Pharmacological and surgical experiments on wing pattern development of Lepidoptera, with a focus on the eyespots of saturniid moths

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Abstract: The outstanding diversity of wing color patterns found in Lepidoptera has fascinated humans for centuries, but we know little about the common developmental mechanisms that shape this diversity across the order. For instance, the eyespot is a pattern element found in numerous lineages that may be separated by over 100 million years of evolution, but whether it is the result of homologous developmental mechanisms or convergent evolution remains unclear. Here, we review published data on the effects of the medical drug heparin, known to affect wing pattern development in Lepidoptera. We then report on novel experiments using this drug with 38 individuals of *Antheraea polyphemus* and 88 individuals of *Automeris io*, discussing the commonalities and differences between these two species that represent two major lineages within Saturniidae, and between saturniid moths and nymphalid butterflies. Lastly, we report observations of localized changes in wing scale color resulting from between-pupae transplants of presumed eyespot organizers based on preliminary results involving 18 transplants performed on *A. io* and *Actias luna*. The latter surgical procedures were accompanied by control cuts, cross-vein disruptions, and point injuries with strong but conflicting evidence for wound-induced color patterns.

Key words: evo-devo, HS-GAGs, insect physiology, metamorphosis, positional information, Saturniidae, wound-induced responses

INTRODUCTION

Lepidoptera wing color patterns can show striking examples of two extremes of variation: from sexual or seasonal forms so different that they were once classified as different species, to species converging on similar, or almost identical, morphs. This kind of variation, in addition to the rapid response of Lepidoptera wing patterns to natural selection resulting from environmental changes (e.g., industrial melanism in the peppered moth, *Biston betularia* Linnaeus, 1758), have produced textbook examples of evolution in action. The spectacular color patterns of moths and butterflies have no doubt also contributed to making Lepidoptera one of the few flagship invertebrates used for conservation efforts (e.g., New, 1997). In addition to the visual appeal and ecological and evolutionary interest of Lepidoptera, the order has proved to be experimentally tractable; from Goldschmidt's (1940) 'hopeful monsters' to pharmacological/surgical manipulations and a working set of modern developmental tools (expression patterns and levels at the gene or genome scale, gene editing with CRISPR-Cas9, etc.), we have at hand a model system to dissect the genetic and developmental mechanisms behind ecological and evolutionary ("eco-evo-devo") questions.

The bulk of what is known about developmental changes that lead to morphological variation with "eco-evo" relevance in Lepidoptera has been discovered in butterflies (Nijhout, 1991; Jiggins, 2017; Sekimura & Nijhout, 2017), although moths were used as the models for early physiological, genetic and developmental studies (e.g., Goldschmidt, 1942; Caspari, 1949; Williams, 1946). Butterfly wing color variation has been organized into a scheme of presumably homologous pattern elements (Schwantwitsch, 1924), today known as the Nymphalid Groundplan (reviewed in Nijhout, 1991; Sekimura & Nijhout, 2017). Based on their morphology and position, pattern elements are divided into three symmetry systems, namely basal, central and border, found respectively at the proximal, medial, and distal/marginal regions of the wing. Pattern elements of other non-nymphalid families have similar morphologies at corresponding positions (Martin & Reed, 2010), and the Nymphalid Groundplan has since been examined in relation to other Lepidoptera (e.g., Gawne & Nijhout, 2019; Schachat, 2020).

There are around 17,500 described butterfly species, while, overall, Lepidoptera comprises over 15,500 genera and 157,400 species (Nieukerken *et al.*, 2011). Despite over 100 million years of divergence between butterflies and moths (Espeland *et al.*, 2018; Chazot *et al.*, 2019), it has been postulated that some wing color pattern elements shared by them may be homologous (e.g., Martin & Reed, 2010). However, homology of wing pattern elements throughout Lepidoptera remains a hypothesis

to be thoroughly tested by the comparison of the developmental mechanisms behind the formation of such elements between these groups (de Beer, 1971; Wagner, 1989; Abouheif, 1997; Weiss & Fullerton, 2000; Young & Wagner, 2011). Attempting to answer this question would not only increase the phylogenetic breadth of comparative insect development studies but would also expand the research tools optimized in butterflies to Lepidoptera clades with important (e.g., behavioral) differences that also have an order of magnitude more species, represented by over 100 families.

One of the cases supporting possible homology between butterfly and moth developmental mechanisms is the expression pattern for two butterfly eyespot genes, *Distal-less* (*Dll*) and *Engrailed* (*En*), that has been found in the saturniid moths *Antheraea polyphemus* (Cramer, 1776) and *Saturnia pavonia* (Linnaeus, 1758) (Monteiro *et al.*, 2006). Nymphalid eyespots are serially repeated pattern elements that resemble vertebrate eyes, found at the distal region of the wing and hence named "border ocelli" in the Nymphalid Groundplan. In Nymphalidae, such as *Bicyclus anynana* (Butler, 1879) and *Junonia coenia* Hübner, 1822, they are the pattern elements for which we have a lot of knowledge about comparative wing pattern development (e.g., Beldade & Brakefield, 2002; Nijhout, 2010; Beldade & Peralta, 2017). In this family, eyespots are concentrically organized and are hence known as "bulls-eye" eyespots. Eyespots are also found in some Saturniidae, but instead of being at the wing margins, they are positioned in the middle of the wing with the M_2-M_3 cross-vein in the center. In *Automeris io* (Fabricius, 1775) (Fig. 1), this eyespot center is covered with brightly colored, white/UV-reflecting scales, which are surrounded by concentric circles of iridescent black-blue scales mixed with white scales similar to those in the center (here referred to as the gray spot), and a black disc. In other saturniids, the center may be surrounded by a clear (i.e. scale-less) elongated window bordered by yellow scales, and the iridescent gray part shifted basally, as in Polyphemus Moth *Antheraea polyphemus*. The concentric organization can also be greatly reduced, as in the forewing of Luna Moth, *Actias luna* (Linnaeus, 1758), where colorful elements surrounding the cross-vein and the adjacent clear windows are minimal. A few saturniids have both medial eyespots and additional nymphalid-like distal ones, as, for example, on the forewings of Cecropia Moth, *Hyalophora cecropia* (Linnaeus, 1758). While the phenotypic similarity between eyespots of saturniids and nymphalids can be striking, convergent evolution is so common in insects that one cannot assume a homologous developmental

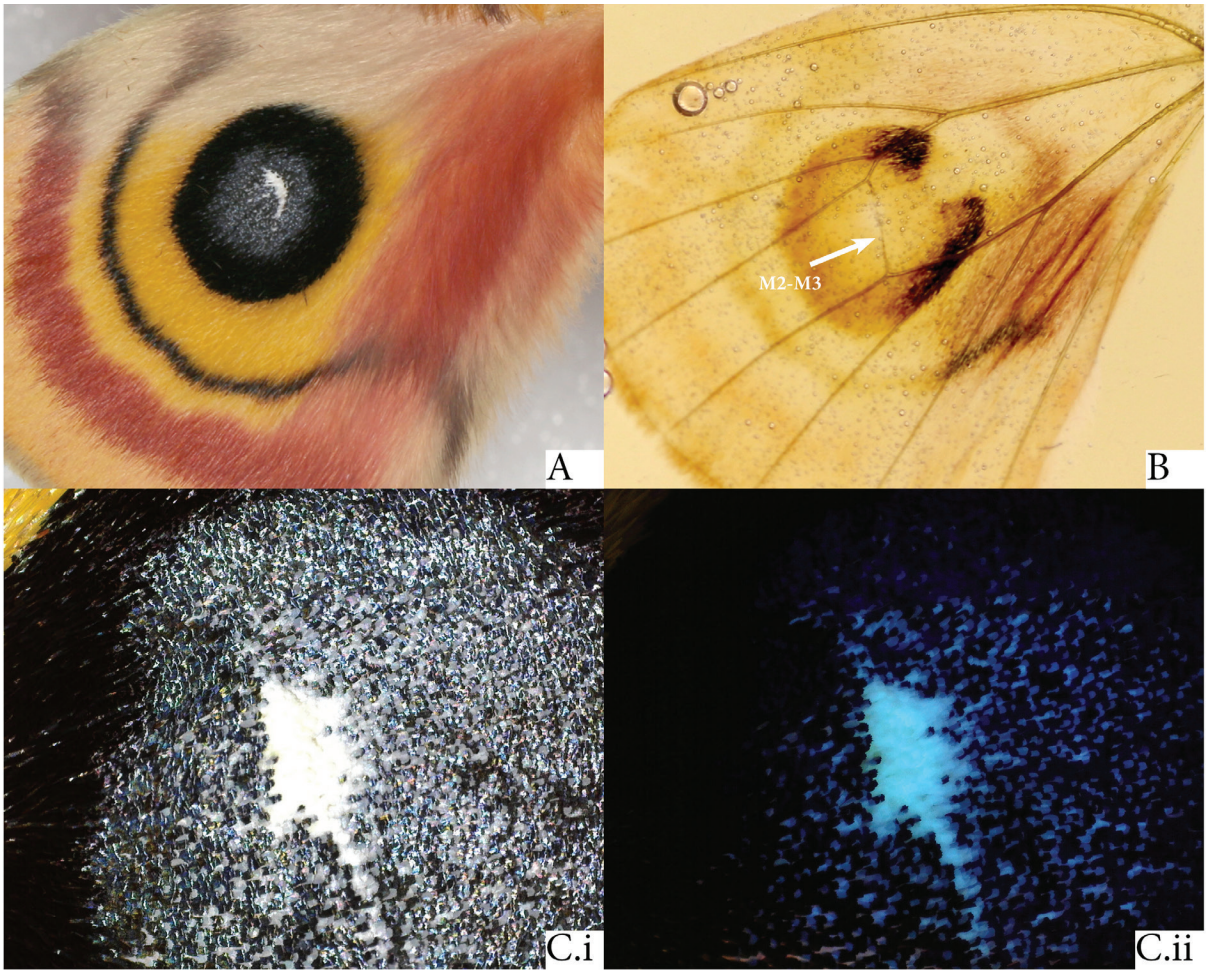


Figure 1. A dorsal hindwing eyespot of *Automeris io*: **A**. intact hindwing, **B**. cleared with bleach to show the underlying venation; **C**. close-up of the same eyespot center under **(i)** white light and **(ii)** UV light.

process. Even comparing gene expression in butterfly wings during the development of a particular pattern element might not satisfactorily answer the question of homology, since homologous eyespots of nymphalids may exhibit dynamic combinations of gene expression during their ca. 90-million long evolutionary history (Shirai *et al.*, 2012; Oliver *et al.*, 2012; see also references above on homology).

Nevertheless, comparative expression patterns have shown that Nymphalidae and Saturniidae share at least two genes associated with eyespot determination (Monteiro *et al.*, 2006). After 2-3 days from the moment the caterpillars began to spin their cocoon, in the prepupal stage, Monteiro *et al.* (2006) found *Dll* and *En* to be expressed in the center of the eyespot of *S. pavonia*, and *En* in the "line marking the elongated central axis of the future discal-cell eyespot" (i.e., the eyespot center) of *A. polyphemus*. Thus, there may be common developmental mechanisms underlying eyespot formation despite the different locations of medial eyespots in moths and distal eyespots in nymphalids. Additionally, the likelihood of some developmental homology in wing pattern development is supported by the finding that heparin injections can affect wing pattern formation in butterflies and moths when they are performed at the stage when the previously mentioned genes have been found to be expressed, the prepupal stage (Sourakov, 2017, 2018b).

Heparin is a highly sulfated form of heparan sulfate glycosaminoglycans (HS-GAGs), known to interact with signaling pathways (Wnt, Hedgehog, Decapentaplegic, Transformation Growth Factor β) from a number of experiments

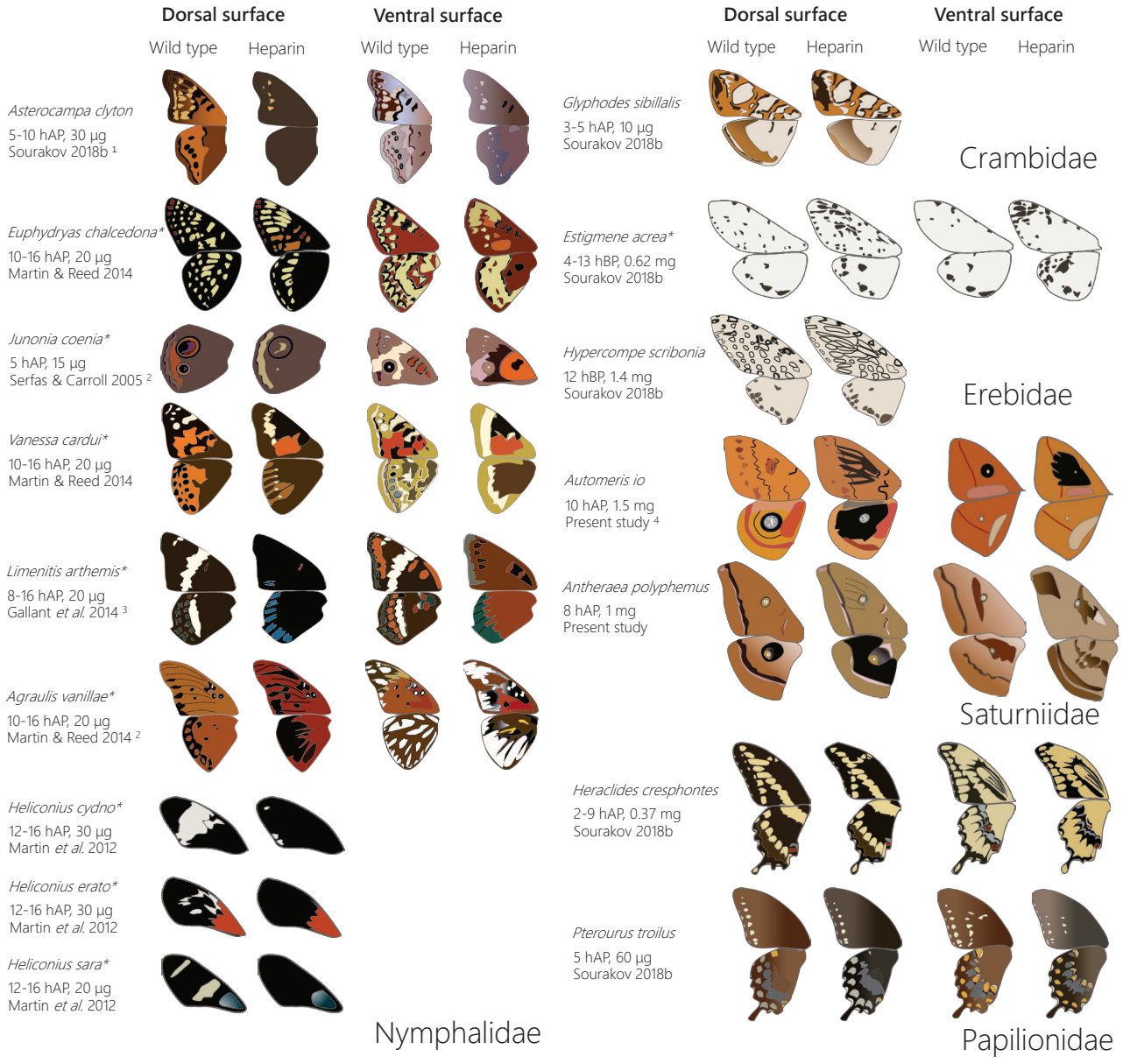


Figure 2. Schematic summary of all Lepidopteran species injected with heparin (nymphalids at the left and other families at the right), compared to the wild-type phenotype (* when it corresponds to an experimental control with such phenotype), for dorsal and ventral wing surfaces, as available. The strongest published effect is represented for each species, with corresponding dosage and time of injection (in hours before or after pupation, respectively, hBP and hAP), and the reference we used for the schematic color pattern changes, without implying changes in wing size or shape. For additional references: [1] Sourakov, 2018a, [2] Sourakov, 2018b, [3] Imhoff, 2016, [4] Sourakov, 2017.

with mammals (e.g., Bradley & Brown, 1990) and insects, such as flies (e.g., Reichsman *et al.*, 1996; Baeg *et al.*, 2001; Perrimon & Häcker, 2004; Selleck, 2000; Nybakken & Perrimon, 2002; Princivalle & de Agostini, 2002). Developmental disruption through pharmacological experiments was pioneered in butterflies with the Common Buckeye, *J. coenia*; eyespots were among the target pattern elements (Serfas & Carroll, 2005). Such experiments were shown to change Lepidoptera wing color patterns, which include eyespots, as well as leading to a complete overwriting of all wing patterns or an expansion of some of the pattern elements at the expense of others, depending on the stage of development, the species, and the dosage (Fig. 2).

While heparin may be disrupting the action of members of any of the major signaling pathways, most of which were shown to be expressed during butterfly eyespot development (latest review in Beldade & Peralta, 2017, see also Özsu & Monteiro, 2017), the best-studied candidate pathway is Wnt, specifically secreted ligands Wnt 1 (or wingless, wg) and WntA, which plays a large and diverse role in nymphalid wing color pattern determination (Mazo-Vargas *et al.*, 2017). While other species, including moths, have been experimented upon with heparin and transformed phenotypes have been achieved (e.g., Sourakov, 2017, 2018a), it is not yet clear if heparin affects a color, a pattern element, a symmetry system, or patterns associated with a gene or a pathway.

In addition to the pharmacological disruption of development, it is also possible to transplant the presumable eyespot organizer during the time when signaling is occurring to the same or a conspecific animal but in a different wing region. The organizer hypothesis proposes that during the development of eyespots, a concentration-dependent, signal-response mechanism determines different colors by the distance from the source (Nijhout, 1990; Monteiro *et al*., 2001; Otaki, 2011). This hypothesis has been validated by two types of surgical manipulations: (a) the transplantation of competent cells which induced ectopic eyespots and (b) cauteries of these cells which reduced or ablated the eyespot (Nijhout, 1980; French & Brakefield, 1995). These competent cells, collectively called the "organizer" (the future eyespot center, or focus), presumably produce signaling molecules that diffuse to and react with surrounding cells, determining their cell fate, or color. This method has been pioneered using Common Buckeye (Nijhout, 1980), and has since been applied to other nymphalid models. However, until the present study, it was never attempted with moths, perhaps due to the difficulty in rearing relevant moth species (e.g., with eyespots) at the scale required by developmental studies.

MATERIAL AND METHODS

In the present study, we aimed to investigate the possible homologous development of Lepidoptera eyespots by using pharmacological as well as surgical manipulations in saturniid moths. Here, we build on previous research on heparin injections in *Automeris io* (Sourakov, 2017) by greatly increasing the number of specimens, varying the heparin dosage injected, and also injecting a single dosage at different developmental stages (time before and after pupation). We also conducted heparin injections on *Antheraea polyphemus*, representing a different evolutionary lineage: *A. io* is placed in Hemileucinae, while *A. polyphemus* is in Saturniinae (Regier *et al.*, 2008). We discuss the evidence for broad Lepidoptera eyespot homology based on a review of publications on heparin injections in the order (Fig. 2). Finally, we attempted a limited number of surgical manipulations by ablating eyespot organizers as well as transplanting potential organizers among conspecific pupae in *A. io* and *Actias luna.* Multiple experiments were conducted in the present study, some conclusive, some preliminary. They are summarized in Table 1 and detailed below.

Heparin injections experiments (H1-H3)

We obtained eggs of *A. io* and *A. polyphemus* in 2017-2018 by keeping female moths caught in Gainesville, Florida (c. 29°38' N, 82°22' W) in paper bags/cages. We reared larvae on *Celtis laevigata* Willdenow (Cannabaceae) and *Quercus nigra* L. (Fagaceae), respectively, at indoor temperatures (around

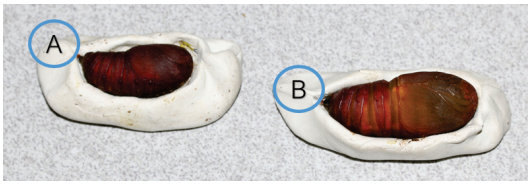
Table 1. Experiments performed in the present study. Unmanipulated control individuals are always siblings to the experimental ("study") individuals.

Experiment	Goal	Manipulation	Species	# of study individuals	Control 1	Control 2
H1	timing and effect of heparin injections	heparin injection of larva, prepupa and pupa	*A. io*	88	>100 unmanipulated	8 phosphate buffer injection
H2	effect of heparin - pilot	heparin injection of prepupa and pupa	*A. polyphemus*	4	1 unmanipulated	
H3	effect of heparin	heparin injection of prepupae and pupa	*A. polyphemus*	34	16 unmanipulated	3 water injection
T1	necessity of eyespot organizer	cut M_2-M_3 cross-vein of pupa	*A. io*	5	unmanipulated wing of each study specimen	
T2	necessity of eyespot organizer	cut M_2-M_3 cross-vein of pupa	*A. luna*	2	unmanipulated wing of each study specimen	
T3	sufficiency of eyespot organizer	transplant M_2-M_3 cross-vein tissue from donor to host pupa	*A. io*	18	unmanipulated wing of each study specimen	3 surgical cuts without any tissue transplanted
T4	sufficiency of eyespot organizer	transplant M_2-M_3 cross-vein tissue from donor to host pupa	*A. luna*	1	unmanipulated wing of each study specimen	
T5	wound-induced response	needle injury to FW of pupa	*A. io*	4	unmanipulated wing of each study specimen	
T6	wound-induced response	needle injury to HW of pupa	*A. io*	1	unmanipulated wing of each study specimen	

Figure 3. The gradient of effects of heparin injections on dorsal surfaces of *Automeris io* (A-D) and *Antheraea polyphemus* (E-G). **A.** normal pattern (unmanipulated wild-type); **B.** 5 ul of 30% (ca. 2 mg*) heparin as prepupa within 1 dBP; **C.** 10 ul of 30% (ca. 4 mg*) heparin as prepupa within 1 dBP; **D.** 4 ul of 27% (1.5mg) heparin as pupa at 10 hAP. **E.** normal pattern (unmanipulated wild-type); **F)** 5 ul of 16% (ca. 1mg) heparin as prepupa 2 hBP; **G.** 5 ul of 16% (ca. 1mg) heparin as pupa 8 hAP. **E.i, Gi,** close-up of FW eyespot, **E.ii, Gii,** close-up of HW eyespot. (*some heparin may have been expelled due to bleeding). Additional experimental specimens are shown in Figs. S2, S3 of Supplementary Material.

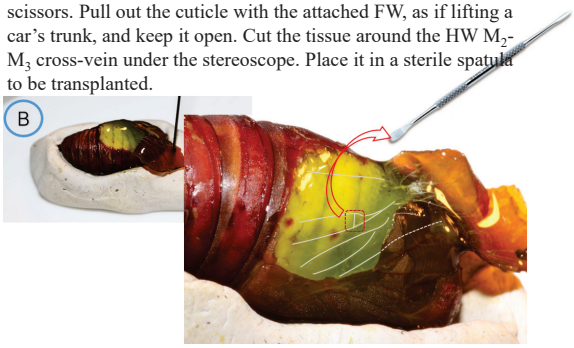
26ºC, 65% humidity). Total developmental time during the larval stage is ca. 2-3 months and 1.5 months, respectively. The prepupal stages last 4-7 days, and adults emerge from pupae within a month, unless they go into diapause, in which case they may take 8-12 month to emerge. Below, we use 'FW' for forewing, 'HW' for hindwing, 'D' for the dorsal wing surface, and 'V' for the ventral. We refer to times of manipulations in days (d) or hours (h) before pupation (BP) or after pupation (AP), using the notation dBP, dAP, hBP, hAP; specific times are detailed in the Results and Discussion sections and in Supplementary Material, Table S1. We determined the injection time in relation to pupation by time-lapse photography using a Dinolite camera (AnMo Electronics Co., Taiwan) connected to a computer, set to take photos every 30 minutes. Sometimes, especially in *A. io*, the color of pupae was used to determine the time since pupation using a previously created photographic calibration (see, Supplementary Material in Sourakov, 2017). This method can be applied with relative precision up to 5-6 hAP. We cut open the *A. polyphemus* cocoons 3 dBP with the top half removed (Fig. S1 of Supplementary Material) and lined

1. Pupae in modeling clay: A is the host and B the donor.



2. Prepare the host by making a fine incision in the FW through the cuticle, at the distal half of the wing (we attempted anterior and posterior regions). This is the procedure of control individuals.

3. Cut the cuticle around the entire FW of the donor with fine scissors. Pull out the cuticle with the attached FW, as if lifting a car's trunk, and keep it open. Cut the tissue around the HW M_2-M_3 cross-vein under the stereoscope. Place it in a sterile spatula to be transplanted.



4. Gently place the donor HW cross-vein inside the host FW incision under the steroscope. Hemolymph is important to keep the incision moist, but avoid "bleeding."

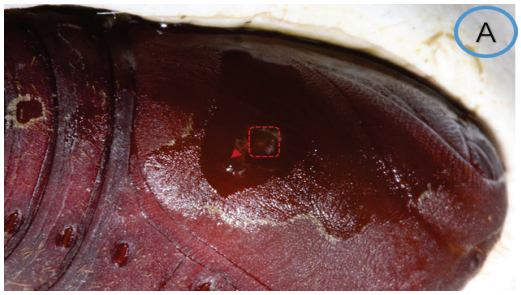


Figure 4. Protocol highlighting the differences in the surgical procedures performed in saturniids, when compared to standard techniques in butterflies (*c.f.* Brakefield *et al*., 2009).

up the prepupae in a tray to check their pupation time.

Heparin sodium salt (porcine, MP Biomedicals, Inc.) was diluted in distilled water at different concentrations varying from 3% to 43%. The corresponding amounts of heparin in a certain volume of solution delivered by injections are detailed in figure legends (Fig. 3 and Figs. S2, S3 and Table S1 of Supplementary Material). We injected *A. io* and *A. polyphemus* immatures using either a sterile 0.3 ml hypodermic syringe (volume measured with a micropipette) or with a 10 ul syringe. We injected *A. polyphemus* pupae through the cuticle, always attempting to keep the needle parallel to the surface of the pupa, so that the injected solution was delivered within the wing compartment (Fig. S1B of Supplementary Material). This was easier to do with *A. polyphemus*, whose pupal wings bulge slightly, than with *A. io*, whose pupal wings are flush with the surface of the rest of the pupa. In a few cases (indicated in the figure legend of Fig. S3 of Supplementary Material), the injection was made deep through the abdomen. Deep injections can cause rapid melanization and death of the prepupae probably due to a punctured gut, so prepupae were injected in the mid-section laterally, under the epidermis, avoiding penetrating the gut (Fig. S1A of Supplementary Material).

A. io experiments (H1) were conducted in 2017-2018 and 88 individuals from 5 broods were injected with heparin. Three transformed individuals were previously reported in Sourakov (2017), including injections at the prepupal stage within 1 dBP, and they showed an effect similar to that achieved by injecting early pupae at 5 hAP. To determine at which developmental stage heparin begins to affect wing pattern, we injected *A. io* starting at the late larval stage at 11 and 8 dBP, when the caterpillar was still feeding. We also injected prepupae after cocoon spinning at 8, 7.5, 7, 5.5, 5, 4, 3, and 2.6 dBP, and pupae until 24 hAP under the same dosage (4 ul of 27%, or 1.5 mg). In order to further investigate the effect of heparin, we added observations on 24 more heparin-injected *A. io* (with different dosages) that successfully emerged (e.g., Fig. 3 A-D and Fig. S3 of Supplementary Material). Over 100 unmanipulated *A. io* individuals from the same broods served as unmanipulated 'controls.' As additional controls, in November 2018, 7 prepupae (1-3 dBP) and 1 pupa (10-15 hAP) of *A. io* were injected with phosphate buffer and compared to 10 unmanipulated siblings (Fig. S4 of Supplementary Material).

For *A. polyphemus* (H2, H3), we conducted a pilot study (H2) in October-November 2017 involving five siblings, four of which were injected with different volumes of 20% heparin (ca. 5 to 20 ul; see figure captions for dosage) and one which was left unmanipulated – four out of five individuals emerged. After observing remarkable wing pattern transformations in the pilot study (Fig. S2 of Supplementary Material), we conducted the second trial (H3) in June-July 2018, injecting 5 ul of solution at different concentrations. Injections were made into 34 individuals with 16 left as unmanipulated 'controls.' In *A. polyphemus*, we made prepupal injections (starting from 44 hBP) and pupal injections (until 13.5 hAP). To help confirm that the change in pattern was caused by heparin and not by the solvent (water) and/or mechanical injury with the needle, in July 2019 we injected 5 ul of distilled water into three *A. polyphemus* pupae (5, 12, and 14 hAP), leaving one unmanipulated (Fig. S5 of Supplementary Material) – three out of four individuals emerged. For the second trial with *A. polyphemus* (H3), 15 unmanipulated and 31 injected pupae or prepupae successfully emerged: one unmanipulated and three injected individuals died as pupae (6% mortality). Heparin concentrations ranged from 4% to 16%, and the times of injections in successfully emerged individuals varied from 44 hBP until 13.5 hAP (see Supplementary Materials). Among the three dead experimental individuals, one fell outside this range, injected at 21 hAP, and the other two were injected shortly after pupation, at 1.5 and 4 hAP.

Organizer tissue transplanting and disruption experiments (T1-T6)

We conducted surgical manipulations involving between-pupal transplants of potential eyespot organizers to test for their sufficiency to form eyespots, as well as disrupting such organizers to test for their necessity in eyespot development. We used only the pupal stage since prepupal wings are too difficult to handle. We did all manipulations with pupae held still in modelling clay, following standard techniques (reviewed in Brakefield *et al*., 2009), unless otherwise stated (Fig. 4).

Firstly (T1, T2), we disrupted the M_2-M_3 cross-vein to determine whether the cross-vein is involved with the formation of the eyespot around it. If the disruption leads to loss or reduction of the adult eyespot, this simple surgical manipulation demonstrates the necessity of the intact cross-vein in organizing the future eyespot. We did cross-vein disruptions in pupae of 5 *A. io* (T1) (1 to 4 hAP, HW cross-vein) and of 2 *A. luna* (T2) (4 hAP, FW cross-vein). Using a sharp sterile needle, the FW cross-vein was cut through the pupal case, since it was easily visible under the stereoscope. For the HW cross-vein, we cut through the cuticle, the FW, and the peripodial membrane to assess the HW and, under the stereoscope, we located and cut the HW cross-vein. While we did not have additional individuals of either species to perform control vein disruptions, the contralateral wing that was left intact served adequately for comparison.

Secondly (T3), we transplanted the supposed organizer tissue (1-2 mm^2 that included the M_2-M_3 HW cross-vein) aiming to induce the formation of an ectopic eyespot in the host, which would demonstrate that the cross-vein is sufficient to determine the cell fate of host cells (Nijhout, 1980; French & Brakefield, 1995). *Automeris io* has a very prominent concentrically organized HW eyespot, so we cut the cuticle around the entire FW of donor pupa (4 to 12 hAP) with fine scissors and held it opened (Fig. 4). We then cut the tissue around the HW M_2-M_3 cross-vein (which removes the tissue of both wing surfaces, dorsal and ventral) and placed it on a sterile spatula to be transplanted. Because of this manipulation, chances of donor survival were low, so we transplanted its tissue to another (host) individual (as opposed to a transplant within the donor). Whenever possible, we used the donor's right and left HW M_2-M_3 cross-veins for two different transplants.

We previously prepared the host pupa (18 individuals of 5 to 14 hAP) by making a fine incision in the FW through the cuticle (1-2 mm), at the distal half of the wing (the responsive region in butterflies, *c.f.* French and Brakefield, 1995), and we gently placed the transplanted tissue inside the wing incision. Some hemolymph was important to keep the cuticle at the incision site moist, but we avoided too much hemolymph ("bleeding") by removing the excess with paper towel, especially at the thin cuticular junctions at which the pupal case opens at eclosion. If this is not taken into consideration, the local melanization due to "bleeding" can make the pupal case harder or impossible to break out of by the emerging moth. We made control incisions in 3 individuals (5, 10 and 10 hAP) by repeating the procedure above without inserting any donor tissue, to check whether there is wound-induced response due only to damage of the host FW, known to occur in butterflies (e.g., Nijhout, 1985; Brakefield & French, 1995). A single transplant in *A. luna* (T4) was possible for lack of individuals (we had no control in this species), using a 5 hAP donor to a 10 hAP host, FW to FW transplant.

Additionally (T5, T6), 4 *A. io* pupae (T5, 2, 4, 4, and 17 hAP) received a FW injury with a sterile needle inside the discal cell to test if this would lead to a wound-induced response (*op. cit.*) inside the FW discal cell. An additional individual (T6, 4 hAP) was punctured inside the discal cell, aiming to reach the HW, through the FW.

We had at hand a limited sample and we did not have enough comparable results to statistically treat the data (that is, enough experimental *versus* control specimens, different types of controls, also controlling for donor and host hAP, wing region, wound-induced response, gender, etc.).

We deposited spread experimental specimens in the collection of the McGuire Center for Lepidoptera and Biodiversity, Florida Museum of Natural History, University of Florida under MGCL Accession Numbers for the following voucher specimens: *A. io*: 286779, 289217-20, 291955-56, 291645-6, 292276, 292055-6, 292280, 291957, 292284, 286790, 291637, 291644, 286791, 292279, 292281, 291648, 292282-3, 291690, 291953, 291692, 291682-3; *A. polyphemus*: 291924-27, 292961-62, 292083, 292104-111, 292236-254, 292260-274 (Table S1 of Supplementary Material); and *A. luna* 291928-30. We also used the MGCL collection to assess the presence of aberrations in wild populations of *A. polyphemus*, for which 797 specimens were examined (see Fig. 5 and Fig. S6 of Supplementary Material for examples).

RESULTS

Heparin injection experiments (Table 1, H1-H3)

Heparin injections caused noticeable transformations of the wing patterns in both *A. io* and *A. polyphemus* (Fig. 3 and Figs. S2 and S3 of Supplementary Material). To illustrate different degrees of the response to heparin in *A. io*, the 3 males (one unmanipulated control and two experimental prepupae injected with 5 ul and 10 ul of 30% heparin solution, respectively) from Sourakov (2017) are shown alongside a male pupa which was injected with 4 ul of 27% solution at 10 hAP (Fig. 3A-D). The effect of heparin in these three individuals range from a slight proximal expansion of black scales around the eyespot to an extreme expansion of black beyond the borders of several pattern elements in the HWd. The injection did not seem to affect the central white dash nor the gray spot at the center of the eyespot and made no effect on the corresponding ventral pattern of the HW. However, whenever the FWd responded to heparin at the discal cell (at the "DII" pattern element) as black finger-like streaks above the venation (Fig. 3C,D), it was always accompanied by a substantial expansion of the black disc surrounding the corresponding ventral pattern - the FWv eyespot (Fig. S3A,B) of Supplementary Material). If such disruption of the FWd did not occur, the FWv eyespot was not affected.

The lowest amount of injected heparin which led to wing pattern change occurred when 1 ul of 3% (0.03 mg) was injected at approximately 8 hAP deep into the pupal abdomen (Fig. S3I, J of Supplementary Material). The manner in which the

injections were made in these two specimens ensured that no heparin was lost due to bleeding. In the present study, injections for the timing experiment (Table 1, H1) resulted in specimens with wild-type patterns, with the exception of 1) the individual injected at 3 dBP (specifically, 75 hBP), which showed the expected expansion of the black disc around the HW eyespot, despite the wings not having fully expanded on one side (Fig. S3G of Supplementary Material); and 2) the one injected at 2.6 dBP (specifically, 63 hBP; Fig. S3K of Supplementary Material), where the black disc also expanded, but the formation of the M_2-M_3 cross-vein was seemingly aborted, and the center of the eyespot did not form. After pupation, HWd eyespot expansion was achieved in specimens injected from 5 to 10 hAP (Fig. 3D, Fig. S3 of Supplementary Material).

In 21 of the heparin-injected *A. polyphemus* (Table 1, H2-H3), the black region inside the HWd eyespot changed, sometimes significantly, resembling responses found in *A. io* (Fig. 3E-G and Fig. S2 of Supplementary Material). However, while in *A. io* the black disc expanded proximally and, with even further increase in heparin's effect also expanding distally, in *A. polyphemus* the black disc did not expand as much basally, and certainly not inside the discal cell, as if prevented from doing so by an invisible border. While in the parental and unmanipulated sibling groups of *A. polyphemus*, the proximal gray region of the eyespot was confined to a narrow semi-circle proximal to the yellow eyespot ring (e.g., Fig. 3E and Fig. S2A of Supplementary Material), in 11 experimental specimens, it expanded basally (e.g., Fig. 3F,G and Fig. S2B,C of Supplementary Material), sometimes touching the proximal pink contour, widening it into a lighter pink. In the most extreme case of heparin-induced transformation, in which the HWd background color became mostly black (e.g., Fig. 3G), the area around the normally-simple FWd eyespot (Fig. 3E.i) acquired the gray spot pattern and resembled HWd eyespots (Fig. 3G.i). As in *A. io*, the central part of the eyespot, here composed of the scale-less center and yellow ring, does not seem to have changed with heparin, even when the strongest effects were observed.

To understand at what point during development the wing pattern is subjected to change in *A. polyphemus*, we injected 6 pupae before 5 hAP, none of which showed any difference to unmanipulated control siblings, independent of the dosage (2.5, 3.25 and 4 hAP injected 3.7% heparin; 2 individuals of 4 hAP injected 5% heparin; and 2 hAP injected 15.7% heparin). Individuals injected at 5 hAP varied in their responses to heparin injection, seemingly correlated with the concentration (3.7% and 5% no effect, and 13% and 25.7% some effect). However, increase in volume at a high concentration (10 ul at 21%) can still produce the maximum effect, as shown by an individual at 5 hAP (Fig. S2C). From 4 to 36 hBP and 9 to 13.5 hAP there were noticeable changes, but not in a consistent fashion. We observed the strongest effects from injections into prepupae at 1 to 2 hBP, and 8 to 9 hAP, even at smaller doses of heparin. In five specimens, the formation of the adult HW was completely aborted (this happened when injections were made at 2.5, 4, 5, 9.5 and 10.5 hAP). None of these specimens showed any signs of transformation in the healthy wings. None of our control water/buffer injections, whether into *A. io* or *A. polyphemus,* resulted in transformations similar to those caused by heparin (Figs. S4 and S5 of Supplementary Material). Photographs of all individuals from *A. polyphemus* study and the corresponding injection data (Table S1) can be found in the Supplementary Materials.

Organizer tissue transplanting and disruption experiments (Table 1, T1-T6)

To explore whether the M_2-M_3 cross-vein acts as an organizer in *A. io*, we disrupted this cross-vein in 5 *A. io* pupae from 1 to 4 hAP (T1) but without any result. While we clearly observed disrupted, disconnected cross-veins under a microscope following the procedure, all resulting adult moths were normal and showed an intact cross-vein.

In an attempt to disrupt the M_2-M_3 cross-vein of a Luna Moth pupa at 4 hAP (T2), the resulting adult had an eyespot with normal phenotype, but a wound-induced eyespot-like color pattern appeared inside the FW discal cell (Fig. 6A), which was not observed in the contralateral wing. This novel element, arranged in a circular pattern, is composed of a mixture of black and blue scales surrounded by a white border reminiscent of the scales surrounding the eyespot basally. The second Luna Moth in which we disrupted the cross-vein (T2) showed no abnormalities, which may be due, as in *A. io*, to regeneration.

The eyespot-like response in the *A. luna* individual (T2, Fig. 6A), compared with the single Luna Moth transplant where only white scales appeared at the transplanted site located distally (T4, Fig. 7C), suggests that the proximal (vs. distal) part of the wing may be more responsive to wound-induced color changes. Two additional *A. io* experimental individuals may support this hypothesis, in which black spots appeared proximally, as a response to injury in the HWd. One of them was meant to be a HW vein disruption (at 4 hAP, T1), which was made through the FW; the M_2-M_3 cross-veins of both wings overlap inside a pupa so veins could be cut on both wings. Only a slight scar appeared with little or no scale color changes on the dorsal and ventral FW, consistent with other control cuts, but on the HW, while the cross-vein was intact, the black spot appeared (Fig. 6B). Following this result, we later tried to replicate it by cutting another female pupa roughly at the same spot at 4 hAP intentionally reaching the HW (T6, cross-vein was not targeted this time). The result was similar – a local change of scale color to black (Fig. 6C).

Sixteen of the 18 host individuals of *A. io* receiving transplants of HW M_2-M_3 cross-vein tissue (T3) survived, of which 6 had holes in their wings and the remaining 10 had intact wings. Among the 10 specimens with intact wings, 8 demonstrated localized changes in scale color at the transplant site: from normally mahogany-brown to black in 5 females (Fig. 7A), and from normally amber color to brown in 3 males (Fig. 7B). Despite clearly not looking like an eyespot, these changes do not appear to be due to injury, as the experimental controls showed no such color change: 1) the control cuts (n = 3, at 5, 5, and 10-12 hAP) in the FW resulted in visible wounds but never showed any color change around the wound (Fig. 8C,D); and 2) individuals in which we made wounds inside the FW discal cell (T5) produced no visible effect on the resulting adult moths (Fig. S7 of Supplementary Material).

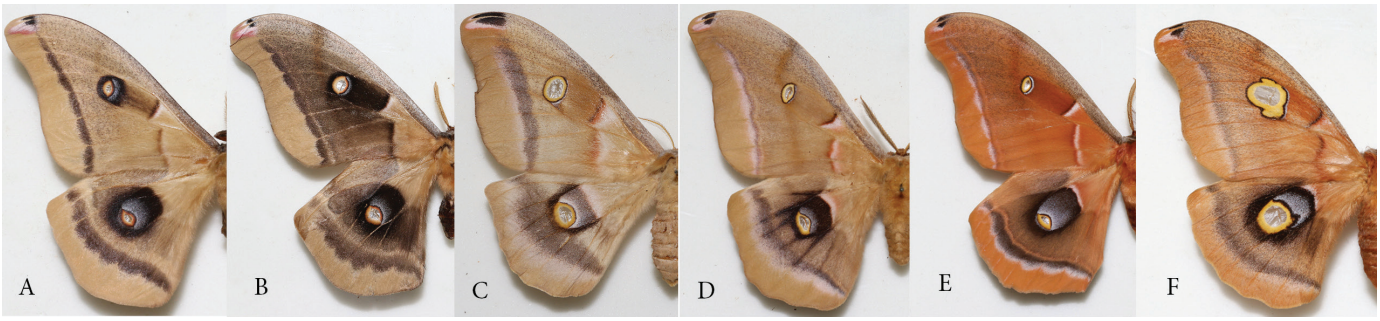


Figure 5. Several unusual *Antheraea* phenotypes from the MGCL collection including sister species and rare/unique aberrations: **A-B**. *A. oculea*: **A**. Arizona, Aug 1999 (ex ova); **B**. Pima Co, Arizona, 7 Aug, 2005, demonstrating melanism around not only HWd but also FWd eyespot (A) and throughout the wing (B); **C-F**. *A. polyphemus*: **C.** Chicago, Illinois, 3 June 1921, demonstrating an eyespot that lacks distal (within discal cell) portion of the black disc; **D**. Salt Lake Co., Utah, 5 Sept 1987 (ex ova) demonstrating HWd black disc expansion along veins; **E**. Florida, demonstrating a combination of slight black disc diffusion combined with an enlarged gray spot; **F**. Quebec, June 2005 (ex ova) demonstrating a unique aberration in which the clear window and the yellow ring forming the center of the eyespot, but not the black disc, are expanded, suggesting that the central elements of the eyespot are under different controls from the black disc.

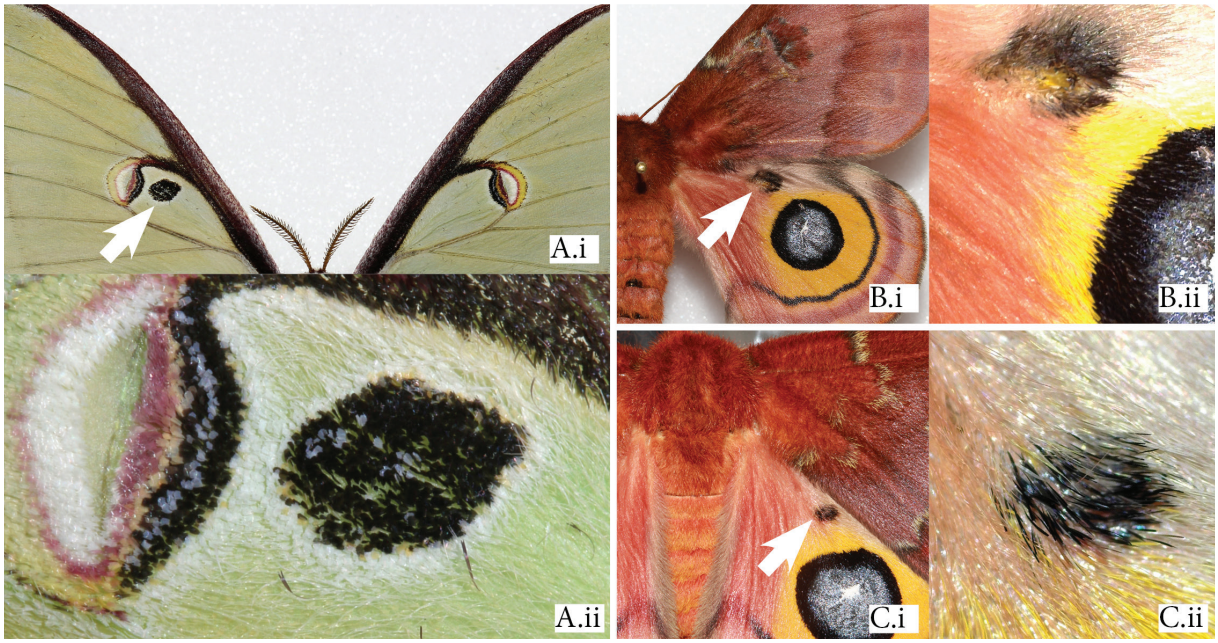


Figure 6. Wound-induced responses in: **A**. *Actias luna*: Wound-induced response to a cut inside the FWd discal cell of a pupa at 4 hAP; and, **B,C**. *Automeris io*: Wound-induced response to a cut to the proximal part of HWv of pupae at 4 hAP.

The change in scale color due to transplant sometimes occurred not only on the FWd, but also on the corresponding surface of FWv (Fig. 8A). In one of the transplants (from a 4 hAP donor into a 9 hAP host) the individual lost the scales dorsally, but formed a ring of black scales in the corresponding spot ventrally (Fig. 8B).

DISCUSSION

Eyespot development and heparin influence on wing pattern

Signaling molecules in butterfly wing development presumably define the boundaries of pattern elements by positional cues achieved through reaction-diffusion, to which cells respond according to threshold-dependent signal levels (Nijhout, 1990; Monteiro *et al*., 2011; Otaki, 2011). Heparin does not alter the production or degradation of signaling molecules. Instead, heparin and other HS-GAGs act on the extracellular space, binding to signaling molecules and aiding the assembly with their receptors (reviewed in Selleck, 2000; Nybakken & Perrimon, 2002; Princivalle & de Agostini, 2002; Perrimon & Häcker, 2004). When we inject heparin into developing Lepidoptera, it overstimulates the signaling pathway(s), acting as a "gain-of-function" experiment along the space into which signaling molecules can act and diffuse. Specifically, this could mean an enlargement of patterns defined by these signaling molecules or, as we argue here, a disruption of pattern boundaries. The difference between the former and the latter is that an enlargement of pattern element should not alter its shape, though the proportions may be skewed, and it would still resemble the wild-type pattern because boundaries

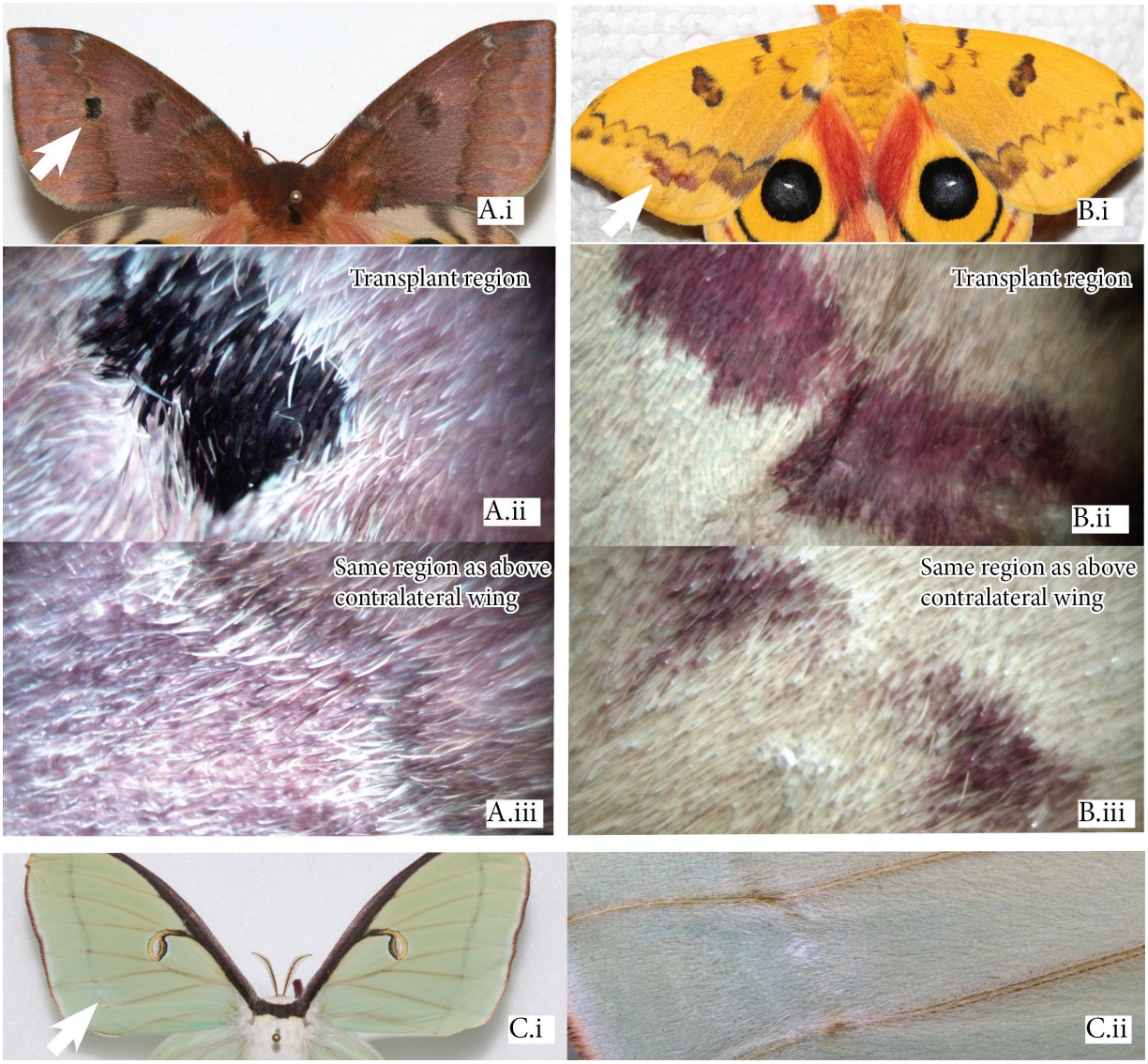


Figure 7. Transplants in *Automeris io*: **A**. local change in scale color in 1 of 5 females, resulting from M_2-M_3 cross-vein transplant (7 hAP donor pupa into a 14 hAP host pupa), **B**. 1 of 3 males where a transplant into the distal part of the wing (8 hAP donor into a 8 hAP host) produced local change in color from yellow to brown, expanding the wing pattern element, with (ii *vs*. iii) transplant region under microscope *vs*. corresponding area on the contralateral wing; and *Actias luna*: **C**. FW cross-vein to FW distal region transplant (5 hAP donor to a 10 hAP host).

are maintained. In contrast, if it is the disruption of pattern boundaries that occurs, we should observe a smearing of the color — that is, a loss of the distinct shape of the pattern element.

The most extreme heparin-induced color changes so far achieved in Lepidoptera (Fig. 2) suggest a breakdown of the boundaries in pattern elements, disrupted in different ways in different species. A lack of consistency among species is seen in: (a) the colors that are affected (such as black, red, orange, or pigment-based *versus* structural colors), (b) the color to which a pattern is transformed, (c) the direction in which the pattern element expands (e.g., proximally, distally), (d) the type of pattern element (e.g., bands, eyespots), or (e) the symmetry system of the 'Nymphalid Groundplan' that is disturbed. While pattern elements in every symmetry system can be influenced by heparin, not all elements in the system are always affected. There is no consistency in the way a given element changes in different species, for example, in eyespots, they may expand (saturniids) or disappear (nymphalids), with some or all rings affected. Tentatively we suggest that a single component of this pattern element, the eyespot center (when there is one), may, consistently across species, remain unaltered by heparin injections. Therefore, we suggest that what underlies the breakdown of boundaries in a wing pattern element is the underlying developmental mechanism determining this element. If butterflies and moths have homologous wing patterns, including the eyespot, we could expect conservation of the molecular pathway(s) responsible for determining pattern elements. Furthermore, we could also expect that this pathway acts by reaction-diffusion, in a concentration-dependent

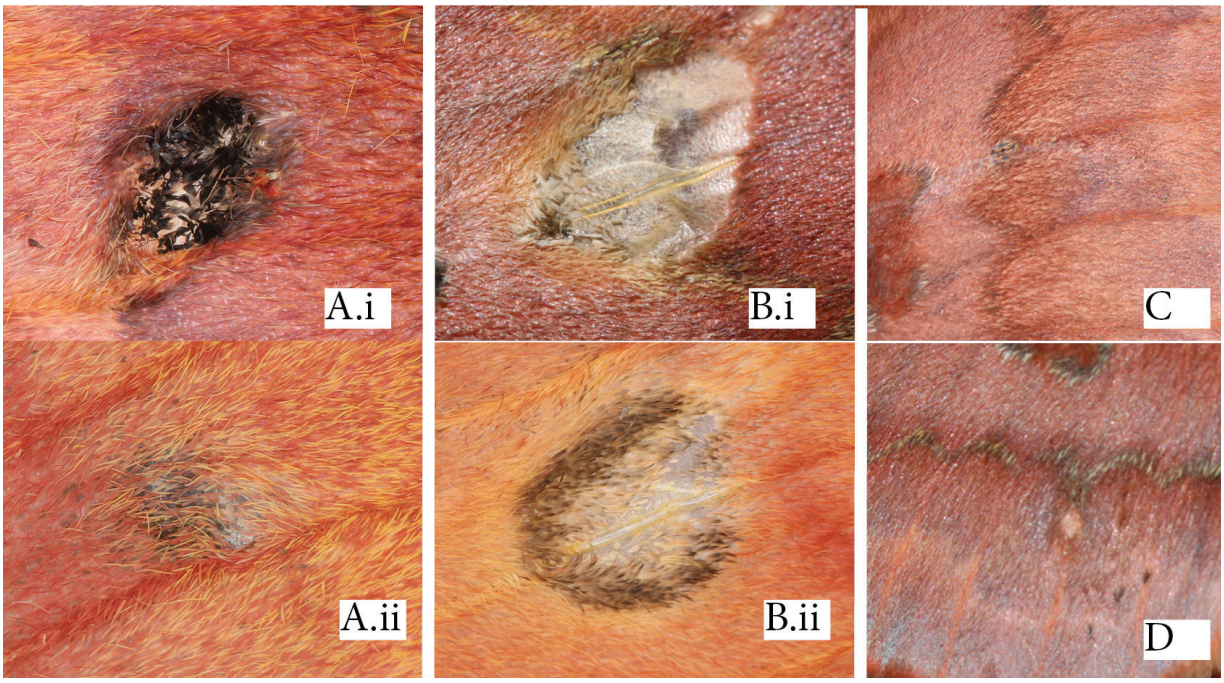


Figure 8. Additional examples of affected wings (both FWd and FWv) in *Automeris io* M_2-M_3 cross-vein transplant (A,B) vs. control cuts (C,D): **A**. from a 4 hAP donor pupa into a 7 hAP host pupa, affecting both wing surfaces; and, **B**. from a 4 hAP donor pupa into a 9 hAP host pupa, removing scales in both surfaces but generating a larger response in the ventral surface; **(i)** dorsal, **(ii)** ventral; **C**. Cut made at 5 hAP, **D**. Cut made at 10-12 hAP.

manner. Thus, the first place to search for candidate pathways is among signaling pathways, especially those known to interact with heparin.

It has been suggested, based on associations of expanded pattern elements in the adult butterfly and *Wnt* expression in larval wings, that heparin targets the Wnt pathway (Martin & Reed, 2010, 2014; Martin *et al.*, 2012; Gallant *et al*., 2014). Although Wnt loss-of-function experiments (such as *WntA* knock-out using CRISPR-Cas9, Mazo-Vargas *et al.*, 2017; or *wg* knocked-down with RNAi, Özsu *et al.*, 2017), are not the exact opposites of gain-of-function experiments (such as heparin injections), it does appear that Wnt is indeed the strongest candidate to be the target of heparin in Nymphalidae. This conclusion is clearly supported by experiments on *Agraulis vanillae*, where the effects of *WntA* transgenic loss-of-function is complimentary to heparin gain-of-function (Martin & Reed, 2014).

WntA and *wg* determine the boundaries of a diverse array of pattern elements in different symmetry systems that have different colors, which might explain why heparin does not consistently act on a fixed location on the wing, on a single type of pattern element, nor on a particular color. If heparin indeed acts on members of the Wnt pathway, it seems plausible that saturniid eyespots are formed through a signaling process, with reaction-diffusion, homologous to that presumably found in nymphalid eyespots. In future, this potential homology should be examined at the gene level, focusing on *WntA* and *wg* which, in the case of the eyespot of Io and Polyphemus moths, should be expressed at the location of the black disc (affected by heparin) and of the focus (the center of production of signaling molecules – notice that the eyespot center did not seem to be affected by heparin, perhaps because it might be insensitive to higher signaling levels since it already has the highest level of signaling molecules among eyespot rings). However, if other signaling molecules are detected at the location of *Wnt* expression, that could mean that heparin binds to signaling molecules other than Wnt. Other signaling pathways (e.g. dpp, Notch, and more recently, Toll) have been implicated in butterfly eyespot development in studies using transcriptomics (Özsu & Monteiro, 2017).

What remains unexplained from this gain-of-function experiment, that is, heparin injection presumably increasing the action of members of Wnt pathway, is that some pattern elements are reduced or are altogether eliminated (Martin & Reed, 2010, 2014; Martin *et al*., 2012; Gallant *et al.*, 2014). These happen to be Wnt-negative patterns, but the absence of Wnt signaling in wild-types does not mean the adult patterns should be reduced when injected with a drug that enhances a signal they normally lack. Perhaps this reduction or elimination is caused by the systemic action of heparin binding to different signaling pathways (not just Wnt), as demonstrated in the literature for other model organisms. This explanation assumes that the binding site of the conjugate heparin+signaling molecule(s) to extracellular receptors is generic, and that signal transduction only occurs when the conjugate binds to the Wnt receptor, Frizzled. In pattern elements where reaction-diffusion does not involve the Wnt pathway (Wnt-negative patterns), receptors would remain clogged, which would lead to an absence of their normal signaling levels. Such cells would receive no developmental instruction (of “cell fate”), causing

them to adopt the background color (the "default" color) or the color of a neighboring inductive pattern element, thereby "disappearing."

Heparin injection experiments

Our experiments demonstrated that eyespots of Saturniidae are affected by heparin (H1-H3), as control injections with water, buffer and other control solutions performed on the saturniid species studied here, as well as other species, did not produce similar effects on wing pattern (e.g., Serfas & Carroll, 2005; Sourakov, 2017, 2018).

It is not easy to judge how much heparin makes its way into the organism if there is bleeding from the injection site. Hence, it was important, in our opinion, to also make several injections deep into the pupal abdomen of *A. io*, where no bleeding occurred. These injections also demonstrated the lowest amount (0.03 mg) at which transformation can be achieved in an 8 hAP *A. io* pupa. It is interesting to note that, under this dosage, the wing pattern change (HWd eyespot black disc expansion) in the female specimen (e.g., Fig. S3I of Supplementary Material) is much smaller than in the male specimen (e.g., Fig. S3J of Supplementary Material). This could be explained by the difference in the body mass of the two individuals: females are at least twice as heavy as males in this species, and the former, in our experience, are much more likely to survive higher dosages of heparin. It should be noted that, when designing heparin injection experiments, one should take into consideration the relative size of the species and the stage of development, as can be observed from the dosages used here and by previous studies (Fig. 2, where some species had mg and others ug of injected heparin). Sourakov (2018b) discussed how change of weight during development of immature stages in Lepidoptera, together with intraspecific variation, might affect the results of heparin injections, illustrating the issue with a variety of examples.

Among the 797 *A. polyphemus* examined at the MGCL, which span the entire United States as well as Mexico (see Fig. S6 of Supplementary Material), the gray spot of HWd eyespot is expanded in 5.5% of individuals (e.g., Fig. S6C,I,S). However, the gray spot expansion and black disc expansion together (such as in heparin-injected individuals shown in Fig. 3F and Fig. S2B of Supplementary Material) were only present in 1% of MGCL specimens (e.g., Fig. 5E). The latter figure shows cases of unique eyespot aberrations found in MGCL holdings of Polyphemus Moth, and a larger variety are also illustrated in Supplementary Material Fig. S6.

In Western Polyphemus Moth, *Antheraea oculea*, formerly a subspecies of *A. polyphemus*, the increase of HWd melanism, including expansion of the black disc outside of the eyespot, is typical, although melanization affects both FW and HW (Fig. 5A,B). It is possible that the phenotypic differences between the *A. polyphemus* and *A. oculea* are based on similar developmental mechanisms that were affected by heparin in our experiment. The genetic difference between them may manifest itself in *Wnt* genes, as has been recently found in closely related species of Buckeye butterflies (Lalonde & Marcus, 2019). As for *Automeris*, in extreme cases, heparin results in a dramatic expansion of the black disc of the HWd eyespot combined with the expansion of the black disc on the FWv and changes in the "DII" pattern element into black finger-like streaks (as seen in Fig. 3C,D), which is even more rare in nature. We are aware of only a single similar case that appears to be natural, in a specimen *Automeris zugana* H. Druce, 1886 collected in Colombia in 1967 and illustrated by Lemaire (2002, plate 55). Similarly, when searching the collection for aberrations similar to those caused by heparin in *Agraulis vanillae* (see Fig. 2), only a single such aberration was found in the 1000 specimens examined (Sourakov, 2018b). Despite the rarity of these aberrations in nature, the fact that they do occur means that they reflect phenotypic diversity potentially available for selection, and hence such aberrations should be seen as a window into the mechanisms by which wing pattern diversity may evolve, instead of being treated just as curious artifacts.

How does the timing of injections relate to wing pattern change?

Based on the time series results (H1), we suggest that signaling molecules affected by heparin in *A. io* are active from approximately 3 dBP and extend to at least 10 hAP, with an inactive period right after pupation (until about 5 hAP). While we did not test the whole spectrum of development in *A. polyphemus*, heparin had an effect from 1.5 dBP to 13.5 hAP in this species, also with the possibility of an inactive period right after pupation.

Injections made at 20 hAP had no effect on the wing patterns of Buckeye butterflies (Serfas & Carroll 2005), but those made at the prepupal stage (2.5-20 hBP) by Sourakov (2018b), a stage hardly investigated in butterflies, did show similar results to the ones obtained at early pupal stage by Serfas and Carroll (2005). Other species that showed an effect on wing pattern from prepupal injections include *A. vanillae* (1-48 hBP), *A. clyton* (1-20 hBP), and tiger moths, *E. acrea* and *H. scribonia* (12-13 hBP) (Sourakov 2018b). The results of numerous injections suggest that heparin injected too early (e.g., 6-9 dBP) has no effect on wing pattern, but that already at 3 dBP heparin may have a significant effect on wing pattern elements. If heparin action is informative about the signaling molecule Wnt, the action of this signaling molecule starts in the prepupal stage, earlier than current data suggest, e.g at 10 hAP for *wg* (Özsu *et al.*, 2017, and 10.5 hAP for wg protein; see below). Expression patterns of *Dll* and *En* (that respond to Wnt in *Drosophila* (Arias, 2003)) were found to be active at 3 days after the start of cocoon-spinning in *A. polyphemus* (approximately 4 dBP in our experiments) and 2 days after cocoon-spinning in *S. pavonia* (Monteiro *et al.*, 2006). Handling prepupal wings for assays of expression patterns is technically difficult, but perhaps transcriptomics could be a strategy to inspect whether signaling molecules are already active in Lepidoptera wings at this stage.

Previous research on imaginal disk development conducted on Buckeye butterflies (Miner *et al*., 2000) suggests that neither size nor stage of larval development (days into the final instar after molting) are good predictors of the development of the wing disk. A much better predictor turns out to be the rate of development, and both starvation and Juvenile Hormone (JH) presence can inhibit wing disk growth. Hence, when conducting heparin injection in larvae of a similar developmental stage we

may be experimenting on quite different animals from the point of view of their wing disk development. Research on the sphinx moth *Manduca sexta* (Browder *et al.*, 2001) demonstrates that JH correlates with larval weight and peaks after the larvae pass the critical weight at which JH secretion ceases. In Sourakov (2018b, Fig 8), a sharp decline in larval/prepupal weight was demonstrated for a number of Lepidoptera after they stopped feeding and began to prepare for pupation (spin silk pads or cocoons). This beginning of weight-loss, according to Browder *et al.* (2011), correlates with the end of JH secretion and perhaps gives rise to the stage at which heparin becomes active as a wing-pattern-altering agent. The role of JH in affecting the ability of heparin to change wing pattern may extend beyond this point. Nijhout and Wheeler (1982) point out that JH titers are affected not only by the activity of the *corpora alata* but also by variation in the activity of enzymes that break it down, so it is not easy to determine based solely on the larval relative size or days prior to pupation how much JH it has. In the developmental stages of interest from the point of view of the present experiments (prepupa a day before pupation – early pupa), a sharp decline/absence of JH and changes in ecdysone secretion have been demonstrated (Nijhout and Wheeler, 1982, p. 115, Fig. 1). Thus, it is reasonable to hypothesize (and warrants further investigation) that titers of either or both of these hormones play a role in making developing Lepidoptera wings sensitive to Wnt signaling and thus to heparin.

Evidence also continues to grow that while wing patterns are sensitive to heparin in late prepupae and early pupae, they may be less responsive immediately after pupation, when the pupa is still soft and just beginning the tanning process. Could there be a 'buffer' time in the transition from prepupa to pupa during which no signaling occurs? In support of this hypothesis, during the first hours of pupation (3-6 hAP), no wg expression was found in the butterfly *Bicyclus anynana* (when several other eyespot genes were found to be expressed, Özsu & Monteiro, 2017), the earliest moment wg was ever detected being 10 hAP (Monteiro *et al.*, 2006, Özsu *et al.*, 2017). If one is to compare these results to the timing of the hormonal activity in developing Lepidoptera (Nijhout & Wheeler 1982, p. 115, Fig. 1), one can hypothesize that the insensitive period to heparin overlaps with the insensitive period to JH and low levels of ecdysone. The idea of a 'buffer' time regulated by ecdysone might also relate to developmental milestones, as demonstrated in *Drosophila* wing discs (Oliveira *et al.*, 2014). Milestones are "checkpoint" moments, such as at pupariation, when wing and whole-body development align, ensuring their coordination in the face of environmental or physiological variation. Judging by our results, a refractory period between 0 to 5 hAP, possibly regulated by hormones, may exist in Lepidoptera, when no signal-response occurs. It would be interesting to evaluate whether the first five hours of pupation also serve as a checkpoint in Lepidoptera wing pattern development.

Organizer tissue transplanting and disruption experiments

We showed, though on a limited scale and with the central drawback of lacking some experimental controls, that transplants of pupal tissue taken from the center of a developing saturniid eyespot might lead to localized change of wing scale color in another individual (T3-T4). While one could argue that melanization could potentially occur in the transplant spot in response to injury, this explanation is not consistent with the results obtained by us in males of *Automeris io*, where the changed scales are brown, not black (Fig. 7B.ii). The single Luna Moth transplant could also serve as evidence against melanization through injury: the changed scales in the transplanted site are white, not black (Fig. 7C). If the signal from the transplanted tissue influenced the FWd inducing localized color changes, that would support the M_2-M_3 cross-vein signaling hypothesis. The same distal wing regions did not react to control cuts or injuries in a similar way. Instead, at most, they showed a small scar, but mostly completely regenerated (T3-T6). Additionally, based on observations of cross-vein disruptions at early stages of pupal development (T1-T2) with not a single change detected, we suggest that *A.io* wing veins and membranes might have a high capacity for regeneration.

However, when injuries were made in other wing areas, such as the discal cell of *A. luna* FWd (T2, Fig. 6A) and the anterior region of *A. io* HWd (T1, T6; Fig. 6B,C), we observed wound-induced responses. These results suggest that the responsive region for eyespot formation may be within the discal cell, and not at the distal region of the wing as in the butterfly *Bicyclus anynana* (Brakefield & French, 1995), which is also the region used in the present study for transplants. We thus suggest that future attempts to transplant tissue in Saturniidae should be done in the medial region and within the discal cell or, more generally, that transplants should target the wing region/symmetry system and wing surface that bear eyespots. This should be tested and the best region optimized before performing future transplants with appropriate controls. Surgical manipulations involving between-pupae transplants of potential eyespot organizers show promise for future exploration of wing pattern formation in saturniid moths, including a more in-depth exploration of wound-induced color changes in Lepidoptera.

The 18 inter-pupal transplants conducted on Io Moth required many host pupae and a number of additional donor pupae at a similar stage of development. This translates into a substantial rearing and pupa-monitoring effort, which, in case of this species, required much time and planning, as Io Moth larvae were raised on a natural hostplant (no artificial diet exists for this species), and larvae develop over period of 60-90 days. In addition to their long larval development and the large amounts of hostplant material needed to rear numerous saturniid moths, there was a challenge of transplanting the M_2-M_3 cross-vein from the HW, and not the FW as usually done in butterflies. In early pupae, the developing FW is attached to the cuticle at the time when surgeries are typically made. When this type of manipulation is normally conducted, the donor FW tissue attached to the cuticle is rotated 180° and scale orientation in the adult identifies where the transplanted tissue was placed. Handling the hardening cuticle (and the "hitchhiked" FW) is much easier than a loose developing wing tissue, which was the case in our saturniid transplants. More importantly, we left the donor tissue "floating" on the host wing tissue, anchored in a cut we had made, which made it impossible to judge, upon the host emergence, whether the tissue at the transplanted site was (a) the

regenerated host tissue, (b) partially incorporated transplanted donor tissue, or (c) the host tissue affected by signaling from the transplant. Although we did not have conclusive results, we report our methods and preliminary results to prevent other researchers from potentially losing precious specimens in trials that we already attempted. However, we strongly suggest that future experiments need to have more adequate controls, for instance involving not just a control cut, as was done here, but also insertion of tissue from a different part of the donor's wing to see whether it too leads to local color change.

CONCLUSIONS

Our results provide evidence both for and against homologous development of eyespots within Saturniidae, and between saturniid moths and nymphalid butterflies. Evidence suggesting a lack of homology includes: 1. The gray spot of the Polyphemus Moth eyespot expanded basally under the influence of heparin, while in Io Moth it was not affected, which could suggest that these eyespots may be non-homologous in the two species. However, it should be noted that the black disc expanded in both species and potentially could be formed by a homologous process, although in Io Moth the disc expanded basally, and in Polyphemus Moth not much so, and not inside the discal cell. 2. In saturniid moths, an expansion of some of the colored rings occurred under the influence of heparin, while in nymphalid butterflies, there is a tendency for the whole or parts of the eyespot to shrink or disappear.

Evidence supporting homologous eyespot development in butterflies and moths includes: 1. Heparin seems to disrupt the boundaries of pattern elements, including eyespots. Such pattern elements are most likely those defined by Wnt signaling, independent of where they are in the wing. 2. In both Saturniidae and Nymphalidae the eyespots are affected by heparin, and thus the Wnt pathway might be involved in their formation, possibly acting by homologous reaction-diffusion mechanisms. In Saturniidae, the eyespot center (the cross-vein and scales immediately surrounding it) seemed to be unaffected by heparin. The same might be true for Nymphalidae eyespot centers, and the insensitivity to heparin in both groups may be because the focus is the center of production of signaling molecules involved in reaction-diffusion, which already has the highest signaling level. 3. Reaction-diffusion may begin at the same stage for both butterflies and moths, in prepupae and not at early pupal stage as was previously thought. A refractory period immediately following pupation may exist, when no signal-response occurs, possibly regulated by hormones. 4. The limited transplant experiments on Io Moth and Luna Moth indicate that there may be a signal coming from the center of the developing eyespot at the early pupal stage that determines the color of the surrounding cells. These surgical experiments need to be repeated and expanded with proper controls, but if these results are confirmed, they could support the eyespot organizer theory formulated based on transplant experiments with nymphalid butterflies. 5. Wounds in the developing wing membrane of Saturniidae changed the color of surrounding scales in the medial part of the wing, but not in the distal part of the wing. In Nymphalidae the same effect is mostly observed in the distal part of the wing. We conclude that wound-healing pathway may have been at the base of eyespot evolution in Lepidoptera, but it manifests itself now in different wing regions in nymphalids and saturniids - likely those regions that bear eyespots. A more in-depth and systematic exploration of both wing regions across Lepidoptera species with and without eyespots would be needed to confirm that.

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