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Co-option of Hedgehog and Wingless pathways in the formation of evolutionary novelties

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1. Resumo

A multiplicidade de padrões e formas que pode ser encontrada na natureza é modelada não só pelas forças evolutivas, selecção e deriva, mas também pelo desenvolvimento que pode funcionar como filtro da variabilidade genética conseguida por mutação. Assim, a ligação entre os níveis fenotípico, sobre o qual a selecção e deriva actuam, e genético é efectuada pela ontogenia. O desenvolvimento traduz genótipos em fenótipos à medida que uma única célula inicial se divide em várias células com organização espacial característica. A informação posicional definida ao longo deste processo, conseguida por exemplo pela utilização de moléculas sinalizadoras como morfogénios, determina o destino celular. Estudar a mecânica do desenvolvimento, como interacções entre redes genéticas ou comunicação celular, e compreender que alterações ocorreram ao longo da evolução é a via para desvendar as leis que estão na base da formação de diversidade. Este objectivo só pode ser conseguido pela ligação entre as disciplinas evolução e desenvolvimento a que se deu o nome de evo-devo.

A diversidade pode ser conseguida pela criação de novidades evolutivas definidas como características com valor adaptativo específicas de uma linhagem evolutiva e que podem ser geradas pela aquisição de novos genes ou novas estratégias. Dados recentes sugerem que a reciclagem de circuitos genéticos é o mecanismo mais comum. A esta reciclagem e reutilização dá-se o nome de co-opção. Como disse Lavoisier, "na Natureza, nada se perde, tudo se transforma". Alguns exemplos de co-opção na geração de novidades evolutivas são encontrados nos padrões coloridos de asas de borboletas.

As borboletas são bem conhecidas pela sua beleza e pela multiplicidade de padrões de cor e forma das asas. O estudo dos mecanismos que estão na base desta grande variabilidade fenotípica pode abrir portas na compreensão do processo evolutivo. Actualmente, a borboleta africana *Bicyclus anynana* é muito utilizada como modelo no estudo da ligação entre evolução e desenvolvimento. Nas suas asas podem ser encontrados padrões relativamente simples constituídos por círculos concêntricos altamente conspícuos a que se dá o nome de ocelos. Estes padrões são de extrema importância para a *fitness* do organismo sendo alvo de selecção natural e sexual. Durante o seu desenvolvimento, podem ser observadas quatro fases bem definidas da formação dos ocelos: pré-padronização, determinação do foco, sinalização e diferenciação celular. Contudo, muitos dos genes envolvidos em cada um dos estádios ainda estão por identificar.

Estudos em *Junonia coenia*, uma outra borboleta estudada em laboratório, revelaram a presença do mRNA da molécula sinalizadora Hedgehog na zona do ocelo durante a fase de determinação do foco em larvas. Por sua vez, durante o desenvolvimento dos ocelos em pupas, a proteína Wingless foi detectada na fase de sinalização. Ambas as moléculas são altamente conservadas ao longo da evolução e importantes na definição dos eixos nas asas de todos os insectos. Neste projecto propomos o estudo da co-opção das vias de sinalização Hedgehog e Wingless na criação de novidades evolutivas.

Em 2006, Marcus e Evans, recorrendo à modelação computacional, propuseram dois possíveis modelos para a rede genética envolvida na determinação do foco. O sinalizador Hedgehog está presente em ambos os modelos e mais tarde, os mesmos autores, previram que a rede genética envolvida poderia ser definida pela observação da expressão do gene *hedgehog* no mutante *Cyclops*. Este apresenta alterações no número e forma dos ocelos tendo sido obtido por mutação espontânea numa população laboratorial de *Bicyclus anynana*. A expressão de *hedgehog* apenas foi encontrada na zona posterior da asa larvar à semelhança do que acontece em *Drosophila*, demonstrando a conservação evolutiva da função deste gene na determinação do eixo Antero-Posterior. Contudo, não foi verificada a maior acumulação do seu mRNA na zona do futuro ocelo, ao contrário do que tinha sido verificado em *Junonia coenia*. Consequentemente, não foi possível validar o modelo da rede génica correspondente à fase de determinação do foco.

Numa tentativa de compreender qual a importância da via de sinalização Hedgehog na formação dos ocelos foram analisadas asas larvares quanto à expressão de *patched*. Este gene codifica uma proteína transmembranar que funciona como receptor de Hedgehog e seria de esperar que, sendo Hedgehog importante na determinação do foco, Patched deveria ser produzido na zona do ocelo. Os resultados revelaram variabilidade entre indivíduos no número e posição dos ocelos em que o gene estava a ser expresso. Se esta variabilidade é sinónimo de diversidade biológica no *timing* de expressão, não se sabe. Contudo, estudos recentes de perda de função são indicativos de que Hedgehog pode não ser funcional na formação dos ocelos. Sendo assim, a expressão de Patched na zona do foco pode ser explicada pela partilha de reguladores de expressão com genes que são importantes para a formação destes padrões na asa de *B. anynana*. Proteínas reguladoras que activam na zona do foco a expressão de genes envolvidos na formação dos ocelos podem também induzir a produção do mRNA de *patched* sem que este seja funcional na região.

No estudo do padrão de expressão de *wingless* durante o desenvolvimento de pupas foi observada a presença do seu mRNA e do anti-transcripto ao mesmo tempo na área do futuro ocelo. Um modelo explicativo foi proposto e postula que, as células produtoras do mRNA e da cadeia complementar, que funcionaria como repressor da tradução de wingless, são diferentes. Assim, este mecanismo de regulação da expressão génica seria utilizado com a finalidade de se obter uma precisa e adequada produção de morfogénio sendo que, em caso de erroneamente uma célula iniciar a transcrição de *wingless*, a sua tradução em proteína iria ser impedida pela existência da cadeia complementar ao seu mRNA. Tendo em conta que, os padrões das asas das borboletas são de extrema importância para a sua *fitness* e que qualquer alteração pode comprometer a sua sobrevivência, é compreensível como, ao longo da evolução, este tipo de mecanismos "protectores" do fenótipo se podem ter mantido na população.

Para se compreender uma via de sinalização deve-se olhar para todos os componentes do sistema: molécula sinalizadora e suas propriedades, modelação, recepção e transdução do sinal. Seguindo esta linha de pensamento neste projecto foram também analisados os padrões de expressão de Frizzled (receptor de Wingless) e Groucho (regulador negativo da transcrição dos genes alvo da via Wingless). Contudo, os resultados obtidos não foram conclusivos.

Para o futuro, é importante actualizar os dados utilizados por Marcus e Evans na formulação dos modelos visto que, tudo aponta para a não funcionalidade de Hedgehog na formação dos ocelos em *Bicyclus anynana*. Por outro lado, testes funcionais são o próximo passo, não só para validar os resultados obtidos para *hedgehog* mas também *wingless*. Estudos de expressão devem estar sempre acompanhados por este tipo de análise uma vez que a existência de mRNA/proteína num determinado local e fase do desenvolvimento não significa obrigatoriamente funcionalidade nem informa quanto à função específica.

Palavras chave: *Bicyclus anynana*, ocelos, co-opção, *hedgehog*, *wingless*

2. Abstract

The world is like a biological diversity accumulator and understanding the mechanisms responsible for the generation of this diversity is a fascinating theme in biology. The diversification of patterns and shapes can be accomplished by the origin of novelties – lineage specific traits of new adaptive value. Much recent data suggest that novelties can arise from co-option: recycling of shared genetic circuitry. One of the most beautiful examples of this is found in butterfly wing patterns. Some wings have conspicuous circular paintings called eyespots development of which is divided in four main stages: prepattern, focus determination, focal signaling and cellular differentiation. There are some gaps in our knowledge about genes involved in this pattern design. Experiments with *Junonia coenia* revealed the expression of *hedgehog* around the foci in larvae, and, in *Bicyclus anynana* pupae, imunohistochemistry analysis showed the expression of Wingless, also a morphogen. Here, we studied the co-option of those two signaling pathways in formation of novelties using *in situ* hybridization to detect spatial patterns of gene expression. *hedgehog* was found only in the posterior area of larval wings with no higher expression around the foci as observed for *J. coenia*. On the other hand, it was detected that both sense and anti-sense transcripts for *wingless* were being expressed in eyespot foci at the same time. A model was designed to explain this finding that requires that different cells should be expressing *wingless* mRNA and anti-transcript. To have a better comprehension about Hedgehog and Wingless pathways involvement in eyespot formation we also analyzed expression of other genes: *patched* (hedgehog receptor), *frizzled* (wingless receptor) and *groucho* (a negative regulator of wingless signal transduction). However, there is still a lot to be done and the necessity of functional tests is clear.

Key words: *Bicyclus anynana*, eyespots, co-option, *hedgehog*, *wingless*.

3. Introduction

3.1. Shaping variation: development and evolution

Development plays a crucial role in shaping the biotic diversity by a non-linear relation between genes and phenotypes [1]. The robustness of the system can be achieved by architectural constraints in a way that genetic differences are not always translated into phenotypic variation during development, a phenomenon called canalization [1, 2]. Looking at gene regulatory dynamics during ontogeny, responsible for the phenotypic variation construction and canalization [1], and seeing which alterations occurred through time is essential to get a better comprehension about the astonishing diversity that can be observed. On the other hand, evolutionary processes as selection and genetic drift also play an active role in shaping variation operating as phenotypic filters while mutation contributes to the formation of variants that might or not translate into phenotypes [3]. The integration of development and evolution (evo-devo) can constitute a revolution in our way of thinking about biodiversity [4].

The diversification of patterns and shapes can be accomplished by the origin of novelties – lineage specific traits of new adaptive value [5] – as a result of novel genes or reutilization of shared genetic circuitry. Much recent data suggest that co-option takes place: recycling of shared genetic circuitry [6]. This concept can be applied for a single gene but it is also possible that multiple genes connected by regulatory linkages evolve as a unit to produce a novel function. However, how many key regulatory genes are needed to get a gene network involved in new contexts is not known [6].

Some authors consider that there are essentially three ways to undergo co-option even in the presence of selective forces acting to maintain the current function of the gene: a) evolution of novel protein potential by changes in the protein-coding sequence not required for the current function; b) evolution of new expression patterns, in other tissues or developmental stages, by alterations in the regulatory region of the gene; and c) duplication event followed by independent modifications in the amino acid and/or regulatory sequences [6].

One of our favorite examples of morphological novelties and gene co-option is represented by butterfly eyespots (**FIG.1**) [6]. These striking pattern elements, composed of concentric rings of pigmented scales [7], evolved in the Lepidoptera, an order that includes moths and butterflies, presumably from simpler spot patterns [8]. The expression of conserved signaling molecules such as Hedgehog [9] and Wingless [10] during eyespot development was already shown.

3.2. Pattern formation

Development translates genotypes into phenotypes while many different cells with characteristic spatial organization appear from a single initial cell [11, 12]. During this process, cellular communication is fundamental to determine cell fate via positional information and can be achieved by the use of signaling molecules like morphogens [12, 13]. Wolpert, in 1969, already described the importance of those proteins for spatial patterning during cellular differentiation [12]. In this kind of communication there are signaling cells – signal producers – the signal itself, cells that receive and interpret the information depending on the amount of morphogen received and the consequence – response. If some components of the system are changed, like signal strength, morphogen properties or sensitivity of the responding cells, pattern modifications can be observed.

Butterfly wings are a good example of morphogen importance in establishing cellular positional information and fate. Each wing surface is two-dimensional [14] with cellular pigmentation depending on position on the wing blade [8, 15]. Some butterfly species also have conspicuous elements in their wings – eyespots – which development was already associated with signaling molecules as Hedgehog [9] and Wingless [10]. Changes in the signal strength (amount of signal produced) can induce changes in eyespot size [16] while modifications in the way cells respond to the signal (alterations in the response threshold) can bring differences in eyespot color composition [17].

3.3. Butterflies in evo-devo

We are still trying to understand the mechanisms involved in formation of diversity and insects are recognized as comprising a large proportion of this variety [18, 19]. Lepidoptera is included and the amazingly diverse colorful mosaics found in butterfly wings are intriguing and still fascinate scientists [14] also due to their adaptive value:

wing-color patterns are often used in inter and intra-specific visual communication [20]. Predator avoidance can be accomplished by the utilization of apostematic warning coloration [14, 21, 22] or crypsis [14, 23, 24] while the same patterns can as well be used for intra-specific recognition, mate localization and sexual selection [14, 25, 26]. If we take into account all of those characteristics and that butterflies are also developmentally tractable it is easy to understand why they are a useful animal model for the study of evolution, development and the interaction of both.

Bicyclus anynana is a small African butterfly ideal to look at the reciprocal interactions between evolution and development [20]. This brownish insect has extremely conspicuous elements in their wings, called eyespots [7], composed of concentric differently colored rings (a white pupil, black disc and gold outer circle). They are a structurally simple trait [20] and easily modified by mutation [15, 27, 28]. Each wing – fore and hindwing – has a singular pattern differing in the number and size of eyespots. The forewing is composed by a simple mosaic with only two eyespots while the hindwing has seven [29].

3.4. Eyespot development

Holometabolous insects are distinguished by its characteristic life history that is divided into discrete developmental stages, including a feeding phase (larvae) and a quiescent one (pupae) from which the adult hatch [30]. Inside the larval body the wings are already being developed in well organized structures called wing imaginal discs, a morphologically recognizable wing anlage [31-33].

Molecular and transplantation studies indicate that the development of eyespots occurs in four stages [34]. In mid-fifth instar larvae, axis determination signals are used to define the future eyespot place – prepattern phase. Genes responding in a threshold way to gradients established during wing formation start to be expressed in a broad distal band and at high levels in stripes down the middle of each wing cell [15, 34] (**FIG.2a**). Then, during the focal determination phase, some genes' expression enlarges at the proximal tips of these stripes [9, 34-36] (**FIG.2b**). By doing *in situ* hybridizations in the butterfly *Junonia coenia* (**FIG.1**), *hedgehog* (*hh*) expression was already found in a two-band pattern around the eyespot focus area [9]. Latter, merging all the knowledge about genes implicated in this phase, Marcus and Evans proposed two possible models using computer science and modulation. They represent two potential gene networks implicated in the determination of the eyespot focus, a place that later operates as an organizing centre around which the color rings are formed

[14]. The Hedgehog signaling pathway is involved in both gene networks [37] (**FIG.2e**). Following this phase a third eyespot developmental stage begins early after pupation – focal signaling [34] (**FIG.2c**). Nijhout proposed that the focus, consisted of signaling cells, organizes the differentiation of butterfly eyespot pattern by producing a long range diffusible protein – morphogen – that is interpreted in a threshold-like fashion by the surrounding epidermal cells [10] (**FIG.2f**). Previous works propose Wingless as the candidate morphogen for eyespots development and the presence of the protein was confirmed by immunohistochemistry experiments in *Bicyclus anynana* [10]. The last phase corresponds to cell differentiation (**FIG.2d**) and depending on the amount of morphogen received, cells follow a certain fate by producing a particular pigment in rings around the central focus [34] (**FIG.2f**).
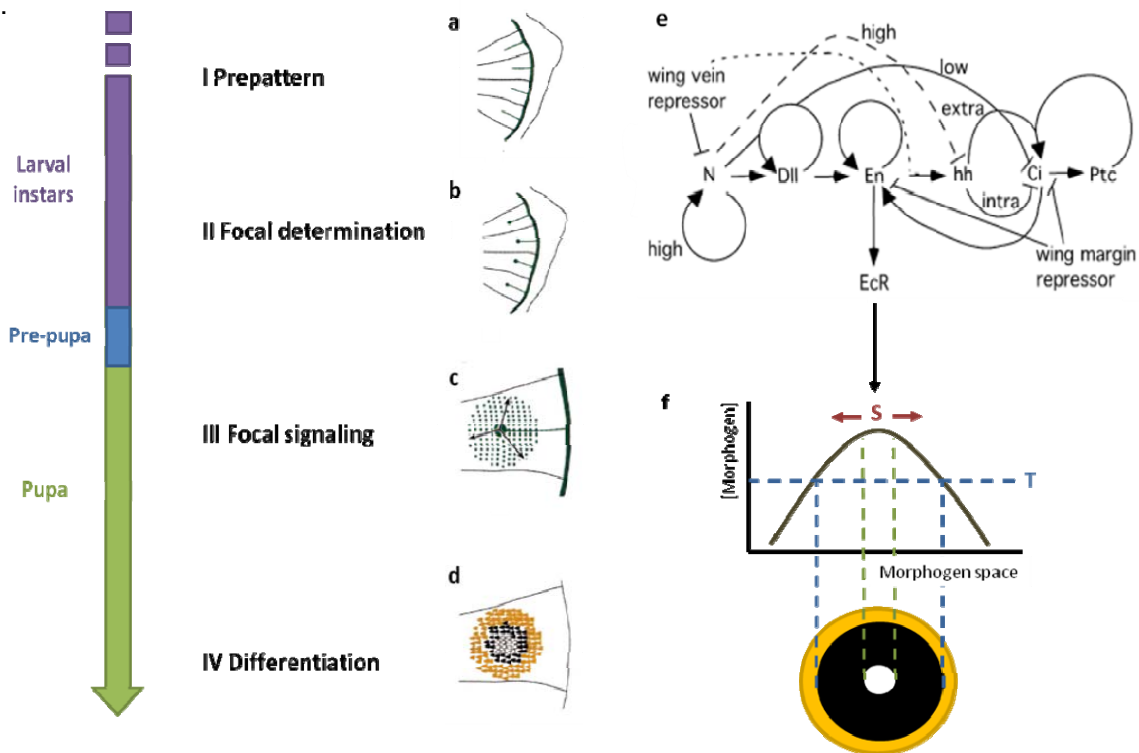

FIG.2 Eyespot developmental steps

The developmental time is defined by the vertical arrow, in early stages larval instars develop until become pre-pupae that latter differentiate into pupae. Early during larval development the wing patterning genes are interpreted in a threshold way and some start to be highly expressed as a distal band that extends through the middle of each wing cell – prepattern phase (**I, a**). Latter, some of those genes start to have bigger expression at the tip of each wing cell stripe – focal determination stage (**II, b**). During those two phases a gene network is activated and by computational science there were proposed two possible models that differ from each other in the way *hedgehog* is activated in the foci (see the dashed arrows in **e**). The third stage corresponds to the focal signaling and starts early after pupation (**III**). A morphogen – the signaling molecule (**S**) – is produced in the foci and secreted to the surrounding cells (**c**). Then, during differentiation (**IV**) cells follow a certain pathway and start to produce a concrete pigment (**d**) depending on the signals received as they respond in a threshold (**T**) manner (**f**). Pictures **a**, **b**, **c** and **d** are taken from Brakefield, P.M. *et al.* (1996), **e** from Evans, T.M. & Marcus, J.M. (2006) and finally **f** was adapted from Beldade, P. & Brakefield, P.M. (2002).

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3.5. Aims of the project

The project is focused on the study of two conserved signaling pathways implicated in establishing the focus in larval wings – Hedgehog pathway – and in focal signaling of pupal wings – Wingless pathway.

4. Materials and Methods

4.1. Experimental animals and dissections

Bicyclus anynana laboratory stocks of *wild type* (*wt*) and *Cyclops* butterflies were maintained at 26°C ± 1°C with high humidity. The latter has abnormalities in wing venation and the mutation is recessive lethal, then there is no pure breeding line (*wild type* plus *Cyclops* segregating). Larvae were fed on maize plants and adults with banana. Final instar larvae stop feeding and form immobile prepupae (light green larvae attached to the maize leaves by the most posterior part of the body) about 24 hours before pupation, which usually occurs within a few hours of the onset of darkness [7, 11]. The light was turned off at around 4pm to regulate the pupation time so that prepupae collected at the end of the day, would start to pupate at 10pm. Pupation times were scored by means of time-lapse photography with a digital camera (Nikon). The time-lapse was set to take pictures every 1 hour with a time stamp on each photo and the hour post-pupation (hpp) defined for each pupa was of 30 minutes before the hour associated to the photograph.

For the study of Hedgehog pathway involvement in eyespots development we dissected 5th instar larvae. Wing imaginal discs that are in the second and third segments were removed by doing a lateral incision. Pupal wings were used to investigate Wingless pathway participation in eyespot pattern formation. During the project some problems to dissect pupal wings were detected. From 13 to 17 hours post-pupation (hpp) the wings are too fragile making it difficult to remove them without damage. The best way to proceed is to not take out the cuticle from the forewing and the hindwing should be placed by suction using a pipette with a bigger tip (the hole should be enlarged by cut induction). All the dissections were made in fresh PBS in a microscope (Leica).

4.2. Target genes

Here, in the Hedgehog pathway research we studied expression patterns of the following genes: *hedgehog* (*hh*) coding for signaling protein and *patched* (*ptc*) coding for its receptor. For Wingless pathway, expression patterns of *wingless* (*wg*), *frizzled* (*fz*) and *groucho* (*gro*) were analyzed. Wingless is a signaling molecule (morphogen), Frizzled is Wingless receptor and Groucho a negative regulator of Wingless signaling pathway. To check the efficiency of *in situ* hybridization protocols we used the probe against *Antennapedia* (*Antp*), a control gene with known eyespot-associated expression.

The probes for *hedgehog* (482bp), *patched* (477bp), *wingless* (315bp), *wingless2* (411bp), *frizzled* (555bp) and *groucho* (407bp) were already available. [*Antennapedia* (154bp) probe was amplified by PCR using primers designed in program Primer3: forward 5' CCTGGAGCTGGAGAAGGAAT 3' and reverse 5' GCCCTTGGTCTTGTTCTCCT 3'] (see details in **Annex7.1**). The size of the obtained PCR product was checked on an electrophoresis gel. This 154bp fragment was cloned into the pCRII-TOPO® vector using the TOPO TA Cloning kit (Invitrogen) and QIAprep Miniprep (QIAGEN) was used to extract the plasmid from the colonies that showed an efficient cloning. The measurement of the DNA concentration in the solution was done by using the NanoDrop equipment. To be sure that the amplified sequence was really *Antennapedia* the DNA solution was sent for sequencing. This sequence was used to generate antisense (AS, a complementary sequence to mRNA) and control sense (S, same sequence as mRNA) RNA probes Dig-labeled.

4.3. *In situ* hybridizations

Whole-mount *in situ* hybridizations of larval wing discs were performed as described in Annex (see **Annex7.2**). The tissue was fixed during 25-40 minutes, treated with proteinase K (25µg/mL) for 4 minutes and post-fixed during 20 minutes. It was used a probe concentration of 1µL/mL in hybridization solution. After doing *in situ* hybridizations the wings became opaque and stage identification was difficult as tracheas are not seen in the dark field. We used the phase contrast microscope and then the 5th instar larval wing staging was possible. Another protocol was used for *in situ* hybridizations of pupal wings as described in Annex (see **Annex7.3**). The tissues were fixed during 2 hours and digested with proteinase K for 3 minutes. There were some difficulties to mount pupal wings, mainly the young forewings, on slides. Two pieces of lab tape were put overlapped in each side of the slide in such a way that when the lamella is laid there is a space between it and the slide. That prevents destruction of tissue and there is some freedom to unfold the wing.

For the analysis of *hedgehog* and *Antennapedia* gene expression in larvae, *wingless*, *wingless2* and *frizzled* in pupae we used left anterior and posterior wings to see the expression of the gene (AS probe) while the right ones were utilized as control wings (S probe). We used ± 40 larval wing discs per treatment while only a maximum of 6 pupal forewings were put in each well. In the *wingless* experiments, to check the protocol efficiency we added also some larval wings as a control. In the *hedgehog* and *patched* experiment to have a correlation between *hh* and *ptc* gene expression in the

same individual we put in each well wings of only one larvae. The left side was used to look at *hh* expression while in the right we checked *ptc*. We used 18 5th instar wings (9 individuals) with different developmental times. To see if the *ptc* probe was working, some larvae were dissected using the left side for the AS probe and the right one for the S probe as a control. Also for the study of *wingless* and *groucho* gene expression we used only one individual per well. The left hindwings were used to see the gene expression using the AS probe and the right ones to look at the anti-mRNA transcript using the S probe.

Images were captured with different microscopes. The pictures from **FIG.3a**, **b**, **c**, **d**, **FIG.5 a**, **b**, **FIG.9**, **FIG.8** and **FIG.9a**, **b**, **c**, **d** were taken with a microscope (Leica) while the images from **FIG.3 e**, **f**, **FIG.4**, **FIG.5c**, **d**, **FIG.6** and all the pictures from the **Annex** were taken in a DIC/fluorescence microscope (BioRad). All the pictures are oriented as the following description: proximal-distal axis horizontally with proximal to the left and anterior-posterior axis vertically with anterior at the top.

4.4. DAPI and larval staging

Larval and pupal wings were fixed in the same fix buffer solution as used for *in situ* hybridizations (25-40 minutes for larval wings and 40-70 minutes for pupae). Wings were immersed in DAPI solution (1µL DAPI in 1mL PBT) for 5 minutes and three washes of 5 minutes long (using PBS) were done before and after the addition of DAPI. Images were captured with a DIC/fluorescent microscope (BioRad). For the staging images the dark field option was used and for DAPI the fluorescence one.
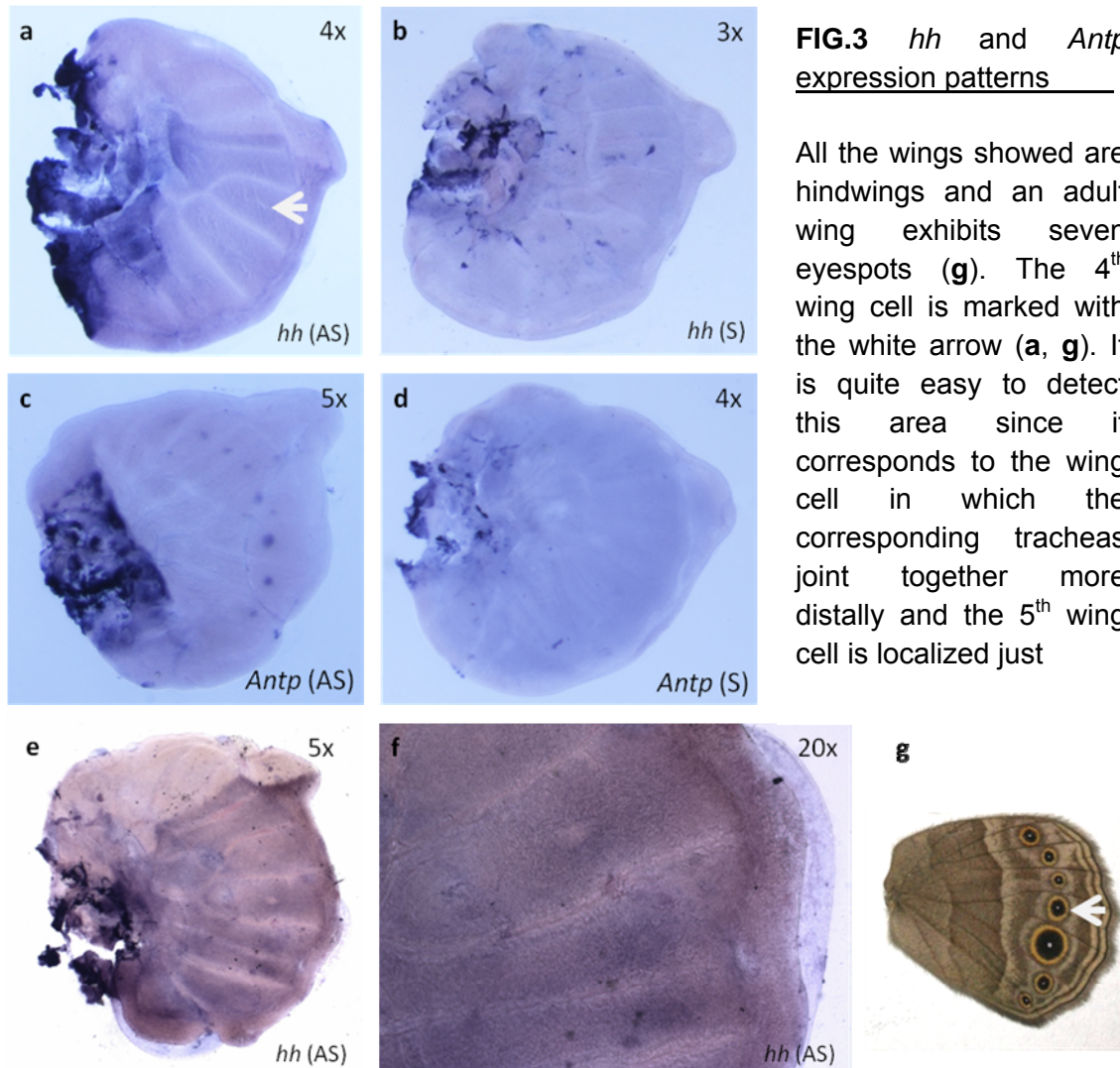
5. Results and Discussion

5.1. Hedgehog signaling in focal determination

Hedgehog is a conserved signaling molecule important to establish the Antero-Posterior axis of Drosophila wing discs and is expressed in the posterior compartment of the wings [38, 39]. The same type of pattern was found in *Junonia coenia* larval wings, as well as a higher *hh* expression in two bands around the eyespot focus[9]. Those results suggest that this signaling molecule can be involved in eyespot formation and that its function in the AP axis definition is conserved between Diptera and Lepidoptera despite 230-280 million years of separation [15, 40].

In 2006, Marcus and Evans collected all the information about genes expressed during eyespot focus determination and, using computer modeling, proposed two possible models for the gene network implicated in this developmental phase [37]. Those models differ in the way Hedgehog expression is regulated (**FIG.2e**) and in 2008 using the same modeling they predicted that *hedgehog* expression in the *Cyclops* mutant could help distinguish between the two models [41].

Here, we did *in situ* hybridization experiments in 5th instar larvae of *Bicyclus anynana wild type* (*wt*) and *Cyclops* mutant to check *hedgehog* (*hh*) gene expression. It was observed that, similarly to observations in *J. coenia*, *hedgehog* is expressed in the posterior area of the *Bicyclus anynana* wing disc except in veins lacunae, but contrarly to what was observed in *J. coenia* there was no higher expression near developing eyespot foci in *wt* and *Cyclops* butterflies (**FIG.3a, b**).

We know that the probe is working because the expression of the gene in the posterior compartment of the wing can be detected. Also the protocol efficiency was tested by looking at *Antennapedia* (*Antp*) gene expression in larval wing discs and it was observed that this Hox gene is expressed in eyespot foci (**FIG.3c, d**). The absence of higher *hh* expression in the eyespot could be due to timing problems. The 5th instar larvae developmental period can be subdivided into 17 stages according to the tracheal development [42]. Subtle thread-like tracheoles extended from the basal mass (stage 0.5) continue to grow until all the major tracheal branches are extended (stage 1.5) and reach the border lacuna (stage 2.5). Then, we can see moderate levels of tracheole growth into intervenous and peripheral tissue (stage 3.5) (**Annex FIG.1**).

FIG.3 *hh* and *Antp* expression patterns

All the wings showed are hindwings and an adult wing exhibits seven eyespots (**g**). The 4th wing cell is marked with the white arrow (**a**, **g**). It is quite easy to detect this area since it corresponds to the wing cell in which the corresponding tracheas joint together more distally and the 5th wing cell is localized just beneath. The *hedgehog* expression is localized only in posterior without a bigger expression near the eyespot (**a**) while *Antennapedia* is found in all the foci (**c**). Sometimes it could be slightly seen that in the foci there is a downregulation of *hh* (**e**, **f**). All the controls did not show any gene expression as expected (**b**, **d**). Picture **g** is taken from Brakefield, P.M. *et al.* (1996).

We propose that *hh* expression during focus determination is confined to a specific stage during the 5th instar development. To explore this suggestion 92 wings (from 46 larvae) at different developmental stages were analyzed. In almost all the wings *hh* expression was found in posterior (only 16 wings did not show *hh* expression) but no obvious higher expression near the foci was observed **(Table.1)**. Still, sometimes it was perceived, not obviously, a downregulation of *hh* in the focus and a slightly superior expression around it. This is evident in the posterior area of the wing where *hh* is expressed but absent in the eyespot region (**FIG.3e, f**).

Table.1 *hedgehog* expression along time

Several larval wings from different 5th instar stages were analyzed. All of them were treated with *in situ* hybridization to check for *hh* expression and the results are resumed below. In none wing it was found a bigger expression near the future focus.

Group stages*	Wings with only posterior hh expression	No expression	Total of wings
0-0.75	2	0	2
1-1.75	8	0	8
2-2.75	34	1	35
3-3.75	32	10	42
4	0	5	5
Total of wings	76	16	92

* the staging is based on the tracheal development (**Annex FIG.1**). See Materials and Methods 6.7 section for more detail.

We observed that sometimes it seems that there is a downregulation of *hh* expression in posterior at the foci area and a slightly superior expression around it. To understand the participation of this gene in eyespot formation we decided to investigate which cells are able to receive *hh* signal by producing its receptor – *patched* [43]. This objective was accomplished by doing *in situ* hybridizations to check *patched* (*ptc*) expression in 5th instar larval wings with different developmental time. In those experiments we used the same individual to see *hh* and *ptc* gene expression so that a correlation between both expression patterns could be obtained. It is expected that, if *hh* plays an important role in eyespot formation then, *ptc* expression should be seen in the focus at the same time that *hh* is expressed around it.

Similarly to what happened with *hedgehog*, *patched* expression pattern in *B. anynana* resembles what is observed in *Drosophila* imaginal wing discs: an anterior-posterior compartment boundary [44]. Moreover, this receptor is expressed in veins lacunae and in some eyespot area. There was phenotypic variability in which wing cells *ptc* was being expressed (**FIG.4**), no correlation between the two genes expression (slightly bigger expression of *hh* around the eyespot in one wing cell does not correspond to a superior expression of *ptc* in the focus at the same region of the wing) and finally no temporal guide line.

We know that the probe is working properly because the anterior-posterior compartment boundary can be seen. We also did *in situ* hybridizations using the left side for the anti-sense probe and the right one for the sense as a control where no gene expression was found (**Annex FIG.2**). Still, no conclusions can be drawn about *hh* importance in the eyespot formation and the necessity of functional tests is evident.
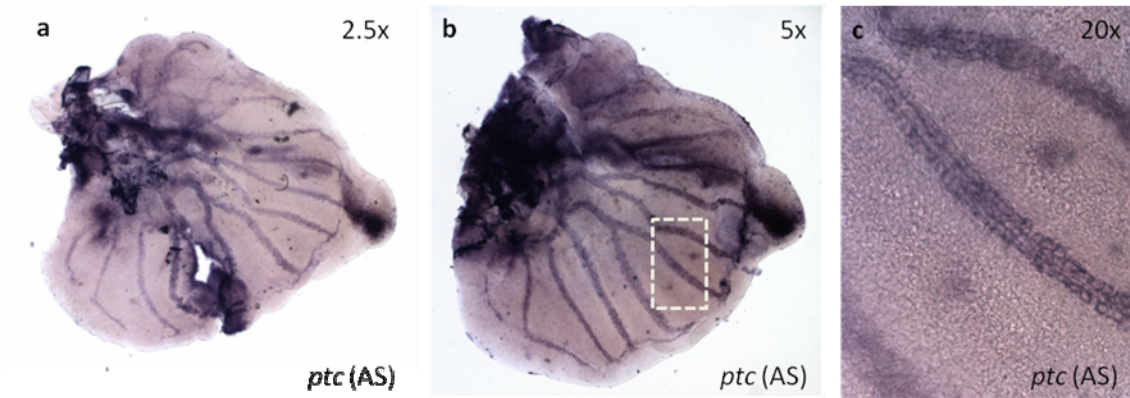
FIG.4 *Patched* expression in larval wing discs

The larval wing discs were used to do *in situ* hybridizations to check *ptc* gene expression. The pattern observed is the same found in *Drosophila*, as a band defining the limit between anterior and posterior (**a, b**). The wing cell in which the gene was being expressed in the foci is not constant, appearing only in the 2nd wing cell (**a**), in the 5th, 4th and 2nd (**b, c**) and in many other combinations. (**c**) is a detailed picture and the area correspond to the marked white square in (**b**). All of those wings are hindwings and the control did not show gene expression as predicted (**Annex FIG.2**).

Accepting the hypothesis that *hh* has a role in eyespot patterning there is always the possibility that the results obtained are a consequence of technical problems. In that case, *in situ* hybridizations must not be the ideal method to study those genes expression, for example, if the mRNA is instable then the efficiency of the technique to detect gene expression is lowered. As soon as antibodies are available for *patched*, imunohistochemistry experiments, based on the protein detection, should be done to check this possibility. However, a more interesting explanation is associated with the recent idea that there is more intra-populational biological variability on the timing of gene expression during development than previously thought [42]. In that case, the timing for *ptc* gene expression can potentially be different between individuals and also eyespots without changing the phenotype. This idea is supported by the ability of eyespots to develop and evolve independently from each other [35, 45].

Nevertheless, is also possible that *hedgehog* is not in *Bicyclus anynana* eyespot building structure. Recently, Antónia Monteiro did loss-of-function experiments for *hh* that support this suggestion. The adults from the treatment showed an effect on wing size but not on eyespots (not published). During evolution, eyespots could have appeared by the reutilization of a conserved gene circuitry. Still, it is possible that only some genes of the network are really involved in this pattern formation. Therefore,

patched can be expressed in the focus because it belongs to the gene circuitry co-opted without being functional in that region.

Sudies in *Junonia coenia* revealed the presence of *hedgehog* during eyespot focus determination [9]. We do not know if *hh* is important for *J. coenia* eyespot formation and it lost function in the lineage Satyrinae or if it does not play a role also in Nymphalinae eyespot development. Next, it would be interesting to do functional tests for this gene in *J. coenia* larvae.

5.2. Wingless signaling in focal signaling

Another important developmental phase in eyespot formation is the focal signaling and Wingless is proposed as the candidate morphogen produced at the eyespot center and that is diffused away to create a concentration-gradient that will induce cells at different distances from the focus to produce different color pigments. Antónia Monteiro, using immunohistochemistry, already showed *wg* expression in *B. anynana* pupal wings but she was doing protein detection [10]. Here, we propose to see which cells in the pupal wing are producing *wingless* and *in situ* hybridizations were done. It is expected that if this signaling molecule has a role in eyespot formation then, focal cells should express *wingless*.

We observed that *wg* mRNA (**FIG.5a**) and also the anti-sense transcript (**FIG.5b**) are produced in focal cells of pupae and we did two types of controls to check the probe and protocol efficiencies. To test probe specificity, *wg* expression was analyzed in larvae. As expected, the mRNA is present along the wing margin (**FIG.5c**) and the anti-sense transcript was not found in larval wings (**FIG.5d**). Still, it was necessary to confirm the protocol effectiveness for pupal wings. We verified it by looking at *Antennapedia* (*Antp*) expression. It was observed that this Hox gene is specifically expressed in focal cells (**Annex FIG.3**) and no staining in the control.

To make sure that the same organism expresses the *wg* gene and the complementary sequence simultaneously, *in situ* hybridizations were performed with one individual per well and the same type of pattern was observed in all the wings analyzed: expression in foci cells of both *wingless* sense and anti-transcript (**FIG.6**). It was also observed that this expression pattern is not very transient as it was found in wings with 14h38m, 14h55m, 16h43m and 18h37m (time post-pupation).
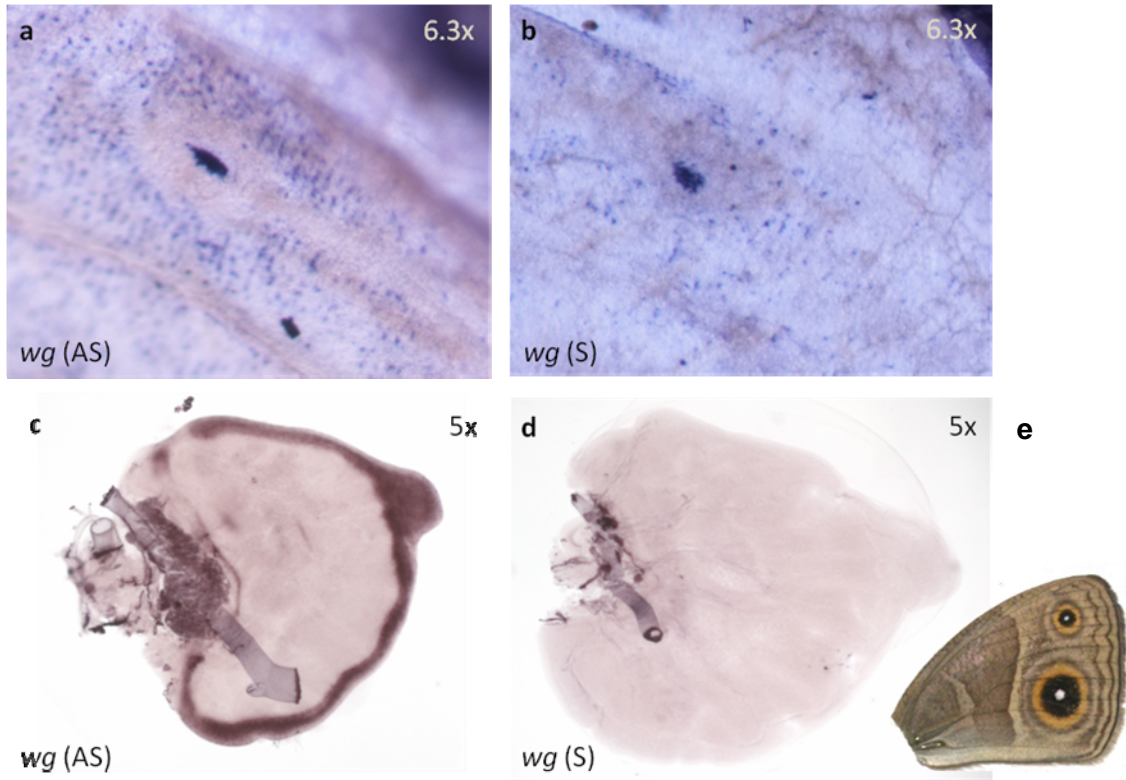
FIG.5 _wingless_ expression pattern in larval and pupal wings

All the wings represented are forewings except the one in (**c**) but the same expression pattern was found in larval fore and hindwings (data not shown). Pupal wings were used to test _wingless_ gene expression using the anti-sense (**a**) and the sense probe (**b**) revealing a similar expression pattern in both. Larval wings were used as a probe control and the results are the expected ones, gene expression using the anti-sense probe (**c**) and no gene detection using the sense probe (**d**). In adults the forewings present two eyespots painted (**e**). The picture **e** is taken from Brakefield, P.M. _et al._ (1996).
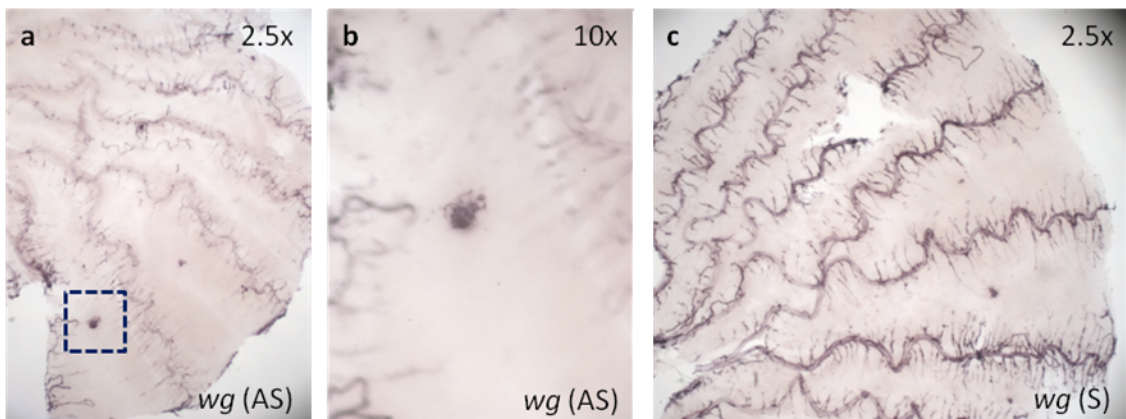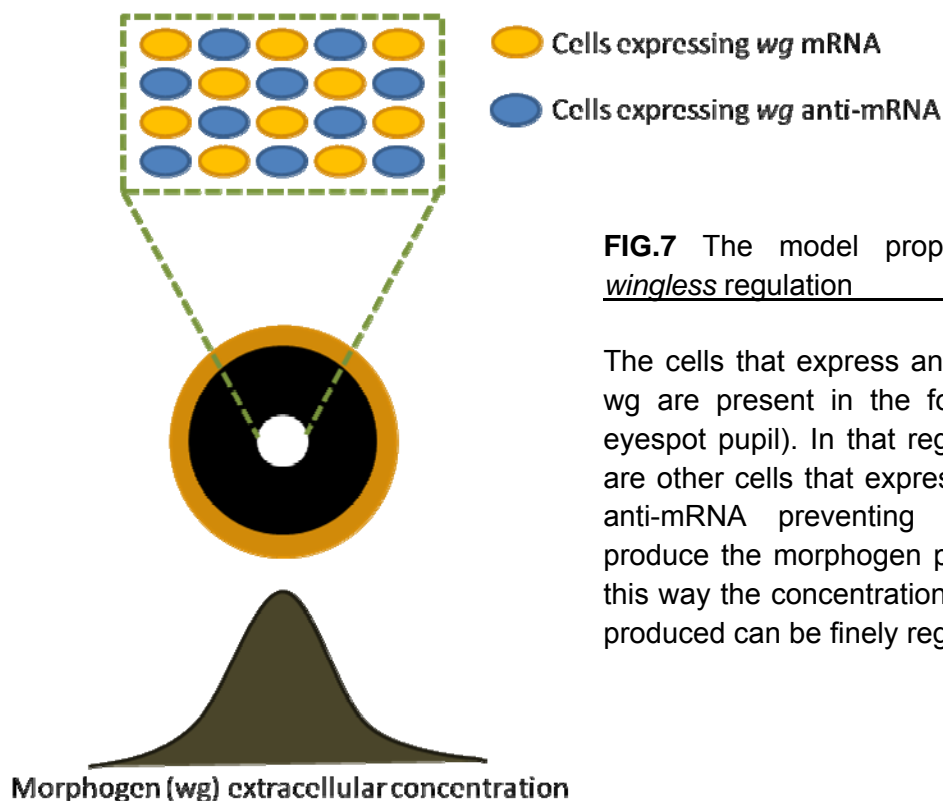

FIG.6 Individual _in situ_ hybridizations for _wingless_ using pupal hindwings

The hindwings belong to a pupa with 18h37m post-pupation. _wingless_ gene expression using the anti-sense (**a**, **b**) and sense (**c**) probes. (**b**) is a detailed picture and the correspondent area is defined by the blue square in (**a**).

A recent study using the centipede *Strigamia maritime* to search for gene expression of some Hox genes suggests that anti-sense transcripts (detected by using the sense probe) are regulating gene translation by inhibition [46]. They observed complementary gene expression patterns for sense and anti-sense probes [46] while here both seem to be expressed in the same place – the focus. It is difficult to explain this observation but there is a possible model: *wingless* sense and anti-sense can be expressed in the same area but in different cells (**FIG.7**). Why only some focal cells would produce *wg* mRNA can be explained easily. It was found that Notch is being expressed during focus determination in different butterfly species [36, 42, 47], suggesting that this gene is important for eyespot pattern formation. Notch signaling has a lateral inhibition ability that results in a mosaic-like expression pattern [48, 49]. Also, it is already known that the activation of this signaling pathway results in *wingless* expression [50-53]. If Notch importance in eyespot formation is proved, then, part of our mosaic model can be explained as following (**FIG.7**): Notch is expressed in a mosaic-like pattern, only cells that express this receptor start to produce *wg* mRNA and consequently the morphogen expression pattern is mosaic-like. Still, the mechanisms whereby the other cells start to express the anti-transcript RNA remain unknown.

○ Cells expressing *wg* mRNA

○ Cells expressing *wg* anti-mRNA

FIG.7 The model proposed for *wingless* regulation

The cells that express and secrete wg are present in the foci (white eyespot pupil). In that region there are other cells that express the *wg* anti-mRNA preventing them to produce the morphogen protein. In this way the concentration of signal produced can be finely regulated.

Morphogen (wg) extracellular concentration

Why it would be advantageous to have a system like this to regulate the expression of the morphogen is unclear but it is possible that it is functioning as a concentration modulator so that the amount of protein secreted can be finely regulated. According to

this idea, if the "wrong cell" starts, by error, to translate the *wg* gene, then, that cell, due to the presence of the anti-sense RNA will not produce the protein [54-56]. This sequence has the ability to bind to the *wg* mRNA preventing the translation step and using this strategy the amount of signal produced can be controlled. If we think about the importance of those colorful wing patterns for butterfly fitness [22, 25, 57, 58] then, it is comprehensible why individuals that are already prepared to hide possible genetic mistakes related to eyespot formation could have had a higher fitness. Having this kind of regulation the phenotype can be preserved in case a wrong cell starts to express *wg*. On the other hand, we can also look at this type of regulation as a diversity generator since if cells start to produce wrongly the anti-transcript then it can be translated into phenotypic differences.

Still, the involvement of *wingless* in this novelty formation and also the model has to be validated. Then, a second probe for *wingless* was designed and it worked in larval wings showing the typical expression in the wing lacuna and no coloration in the control (**Annex FIG.4**) but we still have to use it for pupal *in situ* hybridizations. It would be interesting to do double *in situ* hybridizations using the sense and anti-sense probes for *wg* labeled with different colors. Using this technique it can be detected whether the same or different cells are producing those RNAs. We also need functional assays (loss-of-function or ectopic expression) to confirm *wg* role in eyespot formation. A recent technique, often used to study the digit formation, is based on the application of beads (100-150µm) impregnated with protein to simulate a signal diffusion [59]. The insertion of it impregnated with potential signaling molecules in different wing places and developmental timing has the potential to be an important source of knowledge about eyespot formation.

Additionally, it was observed that *wg* expression pattern in pupal forewings is different between eyespots and changes along time (**FIG.8a, b, c, d**). In young pupa, *wingless* is expressed only in the anterior eyespot focus with a spoon-like pattern around it without staining (**FIG.8a, b**). Only latter in development the same type of pattern is found in the posterior eyespot (**FIG.8d**) and by then, in the anterior, the spoon-like shape without *wg* expression is lost while this gene continues to be expressed in the foci (**FIG.8c**). The diameter of *wingless* expression in the anterior eyespot focus from young pupa is smaller than the one observed in the posterior eyespot from older pupa (**FIG.8a, d**).
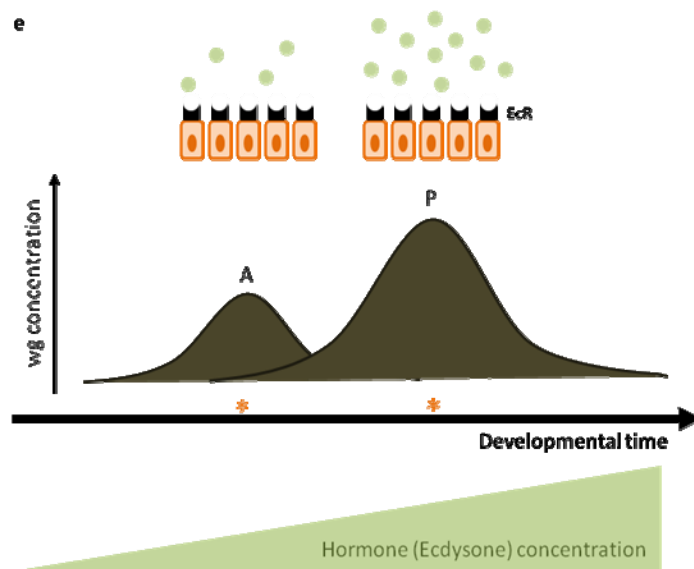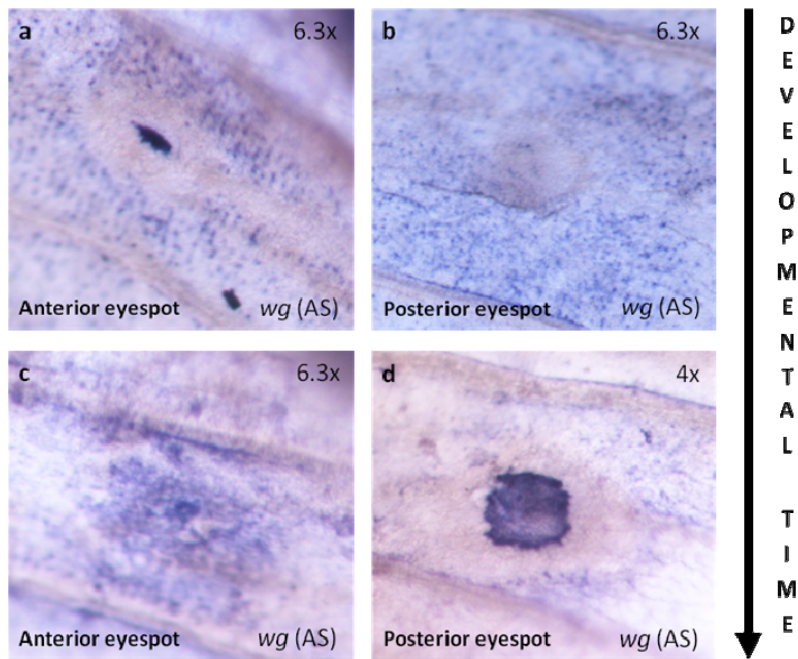
FIG.8 Pupal forewing wingless expression along time in the anterior and posterior eyespots .

In each line it is present the anterior and posterior views of the same forewing (**a**, **c**) and (**b**, **d**) respectively. The developmental time is represented by the vertical arrow and consequently (**a**, **b**) belong to a younger wing comparing to (**c**, **d**). Then, it can be noticed that the posterior eyespot (**d**) shows the same pattern as the anterior one (**a**) latter in the development. Those results can be explained by the proposed model (**e**). In the anterior eyespot (A) the production of wingless is lower and earlier than the posterior (P) one and there is a time lag between both concerning to the activation of the signal production (*). The hormone concentration arises during development and then, an earlier expression of EcR leads to the activation of fewer cells due to a less amount of ecdysone when comparing with a latter developmental time.

The adult *B. anynana* butterfly has phenotypic differences between those two eyespots as the anterior is smaller than the posterior and those dissimilarities are correlated with the diameter of wingless expression in foci. We propose that the development of the posterior eyespot in the forewing is delayed in comparison with the anterior and that this timing difference is responsible for the diameter size discrepancies in the adult. Heterochrony is a phenomenon related with changes in the timing of a suite of characters relative to others during development [60] and it was already associated with eyespot development [28]. Here, we propose that the different eyespot size in forewings is correlated with differences in the timing and pattern of *wingless* expression.

Butterfly wing color patterns are defined during late larval and early pupal development, metamorphosis phase controlled by ecdysteroid hormones via their nuclear hormone receptors [61]. It was already demonstrated that eyespot size is associated with Ecdysone concentration [62] and it is suggested that Ecdysone receptor (EcR) action is involved in all important events leading to the wing color pattern: acting together with other genes during focal signaling and defining the eyespot area [61]. After pupation, the hormone concentration in the hemolymph grows with time [62]. We propose that if the EcR foci expression is activated early in development then, due to a less amount of Ecdysone, fewer cells are going to be activated producing the signal – Wingless expression. On the other hand, a latter EcR expression leads to a higher number of cells activated due to a superior amount of hormone and consequently a larger diameter of *wingless* expression (**FIG.8e**). However, a recent study in *Drosophila* showed that Ecdysone activates gene expression of *crooked legs* (*crol*). This transcription factor represses *wingless* expression in *D. melanogaster* wing imaginal discs [63].

We still need to validate the hypothesis that Wingless is important for eyespot formation, but assuming that this is true, the signal regulation by Crol should have changed in butterfly lineage. The model proposed can be easily tested by doing immunohistochemistry experiments to look at the behavior of EcR expression in forewings along development. However, the differences of pattern and timing observed for *wingless* expression in anterior and posterior forewing eyespots can be due to biological variability without playing any function in eyespot size dissimilarities.

To understand a signaling mechanism it is necessary to look at all components of the system in an integrative way. It is not only important to investigate the signal molecule

itself but also the elements that modulate the way a morphogen is spread, the signal detection and ultimately the cellular response. Following this idea, more pupal *in situ* hybridizations were performed to explore *frizzled* (wingless receptor) [64] and *groucho* (negative regulator of Wingless signaling pathway) [53, 65] gene expression.

For Frizzled it was expected that, if Wingless is the signaling molecule spread from the foci then the receptor should be expressed in all the eyespot area. We observed staining in the focus but it did not look like cellular expression (**FIG.9**). There is the possibility that the localization of the probe in that region is due to a higher cell density in the focus, confirmed by DAPI staining (**Annex FIG.5a**). It is necessary to do more *in situ* hybridizations for *frizzled* (*fz*) in pupal wings, using a different probe, to get a better understanding about Wingless signal detection.
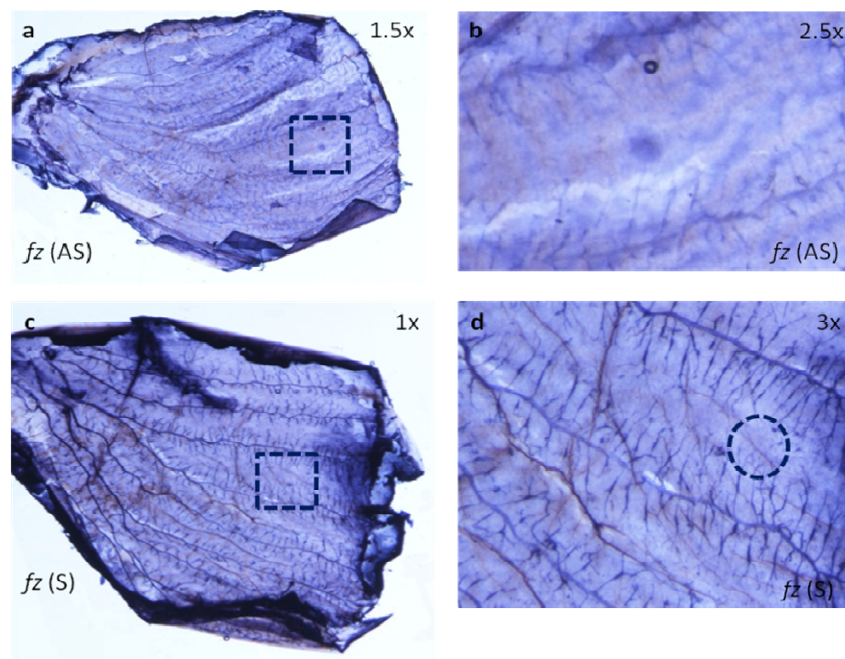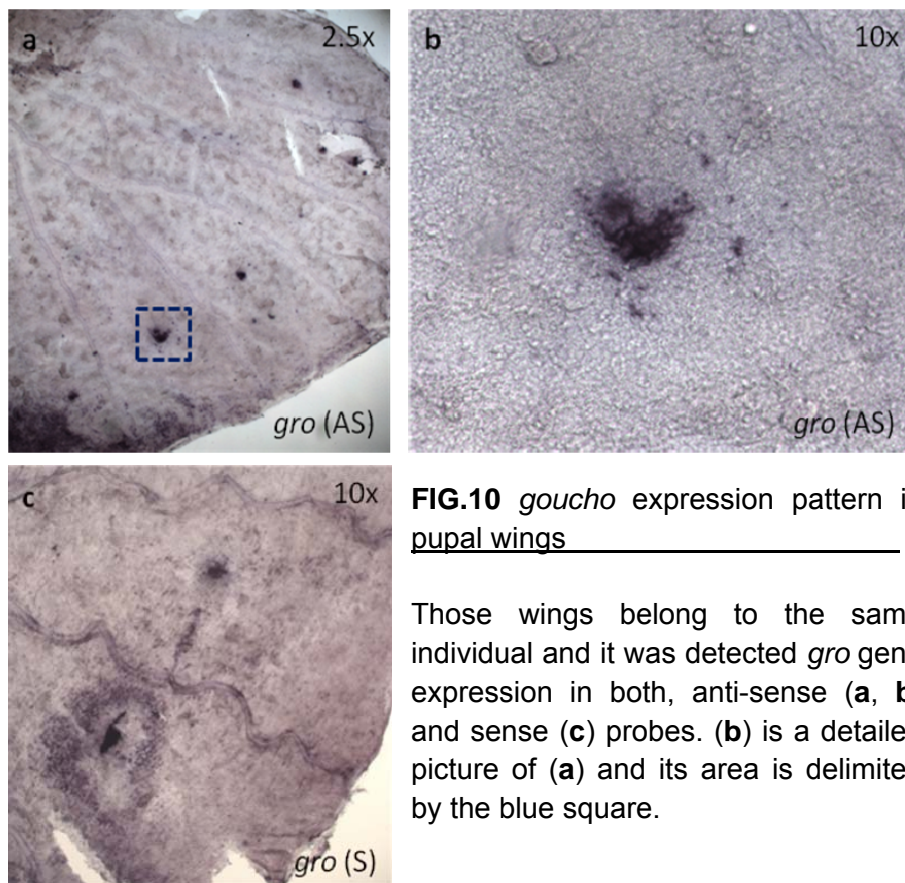

FIG.9 *frizzled* expression in pupal wings .

Detection of *fz* expression using an anti-sense probe (**a**, **b**). The sense probe was used as a control (**c**, **d**). It was found gene expression in the foci (**a**) but it doesn't seem to be cellular (**b**). In the control it was not detected *frizzled* in the foci (**c**, **d**) which area is delimited by the blue circle (**d**). (**b**, **d**) are detailed pictures of (**a**, **c**) which localization is enclosed by the blue squares respectively.

Groucho is an important negative regulator of several signaling mechanisms including Notch and Wingless [53, 66]. This transcription factor is sequestered by other proteins that are responsible to direct Groucho to a specific signaling pathway which target

genes are going to be repressed [53]. Therefore, *groucho* (*gro*) can be expressed in a cell without acting as a negative regulator for Wingless target genes because it is repressing the transcription of genes from other signaling pathway. Still, we decided to look at this gene expression doing *in situ* hybridization experiments in pupal wings. Knowing which cells are expressing *gro* it is possible to identify cellular regions where signal transduction of important signaling pathways as Notch and Wingless can be inhibited. It was observed that *goucho* mRNA and also the anti-transcript are both expressed during eyespot development (**FIG.10**). The model proposed for wingless gene expression (different cells are producing the sense and anti-transcript) can also be applied in this context. This idea has to be validated and it would be interesting to do loss-of-function tests and double *in situ* hybridizations.

FIG.10 *goucho* expression pattern in pupal wings

Those wings belong to the same individual and it was detected *gro* gene expression in both, anti-sense (**a**, **b**) and sense (**c**) probes. (**b**) is a detailed picture of (**a**) and its area is delimited by the blue square.

It would be also appealing to explore the wingless pathway by studying the involvement of other genes in eyespot formation like, *shaggy* [67], *Apc* and *armadillo* [68] concerned to the Wg signal transduction. The *Dally-like* gene is as well interesting to research since it works as a binding protein involved in shaping the extracellular wg morphogen gradient [69]. However, loss-of-function tests are always needed to confirm the participation of each gene in eyespot formation.

6. Final remarks

We need to do functional assays for all the genes analyzed in this project as loss-of-function tests (e.g. iRNA) or ectopic expression (e.g. beads). Furthermore, it would be interesting to look at other components of Hedgehog and Wingless signaling pathways.

The results obtained show variation in gene expression at different biological levels: between eyespots, individuals and species. Different eyespots are regulated by the same gene circuitry however, it seems that they can develop independently from each other as some genes start to be expressed in different eyespots at different developmental time. Also, different individuals with the same developmental age show dissimilarities in gene expression. This intra-populational biological variability in the timing of gene expression during development that is not translated into phenotypic variation was already observed by Reed [42]. Additionally, we can start wondering about the gene networks involved in the eyespot formation of different species. *hedgehog* expression was found near the eyespot foci in *Junonia coenia* [9] but not in *Bicyclus anynana*. If there is a different basis to build the same trait, we do not know, but functional tests using both species would solve the question.
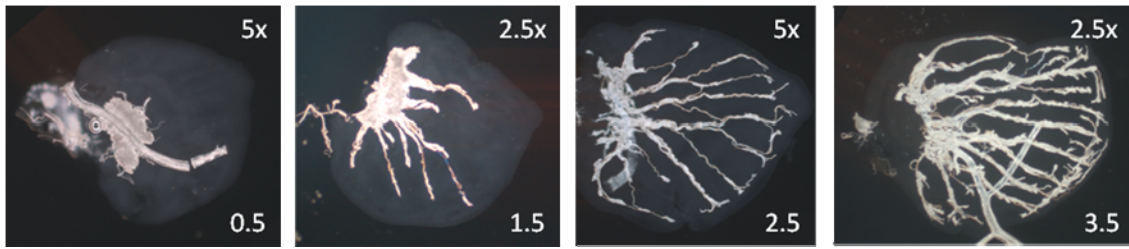
7. Annex

FIG.1 Larval wing discs staging   .

Those wings belong to 5th instar larvae and the staging is based on the tracheal development. Only the middle stages are shown: **0.5** (subtle thread-like tracheoles extended from basal mass are discernable), **1.5** (extension of all major tracheal branches), **2.5** (extension of most tracheae into border lacuna) and **3.5** (moderate levels of tracheole growth into intervenous and peripheral tissue) [42].
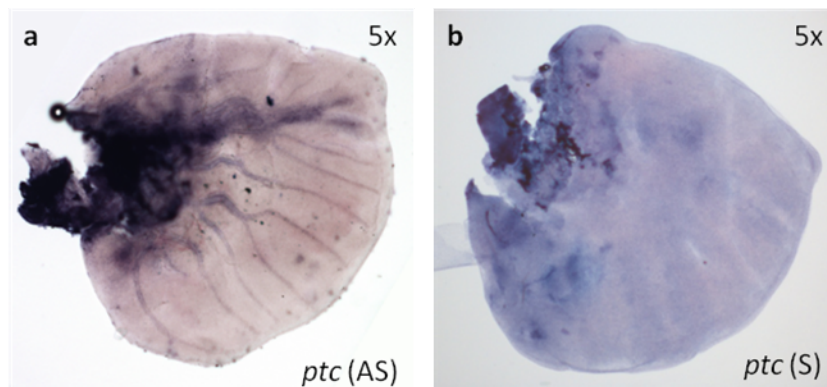

FIG.2 *pactched* expression in larval wing discs   .

patched in *B. anynana* larval wing discs appear in the veins, as a band defining the limit between anterior and posterior and also in some foci (**a**). The control, as expected, did not show any gene expression (**b**).
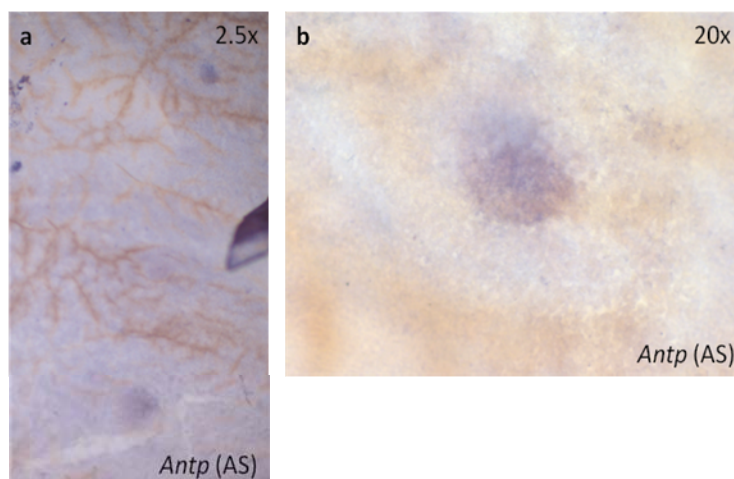

FIG.3 *Antennapedia* gene expression in pupal wings

In situ hybridizations were made using pupal forewings to look for *Antp* gene expression as a protocol control. *Antp* is being expressed in the foci (**a**, **b**) and it was not found gene detection using the sense probe (data not shown).
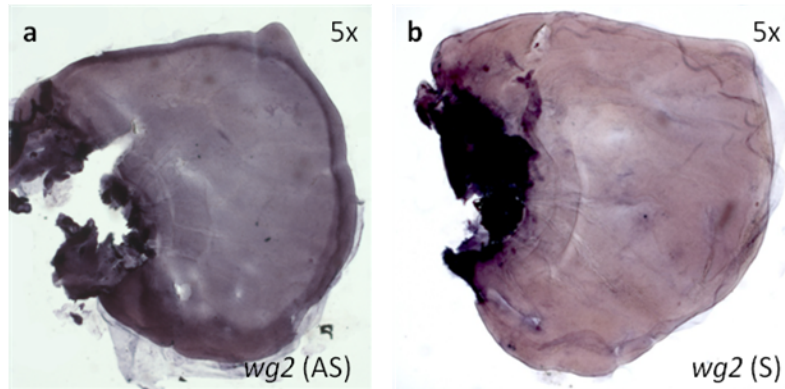
FIG.4 *wingless* expression pattern in larval wing discs using a second probe (*wg2*)   .

wingless gene expression using another designed anti-sense probe (**a**) and the control, by the utilization of a sense probe (**b**). It can be seen, even with background, that *wg* is being expressed as a band in the distal lacuna (**a**) while there is no probe detection in the control (**b**).
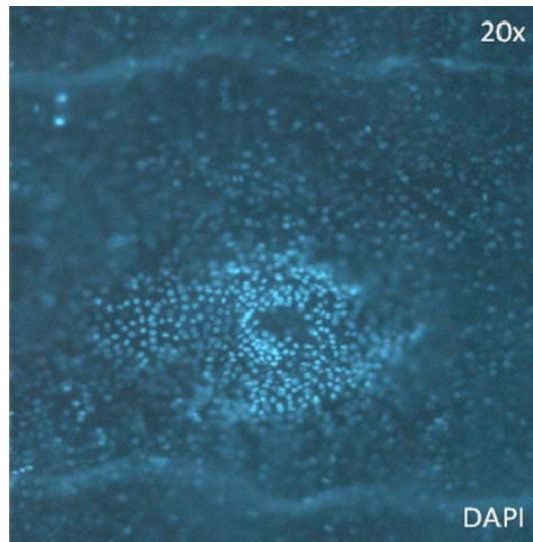
FIG.5 Pupa with stained nuclei   .

The nuclei were stained with DAPI and they can be seen by the blue fluorescence. It can be noticed that cells are not yet organized as defined lines. In this pupal wing (14hpp) we can observe a higher cellular density near the focus. It seems that there are not cells in the foci but changing the focal frame they can be seen in the same density as in the surroundings (data not shown). Those cells belong to a different cellular layer.

7.1. Probe sequences

hedgehog

```
TGACCCCTCTCGTCTTCAACCAGCACGAGCCCAACATCAGTGAGAATTCCAAATC
CGCCAGTGGCCCTCCCGAGGGCCGCATCACGAGGGAGGACGAAAAGTTCAAAGA
CTTAGTGCCCAATTATAACCCGGACATAGAGTTTAAGGATGACGAGGGCACCGGA
GCCGACCGCCACATGACACAGCGGTGCAAAGAGAAGTTGAACACGCTCGCCATC
AGTGTGATGAACCAGTGGCCCGGGGTTCGACTCCGAGTCATCGAGGGCTGGGAC
GAGGAGAACTCGGCTCATCTAGAAAACTCACTGCACTACGAGGGCCGGGCAGTG
GACATCACCACCAGCGACCGGGATCGCAGCAAGTACGGCATGCTGGCACGCCTT
GCTGTGGAAGCCGACTTCGACTGGGTGTTCTATGAGAGCCGGTCCTACATACATT
GTTCTGTCAAGACAGAATCATCAGTGGGCACTGGAGCTGGTTGTTTT
```

patched

```
CGTACTTGATGCTTGGCAGAGAAAGTTTGCAGCTGAAGTAAAAAAGATGACTACCT
CAAGTTCAGTGTCAGCAGCGTACAGTTTTTACCCGTTTTCGACCTCAACATTGAAT
GACATACTCGGAAAATTCTCGGAAGTCTCACTAAAGAACATTATTTTGGGATACAT
GTTTATGTTAATTTATGTTGCTGTAACGTTAATACAATGGCGAGATCCAATTCGTTC
TCAAGCTGGAGTGGGTATAGCCGGAGTATTGCTTCTGTCGATCACAATAGCCGCT
GGCTTAGGCTTTTGTGCATTATTAGGCATACCATTCTATGCATCGAGTACACAAAT
AGTGCCGTTCCTAGCTCTCGGACTAGGTGTTCAAGATATGTTCCTTCTCACTCACA
CATACGTTGAACAAGCGGGAGATGTGCCGAGAGAAGAGAGAACCGGACTGGTAC
TGAAAAAGAGCGGACTGAGCGTTCTACTGGC
```

wingless

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GTCATGATGCCCAATACCGAGGTGGAGGCGCCGTCGCAGAGGAACGACGCCG
CACCTCACAGGGTCCCGCGCCGTGACCGCTACAGGTTCCAACTTCGGCCGCACA
ACCCTGACCACAAAACACCCGGGGTCAAGGACCTTGTATACTTGGAATCTTCACC
AGGTTTCTGCGAAAAGAACCCCAGACTGGGCATCCCGGGTACGCACGAGCGTGC
CTGCAACGACACTAGCATCGGCGTCGACGGTTGCGACCTGATGTGCTGCGGGCG
CGGCTACCGGACCGAGACCATGTTCGTAGTGGAACGGTGCAACTGC
```

wingless2

```
TCATCTACGCCATCACAAGCGCAGGGGTGACGCACGCGGTGTCGCGCGCATGCG
CCGAAGGCTCCATCGAGTCCTGCACGTGCGACTATTCCCATGTGGACCGCTCGC
CGCACCGCGCGCGCGCCGCCGCCGCCGCCAACGTGAGGGTCTGGAAATGGGGC
GGCTGCAGCGACAACATCGGCTTCGGCTTCAAGTTCAGCCGAGAGTTCGTTGACA
CCGGGGAAAGGGGCAAGACGCTTAGGGAGAAGATGAACTTGCACAACAATGAGG
CCGGCAGGATGCACGTGCAAACGGAGATGCGCCAGGAGTGCAAGTGCCACGGT
```

ATGTCTGGGTCCTGCACGGTGAAGACGTGCTGGATGAGGCTGCCGACGTTCCGG
TCTGTAGGCGACGCCCTGAAAGACAGCTTCGACG

frizzled

TGCCCATTTACACATCAGGAGATGGAAAGGCCTAAATTTGAAGTGTTCATGATAAA
ATATCTTATGACAATGATTGTTGGCATCACATCAAGTTTTTGGATTTGGTCTGGTAA
AACTCTAGTTTCATGGCATCAATTTTTTGATAAAATAAGGGGAAGACGAGTTGAAG
CATATGTTTGATCTTATATTTTGATAGATCATGGATTATGCATTTTATTTTGATATCA
TAACAAATGTTTTGTATTTGTTTGTTTTAAAGTTACAAAGAGATTGTCTCATGTTAGA
TGGCTCTTGGCAAATTAAATATTTAATTAAGTTTAGCATTGCATCTGAATCTTTACG
TATGTACTTAATGTAAAAGAAGATTCTTTCCAGGGACACTTATTTCCAAATCACTAT
TGATTATAACAAAACTTTCTTTCATTTTTGGAGTAAACTACTATATAAAGTGCAAGTT
TATTTGCACAGTTGTCAAAATTTAATTCAAAGCTTAAAACTTTGCACATTTTTGAACA
TAATATTGGCAGACTCAACCAAGCAGCCAGGAACCACATA

groucho

ACCGGACAAGTACCAACTGCACCTGCACGAGTCGTGCGTGCTGTCGCTGCGCTT
CGCGTCGTGCGGCAAGTGGTTCGTGTCCACCGGCAAGGACAACCTGCTCAACGC
CTGGCGCACGCCCTACGGCGCCAGCATCTTCCAGTCGAAGGAGTCGTCGTCGGT
GCTGAGCTGCGACATCTCGTCGGACGACAAGTACATAGTGACGGGGTCGGGCGA
CAAGAAGGCCACAGTGTACGAAGTGATCTATTAGTGCGCGGACGCTAGTGATGTG
ACTGAAGTGACAGCGACAGTGGTGATATAGCTTTAGTGTATAGAGTATATTGGGAA
GCCTGTATGTACTTATGCGACCTGTTTATATAGATTAGTGTGCGCGTTTTTATGTTT
ATTTGTTGAGCGCCCGTAGTGTA

Antennapedia

5'-CCTGGAGCTGGAGAAGGAATTCCACTTCAACCGATACCTGACGCGGAGGAGAC
GGATCGAGATCGCGCACGCCCTCTGTCTCACCGAGCGCCAAATCAAGATCTGGTT
CCAGAACCGGCGCATGAAGTGGAAAAAGGAGAACAAGACCAAGGGC 3'

7.2. Larval *in situ* protocol

<u>Day 1</u>

- Freshly prepare the **Fix solution**
- Clean Dissection area. Cool the fix vials, put **PBS** and **PBT** on ice.
- Dissection in PBS
- Fixation 25'-40'
- R, $4xW_{5'-15'}$ in PBT
- **4' proteinase K** at RT
- R, $W_{5'}$ in **stop solution**
- $2x W_{5'}$ in PBT
- **Post-fix** 20'
- R, $4x W_{5'-15'}$ in PBT
- $2x W_{5'}$ in 50:50 PBT:PreHyb

Not on ice. Rocking if possible

- $W_{10'}$ **PreHyb** at RT
- Incubate at least 1h in PreHyb at $65^{o}C$
- Prewarm **Hyb**
- Heat denature the probe (1µl probe/ml of Hyb) 5' at $80^{o}C$
- Transfer at $65^{o}C$ and add 900µl of Hyb
- Incubate the wing discs at $65^{o}C$ overnight

<u>Day 2</u>

- $10xW_{30'}$ in PreHyb at $65^{o}C$, transfer at RT
- $W_{5'}$ in PreHyb:TBT 50:50 (now all the protocol at RT)
- R, $4x W_{15'}$ in **TBT**
- $W_{60'}$ in TBT-BSA
- Incubate with antibody anti-DIG (Roche) 1:2000 in TBT-BSA overnight in the fridge ($4^{o}C$). Note: prior to each use, centrifuge the original vial of Dig antibody for 5', then pipet carefully the required amount of antibody from the surface.

<u>Day 3</u>

- $3xW_{5'}$, $7xW_{15'}$ in TBT

- 3xW$_{5'}$ in fresh **AP buffer**
- Incubate in **staining solution** (protect from light, rocking). Check from time to time. Continue staining until get a convenient signal with little background, wings can be left at 4°C overnight or washed and start over to do not get over-stained samples.
- 2xW$_{5'}$ in PBT EDTA 2mM
- Additional washes in PBT
- Preincubate in Glycerol 80% PBT 2mM EDTA. Mount, take pictures and keep the wings in the fridge protected from the light.

R – rinse; W – wash; RT – room temperature

Solutions

Fix solution

750µL PBT; 50mM EGTA; 250µL formaldehyde 37%

PBS (10x)

18.6mM NaH$_2$PO$_4$; 84.1mM Na$_2$HPO$_4$; 1750.0mM NaCl

Mix phosphates in about 800mL of dH$_2$O for a 1L total volume. Check the pH, it should be 7.4, if it is more than that then start over, otherwise, adjust pH to 7.4 with NaOH. Add the NaCl and the rest of the dH$_2$O. Prepare 1x PBS by diluting 1:10 with dH$_2$O and check the pH again. Both 1x and 10x PBS can be kept indefinitely at room temperature.

PBT

PBS 0.1% Tween20

proteinase K

25µg/mL in PBT

The stock solution is at 20mg/mL stored in the fridge.

Stop solution

10mL glycine 10g/L (this stock solution can be filtered, autoclaved and kept at 4°C); 5mL PBS 10x; 200µL Tween20 25%

Fill up with sterile dH$_2$O until 50mL.

Post-fix solution (freshly prepared)

150µL formaldehyde 37%; 850µL PBT

PreHyb

10mL sterile dH$_2$O; 25mL formamide; 12.5mL 20x SSC (check that pH is 4.5 prior to addiction); 200µL Tween20 25%; 250µL tRNA (heat denature 5' at 80°C prior to addiction).

Lower pH to 5-6, fill up to 50mL with sterile dH$_2$O. Store at -20°C.

Hyb

5mL sterile dH$_2$O; 5mL glycine 10g/L; 25mL formamide; 12.5mL 20x SSC pH4.5; 200µL Tween20 25%; 250µL tRNA (heat denature 5' at 80°C prior to addiction).

Lower pH to 5-6, fill up to 50mL with sterile dH$_2$O. Store at -20°C.

TBS

125mL Tris 1M pH 7.5; 40g NaCl; 1g KCl

Add dH$_2$O to mix and bring up to 500mL. Filter, autoclave and store at 4°C.

TBT

50mL TBS; 200µL Tween20 25%

AP buffer (freshly prepared)

250µL of MgCl$_2$ 1M; 5mL of NaCl 1M; 5mL of Tris 1M (pH9.5); 200µL Tween20 25%

Staining solution (freshly prepared, protect from light)

1mL AP buffer (check that pH is 9.5); 3.5µL BCIP (protect from light); 4.5µL NBT (protect from light).

7.3. Pupal *in situ* protocol

Day 1

- Dissect wings from time-staged pupae in PBS
- Move wings directly to **Fix Buffer** in wells of 24 well culture plate at room temperature
- Fix discs for 2 hours
- 3x $W_{5'}$ minutes in **PBT**
- Incubate wings for 3 minutes in **Proteinase K solution**
- $RW_{5'}$ in **Digestion Stop Buffer**
- 5x $W_{5'}$ minutes in **PBT**
- 2x $W_{5'}$ minutes in 50:50 **PBT : PHB**
- $W_{10'}$ minutes in **PHB**
- Incubate in **PHB** at least 1 hour at 55°C.
- Heat-denature RNA probe (20-50ng needed per well) (80°C for 5 minutes) and add to **hybridization buffer** (100μL needed per well)
- Add probe to wells and incubate 48 hours at 55°C

Day 3

- 6x $W_{30'}$ at 55°C with **PHB**
- Transfer in 55°C **PHB**, let cool down at room temperature
- $W_{5'}$ in 50:50 **PBT : PHB**
- 4x$W_{5'}$ in **PBT**
- Incubate wings for 1 hour in **Block Buffer** at 4°C
- Incubate wings in a 1:2000 dilution of **anti-Dig** antibody overnight at 4°C

Day 4

- 10x$W_{18'}$ in **PBT** at room temperature
- RWW in **staining buffer without MgCl2**
- W in **Staining buffer with MgCl2**
- Transfer the wings to glass dishes
- Replace with **NBT/BCIP solution**

- 2xW$_{5'}$ in PBT EDTA 2mM
- Additional washes in PBT
- Preincubate in Glycerol 80% PBT 2mM EDTA. Mount, take pictures and keep the wings in the fridge protected from the light.

R – rinse; W – wash; RT – room temperature

Solutions

Fix Buffer

4% Formaldehyde in PBS

PBT

0.1% Tween 20 in PBS

Proteinase K solution

2.5μg/mL Proteinase K in PBT

Digestion Stop Buffer

2mg/mL glycine in PBT

Pre-Hybridization Buffer (PHB) (50 ml)

12 mL DEPC treated water

25 mL Formamide

12.5 mL 20 x SSC

50 μL Tween 20

500 μL 10 mg/ml salmon sperm (Rnase free) — heat denature

prior to addition to solution

Hybridization Buffer

Add 1 mg/mL glycogen to prehybridization buffer

Block Buffer

PBT + 0.5x Roche Blocking (stock aliquots are 10x)

Anti-DIG antibody conjugated to Alkaline Phosphatase (Roche Applied Science
Cat. No. 11 093 274 910)

Staining Buffer

- 49 mL TrisHCl 100mM (pH=9.5)
- 1mL NaCl 5M
- 250µL Tween20
- 2.5mL MgCl$_2$

8. References

1. Borenstein, E. and D.C. Krakauer, *An End to Endless Forms: Epistasis, Phenotype Distribution Bias, and Nonuniform Evolution.* Plos Computational Biology, 2008. **4**(10).

2. Wilkins, A.S., *Genetic analysis of animal development.* New York: John Wiley and Sons, 1986.

3. Gilbert, S.F., J.M. Opitz, and R.A. Raff, *Resynthesizing evolutionary and developmental biology.* Developmental Biology, 1996. **173**(2): p. 357-372.

4. Gilbert, S.F., *The morphogenesis of evolutionary developmental biology.* International Journal of Developmental Biology, 2003. **47**(7-8): p. 467-477.

5. Saenko, S.V., et al., *Conserved developmental processes and the formation of evolutionary novelties: examples from butterfly wings.* Philosophical Transactions of the Royal Society B-Biological Sciences, 2008. **363**(1496): p. 1549-1555.

6. True, J.R. and S.B. Carroll, *Gene co-option in physiological and morphological evolution.* Annu. Rev. Cell. Dev. Biol., 2002. **18**: p. 53-80.

7. French, V. and P.M. Brakefield, *The development of eyespot patterns on butterfly wings - morphogen sources or sinks?* Development, 1992. **116**(1): p. 103-&.

8. Nijhout, H.F., *The Development and Evolution of Butterfly Wing Patterns.* Smithsonian Institution Press, Washington, 1991.

9. Keys, D.N., et al., *Recruitment of a hedgehog regulatory circuit in butterfly eyespot evolution.* Science, 1999. **283**(5401): p. 532-534.

10. Monteiro, A., et al., *Comparative insights into questions of lepidopteran wing pattern homology.* Bmc Developmental Biology, 2006. **6**.

11. French, V. and P.M. Brakefield, *Eyespot development on butterfly wings - the focal signal.* Developmental Biology, 1995. **168**(1): p. 112-123.

12. Wolpert, L., *Positional information and spacial pattern of cellular differentiation.* Journal of Theoretical Biology, 1969. **25**(1): p. 1-&.

13. Kerszberg, M. and L. Wolpert, *Mechanisms for positional signalling by morphogen transport: a theoretical study.* Journal of Theoretical Biology, 1998. **191**(1): p. 103-114.

14. Beldade, P. and P.M. Brakefield, *The genetics and evo-devo of butterfly wing patterns.* Nature Reviews Genetics, 2002. **3**(6): p. 442-452.

15. McMillan, W.O., A. Monteiro, and D.D. Kapan, *Development and evolution on the wing.* Trends in Ecology & Evolution, 2002. **17**(3): p. 125-133.

16. Monteiro, A.F., P.M. Brakefield, and V. French, *The evolutionay genetics and developmental basis of wing pattern variation in the butterfly Bicyclus anynana.* Evolution, 1994. **48**(4): p. 1147-1157.

17. Monteiro, A., P.M. Brakefield, and V. French, *Butterfly eyespots: The genetics and development of the color rings.* Evolution, 1997. **51**(4): p. 1207-1216.

18. Mitter, C., B. Farrell, and B. Wiegmann, *The phylogenetic study of adaptive zones - has phytophagy promoted insect diversification.* American Naturalist, 1988. **132**(1): p. 107-128.

19. Nylin, S. and N. Wahlberg, *Does plasticity drive speciation? Host-plant shifts and diversification in nymphaline butterflies (Lepidoptera : Nymphalidae) during the tertiary.* Biological Journal of the Linnean Society, 2008. **94**(1): p. 115-130.

20. Nijhout, H.F., *Focus on butterfly eyespot development.* Nature, 1996. **384**(6606): p. 209-210.

21. Mallet, J. and M. Joron, *Evolution of diversity in warning color and mimicry: Polymorphisms, shifting balance, and speciation.* Annual Review of Ecology and Systematics, 1999. **30**: p. 201-233.

22. Stevens, M., C.J. Hardman, and C.L. Stubbins, *Conspicuousness, not eye mimicry, makes "eyespots" effective antipredator signals.* Behavioral Ecology, 2008. **19**(3): p. 525-531.

23.   Endler, J.A., *Progressive background matching in moths and a quantitative measure of crypsis.* Biol. J. Linn. Soc., 1984. **22**: p. 187-231.

24.   Bond, A.B. and A.C. Kamil, *Visual predators select for crypticity and polymorphism in virtual prey.* Nature, 2002. **415**(6872): p. 609-613.

25.   Robertson, K.A. and A. Monteiro, *Female Bicyclus anynana butterflies choose males on the basis of their dorsal UV-reflective eyespot pupils.* Proceedings of the Royal Society B-Biological Sciences, 2005. **272**(1572): p. 1541-1546.

26.   Jiggins, C.D., et al., *Reproductive isolation caused by colour pattern mimicry.* Nature, 2001. **411**(6835): p. 302-305.

27.   Monteiro, A., et al., *The combined effect of two mutations that alter serially homologous color pattern elements on the fore and hindwings of a butterfly.* Bmc Genetics, 2007. **8**.

28.   Koch, P.B., et al., *Butterfly wing pattern mutants: developmental heterochrony and co-ordinately regulated phenotypes.* Development Genes and Evolution, 2000. **210**(11): p. 536-544.

29.   Brakefield, P.M. and N. Reitsma, *Phenotypic plasticity, seasonal clima and the population biology of Bicyclus butterflies (Satyridae) in Malawi.* Ecological Entomology, 1991. **16**(3): p. 291-303.

30.   Wiegmann, B.M., et al., *Single-copy nuclear genes resolve the phylogeny of the holometabolous insects.* Bmc Biology, 2009. **7**.

31.   Robb, J.A., *Maintenance of imaginal discs of Drosophila melanogaster in chemically defined media.* Journal of Cell Biology, 1969. **41**(3): p. 876-&.

32.   Gehring, W., *Clonal analysis of determination dynamics in cultures of imaginal disks in Drosophila melanogaster.* Developmental Biology, 1967. **16**(5): p. 438-&.

33.   Rudel, D. and R.J. Sommer, *The evolution of developmental mechanisms.* Developmental Biology, 2003. **264**(1): p. 15-37.

34.   Brakefield, P.M., et al., *Development, plasticity and evolution of butterfly eyespot patterns.* Nature, 1996. **384**(6606): p. 236-242.

35.   Monteiro, A., et al., *Mutants highlight the modular control of butterfly eyespot patterns.* Evolution & Development, 2003. **5**(2): p. 180-187.

36.   Reed, R.D. and M.S. Serfas, *Butterfly wing pattern evolution is associated with changes in a Notch/Distal-less temporal pattern formation process.* Current Biology, 2004. **14**(13): p. 1159-1166.

37.   Evans, T.M. and J.M. Marcus, *A simulation study of the genetic regulatory hierarchy for butterfly eyespot focus determination.* Evolution & Development, 2006. **8**(3): p. 273-283.

38.   Kojima, T., et al., *Induction of a mirror-image duplication of anterior wing structures by localized Hedgehog expression in the anterior compartment of Drosophila melanogaster wing imaginal disks.* Gene, 1994. **148**(2): p. 211-217.

39.   Lee, J.J., et al., *Secretion and localized transcription suggest a role in positional signaling for products of the segmentation gene Hedgehog.* Cell, 1992. **71**(1): p. 33-50.

40.   Wooton, R.J., *Palaeozoic insects.* Annu. Rev. Entomol., 1981. **26**: p. 319-344.

41.   Marcus, J.M. and T.M. Evans, *A simulation study of mutations in the genetic regulatory hierarchy for butterfly eyespot focus determination.* Biosystems, 2008. **93**(3): p. 250-255.

42.   Reed, R.D., P.H. Chen, and H.F. Nijhout, *Cryptic variation in butterfly eyespot development: the importance of sample size in gene expression studies.* Evolution & Development, 2007. **9**(1): p. 2-9.

43.   Ingham, P.W., A.M. Taylor, and Y. Nakano, *Role of the Drosophila Patched gene in positional signaling.* Nature, 1991. **353**(6340): p. 184-187.

44.   Capdevila, J., et al., *The Drosophila segment polarity gene patched interacts with decapentaplegic in wing development.* Embo Journal, 1994. **13**(1): p. 71-82.

45.   Beldade, P., K. Koops, and P.M. Brakefield, *Developmental constraints versus flexibility in morphological evolution.* Nature, 2002. **416**(6883): p. 844-847.

46.   Brena, C., et al., *Expression of trunk Hox genes in the centipede Strigamia maritima: sense and anti-sense transcripts.* Evolution & Development, 2006. **8**(3): p. 252-265.

47.   French, V. and P.M. Brakefield, *Pattern formation: A focus on notch in butterfly eyespots.* Current Biology, 2004. **14**(16): p. R663-R665.

48.   Heitzler, P. and P. Simpson, *The choice of cell fate in the epidermis of Drosophila.* Cell, 1991. **64**(6): p. 1083-1092.

49.   Heitzler, P. and P. Simpson, *The choice between epidermal or neural cell fate in the epidermis of Drosophila.* Journal of Neurogenetics, 1991. **7**(2-3): p. 126-126.

50.   Zhou, J., et al., *Notch and Wingless Signaling Cooperate in Regulation of Dendritic Cell Differentiation.* Immunity, 2009. **30**(6): p. 845-859.

51.   Rafel, N. and M. Milan, *Notch signalling coordinates tissue growth and wing fate specification in Drosophila.* Development, 2008. **135**(24): p. 3995-4001.

52.   Rulifson, E.J., et al., *wingless refines its own expression domain on the Drosophila wing margin.* Nature, 1996. **384**(6604): p. 72-74.

53.   Benitez, E., et al., *Lines is required for normal operation of Wingless, Hedgehog and Notch pathways during wing development.* Development, 2009. **136**(7): p. 1211-1221.

54.   Li, T. and L.E. Hightower, *Effects of dexamethasone, heat-shock, and serum responses on the inhibition of HSC70 synthesis by antisense RNA in NIH 3T3 cells.* Journal of Cellular Physiology, 1995. **164**(2): p. 344-355.

55.   Bechler, K., *Influence of capping and polyadenylation on mRNA expression and on antisense RNA mediated inhibition of gene expression.* Biochemical and Biophysical Research Communications, 1997. **241**(1): p. 193-199.

56.   Denhardt, D.T., *Mechanism of action of antisense RNA - sometime inhibition of transcription, processing, transport, or translation.* Annals of the New York Academy of Sciences, 1992. **660**: p. 70-76.

57.   Stevens, M., et al., *Field experiments on the effectiveness of 'eyespots' as predator deterrents.* Animal Behaviour, 2007. **74**: p. 1215-1227.

58.   Vlieger, L. and P.M. Brakefield, *The deflection hypothesis: eyespots on the margins of butterfly wings do not influence predation by lizards.* Biological Journal of the Linnean Society, 2007. **92**(4): p. 661-667.

59.   Merino, R., et al., *Control of digit formation by activin signalling.* Development, 1999. **126**: p. 2161-2170.

60.   Schlosser, G., *Using heterochrony plots to detect the dissociated coevolution of characters.* Journal of Experimental Zoology, 2001. **291**(3): p. 282-304.

61.   Koch, P.B., et al., *Localization of ecdysone receptor protein during colour pattern formation in wings of the butterfly Precis coenia (Lepidoptera : Nymphalidae) and co-expression with Distal-less protein.* Development Genes and Evolution, 2003. **212**(12): p. 571-584.

62.   Koch, P.B., P.M. Brakefield, and F. Kesbeke, *Ecdysteroids control eyespot size and wing color pattern in the polyphenic butterfly Bicyclus anynana (Lepidoptera: Satyridae).* Journal of Insect Physiology, 1996. **42**(3): p. 223-230.

63.   Mitchell, N., et al., *The Ecdysone-inducible zinc-finger transcription factor Crol regulates Wg transcription and cell cycle progression in Drosophila.* Development, 2008. **135**(16): p. 2707-2716.

64.   Bhanot, P., et al., *A new member of the frizzled family from Drosophila functions as a Wingless receptor.* Nature, 1996. **382**(6588): p. 225-230.

65.   Cavallo, R.A., et al., *Drosophila Tcf and Groucho interact to repress Wingless signalling activity.* Nature, 1998. **395**(6702): p. 604-608.

66.   Buscarlet, M. and S. Stifani, *The 'Marx' of Groucho on development and disease.* Trends in Cell Biology, 2007. **17**(7): p. 353-361.

67.   Bourouis, M., *Targeted increase in shaggy activity levels blocks wingless signaling.* Genesis, 2002. **34**(1-2): p. 99-102.

68.   Tolwinski, N.S., *Membrane Bound Axin Is Sufficient for Wingless Signaling in Drosophila Embryos.* Genetics, 2009. **181**(3): p. 1169-1173.

69.   Han, C., et al., *Drosophila glypicans Dally and Dally-like shape the extracellular Wingless morphogen gradient in the wing disc.* Development, 2005. **132**(4): p. 667-679.

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