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A simulation study of the genetic regulatory hierarchy for butterfly eyespot focus determination

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SUMMARY The color patterns on the wings of butterflies have been an important model system in evolutionary developmental biology. Two types of models have been used to study these patterns. The first type of model employs computational techniques and generalized mechanisms of pattern formation to make predictions about how color patterns will vary as parameters of the model are changed. These generalized mechanisms include diffusion gradient, reaction-diffusion, lateral inhibition, and threshold responses. The second type of model uses known genetic interactions from *Drosophila melanogaster* and patterns of candidate gene expression in one of several butterfly species (most often *Junonia* (*Precis*) *coenia* or *Bicyclus anynana*) to propose specific genetic regulatory hierarchies that appear to be involved in color pattern formation. This study combines these two approaches using computational techniques to test proposed genetic regulatory hierarchies for the determination of butterfly eyespot foci (also known as border ocelli foci). Two computer programs, STELLA 8.1 and Delphi 2.0, were used to simulate the determination of eyespot foci. Both programs revealed weaknesses in a genetic model previously proposed for eyespot focus determination. On the basis of these simulations, we propose two revised models for eyespot focus determination and identify components of the genetic regulatory hierarchy that are particularly sensitive to changes in model parameter values. These components may play a key role in the evolution of butterfly eyespots. Simulations like these may be useful tools for the study of other evolutionary developmental model systems and reveal similar sensitive components of the relevant genetic regulatory hierarchies.

INTRODUCTION

Butterfly wing color patterns are an attractive model system for exploring the relationship between developmental genetics and evolution. Such patterns are very suitable for study because they are highly variable, consist of clearly defined subunits, exist in two dimensions, are structurally simple, and at least some patterns are clearly associated with fitness benefits associated with natural or sexual selection (Nijhout 1991; Brakefield et al. 1996; Beldade and Brakefield, 2002). For these reasons, butterfly color pattern formation has been studied by researchers interested in modeling developmental processes. These models can be divided into two types. The first type employs generalized mechanisms of pattern formation to make predictions about how color patterns will vary as parameters of the model are changed. These generalized mechanisms include diffusion gradient (Nijhout 1978; Bard and French 1984), reaction-diffusion (Murray 1981, 1989; Nijhout 1990), lateral inhibition (Nijhout 1990), and threshold responses (Nijhout 1991). Such models have been used as the basis for simulations of the microevolution of color patterns (Nijhout and Paulsen 1997), for understanding fluctuating asymmetry in terms of classical quantitative genetic theory (Klingenberg and Nijhout 1999), to test the suitability of proposed ground plans as a basis for understanding the evolution of pattern polymorphisms (Sekimura et al. 2000), and to understand the responses of wing patterns to surgical perturbations (Brakefield and French 1995; French and Brakefield 1995).

The second group of models proposes regulatory interactions between specific gene products to account for the formation of particular color patterns. These models rely heavily on the study of expression patterns of candidate genes and the coincidence of these gene expression patterns with color patterns in adult butterfly wings (usually *Bicyclus anynana* or *Junonia* (*Precis*) *coenia*) (Carroll et al. 1994; Brakefield et al. 1996). A synthesis of many of these proposed genetic interactions was recently proposed by Marcus (2005). Unfortunately, in most cases, the various hypotheses for genetic interactions are supported solely by correlations between temporal–spatial gene expression patterns and the shape, size, location of color pattern elements, and known genetic interactions of candidate genes from *Drosophila melanogaster*, a species which diverged from the butterfly lineage over 200

million years ago (Kristensen and Skalski 1999), making the proposed networks of genetic interactions highly speculative.

Experimental manipulation of gene expression patterns within developing butterfly wings has proven to be difficult (Marcus 2005), though there has been some progress in developing techniques that may ultimately make such manipulations easier (Lewis et al. 1999; Weatherbee et al. 1999; Marcus et al. 2004). Until methods are developed to permit the routine manipulation of gene expression in butterflies, the development of other types of tests for the proposed genetic regulatory networks is highly desirable. One such test involves the unification of the computational techniques previously used in the generalized developmental models with the genetic regulatory hierarchies that have been proposed for the pattern formation in this system.

This approach has already provided useful insights in other model systems such as cell cycle regulation (Novak and Tyson 1993, 1995), folate metabolism (Nijhout et al. 2004), MAP kinase signal transduction cascades (Huang and Ferrell 1996), Notch–delta interactions (Collier et al. 1996), *Drosophila* embryogenesis (Reinitz and Sharp 1995; Bodnar 1997; Sharp and Reinitz 1998; von Dassow et al. 2000; Bodnar and Bradley 2001), and flower development in *Arabidopsis* (Espinosa-Soto et al. 2004). In this article, we implement the first simulation model of butterfly color pattern formation that employs specific genetic interactions in order to test whether the hypothesized interactions are actually capable of producing the gene expression patterns that have been observed in vivo. Further, our modeling efforts include components of both diffusion gradient and threshold response models, and are the first simulations to combine both of these mechanisms in order to explain butterfly eyespot development.

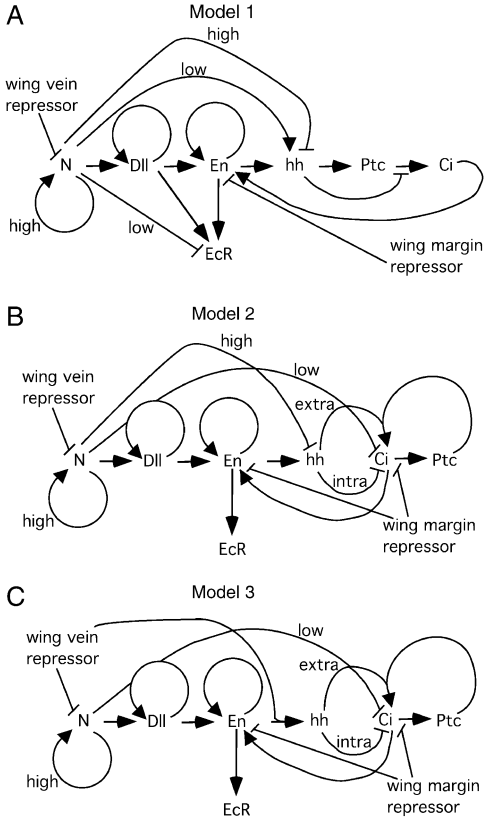

Fig. 1. (A) Diagram of Model 1 genetic regulatory network for eyespot focus determination as proposed by Marcus (2005). (B) Diagram of Model 2 genetic regulatory network for eyespot focus determination. (C) Diagram of Model 3 genetic regulatory network for eyespot focus determination.

MATERIALS AND METHODS

The general structure of Model 1 (Fig. 1A) stemmed from documented patterns of expression of many genes known to be expressed in the developing eyespot foci of *B. anynana* and *J. coenia*. The expression of Notch (N) preceding the co-expression of Notch and Distal-less (Dll), coupled with temporal–spatial observations in butterfly wing imaginal discs suggests the positive regulation of the Dll transcription factor by the membrane-bound receptor *notch* (Reed and Serfas 2004). The expression of Dll appears to precede and induce the expression of a protein in the presumptive eyespot focus that is recognized by a monoclonal antibody that binds to both the Engrailed (En) and Invected transcription factors in *D. melanogaster* (Patel et al. 1989; Monteiro et al. 2003; R. Reed, pers. comm.). The protein(s) recognized by this antibody in the eyespot focus may be homologs of either Engrailed or Invected, or may be paralogs that share an epitope recognized by this antibody. Since these proteins are almost certainly within the engrailed family of transcription factors, we will refer to them in this article as En.

Observations on the development of eyespot foci have revealed apparent associations in the expression patterns of *hedgehog* (*hh*) transcript and En protein. In *J. coenia*, transcription of *hh* has been noted on either side of the mid-line of the wing cell (defined as a region of wing tissue bordered by the wing margin and by a series of wing veins that contains a field of cytological cells), corresponding to areas of focal development within the wing cell expressing En. Co-expression of the transcript of the hedgehog receptor *Patched* (*Ptc*) and the transcription factor Cubitus interruptus (Ci) has been observed to coincide with the expression of En (Keys et al. 1999). Through temporal and spatial observations of the co-expression of Ecdysone Receptor protein (EcR) and Dll, it has been hypothesized that Dll acts in the up-regulation of EcR (Koch et al. 2003). Collectively, these observations led to the generation of Model 1 by Marcus (2005).

We tested the genetic network described in Model 1 using the STELLA 8.1 (2003) and Delphi 2.0 (1996) computer programs. STELLA runs a functional time course by which interactions within and between cells take place based on the thresholds of stocks upstream and downstream in the model. In essence, STELLA generates a working flow chart by which the proposed genetic model could be tested against to see if the interactions hypothesized could in fact generate a plausible outcome resembling that which has been found experimentally (Hargrove et al. 1993).

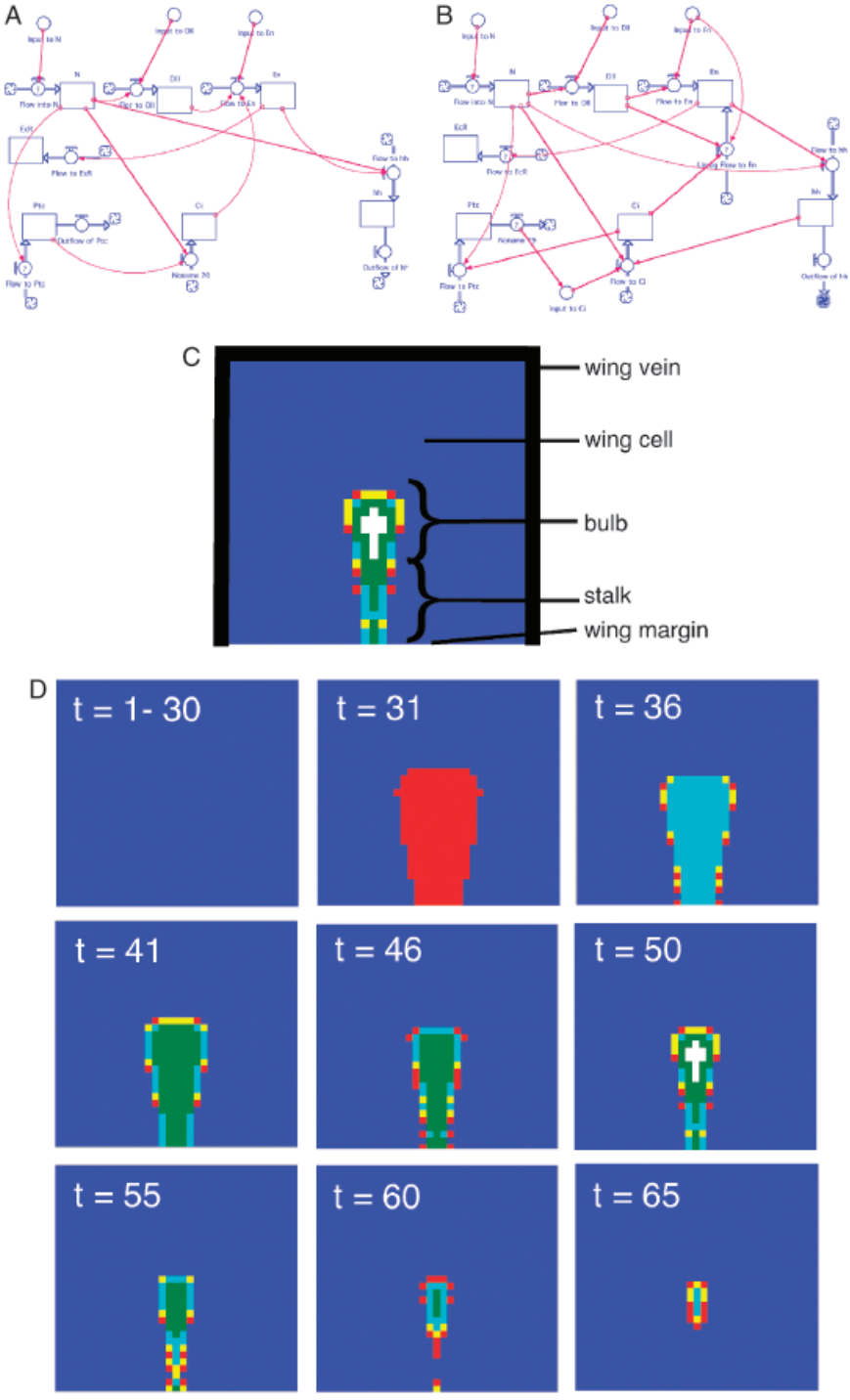

Fig. 2. (A) STELLA diagram for Model 1. (B) STELLA diagram for Model 2. Our STELLA models did not include the effects of either repressor, so we could not use STELLA to investigate Model 3 because the role of the wing margin repressor is essential to the function of this model. (C) A sample Delphi model output defining the terminology used here and in Fig. 4 to describe patterns of gene expression. (D) Time series of Distal-less expression output for all three models as implemented in Delphi. The time series was extended beyond 50 time steps to show the complete retraction of the stalk, as shown by both previous models (e.g., Nijhout 1991, 1994a) and in direct observations of Distal-less expression (e.g., Carroll et al. 1994). The time series for Notch in the models shows a similar pattern to that seen for Distal-less (data not shown) and is also similar to direct observations of Notch expression (Reed and Serfas 2004).

Several STELLA objects were used in the layout of the pathway to be tested. Stocks served as reservoirs by which gene products could be assessed. Connecters served to illustrate and functionally allow the program to assess where interactions were taking place within the model (Figs. 2, A and B). Inflows allowed a space to designate the type and outcome of interactions leading to the product which is then added to the stocks. Outflows were utilized in places that required the removal of a product. From these objects a nine cell square model, consisting of three rows of three cytological cells each, with the center cell of the middle of the three rows being the center of the eyespot focus, connected to a chain of three additional cells representing the stalk. The stalk is a finger of expression protruding along a presumptive midline into the wing cell from the wing margin, where the most proximal region will broad-

en to form a bulb shaped zone that marks the future eyespot foci (see Fig. 2C). In our STELLA model, a threshold of Notch within the proximal region of the stalk, acts as a trigger for the genetic interactions within the nine-cell region. Within each cell the presence of Notch (N) above a threshold value turns on the production of Dll. A threshold of Dll then up-regulates the production of En. The presence of En coupled with a low value of N within a cell will turn on the production of hh. In, Model 1, the presence of hh turns on the production of *Ptc* in neighboring cells (proposed by diffusion) by which Ci is then up-regulated. The presence of Ci further up-regulates the production of En. The equations and thresholds implemented in our models are listed in Appendix A.

The absolute values of the thresholds implemented are arbitrary, although in some cases the relative values of particular parameters may be of importance. The models are robust to minor changes to the thresholds without compromizing the output; however, changes to the thresholds can alter the temporal appearance of expression patterns. In few cases, drastic change to the thresholds moved the appearance of an expression pattern outside of the time course of the simulation, or changed the degree of expression during the time course such that overlapping expression patterns no longer fit within the thresholds downstream of the change/s, resulting in a failure to produce an eyespot focus. The temporal shift of expression patterns was not an issue for most aspects of our models. However, one very important threshold that had to be conserved throughout the models was the relationship between N and hh. The threshold by which Notch up-regulates the expression of Dll must be sufficiently low so that Dll will have the opportunity to up-regulate En, and subsequently hh before Notch exceeds its threshold for initiating repression of hh (Fig. 1).

The STELLA output (Fig. 3) of Model 1 (Fig. 1A), revealed inconsistencies between the model output and known gene expression patterns in the developing eyespot. In order to correct these inconsistencies, Model 2 (Fig. 1B) was generated with slightly altered genetic interactions from Model 1 (see Results). Following the generation of the two models in STELLA, the basic equations determining each step in the process within STELLA were exported and implemented into a PASCAL application written within the Delphi 2.0 programming environment in order to place the models within a spatial modeling environment. The program in Delphi was written such that a large grid of 35 × 35 cytological cells, representing a wing cell (a field of cytological cells bordered by the wing margin and by a series of wing veins) in which an eyespot focus will develop, would display gradients of the different products proposed by the genetic network. The position and relative amount of the products could then be spatially compared with the relative amounts and positions of the products found experimentally within developing butterfly wing imaginal discs.

Three factors, in the genetic model, were selected to diffuse within the wing cell on the basis of theorized and known diffusion patterns. It has been hypothesized that an as of yet unknown repressor is secreted from the incipient wing veins which participates in patterning the wing cell (Nijhout 1991, 1994b; Koch and Nijhout 2002). Similar diffusible repressors are known to participate in wing vein patterning in *Drosophila* (Biehs et al. 1998; Marcus 2001). We hypothesize that the role of this wing vein repressor functions in a direct down-regulation of Notch, such that N is contained within a

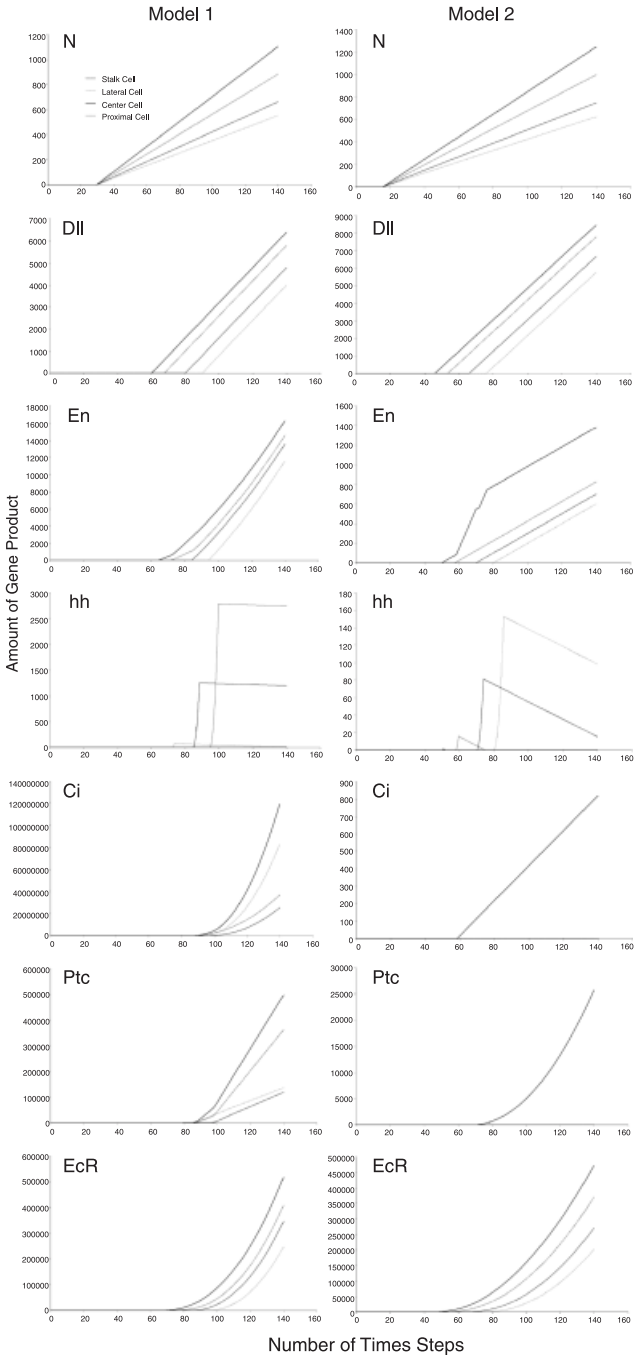

Fig. 3. STELLA 8.1 simulations of eyespot focus development.

stalk-like domain of expression in the midline of the wing cell, the terminus of which later becomes the eyespot focus (Reed and Serfas 2004). We also hypothesized the existence of a second repressor that diffused from the wing margin, as has been described previously but of which is currently unidentified (Nijhout 1990). Ligands that diffuse from the wing margin are also known in *Drosophila* (e.g., wingless; Phillips and Whittle 1993; Blair 1994). This wing margin repressor was utilized to define a region of En

expression within the co-expression of Notch and Dll of the eyespot similar to that observed in developing wing imaginal discs. Without the interaction of this wing margin repressor, En expression would occur over the entire domain of Dll expression, which has not been observed in butterfly eyespot development (Monteiro et al. 2003). The Delphi model implements a diffusion model for both of these hypothetical repressors. The hh gene product is also known to diffuse (Peifer and Bejsovec 1992), but Keys et al. (1999) noted that cells in which hh transcription occurs do not express Ci. In STELLA, it was straightforward to implement these conditions such that hh being produced by a cell has an impact on the genetic interactions within that cell, whereas hh that has diffused out of the cell has a different function. In order to account for both the intracellular and extracellular effects of hh expression in Delphi, our models required two categories of hh expression that had to be tracked separately: the first was hh generated within a cell, while the second was hh being received by a cell via diffusion from nearby cells.

In order to distinguish the two forms of hh in the Delphi simulation, the designations hh initiation and hh diffusion were used. Hh initiation is the stocked form of hh and does not diffuse from the cells in which it is generated. Hh diffusion is based on the diffusion pattern that is set up from the values of hh initiation, with hh initiation being the focal point for diffusion. A general algorithm was used to determine diffusing gradients within Delphi.

The diffusion gradient was calculated for each diffusing gene product by taking the average difference between each cytological cell and its four perpendicular neighbors and adding that value to the previous value for that cytological cell.

$$\text{New } C = \text{Old } C + (((c[x-1,y] + c[x+1,y] + c[x,y-1] + c[x,y+1]) - 4 \times c[x,y])/4)$$

The implementation of Model 1 in Delphi revealed two additional inconsistencies between Model 1 and known gene expression patterns in the developing butterfly wing (see Results). Additional adjustments were made in Model 2 so that the output of Model 2 more closely resembles what is observed in the developing wing imaginal disc. Exploration of the parameter space of Model 2 in our Delphi simulations revealed that there was an additional very similar genetic regulatory network that was capable of generating the observed patterns of gene expression in the developing butterfly wing, which we refer to as Model 3 (Fig. 1C). In this model, the wing vein repressor plays a second role in the regulatory network as an activator of hh in the presence of En (in places of overlap of En and the wing vein repressor). Because the wing vein repressor plays a key role in Model 3, we were not able to perceive this model in our STELLA model, which did not include the implementation of the wing vein repressor.

RESULTS

The output of Model 1 in STELLA revealed the co-expression of hh transcripts and Ci in the same cells (Fig. 3), something that has not been observed in the developing eyespot (Keys et al. 1999). This observation of Model 1, led to the proposed independent intracellular and extracellular effects of hh in Model 2 (Fig. 1B). That is to say, the function of hh being produced within a cytological cell (intracellular hh) inhibits the formation of Ci in that cytological cell, while extracellular hh that is diffusing into the nearby tissue has a separate function on Ci via the transduction of the hh signal through *Ptc*.

In Delphi, an early version of Model 2 revealed that unless the presence of Notch was a requirement for Cubitus interruptus expression, Ci would be expressed in much of the wing cell, which is not observed in vivo (Keys et al. 1999). Also, it was observed in Model 2 that unless we included a down-regulatory effect of the margin repressor on Cubitus interruptus, Ci and *Ptc* accumulated within the domain of expression of N and Dll in the midline of the wing cell (Fig. 4), rather than solely in the eyespot focus as is observed in vitro (Keys et al. 1999). Since, in Model 2, *Ptc* is a target of Ci, the limitation of Ci by the wing margin repressor worked to correct the inappropriate expression of *Ptc* in the stalk.

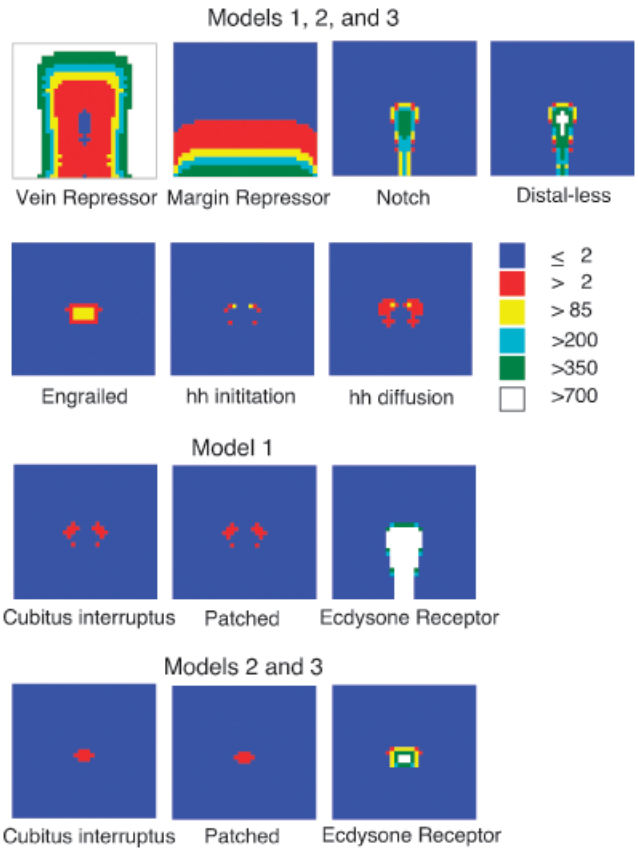

Fig. 4. Delphi 2.0 simulations of eyespot focus development after 50 time steps. Output for the wing vein repressor, the wing margin repressor, Notch, Distal-less, Engrailed, *hedgehog* (*hh*) initiation, and hh diffusion were the same for Models 1–3. Expression patterns of Cubitus interruptus, *Patched*, and the Ecdysone Receptor differed between the models, with Models 2 and 3 more closely resembling the expression patterns previously observed by Keys et al. (1999) and Koch et al. (2003).

Furthermore, the genetic network implemented in Model 1 in Delphi resulted in the misexpression of EcR in the stalk of the eyespot. In vivo, EcR expression is only found late in the eyespot focus, and not in the stalk of N and Dll expression leading out to the focus (Koch et al. 2003). Because of the temporal separation in the appearance of Dll and EcR observed by Koch et al. (2003), we hypothesize that Dll is upregulating EcR indirectly through En, which would both limit EcR to the eyespot and would more closely match the temporal appearance of EcR in the eyespot. By incorporating these adjustments into the genetic regulatory network implemented in Model 2, we have been able to recreate time series expression data for most of the genes thought to play a role in determining the eye spot foci in butterflies (Figs. 2D and 4).

DISCUSSION

Interpreting model outputs

A comparison of the output from the three models in STELLA and in Delphi shows that Models 2 and 3 are better able to reproduce the gene expression patterns described thus far in the developing butterfly wing. The co-expression of hh and Ci in Model 1 in STELLA does not match in vivo observations, while Model 2 in STELLA better matches the observed expression patterns. The co-expression of hh and Ci was also apparent in the Delphi simulation of Model 1, whereas Models 2 and 3 showed that the expression pattern of hh and Ci closely resembled the expression patterns observed in vivo. Furthermore, in Model 2 in STELLA, the expression pattern of *Ptc* and Ci occurs in the eyespot focus, as occurs in vivo, while Model 1 in STELLA shows a pattern of expression of *Ptc* and Ci over the entire nine-cell model. The Delphi programming environment further emphasized the distinctions between the appropriate expression pattern of *Ptc* and Ci in Models 2 and 3, and the misexpression of *Ptc* and Ci in Model 1. Within the Delphi programming environment, we also noted a misexpression of EcR within the stalk of Model 1 but which was appropriately expressed only in the forming eyespot in Models 2 and 3 through the removal of the direct interaction of Dll on EcR. The iterative process by which we derived Models 2 and 3 from Model 1 allowed us to identify deficiencies in Model 1 that prevent it from generating the patterns of gene expression that resemble the patterns observed in vitro. The use of two different simulation environments revealed weaknesses in Model 1 not apparent when using only one of these environments. The larger spatial simulations that were possible in Delphi were particularly useful in identifying problems with the original model.

In addition to verifying some of the suspected genetic interactions, and eliminating others, our simulations suggested interactions that had not been suggested previously. For instance, the interaction between the wing margin repressor and Ci, that without this interaction Ci expression would be seen over the majority if the region of Notch and Dll expression in both Models in Delphi. Likewise, the repressive interaction between intracellular hh and Ci within Model 2 in STELLA was necessary to inhibit the co-expression of hh and Ci. However, within Models 2 and 3 in Delphi, this repressive interaction between intracellular hh and Ci was not necessary because the zone of expression of intracellular hh lies outside the region of Notch expression that contains the appropriate threshold to allow the formation of active Ci. Simulation approaches to genetic hierarchies are very useful in suggesting additional genetic interactions necessary for models of developmental processes to work, as has been previously suggested by Bodnar (1997).

Model thresholds and the evolution of eyespot foci

Our simulations also revealed that in order for our Model 2 to successfully produce an eyespot focus, the low threshold at which N up-regulates Dll and thus initiates the up-regulation of downstream genes to produce hh, and the higher threshold at which N begins to repress hh, must be carefully balanced. Similarly, in Model 3, the same low threshold at which N upregulates Dll must be calibrated with respect to the threshold at which the wing vein repressor acts in a combinatorial fashion with En to upregulate hh. Regardless of which model is correct, if the thresholds necessary to up-regulate and downregulate hh are out of balance, these genetic regulatory hierarchies will fail to produce an eyespot focus, and since the focus is the organizer for the rest of the eyespot, the entire eyespot will fail to form (Brakefield et al. 1996). This suggests that a mechanism for the disappearance and reappearance of eyespots in butterfly lineages, which can be produced by selection in the laboratory (Beldade et al. 2002b), and which is an important feature of the differentiation of species in many groups of butterflies (Arbesman et al. 2003), may be the alteration of the previously described thresholds with respect to one another. And just as small changes in these thresholds relative to one another can cause the failure of eyespot foci to form in lineages that had previously produced eyespots, similar small changes in the opposite direction might restore the production of eyespot foci in lineages that had lost eyespots. This is therefore a possible explanation for the complicated pattern of gains, losses, and atavistic regains of eyespot patterns during the evolutionary history of butterflies (Nijhout 1991).

It is likely that, as many of the gene products involved are transcription factors (Panganiban et al. 1994; Schroeter et al. 1998; Keys et al. 1999), the relevant thresholds may be caused by variation in the ability of these gene products to bind to regulatory sequences of downstream targets and recruit RNA polymerase to the promoters of those targets. This variation may be because of evolution of the transcription factors

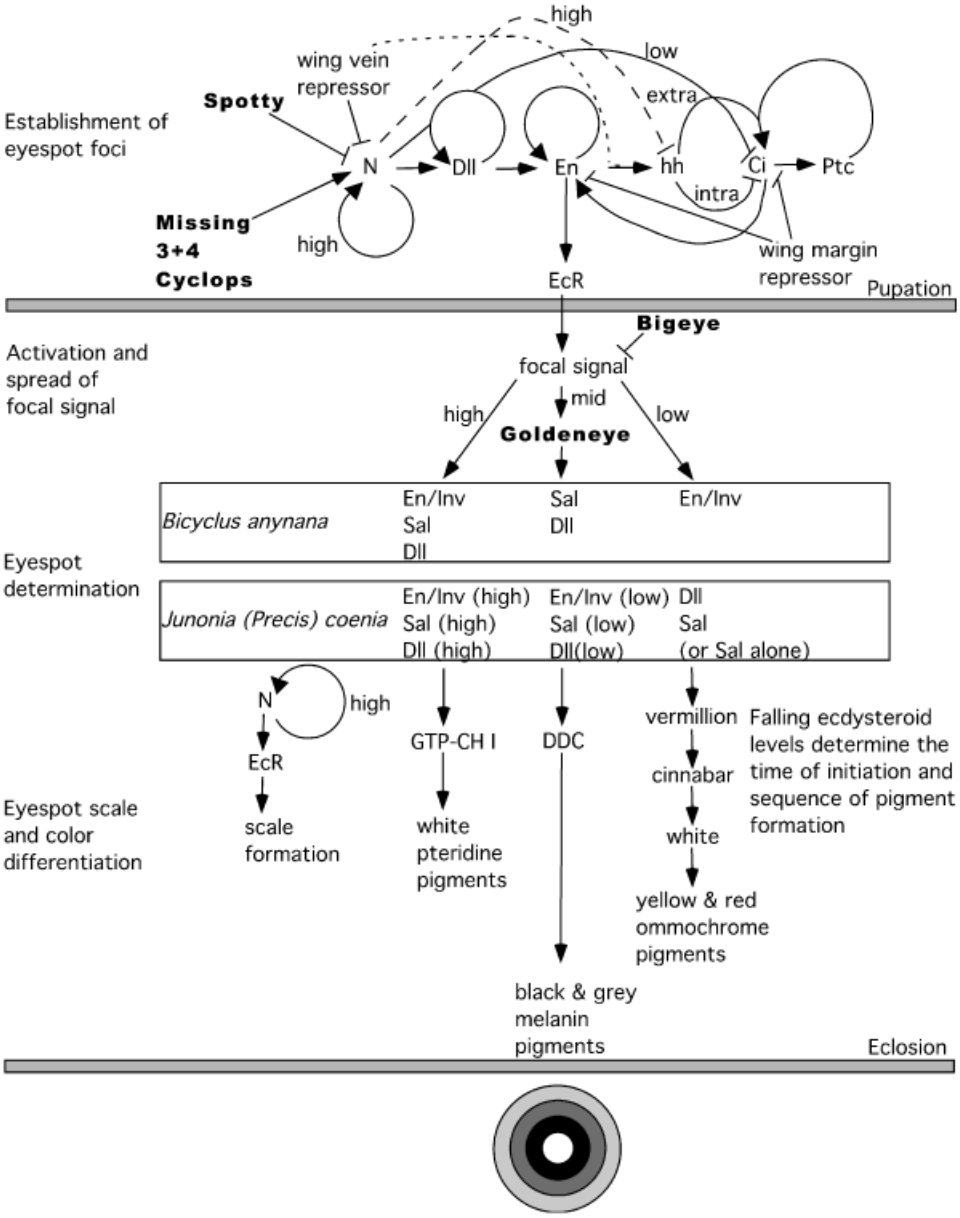

Fig. 5. Revised model for eyespot determination and differentiation. In the eyespot focus determination stage of the model, interactions indicated by large dashes occur only in Model 2, interactions indicated by small dashes occur only in Model 3, and interactions indicated by unbroken lines occur in both Models 2 and 3. Interactions that occur after pupation were not tested in the simulations described here. Names in bold are spontaneous mutants that have not been characterized at the molecular level. Other presumptive components of the pathway are either known from expression patterns of gene products, or are inferred on the basis of perturbation experiments and simulation studies. Several gene products (Notch (N), Distalless (Dll), Engrailed (En)) in addition to Ecdysone Receptor protein (EcR) that are expressed during the establishment of eyespot focus continue to be expressed after pupation. None of these genes alone (N, Dll, En, EcR) are likely to be sufficient to cause eyespot differentiation because all of them are expressed in other domains in the wing imaginal disc that do not differentiate into eyespots (Carroll et al. 1994; Keys et al. 1999; Koch et al. 2003; Reed 2004; Reed and Serfas 2004). Some combination of these gene products may initiate focal signaling or the mechanism may require additional unknown factors. References to most of the experimental data on which this model is based can be found in Marcus (2005), to which data from Reed and Nagy (2005) have been added.

themselves, their binding sites, or evolution of the domains of expression of transcriptional cofactors (Wray et al. 2003). Similarly, changes in these thresholds can also result in alterations in the size of the resultant eyespot focus, which may be the mechanistic explanation for why allelic sequence variation in Dll correlates with eyespot size in selected lines of *Bicyclus* (Beldade et al. 2002a). A final model for the entirety of eyespot development that incorporates both our revised models for eyespot focus determination as well as other events downstream of focus determination (Marcus 2005), including recently published results from Reed and Nagy (2005), is included in Fig. 5. It will be particularly interesting to integrate the results of these simulation studies with a study of gene expression patterns in butterflies with known mutations that alter eyespot focus shape, size and number (Brakefield 2001), as this may help us differentiate between our improved alternative Models 2 and 3.

Candidates for the repressors and the initiator of focal signaling

It is tempting to speculate on the identity of the margin and vein repressors, because of their apparent importance in the formation of eyespot color patterns. Wingless is known to diffuse from the wing margin in *Drosophila* (Phillips and Whittle 1993; Blair 1994), and shows a similar domain of expression at the wing margin in butterflies (Carroll et al. 1994). The expected expression pattern of the margin repres-

sor in our Delphi Models (Fig. 4) shows similarities to the observed expression pattern of wingless transcripts in developing butterfly wings (Carroll et al. 1994) so it is possible that wingless may play the role of the margin repressor, as has been suggested by McMillan et al. (2002).

The ligand decapentaplegic (dpp) is known to be secreted by incipient wing vein cells in *Drosophila* and is thought to participate in long-range signaling necessary for the patterning of the developing wing (Biehs et al. 1998; Marcus 2001; Ralston and Blair 2005). Again there is a similarity between the expected expression pattern of the wing vein repressor and the observed expression pattern of dpp transcripts (Carroll et al. 1994; McMillan et al. 2002), so it is possible that dpp may play the role of the wing vein repressor. While in both the case of wingless and dpp, the similarities between expression patterns and the distributions of the margin and wing vein repressors are perhaps suggestive, it is inappropriate to attribute specific roles to particular gene products until functional tests of the roles of the gene products are conducted in developing butterfly wings. Therefore we have not assigned gene identities to either of the repressors in our models.

The identity of the gene product that specifies focal cell fate and that initiates focal signaling to pattern the surrounding tissue into an eyespot has also been the subject of considerable speculation. To date, there are four gene products that are known to be expressed in eyespot foci at pupation (N, Dll, En, EcR), but none of these genes alone are likely to be sufficient to specify the eyespot focus, because all of them are also expressed in other parts of the developing wing imaginal disc (N at the wing margin and in incipient scale cells, Dll at the wing margin, En in the posterior compartment, EcR at the wing margin and in incipient wing veins and scales) that do not differentiate into eyespots (Carroll et al. 1994; Keys et al. 1999; Koch et al. 2003; Reed 2004; Reed and Serfas 2004). A combination of these gene products may be sufficient to specify focal cell fate and initiate focal signaling or the mechanism may require additional unknown factors (Fig. 5).

Reality check

It is important for simulation studies of biological phenomenon to have some clear relationship to processes that occur in the real world. In our models, we present some hypothesized genetic interactions, such as the upregulation of En by Dll, which at least on the surface occur via a very different mechanism from that thought to operate during *Drosophila* embryonic development (Mann 1994). Other components of our models, for example, the interactions between En, hh, *Ptc,* and Ci, for the most part behave in a fashion very similar to what is thought to occur in *Drosophila* (Keys et al. 1999). Yet, even in this conserved set of genetic interactions, Keys et al. (1999) have noted some important differences. However, for the most part, the combinations of gene products being examined here have never been studied simultaneously by researchers in traditional genetic and developmental model organisms, so direct interactions between them are largely unknown. Further, perhaps unlike the genetic regulatory models presented for many other systems, our models of genetic regulatory hierarchies (Figs. 1 and 5) are not necessarily intended to imply direct interactions of gene products.

Instead, Models 2 and 3 (Fig. 1, B and C) are intended to summarize what is known about gene expression in butterfly eyespot foci, and at the same time represent genetic regulatory hierarchies that are capable of generating an eyespot focus. However, there may be many more players in this genetic network that have not been identified. In particular, it is expected that in some cases the proposed genetic interactions may prove to be highly indirect, with many intermediate gene products participating in the various regulatory interactions. Models 2 and 3 can therefore be said to be consistent with what has been observed in real butterfly imaginal discs, while Model 1 (Fig. 1A), originally presented by Marcus (2005), clearly is not. As data from more gene products are collected from additional butterfly species, it may be possible to further refine Models 2 and 3, and perhaps also differentiate between them to produce more robust models for butterfly color pattern development.

Applications of computer simulations to other model systems

The use of computer simulation to test proposed genetic regulatory hierarchies, as has been implemented many times for traditional genetic and developmental model systems (Reinitz and Sharp 1995; Collier et al. 1996; Huang and Ferrell 1996; Bodnar 1997; Sharp and Reinitz 1998; von Dassow et al. 2000; Bodnar and Bradley 2001; Espinosa-Soto et al. 2004), has not yet been used extensively in the field of evolution and development which often uses organisms in which there are fewer experimental tools available. Yet, because in vitro and in vivo tests of proposed genetic interactions are much more difficult in these nontraditional model organisms, the in silico approach used here may actually be even more useful than in traditional model systems because it allows researchers to eliminate unlikely hypotheses using only a computer. Then, experimental resources can be devoted to the most robust hypotheses that have withstood the initial testing by simulation on the computer. Finally, most of the simulated genetic hierarchies that have been studied so far have been for traits that are relatively well conserved phylogenetically.

As regulatory networks for more evolutionarily labile traits such as butterfly color patterns are studied in this fashion, they will provide an interesting contrast to the more evolutionarily conservative examples of genetic networks examined so far. Specific evolutionary developmental model systems in which these kinds of simulations might be particularly inter-

esting and informative include the evolution of *Drosophila* body pigmentation (Hollocher et al. 2000; Wittkopp et al. 2003); soldier ant caste morphology (Abouheif and Wray 2002); sword growth in swordtail fish (Marcus and McCune 1999); eyelessness in troglodytic fish (Jeffrey 2005); and the development of armored and unarmored forms in freshwater populations of stickleback fish (Shapiro et al. 2004). All of these model systems share with butterfly color patterns features of rapid evolution, parallel evolution of similar phenotypes by multiple lineages, and considerable phenotypic diversity. Many of these systems may also include examples of atavism, in which a lineage can regain a morphological phenotype that it had previously lost. Simulation studies in these systems may reveal points in the genetic regulatory hierarchies that control the traits being considered that are particularly sensitive to change, and which in turn may have had a key role in the evolution of phenotype.

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APPENDIX A

Threshold equations for the simulation models implemented in this article are presented here. The same equations for a given model were used in both STELLA 8.1 and Delphi 2.0 simulation models. In the PASCAL code below, $c[x,y,z]$ denotes a cytological cell within the wing cell grid, the z value denotes the gene product that will be tabulated within each cytological cell (Table A1).

Table A1. Key to z values for the three simulation models

z	Gene product
0	Wing vein repressor
1	Wing margin repressor
2	Notch
3	Distal-less
4	Engrailed
5	Extracellular diffusing hedgehog
6	Cubitus interruptus
7	*Patched*
8	Ecdysone receptor
9	*hedgehog* initiation

Model 1

If ($c[x,y,0]>1$) then $c[x,y,2]:=c[x,y,2]-10$ else (if ($c[x,y,0]<1$) then $c[x,y,2]:=c[x,y,2]+10$);{the presence of Notch up-regulates the production of Dll while the presence of the wing vein repressor down-regulates the production of Dll}

If ($c[x,y,0]>1$) then $c[x,y,3]:=c[x,y,3]-40$ else (if ($c[x,y,2]>300$) then $c[x,y,3]:=c[x,y,3]+40$); {the presence of the wing margin repressor down-regulates the production of En, the presence of Dll up-regulates the production of En}

If ($c[x,y,1]>1$) then $c[x,y,4]:=0$ else (if ($c[x,y,3]>350$) then $c[x,y,4]:=c[x,y,4]+10+c[x,y,6]$); {the presence of En up-regulates the production of *hedgehog* as long as Notch is in low concentrations}

If ($c[x,y,2]<350$) then $c[x,y,9]:=c[x,y,9]+(c[x,y,4])$; {the absence or near absence of Notch inhibits the formation of *Ptc*}

If ($c[x,y,2]<5$) then $c[x,y,7]:=0$; {the presence of diffusing hh upregulates the production of *Ptc*}

If ($c[x,y,5]>0$) then $c[x,y,7]:=c[x,y,7]+c[x,y,5]$; {the presence of *Ptc* coupled with moderate to high levels of Notch up-regulates the production of Ci}

If (($c[x,y,7]>0$) and ($c[x,y,2]>200$)) then $c[x,y,6]:=c[x,y,6]+c[x,y,7]$;

newc$[x,y,5]:=$ new $c[x,y,5]+c[x,y,9]$; {this allows for the proper diffusion of hh}

If ($c[x,y,2]>350$) then $c[x,y,8]:=c[x,y,8]+c[x,y,4]$; {in cells with moderate to high levels of Notch, En up-regulates the production of EcR}

Model 2

If ($c[x,y,0]>1$) then $c[x,y,2]:=c[x,y,2]-10$ else (if($c[x,y,0]<1$) then $c[x,y,2]:=c[x,y,2]+10$);{the presence of Notch up-regu-

lates the production of Dll while the presence of the wing vein repressor down-regulates the production of Dll}

If ($c[x,y,0]>1$) then $c[x,y,3]:=c[x,y,3]-40$ else (if ($c[x,y,2]>300$) then $c[x,y,3]:=c[x,y,3]+40$); {the presence of the wing margin repressor down-regulates the production of En, the presence of Dll up-regulates the production of En}

If ($c[x,y,1]>1$) then $c[x,y,4]:=0$ else (if ($c[x,y,3]>350$) then $c[x,y,4]:=c[x,y,4]+10+c[x,y,6]$); {the presence of En up-regulates the production of hh as long as Notch is in low concentrations}

If ($c[x,y,2]<350$) then $c[x,y,9]:=c[x,y,9]+(c[x,y,4])$; {the absence or near absence of Notch inhibits the formation of *Ptc*}

If ($c[x,y,2]<5$) then $c[x,y,7]:=0$;

If ($c[x,y,5]>1$) then {in cells receiving hh, this simulates the removal of *Ptc*}

begin

$c[x,y,7]:=c[x,y,7]-100$;

Patchdump: = true; {this sets up the removal of *Ptc* as a variable by which Ci is up-regulated}

end; {cells producing hh do not express Ci, while only cells expressing moderate to high levels of Notch and an absence of the wing margin repressor have an up regulation of Ci}

If ((($c[x,y,9]=0$) and ($c[x,y,2]>3$ 50) and ($c[x,y,1]<1$))and Patchdump = true) then $c[x,y,6]:=c[x,y,6]+10$; {in cells with moderate to high levels of Notch, Ci up-regulates the production of *Ptc*}

If ($c[x,y,2]>100$) then $c[x,y,7]:=c[x,y,6]+c[x,y,7]$;

new$c[x,y,5]:=$ new$c[x,y,5]+c[x,y,9]$; {this allows for the proper diffusion of hh}

If ($c[x,y,2]>350$) then $c[x,y,8]:=c[x,y,8]+c[x,y,4]$; {in cells with moderate to high levels of Notch, En up-regulates the production of EcR}

Model 3

If ($c[x,y,0]>1$) then $c[x,y,2]:=c[x,y,2]-40$ else (if($c[x,y,0]<1$) then $c[x,y,2]:=c[x,y,2]+10$); {the presence of the wing vein repressor down-regulates the production of Notch}

If ($c[x,y,0]>1$) then $c[x,y,3]:=c[x,y,3]-40$ else (if ($c[x,y,2]>300$) then $c[x,y,3]:=c[x,y,3]+40$);{the presence of Notch up-regulates the production of Dll while the presence of the wing vein repressor down-regulates the production of Dll}

If ($c[x,y,1]>1$) then $c[x,y,4]:=c[x,y,4]+0$ else (if ($c[x,y,3]>350$) then (if (($c[x,y,5]>c[x,y,7]$) and ($c[x,y,6]>1$)) then $c[x,y,4]:=c[x,y,4]+40$ else $c[x,y,4]:=c[x,y,4]+10$)); {the presence of the wing margin repressor down-regulates the production of En, the presence of Dll up-regulates the production of En while the presence of activating Ci further up-regulates the production of En}

If ($c[x,y,0]>3$) then $c[x,y,9]:=c[x,y,9]+(c[x,y,4])$; {the presence of the wing vein repressor up-regulates the production of hedge-hog as long as Notch is in low concentrations}

If ($c[x,y,2]<5$) then $c[x,y,7]:=0$; {the absence or near absence of Notch inhibits the formation of *Ptc*}

If ($c[x,y,5]>1$) then {in cells recieving hh, this simulates the removal of *Ptc*}

begin

$c[x,y,7]:=c[x,y,7]-100$;

Patchdump: = true; {this sets up the removal of *Ptc* as a variable by which Ci is up-regulated}

end;

If ((($c[x,y,9]=0$) and ($c[x,y,2]>350$) and ($c[x,y,1]<1$))and Patchdump = true) then $c[x,y,6]:=c[x,y,6]+10$; {cells producing hh do not express Ci, while only cells expressing moderate to high levels of Notch and an absence of the wing margin repressor have an up regulation of Ci}

If ($c[x,y,2]>100$) then $c[x,y,7]:=c[x,y,6]+c[x,y,7]$; {in cells with moderate to high levels of Notch, Ci up-regulates the produciton of *Ptc*}

new$c[x,y,5]:=$ new$c[x,y,5]+c[x,y,9]$; {this allows for the proper diffusion of hh}

If ($c[x,y,2]>350$) then $c[x,y,8]:=c[x,y,8]+c[x,y,4]$; {in cells with moderate to high levels of Notch, En up-regulates the production of EcR}