Pattern Formation and Wound Healing: Experimental and Theoretical Approaches

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Pattern formation and wound healing are intricately connected mechanisms in organism development and of significant importance for biomedicine. These mechanisms can be studied both experimentally and theoretically using butterfly wing patterns. Butterfly wings are nearly two-dimensional structures, covered by a single layer of overlapping monochromatic scales. Many colors and kinds of scales generate highly diverse mosaics. This extraordinary variation makes butterfly wing patterns an excellent model to study the role of positional information in cell fate determination both in “normal” patterns and in patterns induce by damage. In this thesis, mathematical modelling is used to describe pattern formation. The model takes into account positional information via signalling molecules and activation of spatially defined expression patterns of transcription factors. The simplest gene regulatory network found to be compatible with experimental results is proposed to describe pattern formation in butterfly wings. The model is validated by reproducing known variants of wing pattern morphology (as size or colour composition) with one signalling source. This was accomplished by varying parameters such as transcription factor binding affinities or protein production rate. Predictions are made about morphology variation, damage-induced pattern formation and possible experimental tests. The latter involves the implementation of two signalling sources. In conclusion, the mathematical model has proven very insightful for the interpretation of the mechanisms underlying this type of pattern formation.

Keywords: Wound healing, pattern formation, morphogen, computational modelling, genetic regulatory network.

I. Introduction

Pattern formation, tissue reorganization and wound healing are key mechanisms in biology and medicine. Wound healing is an evolutionarily conserved and well studied mechanism in model organisms [1] that, in skin, aims at barrier restoration. Commonalities between the mechanisms of pattern formation and those involved in developmental processes (such as dorsal closure [2-4] or response to wounding (by analysis of damage-induced pattern formation [5, 6]) offer unique opportunities to exploit information available for model systems to increase our knowledge about developmental patterning and the ability to heal wounds.

A key challenge in biology is to understand how phenotypic diversity and patterns are generated by the modification of developmental pathways. The allocation of cell fates according to these spatial patterns is a key event in embryonic development – for instance cartilage in the vertebrate limb or veins in the insect wing. Signalling is essential during embryogenesis to establish cells positional information and to promote cell to cell communication. This ensures that correct types of cells and tissues appear at the right place in the right time during embryogenesis [7]. Cells fate depends on these signals that where named by Turing (1952) [8] as morphogens: diffusible substances that spread from a localized source. In this way, depending on the distance to the morphogen source, cell will be divided into different groups. Some of the best-studied morphogens belong to the Hedgehog family [9], Notch [10], or Wingless/Wnt [11] that diffuse within early Drosophila melanogaster embryos.

The epidermis protects the animals from external threats as its function to repair after damage is essential to experience and survive to an occasionally hostile environment. Signals from epidermal wounds lead to tissue repair, immune responses, pigmentation and pattern formation – colours and others [12, 13]. In several organisms, like Xenopus laevis and Bicyclus anynana, wound healing and patterning appear to be key biological processes with several similarities [6, 14, 15]. Nevertheless, many aspects of these responses seem to be highly diverse [12], although evolutionarily conserved [16].

Wound healing must occur to restore health after a trauma and its study helps to understand about commonalities between some diseases and these mechanisms of survival. Some wounds, such as the common foot ulcers of diabetics, heal slowly and not completely [17], whereas others display an exaggerated response that results in disfiguring keloid scars [18]. Nevertheless, the importance of wound healing is not yet fully illustrated. Wound healing is a unique process that connects medicine and developmental biology. In development, the study of wound healing offers advantages due to the commonalities between this and embryonic developmental processes. One example is dorsal closure in Drosophila melanogaster, a process...
similar to wound healing [4]. An important advancement in biology was the inclusion of mathematical models to explain observed events like embryonic wound healing [19]. This has brought new ideas and discoveries. For example, scarless wound healing in Drosophila embryos, involving actin cable formation and filopodial extension [15, 20]. Challenges like to inducing wounds in adults that heal without scars looks possible from these new insights.

In summary, one important wound healing research achievement is to find ways to speed or alter the healing process. Another one is to discover how damaged cells can respond to wounding and, in particular, which information is relayed to neighbouring cells as part of this response.

II. Butterfly as a model, wing wounds and genetic expression

Butterfly wing colour patterns are more than impressive examples of diversity. They are emerging as exceptional model systems, linking the developmental and genetic processes that generate morphological variation with the wound healing processes that are essential to survival. Almost all of the 12 000 described butterfly species can be distinguished by differences in wing patterns, which constitute their most remarkable phenotypic feature. The patterns are essentially two dimensional structures, greatly simplifying the conceptualization and modelling of pattern formation and change [21]. Additionally, butterfly wings are relatively large and easy to manipulate [22]. Developing wing tissue can be damaged or transplanted, revealing the developmental processes that give rise to these patterns [21, 23, 24].

The development of a butterfly wing starts in the larval body as an imaginal wing disc with a double layer of cells. It suffers a large tissue growth at pupation, to form the pupal wing [25]. During pupal development, a group of cell differentiate to produce colourless cuticular projections (scales). Scales pigmentation occurs only in the last days of pupal stage and each scale contains a single colour pigment. Scales have a characteristic morphology [21] (figure 1). Different types of pattern elements can be recognized on butterfly wings, including bands, stripes and concentric rings with intense and contrasting coloration called eyespots (figure 1). In B. anynana, the butterfly “lab rat”, each eyespot is composed of a white pupil, a black disc and a golden outer ring. Eyespot diversity across and within species, the variation for different aspects of pattern morphology, together with the easy manipulation and consequent tractability, reveal to be important features that make the eyespots suitable for studies in evolutionary developmental biology.

Differences between the signal-response components are recurrent in developmental biology for patterning and to produce diversity. In B. anynana, the focus of the eyespot consists of signalling cells proposed to be a source for a morphogen [26], whose levels determine the pigmentation of surrounding cells (like sensitivity thresholds) [27] (figure 2a). Experiments involving grafting and destruction of focal cells in early pupal stages establish the focal cells as the organizer centre, that triggers the developmental eyespot pathway [5, 28-30]. In the case of grafting experiments, transplantation of the focus can induce eyespot development in the new unfated areas forming an ectopic eyespot (figure 2b). In the second case, an inhibition of normal pattern development is observed [28, 31] (figure 2c). Related to the morphology of the eyespot, changes in eyespot size have been associated

Figure 1. Pattern element of wildtype butterfly wings. Top-bottom corresponds to proximal-distal axis and left-right to posterior-anterior axis. In the figure is represented one layer of partly overlapping monochromatic scales arranged in rows; an eyespot composed by concentric rings around a white centre (a black and a golden ring); hairs, brown scales and a large diversity in scale shape around the eyespot.
mostly to changes in focal signal strength [32], while changes in eyespot colour composition (proportion of black to gold) seem to depend on sensitivity to threshold values [33].

There are several parameters that can determine eyespot location. Eyespots usually occur in the distal part of the wing, centred midway between adjacent wing veins. Venation mutants show the importance of wing veins in eyespot formation [35]. There is some evidence that wing veins and the wing margin have an active role in the determination of butterfly wing patterns [36, 37] but their function in eyespot formation is not yet known. Models of eyespot formation have suggested that the wing veins and the margin act as sources of diffusible molecules involved in the determination of the eyespot organizer [21, 38].

Eyespot development begins at the larval stage with the expression of the first known wing patterning genes at the presumptive foci of each eyespot [26, 39, 40]. The morphogen concentration provides positional information which defines a cellular territory, corresponding to the emergent eyespot [5, 28, 30, 31, 41]. Based on the experiments of key regulatory genes in B. anynana, different stages of eyespot development have been proposed [26]: Freepattern (a genetic prepatterning system is presumably laid down by morphogens which diffuse from the wing margin and/or wing veins in larval wings – mid fifth instar); Focal determination (the establishment of localized central organizers in larval wings in larval wings – late fifth instar); Focal signalling (signalling cascade leading to positional information of the future eyespot rings and pigment production in pupal wings – 24h after pupation); Differentiation and pigment production (individual cells interpret the positional information in the first stages and respond by producing colour pigments – end of pupal stage).

In larval stages, a large number of genes appear to be involved in the differentiation of the focal cells like Distal-less (Dil), Notch (N), engrailed (en), hedgehog (hh), cubitus interruptus (ci), patched (patch) and spalt (sal) [40, 42, 43]. In early pupal stages, with an already defined focus, the determination of the surrounding pattern appear to have an influence of three transcription factors: Dil and sal mapping to the black scales, and en to the gold ring of scales (figure 3) [26, 29]. However, it is not yet known what signal, presumably produced in the focal cells, is responsible for the expression of these transcription factors. Beldade et al. [44] began to test candidate signalling molecules to know if they do have a functional role in pattern formation by demonstrating a link between DNA polymorphisms in the candidate gene Dil and eyespot size in B. anynana. Monteiro et al. [6] tested if wingless (wg) and Transforming Growth Factor-beta (TGF-β) signalling pathways were involved as possible morphogens in eyespot determination. Ligands of those pathways are known morphogens in other systems [38, 45, 46]. However, the results were not conclusive regarding the importance of these ligands as morphogens responsible for triggering the other transcription factors expression.

Wound healing has been repeatedly studied in butterflies due to the visible wing scale coloration effects [5, 30, 47-50]. During pupal development, there is a time window where inflicted damage will generate pattern. Around the wound, cells will differentiate to produce a different pigment from the current one. The resultant patterns is generally circular around the wound site, and can have different sizes or colours [5, 32, 33, 48, 49]. In B. anynana, damage-induce eyespots consisting of a ring of gold scales, sometimes containing a black central disc, without the usually white centre, appear on the adult wing following pupal wing wounding [30].

Preliminary data (Beldade, personal communication) tried to address the possibility that native and ectopic eyespots share developmental pathways. Commonalities between native and damage-induce patterns on protein expression level of the Engrailed protein were explored but the results were unclear. One wing (out of 32) at 28-29 hours after pupation showed a small circle of elevated en expression, which could represent the formation of an ectopic eyespot. The technique is difficult to perform and it the time after pupation when the en expression starts is unknown. An effort to know more about the genes involved in the differentiation of these ectopic coloration patterns must be done in order to clarify how much of the same genetic circuitry are natural eyespots and damage-induce patterns using.

III. Modelling and theoretical approaches

Regardless of the importance of genes, a genetics study alone cannot provide the mechanistic understanding about patterning formation. In recent years, mathematical models have been created to validate hypotheses based on experimental data, and, additionally, to design testable experimental predictions. Two great examples are the modelling of tentacle patterns on the radially symmetrical Hydra, and the gastrulation of animal embryos [8, 51]. A considerable number of other biological processes in biomedicine have been addressed by mathematical modelling as tumour formation, several developmental processes or wound healing mechanisms. In development, structures can form as a result of response to chemical

Figure 3. Eyespot of an adult butterfly and its correspondent protein expression. Double labeling for en (green) and sal (purple) expression at 16-20 hr after pupation is shown on the right. sal is mapping the black scales, and en the gold ring of scales [29].
signalling, cell–cell interaction, tissue movement and
rearrangement, or most likely, a combination of all. The
complexity of these systems supports and explains the
need for modelling as it is fundamental to understand the
combined effects of signalling cues involved in the
regulation of cellular processes. The number of examples
of studies in pattern formation complex systems is
enormous and diverse. Khain and Sander [52] have
formulated a model for tumour growth using two coupled
reaction-diffusion equations for cell and nutrient
concentrations in which depend the brain tumour growth.
In chemistry, one example of pattern formation is the
spontaneous generation of propagating fronts, target
patterns, spiral waves and toroids [53]. This was first
described by the Belousov-Zhabotinsky (BZ) reaction and
has been analysed in great detail since then. In this
reaction, the periodic oscillations can be visualized as
colour changes between reddish-orange and blue [54] as a
result of a redox reaction. Similar types of patterning arise
in physiology with the electrical activity in the heart [55]
which stimulates muscle contraction during heart beat.

Regarding pattern formation by chemical signalling, Turing (1952) [8] was the first who realized that the
interaction of two substances with different diffusion rates
could originate patterns. He established an
activator/inhibitor reaction-diffusion mechanism where a
system of reacting and diffusing chemicals that can
progress from an initially uniform spatial distribution,
linearly stable in the absence of diffusion, to concentration
profiles linearly unstable in the presence of diffusion.
Additionally, Meinhardt [56] has shown that Turing-type
models (with two or more chemicals) can exhibit the
spectacular variety of patterns seen in sea shells, while
Nijhout [57] has shown that such models, together with a
small number of sources and sinks, can exhibit the wide
array of pigmentation patterns observed in certain butterfly
wings.

There are many differences in eyespots among different
butterfly species. They differ in number, location, size and
pigmentation. The establishment of localized central
organizers in larval wings – foci determination – remains
unclear, as well as the mechanism by which the focus
induces a specific pattern of pigment synthesis in their
surroundings in pupal wings – eyespot determination.
Evans and Marcus tested genetic regulatory hierarchies
hypothesized to underlie the formation of butterfly eyespot
foi [38, 58]. The computational approach included several
known genes that have some role in the step: Distal-less, Notch, engrailed, hedgehog, cubitus interruptus and
patched. It allowed the proposal of predictions about gene
expression patterns and experiments that would distinguish
between the two hypothesized genetic regulatory
hierarchies that may underlie all butterfly eyespot foci
formation.

This project aims to explore the next step in eyespot
development, the focal signalling, where positional
information is given to the surrounding cells. There are
already few models concerning this type of signalling.
However, patterns complexity and the unclear mechanisms
on which this step depends may have difficult the design of
new useful computational models. Brunetti et al. [29]
proposed a gene regulatory network that would generate
the eyespot rings. It was composed by a morphogen which
activates the genes engrailed and spalt. This network did
not have in consideration some eyespot characteristics as
the white centre or the well defined ring limits. In 2004,
Dilão and Sainhas [59] proposed a reaction–diffusion
model that accounted for eyespot development. The model
considered two diffusive morphogens and three non-
diffusive pigment precursors. The first morphogen was
produced in the focus and determined the differentiation of
the first eyespot ring. A second morphogen was then
produced, modifying the chromatic properties of the wing
background pigment precursor, inducing the differentiation of
a second ring. The model simulated the general
structural organization of eyespots, their phenotypic
plasticity and seasonal variability, and predicted effects of
microsurgical manipulations on pupal wings. Although the
results of this simulation fitted the known final pattern, the
model did not take into account the temporal progression of
the proteins involved, neither the possibility of the
presence just one colour ring in the eyespot, denoting that
the gene network needed some changes.

This project was designed to better understand biological
pattern formation. Its development had the
several specific steps. The first was to build a
computational model and to propose a genetic network.
The second consisted in the validation of the model by
choosing parameter values to match known morphology
variation and it’s described genetics and developing
underpinnings. Finally, the results from the model were
used to make predictions about morphology variation,
possible experimental tests and damage-induced pattern
formation.

IV. Materials and Methods

One-dimensional Computational Model

Computational modelling was chosen for being a great
method of showing possible scenarios that underlie
specific pattern formation and, in association with
experiments, for making testable predictions. The
mathematical approach to the pattern formation starts with
spatial definition: a butterfly wing region as a cell layer
with radial symmetry around the eyespot focus. The mean
number of scales in an eyespot radius is about 50 (despite
the great diversity). One dimensional spatial region was
defined as having 100 cells, from the centre of the eyespot
pattern until after the end of the eyespot radius being
enough to simulate the expected pattern. The next step is to
implement a gene regulatory network that can describe the
known patterns. Gene regulation expression is described
individually in each cell by differential equations,
following the method described by Alves and Dilão (2005)
[60]. Moreover, it is necessary to define time limits to the
simulation and to treat simulation results. The GeNetSim is a
Mathematica package which is used to assemble the
system of equations needed to model the gene regulatory
network in a repeatable and fast way and without errors.
This application is based on a theoretical framework proposed by Alves and Dilão [60]. This package was adapted so it could be used with a gene regulatory network proposed for the eyespot pattern formation. The model simulates the variation in protein concentration over time (t). In this simplified model for protein production, it is assumed that the gene concentration remains the same, mRNA concentration is constant during transcription and that the concentrations of nucleotides, aminoacids and catalysts involved in transcription and translation are not limiting factors.

In the pattern formation gene regulatory network, several genes will interact and be regulated. The interaction between the regulator and inducer or corepressor molecules is not considered in this model so regulatory binding sites are considered independent for each protein (domain overlapping is not possible). Only the interaction between activator or repressor molecules and the respective DNA binding sites is considered. In the network, the amount of transcription factors bound to their specific sites in a certain moment will depend on their concentration and binding affinity, assuming a constant concentration of the DNA molecules. In this case, each binding site has only two possible states: occupied by the respective protein or free. Given the regulatory network structure, the package GeNetSim automatically generates the equations and then the system is solved numerically using Mathematica built in algorithms. The GeNetSim package was adapted to write the equations defining the model to describe eyespots pattern formation. Several parameters were used in the package to establish the proposed gene regulatory network for eyespot patterning. It is composed by three types of information: “Data” where the gene regulatory network is defined; “Constant values” which has the values for the production and degradation rates and binding affinities; and “Initial conditions” where the proteins that have initial concentration different from zero are defined. As a simplification and for the specific questions of eyespot patterning, the value of unbind was assumed to be equal for all proteins. The units of each property are not explicit (appear as “temporal units” or “concentration units”, for example). Due to the lack of information, what was studied were the relative values between the parameters. For example, if the value of the diffusion coefficient is used in μm²s⁻¹, consequently, time will appear in seconds and spatial parameters in μm. A fundamental step in this process is to define the initial conditions of the non-zero initial proteins. Both the concentration gradient of the proteins Signal and M are calculated by a reaction-diffusion equation. The limits were chosen based on the type of results obtained. The protein concentration would achieve a stable pattern at approximately tmax=100 temporal units and do not change until the end of the simulation. The classical question arises: how is it decided the developmental terminus for an organism by nature? It can be time, or positional information of cells, or to reach a certain number of cells, or other unknown signal. As a simplification, for comparison with the experimental results, the numerical integrations were done between 0 and 100 temporal units, to allow all different profiles of protein concentration. The results obtained are the protein concentration values in one line of cells from the centre of the eyespot to 100 cells away, after a specific time – usually 100 temporal units. To be easier for comparison and visualization, this 1D graphic is rotated around the centre, as shown in figure 16b, representing in this case one fourth of an eyespot pattern.

There are several types of thresholds affecting experimental images: Immunofluorescence labelling implies detection thresholds; the protocol itself determines the type of protein that is more enhanced; self-enhancement mechanisms present in the cells; and cells sensitivity to a certain amount of protein. This means that what is shown in the experimental results may not correspond to the exact behaviour. In one dimension, it is more difficult to implement this than in two dimensions. The model considers a parameter of “cell sensitivity” to the amount of protein (as a threshold). This approach does not influence the linear graphic, just the rotation around the eyespot centre. Based on the results obtained, a value of protein concentration was chosen in the linear graphic as [concentration] = 4. All the rotated graphics then present the proteins with concentration equal or higher than 4. Another way of representing the results is by showing the exact protein concentration values, although is doubtful that the mechanisms work like this.

Two-dimensional Computational Model

To understand wound healing mechanisms, an approach to damage-induced eyespot pattern must be implemented. This needs a two dimensional model is need in order to see the effects of the damage to the surrounding pattern, being the one line approach insufficient. To do this, the model must be defined in a two dimensional grid so it can incorporate asymmetric borders as a result of cell independent self-enhancement mechanisms. To implement a 2 dimensional model, the diffusion of the morphogen had to be solved by a different mathematical approach. The morphogen diffusion was defined by the Laplace equations which were solved by approximate finite-difference methods. The simulations were performed with zero flux boundary conditions in a two dimensional spatial region. Due to this project’s short schedule, this was not completed. However preliminary results of the 2D model show some interesting and promising results, being an important subject for future work.

Experiments

To understand how the specific concentric ring pattern is formed, the distance between normal concentric pattern and damage-induce patterns was changed and the resulting data observed. This was done on the dorsal wing of B. anynana with the goal of correlating distance between the two types of patterns (normal and damage-induced) with the observed effects of overlapping patterns. The data is further used to improve the mathematical model resulting in more accurate predictions.
Operations were performed on *Bicyclus anynana* pupae. All butterflies were reared at 27° C with high humidity and a 12:12 hour photoperiod; the larvae were fed on maize plants sprayed with Fumidil B (an anti-microsporidia compound). Larvae reared in these conditions develop into butterflies with full eyespot patterns. The pre-pupae were collected and timed for their individual pupation times within ±30 minutes with a time lapse camera (NIKON 450).

Cautery experiments were performed on 75 pupae. Damage was inflicted in one pupal dorsal forewing of each pupa using a sharpened tungsten needle (World Precision Instruments, catalogue #501317) with 0.25 mm of diameter. In the butterflies, the left and right forewings are very similar [30], but there is considerable variability among individuals. The experimental manipulated pattern was therefore always compared to its contralateral control wing. The cuticle and underlying epidermis of the dorsal forewing were pierced at different sites identified next to the wing veins and cuticular marks. Pupae were cauterised at 12 hours and then returned to 27°C until eclosion (about 7 days). After adult emergence and wing expansion, individuals were immediately frozen and both forewings were removed for analysis. The wings were photographed with a Kodak camera (Kodak EasyShare Z650). A quantitative analysis was not performed, due to the low achieved successful rate (number of adult butterfly wings with damage-induce pattern in relation to the 75 initial total).

From the 75 pupae used, the dead pupae and adult butterflies with malformed wings were taken into account to calculate the survival rate. Only adults with fully eclosed and well formed wings must be considered, in order to have well formed damage-induced patterns and the contralateral control wing stretched. The normal and damage-induce patterns were analysed from the pictures. The resulting patterns were divided into different types depending on colour composition [5] (from only golden patterns, to fully formed eyespot).

V. Theoretical Results and Discussion

In this section simulated results are structured in three groups accordingly with the model progression and will be presented and discussed. In the first group, Model construction, is explained which are the components of the gene regulatory network and how they are related. Model validation describes changes in parameters values in a fixed regulatory network, in order to match variants of eyespot morphology (size, colour composition). The last one, Model extension, tries to approach damage-induced eyespot patterns and some more broad questions. This section also suggests possible experiments to elucidate the underlying biological processes in pattern formation.

The construction of the model was an active and iterative process, as previously discussed. The model started based on experimental information. Then, improvements to the model were made by running it and comparing the theoretical results with experimental data. Considering the focal cells as the initial morphogen sources, the proposed model predicts the patterns of gene expression underlying the colour determination in the surrounding future wing scale cells. The results in this work explore how the eyespot patterns may vary depending on model parameters such as transcription factor binding affinities, morphogen concentration and diffusion coefficients.

**Model Construction – Gene Regulatory Network**

After exploring several possibilities, the simplest gene regulatory network found that was compatible with the experimental data and could generate robust patterns, was proposed to describe eyespot development (figure 4). Engrailed is correlated with the golden rings and Spalt is expressed in the same location of the future black rings. Mutual inhibition was considered so that the genes could be mutually exclusive, leading to sharp colour limits. At this time, the hypothesis was the presence of the same morphogen (M) and a less diffusible protein ("Signal") in the centre of the eyespot. These two molecules are responsible for the activation of *en* and *sal*. The transcription factors En and Sal are both expressed at the focus, but appear in non-overlapping domains around the centre. To provide an interpretation for this expression pattern, the contribution of a non diffusible protein named “AA” was postulated which production would be activated by M and repressed by Signal. Signal is responsible only for the centre of the eyespot and it is a molecule with a limited action (low diffusion coefficient), appearing before M. It was assumed that En and Sal do not diffuse between neighbour cells, as suggested by experimental data [61].

The proposed gene regulatory network is hypothetical and certainly not complete and may not be the only possibility. There are several parameters missing, like, for example, the gene *Distal-less* which has a role in size regulation [44]. Some others must be added like the contribution of more genes and interactions.

![Figure 4. Proposed gene regulatory network for generation of butterfly eyespot colour patterns. The two transcription factors M and Signal are responsible for the activation of en and sal. The production of protein AA is activated by M and repressed by Signal. En inhibits both AA and Sal as Sal and AA inhibit both En. Sal and En have auto-activation.](image)
Model Validation

The first step was to choose a set of parameters so that the model could simulate the formation of a normal eyespot pattern. The correct colour proportions were achieved by manipulating the binding affinities between the intervenients. This process revealed itself to be problematic due to the lack of information about the underlying mechanisms that give rise to these patterns. The chosen mathematical model is described by differential equations that represent decay in biochemical concentration over time. These equations include parameters, such as decay rates, rates of reaction and diffusion, production and degradation, and binding affinities, which are unknown and must be identified using the available data, namely the observed patterns. The process of parameter identification requires solving the model’s differential equations: a guess is made for the numerical values of the parameters (starting, for example, with all values equal to 1), the equations are solved, and the resulting model is simulated and compared with data available from previous studies [6, 29]. If the results do not match, a new assumption is made and the process continues. Upon testing some parameters, it was revealed that increasing the values of self-activation of sal in the normal native eyespot reduces the size of the domain of co-expression (in the eyespot centre). The model could account for “normal” eyespot development and simulating temporal progression of En and Sal proteins, the results are in agreement with the experimental data available.

Changes in model parameter values matched known variation in eyespot morphology: changes in signal strength lead to changes in eyespot size while changes in the binding affinity values of en and sal resulted in variations in the proportion of black to gold (figure 5).

Model Extension: studies with two morphogen sources

Pattern resulting from the interaction of a native and an ectopic eyespot was also analyzed. Could ectopic eyespots be obtained with the same morphogen signal? The results of the mathematical model suggest that the signal could be at least very similar to the native one, in these conditions. It is very complex to propose a general experimental approach to study this question because no morphogen signal has yet been identified in B. anynana.

Considering two distinct morphogen sources, the results suggested that a putative second morphogen (defined as being the signal molecule responsible for damage-induce pattern) could be very similar to that produced in native eyespot centres. Additional results lead to consider that changes in the production rate of the second morphogen could explain diversity in damage-induced patterns. Finally, simulating different distances between the two eyespots sources, showed very similar results to both the experimental results obtained during this thesis and other results available.

Other interesting questions to study with the approach with two morphogen sources remain without answer:

![Figure 5](image_url)

Figure 5. Changes in the binding affinities of the gene regulatory network in order to achieve different colour compositions. (a-e) Results from simulations, (f, i) Changes in the parameters of the gene regulatory network. (g, h) predictions of the behaviour of eyespot depending on thresholds [38]. With the proposed mathematical model, several different colour composition patterns were simulated. (a-b) are black mutants and they were achieved with the (f) changes. (a) is not an already observed mutant. (c) is a normal native eyespot. (d-e) are goldeneye mutants resulting from differences in En-AA binding affinities (i). The protein gradients are calculated with the following kinetic constants: PRODsignal = PRODm = PRODsal = PRODen = PRODaa = 1.0, DEGsignal = DEGm = DEGsal = DEGen = DEGaa = 1.0, salsignal = 0.05, salm = 0.05, salen = 0.01, salsal = 0.0001, salaa = 0.001, ensignal = 1.0, enen = 0.0001, ensal = 0.01, aasignal = aam = 1.0. In (a) enm = 1.0, enaa = 2.0 and aacn = 1.0. In (b) enm = 3.3, enaa = 2.0 and aen = 1.0. In (c) enm = 4.0, enaa = 2.0 and aen = 1.0. In (d) enm = 4.0, enaa = 1.7 and aen = 1.2. In (e) enm = 4.0, enaa = 1.5 and aen = 1.2.
– Testing changes in binding affinities with two sources may give better interpretation about known variants of eyespot morphology (size, colour composition);
– What is the role of time in damage-induced pattern, how it influences organismal developmental stages? Differences in the initial time of the second source could explain the other damage-induced patterns obtained.

In summary, the mathematical model has proven very insightful for the interpretation of the mechanisms underlying eyespot pattern formation.

VI. Experimental Results and Discussion

This section presents the experimental results concerning changes in the distance between normal pattern and damage-induced pattern. The data was used to make comparisons with the mathematical model and to suggest new and interesting questions.

The success rate (number of adult butterfly wings with damage-induced pattern in relation to the 75 initial pupae) was low: 28%. Possible reasons for this low success rate include pupae death after damage (13%), adult inability to stretch the wings, inability to heal the wound in the wing resulting in a hole, no pattern formation or formation of a grey patch (essentially grey scales around the wound site) instead of a complete ectopic eyespot. Regarding the 21 individuals, female/male ratio was balanced: 52% females and 48% males. Male wings are smaller and darker, and eyespots are vague, lowering the contrast between wing background colour and eyespots, increasing the difficulty to identify ectopic eyespots.

Individuals considered to study eyespot development were divided in groups, depending on their pattern type:
– Gold patch (an area, band or spot with golden scales) with 8 elements;
– Black patch (usually just the ring of black scales) with 6 elements;
– Gold/black patch (a patch of golden scales mixed with black scales) with 5 elements;
– Ectopic eyespot with some gold (a black patch with a partly present golden ring) with 1 element;
– Full ectopic eyespot (a complete and fully developed eyespot with both black and golden rings) with 1 element.

Figure 6 shows one example of each type. The images shown represent the best specimens. The type of results acquire confirms the predictions in previous section where were expected either small golden damage-induced eyespots or, large black ones with little number of golden scales. Analysing other damage-induced eyespots (Beldade, unpublished results), for example, big golden ectopic damage-induced eyespots are never observed. This means that predictions generated with the model described a valid interpretation to what is behind the complex patterns involved.

As many individuals were lost, no statistical analysis has been performed. There are several different contributions to the low survival rate: pupae death, to the inability to stretch wings or to heal the inflicted wounds. Due to these reasons, it seems that the healing process of the pupae was somehow compromised. One argument is that the treatment with Fumidil B against the microsporidia infection affects the healing process. It is not known to what extent this treatment may change the normal development. Another possibility is the influence of the infection itself. A new experiment, with a larger, new and
clean stock of *B. anynana* may clear the infection influence and that of the antifungal substance. These two reasons may also explain the few ectopic eyespots formed (only one complete) since they are a consequence of the healing mechanisms. The presence of Furmidil B or even the infection may affect the normal developmental timing, altering the best time for cautery, recorded for 12h after pupation [5] so the inflicted damage won’t have the expected result. A more recent study about the influence of time in wing pattern formation should be done to clear this question.

However, even if the healing was completed, the experiment design is still subject to some limitations, which should be taken into account. It appeared to be quite difficult to standardize the amount of damage given to the pupae, one of the assumptions the experiment is based on. The number of dead cells may vary a lot in each essay so the conditions are always different and it is difficult to draw conclusions from this kind of experiments. Wounds seem to induce a signal that elicits the same response as the focal signal does (eyespot formation). So, if the extent of the damage is controlled, the strength of the signal should also be. A proposal to test this is the use of laser damage. In studies of wound healing in *Drosophila melanogaster*, laser ablation from a nitrogen laser-pumped dye laser (model no. VSL-337ND-S; Laser Science Inc., Franklin, MA) [62] is used. The focal point of a laser is very sharp in comparison with the tungsten needle (laser beam has μm in diameter while the tungsten needle has 0.25 mm). Using a standard procedure with laser, no changes in the applied strength or size of the inflicted wound would be introduced. Different types of laser energy could be used (maintaining the normal pupal development) in order to test different types of damage.

Different types of ectopic pattern can be formed after wound healing but it is still unknown how and why this occurs. The time window used was 12h after pupation with an uncertainty of 30 minutes. In this hour window very different development pupal stages can appear. As the inflicted damage depends on time [5], it will also cause different damage-induced eyespot patterns. Nevertheless, even if the pupation time was observed every 10 minutes, the diversity between individuals is very high, always causing large differences in healing mechanisms and, consequently, different damage-induced eyespots.

Future guidelines and new work directions can be taken from the developed work: working only with females due to the difficulty in observing males eyespots; collecting pupation times in shorter intervals and, additionally, using the infection to study the healing mechanism.

VII. Conclusions

Mathematical modelling of pattern formation can be used in two approach styles depending on the amount of information that is available about the actual mechanisms of pattern formation that operate in a given scenario.

In this case there is still much information missing about modelling pattern formation and wound healing in butterflies. An approach was to validate the model with known pattern diversity and spontaneous mutants, which was well succeeded. Afterwards, the directions are to explore some possible and executable predictions.

To understand how close ectopic and native eyespots are, it is crucial to extend the knowledge about the expression of other proteins besides En, Sal and Dll and to include that information in the model. Moreover, several suggestions about how to improve the model have been addressed. Among these, it is important to point out that the model could be extended to represent a realistic two-dimensional layer of cells, it could include wing borders, veins and even the whole wing and it could be broadened to several other species such as *Junonia coenia* (figure 7c). Finally, other mechanisms of morphogen transport should be considered and other gene networks should be tested.

In conclusion, mathematical modelling can be used as a tool to help understanding phenomena within the life sciences, including patterning. The mathematical model used in this project was successful in describing pattern formation for what it was designed, in making experimentally testable predictions and it also succeed in allowing an intuitive understanding of why the system behaves as it does. Several questions arose within and outside the proposed objectives, confirming life sciences as a source of an enormous number of novel, exciting and challenging problems for mathematical and computational approaches.

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**Figure 7.** The model can be extended to simulate other types of eyespot pattern. Three different butterfly species with different eyespots: (a) *Callicore aegina*, (b) *Caligo memnon* and (c) *Junonia coenia*.  
*Callicore aegina*, (b) *Caligo memnon* and (c) *Junonia coenia.*
References


