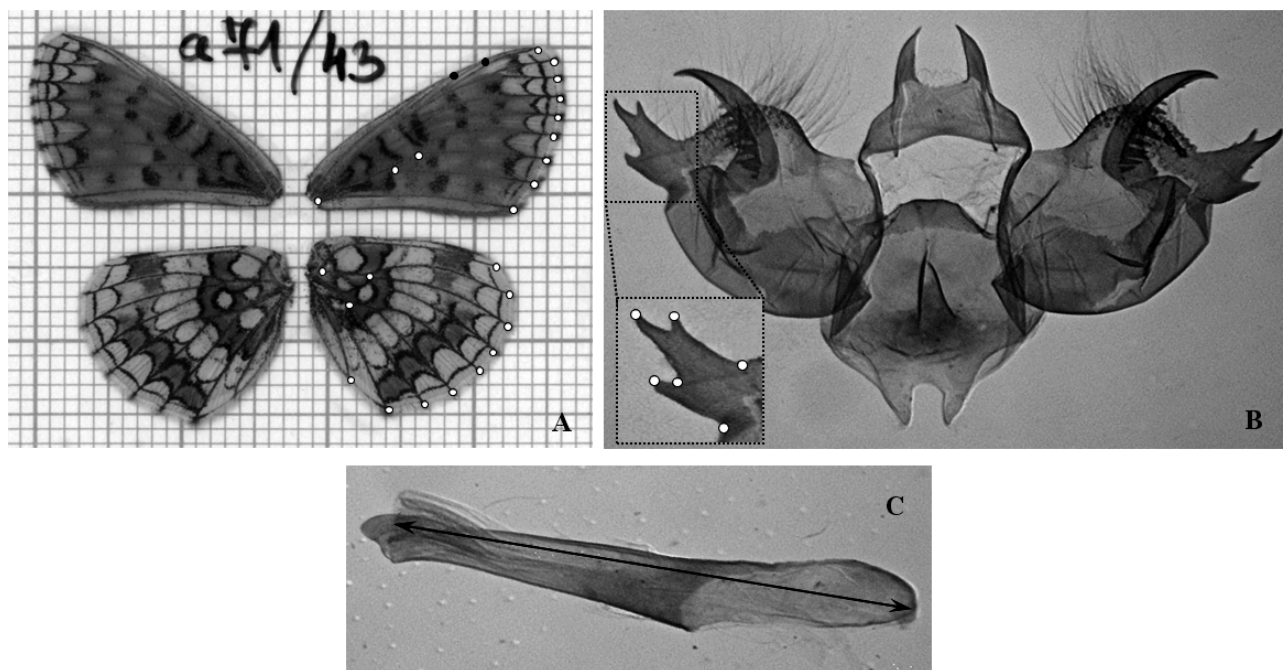


Fig. 1. Features measured on wings and genitalia. Black dots illustrate the two sliding landmarks on the forewing. A – wing; B – external genitalia; C – aedeagus.

Geometric morphometry

Male wings were fixed on transparent films and digitalised using Canon CanoScreen 5200F scanner. Landmark-based geometric morphometric analyses were used to quantify the variation in the shape of wing and reveal any asymmetry. Accordingly, we recorded 14 landmarks on the forewing and 12 on the hindwing at characteristic meeting points of veins (Fig. 1A). Landmark points were assigned as interception points of the veins with insignificant topological variability, following the recommendations of Sanzana et al. (2013) and Bereczki et al. (2014).

The preparation procedure of male external genitalia was carried out based on (Bátori, 2012b). Slides were digitalised using an Olympus C-4000 Zoom camera and a Canon stereomicroscope. Landmark-based geometric morphometric analyses were used to quantify the variation in the shape of processus posterior on the valvae. We recorded 6 landmarks at the tips and origin of the main processi (Fig. 1B). In both cases (wing and external genitalia) TpsDig 2.1 was used to digitalise landmarks (Zelditch et al., 2012).

In the following analyses shape and size variables were treated as dependent metrics and calculated across individuals grouped by year-of-catch: (i) degree of bilateral asymmetry of both wings and processus posterior by retrieving the Goodall’s F-statistic provided by the component of fluctuating asymmetry as a result of Procrustes ANOVA fitted on shape variation (ii) centroid size of both wings and processus posterior calculated by using Procrustes-transformed coordinates (iii) aedeagus length (henceforth morphometric variables). For geometric morphometry we apply the frequently used centroid size, which is defined as the square root of the summed squared distances of each landmark to the centroid.

Traditional morphometry

Traditional morphometry was used to detect changes in the length of the aedeagus (Fig. 1C). Length of the aedeagus was measured using the Image J 1.36 program (Kizic & Borovac, 2001).

Morphometric characteristics of wings and genitalia were chosen from a large set of characters for which the measurement errors were estimated using a series of hierarchical ANOVA. The measurement errors of the characters used in this study accounted for less than 10% of the total variance.

All measurements were repeated three times on each individual to control for measurement error.

Statistical analyses

In the first step, we used Procrustes generalized least squares (ProcGLS) to determine the shape of both fore- and hindwings and male genitalia. Procrustes transformation aims to rescale coordinates to unit centroid size and rotate in order to minimize the sum of squared distances among corresponding landmarks. In the case of forewings we included two sliding landmarks to control for position variance of these unfixed reference points, as indicated by Fig. 1A (Adams & Otárola-Castillo, 2013).

To assess measurement error, the data was subjected to Procrustes ANOVA (Klingenberg & McIntyre, 1998), which indicate significantly lower levels of measurement error (<3.0% on average) than differences between individuals, and between left and right sides of individuals ($p < 0.0001$) (White & Searle, 2008).

In the following step we assessed the effect of climatic variables on all morphometric variables by formulating Procrustes full Linear Mixed Models (LMM) including all climate proxies added as fixed factors and individual ID as a random factor.

After model fitting, the relative importance of environmental variables was determined using an information-theoretic model-comparison, which provides robust measures of climatic responsiveness in terms of body metrics independent of sample size and is thus a robust estimate of the importance of predictors unbiased by the significance levels of a large number of covariates (Burnham & Anderson, 2002). This approach, therefore, excludes the possibility of significant relationships emerging from a large set of predictors, since the importance of each predictor is determined by a subset of models with substantial support, based on their information content (Burnham & Anderson, 2002). The model selection procedure was as follows. First we obtained

Table 2. Temporal trends in the climatic variables measured based on linear regressions as a function of years. Significant relationships ($p < 0.05$) are indicated in bold.

Climatic predictor	Parameter estimate	Adjusted R ²	p
Temperature mean			
March	4.0126	0.0593	0.0706
April	3.7312	0.1548	0.0058
May	2.2243	0.0434	0.0985
May to April	1.1768	0.0050	0.3771
March to April	5.5894	0.1961	0.0019
March to May	5.5894	0.1961	0.0019
July	2.2841	0.0511	0.0872
August to February	1.2370	0.0072	0.2190
Precipitation totals			
March	0.0055	0.0277	0.9613
April	0.1094	0.0186	0.1975
May	-0.0010	-0.0270	0.9846
Complete life cycle (previous May to April)	-0.0185	0.3914	< 0.0001
March to April	-0.0169	0.3277	< 0.0001
March to May	-0.0169	0.3277	< 0.0001
July	0.1770	0.0223	0.1218
August to February	-0.0114	0.0212	0.8991

the values of Akaike’s information criterion corrected for small sample size (AIC_c) and the corresponding Akaike weight of each model (ω). Second, we selected models with substantial support ($\Delta_i = AIC_i - AIC_{min} < 2.0$) and calculated model-averaged parameter estimates (β) and unconditional standard errors (SE_u ; Burnham & Anderson, 2002) of each variable using the sums of their Akaike weights across all the models containing the given predictor that had substantial support. Akaike differences in the range 0–2 indicate a substantial level of empirical support for a given model, whereas $\Delta_i > 7$ implies very weak support (Burnham & Anderson, 2002).

All statistical analyses were carried out in the R statistical programming environment (R Development Core Team, 2013), using the specific packages “geomorph” (Adams & Otárola-Castillo, 2013), “shapes” (Dryden, 2007), “MuMIn” (Barton, 2011) and “nlme” (Pinheiro et al., 2010).

RESULTS

Climatic patterns

First we investigated the temporal trends in the predictors of climate change. Spring temperatures increased in all periods: there were significant positive temporal trends in the mean temperatures of April, March to April and March to May ($b = 3.731$, $p = 0.0058$; $b = 5.589$, $p = 0.002$; $b = 5.589$, $p = 0.002$, respectively, Table 2). Significant negative temporal trends were recorded in the sum of the precipitation from March to May ($b = -0.161$, $p < 0.0001$, Table 2). There were no clear temporal trends in summer,

autumn and winter climatic variables, and those recorded over the pre-diapausal and diapausal periods.

Effects of changes in climate on the morphology of wings and genitalia

In the case of the relationship between forewing centroid size and April mean temperature: forewing size increased with temperature ($\beta = 16.446 \pm 2.033$, Table 3, Fig. 2, Appendix 1). In contrast, there was no correlation between hindwing centroid size and processus posterior, and climatic predictors in the best supported models fitted to both these metrics. Similarly, aedeagus length was not associated with any of the climatic variables.

Fluctuating asymmetry of both wing measurements and processus posterior was not associated with any of the climatic predictors measured as none of these climatic predictors were supported variables. In addition, there was no correlation between the body metrics measured and climatic parameters recorded in the pre-diapausal and diapausal periods (Table 3, Appendix 1).

DISCUSSION

Our study provides two main findings. First, wing and genital traits exhibit similar degrees of stability in response to variations in climate, although with contrasting patterns between forewing size and other body measurements and variation in genitalia. Second, our results suggest that glob-

Table 3. Model-averaged parameters calculated across best supported models fitted to centroid size of forewing.

Variable	β	SE _u	Adjusted SE	Lower CI	Upper CI
Intercept	994.014	154.199	155.451	691.295	1296.733
Temperature means					
March	-18.131	21.697	21.724	-60.710	24.449
April	16.446	8.621	8.645	9.133	51.030
March to April	-1.322	28.605	28.645	-57.466	54.821
Cumulative temperature (March to April)	0.341	0.275	0.276	-0.200	0.881

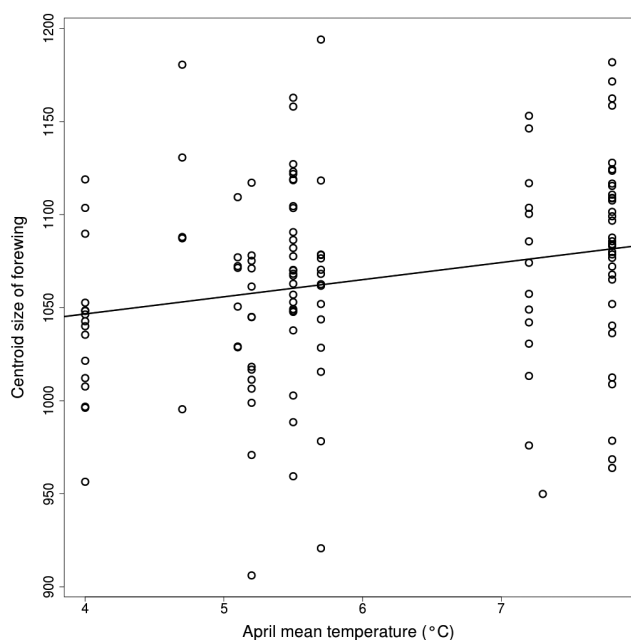


Fig. 2. Centroid size of forewing as a function of April mean temperature.

al changes in climate may affect lepidopteran body metrics over longer periods of time.

Our study is in accordance with others in indicating that insect morphology might be modified by environmental changes, especially in terms of an increased variability in phenotype (Dennis & Shreeve, 1989; Berwaerts, 1998; Talloen et al., 2009; Gibbs et al., 2011). Indeed, the recent work of Xi et al. (2015) support the hypothesis that there has been changes in morphology resulting from recent changes in climate. However, the regional and taxonomic variance in the degree of climate-induced changes in body metrics is still not yet fully understood (Xi et al., 2015).

Our finding that some characteristics of lepidopteran wings vary with the season and possibly with changes in climate implies that they might be an important part of their adaptation to changes in their microclimates, which is confirmed by a recent experimental study (Gibbs et al., 2011). Changes in the shape and size of wings affect thermoregulation in ectotherms (Willmer, 1991; De Keyser et al., 2015). However, relationships between thermal preferences and body metrics are less clear in ectotherms than endotherms, as a recent study demonstrates that there is no evidence for Bergmann's rule applying to insects (Shelomi, 2012). In contrast, increase in body size in lepidopterans induced by high temperatures is predicted by various hypotheses. Firstly, warmer and earlier springs may lead to the larvae spending more time feeding, which result in increased forewing length (Mega, 2014). Secondly, larval size might be indirectly affected by physical characteristics of the food (Dennis, 1991; García-Barros, 2000). Specifically, warm springs might induce earlier and faster development of food plants, which results in an early abundance of food for the larvae, which thus grow larger. However, as the effects of seasonal changes in climate on larval development via the effect on the food plants of the change

in climate are still unknown, further studies on the cascading effects of climatic processes on trophic networks are needed (García-Barros, 2000).

Due to this complexity the relationships between temporal trends in climatic predictors and their effects on morphometry is not straightforward (Parmesan & Yohe, 2003). This might account for why we did not record temporal trends in various morphological traits that are known to be affected by changes in climate.

The greater degree of plasticity in the size of the forewing than of the hindwing in response to changes in temperature is in line with the results of an experimental study that indicates the asymmetric resistance to aerodynamic twisting of the broad forewings of butterflies is a consequence of the curved section of the leading edge (Wootton, 1993). Another investigation indicates that hindwings are not necessary for flight but essential for the execution of evasive flight in butterflies (Jantzen & Eisner, 2008). Thus, the asymmetrical aerodynamic behaviour of the fore- and hindwings might require different allocations of a resource, as these organs compete for a haemolymph-borne resource, such as a nutrient or growth factor (Klingenberg & Nijhout, 1998).

Our finding that forewing size was associated with the temperatures during larval development in spring indicates that environmental conditions experienced by caterpillars can affect adult morphology. Indeed, recent studies have shown that environmental conditions during a specific period in an individual's life cycle are reflected in fitness during the breeding season. For instance, a low availability of food during the pre-hibernation period might result in reduced breeding success (Boggs, 2009; Harrison et al., 2011). This result parallels that of a number of studies that demonstrate that for a large number of lepidopterans warm springs accelerate larval development indirectly by advancing food plant phenology (Altermatt, 2010b). As the species studied is an oligophagous specialist feeding on plants that contain iridoid-glycosides, we assume that larval phenology of *M. athalia* is less constrained by the responsiveness of its food plants to changes in climate than lepidopterans that are more host specific (Warren, 1987a). However, as the effectiveness of insect digestion depends on temporal changes in temperature (Bale et al., 2002), studies on food plant utilisation and nutrient cycle of host specific species is required.

Although the mechanisms underlying how the climatic conditions experienced early on in development can affect later stages of development are not fully understood, possible processes include phenotypic plasticity, microevolutionary processes and epigenetic modifications of morphological traits (Scaven & Rafferty, 2013). More specifically, the variation in body size, growth rate and development time of dung flies remained the same after several generations of artificial selection for body size, indicating that the largest fraction of the phenotypic variance is heritable or environmentally induced plasticity (Blanckenhorn et al., 2009). Similarly, another experimental study shows that morphological alterations might be linked to thermal con-

ditions (Gibbs et al., 2011; Harper, 2011). Such a mechanism might account for the rapid changes in morphological characters in response to changes in climate recorded for *M. athalia* in this study.

In contrast to forewing morphology, measurements of all other body parts, including hindwing, size of the processus posterior and length of the aedeagus, were not associated over the period of this study with any of the climatic predictors measured, probably because of their stricter genetic control (reviewed in e.g. Simmons, 2014). In addition, forewing dimensions are robust predictors of body size (Miller, 1977; Noriyuki et al., 2010; Mega, 2014) and are associated with changes in climate (Mega, 2014; Xi et al., 2015).

Changes in climate did not affect fluctuating asymmetry and the variance in the morphology of both wings and genitalia. Our study did not reveal an increase in fluctuating asymmetry with increase in the unfavourableness of the environment. This finding parallels an investigation on fruit flies carried out by Joubert & Bijlsma (2010), which indicates that the effects of temperature on fluctuating asymmetry is far from unequivocal and might result from a combination of the effects associated with several different environmental predictors.

The robustness of our results is indicated by the fact that we were able to detect biologically relevant trends as a function of macroclimatic conditions in spite of the fact that microclimate has more direct effects on insect development than macroclimate.

In summary, our study highlights the importance of responsiveness of various aspects wing morphology of a lepidopteran that is decreasing throughout Europe to changes in climate, which might be important in determining its ability to adapt to an acceleration in climate change.

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