

BUTTERFLY EYESPOT PATTERNS: EVIDENCE FOR SPECIFICATION BY A MORPHOGEN DIFFUSION GRADIENT

Antónia Monteiro^{1,*}, Vernon French², Gijs Smit¹,
Paul M. Brakefield¹ and Johan A.J. Metz^{3,4}

¹Section of Evolutionary Biology, Institute of Evolutionary and Ecological Sciences, University of Leiden, Kaiserstraat 63, 2311 GP Leiden, The Netherlands.

²Institute of Cell, Animal and Population Biology, University of Edinburgh, Kings Buildings, West Mains Road, Edinburgh EH9 3JT, UK.

³Theoretical Evolutionary Biology, Institute of Evolutionary and Ecological Sciences, University of Leiden, Kaiserstraat 63, 2311 GP Leiden, The Netherlands.

⁴Adaptive Dynamics Network, International Institute for Applied Systems Analysis, 2361 Laxenburg, Austria.

*Author for Correspondence: Antónia Monteiro, Tel: +31 71 5274853.
Fax: +31 71 5274900. E-mail: monteiro@rulsfb.leidenuniv.nl

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ABSTRACT

In this paper we describe a test for Nijhout's (1978, 1980a) hypothesis that the eyespot patterns on butterfly wings are the result of a threshold reaction of the epidermal cells to a concentration gradient of a diffusing degradable morphogen produced by focal cells at the centre of the future eyespot. The wings of the nymphalid butterfly, *Bicyclus anynana*, have a series of eyespots, each composed of a white pupil, a black disc and a gold outer ring. In earlier extirpation and transplantation experiments (Nijhout 1980a; French and Brakefield, 1995) it has been established that these eyespots are indeed organised around groups of signalling cells active during the first hours of pupal development. If these cells were to supply the positional information for eyespot formation in accordance with Nijhout's diffusion-degradation gradient model, then, when two foci are close together, the signals should sum, and this effect should be apparent in the detailed shape of the resulting pigment pattern. We give an equation for the form of the contours that would be obtained in this manner. We use this to test the morphogen gradient hypothesis on measurements of the outlines of fused eyespots obtained either by grafting focal cells close together, or by using a mutation (*Spotty*) that produces adjacent fused eyespots. The contours of the fused patterns were found to satisfy our equation, thus corroborating Nijhout's hypothesis to the extent possible with this particular type of experiment.

1. INTRODUCTION

A butterfly wing starts developing as a double sheet of epidermal cells (or epidermal sac), that grows first inside the larval body as an imaginal disc and later



evaginates, at pupation, to form the pupal wing (Nijhout, 1980b). During further development of this pupal wing there is differentiation of a group of large cells, neatly arranged in rows, which will produce colourless cuticular projections, the scales. Just before adult emergence each scale cell starts synthesising and incorporating a specific pigment in its cuticular projection and a mosaic of coloured scales is formed. The resulting patterns are complicated and varied and seem to be produced by apparently rich and unconstrained developmental mechanisms.

One of these patterns, the eyespot, has been the object of considerable study. Nijhout (1980a) identified cells at its centre, the focus, that organise the pattern during the first 48 hours of pupal development. Damaging the focus can remove the adult eyespot or reduce its size (Nijhout, 1985; Brakefield and French, 1995). Grafting the focus to another site on the wing induces the formation of an ectopic eyespot (Nijhout, 1980a; French and Brakefield, 1995). Nijhout (1978, 1980a) proposed that the different colour rings in an eyespot result from a concentration-dependent response to a single morphogen being produced at and diffusing and degrading away from the focus. High concentrations closer to the source would activate genes, and later pigment synthesis pathways, different from those activated by lower concentrations further away. If Nijhout's hypothesis is correct, the contours of adult eyespot patterns are spatial indicators of where a threshold-dependent response of genes involved in pigment synthesis pathways took place.

Here we attempt to test whether the form of experimentally manipulated eyespot patterns in the nymphalid butterfly *Bicyclus anynana* are compatible with Nijhout's model. According to Nijhout's model, epidermal cells beyond the sharp outer contour of unmanipulated eyespots still receive low levels of morphogen, experiencing part of the "tail" of the concentration gradient. These low concentrations however, do not enable these outlying cells to differentiate into part of the pattern. This implies that if a sub-threshold area of the wing receives morphogen from a second source (also individually influencing this area at sub-threshold levels), the two concentrations can sum, pass the threshold level, and thus produce an extra area of pattern.

In the experiments described below, we used naturally occurring and experimentally manipulated fused eyespot patterns to detect whether this additive effect is present. We give an equation which should be satisfied by the contour of a fused pattern, established by diffusion from two point sources combined with degradation, and test whether this equation is satisfied by the points on a contour of real fused eyespot patterns.

2. MATERIALS AND METHODS

The butterflies

We used four different lines of *B. anynana*: the STOCK, the selected HIGH and LOW lines and the *Spotty* mutant line. The HIGH and LOW lines were previously selected for an increase and decrease, respectively, of the size of the large posterior eyespot on the dorsal forewing (Monteiro *et al.*, 1994). The size difference in these lines was found to be mainly due to differences in the focal cells. *Spotty* is a single, autosomal allele showing incomplete dominance that causes the appearance of two extra eyespots in both the dorsal and the ventral surfaces of the forewing, between the

normal anterior and posterior eyespots (Figure 1a). A homozygous mutant line was used for the experiments below. These individuals have the black and gold regions of the eyespots fused and display the characteristic pattern of an outer gold ring enclosing a single black ellipse with a row of four white pupils (Brakefield and French, 1993).

All butterflies were reared at 28°C, 12L:12D light cycle and high (90%) relative humidity. Pre-pupae from all lines were timed for their individual pupation times within ± 15 minutes. Signalling from the foci and differentiation of a dorsal wing eyespot at this temperature in *Bicyclus* lasts around 24 hours (French and Brakefield, 1995).

Grafting and wing damage operations

We aimed to analyse fusion patterns between the normal eyespots on the dorsal forewing of *B. anynana* and additional ectopic eyespots induced by grafting a focus into an adjacent, more distal, position on the same wing-cell (area between two wing veins). The foci used for grafting came from the HIGH line in order to produce large ectopic eyespots. Both posterior foci of HIGH line donor pupae (from left and right wings) were grafted into distal positions, next to the normal anterior and posterior foci, respectively, of STOCK pupae (Figure 1b). Grafting operations were performed 3 to 5 hours after pupation. Control operations were made in order to measure the proximal-distal symmetry of isolated ectopic eyespots, unfused with the normal eyespots. In these operations, the anterior and posterior foci in the STOCK host pupa were pierced with a fine tungsten needle before grafting the two foci into distal positions (Figure 1c). The eyespots resulting from the damaged foci become very reduced or absent and cannot fuse with the eyespots from the grafted foci. In some control operations, LOW line pupae were used as hosts for HIGH line foci. Here, no damage was applied to the foci of the host pupae since the dorsal eyespots characteristic of the LOW line were too small to fuse with the ectopic eyespots from grafted foci.

Butterflies from the *Spotty* line were used to study fusion patterns between eyespots in adjacent wing-cells, i.e., along the anterior-posterior axis of the wing. The fusion patterns were studied in two pairs of eyespots: the normal anterior eyespot and the one just posterior to it (in wing-cells II and III; see Figure 1a) and the normal large posterior eyespot and the one just anterior to it (in wing-cells IV and V). In order to obtain isolated two-eyespot fusion patterns, instead of the fusion of all four eyespots, two of the foci were damaged early in development. Using a fine tungsten needle, at 4-5 hours after pupation, the two most anterior foci were damaged in one wing and the two most posterior foci were damaged in the other wing (Figure 1c).

Measuring the fused patterns

After adult emergence, the eyespot patterns in operated wings were drawn on paper with a camera lucida attachment. Contours of the white pupil, the black disc and the outer gold ring of the eyespots were drawn, along with the position of the veins and the distal wing margin.

From the most symmetric looking patterns (symmetry is important since we assume a gross scale homogeneity of the medium where diffusion takes place) we took several measurements from points along the outer contour line of each fusion pattern (at approximately 30 points), at similarly spaced intervals, to the centre of each white

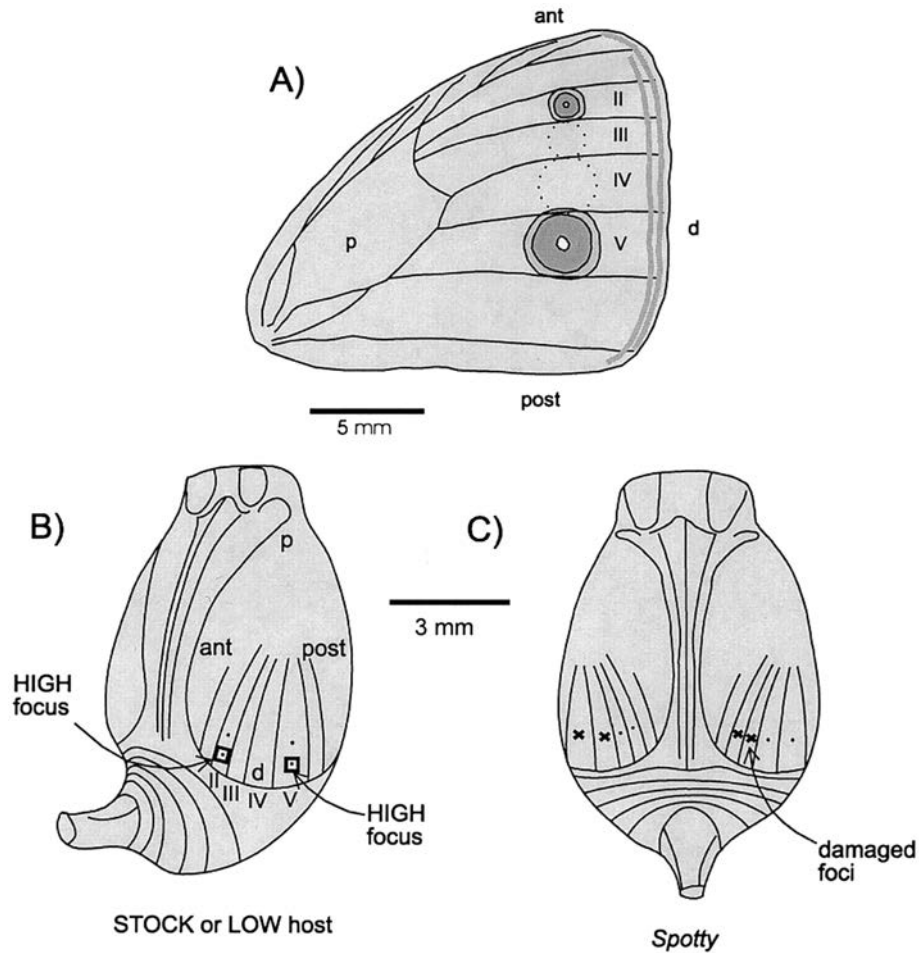


Figure 1. Schematic representation of pupae from *Bicyclus anynana* and dorsal surface of adult forewing. A) Adult wing with normal anterior and posterior eyespot patterns. *Spotty* mutants have two additional eyespots (represented by the dashed circles) on wing-cells III and IV. B) Foci from the HIGH line were grafted onto two distal sites on the wing of STOCK or LOW host pupae. Dots on the wing indicate foci; squares, pieces of grafted tissue; wing-cells are labelled from II-V; p, proximal; d, distal; ant, anterior; post, posterior. C) Pupa from *Spotty* line showing wing-cells where damage was applied to foci, by piercing with a fine needle. The undamaged foci, on right and left wings, will fuse together in pairs.

pupil (r_1 and r_2 ; Figure 2), in order to test whether the eyespot contours satisfied the analytical relationship predicted by the diffusion model to be described below. Note that the assumption of gross scale homogeneity of the diffusion medium is only made in order to arrive at a closed solution of the diffusion equations that can be used as a vehicle for data analysis. What matters is whether the right amount of additional pigmented area is in the right location, compared to what single eyespots would look

like if there had been no signal addition. There is no reason to suspect that selecting for pattern symmetry will produce a bias for this additional area. However, by doing so we remove substantial parameter noise that otherwise would make the discriminatory power of our data analysis technique very slim.

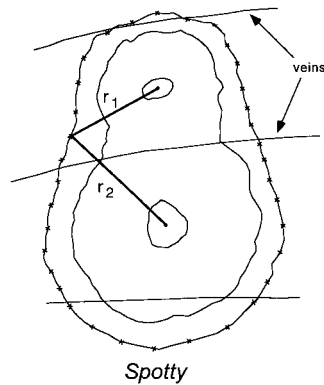


Figure 2. Drawing of a *Spotty* fusion pattern between eyespots IV and V. Distances r_1 and r_2 were taken from approximately equidistant points (marked with crosses) from the outer contour of the fused eyespots to both pupil centres.

Modelling

Assumptions

In our reference model it is assumed in accordance with Nijhout (1978) that the coloured eyespot regions correspond to different, adjacent, concentration ranges in the stationary profile generated by a process in which a morphogen diffuses away from either one or two point sources while decaying at a constant relative rate. We shall refer to the values of the morphogen concentration at the boundary between two such ranges as thresholds.

It can be shown that the idealisation of a spatially extended area of focal cells as a point source approximates well the stationary concentration gradient for roughly circular sources both of the constant strength type assumed by Nijhout (1978), and of a constant level type, in which a constant morphogen concentration is maintained inside the source cells, as considered by Bard and French (1984), provided our interest is in patterns that are considerably larger than the source diameter.

An equation satisfied by the contours

A single point source gives rise to an equilibrium morphogen concentration m of the form $m = cK_0(\lambda r)$, with r the radial distance from the source in cm, $c = S/(dD)$, S the source strength in mol/sec, D the diffusion coefficient in cm^2/sec , d the thickness of the wing tissue, $\lambda = \sqrt{k/D}$, k the decay constant in sec^{-1} , and K_0 the so-called modified Bessel function of the second kind of order zero. Graphs of K_0 , tables and handy approximation formulas can be found in Abramowitz and Stegun (1965). Let T denote the threshold value of morphogen concentration bounding the outer contour of a single eyespot. Then the points on the contour satisfy $m = T$. By dividing through by T we find that the points on the model contour satisfy the equation

$$p K_0(\lambda r) = 1, \quad (1)$$

with $p = c/T$. Not unexpectedly, the contour is a circle with radius depending on the two parameters p and λ .

The model formulation excluded any interaction between the morphogen molecules away from the source. We can, therefore, get the local morphogen concentration due to more than one point source by simply adding the contributions from the various sources. In the case of two sources $m = c_1 K_0(\lambda r_1) + c_2 K_0(\lambda r_2)$, with r_1 and r_2 being the radial distances from each point along the contour to the respective sources, and c_1 and c_2 the corresponding source strengths. The model contour satisfies

$$p_1 K_0(\lambda r_1) + p_2 K_0(\lambda r_2) = 1, \quad (2)$$

with $p_i = c_i/T$. Unfortunately, equation (2) is more easily written down than solved but it permits the derivation of a statistical method, to be described below, to determine λ from a fusion contour of two eyespots, and to test the diffusion model in an analytical manner.

Statistics

Estimation of λ

In the case of a single point source it is not possible to estimate p and λ separately from an estimate of the radius. Given a radius and the value of either λ or p , we can calculate the value of the other parameter by taking recourse to tables of K_0 . However, if we have more than one source it is possible to do better.

From a drawn fusion contour of two eyespots we can determine the distance for several points along that contour to the centre of the two white pupils (r_1 and r_2 from expression (2), above). Expression (2), which describes the relation between all these variables, has the form of a linear equation in the transformed radii $K_0(\lambda r_1)$ and $K_0(\lambda r_2)$. If the diffusion gradient hypothesis is correct, a plot of $K_0(\lambda r_1)$ against $K_0(\lambda r_2)$ should show a straight line, provided λ has been given the right value. Therefore, λ can be estimated as the value that maximises the correlation coefficient between the two Bessel transformed radii. Once λ is estimated in this manner, the strength of a source relative to the threshold level (p_i) can be estimated from an orthogonal regression (see e.g. Sprent, 1969).

Testing the model

Once the correct λ is found, the plot of the two Bessel transformed radii provides an informal test to determine whether or not eyespot development is consistent with Nijhout's diffusion model. If and only if the points in the plot fall on a straight line, the diffusion model is corroborated. This test is the strongest possible one that can be achieved with the type of data under consideration.

3. RESULTS

The grafting of a focus distal to the normal anterior or posterior eyespot resulted in the formation of an ectopic eyespot (with a white pupil, black disc and outer gold ring) that fused with the adjacent eyespot (Figure 3a, b). The damage to pairs of foci in

Spotty individuals greatly reduced these eyespots and resulted in restricted fusion patterns involving the undamaged anterior or posterior pairs of eyespots (Figure 3c). Note that in both type of experiments the colour pattern crosses the veins and these do not seem to play any role in limiting the diffusion of the putative morphogen.

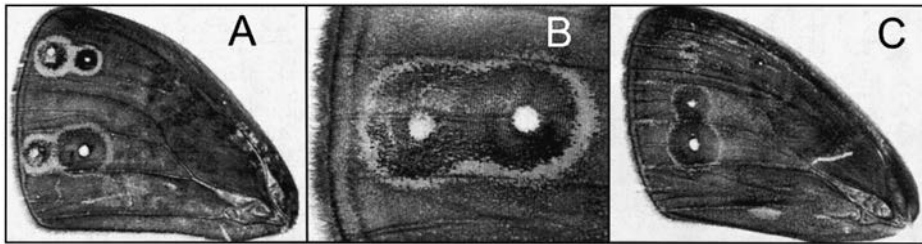


Figure 3. The operated wings of *Bicyclus anynana*. A) A STOCK wing showing ectopic eyespots, induced by grafted HIGH foci, fusing with the normal anterior and posterior eyespots. B) A posterior eyespot fused with an eyespot induced by a piece of rotated focal epidermis. C) *Spotty* wing where the two most anterior eyespots are reduced in size, due to early damage to their foci, and were unable to fuse with the two most posterior eyespots.

Estimating λ and testing the diffusion model with Bessel plots

Table 1. Estimated λ s and correlation coefficients for the most symmetric fused patterns.

Fused pattern	Estimated λ	Correlation coefficient	n
<i>Spotty</i> III + IV	0.35*	0.97	27
	0.45	0.97	27
	0.65	0.96	28
	0.55	0.96	28
	0.53	0.96	25
<i>Spotty</i> V + VI	0.51*	0.97	30
	0.53	0.94	32
	0.95	0.91	34
	0.61	0.94	35
	0.86	0.94	30
STOCK VI + ectopic eyespot	0.89*	0.98	31
	0.75	0.96	34
	0.64	0.97	33
	0.79	0.97	34
	0.84	0.94	33
Control	3.62*	0.83	29

n is the number of points along the contour used to calculate r_1 and r_2 (see “Estimation of λ ” in the M and M section). Control refers to a drawn pattern of two circles that intersect. Patterns shown in Figure 4 are identified by an asterisk (*).

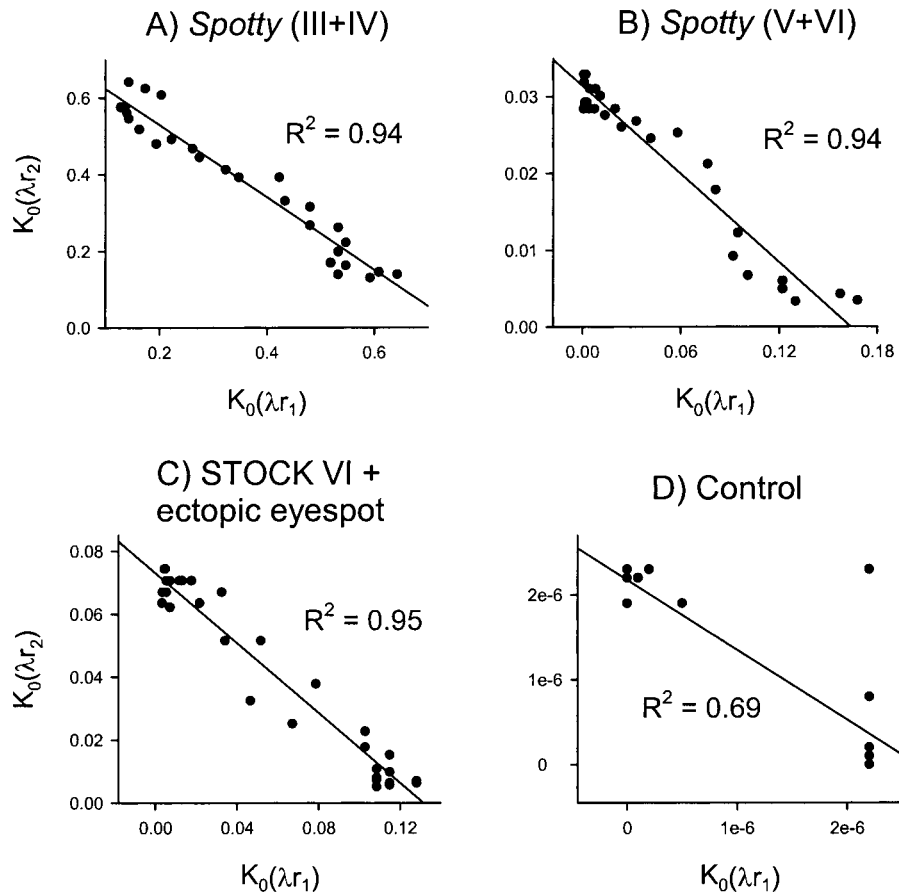


Figure 4. Bessel plots for λ maximising the correlation coefficient between the two Bessel transformed radii. r_1 and r_2 are the distances from several points along a fused contour to the centre of each white pupil (or to the centre of the drawn circles in the control pattern). A) and B) *Spotty* fused patterns. C) A large posterior eyespot fused with an ectopic eyespot. D) The control pattern is a drawing of two intersecting circles (to introduce some noise in the measurements the central point of one of the circles was moved slightly off-centre).

The parameter λ was estimated from the most symmetric fused patterns (Table 1). The plots of the Bessel transformed radii (Figure 4) show that the data from the fused eyespots fits well onto a straight line, producing high correlation coefficients (Table 1).

In order to have an idea about the shape of the plot of the Bessel transformed radii for another type of signalling model, we performed the same operation for an artificial fusion pattern produced by union of two drawn intersecting circles. This control pattern can represent a model based on a simple cell to cell relay system. In this case, points will always be found lying on two straight lines parallel to the axes (apart from

some measurement error), whatever value of λ is chosen (the “optimal” λ will depend on the location of the chosen measurement points along the contour). The results are depicted in Figure 4d for comparison.

The Bessel plots provide the best possible test of the diffusion model, as this technique uses all pattern information available in the fused eyespot data. As such, it has also the greatest possibility to lead to a rejection of the diffusion model due to confounding factors such as a wrong guess about the precise location of the foci. The results of Figure 4 corroborate the diffusion model.

4. DISCUSSION

When two eyespot foci are close on the wing surface they can produce a fused pattern in which more cells than just those expected from the intersection of two circles, differentiate as part of the pattern. The observed pattern is consistent with the presence of a long-range morphogen gradient established from each focus, and influencing cells beyond the outer contour of the eyespot. The outer contour would correspond to a threshold morphogen concentration, below which no pattern is produced, but not to an abrupt end of a signal. In the area of overlap of the two gradients, an additive effect would raise the morphogen levels above the threshold and produce the extra area of pattern observed.

An alternative model for long-range patterning from a signalling group of cells is the cascade model. In this model signalling occurs via a sequence of short-range interactions where cells receiving signal A generate signal B, which is perceived by the neighbouring cells, leading them to produce signal C and so on. Propagation of a signal (and therefore determination of its final range), will depend mainly on the type of signal rather than on the amount received (reviews in Perrimon, 1995; Blair, 1995). The experiments described in this paper, along with the following lines of evidence, strongly indicate that the focus provides one long-range signal, rather than merely the first of a cascade of short-range signals:

1) Progressive later damage applied to the focus, in early pupal development, leads to a progressive increase in adult eyespot size (Nijhout, 1980a; French and Brakefield, 1992; Monteiro *et al.* 1994). If eyespot size is dependent on a cascade of signals, only the first of which is produced in the focus, it is difficult to envisage how late damage to the focus can still affect eyespot size. With a gradient model, the later the damage the smaller the effect on the final eyespot size, until the complete gradient is established.

2) Damaging a focus often leads to a small pattern on the adult wing consisting only of scale cells containing pigment from the outer colour rings (French and Brakefield, 1992). Early damage to a focus might result in a very shallow concentration gradient, that can only rise above the lower thresholds of gene activation - producing the outermost colour rings in an eyespot. Influencing the production or transmission of signals in a cascade model should always first affect differentiation of cells furthest away from those signals, i.e., the ones producing the outer colour rings, which is not the case.

Morphogen gradients have been known to exist since *bicoid* was discovered in the early syncytial state of the *Drosophila* embryo (Driever and Nusslein-Volhard, 1988a, 1988b). At this stage of development, the egg is still devoid of cell membranes and

large proteins can diffuse freely in the common cytoplasm. But proof has been accumulating that even in solid tissue (Gurdon *et al.*, 1994) or in an epidermal layer (Heemskerk and DiNardo, 1994; Katz *et al.*, 1995; Zecca *et al.*, 1995; Nellen *et al.*, 1996; Lecuit *et al.*, 1996), cells are responding to particular substances, synthesised some distance away, in a concentration dependent manner. More recently however, two research groups have directly visualised the establishment of a morphogen gradient in a cellularised environment (Entchev *et al.*, 2000; Teleman and Cohen, 2000). They accomplished this by fusing the *Drosophila* growth factor Decapentaplegic (Dpp) to the small green fluorescent protein, GFP, and observing the establishment of a fluorescence gradient of Dpp in the pupal *Drosophila* wing. They showed by a series of experiments that Dpp-GFP moves rapidly, at a speed of more than four cells per hour and that it reaches its steady state between 6 and 8 hrs (Entchev *et al.*, 2000). Dpp-GFP diffuses through the epidermis via receptor-mediated endocytosis (forming intracellular punctate structures) rather than through the extracellular space. Additionally, endocytic trafficking and degradation in the receiving cells play an essential, rate limiting role for establishing the Dpp signalling range. Entchev *et al.* (2000) propose a model where the balance between recycling and degradation of the Dpp molecule determined the shape of the gradient. Here, to establish and maintain the gradient it is sufficient to have a similar rate of endocytosis and degradation in all receiving cells. In the present modelling of eyespots, we have also assumed a constant diffusion coefficient, D , and a constant decay constant, k , as well as a constant source strength, S . This leads to a stable morphogen gradient profile that becomes visible in the outer eyespot contour.

It is noteworthy that the average radius of the posterior eyespot in *B. anynana* corresponds to about 0.7 mm or 90 cell diameters on the pupal wing epidermis (French and Brakefield, 1995), which is more than three times the range of the Dpp range in the *Drosophila* wing (up to 25 cell diameters from its source). On the other hand, eyespot signalling in *Bicyclus* takes at least 24h till completion. If the rate of morphogen diffusion in the butterfly tissue is the same as that of Dpp in *Drosophila* (four cell diameters per hour), 24h of signalling would reach up to 96 cell diameters, the average size of an eyespot. Modifications in the ratio between recycling and degradation of the morphogen by the target cells, around the focal signalling cells, would allow the steady state of the morphogen in the butterfly wing to be different to that in the fly wing.

A few undetermined issues however, still remain when simulating focal signalling. The first is whether concentration gradients can be read before equilibrium is reached (Gurdon *et al.*, 1995). The second is whether a focus is a constant level or constant rate source of morphogen. Although these two types of sources are conceptually quite different and constant level sources biologically more plausible (see Nijhout, 1991), the patterns at equilibrium should be essentially the same for foci with a narrow diameter.

Some of the wobbliness about the regression line, especially visible in Figure 4b, may be due to some size asymmetry about the line connecting the centres of two fused eyespots in *Spotty* wings (data not shown). This is probably related to the different epidermal cell arrangements encountered along the proximal-distal axis of the wing (Monteiro *et al.*, 1997), thus producing some non homogeneity in the diffusion medium.

In summary, we conclude that the present experiments provide an inexpensive way of testing the morphogen gradient hypothesis for butterfly eyespot formation. A better test, using more sophisticated molecular techniques, will become possible only when the putative morphogen is identified in this system. Carroll *et al.* (1994) have started cloning *Drosophila* homologues of important patterning genes in the imaginal disc of *Precis coenia*, and looking at their expression patterns. The dorsal-ventral, anterior-posterior and proximal-distal axis of the butterfly wing disc and that of *Drosophila* are specified in a similar way. For instance, *apterous* is expressed in dorsal cells, *engrailed* in the posterior domain, and *wingless* in presumptive wing-margin cells. *Distal-less (Dll)* is a gene expressed in the centre of the leg imaginal disc in the fruitfly, that gives rise to the most distal structures of the leg and also in a distal zone in the wing disc of *P. coenia*. *Dll* is also expressed in the future centres of the eyespot patterns in *P. coenia* and *Bicyclus anynana* (Brakefield *et al.*, 1996). Additionally, *Spalt* and *Engrailed*, have recently been found to be expressed in two concentric circles in the pupal wing of *B. anynana*, correlating to the rings of black and gold scales respectively (Brunetti *et al.*, in prep.). To get a handle on the morphogen underlying the establishment of eyespot patterns research into the upstream regulators of the latter genes is needed.

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