Universidade de Lisboa
Faculdade de Ciências
Departamento de Biologia Vegetal

Wound response and pigmentation pattern formation

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Mestrado em Biologia Molecular Humana

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Resumo

Os animais são revestidos pela epiderme e quando esta sofre algum dano tem de ser rápida e eficazmente reparada. O processo de cicatrização é complexo e absolutamente vital e requer a activação de um panóplia de vias de sinalização relacionadas com a coagulação, reparação de tecidos e imunidade. Em muitos animais a epiderme cicatrizada adquire uma coloração diferente. Por exemplo nos artrópodes a coagulação da hemolinfa está associada ao aparecimento de uma marca escura, a qual é consequência da activação da via de sinalização da biosíntese da melanina. Nos insectos esta via é extremamente importante na defesa contra possíveis microorganismos invasores assim como na cicatrização de feridas e na pigmentação do corpo dos insectos. Um caso muito interessante onde uma ferida origina um padrão de cores, é o da borboleta *Bicyclus anynana.* Nesta borboleta uma ferida nas asas da pupa em desenvolvimento pode conduzir ao aparecimento de um padrão de cores à volta da ferida, os quais se assemelham aos padrões nativos desta borboleta – os ocelos (anéis de cores concêntricos em que o centro, focus, é branco). Os mecanismos moleculares que estão na base da formação destes padrões são ainda desconhecidos. No entanto, é provável que a formação dos padrões nativos e dos induzidos por ferida tenha genes e vias de sinalização em comum. Neste estudo nós procurámos saber quais os mecanismos genéticos, a estrutura celular, e a expressão genética associada a estes padrões de pigmentação induzidos por ferida em *B. anynana*.

Numa primeira experiência nós induzimos feridas em pupas em condições de esterilidade e não esterilidade e verificámos que o número e o tamanho dos padrões induzidos por ferida não diferia entre os dois grupos. Assim concluímos que a formação dos padrões induzidos por ferida nas asas de *B. anynana* não é dependente do processo de infecção, o qual induz a activação das vias de sinalização associadas à imunidade e ao combate a microorganismos. Este resultado sugere que os genes e as vias de sinalização, que possam ser partilhados pela formação de padrões nativos e induzidos por ferida, são maioritariamente relacionados com os mecanismos de reparação de tecidos. No entanto, uma ferida mesmo em condições de esterilidade pode activar (em baixos níveis) as mesmas vias de sinalização, o que pode ser suficiente para activar a formação dos padrões induzidos por feridas. A quantificação dos níveis de expressão de genes associados à resposta imune, em asas feridas de *B. anynana* em condições de esterilidade e não esterilidade, poderá esclarecer a extensão da activação destas vias após a indução de feridas estéreis.

Nesta experiência, foi também possível concluir que as fêmeas são mais susceptíveis a formar mais e maiores padrões induzidos por ferida que os machos. Esta diferença pode dever-se ao facto de existir heterocronia e dimorfismo sexual entre os géneros. As horas mais propícias para a formação de padrões induzidos por ferida em *B. anynana* são entre as 12 e as 18 horas após pupação. Um estudo extensivo onde o intervalo de tempo ideal, quer para fêmeas quer para machos, fosse mais reduzido (por exemplo 2 em vez das actuais 6 horas) seria útil para perceber a heterocronia entre os géneros e entender melhor as diferenças observadas.

As feridas induzidas no terceiro compartimento formaram mais padrões que as induzidas no quarto compartimento. Isto sugere que existe compartimentalização da resposta à ferida ao longo do eixo anterior-posterior da asa em desenvolvimento da *B. anynana*. O estudo destes padrões em mutantes ou linhas de selecção artificial com diferentes propriedades na epiderme das asas poderia ajudar a perceber a extensão da compartimentalização da resposta às feridas nas asas desta borboleta.

Numa segunda experiência tentámos perceber qual a estrutura celular da asas em desenvolvimento, assim como as alterações que uma ferida provoca na mesma. Verificámos que, à semelhança de outras borboletas, as células que vão produzir as escamas, estruturas onde os pigmentos serão depositados numa fase mais tardia do desenvolvimento, são determinadas por um mecanismo denominado de inibição lateral por Notch. Este processo ocorre pelo menos entre as 10 e 16 horas após pupação. Para saber a extensão do intervalo de tempo total deste processo será necessário explorar a expressão de *Notch* em horas inferiores e superiores às aqui testadas.

Nos foci dos futuros ocelos detectámos que existiam mais células produtoras de escamas, as quais se organizavam de uma forma diferente do resto do epitelio. A forma como estas células estão organizadas pode ser crucial para a formação do ocelo. Para saber se esta organização é essencial para a formação dos ocelos poder-se-iam fazer feridas no focus às 6 horas após pupação (levam à não formação do ocelo) e às 12 horas (levam à formação de um ocelo maior) e perceber como é que estas feridas afectam a organização das células no focus.

No local das onde as feridas foram feitas verificou-se um aumento do número de células. Este aumento do número de células em tecidos lesados é comum nos animais e é indicativo de uma resposta inflamatória. Apesar de ser claramente visível um aumento do número de células à volta do local da ferida, com esta experiência não nos foi possível distinguir entre proliferação, divisão e migração celular. Futuras experiências deverão ser realizadas para testar a presença de marcadores de proliferação e divisão celular assim como marcadores para as células da hemolinfa. Uma hipótese que considerámos foi o facto do aumento do número de células no local

da ferida ser suficiente para induzir a formação dos padrões induzidos por ferida. Para testar esta hipótese será necessário adquirir mutantes ou técnicas para induzir uma redução na capacidade de proliferação e migração celular e depois investigar nestes indivíduos se a capacidade de formação de padrões induzidos por ferida é menor que nos indivíduos wild type.

Em asas tardias de pupa nós observámos que os pigmentos dourados dos ocelos e dos patrões induzidos por ferida eram depositados nas escamas ao mesmo tempo (ou em tempos muito semelhantes). O mesmo não se verificou para os pigmentos pretos, onde apesar de já serem visíveis nos padrões nativos da asas nunca foram detectados à volta do local da ferida. No entanto esta heterocronia na deposição do pigmento preto nos padrões nativos e induzidos por ferida é difícil de provar, porque não é possível, num estudo desta natureza, ter a certeza qual seria o fenótipo adulto. Um estudo mais pormenorizado que contemplasse mais tempos experimentais e mais indivíduos, assim como outros mutantes ou linhas de selecção com diferentes cores de padrões das asas, ajudaria a esclarecer esta questão.

Na terceira experiência a expressão de vários genes relacionados com a cicatrização de feridas, formação dos ocelos e a biosíntese da melanina foi comparada nos padrões nativos e à volta das feridas. Nenhum dos genes testados foi detectado à volta do focus dos ocelos e apenas a expressão do gene *ple* foi detectada à volta das feridas. O aumento da expressão do *ple* pode indicar a activação da via de sinalização ERK/MAPK, a qual está implicada na regulação da transcrição de componentes da epiderme assim como na morte, proliferação e diferenciação celular. Os dois últimos processos estão de acordo com o aumento do número de células observado na segunda experiência, deste modo esta via de sinalização pode ter um papel fundamental na resposta inflamatória que se segue a uma ferida e também na formação dos padrões induzidos por ferida. No entanto, serão necessários mais estudos para comprovar a proliferação ou migração celular à volta da ferida.

Resumindo, com este projecto foi possível concluir que:

- a formação dos padrões induzidos por ferida não é dependente do processo de infecção;
- as fêmeas são mais sensíveis a formar padrões induzidos por ferida que os machos;
- a resposta à ferida varia consoante a posição onde esta é induzida, o que sugere compartimentalização da asa na sua capacidade de reposta à ferida;
- 10 horas após pupação a determinação das células que vão ser as produtoras de escamas já está a ocorrer;

- os foci dos ocelos tem uma organização celular que é diferente do resto da asa;
- as feridas nas asas em desenvolvimento induziram um aumento do número de células à volta das mesmas;
- o pigmento preto dos padrões induzidos por ferida nunca foi detectado, o que pode indicar que a sua síntese nos padrões induzidos por ferida é posterior à dos padrões nativos;
- a expressão do gene *ple* está aumentada à volta das feridas, o que sugere a activação da via de sinalização ERK/MAPK a qual pode ser responsável pela activação da proliferação celular à volta feridas.

Palavras chave: cicatrização de feridas, imunidade, reparação de tecidos, formação de padrões, expressão genética.

Abstract

Animals are protected by an outer barrier (epidermis) that, when injured, must be rapidly and efficiently repaired. The healing of the epidermis is a vital and complex process which triggers pathways associated with scab formation, tissue repair, and immunity. In a wide range of animals, the healed epidermis can display new pigmentation patterns. In arthropods the blood (hemolymph) coagulation leads to the formation of a black-brown mark, which results in activation of melanin biosynthesis pathway. For insects, melanogenesis is vital for defense against pathogens, wound healing and body coloration. An interesting case of wound-induced pigmentation patterns occurs in the butterfly *Bicyclus anynana.* Damaging on the pupal wing epidermis can result in formation of rings of colour resembling those formed in the native concentric colour rings - eyespots. The molecular underpinnings of this process are largely unknown. However, the genes and pathways involved in development of native eyespots can be also involved in the wound-induced patterns. Here we studied the genetic mechanisms, the cellular dynamics and the gene expression associated with the wound-induced pigmentation patterns in *B. anynana*. We could conclude that the wound-induced pattern formation does not depend of infection process; the females are more sensitive to the wound signals than males; the wound response in the two wing compartments analyzed were different, which indicates the compartmentalization of the wing epithelium; 10 hours post pupation the differentiation of scale forming cells has already started; the eyespot foci have a different cell organization; the wound induces a local increase in cell number; the black pigment of the wound-induced patterns could be synthesized after the black pigment of the native eyespots; *ple* is up-regulated around the wound site 4 hours post wounding and ERK/MAPK pathway could be activating cell proliferation at the wound site.

Key words: wound healing, immunity, tissue repair, pattern formation, gene expression.

1- Introduction

Animals are protected by an outer barrier (epidermis) that, when injured, must be rapidly and efficiently repaired to avoid infection and dehydration. The healing of the epidermis is a vital and complex process which triggers pathways associated with scab formation, tissue repair, and immunity [1, 13]. Several cellular and molecular studies have identified different processes (e.g. movement and fusion of cells, cytoskeleton changes), and genetic pathways (e.g. JNK, MAPK/ERK) involved in wound healing in different animals [1, 7, 14, 15]. These highlight the evolutionary conservation of the wound response process, despite sometimes fundamental differences in epidermal organization between animals [1, 7, 16]. However, there are also particularities of the healing process for specific development stages (e.g. [17-20]), tissues (e.g. [21, 22]) and species (e.g. [16, 23-25]). On the one hand, the study of the commonalities between morphogenetic movements (such as dorsal closure [26-28]), pattern formation, and wound-induced pigmentation patterns [4, 29] could be a new promising approach to investigate developmental patterning and the wound healing process. On the other hand, the study of particularities of wound response will contribute to a deeper understanding of the cellular, molecular and genetic mechanisms of wound healing, and also of its evolution.

1.1- Wound response

Wound healing is a complex process that triggers a plethora of signalling pathways that lead to closing the opening and to reducing the risk of infection. Many different molecules have an active role during healing, including receptors, matrix proteins, and proteases [30, 31]. In mammals, immediately after wounding, the wound edges retract owing to natural tissue tension and an inflammatory response follows [16]. The extracellular matrix is continually remodelled, rapidly at first and at a slower rate thereafter [30, 31]. In most cases the healing of adult tissues results in the formation of a scar, which differs in appearance from the surrounding epidermis and, sometimes, involves tissue/organ loss of function [32, 33].

The molecular mechanisms and pathways associated with infection control and regeneration of epidermal barriers, such as Toll and Imd (Immune Deficiency), Extracellular-Regulated Kinase / Mitogen-Activated Protein Kinase (ERK/MAPK) and Jun N-terminal Kinase (JNK) pathways, are involved in wound response and healing

(Figure 1A) through activation of transcription factors, such as NF-kB, AP1 and *grainy head* [1]:

- Toll and Imd are related to innate immune response and are up regulated after epidermal injury, and lead to activation of the inflammatory response at the wound site [34].
- The ERK/MAPK pathway activates phagocytosis [35], cell proliferation[36, 37] and the transcriptional factor *grh* [1]. This response regulates transcription of surface barrier components which are essential for re-establishing the outer epidermal layer [1].
- The JNK pathway is essential for closing epithelial gaps during dorsal closure in *Drosophila melanogaster* embryogenesis [14], and also for wound healing [14, 38].

One of the goals of wound healing research is to find ways to speed or alter the healing process, and for that we need a better understanding of the fundamental cellular and molecular mechanisms by which cells sense tissue damage and signal to neighbouring healthy cells to contain and repair that damage [38]. However, the complexity of wound healing makes it difficult to reveal the roles of all molecules

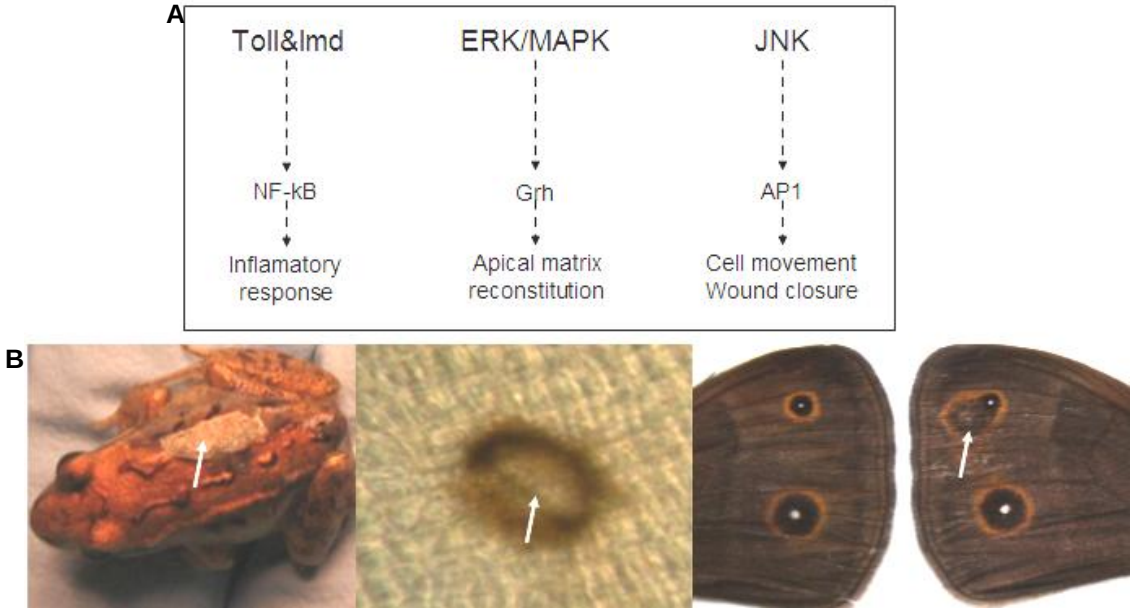


Figure 1: Genetic mechanisms (A) and pigmentation (B) patterns associated to the wound healing process. Epidermal wounding activates Toll and Imd, ERK/MAPK and JNK pathways in cells surrounding the wound gap, which lead to the activation of inflammatory response, reconstitution of apical matrix and cell movement and wound closure (A)(adapted from [1]). Pigmentation patterns are associated with wound healing (B) in different systems; from the left to the right the amphibian, *Rana sylvatica* (from *U.S. Fish and Wildlife Service*), the crustacean, *Daphnia magna* (from Dieter Ebert's webpage) and the insect *Bicyclus anynana*. The wound site is marked with white arrows.

involved, so this process is not yet fully understood and new models suitable to study it could be an advantage.

1.2- Insects melanogenesis and wound response

In a wide range of animals, the healed epidermis can display new pigmentation patterns which result from a new arrangement of melanin-containing cells (melanocytes in vertebrates or crystal cells in invertebrates) [13]. The *Rana sylvatica* , *Daphia magna* and *Bicyclus anynana* (Figure 1B) are some examples where an epidermal wound can lead to the formation of a new pigmentation pattern at the wound site [4, 39-41]. In arthropods the blood (hemolymph) coagulation also leads to the formation of a black-brown mark, which results in activation of melanin biosynthesis pathway. The main function of melanin is body coloration [42, 43], UV photoprotection (mammals) [44] and immunity (insects) [13].

For insects, melanogenesis is vital for both defense against pathogens and wound healing [15, 42], and can be used as an indicator of immune investment [42]. Melanin is involved in a sequence of immunity events immediately upon wounding [38, 45]. This involves a number of mechanistically and functionally inter-connected phenomena, such as melanin scab formation and encapsulation of pathogens [13, 34, 43, 46]. Immediately after a puncture there is hemolymph loss but within an hour (or less) a black-brown plug forms in the wound gap and the hemolymph loss ends [38]. Over the ensuing couple of hours the outer part of the plug melanizes to form a scab, and epidermal cells surrounding the plug orient towards it and then fuse to form a syncytium [38]. Subsequently, more-peripheral cells also orient towards and fuse with the central syncytium. During this time, the JNK pathway is activated in a gradient emanating out from the wound site, and the epidermal cells spread along or through the wound plug to re-establish a continuous epithelium and its basal lamina and apical cuticle [38]. The melanin accumulation at the wound site leads to a darker mark which is visible and can remain even after the epidermis has healed. Thus, melanogenesis is a key mechanism that bridges insect immunity, wound healing and body coloration.

Melanin synthesis is mediated by a serine proteinase cascade in mammals and in insects, despite the different cellular location of this pigment in both [13]. In insects, the synthesis of melanin requires an enzymatic cascade that converts tyrosine in dopa, dopamine, and N- beta-analyl-dopamine into black, brown, and yellow pigments [43, 47]. It is known that after wounding several melanogenic enzymes, such as dopa-decarboxylase and phenoloxidases have elevated activity [13]. However, the details of the mechanisms that activate melanin biosynthesis around wound sites, and the

pleiotropic effects of melanogenesis genes on pigmentation and immunity are not completely understood [13, 43].

1.3- Particularities of wound response and pigmentation in Lepidopterans

The wings of the Lepidoptera (the insect order of butterflies and moths) are covered with thousands of overlapping coloured scales, which are organized in rows within a well defined network of wing veins [48]. The colour pattern on each wing surface is, then, a two-dimensional sheet of scales. This two-dimensional arrangement makes their developmental dissection much simpler relative to three-dimensional morphological structures, such as appendages or bristles [48, 49]. The butterfly *B. anynana*, as many butterflies of the Nymphalid family, has its wings decorated with series of particular pattern elements formed by concentric rings of different colours which are called eyespots, which are evolutionary novelties (i.e. lineage-restricted traits of ecological value [48, 50]) (Figure 2A-C).

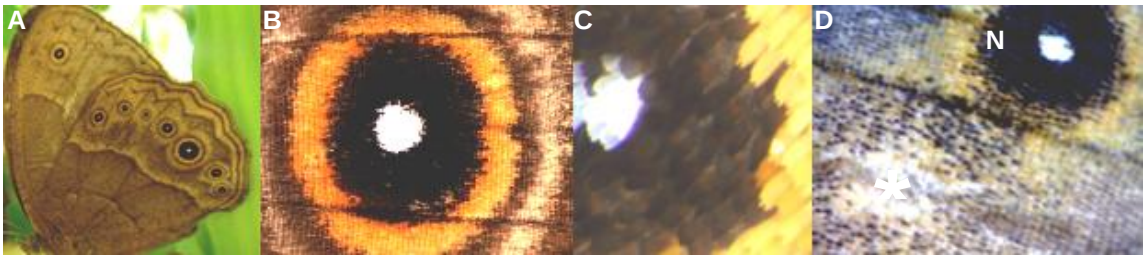


Figure 2- *B. anynana* wing colour patterns. The adult butterfly (A) in the distal part of their wings has eyespots (B). These butterfly colour patterns are formed by overlapping scales (C) which are organized in rows. Wounding developing wings induces a colour patterns around the wound site (*) (D) that resemble the native eyespots (N).

Wing colour patterns are used in inter and intra-specific visual communication [51]. For example, predator avoidance can be accomplished by apostematic warning coloration [48] or crypsis [48, 52], while the same patterns can also be used for intra-specific recognition, mate localization and sexual selection [48, 53]. The recent increase in genetic resources and analytical tools makes butterflies very suitable for genetic studies [54, 55], and a great opportunity to link the evolutionary and developmental mechanisms that generate the morphological variation. In addition they are also interesting to study the wound healing process, because wounding to the pupal wing epidermis during a specific time window leads to the formation of ectopic pigmentation patterns resembling native pattern elements [4, 56] (Figure 2D). The molecular underpinnings of this process are largely unknown, but exploratory studies of

wound-induced eyespot formation in *B. anynana* have lead to the suggestion that the evolutionary origin of eyespots (in butterflies) might be related to the co-option of genetic circuitry for wound response (shared by all insects) [4]. Monteiro and colleagues [4] have investigated if the damage-induced ectopic eyespot recruited the same genetic mechanism used for the formation of native eyespots. The eyespot ring genes *engrailed, spalt* and *Distal-less* were shown to be up-regulated in cells surrounding wound sites [4, 11]. Aside this first study little is known about the genetic mechanisms behind the wound-induced eyespots patterns.

1.4- Native and wound-induced eyespots development in butterfly

Several studies in a few lab models have started to describe aspects of the cellular signalling [29, 50, 57-59] and the genetic pathways [4, 11, 48, 58, 60-63] underlying eyespot formation. Experiments of grafting or destruction of focal cells (the cell of the centre of the eyespot) in early pupa developing wings lead to misplacing or eliminating the corresponding eyespots, and establishes the focal cells as the organizer centre that triggers the developmental pathways for eyespot formation [11, 29, 57]. These centres are established in the final larval instar [2] (Figure 3A). In the young pupal wings, signalling from the focus (Figure 3B) presumably established a concentration gradient of a morphogen, which, in turn, provides information to the

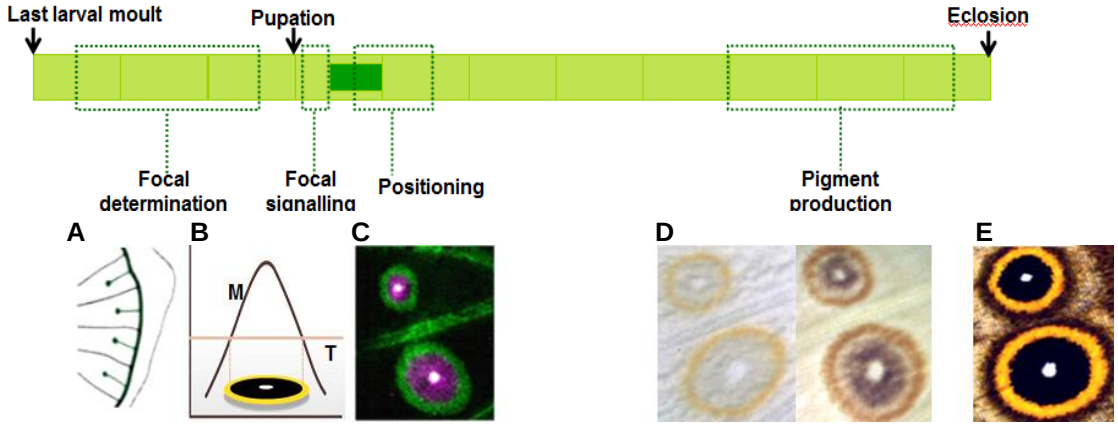


Figure 3- Different phases of eyespot development. During larval development the focal determination (A) occurs and some wing patterning genes (e.g. *Distal-less* [2], engrailed [5] and *hedgehog* [10] are expressed in the future foci cells. Early after pupation the focal signaling (B) establishes a concentration gradient, a signaling molecule(s), morphogen (M), is diffused from the foci to the surrounding cells that respond in a threshold-like (T) manner. This leads to the activation of transcription factors (C), such as Engrailed (green) and Spalt (purple) that reproduce the areas where the different pigments will later be synthesized (D) [11]. Later on, the pigment production occurs (D), according to the signals received before, and follows the maturation of scales that will correspond to the different colour rings of the adult eyespot (E) (adapted from [2] and [12]).

surrounding cells to express different transcription factors (Figure 3C) corresponding to the adult eyespot colour rings (Figure 3E). Latter on, pupal wings are coloured through synthesis of pigments in specialized wing scale cells in a stereotyped developmental succession [64, 65]. The scales that mature first are those at the centre of the eyespots (white focus of adult eyespots), followed by the external golden ring, then the middle black disc and finally the brown background across the whole wing [64, 65].

Homologs of many patterning genes involved in *Drosophila* wing development have been implicated in butterfly wing colour pattern specification [66]. In the last larval instar, wing development genes used in all insects, such as *engrailed* [4, 10], *hedgehog* [10], *Distal-less* [2, 67], and others such as *Notch* [5] and *spalt* [4] are recruited to establish the organizing centres of butterfly eyespots. Later on, during early pupal stage the fate of wing cells is specified. The genes *wingless* and phosphorylated Smad (a signal transducer from the Transforming Growth Factor - β (TGF-β) signalling pathway), which are expressed in the foci and are candidate morphogens [4], probably fate the surrounding cells to express specific transcription factors such as *engrailed, spalt* (Figure 3C) and *Distal-less* [11]. All three genes are expressed in the foci, *engrailed* is expressed in the future golden ring area, and the *spalt* and *Distal-less* in the black ring area [11]. Thus, these genes are part of the genetic machinery that fate the cells to express different pigment synthesis genes, such as the ones that encode enzymes involved in melanogenesis, to produce colour rings in the late pupal stage (Figure 3E). After focal signalling the scale forming cells are differentiated through Notch lateral inhibition [68]. This is a mechanism used in developing tissues to determine cell fate [68-70], and in butterflies the cells that do not express Notch are fated to be the scale forming cells [68].

In *B. anynana*, wounding inflicted during pupal development can lead to the formation of wound-induced pigmentation patterns that resemble native eyespots(Figure 2D). Thus, the wound sites behave like eyespot organizing centres. The wound–induced patterns can range from a simple golden patch, to a black patch, to an ectopic eyespot with rings of different colours [8, 9, 29] (examples in Figure 9 in results). However the wound-induced patterns never have the white central pupil which is always present in native eyespots [29]. There are different possible models that explain wound-induced pattern formation in *B. anynana.* The wounds can induce the formation of a concentration gradient of a morphogen molecule (through morphogen diffusion or degradation (Figure 4C(i) and (ii), respectively) resembling the signals of eyespot foci in early developing wings [4, 8, 9] – activation of focal signals model; or they can induce a dip of thresholds (levels below the basal levels of morphogen (Figure 4C(iii)) [8, 9] – threshold reduction model. Thus, when a wound induces an

increase (C(i)) or a decrease (C(ii)) in a morphogen level higher than the sensitive thresholds that specify the black and the golden colours or a decrease in that sensitive thresholds (C(iii)) lower than the morphogen level it provides information to form an ectopic eyespot centred at the wound site. So far, none of those models can be excluded. The expression of *engrailed, spalt* and *Distal-less* was detected 12 hours post wounding around the wound site [4], which resembles the native eyespot formation. This strongly suggests that the wounds induce the same (or similar) signals of the native eyespot foci and supports the focal signal activation model.

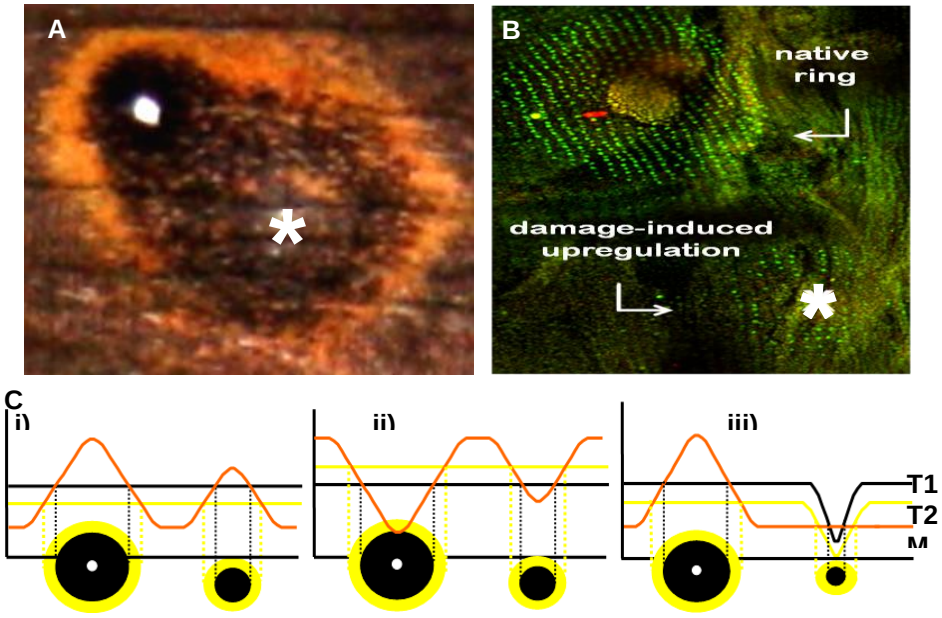


Figure 4- Wound-induced pigmentation pattern formation. The wounding on developing wings induces ectopic eyespots (A) centred at the wound site (*), which can resemble native eyespots (N). The genetic mechanisms underlying the formation a wound-induced ectopic eyespot are not known, but it its known that some “eyespot” genes are up-regulated around the wound sites. In (B) we can see the expression of Engrailed protein in the area corresponding to the golden ring of the native eyespot and around the wound site 12 hours post wounding. There are alternative models that can explain the formation of these patterns, which are represented in (C). X axis represents the wing epidermis and Y axis the concentration levels of morphogen (M) and sensitive thresholds (T); orange curve is eyespot-inducing signal (M) and black and yellow are sensitivity thresholds (concentration values which correspond to colour switch; T1 and T2 establish the limits of black and the golden rings respectively); native eyespot to the left and damage-induced eyespot (without white centre) to the right. The wound induces the activation of the same signals (or similar) to those underlying form a native eyespot (C(i) and (ii)). These signals establish a concentration gradient through diffusion (C(i)) [4] or degradation (C(ii)) [8, 9] of a M. The surrounding cells respond differently, according to their position, in a threshold-like manner (T1 and T2). Other model suggests that the wound induces a degradation of T levels (C(iii)) that makes the surrounding cells sensitive to the M basal levels [8].

The study of the molecular, cellular and genetic mechanisms behind the formation of wound-induced patterns could give new insights both into wound response (healing of developing tissues and involvement of melanin-pathway genes) and into the origin of evolutionary novelties (such as are butterfly eyespots) by recruitment of conserved genetic circuitry. In this project we elucidate some aspects of eyespot formation and its commonalities with the wound response process.

2- Aims

This project was designed to clarify the genetic underpinnings of wound-induced colour pattern formation in the butterfly *B. anynana*. Studies in model systems have identified pathways and cellular mechanisms implicated in wound response [7, 14, 15], while studies in *B. anynana* have revealed pathways and processes involved in native eyespot formation [4, 11, 48]. However, the commonalities between both sets remain unclear. Wounding leads to activation of tissue repair and immunity [16], but the contribution of these processes to the formation of wound-induced patterns, and the effects of the wounds on epidermis organization and on gene expression are unknown. Three main aims were defined to better understand these issues:

Aim 1: Disentangle the role of tissue repair and immunity in induction of pigmentation patterns.

Aim 2: Characterize the cellular dynamics of the developing wing epidermis around eyespots and wound sites.

Aim 3: Compare expression of “eyespot” and “wound response” genes around developing native eyespots and around wound sites.

3- Materials and Methods

3.1- Wound induction and wing dissections

The *Bicyclus anynana* laboratory stock was maintained at 27ºC (+/- 0.5ºC) with 70% (+/-1%) humidity as described in [71]. Pupation times were scored through time-lapse photography with a digital camera (*Canon 1000D*) via remote capture software (*GB Timelapse, Granite Bay Software*). A photo of pre-pupae collected onto 25 well plates was taken each 10 min, so the exact time of pupation was obtained with an error of ±10 minutes.

Previous studies [29] revealed that 12 hours after pupation is the best time to induce pigmentation patterns by wounding (for the above rearing conditions) and the third and fourth wing compartments are the most sensitive to wound signals [29]. Thus, the wounds were inflicted under a stereoscope (*Leica*) in 12 hours pupal wings by inserting a fine tungsten probe of 0.25 mm diameter (*World Precision Instruments*) [71] distally in third and fourth wing compartments (Figure 5). The veins are visible through the hardened pupal cuticle, so it is easy to have a precise location of the wound, on the underlying dorsal epidermis of the forewing [29] (Figure 5).

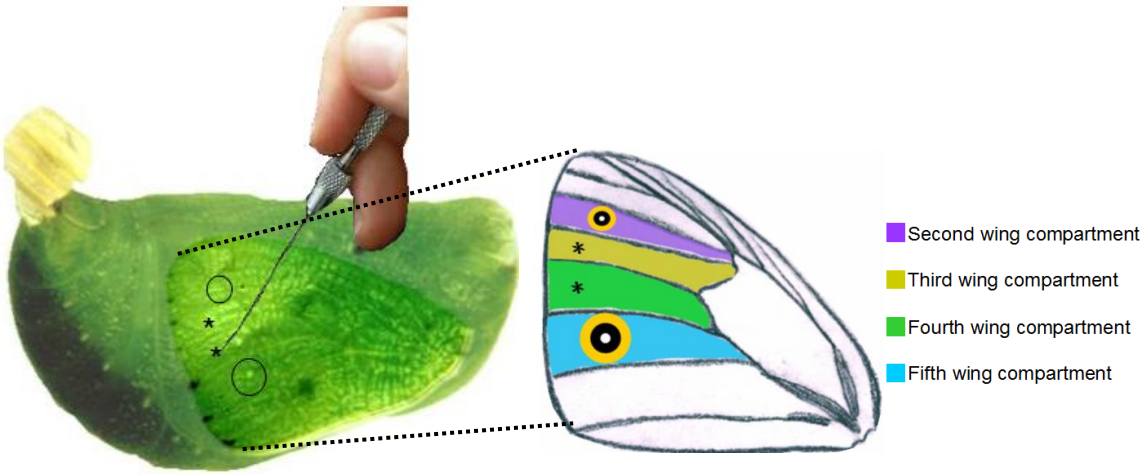


Figure 5- *B. anynana* wings are visible under the hard cuticle that at 12 hours post pupation is still attached to the dorsal surface of the forewing that lies underneath it. Wounding the wings in specific locations is technically easy. The second, third, fourth and fifth wing compartments of the forewings have a white spot in the cuticle corresponding to presumptive native eyespot organizing centers (visible here for the fourth and fifth wing compartments). Eyespots can appear in all these compartments, but typically appear only on the second and fifth compartments - the future eyespot areas are marked with black circles. In this study wounds were inflicted to third and fourth wing compartments - wounding site are marked with (*).

Manipulated pupae were either 1) left to eclose for analysis of adult phenotypes (*ImageJ software*), to investigate the role of tissue repair and immunity in induction of pigmentation patterns (aim 1), or 2) used to dissect developing pupal wings at different times post-wounding, to investigate the cellular dynamics (aim 2) and patterns of gene expression (aim 3) around eyespots and wound sites. All dissections were made under a stereoscope (*Leica*) in cold fresh 1XPBS buffer.

3.2- Experimental design and methods for Aim 1

Three groups were formed in this experiment:

- NON STERILE WOUND: the pupae were wounded, but not sterilized:
- STERILE WOUND: the pupae were wounded and sterilized;
- NO WOUND: the pupae were sterilized, but not wounded (Figure 6).

The wound of the NON STERILE WOUND group presumably induces both tissue repair and immunity (induced by infection) processes (the cuticle has a huge number of microorganisms, which can induce infection at the wounding (Annex 6.1)), while in the STERILE WOUND group the induction of immunity should be reduced or absent (infection avoided through sterilization of pupa cuticle and needle (Annex 6.1)). The NO WOUND group was established to confirm that the sterilization treatment alone does not interfere with native pigmentation or induce any ectopic pigmentation effects. The wound-induced patterns of the NON STERILE and STERILE WOUND groups were characterized in terms of what type of pattern is induced and their size and were compared between the two groups to understand the effect of sterilization in wound-induced pattern formation.

The sterilization was achieved through washing the pupae for a few seconds in 70% ethanol solution followed with a washing in sterile water; this double washing was repeated three times. To verify if the cuticle was really sterile the pupae were immersed into an eppendorf with water for a few seconds (sterile check 1) and were then put in a sterile Petri dish until wounding. The wounding was performed in a sterile environment (with a busen burner on each side of the stereo-microscope) and, after wounding, the pupae were placed again into the sterile Petri dish and put in a growth chamber. Approximately 24 hours after wounding the pupae were immersed into another eppendorf with water for a few seconds (sterile check 2) and put again in the growth chamber until eclosion. Both eppendorfs (sterile check 1 before wounding, and sterile check 2 after healing) were centrifuged and the sediment particles were plated in LB medium. After an overnight at 37ºC the plates were checked for presence of

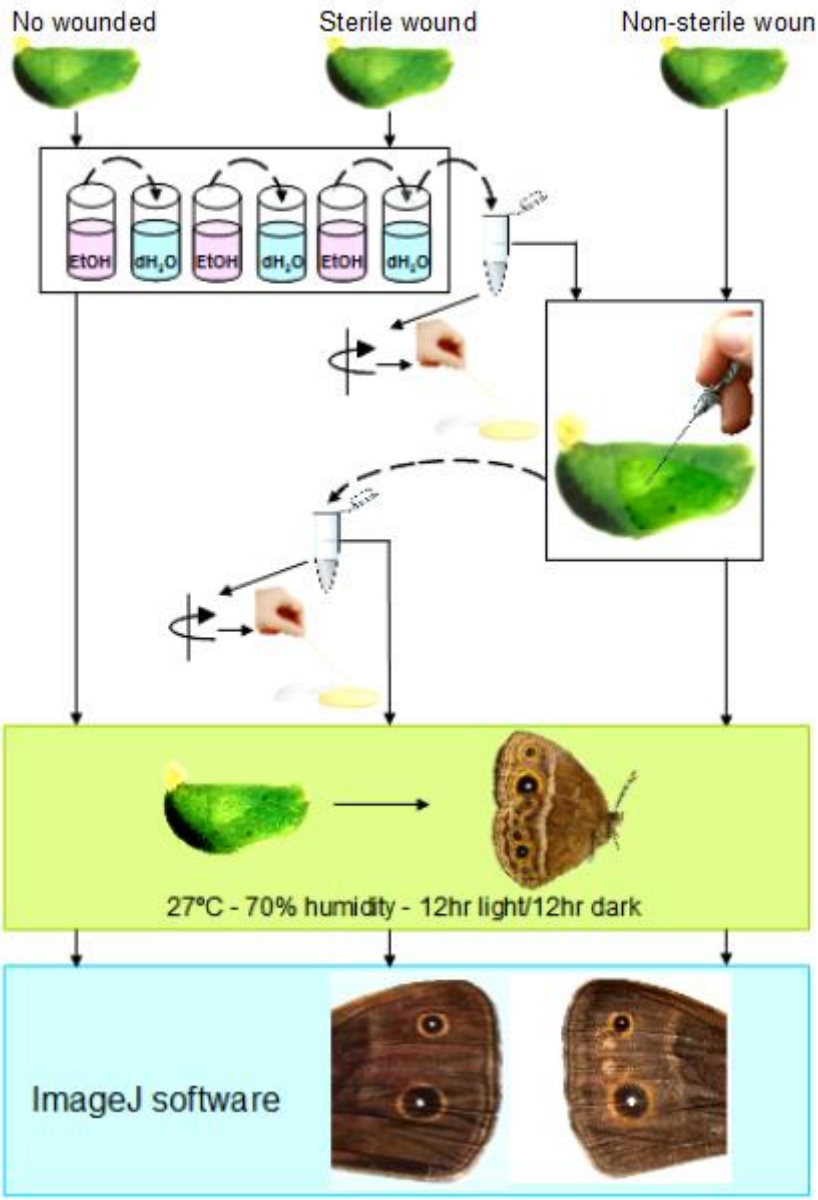


Figure 6- Experimental design of experiment of aim 1 (see details in the text).

microorganisms. Only the pupae with double negatives (i.e. no colonies detected after plating and incubating sterile check 1 and 2 collections) were considered sterile and analyzed further in STERILE WOUND group (Figure 6). After adult eclosion and wing extension, the butterflies were put at -20ºC for approximately 30 minutes to be killed. The two forewings of each individual were then removed and stored at 4ºC, and digital photos of both were then taken simultaneously in light controlled conditions (*Canon 1000D*).

The wound-induced patterns were classified in relation to their presence/absence, colour (golden and black scales and an additional category for light brown scales), composition (one or more colour rings) and size (the diameter). Because the wound-induced patterns have irregular forms and sometimes are fused

with the native eyespots the diameter was measured (using *ImageJ software*) in three replicates and in three different directions (Anterior to Posterior, Anterior-Proximal to Posterior-Distal and Anterior-Distal to Posterior-Proximal corresponding to the axis 1, 2 and 3 of (Figure 7)). The mean of these measurements were used as the size of the wound-induced patterns.

The statistical analyses were made comparing the results between wound type treatments (NON STERILE WOUND versus STERILE WOUND), between genders (female and male) and between wing compartments (third and fourth). The SPSS statistical software was used to compare the number and size of wound-induced patterns between groups.

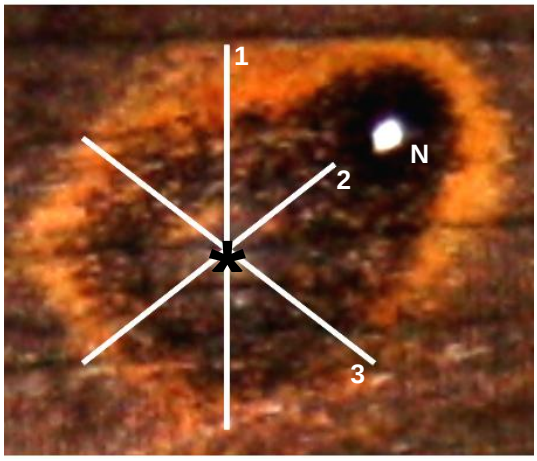


Figure 7- Scheme of wound induced pigmentation patterns size measurements. The size of wound-induced pigmentation patterns was obtained through measurement of their diameter. The diameter of wound-induced patterns was measured in three different directions; Anterior to Posterior (1), Anterior-Proximal to Posterior-Distal (2) and Anterior-Distal to Posterior-Proximal (3). In some cases the wound-induced patterns were fused with the native eyespot and it was difficult establish the limits of each pattern. The wound site is marked (*) and the native eyespot (N).

3.3- Experimental design and methods for Aim 2

The dissection of intact wings [71] is difficult for pupae younger than 24 hours because the wings are too fragile. Thus, the forewings were dissected attached to the cuticle and the staining protocols were run with both. The cuticle was removed during staining (last day) of *in situ* hybridization (ISH) protocol [71] and it was never removed in the immunohistochemistry (IHC) protocol [71].

DAPI (*Invitrogen*) and Rhodamine-labelled Phalloidin (*Invitrogen*) were used for nuclear (DAPI binds to DNA) and cell membrane (phalloidin binds to Actin) stainings, respectively. Dissected pupal wings of different ages (10 to 40 hours post pupation) were fixed for 40-70 minutes in fix buffer and were immersed first in Rhodamin-Phalloidin solution (1 µL in 200mL PBT) for 2 hours, and after washing the wings were immersed in DAPI solution (1µL DAPI in 1mL PBT) for 5 minutes (Annex 6.2.1).

Antibody staining were use to detect proteins in wounded pupal wings in the right dorsal forewing, which were fixed in fix buffer for 30-35 minutes and after washing

they were incubated with primary antibody one overnight. In the next day the wings were washed and incubated with secondary antibody for two hours (Annex 6.2.2). A control staining was performed using the secondary antibodies only. Seven antibodies were tested (Ecdysone Receptor: 10F1, 15C3, 9B9, 6B7; Engrailed: 4D9, and Delta-extracellular domain: C494.9B from *Hydridoma Bank*; at least six wings were tested for each antibody), but the only antibody successfully used was Notch (C17.9C6-s from *Hydridoma Bank*). All fluorescent staining images were captured with a DIC/fluorescent microscope (*Axio Imager, Zeiss*).

To characterize the dynamics of pigments deposition in wounded wings, 69 pupae were dissected [72] at different time points covering the last 48 hours of pupal development. For this experiment, I used the pupae wounded at 12 hours post pupation but which, despite having been sterilized did have microorganisms in some of the sterility checks (see above in Figure 6). After dissection the wings were put in 100% glycerol and later were photographed (*Leica*). Photos were analyzed for the presence of the pigments in the scales around the wound site.

3.4- Experimental design and methods for Aim 3

We generated a list of target genes ("wound response" and "eyespot") to investigate patterns of expression in wounded wings. This list included genes of the pathways associated with wound healing, such as JNK and ERK/MAPK, and genes associated with pigmentation patterns, such as enzymes involved in melanogenesis, as well as signalling molecules presumably involved in establishing (Hedgehog) and signalling (Wingless) from the eyespot-organizing centres (Annex 6.3.1). For those genes for which we did not have sequence available for *B. anynana* (to design probes for detection of mRNA) and for which no cross-reactive antibody was available (to detect protein product), we designed two pairs of degenerated primers using CODEHOP software. cDNA of young pupal wings (12-36 hours post pupation) was used as template to amplify the target genes. The much intense amplified bands were cloned into the pGEM-T Easy® vector (*Promega*). Eight positive colonies (i.e. colonies with a plasmid with the fragment inserted), were picked and used as a template in a PCR reaction where the M13 primers (M13.F- GCCAGGGTTTTCCCAGTCACGA and M13.R- GAGCGGATAACAATTTCACACAGG) were used to amplify the complete inserted fragment. The size of the inserted fragments was checked in a 1% agarose gel, and at least two fragments of the expected size (the same size observed in the degenerated PCR plus 200 bp corresponding to the amplified fraction of the plasmid)

were sequenced. The sequences were used to attempt to identify homologs in GenBank (*tblastx* score>100 and *e-value*<1E-8).

We obtained the *B. anynana* orthologs for eight of the candidate genes. The sequences of target genes were amplified by PCR using primers designed in program *Primer3* (Annex 6.3.2). The size of the PCR product obtained was checked on a 1% agarose gel. For the fragments with the correct size, the plasmid was extracted from the bacteria using NucleoSpin® Plasmid Miniprep (*Macherey-Nagel*). 1µL of plasmid solution was run on agarose gel and the DNA concentration was measured using the *NanoDrop* equipment. To verify if the amplified sequence was really the target gene, plasmid insert was sequenced. Eight sequences were confirmed (the list of probe sequences in Annex 6.3.3), and were used to generate anti-sense (AS, a complementary sequence to mRNA) RNA probes Dig-labelled (*Roche*).

Whole-mount *in situ* hybridizations of young pupal wings were performed as described in 7.1.3. In this protocol pupae wounded in the right forewing were used to see the expression of the target genes around wound sites and in the area where native eyespots are developing. The left forewing was not wounded and was used as a control to characterize gene expression on the undamaged wing. All wings were treated separately. All images were captured with a stereoscope associated with a camera (*Leica*) with 2X magnification.

4. Results and Discussion

In this work we analyzed many pupal and adult wings: aim 1) 636 individuals were analyzed to understand the role of immunity in pigmentation pattern formation, aim 2) 28 young pupal wings were analyzed to characterize the cellular structure of the wing epidermis, and 36 late pupal wings were analyzed to characterize pigment deposition around wound sites, aim 3) 77 pupal wings were used for analysis of gene expression patterns, but only eight wings produced successful stainings (the other pupal wings were degraded or the staining protocol did not work).

4.1- Aim 1- Disentangle the role of tissue repair and immunity in induction of pigmentation patterns

Experimental animals

Of the 636 pupae manipulated in this study, 220 pupae (35%) died before eclosion (Figure 8A), and of the 416 eclosed adults, 73 could not stretch their wings, had wing deformities, or still had an unclosed hole around the wound site (i.e. wound had not healed) (Figure 8B). The differences in survival and wing deformities between treatments were statistically significant (Figure 8); the probability that a pupa dies being higher in the STERILE WOUND group than in the other groups. This could be result of more manipulation in this group. The young pupae are extremely fragile and the manipulation with forceps during sterilization, wounding and sterile checks could be enough to induce internal damage on pupae. In the STERILE WOUND group the pupae were manipulated with forceps for three additional steps when compared with the NON STERILE WOUND group (sterilization and sterility checks) and NO WOUND group (wounding and sterility checks). The probability of the wounded wings (STERILE WOUND and NON STERILE WOUND groups) having deformities is higher than in the NO WOUND group, so the wound leads to disruption of the epithelium and probably leads to epidermis being attached to the cuticle via melanic scab.

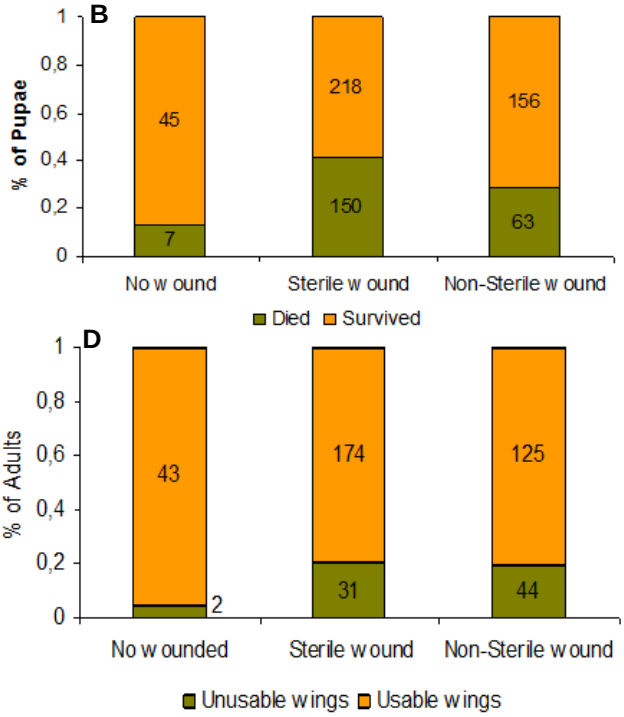
Of the 368 sterilized pupae, only 266 (62%) had absence of microorganisms in both sterile checks 1 and 2. The total of wings analyzed was of 43 individuals for the NO WOUND group, 125 for the NON STERILE WOUND group, and 174 for the STERILE WOUND group. None of the 43 pupae that were sterilized but not wounded (NO WOUND

group) produced wings with disturbed native patterns or any induced ectopic pigmentation patterns. So, we could conclude that the sterilization treatment did not affect wing pigmentation.

A

Survival			
Group	Died	Survived	Total
NO WOUND	7	45	52
STERILE WOUND	150	218	368
NON STERILE WOUND	63	153	216
Total	220	416	636

DF	α	χ^2	*ρ-value*
2	0,05	19,772	<0,001***



C

Usable wings			
Group	Unusable	Usable	Total
NO WOUND	2	43	45
STERILE WOUND	44	174	218
NON STERILE WOUND	27	125	152
Total	73	343	415

DF	α	χ^2	*ρ-value*
2	0,05	6,379	0,041*

Figure 8- Pupal survival and the usable wings rate. The survival rate between groups were statistically significant (χ^2= 19,772; *ρ-value* <0,001 for α=0,05), and the differences in adult wing deformities were also statistically significant between groups (χ^2= 6,379; *ρ-value* <0,041 for α=0,05). The table A and C shows the pupae analyzed in each group and the respective χ^2 test statistical values, and the graphic B and C represent the individual proportions.

Classification of wound induced patterns by colour

The wound induced patterns obtained were very diverse in size, shape and colour. To analyse the colour of the wound induced patterns, they were classified in groups:

- Ectopic eyespot - at least two rings of different colours; brown and gold, brown and black or gold and black (Figure 9A(i));

- Black patch - an area, or spot with just black scales (Figure 9A(ii));

- Gold patch - an area, or spot with just golden scales (Figure 9A(iii));

- Light brown patch - an area, or spot with just lighter brown scales (lighter than the wing background) (Figure 9A(iv));

- Absence of response – no local changes in pigmentation around healed wound.

The formation of these patterns can be explained through the activation of focal signals model and threshold reduction model (Figure 9B and C). The wound leads to the formation of an ectopic eyespot when it induces an increase of the morphogen (M) level higher than sensitive threshold for the black colour (T1) and yellow colour (T2) (Figure 9B(i)) or a decrease in T1 and T2 lower than the M level (Figure 9C(i)). In cases that the T1 and T2 are very similar (Figure 6B and C (ii)), the golden ring of native eyespots are reduced and the wound induces a black patch [9, 12]. When the wound induces an increase of the M level higher than T2 but lower than T1 (Figure 9B(iii)) or a decrease in T1 and T2, but only the T2 is lower than the M level (Figure 9C(iii)), the native eyespots have larger golden rings and the wound forms a golden patch [9, 12]. In this study the rings of the native eyespots were not measured, but the

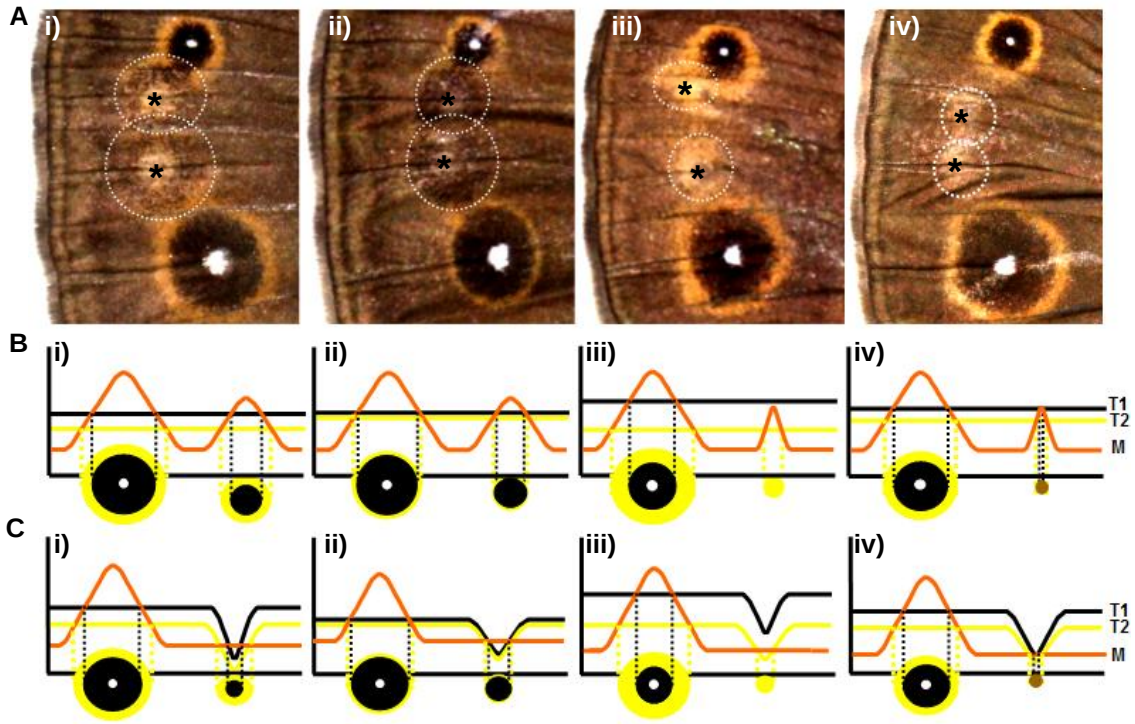


Figure 9- Examples of wound-induced pigmentation patterns in *B. anynana* and respective models. Wound-induced pigmentation patterns can range from a ectopic eyespot (with rings of different colours) (A(i)), to a black patch (A(ii)), to a golden patch (A(iii)) and light brown patches (A(iv). The wound site is marked (*) and the size of wound-induced patterns is limited with a white dashed circle. Both hypothetical models (activation of focal signals model (B) and threshold reduction (C) – see Figure 4) of wound-induced patterns suggested that the wings, which produced black and golden patches should be those with a tiny and a larger golden ring, respectively that was confirmed (compare A, B, C (ii) and (iii)). The hypothetical model for light brown patches suggests that the wound induce an increase of morphogen for the same levels of the treshold1 (B(iv)) or a deep of threshold 1 and 2 for the same and lower morphogen levels (C(iv)), respectively.

observation of the phenotypes seems to corrugate this hypothesis, but an accurate measurement of the native eyespots should be performed for a stronger support. The formation of the light brown patches has not described yet, but could be the case that the wound increases the M to the similar level of the T1 (Figure 9C(iv)) or decreases the T1 and T2 for levels that are very close to the M level (Figure 9C(iv)), and the surrounding cells do not have clear information to form the black ring. These hypothesis need to be further tested. According to these models, the ectopic eyespots are the result of a much more intense wound response of an epithelium with a normal threshold levels, and are larger than the other wound induced patterns. This was expected and was confirmed in our results (Annex 6.4).

Figure 10 summarizes the number of individuals in each class of wound induced pigmentation patterns obtained for each treatment, gender and wing compartment. These results show that males have more black patches and females more gold patches and ectopic eyespots. Male wings are darker, and possibly the wound induced patterns follow this trend. The results also show that, relative to the third wing compartment, the fourth wing compartment had more instances of wounds resulting in no local changes in pigmentation ("absence of response" class). This suggests that the fourth wing compartment is less responsive to the wound signals.

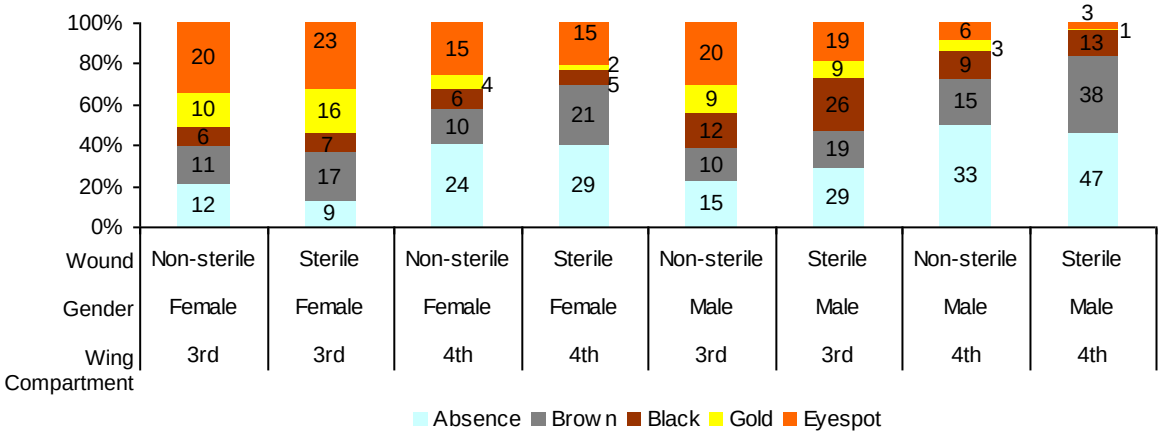
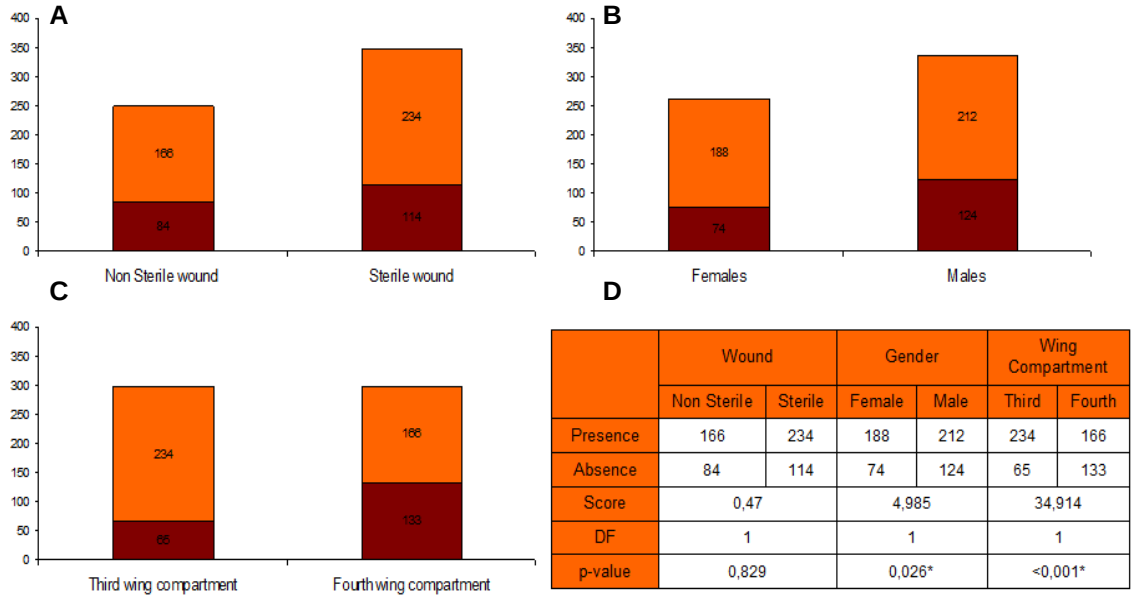


Figure 10- Wound –induced pigmentation pattern distribution *per* type of wound, gender and wing compartment. Females have higher percentage of ectopic eyespots and golden patches while males have higher percentage of black and brown. The absence of wound-induced patterns was higher in the fourth than in the third compartment.

Qualitative analysis of wound induced pigmentation patterns

The adult phenotypes were analyzed statistically for the qualitative effect, i.e. for each wound site on each butterfly wing; we determined which class of pigmentation effect was produced. The differences of presence/absence of wound induced patterns between NON STERILE and STERILE WOUND groups were not significant different (Figure 11A). Thus, we can conclude that to form an ectopic pattern the infection is not required. We had anticipated three scenarios to explain the possible result: the shared pathways of wound response and pigmentation pattern formation are 1) pathways involved in immunity and in tissue repair, which are activated upon wounding; 2) pathways associated just with immunity or 3) those associated only with tissue repair. If first and second scenarios were correct, we would expect wound induced pigmentation patterns of NON STERILE WOUND group to be more extensive (in frequency and in size (see below)) [73, 74], because the infection would increase the strength of the signal (pathways associated with immunity) at the wound site. Instead we obtained the same number (Figure 11A) and the same size (see below in Figure 12A) of wound induced patterns in NON STERILE and STERILE WOUND groups. However, in Drosophila it has been demonstrated that sterile injury *per se*, in the absence of pathogens, triggers



D

	Wound		Gender		Wing Compartment	
	Non Sterile	Sterile	Female	Male	Third	Fourth
Presence	166	234	188	212	234	166
Absence	84	114	74	124	65	133
Score	0,47		4,985		34,914	
DF	1		1		1	
p-value	0,829		0,026*		<0,001*	

Figure 11- Wound-induced pigmentation patterns in different groups (NON STERILE and STERILE WOUND) (A), genders (B) and wing compartment (C). The number of wound-induced patterns in STERILE WOUND group was not significantly different of the NON STERILE WOUND group, so the sterility had no effect in wound-induced pattern formation (A). The females (B) as the third wing compartment (C) were statistically significant more sensitive to the wound signals and formed more wound-induced patterns than males and fourth wing compartment, respectively. The orange in the graphics is the presence of wound induced patterns and the brown the absence. Regression binominal logistic was the statistic test used to test the significance of these results and is summarized in table (D).

induction of a ‘pathogen response', supposedly to prime the organism for what is likely to be an increased risk of infection [46, 75]. If the wound *per se* activates immune system pathways, this could be enough to form wound induced pigmentation patterns and explain our results. We also not controlled if the infection effectively occurred in NON STERILE WOUND group, but with the huge number of microorganisms in the cuticle (Annex 6.1) the infection seems very probable. The third scenario can explain the same number and size of wound induced pigmentation patterns in both groups, because the pathways related with tissue repair should be activated in a similar way in both groups to close the epithelium opening.

We found that males and females differ in the types of wound induced produced; with response appearing to be stronger in females relative to males (Figure 11B). These differences can be a result of the heterochrony (differences in developmental timing [76]) and sexual dimorphism[77] between females and males. Those can potentially lead to changes in size and shape of the eyespots [71] and also the wound induced patterns. The total developmental time is shorter in males than in females, but de pupal developmental time is longer in males than in females (heterochronic differences) and adult wings and eyespots are larger in females than in males (sexual dimorphic differences) [72]. The developmental particularities for each gender can lead to different epidermal sensitivity to the same wound signals.

We observed that wounding to the third wing compartment lead to the formation of significantly more eyespot than wounding to the fourth wing compartment (Figure 11C). Brakefield & French [29] had characterized differences in response to damage on different wing locations and at different developmental time points and had not detected significant differences in wound induced patterns in these two locations at 12 hours post pupation wounding. Additionally, other studies have suggested that the epidermal response to eyespot-inducing signals is a wing-wide property [12, 74]. However, in this study we found significant differences in the type (Figure 11C), but not size (see below in Figure 12C), of the wound induced patterns produced in the third *vs* the fourth wing compartments.The wounds made in the third wing compartment produced more ectopic patterns than those made on fourth wing compartment, which could indicate that there are different sensitivities to the wound signals along the Anterior-Posterior wing axis, and consequently there is compartmentalization of wound response in *B. anynana* developing wings.

Quantitave analysis of wound induced pigmentation patterns

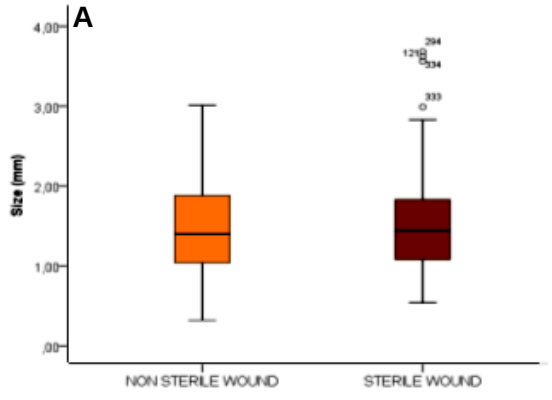
The size of the wound induced patterns was measured. Before the size of the wound induced patterns could be analysed, the normality of the distribution of the size variable was tested using the Kolmogorov-Smirnov test, which tests the null hypothesis of the data has a standard normal distribution. The "diameter" values (see Material and Methods section) were not distributed normally in all the groups (*p-value* <0,05*), but their transformation to "square root of diameter" was (Table 1).

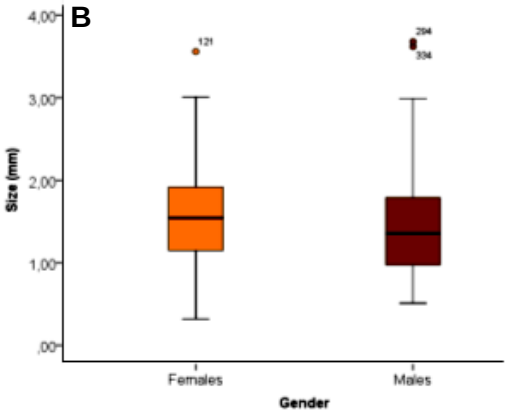
Table1: Test of normality distribution of "diameter" through the Kolmogorov-Smirnov (K-S) test.

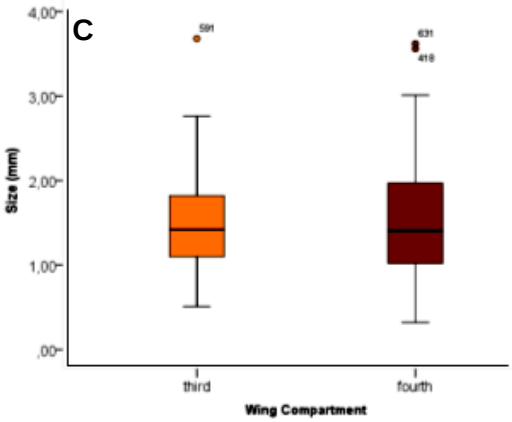
Group	Variable	Class	Tests of Normality Kolmogorov-Smirnov		
			K-S	DF	*p-value*
WOUND	SIZE	Non sterile	0,063	166	0,200
		Sterile	0,063	234	0,025*
	SQRTsize	Non sterile	0,051	166	0,200
		Sterile	0,039	234	0,200
GENDER	SIZE	Female	0,063	188	0,064
		Male	0,066	212	0,024*
	SQRTsize	Female	0,043	188	0,200
		Male	0,055	212	0,200
WING COMPARTMENT	SIZE	Third	0,056	234	0,074
		Fourth	0,073	166	0,029*
	SQRTsize	Third	0,031	234	0,200
		Fourth	0,056	166	0,200

166 and 234 wound induced patterns (of all colour classes defined above) were measured in the NON STERILE and STERILE WOUND groups and the mean of wound-induced patterns "diameter" was 1,47 mm (SE=0,0408) and 1,50 mm (SE=0,0377), respectively. The ANOVA analysis between groups revealed that the size of wound induced patterns of NON STERILE and STERILE WOUND groups did not have significant differences (Figure 12A).

188 and 212 wound induced patterns were measured in females and in males, respectively. The size of female's wound induced patters (1,56 mm; SE=0,0393) were significantly larger than those of males (1,44 mm; SE=0,0389) (F=5,419; p=0,020; the ANOVA test were made with "square root of size") (Figure 12B). These differences were also observed in previous studies [29], but were not statistically significant perhaps because the sample size was smaller. These differences can also be the result of the developmental heterochrony and sexual dimorphism explained above.







D

	Wound		Gender		Wing Compartment	
	Non Sterile	Sterile	Female	Male	Third	Fourth
N	166	234	188	212	234	166
Mean	1,47	1,50	1,55	1,43	1,48	1,51
SD	0,525	0,577	0,538	0,566	0,523	0,600
SE	0,041	0,038	0,039	0,039	0,034	0,047
DF	398		398		398	
F	0,214		5,419		0,173	
p-value	0,644		0,020		0,678	

Figure 12- wound-induced pigmentation patterns size in different groups (NON STERILE and STERILE WOUND (A), genders (B) and wing compartment (C)). The wound-induced pattern size in STERILE WOUND group was not significantly different of the size in NON STERILE WOUND group, so the sterility had no effect in wound-induced pattern size (A). The wound-induced patterns were bigger in females than in males (B), and the wound pattern size was also not different in the third and fourth wing compartment. The number (N), mean of the wound-induced patterns "diameter" and respective standard deviation and error, for each analysed group, are described in table (C). The three last rows are the results of the ANOVA test (for the wound-induced patterns "square root of diameter") with corresponding degrees of freedom (DF), statistic value (*F*) and the significance value (*p-value*).

234 wound induced patterns were measured in the third wing compartment and 166 in the fourth wing compartment, with mean size of 1,48 mm (SE=0,0342) and 1,51 mm (SE=0,0466), respectively. The "square root of the diameter" were not significantly different in third and fourth wing compartment (Figure 12C), but the size mean of the wound induced patterns of the fourth wing compartment were larger than the third. Thus, it seems that when the wing epithelia in the fourth wing compartment can process the wound signals, it has the same "power" to develop a wound induced pattern of the same size (or larger) than the third wing compartment on the same wing. The differences between third and fourth wing compartments appear to be mainly in

the probability of producing different types of response (formation of wound induced patterns *vs* absence of response) than the size of the area around the wound that is affected. Possibly only the severe wounds, which probably induce stronger signals, can induce patterns in the fourth wing epithelia. Other hypothesis is that each wing compartment has the “eyespot large” specification, i.e. the larger compartments develop larger eyespots. The fourth wing compartment is larger (distance between veins along the A-P axis) than the third (see scheme in Figure 5), the same is true for the second and fifth wing compartments and the second wing compartment native eyespots are smaller than those of the fifth. Possible the size of eyespots, which depends of the strength of the signal [57, 73], is also dependent of the wing compartment height, i.e. is dependent of size inhibitory signals from the wing veins. Thus, the smaller wing compartment has smaller eyespots and smaller wound induced patterns, so the fourth wing compartment epithelium is less sensitive to the wound signals, but in the cases when such signals are “processed” the response induces the formation of a wound induced pattern with the same size (or larger) than in the third wing compartment.

4.2- Aim 2: Characterize the cellular dynamics of the developing wing epidermis around eyespots and wound sites

Cellular structure of the young pupal wing

To understand the possible effects of wounding on the wing epidermis we analysed the cellular structure and organization of the undamaged young pupal wing (10, 16, 20 and 40 hours post pupation). The nuclei were localized through staining of DNA with DAPI, the cellular membrane and scales through staining of Actin with Rodanine-Phalloidin, and the Notch protein, which is important for scale forming cells determination [68], through a specific antibody staining (see Material and Methods). In pupal wings 10 and 16 hours post pupation, we observed expression patterns of *Notch* consistent (in four and seven in six and 10 tested wings, respectively; Figure 13A) with its role in the process of lateral inhibition, which underlies specification of which of the epithelial cells will produce scales [68]. The cells that do not express *Notch* are presumably those that will be the scale forming cells. In Figure 13A (white arrows), we can see that these cells have larger nuclei. The pupal wings are a double epithelium

(dorsal and ventral sheets) with an organized cell structure, where the scale forming cells appear in rows [68]. Reed observed the lateral inhibition by *Notch* at 16 hours post pupation in the butterfly *Heliconius erato petiverana* [68], here we observed that at 10 hours after pupation the process already started in *B. anynana* and Notch protein can still be detected in the same pattern at least until 16 hours post pupation. The anti-Notch stainings for 18, 20 and 24 hour old pupal wings were inconclusive because for these development times Notch expression seems to be increased in the whole wing epithelium (data not shown) and this makes it difficult to distinguish individual cells.

Through nuclear staining with DAPI we observed that the area where eyespot organizing centres are localized has more cells with larger nuclei and a different organization compared to the rest of the wing epidermis (Figure 13B). This cell organization is already visible in 10 hour old pupal wings (data not shown), but become more extreme 40 hours after pupation (Figure 13B). This higher density of cells with large nuclei (presumably, scale-forming cells) at the focus is not seen in other part of the wing, and might, thus, be related to the "special" function of foci in morphogen production and diffusion. In adult wings it also seems that the white focus has more cells than the rest of the wing. However we could not appropriately quantify this because the contrast of the focal white scales is weak and it is hard to count them.

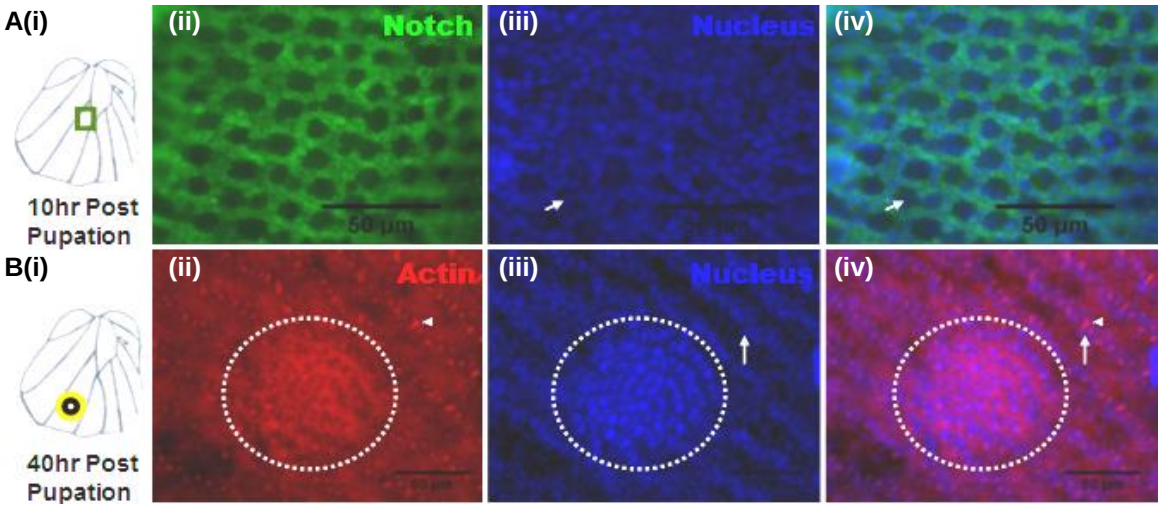


Figure 13- Notch lateral inhibition (A) and cellular structure of a native eyespot (B). In the A and B (i) are the scheme of the wing localization and age of the wings used. Notch lateral inhibition is involved in determining which epithelial cells are the scale forming cells in young pupal wings (A). DAPI staining reveals all nuclei (blue), but only the smaller nuclei are expressing Notch (green) (cells with larger nuclei - arrows). In the developing eyespot focus (white dashed circles) there are more cells with larger nuclei (B), which are differently organized compared to the surrounding epithelium (see iii). In the left image, Actin staining reveals actin accumulation in the focus and in the forming scales (arrow head), which are organized in rows as the larger nucleus cells (arrows). The images on the right (iv) are the merged of (ii) and (iii).

Cellular structure at the wound site

Through nuclei staining with DAPI, we could see an increase of cell number around the wound site 4 hours post wounding (Figure 14A, G and H). This increase could be a result of 1) cell division, 2) cell migration and/or 3) the visible effect of the epithelial distortion (folded back in itself) as a consequence of the mechanical wounding. The first hypothesis could be tested through wing staining with cell division and cell proliferation markers, such as BrdU, Ki67 and PCNA (Proliferating cell nuclear antigen) [78-80]. If there were up-regulation of these markers around the wound sites we could conclude that the wound healing activates the cell proliferation in butterfly pupal wings. The third hypothesis could be tested through a detailed description of the wing structure at the wound site in a confocal microscope, which can provide clues about the 3D epithelium structure at the wound site. To test the second hypothesis one could try to use markers for hemolymph cells, such as crystal cells and plasmatocytes, which are already available for Drosophila systems, and compare their expression in undamaged wings and the in damaged wing around the wound sites. If the hemolymph markers were up-regulated in the wounded wings, particularly around the wound site, and not in the undamaged wings, we could conclude that the wound induced the migration of the hemolymph cells to the wound site.

An actin cable was visible around the wound site 2 hours after wounding (four wings in four tested) (Figure 14B), as has been described in Drosophila wound healing [6, 7]. However, 4 hours after wounding, we could no longer clearly see any Actin cable around the wound (eight wings) because of a high level of fluorescence (Figure 14D and E). This increased level of fluorescent signal could correspond to a real increase of *actin* expression at the wound site, or could be just unspecific biding to molecules deposited at the wound site during the healing process, such as those that form the melanic scab.

The effects of wounds in Notch lateral inhibition were difficult to address because Notch fluorescence signal was too intense at the wound site (eight wings, data not shown). It is difficult to distinguish if the fluorescence intensity is a result of 1) higher expression levels of Notch; 2) a cell concentration effect or 3) unspecific antibody binding. To test the hypothesis of unspecific binding to the healing wound, the affinity of the Notch antibody, DAPI marker and Rodamine-Phalloidin marker to the cuticle and melanic scab was tested through staining of wounded cuticle (i.e. damaged cuticle stripped of the wing, scheme in Figure 14C). Two 16 hour and four 36 hour pupal cuticles (4 and 24 hours post wounding, respectively) were tested and in both time points we observed that part of the wing epithelium was attached to the melanic scab (Figure 14I; the nuclei are visible – white arrows). This indicates that the wing

epithelium could not be completely removed at the wound site. The cuticle opening seemed to have high affinity to anti-Actin Rodamine-Phalloidin marker and to anti-

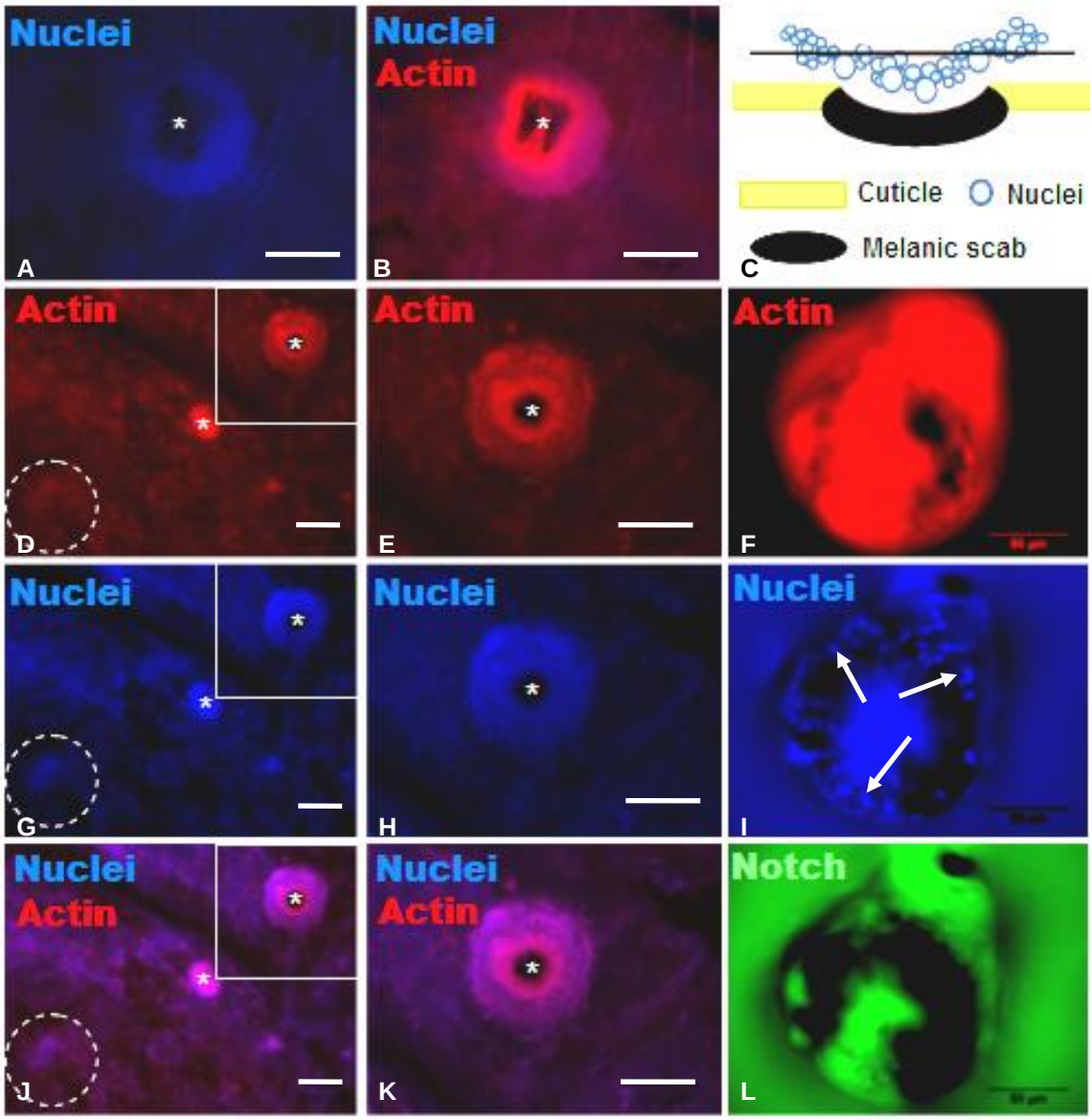


Figure 14- Cellular structure around epidermal wounds on pupal wings. Around the wound, there is an increase in cell number (A,G and H) and the formation of an Actin cable (B), similar to what has been described for other model organisms [6, 7]. In (B) we can see very clearly the Actin cable and the increased cell density around the wound site (2 hours post wounding). 4 hours post pupation the Actin cable is less clear (D and E) and we only can see a large "spot" of Actin being impossible to assess whether it is cellular expression of *actin* or just unspecific biding (the white scale bar correspond to 200µm). To answer this question, a staining to the wounded cuticle was performed as indicates the scheme (C). All markers (Rodamin-Phalloidin, anti-Notch antibody, and DAPI) seem to have high affinity to the wound site. However, any cellular structure was visible in the Actin and Notch staining, but the nucleus of the cells are clearly stained (I) (white arrows), these cells probably migrated towards the cuticle opening as in scheme (C) (the scale bar correspond to 50µm).

Notch antibody (Figure 14F and L). We could detect some cell nuclei at the cuticle wound site, but the Actin and Notch stainings did not revealed any cellular specific structure (cell membrane or nucleus), so we conclude that the fluorescence signal of Actin and Notch corresponds to unspecific binding to the melanic scab in the cuticle.

Cell maturation in later pupal stages

The pupae with sterile treatment but for each the sterility checks 1 and/or 2 were negative were used to investigate the cell maturation process around the wound sites. The developing wings were removed from 69 pupae between 96 and 144 hours post pupation, but only 36 were used to obtain information about the dynamics of pigment production (25 had already completed the pigmentation deposition: females between 114 and 143 hours and males 129 and 144 hours post pupation, and eight did not have any pigmentation on the wings: females and males between 96 and 120 hours post pupation). In total 14 females between 115 and 119 hours post pupation and 22 males between 118 and 137 hours post pupation were analysed. In this exploratory analysis we observed that the yellow pigmented scales matured at the same time (or similar time) in native eyespots and around wound sites (Golden patches and golden ring in Figure 16A and C, respectively). However, the black scales seemed to have different maturation rates in native versus wound induced eyespots. In 22 cases, we could clearly see the black ring in native eyespots but no black pigment around the wound site.

It is difficult to establish the exact physiological developmental time of each pupa because we have different developmental rates for males and females and same gender inter-individual variation. Additionally, it is also difficult to confirm the results obtained because we can never know how the adult pattern would look like for the pupal wings analysed. An example is the wing in Figure 16C, it looks like the adult would have two ectopic eyespots, but we cannot be sure. Still, our results are suggestive of developmental heterogeneity between eyespots and woun induced patterns in the timing of the production of the black pigment. This appears to occur first in native posterior eyespot, and then in the native anterior eyespot (Figure 16C). It seems that only after the black pigmentation is complete in both eyespots the synthesis of this pigment occurs in wound induced patterns (if at all).

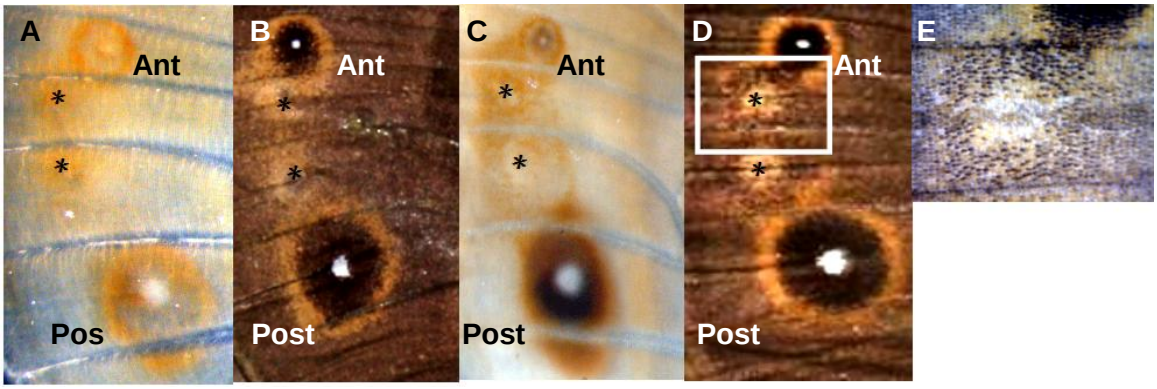


Figure 15- Pigment production around wound sites. Section of distal dorsal forewings showing pigments deposited around wound sites (marked *) and native eyespots (anterior (Ant) and posterior (Post)) in developing wings (A and C) and adult wings (B and D). The yellow pigment of golden patches (A) and the golden ring of damage-induced eyespots (C) and native eyespots seems to be produced at the same time. In contract, the black pigment is not visible around the wound sites, but is clearly visible the full and partial black ring of the Posterior and Anterior eyespot, respectively. (E) is a magnification of the area delimited by a white square in (D) showing the black scales which are harder to see in (D)

4.3 Aim 3: Compare expression of "eyespot" and "wound response" genes around developing native eyespots and around wound sites

Characterization of gene expression around the wound site

We had 27 candidates (Annex 6.3.1) of which *B. anynana* sequence were available for 21. We cloned eight new: *Shaggy*, *Casein-kinase 1*α and *Double-time* from the Wingless signalling network; *c-JNK* and *hemipterous* (*hep*) from the JNK pathway, *hedgehog* (*hh*) from the Hedgehog pathway, *grainyhead* (*grh*) from the ERK/MAPK pathway, and *black cells* (*bc*) from the melanin biosynthesis pathway (Annex 6.3.2). Of 21 for which sequence was then available, we looked at seven: *Dopadecarboxilase* (*Ddc*) and *pale* (*ple*) involved in melanogenesis [43, 81]; *engrailed* (*en*) and *wingless* (*wg*) two genes previously implicated in native eyespot formation [4, 11]; *c-JNK* and *hep* from the JNK pathway; and *grh* from the ERK/MAPK pathway involved in wound healing (see Figure 1). The sequences of the riboprobes as well as of the primer pairs used to amplify them are provided in Annex 6.3.2 and 6.3.3. These genes were tested for pupal wings at of different times post wounding: 4 hours (four replicates), 24 (three replicates), 48 hours (two replicates), 72 hours and 96 hours (one

replicate). However, due to technical problems, such as dissection of intact wings and degradation of wing tissue during the procedures, we only had reasonable wings for the first time point (where the wing was attached to the cuticle), so the results below are only for this time point.

From the seven tested only two genes (*ple* and *en*) were found to be expressed at the wound site (Figure 17H and K). For the other genes we did not find any differential expression at the wound site compared to the rest of the wing (e.g. *grh* and *c-JNK*; Figure 17B and E, respectively)). Our results show that 4 hours after wounding (16 hours post pupation) *ple* is clearly over-expressed around the wound sites, but not at the wound edges (injured cells) (Figure 73I) and *en* is expressed in the whole wing and over-expressed in the wound edges. *ple* and *en* did not have any differential expression in the native eyespots. The absence of *en* over-expression in the foci and in the golden ring of the native eyespots was unexpected because it was well documented that the Engrailed protein localizes to these locations at around the same developmental stages [4, 11]. Even if it is conceivable that local wounding can induce changes in gene expression even in relatively distant wing areas, we think that our lack of detecting en mRNA around native eyespots indicates that the anti-engrailed riboprobe we used was not in good conditions. This is also supported by the fact that the undamaged control wing of the same individual pupae also did not show signs of en expression associated to the native eyespots.

The up-regulation of *ple* around the wound site a few hours post wounding indicates that the ERK/MAPK and/or the melanin biosynthesis pathways could be involved in wound response in *B. anynana*. The activation of the melanin biosynthesis pathway upon wounding is largely described in insects [13, 38, 45, 82, 83], and this pathway should be activated immediately after wounding to form the melanic scab. The melanic scab is visible 2 hours post wounding in Drosophila [38], and in *B. anynana* it is clearly visible 4 hours post wounding, so we assume that the up-regulation of pale 4 hours post wounding is not a consequence melanin biosynthesis pathway activation, at least to form the melanic scab. However, we can not exclude the possibility that this pathway is up-regulated at this time point to control the infection through encapsulation and nodulation [84-86], and consequently it is up-regulating *ple.*

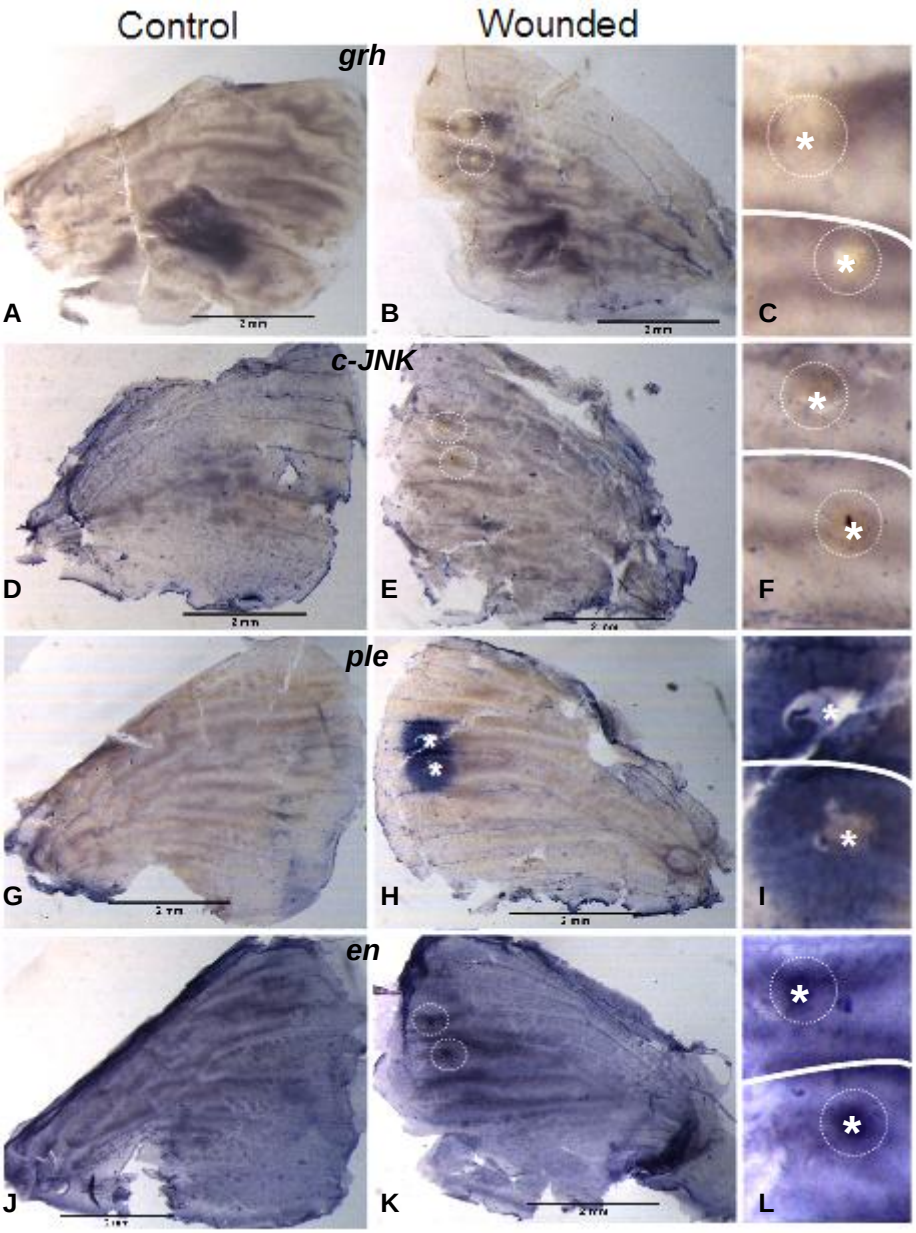


Figure 16- Gene expression around the wound sites. *grh* (A-C) and *c-JNK* (D-F) are not up regulated, but *pale* (*ple*) (G-I) is up-regulated at the wound site 4 hours post wounding. The *engrailed* (*en*) (J-L) seems to be up-regulated at the wound edges, but this could not be confirmed (see details in the text). From the left to the right the images refer to the CTR wing, wounded wing and magnification of the wound sites and the white line is marking the wing vein. The size bars correspond to 2mm. in the third column of images are the amplified wound areas of the central column.

Wang [3] described that, for Drosophila, the wound induces the formation of an actin cable through *stitcher* (*stit*) kinase, which also activates the ERK pathway. This pathway induces the activation of *grh* and this transcription factor activates the *Ddc* and *ple* genes and also up-regulates, in a positive feedback, the *stit* kinase (Figure 18). However, in our work we did not observe the *Ddc* and *grh* expression 4 hours post wounding (Figure17B, C, E and F). Probably, *grh* activates *ple*, as in Drosophila [3] (Figure 18), and *ple* activates *Ddc*, so the *grh* expression should be detected before the time point tested (4 hours post wounding) and *Ddc* after *ple* expression. This signaling pathway also mediates the regulation of cell proliferation, differentiation and survival [36, 37]. Thus, the increase in cell number that we saw around wound sites (Figure 14) could be mediated by ERK/MAPK. Figure 18 summarizes a model of a wound response cascade in *B. anynana*: this cascade is initiated by wounding that leads to the formation of an actin cable, cell proliferation and activation of *Ddc* and *ple*. Further studies including the genes involved in this pathway and targeting closer time points after wounding are necessary to a better understanding of the role and the dynamics of the ERK/MAPK pathway in wound healing process.

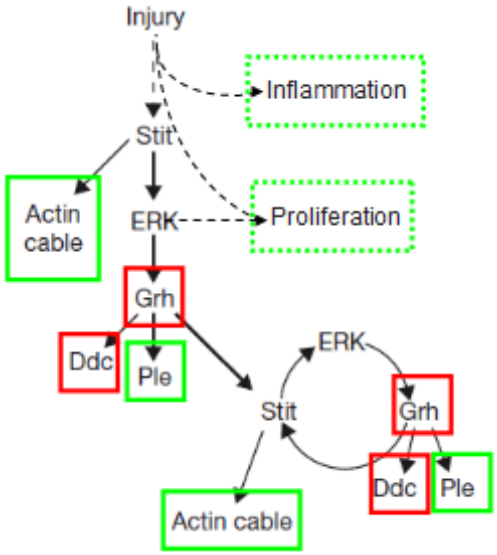


Figure 17- The wound induces the activation of the ERK pathway. The wound leads to the actin cable formation, inflammation and/or proliferation and activation of *Ddc* and *ple* genes. The activation of the last two is mediated by the ERK pathway, which is also involved in activation of proliferation. In this work were detected the actin cable and the *ple* expression (green boxes), it were also observed the increase of the cell number around the wound site, which could be consequence of inflammation and/or cell proliferation (green dashed boxes). The expression of *grh* and *Ddc* should be also increased, but in this work this were not observed (red boxes). Adapted from [3]

5- Conclusions and Perspectives

With this project we could conclude that wound induced pattern formation in *B. anynana* wings does not depend on infection, which typically leads to an increase in activation of pathways associated with immunity. We observed the same number and size of wound induced patterns in response to sterile and non-sterile wounds. This suggests that the commonalities between wound response and pigmentation pattern formation are mainly related with tissue repair mechanisms. However, it is possible that immunity pathways are activated also in sterile wounds, even if at lower levels relative to when infection does occur [46, 75]. To clarify this question the expression levels of genes related with 'pathogen response' should be quantified in sterile and non sterile wounded wings.

Females are more sensitive to the wound signal and produce more and larger wound induced patterns than males. This could be the result of the developmental heterochrony and sexual dimorphism between both genders. Brakefield and French [29] surveyed the effects of wounds for different developmental times and wing locations. They report on the ideal times and locations for induction of ectopic eyespots, but do not make a distinction between males and females. Wounds inflicted on pupal wings 12 hours post pupation wounds were the more likely to generate ectopic eyespots, followed by wounds inflicted at 18 hours post pupation [29]. An extensive study of wound induced eyespots between 10 and 18 hours after pupation (maybe for each two hour intervals) in males and females could help unravel heterochony between them and help explain our result of gender differences in response to wounding. The differences in size between genders were also detected by Brakefield and French [29], and they eliminated the possible sexual dimorphism effect making a recalculation of wound induced pattern size, which became a function of the size of the respective anterior native eyespot. To understand if the results obtained are just the effect of the sexual dimorphism, the anterior native eyespots should be measured and the ectopic patterns size should be recalculated as described by Brakefield and French [29].

The wounds made on the third wing compartment induced more ectopic patterns than those made on the fourth wing compartment. This could be indicating that there is compartmentalization of the properties of wound response along the Anterior-Posterior wing axis in *B. anynana* developing wings. The study of the wound epidermal response in different genetic backgrounds with different epidermal properties, such as the selected lines where the native eyespots are bigger (AP) or smaller (ap) than the

wild type [87], could give new insights in the compartmentalization of wound response and the wing epithelium.

In *B. anynana* the scale forming cells are determined through Notch lateral inhibition, as in *Heliconius erato petiverana* [68]. This process was detected in pupae with 10 and 16 hours post pupation, but the time window of Notch lateral inhibition could be larger. Therefore, to know the extension in time of this process, more test for closer time points in pupae younger than 10 hours and older than 16 are necessary.

In the presumptive eyespot foci we detected a higher concentration of presumable scale forming cells, which have a different organization from the rest of the wing epithelium. This specific structure could be essential for the eyespot-organizing properties of this area. It is known that focal wounds in early pupal wings (one to six hours post pupation) lead to reduction or even absence of adult eyespots, and in pupal wings with 12 hours post pupation leads to an enlargement of the eyespot [29]. It would be interesting check if this specific cell organization is maintained or not after early and 12 hours post pupation focal wounding.

The observed increase in cell number around the wound site is characteristic of the native eyespot foci (Figure 18) and also of the wound healing process (Figure 14), which could indicate a possible inflammatory response at the wound site [16, 88]. However, we were not able to distinguish whether the increase of cell number was due to cellular proliferation or migration, but it is clear that the wound induced an increase in cell number. According to the observed results we construct a scheme that represents the cell organization at the eyespot focus and the wound site areas (Figure 15).

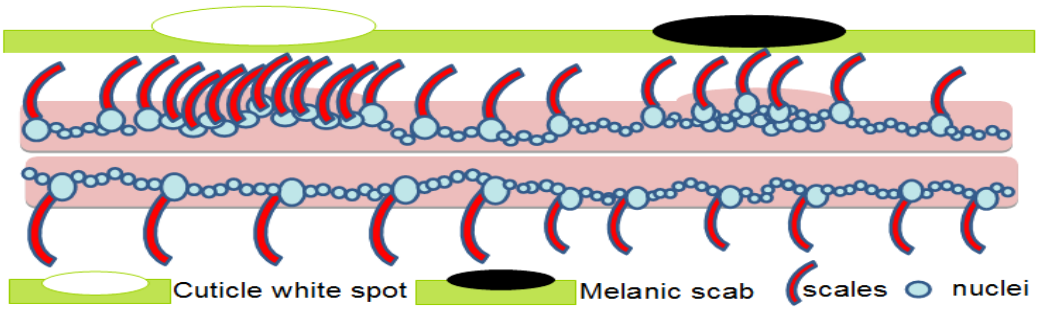


Figure 18- Scheme of wing epithelium with a focus and a wound site area, underlying the cuticle which has corresponding visible land marks (white spot at focal area (see Figure 5) and melanic scab at the wound site). The areas of the eyespot focus and around the wound site both have higher cell density, but can differ. The cells in the eyespot focus seem to be differentiated in scale forming cells, so it is expected a higher number of the scales at the adult eyespot focus. The cells at the wound site do not seem to be differentiated, at least in the studied time points, so a higher scale number is not expected around the wound site in adult wings.

Wing stainings with cell proliferation and hemolymph makers should be performed to clarify this. The increase of cell number around the wound sites could potentially be sufficient to mimicry the signals of the eyespot foci. The new cell (cell division) or the recruited cells (cell migration) around the wound site could transmit to the surrounding cells the same (or similar) as an eyespot focus. To test this hypothesis it will be necessary a mutant with reduced cell migration and cell division ability, but so far this mutant does not exist. However, we can test if an existing lab population with reduced dorsal eyespots [87] is less sensitive to the wound signals. Through grafting experiments Beldade and colleagues [74] already tested the epithelial threshold sensitivity of these selected line to the focal signals, and verified that it is reduced, so the sensitivity to the wound signal is possible also reduced in this lab population. In this case the cells around the wound site could be counted, and if there were significant differences comparing with the wild type situation, this could be indicating that the amount the cells at the wound site is an important factor to the formation of the wound induced patterns.

In late pupal wings we observed that the golden pigment is synthesized at the same time (or very similar) in the native and in the wound induced patterns. However, the black pigment was never observed around wound sites until the wing background (typically the last to be coloured [64, 65]) was almost completed coloured. Because only 36 pupae were analyzed, a study with more individuals and more time points should be performed, and include also other lab populations with altered pigmentation [12]. In particular, in lines where native eyespots have relatively higher black-to-gold ratios, the probability of obtaining wound induced eyespot with a larger black ring are higher [12] and so should be the chances of detecting the deposition of the black pigment.

The *ple* is up-regulated around the wound site 4 hours post wounding, which could indicate the activation of the ERK/MAPK pathway during the healing process. This signaling pathway is involved in transmission of signals that regulate the transcription of surface barrier components [1] cell proliferation, differentiation and survival [36, 37]. The up-regulation of *ple* could be involved in potential cell proliferation around the wound site which would be consistent with increased cell densities observed for those locations. The detection of up-regulation of cell proliferation markers is needed to confirm this.

To sum up, with this project we conclude that:

- the wound-induced pattern formation does not depend of infection process;
- the females are more sensitive to the wound signals than males;

- the wound response in the two wing compartments analyzed were different, which indicates the compartmentalization of the wing epithelium to the wound signals;
- 10 hours post pupation the differentiation of scale forming cells has already started;
- the eyespot foci have a different cell organization;
- the wound induces a local increase in cell number;
- the black pigment of the wound induced patterns could be synthesized after the black pigment of the native eyespots;
- *ple* is up-regulated around the wound site 4 hours post wounding and ERK/MAPK pathway could be activating cell proliferation at the wound site.

6- Annexes

6.1- Pupal cuticle sterility test

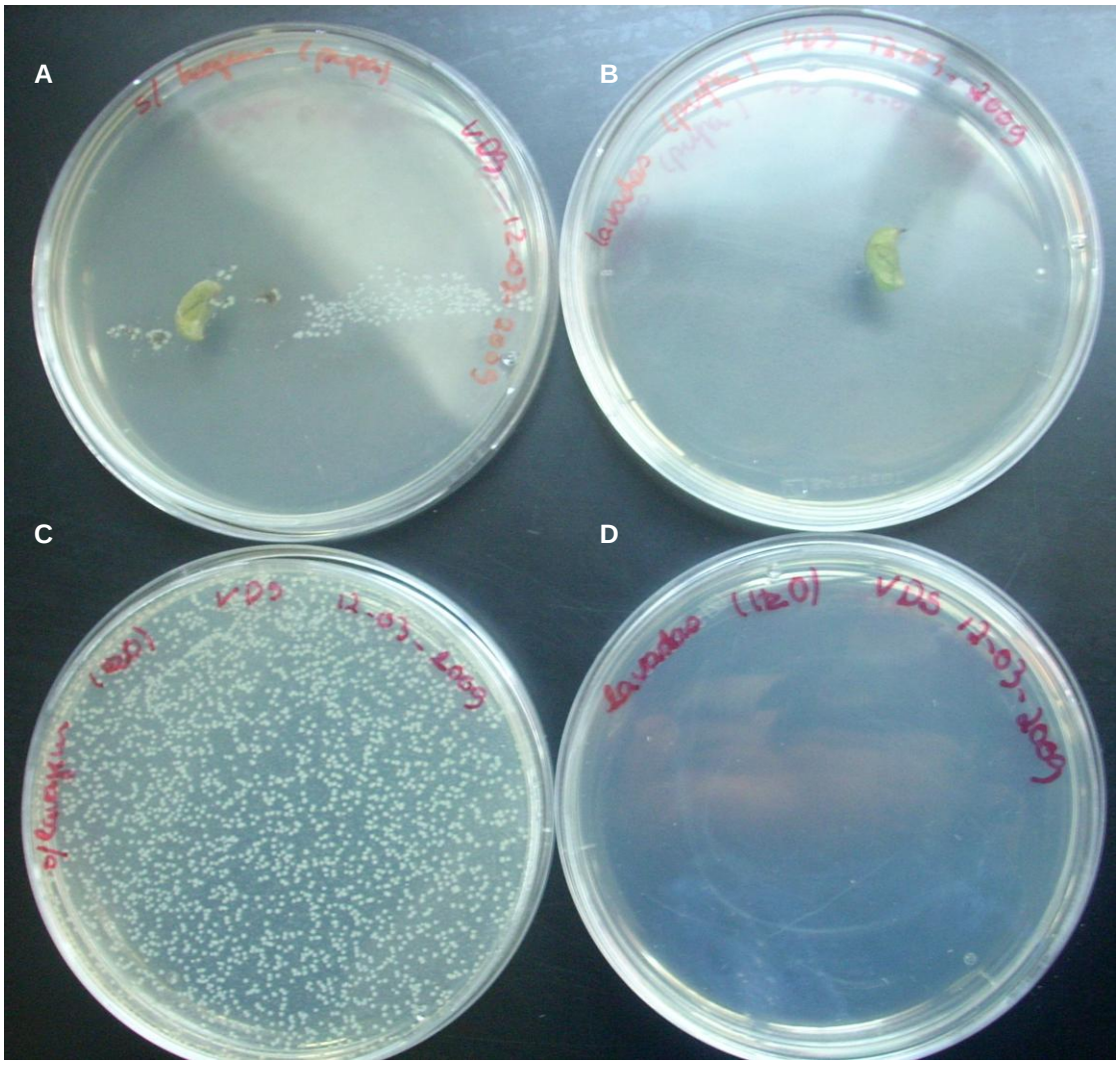


Figure A1- The pupal cuticle is a contaminated surface. In A and C is shown the grown bacteria (LB medium) from the NON STERILE pupa and in B and D from the STERILE pupa. The sterilization was achieved through washing the pupae for a few seconds in 70% ethanol solution followed with a washing in sterile water; this double washing was repeated three times. To verify the presence and the surviving microorganisms on the NO STERILE and the STERILE pupal cuticle, the pupae were immersed into an eppendorf with sterile water for a few seconds and were then put in a sterile Petri dish with LB medium (A and B). The eppendorfs were centrifuged and the sediment particles were plated in LB medium (C and D). After an overnight at 37ºC the plates were checked for presence of microorganisms, and the NON STERILE pupae plates had microorganisms (A and C) and the STERILE pupae plates did not have microorganisms. This test was done with three NON STERILE and STERILE pupae and the results were the same for the three replicates.

6.2 Protocols

6.2.2- DAPI and Rhodamin Phalloidin staining

1- Dissect larval or pupal wing discs in ice-cold PBS (1x) in a Petri dish filled with Sylgard. Perform all steps on ice.
2- Fix the discs for 30 – 35 min in FB.
3- Wash 4 x 5 min with PBS 1x.
4- Incubate 2 hours (or longer) in Rodamine Phalloidine (dilution 1:100)
5- Wash 4 x 5 min with PBS 1x.
6- Incubate 5min in DAPI (dilution 1:1000)
7- Wash 2 x 5 min with PBS 1x.
8- Incubate in glycerol in PBS (50%) for 30min
9- Incubate in glycerol in PBS (80%) for 1h. Mount discs in 100% glycerol. Seal cover slips with nail polish.

<u>Stock Solutions</u>

→PBS 10x, 1 liter: 10.68 g K_2HPO_4
5.28 g KH_2PO_4
81.8 g NaCl
Bring up volume to 1L, autoclave to sterilize. After diluting to 1x, adjust pH to 7.0 with 1N NaOH.

→PIPES (MW = 302.37) pH 6.9, 0.5M
Dissolve 4.53 gram PIPES in milliQ, bring up volume to 20 ml. Adjust pH to 6.9 with 4N NaOH, then bring final volume to 30 ml.

→EGTA (MW = 380.4) pH 6.9, 0.5M
Dissolve 1.902 gram EDTA in a very small volume of milliQ, bring volume up to 10 ml with NaOH 5N, adjust pH to 6.9.

→$MgSO_4$ (MW = 246.48), 1 M
Sterilize by autoclaving.

→Tris (MW = 121.14) pH 6.8, 1 M
Sterilize by autoclaving.

→NaCl (MW = 58.44), 5 M
Sterilize by autoclaving.

→IGEPAL CA630, 20%
Add 20 ml of 100% IGEPAL to 80 ml milliQ. Store at RT. May develop sediment on storage; a clear liquid can be obtained on heating to 40^0C.

→Mounting medium
80% glycerol in PBS

Buffers

Make buffers fresh every 2-3 weeks and store at 4^0C.

→Fix buffer (FB)	0.1 M PIPES	For 50 ml FB: 10 ml 0.5M stock
	1 mM EGTA	0.1 ml 0.5M stock
	1.0% Triton x-100	2.5 ml 20% solution
	2 mM $MgSO_4$	0.1 ml 1M stock

Add 1 part of 37% formaldehyde to 3 parts of FB just prior to the addition of the wing discs.

→Block buffer (BB)	50mM Tris	For 50 ml BB: 2.5 ml 1M stock
	150 mM NaCl	1.5 ml 5M stock
	0.5% IGEPAL	1.25 ml 20% solution
	5 mg/ml BSA	0.25 g

→Wash buffer (WB)	50 mM Tris	For 200 ml WB: 10 ml 1M stock
	150 mM NaCl	6 ml 5M stock
	0.5% IGEPAL	5 ml 20% solution
	1 mg/ml BSA	0.2 g

6.2.3 - Antibody staining of butterfly wing discs

Day 1

1- Dissect larval or pupal wing discs in ice-cold PBS (1x) in a Petri dish filled with Sylgard. Perform all steps **on ice**.

2- Fix the discs for 30 – 35 min in FB.

3- Wash 4 x 5 min with PBS 1x.

4- Incubate 1 – 2 hours (or longer) in BB.

5- Incubate with primary antibody diluted in WB for 18 – 24 hours (in the fridge).

Day 2

6- Wash 4 x 15 min in WB.

7- Incubate with secondary antibody (1:200) and Rhodanine Phalloidin (1:200) (Invitrogen) diluted in WB for 1 – 2 hours (or overnight). Keep in the dark because of the fluorescense.

8- Wash 4 x 15 min in WB.

9- Incubate with DAPI solution (1:1000) for 5 min.

10- Wash 3 x 5 min in WB.

11- Incubate in glycerol in PBS (50%) for 30 min.

12- Incubate in glycerol in PBS (80%) at least for 1 hour.

13- Mount discs in 100% glycerol. Seal cover slips with nail polish.

14- The slides can be kept at -20^{0}C in the dark for several months.

Stock solutions

→PBS 10x, 1 liter

10.68 g K_2HPO_4

5.28 g KH_2PO_4

81.8 g NaCl

Bring up volume to 1 liter, autoclave to sterilize. After diluting to 1x, adjust pH to 7.0 with 1N NaOH.

→Fix buffer (FB)

0.1 M PIPES (pH6.9)

1 mM EGTA (pH6.9)

1.0% Triton x-100

2 mM $MgSO_4$

Add 1 part of 37% formaldehyde to 3 parts of FB just prior to the addition of the wing discs.

→Block buffer (BB)
50mM Tris (pH6.8)
150 mM NaCl
0.5% IGEPAL
5 mg/ml BSA

→Wash buffer (WB)
50 mM Tris
150 mM NaCl
0.5% IGEPAL
1 mg/ml BSA

→Mounting medium
80% glycerol in PBS

6.2.3-In-situ hybridization protocol for *Bicyclus anynana* late pupal wings

Everything should be done in RNase-free conditions and on ice until the end of hybridization.

R: Rinse (quick wash); W: tissue washing, 5' (or longer)

Day 1

1- Time your pupae
2- Treat your baskets + 24-well plates with 4-5% H_2O_2 to destroy RNAses
3- Put on ice 50mL Falcon tubes of PBS, PBT (PBS + 0.1 % Tween 20), PBT +2mg/mL Glycine, freshly prepared 9% formaldehyde in PBT.
4- Pin the pupa laterally, submerge with cold PBS. Cut the wings with fine scissors and put it in a basket with PBS.
5- Fix all the wings at the same time with Fix Buffer (9% Formaldehyde in PBS) for 1hour
6- Stop the fixation with RRW_5W in PBS
7- W_5 W_5 W_5 with PBT
8- Incubate 2' on Proteinase K solution (2.5µg/mL Proteinase K in PBT)
9- Immediately RR with Digestion Stop Buffer
10- W_5 W_5 with PBT
11- Post fix 20' with 50% of 9% Formaldehyde : 50% of PBT
12- $RW_{10}W_5$ in PBT
13- W_5 in PBT:preHB
14- Warm a heating block at 80°C
15- Transfer in preHB and put at 55°C (for short probes like ~300bp) at least 1 hour, pre-warm at the same time one serial of wells with 0.95 mL HB
16- Thaw your probe aliquots
17- Add 1 µL (or less - 20-50ng needed per well) of probe to the 49uLof HB and denature 5' at 80°C
18- Add 50 µL of probe in HB wells
19- Homogenize and transfer the baskets in these wells and incubate 48h at 55°C

Day 3 (Post-Hybridization)

20- Pre-warm the serials of wells with preHB that will be used for post-hyb washes
21- 6x W_{30} in 55°C preHB
22- Transfer in 55°C preHB, let cool down at room temperature
23- W_5 in PBT:preHB
24- W_5 W_5 in PBT

25- W_{60} in Block Buffer

26- Make 1:2000 dilution of anti-Dig antibody (Roche, stored at 4°C in a metallic box in the chem. lab) in PBT. Take the tube with antibody out of the fridge, spin for 5 min, pipet the necessary amount of the antibody carefully from the surface into the tube with PBT

27- Incubate your wings in this solution overnight at 4°C

Day 4 (Staining)

28- R and 10x W_{15} in PBT

29- freshly prepare the following solutions, thoroughly vortex between addition of each salt stock solution, protect the NBT/BCIP from light with aluminium sheet

30- R W_5 W_5 in Staining Buffer without $MgCl_2$ to get rid of PBS (precipitates with Mg^{2+}) so fill well the wells

31- W_5 in Staining Buffer with $MgCl_2$

32- Transfer your samples to very clean glass dishes with staining buffer

33- Replace with staining solution (50µL of NBT/BCIP stock solution for 5mL of staining buffer)

34- Protect the staining reaction from light except when you monitor it, with full-power light and a white background

35- W_5 W_5 W_5 W in H_2O at pH=7 or PBT + 50mM EDTA

In-situ hybridization solutions

Everything must be RNAse free!

Use stock solutions in Falcon tubes (50 and 12 mL) that you can store at 4°C.

→10X PBS (pH = 7) 1L Autoclave and store at 4°C. After diluting to 1x, adjust pH.

81.8g NaCl

10.68 g K_2HPO_4

5.28g KH_2PO_4

→PBT (200mL)

200mL of PBS

200uL of Tween 20 (Store your Tween 20 protected from light in a 12mL vial at the concentration of 20% (add 250 µL of it in 50mL PBS to make 50mL PBT vials))

→Fix Buffer (9% Formaldehyde in PBS) (100mL)

24,3mL of 37% Formaldehyde

75,7mL of PBS

→Digestion Stop Buffer (10 mL)

20mg of glycine

fill ful to 10mL with PBT

→preHB (200 mL)

50 mL H_2O (milliQ, autoclaved)

100 mL Formamide

20 µL Tween 20

50 mL 20 x SSC (Adjust pH to 5,5 with HCl)

SSC 20x stock 1L

175g NaCl

88.2 g Na3-citrate

add H_2O up to 450 ml, adjust pH to 7.0 with 1M HCl and add H_2O to 1L

→HB (Hybridization Buffer) 50mL

50mL preHB

250 µL of heat denatured (5' at 80°) yeast tRNA (20mg/mL)

100 µL of Digestion Stop Buffer

→Block Buffer (1mL)

0,95mL of PBT

50uL of 10x Roche Blocking

→Staining Buffer (50mL)

49 mL Tris-HCl 100mM (pH=9.5)

1 mL NaCl 5M (29.22 g in 100 mL H_2O, autoclave)

250µL Tween 20 (20% conc.)

→Staining Buffer with $MgCl_2$ (1mL)

0,95mL of staining buffer

50uL 1M MgCl2 (20.33g MgCl2-6H2O + H_2O (milliQ, autoclaved) 100mL and autoclave)

6.3- Target genes

6.3.1- Initial list of target genes

Table A1- Initial list of target genes

Pathway	Target genes
JNK	*bsk* (*basket*), *puc* (*puckered*), *hep* (*hemipterous*)
ERK/MAPK	*grh*, *ple* (*pale*), *Ddc*
Wg	*wg, Sgg* (*Shaggy*), *CkIIα* (*Casein kinase II α subunit*), *fz* (*frizzled*), *gro* (*groucho*), *Roh1, DBT* (*Double-time*)
TGF-β	*sal, dpp, Smad*
Hh	*Hh, patch, ci, en*
Notch	*N, Ser* (*Serrate*), *Dl* (*Delta*)
Melanogenesis	*pale*, *Ddc*, *Ebony*, *Bc* (*black cells*)

6.3.2- List of primers used to amplify the target gene sequences

Table A2- List of primers used to amplify the target gene sequences

Gene	Oligo name	Oligo sequence (5' to 3')
Shaggy	Sgg.F1	TGAGCTATGCGGACATGAAG
	Sgg.R5	GAACCAAAGTCGCACAGCTT
Black cells	b.choco.F1	GCAGCGCAGTATTTGTTTCA
	b.choco.R1	CAGCACATGGCTCTTTTCAA
c-JNK	c-JK.F1	ATACTGGGCATGGGCTACAC
	c-JK.R1	ATCAGCTGCTTCCATTGGTC
Hemipterous	Hep.F1	GACTGCAAGTACATCGTGCAA
	Hep.R1	GGCCCTGATGTCGTAGTCC
Dopa-decarboxilase	Ddc.c3202.F1	GACAAGTGGTGCCATCAGTG
	Ddc.c3202.R2	GAAGGCTGAACCAGCGTAAG
Engrailed	En.PTF1	AGCAGAAGAGGCCAGATTCA
	En.PTR1	TCCTCTGACCTGATGCCTTC
Pale	ple.c5608.F1	TCCTCGGACACATTCCTCTC
	ple.c5608.R1	GCACACAAGTCCCACGTCTA
Double-time	Dbt.F1	TCCCTCAAGACTGTGCTCCT
	Dbt.R1	ACTCGATCGGGTGATTTTTG

Casein-kinase 1α	Ck1a.F1	AAACTTCCTCATGGGCA
	Ck1a.R1	TTGCCTCAGCATGGTCC
Hedgehog	Hh.F1	TCACTATAGGGCAAGCAGTGG
	Hh.R4	ACTGATGGCGAGCGTGTT
Grainy head	grh.F1	TTTTATGGCATCACGTTGGA
	grh.R1	CTTGTCGCAGAACACCTTGA

6.3.3- Target gene sequences

The sequences are in the positive sense (5' to 3'). The hedgehog sequence was submitted to GeneBank, and the accession number is HQ020407.

Shaggy

GAACCAAAGTCGCACAGCTTCAGTACCCCCGTCTTGGGGTCCAGCAACAGGTTCTGAGGC
TTGATGTCCCGGTGACAGATGCCCAGCGAGTGGATATACGCCAGGCTTCTGAACAACTGG
TACATGTACAGCTTTATAAAACTAATTGGTATGGTTTGTTCATCTTTGGAGTAGTGACGCGC
AACTTTATACACAGTCTCTGGTATATACTCCAGCACCAAATTCAGGTACACTTCGTCTTTCTT
TTCTCCGCTTGAGTAAAAGAAGTATTTCAATTTGACAATATTACAGTGCTCCAGCCGTCGCA
TGATTTGCAGTTCTCGATTCTTAAACCTTTTGTCCTGGAGCACTTTCTTGATGGCAATGAGC
TCGCCGGTGTCGCATAGTTTGGCCTGGTAGACGACGCCGAAGCTGCCGTTGCCGATGAG
CTTCATGTCCGCATAGCTCAA

Black cells

CAGCACATGGCTCTTTTCAAGAACAGGATGGAAAGTGAACGCTAAACGAAAGAAGTTCTTG
CGTTCTCGAAGCGGAGAATACGCTACCATTGCTTGAGATCTCAGTGTCAACTGTTCCTTGA
TCTTTGGGGTGATCTTATGCATAGTGTTCCACCATTCCTCATTCTCCATTTTGTTCTTCATAA
ACGTTGGAATGTACCAGAAGCATACATTAGGGCACTGCATATGCTTTGTCACCAGTCTAAA
GTTACTTCTCTGAGCAACAGTCTGTATACAAAACTCTGCTATTTCCATTACGTGGTCTGTGA
GGTGATTGAGGCCAATGTCCCCACGAGCTTTCCAAATCATCCACAGTTTGAACGCGTCGAT
CTTTCTTCCACACTGGATACTCTTGTCACCAGTGTCATAACTGACGTCATAAAACTTATCCT
GTTGAAACAAATACTGCGCTG

c-JNK

ATACTGGGCATGGGCTACACCGAGAACGTGGACATCTGGTCGGTGGGCTGCATCATGGG
CGAGATGATCCGCGGCGGCGTGCTGTTCCCCGGCACCGACCACATCGACCAGTGGAACA
AGATTATCGAGCAACTGGGCACGCCGTCGGCGGCGTTCATGGCGCGCCTGCAGCCCACG
GTGCGCAACTACGTGGAGAACCGGCCGCGCTACGCCGGCTACAGCTTCGAGCGCCTGTT
CCCCGACATCCTGTTCCCGTCCGACTCCAGCGAGCACAACCGGCTCAAGGCGTCGCAGG
CGCGCGACCTGCTCTCGCGCATGCTGGTCATCGACCCCGAGCGCCGCATCTCCGTGGAC
GAGGCGCTGCTGCACCCCTACATCAACGTGTGGTACGACGAGGGCGAGGTCAACGCGCC
CGCGCCCGCCGCCTACGACCACTCGGTGGACGAGCGCGAGCACACCGTGGACCAATGGA
AGCAGCTGATA

Hemipterous

ACTGCAAGTACATTCGTGCAATGCCTCGGCTGCTTCGTGACGGACGCCGACGTGTGGATC
TGCATGGAGCTGATGGCCTCGTGCTTCGACAAGCTGCTCAAGAGGCTCGGCGAGCCCATA
CCCGAGGCTATACTGGGGAAAGTTACTGTGGCGACGGTGAACGCGCCGTCGTACCTGAA

GGACACCCACGGCGTGATCCACCGCGACGTGAAGCCGAGCAACATCCTGCTGGACGAGC
GCGGCAACGTCAAGCTCTGCAATTTCGGAATAGGGGCCCCCTTGTTAATCTCAAGGCAAAA
AGCCGCACAGCCGGCTGCTGCGCGGCCTACATGGCGCCGGAGCGGATAGACCCCCCGG
ACCCCACGCGGCCGGACTACGACATCAGGGCCAA

Dopa-decarboxilase

GACAAGTGGTGCCATTAGTGAAGCCTGGCTACCTGCGTCCACTGGTGCCGGAGCAGGCG
CCTGAGAAGCCCGAACCCTGGACGGCTGTCATGGATGACATCGAGCGCGTGATCATGTCC
GGGGTCACACACTGGCACTCCCCGCGCTTCCACGCTTACTTCCCTACCGCTAACTCGTAC
CCATCCATCGTAGCTGATATGCTTAGCGGAGCTATTGCGTGCATCGGATTTACTTGGATTG
CTAGTCCCGCTTGCATTGAACTGGAAGTAGTGGTGTTGGATTGGCTGGGTCAGATGATTG
GACTCCCCGAAGAGTTCCTAGCGCGATCTGGCGGCGAAGCAGGCGGCGTGATCCAAGGC
ACGGCCAGTGAAGCTACTCTGGTCGCGCTTCTTGGAGCGAAGTCCCGTACCATGCATAGA
TTGAAGGAGCAACACCCTGAATGGACAGAAGTTGAAATTCTTTCCAAACTTGTGGGATATT
GTAACAAGCAAGCCCATTCGTCTGTAGAGCGCGCTGGTCTTCTGGGTGGAGTGAAACTTC
GCAGTTTAGCGCCCGACAATAAGCGACGTCTACGTGGGGACACCCTAAAGGAGGCTATAG
AAGAGGATACTCGTAATGGACTTATACCTTTTTATGTCGTGGCGACACTAGGAACGACT

Engrailed

AGCAGAAGAGGCCAGATTCAGCCAGCTCTATTGTCTCTTCCACATCTAGCGGGGCTTTATC
GACGTGTGGCAGCACTGACGCCAACAGCAGTCAAGGCGGGAACAGCAATCTATGGCCTG
CATGGGTGTATTGTACGAGTTACAGCGATAGGCCTAGTTCTGGTCCAAGAAGTAGAAGAGT
TAAGAATAAGTCGGTCCCAGAAAAAGAAGAGACCCAGAACAGCGTTCAGTGCGGCGCAAC
TTTGCTTCGACTAAAGCACGAGTTTCGCAGAGAACCCGCTACCTCACAGAAGAGAAGGAG
ACAGACCCTCGCAGCAGAGCTTAGGGCTGGCGGAAGCCCAAGATCAAGATCTGGTTCCAG
AACAAGCGCGCCAAGATCAAGAAGGCATCAGGTCAGAGGA

Pale

GCACACAAGTCCCACGTCTACACGACACTCTTACACCTACAATATCACTAGCTATACAAGG
GTCGCAGCGCACTGGGCGCAGCGGGTTCACTCGAACTGCGAGCCCCGCAGCTTGTTGAC
GGCGTTGGTGAGATGCAGCATCTCCGTGTTGAGCTGCCAGATGAGTGTCTCGAGCTTCTC
CACCGAATCCAGCACCTCCACGCGCTCTGTGTGCGGGTTGAAGCGGACCTCGAAGGGTC
GCGACATGGGCCGCCACCCCACGGCCTGACTAATTTTTGGGGCTTTCAGACTTTTCACCC
ACGTAGTAAATTGATTGGTTGGTACTCTTGGTCCTGGTAGGGTTGGAGGGACGTGGAGGC
GGGCTCGAACGGGCGCAGCTCGGGCTTGTCGCTGAGGGCGTGCAGGAGTTCTCCGATGG
AGGACAGCAGCGCCGCGCCGTACGCCTTCAACTGTTGGTTCTCTTTGCACAGACCGAATT
CGACAGTGAACCAGTACACCGTGCACAGTTTTTCAATTTCGGAGTCTGAAGCACCAAGAGA
TGCAAGACCAATTTCTTGTGAGAACTGAGCGAAGCTTGGGTCGGCCAGGAGAGGAATGTG
TCCGAGGAAAT

Double-Time

ACTCGATCGGGTGATTTTTGCACAGCTCGTCAAACGGTGTGGACAGTTTCTTCTCCGATAT
TCTCTCGTACTTCTGCCGTTTGGTGGCCGCCTTCAACCCCTGCCACGGCAGGCTGCCGCG
GTTGAAGTACATGAGCACGTAGCCCAGCGACTCCAGGTCGTCGCGCCGGGACTGCTCTAT
GCCTAAGTGAGTGTTGATTGATGCATACCTTGCTGTGCCAGTCAGATTTTTGTTTTCTCTGT
ATGGGATATGTTGATTGGTGCGTCCATCTTTGTACTTCTTGGCTAGTCCAAAATCTATAATG
TATACTAAGTTTCCCCTTTTGCCGAGGCCCATTAAAAAGTTGTCTGGCTTAATATCTCTATG
AATGAAGTTCTTATAGTGTATGTGCTCAATGCGTGTGATGAGCTGGTCTGCTAGAAGGAGC
ACAGTCTTGAGGGA

Casein-kinase 1α

AAACTTCCTCATGGGCATCGGCCGGCACTGCAACAAGCTGTACATGATCGACTTCGGGCT
CGCTAAGAAGTTCCGCGACATGCGCACGCGCGCGCACATCTCCTACCGCGAGGACAAGA
ACCTGACGGGCACCGCCCGCTACGCGTCCATCAATGCGCATCTCGGCATCGAGCAGTCG

CGACGAGATGACATGGAGTCACTCGGCTACGTACTTATGTATTTCAATAGGGGATCGCTGC
CATGGCAAGGACTGAAGGCGATCACCAAGAAGCAGAAGTATGAGAGAATCAGCGAGAAAA
AGATGTCCACTCCGGTTGAAGTGCTTTGCAAGGGATTCCCGGCCGAGTTTGCGATGTATCT
AAATTACTGCCGTGGACTTAGCTTCGATGAACCCCCAGACTACATGTATCTCAGACAACTG
TTCCGCATCCTGTTCCGCACGATGAACTACCAGTACGACTACACGTATGACTGGACCATGC
TGAGGCAA

Grainy Head

CTTGTCGCAGAACACCTTGATCTGGCAATATCCTCTGTGATAAACTTGCGTATCTCTTGGAT
CCTCGAATGTATCAATTTGAATATGAAGTGGTAAGCCCTTAACACCCTTTTGACTGCTGAAG
TCCGTACTCAAACACTGAACAGCGATGTTGATCTTAGCAGCACTCTCCAGTGGGTTCCAGT
AGACAGCGATGGCGTTGTGTGAGACCTCCTCGATGCAGCCGGCGAGCCCGATGCTGTTCT
TCGTGTCGGCGTCGAGTATCCTTTGTTTGACTGAATGCTGTCTCCCGTGCCAGAACTGCCA
TGCCTTAAATTTCGCCCTTGGGGGGTTTTTTCTTCTCAAAACATAAGCATAACGACGCTCTT
AACAGTTTGGTTCTTGAGAGGTTTGTCAGGATCGTGGATGTACTCCAACGTGATGCCATAA
AACTGGCCTTTGTTGATATACGTGATGCGGTCATCTTCCCGCCTCTGGGAACTGCTGCTCG
CCGTCTCCAGATGGTACTTGAAACCGTATGGTGAAAATAATTTAGCGATCTGTATCTTGTCA
GCGTCGTTCTGCCAGCCGTAATCGTGCCAGGGCCGGCA

6.4- The mean size of different classes of wound-induced pigmentation pattern

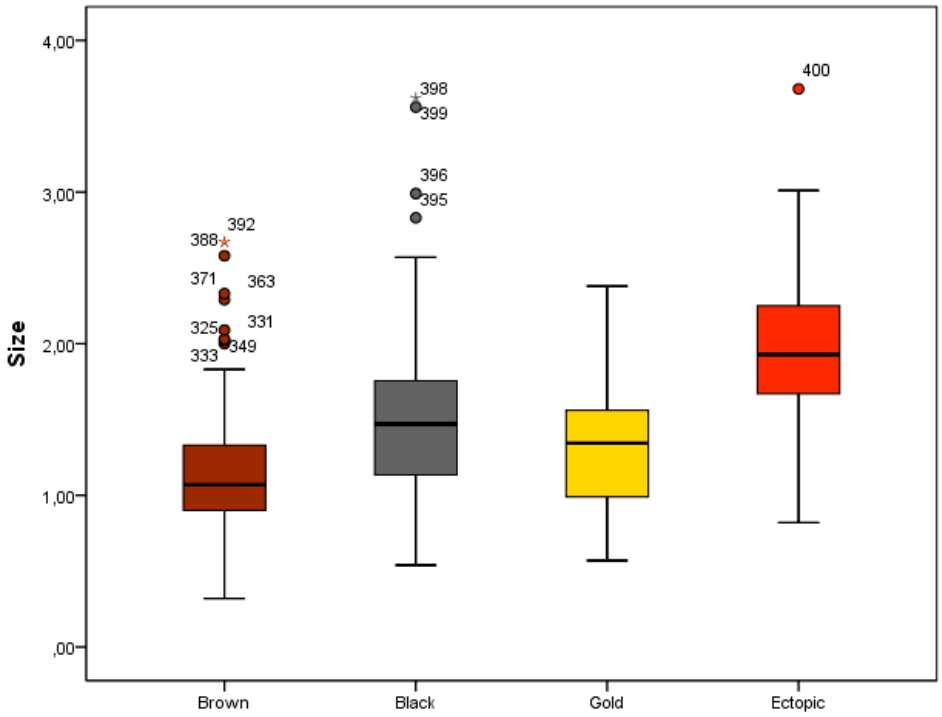


Figure A2-The bigger wound-induced patterns were the ectopic eyespots (orange) with 1,94mm (SE=0,0385), followed by black patches (grey) 1,52mm (SE=0,0645), golden patches (yellow) 1,32mm(SE=0,0527) and light brown patches (brown) 1,15mm(SE=0,0336).

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