

“Development, Plasticity and Evolution of Butterfly Eyespot Patterns” (1996), by Paul M. Brakefield, et al.

Paul M. Brakefield and his research team in Leiden, the Netherlands, examined the development, plasticity, and evolution of butterfly eyespot patterns, and published their findings in *Nature* in 1996. Eyespots are eye-shaped color patterns that appear on the wings of some butterflies and birds as well as on the skin of some fish and reptiles. In butterflies, such as the peacock butterfly (*Aglais io*), the eyespots resemble the eyes of birds and help butterflies deter potential predators. Brakefield's research team described the stages through which eyespots develop, identified the genes and environmental signals that affect eye-spot appearance in some species, and demonstrated that small genetic variations can change butterfly eyespot color and shape. The research focused on a few butterfly species, but it contributed to more general claims of how the environment may affect the development of coloration and how specific color patterns may have evolved.

At the time of experiment, Brakefield was a Chair in Evolutionary Biology at the University of Leiden, in Leiden, the Netherlands, though in 2010 he moved to Cambridge, UK to direct the University Museum of Zoology. While in Leiden, he pursued a research program in evolutionary developmental biology and had started working on butterfly eyespots in collaboration with Vernon French, who was at the University of Edinburgh in Edinburgh, Scotland. Brakefield and French joined forces with Fanja Kesbeke, Pieter Wijngaarden, and Antonia Monteiro in Leiden, and with Julie Gates, Dave Keys, and Sean Carroll at the University of Wisconsin in Madison, Wisconsin for this research project. Together, this team spanned the disciplines of developmental biology, evolutionary developmental biology, molecular biology, and genetics.

Eyespots are diverse among species of butterfly in their number, size, shape, color, and position. To understand this variation, Brakefield's team addressed two main issues in their experiment: how eyespots are generated developmentally and evolutionarily, and how phenotypic plasticity, or the responsiveness of genetic expression to the environment, alters the generation of eyespots. Many species of butterfly display different eyespots depending on the season during which they develop. However, before Brakefield's study, scientists knew little about how eyespot plasticity related to environmental factors, genetic material, and developmental stages.

Brakefield's experiment consisted of three parts. First, the team defined the developmental stages of eyespot formation, specifically stages critical to development, and they identified what elements of the eyespot pattern varied across organisms due to genetic mutations. Second, they examined differences in color pattern development among three species of butterfly to determine how the developmental pathway has been modified through evolution. Lastly, using seasonal morphs of the same species and artificial selection in laboratory manipulations, the team demonstrated that environmental and genetic factors both account for the phenotypic plasticity of butterfly eyespot pattern and that such plasticity can evolve rapidly and independently from other morphological features.

In the first part of the experiment, Brakefield's team described four successive stages in the development of butterfly eyespots. Those four stages of prepattern, focal determination, focal signaling, and differentiation are regulated by the *Distal-less* gene (*Dll*), a gene that helps develop organs or tissues in many other animals. The first two stages occur in the late caterpillar (fifth instar) before it begins to pupate; the final two stages occur during pupation, while the caterpillar is developing into the adult butterfly form. In the prepattern stage, the *Dll* gene is activated to produce proteins in specific cells, forming large bands and smaller stripes in the wing. The focal determination stage exhibits initial spot formation, as small spots expand from the ends of the stripes. In the focal signal-

ing stage, cells surrounding the small dots begin to express the Dll gene, giving rise to an enlarged dot, which will become the future eyespot. In the last stage, called the differentiation stage, the color of the eyespot emerges.

Brakefield and his team showed that abnormal expression of the Dll gene in the four stages results in abnormal traits. Using three mutant types of the Squinting Bush-brown butterfly, *Bicyclus anynana*, they identified the effects of specific mutations in the Dll regulatory gene at different times during development. Normal butterflies have seven eyespots on the hindwing, whereas Cyclops mutants display only one big elliptical eyespot on the hindwing. Spotty mutants display two additional eyespots in the forewing, and Bigeye mutants have enlarged eyespots. Brakefield's team detected abnormal Dll expression in stage one in Cyclops, in stage two in Spotty, and after stage three in Bigeye, respectively. This process revealed that Dll regulates the location, number, and size of eyespots during these developmental stages, but likely does not regulate the color of the pattern.

To see how eyespots form the researchers next grafted the eyespot's center (or focus) to different parts of the wing. The eyespot focus signals surrounding cells to produce specific proteins during the third stage of development. Researchers grafted this tissue to see if eyespot coloration is controlled completely by the signals from the eyespot's center, regardless of location of cells receiving the signal. Alternatively, they thought eyespot coloration may be controlled by the location of the cells receiving the signal from the eyespot's center, as cells in different locations may produce different proteins in response to the same signal. The team used the Buckeye butterfly, *Precis coenia*, which has a white eyespot on the posterior hindwing. They transplanted tissue from the center of the eyespot to specific sites on the wing. Tissue from the same source caused different effects in different locations, indicating that the varied positions of the receiving cells, rather than varied protein signals from the focal cells, dictate color pattern.

In the second part of the experiment, Brakefield's team conducted a comparative analysis on different species of butterfly, using the Squinting Bush-brown, the Buckeye, and the Monarch, *Danaus plexippus*, to study the developmental trajectories that cause large differences in patterns. The Squinting Bush-brown and the Buckeye both have eyespots, whereas the Monarch does not. Brakefield's team found that all the species displayed the banded pattern of Dll expression, but the Monarch never displayed the smaller stripes present during stage one (prepattern) of development in butterflies with eyespots. The lack of smaller, midline stripes in Monarchs means divergence between butterflies with and without eyespots occurs before stage one of development is completed. The Squinting Bush-brown and the Buckeye both develop the banded pattern and smaller stripes of Dll expression, but the Buckeye develops only two eyespots, whereas the Brown-bush forms seven eyespots. In the Buckeye, several of the smaller stripes fade during stage two of eyespot formation (focal determination), leading to the differences in eyespot number between the two species. Fading stripes during focal determination in the Buckeye shows that the development of these two species with eyespots diverges during stage two.

In the last part of the experiment, Brakefield's team studied the phenotypic plasticity of eyespot coloration, or the developing eyespot's ability to change its appearance according to changes in the environment. In Squinting Bush-browns, as well as in many other butterflies, seasonal changes can trigger the development of different colors or traits, including varied eyespot pattern. Bush-browns that develop in the wet season exhibit big eyespots, and those that develop in the dry season exhibit small or no eyespots. Bush-browns may need to hide among dead brown leaves in the dry season, and large eyespots would make them too conspicuous, so selection has favored the association between smaller eyespots or no eyespots and the dry season. In the wet season, however, eyespots may deflect the attention of predators toward the wings rather than the body, increasing butterfly survival and favoring large eyespots. The change of color and pattern development according to the season may be an adaptation to the African environment and has been linked to developmental temperature in the laboratory. Bush-browns raised in intermediate temperatures show a range of patterns between the two seasonal forms, with differences in the prominence of eyespots.

The pattern variation between seasonal forms enabled Brakefield's team to use artificial selection to investigate the number of genes underlying eyespot formation. The researchers obtained a genetic line of Bush-brown butterfly that had been raised in intermediate temperatures to show variation

between individuals in the seasonal form of eyespot pattern. Then, they selected for either the wet-season or the dry-season color pattern. After around twenty generations of artificial selection, they obtained butterflies that only exhibited the wet-season form or the dry-season form, regardless of the conditions during development. Then, by crossbreeding and inbreeding the lines and observing the pattern variation of subsequent generations, they were able to demonstrate for the first time that a minimum of five or six genes are involved in creating the differences in eyespot pattern.

In the conclusion to their published results, Brakefield and colleagues suggested that the developmental pathway of the eyespot formation is highly modular, meaning that it is independent from developmental pathways for other wing features, such as wing shape or the color of the rest of the wing. The team's experiment indicated that the plasticity of the eyespot might be due to a developmental pathway that could easily change due to small genetic variations, and thus is very flexible. The illumination of the developmental pathway and genetic bases of butterfly eyespot formation influences research into color formation in other eyespot-bearing organisms, as these mechanisms may also be uncoupled from control of morphology in other species.

As of 2013, "Development, Plasticity and Evolution" has been cited hundreds of times. According to Sean Carroll, Brakefield's experiment became a classic experiment in evo-devo, or evolutionary developmental biology, which looks at developmental phenomena from an evolutionary perspective.

Sources

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