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Morphometrics and the role of the phenotype in studies of the evolution of developmental mechanisms

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Abstract

Developmental mechanisms are usually assumed to evolve by natural selection of the morphological traits they produce. Therefore, information on phenotypic traits is an important component of comparative studies of development. Morphometrics permits the rigorous quantitative analysis of variation in organismal size and shape, and is increasingly being used in developmental contexts. The new methods of morphometrics combine a geometric concept of shape with the procedures of multivariate statistics, and constitute a powerful and flexible set of tools for analyzing morphological variation. This paper briefly reviews these methods and provides examples of their application in studies of genetic variation and developmental modularity. The results of morphometric analyses can be readily interpreted in relation to the geometry and anatomical structure of the parts under study. Genetic studies of shape in the mouse mandible found two recurrent patterns in environmental and genetic variation from different origins, suggesting that the development system 'channels' the phenotypic expression of variation in similar ways. Moreover, by analyzing the correlations of left-right asymmetries of morphometric traits, it is possible to delimit the spatial extent of developmental modules. These methods complement the experimental approaches of developmental biology and genetics, and can be expected to be especially fruitful in combination with them.

Keywords: Genetic architecture; Modularity; Morphological integration; Quantitative genetics; Quantitative trait loci; Shape; Size

1. Introduction

The central issue of comparative developmental biology is the evolutionary change of developmental mechanisms. This evolutionary change is usually assumed to be adaptive, that is, by natural selection. This means that some variants of the developmental processes in question must produce variant morphological structures that improve the function of the organism and are therefore favored by selection. To understand this adaptive aspect of the evolution of development fully, it is of crucial importance to consider the relationship between developmental processes and the resulting morphological traits and organismal function. It is at the level of morphological expression and function that developmental changes have consequences for organismal fitness, and therefore this level is critical for establishing the link between developmental and evolutionary processes.

Comparative studies of developmental mechanisms have made momentous progress in the past two decades, as is documented by the contributions in this issue (see also Raff, 1996; Arthur, 1997; Gerhart and Kirschner, 1997; Hall, 1999; Davidson, 2001). It is perhaps less well known by the readership of this journal that the field of morphometrics has undergone a similarly rapid change, and now provides a solid framework of powerful methods to quantify the size and shape of organisms (e.g. Bookstein, 1996; Dryden and Mardia, 1998). It is therefore now feasible to combine developmental and morphometric approaches to study the evolution of development with explicit reference to the morphological outcome of developmental changes.

In this paper, I briefly review the new methods of geometric morphometrics and summarize some applications of this approach at the interface of development and evolution. These applications concern the genetic basis of morphological variation as well as the spatial structure of developmental interactions and modularity. Applying morphometrics, which traditionally has been used in systematics and evolutionary biology, in such new developmental contexts opens up a wide and unexplored range for

Abbreviations: EGF, epidermal growth factor; FA, fluctuating asymmetry; QTL, quantitative trait locus; TGFβ2, transforming growth factor β2

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future studies. Therefore, the present overview cannot be exhaustive, but only give a preliminary glimpse on the future potential of this approach.

2. Morphometric methods for quantifying phenotypes

The purpose of morphometrics is to quantify the size and shape of organisms with the methods of multivariate statistics. Such analyses have long existed, and these traditional studies usually have represented morphological form by sets of length measurements of various body parts (Pimentel, 1979; Reyment et al., 1984; Bookstein et al., 1985). These methods are still in use, particularly in studies of growth and evolution (for a review, see Klingenberg, 1996), but also in studies of the genetic basis of morphological variation (Cheverud et al., 1997; Leamy et al., 1999; Weber et al., 1999).

The recent innovations in morphometrics have primarily concerned the way in which size and shape are characterized. Morphometric methods have been developed for analyzing the outline of a part (e.g. Liu et al., 1996; Laurie et al., 1997; Currie et al., 2000). However, the most prevalent approach is to consider the geometric configuration of morphological landmarks, that is, a set of corresponding points that can be precisely located on each of the specimens under study (e.g. Bookstein, 1991, chapter 3). For instance, landmarks can be at a suture point where different skull bones abut, at the intersection of the veins on insect wings, or at the tip of a protrusion such as the angular or coronoid process of a mammalian mandible. The data for such studies are the coordinates of these landmarks, which can either be collected in two dimensions from digital images or located in three dimensions with specialized devices (Dean, 1996) or from computed tomography (Spoor et al., 2000). Such data can easily be obtained from non-model organisms or even from fossils, making the approach particularly useful in evolutionary contexts (e.g. O'Higgins, 2000; Klingenberg et al., 2001a).

Morphometric analyses of landmark data use a mathematical definition of shape. Shape encompasses all features of landmark configurations except for overall size, position, and orientation. These extraneous factors are removed by the Procrustes method (Fig. 1), which scales all configurations to unit size, superimposes them by their centroids (centers of gravity), and rotates them to an orientation of

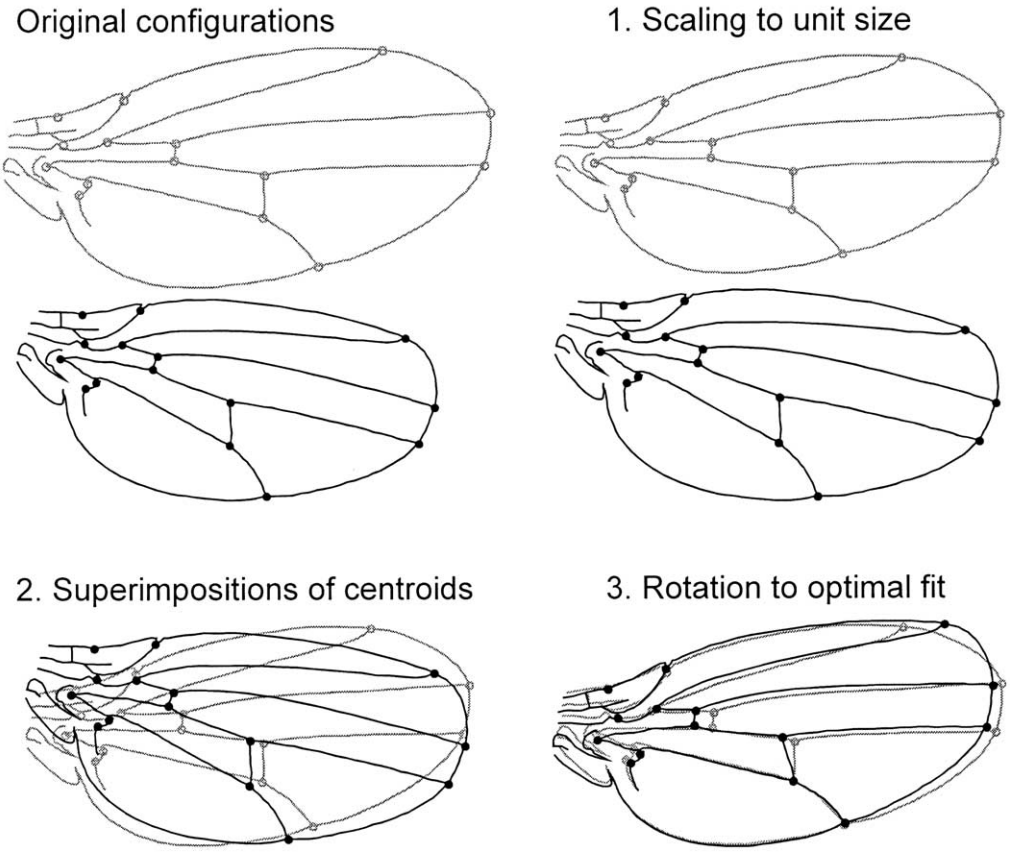

Fig. 1. The Procrustes superimposition. The diagrams show the outlines of two fly wings, and the landmarks are indicated as dots. The original configurations, possibly after reflection of the wings from one body side, are (1) scaled so that they all have the same size. They are then superimposed (2) so that their centroids, or centers of gravity, are all in the same location. Finally, a rotation (3) of the configurations around the shared centroid produces an optimal alignment (an optimal fit according to a least-squares criterion). The remaining differences in the locations of corresponding landmarks are due to variation of shape, and can be used as input for multivariate analyses (for further details, see Dryden and Mardia, 1998; Klingenberg and McIntyre, 1998).

optimal fit to a consensus configuration (Dryden and Mardia, 1998). Moreover, in studies of paired structures such as limbs or wings, reflection is also removed so that parts from the left and right sides, which are mirror images of each other, can be used in the same analysis (Klingenberg and McIntyre, 1998; Auffray et al., 1999). The variation in the landmark coordinates that remains after Procrustes superimposition is a complete and non-redundant description of the variation in shape, and can be used as input for the standard methods of multivariate statistics. In addition to the usual statistical tests and tabular presentation of results from the multivariate analyses, it is also possible to display results graphically so that they can be interpreted easily in relation to the geometric and anatomical structure of the part under study. This way of characterizing, comparing, and interpreting patterns of shape variation with statistical and graphical means is central to morphometric studies.

Moreover, the methods of geometric morphometrics also have a remarkable statistical power. Therefore, they can be used to uncover even very small morphological variation that would go undetected without measurement or when analyzed with less effective methods. As a case in point, a study using this approach found that the average shapes of left and right wings differ subtly, but consistently in three species of flies (Klingenberg et al., 1998). This finding contradicted earlier studies using different methods, which repeatedly had failed to find such directional asymmetries and thus concluded that the left and right body sides of flies were developmentally identical (Tuinstra et al., 1990). Moreover, it also preceded the first demonstration of a developmental asymmetry in *Drosophila* (Ligoxygakis et al., 2001). The study of phenotypic variation can therefore be an effective exploratory strategy.

3. Genetic architecture of size and shape

One application of the new morphometric approaches is the study of genetic architecture of size and shape, and a variety of experimental protocols and morphometric analyses have been used for this purpose. Understanding the amount and developmental origin of genetic variation in laboratory and natural populations is a precondition for studies of evolution. Although these studies are by no means restricted to the classical model organisms (e.g. Arnqvist and Thornhill, 1998; Currie et al., 2000), I review a selection of studies mostly in *Drosophila* or mouse, emphasizing those that relate phenotypic data to the evolution of developmental mechanisms.

The *Drosophila* wing is an excellent system to study the development, genetics, and evolution of a morphological form. The wing is structurally simple, as it develops from an imaginal disc, a single epithelial sheet that folds over during metamorphosis and thus forms the two-layered wing, and its development is known in great detail (e.g. García-Bellido and de Celis, 1992; Sturtevant and Bier, 1995; Biehs et al., 1998; de Celis, 1998; de Celis and Barrio, 2000). Despite the structural simplicity of the wing, however, the intersections of wing veins define many landmarks suitable for morphometrics. Accordingly, a number of studies have used geometric morphometrics to study issues such as geographic variation (Gilchrist et al., 2000), allometry (Baylac and Penin, 1998), as well as left-right asymmetry and morphological integration (Klingenberg et al., 1998; Klingenberg and Zaklan, 2000) in *Drosophila* wings. Moreover, a number of studies has focused on quantitative genetic variation of wing morphology using angles (Whitlock and Fowler, 1999; Phillips et al., 2001) or distances between landmarks (e.g. Cowley et al., 1986; Cowley and Atchley, 1990; Weber et al., 1999).

Gibson and co-authors investigated natural genetic variation of several shape variables derived with geometric morphometrics (relative warps; e.g. Bookstein, 1991) for the intervein areas in fly wings (Birdsall et al., 2000; Palsson and Gibson, 2000; Zimmerman et al., 2000). They analyzed the effects of genetic and environmental factors, and found specific responses of different intervein regions to the same conditions, and ample genetic variation in these responses to environmental variation (Birdsall et al., 2000). Zimmerman et al. (2000) identified a number of quantitative trait loci (QTLs) for size and for each shape variable, and listed possible candidate genes for most of them. Quantitative complementation tests showed that the shape variables are affected by natural variation in genes of the Decapentaplegic, Hedgehog, and EGF signaling pathways (Palsson and Gibson, 2000). The same study also found substantial natural variation at the *Plexate* locus, revealing a subthreshold pattern of wing veins that possibly represented a reversal to an ancestral condition. This line of investigation, therefore, used mapping techniques and the identity of the genes in question to link the measured phenotypic effects to the information about their developmental roles (e.g. Sturtevant and Bier, 1995; de Celis, 1998).

The mouse mandible is another system that has long been investigated as a model for development and morphological variation in complex structures (e.g. Atchley and Hall, 1991). Studies of genetic variation in mandible form have used traditional approaches based on distance measurements (e.g. Atchley et al., 1985b, 1985a) or various methods for comparing geometric configurations of landmarks among different mouse strains (Bailey, 1985, 1986; Cheverud et al., 1991).

A series of studies has analyzed QTLs affecting morphometric variation of the mandible in the same set of mice, but with different analytic methods (Cheverud et al., 1997; Leamy et al., 1997; Mezey et al., 2000; Cheverud, 2001; Klingenberg et al., 2001b). Analyses of distances between landmarks found a division of the mandible into two parts: of the QTLs with statistically significant effects on multiple distances, most had effects that were confined either to the posterior part (50%) or the anterior part (27%) of the mandible, and only a minority of these QTLs (23%) affected

distances in both the anterior and posterior parts (Cheverud et al., 1997). Mezey et al. (2000) developed tests for aggregation of QTL effects within anterior and posterior parts and for the separation between them, and found both tests to be statistically significant. An analysis of the same data using the Procrustes method found a similar number of QTLs affecting either size or shape, and also found that most QTL effects on shape were concentrated particularly in the posterior part of the mandible (Klingenberg et al., 2001b). However, the separation of anterior and posterior parts of the mandible was far from complete; for instance, there was no clear-cut separation between groups of QTLs with effects primarily in one or the other part. Similarly, a quantitative genetic study, using the Procrustes approach to investigate the aggregate effect of the whole genome, showed that simulated selection on a single landmark can elicit a response throughout the whole mandible, and therefore also emphasized the genetic and developmental coherence of the mandible (Klingenberg and Leamy, 2001).

The bulk of the variation in the QTL effects on mandible shape was made up of two patterns that recurred to varying degrees in the effects of most QTLs: an opposite anterior-posterior shift of the angular and coronoid processes, and a dorso-ventral contraction or expansion of the same processes (Klingenberg et al., 2001b). Interestingly, these patterns resembled those found in much more extreme form in the phenotypes resulting from multiple knockout experiments for genes such as *Dlx5* (Depew et al., 1999) and TGFβ2 (Sanford et al., 1997) for the former pattern, or for genes such as *goosecoid* (Rivera-Pérez et al., 1999) or *Ptx1* (Lanctôt et al., 1999) for the latter pattern (Klingenberg et al., 2001b). Moreover, similar patterns also appear in the aggregate effects of all genic variation in a population, as they were uncovered by standard quantitative genetics (Klingenberg and Leamy, 2001). This correspondence in the phenotypic manifestation of genetic variation from widely different origins suggests that the developmental processes that build the mandible 'channel' variation into these fixed patterns. Clearly, if different genetic effects can have a similar phenotypic outcome, this will profoundly affect the evolution of developmental mechanisms, as some genetic changes will be 'sheltered' from the effects of natural selection, whereas some phenotypic changes will be difficult to achieve.

4. Delimiting morphological modules

Organisms are made up of structural elements, or parts, that are morphologically and developmentally distinct from one another to some degree. These elements are internally coherent through manifold connections and interactions among their components, while they are relatively autonomous from other such elements, from which they are often set apart by recognizable boundaries or interfaces. This pattern of strong internal connections and weaker or fewer external links is often called modularity, and has recently become one of the dominant themes in evolutionary developmental biology (e.g. Raff, 1996; Wagner, 1996; Gerhart and Kirschner, 1997; Kirschner and Gerhart, 1998; von Dassow and Munro, 1999; Raff and Sly, 2000; von Dassow et al., 2000).

The concept of modularity has diverse meanings as it can be applied to different domains of organization. Developmental modularity has often been discussed for the organization of gene regulatory networks (von Dassow and Munro, 1999; von Dassow et al., 2000) and for morphological structure (Cheverud, 1996; Wagner, 1996; Wagner and Altenberg, 1996). A recent study even extended the notion of modularity to the different life stages of insects (Yang, 2001). The concept of modularity must be applied in fundamentally different ways in each of these domains. In this paper, I will only deal with modularity in the morphological realm. Modularity in this sense is related to the more traditional concept of morphological integration (Olson and Miller, 1958; Cheverud, 1982; Zelditch, 1987), as modules are structural units that are internally integrated by developmental interactions. Modularity in this sense is also closely related to the concept of morphogenetic fields (Gilbert et al., 1996; Raff, 1996). Because they are constituted by the localized developmental processes that take place within them, morphological modules have concrete spatial dimensions.

Is it possible to infer the spatial extent of developmental modules from morphological data? Any such inferences must be based on covariation between morphological traits, and thus it is important to understand how covariation observed in the phenotype originates. A critical determinant of morphological covariation is the developmental origin of the traits in question (Sakai and Shimamoto, 1965; Riska, 1986; Klingenberg et al., 2001a; Klingenberg, 2002). For instance, two structures derived by fission of a common developmental precursor can be correlated because they share the variation accrued before partitioning of the precursor (Riska, 1986). Other kinds of developmental interactions, such as inductive signaling between different precursors, can also generate morphological covariation. This covariation among traits due to such direct developmental interactions among traits provides the information required to delimit developmental modules, because such covariation will primarily occur within modules, and only to a lesser degree between them (Klingenberg et al., 2001a).

Covariation from direct developmental interactions, however, needs to be distinguished from covariation due to other sources. Environmental variation that affects more than one developmental pathway simultaneously will produce joint variation in the structures derived from them, even if the pathways are separate and do not interact (Klingenberg, 2002). For example, a heat shock may simultaneously affect many processes in different organs. Allelic variation in genes that participate in several different developmental processes may also lead to covariation among traits without a direct

developmental connection (e.g. Distal-less is involved in specifying the tips of appendages and the eyespots on the wings of butterflies; Carroll et al., 1994; Panganiban et al., 1994). Therefore, a study of mutant phenotypes alone is not evidence for direct developmental interactions. Covariation among traits that originates from parallel variation of developmental pathways without direct interaction must rely on an outside source of shared environmental or genetic variation, and does not reflect developmental modularity (Klingenberg et al., 2001a). To identify developmental modules from morphological data, it is essential to control rigorously for these external origins of covariation.

A most effective way to control for environmental and genetic effects is to focus on the small amount of random variation between the left and right sides of individuals: fluctuating asymmetry (FA; Klingenberg and Zaklan, 2000; Klingenberg et al., 2001a; Klingenberg, 2002). FA originates from small random differences in the development of left and right body sides of each individual, which share the same genome and experience virtually identical environmental conditions. Therefore, the study of FA controls almost completely for the factors that might cause parallel variation of separate pathways, and accordingly, covariation between the asymmetries of different traits is due to the direct interactions among developing parts. This covariation makes the left-right asymmetry in one trait at least partly predictable from the asymmetry of another trait. For random developmental deviations to produce such a consistent correlation among traits, the deviations themselves or their developmental effects must be transmitted between the traits by developmental interactions. Accordingly, covariation of FA will be confined primarily within developmental modules, and FA will be uncorrelated between them.

The methods of geometric morphometrics are well suited for delimiting developmental modules in this way, by analyzing the covariation among the relative positions of landmarks for FA (Klingenberg and McIntyre, 1998; Auffray et al., 1999; Debat et al., 2000). These analyses are based on the Procrustes method (Fig. 1) with a reflection of all the configurations from one body side at the outset of the analysis. The asymmetries are computed as the coordinate differences between the superimposed configurations of left and right body sides for each individual, and entered into further multivariate analyses of the statistical associations among the coordinates of different landmarks.

Analyses of this sort in the wing of *Drosophila* demonstrated that the entire wing is a single coherent developmental module (Klingenberg and Zaklan, 2000). The features of overall variation extracted by multivariate analysis were not localized in particular parts of the wing, but each involved variation of landmarks throughout the entire wing. Moreover, an analysis specifically focusing on the variation shared between the anterior and posterior compartments indicated that this component of variation accounted for nearly all the variation throughout the whole wing, showing that the wing is a completely integrated unit. These analyses clearly refuted the conclusions of some earlier studies suggesting that the anterior and posterior compartments are separate units of morphological variation (e.g. Cavicchi et al., 1981, 1991; Thompson and Woodruff, 1982; Pezzoli et al., 1997; Baylac and Penin, 1998). Quite to the contrary, the geometric approach indicated that developmental interactions span the whole wing blade, as might be expected, given the role of the compartment boundary as a source of patterning signals that jointly affect both compartments (Lawrence and Struhl, 1996; Strigini and Cohen, 1999; Entchev et al., 2000; Milán and Cohen, 2000; Teleman and Cohen, 2000). The results are also consistent with the view of the entire imaginal discs as morphogenetic fields, and thus as model examples of developmental modules (Gilbert et al., 1996; Raff, 1996).

If each wing, derived from a single imaginal disc, is a self-contained module, then correlations of FA should be confined to each wing and should be uncorrelated to the FA of parts originating from different imaginal discs. Particularly, for insects with two wing pairs, one would expect FA in the fore- and hindwings to be uncorrelated. Klingenberg et al. (2001a) used the methods of geometric morphometrics to test this prediction for bumblebees (*Bombus empatiens*). In bees reared under normal conditions, FA for shape in the two wing pairs was indeed uncorrelated. If the bees were reared under elevated CO_2 levels, however, there was a correlation between the FA of fore- and hindwings, suggesting a link between the developing fore- and hindwings on either body side. It is possible that variability in the gas exchange established a link between fore- and hindwing imaginal discs through the main tracheal tubes, which run in anterior-posterior direction along either body side (for a more detailed discussion, see Klingenberg et al., 2001a). This result underscores that the experimental conditions can affect the interactions between parts, and therefore require careful consideration and control. Overall, however, the study supports the argument that developmental interactions, and therefore modules, can be delimited via the spatial distribution of correlated FA.

These patterns of variation for FA can also be compared to the patterns for individual variation, focusing on the variation among individuals in the left-right averages of landmark positions. In the studies undertaken so far, comparisons showed correspondence between the patterns of individual variation and FA in mouse mandibles (Leamy, 1993) and insect wings (Klingenberg and McIntyre, 1998; Klingenberg and Zaklan, 2000; Klingenberg et al., 2001a). This correspondence of patterns indicates that the variation at both levels may be expressed through the same developmental pathways, and it suggests that direct developmental interactions account for a substantial part of the correlations among individuals. In contrast, Debat et al. (2000) found a marked difference between patterns of individual variation and FA in mouse cranial shape, and suggested that different developmental processes are involved.

5. Conclusions and outlook

The combination of morphometrics and development is new, and there are only a few case studies so far. The studies reviewed here indicate that morphometrics is a promising addition to the approaches currently used in evolutionary developmental biology and genetics. These methods are highly sensitive, and can demonstrate even very subtle morphological variation. Because the geometric approach is based on a complete characterization of shape, they can identify those features of shape that differ between genotypes or experimental treatments, or those that are most variable or most stable. As biologists become increasingly interested in the developmental control of organismal size and shape (Conlon and Raff, 1999; Day and Lawrence, 2000), such quantitative 'phenotyping' is poised to become a widespread addition to the toolbox of experimental protocols now commonly in use.

Geometric morphometrics provides another novel source of evidence: the patterns of shape variation in relation to the geometry and anatomy of the structure under study. Which landmarks shift in which directions in response to a mutation or experimental treatment is important information about the underlying processes. Moreover, similarity and difference of these patterns can be assessed even among studies of widely different experimental designs, as illustrated for classical quantitative genetics, QTL analyses, and gene knockout experiments. Likewise, even variation of non-genetic origin, such as fluctuating asymmetry, can yield new information about underlying developmental mechanisms via the patterns of morphometric covariation. Because all these studies are carried out 'in the currency' of morphometric variation, effects from studies of very different experimental contexts can be compared directly.

With their emphasis on phenotypic outcome, morphometric analyses of developmental variation provide a new connection of development to evolution. A promising approach will be to combine morphometrics with the molecular approaches of developmental genetics to focus on specific developmental mechanisms, such as gene regulation and signalling processes. Studies of this sort will be able to test predictions on developmental processes derived from molecular-level information. Relating the molecular mechanisms of development to the phenotypes that are the direct targets of natural selection will improve our understanding of the processes by which developmental mechanisms evolve. Such a unified understanding requires a merging of developmental with quantitative and evolutionary genetics; as shown in the examples reviewed in this paper, this merger is well underway.

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